Research Highlights

Highlights from the latest articles in regenerative medicine

Thought you knew VEGF? Think again

Evaluation of: Stratman AN, Davis MJ, Davis GE. VEGF and FGF prime vascular tube morphogenesis and sprouting directed by hematopoietic stem cell cytokines. *Blood* 117(14), 3709–3719 (2011).

It is well established that VEGF and FGF play an important role in angiogenesis and vasculogenesis of endothelial cells (ECs). Indeed, most studies to date use VEGF as the one magic ingredient in any blood vessel-making potion. However, most *in vitro* 3D culture models studied also contain serum or other substances provided by co-cultured cells. Similarly, *in vivo* studies can be confounded by compounds secreted by the surrounding host tissue, making it impossible to distinguish exactly which factors are involved in the vascularization process and at which step.

In this study, the authors imposed a well-defined serum-free environment on human umbilical vein ECs. The culture media only contained reduced serum supplement, ascorbic acid and FGF2 at 40 ng/ml (necessary for EC survival). They employed a high-throughput screening process to demonstrate that hematopoietic stem cell cytokines IL-3, stem cell factor (SCF) and stromal-derived factor-1 α (SDF-1 α) have synergistic control over human vascular tube morphogenesis in 3D collagen matrices. Furthermore, they show that these three cytokines are also necessary for pericyte recruitment and vascular membrane assembly, key steps in blood vessel maturation. These results were also supported in *ex vivo* quail vitelline vein explants in 3D collagen matrices.

Strikingly, combinations of VEGF, FGF and angiopoietins did not support vascular tube morphogenesis or sprouting in serum-free conditions. However, VEGF and FGF enhanced both human and embryonic quail EC vascularization when used during an EC priming step (in serum-free conditions) in a two-step process followed by the addition of the hematopoietic cytokines IL-3, SCF and SDF-1 α . It was further demonstrated that VEGF and FGF regulate EC tube morphogenesis and sprouting through upstream upregulation of hematopoietic cytokine receptors c-Kit, IL-3R α and CXCR4. The authors also show that the priming and morphogenesis steps are functionally separable, and that they are both controlled by the transcriptional regulator Runx1 via regulation of the expression of the receptors involved in each step. Lastly, the authors show that a functional blockade of c-Kit and CXCR4, as well as the use of blocking antibodies for SCF and IL-3, result in a high frequency of brain and abdominal hemorrhage in developing quail embryos.

All together, the work presented in this article is of critical importance in understanding the distinctive role of proangiogenic factors VEGF and FGF, and redirects the spotlight to the hematopoietic cytokines SCF, IL-3 and SDF-1 α . This new knowledge may revolutionize angiogenesis research, proving the importance of these three molecules under well-defined serum-free conditions in 3D collagen matrices, and revealing the signaling pathways activated by VEGF and FGF during EC tube morphogenesis and sprouting. It also makes evident the four dimensions of the vascularization problem, pointing out a distinct two-step process involving two different sets of factors at different times.

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Improving VEGF delivery in implants

Evaluation of: Singh S, Wu BM, Dunn JCY. The enhancement of VEGF-mediated angiogenesis by polycaprolactone scaffolds with surface crosslinked heparin. *Biomaterials* 32(8), 2059–2069 (2011).

A current problem of tissue engineered scaffolds is the limit on their thickness imposed by the diffusion limit of oxygen and other nutrients to the center of the scaffold. Without any pre-existing blood vessels, cells seeded within these implants can experience hypoxia-induced cell death before angiogenesis can take place. Accelerating this process has been attempted by entrapping pro-angiogenic growth factors in the scaffolds before implantation or by direct postimplantation delivery of these factors into the host. However, growth factor diffusion to the center of the implants (in the case of postimplantation delivery) as well as initial burst release kinetics (in the case of growth factor entrapment) have limited the success of these approaches.

In this work, the authors prove the efficacy of surface crosslinked heparin in accelerating VEGF-induced angiogenesis in polycaprolactone (PCL) scaffolds *in vivo*.

Heparin has been shown to play a role in modulating the diffusion and retention of VEGF due to its negatively charged sulfate groups that can bind growth factors and preserve their bioactivity against degradation. The authors prepared PCL scaffolds by solvent casting and particle leaching, and then crosslinked heparin onto the scaffold's surface. They then showed that these scaffolds could be loaded with VEGF and have better sustained release kinetics than scaffolds without heparin. They also confirmed the bioactivity of bound VEGF by the phosphorylation of VEGFR2 using endothelial progenitor cells.

VEGF-loaded scaffolds were implanted subcutaneously into nonobese diabetic-severe combined immunodeficiency mice. The heparin-VEGF scaffold explants retrieved at day 14 were examined for blood vessel ingrowth. They found a threefold increase in vessel density and percentage area occupied by vessels compared with scaffolds with just heparin or VEGF alone, and a 12-fold increase of these parameters over scaffolds without both heparin and VEGF. Heparin-VEGF scaffolds also presented an increase in CD31 and α SMA-positive cells, and displayed an increase in perfused area as proven by intravenous injection of endothelium-binding biotinylated lectin.

The authors also analyzed the differences in blood vessel ingrowth of scaffolds with varying VEGF loading concentrations (ranging from 0 to 10 μ g) and the amount of heparin crosslinked to the scaffolds (1.5 μ g/scaffold vs 7.5 μ g/scaffold). Notably, although scaffolds with higher heparin content showed an increase in the amount of VEGF retained *in vitro*, no significant difference in blood vessels ingrowth was observed *in vivo* between low and high heparin PCL scaffolds. This may be due to the fact that both high and low heparin scaffolds showed a similar release rate of VEGF within the time period of the experiment.

As demonstrated earlier, using the relevant growth factors along the different stages of vascular development is of crucial importance in angiogenic therapy. Another important parameter is the use of 3D scaffolds and how their properties can be exploited to further aid EC tube morphogenesis. This study shows how heparin can be useful in mediating the delivery of VEGF to enhance its activity by controlling its release. Now the need is to fit this piece of the puzzle in the right place and time to coordinate the delivery of different factors at different steps in the vascularization process.

Growth factor gradients in 3D biomimetic microenvironments guide endothelial cell sprouting

Evaluation of: Shin Y, Jeon JS, Han S *et al.* *In vitro* 3D collective sprouting angiogenesis under orchestrated ANG-1 and VEGF gradients. *Lab Chip* 11(13), 2175–2181 (2011).

Understanding the role of the 3D extracellular matrix in vascular morphogenesis has been possible using natural extracellular matrices as *in vitro* models, such as

collagen, to study the underlying molecular mechanisms. However, little is known about the effect of growth factor gradients on tip and stalk cell interactions in 3D physiologically relevant environments. Microfluidics has been shown to be useful in generating gradients *in vitro*, and in this article the authors capitalize on this feature and couple it with a 3D type 1 collagen scaffold to study the effects of diffusion-based VEGF and angiopoietin-1

(ANG-1) gradients on endothelial cell (EC) sprouting angiogenesis from a pre-existing EC monolayer.

Their device consists of a microchannel where type 1 collagen gel has been polymerized, and three reservoirs connected to this collagen gel scaffold. The first reservoir, to the left of the scaffold, contains the control media. The second reservoir, to the right, contains the condition media. The third, located at the

bottom of the scaffold (in between the other two), contains a monolayer of human dermal microvascular ECs. Diffusion through the system was visualized using Texas Red-conjugated dextran, and modeled with a conventional simulator to account for the rates of consumption of the growth factors.

VEGF was added to the right (condition) reservoir, producing two linear gradients: a horizontal profile of VEGF diffusing from the right to the left channel, and a vertical profile from its diffusion toward the bottom end of the collagen scaffold, where the EC monolayer was located. *In situ* microscopy demonstrated that after 3–4 days, tip cells had started migrating in the direction of the increasing VEGF gradient (toward the top and right of the scaffold), and that these tip cells were followed by stalk cells. However, by day 8 it was observed that most tip cells had separated from their stalk cells, and that the stalk cells left behind eventually regressed.

To address this issue the authors utilized ANG-1, which has been shown to stabilize stalk cells during sprouting angiogenesis. When added to the EC monolayer channel media, ANG-1 diffusion created a vertical gradient of this factor increasing toward the top of the scaffold, away from the cell monolayer. Upon incorporation of the previously described VEGF gradients, it was shown that these conditions induced sprouting angiogenesis and significantly increased the number of tip cells that maintained the connection to their stalk cells compared to the system with VEGF alone, producing more stable tube-like structures.

The device described in this article proved useful in distinguishing the effects of gradients of known angiogenic factors VEGF and ANG-1 on tip and stalk ECs. It highlights the importance of presenting early stage angiogenic sprouts with a coordinated distribution of these two growth factors in a 3D environment.

Dealing with immature blood vessels? Give them some FGF9

Evaluation of: Frontini MJ, Nong Z, Gros R *et al.* Fibroblast growth factor 9 delivery during angiogenesis produces durable, vasoresponsive microvessels wrapped by smooth muscle cells. *Nat. Biotech.* 29(5), 421–427 (2011).

A current challenge in therapeutic angiogenesis is the regression phenomenon experienced by newly developed blood vessels. Often these vessels are not vasoreactive as they lack wrapping by smooth muscle cells (SMCs). This maturation step is necessary for blood vessels stability, and without it, new vasculature tends to collapse soon after formation. In this study, the authors applied a microarray expression analysis on human internal thoracic artery (HITC6) SMCs undergoing cord morphogenesis and discovered that FGF9 was the most upregulated factor. They

proceeded to study the effects of this factor on SMCs and their involvement during vasculature development.

In their work they found that SMC exposure to FGF9 activated FGF receptor signaling, promoted cell elongation, decreased apoptosis, improved longevity, increased the expression of PDGFR β and sonic hedgehog (SHH), and augmented the SMC chemotactic response to PDGF-BB, a growth factor necessary for mural cell recruitment. They also showed that treatment with cyclopamine, which blocks SHH signaling, diminished the increase in PDGFR β expression by FGF9. Since PDGFR β has been shown to regulate SMC migration, these results suggest that FGF9 directs SMC recruitment to developing blood vessels via SHH-PDGFR β signaling.

Indeed, Frontini and his colleagues used subcutaneous growth factor-reduced Matrigel™ plug implants in mice to show that FGF9 does not stimulate

angiogenesis, but instead promotes multilayered muscularization of neovessels. Furthermore, the authors used intravital microscopy to demonstrate that this maturation supported vasoconstriction and vasodilation in response to chemical stimuli. Treatment with FGF9 also enhanced flow through the developing microvasculature in the implants, and resisted the regression phenomenon. Moreover, this muscularization was shown to be long-lasting; implants containing FGF9 and FGF2 collected 1 year after implantation had more and wider blood vessels than those containing FGF2 alone.

These results propose a solution to the regression problem found in immature, nonvasoreactive, developing blood vessels and provide a novel outlook for stable therapeutic angiogenesis. In fact, the authors showed that direct delivery of FGF9 to ischemic mouse hindlimbs stimulates investment of smooth muscle

α -actin-positive cells on neovasculature. This maturation came hand-in-hand with reduced muscle necrosis and recovery of limb function.

The work presented in this article reiterates the importance of angiogenic sprout maturation and vasoconstriction, the lack of which leads to the collapse of developing vascular networks. It proposes FGF9 as one critical growth factor necessary for the recruitment of SMCs, and highlights the investment of mural cells in vascular development as a critical process following angiogenesis.

All together, these articles show the importance of both spatial and time distribution of several growth factors in the process of blood vessel formation. This knowledge should help design a controlled step-wise procedure in which the appropriate growth factors are used at specific time points to first induce EC tube morphogenesis and sprouting, then produce quiescence in the EC to stabilize the neovasculature, and finally promote SMC recruitment for the maturation and subsequent vasoreactivity of the developing blood vessels.