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Tissue Kallikrein Is Essential for Invasive Capacity of Circulating Proangiogenic Cells

Gaia Spinetti, **Orazio Fortunato**, **Daniela Cordella**, **Paola Portararo**, **Nicolle Kränkel**, **Rajesh Katare**, **Graciela B. Sala-Newby**, **Christine Richer**, **Marie-Pascale Vincent**, **Francois Alhenc-Gelas**, **Giancarlo Tonolo**, **Sara Cherchi**, **Costanza Emanueli**, and **Paolo Madeddu**
IRCCS MultiMedica (G.S., O.F., D.C., P.P.), Milan, Italy; University of Bristol (N.K., R.K., G.B.S.-N., C.E., P.M.), United Kingdom; INSERM U652/U872 (C.R., M.-P.V., F.A.-G.), Paris, France; and OC San Giovanni di Dio (G.T., S.C.), Olbia, Italy.

Abstract

Rationale—Homing of proangiogenic cells (PACs) is guided by chemoattractants and requires proteases to disrupt the extracellular matrix. The possibility that PAC recruitment involves an interaction between proteases and chemotactic factor receptors remains largely unexplored.

Objective—To determine the role of human tissue kallikrein (hK1) in PAC invasion and its dependency on kinin receptor signaling.

Methods and Results—Human mononuclear cells (MNCs) and culture-selected PACs express and release mature hK1 protein. HK1 gene (*KLK1*) silencing reduced PACs migratory, invasive, and proangiogenic activities. *KLK1*-knockout mouse bone marrow–derived MNCs showed similar impairments and were unable to support reparative angiogenesis in a mouse model of peripheral ischemia. Conversely, adenovirus-mediated *KLK1* (*Ad.KLK1*) gene transfer enhanced PAC-associated functions, whereas the catalytically inactive variant *R53H-KLK1* was ineffective. HK1-induced effects are mediated by a kinin B_2 receptor (B_2R)-dependent mechanism involving inducible nitric oxide synthase and metalloproteinase-2 (MMP2). Lower hK1 protein levels were observed in PACs from type 2 diabetic (T2D) patients, whereas *KLK1* mRNA levels were similar to those of healthy subjects, suggesting a post-transcriptional defect. Furthermore, B_2R is normally expressed on T2D-PACs but remains uncoupled from downstream signaling. Importantly, whereas *Ad.KLK1* alone could not restore T2D-PAC invasion capacity, combined *KLK1* and *B_2R* expression rescued the diabetic phenotype.

Conclusions—This study reveals new interactive components of the PACs invasive machinery, acting via protease- and kinin receptor–dependent mechanisms.

Keywords

kallikrein–kinin system; angiogenesis; circulating proangiogenic cells; cell invasion

Circulating proangiogenic cells (PACs), a subset of mononuclear cells (MNCs), formerly referred to as endothelial progenitor cells, cooperate with resident endothelial cells (ECs) in the promotion of reparative neovascularization. Protease-rich PACs, at the forefront of the advancing endothelial bud, drill holes in the extracellular matrix for new vascular structures

Correspondence to Paolo Madeddu, Chair of Experimental Cardiovascular Medicine, University of Bristol, Bristol BS28HW, United Kingdom. madeddu@yahoo.com.

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to expand and liberate extracellular matrix–bound growth factors and cytokines instrumental to tissue remodeling.[1] A reduction in the expression of metalloproteinase (MMP)9 and cathepsin L reportedly contributes to the impaired invasive capacity of PACs in diabetes.[2] The role of serine proteases in PAC-associated functions remains undefined.

Components of the kallikrein–kinin system (KKS), including the serine protease human tissue kallikrein (hK1), the substrate kininogen, and kinin receptors B_2 (B_2R) (constitutive) and B_1 (B_1R) (inducible), are expressed in vascular cells and leukocytes.[3] Genetic defects of the KKS result in impairment of reparative neovascularization and attenuation of inflammatory response.[4–8] Forced hK1 gene (*KLK1*) expression, but not its loss-of-function polymorphic variant *R53H-KLK1*, induces capillary and arteriole growth by kinin receptor–dependent mechanisms.[6,9,10] Noteworthy, hK1 may also elicit angiogenic and cardioprotective actions through the B_2R via a kinin-independent mechanism.[4,11,12]

Recent studies identified a role for B_2R and downstream phosphoinositide 3-kinase (PI3K)γ/Akt promigratory signaling in the recruitment of distinct populations of proangiogenic cells.[13–15] Here, we newly show that hK1 and B_2R constitute essential cooperative elements of PACs invasive machinery.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Human Studies

Online Table I summarizes diabetic patients and healthy control subjects characteristics.

Experimental Animals

KLK1-knockout mice ($KLK^{-/-}$) were generated by targeted gene inactivation[16] and backcrossed to a pure C57/BL6 genetic background. Six-month-old male $KLK^{-/-}$ mice from heterozygous crossing and wild-type (WT) littermates were studied.

Cell Isolation and Characterization

MNCs were isolated and PACs enriched as described previously.[17] Antigenic profile was assessed using a FACS Canto flow cytometer and FACS Diva software (Becton, Dickinson and Company). Migration, invasion, and networking assays were performed as described.[13,18]

Adenoviral Vectors and KLK1 RNA Silencing

Adenoviral vectors carrying *KLK1* or *R53H-KLK1* were generated as described.[4] *Ad.B$_2$R* was prepared by subcloning *B$_2$R* gene into a modified pDC515 shuttle vector (Microbix) followed by site-specific recombination of shuttle and genomic plasmids in 293 cells. For *KLK1* knockdown, specific small interfering RNA probes were obtained from Dharmacon.

Reverse Transcription–Polymerase Chain Reaction

RNA was extracted using TRIzol reagent (Invitrogen), reverse-transcribed, and amplified using standard techniques.

Protein Levels and Activity

Immunoreactive hK1 and bradykinin (BK) were measured by ELISA.[9] HK1 enzymatic activity was assayed using colorimetric substrate S-2266 (Kabi Diagnostica). Western blotting (WB) and both in situ- and PAGE-zymographies were performed as described.[18,19]

Immunocytochemistry

Samples were fixed, incubated with appropriate primary and secondary, fluorescence-conjugated antibodies, and microphotographs were acquired by a computer-imaging program.

Statistical Analysis

All results are expressed as means±SEM. Student *t* test was used for 2-group comparisons and ANOVA, followed by Bonferroni post hoc test, was used for multiple comparisons. A probability value of <0.05 was taken as statistically significant.

Results

Human PACs Express KLK1 mRNA and hK1 Protein

Q-RT-PCR showed that peripheral blood (PB) MNCs and culture-enriched PACs express *KLK1* mRNA (Figure 1A). Flow cytometry confirmed hK1 expression on distinct PB-MNC subpopulations, including $CD34^{pos}$ cells (30±5%), $CD19^{pos}$ B lymphocytes (23±7%) and $CD3^{pos}$ T lymphocytes (10±1%) (Figure 1B, i). HK1 was also abundant in "nonclassic" $CD16^{pos}/CD14^{low}$ monocytes (43±5%), but less in $CD16^{pos}/CD14^{high}$ (9±3%) and $CD16^{neg}/CD14^{high}$ monocytes (1.0±0.3%) (Figure 1B, ii). Of note, MNCs cultured in the presence of hK1 inhibitor kallistatin lead to significantly lower PACs yields (P<0.05 versus control; Figure 1C), suggesting a role of hK1 in the acquisition of PAC phenotype by MNCs. However, a cooperative contribution of hK1-negative MNCs in PAC formation cannot be excluded. In fact, immunocytochemistry demonstrated that PACs comprise both hK1 positive (60±19%) and hK1 negative elements (Figure 1D). Flow cytometry identified hK1 expression on both PACs cell membrane and cytosol (Figure 1E). Moreover, PACs secrete hK1 as documented by measuring immunoreactive protein (ELISA) and enzymatic activity (amidolytic assay) in conditioned medium (Figure 1F). We verified that PACs bind UEAI (*Ulex europaeus* I lectin), take up acetylated low-density lipoprotein (data not shown), and coexpress markers of endothelial/progenitor (CD34, CXCR4, and KDR) and hematopoietic cells (CD45, CD14, CD11b) (Figure 1G).

KLK1 Silencing Reduces PACs Invasive and Proangiogenic Capacities

To investigate endogenous hK1 functional relevance, human PACs were transfected with a pool of 4 specific small interfering *KLK1* RNAs (siKLK1) (50 to 100 nmol/L) or scrambled sequences (control). Efficient *KLK1* silencing was confirmed by reduction of mRNA, protein levels and enzymatic activity (P<0.05 versus control for all comparisons; Figure 2A). Of note, *KLK1* silencing reduced PAC migration and invasion abilities (Figure 2B) as well as their capacity to stimulate human umbilical vein endothelial cells (HUVECs) networking on Matrigel (Figure 2C; P<0.05 versus control for all comparisons). HK1 has been implicated in the activation of MMPs,[4] crucial factors for PACs invasive activity.[20] Using gel zymography, we observed reduced MMP2 gelatinolytic activity in siKLK1-PAC CM compared to control (P<0.05; Figure 2D).

HK1 Overexpression Enhances the Invasive and Proangiogenic Capacities and MMP2 Activity of PACs

Next, we investigated whether hK1-forced expression enhances PACs functions. HK1 overexpression after *Ad.KLK1* infection was confirmed using Q-RT-PCR and WB of PACs lysates and by ELISA and amidolytic assay on PAC CM ($P<0.05$ versus *Ad.Null* for all comparisons; Online Figure I, A through D). Increased hK1 expression did not result however in increased levels of BK, the product of kininogen cleavage by hK1, in *Ad.KLK1*-PAC CM (Online Figure I, E).

Forced hK1 expression conferred PACs with increased spontaneous motility (1.6-fold), invasive capacity (1.8-fold) and proangiogenic activity (1.4-fold) ($P<0.05$ versus *Ad.Null* for all comparisons; Figure 3A and 3B). In contrast, those functions remained unaltered in PACs infected with the catalytically defective polymorphic variant *R53H-KLK1* (P=NS versus *Ad.Null*; Figure 3A and 3B). Furthermore, *KLK1*-forced expression did not modify PACs ability to migrate toward SDF-1α and BK (data not shown).

To verify hK1/MMP2 functional association, we used both gel- and in situ-zymography, the latter using a FITC-conjugated gelatin system. *Ad.KLK1*-PACs showed enhanced MMP2 activity in both assays ($P<0.05$ versus *Ad.Null* or *Ad.R53H*; Figure 3C and 3D).

PACs Functional Enhancement by hK1 Is Mediated by B_2R- and MMP-Dependent Mechanisms

For mechanistic interpretation of hK1-induced effects, we used hK1 and MMPs inhibitors and B_2R signaling antagonists. Enhanced *Ad.KLK1*-PAC migration and invasion were both abrogated by the serine protease inhibitor aprotinin, the specific hK1 inhibitor kallistatin or the B_2R antagonist icatibant ($P<0.05$ versus *Ad.KLK1*-PACs exposed to inhibitor vehicle (ctr) and P=NS versus *Ad.Null*-PACs; Figure 4A, i and ii). Consistent with the data from icatibant, the B_2R-selective antagonist MEN16132 (MEN), significantly inhibited the hK1-induced PACs migratory activity (Figure 4A, iii). No inhibition was instead seen after B_1R blockade with L-dAL-BK (P=NS versus ctr and $P<0.05$ versus *Ad.Null*-PACs). Of note, both kallistatin and icatibant effectively decreased hK1-dependent MMP2 activation (Figure 4B, i). In addition, hK1-transduced PACs invasive capacity was significantly reduced by MMP inhibitor, GM6001 ($P<0.05$ versus ctr and P=NS versus *Ad.Null*-PACs; Figure 4B, ii).

B_2R-dependent activation of PAC migration is reportedly dependent on the PI3K/protein kinase B (PKB/Akt) signaling pathway.[13] We therefore investigated whether hK1-forced expression triggers a similar signaling mechanism. Akt phosphorylation (WB) and activity (ELISA) were both increased in *Ad.KLK1*-PACs, with this effect being blocked by icatibant ($P<0.05$ versus ctr and P=NS versus *Ad.Null*-PACs; Figure 4C, i and ii). Unexpectedly, we did not find any change in the phosphorylation levels of eNOS (endothelial nitric oxide synthase) (at Ser1177 or Ser113) in *Ad.KLK1*-PACs (data not shown). At variance, we found inducible (i)NOS to be increased following *Ad.KLK1* infection ($P<0.05$ versus *Ad.Null*; Figure 4C, iii). Furthermore, *Ad.KLK1*-PACs enhanced invasive capacity was significantly blunted by the PI3Kγ inhibitor AS605240,[14,15] the NOS inhibitor N5-(1-Iminoethyl)-$_L$-ornithine dihydrochloride (LNIO)[21] or the nitric oxide (NO) scavenger 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO)[22] ($P<0.05$ versus ctr and P=NS versus *Ad.Null*-PACs; Figure 4C, iv).

Finally, *Ad.KLK1*-PACs ability to promote HUVEC networking was abolished by aprotinin and kallistatin, but preserved following B_2R or B_1R blockade or MMP inhibition (Figure 4D). In control experiments, BK (3×10^{-8} mol/L) did not affect HUVEC networking in the absence of PACs but increase network formation by 1.7-fold when PACs and HUVECs

were cocultured, this effect blocked by icatibant (data not shown). Altogether, these data suggest that, although potentially responsive to kinin stimulation, *Ad.KLK1*-PACs may promote endothelial networking by hK1 direct effect on HUVECs or via proteolytic degradation of Matrigel.

Impaired Motility of KLK1-Knockout Lin^{neg} MNCs and PACs

Next, we used null-mutant mice to further verify the importance of endogenous hK1 in MNC and PAC motility. Bone marrow (BM) lineage negative (Lin^{neg}) MNCs and PACs of wild type (WT) or *KLK*$^{-/-}$ mice were tested for migratory and proangiogenic activities. *KLK*$^{-/-}$Lin^{neg}MNCs and PACs showed normal spontaneous migration but were unresponsive to SDF-1α (P<0.05 versus WT; Figure 5A and 5B). Furthermore, *KLK*$^{-/-}$ PACs were less potent in promoting HUVEC networking (Figure 5C; P<0.05 versus WT). Of note, *Ad.KLK1* increased *KLK*$^{-/-}$ PACs spontaneous and SDF-1α–stimulated migration (P<0.05 versus *Ad.Null*; Figure 5B). In contrast, in WT PACs, *Ad.KLK1* supported spontaneous (P<0.05 versus *Ad.Null*) but not SDF-1α–stimulated migration suggesting saturation of the cell migratory capacity (P=NS versus *Ad.Null*; Figure 5B). Finally, *Ad.KLK1* increased *KLK*$^{-/-}$ PACs supporting action on HUVEC network formation, although to a lesser extent when compared to WT PACs (P<0.05 versus *Ad.Null*; Figure 5C).

KLK1 Gene Deletion Impairs In Vivo Proangiogenic Action of BM-MNCs

Next, we evaluated whether *KLK1* gene deletion negatively impacts on reparative angiogenesis. WT or *KLK*$^{-/-}$ BM-MNCs were intravenously injected in WT mice, 1 day after operative induction of unilateral limb ischemia. We observed a significant treatment-related effect on superficial blood flow recovery as assessed by Doppler flowmetry (P<0.01, ANOVA with repeated measurements). Post hoc analysis revealed that perfusion was improved by WT BM-MNCs but not by *KLK*$^{-/-}$ BM-MNCs as compared with vehicle (Figure 6A). Similarly, adductor muscle blood flow measurement using fluorescent microspheres denoted a significant improvement at 3 weeks with WT BM-MNCs, but not with *KLK*$^{-/-}$ BM-MNCs, over the vehicle (Figure 6B).

Immunofluorescence staining showed that capillary and arteriole density in the ischemic adductor muscle was significantly higher in WT BM-MNC–injected group compared with vehicle- or *KLK*$^{-/-}$ BM-MNC–treated groups (Figure 6C).

KLK1 Downregulation in Type 2 Diabetic PACs

PACs functionality is impaired in diabetes, but underpinning mechanisms remain largely undefined.[23] Because gene titration studies showed an effect of hK1 on distinct PACs activities, we next asked whether a defect in hK1 expression/activity is implicated in type 2 diabetes (T2D)-PACs dysfunctional phenotype. Consistently, we found that diabetes reduces the abundance of hK1^{pos} cells within the PB-MNC pool, particularly in the CD34^{pos} subfraction. The intensity of the fluorescent signal was diminished in all studied populations (Online Figure II, A). These changes were seen in the context of an overall decrease of specific PB-MNC subpopulations (Online Figure II, B) and were mirrored by a remarkable reduction in EC- and monocyte-specific markers, ie, CD34, KDR, CXCR4, and CD14, on T2D-PACs compared with PACs from age-matched healthy controls (Online Figure II, C). Of note, whereas *KLK1* mRNA levels were comparable in T2D- and healthy PACs, hK1 protein levels were significantly reduced in T2D-PACs (−57%, P<0.05 versus healthy PACs; Figure 7A, i and ii), suggesting a post-transcriptional defect in hK1 synthesis. We next investigated microRNA (miR)-637, the only miR potentially implicated in inhibition of hK1 protein synthesis according to bioinformatics screening. However, we found no difference in mir637 expression between healthy and T2D-PACs (Figure 7A, iii).

KLK1 and B_2R Genetic Engineering Rescues T2D-PACs Invasive Capacity

T2D-PACs manifested a remarkable reduction in migratory, invasive and network-forming potential (P<0.05 versus healthy PACs for all comparisons; Figure 7B and 7C), in line with previous reports.[24,25] *Ad.KLK1* infection of T2D-PACs resulted in a marked increase in *KLK1* mRNA and secreted hK1 protein, similar to that of healthy *Ad.KLK1*-PACs (data not shown). *Ad.KLK1* was able to enhance healthy PACs functions (P<0.05 versus *Ad.Null*), but failed to correct the defects observed in T2D-PACs (P=NS versus *Ad.Null*) (Figure 7B and 7C). Furthermore, *Ad.KLK1* failed to increase MMP2 activity in T2D-PAC CM (Figure 7D).

To determine the missing element accountable for the "diabetic block," we investigated the status of B_2R in healthy and T2D-PACs. Flow cytometric analysis did not detect significant differences in B_2R expression between the 2 groups (Figure 8A, i). Nevertheless, B_2R-associated biological functions were remarkably impaired in diabetes. In fact, stimulation with the B_2R ligand BK failed to increase T2D-PAC invasion capacity compared to healthy PACs, with this deficit being associated with reduced Akt activity (Figure 8A, ii and iii).

Ad.B_2R infection resulted in B_2R increased mRNA and protein expression in PACs (Figure 8B and 8C). Both *Ad.KLK1* and *Ad.B_2R* increased the invasive capacity of healthy PACs (*P<0.05 versus *Ad.Null*), with no additive effect after combined infection (Figure 8D). In diabetes, neither *Ad.KLK1* nor *Ad.B_2R* alone improved PAC invasiveness, but in combination they remarkably rescued the diabetic phenotype (P<0.05 versus *Ad.Null* +*Ad.Null*; Figure 8D), thus suggesting a cooperative hK1-B_2R interaction.

Discussion

Classic chemoattractants/receptors pairs, like SDF-1α/CXCR4 and vascular endothelial growth factor/KDR, have been recently joined by the kinin/B_2R pair as a potent mediator of PACs chemotaxis.[13] Furthermore, PACs use proteases, like MMPs and cathepsins, to disrupt the extracellular matrix in the initiation of reparative neovascularization.[2,3,26] However, the possibility that proteases could cooperate with chemokine receptors during PACs tissue invasion remains largely unexplored. Here, we reveal that hK1 coupling to B_2R signaling is essential for PACs invasive capacity.

Despite persisting ambiguity about the precise circulating angiogenic/endothelial progenitors characteristics, CD34 is widely used for proangiogenic cells separation in human clinical trials.[27] Furthermore, CD34 progenitors level strictly correlates to cardiovascular risk, especially in patients with metabolic syndrome or diabetes.[23] Here, we demonstrate for the first time that, among freshly isolated MNCs, $CD34^{pos}$ cells represent the subpopulation that most abundantly bears membrane-bound hK1. Furthermore, hK1 was abundantly expressed by nonclassic $CD16^{pos}/CD14^{low}$ monocytes compared to $CD16^{neg}/CD14^{high}$ monocytes. The respective role of these 2 populations in postischemic reparative processes is matter of debate.[28] The presence of hK1 in $CD16^{pos}$ monocytes might add to their angiogenic potential, however this remains to be explored. Interestingly, B_2R is also highly abundant in $CD34^{pos}$ cells,[13] and CD16 monocytes.[29] HK1/B_2R coexpression on specific progenitors and monocyte fractions suggests that KKS components might cooperate during recruitment and homing. Importantly, hK1 inhibition by kallistatin results in remarkable reduction of PACs enrichment from cultured MNCs, in line with our previous observation of reduced $Flk1^{pos}cKit^{pos}$ progenitors production in BM of *KLK1*$^{-/-}$ mice following limb ischemia induction.[4] Thus, hK1 might be a potential biomarker of MNCs differentiation into cellular phenotypes implicated in the modulation of vascular functions.

HK1 relevance in PACs functions was strengthened using silencing and viral vector–mediated overexpression approaches. Importantly, *KLK1* gene titration data indicate that PAC migration, invasion, and proangiogenic activities concordantly increase with hK1 levels. The most acknowledged pathway mediating PAC migration centers on the kinase Akt. B_2R and CXCR4 receptors engagement by kinin and SDF-1α on human PACs is associated with polarized recruitment and activation of PI3Kγ on the cell membrane, phosphorylation/activation of Akt and acquisition of a migratory phenotype.[13,30] Of note, hK1 overexpression activates Akt in PACs and B_2R blockade by icatibant suppresses Akt activation and invasive capacity of hK1-transduced PACs. Surprisingly, neither eNOS protein expression nor activation by phosphorylation on the 2 known regulating serine residues (Ser113 and Ser1177) was modulated by hK1-forced expression. Conversely, we here report for the first time that NOS inducible form, iNOS, is indeed constitutively expressed by PACs and enhanced by hK1 transduction. Furthermore, we show that NOS inhibition or NO scavenge both abrogate the enhanced invasive capacity of hK1-transduced PACs. This data indicates that PACs are equipped with sensors, eg, the B_2R, to capture chemokine guidance signals, and enzymatic machinery, eg, hK1, coupled to B_2R to enhance PAC motility. Furthermore, PACs-secreted hK1 could modify the extracellular environment and thus facilitate PACs and vascular cells tissue invasion. This latter mechanism is seemingly B_2R-independent because kallistatin but not icatibant is able to block hK1-transduced PACs promoting EC networking on Matrigel. It would be interesting to verify whether the same signaling mechanism drives the homing of proinflammatory cells in ischemic tissues.

Furthermore, we found that hK1 overexpression remarkably activates MMP2 through the B_2R. Another MMP, MMP9, was shown to act as a downstream modulator of hK1 angiogenic activity in ischemia.[4] It is likely that the interaction of hK1 with different MMPs is context-dependent. For example, unlike MMP9, MMP2 is activated under nonischemic conditions, such as aging-related vascular remodeling.[31]

To gain insight into *KLK1* inherited defects, we verified the functional phenotype of BM-MNCs and PACs from *KLK1*$^{-/-}$ mice. These mutant mice are reportedly unable to mount proper reparative angiogenesis and to mobilize Lin^{neg} progenitors into the circulation after ischemia.[4]

Our present data show for the first time that *KLK1*$^{-/-}$ PACs are defective in their proangiogenic function, with such functional defect being ameliorated by hK1 transduction. In a mouse model of unilateral limb ischemia, *KLK1*$^{-/-}$ BM-MNCs failed to stimulate vascular repair thus highlighting hK1 essential role in promotion of postischemic neovascularization. Furthermore, we found that engineering human PACs with wild type *KLK1* improves their invasive and proangiogenic capacities, whereas gene transfer with the loss-of-function polymorphic *R53H-KLK1* variant[32] is ineffective. This data complements previous work from our group demonstrating altered angiogenic/arteriogenic capacity of *R53H-KLK1* in limb ischemia model.[4] *R53H-KLK1* polymorphism is especially common in Afro-Caribbean individuals, but also present as a heterozygous trait in 7% of whites. It would be of paramount importance to verify whether subjects carrying *R53H-KLK1* mutation are prone to more complicated postischemic outcomes because of reparative angiogenesis defects.

Finally, we investigated whether hK1 is implicated in T2D-PACs dysfunction.[25,33] When comparing T2D- and healthy PACs, we found an unexpected diversity in EC- and monocyte-specific surface markers expression, with decreased $CD34^{pos}$, KDR^{pos}, $CXR4^{pos}$, and $CD14^{pos}$ subsets in diabetes. Because these antigens are at least in part associated with highly chemotactic, proangiogenic MNCs, our results provide a possible key of

interpretation for the reduced migratory and proangiogenic activity of T2D-MNCs. Of note, typical T2D-PACs dysfunction was associated with decreased hK1 protein, but normal *KLK1* mRNA levels. This result points at a post-transcriptional alteration of hK1 in diabetes, in agreement with data showing that high glucose decreases PACs-cathepsin L activity without affecting its mRNA levels.[2] Different factors could cooperate to modulate hK1 expression in diabetes; ie, glycoxidation could interfere with protein translation/degradation processes.[34] In addition, miRs have been implicated in gene expression inhibition under different disease conditions, including diabetes.[35] Of note, *KLK1* does not possess a strongly predicted interacting miR, but bioinformatics analysis showed miR637 to be associated, even if only poorly conserved.[36] However, we could not find any change of miR637 expression in T2D-PACs, thus further studies are needed to unravel the post-transcriptional mechanism of hK1 downregulation.

Surprisingly, hK1-forced expression could not revert the defective phenotype of T2D-PACs. Investigating possible causes for this failure, we found that the B_2R is normally expressed on T2D-PACs, however it remains uncoupled from its downstream effector Akt. Noteworthy, combined hK1-B_2R transduction did effectively rescue the impaired T2D-PACs invasive capacity. These data, supporting a key role of B_2R in hK1-associated induction of PACs motility, introduces the new concept that implementation of functionally imperfect receptors by gene therapy could provide a means to restore proper coupling between components of the invasive machinery. It remains to be determined whether re-exposing transduced PACs to the diabetic environment may jeopardize their restored invasive function.

Taken together our results show for the first time that human PACs are equipped with active endogenous hK1 and that hK1 is crucial for PAC invasive and proangiogenic activities by both protease- and kinin receptor–mediated mechanisms. Importantly, PACs biological functions are impaired in conditions of hK1 deficit and restored by forced hK1 expression. Finally, this study demonstrates that KKS faulty components need to be repaired to restore the pristine PACs function in diabetes, thus providing the first proof of concept for multiple gene engineering of a pathway crucially implicated in angiogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

Ad	adenoviral vector
BM	bone marrow
BK	bradykinin
B1R	kinin B1 receptor
B2R	kinin B2 receptor
CM	conditioned medium

EC	endothelial cell
eNOS	endothelial nitric oxide synthase
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
hK1	human tissue kallikrein
HUVEC	human umbilical vein endothelial cell
iNOS	inducible nitric oxide synthase
KLK1	hK1 gene
***KLK*$^{-/-}$**	*KLK1*-knockout mice
KKS	kallikrein–kinin system
Lin^{neg}	lineage negative
miR	microRNA
MMP	matrix metalloproteinase
MNC	mononuclear cell
NOS	nitric oxide synthase
PAC	circulating proangiogenic cell
PB	peripheral blood
PI3K	phosphoinositide 3-kinase
T2D	type 2 diabetes
Q-RT-PCR	quantitative RT-PCR
SDF	stromal cell-derived factor
WB	Western blot
WT	wild type

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Novelty and Significance

What Is Known?

- Proangiogenic cells contribute to extracellular matrix remodeling during tissue healing. This mechanism is impaired by diabetes.
- Kinin is a potent chemoattractant for circulating leukocytes and proangiogenic cells.

What New Information Does This Article Contribute?

- Distinct subpopulations of circulating mononuclear cells, including $CD34^{pos}$ cells and $CD16^{pos}/CD14^{low}$ monocytes, express kallikrein, a kinin-generating enzyme.
- Gene titration studies indicate that kallikrein levels are relevant for migration, invasion, and promotion of endothelial network formation activities of human proangiogenic cells.
- Enhancement of proangiogenic cell activities by kallikrein overexpression implicates a mechanism encompassing the kinin B2 receptor, phosphoinositide 3-kinase (PI3K), Akt, and inducible nitric oxide synthase (iNOS).
- Proangiogenic cells that express an enzymatically inactive polymorphic variant of the kallikrein gene or an epigenetically modified version of the wild-type gene are defective in their invasive capacity.
- Functionally defective diabetic proangiogenic cells are rescued by combined transfer of kallikrein and kinin B2 receptor genes.

Blood-borne cells participate in the remodeling of an injured tissue by guiding the formation of new vessels. Invading cells use proteases to drill holes in the extracellular matrix for provisional vessels to expand and organize in a network. Findings of this study indicate that human proangiogenic cells are equipped with sensors to capture chemokine guidance signals and with enzymatic machinery, eg, kallikrein, which by coupling to kinin B2 receptor, triggers the PI3K-Akt-iNOS pathway to boost cell motion. We show that engineering human proangiogenic cells with wild-type kallikrein gene improves invasive and proangiogenic capacities, whereas gene transfer with the loss-of-function polymorphic *R53H* variant is unproductive. Finally, this study demonstrates that diabetes induces epigenetic modifications in the motility program and that this requires correction of multiple faulty components for functional rescue.

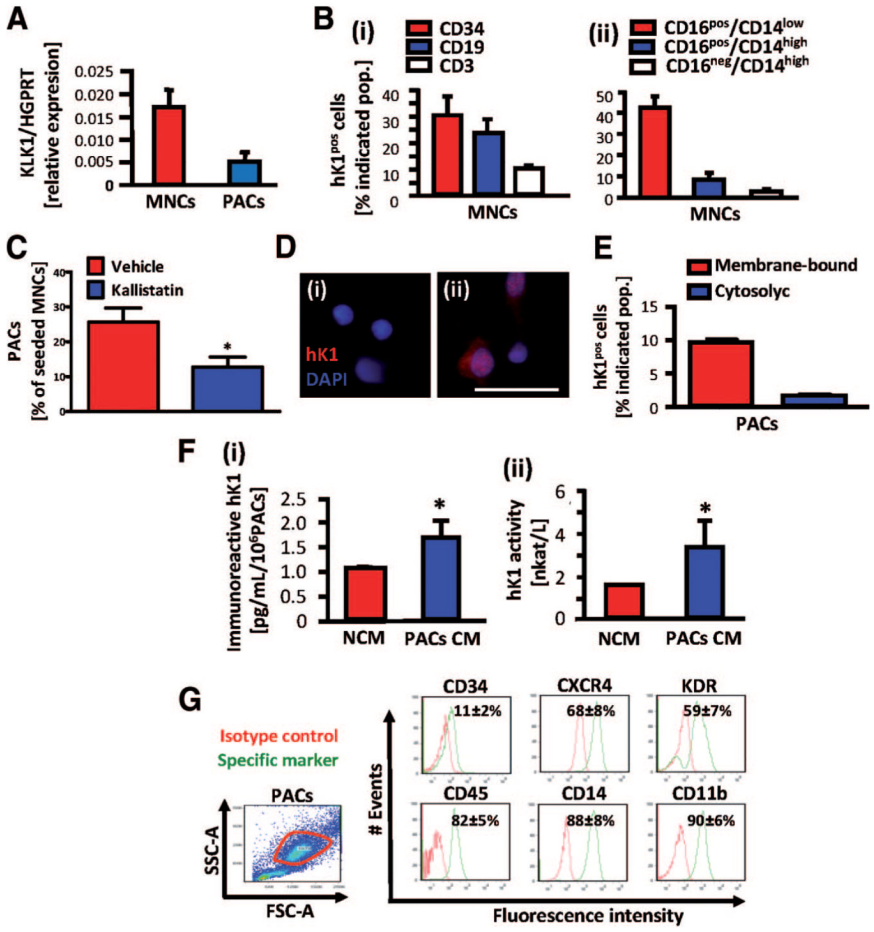

Figure 1. Human PB-MNCs and PACs express *KLK1* gene and hK1 protein

A, Q-RT-PCR *KLK1* expression analysis normalized to hypoxanthine-guanine phosphoribosyltransferase (HGPRT) (n=3 assayed in triplicate). **B,** Flow cytometric quantification of hK1-expressing MNCs (n=4 to 7). **C,** Bar graph shows reduced PACs arising from kallistatin-cultured MNCs (**P*<0.05 vs vehicle, n=4). **D,** PACs stain positive for hK1: TRITC-conjugated secondary antibody only **(i)**; and specific antihK1 antibody plus TRITC-conjugated secondary antibody **(ii)**. **Scale bar,** 20 μm. **E,** Flow cytometric analysis of hK1-expressing PACs on cell membrane or cytosol. **F,** Immunoreactive hK1 in PACs conditioned medium (PAC CM) and nonconditioned medium (NCM) assessed by ELISA **(i)**; and PAC CM hK1 enzymatic activity by amidolytic assay **(ii)**. (**P*<0.05 vs NCM, n=3). **G,** PAC antigenic profile: representative scattergrams and average data (n=3).

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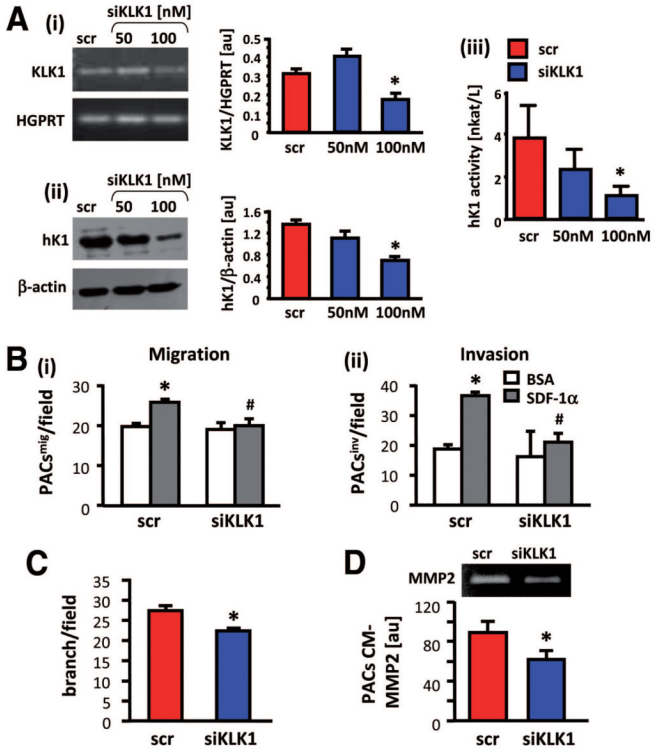

Figure 2. *KLK1* silencing impairs PACs function

A, Representative gels and bar graph showing reduction of *KLK1* mRNA **(i)** and protein levels **(ii)** in siKLK1 RNA (siKLK)-transfected PACs compared with scramble small interfering RNA (scr). Normalization: HGPRT and *β*-actin. **iii,** Bar graph showing reduced hK1 amidolytic activity in siKLK-PAC CM (**P*<0.05 vs scr, n=3). **B,** Bar graph showing the effect of *KLK1* silencing on PAC migration **(i)** and invasion **(ii)**; average number of migrated (PAC-s^{mig}) and invaded PACs (PACs^{inv}) is shown (**P*<0.05 vs BSA and #*P*<0.05 vs SDF-1α–stimulated scr-PACs, n=3). **C,** Reduced HUVEC networking in the presence of siKLK1-PACs (**P*<0.05 vs scr-PACs, n=3). **D,** Representative gel zymography and bar graph showing reduced MMP2 activity in siKLK1-PAC CM (**P*<0.05 vs scr-PACs, n=3).

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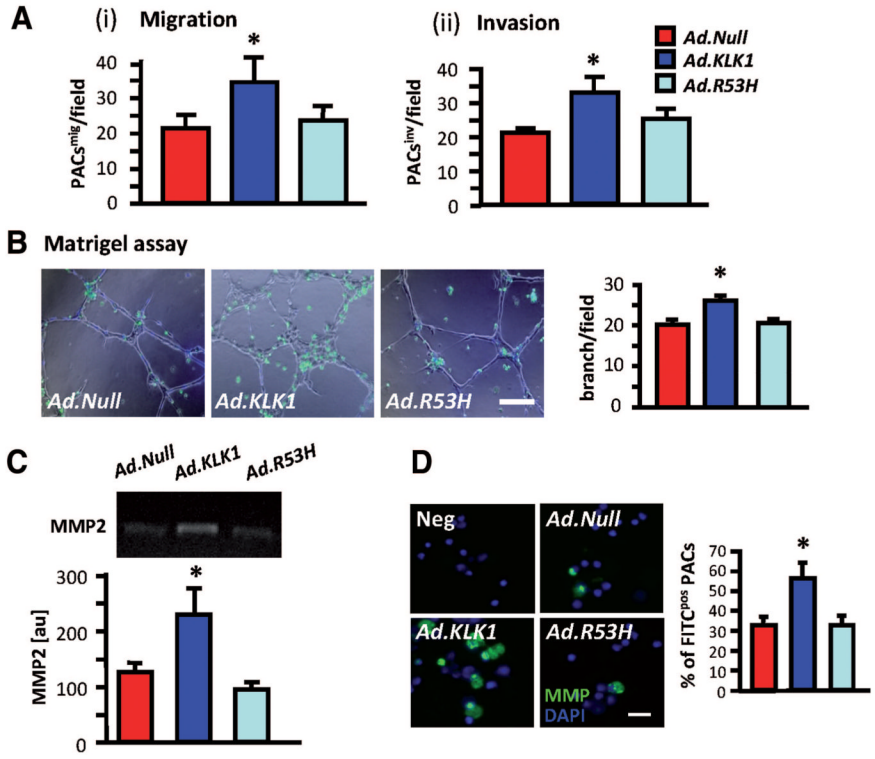

Figure 3. *KLK1* overexpression enhances PACs functions

A, Increased migratory (PACs^{mig}) **(i)** and invasive activity (PACs^{inv}) **(ii)** of *Ad.KLK1*-infected PACs (**P*<0.05 vs *Ad.Null* or *Ad.R53H*, n=4 to 6). **B,** Representative photographs and bar graph showing increased HUVEC networking in the presence of *Ad.KLK1*-PACs (**P*<0.05 vs *Ad.Null* or *Ad.R53H*, n=4). **Scale bar**, 80 μm. **C,** MMP2 activity measured by gel zymography in PAC CM: representative experiment and bar graph showing densitometry data (**P*<0.05 vs *Ad.Null* or *Ad.R53H*; n=3). **D,** In situ zymography analysis of MMP2 activity: representative microphotographs and bar graph showing abundance of FITC^{pos} PACs (**P*<0.05 vs *Ad.Null* or *Ad.R53H*, n=3). In negative control (Neg), FITC-conjugated reagent was omitted. **Scale bar,** 20 μm.

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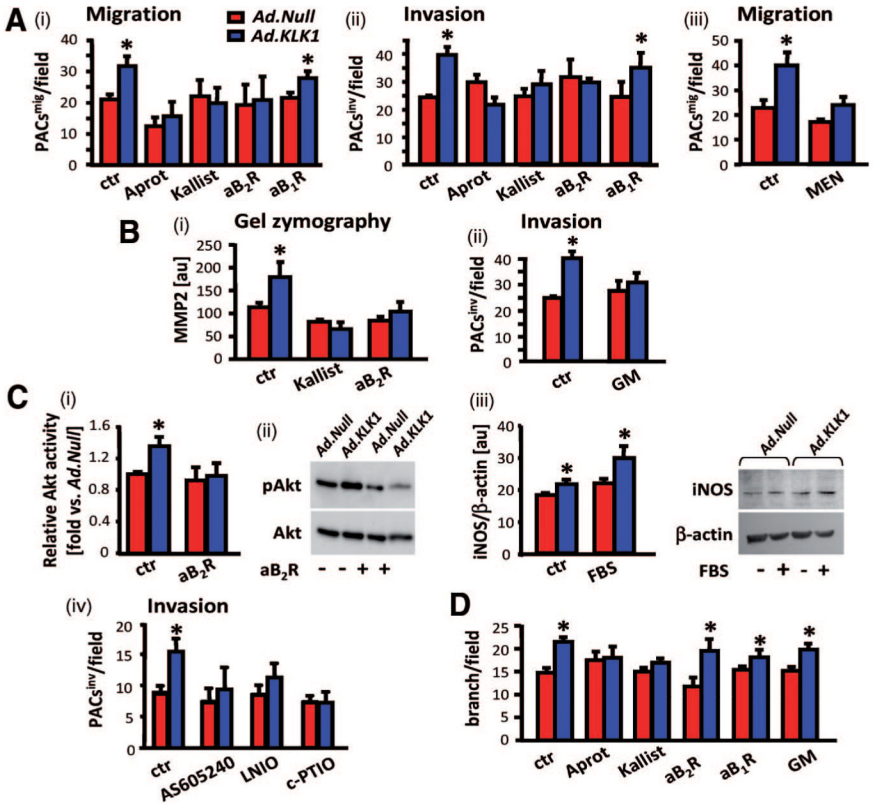

Figure 4. Mechanisms of hK1-induced effects

A, Migration **(i)** and invasion **(ii)** is increased in *Ad.KLK1*-PACs (control, ctr) (**P*<0.05 vs *Ad.Null*) and inhibited by aprotinin (Aprot) (10^{-9} mol/L), kallistatin (Kallist) (10^{-9} mol/L) or icatibant (aB^2R, 2×10^{-7} mol/L), but not by L-dAL-BK (aB_1R, 3×10^{-8} mol/L) (n=4 in triplicate). **iii**, B_2R antagonist MEN16132 (MEN) (10^{-6} mol/L) inhibitory effect on PAC migration (**P*<0.05 vs *Ad.Null*, n=3 in triplicate). **B,** MMP2 activity (gel zymography) is increased in *Ad.KLK1*-PAC CM (**P*<0.05 vs *Ad.Null*) and inhibited by kallistatin and icatibant (n=3) **(i)** and increased *Ad.KLK1*-PAC invasion is inhibited by MMP inhibitor GM6001 (GM) (15×10^{-3} mol/L) (n=3) **(ii)**. **C,** Bar graph showing Akt activity (ELISA) **(i)** and representative WB of phosphorylated and total Akt in PACs lysates **(ii)**. *Ad.KLK1*-induced Akt activation (**P*<0.05 vs *Ad.Null*) is inhibited by icatibant (2×10^{-7} mol/L) (n=3). **iii**, iNOS protein levels in PACs lysates: bar graph showing densitometry data and representative WB (**P*<0.05 vs *Ad.Null*; n=3). **iv,** Bar graph showing inhibition of *Ad.KLK1*-induced PAC invasion by the PI3Kγ inhibitor AS605240 (10^{-6} mol/L), the NOS inhibitor (LNIO) (10^{-4} mol/L), or the NO scavenger (PTIO) (10^{-4} mol/L) (n=3). **D,** Enhanced HUVEC networking by *Ad.KLK1*-PACs (**P*<0.05 vs *Ad.Null*) is inhibited by aprotinin and kallistatin but not by icatibant, L-dAL-BK, or GM (n=4).

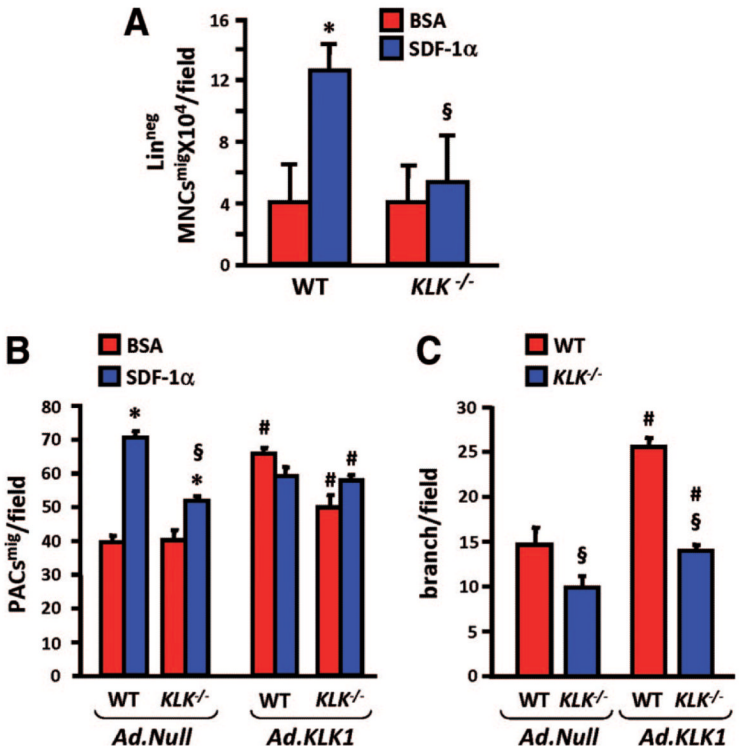

Figure 5. Deletion of the *KLK1* gene results in MNCs and PACs dysfunction

A, Immunomagnetically sorted lineage negative (Lin^{neg})-BM-MNCs from kallikrein knockout mice ($KLK^{-/-}$) show reduced migration toward SDF-1α compared to corresponding cells from WT mice (*P<0.05 vs BSA, §P<0.05 vs WT; MNCs from n=7 mice per group were pooled and assayed in triplicate). **B,** *Ad.KLK1* improves spontaneous and SDF-1α–stimulated migration of $KLK^{-/-}$PACs (*P<0.05 vs BSA, §P<0.05 vs *Ad.Null*-WT stimulated with SDF-1α, #P<0.05 vs corresponding *Ad.Null* group, n=7 mice per group pooled and assayed in triplicate). **C,** ECs networking on Matrigel. Same symbols as in **B**.

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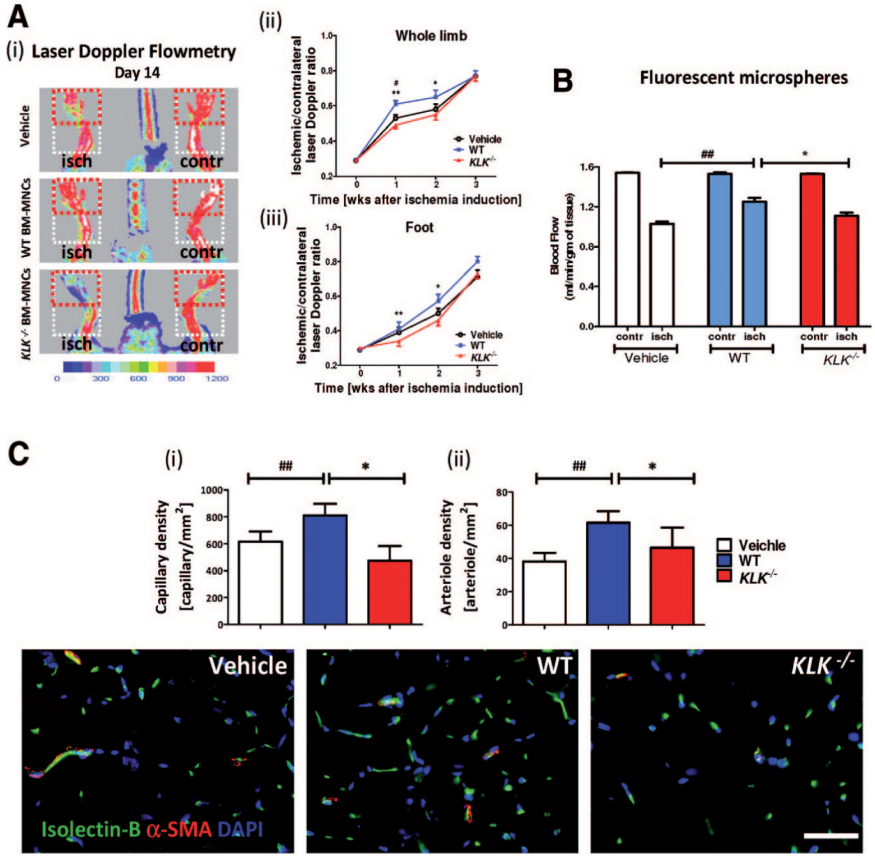

Figure 6. BM-MNCs from *KLK*$^{-/-}$ mice show reduced proangiogenic potential in vivo

A, i, Representative laser Doppler flowmetric images at 14 days after ischemia induction; **dotted squares** delimit the area of measurement in ischemic (isch) and contralateral (contr) limb **(white)** or foot **(red)**. Time course of the ischemic-to-contralateral blood flow ratio in whole limb **(ii)** and foot **(iii)** (**P*<0.05 and ***P*<0.01, *KLK*$^{-/-}$ BM-MNCs vs WT BM-MNCs; #*P*<0.05, WT BM-MNCs vs vehicle; 16 to 20 mice per group). **B,** Bar graph showing adductor muscle blood flow 3 weeks from induction of unilateral ischemia, as assessed by fluorescent microspheres. **C,** Bar graphs and representative pictures showing capillary **(i)** and arteriole density **(ii)** in adductor muscles of mice injected with *KLK*$^{-/-}$ BM-MNCs, WT BM-MNCs, or vehicle at 3 weeks postischemia (**P*<0.05, *KLK*$^{-/-}$ BM-MNCs vs WT BM-MNCs; ##*P*<0.01 WT BM-MNCs cells vs vehicle; n=5 mice per group). **Scale bars,** 50 μm.

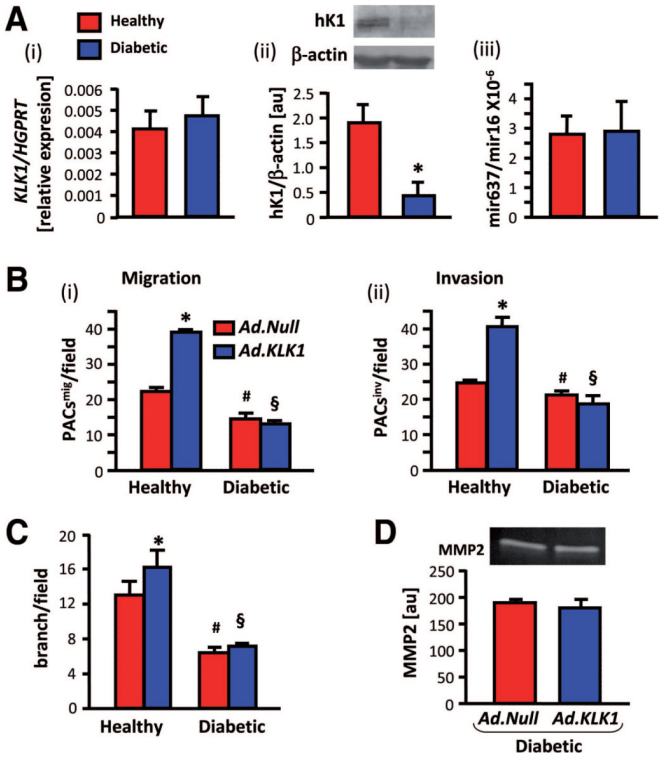

Figure 7. Reduced hK1 expression in T2D-PACs translates into reduced invasive and proangiogenic activities

A, T2D-PACs show normal *KLK1* mRNA (Q-RT-PCR) **(i)** but reduced protein expression (WB) **(ii)**. Q-RT-PCR data show similar levels of mir637 in healthy and T2D-PACs **(iii)** (**P*<0.05 vs healthy, n=5 subjects per group, in triplicates). **B,** Spontaneous migration **(i)** and invasion **(ii)** of *Ad.KLK1*- or *Ad.Null*-infected PACs (**P*<0.05 vs *Ad.Null*-PACs within healthy group, #*P*<0.05 vs *Ad.Null*-healthy PACs, §*P*<0.05 vs *Ad.KLK1*-healthy PACs, n=3 per group). **C,** HUVEC networking in the presence of *Ad.Null*- or *Ad.KLK1*-PACs. n=3; same symbols as in **B**. **D,** Gel zymography measuring MMP2 activity in *Ad.Null*- and *Ad.KLK1*-infected T2D-PAC CM. Representative experiment and average densitometry data (n=3 subjects).

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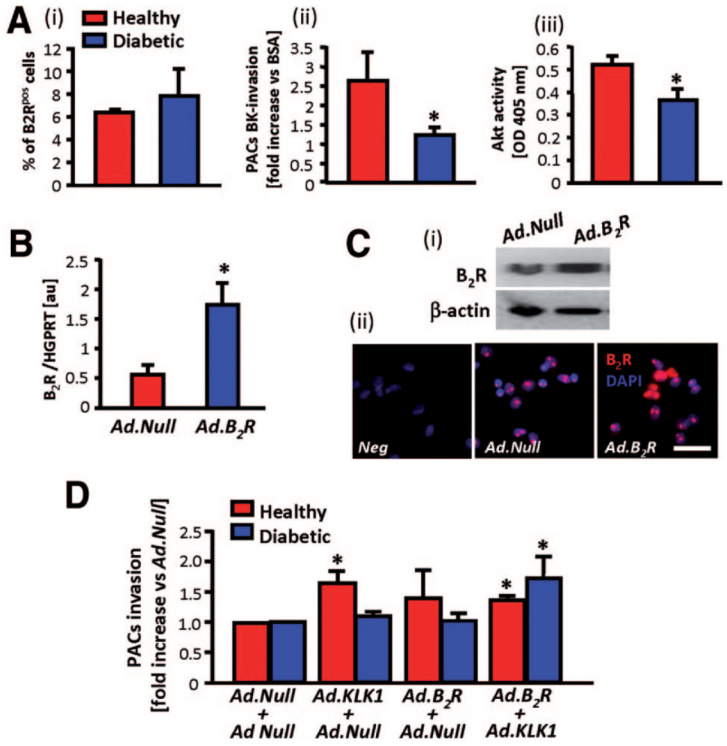

Figure 8. Combined *KLK1-B*$_2$R overexpression rescues diabetes-induced PACs dysfunction

A, Bar graph showing similar abundance of B_2R expressing cells within healthy or T2D-PACs (flow cytometry) (n=3) **(i)**; PAC invasion in response to BK (fold increase vs BSA; $P<0.05$ vs healthy PACs, n=3) **(ii)**; Akt activity in PACs lysates ($P<0.05$ vs healthy PACs, n=3) **(iii)**. **B,** B_2R-forced expression in PACs. B_2R mRNA expression levels normalized to HGPRT (n=3, $*P<0.05$). **C,** Representative WB of B_2R in PACs lysates normalized to β-actin **(i)** and fluorescence immunocytochemistry of B_2R-positive PACs **(ii)**. Neg indicates TRITC-conjugated secondary antibody only; *Ad.Null* and *Ad.*B_2R, transduced PACs stained with specific anti-B_2R antibody plus TRITC-conjugated secondary antibody. **Scale bar,** 20 μm. **D,** Effect of *Ad.Null*, *Ad.KLK1*, *Ad.*B_2R, or double *Ad.KLK1*+*Ad.*B_2R infection on spontaneous invasive activity of PACs from healthy (n=3) and diabetic subjects (n=5) (fold increase vs *Ad.Null*, $*P<0.05$ vs *Ad.Null*+*Ad.Null*).

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