Published in final edited form as:

Microvasc Res. 2010 January ; 79(1): 10. doi:10.1016/j.mvr.2009.10.001.

Implanted Microvessels Progress through Distinct Neovascularization Phenotypes

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Abstract

We have previously demonstrated that implanted microvessels form a new microcirculation with minimal host-derived vessel investment. Our objective was to define the vascular phenotypes present during neovascularization in these implants and identify post-angiogenesis events. Morphological, functional and transcriptional assessments identified three distinct vascular phenotypes in the implants: sprouting angiogenesis, neovascular remodeling, and network maturation. A sprouting angiogenic phenotype appeared first, characterized by high proliferation and low mural cell coverage. This was followed by a neovascular remodeling phenotype characterized by a perfused, poorly organized neovascular network, reduced proliferation, and re-associated mural cells. The last phenotype included a vascular network organized into a stereotypical tree structure containing vessels with normal perivascular cell associations. In addition, proliferation was low and was restricted to the walls of larger microvessels. The transition from angiogenesis to neovascular remodeling coincided with the appearance of blood flow in the implant neovasculature. Analysis of vascular-specific and global gene expression indicates that the intermediate, neovascular remodeling phenotype is transcriptionally distinct from the other two phenotypes. Therefore, this vascular phenotype likely is not simply a transitional phenotype but a distinct vascular phenotype involving unique cellular and vascular processes. Furthermore, this neovascular remodeling phase may be a normal aspect of the general neovascularization process. Given that this phenotype is arguably dysfunctional, many of the microvasculatures present within compromised or diseased tissues may not represent a failure to progress appropriately through a normally occurring neovascularization phenotype.

Keywords

neovascularization; neovessel; sprouting; angiogenesis; microvascular remodeling; microcirculation; structural adaptation; gene expression

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INTRODUCTION

Tissue neovascularization is the process by which vessel segment number and blood perfusion pathway lengths are increased and organized into a functional vascular bed. Perturbation of the vascularization process is often an important contributor to a disease condition. For example, the solid tumor microvascular network is generally poorly formed (Ryschich et al., 2002) resulting in disorganized flow paths and compromised perfusion (Secomb et al., 2004). This, in turn, contributes to the dysfunctional tumor microenvironment and the therapeutic complications often associated with treating cancer (Bhujwalla et al., 2001). Similarly, unstable vascular networks often associated with ischemic tissues can lead to further tissue insult, such as in myocardial infarct no-reflow, due to un-sustained and diminishing tissue perfusion (Ito, 2006).

Therapeutic angiogenesis is a treatment strategy designed to improve the vascularization of ischemic tissues. But here too, problems related to maintaining the newly formed vasculature compromises therapeutic efficacy (Simons et al., 2000; Bouis et al., 2006). It is becoming clear that good tissue vascularization does not depend solely on the addition of new vessel segments to a network (i.e. angiogenesis). The post-angiogenesis maturation events necessary to form an effective perfusion network and establish microvascular stability are proving just as, if not more, important. However, little is known concerning the mechanisms mediating post-angiogenesis maturation.

Previously, we described neovascularization occurring in implanted microvascular constructs comprised of isolated, intact microvessel elements derived from adipose tissue (Shepherd et al., 2004). The sprouting angiogenesis-derived neovessels present within the implant developed into mature, functional microcirculatory beds; the vessels of which were derived from the microvessel elements originally placed into the construct (Shepherd et al., 2004). Although it was clear that the implanted constructs had vascularized, it was not clear as to how individual neovessel elements and networks changed over time leading to the stable microcirculation. In this study, we characterized neovascularization in the implanted constructs in greater detail to better understand the progressive nature of vascularization and begin identifying putative, key regulatory points in neovascular progression.

MATERIALS AND METHODS

Microvessel isolation and implantation

Microvessel fragments (MF) were isolated from male (epididymal fat) and female (uterine horn fat) *tie2:GFP^{Sato}* mice (Motoike et al., 2000) by limited collagenase digestion and selective screening as previously described (Shepherd et al., 2004; Hoying et al., 1996). The type and lot number of collagenase used was pre-determined to optimize fragment yield while maintaining microvessel structure. Microvascular constructs were prepared at a density of 20,000 fragments per ml of 3 mg/ml type I collagen solution and immediately implanted in subcutaneous pockets on the flanks of anesthetized SCID mice as previously described (Shepherd et al., 2004). Two constructs were implanted per SCID mouse, one per flank, in subcutaneous pockets made through individual small incisions using a small, curved hemostat and were harvested together after 1 week, 2 weeks, 3 weeks, or 4 weeks.

Vessel density, perivascular cell identification, proliferation, and apoptosis

Implants were removed with the associated tissue attached, fixed in 2% paraformaldehyde/PBS and processed into paraffin. Vessel density was determined by counting discreet, GS-1-positive structures and perivascular cells were identified as previously described (Shepherd et al., 2004). For the proliferation measurements, 100 μ l of a BrdU (30 mg/kg body weight in

saline) solution was injected IP 24 hours and again at 8 hours prior to harvesting the constructs. The ratio of the total number of BrdU-positive nuclei (detected by immunohistochemistry) to total nuclei (DAPI-positive) was determined for 5 different fields per each of 5 sections from three different implants per time point.

To assess apoptosis, the DeadEnd Fluorometric TUNEL assay (Promega) was used following manufacturer's instructions to evaluate cell viability in non-perfused implants. Briefly, samples were embedded in paraffin blocks, sectioned at 6µm depths, fixed with 4% formaldehyde and permeabilized with Triton X-100. Sections were incubated for 1 hour at 37°C with a combination of fluorescent nucleotide mix and recombinant terminal deoxynucleotidyl transferase. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Five different fields of 5 non-consecutive sections were utilized for the evaluation of apoptosis in the constructs.

Vessel diameter measurement

Implants were removed, fixed in 4% paraformaldehyde/PBS for 2 hours and imaged with an Olympus BX61SWI Confocal Microscope. After volume reconstruction on Amira 5.2 (Visage Imaging, Inc, CA) inner vessel diameter was measured for each vessel segment present in different time points. Significance was calculated using Kruskal-Wallis One Way Analysis of Variance on Ranks. Differences were considered significant if $p < 0.05$.

Dextran perfusion

Blood flow in constructs was visualized by using a rhodamine-conjugated dextran (2 million MW) injected (300 µl of 50 mg/ml dextran in saline) via the tail vein following exposure of the implanted construct. After 5 min., the constructs were examined by epifluorescence using a Zeiss ACM intravital microscope fitted with a 63× water immersion objective (Zeiss) and videotaped via a SIT camera. The movement of blood cells (seen as dark objects in a background of fluorescence) served as an indicator of blood flow. GFP fluorescence identified implant vascular fields. During the entire procedure, the exposed tissues were bathed with Hanks balanced salt solution. Following real-time imaging, constructs were explanted, fixed in 2% paraformaldehyde for 3 hours, and then imaged by confocal microscopy.

Microarray hybridization and real-time PCR

The RNA isolation, microarray hybridizations and data extraction/analysis were performed as previously described (Schwartz et al., 2005) using custom made cDNA microarrays (Genomic Research Laboratory, <http://www.grl.steelecenter.arizona.edu/>) derived from the NIA 15K mouse cDNA clone set (<http://lgsun.grc.nia.nih.gov/cDNA/15k.html>) with each clone being printed in duplicate and a custom ANOVA-based analysis software package (Greer et al., 2006). For each time-point, total RNA was pooled from 4- 6 implanted constructs prepared with microvessel fragments harvested from adipose collected from 10 – 12 *tie2:GFP^{Sato}* mice. For the day 0 time point, microvessel fragments were isolated and then immediately processed for RNA. Each sample was single-pass amplified (O'Dell et al., 1998) and hybridized to 4 separate microarrays with 4 separate but different time-point samples according to an interwoven loop experimental design (Greer et al., 2006) (Supplemental Figure 1) resulting in 4 measurement replicates (2 with the red label and 2 with the green label preparations) for each sample. This entire process was repeated a second time, using new constructs and new RNA samples for the second round. Microarray measurements from both runs were combined for subsequent analysis. Specific transcripts were measured by Sybr green-based real-time PCR on amplified RNA with a Corbett RotorGene thermocycler and primers (Supplemental Table 1) designed from Genbank sequences using Primer3 software (<http://frodo.wi.mit.edu/>) as previously described (Schwartz et al., 2005). Only genes that were consistently, confidently measured (background subtracted intensity > 2 * local background standard deviation) for at

least one sample were used in the gene-by-gene ANOVA analysis. Genes were identified as differentially expressed based on a shrunk ANOVA p-value based on the James-Stein shrinkage concept (Cui et al., 2005) of less than or equal to 0.05 for the time-point term after FDR adjustment (Benjamini and Hochberg, 1995). As part of the ANOVA, estimates of differences in gene expression between each of the explanted samples and the day 0 sample were generated. These values were mean centered and unit normalized and then clustered using the Ward's minimum variance algorithm. Expression changes of 15 randomly selected genes (8 known transcripts and 7 novel transcripts) from the microarray gene set measured by qRT-PCR closely correlated (Pearson's correlation method; correlation coefficient, r^2 of 0.8153) with those made by the microarray (Supplemental Figure 1). Raw expression data (as tab delimited files) and CARMA outputs (as PDF files) are available as online supplements.

RESULTS

By 7 days of subcutaneous implantation, the freshly prepared, non-cultured microvascular constructs (prepared from *tie2:GFP^{Sato}* mice) contained arrays of neovessels originating from the parent microvessel fragments initially seeded into the constructs (Figure 1A). By 14 days, neovessels of irregular morphology are present within an atypical network (Figure 1B). By 21 days, a more recognizable vascular tree has formed (e.g. large vessels branching into smaller vessels) (Figure 1C). Finally, by 28 days post-implantation, construct vessels comprised a classical vascular tree with smooth branch sites, typical heterogeneity in vessel diameters, and contiguous lumens (Figure 1D). Vessel density in the implants, as measured by the density of vessel segments (inter-nodal elements), is highest at day 7 but reduced to a similar level across all other later time points (Figure 1E).

As measured by BrdU incorporation, proliferation is highest at day 7 of implantation, is diminished by day 14, and is significantly lower than day 7 by 21 and 28 days post implantation (Figure 2). At 7 days, proliferating cells are distributed throughout the construct (Figure 2A). While in day 28 implants, proliferating cells are restricted to the wall of large-caliber vessels (Figure 2B), suggesting vessel wall remodeling is occurring in these later time points. Concomitantly, apoptosis within the implants, assessed with a TUNEL assay, is $5.1 \pm 1.1\%$ at day 7, increases to $18.1 \pm 6.2\%$ at day 14 and decreases significantly by days 21 and 28 post-implantation (2 ± 0.7 and $1.2 \pm 0.9\%$, respectively) (Figure 2).

An analysis of vessel diameter, as an indicator of hierarchical organization, showed that vessels in early-stage constructs (days 7 and 14 post implantation) were of uniformly small caliber (Figure 3A). However, in the later time points (days 21 and 28), vessel diameters were more heterogeneously distributed with a larger proportion of diameters being larger than $15 \mu\text{m}$ (Figure 3B).

Previously, we have shown that there is a progressive increase in perivascular cell coverage in implanted constructs (Shepherd et al., 2004). To complement this earlier work, we next evaluated the morphology of perivascular cell-association with the neovasculature within the implants. Consistent with the previous analysis, day 7 implants contained α -actin-positive cells that were diffusely distributed along the neovessels and throughout the construct interstitium (Figure 3B). In the day 14 implants, perivascular cells were associated with neovessels of an immature network, but they exhibited an immature (as compared to day 28) morphology (Figure 3B). In the later stages, when the construct vasculature resembled a more typical circulatory network, the morphology of the perivascular cells associated with the vessels was characteristic of mature, differentiated vessels (Figure 3B). In addition, α -actin-positive cells were seldom observed within the inter-vessel spaces.

To assess flow dynamics during vascularization, we examined blood perfusion within the implants by confocal microscopy and real-time intravital video imaging following the intravascular injection of rhodamine-conjugated dextran into the host mouse vasculature. While the dextran blood tracer was present in implant vessels for all time points (Figure 4), there was a progressive increase in the % of construct vessels perfused over the time-points examined reaching $91.4 \pm 4.1\%$ at day 28 implants (Figure 4). Interestingly, rhodamine fluorescence in the day 7 implants was restricted to isolated regions of GFP-positive, often dead-ended vessels located at the construct periphery (Figure 4). Presumably, the dextran tracer entered these vessels due to the leakage of plasma through the walls of dead-ended vessels (Guerreiro-Lucas et al., 2008) that had inosculated with the host circulation. Intravital imaging of rhodamine-dextran perfusion within the constructs of day 14, day 21 and day 28 implants clearly indicated a network of vessels. However, there were flow pathways in the day 14 constructs that were disorganized or involved sluggish flow (Figure 5A, B). In one instance, blood flow from a smaller caliber vessel drained into that of a larger vessel and then flowed out of that same larger vessel through an adjacent, downstream branch (Figure 5A). Interestingly, this same downstream vessel spontaneously constricted at the branch point during the observation period (Figure 5A), suggesting vasoactive dysfunction. Qualitatively, perfusion within day 28 implants appeared consistent and oriented normally with large vessels feeding smaller, branching vessels on the arterial side and small vessels draining into larger vessels on the venous side (Figure 5C, D).

The vessel morphologies, flow patterns, and other characteristics of the implant vasculature, suggested that there were three general neovascularization phenotypes represented by day 7, day 14, and day 21/28 implants, respectively. We used vascular-related and global gene expression measurements to further classify the different neovascular phenotypes or phases in the implants at the transcriptional level. The expression patterns of genes exhibiting differential expression between at least two time points, including day 0, (Supplemental Data 1) were clustered into 5 general patterns over the time course examined (day 7 through day 28) (Figure 6). Additionally, select vascular-related genes not detected by the microarray were measured by real-time PCR (Supplemental Figure 2) and fit into the clusters. The first pattern is comprised of genes, including *VegfA*, for which transcript levels, although different from day 0, did not change over the course of implantation (pattern A, Figure 6). A pattern indicative of angiogenesis involved up-regulated gene expression at day 7 followed by a progressive down regulation for the remaining days (pattern B, Figure 6). As expected, known angiogenesis-related genes such as *Mmp14* are clustered in this group. Conversely, there were two patterns indicative of post-angiogenesis maturation in which gene expression was down-regulated by day 7 or day 7 and day 14 followed by up-regulation by day 28 (patterns D and E, Figure 6). Anti-angiogenesis genes, such as *Thbs2* (thrombospondin-2), and maturation/stabilization genes, such as *Pdgfb*, *Pdgfrb*, *Tgfb1*, *Ang1* and genes related to muscle differentiation (e.g. *Myo* genes and *Tgfb1*), belong to one of these two groups. An additional pattern reflected a unique up-regulation of gene expression at only the day 14 time point (pattern C, Figure 6). In addition to the vascular destabilizing *Ang2*, genes related to vascular function (phospholipase A2 (Bhagyalakshmi and Frangos, 1989)) and vascular network morphology (glomulin (Brouillard and Vikkula, 2007)) are included in this group.

Transcriptional reversal was commonly observed between the 1st week and the 3rd and 4th weeks, suggesting that the respective gene expression programs simply transitioned from one to the other. However, upregulation of genes specific to the 2nd week suggested that a gene regulation event unique to this phase might be present. We further analyzed the gene expression data by performing a principle component analysis (PCA), which reduces the dimensionality of a dataset to the prominent components or features of the data, to look for additional, emerging patterns using an NIA array analysis tool (Sharov et al., 2005) on the same mean-centered unit-normalized data used for the hierarchical clustering. The analysis identified 5 principal

components (PC) within the data (Supplemental Figure 3). By discarding components accounting for less than $(70/n)\%$ of the overall variability, where n is the number of conditions ($70/6 = 11.67\%$), components 4 and 5 were considered not significant and discarded. Of the remaining 3 components, principal component (PC)1 is largely explained by changes in gene expression at day 14; PC2 is almost completely explained by expression values from day 7; and data for days 21 and 28 contribute significantly to PC3 (Figure 7). Plotting the data on 3-dimensional coordinates with each coordinate representing one of the 3 main principal components shows that day 21 and day 28 plot near each other along the PC3 axis (i.e. day 21 and 28 values are near zero for the other 2 axes or components); the day 7 time point plots high on the PC2 axis but within the plane defined by the PC2 and PC3 axes; and the day 14 time point plots almost exclusively along the PC1 axis and coordinately separate from the other time points (Figure 7). Interestingly, the day 0 time point, included as a reference, plots along all 3 components; however, the plot point lies predominately in the plane defined by axes PC1 and PC3. Excluding considerations of day 0 events (which likely do not reflect neovascularization activity), the PCA results predict that 3 distinct transcriptional programs occur in vascularization and that each appears to coincide with the 3 distinct, neovascular phenotypes.

DISCUSSION

Previously, we have shown that isolated microvessel fragments suspended in 3D collagen I gels spontaneously form a stereotypical microcirculation when implanted (Shepherd et al., 2004). Here, we demonstrate that the formation of the new microvasculature in the implants occurs via a regimented neovascularization process involving three morphologically and transcriptionally defined vascular phenotypes (Table 1). The 1st phenotype, which coincides with the first week of implantation, is characterized by the presence of uniformly narrow neovessels relatively free of perivascular cells and extending from parent microvessel fragments. In addition, vessel segment density is elevated reflecting the relatively high cell proliferation and low apoptosis that is present. Consequently, we consider this early vasculature to have an *angiogenesis* phenotype to reflect the net production of new vessel segments. While not explicitly examined, it appears that sprouting angiogenesis and not intussusception is the primary vascular process relevant to this phenotype. This early phenotype preceded a second, distinct vasculature characterized by similarly narrow vessels which now have loose perivascular cell coverage and are arranged in a perfused (albeit limited) network. Furthermore, moderate cell proliferation coupled with a relatively high apoptosis suggests that considerable turnover is a feature of this vasculature. However, the reduced number of vessel segments in this phase as compared to the angiogenesis phase indicates that vascular pruning is occurring. Based on this, we consider this vasculature to have a *neovascular remodeling* phenotype to reflect the dynamic aspects of forming a new, perfusion-competent network from the neovessels produced during the angiogenesis phase. The subsequent and 3rd phase defined over the time course examined contained a vasculature characterized by a hierarchical (based on diameters) network of vessels. Furthermore, the presence of mature perivascular cell associations and the relatively low cell turnover (low proliferation and low apoptosis) suggests that the vasculature in this phase is stabilizing. However, proliferation in the walls of larger vessels indicate structural adaptation (Pries et al., 2005) and, therefore, continued adjustments in network organization/architecture (Pries et al., 1998). All of these features are characteristics of a *vascular maturation* phenotype.

Progression through the three defined phenotypes presumably involves two important transitional activities (Figure 8). A key feature of the angiogenesis phenotype is the presence of numerous individual neovessels while networks are a feature of the neovascular remodeling phenotype. Therefore, it seems likely that the network assembly marks a transition point between these two phenotypes. Presumably, any network assembly process in the construct depends on the ability of the sprouted neovessels to locate, extend toward and join with each

other. How this occurs within the construct is not yet known. Previously, we have shown in the microvascular construct that collagen fibrils preceding an advancing neovessel tip are aligned in the direction of neovessel growth and that neovessels align parallel to stress fields in the constructs (Kirkpatrick et al., 2007; Krishnan et al., 2008). Perhaps the alignment of collagen fibrils by an advancing neovessel combined with stress fields associated with this alignment creates a physical gradient of guidance cues directing neovessels to one another. Gradients of angiogenesis factors produced by the advancing neovessel tip could also be important. However, it's not clear how the gradient originating from one advancing tip would interface with the gradient from another advancing tip and still provide directional cues. With the neovascular remodeling phenotype, vessels have an immature morphology (lack formal perivascular structure and are uniformly narrow) that may be interpreted as being capillary-like or possibly even "generic" in character. In contrast, vessels in the later, mature vasculature have morphologies typical of specific microvessel types (i.e. arterioles, capillaries, and venules). Therefore, in transitioning to the vascular maturation phenotype, the immature vessels comprising the neovascular remodeling vasculature must be undergoing vessel-type specification as they take on a vascular maturation phenotype. In the developing vasculature, blood flow through the early vascular plexus is important in determining arterial-venous specification (Le et al., 2004). However, endothelial cells of the early plexus may be pre-specified as they express artery-side and venous-side markers prior to blood flow (Wang et al., 1998; Shin et al., 2001). Whether similar processes are active in the vasculature of the microvascular construct remains to be determined. Certainly, all three vessel types are represented in the fragment isolate used to initially make the construct (Hoying et al., 1996) suggesting that pre-specification is possible (neovessel sprouts could retain the type-specific aspects of their parent fragments). More targeted experiments are necessary before we can begin to determine the relevant mechanisms.

Vascular perfusion in the construct progressively increases throughout construct neovascularization. While the changes in perfusion levels may simply reflect changes in vascular structure and network architecture, it's possible that intravascular perfusion may serve as an important neovascularization cue. The appearance of blood, albeit to a very limited extent, in the dead-ended angiogenic neovessels of the implant precedes the appearance of the neovascular remodeling phenotype, suggesting that intravascular perfusion might initiate the first transition from angiogenesis to neovascular remodeling. Shear stress can suppress angiogenesis and related cell activities (Milkiewicz et al., 2001; Hudlicka et al., 2000; Tressel et al., 2007). In vascular development, a vascular plexus (i.e. simple network) appears before intravascular perfusion occurs (Jones et al., 2006) suggesting that blood flow into or through a neovessel is not essential for the formation of new intervascular connections. Whether the cues that prompt network assembly, independent of blood flow, in the embryo are also present in the implanted constructs remains to be determined. We have evidence that if the neovessels of the implanted construct failed to anastomose with the host circulation, and therefore were not perfused, then the construct vasculatures failed to form a microcirculation and subsequently regressed (Supplemental Figure 4). Interestingly, a neovascular remodeling-like phenotype was present early after angiogenesis suggesting that intravascular blood perfusion is not essential for early network formation. However, this preliminary evidence does suggest that the subsequent network stabilization and maturation characteristic of the vascular maturation phenotype does depend on blood perfusion. Shear stress associated with blood flow is known to promote a mature endothelial cell phenotype (Corson et al., 1996; Topper et al., 1996). Furthermore, hemodynamic forces influence vessel wall structure, individual vessel phenotypes, and vascular structural adaptation (Price et al., 2002; Skalak and Price, 1996; Sullivan and Hoying, 2002; Pries et al., 2005; Skalak, 2005; Le et al., 2005; Le et al., 2004). In addition, changes in pO₂ and plasma factors regulate vascular form and function (Rodriguez-Manzanique et al., 2001; Streit et al., 1999). The roles of the many different

components of blood and blood perfusion that are critical in neovascular progression in the construct remain to be determined.

There are a number of interesting observations related to the genes expressed in the constructs. For example, *VegfA* transcription did not change throughout the time course of neovascularization. Given its quintessential role in many instances of angiogenesis, one might expect *VegfA* expression to mirror that of *Mmp14* (a known angiogenesis-related gene) in which expression was highest in the early, angiogenesis-phase. However, *VegfA* is a regulator of a variety of different vascular cell activities and, therefore, may be playing different roles throughout the neovascularization process. The expression pattern observed in the implants reflects this. It's possible that regulation of VEGF-A activity in the implanted construct may rely less on transcription and more on other vascular mediators such as the angiopoietins, which are known to modulate vascular cell responses to VEGF-A (Visconti et al., 2002; Gale et al., 2002). Alternatively, or additionally, angiogenic factors other than VEGF-A, such as IL-3 (Dentelli et al., 1999) (IL3-R gene expression in the construct reflected the angiogenesis phase pattern) may be primary stimulators of angiogenesis in this model. Interestingly, Thrombospondin-2, a protein often thought to serve as an angiogenesis inhibitor, had a gene expression pattern consistent with the post-angiogenesis maturation phase; which raises the question as to whether Thrombospondin-2, and related genes, may play a role in vascular maturation beyond simply arresting/preventing the formation of new vessels. Additionally, *Glmm* (glomulin) a gene involved in proper vascular formation (Brouillard and Vikkula, 2007) is expressed at the highest levels in the neovascular remodeling phenotype suggesting that network patterning may be a central process in this phase. Many of the genes identified as expressed in the constructs have not been previously associated with the vasculature and may likely provide new avenues for investigation. As one example, the gene for SET and MYND domain 1 was strongly up-regulated in the neovascular remodeling phase at day 14. The SET and MYND family of proteins are modulators of chromatin function and act to either activate or repress transcription (Rea et al., 2000). SET and MYND domain 1 has been shown to be important in muscle differentiation in mouse and zebrafish development (Du et al., 2006; Gottlieb et al., 2002). Whether SET and MYND domain 1 plays a similar role in the differentiation of neovessels into mature vessel types and how chromatin regulation may mediate neovascularization remains to be determined. Finally, we identified a number of known or suspected ncRNAs with expression patterns that mimicked changes in protein-coding gene transcription and coincided with the distinct phenotypic changes in vessels undergoing vascularization (Supplemental Figure 5). Given the emerging regulatory role of ncRNAs in endothelial cell biology (Kuehbach et al., 2008; Kuehbach et al., 2007), it is likely that many of the ncRNAs identified here will prove relevant to neovascularization. Further examination of all the genes and gene classes identified, including novel neovascularization genes and known vascularization genes with new functionalities, may uncover new molecular avenues for therapies targeting the vasculature.

A recent study examining the transcriptional network governing the angiogenic switch in a model of chronic pancreatitis/cancer described a transitional pro-angiogenesis phenotype which shared transcriptional features of both quiescence and angiogenesis (Abdollahi et al., 2007). Although the pancreatitis study was examining the switch to angiogenesis, and not post-angiogenesis events as in our study, we observed similar types of transitional transcriptional patterns in the neovascularizing constructs. For example, there was a subset of genes expressed at day 14 in a similar manner as day 7 (Figure 6, pattern E) and a subset of genes similarly expressed at the day 14 and day 21/28 time-points (Figure 6, pattern D). However, we also observed a subset of genes that were uniquely up-regulated at day 14 (Figure 6, pattern C). This and the PCA analysis suggesting that day 14 global expression differs from day 7 and day 21/28 expression suggests that the neovascular remodeling phase is not simply a transition

state between angiogenesis and network maturation (i.e. an “average” of the two phenotypes), but a distinct vascular phenotype involving cellular and vascular processes unique to this phase.

The neovascular remodeling phenotype is reminiscent of microvasculatures observed in many pathological conditions. For example, the disorganized flow pathways and immature vascular morphologies present are very similar to many tumor microvasculatures (Ryschich et al., 2002). Given that both the preceding angiogenesis phase and subsequent vascular maturation phase appear to recapitulate normal vascularization processes (Sullivan et al., 2002; Yu et al., 2005; Kawata et al., 2001), it is likely that this neovascular remodeling phenotype is also a normal aspect of the general neovascularization process. Any dysfunctional aspect present within the neovascular remodeling vasculature may simply reflect an inherent and necessary instability in the neovascular network patterning and neovessel specification, which may be prone to further instability in the disease state. Indeed, the normalized expression patterns of *Ang1* and *Ang2* are consistent with the idea that stability (typically associated with a high *Ang1/Ang2* expression ratio) within the implant vasculature does not occur until after day 14. If this is indeed the case, then many of the microvasculatures present within compromised or diseased tissues may not represent a bona fide abnormal phenotype, such as observed in vascular malformations (Sundine and Wirth, 2007; Azuma, 2000; Liechty and Flake, 2008), but instead represent a failure to progress appropriately through a normally occurring neovascularization process. This suggests that therapies designed to promote neovascular progression towards stability might prove effective in a number of disease conditions and treatments, including therapeutic angiogenesis. Recent reports describing the stabilization of vessels contributing a therapeutic benefit to treating tumors in animal models supports this hypothesis (Tong et al., 2004; Hamzah et al., 2008). Also, our findings suggest that therapies intended to arrest angiogenesis may prove ineffective if the neovascularature has indeed arrested in this post-angiogenesis phase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

ACKNOWLEDGEMENTS

We thank Adam Hoying for the manufacture of the custom cDNA microarrays and additional technical expertise in scanning the hybridizations. This work was supported by NIH grants #HL077683, #HL67067, #EB007556 and an ABRC grant #06-027.

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Figure 1.

Neovessel and network morphology in implanted microvascular constructs. A-D) Epifluorescence images of GFP-positive vessels in explanted constructs that were implanted for 7 (A), 14 (B) or 21 (C) days. Arrows in the d7 panel point to parent microvessel fragments that have given rise to numerous neovessel sprouts. Panel D is a light micrograph of a construct implanted for 28 days and filled, via the host circulation, with dialyzed ink followed by clearance with glycerol. E) Vessel segment quantification in implants. * $p < 0.05$ as compared to day 7.

Figure 2.

Cell proliferation in implanted constructs. Analysis of proliferation (A-C) and apoptosis (D-F) on histology sections of explanted constructs implanted for 7 (d7) or 28 (d28) days. (A and B) Sections immunostained for BrdU to identify proliferating cells (arrows). (C) The percentage of all cell nuclei positive for BrdU at each time point. * $p < 0.05$ as compared to day 7 implants. (D and E) Sections stained by TUNEL show apoptotic cell nucleus in green (arrows) and nuclei staining by DAPI in blue. (F) The percentage of cell nuclei that were TUNEL positive at each time point. * $p < 0.05$ as compared to day 14 implants.

Figure 3.

Representative confocal images of microvessels in constructs en bloc immunostained for smooth muscle alpha actin (red) to identify perivascular cell morphology (A) in explants 7, 14, and 28 days after implantation. Endothelial cells were identified either by endogenous GFP fluorescence or staining with the lectin GS-1 conjugated to Alexa 488. A = arteriole; V = venule; arrow in day 7 panel indicates parent microvessel fragment. Characterization of network architecture in constructs at 7, 14, 21 and 28 days post implantation (B). Vessel diameter distribution in implants throughout network development. Measurements and statistical analysis were performed as described in the *Methods* section. Closed icons represent a significant difference in vessel diameter distribution ($p < 0.05$) as compared to its respective open icon.

Figure 4.

Perfusion of microvessels within implanted constructs. A-C) Merged representative confocal fluorescence images of implanted constructs explanted following rhodamine dextran injection into the host circulation showing the presence of the blood tracer dextran (red) inside GFP-positive construct vessels (green). D) Quantification of vessel perfusion. Vessels were considered perfused if positive for rhodamine-dextran. * $p < 0.05$.

Figure 5.

Blood perfusion and vessel morphology in constructs implanted for 14 or 28 days. A - D) Representative still images from real-time epifluorescence video-microscopy of exposed, day 14 (A, B) or day 28 (C, D) implanted constructs following an injection of a rhodamine-dextran blood tracer into the host circulation. Open arrow in panel B show vessels within the developing network lacking dextran fluorescence and, therefore, considered not perfused by blood. Panels A and B are also examples of perfused microvessels with atypical morphologies while panels C and D are examples of mature network morphologies. Closed arrows show the direction of blood flow as seen in the videos. The asterisk in A is adjacent to a vessel connection that spontaneously constricted during visualization. Micron bar shown is the same for all panels.

Figure 6.

Gene expression patterns in the implanted microvascular constructs. Each data point in the plots is the mean and standard deviation of the relative mean-centered and unit-normalized expression values (scaled from 1.0 to −1.0) for each time point (d7, d14, d21, and d28) for the total number of the genes (lower right corner) in the pattern. In all cases, expression was normalized to the day 0 measurements, which was set to “0”. Letters in the upper right corner refer to the pattern ID and are addressed in the text. Select genes belonging to each pattern are shown to the right of the pattern. Gene transcripts measured by real-time PCR (see Supplemental Figure 2) are shown in italics.

Figure 7.

Graphical results of the principal component analysis of all of the differentially expressed genes in the neovascularizing constructs. A) Plots of the relative impact of gene expression (Y-axis) occurring at each time point (X-axis) on the 3 primary principal components. The higher the Y-axis value for a given time point, the greater the contribution of that time point to the principal component. B) A 3-coordinate plot of the overall expression for each time point on the three main principal components (corresponding to the three different axes). Overall gene expression for the day 21 and 28 time points map together (blue circle) along the 3rd principal component (PC 3) axis. While those for day 14 (red circle) and day 7 (arrow) map on the 1st and 2nd principal component (PC1 and PC2) axes, respectively. In this plot, the larger the data point, the closer it is to the reader.

Figure 8.

Summary of the three phenotypes and two transitional activities in the neovascularizing microvascular construct.

Table 1

Summary of phenotype characteristics in implanted microvascular constructs*.

	Phase 1 (7 days)	Phase 2 (14 days)	Phase 3 (21+28 days)
Vessel morphology	homogeneous diameters	homogeneous diameters	heterogeneous diameters
	perivascular regression	perivascular immaturity	perivascular maturation
Cell activity	PROLIFERATION	PROLIFERATION	proliferation
	apoptosis	APOPTOSIS	apoptosis
Network	perfusion	PERFUSION	PERFUSION
Molecular activity	angiogenesis (<i>Mmp14</i>)	destabilization (<i>Ang2</i>)	stabilization (<i>Thsp2</i> , <i>Ang1</i>)
	principal component #2	principal component #1	principal component #3
Vascular phenotype	Sprouting Angiogenesis	Neovascular Remodeling	Vascular Maturation

* ALL-CAPITAL font indicates relatively high levels of activity