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Novel mechanisms and signaling pathways in angiogenesis

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Appendix

A

Summary in English

Nederlandse Samenvatting

List of Abbreviation

Publications

Acknowledgments

Curriculum Vitae

SUMMARY IN ENGLISH

Angiogenesis, the process of new blood vessel formation from existing blood vessels, is essential for wound healing, embryonic development, as well as organ function regulation. Vascular Endothelial Growth Factor Receptor (VEGFR) and NOTCH signaling are among the main pathways that work in coordination to orchestrate the complex process of angiogenesis. Understanding the underlying molecular and cellular mechanisms is crucial for developing angiogenesis-based therapies for treating diseases like cancer and cardiovascular disease. Approaches to control angiogenesis encompass targeting specific angiogenic signalling molecules and suppressing their key intracellular transcriptional effectors. Within the scope of this thesis I investigated the precise control of angiogenesis and the potential effects of pharmacological intervention of signaling pathways involved in angiogenesis. The obtained insights have shed new light on the basic mechanism underlying angiogenesis and may aid drug discovery endeavors focused on manipulating angiogenic signaling pathways for therapeutic gain.

The NOTCH receptor is a transmembrane receptor that consists of NOTCH extracellular domain (NECD), the transmembrane (TM) domain, and (the NOTCH intracellular domain (NICD). In mammals four NOTCH receptors are found that are highly conserved. NOTCH signaling is initiated when DELL or JAGGED ligands, which are also transmembrane proteins, engage with Notch receptors and thereby induce a proteolytic cleave resulting in the release of the intracellular domain, which translocate to the nucleus and directs specific transcriptional responses. This pathway is regulated in multiple ways and new mechanisms continue to be discovered. Besides, the aforementioned control of angiogenesis, NOTCH signaling also plays a pivotal role in cell differentiation, tissue growth, tissue remodeling, and apoptosis in various cell types, including neuronal and also cancer cells.

The research in chapter 2 has shed light on how NOTCH ligands influence the formation of endothelial cell tubes. Previously, the cleavage of the intracellular domain (ICD) of the NOTCH ligand (DLL4) was poorly understood. We provided evidence that DLL4 ICD which is highly conserved throughout vertebrate is proteolytically cleaved. In addition to that, our data highlights two previously unreported roles for DLL4 ICD. Firstly, it is required for normal DLL4 subcellular localization. Secondly, the untethered ICD interacts with JUN transcription factor and can prevent Jun from binding to DNA, which, in turn, regulates the activation of JUN target genes, including DLL4 itself. Our research also revealed that JUN plays a significant role in promoting the formation of endothelial cell tubes, while DLL4 acts to restrain this process. These findings suggest the intriguing possibility of bidirectional signaling of NOTCH/DLL4 pathway and propose that the DLL4 ICD

may serve as a crucial point of interaction between NOTCH and receptor tyrosine kinase (RTK) signaling.

Previous studies have shown that NOTCH receptors are regulated by two primary mechanisms. One is by direct cell-cell contact, during which NOTCH ligands from one cell interact with NOTCH receptor in another cell, leading to intercellular (trans) NOTCH activation. The second mechanism involves inhibition of receptor function by ligand-receptor interactions within a single cell, known as cis inhibition. Moreover, recent findings have also indicated the occurrence of cis activation upon ligand-receptor interaction within one cell. In Chapter 3 our research demonstrates that NOTCH ligands, such as DLL4 can self-associate effectively, in both biochemical assays and *in vitro* cell culture studies. We show that the specific region responsible for this binding is the membrane proximal epidermal growth factor (EGF)-like repeat of DLL4. Our findings suggest that DLL4 ligand dimerization is crucial for inhibiting NOTCH receptor activity within the same cell (cis inhibition). Our results of NOTCH ligand dimerization/oligomerization are of great importance for understanding the dynamics of the NOTCH signaling, as the regulation of dimer formation and its disassembly may serve as an additional point of control for the strength and specificity of NOTCH signaling.

To strengthen these findings, we have employed powerful mathematical modelling approaches to extended existing models of NOTCH signaling, centered on the use of regular differential equations. The generated computational simulations were greatly supportive of the idea of dimerization-dependent cis inhibition of NOTCH signalling, as revealed in our biochemical and cell biological based study. Importantly, our refined mathematical model recapitulates published experimental data, and improves predictions based upon published mechanisms of NOTCH-driven tissue development. Our model introduces a new perspective for the understanding of the biological processes, which are controlled by NOTCH, underpinning embryo development and angiogenesis.

In chapter 4 we explore the connection between aberrant function of the transcription factor FOS and vascular tumors such as epithelioid hemangioma, which is an aggressive tumor characterized by vascular neoplasms in bones and soft tissue. Recent research has highlighted FOS translocations as a potential contributor to tumorigenesis, though to date no such mutations had been identified as causal mutations in human cancer. Through analysis of human epithelioid hemangioma tumor cell lysates, we revealed a truncated FOS (mutant FOS) protein *in vivo*, which resulted from a FOS-MBNL1 translocation. The finding led us to investigate the molecular underpinnings of a possible role of FOS translocations in vascular tumor evolution and also tumor angiogenesis. Our study represents the first comprehensive molecular and biochemical evaluation of a mutant FOS protein identified within this human vascular tumor. We

provided evidence that the mutant FOS protein was much more stable, due to a C-terminus truncation, and this could drive the emergence of vascular neoplasms. We found that the mutant FOS protein potently stimulated endothelial sprouting, and stimulated vascular tumor formation by disturbing matrix metalloproteinase (MMP) production and the NOTCH signaling pathway, which are key players of angiogenesis. Our study also introduced the concept of ubiquitin-independent proteasomal degradation (UIPD), and revealed the role of the unstructured FOS C-terminus in mediating protein degradation. This is the first link between FOS mutations and natural tumor development. Moreover, our results provide therapeutic avenues for the treatment of epithelioid hemangiomas with mutant FOS proteins by targeting mutant FOS or proteins whose expression is triggered by FOS.

Malfunction of multiple ETS transcription factors have been implicated in contributing to the emergence of various tumors, including Ewing's sarcoma (ES) and prostate cancer. This research described in chapter 5 demonstrates the feasibility of developing molecular inhibitors to reduce ETS factor transcriptional activity through the disruption of ETS protein-DNA interactions. Our unique approach involved obtaining pure protein preparations of ETS factors and conducting functional -high throughput screens to identify novel small molecules inhibitors of ETS function. Selection of the molecules that inhibit ETS-DNA interaction may enable generation of drug-like molecules that will be able to target specific ETS proteins. Initially an HTRF assay was performed which measured the ability of compounds to disrupt binding of a purified ETS DNA-binding domain to its specific consensus DNA binding site resulting in the identification of five novel compounds. Subsequently, we interrogated the inhibitors in different biochemical/biological assays (such as cell proliferation, cytotoxicity and endothelial sprouting assay using primary human endothelial cells as well as, zebrafish and mouse Metatarsal). Our present results offer promising initial indications that these compounds have the potential to impede tumor cell growth, inhibit angiogenesis and that the observed effects could result from inhibition of the activity of ETS transcription factors. This ongoing study has the potential to provide novel therapeutic agents that target ETS proteins.