

Endothelial cell repopulation after stenting determines in-stent neointima formation: effects of bare-metal vs. drug-eluting stents and genetic endothelial cell modification

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Received 8 November 2011; revised 6 June 2012; accepted 19 July 2012; online publish-ahead-of-print 24 September 2012

Aims

Understanding endothelial cell repopulation post-stenting and how this modulates in-stent restenosis is critical to improving arterial healing post-stenting. We used a novel murine stent model to investigate endothelial cell repopulation post-stenting, comparing the response of drug-eluting stents with a primary genetic modification to improve endothelial cell function.

Methods and results

Endothelial cell repopulation was assessed *en face* in stented arteries in ApoE^{-/-} mice with endothelial-specific LacZ expression. Stent deployment resulted in near-complete denudation of endothelium, but was followed by endothelial cell repopulation, by cells originating from both bone marrow-derived endothelial progenitor cells and from the adjacent vasculature. Paclitaxel-eluting stents reduced neointima formation (0.423 ± 0.065 vs. 0.240 ± 0.040 mm², $P = 0.038$), but decreased endothelial cell repopulation (238 ± 17 vs. 154 ± 22 nuclei/mm², $P = 0.018$), despite complete strut coverage. To test the effects of selectively improving endothelial cell function, we used transgenic mice with endothelial-specific overexpression of GTP-cyclohydrolase 1 (GCH-Tg) as a model of enhanced endothelial cell function and increased NO production. GCH-Tg ApoE^{-/-} mice had less neointima formation compared with ApoE^{-/-} littermates (0.52 ± 0.08 vs. 0.26 ± 0.09 mm², $P = 0.039$). In contrast to paclitaxel-eluting stents, reduced neointima formation in GCH-Tg mice was accompanied by increased endothelial cell coverage (156 ± 17 vs. 209 ± 23 nuclei/mm², $P = 0.043$).

Conclusion

Drug-eluting stents reduce not only neointima formation but also endothelial cell repopulation, independent of strut coverage. In contrast, selective targeting of endothelial cell function is sufficient to improve endothelial cell repopulation and reduce neointima formation. Targeting endothelial cell function is a rational therapeutic strategy to improve vascular healing and decrease neointima formation after stenting.

Keywords

Stent • Restenosis • Re-endothelialization • Endothelium • Mouse • Artery

Introduction

Percutaneous coronary intervention with stenting is the commonest means of revascularization in patients with coronary artery disease.

In-stent restenosis, due to neointima formation, has been greatly reduced by drug-eluting stents (DES) which inhibit vascular smooth muscle cell (VSMC) proliferation.^{1,2} However, the reduction of neointima formation may lead to persistent exposure of

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stent struts, and there are potentially detrimental effects of anti-proliferative drugs on endothelial cell repopulation after stenting, leading to clinical concerns over delayed arterial healing, poor re-endothelialization, and late stent thrombosis after DES deployment.^{3,4}

Increasing attention has focused on the importance of endothelial regeneration after stenting. The endothelial cell layer is critical in modulating local haemostasis, thrombolysis, and VSMC proliferation^{5,6} in response to vascular injury. Experimental models of vascular injury have shown that the rate of endothelial cell repopulation post-injury is a critical factor in determining subsequent neointima formation,^{7,8} and is presumed to be a potential therapeutic strategy. Indeed, recent clinical studies have tested the potential value of strategies aimed at increasing endothelial cell capture and re-endothelialization after stenting.⁹ However, both experimental and clinical studies have used 'strut coverage' as a surrogate for presumed endothelial cell repopulation, a measure that is also affected by neointima formation. Thus, it has so far proved difficult to separate the direct effects of anti-proliferative DES on neointima formation from those due to changes in endothelial cell repopulation.

Some studies have attempted to evaluate endothelial cell repopulation using *en face* SEM, Evans Blue dye, and transverse sections,^{10–12} but these approaches have technical limitations in stented vessels. No study has been able to investigate the origin of repopulating endothelial cells after stenting, how endothelial cell repopulation is related to neointima formation and strut coverage in bare-metal vs. DES, and whether a selective and specific endothelial cell intervention is sufficient to alter endothelial cell repopulation and neointima formation. Furthermore, experimental studies in healthy animals do not model the effects of endothelial cell dysfunction that is typical of atherosclerotic vascular disease states. These are all critical requirements to better understand the potential of endothelial cell repopulation as a therapeutic target to improve vascular healing after stenting.

We recently developed a novel mouse model of stenting, using a balloon-expandable slotted tube stent in mouse aorta, combined with isogenic grafting of the stented aorta from donor to recipient animals in order to test local vs. systemic effects on the response to stenting.⁶ This approach allows the use of genetic models of atherosclerosis, the incorporation of cell-specific genetic markers to identify and track endothelial cells, and endothelial cell-targeted transgenes to test the effects of altered endothelial cell function. We used these models to rigorously test the effects of stenting on endothelial cell repopulation and neointima formation in atherosclerotic ApoE^{-/-} mice, after both bare metal and DES deployment, and in transgenic animals with improved endothelial cell function.^{13–15}

Materials and methods

Animals

ApoE^{-/-} mice were bred with ApoE^{-/-} mice which heterozygously expressed β -Gal under the control of the endothelial-specific *Tie-2* promoter (ApoE^{-/-} Lac Z; Jackson Laboratories, Bar Harbor, MI) to generate ApoE^{-/-} mice and ApoE^{-/-} Lac Z littermates.⁷ Mice over-expressing human GTP cyclohydrolase (GCH-Tg) targeted to the vascular endothelium under the *Tie-2* promoter,¹⁶ were crossed with

ApoE^{-/-} LacZ mice to generate GCH-Tg LacZ ApoE^{-/-} mice and LacZ ApoE^{-/-} littermates. Animals were housed in individually ventilated cages; standard chow and water were available *ad libitum*. All animal procedures were carried out in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986.

Arterial stent-graft procedure

Mice were treated pre- and post-operatively with aspirin (10 mg/kg/day in drinking water). Stent arterial grafts or balloon angioplasty grafts were carried out as described previously.⁶ Briefly, a stainless-steel stent (5 cell, 2.5 × 0.8 mm; strut thickness 0.06 mm; Cambus Medical, Ireland) was crimped onto a 1.25 × 13 mm balloon angioplasty catheter (Avion Plus, Invatec International) or for balloon angioplasty grafts a balloon with no stent were deployed (8 atm, 30 s) into the thoracic aorta, resulting in a balloon to vessel ratio of 1.5:1 and a metal-to-artery ratio of 15%. Recipient mice (male: 20–22 weeks of age) were anaesthetized with 2% isoflurane. The right common carotid artery was isolated, ligated, and divided between ties. Polyethylene cuffs (0.65 mm diameter) were placed over the end and anchored by clamps. The artery was everted over the cuffs secured with suture and the stented/balloon angioplasty aorta was interposition grafted.

Paclitaxel-coated stents and their polymer controls were obtained from Nanocopoeia (USA). Stainless-steel stents were fully coated using the ElectroNanosprayTM process with TPE4 (provided by J. Puskas, University of Akron, Ohio) to a target coat weight of 60 ± 10 μ g. Paclitaxel stents were coated to the same target weight as polymer only stents with the addition of 10% wt paclitaxel; 90% TPE4 to give a drug dosage of 100 μ g paclitaxel/cm², resulting in a total dose of 30 μ g, with ~10% release of drug in 28 days.

En face X-gal staining

Preparation and quantification of *en face* X-gal staining were as described previously.¹⁷ Briefly, mice were anaesthetized and perfusion fixed *in situ* (4% formaldehyde/0.25% glutaraldehyde) and stained overnight at 37°C in X-gal (50 mg/ml) solution. Vessels were fixed overnight in 4% paraformaldehyde prior to photographing. Images were analysed using Image Pro Plus (Media Cybernetics, USA). The number of X-gal-stained nuclei were counted and normalized for surface area.

Generation of bone marrow chimeras

Chimeric mice were generated in a manner similar to that described previously.¹⁷ Briefly, donor ApoE^{-/-} and ApoE^{-/-} LacZ mice were killed and a single-cell suspension of bone marrow prepared. Twelve-week-old ApoE^{-/-} mice received a lethal dose of whole body irradiation (2 × 5 Gy) followed by an intravenous injection of 1 × 10⁷ bone-marrow cells in 0.2 mL phosphate-buffered saline from either LacZ-positive or LacZ-negative donor mice. DNA was extracted from blood samples and the absence or presence of the LacZ transgene assessed using PCR. Once reconstitution was confirmed stented arterial grafts were carried out 6 weeks after bone marrow transfer. Grafts were harvested 28 days after surgery and endothelial progenitor cell (EPC)-driven repopulation of the stented segment assessed by *en face* X-gal staining. At the time of harvest, DNA was extracted from bone marrow samples and the absence or presence of the LacZ transgene assessed using standard PCR.

In order to assess the efficiency of the bone marrow transplant mice with differential CD45 antigens (CD45.1 and CD45.2 mice) were used; CD45.1 mice were irradiated and reconstituted with CD45.2 bone marrow in order to quantify reconstitution. Efficiency of bone marrow reconstitution was assessed 4 weeks after bone marrow

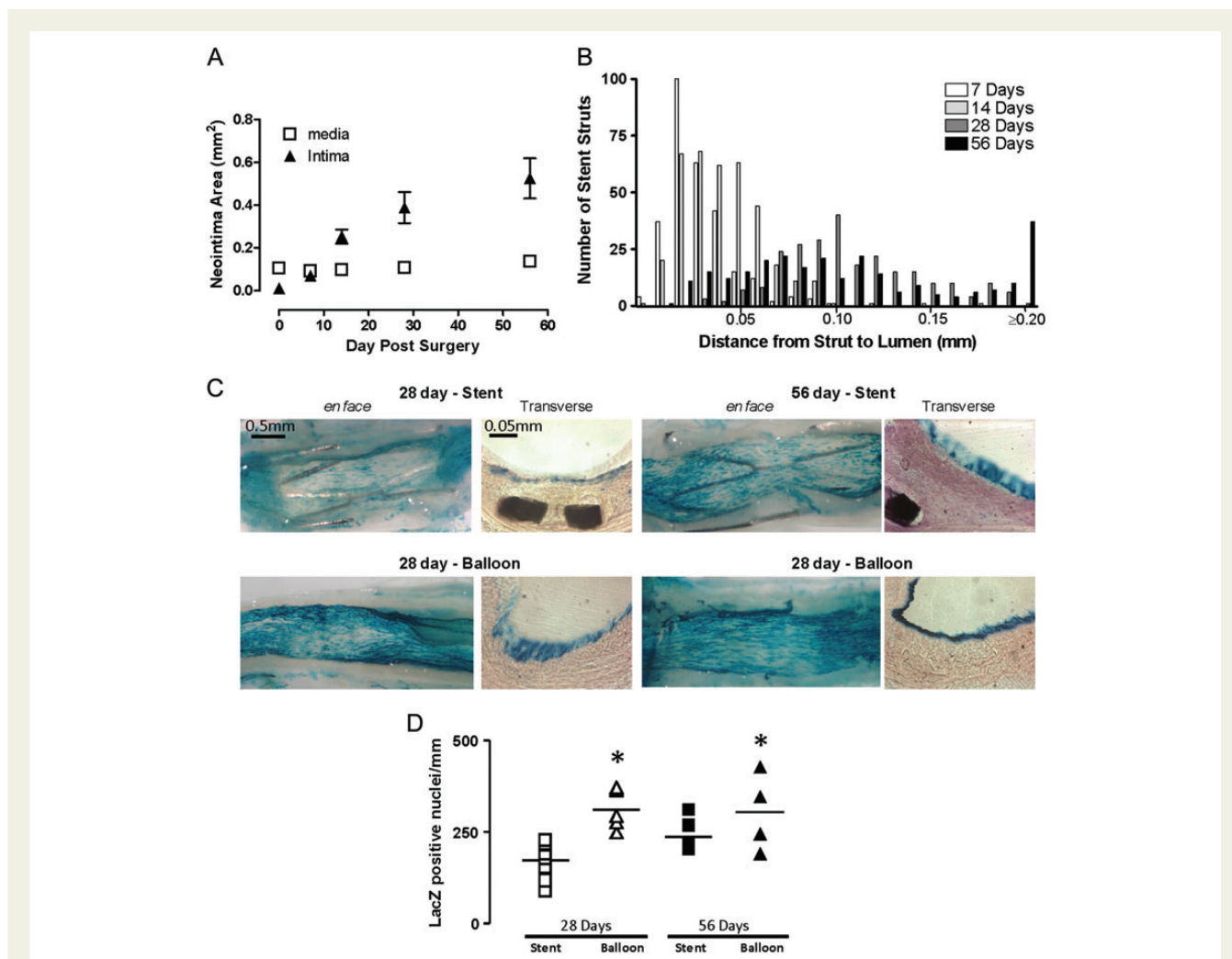


Figure 1 Time course of neointima formation and endothelial cell repopulation. (A) Time course of neointima formation after deployment of a bare-metal stent in ApoE^{-/-} mice, neointima area increases significantly over time ($P < 0.001$; $n = 4-5$ per time point). (B) Frequency distribution of strut-level intimal thickness after deployment of a bare-metal stent in ApoE^{-/-} mice at 7, 14, 28, and 56 days; $n = 3$ per group. (C) Images of X-gal-stained *en face* and transverse sections at 28 and 56 day after stenting or balloon angioplasty in Tie2-LacZ ApoE^{-/-} mice. (D) Quantification of *en face* endothelial cell repopulation 28 and 56 days post-stenting or balloon angioplasty, * $P = 0.009$ balloon vs. stent, $n = 6-8$ per group.

transplant in blood leucocytes, quantified by flow cytometry using specific antibodies, and appropriated isotype controls; neutrophils (7/4 positive Ly-6G negative), monocytes (7/4 high Ly6G negative), and T-cells (CD3 positive and either CD8 or CD4 positive). Percentage repopulation was calculated in blood samples stained for either the presence of the donor CD45.2 or the recipient CD45.1 allele. Flow cytometry analysis was performed with FACS-Calibur (BD Biosciences, USA) using a Cyan ADP analyser (Beckman Coulter, USA), data were analysed using Flowjo software (TreeStar, USA). All antibodies were purchased from BD Pharmingen with the exception of F4/80 (AbD Serotec) and CD45.1, CD45.2, and 7/4 (Biolegend).

Quantification of neointima formation and strut coverage

Mice were anaesthetized and stents fixed *in situ* and overnight *in vitro* (4% formaldehyde/0.25% glutaraldehyde). Neointima formation was quantified using optical coherence tomography (OCT; Imagewire, Lightlab Imaging Inc., USA) with an axial resolution of 10 μm and a

transverse resolution of 25 μm using an automated pullback of 0.5 mm/s. Images were quantified using Image Pro Plus (Media Cybernetics), using the know width of the OCT probe as a calibration marker. The centre point of the stent was identified by the cell-strut geometry and neointima area and neointima thickness were quantified at the mid-point of the stent and 500 μm either side of the mid-point, taking the average of the three measurements as the value for each stented vessel. Single-strut analysis was assessed by identification of the mid-point of the stent and quantifying the strut to lumen length for every visible stent strut every 100 μm from the mid-point of the stent to 800 μm either side of the mid-point (~80–100 struts per stent).

Endothelial progenitor cell quantification

Endothelial progenitor cells were isolated and quantified from bone marrow and blood as described previously.¹⁸ Briefly, 28 day post-stenting animals were culled and low-density bone marrow mononuclear cells were isolated with density gradient centrifugation (LSM

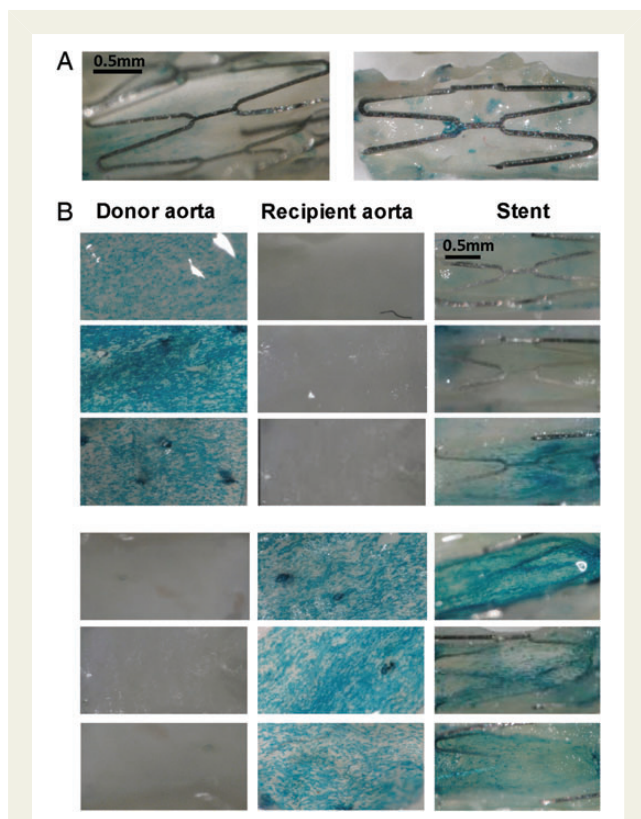


Figure 2 Origin of repopulating endothelial cells. (A) Images of *en face* X-gal-stained aortas from ApoE^{-/-} mice immediately after stent deployment. (B) Representative *en face* X-gal-stained aortas from donor mice and aorta and 28 day stent from recipient mice. Donor and recipient mice were either ApoE^{-/-} Tie2-LacZ positive (endothelial cells stain blue) or negative (endothelial cells not stained). Contribution of donor endothelial cells to repopulation was assessed by grafting a stent aorta from a Tie2-LacZ mouse into a Tie2-LacZ-negative recipient. The contribution of recipient-derived endothelial cells to repopulation was assessed by grafting the stent aorta from a Tie2-LacZ-negative mouse into a Tie2-LacZ-positive recipient. Representative photos of $n = 5$ per group.

1077, PAA Laboratories, Austria). Lineage negative cells were enriched using a lineage cell depletion kit (Miltenyi, Germany). Lineage negative cells were stained for Sca-1 and flk-1 using directly conjugated antibodies and appropriate IgG controls (2 $\mu\text{g}/\text{ml}$; BD Biosciences) flow cytometry analysis was performed with FACS-Calibur (BD Biosciences) using a Cyan ADP analyser (Beckman Coulter), and data were analysed using FlowJo software (TreeStar).

Tissue preparation for injury and inflammation scores

Fixed grafts were embedded in methyl methacrylate resin (T9100, TAAB Laboratories, UK). Transverse sections were cut using an Isomet 5000 diamond-coated rotary saw and polished (30 μm) using a Metaserv 2000 polisher (Buehler, UK). Arterial injury was determined on Verhoeff's Van Gieson stained sections using the Schwartz injury score.¹⁹ Inflammation score sections were determined in stained haematoxylin and eosin-stained sections using the scoring system of Kornowski *et al.*²⁰

Isolation of primary endothelial cells and vascular smooth muscle cell and western blotting

Primary endothelial cells were isolated from lungs by immunoselection with CD31 antibody-coated magnetic beads as described previously.²¹ Vascular smooth muscle cells were isolated from the thoracic aorta as described previously.²² Western blotting was carried out on primary endothelial and VSMC (15 μg protein) using standard techniques and anti-eNOS (BD Transduction Laboratories), mouse GCH, human GCH, smooth muscle cell α -actin (Santa Cruz), and GAPDH (Chemicon, USA) antibodies.

Analysis of NO synthesis by eNOS

Cellular NO synthesis by eNOS was assessed in primary endothelial cells by measuring the conversion of ¹⁴C L-arginine to citrulline with high-performance liquid chromatography detection, in the presence and absence of *N*-monomethyl-L-arginine, as described previously.²³

Statistical analysis

Data are presented as mean \pm SEM. Groups were compared using the Mann–Whitney U-test for non-parametric data or the Student's two-sided *t*-test for parametric data. When comparing multiple groups, data were analysed by analysis of variance with Newman–Keuls post-test for parametric data or Kruskal–Wallis test with Dunns post-test for non-parametric data. A value of $P < 0.05$ was considered statistically significant. Statistical analysis was carried out using GraphPad Prism version 4 (GraphPad Software, USA).

Results

Time course of endothelial cell repopulation and the origin of repopulating endothelial cells

We first used Tie2-LacZ ApoE^{-/-} mice to track the origin and time course of endothelial cell repopulation after stenting, in comparison with balloon angioplasty. Stent deployment resulted in a time-dependent increase in neointima formation from 0 to 56 days ($P < 0.001$; Figure 1A). Strut analysis revealed right ward shifting bell-shape curve at 7, 14, and 28 days with a modal thickness of 0.02, 0.005, and 0.1 mm, respectively. However, at 56 days the bell-shaped distribution was less apparent with a much wider distribution of strut to lumen distance observed (Figure 1B). Stent deployment did not result in medial hypertrophy, as the medial thickness was similar at all time points. Total endothelial cell repopulation was assessed *en face* and in transverse sections of X-gal-stained stents (Figure 1B). A time-dependent increase in endothelial cell repopulation was observed from 0 to 56 days after arterial stent deployment. Compared with balloon angioplasty alone, stenting resulted in a significant reduction in endothelial cell repopulation at both 28 and 56 days post-implant (172.6 vs. 310.6 nuclei/mm, 28 days post-implantation, $n = 6-8$, $P = 0.009$; Figure 1C).

Endothelial cell survival in the stented vessel was assessed immediately after stent deployment. *En face* X-gal staining revealed that stent deployment resulted in near-complete denudation of the endothelial cell layer, apart from occasional surviving endothelial cells that were observed between stent struts (Figure 2A). We next aimed to determine the origin of repopulating endothelial

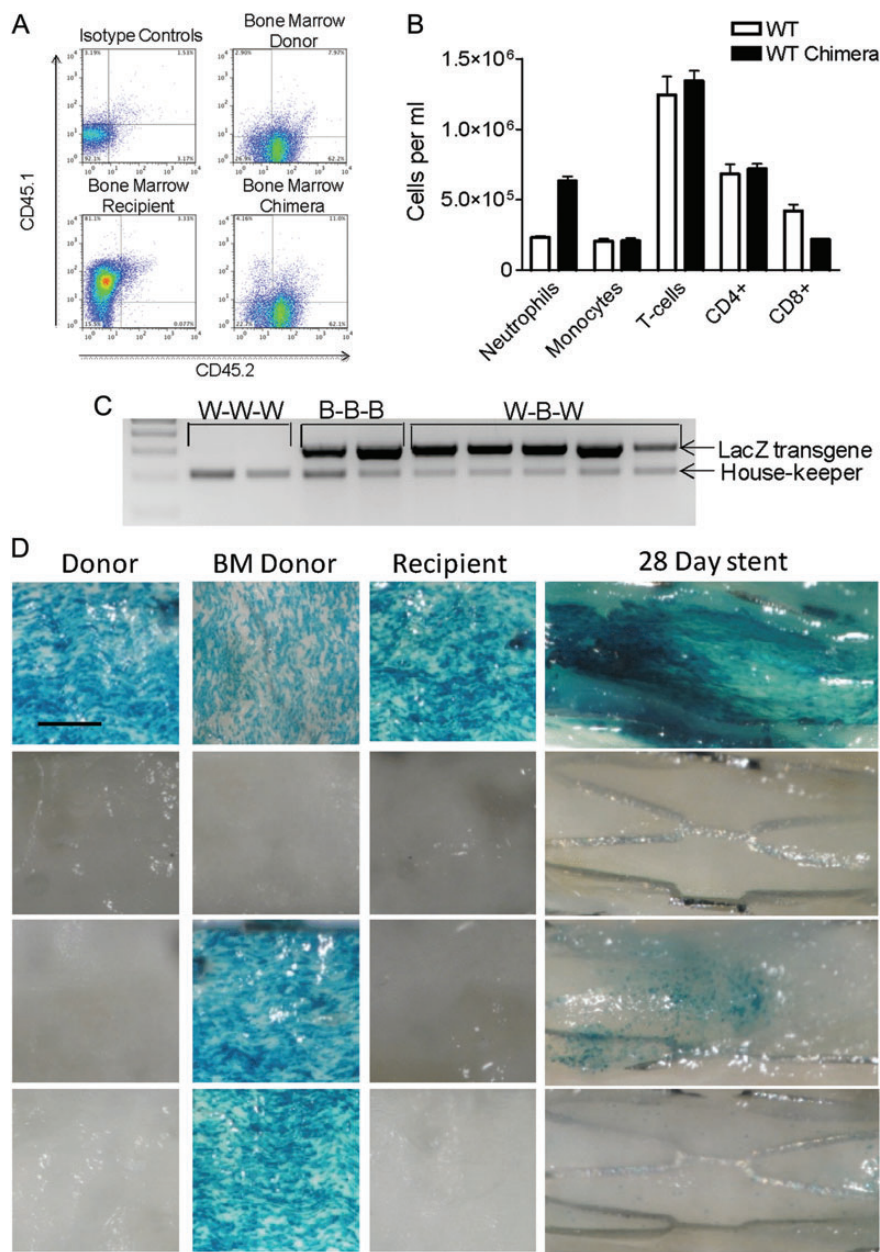


Figure 3 Contribution of endothelial progenitor cells to endothelial cell repopulation. (A) Representative flow cytometry plots of bone marrow from CD45.1 mice irradiated and reconstituted with CD45.2, and donor and recipient controls bone marrow antibody stained for the presence of donor CD45.2 and recipient CD45.1 allele, 6 weeks post-transplant. (B) Quantification of blood leucocytes from irradiated (6 weeks post-irradiation) and non-irradiated mice, $n = 3/\text{group}$. (C) Representative PCR gel for the LacZ transgene (315bp) and a house keeping gene (202bp) from bone marrow of chimera mice, 10 weeks after transplant. W-W-W = LacZ-negative aorta and bone marrow donor and recipient, B-B-B = LacZ-positive aortic and bone marrow donor and recipient, W-B-W = LacZ-negative aortic donor, LacZ-positive bone marrow donor and LacZ-negative recipient. (D) Representative *en face* X-gal-stained aortas from graft donor and bone marrow donor mice and the aorta and 28-day stent from recipient mice. Donor and recipient mice were either ApoE^{-/-} Tie2-LacZ positive (endothelial cells stain blue) or negative (endothelial cells not stained). Contribution of circulating endothelial progenitor cells to endothelial cell repopulation was assessed by grafting a stent aorta for a Tie2-LacZ-negative mouse into a Tie2-LacZ-negative recipient that had been reconstituted with bone marrow from a Tie2-LacZ-positive mouse, representative photos of $n = 5$ per group.

cells in stented vessels. To establish the role of endothelial cells derived from the stented vessel wall, we grafted stented aortas from donor Tie2 LacZ ApoE^{-/-} mice into recipient ApoE^{-/-}

littermates. To establish the contribution of endothelial cells derived from systemic sources, we grafted stented aortas from donor ApoE^{-/-} mice into recipient Tie2 LacZ ApoE^{-/-} mice.

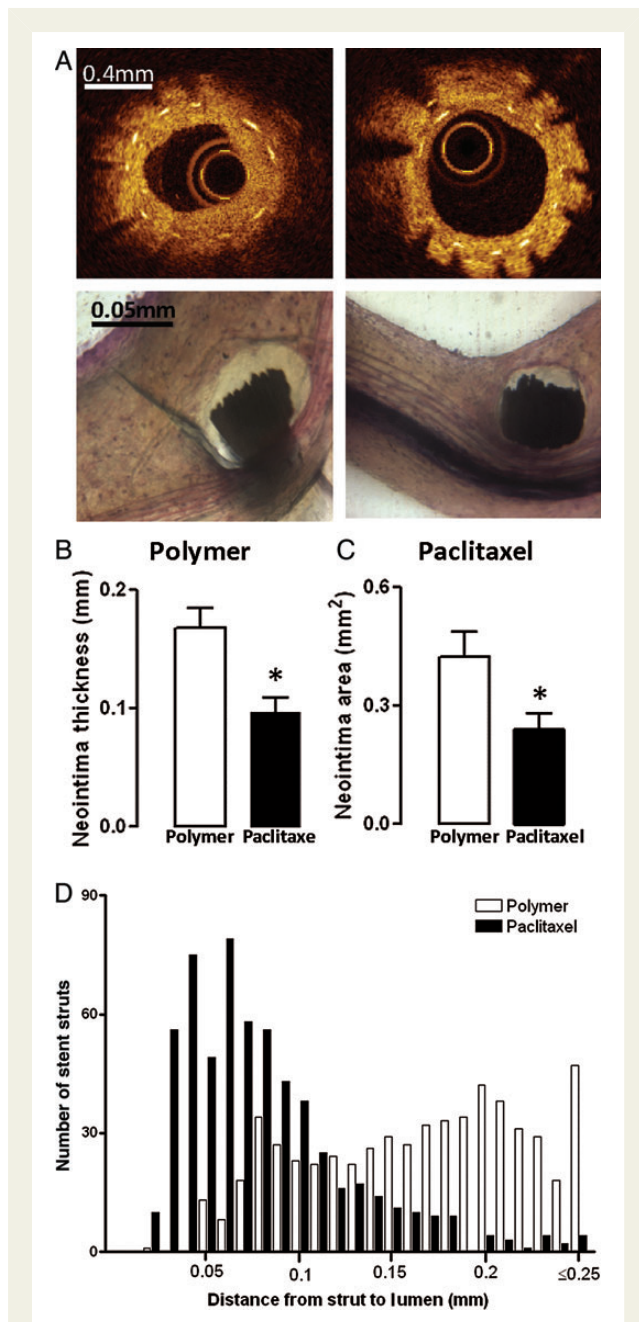


Figure 4 Neointima formation 28 days after deployment of either paclitaxel-eluting or control stents in ApoE^{-/-} mice. (A) Representative optical coherence tomography images (upper panels) and haematoxylin- and eosin-stained transverse sections (lower panels) of 28-day polymer and paclitaxel stents. (B) Quantification of neointima area using optical coherence tomography imaging, **P* = 0.038 paclitaxel vs. polymer 28-day stents, *n* = 8–7/group. (C) Quantification of neointima thickness (distance from strut to lumen) using optical coherence tomography imaging, **P* = 0.030 paclitaxel vs. polymer, *n* = 9/group. (D) Frequency distribution of strut-level intimal thickness. *n* = 7–8 per group.

En face analysis of endothelial cell repopulation showed that endothelial cells from both the stented vessel and recipient animal were responsible for repopulation, with the majority of the endothelial

cells being derived from the recipient animal rather than from the local stented vessel wall (Figure 2B).

To determine whether the recipient-derived endothelial cells were from circulating EPCs or from adjacent vasculature, we created bone marrow chimeras which expressed LacZ only in bone-marrow-derived cells. In order to establish the efficiency of our bone marrow transfer we used mice with a differential CD45 antigen; CD45.1 and CD45.2 mice. Six weeks after bone marrow transplant 95% of bone marrow cells from CD45.1 mice expressed the donor CD45.2 antigen showing high efficiency of bone marrow reconstitution (Figure 3A). Importantly, 6 weeks after bone marrow transplant chimeric mice had similar numbers of leucocytes to non-irradiated mice (Figure 3B). We irradiated LacZ-negative ApoE^{-/-} mice and reconstituted them with bone marrow from LacZ-positive ApoE^{-/-} mice, confirming by PCR the presence of LacZ in the bone marrow of LacZ-negative mice engrafted with LacZ-positive bone marrow (Figure 3C). Six weeks after reconstitution mice were grafted with stented aortas from LacZ-negative mice; hence, any LacZ-positive endothelial cells must have originated from the bone marrow. *En face* analysis of endothelial cell repopulation showed that circulating EPCs contributed to endothelial cell repopulation. However, the contribution of EPCs to repopulation was variable, with three out of five grafts showing a moderate contribution and two out of five grafts showing minimal contribution with only a few sporadic EPC-derived endothelial cells observed (Figure 3D). Importantly, ApoE^{-/-} LacZ-negative mice reconstituted with ApoE^{-/-} LacZ-negative bone marrow did not show any LacZ-positive endothelial cells in the stented artery, and bone marrow from these mice was negative for the presence of the LacZ transgene.

Paclitaxel-eluting stents have reduced neointima formation and reduced endothelial cell repopulation compared with polymer only stents

To test the effects of DES on endothelial cell repopulation, we developed polymer-coated stents for deployment in mouse aorta, with or without elution of paclitaxel, an antiproliferative drug widely used clinically.¹ No significant differences were observed in either neointima area, neointima thickness, or strut coverage after deployment of a bare-metal stent compared with deployment of a polymer-coated bare-metal stent (see Supplementary material online, Figure S1).

Optical coherence tomography of stented vessels harvested after 28 days revealed significantly reduced neointima formation in paclitaxel-eluting stents compared with control stents, as assessed by both neointimal area (0.240 ± 0.040 vs. 0.423 ± 0.065 mm²; *P* = 0.038, *n* = 7–8) and by mean neointimal thickness (0.097 ± 0.012 vs. 0.168 ± 0.016 mm; *P* = 0.030, *n* = 7–8). We also quantified coverage of individual stent struts, revealing a significant reduction in paclitaxel-eluting stents (Figure 4, and Supplementary material online, OCT Videos S1 and S2). However, there was no evidence of either uncovered struts or strut malapposition in this model, in either paclitaxel-eluting or control stents. The differences in neointima formation were not due to any differences in

Table 1 Neointima formation and injury and inflammatory scores in 28-day stent

	Bare-metal stent	Bare-metal stent	Polymer only stent	Paclitaxel-eluting stent
Genetic background of mouse	ApoE ^{-/-}	ApoE ^{-/-} GCH-Tg	ApoE ^{-/-}	ApoE ^{-/-}
Stent coating	None	None	TPE4 polymer	90% TPE4 polymer 10% paclitaxel
Stent expansion	0.93 ± 0.03	0.87 ± 0.02	0.98 ± 0.06	0.91 ± 0.02
Neointima area	0.43 ± 0.05	0.26 ± 0.04*	0.423 ± 0.065	0.240 ± 0.040\$
Distance strut to lumen	0.11 ± 0.01	0.07 ± 0.01*	0.16 ± 0.02	0.08 ± 0.01\$
Injury score	1.94 ± 0.20	1.90 ± 0.23	2.00 ± 0.26	1.57 ± 0.2
Inflammatory score	2.00 ± 0.40	1.33 ± 0.33	1.50 ± 0.34	1.83 ± 0.17

* $P < 0.05$ ApoE^{-/-} GCH-Tg vs. ApoE^{-/-}, \$ $P < 0.05$, paclitaxel vs. polymer only.

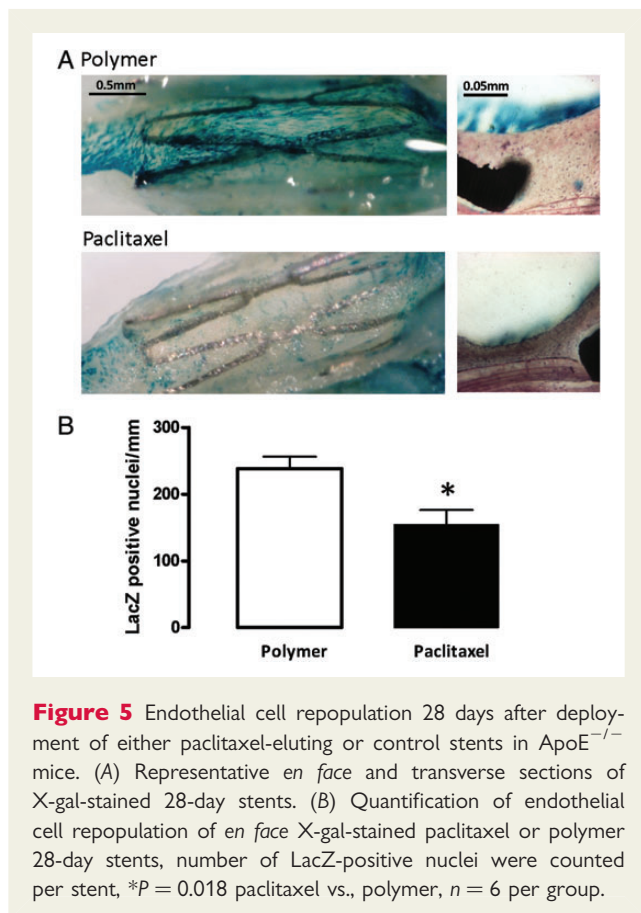


Figure 5 Endothelial cell repopulation 28 days after deployment of either paclitaxel-eluting or control stents in ApoE^{-/-} mice. (A) Representative *en face* and transverse sections of X-gal-stained 28-day stents. (B) Quantification of endothelial cell repopulation of *en face* X-gal-stained paclitaxel or polymer 28-day stents, number of LacZ-positive nuclei were counted per stent, * $P = 0.018$ paclitaxel vs., polymer, $n = 6$ per group.

arterial injury, since both the inflammatory score and injury score were similar between the two groups (Table 1; Figure 4A).

We next assessed how decreased neointima formation in paclitaxel-eluting stents would affect endothelial cell repopulation. *En face* X-Gal staining in ApoE^{-/-} LacZ mice 28 days after stenting revealed significantly fewer endothelial cells in paclitaxel-eluting stents compared with control stents (154.2 vs. 238.5 nuclei/mm; $P = 0.018$, $n = 6$; Figure 5). To ensure that changes observed in endothelial cell repopulation after implantation of a paclitaxel-eluting stent were not due to systemic effects of paclitaxel on EPCs, we assessed numbers of EPCs in both peripheral blood

and bone marrow. There were no differences in the numbers of either peripheral blood or bone-marrow-derived EPCs between animals receiving paclitaxel-eluting stents or control stents (see Supplementary material online, Figure S2). These observations indicate that paclitaxel-eluting stents reduce neointima formation, but result in local impairment of endothelial cell repopulation, despite adequate strut coverage by neointima.

Increased endothelial cell repopulation after stent deployment in GCH-Tg mice decreases neointima formation

We next sought to determine whether selective targeting of endothelial cell function is sufficient to drive alterations in endothelial cell repopulation and alter neointima formation after stenting. We used an endothelial cell-targeted genetic model of enhanced eNOS function,^{13–15} the GCH-Tg mouse, to augment endothelial cell function without recourse to systemic or non-specific treatments that could exert confounding effects on other cell types involved in the response to stenting.

Primary endothelial cells and VSMC from GCH-Tg ApoE^{-/-} mice and their ApoE^{-/-} littermates were assessed for the presence of the GCH transgene and native GCH. Western blot analysis revealed that the human GCH transgene was expressed only in endothelial cells from GCH-Tg ApoE^{-/-} mice and was not present in VSMC, or endothelial cells or VSMC from ApoE^{-/-} mice. Endothelial-specific overexpression of human GCH did not alter the expression of native mouse GCH or expression of eNOS between the two groups (Figure 6A). However, endothelial-specific overexpression of GCH resulted in a significant increase in NO production in primary endothelial cells from GCH-Tg ApoE^{-/-} mice compared with ApoE^{-/-} littermates (0.18 ± 0.03 vs. $0.06 \pm 0.03\%$, $n = 3$; $P = 0.002$; Figure 6B).

We first quantified endothelial cell repopulation in GCH-Tg LacZ ApoE^{-/-} and LacZ ApoE^{-/-} littermate mice, 28 days after stenting. Stented vessels from GCH-Tg LacZ ApoE^{-/-} mice had significantly increased endothelial cell repopulation compared with LacZ ApoE^{-/-} littermates (208.6 ± 12.8 vs. 156.9 ± 17.4 nuclei/mm, $n = 8–11$; $P = 0.043$; Figure 6C), confirming that the cell-specific genetic modification in GCH-Tg ApoE^{-/-} mice is sufficient to enhance endothelial cell repopulation. We next evaluated the effects of this increased endothelial cell repopulation on

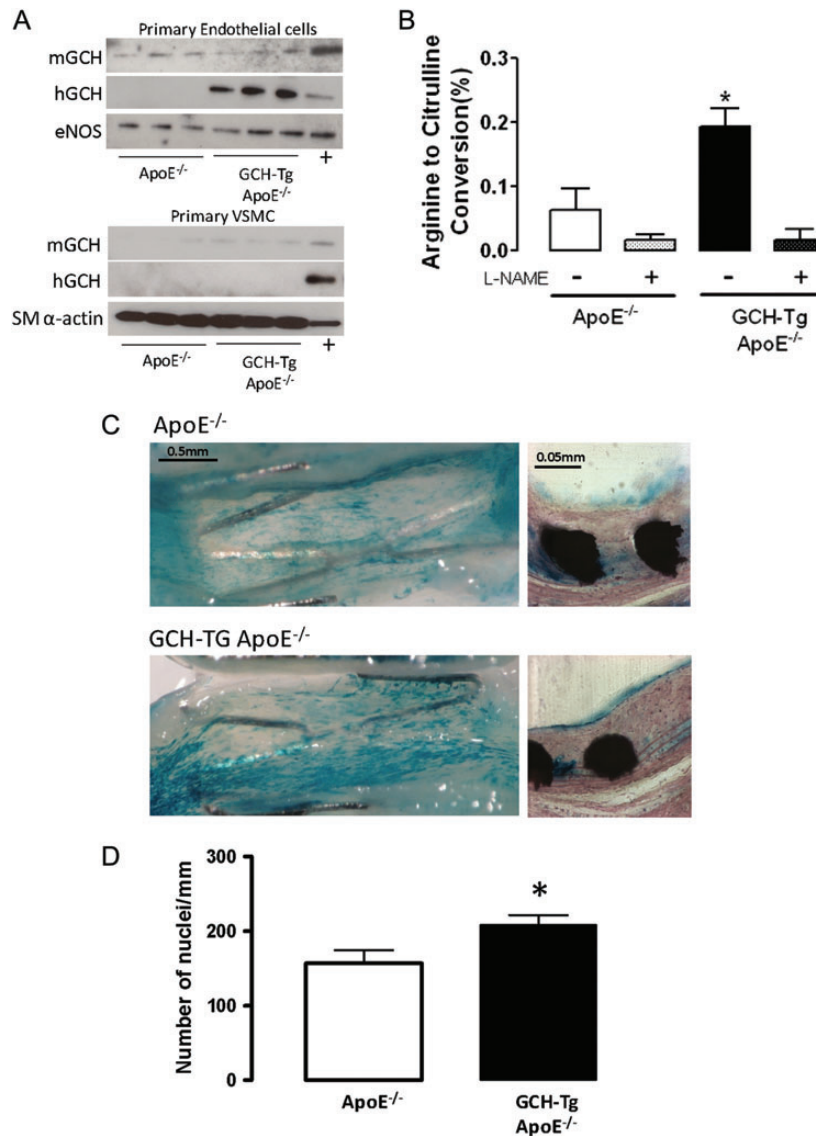


Figure 6 Endothelial cell repopulation 28 days after stenting in either ApoE^{-/-} or GCH-Tg ApoE^{-/-} mice. (A) Western blot analysis from human GCH (hGCH), mouse GCH (mGCH), eNOS, and smooth muscle α -actin (SM α -actin) in primary endothelial cells and vascular smooth muscle cell (VSMC) from GCH-Tg ApoE^{-/-} mice and ApoE^{-/-} littermates, $n = 3$ /group. + Denotes positive control samples GCH-Tg ApoE^{-/-} aorta. (B) Primary endothelial cells from GCH-Tg ApoE^{-/-} mice and ApoE^{-/-} littermates were incubated with calcium ionophore (1 μ mol/L) in the presence (+) or absence (-) of L-NAME (100 μ M) for 30 min L-[14C]citrulline accumulation was then quantified by high-performance liquid chromatography as an indicator of eNOS activity. GCH-Tg ApoE^{-/-} mice had significantly greater eNOS activity compared with their ApoE^{-/-} littermates * $P = 0.002$, $n = 3$ /group. (C) Representative *en face* and transverse sections of X-gal-stained 28-day stents. (D) Quantification of endothelial cell repopulation of *en face* X-gal stained 28-day bare-metal stents from Tie2-LacZ ApoE^{-/-} or Tie2-LacZ GCH-Tg ApoE^{-/-} mice, number of LacZ-positive nuclei were counted per stent, * $P = 0.043$ GCH-Tg ApoE^{-/-} vs. ApoE^{-/-}, $n = 7-10$ per group.

neointima formation. Both neointimal area ($P = 0.039$) and neointimal thickness ($P = 0.029$) were significantly reduced in GCH-Tg ApoE^{-/-} mice compared with ApoE^{-/-} mice (Figure 7B and C). Individual strut coverage was also reduced in stented vessels from GCH-Tg ApoE^{-/-} (Figure 7D), with no evidence of uncovered struts or strut malapposition (see Supplementary material

online, Videos S3 and S4). The difference in neointima formation was not due to differences in arterial injury, since both the inflammatory and injury scores were similar between the two groups (Table 1, Figure 7A). Thus, a primary and selective targeting of endothelial cell function is sufficient to both increasing endothelial cell repopulation and reducing neointima formation after stenting.

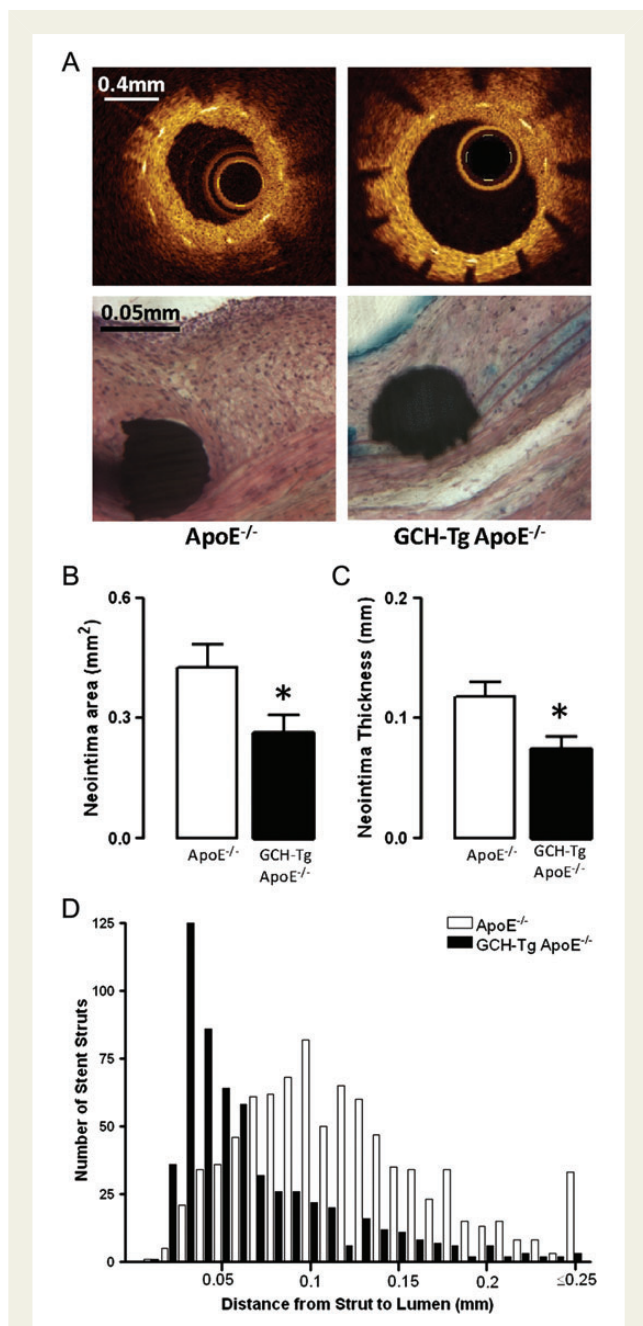


Figure 7 Neointima formation 28 days after stenting in either ApoE^{-/-} or GCH-Tg ApoE^{-/-} mice. (A) Representative optical coherence tomography images (upper panels) and haematoxylin- and eosin- stained transverse sections (lower panels) from ApoE^{-/-} or GCH-Tg ApoE^{-/-} mice after 28 days after bare-metal stent deployment. (B) Quantification of neointima area using optical coherence tomography images, **P* = 0.039 GCH-Tg ApoE^{-/-} vs. ApoE^{-/-}, *n* = 11–8/group. (C) Quantification of neointima thickness (distance from strut to lumen) using optical coherence tomography images, **P* = 0.029 GCH-Tg ApoE^{-/-} vs. ApoE^{-/-}, *n* = 11–8/group. (D) Frequency distribution of strut-level intimal thickness. *n* = 8–11 per group.

Discussion

In this study, we report a novel mouse model of stenting that incorporates specific genetic tracking of endothelial cells and selective genetic modification of endothelial cell function, to define the relationships between endothelial cell repopulation and neointima formation after stenting, in both bare metal and DES. We have shown that, first, stenting results in reduced endothelial cell repopulation compared with balloon angioplasty alone, and is driven principally by endothelial cells derived from outside the stented vessels with contributions from both bone-marrow-derived EPCs and from endothelial cells from the adjacent vasculature. Second, the reduction in neointima formation by paclitaxel is associated with reduced endothelial cell repopulation, despite complete stent strut coverage. Third, selective targeting of endothelial cell function is sufficient to reduce neointima formation by both increasing endothelial cell coverage and increasing endothelial cell NO production. Thus, targeting endothelial cell function can achieve a similar reduction in neointima formation after bare-metal stenting to that observed after paclitaxel-eluting stent deployment.

Our study provides new and definitive insights into the nature of endothelial cell repopulation and neointima formation after stenting. Although many studies have revealed delayed vessel healing and incomplete strut coverage following DES in both humans²⁴ and animals,¹¹ the specific presence of endothelial cells in relation to neointima formation and strut coverage has been demonstrated in very few. This is important as the relationship between strut coverage and endothelial cell repopulation is still unclear. Some strut coverage may be a necessary requirement for endothelial cell repopulation. However, strut coverage with fibrin, proteoglycan-rich matrix, or VSMC will not result in the anti-thrombogenic, anti-inflammatory environment achieved with a mature endothelial cell layer. Most importantly, the lack of strut coverage and strut malapposition reported after drug-eluting stenting may be either a cause or a consequence of impaired endothelial cell repopulation. In our study we show that incomplete endothelial cell repopulation was still observed, despite complete strut coverage. Thus, we can report a direct and inhibitory effect of DESs on endothelial cell repopulation, despite complete strut coverage and no difference in circulating EPCs. This is important as it suggests that strut coverage is not a consistent marker of endothelial cell repopulation. These observations are in keeping with previous reports of the direct effects of anti-proliferative drugs on endothelial cell growth,²⁵ and suggest that strut coverage and/or optimal strut apposition will not be sufficient to restore normal endothelial cell repopulation after drug-eluting stenting. In our study, no uncovered or malapposed struts were observed, most likely due to the mechanical and geometric characteristics of stenting the mouse aorta, the dosage of paclitaxel used, and the differences in disease pathology in the mouse.

Re-endothelialization is a key step in the vascular repair response following injury, and is critical for restoring and maintaining anti-atherogenic, anti-thrombotic, and anti-proliferative effects on the vessel wall.⁵ This study is the first to investigate the origins

of repopulating endothelial cells after arterial stenting. We have shown that stent deployment results in immediate and near-complete loss of the endothelial cell layer. We have also shown that, when present, surviving endothelial cells from within the stented segment have the capability to contribute to re-endothelialization. However, although endothelial cells derived locally from the stented vessel segment contributed to endothelial cell repopulation in some animals, the majority of repopulating endothelial cells were derived from outside the stented vessel segment. Further investigation revealed that bone marrow-derived EPCs contribute to endothelial cell repopulation in some stented vessels, whereas in others a minimal contribution of EPCs was observed. As the majority of repopulating endothelial cells are derived from outside the stented vessel, this indicates that endothelial cells from the adjacent vasculature may play a significant role in repopulation. These observations help to clarify the apparently different results of previous vascular injury models showing the major source of recipient-derived cells being either from the flanking vasculature²⁶ or EPCs.¹⁷ It may also help to explain the variable results from recent studies which have used EPC capture stents.²⁷

Endothelial cell repopulation post-stenting was significantly decreased when compared with balloon angioplasty, in keeping with previous studies using dye exclusion methods,¹² and suggests that stent-induced vascular injury, and the presence of struts within the artery wall delays the endothelial healing response. Furthermore, we observed incomplete endothelial cell re-population even at 56 days post-stenting, significantly later than other animal models where strut coverage is typically complete by 28 days post-stenting.^{11,28} This is an important aspect of the mouse model used in our studies, since the use of hyperlipidemic ApoE^{-/-} mice, in which re-endothelialization after vascular injury is impaired compared with WT mice,¹⁷ provides a relevant model of endothelial cell repopulation which enables specific tracking and quantification of endothelial cells. This model could be used to evaluate endothelial cell functionality such as the formation of tight junctions, or to compare the effects of other drugs or interventions on endothelial cell repopulation after stenting.

In this study we have shown using an endothelial cell-targeted genetic intervention, the GCH-Tg mouse, it is possible to achieve a similar level of neointima formation observed with a paclitaxel-coated stent compared with a bare-metal stent solely by altering endothelial cell function. In this study, we show that endothelial-specific overexpression of GTP cyclohydrolase-1 results in increased endothelial cell repopulation and reduced neointima formation 28 days after deployment of a bare-metal stent. The reduction in neointima formation observed in this study could be due to multiple effects on endothelial cell function. Increased endothelial cell recruitment due to either an increased proliferative or survival capacity of the endothelial cells would result in decreased neointima formation. However, we also observed an increased NO production from isolated endothelial cells from GCH-Tg ApoE^{-/-} mice. Although NO production could not be directly measured from repopulating endothelial cells, the reduced neointima formation could also be due to an enhanced anti-thrombotic, inflammatory, and proliferative capacity of the recruited endothelial cells resulting from increased

NO production. Previous studies have sought to increase endothelial cell repopulation by increasing progenitor cell mobilization, which resulting in increased strut coverage but at the expense of non-specific effects of granulocyte colony-stimulating factor on VSMCs.²⁹ More promising results from EPC capture stents have been reported with decrease thrombogenicity and improved strut coverage.⁹ In addition, studies have attempted to improve the vascular healing response by increasing NO production by adenovirus-mediated delivery of eNOS.³⁰ However, both the EPC capture stent and the use of adenoviral eNOS-coated stents can result in additional non-endothelial cell-related effects such as capture of non-endothelial cells and non-specific adenoviral-mediated side effects. In this study, we have shown that an endothelial-specific intervention is sufficient to improve endothelial cell function by increasing NO production and endothelial cell recruitment resulting in decreased neointima formation. Current approaches and technologies applied to enhance endothelial repopulation after stenting have had limited clinical success. However, the current study provides strong direct support for the notion that endothelial cell function is a valid target to improve vascular healing and reduce neointima formation after stenting. In particular, we demonstrate proof of concept that biological strategies to improve systemic endothelial cell function, irrespective of local endothelial cell capture or local vessel wall effects can exert beneficial effects on endothelial cell repopulation and neointima formation.

There are several limitations to this study. We investigated endothelial cell repopulation in first-generation paclitaxel-eluting stents due to the wealth of pre-clinical and clinical data available on this platform. It would be interesting to compare newer drugs such as everolimus and the vascular healing response with these stent platforms. In our study we used the atherosclerotic ApoE^{-/-} mouse with a specific endothelial cell tracker. Unlike the rabbit and pig model this allowed us to directly measure endothelial cell repopulation. Using our model we observed similar results with our paclitaxel-eluting stents as observed in the clinical setting. However, this study was limited to observations in pre-clinical animal models thus the relevance to human needs to be interpreted with caution.

In conclusion, we have shown definitively that endothelial cell repopulation is decreased after stenting in a mouse model of atherosclerosis, and is further diminished after drug-eluting stenting. Reduced endothelial cell repopulation after drug-eluting stenting is not caused by lack of strut coverage, and is driven principally by endothelial cells that originate from outside the stented vessel segment, including bone-marrow-derived EPCs. Augmentation of systemic endothelial cell function is sufficient to improve endothelial cell repopulation and decrease neointima formation post-stenting, thus identifying systemic endothelial cell function as a rational therapeutic target to aid vascular healing and decrease neointima formation after stenting.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

Acknowledgements

We are grateful to Professor Steven S. Gross of Weill Medical College of Cornell University (New York, USA) for the kind gift of rat anti-mouse GTPCH antibody. Rabbit anti-human GTPCH was provided by Gabriele Werner-Felmayer of the Institute for Medical Chemistry and Biochemistry (Innsbruck, Austria).

Funding

This work was supported by the British Heart Foundation (PG/05/141/20098, RG/07/003/23133) and the NIHR Oxford Biomedical Research Centre. Wellcome Trust Core Award Grant (090532/Z/09/Z).

Conflict of interest: none declared.

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