# Adiponectin Pretreatment Counteracts the Detrimental Effect of a Diabetic Environment on Endothelial Progenitors

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**OBJECTIVE**—It has been shown that vascular progenitors from patients with diabetes are dysfunctional. However, therapeutic strategies to counteract their reduced functional capacity are still lacking. Because adiponectin has reported salutary effects on endothelial function, we investigated the functional effects of globular adiponectin (gAcrp), the active domain of adiponectin, on isolated endothelial colony-forming cells (ECFC).

**RESEARCH DESIGN AND METHODS**—ECFC were isolated from peripheral blood of type 2 diabetic patients (dmECFC) and compared with ECFC of healthy young volunteers (yECFC) and nondiabetic age-matched control subjects (hECFC). Cells were treated with gAcrp for 48 h followed by assessment of cell counts, cell cycle analysis, and migration capacity. For in vivo evaluation, human ECFC were injected into normoglycemic or streptozotocin-induced hyperglycemic *nu/nu* mice after hind limb ischemia.

**RESULTS**—Whereas dmECFC were functionally impaired compared with yECFC and hECFC, gAcrp significantly enhanced their in vitro proliferation and migratory activity. In vitro effects were significantly stronger in hECFC compared with dmECFC and were mediated through the cyclooxygenase-2 pathway. Most important, however, we observed a profound and sustained increase of the in vivo neovascularization in mice receiving gAcrp-pretreated dmECFC compared with untreated dmECFC under both normoglycemic and hyperglycemic conditions.

**CONCLUSIONS**—Pretreatment of ECFC with gAcrp enhanced the functional capacity of ECFC in vitro and in vivo in normoglycemic and hyperglycemic environments. Therefore, preconditioning of dmECFC with gAcrp may be a novel approach to counteract their functional impairment in diabetes. ***Diabetes* 60:652–661, 2011**

Cardiovascular disease leading to coronary artery disease and stroke remains the most frequent cause of death in patients with diabetes mellitus, whereas impaired perfusion of the extremities leads to foot ulcers and amputations resulting in high morbidity and subsequent socioeconomic burden (1). Emerging evidence from experimental and early clinical studies suggests that endothelial progenitors, mobilized from the bone marrow into peripheral blood, travel to sites of tissue injury, and contribute to neovascularization, thereby improving blood flow and promoting tissue regeneration (2–4). Although bone marrow and peripheral blood-derived endothelial progenitors have convincingly been shown to improve neovascularization in different experimental and clinical settings, the clinical utility of so-called early endothelial progenitors is still limited because of their low proliferation rate and a rather monocytic phenotype (5–11).

In contrast, endothelial colony-forming cells (ECFC), a highly homogeneous subpopulation of endothelial progenitors, bear a high proliferative capacity and strong endothelial commitment (9). ECFC can be derived from cord blood and peripheral blood of healthy donors or patients and are therefore more suitable for cell-based vascular regeneration. Although cord blood-derived ECFC demonstrate a high proliferative capacity compared with peripheral blood-derived ECFC, their clinical use is limited by the need for immunosuppressive therapy. On the other hand, progenitors isolated from patients with cardiovascular risk factors including diabetes are limited in numbers, dysfunctional, and linked to impaired vascular homeostasis and vascular function (12–14). In addition, endothelial progenitor cells (EPC) are also functionally impaired in response to high glucose levels in vitro (15–17). Therefore, cell therapeutic approaches taking advantage of the neovascularization capacity of autologous endothelial progenitors have to include strategies aiming for the restoration of the impaired functional capacity of diabetic patient-derived endothelial progenitors before their in vivo administration.

Several studies reported a close association between circulating levels of adiponectin, a hormone originating from adipocytes (also known as adipocyte complement-related protein of 30 kDa [Acrp30]), and type 2 diabetes mellitus. While lower serum adiponectin levels were found in type 2 diabetes (18), people with high serum adiponectin concentrations are at lower risk for developing diabetes (19,20), and increasing adiponectin concentrations are linked to a lower incidence of developing insulin-resistance and type 2 diabetes (21–23). Therefore, we examined the effects of treating type 2 diabetic patient-derived ECFC with the active domain of adiponectin, termed "globular adiponectin" (gAcrp), with respect to their number and function. We found that gAcrp is an effective in vitro pretreatment modality to functionally rescue type 2 diabetic patient-derived ECFC for advanced in vivo cell therapy applications. Most important, this pretreatment effect was also preserved in hyperglycemic mice with hind limb

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ischemia, further emphasizing the clinical relevance of this novel approach.

## RESEARCH DESIGN AND METHODS

**Isolation of human progenitor cells.** Peripheral blood was obtained after informed consent from type 2 diabetic patients with A1C >7.5% ($n$ = 8; male and postmenopausal female, age 60 ± 5.8 years), nondiabetic age-matched control subjects ($n$ = 5; male and postmenopausal female, 66 ± 3.6 years) (Table 1), and young healthy subjects ($n$ = 5; male, age 26.4 ± 0.8 years) as a source for fully functional ECFC as positive controls. ECFC were isolated from peripheral blood by Ficoll density-gradient centrifugation as previously described (24). Briefly, mononuclear cells were cultured in 6-well tissue culture plates precoated with type 1 rat tail collagen (BD Biosciences, Bedford, MA) with endothelial basal medium-2 supplemented with 10% FBS and EGM-2 SingleQuots (human epidermal growth factor, human fibroblast growth factor-B, vascular endothelial growth factor [VEGF], ascorbic acid, hydrocortisone, Long R3-IGF-1, heparin, gentamicin/amphotericin). After 24 h, nonadherent cells and debris were removed. Medium was changed daily during the first 7 days and then every other day until colonies appeared. Colonies were picked and further expanded for the experiments. Expanded cells were then pretreated with control or human recombinant gAcrp30 (1 μg/mL; Peprotech, Hamburg, Germany) in the presence or absence of parecoxib (100 μmol/L; Dynastat; Pfizer Inc., New York, NY) and superoxide dismutase (SOD) (300 units/mL; Sigma, Steinheim, Germany), respectively, during the final 48 h. To assess population-doubling times, ECFC derived from type 2 diabetic patients or healthy donors were cultured for at least 25 days. Cells were regularly split at 80% confluence, cell numbers were counted, and 500,000 cells were replated in a fresh dish. Population doubling time was calculated as follows: $T_d = (t_2 - t_1) \times \log(2)/\log(q_2/q_1)$; ($T_d$: population doubling time; $t_2$: end-time point; $t_1$: start-time point; $q_2$: end population size; $q_1$: start population size).

**Flow cytometry.** Surface expression of CD31, CD34, and VEGF receptor 2 (VEGFR-2) was assessed by flow cytometry. Cells were preincubated with Flebogamma (Grifols, Langen, Germany) to prevent unspecific binding of antibodies and were stained using antibodies against CD31, CD34 (both eBioscience, San Diego, CA) and VEGFR-2 (ReliaTech, Wolfenbüttel, Germany). Each analysis included $10^5$ gated events. Cell cycle analysis was performed using a BrdU Flow Kit (BD Biosciences) according to the manufacturer's instructions. Cells were pulsed with BrdU for 2 h. All samples were analyzed using a FACSCalibur, and data were analyzed with CELLQuest Pro software (both BD Biosciences) or FlowJo (Tree Star, Ashland, Oregon).

**mRNA expression.** For the detection of the specific adiponectin receptors AdipoR1 and AdipoR2, total RNA from both healthy donors and diabetic patient-derived ECFC was isolated using the RNeasy kit (Qiagen, Hilden, Germany). Quantification of AdipoR1 and AdipoR2 mRNA was performed by RT-PCR of 100 ng RNA using previously described primers (25).

**Knock-down of AdipoR1 and AdipoR2.** To determine the functional relevance of AdipoR1 and AdipoR2, we transfected ECFC with Accell small interfering (si)RNA (Thermo Scientific, Waltham, MA) for AdipoR1 or AdipoR2 according to the manufacturer's instructions. Then, changes in cell numbers in response to gAcrp were assessed.

**Assessment of in vitro migratory capacity of progenitor cells.** Cells were pretreated for 48 h with 1 μg/mL gAcrp, 100 μmol/L parecoxib, 300 units/mL SOD, or combinations of those. A total of $5 \times 10^5$ cells each was resuspended in 250 μL Dulbecco's modified Eagle's medium + 5% FCS and placed in the upper chamber of a modified Boyden chamber filled with Matrigel (BioCoat invasion assay, 8-μm pore size, BD Biosciences). The upper chamber was placed in a 24-well culture dish containing 500 μL EGM-2. After 12 hours of incubation at 37°C with 5% $CO_2$, transmigrated cells were stained with di aminido phenyl indol, and 4–6 separate visual fields were recorded with an AxioScope 40 with AxioCam MRc 5 (Carl Zeiss, Jena, Germany) at 10× magnification and counted with ImageJ (National Institutes of Health, Bethesda, MD).

**Live imaging of superoxide production in vitro.** Cells were stimulated for 12 h with 1 μg/mL gAcrp and incubated with MitoSOX Red (Invitrogen, Barcelona, Spain; 5 μmol/L) for 10 min before analysis. Levels of mitochondrial superoxide in the presence or absence of gAcrp were measured by quantification of MitoSOX Red intensity using a Leica SP2 confocal microscope (Leica, Wetzlar, Germany).

**Measurement of prostaglandin $E_2$ and nitric oxide levels in cell culture supernatants.** To determine levels of prostaglandin $E_2$ ($PGE_2$) and nitric oxide, we used a $PGE_2$ ELISA (Cayman Chemical, Ann Arbor, MI) and a fluorometric nitric oxide assay kit (Biovision, Mountain View, CA), respectively, according to the manufacturers' instructions.

**Animal models.** All animal experiments were conducted in accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee of the Centro Nacional de Investigaciones Oncológicas and by the Administrative Panel on Laboratory Animal Care (Government of Upper Bavaria, Germany). Female athymic NMRI *nu/nu* mice (Janvier, Le Genest-Saint-Isle, France) were used. For induction of diabetes, animals were treated according to the low-dose streptozotocin induction protocol of the Animal Models of Diabetic Complications Consortium. Briefly, animals were fasted for 4 h and afterward intraperitoneally injected with streptozotocin (50 mg/kg; Sigma). This procedure was repeated daily for 5 days. On day 28, hyperglycemia was confirmed by urine glucose analyses with Combur 10 urine test strips (Roche, Grenzach-Wyhlen, Germany). Only animals with a minimum glucose grade of 3+ (>750 mg/dL) were used for experiments.

Normoglycemic and hyperglycemic mice were subjected to hind limb ischemia followed by intravenous administration of human progenitor cells ($5 \times 10^5$ cells per mouse) (26) after 24 h. To achieve hind limb ischemia, the proximal portion of the right femoral artery, including the superficial and deep branches, was irreversibly disrupted using electrical coagulation. The overlying skin was closed using surgical staples. After 1 and 2 weeks, respectively, limb perfusion in the ischemic (right) and nonischemic (left) limb was assessed using an O2C laser Doppler blood flow analyzer with a LFM-2 micro probe (2 mm tissue penetration; Lea Medizintechnik, Giessen, Germany). Before analysis, mice were placed on a heating pad at 37°C to minimize variations in ambient temperature. Relative perfusion was calculated as the ratio of blood flow in the ischemic and nonischemic limbs. Furthermore, we assessed the blood flow by intravital near-infrared fluorescence imaging (27). After intravenous injection of the near-infrared fluorophore indocyanine green (Sigma), images were obtained by an In Vivo Imaging System-200 (Caliper Life Sciences, Hopkinton, MA) and analyzed using Living Image 3.2.

**Statistical analysis.** Results for continuous variables are expressed as means ± SEM. Overall comparison of continuous variables was performed with the Kruskal-Wallis test followed by post hoc pairwise comparison using the Mann-Whitney test. Comparison of categoric variables was done using the Pearson $\chi^2$ test. $P < 0.05$ was considered statistically significant. All analyses were performed with SPSS 16.0 (SPSS Inc., Chicago, IL).

TABLE 1
Patient characteristics of diabetic patients and age-matched nondiabetic control subjects

| | Nondiabetic control subjects; $n$ = 5 | Diabetic patients; $n$ = 8 | $P$ value |
|---|---|---|---|
| Height (m) | 1.69 ± 0.05 | 1.72 ± 0.03 | NS |
| Weight (kg) | 75.6 ± 6.1 | 103 ± 7.9 | NS |
| BMI (kg/m$^2$) | 26.3 ± 1.3 | 34.9 ± 2.4 | 0.03 |
| Age (years) | 66 ± 3.6 | 60 ± 5.5 | NS |
| Hypertension | 3 (60%) | 7 (88%) | NS |
| Hypercholesterinemia | 3 (60%) | 6 (75%) | NS |
| Hypertriglyceridemia | 0 (0%) | 3 (38%) | NS |
| CAD | 0 (0%) | 1 (13%) | NS |
| MI | 0 (0%) | 0 (0%) | — |
| PAD | 0 (0%) | 0 (0%) | — |
| Stroke | 0 (0%) | 1 (13%) | NS |
| Renal insufficiency | 0 (0%) | 1 (13%) | NS |
| Antidiabetic medication | 0 (0%) | 8 (100%) | 0.001 |
| Antihypertensive medication | 3 (60%) | 6 (75%) | NS |
| Lipid-lowering medication | 1 (20%) | 3 (38%) | NS |

Results for continuous variables are expressed as means ± SEM. Comparisons between groups were analyzed by the nonparametric Mann-Whitney $U$ test. Comparison of categoric variables was tested by the Pearson $\chi^2$ test. CAD, coronary artery disease; MI, myocardial infarction; PAD, peripheral artery disease.

## RESULTS

**Reduced population doubling time in type 2 diabetic patient-derived ECFC.** First, we determined differences in proliferation between diabetic ECFC (dmECFC) and

ECFC derived from young nondiabetic controls (yECFC) as maximally functional positive controls. To assess population doubling time, we kept ECFC in culture over a period of 1 month. Cells were passaged and cell numbers were recorded during splitting of the cells every time cells had reached 80% confluency. After only 5 days, yECFC were already doubled in numbers compared with dmECFC. On day 15, the population of yECFC exceeded that of dmECFC by 2.9-fold, followed by 3.2-fold excess after 25 days. The resulting calculated population doubling time was 6.7 days for dmECFC compared with 4.6 days for yECFC ($P < 0.05$) (Fig. 1*A*). We did not find significant functional differences between yECFC and ECFC derived from aged, but nondiabetic individuals (hECFC) as assessed by cell numbers after 48 h of culture (Fig. 1*B*). Because the baseline characteristics of donors for hECFC and dmECFC only significantly differed for BMI and antidiabetic medication (Table 1), these data are consistent with the hypothesis that the impaired function of dmECFC is most likely related to the diabetic environment.

**Phenotypic evaluation of hECFC and dmECFC.** By conventional microscopy, dmECFC showed a similar gross morphology compared with hECFC and previously described cord-blood derived ECFC (9) (Supplementary Fig. 1*A*). As assessed by flow cytometry, expression of the endothelial markers CD31 and VEGFR-2, as well as the

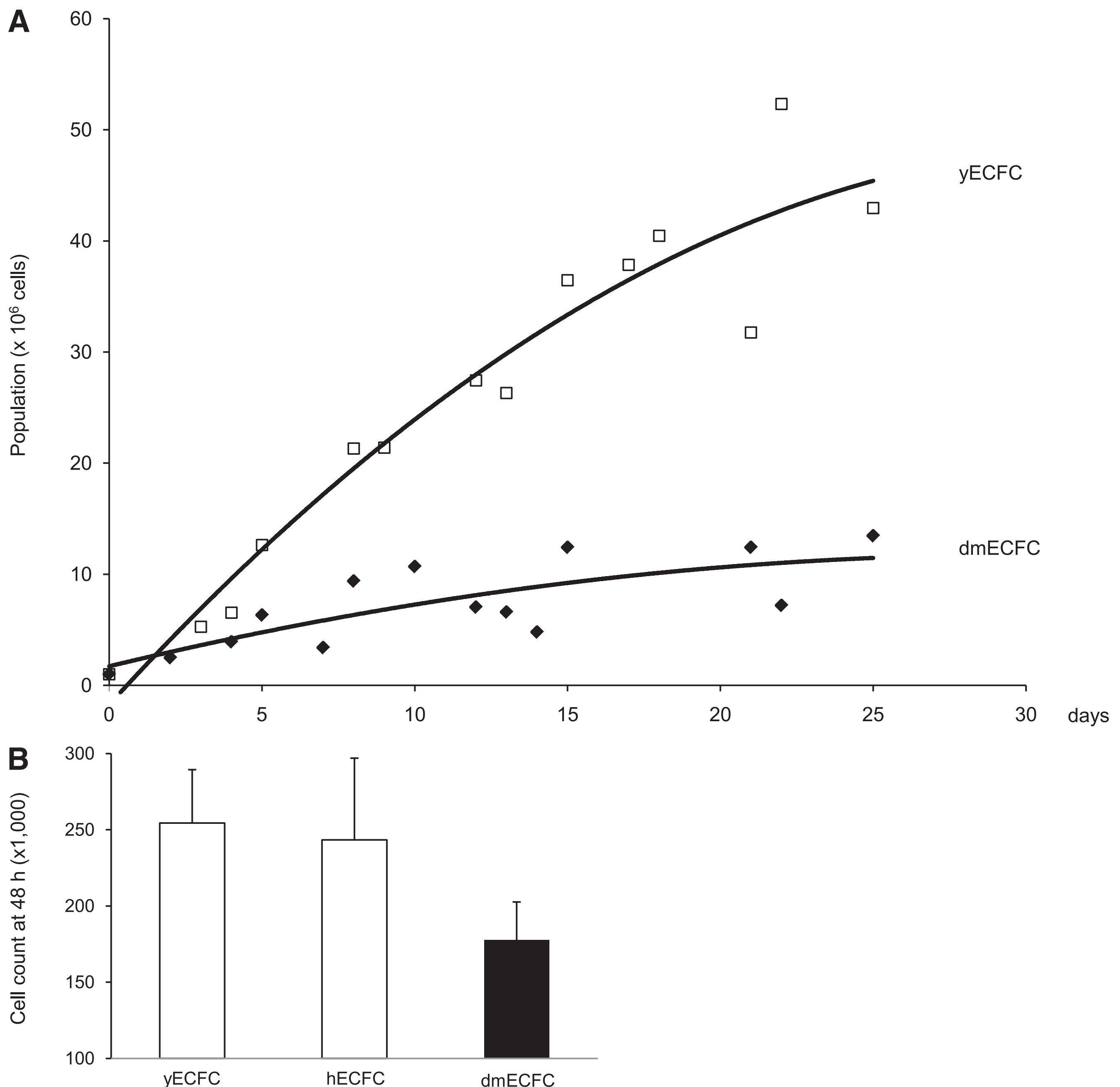



**FIG. 1. Characterization of nondiabetic ECFC vs. diabetic ECFC. *A*: Proliferation of yECFC (white squares; $n = 5$) and dmECFC (black diamonds; $n = 4$) was compared by population doubling curves ($P < 0.01$ dmECFC vs. yECFC). *B*: Cell numbers for yECFC ($n = 5$), hECFC ($n = 3$), and dmECFC ($n = 8$) compared after 48 h in culture.**

progenitor cell marker CD34, was similar (Supplementary Fig. 1*B*) and unchanged over a period of 1 month in both diabetic dmECFC and nondiabetic hECFC (Supplementary Fig. 1*C*).

**gAcrp stimulates ECFC proliferation.** Next, we investigated the effects of gAcrp pretreatment on the proliferative capacity of ECFC. In vitro pretreatment with gAcrp for 48 h led to a significant increase in cell numbers for nondiabetic age-matched control subjects (hECFC) (48.4 ± 14.1% increase compared with control treated hECFC) and dmECFC (18 ± 2.5% increase compared with control treated dmECFC) (Fig. 2*A*). Therefore, ECFC derived from hyperglycemic patients were significantly ($P < 0.05$) less responsive to gAcrp treatment compared with ECFC derived from normoglycemic environments. To investigate the specific mechanism of the modestly higher yield of dmECFC in the gAcrp-treated group, we performed cell cycle analyses. Stimulation with gAcrp increased the fraction of dmECFC in S-phase to 195.4 ± 48.5% of control ($P < 0.05$, Fig. 2*B* and *C*).

**Mechanisms of impaired proliferation of dmECFC.** First, we examined whether hECFC and dmECFC are similarly endowed with the specific adiponectin receptors AdipoR1 and AdipoR2 by RT-PCR (28). Indeed, hECFC and dmECFC both express AdipoR1 and AdipoR2 at similar and comparably high levels, which also did not differ from the expression levels of yECFC (Supplementary Fig. 2). Therefore, we investigated the effects of gAcrp on expansion of ECFC in vitro. To elucidate mechanisms underlying the reduced response of dmECFC to gAcrp compared with hECFC, we first validated that adiponectin receptors are operative in dmECFC. For this purpose, we silenced AdipoR1 and AdipoR2 mRNA by specific small interfering RNA (Fig. 3*A*). Silencing of the individual adiponectin receptors AdipoR1 or AdipoR2 or a combination of those resulted in a significant decrease in cell numbers ($P < 0.05$), which could not be reversed by gAcrp treatment. These data confirm that dmECFC express functional adiponectin receptors AdipoR1 or AdipoR2. Then, we focused on enhanced oxidative stress as a mechanism of impaired gAcrp response in dmECFC because EPC dysfunction in patients with type 2 diabetes has been linked to excessive generation of reactive oxygen species (29). To compensate the less pronounced stimulatory effect of gAcrp on dmECFC compared with hECFC, we also treated the cells with SOD to reduce oxidative stress. Indeed, whereas SOD alone had no effects on dmECFC yield, SOD pretreatment resulted in enhanced stimulation of dmECFC (33.5 ± 8.7% increase in SOD + gAcrp) (Fig. 3*B*). To clarify the effect of gAcrp administration on oxidative stress in ECFC, we pretreated hECFC and dmECFC for 12 h with gAcrp and performed live imaging of the intracellular production of superoxide anions using MitoSOX Red (Fig. 3*C*). Quantification of the signal intensity demonstrated that pretreatment with gAcrp significantly reduced superoxide levels in diabetic ECFC ($P < 0.01$). Therefore, we conclude that the effects of gAcrp in dmECFC are at least in part superoxide-dependent/related.

Furthermore, adiponectin has been reported to induce activation of the cyclooxygenase 2 (COX-2) pathway and subsequently promotes $PGE_2$ synthesis in stromal cells (30). We consistently show that gAcrp induces $PGE_2$ production (Fig. 3*D*). We recently demonstrated that $PGE_2$ stimulates proliferation and function of EPC, an effect that was abrogated after inhibition of COX-2 (31). Thus, we hypothesized that gAcrp-induced effects in ECFC are mediated via the COX-2 pathway. Administration of the selective COX-2 inhibitor parecoxib alone had no effects on cell proliferation, but potently blocked the gAcrp-mediated increase in cell numbers when simultaneously

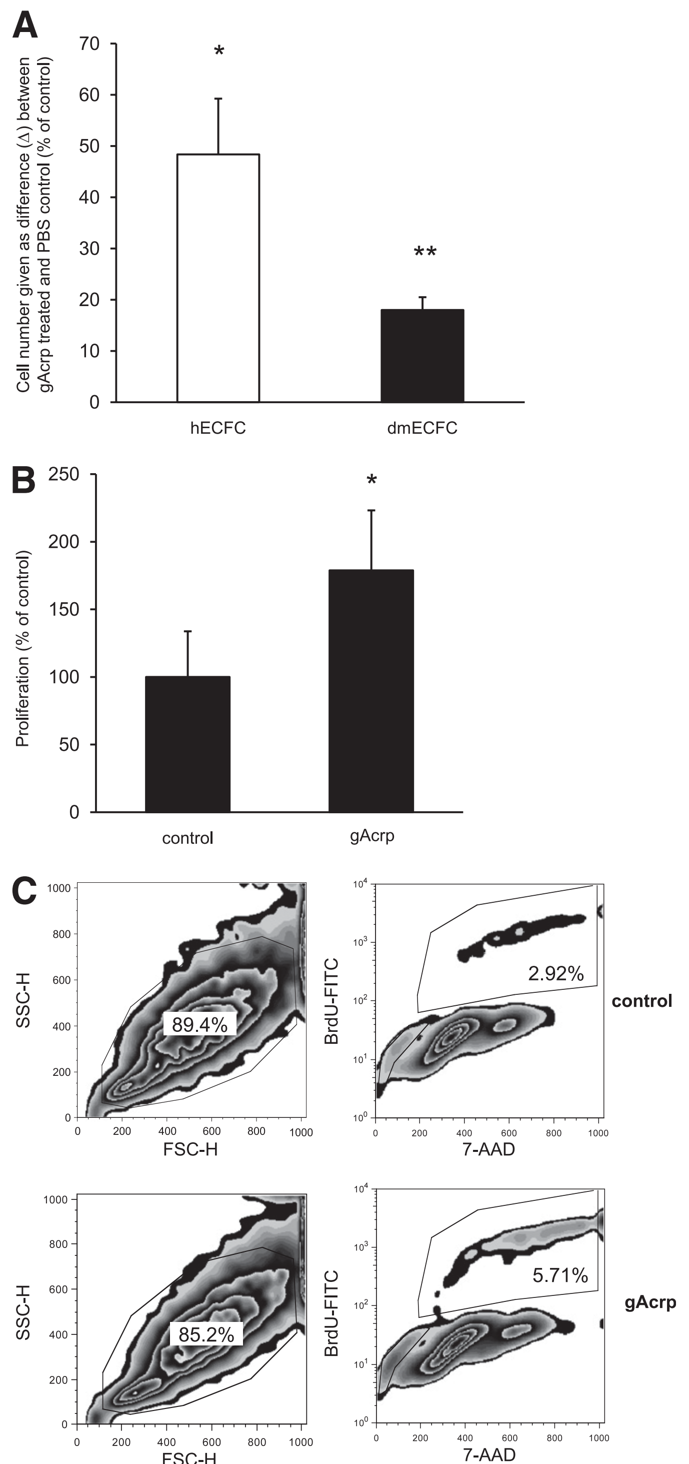



**FIG. 2. Effect of pretreatment with gAcrp on ECFC numbers and proliferation. *A*: Cell numbers for hECFC (*n* = 3) and dmECFC (*n* = 8) after stimulation with 1 μg/mL gAcrp given as difference between gAcrp treatment and PBS control group (*P < 0.05 vs. control, **P < 0.05 vs. dmECFC and control). *B*: Proliferation measured as BrdU incorporation after stimulation with gAcrp (*n* = 3, *P < 0.05). *C*: Representative pictures of the gating strategy used for the evaluation of BrdU incorporation by flow cytometry. *Upper*: control. *Lower*: gAcrp stimulation.**

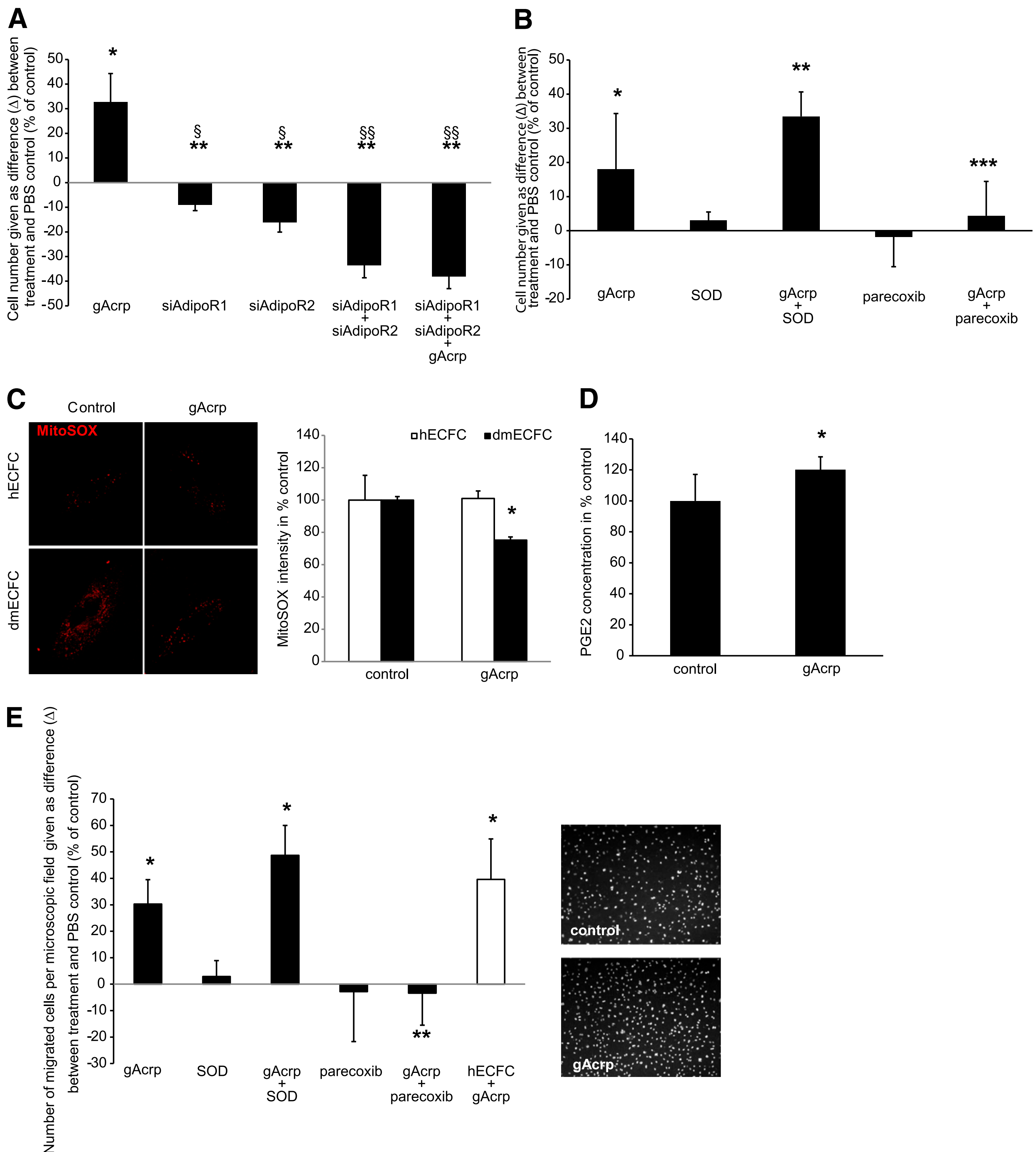



**FIG. 3. Mechanism of reduced gAcrp response in dmECFC.** ***A*****: Expression of functional adiponectin receptors on dmECFC cells. Selective silencing of AdipoR1 and AdipoR2 was performed using small interfering RNA. Stimulation of the cells by gAcrp was assessed as ECFC yield (****P*** **< 0.05 vs. control, *****P*** **< 0.05 vs. gAcrp, §*****P*** **< 0.05 vs. AdipoR1 + AdipoR2 + gAcrp, §§*****P*** **< 0.05 vs. AdipoR1 + gAcrp and AdipoR2 + gAcrp,** ***n*** **= 3).** ***B*****: Effect of treatment of dmECFC with gAcrp (*****n*** **= 7), SOD (*****n*** **= 4), parecoxib (*****n*** **= 6), alone or in combination with gAcrp on cell numbers, given as difference between treatment groups and PBS control (****P*** **< 0.001 vs. control; *****P*** **< 0.05; ******P*** **< 0.01 vs. control,** ***P*** **< 0.05 vs. gAcrp).** ***C*****: Effect of pre-treatment with gAcrp on intracellular superoxide production in ECFC. Representative live images (*****left*****) of untreated or gAcrp-treated hECFC and dmECFC after incubation with MitoSOX Red and quantification of MitoSOX Red intensity (*****right*****) (****P*** **< 0.01 vs. control,** ***n*** **= 3).** ***D*****: Production of $PGE_2$ in supernatants of dmECFC treated with or without gAcrp as measured by ELISA. ****P*** **< 0.01 vs. control.** ***E*****: Migratory activity of ECFC after gAcrp (*****n*** **= 4) preconditioning of dmECFC in the presence or absence of SOD or parecoxib (each** ***n*** **= 3), given as difference between treatment groups and PBS control. The last white bar shows comparison with hECFC treated with gAcrp (****P*** **< 0.01 vs. control; *****P*** **< 0.05 vs. gAcrp,** ***n*** **= 3). Representative images for migrated dmECFC pretreated with control (*****upper right*****) or gAcrp (*****lower right*****). (A high-quality color representation of this figure is available in the online issue.)**

administered with gAcrp (Fig. 3*B*), suggesting that the stimulatory effect of gAcrp is at least in part mediated via the COX-2 pathway. Moreover, we recently showed that prostaglandin $E_1$ and $PGE_2$ stimulate progenitor cell proliferation and function via induction of endothelial nitric oxide synthase (eNOS) (31). To investigate whether gAcrp-induced ECFC numbers might be dependent on the eNOS pathway, we collected supernatants for a fluorometric nitric oxide assay demonstrating that gAcrp treatment results in a significant increase in nitrite levels indicative for enhanced nitric oxide production (142.2 ± 21.8% control, $P < 0.05$, Supplementary Fig. 3). Therefore, we provide evidence that gAcrp directly stimulates nitric oxide production, which suggests involvement of the eNOS pathway in gAcrp-induced functional improvements of diabetic patient-derived ECFC.

**gAcrp stimulation enhances migratory activity of ECFC.** Migration is a key feature of EPC crucial for their in vivo cell therapeutic effects (32). ECFC pretreated with gAcrp showed a similarly increased migratory activity for nondiabetic age-matched control subjects (hECFC: 39.6 ± 15.3% control increase) and for dmECFC (30.3 ± 9.2% control increase) (Fig. 3*E*). Likewise, pretreatment with parecoxib alone did not affect migratory activity, whereas the stimulatory effect of gAcrp vanished when combined with parecoxib. On a functional basis, simultaneous treatment with gAcrp and SOD compared with gAcrp alone showed a further trend to improved migratory activity (48.7 ± 11.3% increase for gAcrp + SOD vs. 30.3 ± 9.2% increase for gAcrp alone; $P = 0.289$).

**In vivo neovascularization capacity of dmECFC is impaired and can be rescued by gAcrp.** Smadja et al. (33) showed that cord blood-derived ECFC are capable of enhancing neovascularization in an in vivo hind limb ischemia model, but data for hECFC and dmECFC were still lacking. Therefore, we set out to evaluate whether ECFC from nondiabetic donors and patients with type 2 diabetes are capable of enhancing neovascularization in a normoglycemic *nu/nu* mouse hind limb ischemia model as the most permissive environment (Fig. 4*A*). Indeed, we observed that nondiabetic age-matched controls (hECFC) significantly ($P < 0.05$) enhance hind limb perfusion (relative blood flow in ischemic vs. nonischemic hind limb; 60.9 ± 10.1% for hECFC vs. 20.1 ± 7.5% for untreated control) (Fig. 4*B* and *C*). Consistent with the above in vitro data, dmECFC-treated animals also showed augmented blood flow; however, the difference did not reach a level of statistical significance relative to control because of their reduced in vivo neovascularization capacity (relative blood flow: 34.9 ± 6.6% in dmECFC vs. 20.1 ± 7.5% in untreated control) (Fig. 4*B* and *C*).

Next, we evaluated the in vivo relevance of our in vitro pretreatment protocol using gAcrp. Pretreated dmECFC significantly facilitated hind limb perfusion (relative blood flow: 65.2 ± 5.1%, $P < 0.01$) versus control dmECFC. The effects of gAcrp-pretreated dmECFC no longer differed from those observed for hECFC ($P = 0.67$) (Fig. 4*B* and *C*).

**Preconditioning effects of gAcrp are maintained in hyperglycemia.** Because a hyperglycemic environment has been shown to negatively affect endothelial progenitors in vitro (15,17), we further assessed whether the in vivo neovascularization capacity of ECFC pretreatment with gAcrp is sustained in a hyperglycemic environment in vivo (Fig. 5*A*). We observed consistent treatment effects as seen in the normoglycemic hind limb ischemia model. Although untreated control animals showed poor hind limb perfusion after ischemia (relative perfusion: 18.5 ± 2.5%), the administration of untreated dmECFC improved relative limb perfusion to 45.3 ± 5.5% ($P < 0.05$ vs. control) (Fig. 5*B*). Most important, animals showed even further and significant improvements in limb perfusion after infusion of dmECFC that were pretreated with gAcrp (71.8 ± 2.9%, $P < 0.05$ vs. control, $P < 0.05$ vs. untreated dmECFC). Thus, gAcrp-pretreated dmECFC restore the drastically impaired blood flow in an ischemic hind limb model, an effect that also persists in a clinically most relevant hyperglycemic environment.

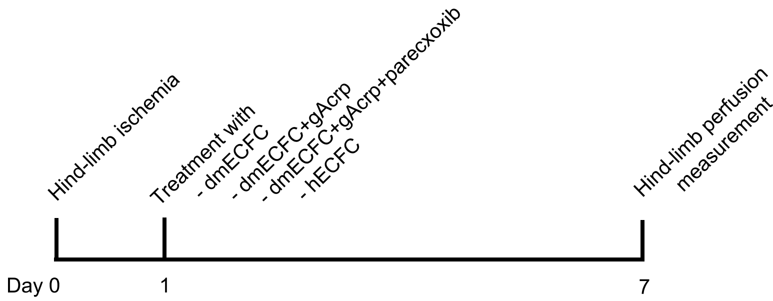
## DISCUSSION

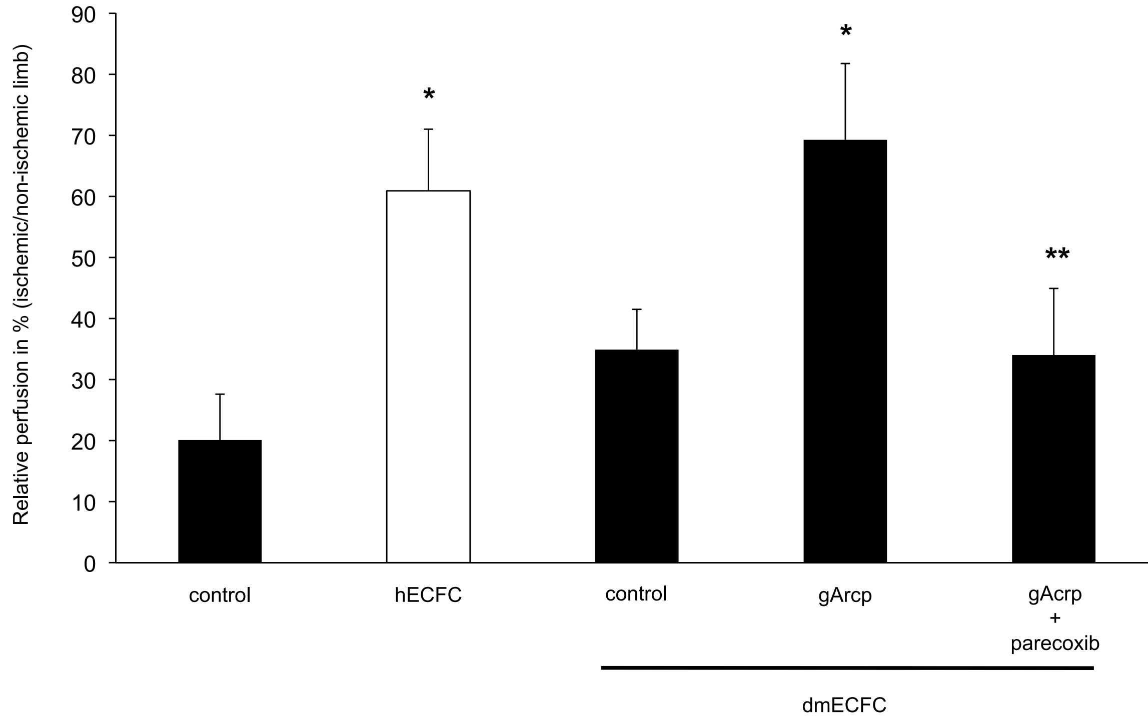
We provide evidence for the salutary effects of gAcrp on number and function of EPC derived from patients with type 2 diabetes. We found that gAcrp-pretreated ECFC are more suitable for cell therapy compared with their untreated dysfunctional counterparts. Most strikingly, these effects were observed in vivo both under nondiabetic conditions and in the hyperglycemic environment of diabetic mice. On the basis of the presented data, we established gAcrp pretreatment for diabetic patient-derived ECFC as a novel way of tailored cell optimization before their use for autologous cell therapy.

Impaired proliferation and function of EPC in patients with type 2 diabetes has been described (16,34–36). Ingram et al. (17) provided the first evidence that cord blood-derived ECFC from newborns of mothers with gestational diabetes also displayed lower proliferation rates and reduced neovascularization potency both in vitro and in vivo. Our experiments further emphasize these findings and add new evidence for an impairment of peripheral blood-derived ECFC in adult patients. Indeed, we observed a reduced proliferation rate of dmECFC in vitro and, more important, a reduced neovascularization capacity in vivo compared with nondiabetic ECFC. Therefore, optimization strategies are absolutely crucial for the clinical use of these cells in an autologous setting.

In 1995, Scherer et al. (37) first described a serum protein similar to the complement component C1q that is produced exclusively in adipocytes and then introduced it as adipocyte complement-related protein of 30 kDa (Acrp30), which was later termed "adiponectin." Several studies demonstrated a correlation between adiponectin and type 2 diabetes mellitus. Hotta et al. (18) found lower serum adiponectin levels in patients with type 2 diabetes compared with BMI-matched control subjects. Reciprocally, people with high serum concentrations seem to have a lower risk of developing diabetes (19,20), and increasing adiponectin concentrations seem to minimize chances to develop insulin resistance and type 2 diabetes (21–23). Only recently, Shibata et al. (38) demonstrated a correlation between adiponectin levels and the number of blood-borne early EPC. In addition, they also reported a beneficial effect of adiponectin on the number of early EPC in vitro. Stimulatory effects of adiponectin have also been shown on hematopoietic stem cells, osteoblasts, and mesangioblasts (38–41). In the current study, we now describe for the first time a strong effect of gAcrp pretreatment on highly purified ECFC before their administration in ischemic models in vivo.

Shibata et al. (42) demonstrated a protective effect of adiponectin for myocardial ischemia–reperfusion injury mediated by inhibition of apoptosis through AMP-activated



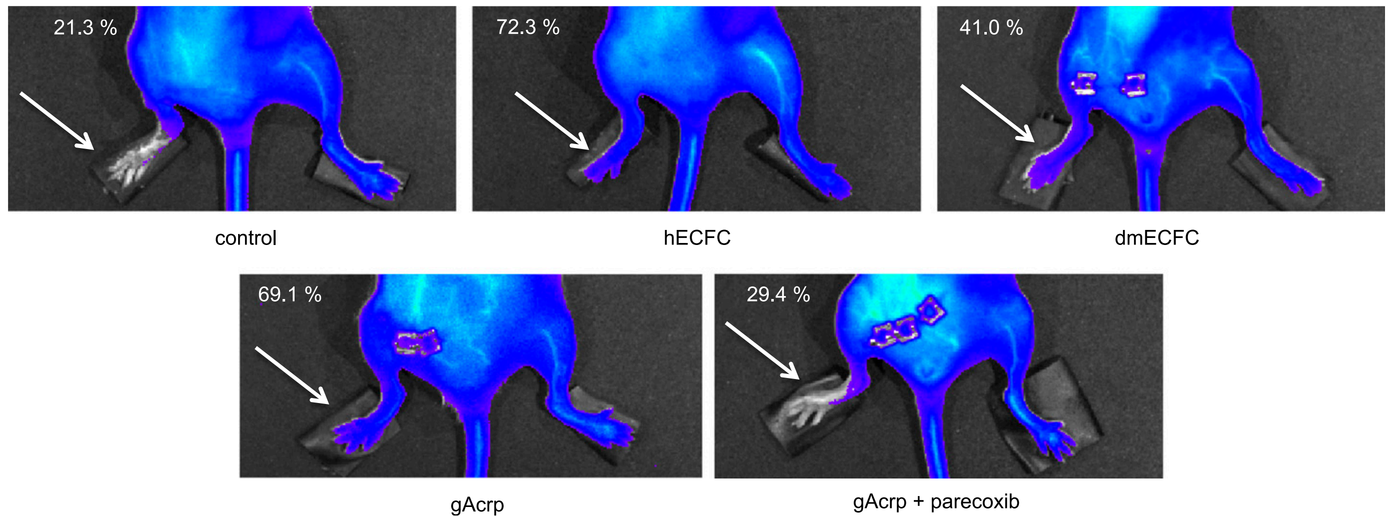




**FIG. 4. Perfusion in a hind limb ischemia model after dmECFC pretreatment with gAcrp. *A*: Experimental setup for the evaluation of efficacy of treatment with dmECFC or hECFC in a normoglycemic NMRI *nu/nu* model. *B*: Quantification of hind limb perfusion after injection of dmECFC treated with gAcrp in the presence or absence of parecoxib compared with hECFC (*$P$ < 0.05 vs. control, $P$ < 0.05 vs. dmECFC, **$P$ < 0.05 vs. gAcrp, $n$ = 4–8). *C*: Representative pictures from hind limb perfusion measurements, as assessed by indocyanine green injection. Arrows indicate the ischemic hind limb. Relative perfusion between ischemic and nonischemic hind limbs is given in percent. (A high-quality digital representation of this figure is available in the online issue.)**

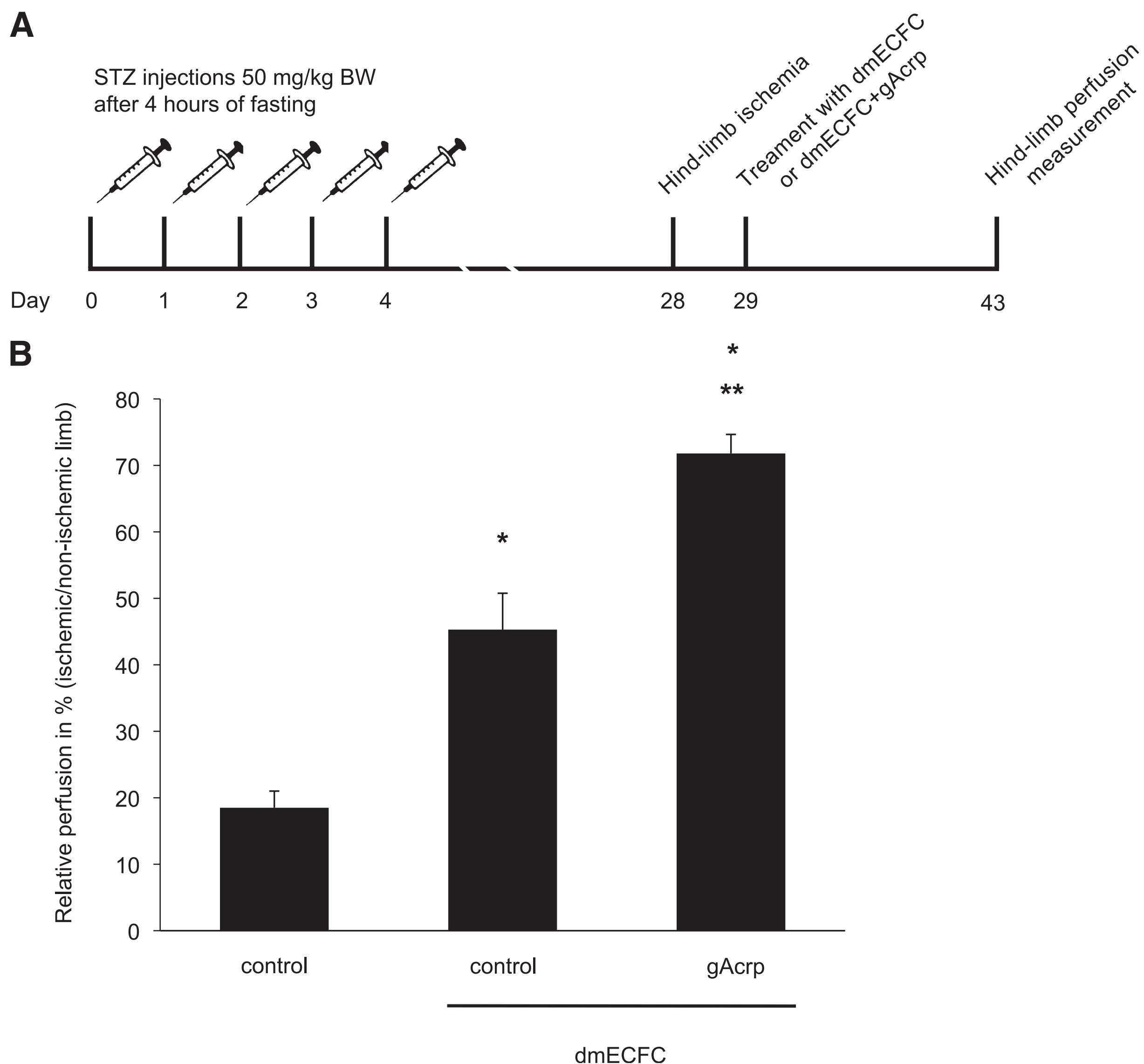



**FIG. 5. Perfusion in a diabetic hind limb ischemia model after dmECFC pretreatment with gAcrp. *A*: NMRI *ν/ν* were treated according to the low-dose streptozotocin protocol of the Animal Models of Diabetic Complications Consortium to create hyperglycemia before hind limb surgery. *B*: Relative perfusion as assessed by O2C laser Doppler measurements in hyperglycemic mice (**P* < 0.05 vs. control; ***P* < 0.05 vs. dmECFC, *n* = 3–6). After 2 weeks, hind limb perfusion was measured via laser Doppler in both the ischemic and nonischemic legs. STZ, streptozotocin.**

protein kinase and by inhibition of tumor necrosis factor-α through COX-2–dependent pathways. In addition, stromal cells and lymphocyte precursors are influenced by adiponectin through the COX-2 pathway (30). Moreover, we recently demonstrated that prostaglandin $E_1$ and $PGE_2$ stimulate progenitor cell proliferation and function through induction of eNOS (31). Consequently, we postulated that the observed effects of gAcrp are primarily mediated through induction of COX-2, followed by a consecutive increase of $PGE_2$ secretion and eNOS expression leading to enhanced ECFC proliferation and, most important, improved function. Indeed, inhibition of the COX-2 pathway virtually abrogated adiponectin-induced effects on ECFC.

Of note, not only the native proliferation of dmECFC was impaired in comparison with nondiabetic ECFC but also the gAcrp-mediated increase in proliferation was less pronounced in dmECFC. Oxidative stress has been identified as one of the major obstacles for progenitor cell proliferation by forcing them into senescence (43). By expressing antioxidative enzymes at high levels, in particular SOD, progenitor cells are usually well protected against oxidative stress (44). Still, Ceradini et al. (45) demonstrated in a streptozotocin-induced diabetes murine model that mobilization and migratory activity of bone marrow-derived EPC are vastly reduced because of oxidative stress. Addition of SOD was able to reverse the observed negative effects. Consistently, we now show that gAcrp-enhanced dmECFC cell numbers can be further increased by subsidiary addition of SOD. Nevertheless, gAcrp alone already showed a significant increase in cell number, which is supported by other antioxidative features of adiponectin, as previously shown (46), and was sufficient to generate strong in vivo cell therapeutic effects. In line with these data, our study clearly demonstrates that gAcrp treatment reduces reactive oxygen levels in diabetic ECFC. Moreover, strong invasive characteristics, which can be assessed in vitro as cell migration in a

Matrigel-coated modified Boyden chamber, are important features mandatory for systemically infused progenitor cells to invade the ischemic tissue. Notably, gAcrp treatment of dmECFC alone also provoked a marked increase in migratory activity that was comparable to that observed in nondiabetic ECFC. This increased migratory activity could be further enhanced by simultaneous treatment with SOD, though not on a statistically significant level. gAcrp-pretreated dmECFC significantly improved neovascularization in a murine hind limb ischemia model, whereas untreated dmECFC showed inferior results with respect to neovascularization improvements. These data suggest that, although proliferation of dmECFC is still limited by oxidative stress, gAcrp pretreatment still leads to a robust functional restoration of dmECFC leading to strong in vivo regenerative activity.

One may speculate that reexposure of gAcrp-pretreated ECFC to a hyperglycemic environment reverses the gained function of ECFC via aforementioned or yet unknown mechanisms. A recently published study of Eren et al. (47) studied the question whether the success of vascular cell therapy relies on adiponectinemia in the cell donor or in the cell recipient. The authors concluded that adiponectinemia in the recipient is essential for the proangiogenic effects of cell therapy. These data suggest that adiponectin-pretreated dmECFC might be ineffective in a hyperglycemic environment associated with depressed adiponectin blood levels. However, our data clearly show that optimization of vascular cell therapy with adiponectin indeed withstands an unfavorable hyperglycemic environment in vivo.

In conclusion, we demonstrate for the first time that treatment effects of ex vivo gAcrp pretreatment of ECFC are preserved even after retransfer of dmECFC to a hyperglycemic environment. We demonstrate feasibility and efficacy of an ex vivo pretreatment protocol aiming for progenitor cell optimization before their use for cell therapy. Furthermore, our present data corroborate our previous findings regarding the importance of the COX-2 pathway for the neovascularization capacity of isolated ECFC (31), and we thus provide important new data that the activation of the COX-2 pathway, which can safely be achieved by gAcrp administration, may be a major target for future EPC optimization strategies.

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S.F.L. researched data, contributed to discussion, wrote the article, and reviewed and edited the article. T.M.S. researched data and reviewed and edited the article. P.C.H. researched data and reviewed and edited the article. J.S. reviewed and edited the article. A.A. researched data, contributed to discussion, and reviewed and edited the article. C.H. researched data, contributed to discussion, wrote the article, and reviewed and edited the article.

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