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Screening assay for blood vessel maturation inhibitors

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ABSTRACT

In cancer patients, the development of resistance to anti-angiogenic agents targeting the VEGF pathway is common. Increased pericyte coverage of the tumor vasculature undergoing VEGF targeted therapy has been suggested to play an important role in resistance. Therefore, reducing the pericytes coverage of the tumor vasculature has been suggested to be a therapeutic approach in breaking the resistance to and increasing the efficacy of anti-angiogenic therapies. To screen compound libraries, a simple *in vitro* assay of blood vessel maturation demonstrating endothelial cells and pericytes association while forming lumenized vascular structures is needed. Unfortunately, previously described 3-dimensional, matrix based assays are laborious and challenging from an image and data acquisition perspective. For these reasons they generally lack the scalability needed to perform in a high-throughput environment. With this work, we have developed a novel *in vitro* blood vessel maturation assay, in which lumenized, vascular structures form in one optical plane and mesenchymal progenitor cells (10T1/2) differentiate into pericyte-like cells, which associate with the endothelial vessels (HUVECs). The differentiation of the 10T1/2 cells into pericyte-like cells is visualized using a GFP reporter controlled by the alpha smooth muscle actin promoter (SMP-8). The organization of these vascular structures and their recruited mural cells in one optical plane allows for automated data capture and subsequent image analysis. The ability of this assay to screen for inhibitors of pericytes recruitment was validated. In summary, this novel assay of *in vitro* blood vessel maturation provides a valuable tool to screen for new agents with therapeutic potential.

1. Introduction

Angiogenesis is critical for tumor progression and metastasis. Currently employed antiangiogenic therapies, however, are primarily focused on the vascular-endothelial growth factor (VEGF) pathway. While clinical benefit is being observed in several solid tumor types, unfortunately, the emergence of resistance is common. One of the potential mechanisms of resistance is the increased coverage of blood vessel with pericytes [1]. These mural cells are believed to make endothelial cells less dependent on growth factor support and protect the tumor vasculature from VEGF withdrawal [2]. Hence, targeting blood vessel maturation may sensitize tumors to VEGF pathway inhibition and prevent or delay the occurrence of resistance.

In order to screen chemical libraries for inhibitors of pericyte recruitment, an *in vitro* assay, which reliably produces lumenized structures of endothelial cells associated with mural cells, is required. Although three-dimensional endothelial cell and pericyte models have been developed and utilized for studying biological questions [3], the methods are too complex to be used in a semi-high throughput fashion [4]. We present here the development of a blood vessel maturation assay, which features the development of lumenized, vascular structures in one optical plane. This format allows for the study of endothelial cell/pericyte interactions and is suitable for the interrogation of chemical compound libraries in semi-high-throughput fashion at the same time.

2. Material and methods

2.1. Materials

Unless otherwise stated, Reagents were from Sigma–Aldrich (St. Louis, MO). Dulbecco's Modified Medium (DMEM) was from Mediatech (Manassas, VA). RPMI Medium 1640 and trypsin were from Invitrogen (Grand Island, NY). Smooth muscle cell medium

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was from ScienCell (Carlsbad, CA). EGM2 Medium was from Lonza (Walkersville, MD). EX-CELL® 293 Serum-Free Medium was from SAFC Biosciences (Lenexa, KS). Fetal bovine serum (FBS) was from Gemini Bio-Products (West Sacramento, CA). VEGF, IL-3, IFN-γ and TGFβ were from R&D system (Minneapolis, MN). Sunitinib malate and imatinib were from LC Laboratories (Woburn, MA).

2.2. Cell culture

C3H10T1/2 cells (10T1/2) were from ATCC, and cultured in DMEM medium with 10% FBS; Human pulmonary artery smooth muscle cells (SMCs) were from ScienCell and cultured in smooth muscle cell medium; Human bone marrow-derived mesenchymal stem cells (MSCs, a gift from Dr. Brennen, Johns Hopkins) were cultured in RPMI 1640 with 20% FBS. Retinal pericytes derived from Immortomice were cultured in DMEM medium added with IFN-γ (5.6 ng/ml), 10% FBS [5]. Human lung fibroblasts were from ATCC and cultured in RPMI 1640 with 10% FBS. HUVECs were from ATCC and grown in EGM2 medium. HEK293T cells were cultured in DMEM medium with 10% FBS. HEK293F cells were from Invitrogen (Carlsbad, CA), and cultured in EX-CELL® 293 Serum-Free Medium. All the cells were maintained at 37 °C in a 5% CO_2 and 95% air incubator.

2.3. Lentivirus production and transduction

The SMP-8 smooth muscle α-actin (α-SMA) promoter (Gift from Dr. Wayne Wang, University of Maryland) was inserted into pLVX-AcGFP1-N1 Vector (Clontech, CA), after the excision of the CMV promoter, a tdtomato fluorescence gene with a stop codon at the 3′ end was inserted into the pLVX-AcGFP1-N1 Vector. The gene encoding extracellular domain of human endoglin was fused with mouse IgG FC domain followed with a stop codon at the 3′ end, and cloned into pLVX-AcGFP1-N1 Vector. Recombinant lentiviruses were generated using a three plasmid system with psPAX2 and pMD2.G vectors in HEK 293T/17 cells. Forty-eight hours after transfection with $CaCl_2$ and plasmids, the virus-containing cell supernatants were harvested, filtered (0.45 μm), and concentrated using PEG 6000 precipiation [6]. HUVECs, pericytes (immortal) and 10T1/2 cells were transduced with concentrated viruses of tdtomato, GFP and SMP-8 controlled GFP with polybrene (8 μg/ml), respectively. Viruses encoding endoglin-FC and IgG-FC were transduced to HEK293F cells. 48 h after transduction, cells were selected with puromycin (10 μg/ml).

2.4. In vitro coculture model

10T1/2 cells, MSCs, SMCs, pericytes and fibroblasts were seeded into a 96-well plate at a concentration of 1.5×10^4 cells/well to form a feeder layer. 48 h later when the stromal cells have grown confluent, HUVECs (1.5×10^4 cells/well) were seeded on top of the stromal cells. The medium for coculturing was M199 medium added with 50 μg/ml ascorbic acid, 40 ng/ml VEGF, 20 ng/ml IL-3 and reduced-serum II supplement [3]. Pictures were taken everyday to record the tube formation and pericyte association.

2.5. Immunostaining

HUVECs were cocultured on top of 10T1/2 cell monolayer. When vascular tubes were formed, cocultures were fixed with 2% PFA and stained with anti-collagen IV (C1926, Sigma); The 10T1/2 (SMP-8) cells without any treatment were fixed and stained with anti α-SMA antibody (A5228, Sigma). Fluorescence conjugated secondary antibodies were from Invitrogen. Nucleus was counterstained with DAPI.

2.6. Protein purification and gel staining

Conditioned medium from HEK 293F cells producing endoglin-FC and control IgG-FC were collected and concentrated. The proteins were then purified by using a protein A column (GE Healthcare). The concentration of the purified protein was determined by BCA assay (Pierce, Rockford, IL). The protein gel was run by a standard method, and was stained with coomassie brilliant blue (Bio-Rad Laboratories).

2.7. Image analysis

The pictures of the coculture assay were taken under a fluorescence microscope. The dissociation of pericytes and HUVECs was considered to be the results of pericytes recruitment inhibition. The distance between adjacent pericytes and HUVECs was calculated by the NIS-Elements software.

2.8. Statistical analysis

Statistical analysis was done using either Student's *t*-test or ANOVA to compare multiple groups. $P < 0.05$ was considered statistically significant.

3. Results and discussion

With this work we introduce a novel *in vitro* assay for blood vessel maturation. To have a scalable model suitable for drug screening efforts we decided against previously described 3-dimensional assays and took our inspiration from an endothelial cell/stromal coculture format, which was originally introduced as an assay for the quantification of stimulatory and inhibitory agents on angiogenesis [7]. This stromal coculture model produces lumenized vascular structures on top of a stromal cell layer. These blood vessel like structures develop over several days and can be maintained for prolonged periods of time. In general, stromal-endothelial cell cocultures are thought to more faithfully represent the angiogenic process and are now utilized by several *in vitro* assays [7,8]. The objective of this study was to develop a model that not only mimics the process of angiogenesis, but also demonstrates the recruitment of pericytes. To this end, five kinds of stromal cells, fibroblasts, MSCs, pericytes (immortal), SMCs, and 10T1/2 cells, were tested as a stromal cell feeder layer. The stromal cells were seeded at the bottom to form a monolayer. 48 h later, when the stromal cells were confluent, HUVECs (tdtomato) were seeded on top. As shown in Fig. 1, all of the stromal cells tested in this coculture assay showed the ability to support the formation of lumenized structures. Vascular-like structures were typically established by day 3. However, these vascular structures were less well developed when HUVECs were cocultured with MSCs or pericytes (immortalized). (Fig. 1). The pericytes started to apoptose on day 4, which may be because of the reduced serum condition used in this assay. Because HUVECs formed better lumenized vascular structures when incubated in reduced serum condition than with serum (data not shown), the coculture assay was then performed in reduced serum condition in this study. In contrast, high quality vascular structures were observed when HUVECs were cocultured with SMCs, 10T1/2 cells and fibroblasts, which confirms previous studies [7,9]; These structures are generally stable for at least 7 days even without changing media.

Then we studied if the recruitment of pericytes could be observed in the coculture systems. Immortalized pericytes were transduced to overexpress GFP and premixed with red HUVECs (tdtomato) at a ratio of 1:3, then seeded on top of the SMCs, 10T1/2 cells or fibroblasts formed feed layer. Disappointingly,

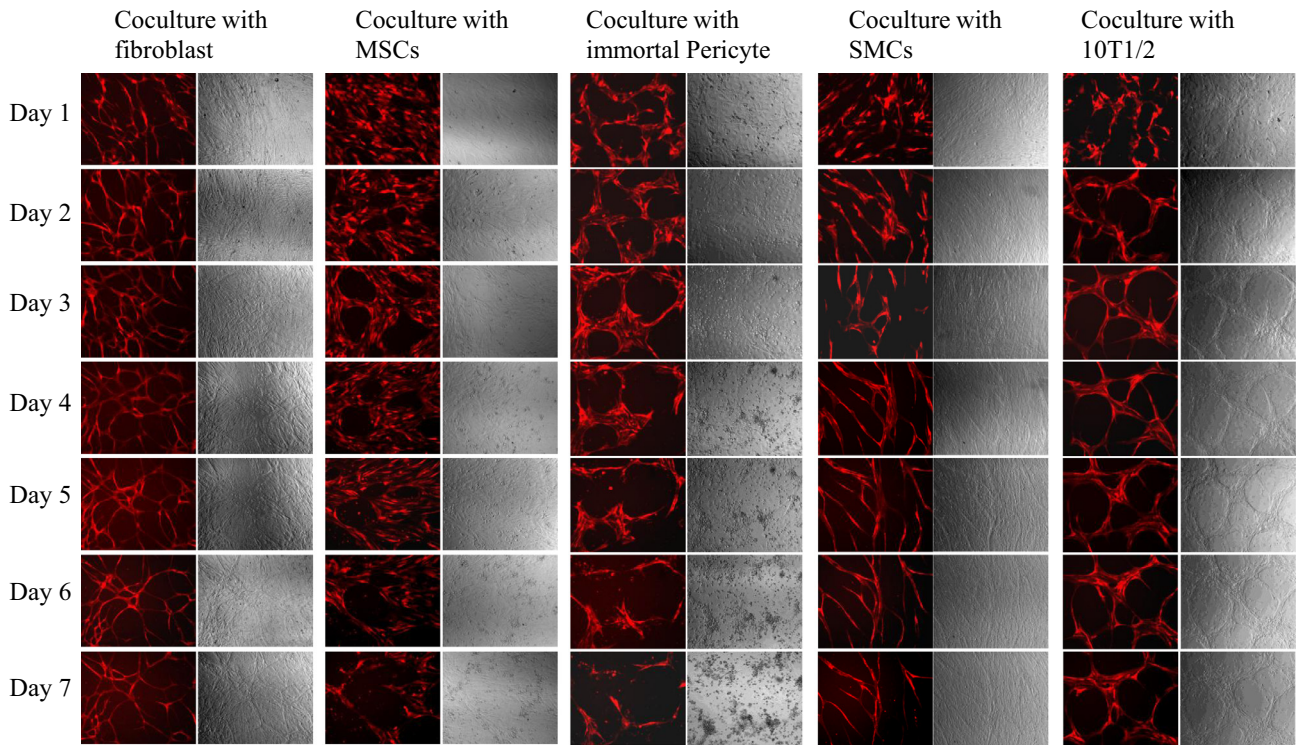

Fig. 1. Establishment of the *in vitro* coculture system. Fibroblasts, MSCs, pericytes, SMCs and 10T1/2 cells were seeded at the bottom of 96-well plate at a concentration of 1.5×10^4 cells/well to form a feed layer. 48 h later, HUVECs (red, tdtomato) were seeded on top of the stromal cells at concentration of 1.5×10^4 cells/well. The media were then changed to coculture media. Vascular structures formed consequently. Pictures were taken under a fluorescence microscope. Magnification: 10x. Results are representative of three separate experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

although vascular structures were formed by HUVECs, the recruitment of the immortalized pericytes could not be observed (Supplementary Fig. 1a). We thus tested if the pericytes could be recruited after first establishing the vascular structures. Therefore, the GFP pericytes (immortal) were added to the coculture assay on day 3 after the tubes have formed. Again, the recruitment of green pericytes could not be observed (Supplementary Fig. 1b). The failure of these cells to associate with the vascular structures is not well understood. Although these immortalized pericytes have classic features of primary pericytes, their dependence on certain growth factors or chemokines might be reduced, which could result in the impaired recruitment to vascular structures. Alternatively, the low serum conditions, which allowed for the development of the best vascular structures, may have contributed to the failure of the pericyte cell line to associate with the endothelial cells.

We then turned to a mesenchymal progenitor cell line. 10T1/2 cells are mouse embryonic mesenchymal cells, which can be induced to undergo differentiation to pericytes and has been validated in several angiogenesis models [10–12]. We thus hypothesized that 10T1/2 cells could differentiate into pericytes, and the 10T1/2 cell derived pericytes could be recruited by the maturing vasculature. In order to track the 10T1/2 cell derived pericytes, 10T1/2 cells were transduced with a GFP reporter controlled by SMP-8 (α-SMA promoter). The functionality of the reporter was validated by TGFβ, an α-SMA inducer [13]. As shown in Fig. 2a, 10T1/2 cells exposed to TGF-beta showed an increased expression of GFP. In addition, when the mesenchymal progenitor cells (10T1/2 SMP-8 cells) were cocultured with HUVECs, a proportion of the 10T1/2 (SMP-8) differentiated to pericytes and associated with vascular structures formed by the HUVECs [11] (Fig. 2b). Importantly, 10T1/2 cells expressing the pericyte markers smooth-muscle actin also demonstrated GFP expression from the smooth muscle actin reporter (Fig. 2c).

The maturation of nascent vasculature, formed by vasculogenesis or angiogenesis, requires recruitment of mural cells and generation of an extracellular matrix [14]. In order to characterize the coculture system, we stained the cells for collagen IV. As demonstrated in Fig. 3a, the endothelial cells express the classic basement membrane protein collagen IV. Importantly, 10T1/2 (SMP-8) derived GFP positive cells and HUVECs (tdtomato) properly aligned in the coculture assay (Fig. 3b), suggesting that this assay format is suitable to study blood vessel maturation *in vitro* and to observe this process in real-time using fluorescence microscopy.

To credential the assay for its ability to identify compounds, which inhibit pericytes recruitment, several compounds known to interfere with pericyte function were tested. Imatinib and sunitinib, both PDGF pathway inhibitors, were used on this coculture model. PDGF is a canonical growth factor that mediates pericytes recruitment, and blockade of PDGF pathway has been known to block pericytes recruitment [15,16]. In Fig. 4a, imatinib was added immediately after seeding of the HUVECs preventing the association of pericytes with endothelial cells partially. In addition, sunitinib, which blocks both, the VEGF and PDGF pathways [17,18], inhibited the formation of well developed vascular structures and blocked the pericytes recruitment in this assay (Fig. 4a and d).

However, once the association of pericytes with endothelial cells was established the disassociation of pericytes with endothelial cells was not observed (Supplementary Fig. 2a). Thus, blockage of the PDGF pathway alone is not sufficient to prevent pericytes coverage and is in line with other reports showing that growth factors such as, HB-EGF [19], stem cell factor and stromal derived factor-1α [4] influence pericytes behavior as well.

Another pathway that has been linked to blood vessel maturation is endoglin. Endoglin binds to TGFβ family members and their receptors and is thought to contribute to TGFβ signaling [20]. Mice deficient in endoglin show vascular abnormalities. In general, these

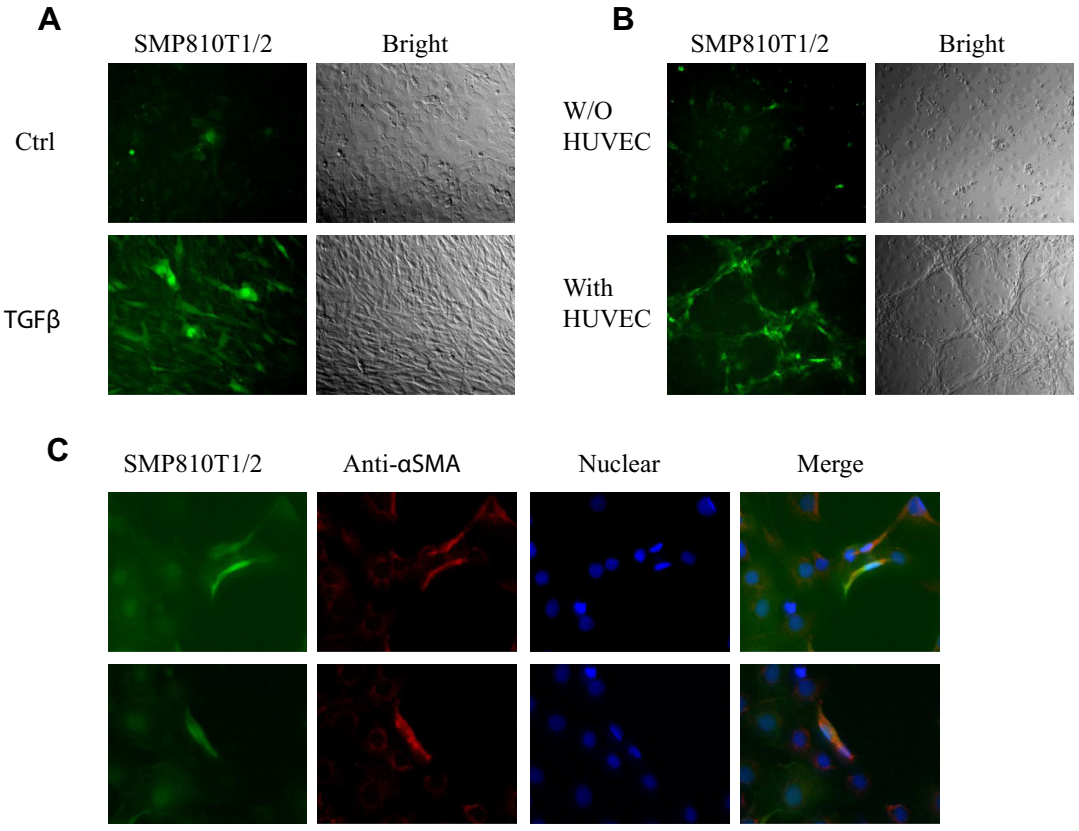

Fig. 2. SMP-8 controlled GFP reporter as an indicator of the 10T1/2 cells derived pericytes. (A) 10T1/2 cells were stably transduced with a lentivirus harboring a GFP reporter controlled by SMP-8 promoter. The 10T1/2 (SMP-8) cells were treated with or without TGF-β (10 ng/ml) for 48 h. Pictures were taken under a fluorescence microscope. Magnification 10x. (B) 10T1/2 (SMP-8) cells were cultured with or without HUVECs for 3 days. Pictures were taken under a fluorescence microscope. Magnification 10x. (C) 10T1/2 (SMP-8) cells were stained for α-SMA. Pictures were taken under a fluorescence microscope. Magnification 30x. Results are representative of 3 separate experiments.

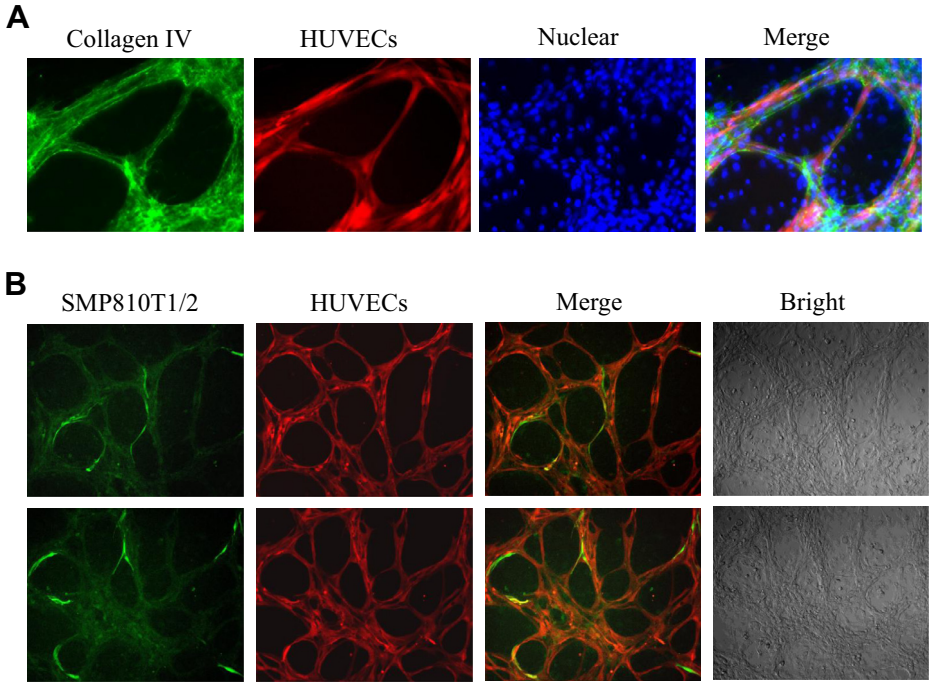

Fig. 3. Establishment of the coculture assay by coculturing HUVECs (tdtomato) on 10T1/2 (SMP-8) cells feeder layer. (A) HUVECs (red, tdtomato) were cocultured on wild type 10T1/2 cells for 3 days, and the coculture system was stained for collagen IV. Pictures were taken under a fluorescence microscope. Magnification 20x. (B) HUVECs (red, tdtomato) were cocultured on 10T1/2 (SMP-8) cells for 3 days. Pictures were taken under a fluorescence microscope. Magnification 10x. Results are representative of 3 separated experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

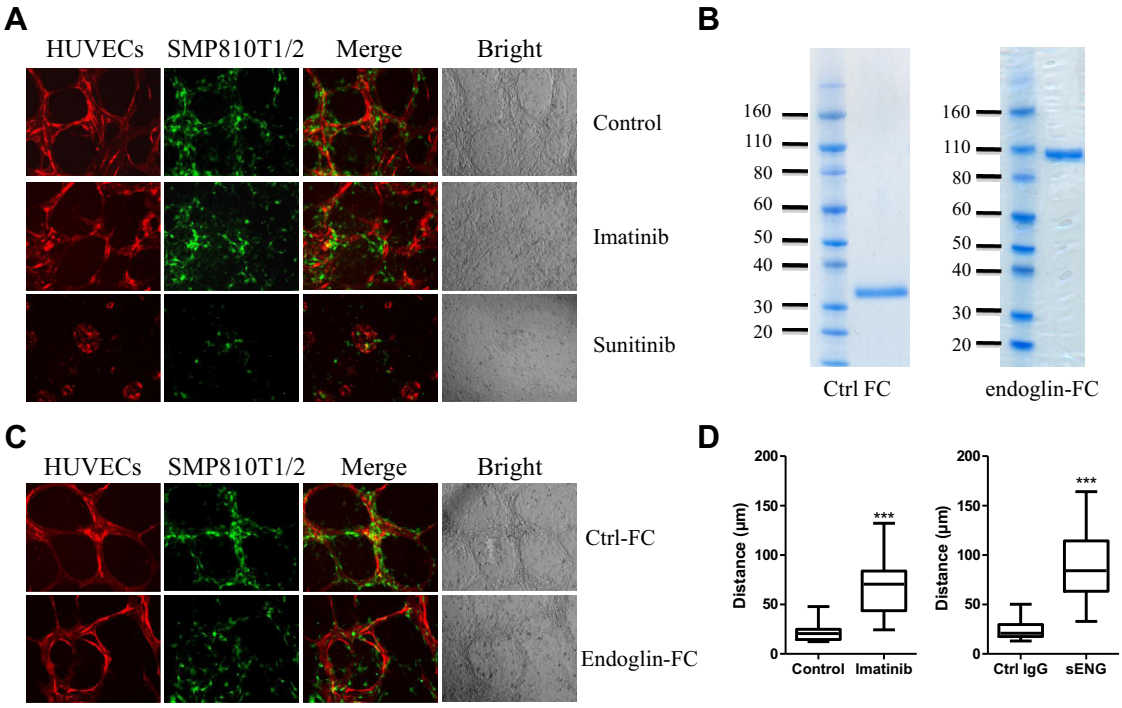

Fig. 4. Validation of the coculture assay for screening pericyte recruitment inhibitors. (A) The coculture assay was treated with imatinib (5 μM) or sunitinib (100 nM) immediately after the seeding of HUVECs (tdtomato) on 10T1/2 (SMP-8) cells. Pictures were taken 3 days later, DMSO was used as control. Magnification 10x. (B) Endoglin-FC and control IgG-FC were produced from HEK 293 F cells and purified by protein A column, the protein gel was stained with coomassie brilliant blue. (C) The coculture assay was treated with control IgG-FC or endoglin-FC (1 μg/ml) immediately after the seeding of HUVECs (tdtomato) on 10T1/2 (SMP-8) cells. Pictures were taken 3 days later. Magnification 10x. (D) The distance between pericyte and HUVECs from figure (A and C), calculated with the NIS-Elements software. Results are representative of 3 separate experiments.

vessels appear immature and are less covered with SMCs [21]. The inherited genetic disorder hemorrhagic telangiectasis results from a mutation in the endoglin gene [22] and these patients have arterial and venous malformations that are characterized by large, leaky vessels with variable SMCs investment [10]. We thus studied if blocking the endoglin pathway could inhibit the pericytes recruitment in this assay. As shown in Fig. 4c, a soluble form of human endoglin inhibited the recruitment of pericytes (Fig. 4d). Furthermore, there was still a modest dissociating effect of soluble endoglin on pericytes, even when matured vascular structures were treated (Supplementary Fig. 2b).

Therefore, this *in vitro* angiogenesis maturation assay enables us to identify drugs and compounds that have the ability to inhibit pericytes recruitment during angiogenesis, and potentially sensitize the tumor vasculature to anti-angiogenic agents.

In conclusion, we have described an *in vitro* blood vessel maturation assay, which reflects known pericyte biology and provides a suitable screen for drugs and natural products that could inhibit the pericytes recruitment and thus have potential clinical applications in angiogenesis related diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.077.

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