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Glycosaminoglycan-based hydrogels to modulate heterocellular communication in *in vitro* angiogenesis models

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Angiogenesis, the outgrowth of blood vessels, is crucial in development, disease and regeneration. Studying angiogenesis *in vitro* remains challenging because the capillary morphogenesis of endothelial cells (ECs) is controlled by multiple exogenous signals. Therefore, a set of *in situ*-forming starPEG-heparin hydrogels was used to identify matrix parameters and cellular interactions that best support EC morphogenesis. We showed that a particular type of soft, matrix metalloproteinase-degradable hydrogel containing covalently bound integrin ligands and reversibly conjugated pro-angiogenic growth factors could boost the development of highly branched, interconnected, and lumenized endothelial capillary networks. Using these effective matrix conditions, 3D heterocellular interactions of ECs with different mural cells were demonstrated that enabled EC network modulation and maintenance of stable vascular capillaries over periods of about one month *in vitro*. The approach was also shown to permit *in vitro* tumor vascularization experiments with unprecedented levels of control over both ECs and tumor cells. In total, the introduced 3D hydrogel co-culture system could offer unique options for dissecting and adjusting biochemical, biophysical, and cell-cell triggers in tissue-related vascularization models.

Angiogenesis describes the outgrowth of new blood vessels and is a research 'hot spot' as emerging therapeutic opportunities for various different pathologic conditions directly relate to this widespread phenomenon. The formation of vascular sprouts is a complex multistep process including: (1) growth factor (GF) gradient formation, (2) endothelial cell (EC) activation, migration, and proliferation, (3) capillary development, and (4) stabilization by mural cells[1]. Studying the process of vascular sprout formation *in vitro* requires 3D models, which recapitulate the consecutive steps of capillary formation. Most angiogenesis research focuses on the biology of ECs, which are the core component of vascular structures. However, their activity *in vivo* is tightly regulated by supporting cell types such as pericytes and smooth muscle cells (SMCs)[2] indicating the importance of the often-neglected heterocellular interactions in *in vitro* models of angiogenesis. Isolated ECs preserve their vasculogenic and angiogenic characteristics in culture. When embedded in a suitable 3D scaffold, ECs temporarily form inter-connected tubular networks resembling vascular capillaries[3], thus enabling *in vitro* vascular sprouting studies. Tissue-derived biopolymer matrices such as collagen[4–6] and fibrin[7,8] were shown to induce EC vascular morphogenesis successfully, and significantly contributed to our knowledge about angiogenesis, but their poor stability limits the development of defined, long term, *in vitro* assays. In search for improvement, synthetic polymer materials were systematically tested and studies using poly(ethylene glycol) (PEG)[9] and hyaluronic acid (HA)[10,11] based hydrogels demonstrated that EC capillary formation is strictly regulated by matrix degradability, integrin ligands (RGD) and GFs. However, in either type of previously reported *in vitro* system, the capillaries developed were unstable and collapsed and degraded within a few days[9,12]. This indicates that cues for maintaining capillary maturity were lacking in the related culture models. Various studies showed that maturity and stability of the capillary structures formed by ECs are dependent on factors provided by supporting cells[4,13] such as SMCs[5,14], mesenchymal stem cells (MSCs)[5,15,16], fibroblasts[7,8], or 10T1/2 cells[9,17,18]. Despite all efforts, these factors have not yet been completely revealed. Clearly, angiogenesis research demands advanced 3D *in vitro* models serving as more faithful mimics of tissue vascularization over prolonged periods.

To address this challenge, we have explored a novel 3D *in vitro* model of heterocellular angiogenesis utilizing *in situ*-forming starPEG-heparin hydrogels as a scaffold. As recently described, these scaffolds are composed of multi-armed PEG and heparin crosslinked via cytocompatible Michael type addition that enabled cells to be embedded in 3D (Fig. 1a)[19]. Based on a rational design strategy, the hydrogel platform allowed the decoupling of

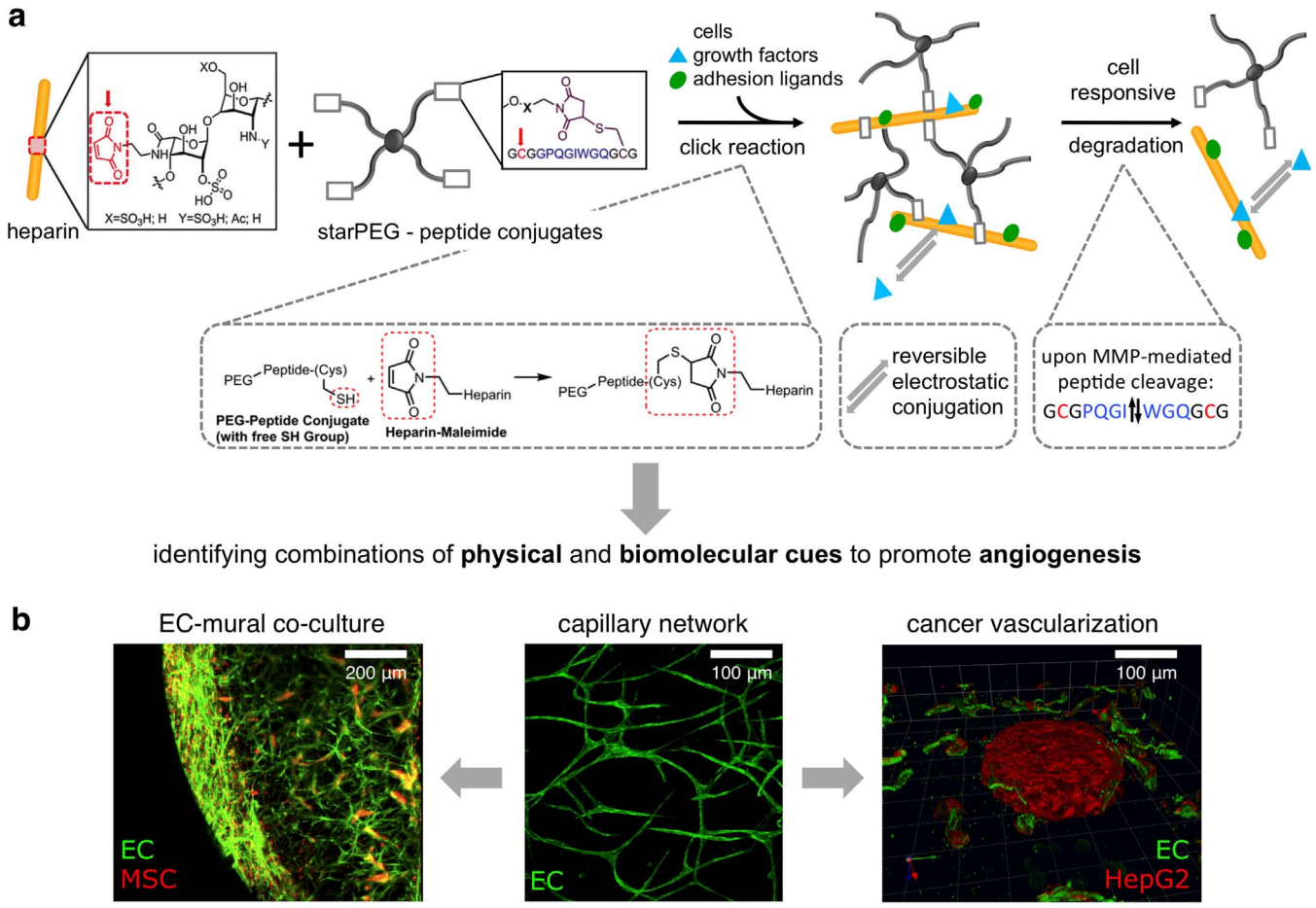

Figure 1 | StarPEG-heparin hydrogels as an extracellular milieu to study heterotypic cell-cell interactions during angiogenic events. (a) Maleimide-functionalized heparin units react with terminal thiol groups of starPEG-peptide conjugates during Michael addition reaction resulting in formation of a modular *in situ* hydrogel platform suitable for displaying multiple adhesion and degradation sites, growth factors, and cell encapsulation. (b) Hydrogels enable studying the role of extracellular milieu and heterocellular cell-cell interactions during vascularization processes as demonstrated by exploring two distinct processes: vascular capillary stabilization by mural cells and tumor vascularization.

their mechanical and biomolecular characteristics[19,20], which is considered a prerequisite for *in vitro* assays suitable for dissecting the cell-instructive roles of biochemical, biophysical, and cell-cell interactions. Adjusting ratio of PEG to heparin allows for control over mechanical properties in a broad range, while maintaining the heparin concentration constant. The highly sulfated glycosaminoglycan heparin is used as a base for the reversible binding and sustained delivery of multiple GFs which show electrostatic affinity toward heparin (e.g., basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), stromal-derived growth factor (SDF) 1α)[21–23] and for the covalent conjugation of adhesion ligands via click chemistry (e.g., RGD, IKVAV)[19]. Network formation through enzymatically cleavable peptide linkers (matrix metalloproteinase (MMP)-sensitive sequences) creates a cell-responsive environment crucial for EC capillary formation[24,25]. The hydrogels can be tuned for adhesiveness and GF delivery irrespective of their intrinsic network properties.

Use of this biohybrid hydrogel platform thus enabled the systematic search and identification of matrix conditions that effectively support vascular network assembly (Fig. 1b). These optimal conditions were further applied to study 3D heterocellular interactions between ECs and other cells. Firstly, the influence of various supporting cell types on EC capillary formation *in vitro* was systematically evaluated. Secondly, heterotypic cell-cell contacts were exemplarily studied in an *in vitro* tumor angiogenesis model. Taken together, we introduce a universal 3D model for the simultaneous investigation of cell-cell and cell-matrix interactions in EC morphogenesis as a holistic, *in vitro* approach to angiogenesis.

Results and Discussion

Mechanical matrix characteristics and bioactive components influence EC network assembly. Shortly after embedment in starPEG-heparin hydrogels, ECs spontaneously initiated vascular morphogenesis and formed multicellular tubular networks within 3 days (Fig. 2a, Supplementary Movie 1), which is a time frame comparable with processes in natural scaffolds[7]. Due to the short polymerization time of the hydrogels, the initial homogenous 3D distribution of ECs (Fig. 2a, **0 h**) resulted in homogenous distribution of capillary structures throughout the construct (Fig. 2a, **72 h**). In order to determine how scaffold properties affect endothelial capillary formation, we varied the storage modulus and $VEGF_{165}$ content of the hydrogels independently. The storage modulus was altered between $G' = 200$ Pa and 850 Pa by increasing the ratio of starPEG to heparin, keeping the heparin concentration constant[19]. As previously shown, heparin-binding GFs such as $VEGF_{165}$ are continuously retained in the starPEG-heparin hydrogels independent of the hydrogel storage modulus, resembling the GAG-GF interaction in extracellular matrices (ECM) *in vivo*[19–24]. This is due to the fact that $VEGF_{165}$ encodes two heparin-binding domains[26]. The variation of hydrogel stiffness and growth factor content significantly affected EC morphogenesis (Fig. 2b). Tubular network development was more pronounced in

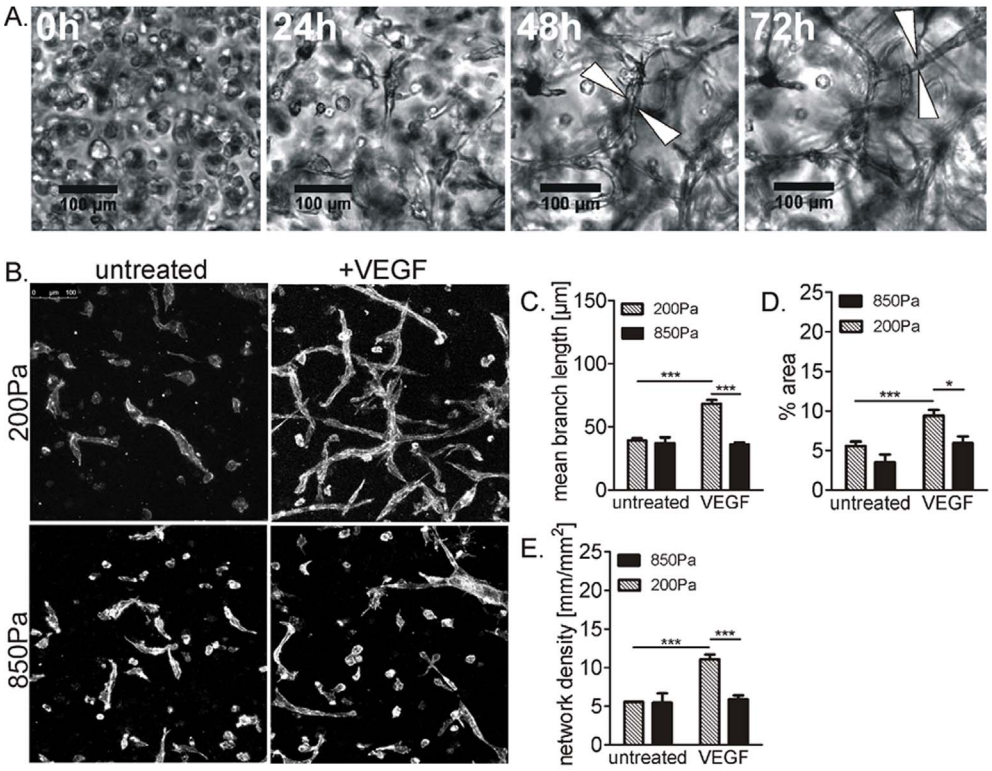

Figure 2 | Vascular morphogenesis of endothelial cells in starPEG-heparin hydrogels. (A) Light microscopy sequential images of endothelial cell morphogenesis over 72 hours, white arrows indicate anastomosed capillaries. (B) Level of capillary network development depends on the stiffness and $VEGF_{165}$ content of the hydrogels. F-actin immunostaining with phalloidin of endothelial cells in hydrogels (200 Pa or 800 Pa, ±5 µg/ml $VEGF_{165}$) at day 3. 200 µm thick section. Scale bar 100 µm. (C) Mean branch length, (D) % area and (E) network density show superior effect of soft hydrogels (200 Pa) containing $VEGF_{165}$. *$p < 0.05$, ***$p < 0.001$, ANOVA.

soft hydrogels (200 Pa) containing $VEGF_{165}$ as quantified by mean branch length (average length of tubes between the anastomosis points) (Fig. 2c), % area (Fig. 2d), and network density (total length of network/area) (Fig. 2e). Importantly, this effect was observed only in the presence of $VEGF_{165}$ (Fig. 2) in a concentration dependent manner (Supplementary Fig. S1). Despite the differences in scaffold composition (ECM-based gels vs. synthetic hydrogels), similar observations on the influence of scaffold stiffness on cells have been made for collagen[6,27], fibrin[28], self-assembling peptides[29], and HA-gelatin hydrogels[10]. In stiffer scaffolds, cell-mediated proteolytic activity is apparently not sufficient to overcome the physical barrier imposed by a dense network, leading to poor cell spreading and migration[30]. Vice versa, if the elasticity of hydrogels becomes compliant upon decease in crosslinking density, an increase of endothelial capillary branching and elongation is observed[10]. Thus, elevated hydrogel stiffness and slower degradation both restrict capillary network formation for hydrogels of higher degrees of crosslinking. Moreover, VEGF increases the expression of MMPs by ECs[24,31] and regulates contractile cell forces[32], thus contributing to the increased EC migration and angiogenesis. These results indicate that the mechanical properties and VEGF concentration co-regulate EC capillary formation in 3D scaffolds.

Lumen formation and tube maturation during vascular morphogenesis. The formation of capillary lumen is a critical functional aspect of vascular morphogenesis. ECs dispersed in supportive 3D matrices generate intracellular vacuoles, which enable them to interconnect with neighboring cells and form multicellular tubes[9,33,34]. The intracellular vacuolation mechanism is genetically programmed and is mainly observed when single ECs initiate morphogenesis in the absence of contact with adjacent cells[10]. As shown with transmission electron microscopy (TEM), 3 days after embedding, ECs in 200 Pa starPEG-heparin hydrogels became highly vacuolated and formed potent intercellular lumen closed by tight junctions (Supplementary Fig. S2a). To further estimate the effect of $VEGF_{165}$ levels on capillary maturation, we evaluated the degree of coalescence (Supplementary Fig. S2b) and lumenization (Supplementary Fig. S2c) of ECs. $VEGF_{165}$ increased the occurrence of EC clusters by about 15% and doubled the amount of lumenized capillaries to almost 100%, thus underlining its fundamental role in vascular morphogenesis. Moreover, as shown with confocal microscopy, cell-secreted collagen IV (Supplementary Fig. S2d) and laminin (Supplementary Fig. S2e) were localized around the tubes, indicating basement membrane formation[9,34]. The basement membrane instructs ECs to polarize and establish basolateral and apical sites required for lumen formation[34], which coincidences with the cytoplasm and the nuclei becoming gradually more associated with the outer, basolateral plasma membrane. These results demonstrate that EC capillaries developing within the hydrogels *in vitro* display functional features of *in vivo* capillaries, such as intercellular lumen and basement membrane, indicating that starPEG-heparin hydrogels can produce a milieu suitable for angiogenesis models.

Delivery of multiple growth factors from GAG-hydrogel enhances EC capillary network formation. As shown above, starPEG-heparin hydrogels sustained high degrees of EC tube formation, even when containing only one growth factor, $VEGF_{165}$. Due to the complexity of the angiogenic and vasculogenic events, single factor delivery is however not successful in forming durable capillary networks and results in collapse and cell apoptosis after 4 days. In several studies of EC morphogenesis, $VEGF_{165}$, bFGF, tumor necrosis factor alpha (TNFα), angiopoietin-1 (Ang-1), SDF-1α, and ascorbic acid have been included in the cell culture medium separately or as a cocktail

in order to promote network formation[4,10,33]. Importantly, starPEG-heparin hydrogels enable the long-term co-delivery of several GFs based on their natural affinity to heparin[21–23]. High concentrations of heparin in the hydrogel system provide a high capacity to bind growth factors and ensure their independent delivery[21]. Moreover, the mesh size of the hydrogel (15–30 nm based on storage modulus data according to rubber elasticity theory[20]) allows for nearly free diffusion of growth factors across the polymer network without steric restrictions. This allowed us to study the influence of simultaneous co-delivery of $VEGF_{165}$, bFGF, and SDF1α on EC morphogenesis (Fig. 3). The factors bFGF and SDF1α either alone or together did not induce EC morphogenesis in soft 200 Pa hydrogels (Supplementary Fig. S3), other than with $VEGF_{165}$. However, bFGF or SDF1α in combination with $VEGF_{165}$ significantly increased the mean branch length of the tubes (Fig. 3b), branching density (Fig. 3c), % area (Fig. 3d) and overall network density (Fig. 3e). Notably, the simultaneous co-delivery of these 3 GFs resulted in EC networks twice as dense, long and branched as those observed with $VEGF_{165}$ alone. Furthermore, GF co-delivery significantly prolonged the network durability to about twice the time achieved in $VEGF_{165}$-only containing gels (10 vs. 4 days) (Supplementary Fig. S4), thus suggesting that the synergistic action of multiple GFs improved the robustness of EC capillary networks. It has been suggested that upon multifactorial stimulation the major function of $VEGF_{165}$ is to prime EC responsiveness to other factors that subsequently control vascular morphogenesis by inducing cytokine receptor expression[35]. Accordingly, $VEGF_{165}$ and bFGF were shown to facilitate the action of pro-morphogenic cytokines such as SDF1α[35]. These results demonstrate that starPEG-heparin hydrogels allowed the systematic study of multi-GF stimulation in EC morphogenesis, enabling a definition of the role of individual molecular components comprising the complex angiogenic machinery.

Heterocellular interplay in GAG-hydrogel based EC co-cultures. It has been convincingly shown that the durability of tubular EC networks in 3D *in vitro* assays is transient due to the action of MMPs, which leads to the rapid remodeling of the hydrogels[9,10,36]. *In vivo* vascular capillaries are stabilized by mural cells, which integrate and coordinate neighboring EC responses[2]. Dysfunction of EC-mural cell interactions is implicated in a variety of vascular diseases, such as diabetic microangiopathy or cancer[37]. In order to evaluate the influence of mural cells on the EC capillary formation and durability systematically *in vitro,* we co-cultured primary ECs with supporting cells (Fig. 4). Firstly, MSCs were used as model-supporting cells in various seeding ratios (0–25%). 10% supporting MSCs were found to be optimal for efficient co-culture (Supplementary Fig. S5a), while higher relative amounts of MSCs (>10%) inhibited tube assembly as similarly reported by others[38,39]. Interestingly, co-culture resulted in hydrogel shrinking, dependent on the amount of MSCs contained (Supplementary Fig. S5b). As MSCs acquire SMC/pericyte phenotypes upon co-culture[40], and apply pulling forces, higher amounts of MSCs cause higher degrees of scaffold shrinking (Supplementary Fig. S5b and c).

Co-culture of ECs with 10% MSCs resulted in capillary networks remaining mature and stable for more than 4 weeks (Fig. 4a–c, Supplementary Movie 2 and 3). Similarly, Koike et al. showed that HUVEC/10T1/2 seeded collagen constructs implanted subcutaneously in mice persist *in vivo* longer than constructs seeded with HUVECs alone (1 year vs. 60 days)[18]. Moon et al. demonstrated that HUVEC/10T1/2 seeded in synthetic PEG hydrogels maintain interconnected network of cells for 4 weeks, however without distinguishing the cell types forming the network[9]. As shown by light sheet microscopy, prolonged co-culture resulted in a regular distribution of supporting cells co-localized with the EC network (Fig. 4b, Supplementary Movie 2) and a tissue-like appearance acquired upon 4 weeks of culture (Fig. 4c, Supplementary Movie 3). As shown by immunostaining, many of the supporting cells were localized in close proximity or directly aligned with the EC tubes (Fig. 4b and d), which resembled the *in vivo* conditions where pericytes are embedded into the EC basement membrane[2]. Long-term co-culture enabled further maturation of EC capillaries leading to the development of a profound lumen diameter of 10–15 μm (Fig. 4e), which corresponded to the size of human capillaries *in vivo*. As such, the introduced 3D *in vitro* model uniquely displayed key features of *in vivo* vascular assembly and angiogenesis.

EC co-cultures have been tested for improving the durability of EC networks before, however with a remarkable heterogeneity of the reported supporting cell types (multipotential cell line 10T1/2[9,17,18], SMCs[5,14,39], human dermal fibroblasts (HDFs)[8], lung fibroblasts[7], MSCs[5,15,16]); scaffolds (natural[15,41] vs. synthetic[9–11]); and culture con-

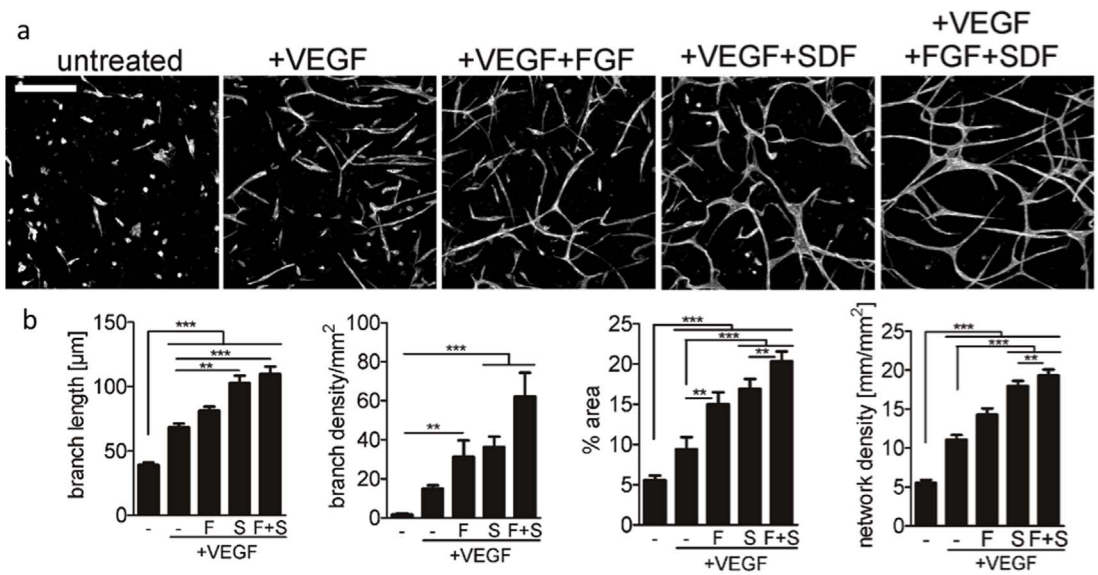

Figure 3 | Capillary network formation is improved by the synergistic effect of $VEGF_{165}$, bFGF and SDF1α. (a) F-actin immunostaining of endothelial cells in hydrogels (200 Pa) with different growth factor content at day 3. 200 μm thick sections. Scale bar 100 μm. (b) Mean branch length, branch density/mm², % area and network density were quantified out of the overlayed images showing superior effect of soft hydrogels (200 Pa) containing $VEGF_{165}$, bFGF and SDF1α. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ANOVA.

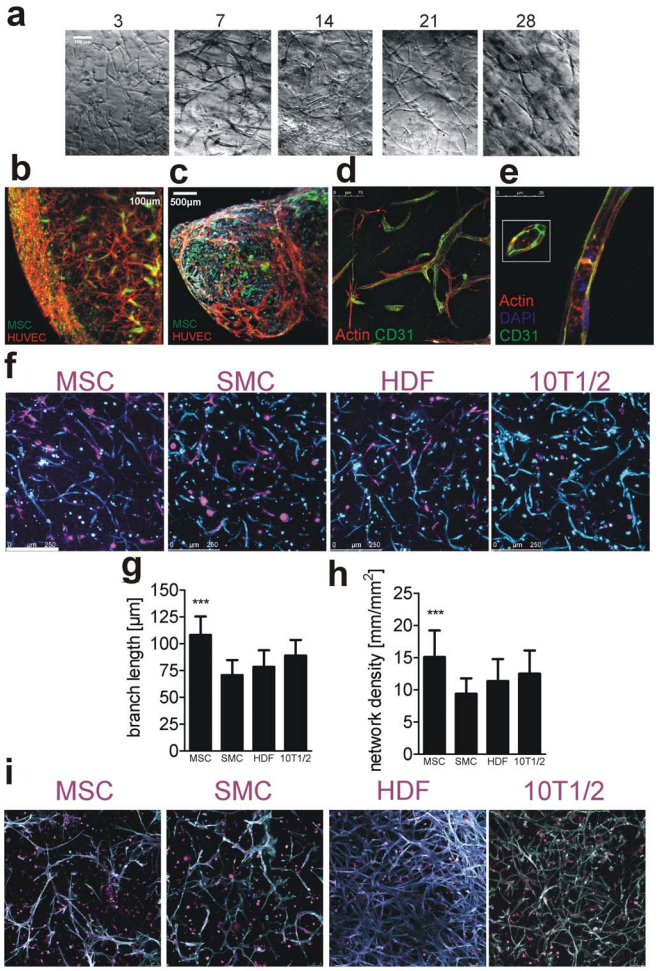

Figure 4 | Heterotypic endothelial cell-supporting cell co-culture. (a) Light microscopy images of endothelial cells co-cultured with 10% MSCs taken over 28 days. Co-culture with supporting cells results in long-lasting capillary networks. Scale bar 100 µm. (b) Light sheet microscopy image of fluorescently labeled endothelial cells (red, CMFDA) co-cultured with 10% MSC (green, CMTMR) at 14 days (scale bar 100 µm) and (c) 28 days (scale bar 500 µm) showing capillary network in gross scale. (d) Immunostaining of endothelial cells (green, CD31) and MSCs (red, F-actin) during the long-term culture showing cell co-localization. Scale bar 75 µm. (e) Confocal longitudinal and cross-section image of a 28 day-old endothelial cell capillary (red-f-actin, green-CD31, blue-nucleus) showing its multicellular composition and internal lumen. Scale bar 25 µm. (f) Confocal images of co-cultured endothelial cells with MSC, SMC, HDF or 10T1/2 after 7 days in hydrogels (200 Pa + $VEGF_{165}$, bFGF, SDF1α). Cells were pre-labelled with fluorescent dyes (endothelial cells-cyan, supporting cells-magenta). 200 µm thick sections. Scale bar 250 µm. (g) Average endothelial cell branch length and (h) average network density upon 7 days of co-culture. *** $p < 0.001$, ANOVA. (i) Endothelial-mural co-culture over 28 days indicates difference in network phenotype depending on the type of mural cell used for the experiment. While MSCs and SMCs maintain the initial density of the network, HDFs and 10T1/2 cells contribute to the overgrowth of the scaffold. Endothelial cells-cyan, mural cells-magenta. Scale bar 100 µm.

ditions. Even a tri-co-culture was evaluated in fibrin-based scaffolds, however without any apparent positive effect over the binary co-cultures[8]. Therefore, we systematically compared various supporting cell types, MSCs, SMCs, HDFs and 10T1/2 (all in amounts of 10%, in hydrogels containing VEGF, bFGF and SDF1α), to evaluate their effect on EC capillary formation (Fig. 4f). Quantification of network parameters after 1 week of co-culture revealed differences in the network characteristics between the various cell types. Co-culture with MSCs resulted in the longest average branch length (Fig. 4g) that contributed to the higher overall network density (Fig. 4h), while no significant differences were observed between SMCs, HDFs, and 10T1/2. However, striking differences were observed after culture periods of one month. While co-cultures of ECs with HDFs or 10T1/2 cells resulted in very dense tissue-like constructs (Figure 4i), MSCs and SMCs sustained the modest character of the networks without any apparent changes. We speculate, that the over-growth of the ECs when co-cultured with HDFs or 10T1/2 can be caused by different growth factor or cell-cell communication patterns as compared to MSCs and SMCs. These observations suggest that the supporting cell type determined the phenotype of capillaries. As blood capillaries supported by tissue-specific mural cells exhibit distinct differences[37], the hydrogel based culture system can be considered suitable for exploring the mechanisms by which ECs recruit tissue-specific mural cells during vessel formation, and gain functional differences depending on the tissue type. In summary, starPEG-heparin hydrogels enabled the long-term *in vitro* investigation of heterotypic cell-cell interactions during EC capillary formation, highlighting the important regulatory role of supporting mural cells in the angiogenic EC microenvironment. The combination of various different mural cells with the multifactor delivery in the hydrogel-based co-cultures of ECs facilitated the most versatile modulation of capillary EC networks, and could offer unprecedented options for generating tissue and disease-specific angiogenesis models.

3D tumor vascularization models based on GAG-hydrogel co-cultures. The established parameters of starPEG-heparin hydrogels were also used to study heterotypic cell-cell interactions during tumor vascularization *in vitro*. Tumor vascularization is a complex process, where interactions between tumor cells and ECs are essential for tumor progression and metastasis[42,43]. However, there is a lack of physiologically relevant 3D *in vitro* models that recapitulate tumor vascularization and tumor-EC interactions in a controlled environment. Therefore, we first established and characterized *in vitro* tumor spheroid formation within the *in situ* starPEG-heparin hydrogels. As an exemplary cancer cell type, single human hepatocarcinoma cells were embedded in hydrogels at various cell densities and grown for several days. This resulted in multicellular spheroid formation with clearly visible cell polarization demonstrated by F-actin staining (Fig. 5a and Supplementary Fig. S6a). The size of the hepatospheroids in this organotypic 3D culture was controlled by the hydrogel biodegradability, stiffness, and RGD content. Non-degradable scaffolds did not permit hepatospheroid formation (Supplementary Fig. S6b). Soft (200 Pa) degradable hydrogels resulted in hepatospheroids of larger diameter when compared with the stiff gels (2.5 kPa) (Fig. 5b). As cell-integrin engagement in 3D is essential in regulating cancer development and growth[44], tumor spheroids were significantly bigger in the cRGD-functionalized soft hydrogels (<1 kPa) but not in stiff hydrogels (>1 kPa) (Supplementary Fig. S6c and d). Finally, tumor spheroids were transferred into hydrogels containing ECs in order to study tumor vascularization (Fig. 5c and d). The migration of ECs into the tumor spheroids was monitored over several days using time-lapse, phase-contrast microscopy, allowing for the observation of heterotypic cell-cell interactions (Supplementary Movie 4). Confocal microscopy showed sprouting of the ECs as well as tumor migration in 2D (Fig. 5c) and 3D (Fig. 5d). Hence, our hydrogel platform presented the opportunity to assess tumor-EC interactions in a 3D matrix where ECs can form tumor-invading sprouts. Measurement of spheroid diameter during progressive tumor angiogenesis showed a slight increase in spheroid size at 3 and 6 days suggesting the importance of tumor-EC contact for cancer progression[43] (Supplementary Fig. S5e). Thus, the proposed 3D

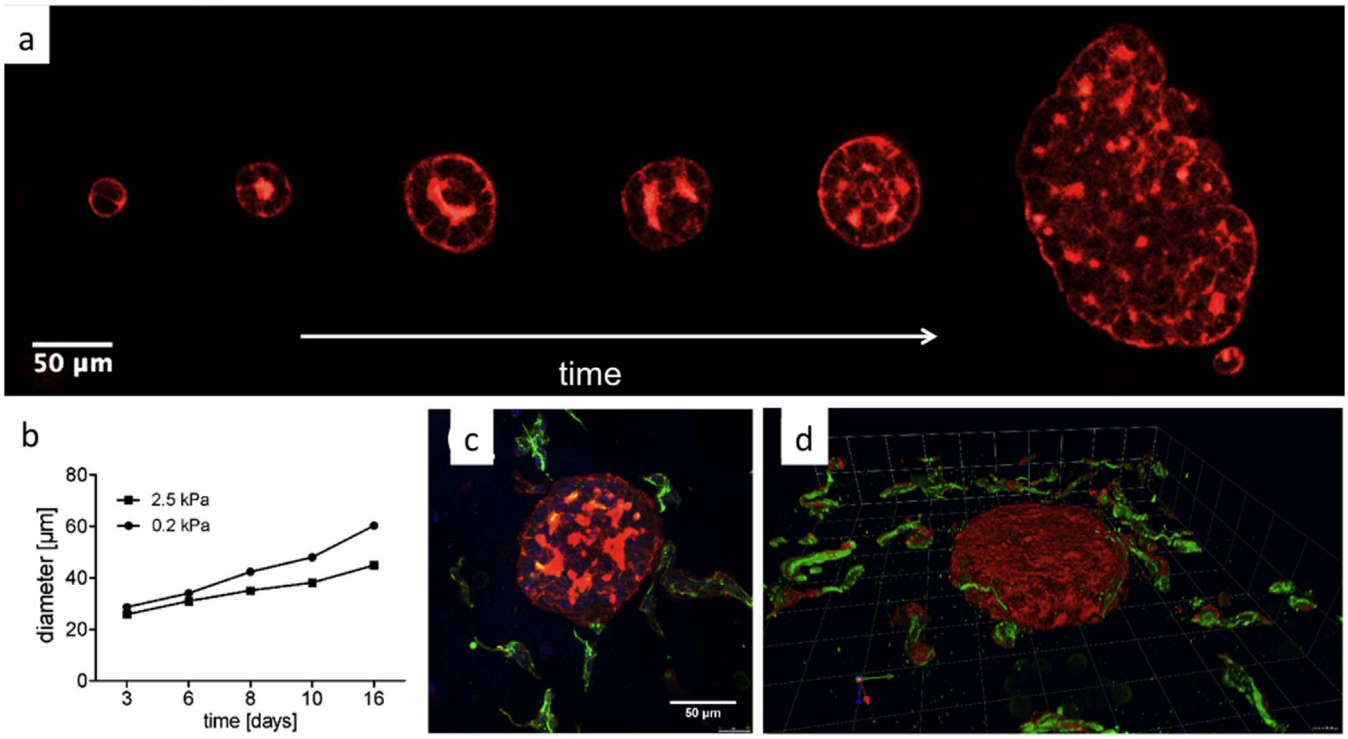

Figure 5 | Heterotypic endothelial cell-tumor culture. (a) Hepatocarcinoma growth in the 0.2 kPa hydrogel over 7 days. Single cell proliferation over the time leads to the development of multicellular tumor spheroids over 150 µm in diameter with distinct foci of F-actin indicating formation of hepatic canaliculi. Confocal cross-section of tumor spheroids immunostained for F-actin (red). Scale bar 50 µm. (b) Growth curve of hepatocarcinoma tumor spheroid registered over 16 days in 0.2 kPa and 2.5 kPa hydrogel indicates that soft hydrogels facilitate faster tumor growth. NS, ANOVA (c) 2D confocal image and (d) 3D reconstruction of tumor spheroid (red-F-actin) co-cultured with endothelial cells (green-CD31) for 3 days in 0.2 kPa hydrogels. Endothelial cells form capillaries in contact with tumor spheroids allowing for observation of heterotypic cell-cell contacts. Scale bar 50 µm.

hydrogel co-culture model to study tumor vascularization offers a new route to understanding the role of ECs in the tumor microenvironment. This can be expected to hugely facilitate the development of therapeutic strategies for angiogenesis-dependent tumors, because previously reported tumor vascularization models fall short in inducing EC capillary formation[43,45], lack a 3D microenvironment[46,47], do not allow for the independent modification of scaffold properties[43,45,47,48], or take weeks to initiate vascular sprouting[49].

Methods

Cell culture. Primary human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords as described elsewhere[24]. Cells were maintained in low-serum Endothelial Cell Growth Medium (PromoCell) with 1% penicillin/streptomycin and utilized within the 3–6th passage.

Primary human aortic smooth muscle cells (SMCs) and primary human dermal fibroblasts (HDFs) were purchased from ATCC. SMCs were maintained in low-serum Smooth Muscle Cell Growth Medium 2 (PromoCell) with 1% penicillin/streptomycin and utilized within the 5–6th passage. HDFs were maintained in low-serum Medium 106 supplemented with Low Serum Growth Supplement (Gibco) with 1% penicillin/streptomycin and utilized within the 7–8th passage.

Human bone marrow mesenchymal stem cells (MSCs) were isolated from healthy donors (male/female, Caucasians 21–35 years of age) after obtaining their informed consent. The study was approved by the institutional review board of the Medical Faculty at the University Hospital Dresden. MSCs were expanded, characterized and cultured as described previously[50]. MSCs were maintained in DMEM (Gibco) with 10% FCS (Biochrom) and 1% penicillin/streptomycin and utilized within the 1–2nd passage.

HepG2 human hepatocarcinoma cells were cultured in RPMI medium (Gibco) supplemented with 10% heat inactivated bovine serum and 50 µg/ml gentamycin (Sigma-Aldrich).

Cell labeling. Cells were pre-labeled using the CellTracker dyes (Invitrogen) according to the manufacturer's instructions. Briefly, cells were incubated for 15 minutes with 10 µm dye diluted in serum free medium (HUVECs – CMFDA, supporting cells – CMTMR). The reaction was stopped by a 30-minute incubation with full medium.

In-situ gel formation. Hydrogels were prepared as described previously[19]. Briefly, 10 mg of heparin-maleimide conjugate (MW 15000) was dissolved in 107 µl serum-free medium. Subsequently, the heparin solution was added to 0.79 mg cRGD (MW 594, Peptides International, Louisville, KY, USA) at a final concentration of 3 mM and incubated at room temperature for 30 minutes. Next, heparin-RGD solution was mixed with cells and human recombinant growth factors: $VEGF_{165}$, bFGF, SDF1α (PeproTech, USA). The final concentration of each growth factor was 5 µg/ml. 10 mg of PEG-MMP conjugate (MW 15920) was dissolved in 372 µl of ice-cold serum-free medium. Heparin-RGD-cell-growth factor solution was mixed gently with PEG-MMP solution in ration 1 : 1 and gel formation occurred within several seconds. The final endothelial cell concentration was 6×10^6/ml, the concentration of supporting cells varied between 5–25% of the endothelial cells. Upon polymerization, gels were immediately immersed in low-serum Endothelial Cell Growth Medium (PromoCell) with 1% penicillin/streptomycin and cultured as floating droplets. 70% of the medium was exchanged every other day. The size of the hydrogel droplets was monitored daily by stereo microscope (Leica S8APO).

Tumor spheroid formation. HepG2 cells were embedded in the hydrogel as a single cell suspension and cultured for 7 days. The RPMI medium (Gibco) supplemented with 10% heat inactivated bovine serum and 50 µg/ml gentamycin (Sigma-Aldrich) was exchanged every other day. The diameter of spheroids was monitored daily by light microscopy (Olympus IX50; 10×). The spheroid development and canaliculi formation inside the HepG2 spheroids was monitored by PhalloidinCF633 (Biotrend) staining (as described in immunostaining section) and confocal microscopy (Leica SP5 20×, 0.70).

Time-lapse microscopy. Hydrogels were immobilized in the wells of the cell culture plate and subjected to time-lapse microscopy over 72 hours (Zeiss; 10×, 0.25). Images were acquired every 15 minutes and analyzed using 4.8 AxioVision (Zeiss) and 1.47 d ImageJ (NIH) software (30 frames/sec).

Immunostaining. Prior to immunostaining, samples were fixed with 4% paraformaldehyde (PFA, Sigma Aldrich) for 15 minutes at room temperature and blocked with 1% goat serum (Dianova, Germany) in a solution of 0.2% bovine serum albumin (BSA) (Sigma-Aldrich, München, Germany), 0,2% Triton-X100 (Sigma-Aldrich, München, Germany) in PBS. The primary antibody (Collagen IV (Millipore AB748), Laminin (Sigma-Aldrich L9393), CD31 (BD 555444)) was then applied in blocking buffer for 1 hour, following 3 × 15 minute washing with the buffer. The secondary antibody (Molecular Probes) diluted 1 : 200 in blocking buffer and

PhalloidinCF633 was applied and incubated for 40 minutes in the dark. Finally, 0.1 µg/ml DAPI (Sigma-Aldrich, München, Germany) in PBS was applied for 5 minutes followed by 3 × 15 minute wash with PBS. All staining steps were performed at room temperature. Samples were imaged with Leica SP5 confocal microscope (20×, 0.70; 40×, 1.25; 63×, 1.40). 200 µm thick z-stacks were acquired every 2 µm and consecutive images were overlapped using maximum intensity and analyzed with ImageJ.

Transmission electron microscopy (TEM). Hydrogel constructs were fixed in 1% glutaraldehyde for 24 hours, followed by 3 washes in water. Osmium tetroxide was not used, because it was found to disrupt the PEG gel. Samples were dehydrated in graded concentrations of ethanol (50%, 70%, 80%, 90%, 96% and 3 × 100%) for 30–45 minutes each. The ethanol was then replaced by pure propylene oxide (PO) in two steps (1 : 1 Ethanol:PO followed by pure PO), followed by infiltration in epon/araldite (EA) 1 : 3 EA:PO 1 h, 1 : 1 EA:PO 1 h, 3 : 1 EA:PO 1 h, pure EA overnight and final polymerization in pure EA at 60°C. 70 nm-thick sections of the sample were cut, collected on copper grids, and stained in 2% UA in 70% methanol, followed by lead citrate. The sections were observed in a Tecnai-12 transmission electron microscope (FEI, The Netherlands) and imaged with a TVIPS camera (TVIPS, Germany).

Ultramicroscopy. After immunofluorescence wholemount staining, hydrogels were imaged with LaVision Ultramicroscope (LaVision BioTec, Bielefeld). Stacks were captured with a step size of 1 µm and at different magnifications. 3D reconstruction, morphometric analysis and analysis of ultramicroscopy stacks were performed with ImageJ.

Tumor angiogenesis. 7 day old HepG2 spheroids were extracted from the 0.2 kPa hydrogel containing 3 mM cRGD, w/o growth factors by mechanical dispersion and incorporated together with the endothelial cell suspension in a fresh 0.2 kPa hydrogel. The 3D cultures were maintained in low-serum Endothelial Cell Growth Medium (PromoCell) with 1% penicillin/streptomycin. 70% of the medium was exchanged every other day. The spheroids and endothelial cell co-culture was terminated on day 3, when the capillaries were fully developed, and fixed with 4% PFA. Hydrogels were embedded in OCT and subjected to cryosectioning, followed with immunostaining with anti-CD31 antibody and phalloidin as described above. Samples were imaged with Leica SP5 confocal microscope (20×, 0.70; 40×, 1.25; 63×, 1.40). 200 µm thick z-stacks were acquired. Three-dimensional reconstruction was performed using Volocity software.

Statistics. All data were analyzed using the InStat software (GraphPad, CA, USA) and displayed as mean ± SEM. Analysis of variance (ANOVA) was the primary analysis method using the Turkey post-hoc test or Kruskal-Wallis ANOVA on ranks with Dunn's post-hoc modification depending on the results of the normality test. In addition, two-tailed Students' t-tests were employed. Levels of significance were determined when * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, NS-non significant.

Ethics statement. The methods were carried out in accordance with the approved guidelines. The isolation procedure of HUVECs and MSCs was approved by the institutional review board of the Medical Faculty at the University Hospital Dresden.

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Author contributions

K.C. designed and performed experiments, analyzed data and wrote the paper. M.V.T. synthesized PEG- and heparin-peptide conjugates. U.W. and C.W. supervised the project and edited the manuscript.

Additional information

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