Novel mechanisms and signaling pathways in angiogenesis

Forghany, Z.

CHAPTER 1

General Introduction

1. Normal Angiogenesis

1.1 Basic biology of normal angiogenesis

The endothelium expands by the process of angiogenesis, during which the formation of new blood vessels occurs from pre-existing ones. Angiogenesis is a normal physiological process that plays a crucial role in wound healing, embryonic development, and regulation of organ function. Generally speaking, at the tissue level, angiogenesis involves the degradation of the extracellular matrix, migration, and proliferation of endothelial cells (ECs) to form new blood vessels. At the cellular level, the migration of ECs is driven by chemical signals such as growth factors and cytokines. These signals activate intracellular signaling pathways that regulate cellular functions such as proliferation, migration, and survival. At the molecular level, key players in angiogenesis include vascular endothelial growth factor (VEGF) and its receptors, NOTCH signaling as well as other signaling molecules such as integrins and matrix metalloproteinases (MMPs). Understanding the molecular mechanisms underlying angiogenesis is crucial for developing new treatments for various diseases that involve dysregulation of the process, such as cancer and cardiovascular diseases (La Mendola et al., 2022; Carmeliet et al., 2011; Eelen. et al., 2020).

1.2 Angiogenic process

Angiogenesis is a dynamic process related to the remodeling and maturation of the vasculature into a complex and branched network of blood vessels, which occurs continuously throughout life (Eelen et al., 2020; Adair et al., 2010). Beginning in utero and embryonic development, a primitive vascular network is established through vasculogenesis, in which angioblasts aggregate to form tube-like structures (Patel-Hett & D'Amore, 2011). Subsequent angiogenic expansion of the vascular system represents a thorough orchestration of cell proliferation, differentiation, migration, matrix remodeling, and intercellular signaling mechanisms (Eelen et al., 2020). Angiogenesis can be classified as either sprouting or intussusceptive angiogenesis; both are thought to occur in utero and adults (Adair & Montani, 2010). Sprouting angiogenesis occurs when new blood vessels form in previously non-vascularized tissue regions, a common occurrence during embryonic development and also in the tumor microenvironment (TME), which will be described later (Adair & Montani, 2010; Fang & Salven, 2011). More specifically, following stimulation by angiogenic factors, MMP activation occurs, leading to the basement membrane's

degradation (Quintero-Fabián et al., 2019). This leads to a transformation of leading edge ECs from quiescent to pathfinding tip cells, characterized by increased cell-matrix interactions, matrix remodeling, and high migratory potential (Mukwaya et al., 2021). Tip cells are the leading edge cells at the sprouting front of a growing blood vessel. Tip cells dynamically extend filopodia in order to sense and respond to proangiogenic signals, which guide the vessel's direction and ensure proper vessel formation (Adams & Alitalo, 2007). Stalk cells trail behind the tip cells. They provide sprout elongation and contribute to the lumen formation (Pardali et al., 2010). Stalk cells proliferate to elongate the vessel, maintaining its width and guiding the vessel's growth behind the tip cells. Figure 1 depicts six different steps of sprouting angiogenesis, from the breakdown of the basement membrane to lumen formation (Gerhardt et al., 2008; Davis et al., 2005 Horowitz and Simons 2008).

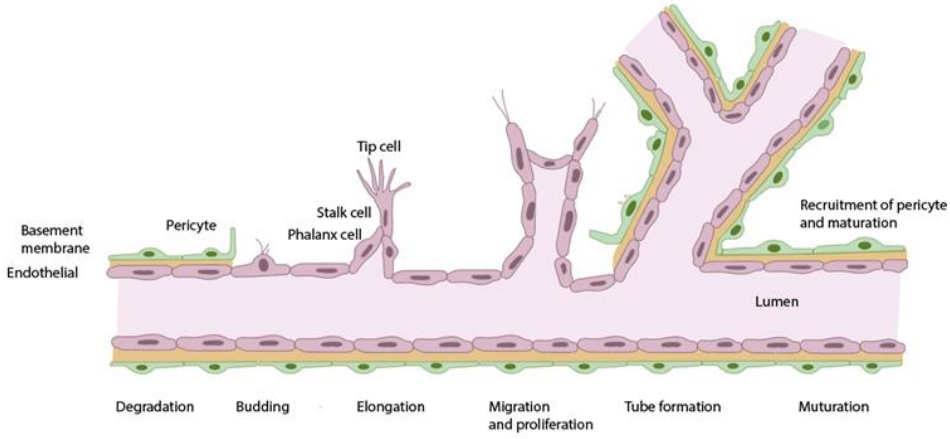

Figure 1. Angiogenesis steps.

- Matrix metalloproteinase (MMP) degradation - MMPs degrade extracellular matrix to create space for new blood vessel growth.
- Budding - Formation of small sprouts from existing blood vessels.
- Elongation - The sprouts grow and lengthen, forming new blood vessels.
- Migration and proliferation - Blood vessel cells move to the site of angiogenesis, multiply, and form a network of new vessels.
- Tube formation - Blood vessels form a lumen and become functional tubes.
- Maturation - Blood vessels mature, forming tight junctions and a functional vasculature (Dufraine et al., 2008; Aspriţoiu et al., 2021).

Intussusceptive angiogenesis is a different mechanism of forming new blood vessels. Instead of sprouting new vessels, this type of angiogenesis involves the splitting of existing blood vessels, creating a new lumen or cavity in a blood vessel. This type of angiogenesis is most commonly seen in tissues that experience rapid growth and increased oxygen demand, such as in the formation

of the placenta and in growing tumors. The process begins with the activation of mural cells, which form a barrier within the vessel, dividing it into two smaller vessels. This results in the formation of new, smaller vessels, which increases the total surface area available for blood flow (Burri et al., 2004; Kurz et al., 2003).
Quiescence, activation, and resolution of vessels are three sequential steps to form a sprouting vessel. It is worth mentioning that the maturation of blood vessels is a transition from an actively growing vascular to a quiescent, completely functional network. Establishing stable and mature blood vessels involves multiple processes, including stabilizing existing vascular tubes, suppressing EC sprouting, and protecting against proangiogenic signals such as VEGF. Cellular differentiation processes such as valve formation, fenestration, and apical-basal polarity are also part of vessel maturation (Pimanda et al., 2006; Cleaver & Melton, 2003).
The following sections will summarize the current knowledge of the known pathways orchestrating angiogenesis. Later, in the section describing pathological angiogenesis, we explore how an understanding of these processes may help find the best approaches to inhibit angiogenesis.

1.3 The role of NOTCH and VEGF signaling on coordination of angiogenesis

The interplay between NOTCH signaling and VEGF signaling in neighboring ECs influences the coordination of angiogenesis. These signaling pathways play a crucial role in regulating the formation and growth of new blood vessels during angiogenesis. The NOTCH signaling pathway is a highly conserved signaling pathway that is involved in a variety of cellular processes, including cell differentiation, proliferation, and survival in ECs and other cell types, including cancer cells (Akil et al., 2021)
The following section provides a brief introduction to the structure of NOTCH ligands and receptors, VEGF signaling, and their roles in tip and stalk cell formation.

1.3.1 The structure of the main components of the NOTCH signaling pathway

NOTCH receptors are classified as type I transmembrane receptors that pass once through the cellular membrane. These receptors are initially synthesized as single polypeptide chains and subsequently undergo proteolytic processing to form heterodimeric configurations displayed on the cell surface. All four NOTCH receptors (NOTCH1-4) in Homo sapiens comprise three distinct domains: (I) the NOTCH extracellular domain (NECD), (II) the transmembrane I domain, and (III) the NOTCH intracellular domain (NICD). The NECD of all NOTCH receptors contains 29-36 Epidermal Growth Factor (EGF) repeats, of which, EGF repeat 11-

12 mediate trans-interactions, whilst EGF repeats 24-29 mediate cis-interactions with NOTCH ligands (Below see trans and cis interactions). Additionally, a subset of the EGF repeats contains calcium-binding sites (Chillakuri et al., 2012; Kopan et al., 2009; Liu et al., 2014). The NICD consists of multiple domains: (I) the RBP association module (RAM) domain, (II) ankyrin (ANK) repeats forming the ANK domain, (III) the transactivation domain (TAD), and (IV) the proline/glutamic acid/serine/threonine (PEST) domain. The ANK domain is flanked by two nuclear localization signals (NLS) that target the NICD to the nucleus (Wang, M., 2011). While there are significant differences in size among various NOTCH family members, particularly compared to the C. elegans orthologs, several fundamental structural characteristics remain consistent across all members (Blaumueller et al., 1997; Logeat et al., 1998).

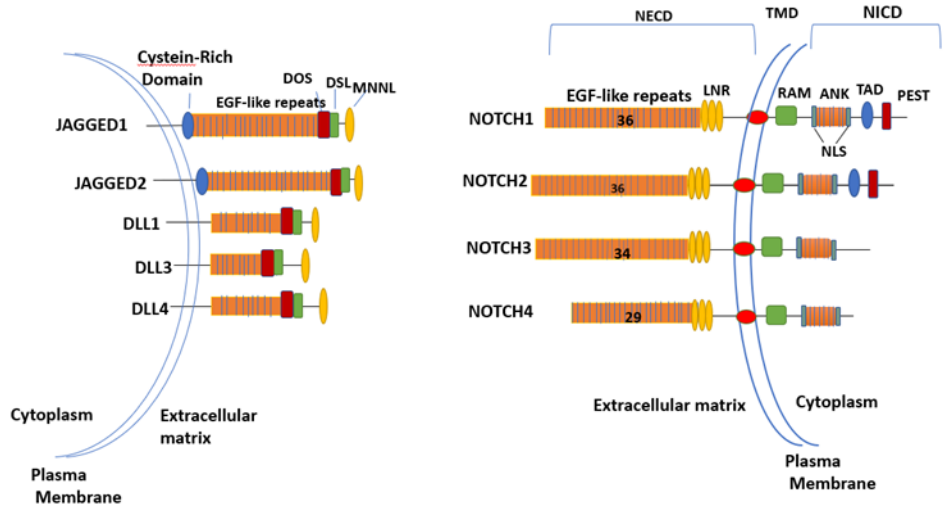

Figure 2. The structure of the NOTCH receptor family and its corresponding ligands**.** NOTCH1~4 consists of extracellular, intracellular and transmembrane domains. The five ligands present in mammals are categorized into two distinct groups: Delta-like (comprising DLLl1, DLL3, and DLL4) and Serrate-like (encompassing JAGGED1 and JAGGED2.

The NOTCH pathway is activated when a ligand binds to the single transmembrane NOTCH receptor on the cell's surface. Ligands are single pass transmembrane polypeptides on the cell surface, and their structure partially resembles that of the receptors (Phng & Gerhardt, 2009; D'Souza et al., 2008). The extracellular domain of the ligands contains multiple EGF-like repeats, which play a role in their interactions with their respective receptors.

For NOTCH canonical signaling responses to occur, ligands and receptors must be expressed on neighboring cells. In general, Notch-dependent signaling output in ECs depend on two central signaling mechanisms: trans-interactions, which

occur when ligands associate with receptors in adjacent cells, and cis-interactions, where ligands can inhibit NOTCH signaling within the same cell. Recently, a cis-activation process has also been identified, which occurs with multiple ligand-receptor pairs (Nandagopal et al., 2019).
The signaling outcomes of the NOTCH pathway and the varied results of specific cellular events rely on different combinations of NOTCH family ligands and receptors (Bigas et al., 2016).
Among the NOTCH ligands found in the vertebrate endothelium, only the absence of DLL4 or JAG1 leads to defects in the vascular system (Shah et al., 2017). Delta-like 4 (DLL4), as one of the key regulators of angiogenesis, is highly expressed in the endothelium of angiogenic blood vessels, as well as in quiescent arteries and capillaries throughout the tissues and organs of mammals (Lobov & Mikhailova, 2018). In addition, DLL4 and JAGGED1 play a crucial role in regulating the selection of tip cells and maintaining a delicate balance that coordinates the sprouting of ECs. Benedito et al. reported that these two ligands have opposing roles in the process of tip cell selection in ECs sprouting. Though Dll4 promotes Notch signaling, which typically inhibits tip cell formation, JAGGED1 plays a proangiogenic role by downregulating the DLL4-Notch signaling pathway (Benedito et al., 2009). Additionally, JAGGED1 has been reported to inhibit NOTCH signaling during embryonic pancreas development (Golson et al., 2009).
Studies have shown that DLL4, which effectively activates NOTCH1, displays a preference for activating NOTCH1 rather than NOTCH2, whereas DLL1, which in general has less affinity for NOTCH1 binding (Andrawes et al., 2013), can activate both NOTCH1 and NOTCH2 (Tveriakhina et al. 2018). It has also been demonstrated that DLL3 functions exclusively in cis-inhibition (Ladi et al., 2005; Chapman et al., 2011).

1.3.2 Intracellular VEGF signaling pathway

The other essential signaling pathway in EC sprouting is the intracellular VEGF signaling pathway (Hellström et al.,2007). VEGF-A is the best characterized and most studied VEGF factor in angiogenesis and acts by signaling through the receptor tyrosine kinase (RTKs) (VEGF receptor 1 [VEGFR1], -2, and- 3 [VEGFR3]) and coreceptors Neuropilin-1 (NP-1), and Neuropilin-2 (NP-2) (Otrock et al., 2007; Cross et al., 2001). While the VEGF165 isoform has been identified as crucial for vascular development (Grünewald, et al., 2010), it's noteworthy that various isoforms of VEGF-A exhibit specific interactions with VEGFR1 and VEGFR2. Among these interactions, the highest affinity is observed towards VEGFR1. However, despite this affinity, it is VEGFR2 that serves as the primary mediator of VEGF-A signaling during vessel branching (Takahashi & Shibuya, 2005; Olsson et al., 2006).

VEGFR-2 receptor consists of 1356 amino acids and comprises several distinct domains. The extracellular portion (amino asid 20-764) contains seven immunoglobulin (Ig)-like folds, with particular significance attributed to Ig domain three due to its role in determining ligand-binding specificity. Following this, there is a 21-amino acid transmembrane (TM) domain (amino acids 765-785), followed by the intracellular domain (amino acids 786-1356). Within the intracellular domain, there are two kinase domains (KD) separated by a kinase insert domain (KID) (Cross et al., 2001).

Under hypoxic conditions, increased transcription of VEGF occurs, leading to a VEGF gradient. The VEGF-A ligand binds to the extracellular Ig domains 2 and 3 on the VEGFR-2 receptor. This binding induces both homo- and heterodimerization of VEGFR-2 and activates the receptor's kinase activity, resulting in self-phosphorylation of the receptors. Subsequently, signaling molecules bind to the phosphorylated VEGFR-2, initiating MAPK (Mitogen activated protein kinase) signaling cascades, which, in turn, promote cell survival, proliferation, and migration of ECs (Olsson et al, 2006; Schweighofer et al., 2009; Kliche et al., 2012; Koch et al., 2012).

1.3.3 Overview of signaling in tip stalk cell formation

During angiogenic sprouting, it is well established that ECs compete dynamically for the tip cell position (Jakobsson et al., 2010). Tip cells are mobile cells that do not undergo proliferation. Instead, they perceive the angiogenic signal and invade the neighboring tissue by elongating multiple filopodia (Kamei et al., 2006; Gerhardt et al., 2003). Following tip cells are stalk cells (De Smet et al., 2009), which undergo proliferation, extend the sprouts, create lumens, and establish blood circulation under suitable conditions. The determination of tip and stalk cell selection relies on the differences in VEGFR levels between neighboring ECs, which are influenced by NOTCH/DLL4 signaling. (Hellström et al. 2007; Lobov et al. 2007; Siekmann and Lawson 2007; Suchting et al. 2007). In brief, the VEGFA ligand–receptor interaction induces dimerization of VEGFR-2 which, in turn, results in the up-regulation of DLL4 expression in leading tip cells. Elevated expression of DLL4 in tip cells leads to amplified NOTCH signaling in trailing stalk cells, which suppresses the tip cell phenotype resulting in decreased VEGFR2 expression within the stalk cells (Pardali et al., 2010) NOTCH activation reduces VEGFR-2 and VEGFR-3 expression in stalk cells, in contrast to tip cells that, upon lateral inhibition, exhibit low NOTCH signaling (Pardali et al. 2010). Besides that, the expression of DLL4 ligand, which is also a target gene regulated by the NOTCH signaling pathway, will be reduced (Uyttendaele et al., 1996; Sainson et al., 2005).

It's worth mentioning that the activity of VEGF-A, which acts through its endothelial receptor VEGFR-2, plays a crucial role in not only inducing tip cell

filopodia, EC migration and proliferation but also promoting EC survival and vascular permeability (Tomlins et al., 2005; Tomlins et al., 2006; Pimanda et al., 2006). As a result, upon VEGF signaling, activation of the intracellular MAPK cascade triggers the phosphorylation and activation of the ETS transcription factor ERG, which is necessary for DLL4 induction. As a consequence of the effect of ETS transcription factors on the *DLL4* promoter, a wave of DLL4 will be generated that mediates NOTCH signaling in the adjacent cells. Upon direct cell-cell interactions in neighboring ECs, a series of proteolytic cleavages of the NOTCH receptor causing the release of the NOTCH intracellular domain (NICD), which translocate to the nucleus where it directly interacts with the CSL transcription factor and thereby triggers the expression of NOTCH target genes such as HES and HEY family of basic helix-loop-helix (bHLH) transcription factors (Weinmaster, 1998; Mumm and Kopan 2000; Nakagawa et al., 2000; Davis and Turner, 2001; Iso et al., 2003) (Figure 3).

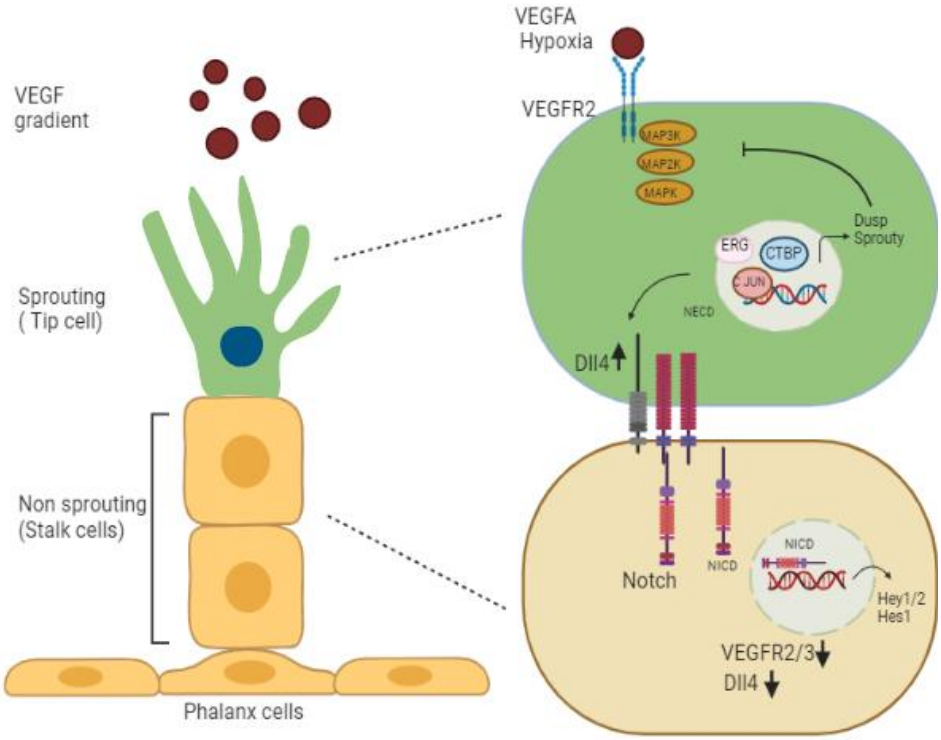

Figure 3. Formation of endothelial tip cells through two signaling pathways: VEGF/VEGFR and DLL4/NOTCH. The specification of tip and stalk cells within the vascular endothelium involves the intricate interplay of the VEGF and Notch signaling pathways. VEGF engages with VEGFR-2, which is expressed on the surface of quiescent ECs in vessels. Upon stimulation by VEGF and subsequent activation of the MAPK signaling pathway, coupled with the transcription factor CJUN, ERG and other transcriptional regulators, DLL4 expression is up-regulated, specifically in the tip cells.

< Figure 3 (continued). This up-regulation of DLL4 leads to the activation of the Notch signaling pathway in the neighboring stalk cells, thereby suppressing the tip cell phenotype. Activation of Notch signaling consequently diminishes the expression of VEGFR2 and orchestrates the expression of various Notch target genes, such as Hey and Hes (Blanco et al., 2013). Some parts of this image were created with Bio Render (https://www.biorender.com).

Among the first evidence of the necessity of the presence of ETS in DLL4 expression is our previous findings that showed in primary ECs, in response to VEGF and following the inactivation of a TEL/C-terminal Binding Protein (CtBP) complex from DLL4 promoter (which harbors a number of conserved consensus transcription factor ETS DNA-binding sites), a signature of genes would be up-regulated, including dual specificity phosphatase DUSP and receptor tyrosine kinase (RTK) antagonist SPROUTY4 as well as DLL4 (Roukens et al., 2010). Nonetheless, the precise mechanism by which SPROUTY proteins control RTK signaling is not fully understood.

In regards to DLL4 expression, *in vitro* studies also confirmed that VEGF-A stimulation consistently augments the expression of DLL4 protein on the surface of human umbilical vein ECs (HUVECs) (Ridgway et al., 2006). Consistent with this idea, there is evidence that shows hypoxic conditions highly induce VEGF-A expression, which, as a consequence, directly up-regulates the mRNA expression of DLL4 in ECs (Patel et al., 2005; Williams et al., 2006),

The NOTCH pathway is linked to other pathways, such as the WNT and HEDGEHOG pathways, through the involvement of ETS transcription factors (Bray, 2016) Briefly, ETS factors play a role in modulating the activity of NOTCH target genes and are involved in the regulation of angiogenesis. Knockdown studies in mice and zebrafish revealed the influence of ETS factors on the regulation of NOTCH signaling through the VEGF pathway. For example, ERG plays a role in maintaining the balance between NOTCH ligands by promoting the expression of DLL4 while inhibiting JAG1 expression (Shah et al., 2017).

Hence, the NOTCH/DLL4 and VEGF signaling pathways are critical players in the regulation of angiogenesis and are linked to other pathways through the involvement of ETS transcription factors. The intricate interplay between signaling pathways in the regulation of angiogenesis highlights the complex nature of this process and underscores the importance of understanding the underlying mechanisms of angiogenesis. In the following section, we elaborate on the function of the TFs ETS in the process of angiogenesis.

1.4 Role of ETS transcription factors in angiogenesis

ETS (E26 Transformation-Specific) transcription factors are a large and diverse protein family which are involved in various biological processes, including control of cellular proliferation, differentiation, hematopoiesis, and angiogenesis (Sementchenko and Watson 2000; Watson and Seth, 2000; Dittmer et al., 2003;

Oikawa et al., 2003). The ETS family is characterized by the presence of a highly conserved DNA-binding domain known as the ETS domain. The ETS domain allows these TFs to bind to specific DNA sequences and regulate the expression of target genes, including the expression of genes involved in the process of angiogenesis, such as NOTCH ligands DLL4 and JAG1, VEGF, and angiopoietin (Shah et al., 2017). It has been well documented that a number of target genes of ETS family transcription factor are expressed in ECs and are responsible for the regulation of angiogenesis. For example, MMP-1, MMP-3, MMP-9, and urokinase-type PA (u-PA) and VEGF and its receptors (Lelievre et al., 2000) (Figure 4).

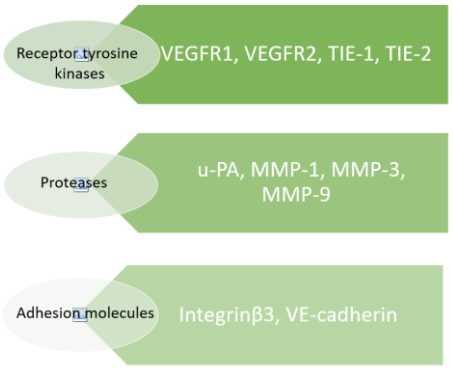

Figure 4. Target genes of ETS Family Transcription Factors in Endothelial cells

Among the members of the ETS family, it has been reported that transcription factors ETS-1, ERG, FLI-1, ELF-1, TEL, and NERF-2 are involved in the expression of proangiogenic genes and the regulation of vascular development (Sato, 2001). Among those, ERG is the most abundantly expressed in resting ECs and has been shown in *in vitro* studies to be essential for angiogenesis (Birdsey et al., 2012). *In vivo* studies in mouse retina showed that ERG orchestrates the NOTCH, and Wnt/β-catenin pathways to facilitate the maturation and stability of blood vessels (Shah,et al,. 2017). Research conducted in Xenopus, mouse, and zebrafish models has demonstrated that ERG, which is expressed in all ECs, plays a crucial role in the differentiation and vascular development of ECs (Baltzinger et al., 1999; Liu et al., 2008). Zebrafish studies have also shown that the highly homologous transcription factors FLI-1 and ERG have also been linked to the regulation of endothelial homeostasis and angiogenesis, as they play a role in the expression of genes defining the endothelial lineage (Liu & Patient, 2008). This aligns with our group's discovery that the disassembly of ETS transcription factor from the *DLL4* promoter can mediate sprouting by controlling DLL4 expression in ECs (Roukens et al., 2010).

Additionally, the transcription of von Willebrand factor is regulated by ERG (McLaughlin et al., 2001). Other functional studies demonstrated that FLI-1 disruption in mouse embryos results in aberrant hematopoiesis and hemorrhage of the animal (Schreiber et al., 2000).
Whereas several studies have investigated the role of FLI-1 in vascular development, Pham et al. took a comprehensive approach to explore the combinatorial function of four members of the ETS family (FLI1, FLI1b, ETS1, and ETSRP) in zebrafish vasculature through using morpholinos to target genes (Pham et al., 2007).
The current available data also shows that ETS proteins, including ETS-1 and ETS-2, have also been found to enhance angiogenesis by interaction with an ETS motif in the VEGFR promotor and via increasing vascular stability through the regulation of NOTCH signaling by ERG (Wei et al., 2009; Niu et al. 2018; Shah et al. 2017). Despite the presence of a conserved ETS binding site in the promoters of proangiogenic genes, the consequences of combining and manipulating these factors remain an area of limited knowledge (Oettgen, 2001). Further research is necessary to gain a deeper understanding of the mechanisms underlying the role of ETS in angiogenesis.
Of note, our group uncovered a new, previously overlooked pathway for the genetic control of angiogenesis. Our research demonstrated the direct role of the TEL (ETV6) in mediating angiogenesis (Roukens et al., 2010). Furthermore, we uncovered the crucial function of the TEL/CtBP transcription repressor complex in this process by bridging the two predominant angiogenic networks: the intracellular VEGF receptor signaling pathway and the intercellular NOTCH/DLL4 pathway, which will be elaborated in this thesis. Our findings suggest that there may be potential for inhibiting angiogenesis in cancer pathology by using inhibitors that target ETS activity (see Chapter 5).

1.5 Role of AP1 in angiogenesis

Activator Protein-1 (AP-1) is a dimeric transcription factor complex composed of the products of the JUN and FOS proto-oncogenes, which play a critical role in regulating various biological processes such as cell differentiation, proliferation, apoptosis, and angiogenesis.
Regarding angiogenesis, several members of the AP-1 family have been shown to differentially regulate genes associated with this process (Angel and Karin, 1991). For example, JUN and JUNB induce the expression of the proliferin gene, FOS induces VEGF-D, and FRA-1 induces urokinase-type plasminogen activator (uPA), uPA receptor (uPAR), and various MMPs (Kustikova et al., 1998; Belguise et al., 2005). Evidence also suggests that the transcription factor FRA-1 promotes vessel development (Schreiber et al., 2000). *In vivo* evidence suggests that the knockout of certain AP-1 family members contributes to the observed lethality

in animals. However, Fra-1-null embryos exhibit a deficiency in placental vascularization, which highlights the role of this factor in stimulating vessel development (Schreiber et al., 2000). In cancer biology, evidence has shown that JUND reduces tumor angiogenesis by protecting cells from oxidative stress (Gerald et al., 2004).
A recent study also demonstrated the impact of deoxy ribozymes targeting CJUN on solid tumor growth and angiogenesis. The findings showed that CJUN activation plays a significant role in the proliferation and angiogenesis of invasive breast cancer (Zhang et al., 2004). Additionally, Fosl1 (Fos-Like1; also known as Fra1) has been shown to play a crucial role in establishing normal placental vascularization in mice (Schreiber et al. 2000). Furthermore, Fra1 is involved in regulating the level of the αvβ3 intergin and the uPA-uPAR complexes on the surface of ECs, contributing to angiogenesis (Galvagni et al., 2013).
Previous studies in our lab have also revealed that DLL4 and JUN expression is highly responsive to VEGFR signaling in HUVECs (Roukens et al., 2010). We have also discovered that the interaction of DLL4 intracellular domain with JUN forms a feedback loop that causes VEGF-dependent changes in DLL4 expression and other angiogenesis-regulating genes (Forghany et al., 2018, Chapter 2) This regulation depends on ETS/AP-1 transcription factors and is controlled by the DLL4 intracellular domains.
Taken together, these findings provide evidence that the AP-1 transcription factor components may play a role in angiogenesis, though the precise mechanistic basis of this remains unclear.

2. Pathological Angiogenesis

2.1 Tumor angiogenesis is a hallmark of cancer, and vascular tumors are a model for deciphering novel mechanisms

Over the past 30 years, pioneering work, starting with the research of Folkman et al., has established that tumor development depends on angiogenesis. As stated before, angiogenesis is indispensable during tissue development and regeneration. Nevertheless, it also has implications in pathological conditions like cancer, termed tumor angiogenesis (Chambers et al., 2002). Tumor angiogenesis is one of the most prominent mechanisms driving tumor growth beyond a few cubic millimeters (Folkman, 1971). It is widely accepted that a critical stage known as the “angiogenic switch” occurs in the initial phase of tumor angiogenesis (De bock et al., 2011). Research on vascularization has a long tradition, with Rudolf Virchow describing tumor vascularization as a hallmark of cancer and a pivotal step in tumor growth in 1863. This phenomenon involves redirecting nearby blood vessels through angiogenesis, facilitated by the vast number of capillaries in the human body. Tumor cells that develop

spontaneously normally lack angiogenic characteristics in the beginning. The transition to angiogenesis typically takes place through a process where certain factors stimulate the formation of new capillaries, which subsequently align towards the tumor (Brossa et al., 2019; Ribatti et al.,2007). Therefore, what triggers vessel formation in both normal and tumor conditions is an essential topic of investigation.

Although a finely balanced equilibrium exists between proangiogenic and anti-angiogenic factors to maintain the homeostasis in physiological angiogenesis (Pollina et al., 2008), tumor growth requires disturbing this balance. Thus, triggering the angiogenic switch is crucial for cancer progression. This transition clearly entails more than just increasing angiogenic activity and is believed to result from a combination of positive and negative regulatory factors. Factors such as progenitor ECs, the interaction between angiogenic factors and their receptors, and the interplay between vasculogenesis and lymph angiogenesis may play a role in triggering this transition (Ribatti et al., 2007). For example, tumor ECs (TEC), shows elevated expression levels of proangiogenic genes like VEGFR, VEGF, platelet-derived growth factor (PDGF), and epidermal growth factor receptor (EGFR). Conversely, expression of endogenous inhibitors, such a tumstatin (Nyberg et al. 2005), thrombospondin-1 (TSP-1) or interferon, may be reduced. Therefore, the transition is not solely driven by increased angiogenic activity but is rather influenced by a complex interplay between positive and negative regulators (Ribatti et al., 2007).

Tumor vessels exhibit several distinct features compared to normal blood vessels. Abnormal structure, increased permeability, poor perfusion, uneven blood flow, and enhanced adaptation are some of the characteristics of tumor vessels that contribute to tumor progression, metastasis, and therapeutic resistance (Siemann et al., 2011). As an example of adaptation, recent studies have reported that TECs consist of heterogeneous populations with variable phenotypes, and they adapt their characteristics in reaction to the tumor microenvironment (TME), such as hypoxic conditions. Therefore, the importance of understanding of these characteristics of vascular tumors and deciphering their mechanisms provides intriguing discoveries that could be utilized to personalize existing cancer treatment (Siemann et al., 2011; Treps et al., 2022).

In many solid tumors, such as sarcomas and breast cancer, the level of angiogenesis and the subsequent vasculature determine the severity of the tumor’s malignancy (Dass and Choong, 2008). This could be viewed as cancer’s Achilles heel: cutting off the blood supply could lead to the failure of tumor development. Over the past years, many angiogenesis inhibitors have been approved by the FDA for cancer treatment, focusing on targeting molecules like VEGF, its receptor, or related factors. For instance, Bevacizumab, recognized as an inhibitor of VEGF's biological activity (Al-Abd et al., 2017), is combined with conventional chemotherapy. This combination, known as the first-line

therapeutic strategy for patients with non-small-cell lung cancer (NSCLC), represents one of the most promising angiogenesis inhibitors for cancer treatment (Sun et al., 2022).

Broadly speaking, the ultimate aim of anti-angiogenic therapy, particularly when combined with other conventional therapies, is to stop tumor growth by maintaining a balance between proliferation and apoptosis rates, thereby limiting the tumor size to just a few millimeters (Szeles et al., 2012). Unlike this conventional therapy, which can affect adjacent tissues, anti-angiogenic therapy targets ECs, thereby inhibiting only the formation of new blood vessels (Szeles et al., 2012). Although this approach may not completely eliminate the tumor, it could effectively stop its growth and maintains it at an early stage. Therefore, it is often used in combination with conventional approaches to achieve complete tumor elimination (Yazdjerdi et al., 2019). Overall, moderating the potential side effects associated with standard cancer treatments, lower the chance of developing drug resistance, and potentially converting cancer into chronic manageable disease would be the aim of utilizing anti-angiogenesis therapy (Ansari et al., 2022). Currently, cancer therapies have demonstrated limited clinical effectiveness due to issues like cytotoxicity and acquired resistance. Despite some progress, existing strategies have failed to fulfil the early promise and high expectations. Thus, it is imperative to expand the spectrum of potential targets for future anti-angiogenesis therapies. To achieve this, one must gain a thorough understanding of the pathways implicated in angiogenesis. By identifying new targets and elucidating the underlying mechanisms of angiogenesis, we can enhance the efficacy of current cancer therapies and ultimately improve patient outcomes. Recently, our lab uncovered a new, previously overlooked pathway for the genetic control of angiogenesis. Specifically, we showed that ETS transcription factors are an essential mediator of angiogenesis. As such, targeting the activity of these proteins through the production of small molecule inhibitors of its function represents an entirely novel approach to the problem of inhibiting illicit vascular development such as tumor angiogenesis. Our work suggests that inhibitors of the activity of ETS transcription factors could potentially inhibit tumor angiogenesis without the problems of acquired resistance and the strong side effects inherent in earlier strategies (see Chapter 5).

2.2 Role of ETS transcription factors in cancer

ETS factors are believed to play a fundamental role in the development and evolution of the majority of tumor types. Since the 1980s, multiple different alterations in ETS factor function have been identified in many different tumor types (reviewed in Sizemore et al., 2017). Activation and dysregulation of ETS TFs occur at both the transcriptional and post-transcriptional levels (Wei et al.,

2023). Such activations of ETS TFs are driven by several mechanisms that include (a) chromosomal rearrangement, which generates ETS gene fusions in Ewing's sarcoma, breast, gastric, head and neck, prostate, and thyroid cancers; (b) gene amplification in breast cancer and melanoma, (c) feed-forward loop signaling, (d) gain-of-function mechanisms, increased ETS factor activity and stability (Sizemore et al., 2017).

In the subsequent section, the involvement of ETS family members in various forms of human cancer is outlined.

For instance, overexpression of ETS-1 was observed in 80% of tumor tissues from esophageal squamous-cell carcinoma (ESCC) patients (Mukherjee et al., 2003). This excessive expression of ETS-1 often coincides with increased VEGF levels, known to trigger tumor angiogenesis (Hashim et al., 2010; Martins et al., 2013), and is strongly associated with lymph node metastasis. In addition, ETS-1 expression has been implicated in angiogenesis in endometrial uterine cancers (Fujimoto et al., 2002), and in ovarian cancers. This contributes to tumor growth and progression, partly through angiogenesis promotion. Moreover, elevated ETS-1 expression correlates strongly with increased microvessel density and VEGF expression in gastric cancer and colorectal carcinoma patients. Similarly, in various types of human brain cancer, ETS-1 expression significantly correlates with tumor grade, unlike in normal brain tissue where its expression is minimal (Kitange et al., 1999). Apart from angiogenesis, ETS factors play roles in DNA repair, genomic stability, and evasion of cell death (Knezevich et al., 1998; Seth and Watson 2005).

Recent findings have also revealed the impact of ETS factors on nucleotide, and steroid metabolism crucial for tumor cell survival. For example, ETS1 regulates genes encoding enzymes in glycolysis and lipid metabolism in ovarian and breast cancer (Verschoor et al. 2013). Additionally, in prostate cancer, TMPRSS2-ERG rearrangements deregulate fatty acid, sphingolipid, and polyamine signaling (Meller et al. 2016). Moreover, ETS factors influence the tumor microenvironment, particularly in extracellular matrix remodeling, angiogenesis, and inflammation (Knezevich et al., 1998; Findlay et al., 2013; Kar & Gutierrez-Hartmann, 2013).

A study by Huang et al. highlighted the critical role of ELF-1 in tumor angiogenesis development. High expression levels of ELF-1 correlate with TIE-2 expression in tumor blood vessels. Blocking ELF-1 using tailored membrane-permeable peptides inhibits ANG-1-mediated EC migration *in vitro* and reduces B16 melanoma tumor growth and tumor-associated angiogenesis in nude mice (Huang et al., 2006). Further studies on TIE-1, TIE-2, and the Ang-2 promoter suggest that ELF-1 acts as a transcriptional regulator of these genes during vascular development, emphasizing its essential role in regulating the ANG-TIE-2 pathway in tumor angiogenesis development (Dube et al. 2001; Hegen et al. 2004).

Overall, since the misregulation of ETS has been reported as a frequent feature in many different types of cancer, the prudent use of ETS inhibitors could selectively target tumor cells. It has been well documented that the ETS family also interacts with other transcription factor complexes, such as the FOS/JUN complex, to form more extensive transcriptional regulatory networks (Basuyaux et al., 1997). This highlights the importance of the ETS family in regulating gene expression and underscores the need for further study of these proteins in both normal and disease states.

2.3 Identification and characterization of novel angiogenesis/tumor cell Inhibitors

For decades, the development of targeted therapy for cancer has been a challenge that is being investigated by researchers around the world. The fundamental challenge of cancer research is the discovery of new treatments that eliminate tumors, are minimally toxic, and are not susceptible to acquired resistance. During the last two decades, the design of new cancer therapies can be broadly categorized into two approaches: those that have sought to target and inhibit tumor cell growth directly and those that target the tumor vasculature and tumor microenvironment.

Published surveys of all drugs approved in the last decade suggest little evidence of significant improvements in patient survival or quality of life. As mentioned before, angiogenesis is coordinated by NOTCH and VEGF signaling (Phng & Gerhardt, 2009). Several lines of evidence indicate that VEGF signaling acts upstream of the NOTCH pathway and impacts the control of the expression of different NOTCH components (Hashim et al. 2010; Mathis 1999; Stone et al. 1995; Lawson et al,.2002; Patel et al. 2005; Ridgway et al. 2006; Lobov et al. 2007). Current data suggests that combining anti-VEGF treatment with chemotherapy and radiation therapy results in greater antitumor effects than either treatment alone (Ziche & Morbidelli, 2004).

Although early results appeared promising regarding the efficacy of anti-VEGF therapy and DLL4/NOTCH targeting antibodies in inhibiting cancer cell growth or angiogenesis deregulation (Wu et al., 2010; Andersson & Lendahl, 2014) clinical studies have revealed that while anti-angiogenic drugs initially benefit patients, their efficacy diminishes over time, resulting in only modest disease-free survival rates. This primarily occurs because of redundancy resulting in the the activation of alternative angiogenic signaling pathways (Brossa et al., 2019). The widespread failure of conventional anti-tumor treatments can be attributed to the acquisition of resistance by tumor cells. This resistance is caused by the intra-tumor heterogeneity and the intrinsic redundancies in signal transduction pathways. When a pathway is inhibited or targeted, another functioning

pathway compensates for the loss of activity, allowing the tumor to evade the effects of treatment (Lei et al., 2023).
Broadly speaking, in the drug discovery process, one can look for potential targets within the "druggable genome." However, the number of possible targets is limited if it is assumed that only certain genes associated with diseases can be targeted by drugs. For example, a comparative analysis of antifungal targets in the yeast genome with human genes suggested that only a small portion of the human genome, about 2-5%, can be targeted by drugs. This translates to approximately 600-1,500 potential drug targets in humans, as indicated by Salami and Crews in 2017 (Figure 5).

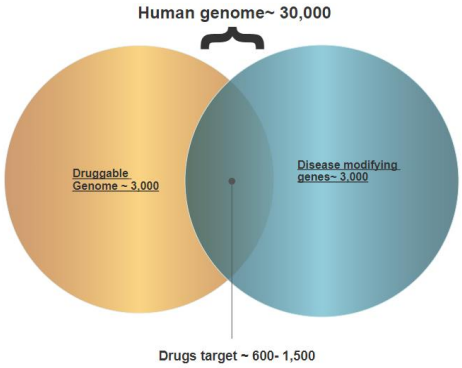

Figure 5. Susceptible drug targets within the human genome. Adapted from (Graham, 2022).

However, there are sound reasons to suppose that many other potential proteins that were previously considered to be undruggable may in fact represent valid targets. To address the issue, we proposed an entirely different methodology in this study: finding a new target beyond the druggable genome. Recently, instead of focusing on the upstream components of cell signaling networks (which were believed to be druggable), we have developed methods for identifying small molecule inhibitors of ETS transcription factors, which act downstream of these pathways. These transcription factors were previously considered "undruggable," but they play a crucial role in driving the behaviors of tumor cells. There are compelling reasons for considering the ETS family of transcription factors as an excellent target. The rationale for targeting ETS factors is simple: firstly, as ETS factors are located downstream of the major receptor signaling pathways, inhibiting this group of proteins could effectively suppress tumor cell growth and alleviate issues of redundancy and resistance (Oikawa et al.,2003). Secondly, because ETS factors are involved in blood vessel growth, selective inhibitors could simultaneously achieve two goals: they could prevent tumor cell proliferation and hinder tumor angiogenesis. Research has already shown that inhibiting ETS activity impedes tumor cell growth (Rahim et al., 2014; Huang et

al., 2021). Given that ETS function is misregulated in tumors, the controlled use of inhibitors might selectively target tumor cells, thereby reducing potential toxicity. In this light, in Chapter 5, we will consider potential approaches for therapeutically targeting oncogenic ETS factors.

3. A brief outline of the thesis

This dissertation has contributed novel perspectives to our comprehension of the mechanisms underlying angiogenesis. Additionally, it has introduced novel methodologies for targeting angiogenesis. **Chapter 1** provides a general introduction to both physiological and pathological angiogenesis, offering an exploration of these critical processes. **Chapter 2** introduces tube formation regulation in ECs through interactions between the intracellular domain of NOTCH ligands and the JUN proto-oncogene. **Chapter 3** presents a new model for controlling the intracellular cis-inhibition of NOTCH receptors by NOTCH ligand dimerization. In **Chapter 4**, we characterized the function of mutant FOS protein in the abnormal vessel growth of epithelioid hemangioma. We also showed through molecular and biochemical analysis how perturbation of normal FOS degradation could lead to this vascular neoplasm. **Chapter 5** builds upon our prior discovery, which identified TEL/CtBP as a promising new target for advancing anti-angiogenic therapies. In collaboration with the European Lead Factory, a consortium of leading academic institutions and pharmaceutical companies, we conducted screens and validated hit small molecules that can putatively inhibit ETS transcription factors by blocking their binding to specific DNA-binding sites and thereby inhibit tumor cell growth and angiogenic sprouting. Finally, **Chapter 6** summarizes the findings of this thesis within the context of contemporary scientific literature.

REFERENCES

1. Adair, T. H., and J. P. Montani. 2010. Angiogenesis (San Rafael (CA)).
2. Adams, R. H., and K. Alitalo. 2007. 'Molecular regulation of angiogenesis and lymphangiogenesis', Nat Rev Mol Cell Biol, 8: 464-78.
3. Al-Abd, A. M., A. J. Alamoudi, A. B. Abdel-Naim, T. A. Neamatallah, and O. M. Ashour. 2017. 'Anti-angiogenic agents for the treatment of solid tumors: Potential pathways, therapy and current strategies - A review', J Adv Res, 8: 591-605.
4. Akil, A., Gutiérrez-García, A. K., Guenter, R., Rose, J. B., Beck, A. W., Chen, H., & Ren, B. (2021). Notch Signaling in Vascular Endothelial Cells, Angiogenesis, and Tumor Progression: An Update and Prospective. Frontiers in cell and developmental biology, 9, 642352.
5. Andersson, ER, and U Lendahl. 2014. 'Therapeutic modulation of Notch signalling--are we there yet?', Nat Rev Drug Discov, 13: 357-78.
6. Andrawes MB, Xu X, Liu H, Ficarro SB, Marto JA, Aster JC, Blacklow SC. Intrinsic selectivity of notch 1 for Delta-like 4 over Delta-like 1(2013)
7. Angel, P, and M Karin. 1991 'The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation.', Biochim Biophys Acta, 1072: 129-57.
8. Ansari, M. J., D. Bokov, A. Markov, A. T. Jalil, M. N. Shalaby, W. Suksatan, S. Chupradit, H. S. Al-Ghamdi, N. Shomali, A. Zamani, A. Mohammadi, and M. Dadashpour. 2022. 'Cancer combination therapies by angiogenesis inhibitors; a comprehensive review', Cell Commun Signal, 20: 49.
9. Aspriţoiu VM, Stoica I, Bleotu C, Diaconu CC. Epigenetic Regulation of Angiogenesis in Development and Tumors Progression: Potential Implications for Cancer Treatment. Front Cell Dev Biol. 2021
10. Baltzinger, M, AM Mager-Heckel, and Remy P. 1999. 'Xl erg: expression pattern and overexpression during development plead for a role in endothelial cell differentiation', Dev Dyn, 216: 420-33.
11. Baluk, P, H Hashizume, and DM McDonald. 2005. 'Cellular abnormalities of blood vessels as targets in cancer', Curr Opin Genet Dev, 15: 102-11.
12. Basuyaux, J. P., Ferreira, E., Stéhelin, D., & Butticè, G. (1997). The Ets transcription factors interact with each other and with the c-Fos/c-Jun complex via distinct protein domains in a DNA-dependent and -independent manner. The Journal of biological chemistry, 272(42), 26188–26195
13. Belguise, K, N Kersual, F Galtier, and D Chalbos. 2005. 'FRA-1 expression level regulates proliferation and invasiveness of breast cancer cells', Oncogene, 24: 1434-44.
14. Bigas, A., Espinosa, L. Notch Signaling in Cell–Cell Communication Pathways. Curr Stem Cell Rep 2, 349–355 (2016).
15. Blanco, R., & Gerhardt, H. (2013). VEGF and Notch in tip and stalk cell selection. Cold Spring Harbor perspectives in medicine, 3 1, a006569.
16. Bray S. J. (2016). Notch signalling in context. Nature reviews. Molecular cell biology, 17(11), 722–735. https://doi.org/10.1038/nrm.2016.94
17. Brenner, J. C., F. Y. Feng, S. Han, S. Patel, S. V. Goyal, L. M. Bou-Maroun, M. Liu, R. Lonigro, J. R. Prensner, S. A. Tomlins, and A. M. Chinnaiyan. 2012. 'PARP-1 inhibition as a targeted strategy to treat Ewing's sarcoma', Cancer Res, 72: 1608-13.
18. Brossa A, Buono L, Fallo S, Fiorio Pla A, Munaron L, Bussolati B. Alternative Strategies to Inhibit Tumor Vascularization. Int J Mol Sci. 2019 Dec 7;20(24):618
19. Burri, P. H., R. Hlushchuk, and V. Djonov. 2004. 'Intussusceptive angiogenesis: its emergence, its characteristics, and its significance', Dev Dyn, 231: 474-88.
20. Carmeliet, P., and R. K. Jain. 2011. 'Molecular mechanisms and clinical applications of angiogenesis', Nature, 473: 298-307.
21. hapman, G., Sparrow, D. B., Kremmer, E., and Dunwoodie, S. L. (2011). Notch inhibition by the ligand DELTA-LIKE 3 defines the mechanism of abnormal vertebral segmentation in

Spondylocostal dysostosis. Hum. Mol. Genet. 20, 905–916.

22. Chambers, A. F., Groom, A. C., & MacDonald, I. C. (2002). Dissemination and growth of cancer cells in metastatic sites. Nature reviews. Cancer, 2(8), 563–572.

23. Chillakuri, Chandramouli R et al. “Notch receptor-ligand binding and activation: insights from molecular studies.” In: Seminars in cell & developmental biology 23.4 (2012), pp. 421–8.

24. Cleaver, O, and DA Melton. 2003. 'Endothelial signaling during development', Nat Med, 9: 661-8.

25. Cross, Michael J. and Claesson-Welsh, Lena. “FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition”. In: TRENDS in Pharmacological Sciences 22.4 (2001).

26. Dass, C. R., and P. F. Choong. 2008. 'Cancer angiogenesis: targeting the heel of Achilles', J Drug Target, 16: 449-54.

27. Davis, RL, and DL Turner. 2001. 'Vertebrate hairy and Enhancer of split related proteins: transcriptional repressors regulating cellular differentiation and embryonic patterning', Oncogene, 20: 8342-57.

28. 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.

29. De Smet, F., Segura, I., De Bock, K., Hohensinner, P. J., & Carmeliet, P. (2009). Mechanisms of vessel branching: filopodia on endothelial tip cells lead the way. Arteriosclerosis, thrombosis, and vascular biology, 29(5), 639–649.

30. DiPietro, LA. 1997. 'Thrombospondin as a regulator of angiogenesis.' in, Regulation of Angiogenesis.

31. Dittmer, J. 2003. 'The biology of the Ets1 proto-oncogene', Mol Cancer: 2:29.

32. D’Souza, B, Miyamoto, a, and Weinmaster, G. “The many facets of Notch ligands.” In: Oncogene27.38 (2008), pp. 5148–67Dube, A, S Thai, J Gaspar, S Rudders, TA Libermann, L Iruela-Arispe, and P Oettgen. 2001. 'Elf-1 is a transcriptional regulator of the Tie2 gene during vascular development', Circ Res, 88: 237-44.

33. Dufraine, J., Y. Funahashi, and J. Kitajewski. 2008. 'Notch signaling regulates tumor angiogenesis by diverse mechanisms', Oncogene, 27: 5132-7.

34. Eelen, G., L. Treps, X. Li, and P. Carmeliet. 2020. 'Basic and Therapeutic Aspects of Angiogenesis Updated', Circ Res, 127: 310-29.

35. Fang, S., and P. Salven. 2011. 'Stem cells in tumor angiogenesis', J Mol Cell Cardiol, 50: 290-5.

36. Findlay, V. J., A. C. LaRue, D. P. Turner, P. M. Watson, and D. K. Watson. 2013. 'Understanding the role of ETS-mediated gene regulation in complex biological processes', Adv Cancer Res, 119: 1-61.

37. Fischetto, R., V. V. Palmieri, M. E. Tripaldi, A. Gaeta, A. Michelucci, M. Delvecchio, R. Francavilla, and P. Giordano. 2019. 'Alagille Syndrome: A Novel Mutation in JAG1 Gene', Front Pediatr, 7: 199.

38. Folkman, J. 1971. 'Tumor angiogenesis: therapeutic implications', N Engl J Med, 285: 1182-6.

39. Forghany, Z, F Robertson, A Lundby, JV Olsen, and Baker DA. 2018. 'Control of endothelial cell tube formation by Notch ligand intracellular domain interactions with activator protein 1 (AP-1)', J Biol Chem, 293: 1229-42.

40. Fujimoto, J., Aoki, I., Toyoki, H., Khatun, S., & Tamaya, T. (2002). Clinical implications of expression of ETS-1 related to angiogenesis in uterine endometrial cancers. Annals of oncology : official journal of the European Society for Medical Oncology, 13(10), 1605–1611. 4

41. Galvagni, F, M Orlandini, and S Oliviero. 2013. 'Role of the AP-1 transcription factor FOSL1 in endothelial cells adhesion and migration', Cell Adh Migr, 7: 408-11.

42. Gerald, D, E Berra, YM Frapart, DA Chan, AJ Giaccia, D Mansuy, J Pouysségur, M Yaniv, and F Mechta-Grigoriou. 2004. 'JunD reduces tumor angiogenesis by protecting cells from oxidative

stress', Cell, 118: 781-94.

43. Gerhardt, H, M Golding, M Fruttiger, C Ruhrberg, A Lundkvist, A Abramsson, M Jeltsch, C Mitchell, K Alitalo, D Shima, and C Betsholtz. 2003. 'VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia', J Cell Biol, 161: 1163-77.

44. Gerhardt, H. 2008. 'VEGF and endothelial guidance in angiogenic sprouting', Organogenesis, 4: 241-6.

45. Golson ML, Le Lay J, Gao N, Brämswig N, Loomes KM, Oakey R, May CL, White P, Kaestner KH. Jagged1 is a competitive inhibitor of notch signaling in the embryonic pancreas. Mechanisms of Development. 2009;126:687–699

46. Graham, H. 2022. 'The mechanism of action and clinical value of PROTACs: A graphical review', Cell Signal, 99: 110446.

47. Grünewald, F. S., Prota, A. E., Giese, A., & Ballmer-Hofer, K. (2010). Structure-function analysis of VEGF receptor activation and the role of coreceptors in angiogenic signaling. Biochimica et biophysica acta, 1804(3), 567–580.

48. Hashim, AF, AA Al-Janabi, LH Mahdi, KM Al-Toriahi, and AA Yasseen. 2010. 'Vascular endothelial growth factor (VEGF) receptor expression correlates with histologic grade and stage of colorectal cancer', Libyan J Med, 5.

49. Hegen, A, S Koidl, K Weindel, D Marmé, HG Augustin, and U Fiedler. 2004. 'Expression of angiopoietin-2 in endothelial cells is controlled by positive and negative regulatory promoter elements', Arterioscler Thromb Vasc Biol, 24: 1803-9.

50. Hellström, M1, LK Phng, JJ Hofmann, E Wallgard, L Coultas, P Lindblom, J Alva, AK Nilsson, L Karlsson, N Gaiano, K Yoon, J Rossant, ML Iruela-Arispe, M Kalén, H Gerhardt, and C Betsholtz. 2007. 'Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis', Nature, 445: 776-80.

51. Hellström, M., Phng, L. K., & Gerhardt, H. (2007). VEGF and Notch signaling: the yin and yang of angiogenic sprouting. Cell adhesion & migration, 1(3), 133–136.

52. Horowitz, A., and M. Simons. 2008. 'Branching morphogenesis', Circ Res, 103: 784-95.

53. Huang, L., Zhai, Y., La, J., Lui, J. W., Moore, S. P. G., Little, E. C., Xiao, S., Haresi, A. J., Brem, C., Bhawan, J., & Lang, D. (2021). Targeting Pan-ETS Factors Inhibits Melanoma Progression. Cancer research, 81(8), 2071–2085.

54. Huang, X, C Brown, W Ni, E Maynard, AC Rigby, and P Oettgen. 2006. 'Critical role for the Ets transcription factor ELF-1 in the development of tumor angiogenesis', Blood, 107: 3153–60.

55. Iso, T, L Kedes, and Y Hamamori. 2003. 'HES and HERP families: multiple effectors of the Notch signaling pathway', J Cell Physiol, 194: 237-55.

56. Jain, RK. 2005. 'Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy', Science, 307: 58-62.

57. Jakobsson L, Franco CA, Bentley K, et al. Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. Nat Cell Biol. 2010;12(10):943-953.

58. Kamei, M., W. B. Saunders, K. J. Bayless, L. Dye, G. E. Davis, and B. M. Weinstein. 2006. 'Endothelial tubes assemble from intracellular vacuoles in vivo', Nature, 442: 453-6.

59. Kar, A., and A. Gutierrez-Hartmann. 2013. 'Molecular mechanisms of ETS transcription factor-mediated tumorigenesis', Crit Rev Biochem Mol Biol, 48: 522-43.

60. Keith, B, and MC Simon. 2014. 'Tumor Angiogenesis.' in J Mendelsohn, P Howley, M Israel, J Gray and C Thompson (eds.), The Molecular Basis of Cancer, (Fourth Edition).

61. Kitange, G., M. Kishikawa, T. Nakayama, S. Naito, M. Iseki, and S. Shibata. 1999. 'Expression of the Ets-1 proto-oncogene correlates with malignant potential in human astrocytic tumors', Mod Pathol, 12: 618-26.

62. Knezevich, S. R., D. E. McFadden, W. Tao, J. F. Lim, and P. H. Sorensen. 1998. 'A novel ETV6-NTRK3 gene fusion in congenital fibrosarcoma', Nat Genet, 18: 184-7.

63. Kliche, Stefanie and Waltenberger, Johannes. “Critical Review VEGF Receptor Signaling and Endothelial Function.” In: International Union of Biochemistry and Molecular Biology Life 52 (2001)

64. Koch, Sina and Claesson-Welsh, Lena. “Signal transduction by vascular endothelial growth factor receptors.” In: Cold Spring Harbor perspectives in medicine 2.7 (2012), a006502.

65. Konstantinidou, M, J Li, B Zhang, Z Wang, S Shaabani, F Ter Brake, K Essa, and A Dömling. 2019. 'PROTACs- a game-changing technology', Expert Opin Drug Discov, 14: 1255-68.

66. Kopan, Raphael and Ilagan, Maria Xenia G. “The canonical Notch signaling pathway: unfolding the activation mechanism.” In: Cell 137.2 (2009), pp. 216–33.

67. Kurz, H., P. H. Burri, and V. G. Djonov. 2003. 'Angiogenesis and vascular remodeling by intussusception: from form to function', News Physiol Sci, 18: 65-70.

68. Kustikova, O, D Kramerov, M Grigorian, V Berezin, E Bock, E Lukanidin, and E Tulchinsky. 1998. 'Fra-1 induces morphological transformation and increases in vitro invasiveness and motility of epithelioid adenocarcinoma cells', Mol Cell Biol, 18: 7095-105.

69. Lawson, ND, AM Vogel, and BM Weinstein. 2002. 'Sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation', Dev Cell, 3: 127-36.

70. La Mendola, D., Trincavelli, M. L., & Martini, C. (2022). Angiogenesis in Disease. International journal of molecular sciences, 23(18), 10962. Lee M, Ellis. (2001). 'Tumor Angiogenesis.' in, Surgical Research.

71. Lelievre, E., V. Mattot, P. Huber, B. Vandenbunder, and F. Soncin. 2000. 'ETS1 lowers capillary endothelial cell density at confluence and induces the expression of VE-cadherin', Oncogene, 19: 2438-46.fbiologicaL

72. Lei, Z. N., Tian, Q., Teng, Q. X., Wurpel, J. N. D., Zeng, L., Pan, Y., & Chen, Z. S. (2023). Understanding and targeting resistance mechanisms in cancer. MedComm, 4(3), e265.Leslie, JD, L Ariza-McNaughton, AL Bermange, R McAdow, SL Johnson, and J Lewis. (2007). 'Endothelial signalling by the Notch ligand Delta-like 4 restricts angiogenesis', development, 134: 839-44.

73. Li, JL, RC Sainson, W Shi, R Leek, LS Harrington, M Preusser, S Biswas, H Turley, E Heikamp, JA Hainfellner, and AL Harris. 2007. 'Delta-like 4 Notch ligand regulates tumor angiogenesis, improves tumor vascular function, and promotes tumor growth in vivo', Cancer Res, 67: 11244-53.

74. Li, M., and C. Chen. 2022. 'Regulation of Metastasis in Ewing Sarcoma', Cancers (Basel), 14.

75. Lincoln, D. W., 2nd, P. G. Phillips, and K. Bove. 2003. 'Estrogen-induced Ets-1 promotes capillary formation in an in vitro tumor angiogenesis model', Breast Cancer Res Treat, 78: 167-78.

76. Liu, F, and R Patient. (2008). 'Genome-wide analysis of the zebrafish ETS family identifies three genes required for hemangioblast differentiation or angiogenesis', Circ Res, 103: 1147-54.

77. Liu, M., S. Xie, and J. Zhou. (2018). 'Use of animal models for the imaging and quantification of angiogenesis', Exp Anim, 67: 1-6.

78. Liu, Zhaoguo et al. “Dll4-Notch signaling in regulation of tumor angiogenesis”. In: Journal of Cancer Research and Clinical Oncology 140.4 (2014), pp. 525–536.

79. Liu, ZJ, T Shirakawa, Y Li, A Soma, M Oka, GP Dotto, RM Fairman, OC Velazquez, and M Herlyn. (2003). 'Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis', Mol Cell Biol, 23: 14-25.

80. Lobov, I, and N Mikhailova. 2018. 'The Role of Dll4/Notch Signaling in Normal and Pathological Ocular Angiogenesis: Dll4 Controls Blood Vessel Sprouting and Vessel Remodeling in Normal and Pathological Conditions', J Ophthalmol: 2018:3565292.

81. Lobov, IB, RA Renard, N Papadopoulos, NW Gale, G Thurston, GD Yancopoulos, and SJ Wiegand. (2007). 'Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting', Proc Natl Acad Sci, 104: 3219-24.

82. Martins, SF, EA Garcia, MA Luz, F Pardal, M Rodrigues, and AL Filho. 2013. 'Clinicopathological correlation and prognostic significance of VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 expression in colorectal cancer', Cancer Genomics Proteomics, 10: 55-67.

83. Mathis, G. 1999. 'HTRF(R) Technology', J Biomol Screen, 4: 309-14McLaughlin, F, VJ Ludbrook, J Cox, I von Carlowitz, S Brown, and Randi AM. (2001). 'Combined genomic and antisense

analysis reveals that the transcription factor Erg is implicated in endothelial cell differentiation', Blood, 98: 3332-9.

84. Meller, S., H. A. Meyer, B. Bethan, D. Dietrich, S. G. Maldonado, M. Lein, M. Montani, R. Reszka, P. Schatz, E. Peter, C. Stephan, K. Jung, B. Kamlage, and G. Kristiansen. 2016. 'Integration of tissue metabolomics, transcriptomics and immunohistochemistry reveals ERG- and gleason score-specific metabolomic alterations in prostate cancer', Oncotarget, 7: 1421-38.

85. Mukherjee, T, A Kumar, M Mathur, TK Chattopadhyay, and R Ralhan. 2003. 'Ets-1 and VEGF expression correlates with tumor angiogenesis, lymph node metastasis, and patient survival in esophageal squamous cell carcinoma', J Cancer Res Clin Oncol, 129: 430-6.

86. Mukwaya, A., Jensen, L., & Lagali, N. (2021). Relapse of pathological angiogenesis: functional role of the basement membrane and potential treatment strategies. Experimental & molecular medicine, 53(2), 189–201. side in', Dev Biol, 228: 151-65.

87. Nakagawa, O, DG McFadden, M Nakagawa, H Yanagisawa, T Hu, D Srivastava, and EN Olson. 2000. 'Members of the HRT family of basic helix-loop-helix proteins act as transcriptional repressors downstream of Notch signaling', Proc Natl Acad Sci, 97: 13655-60.

88. Niu, N., C. Yu, L. Li, Q. Liu, W. Zhang, K. Liang, Y. Zhu, J. Li, X. Zhou, J. Tang, and J. Liu. (2018). 'Dihydroartemisinin enhances VEGFR1 expression through up-regulation of ETS-1 transcription factor', J Cancer, 9: 3366-72.

89. Noguera-Troise, I, C Daly, NJ Papadopoulos, S Coetzee, P Boland, NW Gale, HC Lin, GD Yancopoulos, and G Thurston. (2006). 'Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis', Nature, 444: 1032-7.

90. Nyberg, P, L Xie, and R Kalluri. 2005. 'Endogenous inhibitors of angiogenesis', Cancer Res, 65: 3967-79.

91. Oda, N., M. Abe, and Y. Sato. (1999). 'ETS-1 converts endothelial cells to the angiogenic phenotype by inducing the expression of matrix metalloproteinases and integrin beta3', J Cell Physiol, 178: 121-32.

92. Oettgen, P. 2001. 'Transcriptional regulation of vascular development', Circ Res, 89: 380-8.

93. Oikawa, T, and T Yamada. 2003. 'Molecular biology of the Ets family of transcription factors', Gene, 303: 11-34.

94. Olsson, AK, A Dimberg, J Kreuger, and L Claesson-Welsh. 2006. 'VEGF receptor signalling - in control of vascular function', Nat Rev Mol Cell Biol, 359-71

95. Otrock, Zaher K. et al. “Understanding the biology of angiogenesis: Review of the most important molecular mechanisms”. In: Blood Cells, Molecules, and Diseases 39.2 (2007), pp. 212–220.

96. Walker D M.,. Poczobutt J, Melissa S. Gonzales, Henrick Horita3 and Arthur Gutierrez-Hartmann Papakonstantinou, E., F. Bacopoulou, D. Brouzas, V. Megalooikonomou, D. D'Elia, E. Bongcam-Rudloff, and D. Vlachakis. (2010). ' SE-1 is Required to Maintain the Transformed Phenotype of MCF-7 and ZR-75-1 Human Breast Cancer Cells Open Cancer Journal, 2010

97. Pardali, E., M. J. Goumans, and P. ten Dijke. 2010. 'Signaling by members of the TGF-beta family in vascular morphogenesis and disease', Trends Cell Biol, 20: 556-67.

98. Patel-Hett, S., and P. A. D'Amore. 2011. 'Signal transduction in vasculogenesis and developmental angiogenesis', Int J Dev Biol, 55: 353-63.

99. Patel, NS, Li, JL, D Generali, R Poulsom, DW Cranston, and AL Harris. (2005). 'Up-regulation of delta-like 4 ligand in human tumor vasculature and the role of basal expression in endothelial cell function', Cancer Res, 65: 8690-7.

100. Pham, VN, ND Lawson, JW Mugford, L Dye, D Castranova, B Lo, and BM Weinstein. (2007). 'Combinatorial function of ETS transcription factors in the developing vasculature', Dev Biol, 303: 772-83.

101. Phng, LK, and H Gerhardt. 2009. 'Angiogenesis: a team effort coordinated by notch', Dev Cell, 16: 196-208.

102. Pimanda, JE, WY Chan, IJ Donaldson, M Bowen, AR Green, and B Göttgens. 2006 'Endoglin

expression in the endothelium is regulated by Fli-1, Erg, and Elf-1 acting on the promoter and a -8-kb enhancer', Blood.

103. Pollina, E. A., A. Legesse-Miller, E. M. Haley, T. Goodpaster, J. Randolph-Habecker, and H. A. Coller. (2008). 'Regulating the angiogenic balance in tissues', Cell Cycle, 7: 2056-70.

104. Quintero-Fabián, S., Arreola, R., Becerril-Villanueva, E., Torres-Romero, J. C., Arana-Argáez, V., Lara-Riegos, J., Ramírez-Camacho, M. A., & Alvarez-Sánchez, M. E. (2019). Role of Matrix Metalloproteinases in Angiogenesis and Cancer. Frontiers in oncology, 9, 1370. Ribatti, D., Nico, B., Crivellato, E., Roccaro, A. M., & Vacca, A. (2007). The history of the angiogenic switch concept. Leukemia, 21(1), 44–52.

105. Ribatti, D., Nico, B., Crivellato, E., & Vacca, A. (2007). The structure of the vascular network of tumors. Cancer letters, 248(1), 18–23.

106. Roukens, MG, M Alloul-Ramdhani, B Baan, K Kobayashi, J Peterson-Maduro, H van Dam, S Schulte-Merker, and DA Baker. 2010 'Control of endothelial sprouting by a Tel-CtBP complex', Nat Cell Biol, 12: 933-42.

107. Ruhrberg, C., H. Gerhardt, M. Golding, R. Watson, S. Ioannidou, H. Fujisawa, C. Betsholtz, and D. T. Shima. 2002. 'Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis', Genes Dev, 16: 2684-98.

108. Russo, A. E., D. Priolo, G. Antonelli, M. Libra, J. A. McCubrey, and F. Ferrau. (2017). 'Bevacizumab in the treatment of NSCLC: patient selection and perspectives', Lung Cancer (Auckl), 8: 259-69.

109. Sainson, RC, J Aoto, MN Nakatsu, M Holderfield, E Conn, E Koller, and CC Hughes. (2005). 'Cell-autonomous notch signaling regulates endothelial cell branching and proliferation during vascular tubulogenesis', FASEB J, 19: 1027-9.

110. Salami, J, and CM Crews. 2017. 'Waste disposal-An attractive strategy for cancer therapy', Science, 355: 1163-67.

111. Sato, Y. “Role of ETS family transcription factors in vascular development and angiogenesis.” Cell structure and function vol. 26,1 (2001): 19-24.

112. Schreiber, M, ZQ Wang, W Jochum, I Fetka, C Elliott, and EF Wagner. 2000. 'Placental vascularisation requires the AP-1 component fra1', development, 127: 4937-48.

113. Schweighofer, Bernhard et al. “The VEGF-induced transcriptional response comprises gene clusters at the crossroad of angiogenesis and inflammation”. In: Thrombosis and Haemostasis 102.3 (2009), pp. 544–554.

114. Seth, A., and D. K. Watson. (2005). 'ETS transcription factors and their emerging roles in human cancer', Eur J Cancer, 41: 2462-78.

115. Shah, A. V., G. M. Birdsey, C. Peghaire, M. E. Pitulescu, N. P. Dufton, Y. Yang, I. Weinberg, L. Osuna Almagro, L. Payne, J. C. Mason, H. Gerhardt, R. H. Adams, and A. M. Randi. (2017). 'The endothelial transcription factor ERG mediates Angiopoietin-1-dependent control of Notch signalling and vascular stability', Nat Commun, 8: 16002.

116. Siekmann, AF, and ND Lawson. (2007). 'Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries', Nature, 445: 781-4.

117. Siemann DW. The unique characteristics of tumor vasculature and preclinical evidence for its selective disruption by Tumor-Vascular Disrupting Agents. Cancer Treat Rev. 2011 Feb;37(1):63-74

118. Sizemore, G. M., J. R. Pitarresi, S. Balakrishnan, and M. C. Ostrowski. 2017. 'The ETS family of oncogenic transcription factors in solid tumours', Nat Rev Cancer, 17: 337-51.

119. Smith, BE, SL Wang, S Jaime-Figueroa, A Harbin, J Wang, BD Hamman, and CM Crews. 2019. 'Differential PROTAC substrate specificity dictated by orientation of recruited E3 ligase', Nat Commun, 10: 131.

120. Stone, J, A Itin, T Alon, J Pe'er, H Gnessin, T Chan-Ling, and E Keshet. (1995). 'Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia', J Neurosci, 15: 4738-47.

121. Suchting, S, C Freitas, F le Noble, R Benedito, C Bréant, A Duarte, and A Eichmann. 2007.

'The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching', Proc Natl Acad Sci, 104: 3225-30.

122. Szeles, J. Sápi, D.A. Drexler, I. Harmati, Z. Sápi, L. Kovács Model-based angiogenic inhibition of tumor growth using modern robust control method IFAC Proc., 45 (18) (2012), pp. 113-118,

123. Takahashi, H, and M Shibuya. 2005. 'The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions', Clin Sci (Lond), 109: 227-41.

124. Takahashi N, Hoshi H, Higa A, Hiyama G, Tamura H, Ogawa M, Takagi K, Goda K, Okabe N, Muto S, Suzuki H, Shimomura K, Watanabe S, Takagi M. An In Vitro System for Evaluating Molecular Targeted Drugs Using Lung Patient-Derived Tumor Organoids. Cells. 2019 May 20;8(5):481.

125. Tomlins, S. A., R. Mehra, D. R. Rhodes, L. R. Smith, D. Roulston, B. E. Helgeson, X. Cao, J. T. Wei, M. A. Rubin, R. B. Shah, and A. M. Chinnaiyan. 2006a. 'TMPRSS2:ETV4 gene fusions define a third molecular subtype of prostate cancer', Cancer Res, 66: 3396-400.

126. Treps, L., Ager, A., & Hida, K. (2022). Editorial: Tumor Vessels as Directors of the Tumor Microenvironment: New Findings, Current Challenges & Perspectives. Frontiers in cell and developmental biology, 10, 885670. https://doi.org/10.3389/fcell.2022.885670.

127. Tveriakhina, L., Schuster-Gossler, K., Jarrett, S. M., Andrawes, M. B., Rohrbach, M., Blacklow, S. C., & Gossler, A. (2018). The ectodomains determine ligand function in vivo and selectivity of DLL1 and DLL4 toward NOTCH1 and NOTCH2 in vitro. eLife, 7, e40045.

128. Uyttendaele, H, G Marazzi, G Wu, Q Yan, D Sassoon, and J Kitajewski. 1996. 'Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene', development, 122: 2251-9.

129. Verschoor, M. L., C. P. Verschoor, and G. Singh. 2013. 'Ets-1 global gene expression profile reveals associations with metabolism and oxidative stress in ovarian and breast cancers', Cancer Metab, 1: 17.

130. Wang, Michael M. “Notch signaling and Notch signaling modifiers.” In: The international journal of biochemistry & cell biology 43.11 (2011), pp. 1550–62.

131. Watson, DK, R Li, VI Sementchenko, G Mavrothalassitis, and A Seth. 2001. 'The ETS genes.' in JR Bertino (ed.), Encyclopedia of cancer (San Diego: Academic Press).

132. Watson, DK, and A Seth. 2000. 'Ets gene family.' in Reddy EP Jenkins J (ed.), Oncogene reviews (London: Nature Publishing Group).

133. Wei, Y., Han, S., Wen, J., Liao, J., Liang, J., Yu, J., Chen, X., Xiang, S., Huang, Z., & Zhang, B. (2023). E26 transformation-specific transcription variant 5 in development and cancer: modification, regulation and function. Journal of biomedical science, 30(1), 17.

134. Weinmaster, G. (1998). 'Notch signalling: Direct or what?', Curr Opin Genet Dev, 8: 436-42.

135. Williams, CK, JL Li, M Murga, AL Harris, and G Tosato. 2006. 'Up-regulation of the Notch ligand Delta-like 4 inhibits VEGF-induced endothelial cell function', Blood, 107: 931-9.

136. Wu, Y, C Cain-Hom, L Choy, TJ Hagenbeek, GP de Leon, Y Chen, D Finkle, R Venook, X Wu, J Ridgway, D Schahin-Reed, GJ Dow, A Shelton, S Stawicki, RJ Watts, J Zhang, R Choy, P Howard, L Kadyk, M Yan, J Zha, CA Callahan, and SG Hymowitz. (2010). 'Therapeutic antibody targeting of individual Notch receptors', Nature, 464: 1052-7.

137. Yazdjerdi, P., Meskin, N., Al-Naemi, M., Al Moustafa, A. E., & Kovács, L. (2019). Reinforcement learning-based control of tumor growth under anti-angiogenic therapy. Computer methods and programs in biomedicine, 173, 15–26.

138. Zhang, G, CR Dass, E Sumithran, N Di Girolamo, LQ Sun, and LM Khachigian. 2004. 'Effect of deoxyribozymes targeting c-Jun on solid tumor growth and angiogenesis in rodents', J Natl Cancer Inst, 96: 683-96.

139. Ziche, M, Donnini, S, and L Morbidelli. 2004. 'Development of new drugs in angiogenesis', Curr Drug Targets, 5: 485-93.