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## Enhanced Integrin $\alpha 4\beta 1$ -Mediated Adhesion Contributes to a Mobilization Defect of Endothelial Progenitor Cells in Diabetes

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**Diabetes is associated with a deficit of circulating endothelial progenitor cells (EPCs), which has been attributed to their defective mobilization from the bone marrow. The basis for this mobilization defect is not completely understood, and we sought to determine if hyperglycemic conditions enhanced EPC adhesion. We found that culturing EPCs in high glucose media increased adhesion to bone marrow stromal cells. This enhanced adhesion was associated with decreased expression of protein kinase A regulatory subunit 1 $\beta$  (PRKAR1 $\beta$ ), activation of protein kinase A (PKA), and phosphorylation of  $\alpha 4$ -integrin on serine 988. This potentiated adhesion was reversed by treatment with a PKA inhibitor, overexpression of PRKAR1 $\beta$ , or expression of a phosphorylation-defective  $\alpha 4$ -integrin variant ( $\alpha 4$ [S988A]). Using a model of type 1 diabetes, we showed that  $\alpha 4$ (S988A)-expressing mice have more circulating EPCs than their wild-type counterparts. Moreover, diabetic  $\alpha 4$ (S988A) mice demonstrate enhanced revascularization after hind limb ischemia. Thus, we have identified a novel signaling mechanism activating PKA in diabetes (downregulation of an inhibitory regulatory subunit) that leads to deficits of circulating EPCs and impaired vascular repair, which could be reversed by  $\alpha 4$ -integrin mutation.**

Cardiovascular disease (CVD) is the leading cause of death worldwide (1). A dysfunctional endothelium contributes to the development of CVD by promoting or exacerbating atherosclerosis, hypertension, and thrombosis. Damage to the vasculature is repaired in part by a population of bone

marrow stem cells, the endothelial progenitor cells (EPCs). Several previous studies reported that levels of circulating EPCs are directly associated with vascular health (2), and their abundance and functionality are negatively associated with advanced age (2), smoking (2,3), and a sedentary lifestyle (4). Furthermore, several animal (5–7) and human (8–10) studies have demonstrated an inverse relationship between circulating EPC number and incidence of diabetes.

EPCs are mobilized from the bone marrow by cytokines and growth factors such as stromal cell-derived factor-1 (SDF-1 $\alpha$ ) (6,11) and vascular endothelial growth factor (VEGF) (12,13). Signaling pathways downstream of these agonists disrupt adhesive interactions mediated by C-X-C chemokine receptor 4 (CXCR4) and c-kit, which are partly responsible for maintaining EPCs in the bone marrow. In addition, signals from the sympathetic nervous system have also been implicated in the mobilization of EPCs (14) as well as hematopoietic stem cells (HSCs) (14,15). Diabetes is associated with defective progenitor cell mobilization. Prior reports suggest this may result from deficits of mobilizing agonists (5,16), altered bone marrow structure and responsiveness (7,17), or induced neuropathy and altered expression of intracellular signaling molecules (14).

Stem cell populations are also retained in the bone marrow through  $\alpha 4\beta 1$ -integrin vascular cell adhesion molecule-1 (VCAM-1) interactions. Ablation of these interactions or conditional  $\alpha 4$ -integrin knockdown enhanced circulating levels of both HSCs (18–20) and EPCs (21). Given this prominent role of  $\alpha 4\beta 1$  in bone marrow retention, we hypothesized that diabetes might impact the functional properties of this

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integrin to limit EPC mobilization. To test this possibility, we studied the effects of hyperglycemia on the adhesion of cultured EPCs. We found that growth in high glucose enhanced the adhesion of EPCs to bone marrow stromal cells. This potentiated adhesion was associated with downregulation of the regulatory subunit  $1\beta$  of protein kinase A (PRKAR1 $\beta$ ), consequent activation of protein kinase A (PKA), and phosphorylation of  $\alpha 4$ -integrin on serine 988. Enhanced adhesion was blocked by a PKA inhibitor and PRKAR1 $\beta$  overexpression. EPCs with an alanine substitution at serine 988 (S988A) in the  $\alpha 4$ -integrin subunit were also resistant to high glucose-potentiated adhesion. Furthermore, using a model of type 1 diabetes, we observed that mice expressing the  $\alpha 4$ (S988A) variant had increased levels of circulating EPCs and enhanced revascularization when compared with their wild-type counterparts. Thus, hyperglycemia limits EPC mobilization through PRKAR1 $\beta$  downregulation, activation of PKA, phosphorylation of  $\alpha 4$ -integrin, and potentiated adhesion in the bone marrow. Ablation of this signaling pathway enhanced circulating EPC levels and vascular repair capacity.

## RESEARCH DESIGN AND METHODS

### Reagents

Antibodies for  $\alpha 4$ -integrin immunoprecipitation (PS/2 and HP2/1) and blotting (C-20) were from Millipore, GeneTex, and Santa Cruz Biotechnology, respectively. The phospho- $\alpha 4$ -integrin antibody was generated as previously described (22). PKA subunit antibodies were supplied by Becton Dickinson. An anti-VCAM-1 antibody was from Southern Biotech. H89 was obtained from Millipore, and 8-bromoadenosine cAMP (8-Br-cAMP) was from Enzo Life Sciences. The PRKAR1 $\beta$  cDNA was obtained from GeneCopoeia. Streptozotocin (STZ) and anti-actin and control antibodies were from Sigma-Aldrich.

### Cells and Mice

Endothelial colony-forming cells (ECFCs) were obtained from the Angiogenesis, Endothelial, and Proangiogenic Cell Core of the Simon Cancer Center at the Indiana University School of Medicine (23). They were maintained in EGM-2 media (Lonza) supplemented with 10% FCS and used between passages 3 and 10. To study the effects of high glucose, media was supplemented with 20 mmol/L D-glucose (Sigma-Aldrich) whereas osmotic control media contained an additional 20 mmol/L L-glucose (VWR). Exogenous activators (8-Br-cAMP; 500  $\mu$ mol/L) or inhibitors (H89; 10  $\mu$ mol/L) of PKA were added 1 h prior to cell lysis or biochemical assay. ECFCs were transfected using the TransFectin reagent (Bio-Rad), according to the manufacturer's recommendations, and used for experiments 48 h later.

Mice expressing the S988A  $\alpha 4$ -integrin variant  $\alpha 4$ (S988A) were generated as previously described (24). To induce diabetes, STZ was injected intraperitoneally at a dose of 65 mg/kg for 6 consecutive days. Blood glucose levels were analyzed after 7 days, and only mice having a glucose concentration of  $>350$  mg/dL were used for

subsequent analyses. Control mice received injections of citrate.

Murine bone marrow cells were isolated by flushing femurs and tibiae with media. EPCs from these preparations were cultured on fibronectin-coated plates for 1 week in the above media. Circulating EPCs were detected in lysed whole blood by flow cytometry using APC-conjugated anti-Flk and PE-conjugated anti-Sca antibodies (eBioscience).

### Biochemical Assays

Measurements of  $\alpha 4$ -integrin expression and  $\alpha 4\beta 1$ -integrin affinity were accomplished by flow cytometry using a FITC-conjugated anti- $\alpha 4$  antibody (eBioscience) and a FITC-conjugated affinity ligand (gift from Dr. Larry Sklar, University of New Mexico), respectively. To measure adhesion, harvested cells were incubated with 5  $\mu$ mol/L calcein AM (Life Technologies) for 30 min at 37°C. The cells were then washed, incubated for an additional 30 min, and washed again. Then  $10^5$  cells were added in triplicate to confluent S17 bone marrow stromal cells (gift from Dr. Kenneth Dorschkind, University of California, Los Angeles) in a 24-well plate. At various time points, the wells were aspirated and washed and fluorescence was determined in a plate reader. A percent adhesion was calculated based upon the initial fluorescence of  $10^5$  cell aliquots. Background fluorescence was determined using wells coated with BSA. PKA activity in cell homogenates was determined using the PepTag nonradioactive cAMP-dependent protein kinase assay (Promega). Migration toward SDF-1 $\alpha$  (100 ng/mL) was determined using modifications of a previously described assay (25).

### Hind Limb Ischemia

Revascularization was determined by laser Doppler perfusion imaging (LDPI) in mice subjected to hind limb ischemia (HLI). For HLI, male mice were anesthetized by isoflurane inhalation (3% isoflurane mixed with 100% O<sub>2</sub>), an incision was made through the skin, and the femoral artery and vein were exposed. Sterile 7.0 sutures were threaded under and around the femoral artery and vein and knotted to permanently ligate both blood vessels. Sham HLI surgery was conducted in the same manner, except that blood vessels were not ligated. Three weeks after surgery, mice were anesthetized as above and LDPI (MoorLDI2; Moor Instruments) was recorded for both ischemic and nonischemic hind limbs. LDPI values scale linearly with the product of red blood cell velocity and the number of blood cells within the tissue and are listed in arbitrary units. Two images were taken for every mouse and imported into ImageJ, and areas of blue, green, and red in the region of interest spanning the entire hind limb were enumerated using the moorFLPI-2 Review Software. The value for the ischemic limb was then normalized to the nonischemic limb to calculate a percent recovery of blood perfusion. All procedures were approved by the University of Louisville Institutional Animal Care and Use Committee.

### Bone Marrow Transplantation

For bone marrow transplantation, 7-week recipient male C57BL/6 mice (The Jackson Laboratory) were first conditioned with 950 cGy total body irradiation ( $\gamma$ -cell 40; Nordion, Ontario, Canada). Bone marrow was isolated from tibias and femurs of donor  $\alpha 4$ (WT) or  $\alpha 4$ (S988A) male mice (12 weeks) by flushing with PBS. The cells were resuspended in PBS by gentle aspiration through an 18-gauge needle, filtered through sterile nylon mesh with 100- $\mu$ M pores, centrifuged at 1,000 rpm for 10 min at 4°C, and resuspended in PBS. Twenty-four hours after irradiation, recipients were injected with 0.1 mL PBS containing  $1 \times 10^7$  cells through the retro-orbital plexus with a 27-gauge needle. Five weeks after transplant, recipients were characterized for hematopoietic recovery by assessing complete blood cell counts using a HemaVet 950 (Drew Scientific Inc., Oxford, CT).

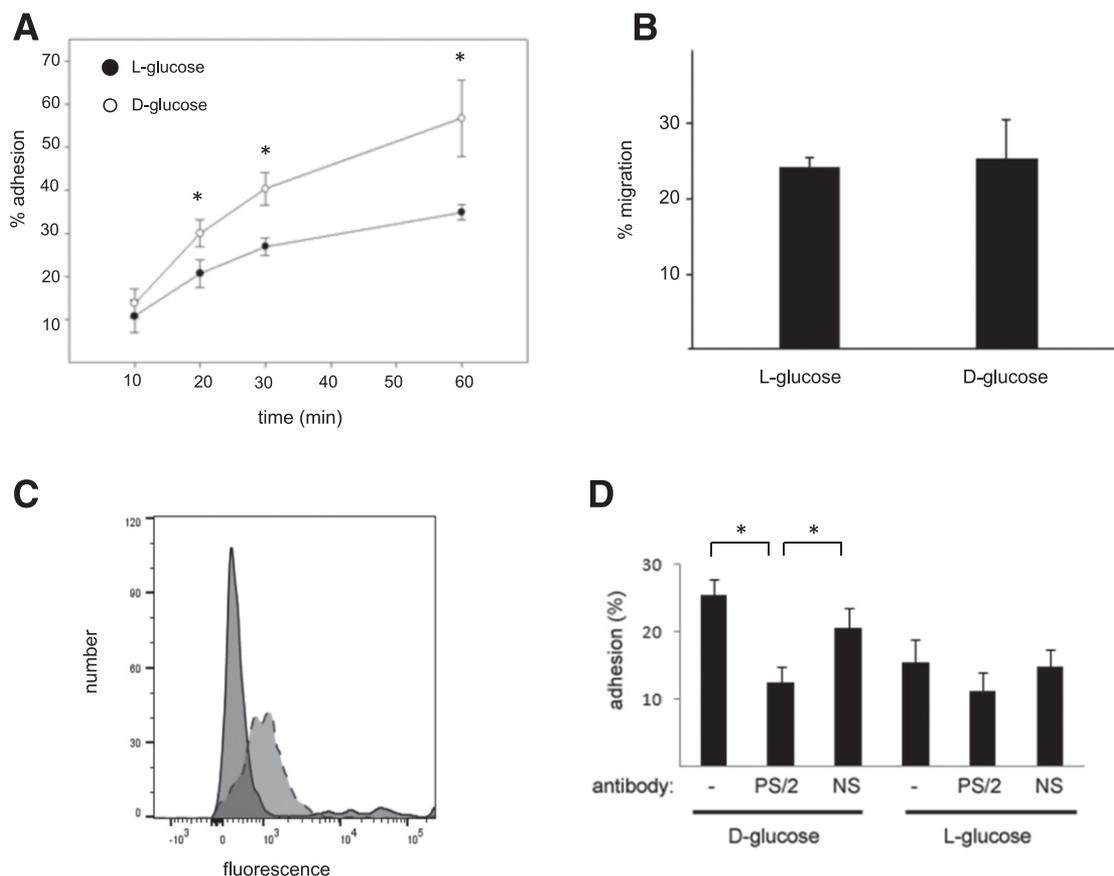
### Immunofluorescence

Skeletal muscle from ischemic limbs was collected, formalin fixed, and paraffin embedded. Sections (5  $\mu$ m) were stained with fluorescently conjugated isolectin (*Griffonia*,

*Bandeiraea simplicifolia* Lectin I; Vector Laboratories) and nuclear counterstained with DAPI. Images were acquired with a fluorescent microscope. Additional sections were stained with Sirius Red (0.1%; RA Lamb LLC, Apex, NC) and Fast Green FCF (0.1%; Sigma-Aldrich). Up to three sections were viewed on an Olympus IOM microscope and imaged with a SPOT camera using SPOT advanced image-capture software. Analysis of isolectin staining and fibrosis was performed using the National Institutes of Health ImageJ software, version 1.45s. Each digital photomicrograph (up to three different sections per slide) was analyzed as previously reported (26). The extent of fibrosis was measured as the percentage of the total area assessed (available at <https://imagej.nih.gov/ij/docs/examples/stained-sections/index.html>).

### Statistical Analysis

Values are expressed as means  $\pm$  SE. Statistical significance ( $P < 0.05$ ) was evaluated using an unpaired Student *t* test. One-way ANOVA with Bonferroni post hoc test was used for comparing multiple groups.



**Figure 1**—Growth in high glucose stimulates ECFC adhesion. **A**: ECFCs were grown in 25 mmol/L D-glucose or the L-glucose osmotic control media for 1 week, and a time course of their adhesion to an S17 cell monolayer was determined.  $n = 4$ . \* $P < 0.05$ . **B**: Cells grown for 1 week in the indicated media were harvested, and SDF-1 $\alpha$ -stimulated migration was analyzed in a transwell assay. **C**: ECFCs express the  $\alpha 4$ -integrin. Solid line, isotype control antibody; dashed line,  $\alpha 4$ -specific antibody. **D**: Cells grown for 1 week in the indicated media were harvested and allowed to adhere for 30 min in the absence or presence of an anti- $\alpha 4\beta 1$  blocking antibody (PS/2) or a nonspecific antibody (NS).  $n = 5$ . \* $P < 0.05$ .

## RESULTS

### Growth in High Glucose Potentiates EPC Adhesion

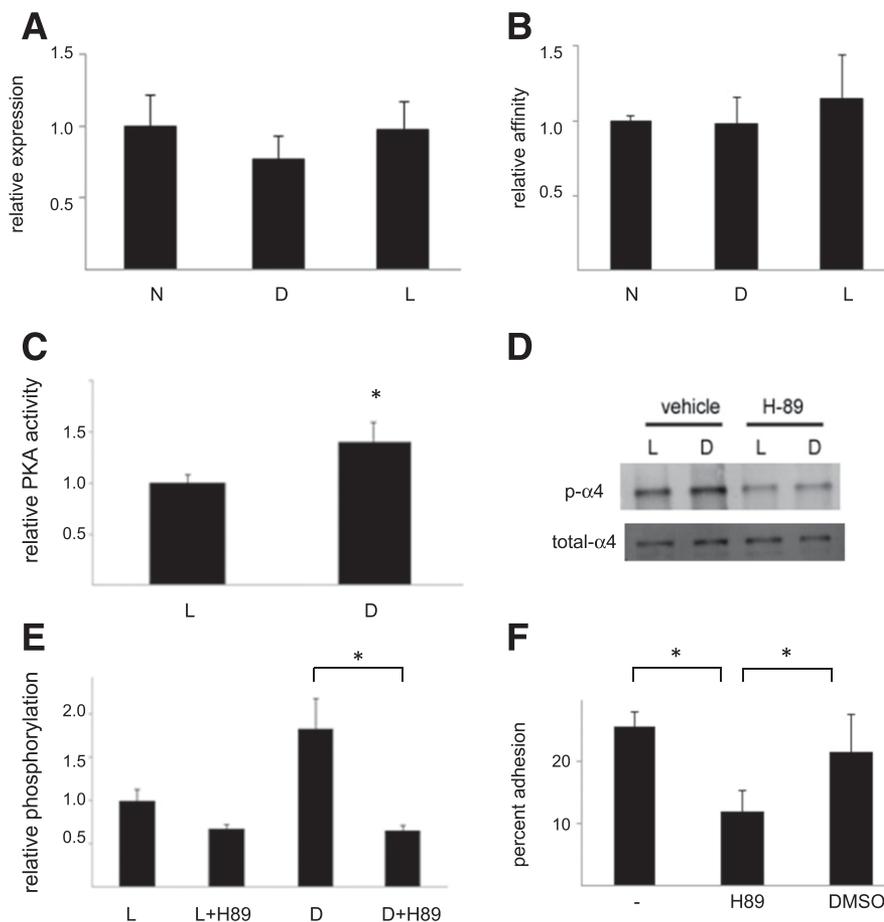
Diabetes has been associated with a deficit of circulating EPCs (5–10). One possible reason for this deficit is enhanced retention of these cells in the bone marrow, as has been suggested for HSCs (17). To test this, we first examined the adhesive properties of cultured EPCs, the ECFCs. To approximate the serum conditions of diabetes, we grew these cells in media containing 25 mmol/L D-glucose for 1 week. These cells demonstrated enhanced adhesion to a monolayer of S17 bone marrow stromal cells compared with those cells grown in the osmotic control media (Fig. 1A). Growth in high glucose media, however, does not induce general cellular dysfunction. Cells cultured in both types of media displayed equivalent SDF-1 $\alpha$ -mediated migration (Fig. 1B).

$\alpha$ 4 $\beta$ 1-Integrin-mediated adhesion is crucial for maintaining HSCs (19,27) and EPCs (21) in the bone marrow, and this integrin is expressed in ECFCs (Fig. 1C). To

determine whether hyperglycemia-stimulated ECFC adhesion is also mediated by  $\alpha$ 4 $\beta$ 1, we performed the adhesion assay in the presence of a function-blocking antibody (PS/2; 10  $\mu$ g/mL). Addition of this antibody reduced ECFC adhesion to background levels (Fig. 1D). Inclusion of a nonspecific antibody had no effect on high glucose-stimulated adhesion. Incubation with either antibody did not affect the adhesion of the osmotic control cells. Thus, growth in high glucose stimulates  $\alpha$ 4 $\beta$ 1-dependent adhesion.

### Growth in High Glucose Stimulates PKA-Dependent $\alpha$ 4-Integrin Phosphorylation

Enhanced  $\alpha$ 4 $\beta$ 1-mediated adhesion might result from an increase in  $\alpha$ 4 $\beta$ 1 expression or ligand binding affinity. However, growth in high glucose media did not appear to change either of these properties (Fig. 2A and B). Alternatively,  $\alpha$ 4 $\beta$ 1-mediated adhesion can be impacted by PKA-dependent phosphorylation of the  $\alpha$ 4-integrin at serine 988 (22,28). To determine whether growth in



**Figure 2**—Growth in high glucose stimulates PKA activity,  $\alpha$ 4-integrin phosphorylation, and PKA-dependent adhesion. **A:**  $\alpha$ 4 expression. ECFCs were grown for 1 week in normal media (N), in media containing 25 mmol/L D-glucose (D), or in the osmotic control media (L), and  $\alpha$ 4-integrin expression was determined by flow cytometry.  $n = 4$ . **B:**  $\alpha$ 4 $\beta$ 1 affinity. The same cells were used to determine  $\alpha$ 4 $\beta$ 1 affinity by flow cytometry.  $n = 3$ . **C:** PKA activity. ECFCs were grown in media containing 25 mmol/L D-glucose and the osmotic control media for 1 week, and PKA activity was then determined.  $n = 10$ . \* $P < 0.05$ . **D:**  $\alpha$ 4 phosphorylation. Cells grown for 1 week in the same media as indicated were treated with DMSO (vehicle) or the PKA inhibitor H89 and levels of phospho- $\alpha$ 4 were determined by Western blotting. Illustrated is a representative blot. **E:** Illustrated are group data for high glucose-stimulated  $\alpha$ 4 phosphorylation.  $n = 8$ . \* $P < 0.05$ . **F:** Cell adhesion. Cells grown in D-glucose media were left untreated or were treated with either H89 or DMSO, and then adhesion to an S17 cell monolayer was determined after 30 min.  $n = 4$ . \* $P < 0.05$ .

high glucose increases adhesion through this mechanism, we first measured PKA activity. Cells grown in 25 mmol/L D-glucose demonstrated a  $1.40 \pm 0.25$ -fold increase in activity compared with cells grown in the osmotic control media (Fig. 2C). Consistently, we also found an increase in  $\alpha 4$ -integrin phosphorylation in the D-glucose-cultured cells (Fig. 2D and E), which was blocked with the PKA inhibitor H89 (Fig. 2D and E). To determine whether there was a causal relationship between PKA activity and increased adhesion, we treated D-glucose-cultured cells with H89 prior to their use in the adhesion assay. We observed that this treatment, but not treatment with vehicle, reduced adhesion (Fig. 2F). To determine whether direct PKA activation stimulated  $\alpha 4$  phosphorylation and adhesion, we treated ECFCs with the PKA activator 8-Br-cAMP. Like the high glucose-cultured cells, these cells demonstrated increased  $\alpha 4$  subunit phosphorylation (Fig. 3A and B) and adhesion (Fig. 3C) compared with vehicle-treated cells. Moreover, 8-Br-cAMP-stimulated adhesion was blocked with an  $\alpha 4$ -integrin function-blocking antibody and with H89 (Fig. 3C). Finally, S17 cells express the  $\alpha 4\beta 1$  ligand VCAM-1, and an anti-VCAM-1 blocking antibody also reduced ECFC adhesion to background levels (Fig. 3C). Collectively, these observations suggest that high glucose stimulates EPC adhesion by increasing the PKA-mediated phosphorylation of the  $\alpha 4$ -integrin.

### PRKAR1 $\beta$ Downregulation Enhances PKA Activity and Potentiates Adhesion

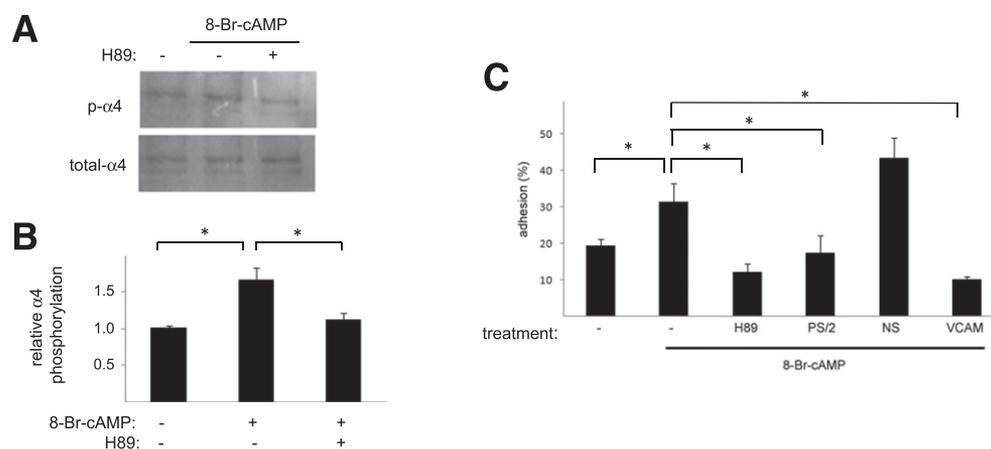
PKA activation may result from increased levels of its cellular activator cAMP. However, quantification of cAMP levels by ELISA indicated no change in the concentration of this secondary messenger (Fig. 4A). Alternatively, PKA activity may be influenced by the stoichiometry of its

catalytic and regulatory subunits. To test this possibility, we first identified which PKA subunits are expressed in ECFCs. In addition to the catalytic subunit (PKA-C), ECFCs predominantly express the regulatory subunits 1 $\beta$  (PRKAR1 $\beta$ ), 2 $\alpha$  (PRKAR2 $\alpha$ ), and 2 $\beta$  (PRKAR2 $\beta$ ) (Fig. 4B). Next we determined whether the expression of these subunits was altered by growth in high glucose. Whereas there were no changes in PKA-C, PRKAR2 $\alpha$ , or PRKAR2 $\beta$ , we did observe a  $33 \pm 12\%$  reduction in PRKAR1 $\beta$  expression (Fig. 4C and D).

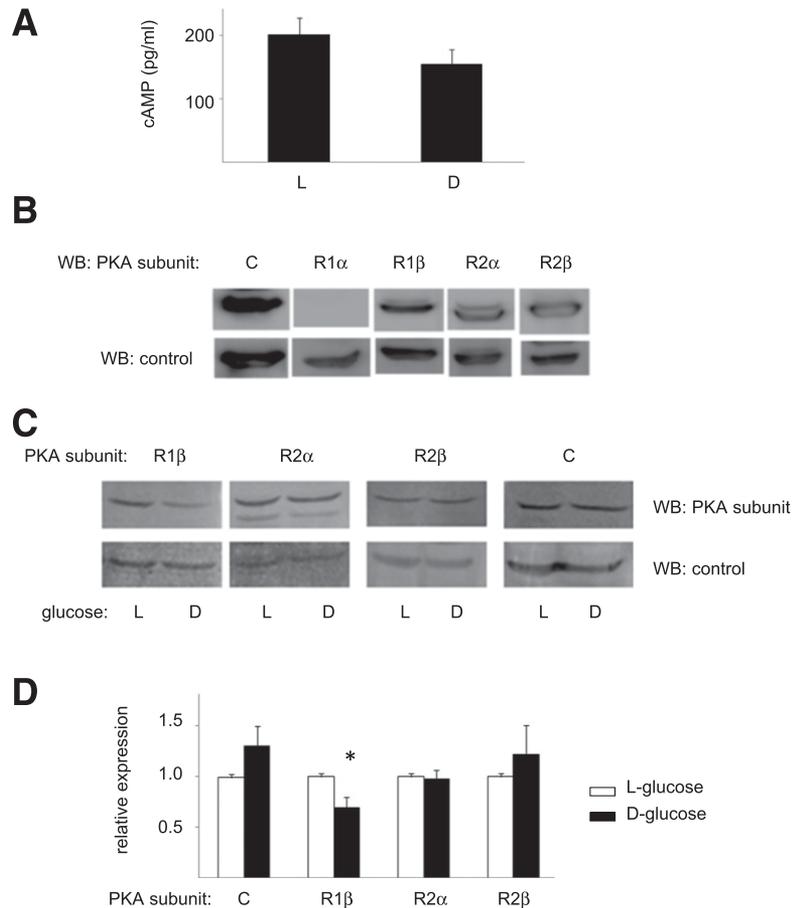
We reasoned that if growth in high glucose downregulates PRKAR1 $\beta$ , resulting in PKA activation,  $\alpha 4$  phosphorylation, and enhanced adhesion, then overexpression of PRKAR1 $\beta$  should reverse these effects. To test this, ECFCs cultured in high glucose media were transfected with PRKAR1 $\beta$  or control vectors. PRKAR1 $\beta$  transfectants demonstrated decreases in PKA activity (Fig. 5A),  $\alpha 4$  phosphorylation (Fig. 5B), and adhesion (Fig. 5C) compared with vector transfectants or untransfected cells. These observations suggest that attenuated expression of PRKAR1 $\beta$  in the presence of high glucose enhances ECFC adhesion.

### Ablation of $\alpha 4$ -Integrin Phosphorylation Reverses Hyperglycemia-Induced Adhesion

To directly test the idea that  $\alpha 4$ -integrin phosphorylation enhances EPC adhesion in hyperglycemic conditions, we used cells isolated from mice expressing the  $\alpha 4$ -integrin S988A variant. This variant has an alanine rather than serine at residue 988 and thus cannot be phosphorylated. Cells from these mice would be predicted to be unresponsive to the effects of hyperglycemia. We tested this possibility by inducing hyperglycemia in  $\alpha 4$ (WT) and  $\alpha 4$ (S988A) mice using the STZ model of type 1 diabetes (Fig. 6A). Four weeks after induction of diabetes, bone



**Figure 3**—Direct PKA stimulation enhances  $\alpha 4$  phosphorylation and adhesion. **A:** PKA-stimulated  $\alpha 4$  phosphorylation. Untreated ECFCs or those treated with 8-Br-cAMP in the presence or absence of H89 were lysed, and levels of phospho- $\alpha 4$  (p- $\alpha 4$ ) and total  $\alpha 4$  determined by Western blotting. Illustrated are representative blots. **B:** Illustrated are grouped data of phospho- $\alpha 4$ -to-total  $\alpha 4$  ratios normalized to untreated samples.  $n = 4$ . \* $P < 0.05$ . **C:** Cell adhesion. Cells variably incubated with 8-Br-cAMP as indicated were allowed to adhere to an S17 cell monolayer for 30 min. Adhesion was done in the presence of anti- $\alpha 4$  (PS/2), nonspecific (NS), or anti-VCAM antibodies, as indicated. The PKA dependence of enhanced adhesion was demonstrated by inhibition with H89.  $n \geq 4$ . \* $P < 0.05$ .



**Figure 4**—Hyperglycemia and PKA activation. **A**: cAMP levels. ECFCs grown for 1 week in 25 mmol/L D-glucose (D) or L-glucose (L) as indicated were analyzed for levels of cAMP by ELISA.  $n = 3$ . **B**: PKA subunits in ECFC. The expression of PKA subunits in ECFC was determined by Western blotting (top panels). In addition to the catalytic subunit C, these cells predominantly express PRKAR1 $\beta$ , PRKAR2 $\alpha$ , and PRKAR2 $\beta$  (R1 $\beta$ , R2 $\alpha$ , and R2 $\beta$ , respectively). Loading control blots (bottom panels) used antibodies for actin (PRKAR1 $\alpha$  [R1 $\alpha$ ], R1 $\beta$ , R2 $\alpha$ , and R2 $\beta$ ) or tubulin (PKA-C). **C**: PKA subunit expression after hyperglycemic growth. ECFCs were cultured for 1 week in 25 mmol/L D-glucose (D) or L-glucose (L) as indicated, and levels of the indicated PKA subunits (top panels) were determined by Western blotting. **D**: Relative expression levels. Illustrated are the relative expression levels of the PKA subunits after hyperglycemic growth.  $n = 4$ . \* $P < 0.05$  vs. L-glucose. C, catalytic subunit C; WB, Western blot.

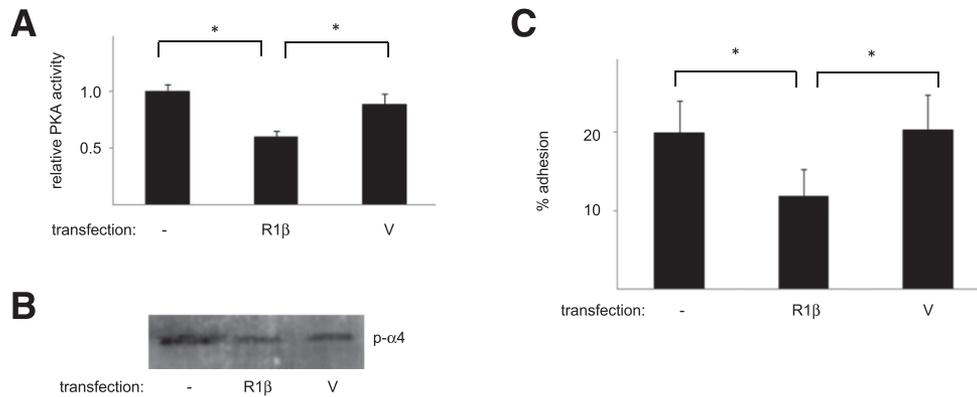
marrow EPCs were isolated, cultured *ex vivo*, and used for cellular and biochemical assays. As expected, cells from both STZ-injected mouse strains demonstrated increased PKA activity compared with their control, citrate-injected counterparts (Fig. 6B). However cells isolated from the diabetic  $\alpha 4$ (S988A) mice demonstrated a  $50 \pm 5\%$  reduction in adhesion compared with cells isolated from the diabetic  $\alpha 4$ (WT) mice (Fig. 6C). These findings indicate that increased adhesion of EPCs in hyperglycemic conditions could be attributable to increased  $\alpha 4$ -integrin phosphorylation.

#### S988A Mice Demonstrate Enhanced Revascularization Potential

The attenuated adhesion of bone marrow EPCs from diabetic  $\alpha 4$ (S988A) mice suggests they may be more abundant in circulation. To explore this, we quantified levels of Flk<sup>+</sup>/Sca<sup>+</sup> cells. Prior to any injection (Fig. 6D), or after citrate injection (Fig. 6E), there was no significant difference in the levels of these cells between  $\alpha 4$ (WT) and

$\alpha 4$ (S988A) mice. After induction of diabetes, however (Fig. 6F), we observed that STZ-treated  $\alpha 4$ (S988A) mice had increased levels of circulating (peripheral blood) Flk<sup>+</sup>/Sca<sup>+</sup> cells compared with STZ-treated  $\alpha 4$ (WT) mice. These levels in the  $\alpha 4$ (S988A) mice were 3.08-fold greater than levels in the  $\alpha 4$ (WT) mice. Reciprocal changes in Flk<sup>+</sup>/Sca<sup>+</sup> cells were observed in the bone marrow of STZ-treated mice. These cells were increased in the bone marrow of wild-type mice compared with circulating levels, but they were decreased in the bone marrow of S988A animals compared with circulating levels (Fig. 6F).

Endogenous or exogenous increases in circulating EPCs promote revascularization (29–32). Thus, increased circulating Flk<sup>+</sup>/Sca<sup>+</sup> cells in diabetic  $\alpha 4$ (S988A) mice suggests that these mice might be protected from revascularization defects. To test this, we subjected diabetic mice of both strains to HLI and examined the return of vascular perfusion after 3 weeks. We found that blood flow in the ischemic limb of  $\alpha 4$ (S988A) mice had returned to  $79.2 \pm 3.1\%$

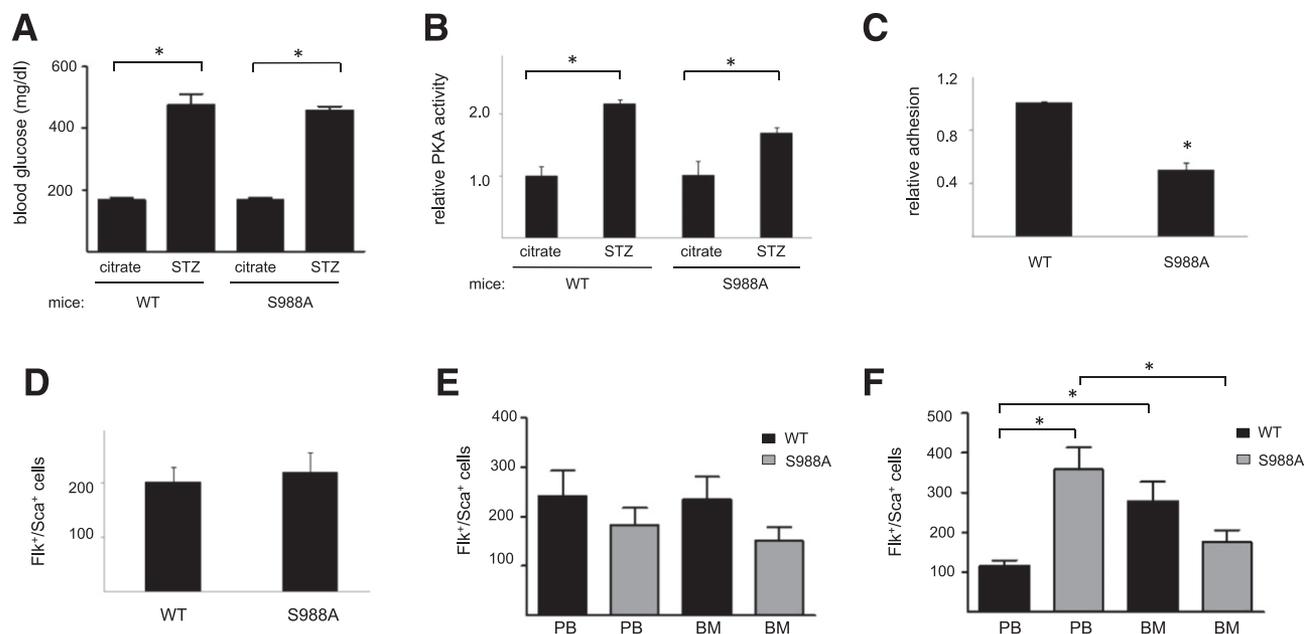


**Figure 5**—Overexpression of PRKAR1β reverses the effect of hyperglycemic culture. EFCs grown in 25 mmol/L D-glucose for 5 days were transfected with PRKAR1β (R1β) or control vector (V) and lysed 2 days later. **A**: PKA activity. The PKA activity of the D-glucose–cultured transfectants was determined as outlined in RESEARCH DESIGN AND METHODS. *n* = 4. \**P* < 0.05. **B**: α4 Phosphorylation (p-α4). Levels of phosphorylated α4-integrin in the transfectants were determined by Western blotting. Illustrated is a representative blot. **C**: Adhesion. The adhesive properties of the D-glucose–cultured transfectants to an S17 cell monolayer were determined and grouped data illustrated. *n* = 6. \**P* < 0.05.

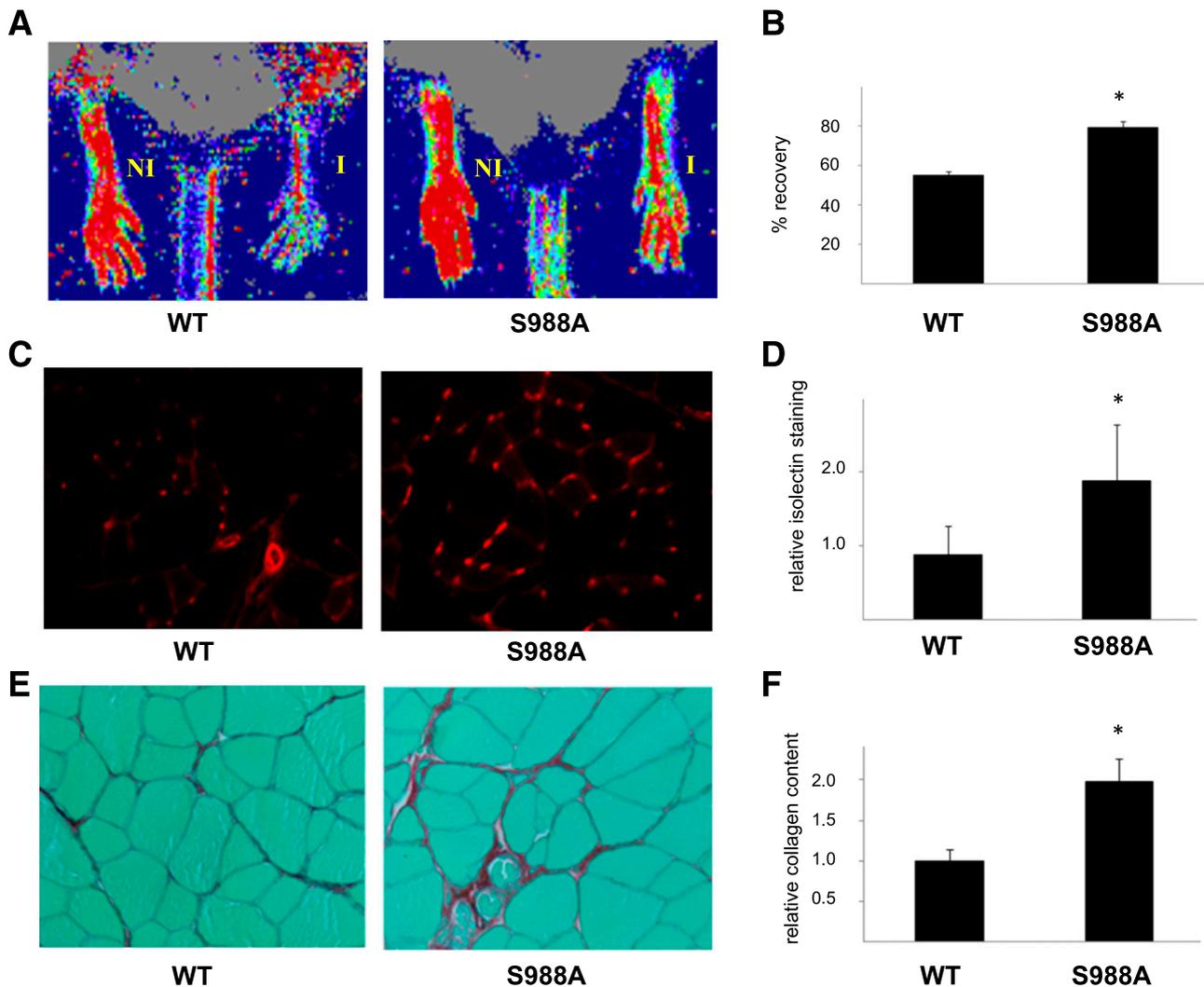
of that in the uninjured limb. In contrast, the α4(WT) mice demonstrated only a 54.8 ± 2.1% recovery (Fig. 7A and B). Immunohistochemical staining of muscle tissue from the injured limb also demonstrated an increase in vascular capillaries (isolectin-positive structures) in the α4(S988A) mice (1.9 ± 0.7-fold) compared with the α4(WT) mice (Fig. 7C and D). In addition, we observed a greater deposition of collagen fibers in the ischemic

muscle of α4(S988A) mice as compared with the α4(WT) mice (Fig. 7E and F).

Finally, we assessed whether the enhanced revascularization potential of the α4(S988A) mice was a consequence of the altered adhesive properties of bone marrow EPCs, or whether this was an unrelated, inherent property of the mice themselves. Hence we performed bone marrow transplants with irradiated wild-type recipient mice receiving



**Figure 6**—Characterization of murine EPCs. **A**: α4(WT) and α4(S988A) mice were injected with citrate or STZ and blood glucose levels determined after 4 weeks. *n* ≥ 8. \**P* < 0.05. **B**: Bone marrow–derived EPCs were then isolated from these mice and cultured, and PKA activity was quantified. *n* = 4. \**P* < 0.05. **C**: The adhesion of cells from the diabetic mouse strains to an S17 cell monolayer was determined as above. *n* = 4. \**P* < 0.05. **D**: Levels of Fik<sup>+</sup>/Sca<sup>+</sup> cells were quantified in the peripheral blood of both α4(WT) and α4(S988A) mice prior to injections. The same cells were then quantified in the peripheral blood (PB) and bone marrow (BM) 4 weeks after injections with citrate (**E**) or STZ (**F**). *n* = 8. \**P* < 0.05.



**Figure 7**—S988A-expressing mice have potentiated repair capacity. Diabetic  $\alpha 4$ (WT) or  $\alpha 4$ (S988A) mice as indicated were subjected to HLI, and blood perfusion was measured after 3 weeks by LDPI. *A*: Illustrated are representative scans. NI, nonischemic limb; I, ischemic limb. *B*: The perfusion in the ischemic limb was normalized to that of the nonischemic limb and a percentage recovery calculated for each mouse. Illustrated are the average recoveries. *C* and *D*: Isolated muscles from the ischemic limbs were formalin fixed and sections stained with *simplicifolia* lectin I (isolectin). Illustrated are representative images (*C*) and quantitative data (*D*). The sections were also stained with Sirius Red and illustrated are representative images (*E*) and quantitative data (*F*). WT,  $n = 5$ ; S988A,  $n = 6$ . \* $P < 0.05$ .

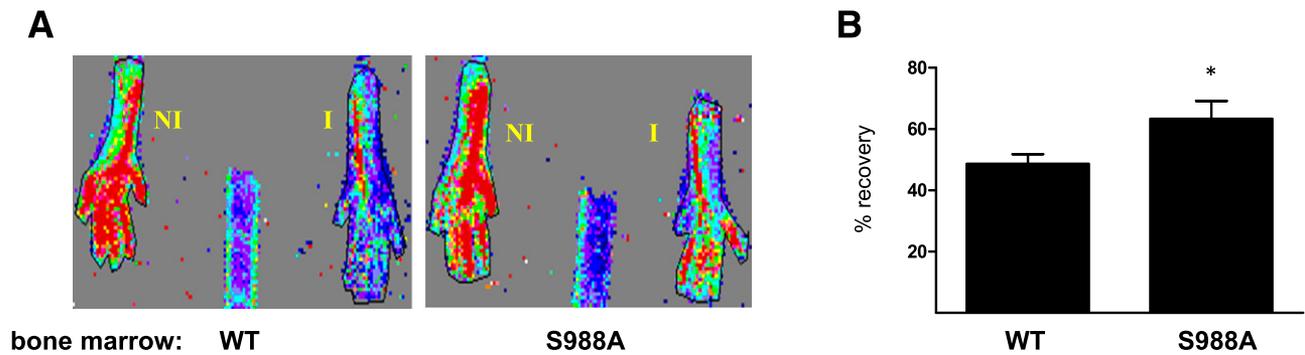
either  $\alpha 4$ (WT) or  $\alpha 4$ (S988A) bone marrow. After repopulation, the mice were made diabetic by STZ injection and subjected to HLI, and vascular perfusion was assessed 3 weeks later. We observed that vascular perfusion in the injured limb of mice receiving the  $\alpha 4$ (S988A) bone marrow had returned to  $63.3 \pm 5.8\%$  of that in the uninjured limb (Fig. 8). In contrast mice receiving  $\alpha 4$ (WT) bone marrow demonstrated only a  $48.7 \pm 3.1\%$  recovery. These findings suggest that the expression of  $\alpha 4$ (S988A) in bone marrow alone is sufficient for enhancing tissue revascularization in diabetic mice.

## DISCUSSION

In this study, we identified a novel mechanism contributing to a deficit of circulating EPCs in diabetes. Specifically, growth in high glucose led to the downregulation of

PRKAR1 $\beta$ , with consequent activation of PKA and  $\alpha 4$ -integrin phosphorylation. This modification of  $\alpha 4$  impacts a pathway regulating cellular adhesion and migration (28). On the basis of these observations, we propose that high levels of phospho- $\alpha 4$ , generated under hyperglycemic conditions, promote an overall increase in EPC adhesion, which presents in vivo as a mobilization defect.

This idea is supported by experiments using ECFCs. First, when grown in high glucose, these cells demonstrated enhanced adhesion to bone marrow stromal cells. Second, this potentiated adhesion was dependent upon VCAM-1 and  $\alpha 4\beta 1$ -integrin, consistent with other studies indicating a major role for this integrin-ligand pair in bone marrow retention (21,33). Third, ECFCs grown in high glucose had enhanced PKA activity and PKA-dependent



**Figure 8**—Mice transplanted with  $\alpha 4$ (S988A) bone marrow display greater revascularization. Irradiated, wild-type C57 mice transplanted with  $\alpha 4$ (WT) or  $\alpha 4$ (S988A) bone marrow as indicated were made diabetic and subjected to HLI. Blood perfusion was measured after 3 weeks by LDPI. **A**: Illustrated are representative scans. NI, nonischemic limb; I, ischemic limb. **B**: The perfusion in the ischemic limb was normalized to that of the nonischemic limb and a percentage recovery calculated for each mouse. WT,  $n = 8$ ; S988A,  $n = 5$ . \* $P < 0.05$ .

$\alpha 4$ -integrin phosphorylation and adhesion. Finally, the observed increase in PKA activity was associated with the downregulation of PRKAR1 $\beta$ , and overexpression of this subunit in high glucose-cultured cells was sufficient to limit PKA activity and reverse the adhesive phenotype.

These cell culture studies were complemented with *in vivo* studies using the STZ model of type 1 diabetes. We found that EPCs from STZ-treated  $\alpha 4$ (S988A) mice had reduced adhesion compared with similar cells from STZ-treated  $\alpha 4$ (WT) mice. Consistent with this attenuated adhesion, diabetic S988A mice demonstrated greater levels of circulating EPCs than their wild-type STZ counterparts. This supports the notion that persistent and systemic vascular injury resulting from hyperglycemia and signaling molecules thereby released (e.g., VEGF) is sufficient to mobilize a more loosely adherent bone marrow cell population. Finally, consistent with quantitative increases of circulating EPCs, we observed that  $\alpha 4$ (S988A) mice, and mice transplanted with  $\alpha 4$ (S988A) bone marrow, displayed enhanced revascularization after injury.

Several mechanisms may contribute to the defective mobilization of EPCs in diabetes. First, as identified here, are increases in progenitor cell adhesiveness. Signaling pathways engaged upon  $\alpha 4$ -integrin phosphorylation result in Rac1 activation, thereby initiating changes in cytoskeletal organization, promoting stable adhesion (28). This signaling mechanism is crucial in migrating cells where phospho- $\alpha 4$  protein displays a polarized distribution (22). Although we have not examined the spatial orientation of phospho- $\alpha 4$  in diabetic EPCs, we propose that levels are uniformly higher throughout the cell, promoting overall adhesion. It follows then that ablation of  $\alpha 4$  phosphorylation, through S988A expression, would decrease adhesive strength, consistent with our observations (Fig. 6C). Further, these loosely adherent cells may be more responsive to mobilizing cues (VEGF and SDF-1 $\alpha$ ), released as a consequence of persistent, diabetes-induced endothelial damage. Although expression of  $\alpha 4$ (S988A) limits bone marrow adhesion and promotes

mobilization, homing and adhesion to the endothelium may not be impaired. Homing involves primarily  $\beta 2$  family integrins (34) and may be independent of  $\alpha 4\beta 1$  (21,34). Thus, the increased availability of EPCs in diabetic S988A mice (Fig. 6F) and their  $\alpha 4\beta 1$ -independent homing to and incorporation at sites of vascular damage may enable the revascularization we observed in these animals (Figs. 7 and 8). Alternatively, paracrine factors released by recruited EPCs may enhance revascularization potential. Although we did not observe an increase in the expression of the  $\alpha 4$  integrin in hyperglycemic conditions, another study has reported this (14). The basis for this discrepancy in  $\alpha 4$  expression between studies is not clear. In addition to  $\alpha 4\beta 1$ , other adhesion molecules may impact progenitor cell retention in diabetes as well. CD11a, CD11c, CD49e, CD62L, and ICAM-1 have all been reported to be increased in bone marrow cells of diabetic mice (14). Interestingly, the same study also reported that CD62L-deficient mice display increased levels of circulating CD34<sup>+</sup>/Flk<sup>+</sup> cells. However, a direct role for CD62L in the bone marrow adhesion of these cells was not demonstrated and there may be other reasons why the CD62L-null animals display enhanced CD34<sup>+</sup>/Flk<sup>+</sup> cell levels.

Second, defective EPC mobilization in diabetes may result from deficits of upstream signaling molecules such as SDF-1 $\alpha$ , VEGF, and nitric oxide (NO) (5,6,16), which trigger EPC mobilization. Indeed, means to promote NO production or signaling have been found to enhance mobilization (6,35,36). Among its effects, NO is believed to activate matrix metalloproteinases (37), which cleave membrane-bound kit ligand (35,37). Soluble kit ligand (kitL; stem cell factor) in turn binds its cell surface receptor, c-kit, enabling cellular release from the bone marrow (35,37). In addition, NO has been reported to affect  $\alpha 4$ -integrin expression (38) and ligand binding affinity (39). Although these properties were not affected in our study, the effects of reduced NO on PKA activity or  $\alpha 4$ -integrin phosphorylation are unknown. Moreover, it is possible that signals downstream of c-kit may impact

PKA activity and  $\alpha 4$ -integrin phosphorylation. Cooperative signaling between growth factor receptors and  $\alpha 4\beta 1$  is not unprecedented. Indeed, it has been demonstrated that interactions between c-kit and  $\alpha 4\beta 1$  signaling pathways are essential for HSC mobilization (27) and in regulating neutrophil retention in the bone marrow as well (40).

A final mechanistic basis of diabetes-impaired mobilization may be alterations of bone marrow structure or responsiveness. Neuropathic outcomes (14) or altered bone marrow physiology and  $\beta$ -adrenergic signaling (17) also appear to impede progenitor cell mobilization in diabetes. Altered stromal properties in diabetic mice have been reported in other studies, although a causal relationship to impaired progenitor cell mobilization was not established (7,41). Thus, diabetes may induce effects on either the progenitor cells themselves or the bone marrow to induce selective retention and defective mobilization.

A significant finding in this study is the novel mechanism upregulating PKA activity in diabetes. Prior studies using animal models of type 1 diabetes have demonstrated variable effects on PKA activity. Whereas some have shown increased activity that is associated with neuronal dysfunction and abnormal cardiac contractility (42,43), others have shown decreased activity that impacts vascular reactivity (44). In addition, a previous study using bovine aortic endothelial cells demonstrated that growth in high glucose media led to an increase in cAMP with presumptive increases in PKA activity, although this was not directly assessed (45). Whereas we did not observe changes of cAMP in ECFCs, we did observe changes in PKA subunit expression resulting in measured increases in enzyme activity. PKA is a tetramer composed of two catalytic subunits and two inhibitory, regulatory subunits. Physiological dissociation of the regulatory subunits, such as that mediated by cAMP, activates PKA. Alternatively, decreased expression of the regulatory subunits can alter tetramer stoichiometry, leading to PKA activation. Such a mechanism seems to be operative in pituitary adenomas and the Carney complex pathologies, which demonstrate reduced levels of PRKAR1 $\alpha$  (46). Increased PKA signaling in these cases appears to drive tumorigenesis. Alterations in PKA regulatory subunit expression have also been observed in lupus T lymphocytes (47,48) and in Bloom syndrome (49), although no pathological causality has been demonstrated in these cases. Finally, a missense mutation in PRKAR1 $\beta$  has been found to segregate with a familial, late-onset neurodegenerative disorder (50). Alterations of localized PKA activity are hypothesized to underlie these neurological defects. Thus our work adds to this understanding of PKA subunit regulation and disease.

Diabetes is a major CVD risk factor and is associated with impaired wound healing, atherosclerosis, hypertension, and increased risk of myocardial infarction. At their core, these outcomes result from a dysfunctional endothelium. Importantly, our studies identify a novel therapeutic target to promote endothelial repair and maintain

its health. Therapeutic strategies to restrain PKA activity and  $\alpha 4$ -integrin phosphorylation in EPCs would enhance their mobilization. The increased availability of EPCs would promote endothelial repair capacity and could ameliorate the cardiovascular consequences of diabetes.

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