Published in final edited form as:

J Biomed Mater Res B Appl Biomater. 2016 November ; 104(8): 1658–1665. doi:10.1002/jbm.b.33510.

Point-of-Care Seeding of Nitinol Stents with Blood-Derived Endothelial Cells

Alexandra E. Jantzen¹, Maria Noviani^{2,3}, James S. Mills⁴, Katherine M. Baker⁵, Fu-Hsiung Lin², George A. Truskey¹, Hardean E. Achneck^{2,3,6,7}

¹Department of Biomedical Engineering, Duke University, Durham, NC;

²Department of Surgery, Duke University Medical Center, Durham, NC;

³Duke-National University of Singapore Graduate Medical School Singapore, Cardiovascular & Metabolic Disorders Program, Singapore;

⁴Department of Medicine - Cardiology, Duke University Medical Center, Durham, NC;

⁵Department of Biology, Duke University, Durham, NC;

⁶Department of Pathology, Duke University Medical Center, Durham, NC,

⁷Hemostemix Inc., Calgary, Alberta, Canada and Ness Ziona, Israel

Abstract

Nitinol-based vascular devices, e.g. peripheral and intracranial stents, are limited by thrombosis and restenosis. To ameliorate these complications, we developed a technology to promote vessel healing by rapidly seeding (*QuickSeeding*) autologous blood-derived endothelial cells (ECs) onto modified self-expanding nitinol stent delivery systems immediately before implantation. Several thousand micropores were laser-drilled into a delivery system sheath surrounding a commercial nitinol stent to allow for exit of an infused cell suspension. As suspension medium flowed outward through the micropores, ECs flowed through the delivery system attaching to the stent surface. The *QuickSeeded* ECs adhered to and spread on the stent surface following 24 hour *in vitro* culture under static or flow conditions. Further, *QuickSeeded* ECs on stents that were deployed into porcine carotid arteries spread to endothelialize stent struts within 48 hours (n=4). The *QuickSeeded* stent struts produced significantly more nitric oxide in *ex vivo* flow circuits after 24 hours, as compared to static conditions (n=5). In conclusion, ECs *QuickSeeded* onto commercial nitinol stents within minutes of implantation spread to form a functional layer *in vitro* and *in vivo*, providing proof of concept that the novel *QuickSeeding* method with modified delivery systems can be used to seed functional autologous endothelium at the point of care.

Keywords

endothelialization; endothelial cells; stem/progenitor cells; stents; thrombogenicity

INTRODUCTION

Percutaneous transluminal angioplasty and stent placement have revolutionized the treatment of cerebrovascular and peripheral vascular diseases, but complications still persist. For example, intracranial stenting to exclude cerebrovascular aneurysms is complicated by thrombosis and in-stent restenosis in as many as 30% of patients.¹ Further, approximately 50% of peripheral arteries that are treated with self-expanding nitinol stents exhibit signs of restenosis within two years.^{2,3} To minimize the risks of in-stent restenosis and thrombosis in peripheral arteries, drug eluting stents have recently been approved.⁴ While drug eluting stents have reduced restenosis rates in coronary arteries, a heightened rate of stent thrombosis has emerged due to delayed endothelialization.^{5–8} Delayed and incomplete re-endothelialization and poor healing of stented arteries have been correlated with in-stent restenosis and thrombosis.⁸ The risk of stent thrombosis should decrease by rapid coverage of stents with healthy endothelium due to the intrinsic anti-coagulant mechanisms of endothelial cells (ECs), as well as paracrine factors released by ECs (e.g., nitric oxide) to inhibit smooth muscle cell (SMC) proliferation and consequently decrease intimal hyperplasia.⁹

Previous approaches to directly seed metal stents with ECs prior to implantation have been hindered by the need to compress an expanded stent back into its delivery catheter after seeding the stent in its expanded state.^{10–13} These seeding methods suffer from incomplete endothelial coverage after recompression of the stent.^{10,12} Furthermore, *ex vivo* culture of cells on the stent struts proved to be impractical for translation into clinical practice.

An alternative strategy is to coat the stent with anti-CD34 antibodies and capture circulating endothelial progenitor cells (EPCs) to cover stents *in vivo*. A recent single center study of the Genous R stent failed to show any improvement in outcomes using this type of capture stent.¹⁴ One potential explanation for this failure is that CD34 is not a specific marker for EPCs and no single antibody specific to EPCs has yet been identified, e.g. anti-CD34 stents also capture cells of the monocytic/ macrophage lineage.¹⁵

Our overall goal is to coat self-expanding nitinol stents with autologous ECs derived from peripheral blood prior to deployment in order to promote rapid endothelial coverage and thereby accelerate vessel healing. We have invented a novel rapid seeding method (*QuickSeeding*), in which an EC suspension is infused into modified stent delivery systems to coat compressed nitinol stents with blood-derived ECs 10 minutes prior to deployment. Since peripheral vascular stents in many cases do not require additional balloon expansion after adequate preparation and angioplasty of the vessel lumen, and cerebrovascular nitinol stents are routinely deployed without any ballooning, autologous blood-derived ECs could be delivered with the self-expanding nitinol stent to a diseased vessel without desquamating or damaging the newly seeded endothelium.¹⁶ This method also eliminates the need to culture the stent and cells together for multiple days, and also the need to manipulate the stent back into a delivery catheter.

We utilized ECs derived from colony-forming cells¹⁵ (also known as late-outgrowth ECs¹⁷) isolated from blood. These ECs are phenotypically and functionally identical to vessel wall

ECs¹⁸ and can be derived from the blood of the patient in need of percutaneous angioplasty.¹⁹ The following studies were designed to test the feasibility and provide proof of concept for *QuickSeeding* autologous ECs onto nitinol vascular stents through modified delivery systems immediately prior to stent deployment at the point of care.

METHODS

Isolation and Culture of ECs

Experiments involving the isolation and use of human cells were reviewed by the Duke University Institutional Review Board. All human ECs (hECs) were isolated from human umbilical cord blood and used at passages 6–10 for *in vitro* studies.^{18,19} All porcine ECs (pECs) were isolated from porcine peripheral blood and used at passages 5–6 for *in vivo* studies.²⁰ See Supplemental Methods.

Delivery System Modification

To enable radial flow of media and cells, micropores were drilled in the outer sheath of commercial nitinol stent delivery systems (Cordis S.M.A.R.T.® CONTROL Vascular Stent System with 30 mm long and 6 mm diameter stents, Cordis Corporation, Bridgewater Township, NJ) using a 193 nm ArF excimer laser in collaboration with Fraunhofer CMI (Brookline, MA). See Supplemental Methods.

Stent Seeding

A schematic of the cell seeding process within the delivery system is shown in Figure 1. The EC suspension was introduced via the side-arm flushing port into the stent delivery system. At the distal end of the delivery system, the tip was blocked by a rubber cap, so that the cell suspension was forced to exit through the circumferential micropores in the sheath. The 2 mL cell suspension was infused using a syringe and a syringe pump (0.4 mL/min) until the entire solution was introduced. Following, the delivery system sat stationary at room temperature for five minutes to enhance cell attachment prior to deployment. Depending on the experiment, stents were deployed into static medium, flow circuit tubing, or a porcine vessel.

In Vitro Flow Experiments, Nitric Oxide Quantification, Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Stents for *in vitro* flow experiments were deployed into clear Tygon PVC tubing (McMaster-Carr, Elmhurst, IL) of 4 mm inner diameter. Stented tubes were exposed to steady, laminar physiological shear stress of 15 dynes/cm² for 24 hours.²¹ Following, ECs were characterized for NO production using nitrite as a surrogate marker, and for expression of cyclooxygenase (COX)-2, endothelial nitric oxide synthase (eNOS), Krüppel-like factor (KLF)-2 and vascular cell adhesion molecule (VCAM)-1 using quantitative RT-PCR. See Supplemental Methods.

Stent Implantation and Explantation in Swine

All animal procedures were approved by the Duke University Animal Care and Use Committee. Immediately prior to stent implantation, angioplasty was performed in the bilateral common carotid arteries (CCA) using a 6×40 mm balloon at a pressure of 1–4 atm for 30–60 sec. Each pig received a stent seeded with its own autologous pECs into one CCA and an untreated bare control stent in the contralateral CCA, implanted in randomized fashion into the right and left carotid arteries. *See Supplemental Methods.*

Two days after stent implantation, the stents were surgically explanted and pigs sacrificed. The stents were fixed in 3.7% paraformaldehyde (30 minutes, room temperature), sectioned and examined for any gross clot in the inner surface; the presence of PKH26-labeled ECs on stents was evaluated with an upright DMRB model microscope (Leica, Solms, Germany), Zeiss 780 upright confocal microscope (Zeiss, Oberkochen, Germany) and Scanning Electron Microscopy (SEM). Cell coverage (number of cells/cm²) was calculated by using ImageJ software Cell Counter to quantify the cells on a predetermined surface area. To test EC functionality *in vivo*, the explanted stents were also tested with a CCK-8 Assay (Sigma-Aldrich, St. Louis, MO). *See Supplemental Methods.*

Computational Modeling of Shear Stress

The geometry of the Cordis S.M.A.R.T.® was generated as a solid model and discretized into a mesh using ANSYS meshing utility (ANSYS, Canonsburg, PA). A steady-state simulation of flow was evaluated to examine shear stress variability along stent struts as expected in steady *in vitro* flow experiments. *See Supplemental Methods.*

RESULTS

EC Isolation and Characterization

The isolated blood-derived hECs and pECs exhibited characteristic endothelial cell cobblestone morphology. Flow cytometry confirmed that the cell population was positive for EC markers CD31 and CD105. Cells were negative for leukocyte markers CD14 and CD45.

Delivery System Micropore Characterization

Micropores drilled into the delivery system were observed with phase contrast microscopy and found to evenly cover the sheath (Figure 1(B)). Approximately 5,000 pores per sheath were repeatedly oriented in groups of 4 between braided wire reinforcements in the sheath. At the sheath outer surface, the micropores were nearly circular as visualized by light microscopy, with a major diameter of 39.4 ± 0.9 μm and minor diameter of 32.4 ± 0.4 μm ($n=4$ systems). The minor diameter aligned along the direction of the stent axis. There were about 16 sets of micropores in each circumferential row and each row of holes was about 390 μm apart. Thus, about 5.5 holes were present along the length of each side of the struts.

hEC Coverage and Spreading inside Nitinol Stents *In Vitro*

hECs *QuickSeeded* immediately before deployment were retained on the stent and spread under both static and flow conditions on the stent surface. The stent surface coverage was calculated as $55,000 \pm 9,500$ cells/cm² ($n=4$) immediately after seeding (Figure 2(A)). The

cells were in a rounded configuration at this time. After 24 hours under both static and flow conditions, large areas of the stent surface were covered with a confluent endothelial cell layer (Figure 2(B, D)) and the cells were fully spread. The EC density was $34,570 \pm 12,600$ cells/cm² after 24 hours and $29,640 \pm 2,610$ cells/cm² after 48 hours. Cell areas were $2,894 \pm 780$ μm^2 after 24 hours and $1,695 \pm 277$ μm^2 after 48 hours, similar to values on planar Ti-coated surfaces *in vitro*.²¹ hEC confluence was confirmed by PECAM stain of cell junctions after 48 hours of static culture (Figure 2(C)). After exposure to flow, the cell density declined to $21,300 \pm 12,600$ cells/cm² while the spread area was $3,100 \pm 900$ μm^2 .

Nitric Oxide Production

NO levels were assessed by directly measuring nitrite in the cell culture medium and adjusting for respective fluid volume under flow and static conditions. As compared to the static condition, nitrite levels indicated a significant increase of NO production after exposure to flow (* $p < 0.01$, $n = 5$). After 24 hours of flow, total nitrite was calculated by subtracting nitrite at 0 hours from nitrite at 24 hours. Total nitrite in media samples of hECs maintained under static condition (controls) was 0.41 ± 0.13 nmol, and total nitrite in the flow condition was 7.83 ± 1.97 nmol (Figure 3).

Gene Expression

ECs *QuickSeeded* onto stents were exposed to static or flow conditions for 24 hours and then evaluated for expression of several genes. The level of expression of inflammatory marker gene VCAM-1 was unchanged from static to flow conditions. In contrast, gene expression of eNOS was significantly downregulated in the flow condition ($p < 0.005$, one-sample t-test). The level of expression of antithrombotic genes KLF-2 and COX-2 relative to static conditions did not increase significantly ($n = 6$; Figure 4; Supplemental Table 2). Further, the coefficients of variation for eNOS, KLF-2 and COX-2 were 1.04, 0.93 and 1.18, respectively, suggesting that the stent geometry increased the variability in gene expression.

Flow and Shear Stress Around Stent

Given the three-dimensional geometry of the stent, we examined the variation in shear stresses occurring during the *in vitro* flow experiments. Computational simulations of steady-state flow over identical stent geometry revealed large variations in shear stress experienced by different stent surfaces (Supplemental Figure 2). Regions of flow reversal are evident. On lateral surfaces of the stent, a large shear stress gradient exists, and shear stresses vary by about an order of magnitude over the strut (Supplemental Table 1) with highest values on the luminal surface and the lowest values on the downstream portion of the strut sides.

Safety of *QuickSeeding* Procedure *In Vivo*

All pigs survived the procedure without complications. A neurological exam did not reveal any evidence of cerebrovascular accidents and there were no deaths.

pEC Retention and Spreading on Nitinol Stents *In Vivo*

Autologous fluorescently labeled pECs were *QuickSeeded* onto the nitinol stents ten minutes before stent implantation into pigs. After two days *in vivo*, pECs spread and formed confluent monolayers on the surface of all the pEC-seeded stents (n=4, Figure 5). The average projected cell area was $1377 \pm 300 \mu\text{m}^2$, similar to values for hECs spread on stents *in vitro*, but about 60% larger than pECs on implanted titanium tubes.²⁰ The pEC coverage on pEC-seeded stents was further supported by SEM images, which showed pECs covering the pEC-seeded stents as compared to fibrous covering on the bare metal stent (Figure 6). As the stent-adherent pECs were positive for fluorescent dye used prior to *QuickSeeding* (Figure 5), our results indicate that the pECs on the stent surface were derived from the *QuickSeeded* pECs, rather than from the bloodstream or local vessel wall.

pEC Viability on Nitinol Stents *In Vivo*

The CCK-8 metabolic assay suggested greater metabolic activity on the pEC-seeded stent segments than on the bare metal control stent segments ($p < 0.1$, n=3), measured by a paired test of absorbance in the medium. Absorbance increased between 3 hours and 21 hours and was greater than the blank condition in both the control stents and pEC-seeded stents. Further, the pEC-seeded stent showed a greater increase in absorbance than the control stent for every pair (n=3), supporting the hypothesis that pECs were covering the pEC-seeded stent surface and were viable and metabolically active (Figure 7).

DISCUSSION

Blood-derived ECs are an easily accessible source of autologous cells from patients and were therefore used in our study for rapid stent endothelialization, which was achieved with the innovative *QuickSeeding* technology. This approach enables rapid cell seeding without the need to recompress the cell-seeded stent. The *QuickSeeding* technology presented here applies to nitinol stents, which are used in cerebrovascular stenting and the majority of peripheral sites because of the flexibility and shape memory of nitinol.^{22,23}

Our method was developed based on the following principles and prior results: 1) Nitinol stents are typically electropolished such that the blood-contacting outer surfaces are free of nickel and almost exclusively comprised of titanium oxide (TiO_2);^{20,23} 2) without precoating of TiO_2 , we had shown that ECs attach rapidly and spread quickly on titanium surfaces under static and fluid flow conditions *in vitro*;²¹ 3) when allowed to adhere for 15 minutes prior to flow exposure (5 minutes of 380 dynes/cm^2), less than 20% of the adherent ECs detach from titanium surfaces *in vitro*;²¹ 4) rapidly seeded pECs form a confluent endothelial lining and prevent thrombosis on the blood-contacting surface of titanium tubes implanted *in vivo*.²⁰

Our *ex vivo* results show that following stent deployment, hECs seeded onto a self-expanding nitinol stents with the *QuickSeeding* method remained adherent under physiological flow conditions. The adherent cells on the stent surface were functional, as demonstrated by the increase in nitrite production over 24 hours; this increase was significantly greater after exposure of the seeded stents to flow, as would be expected for a

healthy layer of ECs. The ECs did not demonstrate a pro-inflammatory phenotype after *QuickSeeding*, deployment or flow, as demonstrated by unchanged gene levels of VCAM-1 at 24 hours.

The levels of antithrombotic genes COX-2 and KLF-2 of ECs increased after exposure of the cell-seeded stents to flow, but the increase was not statistically significant. The non-significant increase in the expression level of these genes was likely due to the large coefficients of variation, which may reflect the range of shear stresses to which the ECs were exposed to on the stent struts. This notion was supported by another study demonstrating much smaller coefficients of variation in COX-2 and KLF-2 gene expression when ECs were exposed to flow on a planar Ti surface.²⁴

The level of eNOS gene was downregulated after exposure of the cell-seeded stents to flow, but the secretion of NO as indicated by nitrite levels increased. In our previous work, we have observed that exposure of blood-derived and aortic hECs to flow shear stress (15 dyne/cm² for 24 hours) caused an overall increase in eNOS gene expression.²⁹ However, the increase was statistically significant only in aortic hECs.²⁹ In contrast to eNOS protein expression, our prior work demonstrated significant increase in NO production after flow in both aortic and blood-derived hECs.²⁹ Thus, blood-derived hECs show an exquisite sensitivity to flow shear stress in the eNOS protein expression. The lower level of eNOS gene expression after flow in our study may reflect the complex flow patterns on the lateral side of the stent struts. Whilst eNOS protein expression does not necessarily associate with NO production, conformational change to eNOS through posttranslational modification could lead to more NO secretion.^{30,31} Therefore, the significant increase in NO secretion after exposure to flow in the present study does indicate that the blood-derived hECs on stents are responsive to flow. The flow-specific changes in NO secretion has been consistently demonstrated in our prior works using several different assays and controls with culture medium and ECs under static conditions.^{18,21}

Our *in vivo* and *in vitro* results demonstrate that pECs *QuickSeeded* onto a stent just minutes before implantation remain adherent even when deployed and exposed to arterial shear stresses for 48 hours. Under these conditions, pECs spread on the stent surface and formed confluent monolayers. Some cells may be lost during deployment in the moving bloodstream, but sufficient numbers of cells remain to form an endothelial coating on the stent surface.

After two days of stent implantation into porcine carotid arteries, all of the *QuickSeeded* stents were endothelialized (n=4) and bare metal control stents were devoid of ECs (n=4). The cells covering the *QuickSeeded* stents were the same pECs seeded onto the stent, rather than colonizing cells from the adjacent vessel wall or circulation, as shown by the presence of the fluorescent dye used to stain pECs prior to implantation. These fluorescently labeled pECs were alive and functional as suggested by higher metabolic activity for the explanted *QuickSeeded* stents, as compared to the bare metal stents. Both the bare metal and seeded stents were covered by erythrocytes and leukocytes, but the additional pEC lining accounted for the higher metabolic activity on the *QuickSeeded* stents (Figure 6, 7).

The presence of adherent leukocytes, as identified by nuclear morphology, on bare metal control implants was not surprising as this is a common occurrence with foreign objects implanted into the vascular system. However, it remains elusive why leukocytes were also found adherent to pECs covering the seeded stents because there should not be an autoimmune reaction against the autologous pECs. We had not previously observed leukocytes attached to pECs seeded on the inner surface of Ti tubes implanted in the porcine inferior vena cava.²⁰ It is conceivable that the autologous cells may have taken up bovine proteins during their *ex vivo* expansion in medium containing bovine serum. If bovine serum proteins were internalized by the pECs, these cells could have become immunogenic due to serum proteins that act as antigenic substrates when implanted.^{25,26} Therefore, the immunogenic effects of xenogeneic serum proteins in an *ex vivo* culture should be investigated in future studies.

This study was designed as proof of principle to establish that 1) nitinol stents can be *QuickSeeded* with autologous blood-derived ECs within minutes of implantation; 2) the seeded cells remain adherent under arterial blood flow; 3) ECs rapidly spread over the stent struts following stent deployment under arterial shear stress *in vivo*.

This study has the following limitations. Firstly, the seeding parameters are not fully optimized. Therefore, the optimal size, number and spatial distribution of micropores in the delivery system wall, which will result in maximum EC coverage, is not yet known. Similarly, infusion parameters, e.g. cell concentration, infusion rate, and “hold time” following infusion, should be modified to enhance EC coverage. Secondly, our technology applies only to self-expanding nitinol stents that do not require post-deployment balloon expansion. Most cerebrovascular nitinol stents fall into this category since they are not ballooned.¹⁶ In addition, peripheral vascular stents also do not require additional balloon expansion in many cases. In cases of peripheral vascular stenting that require additional balloon expansion to achieve adequate stent expansion, e.g., in a calcified vessel, balloon expansion damage to the newly seeded ECs could be minimized by modifying the nitinol surface. For example, the surface of nitinol struts could be sintered or endowed with pores to allow protective niches where *QuickSeeded* ECs would be protected despite balloon expansion.²⁷ Thirdly, the time required to isolate and expand cells is approximately 3–4 weeks. Although peripheral stenting procedures are more likely to be elective when compared to coronary procedures, future improvements in the cell isolation method are needed to increase initial yields²⁸ and to decrease the lead time required to expand the isolated cells.

Finally, given the short duration of the *in vivo* studies, this pilot study did not demonstrate the benefit of our technology in reducing the risks of thrombosis and in-stent restenosis in the *QuickSeeded* stents as compared to bare metal control stents. Nevertheless, this study provides an important first proof of concept that rapidly seeding ECs onto self-expanding nitinol stents at the point of care is feasible and leads to successful cell adhesion and survival after implantation. The implanted stents are lined by *QuickSeeded* ECs that are anticipated to release paracrine factors, e.g., NO, that inhibit SMC proliferation and intimal hyperplasia, thus addressing this critical mode of restenosis. Further, the presented cell seeding therapy appears to be safe, as no adverse events or outcomes were observed in any animals during

the two-day duration of our *in vivo* study. This study avoids many of the pitfalls of previous approaches such as thrombogenic pre-coatings, use of non-autologous cells, modification of the stent itself and cumbersome manipulation of a sterile endothelialized stent back into the delivery catheter.

CONCLUSION

In conclusion, we have demonstrated proof of concept for *QuickSeeding* autologous ECs onto nitinol vascular stents at the point of care immediately prior to stent deployment. Stents implanted for two days into porcine carotid arteries retained the seeded endothelium and showed no observable thrombus or safety problems. Future studies should characterize the technology over longer period of time; specifically, the ability of cells to remain on the stent long term and the potential benefit of the technology to reduce thrombosis and intimal hyperplasia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We are extremely grateful for financial support from the *MORNINGSIDE FOUNDATION*. We also gratefully acknowledge Jason Allen and Thomas Stabler for nitrite measurement; Vrad Levering, Tracy Cheung, Cristina Fernandez for cell isolation; David Chargin and Alexis Sauer-Budge at Fraunhofer CMI for laser processing of stent sheaths; Melissa Ley for exceptional project support.

Funding sources: We are extremely grateful for financial support from NIH 1R21-HL109897-01 and American Heart Association 12BGIA11070002 to HEA, as well as National Science Foundation Graduate Research Fellowship and American Heart Association Predoctoral Fellowship 12PRE11180003 to AEJ, American Heart Association Student Scholarship and Goh Foundation Research Award to MN, and grant 'Short-term implantation of EPC-*QuickSeeded* stent' from the *MORNINGSIDE FOUNDATION*.

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Figure 1.

(A) Schematic of in-catheter seeding of ECs. EC (red) - containing suspension medium were flushed through a stent delivery system from the proximal handle. As the gap near the distal tip of the delivery system was closed with a rubber “cap” (black), the suspension medium flows outward through micropores in the delivery sheath (orange) and ECs would adhere to a stent (gray). (B) Light microscope image of holes machined in outer sheath of a commercial stent delivery system for the process of infusion seeding. Each delivery system had approximately 5000 holes over the area of the stent. The hole diameters were measured at right angles to each other, i.e. major and minor diameter referring to the longer and shorter width, respectively.

Figure 2.

QuickSeeded stents following (A) 0h culture, (B) 24h static culture (luminal view), (C) 48h static culture (lateral view; green: PECAM; blue: Hoechst 34580; curved stent surface caused some areas to be out of focus while others were in focus), (D) 24h flow (lateral view through flow circuit tubing; arrow denoted unsprayed hECs adherent to the tubing).

Figure 3.
Nitrite production of ECs *QuickSeeded* onto nitinol stents following 24h flow or static culture (* $p < 0.01$, $n = 5$).

Figure 4.
Gene expression of ECs on stents following 24h flow. Values were normalized to static conditions (n=6).

Figure 5. Fluorescent images of explanted stents: (A) Bare metal control stent (fluorescent microscopy; the variations in light were from uneven metal surface reflections); (B-D) *QuickSeeded* stents (Leica DMRB); (E-F) *QuickSeeded* stents (Zeiss 780 Upright Confocal).

Figure 6. Representative SEM images of explanted stents: (A) Bare metal control stent; (B) *QuickSeeded* stent. Note: arrows denoted leukocytes.

Figure 7.

CCK-8 metabolic assay showing absorbance increase from 21 to 3 hours (n=3, cell-seeded and bare metal control; n=1 blank). Cell-seeded samples showed more metabolic activity in every pair, consistent with the presence of metabolically active seeded ECs.