Schwann cells promote endothelial cell migration

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Abbreviations: HUVECs, human umbilical vein endothelial cells; rSCs, rat Schwann cells; ECs, endothelial cells; SCs, Schwann cells; IL-8, interleukin-8; VEGF, vascular endothelial growth factor; FAK, focal adhesion kinase; MAPKAPK13, mitogen-activated protein kinase-activated protein kinase 13; ECM, extracellular matrix; VCL, vinculin; PROF, profilin; FGFR3, fibroblasts growth factor receptor-3; CDC42, cell division control 42; PLGA, poly(lactic-co-glycolic acid).

Directed cell migration is a crucial orchestrated process in embryonic development, wound healing, and immune response. The underlying substrate can provide physical and/or chemical cues that promote directed cell migration. Here, using electrospinning we developed substrates of aligned poly(lactic-co-glycolic acid) nanofibres to study the influence of glial cells on endothelial cells (ECs) in a 3-dimensional (3D) co-culture model. ECs build blood vessels and regulate their plasticity in coordination with neurons. Likewise, neurons construct nerves and regulate their circuits in coordination with ECs. In our model, the neuro-vascular cross-talk was assessed using a direct co-culture model of human umbilical vein endothelial cells (HUVECs) and rat Schwann cells (rSCs). The effect of rSCs on ECs behavior was demonstrated by earlier and higher velocity values and genetic expression profiles different of those of HUVECs when seeded alone. We observed 2 different gene expression trends in the co-culture models: (i) a later gene expression of angiogenic factors, such as interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF), and (ii) an higher gene expression of genes involved in actin filaments rearrangement, such as focal adhesion kinase (FAK), Mitogen-activated protein kinase-activated protein kinase 13 (MAPKAPK13), Vinculin (VCL), and Profilin (PROF). These results suggested that the higher ECs migration is mainly due to proteins involved in the actin filaments rearrangement and in the directed cell migration rather than the effect of angiogenic factors. This co-culture model provides an approach to enlighten the neurovascular interactions, with particular focus on endothelial cell migration.

Introduction

During the development stage, blood vessels are formed in a closely coordinated manner with nerves, constructing closed circuits of neurovascular networks.¹–³ The vascular and neural systems mutually regulate their plasticity and affect pattern formation of their networks.²,⁴ In response to their local need, blood vessels can modulate neuronal activities, and vice versa, nerves can guide changes in blood vessel patterns by branching, expanding, and pruning. The development of the neural and vascular systems occurs in similar and parallel patterns through branching, coupling, and guidance within target tissues.² The anatomical similarities and parallelism between the 2 systems are evident and likely due to the remarkable degree of cellular coordination, resulting in the development and maintenance of blood vessels and nerves as a functional neurovascular network.²,³ ECs, neural, and glial cells respond to changing environment producing a wide panel of growth factors and their receptors, including angiogenic factors. These protein pairs transduce cellular information causing ECs and neural cells to proliferate, migrate and differentiate.³ For example, neural cells are known to express angiogenic factors, such as VEGF that induces proliferation of ECs through its receptors. Conversely, ECs produce brain-derived neurotrophic factor (BDNF) that stimulate neurite outgrowth.⁴ Other angiogenic factors generated from neurons and glia have been documented to affect ECs and neuronal cells behavior (Table 1).⁵,⁶ With cell migration being a central step in angiogenesis, here we tailored aligned fibrous substrates that potentiate directed ECs migration.⁷,⁸ Directional control of cell migration is critical to developmental morphogenesis and tissue homeostasis.⁹ Several observations suggest that cells are capable of responding to mechanical and/or chemical cues from the substrates, by exerting contractile forces and then interpreting the substrate deformation to determine a preferred direction of their movement.¹⁰–¹³ Schwann cells (SCs) are the main glia of the peripheral nervous system, their processes are important for the elongation and guidance of regenerating axons¹⁴ and are known to produce neurotrophic factors and their receptors.¹⁵ Gerber et al.⁴ and Mukouyama et al.¹⁶ showed that VEGF-related factors derived from SCs can induce arterial differentiation in ECs precursors, suggesting that peripheral nerves determine the pattern of blood...
vessel branching and arterial differentiation. On the other hand, VEGF prolongs the survival and stimulates the proliferation of SCs in explant cultures of superior cervical and dorsal root ganglia. Gerber et al. and Bates et al. suggested that neuronal cells can induce ECs into arteriole differentiation and promote a specific vascular pattern. The crosstalk between SCs and ECs has been assessed, in most cases, by the influence of SCs in neuroblastoma angiogenesis. Huang et al. showed that conditioned medium from SCs, derived from either adult nerve tissue or stroma-dominant neuroblastoma tumor tissue, inhibits ECs proliferation and migration. Other studies also showed the production of inhibitors of angiogenesis such as TIMP-2, by SCs. More recent studies, using co-culture models of glioblastoma and ECs, suggested an increased activity of metalloproteinases (MMP-9) involved in vascular cell migration and invasion. Glioblastomas are known to produce large amounts of basic fibroblast growth factor and VEGF, which may act to mediate the paracrine control of angiogenesis.

| Factors | Effects on ECs | References |
|---------|----------------|------------|
| IL8 | Induction of angiogenesis, EC survival, differentiation | 29 |
| FGFR3 | FGF-b receptor (induces EC migration, proliferation and angiogenesis) | 31, 4, 30, 58 |
| VEGF | EC Survival, proliferation, and migration. Inhibit apoptosis | 4, 30, 58 |
| VCL | Strengthening the adhesions between cells and ECM | 32 |
| PROF | Dynamic turn-over and restructuring of the actin cytoskeleton | 59 |
| MAPK | Induces directed cell migration | 35 |
| FAK | Promote cell migration and angiogenesis. Focal adhesion turn-over. | 33, 34 |
| CDC42 | Regulates signaling pathways involved in cell morphology and migration. Actin polymerization | 51, 60 |

It is well established that, in the living system, the extracellular matrix (ECM) microenvironmental properties can directly influence the intracellular regulatory mechanisms that govern the migratory phenotype and determine how cell migration proceeds. It was reported that the cells attach and organize well around fibers with diameters smaller than the diameter of the cells. Therefore, to create an ideal scaffold which serves as an artificial ECM for tissue regeneration, it is crucial to replicate the dimensions of natural ECM. When cells are seeded on fibrous substrates, the fiber diameter plays an important role in cell morphology, particularly in cell polarization. The fabricated scaffolds were characterized in terms of their morphology and fiber diameter, which were mainly dependent on the polymer solution, flow rate, concentration, solvent used, and applied electric field. The relationship between the applied voltage and the formation of polymer fibers with a consistent morphology has already been extensively reported by previous studies. As shown in Figure 2A, the produced substrates exhibited a good fiber alignment. The average fiber diameter was 473 ± 111 nm (addressed as electrospun (ESP) fibers onwards, Fig. 2B).

**Cell morphology**

The circularity value gives us an idea about how polarized the cells are. Values close to 0 suggest an elongated cell; values close to 1 suggest a circular cell. Here, HUVECs seeded on fibrous substrates exhibited lower values (0.30 ± 0.12) for circularity when compared with cells seeded on coverslips (0.43 ± 0.15), suggesting a more polarized and elongated morphology in those fibrous substrates (Fig. 2C). Using aligned fibers we expected the cells to exhibit a higher alignment in accordance with the substrate underneath. As shown by the Rose plot diagram the majority of cells exhibited a confined alignment between 30 and 90 degrees (Fig. 2D and F). In contrast when seeded on coverslips, cells exhibited a scattered alignment in several directions (Fig. 2E and G).

**Cell growth on scaffolds – cell viability**

Cell viability was determined using a PrestoBlue® assay. As shown in Figure 3A, rSCs exhibited higher fluorescence values over time, with fluorescence increasing at higher rates (higher values of slope, \( m = 67690 \)) when compared with HUVECs (\( m = 25280 \)) seeded in single culture models. Besides their viability, an interesting correlation that can be made is the influence of cell viability on cell migration. As illustrated in Figure 3B, the percentage of covered area by HUVECs in single culture models seemed not to vary significantly with cell viability: low value of slope (\( m = -4.10^{-6} \)), not significantly different from zero (\( p = 0.45 \)), and negligible value of \( r^2 \) (0.071) suggested a poor dependence between cell viability and covered area. In contrast, HUVECs seeded on coverslips exhibited higher values of slope (\( m = 1.210^{-5} \)), significantly different from zero (\( p < 0.0001 \)), and higher values of \( r^2 \) (0.72), suggesting a higher dependence between cell viability and covered area (cell migration).

**Effect of rSCs on HUVECs migration**

It is well established that there is a cross-talk between the neural and vascular systems. Besides anatomical similarities, there are several angiogenic factors/receptors that affect both systems. Few studies have particularly focused on the role of IL-8 and VEGF. However, to the best of our knowledge, none of...
previous studies have addressed how SCs affect the migration and the gene expression profile of ECs. Here, in order to assess the rSCs influence on HUVECs’ migration, 4 co-culture ratios were chosen, namely 0%, 5%, 10%, 20% of rSCs relatively to HUVECs (rSCs:HUVECs), using a PLGA 50:50 nanofibrillar substrate, with fibers having an approximate diameter of 500 nm.

The covered area represents the displacement of the cell sheet to close the wound. The covered area after 48 hours changed from $1.12 \pm 0.035 \text{ mm}^2$ to $1.64 \pm 0.19 \text{ mm}^2$, when increasing the co-culture ratio from 0% to 5%, to decrease again to $1.13 \pm 0.35 \text{ mm}^2$ when the co-culture ratio was further increased to 20%. The covered area over time for 5% co-culture ratio was always higher than the other culture conditions, though not statistically different ($p > 0.05$, exception made at 24 hours where $p < 0.05$ for 5% versus 10%) (Fig. 4A).

We observed that the average velocity of the cell front varied with the ratio rSCs:HUVECs, decreasing over time, with the highest value measured after 2 hours in all culture models: $9.54 \pm 0.1 \mu\text{m/h}$, $54.5 \pm 15.74 \mu\text{m/h}$, $22.17 \pm 9.44 \mu\text{m/h}$, $9.89 \pm 0.48 \mu\text{m/h}$ for 0%, 5%, 10%, 20% co-culture ratios respectively. The 5% rSCs:HUVECs ratio clearly increased the initial velocity of HUVECs. Cell migration speed in this condition was statistically different when compared to almost all migration speed values in the other culture models, with exception made for the 10% rSCs:HUVECs ratio. When seeded alone, the speed of HUVECs migration increased until 12 hours, when it reached a peak of $15.29 \pm 2.71 \mu\text{m/h}$ (Fig. 4B). The displacement of the cell sheet is in accordance with results above stated, with the 5% ratio exhibiting higher values of displacement over time (Fig. 4C).

**Gene expression – qPCR**

From a wide panel of angiogenic factors/receptors, we selected 3 well-known cell migration inducers (IL-8, FGFR3, and
VEGF\textsuperscript{29-31}; 2 proteins known to be involved in actin filaments dynamic turnover (PROF) and in linkage of integrin adhesion molecules to the actin cytoskeleton (VCL)\textsuperscript{32}; 2 protein kinases (FAK and MAPK) and a small GTPase of the Rho-subfamily that control diverse cellular functions including cell morphology and migration, as well as FAK phosphorylation (CDC42).\textsuperscript{33-35}

The gene expression of different cell migration effectors was analyzed using 5% rSCs:HUVECs co-culture condition compared to HUVECs alone, in the already stated scaffolds.

**Angiogenic factors**

The genetic analysis results for IL-8 showed a delay of 10 hours in the peak of gene expression in the co-culture model. In both models, the peak (7 and 8-fold increase in co-culture at 2 hours and single culture models at 12 hours, respectively) was followed by a decrease in fold induction up to 36 hours, when a slightly up-regulation was observed until 48 hours that corresponded to 3 and 2 fold increase in co-culture and single culture models, respectively (Fig. 5).

Regarding FGFR3, a similar trend was found for HUVECs cultured alone when compared to IL-8 gene expression, with an increase in gene expression until 2 hours (4 fold increase) followed by a decrease in gene expression until 48 hours. However, in the co-culture condition no significant changes in the FGFR3 could be appreciated (Fig. 5).

The co-culture model appeared to induce VEGF expression at earlier time points when compared to single culture models. In both culture models, a peak at 12 hours in the co-culture condition (2 fold increase) and at 24 hours in the single culture condition (2 fold increase) was measured, followed by a decrease in gene expression until 48 hours (Fig. 5).
Actin filaments rearrangement

The VCL and PROF genes exhibited a similar trend for gene expression. A slight upregulation was verified at 2 hours in VCL gene expression, followed by a continuous decrease in gene expression for single culture conditions until 48 hours. In co-culture models, a small upregulation of VCL (2 fold increase) was followed by a relative constant gene expression until 24 hours. Whereas, a continuous up-regulation was exhibited up to 36 hours in PROF gene expression. In both gene expression profiles a considerable peak (3 fold increase and 2 fold increase in VCL and PROF respectively, \( p < 0.05 \)) at 36 hours was verified in the co-culture conditions (Fig. 6).

Protein kinases

MAPK displayed a down regulation in single culture models after 0 hours followed by a plateau with minor oscillations in gene expression. Co-culture conditions exhibited a progressive statistically significant upregulation over time, peaking at 36 hours (7 fold increase) and followed by a down-regulation in gene expression until 48 hours (Fig. 7). FAK expression was continuously down-regulated in single culture condition up to 36 hours,

Figure 3. Analysis of cell viability by PrestoBlue. (A) Each single culture model was individually tested for their viability potential. rSCs have a higher viability rate when compared to HUVECs. (B) For each time-point used in the migration studies the viability potential of the HUVECs single culture model was measured. Viability is plotted against the covered area. The percentage of covered area in fibrous substrates is mainly due to cell migration rather than increased cell viability. (mean ± SD, \( n = 3 \), linear regression). ESP: electrospun.

Figure 4. Effect of rSCs on HUVECs migration. Co-culture models with low ratios of rSCs have a positive effect on HUVECs migration, with increased percentage of covered area over time and higher values of cell migration speed. (A) Wound closure over time. The 5% ratio is the one that showed a higher covered area over time, with higher displacement of the cell sheet (One-way ANOVA, mean ± SD, \( n \geq 2 \), \( \# p < 0.05 \) (5% versus 10 %)). (B) Velocity profile of HUVECs using several rSCs:HUVECs ratios (One-way ANOVA, mean ± SD, \( n \geq 2 \), second order polynomial (quadratic), \( \# p < 0.001 \) (5% vs. 20 %), \( \# p < 0.05 \) (0% versus 20 % and 0% vs. 20 %), \( \# p < 0.05 \) (10% versus 20 %)). (C) Results of the modified scratch wound healing assay; each color represent a different tissue that software will assume and measure as a cell sheet or single cell. The dashed gray vertical line represents the width of the initial gap. Scale bar 1 mm.
followed by a slight increase in gene expression. In co-culture conditions, the initial downregulation until 24 hours was rapidly counterbalanced. FAK expression was then constantly increased up to 48 hours (2 fold increase) (Fig. 7).

Regarding CDC42 there was a slight down regulation followed by a plateau over time, with minor oscillations in both culture models (Fig. 7).

Discussion

HUVEC morphology

Cell migration is usually initiated with cell morphology rearrangements in response to extracellular cues, including physical (mechanotaxis) and/or chemical (chemotaxis) cues from the extracellular matrix, and/or diffusible factors and signals from neighboring cells. Among all the extracellular cues, the fiber diameter of the underneath substrate seems to play a crucial role on cell morphology. The effect of cell morphology on cell migration has been extensively studied. The first step in cell migration is the achievement of direction through the establishment of cell polarity.

When seeded in constraint-free surfaces, the cells exhibited multiple lateral lamellipodia, leading to an area augmentation, and subsequently an increase in cell circularity. In contrast, cell association with aligned fibers seems to provide an important physical cue to initiate cell polarization, by regulating cell shape and orientation of cellular organelles, which results in a decreased cell circularity. Consequently, the fiber alignment per se is a geometrical cue that influences the cell alignment into a restricted direction, the fiber direction.

Cell growth on scaffolds – cell viability

Cell viability in polymeric substrates is a crucial parameter that was taken into account in the process of scaffold manufacturing. As shown in Figure 3B, the correlation between cell viability (an indirect count of cell number) and cell migration seems to be poorer when cells were seeded in ESP fibers ($r^2=0.071$). Consequently, the covered area observed on our substrates seemed to be caused mainly by cell migration and not due to an increase in cell number, suggesting that the migration speed was enhanced by the directionality in aligned substrates, as already shown by

![Figure 5](image_url)

*Figure 5. Influence of rSCs on angiogenic factors' gene expression expressed by HUVECs. The later up-regulation of IL-8 and VEGF in co-culture models seems to be insufficient to decrease HUVECs migration, being masked by significant higher expression of other genes involved in the actin filament rearrangement. PLGA 50:50 ESP fibers used as substrate. (Fold increase was calculated using $\Delta\Delta Ct$ method, mean $\pm$ SD, n = 3, One-way ANOVA Tukey’s multiple comparison Test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, comparisons between single culture model vs. co-culture model).*

![Figure 6](image_url)

*Figure 6. Influence of rSCs on actin turnover proteins' gene expression expressed by HUVECs. In both graphics is exhibited a constant increase in gene expression in the co-culture model, peaking at 36 hours, suggesting a higher actin filaments rearrangement in those culture models. The setup used was the same as previously stated (Fold increase calculated using $\Delta\Delta Ct$ method, mean $\pm$ SD, n = 3, One-way ANOVA Tukey's multiple comparison Test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, comparisons between single culture model versus co-culture model).*
Effect of rSCs on HUVECs migration

Recent studies demonstrated a positive effect of neural cells on ECs in co-culture. Ford et al. showed that ECs and neural progenitor cells (NPCs) together, showed a 3 to 5-fold increase in the number of functional blood vessels. Using the same culture model, Li et al. showed that NPCs play an important role in forming stable blood vessels. Some investigations attempted to correlate the influence of neural cells on angiogenesis or the effect of ECs on neurogenesis.

In our co-culture 3D models, the covered area in 5% ratios samples after 48 hours was superior to single HUVECs culture, suggesting a positive effect of glial cells on ECs migration. Interestingly, in the 5% ratio condition an increase in the initial HUVECs migration speed could also be measured. The rSCs exhibited a higher proliferative rate when compared with HUVECs, which could lead to an overgrowth of glial cells over endothelial cells, reducing the space for endothelial cells to migrate. This could lead to the observed decreased value in migration speed of HUVECs when seeded together with high ratios of rSCs.

Among the genes analyzed, IL-8, FGFR3, and VEGF are known to be a potent angiogenic factors, implicated in EC proliferation and migration both in vivo and in vitro. Thus, their up-regulation at early time points might act as a trigger signal to cells to start migrating. Furthermore, it has been shown that VEGF in mitogenic concentrations induced stress fiber formation and recruitment of vinculin to focal adhesions in a pattern that is typical of migrating ECs, where well-formed focal adhesions and stress fibers aligned in the direction of migration were described.

The complex formed by vinculin and profilin will serve as an anchorage point to actin filaments. The peak observed at 36 hours suggested the formation of strong focal adhesion complexes and, therefore, the end of cell migration. In fact, the recruitment of vinculin to focal adhesion sites has been shown to have a role in strengthening the adhesion between cells and ECM, while reduction of adhesion and increased migration occur with down-regulation of vinculin. We did not expect downregulation of PROF at earlier time points, since during cell migration the turn-over and the restriction of the actin cytoskeleton are constant.

Rousseau et al. showed that MAPK is not an obligatory regulator of cell migration, but seems to be associated with the directionality of cell migration. Other studies showed that MAPKs regulate the expression of metalloproteinases. Those proteins are crucial for the cleavage of basement membrane and subsequent EC migration. The increased upregulation in co-culture models suggests a more directed cell migration toward the denuded gap, resulting in cells completely closing the gap and stop migrating earlier in these co-culture models. Here, at early time points and mainly in co-culture models, we observed a slight up regulation of FAK suggesting an increased focal adhesion turnover. Similarly, after 36 hours, when the gap closure was nearly completed, an increase in FAK gene expression was measured, suggesting an increase in focal adhesion turnover, yet in this case in order to form strong and mature adhesion complexes.

Figure 7. Influence of rSCs on protein kinases’ gene expression expressed by HUVECs. The constant upregulation of these protein kinases suggested an increased substrate phosphorylation and an enhanced directed cell migration in co-culture models. The setup used was the same as previously stated. (Fold increase calculated using \( \Delta\Delta Ct \) method, mean ± SD, n = 3, One-way ANOVA Tukey’s multiple comparison Test, * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \), comparisons between single culture model vs. co-culture model). Note: non-significant differences were observed for FAK between 48 hours versus 2 hours.
earlier time points (2 hours). Another interesting result is the constant increased gene expression of Profilin, and MAPK in coculture models, in contrast with a continuous decrease over time in single culture models. The increased gene expression of Profilin, FAK, VCL and MAPK suggest a pronounced turn-over in actin filaments rearrangements, intrinsic in cell migration. In particular, FAK and MAPK activity suggested an increase in substrate phosphorylation, with an enhanced phosphate group transfer, which will enhance not only the cell migration but the directed cell migration as well.

### Materials and Methods

#### Scaffold fabrication

Scaffolds were manufactured from poly(lactic-co-glycolic acid) with 50:50 ratio between lactic and glycolic acid segments (PLGA 50:50, cat. Number PURASORB PDLG 5002, Corbion Pur Biomaterials). The polymer was dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP, cat. Number 8335101, BioSolve BV) overnight at a final concentration of 25% (wt/v) and then electrosprun using a standard electrospinning apparatus in a chamber with environmental control (25°C, 30% humidity) using a parallel plate collector to obtain aligned fibers.

The electrosprun fibers started to occur around 15 kV, but a more stable jet appeared to be at 17.5 kV. Increasing the voltage resulted on higher electrostatic forces on the apparatus and instability of the jet, which would slip and splay. The parameter to adjust the voltage was based on the instability of the Taylor cone. The advantage of using this technique lies in the simplicity of the setup and the ease of collecting single fibers. The air gap between the electrodes created residual electrostatic repulsion between the spun fibers, which helped the alignment of the fibers.

For all the experiments, the voltage was kept constant at 17.5 kV, the flow rate 1.5 mL/h, and the polymer was pushed out the syringe for 12 minutes. A 14 mm diameter coverslip (thickness 0.13–0.16mm, cat. Number: GG-14-pll, neuVitro) was used as a collector.

#### Scaffold characterization

Scaffolds fiber morphology and diameter were characterized by scanning electron microscopy (SEM) (Philips XL 30 ESEM-FEM). Samples were attached to stubs using double sided carbon tape and gold sputter coated before acquiring images. The fiber diameter was calculated from 12 images using a semi-automated script form ImageJ (National Institutes of Health, Bethesda, MD). Briefly, a Li threshold method was applied to the images followed by the identification of distinctly segmented fibers by the user. The diameter was approximated by creating a selection of the identified fiber length using the Level Set segmentation plugin, following by manual check and adjustment to ensure good approximation of the fiber diameter.

#### Cell culture

Primary human umbilical vein endothelial cells (HUVECs) (cat. Number: C2517A, Lonza, passage 4–8) were cultured in endothelial growth medium (EGM, cat. Number: CC-3162), which consisted of endothelial basic medium supplemented with (%/v/v): 2% foetal bovine serum (FBS), 0.04 % hydrocortisone, 0.4% human fibroblasts growth factor B (hFGFB), 0.1% VEGF, 0.1% R3-Insulin-like Growth Factor-I (R3-IGF-1), 0.1% ascorbic acid, 0.1% human endothelial growth factor (hEGF), 0.1% Gentamicin/Amphotericin-B (GA-1000), and 0.1% heparin (all from Lonza).

Rat Schwann cells (rSCs), cell line RT4-D6P2T (ATCC), were cultured in Dulbecco’s Modified Eagle Medium (DMEM, cat. Number: 41966–029) high glucose supplemented with (%/v/v) 10% FBS (heat inactivated, cat. Number 10500–064, Life Technologies) and 1% Pen/Strep (Life technologiesTM). Cells were grown at 37°C in a humid atmosphere with 5% CO2. The medium was refreshed every other day.

Previously sterilized scaffolds (soaked in 70% ethanol for 2 hours) were incubated in cell culture medium overnight. After media removal, a physical metal barrier was placed in the center of the scaffold, in order to create a gap for the cells to migrate into, and held using O-rings (Eriks, The Netherlands). Cells were then seeded at 2×10^5 cells/cm^2 and returned to the incubator. Upon reaching confluence, the physical barrier was removed using sterile tweezers and the samples washed thrice with Phosphate buffered saline solution (PBS, cat. Number: 10010–023, Life Technologies). Fresh medium was added.

#### Co-culture

In order to evaluate the effect of rSCs on HUVECs’ behavior and cell migration, a direct contact co-culture model was created using 3 cell ratios: 5%, 10% and 20% (%rSCs). The used medium ratio depended on cell ratio, where for example for a 5% cell ratio a correspondent 95%:5% HUVEC:rSC media (%/v/v) ratio was used.

#### Imaging platform

The developed imaging platform is shown in Figure 1. Briefly, after staining with cell tracker and imaging, the original images were cropped in order to all have the same width and height (Fig. 1A). The cropped images were then converted into gray scale images (Fig. 1B). The images were submitted to a blurred Gaussian filter twice (Fig. 1C). The 4th step was the identification of primary objects (i.e. objects that were assumed as tissue) and measurements (Fig. 1D). For this purpose, we used a 3-class thresholding method (Otsu global) in order to: (i) distinguish the background from the foreground and (ii) overcome the issue that the intensity pixel value varies throughout the image. The different colors mean different tissues that have been identified as foreground. The quantified area was the total area of all different tissues. The last 2 steps are the outlining of the identified primary objects (Fig. 1E), and the overlay of that outlined image with the original cropped image. All the steps were done in an automated way.

Figure S1A shows the cell sheet displacement over time prior to the analysis. As the cells move onwards, the area of the denuded gap (green) is measured. From the difference between the gaps at different times resulted 2 new sections (blue). The
area of these sections is summed and then divided by the height of the image and by the interval of time between 2 consecutive images, obtaining the cell velocity (Figure S1B).

Imaging platform – technical specifications

Three classes of thresholding allow defining 3 categories: foreground, background and a middle intensity between the 2. The middle intensity class will be added to the foreground in order to generate the final 2-class output. This thresholding method allows assigning a value to the foreground or background as desired. The threshold value ranges from 0 to 1 (foreground/background). This is a safety precaution when the threshold is calculated automatically, by overriding the automatic threshold. The low value suggests high differences between foreground and background.

A smoothing filter is used only when distinguishing between clumped objects is required. Low values should be used if many objects are merged together that ought to be separated. When too many objects, that have to be merged, are separated the values should be higher, as seen in our studies. Using the value 1, we "transform" the cell layers on each side of the gap as a cell sheet, whose migration would be only assessed toward the denuded gap as a cell sheet, whose migration would be only assessed toward the denuded gap. The following equations were used to assess the percentage of covered area (equation 1) and cell migration velocity (equation 2):

\[ A_0 - A_i \]  \hspace{1cm} (1)

\[ \frac{A_0 - A_i}{h x t} \]  \hspace{1cm} (2)

Where \( A_0 \) is the area of the initial gap, \( A_i \) is the area of the gap after \( i \) hours, \( h \) is the height of the image, and \( t \) is the time occurred between \( A_0 \) and \( A_i \).

Migration assay

Among several cell tracers, Cell Tracker™ Green CMFDA has extensively been used in studies of HUVECs’ live cell tracking. Moreover, the cytotoxicity of these probes have been studied by the supplier in many cell types, including HUVECs, which appears to exert a minimal impact on several cell processes including cell proliferation and motility.

Only HUVECs were treated with 10 \( \mu \)M Cell Tracker™ Green CMFDA (Molecular Probes®, cat. Number: C7025) for 45 minutes at 37 °C. When stained, rSCs and ECs were cultured together, according to the previously mentioned ratios, at a total density 2 \( \times 10^4 \) cells/cm². When the physical barrier was removed, 3 images of each sample were taken (EVOS® XL microscope with 3MP CMOS color camera, cat. Number AME3300, with GFP LED light cube ex.470/22 EM: 525/50 nm). The images were then analyzed using a basic Cell Profiler (Broad Institute) pipeline, developed by the authors.

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Viability assay

Cell viability was assessed using PrestoBlue® assay according to the manufacturer’s protocol (Life Technologies, cat. Number: A-13262). Briefly, 10% (v/v) of PrestoBlue® reagent was added in each well and the samples (n = 3) incubated at 37 °C for 2 hours. Three 100 \( \mu \)L media samples were taken from each well into a Nunc™ 96-well plate (Thermo Scientific, cat. Number: 267350). Fluorescence was measured at 540–570 nm excitation 580–610 nm emission in VICTOR3™ 1420 Multilabel Counter (PerkinElmer). The readout from the scaffolds was corrected with a blank (medium plus PrestoBlue® reagent).

PCR

To analyze the expression of migration markers by HUVECs, the total RNA was isolated using a combination of the TRIzol® method with the NucleoSpin® RNA II isolation kit (Bioké, cat. Number 740955-50). Briefly, scaffolds (n = 3) were washed once with PBS and 500 \( \mu \)l of TRIzol reagent (Invitrogen, cat. Number: 15596026) was added to the samples. After chloroform addition (200 \( \mu \)l) and phase separation by centrifugation (15 mins, 12000g, 4°C), the aqueous phase containing the RNA was collected, mixed with 250 \( \mu \)l of 70% ethanol and loaded onto the RNA binding column of the kit. Subsequent steps were done in accordance with the manufacturer’s protocol. RNA was collected in RNase-free water. The quantity and quality of RNA was analyzed using an ND100 spectrophotometer (Nanodrop technologies). 139.5 ng of RNA were used for first strand cDNA synthesis using iScript (Bio-Rad) according to the manufacturer’s protocol. One \( \mu \)l of undiluted cDNA was used for subsequent analysis. PCR was performed in an iQ5 real time PCR machine (Bio-Rad) using SYBR Green supermix (Bio-Rad) and fold increase calculated using \( \Delta\Delta Ct \) method.57 Primer sequences are shown in Table 2.

| Gene       | Sequence                      |
|------------|-------------------------------|
| Profilin-1 | Forward: TCAAGTTTTTACGTGAATGGGCT  | Reverse: CGAAGATCCATGCTAAATTCGCC |
| MAPKAPK3   | Forward: AGATAATTGGGATATTTGGCAC | Reverse: TGTGATAGATGGTTTTCAGGCT     |
| IL8        | Forward: TTTGCAACAGGAGTCTGAAGA | Reverse: AAACCTTCTGACCCAGTTTCC       |
| FAK        | Forward: GCTTACCTGAGCCCAACCTG  | Reverse: AGCTTCTCATACACCGTACAGC     |
| VEGF       | Forward: AGGGCGAGATCATCAACGAAGT | Reverse: AGGGTTCGATGGTAGGGCA         |
| CDC42      | Forward: CCATCCGGAAATGATCCAGGCTG | Reverse: CTACGCCGGAATCTGACAGGATCG    |
| FGFR3      | Forward: TGGCCTCTGGAGAGAAGATT | Reverse: GCACCGTGACCTAGGAGTTT       |
| VCL        | Forward: CCAAGATGATGAGAACAGACAG | Reverse: AGAGGGTATTTGATACACACGA       |

Table 2. List of primers used.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Supplementary Material

Supplemental data for this article can be accessed on the publisher’s website.

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