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A Vascular Endothelial Growth Factor-Dependent Sprouting Angiogenesis Assay Based on an *In Vitro* Human Blood Vessel Model for the Study of Anti-Angiogenic Drugs

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ABSTRACT

Angiogenesis is the formation of new capillaries from pre-existing blood vessels and participates in proper vasculature development. In pathological conditions such as cancer, abnormal angiogenesis takes place. Angiogenesis is primarily carried out by endothelial cells, the innermost layer of blood vessels. The vascular endothelial growth factor-A (VEGF-A) and its receptor-2 (VEGFR-2) trigger most of the mechanisms activating and regulating angiogenesis, and have been the targets for the development of drugs. However, most experimental assays assessing angiogenesis rely on animal models. We report an *in vitro* model using a microvessel-on-a-chip. It mimics an effective endothelial sprouting angiogenesis event triggered from an initial microvessel using a single angiogenic factor, VEGF-A. The angiogenic sprouting in this model is depends on the Notch signaling, as observed *in vivo*. This model enables the study of anti-angiogenic drugs which target a specific factor/receptor pathway, as demonstrated by the use of the clinically approved sorafenib and sunitinib for targeting the VEGF-A/VEGFR-2 pathway. Furthermore, this model allows testing simultaneously angiogenesis and permeability. It demonstrates that sorafenib impairs the endothelial barrier function, while sunitinib does not. Such *in vitro* human model provides a significant complimentary approach to animal models for the development of effective therapies.

1. Introduction

The vascular system is a complex network made of tubular structures which distribute blood-contained nutrients, oxygen, and cells to and from most organs throughout the body. As a result, it is deeply involved in maintaining homeostasis of the living body. In adults, the formation of the vascular system is mainly ensured by the emergence of new capillaries from existing vessels; an event known as "sprouting angiogenesis". Sprouting angiogenesis is triggered by the lack of oxygen in growing or ischemic tissues (hypoxia) which release angiogenic factors such as vascular endothelial growth factors (VEGF) into the environment. Established neighboring vessels respond to these signals by sprouting new blood vessels that extend in the gradient of angiogenic factors. Within these sprouting capillaries, tip-cells respond to VEGF and form characteristic protruding and actin-rich filopodia. Tip-cells do not proliferate, but migrate toward the source of VEGF, thus giving

Abbreviations: 3D, three-dimensional; BSA, bovine serum albumin; CLSM, confocal laser scanning microcopy; DLL4, Delta-like protein 4; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; EGM-2, endothelial cell growth medium-2; HUVEC, human umbilical vein endothelial cells; kDa, kilodalton; LSFM, light sheet fluorescence microscopy; mRNA, messenger ribonucleic acid; NOCTH1, Neurogenic locus notch homolog protein 1; PBS, phosphate buffered saline; PDMS, polydimethylsiloxane; PFA, paraformaldehyde; siRNA, small interfering ribonucleic acid; UV, ultraviolet; VE-Cad, vascular-endothelial cadherin; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor-2.

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directionality to an angiogenic sprout. Stalk-cells proliferate behind the leading tip-cells and allow for the extension of the angiogenic sprouts (Ribatti and Crivellato, 2012). During the extension of the capillary sprouts, the formation of the vessel lumen in stalk-cells is ensured by endothelial cytoskeleton rearrangements in response, in part, to hydrodynamic forces (Gebala et al., 2016; Charpentier and Conlon, 2014).

The VEGF family of proteins includes five sub-types in vertebrates; VEGF-A, -B, -C, -D and placental growth factor (PlGF). These factors play crucial roles in the formation and maintenance of blood and lymphatic vasculatures through the activation of specific tyrosine kinases VEGF-receptors (VEGF-R). VEGFR-2 is the most studied receptor in human blood vascular endothelial cells activated by VEGF-A and the VEGF-A/VEGFR-2 pathway plays essential roles in angiogenesis. This pathway is involved in the initial determination of the tip/stalk-cell fate through the Delta-like protein 4 (DLL4)/Neurogenic locus notch homolog protein 1 (NOTCH1) (Simons et al., 2016; Hellstrom et al., 2007). VEGF-A binds to its receptor VEGFR-2 on the membrane of endothelial cells, leading to its dimerization. It triggers the activation of the intracellular kinase domain of VEGFR-2, which initiates a signaling cascade leading the responding cell to become a tip-cell and to express DLL4 at its surface. DLL4 interacts with its receptor NOTCH1 on the surface of adjacent endothelial cells. This activates a gamma-secretase complex, causing the downstream activation of molecular pathways that result in a decrease of sensitivity to VEGF-A and stimulates endothelial proliferation, therefore shifting cells toward the stalk-cell fate (Simons et al., 2016; Blanco and Gerhardt, 2013). Upon activation of this system, sprouts form and progress toward the hypoxic tissues (Fig. 1). Angiogenesis takes place in pathological conditions such as inflammation, vaso-proliferative retinopathies, and cancers (Potente et al., 2011; Carmeliet and Jain, 2000) and it was proposed during the 1970s that angiogenesis inhibitors could be designed as anticancer drugs (Folkman, 1971). This eventually led to the development of molecules targeting VEGF-A, its receptors, or downstream effectors of this pathway. Some were approved for clinical use, such as bevacizumab (Avastin), sunitinib (Sutent) and sorafenib (Nexavar), which were among the first available targeted therapies (Niu and Chen, 2010; Carmeliet and Jain, 2011a).

To study angiogenesis and develop more specific therapies, several experimental models were developed using 2D cell culture and animal models (Staton et al., 2004). Recently, sophisticated *in vitro* models have been created to study sprouting angiogenesis of human endothelial cells in a 3D environment simulating the extracellular matrix (ECM). For example, the microbead assay consists in growing endothelial cells to confluence on microbeads which are then embedded into a fibrin gel. Upon treatment with pro-angiogenic factors, capillary formation can be induced (Nakatsu et al., 2003). However, this technique has several limitations, such as the lack of a parent vessel with a lumen. Secondly, although human endothelial cells have been used in this assay, the presence of fibroblasts was required to maintain growth and promote lumen formation. The presence of another cell type in such assay complicates the technique and the analysis of specific processes. For example, it may be difficult to study the direct effect of any drug on endothelial cell sprouting when fibroblasts may also respond to such a drug and trigger an indirect effect. Moreover, due to the spherical structure of the beads, the local inhibition of sprouting in the neighborhood of an existing sprout cannot be easily studied, and until recently such studies relied on the zebrafish animal model (Yokota et al., 2015).

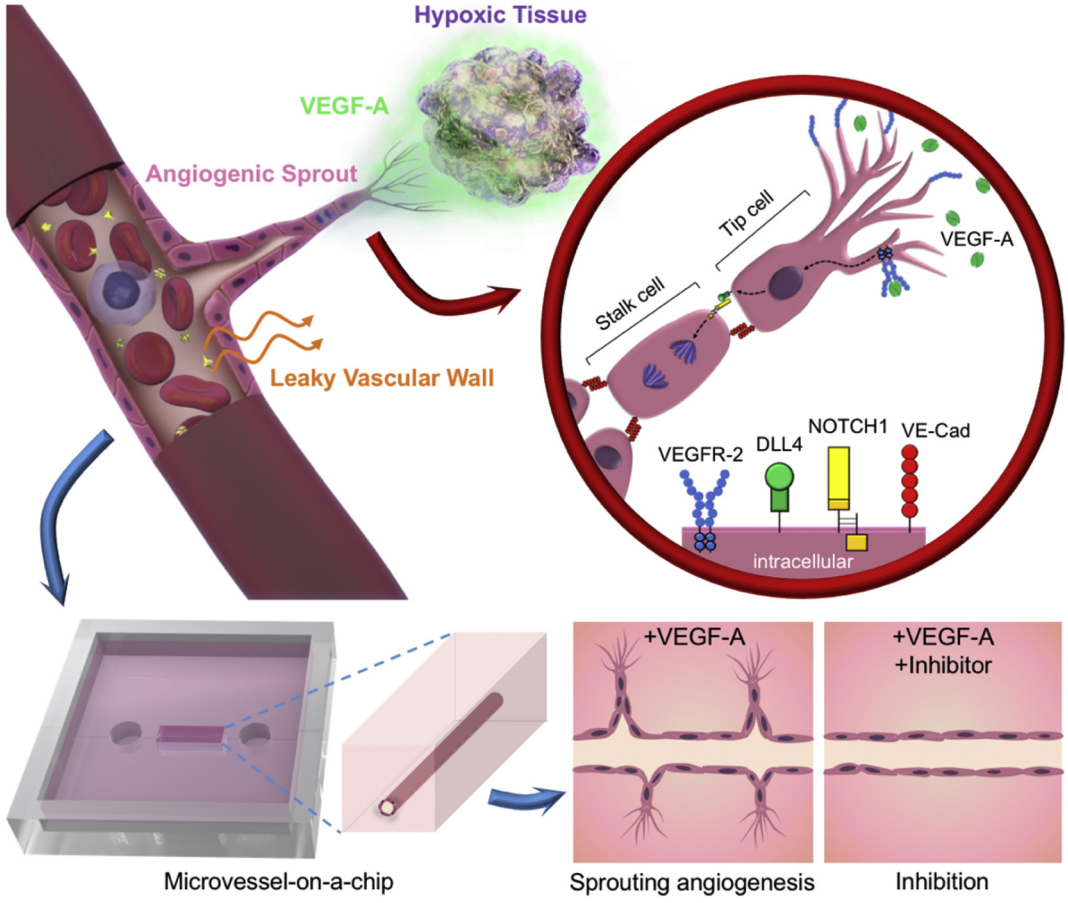

Fig. 1. Concept of the present study: VEGF-induced angiogenesis-on-a-chip for gene and inhibitor study. (Top) *In vivo*, the DLL4/NOTCH1 pathway orients the endothelial cell fate within an angiogenic sprout. The tip-cell migrates and expresses DLL4, while the stalk-cell is inhibited in its migration by the DLL4/NOTCH1 interaction which rather triggers cell proliferation. (VE-Cad: VE-cadherin) (Bottom) Concept of the model: a human microvessel is fabricated on a PDMS chip within a collagen gel scaffold and used to study VEGF-A-induced angiogenesis, permeability and angiogenic inhibitors effects.

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