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Faculdade de Medicina

THE ROLE OF THE DELTA-LIKE 4 NOTCH LIGAND IN TUMOR ANGIOGENESIS

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Doutoramento em Ciências Biomédicas
Especialidade de Ciências Biopatológicas

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To Aleksandra, Nina
and Filip Viktor

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SUMMARY

Clinically significant tumors induce their own vascularization using molecular mechanisms involved in the regulation of physiological angiogenesis. One of these mechanisms is mediated by Delta-like 4 (Dll4)/Notch signaling, which is known to play a fundamental role in the regulation of embryonic angiogenesis and arterial specification. Up-regulated in animal and human tumors, Dll4 represents the focus of the work presented in this thesis, which aimed to characterize its function(s) in the regulation of tumor-driven angiogenesis and validate it as a potential therapeutic target.

To achieve the proposed objectives, Dll4 loss- and gain-of-function phenotypes were characterized in grafted and autochthonous mouse tumor models. Additionally, pharmacological inhibition of Dll4/Notch signaling by soluble Dll4 extracellular domain (sDll4) was assessed as a therapeutic strategy, alone or in combination with the inhibition of downstream Ephrin-B2/EphB4 signaling by soluble extracellular EphB4 (sEphB4).

Loss of Dll4 function by either targeted *Dll4* allele deletion or use of sDll4 was shown to lead to increased, but defective and immature vascular proliferation in subcutaneously grafted malignancies (S180, HT29 and KS-SLK cell lines), as well as in autochthonous insulinomas of transgenic RIP1-*Tag2* mice. The increase in endothelial activation was confirmed to reflect higher endothelial sensitivity to vascular endothelial growth factor A (VEGFA), while retarded vessel maturation, indicated by reduced mural cell recruitment, was found associated with EphrinB2 and Tie2 signaling suppression. This induction of unproductive angiogenesis resulted in significantly reduced tumor growth, creating a new perspective for tumor vasculature targeting. Regarding the suppression of vessel maturation and consequent tumor expansion, greater efficacy against RIP1-*Tag2* insulinomas was observed when Ephrin-B2/EphB4 signaling inhibition by sEphB4 was combined with either *Dll4* allele deletion or sDll4 treatment. Importantly, hepatic vascular alterations arising under chronic Dll4 inhibition were prevented by concomitant sEphB4 treatment.

Apparently in contradiction to data obtained from advanced, aggressive and rapidly expanding tumors, my work demonstrated that *Dll4* down-regulation in pre-malignant, DMBA/TPA-induced chemical skin papillomas, promotes productive, although less mature angiogenesis, as well as neoplasm growth. In both benign and malignant tumors, Dll4 was found to serve as a fundamental suppressor of excessive endothelial proliferation. The observed difference in skin papillomas might be due to the capacity of tumor neo-vessels to mature and gain functionality in early but not in invasive lesions. In these lesions, higher VEGFA levels and decreased VEGFA gradients result in disorganized endothelial proliferation that does not permit functional vessel formation if Dll4/Notch is even partially suppressed. These findings therefore imply that Dll4 requirement to normalize tumor vasculature increases with the tumor grade, being always critical to hold down VEGFA-induced angiogenesis.

Nevertheless, my results reveal that Dll4 functions as a very delicate determinant of tumor success and progression, although vascular response quite expectedly changes with its alteration. By exploring the effects of increased endothelial Dll4 expression to the supra-basal levels in Lewis lung cancer xenografts, chemically-induced skin tumors and transgenic RIP1-*Tag2* insulinomas, this work shows that Dll4 over-expression also decreases tumor growth due to significant suppression of angiogenesis and pronounced reduction of overall tumor blood supply. Although increased Dll4 function consistently improved tumor vascular maturation and functionality, the tumor vessel normalization did not predominate over the tumor-suppressive effects of restricted vessel proliferation, in the experimental settings used. Thereby, *Dll4* over-expression appeared as an alternative that, beside the anti-angiogenic effects, might reduce malignant cell penetration into the bloodstream and ensure increased delivery and effectiveness of co-applied chemotherapy, due to improved vessel maturation and competence.

Collectively, Dll4 plays a prominent role as negative regulator and pro-maturation factor in tumor-driven angiogenesis. Interference with Dll4/Notch may be desirable in cancer patients, while the choice of an agonistic or antagonistic approach will probably depend on tumor type and further toxicity and efficacy assessments, particularly when applied in combination with other anti-angiogenic and cytostatic drugs.

Keywords: Tumor angiogenesis, Dll4, Notch signaling, anti-cancer drug development.

RESUMO

Tumores clinicamente significativos induzem a sua própria vascularização. Para isso, frequentemente utilizam os mecanismos moleculares envolvidos na regulação da angiogénese fisiológica. Uma destas vias é via de sinalização Delta-like 4 (Dll4)/Notch, que se sabe desempenhar um papel fundamental na regulação da angiogénese embrionária e na especificação arterial. Encontrando-se a sua expressão aumentada em tumores humanos e animais, Dll4 constituiu o principal foco deste projecto de Doutoramento, que teve como objectivo caracterizar a sua função na regulação da angiogénese tumoral e validar este ligando de receptores Notch como um potencial alvo terapêutico.

Para atingir os objectivos propostos, fenótipos de perda- e de ganho-de-função de Dll4 foram caracterizados em modelos tumorais enxertados e autóctones em murganhos. Além disso, a inibição farmacológica da via Dll4/Notch com uma proteína solúvel contendo o domínio extracelular de Dll4 (sDll4) foi avaliada como uma estratégia mono-terapêutica, e, subsequentemente, em combinação com a inibição da via de sinalização Ephrin-B2/EphB4, que se sabe actuar a jusante da via Dll4/Notch, realizada com administração de uma proteína solúvel contendo o domínio extracelular de EphB4 (sEphB4).

A perda da função de Dll4 por deleção *monoalélica* de *Dll4* ou por administração de sDll4 resultou em proliferação vascular aumentada, mas defeituosa e imatura, em tumores enxertados subcutaneamente (linhas celulares S180, HT29 e KS-SLK), assim como em insulinomas autóctones de murganhos transgénicos RIP1-*Tag2*. O aumento da activação endotelial foi confirmado como resultante do aumento da sensibilidade endotelial ao factor de crescimento endotelial vascular A (VEGFA), enquanto a maturação vascular retardada, indicada pelo recrutamento diminuído das células murais, foi associada com a supressão da sinalização de EphrinB2 e da função de Tie2. Esta indução da angiogénese não produtiva resultou em redução significativa do crescimento

tumoral, representando a base racional de uma nova, e potencialmente benéfica, estratégia para interferir com a neo-vascularização em tumores. Em relação à supressão da maturação vascular e à consequente expansão tumoral, observou-se uma maior eficácia na regulação do crescimento de insulinomas em murganhos RIP1-Tag2, quando a administração de sEphB4 foi combinada com a deleção *monoalélica* em *Dll4* ou com o tratamento com sDll4. Adicionalmente, as alterações vasculares hepáticas decorrentes da inibição crónica de Dll4 foram impedidas pelo tratamento concomitante com sEphB4.

Aparentemente em contradição com os dados obtidos a partir de tumores avançados, agressivos e em rápida expansão, a deleção de um alelo *Dll4* em papilomas cutâneos pré-malignos, quimicamente induzidos com DMBA/TPA, resultou na promoção de produtiva, embora menos madura, bem como no maior crescimento destas neoplasias. Quer em tumores benignos como malignos, encontra-se documentada a função de Dll4 como importante supressor de proliferação endotelial excessiva. A diferença encontrada nestes estudos deve estar relacionada com a capacidade dos vasos tumorais ganharem funcionalidade no caso de lesões iniciais, mas não em lesões invasivas, onde os níveis mais elevados de VEGFA e diminuição dos gradientes de VEGFA resultam em proliferação endotelial tão desorganizada que praticamente não permitia a formação dos vasos funcionais se a sinalização Dll4/Notch estivesse suprimida, mesmo que parcialmente. Assim, os resultados aqui apresentados sugerem que a importância de Dll4 para normalizar a vascularização tumoral aumenta com o grau de malignidade, sendo sempre crítica para moderar a angiogénese induzida por VEGFA.

No entanto, a função de Dll4 é um determinante critíco da progressão tumoral, embora a resposta vascular mude de um modo bastante previsível com as alterações na expressão de *Dll4*. Através da análise dos efeitos devidos ao aumento da expressão endotelial de Dll4 em enxertos de cancro pulmonar de Lewis, em tumores cutâneos quimicamente induzida e em insulinomas dos murganhos transgénicos RIP1-*Tag2*, é mostrado neste trabalho que Dll4 retarda também o crescimento tumoral devido à supressão significativa de angiogénese e redução acentuada da perfusão sanguínea do tecido neoplásico. Embora a presença aumentada de Dll4 tenha consistentemente melhorado a maturação e funcionalidade vascular nos modelos experimentais utilizados, a normalização dos vasos não predominou sobre os efeitos anti-tumorais decorrentes da restrição da proliferação vascular. Assim, a sobre-expressão de *Dll4* surge como uma alternativa que, além dos efeitos anti-angiogénicos, pode reduzir a penetração de células

malignas na corrente sanguínea e garantir uma maior eficácia da quimioterapia co-aplicada devido à promoção da maturação e competência vascular dos tumores.

Em conclusão, Dll4 desempenha um papel de grande relevo na angiogénese induzida por tumores, quer como um regulador negativo de activação endotelial quer como factor de maturação vascular. Interferência com Dll4/Notch pode ser desejável em doentes oncológicos, embora a escolha de uma estratégia agonista ou antagonista dependerá provavelmente do tipo tumoral e do resultado da avaliação de segurança e eficácia que terá que ser feita, em particular quando aplicada em terapêutica de combinação com outros medicamentos anti-angiogénicos e citostáticos.

Palavras-chave: Angiogênese tumoral, Dll4, sinalização Notch, desenvolvimento de fármacos contra cancro.

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ABBREVIATIONS

ADAM	A disintegrin and maetalloproteinase family
AIDS	Acquired immunodeficiency syndrome
Alb	Albumin
AMD	Age-related macular degeneration
Ang1	Angiopoietin 1
Ang2	Angiopoietin 2
AP	Alkaline phosphatase
B16	Mouse melanoma cell line 16
Bcl-2	B-cell lymphoma 2 protein encoding gene
bFGF	Basic fibroblast growth factor
B-Raf	Murine sarcoma viral oncogene homolog B1
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CD4	Cluster of differentiation glycoprotein 4
CD11	Cluster of differentiation glycoprotein 11
cDNA	Complementary deoxyribonucleic acid
ChoK	Chinese hamster cell line
CIN	Chromosomal instability
c-KIT	Mast/stem cell growth factor receptor
cmm	Cubic millimeter
COL-3	Incyclinide 3
COX-2	Cyclooxygenase 2
Cre	Cre recombinase (topoisomerase) from P1 bacteriophage
CSL	Cbf1/Su(H)/Lag1 DNA-binding protein

D4BE	Dll4 basic-expression mice
D4OE	Dll4 over-expressing mice
DAPI	4′,6-diamidino-2-phenylindole dihydrochloride hydrate
DBP–MAF	Vitamin-D-binding protein–macrophage-activating factor
DCC	Deleted in colorectal cancer receptor
Dll1	Delta-like ligand 1
Dll3	Delta-like ligand 3
Dll4	Delta-like ligand 4
DMBA	7,12-dimethylbenz[a]anthracene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSL	Delta, Serrate, Lag2 ligand family
E	Embryonic day
ECM	Extra-cellular matrix
EDTA	Ethylenediaminetetraacetic acid
EFC-XV	Endostatin-like fragment from type XV collagen
EFNB2	Human gene encoding Ephrin-B2
EGF	Epidermal growth factor
EGFL7	Epidermal growth factor ligand 7
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ENA78	Epithelial neutrophil-activating protein 78
EPC(s)	Endothelial progenitor cell(s)
EphA4	Erythropoietin-producing human hepatocellular carcinoma receptor A4
EphB1	Erythropoietin-producing human hepatocellular carcinoma receptor B1
EphB2	Erythropoietin-producing human hepatocellular carcinoma receptor B2
EphB3	Erythropoietin-producing human hepatocellular carcinoma receptor B3
EphB4	Erythropoietin-producing human hepatocellular carcinoma receptor B4
Ephrin-B2	Eph family receptor interacting protein B2
Ephrin(s)	Eph family receptor interacting protein(s)
Ephs	Erythropoietin-producing human hepatocellular carcinoma receptors
Erb	Erythroblastic leukemia viral oncogene
ERL	NH2-terminal Glu-Leu-Arg sequence

FACS	Fluorescence-activated cell sorter
Fc	Immunoglobulin constant region
FGF	Fibroblast growth factor
FGF2	Fibroblast growth factor 2
Flk-1	Fetal liver kinase 1
Flt-1	Fms-like tyrosine kinase 1
Flt-3	Fms-like tyrosine kinase 3
Flt-4	Fms-like tyrosine kinase 4
Fos	Fibroblast oncogene
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase encoding gene
GCP-2	Granulocyte chemoattractant protein 2
GIST	Gastrointestinal stromal tumor
Glu.Leu.Arg	Glutamine-Leucine-Arginine sequence
Gro	Growth-related oncogene protein
H&E	Hematoxylin and eosin staining
HDAC	Histone deacetylase
HER2	Human Epidermal growth factor Receptor 2
HERP	HES-related repressor proteins
HES	Hairy and enhancers of split transcription factors
HIF	Hypoxia-inducible factor
HP1	Hypoxyprobe-1
HPV	Human papilloma virus
HT29	Human colon cancer cell line 29
HUVECs	Human umbilical vein endothelial cells
Id1	DNA-binding protein inhibitor 1
IFL	Irinotecan, 5-fluorouracil and leucovorin combined chemotherapy
IFN(s)	Interferon(s)
IFN-α	Interferon alpha
Ig	Immunoglobulin
IL(s)	Interleukin(s)
IL-4	Interleukin 4
IL-8	Interleukin 8
IMiDs	Immunomodulatory drugs

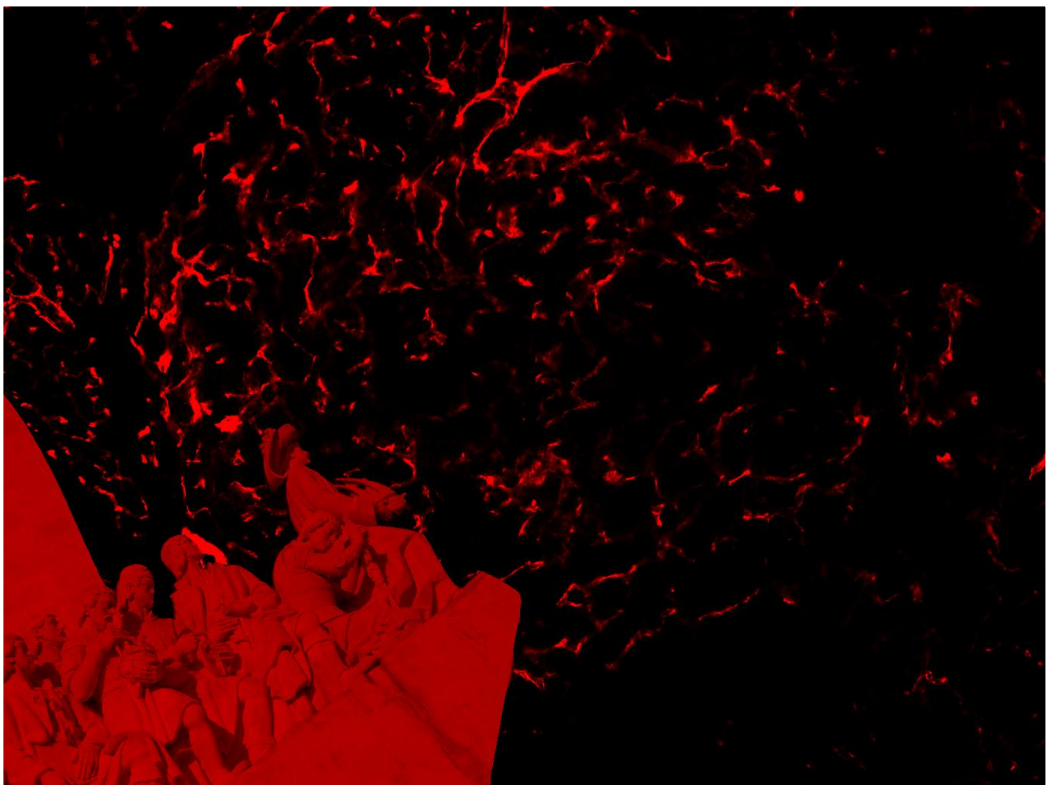
Jag1	Jagged ligand 1
Jag2	Jagged ligand 2
KDR	Kinase domain-containing receptor
KS	Human Kaposi sarcoma cell line
LacZ	β-galactosidase reporter gene
LLC	Lewis lung cancer cell line
Mam	Mastermind co-activator
MCF-7	Michigan Cancer Foundation 7 breast cancer cell line
MHC	Major histocompatibility complex
MIN	Microsatellite instability
MMP(s)	Matrix metalloproteinase(s)
MMP1	Matrix metalloproteinase 1
MMP2	Matrix metalloproteinase 2
MMP9	Matrix metalloproteinase 9
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
Myb	Myeloblastosis oncogene
Myc	Myelocytomatosis oncogene
NG2	Neurogenin 2 chondroitin sulfate proteoglycan
NICD	Notch intra-cellular domain
NK	Natural killer cell
NO	Nitric oxide
p38	Mitogen-activated protein kinase 38
p53	Tumor protein 53 encoding gene
PBS	Phosphate buffered saline
PBST	Tween-20 solution in PBS
PC(s)	Pericyte(s)
PC3	Prostatic adenocarcinoma cell line 3
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFB	Platelet-derived growth factor B
PDGFR-β	Platelet-derived growth factor receptor β
PDZ	Post-synaptic density protein, Drosophila disc large tumor suppressor and

	zonula occludens-1 protein
PECAM	Platelet endothelial cell adhesion molecule
PEDF	Pigment epithelium-derived factor
PEX	Haemopexin C domain autolytic fragment of matrix metalloproteinase 2
PFA	Paraformaldehyde (PFA)
PI3K	Phosphatidyl-inositol-3-kinase
PlGF	Placental growth factor
PPAR-γ	Peroxisome proliferator-activated receptor gama
PTEN	Phosphatase and tensin homolog encoding gene
PTKs	Receptor protein tyrosine kinases
PTN	Pleiotrophin
PTTG1	Pituitary tumor-derived transforming gene 1
PyMT	Polyoma virus middle T oncogene
Ras	Rat sarcoma oncogene
Rb	Retinoblastoma protein encoding gene
RBC	Red blood cell
RET	Glial cell line–derived neurotrophic factor receptor
RIP	Rat insulin promoter
ROBO 1	Roundabout homologue 1
ROBO 2	Roundabout homologue 2
ROBO 3	Roundabout homologue 3
ROBO 4	Roundabout homologue 4
ROS	Reactive oxygen species
RT2	RIP1-*Tag2* transgenic mouse line
RT-PCR	Reverse transcription polymerase chain reaction
S180	Mouse sarcoma 180 cell line
SD	Standard deviation
sDll4	Soluble Dll4 extra-cellular domain
SEM	Standard error of the mean
Sema3A	Semaphorin 3A
Sema3E	Semaphorin 3E
Sema4A	Semaphorin 4A
sEphB4	Soluble monomeric form of the extracellular domain of EphB4

sFlt1	Soluble fms-related tyrosine kinase 1
Src	Sarcoma oncogene
SSC	Squamous cell carcinoma
SV40	Simian vacuolating virus 40
T. f. VEGF	*Trimeresurus flavoviridis* vascular endothelial growth factor F
Tag	Large T antigen
TGF-ß	Transforming growth factor beta
Tie-2	Tyrosine kinase with immunoglobulin-like and EGF-like domains 2
TIMP 1	Tissue inhibitor of metalloproteinase 1
TIMP 2	Tissue inhibitor of metalloproteinase 2
TIMP 3	Tissue inhibitor of metalloproteinase 3
TIMP 4	Tissue inhibitor of metalloproteinase 4
TIMPs	Tissue inhibitors of matrix metalloproteinase
TNF-α	Tumor necrosis factor alpha
TPA	12-O-tetradecanoylphorbol-13-acetate
TSP1	Thrombospondin 1
TSP2	Thrombospondin 2
UNC5	Receptor of the uncoordinated-5 family
US FDA	United States Food and Drug Administration
VEGF	Vascular endothelial growth factor
VEGFA	Vascular endothelial growth factor A
VEGFA	Vascular endothelial growth factor A encoding gene
VEGFB	Vascular endothelial growth factor B
VEGFC	Vascular endothelial growth factor C
VEGFD	Vascular endothelial growth factor D
VEGFE	Vascular endothelial growth factor E
VEGFR1	Vascular endothelial growth factor receptor 1
VEGFR2	Vascular endothelial growth factor receptor 2
VEGFR3	Vascular endothelial growth factor receptor 3
VPF	Vascular permeability factor
WT	Wild-type
α-SMA	Alpha smooth muscle actin
β-gal	β-galactosidase

CHAPTER I

INTRODUCTION



Djokovic D., “Embarkation”, collage (2011)

1
Introduction

In complex vertebrate organisms, the cardiovascular system, composed of the heart, blood vessels and blood itself, delivers oxygen and nutrients to all tissue cells and removes carbon-dioxide and metabolic waste from them (Aaronson et al., 2004). It also distributes water, electrolytes and hormones throughout the body, contributes to the infrastructure of the immune system, establishes hemostasis after injury, and participates in thermoregulation. Deregulated vascular formation and functions contribute to the pathogenesis of different congenital and later onset disorders, including inflammatory, ischemic, neoplastic, and degenerative pathologies (Carmeliet 2003). Thereby, significant efforts have been made over the last decades to better understand the mechanisms regulating vascular growth and homeostasis in both pre- and post-natal settings. Angiogenesis, the predominant mechanism of blood vessel proliferation under adult physiological and pathological conditions (Adams and Alitalo 2007), has been intensely under the focus of biomedical research resulting in the identification of rational bases for novel therapeutic strategies with revolutionary potential regarding the management of a range of human diseases.

This introducing chapter provides a brief overview of the angiogenic process and its regulation. The sites of physiological and pathological angiogenesis are subsequently presented while special attention is paid to the particularities of tumor angiogenesis. The successful attempts to translate laboratory bench results to the angiogenesis-targeting anti-cancer drug development are next summarized. Simultaneously, the major clinical problems arising from the use of currently approved angiogenesis-modulators are reviewed indicating the indisputable need to improve our understanding of tumor-driven angiogenesis. Finally, the attention is focused on Delta-like 4 (Dll4)/Notch signaling which crucial implication in embryonic vascular development (Duarte, Hirashima et al. 2004), together with increased *Dll4* expression levels within human cancers (Mailhos, Modlich et al. 2001; Patel, Dobbie et al. 2006), directed the host laboratory and my professional interest to elucidate this signaling pathway role(s) in tumor vascularization.

1.1. OVERVIEW OF ANGIOGENESIS AND ITS REGULATION

1.1.1. Basic Mechanisms of Blood Vessel Formation

Growing tissues require an increased blood perfusion to guarantee adequate exchange of gases, nutrient supply and removal of waste metabolic products, which is achieved by the proliferation of blood vessels. This is evident during embryogenesis where hierarchically organized arteries, capillaries and veins develop from initial, undifferentiated vascular structures through dynamic growth and remodeling processes (Folkman 1995). Similar vascular expansive phenomena are also seen in the adults, in the female reproductive tract during the ovarian/menstrual cycles and in tissue regenerative processes such as wound healing (Klagsbrun and D'Amore 1991).

There are at least three different mechanisms that contribute to blood vessel formation and growth: **vasculogenesis, angiogenesis** and **intussusceptions** (Adams and Alitalo 2007). Vasculogenesis denominates the formation of blood vessels by the direct assembly of angiogenic progenitor cells while angiogenesis refers to the sprouting of novel capillary blood vessels from the pre-existing vasculature and subsequent stabilization of these sprouts by mural cells. Additionally, the vascular network can be enlarged by splitting a single pre-existing vessel in two new vascular segments through the insertion of a tissue pillar, a process called intussusception or splitting angiogenesis.

Initially, it was though that vasculogenesis occurred only in the embryos where the first vessels developed from endothelial progenitor cells (EPCs, *i.e*. angioblasts), while the adult vessel formation resulted exclusively from division of differentiated endothelial cells and angiogenic sprouting of pre-existing vessels. During early embryogenesis, mesodermal cells differentiate into EPCs and form aggregates, so-called *blood islands*, which subsequent fusion gives origin to the vasculogenic formation of *primary capillary plexi* (Eichmann, Yuan et al. 2005). These primitive vascular structures are extended by angiogenesis and remodelled into an embryonic vascular tree. Recent evidence suggests that the bone-marrow-derived circulating EPCs are capable of penetrating into the endothelium contributing to vasculogenic blood vessel growth in the adults (Rafii, Lyden et al. 2002). Nevertheless, angiogenesis is clearly the most common mechanism engaged in adults for vascular extension under both physiological and pathological conditions (Adams and Alitalo 2007).

1.1.2. The Angiogenic Cascade

Endothelial cells (ECs) are the basic and constant blood vessel components covering, in the form of a monolayer, their luminal surfaces. In favorable pro-angiogenic settings, principally induced by hypoxia, the angiogenic process is initiated by selective activation of individual ECs in differentiated capillaries (Adams and Alitalo 2007). While some ECs are activated others maintain the quiescent state. The selected ECs acquire invasive behavior, degrade the sub-endothelial basement membrane and depart from the vessel of origin following the gradients of pro-angiogenic stimuli. Known as the *tip cells*, they break down the extra-cellular matrix and provide directional migration (*i.e*., lead the growing sprout). Despite their highly migratory phenotype, *tip cells* remain in physically contact with their follower ECs, the *stalk cells*, which proliferate in response to pro-angiogenic factors constituting the endothelial component of the nascent vessel body and extending its length (Iruela-Arispe, 2008). Simultaneously with this endothelial proliferation, mesenchymal cells also proliferate, migrate through the cleaved extra-cellular matrix, incorporate in the new sprout, differentiate into mural cells, *i.e*. pericytes, and mature stabilizing the outgrowth (Papetti and Herman 2002). Both adhesive and repulsive interactions between *tip cells* from adjacent sprouts regulate their fusion and the establishment of novel vascular interconnections (Adams and Alitalo 2007). The vessel wall assembly, lumen acquisition, synthesis and organization of the basement membrane and specification of ECs to arterial or venous fate trigger the resolution of the endothelial activation (Iruela-Arispe, 2008). Once established blood flow improves tissue perfusion that reduces hypoxia-induced pro-angiogenic signals and enhances vascular maturation including the stabilization of EC-EC junctions, matrix deposition and tight pericyte attachment (Adams and Alitalo 2007).

Didactically, the described cellular phenomena can be divided in four consecutive phases representing together so-called *angiogenic cascade*. They are:

1. Stimulation of angiogenic response and selection of sprouting ECs,
2. Sprout outgrowth,
3. Sprout fusion and lumen formation, and
4. Vessel perfusion and maturation

As schematically presented in Figure 1.1., each phase is a tightly regulated process.

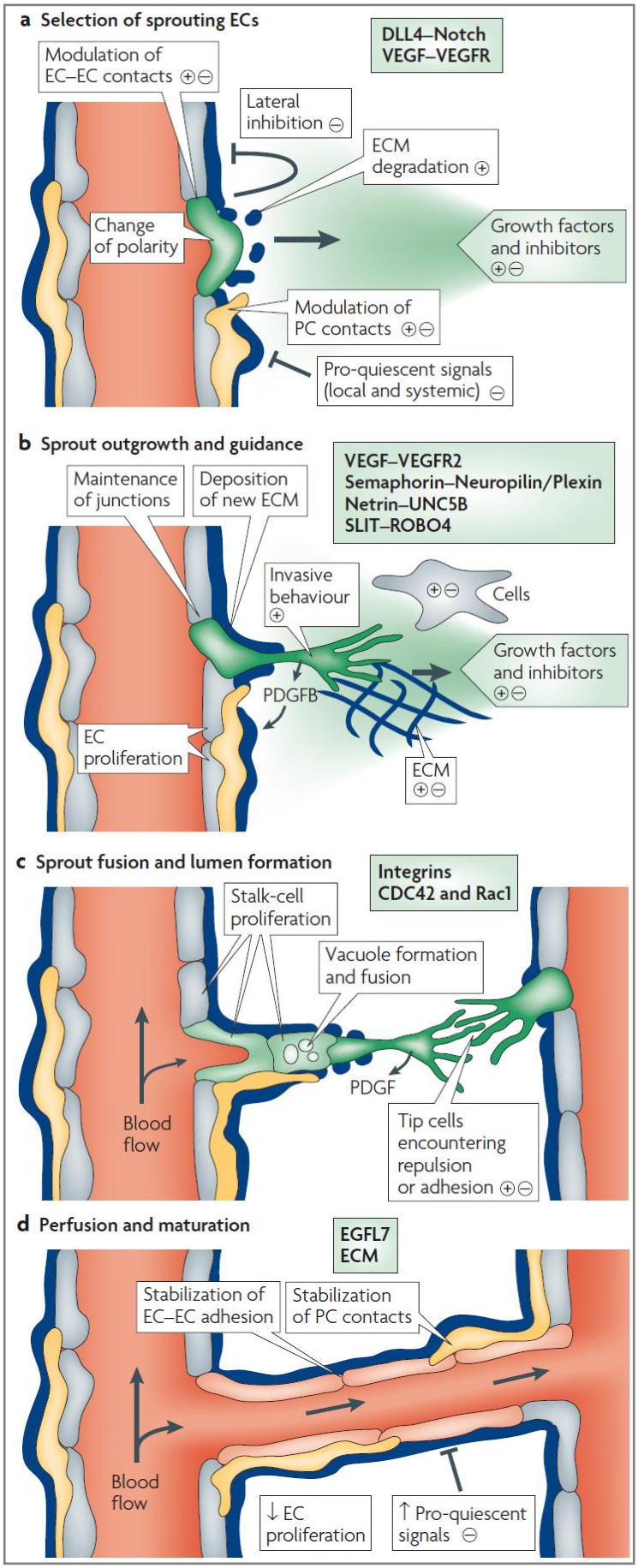


Figure 1.1. – Angiogenic cascade (reproduced from Adams and Alitalo, 2007). **a)** Angiogenic sprouting is controlled by the balance between pro-angiogenic signals (+), such as vascular endothelial growth factor (VEGF), and factors that promote quiescence (–), such as tight mural cell, *i.e.* pericyte (PC; yellow) contact, certain extra-cellular matrix (ECM) molecules or VEGF inhibitors. In conditions that favor angiogenesis, some endothelial cells (ECs) can sprout (green), whereas others fail to respond (grey). Sprouting requires the flipping of apical–basal polarity, the induction of motile and invasive activity, the modulation of cell–cell contacts and local matrix degradation. **b)** The growing EC sprout is guided by VEGF gradients. Other signals may include attractive (+) or repulsive (–) matrix cues and guidepost cells in the tissue environment. Release of platelet-derived growth factor B (PDGFB) by the tip cells promotes the recruitment of PCs to new sprouts. EC–EC junctions need to be maintained after lumen formation to prevent excessive leakage. **c)** Adhesive or repulsive interactions that occur when tip cells encounter each other regulate the fusion of adjacent sprouts and vessels. Lumen formation in stalk ECs involves the fusion of vacuoles but other mechanisms may also contribute. **d)** Fusion processes at the EC–EC interfaces establish a continuous lumen. Blood flow improves oxygen delivery and thereby reduces pro-angiogenic signals that are hypoxia-induced. Perfusion is also likely to promote maturation processes such as the stabilization of cell junctions, matrix deposition and tight PC attachment. Growth factor withdrawal can trigger sprout retraction and endothelial apoptosis. **DLL4**, Delta-like-4 ligand; **EGFL7**, Epidermal growth factor ligand-7; **ROBO4**, Roundabout homologe-4; **VEGF2**, VEGF receptor-2.

1.1.3. Molecular Regulation of Angiogenesis

The angiogenic growth of blood vessels is regulated by different and numerous positively and negatively acting factors. They include soluble polypeptides, cell-cell interactions, cell-matrix interactions and hemodynamic effects (Papetti and Herman 2002). The principal angiogenesis regulators, classified as soluble mediators, membrane-bound molecules, and mechanical forces, are presented in Panel 1.1. Capillary blood vessels remain in the quiescent state when the balance between positive and negative regulatory factors is maintained stable while the shift to the side of pro-angiogenic stimuli triggers angiogenesis.

Panel 1.1. – Angiogenesis regulators (adapted from Papetti and Herman, 2001)

I - SOLUBLE MEDIATORS

Stimulatory Factors	*Inhibitory Factors*
Acidic fibroblast growth factor	Angiostatin
Angiogenin	Antithrombin III fragment
Basic fibroblast growth factor	Endostatin
Fibroblast growth factor 3 and 4	Canstatin
Hepatocyte growth factor	Fragment of platelet factor 4
Interleukin 8	Interferon αand β
Placental growth factor	Interferon-inducible protein 10
Platelet-derived growth factor	Maspin
Pleiotropin	Prolactin fragment
Proliferin	Thrombospondin 1 and 2
Platelet-derived endothelia-cell growth factor	Tumstatin
Transforming growth factor αand β	Vascular endothelial growth inhibitor
Tumor necrosis factor α	Vasostatin
Vascular endothelial growth factor	

II - MEMBRANE-BOUND MOLECULS

Stimulatory Effects	*Inhibitory Effects*
Integrin α5β1	Classe 3 semaphorins /Plexin D1
Integrin αvβ3	Netrin1/ uncoordinated-5B
Integrin, αvβ5,	Slit?/Robo4
Eph-4B/Ephrin-B2	

III - MECHANICAL FORCES (*pro-angiogenic)*

Blood flow/Shear stress

1.1.3.1. Vascular Endothelial Growth Factor A and Stimulation of the Angiogenic Response

Current experimental evidence indicates that the most important pro-angiogenic factor is **vascular endothelial growth factor A** [VEGFA or VEGF; also known as vascular permeability factor (VPF)]. VEGFA is a member of a family of potent angiogenesis and/or lymphangiogenesis positive regulators that also includes VEGFB, VEGFC, VEGFD, placental growth factor (PlGF), Orf virus' VEGFE, and *Trimeresurus flavoviridis* (*T. f.*) svVEGFs (Lyttle, Fraser et al. 1994; Junqueira de Azevedo, Farsky et al. 2001; Ferrara, Gerber et al. 2003). In humans and animals, VEGFA is secreted at low levels by various tissues, but in elevated concentrations, the growth factor is produced in the sites where angiogenesis is needed such as in the placenta, many embryonic/fetal tissues, in the proliferating endometrium, *corpus luteum* as well as in neoplastic tissues (Dvorak, Brown et al. 1995). VEGFA production is principally regulated by the tissue oxygen saturation (Shweiki, Itin et al. 1992). Under hypoxic conditions, VEGFA production is promoted by up-regulated hypoxia-inducible factor (HIF) that binds to the *VEGFA* promoter increasing the gene transcription (Kimura, Weisz et al. 2001).

VEGFA causes diverse effects on the endothelium. It specifically inhibits EC apoptosis (Gerber, McMurtrey et al. 1998) and stimulates EC proliferation (Connolly, Heuvelman et al. 1989), enhances EC migration (Dimmeler, Dernbach et al. 2000), and increases vascular permeability to circulating metabolites (Kitadai, Takahashi et al. 1999). Genetically targeted mouse embryos deficient in *Vegfa* virtually do not develop any vascular structure (Shalaby, Rossant et al. 1995; Ferrara 1999). Studies conducted in mice, on the neonatal corneas and healing bone grafts, indicated that the VEGFA roles are equally important for inducing pos-natal and adult capillary sprouting (Connolly, Heuvelman et al. 1989).

The endothelial responsiveness to VEGFA is regulated by several mechanisms including expression of alternatively spliced VEGFA isoforms. In the first place, alternative splicing of a single *VEGFA* gene can generate different variants of the growth factor composed of 121, 145, 165, 183, 189 or 206 amino acids (Tischer, Mitchell et al. 1991; Robinson and Stringer 2001). Despite the fact that all of these isoforms have the same biological activity, they can be either soluble (*e.g.* VEGFA-121 and the most commonly produced VEGFA-165) or immobilised, cell- or matrix-

associated due to their affinity for heparan sulfates (*e.g.* VEGFA-189 and VEGFA-206) (Houck, Leung et al. 1992). While immobilized VEGFA generates a gradient that is sensed by the *tip cell* providing directional migratory cues, the soluble VEGFA does not form the gradient, so ECs lose directionality becoming less migratory (Tischer, Mitchell et al. 1991; Ruhrberg, Gerhardt et al. 2002; Lee, Jilani et al. 2005). In addition, alternative *VEGFA* splicing can result in the production of " b" isoforms that have anti-angiogenic properties (Ladomery, Harper et al. 2007). Thus, the balance between different VEGFA isoforms alters the EC behaviour in response to the growth factor.

The VEGFA effects depend also on the balance between the different VEGFA receptors. VEGFA binds to at least three tyrosine kinase receptors: Flt-1 (VEGFR1) (de Vries, Escobedo et al. 1992), KDR/Flk-1 (VEGFR2) (Klagsbrun and D'Amore 1991), and Flt-4 (VEGFR3) (Pajusola, Aprelikova et al. 1992). Binding of VEGFA to VEGFR2 leads to the activation of a cascade of downstream signaling pathways and generates the major signals for sprouting angiogenesis (Shibuya and Claesson-Welsh 2006). In contrast, pro-angiogenic VEGFA effects are minimized by VEGFR1, which has ten times higher affinity for the growth factor then VEGFR2, but very weak tyrosine-kinase activity, serving as a VEGFA trapper (Shibuya and Claesson-Welsh 2006). Besides, VEGFR1 appears in a soluble and catalytically inactive form (Shibuya 2006). Therefore, the VEGFR1/VEGFR-2 ratio importantly determines the VEGFA outcome.

Together with VEGFA, different other factors have been demonstrated to counteract pro-quiescent signals and positively influence the process of angiogenesis (Panel 1.1.). Nevertheless, VEGFA is thought to be the initiating angiogenesis stimulus.

1.1.3.2. Delta-like4/Notch Signaling and Acquisition of *Tip vs. Stalk* Endothelial Cell Identities

Stimulating EC survival, proliferation and migration, as presented above, VEGFA plays a fundamental role in the EC activation. Nevertheless, VEGFA signaling, on its own, does not provide an organized endothelial proliferation that is absolutely needed for hierarchical and thereby functional vascular network formation (Iruela-Arispe, 2008). A crucial element in angiogenesis regulation is to establish the leadership between equal ECs under favorable pro-angiogenic conditions and determine which

ECs will acquire the *tip cells* phenotype providing directional migration and which ECs will remain in the quiescent state within the pre-existing vessels or acquire the "follower", *stalk cells* phenotype contributing to the body formation of a novel sprout. Interestingly, it has been documented that the *tip* and the *stalk cells* respond differently to VEGFA stimulation: while the *tip cells* predominantly migrate, the *stalk cells* mostly proliferate (Gerhardt and Betsholtz 2005).

Several studies in zebrafish (Leslie, Ariza-McNaughton et al. 2007; Siekmann and Lawson 2007) and mouse embryos (Duarte, Hirashima et al. 2004; Sainson, Aoto et al. 2005) as well as in mouse neonatal retinas (Claxton and Fruttiger 2004; Hellstrom, Phng et al. 2007; Lobov, Renard et al. 2007; Suchting, Freitas et al. 2007) have indicated that endothelial trans-membrane **Notch receptors**, activated by their **Delta-like 4 (Dll4) ligand** of adjacent ECs, trigger intra-cellular signaling that determines EC fate when exposed to pro-angiogenic stimuli. Most of the referred studies were conducted simultaneously with this PhD project. Since the very first of these findings from developmental and post-embryonic angiogenesis experiments directly inspired our research focusing the Dll4 implication in pathological (tumor-driven) angiogenesis in adult mice, the section *"Dll4/Notch Signaling Pathway and its Involvement in Vascular Biology"* provides more extensive information. Briefly, targeted *Dll4* deletion and pharmacologically achieved Notch signaling blockade enhance ECs to acquire the *tip cell* phenotype resulting in excessive angiogenic branching. Considering the fact that, under VEGFA pressure, *Dll4* has up-regulated expression in the *tip cells*, this implies that activated ECs use the Dll4/Notch system to signal to the neighboring quiescent ECs and suppress their activation. Mechanistically, Dll4/Notch signaling was found to repress VEGFR2 and increases the VEGFA trapper - VEGFR1 (Lobov, Renard et al. 2007; Suchting, Freitas et al. 2007). Thus, binding of Dll4 of already activated ECs (acquiring *tip cells* phenotype) to the Notch on adjacent cells seems to induce the acquisition of the *stalk* identities by enabling ECs to differentially sense the existent VEGFA gradient (Figure 1.2.).

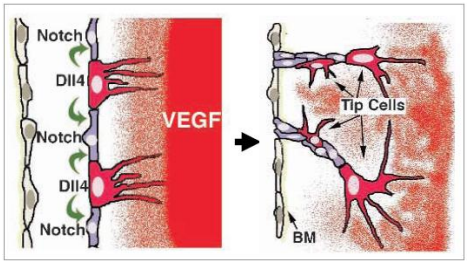


Figure 1.2. – Selection of sprouting endothelial cells (adapted from Iruela-Arispe, 2008). Departing form parental vessels, subset of activated ECs express *Dll4*, activate the Notch receptor in adjacent ECs reducing their sensibility to VEGFA (*left*). The outcome is the formation of a vascular sprout with highly migratory *tip cells* and proliferating *stalk cell* (*right*).

1.1.3.3. Guiding Cues for Sprout Outgrowth

Once determined the fate of ECs and established the leadership between them, organized EC proliferation and migration result in emerging sprout outgrowth. Expressing VEGFR2 in the *tip cells*, EC guidance is controlled by the special concentration gradient of matrix-anchored VEGFA isoforms that functions as a chemo-attractive force (Ruhrberg, Gerhardt et al. 2002; Gerhardt, Golding et al. 2003). In parallel, proliferating vascular sprouts generate the concentration gradient of platelet-derived growth factor B (PDGFB), which up-regulated expression in ECs promotes the recruitment of PDGF receptor β (PDGFR-β)-exhibiting pericytes and, thus, growing vessel stabilization (Gerhardt, Golding et al. 2003). In addition to VEGFA concentration gradient, the endothelial directional sprouting is regulated by attractive and repulsive tissue-derived navigational cues with pronounced analogy to growing nerve fibers (Carmeliet and Tessier-Lavigne 2005). Initially identified as axon guidance regulators, **semaphorins, netrins, slit/robo proteins and ephrins** have been evidenced to play prominent roles in angiogenic vessel growth (Huminiecki, Gorn et al. 2002; Gitler, Lu et al. 2004; Lu, Le Noble et al. 2004; Park, Crouse et al. 2004; Gu, Yoshida et al. 2005; Weinstein 2005; Wilson, Ii et al. 2006; Toyofuku and Kikutani 2007; Sawamiphak, Seidel et al. 2010; Wang, Nakayama et al. 2010)

Semaphorin family includes membrane-bound and secreted proteins that provide navigational signals during neuronal growth, but also, as recently documented, some of them influence vascular directionality (Weinstein 2005). Generally said, membrane-bound semaphorins signal by binding to **plexins** while secreted semaphorins signal through **neuropilins** (Bagri and Tessier-Lavigne 2002). Genetic targeting of Semaphorin4A (Sema4A) showed that it suppresses VEGF-induced EC migration and angiogenesis *in vivo* by interacting with Plexin D1 (Toyofuku and Kikutani 2007). In addition to Sema4A, at least two class 3 semaphorins, Sema3A and Sema 3E, can signal trough Plexin D1 serving as repulsive cues for ECs (Gitler, Lu et al. 2004; Gu, Yoshida et al. 2005).

The second group of ligands involved in both neuronal growth and vascular patterning are the **netrins**. In the nervous system, netrins function either as attractive cues, when they interact with the **family of deleted in colorectal cancer (DCC) receptors**, or as repulsive cues, when they signal through **receptors of the**

uncoordinated-5 (UNC5) family or **UNC5–DCC heterodimers** (Carmeliet and Tessier-Lavigne 2005). In mice and zebrafish, Netrin1-UNC5B signaling was found to result in retraction of the *tip cell* filopodia[1] and inhibit endothelial sprouting (Lu, Le Noble et al. 2004; Park, Crouse et al. 2004). Nevertheless, netrins are also capable of promoting angiogenesis, probably, by using different receptors that remain to be identified (Wilson, Ii et al. 2006).

Slit proteins and **roundabouts (Robo)** represent an additional ligand/receptor family that contributes to neuronal patterning and seems to be involved in the regulation of EC sprouting. Slit/Robo signaling repels growing axons (Brose, Bland et al. 1999). From four Robo receptors identified in mammals (Robo1–4), Robo4, also known as *magic roundabout*, is apparently a vascular-specific receptor, expressed at sites of active angiogenesis, that inhibits EC migration (Huminiecki, Gorn et al. 2002). Robo1 is also actively involved in EC filopodia formation (Sheldon, Andre et al. 2009).

Finally, bidirectional signaling between **Eph receptors** and **ephrin ligands** consists one of the most prominent guidance cues for neuronal axons while **ephrin-B2** reverse signaling regulates endothelial *tip cell* guidance (Egea and Klein 2007; Sawamiphak, Seidel et al. 2010; Wang, Nakayama et al. 2010). It is generally accepted that Ephs (erythropoietin-producing human hepatocellular carcinoma) and ephrins (Eph family receptor interacting proteins) function to translate the density of their "partner" on opposing membranes into precise cellular responses, resulting in cell-cell adhesion or cell-contact repulsion (Campbell and Robbins 2008). Encoded by the gene *EFNB2*, Ephrin-B2 activates several receptors of the Eph tyrosine kinase family, including EphB4 ('forward' signaling), and possesses intrinsic ('reverse') signaling activity (Himanen and Nikolov 2003). Ephrin-B2 is a well-known arterial EC marker while vascular expression of EphB4 is predominantly observed in venous endothelium (Adams and Alitalo, 2007). The involvement of Ephrin-B2 and EphB4 in mouse developmental angiogenesis was suggested by findings that knockout of either protein is embryonically lethal due to vascular arrest at the primitive capillary plexus stage (Adams, Wilkinson et al. 1999). At the time of writing this text, two publications appeared providing at least partial explanation regarding the mechanistic basis of

[1] Filopodia are cellular processes that extend from the leading edge of migrating cells, attach to the extra-cellular matrix and facilitate the cell movement forward (Mattila and Lappalainen, 2008).

ephrin-B2 effects on EC behaviour (Sawamiphak, Seidel et al. 2010; Wang, Nakayama et al. 2010). In cultured vascular ECs, zebrafish and mice, Ephrin-B2 reverse signaling, involving PDZ interactions, increases the number of *tip cells* and their filopodial extensions, promoting motile behaviour and sprouting (Sawamiphak, Seidel et al. 2010; Wang, Nakayama et al. 2010). Ephrin-B2 reverse signaling was found to control VEGFR-2 internalization, which is necessary for activation, downstream signaling of the receptor and VEGFA-induced *tip cell* filopodial extension (Sawamiphak, Seidel et al. 2010). Simultaneously, in cultured lymphatic ECs, Ephrin-B2 was evidenced to induce the internalization of the VEGFC receptor VEGFR3, and, thus, positively regulate this principal pro-lymphangiogenic signaling axis (Wang, Nakayama et al. 2010). Together, these data suggest that Ephrin-B2 activation on *tip cells* directs VEGF-induced migration by stimulating the spatial activation of VEGFR endocytosis.

1.1.3.4. Transformation of Sprouts into Vessels – Sprout Fusion and Lumenization

The onset of blood flow requires the fusion of growing endothelial sprouts between themselves or with pre-existing capillaries as well as the formation of vascular lumen. Strong adhesive interactions among encountering *tip cells* and the firm **EC–EC junctions** enable the fusion of the sprout tips (Adams and Alitalo 2007). The repulsive interactions between *tip cells* of different sprouts probably also occur, preventing, for example, the fusion of incompatible vascular segments. Regarding the lumen formation, high-resolution time-lapse imaging of growing intersegmental vessels in zebrafish has indicated that **pinocytosis**[2], vacuole formation, their intracellular fusion and subsequent intercellular fusion lead to the development of endothelial tubular structures (Kamei, Saunders et al. 2006). Additionally, there is evidence that **Notch signaling** contributes to lumen enlargement by increasing the ratio of *stalk* to *tip cells* (Lobov, Renard et al. 2007; Suchting, Freitas et al. 2007) while **epidermal growth factor-like domain 7** (EGFL7; also known as vascular endothelial statin), a small EC-driven secreted factor, promotes vascular tubulogenesis by suppressing extra-cellular matrix adhesion (Parker, Schmidt et al. 2004). In contrast to immobilized VEGF, the **soluble VEGF isoforms** also favor increased vessel diameters (Tischer, Mitchell et al. 1991).

[2] Pinocytosis is a process by which the extra-cellular or surrounding fluid is internalized into cells in the form of membrane-coated vesicles (Adams and Alitalo, 2007).

1.1.3.5. Vessel Maturation and Termination of the Endothelial Activation

The establishment of blood flow and improved tissue perfusion suppress EC proliferation and promote the maturation of novel vascular connections since increased tissue oxygenation lowers the levels of VEGFA and other pro-angiogenic factors, shifting the balance to anti-angiogenic factor dominancy. Particularly important events during the terminal phase of angiogenic cascade are **the basement membrane formation** and **the incorporation of pericytes** into the newly formed endothelial tubes (Iruela-Arispe, 2008). As previously stated, the PDGFB expression in ECs promotes the recruitment of PDGFR-β-exhibiting pericytes (Gerhardt, Golding et al. 2003). Pericyte-mediated vascular stabilization involves **inhibitors of extra-cellular matrix cleavage by matrix metalloproteinases** (Iruela-Arispe, 2008). Remarkably, pericyte-derived tissue inhibitor of metalloproteinase-3 (TIMP-3), but also EC-derived TIMP-2, induce capillary stabilization upon endothelial-pericyte association, and prevent capillary regression (Davis and Saunders 2006). Nevertheless, capillaries may remain highly stable in the absence of pericytes (Iruela-Arispe, 2008). Therefore, much remains to be understood within this terminal angiogenesis step.

1.2. ADULT ANGIOGENESIS IN HEALTH AND DISEASE

Physiological angiogenesis ensures that normal, developing and growing or regenerating tissues receive an appropriate oxygen and nutrient supply. On the other hand, in various pathological conditions, normal angiogenesis mechanisms are usurped, directly provoking or enhancing the onset of disease.

In contrast to embryogenesis, EC turnover in the adult vasculature is quite minimal (Denekamp 1982). Angiogenesis is rarely observed in healthy adult organs with the exception of the female reproductive tract and during the repair of injured tissue. Each menstrual cycle, various tissues of the female reproductive system are subjected to rigorous vascular bed remodelling (Groothuis 2005). Cyclical changes of angiogenesis and vascular regression are observed in the growing follicles and *corpus luteum* of the ovary, endometrium and the breast tissue. Beside the reproductive function, successful adult tissue repair, including appropriate wound healing, also fundamentally depends on new blood vessel proliferation, with the important contribution of the accompanying inflammatory response that releases pro-angiogenic factors (Hunt et al., 2008). Although several regulators, both inducers and inhibitors, play critical roles, adult physiological angiogenesis, including the reparatory angiogenesis, is mainly triggered and mediated by VEGFA (Groothuis, 2005, Hunt et al., 2008).

Impaired vascular homeostasis, which results from either excessive or insufficient vessel growth, tremendously impacts human health contributing to the onset and/or progress of disorders from virtually all diagnostic categories (Panels 1.2. and 1.3.; Carmelitet, 2003). The best known pathologies characterized or caused by excessive vascular proliferation are cancer, psoriasis, rheumatoid arthritis and diabetic retinopathy, the leading cause of blindness in the developed world. Nevertheless, abnormal angiogenic response is also observed in common diseases such as infections, atherosclerosis, obesity and bronchial asthma. On the other hand, insufficient blood vessel growth and abnormal vascular regression lead to heath and brain ischemia, neurodegenerative lesions, arterial hypertension, pre-eclampsia, respiratory distress syndrome, osteoporosis and many other disorders. Apparently, there are not many other pathophysiological processes that threaten global human well-being such as pathological angiogenesis. Thereby, understanding it´s molecular basis might lead to

therapeutic improvements, particularly in the medical fields, such as oncology, where available treatment options usually do not provide a satisfactory outcome.

Panel 1.2. – Diseases caused/characterized by abnormal or excessive angiogenesis (adapted from Carmelitet, 2003)

Numerous organs – tumors, infectious diseases, autoimmune disorders

Blood vessels – vascular malformations, DiGeorge syndrome, cavernous hemangioma, atherosclerosis, transplant arteriopathy

Adipose tissue – obesity

Skin – psoriasis, warts, allergic dermatitis, scar keloids, pyogenic granulomas, blistering diseases, AIDS-associated Kaposi sarcoma

Eye – persistent hyperplastic vitreous syndrome, diabetic retinopathy, retinopathy of prematurity, choroidal neovascularization

Lung – primary pulmonary hypertension, asthma, nasal polyps

Gastrointestinal tract – inflammatory bowel and periodontal disease, ascites, peritoneal adhesions

Reproductive system – endometriosis, menorrhagia, ovarian cysts, ovarian hyperstimulation

Locomotor system – arthritis, synovitis, osteomyelitis, osteophytes

Panel 1.3. – Diseases caused/characterized by insufficient angiogenesis or vessel regression (adapted from Carmelitet, 2003)

Nervous system – Alzheimer disease, amyotrophic lateral sclerosis, diabetic neuropathy, stoke

Blood vessels – atherosclerosis, hypertension, diabetic vasculopathy, post-traumatic restenosis

Gastrointestinal tract –gastric and oral ulcerations, Crohn disease

Skin – hair loss, skin purpura, teleangiectasia, venous lake formation

Reproductive system – pre-eclampsia, menorrhagia

Lung – neonatal respiratory distress, pulmonary fibrosis, emphysema

Kidney – nephropathy

Locomotor system – osteoporosis, impaired bone fracture healing

1.3. OVERVIEW OF TUMOR ANGIOGENESIS

Tumor denominates a new growth of tissue, neoplasm, in which cell multiplication is uncontrolled and progressive, faster than that of normal tissue, continuing after cessation of the growth stimuli and having no useful physiologic purpose (Dorland 1953). Tumors can be either benign or malignant. While unregulated cell growth without invasion is a characteristic of benign tumors, cancers or malignant tumors, that involve carcinomas originating from epithelial tissues and sarcomas arising from mesenchymal tissues, are defined by two principal features – unrestrained cell proliferation and tissue invasion/metastasis (Fenton and Longo, 2008). Although the cancer incidence has been declining by about 2% each year since 1992 and the application of current treatment options (*i.e.*, surgery, radiotherapy, chemotherapy and biological modalities) results in the cure of nearly two of three patients diagnosed with cancer, malignant neoplasms represent the second leading cause of death world-wide behind heart disease (Longo, 2008).

Cancer is not a single disease, but a group of genetic disorders that result from a series of somatic alterations in DNA, caused by random replication errors, exposure to carcinogens (*e.g.*, radiation), and/or faulty DNA repair processes (Morin et al., 2008). Although most cancers appear sporadically, various histological types cluster in some families that carry a germline mutation in a cancer gene.

The phenotypic characteristics of malignant cells and tissues are summarized in Panel 1.4. One of the most prominent neoplasm features is its neovascularization. Such as normal tissues, both benign tumors and cancers require blood supply to promote their growth (Austen 1997). In 1971, Judah Folkman presented, for the first time, the concept that tumor growth depends on the formation of new blood vessels from the host pre-existing vasculature (Folkman 1971). The hypothesis arose from the observations that neither isolated animal organs nor the vitreous and aqueous humour of the human eye had supported the vascularization of malignant cell agglomerates and their enlargement more than 1-2 mm of diameter (Folkman, Cole et al. 1966; Lutty, Thompson et al. 1983). Subsequently, Folkman and co-workers identified a factor, named “tumor angiogenesis factor” (Folkman, Merler et al. 1971), postulating that the endothelial cells may be switched from a quiescent state to a rapid growth phase by diffusible chemical signals emitting from the tumor cells. Subsequent development of

bioassays for angiogenesis research, increased interest and extensive work, which resulted in 50.002 papers published on this topic from 1971 to January 2011 (*www.ncbi.nlm.nih.gov/pubmed*), did not only confirm Folkman's theory and contributed to better understanding of tumor angiogenesis, but led to the development of angiogenesis inhibitors for clinical use. A total of 1.257.000 patients received prescriptions from United States Food and Drug Administration (US FDA)-approved angiogenesis inhibitors during 2006 (Folkman, 2008). Nevertheless, the survival benefits of these treatments are quite modest (usually measured in months), with the exception of outcome that has been obtained in patients with renal-cell carcinoma (Kerbel 2008). Therefore, increased efforts are needed for better understanding of the tumor-induced angiogenesis process, indicating new therapeutic targets and developing more potent treatment strategies.

Panel 1.4. – Phenotypic characteristics of malignant cells/tissues (reproduced from Fenton and Longo, 2008)

Deregulated cell proliferation: Loss of negative regulators (suppressor oncogenes, *i.e.*, *Rb*, *p53*), and increased positive regulators (oncogenes, *i.e.*, *Ras*, *Myc*). Leads to aberrant cell cycle control and includes loss of normal checkpoint responses.

Failure to differentiate: Arrest at a stage prior to terminal differentiation. May retain stem cell properties. (Frequently observed in leukemias due to transcriptional repression of developmental programs by the gene products of chromosomal translocations.)

Loss of normal apoptosis pathways: Inactivation of *p53*, increases in Bcl-2 family members. This defect enhances the survival of cells with oncogenic mutations and genetic instability and allows clonal expansion and diversification within the tumor without activation of physiologic cell death pathways.

Genetic instability: Defects in DNA repair pathways leading to either single or oligo-nucleotide mutations, (as in microsatellite instability, MIN) or more commonly chromosomal instability (CIN) leading to aneuploidy. Caused by loss of function of *p53*, *BRCA1/*2, mismatch repair genes, and others.

Loss of replicative senescence: Normal cells stop dividing after 25–50 population doublings. Arrest is mediated by the Rb, p16INK4a, and p53 pathways. Further replication leads to telomere loss, with crisis. Surviving cells often harbor gross chromosomal abnormalities.

Increased angiogenesis: Due to increased gene expression of proangiogenic factors (VEGF, FGF, IL-8) by tumor or stromal cells, or loss of negative regulators (endostatin, tumstatin, thrombospondin).

Invasion: Loss of cell-cell contacts (gap junctions, cadherens) and increased production of matrix metalloproteinases (MMPs). Often takes the form of epithelial-to-mesenchymal transition (EMT) with anchored epithelial cells becoming more like motile fibroblasts.

Metastasis: Spread of tumor cells to lymph nodes or distant tissue sites. Limited by the ability of tumor cells to survive in a foreign environment.

Evasion of the immune system: Downregulation of MHC class I and II molecules; induction of T cell tolerance; inhibition of normal dendritic cell and/or T cell function; antigenic loss variants and clonal heterogeneity.

1.3.1. The Concept of the Angiogenic Switch

In an avascular area, the survival and proliferation of tumor cells are jeopardized by hypoxia, nutrient deprivation and waste metabolite accumulation. Therefore, a critical feature of a tumor, be it a primary or metastatic lesion, is the ability to promote its vascularization. All clinically significant solid tumors, including those of the colon, lung, breast, cervix, bladder, prostate and pancreas, progress by means of an avascular stage followed by a vascular stage (Ribatti and Vacca, 2008). The induction of neovascularization in the pathogenesis of hematological malignancies has also been documented (Vacca and Ribatti 2006). In the scientific jargon, the term "angiogenic switch" is used to denominate the transition from the tumor avascular to the vascular phase (Figure 1.3.). It is a stage in neoplasm evolution when the dynamic balance between pro-angiogenic stimuli and anti-angiogenic factors is shifted in favor of vascular proliferation by the effects of the tumor on its environment (Bergers and Benjamin 2003). Nevertheless, a vast majority of tumors reaches a steady state with balanced tumor cell proliferation and death, remaining dormant without the increase of net neoplastic mass and persisting as clinically silent lesions. This notion is supported by the findings that carcinoma *in situ* is found in 98% of the fatal trauma victims, aged 50 to 70 years, but is diagnosed in only 0.1% during life (Ribatti and Vacca, 2008).

While incipient and dormant neoplasms take advantage of oxygen and nutrient diffusion from nearby host vessels, "successful" tumors engage different mechanisms, summarized in Panel 1.5, to gain access to an increased blood supply and to continue to grow (Bergers and Benjamin 2003; Ribatti, Vacca et al. 2003). The most common and, thus, the most important strategy is the induction of angiogenesis by secreting angiogenic inducers and/or by suppressing angiogenic inhibitors.

Panel 1.5. – Tumor mechanisms used to improve its blood supply

Angiogenesis - vascular sprouting from pre-existing host capillaries

Vascular intussusception - division of a pre-existing host vessel into two

Vasculogenesis - *de novo* vessel formation from mobilized vascular progenitor cells

Vascular mimicry - formation of vascular networks by tumor cells without the contribution of vascular cells

Vascular co-option – tumor´s growth along pre-existing host vessels

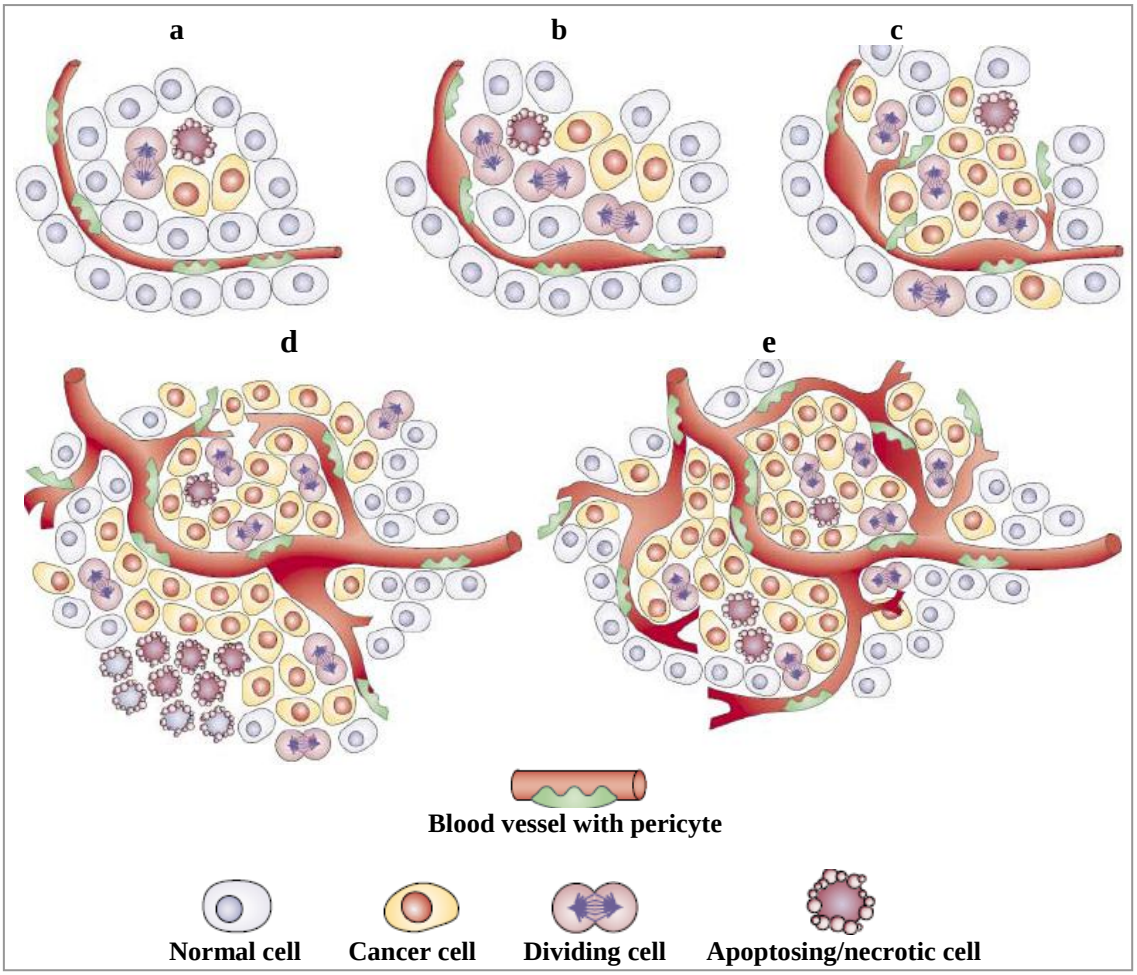


Figure 1.3. – The angiogenic switch (reproduced from Bergers and Benjamin, 2003). Most tumours start growing as avascular nodules (dormant) (a) until they reach a steady-state level of proliferating and apoptosing cells. The initiation of angiogenesis has to occur to ensure exponential tumour growth. The switch begins with perivascular detachment and vessel dilation (b), followed by angiogenic sprouting (c), new vessel formation and maturation, and the recruitment of perivascular cells (d). Blood-vessel formation will continue as long as the tumour grows, and the blood vessels specifically feed hypoxic and necrotic areas of the tumour to provide it with essential nutrients and oxygen (e).

Once the process of sprouting angiogenesis is promoted, tumors can also extend their vascular network by favor of splitting (intussusceptive) angiogenesis (Burri, Hlushchuk et al. 2004) or partaking the process of vasculogenesis, *i.e.* the *de novo* formation of blood vessels from circulating bone marrow-derived endothelial precursor cells (Rafii, Lyden et al. 2002). Sometimes, malignant cells, on their own and without the EC participation, form tubular networks that conduct blood – a phenomenon called vascular mimicry; however, the factual contribution of this type of blood circulation to tumor growth remains unclear (Hendrix, Seftor et al. 2003). Finally, certain tumor types are indeed "successful", in terms of both expansive growth and metastization, although they do not induce new vessel formation. Using a mechanism known as vascular co-option, these tumors grow along pre-existing host vessels and use these blood vessels

for their own needs (Holash, Maisonpierre et al. 1999). A classic example of tumors co-opting host vessels is provided by astrocytoma, which is often found as a non-encapsulated neoplasm progressing alongside blood vessels (Vajkoczy, Farhadi et al. 2002). Importantly, vascular co-option is neither a preferable nor a long-term tumor strategy since it may activate a robust host defense mechanism, in which the co-opted vessels initiate an apoptotic cascade, regress and result in massive tumor necrosis (Holash, Maisonpierre et al. 1999).

The existence and the importance of tumor angiogenic switch was experimentally proven for the first time by Hanahan, who developed RIP1-*Tag* transgenic mice in which a SV40 oncogene, the large T antigen (*Tag*), is expressed under the rat insulin promoter (RIP) (Hanahan 1985). In this model of pancreatic β-islet cell tumorigenesis, all islet cells express the *Tag* oncogene at birth, 75% of islets progress to small foci of proliferating cells, but only 4% become angiogenic and this percentage closely correlates with the incidence of tumor formation. Subsequently, the angiogenic switch was also documented in a large set of other carcinogenesis models in mice (Lacey, Alpert et al. 1986; Kandel, Bossy-Wetzel et al. 1991; Arbeit, Munger et al. 1994; Smith-McCune, Zhu et al. 1997). Initial evidence for the existence of an angiogenic switch in human cancers was obtained from pre-neoplastic lesions leading to breast cancer (Weidner, Folkman et al. 1992). As already mentioned, the angiogenic switch is currently known to occur in almost every human cancer type (Ribatti and Vacca, 2008).

1.3.2. Imbalance Between Pro- and Anti-angiogenic Factors Leading to the Angiogenic Switch

The transition from a pre-vascular to a vascularized tumor stage is regarded as the result of a complex interplay between positively and negatively acting angiogenesis regulators and their receptors on the ECs and other vascular cell. Pro- and anti-angiogenic factors are products or structural components of the tumor cells themselves and the tumor microenvironment, including stromal cells (*e.g.*, endothelial cells, pericytes, smooth muscle cells, myoepithelial cells, fibroblasts and myofibroblasts) and tumor-infiltrating immune cells, such as macrophages, mast cells, dendritic cells, NK cells, and T and B lymphocytes (Coussens and Werb 2001). The expression changes of positive and negative modulators which net balance favors angiogenesis onset and

maintenance can be caused by: 1) the activated oncogenes, 2) lost tumor suppressor genes, 3) transcriptional factors/repressors, and 4) the genes involved in response to cellular stress-generating conditions that include hypoxia, glucose deprivation, formation of reactive oxygen species (ROS), cellular acidosis and iron deficiency (Wicki and Christofori, 2006). Table 1.1. summarizes several cognate factors influencing the expression of pro- and anti-angiogenic effectors responsible for tumor angiogenic switch.

Table 1.1. – Angiogenic switch induction (adapted from Wicki and Christofori, 2006)

Mechanism (examples)	Pathway (effectors)
Environmental and metabolic inducers	
Hypoxia / HIF-1α	VEGF-A↑
Hypoxia / HIF-2α	VEGF-R2↑
Glucose deprivation	VEGF↑, bFGF↑
Reactive oxygen species /NF-κB	VEGF↑, IL-8↑
Cellular acidosis	VEGF↑
Iron defi ciency	VEGF↑
Gain of function (oncogenes)	
Phosphatidyl-inositol-3-kinase (PI3K)	VEGF↑
Human papilloma virus (HPV)	VEGF↑, FGF-BP↑
H-Ras	VEGF↑, TSP-1↓, MMPs↑
EGFR	VEGF↑, bFGF↑, IL-8↑
Erb/B2	VEGF↑
Bcl2	VEGF↑
Src	VEGF↑, TSP-1↓
c-Myb	TSP-2↓
Polyoma virus middle T (PyMT)	TSP-1↓
Fos	VEGF expression
Pituitary tumor-derived transforming gene 1 (PTTG1)	VEGF↑, bFGF↑
Loss of function (tumor suppressor genes)	
p53	VEGF↑, HIF-1α↑, TSP-1↓
PTEN	VEGF↑
Transcription factors/repressors	
Id1	TSP-1↓

1.3.2.1. Pro-angiogenic Factors

Equally as in physiological angiogenesis, **vascular endothelial growth factor A (VEGFA)** plays the role of the principal positive angiogenic regulator in tumors. VEGFA mRNA is highly up-regulated in most human cancers (Ferrara 1999). The

growth factor and its main receptor VEGFR2 are also coherently expressed in metastatic lesions – in malignant and associated ECs, respectively (Takahashi, Kitadai et al. 1995). It is the first secreted and unique pro-angiogenic factor found to be present throughout the entire tumor development (Figure 1.4.). As tumor progresses, secondary angiogenic pathways can also be activated by basic fibroblast growth factor (bFGF or FGF2), angiopoietin-2 (Ang2), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-ß), tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8), placental growth factor (PlGF), pleiotrophin (PTN, also known as heparin binding growth factor 8 or neurite growth-promoting factor 1) or others (Wicki and Christofori, 2006). Nevertheless, VEGFA continues to be up-regulated and tumor angiogenesis remains to rely heavily on this critical angiogenesis stimulus.

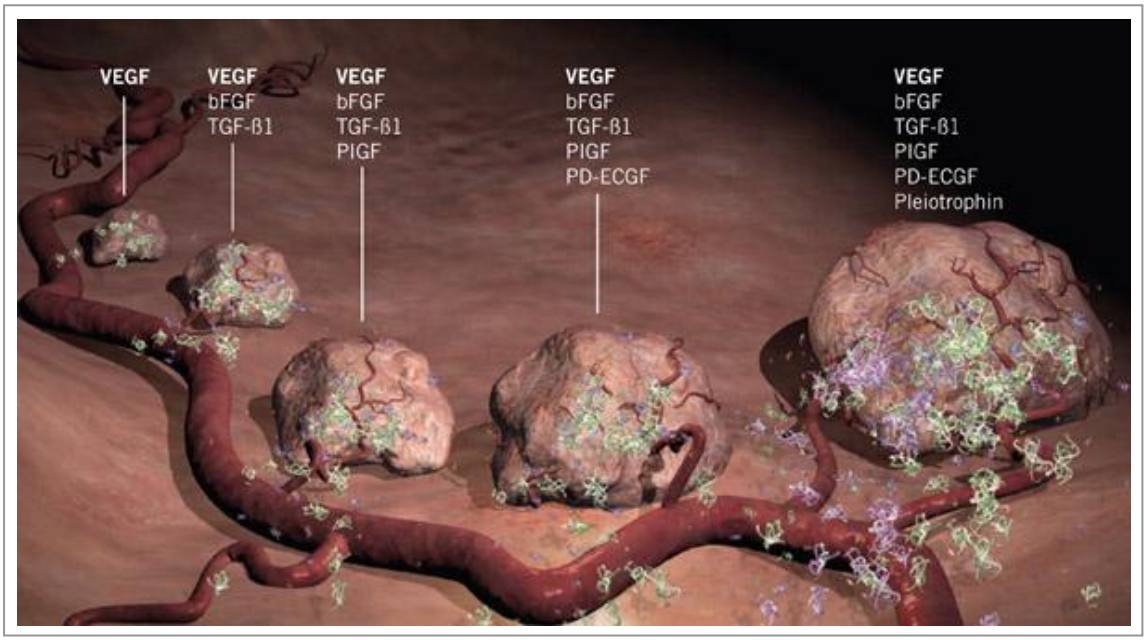


Figure 1.4. – The expression of VEGF during the tumor life cycle (reproduced from www.researchVEGF.com). VEGF is the only pro-angiogenic factor known to be continuously expressed in all phases of the tumor progression. As the tumor matures, secondary angiogenic factors may be increasingly produced.

By analogy to physiological settings, VEGFA production within a neoplasm is predominantly triggered by low oxygen tension (Adair, Gay et al. 1990). Its main sources are the tumor cells, but VEGFA is also secreted by macrophage-lineage cells and mesenchymal cells of tumor microenvironment (Liang, Wu et al. 2006). The elevated VEGFA levels directly correlates with increased tumor microvessel density and poor prognosis, as initially found in breast cancer patients (Toi, Kondo et al. 1996; Nieto, Woods et al. 2007). Beside the direct pro-angiogenic effects, VEGFA increases

vascular permeability and facilitates, in this manner, the penetration of malignant cell to the circulation and the metastization (Saaristo, Karpanen et al. 2000). A causative role for VEGFA in inducing tumor angiogenesis and enhancing tumor progression was emphatically confirmed by preclinical research with VEGFA/VEGFR2 signaling inhibitors (VEGF-neutralizing antibody, soluble VEGFR1, 'VEGF-Trap', and a low-molecular weight chemical tyrosine kinase inhibitor) that significantly decreased implanted tumor vascularization, growth and metastases in mouse models (Asano, Yukita et al. 1995; Kong, Hecht et al. 1998; Wedge, Ogilvie et al. 2000; Wood, Bold et al. 2000; Holash, Davis et al. 2002). Subsequently, clinical trials for the treatment of various cancer types with anti-VEGFA/VEGFR therapies showed statistically significant improvements in disease-free survival, leading to the approvals of some angiogenic inhibitors with anti-VEGFA/VEGFR activity (for details and references, see the section "Current Status of Anti-angiogenic Drugs Development for Oncology Use").

In addition to VEGFA, an important pro-angiogenic factor involved in tumor angiogenesis is **basic fibroblast growth factor (bFGF) or fibroblast growth factor 2 (FGF2)** (Folkman and Shing 1992). FGF2 is increasingly expressed in different carcinomas and sarcomas, including hematological tumors (Presta, Dell'Era et al. 2005). Within tumors, it is produced by various cell types – malignant, stromal and inflammatory cells (Schulze-Osthoff, Risau et al. 1990) and acts on ECs, other stromal and tumor cells via autocrine and paracrine mechanisms (Auguste, Gursel et al. 2001; Polnaszek, Kwabi-Addo et al. 2003). FGF2 induces cellular survival and proliferation (Wesley, McGroarty et al. 2005), stimulates protease production and migration (Yang, Zeisberg et al. 2003), and increases tumor metastatic potential (Suyama, Shapiro et al. 2002). In an FGF2 inhibition study, the administration of a soluble form of the FGF2 receptor to insulinoma-bearing RIP1-*Tag* mice significantly suppressed tumor vessel density and tumor growth (Compagni, Wilgenbus et al. 2000). Nevertheless, with a few exceptions (*e.g.*, melanomas), FGF2 levels do not correlate consistently with microvessel density in human malignancies even in the cases where the factor levels directly reflect the patient prognosis (Presta, Dell'Era et al. 2005). Despite the absence of this correlation, various experimental anti-FGF2 strategies were found to affect tumor growth by acting against both angiogenic endothelium and proliferating tumor cells (Rusnati and Presta 2007). Since the VEGFA signaling inhibition was evidenced to suppress tumor expansion at an earlier time point than FGF2 signaling blockage, it has

been hypothesized that VEGFA initiates tumor angiogenesis while FGF2 joins VEGFA to maintain it active (Compagni, Wilgenbus et al. 2000).

Angiopoietins represent another group of regulators engaged in both normal and tumor angiogenesis. Angiopoietin-1 (Ang1) binds to the endothelial receptor tyrosine kinase Tie-2 and promotes the interaction of ECs with the extra-cellular matix and mural cell recruitment, serving as a maturation factor (Suri, Jones et al. 1996) while angiopoietin-2 (Ang2) also binds to Tie-2 and antagonizes Ang1 activity, having expression often up-regulated prior to vessel sprouting (Tait and Jones 2004). Both *ANG1* and *ANG2* are up-regulated in diverse human cancers, although the *ANG2* up-regulation is more frequent (Wicki and Christofori, 2006). The shift of the Ang1/Ang2 balance in favor of Ang2 is required in tumor development for destabilizing the host vasculature, making the endothelium more prone to sprout. However, it appears that Ang2 produces different effects in early *vs.* late stages of tumor development. In the initial stages, tumor cells, which proliferate around pre-existing blood vessels and do not produce VEGFA, induce *Ang2* expression in the host vessels resulting in their destabilization and regression due to EC apoptosis (Holash, Maisonpierre et al. 1999). On the other hand, in more developed tumors with augmented metabolic demands, increasingly produced VEGFA induces both angiogenesis and *Ang2* expression in ECs of newly formed vessels, facilitating in this way vascular proliferation by maintaining endothelium unstable and plastic (Holash, Wiegand et al. 1999). When tumor ECs are blocked by Ang2, there is no stabilization by the Ang1/Tie2 interaction, contributing to poor association of ECs with underlying stroma, tumor blood vessel leakiness, fragility and hemorrhage (Carmeliet, Mackman et al. 1996).

Platelet-derived growth factor B (PDGF-B) is regarded as an additional angiogenesis regulator with highly significant role in tumor biology, principally in the pericyte investment in tumor vessels. *In vitro*, PDGF-B was documented to have potential to induce EC proliferation, vascular sprouting and tube formation (Battegay, Rupp et al. 1994). It was found capable to enhance the expression of VEGFA and VEGFR2 in ECs (Egeblad and Werb 2002). Nevertheless, genetic and pharmacological studies concluded that PDGF-B basically functions as a mitogenic factor for mural cells serving to recruit pericytes into the tumor vessels (Shaheen, Tseng et al. 2001; Abramsson, Lindblom et al. 2003; Bergers, Song et al. 2003). Although ECs are the main PDGF-B producers, some tumor cells were evidenced to secret the factor. While

both sources of PDGF-B recruit pericytes, the endothelial-derived PDGF-B, remaining in the close contact with the ECs, is required for appropriate vessel wall establishment, vascular stability and functionality (Abramsson, Lindblom et al. 2003). Thereby, high levels of PDGF generated by the tumor cells may be considered a significant contributor to the vascular abnormalities observed in tumors.

A number of other ubiquitously expressed host molecules impact the tumor angiogenesis, such as **pro-angiogenic cytokines, chemokines, and growth factors secreted** by stromal or inflammatory cells, including transforming growth factor β (TGF-β), tumor necrosis factor-α (TNF-α), interleukin 8 (IL-8), growth-related oncogene proteins (Gro) α, β and γ, granulocyte chemoattractant protein 2 (GCP-2) and epithelial neutrophil-activating protein 78 (ENA78) (Wicki and Christofori, 2006). Importantly, **matrix metalloproteases**, particularly MMP2 and MMP9, favor tumor angiogenesis by cleaving the extra-cellular matrix and, thus, providing the path for the migrating and proliferating ECs (Coussens, Tinkle et al. 2000; Fang, Shing et al. 2000). MMP9 also releases sequestered VEGFA, as shown in the Rip-*Tag* and K14HPV16 mouse models (Coussens, Tinkle et al. 2000).

Finally, not only the secreted factors but also several **membrane-bound proteins** prominently mediate tumor angiogenesis, such as **vascular integrins** and **ephrins/Eph receptors**. In contrast to quiescent endothelium, activated ECs over-express specific integrin family members that interact with growth factor membrane receptors and bind to the components of extra-cellular matrix, influencing the EC survival, adhesion and migration. Specifically, integrins αvβ3, αvβ5, and α5β1 facilitate EC spreading and migration in response to VEGFA or FGF2, which in turn up-regulate EC integrin expression (Fenton and Longo, 2008). Sprouting tumor vessels also express high levels of the trans-membrane protein Ephrin-B2 in their *tip cells* (Fenton and Longo, 2008). The bi-directional interplay between Ephrin-B2 in the vasculature and its receptor tyrosine kinase EphB4 expressed on tumor cells promotes blood vessel formation, remodeling and tumor growth (Noren, Lu et al. 2004). Inversely, pharmacological inhibition of Ephrin-B2/EphB4 system was found to inhibit malignant cell migration and invasiveness *in vitro*, and reduce blood vessel density, pericyte recruitment and vessel perfusion in tumors grafted in mice (Scehnet, Ley et al. 2009). Such as in developmental angiogenesis (see the section *"Guiding Cues for Sprout Outgrowth"* of this chapter), Ephrin-B2 "reverse" signaling was demonstrated to promote EC

directional migrations within tumors by inducing VEGFR2 internalization and downstream signaling of the receptor (Sawamiphak, Seidel et al. 2010).

Figure 1.5. summarizes above described factors capable of triggering the angiogenic switch.

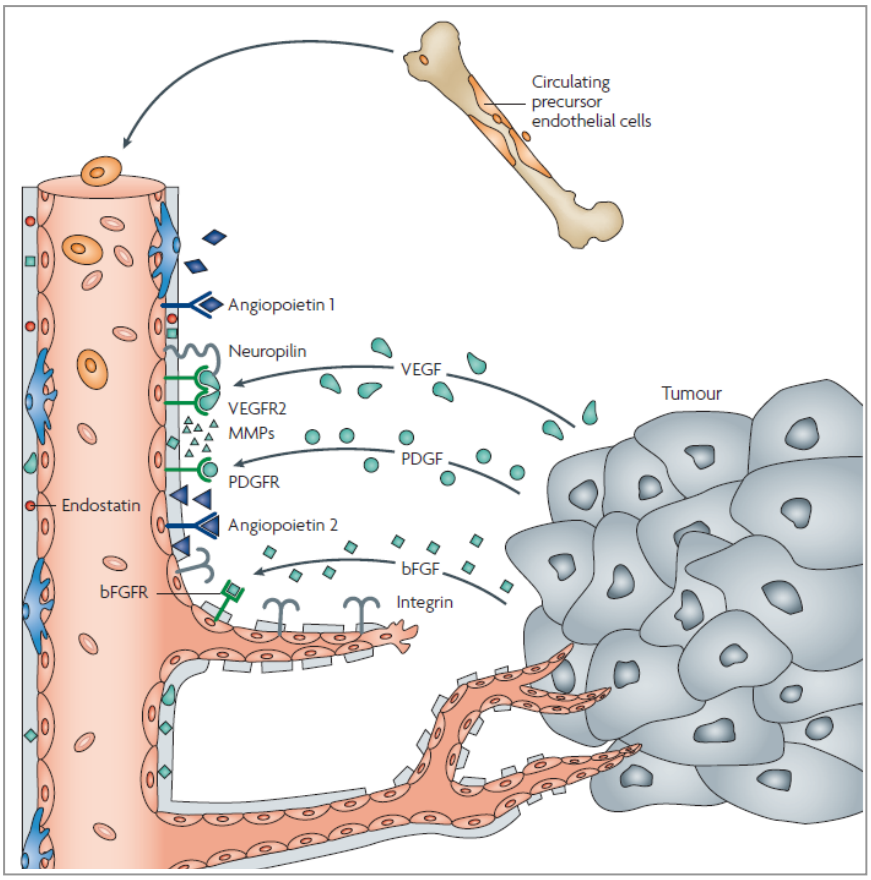


Figure 1.5. – Pro-angiogenic factors and the angiogenic switch (adapted from Folkman, 2007). Angiopoietin 1 (Ang1), expressed by many cells, binds to the endothelial Tie2 receptor and helps maintain a normalized state in blood vessels. Vascular endothelial growth factor A (VEGFA) is secreted by tumor cells and binds to its receptor (VEGFR2) and to neuropilin on endothelial cells. It is the most common of all pro-angiogenic proteins from tumors. Matrix metalloproteinases (MMPs) are released from tumor cells, but also by VEGF-stimulated endothelial cells. MMPs mobilize pro-angiogenic proteins from stroma, but can also cleave endostatin from collagen in the vessel wall and participate in the cleavage of angiostatin from circulating plasminogen (anti-angiogenic factors). Tumor cells secrete angiopoietin 2 (Ang2), which competes with Ang1 for binding to the endothelial Tie2 receptor. Ang2 increases the degradation of vascular basement membrane and migration of endothelial cells, therefore facilitating sprout formation. Platelet-derived growth factor (PDGF), produced by tumor or endothelial cells, is a potent mitogen and chemoattractant for precursors of pericytes and smooth muscle cells. Basic fibroblast growth factor (FGF2) is secreted by other tumours. It appears to be important for maintaining angiogenesis. Integrins on endothelial cells carry signals in both directions. Integrins facilitate endothelial cell binding to extracellular membranes, a requirement for the cells to maintain viability and responsiveness to growth regulatory proteins. Endothelial cells are among the most anchorage-dependent cells. Certain pro-angiogenic proteins up-regulate endothelial integrins and are thought to sustain endothelial cell viability during the intermittent detachments that are required to migrate towards a tumor and to simultaneously increase their sensitivity to growth regulators — both mitogenic (VEGF or bFGF) and anti-mitogenic (endostatin). New endothelial cells, do not all originate from neighboring vessels. A few arrive as precursor bone-marrow-derived endothelial cells. Endothelial growth factors are not all delivered to the local endothelium directly from tumor cells. Some angiogenic regulatory proteins (both pro- and anti-angiogenic) are scavenged by platelets, stored in alpha granules and seem to be released within the tumor vasculature.

1.3.2.2.Anti-angiogenic Factors

Antagonizing the effects of pro-angiogenic stimuli, angiostatic factors maintain vascular quiescence and keep tumors in a dormant state. Thereby, their expressional down-regulation and decreased activity importantly contributes to the tumor angiogenic switch and consequent expansion. Many endogenous inhibitors have been identified and they can be divided into two major groups: 1) proteins and protein-fragments of naturally occurring extra-cellular matrix and basement membrane components, and 2) growth factors, cytokines and other non-matrix-derived proteins that directly repress endothelial cell proliferation and migration (Wicki and Christofori, 2006).

Thrombospondin 1 (TSP1), a large multifunctional extra-cellular matrix glycoprotein, is a prototype of matrix-related angiogenesis inhibitors. Tumor suppressor gene *p53* regulates TSP1 production (Dameron, Volpert et al. 1994). This natural inhibitor of neovascularization and tumorigenesis modulates cell adhesion, proliferation and survival, TGFβ and protease activation (Chen, Herndon et al. 2000). Structurally similar **thrombospondin 2 (TSP2)** has also been found to inhibit both angiogenesis and tumor growth (Streit, Riccardi et al. 1999). Other potent matrix-related anti-angiogenic and tumor-suppressive factors include **endostatin**, a proteolytic cleavage product of collagen XVIII, and the collagen IV fragments **arresten, canstatin** and **tumstatin**. Endostatin specifically inhibits EC proliferation, being reportedly capable of inducing almost complete regression of a wide variety of primary tumors and Lewis lung cancer metastases (O'Reilly, Boehm et al. 1997). Arresten and canstatin achieve angiostatic effects by promoting EC apoptosis while tumstatin functions as a specific EC protein synthesis inhibitor (Ribatti 2009).

The first identified non-matrix-derived angiogenesis inhibitors were **interferons (IFNs)**. In various cancer types, IFN-α and β have been found to inhibit the expression and activity of pro-angiogenic MMP9 and IL-8 (Gupta and Qin 2003). IFN- β also down-regulates FGF2 expression (Nyberg, Xie et al. 2005). Besides, IFNs are directly citotoxic to tumor cells and enhance anti-cancer host immune reaction (Nilsson, Janson et al. 2001). Certain **interleukins (ILs)** also act directly against malignant cell proliferation, amplify immune response and inhibit angiogenesis (Nyberg, Xie et al. 2005). While the ILs possessing a NH2-terminal Glu-Leu-Arg (ERL) sequence (*e.g.*, IL-8), display pro-angiogenic properties, those lacking this motif, such as IL-4, have been found to suppress angiogenesis (Gupta and Qin 2003). Another prominent non-

matrix-derived angiogenesis inhibitor is the plasminogen cleavage product **angiostatin**. It binds to a variety of cell surface molecules hindering EC proliferation, migration (Wicki and Christofori, 2006), consequent tumor growth and metastasis formation (O'Reilly, Holmgren et al. 1994). In addition to angiostatics that modify EC or EC and neoplastic cell behavior, important subgroup of endogenous non-matrix-derived angiogenesis inhibitors affect primarily extra-cellular matrix itself. These are tissue **inhibitors of matrix metalloproteinases (TIMPs)** that prevent extra-cellular matrix degradation required for both new blood vessel sprouting and malignant cell invasion. Although TIMPs have pluripotent effects on EC growth, apoptosis, and differentiation (Ray and Stetler-Stevenson 1994), TIMP-1, 2, 3 and 4 predominantly inhibit neovascularization by blocking matrix turnover induced by MMP-1, MMP-2, and MMP-9 (Gupta and Qin 2003).

Potential therapeutic efficacy of various endogenous angiogenesis inhibitors and their synthetic analogy has been assessed or are currently under investigation in clinic trials (Nyberg, Xie et al. 2005). Recombinant human (rh)-endostatin has already been approved in China for the treatment of advanced non-small cell lung cancer (Folkman 2007).

1.4. CURRENT STATUS OF ANTI-ANGIOGENIC DRUG DEVELOPMENT FOR ONCOLOGY USE

The rationale for developing anti-angiogenic treatment modalities for cancer patients is based on the proven dependence of tumor growth, invasion and metastasis on angiogenesis. Furthermore, unlike the cytotoxic chemotherapy which primary target is the cancer cell with unpredictable mutations, targeting genetically stable ECs in tumor microvasculature and/or the surrounding stroma was anticipated to be less prone to induce acquired drug resistance (Kerbel 1991). Since tumor neovessels are different from the physiological neovasculature, anti-angiogenesis strategies were also expected to have the specificity to discriminate between malignant and normal tissues, resulting in low toxicity (Chau and Figg, 2008).

More than three decades of laboratory research have resulted in the discovery of plenty of potential targets for therapeutic inhibition of tumor angiogenesis (*e.g.*, angiogenic factors or their receptors, EC proliferation, matrix metalloproteinases and EC adhesion, vascular stem cells, pericytes, nitric oxide signaling and hypoxia pathways) and leaded to the development of dozens of drag candidates (Ferrara, Hillan et al. 2005). With the approval of the first anti-angiogenic agents, the concept of suppressing tumor neovascularization was finally translated from the laboratory bench to the clinical practice, currently representing an integral component of certain cancer therapeutic protocols. So far, available anti-angiogenic drugs provide quite minimal survival benefit, in part because of intrinsic refractoriness or evasive resistance (Kerbel 2008). These treatments are also costly (Berenson, 2006) and produce toxic side-effects (Hurwitz, Fehrenbacher et al. 2004). Nevertheless, anti-angiogenesis continues to be a topic of major scientific interest. Improvements are likely to arise from a more complete understanding of the molecular and cellular mechanisms governing tumor angiogenesis and mediating unsatisfactory response to available drugs.

1.4.1. A Brief History of Angiogenesis Inhibitors

Development of the pioneer bioassays for angiogenesis during the 1970s made the discovery of angiogenesis inhibitors possible (Folkman 2007). **Interferon-α** was the first identified substance which low doses were reported to inhibit angiogenesis in 1980 (Brouty-Boye and Zetter 1980). Over the next decade, several other compounds were

discovered to have prominent anti-angiogenic activity (Folkman, 2007; Figure 1.6.), including endogenous inhibitors and the first synthetic anti-angiogenic agent, the fumagillin analogue **TNP-470**[3] (Ingber, Fujita et al. 1990). By the mid-1990s, drug candidates with primary anti-angiogenic activity started entering clinical trials and began to receive US FDA approvals by 2003 (Folkman 2007). Following a phase III study showing a survival benefit, recombinant humanized anti-VEGF monoclonal antibody **bevacizumab** (Avastin®, Genentech) received US FDA approval for colorectal cancer in 2004, being the first drug developed exclusively as an angiogenesis inhibitor (Hurwitz, Fehrenbacher et al. 2004). At the time of this PhD thesis writing, ten drugs, that possess anti-angiogenic activity as the central mechanism contributing to their therapeutic effects, are approved by the FDA in the United States and/or by equivalent institutions in 30 other countries, for the treatment of cancer and age-related macular degeneration[4] (Folkman, 2007; Table 1.2.). There are numerous investigational angiogenesis inhibitors currently in clinical trials for different types of cancer; in the United States, ten of them are in Phase III (Folkman 2007).

[3] TNP-470 is an analogue of the *Aspergillus fumigatus* naturally secreted antibiotic fumagillin. Due to its potent anti-angiogenic activity demonstrated *in vitro* and *in vivo*, TNP-470 was one of the first angiogenesis inhibitors submitted to the clinical trials. Although this compound was found to exhibit severe side-effects such as neurotoxicity and thereby was not approved for systemic use, TNP-470 has one of the highest efficacies for the treatment of the broadest range of tumor types. Novel analogs and formulations of this molecule are considered promising (Mann-Steinberg and Satchi-Fainaro, 2008).

[4] Age-related macular degeneration (AMD) is the leading cause of blindness in individuals 55 years of age and older. Its so called "wet" stage occurs when new and fragile blood vessels form to improve the blood supply to oxygen-deprived retinal tissue. Hemorrhage and the tissue damage lead to severe and rapid loss of central vision (Chau and Figg, 2008). Currently, there are two US FDA-approved anti-angiogenic drugs for treating patients with AMD: pagaptanib (Macugen®, OSI Pharmaceuticals), a pegylated modified oligonucleotide, and ranibizumab (Lucentis®, Genentech) an anti-VEGF antibody (Folkman 2007). While pegaptanib targets the VEGF165 isoform, ranibizumab targets all of the biologically active isoforms of VEGF (Chau and Figg, 2008).

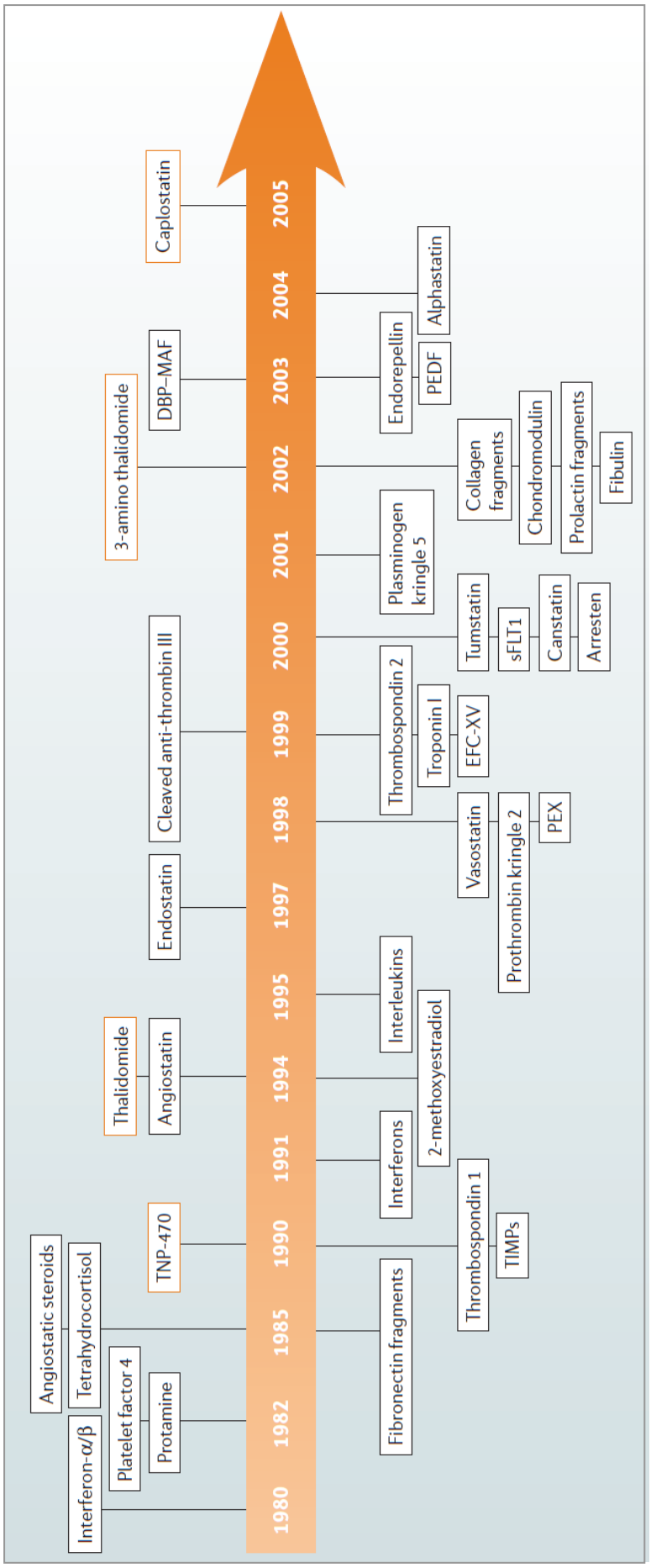


Figure 1.6. – Discovery of angiogenesis inhibitors – timeline (reproduced from Folkman, 2007). Synthetic angiogenesis inhibitors (orange keyline) and endogenous angiogenesis inhibitors that were identified in the Folkman laboratory are depicted above the timeline. Examples of additional endogenous angiogenesis inhibitors discovered in other laboratories are depicted below the timeline. The first drugs with antiangiogenic activity were approved in 2003. DBP–MAF, vitamin-D-binding protein–macrophage-activating factor; EFC-XV, endostatin-like fragment from type XV collagen; PEDF, pigment epithelium-derived factor (also known as SERPINF1); PEX, haemopexin C domain autolytic fragment of matrix metalloproteinase 2; sFLT1, soluble fms-related tyrosine kinase 1; TIMP, tissue inhibitors of matrix metalloproteinase.

Table 1.2. Anti-angiogenic drugs approved for clinical use (adapted from Folkman, 2007)

Drug (Trade name; Company)	Indication*
Bortezomib (Velcade; Millennium Pharma.)	Multiple myeloma (2003)
Thalidomide (Thalomid; Celgene Corporation)	Multiple myeloma (2003‡)
Bevacizumab (Avastin; Genentech)	Colorectal cancer (2004), lung cancer (2006)
Erlotinib (Tarceva; Genentech,OSI Pharm., Roche)	Lung cancer (2004)
Pegaptanib (Macugen; OSI Pharmaceuticals)	Age-related macular degeneration(2004)
Endostatin (Endostar)	Lung cancer (2005§)
Sorafenib (Nexavar; Onyx Pharmaceuticals)	Kidney cancer(2005)
Lenalidomide (Revlimid; Celgene Corporation)	Myelodysplastic syndrome (2005)
Sunitinib (Sutent; Pfizer)	GIST, kidney cancer (2006)
Ranibizumab (Lucentis; Genentech)	Age-related macular degeneration (2006)

*Year of first approval by the US Food and Drug Administration, unless stated otherwise. ‡Australia, approved by US Food and Drug Administration in 2006. §China State Food and Drug Administration. GIST, gastrointestinal stromal tumor.

1.4.2. Classification of Anti-angiogenic Agents

In the first place, angiogenesis inhibitors can be classified as either *exclusive ("pure")* or *inclusive* (Chau and Figg, 2008). The exclusively anti-angiogenic agents have only one known function which is the suppression of angiogenic activity (*e.g.*, **bevacizumab** [Avastin®, Genentech] and **VEGF-Trap**[5]). Other angiogenesis inhibitors have the anti-angiogenic activity *included* with other functions (*e.g.*,

[5] The VEGF Trap is a soluble chimeric receptor containing the key domains of VEGFR1 and VEGFR2 that are fused to the constant region (Fc) of human IgG1. Binding all isoforms of VEGF-A with very high affinity as well as PlGF, a VEGF family member also implicated in pathological angiogenesis, this fully human protein was engineered to optimize VEGF blockade and exhibit improved pharmacokinetic properties, that have not been achieved with bevacizumab (Rudge and Yancopoulos, 2008).

thalidomide[6] [Thalomid®,Celgene Corporation] and its analogues). Actually, the anti-angiogenic activity was discovered as a secondary function of many drugs that had previously received approval for a distinct primary function. Such one agent is **bortezomib** (Velcade®, Millennium Pharmaceuticals), a highly selective reversible inhibitor of the 26S proteasome, initially registered for the treatment of multiple myeloma and subsequently demonstrated to have potent anti-angiogenic activity (Yasui, Hideshima et al. 2006). Other examples of inclusive anti-angiogenic agents are presented in Table 1.3.

Table 1.3. – Inclusive anti-angiogenic compounds: drugs with anti-angiogenic activity as a secondary function (from Chau and Figg, 2008).

Drug Class	Example(s)
Antibiotics	Doxycycline
Bisphosphonates	Zoledronic acid
COX-2 inhibitors	Celecoxib
EGFR small-molecule receptor tyrosine kinase inhibitors	Gefitinib, Erlotinib
EGFR/HER2 monoclonal antibodies	Cetuximab, Panitumumab, Trastuzumab
HDAC inhibitors	Belinostat (PXD101), LBH589, Vorinostat (SAHA)
mTOR inhibitors	Everolimus, Temsirolimus (CCI-779)
PPAR-γ agonists	Rosiglitazone
Proteasome inhibitor	Bortezomib

[6] Thalidomide and its analogues, referred to as "immunomodulatory drugs" (IMiDs), possess anti-angiogenic, anti-inflammatory and immunomodulatory properties. Off the market for several decades due to its teratogenicity and other adverse effects, thalidomide reemerged as an effective treatment for various cancers, neurological, and inflammatory diseases, being currently approved for use in combination with dexamethasone for the treatment of newly diagnosed multiple myeloma patients (Chau and Figg, 2008). Having improved pharmacological characteristics, a thalidomide analogue lenalidomide (Revlimid®, Celgene Corporation) received US FDA approval for treatment of patients with myelodysplastic syndromes associated with 5q deletion and for use in combination with dexamethasone in multiple myeloma patients who have received one prior therapy (Folkman, 2007). IMiDs are being investigated in several Phase II/III trials for treating various tumors including renal cell cancer (Chau and Figg, 2008).

On the other hand, angiogenesis inhibitors can be divided in two categories depending on their mechanism of action: *direct angiogenesis inhibitors* and *indirect angiogenesis inhibitors* (Chau and Figg, 2008). Direct anti-angiogenic agents inhibit ECs forthrightly while indirect angiogenesis inhibitors do that by targeting angiogenic regulators or the components of their signaling pathways in both the neoplastic cells and ECs. An additional group of angiogenesis inhibitors, the *extra-cellular matrix breakdown blockers*, are directed against the matrix metalloproteinases (MMPs), the enzymes that degrade the extra-cellular matrix, required to allow proliferating ECs to invade the surrounding tissues.

Direct angiogenesis inhibitor target ECs themselves and block the endothelium to response to pro-angiogenic factors. Since this anti-angiogenic class acts on genetically stable ECs, direct angiogenesis inhibitors have been thought to be less prone to induce acquired drug resistance (Folkman, 2007). Examples in this category include endogenous anti-angiogenic agents, such as **angiostatin**, **endostatin**, **caplostatin** and **thrombospondin-1 (TSP1)** that specifically block the proliferation and migration of tumor ECs (Chen, Herndon et al. 2000). **Recombinant human (rh)-endostatin** inhibits EC growth by complex mechanisms, being approved in China since 2005 for the treatment of patients with advanced non-small cell lung cancer (Folkman, 2007). Furthermore, the TSP-1 analogue **ABT-510** (Hoekstra, de Vos et al. 2005) and integrin-targeting compounds **abegrin** and **cilengitide** (Nikolopoulos, Blaikie et al. 2004) are currently under investigation in phase I/II studies, showing promising profiles. The recent identification and characterization of the vascular (endothelial) progenitor cell, capability to migrate from bone marrow into circulation and contribute to the tumor vascularization, may also result in development of novel direct inhibitors primarily acting against endothelial progenitors (Rafii, Lyden et al. 2002).

Indirect angiogenesis inhibitors target malignant cells decreasing tumor expression of pro-angiogenic product(s), neutralize the pro-angiogenic factor(s) or block its/their receptor(s) on ECs (Figure 1.7.). Examples of agents that interfere with the major signaling pathways in tumor angiogenesis involve practically all FDA-approved inhibitors such as **erlotinib**[7] (Tarceva®, Genentech – OSI Pharmaceuticals – Roche),

[7] Erlotinib is a protein-tyrosine kinase inhibitor acting in the first line as the epidermal growth factor receptor (EGFR) inhibitor. Erlotinib is used in the treatment of patients with locally advanced or metastatic non-small cell lung cancer after failure of at least one chemotherapy regimen. It may also be

bevacizumab (Avastin®, Genentech), **sorafenib** (Nexavar®, Onyx Pharmaceuticals) and **sunitinib** (Sutent®, Pfizer). Indirect anti-angiogenic agents can inhibit one or multiple pro-angiogenic signaling proteins and/or their receptors (Folkman, 2007). Under the pressure of these inhibitors, tumor cells may increase the expression of pro-angiogenic proteins that are not blocked by the applied indirect inhibitor. Thus, the principal limitation to indirect angiogenesis inhibitors is the development of drug resistance.

Extra-cellular matrix breakdown blockers, the MMP inhibitors (MMPIs), suppress angiogenesis by preventing enzymatic degradation of the extra-cellular matrix. Although an oral MMPI, **incyclinide (COL-3)**, was found active in AIDS-related Kaposi's sarcoma (Dezube, Krown et al. 2006), a broad range of MMPIs failed to achieve any significant response against advanced or metastatic cancers (Lara, Stadler et al. 2006). These unsatisfactory results, the causes of which remain to be clarified, raise serious concerns regarding future developments of MMPIs as anti-cancer drugs.

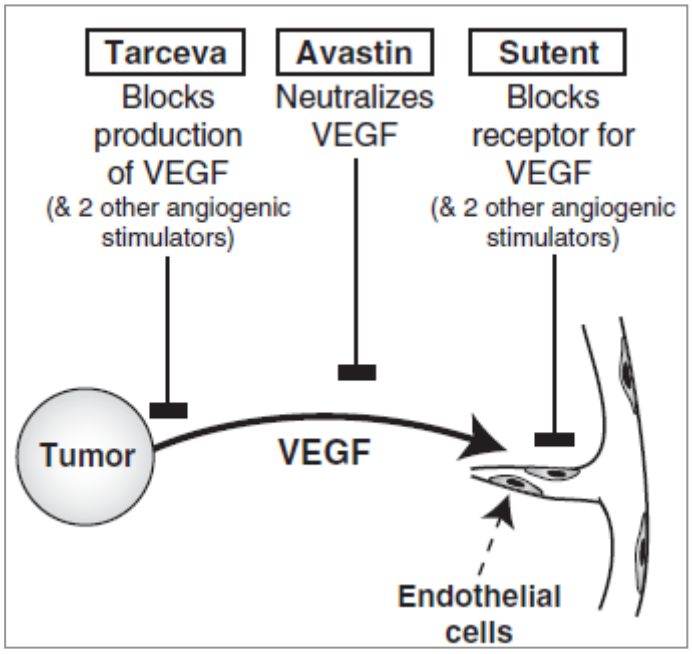


Figure 1.7. – Targets of indirect angiogenesis inhibitor (adapted from Folkman, 2008). Angiogenesis inhibitors currently approved by the FDA use three general mechanisms: blocking the tumour expression of an angiogenic factor, blocking an angiogenic factor after its secretion from a tumour or blocking an endothelial cell receptor.

engaged, in combination with gemcitabine, to treat locally advanced, unresectable or metastatic pancreatic cancer. Other oncology indications for erlotinib are being studied, such as ovarian, head and neck cancers (Folkman, 2007).

1.4.3. US FDA-approved "Pure" Angiogenesis Inhibitors for Cancer Treatment

Currently, there are three exclusive anti-angiogenic drugs that are marketed in the United States and Europe for use in oncology. They are **bevacizumab** (Avastin®, Genentech), and protein-tyrosine kinase inhibitors **sorafenib** (Nexavar®, Onyx Pharmaceuticals) and **sunitinib** (Sutent®, Pfizer).

1.4.3.1. Bevacizumab

Bevacizumab (Avastin®, Genentech) is a humanized monoclonal antibody IgG1, developed from a murine anti-human VEGF monoclonal antibody, which specifically recognizes and inhibits all major isoforms of human VEGF-A (Presta, Chen et al. 1997). The mechanisms underlying bevacizumab´s tumor-suppressive effects involve the regression of existing tumor vessels, inhibition of new vessel formation and vascular normalization (Jain 2005). Since blood vessels in tumors have bizarre defects with abnormal ECs, pericytes and vascular basement membrane (Baluk, Hashizume et al. 2005), bevacizumab-induced normalization of tumor vasculature, (Figure 1.8.), has been considered capable of reducing the risk of malignant cell penetration across the vessel walls to the blood stream and thus decrease the rate of metastization (Jain 2005). Moreover, normalization of tumor perfusion that result in reduced interstitial fluid pressure may increase efficient delivery of cytotoxic chemotherapy (Gerber and Ferrara 2005), while improved oxygen delivery to tumor microenvironment might enhance the radiotherapy effects.

Bevacizumab received US FDA approval for use in 5-fluorouracil-based regimens for treatment of metastatic colorectal cancer in February 2004. The clinical trial that resulted in this approval was a randomized, double-blind, phase III study that evaluated bevacizumab efficacy in combination with bolus irinotecan + 5-fluorouracil + leucovorin chemotherapy ("bolus-IFL") as first-line therapy for previously untreated patients with metastatic colorectal cancer (Hurwitz, Fehrenbacher et al. 2004). On average, the survival was increased from 15.6 months in the bolus-IFL + placebo treated group to 20.3 months in the bolus-IFL + bevacizumab drugged group. In 2006, the drug was approved for use in combination with carboplatin and paclitaxel for first-line treatment of patients with inoperable, locally advanced, recurrent or metastatic non-

squamous, non-small cell lung cancer (Sandler, Gray et al. 2006). The bevacizumab indication was extended to HER2-negative breast cancer in 2008, being recommended its use in combination with paclitexal chemotherapy (Lorusso 2008). Recently, the drug combination with interferon was granted with European approval for the management of renal cell carcinoma (Summers, Cohen et al. 2010). Current trials have been assessing bevacizumab alone or in combination regimens in patients with pancreatic cancer, ovarian cancer, hormone-refractory prostate cancer and other solid tumors (Folkman, 2007).

The bevacizumab adverse effects are presented in Panel 1.6. Occurring to some degree in up to 32% of patients, the most frequent high-grade toxicity is hypertension (Panares and Garcia 2007), which seems to be a class effect observed not only with bevacizumab but also with VEGFR inhibitors (Veronese, Mosenkis et al. 2006). Actually, there is evidence that blood pressure might serve as a biomarker of the efficacy of VEGF signaling inhibitor.

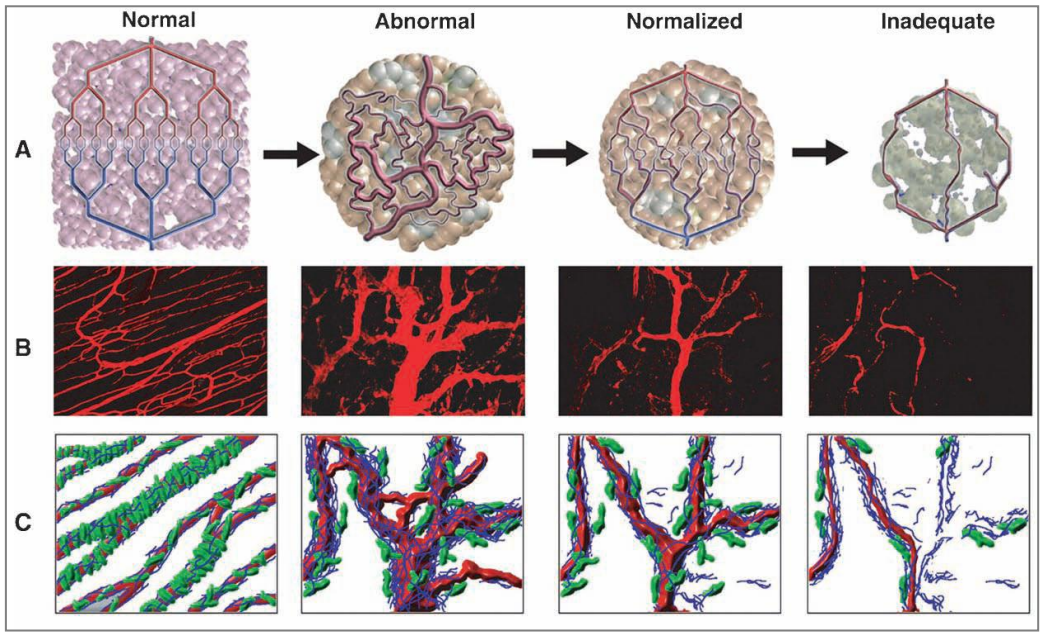


Figure 1.8. - Concept of vessel normalization in the tumor response to anti-angiogenic therapy (reproduced from Jain, 2005). A) Anti-angiogenic therapies such as bevacizumab are proposed to improve initially both the structure and the function of tumor vessels. However, aggressive anti-angiogenic regimens may eventually prune away these vessels, resulting in a vasculature that is both resistant to further treatment and inadequate for delivery of drugs or oxygen. B) Dynamics of vascular normalization induced by VEGFR2 blockade. *Left* Two-photon image showing normal blood vessels in skeletal muscle; subsequent images show human colon carcinoma vasculature in mice at day 0, day 3 and day 5 after administration of VEGR2-specific antibody. C) Diagram depicting the concomitant changes in pericyte (*red*) and basement membrane (*blue*) coverage during vascular normalization.

Panel 1.6. – Serious toxicities associated with bevacizumab (adapted from Panares and Garcia, 2007)

Bleeding 20–53%	Poor wound healing 6.7–10%
Proteinuria 27–38%	Arterial thrombosis 3.8%
Hypertension 22–32%	Reversible posterior leuko-encephalopathy
Gastrointestinal perforation 1.4–11.4%	syndrome <0.1%

1.4.3.2. Protein-tyrosine Kinase Inhibitors: Sorafenib and Sunitinib

Receptor protein tyrosine kinases (PTKs) play key roles in signaling, including angiogenesis-regulating signal transductions, and their enhanced activities are pivotal in many cancers (Levitzki and Mishani 2006). Since PTKs allow a physiological or a cancer signal to be transmitted to a number of elements and each of these downstream elements in turn signals to a set of more signaling elements, the development of PTK inhibitors as anti-angiogenic agents stems from the assumption that the multiple receptor inhibition (*e.g.*, VEGFR2, PDGFR and EGFR) should be advantageous in comparison with exclusive VEGFR2 blockade. There are numerous small-molecule PTK inhibitors in development. Examples of those with demonstrated activity in pre-clinical studies and clinical trials are vatalanib (PTK-787/ZK222584, Novartis), motesanib (AMG706, Amgen), vandetanib (ZD-6474, AstraZeneca) and cediranib (AZD2171, AstraZeneca) (Chau and Figg, 2008). Sorafenib (Nexavar®, Onyx Pharmaceuticals) and Sunitinib (Sutent®, Pfizer) have already been introduced to the clinical practice for the oncology indications presented below.

Sorafenib (Nexavar®, Onyx Pharmaceuticals) is a small molecule that inhibits a broad spectrum of kinases including VEGFR2 and VEGFR3, and, with even greater potency, B-Raf kinase, an oncogene product contributing to the development of many cancers (Chau and Figg, 2008). Beside the VEGF receptors, sorafenib targets involved in tumor angiogenesis are PDGFR, c-KIT and p38 (Wilhelm, Carter et al. 2004). Survival benefits, most likely due to its anti-angiogenic activity, led to the sorafenib approval for the use in patients with advanced renal cell carcinoma (Escudier, Eisen et al. 2007). The US FDA also approved sorafenib for use in patients with hepatocellular carcinoma, when the cancer is inoperable (Llovet, Ricci et al. 2008). In combination with chrmotherapy, sorafenib is being under investigation in clinical trials on other solid

tumors (Chau and Figg, 2008). Although it is generally well tolerated, sorafenib may cause hypertension, gastrointestinal side-effects (nausea, vomitus and diarrhea) as well as the cutaneous alterations (rash, desquamation, hand-foot skin reaction and alopecia) (Escudier, Eisen et al. 2007).

Sunitinib (Sutent®, Pfizer) is a novel, broad-spectrum, orally available PTK inhibitor, inhibiting VEGF receptors 1, 2, and 3, stem cell factor receptor (c-KIT), PDGF receptors, Fms-like tyrosine kinase 3 (Flt-3), colony stimulating factor receptor type 1 receptor, and the glial cell line–derived neurotrophic factor receptor (RET) (Mendel, Laird et al. 2003). In pre-clinical studies, sunitinib was demonstrated to possess robust anti-tumor activity resulting in the regression of colorectal cancer, non-small-cell lung cancer, melanoma, renal carcinoma, and squamous cell carcinoma that was attributed to the inhibition of VEGFR and PDGFR phosphorylation (Chau and Figg, 2008). Based on the phase III studies demonstrating its significant efficacy (*i.e.*, prolonged median time of tumor progression), sunitinib was granted US FDA approval for the treatment of advanced renal cell cancer and gastrointestinal stromal tumor (GIST) after the disease progression with imatinib or in the case of patient intolerance to imatinib (Folkman, 2007). In 2010 and 2011, sunitinib was approved by the European Commission and US FDA, respectively for the treatment of unrespectable or metastatic pancreatic neuroendocrine malignancies (http://www.genengnews.com/gen-news-highlights/pfizer-scores-new-approval-for-sutent-in-europe/81244326/, http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm256237.htm). Ongoing clinical trials investigate sunitinib alone and in association with chemotherapy in various other tumor types. The side-effects observed with sunitinibe use involve diarrhea, mucositis, asthenia, skin abnormalities, altered taste, a decrease in left ventricular ejection fraction and severe hypertension (Chau and Figg, 2008).

1.4.3.3. Efficacy Limitations, Drug Resistance to Angiogenesis Inhibitors and Options

Although pharmacological pre-clinical studies generated enthusiasm regarding the anti-angiogenesis feasibility to suppress and invert the malignant disease progression, marketed angiogenesis inhibitors clearly do not provide desirable clinical efficacy. They do elicit survival benefits in many aggressive tumors; nevertheless, their effects are transitory and followed by tumor recrudescence (Kerbel 2008). Besides, the patients

treated with anti-angiogenic drugs are not exempt from the adverse events, such as severe hypertension.

The life-extension benefits of approved angiogenesis inhibitors are statistically significant, but generally measured in months. As previously presented, bevacizumab, when given intravenously in combination with the standard chemotherapy treatment, prolongs survival by about five months as an initial treatment for metastatic colorectal cancer (Hurwitz, Fehrenbacher et al. 2004). The drug improves progression-free survival for metastatic breast cancer for only one to two months when added to chemotherapy, according to updated results of two trials (AVADO and RIBBON2; www.breastcancer.org). Similarly, in metastatic non-small cell lung cancer patients, the gain with bevacizumab addition was on average less than two months compared with chemotherapy alone (Sandler, Gray et al. 2006). Sorafenib offers extended median survival and the time to progression by nearly three months in patients with advanced renal cell carcinoma (Escudier et al., 2005) and advanced hepatocellular carcinoma (Llovet et al., 2008), while sunitinib application is associated with a longer overall survival for five to six months compared to IFN-α monotherapy in metastatic renal cell carcinoma patients and placebo treatment in imatinib-refractory/intolerant GIST patients (Goodman, Rock et al. 2007).

Completely absent response of some tumor types to angiogenesis inhibitors and their nondurable effects in other cancers point out that both *intrinsic* and *acquired resistance* to these drugs are clinically significant. **Intrinsic resistance or non-responsiveness** refers to "a pre-existing condition characterized by the absence of any, even transitory, beneficial effect of an anti-angiogenic therapy, ranging from the inability to shrink or stabilize tumors to the lack of improvement in quality of life" (Bergers and Hanahan 2008). In contrast, **acquired, adaptive or evasive resistance is defined as** "the ability of a tumor, after an initial response phase, to adapt so as to evade the therapeutic blockade by inducing or accentuating mechanisms that enable neovascularization despite the therapeutic blockade, or reduce dependence on such growth of new blood vessels by other means, leading to renewed tumor growth and progression" (Bergers and Hanahan 2008). Recent studies have commenced providing explanations regarding the mechanisms of such resistance.

Intrinsic resistance occurs in tumors which cells use existing blood vessels (vascular co-option) and do not trigger the angiogenic switching. Diffusely growing

grade II/III astrocytoma may be an example (Holash, Maisonpierre et al. 1999). Another class of pre-existing resistance is observed in characteristically hypovascularized tumors, such as pancreatic ductal adenocarcinoma, which adapt to grow in hypoxic microenvironment resulting from the absence of prominent angiogenesis (Sofuni, Iijima et al. 2005). In contrast with the tumors infirmly dependent on angiogenesis and thereby intrinsically indifferent toward angiogenesis inhibitors, highly angiogenic lesions, such as late-stage breast cancer, may display intrinsic non-responsiveness due to the existence of redundant pro-angiogenic stimuli. While early-stage breast cancer preferentially expresses VEGF-A, high-grade lesions produce a plethora of angiogenic positive regulators, including FGF-2 (Relf, LeJeune et al. 1997). Thus, the pre-existence of multiple pro-angiogenic stimuli may enable continuing angiogenesis in the presence of VEGF signaling inhibitors. As a matter of fact, bevacizumab provides a very modest survival benefit and its efficacy is equivocal in late-stage metastatic breast cancer (Marty and Pivot 2008). With respect to intrinsic resistance mechanisms, it has been also shown that a pre-existing tumor infiltration of inflammatory cells, principally $CD11b^{+}Gr1^{+}$ myeloid cells, which express a number of pro-angiogenic factors, may convey neoplasm non-responsiveness to angiogenesis inhibitors (Shojaei, Wu et al. 2007).

Under the pressure of anti-angiogenic drugs, acquired resistance is most evidently caused by up-regulation of alternative, "uncovered", pro-angiogenic signaling circuits. This phenomenon is probably a consequence of the elevated levels of treatment-induced hypoxia. The examples involve the FGF-2 up-regulation after the tumor treatment with anti–VEGFR-2 antibody (Casanovas, Hicklin et al. 2005), increased PlGF production with bevacizumab application (Willett, Boucher et al. 2005) and sunitinib-associated up-regulation of PlGF and VEGF-A which circulating levels revert during drug-free periods (Motzer, Michaelson et al. 2006). During the course of anti-angiogenic therapy, low oxygen conditions also recruit vascular progenitor cells and pro-angiogenic monocytes from the bone marrow that foster vasculogenic and angiogenic tumor vessel growth enabling tumors to progress (Bergers and Hanahan 2008). Furthermore, anti-angiogenic therapy-induced hypoxia can contribute to the selection and overgrowth of tumor cells with mutations in genes, such as *p53,* that are less dependent on the oxygen supply and angiogenesis (Yu, Rak et al. 2002). Finally, it is increasingly evident, in both animal tumor models and human cancers treated with angiogenesis inhibitors, that

malignant cells are capable to adapt by becoming more invasive, albeit more slowly, giving origin to the multifocal tumor recurrence (Bergers and Hanahan 2008).

Although the targeted therapy resistance remains poorly understood, the known underlying mechanisms indicate that, in many cases, tumors evade vascular-targeting therapy by manipulating with their complex signaling machinery. Thereby, effective anti-angiogenic therapy might rely on combinatorial approaches that simultaneously target multiple pathways. For example, assuming that inhibition of VEGF signaling is absolutely required but not sufficient to permanently block angiogenesis in advanced tumors, R. Sarmiento et al. (2008) proposed two concepts: "first, *vertical inhibition* of the entire VEGF pathway cascade by using neutralizing antibodies for the ligands and the external VEGFR domains in combination with tyrosine kinase inhibitors to block the transduction of signals to the nucleus as well as removing the key VEGF stimulating mechanisms via interfering with hypoxia, NO and related molecules; and second, *horizontal inhibition* by concurrently inhibiting several pro-angiogenic factors (*i.e.*, VEGF, PDGF, bFGF and others) and administering natural anti-angiogenic molecules such as endostatin or TSP1." Although these strategies raise concerns regarding cumulative drug toxicity, they are likely to be powerful tools to overcome drug resistance and ensure the patient full benefit of angiogenesis inhibition.

Collectively, despite the step forward in patient care that was made with the institution of anti-angiogenic therapy as a novel biological modality in cancer treatment, its improvement is greatly needed. The effects obtained with the anti-angiogenic arms that we currently possess seem to be in a great disproportion with the potential of optimal angiogenesis blockade to convert cancer in a chronic manageable condition. It is widely accepted that the saturation of benefit has not been achieved. Since the process of tumor angiogenesis depends on complex molecular machinery involving multiple regulatory pathways, a major challenge for the future seems to be the development of combinatorial therapeutic modalities. This requires much progress in understanding tumor angiogenesis, identification of new and validation of already suggested candidates for therapeutic targeting. Among myriads, some emerging targets, exampled by the Dll4/Notch signaling pathway, draw attention seeming to be particularly worth of validation.

1.5. DLL4/NOTCH SIGNALING PATHWAY AND ITS INVOLVEMENT IN VASCULAR BIOLOGY

The Notch pathway is an evolutionarily conserved signaling system that mediates cell-cell communication in multicellular organisms (Artavanis-Tsakonas, Rand et al. 1999). Using a simple framework, the pathway transduces signals from the cell surface to the nucleus, regulates the expression of particular target genes and, in this fashion, determines cell fate decisions influencing cellular proliferation, differentiation or apoptosis. The Notch signaling pathway is fundamentally important in diverse metazoan developmental and physiological processes and is associated with an array of human inherited and late onset diseases (Weinmaster and Kopan 2006).

1.5.1. Molecular Components of the Notch Pathway

In humans and mice, the Notch family of proteins is composed of four receptors (Notch1-4) that are canonically activated by five ligands of the so-called DSL [Delta, Serrate (Jagged in mammals), Lag2] family: Delta-like (Dll) 1, 3, 4 and Jagged (Jag) 1 and 2 (Lai 2004). The Notch receptors and their ligands are single-pass transmembrane protein molecules with large extracellular domains that basically consist of epidermal growth factor (EGF)-like repeats (Bray 2006).

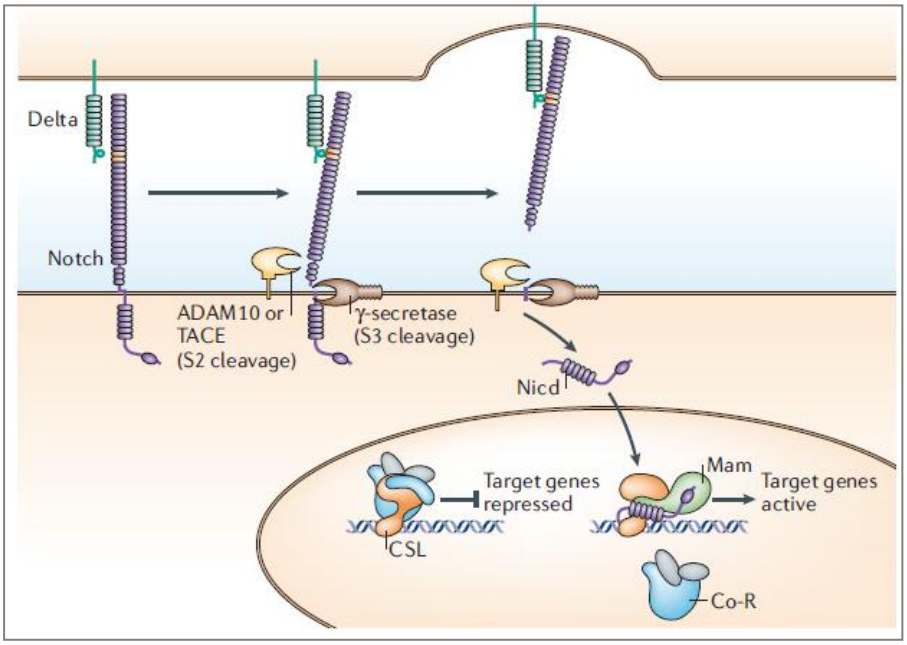


Figure 1.9. – The core Notch signaling (reproduced from Bray, 2006)

As presented in the Figure 1.9, ligand binding promotes two proteolytic cleavages in the Notch receptor that are catalysed by ADAM-family metalloproteases and γ-secretase enzymatic complex, respectively (Bray 2006). The second cleavage results in the release of the Notch intracellular domain (NICD), which translocates to the nucleus where interacts with the DNA-binding protein CSL [named after Cbf1, Su(H) and Lag1] and its co-activator Mastermind (Mam) to promote gene transcription. The known target genes involve different helix-loop-helix type transcription factors known as hairy and enhancers of split (HES – *e.g.*, HES1, 5 and 7), and HES-related repressor proteins (HERP – *e.g.*, HERP1, 2 and 3) (Kopan, Schroeter et al. 1996). HERP are also named HESR, HRT or HEY proteins.

1.5.2. Involvement of the Notch Pathway in Vascular Formation

While Notch signaling regulates many aspects of metazoan development, it is critically involved in the cardiovascular system development. In the first place, it was indicated by the identification of mutations in genes encoding the Notch pathway components and underlining inherited human diseases that exhibit vascular malformations (*i.e.*, Alagille syndrome[8] and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, CADASIL[9]). On the other hand, genetic deletions in experimental animals of several Notch receptors, ligands and downstream target genes produced vascular defects. For example, *Notch1* deletion in mice resulted in embryonic lethal cardiovascular malformations (Swiatek, Lindsell et al.

[8] Alagille syndrome is a heritable (autosomal dominant) multisystem disorder with variable expression, associated with bile duct insufficiency, cardiovascular malformations (most frequently pulmonary stenosis, tetralogy of Fallot, and coarctation of the aorta), abnormalities of the skeleton, eye and kidneys, and a characteristic facial appearance (Krantz et al., 1997). The patients with Alagille syndrome type I have mutations in *JAG1* mapped to chromosome 20p12 (Li et al., 1997), while those negative for *JAG1* aberrations (type II) have significant mutations in *Notch2*, locus 1p13-p11 (McDaniell et al., 2006).

[9] Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a hereditary microangiopathic condition leading to cerebrovascular symptoms in the third to fourth decade of life. The affected gene is *NOTCH3*, located on chromosome 19q13. The principal clinical features of CADASIL involve migraine headaches and the gradual progression of cognitive impairment leading to dementia and increasing disability in mid adulthood **(**Bruening et al., 2001**).**

1994) and these effects were exacerbated by *Notch4* deletion (Krebs, Xue et al. 2000). Moreover, mutations in murine *Notch3* produced marked arterial defects (Domenga, Fardoux et al. 2004). While *Jag1* deletion in mice was associated with a failure of embryonic vascular remodeling, hemorrhages and lethality at approximate embryonic day E10, deletion of *Dll1* resulted in vascular and somitogenesis defects, left-right asymmetry and lethality at approximate E12 (Hrabe de Angelis, McIntyre et al. 1997; Jiang, Lan et al. 1998; Przemeck, Heinzmann et al. 2003). Remarkably, genetic deletion of even one *Dll4* allele in mice resulted in pronounced vascular malformations and significant embryonic lethality at approximate E10.5 (Duarte, Hirashima et al. 2004). Before the discovery of the *Dll4* haploid embryonic lethality, *Vegfa* was the only gene reported to cause such a penetrant embryonic lethality due to aberrant vascular formation.

1.5.3. Dll4/Notch Signaling in Developmental Angiogenesis

In the vasculature, the expression of *Dll4* and its receptor genes, *Notch1* and *Notch4*, is restricted to arterial endothelium. It is one of the earliest genes expressed in arterial ECs, being induced by VEGF signaling and playing an essential role in establishment of the arterial endothelial cell fate (Lawson, Vogel et al. 2002; Duarte, Hirashima et al. 2004; Fischer, Schumacher et al. 2004). Early in development, *Dll4* expression is observed in the major arteries; later, this Notch ligand shifts to smaller arteries and arterioles while in adult mice *Dll4* is expressed in microvascular beds and in small arterioles, being absent or sporadically detected in larger arteries and veins (Gale, Dominguez et al. 2004).

During mouse embryonic development, the requirement for Dll4 is dose-dependent, as the heterozygous lethal phenotype was observed to be partially penetrant (between approximately 60% and 100%, depending on the mouse strain) while deletion of both *Dll4* alleles led to a more severe and fully penetrant (100%) lethal phenotype (Duarte, Hirashima et al. 2004; Krebs, Shutter et al. 2004). *Dll4*-targeted mice appeared at normal Mendelian frequencies at E8.5, began to exhibit increasingly severe defects at E9.5, and embryonic lethality in the heterozygous $Dll4^{+/-}$ mice occurred at approximately E10.5. Haploinsufficiency of *Dll4* was found to cause early pericardial edema, a lack of arterioles in the yolk sac, decreased arterial lumen in proper embryo, the particularly evident reduction of the dorsal aorta caliber, increased number of

vascular sprouts and branches, reduced arterial while augmented venous phenotype, and induced premature fusion between the arterial and venous compartments. The *Dll4*$^{-/-}$ embryos showed more severe and precocious vascular defects than the heterozygotes (Figure 1.10.). Although the major blood vessels were formed in *Dll4*$^{-/-}$ embryos, their later development was completely impaired, none of the studied arterial markers were expressed in the endothelium, and at E10.5 no viable embryo could be identified. The phenotype of the *Dll4*-targeted mice resembles that of the *Notch1* and *Notch4* double-mutant mice, suggesting that the biologic effects of Dll4 appear as the consequence of the activation of Notch1 and Notch4 signaling (Iso, Hamamori et al. 2003).

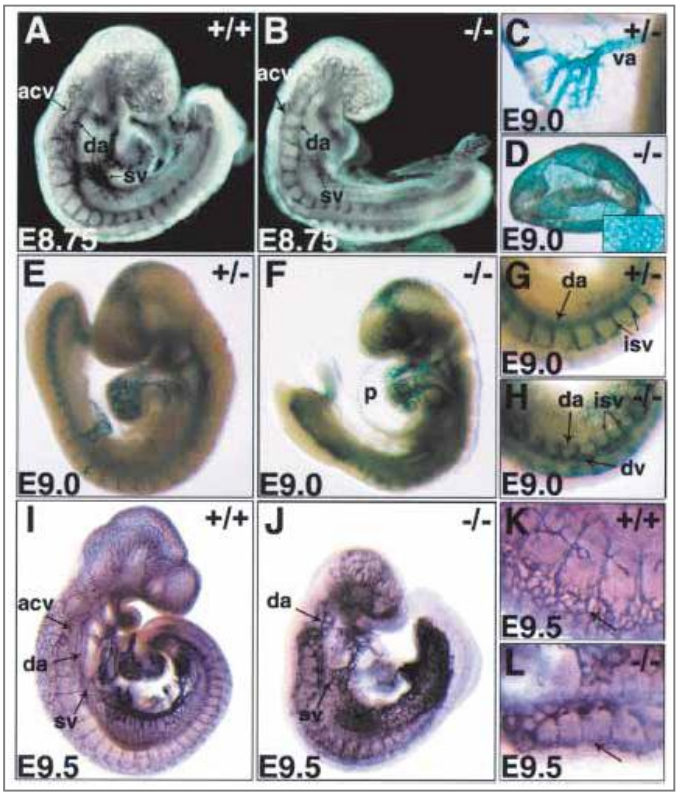


Figure 1.10. – Defective arterial and venous remodeling in *Dll4*$^{-/-}$ embryos (reproduced from Duarte et al., 2004). In this study were generated and analyzed mutants in which *Dll4* coding sequence was partially deleted and substituted by β-galactosidase (*lacZ*) reporter. A,B,I–L) Whole-mount PECAM and C–H) X-gal staining of control and *Dll4*$^{-/-}$ embryos. At E8.75, *Dll4*$^{-/-}$ embryos display reduced dorsal aorta (da), anterior cardinal vein (acv), and sinus venosus (sv). *(C,D)* Presence of vitelline arteries (va) in the yolk sac of a E9.0 *Dll4*$^{+/-}$ embryo contrasts with *Dll4*$^{-/-}$ yolk sac vessels, which appear stalled at the primary capillary plexus stage (*inset*); in *Dll4*$^{-/-}$ embryos the *Dll4-lacZ* reporter expression is observed throughout the yolk sac vessels. (*E–H*) By E9.0, *Dll4*$^{-/-}$ embryos exhibit growth retardation and arrested heart development with pericardial edema (p); the dorsal aorta appears further reduced, and there is an abnormal accumulation of *lacZ*-positive cells in the apical portion of the intersomitic vessels (isv) forming an enlarged dorsal vessel (dv). By E9.5, the *Dll4*$^{-/-}$ embryos are severely retarded with extremely reduced dorsal aortae, a reduced and almost indistinguishable venous structure, sinus venosus, and anterior cardinal vein. (*K,L*; higher magnification of *I,J*, respectively) In the more dorsal region, instead of an intricately branched network between arterial and venous intersomitic vessels, the *Dll4*$^{-/-}$ embryos display a single fused vessel that did not undergo angiogenic remodeling.

Importantly, genetic targeting of the zebrafish homolog of *Dll4* resulted in the phenotype of Dll4 reduction that was very consistent with that observed in mouse embryo (Leslie, Ariza-McNaughton et al. 2007; Siekmann and Lawson 2007). This supports a hypothesis that the Dll4/Notch signaling plays a common role in vascular development in widely different and evolutionary distant species.

1.5.4. Dll4/Notch Signaling in Physiological Postnatal Angiogenesis

To examine the role of Dll4 in normal post-embryonic angiogenesis, several groups used the mouse retina model wherein vasculature develops during the early postnatal period in a stereotypic manner (Claxton and Fruttiger 2004; Hellstrom, Phng et al. 2007; Lobov, Renard et al. 2007; Suchting, Freitas et al. 2007). These studies, in most cases running simultaneously with the present PhD research, found that *Dll4* is strongly expressed in actively proliferating capillary vessels while lower levels of *Dll4* expression were detected in maturing capillaries and in newly formed arterioles and venules. More precisely, Dll4 protein was observed in the leading ECs (*tip cells*) and in some neighboring cells of the expanding vascular plexus.

As presented in the Figure 1.11., heterozygous deletion of *Dll4*, such as the pharmacological inhibition of Notch signaling, revealed a characteristic vascular phenotype, with highly increased number of activated filopodia-extending endothelial *tip cells* and enhanced angiogenic sprouting, giving origin to the formation of a dense and more interconnected superficial capillary plexus (Lobov, Renard et al. 2007; Suchting, Freitas et al. 2007). Retinal expression of Dll4 was demonstrated to be up-regulated by VEGF-A, while Dll4/Notch activation was showed to decrease expression of VEGFR2 and increased expression of VEGFR1, suggesting a reduced responsiveness to VEGF stimulation (Lobov, Renard et al. 2007; Suchting, Freitas et al. 2007). Thus, these retinal angiogenesis-based studies provided a model for function of Dll4 in angiogenesis regulation where Dll4, expressed by activated ECs, signal to adjacent Notch receptor-bearing ECs and reduces their sensibility to VEGF. In this way, Dll4 suppresses the *tip cell* phenotype and promotes the *stalk cell* phenotype, negatively regulating VEGF-induced sprouting and branching, which is absolutely required for normal, coordinated vascular network formation (Figure 1.2.).

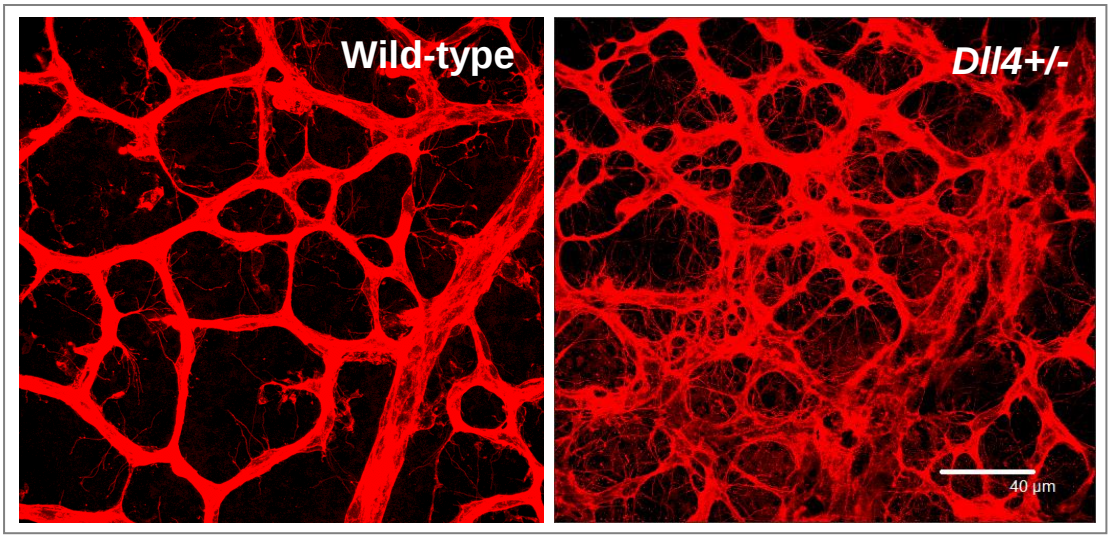


Figure 1.11. – Retinal vasculature in wild-type *vs.* *Dll4*$^{+/-}$ mice at postnatal P10 day (from Suchting et al., 2007).

1.5.5. Dll4 in Neoplastic Vasculature: Potential Drug-target to Be Validated

With the discovery that Dll4/Notch signaling acts as a key regulator of vascular development, numerous studies examined the *Dll4* expression patterns in various tumor types. Indeed, tumor ECs were found to express *Dll4*, both in animal tumor models and in different human tumors (Mailhos, Modlich et al. 2001; Gale, Dominguez et al. 2004; Patel, Dobbie et al. 2006). In addition, *Dll4* expression was shown to be induced by hypoxia, presenting a tendency to correlate with VEGF-A levels (Mailhos, Modlich et al. 2001; Patel, Dobbie et al. 2006).

In human tumor xenografts implanted in mice, *Dll4* was found to be highly expressed in microvessels and small arteries while poorly detectable in venules and larger tumor vessels (Gale, Dominguez et al. 2004). Importantly, *Dll4* expression was clearly more pronounced in the tumor vasculature than in the adjacent normal tissue vessels (Figure 1.12.). Thereby, these results suggested that Dll4 production is induced in tumor vascular beds as compared with normal tissues, but retains its expressional restriction to capillaries and small arteries.

Dll4 expression was also identified in the blood vessels of human clear cell-renal carcinoma, having 9-fold higher levels than in normal kidney tissue and showing a correlation with the levels of VEGF-A (Mailhos, Modlich et al. 2001). Similarly, in human bladder cancer, *Dll4* expression was increased approximately 2-fold in superficial tumors, and approximately 1.5-fold in invasive tumors, compared to normal

bladder tissue, possessing a well correlated relationship with the VEGF-A production (Patel, Dobbie et al. 2006). Furthermore, in a transgenic mouse hepatocarcinoma model, *Dll4*, such as *Notch4,* was progressively up-regulated during the lesion development from hyperplasia to diffuse carcinoma (Hainaud, Contreres et al. 2006). Thus, *Dll4* expression in tumor vessels seemed to follow tumor expansion as a result of augmented VEGFA production.

The *Dll4* expression patterns in tumors vasculature, combined with the wealth of data obtained from embryonic and retinal studies, strongly encouraged the host laboratory and, at the same time, a few other research groups to characterize the mechanistic bases of the Dll4/Notch function in neoplasm-induced angiogenesis and validate Dll4 as a potential anti-angiogenic drug target.

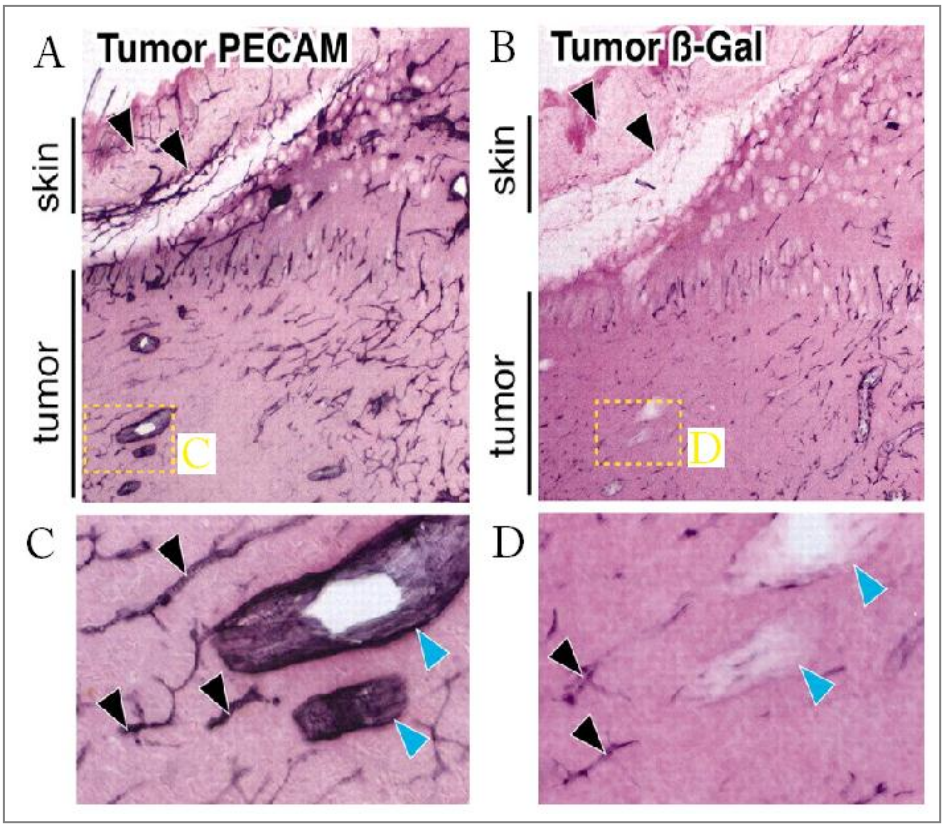


Figure 1.12. – *Dll4* induction during tumor angiogenesis (from Gale et al., 2004). A) Subcutaneous tumor section stained with anti-PECAM (endothelium), demonstrating robust staining in all large and small vessels, both in tumor and overlying skin (arrowheads). B) In contrast, Dll4 reporter analysis reveals stronger expression in tumor vessels as compared with the vessels in the adjacent skin. C,D) High-power views of boxed areas in *A* and *B* highlighting small tumor vessels (black arrowheads) versus large tumor veins (blue arrowheads).

REFERENCES:

Aaronson, P.I., Ward, J.P.T. & Wienera, C.M. (2004) "The cardiovascular system at a glance", 2nd ed. Malden, Mass.: Blackwell Science, p. 8.

Abramsson, A., P. Lindblom, et al. (2003). "Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors." J Clin Invest **112**(8): 1142-51.

Adair, T. H., W. J. Gay, et al. (1990). "Growth regulation of the vascular system: evidence for a metabolic hypothesis." Am J Physiol **259**(3 Pt 2): R393-404.

Adams, R. H. and K. Alitalo (2007). "Molecular regulation of angiogenesis and lymphangiogenesis." Nat Rev Mol Cell Biol **8**(6): 464-78.

Adams, R. H., G. A. Wilkinson, et al. (1999). "Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis." Genes Dev **13**(3): 295-306.

Arbeit, J. M., K. Munger, et al. (1994). "Progressive squamous epithelial neoplasia in K14-human papillomavirus type 16 transgenic mice." J Virol **68**(7): 4358-68.

Artavanis-Tsakonas, S., M. D. Rand, et al. (1999). "Notch signaling: cell fate control and signal integration in development." Science **284**(5415): 770-6.

Asano, M., A. Yukita, et al. (1995). "Inhibition of tumor growth and metastasis by an immunoneutralizing monoclonal antibody to human vascular endothelial growth factor/vascular permeability factor121." Cancer Res **55**(22): 5296-301.

Auguste, P., D. B. Gursel, et al. (2001). "Inhibition of fibroblast growth factor/fibroblast growth factor receptor activity in glioma cells impedes tumor growth by both angiogenesis-dependent and -independent mechanisms." Cancer Res **61**(4): 1717-26.

Austen, B. (1997). "Prognostic and therapeutic implications of tumour angiogenesis." Clin Oncol (R Coll Radiol) **9**(1): 58-61.

Bagri, A. and M. Tessier-Lavigne (2002). "Neuropilins as Semaphorin receptors: in vivo functions in neuronal cell migration and axon guidance." Adv Exp Med Biol **515**: 13-31.

Baluk, P., H. Hashizume, et al. (2005). "Cellular abnormalities of blood vessels as targets in cancer." Curr Opin Genet Dev **15**(1): 102-11.

Battegay, E. J., J. Rupp, et al. (1994). "PDGF-BB modulates endothelial proliferation and angiogenesis in vitro via PDGF beta-receptors." J Cell Biol **125**(4): 917-28.

Bergers, G. and L. E. Benjamin (2003). "Tumorigenesis and the angiogenic switch." Nat Rev Cancer **3**(6): 401-10.

Bergers, G. and D. Hanahan (2008). "Modes of resistance to anti-angiogenic therapy." Nat Rev Cancer **8**(8): 592-603.

Bergers, G., S. Song, et al. (2003). "Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors." J Clin Invest **111**(9): 1287-95.

Berenson, A. (February 15, 2006). "A cancer drug shows promise, at a price that many can't pay." New York Times: February 15.

Bray, S. J. (2006). "Notch signalling: a simple pathway becomes complex." Nat Rev Mol Cell Biol **7**(9): 678-89.

Brose, K., K. S. Bland, et al. (1999). "Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance." Cell **96**(6): 795-806.

Brouty-Boye, D. and B. R. Zetter (1980). "Inhibition of cell motility by interferon." Science **208**(4443): 516-8.

Bruening, R., Dichgans, et al. (2001). "Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy: decrease in regional cerebral blood volume in hyperintense subcortical lesions inversely correlates with disability and cognitive performance." AJNR Am J Neuroradiol **22,** 1268-74.

Burri, P. H., R. Hlushchuk, et al. (2004). "Intussusceptive angiogenesis: its emergence, its characteristics, and its significance." Dev Dyn **231**(3): 474-88.

Campbell, T. N. and S. M. Robbins (2008). "The Eph receptor/ephrin system: an emerging player in the invasion game." Curr Issues Mol Biol **10**(1-2): 61-6.

Carmeliet, P. (2003). "Angiogenesis in health and disease." Nat Med **9**(6): 653-60.

Carmeliet, P., N. Mackman, et al. (1996). "Role of tissue factor in embryonic blood vessel development." Nature **383**(6595): 73-5.

Carmeliet, P. and M. Tessier-Lavigne (2005). "Common mechanisms of nerve and blood vessel wiring." Nature **436**(7048): 193-200.

Casanovas, O., D. J. Hicklin, et al. (2005). "Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors." Cancer Cell **8**(4): 299-309.

Chau, C.H. & Figg, W.D. (2008) “Recent Advances in Angiogenesis Drug Development” in *Angiogenesis: An Integrative Approach From Science to Medicine*. Ed. by William D. Figg & Judah Folkman, Springer Science & Business Media, LLC, pp. 421-430.

Chen, H., M. E. Herndon, et al. (2000). "The cell biology of thrombospondin-1." Matrix Biol **19**(7): 597-614.

Claxton, S. and M. Fruttiger (2004). "Periodic Delta-like 4 expression in developing retinal arteries." Gene Expr Patterns **5**(1): 123-7.

Compagni, A., P. Wilgenbus, et al. (2000). "Fibroblast growth factors are required for efficient tumor angiogenesis." Cancer Res **60**(24): 7163-9.

Connolly, D. T., D. M. Heuvelman, et al. (1989). "Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis." J Clin Invest **84**(5): 1470-8.

Coussens, L. M., C. L. Tinkle, et al. (2000). "MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis." Cell **103**(3): 481-90.

Coussens, L. M. and Z. Werb (2001). "Inflammatory cells and cancer: think different!" J Exp Med **193**(6): F23-6.

Dameron, K. M., O. V. Volpert, et al. (1994). "Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1." Science **265**(5178): 1582-4.

Davis, G. E. and W. B. Saunders (2006). "Molecular balance of capillary tube formation versus regression in wound repair: role of matrix metalloproteinases and their inhibitors." J Investig Dermatol Symp Proc **11**(1): 44-56.

de Vries, C., J. A. Escobedo, et al. (1992). "The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor." Science **255**(5047): 989-91.

Denekamp, J. (1982). "Endothelial cell proliferation as a novel approach to targeting tumour therapy." Br J Cancer **45**(1): 136-9.

Dezube, B. J., S. E. Krown, et al. (2006). "Randomized phase II trial of matrix metalloproteinase inhibitor COL-3 in AIDS-related Kaposi's sarcoma: an AIDS Malignancy Consortium Study." J Clin Oncol **24**(9): 1389-94.

Dimmeler, S., E. Dernbach, et al. (2000). "Phosphorylation of the endothelial nitric oxide synthase at ser-1177 is required for VEGF-induced endothelial cell migration." FEBS Lett **477**(3): 258-62.

Domenga, V., P. Fardoux, et al. (2004). "Notch3 is required for arterial identity and maturation of vascular smooth muscle cells." Genes Dev **18**(22): 2730-5.

Dorland, W. A. N. (1953). American pocket medical dictionary : a dictionary of the principal terms used in medicine, nursing, pharmacy, dentistry,veterinary science, and allied biological subjects. Philadelphia, London, W. B. Saunders company.

Duarte, A., M. Hirashima, et al. (2004). "Dosage-sensitive requirement for mouse Dll4 in artery development." Genes Dev **18**(20): 2474-8.

Dvorak, H. F., L. F. Brown, et al. (1995). "Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis." Am J Pathol **146**(5): 1029-39.

Egea, J. and R. Klein (2007). "Bidirectional Eph-ephrin signaling during axon guidance." Trends Cell Biol **17**(5): 230-8.

Egeblad, M. and Z. Werb (2002). "New functions for the matrix metalloproteinases in cancer progression." Nat Rev Cancer **2**(3): 161-74.

Eichmann, A., L. Yuan, et al. (2005). "Vascular development: from precursor cells to branched arterial and venous networks." Int J Dev Biol **49**(2-3): 259-67.

Escudier, B., T. Eisen, et al. (2007). "Sorafenib in advanced clear-cell renal-cell carcinoma." N Engl J Med **356**(2): 125-34.

Fang, J., Y. Shing, et al. (2000). "Matrix metalloproteinase-2 is required for the switch to the angiogenic phenotype in a tumor model." Proc Natl Acad Sci U S A **97**(8): 3884-9.

Fenton, R.G. & Longo, D.L. (2008) "Cancer Cell Biology and Angiogenesis" in *Harrison's principles of internal medicine*. 17th Ed. by Anthony S. Fauci, New York, The McGraw-Hill Companies, Inc, pp.498-514. by William D. Figg & Judah Folkman, Springer Science & Business Media, LLC, pp. 541-561.

Ferrara, N. (1999). "Molecular and biological properties of vascular endothelial growth factor." J Mol Med **77**(7): 527-43.

Ferrara, N. (1999). "Vascular endothelial growth factor: molecular and biological aspects." Curr Top Microbiol Immunol **237**: 1-30.

Ferrara, N., H. P. Gerber, et al. (2003). "The biology of VEGF and its receptors." Nat Med **9**(6): 669-76.

Ferrara, N., K. J. Hillan, et al. (2005). "Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy." Biochem Biophys Res Commun **333**(2): 328-35.

Fischer, A., N. Schumacher, et al. (2004). "The Notch target genes Hey1 and Hey2 are required for embryonic vascular development." Genes Dev **18**(8): 901-11.

Folkman, J. (1971). "Tumor angiogenesis: therapeutic implications." N Engl J Med **285**(21): 1182-6.

Folkman, J. (1995). "Angiogenesis in cancer, vascular, rheumatoid and other disease." Nat Med **1**(1): 27-31.

Folkman, J. (2007). "Angiogenesis: an organizing principle for drug discovery?" Nat Rev Drug Discov **6**(4): 273-86.

Folkman, J., P. Cole, et al. (1966). "Tumor behavior in isolated perfused organs: in vitro growth and metastases of biopsy material in rabbit thyroid and canine intestinal segment." Ann Surg **164**(3): 491-502.

Folkman, J., E. Merler, et al. (1971). "Isolation of a tumor factor responsible for angiogenesis." J Exp Med **133**(2): 275-88.

Folkman, J. and Y. Shing (1992). "Angiogenesis." J Biol Chem **267**(16): 10931-4.

Gale, N. W., M. G. Dominguez, et al. (2004). "Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development." Proc Natl Acad Sci U S A **101**(45): 15949-54.

Gerber, H. P. and N. Ferrara (2005). "Pharmacology and pharmacodynamics of bevacizumab as monotherapy or in combination with cytotoxic therapy in preclinical studies." Cancer Res **65**(3): 671-80.

Gerber, H. P., A. McMurtrey, et al. (1998). "Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation." J Biol Chem **273**(46): 30336-43.

Gerhardt, H. and C. Betsholtz (2005). "How do endothelial cells orientate?" EXS(94): 3-15.

Gerhardt, H., M. Golding, et al. (2003). "VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia." J Cell Biol **161**(6): 1163-77.

Gitler, A. D., M. M. Lu, et al. (2004). "PlexinD1 and semaphorin signaling are required in endothelial cells for cardiovascular development." Dev Cell **7**(1): 107-16.

Goodman, V. L., E. P. Rock, et al. (2007). "Approval summary: sunitinib for the treatment of imatinib refractory or intolerant gastrointestinal stromal tumors and advanced renal cell carcinoma." Clin Cancer Res **13**(5): 1367-73.

Groothuis, P. G. (2005). "Angiogenesis and vascular remodelling in female reproductive organs." Angiogenesis **8**(2): 87-8.

Gu, C., Y. Yoshida, et al. (2005). "Semaphorin 3E and plexin-D1 control vascular pattern independently of neuropilins." Science **307**(5707): 265-8.

Gupta, M. K. and R. Y. Qin (2003). "Mechanism and its regulation of tumor-induced angiogenesis." World J Gastroenterol **9**(6): 1144-55.

Hainaud, P., J. O. Contreres, et al. (2006). "The role of the vascular endothelial growth factor-Delta-like 4 ligand/Notch4-ephrin B2 cascade in tumor vessel remodeling and endothelial cell functions." Cancer Res **66**(17): 8501-10.

Hanahan, D. (1985). "Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes." Nature **315**(6015): 115-22.

Hellstrom, M., L. K. Phng, et al. (2007). "Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis." Nature **445**(7129): 776-80.

Hendrix, M. J., E. A. Seftor, et al. (2003). "Remodeling of the microenvironment by aggressive melanoma tumor cells." Ann N Y Acad Sci **995**: 151-61.

Himanen, J. P. and D. B. Nikolov (2003). "Eph receptors and ephrins." Int J Biochem Cell Biol **35**(2): 130-4.

Hoekstra, R., F. Y. de Vos, et al. (2005). "Phase I safety, pharmacokinetic, and pharmacodynamic study of the thrombospondin-1-mimetic angiogenesis inhibitor ABT-510 in patients with advanced cancer." J Clin Oncol **23**(22): 5188-97.

Holash, J., S. Davis, et al. (2002). "VEGF-Trap: a VEGF blocker with potent antitumor effects." Proc Natl Acad Sci U S A **99**(17): 11393-8.

Holash, J., P. C. Maisonpierre, et al. (1999). "Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF." Science **284**(5422): 1994-8.

Holash, J., S. J. Wiegand, et al. (1999). "New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF." Oncogene **18**(38): 5356-62.

Houck, K. A., D. W. Leung, et al. (1992). "Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms." J Biol Chem **267**(36): 26031-7.

Hrabe de Angelis, M., J. McIntyre, 2nd, et al. (1997). "Maintenance of somite borders in mice requires the Delta homologue DII1." Nature **386**(6626): 717-21.

Huminiecki, L., M. Gorn, et al. (2002). "Magic roundabout is a new member of the roundabout receptor family that is endothelial specific and expressed at sites of active angiogenesis." Genomics **79**(4): 547-52.

Hunt, T.K., Gimbel, M. & Sen, C.K. (2008) "Revascularization of Wounds: The Oxygen-Hypoxia Paradox" in *Angiogenesis: An Integrative Approach From Science to Medicine*. Ed. by William D. Figg & Judah Folkman, Springer Science & Business Media, LLC, pp. 541-561

Hurwitz, H., L. Fehrenbacher, et al. (2004). "Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer." N Engl J Med **350**(23): 2335-42.

Ingber, D., T. Fujita, et al. (1990). "Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth." Nature **348**(6301): 555-7.

Iruela-Arispe, M.L. (2008) "Endothelial Cell Activation" in *Angiogenesis: An Integrative Approach From Science to Medicine*. Ed. by William D. Figg & Judah Folkman, Springer Science & Business Media, LLC, pp. 35-43.

Iso, T., Y. Hamamori, et al. (2003). "Notch signaling in vascular development." Arterioscler Thromb Vasc Biol **23**(4): 543-53.

Jain, R. K. (2005). "Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy." Science **307**(5706): 58-62.

Jiang, R., Y. Lan, et al. (1998). "Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice." Genes Dev **12**(7): 1046-57.

Junqueira de Azevedo, I. L., S. H. Farsky, et al. (2001). "Molecular cloning and expression of a functional snake venom vascular endothelium growth factor (VEGF) from the Bothrops insularis pit viper. A new member of the VEGF family of proteins." J Biol Chem **276**(43): 39836-42.

Kamei, M., W. B. Saunders, et al. (2006). "Endothelial tubes assemble from intracellular vacuoles in vivo." Nature **442**(7101): 453-6.

Kandel, J., E. Bossy-Wetzel, et al. (1991). "Neovascularization is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma." Cell **66**(6): 1095-104.

Kerbel, R. S. (1991). "Inhibition of tumor angiogenesis as a strategy to circumvent acquired resistance to anti-cancer therapeutic agents." Bioessays **13**(1): 31-6.

Kerbel, R. S. (2008). "Tumor angiogenesis." N Engl J Med **358**(19): 2039-49.

Kimura, H., A. Weisz, et al. (2001). "Identification of hypoxia-inducible factor 1 ancillary sequence and its function in vascular endothelial growth factor gene induction by hypoxia and nitric oxide." J Biol Chem **276**(3): 2292-8.

Kitadai, Y., Y. Takahashi, et al. (1999). "Transfection of interleukin-8 increases angiogenesis and tumorigenesis of human gastric carcinoma cells in nude mice." Br J Cancer **81**(4): 647-53.

Klagsbrun, M. and P. A. D'Amore (1991). "Regulators of angiogenesis." Annu Rev Physiol **53**: 217-39.

Kong, H. L., D. Hecht, et al. (1998). "Regional suppression of tumor growth by in vivo transfer of a cDNA encoding a secreted form of the extracellular domain of the flt-1 vascular endothelial growth factor receptor." Hum Gene Ther **9**(6): 823-33.

Kopan, R., E. H. Schroeter, et al. (1996). "Signal transduction by activated mNotch: importance of proteolytic processing and its regulation by the extracellular domain." Proc Natl Acad Sci U S A **93**(4): 1683-8.

Krantz, I. D., Piccoli, D. A. & Spinner, N. B. (1997). "Alagille syndrome." J Med Genet **34,** 152-7.

Krebs, L. T., J. R. Shutter, et al. (2004). "Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants." Genes Dev **18**(20): 2469-73.

Krebs, L. T., Y. Xue, et al. (2000). "Notch signaling is essential for vascular morphogenesis in mice." Genes Dev **14**(11): 1343-52.

Lacey, M., S. Alpert, et al. (1986). "Bovine papillomavirus genome elicits skin tumours in transgenic mice." Nature **322**(6080): 609-12.

Ladomery, M. R., S. J. Harper, et al. (2007). "Alternative splicing in angiogenesis: the vascular endothelial growth factor paradigm." Cancer Lett **249**(2): 133-42.

Lai, E. C. (2004). "Notch signaling: control of cell communication and cell fate." Development **131**(5): 965-73.

Lara, P. N., Jr., W. M. Stadler, et al. (2006). "A randomized phase II trial of the matrix metalloproteinase inhibitor BMS-275291 in hormone-refractory prostate cancer patients with bone metastases." Clin Cancer Res **12**(5): 1556-63.

Lawson, N. D., A. M. Vogel, et al. (2002). "sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation." Dev Cell **3**(1): 127-36.

Lee, S., S. M. Jilani, et al. (2005). "Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors." J Cell Biol **169**(4): 681-91.

Leslie, J. D., L. Ariza-McNaughton, et al. (2007). "Endothelial signalling by the Notch ligand Delta-like 4 restricts angiogenesis." Development **134**(5): 839-44.

Levitzki, A. and E. Mishani (2006). "Tyrphostins and other tyrosine kinase inhibitors." Annu Rev Biochem **75**: 93-109.

Li, L., Krantz, I. D., et al. (1997). "Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1." Nat Genet **16,** 243-51

Liang, W. C., X. Wu, et al. (2006). "Cross-species vascular endothelial growth factor (VEGF)-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF." J Biol Chem **281**(2): 951-61.

Llovet, J. M., S. Ricci, et al. (2008). "Sorafenib in advanced hepatocellular carcinoma." N Engl J Med **359**(4): 378-90.

Lobov, I. B., R. A. Renard, et al. (2007). "Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting." Proc Natl Acad Sci U S A **104**(9): 3219-24.

Longo, D.L. (2008) "Approaches to the Patient with Cancer" in *Harrison's principles of internal medicine*. 17th Ed. by Anthony S. Fauci, New York, The McGraw-Hill Companies, Inc, pp.479-486.

Lorusso, V. (2008). "Bevacizumab in the treatment of HER2-negative breast cancer." Biologics **2**(4): 813-21.

Lu, X., F. Le Noble, et al. (2004). "The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system." Nature **432**(7014): 179-86.

Lutty, G. A., D. C. Thompson, et al. (1983). "Vitreous: an inhibitor of retinal extract-induced neovascularization." Invest Ophthalmol Vis Sci **24**(1): 52-6.

Lyttle, D. J., K. M. Fraser, et al. (1994). "Homologs of vascular endothelial growth factor are encoded by the poxvirus orf virus." J Virol **68**(1): 84-92.

Mailhos, C., U. Modlich, et al. (2001). "Delta4, an endothelial specific notch ligand expressed at sites of physiological and tumor angiogenesis." Differentiation **69**(2-3): 135-44.

Marty, M. and X. Pivot (2008). "The potential of anti-vascular endothelial growth factor therapy in metastatic breast cancer: clinical experience with anti-angiogenic agents, focusing on bevacizumab." Eur J Cancer **44**(7): 912-20.

Mattila, P. K. & Lappalainen, P. (2008). "Filopodia: molecular architecture and cellular functions." Nat Rev Mol Cell Biol **9**, 446-54.

McDaniell, R., Warthen, D. M., et al. (2006). "NOTCH2 mutations cause Alagille syndrome, a heterogeneous disorder of the notch signaling pathway." Am J Hum Genet **79**,169-73.

Mendel, D. B., A. D. Laird, et al. (2003). "In vivo antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacodynamic relationship." Clin Cancer Res **9**(1): 327-37.

Mann-Steinberg, H. & Satchi-Fainaro, R. (2008) “TNP-470: The Resurrection of the First Synthetic Angiogenesis Inhibitor” in *Angiogenesis: An Integrative Approach From Science to Medicine*. Ed. by William D. Figg & Judah Folkman, Springer Science & Business Media, LLC, pp. 395-414

Morin, P.J., Trent, J.M. & Collins, B.V. (2008) “Cancer Genetics” in *Harrison's principles of internal medicine*. 17th Ed. by Anthony S. Fauci, New York, The McGraw-Hill Companies, Inc, pp.492-498.

Motzer, R. J., M. D. Michaelson, et al. (2006). "Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma." J Clin Oncol **24**(1): 16-24.

Nieto, Y., J. Woods, et al. (2007). "Prognostic analysis of tumour angiogenesis, determined by microvessel density and expression of vascular endothelial growth factor, in high-risk primary breast cancer patients treated with high-dose chemotherapy." Br J Cancer **97**(3): 391-7.

Nikolopoulos, S. N., P. Blaikie, et al. (2004). "Integrin beta4 signaling promotes tumor angiogenesis." Cancer Cell **6**(5): 471-83.

Nilsson, A., E. T. Janson, et al. (2001). "Levels of angiogenic peptides in sera from patients with carcinoid tumours during alpha-interferon treatment." Anticancer Res **21**(6A): 4087-90.

Noren, N. K., M. Lu, et al. (2004). "Interplay between EphB4 on tumor cells and vascular ephrin-B2 regulates tumor growth." Proc Natl Acad Sci U S A **101**(15): 5583-8.

Nyberg, P., L. Xie, et al. (2005). "Endogenous inhibitors of angiogenesis." Cancer Res **65**(10): 3967-79.

O'Reilly, M. S., T. Boehm, et al. (1997). "Endostatin: an endogenous inhibitor of angiogenesis and tumor growth." Cell **88**(2): 277-85.

O'Reilly, M. S., L. Holmgren, et al. (1994). "Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma." Cell **79**(2): 315-28.

Pajusola, K., O. Aprelikova, et al. (1992). "FLT4 receptor tyrosine kinase contains seven immunoglobulin-like loops and is expressed in multiple human tissues and cell lines." Cancer Res **52**(20): 5738-43.

Panares, R. L. and A. A. Garcia (2007). "Bevacizumab in the management of solid tumors." Expert Rev Anticancer Ther **7**(4): 433-45.

Papetti, M. and I. M. Herman (2002). "Mechanisms of normal and tumor-derived angiogenesis." Am J Physiol Cell Physiol **282**(5): C947-70.

Park, K. W., D. Crouse, et al. (2004). "The axonal attractant Netrin-1 is an angiogenic factor." Proc Natl Acad Sci U S A **101**(46): 16210-5.

Parker, L. H., M. Schmidt, et al. (2004). "The endothelial-cell-derived secreted factor Egfl7 regulates vascular tube formation." Nature **428**(6984): 754-8.

Patel, N. S., M. S. Dobbie, et al. (2006). "Up-regulation of endothelial delta-like 4 expression correlates with vessel maturation in bladder cancer." Clin Cancer Res **12**(16): 4836-44.

Polnaszek, N., B. Kwabi-Addo, et al. (2003). "Fibroblast growth factor 2 promotes tumor progression in an autochthonous mouse model of prostate cancer." Cancer Res **63**(18): 5754-60.

Presta, L. G., H. Chen, et al. (1997). "Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders." Cancer Res **57**(20): 4593-9.

Presta, M., P. Dell'Era, et al. (2005). "Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis." Cytokine Growth Factor Rev **16**(2): 159-78.

Przemeck, G. K., U. Heinzmann, et al. (2003). "Node and midline defects are associated with left-right development in Delta1 mutant embryos." Development **130**(1): 3-13.

Rafii, S., D. Lyden, et al. (2002). "Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy?" Nat Rev Cancer **2**(11): 826-35.

Ray, J. M. and W. G. Stetler-Stevenson (1994). "The role of matrix metalloproteases and their inhibitors in tumour invasion, metastasis and angiogenesis." Eur Respir J **7**(11): 2062-72.

Relf, M., S. LeJeune, et al. (1997). "Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor beta-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis." Cancer Res **57**(5): 963-9.

Ribatti, D. (2009). "Endogenous inhibitors of angiogenesis: a historical review." Leuk Res **33**(5): 638-44.

Ribatti, D., A. Vacca, et al. (2003). "New non-angiogenesis dependent pathways for tumour growth." Eur J Cancer **39**(13): 1835-41.

Ribatti, D. & Vacca, A. (2008) “Overview of Angiogenesis During Tumor Growth” in *Angiogenesis: An Integrative Approach From Science to Medicine*. Ed. by William D. Figg & Judah Folkman, Springer Science & Business Media, LLC, pp. 161-168.

Robinson, C. J. and S. E. Stringer (2001). "The splice variants of vascular endothelial growth factor (VEGF) and their receptors." J Cell Sci **114**(Pt 5): 853-65.

Rudgr, J.S. & Yancopoulos, G.D. (2008) “Clincal Development of VEGF Trap” in *Angiogenesis: An Integrative Approach From Science to Medicine*. Ed. by William D. Figg & Judah Folkman, Springer Science & Business Media, LLC, pp. 415-420.

Ruhrberg, C., H. Gerhardt, et al. (2002). "Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis." Genes Dev **16**(20): 2684-98.

Rusnati, M. and M. Presta (2007). "Fibroblast growth factors/fibroblast growth factor receptors as targets for the development of anti-angiogenesis strategies." Curr Pharm Des **13**(20): 2025-44.

Saaristo, A., T. Karpanen, et al. (2000). "Mechanisms of angiogenesis and their use in the inhibition of tumor growth and metastasis." Oncogene **19**(53): 6122-9.

Sainson, R. C., J. Aoto, et al. (2005). "Cell-autonomous notch signaling regulates endothelial cell branching and proliferation during vascular tubulogenesis." FASEB J **19**(8): 1027-9.

Sandler, A., R. Gray, et al. (2006). "Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer." N Engl J Med **355**(24): 2542-50.

Sarmiento, R., Longo, R. & Gasparini,G. (2008) "Challenges of Antiangiogenic Therapy of Tumors" in *Angiogenesis: An Integrative Approach From Science to Medicine*. Ed. by William D. Figg & Judah Folkman, Springer Science & Business Media, LLC, pp. 461-476

Sawamiphak, S., S. Seidel, et al. (2010). "Ephrin-B2 regulates VEGFR2 function in developmental and tumour angiogenesis." Nature **465**(7297): 487-91.

Scehnet, J. S., E. J. Ley, et al. (2009). "The role of Ephs, Ephrins, and growth factors in Kaposi sarcoma and implications of EphrinB2 blockade." Blood **113**(1): 254-63.

Schulze-Osthoff, K., W. Risau, et al. (1990). "In situ detection of basic fibroblast growth factor by highly specific antibodies." Am J Pathol **137**(1): 85-92.

Shaheen, R. M., W. W. Tseng, et al. (2001). "Tyrosine kinase inhibition of multiple angiogenic growth factor receptors improves survival in mice bearing colon cancer liver metastases by inhibition of endothelial cell survival mechanisms." Cancer Res **61**(4): 1464-8.

Shalaby, F., J. Rossant, et al. (1995). "Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice." Nature **376**(6535): 62-6.

Sheldon, H., M. Andre, et al. (2009). "Active involvement of Robo1 and Robo4 in filopodia formation and endothelial cell motility mediated via WASP and other actin nucleation-promoting factors." FASEB J **23**(2): 513-22.

Shibuya, M. (2006). "Differential roles of vascular endothelial growth factor receptor-1 and receptor-2 in angiogenesis." J Biochem Mol Biol **39**(5): 469-78.

Shibuya, M. and L. Claesson-Welsh (2006). "Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis." Exp Cell Res **312**(5): 549-60.

Shojaei, F., X. Wu, et al. (2007). "Tumor refractoriness to anti-VEGF treatment is mediated by CD11b+Gr1+ myeloid cells." Nat Biotechnol **25**(8): 911-20.

Shweiki, D., A. Itin, et al. (1992). "Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis." Nature **359**(6398): 843-5.

Siekmann, A. F. and N. D. Lawson (2007). "Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries." Nature **445**(7129): 781-4.

Smith-McCune, K., Y. H. Zhu, et al. (1997). "Cross-species comparison of angiogenesis during the premalignant stages of squamous carcinogenesis in the human cervix and K14-HPV16 transgenic mice." Cancer Res **57**(7): 1294-300.

Sofuni, A., H. Iijima, et al. (2005). "Differential diagnosis of pancreatic tumors using ultrasound contrast imaging." J Gastroenterol **40**(5): 518-25.

Streit, M., L. Riccardi, et al. (1999). "Thrombospondin-2: a potent endogenous inhibitor of tumor growth and angiogenesis." Proc Natl Acad Sci U S A **96**(26): 14888-93.

Suchting, S., C. Freitas, et al. (2007). "The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching." Proc Natl Acad Sci U S A **104**(9): 3225-30.

Summers, J., M. H. Cohen, et al. (2010). "FDA drug approval summary: bevacizumab plus interferon for advanced renal cell carcinoma." Oncologist **15**(1): 104-11.

Suri, C., P. F. Jones, et al. (1996). "Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis." Cell **87**(7): 1171-80.

Suyama, K., I. Shapiro, et al. (2002). "A signaling pathway leading to metastasis is controlled by N-cadherin and the FGF receptor." Cancer Cell **2**(4): 301-14.

Swiatek, P. J., C. E. Lindsell, et al. (1994). "Notch1 is essential for postimplantation development in mice." Genes Dev **8**(6): 707-19.

Tait, C. R. and P. F. Jones (2004). "Angiopoietins in tumours: the angiogenic switch." J Pathol **204**(1): 1-10.

Takahashi, Y., Y. Kitadai, et al. (1995). "Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer." Cancer Res **55**(18): 3964-8.

Tischer, E., R. Mitchell, et al. (1991). "The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing." J Biol Chem **266**(18): 11947-54.

Toi, M., S. Kondo, et al. (1996). "Quantitative analysis of vascular endothelial growth factor in primary breast cancer." Cancer **77**(6): 1101-6.

Toyofuku, T. and H. Kikutani (2007). "Semaphorin signaling during cardiac development." Adv Exp Med Biol **600**: 109-17.

Vacca, A. and D. Ribatti (2006). "Bone marrow angiogenesis in multiple myeloma." Leukemia **20**(2): 193-9.

Vajkoczy, P., M. Farhadi, et al. (2002). "Microtumor growth initiates angiogenic sprouting with simultaneous expression of VEGF, VEGF receptor-2, and angiopoietin-2." J Clin Invest **109**(6): 777-85.

Veronese, M. L., A. Mosenkis, et al. (2006). "Mechanisms of hypertension associated with BAY 43-9006." J Clin Oncol **24**(9): 1363-9.

Wang, Y., M. Nakayama, et al. (2010). "Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis." Nature **465**(7297): 483-6.

Wedge, S. R., D. J. Ogilvie, et al. (2000). "ZD4190: an orally active inhibitor of vascular endothelial growth factor signaling with broad-spectrum antitumor efficacy." Cancer Res **60**(4): 970-5.

Weidner, N., J. Folkman, et al. (1992). "Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma." J Natl Cancer Inst **84**(24): 1875-87.

Weinmaster, G. and R. Kopan (2006). "A garden of Notch-ly delights." Development **133**(17): 3277-82.

Weinstein, B. M. (2005). "Vessels and nerves: marching to the same tune." Cell **120**(3): 299-302.

Wesley, U. V., M. McGroarty, et al. (2005). "Dipeptidyl peptidase inhibits malignant phenotype of prostate cancer cells by blocking basic fibroblast growth factor signaling pathway." Cancer Res **65**(4): 1325-34.

Wicki, A. & Cristofori, G. (2006) “The molecular basis of the angiogenic switch” in *Tumor Angiogenesis: Mechanisms and Cancer Therapy*. Ed. by Norbert Fusenig and Dieter Marme, Springer Verlag, Heidelberg, pp 67-88.

Wilhelm, S. M., C. Carter, et al. (2004). "BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis." Cancer Res **64**(19): 7099-109.

Willett, C. G., Y. Boucher, et al. (2005). "Surrogate markers for antiangiogenic therapy and dose-limiting toxicities for bevacizumab with radiation and chemotherapy: continued experience of a phase I trial in rectal cancer patients." J Clin Oncol **23**(31): 8136-9.

Wilson, B. D., M. Ii, et al. (2006). "Netrins promote developmental and therapeutic angiogenesis." Science **313**(5787): 640-4.

Wood, J. M., G. Bold, et al. (2000). "PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration." Cancer Res **60**(8): 2178-89.

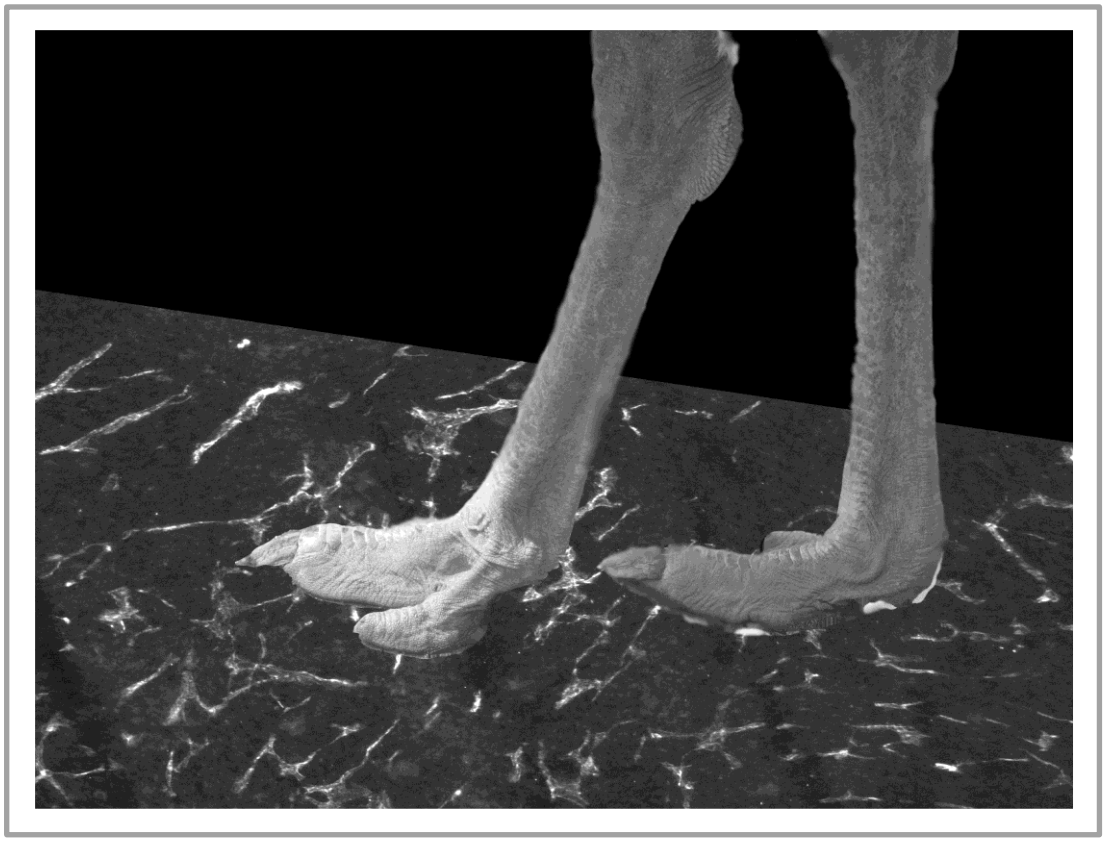
Yang, C., M. Zeisberg, et al. (2003). "Integrin alpha1beta1 and alpha2beta1 are the key regulators of hepatocarcinoma cell invasion across the fibrotic matrix microenvironment." Cancer Res **63**(23): 8312-7.

Yasui, H., T. Hideshima, et al. (2006). "Recent advances in the treatment of Multiple Myeloma." Curr Pharm Biotechnol **7**(5): 381-93.

Yu, J. L., J. W. Rak, et al. (2002). "Effect of p53 status on tumor response to antiangiogenic therapy." Science **295**(5559): 1526-8.

CHAPTER II

RESEARCH DESIGN



Djokovic D., “Step Forward”, collage (2011)

2

Objectives and Experimental Approaches

As presented in the previous chapter, over the years preceding the execution of this work, compelling evidence had indicated an essential involvement of Notch signaling in the regulation of vascular development and in the maintenance of vascular homeostasis. Various genes encoding Notch pathway components were identified as mutated in human diseases exhibiting vascular defects (Weinmaster and Kopan 2006), while mice deficient for various components displayed embryonic lethality accompanied with severe vascular abnormalities (Swiatek, Lindsell et al. 1994; Hrabe de Angelis, McIntyre et al. 1997; Jiang, Lan et al. 1998; Krebs, Xue et al. 2000; Przemeck, Heinzmann et al. 2003; Domenga, Fardoux et al. 2004). The main stimulus for this research arose from the findings that the Notch ligand Dll4 is absolutely required for normal angiogenic sprouting and arterial patterning during embryogenesis (Claxton and Fruttiger 2004; Duarte, Hirashima et al. 2004; Krebs, Shutter et al. 2004). Remarkably, Dll4 haploinsufficiency was observed to result in early embryonic lethality due to impaired vascular formation, a phenotype only previously found in VEGF haploinsufficiency (Duarte, Hirashima et al. 2004).

Facing the necessity to improve angiogenesis-targeted anti-cancer therapy, the pronounced sensitivity of the embryonic vasculature to Dll4 levels opened up the possibility that Dll4 might be a good target for a novel therapeutic strategy. Although the effects of Notch pathway activation on endothelium were shown to be context-dependent (Hofmann and Iruela-Arispe 2007), the demonstrations of increased *Dll4* expression in the vasculature of xenografted and endogenous human tumors pointed to the implication of Dll4/Notch signaling in tumor-induced angiogenesis (Mailhos, Modlich et al. 2001; Gale, Dominguez et al. 2004; Patel, Dobbie et al. 2006). Nevertheless, the exact function of Dll4 during tumor vascularization, its mechanistic basis and the degree of its relevancy, remained to be elucidated. Hence, the main goal of my research was to understand the role of the endothelial Notch ligand Dll4 in the regulation of tumor-driven

neoangiogenesis. Furthermore, we expected to use the insight gained to design novel ways to target tumor vasculature and suppress/invert neoplasm expansion.

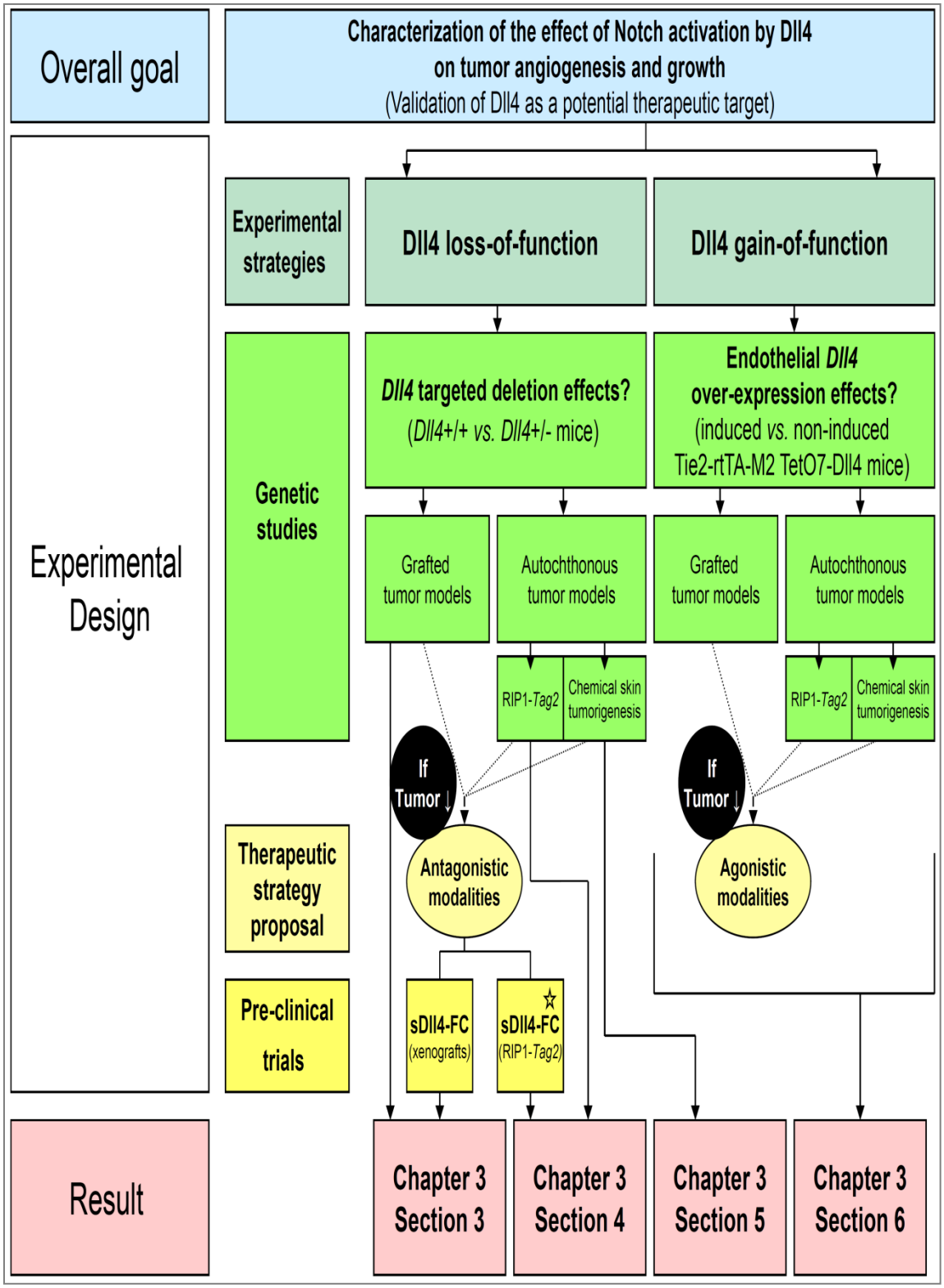


Figure 2.1. – Conceptual framework used for the investigation of Dll4 function in tumor angiogenesis and organization of the obtained results. ☆, sDll4 mono- and combinational therapeutic trials with sEphB4 inhibitor of downstream ephrin-B2/EphB4 signaling.

Specifically, this project aimed:

- To study the effect of Notch activation by Dll4 on tumor neovessel growth and morphology, as well as on tumor kinetics, through the characterization of Dll4 loss-of-function (*i.e.*, targeted *Dll4* gene deletion) phenotypes in xenografted and autochthonous mouse tumor models;
- To further assess Dll4 role(s) in tumor angiogenesis through the characterization of Dll4 gain-of-function (*i.e.*, endothelial *Dll4* overexpression) phenotypes in xenografted and autochthonous mouse tumor models;
- To propose, develop and validate Dll4-based endothelium-targeting tumor therapeutic modality(ies) that, depending on the results obtained from *in vivo* experiments, might use Dll4/Notch signaling agonists or/and antagonists.

In order to achieve the above-listed objectives, the present research followed the experimental design depicted in Figure 2.1. This framework shows that Dll4 function in tumor vasculature was dissected by the use of two opposite experimental approaches and appropriate genetically engineered mouse mutants: 1) **Dll4 knock-out (*Dll4*$^{+/-}$) mice**[1] (Duarte et al., 2004) containing a targeted *Dll4* allele in which an in-frame insertion of the β-galactosidase (*lacZ*) reporter gene replaced the initiation codon and the first three coding exons (Dll4 loss-of-function experiments), and 2) **transgenic Tie2-rtTA-M2 TetO7-Dll4 mice** (Trindade, Kumar et al. 2008) capable of endothelial-specific Dll4 overexpression after tetracycline or doxycycline-induction (Dll4 gain-of-function experiments). Both mouse lines, matched with appropriate control littermates (*i.e.*, *Dll4*$^{+/-}$ *vs. Dll4*$^{+/+}$ and induced *vs.* non-induced Tie2-rtTA-M2 TetO7-Dll4 mice) served directly as the neoplasm-bearing hosts in **grafted tumor**-based experiments (mouse sarcoma S180 and Lewis lung cancer cell implants), as well as in the experiments using **autochthonous** DMBA/TPA chemically-induced skin tumorigenesis model. Upon further breeding with RIP1-*Tag2* mice, *Dll4*$^{+/-}$ *vs. Dll4*$^{+/+}$ and induced *vs.* non-induced Tie2-rtTA-M2 TetO7-Dll4 mice constituted the experimental groups used for evaluation of Dll4 loss- and gain-of-function, respectively, in a **transgenic** insulinoma model. Comparative tumor-kinetics, histological and molecular analyses were subsequently

[1] *Dll4*$^{-/-}$ and most *Dll4*$^{+/-}$ mouse embryos have a lethal phenotype (Duarte et al., 2004). Thus, the Dll4 loss-of-function experiments used a small fraction of *Dll4*+/- mice that had survived gestation.

performed in order to understand the cellular and molecular consequences of Dll4 targeting in the tumor microenvironment[2].

Once the role of Dll4 in tumor-driven angiogenesis was characterized, possible Dll4-based therapeutic strategies were considered. Three possible outcomes were predicted regarding potential therapeutic Dll4-targeting. First, if productive angiogenesis and tumor growth were potentiated in the absence of endothelial *Dll4* expression, an agonistic therapy might be appropriate. Alternatively, if tumor growth was enhanced with increased *Dll4* expression levels, then blocking Dll4 signaling would be indicated. Since functional angiogenesis requires well-balanced regulation and might fail in the absence of an optimal *Dll4* expression, the third possibility anticipated that both Dll4/Notch signaling suppression and stimulation might lead to a favorable anti-cancer outcome. In that scenario, both Dll4/Notch signaling agonists and inhibitors would be useful. To carry out antagonistic experimental therapy, **a systemic Dll4/Notch-inhibitor,** soluble Dll4 extra-cellular domain fused to the IgG Fc (sDll4-Fc)[3], was produced by the group of Prof. Parkash S. Gill from the University of Southern California Keck School of Medicine, Los Angeles (USA). Two grafted tumor models (human colon cancer HT29 and human Kaposi sarcoma KS-IMM cell lines) and the RIP1-*Tag2* model of tumor angiogenesis were used for the trials assessing **the effects and efficacy of** sDll4-Fc **systemic administration. The compound was assessed as a monotherapy as well in the combination with** sEphB4, an inhibitor of downstream ephrin-B2/EphB4 signaling, produced by the same laboratory. The rational basis for such combinational therapy is presented in the Section III – Chapter 4.

[2] Detailed descriptions of the applied techniques are presented in the "Materials and Methods" sections of the publications constituting Chapter III (*Results*). In all tumor models and in experimental setting when it was appropriate, tumor growth was quantified by measuring tumor volumes over time, tumor micro-vessel density was assessed by immunostaining of the endothelial marker PECAM, mural cell recruitment to tumor vessels was examined by immunolocalization of the smooth muscle cell markers SMA and NG2, neovessel functionality was evaluated by perfusing tumor vasculature with endothelium-binding lectin, while RT-PCR and DNA micro-array were used for gene expression analyses.

[3] Soluble Dll4 extra-cellular domain binds to Notch and prevents the receptor endogenous activation that is dependent on the expression of Dll4 in the cellular context. Thereby, soluble Dll4 functions as a Dll4/Notch signaling inhibitor, as proven in the study presented in the Chapter III - Section 3 (*"Inhibition of Dll4-mediated signaling induces proliferation of immature vessels and results in poor tissue perfusion"*).

All mouse tumor models employed in this research are well established experimental systems (for references, see the corresponding sections in Chapter III). Throughout the project, they were introduced in a hierarchical fashion. In addition to the general values influencing a model selection that included its validity, predictability and reproducibility, the tumor angiogenic potential was considered as a primary criterion. For the first-line experiments, we selected grafted tumor models because they are well characterized, reproducible, rapid and technically easy to use (Teicher 2006). The subsequent research included experiments in autochthonous mouse tumor systems since they are believed to model human cancers more closely than transplanted tumors, having as advantages: more natural appearance, orthotopic growth, sequential progression and tumor histology devoid of transplantation introduced changes (Talmadge, Singh et al. 2007). From the spectrum involving spontaneously occurring tumors, chemical, viral or physical carcinogen-induced tumors as well as the transgenic mice produced with a heritable predisposition to develop specific cancers, we selected the model of DMBA/TPA chemically-induced skin tumorigenesis and the RIP1-*Tag2* transgenic mouse model of pancreatic islet carcinogenesis. Both represent reliable tools for angiogenesis research since, in these models, tumors develop in stereotypic multi-stage pathways that include the angiogenic switch before solid tumor formation (Bolontrade, Stern et al. 1998; Bergers, Javaherian et al. 1999). Figures 2.2. and 2.3. depict the multi-step tumor progression in DMBA/TPA-treated and RIP1-*Tag2* mice, respectively. Figure 2.3 also shows the schedule of anti-angiogenic trials targeting discrete stages of carcinogenesis in RIP1-*Tag2* mice.

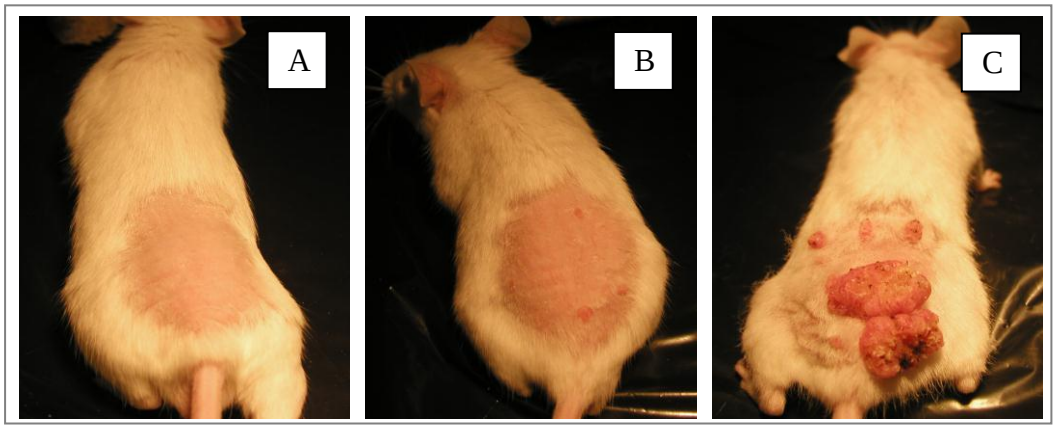


Figure 2.2. – Multi-step DMBA/TPA-induced mouse skin carcinogenesis. Mouse exhibiting normal skin before the tumorigenesis induction (A), skin papillomas (B) and skin papillomas and a squamous carcinoma, 8 and 20 weeks later, respectively.

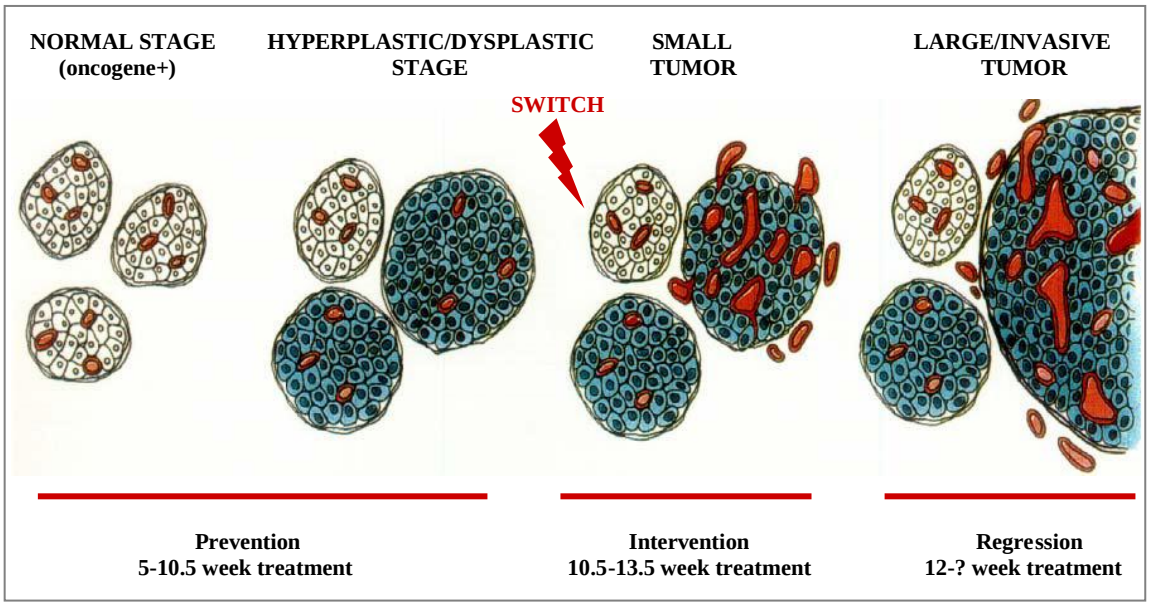


Figure 2.3. – Multi-step progression of tumors in RIP1-*Tag2* mice and anti-angiogenic trials designed to target specific stages of carcinogenesis (adapted from Bergers et al., 1999). The RIP1-*Tag2* mice develop pancreatic insulinomas and islet cell carcinomas in a multi-step pathway that includes an angiogenic switch. Although oncogene expression begins during embryonic development (E8.5), the pancreatic islets initially have a normal anatomical and histological appearance ("normal" stage). Beginning at 4-5 weeks of age, hyperplastic and dysplastic islets begin to appear, reaching ~50% of all islets by 10 weeks of age. Angiogenic islets appear beginning around 6 weeks of age, and represent 10% of all islets at 10.5 weeks. Angiogenic islets are recognized by their dilated blood vessels and microhemorrhaging. Tumors form beginning at 9-10 weeks and represent 2-4% of all the islets by 14 weeks. About half of the tumors at end stage evidence either focal or widespread invasion into the surrounding acinartissue. RIP-Tag2 mice die at approximately 14 weeks of age due to hyperinsulinemiaand tumor burden. In the prevention trials, drug candidates are tested for their ability to block a hallmark of tumor development: the onset of angiogenesis. Thus, 5-week-old transgenic mice, whose islets had not yet activated the angiogenic switch, are treated until the first tumors appear in control mice at 10.5 weeks of age. For intervention studies, designed to address whether angiogenesis inhibitors can slow/stop tumor growth, 10-week-old mice bearing small tumors are treated until end-stage disease (13.5 weeks of age). In the regression trials, anti-angiogenic drug candidates are tested for their ability to extend life-span by inducing regression of large tumors. Thus, regression treatments initiate in 12-week-old mice harbouring substantial tumor burden and with a life expectancy of less than two weeks.

REFERENCES:

Bergers, G., K. Javaherian, et al. (1999). "Effects of angiogenesis inhibitors on multistage carcinogenesis in mice." Science 284(5415): 808-12.

Bolontrade, M. F., M. C. Stern, et al. (1998). "Angiogenesis is an early event in the development of chemically induced skin tumors." Carcinogenesis 19(12): 2107-13.

Claxton, S. and M. Fruttiger (2004). "Periodic Delta-like 4 expression in developing retinal arteries." Gene Expr Patterns 5(1): 123-7.

Domenga, V., P. Fardoux, et al. (2004). "Notch3 is required for arterial identity and maturation of vascular smooth muscle cells." Genes Dev 18(22): 2730-5.

Duarte, A., M. Hirashima, et al. (2004). "Dosage-sensitive requirement for mouse Dll4 in artery development." Genes Dev 18(20): 2474-8.

Gale, N. W., M. G. Dominguez, et al. (2004). "Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development." Proc Natl Acad Sci U S A 101(45): 15949-54.

Hofmann, J. J. and M. L. Iruela-Arispe (2007). "Notch signaling in blood vessels: who is talking to whom about what?" Circ Res 100(11): 1556-68.

Hrabe de Angelis, M., J. McIntyre, 2nd, et al. (1997). "Maintenance of somite borders in mice requires the Delta homologue DII1." Nature 386(6626): 717-21.

Jiang, R., Y. Lan, et al. (1998). "Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice." Genes Dev 12(7): 1046-57.

Krebs, L. T., J. R. Shutter, et al. (2004). "Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants." Genes Dev 18(20): 2469-73.

Krebs, L. T., Y. Xue, et al. (2000). "Notch signaling is essential for vascular morphogenesis in mice." Genes Dev 14(11): 1343-52.

Mailhos, C., U. Modlich, et al. (2001). "Delta4, an endothelial specific notch ligand expressed at sites of physiological and tumor angiogenesis." Differentiation 69(2-3): 135-44.

Patel, N. S., M. S. Dobbie, et al. (2006). "Up-regulation of endothelial delta-like 4 expression correlates with vessel maturation in bladder cancer." Clin Cancer Res 12(16): 4836-44.

Przemeck, G. K., U. Heinzmann, et al. (2003). "Node and midline defects are associated with left-right development in Delta1 mutant embryos." Development 130(1): 3-13.

Swiatek, P. J., C. E. Lindsell, et al. (1994). "Notch1 is essential for postimplantation development in mice." Genes Dev 8(6): 707-19.

Talmadge, J. E., R. K. Singh, et al. (2007). "Murine models to evaluate novel and conventional therapeutic strategies for cancer." Am J Pathol 170(3): 793-804.

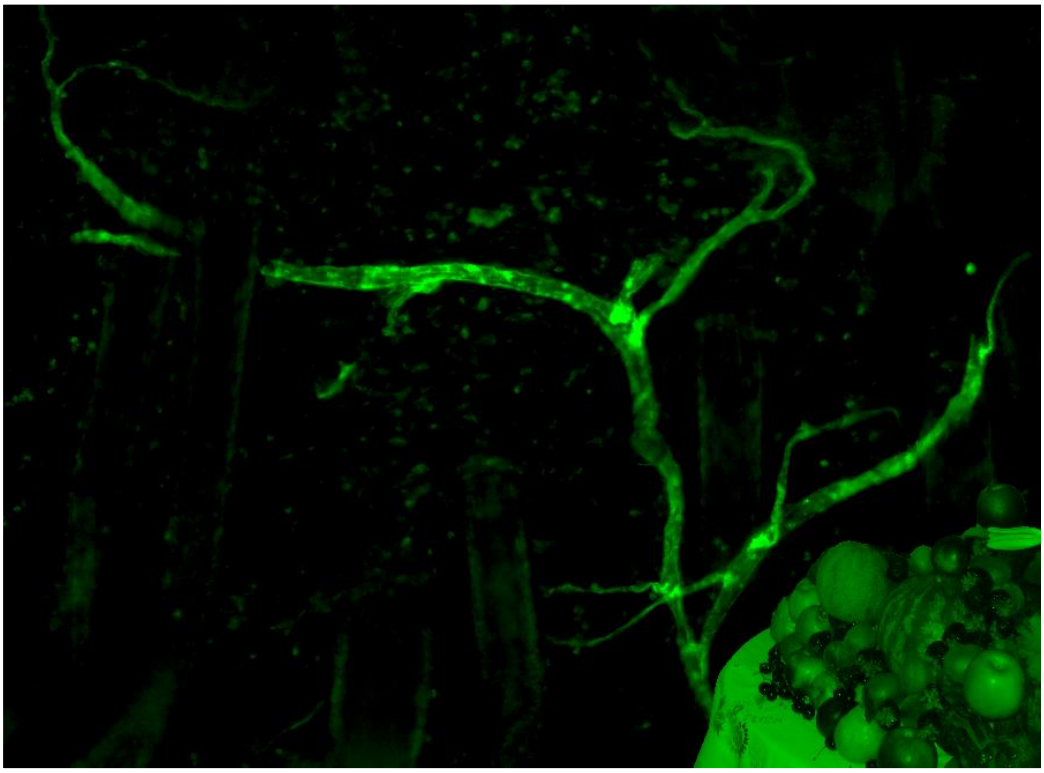
Teicher, B. A. (2006). "Tumor models for efficacy determination." Mol Cancer Ther 5(10): 2435-43.

Trindade, A., S. R. Kumar, et al. (2008). "Overexpression of delta-like 4 induces arterialization and attenuates vessel formation in developing mouse embryos." Blood 112(5): 1720-9.

Weinmaster, G. and R. Kopan (2006). "A garden of Notch-ly delights." Development 133(17): 3277-82.

CHAPTER III

RESULTS



Djokovic D., “The Quiet Life of Fruits and a Tree”, collage (2011)

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Inhibition of Dll4-mediated Signaling Induces Proliferation of Immature Vessels and Results in Poor Tissue Perfusion

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3.1. ABSTRACT

Vascular development is dependent on various growth factors and certain modifiers critical for providing arterial or venous identity, interaction with the surrounding stroma and tissues, hierarchic network formation, and recruitment of pericytes. Notch receptors and ligands (Jagged and Delta-like) play a critical role in this process in addition to VEGF. Dll4 is one of the Notch ligands that regulates arterial specification and maturation events. In the current study, we have shown that loss of function by either targeted allele deletion or use of a soluble form of Dll4 extracellular domain leads to inhibition of Notch signaling, resulting in increased vascular proliferation but defective maturation. Newly forming vessels have thin caliber, a markedly reduced vessel lumen, markedly reduced pericyte recruitment, and deficient vascular perfusion. sDll4 similarly induced defective vascular response in tumor implants leading to reduced tumor growth. Interference with Dll4-Notch signaling may be particularly desirable in tumors that have highly induced Dll4-Notch pathway.

3.2. INTRODUCTION

Primary vasculogenesis serves as the template from which a higher order of branching network is generated by the process defined as angiogenesis (Risau 1997; Folkman 2003; Rossant and Hirashima 2003). During angiogenesis, branching of arterial and venous components is orchestrated such that the capillaries from these 2 compartments fuse in symmetry, anchored in place by interaction with matrix proteins (Carmeliet 2005). Vascular endothelial growth factor (VEGF) is indispensable for the formation of primary vascular network and secondary angiogenesis (Ferrara, Carver-Moore et al. 1996). VEGF however requires the presence of precise quantities of several other constituents within well-defined temporal and spatial constraints to construct and remodel the vascular system. Specifically the Notch signaling pathway is necessary to provide signals for phenotypic determination of arteries and veins, and regulated vessel migration and branching leading to the vascular morphogenesis and remodeling (Bray 2006 and W.J. and P.S.G., unpublished data, December 2006).

In mammals, the Notch family of proteins is composed of 4 single-pass transmembrane receptors (Notch1-4) and 5 membrane-bound ligands (Jagged1, 2 and Dll1, 3, and 4). Mutations of Notch receptors and ligands in mice and humans lead to abnormalities in the vascular system (Iso, Hamamori et al. 2003). The Notch pathway functions through cell-cell interaction such that the extracellular domain of cell membrane–bound ligand interacts with the extracellular domain of the receptor on an adjacent cell. Notch receptor activation requires cleavage of Notch intracellular domain (NICD) and translocation to the nucleus, and activation of target genes (Hainaud, Contreres et al. 2006).

Differentiation of vascular cells to arterial or venous compartments was previously thought to depend on physical factors such as blood pressure and oxygen concentration. Over the past few years, however, the differential and restricted expression of a number of genes in arterial or venous endothelial cells prior to the onset of circulation suggested the potential for genetic determination of the arterial and venous fate of primary endothelial cells. Among these genes are *NOTCH1* (Krebs, Xue et al. 2000), *NOTCH4* (Uyttendaele, Marazzi et al. 1996), *DLL4* (Shutter, Scully et al. 2000), and the Dll4-Notch–regulated genes *EPHB4* and *EFNB2* specifically expressed in venous (Wang, Chen et al. 1998) and arterial endothelial cells, respectively (Gerety, Wang et al. 1999; Duarte, Hirashima et al. 2004).

Vascular expression of Dll4 and its cognate receptors Notch1 and Notch4 is restricted to arterial endothelium. Dll4 is one of the earliest genes expressed in arterial endothelial cells, is induced by VEGF-VEGFR signaling, and is essential for establishment of the arterial endothelial cell fate (Lawson, Vogel et al. 2002; Duarte, Hirashima et al. 2004; Fischer, Schumacher et al. 2004). Haploinsufficiency of Dll4, like that of VEGF, leads to embryonic lethality due to defects in vascular development (Duarte, Hirashima et al. 2004). The observed defects include loss of expression of arterial markers, reduced arterial phenotype and augmented venous phenotypes, reduced arterial lumen, and premature fusion among the arterial and venous compartment leading to short circuiting of the vascular network (Duarte, Hirashima et al. 2004).

In this study, we investigated the role of Dll4 in vascular remodeling at sites of angiogenesis, including tumor vasculature. We show that loss of Dll4 function promotes endothelial cell migration, excessive vascular network formation, and reduction in pericyte recruitment, both in embryos and adult mice. Soluble forms of Dll4 interrupt Dll4-Notch signaling and recapitulate the vascular alterations seen in the gene knock-out mice, including increased vascular network formation, decreased or absent vascular lumen, and reduced recruitment of pericytes resulting in tissue hypoxia and decreased tumor growth.

3.3.MATERIALS AND METHODS

3.3.1. Analysis of Dll4 germ-line mutant mice in embryos and adults

Dll4 knock-out mice were generated in CD1 background and described previously (Duarte, Hirashima et al. 2004). *Dll4*$^{-/-}$ and most *Dll4*$^{+/-}$ mouse embryos have a lethal phenotype. The vasculature of *Dll4*$^{+/-}$ embryos was visualized with platelet endothelial cell adhesion molecule (PECAM) and alpha smooth muscle actin (α-SMA) staining. *Dll4*$^{+/-}$ mice that survived to adulthood were studied for alterations in the vasculature. *Dll4*$^{+/-}$ CD1 male mice and wild-type mice (6-8 weeks old) received a transplant of 5×10^6 tumor cells (S180 mouse sarcoma cell line). Tumors were harvested after 2 weeks for analysis. Dll4 expression was studied in *Dll4*$^{+/-}$ mice in the tumor tissue and adjacent normal tissue by the use of a *lacZ* reporter included in the targeting vector. Whole-mount embryo immunohistochemistry (PECAM antibody was from Pharmingen, San Diego, CA) and *lacZ* staining were carried out by standard techniques (Duarte, Hirashima et al. 2004).

3.3.2. Reverse-transcription–polymerase chain reaction (RT-PCR) analysis

First-strand cDNA was synthesized from total RNA using a SuperScript Preamplification System kit (GIBCOBRL, Grand Island, NY) and used (0.1 μg) for PCR with specific primers for *Dll4*, *GAPDH*, β-actin, *Hey1*, *Hey2*, *Hes1*, and *Hes2* (primer pairs used in this study are available on request); PCR products were visualized by ethidium bromide staining.

3.3.3. Antibodies and other reagents

Anti-PECAM (M20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti–α-SMA (Dako, Carpinteria, CA), IgG-Fc fragment, and anti–human Fc, from Jackson Laboratories (Bar Harbor, ME); Notch1-Fc and Notch3-Fc, from R&D Systems (Minneapolis, MN); hypoxyprobe-1, from Chemicon International (Temecula, CA); rhodamine-labeled ricinus communis agglutinin I (RCA), from Vector Laboratories

(Burlingame, CA); and alkaline phosphatase substrate PNPP, from Sigma Chemicals (St Louis, MO).

3.3.4. Cell culture

Normal human umbilical vein endothelial cells (HUVECs) and human umbilical arterial endothelial cells (HUAECs) were obtained from Cambrex (Walkersville, MD) and maintained in EGM2-supplemented medium (Invitrogen, Carlsbad, CA). For all experiments, HUVECs and HUAECs were used at passages 4 or below and collected from a confluent dish. ChoK cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured under recommended conditions.

3.3.5. Dll4 constructs

Full-length human *Dll4* gene was cloned by PCR amplification from human cDNA (Clontech, Mountain View, CA) made from fetal lung tissues. Both full-length (amino acid residues 1-685) and C-terminally His-tagged extracellular domain (amino acid residues 1-486) proteins were expressed from pcDNA3.1 expression vector (Invitrogen). Fc fusion protein was expressed from pCXFc vector (Invitrogen). AP fusion protein was expressed from pAPtag-2 vector (GeneHunter, Nashville, TN). All proteins were transiently expressed in ChoK cells (ATCC) using Lipofectamine 2000 (Invitrogen). His-tagged and Fc-fusion Dll4 proteins were purified through nickel-NTA column and protein A–Sepharose column (Kertesz, Krasnoperov et al. 2006).

3.3.6. Notch receptor binding and activation pathway

Notch1-Fc and Notch3-Fc (5 μg/mL each) were coated overnight at 4°C in PBS on 96-well plates. Dll4-AP was diluted in PBS and 0.1% Tween-20 (PBST); 50 μL of each dilution was incubated with Notch-Fc and blocked with 5% milk in PBS for one hour. Wells were washed 3 times with PBST, developed with PNPP, and read at OD405. Typically, HUVECs were grown in 100-mm dishes until 80% confluence and were cocultured with ChoK cells transiently expressing full-length Dll4 (1:1 ratio) or ChoK cells transfected with vector alone. Cocultures were treated with either rDll4-His or rDll4-

Fc for a period of 24 hours, cells were harvested, and total RNA was isolated for further analysis (Xia, Kumar et al. 2006).

3.3.7. Cell sorting

For sorting transfected cells, the MACSelect 4.1 transfected cell selection kit (Miltenyi Biotech, Auburn, CA) was used as per manufacturer's instructions. In brief, cells were cotransfected with expression vector containing the plasmid of interest and pMACS 4.1 plasmid. After 36 hours, cells were harvested with 5 mM EDTA and incubated with MACSelect 4 Microbeads for 15 minutes at 4°C. The cell suspension was then passed via an MS+ column in a magnetic field. After 3 washes, the column was removed from the field and selected cells were eluted in culture medium. Selection efficiency was confirmed by fluorescence-activated cell sorter (FACS) analysis of sorted cells with fluorescent Dll4 monoclonal antibody (data not shown).

3.3.8. Endothelial cell (EC) tube formation assay

Matrigel (250 μL; BD Biosciences, Palo Alto, CA) was placed in each well of an ice-cold 24-well plate. The plate was allowed to sit at room temperature for 15 minutes, and at 37°C for 30 minutes for Matrigel to polymerize. HUVECs in EGM2 medium were plated at a concentration of 1×10^4 cells/well with test material at various concentrations in triplicates. After 6-hour and 24-hour incubations, pictures were taken for each concentration using a Bioquant Image Analysis system (Bioquant, Nashville, TN). Length of cords formed and number of junctions were compared among various groups using ImageJ software (NIH, Bethesda, MD). Experiments were repeated twice (Kertesz, Krasnoperov et al. 2006).

3.3.9. Vessel sprouting

Endothelial cell spheroids were generated by suspending equal number of endothelial cells (1000 cells/well) in culture medium containing 0.25% (wt/vol) carboxymethylcellulose and seeded in nonadherent round-bottom 96-well plates. Endothelial cells were suspended to form a single spheroid per well. Spheroids were

embedded into collagen gels and cultured for at least 24 hours. Sprouting was recorded digitally (ocular grid at 100× magnification) using the digital imaging DP-Soft (Olympus, Center Valley, PA) analyzing at least 10 spheroids per experimental group and experiment. Sprouting was also quantitated by measuring the length of the sprouts by ImageJ (Kertesz, Krasnoperov et al. 2006).

3.3.10. Murine Matrigel plug angiogenesis assay

In vivo angiogenesis was assayed using the Matrigel plug assay. Matrigel rapidly forms a solid gel at body temperature, trapping the factors to allow slow release and prolonged exposure to surrounding tissues. Matrigel (8.13 mg/mL, 0.5 mL) in liquid form at 4°C was mixed with vehicle alone (PBS containing 0.25% BSA) or VEGF, or sDll4, or VEGF and sDll4 together. Matrigel (0.5 mL) was injected into the abdominal subcutaneous tissue of female Balb/C nu/nu mice (6 weeks old, 5 mice per group) along the peritoneal midline. On day 6, mice were humanely killed and plugs were recovered, weighed, and divided for hemoglobin measurement and immunohistochemical analysis. Vascular identity of the infiltrating cells was established with PECAM immunostaining. The experiment was repeated 3 times. The vascularized area in each section was calculated using ImageJ. Hemoglobin in one half of the Matrigel plug was measured using the Drabkin method (Drabkin reagent kit 525; Sigma, St Louis, MO) using the manufacturer's recommended protocol.

3.3.11. Immunohistochemistry and immunofluorescence

Sections (5 μm) of formalin-fixed paraffin-embedded tissues were processed using standard methods (Duarte, Hirashima et al. 2004; Kertesz, Krasnoperov et al. 2006). Sections were incubated with primary antibody overnight at 4°C and appropriate secondary antibody for 1 hour at room temperature. Antibody binding was localized with ABC staining kit from Vector Laboratories according to the manufacturer's instructions and peroxidase activity detected using DAB substrate solution (Vector Laboratories). Routine negative controls were exclusion of primary and secondary antibody and substitution of normal IgG isotope for primary antibody. The positive staining area was estimated using ImageJ and analyzed by Student *t* test.

Fluorescent immunostaining was performed in a similar fashion to detect the expression level of EC-specific markers including PECAM. Appropriate fluorescein-conjugated secondary antibodies (Sigma-Aldrich, St Louis, MO) were used and nuclei were counterstained with 4′, 6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI). Slides were mounted with Vectashield antifade mounting solution (Vector Laboratories) and images obtained using an Olympus AX70 fluorescence microscope and Spot v2.2.2 (Diagnostic Instruments, Sterling Heights, MI) digital imaging system.

3.3.12. Murine tumor xenografts

Tumor cells (1.5×10^6) HT29 (human colon cancer cell line) or KS-IMM (human Kaposi sarcoma cancer cell line) were implanted subcutaneously in flanks of male athymic BalbC nu/nu mice (6-8 weeks old, 6 mice/group and repeated twice). For assessing local effects of sDll4, tumor cells were mixed with Matrigel (1:1 vol/vol; BD Biosciences) with or without 5 μg/mL sDll4. Tumor volume was measured on day 14 estimated as $0.52 \times a \times b^2$, where a and b are the largest and smallest lengths of the palpable tumor, respectively. The Student *t* test was used to compare tumor volumes, with $P < .05$ being considered significant. Animals were humanely killed, and tumor and adjacent normal tissues were harvested. Harvested tissues were divided and either fixed in formalin or frozen in OCT for analysis. Distribution and intensity of hypoxia were studied using hypoxyprobe-1 (HP1-100; Chemicon International) infused intraperitoneally at a dose of 60 mg/kg one hour prior to the tumor harvest and localized using recommended protocol. Vessel perfusion was studied using rhodamine-labeled *Ricinus communis* agglutinin 1 (Vector Laboratories) infused 10 to 15 minutes prior to the tumor harvest and analyzed using the manufacturer's recommended protocol. All procedures were approved by our Institutional Animal Care and Use Committees and performed in accordance with the Animal Welfare Act regulations.

3.4. RESULTS

3.4.1. Vascular proliferation in embryonic and adult *Dll4*$^{+/-}$ mutant mice

Dll4$^{-/-}$ and most of *Dll4*$^{+/-}$ mice die in utero due to defective vascular development (Krebs, Shutter et al. 2004). Close examination of the *Dll4*$^{+/-}$ embryos showed normal vasculogenesis until E8.75, when the first vascular defect became apparent. There was increased vascular proliferation appearing like honeycomb and lacking hierarchic arterial branching and maturation (Figure 3.1. A). We next studied vascular response and remodeling in adult *Dll4*$^{+/-}$ mutant and wild-type mice. Mice (6 weeks old) received implants of S180 tumor cells. Tumor and adjacent tissue harvested after 10 days was examined for vascular response by PECAM, and α-SMA immunolocalization. Wild-type mice showed increased vascular response in the tumor (Figure 3.1. B) and the vessels had organized network. In comparison, *Dll4*$^{+/-}$ mice showed an even greater increase in the vascular response (1.5-fold increase, $P < .05$). Furthermore, the vessels showed lack of architecture and loss of hierarchy. Thus vascular response was increased but maturation was lacking. Maturation of newly forming vessels accompanies the recruitment of pericytes. We hypothesized that newly forming vessels in *Dll4*$^{+/-}$ mice may be defective in pericyte recruitment. Thus localization of pericytes with α-SMA antibodies showed abundant signal in tumor vessels in wild-type mice, whereas tumor vessels in *Dll4*$^{+/-}$ mice showed a profound deficiency in pericyte coverage. Reduced recruitment of pericytes may contribute to the lack of vascular hierarchy in *Dll4*$^{+/-}$ mice tumor vessels. Furthermore, these findings reveal a novel function of *Dll4* in the recruitment of pericytes to newly forming vessels. We next wished to determine if defective vascular response in adult mice leads to alteration in gene expression, in particular *Dll4*. To this end, we used the LacZ reporter included in the targeting vector used to generate mutant mice to observe *Dll4* promoter activity. *Dll4*$^{+/-}$ mutant mice showed highly structured *LacZ*-expressing vessels in the normal tissue adjacent to the tumor (Figure 3.1. C), whereas *LacZ* activity was markedly increased in vessels within the tumor vessels (Figure 3.1. C), indicative of *Dll4* activation in the tumor vasculature. PECAM localization in serial sections of the tumor vessels was done to determine the extent of *Dll4* activation in tumor vasculature. Dll4 is expressed in the majority but not in all tumor vessels (Figure 3.2.)

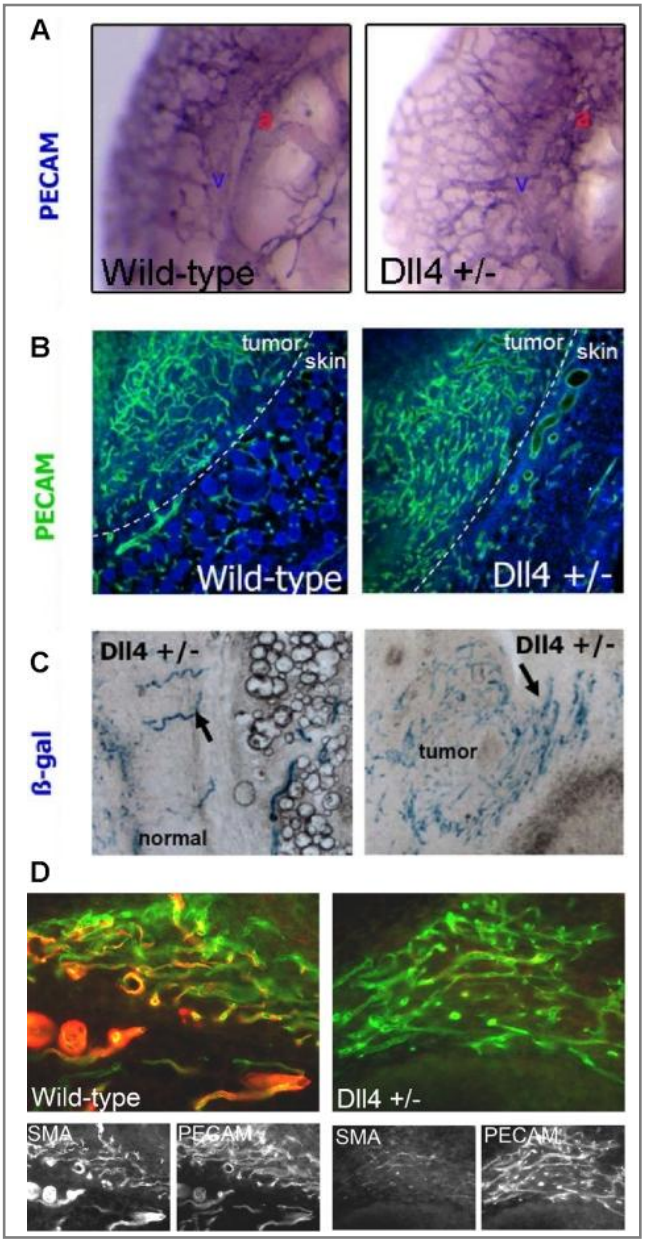

Figure 3.1. - Dll4$^{+/-}$ mutant mice show defective increase in vascular proliferation. (A) The vasculature of wild-type and Dll4$^{+/-}$ embryos were examined using PECAM whole-mount immunostaining. Dorsal aorta and cardinal vein are labeled as a and v, respectively. Absence of large vessels and an increase in vessel branching and density was seen in Dll4$^{+/-}$ embryos at E10.5 compared to wild type. (B) Vascular response in Dll4$^{+/-}$ adult mice was examined as in panel A after tumor implantation. Wild-type mice showed organized vascular proliferation in the tumor (left half), while mutant mice showed markedly increased vascular response that lacks organization and vascular hierarchy. (C) Expression of Dll4 in tumor and normal regions in Dll4$^{+/-}$ mutant mice was examined by β-gal staining. Dll4 expression was observed (arrows) in a few discrete vessels in the normal tissue, while the tumor region showed many β-gal–positive vessels of similar appearance indicative of Dll4 induction in tumor vessels. (D) Pericyte coverage around newly forming vessels was examined by α-SMA localization. In wild-type mice, the vessels showed colocalization of PECAM and α-SMA (left panel). In *Dll4*$^{+/-}$ mice tumor vessels, however, the number of α-SMA–positive cells lining the endothelial cells was profoundly reduced (right panel). Images in panel A were viewed under an Olympus SZX12 stereomicroscope (Tokyo, Japan) with Leica PL Fluotar 0, 5, 20×/0.5 NA dry objective (Wetzlar, Germany), captured with an Olympus C4040 camera, and processed with Olympus DP-Soft 3.2. Images in panels B-D were viewed under a Leica DMRA2 fluorescence microscope with Leica HC PL Fluotar 0, 5, 20×/0.5 NA dry objective, captured using Photometrics CoolSNAP HQ, (Photometrics, Friedland, Denmark), and processed with Metamorph 4.6-5 (Molecular Devices, Sunnyvale, CA).

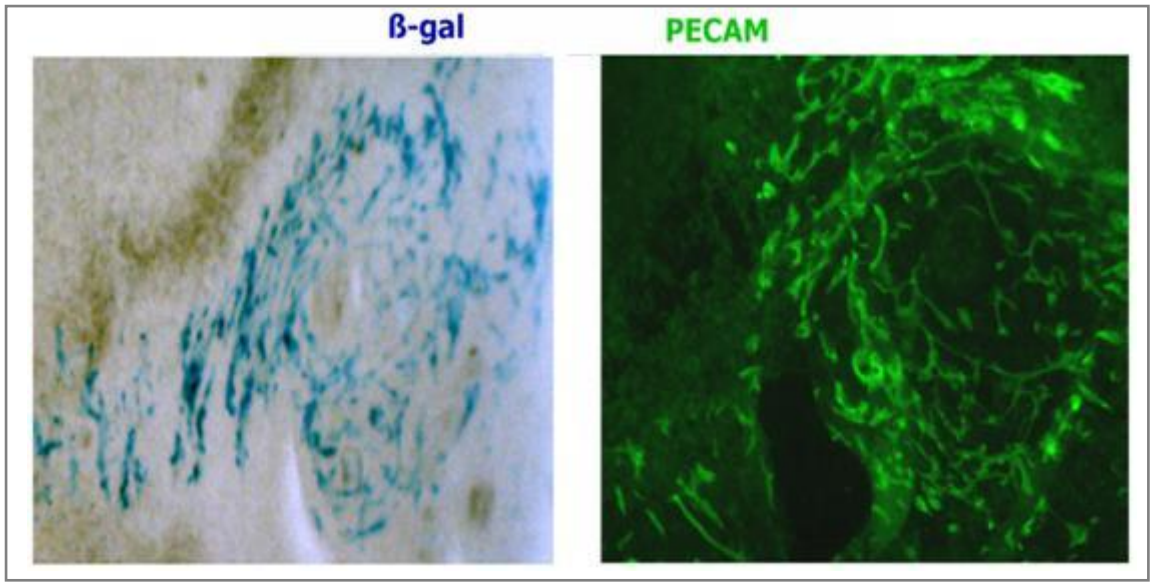

Figure 3.2. - Dll4 is activated in most but not all vessels in the tumor. β-gal (left panel) and PECAM (right panel) immunostaining of adjacent tumor sections show that endothelial-specific PECAM staining is more abundant than *lacZ Dll4* reporter expression.

3.4.2. Soluble Dll4 inhibits Dll4-Notch signaling

We next wished to determine if soluble Dll4 could antagonize Notch activation. Extracellular domain of human Dll4 fused either to AP, Fc, or His tag were expressed in mammalian cells, purified, and determined to bind Notch1-Fc (Figure 3.3. A) and Notch4-Fc (data not shown) but not Notch3-Fc (Figure 3.3. A) or Fc alone (data not shown). Notch activation is dependent on the expression of Dll4 in the cellular context. To test that the soluble forms of Dll4 do not induce Notch activation, we introduced various Dll4 constructs in endothelial cells and examined the induction of downstream Notch-responsive genes *(Hey1*, *Hey2*, *Hes1*, and *Hes2)* by RT-PCR. Representative data for the absence of Notch activation are shown by the lack of Hes2 induction by Dll4-Fc or Dll4-His (Figure 3.3. B). Hes2 is downstream of Notch and induced by full-length Dll4 when presented in the cellular context (Figure 3.3. C). We next determined if sDll4 can inhibit the activity of cellular Dll4 in inducing Notch signaling. To this end, full-length Dll4 (Dll4-FL) was introduced into ChoK cells and cocultured with HUVECs expressing target Notch1 and Notch 4. Dll4-FL induces Notch-regulated genes, *Hey1*, *Hey2*, *Hes1*, and *Hes2* (Figure 3.3. C), in human endothelial cells using human gene-specific primer pairs. In identical experiments, addition of human sDll4-Fc and sDll4-His blocked Dll4-FL–induced activation of *Hey1*, *Hey2*, *Hes1*, and *Hes2* (Figure 3.3. C). Thus soluble Dll4

functions as an antagonist of Dll4-Notch signaling. Quantitation of gene expression showed that Dll4-Fc inhibited Hey1, Hey2, Hes1, and Hes2 to 69%, 26%, 29%, and 46% of control, respectively, while sDll4-His reduced their expression to 48%, 3%, 10%, and 28%, respectively.

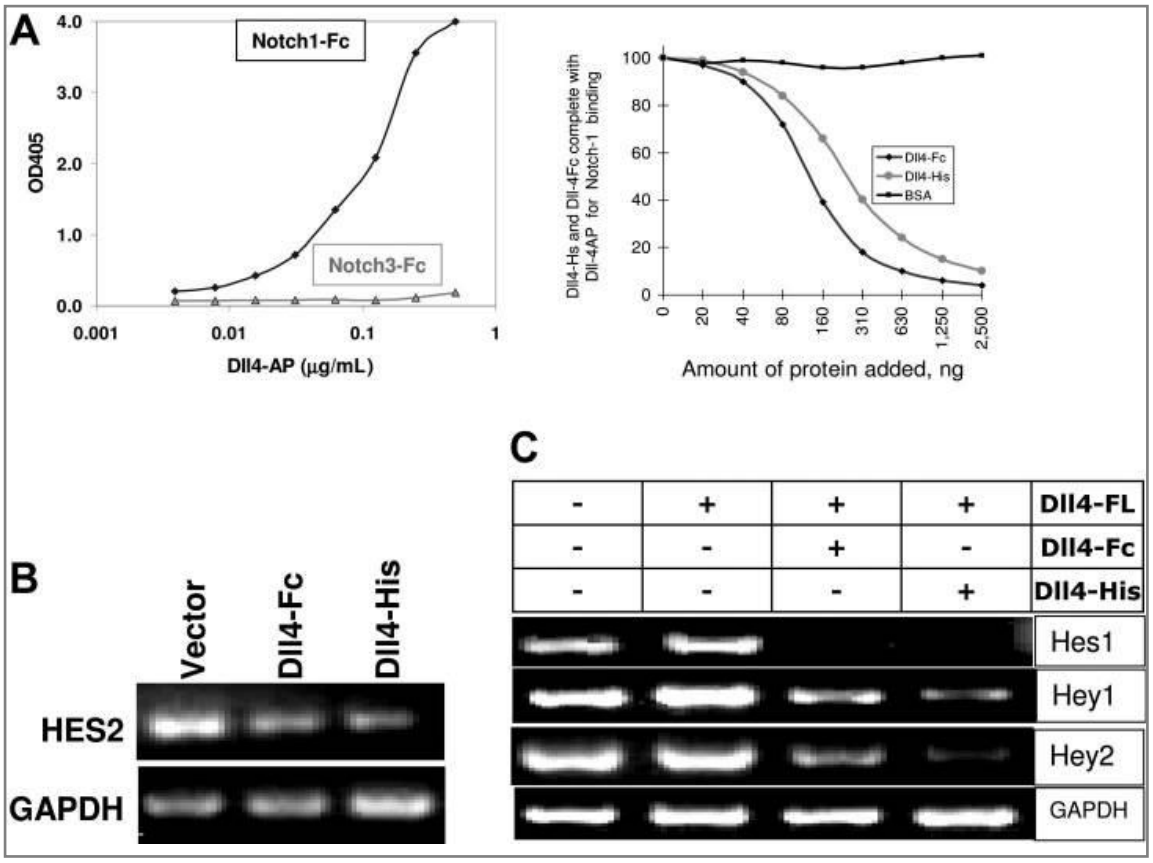

Figure 3.3. - Biochemical properties of sDll4. (A) Notch-Fc fusion protein was coated directly on enzyme-linked immunosorbent assay (ELISA) plates. sDll4-AP was allowed to bind Notch-Fc, and the bound Dll4 was quantitated by the addition of AP substrate. sDll4-AP bound efficiently to Notch1 and not Notch3 (left panel). Binding of sDll-4Fc and sDll4-His to Notch1 was examined. (B) HUVECs were transfected with expression vectors for sDll4-Fc, sDll4-His, or vector alone. Notch-responsive Hes-2 gene expression was not induced by sDll4 proteins. (C) Notch activation measured by the induction in Hes-1, Hey-1, and Hes-2 when HUVECs were cocultivated with ChoK expressing Dll4-FL (full length). Addition of recombinant sDll4-Fc and sDll4-His reduced the induction of Notch responsive genes. Two independent experiments produced similar results.

3.4.3. Soluble Dll4 induces sprouting and tube formation

We intended to determine if soluble Dll4 could mimic the dll4 loss-of-function phenotype. sDll4-Fc and sDll4-His were tested in tube formation assays using endothelial cells placed on polymerized Matrigel to promote the formation of tube-like structures.

Minimal amounts of tubes were formed in the absence of growth factors, and abundant tube formation was observed with the addition of VEGF. Dll4-His showed a dose-dependent induction of tube formation in the absence of additional growth factors (Figure 3.4). Quantitative measurement of junction formation and length of tubes increased in a dose-dependent manner (Figure 3.4). Similar results were seen with Dll4-Fc (data not shown).

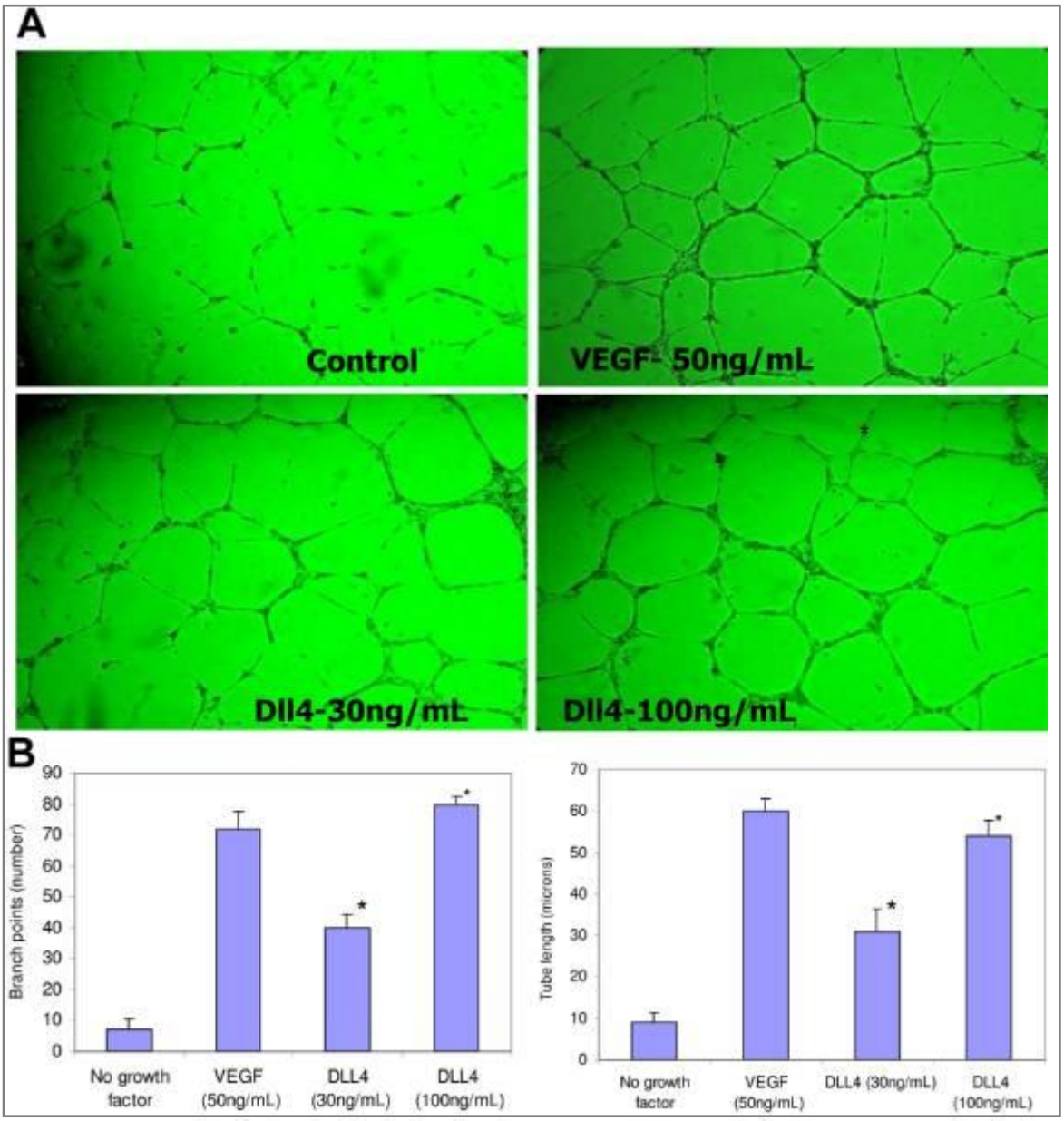

Figure 3.4. - sDll4 induces tubule formation in vitro. (A) HUVECs were cultured on standard Matrigel in growth factor–deficient conditions in triplicates in 2 independent experiments with either sDll4 or VEGF for 24 hours. Shown are representative pictures from triplicate wells repeated twice. (B) Quantitative analysis for tube length and the number of junctions in sDll4-treated HUVECs (Bioquant Image Analysis; mean ± SEM from triplicate wells in 2 repetition experiments). Similar results were seen with human arterial endothelial cell assay (data not shown). *P < .05 compared to no growth factor. Photomicrographs in panel A were taken with a Nikon Plan Fluor ∞, 0.17, 4×/0.12 NA objective and 10× eyepiece and processed with Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MA).

We next extended these studies in an assay in which endothelial cell spheroids placed in collagen show proliferation and outward migration of vessel-like structures. VEGF profoundly increases sprouting, while no sprouts were observed in growth factor–deficient conditions. sDll4-Fc and sDll4-His when examined alone induced sprouting in a dose-dependent manner. Representative data with sDll4-His are shown in Figure 3.5. Similar results were seen with sDll4-Fc (data not shown). These data indicate that inhibition of endogenous Notch signaling leads to increased endothelial cell migration and organization to make tubes and promotes vascular sprouting that is analogous to the observed murine *Dll4*$^{+/-}$ phenotype.

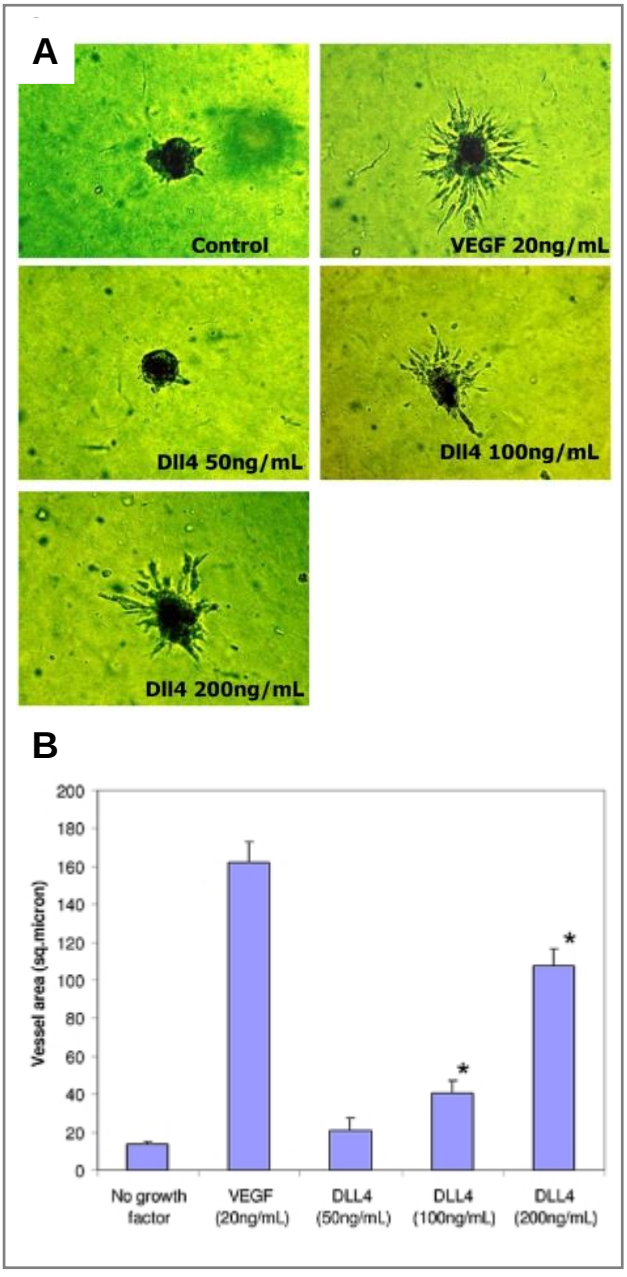

Figure 3.5. - sDll4 induces tubule formation in vitro. (A) HUVECs were cultured on standard Matrigel in growth factor–deficient conditions in triplicates in 2 independent experiments with either sDll4 or VEGF for 24 hours. Shown are representative pictures from triplicate wells repeated twice. (B) Quantitative analysis for tube length and the number of junctions in sDll4-treated HUVECs (Bioquant Image Analysis; mean ± SEM from triplicate wells in 2 repetition experiments). Similar results were seen with human arterial endothelial cell assay (data not shown). **P* < .05 compared to no growth factor. Photomicrographs in panel A were taken with a Nikon Plan Fluor ∞, 0.17, 4×/0.12 NA objective and 10× eyepiece and processed with Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MA).

3.4.4. sDll4 induces vascularization of Matrigel plugs in vivo

To further demonstrate that sDll4 can directly inhibit angiogenesis *in vivo*, we performed a murine Matrigel plug experiment. Matrigel was supplemented with VEGF, sDll4-Fc, sDll4-His, or various combinations, and injected into the ventral abdominal subcutaneous tissue of Balb/C nu/nu mice. Matrigel plugs without growth factors had virtually no vascularization after 6 days (Figure 3.6. A), while VEGF recruited endothelial cells and formed various stages of vascular structures including those with open lumen containing red blood cells throughout the plug. sDll4 in the context of VEGF showed a marked increase in vascular structures (Figure 3.6.), which appeared like thin strings, and mostly lacked lumen.

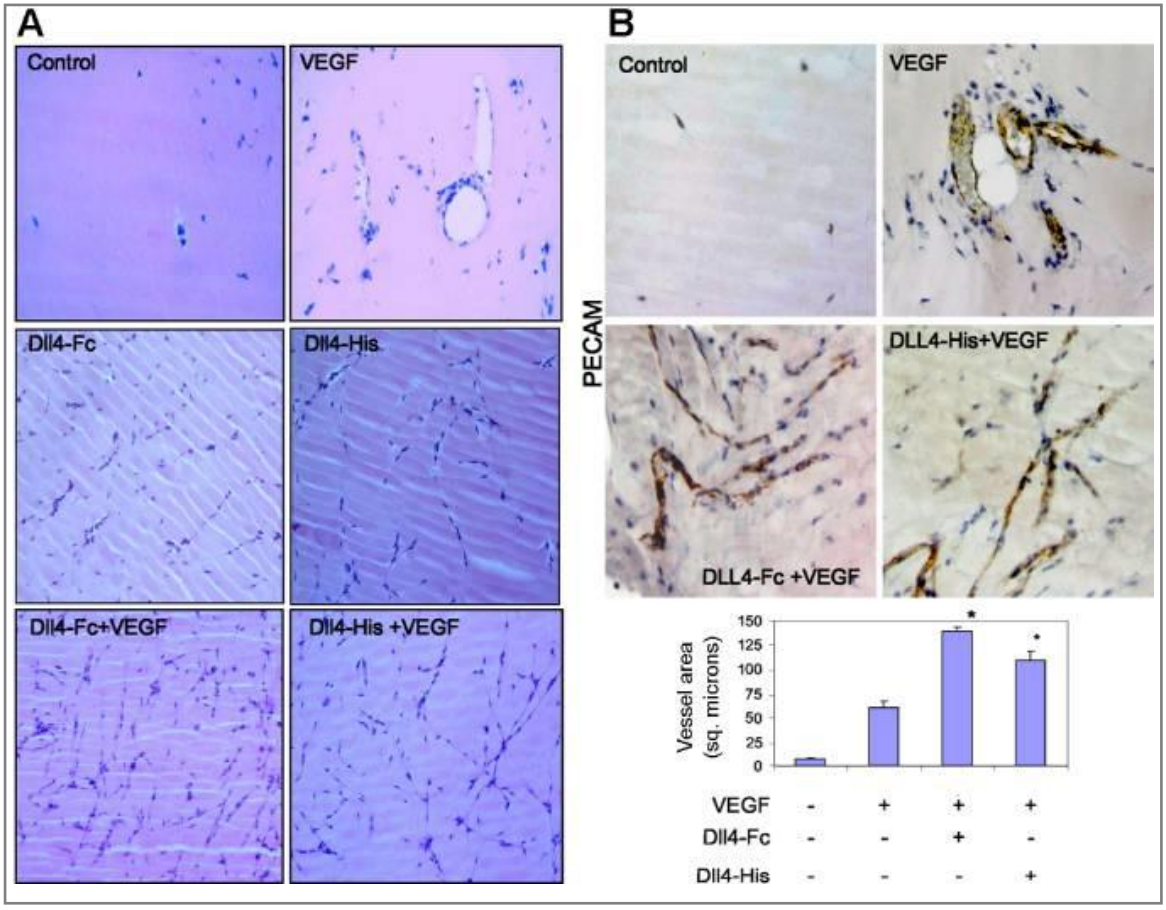

Figure 3.6. - sDll4 induces vessel response but lacks perfusion in murine Matrigel assay. (A) Matrigel was injected subcutaneously into Balb/C nu/nu mice. After 6 days, plugs were removed and processed in paraffin. Individual sections were stained with H&E, and representative photographs at × 20 magnification from triplicate plugs in 2 independent experiments are shown. (B) Matrigel plugs were stained for PECAM. Photomicrographs were taken with an Olympus BX51 microscope with an Olympus UPlan FL ∞, 0.17 20×/0.5 NA dry objective mounted with a Retiga 2000R camera (QImaging, Burnaby, BC, Canada) and processed with Image-Pro Plus 6.0. Quantitation of vascularized area averaged (± SEM) from all plugs (Scion Image software; Scion, Frederick, MD) in bar graph. **P* < .05 compared to no growth factor.

Similarly, sDll4 alone, in the absence of VEGF, was also capable of inducing angiogenesis. Immunochemical examination with PECAM further demonstrates the contrast between VEGF-induced large vessel filled with red blood cells and sDll4-induced vessels that express PECAM but lack lumen and lack perfusion defined by the presence of red blood cells (Figure 3.6. B). Hemoglobin was also quantitated specifically by the Drabkin method using a Drabkin reagent kit and fold change was compared with control measurement represented as 1. Median hemoglobin levels thus were 1, 9.7, 2.5, and 3 g/L/g plug in control, VEGF, Dll4-Fc, and Dll4-His groups, respectively. There was a near 4-fold decrease in hemoglobin concentration in sDll4-containing Matrigel plugs, compared to VEGF alone.

3.4.5. sDll4 inhibits the growth of human tumors in athymic mice

Tumor vessels have distinctive gene expression profile over resting vessels. *Dll4* is one of the genes induced in tumor vessels in certain human and murine tumors (Figure 3.1. C). Dll4 induction may be a generalized feature of tumor vessels, one that could be beneficial for tumor growth. Dll4 expression is seen predominantly in the tumor vasculature. To determine the effect of sDll4 on tumor cells, tumor cell viability in vitro with various concentrations of sDll4 was tested (HT29, MCF-7, SCC-15, B16, PC3, and KS-SLK cell lines), and no effect was observed (data not shown). Given the ability of Dll4 to profoundly affect angiogenesis in vivo, and the observed sDll4 alteration of the vascular response, we speculated that sDll4 may modulate tumor growth in vivo. We therefore examined the activity of sDll4 in vivo in tumor xenograft models. HT29 (human colon carcinoma cell line) and KS-SLK (human Kaposi sarcoma cell line) cells were premixed with Matrigel-containing vehicle or sDll4 and implanted subcutaneously. Compared to control tumors, xenografts supplemented with sDll4 exhibited a significantly reduced tumor growth over 2 weeks (Figure 3.7. A). Similar results were obtained in KS-SLK. Median tumor volume of control tumors at 2 weeks was 585 mm^3, while that of tumors in Matrigel containing sDll4-His was 267 mm^3.

We next studied the effect of sDll4 when produced by tumor cells. HT29 and KS-SLK cells were transfected with expression vectors to produce Dll4-FL, sDll4-Fc, and sDll4-His, and expression of each protein was confirmed in Western blot assays (data not shown). Coexpression of truncated CD4 allowed sorting of transfected cells to more than

90% purity. Equal numbers of cells (1×10^6 per injection site) were implanted in athymic mice (6-8 tumors per group), and tumor volume was measured for 2 weeks. Tumor volume was similar in vector alone and Dll4-FL, while sDll4-Fc and sDll4-His had markedly reduced tumor volume (more than 70% reduction with sDll4-His) (Figure 3.7. B). Tumors harvested at the time were examined for vascular density using PECAM immunostaining. Tumors expressing Dll4-FL or vector alone showed highly structured vessels (Figure 3.7. C). In contrast, sDll4-expressing tumors showed marked changes in the vessel architecture. There were many more branching points in sDll4-expressing tumor vasculature compared to vector alone or Dll4-FL (Figure 3.7.). Similar results were obtained in KS-SLK tumor xenografts. Remarkably, the vessels appeared thin and often lacking apparent lumen. These characteristics were reminiscent of blood vessel branching in *Dll4*$^{+/-}$ mice, and in Matrigel plugs impregnated with sDll4. Consistent with poorly forming thin vessel lacking lumen, we examined the areas of hypoxia. Analysis of hypoxia focused on viable tumor regions only. There were large and wide areas of hypoxia in sDll4-Fc and sDll4-His. Quantitation of these areas showed marked increase in hypoxic regions of sDll4-expressing tumors compared to both Dll4-FL– and vector-expressing tumors (Figure 3.7. D). Tumor perfusion was also measured using fluorescent-labeled lectin, which binds to the luminal surface of the blood vessels. There was very limited perfusion in the sDll4-expressing tumors compared to Dll4-FL– and vector-transfected cells (Figure 3.7. E). In addition, we determined the presence of pericytes on newly forming tumor vessels by localizing α-SMA expression. Tumor vessels in wild-type mice showed normal pericyte coverage, whereas tumor vessels in *Dll4*$^{+/-}$ mice present a dramatic reduction of pericyte coverage as determined by the number of α-SMA–positive cells lining the endothelial cells. sDll4 similarly reduced the number of pericytes in tumor vessels in athymic mice bearing human tumors. Taken together, these data provide strong evidence for the role of Dll4 in pericyte recruitment to newly forming vessels.

Figure 3.7. - sDll4 inhibits the tumor growth in a murine tumor xenograft model. (A) Mice (n = 6/group) were implanted with 1×10^6 HT29 cells in a Matrigel preparation with PBS or sDll4-Fc or sDll4-His (5 μg/mL) and tumor volumes (mean ± SEM) were measured after 2 weeks; tumors were then harvested and analyzed. Tumor volumes were significantly smaller in the sDll4 arm. **P* < .05 compared to control. The experiment was repeated twice. (B) In assessing the effect of endogenous expression of sDll4, HT29 cells were transfected with expression vector with Dll4-FL, sDll4-Fc, sDll4-His, or vector alone. Coexpression of truncated CD4 was done to allow sorting of the transfected cells. Equal numbers of the transfected cells were implanted in mice (n = 6/group). Tumor volumes (mean ± SEM) were assessed. Tumor volumes were significantly smaller in the sDll4 groups. **P* < .05 compared to vehicle. (**Page 97.**)

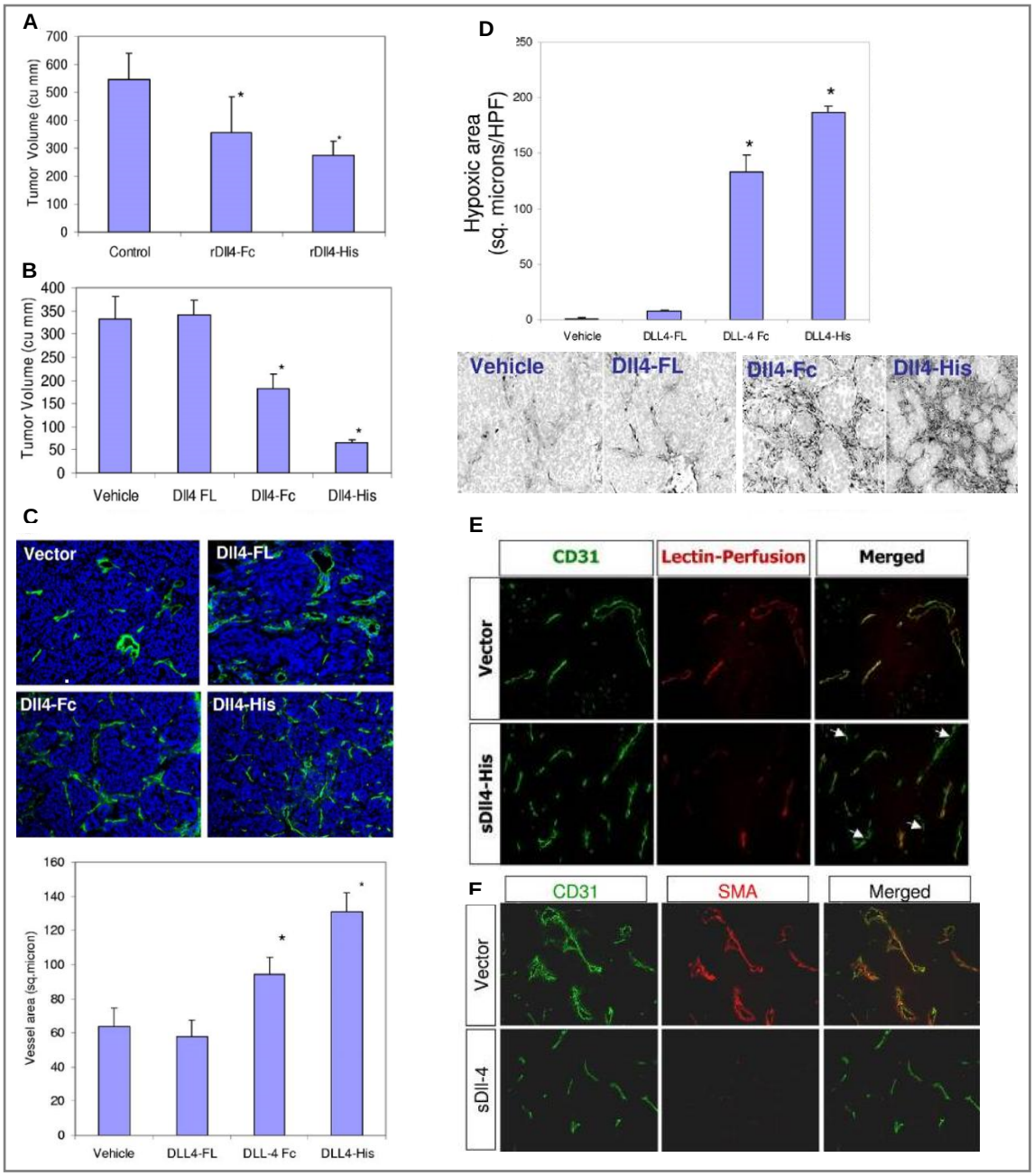

Figure 3.7. - sDll4 inhibits the tumor growth in a murine tumor xenograft model. Legend continuation: (C) Microvasculature was assessed by PECAM immunostaining, and the blood vessel volume was quantitated as described in "Materials and methods." Mean ± SEM. **P* < .05 compared to vehicle. (D) Hypoxy probe was infused prior to tumor harvest; tumor sections were then probed with MAb and fluorescent-labeled secondary antibody as described in "Materials and methods." Hypoxic areas were quantitated (mean ± SEM) using ImageJ as described in "Materials and methods." All values are expressed as mean ± SEM. **P* < .01. Photomicrographs were taken with a Nikon Eclipse 80 microscope with a Nikon Plan Fluor ∞, 0.17 10×/0.3 NA dry objective mounted with a Photometrics CoolSNAP camera and processed with Metamorph V 6.3r2. (E) Vascular perfusion was determined by injecting fluorescent-labeled lectin 10 to 15 minutes prior to killing mice and harvesting tumors. Lectin was localized to perfused areas, while blood vessels were delineated with PECAM staining. Lectin and PECAM colocalized in control group, while sDll4 group showed marked deficiency of perfusion. (F) Localization of α-SMA in tumor vessel. Control group showed colocalization of α-SMA and PECAM, while sDll4 group had paucity of α-SMA–positive cells in the microvessels.

3.5. DISCUSSION

Dll4, Notch1, and *Notch4* are expressed in endothelial cells. *Notch1/Notch4* double-mutant embryos show severe vascular remodeling defects (Krebs, Xue et al. 2000; Duarte, Hirashima et al. 2004), and putative downstream targets of Notch pathway including *Hey1/Hey2* double mutants develop vascular defects with lesser severity (Duarte, Hirashima et al. 2004; Fischer, Schumacher et al. 2004). Dll4 thus profoundly effects vascular development and vessel maturation. Notably, $Dll4^{-/-}$ and most (~ 70% on CD1, 100% on C57BL6 and 129Sv/J genetic backgrounds) $Dll4^{+/-}$ mutant embryos die at midgestation due to defective vascular development. Lethal haploinsufficiency of *Dll4* is analogous to that of *VEGF* (Carmeliet, Ferreira et al. 1996; Ferrara, Carver-Moore et al. 1996), which lies upstream of Notch signaling in arterial development (Lawson, Vogel et al. 2002).

We thus focused on Dll4 in vascular development. Analysis of vasculature in the heterozygote mice has been revealing in that the vasculature develops normally until E8.5 when the defects become apparent and manifest by increased vascular network combined with less mature large vessels that eventually branch and fuse with adjacent vessels and result in short circuit and failing circulatory system. We wished to determine if Dll4 deficiency was manifest in a small fraction of mice that survives to adult life. Use of tumor cell implants in $Dll4^{+/-}$ mice was compared to age-matched wild-type mice for localized vascular response. Vascular response in adult $Dll4^{+/-}$ mice was similar to the embryonic defects including enhanced vascular density, nearly 2-fold higher than in wild-type mice. Secondly, unlike the wild-type mice, the vessels in the mutants have narrow caliber and lack hierarchic branching pattern (Figure3.1 A-B). Dll4 may thus be required for functions in branching and maturation of newly forming vessels. Furthermore, recruitment of pericytes to the newly forming vessels was markedly reduced in $Dll4^{+/-}$ mice tumor vessels. Similar defect was observed in tumor vessels in response to sDll4. It is thus possible that some of the observed defects in Dll4-deficient signaling are due to the paucity of pericytes and smooth muscle.

VEGF and Dll4 have also been shown to be up-regulated by hypoxia (Shweiki, Itin et al. 1992; Mailhos, Modlich et al. 2001), which is one of the environmental factors that regulate vascular patterning and growth. We thus wished to determine if Dll4 expression was changed in tumor vessels compared to those in the adjacent normal tissue. $Dll4^{+/-}$ mice tumor implants were examined for Dll4 expression. LacZ insertion in the *Dll4*

targeting vector allows its expression under the *Dll4* promoter. Thus β-gal staining highlighted a few well-organized vessels in the normal subcutaneous tissue. In the tumor tissue, however, we observed a marked increase in vascular β-gal staining and in the density of vascular structures. This role of Dll4 in modifying vascular response and its overexpression in tumor vessels may provide a therapeutic opportunity to alter tumor response.

Dll4 induces Notch signaling when presented in the cellular context, by activating Notch processing and release of the intracellular domain, which is subsequently translocated to the nucleus to modify expression of target genes including Hey and Hes (Diez, Fischer et al. 2007). Various Dll4 variants containing only the extracellular domain indeed block Dll4-induced Hey and Hes expression. sDll4 thus allowed us to assess the consequences of blocking Dll4-Notch signaling in various distinct functions. sDll4 treatment recapitulates several features seen in *Dll4*$^{+/-}$ mutant mice including increased endothelial cell tube formation and vascular sprouting. Using Matrigel plug assays, we noticed that sDll4 markedly increased endothelial cell migration. Endothelial cells created string-like vascular structures, which do not reveal a prominent lumen and display a notable paucity of red blood cells, unlike those observed upon VEGF treatment, which produces large vascular structures with open lumen filled with red blood cells. sDll4 thus modifies newly forming vessels that are highly deficient in perfusion. Overexpression of Dll4 in tumor vessels and profound alteration of newly forming vessels by disruption of Dll4-Notch signaling provides an opportunity to influence tumor growth. Use of sDll4 indeed showed a marked reduction in tumor growth. Inhibition of tumor growth was also reproduced by engineering tumor cells to produce soluble Dll4. Both sDll4-Fc and sDll4-His markedly reduced the tumor growth combined with changes in tumor vessels similar to those seen in *Dll4*$^{+/-}$ mutant mice and to the effect of sDll4 in the Matrigel assays. Perfusion deficiency was confirmed by examining the areas of hypoxia in the tumor, which showed a marked increase in sDll4-expressing tumor cells. In summary, Dll4 has profound effect in vascular biology. It appears to be essential for regulating migration of vessels in response to VEGF, and necessary for maturation of newly forming vessels (including regulation of lumen formation) and vascular perfusion. Inhibition of Dll4 function thus has a paradoxical effect in inducing an excessive density of newly forming vessels, but defective vessel maturation, lumen formation, recruitment of pericytes, and perfusion. It raises many questions as to how Dll4 regulates endothelial cell migration,

vessel branching, lumen size, and endothelial interaction with pericytes and matrix proteins. Inhibition of Dll4 with soluble protein or neutralizing antibodies or knock down of gene expression with short interference RNA may provide opportunities to treat cancer and vascular proliferative diseases.

REFERENCES:

Bray, S. J. (2006). "Notch signalling: a simple pathway becomes complex." Nat Rev Mol Cell Biol **7**(9): 678-89.

Carmeliet, P. (2005). "Angiogenesis in life, disease and medicine." Nature **438**(7070): 932-6.

Carmeliet, P., V. Ferreira, et al. (1996). "Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele." Nature **380**(6573): 435-9.

Diez, H., A. Fischer, et al. (2007). "Hypoxia-mediated activation of Dll4-Notch-Hey2 signaling in endothelial progenitor cells and adoption of arterial cell fate." Exp Cell Res **313**(1): 1-9.

Duarte, A., M. Hirashima, et al. (2004). "Dosage-sensitive requirement for mouse Dll4 in artery development." Genes Dev **18**(20): 2474-8.

Ferrara, N., K. Carver-Moore, et al. (1996). "Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene." Nature **380**(6573): 439-42.

Fischer, A., N. Schumacher, et al. (2004). "The Notch target genes Hey1 and Hey2 are required for embryonic vascular development." Genes Dev **18**(8): 901-11.

Folkman, J. (2003). "Fundamental concepts of the angiogenic process." Curr Mol Med **3**(7): 643-51.

Gerety, S. S., H. U. Wang, et al. (1999). "Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development." Mol Cell **4**(3): 403-14.

Hainaud, P., J. O. Contreres, et al. (2006). "The role of the vascular endothelial growth factor-Delta-like 4 ligand/Notch4-ephrin B2 cascade in tumor vessel remodeling and endothelial cell functions." Cancer Res **66**(17): 8501-10.

Iso, T., Y. Hamamori, et al. (2003). "Notch signaling in vascular development." Arterioscler Thromb Vasc Biol **23**(4): 543-53.

Kertesz, N., V. Krasnoperov, et al. (2006). "The soluble extracellular domain of EphB4 (sEphB4) antagonizes EphB4-EphrinB2 interaction, modulates angiogenesis, and inhibits tumor growth." Blood **107**(6): 2330-8.

Krebs, L. T., J. R. Shutter, et al. (2004). "Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants." Genes Dev **18**(20): 2469-73.

Krebs, L. T., Y. Xue, et al. (2000). "Notch signaling is essential for vascular morphogenesis in mice." Genes Dev **14**(11): 1343-52.

Lawson, N. D., A. M. Vogel, et al. (2002). "sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation." Dev Cell **3**(1): 127-36.

Loges, S., C. Roncal, et al. (2009). "Development of targeted angiogenic medicine." J Thromb Haemost **7**(1): 21-33.

Mailhos, C., U. Modlich, et al. (2001). "Delta4, an endothelial specific notch ligand expressed at sites of physiological and tumor angiogenesis." Differentiation **69**(2-3): 135-44.

Risau, W. (1997). "Mechanisms of angiogenesis." Nature **386**(6626): 671-4.

Rossant, J. and M. Hirashima (2003). "Vascular development and patterning: making the right choices." Curr Opin Genet Dev **13**(4): 408-12.

Shutter, J. R., S. Scully, et al. (2000). "Dll4, a novel Notch ligand expressed in arterial endothelium." Genes Dev **14**(11): 1313-8.

Shweiki, D., A. Itin, et al. (1992). "Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis." Nature **359**(6398): 843-5.

Uyttendaele, H., G. Marazzi, et al. (1996). "Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene." Development **122**(7): 2251-9.

Wang, H. U., Z. F. Chen, et al. (1998). "Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4." Cell **93**(5): 741-53.

Xia, G., S. R. Kumar, et al. (2006). "Expression and significance of vascular endothelial growth factor receptor 2 in bladder cancer." J Urol 175(4): 1245-52.

4

Combination of Dll4/Notch and Ephrin-B2/EphB4 Targeted Therapy is Highly Effective in Disrupting Tumor Angiogenesis

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4.1. ABSTRACT

Background: Dll4/Notch and Ephrin-B2/EphB4 pathways play critical roles in tumor vessel development and maturation. This study evaluates the efficacy of the inhibition of both signaling pathways, alone and in combination, in reducing the growth of an autochthonous mouse tumor and assesses potential adverse effects.

Methods: We used the transgenic RIP1-Tag2 tumor model to study the effects of 1) inhibition of Dll4/Notch by either *Dll4* allelic deletion or use of a soluble extracellular Dll4 (sDll4), 2) inhibition of Ephrin-B2/EphB4 signaling by a soluble extracellular EphB4 fused to albumin (sEphB4-Alb), and 3) inhibition of both pathways by sEphB4-Alb combined with either *Dll4* allelic deletion or sDll4. To investigate adverse effects, we used inducible endothelial-specific *Dll4* knock-out mice, treated with sEphB4-Alb, and carried out histopathological analysis.

Results: *Dll4* allele deletion or soluble Dll4 treatment resulted in increased tumor vessel density, reduced mural cell recruitment and vessel perfusion which resulted in reduced tumor size. The soluble EphB4 instead reduced vessel density and vessel perfusion, leading to reduction of tumor size. Greater efficacy was observed when sEphB4-Alb was combined with either *Dll4* allele deletion or sDll4 in regards to tumor size, vessel perfusion and mural cell recruitment. Induced endothelial specific *Dll4* loss-of-function caused hepatic vascular alterations, which were prevented by concomitant sEphB4-Alb treatment.

Conclusion: Combination targeting of Dll4/Notch and Ephrin-B2/EphB4 has potential for clinical investigation, providing cumulative efficacy and increased safety over Dll4/Notch inhibition alone.

4.2. BACKGROUND

Targeting tumor angiogenesis, in particular through blocking vascular endothelial growth factor (VEGF) activity, has been successful, with several drugs now approved for use in many different cancer types (Loges, Roncal et al. 2009). Rapid development of resistance to these (Bergers and Hanahan 2008), however, highlights the need for other vascular targeted therapies.

Dll4/Notch pathway, which plays prominent role in angiogenesis, has become such a target. Dll4 is critical for embryonic vascular development and arterial specification and is markedly induced in murine and human tumor vessels (Mailhos, Modlich et al. 2001; Duarte, Hirashima et al. 2004; Gale, Dominguez et al. 2004; Patel, Li et al. 2005; Noguera-Troise, Daly et al. 2006; Scehnet, Jiang et al. 2007). Notch1 and Notch4 are also expressed in tumor vessels (Mailhos, Modlich et al. 2001). Notch ligand expression and Notch activation is induced by VEGF (Williams, Li et al. 2006; Lobov, Renard et al. 2007). Dll4/Notch signaling in turn attenuates VEGF signaling, thus arresting endothelial cell proliferation, followed by recruitment of mural cells and vessel maturation (Noguera-Troise, Daly et al. 2006; Ridgway, Zhang et al. 2006; Williams, Li et al. 2006; Scehnet, Jiang et al. 2007; Suchting, Freitas et al. 2007).

Not surprisingly, targeted *Dll4* allele deletion results in increased vascular proliferation but, unexpectedly, impaired vessel structure and function (Scehnet, Jiang et al. 2007). Overall, inhibition of Dll4/Notch causes reduced tumor growth. Careful evaluation of the tumor vessels reveals increased vessel proliferation, reduced lumen size, reduced mural cell recruitment, increased leakiness and reduced perfusion. Furthermore, tumors resistant to VEGF targeted therapy remain responsive to Dll4/Notch inhibitors (Noguera-Troise, Daly et al. 2006; Ridgway, Zhang et al. 2006).

Another ligand-receptor pair downstream from the VEGF and Notch pathways that plays a critical role in artery-vein endothelium specification is Ephrin-B2 and EphB4. Ephrin-B2 is specifically expressed in arterial angioblasts, endothelial cells, and perivascular mesenchymal cells, whereas EphB4 is expressed in endothelial cells belonging to the venous lineage only. Targeted disruption of either *EphB4* or *EfnB2* results in early lethality in the developing embryo as a result of arrested angiogenesis but not vasculogenesis (Adams, Wilkinson et al. 1999; Gerety, Wang et al. 1999; Gerety and Anderson 2002).

On binding, the receptor and ligand on adjacent cells undergo dimerization and clusterization, activating forward and reverse signaling in receptor-expressing and ligand-expressing cells respectively to achieve vascular maturation. The monomeric form of the extracellular domain of EphB4 functions as an antagonist of EphB4-Ephrin-B2 signaling, thus blocking endothelial cell migration, tube formation and retards angiogenesis in tumor models (Kertesz, Krasnoperov et al. 2006; Scehnet, Ley et al. 2009). Fusion of this protein with albumin at the C-terminus (sEphB4-Alb) results in favorable pharmokinetics for clinical development.

We chose to make use of the RIP1-Tag2 transgenic mouse model (Hanahan 1985), wherein pancreatic islet carcinogenesis occurs secondary to the expression of the SV-40 large T-antigen (Tag) expression under the Rat Insulin Promoter (RIP). In this model, angiogenic islets become hyperplastic and dysplastic by week 5, and acquire angiogenic switch by week 10, progressing to adenomas (insulinomas) and invasive carcinomas (Bergers, Javaherian et al. 1999). Predictable stepwise progression and angiogenic switch permits investigation of tumor angiogenesis and their inhibitors.

This study was undertaken to test the activity of a Dll4/Notch inhibitor and an Ephrin-B2/EphB4 inhibitor, each alone and in combination. We studied the effects of *Dll4* allelic deletion and of systemic administration of sDll4 on the tumor vasculature. In addition we studied the effect of sEphB4-Alb alone, in combination with sDll4 treatment or with *Dll4* allelic deletion. The results validate the efficacy of each inhibitor alone and reveal for the first time that simultaneous inhibition of both pathways has greater efficacy. Furthermore, given the concerns related to potential toxicity caused by therapeutic blockade of Dll4 signaling (Yan, Callahan et al. 2010), conditional Dll4 knockout mice were used to assess the impact of chronic endothelial specific *Dll4* loss-of-function. This was observed to cause hepatic vascular alterations, as previously reported for pharmacological inhibition of Dll4/Notch signaling (Yan, Callahan et al. 2010). Interestingly, these lesions were prevented by systemic Ephrin-B2/EphB4 inhibition, which provides an important advantage to this combination therapy.

4.3. METHODS

4.3.1. Experimental animals

All animal-involving procedures in this study were approved by the Faculty of Veterinary Medicine of Lisbon Ethics and Animal Welfare Committee. The generation of *Dll4*$^{+/-}$ (*Dll4/LacZ*) mice on CD1 background has been reported previously (Duarte, Hirashima et al. 2004). The transgenic RIP1-Tag2 (RT2) mice of CD1 and C57/BL6 backgrounds, used for breeding with the *Dll4*$^{+/-}$ line and in experimental drug trials, respectively, were provided by Dr. Oriol Casanovas. Dll4 conditional knockout mice (*Dll4*$^{lox/lox}$) were generated as previously described (Bergers and Hanahan 2008) and crossed with VE-cadherin-Cre-ERT2 mice, a kind gift from Dr. Ralph Adams, to produce a tamoxifen-inducible endothelial-specific *Dll4* loss-of-function line (*Dll4*$^{lox/lox}$ Cre+). The animals were housed in well ventilated propylene cages with sawdust as bedding, in a room with controlled temperature between 22°C and 25°C and a 12-hours-light/12-hours-dark cycle. The mice were fed with standard laboratory diet and water *ad libitum*. From 12 weeks of age, all RT2 mice received 5% sugar in their water to relieve the hypoglycemia induced by the insulin-secreting tumors.

4.3.2. Experimental design, tumor burden analyses and therapeutic trials

To study the effects of impaired Dll4/Notch signaling on RT2 insulinoma growth, RT2 *Dll4*$^{+/+}$ and RT2 *Dll4*$^{+/-}$ littermates (CD1 background, n = 8 for each group) were sacrificed for tumor measurement, histological analysis of vascular morphology and gene expression analysis at 13.5 weeks of age. The pancreas glands were dissected and the macroscopic tumors (≥1 × 1 mm) were excised. Tumor volume was calculated using the formula $V = 0.52 \times a \times b^2$ where a and b equal the longer and shorter diameter of the tumor, respectively. The volumes of all tumors from each mouse were added to give the overall tumor burden per animal.

The effect of *Dll4* allelic deletion in combination with Ephrin-B2/EphB4 signaling inhibition on the growth of the RT2 insulinoma was assessed by the administration of the soluble extracellular domain of EphB4 fused with albumin (sEphB4-Alb), which was produced as previously described (Scehnet, Ley et al. 2009). Both RT2 Dll4$^{+/+}$ and RT2

$Dll4^{+/-}$ mice (CD1 background, n = 12 for each group) were separated in equal subgroups, treated intraperitoneally (*i.p.*) with vehicle (PBS) or sEphB4-Alb (10 mg/kg) 3×/wk for 3.5 weeks beginning at the age of 10 weeks and finally sacrificed for tumor measurement and histological analysis.

In the therapeutical trials. we assessed the efficacy of a systemically administered Dll4/Notch-inhibitor, soluble Dll4 extracellular domain fused to Fc (sDll4), both alone and in combination with sEphB4-Alb. sDll4 was produced as previously described (Scehnet, Jiang et al. 2007). Vehicle (PBS, *i.p.* 3×/wk), sDll4 (10 mg/kg/day, *i.p.*, 3×/wk), sEphB4-Alb (10 mg/kg/day, *i.p.*, 3×/wk), and the combination of sDll4 (10 mg/kg/day, *i.p.*, 3×/wk) with sEphB4-Alb (10 mg/kg/day, *i.p.*, 3×/wk) treatments were started when RT2 mice (C57BL6 background) reached the age of 10 weeks and continued until mice were 13.5 week-old. Two independent experiments involved 6 animals per treatment group.

4.3.3. Longevity study

To evaluate the effect of *Dll4* allelic deletion in combination with Ephrin-B2/EphB4 signaling inhibition on longevity, the RT2 $Dll4^{+/+}$ and RT2 $Dll4^{+/-}$ mice were separated in two equal groups (n = 10 for each group), treated *i.p.* with vehicle (PBS) or sEphB4-Alb (5 mg/kg, 3×/wk), beginning at the age of 10 weeks and continuously monitored for signs of hypoglycemic shock. The mice were sacrificed if found moribund or if body weight loss exceeded 15%. Survival rate was calculated as the percentage of live mice at the end of each week relative to the initial number of animals in the experimental group.

4.3.4. Assessment of toxicity

Heart, lung, liver, brain, kidney and intestines were collected from sDll4 and sEphB4-Alb treated mice used in the therapeutical trials, fixed in 10% formalin solution for 48 h, dehydrated in alcohol, cleared in xylene, embedded in paraffin, sectioned at 10 μm and stained with hematoxylin (Fluka AG Buchs SG Switzerland) and eosin Y (Sigma Chemicals, St. Louis, MO) to study eventual histopathological alterations. To assess the side effects that might arise from total (100%) inhibition of endothelial-specific Dll4 signaling, 8 week-old $Dll4^{lox/lox}$ Cre+ (n = 10) were treated with tamoxifen (50 mg/kg

daily for 5 days) to produce endothelial-specific Dll4 null individuals, while a group of 8 week-old *Dll4*$^{lox/lox}$ Cre+ (n = 10) were left uninduced (control mice with constitutive *Dll4* expression). Ten weeks later, the mice were sacrificed and heart, lung, liver, brain, kidney and intestines were collected, processed and examined as described above. Since *Dll4* endothelial loss of function has been associated with hepatic lesions, we decided to determine the potential toxicological effect of a combination of Dll4/Notch and Ephrin-B2/EphB4 targeted therapy. Therefore another group of 8-week-old *Dll4*$^{lox/lox}$ Cre+ (n = 10) were treated with tamoxifen (50 mg/kg daily) for 5 days, subsequently divided in two equal subgroups that were injected with vehicle (PBS) or sEphB4-Alb (10 mg/kg) for ten weeks and then sacrificed. Liver samples were processed and examined as described above.

4.3.5. Immunohistochemistry

RT2 insulinomas obtained from tumor burden studies and therapeutical trials were fixed in a 4% paraformaldehyde (PFA) solution at 4°C for 1 h, cryoprotected in 15% sucrose, embedded in 7.5% gelatin, snap frozen in liquid nitrogen and cryosectioned at 10 and 20 μm. Double fluorescent immunostaining to the platelet endothelial cell adhesion molecule (PECAM) and the peri-vascular cell marker alpha smooth muscle actin (α-SMA) was performed on tissue sections to examine tumor vascular density and vessel maturity while double fluorescent immunostaining to PECAM and the pericyte marker neurogenin 2 chondroitin sulfate proteoglycan (NG2) was used to visualize pericyte recruitment. Rat monoclonal anti-mouse PECAM (BD Pharmingen, San Jose, CA), and rabbit polyclonal anti-mouse α-SMA (Abcam, Cambridge, UK) or rabbit polyclonal anti-mouse NG2 (Millipore, Billerica, MA) were used as primary antibodies. Species-specific secondary antibodies conjugated with Alexa Fluor 488 and 555 were from Invitrogen (Carlsbad, CA). Tissue sections were incubated with primary antibody overnight at 4°C and with secondary antibody for 1 hour at room temperature. Nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; Molecular Probes, Eugene, OR). Fluorescent immunostained sections were examined under a Leica DMRA2 fluorescence microscope with Leica HC PL Fluotar 10 and 20X/0.5 NA dry objective, captured using Photometrics CoolSNAP HQ, (Photometrics, Friedland, Denmark), and processed with Metamorph 4.6-5 (Molecular Devices, Sunnyvale, CA). Morphometric

analyses were performed using the NIH ImageJ 1.37 v program. Vessel density corresponds to the percentage of each tumor section field occupied by a PECAM-positive signal. As a measure of vascular maturity, vessel wall assembly was assessed by quantifying the percentage of PECAM-positive structures lined by α-SMA-positive coverage while pericyte recruitment was assessed by quantifying the percentage of PECAM-positive structures lined by NG2-positive coverage.

4.3.6. Vessel perfusion study

To mark vessel perfusion, mice were anesthetized and biotin-conjugated lectin from *Lycopersicon esculentum* (100 μg in 100 μl of PBS; Sigma, St. Luis, MO) was injected via caudal vein and allowed to circulate for 5 minutes before the vasculature was transcardially perfused with 4% PFA in PBS for 3 minutes. Tumor samples were collected and processed as described above. Endothelial cells were stained with PECAM antibody and perfused vessels were visualised by streptavidin-Alexa 488 (Invitrogen, Carlsbad, CA), which binds to biotinylated lectin. The images were obtained and processed as described above. Tumor perfusion was quantified by determining the percentage of PECAM-positive structures that were colocalized with Alexa 488 signals.

4.3.7. Global gene expression and quantitative transcriptional analysis

Tumors from sEphB4-Alb or PBS treated RT2 *Dll4*$^{+/+}$ mice were harvested at week 13.5. RNA was then isolated and used for global gene expression analysis with Illumina MouseRef-8 v2.0 Expression BeadChip (Illumina, San Diego, CA). The genearray data were deposited to NCBI-GEO database *http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE24603*. Genes with expression change between two groups higher than 2 fold and P value smaller than 0.05 were selected and the changes were validated by quantitative RT-PCR.

Using a SuperScript III FirstStrand Synthesis Supermix (Invitrogen, Carlsbad, CA), first-strand cDNA was synthesized from total RT2 *Dll4*$^{+/+}$ and RT2 *Dll4*$^{+/-}$ insulinoma RNA. Real-time PCR analysis was performed as described (Duarte, Hirashima et al. 2004) using specific primers for *β-actin, GAPDH, PECAM, Dll4, Hey2, VEGF-A, VEGFR1, VEGFR2, VEGF-C, VEGFR3, PDGF-β, Ephrin-B2, and Tie2*. Primer pair

sequences are available on request. Gene expression was normalized to *β-actin and GAPDH*.

4.3.8. Statistical analyses

Data processing was carried out using the Statistical Package for the Social Sciences version 15.0 (SPSS v. 15.0; Chicago, IL). Kaplan-Meier product-limit estimation with Breslow generalized Wilcoxon test was used for survival analyses. All other statistical analyses were performed using the Mann-Whitney-Wilcoxon test. All results are presented as mean ± SEM or mean ± SD when more appropriate. *P*-values < 0.05 and <0.01 were considered significant (indicated in the figures with *) and highly significant (indicated with **), respectively.

4.4. RESULTS

4.4.1. Targeted *Dll4* allele deletion reduces tumor growth in RT2 mice

To assess the effect of impaired signaling through the Dll4/Notch pathway in an autochthonous tumor model, we crossed RT2 transgenic mice with $Dll4^{+/-}$ mice and compared tumor burden between RT2 $Dll4^{+/+}$ and RT2 $Dll4^{+/-}$ offspring. The two groups were humanely sacrificed at 13.5 weeks of age. Average tumor numbers per mouse were similar in RT2 $Dll4^{+/+}$ and RT2 $Dll4^{+/-}$ littermates (Figure 4.1. A). In contrast, a significant decrease in tumor growth was observed in RT2 $Dll4^{+/-}$ mice when compared to the RT2 $Dll4^{+/+}$ control group. On average, RT2 $Dll4^{+/-}$ insulinoma volume and overall tumor burden, calculated as a sum of tumor volumes per mouse, were reduced by approximately 50% compared to the control animals (Figure 4.1. A).

4.4.2. Partial Dll4/Notch suppression due to *Dll4* allelic deletion promotes immature and non-functional vessel proliferation in RT2 insulinomas

We also examined the vascular morphology of tumors derived from both RT2 $Dll4^{+/+}$ and RT2 $Dll4^{+/-}$ mice at week 13.5. Tumor endothelium was visualized by immunostaining of PECAM while PECAM/α-SMA co-localization was used to evaluate mural cell recruitment and thereby vessel maturity. In addition, subgroups of RT2 $Dll4^{+/+}$ and RT2 $Dll4^{+/-}$ mice were perfused with endothelium-binding lectin to visualize the perfused regions of the tumor vasculature. The RT2 $Dll4^{+/+}$ tumors had irregular vessel formation and absence of a distinct branching pattern (Figure 4.1. B) as well as a high degree of vessel maturity with a mean α-SMA-positive cell coverage of approximately 75% (Figure 4.1. C). Furthermore, the lectin-perfusion study indicated that nearly 80% (78 ± 4%) of the vessels displayed appropriate lumen formation and were perfused (Figure 4.1. D). In comparison, RT2 $Dll4^{+/-}$ insulinoma showed a 1.8-fold increase in the density of PECAM-positive areas ($p < 0.05$) and formed atypical networks lacking vessel hierarchy or symmetry but displaying pronounced branching and multiple interconnections (Figure 4.1. B). The number of α-SMA-positive cells lining PECAM-positive endothelial cells was greatly reduced (45% reduction, $p < 0.05$, Figure 4.1.C). As anticipated, a significant reduction in the proportion of functional vessels was demonstrated by lectin-perfusion (2-fold decrease, $p < 0.05$, Figure 4.1. D).

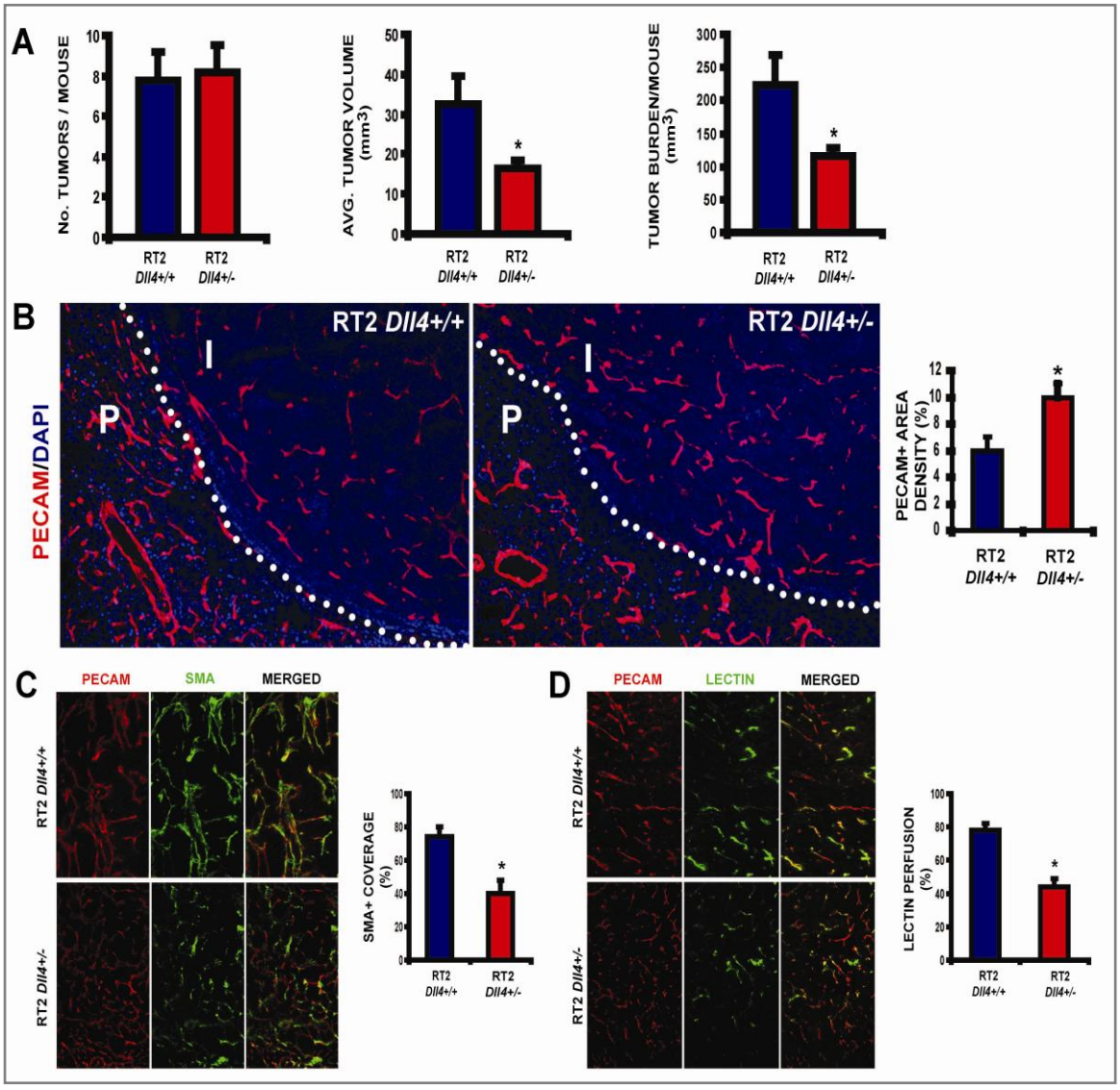

Figure 4.1. - *Dll4* allelic deletion reduced tumor burden in RT2 mice due to increased nonproductive tumor angiogenesis. *A*, Number of tumors per animal, average tumor volume and overall tumor burden, are calculated as the sum of tumor volumes per mouse, in RT2 *Dll4*$^{+/+}$ (n = 8) and RT2 *Dll4*$^{+/-}$ (n = 8) mice. *B*, Vascular response examined in RT2 *Dll4*$^{+/+}$ (n = 5) and RT2 *Dll4*$^{+/-}$ (n = 5) insulinomas by PECAM immunostaining. RT2 *Dll4*$^{+/-}$ mice showed reduced vessel calibers, increased sprouting, branching irregularities, and network disorganization (*left*). Dotted lines mark tumor border. *I* indicates insulinoma while *P* indicates normal pancreatic tissue surrounding the tumor. Vascular density was estimated as percentage of PECAM-positive area per tumor section surface and presented for two experimental groups (*right*). *C*, Mural cell coverage on newly formed vessels was examined by double staining of PECAM and α-SMA. SMA-positive cells lining PECAM-positive endothelium were profoundly reduced in RT2 *Dll4*$^{+/-}$ insulinomas (*left*). The percentage of PECAM-positive structures covered by SMA-positive area was measured as an indicator of vessel maturation (*right*). *D*, Tumor vessel competence evaluated by lectin perfusion. Simultaneous staining of endothelial cells with PECAM and visualization of perfused vessels demonstrate a reduction in the fraction of perfused vessels in RT2 *Dll4+/-* vs. RT2 *Dll4+/+* mice (*left*). The percentage of PECAM-positive area co-localized with lectin was measured to estimate the proportion of functional vessels within insulinomas (*right*). The two group values for each parameter were statistically analyzed using Mann-Whitney-Wilcoxon test. *Error bars* represent SEM. * $P <$ 0.05 was considered significant.

4.4.3. Reduced Dll4/Notch signaling in the RT2 tumor affects expression of vessel growth regulators and/or their receptors

Quantitative RT-PCR was used to analyze RT2 *Dll4*$^{+/+}$ and RT2 *Dll4*$^{+/-}$ tumors for putative differences in the expression of genes known to be involved in physiological and tumor angiogenesis (Figure 4.2.). Relative to *Dll4*$^{+/+}$ insulinomas, *Dll4*$^{+/-}$ tumors showed an approximately 50% reduction in *Dll4* mRNA levels, as expected, as well as reduced *Hey2* expression, consistent with a reduction in Notch signaling. Furthermore, there was increased *Vegf-a* expression and an over 8-fold increase in the VEGFR2/VEGFR1 ratio in the *Dll4*$^{+/-}$ tumors. Similarly, both *Vegf-c* and *Vegfr3* were up-regulated in these tumors. In addition, there was an increase in *PDGFR-β*, and decrease in *Ephrin-B2* and *Tie2 mRNA levels*. The RNA level of PECAM was not significantly increased (1.2 fold), different from 40% increase of the PECAM signal in Figure 4.2. B. One explanation is that quantitative PCR was done in whole tumor tissue and not microdissected vessels, which likely resulted in failure to document PECAM modulation in mRNA level.

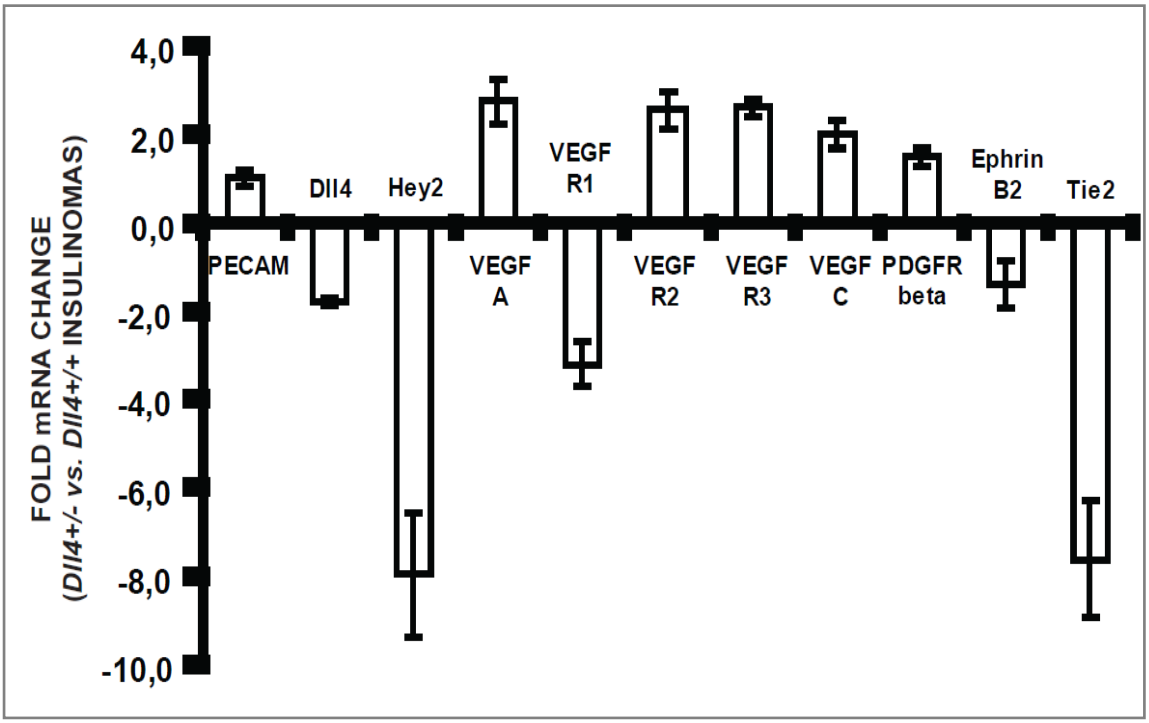

Figure 4.2. -Differential gene expression in RT2 *Dll4*$^{+/+}$ vs. RT2 *Dll4*$^{+/-}$ insulinomas. Total RNA was isolated from harvested insulinomas (2-3 tumors/mouse, n = 5 for each genotype) and gene expression analysis of tumor tissues was performed by quantitative RT-PCR for indicated genes involved in angiogenesis. Gene expression levels were normalized to *β-actin* levels. Error bars represent SD.

4.4.4. sEphB4-Alb potentiates the effects of *Dll4* allelic deletion on RT2 insulinoma growth and survival rate by further antagonizing tumor vessel maturation

sEphB4-Alb has been shown to be a potent inhibitor of tumor angiogenesis by blocking Ephrin-B2 and EphB4 signaling. To examine the effect of sEphB4-Alb in the RT2 system, RT2 mice were treated with sEphB4-Alb or PBS for 3.5 weeks beginning at 10 weeks of age. At 13.5 weeks of age, sEphB4-Alb treated RT2 mice showed reduced mean tumor volumes relative to PBS-treated control mice by approximately 50% ($p < 0.05$) (Figure 4.3. A). At this point tumors were harvested and RNA was isolated and used for global gene expression analysis. Genes with expression changes between the two groups higher than 2-fold and P value smaller than 0.05 are shown in Table 4.1 Selected genes with known role in vascular biology were evaluated by quantitative RT-PCR for validation studies. Of these genes, *Rgs5* and *Psenen* are of special interest. They are up-regulated in sEphB4-Alb treated group compared to the untreated wild type mice and their changes were validated by quantitative RT-PCR (supplementary material, page 129, Figure 4.6. A). Rgs5 is a marker of pericytes and is a negative regulator of pericyte maturation. Rgs5 was also up-regulated in umbilical artery smooth muscle cell co-cultured with human umbilical artery endothelial cell when treated with sEphB4-Alb (supplementary Figure 4.6). Psenen is a critical component of presenilin complex that is required for Notch receptor processing.

To examine whether synchronous suppression of Dll4/Notch and EphB4/Ephrin-B2 signaling pathways might result in greater inhibition of RT2 tumor development, we treated RT2 $Dll4^{+/-}$ mice with sEphB4-Alb. The reduction of the average tumor volume was even more pronounced in sEphB4-Alb treated RT2 $Dll4^{+/-}$ group (approximately 90% reduction, $p < 0.01$) where Dll4/Notch and Ephrin-B2/EphB4 signaling were simultaneously inhibited (Figure 4.3. A).

To test whether these tumor-suppressive effects are reflected on longevity, both $Dll4^{+/+}$ and $Dll4^{+/-}$ RT2 mice were treated with PBS or half dose of sEphB4-Alb (5 mg/kg) beginning at 10 weeks of age and continuously monitored for survival benefit estimation. *Dll4* allelic deletion just as sEphB4-Alb treatment were found to extend the mean lifespan of RT2 mice by 2 weeks (15 ± 1 wk in PBS treated RT2 $Dll4^{+/+}$ *versus* 17 ± 1 wk in PBS treated RT2 $Dll4^{+/-}$ and sEphB4-Alb treated RT2 $Dll4^{+/+}$ mice, $p < 0.05$

Figure 4.3. B). Significantly, an additional survival benefit of 2 weeks, on average, was provided by combinatorial Dll4/Notch and Ephrin-B2/EphB4 inhibition (15 ± 1 wk in PBS-treated RT2 *Dll4*$^{+/+}$ *versus* 19 ± 1 wk in sEphB4-treated RT2 *Dll4*$^{+/-}$, $p < 0.01$, Figure 4.3. B).

Histologicaly, microvessel density assessed by immunostaining of PECAM was significantly increased in PBS treated *Dll4*$^{+/-}$ tumors compared to PBS treated *Dll4*$^{+/+}$ insulinomas, while sEphB4-Alb treatment in RT2 *Dll4*$^{+/+}$ mice caused a statistically significant decrease of tumor vessel density (Figure 4.3. C). Notably, in both PBS treated *Dll4*$^{+/-}$ tumors and sEphB4-Alb treated RT2 *Dll4*$^{+/+}$ tumors, average vessel caliber was reduced, and pericyte recruitment and vessel wall formation were markedly impaired, as indicated by PECAM/NG2 and PECAM/α-SMA immunostaining (Figure 4.3. C and D). Furthermore, the simultaneous suppression of Dll4/Notch and Ephrin-B2/EphB4 signaling in sEphB4-Alb treated RT2 *Dll4*$^{+/-}$ mice drastically reduced the vessel diameters and their mural cell coverage, even though the total PECAM-positive area apparently similar to PBS-treated *Dll4*$^{+/+}$ insulinomas (Figure 4.3. C and D). Therefore, combinational targeting Dll4/Notch signaling EphrinB2-EphB4 has greater efficacy in blocking vessel maturation and perfusion of the tumor.

Table 4.1. – Up-regulated genes in sEphB4-Alb treated RT2 mice

Gene name	Fold Change (P value)	Gene name	Fold Change (P value)
9430028L06Rik	2.11 (0.02)	Lamp2	
Acta2	2.26 (0.05)	Lgals1	2.55 (0.02)
Akr1b8	2.26 (0.02)	Ms4a6d	2.48 (0.02)
Anpep	2.10 (0.03)	Ncf4	2.23 (0.04)
Atp6v1a	2.58 (0.05)	Ndrg4	2.10 (0.04)
BC048546	2.16 (0.01)	Nmu	2.13 (0.04)
Bgn	3.06 (0.05)	P2ry6	2.37 (0.03)
Bmp4	2.21 (0.03)	Pdia3	2.45 (0.04)
C3	2.58 (0.05)	Ppp2r4	2.21 (0.03)
C4b	2.17 (0.02)	Prelp	2.05 (0.03)
Ccdc32	2.44 (0.01)	Prph	2.48 (0.05)
Cd52	2.90 (0.04)	Psenen	2.89 (0.01)
Cfd	2.79 (0.02)	Pyy	2.18 (0.02)
Clec4n	2.30 (0.03)	Rbp1	3.44 (0.01)
Cox7a1	2.63 (0.01)	Rgs5	2.96 (0.02)
Cyba	2.46 (0.01)	Rnasek	2.66 (0.01)
D430039N05Rik	2.66 (0.04)	Samd9l	2.03 (0.03)
Dab2	2.02 (0.05)	Scara3	2.45 (0.05)
Foxp1	2.24 (0.05)	Slc15a3	2.33 (0.05)
Fus	2.42 (0.01)	Slc1a3	2.06 (0.00)
Gde1	2.20 (0.04)	St3gal6	2.46 (0.04)

Table 4.1. – Up-regulated genes in sEphB4-Alb treated RT2 mice (continuation)

Gene name	Fold Change (P value)	Gene name	Fold Change (P value)
H2-DMb1	2.21 (0.04)	Tax1bp3	3.20 (0.04)
Hoxb2	2.40 (0.04)	Usp11	2.00 (0.03)
Il3ra	2.00 (0.05)	Zcchc12	2.03 (0.04)
Ins1	3.00 (0.01)	Zfp36l1	2.06 (0.04)
Iqgap1	2.29 (0.05)		

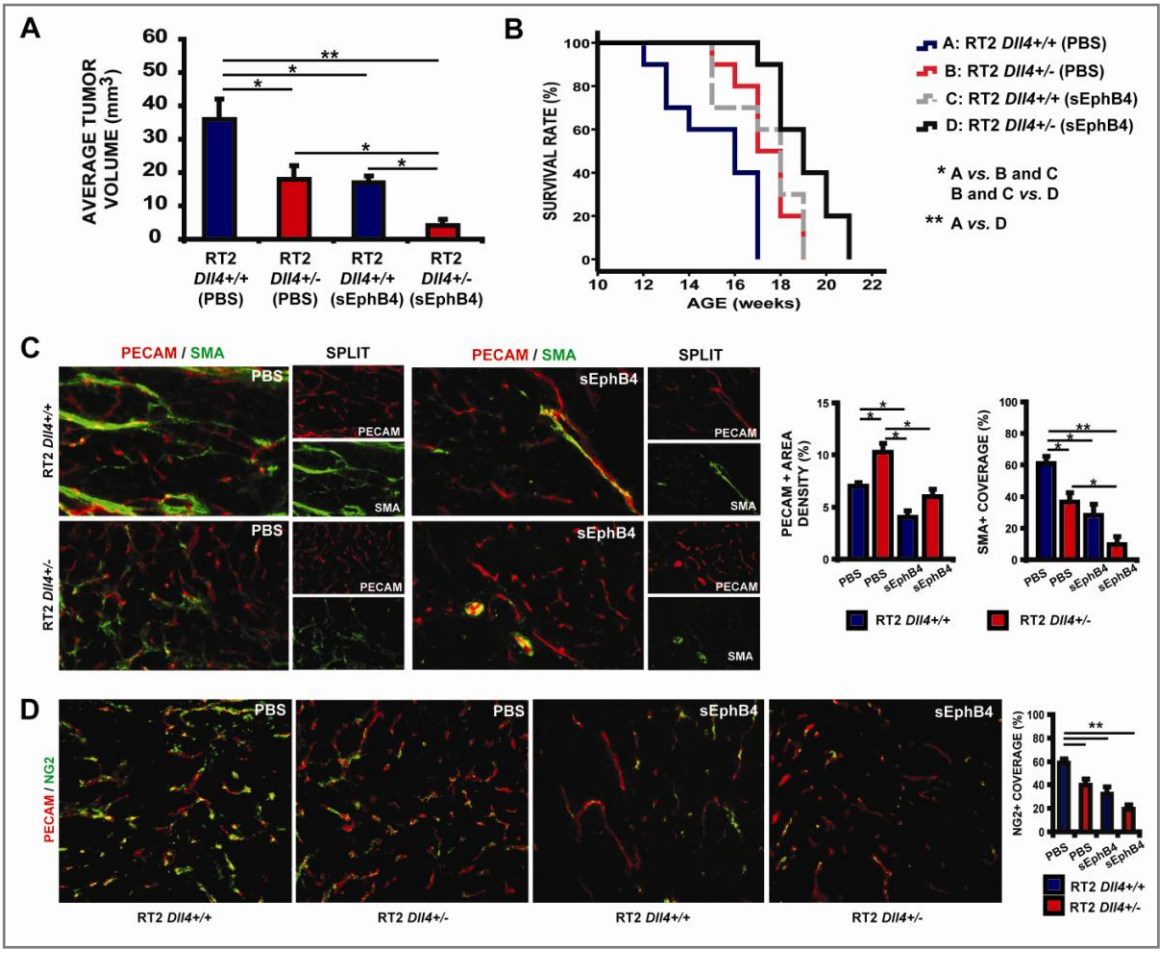

Figure 4.3. - sEphB4-Alb inhibited insulinoma growth and extended longevity in RT2 *Dll4*$^{+/+}$ and *Dll4*$^{+/-}$ mice. *A*, Average tumor volumes developed by 13.5-week old RT2 (*Dll4*$^{+/+}$ or *Dll4*$^{+/-}$) male mice previously treated for 3.5 weeks with drug vehicle (PBS) or sEphB4-Alb (10 mg/kg). All the groups had 6 animals and were treated i.p. 3 times a week. *B*, Survival rates in RT2 *Dll4*$^{+/+}$ and RT2 *Dll4*$^{+/-}$ treated with vehicle (PBS) or sEphB4-Alb (5 mg/kg). All the groups had 10 animals and were treated i.p. 3 times a week. Survival rate was calculated as the percentage of live mice at the end of each week relative to the initial number of the animals per experimental group. *C*, Sections of tumors harvested at the end of the experiment mentioned in *A* were co-stained with anti-PECAM (*red*) and anti-α-SMA (*green*) as described in Fig. 4.1*C*. *D*, Sections of tumors harvested at the end of the experiment mentioned in *A were* co-stained with anti-PECAM (*red*) and anti-NG2 (*green*). The percentage of PECAM-positive structures covered by NG2-positive area was measured as a parameter of pericyte recruitment (*right*). Kaplan-Meier product-limit estimation with Breslow generalized Wilcoxon test was used for survival analyses. Other data were analyzed using Mann-Whitney-Wilcoxon test. *Error bars* represent SEM. * $P < 0.05$ and ** $P < 0.01$ were considered significant and highly significant, respectively.

4.4.5. Both sDll4 and sEphB4-Alb inhibit RT2 tumor growth and act in a cumulative manner

sDll4 is a potent inhibitor of Notch signaling and has been shown to reduce tumor growth in various tumor models (Noguera-Troise, Daly et al. 2006; Scehnet, Jiang et al. 2007). We sought to determine the effect of combined blockade of Dll4/Notch pathway (by sDll4) and Ephrin-B2/EphB4 pathway (by sEphB4-Alb) on tumor angiogenesis. RT2 mice were treated from 10 to 13.5 weeks of age. Compared to control mice treated with the vehicle (PBS), tumor volumes were reduced in both sDll4 treated (81% reduction, $p < 0.01$) and sEphB4-Alb treated animals (60%, $p < 0.05$). As presented in Figure 4.4. A, the combination therapy caused an even more significant tumor suppression (92%, $p < 0.01$), which has significant difference when compared to the therapy with sDll4 or sEphB4-Alb alone ($p < 0.05$).

Harvested tumors were double immunostained for PECAM/α-SMA to analyze microvessel density and maturity and PECAM/lectin to estimate tumor perfusion and vessel functionality (Figure 4.4. B-D). Compared to PBS treated group, sDll4 treated tumors showed an increase in total tumor vessel density of 41% on average ($p < 0.01$), reduced mural cell coverage of 47% ($p < 0.05$), and reduced lectin perfusion of 32% ($p < 0.05$) sEphB4-Alb treatment in contrast caused reduced microvessel density (39% reduction, $p < 0.01$), pericyte recruitment (39%) and lectin perfusion (21%). Finally, the small sized tumors observed in mice treated with both sDll4 and sEphB4-Alb were characterized by decreased vessel diameters (Figure 4.4. B) and markedly impaired vessel maturation with 73% reduction in mural cell coverage compared to PBS-treated insulinomas ($p < 0.01$) (Figure 4.4. C). Meanwhile, lectin perfusion was drastically decreased (79%, $p < 0.01$) (Figure 4.4. D). These results indicate that sDll4/sEphB4-Alb combination therapy has at least a cumulative effect on minimizing tumor vessel competency and blood delivery to neoplasic cells, consequently reducing tumor growth.

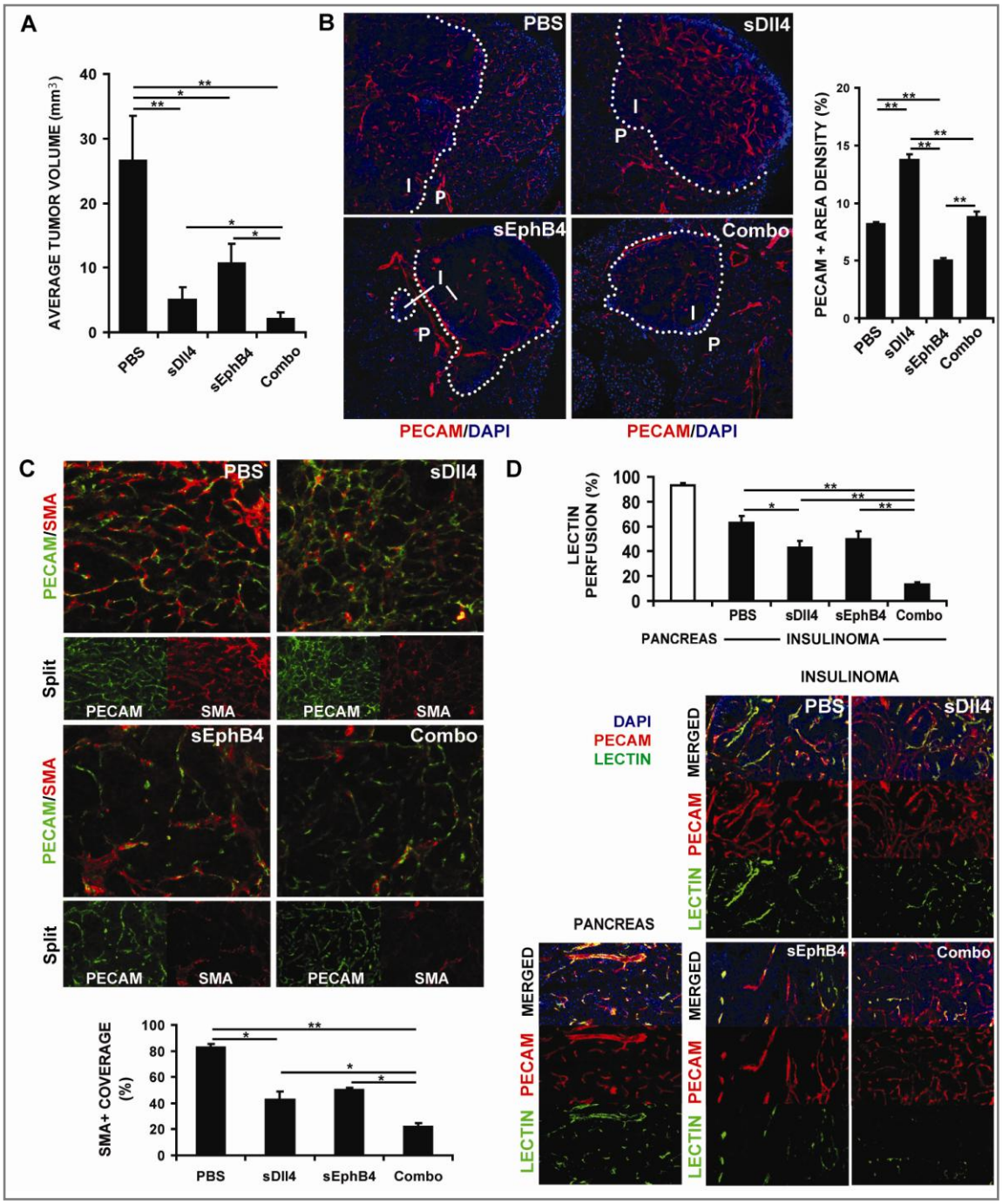

Figure 4.4.- Combination therapy of sDll4-Fc and sEphB4Alb inhibits RT2 tumor growth with greater efficacy than either molecule alone. A, Average tumor volumes developed by 13.5-week old RT2 male mice previously treated for 3.5 weeks with drug vehicle (PBS, i.p. 3×/wk, control group), sEphB4-Alb (10 mg/kg i.p., 3×/wk), sDll4-Fc (10 mg/kg i.p. 3×/wk), or combination of sEphB4-Alb and sDll4-Fc (10 mg/kg, i.p. 3×/wk for both). The results were obtained from two independent trials involved 6 animals per treatment group and per trial. B, Sections of tumors harvested at the end of the experiment mentioned in A were immunostained with PECAM (red) and DAPI (blue) as described in Fig. 4.1B. C. Sections of tumors harvested at the end of the experiment mentioned in A were stained as described in Fig. 4.1C. D, Tumor vessel functionality evaluated by lectin perfusion as described in Fig. 4.1D. Perfusion in normal pancreas tissue was also analyzed and shown on the bottom left. The data were analyzed using Mann-Whitney-Wilcoxon test. Error bars represent SEM. * $P < 0.05$ and ** $P < 0.01$ were considered significant and highly significant, respectively.

4.4.6. Endothelial Dll4/Notch inhibition results in hepatic vascular alterations that can be prevented by concomitant inhibition of Ephrin-B2/EphB4

Chronic Dll4 blockade results in benign vascular proliferative lesions in the liver (Duarte, Hirashima et al. 2004). In contrast, *Dll4* haploinsufficiency and intermittent administration of sDll4 (3 times a week for 3.5 weeks) in RT2 mice significantly reduced tumor growth, but did not cause any vascular lesions in the liver or other vital organs such as heart, brain, lung, kidney, and intestine (data not shown). To confirm if complete and persistent loss of Dll4 causes organ toxicity, we used a conditional, endothelial-specific *Dll4* knock-out mouse line (*Dll4*$^{lox/lox}$ Cre+), in which *Dll4* deletion is dependent on tamoxifen administration. Comparative histological analyses were performed with cardiac, cerebral, pulmonary, renal, intestinal and hepatic samples obtained from mice 10 weeks after tamoxifen-induction or vehicle (PBS)-administration. While control animals presented normal organ histology, the endothelial Dll4 knock-out mice showed alterations in liver architecture. Macroscopically, the liver surface of tamoxifen-induced Dll4$^{lox/lox}$ Cre+ mice had a characteristic micro-nodule. Although general lobular architecture and portal spaces were preserved, we observed some areas with markedly dilated sinusoids. However, the most prominent feature was excessive subcapsular vessel proliferation with a few hemangioma-like structures (Figure 4.5. A and B). To assess whether and to which extent these alterations can be influenced by concomitant Ephrin-B2/EphB4 inhibition, we treated tamoxifen-induced Dll4$^{lox/lox}$ Cre+ mice with PBS or sEphB4-Alb (10 mg/kg). While PBS treated mice developed previously described subcapsular vascular alterations (Figure 4.5 E), sEphB4-Alb treated animals showed a few dilated sinusoids but no evidence of vascular proliferative lesions (Figure 4.5 F).

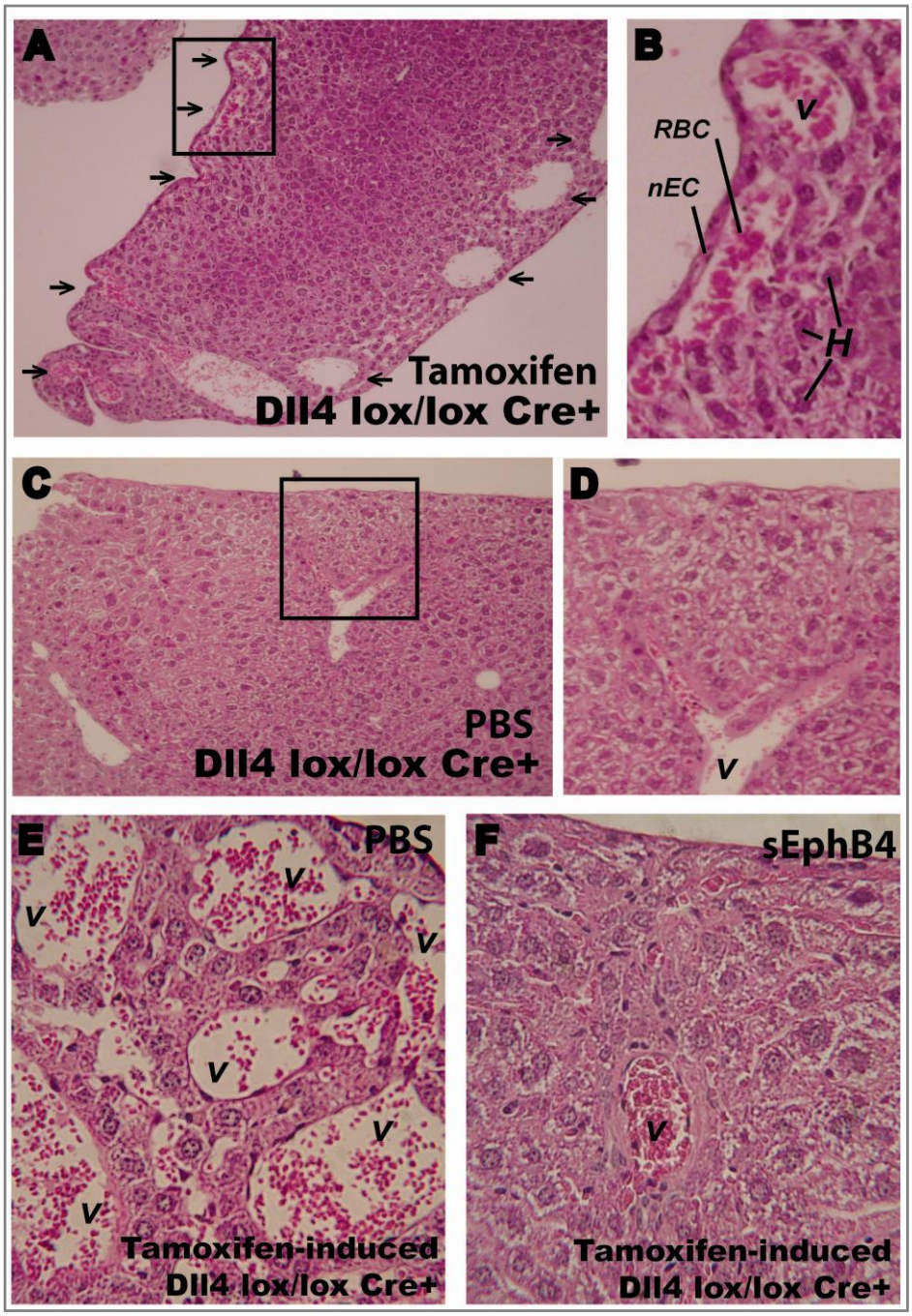

Figure 4.5 - Hepatic vascular lesions observed in endothelialspecific Dll4 knock-out mice are prevented by sEphB4-Alb administration. Representative pictures of hematoxylin and eosin staining of liver sections from induced, tamoxifen-treated (A and B) and non-induced, PBS-treated (C and D) Dll4lox/lox Cre+ mice, and from induced, tamoxifen-treated Dll4lox/lox Cre+ mice administered with PBS vehicle (E) or sEphB4-Alb (F) for 12 weeks to assess the effect of Ephrin-B2/EphB4 blockade combined with total inhibition of Dll4/Notch endothelial signaling. A, Ten weeks after the induction of endothelium-specific Dll4 loss-of-function, all studied 14-week-old Dll4lox/lox Cre+ presented excessive sub-capsular vascular proliferation. The image shows vessels (arrows), some of which extremely dilated, occupying sub-capsular regions throughout the liver. B, Higher magnification of sub-capsular blood vessels (inset in A); C, Age-matched non-induced Dll4lox/lox Cre+ presented normal liver histology. D, Higher magnification of the inset in C showing a single hepatic vessel that cannot be considered sub-capsular. E and F, Excessive vascular proliferation forming an hemangioma-like subcapsular structure in a tamoxifen-induced Dll4lox/lox Cre+ mouse injected with PBS and normal hepatic structure in a sEphB4-treated tamoxifen-induced Dll4lox/lox Cre+ mouse, respectively. V, blood vessel; nEC, endothelial cell nucleus, RBC, red blood cells; H, hepatocytes.

4.5. DISCUSSION

Dll4 allelic deletion or sDll4 treatment reduces the rate of tumor growth in this spontaneous model of insulinoma in the pancreas, while the number of tumors per mouse were unchanged, indicating that the transforming event caused by SV40 large T antigen under insulin promoter is independent on Dll4/Notch pathway, while the tumor growth is promoted by the expression of Dll4 in the vasculature. Similarly, sEphB4-Alb, which blocks Ephrin-B2 mediated forward signaling in venous endothelial cells through its cognate receptors, in particular EphB4, and reverse signaling in the arterial endothelial cells expressing Ephrin-B2, reduces the rate of tumor growth without affecting the number of oncogenic tumor nodules in the pancreas. Combination of sEphB4-Alb and sDll4 had a greater tumor inhibition efficacy than each one alone. This novel finding indicates that the activity of both pathways is non-overlapping even though Dll4/Notch signaling induces Ephrin-B2 (Hainaud, Contreres et al. 2006; Iso, Maeno et al. 2006; Yamanda, Ebihara et al. 2009). The lack of overlapping effects is consistent with the fact that Dll4 targeted therapy has a markedly increased number of tumor vessels while sEphB4-Alb therapy results in significantly reduced tumor vessel density. Inhibition of Dll4-Notch signaling leads to induction of VEGF, probably through hypoxia. Meanwhile, there is also an increase in VEGFR2 level, resulting in a positive loop leading to enhanced VEGF signaling and thus increased vascular density. In addition, VEGFR3 is induced in insulinomas of $Dll4^{+/-}$ RT2 mice compared to the $Dll4^{+/+}$ RT2 littermates. VEGFR3 expression in nascent vessels in addition to lymphatic endothelium may thus also have contributed to the enhanced vascular response. sEphB4-Alb mediated inhibition of Ephrin-B2/EphB4 signaling also results in areas of hypoxia in the tumor and increased expression of VEGF and Dll4 (Scehnet, Ley et al. 2009), which support the combined use of Ephrin-B2, VEGF, and Dll4 inhibitors. However, sEphB4-Alb treatment leads to fewer tumor blood vessels, suggesting that inhibition of Ephrin-B2/EphB4 pathway may reduce VEGF signaling even though VEGF level is increased. This can be explained by the recent findings that Ephrin-B2 controls internalization and signaling of VEGFR2 and VEGFR3 (Sawamiphak, Seidel et al. 2010; Wang, Nakayama et al. 2010). VEGF receptor expression and internalization (trafficking), rather than the VEGF level, determine the net endothelial response. Concomitant inhibition of these two signaling systems results in vessel density below the controls, suggesting that reduction of VEGF signaling by Ephrin-B2/EphB4 inhibition partially overcomes the VEGF/VEGFR2 activation resulting

from Dll4/Notch blockade. More importantly greater anti-tumor efficacy of combined therapy may result from markedly reduced vessel perfusion and maturation.

Interestingly, sEphB4-Alb treatment induces the expression of PSENEN, a critical component of gamma-secretase presenilin complex (Iwatsubo 2004). Presenilin complex is responsible for the cleavage of Notch receptor to release Notch intracellular domain, thus is required for activation of Notch signaling (Selkoe and Kopan 2003). Significance of PSENEN modulation in response to sEphB4-Alb is not known at present.

In *Dll4*$^{+/-}$ mice or sDll4 treated mice, the defects in vessel maturation may be in part be explained by the impaired Notch3 signaling, which is critical for mural cell function (Domenga, Fardoux et al. 2004). Moreover, one critical step of vessel maturation, the recruitment of mural cells to the newly forming vessels, is known to be regulated by Ephrin-B2, PDGFRβ/PDGF-B, Tie-2, and Sphingosine-1 phosphate (Shaheen, Tseng et al. 2001; Abramsson, Lindblom et al. 2003; Bergers, Song et al. 2003; Armulik, Abramsson et al. 2005). We thus examined these factors after Dll4/Notch inhibition. Surprisingly, increased PDGFR-β levels were identified in RT2 *Dll4*$^{+/-}$ vs. RT2 *Dll4*$^{+/+}$ insulinomas even though there was a prominent reduction in mural cell recruitment to the vessels. We believe that defective recruitment results in a feedback increase in PDGFR-β. Angiopoietin receptor Tie2 (Sato, Tozawa et al. 1995; Peters, Kontos et al. 2004) as expected was decreased in Dll4$^{+/-}$ compared to Dll4$^{+/+}$ insulinomas. The impaired mural cell recruitment upon Dll4/Notch inhibition may also be explained by the reduced Ephrin-B2 level. Ephrin-B2 is critical for the recruitment of mural cells to nascent vessels and its absence leads to vessel dilation and bleeding (Adams, Wilkinson et al. 1999; Foo, Turner et al. 2006). In this study, sEphB4-Alb significantly impaired the recruitment of mural cells to blood vessels. The global gene expression analysis identified Rgs5 is up-regulated upon sEphB4-Alb treatment both in vivo and in co-cultured smooth muscle cells and endothelial cells. Rgs5 is a pericyte specific marker. Knockout of Rgs5 in mice promotes the maturation of pericytes and normalization of tumor vessels (Hamzah, Jugold et al. 2008). Rgs5 is also a novel HIF-1-dependent, hypoxia-induced gene that is involved in the induction of endothelial apoptosis (Jin, An et al. 2009). Therefore, up-regulation of Rgs5 is consistent with the decreased vascular density and paucity of pericyte recruitment after sEphB4-Alb treatment which results in immature and non-functional vessels.

Finally, in addition to improved anti-tumor efficacy, simultaneous blockade of Dll4/Notch and Ephrin-B2/EphB4 abolished the liver vascular lesions seen when only

Dll4/Notch is inhibited (Yan, Callahan et al. 2010). sEphB4-Alb blocks this toxicity, consistent with the reduced vascular response upon Ephrin-B2/EphB4 inhibition. Thus, Ephrin-B2/EphB4 blockade combined with Dll4/Notch inhibition provides greater safety and improved efficacy.

4.6. CONCLUSION

In summary, targeting of Dll4/Notch and Ephrin-B2/EphB4 in combination showed marked improvement in tumor growth inhibition. This is primarily through modulation of vascular response in which impaired vessel maturation leads to poor perfusion. Complementary to improved efficacy, combination of sEphB4-Alb with Dll4/Notch inhibition appears to prevent excessive vascular proliferation in the liver, which is seen in extended Dll4/Notch blockade alone. Therefore, this combination is a potential candidate for cancer therapy.

SUPPLEMENTARY MATERIAL:

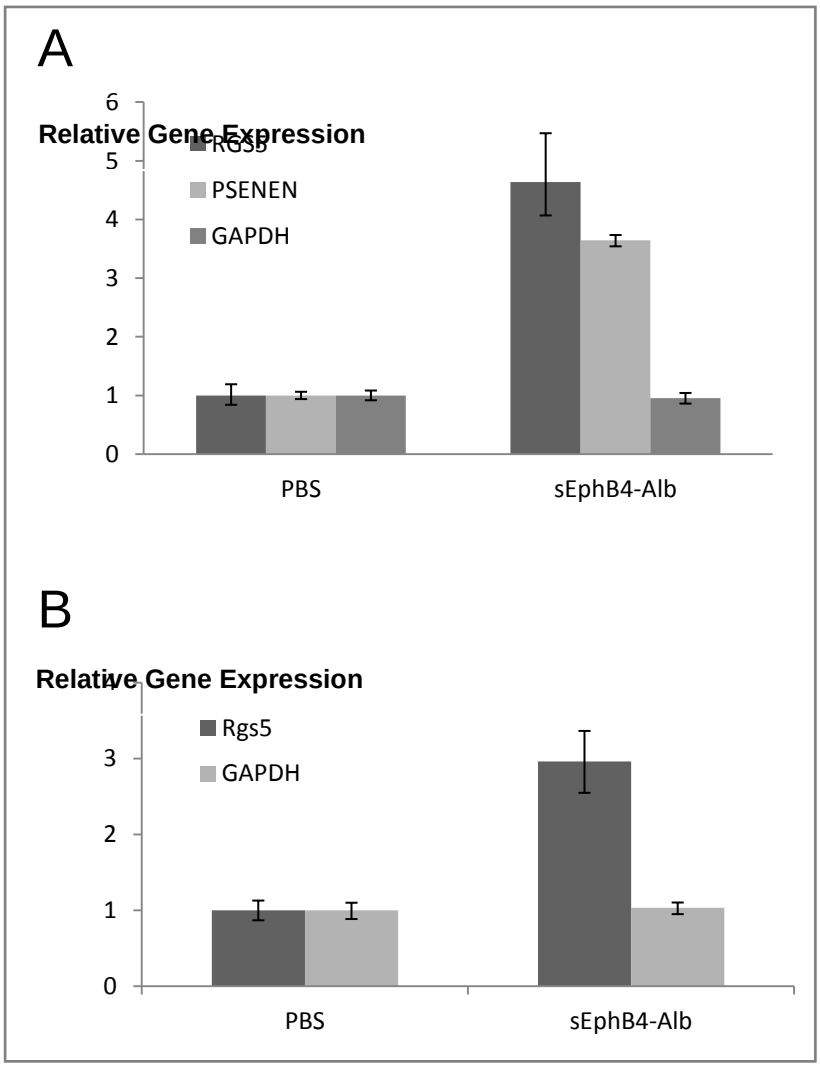

Figure 4.6. – Up-regulation of Rgs5 and PSENEN by sEphB4-Alb. *A.* Total RNA used for global gene analysis (Table 4.1) was used to validate up-regulation of Rgs5 and PSENEN. Gene expression level was normalized to β-actin. Another housekeeping gene GAPDH was also included in this analysis. *B.* Human umbilical artery endothelial cells and smooth muscle cells (both from Lonza) were co-cultured (1:1) for overnight, followed by treatment with sEphB4-Alb (10 ug/ml) for 6 hours. Cells were harvested and total RNA was isolated for quantitative RT-PCR. Gene expression level was normalized to β-actin. Another housekeeping gene GAPDH was also included in this analysis.

REFERENCES:

Abramsson, A., P. Lindblom, et al. (2003). "Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors." J Clin Invest **112**(8): 1142-51.

Adams, R. H., G. A. Wilkinson, et al. (1999). "Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis." Genes Dev **13**(3): 295-306.

Armulik, A., A. Abramsson, et al. (2005). "Endothelial/pericyte interactions." Circ Res **97**(6): 512-23.

Bergers, G. and D. Hanahan (2008). "Modes of resistance to anti-angiogenic therapy." Nat Rev Cancer **8**(8): 592-603.

Bergers, G., K. Javaherian, et al. (1999). "Effects of angiogenesis inhibitors on multistage carcinogenesis in mice." Science **284**(5415): 808-12.

Bergers, G., S. Song, et al. (2003). "Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors." J Clin Invest **111**(9): 1287-95.

Domenga, V., P. Fardoux, et al. (2004). "Notch3 is required for arterial identity and maturation of vascular smooth muscle cells." Genes Dev **18**(22): 2730-5.

Duarte, A., M. Hirashima, et al. (2004). "Dosage-sensitive requirement for mouse Dll4 in artery development." Genes Dev **18**(20): 2474-8.

Foo, S. S., C. J. Turner, et al. (2006). "Ephrin-B2 controls cell motility and adhesion during blood-vessel-wall assembly." Cell **124**(1): 161-73.

Gale, N. W., M. G. Dominguez, et al. (2004). "Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development." Proc Natl Acad Sci U S A **101**(45): 15949-54.

Gerety, S. S. and D. J. Anderson (2002). "Cardiovascular ephrinB2 function is essential for embryonic angiogenesis." Development **129**(6): 1397-410.

Gerety, S. S., H. U. Wang, et al. (1999). "Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development." Mol Cell **4**(3): 403-14.

Hainaud, P., J. O. Contreres, et al. (2006). "The role of the vascular endothelial growth factor-Delta-like 4 ligand/Notch4-ephrin B2 cascade in tumor vessel remodeling and endothelial cell functions." Cancer Res **66**(17): 8501-10.

Hamzah, J., M. Jugold, et al. (2008). "Vascular normalization in Rgs5-deficient tumours promotes immune destruction." Nature **453**(7193): 410-4.

Hanahan, D. (1985). "Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes." Nature **315**(6015): 115-22.

Iso, T., T. Maeno, et al. (2006). "Dll4-selective Notch signaling induces ephrinB2 gene expression in endothelial cells." Biochem Biophys Res Commun **341**(3): 708-14.

Iwatsubo, T. (2004). "The gamma-secretase complex: machinery for intramembrane proteolysis." Curr Opin Neurobiol **14**(3): 379-83.

Jin, Y., X. An, et al. (2009). "RGS5, a hypoxia-inducible apoptotic stimulator in endothelial cells." J Biol Chem **284**(35): 23436-43.

Kertesz, N., V. Krasnoperov, et al. (2006). "The soluble extracellular domain of EphB4 (sEphB4) antagonizes EphB4-EphrinB2 interaction, modulates angiogenesis, and inhibits tumor growth." Blood **107**(6): 2330-8.

Lobov, I. B., R. A. Renard, et al. (2007). "Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting." Proc Natl Acad Sci U S A **104**(9): 3219-24.

Loges, S., C. Roncal, et al. (2009). "Development of targeted angiogenic medicine." J Thromb Haemost **7**(1): 21-33.

Mailhos, C., U. Modlich, et al. (2001). "Delta4, an endothelial specific notch ligand expressed at sites of physiological and tumor angiogenesis." Differentiation **69**(2-3): 135-44.

Noguera-Troise, I., C. Daly, et al. (2006). "Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis." Nature **444**(7122): 1032-7.

Patel, N. S., J. L. Li, et al. (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**(19): 8690-7.

Peters, K. G., C. D. Kontos, et al. (2004). "Functional significance of Tie2 signaling in the adult vasculature." Recent Prog Horm Res **59**: 51-71.

Ridgway, J., G. Zhang, et al. (2006). "Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis." Nature **444**(7122): 1083-7.

Sato, T. N., Y. Tozawa, et al. (1995). "Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation." Nature **376**(6535): 70-4.

Sawamiphak, S., S. Seidel, et al. (2010). "Ephrin-B2 regulates VEGFR2 function in developmental and tumour angiogenesis." Nature **465**(7297): 487-91.

Scehnet, J. S., W. Jiang, et al. (2007). "Inhibition of Dll4-mediated signaling induces proliferation of immature vessels and results in poor tissue perfusion." Blood **109**(11): 4753-60.

Scehnet, J. S., E. J. Ley, et al. (2009). "The role of Ephs, Ephrins, and growth factors in Kaposi sarcoma and implications of EphrinB2 blockade." Blood **113**(1): 254-63.

Selkoe, D. and R. Kopan (2003). "Notch and Presenilin: regulated intramembrane proteolysis links development and degeneration." Annu Rev Neurosci **26**: 565-97.

Shaheen, R. M., W. W. Tseng, et al. (2001). "Tyrosine kinase inhibition of multiple angiogenic growth factor receptors improves survival in mice bearing colon cancer liver metastases by inhibition of endothelial cell survival mechanisms." Cancer Res **61**(4): 1464-8.

Suchting, S., C. Freitas, et al. (2007). "The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching." Proc Natl Acad Sci U S A **104**(9): 3225-30.

Wang, Y., M. Nakayama, et al. (2010). "Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis." Nature **465**(7297): 483-6.

Williams, C. K., J. L. Li, et al. (2006). "Up-regulation of the Notch ligand Delta-like 4 inhibits VEGF-induced endothelial cell function." Blood **107**(3): 931-9.

Yamanda, S., S. Ebihara, et al. (2009). "Role of ephrinB2 in nonproductive angiogenesis induced by Delta-like 4 blockade." Blood **113**(15): 3631-9.

Yan, M., C. A. Callahan, et al. (2010). "Chronic DLL4 blockade induces vascular neoplasms." Nature **463**(7282): E6-7.

5

Dll4 Allelic Deletion Promotes Productive Angiogenesis and Tumor Growth in Chemically-induced Mouse Skin Papillomas

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5.1. ABSTRACT

Dll4/Notch signaling is an important regulator of angiogenesis. In various invasive malignancies, the inhibition of this pathway has been shown to modify the tumor-induced vascular response enhancing non-functional vessel proliferation and limiting tumor growth. This study focused on the role of Dll4/Notch in early tumors and tested the effects of targeted *Dll4* allele deletion on chemical, DMBA/TPA-mediated skin tumorigenesis in mice. In contrast to late tumors, we observed that *Dll4* down-regulation promotes productive, although less mature, angiogenesis in pre-cancerous skin papillomas and consequently stimulates their growth. The increase in endothelial activation was found to be associated with an increase in the VEGFR2 to VEGFR1 ratio in $Dll4^{+/-}$ *vs.* wild-type papilloma-bearing mice while retarded vessel maturation, indicated by reduced mural cell recruitment, was associated to *Tie2* and *EphrinB2* suppression. Importantly, this change in VEGF signaling due to haploid *Dll4* deletion also explains its neutralizing effect on VEGFR-targeting sorafenib that occurred in chemical papillomas. The distinguishing capability of neo-vessels to gain functionality under the condition of partial Dll4/Notch inhibition, seen in early but not in invasive lesions, can be causally related to lower background VEGF levels and thus increased VEGF gradients in early *vs.* advanced tumors that allow the formation of a vascular network even in the presence of a 50% Dll4/Notch suppression. Collectively, Dll4 plays a prominent and protective role in early tumors, being fundamental to suppress VEGF-induced angiogenesis and less critical, in comparison with late tumors, to prevent aberrant vascular development.

5.2. INTRODUCTION

Delta-like 4 (Dll4)-mediated Notch signaling critically influences blood vessel formation in both physiological and pathological settings. During embryonic development, the signaling is absolutely required for normal arterial specification (Duarte, Hirashima et al. 2004). Besides, the Dll4/Notch pathway fundamentally participates in the regulation of embryonic (Duarte, Hirashima et al. 2004; Krebs, Shutter et al. 2004), post-natal developmental (Claxton and Fruttiger 2004; Hellstrom, Phng et al. 2007; Lobov, Renard et al. 2007; Suchting, Freitas et al. 2007) and tumor sprouting angiogenesis (Noguera-Troise, Daly et al. 2006; Ridgway, Zhang et al. 2006; Li, Sainson et al. 2007; Scehnet, Jiang et al. 2007; Djokovic, Trindade et al. 2010; Haller, Brave et al. 2011).

In the vasculature, Dll4 expression is restricted in the endothelial cells (ECs) and up-regulated by vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF) and hypoxia (Li, Sainson et al. 2007; Kuhnert, Kirshner et al. 2011). The ligand binds Notch 1 and Notch 4 receptors of adjacent ECs, triggering γ-secretase proteolytic cleavage of Notch, which cytoplasmic domain subsequently translocates to the nucleus forming a complex with the RPB-Jk transcription factor and determines EC fate by activating target genes such as the members of the *Hes* and *Hey* families of basic helix-loop-helix transcription factors and *EphrinB2* (Iso, Kedes et al. 2003; Iso, Maeno et al. 2006). In sprouting angiogenesis, Dll4/Notch mediates communication between migratory ECs that lead the sprout formation and adjacent ECs that, under the Dll4/Notch control, remain in the quiescent state in pre-existing vasculature or rather proliferate then migrate, forming the trunk of the new vessel (Hellstrom, Phng et al. 2007; Lobov, Renard et al. 2007; Suchting, Freitas et al. 2007). Mechanistically, Dll4/Notch enables the selective EC departure from pre-existing activated endothelium and organized sprout outgrowth by decreasing the VEGFR2/VEGFR1 ratio and reducing the sensitivity of signal-receiving ECs to VEGF that suppresses their invasive phenotype.

In various malignant cell lines grafted in mice, the inhibition of Dll4/Notch signaling has been demonstrated to cause excessive endothelial activation, sprouting and branching, impaired vessel wall assembly and reduced lumina, leading to increasingly aberrant vascular network formation, hypoxia and tumor growth suppression (Ridgway, Zhang et al. 2006; Noguera-Troise, Daly et al. 2007; Scehnet, Jiang et al. 2007).

Additionally, we have previously obtained similar results in transgenic RIP1-*Tag2* model, wherein both *Dll4* allelic deletion and pharmacological Dll4/Notch blockade have been evidenced to inhibit the growth of autochthonous insulinomas due to exaggerated angiogenic formation of non-functional tumor vessels (Djokovic, Trindade et al. 2010). Although these findings indicate that the Dll4/Notch blockade may provide an effective way to improve cancer control, the normalization capacity of the vascular aberrations due to Dll4/Notch inhibition remains undetermined. Moreover, a recent study has suggested that the therapeutic inhibition of Dll4 signaling may face important safety limitations since chronic Dll4/Notch impairment is capable of destabilizing normal endothelium giving origin to the formation of benign vascular tumors (Djokovic, Trindade et al. 2010; Li, Jubb et al. 2010; Yan, Callahan et al. 2010).

Simultaneously, despite the wealth of information regarding the effects of Dll4/Notch inhibition in invasive neoplasms, little is known regarding its role in benign and early, pre-cancerous lesions. Thus, the present study was undertaken to assess the effects of targeted *Dll4* allelic deletion in the incipient stages of tumor pathogenesis. For this purpose, we chose to make use of the classic 7,12-dimethylbenz[a]anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin carcinogenesis model wherein the initiating carcinogen, DMBA, results in an activating mutation in the *H-ras* gene and generation of "initiated" epidermal cells (Demehri, Turkoz et al. 2009). Subsequent and continuous exposure of the skin to a tumor promoting agent, TPA, leads to expansion of the mutant cells and the formation of highly angiogenic squamous papillomas that, in the following stage, develop to squamous cell carcinoma (SSC).

Such as in invasive malignancies, our results show that loss of *Dll4* function in skin papillomas promotes VEGF-induced EC activation, excessive vascular network formation, and reduces mural cell recruitment. However, in these pre-malignant lesions, allelic *Dll4* deletion is associated with quite preserved vessel function. Contradicting previous data regarding the Dll4/Notch role in late tumors, we thus demonstrate that the constitutive signaling is protective in early papillomas, while the Dll4/Notch partial inhibition, in the context of relatively modest VEGF background levels (Larcher, Robles et al. 1996), enhances functional angiogenesis and tumor growth. We correlate the discrepant effects of *Dll4* loss-of-function in early *vs.* late tumors with different VEGF levels that highly increase during the tumor progression, reinforcing the need for Dll4/Notch to maintain a normal vascular organization and function.

5.3. MATERIALS AND METHODS

5.3.1. Mice

Used throughout the study, the CD1 wild-type (WT) and heterozygous *Dll4*$^{+/-}$ mice, previously generated as described (Duarte, Hirashima et al. 2004), were housed in well ventilated propylene cages with sawdust as bedding, in a room with temperature between 22ºC and 25ºC and a 12-hours-light/12-hours-dark cycle. The mice were fed standard laboratory diet and drinking water *ad libitum*. All animal-involving procedures were approved by the Faculty of Veterinary Medicine of Lisbon Ethics and Animal Welfare Committee (Approval ID: PTDC/CVT71084/2006).

5.3.2. Chemically-induced skin tumorigenesis

Male, 8-week old WT and *Dll4*$^{+/-}$ littermates (n=12 for each group) were treated with a single dose of 25µg of 7,12-dimethylbenz[a]anthracene (DMBA; Sigma, St. Luis, MO) in 200µL acetone per mouse applied to shaved dorsal skin. Beginning a week after DMBA-induction, tumor onset and growth was promoted by treating mice twice a week for 19 weeks with 4µg of 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma, St. Luis, MO) in 100 µL of dimethyl sulfoxide (DMSO) per mouse. The appearance of skin lesion was monitored and recorded weekly. Mouse weight and tumor sizes (diameters) were periodically measured and lesion diameters were converted to tumor volume using the following formula: V= length x width x height x 0.52. Tumor burden of each individual mouse was calculated as the sum of its tumor volumes. Twenty weeks after the DMBA-initiation, mice were anesthetized with i.p. 2.5% tribromoethanol (Sigma-Aldrich, St. Louis, MO) and total blood was collected by axillary bleeding from six animals of each genotype for the determination of VEGF, cleaved VEGFR1 and cleaved VEGFR2 concentrations. The remaining WT and *Dll4*$^{+/-}$ mice (n=6 for each genotype) were perfused with biotin-conjugated lectin (Sigma, St. Luis, MO), as described below, for the assessment of tumor vessel functionality. The skin tumors were then dissected from all WT and *Dll4*$^{+/-}$ mice and processed for histological or molecular analyses.

5.3.3. Tumor tissue preparation, histopathology and immunohistochemistry

Skin tumor samples were fixed in 4% paraformaldehyde (PFA) solution at 4ºC for 1h, cryoprotected in 15% sucrose, embedded in 7,5% gelatine, frozen in liquid nitrogen and cryosectioned at 20µm. Skin lesion sections were stained with hematoxylin (Fluka AG Buchs SG Switzerland) and eosin Y (Sigma Chemicals, St. Louis, MO) and subjected to review by a pathologist. Simultaneously, double fluorescent immunostaining to platelet endothelial cell adhesion molecule (PECAM) and pericyte marker alpha smooth muscle actin (α-SMA) was performed on tumor tissue sections to examine tumour vascular density and vessel maturity. Rat monoclonal anti-mouse PECAM (BD Pharmingen, San Jose, CA) and rabbit polyclonal anti-mouse α-SMA (Abcam, Cambridge, UK) were used as primary antibodies and appropriate species-specific antibodies conjugated with Alexa Fluor 488 and 555 (Invitrogen, Carlsbad, CA) were engaged as secondary antibodies. We incubated tissue sections with primary antibody overnight at 4°C and, subsequently, with secondary antibody for 1 hour at room temperature. Nuclei were counterstained with 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; Molecular Probes, Eugene, OR). Fluorescent immunostained sections were examined under a Leica DMRA2 fluorescence microscope with Leica HC PL Fluotar 10 and 20X/0.5 NA dry objective, captured using Photometrics CoolSNAP HQ, (Photometrics, Friedland, Denmark), and processed with Metamorph 4.6-5 (Molecular Devices, Sunnyvale, CA). Morphometric analyses were performed using the NIH ImageJ 1.37v program. To estimate vessel density, we measured the percentage of tumor stroma surface occupied by a PECAM-positive signal. , Mural cell recruitment was assessed as a measure of vascular maturity by quantitating the percentage of PECAM-positive structures lined by α-SMA-positive coverage.

5.3.4. Vessel perfusion study

To visualize the blood-perfused, *i.e.* functional, portion of tumor circulation, the subgroups of WT and *Dll4*$^{+/-}$ mice (n=6 for each group) were anesthetized as described above and biotin-conjugated lectin from *Lycopersicon esculentum* (100µg in 100µl of PBS; Sigma, St. Luis, MO) was injected via caudal vein and allowed to circulate for 5 minutes before we perfused animal vasculature transcardially with 4% PFA in PBS for 3 minutes. Tumor samples were collected and processed as presented above. Tissue

sections (20μm) were stained with rat monoclonal anti-mouse PECAM antibody (BD Pharmingen, San Jose, CA), followed by Alexa 555 goat anti-rat IgG (Invitrogen, Carlsbad, CA). Biotinylated lectin was visualized with Strepatavidin-Alexa 488 (Invitrogen, Carlsbad, CA). The images were obtained and processed as described above. Tumor perfusion was quantified by determining the percentage of PECAM-positive structures that were co-localized with Alexa 488 signals corresponding to lectin-perfused vessels.

5.3.5. Serum VEGF, VEGFR1 and VEGFR2 measurement

Total blood was collected by axillary bleeding from WT and *Dll4*$^{+/-}$ mice (n=6 for each group) as described above. To obtain serum samples, blood was allowed to clot during 45 min. at 37ºC and then centrifuged during 10 min. at 1000 X g. VEGF, VEGFR1 and VEGFR2 serum levels were measured by enzyme-linked immunosorbent assay (ELISA; R&D Systems), as described (Williams, Li et al. 2006).

5.3.6. Quantitative transcriptional analysis

Using a SuperScript III FirstStrand Synthesis Supermix qRTPCR (Invitrogen, Carlsbad, CA), first-strand cDNA was synthesized from total RNA previously isolated with RNeasy Mini Kit *(*Qiagen, Valencia, CA) from skin tumors developed by WT and *Dll4*$^{+/-}$ mice (n=10 tumors for each genotype). Real-time PCR analysis was performed as described (Trindade, Kumar et al. 2008) using specific primers for *β-actin, Dll4, Hey2, PDGF-β, EphrinB2* and *Tie2*. Primer pair sequences are available on request. Gene expression was normalized to β-*actin*.

5.3.7. Sorafenib assay

For the evaluation of combined effect of *Dll4* allelic deletion and sorafenib, 8-week old WT and *Dll4*$^{+/-}$ male mice were separated in two equal sub-groups for each genotype (n=4 for each of four experimental sub-group) and chemical skin tumorigenesis was induced and promoted as described above. Sorafenib was formulated twice a week at 4-fold (4X) of the used dose in a Cremophor EL (Sigma, St. Luis, MO)/ethanol 50:50

solution. The dosing solutions were prepared on the day of use by dilution to 1X with Cremophor EL/ethanol/water mixture (12.5:12.5:75). Beginning 13 weeks after DMBA-induction, we treated a sub-group of WT and a sub-group of *Dll4*$^{+/-}$ mice by oral gavage for 21 days with sorafenib (40mg/kg/day) while the mice from two remaining WT and *Dll4*$^{+/-}$ sub-groups received p.o. vehicle (Cremophor EL/ethanol/water 12.5:12.5:75 mixture). Prior and during the treatments, the weight of all mice and their skin tumors were measured once per week as well as at the experiment end-point.

5.3.8. Statistical analyses

Data processing was carried out using the Statistical Package for the Social Sciences version 15.0 software (SPSS v. 15.0; Chicago, IL). Statistical analyses were performed using Mann-Whitney-Wilcoxon test. All results are presented as mean ± SEM. *P*-values < 0.05 and <0.01 were considered significant (indicated in the figures with *) and highly significant (indicated with **), respectively.

5.4. RESULTS

5.4.1. *Dll4* allelic deletion promotes the growth of chemically-induced skin papillomas

To study the Dll4/Notch function in chemical skin tumorigenesis and evaluate the impact of its suppression, we monitored skin lesion formation and evolution in DMBA/TPA-treated wild-type (WT) mice and their *Dll4*$^{+/-}$ littermates during 20 weeks after the tumor DMBA-induction. As presented in Figure 5.1. A, *Dll4*$^{+/-}$ mice started developing skin tumors as early as week 6 after DMBA-initiation and by week 10.5 of the study, 50% of *Dll4*$^{+/-}$ mice developed at least one lesion (tumor latency). Tumor onset and latency were delayed in WT animals for two and one week, respectively. Regarding tumor multiplicity, increased number of lesions per mouse was observed in *Dll4*$^{+/-}$ mice compared to WT controls throughout the experiment; however, the experimental groups did not differ statistically. On the contrary, we observed a higher frequency of larger tumors, significantly increased mean lesion volume as well as overall tumor burden (calculated as the sum of tumor volumes per mouse) in *Dll4*$^{+/-}$ relative to WT mice at the experiment end-point; $p<0,05$ (Figure 5.1. A and B). Body weights of both WT and *Dll4*$^{+/-}$ mice were not significantly different prior to the start of, or at any time during the course of this study (data not shown), suggesting that the level of carcinogen dosing was not toxic to the animals.

Subsequent diagnostic analysis revealed that two experimental groups were uniform in terms of tumor pathohistology, presenting undistinguished rates of tumor malignant conversion (Table 5.1.). With the exception of a single squamous cell carcinoma (SSC), grade I, observed in each group, all remaining WT and *Dll4*$^{+/-}$ mouse skin alterations were exophytic lesions classified as benign squamous papillomas displaying hyperkeratotic epidermal projections with foci of dyskeratosis and dysplasia, intact basement membrane and superficial dermal inflammation (Figure 5.1. C).

Table 5.1 – Histological diagnosis of DMBA/TPA-treated wild-type and *Dll4*+/- mice

Diagnosis	Wild-type	*Dll4*+/-
Squamous papilloma	68	112
Squamous cell carcinoma grade I	1	1
Total	**69**	**113**

Results shown are from 182 lesions from WT and Dll4$^{+/-}$ *littermates (n=12 for each group) treated with a single initiation dose of 25µg DMBA followed by 4µg TPA twice a week for 19 weeks.*

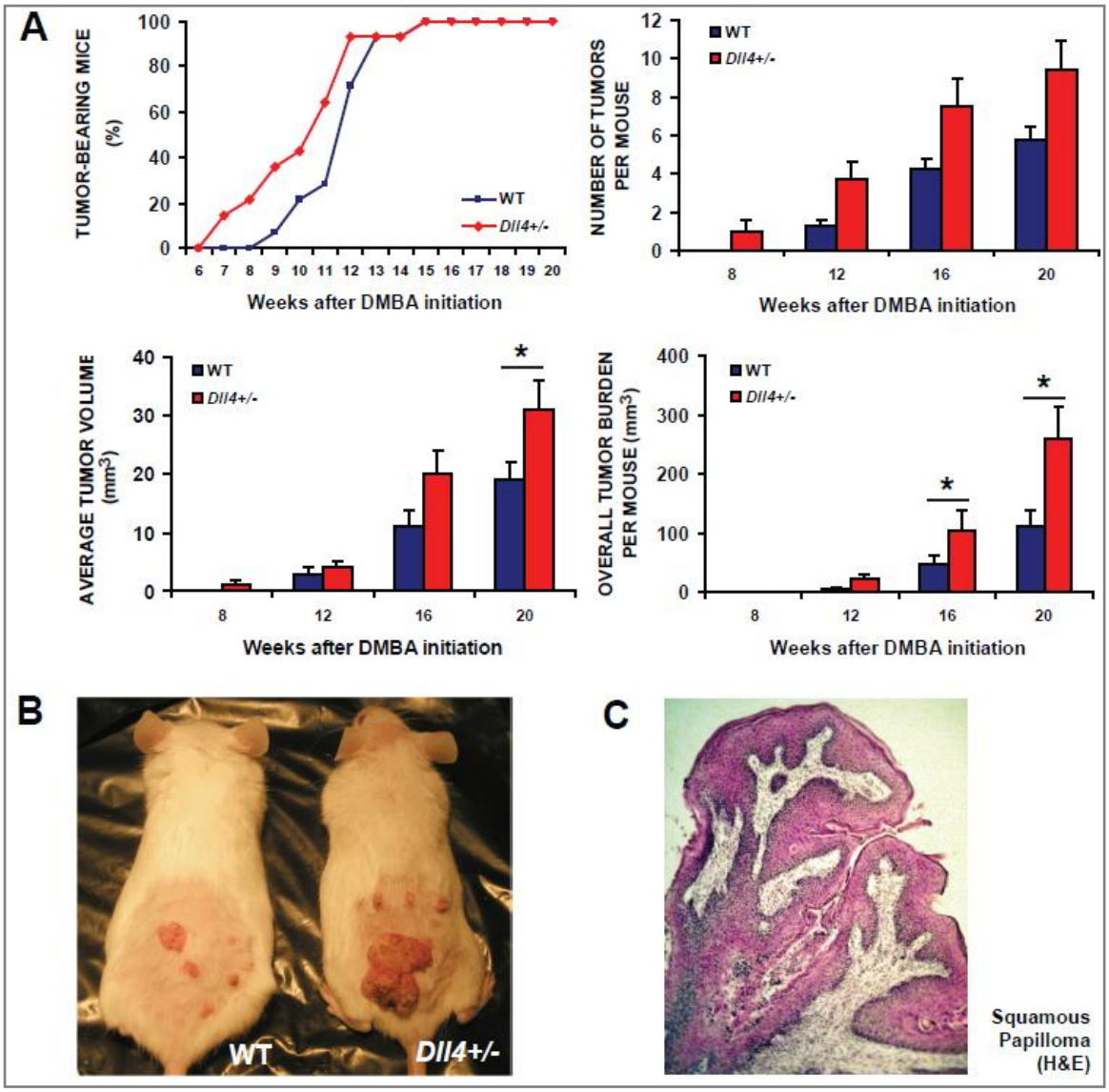

Figure 5.1 - *Dll4* allelic deletion promotes the onset and growth of chemically-induced skin tumors. ***A***, Tumor kinetics in DMBA/TPA-treated WT and *Dll4*$^{+/-}$ mice: tumor incidence (*top left*), average number of lesions per mouse following DMBA/TPA-treatment (*top right*), growth rate measured by average tumor volume per mouse (*below left*), and tumor growth presented as the increase of overall tumor burden, *i.e.* average sum of tumor volumes, per individual mouse for each experimental group (*below right*). *Error bars* represent SEM. * $P < 0.05$ was considered statistically significant. ***B***, Representative WT (*left*) and *Dll4*$^{+/-}$ mouse (*right*) 20 weeks after DMBA-initiation. ***C***, Hematoxylin and eosin staining of a squamous papilloma, representing the pathohistological diagnosis of 98.6% WT and 99.1% *Dll4*$^{+/-}$ skin lesions.

5.4.2. Impaired Dll4/Notch signaling results in excessive, less mature but productive angiogenic response upon chemically-mediated skin tumorigenesis

To better understand the observed, simulative effects of reduced Dll4/Notch signaling on chemical skin papillomas, we examined tumors derived from WT and *Dll4*$^{+/-}$

mice for their vascular morphologies. Tumor endothelium was visualized by immunostaining to PECAM while PECAM/α-SMA immunolocalization was engaged for evaluation of mural cell recruitment and thereby vessel maturity. Besides, subgroups of control, WT and *Dll4*$^{+/-}$ littermates were perfused just before being sacrificed by endothelium-binding lectin for the visualization of thefunctional portions of the tumor circulation. As presented in Figure 5.2. A, the WT papillomas were found to be vascularized lesions characterized by irregular vessel formation. However, the WT tumor vasculature presented elevated degree of both vessel maturity (Figure 5.2. B) and vascular functionality (Figure 5.2. C), as indicated by PECAM/α-SMA and PECAM/lectin co-localization, respectively. In comparison, *Dll4*$^{+/-}$ papillomas showed ~35% increase in density of PECAM–positive area per tumor stroma surface ($p < 0.05$), forming more disorganize endothelial networks with pronounced branching and thin interconnections (Figure 5.2. A). In addition, α-SMA–positive cells lining PECAM–positive endothelium were significantly reduced (31% reduction, $p < 0.05$, Figure 5.2. B). Despite these data showing reduced luminal diameters and reflecting impaired vessel wall assembly in *Dll4*$^{+/-}$ papillomas relative to the WT mouse lesions, *Dll4* allelic deletion insignificantly reduced the portion of lectin-perfused vessels (<10%, Figure 5.2. C). Thus, the 50% reduction of Dll4 function enhanced quite competent neovessel proliferation and is likely to be responsible for the increase in chemically-induced papilloma growth.

5.4.3. *Dll4* down-regulation increases the VEGFR function in chemically-induced skin papillomas

An expected effect of suppressed Dll4/Notch is to reduce the *Vegfr1* expression and increase *Vegfr2* expression (Suchting, Freitas et al. 2007) that potentially explains the increase in angiogenesis and growth of the *Dll4*$^{+/-}$ tumors. We assessed whether this effect could be monitored by the release of cleaved VEGFR1 and VEGFR2 into the serum of mice, and compared assays for controls *vs*. the *Dll4*$^{+/-}$ animals at sacrifice when the tumor bulk was maximal. We investigated VEGF levels also and there was no significant difference in receptor or ligand concentrations in the two groups of animals. Nevertheless, in *Dll4*$^{+/-}$ *vs*. WT mice, we detected a marked and statistically significant increase in serum VGFR2/VGFR1 ratio (Figure 5.3. A), implying increased endothelial sensitivity to VEGF and enhanced VEGF signaling in *Dll4*$^{+/-}$ papillomas.

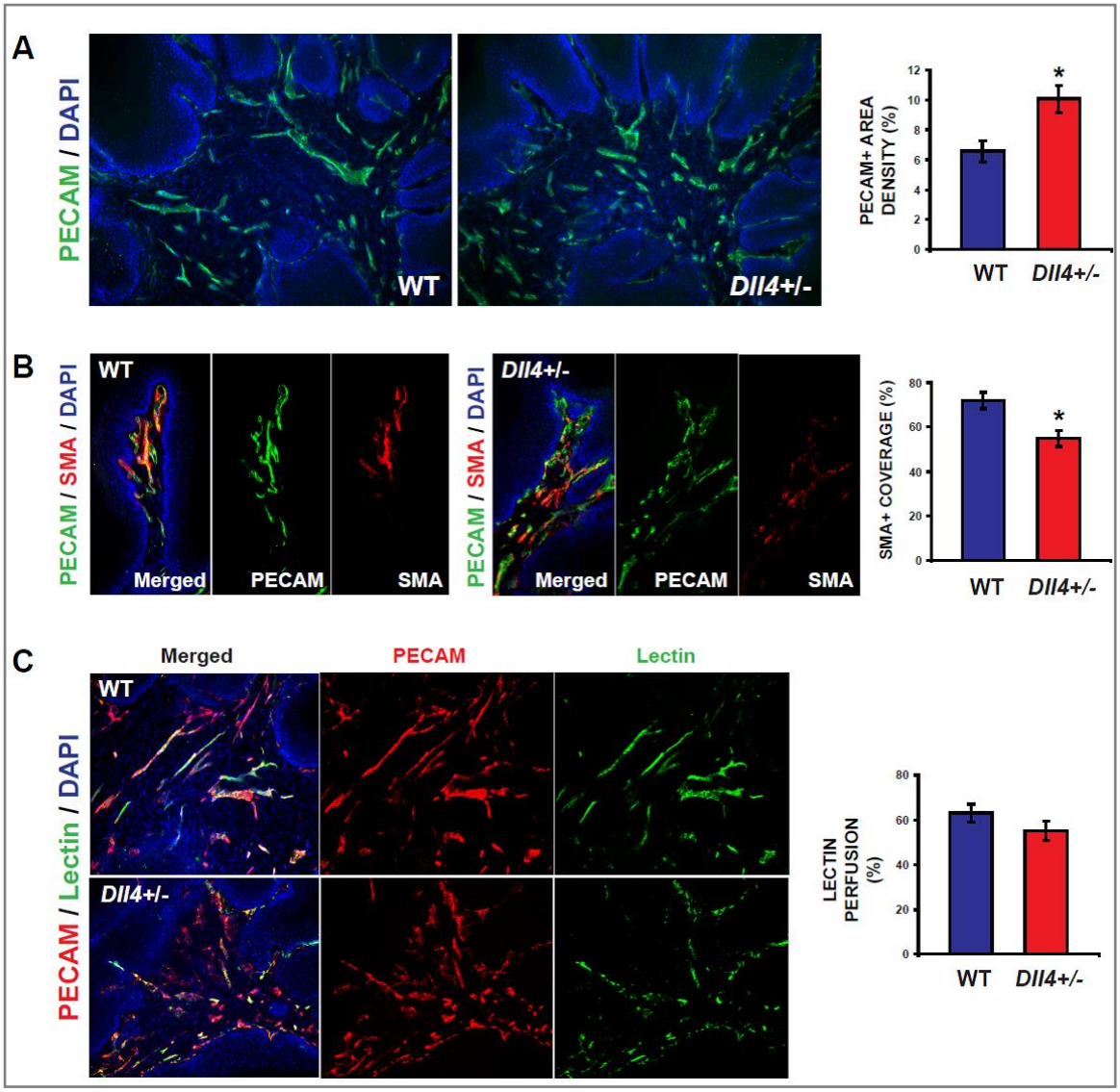

Figure 5.2 – Partial Dll4/Notch inhibition due to haploid *Dll4* deletion enhances less mature but productive angiogenesis in chemically-induced skin papillomas. ***A***, Vascular response examined by PECAM immunostaining indicating increased sprouting and network disorganization with reduced vessel calibers in *Dll4*$^{+/-}$ mice in comparison with WT controls(*left*). Vascular density estimated as percentage of PECAM-positive area per tumor stroma surface and presented for two experimental groups (*right*). ***B***, Mural cell coverage examined by double immunostaining of PECAM (*green*) and α-SMA (*red*) showing a reduced recruitment of, α-SMA-positive cells in *Dll4*+/- tumors (*left*). The percentage of PECAM-positive structures lined by SMA-positive area measured as an indicator of vessel maturation (*right*). ***C***, Tumor vessel competence evaluated by lectin perfusion and subsequent double staining to PECAM and biotinylated lectin demonstrating a reduction in the fraction of perfused vessels in *Dll4*$^{+/-}$ *vs.* WT mice (*left*). Percentage of PECAM-positive area co-localized with lectin measured to estimate the portion of functional vessels within papillomas (*right*). *Error bars* represent SEM. * $P < 0.05$ was considered significant.

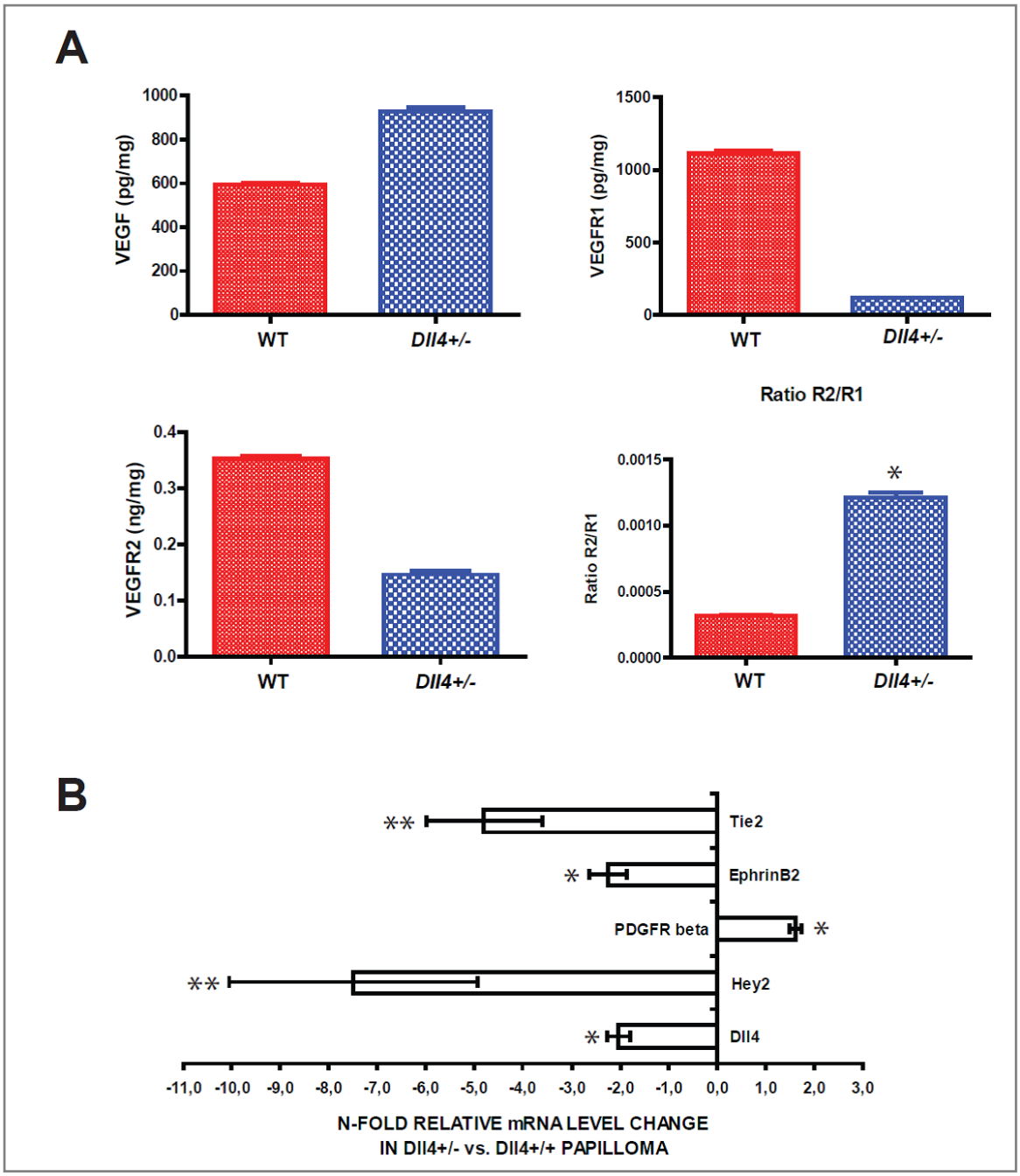

Figure 5.3 – *Dll4* allele deletion affects VEGF/VEGFR signaling as well as the regulators of pericyte recruitment in chemically-induced skin papillomas. ***A***, Average serum level of VEGF, VEGFR1, VEGFR2 measured by ELISA and VEGFR2/VEGFR1 ratio in WT and *Dll4*$^{+/-}$ mice.. ***B***, Differential gene expression in WT *vs.* *Dll4*$^{+/-}$ papillomas determined by quantitative RT-PCR analyses. *Error bars* represent SEM. * $P < 0.05$ and ** $P < 0.01$ were considered significant and highly significant, respectively.

5.4.4. *Dll4* deletion affects the expression of factors regulating the pericyte recruitment in chemical skin papillomas

Although *Dll4* haploinsufficiency was found to promote productive angiogenesis in the used model, it did indeed negatively influenced pericyte recruitment to the proliferating tumor capillaries, the process regulated by PDGFB/PDGFRβ, Tie-2 and EfrinB2/EphB4 (Abramsson, Lindblom et al. 2003; Bergers, Song et al. 2003; Armulik, Abramsson et al. 2005). Thereby, we next used qRT-PCR to analyze WT and $Dll4^{+/-}$ papillomas for differential expression of genes known to be involved in EC/pericyte interactions (Figure 5.3. B). Once we demonstrated that $Dll4^{+/-}$ *vs.* WT tumors had ~2-fold reduced *Dll4* mRNA levels and down-regulated *Hey2* expression confirming the Notch pathway suppression, we compared the *Tie2, EphrinB2* and *Pdgfr-β* mRNA levels between the two tumor groups. Both angiopoietin receptor *Tie2* (Sato, Tozawa et al. 1995; Peters, Kontos et al. 2004) and *EphrinB2* (Foo, Turner et al. 2006) were down-regulated upon *Dll4* allelic deletion being at least a part of explanation for impaired mural cell recruitment in *Dll4+/- vs.* WT papillomas. Unexpectedly, increased *Pdgfr-β* mRNA levels were found, probably as a negative feed-back to retarded vessel maturation.

5.4.5. Partially inhibited Dll4/Notch signaling decreases the tumor-suppressive effect of sorafenib on chemically-induced skin tumors

Considering the fact that *Dll4* allele deletion changes the VEGFR function in chemically-induced papillomas, we finally examined the influence of *Dll4* down-regulation on the efficacy of sorafenib, an approved oral small-molecule-receptor tyrosine kinase inhibitor which targets include VEGF receptors (Kerbel 2008). Beginning at week 13 after the DMBA-initiation, when all WT and $Dll4^{+/-}$ mice developed at least one skin lesion and approached the exponential tumor-growth phase, we treated the mice of both genotypes, appropriately separated in four experimental groups (Figure 5.4. A), with Cremophor EL/ethanol/water 12.5:12.5:75 mixture, the sorafenib vehicle or with sorafenib (40mg/kg/day) for three weeks. Comparing pre- and post-treatment tumor volumes (Figure 5.4. B), we observed that vehicle-treated WT tumors showed a 2.1-fold volume increased over the period of three weeks while vehicle-treated $Dll4^{+/-}$ papillomas presented even more pronounced progression with a 2.6-fold volume expansion ($p < 0.05$). On the other hand, sorafenib treatment effectively suppressed WT animal tumor

growth and resulted, after the three weeks of application, in an average WT papilloma regression of 56% ($p < 0.05$). In contrast, *Dll4*$^{+/-}$ skin lesions continued to progress despite the sorafenib treatment, although the drug application provided a marked tumor growth retardation compared to vehicle-treated *Dll4*$^{+/-}$ papillomas. Importantly, vehicle-treated WT and sorafenib-treated *Dll4*$^{+/-}$ mice presented very comparable tumor volumes at the treatment end-point indicating that 50% Dll4/Notch inhibition virtually neutralized the effects of sorafenib.

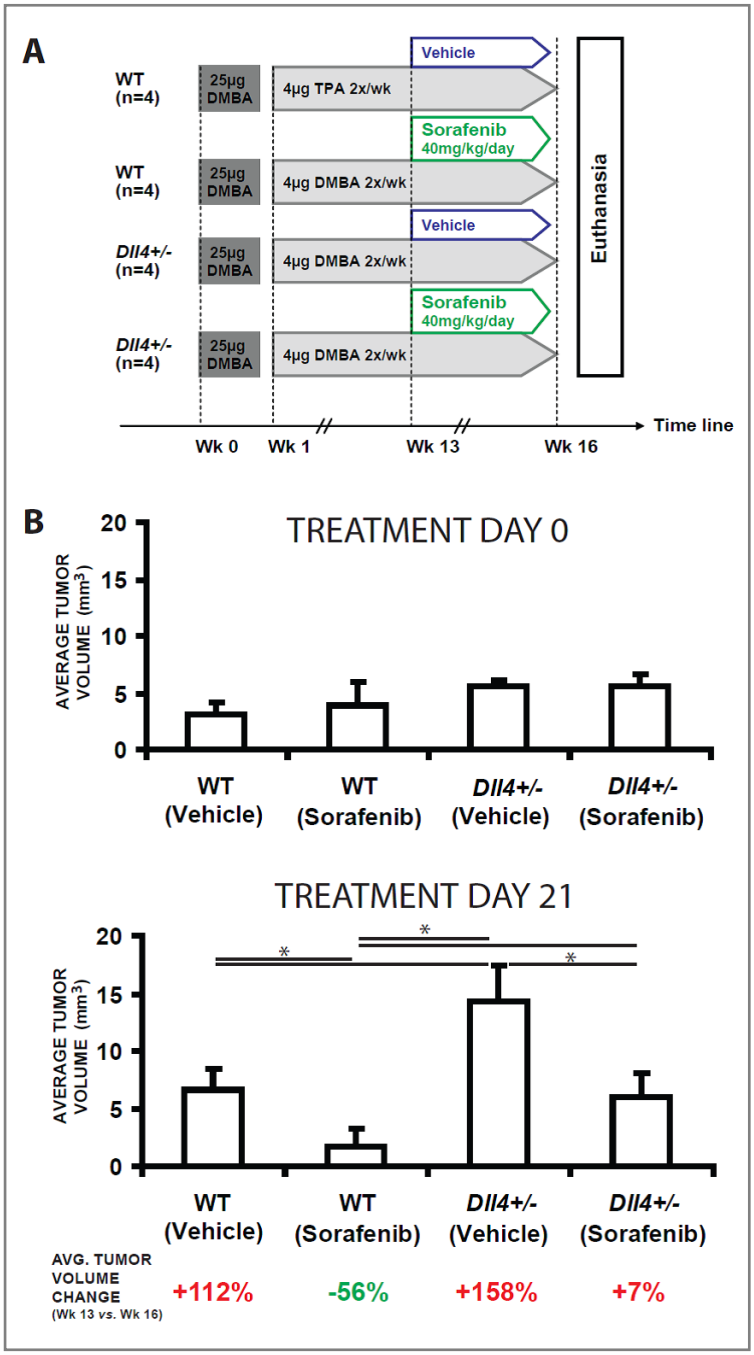

Figure 5.4 – *Dll4* deletion reduces sorafenib efficacy against chemically-induced skin papillomas. ***A***, Schematic diagram outlining the protocol used for the evaluation of combinatorial effect of *Dll4* allelic deletion and sorafenib on mouse chemically-induced tumors. ***B***, Tumor volume changes in WT and *Dll4*$^{+/-}$ mice during the treatment with vehicle and sorafenib, respectively: average tumor volumes at the treatment day 0 (*above*) and treatment end-point (*middle*); percentage change of average tumor volumes during the trial for each experimentalgroups (*bottom*). *Error bars* represent SEM. * $P < 0.05$ was considered significant.

5.5. DISCUSSION

Previous genetic and pharmacological studies show that Dll4/Notch blockade increases tumor-driven angiogenic response in a spectrum of invasive malignancies, but suppresses tumor growth due to the formation of immature and non-functional vessels that reduce tumor perfusion (Djokovic, Trindade et al. ; Noguera-Troise, Daly et al. 2006; Ridgway, Zhang et al. 2006; Li, Sainson et al. 2007; Scehnet, Jiang et al. 2007; Haller, Brave et al. 2011; Kalen, Heikura et al. 2011). Our results demonstrate that low Dll4 levels are associated with an increase in both growth and angiogenesis in chemically-induced skin papillomas. *Dll4* allelic deletion reduced papilloma latency and promoted their multiplicity and growth, although the malignant alteration of these lesions was not found to be influenced by Dll4/Notch since SSC rates did not significantly differ between WT and $Dll4^{+/-}$ mice. Histologically, $Dll4^{+/-}$ papillomas were characterized by increased vascular density, narrower vessel calibers and reduced mural cell recruitment in comparison with the control tumors, but no significant reduction in the portion of perfused vessel was evidenced. Thus, our results initially appear contradictory to substantial data showing that the Dll4/Notch blockade inhibits functional angiogenesis, yet with genetic modification to lower Dll4 there is enhanced productive angiogenesis. With regard to the number of vessels, however, the effects are comparable with the Dll4/Notch inhibition in invasive tumors that produces an increase in vessels, just as *Dll4* haploinsufficiency increased vessels in our experiments. The difference relates to function of the vessels.

We think that different effects of reduced Dll4 on vascular functionality in early *vs*. late tumors are determined by the background level of VEGF and VEGF/VEGFR signaling. One potential explanation for the increase in angiogenesis and growth of the $Dll4^{+/-}$ papillomas is increase in VEGFR function. In the tumors of $Dll4^{+/-}$ *vs*. WT mice, we did indeed find a dramatic increase in the amount of VGFR2 to VGFR1 measured at protein level, but no difference in VEGF concentrations. In the context of VEGF levels which are increased in papillomas, but not to the level of VEGF in invasive tumors (Larcher, Robles et al. 1996), Dll4/Notch signaling at constitutive levels act as a suppressor of VEGF signaling and even a 50% decrease can result in a change of VEGFR2/VEGFR1 ratio and marked increase in responsiveness to VEGF levels. In the context of very high levels of VEGF, Dll4/Notch signaling may be essential to prevent

excessive proliferation, aberrant vessel structure and leakiness. So in this case unrestrained VEGF function is detrimental.

There are some similarities here with the effects of Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2) interacting on the Tie2 receptor. Antagonism of Ang-1 by Ang-2 dissociates pericyte cover, and then in the absence of VEGF vessels regress, but in the presence of VEGF, endothelium becomes activated and vessels proliferate (Lobov, Brooks et al. 2002). This level of VEGF is critical for the effect of the antagonistic pathway. Similarly, Dll4/Notch blockade enhances EC activation and allows the large number of small vessels to proliferate, but against the background of very high VEGF in invasive tumors they fail to become productive while in the background of lower VEGF levels in benign/early lesions the vessels succeed in acquiring functionality. Nevertheless, they still present retarded maturation and we provide data implying that this could be due to down-regulated *Tie2* and *EphrinB2*. As a negative feed-back, increased *Pdgfr-β* expression in *Dll4*$^{+/-}$ *vs.* WT papillomas probably occurred to compensate impaired mural cell recruitment.

Furthermore, we demonstrated that *Dll4* allele deletion in chemically-induced skin papillomas reduced the effectiveness of sorafenib, a small molecule inhibitor of a broad spectrum of receptor tyrosine kinases including VEGFR2, VEGFR3 and B-Raf (Kerbel 2008). Upon the Dll4/Notch inhibition, the papillomas became more sensitive to VEGF due to increased VEGFR function and less responsive to therapeutic VEGFR inhibition. Decreased efficacy of tyrosine kinases inhibitors might also occur and be even more pronounced in invasive lesions with high VEGF levels. However, the effect on the efficacy of VEGF inhibitors is likely to change with the degree of Dll4/Notch inhibition. More pronounced Dll4/Notch suppression than that observed in *Dll4*$^{+/-}$ mice, might increase their efficacy since the abolishment of Dll4 function promotes unproductive vascular response while concomitant VEGF/VEGFR2 inhibition will reduce the rate of endothelial proliferation. Such an outcome was previously observed with combinational blockade of Dll4/Notch signaling, reducing vascular competence, and Ephrin-B2 signaling, reducing endothelial proliferation (Djokovic, Trindade et al. 2010), by interfering with the VEGFR trafficking (Sawamiphak, Seidel et al. 2010).

In summary, the role of Dll4 is fundamental and differs in early and late tumor development. In early papillomas, low levels of Dll4 increase vascularization through change in VEGFR2 and enhance sensitivity to endogenous levels of VEGF, but in

invasive cancers which greatly reduce gradients of VEGF signaling, Dll4 is critical to maintain some normal vascular function and organization, and a loss of this allows a greater numbers of malformed vessels to form. We therefore demonstrate a new role of Dll4 in tumor development which emphasizes the critical relationship between VEGF signaling and Dll4/Notch signaling.

REFERENCES:

Abramsson, A., P. Lindblom, et al. (2003). "Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors." J Clin Invest **112**(8): 1142-51.

Armulik, A., A. Abramsson, et al. (2005). "Endothelial/pericyte interactions." Circ Res **97**(6): 512-23.

Bergers, G., S. Song, et al. (2003). "Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors." J Clin Invest **111**(9): 1287-95.

Claxton, S. and M. Fruttiger (2004). "Periodic Delta-like 4 expression in developing retinal arteries." Gene Expr Patterns **5**(1): 123-7.

Demehri, S., A. Turkoz, et al. (2009). "Epidermal Notch1 loss promotes skin tumorigenesis by impacting the stromal microenvironment." Cancer Cell **16**(1): 55-66.

Djokovic, D., A. Trindade, et al. "Combination of Dll4/Notch and Ephrin-B2/EphB4 targeted therapy is highly effective in disrupting tumor angiogenesis." BMC Cancer **10**: 641.

Djokovic, D., A. Trindade, et al. (2010). "Combination of Dll4/Notch and Ephrin-B2/EphB4 targeted therapy is highly effective in disrupting tumor angiogenesis." BMC Cancer **10**: 641.

Duarte, A., M. Hirashima, et al. (2004). "Dosage-sensitive requirement for mouse Dll4 in artery development." Genes Dev **18**(20): 2474-8.

Foo, S. S., C. J. Turner, et al. (2006). "Ephrin-B2 controls cell motility and adhesion during blood-vessel-wall assembly." Cell **124**(1): 161-73.

Haller, B. K., A. Brave, et al. (2011). "Therapeutic efficacy of a DNA vaccine targeting the endothelial tip cell antigen delta-like ligand 4 in mammary carcinoma." Oncogene **29**(30): 4276-86.

Hellstrom, M., L. K. Phng, et al. (2007). "Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis." Nature **445**(7129): 776-80.

Iso, T., L. Kedes, et al. (2003). "HES and HERP families: multiple effectors of the Notch signaling pathway." J Cell Physiol **194**(3): 237-55.

Iso, T., T. Maeno, et al. (2006). "Dll4-selective Notch signaling induces ephrinB2 gene expression in endothelial cells." Biochem Biophys Res Commun **341**(3): 708-14.

Kalen, M., T. Heikura, et al. (2011). "Gamma-secretase inhibitor treatment promotes VEGF-A-driven blood vessel growth and vascular leakage but disrupts neovascular perfusion." PLoS One **6**(4): e18709.

Kerbel, R. S. (2008). "Tumor angiogenesis." N Engl J Med **358**(19): 2039-49.

Krebs, L. T., J. R. Shutter, et al. (2004). "Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants." Genes Dev **18**(20): 2469-73.

Kuhnert, F., J. R. Kirshner, et al. (2011). "Dll4-Notch signaling as a therapeutic target in tumor angiogenesis." Vasc Cell **3**(1): 20.

Larcher, F., A. I. Robles, et al. (1996). "Up-regulation of vascular endothelial growth factor/vascular permeability factor in mouse skin carcinogenesis correlates with malignant progression state and activated H-ras expression levels." Cancer Res **56**(23): 5391-6.

Li, J. L., A. M. Jubb, et al. (2010). "Targeting DLL4 in tumors shows preclinical activity but potentially significant toxicity." Future Oncol **6**(7): 1099-103.

Li, J. L., R. C. Sainson, et al. (2007). "Delta-like 4 Notch ligand regulates tumor angiogenesis, improves tumor vascular function, and promotes tumor growth in vivo." Cancer Res **67**(23): 11244-53.

Lobov, I. B., P. C. Brooks, et al. (2002). "Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival in vivo." Proc Natl Acad Sci U S A **99**(17): 11205-10.

Lobov, I. B., R. A. Renard, et al. (2007). "Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting." Proc Natl Acad Sci U S A **104**(9): 3219-24.

Noguera-Troise, I., C. Daly, et al. (2006). "Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis." Nature **444**(7122): 1032-7.

Noguera-Troise, I., C. Daly, et al. (2007). "Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis." Novartis Found Symp **283**: 106-20; discussion 121-5, 238-41.

Peters, K. G., C. D. Kontos, et al. (2004). "Functional significance of Tie2 signaling in the adult vasculature." Recent Prog Horm Res **59**: 51-71.

Ridgway, J., G. Zhang, et al. (2006). "Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis." Nature **444**(7122): 1083-7.

Sato, T. N., Y. Tozawa, et al. (1995). "Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation." Nature **376**(6535): 70-4.

Sawamiphak, S., S. Seidel, et al. (2010). "Ephrin-B2 regulates VEGFR2 function in developmental and tumour angiogenesis." Nature **465**(7297): 487-91.

Scehnet, J. S., W. Jiang, et al. (2007). "Inhibition of Dll4-mediated signaling induces proliferation of immature vessels and results in poor tissue perfusion." Blood **109**(11): 4753-60.

Suchting, S., C. Freitas, et al. (2007). "The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching." Proc Natl Acad Sci U S A **104**(9): 3225-30.

Trindade, A., S. R. Kumar, et al. (2008). "Overexpression of delta-like 4 induces arterialization and attenuates vessel formation in developing mouse embryos." Blood **112**(5): 1720-9.

Williams, C. K., J. L. Li, et al. (2006). "Up-regulation of the Notch ligand Delta-like 4 inhibits VEGF-induced endothelial cell function." Blood **107**(3): 931-9.

Yan, M., C. A. Callahan, et al. (2010). "Chronic DLL4 blockade induces vascular neoplasms." Nature **463**(7282): E6-7.

6

Endothelial *Dll4* Overexpression Reduces Vascular Response and Tumor Growth in Grafted and Autochthonous Mouse Models

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6.1. ABSTRACT

The inhibition of Dll4/Notch signaling has been shown to result in excessive, non-functional vessel proliferation and significant tumor growth suppression. However, safety concerns emerged with the identification of side effects resulting from chronic Dll4/Notch blockade. Alternatively, we explored the endothelial *Dll4* overexpression using different mouse models and found that increased Dll4/Notch signaling retards tumor growth by reducing VEGF-induced endothelial proliferation, tumor vessel density and overall tumor blood supply. In addition we observed that *Dll4* overexpression consistently improved tumor vascular maturation and functionality, as indicated by increased vessel calibers, enhanced mural cell recruitment and increased network perfusion. Importantly, the tumor vessel normalization is not more effective than restricted vessel proliferation, but it might reduce bloodstream invasion of malignant cells and guarantee increased delivery and effectiveness of concomitant chemotherapy. By reducing endothelial sensitivity to VEGF, these results additionally imply that Dll4/Notch stimulation in tumor microenvironment may increase the efficacy of approved VEGF signaling inhibitors, which in monotherapy show modest survival benefits. *Dll4* overexpression thus appears as a promising anti-angiogenic modality that might improve cancer control.

6.2. INTRODUCTION

Current angiogenic inhibitors targeting vascular endothelial growth factor (VEGF) signaling produce therapeutic efficacy in many aggressive tumors, but fail to provide enduring clinical benefit in most cases (Jain 2005; Jain, Duda et al. 2006; Saltz, Lenz et al. 2007; Shojaei and Ferrara 2007; Allegra, Yothers et al. 2011; Ebos and Kerbel 2011). In addition, the VEGF inhibitors with documented anti-tumor efficacy have been found prone in mouse models to elicit tumor adaptation and progression to stages of greater malignancy, with heightened invasiveness and in some cases increased lymphatic and distant metastasis (Paez-Ribes, Allen et al. 2009). Therefore, there is an unequivocal need to validate novel therapeutic targets alongside VEGF signaling. The Delta-like 4 (Dll4)/Notch pathway appears as a promising candidate.

Acting downstream of VEGF signaling, Dll4/Notch signaling essentially contributes to proper vascular remodeling during embryonic vascular development (Duarte, Hirashima et al. 2004; Gale, Dominguez et al. 2004; Krebs, Shutter et al. 2004). The endothelial Dll4 ligand interacts with Notch 1 and Notch 4 receptors of adjacent endothelial cells, triggering γ-secretase proteolytic cleavage of Notch intracellular domain (NICD), which subsequently translocates to the nucleus as a complex with the recombination signal binding protein Jκ (RBP-Jκ) and activates effector genes including the members of the *Hes* and *Hey* families of basic helix-loop-helix transcription factors (Bray 2006)and *EphrinB2* (Phng and Gerhardt 2009). Postnatal Dll4 expression in the vascular system is limited to angiogenic endothelia and generally absent in quiescent vessels of normal tissue (Mailhos, Modlich et al. 2001). However, its up-regulation is a hallmark of proliferating tumor vessels in both preclinical murine models (Djokovic, Trindade et al. ; Gale, Dominguez et al. 2004; Hainaud, Contreres et al. 2006; Noguera-Troise, Daly et al. 2007; Scehnet, Jiang et al. 2007) and different human malignancies (Mailhos, Modlich et al. 2001; Patel, Dobbie et al. 2006; Jubb, Turley et al. 2009; Jubb, Soilleux et al. 2011). Besides *Dll4* expression was identified in malignant cells of different types (Martinez, Muller et al. 2009), and the ligand was shown to have an impact on colon cancer stem cell frequency by suppressing apoptosis of tumor cells (Hoey, Yen et al. 2009; Fischer, Yen et al. 2011). Nevertheless, vascular endothelium represents the most prominent and constant site of *Dll4* expression within tumors.

Targeted *Dll4* allele deletion, local overexpression of Dll4/Notch-blockers, systemic application of soluble Dll4/Notch-inhibitors and DNA vaccination were found

to result in a significant suppression of tumor growth in numerous preclinical models,, including malignancies resistant to VEGF inhibitors (Djokovic, Trindade et al. ; Noguera-Troise, Daly et al. 2006; Ridgway, Zhang et al. 2006; Li, Sainson et al. 2007; Scehnet, Jiang et al. 2007; Haller, Brave et al. 2011; Kalen, Heikura et al. 2011). Tumor growth retardation due to Dll4/Notch inhibition is associated with an apparently paradoxical increase of endothelial proliferation, migration and subsequent tumor vessel density, but also excessive branching with defective lumenization and impaired mural cell recruitment, both leading to non-functional vessel formation and subsequent tumor starvation. Nevertheless, the normalization capacity of these vascular aberrations remains undetermined as well as the persistence of beneficial effects attributed to the Dll4/Notch inhibition. Additionally, poor blood perfusion raises concerns that therapeutic Dll4/Notch inhibition may reduce the effectiveness of concomitant chemotherapy while hypoxia can contribute to more malignant cell selection (Hayden 2009). Besides, chronic Dll4 blockade was found to disrupt normal organ vascular homeostasis and induce vascular tumor formation (Djokovic, Trindade et al. ; Li, Jubb et al. 2010; Yan, Callahan et al. 2010). Thus, in certain contexts, the putative side effects of blocking Dll4/Notch pathway may come to limit its clinical usefulness.

The vessel defects induced by the Dll4/Notch inhibition closely resemble vascular abnormalities commonly observed in human malignancies; so increased Dll4 expression in tumor vasculature, that reduces endothelial sensitivity to VEGF (Suchting, Freitas et al. 2007), may be considered a host defense mechanism serving to restrict tumor vascularization and malignant cell access to the bloodstream such as Angiopoietin-2 does in the case of vascular cooption (Holash, Maisonpierre et al. 1999). In this light, amplification of Dll4/Notch signals appears as a reasonable therapeutic option to be tested. Virus-transduced malignant cells that over-express Dll4 activate Notch signaling in co-cultured endothelial cells and restrict VEGF-induced endothelial cell growth (Noguera-Troise, Daly et al. 2006; Li, Sainson et al. 2007). When expressed in tumor cells, Dll4 also functions as a negative regulator of tumor angiogenesis; however, there is no consistent information on the effects of Dll4 over-expression on tumor kinetics. While it was reportedto act as a negative driver of tumor expansion in tested malignant cell lines grafted in mice (Noguera-Troise, Daly et al. 2006; Li, Sainson et al. 2007; Segarra, Williams et al. 2008), *Dll4* expression in transduced human glioblastoma and prostate

cancer cell xenografts was associated with promoted tumor growth, to some extent, due to a reduction of tumor hypoxia and apoptosis (Li, Sainson et al. 2007).

In this study, Dll4 was amplified for the first time in the tumor endothelium, its predominant site of expression, and the consequences were analyzed in both grafted and in more reliable autochthonous tumor models that better reflect host-tumor interaction and wherein the lesions arise and develop resembling the human disease. We consistently found that endothelial *Dll4* overexpression reduces the growth of Lewis Lung Carcinoma (LLC) grafts, chemically-induced murine skin tumors as well as transgenic RIP1-*Tag2* (RT2) mouse insulinomas due to decreased vascular proliferation by modifying the activity of angiogenesis regulators. Importantly, we show that *Dll4* overexpression reduces vascular responsiveness to VEGF seeming reasonable for concomitant application with VEGF-inhibitors and stabilizes tumor circulation opening perspectives for more efficient chemotherapy delivery.

6.3. MATERIALS AND METHODS

6.3.1. Mice

Double heterozygous Tie2-rtTA-M2 TetO7-Dll4 mice were generated as described (Trindade, Kumar et al. 2008) and used as hosts for tumor xenografts and chemically-induced skin tumors. The RIP1-*Tag2* (RT2) mice were kindly provided by Dr. Oriol Casanovas and used for breeding with Tie2-rtTA-M2 TetO7-Dll4 line for generation of triple mutants (RT2 Tie2-rtTA-M2 TetO7-Dll4) capable ofdeveloping pancreatic insulinomas and overexpress endothelial *Dll4* after tetracycline or doxycycline-induction. Restricted in the endothelium by the *Tie-2* promoter, *Dll4* overexpression was activated in inducible Tie2-rtTA-M2 TetO7-Dll4 (xenograft and skin tumorigenesis experiments) and RT2 Tie2-rtTA-M2 TetO7-Dll4 mutants (autochthonous insulinoma experiment) by *per os* administration of the tetracycline analogue doxycycline (2mg/ml in drinking water *ad libitum*; Dll4 over-expression mice, D4OE). Non-induced Tie2-rtTA-M2 TetO7-Dll4 or RT2 Tie2-rtTA-M2 TetO7-Dll4 littermates, receiving just water, served as Dll4 basic-expression controls (D4BE).

The animals were housed in ventilated propylene cages with sawdust bedding, in a room with temperature between 22ºC and 25ºC and a 12-hours-light/12-hours-dark cycle. The mice were fed standard laboratory diet. From 12 weeks of age, RT2 and RT2 Tie2-rtTA TetO7-Dll4 mice received 5% sugar in drinking water to relieve hypoglycemia.All animal-involving procedures of this study were approved by the Faculty of Veterinary Medicine of Lisbon Ethics and Animal Welfare Committee (Approval ID PTDC/CVT71084/2006).

6.3.2. Xenograft mouse model

Male, 8-week old Tie2-rtTA TetO7-Dll4 mice (n=12) were distributed into two equal groups that respectively continued receiving water (control Dll4-basic expression group, D4BE) or started receiving 2mg/ml doxycycline in drinking water (Dll4 over-expression group, D4OE). A week later, Lewis lung carcinoma cells (LLC, 10^6) were implanted subcutaneously into the flank of each mouse. Injected sites were monitored daily. Once palpable, tumor largest (a) and smallest (b) diameters were measured and

tumor volumes were calculated using the formula: $V = a \times b^2 \times 0.52$. Two weeks after the LLC injection, when the largest tumors approached the prescribed maximal size, xenografts were dissected and processed for histological studies.

6.3.3. Chemically-induced skin tumorigenesis model

Male, 8-week old Tie2-rtTA TetO7-Dll4 mice (n=12) were separated into two equal groups and continued receiving water (control *Dll4* basic expression group, D4BE) or started receiving 2mg/ml doxycycline in drinking water (*Dll4* overexpression group, D4OE). A week later, all mice were shaved and treated with a single dose of 25µg of 7,12-dimethylbenz[a]anthracene (DMBA; Sigma, St. Luis, MO) in 200µL acetone per mouse applied to the dorsal skin. Beginning a week after DMBA-induction, tumor onset and growth was promoted by treating mice twice a week for 19 weeks with 4µg of 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma, St. Luis, MO) in 100 µL of dimethyl sulfoxide (DMSO) per mouse. The appearance of skin lesion was monitored and recorded weekly. Mouse weight and tumor sizes (diameters) were periodically measured and lesion diameters were converted to tumor volume using the following formula: V= length x width x height x 0.52. Tumor burden of each individual mouse was calculated as the sum of individual tumor volumes. Twenty weeks after the DMBA-initiation, mice were euthanized and skin tumors were dissected and processed for histological and molecular analyses.

6.3.4. RIP1-*Tag2* (RT2) insulinoma model

RT2 Tie2-rtTA TetO7-Dll4 mice were generated as described above. Approaching the RT2 tumor stage conventionally used for therapeutic intervention assessments, 9.5-week old RT2 Tie2-rtTA TetO7-Dll4 mice (n=16) were distributed into two equal groups receiving just drinking water (control RT2 *Dll4* basic expression group, RT2 D4BE) or 2mg/ml doxycycline in drinking water (RT2 *Dll4* overexpression group, RT2 D4OE). The mice were sacrificed at 13.5 weeks just before most RT2 progenitors die due to tumor burden and hypoglicemia. The pancreata were dissected and macroscopic tumors ($\geq$1 x 1 mm) were excised. Tumor volumes were calculated using the formula: V= length x width x height x 0.52. The volumes of all tumors from each mouse were added to give

the overall tumor burden per animal. Subsequently, insulinoma samples were processed for histological analyses.

6.3.5. Tumor tissue preparation, histopathology and immunohistochemistry

When dissected and measured as described above, tumors (LLC xenografts, chemically-provoked skin lesions and RT2 insulinomas developed in D4BE and D4OE mice) were fixed in 4% paraformaldehyde (PFA) solution at 4ºC for 1h, cryoprotected in 15% sucrose, embedded in 7,5% gelatine, frozen in liquid nitrogen and cryosectioned at 20µm. Skin tumor and insulinoma tissue sections were stained with Hematoxylin and Eosin (H&E) and subjected to review by a pathologist. Simultaneously, double fluorescent immunostaining to platelet endothelial cell adhesion molecule (PECAM) and pericyte marker alpha smooth muscle actin (α-SMA) was performed on xenograft and autochthonous skin and pancreatic tumor sections to examine tumor vascular density and vessel maturity. Rat monoclonal anti-mouse PECAM (BD Pharmingen, San Jose, CA) and rabbit polyclonal anti-mouse α-SMA (Abcam, Cambridge, UK) were used as primary antibodies and species-specific conjugated with Alexa Fluor 488 and 555 (Invitrogen, Carlsbad, CA) were used as secondary antibodies. Tissue sections were incubated with primary antibody overnight at 4°C and appropriate secondary antibody for 1 hour at room temperature. Nuclei were counterstained with 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; Molecular Probes, Eugene, OR). Stained sections were examined under a Leica DMRA2 fluorescence microscope with Leica HC PL Fluotar 10 and 20X/0.5 NA dry objective, captured using Photometrics CoolSNAP HQ, (Photometrics, Friedland, Denmark), and processed with Metamorph 4.6-5 (Molecular Devices, Sunnyvale, CA). The NIH ImageJ 1.37v program was used for morphometric analyses. Vessel density was estimated as the percentage of each tumor section field occupied by a PECAM-positive signal. Mural cell recruitment was assessed by quantitating the percentage of PECAM-positive structures lined by α-SMA-positive coverage.

6.3.6. Perfusion study

To visualize the blood-perfused, i.e. functional, portion of tumor circulation, mice were anesthetized and biotin-conjugated lectin from *Lycopersicon esculentum* (100µg in 100µl of PBS; Sigma, St. Luis, MO) was injected via caudal vein and allowed to circulate for 5 minutes before the animal vasculature was perfused transcardially with 4% PFA in PBS for 3 minutes. Tumor samples were collected and processed as presented above. Tissue sections (20µm) were stained with rat monoclonal anti-mouse PECAM antibody (BD Pharmingen, San Jose, CA), followed by Alexa 555 goat anti-rat IgG (Invitrogen, Carlsbad, CA). Biotinylated lectin was visualized with Strepatavidin-Alexa 488 (Invitrogen, Carlsbad, CA). The images were obtained and processed as described above. Tumor perfusion was quantified by determining the percentage of PECAM-positive structures that were co-localized with Alexa 488 signals corresponding to lectin-perfused vessels.

6.3.7. Quantitative transcriptional analysis

Using a SuperScript III FirstStrand Synthesis Supermix qRTPCR (Invitrogen, Carlsbad, CA), first-strand cDNA was synthesized from total RNA previously isolated with RNeasy Mini Kit *(*Qiagen, Valencia, CA) from skin tumors developed by D4BE and D4OE mice (n=10 for each group). Real-time PCR analysis was performed as described (Trindade, Kumar et al. 2008) using specific primers for *β-actin*, *PECAM*, *Dll4*, *Jag1*, *Hey2*, *Vegf-a*, *Vegfr1*, *Vegfr2*, *Vegf-c*, *Vegfr3*, *Pdgf-β* , *EphrinB2* and *Tie2*. Primer pair sequences are available on request. Gene expression was normalized to β-*actin*.

6.3.8. Statistical analyses

Data processing was carried out by engaging Statistical Package for the Social Sciences version 15.0 (SPSS v. 15.0; Chicago, IL). Statistical analyses were performed using Mann-Whitney-Wilcoxon test. The results are presented as mean ± SEM or mean ± SD when more appropriate. *P*-values < 0.05 and <0.01 were considered significant (indicated in the figures with *) and highly significant (indicated with **), respectively.

6.4. RESULTS

6.4.1. Endothelial *Dll4* overexpression retards xenograft growth and reduces vascular response in LLC-bearing mice

To study the effects of endothelial Dll4 overexpression on tumor growth, LLC cells were injected subcutaneously in D4BE (non-induced Tie2-rtTA TetO7-Dll4) and D4OE (doxycycline-induced Tie2-rtTA TetO7-Dll4) mice. The injected sites were monitored for a period of 15 days. In both mouse groups, the LLC tumors became palpable by day 8. However, D4OE mice revealed retarded xenograft expansion relative to D4BE mice throughout the experiment and displayed significantly smaller average tumor volumes beginning by day 13 (Figure 6.1. A). At the end of the experiment, all xenografts were removed and observed as encapsulated lesions while animals were inspected macroscopically and adjacent tissues as well as distant organs were found to be free of metastases.

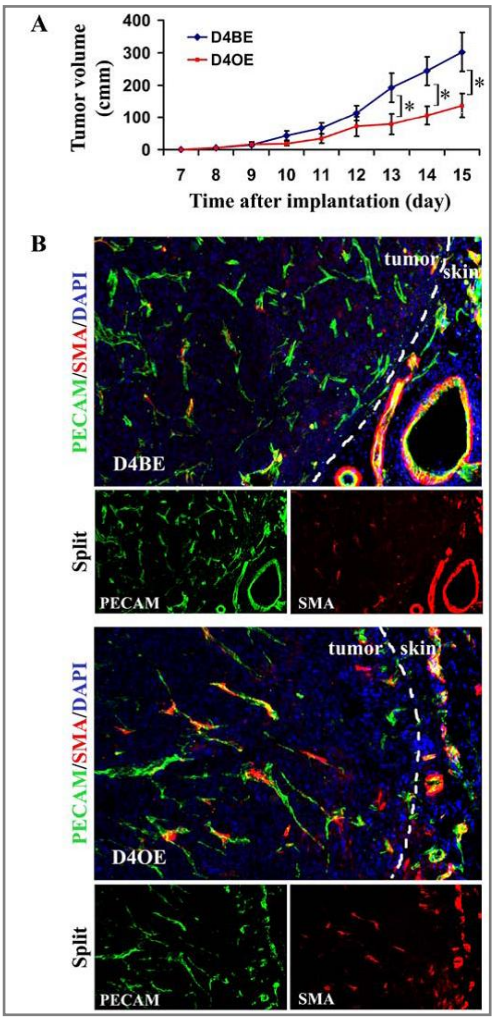

Figure 6.1. – Endothelial *Dll4* overexpression restricts the growth and vascularization of subcutaneous LLC xenografts. ***A***, Delayed LLC xenograft growth due to *Dll4* overexpression in endothelial cells. *D4BE*, mice with *Dll4*-basic expression; *D4OE*, mice with overexpressed endothelial *Dll4*; *cmm*, cubic millimeters. *Error bars* represent SEM. *, $P < 0.05$ was considered significant. *cmm*, cubic millimeters. ***B***, Double PECAM/α-SMA immunostaining used for the examination of the vascular response in D4BE *(above)*and D4OE tumor grafts *(below)* indicating reduced vascular density, but increased vessel calibers and promoted vessel wall assembly as the results of endothelial *Dll4* overexpression *Dotted lines* mark tumor borders.

Subsequently, D4BE and D4OE tumors were analyzed for vascular morphologies by double immunostaining to endothelial marker PECAM and mural cell marker α-SMA. As presented in Figure 6.1. B, control D4BE tumors were found to be vascularized structures which fine and highly branched PECAM-positive (endothelial) networks lacked organized architecture and distinct hierarchy. Besides, newly forming vasculature showed weak α-SMA-positive mural cell recruitment, indicating low maturation rate. In comparison, D4OE tumors showed a reduced vascular response with decreased average PECAM-positive area density compared to D4BE xenografts (1.5-fold decrease, $p < 0.05$). Their vessels were found to be straighter and less branched, presenting, in the same time, wider calibers and highly significant increase in SMA-positive cell coverage relative to the control tumor vessels (>2-fold increase, $p < 0.01$). Thus, endothelial *Dll4* overexpression, that caused the LLC implant growth retardation, was associated with reduced density of the endothelial network but promoted vascular stabilization as indicated by increased lumen diameters and vessel wall maturity.

6.4.2. *Dll4* overexpression inhibits the skin papilloma formation, restricts vessel proliferation and improves vascular functionality upon chemically mediated tumorigenesis

To confirm the beneficial effects of endothelial *Dll4* overexpression in an autochthonous tumor model, and to gain a more thorough insight into the morphological, functional and molecular consequences of increased Dll4/Notch signaling, skin tumorigenesis was initiated in D4BE and D4OE mice by single topical DMBA treatment and then promoted by topical TPA applications twice per week for 19 weeks. As presented in Figure 6.2., D4BE mice started developing skin tumors as early as week 10 after DMBA-initiation and by week 12 of the study, 50% of D4BE mice developed at least one lesion (tumor latency). Tumor onset was delayed in D4OE animals for 2 weeks while tumor latency was extended up to week 17 of DMBA/TPA treatment. Beginning at week 14, we observed a statistically significant difference in the number of lesions per mouse (tumor multiplicity) and in the mean tumor volume between two experimental groups ($p < 0.05$). At the experiment end-point (20 weeks after DMBA-initiation), both tumor multiplicity and overall tumor burden, calculated as the sum of individual tumor volumes per mouse, were reduced with highly significant statistical difference ($p < 0.01$) in D4OE relative to control D4BE mice. Body weight of each mouse used in this study

did not differ significantly prior to the start of, or at any time during the course of the experiment, suggesting that the level of carcinogen dosing was not toxic to the animals. Subsequent histopathological analysis did not reveal any marked difference between D4BE and D4OE tumors. All lesions developed by D4BE as well as D4OE mice were exophytic lesions classified as benign squamous papillomas displaying hyperkeratotic epidermal projections with foci of dyskeratosis and dysplasia, intact basement membrane and superficial dermal inflammation.

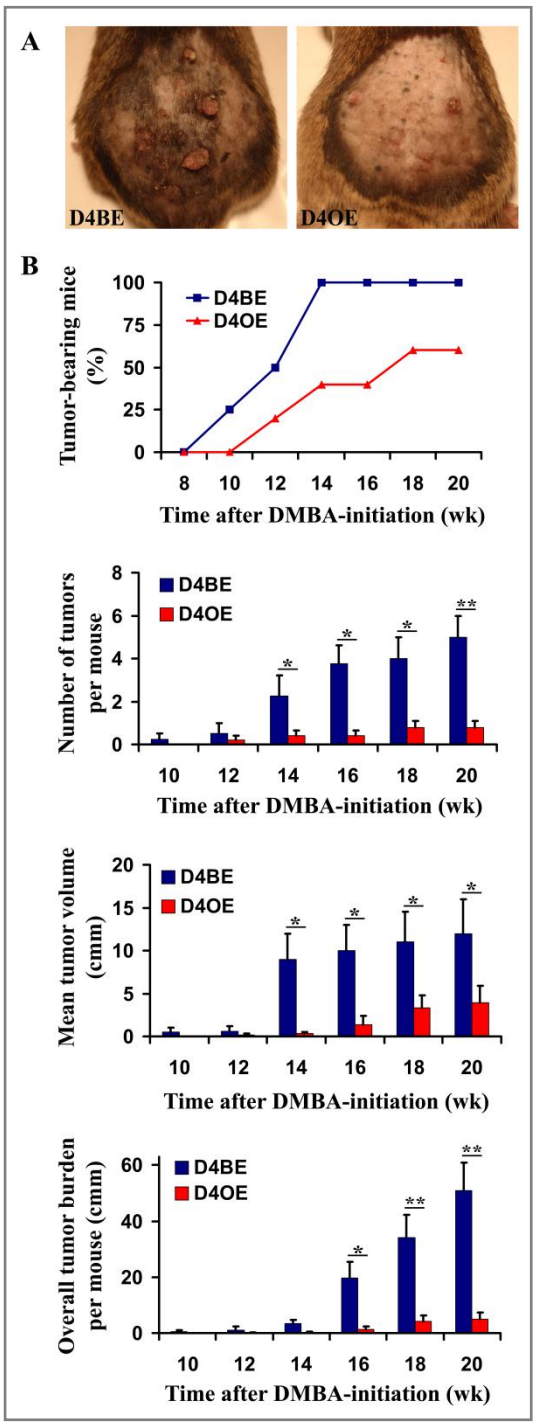

Figure 6.2. – Increased endothelial *Dll4* expression suppresses chemical DMBA/TPA-induced skin tumor onset and development. *A*, Representative pictures of D4BE and D4OE mice taken at the experiment end-point, *i.e.* 20 weeks after the DMBA-initiation, illustrating tumor-suppressing effect of endothelial *Dll4* overexpression. ***B*,** Percentage of tumor-bearing mice, number of tumors per mouse, mean tumor volume and over-all tumor burden per mouse, calculated as the sum of tumor volumes developed by a mouse, throughout the tumor TPA-promotion in D4BE *vs.* D4OE mice. *cmm*, cubic millimeters. *Error bars* represent SEM. *****, $P < 0.05$ and ******, $P < 0.01$ were considered statistically significant and highly significant, respectively.

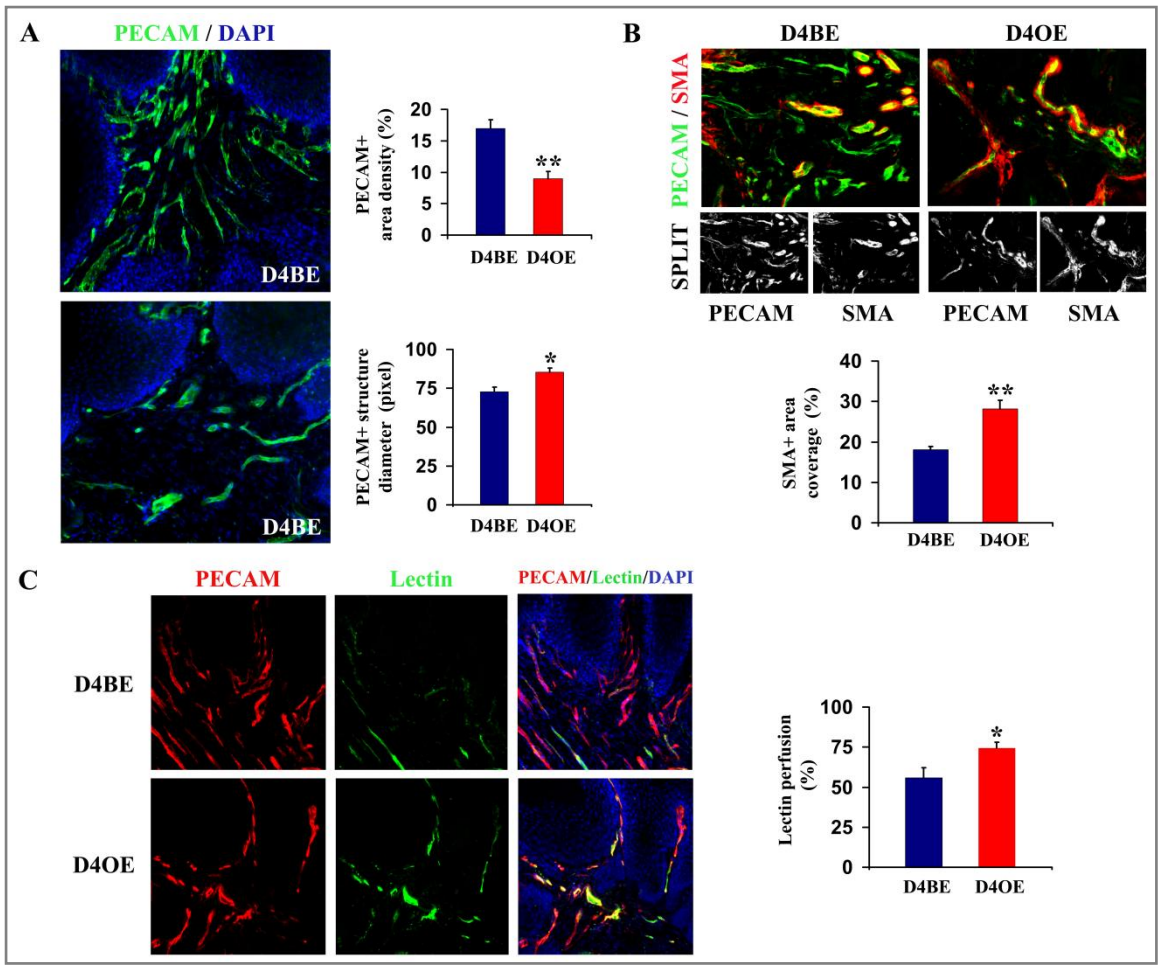

Figure 6.3. – Endothelial *Dll4* overexpression reduces vascular proliferation and promotes vessel maturation and perfusion in chemically-induced skin tumors. ***A,*** Reduced ramification and vascular density, and increased branch diameters in skin papillomas of D4OE *vs.* D4BE mice. PECAM immunostaining of skin tumors collected from DMBA/TPA-treated mice 20 weeks after the DMBA-initiation (*left*). Vascular density estimated as percentage of PECAM-positive area per surface of tumor stroma section and average neovessel caliber estimated by measuring diameters of PECAM-positive structures and presented for two experimental groups (*right*). ***B,*** Promoted vessel wall assembly in D4OE *vs.* D4BE mice. Mural cell recruitment assessed by PECAM/ α-SMA co-immunostaining (*above*). The percentage of PECAM-positive structures covered by α-SMA-positive area indicating the level of vessel maturation in two experimental groups (*below*). ***C,*** Increase in the lectin perfusion associated with endothelial *Dll4* overexpression. Simultaneous staining to PECAM and biotinylated lectin demonstrating the increase in the fraction of competent vessels in D4OE *vs.* D4BE papillomas (*left*). Percentage of PECAM-positive area co-localized with lectin (measured to quantify the portion of functional vessels within skin tumors (*right*). *Error bars* represent SEM. *****, $P < 0.05$ and ******, $P < 0.01$ were considered statistically significant and highly significant, respectively.

Immunostaining to PECAM indicated highly significant microvascular density reduction ($p < 0.01$) in papillomas of D4OE *vs.* D4BE mice (Figure 6.3. A). Comparable to morphological changes observed in subcutaneous xenografts, *Dll4* overexpression was shown to restrict PECAM-network branching but increase papilloma vessel lumen calibers ($p < 0.05$, Figure 6.3. A), α-SMA-positive mural cell recruitment ($p < 0.01$, Figure 6.3. B) and promote vessel competence as indicated by significantly increased lectin perfusion in papillomas developed by D4OE *vs.* D4BE ($p < 0.05$, Figure 6.3. C). In

this way, retarded growth of autochthonous skin tumors under the condition of increased endothelial Dll4/Notch signaling was demonstrated to be based a consequence the reduced tumor vascular density , even though Dll4 over-expression was simultaneously found to augment papilloma vessel functional capacity in papillomas.

6.4.3. Increased Dll4/Notch signaling affects the expression of principal angiogenesis regulators in chemically-induced skin papillomas

To study the molecular changes underlining morphological and functional vessel alterations caused by increase Dll4 expression, we used RT-PCR and analyzed D4BE and D4OE papillomas for differential expression of the main genes implicated in angiogenesis regulation (Figure 6.4.). Compared to D4BE papillomas, D4OE tumors had on average 2.8-fold increased Dll4 mRNA levels and 2.5-fold up-regulated *Hey2* expression confirming the Dll4/Notch pathway over-induction. Increased *Dll4* expression was associated with reduced *Jag1* Notch ligand expression levels. The *Vegf-a* mRNA levels were augmented probably due to increased hypoxia; nevertheless, dramatically reduced sensitivity to this major pro-angiogenic factor was indirectly indicated by ~2-fold increased trapper-receptor *Vegfr1* expression and >2-fold reduced principal receptor *Vegfr2*expression. Similarly, *Vegf-c* was up-regulated while its receptor *Vegfr3* expression was shown to be reduced. As expected, our analysis demonstrated an increase of *Pdgfr-β*, *EphrinB2* and *Tie2* activity accompanying promoted mural cell recruitment and vascular stability.

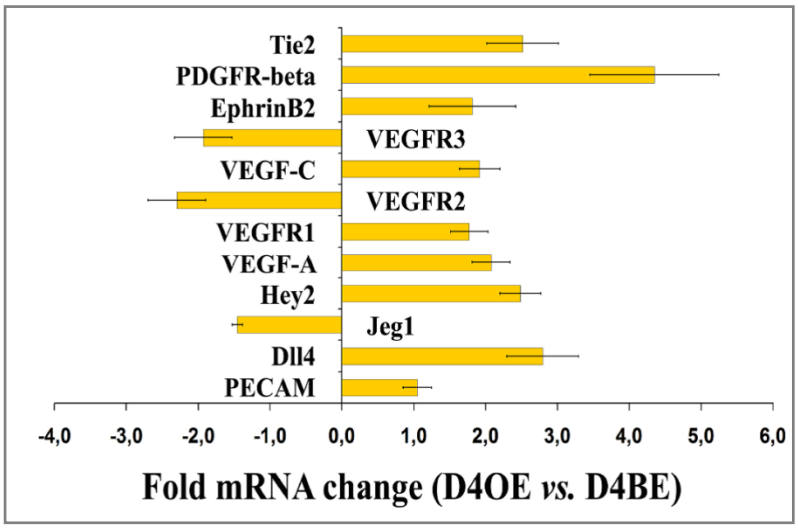

Figure 6.4. – Endothelial *Dll4* overexpression and increased Dll4/Notch signaling modify the expression of major angiogenesis regulators and/or their receptors in chemically-induced skin papillomas. Quantitative RT-PCR data normalized to β-actin levels and presented as x-fold change ofrelative mRNA levels of selected genes in D4OE tumor samples in comparison with the reference D4BE levels. *Error bars* represent SD.

6.4.4. Endothelial Dll4 overexpression reduces RT2 insulinoma growth, decreases vascular density and stabilizes tumor circulation

To confirm that *Dll4* overexpression results in tumor suppression independently on neoplasm histological tip, we also studied its impact on autochthonous RT2 insulinoma development. In this model, pancreatic islet carcinogenesis develops as a result of the simian virus SV-40 large T-antigen expression that is restricted to the insulin-producing β cells of the Langerhans islets due to the control of the Rat Insulin Promoter (Hanahan 1985). Although oncogene expression is detectable as early as the embryonic day E8.5, RT2 are born with normal pancreatic histology and hyperplastic/dysplastic islets begin to appear later, at 4-5 weeks of age, progressing during the next 5 weeks into angiogenic islets, subsequently encapsulated adenomas (insulinomas) and finally invasive carcinomas (Bergers, Javaherian et al. 1999). We generated RT2 Tie2-rtTA-M2 TetO7-Dll4 mice and simulated a therapeutic intervention by inducing endothelial *Dll4* overexpression in a mouse group (RT2 D4OE) *vs*. non-induced control group (RT2 D4BE) during the developing RT2 tumor stage (10 – 13.5 weeks old animals). When dissected in the phase corresponding to RT2 terminal-disease, RT2 D4BE and RT2 D4OE were found not to differ in terms of average number of insulinomas per mouse. On the other hand, average tumor volume and overall tumor burden were significantly reduced in RT2 D4OE compared to RT2 D4BE mice (~50% reduction, $p < 0.05$; Figure 6.5. A). Histologicaly, *Dll4* overexpression in RT2 D4OE mice was demonstrated to decrease insulinoma endothelial density by 32% ($p < 0.05$), increase α-SMA-positive mural cell coverage in ~20% ($p < 0.05$) and result in improved lectin perfusion by 27% ($p < 0.05$) in relation with RT2 D4OE insulinomas (Figure 6.5. B and C). Thus, amplified Dll4/Notch signaling repeated its effects on tumor growth and modified vascular response in RT2 insulinomas in the same way it previously influenced angiogenesis in LLC xenografts and chemically-induced skin tumors.

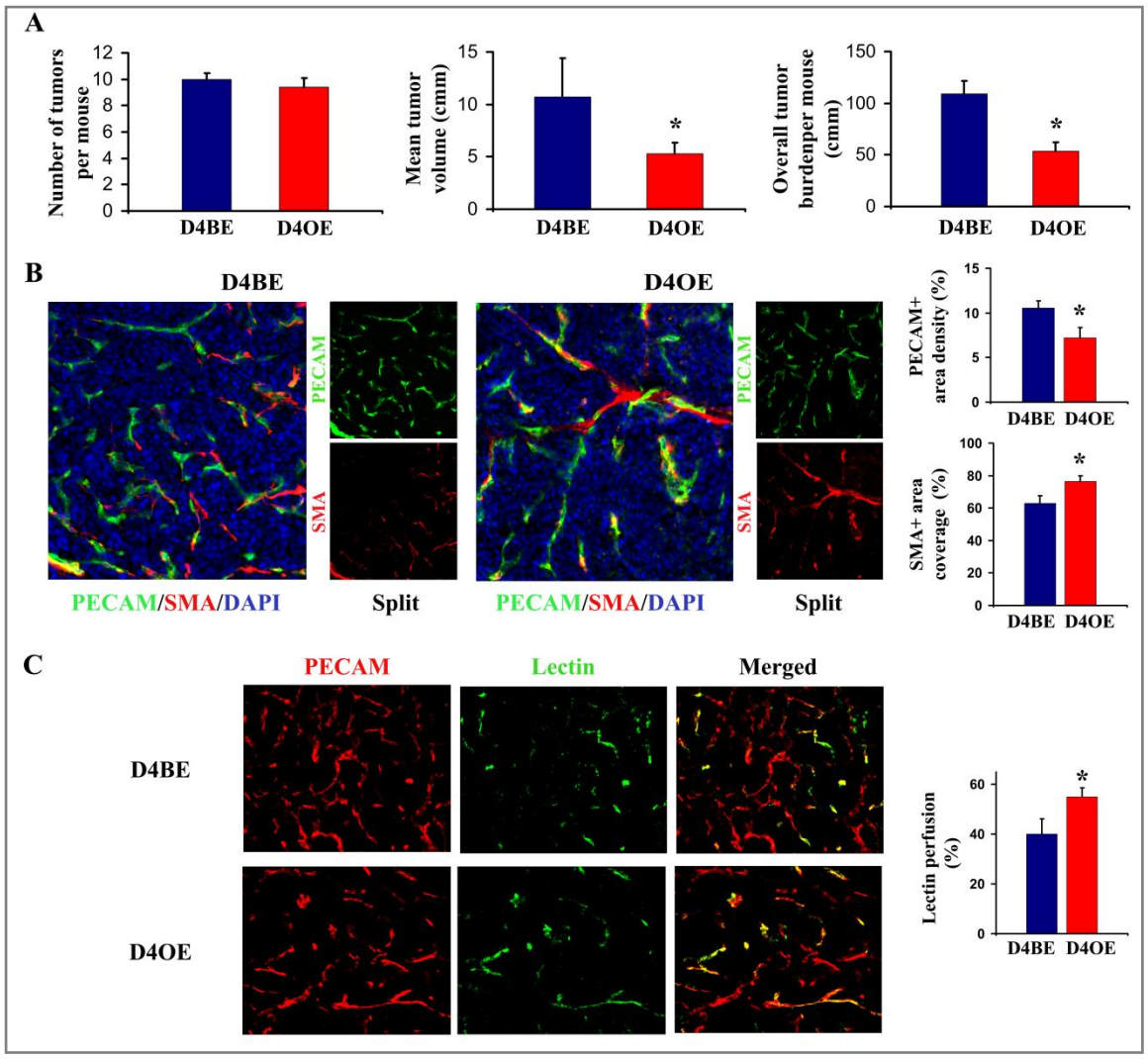

Figure 6.5. – Endothelial Dll4 overexpression negatively affects the RT2 insulinoma kinetics due to decreased vascular density although it enhances vascular maturation and competence. ***A,*** Number of tumors per mouse, mean tumor volume and over-all tumor burden per mouse, calculated as the sum of tumor volumes developed by a mouse, in 13.5-week old RT2 D4BE *vs.* RT2 D4OE mice. ***B,*** Double immunostaining to PECAM/α-SMA indicating reduced microvessel density and increased mural cell recruitment in RT2 D4OE *vs.* RT2 D4BE insulinomas (*left*). Percentage of PECAM-positive area per tumor section surface and percentage of PECAM-positive structures covered by SMA-positive area measured as parameters of vascular density and vessel maturation, respectively, and presented for two experimental groups (*right*). ***C***, Double staining to PECAM and biotinylated lectin demonstrating increased portion of perfused vessels in RT2 D4OE *vs.* RT2 D4BE tumors. Percentage of PECAM-positive area co-localized with lectin signals measured to quantify the portion of competent vessels within pancreatic tumors (*right*). *RT2,* RIP1-*Tag2* mice. *cmm,* cubic millimeters. *Error bars* represent SEM. *, $P < 0.05$ was considered statistically significant.

6.5. DISCUSSION

The inhibition of Dll4/Notch signaling was demonstrated in preclinical models to induce immature and non-functional vessel proliferation on an accelerated rate and result in poor blood supply and consistent growth inhibition of different mouse, rat and human tumors (Djokovic, Trindade et al. ; Noguera-Troise, Daly et al. 2006; Ridgway, Zhang et al. 2006; Scehnet, Jiang et al. 2007). Nevertheless, Dll4/Notch pathway-blockade remains a strategy with still unpredictable clinical usefulness. Basically, vascular defect self-repair and reperfusion over a long-term Dll4/Notch-suppression might convert regressive tumor behavior, particularly in the association with increasing malignant cell invasiveness, previously documented as a consequence of antiangiogenic-induced hypoxia (Hayden 2009). Besides, reduced vessel competence due to Dll4/Notch-inhibition can be expected to limit concomitant chemotherapy effectiveness. Therefore, we examined the effects of the opposite strategy based on endothelial *Dll4* overexpression that was anticipated to reduce vascular response within tumors and suppress their expansion. In addition, *Dll4* overexpression was expected to promote vessel maturation and stabilize neoplasm vasculature reducing the remodeling capacity of tumor vessels and, in this way, the risk of development of therapy resistance.

Our results demonstrate that endothelial *Dll4* overexpression reduces the growth of LLC xenografts, autochthonous chemically-induced skin papillomas and RT2 insulinomas. In all three models, remarkable tumor burden reduction due to *Dll4* overexpression was consistently associated with decreased endothelial density and presumed reduction of the overall tumor blood supply. In contrast, vascular maturity and functionality were improved as evidenced by the formation of larger branches, increased vessel network perfusion and increased mural cell recruitment. However, improved vessel competence was not found to be predominant over the beneficial effects caused by restricted vessel proliferation but might result in better cytostatic or other drug delivery at the tumor site. In addition, the enhanced vessel wall maturation seen in endothelial *Dll4* overexpressing mice may decrease the penetration of the malignant cells into the circulation and metastisization. Both the potential benefit of Dll4 overexpression in increasing chemotherapy effectiveness and its influence on metastasis formation rates are promising and should be explored in future work.

The comparative gene expression analysis of skin tumors developed by wild-type *vs*. *Dll4* overexpression mice confirmed that Dll4/Notch signaling restricts VEGF-

dependent neoangiogenesis. Although *Vegf-a* was found to be up-regulated under the conditions of amplified Dll4/Notch signaling, and this is likely to be due to increased hypoxia, reduced vascular sensitivity to VEGF-A was achieved by reduced *Vegfr2* expression and simultaneous up-regulation of *Vegfr1*, which lacks signaling activity. Explaining, at least partially, the molecular mechanisms leading to reduced vascular response in *Dll4* overexpressing *vs.* control tumors, these findings also point out the potential capacity of endothelial *Dll4* overexpression to increase the efficacy of currently available VEGF signaling-inhibitors whose clinical success has been limited by development of tumor-resistance. Similarly to VEGF-A, VEGF-C, a positive driver of normal lymphangiogenesis and an additional tumor pro-angiogenic factor (Tammela, Zarkada et al. 2008), was also found up-regulated possibly due to more pronounced hypoxia while *Dll4* overexpression in tumor endothelial cells decreased receptor VEGFR3 levels and, thereby, tumor vascular sensitivity to VEGF-C. Interestingly, increased Dll4/Notch signaling in tumor endothelium was found to down-regulate *Jag1* ,, another Notch ligand involved in the control of VEGF-dependent endothelial cell sprouting, was previously demonstrated to antagonize Dll4 signaling and play a pro-angiogenic role in the mouse neonatal retina (Benedito, Roca et al. 2009).

Concerning the improved pericyte recruitment, we found *Dll4* overexpression to influence the expression of both Ephrin-B2 and platelet-derived growth factor receptor beta (PDGFRβ) . In several developing tissues, binding of Ephrin-B2 to its receptor EphB4 modulates adjacent endothelial cell interactions, while Ephrin-B2/EphB4 signaling between endothelial and mural cells controls mural cell motility and adhesion (Adams and Alitalo 2007). In parallel, high levels of platelet-derived growth factor B (PDGFB) in proliferating endothelial cells promote the recruitment of pericytes that express the PDGFRβ (Adams and Alitalo 2007). Our evidence suggests that Dll4/Notch signaling amplification stabilizes tumor vessels by enhancing EphrinB2/EphB4 and PDGF/ PDGFRβ signaling and, therefore, promoting vascular maturation. Induced *Tie2* expression can be considered complementary since Tie2, when activated by angiopoietin 1, is essential to maintain the endothelium in the quiescent state (Wong, Haroon et al. 1997) by promoting mural cell recruitment (Asahara, Chen et al. 1998).

Transduced malignant cells that over-express membrane Dll4 (entire molecule or functional extra-cellular portion) were previously found in subcutaneous tumor grafts to result in reduced tumor vessel density and produce wide, straight and less branched

vessels (Noguera-Troise, Daly et al. 2006; Li, Sainson et al. 2007; Segarra, Williams et al. 2008). Although conflicting data were obtained regarding these neovessel functional capacities and repercussions on tumor expansion, a significant number of tumor lines responded with regression to *Dll4* overexpression while quite minimal Notch activation was noted in several other tumors characterized as unresponsive to Dll4/Notch activation (Segarra, Williams et al. 2008). This study focused on endothelial *Dll4* overexpression, since Dll4/Notch predominantly mediates EC-EC rather than malignant cell-EC communication, as simulated in previous *Dll4* overexpression experiments, even though the Dll4 molecule does appear in a wide range of human malignant cells (Martinez, Muller et al. 2009). In addition, considering the accessibility of the tumor endothelium, therapeutic *Dll4* delivery to the neoplastic cells seems much more complex to implement than endothelial targeting. More importantly, as the generalized Notch1/4 activation by a systemic agonist would certainly produce several side-effects caused by the perturbation of different Notch-dependent physiological processes, selective *Dll4* genetic sequence delivery, *e.g.* using endothelial-specific liposomes, might restrict Notch over-activation to sites of active angiogenesis.

In summary, this study suggests that endothelial *Dll4* overexpression an effective mean to suppress tumor angiogenesis and neoplasm growth, without observed toxic side-effect.. Mechanistically, it has potential to provide synergy with VEGF inhibitors and enhance chemotherapy effectiveness. Therefore, endothelial *Dll4* overexpression is worth further investigation.

REFERENCES:

Adams, R. H. and K. Alitalo (2007). "Molecular regulation of angiogenesis and lymphangiogenesis." Nat Rev Mol Cell Biol **8**(6): 464-78.

Allegra, C. J., G. Yothers, et al. (2011). "Phase III trial assessing bevacizumab in stages II and III carcinoma of the colon: results of NSABP protocol C-08." J Clin Oncol **29**(1): 11-6.

Asahara, T., D. Chen, et al. (1998). "Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal neovascularization." Circ Res **83**(3): 233-40.

Benedito, R., C. Roca, et al. (2009). "The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis." Cell **137**(6): 1124-35.

Bergers, G., K. Javaherian, et al. (1999). "Effects of angiogenesis inhibitors on multistage carcinogenesis in mice." Science **284**(5415): 808-12.

Bray, S. J. (2006). "Notch signalling: a simple pathway becomes complex." Nat Rev Mol Cell Biol **7**(9): 678-89.

Djokovic, D., A. Trindade, et al. "Combination of Dll4/Notch and Ephrin-B2/EphB4 targeted therapy is highly effective in disrupting tumor angiogenesis." BMC Cancer **10**: 641.

Duarte, A., M. Hirashima, et al. (2004). "Dosage-sensitive requirement for mouse Dll4 in artery development." Genes Dev **18**(20): 2474-8.

Ebos, J. M. and R. S. Kerbel (2011). "Antiangiogenic therapy: impact on invasion, disease progression, and metastasis." Nat Rev Clin Oncol **8**(4): 210-21.

Fischer, M., W. C. Yen, et al. (2011). "Anti-DLL4 inhibits growth and reduces tumor-initiating cell frequency in colorectal tumors with oncogenic KRAS mutations." Cancer Res **71**(5): 1520-5.

Gale, N. W., M. G. Dominguez, et al. (2004). "Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development." Proc Natl Acad Sci U S A **101**(45): 15949-54.

Hainaud, P., J. O. Contreres, et al. (2006). "The role of the vascular endothelial growth factor-Delta-like 4 ligand/Notch4-ephrin B2 cascade in tumor vessel remodeling and endothelial cell functions." Cancer Res **66**(17): 8501-10.

Haller, B. K., A. Brave, et al. (2011). "Therapeutic efficacy of a DNA vaccine targeting the endothelial tip cell antigen delta-like ligand 4 in mammary carcinoma." Oncogene **29**(30): 4276-86.

Hanahan, D. (1985). "Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes." Nature **315**(6015): 115-22.

Hayden, E. C. (2009). "Cutting off cancer's supply lines." Nature **458**(7239): 686-7.

Hoey, T., W. C. Yen, et al. (2009). "DLL4 blockade inhibits tumor growth and reduces tumor-initiating cell frequency." Cell Stem Cell **5**(2): 168-77.

Holash, J., P. C. Maisonpierre, et al. (1999). "Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF." Science **284**(5422): 1994-8.

Jain, R. K. (2005). "Antiangiogenic therapy for cancer: current and emerging concepts." Oncology (Williston Park) **19**(4 Suppl 3): 7-16.

Jain, R. K., D. G. Duda, et al. (2006). "Lessons from phase III clinical trials on anti-VEGF therapy for cancer." Nat Clin Pract Oncol **3**(1): 24-40.

Jubb, A. M., E. J. Soilleux, et al. (2011). "Expression of vascular notch ligand delta-like 4 and inflammatory markers in breast cancer." Am J Pathol **176**(4): 2019-28.

Jubb, A. M., H. Turley, et al. (2009). "Expression of delta-like ligand 4 (Dll4) and markers of hypoxia in colon cancer." Br J Cancer **101**(10): 1749-57.

Kalen, M., T. Heikura, et al. (2011). "Gamma-secretase inhibitor treatment promotes VEGF-A-driven blood vessel growth and vascular leakage but disrupts neovascular perfusion." PLoS One **6**(4): e18709.

Krebs, L. T., J. R. Shutter, et al. (2004). "Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants." Genes Dev **18**(20): 2469-73.

Li, J. L., A. M. Jubb, et al. (2010). "Targeting DLL4 in tumors shows preclinical activity but potentially significant toxicity." Future Oncol **6**(7): 1099-103.

Li, J. L., R. C. Sainson, et al. (2007). "Delta-like 4 Notch ligand regulates tumor angiogenesis, improves tumor vascular function, and promotes tumor growth in vivo." Cancer Res **67**(23): 11244-53.

Mailhos, C., U. Modlich, et al. (2001). "Delta4, an endothelial specific notch ligand expressed at sites of physiological and tumor angiogenesis." Differentiation **69**(2-3): 135-44.

Martinez, J. C., M. M. Muller, et al. (2009). "Nuclear and membrane expression of the angiogenesis regulator delta-like ligand 4 (DLL4) in normal and malignant human tissues." Histopathology **54**(5): 598-606.

Noguera-Troise, I., C. Daly, et al. (2006). "Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis." Nature **444**(7122): 1032-7.

Noguera-Troise, I., C. Daly, et al. (2007). "Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis." Novartis Found Symp **283**: 106-20; discussion 121-5, 238-41.

Paez-Ribes, M., E. Allen, et al. (2009). "Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis." Cancer Cell **15**(3): 220-31.

Patel, N. S., M. S. Dobbie, et al. (2006). "Up-regulation of endothelial delta-like 4 expression correlates with vessel maturation in bladder cancer." Clin Cancer Res **12**(16): 4836-44.

Phng, L. K. and H. Gerhardt (2009). "Angiogenesis: a team effort coordinated by notch." Dev Cell **16**(2): 196-208.

Ridgway, J., G. Zhang, et al. (2006). "Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis." Nature **444**(7122): 1083-7.

Saltz, L. B., H. J. Lenz, et al. (2007). "Randomized phase II trial of cetuximab, bevacizumab, and irinotecan compared with cetuximab and bevacizumab alone in irinotecan-refractory colorectal cancer: the BOND-2 study." J Clin Oncol **25**(29): 4557-61.

Scehnet, J. S., W. Jiang, et al. (2007). "Inhibition of Dll4-mediated signaling induces proliferation of immature vessels and results in poor tissue perfusion." Blood **109**(11): 4753-60.

Segarra, M., C. K. Williams, et al. (2008). "Dll4 activation of Notch signaling reduces tumor vascularity and inhibits tumor growth." Blood **112**(5): 1904-11.

Shojaei, F. and N. Ferrara (2007). "Antiangiogenic therapy for cancer: an update." Cancer J **13**(6): 345-8.

Suchting, S., C. Freitas, et al. (2007). "The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching." Proc Natl Acad Sci U S A **104**(9): 3225-30.

Tammela, T., G. Zarkada, et al. (2008). "Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation." Nature **454**(7204): 656-60.

Trindade, A., S. R. Kumar, et al. (2008). "Overexpression of delta-like 4 induces arterialization and attenuates vessel formation in developing mouse embryos." Blood **112**(5): 1720-9.

Wong, A. L., Z. A. Haroon, et al. (1997). "Tie2 expression and phosphorylation in angiogenic and quiescent adult tissues." Circ Res **81**(4): 567-74.

Yan, M., C. A. Callahan, et al. (2010). "Chronic DLL4 blockade induces vascular neoplasms." Nature **463**(7282): E6-7.

CHAPTER IV

GENERAL DISCUSSION AND CONCLUSIONS

Djokovic D., “End Road Star”, collage (2011)

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General Discussion, Open Issues and Conclusions

The results obtained during the course of the present research have provided major insights into the function of Dll4/Notch signaling in tumor-driven angiogenesis, indicating that Dll4 might be a useful therapeutic target. These findings are discussed in specific sections of **Chapter III (Publication I – IV)** , and here, they will be interpreted in a unifying manner and at the light of relevant, concomitant and more recent publications related with the implication of the Dll4/Notch pathway in the regulation of tumor vascular response.

Tumor angiogenesis depends on VEGFA, other growth factors and different modifiers. The Notch ligand Dll4, a critical regulator of embryonic vascular development and arterial specification (Duarte, Hirashima et al. 2004), appears as a prominent marker of angiogenic endothelium within tumor microenvironment (Mailhos, Modlich et al. 2001; Gale, Dominguez et al. 2004; Patel, Dobbie et al. 2006). Its cognate receptor genes, *Notch 1* and *Notch 4*, are also up-regulated in tumor vasculature (Mailhos, Modlich et al. 2001). Notch ligand expression and Notch receptor activation is induced by VEGFA (Williams, Li et al. 2006). Analyzing subcutaneous tumor grafts in *Dll4*$^{+/-}$ mice and exploiting the *LacZ* reporter included in the targeting vector used to generate the mutant mice, we initially confirmed that the expression of *Dll4* was much higher in the neoplasm vasculature than in adjacent normal vessels (**Publication I**). Simultaneously, Noguera-Troise and colleagues demonstrated that the blockade of VEGFA in mice bearing tumor implants resulted in a profound *Dll4* down-regulation (Noguera-Troise, Daly et al. 2006). Thus, the high levels of *Dll4* expression in tumor endothelium observed by us and others were assumed to be a consequence of relatively high levels of VEGFA signaling in tumor vessels in comparison to normal tissue vessels.

Contrary to early belief, *Dll4* expression was not only identified in tumor endothelial cells but also in malignant cells of a range of neoplasm types as well as in reactive tissues (Martinez, Muller et al. 2009). Despite the fact that these results suggest

the Dll4 function to be involved in cellular mechanisms of certain malignant cells themselves, we did not observe extra-vascular Dll4 in the tumor models used throughout the present work. Indeed, Dll4 has been recently found to have an impact on colon cancer stem cell frequency while suppressing apoptosis in tumor cells (Hoey, Yen et al. 2009; Fischer, Yen et al. 2011). Nevertheless, vascular endothelium clearly represents the most prominent and constant site of *Dll4* expression within tumors.

To define the function of increased expression of *Dll4* in tumor vessels, we performed a comparative analysis of the vasculature of tumor cell implants grown in age-matched wild-type and $Dll4^{+/-}$ mice (**Publication I**). In the first place, it was found that the tumor vascular response in $Dll4^{+/-}$ mice was marked by highly increased vascular density. In contrast to the wild-type mice, the neovessels in the mutants had narrow calibers, exaggerated and aberrant branching pattern and lacked any hierarchic organization. Secondly, recruitment of pericytes to the newly forming tumor capillaries was significantly reduced in $Dll4^{+/-}$ mice when compared with wild-type littermates. Thus, tumor angiogenic response was increased but defective and vessel maturation was severely compromised. In parallel, the expression of a blocker of Dll4/Notch signaling (soluble Dll4) by transduced tumor cells was demonstrated to result in the formation of dense, highly branched and markedly irregular networks composed of fine and weakly perfused interconnections, while the expression of the full-length membrane bound form of Dll4 caused Notch activation in the tumor blood vessels and resulted in a reduction of tumor vessel sprouting and straighter branch formation (Noguera-Troise, Daly et al. 2006). Altogether, these data indicate that the Dll4-mediated signaling in tumors serves as a negative regulator of sprouting angiogenesis and an important pro-maturation factor that is required for both regular branching and mural cell recruitment, contributing to the formation of productive tumor vasculature.

Pharmacological Dll4/Notch inhibition, which we subsequently achieved by intraparitoneal administration of soluble extra-cellular Dll4 domain (sDll4) in xenograft-bearing mice, was observed to lead to the defects in tumor vessels that are qualitatively identical to those caused by targeted *Dll4* allele deletion (**Publication I**). Even more importantly, our evidence was that systemic sDll4 application markedly retarded tumor growth. Notch receptors are activated by membrane-associated ligands and sDll4 biding disables the endogenous Notch activation, as we confirmed by detecting that sDll4 treatment down-regulated the expression of the Notch target genes *Hey* and *Hes*. sDll4 modifies newly forming vessels which proliferate excessively, being highly deficient in

maturation and perfusion. Although tumor growth rate generally correlates with vascular density, the formation of such a poorly low-functional vascular network did not provide appropriate conditions to support malignant cell proliferation. Systemically applied soluble Dll4 variants generated by others and antibodies blocking Dll4/Notch signaling were also shown to enhance immature and non-productive vascular responses, resulting in decreased perfusion, tumor hypoxia and suppressed neoplasm expansion, with little or not observed systemic toxicity (Noguera-Troise, Daly et al. 2006; Ridgway, Zhang et al. 2006). In addition to angiogenesis inhibitors, such as VEGFA signaling blockers, that suppress tumor progression by reducing tumor microvascular density, our and other experiments assessing the effects of Dll4/Notch inhibition provided thus a documented basis for a novel concept that tumor starvation and consequent growth retardation may be further amplified by inducing non-productive angiogenic response. While VEGFA stimulates tumor endothelium to proliferate, Dll4/Notch signaling, induced by VEGFA itself (Williams, Li et al. 2006; Noguera-Troise, Daly et al. 2007), suppresses chaotic sprouting and branching, and promotes vessel wall assembly, vessel lumenization and perfusion. In this way, Dll4/Notch ensures the formation of vasculature capable of providing tumors with oxygen and nutrients. Thereby, it can be assumed that combined inhibition of VEGFA and Dll4/Notch signaling pathways, when appropriately balanced, may be more beneficial that VEGFA inhibition alone, resulting in both reduced and non-productive tumor angiogenesis.

To extend the information concerning the effects of Dll4/Notch targeting on tumor angiogenesis that was obtained from tumor xenografts, we used both genetic and pharmacological approaches to evaluate the signaling inhibition in autochthonous tumor models (**Publication II** and **III**). Clinical trials of some anti-angiogenic agents, most notably those testing matrix metalloproteinase (MMP) inhibitors, revealed that ectopic graft models may provide erroneous prediction regarding the real effects on human disease (Coussens, Fingleton et al. 2002; Cristofanilli, Charnsangavej et al. 2002). In the first instance, we chose to make use of transgenic RIP1-*Tag2* (RT2) tumor model of pancreatic islet carcinogenesis wherein β cell tumors (insulinomas) arise and develop in a way that resembles the human disease, host/tumor interactions are more reliably reflected then in grafted models while predictable stepwise progression and angiogenic switch permits investigation of tumor angiogenesis and its inhibitors (Hanahan 1985; Bergers, Javaherian et al. 1999). We analyzed the consequences of *Dll4* allelic deletion on

insulinoma onset, growth, associated vascular response and mouse longevity (**Publication II**). Our results showed that *Dll4* haploinsufficiency did not influence oncogene-driven β-cell transformation since the difference in number of tumors per mouse was not statistically significant between RT2 *Dll4*$^{+/+}$ and RT2 *Dll4*$^{+/-}$ littermates. However, remarkably retarded tumor growth accompanied with increased RT2 *Dll4*$^{+/-}$ mouse longevity in comparison with the control RT2 *Dll4*$^{+/+}$ mice pointed out the importance of Dll4/Notch function for tumor development as well as the beneficial effect of Dll4/Notch inhibition previously observed in xenograft-based experiments. Mechanistically, in autochthonous insulinomas as well as in ectopic grafted tumors, *Dll4* allele deletion resulted in increased vessel sprouting and led to the establishment of dense vascular networks with fragile branches that lacked adequate mural cell stabilization and, clearly, the capacity to provide the tumor with needed blood supply.

This excessive and aberrant vascular response associated with reduced *Dll4* expression levels in RT2 tumor vessels, was next confirmed to reflect increased tumor vessel sensitivity to VEGFA (**Publication II**), as was the case in post-natal retinal angiogenesis upon genetic or pharmacological Dll4/Notch suppression (Lobov, Renard et al. 2007; Suchting, Freitas et al. 2007). We have shown that Dll4/Notch impairment promoted VEGFA production, probably through deteriorated tumor tissue oxygenation. There were also an increase in VEGFR2 levels and reduced expression of *Vegfr1*. As presented in more detail in **Introduction**, VEGFR2 is the VEGFA principal receptor while VEGFR1 binds VEGFA with much greater affinity then VEGFR2 and functions as a VEGFA trapper having extremely weak signal-transducing properties (Shibuya and Claesson-Welsh 2006). Increased VEGFR2/VEGFR1 ratio in *Dll4* haploinsufficient RT2 mice enhanced VEGFA signaling and thus increases endothelial proliferation and vascular density. In addition, *Vegfr3* was induced in insulinomas of RT2 *Dll4*$^{+/-}$ mice compared to the RT2 *Dll4*$^{+/+}$ littermates. The VEGF-C/VEGFR3 system is the main lymphangiogenesis positive regulator (Karpanen, Wirzenius et al. 2006); nevertheless, up-regulated *Vegfr3* expression in novel blood vessels aside of lymphatic endothelium might have contributed to the enhanced vascular response associated with *Dll4* allelic deletion.

Regarding the defective mural cell recruitment related with impaired Dll4/Notch signaling, we focused on platelet-derived growth factor B (PDGF-B)/PDGFRβ, angiopoietin/Tie2, and EphB4/EphrinB2 signaling (**Publication II**). Preserved function of PDGFRβ in pericytes is required for proper mural cell coverage of the tumor vessels

(Shaheen, Tseng et al. 2001; Abramsson, Lindblom et al. 2003; Bergers, Song et al. 2003). Unexpectedly, increased PDGFR-β levels were identified in RT2 *Dll4*$^{+/-}$ *vs.* RT2 *Dll4*$^{+/+}$ insulinomas. This probably occurred due to feedback from defective vessel maturation. On the other hand, the angiopoietin receptor Tie2 (Sato, Tozawa et al. 1995) was decreased in *Dll4*$^{+/-}$ *vs. Dll4*$^{+/+}$ insulinomas. Tie2 serves as an endothelial cell survival factor and promotes perivascular cell recruitment (Peters, Kontos et al. 2004). Thereby, its down-regulation in *Dll4*$^{+/-}$ tumors may represent one of the reasons contributing to the mural cell depletion seen under conditions of Dll4 deficiency. Furthermore, *Dll4* allelic deletion was associated with reduced EphrinB2 level in RT2 neoplastic lesions. EphrinB2 helps the recruitment of mural cells to nascent endothelial sprouts and its absence results in pronounced vessel dilation and bleeding (Adams, Wilkinson et al. 1999; Foo, Turner et al. 2006). Thus, reduced EphrinB2 levels were additionally considered as an important contributor to aberrant vessel wall assembly in *Dll4*$^{+/-}$ insulinomas.

As the *Dll4* allelic deletion resulted in a tumor-suppressive outcome in spontaneous RT2 insulinomas and the morphological and molecular bases of this effect became more apparent, we used the same experimental tool, the RT2 model, to assess the efficacy of sDll4. Facing a great need to improve anti-cancer angiogenesis-modulating therapy that seems possible to be achieved by multiple pathway targeting, the Notch pharmacological suppression by sDll4 was also tested in combination with sEphB4, an antagonist of EphB4/EphrinB2 signaling (**Publication II**). EphrinB2 and EphB4 represent another ligand-receptor pair which interaction on adjacent cells activates bidirectionally signaling in ligand-expressing and receptor-expressing cells (Adams, Wilkinson et al. 1999). While Ephrin-B2 is a marker of both arterial endothelial cells and perivascular mesenchymal cells, EphB4 is restricted in the venous endothelium (Adams and Alitalo 2007). EphB4/EphrinB2 signaling is required for proper arterial-venous endothelium specification (Gerety and Anderson 2002). Other members of Eph receptor femily, including EphB1, EphB2, EphB3 and EphA4, also interact with EphrinB2 (Kullander and Klein 2002). Its activation in vascular *tip cells* promotes motile behaviour and directs VEGF-induced endothelial sprouting by stimulating the spatial activation of VEGFR2 endocytosis (Sawamiphak, Seidel et al. 2010). In addition, mural cells require Ephrin-B2 for normal association with novel endothelial sprouts (Foo, Turner et al. 2006). The soluble monomeric form of the extracellular domain of EphB4 (sEphB4) functions as an

antagonist of EphB4/EphrinB2 signaling, being previously documented to suppress sprouting angiogenesis in tumor models (Kertesz, Krasnoperov et al. 2006; Scehnet, Ley et al. 2009).

In our trials with sDll4 and sEphB4 (**Publication II**), both compounds reduced the rate of insulinomas growth when applied as monotherapies in RT2 mice. sEphB4 was highly efficient while, in the chosen inhibitory concentrations, sDll4 resulted in even more pronounced tumor suppression. As in the case with *Dll4* haploinsufficiency, the number of neoplastic lesions per mouse remained substantially unchanged in sDll4-treated as well as in sEphB4-treated mice when compared with untreated controls and between themselves. Thus, the malignant transformation in the RT2 model was confirmed to be independent on Dll4/Notch and EphB4/EphrinB2 while the tumor growth was retarded by the inhibition of these signaling pathways. Combination of two inhibitors was superior to each antagonist alone. sDll4 resulted in enhanced endothelial proliferation in RT2 insulinomas, consistent with previously observed VEGFA/VEGFR2 activation resulting from Dll4/Notch inhibition while sEphB4 had the opposite effect, in accordance with findings that Ephrin-B2 positively controls VEGFR2 internalization and signalling (Sawamiphak, Seidel et al. 2010). Both compounds, however, were evidenced to compromise tumor neovessel maturation defined by reduced mural cell recruitment, enhanced vessel leakiness and impaired lumenization. Considering the fact that the combination of the two compounds resulted in vessel density slightly below the controls, the vascular competence rather than tumor vessel density determines the neoplasm expansion.

VEGF induces Dll4 and both VEGF and Dll4 induce EphrinB2 (Iso, Maeno et al. 2006; Yamanda, Ebihara et al. 2009). Our evidence is also that *Efnb2* is down-regulated in $Dll4^{+/-}$ mice, representing, simultaneously with reduced *Tie2* expression, an effector mechanism that contributes to impaired vessel wall assembly associated with Dll4 deficiency. Although sDll4 markedly increases and sEphB4 therapy significantly reduces tumor microvessel density, both compounds lead to the paucity of pericyte recruitment and non-productive vessel formation. Importantly, our trials show that simultaneous Dll4/Notch and EphrinB2 inhibition has cumulative efficacy in blocking vessel maturation and perfusion of the tumor. Thus, inhibition of Dll4/Notch and EphrinB2/EphB4 provide effects that are not overlapping even though Dll4/Notch signaling induces Ephrin-B2. In addition, other reasons support the combined use of Dll4 and EphrinB2 inhibitors. sEphB4-mediated inhibition of EphB4-EphrinB2 increases

hypoxia and consequently the expression of *Vegfa* and *Dll4* (Scehnet, Ley et al. 2009). Besides, sEphB4 treatment induces the expression of PSENEN (Iwatsubo 2004), a component of gamma-secretase presenilin complex which is responsible for the cleavage of Notch receptor after ligand binding and activation of the signaling (Selkoe and Kopan 2003). Since EphrinB2 is downstream to Dll4/Notch signaling, up-regulation of PSENEN may be a feedback when EphrinB2 signaling is individually blocked, enhancing the Notch function.

Regarding the safety concerns, we have not observed that *Dll4* allelic deletion in CD1 and RT2 mice could cause vascular or other lesions in vital organs such as heart, lung, kidney, brain, liver and intestinal tract (**Publication I and II)**. Equally, no toxic side-effects were observed to follow the intermittent administration of tumor-suppressive doses of sDll4, alone or in combination with sEphB4, in RT2 mice (**Publication II)**. In contrast, chronic application of Dll4/Notch antagonists has been found to induce benign vascular neoplasms in rat liver as well as prominent atrophy of mouse thymus where Dll4/Notch1 signaling is involved in the regulation of T-cell development (Yan, Callahan et al. 2010). Using a conditional, endothelial specific Dll4 knock-out mouse line, the host laboratory evidenced that these mutants also develop alterations in liver architecture. They include macroscopic micro-nodular liver surface aspect, areas with markedly dilated sinusoids and excessive subcapsular vessel proliferation with sporadic hemangioma-like structure formation (**Publication II**). Encouragingly, endothelial Dll4 knock-out mice did not show evidence of vascular proliferative lesions when treated with sEphB4. In other words, simultaneous blockade of Dll4/Notch and Ephrin-B2/EphB4 abolish hepatic alterations seen when only Dll4/Notch signaling is interrupted. Further specific studies are required to provide a comprehensive understanding of the side effects arising from therapeutic Dll4/Notch inhibition. Special attention should be paid to the sites where physiological and reparatory angiogenesis occur, namely the female reproduction tract, bone fractures and soft tissue traumatic and ischemic lesions.

Despite its impact on normal tissue homeostasis that remains to be better studied, Dll4/Notch inhibition opens up a possibility for improved control of invasive tumor, as strongly suggested by the presented findings. In order to evaluate the Dll4 function in benign tumors and precancerous lesion, we complementary studied the effects of targeted *Dll4* deletion in another autochthonous, DMBA/TPA-induced model of skin tumorigenesis (**Publication III**). In this experimental setting, chemically-mediated

formation of highly angiogenic squamous papillomas is followed by squamous cell carcinoma (SSC) development (Demehri, Turkoz et al. 2009), with tumor kinetics timely more gradual then in RT2 mice. We focused on the early tumorigenesis phase (benign papilloma formation) in wild-type *vs.* *Dll4*$^{+/-}$ mice. In contrast to ectopic malignant xenografts and RT2 insulinomas, in this case Dll4/Notch signaling was evidenced to be protective. Genetic mutation resulting in 50% reduction of constitutional *Dll4* expression reduced squamous papilloma latency, increased tumor multiplicity and enhanced tumor growth while malignant transformation rate did not differ between control mice and *Dll4*+/- mutants. Although the effect of *Dll4* allelic deletion on chemical papillomas was apparently in contradiction with previous data, analytic insight into the vascular response to expressional *Dll4* reduction confirmed Dll4/Notch to prevent excessive endothelial sprouting and promote vascular maturation, since its impairment was followed by increased tumor microvascular density, branching aberrations and inappropriate mural cell recruitment. Reiteratively, our evidence associated the observations of enhanced endothelial activation with increased VEGFR2/VEGFR1 ratio and impaired vessel wall assembly with down-regulated *Tie2* and *Efnb2*. However, altered vascular hierarchy, increased branch malformations and reduced vessel maturation in *Dll4*$^{+/-}$ papillomas relative to the wild-type mouse lesions did not significantly compromise vessel perfusion, as it was the case in advanced RT2 and grafted malignancies. Thus, the difference relates to the vascular function in the tumors.

Since VEGFA is increased in papillomas in comparison with normal tissues, but not to its level in the invasive tumors (Larcher, Robles et al. 1996), we speculate that different impact of *Dll4* haploinsufficiency on the angiogenic response and growth of early lesions with benign behavior *vs.* late, invasive tumors relates to the background level of VEGFA. In both neoplasm types, Dll4/Notch inhibition increases endothelial cell sensitivity to VEGFA and consequently tumor vessel density. In the papillomas of *Dll4*$^{+/-}$ *vs.* wild type mice, we did document a pronounced increase in VGFR2 to VGFR1 measured at protein and mRNA levels. However, under the condition of relatively modest VEGFA background of benign tumors, partially inhibited Dll4/Notch still allows proliferating neovessel networks to maintain a certain organization and gain functionality, promoting at the same time angiogenesis and tumor growth. That is not the case in malignancies with high VEGFA levels and, thus reduced VEGFA gradients, where preserved Dll4/Notch function is essential to suppress excessive endothelial activation, help directional sprouting and prevent vessel malformations and leakiness. Hence, Dll4

functions as a critical negative regulator of neoplasm vascularisation in both early and late tumor development, while its requirement for promoting vascular organization and functionality increases, simultaneously with the VEGFA production, in malignant lesions.

In sequence (**Publication III**), we demonstrated that *Dll4* allelic deletion in skin papilloma reduced the effectiveness of sorafenib, a small molecule inhibitor of a broad spectrum of receptor tyrosine kinases including VEGFR2, VEGFR3 and B-Raf (Kerbel 2008). This is consistent with our findings that VEGFR2 function is promoted upon the Dll4/Notch inhibition. Reduced efficacy of tyrosine kinases inhibitors, such as approved sorafenib and sunitinib, might also occur and be even more pronounced in malignant tumors with high VEGFA levels. Additionally, partial Dll4/Notch inhibition and simultaneous therapeutic neutralization of VEGFA itself (*e.g.,* achieved by bevacizumab) in invasive tumors might reduce vascular malformations and promote vessel functionality mimicking the response observed in early papillomas due to the reduction of functional VEGFA levels in the tumor environment. On the other hand, it cannot be excluded that more pronounced Dll4/Notch suppression than that we had in experiments with $Dll4^{+/-}$ mice, might not only have undesirable repercussions on the effectiveness of VEGFA signaling inhibitors, but also increases their efficacy since the abolishment of Dll4 function with concomitant VEGFA/VEGFR2 inhibition is expected to result in both reduced and non-productive tumor angiogenesis. Considering our previous results, such reduction and functional impairment of tumor angiogenesis is even more likely to be achieved by triple VEGFA/VEGFR, Dll4/Notch and EphrinB2 targeting. All these hypotheses are worth testing in future works.

Another important and unanswered question regarding the Dll4/Notch therapeutic suppression is whether and in to which extent it will influence co-applied chemotherapy effectiveness. Angiogenesis-targeting is basically considered as an adjuvant therapeutic strategy. Reduced vessel competence due to Dll4/Notch inhibition might limit the tumor saturation with cytostatics and, thus, their efficacy. Marketed bevacizumab and sunitinib, also reduce tumor perfusion and presumably cytostatic accumulation in tumor tissue, but when added to standard chemotherapy, do improve disease progression-free survival in patients with various malignancies (Hurwitz, Fehrenbacher et al. 2004; Sandler, Gray et al. 2006; Goodman, Rock et al. 2007). In a similar way, Dll4-based therapy can be expected to offer extended survival and time to progression when associated to the standard chemotherapy treatment.

Lastly, another undetermined aspect of Dll4/Notch inhibition in tumors is the durability of its effects. The normalization capacity of vascular defects associated with impaired Dll4/Notch remains to be examined in future research and in appropriate models. If vascular malformation self-repair occurred over a long-term Dll4/Notch-suppression, it would result in tumor reperfusion. In this scenario, regressive tumor behavior will cease, while the selection of more resistant and invasive malignant cells, previously documented as a consequence of antiangiogenesis-induced hypoxia (Yu, Rak et al. 2002; Hayden 2009), may increase the tumor metastatic potential. Inversely, the demonstration of the persistence of the effects arising from Dll4/Notch inhibition would greatly encourage its clinical applicability.

To sum-up the discussion on results obtained from Dll4 allele loss-of-function experiments and Dll4/Notch inhibition studies, the present research provides data indicating that Dll4-mediate Notch signaling is a crucial regulator of VEGFA-driven tumor angiogenesis, restricting exaggerated endothelial activation and proliferation that disable vessel structural and functional maturation. Its´ importance apparently increases in parallel with the degree of tumor malignancy, presumably due to increased background VEGFA levels. Promoted non-functional angiogenesis in tumors by Dll4/Notch suppression may be desirable while combined interference with Dll4/Notch and EphB4/EphrinB2 may offer greater benefit. Dll4/Notch suppression as a potential therapeutic strategy requires further evaluation, particularly in the direction to better characterize: 1) its efficacy in other tumor types, 2) the normalization capacity of vascular defects associated Dll4/Notch inhibition, *i.e.* the durability of its effects, 3) the adverse consequences arising from Dll4/Notch targeting, and 4) its influence on the effectiveness of co-applied chemotherapy as well as the effects of its association with other available anti-angiogenic compounds.

Following the assessment of the Dll4 role in tumor angiogenesis by antagonizing its signaling, we explored the effects of *Dll4* gain-of-function on tumor vascular response and growth, by using conditional Tie2-rtTA-M2 TetO7-Dll4 mice that overexpress endothelial *Dll4* upon tetracycline or doxycycline-induction (Trindade, Kumar et al. 2008). The endothelial *Dll4* up-regulation was examined in three tumor models: Lewis Lung Cancer (LLC) xenografts, chemically-induced skin papillomas and RIP1-*Tag2* insulinomas. The analyses provided very consistent results leading not only to the confirmation of the cellular and molecular bases underlining the fundamental Dll4 involvement in tumor vascular biology, but also to the establishment of a concept that

endothelial Dll4 function antagonists represent an alternative anti-cancer strategy (**Publication IV**).

In all three models, enhanced Dll4/Notch signaling in endothelial cells restricted their proliferation resulting in reduced tumor vessel density while vascular maturity and competence were promoted as indicated by larger branch formation, increased mural cell recruitment and improved vessel network perfusion. Despite the vascular normalization, the effect of suppressed vascular sprouting was predominant since *Dll4* overexpression significantly inhibited tumor growth in LLC xenografts as well as in autochthonous chemical skin papillomas and RT2 insulinomas. As expected, the increase in Dll4/Notch function reduced VEGFA/VEGFR2 and VEGFC/VEGFR3 signaling, explaining the inhibition of endothelial cell activation. In addition, increased Dll4/Notch down-regulated *Jag1*, a gene encoding another Notch ligand which signaling is pro-angiogenic (Benedito, Roca et al. 2009). On the other hand, our results suggested that the amplification of endothelial Dll4 function stabilized tumor vessels by enhancing *Efnb2* and *Tie2* expression such as the PDGF/PDGFRβ signaling.

Overexpressed *Dll4* in transduced malignant cells has been also found to minimize tumor vessel density and suppress tumor progression in most tested malignant cell lines (Noguera-Troise, Daly et al. 2006; Li, Sainson et al. 2007; Segarra, Williams et al. 2008). Based on these and our results, Dll4 overexpression joins the Dll4 inhibition as a modality that might improve cancer control, being exempt of toxic side-effects, as far as our evidence shows. Unlike the Dll4/Notch blockade, elicited vessel competence seen in endothelial Dll4 overexpressing mice might result in better cytostatic delivery at the tumor site while improved vessel wall assembly might decrease the malignant cell penetration to the circulation and metastization. Additionally, Dll4-antagonizing therapy is likely to improve the efficacy of VEGFR inhibitors (*e.g.* sorafenib and sunitinib) by reducing the receptor expression. In contrast, the development of a Dll4-based agonistic therapy seems more complex that the generation of Dll4/Notch inhibitors, such as sDll4, considering the fact that Dll4 and Notch functionally interact only when presented in the cellular context. The recent advances in the field of gene delivery systems encourage this option. Nevertheless, the main concern is that unspecific Notch1/4 activation would produce several side-effects driven from the perturbation of different Notch-dependent physiological processes outside the vasculature. Selective *Dll4* genetic sequence delivery, *e.g.* with endothelium-specific liposomes, might restrict therapeutic Notch over-activation

in the sites of angiogenesis-active vasculature offering a safe tool for more efficacious cancer treatment.

Finally, it is apparent that some tumor types respond better to Dll4/Notch stimulation and others to Dll4 inhibition. While our results show that RT2 insulinomas respond to both approaches, chemical skin papillomas were promoted by Dll4 blockade and suppressed by endothelial *Dll4* expression (**Publications II and III)**, just as hematological malignancies (Segarra, Williams et al. 2008), and human glioblastoma xenografts in mice presented opposite behavior (Li, Sainson et al. 2007). Thus, development of Dll4/Notch agonists and antagonists is complementary. It is interesting to further investigate which one of two opposite approaches, stimulating or inhibiting Dll4/Notch, is best suited for the treatment of different malignancies.

To recapitulate, endothelial Dll4/Notch signaling serves as a negative modifier of angiogenic vessel growth and, at the same time, a normalization factor in tumor vasculature. The present research shows that both loss and gain of Dll4 function interferes with tumor growth in distinct experimental settings by promoting non-productive angiogenesis or suppressing tumor angiogenic response, respectively. This represents a rational basis for the development of new, antagonistic and agonistic, Dll4/Notch-based therapies that, upon validation, may substantially contribute to control cancer.

REFERENCES:

Abramsson, A., P. Lindblom, et al. (2003). "Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors." J Clin Invest **112**(8): 1142-51.

Adams, R. H. and K. Alitalo (2007). "Molecular regulation of angiogenesis and lymphangiogenesis." Nat Rev Mol Cell Biol **8**(6): 464-78.

Adams, R. H., G. A. Wilkinson, et al. (1999). "Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis." Genes Dev **13**(3): 295-306.

Benedito, R., C. Roca, et al. (2009). "The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis." Cell **137**(6): 1124-35.

Bergers, G., K. Javaherian, et al. (1999). "Effects of angiogenesis inhibitors on multistage carcinogenesis in mice." Science **284**(5415): 808-12.

Bergers, G., S. Song, et al. (2003). "Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors." J Clin Invest **111**(9): 1287-95.

Coussens, L. M., B. Fingleton, et al. (2002). "Matrix metalloproteinase inhibitors and cancer: trials and tribulations." Science **295**(5564): 2387-92.

Cristofanilli, M., C. Charnsangavej, et al. (2002). "Angiogenesis modulation in cancer research: novel clinical approaches." Nat Rev Drug Discov **1**(6): 415-26.

Demehri, S., A. Turkoz, et al. (2009). "Epidermal Notch1 loss promotes skin tumorigenesis by impacting the stromal microenvironment." Cancer Cell **16**(1): 55-66.

Duarte, A., M. Hirashima, et al. (2004). "Dosage-sensitive requirement for mouse Dll4 in artery development." Genes Dev **18**(20): 2474-8.

Fischer, M., W. C. Yen, et al. (2011). "Anti-DLL4 inhibits growth and reduces tumor-initiating cell frequency in colorectal tumors with oncogenic KRAS mutations." Cancer Res **71**(5): 1520-5.

Foo, S. S., C. J. Turner, et al. (2006). "Ephrin-B2 controls cell motility and adhesion during blood-vessel-wall assembly." Cell **124**(1): 161-73.

Gale, N. W., M. G. Dominguez, et al. (2004). "Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development." Proc Natl Acad Sci U S A **101**(45): 15949-54.

Gerety, S. S. and D. J. Anderson (2002). "Cardiovascular ephrinB2 function is essential for embryonic angiogenesis." Development **129**(6): 1397-410.

Goodman, V. L., E. P. Rock, et al. (2007). "Approval summary: sunitinib for the treatment of imatinib refractory or intolerant gastrointestinal stromal tumors and advanced renal cell carcinoma." Clin Cancer Res **13**(5): 1367-73.

Hanahan, D. (1985). "Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes." Nature **315**(6015): 115-22.

Hayden, E. C. (2009). "Cutting off cancer's supply lines." Nature **458**(7239): 686-7.

Hoey, T., W. C. Yen, et al. (2009). "DLL4 blockade inhibits tumor growth and reduces tumor-initiating cell frequency." Cell Stem Cell **5**(2): 168-77.

Hurwitz, H., L. Fehrenbacher, et al. (2004). "Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer." N Engl J Med **350**(23): 2335-42.

Iso, T., T. Maeno, et al. (2006). "Dll4-selective Notch signaling induces ephrinB2 gene expression in endothelial cells." Biochem Biophys Res Commun **341**(3): 708-14.

Iwatsubo, T. (2004). "The gamma-secretase complex: machinery for intramembrane proteolysis." Curr Opin Neurobiol **14**(3): 379-83.

Karpanen, T., M. Wirzenius, et al. (2006). "Lymphangiogenic growth factor responsiveness is modulated by postnatal lymphatic vessel maturation." Am J Pathol **169**(2): 708-18.

Kerbel, R. S. (2008). "Tumor angiogenesis." N Engl J Med **358**(19): 2039-49.

Kertesz, N., V. Krasnoperov, et al. (2006). "The soluble extracellular domain of EphB4 (sEphB4) antagonizes EphB4-EphrinB2 interaction, modulates angiogenesis, and inhibits tumor growth." Blood **107**(6): 2330-8.

Kullander, K. and R. Klein (2002). "Mechanisms and functions of Eph and ephrin signalling." Nat Rev Mol Cell Biol **3**(7): 475-86.

Larcher, F., A. I. Robles, et al. (1996). "Up-regulation of vascular endothelial growth factor/vascular permeability factor in mouse skin carcinogenesis correlates with malignant progression state and activated H-ras expression levels." Cancer Res **56**(23): 5391-6.

Li, J. L., R. C. Sainson, et al. (2007). "Delta-like 4 Notch ligand regulates tumor angiogenesis, improves tumor vascular function, and promotes tumor growth in vivo." Cancer Res **67**(23): 11244-53.

Lobov, I. B., R. A. Renard, et al. (2007). "Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting." Proc Natl Acad Sci U S A **104**(9): 3219-24.

Mailhos, C., U. Modlich, et al. (2001). "Delta4, an endothelial specific notch ligand expressed at sites of physiological and tumor angiogenesis." Differentiation **69**(2-3): 135-44.

Martinez, J. C., M. M. Muller, et al. (2009). "Nuclear and membrane expression of the angiogenesis regulator delta-like ligand 4 (DLL4) in normal and malignant human tissues." Histopathology **54**(5): 598-606.

Noguera-Troise, I., C. Daly, et al. (2006). "Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis." Nature **444**(7122): 1032-7.

Noguera-Troise, I., C. Daly, et al. (2007). "Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis." Novartis Found Symp **283**: 106-20; discussion 121-5, 238-41.

Patel, N. S., M. S. Dobbie, et al. (2006). "Up-regulation of endothelial delta-like 4 expression correlates with vessel maturation in bladder cancer." Clin Cancer Res **12**(16): 4836-44.

Peters, K. G., C. D. Kontos, et al. (2004). "Functional significance of Tie2 signaling in the adult vasculature." Recent Prog Horm Res **59**: 51-71.

Ridgway, J., G. Zhang, et al. (2006). "Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis." Nature **444**(7122): 1083-7.

Sandler, A., R. Gray, et al. (2006). "Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer." N Engl J Med **355**(24): 2542-50.

Sato, T. N., Y. Tozawa, et al. (1995). "Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation." Nature **376**(6535): 70-4.

Sawamiphak, S., S. Seidel, et al. (2010). "Ephrin-B2 regulates VEGFR2 function in developmental and tumour angiogenesis." Nature **465**(7297): 487-91.

Scehnet, J. S., E. J. Ley, et al. (2009). "The role of Ephs, Ephrins, and growth factors in Kaposi sarcoma and implications of EphrinB2 blockade." Blood **113**(1): 254-63.

Segarra, M., C. K. Williams, et al. (2008). "Dll4 activation of Notch signaling reduces tumor vascularity and inhibits tumor growth." Blood **112**(5): 1904-11.

Selkoe, D. and R. Kopan (2003). "Notch and Presenilin: regulated intramembrane proteolysis links development and degeneration." Annu Rev Neurosci **26**: 565-97.

Shaheen, R. M., W. W. Tseng, et al. (2001). "Tyrosine kinase inhibition of multiple angiogenic growth factor receptors improves survival in mice bearing colon cancer liver metastases by inhibition of endothelial cell survival mechanisms." Cancer Res **61**(4): 1464-8.

Shibuya, M. and L. Claesson-Welsh (2006). "Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis." Exp Cell Res **312**(5): 549-60.

Suchting, S., C. Freitas, et al. (2007). "The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching." Proc Natl Acad Sci U S A **104**(9): 3225-30.

Trindade, A., S. R. Kumar, et al. (2008). "Overexpression of delta-like 4 induces arterialization and attenuates vessel formation in developing mouse embryos." Blood **112**(5): 1720-9.

Williams, C. K., J. L. Li, et al. (2006). "Up-regulation of the Notch ligand Delta-like 4 inhibits VEGF-induced endothelial cell function." Blood **107**(3): 931-9.

Yamanda, S., S. Ebihara, et al. (2009). "Role of ephrinB2 in nonproductive angiogenesis induced by Delta-like 4 blockade." Blood **113**(15): 3631-9.

Yan, M., C. A. Callahan, et al. (2010). "Chronic DLL4 blockade induces vascular neoplasms." Nature **463**(7282): E6-7.

Yu, J. L., J. W. Rak, et al. (2002). "Effect of p53 status on tumor response to antiangiogenic therapy." Science **295**(5559): 1526-8.

Appendix – Related Publication

PLoS ONE. Publication in press.

Low-dosage inhibition of Dll4 signaling promotes wound healing by inducing functional neo-angiogenesis

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ABSTRACT: Recent findings regarding Dll4 function in physiological and pathological conditions indicate that this Notch ligand may constitute an important therapeutic target. Dll4 appears to be a major anti-angiogenic agent, occupying a central role in various angiogenic pathways. The first trials of anti-Dll4 therapy in mice demonstrated a paradoxical effect, as it reduced tumor perfusion and growth despite leading to an increase in vascular density. This is seen as the result of insufficient maturation of the newly formed vasculature causing a circulatory defect and increased tumor hypoxia. As Dll4 function is known to be closely dependent on expression levels, we envisioned that the therapeutic anti-Dll4 dosage could be modulated to result in the increase of adequately functional blood vessels. This would be useful in conditions where vascular function is a limiting factor for recovery, like wound healing and tissue hypoxia, especially in diabetic patients. Our experimental results in mice confirm this possibility, revealing that low dosage inhibition of Dll4/Notch signaling causes improved vascular function and accelerated wound healing.