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Global Remodeling of the Vascular Stem Cell Niche in Bone Marrow of Diabetic Patients:

Implication of the microRNA-155/FOXO3a Signaling Pathway

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Abstract

Rationale—The impact of diabetes mellitus on bone marrow (BM) structure is incompletely understood.

Objective—Investigate the effect of type-2 diabetes mellitus (T2DM) on BM microvascular and hematopoietic cell composition in patients without vascular complications.

Methods and Results—Bone samples were obtained from T2DM patients and nondiabetic controls (C) during hip replacement surgery and from T2DM patients undergoing amputation for critical limb ischemia. BM composition was assessed by histomorphometry, immunostaining, and flow cytometry. Expressional studies were performed on $CD34^{pos}$ immunosorted BM progenitor cells (PCs). Diabetes mellitus causes a reduction of hematopoietic tissue, fat deposition, and microvascular rarefaction, especially when associated with critical limb ischemia. Immunohistochemistry documented increased apoptosis and reduced abundance of $CD34^{pos}$-PCs in diabetic groups. Likewise, flow cytometry showed scarcity of BM PCs in T2DM and T2DM +critical limb ischemia compared with C, but similar levels of mature hematopoietic cells. Activation of apoptosis in $CD34^{pos}$-PCs was associated with upregulation and nuclear localization of the proapoptotic factor FOXO3a and induction of FOXO3a targets, p21 and $p27^{kip1}$. Moreover, microRNA-155, which regulates cell survival through inhibition of FOXO3a, was downregulated in diabetic $CD34^{pos}$-PCs and inversely correlated with FOXO3a levels. The effect of diabetes mellitus on anatomic and molecular end points was confirmed when considering background covariates. Furthermore, exposure of healthy $CD34^{pos}$-PCs to high glucose reproduced the transcriptional changes induced by diabetes mellitus, with this effect being reversed by forced expression of microRNA-155.

Conclusions—We provide new anatomic and molecular evidence for the damaging effect of diabetes mellitus on human BM, comprising microvascular rarefaction and shortage of PCs attributable to activation of proapoptotic pathway.

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Keywords

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An imbalance between circulating proinflammatory and proangiogenic cells leading to counterproductive cell recruitment to sites of endothelial injury contributes to vascular complications in patients with diabetes mellitus.[1–6] Studies in animal models suggest that the altered spectrum of circulating cells is consequent to a deregulated control of cell mobilization from bone marrow (BM).[7–10] In line, clinical data show that the BM of diabetic patients has an impaired capacity to release hematopoietic stem cells (HSCs) after stimulation with granulocyte colony-stimulating factor,[11] a defect for which the term diabetic stem cell (SC) mobilopathy was recently proposed.[12] Moreover, diabetes mellitus might impinge on the integrity of SCs/progenitor cells (PCs) by altering the marrow microenvironment, which consists of stromal, endosteal, and microvascular cells.[10,13,14]

Besides providing oxygen and nutrients, the marrow microvasculature plays a key role in the regulation of hematopoiesis.[15,16] Furthermore, vascular sinusoids constitute a dedicated interface for cell exchanges with the peripheral circulation. In a mouse model, we showed that type 1 diabetes mellitus causes microvascular rarefaction, resulting in critical hypoperfusion, depletion of SCs at the level of the endosteal niche, and altered transendothelial cell trafficking.[17] It would be of paramount importance to understand whether similar microvascular pathology occurs in BM of patients with diabetes mellitus. Current knowledge is limited to flow cytometry analysis of aspirates, which showed reduction of $CD34^{pos}$-PCs in BM of diabetic patients.[5] However, this sampling procedure is not suited to define the anatomic structure of the marrow and may provide inaccurate SC counts if the marrow is remodeled. Moreover, the molecular mechanisms underlying diabetes mellitus-induced SC depletion are incompletely understood. We showed that experimental diabetes mellitus causes an elevation in reactive oxygen species, infringes on DNA integrity, and induces apoptosis of $Sca\text{-}1^{pos}c\text{-}Kit^{pos}$ cells.[17] In addition, signaling mechanisms that maintain the self-renewal capacity and prevent senescence of SCs, like the polycomb group gene Bmi-1,[18] are downregulated in BM cells from diabetic mice.[13]

Recent evidence indicates that microRNAs (miRs) regulate the maturation of different hematopoietic lineages.[19–21] In particular, a restricted subset of miRs expressed in $CD34^{pos}$ HSCs is hierarchically organized in a circuitry that controls proliferation, viability, and differentiation.[21,22] Among HSC-associated miRs, miR-221 reportedly regulates terminal stages of erythropoiesis via repression of c-Kit, whereas miR-155 acts upstream of miR-221 holding HSCs at an early stem-progenitor stage through inhibition of differentiation-associated molecules, like CCAAT/enhancer-binding protein-β, cAMP response element-binding protein, JUN and FOS.[22,23] In addition, miR-155 inhibits the forkhead transcription factor FOXO3a.[24] In hematopoietic cells with incurred DNA damage, FOXO3a induces cell cycle arrest and apoptosis, via transcriptional regulation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ and proapoptotic Bcl-2 family member Bim.[25–27] By inhibiting FOXO3a, miR-155 exerts prosurvival effects in HSCs.

The present study examines the damaging action of diabetes mellitus on human BM using femoral bone specimens collected from orthopedic surgery. To determine whether macrovascular disease can further disrupt BM integrity, we also studied patients with type 2 diabetes mellitus (T2DM) undergoing amputation for critical limb ischemia (CLI). Furthermore, we investigated the influence of diabetes mellitus and high glucose (HG) on miR expression in $CD34^{pos}$ immunosorted BM PCs and the ability of miR-155 to reverse FOXO3a upregulation in HG-challenged $CD34^{pos}$ cells.

Methods

A Supplemental Methods section is available in the Online Data Supplement.

Patients and Study Protocol

The study complied with the principles stated in the Declaration of Helsinki and was covered by institutional ethical approval (protocol number 20/2010). Eligible subjects were screened from a consecutive series referring to MultiMedica Hospital for hip replacement surgery (nondiabetic controls and T2DM patients) or limb amputation (T2DM patients with CLI) from August 2010 to November 2012. T2DM and CLI were defined according to the American Diabetes Association and the Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II), respectively.[28] Exclusion criteria were acute infection, immune diseases, current or past hematologic disorders or malignancy, drug-induced diabetes mellitus, unstable angina, recent (within 6 months) myocardial infarction or stroke, liver failure, dialysis because of renal failure, pregnancy, and lack of consent to participate in the study. Online Table I illustrates main clinical data of the 82 subjects who entered the study.

Immunohistochemistry, flow cytometry, and molecular biology analyses were performed on femoral head leftovers from orthopedic surgery and proximal part of amputated femurs. In addition, a 30 mL peripheral blood (PB) sample was obtained by venipuncture the day before interventional procedures.

Results

Characteristics of the Study Population

As shown in Online Table I, groups were similar with regard to age and sex distribution. T2DM patients showed a higher body mass index than controls and T2DM+CLI, whereas the T2DM+CLI group had the highest percentage of smokers and a longer duration of diabetes mellitus. Hypertension was more frequent in the 2 diabetic groups than in controls, and cardiovascular complications were common in T2DM+CLI patients. Insulin was the preferred antidiabetic medication in T2DM+CLI, whereas T2DM patients were mainly treated with oral antidiabetic drugs including glitazones. Between 40% and 52% of diabetic patients were taking statins. Fasting glucose was higher in the 2 diabetic groups, especially in T2DM+CLI.

Diabetes Mellitus Alters BM Composition

Histomorphometry demonstrates a remarkable remodeling of BM from diabetic patients, consisting of decreased hematopoietic tissue, fat deposition, and bone rarefaction (Figure 1A and 1B). The effect of diabetes mellitus was significant, incremental in T2DM+CLI patients and independent of background factors (Online Table III). Multiple regression analysis showed that the dependent variables, hematopoietic and fat fractions, can be predicted by grouping factor, fasting glucose and duration of diabetes mellitus, with no effect of other independent variables except for body mass index, which in association with duration of diabetes mellitus predicts the abundance of fat in BM (Online Table V).

Diabetes Mellitus and CLI Additively Reduce Vascular Density in Human BM

We next assessed the marrow microvasculature by immunohistochemistry. Capillaries were recognized as CD31-positive structures whose lumen-size does not exceed the diameter of an erythrocyte, whereas venous sinusoids were identified as larger irregular structures containing several erythrocytes (Figure 1C). Furthermore, microvessels and arterioles were identified by confocal and fluorescence microscopy using the endothelial marker von

Willebrand factor and the vascular smooth muscle cell marker α-smooth muscle actin (Figure 1D).

Analysis of microvascular density identified a significant difference among groups for all sets of vascular structures (P=0.005; Figure 2E). Pairwise comparison indicates a large decrease of capillary density in BM of T2DM patients (P<0.05 versus controls) and a further reduction of all 3 vascular fractions in T2DM+CLI patients (P<0.01). The effect of diabetes mellitus on BM microvascular density was confirmed when considering background covariates (Online Table III). In multiple regression analysis, the dependent microvascular variables were predicted by grouping factor, duration of diabetes mellitus, and fasting glucose, in linear combination with hypertension as far as capillaries and sinusoids are concerned (Online Table V). Furthermore, we found that vascular density is directly correlated with the hematopoietic fraction and inversely correlated with fat abundance in BM (P<0.01 for both comparisons).

Altogether, these data indicate that diabetes mellitus causes microangiopathy in human BM, with vascular rarefaction being aggravated by CLI. Among associated risk factors, hypertension interacts with diabetes mellitus in influencing capillary and sinusoid density.

Diabetes Mellitus Reduces the Abundance of Hematopoietic Progenitors in BM

BM-derived $CD34^{pos}$ cells are a well-characterized population that have been used for hematopoietic reconstitution and more recently for the treatment of myocardial and peripheral ischemia.[29] We investigated the abundance of $CD45^{pos}CD34^{pos}$ and $CD45^{neg}CD34^{pos}$ cells in BM by confocal microscopy (Figure 2Ai and 2Aii). ANOVA detected a difference among groups with regard to the $CD45^{pos}CD34^{pos}$ fraction (P=0.008) and pairwise comparison showed a large reduction of this cell population in T2DM+CLI compared with controls (P<0.05) (Figure 2Aiii). Likewise, $CD45^{neg}CD34^{pos}$ cells were different among groups (P=0.001) because of a large reduction in T2DM (P=0.008 versus controls) and T2DM+CLI patients (P=0.0001 versus controls) (Figure 2Aiv). The difference among groups was confirmed after consideration of background factors by ANCOVA (Online Table III). Grouping factor was a strong predictor of the dependent variables, $CD45^{neg}CD34^{pos}$ and $CD45^{pos}CD34^{pos}$ cells. Furthermore, duration of diabetes mellitus and fasting glucose predicted the abundance of $CD45^{neg}CD34^{pos}$ cells (Online Table V). Moreover, in situ detection of DNA fragmentation by terminal deoxynucleotidyl transferase dUTP nick end labeling assay indicates activation of apoptosis in BM-mononuclear cells and $CD34^{pos}$-PCs of T2DM and T2DM+CLI patients compared with controls (Figure 2B and Online Figure I).

We next used multicolor flow cytometry for quantification of various cell populations in BM and PB (Figure 3A).[30] $CD45^{dim}CD34^{pos}$ cells were reduced in BM of T2DM (P=0.02 versus controls) and T2DM+CLI patients (P=0.004), but did not differ between the diabetic groups (Figure 3Bi), with these data being confirmed by ANCOVA (Online Table III). In contrast, PB $CD45^{dim}CD34^{pos}$ cells were similar in control and diabetic patients (Figure 3Bii). The relative abundance of $CD45^{dim}CD34^{pos}$ cells in BM could be predicted independently by grouping factor, duration of diabetes mellitus, and fasting glucose in the multiple regression model (Online Table V).

The surface antigen CD133, a prominin 5 transmembrane glycoprotein 1, has been used to identify primitive hematopoietic and nonhematopoietic PCs[31] endowed with proangiogenic and healing activities in animal models[32] and patients with acute myocardial infarction.[33–35] We found that $CD45^{dim}CD133^{pos}CD34^{pos}$ cells are remarkably reduced in BM of T2DM (P=0.02 versus controls) and T2DM+CLI (P=0.01), with no difference between the 2 diabetic groups (P=0.83) (Figure 3Ci and Online Table III). The levels of

$CD45^{dim}CD133^{pos}CD34^{pos}$ cells were predicted by grouping, duration of diabetes mellitus, and fasting glucose (Online Table V). In contrast, no group difference was found in PB $CD45^{dim}CD133^{pos}CD34^{pos}$ cells (P=0.35) (Figure 3Cii). Moreover, there was no difference between diabetic and control subjects with regard to BM and PB $CD133^{pos}CD34^{neg}$ cells (Figure 3Di and 3Dii), which are considered very early hematopoietic precursors.[36]

Differential Effects of Diabetes Mellitus on Endothelial PCs and Lineage Committed Hematopoietic Cells

Several BM cell subpopulations, including hematopoietic and nonhematopoietic SCs, have the capacity to differentiate into endothelial cells (ECs) or promote EC growth by paracrine mechanisms. For instance, BM $CD34^{pos}$ kinase insert domain receptor $(KDR)^{pos}$ cells have been postulated to comprise the hemangioblast, that is, a bipotent cell type able to form both ECs and blood cells.[37–39] Moreover, $CD34^{pos}KDR^{pos}$ cells are reportedly decreased in PB of patients with traditional cardiovascular risk factors including diabetes mellitus.[3,40,41] As shown in Figure 4Ai, $CD34^{pos}KDR^{pos}$ cells were mainly enriched in the $CD45^{dim}$ mononuclear cell fraction. Moreover, we could confirm a large reduction of BM and PB $CD45^{dim}CD34^{pos}KDR^{pos}$ cells in both subgroups of diabetic subjects as compared with controls (P=0.006 for both comparisons), with no difference between T2DM and T2DM +CLI (Figure 4Aii and 4Aiii and Online Table III). The dependent variable, BM $CD45^{dim}CD34^{pos}KDR^{pos}$ cells, was predicted by grouping factor, duration of diabetes mellitus, and fasting glucose, whereas PB $CD45^{dim}CD34^{pos}KDR^{pos}$ cells were predicted by grouping factor and duration of diabetes mellitus (Online Table V).

We also verified the abundance of proangiogenic PCs defined as $CD34^{pos}CD14^{pos}CD45^{dim}KDR^{pos}CXCR4^{pos}$ mononuclear cells.[42,43] This cellular subpopulation was reduced in BM and PB of both diabetic groups, with no difference between T2DM and T2DM+CLI (Figure 4Bi–4Biii). However, this effect was attenuated when considering other covariates (Online Table III). Nonetheless, the reduction of the PB cell fraction was predicted by grouping factor (Online Table V). Furthermore, a recent study showed that BM $CD31^{pos}$ cells are enriched of highly angiogenic cells.[44] However, we found no difference among groups regarding this cell population (data not shown).

We next verified the influence of diabetes mellitus on lineage committed hematopoietic cells (Figure 5A–5D) and mature ECs (Figure 5E). No difference was found in the BM and PB levels of $CD45^{pos}CD19^{pos}$ B-lymphocytes (Figure 5Bi and 5Bii) and $CD45^{pos}CD3^{pos}$ T lymphocytes (Figure 5Ci and 5Cii). In contrast, $CD3^{neg}CD56^{pos}CD16^{pos}$ natural killer (NK) lymphocytes were increased in BM of T2DM patients, with this effect being confirmed after correction for other covariates. In multiple regression analysis, fasting glucose was a predictor of NK lymphocytes (P=0.001). Moreover, PB NK lymphocytes did not differ between diabetic and control subjects (Figure 5Di and 5Dii). Finally, $CD45^{neg}CD31^{pos}CD144^{pos}$ ECs were reduced in BM of T2DM+CLI (Figure 5E), thus, confirming the in situ data from immunohistochemistry.

Altogether, these data indicate that diabetes mellitus causes a selective reduction of endothelial PCs in BM and PB, without altering the abundance of lineage committed hematopoietic cells with exception of NK lymphocytes which were augmented in BM.

Effect of Diabetes Mellitus on Non-HSCs

This fraction represents a heterogeneous population that comprises $CD45^{neg}CXCR4^{pos}CD34^{pos}$ SCs, $CD34^{neg}CD45^{neg}CD14^{neg}CD90^{pos}CD73^{pos}CD105^{pos}$ mesenchymal SCs and $cKit^{pos}$ PCs.[45–47] We could not find any difference between diabetic patients and controls with regard to BM $CD45^{neg}CXCR4^{pos}CD34^{pos}$ SCs and mesenchymal

SCs (data not shown). Surprisingly, cKit^{pos} PCs were increased in BM and PB of T2DM patients (P<0.05 versus controls) but not in T2DM+CLI patients (Online Figure II). However, in multiple regression analysis, diabetes mellitus was not a predictor of cKit^{pos} PCs.

Activation of the Proapoptotic FOXO3a Signaling Pathway in BM CD34^{pos} Cells

We investigated the expression levels of miR-155 and its validated target FOXO3a[24] in CD34^{pos} immunosorted BM PCs. Results indicate a downregulation of miR-155 in CD34^{pos}-PCs from diabetic patients compared with controls (P=0.019) (Figure 6A). In contrast, miR-221 did not differ among groups (data not shown). Moreover, we found FOXO3a mRNA levels to be increased in BM CD34^{pos}-PCs of diabetic patients (P=0.004) (Figure 6B) and inversely correlated to miR-155 levels (R^2=−0.41; P=0.002) (Figure 6C). Of note, in situ analysis of FOXO3a expression showed a remarkable increase of the transcription factor in BM cells from both diabetic groups (P<0.0007) (Figure 6Di and 6Dii), with evidence of nuclear translocation which is required for transcriptional activation of target genes (Figure 6Diii and 6Div). In line, we found that the FOXO3a targets p21 and p27^{kip1} are upregulated in CD34^{pos} cells from diabetic patients (P=0.001 and 0.006, respectively) (Figure 6E and 6F). The effect of diabetes mellitus on miR-115, FOXO3a, and p21 was confirmed when considering background covariates (Online Table VI). P21 and p27^{kip1} inhibit cell cycle progression by binding to, and inactivating, cyclin-dependent kinase complexes. Analysis of cell cycle by flow cytometry confirmed that CD34^{pos} cells from diabetic BM are stalled at the G1 checkpoint and undergo apoptosis with high frequency (Online Figure III).

We next investigated whether in vitro exposure to HG mimics diabetes mellitus in altering the miR-155/FOXO3a/p21/p27^{kip1} signaling pathway in CD34^{pos}-PCs. We found that HG decreases cell counts and miR-155 levels and increases FOXO3a (Online Figure IVA–IVC). Moreover, HG increases the levels of p21 and p27^{kip1} (Online Figure IVD and IVE). The osmotic control mannitol did not alter the expression of miR-155 and related target genes (Online Figure V).

To verify whether miR-155 overexpression can rescue the effect of HG on target genes, we transfected HG-challenged CD34^{pos} cells with pre–miR-155 and confirmed that transfection results in upregulation of related mature miR (Online Figure VI). Of note, forced expression of miR-155 contrasted the diminishing effect of HG on CD34^{pos} cell counts and abrogates the HG-induced upregulation of FOXO3a, p21, and p27^{kip1} (Figure 7A and 7B). Finally, in inductive colony forming unit assays on HG-challenged CD34^{pos} cells, miR-155-transduced cells generated fewer myeloid and erythroid colonies. Furthermore, the colonies generated by miR-155–transduced cells were smaller than controls (Figure 7C).

Discussion

To the best of our knowledge, this is the first study showing the damaging effect of diabetes mellitus on BM SCs and their microvascular environment in human subjects. We demonstrate for the first time the presence of microangiopathy in BM of diabetic patients. Furthermore, we screened a large spectrum of SCs/PCs and mature hematopoietic cells in BM and PB by flow cytometry. Results newly document that the BM of diabetic patients is depleted of hematopoietic and proangiogenic PCs but not of differentiated hematopoietic cells. Finally, we show that both diabetes mellitus and HG alter the miR-155/FOXO3a/p21/p27^{kip1} signaling pathway, which represents a crucial mechanism controlling human CD34^{pos}-PC renewal and differentiation. Importantly, forced expression of miR-155 inhibits the inductive effect of HG on the FOXO3a/p21/ p27^{kip1} trio and reduces differentiation of CD34^{pos}-PCs in a colony forming unit assay.

Diabetic Microangiopathy in Human BM

A prominent feature of diabetic BM consists of substitution of the hematopoietic component with adipose tissue. Interestingly, the effect of diabetes mellitus on marrow composition was independent of background factors, with exception of fat accumulation which was also predicted by the combination of duration of diabetes mellitus and body mass index. Although additional investigation is needed to interpret the relationship between fatty infiltration of the marrow and obesity, evidence indicates that BM adipocytes could contribute to inhibit hematopoiesis in an obese model of T2DM.[48]

We found a direct correlation between vascular rarefaction and marrow remodeling, in line with the concept that a reduction of nurturing vasculature is detrimental for SC homeostasis. Of note, regression analysis indicates that, besides grouping factor, both duration of diabetes mellitus and hypertension are good predictors of capillary and sinusoid rarefaction, whereas arteriole density could be predicted only by variables inherent to diabetes mellitus, that is, disease duration and fasting glucose. The relationship between glycemic control and hypertension in the development of microangiopathy in peripheral organs is well documented. In CLI patients, who showed the most striking microvascular remodeling, analyses were performed on the proximal part of the amputated femoral bone which contains healthy tissue. We cannot exclude that the different anatomic source, in addition to ischemia, might have an impact on the incremental reduction of vascularity observed in this category.

Impact of Diabetes Mellitus on PC Abundance

Comparing marrow aspirates from 10 nondiabetic and 10 diabetic subjects, Fadini et al[5] reported a significant reduction of $CD34^{pos}$ cells in the latter group. Extending those data, we observed a decrease in both $CD34^{pos}CD133^{pos}$ and $CD34^{pos}KDR^{pos}$ PCs in BM of diabetic patients. In contrast, lineage committed hematopoietic cells, including B and T lymphocytes, were similarly abundant or even increased, as in the case of NK subpopulation. Altogether, these findings newly indicate a disparity in the depleting effect of diabetes mellitus on different hematopoietic cell lineages, which might contribute to the altered spectrum of circulating cells. Importantly, the dominant influence of diabetes mellitus on these cellular endpoints was confirmed by analysis of associated risk factors and confounding variables.

Diabetes Mellitus Impinges on Master Molecular Regulators of Hematopoiesis

The present study highlights new molecular mechanisms that may account for HSC depletion in diabetes mellitus. Results of cell cycle analysis show that freshly sorted $CD34^{pos}$-PCs from BM of diabetic patients are held in G_1 phase, the typical restriction checkpoint where HSCs are cycle-arrested to prevent accrual of DNA damage. Moreover, diabetic BM cells show increased apoptosis, as assessed by immunohistochemistry and cell cycle analysis.

FOXO transcription factors are typically involved in enforcing cell cycle checkpoints in hematopoietic cells with DNA damage.[27] A potential mediator of FOXO-induced cell cycle arrest as well as of irreversible progression toward cell apoptosis is $p27^{kip1}$, a cyclin-dependent kinase inhibitor that reportedly reduces proliferation and survival of HSCs.[49] We found that FOXO3a and downstream mediators, p21 and $p27^{kip1}$, are remarkably upregulated in $CD34^{pos}$-PCs from BM of diabetic patients, with these transcriptional changes being associated with reduction of cell viability. In vitro experiments challenging $CD34^{pos}$ cells with HG confirm that glucose is sufficient to activate the proapoptotic signaling pathway in healthy HSCs.

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MiRs regulate major cellular processes, including metabolism, apoptosis, and differentiation and also participate in the pathogenesis of human diseases.[50–52] Seminal studies using conditional deletion of Dicer, which disrupts miR processing, revealed critical roles of miRs during development of hematopoietic cell lineages.[53,54] In particular, miR-155 regulates early stages of hematopoiesis through inhibition of multiple genes implicated in HSC survival and differentiation, such as the human *FOXO3a* gene.[22] Current knowledge, however, is mainly centered on the implication of miR-155 in inflammation and cancer.[55] Intriguingly, a recent study showed that circulating levels of miR-155 are lower in patients with coronary artery disease compared with healthy controls, with an additive diminishing effect of diabetes mellitus.[56] It remains unknown what is the source of circulating miR-155 and whether a reduction of miR-155 expression in BM-derived cells can contribute to this defect.

Here, we report for the first time that miR-155 expression is reduced in BM $CD34^{pos}$ cells from diabetic patients and inversely correlated with levels of its validated target FOXO3a. Importantly, the inhibitory effect on miR-155 and the increase of FOXO3a, p21 and $p27^{kip1}$ by diabetes mellitus were independent of other background factors and all replicated by challenging healthy $CD34^{pos}$ cells with HG. To establish causality, we next asked whether miR-155 would prevent the effects of HG. Results indicate that forced expression of miR-155 is able to reverse the HG-induced upregulation of FOXO3a, p21, and $p27^{kip1}$. Furthermore, miR-155–transduced BM $CD34^{pos}$ cells formed fewer myeloid and erythroid colonies compared with scramble-transfected controls. The latter result replicates data from a previous study showing the ability of miR-155 in blocking differentiation in models of human hematopoiesis.[22] It remains unknown whether the described miR-dependent mechanism is implicated in other deficiencies of diabetic $CD34^{pos}$ cells, including unresponsiveness to granulocyte colony-stimulating factor. Intriguingly, a recent study in healthy primates showed that granulocyte colony-stimulating factor–mobilized $CD34^{pos}$ cells express higher miR-155 levels compared with nonstimulated or Plerixaformobilized cells.[57]

Altogether, these findings suggest that deregulation of the miR-155/FOXO3a/p27 signaling pathway might contribute to BM $CD34^{pos}$ cell depletion in diabetes mellitus. More investigation is warranted to establish whether other HSC-associated miRs participate in determining an imbalance between endothelial progenitors and mature hematopoietic cells.

Clinical Implications

In conclusion, this study draws attention to the BM as a primary target of diabetes mellitus-induced damage. Our data suggest that the severity of systemic vascular disease has an impact on BM remodeling. Conversely, more severe BM pathologies can cause (or contribute to) macroangiopathy, through shortage of vascular regenerative cells. Moreover, it should be acknowledged that this study was conducted on aged subjects and that inference to a younger population is uncertain. Further research is warranted to find specific treatments able to preserve BM integrity in patients with diabetes mellitus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

BM	bone marrow
CLI	critical limb ischemia
EC	endothelial cell
HG	high glucose
HSC	hematopoietic stem cell
KDR	kinase insert domain receptor
miR	microRNA
NK	natural killer lymphocytes
PB	peripheral blood
PC	progenitor cell
SC	stem cell
T2DM	type 2 diabetes mellitus

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Novelty and Significance

What Is Known?

- Diabetes mellitus is associated with reduced levels of circulating progenitor cells.
- Studies in diabetic animal models indicate the presence of microangiopathy in bone marrow endangering resident stem cell integrity.
- A restricted spectrum of microRNAs (miRs) that regulate self-renewal is expressed in hematopoietic stem cells.

What New Information Does This Article Contribute?

- In patients with diabetes mellitus, there is microangiopathy in the bone marrow, which is associated with incremental vascular damage in the presence of critical limb ischemia.
- The bone marrow of diabetic patients is depleted of hematopoietic and proangiogenic progenitor cells but not differentiated hematopoietic cells.
- Hyperglycemia inhibits miR-155, thereby releasing the activity of proapoptotic pathway involving FOXO3a/p21.

Although remodeling of blood vessels in peripheral organs has been extensively studied, little is known about the impact of common diseases on the marrow vascular niche, which is crucial for stem cell homeostasis and mobilization. Therefore, we investigated the effect of diabetes mellitus on bone marrow stem cells and their nurturing vasculature in humans. Results show a profound remodeling of the vascular niche, which is mainly replaced by fat, especially in patients with critical ischemia. Stem cell depletion did not preclude a regular abundance of mature hematopoietic cells, suggesting a defect in stem cell self-renewal. Investigation of underpinning mechanisms revealed that hyperglycemia inhibits a master regulator of hematopoietic cell fate, miR-155, thereby resulting in an unbalance between proliferation and differentiation. This was corrected by forced expression of miR-155. Our findings advance current understanding of pathological mechanisms leading to collapse of the vascular niche and reduced availability of proangiogenic cells. These data provide a key for interpretation of diabetes mellitus-associated defect in stem cell mobilization. In addition, our study reveals a negative circuitry, normally contrasted by a hematopoietic miR-responsible for pauperization of the marrow regenerative potential.

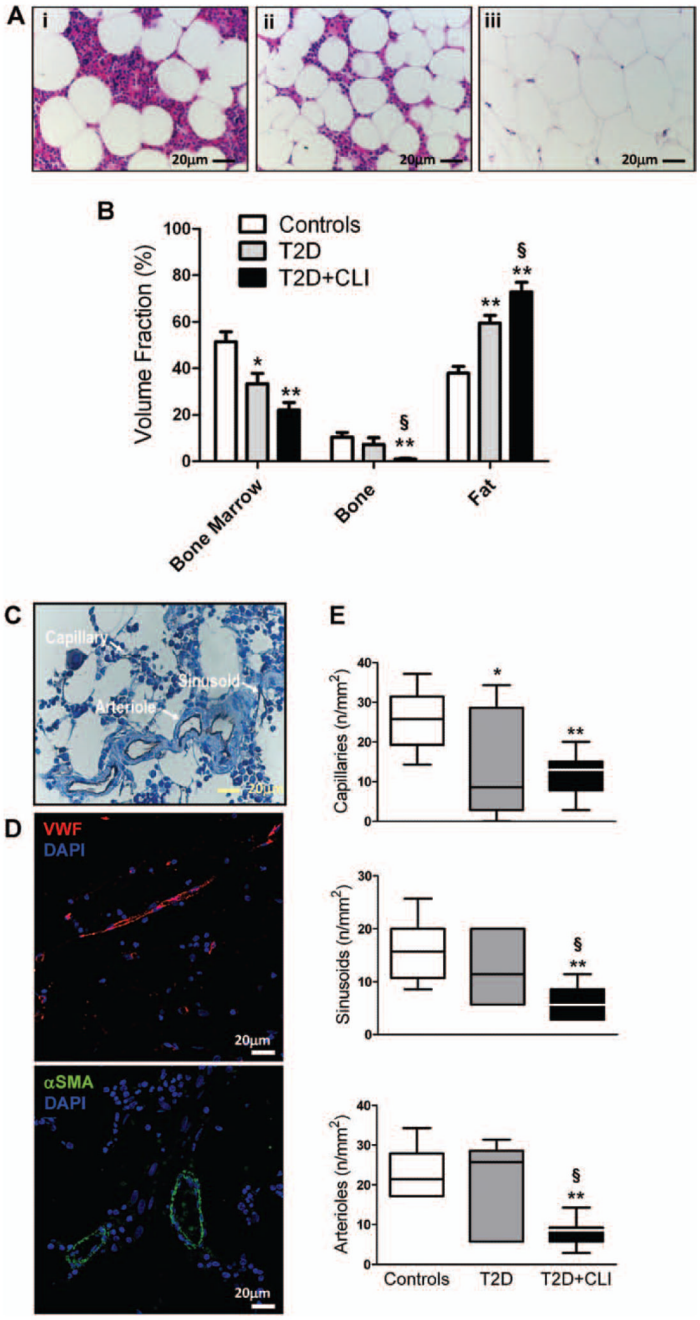

Figure 1. Diabetes mellitus induces bone marrow (BM) remodeling and vascular rarefaction

A and **B**, Histomorphometric analysis shows replacement of marrow with fat and bone rarefaction. **A**, Representative microphotograph of hematoxylin and eosin-stained BM sections: (**i**) Control, (**ii**) type-2 diabetes mellitus (T2DM) patient, and (**iii**) T2DM patient with critical limb ischemia (CLI). **B**, Bar graph showing average data of marrow fractions. **C**, Representative microphotograph of human BM showing CD31-positive vascular structures. **Arrows** indicate the type of vessel. **D**, Confocal microscopy photographs of human BM showing vascular structures stained with the endothelial marker von Willebrand factor (VWF) and the vascular smooth muscle marker α-smooth muscle actin (αSMA).

Nuclei are stained blue by 4′,6-diamidino-2-phenylindole (DAPI). **E**, Bar graph showing average data of microvascular density. *P<0.05 and **P<0.01 versus Controls, §P<0.05 versus T2DM. Controls, n=10; T2DM, n=7; T2DM+CLI, n=10.

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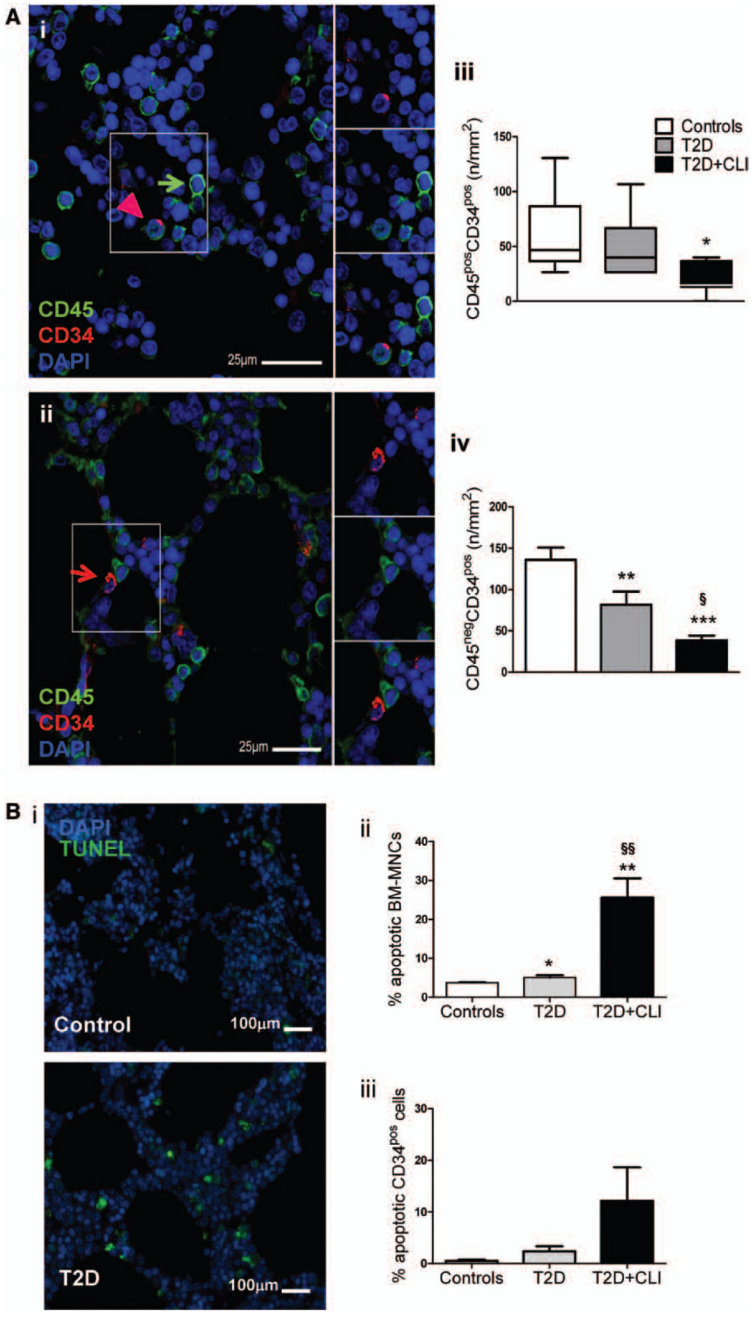

Figure 2. Reduced abundance of CD34^{pos} cells in bone marrow (BM) of type-2 diabetes mellitus (T2DM) patients

A, Representative confocal microscopy photographs of human BM showing the presence of CD45^{pos} (**green arrow**), CD45^{pos}CD34^{pos} (**pink arrowhead**) (**i**), and CD45^{neg}CD34^{pos} cells (**red arrow**) (**ii**). Nuclei are stained blue with 4′,6-diamidino-2-phenylindole (DAPI). Bar graphs showing the average density of CD45^{pos}CD34^{pos} cells (**iii**, median and 5%–95% distribution) and CD34^{pos}CD45^{neg} cells (**iv**, mean±SEM). **B**, Increased abundance of apoptotic mononuclear cells and CD34^{pos} cells in BM from diabetic patients. Representative microphotographs of fluorescent terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive cells (**i**) and bar graphs showing average data (**ii** and **iii**).

*P<0.05, **P<0.01 and ***P<0.001 versus controls, §P<0.05 and §§P<0.01 versus T2DM. Controls, n=10; T2DM, n=7; T2D+critical limb ischemia (CLI), n=8.

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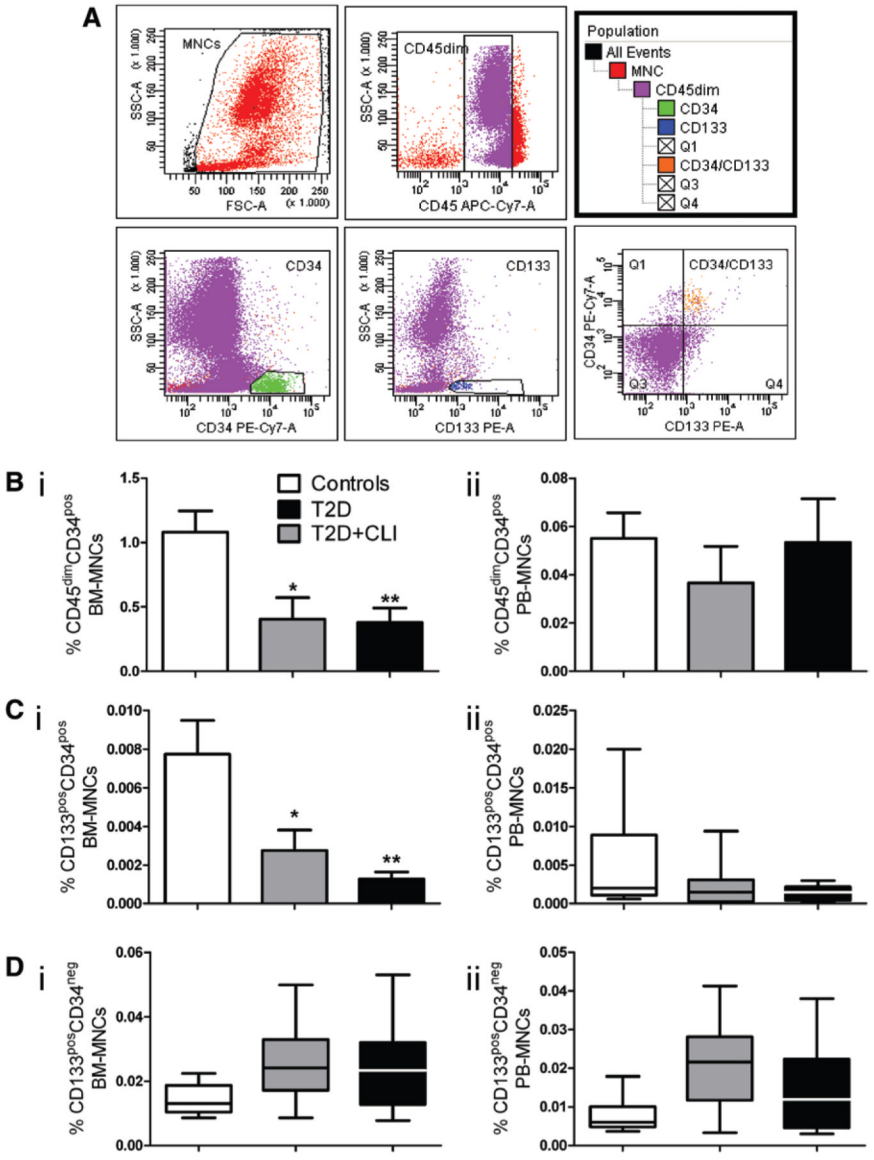

Figure 3. Flow cytometry characterization of hematopoietic cells in bone marrow (BM) and peripheral blood (PB) of type-2 diabetes mellitus (T2DM) patients

A, Gating strategy of multicolor flow cytometry. **B** to **D**, Bar graphs showing the abundance of $CD45^{dim}CD34^{pos}$ (**Bi** and **Bii**), $CD45^{dim}CD133^{pos}CD34^{pos}$ cells (**Ci** and **Cii**) and $CD45^{pos}CD133^{pos}CD34^{neg}$ cells (**Di** and **Dii**). **P*<0.05, ***P*<0.01 and ****P*<0.001 versus controls. Controls, n=8 to 14; T2DM, n=7 to 9; T2DM+critical limb ischemia (CLI), n=7 to 11.

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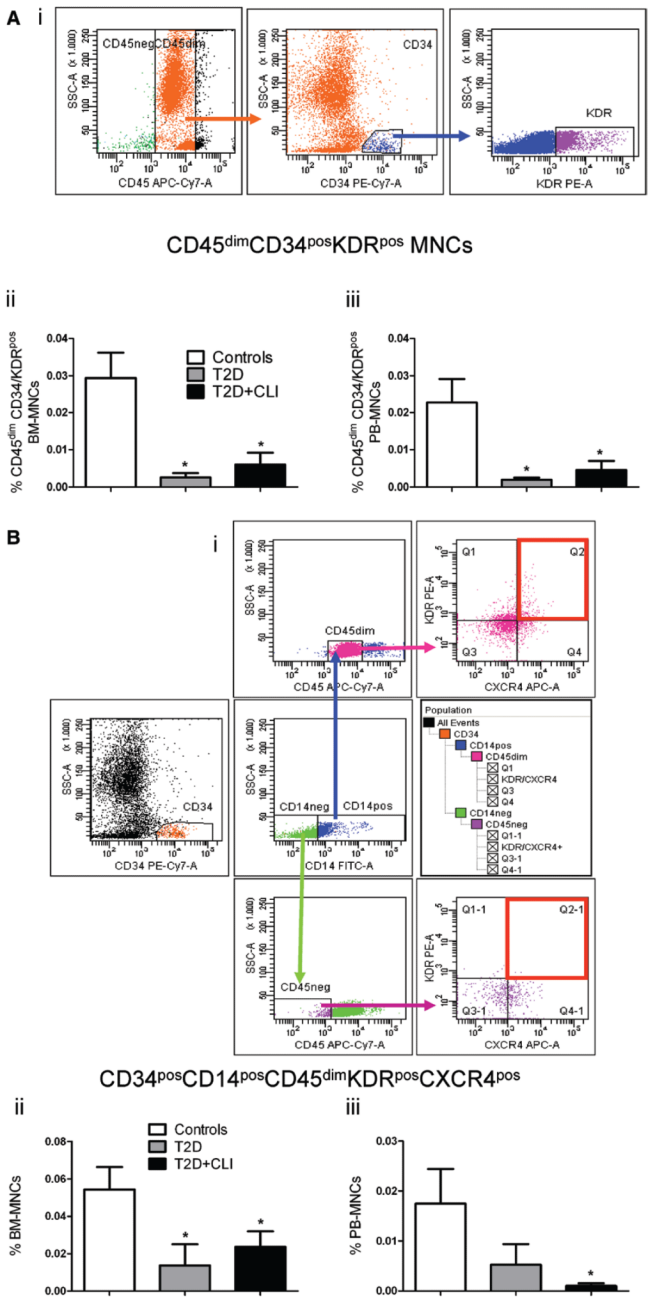

Figure 4. Flow cytometry characterization of bone marrow (BM) and peripheral blood (PB) endothelial progenitors

A, Gating strategy of $CD45^{dim}CD34^{pos}KDR^{pos}$ mononuclear cells (MNCs) (**i**), and bar graphs showing the abundance of this population in BM (**ii**) and PB (**iii**). B, Gating strategy of $CD34^{pos}CD14^{pos}CD45^{dim}KDR^{pos}CXCR4^{pos}$ cells (**i**), and bar graphs showing the abundance of this population in BM (**ii**) and PB (**iii**). *$P<0.05$ versus controls. Controls, n=8 to 11; type-2 diabetes mellitus (T2DM), n=6 to 9; T2DM+critical limb ischemia (CLI), n=7 to 10.

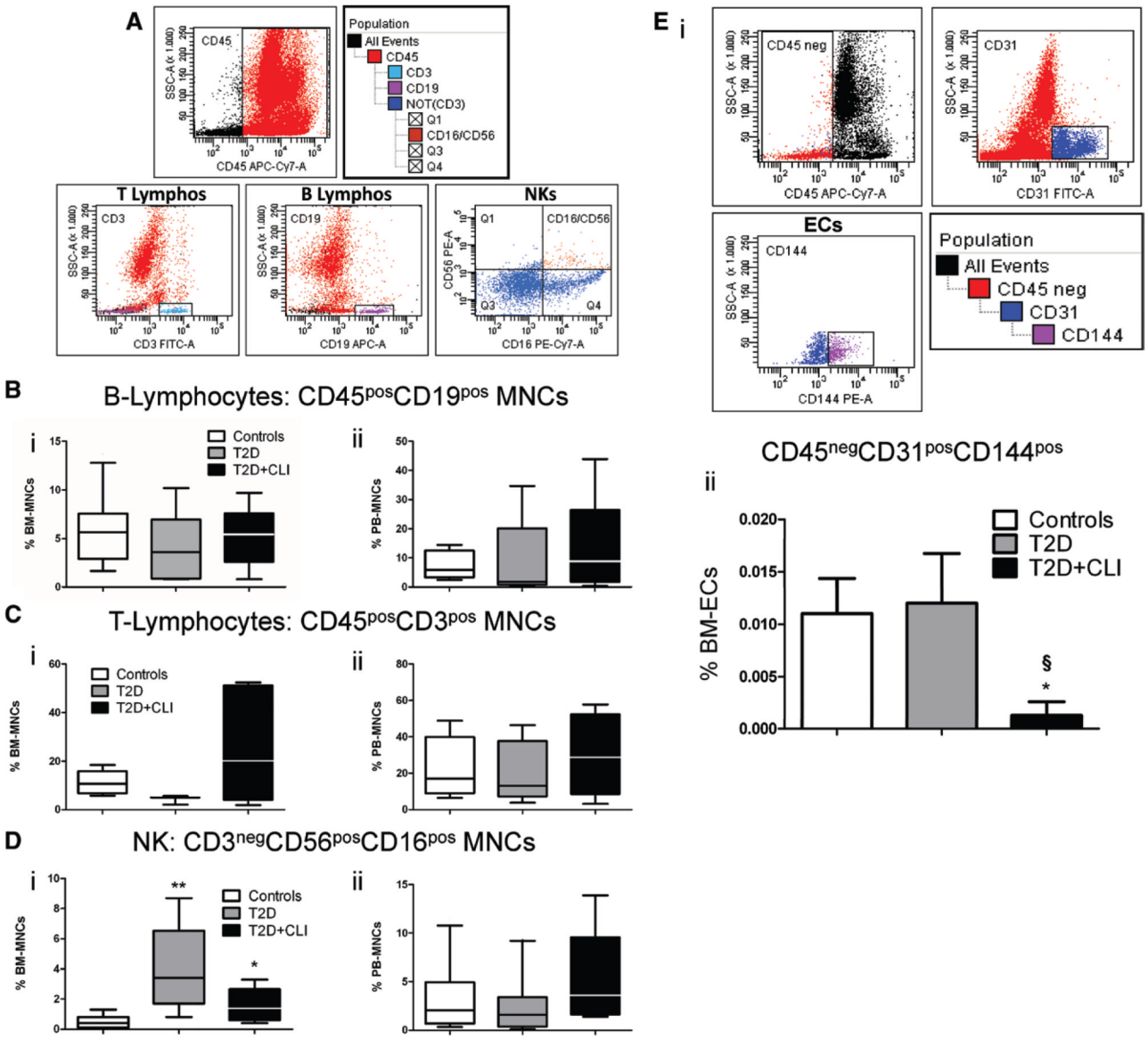

Figure 5. Flow cytometry characterization of lineage committed hematopoietic cells and endothelial cells (ECs)

A, Gating strategy for identification of B-lymphocytes, T-lymphocytes, and natural killer (NK) cells. **B** to **D**, Bar graphs showing the abundance of B-lymphocytes (**B**), T-lymphocytes (**C**) and NK cells (**D**) in bone marrow (BM) (**i**) and peripheral blood (PB) (**ii**). **E**, Gating strategy for identification of BM ECs (**i**) and bar graph showing average values (**ii**) **P*<0.05 and ***P*<0.01 versus controls, §*P*<0.05 versus type-2 diabetes mellitus (T2DM). Controls, n=9 to 16; T2DM, n=7 to 9; T2DM+critical limb ischemia (CLI), n=7 to 8.

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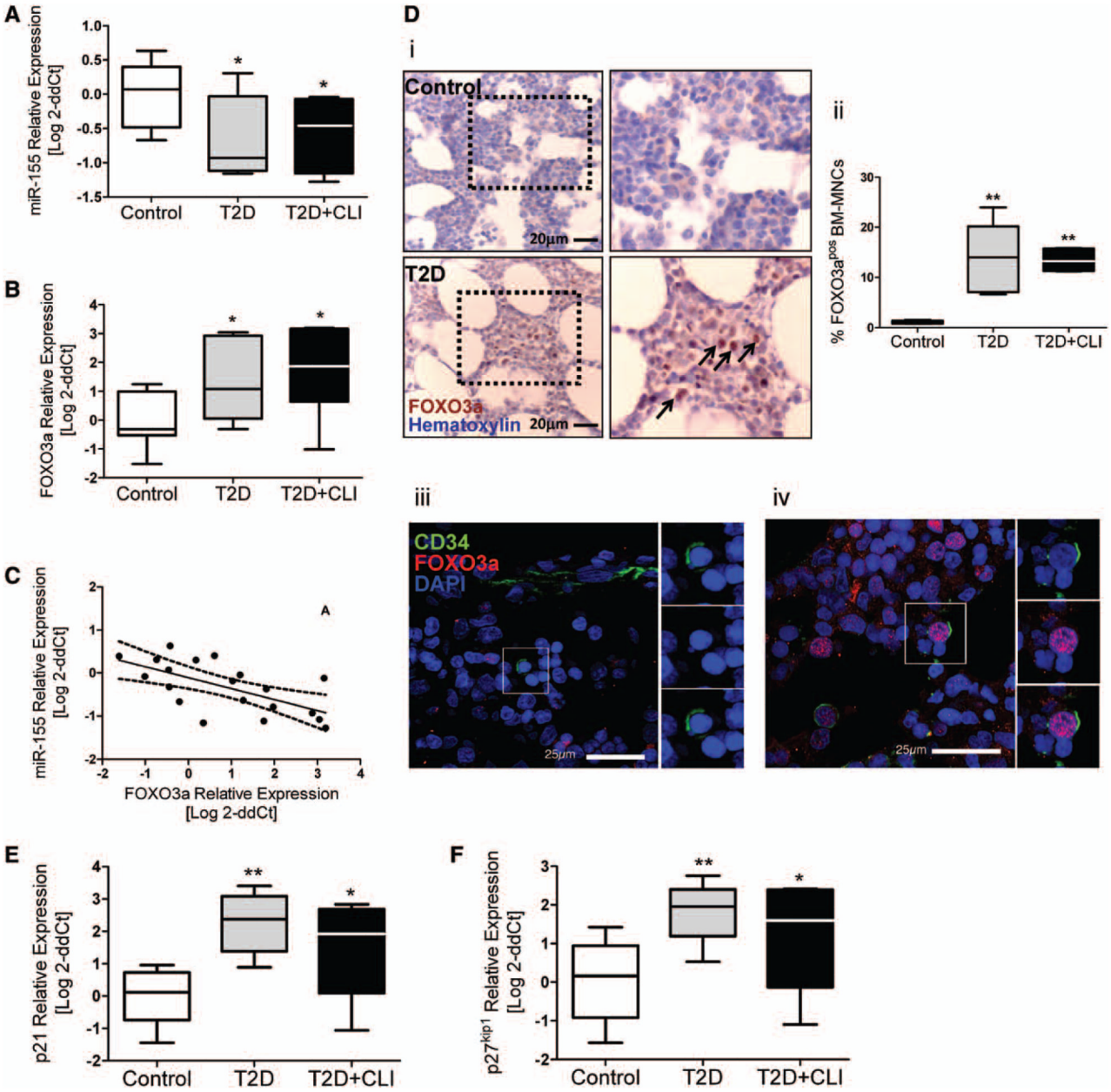

Figure 6. Diabetes mellitus-induced expressional changes in bone marrow (BM) CD34^{pos} cells

A and **B**, Bar graphs showing mRNA levels of microRNA (miR)-155 (**A**) and its target FOXO3a (**B**). Controls, n=10; type-2 diabetes mellitus (T2DM), n=7; T2DM+critical limb ischemia (CLI), n=6. **C**, Graph showing the inverse correlation between miR-155 and FOXO3a mRNA levels in CD34^{pos} cells. **D**, Representative microphotographs (**i**) and bar graph (**ii**) showing the in situ expression of FOXO3a in BM cells. Confocal microphotographs showing FOXO3a (red) localization in the cytoplasm (**iii**) and nucleus (**iv**) of CD34^{pos} cells (green). Nuclei are stained blue with 4′,6-diamidino-2-phenylindole (DAPI). n=5 per group. **E** and **F**, Bar graphs showing mRNA levels of CDKN1A/p21 (**E**) and CDKN1B/p27^{kip1} (**F**). Controls, n=10; T2DM, n=7; T2DM+CLI, n=6. *P<0.05 and **P<0.01 versus controls.

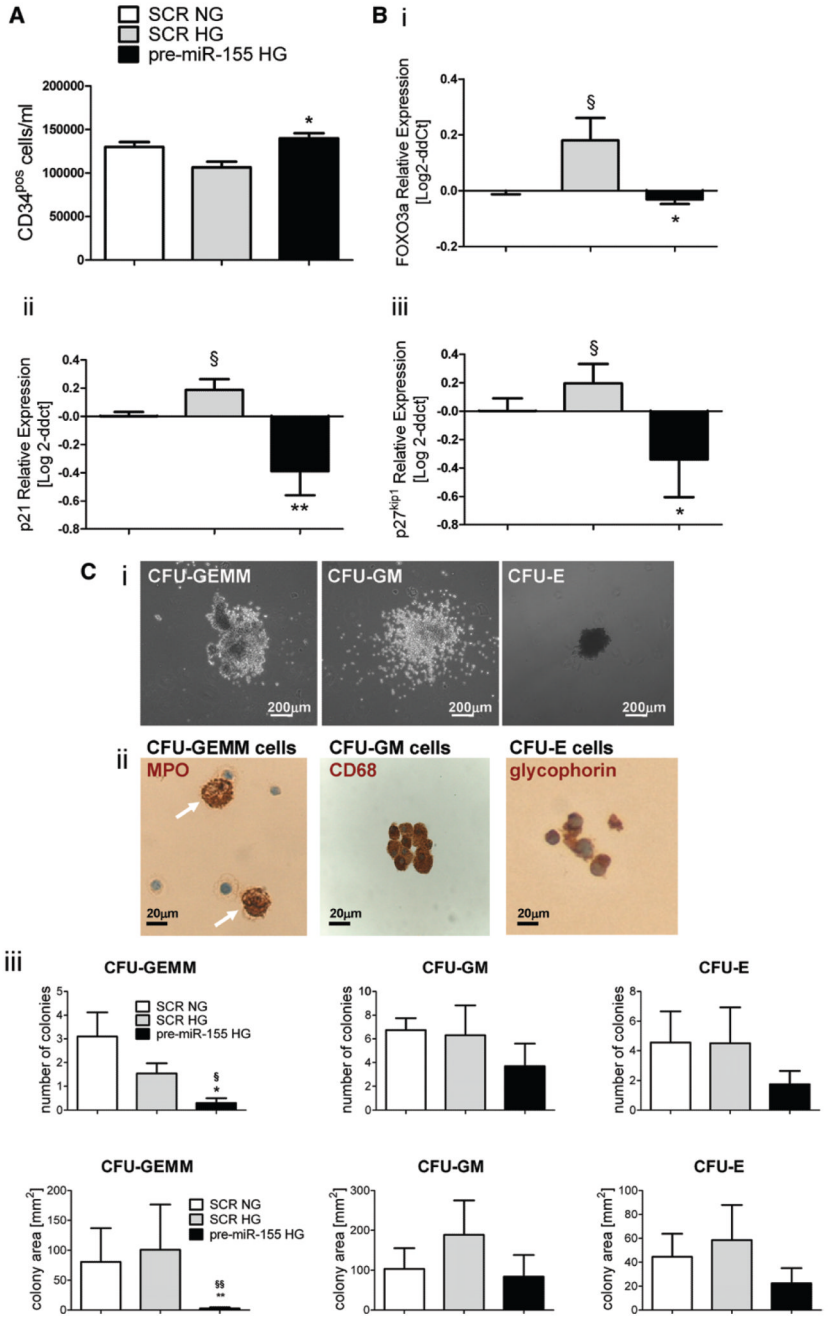

Figure 7. MicroRNA (miR)-155 overexpression in bone marrow (BM) CD34^{pos} cells reverts high glucose (HG)-induced expressional changes and reduces the generation of myeloid and erythroid colonies

A, Bar graphs showing the effect of HG and pre–miR-155 or control scramble (SCR) transfection on CD34^{pos} cell number. *P<0.05 versus SCR HG. Cells were cultured either in normal glucose (NG) (5 mmol/L D-glucose, NG) or HG (25 mmol/L D-glucose, HG) for 48 hours. **B**, Bar graphs showing mRNA levels of (**i**) FOXO3a, (**ii**) CDKN1A/p21, and (**iii**) CDKN1B/p27^{kip1} after pre–miR-155 or control SCR transfection. N=5 healthy donors per group assayed in duplicate. **C**, Colony forming unit (CFU) assay of CD34^{pos} cells. (**i**) Representative images of CFU-granulocytes, erythroid, macrophage, megakaryocyte (CFU-

GEMM), CFU-granulocyte, macrophage (CFU-GM), and CFU-erythroid (CFU-E) colonies. **ii**, Immunocytochemical characterization of CFU-isolated cells by staining for myeloperoxidase (MPO), a marker for granulocytes, CD68, a marker of macrophages, and glyphorin, a marker of erythrocytes. **Arrows** in CFU-GEMM point at MPO^{pos} granulocytes. Nuclei are stained blue with hematoxylin. **iii**, Bar graph showing the effect of miR-155 overexpression on number (top) and area (**bottom**) covered by colonies. N=5 healthy donors per group. **P*<0.05 and ***P*<0.01 versus SCR HG, §*P*<0.05 and §§*P*<0.01 versus SCR NG. Data represent means±SEM.

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