Haploinsufficiency of the Insulin-Like Growth Factor-1 Receptor Enhances Endothelial Repair and Favorably Modifies Angiogenic Progenitor Cell Phenotype


Objectives—Defective endothelial regeneration predisposes to adverse arterial remodeling and is thought to contribute to cardiovascular disease in type 2 diabetes mellitus. We recently demonstrated that the type 1 insulin-like growth factor receptor (IGF1R) is a negative regulator of insulin sensitivity and nitric oxide bioavailability. In this report, we examined partial deletion of the IGF1R as a potential strategy to enhance endothelial repair.

Approach and Results—We assessed endothelial regeneration after wire injury in mice and abundance and function of angiogenic progenitor cells in mice with haploinsufficiency of the IGF1R (IGF1R+/−). Endothelial regeneration after arterial injury was accelerated in IGF1R+/− mice. Although the yield of angiogenic progenitor cells was lower in IGF1R+/− mice, these angiogenic progenitor cells displayed enhanced adhesion, increased secretion of insulin-like growth factor-1, and enhanced angiogenic capacity. To examine the relevance of IGF1R manipulation to cell-based therapy, we transfused IGF1R+/− bone marrow–derived CD117+ cells into wild-type mice. IGF1R+/− cells accelerated endothelial regeneration after arterial injury compared with wild-type cells and did not alter atherosclerotic lesion formation.

Conclusions—Haploinsufficiency of the IGF1R is associated with accelerated endothelial regeneration in vivo and enhanced tube forming and adhesive potential of angiogenic progenitor cells in vitro. Partial deletion of IGF1R in transfused bone marrow–derived CD117+ cells enhanced their capacity to promote endothelial regeneration without altering atherosclerosis. Our data suggest that manipulation of the IGF1R could be exploited as novel therapeutic approach to enhance repair of the arterial wall after injury. (Arterioscler Thromb Vasc Biol. 2014;34:2051-2058.)

Key Word: endothelium

Type 2 diabetes mellitus is a disorder of cell growth and metabolism leading to a portfolio of chronic disorders of human health dominated by atherosclerosis-related diseases, such as myocardial infarction, stroke, and peripheral arterial disease. As a result, individuals with type 2 diabetes mellitus experience cardiovascular event rates clinically manifest 15 years earlier than in patients without diabetes mellitus.1 Effective endothelial repair following structural or functional damage to the endothelium is critical to prevent adverse arterial remodeling, thrombosis, and atherosclerosis.2 Recent studies support a prominent role for impairment of nitric oxide (NO)-dependent endothelial cell repair/replacement in diabetes mellitus–related vascular disease.3,4 Indeed, insulin resistance, the primary metabolic disturbance underpinning type 2 diabetes mellitus, is associated with delayed endothelial repair in the absence of overt glycemia.4 We recently demonstrated that the type 1 insulin-like growth factor receptor (IGF1R), which acts as the principal tyrosine kinase receptor delivering cues for cellular and whole organism growth, is a negative regulator of NO bioavailability and insulin sensitivity in the endothelium.3 In mice with haploinsufficiency of the IGF1R, we observed a phenotype of enhanced whole-body insulin sensitivity, enhanced insulin-stimulated phosphorylation of endothelial nitric oxide synthase (eNOS), and increased basal NO generation.5 In the present report, we examine the effect of partial deletion of the IGF1R on vascular regeneration in this model and investigate whether manipulation of IGF1R abundance could be a strategy to refine cell-based therapy to augment endothelial repair.
Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Effect of Partial IGF1R Deletion on Endothelial Regeneration After Arterial Injury

To investigate the effects of partial deletion of IGF1Rs on endothelial regeneration, we subjected IGF1R<sup>−/−</sup> and wild-type (WT) mice to femoral artery endothelium-denuding wire injury. Femoral arteries were harvested ay days 0, 4, and 7 and the area of endothelial regeneration quantified by Evans blue staining. Wire injury induced immediate and complete denudation of endothelium (Figure 1A and 1B). Four days after injury, IGF1R<sup>+/−</sup> mice displayed significantly enhanced re-endothelialization compared with WT littermates (Figure 1A and 1B). Regenerated area was similar in IGF1R<sup>+/−</sup> and WT mice by day 7 (Figure 1A and 1B). Serum concentration of insulin-like growth factor (IGF)-1 was similar in IGF1R+/− and WT mice (Figure 1C).

Quantification of Angiogenic Progenitor Cells

To investigate whether the accelerated endothelial repair in IGF1R<sup>−/−</sup> mice was contingent on increased abundance of endothelial progenitors, we quantified circulating Sca-1<sup>+</sup>/Flk-1<sup>+</sup> progenitor cells and the yield of angiogenic progenitor cells (APCs) from mononuclear cells in IGF1R<sup>+/−</sup> and WT mice. Sca-1<sup>+</sup>/Flk-1<sup>+</sup> cells were similar in abundance in IGF1R<sup>−/−</sup> mice compared with WT littermates (Figure 2A). Mobilization of progenitor cells from the bone marrow niche in response to proangiogenic cytokines is a prerequisite for effective endothelial repair.<sup>3</sup> Systemic administration of vascular endothelial growth factor increased circulating numbers of Sca-1<sup>+</sup>/Flk-1<sup>+</sup> cells in both groups of mice (Figure 2B). We found no significant difference in APC mobilization following vascular endothelial growth factor administration in IGF1R<sup>+/−</sup> mice compared with WT littermates (Figure 2C). We expanded mononuclear cells derived from peripheral blood, spleen, and bone marrow and counted cells displaying typical properties (eg, uptake of acetylated low-density lipoproteins and binding of Ulex lectin) after 7 days of culture as APCs (Figure 2D). The yield of APCs from WT and IGF1R<sup>+/−</sup> mice was significantly lower in IGF1R<sup>+/−</sup> mice compared with WT; no difference between mice was found in the yield of spleen-derived APCs (Figure 2E). The size and density of APCs, assessed by flow cytometry, was similar in WT and IGF1R<sup>+/−</sup> mice (Figure IA and IB in the online-only Data Supplement). Expression of the leukocyte marker CD11b and the integrin chain CD49e (which is abundantly expressed on monocytes) were similar in APCs from WT and IGF1R<sup>+/−</sup> mice (Figure IC and ID in the online-only Data Supplement). APCs from both genotypes expressed the markers CD68 and Factor XIII-A (Figure IE in the online-only Data Supplement) in keeping with the monocytic origin of these cells.<sup>7</sup>

Adhesion Assay

As adhesion of circulating cells to the damaged vessel wall is a critical step in progenitor cell-mediated vascular repair, we examined the capacity of APCs to adhere to the extracellular matrix glycoprotein protein fibronectin in vitro. APCs from IGF1R<sup>+/−</sup> mice showed significantly enhanced adhesion to fibronectin compared with WT APC (Figure 3A). Incubation of APCs with the NO synthase inhibitor N<sup>ω</sup>-monomethyl-L-arginine did not modify adhesion of WT APCs but significantly inhibited adhesion of IGF1R<sup>+/−</sup> APCs to control levels (Figure 3A).
To evaluate the effect of partial IGF1R deletion on the angiogenic potential of APCs, we performed an in vitro angiogenesis assay using conditioned media from APCs from IGF1R+/− and WT mice. As shown in Figure 3B and 3C, we demonstrated a significant increase in endothelial tube formation when human umbilical vein endothelial cells were cultured with conditioned medium from IGF1R+/− APCs compared with medium from WT APCs. We then measured secretion of angiogenic cytokines by APCs of both genotypes. We observed a significant increase in concentration of IGF-1 in conditioned medium of IGF1R+/− APCs compared with WT APCs (Figure 3D). The concentrations of vascular endothelial growth factor and hepatocyte growth factor were similar in conditioned media from IGF1R+/− and WT APCs (Figure 3E–3F). Concentrations of stromal cell–derived factor-1 and granulocyte colony–stimulating factor were below the limits of detection (not shown).

**Tube-Forming Assay**

To evaluate the effect of partial IGF1R deletion on the angiogenic potential of APCs, we performed an in vitro angiogenesis assay using conditioned media from APCs from IGF1R+/− and WT mice. As shown in Figure 3B and 3C, we demonstrated a significant increase in endothelial tube formation when human umbilical vein endothelial cells were cultured with conditioned medium from IGF1R+/− APCs compared with medium from WT APCs. We then measured secretion of angiogenic cytokines by APCs of both genotypes. We observed a significant increase in concentration of IGF-1 in conditioned medium of IGF1R+/− APCs compared with WT APCs (Figure 3D). The concentrations of vascular endothelial growth factor and hepatocyte growth factor were similar in conditioned media from IGF1R+/− and WT APCs (Figure 3E–3F). Concentrations of stromal cell–derived factor-1 and granulocyte colony–stimulating factor were below the limits of detection (not shown).

**Expression of IGF1R, Insulin Receptor, and eNOS in APC**

Real-time reverse-transcription polymerase chain reaction demonstrated robust expression of the truncated Igf1 allele with deletion of exon 3 (encoding a nonfunctional receptor protein) in APCs from IGF1R−/− mice as expected (Figure 4A). Expression of insulin receptor mRNA was significantly increased in APCs from IGF1R−/− mice (Figure 4B). We found no difference in expression of eNOS mRNA in APC from IGF1R−/− mice compared with WT mice (Figure 4C). eNOS protein expression was similarly unchanged in mononuclear cells derived from bone marrow of IGF1R−/− mice compared with that from WT mice (Figure 4D). However, eNOS Ser1177 phosphorylation was significantly higher in cells derived from IGF1R−/− mice compared with WT mice (Figure 4D).

**Aortic Ring Angiogenesis Assay**

Endothelial repair is thought to be mediated by an orchestrated response of both circulating APCs and proliferation of native vascular endothelial cells. To investigate whether partial deletion of IGF1R impacts on endothelial cell vascular sprouting in the absence of circulating progenitor cells, we performed an in vitro angiogenesis assay in aortic rings. Angiogenic sprouting did not differ significantly in aortic rings harvested from IGF1R+/− mice and WT littermates (Figure II in the online-only Data Supplement).

**Effect of Pharmacology IGF1R Inhibition on Endothelial Regeneration After Arterial Injury**

To determine whether the enhanced endothelial repair mediated by genetic partial deletion of IGF1R could be replicated by pharmacological inhibition of IGF1R, we performed further arterial injury experiments in WT mice treated with PQ401, whichblocks signal transduction by inhibiting IGF1R autophosphorylation. Administration of PQ401 did not
significantly alter endothelial regeneration after arterial injury (Figure III in the online-only Data Supplement).

Effect of Partial Deletion of IGF1R in Transfused CD117+ Cells on Endothelial Regeneration

To evaluate whether enhanced endothelial repair associated with IGF1R downregulation is contingent on changes in the vessel wall or in progenitor cells per se, we performed further injury experiments followed by infusion of bone marrow–derived CD117+ (c-kit+) cells, which we have previously shown to be effective in enhancing arterial endothelial regeneration.4 Splenectomized WT mice were infused with WT or IGF1R+/− bone marrow–derived CD117+ (c-kit+) cells or saline after femoral artery injury. Re-endothelialization was significantly enhanced by infusion of IGF1R+/− CD117+ bone marrow–derived cells when compared with infusion of WT cells or cell-free saline (Figure 5A and 5B). To investigate whether transfused CD117+ cells incorporate into the neoendothelium, we repeated the injury experiments in WT mice transfused with CellTracker-Red-labeled CD117+ cells. Labeling with CellTracker-Red did not alter the regenerative capacity of the cells: endothelial regeneration remained enhanced after trans- 
fusion of cells with partial deletion of IGF1R (Figure 5C and 5D). At day 4 following arterial injury, CellTracker+ cells were detected only rarely in the neoendothelium (in 1 of 4 mice receiving WT cells and 1 of 4 mice receiving IGF1R+/− cells; Figure 5E).

Effect of Partial Deletion of IGF1R in Transfused CD117+ Cells on Atherosclerosis

Because there are conflicting data on the effects of transfusion of bone marrow–derived mononuclear cells on atherosclerosis, with both increased9,10 and reduced11,12 atherosclerotic plaques reported, we performed further experiments to exclude the possibility that partial IGF1R deletion in transfused CD117+ cells could be proatherosclerotic. We quantified atherosclerotic lesion formation in ApoE−/− mice subjected to adventitial carotid artery cuff placement after transfusion of IGF1R+/− or WT CD117+ bone marrow cells. We confirmed that partial IGF1R deletion in transfused cells did not alter atherosclerotic lesion formation (Figure 6A and 6B). To determine whether transfused cells incorporated into plaques, we repeated the experiment using CD117+ cells labeled with CellTracker. At 4 days after transfusion, very few CellTracker+ cells were observed in cuffed carotid arteries (scant CellTracker+ staining was seen in 2 of 3 mice receiving WT cells and 1 of 4 mice receiving IGF1R+/− cells; Figure 6D and 6E). When present, CellTracker+ cells were seen in both the endothelium and adventitia (Figure 6D and 6E).
Discussion
This study builds on our recent reports highlighting IGF1R abundance as a modulator of endothelial NO bioavailability \(^5\) \(^1\) \(^3\) by demonstrating a critical role of the IGF1R in vascular repair. First, we observed that endothelial regeneration is significantly accelerated in IGF1R-deficient mice following denuding wire injury. Second, APCs derived from IGF1R-deficient mice displayed enhanced angiogenic properties in vitro. IGF1R\(^{+/−}\) APCs exhibited increased adhesion to extracellular matrix, an effect blocked by inhibition of NO synthase. APCs from IGF1R\(^{+/−}\) mice also demonstrated increased secretion of IGF-1 into the medium and greater capacity to promote endothelial tube formation. Third, infusion of CD117\(^+\) bone marrow–derived cells deficient in IGF1R into WT mice

Figure 4. mRNA expression and endothelial nitric oxide synthase (eNOS) phosphorylation in cells from IGF1R\(^{−/−}\) and wild-type (WT) mice. A, Real-time polymerase chain reaction (PCR) mRNA expression of the truncated Igf1r allele (\(^*\)P<0.02; n=6 mice per group) in angiogenic progenitor cells (APCs). B, Real-time PCR mRNA expression of the murine insulin receptor in APCs (\(^*\)P<0.05). C, Real-time PCR mRNA expression of eNOS in APCs (P=not significant). D, Representative blots of total eNOS and ser\(^{1177}\) phospho-eNOS expression in bone marrow–derived mononuclear cells. β-actin expression is shown as loading control. Mean data of ser\(^{1177}\) phospho-eNOS expression are presented (P<0.05; n=9–11 mice per group).

Figure 5. Endothelial regeneration in wild-type (WT)-injured mice following infusion of bone marrow–derived CD117\(^+\) (c-kit\(^+)\) cells. A, Percent endothelial regeneration of injured vessels from WT mice in response to cell transfusion at day 4 (P<0.001; n=6 per group). B, Representative images of femoral vessels harvested 4 days following injury and transfusion of bone marrow–derived CD117\(^+\) cells from WT and IGF1R\(^{+/−}\) mice (magnification x20). Saline-infused mice acted as control. C, Percent endothelial regeneration at day 4 of injured vessels from WT mice in response to transfusion of CellTracker-labeled cells (P<0.001; n=4 per group). D, Representative images of injured vessels stained with Evan blue. E, Only rare CellTracker-Red labeled cells were seen in the neoendothelium (arrows). Nuclei are labeled with DAPI (4',6-diamidino-2-phenylindole). Endothelial cells labeled with anti-CD31 (yellow). Magnification x630.
after denuding injury accelerated healing in comparison with infusion of WT APCs, but did not alter atherosclerotic lesion formation. Collectively, these findings support a novel role for the IGF1R in vascular regeneration.

Recent large-scale clinical trials have challenged conventional thinking about therapeutic strategies aiming to reduce the complications of atherosclerosis in patients with type 2 diabetes mellitus. These studies demonstrated that intensive lowering of blood glucose fails to improve cardiovascular mortality in individuals suffering from type 2 diabetes mellitus. We have shown that despite contemporary secondary prevention therapies, mortality after an acute myocardial infarction in patients with type 2 diabetes mellitus has not improved over the last 10 years, in contrast to the enhanced outcomes enjoyed by patients without type 2 diabetes mellitus. Collectively, these data highlight the fundamental importance of identifying novel approaches to prevent/retard cardiovascular complications in patients with type 2 diabetes mellitus.

Recently, new mechanistic insights into the regulation of endothelial function in the context of metabolic disorders have begun to emerge. Critically, we have shown that the IGF1R can reduce NO bioavailability by interacting with insulin receptors to form insulin-resistant hybrid receptors. An increase in hybrid receptors is a hallmark of type 2 diabetes mellitus. Many aspects of the diabetic phenotype have been shown to contribute to the formation of hybrids, suggesting that their formation is not just a random process. We demonstrated that downregulation of IGF1R at the whole-body level, or selectively in the endothelium, confers both enhanced insulin sensitivity and endothelial NO generation. These data raise the possibility that modulation of IGF1R abundance could be of pathological and potential therapeutic importance in vascular remodeling and disease.

Effective endothelial repair in response to mechanical or biochemical injury has been implicated in preventing adverse vascular remodeling and is thought to mitigate against a range of pathologies, including atherosclerosis, restenosis, stent thrombosis, and bypass graft failure. Recruitment of circulating progenitor cells to sites of injury is central to the repair process. IGF-1 is recognized to increase the circulating abundance and enhance the function of endothelial progenitor cells via a mechanism involving phosphoinositide-3-kinase/Akt-mediated phosphorylation of eNOS. Insulin-invoked clonal expansion of endothelial progenitor cell colony-forming units in vitro is also thought to be transduced by the IGF1R rather than the insulin receptor. However, the effects of genetic modulation of IGF1R abundance in progenitor cells and consequences for endothelial repair have not previously been subjected to scrutiny.

In the present study, we have clearly demonstrated that reducing IGF1R at a whole-body level accelerates regeneration of the endothelium after mechanical injury. This observation contrasts with our recent findings in mice with haploinsufficiency of the insulin receptor in which endothelial regeneration was compromised and implies divergent functional consequences of downregulation of the 2 tyrosine kinase receptors on vascular repair. We did not uncover any differences in angiogenic sprouting between vessels from IGF1R−/− and WT mice in vitro, suggesting that alterations in circulating progenitor cells rather than native endothelial cells likely contributed to the enhanced endothelial regeneration observed in vivo. We observed significant reductions in bone marrow–derived and circulating APCs in IGF1R−/− mice. Although at first this seems discordant with the accelerated repair observed in IGF1R−/− mice, it is possible that reduced bone marrow stores and circulating abundance of APCs in IGF1R−/− mice arise as a compensatory response to their enhanced functionality. It is also possible that there is reduced basal endothelial damage in IGF1R−/− mice in keeping with their greater longevity.

The contribution of APCs to endothelial regeneration is dependent not only on their efficient mobilization in to the circulation but also by their attachment to the damaged vascular wall and favorable functional characteristics. Direct evidence that APCs may contribute to endothelial regeneration has emerged from our own studies and those of other laboratories. In keeping with others reports, we demonstrated that APCs display markers of monocytic origin which...
is in accordance with the accepted paradigm that these cells facilitate vascular repair by secreting proangiogenic cytokines, rather than expanding to regenerate damaged endothelium per se. In vitro studies described here are consistent with this paradigm, as we demonstrated that IGF1R-deficient APCs were more adherent to the matrix protein fibronectin and enhanced formation of tubular networks of human umbilical vein endothelial cells more than comparative WT cells. Intriguingly, we found secretion of IGF-1 in to medium was increased in IGF1R+/− cells in comparison with WT cells. IGF-1 exerts potent proangiogenic effects on endothelial cells, which may explain the increased tube formation we observed in vitro. It is less clear whether increased IGF-1 secretion by APCs could explain the enhanced endothelial regeneration occurring in vivo when IGF1R abundance is also reduced. However, we previously observed that vascular responses to IGF-1 were diminished, but not abrogated in IGF1R+/− mice, suggesting that endothelial cells remain responsive to IGF-1. Nevertheless, enhanced functionality of IGF1R+/− APCs may be permissive of their lower abundance in normal vascular homeostasis and may facilitate more expeditious repair in the context of vascular injury.

At the mechanistic level, although APC function is regulated by a range of factors, bioavailability of NO plays a key role. As our in vitro experiments were performed in the absence of exogenous IGF-1, we postulate that the enhanced functions of IGF1R+/− APCs we observed are secondary to increased NO bioavailability facilitated by reduced IGF1R abundance rather than altered responses to IGF-1 per se. This is in keeping with our previous demonstration that reduced IGF1R abundance enhances NO generation and insulin sensitivity by altering IGF1R–insulin receptor stoichiometry and hybrid receptor formation. Our demonstration here that enhanced adhesion of IGF1R+/− APCs is abrogated by NO synthase inhibition and our finding of increased serine phosphorylation of eNOS in APCs from IGF1R+/− mice, despite similar circulating IGF-1 concentrations, are consistent with this contention. It is also noteworthy that inhibition of IGF1R with monoclonal antibodies, which would not be expected to alter IGF–insulin receptor stoichiometry, has been shown to reduce the migratory and angiogenic capacity of APCs in vitro, which contrasts with our finding of enhanced APC function in response to genetic partial deletion of IGF1Rs.

The enhanced repair observed following partial IGF1R deletion in vivo was not replicated by pharmacological IGF1R inhibition with PQ401. The chronicity of IGF1R suppression achieved by genetic IGF1R deletion limits direct comparison with acute pharmacological IGF1R inhibition. It is possible, however, that the pharmacological strategy proved ineffective because inhibition of IGF1R autophosphorylation by PQ401 would not be expected to modulate IGF1R–insulin receptor stoichiometry and hybrid receptor formation, with the consequent enhancement of insulin signaling and NO bioavailability.

Cell-based therapies aimed at enhancing vascular repair represent a novel nonpharmacological approach to treat and prevent diabetes mellitus–related cardiovascular disease. Although some commentators have drawn into question the contribution of progenitor cells to normal vascular homeostasis, our demonstration here that infusion of cells enhances regeneration adds to the evidence that such cells offer therapeutic potential. Bone marrow cells enriched for the stem cell marker CD117 (c-kit) represent a promising population to augment vascular repair, and we have previously shown these cells to accelerate endothelial regeneration in the context of insulin resistance. Here, by demonstrating that down-regulating the IGF1R in infused CD117+ cells enhances their reparative capacity, we raise the intriguing possibility that modulating IGF1R abundance could be a strategy to circumvent the progenitor cells dysfunction which currently limits the utility of cell-based regenerative therapy in diabetes mellitus.

Although infused unselected mononuclear cells and APCs are known to incorporate into the vessel wall, the homing and incorporation of CD117+ cells remains underexplored. Here, we observed robust positive effects of infused CD117+ cells on endothelial regeneration, despite apparently detecting only rare incorporation of infused cells into the injured vessel wall at 4 days after injury. As little is known about the kinetics and survival of infused CD117+ cells, we cannot exclude the possibility that the cells incorporated early after infusion but failed to persist >4 days. It is clear that infused CD117+ cells do not directly contribute to endothelial coverage per se, and it is likely that they augment the formation of the neointima by paracrine or systemic effects of secreted angiogenic factors on native endothelial cells.

Enthusiasm for cell-based therapy may be tempered by reports that mononuclear cell infusion can accelerate and diminish atherosclerosis, induced delay in reendothelialization following arterial injury. Here, we were reassured that partial deletion of IGF1R in CD117+ cells infused in to ApoE−/− mice did not modify atherosclerotic lesion formation, suggesting that this strategy could safely be exploited for therapeutic use to accelerate re-endothelialization.

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Disclosures

None.

References

IGF1R deletion enhanced adhesion, increased insulin-like growth factor-1 secretion, and promoted endothelial tube formation in vitro. Partial optimizing cell-based therapy as a strategy to enhance vascular repair.

We have previously demonstrated that partial deletion of the type 1 insulin-like growth factor receptor (IGF1R) enhanced insulin sensitivity and endothelial nitric oxide bioavailability. Here, we demonstrate that heterozygous deletion of IGF1R in bone marrow-derived CD11c(-k(-)) cells significantly enhanced endothelial regeneration when transfused in to wild-type mice but did not accelerate atherosclerosis in ApoE(-/-) mice. These findings have important implications for modulation of the IGF1R in optimizing cell-based therapy as a strategy to enhance vascular repair.

Significance

Defective endothelial regeneration in response to loss of endothelial structural/functional integrity plays a critical role in adverse vascular remodeling in type 2 diabetes mellitus and insulin resistance. We have previously demonstrated that partial deletion of the type 1 insulin-like growth factor receptor (IGF1R) enhanced insulin sensitivity and endothelial nitric oxide bioavailability. Here, we demonstrate that heterozygous deletion of IGF1R (IGF1R(-/-)) in mice enhanced endothelial regeneration following arterial injury. In angiogenic progenitor cells, partial IGF1R deletion enhanced adhesion, increased insulin-like growth factor-1 secretion, and promoted endothelial tube formation in vitro. Partial deletion of IGF1R in bone marrow-derived CD11c(-k(-)) cells significantly enhanced endothelial regeneration when transfused in to wild-type mice but did not accelerate atherosclerosis in ApoE(-/-) mice. These findings have important implications for modulation of the IGF1R in optimizing cell-based therapy as a strategy to enhance vascular repair.
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Characterisation of APCs from WT and IGF1R<sup>+/−</sup> mice.

Flow cytometry was used to estimate the size and density of APCs. Forward scatter (A) and side scatter (B) were evaluated in 100 000 counted events (n=6 per group). Expression of the leukocyte marker CD11b (C) and the integrin α-5 chain CD49e (D) were also evaluated by flow cytometry (n=6 per group). Immunofluorescence microscopy was used to identify CD68 (green), FXIII-A (red) and nuclei DAPI (blue) in bone-marrow-, blood- and spleen-derived APCs after 4 and 7 days of culture (E – magnification x 200).
Supplementary Figure II

Aortic ring angiogenesis assay. A: representative images of angiogenic sprouting from aortic rings derived from IGF1R$^{+/−}$ and WT mice. B: average sprout length, normalised to aortic ring circumference, in IGF1R$^{+/−}$ and WT mice (n=6-8 per group).
Effect of pharmacological inhibition of IGF1Rs on endothelial regeneration following arterial injury. Wild type C57BL6 mice were treated with PQ401 (10mg/kg intra-peritoneal) or equivalent DMSO after femoral artery wire injury (n=4-5 per group). A: mean data for endothelial regeneration at day 4 following injury. B: representative images of injured vessels from control and PQ401-treated animals.
Materials and Methods

Gene modified mice. Mice with whole body haploinsufficiency of the Igf1r<sup>1</sup>, were obtained from the European mutant mouse archive (Munich, Germany; EMMA Ref:00115), and maintained in the heterozygous state on a C57BL/6 background by mating IGF1R<sup>+/−</sup> males with 9-12 week old female C57BL/6 wild type mice (Harlan Laboratories). Mice were housed in a conventional animal facility with a 12-h light/dark cycle with ad libitum access to standard rodent chow. Genotyping was performed by RT-PCR of tail DNA as previously described<sup>5</sup>. ApoE<sup>−/−</sup> mice were purchased from Charles River Laboratories (Margate, UK). All experiments were performed in 4-6 month old male mice unless otherwise stated and were conducted in accordance with accepted standards of humane animal care under United Kingdom Animals (Scientific Procedures) Act 1986.

Femoral artery endothelial denuding injury. Unilateral femoral artery wire injury was carried out as previously described<sup>2</sup>. Mice were anesthetized with isoflurane (1.5–2%), before a small incision was made in the mid thigh and extended. Having carefully isolated the femoral artery, an arteriotomy was made in the saphenous artery using iris scissors (World-Precision Instruments, Sarasota, FL), and a 0.014-inch-diameter angioplasty guidewire with tapered tip (Hi-Torque Cross-It 200XT, Abbott-Vascular, IL), was introduced. The guidewire was advanced 1.5 cm in to the femoral artery, and three passages performed per mouse, resulting in complete endothelial denudation. The guidewire was removed completely and a suture tightened rapidly immediately distal to the bifurcation of the femoral artery. The skin was closed with a continuous suture. The contralateral artery underwent an identical sham operation, without passage of the wire. Animals received peri-operative analgesia with buprenorphine (0.25 mg/kg s.c.)

Assessment of endothelial regeneration by en face microscopy. Mice were anesthetized at 0, 4 and 7 days after wire injury, and 50 µL of 0.5% Evans blue dye injected into the inferior vena cava. The mice were perfused/fixated with 4% paraformaldehyde in PBS before the femoral arteries (injured and uninjured) were harvested. The vessels were opened longitudinally. The areas stained and unstained in blue were measured in a 5mm injured segment beginning 5mm distal to the aortic bifurcation, and the percentage areas were calculated using ImagePro Plus 7.0 software (Media Cybernetics, Bethesda, MD).

Flow cytometric enumeration of circulating progenitor cells. Saphenous vein blood samples (150µL), were incubated with PharmLyse (BD Biosciences, San Jose, CA), at room temperature. After centrifugation, mononuclear cells were resuspended in fluorescence-activated cell sorter (FACS) buffer and incubated with FcR blocker (BD Biosciences) at 4°C.
Appropriate volumes of the following antibodies, or their respective isotype controls, were then added for 10 minutes at 4°C: fluorescein isothiocyanate (FITC) anti-mouse Sca-1 and phycoerythrin (PE) anti-mouse Flk-1 (BD Biosciences). Circulating progenitor cells were enumerated using flow cytometry (BD FACS Calibur), to quantify dual-stained Sca-1+/Flk-1+ cells. Isotype control specimens were used to define the threshold for antigen presence and to subtract nonspecific fluorescence. The cytometer was set to acquire 100,000 events within the lymphocyte gate, defined by typical light scatter properties. To assess circulating progenitor cell mobilization, mice received an intra-peritoneal injection of 5µg of vascular endothelial growth factor (VEGF), on four consecutive days, as previously described. Sca-1+/Flk-1+ cells were quantified using FACS analysis at baseline and at day 4 after treatment.

Circulating angiogenic progenitor cell isolation and culture. Mononuclear cells from 1mL of blood, obtained from the vena cava under terminal anesthesia, were isolated by Histopaque-1083 (Sigma) density gradient centrifugation. Mononuclear cells were seeded on fibronectin coated 24-well plates (BD Biosciences) at a density of 5x10⁶ cells/well. Cells were cultured in endothelial cell growth (EGM-2), medium supplemented with EGM-2 Bullet kit (Lonza, Basel, Switzerland), in addition to 20% FCS. Spleens obtained from mice under terminal anaesthesia were mechanically minced. Mononuclear cells were isolated by density gradient centrifugation. After washing steps, cells were seeded on fibronectin coated 24-well plates at a seeding density of 8x10⁶ cells/well and cultured as described above. Femurs and tibias were flushed three times in DMEM with a 26-gauge needle to collect bone marrow. Mononuclear cells were isolated by density gradient centrifugation as described above. After washing steps, cells were seeded on fibronectin coated 24-well plates at a seeding density of 1x10⁶ cells/well and cultured as described above. After 4 days of incubation at 37°C in 5% CO₂, non-adherent cells were discarded by gentle washing with PBS and adherent cells resuspended in medium after trypsinisation.

Phenotypic analysis of angiogenic progenitor cells in culture. DiI-ac-LDL+/lectin⁺ angiogenic progenitor cells (APC) were quantified in cultured peripheral blood, spleen, and bone marrow derived mononuclear cells (see above), resulting in the growth of early-outgrowth cells. These cells exhibited typical spindle-shaped morphology on day 4 of culture. At day 7, attached cells from peripheral blood, spleen and bone marrow were stained for the uptake of 1,1'-dioctadecy-3,3,39,39-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (DiI-Ac-LDL) (Molecular Probes, Invitrogen, Carlsbad, CA) and lectin from Ulex europaeus FITC conjugate (Sigma). Cells were first incubated with DiI-Ac-LDL at 37°C for 3 h and later fixed with 4% paraformaldehyde for 10 minutes at room temperature. Cells were washed and reacted with lectin for 1 h at room temperature. After staining, cells were quantified by examining five random high-power fields per well and double-positive cells were identified as APCs. APC size
and density were estimated by analysis of forward and size scatter on flow cytometry (BD FACS Calibur). Flow cytometry was also used to quantify expression of CD11b and CD49e using AF647-labelled anti-CD11b/isotype-control antibodies (Abcam, Cambridge, UK) and PE-labelled anti-49e/isotype-control antibodies (BD Biosciences, San Jose, CA, USA). Expression of monocyte/macrophage markers by APCs was evaluated by fluorescence microscopy using rat monoclonal anti mouse CD68 antibodies (AbD Serotec, Kidlington, UK) and affinity purified polyclonal sheep anti human factor XIII (A-subunit) antibodies (Enzyme research laboratories, Swansea, UK). Images were acquired using multiple labelling grade DyLight conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) in maximum intensity projections of 1.5µm slices through the sample thickness for each channel on a LSM700 confocal microscope (Zeiss, Germany).

**Angiogenic progenitor cell function: adhesion and angiogenesis.** To evaluate adhesion, 50,000 APCs were resuspended in EGM-2 medium, plated onto fibronectin coated 24-well plates, and incubated for 1 h at 37°C. After washing three times with PBS, attached cells were counted. Adhesion was evaluated as the mean number of attached cells per high power field. The contribution of nitric oxide to adhesion was evaluated by repeated the experiments after incubating APCs with the nitric oxide synthase inhibitor L-LMMA (100nmol/L; 30 minutes). The potential for APCs to stimulate angiogenesis by secreting paracrine factors was assessed as previously described. Briefly, conditioned media were obtained by replacing the medium of day 4 APC cultures with serum-free EGM-2 supplemented with 1% FCS and culturing the cells for an additional 24 h. APCs were enumerated, and conditioned media diluted to correct for cell numbers. After 24 h, tube formation by human umbilical vein endothelial cell (HUVEC) (Promocell, Heidelberg, Germany), on matrigel coated 24-well plates (BD Bioscience), in the presence of conditioned media was measured by staining the viable cells with hematoxylin-eosin (Sigma). HUVEC cultures were used at passage 3–5. The number of endothelial tubes was quantified by a blinded observer by averaging the count in multiple high power fields per well. Secretion of angiogenic cytokines by APCs was evaluated by assessing the concentration of insulin-like growth factor 1 (IGF1), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), stromal cell derived factor (SDF)-1 and granulocyte-colony stimulating factor (G-CSF) in day 4 conditioned medium using Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. Conditioned medium was 10x concentrated before analysis (0.5mL concentrator with 3K molecular weight cut-off, Pierce Biotechnology. Rockford, IL, USA).

**mRNA expression in APCs.** RNA was isolated using Tri Reagent (Sigma-Aldrich). After DNAse treatment, 1 μg RNA was reverse transcribed using High Capacity RNA to cDNA kit. Real-time
PCR was performed (Applied Biosystems) using SYBR Green PCR Master Mix and the gene specific primer. Results were normalized to expression of β-actin. Primer sequences were as follows: mIR F:TGGAGAGGTGTGCCCTGGT R:TGAACATCAGGAGGATCTGCAG; mIGF1R with deleted exon F:CGCTGCCAGAAAACATGTACTG R:GGTGCATCCTTGGAGCATTT; eNOS F:CTGGAGCACCACCCAGCT R: AGCGGTGAGGGTCACACAG; beta-actin F: CGTGAAGATGACCACGACG; R: TGGTACGACCAGAGGCATACAG.

**Quantification of serine$^{1177}$ phosphorylated eNOS in bone marrow**

Bone marrow was collected by flushing the femur and tibia from one mouse with DMEM and kept on ice. Marrow was then homogenized and collected through a cell strainer. Homogenate was added to Ficoll and centrifuged at 400g for 30 minutes to collect mononuclear cells. The Buffy coat layer was collected and washed once with PBS and then with PBS/DMEM. The resulting cell pellet was frozen at -80°C. Marrow-derived mononuclear cells were re-suspended in 50µL of cell lysis or RIPA buffer, sonicated and kept on ice for 45 minutes, before protein concentrations were determined using BCA Assay. 50µL of DynaBeads Protein G (Invitrogen) were first coated with anti-eNOS/NOS Type III anti-body (BD Biosciences) according to manufacturer’s instructions. After washing the prepared beads, 20µg of marrow-derived APC lysates were incubated with anti-eNOS-DynaBeads for 30 minutes at room temperature. Following extensive washing, 30µL of 1X NuPAGE LDS sample buffer and 1X reducing buffer were added into each of the resulting sample preparations. Samples were then incubated at 95°C, for 5 minutes before an SDS-PAGE were run and membrane blotted. Levels of total and phosphorylated eNOS were evaluated by incubating the membranes overnight with anti-eNOS and anti-phospho eNOS (pS$^{1177}$) antibodies (Cell Signaling) and chemiluminescence assay carried out the following day. Quantification of β-actin expression (Santa Cruz Biotechnology) was used as a loading control.

**Aortic ring angiogenesis assay.**

Descending thoracic aortas were excised and flushed with ice-cold PBS until free of blood. Surrounding fibroadipose tissue was dissected free, and the aorta sectioned into 1mm rings. Culture plates (24-well) were coated with 200 µL/well of growth factor–reduced Matrigel (BD Bioscience), rings were embedded and the Matrigel was then allowed to polymerize for 30 minutes at 37°C. Rings were incubated with Endothelial Cell Growth Medium MV2 (PromoCell, Heidelberg, Germany), which was replaced daily. Quantitative analysis of endothelial sprouting was performed using images from day 6. The greatest distance from the aortic ring body to the end of the vascular sprouts was measured at three distinct points per ring and in three different rings per treatment group. Sprout lengths were normalised to the circumference of the aortic ring.
Effect of pharmacological IGF1R inhibition on endothelial regeneration after arterial injury. Arterial injury experiments were carried out as above in wild type mice in which IGF1R signalling was inhibited by the pharmacological inhibitor PQ401\(^3\). 16-18 week-old male C57BL6 mice were administered PQ401 (10mg/kg\(^4\) reconstituted in 6% DMSO) (SigmaAldrich, St. Louis, MO) or equivalent DMSO by intra-peritoneal injection immediately after injury. Femoral arteries were harvested at day 4 and endothelial regeneration was quantified as above.

Effect of CD117\(^+\) cell transfusion on endothelial repair. Bone marrow derived cells were subjected to magnetic bead separation to collect CD117\(^+\) (C-kit receptor\(^+\)) cells. In brief, bone marrow derived cells were washed, resuspended, and magnetically labelled with CD117 microbeads (MACS Microbeads, Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). After incubation and additional washing, magnetic cell separation was performed using a separation column placed in a magnetic field of a magnetic bead separator (MACS Separation Columns, Miltenyi). CD117\(^+\) cells were collected in buffer and resuspended in 200\(\mu\)L of MACS buffer for intravenous injection. In order to prevent sequestration of injected cells by the spleen, mice were anaesthetised with isoflurane, and the spleen excised through a left lateral abdominal incision. Splenic vessels were carefully ligated using 6.0 silk. After removal of the spleen the peritoneal wall was closed with 8.0 vicryl sutures before skin closure with 6.0 vicryl. Immediately after splenectomy and arterial injury, wild type mice received an intravenous injection of 5x10\(^5\) CD117\(^+\) bone marrow cells from IGF1R\(^+/−\) or wild type mice. Control animals underwent splenectomy and vascular injury and received a corresponding volume of normal saline without cells. Femoral arteries were harvested and examined at 4 days for Evans blue staining as above. In additional experiments, CD117\(^+\) cells derived from IGF1R\(^+/−\) or wild type mice were labelled with CellTracker Red (Life Technologies, Paisley, UK) before transfusion. Injured arteries were imaged en face at day 4 using on a LSM 510 META confocal microscope (Zeiss, Germany). Endothelial cells were identified using rabbit polycolonal anti-CD31-antibodies and Chromeo642-conjugated goat polyclonal anti-rabbit secondary antibodies (Abcam, Cambridge, UK).

Effect of CD117\(^+\) cell transfusion on atherosclerosis.

Atherosclerotic lesion formation was evaluated in carotid arteries of ApoE\(^−/−\) mice after placement of a peri-adventitial vascular collar as previously described\(^5\). Briefly, unilateral 2mm silastic collars (0.3mm internal diameter) (Dow Corning, Midland, MI, USA) were placed around
the carotid artery in 16-week old male ApoE−/− mice under general anaesthesia. Mice were fed Western diet from 8 weeks of age (21% fat from lard supplemented with 0.15% wt/wt cholesterol (#829100, SDS, Witham, Essex, UK). 5x10⁵ CD117⁺ bone marrow cells from IGF1R+/− or wild type mice were injected via the femoral vein after cuff placement. Carotid arteries were harvested under terminal anaesthesia after perfusion fixation (4% paraformaldehyde in PBS) 4 weeks after collar placement. Sections obtained proximal to the cuffs (where lesion formation was maximal) were stained with Miller Elastin Stain. Lesion intima/media ratio was calculated using ImagePro Plus 7.0 software (Media Cybernetics, Bethesda, MD). Incorporation of CD117⁺ cells into atherosclerotic plaques was evaluated by repeating the experiment using CD117⁺ cells labelled with CellTracker Red (Life Technologies, Paisley, UK). Cellular incorporation was assessed after 4 days in carotid artery cross sections by confocal microscopy as described above. Endothelial cells were identified using rabbit polyclonal anti-CD31-antibodies and Chromeo642-conjugated goat polyclonal anti-rabbit secondary antibodies (Abcam, Cambridge, UK).

Statistics. Results are expressed as mean±SEM. Comparisons within groups were made using paired Students t-tests and between groups using unpaired Students t tests or repeated measures ANOVA, as appropriate; where repeated t-tests were performed a Bonferroni correction was applied. P<0.05 considered statistically significant.

References