Novel mechanisms and signaling pathways in angiogenesis

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CHAPTER 6

General Discussion

This thesis focuses on angiogenesis, the formation of new blood vessels from pre-existing ones. It plays a crucial role in various physiological processes, such as wound healing, tissue repair, tumor growth, invasion, and metastasis (Liu et al., 2023; Bielenberg and Zetter, 2015; Lugano et al., 2020). 2023; Bielenberg and Zetter 2015; Lugano, Ramachandran, and Dimberg 2020). While angiogenesis is mediated by a variety of signaling pathways such as cytokines and extracellular matrix molecules, it is orchestrated mainly by two principal pathways: one comprises intracellular signaling through vascular endothelial growth factor (VEGF) receptors (VEGFR), and the other includes intercellular signaling via the NOTCH/DLL4 pathway. Understanding the mechanisms of this process is crucial for developing therapies for diseases associated with abnormal angiogenesis such as cancer and diabetic retinopathy.

Intriguingly, within the scope of this thesis, we have uncovered new fundamental aspects of NOTCH/DLL4 signaling. This has introduced a new dimension for understanding the molecular mechanisms governing angiogenesis. Interestingly, we have also identified mutant FOS protein in human vascular tumors and conducted functional analyses of this FOS variant. Although, concerning angiogenesis, the NOTCH pathway in general, and DLL4 in particular, have emerged as potential and promising targets for novel therapeutic interventions in cancer. However, the work presented in this thesis provides an excellent methodology for drug screening and has led to the characterization of a novel ETS transcription factor inhibitors.

In the scope of this thesis, we have uncovered new basic features of NOTCH signalling that might strongly influence basic angiogenesis mechanisms. This understanding has the potential to greatly benefit drug discovery efforts aimed at inhibiting angiogenic signaling pathways.

CHAPTER 2

The NOTCH signaling pathway plays a critical role in cell differentiation, tissue growth, tissue remodeling, and apoptosis of most metazoan cell types. However, accumulating evidence suggests that there are multiple, less well-defined layers of regulation in the NOTCH signaling pathway. The work in chapter 2 has provided strong insight into how NOTCH ligands control endothelial cell (EC) tube formation. Until now, little was known about the cleavage of the intracellular domain (ICD) of the NOTCH ligand, DLL4 (DLL4INTRA). The experiments reported in chapter 2 provide a new insight into the potential relationship between the intracellular domain of DLL4 and specific components of the AP-1 transcription factor complex. This study suggested that DLL4INTRA, a component of the NOTCH signaling pathway, can play a direct role in gene regulation and signaling by inhibiting the activity of the JUN transcription factor.

While previous research has focused on the canonical NOTCH signaling pathway (Zhu et al., 2019; Schober et al., 2005), our results demonstrate the possible existence of multiple layers of regulation, which to date are not well-defined. These results build on existing evidence of the effects of ligand ICDs at the cellular level. It has been shown that ectopic expression of the DLL1 ICD causes growth arrest of primary ECs (Fortini, 2009). In addition, inhibition of NOTCH1 signaling by the JAGGED1 ICD can modulate cardiac homeostasis in the postnatal heart (Metrich et al., 2015). However, in this study our results strikingly highlight a potential link between untethered NOTCH ligand ICDs and the immediate-early gene AP-1 transcription factor complex. Our findings provide additional information about the potential role of DLL4INTRA in endothelial cell response through cross-talk with the bZIP domain of JUN. Furthermore, our study suggests that corruption of this mechanisms might play a role in disease processes such as tumor angiogenesis and developmental disorders. This important finding may further highlight the NOTCH pathway as a primary target to inhibit tumor growth therapeutically, and it has also been linked to rare congenital disorders and diseases.

Interestingly, our present data also indicates that DLL4 ICD is required for normal DLL4 subcellular localization. The experiments revealed that JUN does not appear to interact with the full-length version of DLL4, which contains an intracellular domain. This suggests that the overall structure of DLL4 may impede the occurrence of this interaction. Additionally, it is possible that DLL4 forms oligomers or dimers (Consistent with our discovery discussed in Chapter 3), which may have impact on DLL4 interactions and which could make the C terminus inaccessible for interactions with JUN These findings indicate that the intracellular domain of DLL4 may have a dual role, interacting with the bZIP domain of JUN and also encoding a PDZ-binding domain that enables a functional association with multi-PDZ domain protein MUPP1, which is required for normal ligand trafficking, as well as the cAMP responsive element binding protein CREB3. Notably, our results provide evidence for the importance of the JUN bZIP domain in the stimulation of sprouting of ECs. In line with our finding that DLL4INTRA inhibits JUN-driven EC sprouting, it can be concluded that this occurs by inhibiting JUN binding to a consensus AP-1 DNA site and thereby controlling the expression of JUN target genes, including DLL4. This finding suggested that JUN-DLL4INTRA interactions, could form part of a feedback loop in regulating DLL4 expression. This result ties in well with previous studies wherein genome-wide screens have shown cooperation between AP-1 and ETS transcription factor via neighboring AP-1 and ETS DNA-binding sites (Plotnik et al., 2014).

Furthermore it has been reported that ETS transcription factors regulate angiogenesis (Craig and Sumana,s 2016; Randi et al., 2009; Roukens et al., 2010) via control of DLL4 expression (Shah et al., 2017; Roukns et al., 2010).Overall, our

results suggest that upon VEGF stimulation, DLL4INTRA, in association with ETS/AP-1 factors could potentially regulate DLL4 expression.
Our results shed new light on the intracellular domain of the NOTCH ligand DLL4, which might participate in signaling and downstream transcription. These results may lead to a better understanding of the potential bidirectional signaling of NOTCH/DLL4. Furthermore, our key findings highlight the untethered NOTCH ligand ICDs as a point of cross-talk between the NOTCH pathway and other pathways, such as receptor tyrosine kinase (RTK) signaling. It will be of great importance to explore the specific mechanisms of DLL4INTRA cleavage and turnover, as well as determining the possible similar functionality of other NOTCH ligand ICD domains. Further studies should investigate the function and regulation of NOTCH signaling through the DLL4 ICD, which could lead to a better understanding of the pathway and uncover new, potential therapeutic targets.

CHAPTER 3

The NOTCH receptor has been intensely studied since its initial description by Thomas Hunt Morgan in 1917 (Andersson et al., 2011). Although, experimentally and theoretically, numerous research efforts have been directed toward a comprehensive understanding of NOTCH signaling, several gaps in the field still need to be addressed. In Chapter 3, we aimed to provide a new perspective on understanding the mechanism of the NOTCH pathway. The NOTCH signaling pathway is a critical signaling system that plays a role in the development and differentiation of cells. It involves the interaction between NOTCH receptors and ligands expressed on neighboring cells (trans signalling) and within the same cell (cis inhibition).
Different molecules and force requirements are needed to initiate NOTCH signaling. An important open question in the field is how specific output is generated from the combination of Cis and Trans interactions of the various NOTCH ligands and receptors. In this chapter, we embarked on a series of experimental and mathematical modeling studies to understand the mechanism of the NOTCH pathway and answer these questions.
It is assumed that the DLL4 ligand functions as a monomer, but surprisingly, in this study, we have presented evidence that NOTCH ligands can efficiently homo- and heterodimerize. NOTCH ligand dimerization/oligomerization could have a crucial impact on our understanding of the dynamics of NOTCH signaling, as the regulation of dimer formation and its disassembly may serve as an additional point of control for the strength of NOTCH signaling.
Specifically, in Chapter 3, we demonstrated that NOTCH ligands can self-associate and that this dimerization is required explicitly for cis-inhibition of NOTCH receptor activity but not NOTCH receptor transactivation. In light of this, we developed a mathematical model to take into account the role of ligand

dimerization in cis-inhibition and found that the model accurately reproduced previously published experimental data and could improve the predictions. Interestingly, our results suggest that the net output of NOTCH signalling is determined by a balance between ligand monomer-driven transactivation and ligand dimer-mediated cis-inhibition. A new general model adapted from the mutual inactivation model has been developed to explore the potential role of ligand monomers and dimers in net signaling output from the NOTCH pathway.
Importantly, our results shed new light on the cellular mechanism underlying the formation of boundaries in the development of the Drosophila wing's discs, which historically has been an essential model system for understanding NOTCH signalling. Our dimerization model's validity has also been verified in the context of tissue patterning processes. We also analyzed a further extension of our dimerization model, in which we showed that the ligand dimerization mechanism of NOTCH enables veins and inter-vein formation in the Drosophila wing.
The four mammalian NOTCH receptors and the five mammalian NOTCH ligands give rise to at least 20 different receptor-ligand combinations. The finding of ligand dimerization potentially increases the range of possible receptor-ligand combinations and resulting signaling outputs. Additionally, our biochemical experiments showed that ligands can also hetero-oligomerize. Therefore, ligand oligomerization may offer additional complexity in NOTCH signaling and yield diverse signaling outputs (see Fig C in S1 Text chapter 3).
Besides our previous discovery regarding NOTCH/DLL4 bidirectional signaling, understanding the biological function and mechanism of the DLL4 dimerization might be integral to designing robust treatments. In this regard, a more complete understanding of the mechanism of binding different interactions between NOTCH ligands and receptors will require additional analysis. However, from an experimental aspect, several technical hurdles still had to be overcome, including the size and complexity of the extracellular domains of ligands and receptors, which make some experiments more challenging. For instance, NOTCH receptors and ligands are single-pass transmembrane proteins of about 150-300 kDa in size. Hence, the technical challenges associated with purifying sufficient quantities of receptors and ligands have precluded biochemical studies with full-length proteins.
Further work is certainly required to disentangle these complexities. Under these circumstances, recent technological advances such as Cryo-EM might enable a deeper structural understanding of the dimerization process and its role in NOTCH signalling.
Collectively, uncovering this novel dimension of NOTCH signaling opens up entirely new perspectives for understanding the dynamics of the NOTCH pathway and, accordingly, of angiogenesis. It would be interesting to examine

whether the NOTCH receptors themselves can also oligomerize/dimerize and, if so, to delineate the mechanistic consequences. This also has potential implications for understanding the role of NOTCH in disease.

CHAPTER 4

The research in Chapter 4 presented the first functional characterization of mutant FOS proteins discovered in a human tumor, specifically, an epithelioid hemangioma tumor. In order to elucidate the underlying cause of this bone tumor, we analyzed three previously characterized translocations (van et al. 2015). We analyzed human epithelioid cell Lysate and revealed a truncated FOS protein lacking a C-terminal disordered region. Although the involvement of a FOS gene in this type of cancer was established before (Antonescu et al., FOS-MBNL1 translocation), this is the first study to functionally characterize mutant FOS proteins discovered in a human tumor.

To the best of our knowledge, this was the first report that indicates sustained expression of FOS due to deletion of the four C-terminal amino acids of FOS, which could initiate the formation of vascular neoplasms. By taking a mutagenesis approach, we discovered that the terminal short helical region of C terminal FOS can orchestrate the ubiquitin-independent degradation of FOS. The fact that ubiquitination is not a prerequisite for FOS proteasomal degradation and that truncated FOS appears to be resistant to this process, coupled with the fact that FOSΔ protein levels appear to be substantially higher than wild-type FOS protein levels in patient cells, suggests that the highly conserved helical motif is critical for controlling FOS stability. Our data reveals a novel mechanism by which aberrant FOS protein can drive the formation of vascular neoplasms.

Our global transcriptome analyses clarified the mechanistic basis of FOS-driven sprouting. Importantly, we found that both wild-type and mutant FOS control the expression of angiogenesis-related genes, including MMPs and components of the NOTCH-signaling pathway. These findings were among the first to highlight FOS as an activator of endothelial sprouting by direct interaction with the FOS promoter. Our work presented in Chapter 3 has uncovered a new role for FOS in stimulating endothelial cell sprouting. We also demonstrated that a recently reported small molecule inhibitor of FOS (Makino et al., 2017) can inhibit FOS-driven endothelial sprouting (Figure. S5). This finding might help the development of novel treatments for rheumatoid arthritis and open a new dimension for understanding tumor angiogenesis.

CHAPTER 5

Angiogenesis can be controlled through various mechanisms like inhibition of angiogenic signaling pathways, suppression of critical transcriptional regulators, and targeting specific angiogenic molecules (Liu et al., 2023). While anti-

angiogenic therapies have shown promise in treating cancer with combination therapy, there are growing appeals for new targets due to a growing concern about the development of drug resistance and tumor recurrence, among other limitations (Liu et al., 2023). Although these challenges are also evident in anti-angiogenic therapy and chemotherapy, consuming anti-angiogenic combination therapy to its full potential is urgently needed to treat various clinical conditions where treatment options are lacking (Nandagopal et al., 2019; Fleming et al., 2013). For example, maximizing the clinical efficacy of treatment for non-small cell lung cancer (NSCLC) has been achieved through a combination of angiogenesis inhibitors, immune system regulators, and chemotherapy. This combination overcome the resistance issue regarding the treatment of EGFR-mutant NSCLC (Sun et al., 2022). Therefore, the angiogenesis process has been widely observed, and several approaches to inhibit the angiogenic signaling pathways have been considered through the last decades.

Through the use of new technologies, the pool of potential drug targets is expanding. Rather than targeting the upstream components of cell signalling networks (presumed to be druggable), we developed methodologies for identifying small molecule inhibitors of transcription factors downstream of these pathways (previously regarded as undruggable). It is likely that the action of transcription factors, which are downstream of signalling systems, ultimately drive the illicit behaviors of tumor cells. This makes them attractive targets for rational anticancer drug design.

The research presented in Chapter 5 builds on existing evidence of the central role of ETS transcription factors in regulating angiogenesis. To the best of our knowledge, this is the first report of identifying small molecule inhibitors that block the activity of these transcription factors and their association with specific DNA binding sites. As stated in Chapter 5, ETS transcription factors are frequently dysregulated in cancers. Additionally, certain ETS factors are also crucial in angiogenic development. Therefore, blocking the angiogenesis process by targeting ETS proteins could represent a novel approach to cancer treatment.

Through fragment-based screening, we identified potential fragment hits, which were screened by NMR. We selected the fragment hits as an ETS/DNA binding inhibitor, followed by a high-throughput screen to obtain lead-like compounds. Thus, this project aimed to identify potential ETS inhibitors and validate them through various cell-based and biochemical approaches. To investigate the anti-tumor activity of our inhibitors, several cancer types, in particular prostate cancer and Ewing's sarcoma, were studied.

As it has been established that the promotion of cellular proliferation is probably the most important function of ETS factors within cancer, and also ETS mutations are the primary drivers of cellular proliferation, one would expect to see cancer cell proliferation reduction with the treatment of effective ETS inhibitors (Zhu et

al., 2019). Here, we observed that the ETS inhibitors strongly inhibited cellular proliferation and movement of cancer cell lines. Additionally, it has been demonstrated that the treatment with our compounds reduced sprouting vessels in Metatarsal angiogenesis assays, thereby highlighting an additional potential benefit of this anti-angiogenic treatment. The validity of this data can be validated through the use of Xenograft mouse cancer models, which would allow us to determine the impact of our drugs on *in vivo* tumorigenesis. This is the next essential step in our study.

Targeting specific transcription factors could help overcome problems such as drug resistance and toxicity. One way to potentially enhance specificity would be to perform screens with full-length protein and not simply the ETS DBD. With a complimentary effort of characterizing and determining the nature of gene networks, we will address the influence of the novel compounds at the molecular level through transcriptome analyses, proteome analyses, and genome-wide ChIP studies and by examination of the expression of ETS and ERG-regulated genes, such as SOX9, which has been earlier shown to stimulate PCa invasion (Cai et al., 2013; Wang et al., 2008).

Resistance to treatment is a significant problem in oncology. In future studies, we will investigate the potential of cancer cells to develop resistance to the ETS inhibitors THIS. Testing potential synergistic interactions between our ETS inhibitors and other inhibitors, such as the BCL2 inhibitor venetoclax, will be important.

While it was outside the scope of this study to thoroughly examine all possible avenues for further investigation, future research will greatly benefit from incorporating the additional experiments mentioned. The concept that ETS proteins can serve as viable targets for drug development could represent a significant advancement in the field of oncology, as these previously considered undruggable targets are now considered potential options for future treatments.

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