Published in final edited form as:

Exp Cell Res. 2019 March 01; 376(1): 58–66. doi:10.1016/j.yexcr.2019.01.011.

Migration versus Proliferation as Contributor to *In Vitro* Wound Healing of Vascular Endothelial and Smooth Muscle Cells

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Abstract

Wound closure, as a result of collective cell growth, is an essential biological response to injury. In the field of vascular biology, the response of vascular smooth muscle cells (SMCs) and endothelial cells (ECs) to injury and substrate surface is important in therapeutic clinical treatment interventions such as angioplasty and atherectomy. Specifically, the mechanism by which cells close wounds (i.e. proliferation versus migration) in response to injury stimuli is of interest to better modulate recurrent vascular stenosis, prevent thrombus formation, occlusion, and life-threatening cardiovascular events. Here, we examine growth extent and temporal sequence of events following wound or gap introduction to a confluent monolayer of vascular SMCs or ECs. Significant differences in the preferred mechanisms of these cells to close wounds or gaps were observed; after 48 hours, 73% of SMC wound closure was observed to be due to proliferation, while 75% of EC wound closure resulted from migration. These mechanisms were further modulated via addition or removal of extracellular matrix substrate and injury, with ECs more responsive to substrate composition and less to injury, in comparison to SMCs. Our results indicate that ECs and SMCs heal wounds differently, and that the time and mode of injury and associated substrate surface all impact this response.

Keywords

migration; proliferation; wound healing; vascular; smooth muscle cells; endothelial cells

Introduction

Atherosclerotic arterial disease is the leading cause of chronic morbidity and mortality in the United States and the Western world [1,2]. Depending upon disease location, it may manifest as arterial narrowing or occlusion, leading to myocardial infarction (heart), stroke (brain), or peripheral claudication (limbs), often requiring urgent intervention to restore

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Declarations

The authors declare they have no competing interests.

arterial patency and blood flow to ischemic tissues [3]. While effective, many of these treatments i.e. percutaneous intervention - angioplasty and stenting, or bypass graft surgery introduce mechanical injury to the arterial wall, leading to aggressive post-injury wound healing and arterial restenosis (re-narrowing) [4–6]. Restenosis results from a complex biological cascade in response to injury. This cascade begins with endothelial denudation, surface thrombosis, and a subsequent inflammatory response, ultimately leading to neointimal thickening with further thrombosis and smooth muscle cell luminal invasion [7–9]. As major cellular constituents of the arterial wall, vascular endothelial cells (ECs) and smooth muscle cells (SMCs) are important targets for improving the response to injury and vessel restenosis prevention [10].

Vascular ECs form the intimal endoluminal lining of the blood vessel and are an important regulatory barrier between blood and the platelet-activating sub-endothelial layer. Disruption of the endothelium via trauma or vascular intervention often injures this layer, and in chronic cases, impairs the ability of ECs to grow and re-establish endothelial continuity [11–14]. Lack of endothelial coverage increases the thrombogenicity of the arterial surface and eliminates the paracrine control effect exerted upon underlying SMCs. Without EC interaction, SMCs invade the vessel lumen, leading to neointimal thickening, arterial narrowing and progressive occlusion [15–17].

In contrast to ECs, vascular SMCs form the multi-layer media of arteries and under healthy conditions exist in a non-proliferative, contractile state. In response to injury, SMCs typically switch from a contractile phenotype to a synthetic, proliferative state, exhibiting increased growth and invasion into the lumen [18–20]. In a seminal study by Clowes and Schwartz, a significant number of post-angioplasty neointimal SMCs were found to be non-doubling SMCs, suggesting that injury-stimulated cell movement into the arterial lumen was achieved via geographic translocation, i.e. migration of SMCs [21]. More recent work has focused on identifying the time course of SMC migration and proliferation, as this relates to signal transduction and exposure to growth factors (i.e. PDGF, IGF) in the blood and subsequent SMC phenotypic switching [19,22]. Extracellular matrix (ECM) production and breakdown by matrix metalloproteinases (MMPs) are an additional important dynamic that influence phenotypic switching and the relation between both SMCs and ECs and their ability to migrate or proliferate [23–26].

As with any tissue repair, wound healing in the vasculature requires a coordinated effort between multiple cell types and extracellular factors. In culture, SMCs typically are highly proliferative with markedly lower doubling time compared to ECs. However, collective sheet growth of cells involves contact inhibition and different dynamics influencing growth, beyond doubling time. Specifically, the relationship between extracellular factors such as substrate or injury stimulation and their influence on vascular cell migration versus proliferation following wounding remains unclear. We previously reported on the development of multiple 2-dimensional *in vitro* assays to study mechanisms involved in collective cell growth of injured and non-injured (i.e. contact inhibition release) vascular cells on multiple substrate surfaces [27]. In the present study, we aim to further identify which reparative mechanism, i.e. migration vs. proliferation, has the greatest impact over time in the wound healing or “filling the gap” process. We hypothesize that migration and

proliferation each have varying temporal mechanistic dominance in the natural history of arterial cell wound healing *in vitro*. Here, we specifically explore the effects of 1) cell injury and 2) substrate surface on the growth state (i.e. migration and proliferation extent) of both vascular ECs and SMCs during a 48-hour gap- or wound-closure process.

Materials and Methods

Human Vascular Cell Culture

Primary human umbilical artery smooth muscle cells (HUASMCs) were purchased from PromoCell (Heidelberg, Germany) and cultured with Smooth Muscle Cell Growth Medium 2TM (PromoCell, Heidelberg, Germany), supplemented with 1% (v/v) antibiotic-antimycotic, 5% (v/v) fetal bovine serum, 0.5 ng/ml EGF, 2 ng/ml bFGF, and 5 µg/ml insulin.

Primary human umbilical vein endothelial cells (HUVECs) were also purchased from PromoCell and cultured with Endothelial Cell Growth Medium 2TM (PromoCell, Heidelberg, Germany), supplemented with 1% (v/v) penicillin-streptomycin, 2% (v/v) fetal bovine serum, 0.4% (v/v) endothelial cell growth supplement, 0.1 ng/ml EGF, 1 ng/ml bFGF, 90 µg/ml heparin, and 1 µg/ml hydrocortisone.

Media were stored at 4°C for use up to 4 weeks. For experimental use, cells were grown to 80% or greater confluency and only HUASMCs between passages 3–10 and HUVECs between passages 3–6 were used for experiments.

Substrate Surface Preparation

NUNC-treated polystyrene 24-well plates were used for all experiments (VWR, Roskilde, Zealand, DK). In experiments where a gelatin surface was used, 25 µl of dextrose-gelatin-veronal solution (600 mg gelatin/L buffer) was coated on each polystyrene well. The solution was dried in a sterile laminar flow hood for 2 hours and was kept at 4°C for a maximum of 1 week until needed.

Cell Growth Assays

Three growth assays (out-growth, in-growth, and scrape wound) were utilized in this study and have been described in detail in Ammann, et al (2015). The cell suspensions utilized for each assay contained vascular ECs or SMCs suspended in respective growth media (100,000 cells/ml). Cell incubation occurred at physiological conditions (37°C at 5% CO₂).

Out-Growth Assay: Pyrex® Cloning Cylinders (Fisher Scientific, Pittsburg, PA, USA) were sterilized via autoclaving and placed under UV light for 1 hour before use with cells. Individual cylinders were placed into the center of each well of a 24-well plate prior to cell seeding. 50 µl of cell suspension was added to the inside of each cylinder and then incubated for 4 hours. Cylinders were then atraumatically lifted out of each well and 0.5 ml fresh media was added to each well. Plates with cells were re-incubated for desired growth time (0, 4, 24, 48 hours).

In-Growth Assay: PDMS in-growth molds were sterilized with 70% (v/v) ethanol in water and then placed under UV light for 1 hour before use with cells. The molds were inserted

into 24 well-plates prior to cell seeding. 500 µl of cell suspension was added to the wells with the inserts placed. The plates with cells and inserts were incubated for 4 hours. PDMS inserts were then atraumatically lifted out of the wells. Cells were re-incubated for desired growth time (0, 4, 24, 48 hours).

Scrape Wound Assay: Wooden applicator sticks were sterilized by autoclave and then placed under UV light for 1 hour before use with cells. Sticks were selected for uniform end flatness; sticks with irregularities were discarded. 0.5 ml of cells suspension solution were seeded into each well of a 24-well plate. Plates with cells were incubated for 4 hours. The cell surface was then vertically scraped with the end of a sterilized applicator stick in direct contact with the plate surface. Cells were re-incubated for desired growth time (0, 4, 24, 48 hours).

After growth with respective assay, plates were rinsed with 1X phosphate buffered saline (PBS), fixed with SafeFix II™ (Fisher Diagnostics, Middletown, VA, USA), rinsed again with 1X PBS, and stained with 0.1% toluidine blue in 1X PBS for imaging.

Cell Proliferation Assay

The EdU Click-iT™ proliferation kit was purchased from Life Technologies (Carlsbad, CA, USA) for quantifying proliferation of cells via fluorescent-tagged nucleoside analog, 5-ethynyl-2'-deoxyuridine (EdU). In brief, cells were incubated with a 10 µM solution of EdU in corresponding full medium for the desired time point of cell growth (4, 24, or 48 hours). After incubation, media was removed, and cells were fixed with 3.7% (v/v) para-formaldehyde in 1X PBS before being blocked with 3% (w/v) bovine serum albumin (BSA), and permeabilized with 0.5% (v/v) Triton X-100 in 1X PBS. Alexa-fluor dye in reaction buffer provided by Life Technologies and added to the cells to bind with EdU. Dye was aspirated, and wells were rinsed with 1 mL of 3% BSA (w/v) in 1X PBS. Immediately before imaging, cells were mounted with fluoroshield with DAPI (Sigma-Aldrich, St. Louis, MI, USA).

Cell Growth Data Analysis

After growth study, cells were imaged with a Zeiss Axiovert 135 microscope at 4X magnification for quantitative analysis. Each image was taken to include the leading edges of the wound area at each growth time point (0, 4, 24, and 48 hours). The extent of growth was measured and calculated by determining the percentage of growth area into the wound or clear zone area after a specified growth period (4, 24, or 48 hours) in relation to an initial starting point at 0 hours growth. The formula for finding the percent growth when using the in-growth assay or the scrape wound assay is given by:

$$\% \text{ Growth} = \left[(A_{\text{initial}} - A_{\text{growth}}) / A_{\text{initial}} \right] \times 100$$

A_{initial} = Initial clear zone area

A_{growth} = Clear zone area after growth

The formula for finding the percent growth when using the non-injury out-growth assay is given by:

$$\% \text{ Growth} = \left[(A_{\text{growth}} - A_{\text{initial}}) / A_{\text{initial}} \right] \times 100$$

A_{initial} = Initial cell area

A_{growth} = Cell area after growth

Areas needed for calculating the percent growth were measured by tracing the leading-edge boundary of growth with ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, US). Percentage of cell growth was necessary for comparison between different assays and cell types. Every assay was performed on both gelatin and polystyrene surfaces to produce at least 9 samples at every time point (0, 4, 24, and 48 hours). Growth percentage comparisons were statistically assessed using Welch's t-tests and considered significantly different for p-values less than 0.05.

Cell Proliferation Data Analysis

After proliferation study, cells were imaged with fluorescence at 360 nm (blue) and 490 nm (green) excitations using a Zeiss Axiovert 135 microscope at 20X magnification. Each image was taken only at the leading edge, where new growth was present. Images were taken after a specified period of proliferation (4, 24, and 48 hours). The extent of proliferation was calculated by counting total number of cells (C_{Total}) in each image and total number of green cells in each image (C_{Prolif}). The formula for finding the percent proliferation for any growth assays is given by:

$$\% \text{ Proliferation} = \left[C_{\text{prolif}} / C_{\text{Total}} \right] \times 100$$

C_{Total} = Total number of cells

C_{Prolif} = Number of proliferating (green) cells

Every assay was performed on both gelatin and polystyrene surfaces to produce at least 9 samples at every time point (4, 24, and 48 hours). Proliferation percentage comparisons were statistically assessed using Welch's t-tests and considered significantly different for p-values less than 0.05. Average proliferation percentages were applied as a fraction to overall average growth to distinguish between the contribution of migration versus proliferation towards growth.

Results

Injury and/or gap formation of vascular cell populations was effectively achieved utilizing the three growth assays methods described [27]. Two assays, out-growth and in-growth, used non-injury methods to create a cell barrier for defined cell growth (Fig. 1a and Fig. 1b). The third assay, scrape wound, introduced injury prior to growth by scraping a surface of confluent cells (Fig. 1c). All three assays created a discrete cell zone, i.e. a starting point,

from which 2-dimensional growth across the surface could be readily measured and normalized to a 0-hour baseline, yielding percent growth.

Collective Cell Growth on Polystyrene

Initially (at 4 hours), the highest overall EC growth on polystyrene was seen with the out-growth assay in comparison to in-growth and scrape wound assays ($p = 0.02$ out-growth vs. in-growth, $p < 0.001$ out-growth vs. scrape wound; Fig. 2a). However, after longer time points, the scrape wound (injury) assay overtook the non-injury assays and showed the most growth on polystyrene after 24 and 48 hours of growth ($p = 0.11$ scrape wound vs. out-growth, $p = 0.19$ scrape wound vs. in-growth; Fig. 2a).

SMC growth extent on polystyrene was similar to EC growth extent at 4 hours but was much higher after 24 and 48 hours ($p < 0.01$; Fig. 2a and Fig. 2b). Growth differences between assays of SMCs were most pronounced at 24 hours when SMC in-growth showed the significantly highest growth at 24 hours, followed by out-growth assay ($p < 0.04$; Fig. 2b). Like ECs, the scrape wound (injury) assay overtook the non-injury assays and showed the highest growth at 48 hours ($p < 0.001$ scrape wound vs. in-growth; Fig. 2b).

Leading Edge Proliferation on Polystyrene

The clearly defined leading edge exhibited during gap closure with all three assays allowed for regions of new growth to be easily identified. At each time point, cells were fluorescently stained, and regions of new growth were targeted for imaging and quantification of migrating (blue) or proliferating (green) cells, as seen in Figure 3a.

Despite different initial EC collective growth between assays, initial EC proliferation at 4 hours was similar and low ($< 10\%$) across all assays. After 48 hours, EC proliferation with the out-growth assay remained low while in-growth and scrape wound assay proliferation was significantly higher ($p < 0.03$; Fig. 3b).

Like SMC collective growth, differences in SMC proliferation between assays were most pronounced at 24 hours. However, while SMC growth was lowest with scrape wound assay at 24 hours, SMC proliferation was highest ($p < 0.0001$; Fig. 3c). Additionally, the trend of SMC collective growth at 48 hours showed scrape wound (injury) exhibited highest growth, this trend was completely opposite for SMC proliferation at 48 hours, suggesting a majority contribution of SMC migration in the injury assay (Fig. 3c).

Contribution of Migration versus Proliferation to Growth on Polystyrene

EC migration on polystyrene remained the most dominant contributor to overall growth across all assays and time points. Migration contributed up to 90% of the wound closure after 4 hours and only went as low as 60% after 48 hours (Fig. 4a–c).

In contrast, SMC migration is dominant for only up to 24 hours of growth on polystyrene for all assays (Fig. 4d–f). This suggests that after 24 hours, most SMC growth on polystyrene is due to proliferation. Additionally, the amount of relative migration between 24 and 48 hours remains the same for the non-injury assay, but increases for the scrape wound assay on polystyrene, suggesting injury is more stimulating to migration specifically (Fig. 4d–f).

Collective Cell Growth on Gelatin

Similar to EC growth on polystyrene, the highest initial (at 4 hours) EC growth on gelatin was seen with the out-growth assay ($p < 0.01$). Unlike on polystyrene, the non-injury assays exhibited significantly higher growth on gelatin after 48 hours ($p < 0.02$). Overall, non-injury EC growth was significantly higher on gelatin than on polystyrene for every time point but was similar for the scrape wound (injury) assay (Fig. 5a).

Unlike ECs, there were no significant differences in growth of SMCs when comparing between gelatin and polystyrene substrates. On gelatin, differences between assays were also most pronounced after 24 hours of growth, with non-injury in-growth assay showing highest SMC growth at 24 hour and 48 hours ($p < 0.005$ at 24 hours, $p < 0.09$ at 48 hours; Fig. 5b).

Leading Edge Proliferation on Gelatin

The cell growth assays were repeated for each time point on gelatin-coated polystyrene. Again, the leading edge was identified and imaged to differentiate between proliferating (green) and migrating (blue) cells, as shown in representative images in Figure 6a.

Despite exhibiting highest EC growth after 48 hours on gelatin, the out-growth assay exhibited significantly lowest proliferation of all assays after 48 hours, suggesting long-term growth was primarily due to migration ($p < 0.002$; Fig. 6b).

Similar to ECs, all three assays exhibited significantly higher SMC proliferation on gelatin than on polystyrene surface after 48 hours. However, at 24 hours only the non-injury assays had significantly higher SMC proliferation on gelatin, while the scrape wound assay showed a similar amount of SMC proliferation regardless of substrate ($p < 0.02$ at 24 hours; Fig. 6c).

Contribution of Migration versus Proliferation to Growth on Gelatin

EC migration on gelatin, like polystyrene, was the dominant contributor to overall growth across all assays and time points. Migration contributed up to 95% of the wound closure after 4 hours and only went as low as 55% after 48 hours (Fig. 7a–c).

In contrast, SMC migration on gelatin was the dominant factor only initially but was overtaken by proliferation at 48 hours for all assays (Fig. 7d–f). Regardless of substrate, migration is the dominant factor with the scrape wound assay only up to 24 hours, suggesting that 24 hours is a critical time point for differences between injury and non-injury SMC growth dynamics (Fig. 7d–f).

Discussion

In this study, we go beyond traditional methods of quantifying wound closure via cell coverage area by elucidating migratory versus proliferative mechanisms during the gap closure process. A main goal of this study was to identify when each mechanism was dominant for both SMCs and ECs throughout wound closure and how these were affected by injury and substrate variables. For both injury and non-injury assays, we find that the initial response of SMCs and ECs is to fill the gap primarily via migration. Over time, however, we observed that proliferation becomes the main contributor for SMCs after 24 hours, while

migration remains dominant throughout the entire 48 hours for EC gap closure. While substrate can affect the overall extent and rate of growth (Fig. 3 and Fig 6), the temporal dynamics of dominant migration versus proliferation between ECs and SMCs remain the same regardless of substrate (Fig. 4 and Fig. 7).

Influence of Injury on Cell Migration versus Proliferation

Our results support previous findings that injury plays a key role in SMC growth dynamics [28]. SMCs exhibited significant migration over proliferation, evidenced by the fact that SMC growth on polystyrene was highest with injury after 48 hours, but SMC proliferation was lowest. However, the relative contribution of SMC proliferation to growth increases more rapidly and more significantly with the injury scrape wound assay compared to non-injury assays. Additionally, we found that 24 hours is the critical time point in detecting differences of contribution of proliferation between the non-injury and injury assays, with the contribution of proliferation near 50% for injury wounds and only 30% for non-injury. This is similar to the *in vivo* results exhibited in the early work of Clowes and Schwartz [21].

Despite EC migration remaining the dominating factor throughout EC growth, we see the same stimulatory effect of injury on EC proliferation on polystyrene. The release of local cellular contents and the loss of contact inhibition via injury effectively create paracrine effects that stimulate growth, especially proliferation, which is evident for ECs on polystyrene [29,30]. While this is contrary to some previous findings that injury can impair EC growth, this is mostly in cases where the wound is too large or chronic to heal, causing ECs to become senescent and further contributing to their dysfunction [12,31]. ECs did not exhibit complete healing or gap closure for any of the assays after 48 hours. This observation raises the possibility that beyond 48 hours they could exhibit slowed or cessation of growth due to increasing senescence.

Influence of Substrate Surface to Cell Migration versus Proliferation

It is well known that substrate is another important contributor to cell motility dynamics. Cell adhesion, migration and proliferation are mediated by adhesion receptors binding to defined sequences in ECM, with this interaction vital for force transmission needed for cell motion [32]. While ECM basement membrane is vital for healthy physiologic endothelium, previous studies have shown that ECM effects on cell functionality are largely based upon ECM density and stiffness [33–35]. ECM at high densities can crowd and hinder EC growth, especially when local SMCs are injured and switch to their ECM-producing synthetic phenotype [36–39]. From our results, addition of gelatin ECM resulted in a significant increase in overall growth for non-wounding assays. This increase however, is largely due to EC proliferation, not migration. These findings suggest that ECM substrate is a key factor in promoting growth via proliferation, but not migration of ECs.

SMCs are less responsive than ECs to the addition of gelatin ECM and caused SMC overall growth to only slightly increase or stay the same compared to the polystyrene substrate. However, the contribution of migration to overall SMC growth increased at 24-hour and 48-hour time points, while proliferation decreased. This finding is reminiscent of previous

studies that showed addition of ECM can slow motility of vascular SMCs, and is again largely dependent upon ECM density and stiffness [40,41]. Therefore, our findings suggest that ECM causes more SMC migration than proliferation, opposite to our findings with ECs. The influence of ECM substrate, however, is much less significant on SMCs than the influence of ECM on ECs.

Clinical Implications

Current arterial revascularization therapies incorporate pharmacologic agents, e.g. paclitaxel or everolimus, with broad effects modulating both cell proliferation and cell migration mechanisms [42,43]. While the addition of these agents to arterial intervention has vastly improved overall restenosis outcomes, these drugs adversely affect EC growth and establishment of a non-thrombogenic endothelial layer, leading to increased rates of late arterial thrombosis and ischemic events [44]. Our *in vitro* results provide additional mechanistic insight into these findings while elucidating the importance of the wounding environment, particularly injury and substrate, on both EC and SMC behavior. Specifically, our findings reveal that significant injury and ECM exposure, as occurs with interventional injury, are stimulating to EC proliferation, but not migration. As such, present administration of agents limiting EC proliferation is unfortunately halting the preferred growth mechanism of ECs to re-establish a continuous non-thrombogenic endothelial layer. In contrast, these same conditions are stimulating to both SMC migration and proliferation, allowing for SMC migration into the lumen, with resultant progressive narrowing. Our results highlight the importance of considering these mechanisms and underscore the need for agents targeting the appropriate mechanisms operative for a given cell type. To further understanding and reinforce the clinical impact, of our findings, examination of the effects of substrate and injury conditions on cells sourced from coronary arteries, or other arterial beds typically subject to atherosclerosis, warrants future investigation. Similarly, while growth behavior was significantly proliferative in culture and in our study, confirmation of the phenotype of both cell types over time (via protein isoform quantification) should be investigated to better understand adhesive and interacting cell behavior, beyond growth dynamics.

Conclusions

Migration and proliferation are both necessary components for wound or gap closure. Effective wound healing in the vasculature, however, relies on stimulation of one cell type (endothelial) and simultaneous inhibition of another (smooth muscle). Understanding both the temporal sequence and the relative contribution of migration and proliferation can inform the development of clinical or pharmaceutical therapies to both minimize SMC invasion of the lumen of the artery post-intervention while re-establishing a thrombo-protective intimal endothelial layer. Wound closure in the vasculature is also dependent the wound environment and its impact on cell type. While injury is stimulatory to both ECs and SMC proliferation, in our study ECM enhances only EC proliferation and SMC migration. This study also highlights the importance of understanding the environment in which cells are growing, and to use this information as well to inform a time-matched, cell-selective therapy to achieve desired therapeutic outcomes.

Study Limitations

Two of the developed assays described in this study (in-growth and scrape wound) were limited by the availability of space, or size of wound. For most cases, this limited space was not an issue, however, 48 hours did provide enough time to achieve 100% wound closure with some SMC experiments. Because of this, the authors would like to note that the SMC growth area may be, on average, higher after 48 hours with SMCs for in-growth and scrape wound assays.

Additionally, the authors acknowledge that many migration experiments in 2-dimensional space are limited by comparability to the actual conditions of the body or blood vessels. We are encouraged that our results are comparable by the fact that our percentages were similar to those presented previously in *in vivo* conditions [45,46]. Adding 3-dimensionality, flow or shear, and co-culturing to our assays is an aspect we are considering for future work to better mimic *in vivo* blood vessel conditions.

Acknowledgements

This work was performed at The Sarver Heart Center at the University of Arizona. The authors would like to acknowledge the NIH Cardiovascular Biomedical Engineering Grant (T32 HL007955) and the Arizona Center for Accelerated Biomedical Innovation (ACABI) for funding support.

Abbreviations

SMC	smooth muscle cell
EC	endothelial cell
ECM	extracellular matrix
PDGF	platelet-derived growth factor
IGF	insulin-like growth factor
bFGF	basic fibroblast growth factor
HUVEC	human umbilical vein endothelial cell
HUASMC	human umbilical artery smooth muscle cell
PBS	phosphate buffered saline
PDMS	polydimethylsiloxane
DAPI	4,6-diamidino-2-phenylindole

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Highlights

- Early wound healing (< 4 hrs) is migration-dominant for both ECs and SMCs
- Late endothelial cell growth (4 hrs) is migration-dominant
- Late smooth muscle cell growth (4 hrs) is proliferation-dominant
- SMC growth impacted by injury over substrate
- EC growth impacted by substrate over injury

Figure 1. Cell Growth Assay Methods.

(A) Out-Growth Assay. (B) In-Growth Assay. (C) Scrape Wound Assay.

Figure 2. Percent Growth of Human EC and SMC on Polystyrene.

(A) Human EC percent growth on polystyrene after 4, 24 and 48 hours with three growth assays. Images depict growth for each assay after 24 hours. (B) Human SMC percent growth on polystyrene after 4, 24 and 48 hours with three growth assays. Growth percentages based on new cell area over starting cell area ($n = 9$). Black scale bar (lower left in images) represents 1 mm. Values represent average ($n = 9$) $\pm$ standard error. * Indicates statistical significance ($p < 0.05$)

Figure 3. Percent Proliferation of Human EC and SMC on Polystyrene.

(A) Representative images of the EdU assay after 4, 24, and 48 hours of ECs (left column) and SMCs (right column). (B) Human EC percent proliferation at the leading edge on polystyrene after 4 hours, 24 hours, and 48 hours with three growth assays. (C) Human SMC percent proliferation at the leading edge on polystyrene after 4 hours, 24 hours, and 48 hours with three growth assays. Growth percentages based on new cell area over starting cell area ($n = 9$). White scale bar (lower right in images) represents 100 μm . Values represent mean $\pm$ standard error. * Indicates statistical significance ($p < 0.05$).

Figure 4. Percent Contribution to Growth of Human EC and SMC on Polystyrene.

(A-C) Percent contribution of migration and proliferation to the overall growth of ECs at 4, 24, and 48 hours on polystyrene with the out-growth assay (A) in-growth assay (B) and scrape wound assay (C). (D-F) Percent contribution of migration and proliferation to the overall growth of SMCs at 4, 24, and 48 hours on polystyrene with the out-growth assay (D) in-growth assay (E) and scrape wound assay (F). Values represent mean percent.

Figure 5. Percent Growth of Human EC and SMC on Gelatin.

(A) Human EC percent growth on gelatin-coated polystyrene after 4, 24 and 48 hours with three growth assays. Images depict growth for each assay after 24 hours. (B) Human SMC percent growth on gelatin-coated polystyrene after 4, 24 and 48 hours with three growth assays. Growth percentages based on new cell area over starting cell area ($n = 9$). Black scale bar (lower left in images) represents 1 mm. Values represent average ($n = 9$) $\pm$ standard error. * Indicates statistical significance ($p < 0.05$)

Figure 6. Percent Proliferation of Human EC and SMC on Gelatin.

(A) Representative images of the EdU assay after 4, 24, and 48 hours of ECs (left column) and SMCs (right column). (B) Human EC percent proliferation at the leading edge on gelatin-coated polystyrene after 4 hours, 24 hours, and 48 hours with three growth assays. (C) Human SMC percent proliferation at the leading edge on gelatin-coated polystyrene after 4 hours, 24 hours, and 48 hours with three growth assays. Growth percentages based on new cell area over starting cell area ($n = 9$). White scale bar (lower right in images) represents 100 μ m. Values represent mean $\pm$ standard error. * Indicates statistical significance ($p < 0.05$)

Figure 7. Percent Contribution to Growth of Human EC and SMC on Gelatin .

(A-C) Percent contribution of migration and proliferation to the overall growth of ECs at 4, 24, and 48 hours on gelatin-coated polystyrene with the out-growth assay (A) in-growth assay (B) and scrape wound assay (C). (D-F) Percent contribution of migration and proliferation to the overall growth of SMCs at 4, 24, and 48 hours on gelatin-coated polystyrene with the out-growth assay (D) in-growth assay (E) and scrape wound assay (F). Values represent mean percent.