Therapeutic and mechanistic explorations of in-stent restenosis in the rat aortic stenting model
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CHAPTER 3

NON-BONE MARROW ORIGIN OF NEOINTIMAL SMOOTH MUSCLE CELLS IN EXPERIMENTAL IN-STENT RESTENOSIS IN RATS

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ABSTRACT

Aim: To determine the contribution of bone marrow-derived cells in in-stent restenosis and transplant arteriosclerosis. Methods: Nontransgenic rats WT F344\textsuperscript{TG} (n=3) received stent implantation 6 weeks after lethal total body irradiation and suppletion with bone marrow from a R26-hPAP transgenic rat. After 4 weeks the abdominal aortas were harvested, the stent was quickly removed, the abdominal aorta was snap-frozen in liquid nitrogen and 5 µm cryosections for stainings were cut. Additionally DA aortic allografts were transplanted into WT F344\textsuperscript{TG}(n=3) and R26-hPAP\textsuperscript{WT}(n=3) BM-chimeric recipients. Immunohistochemistry (hPAP-staining) and immunofluorescence (hPAP, α-SMA and OX-1) was performed on all sections. Results: Few hPAP positive cells were observed in the neointima Double stainings of hPAP positive areas showed no α-SMA colocalization, OX-1 did show colocalization. Conclusions: Non-BM-derived cells are the predominant source of neointimal cells in ISR and TA. Vascular wall-derived progenitor cells may rather be the source of SMCs that contribute to ISR and TA which may have implications for our quest for new therapeutic targets to treat these vasculopathies.
INTRODUCTION
Development of in-stent restenosis (ISR) is the most common complication associated with coronary stenting, especially in patients treated with bare-metal stents. No adequate treatment modalities are available to treat or prevent development of ISR[1;2]. Recruitment and proliferation of smooth muscle cells (SMCs) in response to vascular injury after stenting are key phenomena that lead to the development of an occlusive neointima culminating in ISR[3;4]. Although it is well established that in humans the neointima of stented vessels is mainly composed of α-smooth muscle actin (SMA) positive cells[5], the origin of neointimal cells in ISR is still a matter of debate. Identifying the anatomical origin and molecular characteristics of the progenitor cells that ultimately form the neointima in ISR is of importance since these cells form a putative target for therapeutic intervention to prevent ISR and related occlusive vascular diseases like transplant arteriosclerosis (TA). Along with the classical theory of inward migration and proliferation of medial SMCs[6], a more recent proposed hypothesis attributes an important role to bone marrow (BM)-derived vascular progenitor cells in the process of neointimal formation[7]. BM contains both hematopoietic and mesenchymal stem cells which have the capacity to self-renew and to differentiate into a variety of cell types including SMCs[8;9]. Given the potential of BM stem cells to give rise to SMCs, SMC progenitors might be recruited from the BM into the circulation in response to vascular injury and home to the site of injury resulting in neointimal formation eventually. In line with this, various animal models of vascular injury (atherosclerosis, wire injury and TA) indeed demonstrate contribution of BM-derived cells in neointimal formation to some extent[10-13]. However, results described so far are not conclusive since we and others demonstrated that in experimental TA and (vein graft) atherosclerosis neointimal SMCs are primarily non-BM-derived[14-16]. The contribution of BM-derived cells in the development of ISR is largely unknown although some recent studies suggest involvement of BM-derived cells based on increased numbers of circulating CD34+ cells shortly after stenting[17] and the presence of neointimal cells expressing stem cell antigens like c-kit[18;19], CD34 and AC133[20]. However, although these markers are indeed expressed on primitive cells residing in the bone marrow, expression is not strictly confined to BM-derived cells. As a result, neointimal cells expressing these markers in ISR are not derived from the BM by definition. Direct evidence of involvement of BM-derived cells in the development of ISR has thus not been reported so far. Since the contribution of BM-derived cells in neointimal formation is most likely dependent on the severity of endovascular injury [11;21], it is of importance that studies on the origin of neointimal cells in specifically ISR are performed in a relevant model of true ISR and not a model of otherwise induced endovascular injury. In this study we therefore determined the contribution of BM-derived cells in ISR in a direct way using our recently developed model of ISR [22] using genetically marked BM chimeric rats.

METHODS
Rats
Male wild-type (WT) Fischer344 (F344) and Dark Agouti (DA) rats were obtained from Harlan Nederland (Horst, The Netherlands). Human Placental Alkaline Phosphatase (hPAP) transgenic F344 rats (R26-hPAP rats) were derived from a breeding nucleus provided by Dr. E.P. Sandgren (University of Wisconsin-Madison, USA)[23]. Rats were kept under clean conventional conditions and were fed standard rat chow and acidified water ad libitum. The investigation conforms 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 the Dutch Law on Experimental Animal Care.
Bone marrow transplantation

Both femora and tibiae of BM donor rats were excised and surrounding muscle and connective tissue were removed and the BM was flushed with sterile PBS. Erythrocytes were lysed in lysing buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM sodium ethylenediaminetetraacetic acid [EDTA]), and the cell suspension was then filtered through a 20 µm cell strainer (Becton Dickinson, Alphen aan den Rijn, The Netherlands). BM recipient rats were lethally γ-irradiated (9 Gy) using a 137Cesium source (IBL 637, CIS Bio International). One hour after irradiation rats were reconstituted with 1-5x10⁷ BM cells by tail vein injection. Three experimental groups were included: 1) hPAP Tg F344 BM -> WT F344 [WT F344TG], stented 6 wks after reconstitution; 2) hPAP Tg F344 BM -> WT F344 [WT F344TG], aorta allografted 6 wks after reconstitution; 3) WT F344 BM -> hPAP Tg F344 [R26-hPAPWT], aorta allografted 6 wks after reconstitution. BM chimeric rats were housed in filtertop cages throughout the duration of the experiment. Rats received drinking water containing neomycin (0.35% wt/vol) starting 1 week before irradiation until 2 weeks after BM reconstitution. Six weeks after BM reconstitution and prior to stenting or allografting the level of chimerism was determined by flowcytometric analysis on PBMCs. The level of chimerism was typically between 80% and 90% (data not shown).

Stent implantation

Chimeric rats (6 wks after BM reconstitution) received a stent in the abdominal aorta as described in detail elsewhere[22]. Briefly, under anesthesia (2% isoflurane [Abbot, Hoofddorp, The Netherlands], 0.4 L/min O₂ and 0.4 L/min N₂O) the abdominal cavity was opened. The aorta was dissected and surrounding connective tissue was removed. Next, two vascular clips were placed onto the aorta distal to the renal arteries and proximal to the aortic bifurcation. A small incision was then made in the distal abdominal aorta and the balloon catheter was inserted and inflated to 9 atm pressure to deploy a pre-mounted 2.5 x 8 mm Micro-Driver stent (Medtronic, Minneapolis, United States, n=3). After deflation and removal of the balloon, the aortic incision was closed with a 9-0 suture. Reperfusion was established by removing the clips and the abdomen was closed with 4-0 sutures. Four weeks after surgery the stented aorta’s were harvested and the stents were carefully removed from the lumen of the aorta. Aortic tissue was snap-frozen in liquid nitrogen and stored at -80°C for cryostat sections.

Aorta transplantation

Since we previously showed non-BM origin of neointimal VSMCs and ECs in transplant arteriosclerosis (TA) using allogeneic BM-chimeric rats [15;21], we also performed aortic allografting in WT F344⁴TG and R26-hPAPWT BM-chimeric rats to test the feasibility of detecting (non)-BM-derived VSMCs and ECs in established neointimal lesions in the hPAP-transgenic F344 rat model. So far, this rat model has not been used to track (non)-BM-derived VSMCs and ECs in neointimal lesions. Under anesthesia (as described above) DA aortic allografts were transplanted into WT F344⁴TG (n=3) and R26-hPAPWT (n=3) BM-chimeric recipients as described previously[24]. Briefly, the abdominal aorta between the left renal artery and the bifurcation was removed from the donor, perfused with saline and subsequently orthotopically transplanted into the recipient via end-to-end anastomosis with total cold and warm ischemic time consistently less than 25 minutes.

Immunohistochemistry

To localize BM-derived hPAP-positive cells in ISR, an indirect immunoperoxidase staining for hPAP was performed on cryosections cut from the stented area. Sections (5 µm) were acetone-fixed (10 min., 4°C). Blockade of endogenous peroxidase (incubation 30 min. with PBS containing 0.03% H₂O₂) was followed by incubation for 60 min at room temperature with the primary polyclonal antibody against hPAP (AHP537HT, AbD Serotec, BioConnect, Huissen, The Netherlands) diluted in 1% BSA/PBS.
Subsequently, the sections were incubated with a second-step horseradish peroxidase-conjugated goat-anti-rabbit antibody (DAKO A/S, Glostrup, Denmark) for 30 min diluted in 1% BSA/PBS supplemented with 1% normal rat serum. Peroxidase activity was developed using chromogen 3-amino-9-ethyl carbazole (AEC, DAKO A/S, Glostrup, Denmark). Sections were counterstained with hematoxylin and mounted in Faramount (DAKO A/S, Glostrup, Denmark). Control slides, in which the primary antibody was replaced with PBS were consistently negative (not shown).

**Immunofluorescence**

To further phenotype hPAP+ cells in ISR triple-immunofluorescent staining was performed using anti-hPAP, α-SMA (SMCs; clone 1A4, mlgG2a, Dako A/S, Glostrup, Denmark) and anti-CD45 (clone OX-1, mlgG1 tissue culture supernatant). Sections were incubated for 1 hr with a mixture of the primary antibodies (diluted in 1% BSA/PBS) followed by incubation with Alexa488-conjugated goat anti-mouse IgG2a (Molecular Probes, Leiden, The Netherlands), Cy5-conjugated goat anti-mouse IgG1 (Molecular Probes, Leiden, The Netherlands) and horseradish peroxidase-conjugated swine-anti-rabbit Ig (Dako A/S, Glostrup, Denmark) in 1% BSA/PBS supplemented with 1% normal rat serum for 30 min. Horseradish peroxidase-conjugated swine-anti-rabbit Ig was detected using the TSA™ Tetramethylrhodamine System (PerkinElmer LAS, Inc., Boston, MA, USA). Nuclei were stained with DAPI and sections were embedded in Citifluor (AF 1, Agar Scientific Ltd., Stansted, UK). To validate this four-color immunofluorescent staining protocol and check for potential crossreactivity of the isotype-specific second-step antibodies, first single stainings for hPAP, SMA and CD45 were performed on hPAP-transgenic F344 spleen sections.

Following a similar immunofluorescence protocol double staining for hPAP (polyclonal rabbit Ig) and α-SMA (mouse IgG2a), and hPAP and RECA-1 (mouse IgG1, endothelium)[25] were performed on 5 µm aortic graft cryosections. Binding of anti-hPAP antibodies was detected using FITC-conjugated goat anti-rabbit Ig (Dako A/S, Glostrup, Denmark) whereas binding of α-SMA and RECA-1 antibodies was detected using horseradish peroxidase-conjugated rabbit-anti-mouse Ig (Dako A/S, Glostrup, Denmark) which was visualized using the TSA™ Tetramethylrhodamine System. All fluorescently labeled sections were analyzed on a Confocal Laserscanning Microscope (TCS SP2, Leica, Microsystems Nederland B.V., Rijswijk, The Netherlands).

**RESULTS**

**Specificity α-hPAP staining**

Since the hPAP-transgenic F344 rat model has not been used before to track (non)-BM-derived VSMCs and ECs in neointimal lesions we first analyzed the specificity and sensitivity of our staining method to detect hPAP-transgenic BM and vascular wall cells. As shown in Figure 1, both BM cells (C) and medial VSMCs and ECs in non-injured aorta (D) from hPAP-transgenic F344 rats stained positive for hPAP using an hPAP-specific polyclonal antibody. For comparison, BM cells (A) and aortic tissue (B) from wildtype F344 rats did not react with the α-hPAP antibody. These results indicate that this staining method is specific and sufficiently sensitive to detect hPAP-expressing BM and vascular cells.

**Development 4-parameter (CD45, SMA, hPAP, DNA) immunofluorescent staining protocol**

To validate a four-parameter immunofluorescent staining protocol and check for potential crossreactivity of the isotype-specific second-step antibodies, single and triple stainings for hPAP, SMA and CD45 were performed on hPAP-transgenic F344 spleen sections. Sections were incubated with one primary antibody and then detected with a cocktail of three fluorochrome-labeled isotype-specific second-step antibodies. As shown in Figure 2, mlgG1 α-CD45 was only detected with α-mlgG1-Cy5 (A-E), mlgG2a α-SMA was only detected with α-mlgG2a-Alexa488 (F-J), and rlgG α-hPAP was only
detected with α-rIgG TRITC (K-O). When incubating sections with a mixture of CD45, hPAP and SMA primary antibodies, expression of all antigens could be demonstrated simultaneously (P-T). This four-parameter immunofluorescent staining protocol was then used to determine the origin of neointimal VSMCs and ECs in TA and ISR.

**Figure 1.** The α-hPAP staining is sufficiently sensitive and specific to detect hPAP-transgenic BM and vascular wall cells. (A) BM cells (magnification x1890) and (B) aortic tissue (magnification x200, inset x630) from wildtype F344 rats did not react with the α-hPAP antibody, whereas (C) BM cells (magnification x1890) and (D) medial VSMCs and ECs (arrowheads inset) in non-injured aorta (magnification x200, inset x630) from hPAP-transgenic F344 rats clearly reacted with the α-hPAP antibody. Abreviations: A: adventitia, M: media.

**Neointimal VSMCs and ECs in TA are non-BM-derived**

Since we previously showed non-BM origin of neointimal VSMCs and ECs in transplant arteriosclerosis (TA) using allogeneic BM-chimeric rats [15;21], we first performed aortic allografting in WT F344\(^{TG}\) and R26-hPAP\(^{WT}\) BM-chimeric rats to test our model system for specificity and sensitivity of detecting (non)-BM-derived VSMCs and ECs in established neointimal lesions. Two months after allografting both the WT-F344\(^{TG}\) (Figure 3A and B) and R26-hPAP\(^{WT}\) (Figure 3C and D) had developed marked TA characterized by a neointima consisting of a packed layer of SMA\(^{+}\) cells covered by ECs at the luminal side. The neointimal cells in allografts transplanted in wild-type recipients reconstituted with hPAP-transgenic BM (WT-F344\(^{TG}\)) expressed SMA but colocalization with hPAP-expression was not observed (0% BM-derived α-SMA\(^{+}\) VSMCs, Figure 3A). Also neointimal ECs did not express hPAP (0% BM-derived RECA-1\(^{+}\) VSMCs, Figure 3B). The BM-derived hPAP\(^{+}\) cells that were detected in the neoointima, media and adventitia expressed CD45
indicating that these cells were infiltrating leukocytes (data not shown). These results suggest a non-BM origin of the neointimal ECs and VSMCs in established TA. Analyses performed on allografts transplanted in hPAP-transgenic recipients reconstituted with WT BM (R26-F344WT) confirmed this premise as shown in Figure 3C and D. Virtually all neointimal SMA⁺ (Figure 3C) and ECs (Figure 3D) coexpressed the hPAP transgene indicating ~100% non-BM origin of these cells in TA. These data confirm our previous observations and indicate that the hPAP-transgenic F344 rat model is sufficiently specific and sensitive to detect (non)-BM-derived VSMCs and ECs in established neointimal lesions.

![Figure 2](image_url)

**Figure 2.** Simultaneous detection of CD45, SMA, hPAP and DNA by immunofluorescent staining on hPAP-transgenic F344 rat spleen. (A-E) Primary incubation with OX1 (α-CD45, mlgG1) and secondary incubation with α-mlgG1-Cy5, α-mlgG2a-Alexa488, and α-rlgG TRITC. OX1 reacted with the lymphocytes present in the white pulp (WP) and red pulp (RP) around the central arteriole (arrowhead) and was only detected with α-mlgG1-Cy5. (F-J) Primary incubation with 1A4 (α-SMA, mlgG2a) and secondary incubation with α-mlgG1-Cy5, α-mlgG2a-Alexa488, and α-rlgG TRITC. 1A4 reacted with the stromal cells present in the white pulp and medial VSMCs in the central arteriole (arrowhead) and was only detected with α-mlgG2a-Alexa488. (K-O) Primary incubation with α-hPAP (rlgG) and secondary incubation with α-mlgG1-Cy5, α-mlgG2a-Alexa488, and α-rlgG TRITC. α-hPAP reacted with all cells present in the white and red pulp and was only detected with α-rlgG-TRITC. (P-T) Primary incubation with OX1, 1A4 and α-hPAP and secondary incubation with α-mlgG1-Cy5, α-mlgG2a-Alexa488, and α-rlgG TRITC. Expression of all antigens could be demonstrated simultaneously. (magnification x200). Abbreviations: RP: red pulp, WP: white pulp, arrowhead: central arteriole.
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Presence of BM-derived hPAP+ cells in ISR
Stenting of the BM-chimeric rats resulted in the development of extensive and maximal ISR after 4 weeks. In this model of ISR at earlier time-points only mild lesions (without SMA-positive VSMCs) are present which are characterized by local thrombus formation around the stent struts with surface adhering leucocytes (1 day) or thrombus-infiltrating leucocytes (3 days and 1 week). Figure 4 shows representative photomicrographs of the histological appearance of the composition of the lesions in developing ISR at 1 (A) and 3 days (B) and 1 (C) and 4 (D) weeks after stenting. Since the aim of this study is to determine the (non)-BM origin of neointimal VSMCs in established ISR, stented aorta’s were analyzed 4 weeks after stenting. After removal of the stents the neointima was still attached to the luminal side of the aortic wall (Figure 5). Immunohistochemistry for hPAP-transgene expression revealed the abundant presence of hPAP+ cells in the adventitia (Figure 5A) and media (Figure 5B and C) whereas the neointima contained a relatively low number of hPAP+ cells (Figure 5B and 5C).

Neointimal VSMCs in ISR are non-BM-derived
To determine the smooth-muscle-like phenotype of the BM-derived neointimal hPAP+ cells in ISR triple staining for hPAP, SMA and CD45 was performed. The neointima consisted primarily of SMA+ VSMCs whereas the media was devoid of SMA+ VSMCs after stenting (Figure 6B). Although the neointima contained considerable numbers of BM-derived hPAP+ cells (Figure 6C) the absence of colocalization of hPAP and SMA expression was consistently observed in all animals analyzed (Figure 6E) indicating a non-BM origin of neointimal VSMCs in ISR (0% BM-derived SMA+ VSMCs). Similar results were obtained for the neointimal ECs which were however only sparsely present due to the mechanical removal of the stents (data not shown). Colocalization of hPAP and CD45 expression in the neointima (Figure 6D and E), media (Figure 6D and E) and adventitia (Figure 7) indicate that the BM-derived hPAP+ cells in ISR were infiltrating leukocytes.

DISCUSSION
In the present study we determined the contribution of BM-derived cells in the development of ISR and TA after respectively experimental stenting and aortic transplantation in rats. In both models no BM-derived neointimal SMCs and ECs were detected and the few neointimal hPAP+ BM-derived cells turned out to be CD45+ infiltrating leukocytes. We conclude that vascular cells originating from the BM are not part of established neointimal lesions in both TA and ISR. Although the origin of neointimal cells has gained considerable interest in the last decade, only a few studies have been reported on the origin of neointimal SMCs after stenting[17-19] [20]. Identification of the anatomical origin of the cells involved in development of ISR is of clinical importance since this may elucidate new targets that can be used for therapeutic intervention in order to prevent or reduce development of ISR. A putative source is the bone marrow. It is generally accepted that the BM contains hematopoietic and mesenchymal stem cells which have the ability of self-renewal and which can differentiate into a variety of cell types including SMCs [8;9]. Furthermore, the human peripheral blood contains CD34+ SMC progenitors[26] and therefore the BM is a putative source of SMCs involved in the development of ISR. In line with this, increased frequencies of circulating CD34+ cells were detected after coronary stenting [17] and which was found to correlate with the late lumen loss i.e. ISR in stented patients[27]. Not only numerical differences but also the differentiation fate of progenitor cells appear to correlate with the development of ISR[17]. Mononuclear cells isolated from patients with and without ISR preferentially differentiated into α-SMA+ and endothelial-like cells in vitro respectively, indicating that differentiation in favor of SMCs may predispose for ISR[17]. Despite these correlative studies direct evidence of involvement of BM-derived cells in the development of ISR has not been reported. In
clinical ISR it is furthermore hard to discriminate between potential BM-derived cells that appeared after stenting or that were already present in the vicinity of the stenotic area before stenting[17;20].

**Figure 4. Kinetics of the development of ISR after experimental stenting in rats.** (A) 1 day after stenting: local thrombus formation around the stent struts (asterisk) with surface adhering leucocytes (arrows). Toluidine blue staining, magnification x200. (B) 3 days after stenting: local thrombus formation around the stent struts (asterisks) with an increased number of infiltrating leucocytes (arrowheads). Toluidine blue-basic fuchsin staining, magnification x200. (C) 1 week after stenting: organized thrombus with surface-adherent leucocytes (arrows) and increased leucocyte infiltration (arrowhead). Toluidine blue-basic fuchsin staining, magnification x200. (D) 4 weeks after stenting: stent struts (asterisk) are completely covered by neointima which mainly consist of VSMCs and extracellular matrix with the absence of large numbers of infiltrating leucocytes. Elastica van Gieson staining, magnification x200. Abbreviations: A: adventitia, M: media, NI: neointima.

In our model[22] no atherosclerosis is present at the time of stenting and therefore allows analysis of the direct effect of stenting on the recruitment of BM-derived cells and the development of ISR as reported in this article. However, in human atherosclerosis it has been shown that in atherosclerotic plaques about 10% of the intimal cells is derived from the BM[13]. In case these BM-derived cells are a main source for the SMA⁺ VSMCs in ISR after stenting of the atherosclerotic lesion, pre-existing atherosclerosis might result in a higher percentage of BM-derived VSMCs in ISR than observed in our study without the presence of pre-existing atherosclerosis. Recently, cells expressing stem cell antigens like CD34, c-kit[18-20] and AC133 have been shown to be present in ISR albeit at low levels (maximal ~11%). Taking into account the indirect way of detecting putative BM-derived cells in these studies, our data are in fact quite similar and support the previously published data that the BM compartment is only marginally involved in the development of established ISR if involved at all. Although the BM has been shown to harbor potential to provide cells that contribute to neointimal formation in various models for vascular injury other than ISR the actual contribution of these cells is relatively low[11;12;28]. We and others indeed showed that a non-BM source predominantly provides the cells involved in neointimal formation in restenosis and TA[11;14;15]. A potential explanation for the differences in the contribution of BM-derived cells between previous reports[11;12;28] and the current
study is the severity of vascular injury since BM contribution in neointimal formation appears to be dependent on the severity of endovascular injury[11;21].

**Figure 5.** BM-derived hPAP⁺ cells are present in ISR. Stenting was performed in hPAP-transgenic BM chimeric rats and analyzed 4 wks after stenting. Photomicrographs of neointima formed in stented aorta immunostained for hPAP (red-brown) and counterstained with hematoxylin. Few BM-derived hPAP⁺ cells are present in the neointima (arrows, B and C), whereas hPAP⁺ cells are abundantly present in the adventitia (asterisks, B and C). Arrowheads indicate the internal elastic lamina. Magnification: A: x20; B & C: x200 Abreviations: Adv: adventitia; M: media; NI: neointima.

**Figure 6.** Neointimal BM-derived cells in ISR represent inflammatory cells but not VSMCs. Triple immunofluorescence staining for (A) Nuclear staining, (B) SMA (VSMCs), (C) hPAP (BM-derived cells) and (D) CD45 (Leukocyte Common Antigen, inflammatory cells). (E) Merged image of A, B C and D showing no colocalization of hPAP (red) and SMA (green) expression and colocalization of hPAP (red) and CD45 (dark blue) expression (magnification x630). Inset shows high-power magnification of hPAP⁻ neointimal VSMCs (magnification x1890). Abreviations: M: media; NI: neointima.
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Figure 3. **Neointimal VSMCs and ECs in TA are non-BM-derived.** DA aortic allografts were transplanted in WT F344\textsuperscript{Tg} (hPAP BM chimeric F344 wild-type rats; A and B) and R26-hPAP\textsuperscript{WT} (wild-type BM chimeric R26-hPAP transgenic rats; C and D) recipients and analyzed 2 months after transplantation. (A) Neointimal SMA\textsuperscript{+} VSMCs (red) do not express hPAP (magnification x630). (B) RECA-1\textsuperscript{+} ECs (red) do not express hPAP (magnification x630). Insets show high-power magnifications of neointimal VSMCs (A; magnification x1890) and ECs (B; magnification x2520) that do not express hPAP. (C) Colocalized expression of SMA (red) and hPAP (green) in neointimal VSMCs (magnification x630). (D) Colocalized expression of RECA-1 (red) and hPAP (green) in neointimal ECs (magnification x630). Insets show high-power magnifications of hPAP\textsuperscript{+} neointimal VSMCs (C; magnification x1890) and ECs (D; magnification x2520).

Abbreviations: M: media; NI: neointima.

Figure 7. **Adventitial BM-derived cells in ISR represent inflammatory cells.** Triple immunofluorescence staining for (A) Nuclear staining, (B) SMA (VSMCs), (C) hPAP (BM-derived cells) and (D) CD45 (Leukocyte Common Antigen, inflammatory cells). (E) Merged image of A, B, C and D showing colocalization of hPAP (red) and CD45 (dark blue) expression (magnification x630). Inset shows high-power magnification of hPAP\textsuperscript{+} inflammatory cells (magnification x1890).
However, we believe that our experimental model of ISR in rats also produces solid mechanical endovascular injury as measured by the injury scores as reported previously [22] which makes differences in severity of endovascular injury a less likely explanation for the observed differences in BM contribution. In this study we for the first time clearly demonstrate that in experimental ISR in rats the neointimal VSMCs are derived from a non-BM source. The BM thus plays a minor role in the development of established ISR. However, our results do not exclude the possibility that early after stenting BM-derived cells are recruited to the injured vascular wall and create a microenvironment in which local progenitor cell niches are activated and mobilized by BM-derived cells in a paracrine fashion. Localized progenitor cell niches in the media[29] and the adventitia[30;31]of the vascular wall have been recently identified. Furthermore, isolated adventitial Sca-1\(^+\) progenitor cells were shown to differentiate into VSMCs \textit{in vitro}, but also to contribute to the development of atherosclerotic lesions \textit{in vivo}[30]. The contribution of vascular wall-derived progenitor cells in the development of ISR and TA is as yet unknown but is currently under investigation.

In conclusion, non-BM-derived cells are the predominant source of neointimal cells in ISR and TA. Vascular wall-derived progenitor cells may rather be the source of SMCs that contribute to ISR and TA which may have implications for our quest for new therapeutic targets to treat these vasculopathies.

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