The transcription factor KLF2 in vascular biology

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Citation for published version (APA):
KLF2 increases endothelial progenitor cells and is reduced by aging/senescence and by hyperglycemia in a p38-dependent manner

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Manuscript in preparation
Abstract

Coronary artery disease (CAD) patients have less circulating endothelial progenitor cells (EPCs), which exhibit impaired neovascularization properties. Inverse correlations were also found between EPC function and risk factors like age and diabetes. Krüppel-like factor 2 (KLF2) is expressed by mature endothelial cells (ECs), is induced by both shear stress and statins and provokes endothelial quiescent differentiation.

Here, we describe that EPCs also express KLF2 at a comparable level to mature ECs. Furthermore, both cell culture induced senescence in EPCs and cell cycle-provoked senescence in HUVECs decrease KLF2 levels. To study the effects of aging on KLF2 levels, we compared progenitor cells of young (4 weeks old) and aged (16-18 months old) C57Bl6 mice. In addition to the 2.5-fold reduction of circulating Sca1+/c-Kit+/Lin- progenitor cells and the 40% reduction of Sca1+/Flk1+ endothelial committed progenitor cells, the spleen-derived EPCs isolated from these aged mice showed a 60% lower level of KLF2 when compared to EPCs isolated from young mice. In accordance with this finding, EPCs isolated from peripheral blood of CAD patients were shown to contain 3-fold less KLF2 than EPCs isolated from healthy controls. Furthermore, we provide evidence that KLF2 levels in EPCs are acutely reduced by 2.5-fold during hyperglycemia, through p38-mediated mRNA destabilization. Finally, lentiviral overexpression of KLF2 increased human EPC numbers by 60% during in vitro culture.

In conclusion, reduced functionality in aged or diseased EPCs is associated with lower KLF2 levels, whereas exogenous overexpression of KLF2 increases EPC numbers ex vivo. These results imply that KLF2 might be an attractive novel target to rejuvenate autologous EPCs before administration to CAD patients.
Introduction

The coordinated growth of new blood vessels is essential for tissue repair after ischemia. This can be mediated by the migration and proliferation of neighbouring endothelial cells (ECs), but also through incorporation of circulating precursor cells, known as endothelial progenitor cells (EPCs). EPCs are circulating bone marrow–derived cells, that can home to sites of neovascularization and differentiate into ECs or promote angiogenesis by excreting paracrine factors, which have been successfully used for therapeutic angiogenesis. This has also been applied for tissue repair after myocardial infarction. However, patients in most need of neovascularization are often burdened with comorbidity factors, like diabetes, known to be detrimental for EPC numbers and quality.

Shear stress, the frictional force generated by blood flow, is thought to be one of the factors that play a role in the differentiation of EPCs to ECs. Furthermore, one of the most-prescribed drug classes, the 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, was shown to improve the function of EPCs especially in coronary artery disease (CAD) patients. Recently, statins were described to induce Krüppel-like factor 2 (KLF2) expression, a transcription factor originally described to be induced by shear stress in ECs, where it induces functional quiescence. KLF2 was also described to be anti-inflammatory and anti-thrombotic, to be essential for the shear stress- and statin-mediated induction of endothelial nitric oxide synthase (eNOS) and thrombomodulin and to govern approximately 30% of the shear stress-induced gene expression pattern. The endothelial-specific KLF2 null mouse exhibits an embryonically lethal phenotype, which is caused by vascular leakage and a lack of peripheral vascular tone, underscoring the crucial importance of KLF2 for proper endothelial function.

KLF2 was also found to be highly expressed in pluripotent stem cells, with loss of KLF2 expression upon differentiation of these cells. Also, KLF2 can be used as one of the four factors described for the generation of induced pluripotent stem cells from dermal fibroblasts. This signifies a possible role for KLF2 in pluripotent stem cell function. Therefore, we hypothesized that KLF2 might be required for the stem/progenitor cell-like phenotype of EPCs.

In search for factors and mechanisms to functionally improve EPCs to aid neovascularization, we analyzed the expression and regulation of KLF2 in EPCs. We conclude that KLF2 levels in aged and senescent EPCs and ECs are lower compared to control cells. Furthermore, we show that KLF2 mRNA is destabilized by p38 in response to elevated glucose levels. Finally, we describe that KLF2 overexpression increases the number of human peripheral-blood derived EPCs during ex vivo culture.
Materials and methods

Reagents, animals and patient inclusion criteria
The KLF2 antibody was described previously. The KLF2 antibody was described previously.\textsuperscript{17} SB203580, glucose and Actinomycin D were purchased from Sigma (St. Louis, MO). C57Bl6 mice were obtained from Jackson Laboratory (Bar Harbor, ME). The animal experiments were approved by the Regional Board of Land Hessen, Germany. For studies with patient material, approval was obtained from the J.W. Goethe University Hospital Institutional Review Board. Informed consent was obtained in accordance with the Declaration of Helsinki. Only patients who did not receive statin therapy and presented with stenosis in at least one branch of the coronary artery, as measured by coronary angiography, were included.

Cell isolation and culture
Ex vivo EPC differentiation from circulating mononuclear cells (MNCs) was assayed as described previously.\textsuperscript{1} In brief, MNCs were isolated by density gradient centrifugation with Biocoll separating solution (density 1.077; Biochrom AG, Berlin, Germany) from human peripheral blooduffy coats or full blood. For the isolation of spleen-derived EPCs, murine MNCs were isolated from homogenized splenic tissue by density gradient centrifugation with Biocoll separating solution. Immediately after isolation, 8 × 10^6 MNCs/ml of medium were plated on culture dishes coated with human fibronectin (Sigma) and maintained in endothelial basal medium (EBM; Cambrex, East Rutherford, NJ) supplemented with EGM SingleQuots (Cambrex) and 20% FCS (GIBCO BRL, Carlsbad, CA). After 3 days in culture, nonadherent cells were removed by thorough washing with PBS. Adherent cells were stained with 2.4 μg/ml Dil-Ac-LDL (Harbor Bio-Products, Norwood, MA) at 37°C for 1 h. HUVECs were purchased from CellSystems and cultured in endothelial basal medium (EBM; Cambrex) supplemented with EGM SingleQuots (Cambrex) and 10% FCS (GIBCO BRL).

Endothelial differentiation of ES cells
ES D3 cells, a 129/Sv-derived embryonic stem (ES) cell line, were cultured as previously described.\textsuperscript{18} To initiate ES cell differentiation and embryonic body formation, ES cells were trypsinized and suspended in IMDM (GIBCO BRL) with 15% FBS, 10 μg/ml insulin (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin, 450 μmol/liter monothioglycerol, and endothelial growth factors including 50 ng/ml recombinant human VEGF (PeproTech, Rocky Hill, NJ), 2 U/ml recombinant human erythropoietin (Cilag AG), 100 ng/ml human basic fibroblast growth factor (Genzyme, Cambridge, MA), and 10 ng/ml murine interleukin 6 (Genzyme).

Flow cytometry analysis
3 x 10^5 murine peripheral blood-derived EPCs were incubated for 30 min at 4°C with FITC- or PE-labeled antibodies (Flk1 (VEGFR2), Sca-1, c-kit and/or an anti–lineage marker antibody cocktail (BD Biosciences, Franklin Lakes, NJ). Cells were then washed and surface expression was quantified using a FACS Calibur (BD Biosciences).

Real-Time Reverse Transcription–Polymerase Chain Reaction
Total RNA from was isolated by using the Qiagen RNA extraction kit. The RT-PCR reaction was composed of LightCycler–RNA MasterPLUS SYBR Green I (Roche), using the
LightCycler (Roche, Basel, Switzerland) real-time thermocycler according to the instructions of the manufacturer (Roche). Amplification was performed with 40 cycles and an annealing temperature of 60°C. The specificity of the amplification reaction was determined by a melting curve analysis.

**Luciferase reporter constructs and assay**

KLF2 promoter fragments were obtained by PCR using KpnI and XhoI restriction sites-containing primers and human chromosomal DNA. Mutation of the transcription factor binding site was accomplished by PCR overlap mutagenesis, with which the core binding sequence for MEF2 (5'-TTTA-3') was mutated into 5'-CGCG-3'. The constructs were cloned into the pGL3 basic Firefly luciferase reporter vector and transferred to the modified pRRL-cPPT-CMV-X2-PRE-SIN lentiviral vector, using the NotI and SalI restriction sites, according to our recently described procedure. A control thymidine kinase promoter driven Renilla luciferase construct was likewise transferred to the modified lentiviral vector. HUVECs were transduced at least 48h before luciferase activity was measured using the dual luciferase reporter assay system following the manufacturer’s protocol (Promega, Madison, WI).

**Statistical Analysis**

Data were analyzed using unpaired student’s t-tests when comparing two conditions, or a one-way ANOVA with Bonferroni correction for multiple comparisons. Probability-values of less than 0.05 were considered significant.
Results

KLF2 is expressed in a bi-phasic manner during endothelial differentiation of embryonic stem cells

Since the transcription factor KLF2 was identified as marker for undifferentiated stem cells\(^1\) as well as a functional endothelial marker gene,\(^\text{10, 17}\) we analyzed its expression in an established model of endothelial differentiation of ES-D3 mouse embryonic stem cells (ESCs).\(^\text{18}\) In this model, ESCs are cultured as embryoid bodies in endothelial differentiation medium containing growth factors and cytokines, like interleukin 6 (IL-6) and vascular endothelial growth factor (VEGF). This leads to an increase in expression of endothelial markers like VEGF receptor 2 (VEGFR2, Flk1), endothelial nitric oxide synthase (eNOS) and tyrosine kinase with immunoglobulin and epidermal growth factor homology domain 2 (Tie2) within 4-10 days (Figure 1). RT-PCR analysis revealed that KLF2, is indeed expressed by mouse ESCs (Figure 1). KLF2 expression levels then drop when endothelial differentiation is initiated, but rise again during endothelial differentiation. Interestingly,

![Figure 1. KLF2 is bi-phasicly expressed in differentiating embryonic stem cells.](image)

mRNA levels of murine KLF2, thrombomodulin (THBD), eNOS, Tie2, CD31, Flk1 and GAPDH were analyzed by RT-PCR and gel-electrophoresis. The first lane represents undifferentiated embryonic stem cells (ESCs) and the second, third and forth lanes represent ESCs under endothelial differentiation conditions for 4, 7 and 11 days respectively.
direct transcriptional targets of KLF2 in endothelial cells, thrombomodulin and eNOS, which are considered endothelial markers, are also expressed in mouse ESCs.

**KLF2 is expressed by endothelial progenitor cells, but is reduced by senescence in vitro**

Next, we analyzed the expression levels of KLF2 in human EPCs and compared them to mature endothelial cells (HUVECs) and human circulating mesoangioblast-like cells, which express endothelial and mesenchymal markers (Dr. Koyanagi, personal communication) using real-time reverse transcriptase polymerase chain reaction (RT-PCR). As can be seen in figure 2A, human EPCs express KLF2 at a comparable level to mature ECs, where KLF2 can be induced by shear stress.20 Notably, mesoangioblast-like cells express considerably lower levels of KLF2, when compared to HUVECs and EPCs. Since eNOS was shown to be a direct transcriptional target of KLF2, while eNOS expression decreased in senescent endothelial cells,21 we assessed the expression of KLF2 in replicative senescent HUVECs, compared to relatively young ECs (Figure 2B). Proliferating young HUVECs (passage 5 and 8) express higher levels of KLF2 mRNA as compared to senescent HUVECs (passage 14 and 16). KLF2 levels in EPCs also markedly decrease during cell culture induced senescence (Figure 2C).

**Age reduces murine progenitor cell numbers and represses KLF2 expression**

To study KLF2 during aging *in vivo*, we compared progenitor cells of young (4 weeks old) and aged (16-18 months old) C57Bl6 mice. First, as a measure for EPC quality, acetylated LDL (Ac-LDL) uptake was measured in EPCs derived from equal amounts of bone marrow mononuclear cells that were isolated from young and aged mice. Results indicate that in aged mice numbers of EPCs are reduced by ~40%, as compared to young mice (Figure 3A). Second, immuno-fluorescent flow cytometry was used to analyze levels of cellular surface expression of Sca1, c-Kit, both stem cell markers, and Flk1 (VEGFR2), an endothelial marker, in combination with a negative selection for lineage markers (Lin'). Aged mice were shown to contain ~60% less Sca1'c-Kit'Lin' progenitor cells as well as ~40% less Sca1'Flk1' endothelial committed progenitor cells, when compared to young control mice (Figure 3B). Third, the spleen-derived EPCs isolated from these aged mice also have ~60% lower levels of KLF2 than EPCs isolated from spleens of young mice (Figure 3C). These results clearly show that in addition to reduced numbers of circulating EPCs in aged mice, also the expression level of KLF2 is markedly reduced in the remaining cells.

**Coronary artery disease patients show reduced circulating progenitor numbers as well as reduced KLF2 expression**

To extrapolate these findings to humans, we performed a pilot experiment in which we
isolated EPCs from four CAD patients and four healthy volunteers. Because statins are known to induce KLF2, CAD patients and control subjects were only included if they were not receiving statin therapy. For practical reasons, the patients and controls were not matched for age or sex, which could be a confounding factor (age 64.5 ± 3.9 years and 75% males for patients and age 26.8 ± 1.1 years and 50% males for control subjects). Human KLF2 levels were analyzed by RT-PCR, revealing a marked 3-fold reduction of KLF2 levels in EPCs isolated from CAD patients as compared to controls (Figure 3D).

Figure 2. KLF2 is expressed in EPCs and is repressed by replicative and cell-culture induced senescence.
(A) In EPCs, HUVECs (static), HUVECs exposed to shear stress and meso-angioblasts, mRNA levels of human KLF2 were measured by real-time RT-PCR. Expression levels were corrected for GAPDH mRNA levels. (N=3 to 9) (B) RT-PCR analysis of KLF2 and GAPDH mRNA levels in HUVECs cultured for 5, 8, 14 or 16 passages. (C) KLF2 and GAPDH mRNA levels in EPCs that were cultured for 3, 4, 5, 7, 10 or 12 days. *P<0.05 compared to HUVECs (static).
Elevated glucose levels reduce KLF2 in EPCs through p38-mediated mRNA destabilization

Next to age and CAD, diabetes is also known to have detrimental effects on EPC numbers and function. Many adverse effects of diabetes, especially those on EPCs, are mediated through glucose via the p38 mitogen activated protein kinase (MAPK) pathway. To assess whether glucose can also reduce KLF2 levels in EPCs, we treated EPCs with 25 mM (450 mg/dL) glucose for 24 hours and analyzed the KLF2 levels by RT-PCR (Figure 4A). This revealed that hyperglycemic conditions reduce KLF2 levels by ~60% in EPCs, as compared...
to normoglycemic conditions. Next, we investigated the role of the p38 MAPK pathway, using the p38 inhibitor SB203580 (1 μM) in combination with glucose treatment. Inhibition of p38 led to a complete restoration of KLF2 mRNA levels to levels measured in untreated EPCs (Figure 4A).

To further understand the mechanism by which glucose reduces KLF2 expression levels, we analyzed the KLF2 promoter activity in EPCs after treatment with glucose and SB203580 (Figure 4B). This revealed that neither glucose nor p38 regulates transcriptional activity of the 367 basepair core23 KLF2 promoter, suggesting that the lowering effect of glucose on KLF2 levels occurs through post-transcriptional effects on the KLF2 mRNA. Therefore, stability of KLF2 mRNA was analyzed by RT-PCR during transcription blockage with Actinomycin D, using EPCs that were first treated with glucose and SB203580 for 24 hours (Figure 4C). KLF2 mRNA is degraded more rapidly in glucose-treated EPCs (half-life of ~20 min.) when

![Figure 4](image)

**Figure 4. Glucose reduces KLF2 mRNA stability in a p38-dependent way.**

(A) KLF2 mRNA levels were measured by conventional RT-PCR (for gel-electrophoresis) and real-time RT-PCR (quantification) in human EPCs. Cells were treated for 24 hours with glucose (25 mM) in the presence or absence of 1 μM SB203580 or left unstimulated (N=5). (B) Luciferase reporter lentivirus containing the proximal 367bp. KLF2 promoter or a mutation thereof (where the MEF2 binding site is mutated) was used to transduce EPCs two days before stimulation. Glucose (25 mM) and/or SB203580 (1 μM) was added to the culture medium 24 hours before luciferase activity measurement. (C) EPCs were cultured under hyperglycemic conditons (25 mM glucose) in the presence or absence of SB203580 (1 μM) for 24 hours or left unstimulated. Then transcription was blocked by the addition of Actinomycin D (200 nM) and RNA was isolated after 60 or 120 minutes (N=3). *P<0.05
compared to vehicle-treated control cells (half-life of ~35 min.). This destabilizing effect of glucose is completely abrogated in the presence of the p38 inhibitor SB203580 (half-life of ~35 min.).

**KLF2 overexpression increases EPC number ex vivo.**

To identify a potential role for KLF2 in EPCs, KLF2 was ectopically overexpressed using our previously described lentiviral overexpression system. Three days after lentiviral transduction with mock or KLF2 overexpression virus, KLF2 mRNA levels were measured using RT-PCR (Figure 5A). KLF2 protein levels were analyzed by Western blot (Figure 5A), which confirmed overexpression of KLF2 protein in KLF2-transduced EPCs, as compared to mock-transduced EPCs. Using Dil-labeled Ac-LDL, we assessed the number of EPCs three days after transduction (Figure 5B). This revealed that lenti-viral overexpression of KLF2 increases the number of EPCs by 60% ex vivo. Importantly, we did not detect increased proliferation rates after KLF2 overexpression (data not shown).

**Figure 5. Lentiviral overexpression of KLF2 increases number of EPCs in vitro.**

(A) Three days after lentiviral transduction of EPCs with mock- or KLF2-lentivirus, protein and RNA was isolated. KLF2 and GAPDH mRNA levels were measured using RT-PCR. KLF2 and α-tubulin protein levels were analyzed by Western blot. (B) Three days after mock- and KLF2-transduction, the number of EPCs that take up Ac-LDL was counted using Dil-labeled Ac-LDL (N=5). *P<0.05
Discussion

Promotion of neovascularisation by EPCs is essential for tissue repair after ischemia. To facilitate this, EPCs differentiate to ECs or promote angiogenesis by excreting paracrine factors. The autologous transfer of these cells has also been successfully used for tissue repair after myocardial infarction. However, the quality and numbers of EPCs that are isolated from patients in most need of neovascularisation, who are often of old age and suffer from diabetes, are usually the limiting factors for successful therapy. Here, we describe the relation between KLF2 levels and EPC function, in the context of age, senescence, hyperglycemia and CAD. Importantly, we demonstrate that overexpression of KLF2 increases the number of EPCs in vitro.

Ageing is associated with a reduced number of circulating EPCs (Figure 3). Moreover, these EPCs display lower survival, migration and proliferation, as well as increased cellular senescence. These observations imply functional impairment of EPCs from aged subjects. We now show that KLF2 expression is reduced in EPCs isolated from aged mice and senescent human EPCs, when compared to young mice and healthy human EPCs, indicating a positive correlation between EPC function and KLF2 expression levels. Similar functional changes have been observed in diabetes: EPCs isolated from patients with type II diabetes showed decreased proliferation and adherence to HUVECs and reduced potential to participate in tube formation. We demonstrate that EPCs cultured under hyperglycemic conditions have lower KLF2 levels and that this is mediated through p38 MAPK signaling.

The p38 MAPK signaling pathway has previously been indicated to be responsible for the detrimental effects of hyperglycemia on EPC function. Post-transcriptional destabilization of mRNA by p38 occurs specifically through AU-rich elements in the 3'UTRs of target mRNAs. The KLF2 3'UTR was previously shown to contain two AU-rich elements and to be destabilized in a phosphoinositide 3-kinase-dependent manner in HUVECs. We now describe a potential mechanism for the p38-dependent deleterious effects of hyperglycemia on EPCs, through the destabilization of KLF2 mRNA.

The HMG-CoA reductase inhibitors (statins) have been shown to induce EPC numbers and function, partly through the inhibition of senescence, and are nowadays used as a standard component of the in vitro differentiation protocol used in clinical trials. Statins are also known to augment one of the best-known inducers of EPC function, eNOS, which in mature ECs was shown to be a direct KLF2 target. Interestingly, the KLF2-induced increase of EPC numbers (Figure 4B) is identical to the previously reported statin-mediated increase in EPC numbers (~1.6 fold). These findings, combined with the results reported here, suggest that the superior EPC function induced by statins, could be critically mediated through KLF2. The mechanism by which KLF2 increases EPC numbers comparably to statins is still elusive and could comprise increased survival, adhesion, differentiation and/or anti-oxidant capacity. However, we exclude that KLF2 alters proliferation of EPCs. In concurrence, KLF2 was shown to induce both adhesion and differentiation in HUVECs as
well as to enhance Nrf2-mediated anti-oxidant gene expression, but to have no effect on proliferation rate in HUVECs.\textsuperscript{10, 32}

Important for functional EPCs are their stem cell-like surface markers, their EC phenotype as well as their monocytic features.\textsuperscript{33} KLF2 seems to be able to positively affect all three of these features. First, KLF2 is expressed in pluripotent ESCs as well as in ESCs differentiated towards the endothelial lineage (Figure 1), which confirms the reported identification of KLF2 as stem cell marker.\textsuperscript{15} Therefore, KLF2 could induce an EPC phenotype more reminiscent of stem cells. Second, KLF2 could also skew EPCs more towards an endothelial phenotype, because KLF2 is highly expressed in endothelial-committed cells (Figures 1 and 2A). In support of this option, p38 inhibition, which rescues glucose-mediated KLF2 inhibition, was shown to modulate the ratio of endothelial and monocyte/macrophage committed cells in favor of the endothelial lineage.\textsuperscript{22} Third, monocytic features are also known to be essential for EPC function\textsuperscript{34} and KLF2 overexpression was shown to prevent monocytes from differentiating to macrophages.\textsuperscript{35} Together, the synergistic effects of KLF2 on these three functions could thus prevent dedifferentiation or maintain proper differentiation of EPCs and thereby augment their functionality.

The observation that EPC quality of patients most in need of neovascularization is not optimal, raised the concept that improving EPC functionality \textit{in vitro}, prior to re-administering these cells to the patient, might serve as a better therapeutic tool for improvement of impaired neovascularization in ageing and diabetes. A number of factors have been proposed for this so-called ‘\textit{in vitro} rejuvenation’ of EPCs, including statins and p38 inhibitors.\textsuperscript{36} We show that restoring KLF2 levels in aged or diabetic EPCs might be a useful strategy for the ‘\textit{in vitro} rejuvenation’ of these EPCs.

In conclusion, the present study demonstrates that KLF2 is expressed by EPCs, but is reduced by old age, senescence, coronary artery disease and hyperglycemia, pointing toward a positive correlation between KLF2 levels and EPC function. Moreover, lentiviral overexpression of KLF2 increases the number of human EPCs when cultured \textit{ex vivo}. Collectively, these results implicate KLF2 as an attractive novel target for the improvement of EPC function prior to autologous transfer in CAD patients.
KLF2 expression and regulation in EPCs

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