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Angiogenic potential of microvessel fragments is independent of the tissue of origin and can be influenced by the cellular composition of the implants

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Abstract

We have demonstrated that microvessel fragments (MFs) isolated from adipose retain angiogenic potential *in vitro* and form a mature, perfused network when implanted. However, adipose-derived microvessels are rich in pro-vascularizing cells that could uniquely drive neovascularization in adipose-derived MFs implants.

Objective—Investigate the ability of microvessel fragments from a different vascular bed to recapitulate adipose-derived microvessel angiogenesis and network formation and analyze adipose-derived vessel plasticity by assessing whether vessel function could be modulated by astrocyte-like cells.

Methods—MFs were isolated by limited collagenase digestion from rodent brain or adipose and assembled into 3D collagen gels in the presence or absence of GRPs. The resulting neovasculatures that formed following implantation were assessed by measuring 3-D vascularity and vessel permeability to small and large molecular tracers.

Results—Similar to adipose-derived MFs, brain-derived MFs can sprout and form a perfused neovascular network when implanted. Furthermore, when co-implanted in the constructs, GRPs caused adipose-derived vessels to express the brain endothelial marker glucose transporter-1 and to significantly reduce microvessel permeability.

Conclusion—Neovascularization involving isolated microvessel elements is independent of the tissue origin and degree of vessel specialization. In addition, adipose-derived vessels have the ability to respond to environmental signals and change vessel characteristics.

Keywords

Angiogenesis; vessel permeability; glial restricted precursors; astrocytes; angiogenesis assay

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Introduction

A microvascular network is critical for the function and survival of most tissues and is essential for nutrient and oxygen delivery as well as removal of waste metabolites. However, vascular disorders are the most frequent cause of human disease and ischemia is the most common consequence of vessel dysfunction, resulting in the disruption of tissue homeostasis. In order to try to reestablish blood flow to affected areas, the formation of new microvascular networks has been used as a therapeutic strategy to re-vascularize ischemic tissues[23,33].

We have previously shown that adipose-derived microvessel fragments isolated by limited digestion with collagenase retain native microvessel structures (endothelial cells surrounded by perivascular cells) and angiogenic potential[12]. When seeded in a three-dimensional collagen type I matrix and implanted subcutaneously *in vivo*, these microvessel fragments progress into a new, mature, hierarchical microvascular network that is fully anastomosed with the host circulation[21]. This approach has been successfully used to treat myocardial ischemia leading to a reduction in the infarct size and improvement in left ventricular function in animals[29].

The ability to spontaneously form a new microvasculature without further manipulation or addition of factors suggests that angiogenesis and network maturation is inherent to the isolated microvessel elements. Increasingly, it is clear that adipose tissue is rich in resident stromal and mesenchymal stem cells that have been shown to be pro-angiogenic[19]. Furthermore, many of these pro-vascular cells may likely reside within the vessel walls of the adipose microvasculature[35]. Because we isolate intact microvessels, it is possible that these regenerative cells intrinsic to adipose may be present in the isolate and could explain the ability of these microvessel fragments to spontaneously form a new microvasculature. It is also possible, because of the enriched numbers of regenerative cells in adipose, that the therapeutic neovascularization capacity of the microvessel fragment system may be unique to adipose-derived microvessels. Here, our goal was to investigate whether a vascular bed from a different, more specialized tissue source such as brain cortex would recapitulate adipose-derived vessel fragment neovascularization. In addition, we analyzed adipose-derived vessel plasticity via the ability of non-vascular cells to influence tissue-specific functions by assessing whether vessels formed from fragments obtained from adipose tissue could be modulated by inclusion of glial restricted precursor cells (GRPs), a cell population unique to the central nervous system.

Material and Methods

Microvessel isolation and construct implantation

Fat microvessel fragments (FMF) were isolated from rat epididymal fat by limited collagenase digestion and selective screening as previously described[12,28]. The collagenase used (type I Worthington Biochemical Company, NJ) was lot-tested to yield high numbers of fragments with intact morphologies. Rodent brain cortex microvessel fragments (BMF) were isolated by identical methods used in FMF isolations except that BMFs were suspended in 15% Dextran100,000 to 200,000MW (Sigma) and centrifuged at 4000xg at 4°C for 20 min. after the collagenase digestion step and before screening. Density centrifugation of the isolate separated the microvessel fragments from axon and myelin fragments. Pelleted BMFs were collected and washed in divalent cation free phosphate buffered saline with 0.1% bovine serum albumin (Invitrogen, CA) (BSA-PBS) and selective screened by size.

To form the microvascular construct, isolated MFs were suspended into liquid collagen type I at a density of 20,000 MFs/ml. The collagen was prepared by mixing sterile, acidified rat tail collagen Type I (BD Biosciences) with ice cold 4x Dulbecco's Modified Eagle's Media (DMEM) (Invitrogen) to yield a final concentration of 3mg/mL collagen and 1x DMEM. The collagen pH was adjusted to 7.4 by the addition of 1N NaOH and kept on ice to prevent polymerization. MF/collagen suspensions were pipetted into wells of a 48-well culture plate (0.2 ml/well) to form a three-dimensional microvascular construct (MVC). MVCs were either cultured in DMEM+10%FBS or implanted subcutaneously on the flanks of SCID mice as previously described[21]. Alternatively, FMFs were seeded in the presence of glial restricted precursor cells or GRPs (5×10^5 /mL of collagen) before implantation. In order to be able to distinguish between the host vasculature and the one formed by implanted vessels, vessel fragments were obtained from rats that ubiquitously and constitutively express green fluorescent protein (RRRC, University of Missouri, Columbia, MO).

Glial Restricted Precursor Cell (GRP) Isolation

Spinal cords from E14 rat embryos were dissected and incubated in trypsin 0.05% (Gibco) for 30 min at 4°C. After washing the cords, dissociated cells were selected by plating the digestate in a Petri dish coated with anti-A2B5 hybridoma supernatant for 1 hour at room temperature. Dishes were washed to remove unbound cells and the A2B5-bound cells were mechanically lifted and plated on fibronectin (Invitrogen) coated plates in DMEM/F12 containing 1% N2 (Gibco), 20 ng/mL FGF₂ (Millipore) and 10 ng/mL PDGFAA (Sigma). Only cells between passages 0 – 2 were used for experiments.

Construct Analysis

MVCs were harvested at either 4 or 8 weeks after implantation and fixed in 4% paraformaldehyde for 2 hours. Samples were permeabilized with 0.5% Triton X-100 and rinsed with PBS. After blocking for 2 hours with 10% goat serum (Sigma), samples were incubated overnight with different fluorescent conjugated antibodies (see figure legends). Following three 15 minutes washes in DCF-PBS, samples were imaged en bloc with an Olympus MPE FV1000 Confocal Microscope and analyzed with Amira 5.2 (Visage Imaging, Inc, CA). Vessels were identified by either constitutive expression of GFP (when fragments were obtained from animals that ubiquitously and constitutively express GFP) or staining with GFP conjugated Griffonia simplicifolia I (GSI) lectin (Vector labs). To evaluate vessel perfusion in the implanted constructs, host mice were perfused intravenously with Dextran-TRITC 2,000,000 MW for 15 min before the constructs were harvested.

For hematoxylin and eosin (H&E) staining, fixed samples were embedded in paraffin blocks, allowed to cool, and sectioned at 6µm depths. Slides were rinsed twice in 100% xylene for 10 minutes, followed with two 3-minute rinses in 100% and one rinse in 95%, 90%, 80%, and 70% ethanol. Samples were then placed in a 1-minute rinse in deionized and distilled water (ddH₂O). Slides were stained for 10 minutes in hematoxylin. After 2 minutes in ddH₂O, slides were dipped in acid ethanol, ddH₂O water, ammonia water, and then rinsed in ddH₂O water for 20 minutes before eosin application for 20 minutes. Samples were then dehydrated in 95% ethanol followed by 100% ethanol. All slides were finally rinsed in 100% xylene prior to permount and coverslip application.

Quantitative Image analysis

To estimate the vascular volume index fraction, individual image slices of confocal image stacks obtained at 5X magnification were brightened and background-subtracted using Matlab (Mathworks). These processed images were then imported into NIH ImageJ as 8-bit image stacks to generate an average Z-projected image and enhanced further (sharpening, level equalization, contrast enhancement, and image normalization). A histogram of the Z-

projected image, the mean pixel intensity and its standard deviation were estimated and used as inputs to a custom Matlab code [14] to generate an automated threshold for each image for uniformity. Additionally, the non-vessel like artifacts of imaging and thresholding were filtered out and the cleaned image was used for estimation of the vascular area fraction per image in Matlab[3]. The vascular area fraction was then weighted to the number of sections used to generate the Z-projected image to give a 'vascular volume index', which was then evaluated for changes with test conditions.

To calculate the percentage of mural cell coverage, image stacks of explanted constructs immunostained for α -actin were acquired containing sequential green (endothelial cell) and red (mural cell) channel slices. Image stacks were imported into NIH ImageJ and converted to 8-bit grayscale images. The images were then imported into Amira® (Visage Imaging, San Diego, CA), corrected for variations introduced by imaging depth, deconvolved (N.A. = 0.5, λ = 0.52 or 0.57 based on color, η = 1.3), and filtered based on methods described earlier[14]. An intensity-threshold value was estimated manually for each image stack and used to binarize the images to select vessel and mural cell features from the respective image stacks. Non-vessel objects (identified in the green channel) were manually de-selected for analysis from this segmented image data. The total volume of structures present after thresholding in each channel of each slice in a stack was then estimated from these segmented images. The number and volume of areas of overlap between the two channels were identified using the 'logical and' operation in Amira®. The segmented and overlap results were always visually compared to the original image stacks at critical steps to assure validity of this process. The percent coverage of vessels by mural cells (expressed as mean $\pm$ SE) was calculated by dividing the total volume of overlapped structures by the total vascular volume. An independent sample t-test was performed to determine significant differences in this ratio between BMF and FMF from a total of 3 representative samples for each condition.

Evaluation of vessel permeability

Low and high molecular weight protein permeability was evaluated by intravenous perfusion of host mice with a combination of 100 μ l of 3,000MW Dextran-TRITC (10mg/ml, Invitrogen) and 50 μ l of the bioluminescent protein luciferase (800 μ g/ml, Sigma). After 45 min, the chest was opened and mice were perfused with saline through the left ventricle until colorless perfusion fluid was obtained from the right atrium. Constructs, as well as fat and brain tissue from host, were removed and Dextran-TRITC and Luciferase were extracted by grinding the tissue with a chilled micro-homogenizer and lysed in reporter lysis buffer (Promega). Protein concentration was detected by BCA assay (Pierce). Low molecular weight permeability was calculated by detection of Dextran-TRITC extravasation in 100 μ l of lysate in a fluorimeter (BioTek). Permeability to high molecular weight proteins was detected with the use of a Luciferase Assay System kit (Promega) [17]. Briefly, 20 μ l of lysate was dispensed into a multiwell plate into a luminometer (Orion Microplate Luminometer, Berthold Detection Systems) with injector that adds 100 μ l of luciferase assay reagent per well. The light produced is immediately read before advancing to the next well for a repeat of the inject-then-read process. Raw values obtained were normalized to protein concentration and to each respective host tissue to account for differences in perfusion/flush between animals. To eliminate differences due to different vessel area and perfusion, vessel permeability was also normalized to vascular index and percentage perfusion (vascular-perfusion index).

Statistical analysis

When not specifically indicated, significance was calculated using Kruskal-Wallis One Way Analysis of Variance on Ranks. Differences were considered significant if $p < 0.05$.

Results

Brain-derived microvessel fragments retain neovascularizing potential

The likely presence of pro-vascular mesenchymal stem cells in association with adipose microvessels[18] and the plasticity of adipose-derived microvascular cells[31] prompted us to address the possibility that only adipose-derived microvessel elements retain spontaneous neovascularizing potential. To address this, we investigated whether or not microvessels isolated from brain cortex, a very different tissue than adipose, exhibited the same neovascularizing behavior. As with fat-derived fragments, isolated BMFs maintained their vessel structure with endothelial cells lining the vessel lumen surrounded by perivascular cells positive for α -smooth muscle actin (Figure 1A–C). Astrocytes, cells normally associated with the microvasculature of the brain, were not present in the isolated BMFs (data not shown). Also similar to FMFs, BMFs formed angiogenic sprouts around day 3 post-seeding (Figure 1D) when cultured *in vitro*. Sprouts continued to elongate and formed an interconnected network of neovessels in a manner qualitatively similar to FMFs[12].

As with FMFs[21], BMFs formed a perfused microvasculature when implanted subcutaneously in SCID mice as a microvascular construct. In histology sections, red blood cells are present in microvessels of the constructs by 4 weeks after implantation in a multicellular vessel wall indicative of a mature morphology (Figures 1E–1F). The presence of mature vessels containing perivascular cells was confirmed by en bloc staining of BMF constructs for smooth muscle actin (Figure 1F). BMF-derived neovasculatures persisted up to 8 weeks after implantation (Figure 2A) and there was a tendency to a higher vascular index at 4 weeks when compared to 8 weeks after implantation and to FMF constructs at both 4 and 8 weeks (Figure 2B).

Vessel perfusion was verified by the presence of the Dextran-TRITC blood tracer injected intravenously into the host mouse. Dextran fluorescence was routinely observed within the microvessels of the implanted constructs (Figure 3) demonstrating that implanted microvessels inosculate with the host circulation and are perfused by the host. Vessel perfusion in BMFs was significantly higher at 8 weeks post implantation ($94.5 \pm 3.1\%$) than at 4 weeks ($40.1 \pm 16\%$). Interestingly, perfusion in BMF constructs after 4 weeks was often regionalized, with areas showing no perfusion (data not shown). Perfusion in BFM implants at 8 weeks was also significantly higher than in FMF implants at both 4 and 8 weeks after implantation.

Fragment-derived neovascularization is modulated by peri-vascular cells

These results demonstrate that the ability of isolated microvessel fragments to neovascularize a tissue space and form a microcirculation is not specific to any one tissue bed. There remains the question as to what role, if any, vascular accessory cells play in neovascularization. As with other tissues, the brain microvasculature is intimately associated with cells unique to the brain that impart specialized functions to the microcirculation[9]. For example, astrocytes interact with microvessels through foot processes to form a series of interconnections between microvessel elements[9]. Through an, as of yet, incompletely described mechanism, astrocytes are important in establishing the blood brain barrier (BBB) [32] characterized by a relatively low permeability to molecules[16].

We next tested the hypothesis that the inclusion of astrocytes into the microvascular constructs would induce adipose-derived microvessel fragments to form a more “brain-like” microcirculation. Because astrocytes have not yet been successfully isolated and cultivated, we used precursor cells that can be induced to form astrocytes called glial restricted precursors or GRPs. GRPs preferentially differentiate into glial fibrillary protein-expressing cells when implanted *in vivo* in neonatal and adult brains[11]. FMFs co-implanted with

GRPs also formed a neovasculature. However, the vascular index of FMF+GRP implants after 4 weeks was significantly higher than of FMF only implants after 4 and 8 weeks and of FMF+GRP after 8 weeks (Figure 4). After 8 weeks of implantation, the difference in vascular index between FMF+GRP and FMF implants disappeared (Figure 4B). The vascular volume index of implanted MVCs formed with FMFs alone did not change appreciably between weeks 4 and 8 (Figure 4B). Interestingly, the inclusion of GRPs with FMFs resulted in a vascular index more similar to that of BMF-derived constructs implanted for 4 weeks (Figures 2B, 4B). Vessel perfusion was significantly higher in the FMF implants when GRPs were included (FMF+GRP, $81.4 \pm 5.4\%$) than in FMF alone implants ($59.4 \pm 8.9\%$) at 4 weeks after implantation (Figure 5). After 8 weeks, FMF implants were still significantly less perfused ($74.5 \pm 5.9\%$) than FMF+GRP ($97 \pm 1.1\%$).

Vascular permeability of construct microvessels is affected by peri-vascular cells

Since reduced vessel permeability due to the presence of the BBB is one of the characteristic features of brain vessels, we performed a permeability assay for low (3kDa, Dextran-TRITC) and high (60 kDa, Luciferase) molecular weight macromolecules in the implanted constructs. With the assay, host adipose tissue was determined to be 20X more permeable than brain tissue to low molecular weight proteins (Figure 6A) and 15X more permeable to high molecular weight proteins (Figure 6A) at 4 weeks after construct implantation. This is in accordance with BBB properties of brain vessels and demonstrates the utility of the assay. More importantly, the microcirculation that formed in the implanted (4 weeks) microvascular constructs by FMFs was relatively very leaky to both molecular species. However, the presence of GRPs significantly reduced macromolecular permeability in the FMF-derived microvasculatures to a level similar to that of the BMF-derived constructs (Figure 6B–6C). Interestingly, there was no significant difference between the percentage of perivascular cell coverage in BMF- and FMF-derived networks (Figure 7), suggesting that vascular bed-specific cell characteristics are responsible for the differences observed.

GRPs form astrocyte-like cells that are associated with construct microvessels

When included with FMFs, GRPs maintained their ability to differentiate into astrocytes in the constituted implants as demonstrated by positive staining for glial fibrillary acidic protein (GFAP), an astrocyte marker (Figure 5, 8). GRPs also expressed aquaporin-4 (AQP4), a water channel protein strongly expressed in the brain in the foot processes of peri-vascular astrocytes[26] (Figure 8A). Furthermore, implanted GRPs closely interacted with the adipose-derived neovessel networks and were (39.7%, 4 weeks) found integrated with the nascent vasculature (Figure 8B, C). In addition, endothelial cells of construct microcirculation express the brain endothelial marker glucose transporter-1 (Glut-1) after 8 weeks post-implantation when co-implanted with GRPs but not in the absence of GRPs (Figure 9).

Discussion

We have previously demonstrated that microvessel fragments derived from adipose and suspended in 3D collagen gels form a stereotypical microcirculation when implanted[21,29]. In this system, neovascularization is spontaneous and does not require the addition of factors or exogenous agents. Because adipose tissue is rich in pro-angiogenic cells[2,19,25,27], we considered the possibility that neovascularization by the adipose-derived microvessel fragments (FMFs) could simply reflect a pro-vascular potential unique to adipose tissue. However, the observation that microvessel fragments from brain cortex (BMFs) can also generate a new microcirculation indicates that the neovascularizing potential of isolated microvessel fragments is not limited to adipose sources and is likely tissue independent. Microvessel fragments from both adipose and brain isolates consisted of intact microvessels

comprised of endothelial cells and perivascular cells positive for α -actin (this study and [12]). Presumably, these two general cell populations are responsible for the neovascularizing potential of the microvessel fragments and likely are necessary for the formation of the new microcirculation (endothelial cells without perivascular cells are inefficient at producing persistent implanted microcirculations[13]). If so, it suggests that regardless of the tissue source, as long as the microvessel fragments contain these requisite cell populations, they should retain the ability to neovascularize a tissue space.

Interestingly, the network that was formed by BMFs, while qualitatively similar, was not identical to that formed by FMFs. BMF-derived microvasculatures displayed a higher vascular density (measured as a vascular index) at the early time point and were significantly less permeable to both high and low molecular weight molecules as compared to adipose-derived microvasculatures. BMF-derived vasculatures also displayed a number of areas of no-perfusion at 4 weeks post-implantation, a possible indicator of vessel network immaturity. However, after 8 weeks, areas displaying no perfusion are not seen in the constructs, suggesting that either perfusion has increased and reached areas that were not perfused or that non-perfused vessel segments were excluded from the neo-vasculature.

While it's clear that the microvasculatures of the respective sources function within very different tissue environments, the isolation of the microvessel fragments removes them from this environment and, consequently, should remove any direct influence the tissue environment might have on a neovascularization response. However, because the isolates are enriched for fragments and are not pure preparations (unpublished observation), there is the possibility that an additional, non-vascular cell type is present in the microvessel fragment isolate of either preparation that may mediate differences in the neovascularization response. If there are additional non-vascular cells involved, then those present in the brain are likely different than those associated with the adipose-derived fragments. Unlike adipose, brain cortex is derived from non-mesenchymal germ tissue[30] and contains a limited number of mesenchymal stem cells[36]. On the other hand, it's becoming clear that certain populations of vascular mural cells (i.e. pericytes) present within the walls of capillaries and distal microvessels of multiple organs, including brain, can act as regenerative units[4–6,8] and may influence the character of the new microvasculature that forms from the fragments. Microvessels formed by BMFs were significantly less permeable than the ones formed by FMFs indicating that brain vessels retain some characteristics of the blood brain barrier (BBB), a vascular structure unique to the CNS[16], after *in vivo* implantation. Yet, we found no evidence that astrocytes, cells closely associated with brain microvessels and known to influence BBB permeability[32] were present in the BMF preparations (data not shown) nor did BMF-derived vessels contain a more extensive perivascular cell coverage (which might have restricted permeability) than FMF-derived vessels. However, we can't rule out possible epigenetic effects on endothelial and perivascular cells in BMF vessels caused by brain parenchyma cells that may persist and contribute to the differences observed between BMF-and FMF-derived neovasculatures. Brain pericytes of the distal microvessels can induce higher electrical resistances and lower permeability in co-culture systems with non-brain endothelial cells *in vitro*[7,20]. Since vessel fragments contain both endothelial and perivascular cells (likely including pericytes), the presence of brain pericytes could explain why brain-derived vessels still form a less permeable vascular bed even in the absence of astrocytes. Whether analogous tissue-specific differences in the ability of the respective microvasculatures to undergo angiogenesis and neovascular remodeling also exist is not clear.

Interestingly, the addition of cells with astrocyte-like functionality, glial restricted precursors (GRPs), to the FMFs resulted in a more brain-like neovascularization response by the adipose fragments. Similar to BMFs, co-implantation of FMFs with GRPs resulted in a

higher initial vascular index, produced a less leaky microvasculature, and induced the expression of the brain endothelial cell marker Glut-1 in the FMF-derived neovessels, the expression of which correlates with the tightening of the blood brain barrier in the mouse embryo[1]. GRPs can produce angiogenic factors[15], which would explain the increased vascularity in the co-implants. It is also possible that, given the fact that in the presence of GRPs FMF-derived neovasculatures are significantly more perfused, GRPs could be enhancing inosculature thereby increasing the number of vessel elements carrying blood which may in turn promote vessel maturation and persistence. We have previously demonstrated the importance of blood perfusion in vessel persistence and maturation in our MVCs where adipose-derived neovessels that are not perfused regress and display a significantly higher number of apoptotic cells after 4 weeks of implantation[21]. Others have shown that co-culture of astrocytes with non-brain endothelial cells *in vitro* can induce various BBB properties (i.e. transendothelial permeability and expression of brain specific markers)[10,22]. All of which is consistent with our observations *in vivo* with the microvascular implants. These findings also emphasize the importance of the presence of astrocytes in the induction of BBB properties *in vivo* and suggest that while the vascular cells (endothelial and perivascular) are required for neovascularization, stromal and other non-vascular cells, such as astrocytes, are required to assign tissue-specific function to a vascular bed.

We have shown that the inclusion of astrocyte precursors with the FMFs lead to a shift in vascular phenotype in the new microvasculature to a more, albeit limited, brain-like character (reduced permeability and upregulation of a BBB marker). This suggests that the implanted microvessels retain some plasticity and can be stimulated to change functional properties. From a therapeutic perspective, this plasticity expands the applicability of adipose-based, autologous microvascular implantation. Adipose is a relatively accessible and dispensable tissue source that can provide large numbers of microvessel fragments suitable for implantation. It could be possible, as our findings suggest, to manipulate the fragments to take on features characteristic of the target implant site. On the other hand, the new tissue environment may provide sufficient stimulus to cause the newly constructed microvasculature to match the tissue site. For example, implantation of FMFs into a CNS position, where all relevant cues are present, may induce a more complete brain microvascular phenotype. Related to this, the vascular index between the microvascular beds derived from the two different tissue sources, while different early on, was similar after 8 weeks. Perhaps this reflects the strong influence of metabolic needs and other characteristics unique to the subcutaneous tissue environment on network maturation and structural adaptation[24,34]. Similarly, we have previously shown that FMFs transplanted onto the cardiac surface as a microvascular implant improved cardiac function following myocardial infarct[29]. While we did not examine the new microvasculature that formed for cardiac-specific characteristics, we did find that the vessel density within the implant was similar to that typically found in epicardium. Therefore, the plasticity of adipose-derived vessel fragments, coupled with the relative abundant availability and ease of harvest of adipose (i.e. via liposuction), could make the adipose-derived microvessel constructs ideally suited for autologous revascularization therapies. Furthermore, the microvessel fragments could serve as a powerful experimental model for the study of vascular cell dynamics and plasticity in neovascularization including brain angiogenesis. Moreover, since this model allows for the reconstitution of different vessel components (vascular + perivascular) and regenerative cells (stem cells or precursors), it can also be used as a tool to investigate different aspects of cell-cell interaction and activity in the context of revascularization and to assess the role of these cells in the neovascularization process.

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Figure 1.

Angiogenic potential of isolated brain cortex microvessel fragments (BMFs). A, Phase contrast micrograph of freshly isolated brain cortex microvessel fragments (arrows) before suspension in collagen matrix. B, Higher magnification of a brain microvessel fragment showing vessel structure comprised of a lumen, endothelial cells and a perivascular cell layer. C, Brain microvessel fragments isolated from tie2-GFP transgenic mice in which GFP is expressed by only endothelial cells (EC) and subsequently stained for α -smooth muscle actin (SMA – red) marking perivascular muscle cells. D, Brain microvessel fragment sprouting (arrows) in culture in 3-dimensional collagen type I gels. E, H&E stain of a section of BMF-derived construct implanted subcutaneously for 4 weeks showing microvessels containing red blood cells (white arrow) and multi-cellular vessel walls (black arrow). F, Confocal image (Z projection) of part of a microvessel network derived from BMFs implanted for 4 weeks. Green, endothelial cells; Blue, α -smooth muscle actin.

Figure 2.

Vessel network formation by brain microvessel fragments implanted subcutaneously for 4 and 8 weeks. A, Confocal images (Z projections) of microvessel networks at 4 and 8 weeks post implantation stained with GFP conjugated Griffonia simplicifolia I (*GSI*) lectin. B, Vascular index of implanted constructs was obtained from volume rendered image stacks of vascular networks as described in methods section. Micron bar shown is the same for all panels.

Figure 3.

Vessel perfusion analysis of BMF-derived constructs implanted for 4 and 8 weeks. A, Confocal images (Z projections) of vessel networks in constructs prepared with GFP-BMFs (green) and implanted subcutaneously. Perfusion was assessed by intravenous injection of high-molecular weight Dextran-TRITC (red) into host mice. B, The % of vessels perfused (fraction of green vessel area exhibiting red fluorescence) was obtained from 3D images of vascular networks as described in methods section. * $p < 0.05$. Micron bar shown is the same for all panels.

Figure 4.

Vessel network formation from microvessel fragments implanted for 4 and 8 weeks. A, Confocal images (Z projections) of networks formed from implanted constructs containing either fat-derived microvessel fragments (FMF) or FMF combined with glial restricted precursor constructs (FMF+GRP) stained with GFP conjugated *Griffonia simplicifolia I* (GSI) lectin. B, Vascular index of implanted constructs was obtained from volume rendered 3D image stacks of vascular networks after implantation. * $p < 0.1$. Micron bar shown is the same for all panels.

Figure 5.

Vessel perfusion analysis of implanted vessel fragment constructs. A, Confocal images (Z projections) of vessel networks formed from GFP vessels perfused with high-molecular weight Dextran-TRITC (red) by intravenous injection of host mice. Differentiated astrocytes were detected by immunostaining for GFAP (blue). B, Vascular index was obtained from volume rendered 3D image stacks of networks formed after *in vivo* implantation. * $p < 0.05$. Micron bar shown is the same for all panels.

Figure 6.

Co-implantation of adipose-derived vessel fragments with GRPs induces a significant change in vessel permeability after 4 weeks. A, Vessel permeability to small molecular weight molecule (Dextran 3,000MW) and high molecular weight protein (Luciferase, ~61 kDa MW) in brain and adipose tissue. B,C Vessel permeability to the small dextran tracer (B) and to the larger luciferase tracer (C) in implanted constructs. Low and high molecular weight molecule permeability was evaluated by intravenous perfusion of host mice with Dextran-TRITC and luciferase. After flushing the blood out, quantification of vessel leakage was performed by analysis of Dextran-TRITC fluorescence and luciferase luminescence. To eliminate differences due to different vessel area and perfusion, vessel leakage was normalized to vascular index and percentage of perfused vessels (vascular-perfusion index).

* $p < 0.05$.

Figure 7. Perivascular cell coverage in neovasculatures after 4 weeks implantation. A, Staining for α -smooth actin (red) in BMF- and FMF-derived neovasculatures (green). B, Percentage of perivascular cell coverage (fraction of red staining overlap with green) was obtained from 3D images of vascular networks as described in methods section.

Figure 8.

GRPs associate with FMFs. A, GRP co-implanted with FMF for 4 weeks express glial fibrillary acidic protein (GFAP) and aquaporin 4 (AQP4). B, C, FMF+GRP implanted for 4 weeks. GRP-derived astrocytes invest adipose derived vessels.

Figure 9. Co-implantation of FMF with GRPs induces expression of the brain endothelial marker GLUT-1. FMF implanted for 8 weeks do not express Glut-1. Expression of Glut-1 is induced in adipose-derived vessels after co-implantation with GRPs for 8 weeks.