Aims A close relationship exists between hyperglycaemia, oxidative stress, and diabetic complications. In fact, high glucose (HG) determines the overproduction of reactive oxygen species (ROS) by the mitochondria. p66ShcA is a gene that regulates the apoptotic responses to oxidative stress. Indeed, p66ShcA knockout (ko) mice display decreased ROS production and increased resistance to ROS-induced cell death in a variety of pathophysiological settings. Reduced endothelial progenitor cell (EPC) number, differentiation, and function are relevant components of the angiogenesis impairment observed in diabetic patients. We examined the role of p66ShcA in the EPC deficit induced by HG.

Methods and results Mouse bone marrow-derived c-kit+ cells differentiate in endothelial-like cells when plated on fibronectin (BM-derived EPCs). We found that cell culture in the presence of HG upregulated p66ShcA protein expression and that HG exposure markedly decreased the number of BM-derived EPCs. Conversely, p66ShcA ko BM-derived EPCs were not sensitive to HG inhibition. Indeed, the resistance of p66ShcA ko BM-derived EPCs to HG was associated with reduced levels of both apoptosis and oxidative stress. To functionally link the HG response to ROS production, p66ShcA ko BM-derived EPCs were reconstituted either with p66ShcA wild-type (wt) or with a p66ShcA allele (p66ShcA qq) that was devoid of its ROS-generating function. We found that only p66ShcA wt and not the qq mutant rescued p66ShcA ko cell sensitivity to HG. One major feature of oxidative stress is its ability to reduce the bio-availability of nitric oxide (NO) that, in turn, plays a crucial role in endothelial differentiation and function. We found that the p66ShcA deletion prevented the HG-induced increase of nitrotyrosine, and that the resistance to HG of p66ShcA ko BM-derived EPCs was prevented by NO synthase inhibition. With a reciprocal approach, the treatment of p66ShcA wt cells with a NO donor prevented the HG-induced deficit. Finally, using a Matrigel plug angiogenesis assay, we demonstrated that p66ShcA ko prevented diabetic impairment of angiogenesis in vivo.

Conclusion p66ShcA deletion rescues the BM-derived EPCs defect induced by HG, indicating p66ShcA as a potential therapeutic target in diabetic vasculopathy.
endothelial markers and have higher proliferative and clonogenic potential. Indeed, the EPCs concept has been recently revisited in humans according to a hierarchical model describing EPCs differentiation of BM-derived CD45− progenitors, characterized by the expression of CD133, VEGFR-2/Flik-1, and CD34 stem cell antigens. This model allowed to discriminate two lineage-separated cellular populations, named CFU-EC (CD45−) and ECFC (CD45−), that seem to play completely different roles in angiogenesis and, respectively, in ischemic tissues repair.14,15

Mouse EPCs are less characterized compared with human EPCs; however, cellular populations present in the BM having similar activities are likely present in mice as well. In the mouse system, the c-kit (CD117) antigen has been used to define cells having angiogenic and myocardial repair potential. Indeed, it has been shown that mouse BM c-kit+ cells are mobilized to peripheral circulation in response to ischaemia and are incorporated in the vascularization at neo-angiogenesis sites.1–3,16 In addition, upon injection in ischemic skeletal muscle or myocardium, they stimulate and participate to the angiogenic process and can differentitate in endothelial and myocardial cells.17–21

We and others have shown that isolated BM c-kit+ cells seeded onto fibronectin or gelatin in the presence of fetal calf serum (FCS), differentiate and express numerous endothelial markers.19–21 Finally, Sata et al.22 showed that BM c-kit+ (Sca+/Lin−) cells yield not only endothelial cells, but also neointimal smooth muscle cells in a mouse model of mechanically injured femoral artery. Thus, BM c-kit+ cells represent a useful experimental model to study the differentiation of precursor cells towards the endothelial lineage and EPCs physiology.

The mammalian adaptor protein ShcA has three isoforms named p46, p52, and p66. They all share a common structure, but p66ShcA has an additional domain at its N-terminus. p52 and p46 are cytoplasmic signal transduction molecules involved in mitogenic signalling from activated tyrosine kinase receptors to Ras. The p66 isoform is devoid of this function and regulates reactive oxygen species (ROS) metabolism and apoptosis.23–26 Both p66ShcA knockout (ko) cells and mice display decreased intracellular ROS levels and are resistant to apoptosis induced by a variety of different stimuli.24–26 Indeed, we demonstrated that p66ShcA deletion increased both skeletal muscle and endothelial cell resistance to ischaemia.27 Intriguingly, we also found that p66ShcA not only modulated cell survival, but also differentiation of skeletal muscle progenitors and skeletal muscle regeneration after hindlimb ischaemia.28

Although the exact mechanism of action of p66ShcA remains to be elucidated, it has been shown that a portion of p66ShcA localizes in the mitochondria where it works as a red-ox enzyme that generates mitochondrial ROS, triggering apoptosis.24–26,29 Mitochondrial ROS production is acknowledged as an important mediator of hyperglycaemic endothelial damage.30 In keeping with this observation, p66ShcA gene expression increases significantly in peripheral blood mononuclear cells of diabetic patients31 and p66ShcA ko mice are resistant to diabetic glomerulopathy,32 to cardiac stem cell aging and heart failure33 and to hyperglycaemia-induced endothelial dysfunction.34

Given the p66ShcA role in transducing glucose signalling, we investigated whether p66ShcA deletion affected EPCs response to increased glucose concentrations (HG). We found that HG decreased EPCs number derived from BM c-kit+ cells with an oxidative-stress-dependent mechanism.

2. Methods

2.1 Animals

All experimental procedures complied with the Guidelines of the Italian National Institutes of Health and with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee. Male 129 Sv-Ev p66ShcA wild-type (wt) and ko, 1–2 months old mice were used.23 Diabetes mellitus was induced by intraperitoneal injection of streptozotocin (STZ, Sigma-Aldrich), as described in Supplementary material online, Methods.

2.2 Cell isolation and culture

BM c-kit+ cells isolation and culture conditions were described previously19 and the details are in Supplementary material online, Methods. Briefly, after Ficoll gradient separation, BM mononuclear cells were separated using an anti-mouse-CD117/c-kit antibody (eBioscience) linked to paramagnetic microbeads (Miltenyi Biotech). c-kit+ cells were then seeded (3×105 cells/well) on glass chamber slides (8 well glass slide-NUNC) coated with fibronectin (20 μg/mL, Sigma-Aldrich) in M199 medium containing 5 mM glucose, supplemented with 20% FCS for 7 days. Medium was changed every 3 days. HG and control cultures were supplemented with 45 mM glucose and mannnitol, respectively.

2.3 Immunofluorescence, Dil-ac-LDL uptake and lectin binding assay

Immunofluorescence, Dil-ac-LDL uptake, and lectin binding assay were described previously28 and were detailed in Supplementary material online, Methods. Briefly, cells were incubated with Dil-ac-LDL (2 μg/mL, Biomedical technologies), and/or with UEA-1 lectin from Ulex europaeus (Sigma-Aldrich). Then cells were fixed with 4% paraformaldehyde and immunofluorescences were performed using relevant antibodies.28 A Zeiss Axioplan2 fluorescence microscope with image analyzer IAS-software (Delta System) was used to acquire images. The same number of optical fields (12–16) was counted for each sample within the same experiment, totalling 500–1500 cells/sample. To make different experiments comparable data were expressed as per cent of the control. Cells were counted by two blinded readers obtaining similar results.

2.4 Flow cytometry

The purity of BM c-kit+ cells was assessed by flow cytometry as described previously19 and as specified in Supplementary material online, Methods.

2.5 Apoptosis

Apoptosis was assessed by measuring the amount of cytoplasmic nucleosomes generated during the apoptotic fragmentation of cellular DNA by Cell Death Detection Elisa (Roche), according to the manufacturer’s instructions.
2.6 Vectors and retrovirus infection
Phoenix-ampk cells were transfected with retroviral vectors together with pMD2-VSVG. Medium containing the pseudo-typed emerging retrovirus was harvested 48 and 72 h after transfection. To assay for infectious virus, exponentially growing BM c-kit+ cells were infected and selected in puromycin-containing medium (2 μg/mL, Sigma-Aldrich). After selection, the cells were seeded on fibronectin-coated chamber slides as described earlier. Retroviral vectors generation is described in Supplementary material online, Methods.

2.7 Oxidative stress
Intracellular ROS production was evaluated incubating the cells with 25 μM 2′,7′-dichlorofluorescin-diacetate (DCFH-DA, Eastman Kodak Co.)28 or 2 μM Dihydroethidium (DHE, Sigma-Aldrich) at 37 °C for 30 and 15 min, respectively. Then, cells were fixed with 4% paraformaldehyde, nuclei were stained with Hoechst 33342, and fluorescence was revealed by fluorescence microscopy, using the same exposure conditions in each experiment. Cells were counted by two-blinded readers obtaining similar results. Nitrotyrosine levels were measured by immunofluorescence and quantified using Scion Image software as described previously.35

2.8 Subcellular fractionation
Subcellular fractionation was performed as described previously27 and as detailed in Supplementary material online, Methods. Cells were incubated in isotonic Extraction Buffer on ice for 30 min. Then, they were disrupted using Glass beads (Sigma Aldrich, G9268) and cell homogenate was centrifuged at 1000 g for 10 min at 4 °C to remove nuclei and unbroken cells. The supernatant was centrifuged at 10000 g for 20 min and the pellet was designated the mitochondrial fraction, whereas the supernatant was used for preparation of cytosolic fraction.

2.9 Western blotting and mRNA quantification by real-time PCR
Western blotting was performed as described previously36 and specified in Supplementary material online, Methods. Briefly, cells were lysed in 2x Laemmlli buffer and boiled for 5 min. Equal amounts of proteins were separated by SDS–PAGE and transferred to nitrocellulose by standard procedures. Proteins of interest were detected using specific antibodies. p66ShcA expression levels were evaluated by densitometric analysis using a GS-710 scanner (Biorad) and Quantity One software.

mRNA was quantified by reverse transcriptase real-time PCR (qPCR).35 Total RNA was extracted using TRIzol (Invitrogen) and then used as a template to synthesize cDNA using reverse transcriptase (Invitrogen) according to manufacturer guide. The SYBR-GREEN qPCR method was used to analyse mRNA levels and relative expression was calculated using the comparative Ct method (2−ΔΔCt).

2.10 Matrigel plug assay
Matrigel plug assays were performed as described previously.37 Briefly, 400 μL of growth factor supplemented basement membrane extract (Matrigel, Cultrex, Trevigen, USA) was injected subcutaneously into mouse abdominal region. Matrigel plugs were harvested 7 days later, fixed in 4% paraformaldehyde, and paraffin embedded for the histological analysis. Three micrometre thick sections were stained with Masson Trichrome Goldner procedure (Bio Optica) and capillaries quantified. Details are in Supplementary material online, Methods.

2.11 Statistical analysis
Variables were analysed by Student’s t-test and one-way ANOVA. A value of P ≤ 0.05 was deemed statistically significant. Mean values are indicated as ±SEM.

3. Results
3.1 p66ShcA is necessary for glucose-induced endothelial progenitor cells apoptosis
BM c-kit+ progenitor cells were isolated from wt or p66ShcA ko mice and then seeded on fibronectin-coated dishes in the presence of 20% FCS for 7 days.19 Under these experimental conditions, 96 ± 1% of the adherent cells expressed the endothelial marker von Willebrand factor/FactorVIII (vWF), 95 ± 1% displayed Ac-LDL uptake and 94 ± 1% were positive for both. Other endothelial markers such as KDR, CD34, and lectin binding were similarly expressed (Supplementary material online, Figure S1). We found that 45 ± 4% of the cells expressed CD45, while only sporadic cells were positive for the macrophage marker MAC3 (Supplementary material online, Figure S1). Thus, these cells display certain features of human early-EPCs and, to highlight their origin, will be referenced as BM-derived EPCs. We tested whether cell culture in the presence of HG concentrations that simulate hyperglycaemia affected the number of BM c-kit+ cells undergoing endothelial differentiation. Figure 1 shows that no significant differences in the number of BM-derived EPCs was observed between the two genotypes in control conditions. As previously reported in similar experimental systems,10–13 HG decreased the number of p66ShcA wt BM-derived EPCs. Indeed, the number of p66ShcA wt BM-derived EPCs. Indeed, the number of p66 ShcA ko mice were markedly resistant to HG treatment (Figure 1A and B). However, BM-derived EPCs obtained from p66ShcA ko mice were markedly resistant to HG inhibition (Figure 1A and B). These data were confirmed when EPCs derived from a different source were assayed. Peripheria blood mononuclear cells were isolated from p66ShcA wt and ko mice and fibronectin adherent early-EPCs (PB-derived EPCs) were counted after 7 days of culture. More than 95% of the cells expressed vWF, KDR, and CD34 and displayed Ac-LDL uptake and lectin binding. Only sporadic cells were positive for the macrophage marker MAC3 (Supplementary material online, Figure S2). HG exposure markedly decreased the number of p66ShcA wt PB-derived EPCs compared with the iso-osmotic control, whereas p66ShcA ko cells were not affected (Supplementary material online, Figure S2F).

To further strengthen these findings an RNAi experimental approach was used. BM-derived EPCs wt were infected with retroviral vectors pSUPER (as a control) or pSUPER-p66ShcA to knock-down p66ShcA expression. Western blotting analysis confirmed a >70% p66ShcA knock-down efficiency.
Then, cells were plated on fibronectin in the presence of either mannitol or HG for 7 days. We found that cells expressing p66shcA shRNA were significantly less sensitive to HG inhibition than controls (Figure 1D and Supplementary material online, Figure S3).

Several mechanisms may contribute to the observed decrease of p66ShcA wt EPCs upon HG exposure. We found that HG increased apoptosis in 4 and 7 days cultures of p66ShcA wt BM-derived EPCs, whereas ko cells were resistant (Supplementary material online, Figure S4). Conversely, proliferation, measured by BrdU incorporation, was not decreased by HG significantly (Supplementary material online, Figure S5). Finally, we did not observe differences in cell senescence, as assessed by measuring senescence-associated beta-galactosidase activity and the expression of the senescence markers p19ARF and p16INK4a (data not shown).

3.2 HG induces p66ShcA expression and oxidative stress

To investigate the effect of HG on p66ShcA protein, we measured its expression in p66ShcA wt BM c-kit+ cells cultured for 24 h in the presence of HG. (Figure 1C). Then, cells were plated on fibronectin in the presence of either mannitol or HG for 7 days. We found that cells expressing p66ShcA shRNA were significantly less sensitive to HG inhibition than controls (Figure 1D and Supplementary material online, Figure S3).

Several mechanisms may contribute to the observed decrease of p66ShcA wt EPCs upon HG exposure. We found that HG increased apoptosis in 4 and 7 days cultures of p66ShcA wt BM-derived EPCs, whereas ko cells were resistant (Supplementary material online, Figure S4). Conversely, proliferation, measured by BrdU incorporation, was not decreased by HG significantly (Supplementary material online, Figure S5). Finally, we did not observe differences in cell senescence, as assessed by measuring senescence-associated beta-galactosidase activity and the expression of the senescence markers p19ARF and p16INK4a (data not shown).
It has been shown that HG treatment increases ROS production in EPCs, whereas treatment with iso-osmotic concentrations of mannitol has no effect. Thus, we tested whether p66<sup>ShcA</sup> deletion decreased HG-induced oxidative stress. Oxidative stress was evaluated by either DCFH or DHE red/ox sensitive fluorescent probes. As expected, the number of DCFH<sup>+</sup> and DHE<sup>+</sup> cells increased upon HG treatment of p66<sup>ShcA</sup> wt BM-derived EPCs. When p66<sup>ShcA</sup> ko BM-derived EPCs were assayed, no significant difference was observed between HG and its iso-osmotic control (Figure 3). Similar data were obtained analysing PB-derived EPCs stained with DCFH (not shown).

### 3.3 p66<sup>ShcA</sup> electron-transfer domain is necessary for HG-induced bone marrow-derived endothelial progenitor cells decrease

To investigate the role of oxidative stress in HG-induced impairment of BM-derived EPCs, cells were treated with the cell permeable antioxidant PEG-catalase. As shown in Supplementary material online, Figure S6, co-incubation with catalase strongly reduced the increase of DCFH<sup>+</sup> and DHE<sup>+</sup> cells (Supplementary material online, Figure S6A–D) caused by HG treatment. In keeping with data obtained in a different experimental system, catalase also attenuated HG-induced EPCs decrease significantly (Supplementary material online, Figure S6E).

Previous studies showed that a fraction of p66<sup>ShcA</sup> displays mitochondrial localization. Thus, p66<sup>ShcA</sup> localization was tested in both BM-derived EPCs and differentiated endothelial cells cultured in the presence or absence of HG. We confirmed that p66<sup>ShcA</sup> was localized, at least in part, in the mitochondria and that its accumulation increased upon HG treatment (Supplementary material online, Figure S7).

To functionally link p66<sup>ShcA</sup>-mediated ROS production to HG response of BM-derived EPCs, we took advantage of a p66<sup>ShcA</sup> allele (p66<sup>ShcA</sup> qq) that is no longer able to bind cytochrome C, to transfer electrons, and to stimulate mitochondrial ROS generation, whereas other p66<sup>ShcA</sup> properties are unaffected.

BM c-kit<sup>+</sup> cells were reconstituted with retroviral vectors encoding p66<sup>ShcA</sup> wt or qq mutant alleles or with backbone vector and the effect of HG was assayed. Western blotting analysis confirmed that exogenous p66<sup>ShcA</sup> wt and qq mutant were expressed with similar efficiency and at levels comparable to those of the endogenous protein in wt cells (Figure 4B). When plated on fibronectin in the presence of either HG or mannitol, cells transduced with the wt allele recovered their sensitivity to HG. However, BM-derived EPCs transduced with the p66<sup>ShcA</sup> qq allele were not significantly different from the cells transduced with vector alone (Figure 4C).

### 3.4 p66<sup>ShcA</sup> regulation of HG-treated bone marrow-derived endothelial progenitor cells is mediated by nitric oxide

Increased concentration of superoxide decreases nitric oxide (NO) bioavailability, inducing both NO-synthase (NOS) uncoupling and the formation peroxynitrite, a powerful toxicant that leads nitrotyrosine accumulation. In keeping with lower oxidative stress levels, we observed that p66<sup>ShcA</sup> ko BM-derived EPCs did not display a significant increase in nitrotyrosine levels upon HG exposure (Figure 5A and Supplementary material online, Figure S8A). To investigate whether NO played a role in p66<sup>ShcA</sup> ko cells resistance to HG, p66<sup>ShcA</sup> ko BM-derived EPCs were treated with the NOS inhibitor L-NAME and their number was assayed in the presence or absence of HG. We observed that while L-NAME treatment had only marginal effects in control cells, it significantly decreased p66<sup>ShcA</sup> ko BM-derived EPCs in the presence of HG (Figure 5B and Supplementary material online, Figure S8B). Using a reciprocal approach, we treated p66<sup>ShcA</sup> wt cells with the NO donor, DETA-NO. In keeping with previous observations, we found that...
DETA-N0 prevented BM-derived EPCs demise induced by HG (Figure 5C and Supplementary material online, Figure S8C).

3.5 Angiogenesis impairment is prevented in diabetic p66ShcA ko mice

We assessed whether p66ShcA played a role in the angiogenesis impairment associated to diabetes mellitus. To this aim, an in vivo Matrigel plug assay was performed in both p66ShcA wt and ko mice, in the presence or absence of experimental diabetes induced by streptozotocin treatment. In this angiogenesis model, host EPCs as well as endothelial cells and smooth muscle cells migrate and form a vascular network in the Matrigel plugs.42 The number of capillaries within the plugs was determined 7 days after the implantation, using histological sections stained with Masson trichrome procedure (Figure 6A). Quantitative analysis revealed that capillary density was similar in normoglycaemic p66ShcA wt and ko mice. Figure 6B shows that in diabetic p66ShcA wt mice capillary density decreased, whereas it did not change significantly in p66ShcA ko mice.

4. Discussion

Understanding the molecular mechanisms underpinning impaired new blood vessels formation following ischaemia observed in type 1 and 2 diabetic patients is of crucial importance. We provided evidences that EPCs deficit induced by increased glucose concentrations in the culture medium was mediated by a mechanism requiring ROS formation and p66ShcA. Specifically, we demonstrated that p66ShcA ability to generate ROS was necessary to transduce HG-sensitivity in BM-derived EPCs. In agreement with these observations, p66ShcA also mediated the angiogenesis impairment induced by diabetes mellitus in a mouse model of angiogenesis.

Decreased stimulation of the ROS-induced signalling may act via different mechanisms. We found that p66ShcA was
necessary for HG-induced apoptosis. Further studies are needed to elucidate the interplay between cell survival and other events, such as adhesion to the substrate, cell cycle regulation, differentiation, and endothelium-specific gene expression.

We also showed that HG resistance conferred by p66\textsuperscript{ShcA} deletion was dependent on the activity of NOS and that a NO donor was sufficient to rescue BM-derived EPCs deficit induced by HG. Our observations are in agreement with NO-bioavailability decrease caused by superoxide that induces both the formation peroxynitrite and NOS uncoupling\textsuperscript{4,26} as well as with numerous reports highlighting the importance of NO in EPCs function.\textsuperscript{1,2,4} Thum et al.\textsuperscript{33} demonstrated that p66\textsuperscript{ShcA} ko mice are resistant to c-kit\textsuperscript{+}-cardiac progenitor cell aging and heart failure. Moreover, gene delivery of Tim44, a component of the complex that inhibits the mitochondrial fraction of p66\textsuperscript{ShcA}, prevents the increase of superoxide generation in smooth muscle cells exposed to HG and improves the neointimal hyperplasia of diabetic rats following balloon injury.\textsuperscript{45} Finally, our observations may provide mechanistic insights to previous studies indicating p66\textsuperscript{ShcA} involvement in diabetic glomerulopathy,\textsuperscript{32} and in endothelial dysfunction.\textsuperscript{34}

We can conclude that, while the p66\textsuperscript{ShcA}-dependent signalling and its physio-pathological implications require further investigation, p66\textsuperscript{ShcA} represents a promising therapeutic target to prevent development and progression of diabetic vasculopathy. The latter point is supported by the observation that not only congenital deletion of p66\textsuperscript{ShcA}, but also acute knock down of its expression by RNAi, increased BM-derived EPCs resistance to high glucose.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

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References


