UNIVERSITÀ DEGLI STUDI DI TORINO

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PhD THESIS

"The role of transcription factor ERG (ETS related gene) in glomerular endothelial cell pathophysiology"

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ABSTRACT

The main topic of this study is the endothelium, which involves elucidating several physiological and pathological processes that affect its function such as: cancer angiogenesis and glomerular endothelial dysfunction. In the first part of this thesis we will consider endothelial physiology, describing the main endothelial functions, such as angiogenesis, glomerular endothelial permeability and endothelial activation to an inflammatory response. From a pathological point of view, we will focus on renal cancer and in particular tumor angiogenesis, since this process is crucial for tumor progression. We will describe the kidney tumor-derived endothelial cells (TECs) properties and their pathological effects. In our review entitled "*Alternative strategies to inhibit tumor vascularization*" (*Brossa et al.,2019*) we studied different anti-angiogenic factors, such as human liver stem cells derived extracellular vesicles (HLSC-EVs) and new targets, such as ERG (ETS family related gene), which both exhibit potential anti-angiogenic properties. In another study "*Extra-cellular vesicles from human liver stem cells inhibit tumor angiogenesis*" (*Lopatina et al*., *2019*), we investigated, the anti-angiogenic effects of HLSC-EVs on renal TECs both *in vitro* and *in vivo*, in comparison with mesenchymal stem cells EVs (MSC-EVs) which also possess anti-angiogenic properties. We demonstrated that HLSC-EVs down-regulate pro-angiogenic genes such as ITGB3, FGF1, EPHB4 and PLAU in renal TECs, because of the the presence of the specific microRNAs (miRNAs) miR-15a, miR-181b, miR-320c and miR-874 in HLSC-EVs, which exhibit an anti-angiogenic potential.

In the second part of the study, the aim was to investigate the role of the endothelial transcription factor ERG in glomerular endothelial damage. Endothelial damage is characterized by an increased permeability of the glomerular filtration barrier (GFB), leading to edema, inflammation and proteinuria, which are also some of the main features of Diabetic Nephropathy (DN). In our work, we showed that ERG activation through phosphorylation in glomerular endothelial cells (GECs), is VEGF dependent. Furthermore, we observed a significant increase in permeability in ERG Knock-Down (ERG KD) GECs compared with control GECs, indicating that ERG is a key regulator in VEGF-induced permeability of GECs. We also studied the expression of ERG in the glomeruli of mice with DN, where we observed a

downregulation of ERG at both molecular and protein level. The study was then extended to understand whether downregulation of ERG in the kidney could be a trigger of Endothelial to mesenchymal transition (EndMT), which is already shown to occur in DN. In our experiments, we found that loss of ERG expression led to EndMT *in vitro* in ERG KD GECs, as well as in the glomeruli of ERG Knock-out mice (ERG KO). We observed an increased expression of TGF-β1, TGF-β2 and α-SMA in ERG KD GECs, an increased expression of SNAIL1 in diabetic mice, and finally an increase of α-SMA in ERG KO mice, which are all considered to be well known markers of EndMT. In addition, this find was extended to the glomeruli of patients with diffuse diabetic glomerulosclerosis whereby ERG was also found to be downregulated.

AIM

The aim of this work is to investigate the endothelium under two different pathological states: cancer tumor angiogenesis, and glomerular endothelial damage. In the context of cancer, we will investigate the anti-angiogenic potential of HLSC-EVs in the treatment of renal tumor progression concentrating on the molecular content enriched in these vesicles including miRNAs and their effects on angiogenesis. Glomerular endothelial damage is a common feature of many types of kidney diseases including Diabetic Nephropathy (DN). We will investigate the role of ERG in glomerular endothelial dysfunction related to DN, and EndMT both *in vitro* and *in vivo* as well as in clinical samples of diffuse diabetic glomerulosclerosis.

THE PHYSIOLOGY OF THE ENDOTHELIUM

The endothelium: structural heterogeneity

The endothelium, a monolayer of endothelial cells, constitutes the inner cellular lining of the blood vessels (arteries, veins and capillaries) and the lymphatic system, and therefore is in direct contact with the blood/lymph and the circulating cells. The endothelial cells (ECs) are anchored to the inner side of an 80-nm-thick basal lamina, that represents the scaffold of arteries, veins and capillaries. The outside of the scaffold is covered with smooth muscle cells or pericytes (Figure a,b). The endothelial cells can synthesize all the proteins constituting the basal lamina and can produce all the enzymes that are necessary for its remodeling, such as matrix metalloproteinases that degrade this extracellular matrix, crucial event for angiogenesis. The ECs are generally thin and slightly elongated, their dimension is about 50–70 μm long, 10–30 μm wide and 0.1–10 μm thick. In the blood vessel wall, endothelial cells are orientated along the axis of the vessel, minimizing the shear stress forces exerted by the flowing blood.

The most important feature of the ECs is their structural heterogeneity : the electron microscopy observations of the several different types of intercellular junctions of ECs led to a classification of the endothelium in: “Continuous endothelium”, “Fenestrated endothelium” and “Discontinuous endothelium (Bennett, Luft, and Hampton 1959) (Figure 2).The continuous endothelium is the most common since it is found in arteries, veins and capillaries of the brain, skin, lung, heart and muscle : here, the ECs are bound tight junctions and anchored to a continuous basal membrane. The tissues involved in trans-endothelial transport or high filtration, like endocrine and exocrine systems, gastrointestinal tract, kidney glomeruli and subpopulations of renal tubules, present a fenestrated endothelium. Fenestrated endothelium is also characterized by a continuous basal membrane (like continuous endothelium), but it has some transcellular wide pores (50–60 nm), which are sealed by diaphragm (5-6 nm). Lastly, the discontinuous endothelium is associated with a poorly structured basal membrane and it presents large nm-wide fenestrations (100-200 nm), without diaphragm. This type of endothelium is predominantly found in

liver sinusoidal vasculature, but also in the spleen and bone marrow. Structural heterogeneity of ECs also includes various cellular shapes and all those structural components needed for the Endocytosis and Transcytosis processes. Endocytosis consists of targeting macromolecules to the lysosomal compartment for degradation. i.e. by clathrin-coated pits; at variance transcytosis refers to the transcellular transfer of molecules across the endothelium, through the caveolae. Moreover, ECs can present various levels of expression and types of intercellular junctions, tight junctions, adherens junctions or gap junctions, and various compositions of the glycocalyx, together with the associated endothelial surface layer (Schött et al. 2016). This heterogeneity in endothelial cells depends on the genetic and environmental components, but also on other causes like location, mediation with soluble factors, cell to cell contact, cell–matrix interactions, pH and pO_2. Furthermore, the origin of ECs has to be considered: ECs, together with hematopoietic cells, derive from the mesoderm by the differentiation of the same precursor hemangioblasts (Choi et al. 1998). Hemangioblasts give rise to angioblasts or endothelial progenitor cells, which can differentiate into endothelial cells of arteries, veins and capillaries, identified by the expression of specific genes. Other cell lineages, such as adipose or neural stem cells, can trans-differentiate into endothelial cells.

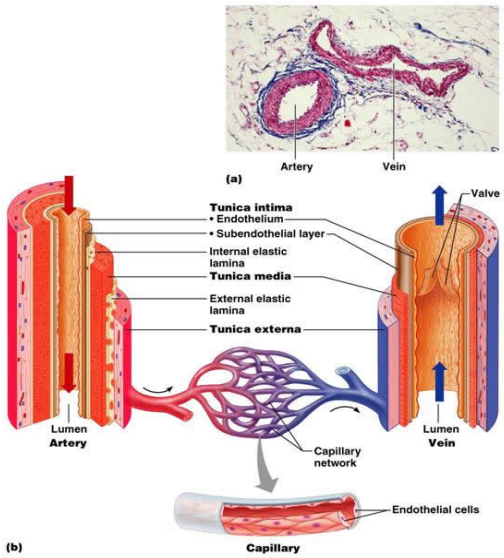

Figure 1. (a) Histology image of vein and artery **(b) Anatomical structure of a blood vessel wall** (artery, vein and capillary). (www.openstax.org)

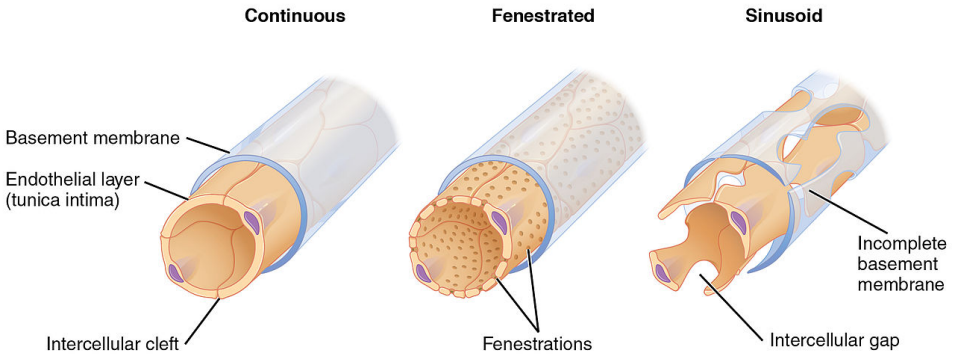

Figure 2. The three principal types of endothelium: Continuous, Fenestrated and Sinusoid (or Discontinuos). (www.openstax.org)

Endothelial permeability: transcellular and paracellular pathways

The endothelium is a semi-permeable barrier that controls the transfer of macromolecules across the blood vessel wall to the surrounding tissues. Endothelium permeability can be basal (at the level of capillaries), or can be induced, when it is associated with inflammation, which predominantly involves post-capillary venules. Fluids and small solutes move passively across the barrier via a paracellular route, while macromolecules use either transcellular or paracellular pathways (Figure 3).

The transcellular transport involves membrane-attached and cytosolic "Caveolae" that migrate across the capillary endothelial cells and shuttle macromolecules from the blood to the interstitium. Transcellular transport of macromolecules may involve

receptor-dependent or independent mechanisms. The “Caveolae” are vesicle carriers, resulting by invaginations in the cell membrane, and they regulate the transcytosis in endothelial cells: an example is the transfer of albumin across endothelium. The key-signaling event regulating transcytosis is the *Src*-dependent tyrosine phosphorylation of Caveolin-1 (a scaffolding protein), which promotes the formation of membrane-attached vesicles. Then the endocytosis, the trans-migration of the vesicle across the endothelium, the fusion of the vesicle to the basolateral can occur. Moreover, ECs express only two among the seven families of glucose transporters: GLUT-1 and GLUT-4. They are expressed mainly in the brain and blood endothelial barrier, and they are crucial in the modulation of glucose transport, including pathological conditions, i.e., diabetes and hypoxaemia. Other isoforms of glucose transporters have also been detected in ECs throughout the body, including umbilical vein, adrenal capillaries, aorta, retina, heart, placenta, the eye, and testis. The most relevant transport systems for amino acids expressed in ECs is the “System y+ cationic amino acid transporter”, which is the transporter of L-arginine, the substrate of the nitric oxide. Several studies have shown that cytokines such as tumour necrosis factor a (TNF) are able to stimulate L -arginine transport in ECs, resulting in increased nitric oxide. Moreover, the paracellular pathway involves ECs intercellular junctions, that are normally impermeable to macromolecules. Tight junctions and adherens junctions interact with the endothelial actin cytoskeleton, and act as a selective barrier to the enter of molecules from the blood circulation.

Endothelial adherens junctions consist of transmembrane VE-cadherins molecules, which are linked to the actin cytoskeleton. In pathological conditions, like acute or chronic inflammation, injury, angiogenesis or tumor metastasis, mediators, cytokines or growth factors such as vascular endothelial growth factor (VEGF) can induce endothelial cell activation: this event leads to an increased contraction of endothelial cells, increase of intercellular space and subsequently increase of endothelial permeability. Alteration of endothelial barrier function causes an abnormal extravasation of fluid and macromolecules, resulting in edema and endothelial dysfunction.

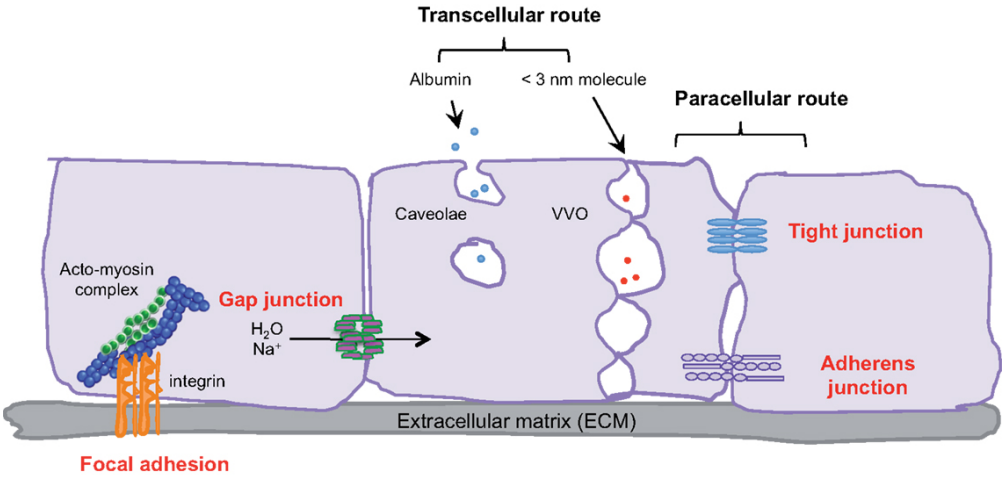

Figure 3. Transcellular and paracellular pathways (Azzi, Hedba and Gavard 2013)

Endothelial activation and inflammation

ECs play an important role in the initiation, amplification, and resolution of the inflammatory response. A dysregulation in the inflammatory response causes a variety of diseases such as atherosclerosis, obesity, diabetes, and hypertension. Several studies have clarified the complex molecular pathways mediating the pro- and anti-inflammatory signaling in endothelial cells. Specifically, they have demonstrated that many macromolecular complexes can regulate the signaling from the membrane receptors to key transcription factors such as nuclear factor-κB (NF-κB). These molecules are associated with receptors such as Toll-like receptors (TLRs), nucleotide-binding oligomerization-domain (NOD)-like receptors (NLRs) to mediate innate immunity responses. Therefore, the endothelium not only provides a highly selective physical barrier to control the vascular permeability, but it also secretes a large number of vasoactive substances to regulate the vascular tone and the remodeling of vessel walls (Tousoulis et al.). Vasodilation increases blood flow and causes the redness (rubor) and an elevated focal heat. Increased endothelial

permeability results in a leakage of plasma proteins and fluid into the tissue, which leads to swelling. Lastly, the mediators released from ECs and leukocytes, such as bradykinin and prostaglandins, contribute to the sensitivity to pain.

Inflammation is pathologically defined as “The local infiltration and activation of leukocytes”, and the endothelium plays a crucial role in regulating this process. Inflammatory activation of ECs is a key pathophysiological step in many diseases including infections, autoimmunity, and cancer, hypertension, coronary heart disease, obesity, and diabetes.

ECs are constantly exposed to various biological, chemical, and mechanical events, but they maintain a quiescent state in which they are not adhesive to circulating leukocytes and they have antithrombotic, anti-inflammatory, and antiproliferative properties. During inflammatory responses, ECs are phenotypically converted and activated, leading to several events: they promote an increase of vessel permeability, induction of leukocyte adhesion, and a prothrombotic process (Pober et al.). ECs can lose their junctions, in order to increase the permeability and they release molecules as the vonWillebrand factor (vWF) (normally stored in Weibel-Palade bodies), P-selectin or E-selectin initiating the “tethering” and “rolling” of leukocytes onto the inflamed endothelium. E-selectin and P-selectin to their low affinity ligands Sialyl-LewisX and P-selectin glycoprotein ligand 1 (PSGL-1). ECs (and vascular residing macrophages) also secrete chemokines such as monocyte chemotactic protein-1 (MCP-1 or CCL2), which binds to the receptor C-C chemokine receptor type 2 (CCR2) and CCR4 on monocytes and T lymphocytes, and interleukin-8 (IL-8), which binds to the IL-8 receptor α (CXCR1 or IL8RA) and β (CXCR2 or IL8RB) on neutrophils (Campbell. Et al.). Induced surface expression of intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) on ECs interacts with integrins counter receptors on leukocyte and, hence, mediates the firm adhesion (arrest) and transmigration of leukocytes into the subendothelial spaces of vessel wall or extravasation into injured tissue, to initiate inflammation process (Cook-Mills et al.). One of the principal regulators of proinflammatory responses in ECs is the Nuclear factor (NF)-κB, activated by various agonists such as oxidized low-density lipoprotein (LDL) or angiotensin II (ANG II) (Pueyo et al.). NF-κB is transcription factor activates transcription of proinflammatory genes like TNF-α, interleukin-1 (IL-1), interleukin-8 (IL-8), E-selectin, VCAM-1, and ICAM-1 by its binding to the promoters or enhancers of the target genes. In mammals, NF-κB

has five members: NF-κB1 (p50 and the precursor p105), NF-κB2 (p52 and the precursor p100), Rel A (p65), Rel B, and c-Rel. In the NH_2 terminal is responsible for DNA binding and interaction with the inhibitor of NF-κB (IκB) (Shih, Wang, and Yang 2015).

Angiogenesis

Formation of the vascular network occurs through three main mechanisms: vasculogenesis, angiogenesis and arteriogenesis. Vasculogenesis is defined as "De novo formation of vessels", during embryogenesis, from the mesoderm-derived endothelial precursors that differentiate and assemble into the primitive vessel plexus (Figure 4). Additionally, after birth, bone-marrow endothelial progenitor cells are recruited to stimulate new vessel growth, by releasing pro-angiogenic stimuli. Angiogenesis is defined as "The sprouting of new capillaries and blood vessels derived from pre-existing blood vessels, and it is a fundamental process in reproduction, development and repair. In the adult, angiogenesis is crucial for reproduction and development, but it is also involved in wound repair, responding to stimuli like inflammation or hypoxia (Félétou 2011). After release of angiogenic factors, the endothelium is activated: ECs destroy the extracellular matrix and the basal lamina, lose their junctions, proliferate and organize in tubular structures new blood vessels. Angiogenic process by ECs finely involves growth factors and chemokines that all together contribute to the tubes formation and also arrest the process at the end.

Among the growth factors released, Vascular Endothelial Growth Factor (VEGF) plays an essential role in this phenomenon, together with other molecules such as angiopoietin-1, integrins, FGF-2, IGF-1, and TNF-α. Ephrins play an important role in the guiding of the forming vessel toward its target. Factors such as thrombospondin-1, canstatin, tumstatin, angiostatin are also released in order to switch off the angiogenic process. Lasly, the angiogenic phenomenon can largely differ, based on the origin, if it is physiological or pathological.

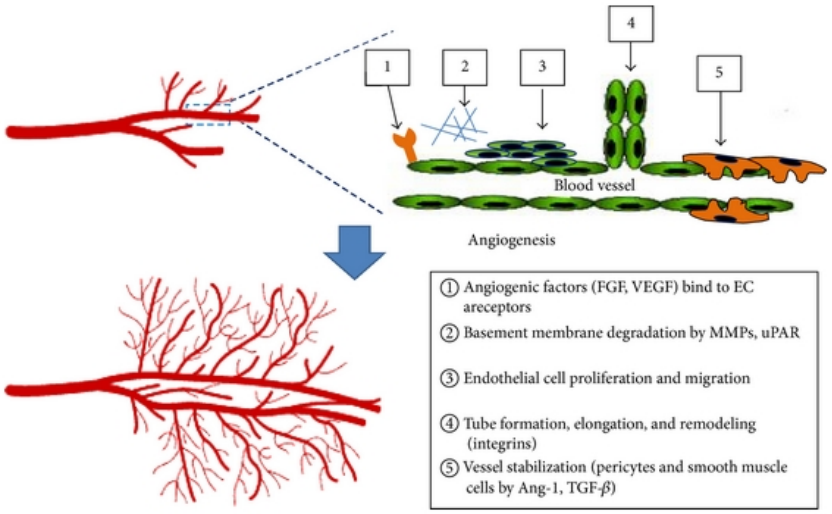

Figure 4. Physiological processes in angiogenesis (Yoo and Kwon 2013)

Vessel sprouting: tip and stalk cells

The angiogenic sprouting is a complex process involving different cell behaviours and regulated by several signalling pathways: it leads to the formation of vessels, critical for the passage of nutrients and oxygen throughout the body (Gerhardt et al. 2003). After an angiogenic stimulus of VEGF has "selected" one endothelial cell, "Sprouting initiation" occurs and the cell starts to extend its filopodia. Then, other ECs are activated and start to migrate out of the parent vessel, but they are still connected to the neighbouring cells. The activated endothelial cells are called a "Tip cells": they start the sprouting but, in the same time, they prevent neighboring endothelial cells from sprouting. The adjacent neighbouring cells that trail the tip cell, assume a secondary position behind them, and start dividing, in order to support sprout elongation. These cells are called "Stalk cells": they increase the surface area and the mass of tip cells, and also, unlike the tip cells, they form the vascular lumen.

Most sprouts can eventually find other sprouts or other vessels and set up cell junctions with one or more endothelial cells in that structure. The newborn blood vessels then may act as the parent vessel for another round of sprouting, setting up again the angiogenic process (Chappell, Wiley, and Bautch 2011).

The tip cells differ from the stalk cells also in the genetic profile: they express higher levels of the Notch ligand Delta Like Ligand (Dll4), VEGFR-2, growth factor subunit B, vascular endothelial growth factor receptor (VEGF-R2) and VEGFR3 (Thurston and Kitajewski 2008). The differences in cell morphology and gene expression indicate that endothelial tip and stalk cells have specialized functions.

Crosstalk between VEGF and Notch signalling pathways

VEGF signaling pathway is crucial in the regulation of sprouting angiogenesis in both physiological and pathological conditions. (Ferrara et al. 2003). In mammals, the VEGF family of growth factors include six different glycoproteins (VEGF-A, -B, -C, -D, -E, and placenta growth factor [PlGF]), that bind to three types of receptors (VEGF-R1/R2/R3).

VEGF-A is the most important in the prominently involved in the angiogenic process: VEGF-A expression is induced by hypoxic conditions (in a hypoxia-inducible factor Hif1/2–dependent manner), cytokines, growth factors, hormones, oncogenes, and tumor-suppressor genes (Dvorak 2005). VEGF-A has different isoforms (expressed in different tissues), which interact mostly with VEGF-R1: binding of VEGF with its receptor VEGF-R2, leads to proangiogenic signalling in the endothelium. Several studies showed that , the homozygous as well the heterozygous and knock-out of VEGF ligands (especially VEGF-A) and VEGF-Rs in mice, lead to embryonic death in utero, caused by an aberrant vascular network formation development (Dumont et al. 1998).

Another signalling pathway that plays a key role in vascular development and angiogenesis sprouting, is the Notch signalling. In vertebrates, there are four Notch receptors (from Notch1 to Notch4) and five Notch transmembrane ligands (Jagged1 and Jagged2, homologs to Serrate, and Delta-like ligand 1 [Dll1], 3 [Dll3], and 4 [Dll4]). Among the four Notch ligands expressed in the endothelium, only loss of Dll4 or Jag1 lead to vascular defects (Hofmann and Luisa Iruela-Arispe 2007). These

two Notch ligands show different spatial expression: Dll4 is highly expressed in tip cells, while Jag1 expression is low/absent in tips cells, but higher in adjacent stalk cells (Benedito et al. 2009). However, both these two Notch ligands are required for the formation of fully functional a vascular networks. Endothelial deletion or overexpression of Jagged1 lead respectively to an *in vivo* reduction or increase in angiogenic sprouting. *In vitro* studies showed that Jag1 regulates negatively Notch activity, indeed Notch signalling results to be more active in the absence of Jag1. Benedito et al. showed that Jag1 interferes with the capacity of Dll4 to activate Notch signalling in tip cells. Moreover, It has been shown that Dll4 is upregulated by VEGF in the angiogenic process, suggesting a crosstalk between VEGF and NOTCH pathways in the regulation of physiological and pathological angiogenesis (Lobov et al. 2007). *In vitro* studies reported that the treatment of human umbilical vein endothelial cells (HUVECs) with VEGF-A, causes an increased expression of Dll4. Treatment of in human umbilical vein endothelial cells (HUVECs) with VEGF-A consistently increases the expression of Dll4 protein (Ridgway et al. 2006). Therefore, the expression of Dll4 mRNA increased in tumor blood vessels, after an increase of VEGF-A, compared with vessels in the surrounding healthy tissue (Patel et al. 2005). In contrast, after blocking of VEGF-A in mice, a reduction of Dll4 endothelial levels was observed (Noguera-Troise et al. 2006) (Thurston and Kitajewski 2008). VEGF stimulated ECs, and acquired phenotypic features, which result in becoming tip cells: they exhibit filopodial projections, increased migratory properties and express high VEGFR2, also known as KDR (Blanco and Gerhardt 2013). In tip cell Dll4 binds and activates Notch receptors on adjacent stalk cells. Notch activity in stalk cells induces the transcription of Notch-dependent genes (such as members of transcription factor families HEY and HES), suppresses filopodia formation and migration capacity and reduces VEGFR2 expression. Importantly, tip and stalk cell phenotypes are dynamic, and tip cells can transform to stalk cell can, and vice versa (Jakobsson et al. 2010). Molecular mechanisms that regulate tip and stalk cell formation and their conversion is not totally known, and how VEGF promotes transcriptional activation of Dll4 gene is still unclear.

However, Fish et al. identified one potential mechanism for the transient VEGF-dependent transcriptional activation of Dll4 in ECs: this involves VEGF stimulation of MAPK/ERK pathway and it it requires the ETS transcription factor ERG, together with its coactivator p300 (Fish, Gutierrez, and Wythe 2016). This

VEGF/ERK/ERG/p300 signalling transcriptional pathway leads to the transcriptional activation of a network of genes necessary to (NDRG HLX, FJX1, EGR3, MAP2) (Prahst et al. 2014) (Pin et al. 2012) (Al-Greene et al. 2013). It was shown that VEGF signalling leads to the activation of MAPK/ERK pathway, through the ERK -dependent phosphorylation of ERG. ERG activation is fundamental for the p300 recruitment to Dll4 enhancers, that finally cause transcriptional Dll4 expression. Moreover, other studies found a linkage between VEGF-A signalling and MEF2 transcriptional factors. In ECs, VEGF-A-induces the release of repressive histone deacetylases and the recruitment of EP300 to MEF2 target gene regulatory elements, that links to MEF2 target gene activation. MEF2 transcriptional activation was found to result in further transcriptional production of Dll4 and many other key genes overexpressed in both angiogenesis physiological and tumor vascularization (Sacilotto, Chouliaras, and Val 2015).

References:

Al-Greene, Nicole T, Anna L Means, Pengcheng Lu, Aixiang Jiang, Carl R Schmidt, A Bapsi Chakravarthy, Nipun B Merchant, et al. 2013. "Four Jointed Box 1 Promotes Angiogenesis and Is Associated with Poor Patient Survival in Colorectal Carcinoma." *PLOS ONE* 8 (7): 1–15. https://doi.org/10.1371/journal.pone.0069660.

Azzi, Sandy, Jagoda Hebda, and Julie GAVARD. 2013. "Vascular Permeability and Drug Delivery in Cancers." *Frontiers in Oncology* 3: 211. https://doi.org/10.3389/fonc.2013.00211.

Benedito, Rui, Cristina Roca, Inga Sörensen, Susanne Adams, Achim Gossler, Marcus Fruttiger, and Ralf H. Adams. 2009. "The Notch Ligands Dll4 and Jagged1 Have Opposing Effects on Angiogenesis." *Cell* 137 (6): 1124–35. https://doi.org/10.1016/j.cell.2009.03.025.

Bennett, H Stanley, John H Luft, and James C Hampton. 1959. "Morphological Classifications of Vertebrate Blood Capillaries." *American Journal of Physiology-Legacy Content* 196 (2): 381–90. https://doi.org/10.1152/ajplegacy.1959.196.2.381.

Blanco, Raquel, and Holger Gerhardt. 2013. “VEGF and Notch in Tip and Stalk Cell Selection.” *Cold Spring Harbor Perspectives in Medicine* 3 (1). https://doi.org/10.1101/cshperspect.a006569.

Chappell, John C., David M. Wiley, and Victoria L. Bautch. 2011. “Regulation of Blood Vessel Sprouting.” *Seminars in Cell and Developmental Biology* 22 (9): 1005–11. https://doi.org/10.1016/j.semcdb.2011.10.006.

Choi, Kyunghee, Marion Kennedy, Alexander Kazarov, John C. Papadimitriou, and Gordon Keller. 1998. “A Common Precursor for Hematopoietic and Endothelial Cells.” *Development* 125 (4): 725–32.

Dumont, Daniel J, Lotta Jussila, Jussi Taipale, Athina Lymboussaki, Tuija Mustonen, Katri Pajusola, Martin Breitman, and Kari Alitalo. 1998. “Cardiovascular Failure in Mouse Embryos Deficient in VEGF Receptor-3.” *Science* 282 (5390): 946–49. https://doi.org/10.1126/science.282.5390.946.

Félétou, Michel. 2011. *The Endothelium: Part 1: Multiple Functions of the Endothelial Cells*. San Rafael (CA). https://doi.org/10.4199/C00031ED1V01Y201105ISP019.

Fish, Jason E, Manuel Cantu Gutierrez, and Joshua D Wythe. 2016. “Dynamic Regulation of VEGF-Inducible Genes by an ERK / ERG / P300 Transcriptional Network” 4.

Gerhardt, Holger, Matthew Golding, Marcus Fruttiger, Christiana Ruhrberg, Andrea Lundkvist, Alexandra Abramsson, Michael Jeltsch, et al. 2003. “VEGF Guides Angiogenic Sprouting Utilizing Endothelial Tip Cell Filopodia.” *Journal of Cell Biology* 161 (6): 1163–77. https://doi.org/10.1083/jcb.200302047.

Hofmann, Jennifer J., and M. Luisa Iruela-Arispe. 2007. “Notch Expression Patterns in the Retina: An Eye on Receptor-Ligand Distribution during Angiogenesis.” *Gene Expression Patterns* 7 (4): 461–70. https://doi.org/10.1016/j.modgep.2006.11.002.

Jakobsson, Lars, Claudio A Franco, Katie Bentley, Russell T Collins, Bas Ponsioen, Irene M Aspalter, Ian Rosewell, et al. 2010. “Endothelial Cells Dynamically Compete for the Tip Cell Position during Angiogenic Sprouting.” *Nature Cell Biology* 12 (10): 943–53. https://doi.org/10.1038/ncb2103.

Lobov, I. B., R. A. Renard, N. Papadopoulos, N. W. Gale, G. Thurston, G. D. Yancopoulos, and S. J. Wiegand. 2007. “Delta-like Ligand 4 (DII4) Is Induced by VEGF as a Negative Regulator of Angiogenic Sprouting.” *Proceedings of*

the National Academy of Sciences of the United States of America 104 (9): 3219–24. https://doi.org/10.1073/pnas.0611206104.

Noguera-Troise, Irene, Christopher Daly, Nicholas J Papadopoulos, Sandra Coetzee, Pat Boland, Nicholas W Gale, Hsin Chieh Lin, George D Yancopoulos, and Gavin Thurston. 2006. "Blockade of Dll4 Inhibits Tumour Growth by Promoting Non-Productive Angiogenesis." *Nature* 444 (7122): 1032–37. https://doi.org/10.1038/nature05355.

Patel, Nilay S, Ji-Liang Li, Daniele Generali, Richard Poulsom, David W Cranston, and Adrian L Harris. 2005. "Up-Regulation of Delta-like 4 Ligand in Human Tumor Vasculature and the Role of Basal Expression in Endothelial Cell Function." *Cancer Research* 65 (19): 8690–97. https://doi.org/10.1158/0008-5472.CAN-05-1208.

Pin, Anne-Laure, François Houle, Maëva Guillonneau, Éric R Paquet, Martin J Simard, and Jacques Huot. 2012. "MiR-20a Represses Endothelial Cell Migration by Targeting MKK3 and Inhibiting P38 MAP Kinase Activation in Response to VEGF." *Angiogenesis* 15 (4): 593–608. https://doi.org/10.1007/s10456-012-9283-z.

Prahst, Claudia, Bahar Kasaai, Filipa Moraes, Espen D Jahnsen, Bruno Larrivee, David Villegas, Luc Pardanaud, et al. 2014. "The H2.0-Like Homeobox Transcription Factor Modulates Yolk Sac Vascular Remodeling in Mouse Embryos." *Arteriosclerosis, Thrombosis, and Vascular Biology* 34 (7): 1468–76. https://doi.org/10.1161/ATVBAHA.114.303626.

Ridgway, John, Gu Zhang, Yan Wu, Scott Stawicki, Wei-Ching Liang, Yvan Chanthery, Joe Kowalski, et al. 2006. "Inhibition of Dll4 Signalling Inhibits Tumour Growth by Deregulating Angiogenesis." *Nature* 444 (7122): 1083–87. https://doi.org/10.1038/nature05313.

Sacilotto, Natalia, Kira M Chouliaras, and Sarah De Val. 2015. "MEF2 Transcription Factors Are Key Regulators of Sprouting Angiogenesis The Dll4 Enhancer Dll4in3 Directs Expression to Endothelial Cells during Sprouting Angiogenesis."

Schött, Ulf, Cristina Solomon, Dietmar Fries, and Peter Bentzer. 2016. "The Endothelial Glycocalyx and Its Disruption, Protection and Regeneration: A Narrative Review." *Scandinavian Journal of Trauma, Resuscitation and Emergency Medicine* 24 (1): 48. https://doi.org/10.1186/s13049-016-0239-y.

Shih, Ruey-Horng, Chen-Yu Wang, and Chuen-Mao Yang. 2015. “NF-KappaB Signaling Pathways in Neurological Inflammation: A Mini Review.” *Frontiers in Molecular Neuroscience* 8 (December): 77. https://doi.org/10.3389/fnmol.2015.00077.

Thurston, G., and J. Kitajewski. 2008. “VEGF and Delta-Notch: Interacting Signalling Pathways in Tumour Angiogenesis.” *British Journal of Cancer* 99 (8): 1204–9. https://doi.org/10.1038/sj.bjc.6604484.

Yoo, So Young, and Sang Mo Kwon. 2013. “Angiogenesis and Its Therapeutic Opportunities.” Edited by Grzegorz Szewczyk. *Mediators of Inflammation* 2013: 127170. https://doi.org/10.1155/2013/127170.

ENDOTHELIUM AND CANCER

Angiogenesis process plays a key role in cancer growth and development, since tumor vessels supply tumors with oxygen and nutrients, and remove waste products from it. Therefore, tumor vessels formation is essential for the metastasis process (Hida et al., 2018).

The tumor blood vessels consist of tumor endothelial cells (TECs) and perivascular cells: TECs line the internal part of blood vessels, while perivascular cells (pericytes in microvessels and smooth muscle cells for arteries and veins) form the external part of which blood vessels externally and can regulate their contraction and relaxation.

Tumor blood vessels have recently become a strategic therapeutic target to block cancer. The first antiangiogenic drug against cancer, Bevacizumab, was approved in 2004 by US Food and Drug Administration (FDA) and it is a neutralizing antibody against VEGF: the benefits of Bevacizumab, as all the antiangiogenic drugs that mainly target VEGF/VEGF-Rs signalling pathways, consist in a minor toxicity compare with the canonical chemotherapy treatment. However they can induce many severe side effects like lethal hemoptysis (Keedy & Sandler, 2007) and intestinal perforation (Saif et al., 2007). The goal of cancer antiangiogenic treatment is to find a safe therapy that will depend on studying about the tumor endothelial cells.

Tumor endothelial cells (TECs) and abnormalities of tumor blood vessels

Tumor angiogenesis results in enhanced vascularization, and consequently in enhanced tumor development: the new fresh vessels network tumor represents a way for tumor cells to escape from the solid tumor, leading to metastasis in distant organs. Tumor blood vessels differ dramatically from the normal vessels (Kindler et al., 2005): first of all, they are not organized in a hierarchical branching pattern which is typical for the normal vasculature, but they are very chaotic (McDonald & Choyke, 2003). Tumor vessels usually are morphologically immature: pericytes coverage is lower, they associate with TECs through loose junctions, and the vessels underlying basement membranes have not a regular thickness (Baluk et al., 2005). Therefore, also the morphology of TECs is irregular: they do not form monolayers and present

long cyto-plasmic projections extending across the lumen. Notably, the wall of TECs present irregular gaps and transcellular fenestrae, resulting in plasma leakage and increased vascular permeability. Cancer tissues are usually hypoxic, although they are usually highly vascularized (Figure 1).

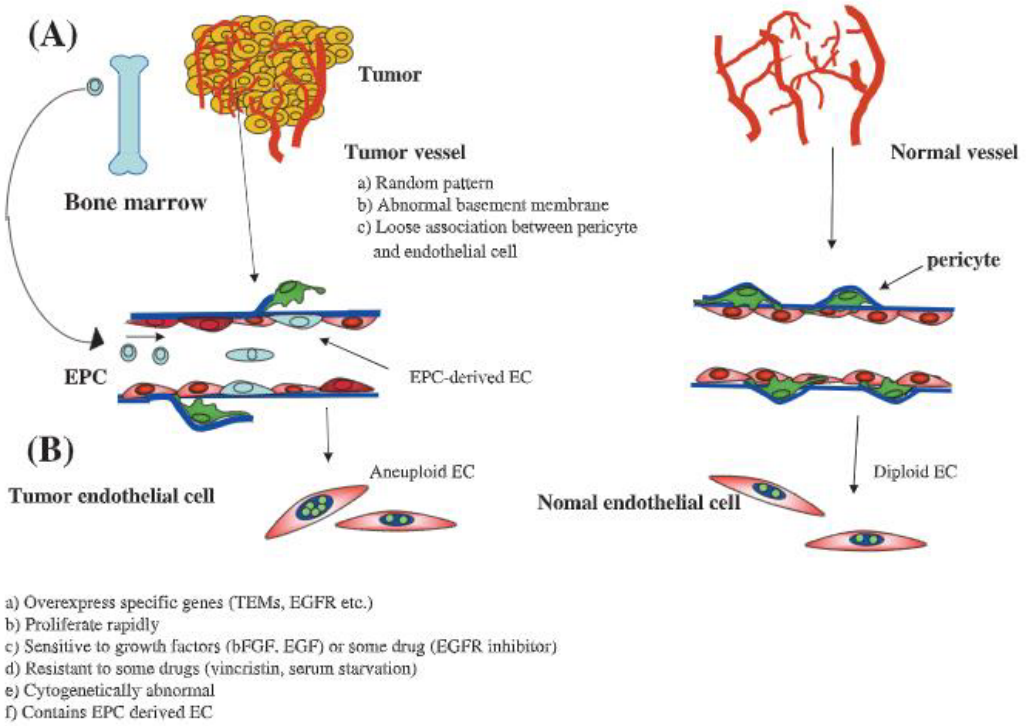

Figure 1 Differences in blood vessels and endothelial cells between tumors and normal tissues (Hida et al., 2008)

Phenotypic, functionals and genetic alterations of tumor endothelial cells (TECs)

In the past, most studies on tumor angiogenesis have been performed using normal endothelial cells (NECs) like HUVECs, due to the technical complexity of TECs isotation and to the fact that, at times, TECs were considered to loose their specific phenotype after isolation of tumor tissue (Hida et al., 2018). Later on, it has been shown that TECs, once isolated from tumors possess a distinct phenotype, different

from normal endothelial cells phenotype, at both molecular and functional levels. In 2000, for the first time, St. Croix et al. could isolate ECs from colon carcinoma and compared their gene expression profile to the one of ECs from normal colonic mucosa: they demonstrated a genetic difference between the two profiles, by the identification of a specific set of tumor endothelial markers (TEMs) (St. Croix et al., 2000). Then, following studies identified other tumor-specific markers, upregulated in isolated TECs of primary tumors, (such as breast, glioma or ovarian cancer) if compared to normal tissue (Parker et al., 2004) (Buckanovich et al., 2007): these TEMs consist of transmembrane proteins associated with the endothelial cell surface. Several different TEMs genes have been identified, including TEM1, TEM5, TEM7, TEM7R, and TEM8. TEM7 and TEM7R are the most specific for tumor endothelial cells, together with TEM8 (Nanda, Buckhaults, et al., 2004). TEM8 interacts with a COOH-terminal C5 domain of collagen alpha 3(VI) region, which is also mainly expressed in tumor endothelium: thus, TEM8 has been reported to be a key target in cancer antiangiogenic therapy (Nanda, Carson-Walter, et al., 2004). Since this study, several other works have been conducted in order to elucidate the molecular differences between TECs and NECs, using different types of analysis (Lu et al., 2007). In some reports, TECs have been compared to NECs in normal conditions, in order to understand the difference in the tissues under physiological angiogenesis. Seaman et al., focusing on angiogenesis in corpo luteum, succeeded in identification of several TEMs such as CD276, a regulator of T cell-mediated immune response (Seaman et al., 2007). Moreover, by comparing placental TECs and NECs genetic profiles, it has been demonstrated that high mobility group box 1 protein (HMGB1O) is expressed mainly during tumor angiogenesis (Van Beijnum et al., 2013). Other studies based on cultured TECs isolated from human renal carcinoma, have reported that they may derive from an intratumor embryonic like vasculogenesis, in which cancer or progenitor stem cells can differentiate into TECs. It has been reported that TECs express typical embryonic gene markers, even if the expression of these markers is usually a restricted feature of normal endothelial cells. Indeed, renal TECs, but not renal NECs, have been found to express the transcription factor Paired-box2 (Pax2) and the protein Pax2, which confer angiogenic, adhesion and invasion properties to TECs. Therefore, renal TECs did not undergo the senescence which is typical of NECs, and were resistant to serum starvation and vincristine-induced

apoptosis (Bussolati et al., 2003). Pax2 has been shown to be involved in renal tumor angiogenesis and its expression can antagonize PTEN tumor suppressor gene expression through Akt pathway (Fonsato et al., 2006). Hence, the expression of embryonic markers by TECs may indicate that they originate from normal adult or cancer stem and progenitor cells differentiated into TECs. Another possibility regarding the origin of TECs, is that they can derive from the normal endothelium or tumor adjacent vessels that change their phenotype due to the tumor microenvironment. In this context, extracellular microvesicles, apoptotic bodies or exosomes released from tumor cells, has been reported to induce epigenetic alteration of NECs, reprogramming them through the transfer of genetic material, such as mRNAs, microRNAs (miRNAs) or oncogenes (Ratajczak et al., 2006).

From a genetical point of view, as mentioned before, TECs may present different sets of genes, compared to NECs. It has been shown that endothelial cells in solid tumors are cytogenetically abnormal: these cells are aneuploid with multiple chromosomes and multiple centrosomes. The aneuploid characteristic of TECs have been observed in culture cells, in contrast to NECs, which remain diploid also in long-term cultures. Hida et al. compared mouse TECs (melanoma and liposarcoma) and NECs (skin and adipose), after isolating the two lines. TECs were isolated from a xenograft model of human tumor growing in nude mice, in order to obtain mouse TECs and understand the effects of tumor microenvironment on ECs behaviour. Mouse TECs express typical endothelial cells markers, such as CD31, VEGF, VEGF-Rs but also TEM1, TEM5, TEM7 and TEM8. Moreover, TECs grew faster, had a lower serum requirement and had larger nuclei, indicating the different DNA content compared to NECs. Karyotype analysis showed that TECs had multiple chromosomes characteristic of aneuploidy, whereas NECs grown under the same conditions were strictly diploid. Lastly, by using multiple-colored fluorescent in situ hybridization (M-FISH) analysis TECs presented structural aberrations such as nonreciprocal translocations, missing chromosomes, marker chromosomes, and double minutes (Hida et al., 2004). Hida et al., observed in TECs also abnormalities in the centrosomes, then associated with aneuploidic characteristics. These defects in centrosomes lead to a loss of polarity and chromosome missegregation, features detected also in aggressive human tumors. In high-risk human papillomavirus (HPV)-associated cervical neoplasia, the two HPV-encoded oncoproteins E6 and E7 are able to induce centrosome-related mitotic disturbances (Duensing & Münger,

2002). How TECs become cytogenetically abnormal is still not well understood: however, many studies have suggested hypotheses about the mechanisms involved. First of all, the interactions between tumor environment and TECs was considered crucial: growth factors or cytokines (for example VEGF or EGFR), released by tumor or stromal cells within the tumors, were identified causing genetic instability in TECs, through the activation of Akt signalling pathway, which is known to promote genetic instability. Other studies were focused on the crucial roles of tumor suppressors, like p53. The loss or mutational inactivation of p53 can cause abnormal amplification of centrosomes, leading to increased frequency of defective mitoses and resulting in missegregation of chromosomes into daughter cells (Vogelstein & Kinzler, 2004). A number of studies described the involvement of oncogenes: Fest et al., showed that overexpression of cMyc in nonadherent murine pro-B lymphocytic cells induced structural and genomic instability such as gene amplification, chromosomal breakage and deletions, increased aneuploidy and polyploidization (Fest et al., 2002). Transdifferentiation of tumor cells, cancer stem cells or vascular progenitor cells into endothelial cells is a possibly alternative mechanism: Streubel at al., suggested that microvascular endothelial cells in B-cell lymphomas are in part tumor related. However, how TECs in lymphoma acquires the specific genetic alterations of lymphoma cells remains to be elucidated (Streubel et al., 2004). Lastly, it has been suggested that cell fusion may be involved: malignant tumor cells or stem cells may fuse with normal endothelial cells or circulating vascular progenitor cells, resulting in aberrant endothelial cells. In fact, endothelial Progenitor Cells (EPC) , a subset of stem cells derived from bone marrow, has shown to be incorporated into tumor blood vessels (Rafii & Lyden, 2003). Cytogenetic abnormalities of TECs and their aneuploid character may suggest that acquired drug resistance to anticancer therapy can be a consequence of aneuploidy: indeed, it has been shown that some antiangiogenic drugs lose their efficacy over time, due to acquired resistance. Kerbel et al., suggested that TECs can develop resistance to many types of antiangiogenic drugs, including those specific for endothelial cells (Kerbel et al., 2001). Other examples are the renal carcinoma-derived TECs resistance to vincristine (Bussolati et al., 2003), hepatocellular carcinoma-derived TEC resistance to 5-fluorouracil and adriamycin (Xiong et al., 2009) and tumor-derived VEGF-mediated TEC resistance to paclitaxel with ATP-binding Cassette Sub-family B Member 1 (ABCB1) upregulation (Akiyama et al., 2012).

On the other hand, TECs may be more sensitive to specific drugs in comparison with normal ECs. Amin et al. found that Epidermal Growth Factor Receptor (EGFR) is not expressed in TECs, while high levels of EGF protein 1 were detected. EGFR is activated by EGF and other ligands, after phosphorylation, and it leads to tumor endothelial cells proliferation. In contrast, in NECs this EGF phosphorylation does not occur since NECs do not express EGFR. Subsequently, EGFR kinase inhibitors may represent possible therapeutic molecules, which target EGFR in TECs, in order to block TECs proliferation (Amin et al., 2006).

Mechanisms of tumor angiogenesis

Although tumor-associated angiogenesis has traditionally been defined as the sprouting of new vessels from preexisting vessels, it is becoming clear that blood vessels supporting tumor growth can also originate from cells recruited from bone marrow or can even differentiate from tumor stem cells. However, in all these cases, the tumor environment plays a pivotal role in cancer development: any stimulus from the environment, such as hypoxia and nutrient deprivation, triggers an "angiogenic switch" and allows tumor progression (Folkman & Hanahan, 1991). The "angiogenic switch" consists of a combination of signals in the tumor microenvironment, which lead to changes in multiple cell types. Tumor cells start to release cytokines and growth factors that activate the quiescent cells around them, promoting a cascade of events (Hida et al., 2018). The most relevant growth factor released is VEGF (with their associated receptors), and its expression can be induced by several events occurring in cells, such as activation of oncogenes or tumor suppressor genes mutations, or hypoxia. Upregulated levels of VEGF stimulate tumor ECs migration and proliferation, resulting in induction of tumor angiogenesis (Senger et al., 1983). Among VEGF-Rs, VEGFR-2 is mostly expressed in ECs and it plays a key role in angiogenic signaling. VEGF-R2, expressed in monocytes and macrophages, is also involved in angiogenesis by stimulating mobilization of these cells from bone marrow. In addition to VEGF, cancer cells secrete other factors crucial tumor expansion and angiogenesis, such as basic fibroblast growth factor (bFGF), angiopoietins (Ang), hepatocyte growth factor, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (Du et al., 2008). Although the

induction of angiogenesis may initially provide the tumor with more oxygen and nutrients, finally it results to be poor, with leaky vessels and consequent irregular blood flow, and increased permeability. Exposition of vessels basal lamina due to vascular leak, leads to recruitment and activation of platelets, which also can release angiogenic and permeability factors within the environment. High levels of PDGF released by platelets lead to ECs and perivascular cells activation. Perivascular cells, when persistently exposed to stimulatory signals, lose their capacity in regulating vessels stability and maturation. Tumor associated fibroblasts recruitment also occurs, leading to aberrant deposits of extracellular matrix (ECM) and releasing of metalloproteinases (MMPs), which remodel the ECM. The action of MMPs, like MMP-2, MMP-9 and MT1-MMP, consists of the proteolytic degradation of ECM: the effect is balanced by the expression of natural tissue inhibitors of MMPs, and regulated by the availability of its substrate (Deryugina & Quigley, 2010). ECM also produce molecules which regulate the release or the sequestration of soluble factors in the matrigel, such as bone morphogenetic proteins (BMPs), thrombospondins (TSPs), a disintegrin and metalloprotease (ADAM) family members, secreted protein, acidic and rich in cysteine (SPARC), syndecans and perlecan.

In this dynamic environment, cancer cells, integrins and receptors on the surface of endothelial cells bind factors that promote several signaling pathways, leading to phenotypic events, like cell migration, invasion, survival and proliferation, all involved in vessels sprouting. Receptors from different pathways can crosstalk in order to suppress or activate cells, and the interaction between ECs and tumor environment can regulate the cells faith, including their possibility to be tip or stalk cell. The principal pathways involved in cancer angiogenesis, like Notch, Semaphorins, Slits involve all their ligands and receptors, which drive the signals in both paracrine and autocrine ways. The response of ligands on different receptors, lead to activation of several different pathways: for example, Slit ligands bind to ROBO4-R that inhibit the signalling of VEGF and pathways with opposite effects in angiogenesis (Jones et al., 2008). Crosstalk between growth factors and integrins also plays a crucial role: integrin $\alpha_v\beta_3$ binds to angiogenic factors including VEGF-R2, the hepatocyte growth factor receptor c-Met, FGF-R1, PDGF-R and EGF receptors, while integrin $\alpha_5\beta_1$ interacts with some of these same receptors, as well as TIE2, which binds angiopoietins (Serini et al., 2008) (Somanath et al., 2009). In addition, some integrins can mediate directly with the ligand: in endothelial cells integrins

binds to VEGF, causing in vitro ECs adhesion and spread, independently of VEGF receptor binding (Hutchings et al., 2003).

Anti-angiogenic strategies for cancer treatment

As described above, tumor angiogenesis process is essential for tumor growth, progression, and metastasis: thus, anti-angiogenesis strategies have been developed overtime, in order to inhibit tumor vascularization. The classic anti-angiogenic drugs mainly have four different targets: they act on principal endogenous proangiogenic factors, they are natural angiogenesis inhibitors and then can be clinically administered, they can inhibit molecules which promote TECs invasion within tumor environment, and they can inhibit TECs proliferation (Mousa & Davis, 2017). At present, among the currently available anti-angiogenic drugs, the most effective in the treatment of various type of solid cancer are bevacizumab, sunitinib, sorafenib, pazopanib, axitinib, regorafenib, cabozantinib, nintedanib, ranibizumab, lenvatinib, vandetanib, and aflibercept (Wang et al., 2014), (Kikuchi et al., 2019). Bevacizumab was the first anti-angiogenic drug approved in 2004 by FDA for clinical application, as a first-line treatment for metastatic colorectal cancer in combination with chemotherapy. Bevacizumab is a humanized monoclonal IgG antibody against all the isoforms of VEGF-A: binding to VEGF-a, it blocks TECs vascularization, resulting in inhibition of tumor growth and cancer cells metastatic ability. (Ferrara et al., 2004). Nowadays, it is used for many types of cancer including, lung, breast, fallopian tubes, carcinoma, ovarian and peritoneal cancer. However, treatment with bevacizumab has been associated with several side effects, including hypertension, proteinuria, and gastrointestinal perforations and bleeding. Differently, most of the anti-angiogenic drugs mentioned above consist of anti-angiogenic receptor tyrosine kinase inhibitors (TKIs). TKIs target proangiogenic receptors, mainly VEGF-Rs family and PDGF-Rs but also FGF-Rs, c-Kit, and c-Met, and they can block kinase activity of receptor and so the transduction of downstream signal involved in cell proliferation, migration, and survival. The tyrosine kinase receptors group is the major subclass of the human protein kinases and can be subdivided into receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (nRTKs) (Manning et al., 2002) (Ling et al., 2018). RTKs family present an extracellular domain which binds

specific ligands, and an intracellular region, containing a protein tyrosine kinase domain, called kinase domain (Liu et al., 2018). The ligand's binding to the extracellular domain induces auto-phosphorylation of the intracellular domain, and finally receptor's tyrosine kinase activation (Beniston, 2011). The RTK family includes the insulin receptor and the receptors for many growth factor families such as VEGF, FGF, PDGF, and EGF. VEGF-A with the high-affinity binding to its RTKs VEGF-R1 and VEGF-R2 is the most important factor related to angiogenesis. Moreover, PDGF with its main receptors PDGFR-α and PDGFR-β is also a key regulator in tumor vascularization (Andrae et al., 2008). The first anti-angiogenic receptor TKI approved by FDA in 2005 was Sorafenib, which targets VEGFR-1/2/3, PDGFR-β, and c-Kit receptor. It was initially approved for treatment of advanced renal cell carcinoma (RCC) based on a phase III clinical trial, where 903 patients resistant to standard anticancer therapy, were randomly assigned into two groups: sorafenib or placebo. The study demonstrated a significant improvement in median progression-free survival (PFS) in sorafenib group compared with placebo group, and the partial response was elevated from 2% to 10% (Escudier et al., 2007). Currently, Sorafenib is recognized as a standard treatment for patients with RCC, advanced hepatocellular carcinoma (HCC), liver and thyroid cancer. Pazopanib and Axitinib are both multi-kinase inhibitors of VEGFR-1/2/3, PDGFR-α/β, and c-Kit receptor (Frampton, 2017).Axitinib was approved by the FDA in 2012 for the second-line treatment of patients with advanced RCC (Zarrabi et al., 2017). Cabozantinib is a small pan-tyrosine kinase inhibitor forVEGFR-1/2/3, c-Kit receptor, c-Met, and FLT-3 approved in 2012 for metastatic medullary thyroid cancer (MTC) (Ranieri et al., 2017). Nintedanib is a multiple angio-kinase inhibitor targeting VEGFR-1/2/3, PDGFR-α/β, and FGFR-1/2 (Dhillon, 2015). Apatinib can simultaneously suppress the kinase activities of VEGFR-2, c-Kit, and c-Src and was approved by the China Food and Drug Administration (CFDA) for the treatment of advanced gastric cancer (GC) in October 2014 (Aoyama & Yoshikawa, 2016). Endostar is a new human recombinant more effective and stable version of endostatin (rh-endostatin) expressed in Escherichia coli. It was approved by FDA in 2005 for treatment of non-small cell lung cancer. Additionally, other strategies inhibit mTOR (the mammalian target of rapamycin), acting at the catalytic subunit of two protein kinase complexes: mTOR complexes 1 and 2 (mTORC1/2). mTOR plays a key role in cell growth and proliferation and mTORC1 signaling is activated by

several oncogenic signaling pathways, and upregulated in many types of cancers (Giuliano & Pagès, 2013).

Although these clinically approved anti-angiogenic drugs have shown their effectiveness in inhibiting tumor angiogenesis, cancer progression and metastasis still occur after treatment. This is mainly due to the expression of alternative angiogenic pathways: since tumor angiogenesis is regulated by several different pathways, many interconnected pathways can compensate for the effect of single inhibition of VEGF signaling. Indeed, multi-targeted TKIs have a therapeutic advantage as compared to monoclonal antibodies since they can block multiple angiogenic signaling pathways simultaneously (Teleanu et al., 2019). However, also in the case of TKIs, the therapeutic effect is not complete: the administration of anti-angiogenic drugs can result in the development of resistance, mainly caused by revascularization, tumor vasculature protection, higher capacity in metastasis through new models of vascularization. For example, treatment with anti-VEGF leads to the upregulation of placental growth factor (PlGF), which binds to VEGF-R1 and causes transphosphorylation of VEGF-R2 (Fischer et al., 2007). PlGF leads to the recruitment of bone marrow endothelial progenitor cells, which confers resistance to anti-VEGF therapies (Loges et al., 2009). Despite the cessation of anti-VEGF therapy, it has been shown in multiple mouse models of cancer that the tumor became too aggressive and metastatic to regress. This suggested that the tumor has probably gained new strategies to trace VEGF, and express new angiogenic pathways (Pàez-Ribes et al., 2009). Moreover, anti-angiogenic treatment may increase hypoxia at both tumor and metastatic sites, promoting cancer stem cells survival and maintaining tumor growth and resistance to therapy (Mancuso et al., 2006). Notably, although anti-angiogenic drugs therapy has shown to inhibit tumor progression, there are only a few examples supporting the efficacy of any antiangiogenic monotherapy; thus, novel strategies have been focusing on combining anti-angiogenic agents with chemotherapy or immunotherapy. Association of different types of drugs has led to improved therapeutic effects and reduction of side effects (Letellier et al., 2017). Clinical studies have proved that the combination of bevacizumab and conventional chemotherapy can increase the survival and response rates in patients with gastrointestinal cancer, non-small cell lung cancer, breast cancer (Robert et al., 2011) and ovarian cancer. Specifically, administration of bevacizumab combined with gemcitabine and carboplatin for the treatment of recurrent ovarian cancer (OCEANS

trial), has shown to improve median progression-free survival (Wieser & Marth, 2019). Similar results have been observed using the combination of bevacizumab with PEGylated liposomal doxorubicin, weekly paclitaxel, or topotecan for the therapy of platinum-resistant ovarian cancer. Similarly, there are emerging evidences on beneficial effects in cancer, by combinating TKIs and immunotherapy. A study conducted by Choueiri et al. (JAVELIN Renal 100), has reported the improvement of patients with advanced RCC, after a combined therapy with axitinib and avelumab (a PD-L1 mAb), compared with sunitinib (Motzer et al., 2019). Other combinations like lenvatinib with pembrolizumab or SHR 1210 with apatinib in patients with HCC have been shown to be therapeutically effective (Kudo, 2018).

In the light of the latest observations, anti-angiogenic therapy cannot eradicate tumors fully the tumors, even if different drugs are combined. Anti-angiogenic drugs resistance remains a challenge in anti-cancer therapy, resulting in the study of new, alternative strategies in order to obtain a more substantial and lasting therapeutic effect. In this respect, in the next chapter some of alternative antiangiogenic strategies will be described, referring to the review of 2019 entitled "Alternative Strategies to inhibit tumor vascularization" (Alessia Brossa, Lola Buono, **Sofia Fallo**, Alessandra Fiorio Pla, Luca Munaron and Benedetta Bussolati, Dec 2019). These alternative strategies include the use of new mAbs, the study of new possible targets such as Calcium permeable channels and the endothelial transcription factor ERG, the vaccinations with endothelial antigens, the use of extracellular vesicles and new drugs with vascular normalizing effects.

Extracellular vesicles as anti-angiogenic treatment

Extracellular vesicles (EVs) are small-membrane bound vesicles (50 nm-1um), enclosed by a lipid bilayer ranging from 30 m to 2000 nm in diameter, and secreted by a wide variety of cells (Figure 2). In the past decade, they emerged as a new mechanism of cell-to-cell communication, due to their capability to transfer proteins, lipids and nucleic acids to target cells, resulting in modifying their function and phenotype. They have unique molecular profiles acquired from originating cells and they are present in a number of body fluids, including blood and urine. Generally, EVs are produced by all types of cells in basal condition; however, their production

is higher in specific cell types (stem cells, progenitor cells and cancer cells) and may increase during cell proliferation and activation, or under stress conditions (Yáñez-Mó et al., 2015). Since EVs are vehicles for crosstalk between cells, they can influence several physiological and pathological functions of both recipient and parent cells. EVs target specific cells binding to surface receptors or bioactive lipids, merging their membrane contents into the recipient cell plasma membrane and finally delivering their bio-active cargo including oncogenes, transcription factors, DNA and extracellular secreted RNA (exRNA), including long-non-coding RNA, messenger-RNA (mRNA) and microRNAs (miRNAs), which allow them to produce epigenetic changes in target cells both locally and systemically (Camussi & J., 2013). Cancer-derived EVs play critical roles in tumorigenesis: they activate all cellular mechanisms altered in cancer, such as cell proliferation, migration, invasion, apoptosis and angiogenesis. Therefore, EVs can induce metastasis, evasion of host immune defense, chemo-resistance and promote a pre-metastatic niche inducing tumor formation (Tompkins et al., 2015). On the other hand, EVs secreted by normal cells have their innate therapeutic potential: several studies have previously demonstrated that EVs released by stem cells or progenitor cells may deliver informations to target cells on tissue regeneration or on immune response modulation, including activation of angiogenic programmes, suppression of apoptosis and stimulation of cell proliferation. Several studies, have reported the therapeutic application of EVs, isolated from multipotent stem cells, such as mesenchymal stem cells (MSCs) or human liver stem cells (HLSCs), in the field of regenerative medicine (EL Andaloussi et al., 2013) Administration of EVs has been clinically used for the treatment of various diseases, including cardiovascular disease, kidney fibrosis and also in model of acute kidney injury (AKI) In regard to these studies, MSC-EVs therapy has shown an increased survival in a lethal mouse model of AKI and an amelioration of Kidney injury in a mouse model of aristolochic acid Nephropathy (Bruno et al., 2012) (Kholia et al., 2020). Moreover, also HLSC-EVs somministration led to a remarkable improvement of survival in the mouse model of AKI (Sanchez et al., 2014).

Regarding the potential application of EVs for cancer therapy, several studies have been conducted on the anticancer and anti-angiogenic effect of stem cellsA-derived EVs, mainly based on the delivery of their miRNAs cargo into tumor target cells. microRNAs are are endogenous ~23-nt RNAs, and are the most investigated and best

characterized molecules in the class of small regulatory noncoding RNAs (Bartel, 2009): they are involved in several important physiological and pathological processes and functions through the post-transcriptional regulation of mRNAs. A great number of miRNAs with anti-tumor and anti-angiogenic properties have been described, such as let-7 family, miRNA200 family, miRNA15-16 cluster, and miRNA 451 (Lopatina et al., 2016). Among the possible EVs sources, most studies for anticancer therapy have employed EVs isolated from stem cells such as mesenchymal stem cells isolated from different sources, adipose stem cells (ASCs) or, more recently, HLSCs. Indeed, Camussi et al., has shown the emergent and promising therapeutic role of HLSC-EVs in vitro in HepG2 hepatoma and primary HCC, by inhibition of tumor growth and stimulation of apoptosis. The study was confirmed also in vivo, where after injection of HLSC-EVs in SCID mice with HepG2, the tumor size importantly reduced. Anti-tumor effects of HLSC-EVs were observed also in lymphoblastoma and glioblastoma (Fonsato et al., 2012). However, few studies investigated their direct effects on tumor angiogenesis. Lee et al. showed that EVs isolated from mesenchymal stem cells (MSC) were able to inhibit tumor growth and angiogenesis in a murine model of breast cancer, by downregulating VEGF production in breast cancer cells (Lee et al., 2014). EVs isolated from cardiosphere-derived cells were able to inhibit tumor angiogenesis in a murine model of fibrosarcoma (Grigorian-Shamagian et al., 2017). More recently, Lopatina et al. have showed both in vivo and in vivo the antitumor potential of HLSC-EVs, which exhibit direct anti-angiogenic effects on TECs isolated from human renal carcinoma (HRC). The work of Lopatina et al. will be described in the third chapter of the thesis, in the article entitled “Extracellular vesicles from human liver stem cells inhibit tumor angiogenesis” (Tatiana Lopatina, Cristina Grange, Valentina Fonsato, Marta Tapparo, Alessia Brossa, **Sofia Fallo**, Adriana Pitino, Maria Beatriz Herrera-Sanchez, Sharad Kholia, Giovanni Camussi, Benedetta Bussolati.Pub. Aug 2018).

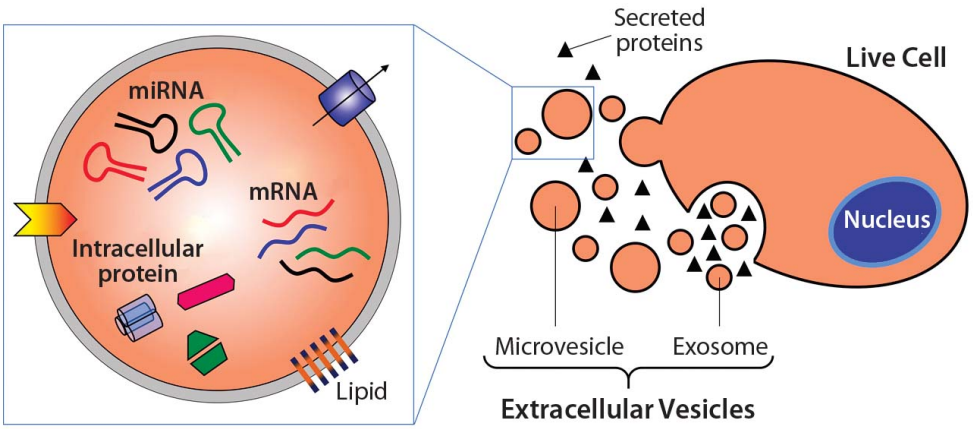

Figure 2 Representation of Extracellular vesicles. Extracelluar vesicles are essentially membrane-bound samples of cellular cytosol that transport cargoes such as miRNA, mRNA, and proteins. Exosomes form from fusion of multivesicular bodies with the plasma membrane, whereas microvesicles bud directly from live cells. Apoptotic bodies result from outward blebbing of the cell surface during apoptosis but are not secreted from healthy cells. (Brindley D.A)

References:

Akiyama, K., Ohga, N., Hida, Y., Kawamoto, T., Sadamoto, Y., Ishikawa, S., Maishi, N., Akino, T., Kondoh, M., Matsuda, A., Inoue, N., Shindoh, M., & Hida, K. (2012). Tumor endothelial cells acquire drug resistance by MDR1 up-regulation via VEGF signaling in tumor microenvironment. *American Journal of Pathology*, *180*(3), 1283–1293. https://doi.org/10.1016/j.ajpath.2011.11.029

Amin, D. N., Hida, K., Bielenberg, D. R., & Klagsbrun, M. (2006). Tumor endothelial cells express epidermal growth factor receptor (EGFR) but not ErbB3 and are responsive to EGF and to EGFR kinase inhibitors. *Cancer Research*, *66*(4), 2173–2180. https://doi.org/10.1158/0008-5472.CAN-05-3387

Andrae, J., Gallini, R., & Betsholtz, C. (2008). Role of platelet-derived growth factors in physiology and medicine. *Genes & Development*, *22*(10), 1276–1312. https://doi.org/10.1101/gad.1653708

Aoyama, T., & Yoshikawa, T. (2016). Apatinib — new third-line option for refractory gastric or GEJ cancer. *Nature Reviews Clinical Oncology*, *13*(5), 268–270. https://doi.org/10.1038/nrclinonc.2016.53

Baluk, P., Hashizume, H., & McDonald, D. M. (2005). Cellular abnormalities of blood vessels as targets in cancer. *Current Opinion in Genetics & Development*, *15*(1), 102–111. https://doi.org/https://doi.org/10.1016/j.gde.2004.12.005

Bartel, D. P. (2009). MicroRNAs: Target Recognition and Regulatory Functions. *Cell*, *136*(2), 215–233. https://doi.org/10.1016/j.cell.2009.01.002

Beniston, M. (2011). Alps. *Encyclopedia of Earth Sciences Series*, *Part 3*, 35–38. https://doi.org/10.1007/978-90-481-2642-2_16

Bruno, S., Grange, C., Collino, F., Deregibus, M. C., Cantaluppi, V., Biancone, L., Tetta, C., & Camussi, G. (2012). Microvesicles derived from mesenchymal stem cells enhance survival in a lethal model of acute kidney injury. *PLoS ONE*, *7*(3). https://doi.org/10.1371/journal.pone.0033115

Buckanovich, R. J., Sasaroli, D., O'Brien-Jenkins, A., Botbyl, J., Hammond, R., Katsaros, D., Sandaltzopoulos, R., Liotta, L. A., Gimotty, P. A., & Coukos, G. (2007). Tumor vascular proteins as biomarkers in ovarian cancer. *Journal of Clinical Oncology*, *25*(7), 852–861. https://doi.org/10.1200/JCO.2006.08.8583

Bussolati, B., Deambrosis, I., Russo, S., Deregibus, M. C., & Camussi, G. (2003). Altered angiogenesis and survival in human tumor-derived endothelial cells. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *17*(9), 1159–1161. https://doi.org/10.1096/fj.02-0557fje

Camussi, G., & J., P. (2013). Perspectives on the Potential Therapeutic Uses of Vesicles. *Exosomes and Microvesicles*, 1. https://doi.org/10.5772/57393

Deryugina, E. I., & Quigley, J. P. (2010). Pleiotropic roles of matrix metalloproteinases in tumor angiogenesis: Contrasting, overlapping and compensatory functions. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1803*(1), 103–120. https://doi.org/10.1016/j.bbamcr.2009.09.017

Dhillon, S. (2015). Nintedanib: A Review of Its Use as Second-Line Treatment in Adults with Advanced Non-Small Cell Lung Cancer of Adenocarcinoma

Histology. *Targeted Oncology*, *10*(2), 303–310. https://doi.org/10.1007/s11523-015-0367-8

Du, R., Lu, K. V., Petritsch, C., Liu, P., Ganss, R., Passegué, E., Song, H., VandenBerg, S., Johnson, R. S., Werb, Z., & Bergers, G. (2008). HIF1α Induces the Recruitment of Bone Marrow-Derived Vascular Modulatory Cells to Regulate Tumor Angiogenesis and Invasion. *Cancer Cell*, *13*(3), 206–220. https://doi.org/10.1016/j.ccr.2008.01.034

Duensing, S., & Münger, K. (2002). Human papillomaviruses and centrosome duplication errors: Modeling the origins of genomic instability. *Oncogene*, *21*(40 REV. ISS. 4), 6241–6248. https://doi.org/10.1038/sj.onc.1205709

EL Andaloussi, S., Mäger, I., Breakefield, X. O., & Wood, M. J. A. (2013). Extracellular vesicles: biology and emerging therapeutic opportunities. *Nature Reviews Drug Discovery*, *12*(5), 347–357. https://doi.org/10.1038/nrd3978

Escudier, B., Eisen, T., Stadler, W. M., Szczylik, C., Oudard, S., Siebels, M., Negrier, S., Chevreau, C., Solska, E., Desai, A. A., Rolland, F., Demkow, T., Hutson, T. E., Gore, M., Freeman, S., Schwartz, B., Shan, M., Simantov, R., & Bukowski, R. M. (2007). Sorafenib in Advanced Clear-Cell Renal-Cell Carcinoma. *New England Journal of Medicine*, *356*(2), 125–134. https://doi.org/10.1056/NEJMoa060655

Ferrara, N., Hillan, K. J., Gerber, H.-P., & Novotny, W. (2004). Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nature Reviews Drug Discovery*, *3*(5), 391–400. https://doi.org/10.1038/nrd1381

Fest, T., Mougey, V., Dalstein, V., Hagerty, M., Milette, D., Silva, S., & Mai, S. (2002). c-MYC overexpression in Ba/F3 cells simultaneously elicits genomic instability and apoptosis. *Oncogene*, *21*(19), 2981–2990. https://doi.org/10.1038/sj/onc/1205274

Fischer, C., Jonckx, B., Mazzone, M., Zacchigna, S., Loges, S., Pattarini, L., Chorianopoulos, E., Liesenborghs, L., Koch, M., De Mol, M., Autiero, M., Wyns, S., Plaisance, S., Moons, L., van Rooijen, N., Giacca, M., Stassen, J. M., Dewerchin, M., Collen, D., & Carmeliet, P. (2007). Anti-PlGF Inhibits Growth of VEGF(R)-Inhibitor-Resistant Tumors without Affecting Healthy Vessels. *Cell*, *131*(3), 463–475. https://doi.org/10.1016/j.cell.2007.08.038

Folkman, J., & Hanahan, D. (1991). Switch to the angiogenic phenotype during

tumorigenesis. *Princess Takamatsu Symposia*, *22*, 339—347. http://europepmc.org/abstract/MED/1726933

Fonsato, V., Buttiglieri, S., Deregibus, M. C., Puntorieri, V., Bussolati, B., & Camussi, G. (2006). Expression of Pax2 in human renal tumor-derived endothelial cells sustains apoptosis resistance and angiogenesis. *American Journal of Pathology*, *168*(2), 706–713. https://doi.org/10.2353/ajpath.2006.050776

Fonsato, V., Collino, F., Herrera, M. B., Cavallari, C., Deregibus, M. C., Cisterna, B., Bruno, S., Romagnoli, R., Salizzoni, M., Tetta, C., & Camussi, G. (2012). Human liver stem cell-derived microvesicles inhibit hepatoma growth in SCID mice by delivering antitumor microRNAs. *Stem Cells (Dayton, Ohio)*, *30*(9), 1985–1998. https://doi.org/10.1002/stem.1161

Frampton, J. E. (2017). Pazopanib: a Review in Advanced Renal Cell Carcinoma. *Targeted Oncology*, *12*(4), 543–554. https://doi.org/10.1007/s11523-017-0511-8

Giuliano, S., & Pagès, G. (2013). Mechanisms of resistance to anti-angiogenesis therapies. *Biochimie*, *95*(6), 1110–1119. https://doi.org/https://doi.org/10.1016/j.biochi.2013.03.002

Grigorian-Shamagian, L., Fereydooni, S., Liu, W., Echavez, A., & Marbán, E. (2017). Harnessing the heart's resistance to malignant tumors: Cardiacderived extracellular vesicles decrease fibrosarcoma growth and leukemia-related mortality in rodents. *Oncotarget*, *8*(59), 99624–99636. https://doi.org/10.18632/oncotarget.20454

Hida, K., Hida, Y., Amin, D. N., Flint, A. F., Panigrahy, D., Morton, C. C., & Klagsbrun, M. (2004). Tumor-associated endothelial cells with cytogenetic abnormalities. *Cancer Research*, *64*(22), 8249–8255. https://doi.org/10.1158/0008-5472.CAN-04-1567

Hida, K., Hida, Y., & Shindoh, M. (2008). Understanding tumor endothelial cell abnormalities to develop ideal anti-angiogenic therapies. *Cancer Science*, *99*(3), 459–466. https://doi.org/https://doi.org/10.1111/j.1349-7006.2007.00704.x

Hida, K., Maishi, N., Annan, D. A., & Hida, Y. (2018). Contribution of tumor endothelial cells in cancer progression. *International Journal of Molecular Sciences*, *19*(5). https://doi.org/10.3390/ijms19051272

HUTCHINGS, H., ORTEGA, N., & PLOUËT, J. (2003). Extracellular matrix-bound vascular endothelial growth factor promotes endothelial cell adhesion,

migration, and survival through integrin ligation. *The FASEB Journal*, *17*(11), 1520–1522. https://doi.org/10.1096/fj.02-0691fje

Jones, C. A., London, N. R., Chen, H., Park, K. W., Sauvaget, D., Stockton, R. A., Wythe, J. D., Suh, W., Larrieu-Lahargue, F., Mukouyama, Y. S., Lindblom, P., Seth, P., Frias, A., Nishiya, N., Ginsberg, M. H., Gerhardt, H., Zhang, K., & Li, D. Y. (2008). Robo4 stabilizes the vascular network by inhibiting pathologic angiogenesis and endothelial hyperpermeability. *Nature Medicine*, *14*(4), 448–453. https://doi.org/10.1038/nm1742

Keedy, V. L., & Sandler, A. B. (2007). Inhibition of angiogenesis in the treatment of non-small cell lung cancer. *Cancer Science*, *98*(12), 1825–1830. https://doi.org/10.1111/j.1349-7006.2007.00620.x

Kerbel, R. S., Yu, J., Tran, J., Man, S., Viloria-Petit, A., Klement, G., Coomber, B. L., & Rak, J. (2001). Possible Mechanisms of Acquired Resistance to Anti-angiogenic Drugs: Implications for the Use of Combination Therapy Approaches. *Cancer and Metastasis Reviews*, *20*(1), 79–86. https://doi.org/10.1023/A:1013172910858

Kholia, S., Herrera Sanchez, M. B., Cedrino, M., Papadimitriou, E., Tapparo, M., Deregibus, M. C., Bruno, S., Antico, F., Brizzi, M. F., Quesenberry, P. J., & Camussi, G. (2020). Mesenchymal Stem Cell Derived Extracellular Vesicles Ameliorate Kidney Injury in Aristolochic Acid Nephropathy. *Frontiers in Cell and Developmental Biology*, *8*(March), 1–17. https://doi.org/10.3389/fcell.2020.00188

Kikuchi, R., Stevens, M., Harada, K., Oltean, S., & Murohara, T. (2019). *Chapter One - Anti-angiogenic isoform of vascular endothelial growth factor-A in cardiovascular and renal disease* (G. S. Makowski (ed.); Vol. 88, pp. 1–33). Elsevier. https://doi.org/https://doi.org/10.1016/bs.acc.2018.10.001

Kindler, H. L., Friberg, G., Singh, D. A., Locker, G., Nattam, S., Kozloff, M., Taber, D. A., Karrison, T., Dachman, A., Stadler, W. M., & Vokes, E. E. (2005). Phase II Trial of Bevacizumab Plus Gemcitabine in Patients With Advanced Pancreatic Cancer. *Journal of Clinical Oncology*, *23*(31), 8033–8040. https://doi.org/10.1200/JCO.2005.01.9661

Kudo, M. (2018). Systemic Therapy for Hepatocellular Carcinoma: Latest Advances. *Cancers*, *10*(11), 412. https://doi.org/10.3390/cancers10110412

Lee, J.-K., Park, S.-R., Jung, B.-K., Jeon, Y.-K., Lee, Y.-S., Kim, M.-K., Kim, Y.-G.,

Jang, J.-Y., & Kim, C.-W. (2014). Exosomes Derived from Mesenchymal Stem Cells Suppress Angiogenesis by Down-Regulating VEGF Expression in Breast Cancer Cells. *PLOS ONE*, *8*(12), 1–11. https://doi.org/10.1371/journal.pone.0084256

Letellier, C., Sasmal, S. K., Draghi, C., Denis, F., & Ghosh, D. (2017). A chemotherapy combined with an anti-angiogenic drug applied to a cancer model including angiogenesis. *Chaos, Solitons & Fractals*, *99*, 297–311. https://doi.org/https://doi.org/10.1016/j.chaos.2017.04.013

Ling, Y., Xie, Q., Zhang, Z., & Zhang, H. (2018). Protein kinase inhibitors for acute leukemia. *Biomarker Research*, *6*, 8. https://doi.org/10.1186/s40364-018-0123-1

Liu, Q., Yu, S., Zhao, W., Qin, S., Chu, Q., & Wu, K. (2018). EGFR-TKIs resistance via EGFR-independent signaling pathways. *Molecular Cancer*, *17*(1), 53. https://doi.org/10.1186/s12943-018-0793-1

Loges, S., Schmidt, T., & Carmeliet, P. (2009). {\textquotedblleft}Antimyeloangiogenic{\textquotedblright} Therapy for Cancer by Inhibiting PlGF. *Clinical Cancer Research*, *15*(11), 3648–3653. https://doi.org/10.1158/1078-0432.CCR-08-2276

Lopatina, T., Gai, C., Deregibus, M. C., Kholia, S., & Camussi, G. (2016). Cross Talk between Cancer and Mesenchymal Stem Cells through Extracellular Vesicles Carrying Nucleic Acids. *Frontiers in Oncology*, *6*, 125. https://doi.org/10.3389/fonc.2016.00125

Lopatina T., Grange C., Fonsato V., Tapparo M., Brossa A, Fallo S., Pitino A. Herrera-Sanchez M.B., Kholia S., Camussi G. and Bussolati B (2018) Extracellular vesicles from human liver stem cells inhibit tumor angiogenesis. *International Journal of Cancer*

Lu, C., Bonome, T., Li, Y., Kamat, A. A., Han, L. Y., Schmandt, R., Coleman, R. L., Gershenson, D. M., Jaffe, R. B., Birrer, M. J., & Sood, A. K. (2007). Gene alterations identified by expression profiling in tumor-associated endothelial cells from invasive ovarian carcinoma. *Cancer Research*, *67*(4), 1757–1768. https://doi.org/10.1158/0008-5472.CAN-06-3700

Mancuso, M. R., Davis, R., Norberg, S. M., O'Brien, S., Sennino, B., Nakahara, T., Yao, V. J., Inai, T., Brooks, P., Freimark, B., Shalinsky, D. R., Hu-Lowe, D. D., & McDonald, D. M. (2006). Rapid vascular regrowth in tumors after reversal of VEGF inhibition. *The Journal of Clinical Investigation*, *116*(10), 2610–2621.

https://doi.org/10.1172/JCI24612

Manning, G., Whyte, D. B., Martinez, R., Hunter, T., & Sudarsanam, S. (2002). The Protein Kinase Complement of the Human Genome. *Science*, *298*(5600), 1912–1934. https://doi.org/10.1126/science.1075762

McDonald, D. M., & Choyke, P. L. (2003). Imaging of angiogenesis: from microscope to clinic. *Nature Medicine*, *9*(6), 713–725. https://doi.org/10.1038/nm0603-713

Motzer, R. J., Penkov, K., Haanen, J., Rini, B., Albiges, L., Campbell, M. T., Venugopal, B., Kollmannsberger, C., Negrier, S., Uemura, M., Lee, J. L., Vasiliev, A., Miller Jr, W. H., Gurney, H., Schmidinger, M., Larkin, J., Atkins, M. B., Bedke, J., Alekseev, B., … Choueiri, T. K. (2019). Avelumab plus Axitinib versus Sunitinib for Advanced Renal-Cell Carcinoma. *The New England Journal of Medicine*, *380*(12), 1103–1115. https://doi.org/10.1056/NEJMoa1816047

Mousa, S. A., & Davis, P. J. (2017). Chapter 1 - Angiogenesis and Anti-Angiogenesis Strategies in Cancer. In S. A. Mousa & P. J. Davis (Eds.), *Anti-Angiogenesis Strategies in Cancer Therapeutics* (pp. 1–19). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-802576-5.00001-2

Nanda, A., Buckhaults, P., Seaman, S., Agrawal, N., Boutin, P., Shankara, S., Nacht, M., Teicher, B., Stampfl, J., Singh, S., Vogelstein, B., Kinzler, K. W., & St. Croix, B. (2004). Identification of a binding partner for the endothelial cell surface proteins TEM7 and TEM7R. *Cancer Research*, *64*(23), 8507–8511. https://doi.org/10.1158/0008-5472.CAN-04-2716

Nanda, A., Carson-Walter, E. B., Seaman, S., Barber, T. D., Stampfl, J., Singh, S., Vogelstein, B., Kinzler, K. W., & St. Croix, B. (2004). TEM8 Interacts with the Cleaved C5 Domain of Collagen α3(VI). *Cancer Research*, *64*(3), 817–820. https://doi.org/10.1158/0008-5472.CAN-03-2408

Pàez-Ribes, M., Allen, E., Hudock, J., Takeda, T., Okuyama, H., Viñals, F., Inoue, M., Bergers, G., Hanahan, D., & Casanovas, O. (2009). Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell*, *15*(3), 220–231. https://doi.org/10.1016/j.ccr.2009.01.027

Parker, B. S., Argani, P., Cook, B. P., Liangfeng, H., Chartrand, S. D., Zhang, M., Saha, S., Bardelli, A., Jiang, Y., St. Martin, T. B., Nacht, M., Teicher, B. A.,

Klinger, K. W., Sukumar, S., & Madden, S. L. (2004). Erratum: Alterations in vascular gene expression in invasive breast carcinoma (Cancer Research (November 1, 2004) 64, 1 (7857-7866)). *Cancer Research*, *64*(23), 8794. https://doi.org/10.1158/0008-5472.CAN-64-23-COR

Rafii, S., & Lyden, D. (2003). Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. *Nature Medicine*, *9*(6), 702–712. https://doi.org/10.1038/nm0603-702

Ranieri, G., Marech, I., Asabella, A. N., Di Palo, A., Porcelli, M., Lavelli, V., Rubini, G., Ferrari, C., & Gadaleta, C. D. (2017). Tyrosine-Kinase inhibitors therapies with mainly anti-angiogenic activity in advanced renal cell carcinoma: Value of PET/CT in response evaluation. *International Journal of Molecular Sciences*, *18*(9), 1–22. https://doi.org/10.3390/ijms18091937

Ratajczak, J., Wysoczynski, M., Hayek, F., Janowska-Wieczorek, A., & Ratajczak, M. Z. (2006). Membrane-derived microvesicles: Important and underappreciated mediators of cell-to-cell communication. *Leukemia*, *20*(9), 1487–1495. https://doi.org/10.1038/sj.leu.2404296

Robert, N. J., Diéras, V., Glaspy, J., Brufsky, A. M., Bondarenko, I., Lipatov, O. N., Perez, E. A., Yardley, D. A., Chan, S. Y. T., Zhou, X., Phan, S.-C., & O'Shaughnessy, J. (2011). RIBBON-1: Randomized, Double-Blind, Placebo-Controlled, Phase III Trial of Chemotherapy With or Without Bevacizumab for First-Line Treatment of Human Epidermal Growth Factor Receptor 2–Negative, Locally Recurrent or Metastatic Breast Cancer. *Journal of Clinical Oncology*, *29*(10), 1252–1260. https://doi.org/10.1200/JCO.2010.28.0982

Saif, M. W., Elfiky, A., & Salem, R. R. (2007). Gastrointestinal Perforation Due to Bevacizumab in Colorectal Cancer. *Annals of Surgical Oncology*, *14*(6), 1860–1869. https://doi.org/10.1245/s10434-006-9337-9

Sanchez, M. B. H., Bruno, S., Grange, C., Tapparo, M., Cantaluppi, V., Tetta, C., & Camussi, G. (2014). Human liver stem cells and derived extracellular vesicles improve recovery in a murine model of acute kidney injury. *Stem Cell Research and Therapy*, *5*(6), 1–11. https://doi.org/10.1186/scrt514

Seaman, S., Stevens, J., Yang, M. Y., Logsdon, D., Graff-Cherry, C., & St. Croix, B. (2007). Genes that Distinguish Physiological and Pathological Angiogenesis. *Cancer Cell*, *11*(6), 539–554. https://doi.org/10.1016/j.ccr.2007.04.017

Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S., & Dvorak, H. F. (1983). Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science*, *219*(4587), 983 LP – 985. https://doi.org/10.1126/science.6823562

Serini, G., Napione, L., Arese, M., & Bussolino, F. (2008). Besides adhesion: New perspectives of integrin functions in angiogenesis. *Cardiovascular Research*, *78*(2), 213–222. https://doi.org/10.1093/cvr/cvn045

Somanath, P. R., Ciocea, A., & Byzova, T. V. (2009). Integrin and growth factor receptor alliance in angiogenesis. *Cell Biochemistry and Biophysics*, *53*(2), 53–64. https://doi.org/10.1007/s12013-008-9040-5

St. Croix, B., Rago, C., Velculescu, V., Traverso, G., Romans, K. E., Montegomery, E., Lal, A., Riggins, G. J., Lengauer, C., Vogelstein, B., & Kinzler, K. W. (2000). Genes expressed in human tumor endothelium. *Science*, *289*(5482), 1197–1202. https://doi.org/10.1126/science.289.5482.1197

Streubel, B., Chott, A., Huber, D., Exner, M., Jäger, U., Wagner, O., & Schwarzinger, I. (2004). Lymphoma-specific genetic aberrations in microvascular endothelial cells in B-cell lymphomas. *New England Journal of Medicine*, *351*(3), 250–259. https://doi.org/10.1056/NEJMoa033153

Teleanu, R. I., Chircov, C., Grumezescu, A. M., & Teleanu, D. M. (2019). Tumor Angiogenesis and Anti-Angiogenic Strategies for Cancer Treatment. *Journal of Clinical Medicine*, *9*(1), 84. https://doi.org/10.3390/jcm9010084

Tompkins, A. J., Chatterjee, D., Maddox, M., Wang, J., Arciero, E., Camussi, G., Quesenberry, P. J., & Renzulli, J. F. (2015). The emergence of extracellular vesicles in urology: fertility, cancer, biomarkers and targeted pharmacotherapy. In *Journal of extracellular vesicles* (Vol. 4, p. 23815). https://doi.org/10.3402/jev.v4.23815

Van Beijnum, J. R., Nowak-Sliwinska, P., Van Den Boezem, E., Hautvast, P., Buurman, W. A., & Griffioen, A. W. (2013). Tumor angiogenesis is enforced by autocrine regulation of high-mobility group box 1. *Oncogene*, *32*(3), 363–374. https://doi.org/10.1038/onc.2012.49

Vogelstein, B., & Kinzler, K. W. (2004). Cancer genes and the pathways they control. *Nature Medicine*, *10*(8), 789–799. https://doi.org/10.1038/nm1087

Wang, Q., Zorn, J. A., & Kuriyan, J. (2014). Chapter Two - A Structural Atlas of Kinases Inhibited by Clinically Approved Drugs. In K. M. Shokat (Ed.), *Protein*

Kinase Inhibitors in Research and Medicine (Vol. 548, pp. 23–67). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-397918-6.00002-1

Wieser, V., & Marth, C. (2019). Resistance to chemotherapy and anti-angiogenic therapy in ovarian cancer. *Memo - Magazine of European Medical Oncology*, *12*(2), 144–148. https://doi.org/10.1007/s12254-019-0478-5

Xiong, Y. Q., Sun, H. C., Zhang, W., Zhu, X. D., Zhuang, P. Y., Zhang, J. B., Wang, L., Wu, W. Z., Qin, L. X., & Tang, Z. Y. (2009). Human hepatocellular carcinoma tumor-derived endothelial cells manifest increased angiogenesis capability and drug resistance compared with normal endothelial cells. *Clinical Cancer Research*, *15*(15), 4838–4846. https://doi.org/10.1158/1078-0432.CCR-08-2780

Yáñez-Mó, M., Siljander, P. R.-M., Andreu, Z., Zavec, A. B., Borràs, F. E., Buzas, E. I., Buzas, K., Casal, E., Cappello, F., Carvalho, J., Colás, E., Cordeiro-da Silva, A., Fais, S., Falcon-Perez, J. M., Ghobrial, I. M., Giebel, B., Gimona, M., Graner, M., Gursel, I., … De Wever, O. (2015). Biological properties of extracellular vesicles and their physiological functions. *Journal of Extracellular Vesicles*, *4*, 27066. https://doi.org/10.3402/jev.v4.27066

Zarrabi, K., Fang, C., & Wu, S. (2017). New treatment options for metastatic renal cell carcinoma with prior anti-angiogenesis therapy. *Journal of Hematology and Oncology*, *10*(1), 1–12. https://doi.org/10.1186/s13045-016-0374-y

THE GLOMERULAR FILTRATION BARRIER (GFB) AND ENDOTHELIAL DYSFUNCTION

The renal glomerulus is an intricate elegant structure, in which the renal function takes place, by blood filtering and concentration of waste metabolites into the ultrafiltrate or urine. The glomerulus is a tuft of capillaries surrounded by the Bowman's capsule, situated in the Nephron, that is the structural and functional unit of the kidney (Figure 1 a). This capillary system receives the blood from the afferent arteriole of the renal arterial circulation. After passing through the capillaries and undergoing the process of Ultrafiltration, the blood exits into efferent arterioles. The glomerulus and its surrounding Bowman's capsule represent the basic filtration unit of the kidney. The rate at which blood is filtered through all of the glomeruli, and thus the measure of the overall kidney function, is defined as the glomerular filtration rate (GFR) (Salgado et al., 2010).The glomerulus is structurally supported by the mesangium: it is situated in the space between the capillaries, and it is continuous with the smooth muscles of the arterioles, and also with the podocytes. The mesangium consists of the intraglomerular mesangial cells and in the mesangial matrix, an amorphous basement membrane. The intraglomerular mesangial cells are specialized pericytes that, thanks to actin and myosin filaments, participate in the filtration process (Singh et al., 2007).

The glomerulus, synonymous with the glomerular filtration barrier (GFB), is classically described as a three-layer structure (Figure 1 b):

- Endothelial cells of glomerular capillaries
- Glomerular basement membrane (GBM)
- Podocytes (specific epithelial cells)

The glomerular endothelial cells are very specialized cells that are extraordinarily flattened and present gaps, called fenestrae. In this form, they allow the formation of glomerular ultrafiltrate at a prodigious rate. The fenestrae**,** which are pores about 50-100 nm in size, do not restrict the movement of water, proteins or large molecules but instead they limit the filtration of cellular components.

Surrounding the luminal surface of the endothelial cells is glycocalyx consisting of negatively charged glycosaminoglycans, which function to hinder the diffusion of negatively charged molecules.

The GBM is situated between the endothelium and the podocytes: it is made by collagen (types I, IV, VI, and XVIIII subunits), laminins (α5, β2, and γ1) and heparan sulfate proteoglycans. The most abundant collagen type is collagen IV that, with its poligonal reticulum, represents a physical barrier against the passage of the large molecules, like proteins such as albumin and globulin, into the filtrate. The GBM is thicker compared to the basement membranes of other tissues, and it consists of 3 layers: they are an inner thin layer (lamina rara interna)**,** a thick layer (lamina densa) and an outer dense layer (lamina rara externa). The two layers of lamina rara are rich in heparan sulfate, that gives them a negative charge necessary for the formation of the charge barrier. Hence, the GBM functions as a mediator for the cellular communication between podocytes and GECs, since it is a way for the passage of pro-angiogenic ligands and secreted factors (Abrahamson, 2012).

Podocytes are highly specialized epithelial cells, that present processes called *foot processes* (FPs) These foot processes or pedicels, wrap around the capillaries and leave gaps, called slits diaphragm (STs*)*, between them. The foot processes increase the surface area of the cells, enabling ultrafiltration; on the other hand, they prevent large molecules such as proteins,from entering in the ultrafiltrate. In contrast, small molecules like water, glucose and ionic salts can pass through the slits, forming the ultrafiltrate,that is further processed to produce urine. The slits diaphragm are composed by a great number of cell-surface proteins including Nephrin1/2, podocalyxin, P-cadherin, podocin, ZO-1, CD2AP and and catenins, that have a key importance in the establishment and maintenance of the GFB. Indeed, any mutations genes encoding for these type of proteins, can cause congenital nephropathy characterized by the collapse of FPs and the absence of SDs (Quaggin & Kreidberg, 2008). Moreover, similar to the glycocalyx around endothelial cells, negatively charged glycoproteins cover the podocytes, restricting filtration of large anions (Reiser & Altintas, 2016). Lastly, podocytes produce growth factors, like VEGF, that is required for the GECs homeostasis.

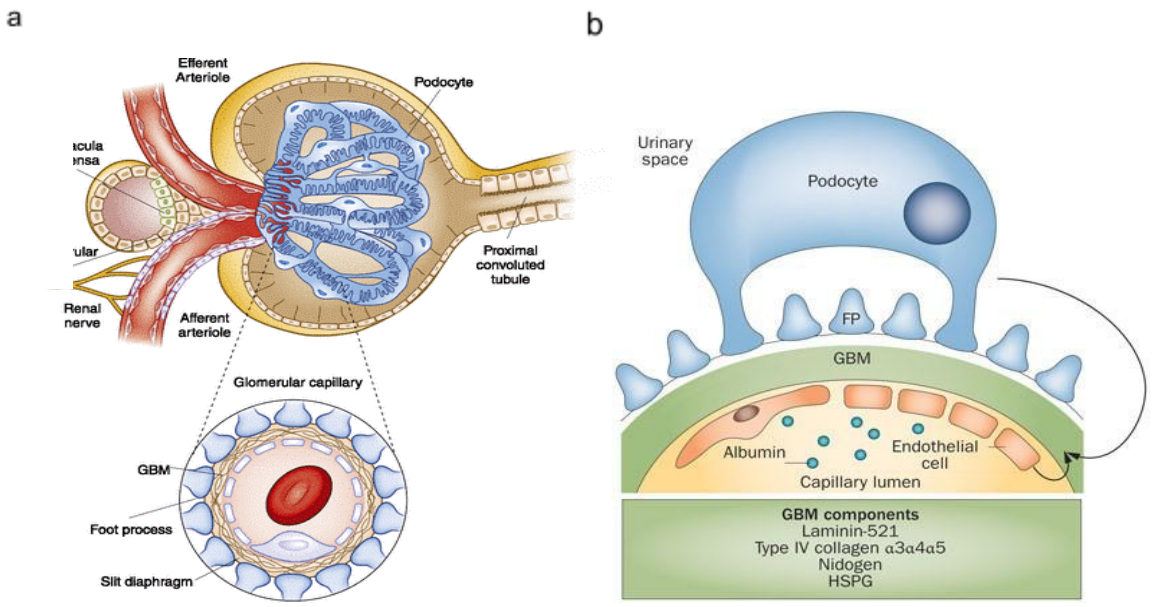

Figure 1 (a) Structure of a renal glomerulus surronded by the Bowmann's capsule (Pollak et al., 2014) (b) Schematic representation of the three-layer structure of the glomerular filtration barrier.

The glomerular filtration

Human blood filtration by the kidneys generates on average 1 liter of urine per day. Urine is produced and concentrated along the length of nephrons: they are from 1 to 2, 5 millions for each kidney. Despite their relatively small size, the kidneys receive approximately 20% of the cardiac input: the blood enters in the kidneys, through the afferent arteriole, that receive it from the renal arteries. After filtration occurs in the nephron, the blood moves through a small network of small veins, venules, that converge, at the end, into the renal veins which exit the kidney. The kidneys excrete a variety of waste products produced by metabolism into the urine: the nephron processes the blood supplied to it via the four stages of filtration, reabsorption, secretion and excretion: the consequence of these processes is the production of urine. The filtration process, that produces the ultrafiltrate, is a mechanism where large molecules, like proteins, are retained, while materials of smaller molecular weights are filtered. The benefits of the filtration process are that it eliminates the

excess fluid, solutes, and waste metabolites, and that it serves to detoxify the system (Carrol et al. book, The renal system). The volume of blood filtered from the glomerular capillaries into the Bowman's capsule per unit time is defined as Glomerular filtration rate (GFR). The GFR is based on the differential basal tone of the afferent and efferent arterioles: it depends on the difference between the higher blood pressure created by vasoconstriction of the input or afferent arteriole, versus the lower blood pressure created by lesser vasoconstriction of the output or efferent arteriole. The GFR is about 125 mL/min filtrate in men (range of 90 to 140 mL/min) and 105 mL/min filtrate in women (range of 80 to 125 mL/min). The estimated GFR (eGFR) can be calculated using several formulas. The following formula only applies for GFR calculation when it is equal to the Clearance Rate:

eGFR= Urine Concentration x Urine Flow/ Plasma Concentration

Glomerular filtration is carefully controlled by two different mechanisms: the kidney can control itself locally through intrinsic mechanism, the renal autoregulation, and through other two extrinsic controls, from the nervous and from the endocrine systems. Autoregulation include myogenic mechanism and tubuloglomerular feedback: the first one regulates the vasoconstriction or the vasorelaxation of the afferent arteriole in response to the changes of the body pressure, in order to bring the GFR back to normal levels. The tubule-glomerular feedback mechanism involves the juxtaglomerular (JG) cells, or granular cells, from the juxtaglomerular apparatus (JGA) and a paracrine signaling mechanism regulated by the macula densa cells. The JG cells are modified, smooth muscle cells lining the afferent arteriole that can contract or relax, in response to the paracrine secretion released by the macula densa, that respond to changes in the fluid flow rate and Na^+ concentration. Regarding the extrinsic mechanisms, the kidneys are innervated by the sympathetic neurons of the autonomic nervous system via the celiac plexus and splanchnic nerves. Reduction of sympathetic stimulation results in vasodilation and increased blood flow through the kidneys during resting conditions. Under conditions of stress, sympathetic nervous activity increases, resulting in the direct vasoconstriction of afferent arterioles (norepinephrine effect) as well as in a stimulation of the adrenal medulla that produces a generalized vasoconstriction through the release of epinephrine. Lastly, there is the hormone system of renin-angiotensin-aldosterone (SRAA): renin,

produced by the JGA, enzymatically converts angiotensinogen (made by the liver, freely circulating) into angiotensin I. Its release is stimulated by paracrine signals from the JGA in response to decreased extracellular fluid volume. Angiotensin I is then converted by the enzyme Angiotensin-converting enzyme (ACE) (situated in the lung) into active angiotensin II. Angiotensin II is a potent vasoconstrictor that acts systemically in both the afferent and efferent arterioles of the glomerulus. In response to angiotensin II, Aldosterone is released by the adrenal cortex: it promotes water and Na+ reabsorption by the nephron, and also K+ excretion. This increases the volume of extracellular fluid in the body, which thus leads to the increase of blood pressure.

The glomerular endothelial dysfunction

Many diseases affect kidney function by acting on the glomeruli. Several genetic or environmental causes can lead to glomerular diseases; however, they can be divided into two main groups: glomerulonephritis and glomerulosclerosis. Glomerulonephritis consists mainly of inflammation of the membrane tissue, instead glomerulosclerosis causes the scarring and the hardening of the small blood vessels within the kidney. The two groups can overlap, and they can also occur in other more complex diseases like Nephrotic and Nephritic Syndrome, Lupus Nephropathy, Focal segmental glomerulosclerosis (FSGS) and Diabetic nephropathy (DN). Most forms of glomerular disease develop gradually, often causing no symptoms for many years: Chronic kidney disease (CKD) is the slowest, gradual loss of kidney function. Some forms of CKD can be controlled or slowed down, like DN, but CKD cannot be cured. In many cases, CKD leads to kidney failure, which leads to the acute or chronic loss of at least 85% kidney function. The End-stage renal disease (ESRD) consists in kidney failure, treated only by dialysis or kidney transplant.

The Endothelial Dysfunction, in the glomerular endothelium, occurs when the endothelium reduces its capacity to maintain homeostasis, and leads to the development of pathological inflammatory processes and vascular disease (Esper et al., 2006). The endothelial dysfunction in the kidney can lead to Glomerular Endotheliosis: it represents a specific variant of thrombotic microangiopathy, and it

is characterized by glomerular endothelial swelling, with loss of endothelial fenestrae, reduction of endothelial tight junction proteins expression (Ve-cadherin, ZO-1), and occlusion of the capillary lumens (Xu et al., 2014). All these features can cause overall damage of the GFB, with the resultant increase of glomerular permeability. The hyperfiltration of water, large-size molecules (like proteins), waste products, and sometimes red blood cells, can cross the barrier, leading to edema, proteinuria and hematuria. The next part of my report will consider three types of pathological processes, in which Glomerular Endotheliosis represents one of the main feature: they are Preeclampsia, Endothelial to Mesenchymal Transition (which mainly leads to Fibrosis) and Diabetic nephropathy (result of Diabetes).

Endothelial to mesenchymal transition (EndMT)

Endothelial to mesenchymal transition (EndMT) is a complex biological process, recently recognized as a type of cellular transdifferentiation. During EndMT, endothelial cells lose their phenotypic profile and progressively evolve into cells with a mesenchymal phenotype, which include a spindle-shaped elongated cell morphology, loss of cell-cell junctions and polarity, and the acquisition of cellular motility coupled to invasive and contractile properties (Figure 2). At the genetic level, EndMT leads to the increase of expression and production of mesenchymal cell-specific genes and the corresponding proteins, including α-smooth muscle actin (α-SMA), extra domain A (EDA) fibronectin, N-cadherin, vimentin, fibroblast-specific protein-1 (FSP-1; also known as S100A4 protein), fibroblast activating protein (FAP), and fibrillar collagens type I and type III. Expression of mesenchymal cell-specific genes occurs in parallel to the progressive reduction and the eventual loss of endothelial cell-specific proteins, including von Willebrand factor (vWF), CD31/platelet-endothelial cell adhesion molecule-1 (CD31/PECAM-1), and vascular endothelial cadherin (VE-cadherin) (Piera-Velazquez & Jimenez, 2012) (Piera-Velazquez et al., 2011) (Sanchez-Duffhues et al., 2016). It has been shown that EndMT consists of a trans-differentiation process which does not require DNA replication and, therefore, is mediated by the combined effects of activation of previously silent genes and/or silencing of genes expressed in the cells which display the original phenotype. This concept, named “Epigenetic landscape” was firstly introduced by Waddington in 1942, referring to

important changes caused by DNA-sequence independent mechanisms and pathways, which lead to the formation of a specific cellular phenotype (Boland et al., 2014) (Dambacher et al., 2013). It has been shown that the main inducers of the trigger of EndMT are the transforming growth factor-β (TGF-β) family, and mainly the TGF-β1 isoforms (Medici et al., 2011) (Mihira et al., 2011) (van Meeteren & ten Dijke, 2012). Both canonical and non-canonical TGF-β signaling pathways, as well as other different pathways, can modulate the EndMT process by multiple molecular mechanisms, depending on the physiological or pathological status of the cells. TGF-β1, TGF-β2 and TGF-β3 are the principal inducers and initiators of EndMT under most physiological and pathological conditions (Pardali et al., 2017). However, from a direct comparison of their effects on EndMT in human microvascular ECs, TGF-β2 turned out to be the most potent (Sabbineni et al., 2018). Moreover, it has subsequently shown that both Smad-dependent or canonical and Smad-independent or non canonical TGF-β signaling pathways may be involved in the EndMB and that this process is mediated by numerous transcriptional regulators such as Snail1, Snail2 (or Slug), Twist, and some members of Zeb family of proteins. TGF-β binding to its two types of receptors (ALK5 and TGF-β receptor type II) leads to the phosphorylation of Smad2/Smad3, which forms a complex with the co-Smad, Smad4, that allows its translocation to the nucleus. Inside the nucleus, the Smad2/Smad3/Smad4 complex binds the promoter regions of various TGF-β target genes, stimulating their transcription. By contrast, Smad6 and Smad7 are potent negative regulators of TGF-β induced signaling cascades (Nakao et al., 1997) (Imamura et al., 1997). Although the canonical Smad2/3-mediated pathway is the principal inducer of EndMT, several other molecular Smad2/3-dependent pathways can regulate the EndMT process:these pathways involve molecules modulated by TGF-β, such as Toll-like receptor (TLR) and proteins such as TLR5, the Friend leukemia virus integration 1 (Fli-1) protein, and SIRTUIN 1 and 3 (SIRT1/SIRT3). SIRT3 is a key regulator of EndMT in mice glomerular endothelial cells: the results of an *in vitro* study indeed confirmed that transgenic mice with SIRT3 deficiency can lead to development of EndMT, coupled to renal disease and fibrosis (Maynard et al., 2003) (Lin et al., 2018). Besides the canonical TGF- β pathways, there are a number of non-canonical Smad2/3-independent signaling pathways activated by TGF- β, such as the three well known mitogen-activated protein kinase MAPK pathways: extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun NH -terminal

kinases (JNK) (Moustakas & Heldin, 2005) (Zhang, 2009). In addition, the noncanonical pathways activated by TGF-β also include the phosphatidylinositol 3-kinase (PI3K), RhoA, Rac, protein kinase C Abl kinase (c-Abl), the PKC-δ isoform of the PKC family of kinases and the Janus kinase 2 (JAK2). Regarding JAK2, it has been shown that high levels of phosphorylated JAK2 (JAK2p) are correlated with EndMT during the pulmonary arterial hypertension (PAH) associated with idiopathic pulmonary fibrosis (IPF) in patients (Milara et al., 2018.). EndMT can be also regulated by signalling pathways, which are TGF- β independent. These pathways include Notch signaling, the canonical Wnt pathway and other signalling cascades mediated by inflammatory cytokines such as caveolin-1 (CAV1), endothelin-1 (ET-1), the hypoxia inducible factor (HIF)-1. Finally, EndMT can be induced by pathological conditions such as metabolic alterations like hyperglycemia, hypoxia, and shear stress and oxidative stress. Indeed, Murdoch at al., reported that the reactive oxygen species (ROS)- generating enzyme, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-2 (NOX2) is a major driver of endothelial dysfunction, since it drives to EndMT mediated by Angiotensin II activation. NOX2 overexpression in transgenic mice (TG) increase NOX2 protein level and NOX2 activation in response to AngII, leading to diastolic dysfunction, hypertension, cardiac hypertrophy and fibrosis and proinflammatory effects induced by superoxide-mediated inactivation of nitric oxide (during chronic AngII elevation).

Regarding hyperglycemia, some studies investigated the key role of EndMT in the severe vascular pathology of diabetes, mainly affecting the microvascular system of kidneys (Li et al., 2009) (Li et al., 2010). High glucose levels on cultured human aortic ECs can induce EndMT, through a mechanism mediated by angiotensin II (R. Tang et al., 2010). This was further confirmed by the abrogation of high-glucose-induced EndMT after treatment of ECs with an angiotensin II receptor 1 inhibitor (Ibesartam) (R.-N. Tang et al., 2013). Zeisberg at al., conducted for the first time a *in vivo* study on three different mouse model (unilateral ureteral obstruction, streptozotocin induced diabetic nephropathy and alpha4 chain of collagen type 4 (COL4A3) knockout mice), which confirmed the contribution of EndMT in renal fibrosis. They found that myofibroblasts co-express CD31, and α-SMA and fibroblast specific protein 1 (FSP-1) in all three models and that these activated fibroblasts originated from EC. Furthermore, 6 months after a single injection of STZ kidneys developed glomerulosclerosis and tubulointerstitial fibrosis.

Li et al., also confirmed that EndMT occurs and contributes to generation of fibroblasts and myofibroblasts in early diabetic renal fibrosis. Using endothelial-lineage tracing transgenic mice, they revealed α-SMA positive cells (myofibroblasts) of endothelial origin from mice induced with STZ. They also observed that only one month after STZ injection without albuminuria, renal EC underwent EndMT and contributed to the accumulation of renal myofibroblasts. These findings suggest that EC may play a role in renal fibrosis through the process of EndMT.

In conclusion, EndMT appears to be especially important in the pathogenesis of numerous human disorders, although further studies are required to elucidate the molecular mechanisms of this process. However, EndMT may provide a novel therapeutic approach for the treatment of several human diseases.

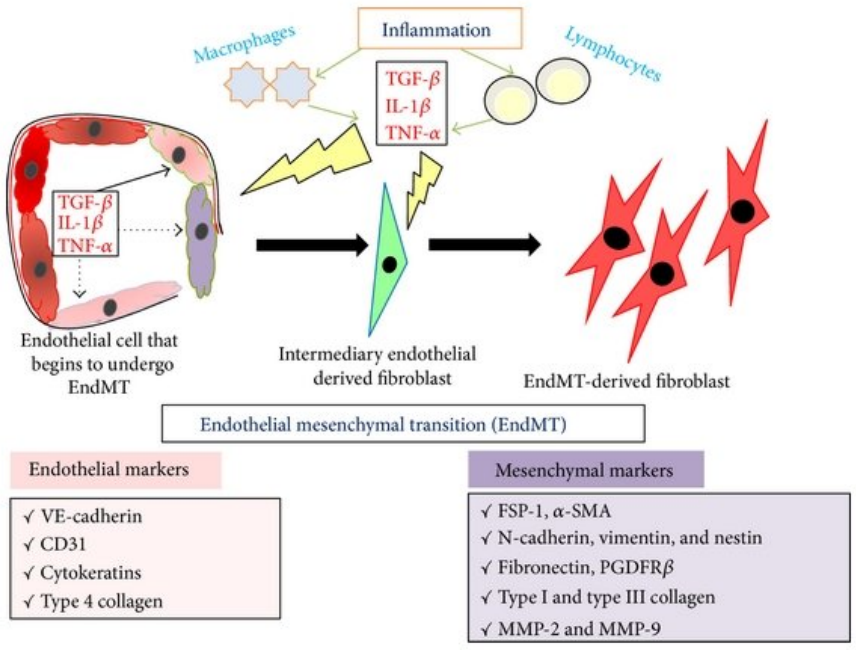

Figure 2. Biochemical changes during EndMT (Shu et al., 2020)

Diabetic nephropathy (DN)

Diabetic nephropathy (DN) or diabetic kidney disease (DKD) is the leading cause of chronic kidney disease (CKD) and end-stage renal failure (ESRF). It affects 40% of type 1 and type 2 diabetic mellitus (DM) patients, and it consists of the primary microvascular complication of this pathology. DN syndrome is defined by a pathological quantity of urine albumin excretion (UAE). Based on UAE values, it is categorized into two stages: microalbuminuria (UAE >20 μg/min and <199 μg/min) and macroalbuminuria (UAE >200 μg/min), although the progression is variable. The exact causes of DN are still unknown, however the main modifiable factor risks for the development of the pathology include hypertension, glycemia, and dyslipidemia. DN is characterized by structural and functional changes, mainly in the glomeruli. They present mesangial expansion, thickening of the basement membrane, and accumulation of extracellular matrix in tubular and glomerular membranes. In early DN, tubular hypertrophy occurs, accompanied with interstitial fibrosis and tubular atrophy. In advanced cases, kidneys present also infiltrates macrophages and T-lymphocytes. Moreover, there is podocyte injury and loss and podocyturia, and a reduction of endothelial cell fenestration, due to the loss of cell-to-cell junctions and adhesive proteins (Weil et al., 2012). Other pathological features of DN include glomerular endothelial lesions, accompanied by glomerular hyperfiltration, glomerular hypertrophy, glomerulosclerosis, and tubule-interstitial inflammation and fibrosis. Subsequently, all these pathological events lead to glomerular hyperfiltration and increase of albumin excretion, resulting in proteinuria and reduction of glomerular filtration rate (GFR). In DN, several different mechanisms are involved, which have been observed and investigated. First of all, activation of immune system and inflammation play a key role in the pathogenesis of DM, and therefore of DN (Navarro-González & Mora-Fernández, 2008) (Navarro-González et al., 2009). Macrophages, monocytes, and lymphocytes are recognized as the principal inflammatory cells involved in diabetic kidney damage, as well as other factors such as chemokines, growth factors, adhesion molecules, nuclear factors and cytokines. Among cytokines, TGF-β1 plays a key role in the pathogenesis of DN, since it is related to renal damage and promotion of renal fibrosis (Figure 3). Upregulation of TGF-β1 and of its downstream cytokine CTGF, promotes extracellular matrix proliferation and therefore, it causes glomerulosclerosis and glomerulonephritis;

moreover, it induces EndMT process and the transformation of endothelial cells into fibroblasts, resulting in chronic inflammation and renal fibrosis. Proinflammatory and profibrotic activity of TGF-β1 has been investigated in DM patients with DN: TGF-β1 and CTGF levels were higher, as well as UAE, compared to normal individuals (El Mesallamy et al., 2012). In addition, nephropathy in diabetic mice and in humans is associated with high levels of angiotensin converting enzyme (ACE), leading to elevated levels of endothelin-1 and urotensin II (Huang et al., 2001) (Rudberg et al., 2000). Dysregulation of nitric oxide and nitric oxide synthase has been described in DN, resulting in vasodilation. It has been shown that nitric oxide synthase knockout mice develop more glomerular lesions and proteinuria, respect with control mice (Kanetsuna et al., 2007). The responsible mechanism for nitric oxide dysregulation and therefore endothelial dysfunction can be the activation of PKC. Activation of intracellular kinases such as PKC and MAPK promote the nuclear transcription of factors including NF-κB, which regulate gene expression of various cytokines, chemokines, and adhesion molecules. Activation of p38α isoform of the p38 MAPK pathway is strongly associated with renal inflammation and DN (Sakai et al., 2005). In addition, toll-like receptors (TLR2, TLR4) and B7-1 play an important role in inflammation and injury in DN (Fiorina et al., 2014). To conclude, inflammation plays an essential role in the development of DN, involving increased chemokine production, infiltration of inflammatory cells into the kidney, pro-inflammatory cytokine production, and tissue damage. Therefore, a better understanding of inflammatory response in diabetic kidneys is essential in order to identify new anti-inflammatory strategies for the potential treatment of human DN.

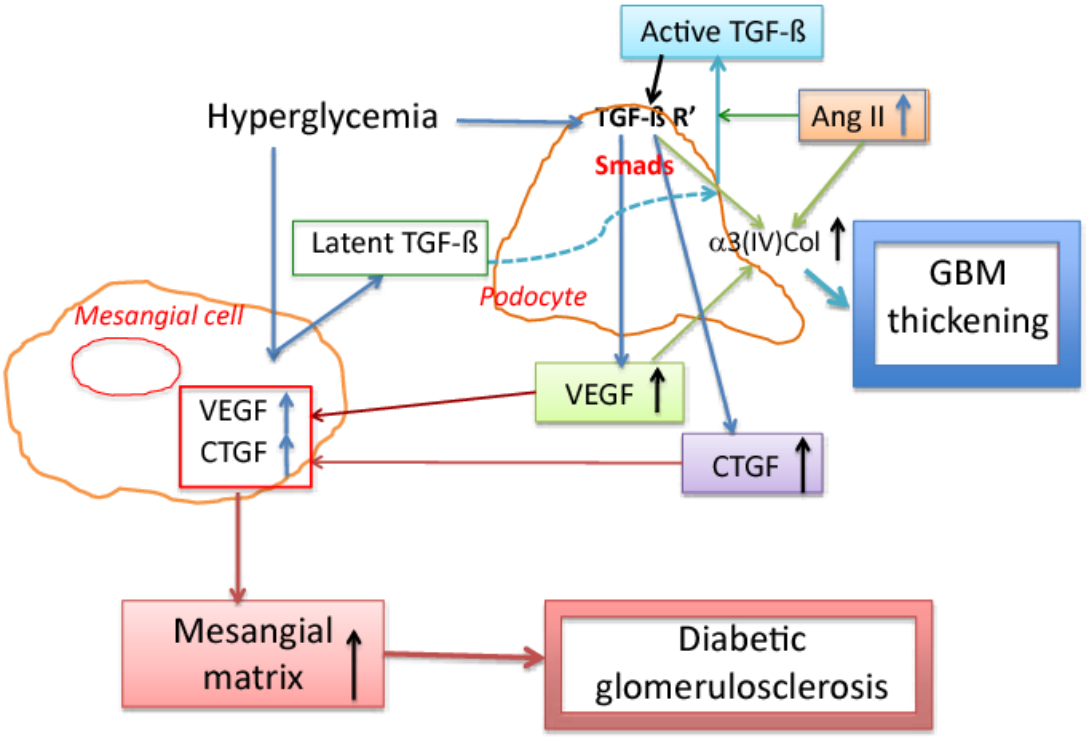

Figure 3 Schematic illustration of proposed mechanism of TGF-β action in podocytes and mesangial cells leading to the development of diabetic nephropathy. High glucose stimulates TGF-β secretion in mesangial cells and TGF-β receptor (TGF-R) expression in podocytes. Soluble forms of latent TGF-β complex released from mesangial matrix may be localized to the podocyte surface and activated by angiotensin (Ang) II. Activated TGF-β/Smad signalling pathway in podocytes may induce 3(IV) collagen, connective tissue growth factor (CTGF) and vascular endothelial growth factor (VEGF) overexpression, leading to glomerular basement membrane (GBM) thickening and mesangial matrix expansion, culminating in diabetic glomerulosclerosis.(Lee, 2013)

References

Abrahamson, D. R. (2012). Role of the podocyte (and glomerular endothelium) in building the GBM. *Seminars in Nephrology*, *32*(4), 342–349. https://doi.org/10.1016/j.semnephrol.2012.06.005

Boland, M. J., Nazor, K. L., & Loring, J. F. (2014). Epigenetic regulation of pluripotency and differentiation. *Circulation Research*, *115*(2), 311–324. https://doi.org/10.1161/CIRCRESAHA.115.301517

Dambacher, S., de Almeida, G. P., & Schotta, G. (2013). Dynamic changes of the epigenetic landscape during cellular differentiation. *Epigenomics*, *5*(6), 701–713. https://doi.org/10.2217/epi.13.67

El Mesallamy, H. O., Ahmed, H. H., Bassyouni, A. A., & Ahmed, A. S. (2012). Clinical significance of inflammatory and fibrogenic cytokines in diabetic nephropathy. *Clinical Biochemistry*, *45*(9), 646–650. https://doi.org/https://doi.org/10.1016/j.clinbiochem.2012.02.021

Esper, R. J., Nordaby, R. A., Vilariño, J. O., Paragano, A., Cacharrón, J. L., & Machado, R. A. (2006). Endothelial dysfunction: a comprehensive appraisal. *Cardiovascular Diabetology*, *5*, 4. https://doi.org/10.1186/1475-2840-5-4

Fiorina, P., Vergani, A., Bassi, R., Niewczas, M. A., Altintas, M. M., Pezzolesi, M. G., D'Addio, F., Chin, M., Tezza, S., Ben Nasr, M., Mattinzoli, D., Ikehata, M., Corradi, D., Schumacher, V., Buvall, L., Yu, C.-C., Chang, J.-M., La Rosa, S., Finzi, G., … Sayegh, M. H. (2014). Role of podocyte B7-1 in diabetic nephropathy. *Journal of the American Society of Nephrology : JASN*, *25*(7), 1415–1429. https://doi.org/10.1681/ASN.2013050518

Huang, W., Gallois, Y., Bouby, N., Bruneval, P., Heudes, D., Belair, M. F., Krege, J. H., Meneton, P., Marre, M., Smithies, O., & Alhenc-Gelas, F. (2001). Genetically increased angiotensin I-converting enzyme level and renal complications in the diabetic mouse. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(23), 13330–13334. https://doi.org/10.1073/pnas.231476798

Imamura, T., Takase, M., Nishihara, A., Oeda, E., Hanai, J., Kawabata, M., & Miyazono, K. (1997). Smad6 inhibits signalling by the TGF-β superfamily. *Nature*, *389*(6651), 622–626. https://doi.org/10.1038/39355

Kanetsuna, Y., Takahashi, K., Nagata, M., Gannon, M. A., Breyer, M. D., Harris, R. C., & Takahashi, T. (2007). Deficiency of endothelial nitric-oxide synthase confers susceptibility to diabetic nephropathy in nephropathy-resistant inbred mice. *The American Journal of Pathology*, *170*(5), 1473–1484. https://doi.org/10.2353/ajpath.2007.060481

Lee, H. (2013). Pathogenic Role of TGF- ◆ in Diabetic Nephropathy. *Journal of Diabetes & Metabolism*, *2013*, 1–7.

Li, J., Qu, X., & Bertram, J. F. (2009). Endothelial-myofibroblast transition contributes to the early development of diabetic renal interstitial fibrosis in

streptozotocin-induced diabetic mice. *The American Journal of Pathology*, *175*(4), 1380–1388. https://doi.org/10.2353/ajpath.2009.090096

Li, J., Qu, X., Yao, J., Caruana, G., Ricardo, S. D., Yamamoto, Y., Yamamoto, H., & Bertram, J. F. (2010). Blockade of endothelial-mesenchymal transition by a Smad3 inhibitor delays the early development of streptozotocin-induced diabetic nephropathy. *Diabetes*, *59*(10), 2612–2624. https://doi.org/10.2337/db09-1631

Lin, J., Zheng, Y., Zhang, Z., Shen, W., Li, X., Wei, T., Ruan, C., Chen, X., Zhu, D., & Gao, P. (2018). Suppression of Endothelial-to-Mesenchymal Transition by SIRT (Sirtuin) 3 Alleviated the Development of Hypertensive Renal Injury. *Hypertension*, *72*(2), 350–360. https://doi.org/10.1161/HYPERTENSIONAHA.118.10482

Maynard, S. E., Min, J.-Y., Merchan, J., Lim, K.-H., Li, J., Mondal, S., Libermann, T. A., Morgan, J. P., Sellke, F. W., Stillman, I. E., Epstein, F. H., Sukhatme, V. P., & Karumanchi, S. A. (2003). Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *The Journal of Clinical Investigation*, *111*(5), 649–658. https://doi.org/10.1172/JCI17189

Medici, D., Potenta, S., & Kalluri, R. (2011). Transforming growth factor-β2 promotes Snail-mediated endothelial-mesenchymal transition through convergence of Smad-dependent and Smad-independent signalling. *The Biochemical Journal*, *437*(3), 515–520. https://doi.org/10.1042/BJ20101500

Mihira, H., Suzuki, H. I., Akatsu, Y., Yoshimatsu, Y., Igarashi, T., Miyazono, K., & Watabe, T. (2011). TGF-β-induced mesenchymal transition of MS-1 endothelial cells requires Smad-dependent cooperative activation of Rho signals and MRTF-A. *The Journal of Biochemistry*, *151*(2), 145–156. https://doi.org/10.1093/jb/mvr121

Milara, J., Ballester, B., Morell, A., Ortiz, J. L., Escrivá, J., Fernández, E., Perez-Vizcaino, F., Cogolludo, A., Pastor, E., Artigues, E., Morcillo, E., & Cortijo, J. (2018). JAK2 mediates lung fibrosis, pulmonary vascular remodelling and hypertension in idiopathic pulmonary fibrosis: an experimental study. *Thorax*, *73*(6), 519 LP – 529. https://doi.org/10.1136/thoraxjnl-2017-210728

Moustakas, A., & Heldin, C.-H. (2005). Non-Smad TGF-β signals. *Journal of Cell*

Science, *118*(16), 3573–3584. https://doi.org/10.1242/jcs.02554

Murdoch C.E., Sanjay C., Lingfang Z., Bin Y., Ivetic A., Walker S., Vanhuotte D., Heymans S., Grieve D., Cave A., Brewer A., Zhang M., Shah A. M., (2014) Endothelial NADPH-2 oxidase-2 promotes interstitial cardiac fibrosis and diastolic dysfunction through proinflammatory effects and endothelial-mesenchymal transition *Journal of American College of Cardiology*

Nakao, A., Afrakhte, M., Morn, A., Nakayama, T., Christian, J. L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N.-E., Heldin, C.-H., & Dijke, P. ten. (1997). Identification of Smad7, a TGFβ-inducible antagonist of TGF-β signalling. *Nature*, *389*(6651), 631–635. https://doi.org/10.1038/39369

Navarro-González, J. F., Jarque, A., Muros, M., Mora, C., & García, J. (2009). Tumor necrosis factor-α as a therapeutic target for diabetic nephropathy. *Cytokine & Growth Factor Reviews*, *20*(2), 165–173. https://doi.org/https://doi.org/10.1016/j.cytogfr.2009.02.005

Navarro-González, J. F., & Mora-Fernández, C. (2008). The Role of Inflammatory Cytokines in Diabetic Nephropathy. *Journal of the American Society of Nephrology*, *19*(3), 433 LP – 442. https://doi.org/10.1681/ASN.2007091048

Pardali, E., Sanchez-Duffhues, G., Gomez-Puerto, M. C., & Ten Dijke, P. (2017). TGF-β-Induced Endothelial-Mesenchymal Transition in Fibrotic Diseases. *International Journal of Molecular Sciences*, *18*(10), 2157. https://doi.org/10.3390/ijms18102157

Piera-Velazquez, S., & Jimenez, S. A. (2012). Molecular mechanisms of endothelial to mesenchymal cell transition (EndoMT) in experimentally induced fibrotic diseases. *Fibrogenesis & Tissue Repair*, *5*(Suppl 1), S7–S7. https://doi.org/10.1186/1755-1536-5-S1-S7

Piera-Velazquez, S., Li, Z., & Jimenez, S. A. (2011). Role of endothelial-mesenchymal transition (EndoMT) in the pathogenesis of fibrotic disorders. *The American Journal of Pathology*, *179*(3), 1074–1080. https://doi.org/10.1016/j.ajpath.2011.06.001

Quaggin, S. E., & Kreidberg, J. A. (2008). Development of the renal glomerulus: good neighbors and good fences. *Development*, *135*(4), 609–620. https://doi.org/10.1242/dev.001081

Reiser, J., & Altintas, M. M. (2016). Podocytes. *F1000Research*, *5*, F1000 Faculty Rev-114. https://doi.org/10.12688/f1000research.7255.1

Rudberg, S., Rasmussen, L. M., Bangstad, H. J., & Osterby, R. (2000). Influence of insertion/deletion polymorphism in the ACE-I gene on the progression of diabetic glomerulopathy in type 1 diabetic patients with microalbuminuria. *Diabetes Care*, *23*(4), 544–548. https://doi.org/10.2337/diacare.23.4.544

Sabbineni, H., Verma, A., & Somanath, P. R. (2018). Isoform-specific effects of transforming growth factor β on endothelial-to-mesenchymal transition. *Journal of Cellular Physiology*, *233*(11), 8418–8428. https://doi.org/10.1002/jcp.26801

Sakai, N., Wada, T., Furuichi, K., Iwata, Y., Yoshimoto, K., Kitagawa, K., Kokubo, S., Kobayashi, M., Hara, A., Yamahana, J., Okumura, T., Takasawa, K., Takeda, S.-I., Yoshimura, M., Kida, H., & Yokoyama, H. (2005). Involvement of extracellular signal-regulated kinase and p38 in human diabetic nephropathy. *American Journal of Kidney Diseases*, *45*(1), 54–65. https://doi.org/10.1053/j.ajkd.2004.08.039

Salgado, J. V., Neves, F. A., Bastos, M. G., França, A. K., Brito, D. J., Santos, E. M., & Salgado Filho, N. (2010). Monitoring renal function: Measured and estimated glomerular filtration rates - A review. *Brazilian Journal of Medical and Biological Research*, *43*(6), 528–536. https://doi.org/10.1590/S0100-879X2010007500040

Sanchez-Duffhues, G., Orlova, V., & ten Dijke, P. (2016). In Brief: Endothelial-to-mesenchymal transition. *The Journal of Pathology*, *238*(3), 378–380. https://doi.org/10.1002/path.4653

Shu, D. Y., Butcher, E., & Saint-geniez, M. (2020). *EMT and EndMT : Emerging Roles in Age-Related Macular Degeneration*.

Singh, A., Satchell, S. C., Neal, C. R., McKenzie, E. A., Tooke, J. E., & Mathieson, P. W. (2007). Glomerular Endothelial Glycocalyx Constitutes a Barrier to Protein Permeability. *Journal of the American Society of Nephrology*, *18*(11), 2885–2893. https://doi.org/10.1681/ASN.2007010119

Tang, R.-N., Lv, L.-L., Zhang, J.-D., Dai, H.-Y., Li, Q., Zheng, M., Ni, J., Ma, K.-L., & Liu, B.-C. (2013). Effects of angiotensin II receptor blocker on myocardial endothelial-to-mesenchymal transition in diabetic rats. *International Journal of Cardiology*, *162*(2), 92–99. https://doi.org/10.1016/j.ijcard.2011.06.052

Tang, R., Li, Q., Lv, L., Dai, H., Zheng, M., Ma, K., & Liu, B. (2010). Angiotensin II mediates the high-glucose-induced endothelial-to-mesenchymal transition in human aortic endothelial cells. *Cardiovascular Diabetology*, *9*, 31.

https://doi.org/10.1186/1475-2840-9-31

van Meeteren, L. A., & ten Dijke, P. (2012). Regulation of endothelial cell plasticity by TGF-β. *Cell and Tissue Research*, *347*(1), 177–186. https://doi.org/10.1007/s00441-011-1222-6

Weil, E. J., Lemley, K. V, Mason, C. C., Yee, B., Jones, L. I., Blouch, K., Lovato, T., Richardson, M., Myers, B. D., & Nelson, R. G. (2012). Podocyte detachment and reduced glomerular capillary endothelial fenestration promote kidney disease in type 2 diabetic nephropathy. *Kidney International*, *82*(9), 1010–1017. https://doi.org/10.1038/ki.2012.234

Xu, C., Chang, A., Hack, B. K., Eadon, M. T., Alper, S. L., & Cunningham, P. N. (2014). TNF-mediated damage to glomerular endothelium is an important determinant of acute kidney injury in sepsis. *Kidney International*, *85*(1), 72–81. https://doi.org/https://doi.org/10.1038/ki.2013.286

Zeisberg EM, Potenta SE, Sugimoto H, et al. Fibroblasts in kidney fibrosis emerge via endothelial-to-mesenchymal transition. J Am Soc Nephrol JASN. 2008;19:2282–7.

Zhang, Y. E. (2009). Non-Smad pathways in TGF-beta signaling. *Cell Research*, *19*(1), 128–139. https://doi.org/10.1038/cr.2008.328

REVIEW: “Alternative strategies to inhibit tumor vascularization”

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Review

Alternative Strategies to Inhibit Tumor Vascularization

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Abstract: Endothelial cells present in tumors show different origin, phenotype, and genotype with respect to the normal counterpart. Various mechanisms of intra-tumor vasculogenesis sustain the complexity of tumor vasculature, which can be further modified by signals deriving from the tumor microenvironment. As a result, resistance to anti-VEGF therapy and activation of compensatory pathways remain a challenge in the treatment of cancer patients, revealing the need to explore alternative strategies to the classical anti-angiogenic drugs. In this review, we will describe some alternative strategies to inhibit tumor vascularization, including targeting of antigens and signaling pathways overexpressed by tumor endothelial cells, the development of endothelial vaccinations, and the use of extracellular vesicles. In addition, anti-angiogenic drugs with normalizing effects on tumor vessels will be discussed. Finally, we will present the concept of endothelial demesenchymalization as an alternative approach to restore normal endothelial cell phenotype.

Keywords: tumor vasculogenesis; endothelial cells; anti-angiogenic drugs; normalization; endothelial demesenchymalization; endothelial vaccination

1. Introduction

1.1. Tumor Endothelial Cell Characterization

In 1971, Judah Folkman observed that solid tumors show a diffuse vascular network, often hemorrhagic, and that poorly vascularized tumors were unable to grow beyond 2–3 mm [1]. These observations led Folkman to hypothesize that to grow and expand, tumors need new blood vessels, introducing the concept of tumor angiogenesis [1]. It is now well established that, during the early phases of tumor angiogenesis, a process called "angiogenic switch" occurs, characterized by overexpression of pro-angiogenic factors, neoangiogenesis, and tumor cell survival [2]. Indeed, tumor angiogenesis significantly differs from physiological angiogenesis. Tumor vessels have an irregular aspect, are dilated and tortuous, and this chaotic organization results in the absence of distinct venules, arterioles, and capillaries, with the formation of a leaky and hemorrhagic vascular network [3]. They have an incomplete basal membrane, with large joints and fenestrations which increase the interstitial fluid pressure, possibly resulting in intra-tumor bleeding [4].

Tumor endothelial cells (TEC) themselves substantially differ from the normal counterpart. In 2000, Croix et al. identified for the first time genes differentially expressed in TEC with respect to normal endothelial cells, most of which are involved in the formation of collagen, in angiogenesis and in the wound healing process, demonstrating that tumor endothelium is different from normal endothelium

at a molecular level [5]. In addition, TEC can be aneuploid, express embryonic markers, and can undergo endothelial–mesenchymal transition. Hida et al. first demonstrated that freshly isolated TEC present structural aberrations, such as nonreciprocal translocations, missing chromosomes, and have multiple centrosomes [6]. Functionally, TEC display an increased proliferation rate and delayed senescence with respect to normal endothelial cells due to autocrine production of proangiogenic factors [7] and are resistant to classical anti-angiogenic drugs [8,9].

1.2. Tumor Endothelial Cell Origin

The evidence that tumor vessels differ from normal vessels, both, genotypically and functionally suggests that tumor vasculature could either be modified by factors deriving from the tumor microenvironment or directly originate by intra-tumor vasculogenesis as alternative mechanisms other than the recruitment from pre-existing vessels in adjacent tissues [10]. The strategies of intra-tumor vasculogenesis are shown in Figure 1.

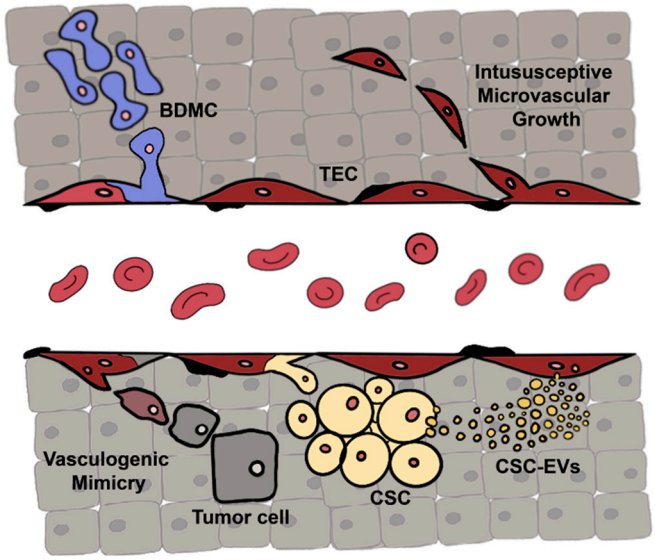

Figure 1. Alternative strategies of tumor vascularization. Tumor vessels may be generated by intra-tumor vasculogenesis as an alternative to endothelial cell recruitment from adjacent vessels. TEC may originate from the recruitment of bone marrow-derived cells (BMDC), such as endothelial progenitor cells, or directly from tumor cells acquiring an endothelial phenotype in a process called vasculogenic mimicry. Moreover, a subpopulation of cancer cells with stem features (CSCs) can directly differentiate into tumor endothelial cells (TEC) or can reprogram normal endothelial cells by the release of extracellular vesicles (CSC-EVs). Finally, intussusceptive microvascular growth allows the generation of a new vessel by the split of a pre-existing one.

There are several pieces of evidence that factors secreted by tumor cells, and *in primis* extracellular vesicles (EVs), may reprogram normal quiescent endothelial cells through the transfer of proteins and genetic material (mRNAs, miRNAs, or proteins) [11–13]. In parallel, the intratumor vasculogenesis might be dependent on the differentiation of normal or cancer stem cells or by endothelial mimicry of differentiated tumor cells [10]. Bone marrow-derived cells, and in particular endothelial progenitor cells, actively participate to tumor growth, not only through the secretion of pro-angiogenic factors but also through their incorporation within the vessels [14,15]. Resident normal tissue stem cells were also shown to differentiate into endothelial cells in the presence of growth factors released by the tumor [15]. Cancer stem cells (CSC), a subpopulation of tumor cells with stem properties, can generate all different tumor cell types, becoming responsible for tumor growth and progression. Several groups demonstrated the ability of CSC to differentiate into endothelial cells and pericytes and thus their contribution to tumor vasculogenesis [16–18].

Differentiated cancer cells themselves can also generate vascular structures by a process called vasculogenic mimicry. First identified in melanoma [19], the presence of vascular mimicry has been subsequently confirmed in a number of tumors, such as lung, breast, prostate, bladder, and renal carcinomas and glioblastoma [20]. Finally, to rapidly adapt to the surrounding microenvironment, tumors may generate new vessels trough intussusceptive microvascular growth. This mechanism, also known as non-sprouting or splitting angiogenesis, is characterized by the generation of new blood vessels by splitting an existing one [21]. The capillary network can, therefore, increase its complexity and vascular surface, generating vessels more rapidly with a minor metabolic demand as compared to sprouting angiogenesis.

Given the different origin, phenotype, and genotype of TEC with respect to the normal counterpart, in the last decades, many researchers focused on the isolation of TEC from solid tumors (Table 1) [22], to obtain an in vitro model resembling tumor angiogenesis.

Table 1. TEC isolation from solid tumors.

Tumor Type	Species	Year	References
Glioblastoma	Human	1999	Alessandri et al. [23]
Colon	Human	2000	St. Croix et al. [5]
Brain tumors	Human	2002	Unger et al. [24]
Renal	Human	2003	Bussolati et al. [7]
Lung	Mouse	2003	Allport et al. [25]
B-Cell lymphoma	Human	2004	Streubel et al. [26]
Liposarcoma and melanoma	Mouse	2004	Hida et al. [27]
Breast	Human	2006	Grange et al. [28]
Breast	Mouse	2006	Amin et al. [29]
Liver	Human	2007	Wu et al. [30]
Ovary	Human	2007	Buckanovitch et al. [31] Lu et al. [32]
Glossal lymphangioma	Human	2010	You et al. [33]
Prostate	Human	2014	Fiorio et al. [8]

1.3. Classic Anti-Angiogenic Therapies

A number of anti-angiogenic drugs have been developed and proposed to limit tumor growth and expansion [34]. At present, the main anti-angiogenic therapies approved by the FDA are described in Table 2 [34]. The use of anti-angiogenic drugs in clinical practice, however, only showed an initial benefit in patients, followed by limited effectiveness and only a moderate disease-free survival [35]. This is mainly due to the expression of alternative angiogenic pathways [36,37]. Although inhibitors of the VEGF pathway are substantially effective in reducing tumor vascularization, after treatment discontinuation the tumor vascular network is able to re-grow, acquiring overexpression of vascular growth factor receptors [36]. This overexpression leads the survived vessels to VEGF-independency and, therefore, to the development of resistance [37]. In addition, anti-angiogenic treatment can lead to the formation of a hypoxic microenvironment, which regulates the cancer stem cell population and can contribute both to the maintenance of the tumor and to the resistance to therapies [36].

Anti-angiogenic agents, such as the monoclonal antibody (mAb) bevacizumab, only showed significant activity when combined with cytotoxic chemotherapy [36]. Moreover, despite the success of the dual blockade of VEGFR and PDGFR by the tyrosine kinase inhibitor (TKI) sunitinib, a combination strategy using bevacizumab and imatinib, another inhibitor of PDGF signaling, was not effective but rather toxic during renal cancer treatment [38]. After 10 years of approval by the FDA of the first anti-VEGF drug, bevacizumab, resistance to anti-VEGF therapy remains a challenge in the treatment of cancer patients, revealing the need to explore alternative strategies to classical anti-angiogenic therapies, to obtain a durable therapeutic effect. In this review, we will describe some alternative strategies to inhibit tumor vascularization, such as the use of new mAbs, the target of alternative signaling pathways, the vaccination with endothelial antigens, and the use of extracellular vesicles

(Figure 2). In addition, the use of alternative anti-angiogenic drugs with normalizing effects on tumor vessels will be described.

Table 2. Main anti-angiogenic drugs for solid tumors treatment.

Drug Name	Type	Targets	Tumor Type	Combined Therapy
Bevacizumab	mAb	VEGF-A	Colorectal, lung, glioblastoma, renal cell carcinoma, breast, brain, ovarian, cervical, fallopian tube, and peritoneal cancer	Fluoropirimidine, Cisplatinum, Paclitaxel, Interferon a-2a
Sorafenib	TKI	VEGFR1/2/3, PDGFR, c-kit	Renal cell carcinoma, liver, thyroid, desmoid tumors	
Sunitinib	TKI	VEGFR1/2/3, PDGFR, c-kit, FLT-3, Ret	Renal cell carcinoma, gastrointestinal stromal, pancreatic neuroendocrine cancer, and leukemia	
Pazopanib	TKI	VEGFR1/2/3, PDGFR, c-kit, FGFR	Renal cell carcinoma and soft tissue sarcoma	
Axitinib	TKI	VEGFR1/2/3, c-kit, PDGFR	Renal cell carcinoma	
Regorafenib	TKI	VEGFR1/2/3, PDGFRα/β, FGFR1/2, Tie2, c-Kit	Metastatic colorectal cancer, advanced gastrointestinal stromal cancer and advanced hepatocellular carcinoma	
Cabozantinib	TKI	c-MET, VEGFR2, AXL, Ret	Medullary thyroid cancer and renal cell carcinoma	
Nintedanib	TKI	VEGFR1/2/3, PDGFR, FLT-3	Idiopatic pulmonary fibrosis, lung cancer	Docetaxel
Levantinib	TKI	VEGFR1/2/3, PDGFR, FGFR, Ret, c-Kit	Thyroid cancer and renal cell carcinoma	Everolimus
Vandetanib	TKI	VEGFR1/2/3, EGFR, and Ret	Medullary thyroid cancer	

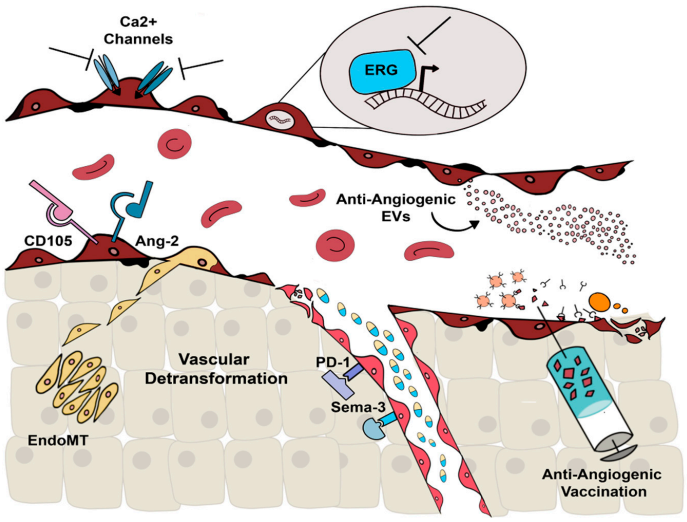

Figure 2. Alternative strategies to target tumor vascularization. Approaches to overcome the resistance to classical anti-angiogenic agents may involve the target of different molecules, such as calcium-permeable channels (Ca^{2+} channels), the transcription factor ERG, endoglin (CD105), or angiopoietin (Ang-2). TEC could also be targeted by stem cell-derived extracellular vesicles with anti-angiogenic effect (anti-angiogenic EVs), or by a specific multi-targeted cytotoxic immune response driven by anti-angiogenic vaccination. The irregular vascular network could be targeted by new normalizing agents, such as Sema 3. Finally, endothelial–mesenchymal transition (EndoMT), involving the downregulation of angiogenic molecules, represents an additional strategy for anti-angiogenic therapy resistance. Vascular detransformation represents, therefore, a novel strategy to block tumor abnormal vascularization.

2. Alternative Molecular Targets

2.1. Alternative Anti-Angiogenic Antibodies

The therapeutic use of classical anti-angiogenic drugs, as the anti-VEGF Ab bevacizumab, lacked the expected results observed in experimental models [35]. As mentioned earlier, anti-VEGF therapy-resistant tumors increase the expression of molecules that activate alternative angiogenic pathways [36,37,39], they can represent a new target of antibody-mediated therapies. For example, Abs against anti-angiopoietin-2 (like nesvacumab, AMG780, MEI3617, and vanucizumab), and Abs anti-integrin αvβ3 have been successfully tested in phase I/II study [40,41]. Another example of an antibody targeting alternative angiogenic pathways is MP0250, a genetically engineered designed ankyrin repeat protein (DARPin®) that specifically binds to VEGF-A, hepatocyte growth factor (HGF), and human serum albumin (HSA) [42,43]. The antibody is currently being studied in phase I and II clinical trials on multiple myeloma relapses (NIH N. NCT03136653) [44] on EGFR-mutated non-small cell lung cancer (NIH N. NCT03418532) and on other neoplasms (NIH N. NCT02194426). With its target specificities, MP0250 may thus help to overcome the resistance due to single targeting mAbs. Moreover, transforming growth factor (TGF-β) pathway has been found over-expressed after anti-VEGF therapy [45], suggesting that it might play an important role in the acquisition of therapy resistance. Endoglin (CD105) is a cell membrane glycoprotein overexpressed on proliferating endothelial cells that binds several factors of the TGF-β superfamily, suggesting that activation of this pathway may be responsible for tumor VEGF-independency [46]. In 1995, CD105 was described as a receptor overexpressed in tumor vasculature [47] and, more recently, it has been shown that high CD105 expression on vessels is correlated with poor prognosis in many solid tumors, such as kidney [48], prostate [49], and ovarian cancer [50]. CD105 was also described as a marker of CSC in renal cell carcinoma [16]. TRC105 (carotuximab) is a novel, clinical-stage antibody against CD105, that inhibits tumor vessel formation through the blockade of CD105. In a recent study, the TRC105 effect on both TEC lines and CSC-TEC was described [51]. In particular, TRC105 alone affected the ability of TEC and CSC-TEC to organize in tubular structures [51]. Moreover, TRC105 increased the effect of the tyrosine kinase inhibitor Sunitinib in inhibiting tumor endothelial proliferation, survival, and new vessel formation [51]. Taken together, these findings indicate that the combined inhibition of VEGF and TGF-β pathways may have potential use in renal carcinoma therapy. Indeed, TRC105 is currently being studied in phase III clinical trial in combination with pazopanib for the treatment of advanced angiosarcoma [52] and in multiple phase I and II clinical trials combination with VEGF inhibitors for the treatment of different solid tumors. For example, clinical trials in phase I and II are testing the efficacy of TRC105 in combination with bevacizumab in refractory gestational trophoblastic neoplasia and choriocarcinoma (NIH N. NCT02396511) [53], metastatic renal cancer (NIH N. NCT01727089), and glioblastoma (NIH N. NCT01564914 and NCT01648348). Other studies tested the combination of TRC105 with tyrosine kinase inhibitors, such as Axitinib in renal cell carcinoma (NIH N. NCT01806064), and sorafenib in hepatocellular carcinoma [54]. Encouraging evidence of activity to date was observed, and the study is now continuing to recruit in the phase II stage to confirm the activity of the combination therapy [54].

2.2. Ca^{2+}-Permeable Channels

Accumulating evidence demonstrates that the development of several cancers involves altered Ca^{2+} homeostasis and aberrant ion channel expression [55,56]. This is not surprising considering the multifaceted role of Ca^{2+} as an ubiquitous second messenger, which is involved in the tuning of multiple fundamental cellular functions [57]. Indeed, ion channels represent good potential pharmacological targets due to their location on the plasma membrane, where they can be easily accessed by drugs. As the first reports suggesting a role for ion channels in cancer progression, the field has undergone an exponential development giving rise to a large consensus in the scientific community to include "channelopathy" among the causal factors in cancer development [58,59]. In particular, it has been

clearly established a key role for Ca^{2+}-permeable channels in tumor vascularization both in vitro and in vivo [60–63]. Many pro-angiogenic growth factors, as well as chemokines, trigger Ca^{2+} signals directly involved in the angiogenic switch by mediating endothelial cells proliferation, migration, and sprouting [64–67].

Among Ca^{2+}-permeable channels, transient receptor potential (TRP) superfamily has been deeply investigated for their functions in endothelial cells where they emerged as important factors contributing to several key vascular processes, such as vascular tone, permeability, and cell migration [68–70]. In addition, different TRP channels have been described as mediators of VEGF-mediated Ca^{2+} signals [71,72]. As previously stated, TEC significantly differ from healthy endothelial cells, showing aberrant phenotypes and physiology. It is, therefore, expected that Ca^{2+} homeostasis is also severely altered in TEC; indeed, Ca^{2+} signals mediated by different growth factors, such as VEGF or ATP and their downstream second messengers (arachidonic acid, nitric oxide, hydrogen sulfide, and cyclic AMP) are drastically remodeled in TEC where they play key roles in cell migration as compared to healthy endothelial cells [73–77]. Intriguingly, recent studies reported that TRP channels are differentially expressed in TEC. In particular, TRPV4 has been shown to exert a proangiogenic role on TEC by promoting cell migration and normalization [78,79]. On the contrary, TRPM8 exerts a protective role in endothelium by inhibiting cell migration via a Rap1/βintegrin mechanism [80]. Comparative TRP expression profile has been recently performed on prostate cancer TEC (PTEC) and their heathy counterpart, as well as on other TEC and endothelial cells. Interestingly, TRPA1, TRPV2, and TRPC3 are overexpressed in PTEC. TRPA1 showed a clear proangiogenic role by promoting an increase in intracellular $[Ca^{2+}]$ and consequent endothelial cells migration in vitro as well as sprouting angiogenesis in the retina in vivo model [81].

2.3. ERG

ERG (ETS related gene) is part of the E-26 transformation specific (ETS) family of transcription factors, and it was first discovered in 1987 by Reddy et al. in human colorectal carcinoma cells [82]. These factors function as either transcription activators or repressors, depending on the target gene or on the post-transcriptional modification required [83]. From the embryonic developmental stage, ERG is widely expressed in a variety of mesodermic tissues, and in particular in the endothelium, where it's highly expressed in the endothelial cells of the majority of adult tissues [83]. In endothelial cells, ERG plays a key role in the regulation of endothelial homeostasis by influencing numerous biological processes, such as vasculogenesis, angiogenesis, junction stability, cell migration, and survival [83]. In fact, ERG has been shown to act as a controller of the balance between pro- and anti-angiogenic processes, by regulating the expression of key genes like VEGFR1, VEGFR2, FZDL4, and EGF-like protein 7 [83]. Furthermore, in vitro studies have shown that ERG is essential for endothelial tube formation [84]. For instance, ERG inhibition studies in human endothelial cells revealed a lowered expression of the adhesion molecule VE-cadherin that resulted in the loss of cell–cell contacts, cell death, and, therefore, malformation of endothelial tubes [84]. These results were confirmed in vivo, whereby a postnatal deletion of ERG in inducible endothelial-specific ERG knockout mice led to defective angiogenesis in the retina, therefore confirming the crucial role of ERG in the regulation of angiogenesis [84,85].

Many studies reported the involvement of abnormal ectopic expression of ERG fusion proteins in many cancer types [86–88], however, limited studies reported the role of ERG in the regulation of tumor neovascularization. For instance, Nagai et al. in a mouse xenograft B16F0 tumor model, which depends on angiogenesis for growth, observed that knocking out endothelial ERG significantly reduced the size of melanoma tumors, and significantly reduced tumor blood vessel density and pericyte coverage compared to controls [89]. This study, therefore, confirms that ERG could play an essential role in tumor angiogenesis and growth and that downregulation of ERG expression could be an effective strategy towards developing new anticancer therapies.

As ERG is largely involved in the biology of cancer, it can be considered as a potential new target for cancer therapies itself. Indeed, ERG is one of the most overexpressed oncogenes in prostate cancer, where a chromosomal translocation results in the fusion of the promoter region of androgen-regulated transmembrane protease serine 2 (TMPRSS2) with the DNA-binding domain of ERG [86]. TMPRSS2-ERG expression leads to the upregulation of the histone deacetylase 1 (HDAC) gene and the downregulation of its target genes. A therapy based on the inhibition of HDAC can be, therefore, effective against prostate cancer development. The HDAC inhibitors can indeed reduce cancer growth by inducing apoptosis of ERG positive prostate cancer cells [88]. Moreover, the development of YK-4-279, a small molecule inhibitor of ETS factors, reduced invasion, motility, and metastasis of ERG positive cells in prostate cancer [90].

The ERG DNA-binding activity can also be targeted by modulators, such as DB1255, which prevents ERG DNA binding [91]. Finally, the degradation of ERG by targeting an ubiquitin-specific peptidase 9, resulted in prostate tumor growth inhibition, both, in vitro and in vivo [92]. Altogether, these therapeutic approaches, developed at present as anti-tumor strategies, might also influence tumor angiogenesis in view of its upregulation in TEC.

3. Extracellular Vesicles

Extracellular vesicles (EVs) are bio-active particles delimited by a lipid bilayer, secreted by a wide variety of cells, nowadays emerging as one of the main effectors of intercellular communication [93]. Depending on the cell source, EVs have been shown to exert multiple effects on specific cell targets by modifying their function and phenotype [93].

The clinical use of EVs for the treatment of cancer is currently under evaluation, being these bioactive molecules an efficient tool to allow the delivery of therapeutic cargos to neoplastic cells [94]. In oncology, the use of EVs has been proposed not only as a biological carrier for anti-tumor drugs but also as an immunomodulator and tumor vaccination [95].

Among the possible EV sources, EVs isolated from stem cells are one of the most studied as an anticancer strategy [96]. However, few studies investigated their direct effects on tumor angiogenesis [97–99]. Lee et al. showed that EVs isolated from mesenchymal stem cells (MSC) were able to inhibit tumor growth and angiogenesis in a murine model of breast cancer, by downregulating VEGF production in breast cancer cells [98]. On the other hand, EVs isolated from cardiosphere-derived cells were able to inhibit tumor angiogenesis in a murine model of fibrosarcoma [99].

More recently, Lopatina et al. showed that EVs derived from human liver stem cells (HLSC) exhibit a direct anti-angiogenic effect on tumor-derived endothelial cells isolated from human renal carcinoma [97]. Treatment of renal tumor endothelial cells with HLSC-EVs in vitro inhibited the angiogenic and migration properties of TEC in a dose-dependent manner. The inhibitory effects on angiogenesis were mainly attributed to a down-regulation of different proangiogenic genes, targets of specific miRNAs enriched in HLSC-EVs. In addition, the anti-angiogenic activity of HLSC-EVs has been observed in vivo in a model of tumor angiogenesis in SCID mice. HLSC-EVs treated tumor endothelial cells showed a limited ability to connect with murine vasculature, and treatment of pre-existent tumor vessels with HLSC-EVs reduced vessel density [97].

These studies reveal a complex effect of EVs on tumor vascularization, which may result from the modulation of multiple targets on different tumor cell types, including a direct effect on TEC and an indirect one on tumor cells.

4. Anti-Angiogenic Vaccination

Cancer vaccines are emerging as one of the most promising tools for tumor eradication. Recently, new vaccination strategies against TEC, rather than against cancer cells, have been proposed [100]. Being TEC phenotypically and genotypically different from a normal endothelium [22], anti-angiogenic vaccination would theoretically target the activated tumor endothelium only, without affecting other angiogenic processes involving normal endothelial cells. On the other hand, targeting one

specific molecule could activate compensatory angiogenic pathways and resistance mechanisms, that could be overcome by the combination with tumor immunotherapy or chemotherapy, or with other endothelial-cell vaccines.

Vaccination protocols involve the use of different vaccine types, such as DNA or peptide vaccines, or directly the injection of blocking antibodies against different immunogenic epitopes of proteins overexpressed by TEC [98–102]. However, both in preclinical and in clinical studies, the efficacy and the observed adverse events were variable, according to vaccine type, route of administration and to the adjuvant choice [98–102]. At present, different anti-angiogenic vaccination protocols, involving the use of peptide-based vaccines, are undergoing clinical trials, as summarized in Table 3 [101–108].

Preclinical studies involving the development of an immune response against different antigens overexpressed by TEC, such as bFGF, angiomotin, endoglin, Robo4, PDGFRβ, Tie-2, and tumor endothelial markers (TEM1 and TEM8) show that endothelial vaccination successfully reduces tumor growth in different tumor models, both in vitro and in vivo [100]. However, TEC genetic instability, together with the activation of compensatory pathways, may lead to an incomplete response to vaccination therapies against specific targets. Therefore, further clinical studies using whole endothelial [106] or placental cells [107] to induce a polyvalent immune response are currently under evaluation.

Table 3. Main anti-angiogenic vaccination approaches currently undergoing clinical trials.

Antigens	Vaccine Type	Tumor Type	Phase	REF/NIH N.
VEGF-A	Recombinant human VEGF-A-121 isoform	Advanced solid tumors	I	Gavilondo 2014 [108]
VEGFRs	VEGFR2-169 peptide	Pancreatic cancer	I	Miyazawa 2010 [101]
	VEGFR1-1084 and VEGFR2-169 peptides		I/II	NCT00655785
	VEGFR1-A2-770 peptide		I/II	NCT00683085
	VEGFR2-169 peptide	Advanced solid tumors	I	Okamoto 2012 [102]
	VEGFR1-1084 peptide		I	Hayashi 2013 [103]
	VEGFR2, VEGFR1, URLC10, TTK, CDCA1 multipeptide	Non small cell lung cancer	I	Suzuki 2013 [104]
Survivin	hTERT/survivin/CMV multipeptide	Breast cancer	I	NCT01660529
	Survivin long peptide	Neuroendocrine tumors	I	NCT03879694
	Salmonella-based Survivin peptide	Multiple myeloma	I/II	NCT03762291
EGF	Recombinant Human EGF-rP64K/Montanide ISA 51 peptide	Non-small cell lung cancer	II	Garcia 2008 [105]
			II/III	NCT00516685
			III	NCT02187367
			III	NCT01444118
		Non-small cell lung cancer, squamous head and neck cancer	I/II	NCT02955290

5. Vascular Normalization and Detransformation

Vessel normalization is defined as a vascular remodeling that leads to the re-acquisition of a normal structure and function of abnormal vessels [109].

The concept of vascular normalization as a therapeutic strategy to improve chemotherapeutic drug delivery to tumor cells was introduced in 1996 when Yuan et al. observed an increase of permeability in tumors treated with a VEGF-neutralizing antibody [110]. Several combinations of classical anti-angiogenic agents and cytotoxic drugs (used in a low dose and continuous protocol, the so-called metronomic dose) were, therefore, studied in clinical trials, but only a marginal increase of antitumor efficacy was observed [111]. Indeed, both the dose and the temporal window of

anti-angiogenic treatment needed to achieve a transient normalized vasculature, which allows an adequate drug delivery to the inner tumor mass showed high variability [112,113].

Alternative strategies to achieve an increased response to anti-tumor therapies involving vascular normalization have been proposed during the past years. Class 3 semaphorins (Sema3) are secreted proteins that regulate cell adhesion through the signaling mediated by their receptors, composed by the dimerization of neuropilins and plexins [114]. In endothelial cells, neuropilins were found to bind VEGF receptors, regulating vascular development [114]. Sema3 acts as a tumor suppressor by blocking tumor cell growth and invasion, as well as by inducing endothelial cell apoptosis [114]. In addition, Sema3A has been identified as a novel normalizing agent that can overcome the resistance to anti-angiogenic therapies by extending the normalization window in mouse models [115].

Moreover, combined therapies involving activation of immune response and classical anti-angiogenic agents used at a normalizing dose are under study. Several groups recently demonstrated that the immunotherapy effect is enhanced by vessel normalization [109,113,116–119]. In particular, the immune checkpoint blockade of the programmed death receptor-1 (PD-1)/PD ligand 1 (PD-L1) pathway, combined with the VEGF-pathway blockade, can enhance both anti-tumor immunity and a structural normalization of tumor vessels [116,118,119]. In particular, Schmittnaegel et al. observed that blocking both angiopoietin and VEGF pathways induced tumor vessel normalization that favored a cytotoxic immune response [118]. Allen et al. contemporarily observed that combination therapy using blocking antibodies against VEGFR2 and PD-L1 resulted in enhanced cytotoxic activity, together with an increased normalizing effect of VEGF blockade on tumor vasculature [119]. Clinical trials investigating the efficacy of a combined therapy that involves the use of immune checkpoint inhibitors and anti-angiogenic agents are currently undergoing [120].

All the anti-angiogenic therapies mentioned above, including normalizing therapies, may induce overtime a transformation of the tumor-associated endothelial cells towards a mesenchymal profile, called endothelial to mesenchymal transition (EndMT) [121]. In the course of EndMT, resident endothelial cells delaminate from an organized cell layer and acquire a mesenchymal phenotype characterized by loss of cell–cell junctions, loss of endothelial markers, the gain of mesenchymal markers, and acquisition of invasive and migratory properties [120]. In cancer, EndMT supports the formation of cancer-associated fibroblasts, which are known to facilitate tumor progression. Furthermore, EndMT could also modify the endothelium abnormally, therefore, assisting tumor-cell extravasation. Lastly, EndMT has also been reported to be induced by events such as hypoxia, high glucose levels, as well as through the release of soluble factors in the tumor microenvironment [121].

Nagai et al. showed that EndMT can be triggered through a reduction in the expression of ERG together with friend leukemia integration 1 transcription factor (FLI1), which has been reported to play a pivotal role in endothelial cell homeostasis. A combined knockdown of both ERG and FLI-1 through short interfering RNA (siRNAs) in endothelial cells, caused the downregulation of endothelial genes accompanied by a consistent upregulation of genes involved in EndMT, such as alphaSMA and CollagenA1 in vitro [89].

It can, therefore, be concluded that dysregulation of angiogenic signaling pathways that play a crucial role in the homeostasis of endothelial cells, can cause an imbalance in endothelial physiology, leading to EndMT, which has been implicated in cancer progression.

As low doses of anti-angiogenic therapies from one side may induce the transformation of aberrant vases towards normal vessels, and from the other side may favor the activation of a mesenchymal phenotype in endothelial cells, an optimal therapy should take into consideration both vascular normalization and endothelial de-mesenchymalization.

For example, the combination of VEGF-targeting agents and TGFβ signaling inhibition, such as Sunitinib and TRC105 [51], as discussed above, could represent a valid therapy to block both endothelial and mesenchymal-related pathways.

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References

1. Judah Folkman Tumor angiogenesis: Therapeutic implications. *N. Engl. J. Med.* **1971**, *262*, 1182–1186.
2. De Bock, K.; Cauwenberghs, S.; Carmeliet, P. Vessel abnormalization: Another hallmark of cancer? Molecular mechanisms and therapeutic implications. *Curr. Opin. Genet. Dev.* **2011**, *21*, 73–79. [CrossRef] [PubMed]
3. Baluk, P.; Hashizume, H.; McDonald, D.M. Cellular abnormalities of blood vessels as targets in cancer. *Curr. Opin. Genet. Dev.* **2005**, *15*, 102–111. [CrossRef] [PubMed]
4. De Sanctis, F.; Ugel, S.; Facciponte, J.; Facciabene, A. The dark side of tumor-associated endothelial cells. *Semin. Immunol.* **2018**, *35*, 35–47. [CrossRef] [PubMed]
5. Miller, J.R.; Moon, R.T.; Dev, G.; Cadigan, K.M.; Nusse, R.; Cells, G.; Hart, M.J.; Santos, R.D.L.; Albert, I.N.; Rubinfeld, B.; et al. Genes Expressed in Human Tumor Endothelium. *Science* **2000**, *289*, 1197–1202.
6. Hida, K.; Klagsbrun, M. A new perspective on tumor endothelial cells: Unexpected chromosome and centrosome abnormalities. *Cancer Res.* **2005**, *65*, 2507–2510. [CrossRef]
7. Bussolati, B.; Deambrosis, I.; Russo, S.; Deregibus, M.C.; Camussi, G. Altered angiogenesis and survival in human tumor-derived endothelial cells. *FASEB J.* **2003**, *17*, 1159–1161. [CrossRef]
8. Pla, A.F.; Brossa, A.; Bernardini, M.; Genova, T.; Grolez, G.; Villers, A.; Leroy, X.; Prevarskaya, N.; Gkika, D.; Bussolati, B. Differential sensitivity of prostate tumor derived endothelial cells to sorafenib and sunitinib. *BMC Cancer* **2014**, *14*, 1–13.
9. Xiong, Y.Q.; Sun, H.C.; Zhang, W.; Zhu, X.D.; Zhuang, P.Y.; Zhang, J.B.; Wang, L.; Wu, W.Z.; Qin, L.X.; Tang, Z.Y. Human hepatocellular carcinoma tumor-derived endothelial cells manifest increased angiogenesis capability and drug resistance compared with normal endothelial cells. *Clin. Cancer Res.* **2009**, *15*, 4838–4846. [CrossRef]
10. Bussolati, B.; Grange, C.; Camussi, G. Tumor exploits alternative strategies to achieve vascularization. *FASEB J.* **2011**, *25*, 2874–2882. [CrossRef]
11. Ratajczak, J.; Wysoczynski, M.; Hayek, F.; Janowska-Wieczorek, A.; Ratajczak, M.Z. Membrane-derived microvesicles: Important and underappreciated mediators of cell-to-cell communication. *Leukemia* **2006**, *20*, 1487–1495. [CrossRef] [PubMed]
12. Skog, J.; Würdinger, T.; van Rijn, S.; Meijer, D.H.; Gainche, L.; Curry, W.T.; Carter, B.S.; Krichevsky, A.M.; Breakefield, X.O. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat. Cell Biol.* **2008**, *10*, 1470–1476. [CrossRef] [PubMed]
13. Bergsmedh, A.; Szeles, A.; Henriksson, M.; Bratt, A.; Folkman, M.J.; Spetz, A.L.; Holmgren, L. Horizontal transfer of oncogenes by uptake of apoptotic bodies. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 6407–6411. [CrossRef] [PubMed]
14. Ding, Y.; Song, N.; Luo, Y. Role of bone marrow-derived cells in angiogenesis: Focus on macrophages and pericytes. *Cancer Microenviron.* **2012**, *5*, 225–236. [CrossRef] [PubMed]
15. Bruno, S.; Bussolati, B.; Grange, C.; Collino, F.; Graziano, M.E.; Ferrando, U.; Camussi, G. CD133+ renal progenitor cells contribute to tumor angiogenesis. *Am. J. Pathol.* **2006**, *169*, 2223–2235. [CrossRef]
16. Bussolati, B.; Bruno, S.; Grange, C.; Ferrando, U.; Camussi, G. Identification of a tumor-initiating stem cell population in human renal carcinomas. *FASEB J.* **2008**, *22*, 3696–3705. [CrossRef]
17. Ricci-Vitiani, L.; Pallini, R.; Biffoni, M.; Todaro, M.; Invernici, G.; Cenci, T.; Maira, G.; Parati, E.A.; Stassi, G.; Larocca, L.M.; et al. Tumour vascularization via endothelial differentiation of glioblastoma stem-like cells. *Nature* **2010**, *468*, 824–830. [CrossRef]
18. Bussolati, B.; Grange, C.; Sapino, A.; Camussi, G. Endothelial cell differentiation of human breast tumour stem/progenitor cells. *J. Cell. Mol. Med.* **2009**, *13*, 309–319. [CrossRef]

19. Maniotis, A.J.; Folberg, R.; Hess, A.; Seftor, E.A.; Gardner, L.M.G.; Pe'er, J.; Trent, J.M.; Meltzer, P.S.; Hendrix, M.J.C. Vascular channel formation by human melanoma cells in vivo and in vitro: Vasculogenic mimicry. *Am. J. Pathol.* **1999**, *155*, 739–752. [CrossRef]
20. Ge, H.; Luo, H. Overview of advances in vasculogenic mimicry — A potential target for tumor therapy. *Cancer Manag. Res.* **2018**, *10*, 2429–2437. [CrossRef]
21. Ribatti, D.; Djonov, V. Intussusceptive microvascular growth in tumors. *Cancer Lett.* **2012**, *316*, 126–131. [CrossRef] [PubMed]
22. Dudley, A.C. Tumor endothelial cells. *Cold Spring Harb. Perspect. Med.* **2012**, *2*, 1–18. [CrossRef] [PubMed]
23. Alessandri, G.; Chirivi, R.G.S.; Fiorentini, S.; Dossi, R.; Bonardelli, S.; Giulini, S.M.; Zanetta, G.; Landoni, F.; Graziotti, P.P.; Turano, A.; et al. Phenotypic and functional characteristics of tumour-derived microvascular endothelial cells. *Clin. Exp. Metastasis* **1999**, *17*, 655–662. [CrossRef] [PubMed]
24. Unger, R.E.; Oltrogge, J.B.; Von Briesen, H.; Engelhardt, B.; Woelki, U.; Schlote, W.; Lorenz, R.; Bratzke, H.; Kirkpatrick, C.J. Isolation and molecular characterization of brain microvascular endothelial cells from human brain tumors. *Vitr. Cell. Dev. Biol. Anim.* **2002**, *38*, 273–281. [CrossRef]
25. Allport, J.R.; Weissleder, R. Murine Lewis lung carcinoma-derived endothelium expresses markers of endothelial activation and requires tumor-specific extracellular matrix in vitro. *Neoplasia* **2003**, *5*, 205–217. [CrossRef]
26. Streubel, B.; Chott, A.; Huber, D.; Exner, M.; Jäger, U.; Wagner, O.; Schwarzinger, I. Lymphoma-specific genetic aberrations in microvascular endothelial cells in B-cell lymphomas. *N. Engl. J. Med.* **2004**, *351*, 250–259. [CrossRef]
27. Hida, K.; Hida, Y.; Amin, D.N.; Flint, A.F.; Panigrahy, D.; Morton, C.C.; Klagsbrun, M. Tumor-associated endothelial cells with cytogenetic abnormalities. *Cancer Res.* **2004**, *64*, 8249–8255. [CrossRef]
28. Grange, C.; Bussolati, B.; Bruno, S.; Fonsato, V.; Sapino, A.; Camussi, G. Isolation and characterization of human breast tumor-derived endothelial cells. *Oncol. Rep.* **2006**, *15*, 381–386. [CrossRef]
29. Amin, D.N.; Hida, K.; Bielenberg, D.R.; Klagsbrun, M. Tumor endothelial cells express epidermal growth factor receptor (EGFR) but not ErbB3 and are responsive to EGF and to EGFR kinase inhibitors. *Cancer Res.* **2006**, *66*, 2173–2180. [CrossRef]
30. Wu, L.Q.; Zhang, W.J.; Niu, J.X.; Ye, L.Y.; Yang, Z.H.; Grau, G.E.; Lou, J.N. Phenotypic and functional differences between human liver cancer endothelial cells and liver sinusoidal endothelial cells. *J. Vasc. Res.* **2007**, *45*, 78–86. [CrossRef]
31. Buckanovich, R.J.; Sasaroli, D.; O'Brien-Jenkins, A.; Botbyl, J.; Hammond, R.; Katsaros, D.; Sandaltzopoulos, R.; Liotta, L.A.; Gimotty, P.A.; Coukos, G. Tumor vascular proteins as biomarkers in ovarian cancer. *J. Clin. Oncol.* **2007**, *25*, 852–861. [CrossRef] [PubMed]
32. Lu, C.; Bonome, T.; Li, Y.; Kamat, A.A.; Han, L.Y.; Schmandt, R.; Coleman, R.L.; Gershenson, D.M.; Jaffe, R.B.; Birrer, M.J.; et al. Gene alterations identified by expression profiling in tumor-associated endothelial cells from invasive ovarian carcinoma. *Cancer Res.* **2007**, *67*, 1757–1768. [CrossRef] [PubMed]
33. You, L.; Wu, M.; Chen, Y.; Xu, G.; Wei, J.; Li, Q.; Song, A.; Zhao, L.; Li, S.; Han, Z.; et al. Isolation and characterization of lymphatic endothelial cells from human glossal lymphangioma. *Oncol. Rep.* **2010**, *25*, 223–230.
34. Qin, S.; Li, A.; Yi, M.; Yu, S.; Zhang, M.; Wu, K. Recent advances on anti-angiogenesis receptor tyrosine kinase inhibitors in cancer therapy. *J. Hematol. Oncol.* **2019**, *12*, 1–11. [CrossRef]
35. Jayson, G.C.; Hicklin, D.J.; Ellis, L.M. Antiangiogenic therapy-evolving view based on clinical trial results. *Nat. Rev. Clin. Oncol.* **2012**, *9*, 297–303. [CrossRef]
36. McIntyre, A.; Harris, A.L. Metabolic and hypoxic adaptation to anti—angiogenic therapy: A target for induced essentiality. *EMBO Mol. Med.* **2015**, *7*, 368–379. [CrossRef]
37. Mancuso, M.R.; Davis, R.; Norberg, S.M.; Brien, S.O.; Sennino, B.; Nakahara, T.; Yao, V.J.; Inai, T.; Brooks, P.; Freimark, B.; et al. Rapid vascular regrowth in tumors after reversal of VEGF inhibition. *J. Clin. Investig.* **2006**, *116*, 2610–2621. [CrossRef]
38. Hainsworth, J.D.; Spigel, D.R.; Sosman, J.A.; Burris, H.A.; Farley, C.; Cucullu, H.; Yost, K.; Hart, L.L.; Sylvester, L.; Waterhouse, D.M.; et al. Treatment of advanced renal cell carcinoma with the combination bevacizumab/erlotinib/imatinib: A phase I/II trial. *Clin. Genitourin. Cancer* **2007**, *5*, 427–432. [CrossRef]
39. Itatani, Y.; Kawada, K.; Yamamoto, T.; Sakai, Y. Resistance to anti-angiogenic therapy in cancer-alterations to anti-VEGF pathway. *Int. J. Mol. Sci.* **2018**, *19*, 1232. [CrossRef]

40. Kong, D.H.; Kim, M.R.; Jang, J.H.; Na, H.J.; Lee, S. A review of anti-angiogenic targets for monoclonal antibody cancer therapy. *Int. J. Mol. Sci.* **2017**, *18*, 1786. [CrossRef]
41. McNeel, D.G.; Eickhoff, J.; Lee, F.T.; King, D.M.; Alberti, D.; Thomas, J.P.; Friedl, A.; Kolesar, J.; Marnocha, R.; Volkman, J.; et al. Phase I trial of a monoclonal antibody specific for α v β 3 integrin (MEDI-522) in patients with advanced malignancies, including an assessment of effect on tumor perfusion. *Clin. Cancer Res.* **2005**, *11*, 7851–7860. [CrossRef] [PubMed]
42. Binz, H.K.; Bakker, T.R.; Phillips, D.J.; Cornelius, A.; Zitt, C.; Göttler, T.; Sigrist, G.; Fiedler, U.; Ekawardhani, S.; Dolado, I.; et al. Design and characterization of MP0250, a tri-specific anti-HGF/anti-VEGF DARPin® drug candidate. *MAbs* **2017**, *9*, 1262–1269. [CrossRef] [PubMed]
43. Fiedler, U.; Ekawardhani, S.; Cornelius, A.; Gilboy, P.; Bakker, T.R.; Dolado, I.; Stumpp, M.T.; Dawson, K.M. MP0250, a VEGF and HGF neutralizing DARPin® molecule shows high anti-tumor efficacy in mouse xenograft and patient-derived tumor models. *Oncotarget* **2017**, *8*, 98371–98383. [CrossRef] [PubMed]
44. Rao, L.; Veirman, K.; de Giannico, D.; Saltarella, I.; Desantis, V.; Frassanito, M.A.; Solimando, A.G.; Ribatti, D.; Prete, M.; Harstrick, A.; et al. Targeting angiogenesis in multiple myeloma by the VEGF and HGF blocking DARPin® protein MP0250: A preclinical study. *Oncotarget* **2018**, *9*, 13366–13381. [CrossRef]
45. Park, S.Y.; Piao, Y.; Jeong, K.J.; Dong, J.; de Groot, J.F. Periostin (POSTN) regulates tumor resistance to antiangiogenic therapy in glioma models. *Physiol. Behav.* **2016**, *176*, 105–111. [CrossRef]
46. Paauwe, M.; Heijkants, R.C.; Oudt, C.H.; Van Pelt, G.W.; Cui, C.; Theuer, C.P.; Hardwick, J.C.H.; Sier, C.F.M.; Hawinkels, L.J.A.C. Endoglin targeting inhibits tumor angiogenesis and metastatic spread in breast cancer. *Oncogene* **2016**, *35*, 4069–4079. [CrossRef]
47. Derbyshire, E.J.; Gazdar, A.F.; King, S.W.; Thorpe, P.E.; Derbyshire, E.J.; King, S.W.; Thorpe, P.E.; Gazdar, A.F.; Vitetta, E.S.; Tazzari, P.L.; et al. Up-Regulation of Endoglin on Vascular Endothelial Cells in Human Solid Tumors: Implications for Diagnosis and Therapy. *Clin. Cancer Res.* **1995**, *1*, 1623–1634.
48. Hu, J.; Guan, W.; Liu, P.; Dai, J.; Tang, K.; Xiao, H.; Qian, Y.; Sharrow, A.C.; Ye, Z.; Wu, L.; et al. Endoglin Is Essential for the Maintenance of Self-Renewal and Chemoresistance in Renal Cancer Stem Cells. *Stem Cell Reports* **2017**, *9*, 464–477. [CrossRef]
49. Madhav, A.; Andres, A.; Duong, F.; Mishra, R.; Haldar, S.; Liu, Z.; Angara, B.; Gottlieb, R.; Zumsteg, Z.S.; Bhowmick, N.A. Antagonizing CD105 enhances radiation sensitivity in prostate cancer. *Oncogene* **2018**, *37*, 4385–4397. [CrossRef]
50. Zhang, J.; Yuan, B.; Zhang, H.; Li, H. Human epithelial ovarian cancer cells expressing cd105, cd44 and cd106 surface markers exhibit increased invasive capacity and drug resistance. *Oncol. Lett.* **2019**, *17*, 5351–5360. [CrossRef]
51. Brossa, A.; Buono, L.; Bussolati, B. Effect of the monoclonal antibody TRC105 in combination with Sunitinib on renal tumor derived endothelial cells. *Oncotarget* **2018**, *9*, 22680–22692. [CrossRef] [PubMed]
52. Mehta, C.R.; Liu, L.; Theuer, C. An adaptive population enrichment phase III trial of TRC105 and pazopanib versus pazopanib alone in patients with advanced angiosarcoma (TAPPAS trial). *Ann. Oncol.* **2019**, *30*, 103–108. [CrossRef] [PubMed]
53. Worley, M.J.; Elias, K.M.; Horowitz, N.S.; Quade, B.J.; Berkowitz, R.S. Durable remission for a woman with refractory choriocarcinoma treated with anti-endoglin monoclonal antibody and bevacizumab: A case from the New England Trophoblastic Disease Center, Brigham and Women's Hospital and Dana-Farber Cancer Institute. *Gynecol. Oncol.* **2018**, *148*, 5–11. [CrossRef] [PubMed]
54. Duffy, A.G.; Ma, C.; Ulahannan, S.V.; Rahma, O.E.; Makarova-Rusher, O.; Cao, L.; Yu, Y.; Kleiner, D.E.; Trepel, J.B.; Lee, M.-J.; et al. Phase I and preliminary Phase II study of TRC105 in combination with Sorafenib in Hepatocellular Carcinoma. *Clin. Cancer Res.* **2017**, *23*, 4633–4641. [CrossRef]
55. Iamshanova, O.; Fiorio Pla, A.; Prevarskaya, N. Molecular mechanisms of tumour invasion: Regulation by calcium signals. *J. Physiol.* **2017**, *595*, 3063–3075. [CrossRef]
56. Monteith, G.R.; Prevarskaya, N.; Roberts-Thomson, S.J. The calcium–cancer signalling nexus. *Nat. Rev. Cancer* **2017**, *17*, 367–380. [CrossRef]
57. Berridge, M.J.; Bootman, M.D.; Roderick, H.L. Calcium signalling: Dynamics, homeostasis and remodelling. *Nat. Rev. Mol. Cell Biol.* **2003**, *4*, 517–529. [CrossRef]
58. Munaron, L.; Arcangeli, A. Editorial: Ion fluxes and cancer. *Recent Pat. Anticancer Drug Discov.* **2013**, *8*, 1–3. [CrossRef]

59. Prevarskaya, N.; Skryma, R.; Shuba, Y. Ion Channels in Cancer: Are Cancer Hallmarks Oncochannelopathies? *Physiol. Rev.* **2018**, *98*, 559–621. [CrossRef]
60. Munaron, L.; Genova, T.; Avanzato, D.; Antoniotti, S.; Pla, A.F. Targeting Calcium Channels to Block Tumor Vascularization. *Recent Pat. Anticancer. Drug Discov.* **2013**, *8*, 27–37. [CrossRef]
61. Moccia, F.; Negri, S.; Shekha, M.; Faris, P.; Guerra, G. Guerra Endothelial Ca^2+ Signaling, Angiogenesis and Vasculogenesis: Just What It Takes to Make a Blood Vessel. *Int. J. Mol. Sci.* **2019**, *20*, 3962. [CrossRef]
62. Farfariello, V.; Iamshanova, O.; Germain, E.; Fliniaux, I.; Prevarskaya, N. Calcium homeostasis in cancer: A focus on senescence. *Biochim. Biophys. Acta* **2015**, *1853*, 1974–1979. [CrossRef] [PubMed]
63. Yokota, Y.; Nakajima, H.; Wakayama, Y.; Muto, A.; Kawakami, K.; Fukuhara, S.; Mochizuki, N. Endothelial Ca^2+ oscillations reflect VEGFR signaling-regulated angiogenic capacity in vivo. *Elife* **2015**, *4*, 1–26. [CrossRef] [PubMed]
64. Dragoni, S.; Laforenza, U.; Bonetti, E.; Lodola, F.; Bottino, C.; Berra-Romani, R.; Carlo Bongio, G.; Cinelli, M.P.; Guerra, G.; Pedrazzoli, P.; et al. Vascular endothelial growth factor stimulates endothelial colony forming cells proliferation and tubulogenesis by inducing oscillations in intracellular Ca^2+ concentration. *Stem Cells* **2011**, *29*, 1898–1907. [CrossRef] [PubMed]
65. Dragoni, S.; Reforgiato, M.; Zuccolo, E.; Poletto, V.; Lodola, F.; Ruffinatti, F.A.; Bonetti, E.; Guerra, G.; Barosi, G.; Rosti, V.; et al. Dysregulation of VEGF-induced proangiogenic Ca^2+ oscillations in primary myelofibrosis-derived endothelial colony-forming cells. *Exp. Hematol.* **2015**, *43*, 1019–1030. [CrossRef] [PubMed]
66. Noren, D.P.; Chou, W.H.; Lee, S.H.; Qutub, A.A.; Warmflash, A.; Wagner, D.S.; Popel, A.S.; Levchenko, A. Endothelial cells decode VEGF-mediated Ca^2+ signaling patterns to produce distinct functional responses. *Sci. Signal.* **2016**, *9*, 1–21. [CrossRef] [PubMed]
67. Savage, A.M.; Kurusamy, S.; Chen, Y.; Jiang, Z.; Chhabria, K.; MacDonald, R.B.; Kim, H.R.; Wilson, H.L.; van Eeden, F.J.M.; Armesilla, A.L.; et al. Tmem33 is essential for VEGF-mediated endothelial calcium oscillations and angiogenesis. *Nat. Commun.* **2019**, *10*. [CrossRef]
68. Nilius, B.; Droogmans, G. Ion channels and their functional role in vascular endothelium. *Physiol. Rev.* **2001**, *81*, 1415–1459. [CrossRef]
69. Thakore, P.; Earley, S. Transient Receptor Potential Channels and Endothelial Cell Calcium Signaling. *Compr. Physiol.* **2019**, *9*, 1249–1277.
70. Smani, T.; Gómez, L.J.; Regodon, S.; Woodard, G.E.; Siegfried, G.; Khatib, A.-M.; Rosado, J.A. TRP Channels in Angiogenesis and Other Endothelial Functions. *Front. Physiol.* **2018**, *9*, 1731. [CrossRef]
71. Pocock, T.M.; Foster, R.R.; Bates, D.O. Evidence of a role for TRPC channels in VEGF-mediated increased vascular permeability in vivo. *Am. J. Physiol. Heart Circ. Physiol.* **2004**, *286*, H1015–H1026. [CrossRef] [PubMed]
72. Cheng, H.-W.; James, A.F.; Foster, R.R.; Hancox, J.C.; Bates, D.O. VEGF activates receptor-operated cation channels in human microvascular endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **2006**, *26*, 1768–1776. [CrossRef] [PubMed]
73. Avanzato, D.; Genova, T.; Fiorio Pla, A.; Bernardini, M.; Bianco, S.; Bussolati, B.; Mancardi, D.; Giraudo, E.; Maione, F.; Cassoni, P.; et al. Activation of P2X7 and P2Y11 purinergic receptors inhibits migration and normalizes tumor-derived endothelial cells via cAMP signaling. *Sci. Rep.* **2016**, *6*, 32602. [CrossRef] [PubMed]
74. Fiorio Pla, A.; Grange, C.; Antoniotti, S.; Tomatis, C.; Merlino, A.; Bussolati, B.; Munaron, L. Arachidonic acid-induced Ca^2+ entry is involved in early steps of tumor angiogenesis. *Mol. Cancer Res.* **2008**, *6*, 535–545. [PubMed]
75. Fiorio Pla, A.; Genova, T.; Pupo, E.; Tomatis, C.; Genazzani, A.; Zaninetti, R.; Munaron, L. Multiple roles of protein kinase a in arachidonic acid-mediated Ca^2+ entry and tumor-derived human endothelial cell migration. *Mol. Cancer Res.* **2010**, *8*, 1466–1476. [CrossRef] [PubMed]
76. Pupo, E.; Pla, A.F.; Avanzato, D.; Moccia, F.; Cruz, J.-E.E.A.; Tanzi, F.; Merlino, A.; Mancardi, D.; Munaron, L. Hydrogen sulfide promotes calcium signals and migration in tumor-derived endothelial cells. *Free Radic. Biol. Med.* **2011**, *51*, 1765–1773. [CrossRef]
77. Scarpellino, G.; Genova, T.; Avanzato, D.; Bernardini, M.; Bianco, S.; Petrillo, S.; Tolosano, E.; de Vieira, J.R.A.; Bussolati, B.; Pla, A.F.; et al. Purinergic Calcium Signals in Tumor-Derived Endothelium. *Cancers* **2019**, *11*, 766. [CrossRef]

78. Adapala, R.K.; Thoppil, R.J.; Ghosh, K.; Cappelli, H.C.; Dudley, A.C.; Paruchuri, S.; Keshamouni, V.; Klagsbrun, M.; Meszaros, J.G.; Chilian, W.M.; et al. Activation of mechanosensitive ion channel TRPV4 normalizes tumor vasculature and improves cancer therapy. *Oncogene* **2015**, 1–9. [CrossRef]
79. Fiorio Pla, A.; Ong, H.L.; Cheng, K.T.; Brossa, A.; Bussolati, B.; Lockwich, T.; Paria, B.; Munaron, L.; Ambudkar, I.S. TRPV4 mediates tumor-derived endothelial cell migration via arachidonic acid-activated actin remodeling. *Oncogene* **2012**, *31*, 200–212. [CrossRef]
80. Genova, T.; Grolez, G.P.; Camillo, C.; Bernardini, M.; Bokhobza, A.; Richard, E.; Scianna, M.; Lemonnier, L.; Valdembri, D.; Munaron, L.; et al. TRPM8 inhibits endothelial cell migration via a nonchannel function by trapping the small GTPase Rap1. *J. Cell Biol.* **2017**, *216*, 2107–2130. [CrossRef]
81. Bernardini, M.; Brossa, A.; Chinigo, G.; Grolez, G.P.; Trimaglio, G.; Allart, L.; Hulot, A.; Marot, G.; Genova, T.; Joshi, A.; et al. Transient Receptor Potential Channel Expression Signatures in Tumor-Derived Endothelial Cells: Functional Roles in Prostate Cancer Angiogenesis. *Cancers.* **2019**, *11*, 956. [CrossRef] [PubMed]
82. Reddy, E.S.P.; Rao, V.N.; Papas, T.S. The erg gene: A human gene related to the ets oncogene. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 6131–6135. [CrossRef] [PubMed]
83. Shah, A.V.; Birdsey, G.M.; Randi, A.M. Regulation of endothelial homeostasis, vascular development and angiogenesis by the transcription factor ERG. *Vascul. Pharmacol.* **2016**, *86*, 3–13. [CrossRef] [PubMed]
84. Birdsey, G.M.; Dryden, N.H.; Amsellem, V.; Gebhardt, F.; Sahnan, K.; Haskard, D.O.; Dejana, E.; Mason, J.C.; Randi, A.M. Transcription factor erg regulates angiogenesis and endothelial apoptosis through VE-cadherin. *Blood* **2008**, *111*, 3498–3506. [CrossRef] [PubMed]
85. Birdsey, G.M.; Shah, A.V.; Dufton, N.; Reynolds, L.E.; Almagro, L.O.; Yang, Y.; Aspalter, I.M.; Khan, S.T.; Mason, J.C.; Dejana, E.; et al. The endothelial transcription factor erg promotes vascular stability and growth through Wnt/β-catenin signaling. *Dev. Cell* **2015**, *32*, 82–96. [CrossRef] [PubMed]
86. Adamo, P.; Ladomery, M.R. The oncogene ERG: A key factor in prostate cancer. *Oncogene* **2016**, *35*, 403–414. [CrossRef]
87. Brenner, J.C.; Ateeq, B.; Chinnaiyan, A.M.; Yocum, A.K.; Cao, Q.; Asangani, I.A.; Patel, S.; Wang, X.; Liang, H.; Yu, J.; et al. Mechanistic Rationale for Inhibition of Poly (ADP-Ribose) Polymerase in ETS Gene Fusion-Positive Prostate Cancer. *Cancer Cell.* **2011**, *23*, 664–678.
88. Björkman, M.; Iljin, K.; Halonen, P.; Sara, H.; Kaivanto, E.; Nees, M.; Kallioniemi, O.P. Defining the molecular action of HDAC inhibitors and synergism with androgen deprivation in ERG-positive prostate cancer. *Int. J. Cancer* **2008**, *123*, 2774–2781. [CrossRef]
89. Nagai, N.; Ohguchi, H.; Nakaki, R.; Matsumura, Y.; Kanki, Y.; Sakai, J.; Aburatani, H.; Minami, T. Downregulation of ERG and FLI1 expression in endothelial cells triggers endothelial-to-mesenchymal transition. *PLoS Genet.* **2018**, *14*, 1007826. [CrossRef]
90. Rahim, S.; Beauchamp, E.M.; Kong, Y.; Brown, M.L.; Toretsky, J.A.; Üren, A. YK-4-279 inhibits ERG and ETV1 mediated prostate cancer cell invasion. *PLoS ONE* **2011**, *6*, e19343. [CrossRef]
91. Nhili, R.; Peixoto, P.; Depauw, S.; Flajollet, S.; Dezitter, X.; Munde, M.M.; Ismail, M.A.; Kumar, A.; Farahat, A.A.; Stephens, C.E.; et al. Targeting the DNA-binding activity of the human ERG transcription factor using new heterocyclic dithiophene diamidines. *Nucleic Acids Res.* **2013**, *41*, 125–138. [CrossRef] [PubMed]
92. Wang, S.; Kollipara, R.K.; Srivastava, N.; Li, R.; Ravindranathan, P.; Hernandez, E.; Freeman, E.; Humphries, C.G.; Kapur, P.; Lotan, Y.; et al. Ablation of the oncogenic transcription factor ERG by deubiquitinase inhibition in prostate cancer. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 4251–4256. [CrossRef] [PubMed]
93. El Andaloussi, S.; Mäger, I.; Breakefield, X.O.; Wood, M.J.A. Extracellular vesicles: Biology and emerging therapeutic opportunities. *Nat. Rev. Drug Discov.* **2013**, *12*, 347–357. [CrossRef] [PubMed]
94. Khawar, M.B.; Abbasi, M.H.; Siddique, Z.; Arif, A.; Sheikh, N. An Update on Novel Therapeutic Warfronts of Extracellular Vesicles (EVs) in Cancer Treatment: Where We Are Standing Right Now and Where to Go in the Future. *Oxid. Med. Cell. Longev.* **2019**, *2019*, 1–21. [CrossRef] [PubMed]
95. Sil, S.; Dagur, R.S.; Liao, K.; Peeples, E.S.; Hu, G.; Periyasamy, P.; Buch, S. Strategies for the use of Extracellular Vesicles for the Delivery of Therapeutics. *J. Neuroimmune Pharmacol.* **2019**, 1–21. [CrossRef] [PubMed]
96. Brossa, A.; Fonsato, V.; Bussolati, B. Anti-tumor activity of stem cell-derived extracellular vesicles. *Oncotarget* **2019**, *10*, 1872–1873. [CrossRef] [PubMed]

97. Lopatina, T.; Grange, C.; Fonsato, V.; Tapparo, M.; Brossa, A.; Fallo, S.; Pitino, A.; Herrera-Sanchez, M.B.; Kholia, S.; Camussi, G.; et al. Extracellular vesicles from human liver stem cells inhibit tumor angiogenesis. *Int. J. Cancer* **2019**, *144*, 322–333. [CrossRef]
98. Lee, J.K.; Park, S.R.; Jung, B.K.; Jeon, Y.K.; Lee, Y.S.; Kim, M.K.; Kim, Y.G.; Jang, J.Y.; Kim, C.W. Exosomes derived from mesenchymal stem cells suppress angiogenesis by down-regulating VEGF expression in breast cancer cells. *PLoS ONE* **2013**, *8*, 84256. [CrossRef]
99. Grigorian-Shamagian, L.; Fereydooni, S.; Liu, W.; Echavez, A.; Marbán, E. Harnessing the heart's resistance to malignant tumors: Cardiacderived extracellular vesicles decrease fibrosarcoma growth and leukemia-related mortality in rodents. *Oncotarget* **2017**, *8*, 99624–99636. [CrossRef]
100. Wagner, S.C.; Ichim, T.E.; Ma, H.; Szymanski, J.; Perez, J.A.; Lopez, J.; Bogin, V.; Patel, A.N.; Marincola, F.M.; Kesari, S. Cancer anti-angiogenesis vaccines: Is the tumor vasculature antigenically unique? *J. Transl. Med.* **2015**, *13*, 1–11. [CrossRef]
101. Miyazawa, M.; Ohsawa, R.; Tsunoda, T.; Hirono, S.; Kawai, M.; Tani, M.; Nakamura, Y.; Yamaue, H. Phase I clinical trial using peptide vaccine for human vascular endothelial growth factor receptor 2 in combination with gemcitabine for patients with advanced pancreatic cancer. *Cancer Sci.* **2010**, *101*, 433–439. [CrossRef] [PubMed]
102. Okamoto, I.; Arao, T.; Miyazaki, M.; Satoh, T.; Okamoto, K.; Tsunoda, T.; Nishio, K.; Nakagawa, K. Clinical phase I study of elpamotide, a peptide vaccine for vascular endothelial growth factor receptor 2, in patients with advanced solid tumors. *Cancer Sci.* **2012**, *103*, 2135–2138. [CrossRef] [PubMed]
103. Hayashi, H.; Kurata, T.; Fujisaka, Y.; Kawakami, H.; Tanaka, K.; Okabe, T.; Takeda, M.; Satoh, T.; Yoshida, K.; Tsunoda, T.; et al. Phase I trial of OTS11101, an anti-angiogenic vaccine targeting vascular endothelial growth factor receptor 1 in solid tumor. *Cancer Sci.* **2013**, *104*, 98–104. [CrossRef] [PubMed]
104. Suzuki, H.; Fukuhara, M.; Yamaura, T.; Mutoh, S.; Okabe, N.; Yaginuma, H.; Hasegawa, T.; Yonechi, A.; Osugi, J.; Hoshino, M.; et al. Multiple therapeutic peptide vaccines consisting of combined novel cancer testis antigens and anti-angiogenic peptides for patients with non-small cell lung cancer. *J. Transl. Med.* **2013**, *11*, 1–10. [CrossRef] [PubMed]
105. Garcia Verdecia, B.; Neninger, E.; De La Torre, A.; Leonard, I.; Martínez, R.; Viada, C.; González, G.; Mazorra, Z.; Lage, A.; Crombet, T. Effective inhibition of the epidermal growth factor/epidermal growth factor receptor binding by anti-epidermal growth factor antibodies is related to better survival in advanced non-small-cell lung cancer patients treated with the epidermal growth factor. *Clin. Cancer Res.* **2008**, *14*, 840–846. [CrossRef]
106. Tanaka, M.; Tsuno, N.H.; Fujii, T.; Todo, T.; Saito, N.; Takahashi, K. Human umbilical vein endothelial cell vaccine therapy in patients with recurrent glioblastoma. *Cancer Sci.* **2013**, *104*, 200–205. [CrossRef]
107. Ichim, T.E.; Li, S.; Ma, H.; Yurova, Y.V.; Szymanski, J.S.; Patel, A.N.; Kesari, S.; Min, W.P.; Wagner, S.C. Induction of tumor inhibitory anti-angiogenic response through immunization with interferon Gamma primed placental endothelial cells: ValloVax^{TM}. *J. Transl. Med.* **2015**, *13*, 1–9. [CrossRef]
108. Gavilondo, J.V.; Hernández-Bernal, F.; Ayala-Ávila, M.; de la Torre, A.V.; de la Torre, J.; Morera-Díaz, Y.; Bequet-Romero, M.; Sánchez, J.; Valenzuela, C.M.; Martín, Y.; et al. Specific active immunotherapy with a VEGF vaccine in patients with advanced solid tumors. Results of the CENTAURO antigen dose escalation phase I clinical trial. *Vaccine* **2014**, *32*, 2241–2250. [CrossRef]
109. Tian, L.; Goldstein, A.; Wang, H.; Ching Lo, H.; Sun Kim, I.; Welte, T.; Sheng, K.; Dobrolecki, L.E.; Zhang, X.; Putluri, N.; et al. Mutual Regulation of Tumour Vessel Normalization and Immunostimulatory Reprogramming. *Nature* **2017**, *544*, 250–254. [CrossRef]
110. Yuan, F.; Chen, Y.; Dellian, M.; Safabakhsh, N.; Ferrara, N.; Jain, R.K. Time-dependent vascular regression and permeability changes in established human tumor xenografts induced by an anti-vascular endothelial growth factor/vascular permeability factor antibody. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 14765–14770. [CrossRef]
111. Arjaans, M.; Schröder, C.P.; Oosting, S.F.; Dafni, U.; Kleibeuker, J.E.; De Vries, E.G.E. VEGF pathway targeting agents, vessel normalization and tumor drug uptake: From bench to bedside. *Oncotarget* **2016**, *7*, 21247–21258. [CrossRef]
112. Ribatti, D.; Annese, T.; Ruggieri, S.; Tamma, R.; Crivellato, E. Limitations of Anti-Angiogenic Treatment of Tumors. *Transl. Oncol.* **2019**, *12*, 981–986. [CrossRef]

113. Huang, Y.; Yuan, J.; Righi, E.; Kamoun, W.S.; Ancukiewicz, M.; Nezivar, J.; Santosuosso, M.; Martin, J.D.; Martin, M.R.; Vianello, F.; et al. Vascular normalizing doses of antiangiogenic treatment reprogram the immunosuppressive tumor microenvironment and enhance immunotherapy. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 17561–17566. [CrossRef]
114. Serini, G.; Bussolino, F.; Maione, F.; Giraudo, E. Class 3 semaphorins: Physiological vascular normalizing agents for anti-cancer therapy. *J. Intern. Med.* **2013**, *273*, 138–155. [CrossRef]
115. Maione, F.; Molla, F.; Meda, C.; Latini, R.; Zentilin, L.; Giacca, M.; Seano, G.; Serini, G.; Bussolino, F.; Giraudo, E. Semaphorin 3A is an endogenous angiogenesis inhibitor that blocks tumor growth and normalizes tumor vasculature in transgenic mouse models. *J. Clin Invest.* **2009**, *119*, 3356–3372. [CrossRef]
116. Shigeta, K.; Datta, M.; Hato, T.; Kitahara, S.; Chen, I.X.; Matsui, A.; Kikuchi, H.; Mamessier, E.; Aoki, S.; Ramjiawan, R.R.; et al. Dual PD –1 and VEGFR —2 blockade promotes vascular normalization and enhances anti–tumor immune responses in HCC. *Hepatology* **2019**, 0–2. [CrossRef]
117. Huang, Y.; Goel, S.; Duda, D.G.; Fukumura, D.; Jain, R.K. Vascular normalization as an emerging strategy to enhance cancer immunotherapy. *Cancer Res.* **2013**, *73*, 2943–2948. [CrossRef]
118. Schmittnaegel, M.; Rigamonti, N.; Kadioglu, E.; Cassará, A.; Rmili, C.W.; Kiialainen, A.; Kienast, Y.; Mueller, H.J.; Ooi, C.H.; Laoui, D.; et al. Dual angiopoietin-2 and VEGFA inhibition elicits antitumor immunity that is enhanced by PD-1 checkpoint blockade. *Sci. Transl. Med.* **2017**, *9*, 9670. [CrossRef]
119. Allen, E.; Jabouille, A.; Rivera, L.B.; Lodewijckx, I.; Missiaen, R.; Steri, V.; Feyen, K.; Tawney, J.; Hanahan, D.; Michael, I.P.; et al. Combined antiangiogenic and anti-PD-L1 therapy stimulates tumor immunity through HEV formation. *Sci. Transl. Med.* **2017**, *12*, 385–413. [CrossRef]
120. Fan, Y. Vascular Detransformation for Cancer Therapy. *Trends in Cancer* **2019**, *5*, 1–4. [CrossRef]
121. Platel, V.; Faure, S.; Corre, I.; Clere, N. Endothelial-to-Mesenchymal Transition (EndoMT): Roles in Tumorigenesis, Metastatic Extravasation and Therapy Resistance. *J. Oncol.* **2019**, *2019*, 1–13. [CrossRef]

ARTICLE: “Extracellular vesicles from human liver stem cells inhibit tumor angiogenesis”

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Extracellular vesicles from human liver stem cells inhibit tumor angiogenesis

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Human liver stem-like cells (HLSC) and derived extracellular vesicles (EVs) were previously shown to exhibit anti-tumor activity. In our study, we investigated whether HLSC-derived EVs (HLSC-EVs) were able to inhibit tumor angiogenesis *in vitro* and *in vivo*, in comparison with EVs derived from mesenchymal stem cells (MSC-EVs). The results obtained indicated that HLSC-EVs, but not MSC-EVs, inhibited the angiogenic properties of tumor-derived endothelial cells (TEC) both *in vitro* and *in vivo* in a model of subcutaneous implantation in Matrigel. Treatment of TEC with HLSC-EVs led to the down-regulation of pro-angiogenic genes. Since HLSC-EVs carry a specific set of microRNAs (miRNAs) that could target these genes, we investigated their potential role by transfecting TEC with HLSC-EV specific miRNAs. We observed that four miRNAs, namely miR-15a, miR-181b, miR-320c and miR-874, significantly inhibited the angiogenic properties of TEC *in vitro*, and decreased the expression of some predicted target genes (ITGB3, FGF1, EPHB4 and PLAU). In parallel, TEC treated with HLSC-EVs significantly enhanced expression of miR-15a, miR-181b, miR-320c and miR-874 associated with the down-regulation of FGF1 and PLAU. In summary, HLSC-EVs possess an anti-tumorigenic effect, based on their ability to inhibit tumor angiogenesis.

Introduction

Tumor vascularization is a fundamental step in tumor growth and metastasis. Solid tumors are in fact unable to grow more than a few millimeters per square in the absence of a vascular supply of oxygen and nutrients. Moreover, the number of metastases was reported to correlate with the vessel density of the primary tumor.[1] Tumor endothelial cells (TEC) are distinct from normal endothelial cells and display a pro-angiogenic phenotype.[2] For instance, TEC demonstrate a higher *in vitro* motility and proliferation independent of serum and enhanced survival through Akt signaling.[3] From a phenotypic point of view, TEC have an altered expression of growth factors and their receptors, including VEGF and EGF receptors,[4] integrins[5,6] and extracellular matrix proteins.[7] TEC are also resistant to chemotherapeutic drugs and less sensitive to anti-angiogenic drugs targeting VEGF.[7] Furthermore, TEC genetically differ from normal endothelial cells.[8–10]

Extracellular vesicles (EVs) are an important mechanism for cell-to-cell communication, and their active cargo may reprogram recipient cells, modifying their function and phenotype. In fact, the activity of EVs seems to rely on the transfer of a number of different factors, including proteins, RNA, DNA and lipids.[11–13] Stem cell-derived EVs and in particular human bone marrow-derived mesenchymal stromal cells (MSC) have been shown to display both pro-tumorigenic and anti-tumorigenic activities, depending on tumor type and stage of development. In analogy, MSC-EVs may also modulate tumor vascularization in a positive or negative manner. For instance, MSC-EVs were reported to be pro-angiogenic after *in vivo* administration into tumor-bearing mice.[14] Other studies[15,16] detected an indirect inhibitory effect of MSC-EVs on VEGF secretion by tumor cells. The mechanisms of this inhibition have been suggested to be due to the VEGF-targeting effect of miR-16[15] and the down-regulation of PDGF/PDGFR axis.[16]

Key words: renal tumor endothelial cells, human liver stem cells, extracellular vesicles, microRNA, tumor angiogenesis, exosomes

Abbreviations: EPHB4: ephrin receptor β4; ITGB3: integrin β3; EGF: epidermal growth factor; EVs: extracellular vesicles; FBS: fetal bovine serum; FGF1: fibroblast growth factor 1; HLSC: human liver stem-like cells; HMEC: human microvascular endothelial cells; miRNA: microRNA; MSC: mesenchymal stem cells; PDGF: platelet-derived growth factor; PLAU: plasminogen activator, urokinase; TEC: tumor endothelial cells; VEGF: vascular endothelial growth factor

Additional Supporting Information may be found in the online version of this article.

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What's new?

Tumor vascularization is a fundamental step in tumor growth and metastasis. In this study, extracellular vesicles (EVs) released from human liver stem-like cells (HLSC) inhibited migration of tumor endothelial cells and significantly reduced vessel-like formation in vitro. Experiments performed in vivo in a SCID mouse model of tumor angiogenesis also showed that HLSC-EVs were able to inhibit vessel formation and growth. This effect appeared to be specific to HLSC-EVs, as bone marrow-derived mesenchymal stem cells EVs displayed no effect. In addition, this anti-angiogenic feature of HLSC-EVs was mediated by transfer of specific anti-angiogenic miRNAs and down-regulation of predicted target genes.

Recently, we showed that the human liver stem-like cells (HLSC), another source of human resident mesenchymal stromal cells isolated from the liver,[17] may display anti-tumor effects. In particular, HLSC-EVs decreased the growth and survival of a number of different tumors, such as hepatocellular carcinoma, lymphoblastoma and glioblastoma.[18]

In our study, we aimed to investigate the effect of MSC-EVs and HLSC-EVs on the angiogenic properties of tumor-derived endothelial cells. We found that HLSC-EVs but not MSC-EVs were able to inhibit tumor angiogenesis *in vitro* and *in vivo* by transfer of specific anti-angiogenic miRNAs and down-regulation of FGF1 and PLAU.

Materials and Methods

Cell cultures

TEC have been previously isolated and cultured in our laboratory from surgical specimens of patients with renal carcinomas.[3] TEC were isolated from digested tissue using anti-CD105 positive selection by magnetic cell sorting (MACS system, Miltenyi Biotech) and grown in EndoGro complete medium (Millipore), as described previously.[3]

HLSC were isolated in our laboratory from human cryopreserved normal hepatocytes obtained from Lonza as described previously.[17] Briefly, cells were plated in hepatocyte serum-free medium (Gibco Hepatozyme-SFM; Invitrogen) at a density of 1.0–1.5 × 10^5 viable cells per cm^2 on collagen-coated culture plates for 2 weeks. After 2 weeks of culture, the medium was substituted to α -minimum essential medium/endothelial cell basal medium-1 (α-MEM/EBM) (3:1) (Gibco/Euroclone) supplemented with L-glutamine (5 mM), Hepes (12 mM, pH 7.4), penicillin (50 IU/mL), streptomycin (50 μg/mL), (all from Sigma) and 10% FBS (Lonza). At this moment, individually attached cells were cloned after 3 weeks and expanded. HLSC were positive for CD73, CD90, CD29 and CD44 and negative for CD45, CD34, CD117 (c-kit) and CD133.[17]

MSC were purchased from Lonza and cultured in MSCBM complete medium (Lonza). HMEC were purchased from ATCC and cultured in EBM complete medium (Lonza).

EV isolation and characterization

Isolation of EVs was performed as described previously[19] with minor modifications. Briefly, confluent HLSC or MSC were cultured in serum-free RPMI for 18 h. Post culture, the medium was centrifuged for 30 min at 3,000g to remove cell debris and apoptotic bodies. After which, the supernatant was ultracentrifuged for 2 h at 100,000g, 4 °C using the Beckman Coulter Optima L-100 K Ultracentrifuge with the rotor type 45 Ti 45,000rpm. The pellet of EVs obtained was resuspended in RPMI supplemented with 10% DMSO. Suspension of HLSC-EVs was then stored at −80 °C until further use. EVs were analyzed using NTA analysis using the NanoSight NS300 system (Malvern Instruments, Ltd) and electron microscopy. Mean size of EVs evaluated by electron microscopy was 90 nm (±20) (Supporting Information Fig. 1).

Viability and migration tests

For proliferation, TEC were seeded in a 96 well plate at the density of 2 × 10^3/well. The next day, cells were treated with HLSC-EVs or MSC-EVs at the concentrations of 1 × 10^3, 5 × 10^3, 10 × 10^3, or 20 × 10^3 EVs per TEC in EndoGro complete medium (Lonza). Proliferation was measured by BrdU incorporation at 24, 48 and 72 h post EVs stimulation using Cell Proliferation ELISA BrdU (colorimetric) kit (Roche, 11647229001) according to the manufacturer's instructions.

Measurement of apoptotic cells was performed on TEC stimulated with HLSC-EVs or MSC-EVs as described above using Muse® Annexin V Dead cell Kit (Millipore, MCH100105) according to manufacturer's instructions.

For the migration test, TEC were seeded in a 24-well plate and grown to confluence. EVs were then added in the concentrations of 1 × 10^{15}, or 5 × 10^{15} or 10 × 10^{15} EVs/well just after the scratch was done. Images were captured using a light microscope with the magnification of 10× at the time points of 0, 3, 7 and 24 h after the scratch. The distance was measured by LAS software (Leica) and, the results were expressed as mean ±SEM of three independent experiments.

Vessel-like structure formation *in vitro*

TEC were seeded onto Matrigel-coated 24-well plates at the density of 25 × 10^3 cells per well and cultured in EndoGro complete medium in the presence of 1 × 10^3, 5 × 10^3, 10 × 10^3 or 20 × 10^3 EVs per TEC. TEC without EVs served as a control. After incubating for 24 h, phase-contrast images (magnification 10×) were recorded and the total length of the network structures was measured using LAS software (Leica). The total length per field was calculated in five random fields and expressed as a ratio respective to the control. Data were expressed as mean ±SEM.

In vivo angiogenesis model

Animal studies were conducted in accordance with the national guidelines and regulations and were approved by the Ethics Committee of the University of Torino (Protocol Number: 338/2016-PR). A model of *in vivo* tumor angiogenesis obtained by injection of TEC incorporated within Matrigel was used to assess the effect of stem cell-derived EVs, as described previously.[3] For this purpose SCID mice (6–8 weeks old) (Charles River Laboratories) were subcutaneously injected with 1×10^6 TEC incorporated within Matrigel, pretreated or not with HLSC-EVs/MSC-EVs (10×10^3 EV per cell): ($n = 8$ each group). After 7 days, Matrigel plugs were excised and vessel density was analyzed by Masson's trichromic reaction staining. To evaluate the influence of EVs on established tumor vessels, Matrigel incorporated with 1×10^6 TEC was subcutaneously injected in SCID mice. HLSC-EVs or MSC-EVs (10×10^3 EVs per cell or 1×10^{10} EVs per plug) were injected twice into Matrigel plugs on day 3 and 7 post injection. Control mice were injected with the vehicle (PBS). At day 10 of the experiment, mice were sacrificed and Matrigel plugs excised for histochemical analysis ($n = 8$ for each group).

Gene expression study and Real-time PCR

miRNA expression levels in HLSC-EVs or MSC-EVs were evaluated using the Applied Biosystems TaqMan® Array Human MicroRNA A/B Cards (Applied Biosystems, Foster City, CA) to profile 754 mature miRNAs by qRT-PCR. The kit used miRNA-specific stem-loop reverse transcription primers and TaqMan probes to detect mature miRNA transcripts in a 2-step real-time reverse-transcription PCR assay. Briefly, single-stranded cDNA was generated from total RNA sample (80 ng) by reverse transcription using a mixture of looped primers (Multiplex RT kit, Applied Biosystems) following the manufacturer's protocol. The RT reactions were then diluted and mixed with a Taqman universal master Mix (Applied) in a ratio of 1:1, and loaded in the TaqMan microfluid card to analyze via qRT-PCR. All reactions were performed using an Applied Biosystems 7900HT real-time PCR instrument equipped with a 384 well reaction plate. Raw Ct values were calculated using the SDS software version 2.3 using automatic baseline and threshold. We analyzed the expression of miRNAs in 3 replicate samples of HLSC-EVs. All miRNAs that were amplified after 35 cycles of PCR were classified as unexpressed. Furthermore, only miRNAs that were detected or undetected in more than two replicate samples were taken into consideration.

qRT-PCR was used to confirm miRNAs or target gene expression in TEC. Briefly, 200 ng of input RNA from all samples were reverse transcribed with the miScript Reverse Transcription Kit and the cDNA was then used to detect and quantify miRNAs or genes of interest by qRT-PCR using the miScript SYBR Green PCR Kit (all from Qiagen). All samples were run in triplicate using 3 ng of cDNA for each reaction as described by the manufacturer's protocol (Qiagen). Relative expression data were then normalized using the mean expression value, calculated on the overall miRNA expression in each array, according to a Ct detection cut-off of 35 PCR cycles as described by Mestdagh *et al.*[20]

PCR analysis of the expression of pro-angiogenic genes in TEC, treated or not with HLSC-EVs, was done using Human Angiogenesis PCR Array (RT^2 Profiler PCR array, 96/well Format, Qiagen) in triplicate according to the manufacturers' instructions. Data were analyzed using SaBioscience (Qiagen) online software and expressed as Relative Quantification ±CI (Confidence interval). To compare the list of genes downregulated in TEC after HLSC-EVs stimulation (miRNAs carried by these EVs) the online software FunRich (http://funrich.org) and MirWalk (http://mirwalk.umm.uni-heidelberg.de) were used.

Cell transfection

Transfection of TEC was performed using HiPerfect reagent (Qiagen). To identify the optimal concentration for transfection, TEC were transfected with a scramble control RNA marked with FITC. FACS analysis performed the day after transfection revealed that more than 60% of TEC were transfected with no effect on their viability and proliferation.

Transfection of TEC was performed using the following mimic miRNAs: miR-15a, miR-20b, miR-23a, miR-93, miR-181b, miR-320c, miR-424 and miR-874 (all from Qiagen, Supporting Information table 1) in concentration 20 μM. The day after transfection fresh growth medium was replaced and at day two the cells were used for *in vitro* experiments (proliferation, apoptosis tests, angiogenesis in vitro assay) or gene expression analysis (Real-time PCR, Western blot, FACS analysis).

To verify miRNA transfer, HLSC were transfected with the fluorescence labeled mimic miR-320c-FAM (Ambion, Supporting Information table 1) using HiPerfect reagent. HLSC transfected with scramble mimic were used as control. After 18 h the medium was changed on FBS-free EndoGro medium for EV collection. Transfected HLSC and their EVs were analyzed by FACS. Then, EVs from transfected and control HLSC were added to TEC. After 24 h incubation, TEC were analyzed by FACS.

To demonstrate the role of the selected miRNAs in the inhibition of tumoral angiogenesis, we transfected TEC with antagomirs for miR-15a, miR-181b, miR-320c and miR-874 (in concentration 50 μM) using HiPerfect reagent (Qiagen, Supporting Information table 1). After 24 h, cells were stimulated or not with HLSC-EV. The day after, transfected cells were used for vessel-like structure formation *in vitro*.

Fluorescence-activated cell sorting (FACS) analysis

FACS analysis of HLSC-EVs and MSC-EVs was performed using CytoFLEX Flow Cytometer (Beckman Coulter) (Supporting Information Fig. 1). The following antibodies were used: FITC-conjugated antibodies against CD63 (Abnova), CD105 (Dako

Cytomation), CD90 (BD Pharmigen), CD44 (Miltenyi Biotech), CD45 (BD Pharmigen), ICAM and VCAM (Serotec), CD31 (BioLegend), integrin subunit α4, α5, α6 (from BD Pharmigen); PE- conjugated antibodies against CD73 (BD Pharmigen), integrin subunit α4, α5 (all from BD Pharmigen) and VE-cadherin (BioLegend). FITC or PE mouse nonimmune isotypic IgG (Dako Cytomation) were used as a control.

Western blot

Protein samples were separated by 4% to 15% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS PAGE) and subjected to immunoblotting with antibodies to PLAU (Abcam, ab131433) or FGF1 (Abcam, ab9588), actin (Santa Cruz, sc-1616) and vinculin (Santa Cruz, sc-7648). The protein bands were visualized with an enhanced chemiluminescence detection kit and ChemiDoc™ XRS+ System (BioRad). Cell lysates (20 μg protein) were loaded per well.

Inhibition of TEC transcription with amanitin

TEC were seeded in 6-well plate (200×10^3/well). After 12 h incubation, cells were incubated with the transcriptional inhibitor α-amanitin (50 μg/mL) either in the absence or in the presence of HLSC-EVs (10×10^3/cell, equal to 2×10^9/well). Twenty-four hours after treatment, TEC were collected for RNA extraction and real-time PCR analysis. Expression of actin, RNU6b and 18S RNA were used for normalization.

Statistics

Data were assessed for normality of distribution using the Kolmogorov–Smirnov test. Statistical analysis was performed using SigmaPlot 11.0 Software. Differences between treatment and control groups were then analyzed using Dunnett's test when the distribution was normal. Data are expressed as mean ±SEM. We considered differences to be significant when $p < 0.05$.

Results

HLSC-EVs inhibit the angiogenic potential and migration of renal TEC *in vitro*

Stimulation with HLSC-EVs significantly inhibited the angiogenic properties of human renal TEC *in vitro* in a dose-depended manner (Figs. 1*a*, 1*b*, and 1*d*). At variance, MSC-EVs did not show pro- or anti-angiogenic effects on TEC (Figs. 1*a*, 1*c*, and 1*d*). Both EVs did not change TEC viability (Supporting Information Fig. 2). We also evaluated the effect of MSC-EVs and HLSC-EVs on the motility of TEC through a wound-healing assay. Both EVs significantly inhibited the migration of TEC at the dose of 10×10^3 EVs per TEC. However, HLSC-EVs were already effective at the lower dose of 1×10^3 EVs per TEC (Fig. 1*e*) compared to MSC-EVs.

As control experiments, we evaluated the effect of MSC-EVs and HLSC-EVs on normal endothelial cells: MSC-EVs were able to enhance the angiogenic property of human microvascular endothelial cells (HMEC), in line with their reported pro-angiogenic activity,[21] whereas HLSC-EVs did not show any effect (Fig. 1*f*). The anti-angiogenic effect of HLSC-EVs was evident on TEC but not on HMEC. As previously shown TEC are significantly more pro-angiogenic than normal endothelial cells and are able to sustain angiogenesis in autocrine manner.[2] In contrast MSC-EV did not display any anti-angiogenic activity on TEC but rather stimulated the angiogenesis in HMEC. This indicates that EVs from MSC and HLSC have different action on normal and tumor angiogenesis.

HLSC-EVs prevent tumor angiogenesis *in vivo*

We subsequently evaluated the effect of MSC-EVs and HLSC-EVs *in vivo* by using a model of human tumor angiogenesis induced by TEC implanted subcutaneously in SCID mice within Matrigel.[3] In this model, TEC organize in patent structures connected with the mouse circulation within 7 days. In a pretreatment setting, TEC were incubated with HLSC-EVs or MSC-EVs for 24 h and implanted subcutaneously into SCID mice. Seven days after implantation, Matrigel plugs were excised and vessel density analyzed by trichrome staining. The analysis of control plugs showed, as expected, the presence of erythrocyte containing vessels (Fig. 2*a*). Plugs of TEC treated with HLSC-EVs for 24 h before implantation did not present vessels (Figs. 2*b* and 2*d*), whereas those treated with MSC-EVs were highly angiogenic (Figs. 2*c* and 2*d*).

To evaluate whether EVs were able to affect formed tumor vessels, HLSC-EVs or MSC-EVs were injected at day 3 and 7 in Matrigel plugs containing an established TEC network. Plugs were explanted at day 10. The treatment with HLSC-EVs significantly reduced vessel density with respect to control and to treatment with MSC-EVs (Fig. 2*e*), confirming the *in vitro* results.

Molecular effects of HLSC-EVs on TEC

Based on these results, a molecular analysis of the changes occurring in TEC after HLSC-EVs stimulation during *in vitro* vessel-like structure organization was conducted using an Angiogenesis PCR array. Briefly, TEC were treated with HLSC-EVs (10×10^3 EVs/TEC) during *in vitro* angiogenesis and subsequently harvested for the PCR array. Among the 84 genes tested, we identified 11 pro-angiogenic factors significantly down-regulated in TEC treated with HLSC-EVs (Fig. 3*a*). In particular, HLSC-EVs down-regulated pro-angiogenic surface receptors including Tie-1, beta 3 integrin (ITGB3), ephrin receptor B4 (EPHB4) and endoglin (ENG or CD105), as well as growth factors such as fibroblast growth factor 1 (FGF1), TGF family members, urokinase-type plasminogen activator (PLAU) and tissue factor (F3). Additionally, Akt1, known to be involved in the pro-angiogenic effects of TEC,[22] was also down-regulated.

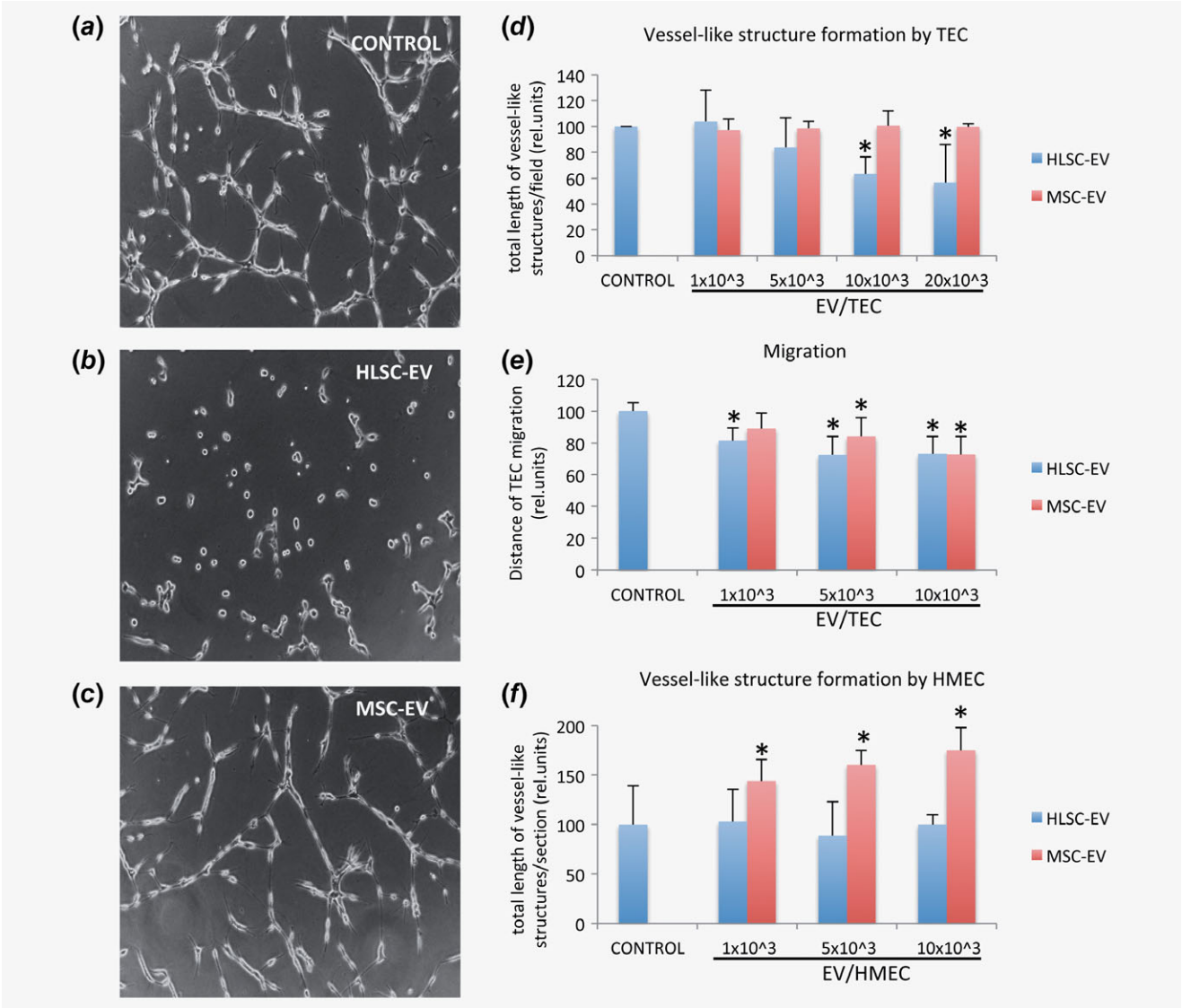

Figure 1. Effect of HLSC-EVs or MSC-EVs on the angiogenic properties of TEC *in vitro*. (*a-c*) Representative micrographs showing the formation of vessel-like structures by control TEC (*a*), by TEC treated with HLSC-EVs (*b*) or with MSC-EVs (*c*); (*d*) diagram of the total length of vessel-like structures per field, formed by control TEC or TEC treated with different doses of EVs; (*e*) diagram of the TEC migration during wound healing assay in the presence or absence of different doses of EVs; (*f*) diagram of the total length of vessel-like structures per field, formed by HMEC, treated with different doses of EVs. Data are expressed as mean ± SEM of 3 experiments performed in duplicate. Statistical analysis was performed using Dunnett's test versus control stimulated with vehicle alone. * - $p < 0.05$. [Color figure can be viewed at wileyonlinelibrary.com]

Identification of anti-angiogenic miRNAs carried by HLSC-EVs

To dissect the possible effectors of the observed gene regulation, we focused on miRNA content of HLSC-EVs. In order to accomplish this, we performed a bioinformatic analysis, followed by *in vitro* functional validation.

Using Funrich V3 software,[23] we predicted miRNAs that could target the 11 down-regulated genes. We identified 136 miRNAs and we matched them with miRNAs carried by HLSC-EVs. Among them, we identified 42 miRNAs expressed by HLSC-EVs (Fig. 3*b*). A subsequent analysis was performed to exclude those also present in MSC-EVs,[24] due to their lacking effect on TEC (Fig. 3*c*). The complete list of HLSC- and MSC-EV microRNAs can be found in the exocarta repository (http://exocarta.org, August 2018 release, number under request). Fifteen out of forty-two miRNAs targeting the modulated genes were identified to be present only in HLSC-EVs and were adopted for further functional studies (Fig. 3*d*). Among them, three described as pro-tumorigenic (has-miR-30e-5p, has-miR-301a-3p, has-miR-212-3p)[25–27] were excluded. Therefore, we took in consideration 8 miRNAs (testing one member only of miR-181, miR-320 and miR-23

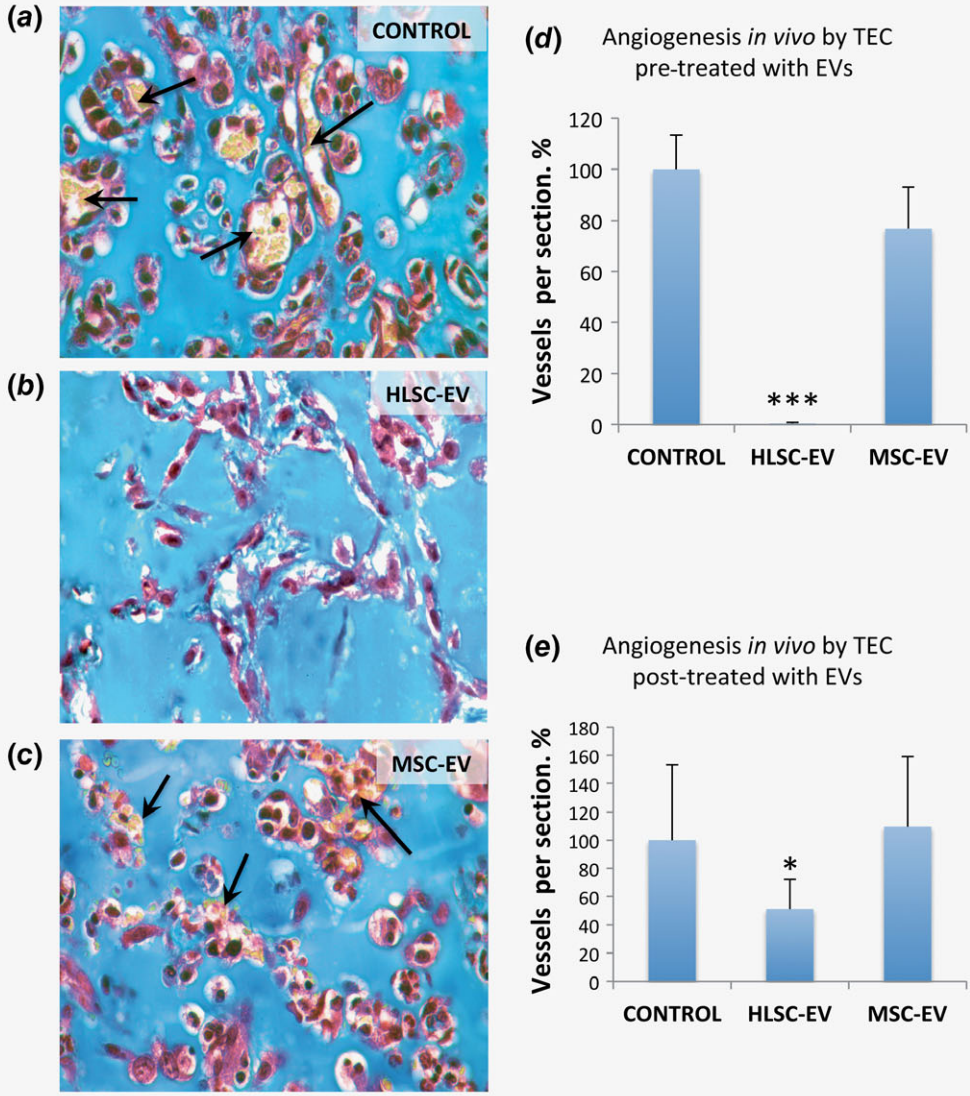

Figure 2. Tumor angiogenesis *in vivo*. Representative images of Matrigel sections, stained with Masson's trichromic reaction (extracellular matrix is stained in blue, cells in red and erythrocytes in yellow): (*a*) Matrigel plugs containing control TEC treated with vehicle alone; (*b*) Matrigel plugs containing TEC, pretreated with 10×10^3 HLSC-EVs per cell; (*c*) Matrigel plugs containing TEC, pretreated with 10×10^3 MSC-EVs per cell. (Original magnification ×20; erythrocytes containing vessels are indicated by arrows). (*d*) Diagram of vessel density in Matrigel containing control TEC or TEC pretreated with HLSC-EVs or MSC-EVs. (e) Diagram of vessel density in TEC contained Matrigel, injected or not with HLSC-EVs or MSC-EVs on day 3 and 7 after TEC injection. Data are expressed as mean ± SEM of 8 experiments performed independently. Statistical analysis was performed using Dunnett's test versus control group stimulated with vehicle alone. * - $p < 0.05$; ***- $p < 0.001$.

families): miR-15a, miR-20b, miR-23a, miR-93, miR-181b, miR-320c, miR-424 and miR-874 (Fig. 3*d*, in bold). According to Mirwalk software (http://mirwalk.umm.uni-heidelberg.de) these miRNAs could target pro-angiogenic genes down-regulated by HLSC-EVs in TEC. Of importance, miR-15a was not expressed and other selected miRNAs were expressed in low-level in control TEC (Ct > 30, not shown).

Effect of HLSC-EV miRNAs on TEC angiogenesis

To demonstrate the specificity of these selected miRNAs on the angiogenic properties of TEC, we transfected cells with the corresponding miRNA mimics. Two days after transfection, angiogenesis *in vitro* assay was performed. Four miRNAs (miR-15a, miR-181b, miR-320c and miR-874) significantly inhibited *in vitro* vessel-like structure formation (Fig. 4*a*), whereas miR-20b, miR-23a, miR-93 and miR-424 had no effect. Furthermore, all mimics had no effect on proliferation or apoptosis (Supporting Information Fig. 3).

To study which miRNA could have a predominant effect on TEC, we transfected TEC with antagomir for the selected four miRNAs (miR-15a, miR-181b, miR-320c and miR-874)

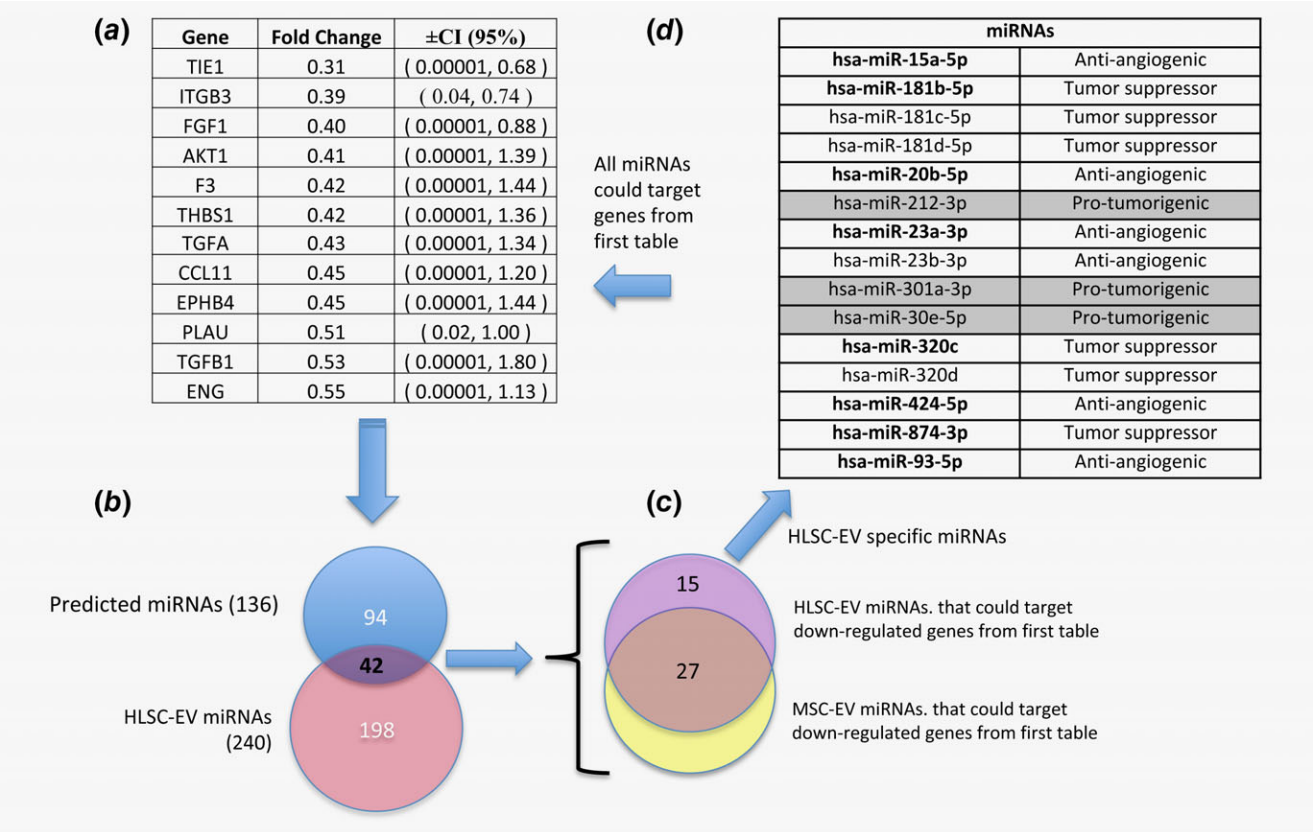

Figure 3. Selection of miRNAs, specific to HLSC-EVs, responsible for the anti-angiogenic effect on TEC. (*a*) List of the genes down-regulated in TEC after treatment with HLSC-EVs ($n = 3$ experiments; data are expressed as average Fold change ±CI); these genes could be targeted by 136 miRNAs (*b*), 42 of which are carried by HLSC-EVs. Among these 42 miRNAs, 27 are also carried by MSC-EVs and were excluded from the study, as MSC-EVs did not show anti-angiogenic effect on TEC (*c*). Fifteen miRNAs, specific for HLSC-EVs, could be relevant towards the biologic action of HLSC-EVs on TEC. From these 15 miRNAs, 8 were selected for further studies (in bold: one only for the families miR-181, miR-23, miR-320) (*d*). Three miRNAs were described as pro-tumorigenic and were excluded (in gray). [Color figure can be viewed at wileyonlinelibrary.com]

and stimulated them with HLSC-EVs. This transfection completely abrogated the *in vitro* anti-angiogenic effect of HLSC-EVs according to angiogenesis *in vitro* assay (Fig. 4*b*). Since miR-181b, miR-320c and miR-874 were expressed at low level in TEC, their down-regulation had enhanced the pro-angiogenic activity of nonstimulated TEC.

We evaluated the effective transfer of these miRNAs by HLSC-EVs in TEC. After 24 h of incubation with HLSC-EVs, the TEC expression of miRNAs was significantly up-regulated (Fig. 5*a*), therefore validating ours *in silico* data. To investigate if the observed miRNA increase was due to transfer or to induction, we blocked the transcription in TEC using α-amanitin. Twenty-four hours after HLSC-EV treatment, the content of the selected miRNAs in TEC was analyzed by Real-time PCR. The expression of miR-181b, miR-320c and miR-874 was significantly enhanced in TEC treated with amanitin and HLSC-EVs, in respect to TEC treated with amanitin only (Fig. 5*b*). The increase of miR-15a did not reach statistical significance. In addition, miRNA transfer was also demonstrated by use of a fluorescent mimic. For this purpose, we transfected HLSC with miRNA-320c-FAM, and incubated TEC with the deriving fluorescent HLSC-EVs (Figs. 5*c*–5*e*). FACS analysis of TEC, treated with these EVs, showed the effective transfer miR-320c-FAM from the transfected HLSC to TEC (mean intensity of TEC, treated with fluorescent HLSC-EV 8,220.6 ±987, whereas mean intensity of control TEC, treated with control EVs, 5,422.4 ±1,235). Altogether, these experiments suggest that HLSC-EVs transfer miRNAs to recipient cells.

Furthermore, we investigated the expression of the predicted pro-angiogenic target proteins after TEC transfection with the four selected miRNA mimics. We detected significant down-regulation of EPHB4, ITGB3, FGF1 and PLAU at mRNA level after transfection (Figs. 6*a*–6*d*). Then we evaluated their protein levels after TEC transfection or HLSC-EV stimulation. We confirmed the down-regulation of FGF1 in TEC transfected with miR-15a or stimulated with HLSC-EVs, as well as down-regulation of PLAU in TEC transfected with miR-181b or stimulated with HLSC-EV (Figs. 6*e* and 6*f*).

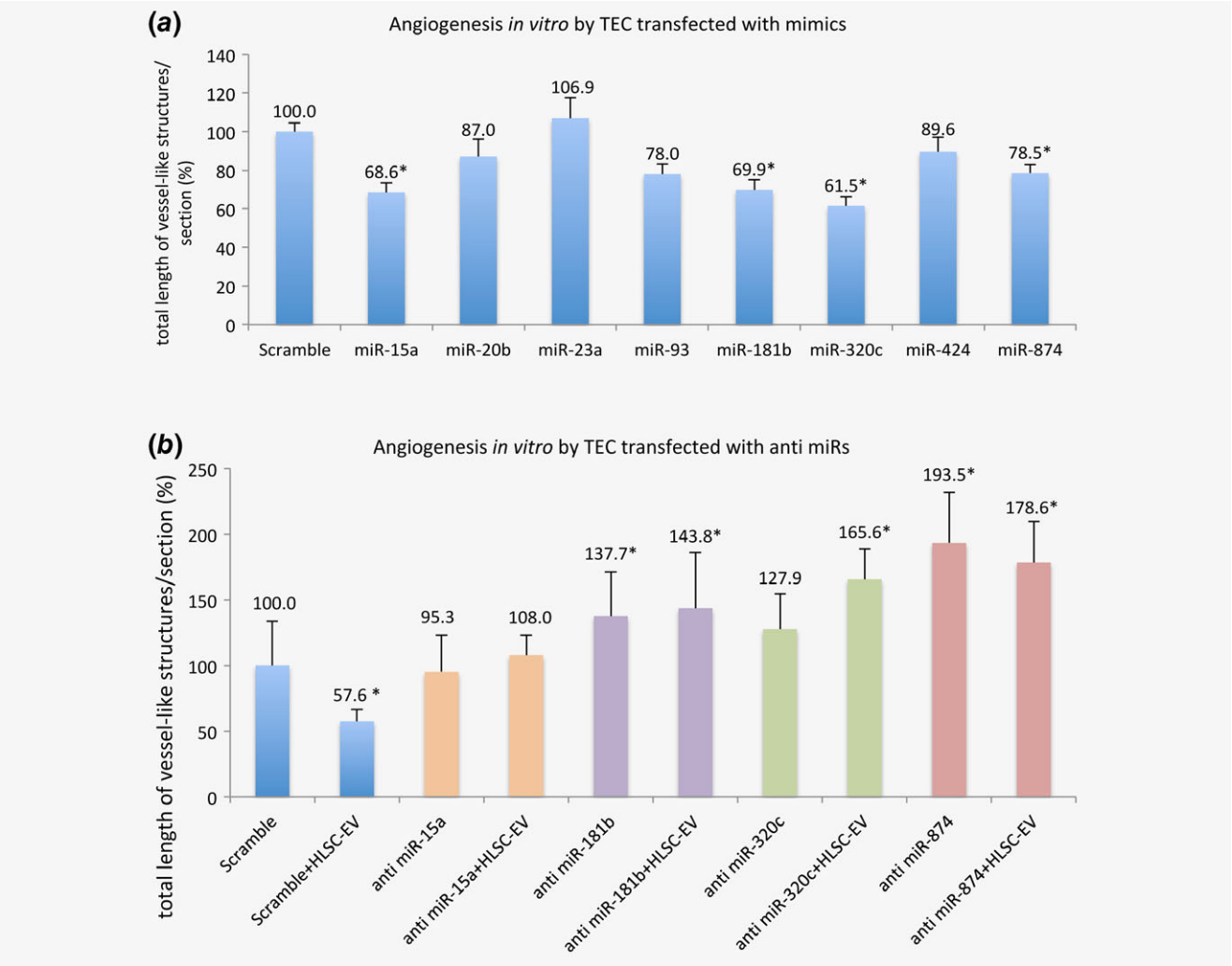

Figure 4. Influence of selected miRNAs on pro-angiogenic properties of HLSC-EVs. The diagrams show: (*a*) *in vitro* vessel-like structure formation by TEC transfected with selected mimic miRNAs or scramble miRNAs; (*b*) *in vitro* vessel-like structure formation by TEC transfected with antagomirs for selected miRNA and stimulated with HLSC-EVs. In different colors are shown TEC transfected with different molecules. Data are normalized to Scramble (100%) and expressed as mean ± SEM of 3 experiments performed independently. Statistical analysis was performed using Dunnett's test versus control transfected with scramble RNA. * - $p < 0.05$ versus scramble. [Color figure can be viewed at wileyonlinelibrary.com]

Discussion

In our study, we found that EVs from a stromal stem cell population obtained from the human liver inhibited migration of tumor endothelial cells and significantly reduced vessel-like formation *in vitro*. Experiments performed *in vivo* in a model of tumor angiogenesis in SCID mice also showed that HLSC-EVs were able to inhibit vessel formation and growth. This effect appeared to be specific to HLSC-EVs, as bone marrow-derived MSC-EVs did not display any effect. In addition, this anti-angiogenic feature of HLSC-EVs was dependent on the presence of a specific miRNA subset.

Tumor angiogenesis has different characteristics and mechanisms in respect to normal angiogenesis. TEC derived from renal carcinoma were shown to be able to form *in vivo* a human vascular network connected with mouse vasculature once implanted within Matrigel in mice.[2,3] These cells maintain a pro-angiogenic program in an autocrine manner.[2,3]. Several studies have previously shown that endothelial cells derived from different tumors are different from the normal endothelium as they express a distinct and unique molecular and functional phenotype.[2] EVs released from stem cells were shown to be able to reprogram target cells by inducing epigenetic changes.[28,29] This observation prompted us to investigate whether EVs derived from MSC and HLSC were able to modify the pro-angiogenic phenotype of TEC.

Previous studies of the effect of MSC and MSC-derived EVs provided conflicting results on *in vivo* tumor growth.[14,15,30] These contradictory results probably depend on cell growth conditions and on timing of administration.[31] MSC-EVs are described as strictly pro-angiogenic for healthy

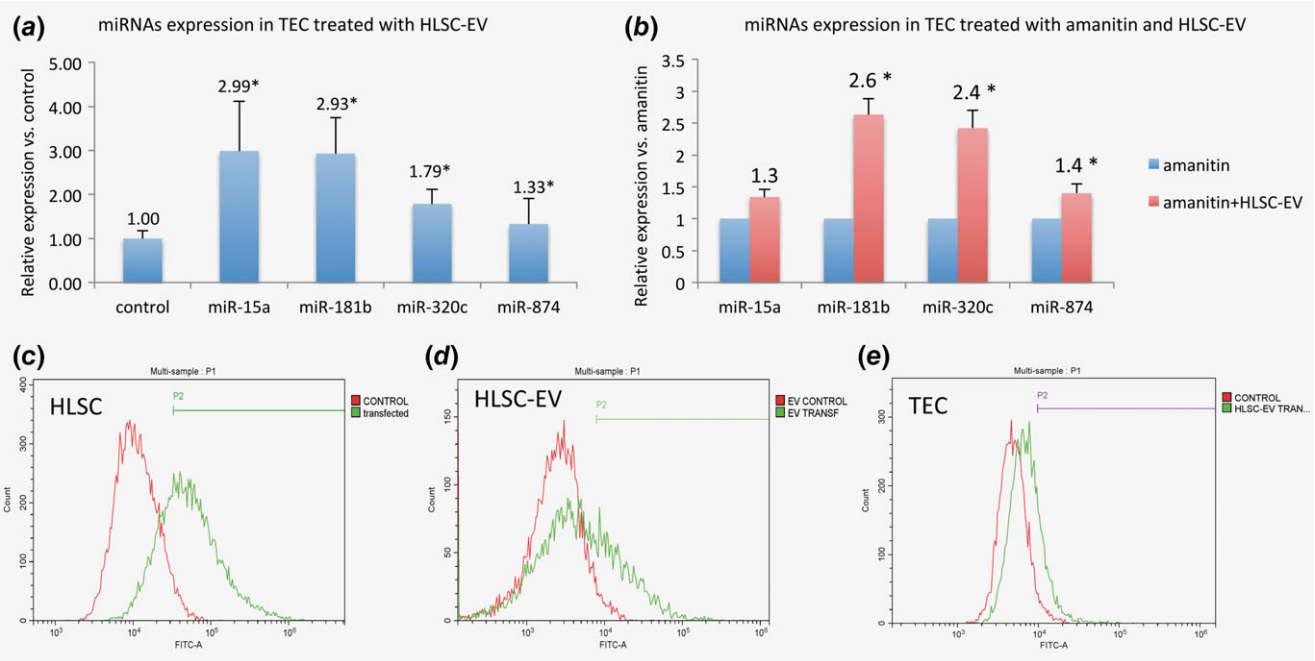

Figure 5. Transfer of miRNAs by HLSC-EVs to TEC. (*a*) enhanced expression of the selected miRNAs in TEC after 24 h stimulation with HLSC-EVs; data are expressed as mean ± SEM of 3 experiments performed independently. * - $p < 0.05$ versus control. (*b*) enhanced expression of the selected miRNAs in TEC, treated for 24 h with HLSC-EVs in the presence or absence of α-amanitin; data are expressed as mean ± SEM of 3 experiments performed independently. * - $p < 0.05$ versus α-amanitin. (*c*) representative image of FACS analysis of HLSC transfected with miR-320c-FAM; (*d*) representative image of FACS analysis of HLSC-EVs derived from cells transfected with miR-320c-FAM; (*e*) representative image of FACS analysis of TEC incubated with miR-320c-FAM carrying HLSC-EVs showing increase in TEC fluorescence after HLSC-EV incubation. [Color figure can be viewed at wileyonlinelibrary.com]

endothelial cells.[32,33] Lindoso *et al.* showed that EVs derived from MSC primed by tumor cells acquired a pro-angiogenic and pro-tumorigenic activity.[34] In our study, we found that MSC-EVs were able to inhibit migration but not influence proliferation and angiogenesis of TEC. Pretreatment of TEC with MSC-EVs was unable to modify the formation of an *in vivo* vascular network. In contrast to MSC-EVs, HLSC-EVs were found to possess an intrinsic anti-angiogenic activity. Previous studies have shown that HLSC-EVs inhibited tumor growth both *in vitro* and *in vivo* and the mechanism was related to the delivery of anti-tumor miRNAs that were able to down-regulate oncogenic targets.[18] Herein, we have found that HLSC-EVs were able to almost completely abrogate tumor angiogenesis *in vitro* and *in vivo* without affecting normal endothelial cells. The specificity of this effect on tumor angiogenesis could be possibly related to reduced angiogenic properties of HMEC and therefore to the expression of a diverse gene profile differently modified by HLSC-EV treatment.

EVs are complex structures composed of specific functional proteins, lipids and nucleic acids. The biological activity of EVs depends on the coordinated action of all these components. However, a number of reports have indicated the relevant role of EV mediated miRNA transfer in inducing epigenetic changes in target cells. In our study, we identified four miRNAs carried by HLSC-EVs, but not by MSC-EVs, with an anti-angiogenic function. These miRNAs, miR-15a, mir-181b, miR-320c and miR-874, were able to inhibit tumor angiogenesis when transfected in TEC mainly by down-regulating the expression of their target genes (FGF1, PLAU, ITGB3 and EPHB4). In parallel, when TEC were stimulated with HLSC-EVs, a significantly enhanced expression of these miRNAs was observed. EVs represent a heterogeneous population, composed of vesicles that differ for biogenesis, size, molecular composition and possibly function. For instance, it was recently shown that the regenerative properties of MSC-EVs were restricted to the exosomal-enriched population.[35,36] The exact function of HLSC-EV subpopulations would be of interest, since potential subsets, including exosomes, may carry the specific microRNAs.

The effective down-regulation of the predicted target genes was observed only for FGF1 and PLAU. These miRNAs were previously described to have different function in tumors. MiR-15a is a well-known tumor suppressor. This miRNA inhibits cell proliferation, promotes apoptosis of cancer cells and suppresses tumor growth by targeting multiple oncogenes, including BCL2, MCL1, CCND1 and WNT3A.[37] MiR-181b could play a contradictory role in tumor development depending on the type of tumor and cell being studied.[38] Mir-874 is described not only as a tumor

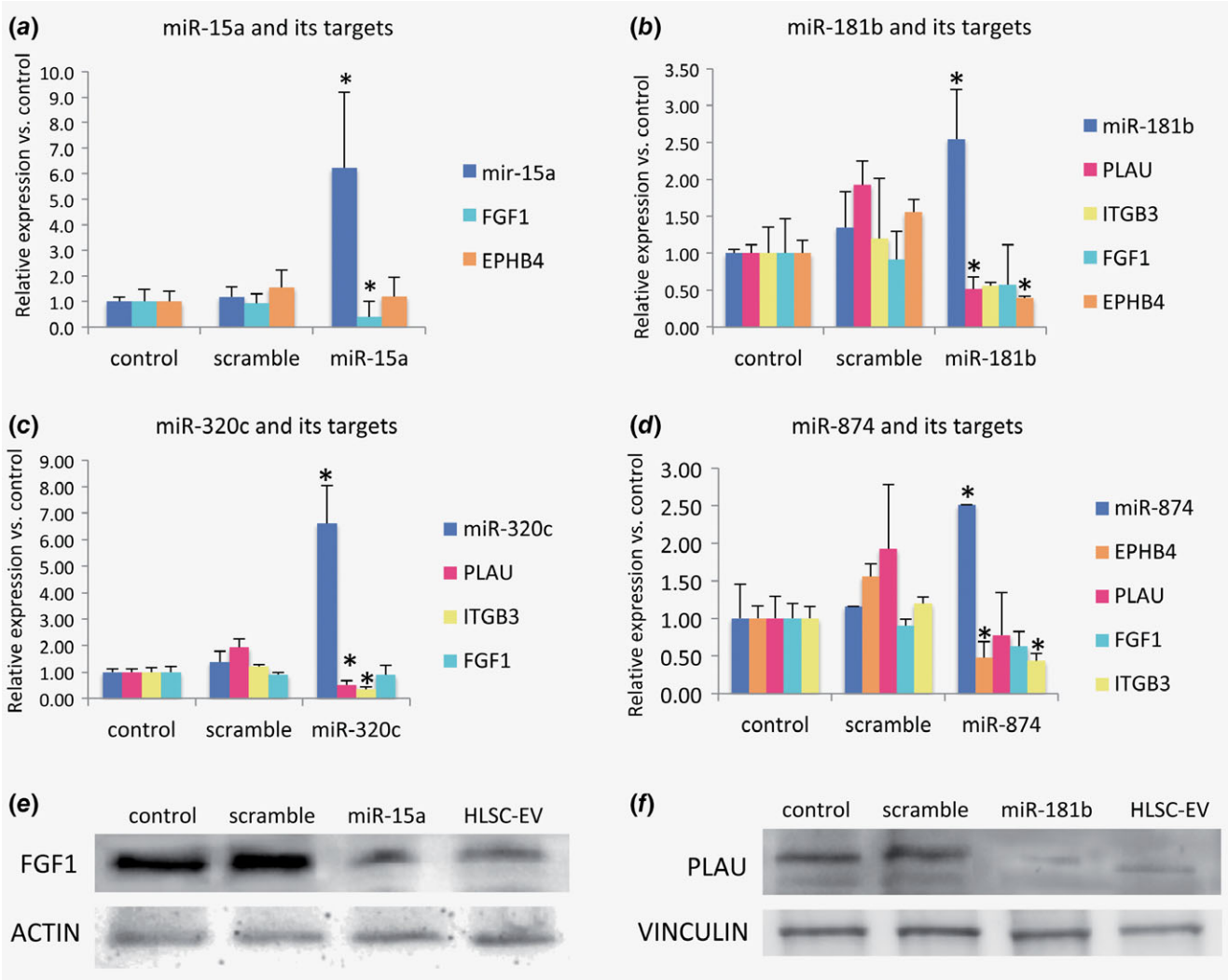

Figure 6. Modulation of miRNA targets in TEC transfected with the selected mimic miRNAs. (*a*) TEC expression of miR-15a and its target genes FGF1, EPHB4; (*b*) expression of miR-181b and its target genes PLAU, ITGB3, FGF1, EPHB4; (*c*) expression of miR-320c and its target genes PLAU, ITGB4, FGF1; (*d*) expression of miR-874 and its target genes EPHB4, PLAU, ITGB3, FGF1; Data are expressed as mean RQ ± SEM of 5 experiments performed. Statistical analysis was performed using Dunnett's test versus control transfected with scramble RNA. * - $p < 0.05$. (*e*) Representative image of Western blot analysis of the FGF1 expression in control TEC, TEC transfected with scrambled sequence or miR-15a or stimulated with HLSC-EVs. (*f*) Representative image of Western blots showing the expression of PLAU in control TEC, TEC transfected with scrambled sequence or miR-181b or stimulated with HLSC-EVs.

suppressor[39] but also as an inhibitor of tumor angiogenesis through STAT3/VEGF-A pathway.[40] miR-320c, on the other hand, has been shown to be down-regulated in many types of cancer, such as myeloma,[41] colorectal cancer[42] and bladder cancer.[43] Predicted targets that we have found by FunRich online software for every miRNA have not been previously described or confirmed, except for EPHB4 as a target for miR-181.[44] After the transfection of TEC with the selected miRNAs or stimulation with HLSC-EVs, two predicted pro-angiogenic genes were significantly down-regulated at mRNA and protein level. FGF1 is one of the most important pro-angiogenic factor involved in tumor angiogenesis.[45] FGF1 is able to regulate angiogenesis independently from VEGF[46] and an enhanced expression of this factor has been reported in different types of tumors.[47,48] PLAU is a gene that codes for urokinase-type plasminogen activator (uPA), an enzyme that activates plasmin from plasminogen. Plasmin participates in the proteolytic processes of extracellular matrix degradation which is important for angiogenesis and cancer progression.[49] FGF1 and PLAU are connected through the receptors of FGF1 (FGFRs) that could activate uPA and enhance the expression of its receptor uPAR. Furthermore, FGF1, uPA and uPAR are all linked through FGFRs creating a positive feedback loop. In fact, cells overexpressing FGFRs were shown to be more invasive and tumorigenic.[50]

In conclusion, we have shown that HLSC-EVs specifically and significantly inhibit tumor angiogenesis *in vitro* and *in vivo*. Furthermore, based on the bioinformatic analysis and the characterization of anti-angiogenic miRNAs carried by HLSC-EVs, we postulate that EV mediated transfer of miRNAs may be involved in the inhibition of tumor angiogenesis through down-regulation of a number of genes including FGF1 and PLAU.

Acknowledgments

B.B. T.L., B.B. and G.C. are named as inventors in a related patent application # 170095.

References

1. Liotta LA, Saidel MG, Kleinerman J. The significance of hematogenous tumor cell clumps in the metastatic process. *Cancer Research* 1976;36(3):889–94.
2. Bussolati B, Grange C, Camussi G. Tumor exploits alternative strategies to achieve vascularization. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology* 2011;25(9):2874–82.
3. Bussolati B, Deambrosis I, Russo S, et al. Altered angiogenesis and survival in human tumor-derived endothelial cells. *FASEB J* 2003;17(9):1159–61.
4. Hida K, Hida Y, Shindoh M. Understanding tumor endothelial cell abnormalities to develop ideal anti-angiogenic therapies. *Cancer Sci* 2008;99(3):459–66.
5. Goel HL, Moro L, Murphy-Ullrich JE, et al. Beta1 integrin cytoplasmic variants differentially regulate expression of the antiangiogenic extracellular matrix protein thrombospondin 1. *Cancer Res* 2009;69(13):5374–82.
6. Sayeed A, Fedele C, Trerotola M, et al. IGF-IR promotes prostate cancer growth by stabilizing alpha5beta1 integrin protein levels. *PLoS One* 2013;8(10):e76513.
7. Dutta A, Li J, Lu H, et al. Integrin alphavbeta6 promotes an osteolytic program in cancer cells by upregulating MMP2. *Cancer Res* 2014;74(5):1598–608.
8. Akino T, Hida K, Hida Y, et al. Cytogenetic abnormalities of tumor-associated endothelial cells in human malignant tumors. *Am J Pathol* 2009;175(6):2657–67.
9. Maishi N, Ohba Y, Akiyama K, et al. Tumour endothelial cells in high metastatic tumours promote metastasis via epigenetic dysregulation of biglycan. *Sci Rep* 2016;6:28039.
10. Pezzolo A, Parodi F, Corrias MV, et al. Tumor origin of endothelial cells in human neuroblastoma. *J Clin Oncol* 2007;25(4):376–83.
11. Ratajczak MZ, Ratajczak J. Extracellular microvesicles as game changers in better understanding the complexity of cellular interactions-from bench to clinical applications. *Am J Med Sci* 2017;354(5):449–52.
12. DeRita RM, Zerlanko B, Singh A, et al. c-Src, Insulin-like growth factor I receptor, G-protein-coupled receptor kinases and focal adhesion kinase are enriched into prostate cancer cell exosomes. *J Cellul Biochem* 2017;118(1):66–73.
13. Quesenberry PJ, Goldberg LR, Aliotta JM, et al. Cellular phenotype and extracellular vesicles: basic and clinical considerations. *Stem Cells Dev* 2014;23(13):1429–36.
14. Zhu W, Huang L, Li Y, et al. Exosomes derived from human bone marrow mesenchymal stem cells promote tumor growth in vivo. *Cancer Lett* 2012;315(1):28–37.
15. Lee JK, Park SR, Jung BK, et al. Exosomes derived from mesenchymal stem cells suppress angiogenesis by down-regulating VEGF expression in breast cancer cells. *PLoS One* 2013;8(12):e84256.
16. Ho IA, Toh HC, Ng WH, et al. Human bone marrow-derived mesenchymal stem cells suppress human glioma growth through inhibition of angiogenesis. *Stem Cells* 2013;31(1):146–55.
17. Herrera MB, Bruno S, Buttiglieri S, et al. Isolation and characterization of a stem cell population from adult human liver. *Stem Cells* 2006;24(12):2840–50.
18. Fonsato V, Collino F, Herrera MB, et al. Human liver stem cell-derived microvesicles inhibit hepatoma growth in SCID mice by delivering antitumor microRNAs. *Stem Cells* 2012;30(9):1985–98.
19. Herrera MB, Fonsato V, Gatti S, et al. Human liver stem cell-derived microvesicles accelerate hepatic regeneration in hepatectomized rats. *J Cellul Mol Med* 2010;14(6B):1605–18.
20. Mestdagh P, Van Vlierberghe P, De Weer A, et al. A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol* 2009;10(6):R64.
21. Bronckaers A, Hilkens P, Martens W, et al. Mesenchymal stem/stromal cells as a pharmacological and therapeutic approach to accelerate angiogenesis. *Pharmacol Therap* 2014;143(2):181–96.
22. Bussolati B, Assenzio B, Deregibus MC, et al. The proangiogenic phenotype of human tumor-derived endothelial cells depends on thrombospondin-1 downregulation via phosphatidylinositol 3-kinase/Akt pathway. *J Mol Med* 2006;84(10):852–63.
23. Pathan M, Keerthikumar S, Ang CS, et al. FunRich: An open access standalone functional enrichment and interaction network analysis tool. *Proteomics* 2015;15(15):2597–601.
24. Collino F, Bruno S, Incarnato D, et al. AKI recovery induced by mesenchymal stromal cell-derived extracellular vesicles carrying microRNAs. *J Am Soc Nephrol* 2015;26(10):2349–60.
25. Yu F, Deng H, Yao H, et al. Mir-30 reduction maintains self-renewal and inhibits apoptosis in breast tumor-initiating cells. *Oncogene* 2010;29(29):4194–204.
26. Liang B, Yin JJ, Zhan XR. MiR-301a promotes cell proliferation by directly targeting TIMP2 in multiple myeloma. *Int J Clin Exp Pathol* 2015;8(8):9168–74.
27. Ma C, Nong K, Wu B, et al. miR-212 promotes pancreatic cancer cell growth and invasion by targeting the hedgehog signaling pathway receptor patched-1. *J Exp Clin Cancer Res* 2014;33:54.
28. Ratajczak J, Wysoczynski M, Hayek F, et al. Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. *Leukemia* 2006;20(9):1487–95.
29. Deregibus MC, Cantaluppi V, Calogero R, et al. Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA. *Blood* 2007;110(7):2440–8.
30. Katakowski M, Buller B, Zheng X, et al. Exosomes from marrow stromal cells expressing miR-146b inhibit glioma growth. *Cancer Lett* 2013;335(1):201–4.
31. Hong IS, Lee HY, Kang KS. Mesenchymal stem cells and cancer: friends or enemies? *Mutat Res* 2014;768:98–106.
32. Anderson JD, Johansson HJ, Graham CS, et al. Comprehensive proteomic analysis of mesenchymal stem cell exosomes reveals modulation of angiogenesis via nuclear factor-kappaβ signaling. *Stem Cells* 2016;34(3):601–13.
33. Merino-Gonzalez C, Zuniga FA, Escudero C, et al. Mesenchymal stem cell-derived extracellular vesicles promote angiogenesis: potencial clinical application. *Front Physiol* 2016;7:24.
34. Lindoso RS, Collino F, Camussi G. Extracellular vesicles derived from renal cancer stem cells induce a pro-tumorigenic phenotype in mesenchymal stromal cells. *Oncotarget* 2015;6(10):7959–69.
35. Bruno S, Tapparo M, Collino F, et al. Renal regenerative potential of different extracellular vesicle populations derived from bone marrow mesenchymal stromal cells. *Tissue Eng Part A* 2017;23(21–22):1262–73.
36. Collino F, Pomatto M, Bruno S, et al. Exosome and microvesicle-enriched fractions isolated from mesenchymal stem cells by gradient separation showed different molecular signatures and functions on renal tubular epithelial cells. *Stem Cell Rev* 2017;13(2):226–43.
37. Aqeilan RI, Calin GA, Croce CM. miR-15a and miR-16-1 in cancer: discovery, function and future perspectives. *Cell Death Diff* 2010;17(2):215–20.
38. Liu J, Shi W, Wu C, et al. miR-181b as a key regulator of the oncogenic process and its clinical implications in cancer (Review). *Biomed Rep* 2014;2(1):7–11.
39. Jiang T, Guan LY, Ye YS, et al. MiR-874 inhibits metastasis and epithelial-mesenchymal transition in hepatocellular carcinoma by targeting SOX12. *Am J Cancer Res* 2017;7(6):1310–21.
40. Zhang X, Tang J, Zhi X, et al. miR-874 functions as a tumor suppressor by inhibiting angiogenesis through STAT3/VEGF-A pathway in gastric cancer. *Oncotarget* 2015;6(3):1605–17.
41. Alzrigat M, Jernberg-Wiklund H. The miR-125a and miR-320c are potential tumor suppressor microRNAs epigenetically silenced by the polycomb repressive complex 2 in multiple myeloma. *RNA Dis* 2017;4(2):e1529.
42. Vishnubalaji R, Hamam R, Yue S, et al. MicroRNA-320 suppresses colorectal cancer by targeting SOX4, FOXM1, and FOXQ1. *Oncotarget* 2016;7(24):35789–802.
43. Wang X, Wu J, Lin Y, et al. MicroRNA-320c inhibits tumorous behaviors of bladder cancer by

targeting Cyclin-dependent kinase 6. *J Exp Clin Cancer Res* 2014;33:69.
44. Foo CH, Rootes CL, Cowley K, et al. Dual microRNA screens reveal that the immune-responsive miR-181 promotes henipavirus entry and cell-cell fusion. *PLoS Pathogens* 2016;12(10): e1005974.
45. Raju R, Palapetta SM, Sandhya VK, et al. A network map of FGF-1/FGFR signaling system. *J Sig Transduct* 2014;2014:962962.
46. Cao R, Brakenhielm E, Pawliuk R, et al. Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2. *Nat Med* 2003;9(5):604–13.
47. Mori S, Tran V, Nishikawa K, et al. A dominant-negative FGF1 mutant (the R50E mutant) suppresses tumorigenesis and angiogenesis. *PLoS One* 2013;8(2):e57927.
48. Li J, Wei Z, Li H, et al. Clinicopathological significance of fibroblast growth factor 1 in non-small cell lung cancer. *Hum Pathol* 2015; 46(12):1821–8.
49. Tang L, Han X. The urokinase plasminogen activator system in breast cancer invasion and metastasis. *Biomed Pharmacother* 2013;67(2): 179–82.
50. Billottet C, Janji B, Thiery JP, et al. Rapid tumor development and potent vascularization are independent events in carcinoma producing FGF-1 or FGF-2. *Oncogene* 2002;21(53):8128–39.

THE TRANSCRIPTION FACTOR ERG (ETS related gene)

The ETS (E-26 transformation specific) transcription factors family

The ETS (E-26 transformation specific) family consists of 28 mammalian transcription factors. The first identified ETS factor was ETS1, which was discovered as a homolog of the avian leukaemia virus E26 oncogene in 1983 (Leprince et al., 1983). Subsequent analyses have identified a total of 27 and 26 ETS-family members in human and mouse genomes, respectively (Bult et al., 2008). All ETS family members have in common a highly conserved 85 amino acid DNA-binding domain (ETS domain or EBD), that presents a winged helix-turn-helix (HTH) structure, binding to the specific DNA core sequence 5'GGA(A/T)3'. The direct contact with DNA is made between two arginines within the third helix and the two guanines of the GGA(A/T) sequence. The HTH motif is the major structural motif capable of binding DNA: each monomer incorporates two α helices, joined by a short strand of amino acids, that bind to the major groove of DNA. The ETS family is further divided in 12 subfamilies, (ELF, ELG, ERG, ETS, ERF, ESE, ETS, PDEF, PEA3, ER71, SPI, TCF, TEL) based on the homology of their ETS domain and the presence of another conserved domain (Figure 1 a). Other domains are also present and vary from ETS member to ETS member, including the 65 amino acid monomeric pointed domain (PNT), consisting of four α-helices and a short β-helix (Hollenhorst et al., 2011), and representing a site of interaction with kinases and transcriptional regulators, and of dimerization with other ETS transcription factors (Figure 1 b). The ETS family members can function as either activators or repressors of the transcription process, depending on the target gene or the post-transcriptional modification. They can also act together with some other transcription factors: this ability is shown through the presence of composite DNA binding sites, such as FOXC/ETS and AP-1/ETS (De Val et al., 2008). Some ETS factors, like ETV-2, are expressed only in specific time periods during development, and they are ubiquitous (ELK-1); in contrast, others factors such as ERG, FLI-1 and ETV-1, are expressed during the entire development and adulthood, and they are tissue-specific. ETS members are involved in a wide variety of functions, such as cell differentiation, cell cycle control, cell proliferation, apoptosis and angiogenesis. They can also act as proto oncogenes (ETS-1, ETS-2, SPI1, FLI-1), for examples through gene fusion processes

(ERG to EWG gene, TEL to JACK2 protein) and therefore associated with progression of several different types of cancer (Seth & Watson, 2005).

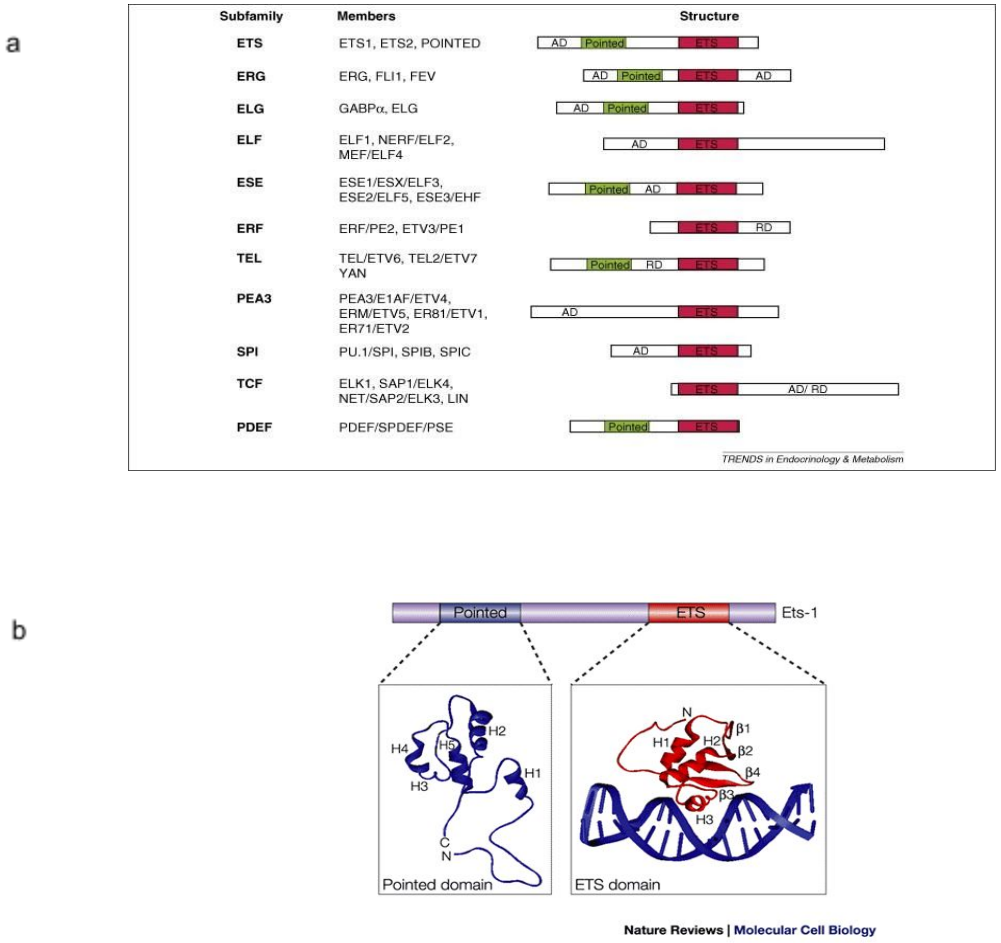

Figure 1 (a) The 12 subfamilies and family members of ETS family. (b) Structures of the pointed domain (blue) and ETS domain (red): location of helices (H) and β-strands (β) are shown

ETS family in the endothelium

A number of ETS family members are expressed in the endothelial cells (ECs), during development but also adulthood. In general, they act as promoters or enhancers of genes including vascular endothelial growth factor receptor1 (VEGF-R1), vascular endothelial growth factor receptor 2 (VEGF-R2), TIE1, TIE2, endothelial nitric oxide synthase

(eNOS). All these genes are crucial for endothelial homeostasis and processes like angiogenesis. Angiogenesis is a complex phenomenon that requires migration, proliferation, and tubular morphogenesis of ECs: Salto Y. *et al*. observed that ETS-1 is able to promote angiogenesis by inducing the expression of matrix metalloproteinases and integrin β3 in ECs (Iwasaka et al., 1996). Another ETS member, ERG, mediates ECs junctional integrity by regulating the transcription of endothelial junctional adhesion molecules such as VE-cadherin (Ve-cadh) and CLAUDIN5 (CLDN5) through binding to their promoters (Birdsey et al., 2008).

ERG: genomic structure and isoforms

The ETS family member ERG was first discovered in 1987 by Reddy *et al*. in human colorectal carcinoma cells. Full-length ERG is a 486 amino acid 54 kDa transcription factor and It is situated in the long arm of chromosome 21 at position 22.2 (21q22.2). As all members of ETS family, it presents the ETS domain, which recognises specific DNA sequences. Zammarchi *et al.* in 2013 gave a detailed description of ERG gene and exon/intron structure, which is shown in Figure 2 A (Zammarchi et al., 2013).The ERG locus is approximately 300 kb long and includes at least 12 exons. There are three alternative promoters (PI-III) and three alternative first exons (1a, 1b and 1c) and translation start sites. In addition, 30 alternative ERG transcripts (ERG isoforms) are expressed and encode at least 15 protein variants (Figure 2 B). The protein variants can include three different N-termini, two alternative transactivation domains and three different C-termini. ERG2, and ERG3 are the main isoforms expressed in most endothelial, myeloid and lymphoid haematopoietic progenitor cells (Rainis et al., 2005). ERG8 was shown to interact with other ERG isoforms to inhibit their transcriptional activity (Rastogi et al., 2014). Furthermore, knockdown of ERG8 in EC results in upregulation of endogenous ERG transcriptional activity, suggesting that ERG8 functions as an inhibitor of ERG's active isoforms (Ginsberg et al., 2012).

In cancer, ERG promoters can cooperate between each other: ERG binds ETS motifs within its own promoter. This mechanism has been found in prostate cancer, where it results in an increase of tumor cells invasiveness (Thurston & Kitajewski, 2008) (Thurston & Kitajewski, 2008) (Mani et al., 2009) (Mani et al., 2011). In respect to the genomic structure, ERG contains the PNT domain. PNT is localized on the N-terminus

of ERG protein and it mediates heterodimerization with protein partners, including other members of the ETS family (ETS1 and 2, ETV1, ETV6, FLI1 and ELK3), and with associated factors including DNA-dependent protein kinases, the androgen receptor (AR) and the AP-1 complex (Salek-Ardakani et al., 2009). Moreover, some studies have shown that ERG may also form homodimers with itself through both the PNT and the ETS binding domains (Verger et al., 2001). Therefore, ERG contains another transcriptional activation domain (TAD): TAD is located in the C-terminus of the protein, near the ETS-binding site. TAD can be inhibited by the negative regulatory domain (NID) and the C-terminal inhibitory domain (CID), by a mechanism of autoinhibition, activated by the ETS domain.

The binding specificity of ERG, like that of other ETS members, is not fully known, although all they share a GGA(A/T) core sequence. In general, ETS transcription factor-binding targets include sequences of 15–20 bp in length (Shore et al., 1996). Some classifications of ETS members, based on the similarity of the ETS binding domain, have been proposed: Wei et al., defined five classes (I, IIa, IIb, III and IV), based on the sequences which surround the main core sequence GGA(A/T). ERG belongs to class I, containing the largest number of ETS factors (ERG, ETS1 and 2, ETV1–5, ELK1, ELK3, ELK4, ERF, FEV, FLI1 and GABPα) (Wei et al., 2010). Binding specificity is also affected by post-translational modifications and protein–protein interactions. It has been demonstrated that ERG can cooperate with other proteins, to alter DNA structure locally: for example, ERG works with the SRY-related HMG box transcription factor SoxD to bind the major and minor groove of DNA, or with the AP-1 complex (Fos+Jun), to form a pincer-like structure around the major groove of a DNA double helix.

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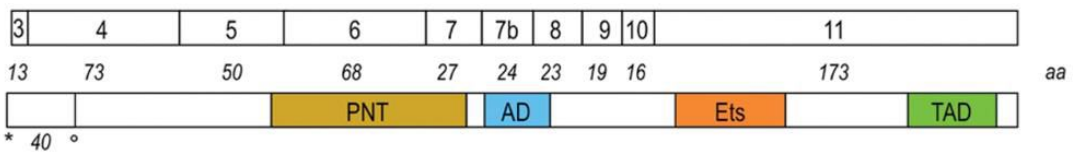


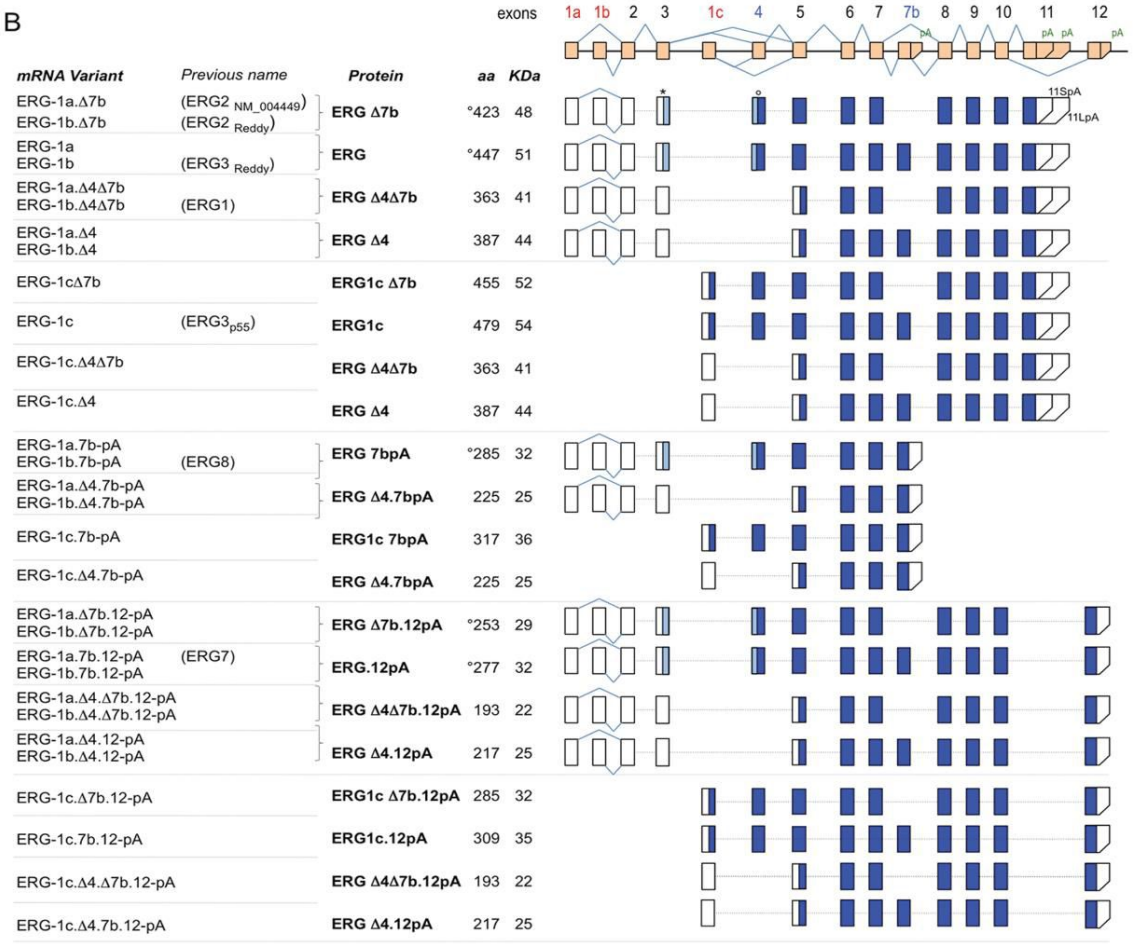


Figure 2 (A) Human ERG gene structure. Alignment of the exons forming the main ORF (Open Reading Frame) with the protein domains. Numbers indicate size in amino acids. PNT = pointed domain, AD = alternative domain, Ets = Ets domain, TAD = transactivation domain. **(B) Human ERG main variants.** Alignment of exons forming the 30 main RNA variants of human ERG. Blue indicates the ORF, light blue the additional region from the ATG in exon 3. For each variant, the proposed name is indicated next to previous nomenclature (if available). The proposed protein name is reported along the predicted size in aa and KDa. Variants derived from the alternative usage of promoter 1a and 1b are paired as they lead to related mRNAs and identical proteins. (Zanmarchi et al. 2013)

ERG: localization, target-genes and regulation of endothelial pathways

ERG is widely expressed from the embryonic developmental stage in a variety of mesodermal tissues, in particular the endothelium, where it remains highly expressed in the endothelial cells, and in the majority of adult tissues. Genomic studies on ECs showed the ERG, among the ETS family members, is the most expressed in differentiated quiescent ECs, with no differences between arteries, veins and micro

endothelium. Some studies have shown that, during mouse embryonic development, ERG is initially expressed in ECs, mainly in the amniotic membrane, in the blood vessels surrounding the neural tube, in the vasculature of the heart and in (Wythe et al., 2013). ERG is located mainly in the nucleus of ECs, although some isoforms, like ERG8, were found in the cytoplasm. ERG plays a key role in the regulation of endothelial homeostasis by influencing numerous biological processes, such as vasculogenesis, angiogenesis, junction stability, cell migration and survival. ERG influences endothelial homeostasis through regulation of multiple ECs genes that directly or indirectly control the various endothelial processes mentioned above. For example, Fish et al., found the NOTCH/VEGF/MAPK transcriptional pathway that induced one of the earliest essential ligands for artery specification DLL4 (Figure 3 a) (Fish et al., 2017). Importantly, they found that VEGF signaling activates MAP kinase (MAPK)-dependent ERG in arterial endothelium to drive expression of DLL4, as well as Notch4. Moreover, ERG mediates ECs junctional integrity and stability by regulating the transcription of endothelial adherens glycoprotein VE-cadherin and the tight junction protein claudin protein 5 (CLDN5), by binding to their promoters. Indeed, knockdown of ERG in human umbilical vein ECs is associated with significant increases in endothelial permeability, as a consequence of changes in cell structure, loss of cell-to-cell contact and angiogenesis inhibition (Birdsey et al., 2008) (McLaughlin et al., 2001). ERG also inhibits vascular inflammation, suppressing genes such as ICAM-1, interleukin-8 (IL-8) and vascular cell adhesion protein (VCAM). The Wnt/beta-catenin signalling pathway plays an important role in ECs where it supports ECs proliferation, junctional stabilization, ECs survival and overall vessel stability. All these processes, together with the transcriptional control of EC-specific genes (angiopoietin 2, endoglin, vWF, VEGF-A and VE-cadherin), are maintained through a stable localization and activity of beta-catenin, which has been shown to be tightly regulated by ERG (Vijayaraj et al., 2012). Birdsey *et al*. reported that ERG may promote β-catenin stability, by regulating transcription of both VE-cadherin and the Wnt receptor Frizzled-4 (Figure 3 a). (Birdsey et al., 2015). ERG has also been shown to be a key regulator of the Angiogenesis process. Angiogenesis is defined as the formation of new blood vessels from existing ones: it is crucial for the development of physiological functions but also pathological conditions including cancer. Angiogenesis is regulated by maintaining a delicate balance between its promoting and inhibiting factors. It has been demonstrated that ERG finely acts as a controller of this balance, as

it binds to the enhancers or promoters of ECs pro-angiogenic genes such as VEGF-R1, VEGF-R2, FZDL4 and EGF-like protein 7 (EGFL7). For instance, *in vitro* studies have shown that ERG is essential for endothelial tube formation: depletion of ERG in HUVECs led to a lower expression of the adhesion molecule VE-cadherin, that resulted in loss of cell-cell contacts, cell death and therefore malformation of endothelial vessels. These effects were further confirmed i*n vivo,* whereby a postnatal deletion of ERG in inducible endothelial-specific ERG knock-out mice $Erg^{iEC\text{-}KO}$, also led to defective angiogenesis in the retina (Birdsey et al.). Recently it has been shown that, depletion of ERG in HUVECs or in ERG KO mice model can lead to the complex process of "Endothelial to Mesenchymal Transition" (EndMT), in which ECs adopt a mesenchymal phenotype, displaying typical mesenchymal cell morphology and functions, including acquisition of cellular motility and contractile properties. ERG can regulate the canonical TGF-β/SMAD signalling pathway by promoting SMAD1/5/8 signalling, which mediates ECs homeostasis, andeby repressing SMAD2/3 signalling, which is a key regulator of EndMT (Figure 3b). Dunfton et al., have shown the negative correlation between EndMT and ERG: they have reported that, the ablation of ERG (using ERG siRNAs) can cause EndMT in HUVECs and it is correlated with EndMT in end-stage liver fibrosis patients. Moreover, ERG depletion can lead spontaneous liver fibrogenesis in mice $Erg^{iEC\text{-}KO}$ and in EC-specific constitutive hemi-deficient mice $Erg^{cEC\text{-}Het}$, in a SMAD3-dependent manner, confirming the key role of ERG in the signalling pathway (Dufton et al., 2017). It has been also demonstrated that ERG is a mediator in Ang1-dependent regulation of Notch ligands (β-catenin and DLL4), and it is required for the stabilizing effects of Ang1 *in vivo.* Thus, ERG coordinates the Ang1, Notch and Wnt/β-catenin pathways to promote vascular maturation and stability (Birdsey et al., 2015). The involvement of ERG in the pathways described above, confirmed the crucial role of ERG (and of others ETS transcription factors) in the endothelial function. Furthermore, ERG plays an important role in haematopoiesis, normal haematopoietic stem cell function and in the maintenance of peripheral blood platelets (Thoms et al., 2011). ERG expression is found in B-lymphocytes and during the entire maturation process and in T-lymphocytes (Anderson et al., 1999). The abnormal expression of ERG in T cells causes T-cell acute lymphoblastic leukaemia, and the aberrant genetic modification in the DNA-binding domain of ERG, can lead to reduction of mature platelets, erythrocytes, and leukocytes. Lastly, although the role of ERG is mainly specific for the endothelium, its ectopic expression in non-endothelial

tissues, can largely promote the oncogenesis process.

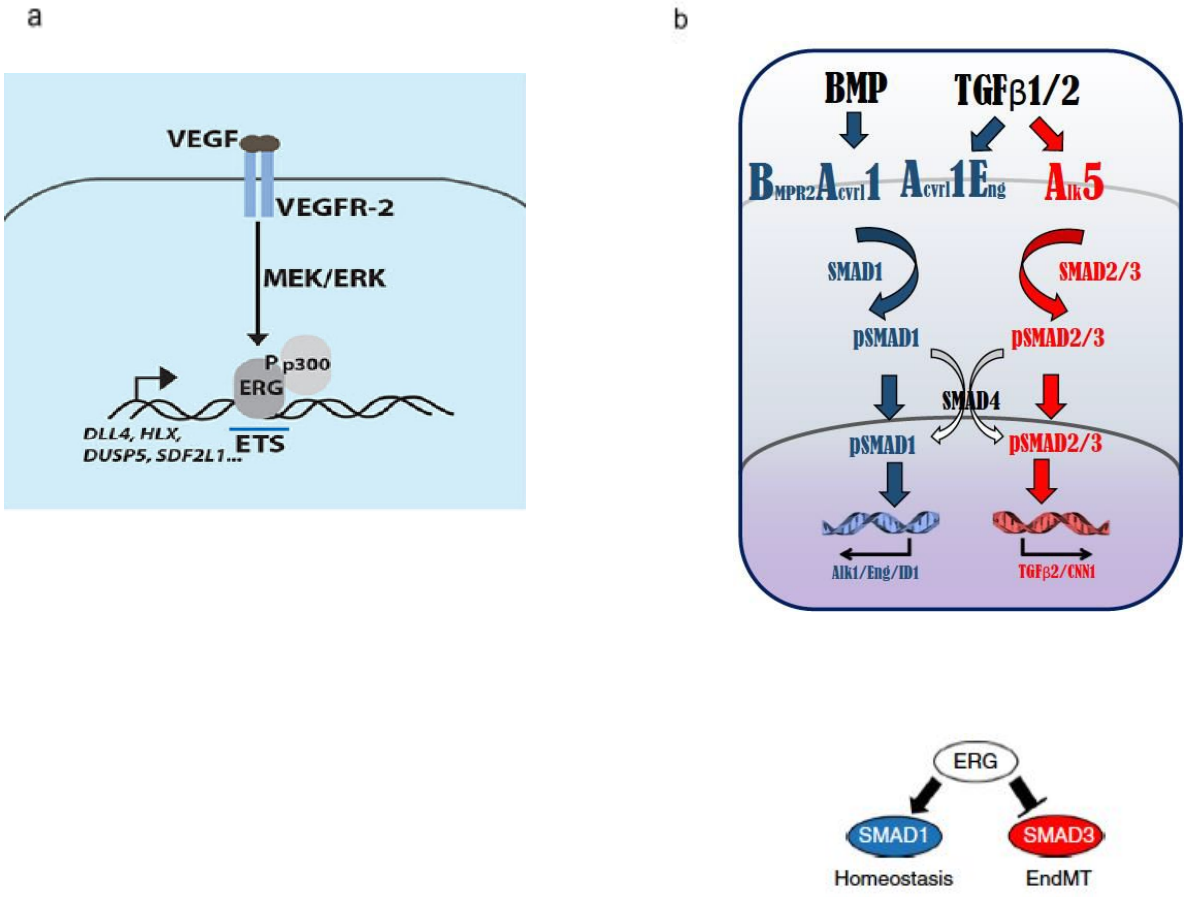


Figure 3 (a) Schematic of the VEGF/MEK/ERK/ERG/p300 transcriptional pathway. Image taken from *Fish et al., 2017*. **(b) Schematic of the SMAD-dependent TGF/BMP canonical signalling pathways in ECs and schematic of ERG regulation of SMAD1 and SMAD3.** ERG controls canonical TGFβ-SMAD signalling, driving the SMAD1 pathway while repressing SMAD3 activity. Image taken from *Dufton et al., 2017*.

ERG and cancer: tumour neovascularization and vascular malignancies

While several studies have reported the involvement of abnormal ectopic expression of ERG fusion proteins in many types of cancer, there are few studies on the role of ERG in the regulation of tumour neovascularization. Birdsey et al., using a mouse xenograft

B16F0 tumor model, which depends on angiogenesis for growth, observed that knocking out endothelial ERG, significantly reduced the size of the melanoma tumors, tumour blood vessel density and pericyte coverage of blood vessels compared to controls (Birdsey et al., 2008). This study therefore confirmed that ERG could play an essential role in tumour angiogenesis and growth, and that, downregulating the expression of ERG in tumours, could represent an effective strategy for developing new anticancer therapies. Other studies on vascular malignancies have only reported ERG as a marker for tumour endothelial cells, and an active role of ERG in these malignancies has to be further elucidated.

The role of ERG in cancer

ERG has been linked to multiple cancers through the formation of oncogenic ERG fusion proteins as a result of chromosomal translocation. In several types of human myeloid leukemias, TLS/FUS gene shows recurrent chromosomal translocation t(16;21)(p11:q22). The TLS/FUS gene is fused to the transcriptional activator ERG, causing the replacement of the RNA-binding domain of TLS/FUS with the DNA-binding domain of ERG (Prasad et al., 1994). In Ewing sarcoma (ES), there is a canonical fusion between the EWSR1 gene and two members of the ETS family: FLI1 and ERG. The EWSR1-FLI1 fusion has been identified in 90% of cases, while EWSR1-Erg fusion has a low occurrence rate (in 5-10% of cases), and represents the second most common molecular alteration (Chen et al., 2016). The ERG fusion results in a loss of endogenous ERG promoter activity, leading to a dysregulation of ERG and its target genes. Moreover, ERG has been reported to be one of the most overexpressed oncogene in prostate cancer: A combined loss of PTEN and TP53 associated with an overexpression of ERG is considered to be the principal driver of high grade prostate intraepithelial neoplasia (PIN) to invasive carcinoma (Tomlins et al., 2005). In prostate cancer, a chromosomal translocation results in the fusion between the androgen receptor-regulated gene promoter of transmembrane protease serine 2 (TMPRSS-)2 and the DNA-binding domain of ERG. This leads to the overexpression of TMPRSS: ERG fusion protein. The chromosomal translocation can be distinguished into two main types: ETS^{+ve}, which involves fusion with ERG or another ETS gene, and ETS^{-ve} in which the fusion is not with ERG/ETS. The ETS^{+ve} fusion leads to an abnormal

overexpression of ERG protein in the prostate epithelium, and has been correlated with increased cell invasiveness, a poor prognosis and an increased level of malignancy (Adamo & Ladomery, 2016).

ERG as anticancer therapy

Since ERG is largely involved in the biology of cancer, it can be considered to be a marker for diagnosis and prognosis as well as a potential target for cancer therapies.
ERG gene fusion TMPRSS2 interacts in a DNA-independent manner with the enzyme poly (ADP-ribose) polymerase 1 (PARP1) and the catalytic subunit of DNA protein kinase (DNA-PKcs). ETS gene-mediated transcription and cell invasion requires PARP1 and DNA-PKcs expression and activity. Pharmacological treatment of PARP1 with olaparib has been shown to significantly inhibit ETS-positive, but not ETS-negative, prostate cancer cells (Brenner et al., 2011). Similarly, TMPRSS2:ERG leads to upregulation of histone deacetylase 1 (HDAC1) gene and downregulation of its target genes. A therapy based on the inhibition of HDAC, especially in combination with anti-androgens, can be effective against prostate cancer development. The HDAC inhibitors Trichostatin A (TSA), MS-275 and suberoylanilide hydroxamic (SAHA), with or without androgen deprivation, can reduce cancer growth and induce apoptosis of ERG positive prostate cancer cells (Björkman et al., 2008). A different strategy could involve targeting of ERG itself. For instance, the development of YK-4-279, a small inhibitor of EWS-FLI1 oncoprotein in Ewing's Sarcoma has been reported. Since ERG and ETV1 belong to the same class of ETS factors as FLI1, YK-4-279 also works in ERG positive cells of prostate cancers and has showed its ability to reduce invasion, motility and metastasis (Rahim et al., 2011).The DNA-binding ability of ERG can also be targeted by modulators such as DB1255 di-(phenyl-thiophene-amidine), that prevent ERG binding to the domain. Lastly, the degradation of the ERG gene could also represent an alternative strategy. Kittler *et al*. showed indeed that the deubiquitinase enzyme ubiquitin-specific peptidase 9, X-linked (USP9X) can bind to ERG in ERG positive prostate cancer inducing its de-ubiquitination *in vitro*. Knocking down USP9X also led to the ubiquitination and degradation of ERG. Hence, targeting USP9X with

inhibitors like the second-generation tyrphostin derivative WP1130, resulted in ERG degradation both *in vivo* and *in vitro.*

ERG and microRNAs

ERG has been reported to be a potential target of miRNA-145*,* which has consistently been found to be down-regulated in prostate cancer. In tumour samples, ERG protein expression is found to be elevated, together with an increased expression of ERG splicing isoforms. This suggests that the upregulation of ERG oncoprotein, caused by downregulation of miRNA 145, as well as miRNA 221 are relevant for the development of prostate cancer (Hart et al., 2013). ERG can also act as a regulator of miRNAs: for instance, Zhang *et al*. showed that the miRNA 200b subfamily (miR-200b, 200a and miRNA 429) is an ERG gene target, therefore implicating an important role in TMPRSS2/ERG-dependent prostate cancer development. Knockdown of ERG in prostate cancer cells reduced expression of these three miRNAs, and caused an overexpression of the miR-200b/a/429 gene, inhibited prostate cancer cell growth and invasion (Zhang et al., 2016). Furthermore, it has been reported the linkage between ERG and miRNA-126: loss of ERG and FLT1 induces EndMT coupled with dynamic epigenetic changes in ECs, while miR-126 blocks TGFβ-induced EndMT by targeting PIK3R2 mRNA (Zhang et al., 2016). Nagai et al. showed that, among microRNAs related to EndMT/EMT and targeted by ERG and FLT1, the most promising direct target was mR126. They demonstrated that inhibition of miR126 in HUVECs, induced partial EndMT, with downregulation of CDH5 and upregulation of TAGLN, COL1A1 and SNAI2 expression. This study thus suggested that EndMT, mediated by downregulation of ERG/FLT1, is partially dependent on the reduction of miR-126 expression, under the control of these transcription factors (Nagai et al., 2018).

References:

Adamo, P., & Ladomery, M. R. (2016). The oncogene ERG: a key factor in prostate cancer. *Oncogene*, *35*(4), 403–414. https://doi.org/10.1038/onc.2015.109

Anderson, M. K., Hernandez-Hoyos, G., Diamond, R. A., & Rothenberg, E. V. (1999). Precise developmental regulation of Ets family transcription factors during specification and commitment to the T cell lineage. *Development*, *126*(14), 3131–3148. https://dev.biologists.org/content/126/14/3131

Birdsey, G. M., Dryden, N. H., Amsellem, V., Gebhardt, F., Sahnan, K., Haskard, D. O., Dejana, E., Mason, J. C., & Randi, A. M. (2008). Transcription factor Erg regulates angiogenesis and endothelial apoptosis through VE-cadherin. *Blood*, *111*(7), 3498–3506. https://doi.org/10.1182/blood-2007-08-105346

Birdsey, G. M., Shah, A. V, Dufton, N., Reynolds, L. E., Osuna Almagro, L., Yang, Y., Aspalter, I. M., Khan, S. T., Mason, J. C., Dejana, E., Göttgens, B., Hodivala-Dilke, K., Gerhardt, H., Adams, R. H., & Randi, A. M. (2015). The endothelial transcription factor ERG promotes vascular stability and growth through Wnt/β-catenin signaling. *Developmental Cell*, *32*(1), 82–96. https://doi.org/10.1016/j.devcel.2014.11.016

Björkman, M., Iljin, K., Halonen, P., Sara, H., Kaivanto, E., Nees, M., & Kallioniemi, O. P. (2008). Defining the molecular action of HDAC inhibitors and synergism with androgen deprivation in ERG-positive prostate cancer. *International Journal of Cancer*, *123*(12), 2774–2781. https://doi.org/10.1002/ijc.23885

Brenner, J. C., Ateeq, B., Li, Y., Yocum, A. K., Cao, Q., Asangani, I. A., Patel, S., Wang, X., Liang, H., Yu, J., Palanisamy, N., Siddiqui, J., Yan, W., Cao, X., Mehra, R., Sabolch, A., Basrur, V., Lonigro, R. J., Yang, J., … Chinnaiyan, A. M. (2011). Mechanistic Rationale for Inhibition of Poly(ADP-Ribose) Polymerase in ETS Gene Fusion-Positive Prostate Cancer. *Cancer Cell*, *19*(5), 664–678. https://doi.org/https://doi.org/10.1016/j.ccr.2011.04.010

Bult, C. J., Eppig, J. T., Kadin, J. A., Richardson, J. E., & Blake, J. A. (2008). The Mouse Genome Database (MGD): mouse biology and model systems. *Nucleic Acids Research*, *36*(Database issue), D724-8. https://doi.org/10.1093/nar/gkm961

Chen, S., Deniz, K., Sung, Y.-S., Zhang, L., Dry, S., & Antonescu, C. R. (2016). Ewing sarcoma with ERG gene rearrangements: A molecular study focusing on the prevalence of FUS-ERG and common pitfalls in detecting EWSR1-ERG fusions by FISH. *Genes, Chromosomes & Cancer*, *55*(4), 340–349. https://doi.org/10.1002/gcc.22336

De Val, S., Chi, N. C., Meadows, S. M., Minovitsky, S., Anderson, J. P., Harris, I. S., Ehlers, M. L., Agarwal, P., Visel, A., Xu, S.-M., Pennacchio, L. A., Dubchak, I., Krieg, P. A., Stainier, D. Y. R., & Black, B. L. (2008). Combinatorial regulation of endothelial gene expression by ets and forkhead transcription factors. *Cell*, *135*(6), 1053–1064. https://doi.org/10.1016/j.cell.2008.10.049

Dufton, N. P., Peghaire, C. R., Osuna-Almagro, L., Raimondi, C., Kalna, V., Chauhan, A., Webb, G., Yang, Y., Birdsey, G. M., Lalor, P., Mason, J. C., Adams, D. H., & Randi, A. M. (2017). Dynamic regulation of canonical TGFβ signalling by endothelial transcription factor ERG protects from liver fibrogenesis. *Nature Communications*, *8*(1), 895. https://doi.org/10.1038/s41467-017-01169-0

Fish, J. E., Cantu Gutierrez, M., Dang, L. T., Khyzha, N., Chen, Z., Veitch, S., Cheng, H. S., Khor, M., Antounians, L., Njock, M.-S., Boudreau, E., Herman, A. M., Rhyner, A. M., Ruiz, O. E., Eisenhoffer, G. T., Medina-Rivera, A., Wilson, M. D., & Wythe, J. D. (2017). Dynamic regulation of VEGF-inducible genes by an ERK/ERG/p300 transcriptional network. *Development (Cambridge, England)*, *144*(13), 2428–2444. https://doi.org/10.1242/dev.146050

Ginsberg, M., James, D., Ding, B.-S., Nolan, D., Geng, F., Butler, J. M., Schachterle, W., Pulijaal, V. R., Mathew, S., Chasen, S. T., Xiang, J., Rosenwaks, Z., Shido, K., Elemento, O., Rabbany, S. Y., & Rafii, S. (2012). Efficient direct reprogramming of mature amniotic cells into endothelial cells by ETS factors and TGFβ suppression. *Cell*, *151*(3), 559–575. https://doi.org/10.1016/j.cell.2012.09.032

Hart, M., Wach, S., Nolte, E., Szczyrba, J., Menon, R., Taubert, H., Hartmann, A., Stoehr, R., Wieland, W., Grässer, F. A., & Wullich, B. (2013). The proto-oncogene ERG is a target of microRNA miR-145 in prostate cancer. *The FEBS Journal*, *280*(9), 2105–2116. https://doi.org/10.1111/febs.12236

Iwasaka, C., Tanaka, K., Abe, M., & Sato, Y. (1996). Ets-1 regulates angiogenesis by inducing the expression of urokinase-type plasminogen activator and matrix metalloproteinase-1 and the migration of vascular endothelial cells. *Journal of Cellular Physiology*, *169*(3), 522–531. https://doi.org/10.1002/(SICI)1097-4652(199612)169:3<522::AID-JCP12>3.0.CO;2-7

Leprince, D., Gegonne, A., Coll, J., de Taisne, C., Schneeberger, A., Lagrou, C., & Stehelin, D. (1983). A putative second cell-derived oncogene of the avian leukaemia retrovirus E26. *Nature*, *306*(5941), 395–397.

https://doi.org/10.1038/306395a0

Mani, R.-S., Iyer, M. K., Cao, Q., Brenner, J. C., Wang, L., Ghosh, A., Cao, X., Lonigro, R. J., Tomlins, S. A., Varambally, S., & Chinnaiyan, A. M. (2011). TMPRSS2{\textendash}ERG-Mediated Feed-Forward Regulation of Wild-Type ERG in Human Prostate Cancers. *Cancer Research*, *71*(16), 5387–5392. https://doi.org/10.1158/0008-5472.CAN-11-0876

Mani, R.-S., Tomlins, S. A., Callahan, K., Ghosh, A., Nyati, M. K., Varambally, S., Palanisamy, N., & Chinnaiyan, A. M. (2009). Induced chromosomal proximity and gene fusions in prostate cancer. *Science (New York, N.Y.)*, *326*(5957), 1230. https://doi.org/10.1126/science.1178124

McLaughlin, F., Ludbrook, V. J., Cox, J., von Carlowitz, I., Brown, S., & Randi, A. M. (2001). Combined genomic and antisense analysis reveals that the transcription factor Erg is implicated in endothelial cell differentiation. *Blood*, *98*(12), 3332–3339. https://doi.org/10.1182/blood.V98.12.3332

Nagai, N., Ohguchi, H., Nakaki, R., Matsumura, Y., Kanki, Y., Sakai, J., Aburatani, H., & Minami, T. (2018). Downregulation of ERG and FLI1 expression in endothelial cells triggers endothelial-to-mesenchymal transition. *PLoS Genetics*, *14*(11), e1007826. https://doi.org/10.1371/journal.pgen.1007826

Prasad, D. D., Ouchida, M., Lee, L., Rao, V. N., & Reddy, E. S. (1994). TLS/FUS fusion domain of TLS/FUS-erg chimeric protein resulting from the t(16;21) chromosomal translocation in human myeloid leukemia functions as a transcriptional activation domain. *Oncogene*, *9*(12), 3717—3729. http://europepmc.org/abstract/MED/7970732

Rahim, S., Beauchamp, E. M., Kong, Y., Brown, M. L., Toretsky, J. A., & Üren, A. (2011). YK-4-279 Inhibits ERG and ETV1 Mediated Prostate Cancer Cell Invasion. *PLOS ONE*, *6*(4), 1–8. https://doi.org/10.1371/journal.pone.0019343

Rainis, L., Toki, T., Pimanda, J. E., Rosenthal, E., Machol, K., Strehl, S., Göttgens, B., Ito, E., & Izraeli, S. (2005). The Proto-Oncogene ERG in Megakaryoblastic Leukemias. *Cancer Research*, *65*(17), 7596–7602. https://doi.org/10.1158/0008-5472.CAN-05-0147

Rastogi, A., Tan, S.-H., Mohamed, A. A., Chen, Y., Hu, Y., Petrovics, G., Sreenath, T., Kagan, J., Srivastava, S., McLeod, D. G., Sesterhenn, I. A., Srivastava, S., Dobi, A., & Srinivasan, A. (2014). Functional antagonism of TMPRSS2-ERG splice variants in prostate cancer. *Genes & Cancer*, *5*(7–8), 273–284.

https://doi.org/10.18632/genesandcancer.25

Salek-Ardakani, S., Smooha, G., de Boer, J., Sebire, N. J., Morrow, M., Rainis, L., Lee, S., Williams, O., Izraeli, S., & Brady, H. J. M. (2009). ERG Is a Megakaryocytic Oncogene. *Cancer Research*, *69*(11), 4665–4673. https://doi.org/10.1158/0008-5472.CAN-09-0075

Seth, A., & Watson, D. K. (2005). ETS transcription factors and their emerging roles in human cancer. *European Journal of Cancer (Oxford, England : 1990)*, *41*(16), 2462–2478. https://doi.org/10.1016/j.ejca.2005.08.013

Shore, P., Whitmarsh, A. J., Bhaskaran, R., Davis, R. J., Waltho, J. P., & Sharrocks, A. D. (1996). Determinants of DNA-binding specificity of ETS-domain transcription factors. *Molecular and Cellular Biology*, *16*(7), 3338–3349. https://doi.org/10.1128/mcb.16.7.3338

Thoms, J. A. I., Birger, Y., Foster, S., Knezevic, K., Kirschenbaum, Y., Chandrakanthan, V., Jonquieres, G., Spensberger, D., Wong, J. W., Oram, S. H., Kinston, S. J., Groner, Y., Lock, R., MacKenzie, K. L., Göttgens, B., Izraeli, S., & Pimanda, J. E. (2011). ERG promotes T-acute lymphoblastic leukemia and is transcriptionally regulated in leukemic cells by a stem cell enhancer. *Blood*, *117*(26), 7079–7089. https://doi.org/10.1182/blood-2010-12-317990

Thurston, G., & Kitajewski, J. (2008). VEGF and Delta-Notch: Interacting signalling pathways in tumour angiogenesis. *British Journal of Cancer*, *99*(8), 1204–1209. https://doi.org/10.1038/sj.bjc.6604484

Tomlins, S. A., Rhodes, D. R., Perner, S., Dhanasekaran, S. M., Mehra, R., Sun, X.-W., Varambally, S., Cao, X., Tchinda, J., Kuefer, R., Lee, C., Montie, J. E., Shah, R. B., Pienta, K. J., Rubin, M. A., & Chinnaiyan, A. M. (2005). Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science (New York, N.Y.)*, *310*(5748), 644–648. https://doi.org/10.1126/science.1117679

Verger, A., Buisine, E., Carrère, S., Wintjens, R., Flourens, A., Coll, J., Stéhelin, D., & Duterque-Coquillaud, M. (2001). Identification of Amino Acid Residues in the ETS Transcription Factor Erg That Mediate Erg-Jun/Fos-DNA Ternary Complex Formation. *Journal of Biological Chemistry*, *276*(20), 17181–17189. https://doi.org/10.1074/jbc.M010208200

Vijayaraj, P., Le Bras, A., Mitchell, N., Kondo, M., Juliao, S., Wasserman, M., Beeler, D., Spokes, K., Aird, W. C., Baldwin, H. S., & Oettgen, P. (2012). Erg is a crucial regulator of endocardial-mesenchymal transformation during cardiac valve

morphogenesis. *Development (Cambridge, England)*, *139*(21), 3973–3985. https://doi.org/10.1242/dev.081596

Wei, G.-H., Badis, G., Berger, M. F., Kivioja, T., Palin, K., Enge, M., Bonke, M., Jolma, A., Varjosalo, M., Gehrke, A. R., Yan, J., Talukder, S., Turunen, M., Taipale, M., Stunnenberg, H. G., Ukkonen, E., Hughes, T. R., Bulyk, M. L., & Taipale, J. (2010). Genome-wide analysis of ETS-family DNA-binding in vitro and in vivo. *The EMBO Journal*, *29*(13), 2147–2160. https://doi.org/10.1038/emboj.2010.106

Wythe, J. D., Dang, L. T. H., Devine, W. P., Boudreau, E., Artap, S. T., He, D., Schachterle, W., Stainier, D. Y. R., Oettgen, P., Black, B. L., Bruneau, B. G., & Fish, J. E. (2013). ETS factors regulate Vegf-dependent arterial specification. *Developmental Cell*, *26*(1), 45–58. https://doi.org/10.1016/j.devcel.2013.06.007

Zammarchi, F., Boutsalis, G., & Cartegni, L. (2013). 5′ UTR Control of Native ERG and of Tmprss2:ERG Variants Activity in Prostate Cancer. *PLoS ONE*, *8*(3). https://doi.org/10.1371/journal.pone.0049721

Zhang, Z., Lanz, R. B., Xiao, L., Wang, L., Hartig, S. M., Ittmann, M. M., Feng, Q., & He, B. (2016). The tumor suppressive miR-200b subfamily is an ERG target gene in human prostate tumors. *Oncotarget*, *7*(25), 37993–38003. https://doi.org/10.18632/oncotarget.9366

MATERIAL AND METHODS

Immortalization of glomerular endothelial cells (GECs)

Primary Kidney glomerular endothelial cells (GECs) purchased from Clinisciences (Clinisciences Italy, Cat. No. H6014G) were infected (at passage 2) with a retrovirus containing pBABE-puro-hTERT plasmid (Addgene plasmid #1771) and selected using antibiotic resistance (1 μg/mL puromycin, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) for two weeks (Bernardini et al., 2019).

Cell cultures

Glomerular endothelial cells (GECs)

GECs were cultured in EndoGRO VEGF medium (Merck Millipore, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). Prior to seeding, the plastic was coated with a layer of attachment factor as per manufacturer's protocol (Cat. No. S006100; Gibco; Thermofisher Scientific). The media was replaced every two days after one wash with PBS and cells subcultured once a confluency of 90% was achieved.

Podocytes

Primary cultures of human podocytes were established and lines of differentiated podocytes we obtained by infection with a hybrid Adeno5/SV40 Virus (Collino et al., 2008). Podocytes were characterized for the positive expression of nephrin, podocin and synaptopodin and for negative expression of the Willebrand factor, CD31 and α-SMA, as previously described (Conaldi PG et al., 1997).

VEGF assay

GECs that were used for experiments that involved treatment with Vascular Endothelial Growth Factor (VEGF) were cultured in a separate EndoGRO medium in which the VEGF component was omitted from the cell kit medium. For this assay, GECs ($50x10^4$) were seeded 24 h before experiments in endothelial basal medium-1 (EBM) (Lonza, Basel, Switzerland) supplemented with L-glutamine (5 mM), HEPES (12 mM, pH 7.4), penicillin (50 IU/ml), streptomycin (50 µg/ml) (all from Sigma, St. Louis, MO, USA), and 2% FBS for synchronization and acclimatization. Post 24 h, the cells were treated with VEGF (5 ng/ml) (Vascular Endothelial Growth Factor human, 10 ug/ml, Cat. No. V7259, Sigma-Aldrich) in the presence or absence of anti-VEGF antibody (0.12 µg/ml) (VEGF Monoclonal Antibody, 500 µl, Cat. No. MA5-23719, Thermofisher Scientific) for 30 minutes. After 30 minutes, the experiment was stopped on ice and cells subjected to protein isolation using RIPA buffer.

Co-culture model (Permeability assay)

GECs were seeded on the lower side of a 24 well-plate cell culture insert (Transparent PET membrane 0.4 µm pore size, Cat. No. 353095; Falcon-Corning, Glendale, AZ, USA), at a density of 5×10^4 cells in Endogro with 10% FBS. After 3 h, podocytes were seeded on the upper side of the insert at the same density in DMEM High Glucose with 10% FBS. The Transwells were coated with Collagen IV (Collagen IV, 10 mg, Cat. No. C7521; Sigma-Aldrich) on both sides prior to seeding cells. The following day, GECs were treated with or without VEGF for 24 h at 37C. Post VEGF treatment, the media in the Transwell was replaced with complete EBM whereas media on the underside of the Transwell (containing GECs) was replaced with complete EBM supplemented with FITC-BSA (1 mg/ml, Sigma) and the plate incubated at 37C for 6 h. Post incubation, 100 µl of medium was taken from the podocyte compartment (which would determine the passage of FITC-BSA from the lower to the upper chamber through GECS and podocytes) and assessed for fluorescence using a fluorescence reader. All experiments were performed in triplicate. When permeability was tested in

GECs silenced for ERG, the assay was performed on GECs after 3 days of transfection with siRNA.

Western Blot

GECs were lysed in Radioimmunoprecipitation assay buffer (RIPA) (RIPA Buffer, 500 ml Cat. No. R2078 Sigma-Aldrich; Life Science) supplemented with protease inhibitor (Cat. No. P8340; Sigma-Aldrich), phosphatase inhibitor cocktail 2 (Cat. NO. P5726; Sigma-Aldrich), phosphatase inhibitor cocktail 3 (Cat. No. P0044; Sigma-Aldrich), and Phenylmethanesulfonyl Fluoride (PMSF) (Cat. No. 93282; Sigma-Aldrich) (all diluted at 1:100). Briefly, cells to be lysed were detached, washed once with PBS and resuspended in RIPA lysis buffer. For some experiments, RIPA buffer was added directly to the wells containing cells following a single wash with PBS. The Eppendorf or plate was then incubated on ice for 30 minutes on a shaker to allow cell lysis. Post incubation, the lysate was centrifuged at 13000 RPM for 15 min at 4° C. The supernatant was collected and quantified using the Bradford method (Protein Assay Dye Reagent Concentrated, Cat. No. 500-0006; Biorad) as per manufacturer's protocol. Total cell lysate (30-50µg/ml per sample) was solubilized in 4x Laemmli buffer at 95° C, and separated by electrophoresis in a 4-20% gradient sodium dodecyl sulfate (SDS)-polyacrylamide gel (mini-PROTEAN TGX precast gels; Cat. No. 4568094; Biorad). Transfer of proteins from the gel onto nitrocellulose membrane (iBlot 2NC Regular stacks Cat. No. IB23001, Invitrogen, Thermofisher) was performed using the iBlot™ Dry Blotting System with the 7 min. quick transfer program. Post transfer, the membrane was blocked for 1 h in blocking solution (5% Bovine Serum Albumin, Sigma-Aldrich, powdered in TBS/ 0.1% Tween) at room temperature on a shaker. This was followed by an overnight incubation with primary antibodies (resuspended in 5%BSA/TBS-Tween) at 4̊C on a shaker. The following day, the membrane was washed three times for 15 min with wash buffer (TBS 0,1% Tween), and then incubated with the respective horseradish peroxidase-conjugated secondary antibodies (1:3000–1:8000 dilutions) for 1 h at room temperature on a shaker. Post incubation, the membranes were washed three times for 15 min on a shaker. For the development, the membranes were incubated with ECL chemiluminescence reagent (Bio-Rad) and images acquired via a Chemidoc machine (Bio-Rad) as per manufacturer's protocol.

For western blotting, the following antibodies were used: Vinculin (Cat. No. V4505; 1:8000 dilution; Sigma-Aldrich, St Louis, MO, USA), GAPDH (Cat. No. ab37168; 1:1500; Abcam, Cambridge, UK), ERG (Cat. No. ab133264; 1:1000 dilution; Abcam, Cambridge, UK), ERG1/2/3 (Cat. No. sc271048; 1:500; Santa-Cruz, Dallas, TX, USA), DLL-4 (Cat. No. 96406S; 1:500; Cells Signalling Technologies, Boston, MA, USA), TGF-β1 (Cat. No. E-ab 33090; 1:1000; R&D System, Minneapolis, USA), TGF-β2 (Cat. No. ab36495; 1:1000; Abcam, Cambridge, UK), α-SMA (Cat. No. A5228; 1:1000; Sigma-Aldrich, St Louis, MO, USA), VE-cadherin (Cat. No. ab33168; 1:500; Abcam, Cambridge, UK), phospho-ERG (1:500; kindly gifted by Professor Hollenhorst, University of Indiana, USA). The antibodies were blotted overnight at 4°C in 5% BSA/TBS-Tween 0,1%. The secondary antibodies used are as follows: Goat anti-mouse (1:3000) and Goat anti-rabbit (1:3000) horseradish peroxidase (HRP)-conjugated antibodies. Densitometry analysis was performed using Image lab analysis software ver 5.2.1 (Bio-Rad laboratories).

Protein Immunoprecipitation

In order to conjugate the beads with ERG antibody, 100 µl of magnetic beads (Sure beads Protein A, Magnetic beads; Cat. No. 181-4013; Bio-rad Laboratories) per sample were washed three times with PBS 0,1% Tween, and then co-incubated in 200 µl of 0.1% Tween/PBS containing anti-ERG antibody (1:4 dilution) (per each sample) overnight at 4°C, on a rotator. The following day, GECs ($10x10^5$) were treated with VEGF (5 ng/ml) in the presence or absence of anti-VEGF antibody (0.12 ug/ml) for 30 min at 37C. Post incubation, proteins were extracted as described previously (WB section) and left on ice. The beads left overnight with anti-ERG antibody were washed three times with PBS and co-incubated with protein lysates (1 mg/sample) from above at 4°C for 2 h. Post incubation, the beads were magnetized and supernatant discarded. After three washes with PBS, 50 µl of NP-40 cell lysis buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1% Nonidet P40 (NP40), 0.02% NaN_3) together with 4x Laemmli buffer (with β-mercaptoethanol 1:10) were added to the beads and boiled for 10 minutes at 95°C. The heated samples were than subjected to Western blotting as mentioned above (Western blot section). The Membranes were incubated with primary antibodies for phospho-ERG and ERG overnight at 4° C on a

shaker. The following day, after incubation with the respective secondary antibodies, the membranes were developed using ECL as mentioned above.

Transfection of GECs

The transfection of GECs was performed using the HiPerfect reagent (Qiagen) with either negative control siRNA (Negative control #1, Silencer Select, Pre-designed siRNA, 40 nmole, Cat. No. 4390844; Thermofisher Scientific) or siRNA ERG (ERG Silencer Select Pre-designed siRNA, 40 nmole, Cat. No. 4392422; Thermofisher Scientific) at a concentration of 20 µM. Briefly, for each sample, 3.5 ul of HiPerfect reagent, 20 µM of negative control siRNA or 20 µM of siRNA ERG was co-incubated in 100 µl of serum free media at room temperature for 10 min to form complexes. Post incubation, the HiPerfect complex was added dropwise to 200 µl of GECs cell suspension (in Endogro 10% FBS). After 3 hours of incubation at at 37C 200 µl of EndoGro 10% FBS were added to each well in order to reach a total medium volume of 500 µl for each well. Therefore the 24 well plate was incubated for 3 days at 37C. After 72 h, cells were washed once with PBS and assessed for ERG inhibition at a molecular (Real-time PCR). Subsequently, cells were also used for *in vitro* experiments (permeability assay). For WB analysis, after 72 hours post transfection, the media was replaced, and GECs were kept in culture for a further 72h before protein extraction and analysis.

RNA isolation and Real time PCR (q-RT PCR)

Total RNA was isolated from different cell preparations or from mice kidney glomeruli, using Trizol (TRIzol Reagent, 200 ml, Cat. No. 15596018, Thermo Fisher Scientific) according to the manufacturer's protocol or RNA extraction Kit (miRNeasy mini-kit (50) Cat. No. 16601432; Qiagen). The RNA was then quantified with a spectrophotometer (Nanodrop ND-1000) and subjected to retro-transcription. For gene expression analysis, quantitative real-time PCR was performed. Briefly, first-strand cDNA was produced from 200 ng of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as per manufacturer's protocol. Real-time PCR

experiments were performed in 20-µl reaction mixture containing 5 ng of cDNA template, the sequence-specific oligonucleotide primers (purchased from MWG-Biotech) and SYBR Green (Power SYBR Green PCR Master Mix, 4368708, Thermo Fisher Scientific) and assembled into a 96-well StepOne Real Time System (Applied Biosystems). GAPDH or β-Actin was used as a house-keeping gene for normalization of RNA inputs. Fold change expression (RQ) with respect to control was calculated for all samples. The sequence-specific oligonucleotide human and mouse primers used are listed in the table below:

Target genes	Forward primer	Reverse primer
h-GAPDH	CCGCTTCGCTCTCTGCTC	CGACCAAATCCGTTGACTCC
h-β-Actin	TGAAGATCAAGATCA	CACATCTGCTGGAAGGTGGAC
h-ERG	CGTGCCAGCAGATCCTAC	CAAGATGTTGACGTCTGGAAGG
h-Dll4	GGCAGCTGTAAGGACCAGGAG	TCACAAGCATAGTTGGCCCC
h-TGFβ1	GCCCTGGACACCAACTATTGCT	AGGCTCCAAATGTAGGGGCAGG
h-TGFβ2	GTCCCTGCTGCACTTTTGTA	TGCCATCAATACCTGCAAAT
h-α-SMA	ACAGGAATACGATGAAGCCG	GCTTTGGCTAGGAATGATTTGG
h-CNN-1	CTGGCTGCAGCTTATTGATG	CTGAGAGAGTGGATCGAGGG
m-GAPDH	TGT CAA GCT CAT TTC CTG GTA TGA	TCTTACTCCTTGGAGGCCATG
m-ERG	TCACATCTCCACTACCTCAGAGAGACT	TGGCATGCATTAACCGTG
m-SNAIL1	CCACACTGGTGAGAAGCCATT	CTCTTGGTGCTTGTGGAGCA

Figure 1 Sequence-specific oligonucleotide human and mouse primers.

Mouse model of diabetic nephropathy

Animal studies were conducted in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals. All procedures were approved by the Ethics Committee of the University of Turin and the Italian Health. Eight-week-old male NSG mice were purchased from the animal facility at the Molecular Biotechnology Centre. Diabetes was induced via the intraperitoneal injection of STZ (37 mg/kg), dissolved in freshly made 0.1 mol/l citrate buffer, at pH 4.5, for 4 consecutive days in order to avoid acute STZ toxicity, according to the Animal Models of Diabetic Complications Consortium guidelines.

Glucose levels were measured, after 4 hours of fasting, in blood from the tail-vein using a blood glucometer (GlucoMen LX Plus+, A. Menarini diagnostics, Florence, IT). The onset of diabetes was established by measuring glycaemia (up to 250 mg/ml) 10 days after STZ injection (T0). Glycaemia was monitored every 2 weeks and body weight and water up-take every week. At day 28 (T28) or at day 60 (T60), urine (12 hours collection in metabolic cage) and blood were collected for the evaluation of albuminuria, creatinuria, plasma creatinine and BUN. Mice were sacrificed either 28- or 60-days post diabetes and the kidneys collected for histological analysis.

For the molecular analysis of glomeruli, mice kidneys were minced into pieces and then directly resuspended in Trizol for RNA isolation. For protein analysis, glomeruli were isolated after kidney collection. Briefly, kidneys were minced into small pieces and pressed through a 100 μm cell strainer (Corning Cell Strainer 100 μm Nylon; Cat. No. 431752) using a syringe plunger. After three washes with PBS, the glomeruli which remained on the strainer were collected and resuspended in RIPA buffer ready for protein extraction as mentioned above.

ERG Knock-out mouse model

A Cre/LoxP strategy was used to develop an inducible homozygous deletion of Erg using Pdgfb-iCreER-eGFP/Ergfl/fl mice (Erg $^{iEC-KO}$). All experiments with Erg-deficient mice were conducted with age and gender matched animals at Imperial College London in accordance with the UK Animals (Scientific Procedures) act of 1986. All animals used were retained on a C57BL/6 background. Both male and female mice were used for experiments and were 8–10-weeks old (Dufton et al., 2017). Endothelial deletion was induced by tamoxifen injection (five injections of 0.5 mg daily). All experiments were conducted using littermate controls; mice were monitored, and kidney issues were harvested 30 days post-tamoxifen injection. The set-up of the model was performed at Imperial College London in the laboratory of Prof. Randi. Histological analysis for the evaluation of ERG expression was performed at the University of Turin.

Human kidney tissue sections

Human kidney tissue was collected from diabetic patients with diffuse diabetic glomerulosclerosis from the hospital of Molinette in Turin. Healthy tissues were used as control. All tissues were accessed with informed written patient consent and research ethics committee approval provided by University of Turin. Tissue was formalin fixed upon collection from patients and paraffin embedded for immunohistochemical analysis.

Immunofluorescence

Renal tissues from diabetic and healthy mice were embedded in paraffin and 5-µm-thick sections were subjected to immunofluorescence staining. Briefly, tissue sections were incubated in boiling citrate buffer (pH6) for 20 min for antigen retrieval, after which they were permeabilized with PBS-0.2% Triton X-100 (Cat. No. T8787; Sigma-Aldrich) for 8 minutes at 4°C or at room temperature (RT) respectively, depending on the specific primary antibodies. The slides were then blocked in PBS-3 % bovine serum albumin (BSA; Sigma-Aldrich) for 1 h at room temperature followed by incubation with primary antibodies overnight at 4 °C in a humidified chamber: sections were labeled for ERG (Cat. No. 97249S; 1:100; Cells Signalling), CD31 (Cat. No. 3528s; 1:100; Cells Signalling) and α-SMA (1:200; Sigma-Aldrich). The following day, the sections were washed with PBS-0,1% Triton and labelled with the respective fluorescence conjugated secondary antibodies for 1 h at RT in the dark in a humidified chamber. The secondary antibodies used are as follows: AlexaFluor 488-labeled chicken anti-rabbit (Cat. No. A21441; 1:1000; Invitrogen, Thermo Fisher Scientific), AlexaFluor 488-labeled rabbit anti-mouse (Cat. No. A11059; 1:1000; Invitrogen, Thermo Fisher Scientific) and Alexa Fluor 594-labeled chicken anti-goat (Cat. No. A21468; 1:1000; Life Tech, Thermo Fisher Scientific). Post incubation, the slides were washed twice followed by incubation with Hoechst 33258 dye (Sigma-Aldrich, 1:5000) for nuclear staining for 8 minutes. After the final wash, the coverslips were mounted with Fluoromount mounting medium (Fluromount Aqueous Mounting Medium, F4680, Sigma-Aldrich).

Glomerular expression of ERG or α-SMA in diabetic, ERG KO and control mice was calculated by counting ERG or α-SMA positive cells versus all cells stained with DAPI, from 12 random pictures per section at 400x magnification (expressed as mean values ± SEM). For ERG analysis we counted at least 50 cells stained with Dapi per glomerulus and then compared with cells positive for ERG (at least 12 glomeruli for sample). For α-SMA analysis we counted at least 150 glomeruli for sample and then compared with glomeruli positive for α-SMA (at least 12 random pictures for sample). We compared 3 mice (n=3) for every condition (DN mice vs healthy mice, ERG KO mice vs WT mice). Confocal microscopy analysis was performed using a Zeiss LSM 5 Pascal model confocal microscope (Carl Zeiss International).

Statistics

All data are reported as either mean ± SD or mean ± SEM. At least three replicates were performed for each experiment. Two-tailed or one-tailed Student's t-tests were performed for analysis when two groups of data were compared, while one-way ANOVA following Bonferroni or Dunnett's multiple comparison t-test was applied when comparing more than two groups of data. P-values of <0.05 were considered statistically significant (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.001). All statistical analyses were performed using GraphPad Prism software version 6.01 (GraphPad Software, Inc, La Jolla, CA, USA).

References:

Bernardini, M., Brossa, A., Chinigo, G., Grolez, G. P., Trimaglio, G., Allart, L., Hulot, A., Marot, G., Genova, T., Joshi, A., Mattot, V., Fromont, G., Munaron, L., Bussolati, B., Prevarskaya, N., Fiorio Pla, A., & Gkika, D. (2019). Transient Receptor Potential Channel Expression Signatures in Tumor-Derived Endothelial Cells: Functional Roles in Prostate Cancer Angiogenesis. *Cancers*, *11*(7). https://doi.org/10.3390/cancers11070956

Collino F., Bussolati B., Gerbaudo G., Marozio L., Pelissetto S., Benedetto C. and Camussi G. Preeclamptic sera induce nephrin shedding from podocytes through endothelin-1 release by endothelial glomerular cells *Am J Physiol Renal Physiol294*: F1185–F1194, 2008.First published February 20, 2008;

doi:10.1152/ajprenal.00442.2007

Conaldi PG, Biancone L., Bottelli A., De Martino A., Camussi G. and Toniolo A. Distinct pathogenic effects of group B coxsackieviruses on human glomerular and tubular kidney cells.*J Virol 71*: 9180–9187, 1997.

Dufton, N. P., Peghaire, C. R., Osuna-Almagro, L., Raimondi, C., Kalna, V., Chauhan, A., Webb, G., Yang, Y., Birdsey, G. M., Lalor, P., Mason, J. C., Adams, D. H., & Randi, A. M. (2017). Dynamic regulation of canonical TGFβ signalling by endothelial transcription factor ERG protects from liver fibrogenesis. *Nature Communications*, *8*(1), 895. https://doi.org/10.1038/s41467-017-01169-0

RESULTS

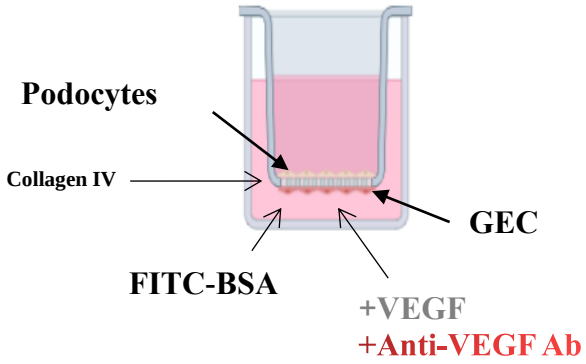
Setting of the *in vitro* permeability assay

In order to mimic the glomerular filtration barrier (GFB), an *in vitro* permeability assay was set up in which $50x10^3$ GECs and $50x10^3$ podocytes were seeded respectively on the under and upper side of a transwell of a 24 well-plate (Figure 1 a). Both surfaces of the transwell were coated with Collagen IV before seeding the cells. Therefore, FITC-BSA (1 mg/ml) was added to the lower chamber of the well and incubated for 6 hours at 37° C. Post incubation, transfer of FITC-BSA from the lower chamber to the upper chamber was measured by collecting the supernatant from the upper chamber and analyzed by a fluorescence reader.

VEGF inhibition increases permeability in the co-culture model

GECs were treated with VEGF (5 ng/ml) in the presence or absence of anti-VEGF antibody (0.12 μg/ml) for 24 h. As shown in Figure 1 b, the treatment with VEGF plus anti-VEGF Ab caused a significant rise in GECs permeability in comparison with control and with GECs treated with VEGF only, in which a subtle reduction in permeability was observed. Hence, the blocking of VEGF activity in GECs could lead to the loss of endothelial integrity, compromising ECs permeability.

a



b

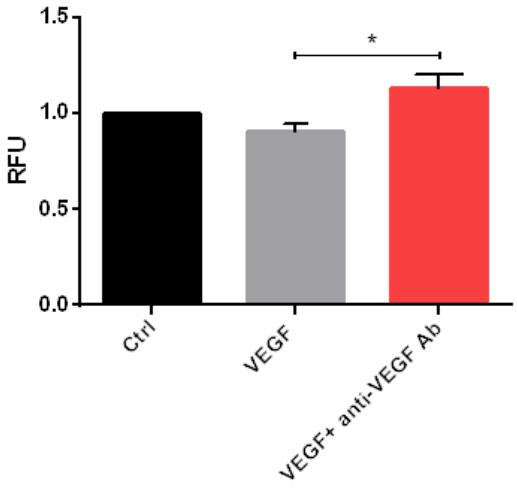
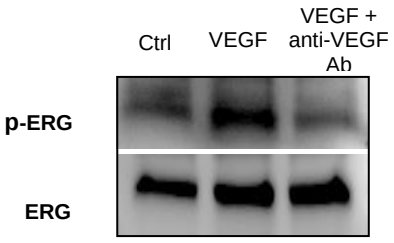


Figure 1 (a) Schematic representation of the *in vitro* co-culture model. GECs and podocytes were pre-seeded in the lower and upper part of a transwell chamber 24 h before the experiment. The transwell was coated at both side with Collagen IV. Lastly, FITC- BSA (1 mg/ml) was added in the lower part of the chamber, and the filtration, indicating albumin passage from the GECs compartment to the podocytes compartment, was measured over 6 h. **b) Anti-VEGF antibody treatment compromises permeability in GECs.** After 24h of co-culture, GECs were treated with VEGF (5 ng/ml) in the presence or absence of anti-VEGF Ab (0.12 µg/ml) GECs treated with VEGF plus anti-VEGF antibody show a significant increase in permeability (as observed through increased fluorescence in the upper chamber) compared to GECs treated VEGF only. Data are expressed as the mean fluorescence of FITC-BSA (collected from the upper chamber) from three independent experiments performed in triplicate. Statistical analysis was performed using one way ANOVA with Bonferroni's multiple comparison test *$p < 0.05$.

ERG activation in GEC is VEGF dependent

The next step was to understand the role of ERG in regulating permeability of GECs. In order to do this, we investigated the activation of ERG at a protein level following stimulation with VEGF. Briefly, GECs were treated with VEGF (5 ng/ml) in the presence or absence of anti-VEGF antibody (0.12 μg/ml) for 30 minutes. After the incubation period, cells were lysed in RIPA buffer and protein isolated and quantified. The total protein, 1 mg/ml from each treatment, was immunoprecipitated with anti-ERG antibody beads and subjected to Western blot analyses to identify phosphorylation of ERG. Experimental analyses revealed an increase in phosphorylated ERG (p-ERG) in GECs treated with VEGF. On the contrary, the anti-VEGF Ab treatment reverted the effect induced by VEGF on p-ERG expression, indicating that activation of ERG is VEGF dependent (Figure 2 a, b).

a



b

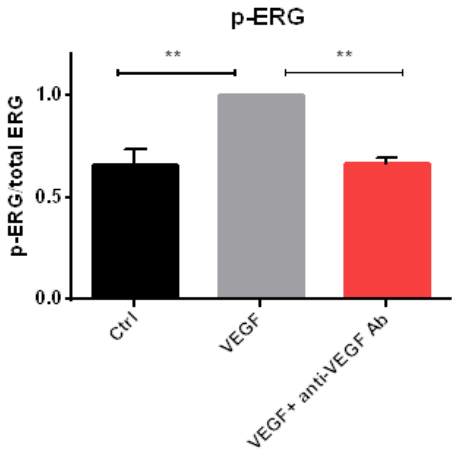


Figure 2. Treatment with VEGF induces phosphorylation of ERG in GECs. Western Blot representative images **(a)** and quantification **(b)** depicting phosphorylation of ERG in immunoprecipitated protein lysates from GECs treated with VEGF (5 ng/ml) or VEGF plus anti-VEGF antibody (0.12 µg/ml) for 24h. VEGF treatment activates ERG through phosphorylation, which is significantly reduced in the presence of anti-VEGF antibody. Data are expressed as mean ± SEM of band intensity normalized to ERG of three independent experiments. Statistical analysis was performed using one way ANOVA with Bonferroni's multiple comparison test $^{**}p < 0.01$.

Phosphorylation of ERG in GEC leads to the activation of the endothelial gene Delta-like 4 (DLL4)

To investigate the effects of ERG phosphorylation, we considered both molecular and protein expression of Dll4, a well-known endothelial protein crucial for ECs homeostasis and barrier function, and activated by ERG in HUVECs (Fish et al. 2017). GECs were treated with VEGF (5 ng/ml) in the presence or absence of anti-VEGF Ab (0.12 µg/ml) for 30 minutes. As showed in Figure 3 (a-c) VEGF treatment resulted in a significant upregulation of Dll4 both at gene (Figure 3 a) and protein level (Figure 3 b, c). On the other hand, GECs treated with anti-VEGF Ab antibody showed a significant reduction in Dll4 expression both at a gene (Figure 3 a) and protein level (Figure 3 b) compared to GECS treated with VEGF alone.

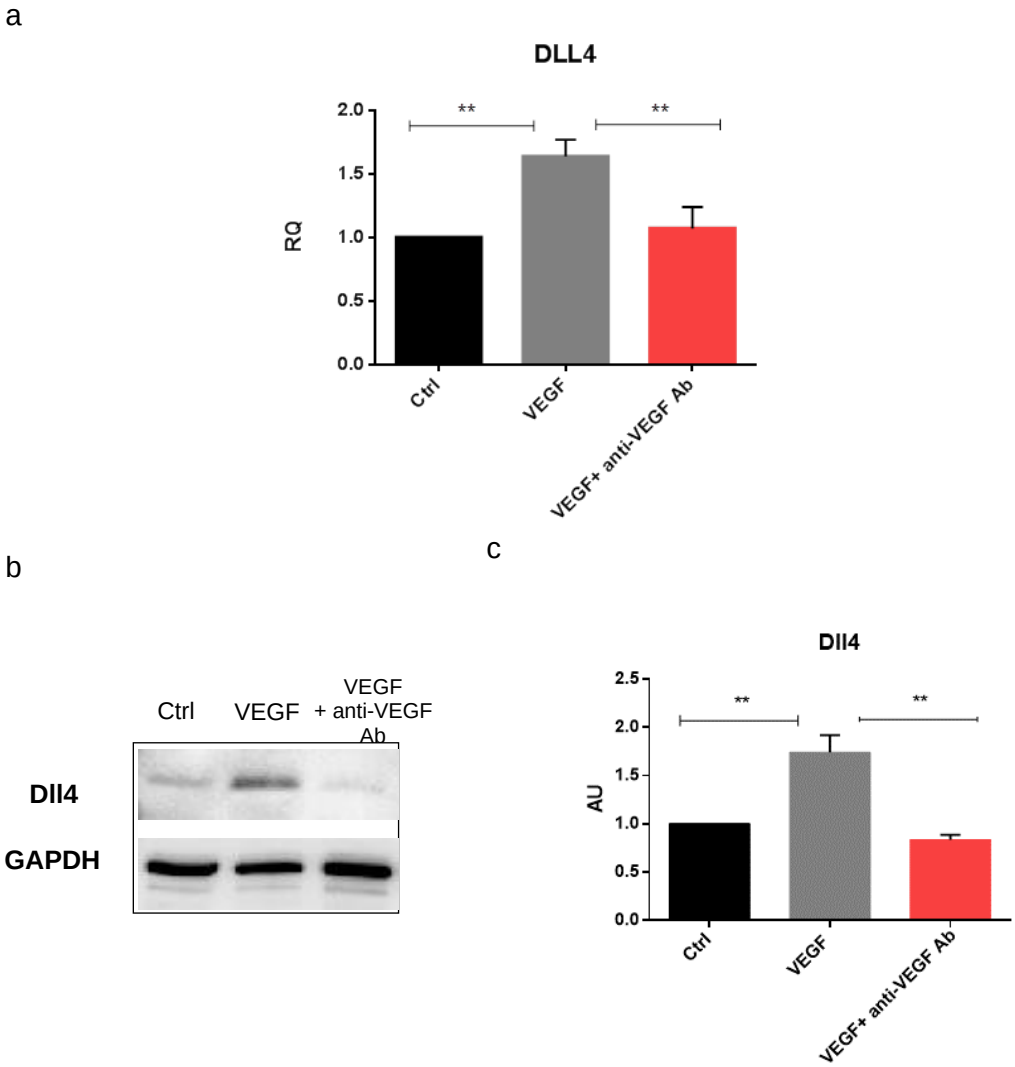


Figure 3. Treatment with VEGF induces upregulation of Dll4 in GECs. Pre-seeded GECs were treated with VEGF in the presence or absence of anti-VEGF antibody for 30 minutes. Following incubation, the RNA and protein were analyzed for the expression of Dll4. Data analysis revealed a significant upregulation of DLL4 gene **(a)**, and protein **(b, c)** in cells treated with VEGF which was significantly downregulated in the presence of anti-VEGF antibody. Data for RT-qPCR are shown as relative quantification normalized to β-Actin housekeeping gene and to control GECs from three independent experiments. Statistical analysis was performed using Dunnett's multiple comparison test **$p < 0.01$. The Western blot analysis (representative images and quantification) are expressed as mean ± SEM of band intensity normalized to GAPDH of three independent experiments. Statistical analysis was performed using one way ANOVA with Bonferroni's multiple comparison test **$p < 0.01$.

Transfection of GEC with siRNA ERG

To investigate the role played by ERG in VEGF mediated permeability of GECs, we downregulated the expression of ERG in ECs. Briefly, $50x10^3$ cells/well were seeded in a 24 well-plate (400 µl of GECs media) and treated with transfection mix (100 µl/well) composed of: negative control siRNA or siRNA ERG (20 µM – calculated to a final volume of 500 µl), the transfection agent HiPerfect (3.5 µl/well) and serum free media (100 µl /well) for 72 h at 37° C. The transfection mix was incubated at room temperature with all the components prior to adding it to the cells. Post experimental analysis revealed a significant downregulation of ERG both at a molecular level (72 h post transfection) (Figure 4 a), and at a protein level (6 days after transfection) (Figure 4 b, c) in cells transfected with siRNA ERG (ERG knock down GECs). No downregulation of ERG was observed in cells transfected with scramble siRNA.

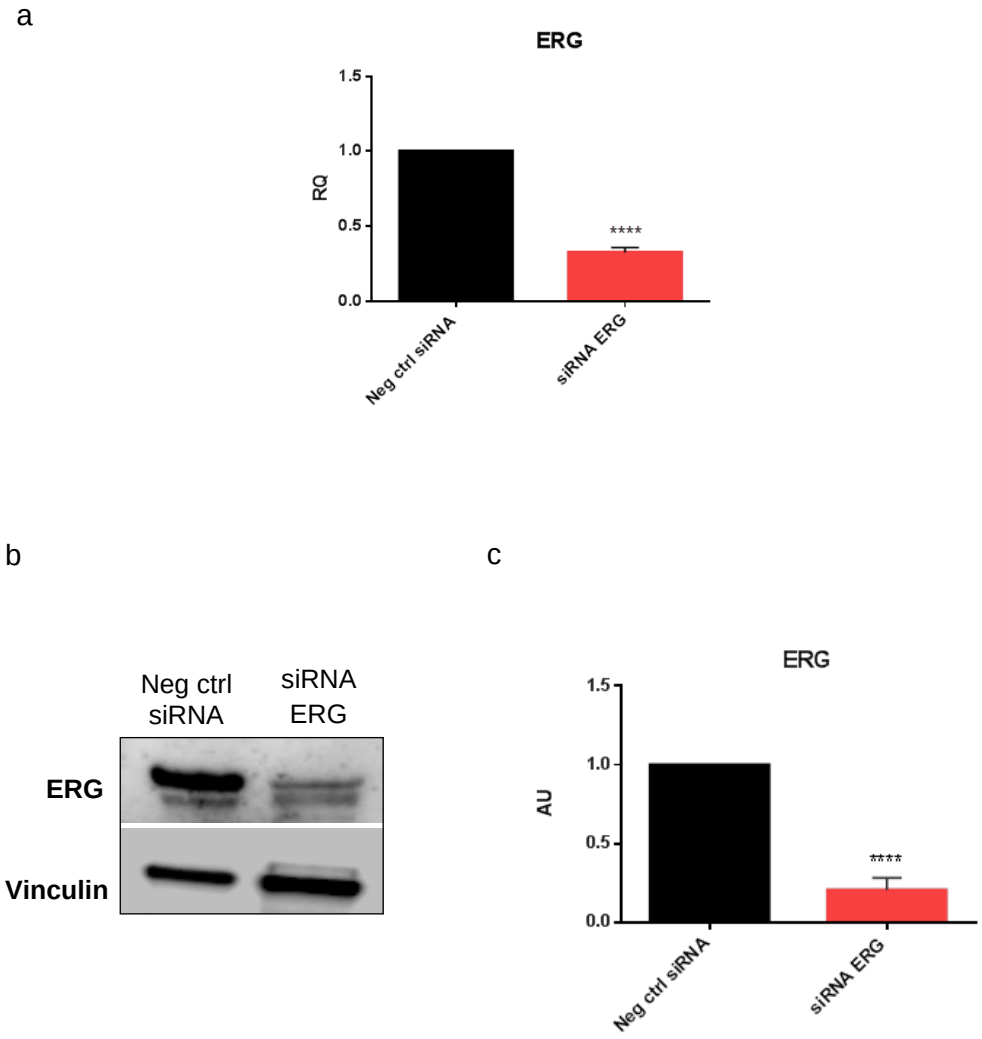
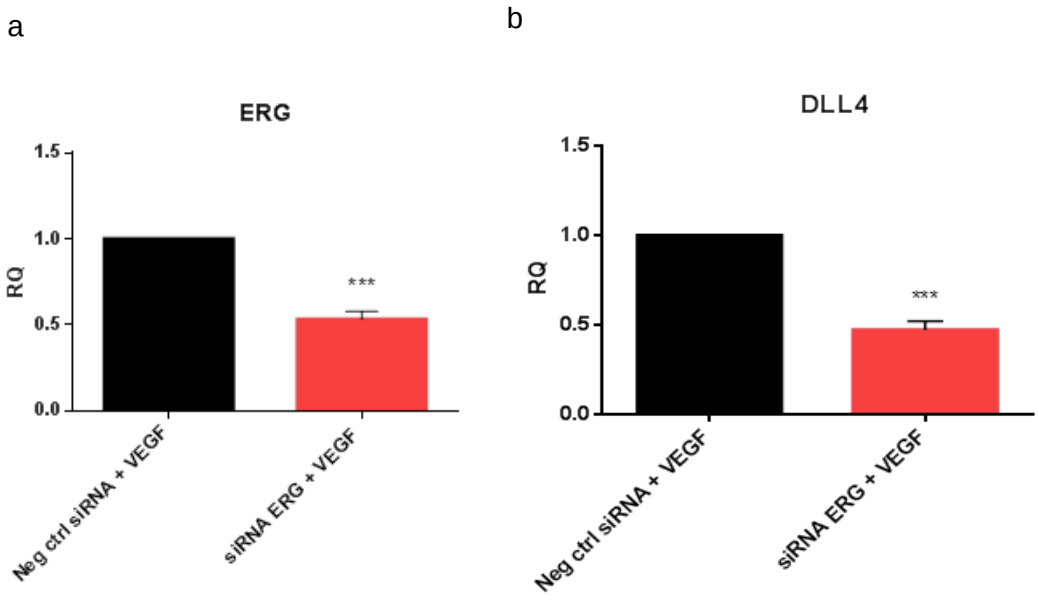


Figure 4. ERG expression is downregulated after siRNA at both molecular and protein level. GECs were treated with negative control siRNA or siRNA ERG (20 µM) for 72h. Post incubation, RNA from the treatments was subjected to RT-qPCR analysis to validate the silencing of ERG. **(a)** RT-qPCR analysis revealed a significant downregulation of the ERG gene in GECs transfected with siRNA ERG compared to GECs transfected with scramble siRNA (Neg ctrl siRNA). Data are shown as relative quantification, normalized to β-actin and to control GECs of three independent experiments. Statistical analysis was performed using Dunnett's multiple comparison test ****$p < 0.0001$. Western blot analysis (representative images **(b)** and quantification **(c)**) of transfected cells cultured under normal conditions 6 days post transfection revealed a significant downregulation of ERG protein in GECs transfected with siRNA ERG therefore confirming the efficiency of ERG inhibition over a long period of time. Data are expressed as mean ± SEM of band intensity normalized to Vinculin from three independent experiments. Statistical analysis was performed using Student's T-test ****$p < 0.0001$.

Knocking down of ERG in GECs prevents DLL4 activation

To confirm the essential role of ERG in VEGF induced Dll4 activation observed earlier, the expression of Dll4 was assessed in ERG deficient GECs post treatment with VEGF. After three and six days post transfection, GECs were treated with VEGF (5 ng/ml) for 30 minutes. Following treatment, RNA and protein from the cells were isolated and analyzed for both ERG and Dll4 expression. As showed in Figure 5, GECs silenced for ERG (Figure 5 a, d) failed to upregulate Dll4 expression after VEGF stimulation both at a molecular (Figure 5 b) and protein (Figure 5 c, f) level compared with GECs treated with negative control siRNA, confirming a crucial role of ERG in VEGF induced activation of Dll4.



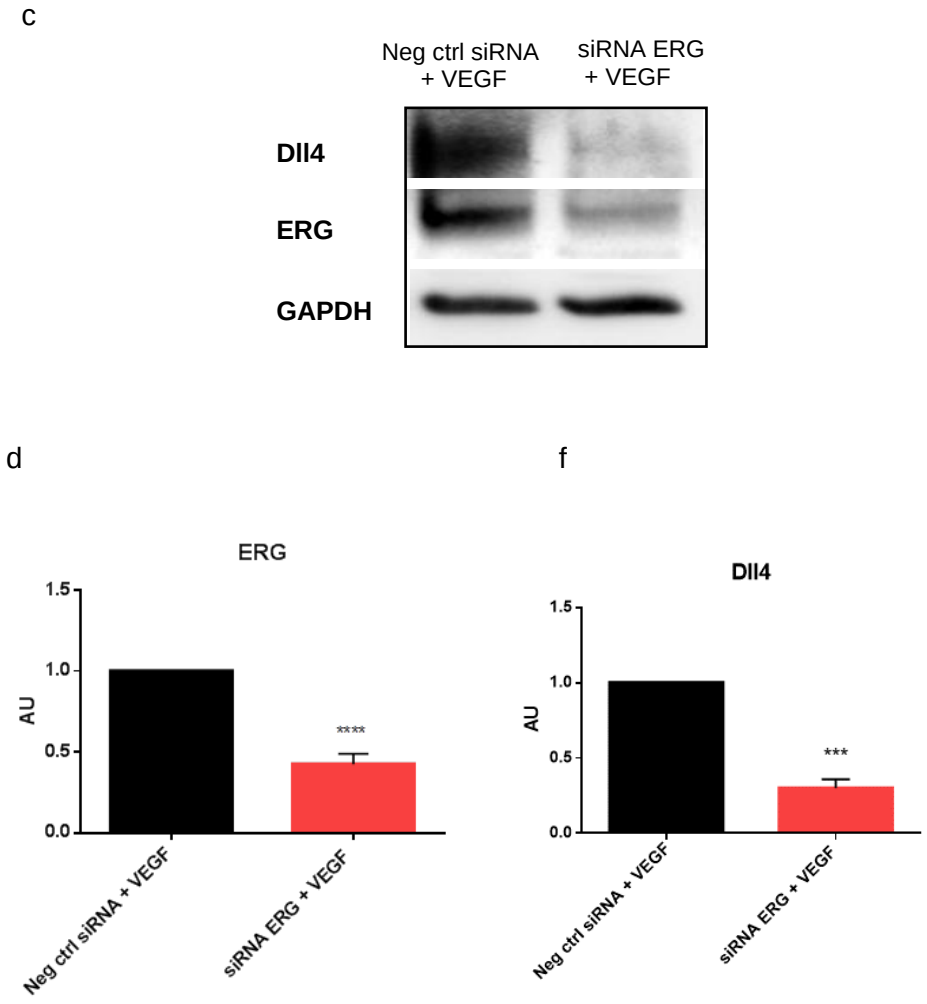


Figure 5. ERG is essential for Dll4 activation in GECs. GECs were treated with VEGF for 30 min three days or six days after transfection with siRNA ERG or negative ctrl siRNA. Post experimental analysis revealed a significant downregulation DLL4 **(b)** at a molecular level in GECs silenced for ERG **(a)** three days after transfection following VEGF treatment **(a)**. Data are shown as relative quantification, normalized to β-actin and to control GECs of three independent experiments. Statistical analysis was performed using unpaired Student's t-test. ***p < 0,0001. **(c)** A significant downregulation was also observed at a protein level in Dll4 (representative image and quantification) in ERG silenced GECs 6 days after transfection following VEGF treatment. Data are expressed as mean ± SEM of band intensity normalized to GAPDH of three independent experiments. Statistical analysis was performed using unpaired Student's t-test. ***p < 0,0001.

Knocking down of ERG increases VEGF induced permeability in GEC

After observing an increase in permeability in GECs following total inhibition of VEGF with an anti-VEGF antibody, we wanted to understand if ERG had a role to play in VEGF mediated permeability. Three days after transfection, we set a permeability assay plating GECs in the bottom side and podocytes on the upper side of the transwell. ERG knock down (KD) GECs or negative control siRNA were treated with or without VEGF (5 ng/ml) for 24 hours. Following treatment, the media in the lower chamber was replaced with fresh media supplemented with FITC-BSA (the upper chamber media was replaced with only fresh media) and incubated for a further 6 hours. Post incubation, the supernatant from the upper chamber was collected and analyzed in a fluorescence reader as mentioned before. Data analysis revealed a significant increase in permeability in GECs silenced for ERG following treatment with VEGF, compared to control GECs (GECs transfected with negative ctrl siRNA) treated with VEGF, suggesting that ERG may play a role in VEGF mediated permeability of GECs. In addition, the increase of permeability is observed also in ERG KD GECs treated with VEGF respect with control GECs (negative ctrl siRNA), indicating an interdependent role between ERG and VEGF towards the regulation of permeability (Figure 6).

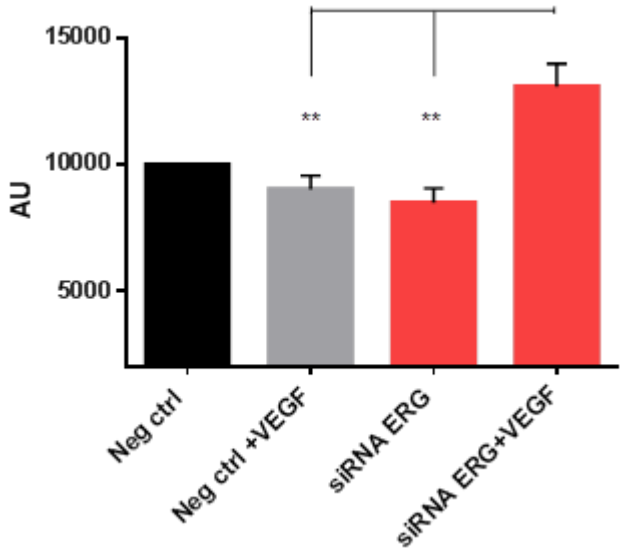


Figure 6. Knocking down of ERG compromises VEGF mediated permeability in GECs. 72 h post transfection with siRNA ERG or negative control siRNA GECs and podocytes pre-seeded in the lower and upper part of a transwell membrane respectively were treated with VEGF for 24h. ERG KD GECs treated with VEGF showed an increase in permeability compared to negative control siRNA GECs. Similarly, also ERG KD GECs treated with VEGF show an increase in permeability respect with negative ctrl siRNA ERG treated with VEGF. Data are expressed as the mean fluorescence intensity of filtered BSA-FITC from three independent experiments performed in triplicate. Statistical analysis was performed using one way ANOVA with Tukeys multiple comparison test $^{**}p < 0.01$.

The role of ERG in regulating EndMT in GECs

Hyperpermeability and endothelial dysfunction are common features of Diabetic nephropathy (DN), and one of the main causes of loss of function in DN is the phenomenon of endothelial to mesenchymal transition (EndMT). Since ERG is considered to be an important factor in the homeostasis of endothelial cells and our results demonstrated that knocking down of ERG in GECs led to hyperpermeability, we

wanted to investigate whether ERG would have a role to play in the process of EndMT. Following transfection of GECs, we analyzed the cells both at a molecular and protein level for the expression of well-known EndMT markers. Three days post transfection, we observed a significant upregulation of the EndMT genes: TGF-β1, TGF-β2, α-SMA and CNN-1 (Figure 7 a-d). Notably, an upregulation of prominent EndMT markers such as TGF-β1, TGF-β2 and α-SMA was also observed at protein level, six days after transfection (Figure 8a-c). Taken collectively, this data therefore suggest a significant role of ERG in the process of EndMT in GECs.

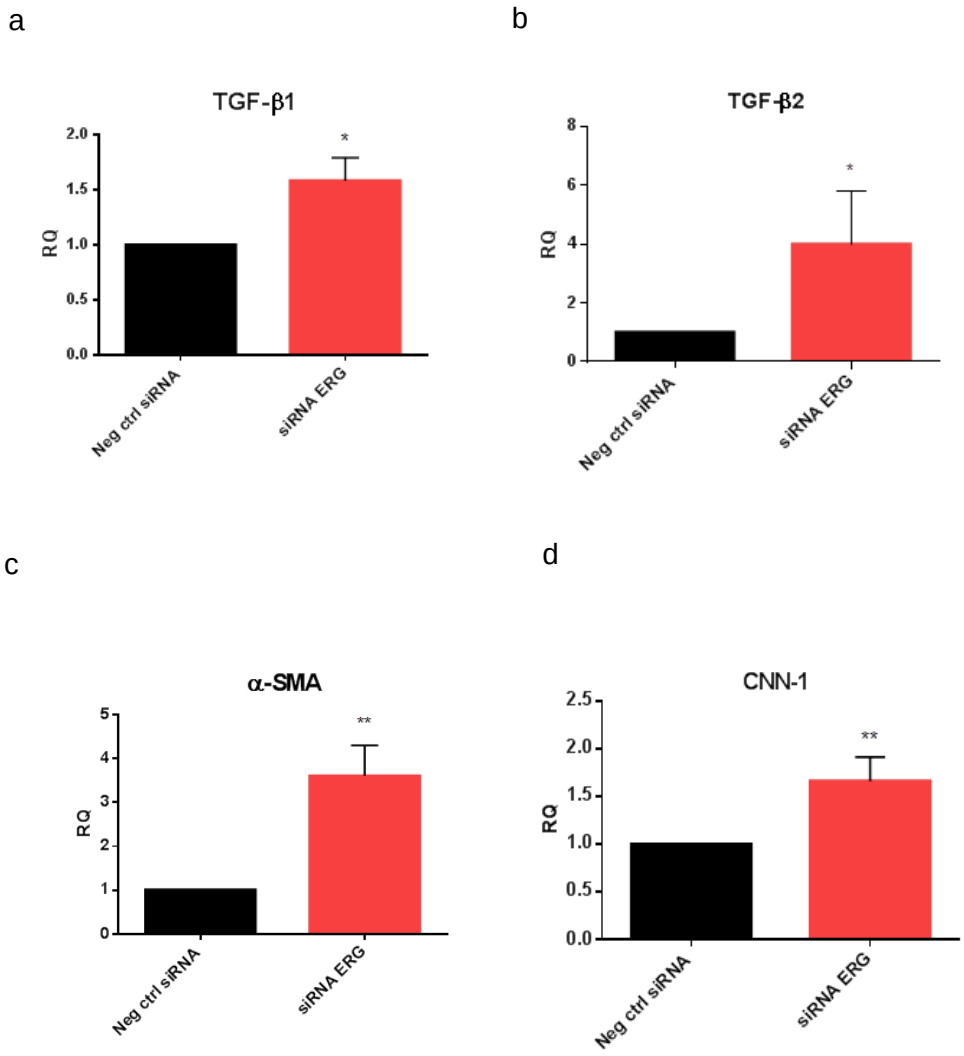


Figure 7 Silencing of ERG in GECs upregulates EndMT markers at a molecular level. RT-qPCR was performed on GECs three days after transfection with negative control siRNA or siRNA ERG. GECs treated with siRNA ERG show an upregulation of TGF-β1 (**a**), TGF-β2 (**b**), α-SMA (**c**) and CNN-1 (**d**) compared with GECs treated with negative control siRNA. Data are shown as relative quantification, normalized to β-Actin and to control GECs of three independent experiments. Statistical analysis was performed using unpaired Student's t-test. *$p < 0.05$, **$p < 0.01$.

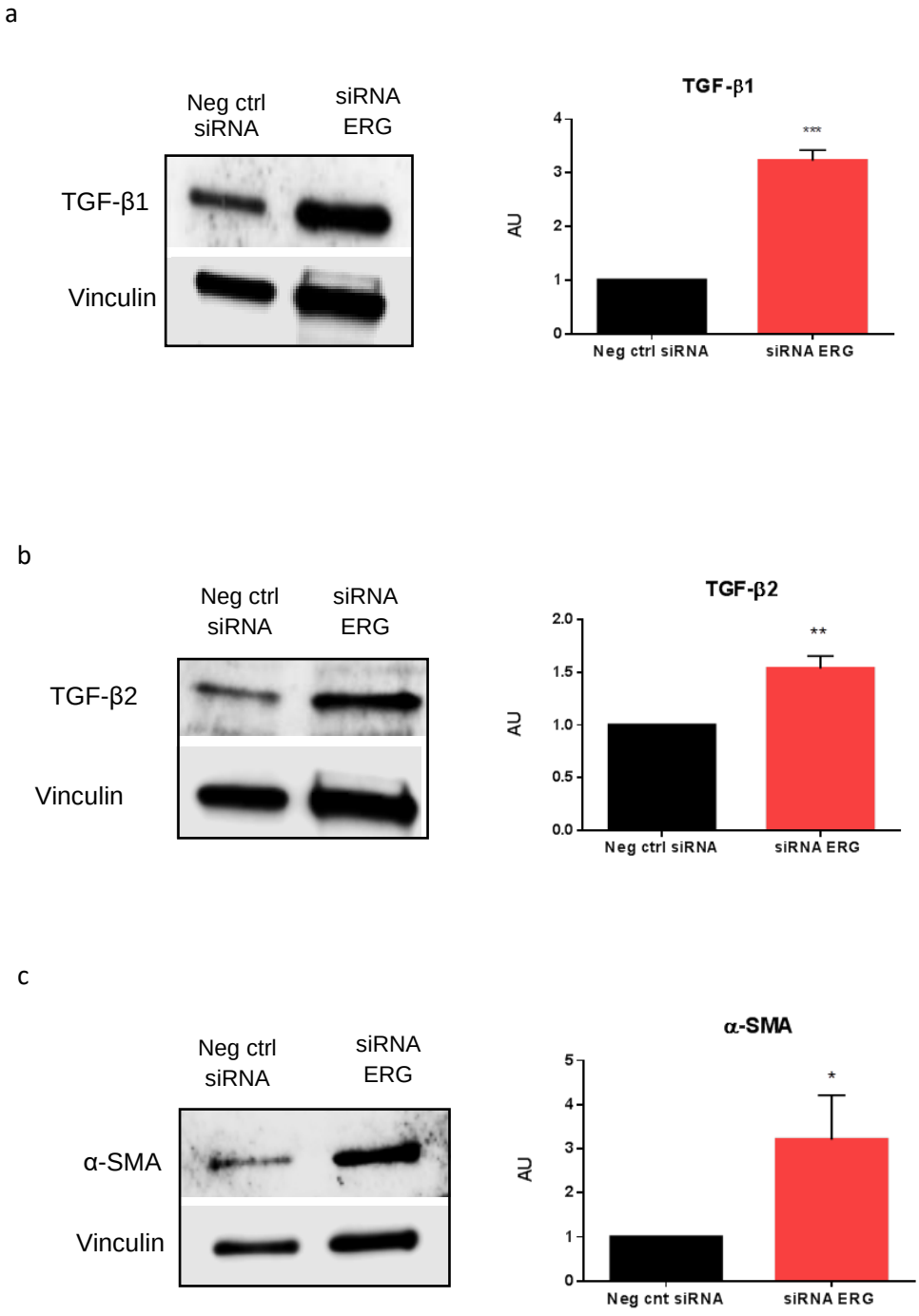
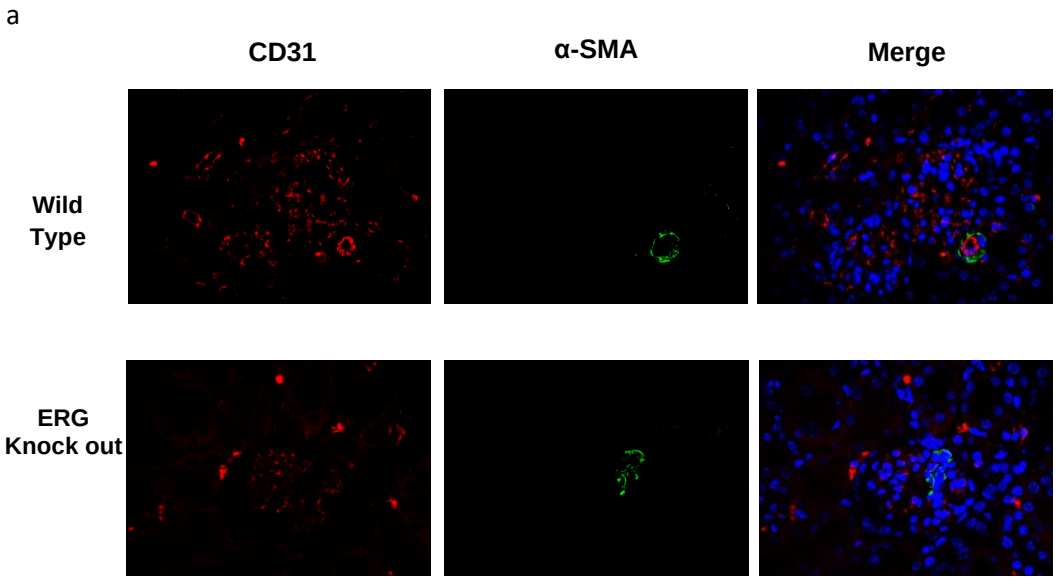


Figure 8. Silencing of ERG in GECs causes a protein upregulation of EndMT markers. Western blot analysis (representative images and quantification) revealed a significant upregulation of TGF-β1 (**a**), TGF-β2 (**b**), and α-SMA **(c)** in ERG KD GECs with respect to GECs transfected with a control siRNA, six days post transfection. Data are expressed as mean ± SEM band intensity normalized to Vinculin from three independent experiments. Statistical analysis was performed using unpaired Student's T-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$.

Endothelial to Mesenchymal transition in ERG Knock out mice

As EndMT was observed in GECs knocked down for ERG, we wanted to investigate whether this process was observed *in vivo* in mice permanently silenced for ERG: a Cre/LoxP strategy was used to develop a inducible EC-specific homozygous deletion of Erg using Tie2-Cre-Ergfl/+ ($Erg^{iEC\text{-}ko}$) (Birdsey et al. 2015). All animals used were retained on a C57BL/6 background. Immunofluorescence staining revealed the expression of the EndMT marker α-SMA only in glomeruli $Erg^{iEC\text{-}ko}$ mice compared to Wild Type (WT) mice (Figure 9 a,b). In particular, whereas control mice only expressed α-SMA in the smooth muscle cells of the peritubular arterioles, ERG KO mice expressed α-SMA in glomeruli also, as a focal process. These data confirm the role of ERG deletion in EndMT transition of GECs observed *in vitro*.



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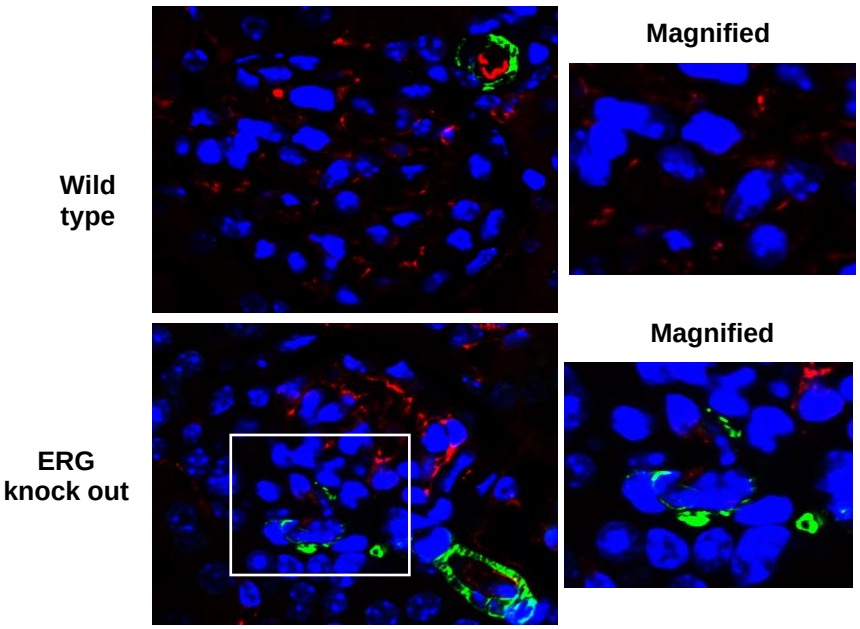

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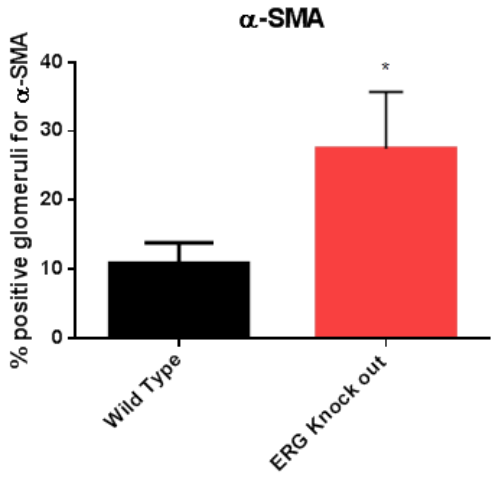

Figure 9. α-SMA is present in glomeruli of ERG knock-out mice ($Erg^{iEC\text{-}ko}$) (a,b) Immunofluorescence staining of α-SMA (green) and CD31 (red) in glomeruli from ERG KO mice (n=3) compared with Wild Type mice (n=3). DAPI for nuclear staining is in blue (Original magnification: 400×). **(a,b)** α-SMA is expressed in the glomeruli of ERG KO mice with respect to glomeruli of WT (in figure b, photos are magnified and α-SMA highlighted in white squares), in which α-SMA is expressed only in endothelial peritubular capillaries. **(c)** Histogram depicts the percentage of glomeruli positive for α-SMA in ERG KO mice compared to WT mice. Data represent mean ± SEM of percentage glomeruli positive for α-SMA versus total number of glomeruli measured by counting glomeruli from 12 images taken at random from 3 independent samples from ERG KO and WT mice. Statistical analysis was performed using unpaired Student's T-test *p <

Expression of ERG is reduced in glomeruli of mice with Diabetic nephropathy

As both EndMT and hyperpermeability are considered to be hallmarks of DN, and as our ERG knock out studies revealed an important role of ERG in regulating these processes in GECs, we wanted to evaluate the expression of ERG in a pathological setting such as diabetes *in vivo* whereby the kidney glomeruli are compromised. Briefly, male immunodeficient NSG (Nod SCID gamma) mice 6-8 weeks old were injected intraperitoneally with Streptozotomicin (STZ) (37 mg/kg) for 4 consecutive days and sacrificed one month after the onset of diabetes (Grange et al. 2019). Post sacrifice, kidneys were collected for histological, molecular and protein analysis (glomeruli from healthy mice served as controls). The principal histological features associated with DN that were observed in our model include: extensive mesangial expansion due to increased extracellular matrix production (Figure 10 D). In addition, Masson's trichrome staining revealed increased tubular damage and collagen deposition (blue fibers) in both glomerular and interstitial renal spaces (Figure 10 E) which are hallmarks of interstitial fibrosis. Moreover, PAS staining showed thickening of the glomerular basement membrane together with an increase in the Bowman's capsule space (Fig 10 F). The above mentioned features were absent in healthy mice which served as controls (Figure 10 A-C).

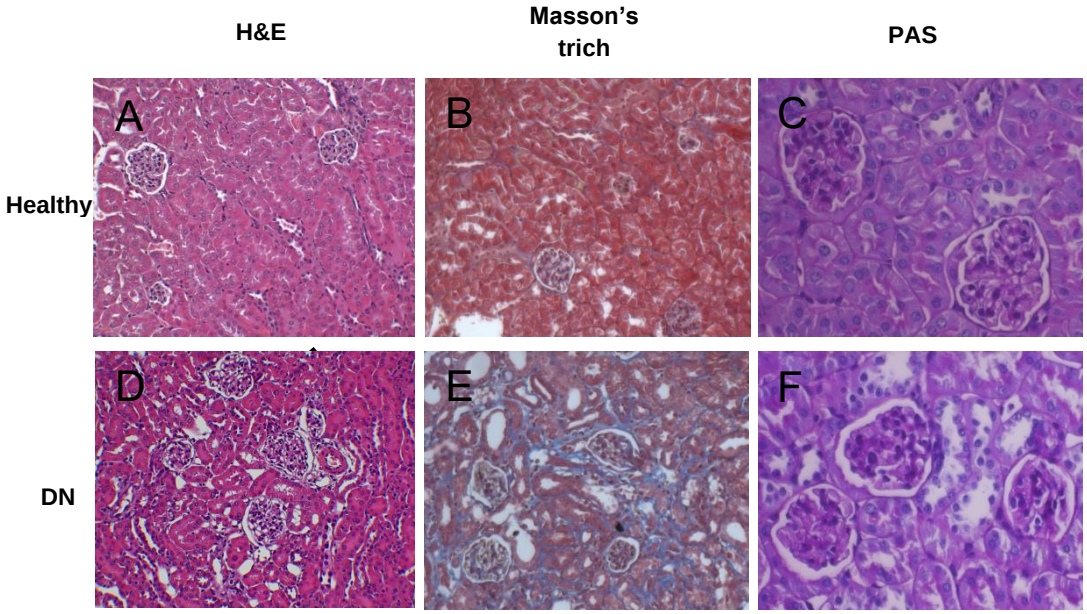

Figure 10. Representative images of renal hematoxylin and eosin (H&E) (A, D), Masson's trichrome (B, E) and PAS (C, F) stained sections of healthy and diabetic mice (magnification: 200X, 200X, 400X respectively). The principal histological changes features associated to DN are: increased extracellular matrix production **(D)**, increased tubular damage and collagen deposition (blue fibers) in both glomerular and interstitial renal spaces shown by Masson's trichrome staining **(E)**, and thickening of the glomerular basement membrane together with an increase of Bowman's capsule space revealed by PAS staining **(F)**. None of these features are found in healthy mice sections **(A, B, C)**.

Regarding the molecular analysis, a significant downregulation in the expression of ERG gene in glomeruli of diabetic mice compared to healthy controls was observed (Figure 11 a). On the other hand, there was an upregulation of the EndMT gene SNAIL1 in the diabetic glomeruli compared to healthy controls (Figure 11 b). Moreover, ERG was also found to be downregulated at a protein level in the glomeruli of DN mice compared to healthy controls as observed by Western blot analysis, calculated as the mean of ERG expression normalized versus total VE-cadherin of diabetic mice experimental group (n=6) versus healthy mice experimental group (n=6) (Figure 11 d,e). In addition, this was further confirmed via immunofluorescence

staining of ERG in kidney tissues of diabetic and healthy mice. As shown in Figure 11 f,g ERG expression (highlighted by white circles) in the glomeruli of diabetic mice is significantly reduced with respect to the glomeruli of control mice. These results therefore suggest the involvement of ERG in DN, indicating ERG downregulation as a possible marker of endothelial damage and as a partial contributor to pathological processes such as hyperpermeability and EndMT observed in DN.

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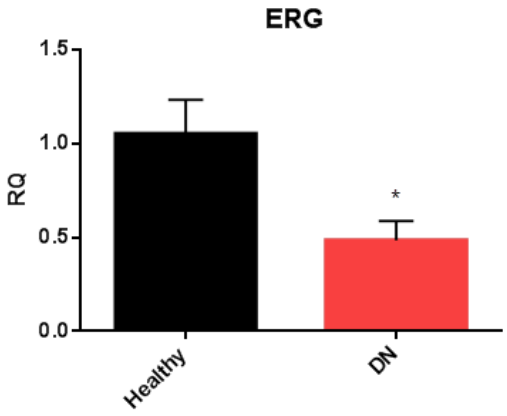

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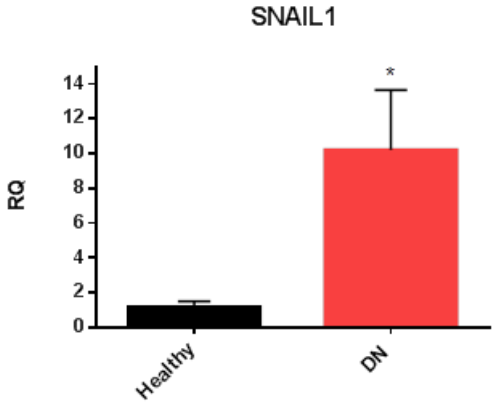

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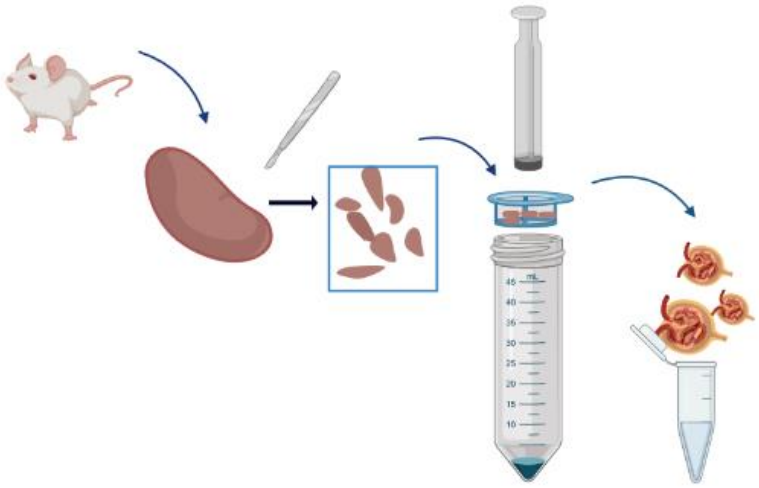

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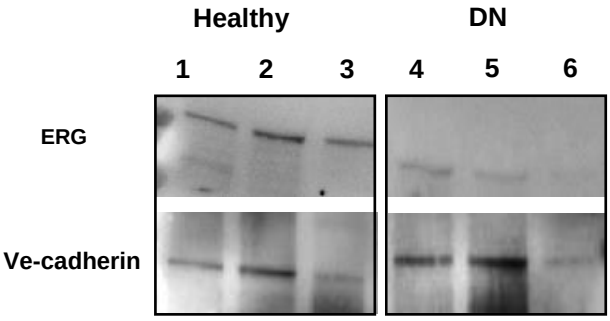

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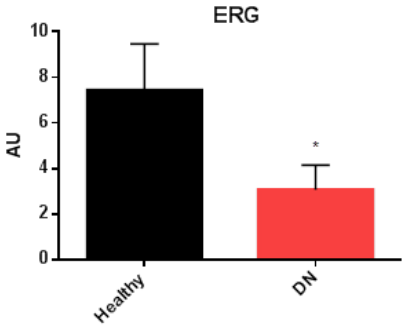

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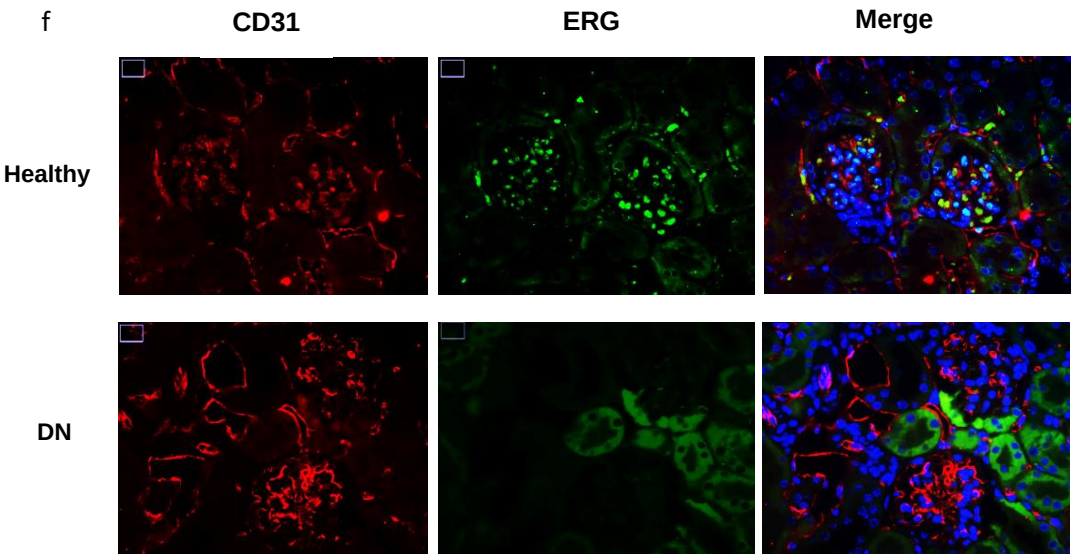
CD31
ERG
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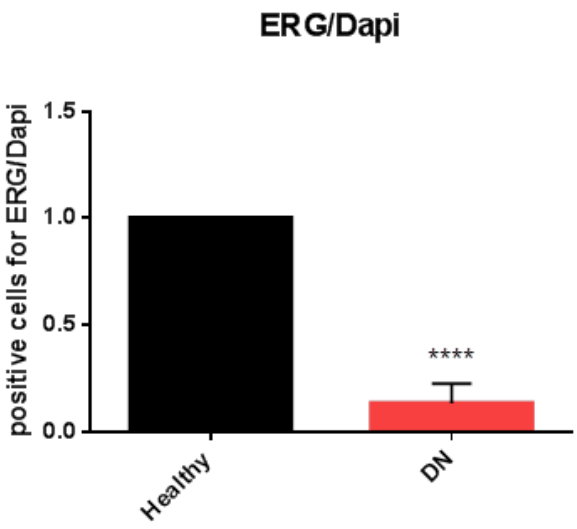
g


ERG/Dapi
positive cells for ERG/Dapi
1.5
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Healthy
DN

Figure 11. (a, d, e) Molecular and protein expression of ERG in glomeruli of mice with diabetic nephropathy is reduced compared to healthy mice's glomeruli. **(a)** RT-qPCR of glomeruli extracted from diabetic mice showed a downregulation in the ERG gene **(b) SNAIL1 molecular expression is upregulated in DN mice compared to controls.** Data are expressed as mean ± SEM of n=6 mice per treatment. Statistical analysis was performed using unpaired Student's t-test *p < 0.05**. (c) Schematic representation of glomeruli isolation from mouse kidney.** After kidney removal from mice, kidneys were minced into small pieces and pressed in a 100 μm cells strainer using a syringe plunger. After three washes with PBS, the glomeruli which remain on the strainer were collected and resuspended in RIPA buffer. **(d, e)** Western blot analysis (representative images and quantification) shows a downregulation of ERG expression in diabetic mice with respect to controls. Data are expressed as mean ± SEM of band intensity normalized to Ve-cadherin of n=6 mice per treatment. Statistical analysis was performed using Student's unpaired t-test. *p < 0.05. The glomeruli were extracted from 12 mice (6 diabetic and 6 controls). **(f)** Immunofluorescent staining of ERG (green) and CD31 (red) shows a decreased expression of ERG in glomeruli from diabetic mice (n=3) compared to control mice (n=3). DAPI for nuclear staining is in blue. (Original magnification: 400×). The expression of ERG in DN mice is decreased respect with ERG expression in healthy mice which is abundant. **(g)** Histogram depicting cells positive for ERG with respect to total number of cells in the glomeruli from diabetic and control experimental groups (n=3). Data represent mean ± SEM of cells positive for ERG versus total cells stained with DAPI measured from 12 images taken in a high-power field at random. Statistical analysis was performed using Student's unpaired t-test ****p < 0.0001.

Expression of ERG is reduced in the glomeruli of patients with diffuse diabetic glomerulosclerosis (DGS)

Since we observed ERG downregulation in glomeruli of diabetic mice, we wanted to further investigate whether ERG would be downregulated in the glomeruli of patients with diffuse diabetic glomerulosclerosis (DGS). It consists of a gradual and inexorable scarring of the renal glomerulus, characterized by thickening of the glomerular basement membrane with increased permeability (Qian et al. 2008). Through immunofluorescence staining, we found a downregulation in the expression of ERG in the glomeruli of patients with DGS compared to healthy patients (Figure 12 a,b). ERG downregulation in sclerotic glomeruli could further confirm the link between the downregulation of ERG and EndMT.

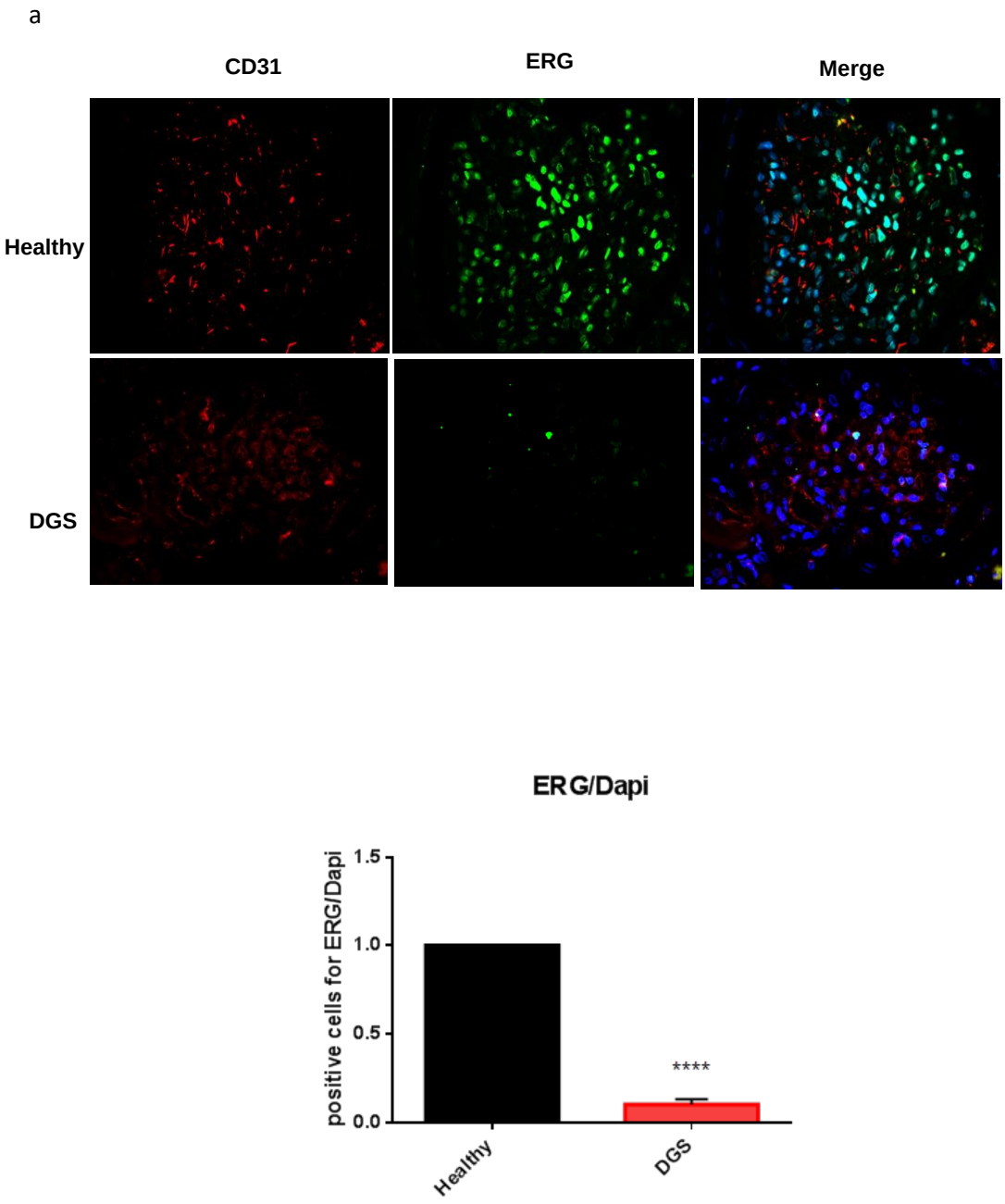

Figure 12. The expression of ERG is reduced in the glomeruli of patients with DGS compared with healthy patients. (a) Immunofluorescence staining of ERG (green) and CD31 (red) in a glomerulus from patients with diffuse diabetic glomerulosclerosis (n=3) compared with one from healthy patients (n=3). DAPI for nuclear staining is in blue (Original magnification: 400×). **(b)** Histogram depicting cells positive for ERG with respect to total cells in the human glomerulus from diabetic and control experimental groups (n=3). Data represents mean ± SEM of cells positive for ERG versus cells stained with DAPI measured from 12 images taken on a high-power field at random from 3 samples per experimental group. Statistical analysis was performed using Student's unpaired T-test. Statistical analysis was performed using Student's unpaired t-test ****$p < 0.0001$.

References:

Birdsey, Graeme M, Aarti V Shah, Neil Dufton, Louise E Reynolds, Lourdes Osuna Almagro, Youwen Yang, Irene M Aspalter, et al. 2015. "The Endothelial Transcription Factor ERG Promotes Vascular Stability and Growth through Wnt/β-Catenin Signaling." *Developmental Cell* 32 (1): 82–96. https://doi.org/10.1016/j.devcel.2014.11.016.

Fish, Jason E, Manuel Cantu Gutierrez, Lan T Dang, Nadiya Khyzha, Zhiqi Chen, Shawn Veitch, Henry S Cheng, et al. 2017. "Dynamic Regulation of VEGF-Inducible Genes by an ERK/ERG/P300 Transcriptional Network." *Development (Cambridge, England)* 144 (13): 2428–44. https://doi.org/10.1242/dev.146050.

Grange, Cristina, Stefania Tritta, Marta Tapparo, Massimo Cedrino, Ciro Tetta, Giovanni Camussi, and Maria Felice Brizzi. 2019. "Stem Cell-Derived Extracellular Vesicles Inhibit and Revert Fibrosis Progression in a Mouse Model of Diabetic Nephropathy." *Scientific Reports* 9 (1): 4468. https://doi.org/10.1038/s41598-019-41100-9.

Qian, Ying, Eva Feldman, Subramanian Pennathur, Matthias Kretzler, and Frank C Brosius 3rd. 2008. "From Fibrosis to Sclerosis: Mechanisms of Glomerulosclerosis in Diabetic Nephropathy." *Diabetes* 57 (6): 1439–45. https://doi.org/10.2337/db08-0061.

DISCUSSION

In the current work, we report the role of the transcription factor ERG in glomerular endothelial function in physiological and pathological conditions, both *in vitro* and *in vivo*. We show for the first time the downregulation of ERG in the glomeruli of kidneys from mice with diabetic nephropathy and in kidneys of patients with diffuse diabetic glomerulosclerosis. We also reveal the functional role of ERG in the highly specialized glomerular endothelial cells (GEC), wherein it is involved in the regulation of VEGF induced permeability and activation of genes such as DLL4. In particular, DLL4 has an important role in sprouting angiogenesis and switching of endothelial cells (ECs) into migratory tip cells or proliferating stalk cells (DLL4-NOTCH 1 signalling pathway) (Tiemeijer et al. 2018). Lastly, we also report for the first time in literature, the regulatory role of ERG in the endothelial to mesenchymal transition (EndMT) in GECs, through knock-down experiments *in vitro,* and *in vivo* in a model of ERG knock-out mice.

Previous works have shown ERG to be a crucial regulator of endothelial homeostasis, particularly influencing multiple endothelial genes and pathways as a promoter/enhancer. For instance, Fish et al. found that the VEGF-induced activation of MAPK through the NOTCH/VEGF/MAPK cascade, was ERG dependent. Moreover, this transcriptional pathway was responsible for inducing DLL4 which is one of the earliest essential ligands for endothelial specification, therefore confirming a significant role of ERG in vascular homeostasis (Fish, Gutierrez, and Wythe 2016). In another study, Birdsey at al. described the regulatory role of ERG in the Wnt/β-catenin pathway which is involved in maintaining vascular stability (Birdsey et al. 2015). They demonstrated that downregulation of ERG in HUVECs decreased Wnt signalling by regulating transcription of both CDH5 (Ve-cadherin) and the Wnt receptor Frizzled-4. Moreover, they also reported that constitutive endothelial deletion of ERG (Erg $^{cEC\text{-}KO}$) in mice, led to embryonic lethality with vascular defects, whereas an inducible endothelial deletion of ERG (Erg $^{iEC\text{-}KO}$) caused pathological angiogenesis in the postnatal retina and compromised vascular stability. On the other hand, an over-expression of ERG *in vivo* reduced permeability and increased stability of VEGF-induced angiogenesis. Furthermore, ERG has also been shown to mediate

junctional integrity and stability of ECs by binding to the promoter genes of both endothelial adherent glycoproteins and tight junction proteins. For instance, a significant downregulation of VE-cadherin was observed after silencing ERG gene in HUVECs, which led to changes in cell structure, loss of cell-to-cell contact, angiogenesis inhibition, and apoptosis ultimately compromising endothelial permeability (Birdsey et al. 2015). In this case, apoptosis was partially rescued by the overexpression of VE-cadherin, suggesting it could play a role in the ERG-dependent survival signals. These results are in line with the data of the present study, showing the essential role of ERG in glomerular endothelial cells homeostasis.

Here, we report similar findings in GECs, where treatment with VEGF led to an increased expression of phosphorylated ERG, which was abrogated in the presence of anti-VEGF antibody. A similar trend was also observed with Dll4, as VEGF increased the activation of Dll4 both at a gene and protein level and was abolished in the presence of anti-VEGF antibody, thus confirming pre-existing results (Fish et al. 2017). The role of VEGF as a key regulator of vascular permeability in the kidney is very well known (Esser et al. 1998). VEGF-A is mainly produced by podocytes and is required, by the adjacent GECs, to regulate permeability through paracrine signalling, via VEGF-R2 activation (Bartlett, Jeansson, and Quaggin 2016). We observed, after treating cells with VEGF together with the anti-VEGF antibody, an increased permeability of GECs in a significant way. We speculate that the anti-VEGF antibody sequestered the VEGF produced by podocytes, which were cultured together with the GECs in the permeability assay. Indeed, ERG silencing in GECs, exhibited a significant increase in permeability following treatment with VEGF, compared to negative control siRNA. In addition, this effect was VEGF-mediated, as no increase in permeability was observed in ERG silenced GECs in the absence of VEGF; therefore, not only it was confirmed the interdependence of ERG and VEGF, but also a regulatory role of ERG in VEGF-mediated permeability.

Hyperpermeability, together with glomerular endothelial swelling and proteinuria, are some of the main features of several kidney pathologies, like diabetic nephropathy (DN). Moreover, cultured GECs stimulated with high levels of glucose, exhibit an increase in permeability (Peng et al. 2016). Taking these data into consideration, together with our recent findings on the role of ERG in

VEGF-mediated permeability in GECs, we decided to investigate if ERG had a role to play in DN. Here, we were able to demonstrate for the first time *in vivo,* that the expression of ERG is significantly downregulated in the glomeruli of diabetic mice compared to healthy animals, both at molecular and protein level. In addition, this finding was extended to the glomeruli of patients with diffuse diabetic glomerulosclerosis, wherein ERG was found to be downregulated compared to healthy patients. One explanation for this result could be the onset of endothelial to mesenchymal transition (EndMT), which is commonly observed in these pathologies. For instance, Peng et al. reported that GECs exposed to high glucose (HG) conditions *in vitro*, ECs of diabetic mice kidneys, as well as ECs of patients with DN, all underwent EndMT; as such, typical endothelial marker CD31 was downregulated and an increase in the mesenchymal marker α-SMA was observed. EndMT is a gradual, reversible, and dynamic inflammatory-like process, which is correlated with DN, resulting in hyperfiltration and albuminuria. In response to injury, ECs are converted to mesenchymal-like-cells, losing their endothelial characteristics and also downregulating their typical markers, like VE-cadherin, platelet/EC adhesion molecule-1 (CD31/PECAM-1), and von Willebrand factor (vVF), and acquiring a mesenchymal profile including markers such as N-cadherin, α-SMA, vimentin, fibronectin among others which eventually contributes to interstitial fibrosis in DN and other kidney diseases.

Notably, transforming growth factor β (TGF-β), a multifunctional cytokine with pleiotropic physiological roles, is one of the most prominent EndMT inducers. The regulation of TGF-β receptor balance, between SMAD1 and SMAD3 signalling, is crucial in maintaining ECs homeostasis. A recent study by Dufton at al., reported that ERG could play a key role in maintaining this balance, by promoting the SMAD1 pathway and repressing SMAD2/3 activity. Indeed, they revealed that a reduction of ERG expression in HUVECs *in vitro,* as well as knock out of ERG in endothelial cells *in vivo* in hemi-deficient (ERG $^{cEC\text{-}Het}$) and inducible homozygous deficient (ERG $^{iEC\text{-}KO}$) mice, led to spontaneous liver fibrosis, in a SMAD2/3 dependent manner (Dufton et al. 2017). Furthermore, they also observed a correlation between loss of ERG and EndMT in tissues from patients with end-stage liver fibrosis. These findings therefore revealed a correlation between ERG and

EndMT at a cellular and tissue level. Based on these results, we investigated whether GECs would undergo EndMT after ERG knock down (KD), and whether EndMT was extended to the kidneys of ERG knockout (KO) mice. Our studies showed that, after knocking down of ERG in GECs, a significant upregulation of well-known mesenchymal markers such as TGF-β1, TGF-β2, α-SMA and CNN-1 was observed 3 days post siRNA ERG transfection at a molecular level. Interestingly, expression of TGF-β1, TGF-β2 and α-SMA was observed at a protein level 6 days post transfection, suggesting an essential role of ERG in regulating EndMT in GECs. *In vivo*, we found an upregulation of α-SMA expression in the glomeruli of ERG knockout (ERG $^{iEC\text{-}KO}$) mice compared to wild type (WT) mice, therefore suggesting spontaneous EndMT in the glomeruli in a focal and segmental pattern. In addition, we also observed a significant upregulation of the EndMT marker SNAIL1 at a gene level in the glomeruli of mice with diabetic nephropathy.

One of the limitations of this work is the understanding of how ERG is downregulated in diabetic nephropathy: we observed clearly that ERG is downregulated in the glomeruli of diabetic mice and patients but we could not study the pathways or molecular mechanism this downregulation is achieved, due to time constraints. Moreover, we also observed an upregulation of SNAIL1, which was recently reported to be a prerequisite for EndMT (Kokudo et al. 2008). However, as the upregulation of SNAIL1 cannot solely be attributed to a downregulation of ERG, other pathological factors can also contribute towards EndMT, which need to be investigated. In addition, regarding EndMT following silencing of ERG in GEC, we found an upregulation of EndMT genes and protein such as TGF-β1, TGF-β2, α-SMA and CNN-1 but we could not observe a downregulation of typical endothelial markers. We speculate that, as EndMT is a transition process, endothelial genes may require a longer period to be downregulated, as mesenchymal are manifested. As this work is at an early stage, further studies are required to understand the complex role of ERG in GEC both *in vitro* and *in vivo*, in physiological and pathological environments.

In conclusion, we elucidate for the first time the role of ERG in the homeostasis of GEC, observing that ERG has a key role in the regulation of glomerular filtration, in a VEGF dependent manner. In addition, we also showed how the downregulation of ERG *in vitro* and *in vivo*, through silencing or knock-out, could contribute towards both glomerular endothelial permeability dysfunction and the process of EndMT, both of which are correlated in kidney diseases such as diabetic nephropathy. Indeed, we also report a downregulation of ERG in the glomeruli of mice with DN at both molecular and protein level. Therefore, ERG may represent a promising biomarker to monitor glomerular endothelial pathologies and its modulation could be of interest for glomeruli related pathologies. Nonetheless, further work is required to understand the regulatory function of ERG in DN and the pathways it mediates.

References:

Bartlett, Christina S, Marie Jeansson, and Susan E Quaggin. 2016. "Vascular Growth Factors and Glomerular Disease." *Annual Review of Physiology* 78: 437–61. https://doi.org/10.1146/annurev-physiol-021115-105412.

Birdsey, Graeme M, Aarti V Shah, Neil Dufton, Louise E Reynolds, Lourdes Osuna Almagro, Youwen Yang, Irene M Aspalter, et al. 2015. "The Endothelial Transcription Factor ERG Promotes Vascular Stability and Growth through Wnt/β-Catenin Signaling." *Developmental Cell* 32 (1): 82–96. https://doi.org/10.1016/j.devcel.2014.11.016.

Blanco, Raquel, and Holger Gerhardt. 2013. "VEGF and Notch in Tip and Stalk Cell Selection." *Cold Spring Harbor Perspectives in Medicine* 3 (1). https://doi.org/10.1101/cshperspect.a006569.

Dufton, Neil P, Claire R Peghaire, Lourdes Osuna-Almagro, Claudio Raimondi, Viktoria Kalna, Abhishek Chauhan, Gwilym Webb, et al. 2017. "Dynamic Regulation of Canonical TGFβ Signalling by Endothelial Transcription Factor ERG Protects from Liver Fibrogenesis." *Nature Communications* 8 (1): 895. https://doi.org/10.1038/s41467-017-01169-0.

Esper, Ricardo J, Roberto A Nordaby, Jorge O Vilariño, Antonio Paragano, José L Cacharrón, and Rogelio A Machado. 2006. "Endothelial Dysfunction: A

Comprehensive Appraisal." *Cardiovascular Diabetology* 5 (February): 4. https://doi.org/10.1186/1475-2840-5-4.

Esser, Sybille, Karen Wolburg, Hartwig Wolburg, Georg Breier, Teymuras Kurzchalia, and Werner Risau. 1998. "Vascular Endothelial Growth Factor Induces Endothelial Fenestrations in Vitro." *Journal of Cell Biology* 140 (4): 947–59. https://doi.org/10.1083/jcb.140.4.947.

Fish, Jason E, Manuel Cantu Gutierrez, Lan T Dang, Nadiya Khyzha, Zhiqi Chen, Shawn Veitch, Henry S Cheng, et al. 2017. "Dynamic Regulation of VEGF-Inducible Genes by an ERK/ERG/P300 Transcriptional Network." *Development (Cambridge, England)* 144 (13): 2428–44. https://doi.org/10.1242/dev.146050.

Fish, Jason E, Manuel Cantu Gutierrez, and Joshua D Wythe. 2016. "Dynamic Regulation of VEGF-Inducible Genes by an ERK / ERG / P300 Transcriptional Network" 4.

Goumans, Marie-José, Zhen Liu, and Peter ten Dijke. 2009. "TGF-β Signaling in Vascular Biology and Dysfunction." *Cell Research* 19 (1): 116–27. https://doi.org/10.1038/cr.2008.326.

Kokudo, Takashi, Yuka Suzuki, Yasuhiro Yoshimatsu, Tomoko Yamazaki, Tetsuro Watabe, and Kohei Miyazono. 2008. "Snail Is Required for TGFbeta-Induced Endothelial-Mesenchymal Transition of Embryonic Stem Cell-Derived Endothelial Cells." *Journal of Cell Science* 121 (Pt 20): 3317–24. https://doi.org/10.1242/jcs.028282.

Lal, B K, S Varma, P J Pappas, R W 2nd Hobson, and W N Durán. 2001. "VEGF Increases Permeability of the Endothelial Cell Monolayer by Activation of PKB/Akt, Endothelial Nitric-Oxide Synthase, and MAP Kinase Pathways." *Microvascular Research* 62 (3): 252–62. https://doi.org/10.1006/mvre.2001.2338.

Ourradi, Khadija, Thomas Blythe, Caroline Jarrett, Shaney L Barratt, Gavin I Welsh, and Ann B Millar. 2017. "VEGF Isoforms Have Differential Effects on Permeability of Human Pulmonary Microvascular Endothelial Cells." *Respiratory Research* 18 (1): 116. https://doi.org/10.1186/s12931-017-0602-1.

Peng, Hui, Yuanqing Li, Cheng Wang, Jun Zhang, Yanru Chen, Wenfang Chen, Jin

Cao, Yanlin Wang, Zhaoyong Hu, and Tanqi Lou. 2016. “ROCK1 Induces Endothelial-to-Mesenchymal Transition in Glomeruli to Aggravate Albuminuria in Diabetic Nephropathy.” *Scientific Reports* 6 (1): 20304. https://doi.org/10.1038/srep20304.

Tiemeijer, L A, J-P Frimat, O M J A Stassen, C V C Bouten, and C M Sahlgren. 2018. “Spatial Patterning of the Notch Ligand Dll4 Controls Endothelial Sprouting in Vitro.” *Scientific Reports* 8 (1): 6392.

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