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An Ex Vivo Vessel Injury Model to Study Remodeling

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Abstract

Objective: Invasive coronary interventions can fail due to intimal hyperplasia and restenosis. Endothelial cell (EC) seeding to the vessel lumen, accelerating re-endothelialization, or local release of mTOR pathway inhibitors have helped reduce intimal hyperplasia after vessel injury. While animal models are powerful tools, they are complex and expensive, and not always reflective of human physiology. Therefore, we developed an *in vitro* 3D vascular model validating previous *in vivo* animal models and utilizing isolated human arteries to study vascular remodeling after injury. **Approach:** We utilized a bioreactor that enables the control of intramural pressure and shear stress in vessel conduits to investigate the vascular response in both rat and human arteries to intraluminal injury. **Results:** Culturing rat aorta segments *in vitro*, we show that vigorous removal of luminal ECs results in vessel injury, causing medial proliferation by Day-4 and neointima formation, with the observation of $SCA1^+$ cells (stem cell antigen-1) in the intima by Day-7, in the absence of flow. Conversely, when endothelial-denuded rat aortae and human umbilical arteries were subjected to arterial shear stress, pre-seeding with human umbilical ECs decreased the number and proliferation of smooth muscle cell (SMC) significantly in the media of both rat and human vessels. **Conclusion:** Our bioreactor system provides a novel platform for correlating *ex vivo* findings with vascular outcomes *in vivo*. The present *in vitro* human arterial injury model can be helpful in the study of EC-SMC interactions and vascular remodeling, by allowing for the separation of mechanical, cellular, and soluble factors.

Keywords

human vessel, bioreactor, vessel injury, endothelial cell, neointima, SCA1

Introduction

Cardiovascular diseases, affecting one-third of the US population, are the leading cause of mortality globally[1,2]. Balloon angioplasty, stenting, and autologous coronary bypass grafting are the most commonly used methods to treat coronary occlusion[3]. These interventions result in partial or complete endothelium denudation (due to the mechanical trauma of vessel harvest or manipulation) and smooth muscle injuries, which cause short- and long-term complications such as inflammation, thrombosis or excessive smooth muscle cell (SMC) growth, or intimal hyperplasia[4–7]. Furthermore, endothelial cells (ECs) of vein grafts cannot adapt to arterial flow after coronary bypass[8,9]. In the absence of a functional endothelium, platelet adhesion and activation on the vessel lumen is followed by localization of monocytes, neutrophils, and T-lymphocytes in the subendothelium[10,11]. Foam cells derived from monocytes, and other immune cells, accumulate to form plaques and fatty streaks, which result in endothelial damage and, subsequently, intimal hyperplasia and the vessel occlusion[11–13].

In addition, during the surgical preparation of vein grafts (pressurizing vein grafts to overcome vasospasm or to check the competence of side branches, etc.), the vessel wall is distended and stretched[14–17]. Mechanical injury results in SMC migration and proliferation through increase of matrix metalloproteinases production[18], and local secretion of growth factors such as basic fibroblast growth factor (bFGF)[19], platelet

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derived growth factor (PDGF)[20,21] and transforming growth factor-β (TGF-β)[22]. Moreover, some *ex vivo* vessel culture studies have shown that the disruption of extracellular matrix in the media triggers SMC proliferation by degradation of cyclin-dependent kinase inhibitors[23]. In addition to medial SMCs, the migration and proliferation of adventitial cells can contribute to formation of intimal hyperplasia[24–26].

Therapeutic use of ECs, or elements of their secretome, may be an effective biological approach to treat vascular injuries caused by the aforementioned interventions. In animal models, local delivery of endothelial or endothelial progenitor cells to the injury site[27–31], injection of intra-arterial VEGF[32] or VEGF gene delivery[33], all result in reduction of intimal hyperplasia after balloon injury in animal models. However, all of these studies employ animal models that are not ideally representative of human physiology. The ability to mimic vessel injury on human tissues, and to re-endothelialize these vessels *ex vivo* to study the EC-SMC interaction and vessel remodeling, would be a useful tool for enhancing our understanding of arterial injury and repair processes. Perfusion bioreactors have been used previously to study cell viability and proliferation in *ex vivo* vessel cultures[34–36]. Although most of the reported data is in animal tissues, recent studies have used human saphenous veins in bioreactors to assess vein adaptation to arterial pressure[37–39].

Here, we created an ex vivo vessel culture system that is able to validate the previous *in vivo* works and to isolate different factors affecting vessel remodeling. To this aim, we studied the effect of vessel wall injury and pre-endothelilalization of injured vessels, where we applied arterial shear and pressure in perfusion bioreactors. We first validated the *ex vivo* bioreactor system for creating vessel injury and remodeling in rats, and then studied human vessel injury and remodeling using umbilical arteries. Our results show that culture of human umbilical arteries in perfusion bioreactors is an effective *in vitro* model for EC-SMC co-culture, or for drug testing studies, and that arterial shear stress and the presence of endothelium interact to impact the extent of SMC remodeling *ex vivo* after injury.

Materials and Methods

This study was approved by the Yale University Institutional Animal Care and Use Committee. All animal care complied with the Guide for the Care and Use of Laboratory Animals. Human tissues and cell populations were obtained using protocols approved by the Yale University Human Investigation Committee, and were discarded tissues.

Perfusion bioreactor design

In order to create an *in vitro* system that mimics arterial pressure and flow, we designed a bioreactor as shown in Fig. 1A. The main body of the bioreactor is a glass medium reservoir fitted with a silicone stopper. The vessels are cannulated with glass pipettes, secured with silk sutures, and placed into the bioreactor. PharMed silicone tubing (Westlake, OH, USA) was attached to the glass pipettes, reservoir inlet and outlet to complete the perfusion loop. Rat aortas and umbilical arteries were isolated under sterile conditions. Each bioreactor and tubing component was autoclaved and handled with sterile gloves in the hood. Following mounting of the vessel to bioreactor and assembling all components, each connection was sealed with 70% ethanol-soaked parafilm (Fig. 2). Culture medium was drawn from the media reservoir and pumped through the vessel and then drained back to the reservoir via a Masterflex L/S roller pump (Vernon Hills, IL, USA). Lengths of the tubing and the attachment configurations were optimized to achieve 50–70 mmHg diastolic pressures, and 90–130 mmHg systolic pressures, at a target shear stress of 20 dyne/cm^2. The intraluminal shear was calculated according to Equation 1, where Q, μ, and r are the volumetric flow rate, dynamic viscosity of the fluid, and the lumen radius, respectively[40].

$$\tau = \frac{4Q\mu}{\pi r^3} \quad (1)$$

This simple bioreactor system enables us to tune the flow rate, intramural pressure and wall shear stress effectively by adjusting pump speed, tubing resistance and media viscosity. We were able to create similar shear stress (20 dyne/cm^2) and intramural pressure (50–70 mmHg diastolic and 90–130 mmHg systolic) in both rat and human vessels. Compared to other bioreactor systems, where a separate media reservoir is used[37,38,41], our system utilized a close loop system that circulates the same media in and out of the vessels, thereby conserving reagents and cost.

Rat aorta preparation

Sprague-Dawley rats weighing 250–300 g were euthanized via intraperitoneal injection of 150 mg/kg sodium pentobarbital (Sigma, St. Louis, MO, USA). Only male rats were used due to larger aorta sizes. The descending thoracic aorta was dissected free and placed in ice-cold Dulbecco's modified eagle's medium (DMEM) with 10% FBS (Hyclone, Marlborough, MA, USA), and then the surrounding connective tissues and fat were removed. For gentle removal of luminal ECs without inducing extensive medial injury, arteries were flushed with 0.25% trypsin EDTA (Life Technologies, Grand Island, NY, USA), PBS, (Invitrogen, Carlsbad, CA, USA) and DMEM with 10% FBS to deactivate trypsin, consecutively. Medial injury was created by inserting a 14-gauge sterile needle into the vessel. Aortas were then stored in ice-cold DMEM with 10% FBS until being mounted to perfusion bioreactors (less than 6 h) or cut into segments of 0.3–0.5 cm length for static ring culture.

Human umbilical artery preparation

Anonymized human umbilical cords were obtained from Yale–New Haven Children's Hospital (New Haven,

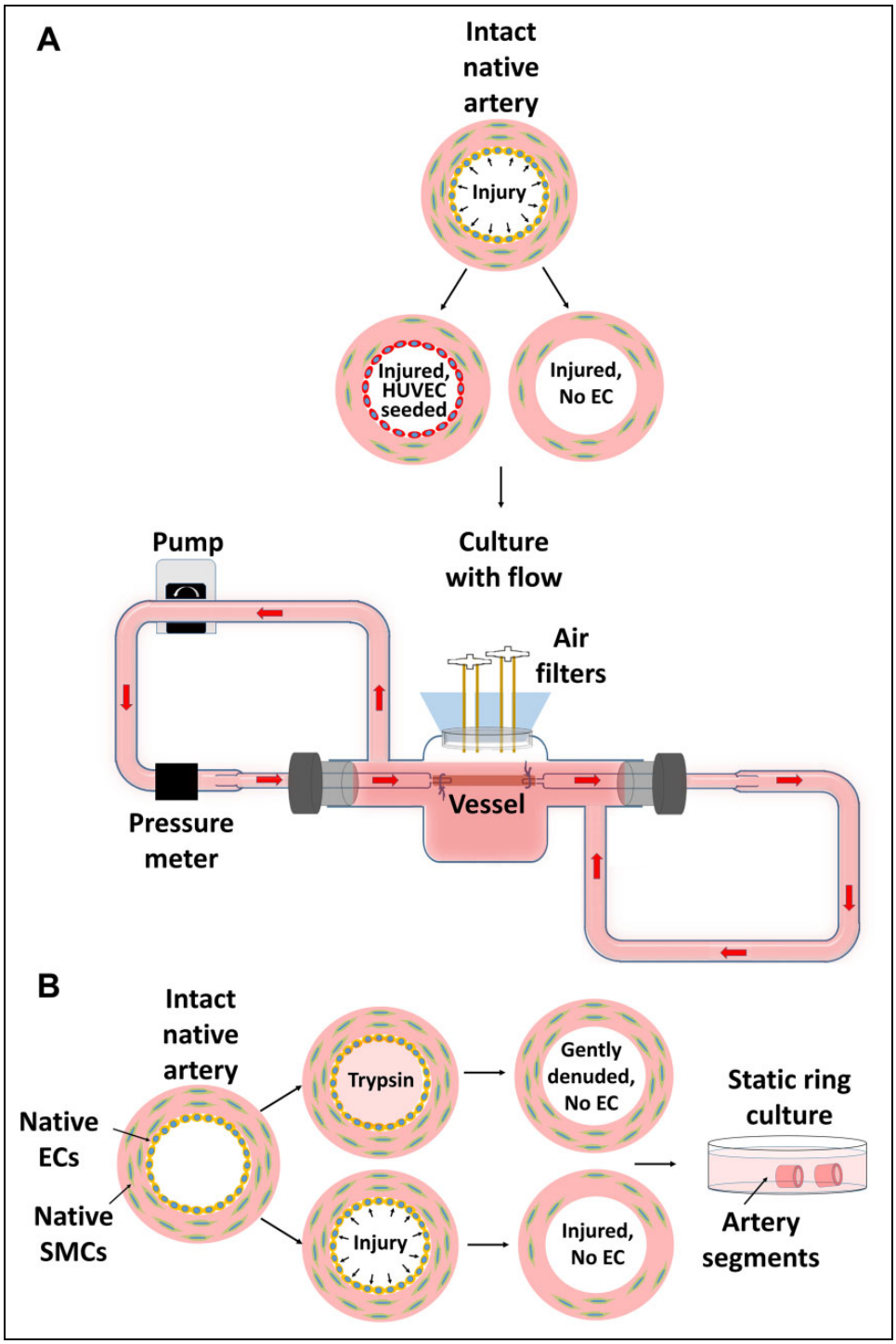

Fig. 1. Intact vessels were either gently denuded via flushing with trypsin or injured by inserting 14-gauge needle into lumen. A: Injured vessels (5 cm in length), either seeded with HUVECs onto luminal surface or without ECs, were cultured with flow in perfusion bioreactors. B: Segments of both gently-denuded and injured arteries were cultured statically in 6-well plates.

CT, USA). Arteries were isolated from human umbilical cords (20–30 cm in length) using sharp dissection in a sterile hood immediately after delivery as described[42]. Briefly, a pair of Metzenbaum scissors were inserted into the Wharton's jelly surrounding the arteries, and the tissue was dissected from the arterial vessels. Intact arteries were separated from the umbilical vein and then cut into segments of 5 cm length. Gentle denudation and medial injury induction were performed as described for rat aortas.

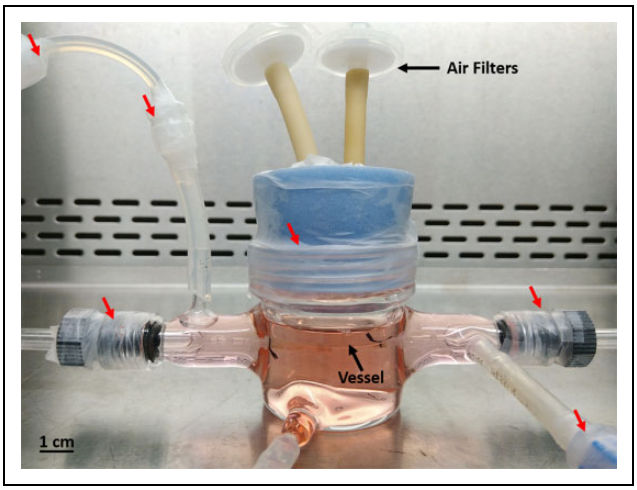

Fig. 2. Vessel in the bioreactor. Every connection and cap are sealed with ethanol soaked parafilm (red arrows) to prevent contamination.

Static vessel segment culture

Gently denuded and medially-injured rat aorta segments were cultured in 6-well tissue culture plates with DMEM: Vasculife (1:1) containing 5% FBS for up to 14 days (Vasculife, Lifeline Technologies, Frederick, MD, USA). The medium was changed every 2 days. Samples were fixed with 10% neutral buffered formalin at indicated times and processed for immunofluorescence staining analysis.

Culture under arterial flow in perfusion bioreactor

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins using 0.1% collagenase, and were cultured in Vasculife at 37 °C and 5% CO_2 until sub-confluence. Cells at passage 3–4 were used in all experiments in this study. To seed endothelium onto denuded arteries in vitro, fibronectin in PBS (50 μg/mL) was delivered through the glass cannulae in the lumen of vessels mounted in perfusion bioreactors. Fibronectin was applied for 1 h at 37 °C and then replaced with HUVECs at 3 x 10^6 cell/mL. HUVECs were allowed to adhere for 3 h at 37 °C and 5% CO_2, after which the non-adhered cells were flushed and vessels were perfused with co-culture medium: DMEM: Vasculife (1:1) with 5% FBS. The viscosity of the medium was brought to 3.8 cp by addition of 3% dextran (MW 1.5–2.8 x 10^6, Sigma, St. Louis, MO, USA)[43]. The intraluminal shear was increased gradually from 1.5 dyne/cm^2 to 20 dyne/cm^2 over 80 h. The vessels were cultured with flow for 4 or 7 days. To assure uniform shear stress application, we analyzed only the 1.5-cm-long central part of the vessels to avoid the effect of flow distortion in the inlet and outlet regions.

Labeling adventitial cells

To track possible adventitial cell migration, the cells in this regions were stained with FastDil dye (Invitrogen, D7756, Carlsbad, CA, USA). Freshly isolated intact rat aortas were ligated from both ends to isolate intraluminal space from staining. FastDil dye was diluted in culture media with 12 μg/mL final concentration and applied gently to the outer surface of the intact vessel by a sterile cotton swab. The vessels were incubated at 37 °C for 5 min, and then briefly rinsed with PBS. Ligated ends were cut and the vessels were injured, cut into segments and cultured statically as described above. Non-injured vessels were also cultured as control. Samples were frozen in optimum cutting temperature (OCT) compound at Day-1, Day-4, and Day-7 time points, cut in 5-μm-thick sections and imaged with 555 nm fluorescent excitation.

Histology and immunostaining

Vessels were fixed with 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5 μm thickness. Tissue slides were stained for hematoxylin and eosin (H&E) as described previously[42]. Cell death was by labeling DNA strand breaks (TUNEL stain, Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. For immunofluorescence, sections were incubated in sodium citrate buffer (pH 6) at 75 °C for 20 min for antigen retrieval, permeabilized, and blocked with PBS containing 5% BSA, 0.75% glycine, and 0.2% Triton X-100 for 1 h, and subsequently incubated in primary antibodies against ki67 (Abcam, ab16667, rabbit monoclonal, 0.5 μg/mL, Cambridge, MA, USA), alpha-smooth muscle actin (αSMA) (Dako M081, mouse monoclonal, 70 μg/mL, Agilent Technologies, Santa Clara, CA, USA), platelet endothelial cell adhesion molecule (CD31) (Abcam ab28364, rabbit polyclonal, 20 μg/mL, Cambridge, MA, USA and Santa Cruz, sc-1506, goat polyclonal 4μg/mL, Dallas, TX, USA), von Willebrand factor (vWF) (Abcam, ab6994, rabbit polyclonal, 20 μg/mL, Cambridge, MA, USA), stem cell antigen-1 (SCA1) (Millipore, ab4336, rabbit polyclonal, 2.5 μg/mL Burlington, MA, USA) overnight at 4 °C. After washing slides with 0.2% Triton x-100 in PBS, secondary antibodies (Alexafluor 555, 647, 546or 488) were applied at 1:500 dilution for 1 h. For negative control, secondary antibodies were applied without incubating samples with primary antibodies (Supplementary Fig. 1). The slides were visualized using a Zeiss Axiovert 200 m fluorescence microscope. Cell number was quantified by counting DAPI stain in nuclei. Proliferating and apoptotic cells were quantified by counting nuclei that are co-localized with $ki67^+$ and $TUNEL^+$ stain, respectively. Cells with cytoplasmic $SCA1^+$ stain was counted to quantify SCA1 expression. Cells expressing the related markers were counted in five randomly taken 20× magnification images of each vessel section, and the numbers normalized to the average number of total nuclei in freshly isolated vessel sections.

Statistical analysis

Data were expressed as mean ± SD and analyzed using one-way ANOVA test with Tukey's multiple comparison using GraphPad Prism-7.01 (GraphPad Software, Inc., La Jolla, CA, USA). A p value of <0.05 was considered statistically significant.

Results

Effect of gentle denudation and medial injury on EC and SMC viability in rat aortas

To differentiate the effect of EC denudation with and without medial injury, freshly isolated rat aortas were either gently flushed with trypsin to remove native ECs, or injured by inserting a 14-gauge (2.05 mm) needle to the lumen (that mimics balloon injury) and moving back and forth three times. The needle diameter is 1.5-fold larger than that of the intact rat aorta (1.4 ± 0.2 mm). The aortas shrank to their initial diameter immediately after needle withdrawal. In the native vessels, there was a confluent layer of ECs shown by CD31 and vWF staining, which was lost after trypsin or needle injury (Fig. 3 A–F, Supplementary Fig. 2), indicating that both of the treatments removed the native ECs successfully. The treated vessels were then cultured in 6-well culture plates for 24 h, and cell apoptosis was evaluated by TUNEL staining. SMC death in freshly isolated aortas was less than 0.5% of total nuclei, consistent with many prior observations[44,45]. As expected, medial injury induced by stretching the vessel wall caused significantly higher SMC death than did gentle EC denudation (40 ± 8% vs. 0.75 ± 2%, $n = 4$, $p < 0.01$) (Fig. 3 G–J). In addition, the αSMA intracellular staining appeared to be altered in needle-treated group (Fig. 3 D–F). These results indicate that the treatment with trypsin could remove ECs without extensive SMC injury, while needle treatment could mimic the vessel wall injury in ways similar to invasive percutaneous interventions.

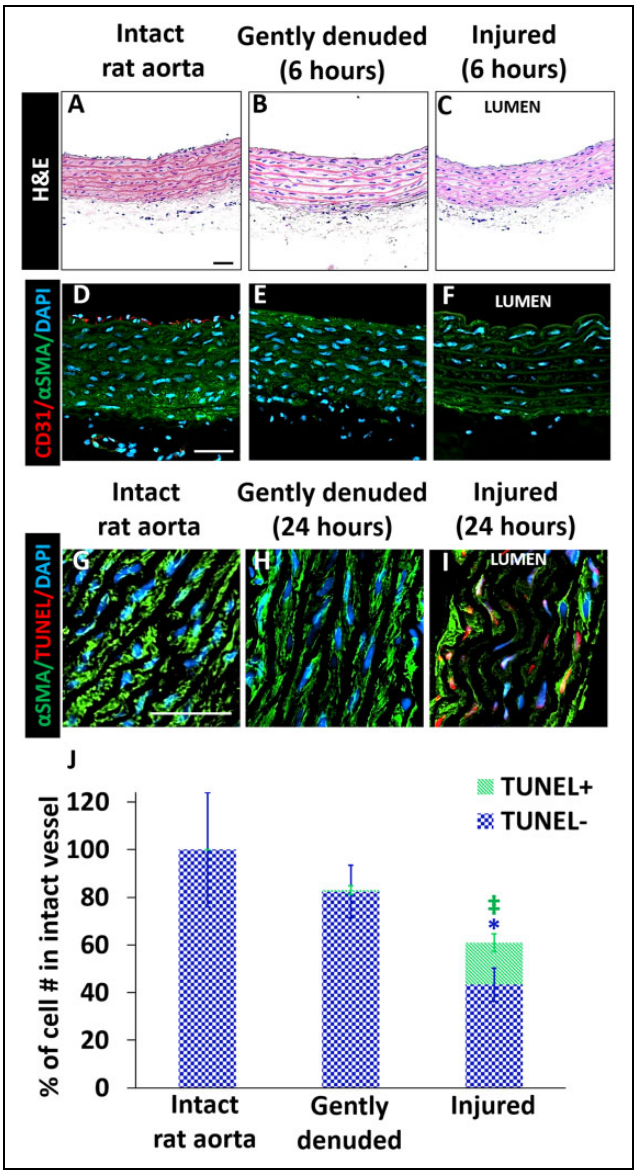

Fig. 3. Characterization of intact, gently denuded and injured rat aortas. (A–C): H&E; (D–F): αSMA (green)/CD31 (red); (G–I): αSMA (green)/TUNEL stain (red). (J): Average number of nuclei and TUNEL$^+$ cells per area was normalized to average cell number in the intact rat aorta. The total bar height represents the total cell number. The total number of cells in the media+intima of intact native vessel was set as 100%. $n = 4$ for each group, one-way ANOVA with Tukey test for multiple comparison. $^*p < 0.01$, $^‡p < 0.0001$. Scale bars: 50 μm.

Effect of gentle denudation and medial injury on proliferation, SCA1$^+$ cells and neointima formation in rat aortas

To probe cellular proliferation and migration in response to EC removal and medial injury, we cultured gently denuded and medially-injured rat aorta segments in 6-well culture plates for 4 and 7 days. As shown by H&E staining (Fig. 4 A–D), the wall thickness was 0.16 ± 0.02 mm in the freshly isolated vessel, but decreased to 0.10 ± 0.01 mm following both procedures. The wall thickness of the injured rat aorta at days 4 and 7 (0.12 ± 0.02 mm and 0.14 ± 0.02 mm, respectively) was greater than that of the gently denuded aorta (0.10 ± 0.02 mm and 0.10 ± 0.01 mm, respectively). The vessel walls in injured aortas started to thicken as *de novo* cell layers formed over the original vessel wall, which was bound by native connective tissue, indicating very early stages of intimal hyperplasia.

To assess the degree of SMC damage in the media, the endothelial recovery and recruitment of progenitor cells in vessel repair, we stained tissues for αSMA, CD31, and SCA1, respectively. The αSMA staining was altered in injured rat aorta as compared to gently denuded aortas, and, interestingly, CD31 immunostaining was evident after 7 days

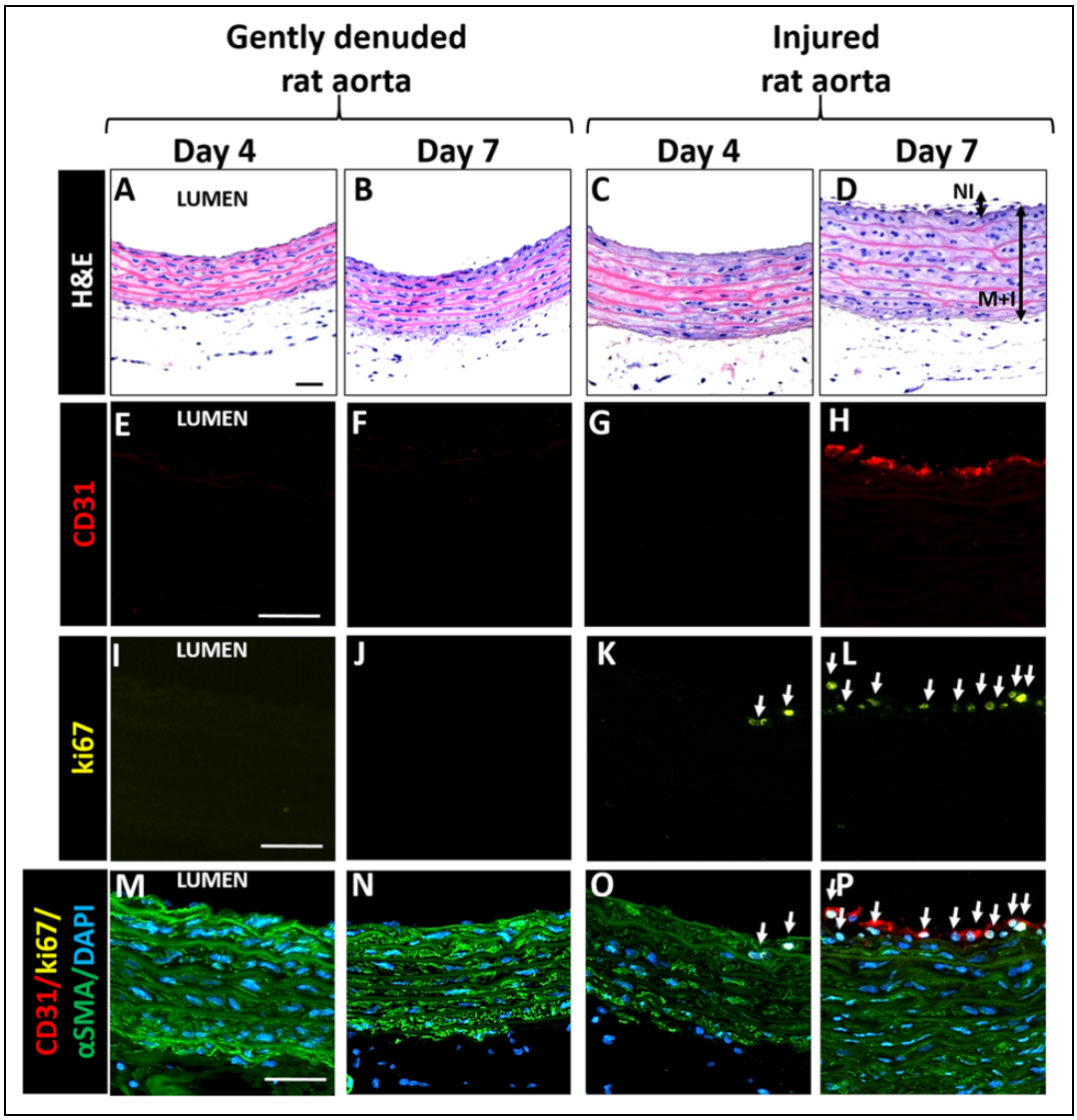

Fig. 4. Characterization of statically cultured vessel segments 4–7 days following either gently denuding or injury of rat aortas. A–D: H&E; E– H: CD31 (red); I–L: ki67 (yellow), M–P: Merged: CD31 (red)/ki67 (yellow)/aSMA (green)/DAPI (blue). White arrows indicate ki67þ cells (K, L, O and P). Injury but not gentle denudation causes formation of neointima (D), where some of the cells are both proliferatingand CD31þ by day 7 (white stain in H). Scale bars: 50 mm. NI: Neointima, MþI: MediaþIntima.

in the injured rat aortas (Fig. 4 D and E) but not in the gently denuded aortas. Most of the $CD31^+$ cells seen in the injured aortas were also positive for SCA1 ($SCA1^+$) (Fig. 5 I, J, N, and O).

In native fresh aortas, 14% cells were $SCA1^+$, and were mostly located in the near-adventitia region. Four days later, medial injury increased the percentage of the $SCA1^+$ cells in the media of rat aortas to 29.9%, while in gently denuded group the fraction of $SCA1^+$ cells was 4% (Fig. 5). By day 7, the neointma cells in the injured rat aortas were mostly $SCA1^+/CD31^+$ but $\alpha SMA^-/vWF^-$ (Fig. 5 D, E, I, J, N, and O and Supplementary Fig. 2). We labeled the adventitial cells with FastDil dye to investigate the possibility of adventitial cell migration toward lumen after injury. As shown in Fig. 5, labeled cells stayed in the adventitia during the entire culture period, but new $SCA1^+$ cells appeared in the media 4 days, and in the neointima 7 days, after the injury. These results imply that the medial injury trigger the activation and proliferation of $SCA1^+$ cells, which might contribute to ongoing vessel wall repair during *ex vivo* culture.

There was an apparent decrease in cell number in the media sections of the injured rat aortas as compared to those of the gently denuded aortas. In the injured rat aortas, the total cell number in the media layer reduced to $28 \pm 7\%$ of that in the intact rat aortas on day 4 of culture, and to $18 \pm 4\%$ on day 7 of culture, while cell number in the media layer of gently denuded aortas did not change (Fig. 6, $n = 4$–6). To quantify cell

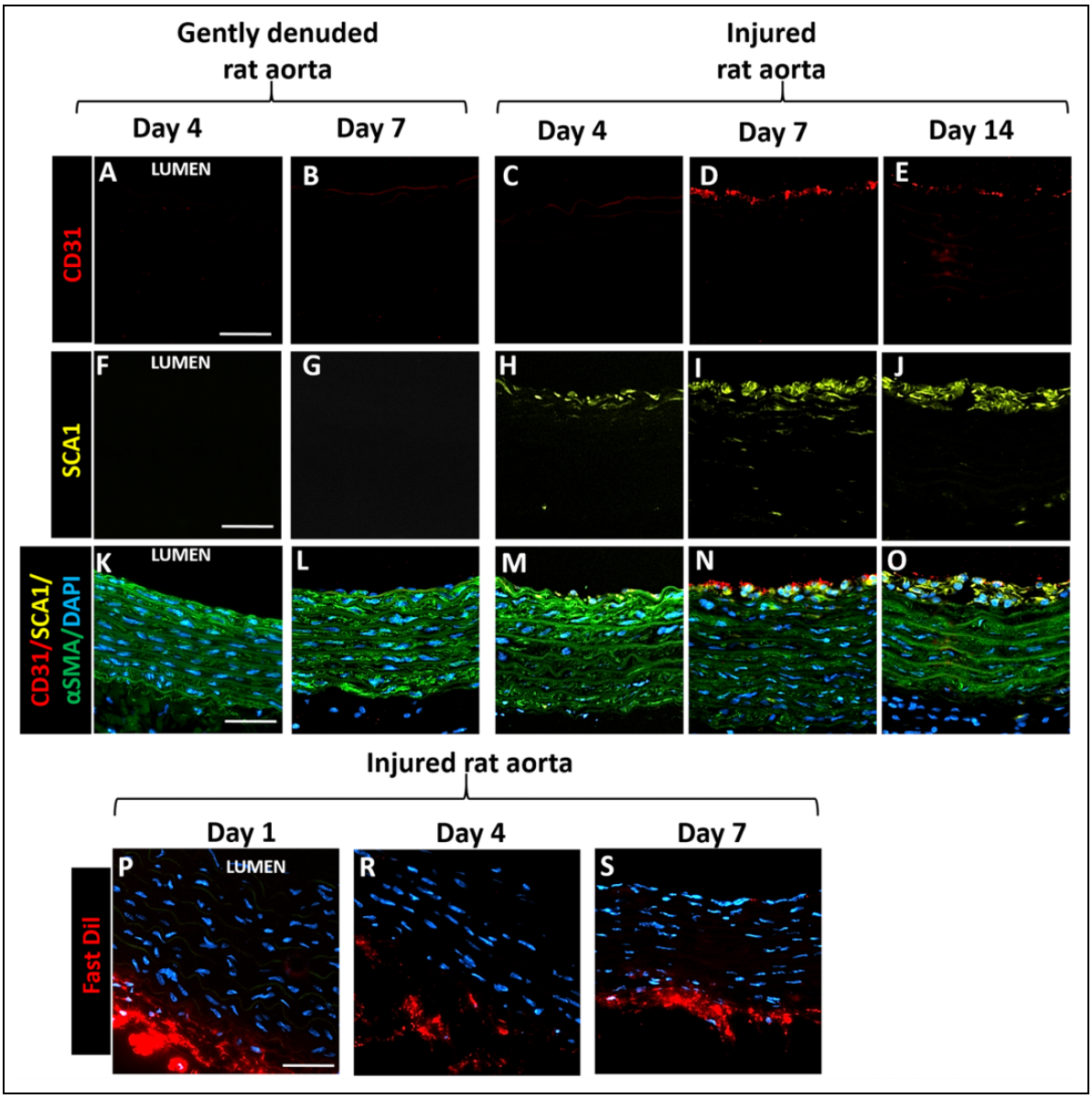

Fig. 5. Distribution of SCA1$_þ$ cells in statically cultured rat aorta segments 4–14 days following either gently denuding or injury. A–E: CD31 (red); F–J: SCA1 (yellow), K–O: Merged, CD31 (red)/SCA1 (yellow)/aSMA (green)/DAPI (blue). SCA1$_þ$ cells appeared in the media layer of injured rat aortas 4 days after injury (H). By day 7 SCA1$_þ$ cells formed neointima-like layer, and started to express CD31 but not aSMA on the lumen surface (N). By day 14, neointima layer became thicker (O). P–S: FastDil (red) labeled adventitia cells. Labeled adventitial cells did not migrate toward lumen. Scale bars: 50 mm.

proliferation, vessel segments were stained for ki67. In the medially-injured group, 6.6% of the cells in media layer were proliferating by day 4 (Fig. 5 K) while both in gently denuded aortas and freshly isolated aortas the number of ki67$^+$ cells in the media region were less than 0.5%. By day 7, neointima-like cell layers on the luminal surface were observed in the injured but not the gently denuded rat aortas, and 14.8% of the neointimal cells were ki67$^+$ (Fig. 6). Thus, the medial injury was necessary to trigger medial proliferation, along with neointima-like cell accumulation on the lumen.

Effect of arterial flow on remodeling of injured rat aortas

To determine whether exogenously-seeded and arterial-shear stimulated ECs can reduce medial cell proliferation and migration to the lumen[22,26], 5-cm-long injured rat aortas were cultured in perfusion bioreactors at 20 dyne/cm^2 (arterial shear) for 4 and 7 days[46], with and without HUVECs seeded onto the vessel lumen. After 7 days of culture under arterial shear, the diameter of the rat aortas remained similar as that of freshly isolated ones (1.5 $\pm$ 0.3 mm), either with or without seeded HUVECs. Regardless of HUVEC seeding,

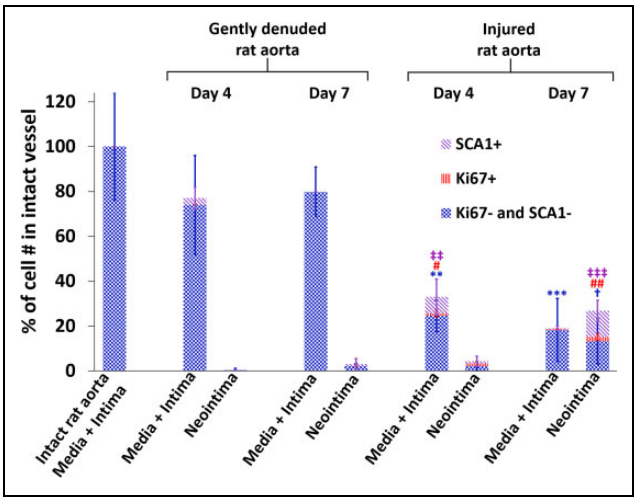

Fig. 6. Distribution of ki67$^+$ and SCA1$^+$ cells at day 4 and day 7 as a response to gently denuding or injury. Numbers were normalized to the average cell number in intact rat aorta. The total bar height represents the total cell number. The total number of cells in the media+intima of intact native vessel was set as 100%. $n = 4$–6 per group. $^{**}p < 0.01$, $^{***}p < 0.0001$; total number of the cells in the media+intima was significantly less than gently denuded groups. $^{\dagger}p < 0.05$; significantly more cells in neointima than any other group. $^{\#}p < 0.05$; significantly more ki67$^+$ cells in the media+intima than any other group, $^{\#\#}p < 0.001$; significantly more ki67$^+$ cells in the neointima than any other group. $^{\ddagger\ddagger}p < 0.01$; significantly more SCA1$^+$ cells in the media+intima than any other group. $^{\ddagger\ddagger\ddagger}p < 0.0001$ significantly more SCA1$^+$ cells in the neointima than any other group (one-way ANOVA with Tukey test for multiple comparison).

arterial flow did not change the wall thickness, which was 0.10 ± 0.02 mm immediately after vessel injury.

In contrast to static culture, neither proliferation nor neointima formation was observed in aortas cultured with arterial flow, either in the absence or the presence of ECs (Fig. 7). These results suggest that the cyclic distension created by intramural pressure alone might inhibit SMC proliferation[47] and migration in medially-injured rat aortas whereas adventitial cells can leak into the lumen through severely damaged vessel walls.

Under arterial shear stress, the total cell number decreased to 20% of that of freshly isolated aortas on the day-4 of culture, regardless of HUVEC seeding (Fig. 7). In aortas without HUVEC seeding, cell number did not significantly change until day-7 of culture, but in HUVEC-seeded rat aortas, severe SMC loss was observed (98.4 ± 1.4%) (Fig. 7 M). These results suggest that ECs might trigger SMC death in injured rat aortas under arterial flow.

Interestingly, when the vessel wall was broken by medial injury, adventitial cells migrated through the gap in the vessel matrix and invaded toward the lumen as shown in Fig. 8. These invading cells were stained negative for αSMA, but positive for SCA1 (Fig. 8 B).

Remodeling of injured human umbilical arteries cultured in perfusion bioreactor with flow

We have demonstrated that our perfusion bioreactor was able to generate arterial shear stress and pressure, and culture rat aortae *ex vivo*. In order to determine whether this bioreactor model can be used to provide information on human vessel injury and remodeling *ex vivo*, we cultured injured human umbilical arteries in these bioreactors for 4 and 7 days. To understand the contribution of ECs in vascular remodeling after injury in human vessels, human umbilical arteries were used for proof-of-concept testing. We cultured 5-cm-long injured human umbilical arteries under 20 dyne/cm^2 shear stress, with and without HUVECs seeded onto the luminal surface for 4 and 7 days. The medial injury created with a 14-gauge needle (2.05 mm) increased the inner diameters of the umbilical arteries from 0.59 ± 0.88 mm to 0.92 ± 0.12 mm. Arterial flow did not significantly increase the inner diameter without ECs (1.01 ± 0.1 mm). However, in the presence of seeded HUVECs, the inner diameters of the umbilical arteries were significantly larger than those of non-EC-seeded arteries: 1.42 ± 0.14 mm on day 4 and 2.03 ± 0.17 mm on day 7 (Fig. 9). These results showed that seeded ECs caused significant arterial dilatation under flow conditions.

Similar to rat aortas cultured with arterial flow, we did not observe *de novo* cell layers at the luminal surface in umbilical arteries that were cultured in bioreactors. The wall thickness of freshly isolated umbilical arteries was almost uniform along the circumference (0.61 ± 0.1 mm), and seeded HUVECs decreased the wall thickness circumferentially to 0.22 ± 0.07 mm while dilating the vessel wall as described above (Fig. 9 C and D). In No-EC-seeded groups, however, the wall thickness increased to 1.08 ± 0.1 mm on one-half of the vessel, and decreased to 0.24 ± 0.02 mm on the other half (Fig. 9 A and B). These results indicate that two vessel types remodeled differently as a response to *in vitro* arterial flow.

To determine cell proliferation in umbilical arteries cultured under arterial flow, we stained cells with Ki67. There were significantly more proliferating cells (6.9%) in vessels without ECs, as compared to the HUVEC-seeded group on day 7 (Fig. 9 J and Fig. 9 M). In HUVEC-seeded arteries, the total cell number decreased significantly (to levels of 2% of original native vessels) after 7 days (Fig. 9 C, D, H, G and M). Therefore, these results indicate that exogenously-seeded ECs caused severe SMC loss and significant vessel thinning in injured human umbilical arteries cultured with arterial flow.

Discussion

In the present study, we built an *ex vivo* bioreactor system that can create arterial flow and pressure. Most of our findings regarding the cellular responses to either vascular wall damage or endothelial denudation were reported in early

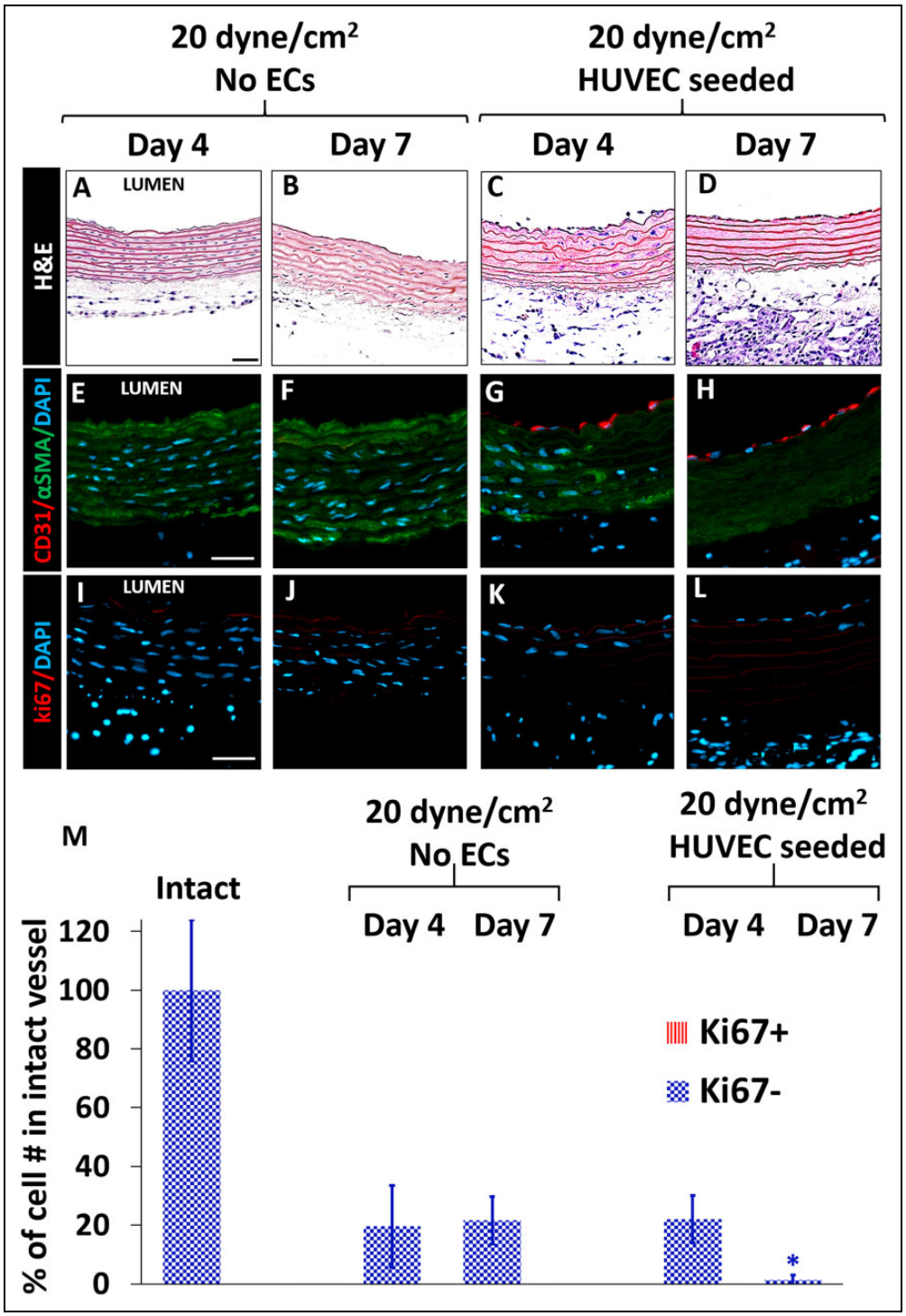

Fig. 7. Injured rat aortas cultured under arterial shear either with or without HUVECs seeded in the lumen for 4 and 7 days. A–D: H&E; E–H: αSMA (green), CD31 (red); I–L: ki67 (red). Neither proliferation in the media layer nor neointima formation was observed in culture with flow. In the HUVEC-seeded groups, number of SMCs in the media layer was less than that in No-EC group on day 4 and day 7, respectively. Scale bars: 50 μm. M: The total bar height represents the total cell number. The total number of cells in the media+intima of intact native vessel was set as 100%. *$p < 0.05$; significantly less number of cells in media+intima than no-EC groups. ($n = 3$–7 for each rat group, one-way ANOVA with Tukey test for multiple comparison).

studies. Here, we show the ability of our bioreactor culture method to validate previous findings, and, also, to isolate the effect of different factors by adding or withdrawing components like flow, pressure, growth factor, or ECs. Rat aortas and human umbilical arteries were injured (via needle application) or gently denuded (with trypsin), and cultured

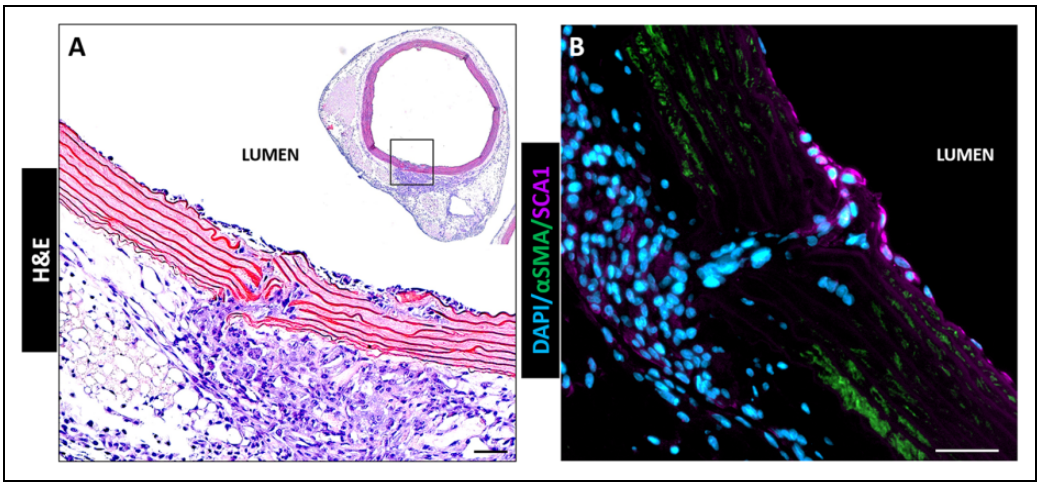

Fig. 8. Medially-injured rat aorta in perfusion bioreactor after 7 days of culture. Adventitial cells migrated through broken vessel wall and invaded luminal surface. The cells invading the lumen stained SCA1 but not αSMA (B). Scale bars: 50 μm.

ex vivo to study the early stages of vessel remodeling after injury. We assessed the effect of arterial flow, as well as exogenously-seeded ECs, on the cellular response to injury in the *ex vivo* bioreactor system.

The vascular injury created by needle application, which mimics wall distention upon stenting or balloon injury, successfully triggered proliferation in the media region of the rat aorta segments within 4 days post injury. Without injury, gentle removal of native ECs did not result in media proliferation, even in the presence of 30% FBS (Supplementary Fig. 3). These observations are consistent with studies reporting that application of high serum or exogenous growth factors is not enough to trigger SMC dedifferentiation and proliferation in vessel segments cultured *ex vivo,* unless medial injury is created[20,48]. Following endothelial denudation *in vivo*, PDGF release from adhered platelets and vascular SMCs acts as a chemoattractant for SMC migration to the lumen[10,20,48,49] but early medial proliferation is caused by bFGF released from injured SMCs and ECs[19]. It has been reported that SMC proliferation in the rat balloon injury model is proportional with the degree of balloon injury, since degradation of arterial heparan sulfate proteoglycans and cyclin-dependent kinase inhibitors surrounding the SMC surface are required for SMCs to enter the cell cycle[16,23,50].

In this study, we also found that the number of $SCA1^+$ cells in the media was significantly higher than that in gently denuded group in the rat vessels. By day 7, we observed neointima-like cell accumulation on the luminal surface of the medially-injured rat aortas. The majority of these cells were $SCA1^+$ and $CD31^+$ and 14.8% were proliferating. Majesky and other investigators have reported that $SCA1^+$ cells are important mediators of vascular repair[24,26,51]. An "outside-in" model of vascular repair suggests that inflammation originates from the adventitia, and moves inward toward the intima[24,26,51,52]. Mesenchymal stem/progenitor cells ($SCA1^+$ /$Gli\text{-}1^+$) residing in the adventitia can migrate into the media 3–4 days after injury, and thence to the intima after 7–14 days[22,51]. However, in our study, the labeled adventitial cells did not migrate through the vessel wall, except leaking through the broken vessel wall. Therefore, $SCA1^+$ cells residing in the medial region might become activated and migrate to the lumen upon injury, and contribute to the restoration of the endothelium, as well as formation of neointima. This observation is in agreement with the recent findings showing that mature SMCs can serve as a source of adventitial SCA1 cells[53]. Formation of neointima 7 days after medial injury has been reported frequently in *in vitro* and *in vivo* studies[19,51,54,55]. In this study, cells in neointima were also $CD31^+$ at 2 weeks following the injury, indicating that SCA1+ cells might assume EC phenotype to repair endothelium[22]. Further research is needed to clarify the factors (such as growth factors) causing $SCA1^+$ cell activation and migration to the luminal surface for vascular remodeling/repair, which could be elucidated using our *ex vivo* bioreactor system. Our observations using the *ex vivo* injury model are very similar to results reported from *in vivo* balloon injury studies.

Physiological shear stress is known to inhibit vascular SMC proliferation and neointimal thickening[56,57]. In this study, when injured rat aortas and human umbilical arteries were cultured under arterial shear stress in the bioreactors, unlike static culture, there was no SMC proliferation or neointima formation 7 days after the injury, we attribute this difference to the effect of shear stress and the cyclic strain on the SMC proliferation. Interestingly, we observed some proliferating SMCs in the media layer in umbilical arteries on day 4. Since rat and human vessels were exposed to similar shear stress, this difference might arise mainly from the difference in the mechanical properties of the vessels (aorta versus umbilical artery), and species. Even for the same species, vessels from different regions of the body would have different mechanotransduction properties, and show

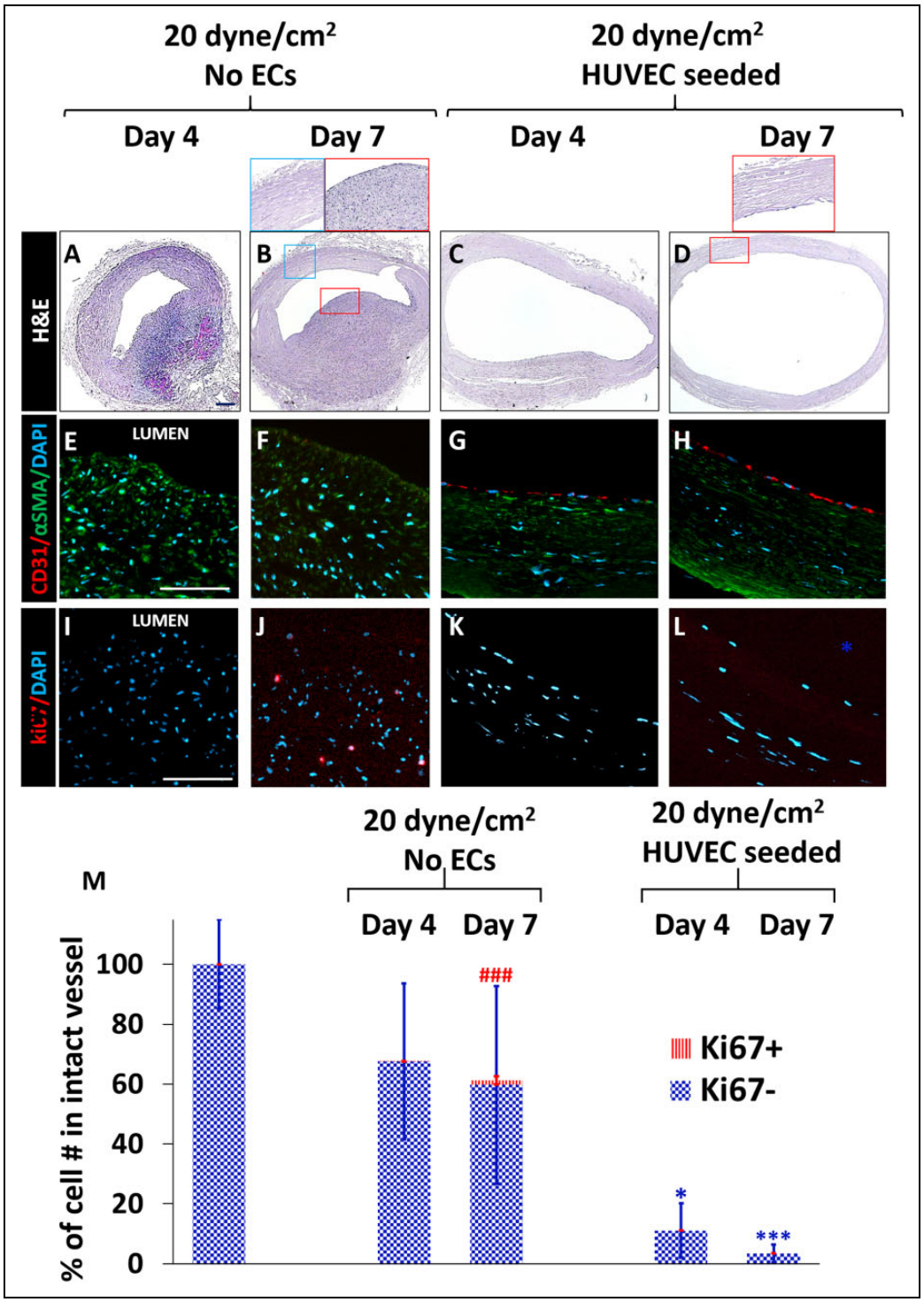

Fig. 9. Injured human umbilical arteries cultured under arterial shear either with or without HUVECs seeded in the lumen for 4 and 7 days. A–D: H&E; E–H: αSMA (green), CD31 (red); I–L: ki67 (red). In the absence of HUVECs, ki67^{+} cells were observed on day 4 and day 7. In the HUVEC-seeded group, similar to the rat aorta, the number of SMCs in the media layer was decreased significantly compared to No-EC group on day 4 ($^{*}p < 0.01$) and Day 7 ($^{***}p < 0.001$), respectively. Scale bars; A–D: 200 μm, E–L: 100 μm. M: The total bar height represents the total cell number. The total number of cells in the media+intima of intact native vessel was set as 100%. $^{\#\#\#}p < 0.0001$; significantly more ki67^{+} cells than other groups in media+intima. ($n = 3$–8, one-way ANOVA with Tukey test for multiple comparison).

different responses to ECs and the applied shear. For instance, the remodeling of human coronary artery and human iliac artery in the same bioreactor settings might be dramatically different[58].

Mature EC or EC progenitor cell transplantation to the injured vessel lumen[27–31], or accelerating re-endothelialization by VEGF or VEGF gene delivery[32,33], may be promising to combat restenosis after balloon injury or in-stent stenosis. To compare the effect of ECs and arterial flow on vascular remodeling, we seeded HUVECs in the lumen of rat aortas and human umbilical arteries immediately after injury. We observed severe SMC death in both rat and human arteries, but dramatic dilation only in umbilical arteries in the presence of HUVECs and arterial shear. In the absence of ECs, SMCs were viable in both species, while proliferating only in umbilical arteries. Interestingly, when HUVEC-seeded and injured vessels were cultured without arterial shear, SMC death was not observed (Supplementary Fig. 4). Also, we did not observe SMC death when we cultured intact or gently-denuded (non-injured) and HUVEC-seeded rat aortas with arterial flow (Supplementary Fig. 5). Neither ECs without flow nor flow without ECs caused SMC death, suggesting a synergistic effect of ECs and the flow/stretch imposed on SMCs of the injured arteries but not of the non-injured arteries, resulting in significant SMC death. It is known that shear stress increases nitric oxide production in ECs[59]. Thus, it can be speculated that cyclic stretch and nitric oxide production enhanced by shear stress on ECs can trigger apoptosis in injured SMCs in a synergistic fashion. Unlike *in vivo* balloon injury models, our vessels in perfusion bioreactors lacked surrounding connective tissue to prevent excessive periodic distension caused by pulsatile arterial flow. It is known that cyclic stretch enhances p53 upregulated modulator of apoptosis (PUMA) protein and gene expression in vascular SMCs[60], and that p53 is activated by nitric oxide[61]. Furthermore, the p53 gene promotes SMC apoptosis in mechanically injured vessels but has no effect on native atherosclerotic vessels[62]. These findings are parallel to our observations that only injured SMCs were sensitive to flow/EC combination. Although elucidation of this mechanism grants further research focusing on nitric oxide production and apoptosis pathways, the present *ex vivo* vessel injury model can be a very useful platform to isolate factors triggering excessive SMC proliferation or apoptosis, since it enables us to separate components such as shear, stretch, endothelium, and soluble factors.

Conclusions

In the present *ex vivo* vessel injury model, we successfully cultured rat and human arteries *ex vivo*, to study the effects of seeded ECs under arterial flow conditions created in perfusion bioreactors. Although our system does not fully represent an ideal model of arterial injury, it was able to validate the well-established findings from previous *in vivo* studies. In addition, we observed interesting vascular response, which revealed the complexity of the problem that was not known before, and opens additional investigations in the future. The present *ex vivo* vessel culture model might be very useful for future studies focusing on the effects of different shear levels, EC types (arterial versus venous), growth factors on remodeling of different vessel types.

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Ethical Approval

This study was approved by the Yale University Institutional Animal Care and Use Committee.

Statement of Human and Animal Rights

All animal care complied with the Guide for the Care and Use of Laboratory Animals. Human tissues and cell populations were obtained using protocols approved by the Yale University Human Investigation Committee, and were discarded tissues.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Disclosures

L.E.N. is a founder and shareholder in Humacyte, Inc., which is a regenerative medicine company. Humacyte produces engineered blood vessels from allogeneic smooth muscle cells for vascular surgery. L.E.N.'s spouse has equity in Humacyte, and L.E.N. serves on Humacyte's Board of Directors. L.E.N. is an inventor on patents that are licensed to Humacyte and that produce royalties for L.E.N. L.E.N. has received an unrestricted research gift to support research in her laboratory at Yale. Humacyte did not influence the conduct, description, or interpretation of the findings in this report. The other authors report no conflicts.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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