UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

THE ROLE OF ENDOTHELIAL DLL4 IN CANCER METASTASIS

LILIANA MENDONÇA DA SILVA DIAS

Orientador: Professor Doutor António Freitas Duarte
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Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na Especialidade de Ciências Biológicas e Biomédicas

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2019

"Tudo vale a pena quando a alma não é pequena"

Fernando Pessoa (*in* Mar Português, Mensagem, 1934)

To my beloved daughters: Sara, Alice and Clara.

You are my Sunshine, my Ocean and my Sand.

To my love: André. To the deep Universe and back.

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Title – The role of endothelial Dll4 in cancer metastasis

Abstract

The metastatic spread of cancer is still the major barrier to the treatment of this disease. Cancer-mortality is mainly due to recurrence and metastasis. Although much has been done in the field of cancer treatment, prevention and approach to metastases are still areas not fully explored.

Over and under-expression of Dll4/Notch signaling has been demonstrated to impair tumor growth through opposing patterns of vascular modulation in different mouse tumor models and human cancer xenografts. Recent evidence implicates Dll4/Notch pathway in the metastasis mechanism, but less is known about the specific role of endothelial Dll4. For this reason, we proposed to investigate how endothelial Dll4 expression interferes with the metastatic process. To address it we used a spontaneous metastasis mouse model based on Lewis Lung Carcinoma (LLC) subcutaneous transplants in endothelial-specific *Dll4* loss-of-function (eDll4cKO) and endothelial-specific *Dll4* over-expression (D4OE) mice.

Results demonstrated that eDll4cKO is responsible for the vascular function regression that leads to tumor growth reduction. Early steps of epithelial-to-mesenchymal transition (EMT) and cancer stem cell selection were also inhibited by eDll4cKO, leading to a substantial reduction of circulating tumor cells and reduction in the number and burden of macro-metastases. Intravasation and extravasation were also compromised by eDll4cKO, possibly due to blockade of the metastatic niche.

In the case of the D4OE mice we observed that tumor growth reduction was achieved by vessel proliferation restriction along with an improved vascular maturation, which allowed a more efficient delivery of chemotherapy. This last effect of vessel normalization seemed to prevent metastasis formation even though EMT markers were increased.

In conclusion, despite the opposite vascular architecture phenotypes of eDll4cKO and D4OE, both lead to a reduction in metastasis. This is in line with the concept of Dll4 dosage observed in the wound-healing context and represents a promising therapeutic approach in metastasis prevention/ treatment.

Keywords: Dll4/ Notch, LLC, tumor growth, metastasis, EMT.

Título – A importância da expressão endotelial do ligando Dll4 no processo de metastização

Resumo

O cancro é, atualmente, uma das principais causas de morte em todo o mundo, a par das doenças cardiovasculares e da doença pulmonar obstrutiva crónica. A mortalidade por cancro está frequentemente associada à metastização, quer seja pelo compromisso hemodinâmico, quer seja pelas consequências do seu tratamento. Apesar dos avanços no tratamento do cancro, com novos agentes biológicos e imunomodeladores, a investigação do mecanismo da metastização, prevenção e abordagem das metástases continuam a ser áreas pouco exploradas.

A via Notch é uma via de sinalização intercelular altamente conservada que está envolvida na determinação do destino, regulação da proliferação e diferenciação celulares. O ligando Dll4 tem uma expressão predominantemente endotelial arterial e capilar, crítica para o correto desenvolvimento embrionário, mas restringindo-se às pequenas artérias e capilares no adulto em homeostasia. Ele atua como agente anti-angiogénico em situações de "stress" fisiológico e em contexto tumoral, promovendo a ocorrência de pausas ritmadas na fase de crescimento vascular ativo, que são conducentes à maturação funcional da vasculatura nascente.

Os efeitos do aumento e diminuição da expressão do ligando Dll4 sobre a dinâmica tumoral já foram extensamente estudados em modelos tumorais de ratinhos transgénicos e com xenografos. Geralmente, a ativação aberrante da via de sinalização Dll4/Notch está associada a mau prognóstico e probabilidade de metastização. Por outro lado, as terapias de bloqueio Dll4/Notch têm-se revelado eficazes no tratamento de modelos tumorais resistentes às terapias baseadas no "vascular endotelial growth factor" (Vegf), em modelos pré-clínicos. Estudos recentes implicam a ativação da via Notch nas células endoteliais, com alteração do seu fenótipo de forma a favorecer a transmigração das células tumorais, bem como a secreção de "vascular cell adhesion molecule-1", que promove a adesão de leucócitos e potencia os fenómenos de intravasação e extravasação.

Nesse sentido, surge a oportunidade de explorar o papel do ligando Dll4, em concreto, de que forma é que a sua expressão endotelial influencia as várias etapas da cascata da metastização. Para isso, usamos um modelo murino de metastização espontânea que consistiu na injeção subcutânea de células Lewis Lung Carcinoma (LLC) em dois tipos de ratinhos transgénicos: ratinhos com perda-de-função ou sub-expressão endotelial-específica de Dll4 e ratinhos com ganho-de-função ou sobre-expressão endotelial-específica de Dll4. Relativamente ao estudo da influência do ganho-de-função endotelial-específico de Dll4 sobre o tumor primário, foram também usados outros ratinhos: o modelo de papiloma da pele induzido quimicamente e o modelo murino transgénico RIP-Tag2, com

insulinoma. Para avaliar a angiogénese do tumor primário foram determinados parâmetros como: densidade, maturidade, funcionalidade e permeabilidade vasculares. Depois de explorado o efeito de Dll4 sobre o fenómeno de transição-epitélio-mesenquimal (EMT), *in vitro*, procurou-se explorar o mesmo, *in vivo*, com recurso aos ratinhos mutantes endoteliais-específicos citados. EMT foi caracterizada através da marcação imunofluorescente para Snail-1 e Twist. As células tumorais estaminais (CSC) foram exploradas através da marcação imunofluorescente para p63 e CD49f. A hipóxia tumoral foi avaliada através do teste com "Hypoxyprobe". O circuito das células tumorais metastáticas foi analisado com recurso à marcação das células com uma proteína verde fluorescente, recorrendo às técnicas de imunofluorescência e "fluorescent associated cell sorting". A análise da expressão génica foi feita através do "quantitative real time polymerase chain reaction", tanto nos ratinhos com perda, como ganho-de-função endotelial-específico de Dll4.

Quanto ao estudo da perda-de-função endotelial-específica de Dll4 foi possível avaliar, genericamente, todas as etapas da metastização. Começando pelo tumor primário, verifica-se que ocorre uma redução considerável do seu crescimento, em parte devido à angiogénese que, apesar de aumentada, é desorganizada e menos funcional. A árvore vascular apresenta inúmeras ramificações e um revestimento peri-vascular insuficiente (redução de células de músculo liso, dos marcadores α-SMA e Pdgfr-β), indicativo de imaturidade vascular. Observa-se um aumento da permeabilidade, que leva a fenómenos de exsudação e consequente má perfusão do tumor. Este endotélio disfuncional acaba por sofrer regressão, condicionando o crescimento tumoral. Constata-se que eventos iniciais como a EMT e a seleção de clones de células tumorais estaminais (CSC) são inibidos pelo bloqueio endotelial-específico de Dll4, conduzindo a uma redução do número de células tumorais circulantes e do número e crescimento das macro-metástases. Os fenómenos posteriores de intravasação e extravasação estão também reduzidos, provavelmente pela não sinalização do tumor primário à medula óssea, via Dll4/ Notch1/ Hey1/ TGF-*β*. Pelo que, há uma redução da mobilização de células mieloides $Cd11c^{+}$/$VEGFR\text{-}1^{+}$ para o nicho metastático (pulmão) e fraca deposição de fibronectina, que seria essencial para a acomodação das células tumorais circulantes/metastáticas. De salientar, que não ocorre sinalização parácrina entre o endotélio pulmonar e as células tumorais metastáticas, já que não se verifica marcação para N1ICD pulmonar.

No caso da avaliação do papel do ganho-de-função endotelial-específico de Dll4, verificou-se que a redução do crescimento tumoral resulta duma diminuição da angiogénese (com redução concordante da expressão dos receptores pro-angiogénicos Vgfr1 e Vgfr2), mas também da sua normalização. Trata-se duma árvore vascular com poucas ramificações, com características de maturidade evidenciadas pelo grande revestimento peri-vascular (aumento de células de músculo liso, dos marcadores α-SMA, Pdgfr-β e Ephrin-B2),

permitindo um fluxo sanguíneo eficiente, ainda que inferior ao normal, pela redução da densidade vascular. Este efeito, aparentemente paradoxal (contra-intuitivo), acaba por promover um melhor aporte da doxorrubicina (quimioterápico), com bloqueio do crescimento e/ou indução da apoptose tumoral. Esta normalização parece também inibir a metastização, já que apesar de haver um aumento da expressão dos marcadores de EMT, essas células tumorais acabam por ficar "aprisionadas" no endotélio tumoral, dado que se constata uma redução do número de macro-metástases. No entanto, este modelo carece de clarificação no número de células tumorais circulantes, bem como nas restantes etapas da metastização que não chegaram a ser exploradas.

Em ambos os modelos, verifica-se que é a função endotelial Dll4/Notch, e não a hipóxia tumoral, que determina a sinalização para EMT e seleção de CSC.

Concluindo, observa-se no contexto da dinâmica tumoral fenótipos opostos para os modelos de perda e ganho-de-função endotelial-específico de Dll4, em linha com o que já foi descrito para o contexto da cicatrização de feridas. Estamos novamente perante o conceito de dose-dependência de Dll4, com aparente repercussão também na metastização. Isto porque o fenótipo final é o mesmo, de redução do número de macro-metástases, apesar das diferenças na angiogénese tumoral e no evento inicial de EMT, ficando por explorar a restante cascata da metastização.

O uso do ligando Dll4, na forma de sub ou sobre-expressão, pode constituir uma nova abordagem terapêutica ao tratamento dos cancros da mama e colo-rectal metastizados, a par de outros anti-angiogénicos já utilizados. Mas mais interessante será explorar a sua abordagem como fármacos antagonistas ou agonistas, numa estratégia de prevenção da metastização.

Palavras-chave: Dll4/ Notch, LLC, crescimento tumoral, metastização, EMT.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABREVIATIONS

ABC - Advanced Breast Cancer

ADAM - A Disintegrin And Metalloproteinase

Ang - angiopoietins

ANK - ankyrin

α- SMA - alpha smooth muscle actin

BMDC - bone marrow-derived cell

BSA - Bovine Serum Albumine

CAFs - carcinoma-associated fibroblasts

cDNA - complementary Deoxyribonucleic acid

CRD - cysteine-rich domain

CRC - colorectal cancer

CSC - Cancer Stem Cell

CTCs - circulating tumor cells

CSL - CBF-1 Suppressor of Hairless-LAG1

DAPI - 4´,6-diamidino-2-phenylindole dihydrochloride hydrate

DCs - dendritic cells

$Dll4^{lox/lox}$ - Dll4 conditional knockout mice

DMBA - 7,12-dimethylbenz[a]anthracene

DMSO - dimethyl sulfoxide

DPBS - Dulbecco's phosphate-buffered saline

DSL - Delta-Serrate-LAG2

DTC - disseminated tumor cell

D4BE - Dll4 basic expression control

D4BE + Doxy - Doxycycline control

D4OE - Dll4 over-expression

ECM - extracellular matrix

eDll4cKO - endothelial-specific inducible Dll4 loss-of-function

EGF - epidermal growth factor

EMT - Epithelial-to-mesenchymal transition

EPCs - endothelial progenitor cells

eNOS - endothelial nitric oxide synthase

FACS - Fluorescent-activated cell sorting

FGFs - fibroblast growth factors

GFP - green fluorescent protein

HD - heterodimerization domain

H&E - Hematoxylin and Eosin

HIF1-α - hypoxia-inducible factor 1 alpha

HPCs - hematopoietic progenitor cells

LLC - Lewis Lung Carcinoma

LOX - lysyl oxidase

MAML1- Mastermind-like transcriptional co-activator 1

mCRC - metastatic CRC

MET - mesenchymal-to-epithelial transition

MMP - matrix metalloproteinase

MTCs - metastatic tumor cells

NECD - Notch extracellular domain

NICD - Notch intracellular domain

NRR - negative regulatory region

NS - Not Statistically Significant

NT - amino-terminal

PBS - Phosphate buffered saline

PCR - Polymerase chain reaction

PECAM - platelet-endothelial cell adhesion molecule

PEST - Proline, glutamate, serine, threonine

PDGF - platelet-derived growth factor

PFA - paraformaldehyde

PlGF - placental cancer growth factor

RBC - Red blood cell

RBP-Jκ - recombination signal binding protein Jκ

RCC - renal cell carcinoma

RNA - Ribonucleic acid

RTKs - Receptor Tyrosine Kinases

RT2 - RIP1-Tag2

S.c. - subcutaneous

SCID - severe combined immunodeficiency

SD - Standard deviation

SDF-1 - stromal-derived growth factor

SEM - Standard error mean

TAMs - Tumor-Associated Macrophages

TDSFs - tumor-derived secreted factors

TGF- β - transforming growth factor beta

Tie - tyrosine kinase with immunoglobulin-like and epidermal growth factor-like domain

TPA - 12-O-tetradecanoylphorbol-13-acetate

uPA - urokinase-type plasminogen activator

VCAM1 - vascular cell adhesion molecule-1

VEGF - Vascular endothelial growth factor.

INTRODUCTION

Cancer is a major public health problem throughout the world. The latest statistics report in 2017 indicate that cancer is the second most common cause of death in Portugal, with an incidence increase of 3% a year (Direção-Geral da Saúde, 2017). Lung cancer accounts for the highest mortality. A similar scenario is found in North America and Europe, where cancer is responsible for the loss of 169,3 million years of life (Ferlay et al., 2015). The main reason for the high mortality of cancer is due to the highly invasive behavior of cancer cells, which usually results in cancer progression and metastasis. Metastasis is responsible for the vast majority of cancer-related death, so it is clear that the mechanism of cancer metastasis deserves urgent attention.

The term *metastasis* was first formulated by Joseph-Claude-Anthelme Récamier (Recamier, 1829; Talmadge & Fidler, 2010) as the transfer of tumor cells from one part of the body to another. Nowadays, this functional perception can be completemented genomically since analysis of matched sets of patient's primary tumor and distant metastasis reveal mutations common to both and, almost universally, mutations that are distinct to a metastasis (Brosnan & Iacobuzio-Donahue, 2012)

The metastatic cascade begins with tumor cells invasion of the tissue surrounding the primary tumor. Tumor cells enter the bloodstream, either directly or via lymphatic system; angiogenesis is therefore crucial for tumor cells escape (Folkman, 2002). Tumor cells then extravasate the bloodstream to land on "foreign soil" and not just simply the first capillary bed encountered. Paget, over a century ago (Paget, 1889), described metastasis in botanical terms as the interaction of "seeds" (tumor cells) and "congenial soil" (the metastatic microenvironment). How a foreign tissue becomes congenial contributes to metastatic colonization, that is, the progressive outgrowth of tumor cells at the distant site. The metastatic soil can be altered by bone marrow-derived cells before tumor cell arrival, termed the pre-metastatic niche (Kaplan et al., 2005). Eventually the cellular composition, immune status, blood supply modulated essentially by angiogenesis, extracellular matrix (ECM) and virtually every other aspect of the metastatic site can be altered to favor colonization (Steeg, 2016).

The spread of tumor cells from organ to organ is incredibly inefficient; only 0.01% of cells that leave the primary site go on to create a distant metastasis (Fidler, 1970). Why, then, do so many cancer patients succumb to metastatic disease? Because tumors are composed of billions of tumor cells, and 1-4 million tumor cells can be shed into the circulation each day from a one-gram tumor (Butler & Gullino, 1975). Additionally, hundreds of metastatic colonies can arise from a "dormant state" in an individual organ and the unrestricted growth of these colonies can lead to replacement of normal tissue by tumor in essential organs, such as the lung, liver, brain and bone marrow (Bielenberg & Zetter, 2015; Hensel, Flaig, & Theodorescu, 2012). Is also important to consider that clinical metastatic disease results from several selective forces. Pathways that fuel initial tumorigenesis, described as the "trunk" of a cancer

evolutionary tree, can also endow tumor cells with metastatic properties and *de novo* drug resistance. The “limbs” are the events that promote resistance to therapy and selection of other pathways that induce or accelerate metastasis to distant organs (Steeg, 2016).

The link between metastasis and angiogenesis is now obvious in many steps of the metastasis process and has been studied since Folkman and colleagues showed that the surgical excision of a murine primary tumor could stimulate the growth of dormant metastases (O’Reilly et al., 1994).

The Notch signaling pathway is evolutionarily conserved in mammals and plays an important role in cell development and differentiation. In recent years, increasing evidence has shown that aberrant activation of Notch is associated with tumor development, angiogenesis and metastasis. For example, high tumor cell co-expression of Notch-1 and Vascular endothelial growth factor (VEGF) A in lung cancer is associated to a poor prognosis, because it increases the probability of metastasis through tumor angiogenesis regulation via Notch-VEGF signaling (Donnem et al., 2010) In agreement, blockade of the DLL4-Notch pathway is associated with poorly functional angiogenesis and reduced tumor growth in immunodeficient mice with renal cancer xenografts (Djokovic et al., 2010; Miles et al., 2014; Scehnet et al., 2007). But the role of Notch signaling in the metastasis angiogenesis setting is only now being explored. A recent report described that activated Notch1 receptor, observed in endothelial cells of primary tumors, is associated with increased metastasis rates and leads to a senescence-like, pro-inflammatory endothelial cell phenotype that facilitates tumor cells intravasation as well as extravasation at distant sites. They suggest that the vascular cell adhesion molecule-1 (VCAM-1) secreted by these Notch activated endothelial cells, mediate the metastasis process through multiple mechanisms involving crosstalk with tumor and immune cells. This report did not make clear which Notch ligand is implicated (Wieland et al., 2017) but other reports showed the relationship between DLL4 function and metastasis (Huang et al., 2014; Kuramoto et al., 2012; Xu et al., 2016).

Therefore, we proposed to study the specific role of endothelial *Dll4* in the tumor metastasis process. The selected approach to produce a murine spontaneous metastasis model was to carry out subcutaneous injection of Lewis Lung Carcinoma (LLC) cells in endothelial-specific Dll4 loss-of-function mice, with previous validation *in vitro*. Early events like epithelial-to-mesenchymal transition, invasion and tumor angiogenesis were characterized with this model. But to address the steps of intravasation, bloodstream circulation, extravasation and colonization in the metastatic niche we had to mark LLC cells with a fluorescent protein. Then we asked the opposite question: what happens when *Dll4* is overexpressed, since there were reported side effects from the chronic Dll4/Notch blockade. To answer that question we transplanted the same LLC in a transgenic mouse model of endothelial-specific Dll4 over-expression.

This work resulted in three articles that were organized in four chapters, as follows:

1. **Exploratory experiments.**

2. **Metastasis is impaired by endothelial-specific *Dll4* loss-of-function through inhibition of epithelial-to-mesenchymal transition and reduction of cancer stem cells and circulating tumor cells.**

Liliana Mendonça, Alexandre Trindade, Catarina Carvalho, Jorge Correia, Marina Badenes, Joana Gigante, António Duarte. Accepted in Clinical & Experimental Metastasis.

3. **Endothelial Dll4 loss-of-function reduces metastasis through intravasation-extravasation and metastatic niche inhibition.**

Liliana Mendonça, Alexandre Trindade, Marina Badenes, Joana Gigante, António Duarte. Manuscript in preparation.

4. **Endothelial Dll4 overexpression reduces vascular response and inhibits tumor growth and metastasization *in vivo*.**

Alexandre Trindade*, Dusan Djokovic*, Joana Gigante, Liliana Mendonça and António Duarte. *These authors contributed equally to this work. *BMC Cancer* 2017; 17:189 DOI: 10.1186/s12885-017-3171-2.

LITERATURE REVIEW

1. Notch signaling pathway

The Notch signaling pathway is a highly conserved pathway throughout the animal kingdom (Artavanis-Tsakonas, Rand, & Lake, 1999; Weinmaster, Roberts, & Lemke, 1991). Although its relatively simple mechanism, it has been implicated in different developmental and disease contexts, from stem cell regulation and heart morphogenesis to cancer and cardiomyopathies (S J Bray, 2006; Sarah J. Bray, 2016).

The canonical Notch signaling pathway functions as a mediator of short-range cell-cell communication, as both receptors and ligands are transmembrane proteins. In mammals, there have been described four Notch receptors (Notch 1-4) and five ligands (Delta-like 1, 3, 4 and Jagged 1, 2) (Fleming, 1998; Sainson & Harris, 2008).

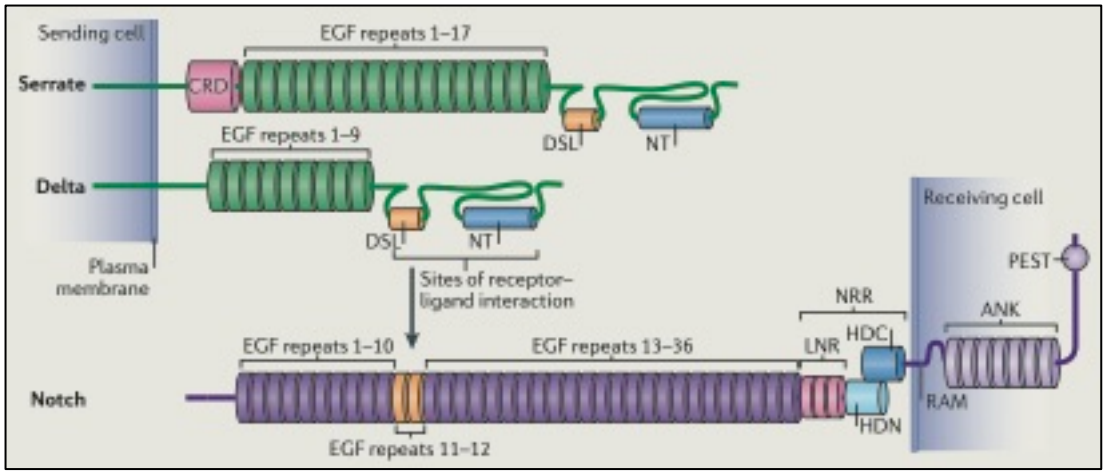
1.1. Notch receptors

The mature Notch receptor is produced through a furin cleavage (S1 cleavage) during biosynthesis (Logeat et al., 1998). Notch extracellular domain (NECD) consists of 29 to 36 epidermal growth factor (EGF)-like repeats. EGF-repeats 11 and 12 are essential for ligand binding (Rebay et al., 1991). A negative regulatory region (NRR), composed of three cysteine-rich Lin12/Notch repeats and the heterodimerization domain (HD), follows NECD. The NICD consists of RAM domain, ankyrin (ANK) activation domain and a C-terminal Pro Glu Ser Thr (PEST) domain (Fig.1). The RAM and ANK domains are essential for interacting with the DNA-binding protein CBF-1 Suppressor of Hairless-LAG1 (CSL; also known as RBPj) in the nucleus (Hori, Sen, & Artavanis-Tsakonas, 2013; Kopan et al., 2009). Notch1 is broadly expressed in many tissues, including the heart and vascular endothelial cells, while Notch4 expression is restricted to vascular endothelial cells (Krebs et al., 2000; Wu et al., 2005), and Notch3 is predominantly expressed in vascular smooth muscle cells (Joutel et al., 2000).

Notch1$^{-/-}$ mutant mice die during early embryogenesis, with severe vascular and cardiovascular defects (Swiatek, Lindsell, del Amo, Weinmaster, & Gridley, 1994). It negatively regulates endothelial cell proliferation and migration, and angiogenic sprouting (Krebs et al., 2000; Swiatek et al., 1994). Apart from other functions, it has also been implicated in several types of cancer, from chronic lymphocytic leukemia (Rossi et al., 2012) to head and neck squamous cell carcinoma (Rettig et al., 2015).

Notch4$^{-/-}$ mutant mice display no conspicuous phenotype, but the vascular defects observed in compound *Notch1*$^{-/-}$*; Notch4*$^{-/-}$ mutant embryos are more severe than those in Notch1$^{-/-}$ mutants, which suggests that the functions of Notch1 and Notch4 overlap during vascular development (Krebs et al., 2000).

Figure 1 - Mechanistic features of Notch signaling. *In* (Sarah J. Bray, 2016).



1.2. Notch ligands

Notch ligands are also themselves transmembrane proteins. They share a largely similar structure, with an extracellular domain comprised primarily of multiple EGF repeats. Serrate (in Drosophila) and its Jagged orthologues also contain a cysteine-rich domain (CRD). Binding by canonical Notch ligands involves the extracellular Delta-Serrate-LAG2 (DSL) conserved domain and amino-terminal (NT) domain (D'Souza, Miyamoto, & Weinmaster, 2008) (Fig.1).

Four of the five known mammalian Notch ligands (Dll1, Dll4, Jagged1 and Jagged2) are expressed in vascular endothelial cells (Beckers, Clark, Wünsch, Hrabé De Angelis, & Gossler, 1999; Krebs et al., 2000; Sörensen, Adams, & Gossler, 2009; Villa et al., 2001); Jagged1 is also expressed in smooth muscle cells surrounding the arteries and plays an important role in smooth muscle cell maturation (H. Liu, Kennard, & Lilly, 2009).

Dll1 is not critically involved in arterial-cell specification; however, analyses in hypomorphic and endothelial-specific *Dll1* mutant mice indicate that Dll1 is required for the maintenance of arterial identity (Sörensen et al., 2009).

Dll4 ligand revealed a highly selective expression within the vascular endothelium (Shutter et al., 2000). In the developing embryo, there is strong expression of Dll4 mainly restricted to large arteries (Duarte et al., 2004). But it can also be detected in the nervous system, gut, renal glomerulus, thymus and airway endothelium (Rui Benedito & Duarte, 2005; Mailhos et al., 2001; Shutter et al., 2000). *Dll4*$^{+/-}$ mice mutants display incompletely penetrant, strain dependent, lethal haplo-insufficiency and *Dll4*$^{-/-}$ embryos die before embryonic day 10.5 (E10.5), in both cases due to major vascular abnormalities. Those include stenosis and atresia of the aorta, with defective branching, arterial regression, arterial-venous fusions, arterial venularisation, enlargement of the pericardial sac and failure to remodel the yolk sac vasculature (R Benedito et al., 2008; Duarte et al., 2004). The opposite is seen in Dll4 overexpression mutant embryos, which show arterialization of the venous endothelium, with

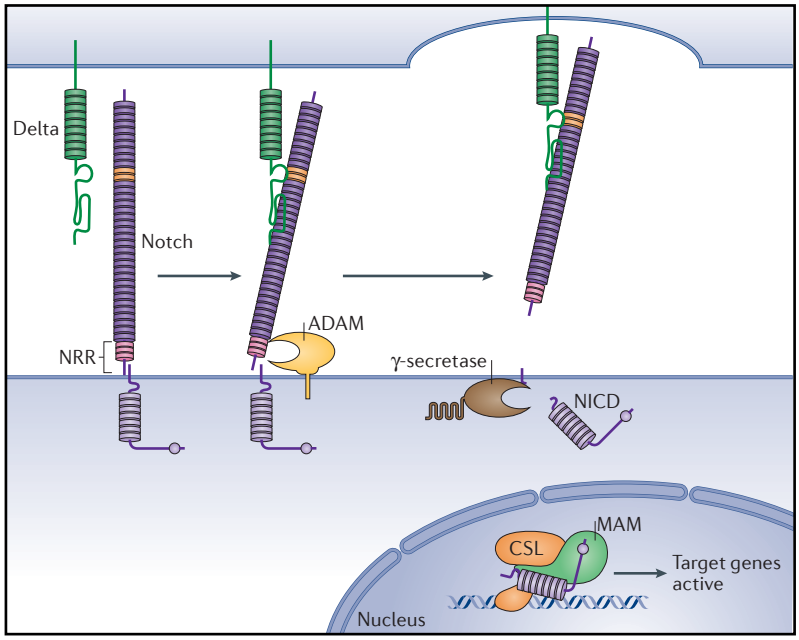
arterial specific markers like Ephrin-B2 being also expressed in veins. Their vascular system exhibits a premature fusion between arteries and veins due to loss of repulsive signal between arterial and venous vascular beds, enlarged arteries with excess fibronectin accumulation and decreased vessel branching (Trindade et al., 2008). This means that Dll4 dose and location are critical for proper vessel formation, as either gain- or loss-of-function leads to profound vascular defects. In the adult, Dll4 endothelium expression is weak and restricted to small arteries and microvessels (Rui Benedito & Duarte, 2005). Additionally is found in the kidney, lung, heart, ovaries, testes and gut (Mailhos et al., 2001; Shutter et al., 2000). Most importantly, Dll4 is an essential regulator of physiological and tumoral angiogenesis, since it acts as an anti-angiogenic factor to “normalize” the vasculature proliferation (Noguera-Troise et al., 2006; Ridgway et al., 2006; Trindade et al., 2012) and was recently linked to several steps of the metastatic process (Kuramoto et al., 2012; Xu et al., 2016).

Jagged1 does not play a critical role in arterial development (High et al., 2008; Robert-Moreno et al., 2008; Xue et al., 1999), but is required for the definitive hematopoietic program in the dorsal aorta. Jagged1 and Notch1 expression overlap in the dorsal aorta, while Jagged2 expression occurs in endothelial cells adjacent to Notch1-positive endothelial cells, and Dll4 is expressed in both Notch1-positive and Notch1-negative endothelial cells (Robert-Moreno et al., 2008).

1.3. Notch signaling pathway activation

Notch receptor activation is mediated by a sequence of proteolytic events. Ligand binding exposes the cleavage site (S2), in the NRR region, for ADAM-family metalloproteases (mainly ADAM10, in physiological conditions) (Gordon, Arnett, & Blacklow, 2008; van Tetering et al., 2009). Cleavage renders the remaining transmembrane-intracellular fragment a substrate for the γ-secretase complex, which catalyses intramembrane proteolysis to release the NICD (Struhl & Greenwald, 1999). Once released, the NICD enters the nucleus and, together with the CSL and the co-activator Mastermind (Mam; Mastermind-like transcriptional co-activator 1 (MAML1) in human), induces transcription of target genes (Iso, Kedes, & Hamamori, 2003; Kitagawa, 2015) (Fig.2).

Figure 2 - Ligand binding leads to exposure of the cleavage site in Notch. *In* (Sarah J. Bray, 2016).



The main Notch effectors in the vascular system belong to the Hey family, namely *Hey1* and *Hey2*. Loss of *Hey2* in the mouse leads to cardiac defects with high postnatal lethality (Gessler et al., 2002). The combined loss of *Hey1* and *Hey2* results in embryonic death after E9.5, with a global lack of vascular remodeling and massive hemorrhage (A. Fischer, Schumacher, Maier, Sendtner, & Gessler, 2004).

Another important Notch effector is *Nrarp*, a small protein with two ankyrin repeats originally identified in *Xenopus* embryos (Lamar et al., 2001). The levels of Notch1 signaling regulate *Nrarp* transcript levels (Krebs, Deftos, Bevan, & Gridley, 2001). This effector acts as a negative feedback regulator of Notch signaling but also as link between Notch and Lef1-dependent Wnt signaling in endothelial cells to control stability of new vessel connections in mouse and zebrafish (Li-Kun Phng et al., 2009).

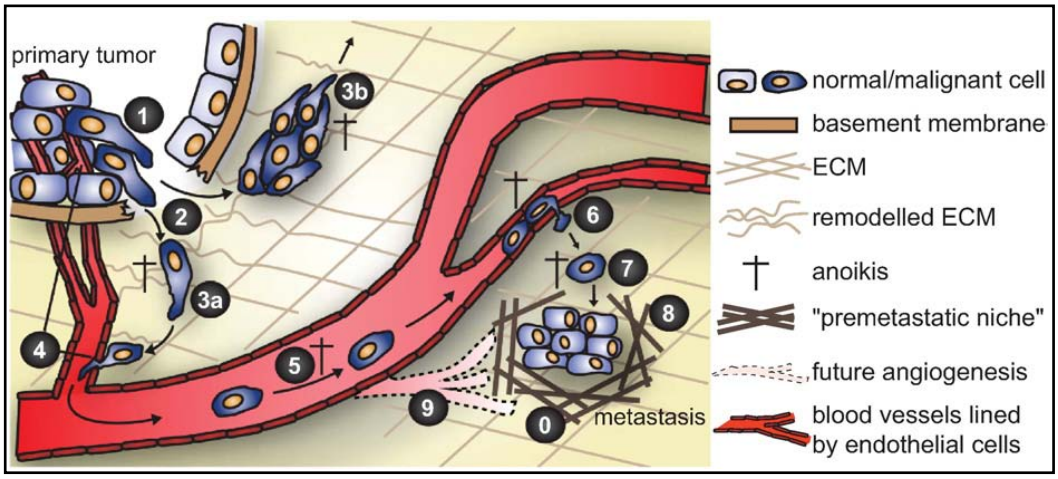
The different outcome upon Notch signaling relies on the deployment of different levels of control that adapt the pathway to each context. In terms of ligand-receptor dynamics: 1. The spatial and temporal ligand distribution, rather than the classic paradigm of lateral inhibition, is crucial but also the transcriptional and microRNAs modulation (Collier, Monk, Maini, & Lewis, 1996; Vallejo, Caparros, & Dominguez, 2011); 2. The amount or duration of the NICD "signal" produced, that will depend on the specific ligand-receptor pairs engaged, as seen in hematopoietic stem cells specification that involves low-strength JAG1-dependent signal, whereas specification of endothelial arterial cells requires high-strength DLL4 signal (Gama-Norton et al., 2015); 3. The balance between *cis* interactions and *trans* interactions, with *cis*-inhibition helping stabilization of tip and stalk cell fates during angiogenesis, for example (Boareto et al., 2015); 4. The receptor activity modifications by glycosylation or fringe-

mediated, that affects ligand-receptor interactions. This is documented by the high levels of JAG1 present in stalk cells, through Fringe action, making JAG1 an effective competitor and preventing DLL4 from signaling between adjacent stalk cells, which avoids excessive sprouting (Rui Benedito et al., 2009). Relative to architecture context: 1. The factors that modulate endocytosis and trafficking of receptors and ligands have a number of important consequences on the pathway activity (Yamamoto, Charng, & Bellen, 2010); 2. The tissue organization influence the pattern of signaling and affect the extent of the adhesive contacts between cells, but transient interactions can be sufficient to deliver Notch activity and switch cells to a specific cell fate (Jakobsson et al., 2010); 3. Paracrine activation of Notch-Dll4 signaling is also possible, as demonstrated for Fibulin-3 in endothelial cells to promote glioma angiogenesis (Nandhu et al., 2014); 4. Additionally, it has been demonstrated that Dll4 protein can be incorporated into endothelial and tumor exosomes, that in turn can transfer it to other endothelial cells, incorporating the membrane, which results in an inhibition of Notch signaling (Sheldon et al., 2010). Among nuclear modulation there are several mechanisms of non-canonical Notch signaling that involve interactions of Notch with non-CSL transcription factors, such as β-catenin (Hayward et al., 2005), HIF1-α (hypoxia-inducible factor 1 alpha) (Gustafsson et al., 2005), NF-κB (Guan et al., 1996) and estrogen receptor ERα (Hao et al., 2010). Other examples of nuclear context regulation are the role of cooperating transcription factors, like Sox and Fox transcription factors for DLL4 expression during arterial specification (Sacilotto et al., 2013), the chromatin regulatory complexes or the factors that modify the stability or activity of NICD (Sarah J. Bray, 2016).

2. Metastatic process

Metastasis, the spread of malignant cells from a primary tumor to distant sites, is the main cause of cancer-related deaths from solid tumors (Gupta et al., 2006). It is a complex biological process that involves a series of discrete steps, which have been modeled into a “metastatic cascade” (Thomas R Geiger & Peeper, 2009) (Fig.3).

Figure 3 - The metastatic cascade. *In* (Thomas R Geiger & Peeper, 2009).



0) Being induced by a distant tumor and mediated by bone marrow-derived cells, a "pre-metastatic niche" forms before metastasis becomes evident. **1)** Cells in the primary tumor undergo Epithelial-to-mesenchymal transition (EMT) and acquire invasive properties. **2)** Degradation of basement membranes and remodeling of the Extracellular matrix (ECM) by proteinases facilitate tumor cell invasion. **3)** Tumor cells invade surrounding tissue as single cells **(3a)** or collectively **(3b)**. **4)** Intravasation of tumor cells into newly formed vessels within or nearby the tumor. **5)** Tumor cells are transported through the vasculature and arrest in a capillary bed where they extravasate **(6)**. **7)** Extravasated tumor cells can stay dormant for years. **8)** Eventually, some disseminated cells grow out to a secondary tumor/ macro-metastasis, requiring ongoing ECM remodeling and angiogenesis **(9)**. Cells outside their normal microenvironment undergo anoikis ("detachment-induced apoptosis"). Anoikis could hamper metastasis at several steps of the cascade, as indicated in the scheme. Not all steps of the metastatic cascade necessarily occur in a linear way. For example, premalignant tumors can already be vascularized while the timing of induction of the pre-metastatic niche remains elusive.

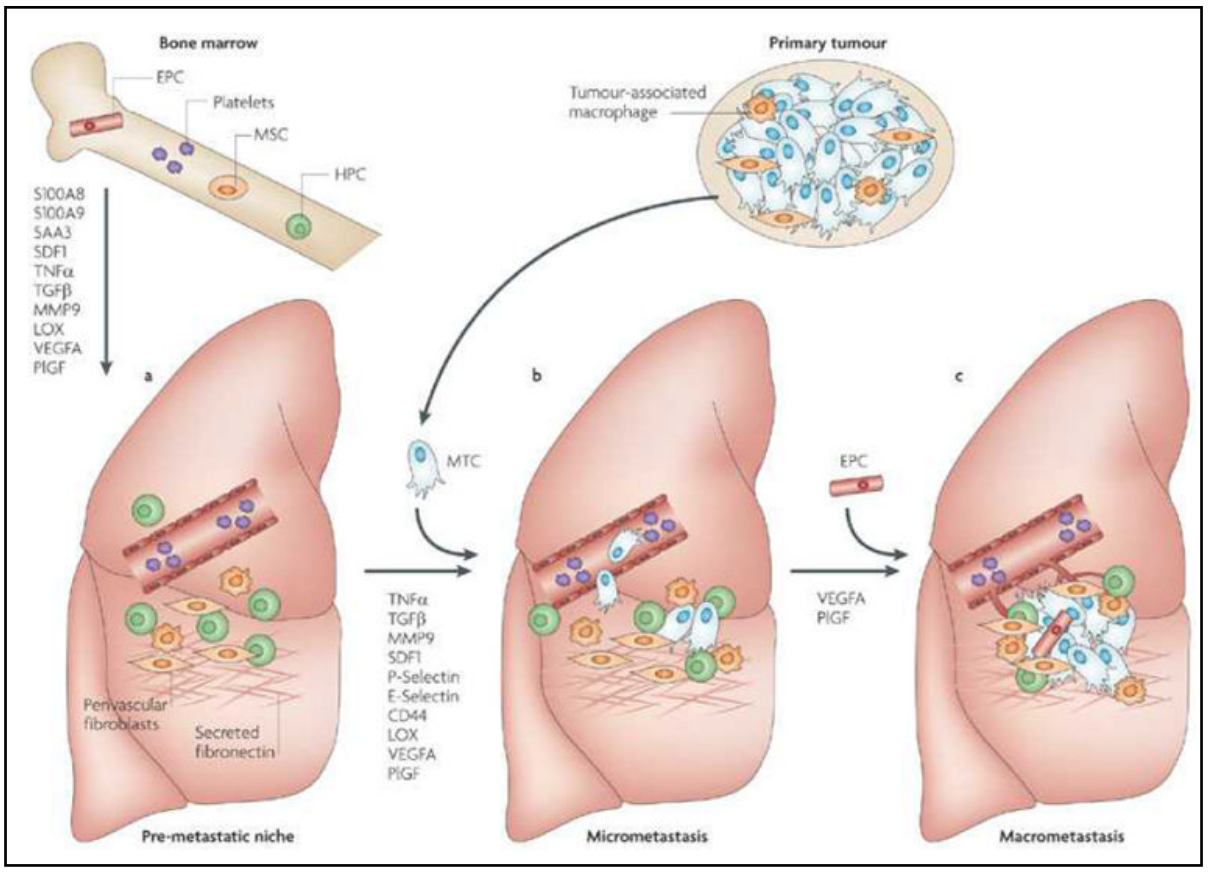
Depending on the cancer type, metastasis may be achieved by just a rare minority of tumor-initiating cells that reach, survive and eventually overtake a distant tissue microenvironment over a long period of time, as in certain types of breast cancer (Pantel & Brakenhoff, 2004; Zhang et al., 2009), or it may represent a relatively common occurrence among primary tumor cell populations that are primed to perform many of the necessary steps and prone to forming rapidly growing metastatic colonies as, for example, in lung adenocarcinoma (Nguyen, Bos, & Massagué, 2009; Quintana et al., 2008).

2.1. Pre-metastatic niche

Steven Paget's "seed and soil" hypothesis, proposed over a century ago, still forms the basis of our understanding of the metastatic process. He suggested that factors inherent to certain tumor types (the seeds) might allow them to preferentially grow in certain organs (the soil) (Paget, 1889). It is now well established that specific organs are predisposed to metastases in certain cancers and that signaling between cytokines, chemokines, and their receptors regulates tumor cell homing to secondary organs, the so called "metastatic niche" (Joyce &

Pollard, 2009; Psaila & Lyden, 2009) (Fig.4). This microenvironment made on the crosstalk between metastatic tumor cells, stromal and bone marrow-derived cell (BMDC) lineages dictates whether a disseminated tumor cell (DTC) survives and proliferates, becomes quiescent, or dies at metastatic sites.

Figure 4 - A model of the evolution of a metastatic niche. *In* (Psaila & Lyden, 2009).



a) In response to growth factors secreted by the primary tumor including vascular endothelial growth factor (VEGF), placental growth factor (PlGF) and transforming growth factor beta (TGF- β), inflammatory S100 chemokines and serum amyloid A-3 are upregulated in pre-metastatic sites leading to clustering of bone marrow-derived hematopoietic progenitor cells (HPCs). Platelet-deployed stromal-derived growth factor (SDF)-1 is also chemotactic for $CXCR4^{+}$ HPCs and metastatic tumor cells (MTCs). HPCs secrete a variety of pre-metastatic factors including TNF-α, matrix metalloproteinase (MMP)-9 and TGF-β. Activated fibroblasts secrete fibronectin, an important adhesion protein in the niche, and lysyl oxidase (LOX) expression is increased, modifying the local extracellular matrix. **b)** MTCs engraft the niche to populate micro-metastases. The site specific expression of adhesion integrins on activated endothelial cells such as P- selectin and E-selectin may enhance MTC adhesion and extravasation at these sites, and cell-cell interactions such as CD44 ligation in the metastatic niche may promote MTC survival and enable proliferation. **c)** Recruitment of endothelial progenitor cells (EPCs) to the early metastatic niche mediates the angiogenic switch and enables progression to macro-metastases.

Recent evidence suggests the primary tumor itself is able to influence and alter the environment of secondary organs by promoting the formation of the supportive metastatic

microenvironments, termed “pre-metastatic niches”, prior to tumor cell dissemination. Kaplan and colleagues first described the pre-metastatic niche in 2005 (Kaplan et al., 2005). They demonstrated that tumor-derived VEGF and placental growth factor (PlGF) promoted the recruitment of VEGFR1+ hematopoietic progenitor cells (HPCs) to secondary organs. Once there, these clusters of VEGFR1+ HPCs expressing the fibronectin receptor integrin VLA-4 interact with resident fibroblasts to stimulate fibronectin production and secrete matrix metalloproteinase (MMP)-9 to create pre-metastatic niches for disseminating CXCR4+ tumor cells. Subsequent research has identified various tumor-derived secreted factors (TDSFs) and BMDCs important in pre-metastatic niche formation in different tumor models (Psaila & Lyden, 2009). Although it is likely that pre-metastatic niches are not essential for metastases to form, various studies suggest that they greatly enhance the likelihood of metastatic progression (Psaila & Lyden, 2009).

2.2. Epithelial-to-mesenchymal transition (EMT) and Cancer Stem Cells (CSC)

During the transformation of a tumor *in situ* to an invasive carcinoma, epithelial tumor cells are released from their neighbors and breach the basement membrane barrier. The process underlying this phenomenon has often been suggested to involve EMT (Christofori, 2006; Thiery, 2002). EMT is a unique process by which epithelial cells undergo remarkable morphologic changes characterized by a transition from epithelial cobblestone phenotype to elongated fibroblastic phenotype (mesenchymal phenotype) leading to increased motility and invasion (Christiansen & Rajasekaran, 2006; Klymkowsky & Savagner, 2009). Cells lose epithelial cell-cell junction, cell polarity, actin cytoskeleton reorganization and the expression of proteins that promote cell-cell contact such as E-cadherin and γ-catenin, and gain the expression of mesenchymal markers such as vimentin, fibronectin, α-smooth muscle actin (SMA), fibrillar collagen (type I and III), fibroblast-specific protein-1, N-cadherin as well as increased activity of matrix metalloproteinases like MMP-2, MMP-3 and MMP-9 (Min, Eddy, Sherr, & Sonenshein, 2008; Moreno-Bueno, Portillo, & Cano, 2008). Although EMT was originally identified as a crucial differentiation and morphogenetic process during embryogenesis, it has now been well illustrated in many patho-physiological conditions including tumor progression and metastasis (Wang, Li, Kong, & Sarkar, 2010).

The key signaling pathways and molecules inducing EMT include Receptor Tyrosine Kinases (RTKs), the transforming growth factor beta (TGF- β) superfamily, WNT, NOTCH and Hedgehog pathways (Gregory, Bracken, Bert, & Goodall, 2008; Peter, 2009) and NFκB (Cannito et al., 2008). Several of the EMT-inducing pathways play prominent roles in development and stem cell self- renewal (Sahlgren, Gustafsson, Jin, Poellinger, & Lendahl, 2008; Timmerman et al., 2004). Many studies have demonstrated that loss of epithelial phenotype through EMT can promote the acquisition of a stem-like phenotype and drug resistance (Kong, Li, Wang, & Sarkar, 2011). In fact, recent progress in tumor stem cell biology suggests that cells that mediate metastasis are cancer stem cells (Kakarala & Wicha,

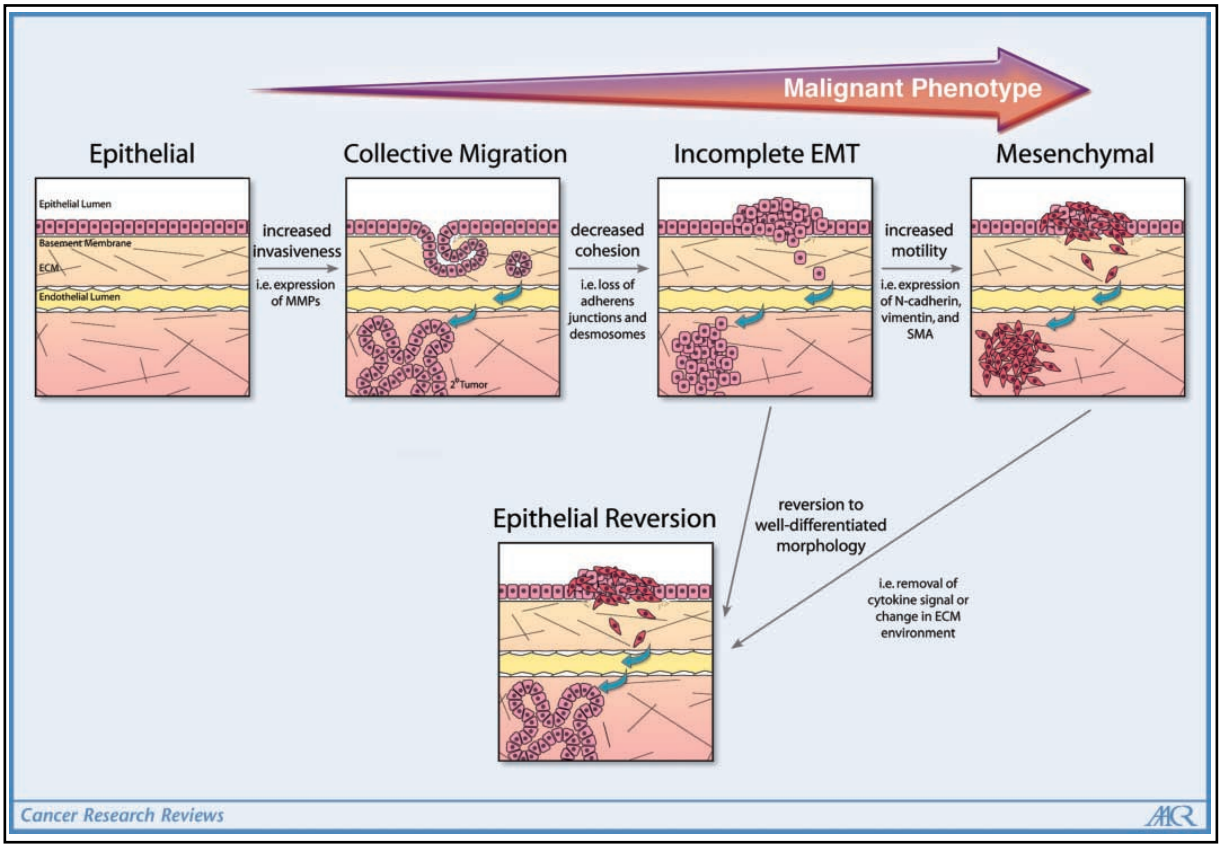
2008). Notch signaling regulates both the formation of CSC and the acquisition of the EMT phenotype (Wang, Li, Banerjee, & Sarkar, 2009; Wang, Li, Kong, Ahmad, et al., 2010).

Notch activation triggers mesenchymal transformation not only in epithelial but also in endothelial cells (endoMT). These changes include downregulation of endothelial markers (vascular endothelial-cadherin, tyrosine kinase with immunoglobulin-like and epidermal growth factor-like domain (Tie) 1, Tie2, platelet-endothelial cell adhesion molecule (PECAM) 1, and endothelial nitric oxide synthase (eNOS) and upregulation of mesenchymal markers (α-SMA, fibronectin, and platelet-derived growth factor receptors) (Noseda et al., 2004). Notch also cross-talks with several transcription and growth factors relevant to EMT, including Snail-1, Slug, and TGF-β. Snail-1 has been shown to be activated during EMT and act as repressor of E-cadherin expression. The relation with Notch pathway was demonstrated by Timmerman et al. in immortalized endothelial cells that expressed Snail-1 under the overexpression of Notch-1, resulting in EMT and oncogenic transformation (Timmerman et al., 2004). Notch signaling also controls Snail-1 expression under hypoxic conditions, both directly via transcriptional activation and indirectly via lysyl oxidase (LOX) (Sahlgren et al., 2008). In this context, EMT is promoted by direct regulation of *Twist* expression, through the induction of the hypoxia inducible factor 1-alpha (Hif1-α) (Yang & Wu, 2008). Moreover, it has been reported that Jagged-mediated activation of Notch induces Slug expression, with subsequent repression of E-cadherin and EMT promotion (Leong et al., 2007; Niessen et al., 2008). Additionally, Jagged-1 or Hey-1 inhibition can arrest *TGF*-β-induced EMT (Espinoza, Pochampally, Xing, Watabe, & Miele, 2013).

2.3. Invasion and cell migration

The EMT program orchestrates a particular type of invasiveness that has been termed ‘‘mesenchymal.’’ However, there are at least two other forms of invasion, that have been observed in tumors with incomplete or no EMT (Christiansen & Rajasekaran, 2006; P. Friedl & Wolf, 2008; Peter Friedl & Wolf, 2010) (Fig.5).

Figure 5 - Epithelial to malignant transition encompasses a wide range of metastatic phenotypes. *In* (Christiansen & Rajasekaran, 2006).



‘‘Collective invasion’’ involves nodules of cancer cells moving *en masse* into adjacent tissues and is characteristic of, for example, squamous cell carcinomas; interestingly, such cancers are rarely metastatic (Douglas Hanahan & Weinberg, 2011); conversely, a selective advantage could be that those cell clones with different properties (like survival, migration, protease secretion) could collaborate and support each other in order to successfully metastasize (Clark & Vignjevic, 2015; Thomas R Geiger & Peeper, 2009).

The ‘‘amoeboid’’ form of invasion, in which individual cancer cells show morphological plasticity, enabling them to slither through existing interstices in the extracellular matrix rather than clearing a path for themselves, as occurs in both the mesenchymal and collective forms of invasion, is not so well understood (Madsen & Sahai, 2010; Sabeh, Shimizu-Hirota, & Weiss, 2009).

What has become clear over time is that invasion is a plastic process and that tumor cells can adapt to different conditions by switching their properties and requirements. For example, the Friedl laboratory showed that upon blocking protease function, cells could switch from mesenchymal to amoeboid migration *in vitro* and *in vivo*. This switch makes the cells independent of proteases and enables them to continue to invade in the presence of protease inhibitors (Wolf et al., 2003).

Furthermore, invasion could be enhanced by the cross-talk between cancer cells and cells of the neoplastic stroma, with inflammatory cells producing the extracellular matrix-degrading

enzymes and other factors that enable invasive growth and obviate the need of EMT program activation (Joyce & Pollard, 2009; Kessenbrock, Plaks, & Werb, 2010; B.-Z. Qian & Pollard, 2010).

When tumor cells lose contact with the basement membrane during invasion they hit another barrier against metastasis: anoikis (cell death induced by inappropriate or loss of cell adhesion). This process, first described by Meredith and Frisch, is thought to ensure tissue homeostasis and normal development (Frisch & Francis, 1994; Meredith, Fazeli, & Schwartz, 1993). Similarly, anoikis could hamper metastasis by inducing apoptosis when tumor cells enter "foreign" environments, particularly during tissue invasion, transport through blood and lymph vessels and after extravasation at distant anatomical sites (Liotta & Kohn, 2004). Anoikis suppression, therefore, is likely to be a prerequisite for tumor cells to successfully metastasize to distant sites (T. R. Geiger & Peeper, 2005; Zhu et al., 2001). Plus, it has been demonstrated that Twist and Snail are required for anoikis suppression in different cell systems (Smit, Geiger, Song, Gitelman, & Peeper, 2009) and that Slug is fundamental for Notch-mediated repression of E-cadherin, which resulted in β-catenin activation and resistance to anoikis, connecting this event to EMT (Leong et al., 2007).

2.3.1. Dll4-Notch signaling in EMT, CSC and invasion

There are several reports linking DLL4 function to the metastasis process.

Multivariate logistic analysis of renal cell carcinoma (RCC) specimens showed that tumor hematogenous metastasis not only depended on angiogenesis but also was associated with tumor size and DLL4 density. Functionally, the migration and invasion capacities of RCC cells were directly enhanced by DLL4–Notch binding. In addition, it was demonstrated that MMP secretion, involved in the basement membrane disruption and invasion, was Notch dependent because inhibition of the Notch effector Hey1, decreased both MMP2 and MMP9 (Huang et al., 2014).

In another study, Dll4-Notch signaling blockade has shown to inhibit liver micro-metastasis of human small cell lung cancer cells expressing high levels of *Dll4*. The authors suggest that was achieved by suppressing the early steps of liver metastasis through Notch1/ NF-κB signaling attenuation, without significantly affecting angiogenesis (Kuramoto et al., 2012).

Xu et al. demonstrated that MMGZ01, a novel human DLL4-specific monoclonal antibody, induces tumor growth inhibition through various mechanisms, namely inhibition of tumor cell proliferation, promotion of tumor cell apoptosis and reduction of the cancer stem-like cell population, leading to the reversal of EMT (Xu et al., 2016).

Recently, Wieland et al. have shown that tumor cells can stimulate Notch1 signaling activity of endothelial cells, leading to its morphology and function alterations in a way that promotes tumor cells transmigration, intravasation as well as homing at distant sites. VCAM-1 secreted by this senescent, pro-inflammatory endothelium seems to be the implicated chemokine. VCAM-1 is an adhesion factor for leukocytes and also transmits signals into the endothelial

cells to induce stress fiber formation and opening of endothelial cells junctions. Notch activation with immobilized DLL4 ligands also resulted in stronger induction of a senescence-associated protein in cultured endothelial cells, compared to control, but clear demonstration that Dll4 is the responsible ligand for this phenotype was missing (Wieland et al., 2017).

2.4. Angiogenesis

Similar to normal tissues, tumors require vasculature to ensure sustenance in the form of nutrients and oxygen as well as elimination of metabolic wastes and carbon dioxide.

Embryonic vascularization involves the differentiation of new endothelial cells from progenitor cells and their assembly into tubes (vasculogenesis) and the sprouting of new vessels from existing ones (angiogenesis). In the adult, normal vasculature is largely quiescent, with physiologic angiogenesis being activated only during processes such as wound healing and the female reproductive cycle. But, during tumor progression, an “angiogenic switch” is almost always turned on, leading to a continuous sprouting of new vessels (neo-angiogenesis) in order to maintain the expanding neoplastic growth. Neoangiogenesis can be observed already at premalignant stages of tumorigenesis, before local invasion occurs, as is seen in several cancer mouse models and in human breast and cervical cancer (D Hanahan & Folkman, 1996; Douglas Hanahan & Weinberg, 2011). Besides neo-angiogenesis, tumor vasculature can be generated by the co-option of existing blood vessels (William P J Leenders, Küsters, & de Waal, 2002), vasculogenic mimicry (in which poorly differentiated, highly malignant tumor cells can form a primitive vascular system) (Hendrix, Seftor, Kirschmann, Quaranta, & Seftor, 2003) or a combination of these processes.

The growth of new vessels is tightly held in check by a delicate balance of angiogenic activators (VEGFA, angiopoietins (Ang), platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs) and epidermal growth factor (EGF)) and angiogenic inhibitors (thrombospondin 1, angiostatin, endostatin, tumstatin and Dll4) (Gabriele Bergers & Benjamin, 2003; Kalluri, 2003; Suchting et al., 2007).

VEGF signaling cascade is accepted as the most relevant pathway in the regulation of angiogenesis. There are four *Vegf* genes known in the mouse, *Vegf-A*, *B*, *C* and *D* and one growth factor associated, PlGF (Neufeld, Cohen, Gengrinovitch, & Poltorak, 1999). *Vegf-A* is the family gene more important for embryonic development being a powerful inducer of angiogenesis (D Hanahan & Folkman, 1996; Shweiki, Itin, Soffer, & Keshet, 1992), also involved in vascular permeability regulation (Senger et al., 1983). VEGFA has been shown to interact with VEGFR1 and VEGFR2. Following ligand-receptor activation occurs receptor dimerization followed by trans/auto-phosphorylation of tyrosine residues in the cytoplasmic kinase domain. However, even though VEGFR1 was firstly identified (Shibuya et al., 1990), it was shown that VEGFA stimulates only very weak auto-phosphorylation of VEGFR1 (Waltenberger, Claesson-Welsh, Siegbahn, Shibuya, & Heldin, 1994), and mouse embryos with only *Vegfr-1* tyrosine kinase deleted formed normal vascular structures and survived

with defects in macrophage migration (S Hiratsuka, Minowa, Kuno, Noda, & Shibuya, 1998). These observations suggest this receptor functions as a decoy for VEGFA, or negative regulator, limiting its availability for VEGFR2 and not as a VEGF signal transducer (S Hiratsuka et al., 1998; Sachie Hiratsuka, Nakao, Nakamura, Katsuki, & Maru, 2005; Veikkola, Karkkainen, Claesson-Welsh, & Alitalo, 2000). Contrarily, activation of VEGFR2 by VEGFA stimulates a number of signal transduction pathways that later drive mitogenesis, migration, and survival of endothelial cells (Karamysheva, 2008). Moreover, VEGFA does not interact with receptor VEGFR3, its ligands being two other members of this family, VEGFC and VEGFD (Achen et al., 1998; Lee et al., 1996), mainly involved in lymphangiogenesis (Alitalo & Carmeliet, 2002; Kaipainen et al., 1995). In general, VEGF gene expression can by upregulated both by hypoxia and by oncogene signaling (Carmeliet, 2005; Ferrara, 2009; Gabhann & Popel, 2008). Additionally, VEGF ligands can be sequestered in the extracellular matrix in latent forms that are subject to release and activation by extracellular matrix-degrading proteases (e.g., MMP-9) (Kessenbrock et al., 2010).

The angiopoietin signaling is also involved in the regulation of angiogenesis and most specifically on the control of endothelium and surrounding support cells interactions. This pathway is composed of two tyrosine kinase receptors Tie1 and Tie2 (*Tek*) and four ligands, angiopoietins 1-4 (Ang1-4) (Ward & Dumont, 2002). Although both bind to Tie2, Ang1 and Ang2 exert different effects in the Tie2-signaling cascade (Davis et al., 1996; Maisonpierre et al., 1997). While Ang1 is able to stimulate Tie2 phosphorylation, interaction with Ang2 does not result in activation of the receptor. Therefore, similarly to what was described to Vegfr1, Ang2 is a competitive inhibitor, or antagonist, of Ang1 (Maisonpierre et al., 1997). Angiopoietin 1 was shown in mesenchymal cells, including pericytes and smooth muscle cells, while Tie2 is expressed on the surface of endothelial cells (Ramsauer & D'Amore, 2002). As a result, Ang1/Tie2 signaling promotes the association between endothelial cells and perivascular support cells, contributing to stabilization of the maturing vascular system and decreased vascular permeability (Armulik, Abramsson, & Betsholtz, 2005).

PDGF pathway participates in pericytes recruitment to form the walls of newly formed vessels (Betsholtz, 2004). This family has four different PDGF isoforms (A-D) establishing functional homodimers (PDGF-AA, PDGF-BB, PDGF-CC, and PDGF-DD) or a heterodimer PDGF-AB (Fredriksson, Li, & Eriksson, 2004). PDGF receptors (PDGFRα and PDGFRβ), similar to VEGF receptors, belong to the superfamily of receptor tyrosine kinases and are transmembrane proteins whose intracellular region contains the tyrosine kinase domain (Betsholtz, 2004). Upon interaction with their ligands, PDGFR receptors form homo- or heterodimers (Betsholtz, 2004; Fredriksson et al., 2004). PDGF-AA binds only PDGFRα, while PDGF-BB exhibits higher affinity for receptor PDGFRβ but is also able to bind PDGFRα and PDGFR heterodimers (Betsholtz, 2004). During angiogenesis, endothelial cells express PDGF-BB, creating a concentration gradient that stimulates the recruitment of

pericytes, PDGFRβ responsive, and thus assisting in the capillary wall formation (Gabriele Bergers & Song, 2005; Gerhardt et al., 2003).

Intra-tumor hypoxic conditions promote not only sustained angiogenesis, but also is described as an inducer of an invasive and metastatic phenotype (Sullivan & Graham, 2007). In this process, the hypoxia-inducible factors (HIF1-α, HIF2-α) play central roles (Harris, 2002). Under normoxic conditions, the tumor suppressor VHL, an E3 ubiquitin ligase, targets HIF1-α for degradation. During hypoxia, HIF1-α accumulates due to increased protein stability. Furthermore, it can occur by genetic alterations in oncogenes (like RAS or PI3K) or tumor suppressor genes (like VHL or PTEN). HIF1-α regulates numerous target genes including many that are involved in angiogenesis (for example VEGF), cell proliferation and glucose metabolism (Semenza, 2003).

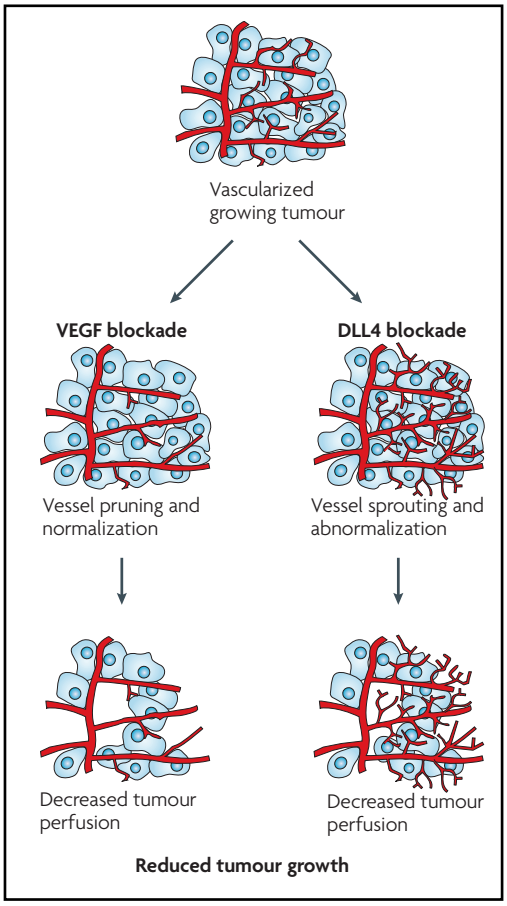
The blood vessels produced within tumors by chronically activated angiogenesis and an unbalanced mix of pro-angiogenic signals are typically aberrant: tumor neo-vasculature is marked by precocious capillary sprouting, convoluted and excessive vessel branching, distorted and enlarged vessels, erratic blood flow, micro-hemorrhaging, leakiness, and abnormal levels of endothelial cell proliferation and apoptosis (Baluk, Hashizume, & McDonald, 2005; Nagy, Chang, Shih, Dvorak, & Dvorak, 2010). In one hand, this leads to a high interstitial (tissue) pressure in solid tumors, with deficient supply of nutrients and oxygen. Anti-angiogenic therapeutics can induce “normalization” of the tumor vasculature, conceivably increasing the efficacy of cytotoxic agents, because of a better perfusion of the tumor (R. K. Jain, 2005). Consistent with this hypothesis, anti-VEGF treatment with bevacizumab significantly improves survival among chemotherapy-treated patients with metastatic colorectal cancer (Giantonio et al., 2007; Hurwitz et al., 2004). Bevacizumab is a humanized monoclonal antibody to VEGF that effectively prevents it from binding to its receptors. On the other hand, the overall immature state and dysfunctionality of angiogenic vessels appear to be at odds with the ability of the same vascular networks to sustain active intravasation of tumor cells and their dissemination to secondary sites. Therefore, the pericyte-mediated vessel stabilization and architectural support are essential for the functionality of tumor angiogenic vasculature and its ability to sustain tumor cell dissemination (Raza, Franklin, & Dudek, 2010).

2.4.1. Dll4-Notch signaling in tumor angiogenesis

Studies in humans and mice have demonstrated that Dll4 is strongly expressed by the tumor vasculature and generally not by the tumor cells themselves. Furthermore, in various mouse models (Gale et al., 2004; Mailhos et al., 2001; Noguera-Troise et al., 2006) and in human tumors from kidney, bladder, colon, brain and breast (A M Jubb et al., 2009; Adrian M. Jubb et al., 2010; N. S. Patel et al., 2006; Nilay S Patel et al., 2005) there is a robust Dll4 expression in the majority of tumor vessels, but significantly lower or no expression in the vasculature of adjacent normal tissue.

Although Dll4 is an anti-angiogenic molecule, in 2006, two reports described the paradoxical phenotype of inhibiting Dll4 in restricting tumor growth (Noguera-Troise et al., 2006; Ridgway et al., 2006) (Fig. 6). Using soluble forms and antibodies to block the binding of mouse Dll4 to Notch1 receptor (Noguera-Troise et al., 2006) or a humanized phage antibody that blocks mouse and human Dll4 binding to Notch1 with high affinity (Ridgway et al., 2006), they observed that tumor growth reduction was associated with an increase in vessel density, but an overall decreased perfusion.

Figure 6 - Comparison of VEGF versus DLL4-Notch inhibition in tumor angiogenesis. *In* (Thurston, Noguera-Troise, & Yancopoulos, 2007).



These two studies were the first to demonstrate that for tumor development the amount of vessels present is of less importance than the ability of the neo-vasculature to effectively deliver nutrients and oxygen to the actively dividing tumor cells (Thurston et al., 2007). This is due in part to the maturation status of the newly formed vessels, and given that *Dll4* loss-of-function mutants presented decreased mural cell recruitment, the vessel walls become leaky, and thus less perfused and functional (Scehnet et al., 2007; Trindade et al., 2012).

Additionally, Dll4 has been shown to help regulate the cellular actions of VEGF. Mechanistically, tumor-derived VEGF induces Dll4 expression in sprouting endothelial cells (tip cells), which then provide signals to adjacent downstream Notch receptor-bearing endothelial cells (stalk cells) to down-regulate VEGF-induced sprouting and branching

(Hellström et al., 2007). But, when Dll4 is blocked, it induces an increase in tumor vasculature, characterized by sprouting and proliferating small vessel branches. These vessels were poorly functional, also resulting in decreased tumor perfusion and decreased tumor growth. This created the idea of tumor growth reduction by “abnormalization” of tumor vasculature (Thurston et al., 2007).

Tumors responsive to anti-Dll4 therapy include those that are resistant to inhibition with anti-VEGF, highlighting the potential utility of this approach (Ji-Liang Li et al., 2011). However, in rodents Dll4 inhibition or loss of function has been associated with certain adverse events including vascular hyperproliferation in the liver and benign vascular neoplasms (Djokovic et al., 2010; Yan et al., 2010).

Contrarily, when expressed in tumor cells (human glioblastoma and prostate cancer), Dll4 dramatically inhibited tumor angiogenesis, but surprisingly improved the structure and function of tumor vasculature by inducing larger vessels with large lumen and increased vessel perfusion and tumor oxygenation, resulting in increased tumor growth *in vivo*. The mechanism underlying DLL4 stimulation of tumor growth was related to a marked reduction in apoptosis and intra-tumoral hypoxia (J.-L. Li et al., 2007).

2.5. Intravasation and transport through vessels

Intravasation starts when tumor cells orientate themselves towards vessels, followed by directional cell migration (C. Y. Li et al., 2000; Sahai, 2007).

Tumor-Associated Macrophages (TAMs) play a crucial role in this process, through a paracrine signaling loop relying on the CSF1 receptor (expressed on macrophages) and EGFR (expressed on tumor cells) (Lin, Nguyen, Russell, & Pollard, 2001; J. B. Wyckoff et al., 2007; J. Wyckoff et al., 2004).

Although frequently neglected, it was suggested that intravasation does represent a critical step of the metastatic cascade. Studies demonstrated a direct correlation between the number of intravasated cells and the number of lung metastases (J. B. Wyckoff, Jones, Condeelis, & Segall, 2000) and that the number of circulating tumor cells in peripheral blood can be a prognostic factor in breast cancer (Cristofanilli et al., 2004; Stathopoulou et al., 2002). The same has been shown in colorectal cancer for the presence of disseminated tumor cells in the bone marrow, providing a transient “hiding place” for tumor cells (Pantel, Brakenhoff, & Brandt, 2008).

However, it is undisputed that only a very small proportion of tumor cells entering the vasculature will eventually form a full-blown metastasis. Plus, although both metastatic and non-metastatic cells are motile in the primary tumor, only metastatic cells are polarized toward blood vessels, while non-metastatic cells experience fragmentation during intravasation (J. B. Wyckoff et al., 2000).

The physical mechanisms underlying tumor cell intravasation to vessels are likely to be similar to those contributing to extravasation. This involves tumor cells protrude membrane

extensions through gaps in the endothelial wall of lymph vessels (Carr, Carr, Dreher, & Betts, 1980), clusters of tumor cells may enter into “leaky” lymph vessels passively (Cavallaro, Niedermeyer, Fuxa, & Christofori, 2001) and, finally, tumor cells can line blood vessels and replace endothelial cells in “mosaic vessels” or “vascular mimicry” (Chang et al., 2000).

But intravasation occurs mainly through hematogenous routes (Chiang, Cabrera, & Segall, 2016). For that, vessels must have three characteristics, namely: accessible points for vessel wall penetration, given by the deficient pericyte coverage and abnormal endothelial cell lining; enough lumen space for tumor cell circulate after intravasation; and an adequate rate of blood flow to move those cells to distant sites (Deryugina & Quigley, 2015).

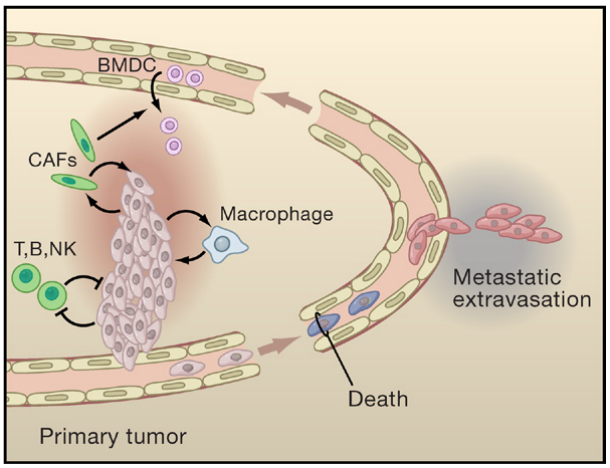
The effect of anti-angiogenic treatments over tumor metastasis was clarified by experiences using the chick embryo spontaneous metastasis model. Contrarily to murine metastasis models, the anti-VEGF treatment did not significantly affect tumor growth or induced hypoxia. They observed a simultaneous reduction in vessel density within the tumor and intravasation, which suggests that decreasing the number of intra-tumoral angiogenic vessels directly limits the number of vascular points of entry available to the escaping tumor cells (Conn et al., 2009).

Furthermore, it appears that interfering with urokinase-type plasminogen activator (uPA) activation and activity might block intravasation/ metastasis by at least two separate mechanisms: 1) decreasing extracellular matrix invasion and 2) decreasing tumor angiogenesis (through VEGF reduction) (Conn et al., 2009). Because disrupting the tumor vasculature alone can actually increase the invasive behavior of tumor cells due to hypoxia-response programs (Brahimi-Horn, Chiche, & Pouysségur, 2007; Ebos et al., 2009; Pàez-Ribes et al., 2009), targeting a protease pathway like uPA could be one potential way to overcome the problem of possible increased tumor invasiveness after anti-angiogenic treatment.

2.6. Extravasation and outgrowth of micro to macro-metastases

After getting access to the blood vessel lumen and withstanding the circulation pressures, metastatic cells will at some point escape once again, but this time out of the endothelial vasculature and into a target tissue in a process called extravasation (Gupta et al., 2006) (Fig. 7). This precise moment may vary from tumor to tumor. In some cases, considerable growth within the intra-vascular space occurs until the lesion physically bursts through the limiting surrounding vasculature (Al-Mehdi et al., 2000).

Figure 7 - Distinct fates for disseminated cancer cells. *In* (Gupta et al., 2006).

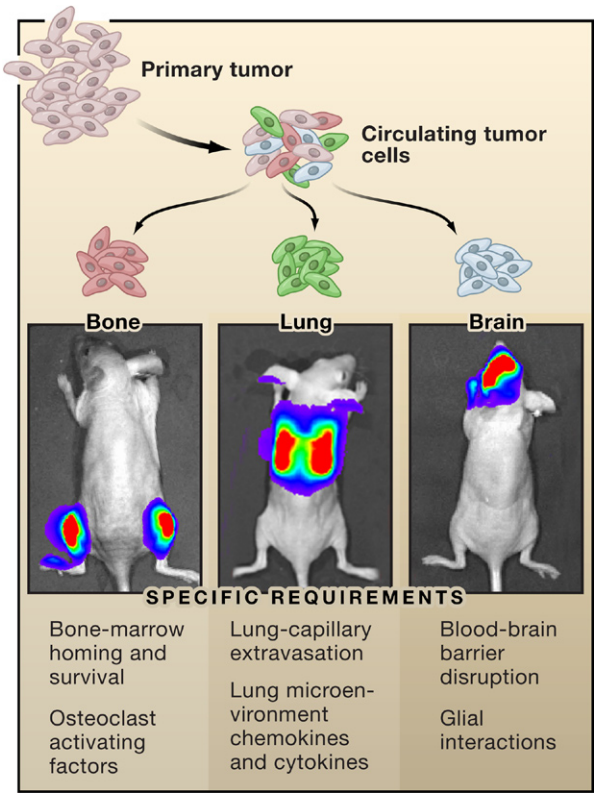


The tumor-suppressive activities of lymphocytes (T, B, and NK cells) are kept in check in part through the release of immuno-suppressive cytokines (e.g., TGFβ, IL-10, and IL-23). Carcinoma-associated fibroblasts (CAFs) can secrete factors that promote tumor-cell growth and invasiveness and also promote angiogenesis through recruitment of endothelial precursor cells (EPCs) from circulation, which are bone marrow derived cells (BMDC). Activated macrophages are also recruited to tumors and release many pro-tumorigenic growth factors. When cancer cells invade the blood stream, many of them will die from stresses associated with circulatory passage. Those that are able to resist death may attach to capillaries within a secondary organ through adhesion receptors. Subsequently, disseminated cancer cells may or may not thrive at the secondary site, with or without metastatic extravasation.

The cytoskeletal anchoring protein ezrin possibly facilitate this escape in metastatic osteosarcoma cells, through anoikis suppression (Khanna et al., 2004). Integrins (Felding-Habermann et al., 2001) as well as selectin proteins (Witz, 2008) play an important role in the interaction of tumor cells with platelets and leukocytes, but also with endothelial cells. Conceivably, formation of tumor cell aggregates facilitated by platelets protects also against anoikis and sheer stress inside the vasculature. Metastatic cells can produce “signals” that actively induce changes in the vascular permeability, like VEGF (Weis & Cheresh, 2005). Indeed, the Src kinases activation in endothelial cells exposed to VEGF induces disruptions in endothelial cell junctions, which can enable metastatic extravasation.

Of the millions of cancer cells that enter the circulation, very few will successfully engraft, survive and proliferate at secondary sites (Luzzi et al., 1998). It is thought that during hematogenous dissemination, the initial localization and extravasation of cells at secondary sites occurs very efficiently, while the initiation and persistence of growth is highly inefficient (Ann F. Chambers, Groom, & MacDonald, 2002). The anatomic architecture of the blood flow is not sufficient to explain this phenomenon. Most probably it is affected by both the receptiveness of the local microenvironment and also by cell-intrinsic factors responsible for a survival advantage in specific environments (Langley & Fidler, 2007) (Fig. 8).

Figure 8 - Patterns of metastatic colonization. *In* (Gupta et al., 2006).



In line with the "seed and soil" hypothesis, distinct genetic "signatures" of tumor cell subpopulations were recently identified that correlate with a propensity for metastasis to specific organs (Minn et al., 2005), while other studies have characterized "metastasis suppressor genes" that when re-expressed in malignant cells prevent metastasis, without affecting their growth at the primary tumor site (Steeg, 2003).

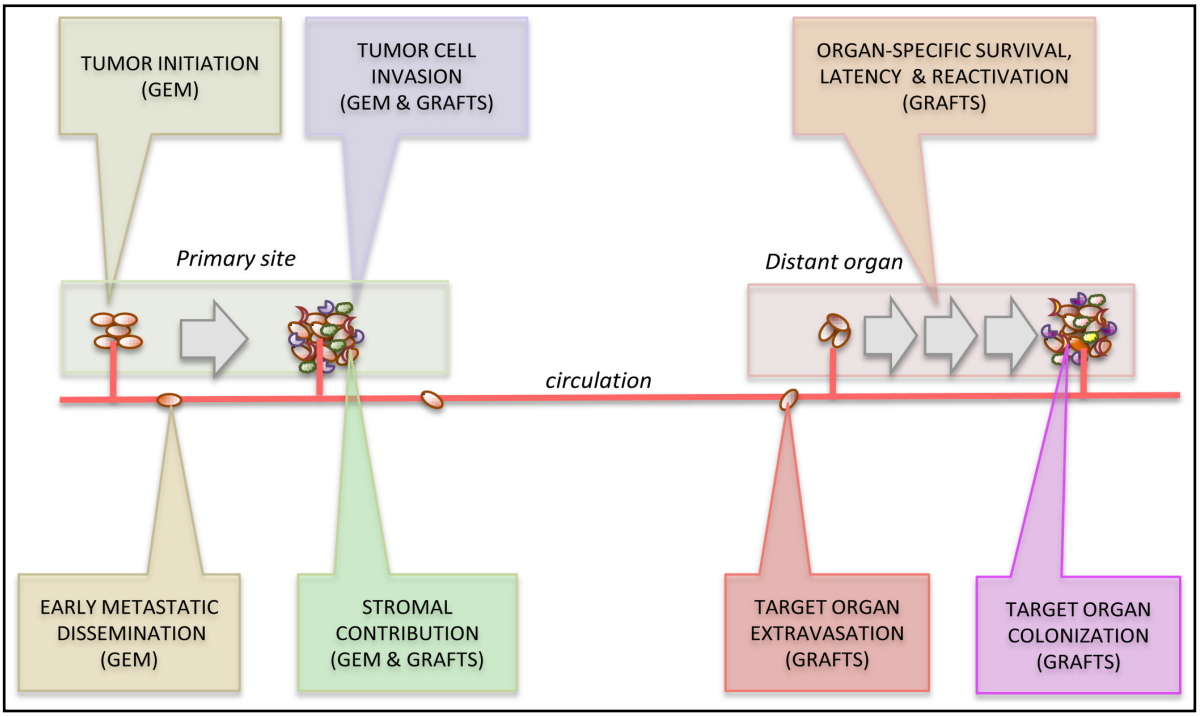
The interactions between tumor cells and molecular components of the local microenvironment or metastatic niche like the hyaluronic acid receptor CD44, fibronectin, collagen I, osteopontin and laminin are crucial in the evasion of cell death during micro-metastases formation (Q. Yu, Toole, & Stamenkovic, 1997). Subsequently, the assembly of a functional vasculature is required to enable further cellular expansion and progression to macro-metastases, a process for which activation of the angiogenic switch is required (Holmgren, O'Reilly, & Folkman, 1995; Naumov, Akslen, & Folkman, 2006). Bone marrow-derived endothelial progenitor cells (EPCs) were identified as critical regulators of this process (Gao et al., 2008). In addition to EPCs, hematopoietic and mesenchymal cells aid in macro-metastatic progression. TAMs potentiate the angiogenic stimulus by expression of VEGF and angiopoietins, accelerate recruitment of other inflammatory cells, and secrete proteases furthering matrix remodeling (Pollard, 2004). But the signals that initiate EPC recruitment and the angiogenic switch in the setting of dormant micro-metastases and the molecular pathways underlying macro-metastatic progression remain unclear. Plus, it is not known if all disseminated cancer cells undergo the process of mesenchymal-to-epithelial transition (MET), as a mechanism to help tumor cells in the secondary organ to undergo "re-

differentiation" to an epithelial phenotype similar to the primary tumor (Mego, Reuben, & Mani, 2017).

3. Mouse models of metastasis

The *in vitro* model systems were the first used to study metastatic cells characteristics like migration and invasiveness. Technological advances such as fluorescent or bioluminescent reporter molecules and sophisticated microscopy have allowed sensitive and accurate analysis of these processes and their molecular underpinnings at the single-cell or cell-cluster levels (A F Chambers, Naumov, Vantyghem, & Tuck, 2000; C. W. Wong et al., 2002). Additionally, *in vitro* model systems helped in the search of candidate metastasis genes in particular steps of the metastatic cascade (A F Chambers, Schmidt, MacDonald, Morris, & Groom, 1992). However, these models can provide surrogate systems for the analysis of only a limited set of events in the metastatic cascade, which *in vivo* involves multiple steps within specific tissue contexts. To better understand tumor development and progression *in vivo*, two general strategies have been pursued in mice: transplantable tumor model systems and genetically engineered models of cancer (Bos, Nguyen, & Massagué, 2010) (Fig. 9).

Figure 9 - Contribution of different mouse models to the various steps of metastatic dissemination. *In* (Bos et al., 2010).



GEM: Genetically engineered mouse models; GRAFTS: xeno-or allograft transplantation.

3.1. Transplantable tumor model systems

Transplantable models can be divided into two broad groups, syngeneic/allograft models and xenograft models.

Syngeneic/allograft transplantable models usually refer to mouse or rat (murine) cancer cell lines or tissues that result in tumors in inbred animals of the same genetic background as the derived cell line or tissue. Traditionally syngeneic cell lines derive from carcinogen-induced tumors or tumors that spontaneously develop in a particular mouse or rat (Brown, Buchmann, & Balmain, 1990; Double, Ball, & Cowen, 1975). The advantage of syngeneic models is that the transplanted tissues, the tumor microenvironment, and the host are from the same strain of the same species and are immunocompetent. This is particularly important when considering the close interaction between tumor and host characterized by the process of metastasis. These model systems lack many of the important features of human tumors. For example, they are usually derived from homozygously inbred mice and therefore lack the genetic complexity of human tumors. In addition, due to species-specific differences in oncogenesis, they may not bear the same constellation of mutations observed in human patients.

Xenograft models are made with human cancer cell lines or tissues that can be transplanted into immunocompromised animals and effectively grow tumors. These tumors are a mosaic of human cancer cells and murine stromal cells. Several lines of evidence suggest the importance of cancer cell-stromal cell interactions in the biology of cancer progression and metastasis. For some pathways, species specificity does not allow this interaction to occur across species boundaries (Cooper et al., 2003; De Wever & Mareel, 2003; Schmidt-Hansen et al., 2004). Furthermore, in order for human tumors to grow in mice the murine host must be immunocompromised to prevent immune rejection. This eliminates the ability to examine the role of the immune system in tumor progression in xenografts.

Both models can be subjected to one or two experimental approaches: experimental metastasis assays and spontaneous metastasis assays. These assays differ in the way that cells are delivered to the recipient animals.

3.1.1. Experimental metastasis

In the experimental metastasis assay tumor cells are directly injected into the systemic circulation. Depending on the site of injection and tropism of the tumor cell, distant metastases may (or may not) develop at a number of anatomic locations throughout the body. The most common site of tumor cell injection is the lateral tail vein in mice that normally results in pulmonary metastases. In contrast, intrasplenic or portal vein injection of tumor cells is the typical site employed for developing metastasis in the liver. The influence of injection site on end-organ target is in part explained by the first capillary bed that tumor cells face following injection of cells (Khanna & Hunter, 2005).

Experimental metastasis models made possible the development of clonally related variants of tumor cell lines that differ in their metastatic potential. The best example is the B16F10 cells (melanoma) (Poste, Doll, Hart, & Fidler, 1980).

There are several advantages of these models. The time course for model maturity is generally rapid, the biology of metastasis is reproducible and consistent, and the user has control of the number and type of cells that are introduced to the circulation. But it has also disadvantages. Because cells circulate, often in high numbers, as single cells or small clusters of platelets they may not arrest or interact with target tissues at distant sites in the same ways as tumor cells that spontaneously metastasize (as emboli). Furthermore, the process of spontaneous metastasis from a primary site may be associated with selection events that yield a distinct profile of successful metastasis. As a result, experimental metastases have often been described as multiple primary tumors developing in the lung (Khanna & Hunter, 2005).

3.1.2. Spontaneous metastasis

Historically, transplantable tumor models were characterized by and selected for rapid primary tumor growth at subcutaneous (s.c.) (heterotopic) sites. In this scenario, it was uncommon to observe spontaneous metastasis to distant sites. Nowadays, this was overstepped with the selection of highly metastatic cell sublines, like LLC cells (Ke et al., 2008). Still, efforts based on the "seed and soil" hypothesis led to the development of tumor cells orthotopic transplantation into mice (Hoffman, 1999; Killion, Radinsky, & Fidler, 1998). Orthotopic transplantation refers to the delivery of cancer cells to the anatomic location or tissue from which a tumor was derived. This technique has resulted in tumor models that may more closely resemble human cancers including tumor histology, vascularity, gene expression, responsiveness to chemotherapy and metastatic biology (Bibby, 2004; Rashidi et al., 2000).

The fact that spontaneous metastases arise from a primary transplanted tumor provides an opportunity to study the metastatic process and many aspects of the metastatic cascade that are bypassed using experimental metastasis models.

3.2. Genetically engineered models of cancer

Genetically engineered models driven by the introduction of oncogenic mutations in a tissue-specific manner can faithfully recapitulate important aspects of tumor initiation, local progression, and response to therapy (Frese & Tuveson, 2007; Jonkers & Berns, 2002). In these models, cancer develops with high penetrance in a stepwise way enabling the study of tumor initiation and early steps of metastatic dissemination. Unfortunately the majority of the spontaneously arising tumors, at least in mouse models, do not metastasize, metastasize with a very long latency or are characterized by intravascular metastases alone, precluding easy and efficient analysis. Fortunately a number of genetically engineered models have been developed that produce *bona fide* metastatic disease (Bos et al., 2010).

While it is possible that the constitutive activation or loss of a gene in these models may not completely replicate native metastasis, the tumors do arise in their normal context, more

closely replicating the clinical setting and in a system possessing a functional immune system. Meantime, although transgenic tumors metastasize at a higher frequency than spontaneously arising or chemically induced tumors the latency for metastases for most is still measured in months. Also, the relative penetrance for metastatic disease is often significantly lower than that of tumor incidence. As a consequence, large numbers of animals are often required and for long periods of time in order to generate enough population-based data for analysis. Plus, due to the variability in tumor dissemination and penetrance of metastatic disease it is difficult to stage animals or detect the presence of metastatic disease before death (Khanna & Hunter, 2005).

3.3. Imaging metastasis in the mouse

Tracking cancer cells in real time in whole animals has provided a tremendous advantage in the dynamic monitoring of metastatic development.

Imaging studies for metastasis have included the use of bioluminescence and fluorescent imaging techniques to evaluate the fate of single metastatic cells within the mouse. These single-cell imaging studies have shed new light on the biology of metastasis and the interactions of metastatic cells with their microenvironment early in the process of metastasis (Khanna et al., 2004). In general, transplantation models of cancer offer greater flexibility for imaging strategies. Tumor cells may be transfected to express targets for imaging (e.g. green fluorescent protein (GFP), luciferase) or may be labeled immediately before delivery to mice (e.g. quantum dots) (Byron Ballou, B. Christoffer Lagerholm, Lauren A. Ernst, Marcel P. Bruchez, & Alan S. Waggoner†, 2003; Jenkins et al., 2003). In addition, the same tissues can be further analyzed by histological detection of GFP in frozen sections, or immunohistochemical detection of the reporter (Ponomarev et al., 2004). The development of more advanced reporter systems based on similar technology has also permitted the *in vivo* monitoring of gene pathway activity and inhibition (Korpal et al., 2009).

EXPERIMENTAL WORK

Chapter I. Exploratory experiences

Based on a report (Xu et al., 2016) demonstrating the effect of systemic *Dll4* inhibition over EMT in breast cancer, we did a preliminary trial with LLC cells *in vitro.*

1. Methods

1.1. Lewis Lung Carcinoma cells *in vitro* assay with anti-Dll4 antibody

Sterile coverslips were placed into the wells of a 24-well plate. A highly metastatic sub-line of LLC cells, kindly provided by Dr. Parkash Gill, was used. Cells were plated in a concentration of 2×10^4 cells/well. To induce the EMT process, half the wells were treated with 10 ng/ml recombinant human TGF-β1 (R&D Systems 240-B-010) for 48h (Haynes, Srivastava, Madson, Wittmann, & Barber, 2011). Then arbitrary wells EMT-induced were treated with 10 µg/ml of anti-Dll4 antibody (Yamanda et al., 2009) for 48h (cells reached 80-90% of confluence).

For the immunostaining assay the following process was applied (Tang, Herr, Johnson, Resnik, & Aho, 2013): fixation with 4% Paraformaldehyde (PFA) for 20 min (room temperature); incubation with blocking buffer (Phosphate buffered saline (PBS) with 1% Bovine Serum Albumine (BSA), 10% donkey serum and 0.3% Triton X-100) for 1h, at room temperature; incubation with the primary antibodies Alexa-488 conjugated mouse anti-E-cadherin, goat polyclonal anti-Snail and Rhodamine Phalloidin, an anti-F-actin probe conjugated to TRITC (Molecular Probes by Life Technologies R415), overnight at 4ºC; and 1h incubation at room temperature with fluorescently-tagged specific secondary antibodies (Invitrogen). In the end, cells were rinsed in deionized water and mounted, with coverslips face down on a slide, using 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) Fluoromount-G™ (SouthernBiotech) as mounting media.

EMT was quantified by determining the area (in pixels) of cells (through nuclei marked with DAPI) stained against E-cadherin, F-actin and Snail-1, in 20 fields per sample.

1.2. Statistical analysis

Data processing was carried out using the Statistical Package for the Social Sciences software, version 17.0 (SPSS v. 17.0; Chicago, IL) and GraphPad Prism, version 6.0f (GraphPad Software). Statistical analyses were performed using Student's t-test. All results are presented as mean ± Standard error mean (SEM). *P*-values < 0.05 and <0.01 were considered significant (indicated in the figures with * and **, respectively), and *P*-values <0.001 and <0.0001 were considered highly significant (indicated with *** and ****, respectively).

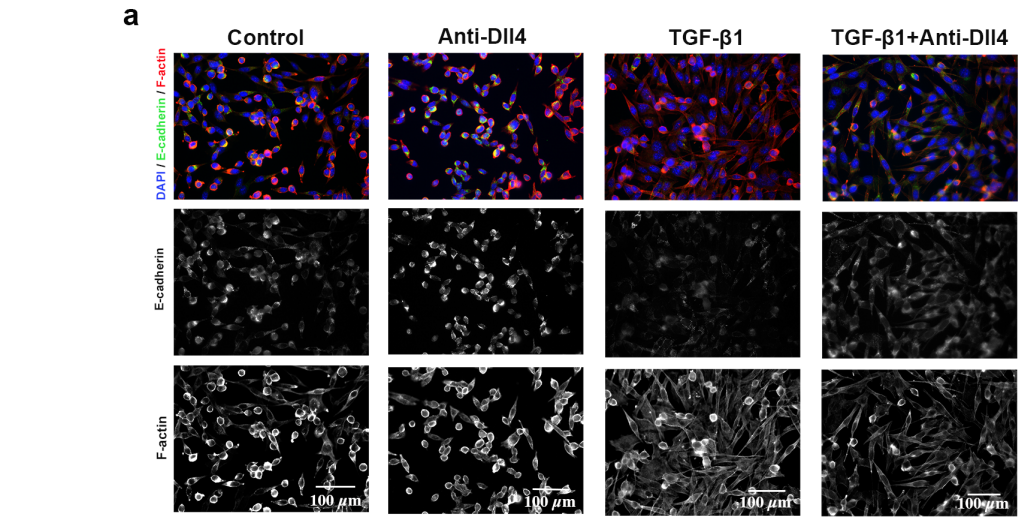
2. Results

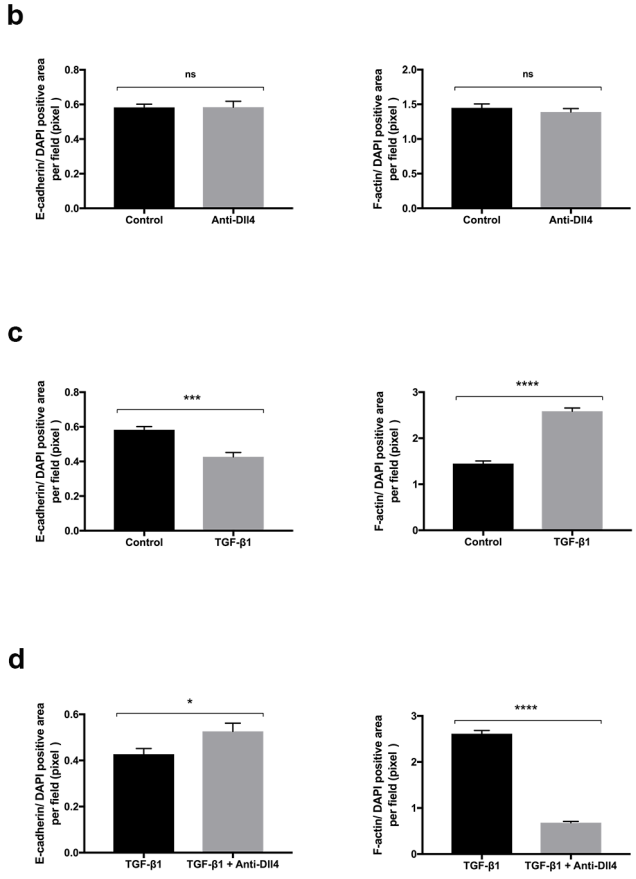
2.1. Blocking *Dll4* stops epithelial-to-mesenchymal transition of LLC *in vitro*

Firstly, we characterized the cells by immunostaining against E-cadherin (epithelial marker), F-actin and Snail-1 (both mesenchymal markers), after culture in standard conditions (Figure 1a and 2a, Control). The same analysis was done after anti-Dll4 treatment alone, as a negative control (Figure 1a and 2a, Anti-Dll4). Despite LLC cells expressing Dll4, no differences were observed (Figure 1b and 2b). Secondly, the immunostainings were repeated after TGF-β1 stimulation alone, which stimulates EMT (Figure 1a and 2a, TGF-β1). As shown in Figures 1c and 2c, TGF-β1 stimulation was effective in EMT induction, since there was a significant decrease in cells with a more epithelial phenotype *versus* an increase in cells with a more mesenchymal phenotype.

Finally, we tested the effect of anti-Dll4 treatment on LLC cells stimulated with TGF-β1 (Figure 1a and 2a, TGF-β1+ Anti-Dll4). We observed that blocking Dll4/Notch signalling in LLC cells with an anti-Dll4 antibody stops EMT induction, because TGF-β1 plus Anti-Dll4 treated cells had an increase in the epithelial-like cell number and a decrease in the mesenchymal-like cell count, compared to the cells stimulated with TGF-β1 alone (Figure 1d and 2d).

Figure I-1 - *In vitro* LLC cells EMT response to anti-Dll4 antibody treatment.

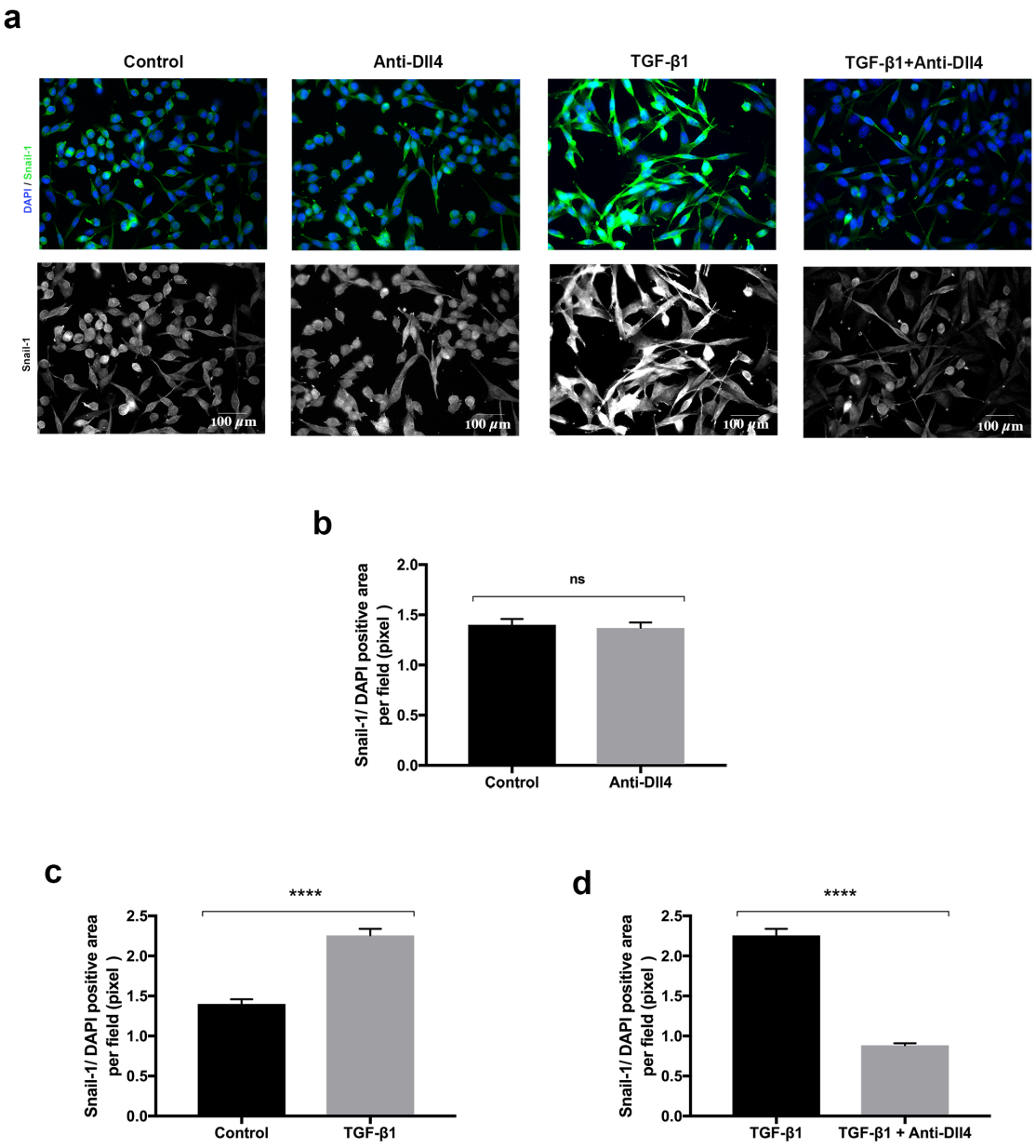




a) Immunostaining images (40x amplification) of LLC cells marked for E-cadherin (green) and F-actin (red), to evaluate epithelial and mesenchymal characteristics (EMT), respectively. "Control" images represent LLC cells in standard conditions. "Anti-Dll4" images display LLC cells in standard conditions submitted to anti-Dll4 antibody treatment alone. "TGF-β1" images represent LLC cells stimulated with TGF-β1, to induce EMT. "TGF-β1+Anti-Dll4" images display LLC cells stimulated with TGF-β1 and then treated with anti-Dll4 antibody. 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; blue) stains nuclei. Immunostaining assay was performed 72h after plating 2×10^4 cells/well, in a 24-well plate.

b) Graphs demonstrate that there isn't difference in the number of LLC cells undergoing EMT between "Control" and "Anti-Dll4" conditions. **c)** LLC cells undergo EMT after TGF-β1 exposure. **d)** The anti-Dll4 antibody is effective in reverting the EMT process after TGF-β1 stimulation. EMT quantification of LLC cells was assessed by the number of epithelial cells (E-cadherin/DAPI positive area per field, in pixels) and mesenchymal cells (F-actin/DAPI positive area per field, in pixels). Error bars represent SEM; ^{ns} Not Statistically Significant; *P<0.05; ***P<0.001; ****P<0.0001.

Figure I-2 - LLC cells TGF-β1 induced marked with Snail-1 after anti-Dll4 antibody treatment.



a) Snail-1 (green) was also used to confirm mesenchymal phenotype of LLC cells (40x amplification immunostaining images) in "Control" (standard conditions), "Anti-Dll4" (anti-Dll4 treatment alone), "TGF-β1" (TGF-β1 stimulation alone) and "TGF-β1+Anti-Dl4 (TGF-β1 induced cells treated with anti-Dll4). **b)** Graphs illustrate that anti-Dll4 treatment has no effect in LLC cells in standard conditions but reduces significantly the EMT process in LLC cells stimulated with TGF-β1. Mesenchymal cells count is given by Snail-1/DAPI positive area per field, in pixels. 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; blue) stains nuclei. Immunostaining assay was performed 72h after plating 2×10^4 cells/well, in a 24-well plate. Error bars represent SEM; ^{ns} Not Statistically Significant; ****P<0.0001.

As expected, Dll4 blockade arrested EMT. This result prompted us to explore the role of endothelial-specific Dll4 in the EMT mechanism, as a crucial step in the metastatic process.

Chapter II. Metastasis is impaired by endothelial-specific *Dll4* loss-of-function through inhibition of epithelial-to-mesenchymal transition and reduction of cancer stem cells and circulating tumor cells

Liliana Mendonça, Alexandre Trindade, Catarina Carvalho, Jorge Correia, Marina Badenes, Joana Gigante, António Duarte.

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1. Abstract

Systemic inhibition of Dll4 has been shown to thoroughly reduce cancer metastasis. The exact cause of this effect and whether it is endothelial mediated remains to be clarified. Therefore, we proposed to analyze the impact of endothelial *Dll4* loss-of-function on metastasis induction on three early steps of the metastatic process, regulation of epithelial-to-mesenchymal transition (EMT), cancer stem cell (CSC) frequency and circulating tumor cell (CTC) number. For this, Lewis Lung Carcinoma (LLC) cells were used to model mouse tumor metastasis *in vivo,* by subcutaneous transplantation into endothelial-specific *Dll4* loss-of-function mice.

We observed that endothelial-specific *Dll4* loss-of-function is responsible for the tumor vascular regression that leads to the reduction of tumor burden. It induces an increase in tumoral blood vessel density, but the neovessels are poorly perfused, with increased leakage and reduced perivascular maturation. Unexpectedly, although hypoxia was increased in the tumor, the number and burden of macro-metastasis was significantly reduced. This is likely to be a consequence of the observed reduction in both EMT and CSC numbers caused by the endothelial-specific *Dll4* loss-of-function. This multifactorial context may explain the concomitantly observed reduction of the circulating tumor cell count. Furthermore, our results suggest that endothelial Dll4/Notch-function mediates tumor hypoxia-driven increase of EMT. Therefore, it appears that endothelial *Dll4* may constitute a promising target to prevent metastasis.

Keywords: Dll4/Notch, tumor-angiogenesis, tumor-metastasis, epithelial-to-mesenchymal-transition, cancer-stem-cells, circulating-tumor-cells.

2. Introduction

Most current cancer therapies fail to control the metastatic process (Ma & Adjei, 2009), making metastasis-based research a priority to improve patient prognosis.

The classical view of the metastatic cascade, starting from a primary, epithelial-neoplastic lesion includes the following steps. 1) Creation of a "pre-metastatic niche" at the target site. 2) Epithelial-to-mesenchymal transition (EMT) and breach of the basement membrane barrier. 3) Invasion of the neighbouring tissue. 4) Intravasation into pre-existing and newly formed blood and lymph vessels. 5) Transport through vessels. 6) Extravasation from vessels. 7) Establishment of disseminated cells at a secondary anatomical site. 8) Outgrowth of micro-metastases to macro-metastases/secondary tumors, that will be angiogenesis-limited until they undergo their own angiogenic switch (Abdollahi & Folkman; Thomas R Geiger & Peeper, 2009).

Angiogenesis is one of the hallmarks of cancer and constitutes an important area of interest in oncological drug development. However, the suggestion that anti-angiogenic drugs, mostly targeting the VEGF pathway, could end up increasing the invasiveness of the tumor and the frequency of metastasis is a major cause for concern requiring thorough analysis and clarification (Ribatti, 2011; Saranadasa & Wang, 2011).

Dll4/Notch pathway-blocking therapies have been shown to be effective in pre-clinical models resistant to VEGF-based anti-angiogenic therapies. Dll4 is an endothelial-specific Notch ligand, with expression restricted to small arteries and capillaries in the adult (Rui Benedito & Duarte, 2005; Duarte et al., 2004; Noguera-Troise et al., 2006). Histological observation of tumors treated with Dll4/Notch inhibitors revealed that the reduced tumor growth was associated with an increase in tumor vascular density. However, those blood vessels were poorly perfused, leading to increased tumor hypoxia. It appears that excessive branching results in a highly chaotic vascular network lacking the essential hierarchy for efficient directional blood flow (S. K. Liu et al., 2011; Noguera-Troise et al., 2006; Ridgway et al., 2006; Scehnet et al., 2007; Yamanda et al., 2009).

Notch pathway genes cross-talk with several transcription and growth factors relevant to EMT, including Snail1, Slug, and TGF-β.

The acquisition of the EMT phenotype implies a reduction in cell-to-cell adhesion mediated in part by the loss of E-cadherin expression. Snail-1 has been shown to be activated during EMT and act as repressor of E-cadherin expression. The relationship with the Notch pathway was demonstrated by Timmerman et al. in immortalized endothelial cells, that expressed Snail1 under the overexpression of Notch-1, resulting in EMT and oncogenic transformation (Timmerman et al., 2004). Notch signalling also controls Snail1 expression under hypoxic conditions, both directly via transcriptional activation and indirectly via lysyl oxidase (LOX) (Sahlgren et al., 2008). In this context, EMT is promoted by direct regulation of Twist expression, through the induction of the hypoxia inducible factor 1 alpha (Hif-1 α) (Yang &

Wu, 2008). Moreover, it has been reported that Jagged-mediated activation of Notch induces Slug expression, with subsequent repression of E-cadherin and EMT promotion (Leong et al., 2007; Niessen et al., 2008). Additionally, Jagged1 or Hey1 blocking can arrest TGF-β -induced EMT (Espinoza et al., 2013).

There are several reports linking DLL4 function to the metastasis process. The migration and invasion capacities of renal cell carcinoma (RCC) were directly enhanced by DLL4-Notch binding, through Hey1 effector promotion of MMP9 secretion, involved in basement membrane disruption (Huang et al., 2014). In another study, DLL4/NOTCH signalling blockade was shown to inhibit liver micro-metastasis of human small cell lung cancer cells expressing high levels of *DLL4*. The authors suggest that was achieved by suppressing the early steps of liver metastasis, through NOTCH 1/ NF-κB signaling attenuation, without significantly affecting angiogenesis (Kuramoto et al., 2012). More recently, Xu et al. demonstrated that blocking Dll4 leads to tumor growth inhibition through EMT reversal and suppression of putative breast cancer stem cell-like (CSC) characteristics (Xu et al., 2016). However, these studies used systemic Dll4 blockade, which makes it impossible to distinguish between non-cell-autonomous endothelial Dll4 functions from other, non-endothelial, cell-autonomous Dll4 functions in the tumor milieu. Finally, it was suggested that Dll4 may be the ligand that activates Notch-1 in endothelial cells from primay tumor and metastatic niche, which stimulates vascular cell adhesion molecule-1 (VCAM1) expression that is implicated in neutrophil infiltration and metastasis (Wieland et al., 2017).

Another early step of the metastatic cascade involves intravasation of metastatic cells into adjacent blood and lymphatic vessels with circulation to distant sites. This entry occurs mainly through hematogenous routes (Chiang et al., 2016). For that, vessels must have three characteristics: accessible points for vessel wall penetration; enough lumen space; and sufficient rate of blood flow (Deryugina & Quigley, 2015), with increased intravasation correlating with increased vessel diameter in some tumors (Yamamura, Tsukikawa, Yamada, & Yamaguchi, 2001). The connection between disrupting tumor vasculature, through endothelial Dll4 inhibition, and the metastatic process should be elucidated. Additionally, there are also several studies showing that TGF-G signaling can enhance intravasation, at least in part, through the induction of EMT (T. Tsuji et al., 2008; Takanori Tsuji, Ibaragi, & Hu, 2009).

Here we provide evidence that EMT, CSC and CTC frequency are impaired by endothelial-specific *Dll4* loss-of-function, therein compromising the first steps of the metastatic process. For that, we performed tumor burden, vasculature, macro-metastasis, hypoxia, EMT, CSC and Green Fluorescent Protein (GFP)- marked tumor cell analysis in a tumor transplant endothelial-specific *Dll4* loss-of-function mouse model. These findings were further supported by gene expression results.

3. Methods

3.1. Experimental animals

Animals were housed in 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. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed and the studies have been approved by the Ethics and Animal Welfare Committee of the Faculty of Veterinary Medicine of Lisbon. Dll4 conditional knockout mice (*Dll4*$^{lox/lox}$) were generated by flanking the first three exons of the Dll4 allele with two loxP sites. This was done in our laboratory, in collaboration with Dr. Freddy Radtke, as reported (Koch et al., 2008). These mice were then crossed with *VE-cadherin-Cre-ERT2* mice, a kind gift by Dr. Ralf Adams, which express tamoxifen-inducible Cre recombinase under the control of the VE-cadherin endothelial promoter. Tamoxifen was administrated in a dosage of 50 mg/kg, daily intra-peritoneal (i.p.) injections for 5 days. Hence we obtained endothelial-specific inducible Dll4 loss-of-function (eDll4cKO) mice. Control mice have the same genotype but are not induced and are given saline i.p. injections instead.

3.2. Lewis Lung Carcinoma subcutaneous tumor transplant mouse model

A highly metastatic sub-line of LLC cells, kindly provided by Dr. Parkash Gill, was used. These cells were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 medium supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin, in 100mm tissue culture dishes coated with poly-D-Lysine hydrobromide, at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

When cells reached sub-confluence, they were detached by 5 min treatment with 0.25% Trypsin-EDTA and resuspended in PBS to a cell concentration of 1×10^7/ml. For the transplant tumor model, cells (1×10^6/mouse) were inoculated subcutaneously, in the right flank of the mouse under anaesthesia (2.5% Avertin). The test group, named LLC-eDll4cKO mice, was induced with tamoxifen at 6 weeks of age and the control group, named LLC-control mice, were not tamoxifen-induced, based on data from our laboratory demonstrating no differences in tumor growth dynamics or tumor vascular phenotypes when tamoxifen is administered to control group (Ana-Rita Pedrosa, Trindade, Carvalho, et al., 2015). Both groups received LLC cells at 8 weeks of age. Each group had 5 animals, all male and this experiment was repeated twice.

This model reproduces the spontaneous metastasis process (Bos et al., 2010; Cai et al., 2008).

One week after the LLC cell injection, the injection site was evaluated for measurable tumor. The tumor was measured biweekly with a calliper until the end-point, 6 weeks after LLC

injection. Tumor volume was calculated using the formula V = 0.52 x *a* x b^2, where *a* and *b* equal the longer and shorter length of the tumor, respectively (Djokovic et al., 2010).
At the end-point, the mice were humanely euthanized and macro-metastases were observed and counted under a dissection microscope. Their volume was calculated using the above-mentioned formula. Even though all organs and lymph nodes, were inspected, because these cells have tropism for the lungs and given our interest in addressing haematogenous metastasis, we focused on the lungs. These were resected and fixed in Bouin's fixative, for posterior metastasis histological confirmation.

3.3. Fluorescent-activated cell sorting of LLC-GFP

LLC cells were transduced with GFP (AntiCancer Incorporated, San Diego, California, USA) (Rashidi, Moossa, & Hoffman, 2013) and cultured as described. The aforementioned tumor transplant model was done this time with these LLC-GFP cells in groups with 4 animals, all males with two repetitions (test group, named GFP-eDll4cKO and control group, named GFP-control).
At the dissection day, mice of each group were anesthetized (2.5% Avertin) and 1ml of blood (total mouse blood volume) was recovered by cardiac punction (to heparin coated eppendorfs). After erythrocytes lysis with 1x RBC lysis buffer (10x RBC lysis buffer multi-species, eBioscience), the remaining blood cells were washed with Dulbecco's phosphate-buffered saline (DPBS) (1x) no calcium, no magnesium (Gibco) and resuspended in FACS (1x) buffer (eFluor® NC Flow Cytometry Staining Buffer (5X), eBioscience). These samples were used for cytometric count of GFP- positive cancer cells travelling in the peripheric blood, having exited the primary tumor and done intravasation successfully. Cells were sorted in a FACSCalibur cytometer (BD, Enzifarma) and analysed using BD FlowJo sotfware (version 10.0, BD Bioscence).

3.4. Tissue preparation and immunofluorescence

Tumor samples were collected and fixed with 4% PFA solution at 4°C for 1h, cryoprotected in 15% sucrose, embedded in 7.5% gelatine, frozen in liquid nitrogen, cryosectioned at 10 and 20 μm and immunostained. Immunostaining was performed using the following protocol: tissue slides were permeabilized in 3% H_2O_2 methanol solution for 40 min and PBS-Triton 0.5% solution 2 x 10 min; blocking was performed for 1h (room temperature) with 5% BSA in PBS-W 0.1% solution; after blocking, slides were incubated overnight at 4ºC with specific primary antibodies followed by 1h incubation at room-temperature with fluorescently-tagged specific secondary antibodies (Invitrogen). Nuclei were counterstained with 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; Molecular Probes, Eugene, OR). Fluorescent immunostained sections of the tumor tissues (avoiding necrotic central area) were examined under a Leica DMRA2 fluorescence microscope with Leica HC PL Fluotar 20x and 40x/0.5 NA dry objectives (Leica, Heidelberg, Germany), 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 (NIH, Bethesda, MA, USA).

To confirm the endothelial specific Dll4 loss-of-function we quantified the positive signal of the endothelium (platelet endothelial cell adhesion molecule, PECAM-1 staining) (BD Pharmingen, San Jose, CA), co-marked with a goat anti-Dll4 (R&D) and with a rabbit anti-Notch 1 intracellular domain (N1ICD) (Cell Signalling) primary antibodies, on tumor tissue sections (10 μ m). We used as secondary antibodies an anti-goat or an anti-rabbit conjugated with Alexa Fluor 488 (Invitrogen, Carlsbad, CA) and an anti-rat conjugated with Alexa Fluor 594 (Invitrogen, Carlsbad, CA).

To assess vascular perfusion, Avertin (2.5%) anesthetized mice were injected with biotin-conjugated lectin from *Lycopersicon esculentum* (100 μ g in 100 μ l of PBS; Sigma, St. Luis, MO) via caudal vein and allowed to circulate for 10 minutes, before the vasculature was transcardially perfused with 4% PFA in PBS for 3 minutes. Tumor tissue sections (20 μ m) were stained with rat monoclonal anti-mouse platelet endothelial cell adhesion molecule, PECAM-1 (BD Pharmingen, San Jose, CA), followed by Alexa 594 goat anti-rat IgG (Invitrogen, Carlsbad, CA). Biotinylated lectin was visualized with Streptavidin-Alexa 488 (Invitrogen, Carlsbad, CA). Tumor perfusion area was quantified by determining the area (in pixels) of PECAM-1-positive structures that were co-localized with Alexa 488 signals (Invitrogen, Carlsbad, CA).

In order to visualize vascular extravasation, Avertin-anesthetized mice were injected with 1% Evans' Blue dye solution (Sigma, St. Luis, MO) via caudal vein, and perfused transcardially 10 minutes later with 4% PFA in PBS for 3 minutes. Tumor tissue sections (20 μ m) were stained with rat monoclonal anti-mouse PECAM-1, followed by Alexa 488 goat anti-rat IgG. Extravasation was visualized by observing Evans' Blue red fluorescence in contrast with green fluorescent vascular structures (Gratton et al., 2003). Tumor vascular extravasation area was quantified by determining the tumor section field of Evans' Blue red positive signal per vessel area (given by vascular density measurements).

To examine tumor vascular density and vessel maturity, judged by the recruitment of smooth muscle cells and pericytes, we did double fluorescent immunostaining on tumor tissue sections (20 μ m). PECAM-1 with alpha smooth muscle actin (α-SMA) was used to assess perivascular smooth cells, and PECAM-1 with Pdgfr-β was used to address perivascular pericytes. The primary antibodies used were rat monoclonal anti-mouse PECAM-1, mouse monoclonal anti-SMA Cy3 conjugate (Sigma Aldrich, USA), rabbit monoclonal anti-Pdgfr-β (Cell signalling Technology) and the secondary antibody was anti-rat conjugated with Alexa Fluor 488 and anti-rabbit conjugated with Alexa Fluor 594 (Invitrogen, Carlsbad, CA).

For tumor vascular regression analysis, double fluorescent immunostaining for endothelium

and basal membrane was done on tumor tissue sections (20 μ m), using rat monoclonal anti-mouse PECAM-1 and rabbit anti-mouse Collagen IV (Abcam Cambridge, UK), respectively. The secondary antibodies were anti-rat conjugated with Alexa Fluor 488 and anti-rabbit conjugated with Alexa Fluor 594 (Invitrogen, Carlsbad, CA), respectively.

Then we characterized the EMT phenotype by using the antibodies Alexa-488 conjugated mouse anti-E-cadherin, goat polyclonal anti-Snail and rabbit polyclonal anti-Twist (Abcam, Cambridge, UK) on tumor tissue sections (10 μ m). These markers were detected with the secondary antibodies Alexa-488 donkey anti-goat and Alexa-488 donkey anti-rabbit (Invitrogen, Carlsbad, CA), respectively. In the Twist images were co-stained for PECAM-1 to investigate the relation of tumor mesenchymal cells with the blood vessels.

For CSC analysis we used a PE rat anti-human CD49f antibody (BD Pharmigen), which marks the integrin subunit α 6 that enhances multipotency and maintains stemness (Atkinson et al., 2013; K.-R. Yu et al., 2012) and a rabbit anti-p63 antibody (Novus), that marks a transcription factor essential for proliferative potential of stem cells in stratified epithelia (Senoo, Pinto, Crum, & McKeon, 2007). The immunostainings were done separately, on tumor tissue sections with 10 μ m, and additional secondary antibodies used were Alexa-488 donkey anti-rat and Alexa-488 donkey anti-rabbit (Invitrogen, Carlsbad, CA). In the p63 images we co-stained for PECAM-1 to investigate the relation of CSC with the blood vessels.

The Hypoxyprobe^{TM}-1 Plus Kit (Hypoxyprobe, Inc, USA) was used for hyoxia evaluation (cells with a oxygen pressure of 10 mmHg), in a concentration of 60mg/kg body weight. The mice were sacrificed 1 hour after pimonidazole hydrochloride (Hypoxyprobe^{TM}-1) intra-peritoneal administration. For protein adducts detection of pimonidazole hydrochloride in hypoxic cells of tumor tissue sections (20 μ m), was used a primary antibody reagent consisting in a conjugate of Hypoxyprobe^{TM}-1 MAb1 (mouse IgG_1) covalently bound to FITC. Each analysis was repeated in 10 fields per sample.

3.5. Real-time PCR analysis

Tumor samples were collected at the end-point of each experiment and snap frozen in liquid nitrogen until Ribonucleic acid (RNA) extraction (Qiagen RNeasy). A total of 300 ng RNA per reaction was used to generate complementary Deoxyribonucleic acid (cDNA) with the SuperScript III First Strand Synthesis Supermix Q RT-PCR Kit (Invitrogen, Carlsbad, CA). Relative quantification real-time Polymerase chain reaction (PCR) analysis was performed as described (Trindade et al., 2012), using Sybergreen Fastmix ROX dye (Qiagen). Primer pair sequences are available on request. The housekeeping gene β -actin was used as endogenous control.

3.6. Statistical analysis

Data processing was carried out using the Statistical Package for the Social Sciences software, version 17.0 (SPSS v. 17.0; Chicago, IL) and GraphPad Prism, version 6.0f (GraphPad Software). Statistical analyses were performed using Mann-Whitney-Wilcoxon test and Student's t-test. All results are presented as mean ± Standard error mean (SEM). *P*-values < 0.05 and <0.01 were considered significant (indicated in the figures with * and **, respectively), and *P*-values <0.001 and <0.0001 were considered highly significant (indicated with *** and ****, respectively).

4. Results

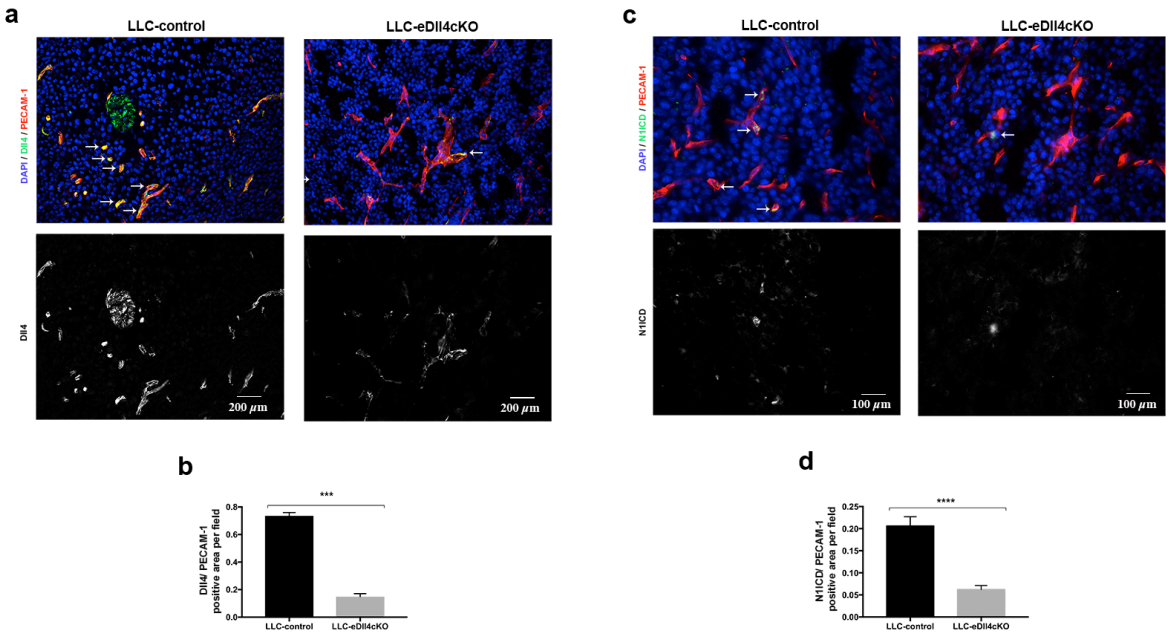
4.1. Endothelial-specific *Dll4* loss-of-function impairs tumor growth and metastasis in a subcutaneous LLC tumor transplant

Given a recent report regarding the effect of *Dll4* inhibition on EMT and subsequent metastasis in breast cancer (Xu et al., 2016), we wanted to determine if this effect was endothelial-mediated. For that we used subcutaneous LLC tumor transplants in endothelial-specific inducible *Dll4* loss-of-function (eDll4cKO) mice.

The endothelial-specific *Dll4* loss-of-function was confirmed by co-stainings for Dll4 plus PECAM-1 (Figure 1a) and for the intracellular domain of Notch1 (N1ICD) plus PECAM-1 (Figure 1c) in tumor samples, collected at the end-point. The LLC-eDll4cKO group revealed a significant reduction in endothelial cells marked with either Dll4 or N1ICD, relative to LLC-control (76% and 70% reduction in Dll4 and N1ICD areas, respectively) ($P < 0.001$ and $P < 0.0001$, respectively; n = 8) (Figures 1b and 1d).

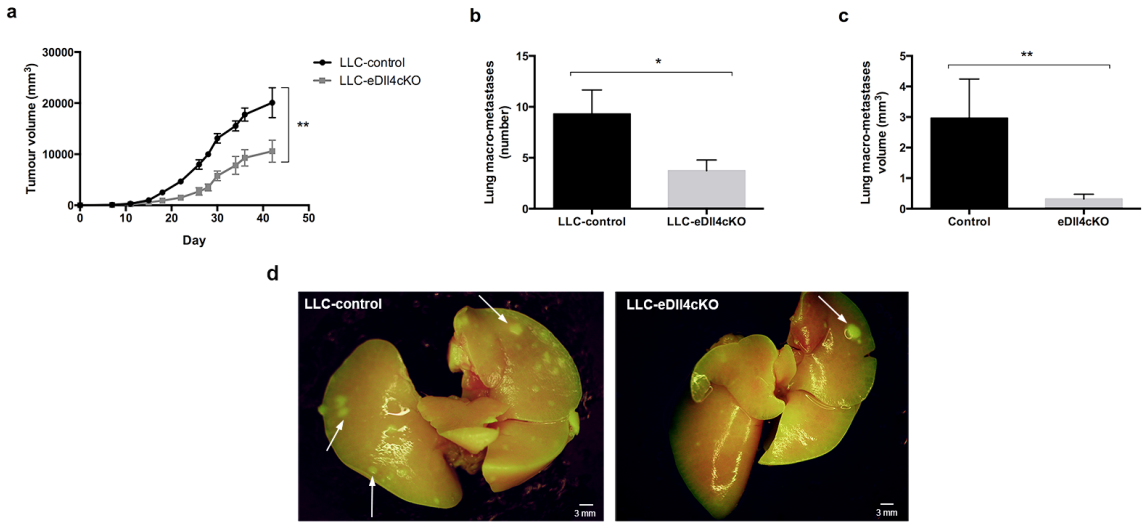
We addressed tumor burden by measuring tumor volume until the end-point (6 weeks after LLC cells injection). As shown in Figure 2a, LLC-eDll4cKO mice displayed a reduction in tumor burden, starting from the first week, and the end-point tumor volume was significantly reduced when compared to LLC-control mice (47% tumor volume reduction) ($P < 0.01$, n = 10). When the lungs were examined for macro-metastases, under a dissection microscope, LLC-eDll4cKO mice displayed a significant decrease in the number (60% reduction) ($P < 0.05$, n = 10) and burden (89% volume reduction) ($P < 0.01$, n = 10) of lung macro-metastases (Figures 2b, 2c and 2d).

Figure II-1 - Immunostaining of Dll4 and Notch1 intracellular domain in LLC tumors transplanted to endothelial-specific Dll4 loss-of-function mice.



a) Immunostaining images (20x amplification) of LLC tumor samples collected at the end-point (6 weeks after LLC cell injection) from two mouse groups, LLC-eDll4cKO and LLC-control, marked for Dll4 (green) and PECAM-1 (red). **b**) Endothelial Dll4-positive area (area of Dll4/ PECAM-1 co-staining per field, in pixels; white arrows) showing significant reduction of Dll4 expression in LLC-eDll4cKO endothelial cells (EC), relative to LLC-control EC. **c)** Immunostaining images (40x amplification) of LLC tumor samples from LLC-eDll4cKO *vs.* LLC-control groups, marked for Notch1 intracellular domain, N1ICD (green) and PECAM-1 (red). **d)** N1ICD-positive area (area of N1ICD/ PECAM-1 co-staining per field, in pixels; white arrows) showing significantly decreased N1ICD in LLC-eDll4cKO endothelial cells (EC), relative to LLC-control EC. LLC tumor samples were collected at the end-point. 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; blue) stains nuclei. Results are representative of 2 independent experiments, each with n=4 mice per group. Error bars represent SEM; ****P*<0.001; *****P*<0.0001.

Figure II-2 - Effect of endothelial-specific Dll4 loss-of-function mice in LLC tumor burden and metastasis formation.



a) LLC tumor growth from day 0, when LLC cells were injected, until the endpoint, 6 weeks later, in two mouse groups, LLC eDll4cKO and LLCcontrol. Endothelial specific loss of *Dll4* led to a decrease in the growth rate of LLC tumors, with an average final volume that was half of that of the respective controls. **b)** LLC tumor lung macro metastases number evaluated at the end point, showing a significant reduction in LLC eDll4cKO mice relative to LLC control. **c)** LLC eDll4cKO mice exhibited reduced macro metastasis burden (volume in mm^3), compared to LLC control mice. **d)** LLC tumor lung macro metastasis stereoscope photographs illustrative of the two mouse groups (the white arrows indicate macro metastasis). Results are representative of 2 independent experiments, each with n=5 mice per group. Error bars represent SEM; **P*<0.05; ***P*<0.01.

4.2. LLC tumor burden reduction is a consequence of the endothelial-specific *Dll4* loss-of function effect on tumor vasculature

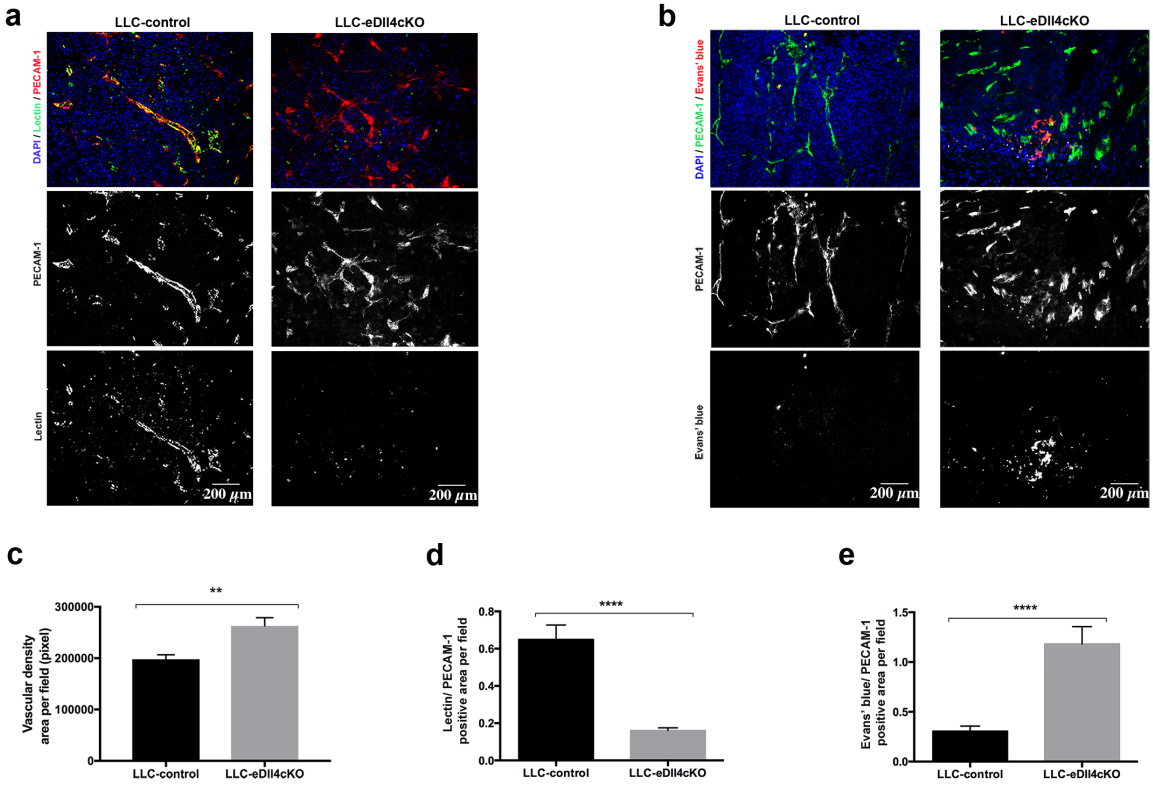
Aiming to understand the possible mechanisms behind tumor burden and metastasis reduction in endothelial-specific *Dll4* loss-of-function mice we started to analyse the LLC tumor vascular phenotype, in tumor samples obtained at the end-point.

LLC tumor vessel functionality was evaluated by analysing biotinylated lectin perfusion and Evans' Blue dye vascular leakage. Despite an increase in vascular density (Figures 3a, 3b and 3c), endothelial-specific *Dll4* loss-of-function was associated with a decrease in the amount of perfused, lectin-containing vessels (75% decrease in vascular perfusion area) (*P* < 0.0001, n = 10) (Figures 3a and 3d). At the same time, Evans' Blue extravasation was significantly increased in comparison with controls (285% increase in vascular extravasation area) (*P* < 0.0001, n = 10) (Figures 3b and 3e).

With regard to vessel maturation we stained blood vessels against PECAM-1 plus α-SMA (perivascular smooth muscle), and against PECAM-1 plus Pdgfr-β (pericyte marker). Vascular density was confirmed to be higher in LLC-eDll4cKO mice (Figures 4a, 4b and 4c), but there was a significant decrease in perivascular smooth muscle (69% decrease in

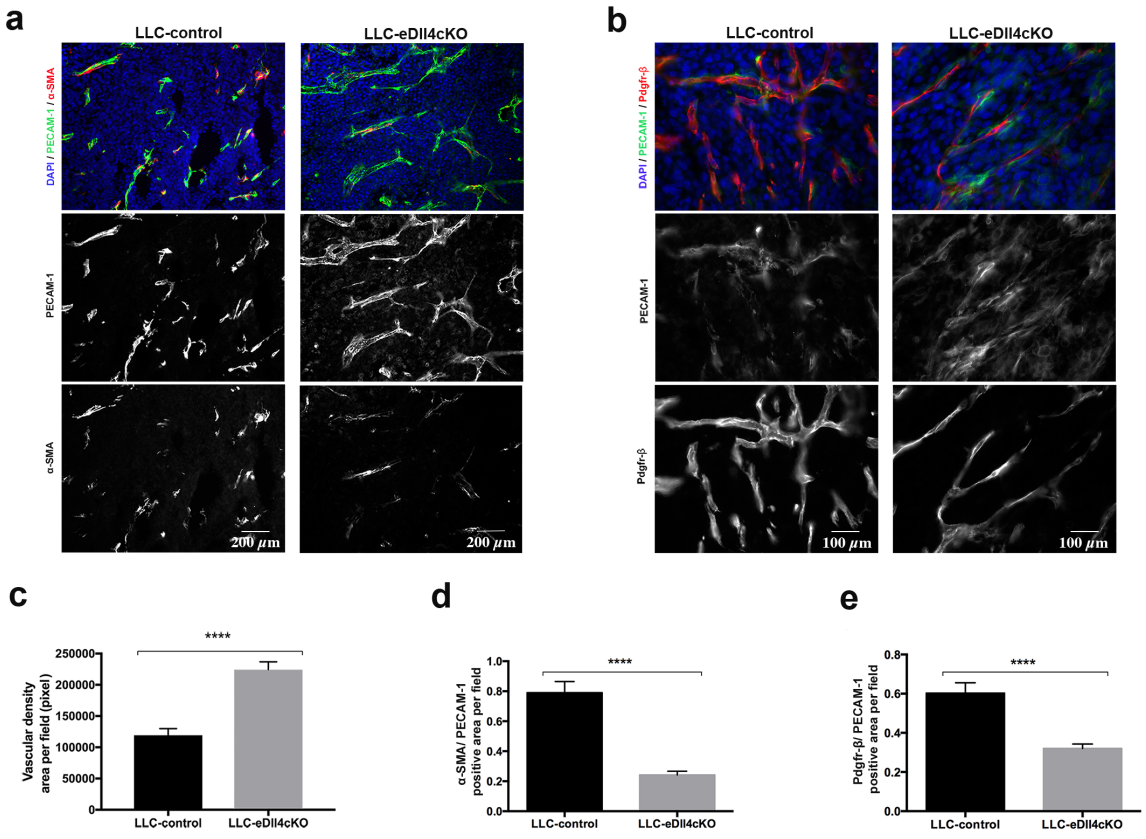
perivascular α-SMA area) ($P < 0.0001$, n = 10) (Figures 4a and 4d) as well as a decrease in pericyte coverage (47% decrease in pericyte area) ($P < 0.0001$, n = 10) (Figures 4b and 4e). This indicates that these vessels are less mature than their equivalent in LLC-control mice. Given these results we hypothesised that this endothelium might experience a regression phenotype. We stained blood vessels against PECAM-1 (endothelium) and Collagen IV (basal membrane). In LLC-eDll4cKO mice, although vascular density was higher (Figures 5a and 5b), we observed an increased vascular regression area (61% increase in vascular regression area) ($P < 0.0001$, n = 10), relative to LLC-control mice (Figures 5a and 5c).

Figure II-3 - Effect of endothelial *Dll4* loss-of-function in LLC tumor vascular perfusion and extravasation.



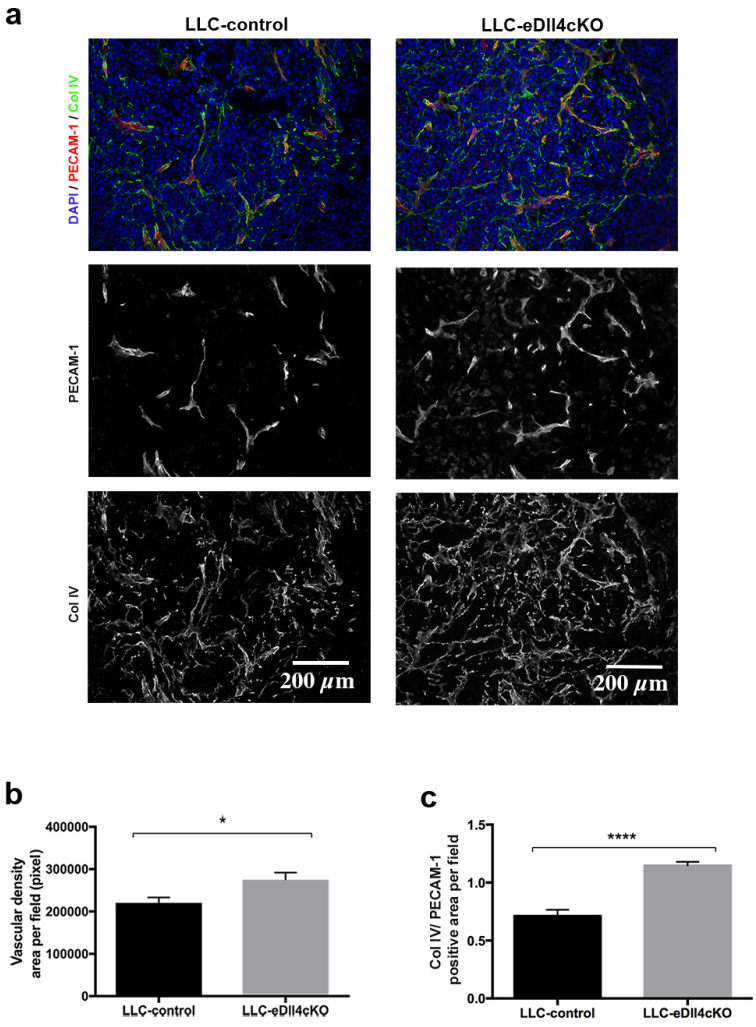
a) Lectin (green) and PECAM-1 (red) immunostaining images (20x amplification) of LLC tumor samples to evaluate the co-localization of both signals, indicative of vessel perfusion, in LLC-eDll4cKO and LLC-control mouse groups. **b)** Evans' blue (red) and PECAM-1 (green) immunostaining images (20x amplification) of LLC tumor samples in the two mouse groups showing the extravasation area. **c)** LLC-eDll4cKO group exhibits an increased vascular density area (area of PECAM-1 staining per field, in pixels), relative to control. **d)** Perfused blood vessels area (area of Lectin/ PECAM-1 co-staining per field, in pixels) showing decreased lectin labelling in LLC-eDll4cKO, compared to LLC-control. **e)** Vascular extravasation area (area of Evans' blue/ PECAM-1 co-staining per field, in pixels) of LLC-eDll4cKO mice is significantly increased, relative to LLC-control mice. 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; blue) stains nuclei. LLC tumor samples were collected at the end-point. Results are representative of 2 independent experiments, each with n=5 mice per group. Error bars represent SEM; ***P*<0.01; *****P*<0.0001.

Figure II-4 - Effect of endothelial Dll4 loss-of-function in LLC tumor vascular maturation.



a) Immunostaining images (20x amplification) marked for PECAM-1 (green) and α-SMA (red), to evaluate vascular density and smooth muscle actin coverage of LLC tumor samples in LLC-eDll4cKO and LLC-control mouse groups. **b)** Immunostaining images (40x amplification) marked for PECAM-1 (green) and Pdgfr-β (red), to evaluate vascular density and pericyte coverage of LLC tumor samples in the two mouse groups. **c)** Vascular density (area of PECAM-1 staining per field, in pixels) is significantly higher in LLC-eDll4cKO mice. **d)** Perivascular smooth muscle area (area of α-SMA/ PECAM-1 co-staining per field, in pixels) is decreased to less than a half in LLC-eDll4cKO, compared to LLC-control. **e)** Pericyte coverage area (area of Pdgfr-β / PECAM-1 co-staining per field, in pixels) of LLC-eDll4cKO mice is reduced, relative to LLC-control mice. 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; blue) stains nuclei. LLC tumor samples were collected at the end-point. Results are representative of 2 independent experiments, each with n=5 mice per group. Error bars represent SEM; ****P<0.0001.

Figure II-5 - Vascular regression in LLC tumors transplanted to endothelial-specific *Dll4* loss-of-function mice.



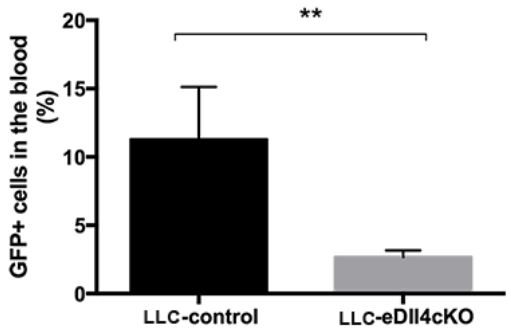
a) Vascular regression area immunostaining images (20x amplification), obtained with co-staining for PECAM-1 (green), that addresses vascular density, and Collagen IV (red), that stains the basal membrane, of LLC tumor samples collected in LLC-eDll4cKO and LLC-control mouse groups. **b)** LLC-eDll4cKO group exhibits increased vascular density area (area of PECAM-1 staining per field, in pixels), relative to control. **c)** Vascular regression area (area of Col IV/ PECAM-1 co-staining per field, in pixels) is highly increased in the tumors of LLC-eDll4cKO mice, compared to LLC-control mice. 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; blue) stains nuclei. LLC tumor samples were collected at the end-point. Results are representative of 2 independent experiments, each with n=5 mice per group. Error bars represent SEM; **P*<0.05; *****P*<0.0001.

4.3. Endothelial-specific *Dll4* loss-of-function inhibits metastasis by affecting epithelial-to-mesenchymal transition and reducing the number of cancer stem cells causing a reduction of circulating tumor cells

To verify if the observed reduction in metastasis was caused by a reduction in CTC number we quantified circulating LLC tumor cells expressing GFP. For that, blood samples were collected at the end-point and processed for FACS. Cytometry analysis demonstrated that the LLC-GFP cells percentage was substantially decreased in the LLC-eDll4cKO group, relative to LLC-control (76% decrease in GFP cells detected in blood) ($P < 0.01$) (Figure 6).

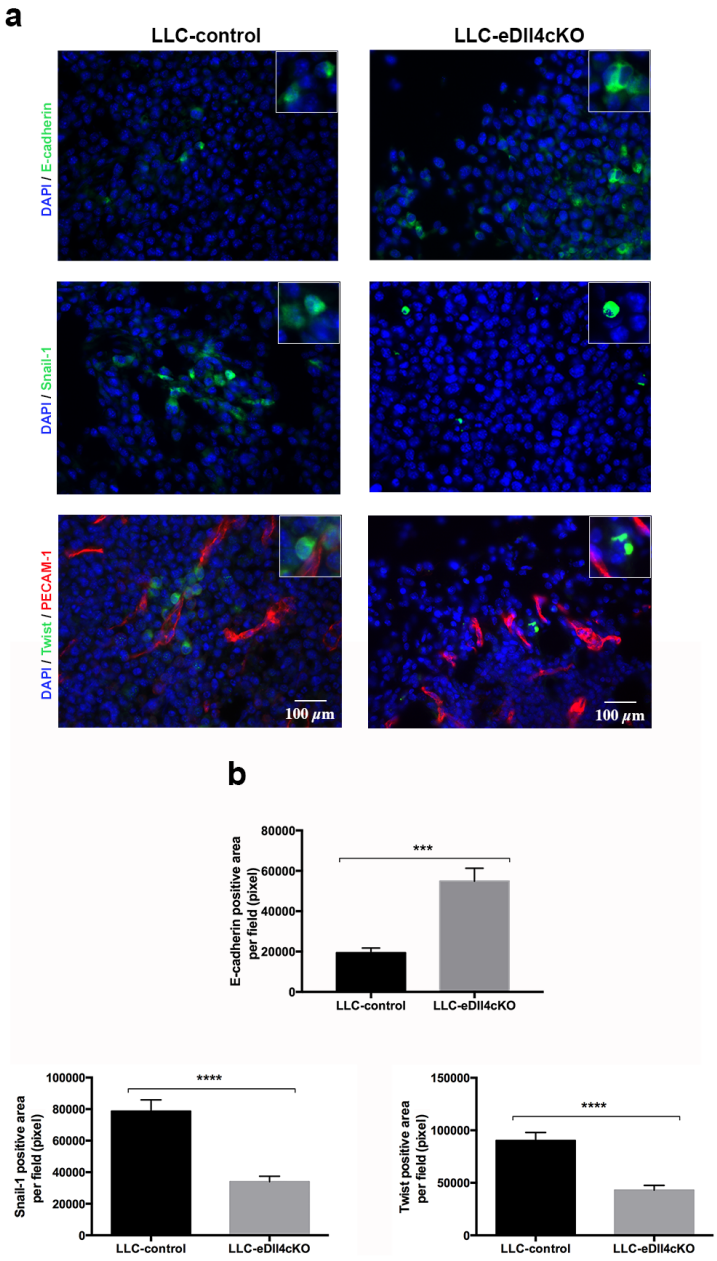
To observe if metastasis was being affected in early steps of the metastatic cascade we started to characterize EMT in tumor samples, at the end-point, by immunostaining against E-cadherin (epithelial marker), Snail1 and Twist (both mesenchymal markers). LLC-eDll4cKO mice tumors revealed less EMT than LLC-control mice tumors (182% increase in E-cadherin area, 57% decrease in Snail1 area and 52% decrease in Twist area) with statistical significance ($P < 0.001$ for E-cadherin area and $P < 0.001$ for the others, n = 8) (Figures 7a and 7b). Additionally, we observed that tumor cells adopting a mesenchymal phenotype (Twist positive cells) were preferentially located near blood vessels (PECAM-1 positive cells), mainly in the LLC-control group (Figure 7a).

Figure II-6 - Blood circulation of LLC-GFP cells in endothelial-specific Dll4 loss-of-function mice



Graph representative of the LLC-GFP positive cells percentage (%) in the bloodstream of two mouse groups, showing an important reduction of GFP positive cells in LLC-eDll4cKO, compared to LLC-control mice. Blood samples were collected at the end-point and treated for flow cytometry. Results are representative of 2 independent experiments, each with n=4 mice per group. Error bars represent SEM; $^{**}P<0.01$.

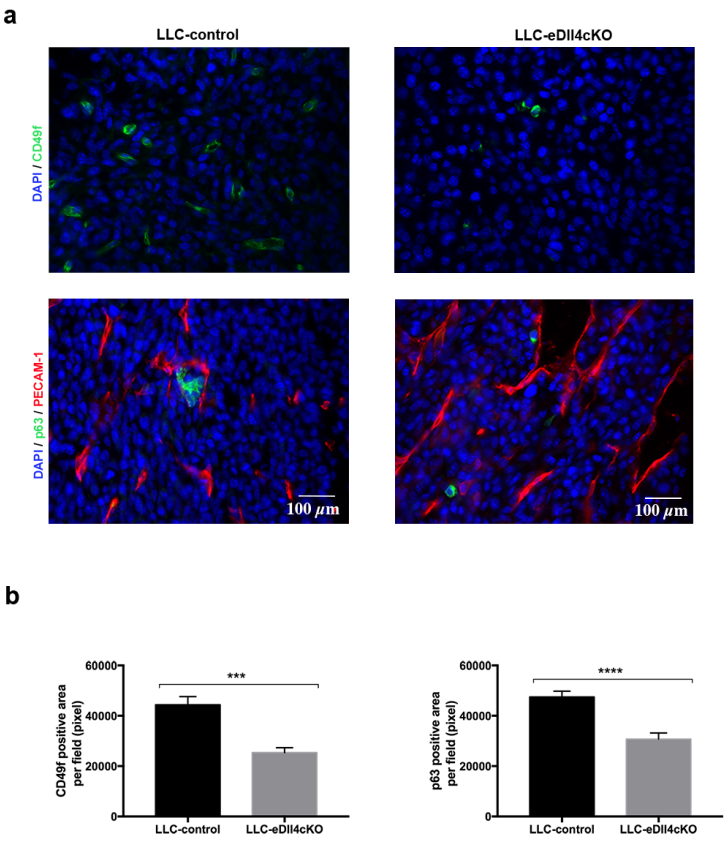
Figure II-7 - Effect of endothelial *Dll4* loss-of-function in LLC tumor epithelial-to-mesenchymal transition.



a) Immunostaining images (40x amplification) of LLC tumor samples from LLC-eDll4cKO and LLC-control groups, marked for E-cadherin (green), **top**, for epithelial phenotype and stained for Snail1 (green), **middle**, and Twist (green), **bottom**, to assess epithelial-to-mesenchymal transition. PECAM-1 (red) was used in conjunction with Twist shows that mesenchymal tumor cells tend to be located near the blood vessels, most noticeably in the LLC-control group. **b)** Tumor area in EMT is significantly reduced in the LLC-eDll4cKO mice (increase in E-cadherin staining area per field, in pixels and decrease in Snail1 and Twist staining areas per field, in pixels), compared to the LLC-control mice. LLC tumor samples were collected at the end-point. 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; blue) stains nuclei. Results are representative of 2 independent experiments, each with n=4 mice per group. Error bars represent SEM; ****P*<0.001; *****P*<0.0001.

Other early mechanism, involving the reduction of cancer stem cells by *Dll4* blockade was recently proposed (Xu et al., 2016). For CSC evaluation we used two staining markers for basal/stem cells, CD49f and p63, in LLC tumors. Both cell populations were severely reduced in the LLC-eDll4cKO group (43% reduction in CD49f area and 35% reduction in p63 area) ($P < 0.001$ and $P < 0.0001$, respectively; n = 8) (Figures 8a and 8b), demonstrating that the Dll4 effect on CSCs is also endothelial-specific. These stem-like tumor cells also tended to localize close to blood vessels (PECAM-1 positive cells) (Figure 8a).

Figure II-8 - Cancer stem-like phenotype in LLC tumors transplanted to endothelial-specific *Dll4* loss-of-function mice.



a) Representative immunofluorescence images (40x amplification) of two cancer stem cell markers, CD49f (green, **top**) and p63 (green, **bottom**) in LLC-eDll4cKO and LLC-control mice. p63 positive cells tend to co-localize with the endothelium, stained with PECAM-1 (red). **b)** Quantification of CD49f and p63 positive area per field (pixels) demonstrating an important decrease of both CSC markers in LLC-eDll4cKO group, relative to LLC-control group. LLC tumor samples were collected at the end-point. 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; blue) stains nuclei. Results are representative of 2 independent experiments, each with n=4 mice per group. Error bars represent SEM; ***P<0.001; ****P<0.0001.

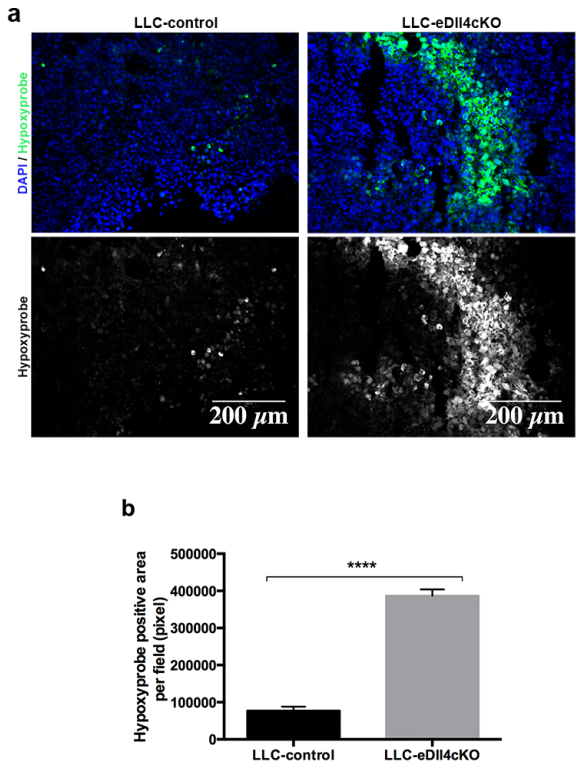
4.4. Hypoxia fails to drive metastasis in endothelial-specific *Dll4* loss-of-function LLC tumors

Previous findings relative to tumor hypoxia raised questions about the recruitment of Hif1-α to the lysyl oxidase (LOX) promoter, which stabilizes the Snail1 protein, leading to an increase in EMT and metastasis (Sahlgren et al., 2008).

To assess tumor hypoxia levels we carried out immunostaining against pimonidazole hydrochloride protein adducts (Hypoxyprobe™-1 test) in LLC-eDll4cKO mice tumors *versus* LLC-control mice tumors. As shown in Figures 9a and 9b, endothelial-specific *Dll4* loss-of-function leads to a significant increase in hypoxia (399% increase in Hypoxyprobe area) ($p < 0.0001$, n = 8).

Therefore, even though hypoxia is greatly increased in these tumors, EMT is reduced by endothelial-specific *Dll4* loss-of-function as describe above. This suggests that endothelial specific Dll4 may be involved in the process of hypoxia induction of EMT (Ioannou, Simos, & Koukoulis, 2013).

Figure II-9 - Hypoxic levels of LLC tumors transplanted to endothelial-specific *Dll4* loss-of-function mice.



a) Immunostaining images (20x amplification) of LLC tumor hypoxia evaluated by Pimonidazole Hydrochloride (Hypoxyprobe™-1 MAb1-FITC) (green) in LLC-eDll4cKO and LLC-control mouse groups. **b)** LLC-eDll4cKO mice shows a great increase in the tumor hypoxic area (area of hypoxyprobe staining per field, in pixels), when compared to LLC-control mice. LLC tumor samples were collected at the end-point. 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; blue) stains nuclei. Results are representative of 2 independent experiments, each with n=4 mice per group. Error bars represent SEM; ****P<0.0001.

4.5. Regulation of angiogenic and epithelial-to-mesenchymal transition gene expression by Dll4/Notch signalling inhibition

To validate the preceding results we performed quantitative reverse transcriptase polymerase chain reaction analysis of relevant genes in EMT, angiogenesis and Notch pathway (Figure 10). RNA was extracted from the whole tumor, that is mainly composed by LLC tumor cells (Janker, Weder, Jang, & Jungraithmayr, 2018). Since these cells express *Dll4* (Ohnuki et al., 2014), and to compensate for variations in vascular density between samples, we normalized all Notch pathway and angiogenesis genes for PECAM-1 mRNA levels (Badenes, Trindade, Pissarra, Lopes-da-Costa, & Duarte, 2017; Djokovic et al., 2015; A-R. Pedrosa, Trindade, Fernandes, et al., 2015; Ana-Rita Pedrosa, Trindade, Carvalho, et al., 2015; Trindade et al., 2012; Trindade, Djokovic, Gigante, Mendonça, & Duarte, 2017). This procedure did not change the trend of Notch-target gene expression (data not shown).

As expected, *Dll4* ($P < 0.01$) was significantly downregulated by 2.9-fold and PECAM-1 upregulated by 5.3-fold in LLC-eDll4cKO mice, compared to LLC-control mice. Likewise, *Notch1* and *Hey1* (vascular cell Notch effector) tend to be downregulated. In summary, we can assume that the Notch pathway is being essentially downregulated by *Dll4* deletion in LLC-eDll4cKO mice, relative to LLC-control mice.

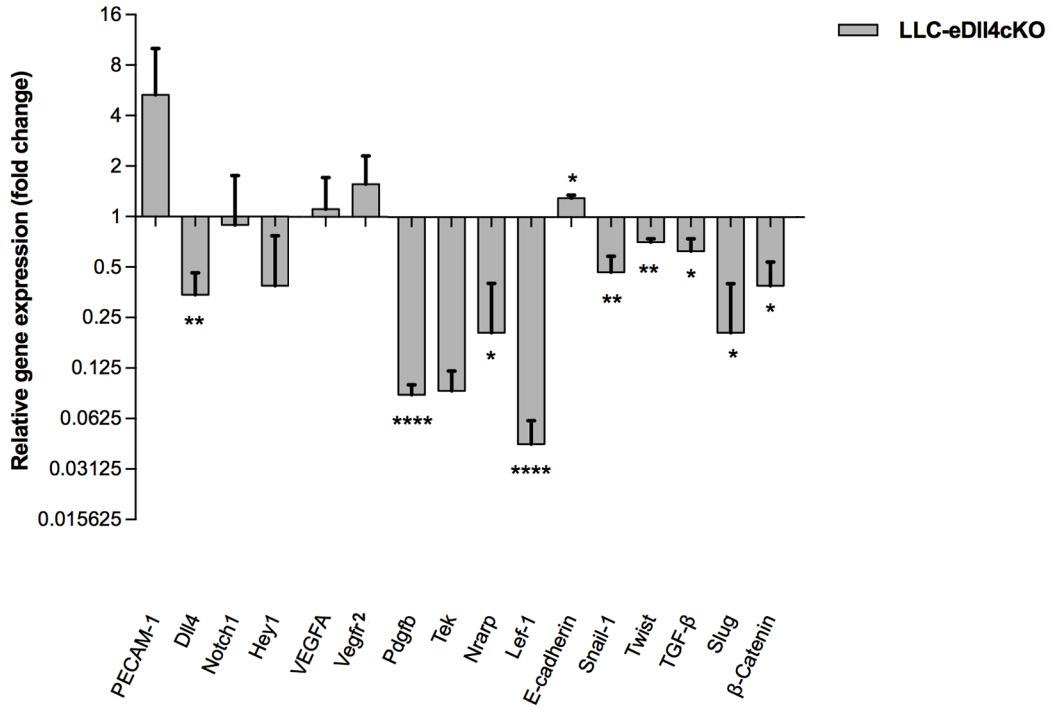
Both inhibition of Dll4/Notch1 signalling and increased hypoxia may explain the discrete upregulation of *Vegfr2* mRNA expression in the LLC-eDll4cKO group (*VEGFA* transcript levels shows the same tendency).

Moreover, there was a significant decrease in *Pdgfb* ($P < 0.0001$) and *Tek* transcript levels, which control the recruitment of perivascular cells and vascular permeability, respectively (Thomas & Augustin, 2009; Winkler, Bell, & Zlokovic, 2010).

Nrarp ($P < 0.05$), a gene known to be rapidly upregulated in response to Dll4/Notch signalling, which in turn is associated with *Lef1* ($P < 0.0001$) transcript levels reduction by downregulating the Wnt pathway (Li-Kun Phng et al., 2009), was strongly downregulated in LLC-eDll4cKO mice.

Apparently, Dll4/Notch signalling downregulation leads to a significant decrease in *Snail1* expression ($P < 0.01$). *Twist* ($P < 0.01$) and *TGF-*β ($P < 0.05$) transcript levels follow the same trend, but *E-cadherin* ($P < 0.05$) is upregulated, concomitantly with the observed EMT inhibition. Additionally, the low levels of *Slug* ($P < 0.05$) transcripts are accompanied by downregulated β*-Catenin* ($P < 0.05$) transcription, which may contribute to the upregulation of E-cadherin.

Figure II-10 - Gene expression analysis of LLC tumors in endothelial *Dll4* loss-of-function mice.



RNA was isolated from LLC tumor samples collected at the end-point, and gene transcript analysis was performed by quantitative real-time reverse transcriptase polymerase chain reaction for genes involved in the Notch pathway, angiogenesis and EMT. Notch pathway/angiogenesis gene transcript levels were normalized to *Pecam-1* mRNA levels, and the housekeeping gene *β-actin* was used as endogenous control. Gray bars represent the gene expression levels of samples collected from LLC-eDll4cKO mice, relative to the respective LLC-control mice. n=4 mice per group. Error bars represent SEM; **P*<0.05; ***P*<0.01; and *****P*<0.0001.

5. Discussion

Interaction between endothelial and cancer cells was reported to promote tumor progression by generating new vessels or an invasive phenotype of cancer cells (Kaessmeyer, Bhoola, Baltic, Thompson, & Plendl, 2014; D. Liu et al., 2010). Dll4 signaling inhibition has been extensively reported to prevent tumor growth by deregulating angiogenesis and promoting non–productive angiogenesis (Noguera-Troise et al., 2006; Ridgway et al., 2006). These studies used systemic Dll4 blockade, so it is unclear which cell type is leading the Dll4 cross talk to the tumor. A few investigations focused on the Dll4 effect via cell-to-cell communication. For example, it was reported that endothelial Dll4-Tumor-Notch interactions made the tumor overcome dormancy (Indraccolo et al., 2009). Others have shown that Dll4/Notch mediated cross–talk between endothelial cells and tumors, which suppressed lung cancer growth (Ding et al., 2012).

The present findings are the first to support a direct effect of endothelial *Dll4* loss-of-function in the regulation of tumor growth. To assess the specific role of endothelial Dll4 we transplanted LLC cells into endothelial-specific inducible *Dll4* loss-of-function mice and

evaluated tumor samples at the end-point. An important reduction of Dll4 and N1ICD endothelial staining in the LLC-eDll4cKO mice, supported by transcription analysis (downregulation of *Dll4*, *Notch1* and *Hey1* genes), clearly demonstrated the endothelial Dll4/Notch signalling blockade. *In vivo* endothelial *Dll4* loss-of-function inhibited the vascular maturation process, with tumor vascular beds presenting reduced perivascular cell coverage (smooth muscle cell layer and pericytes). Transcription results point to a downregulation of PDGF-B/PDGFR β pathway, which is in agreement with our previous studies (Ana-Rita Pedrosa, Trindade, Fernandes, et al., 2015). We believe there is a connection between reduced tumor vascular maturation and the observed vascular leakiness increase and functionality reduction from endothelial *Dll4* loss-of-function, marking the endothelium to a regression fate. Therefore, a progressively hypoxic and starvation environment arrests tumor growth.

Surprisingly, in this scenario the tumor hypoxia does not seem relevant in selecting resistant cell clones to escape the primary tumor (Sahlgren et al., 2008). We observed, instead, a significant reduction in the number and burden of distant site macro-metastasis and of the number of circulating tumor cells.

Xu et al. recently reported a reversal of EMT markers in breast tumor xenograft mouse models, after blocking Dll4/Notch signaling with an anti-Dll4 monoclonal antibody (Xu et al., 2016). The *Dll4* endothelial effect was not fully demonstrated although there is evidence from previous studies suggesting in that direction. Namely, Huang et al. proposed that Dll4/Notch/Hey1/MMP9 cascade mediates a direct interplay between endothelial cells and tumor cells, which eventually promotes RCC hematogenous metastasis (Huang et al., 2014).

Therefore we explored EMT, one of the first events that trigger the metastatic process. An important reduction of EMT in LLC-eDll4cKO mice was observed, both in immunostaining for E-cadherin (increased), Snail1 (reduced) and Twist (reduced), and gene expression analysis with *Snail1*, *Twist*, *Slug* and *TGF-*β being downregulated and *E-cadherin* upregulated.

Accumulating evidence demonstrates that the inhibition of Notch signalling reduces the number of cancer stem cells, indicating the critical role of Notch signalling in the maintenance of the cancer stem cell phenotype (Rofstad & Mathiesen, 2010). Specifically, Dll4 has been implicated in the reduction of $CD44^{+}/CD24^{-}$ breast cancer cell subpopulation (Xu et al., 2016). Here we show that Dll4 effect over CSC may also be endothelial specific, since LLC-eDll4cKO mice had reduced CD49f and p63 positive areas in the tumor immunostaining.

An interesting finding, related to these more mesenchymal and stem-like tumor cells, was its localization close to blood vessels. In fact, we observed a significant reduction in the number of circulating LLC-GFP tumor cells in the endothelial Dll4 knockout mice. However, we cannot be sure if this reduction is just caused by EMT inhibition itself or also by the eDll4cKO vasculature being less permeable and/or failing to drive forward metastatic tumor cells.

Although we couldn't use GFP-marked tumor cells to assess EMT and CSC, we can assume that the majority of cells in this type of xenotransplant are tumor cells and considering that the stained nuclei are unlikely to be endothelial cells or leukocytes, given their shape, it would be fair to assume that counts are predominantly of tumor cells.

Endothelial loss of Dll4 function did play a part in regulating metastasis growth as the metastatic tumor burden was much more highly reduced than the metastasis count. This means that, not only endothelial loss of Dll4 produced less distant site metastasis as, in average individual metastatic foci were smaller than their control counterpart. Despite this, we cannot rule out a causative role for the impaired angiogenesis in the reduced metastatic dissemination.

Further research is needed to elucidate other possible mechanisms for metastasis suppression, for instance, involving the attenuation of the NF-κB signaling under endothelial specific *Dll4* loss-of-function (Kuramoto et al., 2012).

Because the transcriptional analysis was performed on whole tumor samples, which by itself cannot differentiate between mRNAs from tumor cells *versus* stromal cells, the results from angiogenesis and endothelial related genes were normalized, as previously (Badenes et al., 2017; Djokovic et al., 2015; A-R. Pedrosa, Trindade, Fernandes, et al., 2015; Ana-Rita Pedrosa, Trindade, Carvalho, et al., 2015; Trindade et al., 2012, 2017), to compensate for differences in endothelial cell representativeness in the different samples. In relation to non-angiogenesis related genes, given the vast majority of tumor cells compared to stromal cells in this type of xenotransplants, stromal-derived transcripts may have a limited quantitative effect. So it is unlikely to affect the overall measurements in a way that changes the interpretation of the data in qualitative terms.

In summary, our study went beyond what was known about Dll4 blockade in tumor growth and metastasis. We have discriminated the Dll4 function and demonstrated that both events are compromised by endothelial specific *Dll4* loss-of-function. Regarding tumor growth, endothelial *Dll4* knockout stimulates the development of a dense but leaky vasculature, immature and non-functional, which ends up undergoing regression, thus limiting tumor burden. Most importantly, the metastasis process is impaired, at least in three early events, by the specific effect of endothelial Dll4 inhibition. Not only endothelial Dll4 inhibition is responsible for a reduction in EMT, through a possible downregulation of Dll4/Notch1/Hey1/Snail-1 (Twist, Slug and/or TGF-β) pathway, and involved in the decrease of CSC clone selection in the primary tumor, but also determinant in limiting the number of circulating metastatic cells. Apparently, in this context, hypoxia is not the determinant key for metastasis initiation. This is in agreement with observations made in melanoma xenograft metastasis, that the metastatic propensity was governed by the constitutive angiogenic potential of the tumor cells rather than differences in hypoxia-induced angiogenesis (Rofstad & Mathiesen, 2010).

Taken together, the current results unveil the role of endothelial *Dll4* as a potential therapeutic target in controlling the metastasis process in its initial phases.

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Chapter III. Endothelial Dll4 loss-of-function reduces metastasis through the inhibition of intravasation-extravasation and metastatic niche formation

Liliana Mendonça, Alexandre Trindade, Marina Badenes, Joana Gigante, António Duarte.

Manuscript in preparation.

1. Abstract

Endothelial *Dll4* loss-of-function has been demonstrated to impair metastasis through inhibition of epithelial-to-mesenchymal transition and reduction of cancer stem cell number and circulating tumor cells (CTCs). However, the metastatic process involves several other distinct steps.

Therefore, we proposed to analyse the impact of endothelial Dll4 loss-of-function in the intravasation, extravasation and metastatic niche formation. For that, we used Lewis lung carcinoma (LLC) cells marked with a green fluorescent protein (GFP) transplanted into endothelial-specific *Dll4* conditional loss-of-function mice.

We observed an important reduction of LLC-GFP cells intravasated into the tumor vasculature of endothelial-specific Dll4 loss-of-function mice, in line with the previous result showing less CTCs. In addition, the percentage of LLC-GFP cells that extravasate to the lungs is decreased. The recruitment of myeloid Cd11c/ VEGFR-1 positive cells and fibronectin deposition in the lung, as pre-conditioning for the LLC-GFP cell engraftment, is reduced in the endothelial-specific Dll4 loss-of-function mice. This phenotype does not seem to be influenced by endothelial Dll4/Notch regulation in the lung, as Dll4 and N1ICD lung immunostainings are not changed and the lung vasculature does not undergo remodelling.

Our findings clearly suggest that endothelial *Dll4* loss-of-function can also arrest metastasis by limiting the intravasation-extravasation process and the formation of the metastatic niche. Opposing the theory of vascular leakiness favouring metastasis, in endothelial *Dll4* loss-of-function an important reduction in intravasation occurs. Whether EMT and /or less conductive vasculature determined this effect we cannot be sure. In addition, the primary tumor under endothelial Dll4 deletion fails to signal for the mobilization of myeloid Cd11c/VEGFR-1 positive cells. So, the metastatic niche is less suitable, with decreased myeloid cells and fibronectin deposition. This may explain why, less metastatic cells extravasate and subsequently fail to engraft, assuming that lung vasculature is in a quiescent state in an early phase.

Keywords: Dll4/Notch, metastasis, intravasation, extravasation, metastatic niche, Lewis-lung-carcinoma, GFP.

2. Introduction

Metastases are the main cause of cancer-related deaths from solid tumors (Gupta et al., 2006). The difficulty in predicting metastasis and the late diagnosis, when metastases are already disseminated, limits the effectiveness of cancer therapeutics. Therefore, a cure for metastatic disease remains elusive.

The process of metastasis is defined by distinct steps involving local invasion, intravasation into adjacent blood and lymphatic vessels, transit through circulation, extravasation into the parenchyma of distant organs, with colonization and formation of micro-metastases that will progress to macro-metastases. This conceptual view of metastasis, conceived as a late event in tumorigenesis, has been reformulated with the discovery of the pre-metastatic niche (Kaplan et al., 2005), the importance of microenvironment, different forms of evasion in distinct cancers and the genetic make-up of individuals (Eccles, Welch, & Welch, n.d.; Thomas R Geiger & Peeper, 2009).

The metastatic niche model suggests that a suitably conducive microenvironment (pre-metastatic niche) must evolve in order for tumor cells to be able to engraft (metastatic niche) and proliferate at secondary sites (micro- to macro- metastatic transition). This idea builds on Paget's seed and soil hypothesis by suggesting a temporal evolution for the development of the soil, and incorporates key cellular and molecular components of the metastatic microenvironment (Psaila & Lyden, 2009).

The components crucial to pre-metastatic niche formation include tumor-derived secreted factors (TDSFs) and bone marrow-derived cells (BMDCs). TDSFs from the primary tumor promote the mobilization and recruitment of BMDCs that interact with the local stroma and extracellular matrix at secondary organs, to help create microenvironments suitable for colonization by metastasizing tumor cells (Sceneay, Smyth, & Möller, 2013). Among these BMDCs is a common myeloid progenitor that express the VEGF receptor 1 (VEGFR-1) and also the fibronectin receptor VLA-4 (Kaplan et al., 2005). It can give rise to a variety of monocytic and granulocytic cell subtypes including macrophages, dendritic cells (DCs), neutrophils, and myeloid-derived suppressor cells, with distinct roles depending on the primary tumor and target site for metastasis. For example, phenotype analysis revealed that the metastasis-associated macrophages found in the lung differ from the Cd11c-positive lung interstitial resident macrophages. Instead, they are characterized by cell surface expression of Cd11b, F4/80, VEGFR-1, and CCR2, absence of Gr1 and low Cd11c. These cells were demonstrated to mediate metastatic breast cancer cell extravasation, establishment and growth (B. Qian et al., 2009).

A variety of TDSFs including VEGF, PlGF, TNF-α and TGF-β have been shown to drive pre-metastatic niche formation in various tumor models. But sometimes they simply result as a consequence of systemic disturbances caused by the presence of the primary tumor (Sceneay et al., 2013). In our previous work we found that endothelial-specific Dll4 loss-of-

function was responsible for the downregulation of TGF-*β*, leading to a decrease in the epithelial-to-mesenchymal transition (EMT) and invasion of Lewis lung carcinoma (LLC). Conversely, VEGFA and VEGFR-2 exhibit an upregulation tendency, in response to downregulation of Dll4 upstream.

In this context, the steps of intravasation and extravasation were considered as secondary. However, the Condeelis laboratory showed a direct correlation between the number of intravasated cells and the number of lung metastases in an orthotopic breast tumor rat model, suggesting that intravasation does represent a critical step of the metastatic cascade (J. B. Wyckoff et al., 2000). Intravasation of tumor cells into the circulation can occur through entry into blood vessels or lymphatic vessels, with the majority of entry occurring through hematogenous (blood vessel) routes (Chiang et al., 2016). Properties of the vasculature, which are important for intravasation, include microvessel density and also diameter of the vasculature, with increased intravasation correlating with increased vessel diameter in some tumors (Yamamura et al., 2001). The primary tumor “angiogenic switch” may also affect intravasation. In fact, it was showed in the “human tumor-chick embryo” spontaneous metastasis model that increased secretion of VEGF increases angiogenesis and intravasation (Conn et al., 2009). Another study pointed out that these vessels have three characteristics, namely: accessible points for vessel wall penetration; enough lumen space; and sufficient rate of blood flow (Deryugina & Quigley, 2015). Wyckoff et al. demonstrated that increased cell orientation of metastatic cells toward blood vessels could increase the efficiency with which they can then intravasate. This orientation could be induced by chemoattractant diffusing from the blood vessel, like epidermal or platelet-derived growth factors (EGF or PDGF), present in platelets and smooth muscle cells. They unveiled another mechanism that involves fragmentation occurring during intravasation. If the shear forces in the blood vessels are causing non-metastatic cells to fragment as they enter the blood vessel, that event will eliminate the possibility of metastasis (J. B. Wyckoff et al., 2000). Increased number of immune system cells could contribute to increased metastasis by production of chemotactic factors or degradation of extracellular barriers (Dong, Kumar, Yang, & Fidler, 1997; Nielsen et al., 1996). There are also several studies showing that TGF-*β* signaling can enhance intravasation, at least in part, through induction of EMT (T. Tsuji et al., 2008; Takanori Tsuji et al., 2009). Finally, another intriguing potential mechanism for intravasation involves vascular mimicry by tumor cells, where tumor cells line blood vessels and replace endothelial cells (Seftor et al., 2012). These physical mechanisms underlying tumor cell intravasation to vessels are likely to be similar to those contributing to extravasation and represent an opportunity for Dll4/Notch pathway-blocking therapies.

Dll4 is a Notch ligand, with vascular expression restricted to small arteries and capillaries in adults (Rui Benedito & Duarte, 2005; Duarte et al., 2004; Noguera-Troise et al., 2006). Several reports demonstrate that Dll4/Notch inhibitors reduce tumor growth through a

reduction of the net blood flow due to a highly chaotic vasculature (S. K. Liu et al., 2011; Noguera-Troise et al., 2006; Ridgway et al., 2006; Scehnet et al., 2007; Yamanda et al., 2009). We recently demonstrated that this effect is endothelial-mediated and is associated with a reduction in distant metastasis. Metastasis was impaired by the endothelial Dll4 loss-of-function effect on EMT, cancer stem cell (CSC) and circulating tumor cell (CTC) reduction. The remaining steps of the metastatic cascade had yet to be explored.

In this study we provide evidence that intravasation, circulation in the bloodstream, extravasation and engraftment (metastatic niche) at a distant organ are also compromised by the endothelial Dll4 knockout. For that, we used a line of LLC transduced with green fluorescent protein (GFP), to allow the tracking of the metastatic cells in those processes, transplanted in the endothelial-specific Dll4 loss-of-function mice.

3. Methods

3.1. Experimental animals

The Ethics and Animal Welfare Committee of the Faculty of Veterinary Medicine of Lisbon has approved all the procedures involving animals used in this study.

Animals were housed in ventilated polypropylene 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.

Dll4 conditional knockout mice (*Dll4$^{lox/lox}$*) were generated by flanking the first three exons of the Dll4 allele with two loxP sites. This was done in our laboratory, in collaboration with Dr. Freddy Radtke, as reported (Koch et al., 2008). These mice were then crossed with *VE-cadherin-Cre-ERT2* mice, a kind gift by Dr. Ralph Adams, which express tamoxifen-inducible Cre recombinase under the control of the VE-cadherin endothelial promoter. Tamoxifen (Sigma) was administrated via intra-peritoneal (i.p.) injection in a dosage of 50 mg/kg, daily for 5 days. Hence we obtained endothelial-specific inducible Dll4 loss-of-function (eDll4cKO) mice. Control mice have the same genotype but are not induced and given saline i.p. injections instead.

3.2. LLC-GFP subcutaneous tumor transplant mouse model

LLC cells were transduced with GFP (AntiCancer Incorporated, San Diego, California, USA). These cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% foetal bovine serum (Gibco) and 1% penicillin/streptomycin, in 100mm tissue culture dishes coated with poly-D-Lysine Hydrobromide (Sigma), at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

When cells reached sub-confluence, they were detached by 5 min treatment with 0.25% Trypsin-EDTA (Gibco) and resuspended in PBS to a cell concentration of 1×10^7/ml. For the transplant tumor model, cells (1×10^6/mouse) were inoculated subcutaneously (SC), in the

right flank of the mouse under anaesthesia (2.5% Avertin). This model reproduces the spontaneous metastasis process(Bos et al., 2010; Cai et al., 2008).

The test group, named GFP-eDll4cKO mice, was induced with tamoxifen at 6 weeks of age, as previously described, and the control group, named GFP-control mice, were not tamoxifen-induced. Both received LLC-GFP cells at 8 weeks of age. Each group had 4 animals, all males and this experiment was repeated two times.

LLC-GFP have been shown to rapidly metastasize (Rashidi et al., 2013) and because we wanted to assess the movement of these cells towards the lung, we defined as end-point 9-14 days after cell injection.

3.3. Fluorescent-activated cell sorting of LLC-GFP

At the dissection day, mice of each group were anesthetized (2.5% Avertin) and 1ml of blood (total mouse blood volume) was recovered by cardiac punction (to heparin coated eppendorfs). After erythrocytes lysis with 1x RBC lysis buffer (10x RBC lysis buffer multi-species, eBioscience), the remaining blood cells were washed with Dulbecco's phosphate-buffered saline (DPBS) (1x) no calcium, no magnesium (Gibco) and resuspended in FACS (1x) buffer (eFluor® NC Flow Cytometry Staining Buffer (5x), eBioscience). These samples were used for cytometric count of GFP-positive cancer cells travelling in the peripheric blood, having exited the primary tumor and done intravasation successfully.

To access extravasation we collected the lungs at the end-point. Two samples of each principal lung lobe were dissected so that a sample of the right and left lobes could be processed to immunofluorescence and the remaining tissue could be used for flow cytometry. For that, lung samples (2-3 mm^3) were digested with 1% collagenase (type I from *Clostridium histolyticum*, Sigma) and 2,4U/ml of dispase (Gibco), under an incubation period of 1h at 37ºC, with gentle agitation. DNAse I (Sigma) was added during digestion to eliminate DNA residues. These cells were also exposed to RBC lysis buffer (1x) and after washing with DPBS (1x), digested cells were resuspended in FACS buffer. All GFP-positive cancer cells detected are mainly extravasated cells, and not from blood circulation, since exsanguination was carried out at the time of euthanasia.

Cells were sorted in a FACSCalibur cytometer (BD, Enzifarma) and analysed using BD FlowJo software (version 10.0, BD Bioscence).

3.4. Tissue preparation and immunofluorescence

For intravasation analysis we collected tumor samples at the end-point. They were fixed with 4% paraformaldehyde (PFA) solution at 4°C for 1h, cryoprotected in 15% sucrose, embedded in 7.5% gelatin, frozen in liquid nitrogen and cryosectioned at 14 and 20µm.

Immunostaining was performed using the following protocol: tissue slides were permeabilized in 3% H_2O_2 methanol solution for 40 min and PBS-Triton 0.5% solution 2 x 10 min; blocking was performed for 1h (room temperature) with 5% BSA in PBS-W 0.1%

solution; after blocking, slides were incubated overnight at 4ºC with specific primary antibodies followed by 1h incubation at room-temperature with fluorescently-tagged specific secondary antibodies (Invitrogen).

Fluorescent immunostained sections were examined under a Leica DMRA2 fluorescence microscope with Leica HC PL Fluotar 20x and 40x/0.5 NA dry objectives (Leica, Heidelberg, Germany), 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 (NIH, Bethesda, MA, USA).

To evaluate intravasated cancer cells we amplified the GFP signal with anti-GFP antibody (FITC) staining (Abcam, Cambridge, UK) of tumor sections (20μm). Then we counted the GFP positive cells co-stained with rat monoclonal antibody anti-mouse platelet endothelial cell adhesion molecule, PECAM-1 (BD Pharmingen, San Jose, CA), followed by Alexa 594 goat anti-rat IgG antibody (Invitrogen, Carlsbad, CA), that marks the endothelium.

Lungs were also collected and processed as described. By doing immunofluorescence of lung sections (14μm) against GFP we determined the relative area per field (in pixels) density of GFP-positive cancer cells that successful arrived at the lung.

In order to determine if endothelial Dll4 was changed in the lung and, by consequence Notch1, we did double fluorescent immunostaining on lung sections (14μm) with goat polyclonal anti-mouse Dll4 (R&D) and rabbit anti-Notch1 cleaved (N1ICD) (Cell Signalling) primary antibodies. For secondary antibodies, donkey anti-goat Alexa Fluor 488 and donkey anti-rabbit Alexa Fluor 555 were used. The positive signals of each marker were quantified (area per field, in pixels).

For vasculature analysis, we addressed lung vascular density (area per field, in pixels, of endothelial cells marked with PECAM-1) and vessel maturity (smooth muscle cells given by the ratio of PECAM-1 and alpha smooth muscle actin, α-SMA). The primary antibodies used were rat monoclonal anti-mouse PECAM-1, mouse monoclonal anti-SMA Cy3 conjugate (Sigma Aldrich, USA) and the secondary antibody used was anti-rat conjugated with Alexa Fluor 488 (Invitrogen, Carlsbad, CA).

Finally, to access the metastatic niche we stained lung sections (14μm) against myeloid cells VEGFR-1 positive and fibronectin. The myeloid cells (dendritic cells) were counted after co-staining with hamster monoclonal anti-mouse Cd11c (BD Pharmigen) and rabbit polyclonal anti-mouse Flt-1 (H-225) (Santa Cruz Biotechnology) primary antibodies. Additional secondary antibodies used were Alexa-488 goat anti-hamster and Alexa-555 donkey anti-rabbit (Invitrogen, Carlsbad, CA). Fibronectin was detected with the rabbit polyclonal anti-human fibronectin (Sigma), followed by Alexa-555 donkey anti-rabbit (Invitrogen, Carlsbad, CA). Its area per field (pixels) was quantified.

In all tissue sections nuclei were counterstained with 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; Molecular Probes, Eugene, OR).

Each analysis was repeated in 10 fields per sample.

3.5. Statistical analysis

Data processing was carried out using the Statistical Package for the Social Sciences software, version 17.0 (SPSS v. 17.0; Chicago, IL) and GraphPad Prism, version 6.0f (GraphPad Software). Statistical analyses were performed using Mann-Whitney-Wilcoxon test. All results are presented as mean ± SEM. *P*-values <0.01 was considered significant (indicated in the figures with **), and *P*-values <0.001 and <0.0001 were considered highly significant (indicated with *** and ****, respectively).

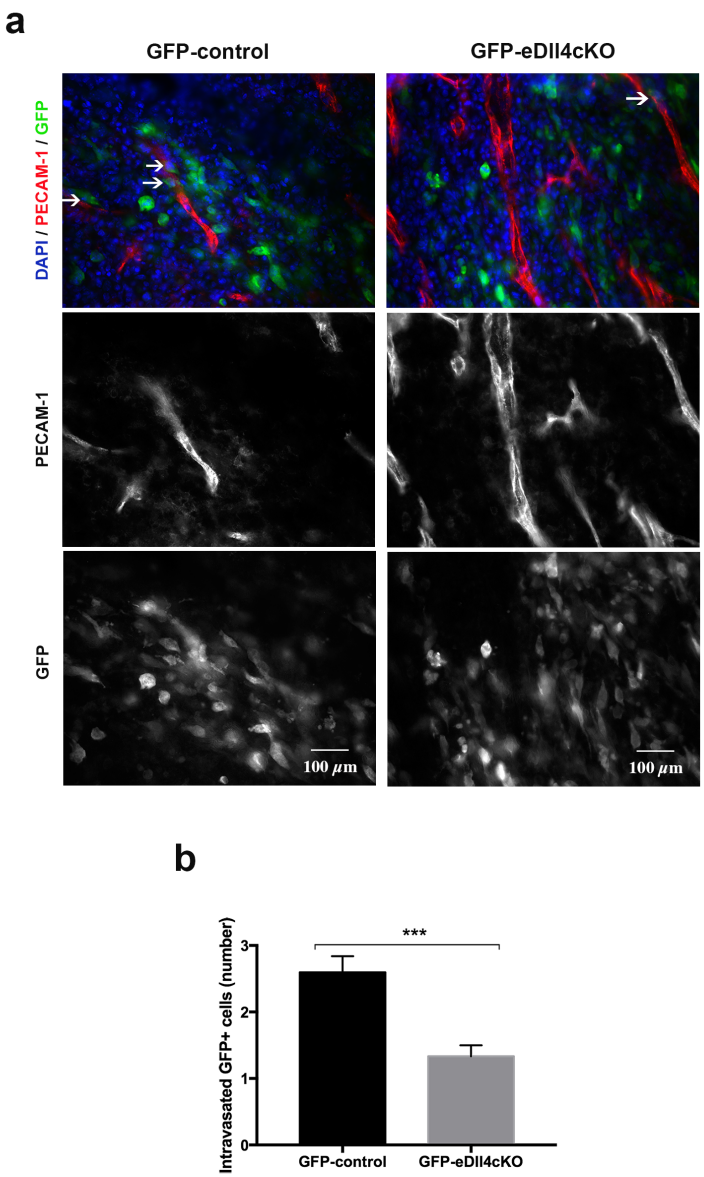
4. Results

4.1. Loss of endothelial Dll4 in Lewis Lung Carcinoma GFP tumor leads to intravasation reduction

Based on previous work, were we demonstrated a significant reduction of CTCs in endothelial Dll4 loss-of-function mice we wanted to investigate if intravasation was contributing factor. For that, we accessed the number of LLC-GFP positive cells inside the tumor vasculature.

Briefly, LLC cells were transduced with a GFP expression vector (AntiCancer Incorporated, San Diego, California, USA), cultured in appropriate media, resuspended in PBS and SC injected in the eDll4cKO mice (1×10^6/mouse). In one group, GFP-eDll4cKO, recombination was induced with tamoxifen, as previously described, whereas the control group, GFP-control, was not induced. The end-point was defined to 9-14 days after cell injection, due to rapid metastatic capacity of these cells. At the end-point we collected tumor samples and produced histological sections. To identify LLC-GFP positive cells inside the tumor vessels these sections were co-stained for GFP and PECAM-1, which marks the endothelium. As shown in Figures 1a and 1b, GFP-eDll4cKO mice displayed a significant reduction in the number of intravasated LLC-GFP cells, when compared to GFP-control mice.

Figure III-1 - Effect of endothelial Dll4 loss-of-function in Lewis lung carcinoma-GFP tumor intravasation.

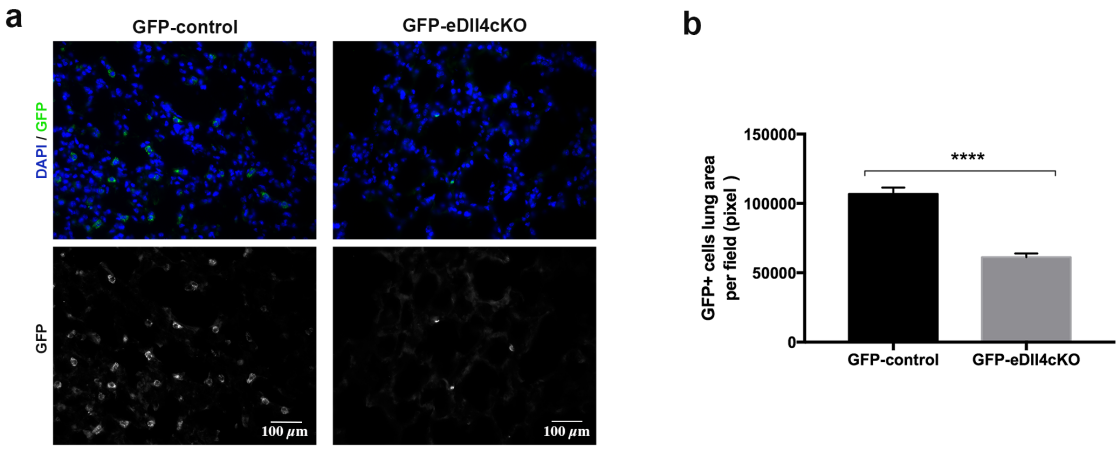


a) GFP (green) and PECAM-1 (red) immunostaining images (40x amplification) of LLC-GFP tumor samples to evaluate the co-localization of both signals, indicative of LLC-GFP cells intravasating blood vessels (white arrows), in GFP-eDll4cKO and GFP-control mouse groups. **b)** Intravasated LLC-GFP positive cells (number) are significantly decreased in GFP-eDll4cKO mice, compared with GFP-control. 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; blue) stains nuclei. LLC-GFP tumor samples were collected at the end-point. Results are representative of 2 independent experiments, each with n=4 mice per group. Error bars represent SEM; ***P<0.001.

4.2. Extravasation of LLC-GFP tumor cells is compromised by endothelial-specific Dll4 loss-of-function

The next step in the metastatic cascade is the extravasation from the blood vessels, when CTCs find a suitable niche in a target organ and suffer endothelial transmigration to its stroma. First, we immunofluorescence imaged the LLC-GFP cells area density in the lung, that is the target organ of metastasizing LLC. In Figures 2a and 2b we can see that GFP-eDll4cKO mice have fewer LLC-GFP cells in the lung, than the respective controls, with statistical significance.

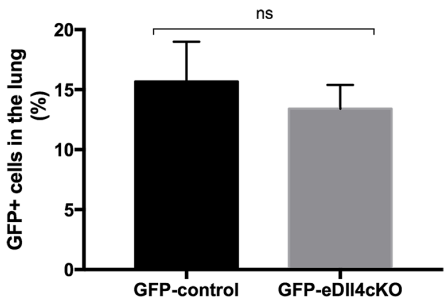
Figure III-2 - Endothelial Dll4 loss-of-function effect on LLC-GFP cells mobilization to the lung.



a) Immunostaining images (40x amplification) of lung samples marked for GFP (green) in GFP-eDll4cKO and GFP-control mice. **b)** The GFP-eDll4cKO shows a significant decrease in LLC-GFP cells on the lung (area of GFP staining per field, in pixels), compared to GFP-control group. 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; blue) stains nuclei. Lung samples were collected at the end-point. Results are representative of 2 independent experiments, each with n=4 mice per group. Error bars represent SEM; ****P<0.0001.

Then, to determine how many of these cells are really being arrested at the local capillaries and extravasate into the lung parenchyma, we sorted the cells by FACS, using the lung samples collected after total exsanguination of the mice, at the end-point. Although the difference was not statistically significant, there was a decrease in LLC-GFP cells extravasated in the GFP-eDll4cKO group, compared with GFP-control (Figure 3).

Figure III-3 - Extravasation of LLC-GFP cells into the lung of endothelial Dll4 loss-of-function.

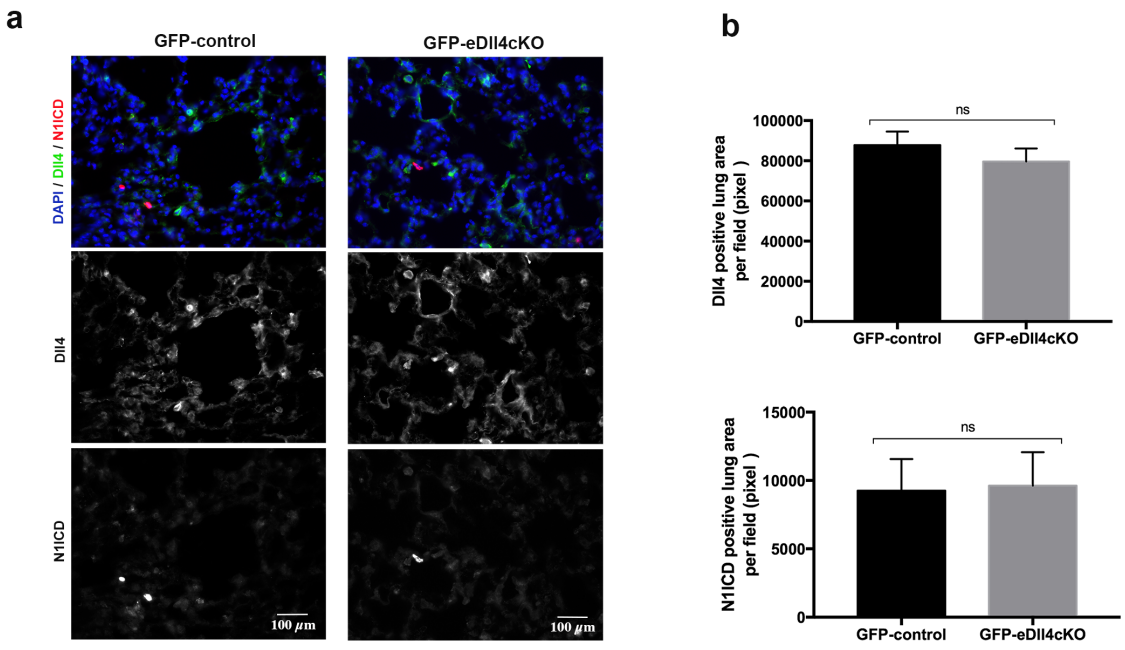


Graph representative of the LLC-GFP positive cells percentage (%) in the lung of two mouse groups (flow cytometry). LLC-GFP positive cells that extravasate to the lung (%) are slightly reduced in GFP-eDll4cKO mice, relative to GFP-control mice. Results are representative of 2 independent experiments, each with n=4 mice per group. Error bars represent SEM; ns Not Statistically Significant.

4.3. Lung vasculature is not affected by the endothelial-specific Dll4 loss-of-function

We questioned if the extravasation reduction observed in the GFP-eDll4cKO mice was being influenced by the endothelial Dll4 knockout, along with the reduction seen in the bloodstream. Given that *Dll4* is expressed only in some regions of the lung endothelium (Rui Benedito & Duarte, 2005; Rui Benedito et al., 2012), we stained the lungs for Dll4 and also for N1ICD. Immunofluorescence images show a basal staining for Dll4 in the lung endothelium, equivalent in both groups. Additionally, no differences are observed in the staining for N1ICD (Figures 4a and 4b).

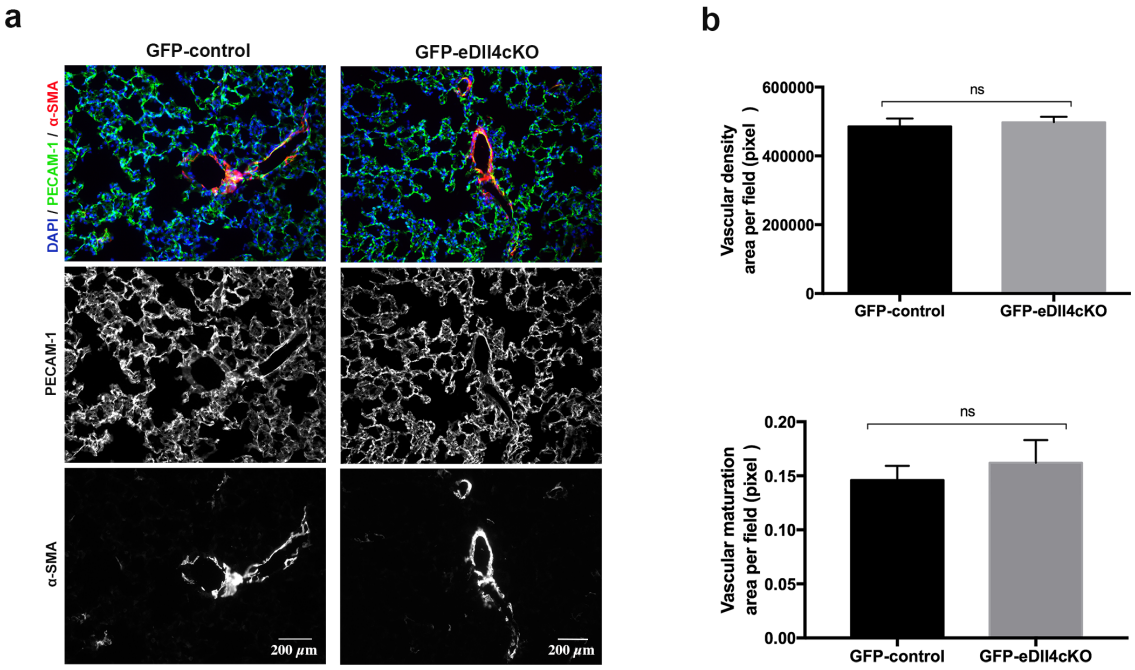
Figure III-4 - Immunostaining of Dll4 and Notch1 intracellular domain in the lungs of endothelial-specific Dll4 loss-of-function mice.



a) Immunostaining images (40x amplification) of lung samples from GFP-eDll4cKO *vs* GFP-control groups, marked for Dll4 (green) and Notch1 intracellular domain, N1ICD (red). **b)** Dll4 and N1ICD-positive lung areas (area per field, in pixels) have no significant differences between the two groups. 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; blue) stains nuclei. Lung samples were collected at the end-point. Results are representative of 2 independent experiments, each with n=4 mice per group. Error bars represent SEM; ^{ns} Not Statistically Significant.

Later we checked the vasculature, addressing both density and maturation with immunostainings for PECAM-1 plus α-SMA (smooth muscle actin). As expected, lung vasculature is similar between groups, in terms of both density and maturation (Figures 5a and 5b).

Figure III-5 - Lung vasculature phenotype of endothelial-specific Dll4 loss-of-function.



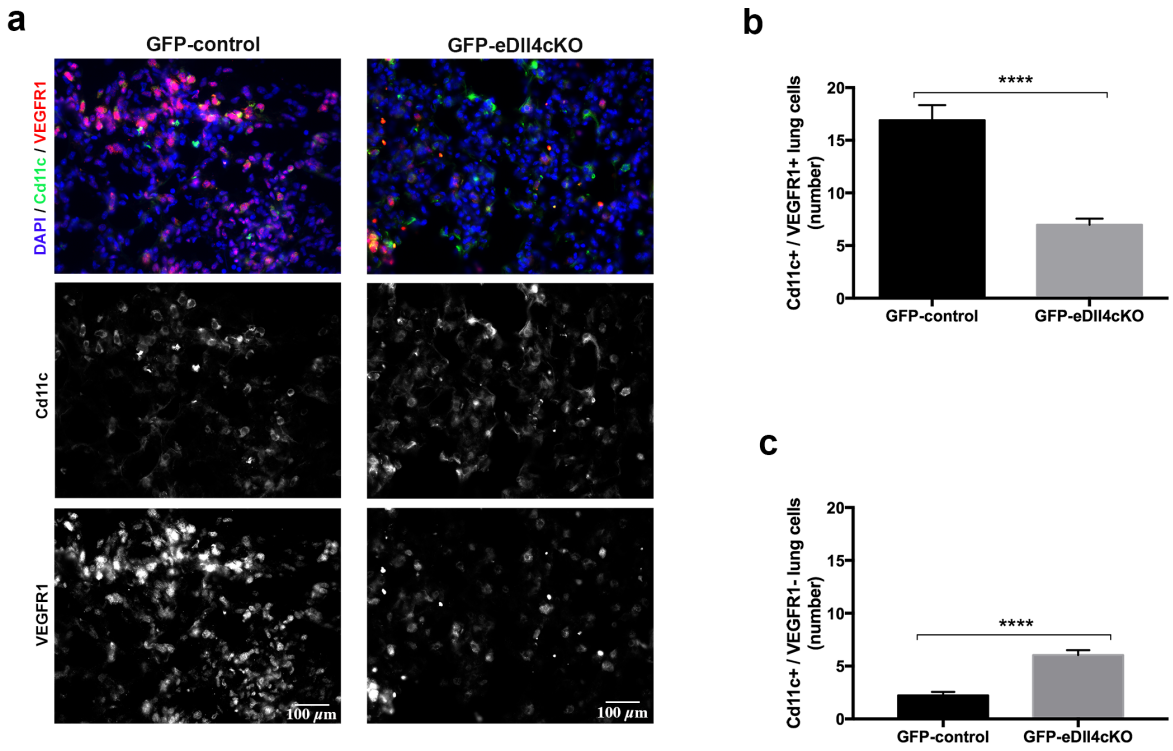
a) Immunostaining images (20x amplification) marked for PECAM-1 (green) and α-SMA (red), to evaluate vascular density and perivascular smooth muscle actin coverage of lung samples in GFP-eDll4cKO and GFP-control mouse groups. **b)** Vascular density (area of PECAM-1 staining per field, in pixels) and vascular maturation (area of α-SMA/ PECAM-1 co-staining per field, in pixels) aren't different between the two groups. 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; blue) stains nuclei. Lung samples were collected at the end-point. Results are representative of 2 independent experiments, each with n=4 mice per group. Error bars represent SEM; ^{ns} Not Statistically Significant.

4.4. Endothelial *Dll4* knockout in LLC-GFP tumors modifies the metastatic niche

Another mechanism involved in the ability of tumor cells to extravasate and engraft at a particular microenvironment is the concept of metastatic niche. This involves the tissue parenchyma pre-conditioning at target sites of metastasis, to make the microenvironment not just permissive but essential to enable a disseminating tumor cell to spawn a secondary tumor growth (Psaila & Lyden, 2009).

Among other possible changes, we evaluated by immunofluorescence the mobilization of VEGFR-1 positive myeloid cells (Cd11c positive) and the deposition of fibronectin in the lung. As shown in Figures 6a and 6b, GFP-eDll4cKO mice displayed a great reduction in the number of Cd11c/VEGR-1 positive cells, relative to GFP-control mice. We also addressed the lung resident DC (myeloid cells Cd11c positive but VEGFR-1 negative). Interestingly, these cells were increased in the GFP-eDll4cKO mice, relative to GFP-control (Figures 6a and 6c).

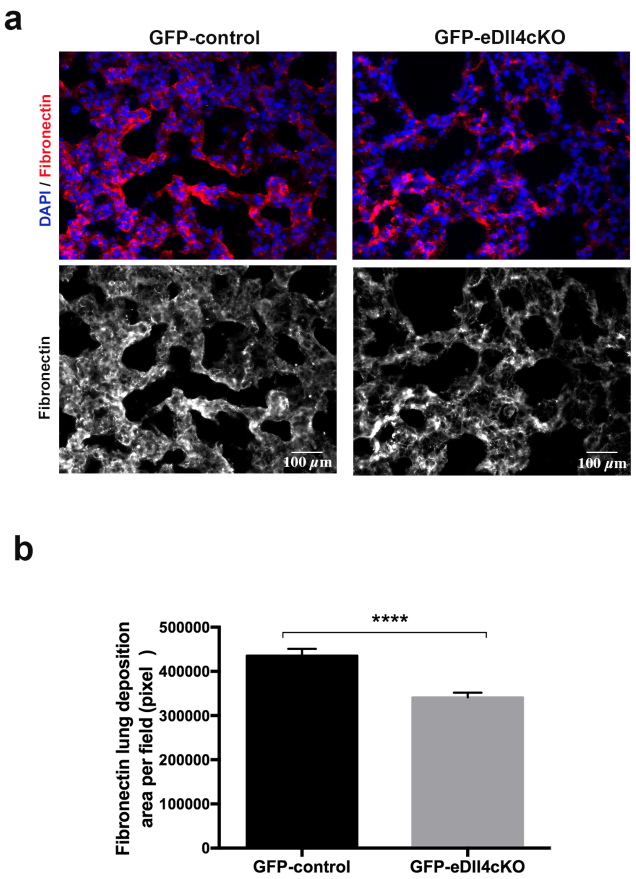
Figure III-6 - Effect of endothelial Dll4 loss-of-function in the recruitment of myeloid cells to the metastatic niche.



a) Cd11c (green) and VEGFR-1 (red) immunostaining images (40x amplification) of lung samples to evaluate the co-localization of both signals, indicative of myeloid cells mobilized to the lungs, in GFP-eDll4cKO and GFP-control mice. **b)** Myeloid cells Cd11c/ VEGFR-1 positive cells are significantly decreased (number) in GFP-eDll4cKO mice, compared with GFP-control. **c)** Lung resident dendritic cells (Cd11c positive/ VEGFR-1 negative) are significantly increased (number) in GFP-eDll4cKO mice, relative to controls. 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; blue) stains nuclei. Lung samples samples were collected at the end-point. Results are representative of 2 independent experiments, each with n=4 mice per group. Error bars represent SEM; ****P<0.0001.

Concerning the fibronectin deposition area in the lung, the GFP-eDll4cKO group presented a significant decrease, compared to GFP-control group (Figures 7a and 7b).

Figure III-7 - Fibronectin deposition in the metastatic niche of endothelial-specific Dll4 loss-of-function mice.



a) Immunostaining images (40x amplification) of lung samples from GFP-eDll4cKO *vs* GFP-control groups, marked for fibronectin (red). **b)** The area per field (pixels) of fibronectin in the metastatic niche (lung) is significantly reduced in the GFP-eDll4cKO group, compared to GFP-control group. 4´,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; blue) stains nuclei. Lung samples samples were collected at the end-point. Results are representative of 2 independent experiments, each with n=4 mice per group. Error bars represent SEM; ****P<0.0001.

5. Discussion

Since the Dll4 paradox establishment (Thurston et al., 2007), there have been numerous works addressing whether the increased leakiness and vessel permeability would facilitate metastatic dissemination of the escaping tumor cells. Exemplifying such apparent contradiction, the diminishment of pericyte recruitment to primary tumors developing in mice genetically devoid of MMP-9 (critical angiogenic enzyme), has been associated with collapsed morphology of tumor vasculature and substantial inhibition of the metastatic process (Chantrain et al., 2006).

This is in agreement with our previous work, were we show that endothelial Dll4 loss-of-function inhibits LLC tumor vascular maturation process, with tumor vascular beds presenting reduced perivascular cell coverage (both pericytes and smooth muscle cells). In turn, this leads to vascular leakiness increase and functionality reduction, marking the endothelium to a regression fate. Surprisingly, we found a significant reduction in the number and burden of distant metastases. We demonstrated that endothelial Dll4 loss-of-function is reducing metastasis by inhibiting three early steps in the metastatic cascade, EMT, CSC and CTCs frequency.

The present study reveals that endothelial Dll4 is also fundamental in the subsequent steps of metastasis. We observed a significant reduction in the number of intravasated metastatic cells in the endothelial Dll4 knockout mice, which is in line with the reduced CTCs already described. We cannot be sure if this reduction is just caused by EMT inhibition itself or also by the eDll4cKO vasculature being less permeable to metastatic tumor cells.

In our past expression analysis of LLC tumors we identified, at least, one documented TDSFs, TGF-β that is being significantly downregulated by endothelial Dll4 loss-of-function. The downregulation of Dll4/Notch1/TGF-β can inhibit metastasis through the reduction of EMT, but also through the reduction of the "signal" for the metastatic cells to evade.

Meanwhile, the extravasation is compromised in the endothelial Dll4 knockout mice not only because of the reduction of metastatic cells in circulation, but above all because the metastatic niche has less myeloid Cd11c/VEFR-1 positive cells and fibronectin deposition, which prevents metastatic cell engraftment. We demonstrate that the lung vasculature is in a quiescent state with no differences in vessel density and maturation between GFP-eDll4cKO and GFP-control mice. Both Dll4 and N1ICD have similar patterns. Indeed, N1ICD is almost absent, which may indicate that lung endothelial Dll4/Notch pathway is not being activated in any group. This excludes the hypothesis of a paracrine crosstalk between endothelial and metastatic cells, as another mechanism to prepare the metastatic niche.

The role of possible exosomes secreted by the primary tumor and/or other factors, in this model, remains to elucidate. Another handicap is the proliferation to micro and macro-metastases and the necessary angiogenic switch. For that, a late end-point should be tested.

An interesting result was the increased number of myeloid Cd11c positive/ VEGFR-1 negative cells in the endothelial Dll4 loss-of-function group. These cells can be assumed to be lung-resident dendritic cells, which recently have been shown to mediate potent anti-metastatic effects (Headley et al., 2016).

In summary, it seems quite evident that the pre-metastatic niche, already considered as the zero step of the metastatic cascade, is a major limiting step of the whole process. Hence, the metastasis is truly an early, not late, event in tumorigenesis. We are the first to show that endothelial-specific Dll4 loss-of-function inhibits intravasation, circulation and extravasation of metastatic cells, mainly by conditioning the pre-metastatic niche. The aforementioned result involves the downregulation of TGF-β from the primary tumor, with reduced bone marrow mobilization of the myeloid Cd11c/ VEGFR-1 positive cells and consequent decreased fibronectin deposition in the lung, the target organ for metastasis in this model (Kaplan et al., 2005; Petty & Yang, 2017; B.-Z. Qian & Pollard, 2010; B. Qian et al., 2009).

Chapter IV. Endothelial Dll4 overexpression reduces vascular response and inhibits tumor growth and metastasization *in vivo*.

Alexandre Trindade[*], Dusan Djokovic[*], Joana Gigante, Liliana Mendonça and António Duarte.

[*]These authors contributed equally to this work.

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1. Abstract

Background- The inhibition of Delta-like 4 (Dll4)/Notch signaling has been shown to result in excessive, nonfunctional 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 tumor models.

Methods- We used a transgenic mouse model of endothelial-specific Dll4 overexpression, previously produced. Growth kinetics and vascular histopathology of several types of solid tumors was evaluated, namely Lewis Lung Carcinoma xenografts, chemically-induced skin papillomas and RIP1-Tag2 insulinomas.

Results- We found that increased Dll4/Notch signaling reduces tumor growth by reducing vascular endothelial growth factor (VEGF)-induced endothelial proliferation, tumor vessel density and overall tumor blood supply. In addition, 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 was found to prevent metastasis formation and allow for increased delivery to the tumor of concomitant chemotherapy, improving its efficacy.

Conclusions- By reducing endothelial sensitivity to VEGF, these results imply that Dll4/Notch stimulation in tumor microenvironment could be beneficial to solid cancer patient treatment by reducing primary tumor size, improving tumor drug delivery and reducing metastization. Endothelial specific Dll4 overexpression thus appears as a promising anti-angiogenic modality that might improve cancer control.

Keywords: Angiogenesis, Dll4, Notch, Overexpression, Tumor, Metastasis, Drug-delivery.

2. Background

Current angiogenic inhibitors targeting VEGF signaling show therapeutic efficacy in many aggressive tumors, but fail to provide enduring clinical benefit in most cases (Aravantinos & Pectasides, 2014; Chen et al., 2014; Ebos & Kerbel, 2011; Rakesh K Jain, 2005; Rakesh K Jain, Duda, Clark, & Loeffler, 2006; Saltz et al., 2007; Shojaei et al., 2007) .

In addition, the VEGF inhibitors with documented anti-tumor efficacy have been found in mouse models to be prone to elicit tumor adaptation and progression to stages of greater malignancy, with heightened invasiveness and in some cases increased lymphatic and distant metastasis (Pàez-Ribes et al., 2009). Therefore, there is a need to validate novel therapeutic targets alongside VEGF signaling inhibition. In this context, Dll4/Notch signaling appears as a promising candidate.

Acting downstream of VEGF signaling, Dll4/Notch signaling essentially contributes to proper vascular remodeling during embryonic vascular development (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004). The endothelial ligand Dll4 interacts with Notch 1 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 (S J Bray, 2006) and EphrinB2 (L.-K. Phng & Gerhardt, 2009). In the post- natal period Dll4 has a low level of expression in quiescent blood vessels of normal tissue (Mailhos et al., 2001). However, its up- regulation is a hallmark of proliferating tumor vessels in both preclinical murine models (Djokovic et al., 2010; Gale et al., 2004; Hainaud et al., 2006; Noguera-Troise et al., 2006; Scehnet et al., 2007) and different human malignancies (A M Jubb et al., 2009; Adrian M. Jubb et al., 2010; Mailhos et al., 2001; N. S. Patel et al., 2006). Besides, Dll4 expression was identified in malignant cells of different types (Martinez et al., 2009), and the ligand was shown to have an impact on colon cancer stem cell frequency by suppressing apoptosis of tumor cells (M. Fischer et al., 2011; Hoey et al., 2009). 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 et al., 2010; Haller et al., 2010; Kalén et al., 2011; J.-L. Li et al., 2007; Noguera-Troise et al., 2006; Ridgway et al., 2006; Scehnet et al., 2007). 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 re- duce 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 et al., 2010; Ji-Liang Li, Jubb, & Harris, 2010; Yan 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 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 et al., 1999). Thus, amplification of Dll4/Notch signals appears as a rational 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 (J.-L. Li et al., 2007; Noguera-Troise et al., 2006). 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 reported to act as a negative driver of tumor expansion in tested malignant cell lines grafted in mice (J.-L. Li et al., 2007; Noguera-Troise et al., 2006; Segarra et al., 2008), Dll4 expression in transduced human glioblastoma, prostate and gastric cancer cell xenografts was associated with promoted tumor growth, to some extent, due to a reduction of tumor hypoxia and apoptosis or increased secretion of matrix metalloproteinase-2 (G.-G. Li et al., 2013; J.-L. Li 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 representative 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 indicated for concomitant application with VEGF-inhibitors and stabilizes tumor circulation allowing for more efficient chemotherapy delivery while at the same time reducing the formation of distant-site metastasis.

3. Methods

3.1. Experimental animals

Double heterozygous Tie2-rtTA-M2 TetO7-Dll4 mice were generated as described (Trindade 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 (Catalan Institute of Oncology) and used for breeding with Tie2-rtTA-M2 TetO7-Dll4 line for generation of triple mutants (RT2 Tie2-rtTA-M2 TetO7-Dll4) capable of developing pancreatic insulinomas and overexpress endothelial Dll4 after tetracycline or doxycycline-induction.

Restricted to 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 administration *per os* of the tetracycline analogue doxycycline (2 mg/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). Non-induced TetO7-Dll4 littermates, receiving 2 mg/ml doxycycline in drinking water ad libitum, served as Doxycycline control mice (D4BE + Doxy).

The animals were housed in ventilated propylene cages with sawdust bedding, in room with temperature be- tween 22 °C and 25 °C 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/CVT71084/2012).

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 2 mg/ml doxycycline in drinking water (Dll4 over-expression group, D4OE). A week later, Lewis lung carcinoma cells (LLC - ATCC® CRL- 1642™, 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, xenografts were dissected and processed for histological studies. For the metastasization experiment the basic protocol described above was used but the experiment was run for 6 weeks after the LLC injection.

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 respectively water (control Dll4 basic expression group, D4BE) or

started receiving 2 mg/ml doxycycline in drinking water (Dll4 overexpression group, D4OE). A week later, all mice were shaved and treated with a single dose of 25 μg of 7,12-dimethylbenz[a]anthracene (DMBA; Sigma, St. Luis, MO) in 200 μL 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 4 μg 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 × width × height × 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.

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 that respectively started receiving water (control RT2 Dll4 basic expression group, RT2 D4BE) or 2 mg/ml doxycycline in drinking water (RT2 Dll4 overexpression group, RT2 D4OE). The mice were sacrificed at the age of 13.5 weeks just before most RT2 progenitors die due to tumor burden and hypoglicemia. The pancreata were dissected and macroscopic tumors (≥1 × 1 mm) were excised. Tumor volumes were calculated using the formula: V = length × width × height × 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.

3.5. Tumor tissue preparation, histopathology and immunohistochemistry

When dissected and measured as described above, tumors (LLC xenografts, skin lesions and RT2 insulinomas developed in D4BE and D4OE mice) were fixed in 4% paraformaldehyde (PFA) solution at 4 °C for 1 h, cryoprotected in 15% sucrose, embedded in 7.5% gelatin, frozen in liquid nitrogen and cryosectioned at 20 μm. For the metastasization experiment, lungs were dissected and macro-metastases were observed under a dissection microscope. The lungs were resected for fixation in Bouin's fixative for posterior metastasis histological confirmation. 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 h 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.

3.6. Perfusion study

To visualize the blood-perfused, i.e. functional, portion of the tumor circulation, 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 min before the animal vasculature was perfused transcardially with 4% PFA in PBS for 3 min. Tumor samples were collected and processed as presented above.

Tissue sections (20 µm) 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. In all cases, signal positive areas per microscopic field (n = 20 per mouse sample) were determined by the percentage of black pixels per field after transforming the RGB images into binary files.

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 in D4BE and D4OE mice (n = 10 for each group). Real-time PCR analysis was performed as described (Trindade et al., 2008) using specific primers for β-actin, Pecam, Dll4, Hey2, Vegf-a, Vegfr1, Vegfr2, Vegf-c, Vegfr3, Pdgf-β, EphrinB2 and Tie2. Gene expression was normalized to β-actin. Primer pair sequences are available upon request.

3.8. Total tumor doxorubicin quantitation

Total tumor doxorubicin was quantified using a method similar to Mayer et al. (Mayer, Dougherty, Harasym, & Bally, 1997). At endpoint, 10% w/v tumor homogenates were prepared in tissue lysis buffer. Samples of the homogenate (200 uL) were placed in 2- mL microcentrifuge tubes, and 100 uL of 10% (v/v) Triton X-100, 200 uL of water, and 1500 uL of acidified isopropanol (0.75 N HCl) were added. After vortexing, doxorubicin was extracted overnight at -20 °C. The next day, the tubes were first vortexed and then centrifuged at 15,000 g for 20 min, and stored at -80 °C until analysis. Doxorubicin was quantified fluorometrically (kex = 470 nm, kem = 590 nm). To correct for nonspecific background fluorescence, the samples were compared with a standard curve made from the fluorescent emission of known amounts of doxorubicin added to acidified isopropanol extracts of homogenized tumor tissue from untreated mice. The data are expressed as microequivalents of doxorubicin/g tissue.

3.9. 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.

4. Results

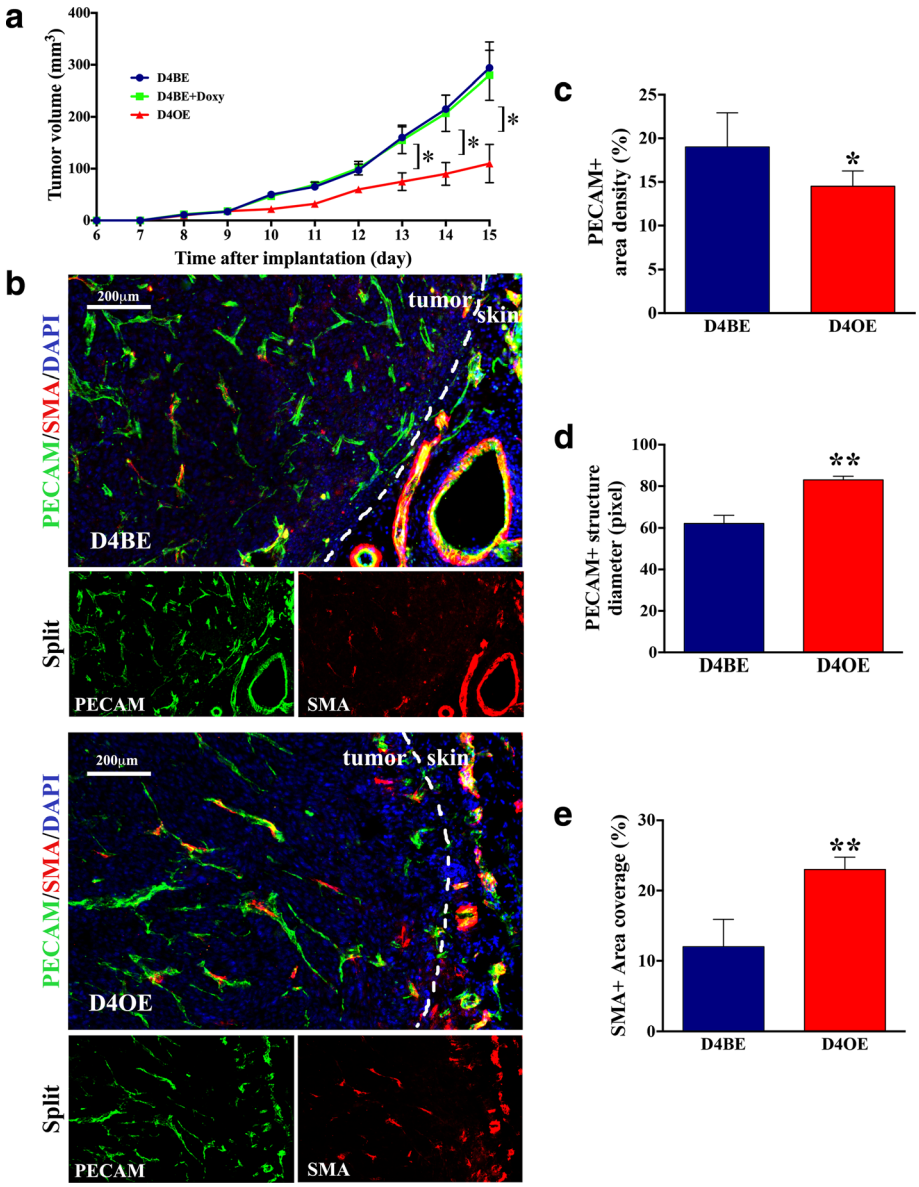
4.1. Endothelial Dll4 overexpression retards xenograft growth and reduces vascular response in LLC-bearing mice

To study the effects of endothelial Dll4 overexpression on tumor growth, LLC cells were injected subcutaneously in D4BE (non-induced Tie2-rtTA TetO7-Dll4), in D4BE + Doxy (doxycycline-induced TetO7-Dll4, which served as induction controls) and D4OE (doxycycline-induced Tie2-rtTA TetO7-Dll4) mice (n = 6). D4OE mice have been previously reported to efficiently overexpress Dll4 in the mouse embryonic (Trindade et al., 2008)and adult vasculature (Trindade et al., 2012) producing angiogenic arrest in active angiogenic areas while not affecting quiescent vasculatures (Trindade et al., 2012). The injected sites were monitored for a period of 15 days. In all mouse groups, the LLC tumors became palpable by day 8. D4BE + Doxy mice xenografts always grew at a similar rate to D4BE mice xenografts and were not further analyzed. However, D4OE mice revealed retarded xenograft expansion relative to D4BE mice throughout the experiment and displayed significantly smaller aver- age tumor volumes beginning by day 13 (Fig. 1a). 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 metastasis.

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 Fig. 1b, 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 de- crease, $p < 0.05$; Fig. 1c). Their vessels were found to be straighter and less branched, presenting, at the same time, wider calibers (Fig. 1d) and an highly significant increase in SMA-positive cell coverage relative to the control tumor vessels (>2-fold increase, $p < 0.01$; Fig. 1e). 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.

Figure IV-1 - Endothelial Dll4 over-expression affects LLC subcutaneous xenograft growth and vasculature.



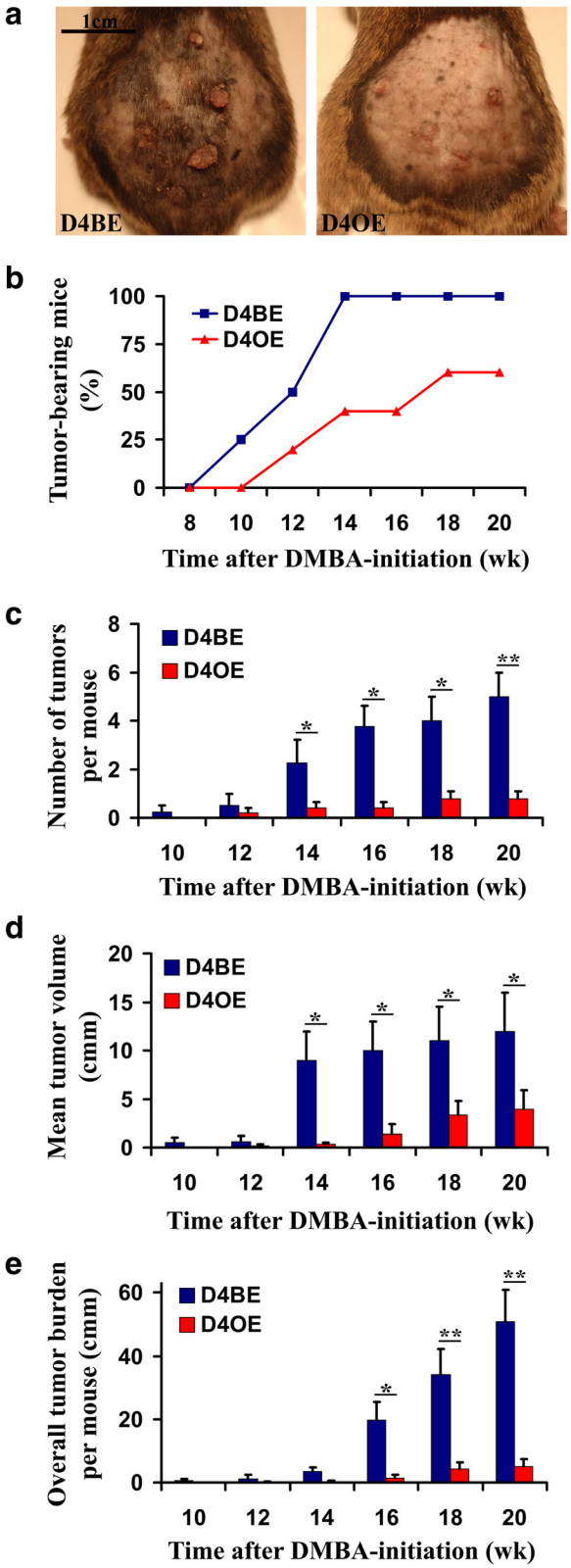
a) Dll4 over-expressed in endothelial cells retarded xenograft volume increase after subcutaneous LLC implantation in D4BE, D4BE + Doxy and D4OE littermates. Error bars represent SEM. *, $P < 0.05$ was considered significant. **b)** Vascular response was examined in D4BE and D4OE tumor grafts using double immunostaining to endothelial PECAM and mural cell marker α-SMA. In relation with D4BE controls (above), the LLC xenografts developed in D4OE mice presented reduced vascular density **(c)**, but increased vessel calibers **(d)** and promoted vessel wall assembly **(e)**. Contrary to the D4BE tumor vessels that showed weak SMA staining and its co-localization with PECAM signals, SMA-positive cells lining PECAM-positive endothelium were distinctively increased in D4OE xenografts indicating improved vessel wall maturation. Dot lines mark tumor borders. Results are representative of n = 6 per mice group.

4.2. Dll4 overexpression inhibits the skin papilloma formation, restricts vessel proliferation and improves vascular functionality

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 (n = 10) by a single topical DMBA treatment and then promoted by topical TPA applications twice per week for 19 weeks. As presented in Fig. 2a, b, 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, Fig. 2c) and in the mean tumor volume between two experimental groups ($p < 0.05$; Fig. 2d). 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 ($p < 0.01$; Fig. 2e) 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 IV-2 - Endothelial Dll4 over-expression suppresses chemically- induced skin tumor onset and development.

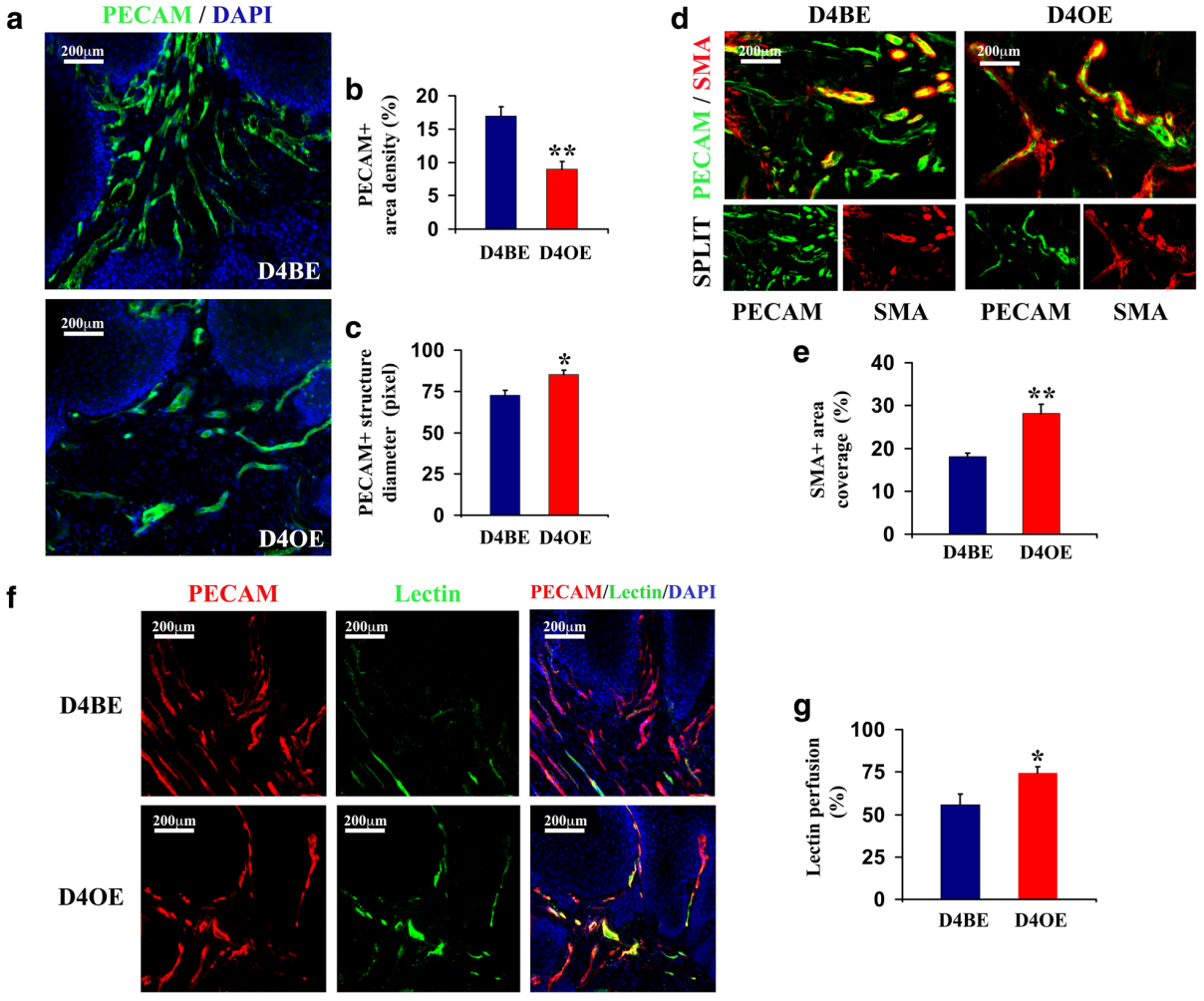


D4BE and D4OE mice were treated topically with a single dose of DMBA followed by two weekly applications of TPA for 19 weeks. **a)** Representative pictures of D4BE and D4OE mice taken at the experiment end- point, i.e. 20 weeks after the DMBA-initiation, illustrate tumor-suppressing effect of endothelial Dll4 over-expression. **b)** Percentage of tumor-bearing mice, number of tumors per mouse **(c)**, mean tumor volume **(d)** and over-all tumor burden per mouse **(e)**, calculated as the sum of tumor volumes developed by a mouse, throughout the tumor TPA-promotion in D4BE vs. D4OE mice. Error bars represent SEM. *, $P < 0.05$ and **, $P < 0.01$ were considered statistically significant and highly significant, respectively. Results are representative of n = 10 per mice group.

Immunostaining to PECAM indicated highly significant microvascular density reduction ($p < 0.01$) in papilomas of D4OE vs. D4BE mice (Fig. 3a,b). Comparable to morphological changes observed in subcutaneous xenografts, Dll4 overexpression was shown to restrict

PECAM-network branching but increase papilloma vessel lumen calibers ($p < 0.05$, Fig. 3c), α-SMA-positive mural cell recruitment ($p < 0.01$, Fig. 3d,e) and promote vessel competence as indicated by significantly increased lectin perfusion in papillomas developed by D4OE vs. D4BE ($p < 0.05$, Fig. 3f,g). In this way, retarded growth of autochthonous skin tumors under the condition of increased endothelial Dll4/Notch signaling was demonstrated to be a consequence of reduced tumor vascular density, even though Dll4 over-expression was simultaneously found to augment individual papilloma vessel functional capacity in papillomas.

Figure IV-3 - Vascular response upon skin chemical tumorigenesis in D4BE and D4OE mice.



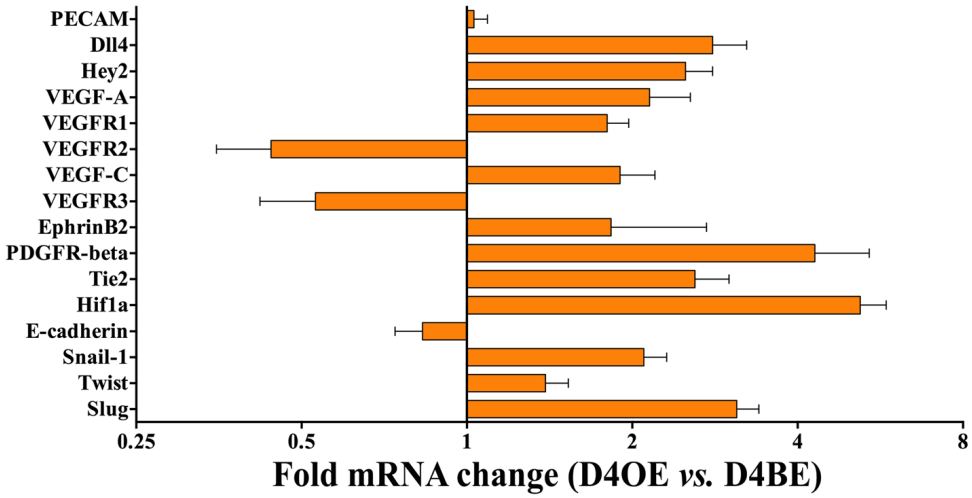
a) Tumor endothelium was visualized by section PECAM immunostaining of skin tumors collected from DMBA/TPA-treated mice 20 weeks after the DMBA-initiation. Endothelial Dll4 over-expression was found to reduce endothelial system density by reducing its ramification **(b)**, although branch diameters were observed increased in D4OE vs. D4BE mice **(c)**. **d)** Mural cell recruitment was assessed by PECAM/SMA co-immunostaining (above). **e)** Endothelial Dll4 over-expression promoted vessel wall assembly which contributed to the normalization of tumor vasculature. **f)** To evaluate tumor vessel competence, sub-groups of both D4BE and D4OE mice were perfused before being sacrificed with i.v. Injected lectin as presented in “Materials and Methods”. Simultaneous immunostaining to

PECAM and biotinylated lectin visualization with Streptavidin-Alexa 488 demonstrate increased fraction of competent vessels in D4OE vs. D4BE skin tumors **(f)**. Percentage of PECAM-positive area co-localized with lectin (Alexa 488 signal) was measured to quantify the portion of functional vessels within skin tumors **(g)**. The images presented in panels **a-g** were captured and processed as described in Fig. 1. Error bars represent SEM. *, $P < 0.05$ and **, $P < 0.01$ were considered statistically significant and highly significant, respectively. Results are representative of n = 10 per mice group.

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 increased Dll4 expression, we used qRTPCR and analyzed D4BE and D4OE papillomas for differential expression of the main genes implicated in angiogenesis regulation (Fig. 4.). Compared to D4BE papillomas, D4OE tumors had on average 2.8-fold increased Dll4 mRNA levels and 2.5-fold up-regulated Hey2 transcription confirming the Dll4/Notch pathway over-induction. 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 a ~2-fold increase of the trapper-receptor Vegfr1 transcription and a >2-fold reduction of the main signaling receptor Vegfr2 transcription. Similarly, Vegf-c was up-regulated while the transcription of its receptor Vegfr3 was shown to be reduced. As expected, our analysis demonstrated an increase of Pdgfr-β, EphrinB2 and Tie2 activity accompanying increased mural cell recruitment and vascular stability. To evaluate the impact of endothelial Dll4 overexpression on epithelial homeostasis we also evaluated the transcriptional changes of E- cadherin, as an epithelial marker, Snail-1, Twist and Slug as Epithelial-to-mesenchymal transition (EMT) markers and Hif1-a as a hypoxia marker. The results demonstrated that D4OE mice tumors display increased EMT markers and Hif1-a transcription.

Figure IV-4 - Differential gene activity between chemically-induced skin tumors of D4BE and D4OE mice.



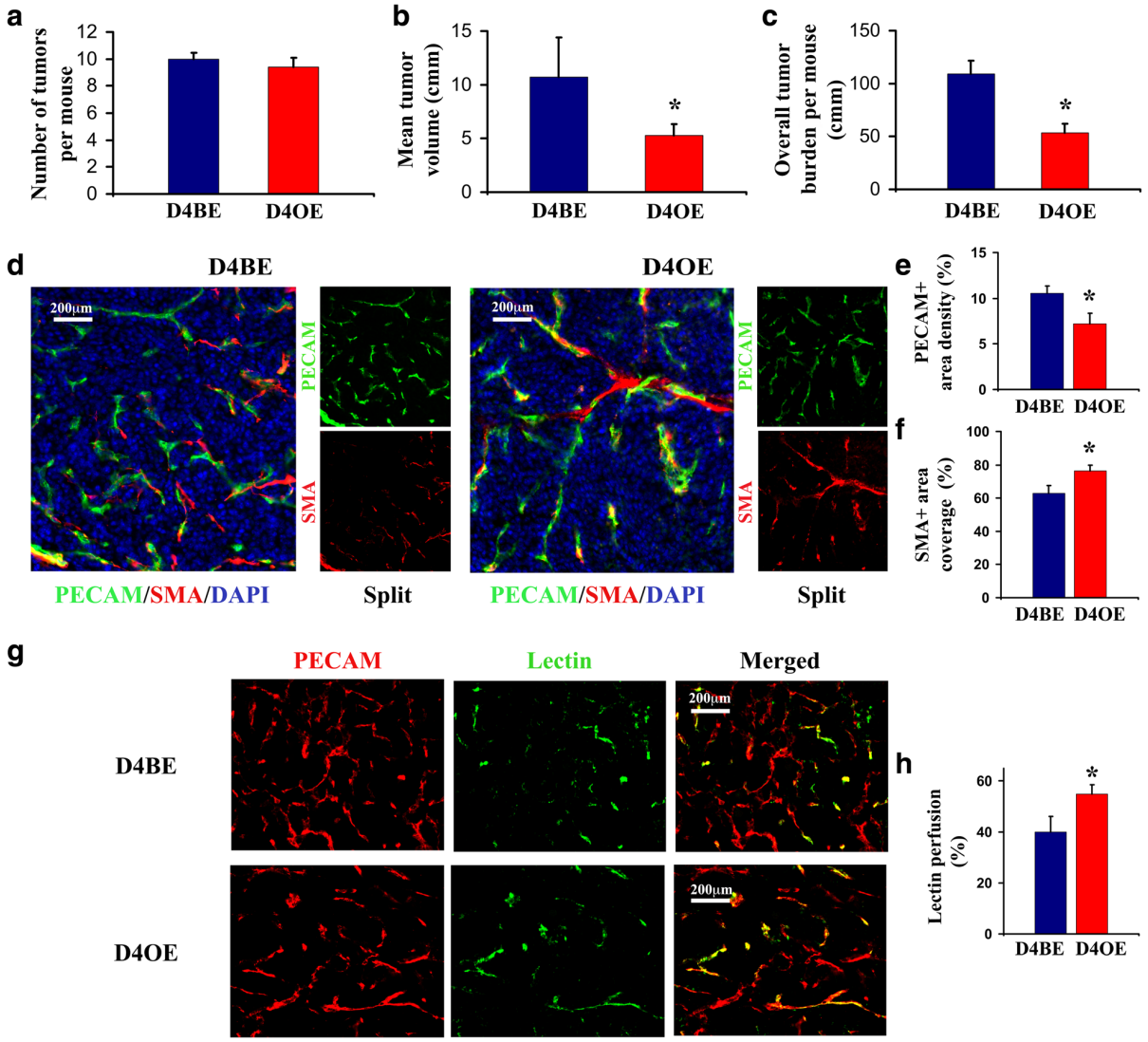
Expressions of selected genes in tumor tissue samples were examined using quantitative RT-PCR. Tests were done in triplicate. All RT-PCR data were normalized to β-actin levels. For each gene, relative mRNA levels of D4OE tumor samples were compared to the reference D4BE levels and presented as x-fold change. Error bars represent SEM.

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 of neoplasm histological type, 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 (Douglas Hanahan, 1985). Although oncogene expression is detectable as early as embryonic day E8.5, RT2 mice 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 (G Bergers, Javaherian, Lo, Folkman, & Hanahan, 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) (n = 10) 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 mice were found not to differ in terms of average number of insulinomas per individual (Fig. 5a). However, average tumor volume (Fig. 5b) and overall tumor burden were significantly reduced in RT2 D4OE compared to RT2 D4BE mice (~50% reduction, $p < 0.05$; Fig. 5c). Histologically, Dll4 overexpression in RT2 D4OE mice was found to decrease insulinoma endothelial density by 32% ($p < 0.05$; Fig. 5d,e), increase α-SMA- positive mural cell coverage by ~20% ($p < 0.05$; Fig. 5d,f) and result in improved lectin perfusion by 27% ($p <$

0.05) in relation with RT2 D4BE insulinomas (Fig. 5g, h). 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 IV-5 - The endothelial Dll4 over expression effects on RT2 insulinoma kinetics and vascular response.



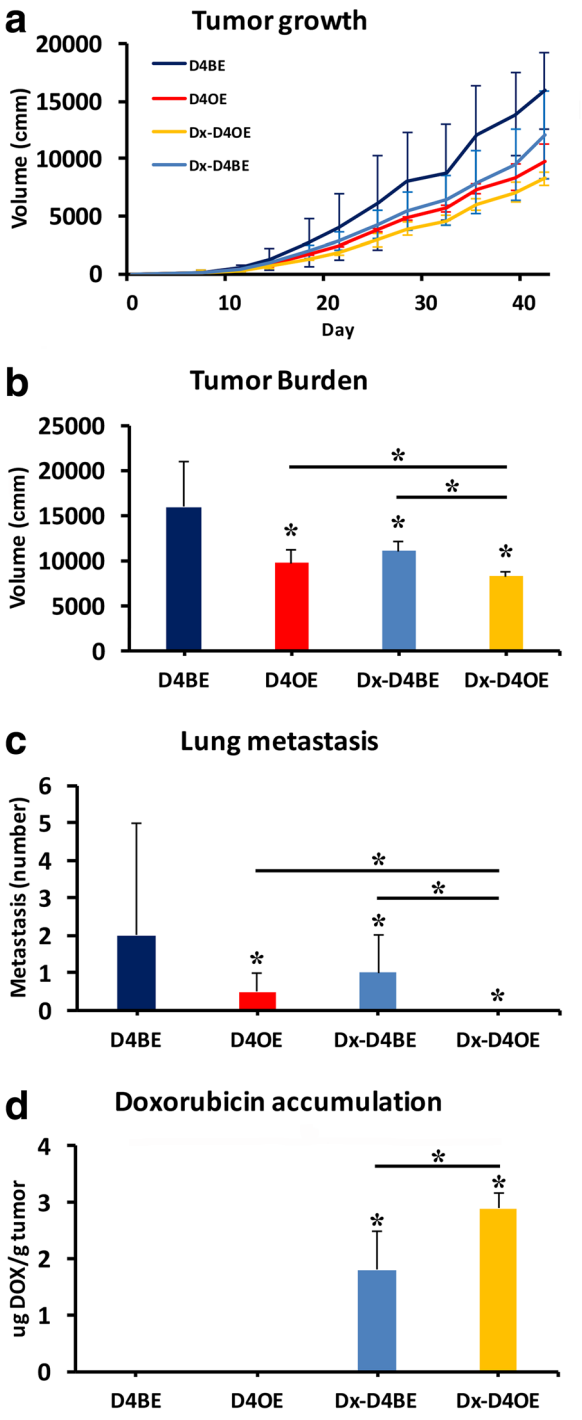
Dll4 over-expression in endothelial cells was induced in 9.5-week old RT2 Tie2-rtTA TetO7-Dll4 mice, maintained for 4 weeks by doxycycline p.o. application and assessed as a potential therapeutic strategy in comparison with non-induced RT2 Tie2-rtTA TetO7-Dll4 littermates. Increased endothelial Dll4 expression was demonstrated to be a powerful tumor-suppressor intervention in RT2 mice recapitulating the vascular phenotype previously observed in grafted and autochthonous skin tumors. **a)** Number of tumors per mouse, mean tumor volume **(b)** and over-all tumor burden per mouse **(c)**, calculated as the sum of tumor volumes developed by a mouse, in 13.5-week old RT2 D4BE vs. RT2 D4OE mice. **d)** Double immunostaining to PECAM/SMA indicating reduced microvessel density **(e)** and increased mural cell recruitment **(f)** in RT2 D4OE vs. RT2 D4BE insulinomas (left). **g)** Vascular functionality was examined by mouse lectin perfusion. Simultaneous immunostaining to PECAM and

biotinylated lectin visualization with Streptavidin-Alexa 488 demonstrate increased portion of perfused vessels in RT2 D4OE vs. RT2 D4BE tumors **(h)**. Percentage of PECAM-positive area co-localized with lectin signals was measured to quantify the portion of competent vessels within pancreatic tumors **(h)**. The images presented in panels **d** and **g** were captured and processed as described in Fig. 1. Error bars represent SEM. *, $P < 0.05$ was considered statistically significant. Results are representative of n = 10 per mice group.

4.5. Endothelial Dll4 overexpression reduces metastasis formation and improves accumulation of concomitantly administrated chemotherapy in primary tumor

To test if the vascular normalization induced by Dll4 overexpression had an impact on tumor drug delivery and the formation of distant site metastasis, we used a subcutaneous Lewis Lung Carcinoma (LLC) tumor transplant mouse model. Mice were palpated daily until detection of first solid masses was possible, which happened at day 7. At day 8, we started administrating doxycycline in the drinking water to test mice, separating Dll4BE and Dll4OE test groups (n = 10), and tumor volume was measured until the end-point (6 weeks after LLC cells injection). At day 11 we started administrating doxorubicin (2 mg/kg, 3x a week) to Dll4BE and Dll4OE mice separating further two groups Dx-Dll4BE and Dx-Dll4OE (n = 5). Doxorubicin is a member of the anthraciclines family of chemotherapeutic drugs. As shown in Fig. 6a, tumor growth was reduced by both endothelial Dll4 overexpression and doxorubicin administration to Dll4BE mice, while the concomitant administration of doxorubicin to Dll4OE mice further reduced the tumor growth by 15% at endpoint ($p < 0.05$; Fig. 6b). Lung metastases were counted at endpoint. While Dll4OE mice displayed a 4x reduced number of metastatic foci when compared to control mice, no metastatic foci were detected in Dll4OE mice administered with doxorubicin ($p < 0.05$; Fig. 6c). Evaluation of doxorubicin concentration in the primary tumor revealed that endothelial Dll4 overexpression improved drug accumulation by 60% ($p < 0.01$; Fig. 6d).

Figure IV-6 - Endothelial Dll4 over expression improves tumor drug delivery and reduces metastasis formation.



a, b) doxorubicin administration (Dx- D4BE) and Dll4 over-expression in endothelial cells (D4OE) retarded xenograft volume increase after subcutaneous LLC implantation in D4BE and D4OE littermates. Doxorubicin administration to Dll4 endothelial overexpressing mice (Dx-D4OE) resulted in the highest tumor growth regression. Upon endpoint lungs were dissected and metastatic foci were counted under a dissection microscope. Both D4OE and Dx-D4BE mice displayed reduced numbers of lung metastasis but Dx-D4OE mice had no detectable lung metastatic foci **(c)**. **d)** Primary tumor samples were also recovered at endpoint to evaluate doxorubicin concentration. Doxorubicin accumulation in the primary tumor was increased by 60% in D4OE mice when compared to D4BE. Error bars represent SEM. *, $P < 0.05$was considered significant. Results are representative of n = 10 per mice group.

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 et al., 2010; Noguera-Troise et al., 2006; Ridgway et al., 2006; Scehnet et al., 2007). Nevertheless, Dll4/Notch signaling-blockade remains a strategy with unpredictable

clinical usefulness. Basically, vascular defects self-repair and reperfusion over long-term Dll4/Notch-suppression may revert tumor growth, particularly in association with increasing malignant cell invasiveness, previously documented as a consequence of anti-angiogenic-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, which was anticipated to reduce vascular response within tumors and suppress their expansion. In addition, Dll4 overexpression was expected to promote vessel maturation and stabilize the tumor vasculature by reducing its remodeling capacity and, in this way, the risk of development of therapy resistance and also improve tumor drug delivery.

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 presumably reduced 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 could 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 help to prevent the penetration of the malignant cells into the circulation and subsequent metastasization.

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, which is likely to be due to increased hypoxia revealed by elevated Hif1-a transcription, reduced vascular sensitivity to VEGF-A was achieved by reduced Vegfr2 transcription and simultaneous up-regulation of Vegfr1, which lacks significant signaling activity in endothelial cells. 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 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.

Concerning the improved perivascular cell recruitment, we found Dll4 overexpression to influence the transcription 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 & Alitalo, 2007; Djokovic et al., 2010). 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 & 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 transcription can be considered complementary since Tie2, when activated by angiopoietin1, is essential to maintain the endothelium in the quiescent state (A. L. Wong et al., 1997) by promoting mural cell recruitment (Asahara et al., 1998).

Epithelial homeostasis was also revealed to be changed from endothelial Dll4 overexpression. Epithelial marker E-cadherin transcription was downregulated while EMT markers Snail-1, Twist and Slug were all upregulated. This is indicative of a higher pressure towards the meta- static phenotype in D4OE mice. Both the potential benefit of Dll4 overexpression in increasing chemotherapy effectiveness and its influence on metastasis formation were also evaluated in this study by use of a metastasizing LLC xenograft protocol. We understand there are advantages to using an orthotopic mouse model of metastization (Martinez et al., 2009) instead of a xenograft protocol, especially because the tumor microenvironment is so different but the protocol we chose allowed us to explore the lung tropism of the LLC cells to more effectively restrict and direct circulating tumor cells to the lungs. Results revealed that endothelial Dll4 overexpression and the independent administration of doxorubicin, a common chemotherapeutic drug, were equally effective in reducing tumor burden and the formation of distant-site metastasis. It is also worth making note that results were always found to be better for the D4OE group but never significantly different from independent administration of doxorubicin. However, the administration of doxorubicin to D4OE mice resulted in the highest reduction of tumor growth and endpoint tumor burden, with no detectable metastasis found in the lungs of test mice. Evaluation of primary tumor drug accumulation revealed that doxorubicin accumulation was increased by 60% when endothelial cells were overexpressing Dll4. The highly significant decrease in metastasis formation in D4OE mice contrasts with the increase in EMT markers previously observed. This could be indicative that tumor cells that become malignant could be failing to effectively intravasate the highly smooth muscle cell covered neovasculature of the primary tumor and become trapped.

The results presented here represent the opposite to those described by Dll4/Notch genetic or pharmacologic inhibition when we look at the endothelial or smooth muscle cell layer phenotypes. Nevertheless, in both cases we observe a reduction of tumor growth. Probably because in both cases, despite opposing vascular phenotypes, tumor hypoxia is increased.

Something similar was previously reported by us in a wound healing setting (Trindade et al., 2012). Also in that case, both endothelial Dll4 loss- and gain-of-function resulted in impaired wound regeneration despite having opposing vascular phenotypes. As in that case, tissue dynamics depend more on vascular function than morphology.

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 pro- duce wide, straight and less branched vessels (J.-L. Li et al., 2007; Noguera-Troise et al., 2006; Segarra 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 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 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 could 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.

6. Conclusions

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

DISCUSSION and CONCLUSIONS

The main objective of this thesis was to study how endothelial Dll4 interferes with tumor metastases formation. Chapter II addressed the steps of primary tumor growth, angiogenesis and hypoxia status, EMT and selection of CSC, tumor cells in the bloodstream and macro-metastases detection. To unveil the direct contribution of endothelial Dll4, mice with endothelial-specific inducible *Dll4* loss-of-function were generated and used for the spontaneous metastasis model of LLC subcutaneous transplantation. The systemic effect of blocking Dll4 in LLC cells, particularly the EMT arrest (Xu et al., 2016) was first tested *in vitro*. *In vivo* validation of endothelial *Dll4* knockout was demonstrated by immunostaining for N1ICD in the primary tumor, at the endpoint. Chapter III explored the following steps of the metastatic cascade that involved the circulation of tumor cells, namely the intravasation from the primary tumor to blood vessels and its transportation to a distant organ-site (lung, in this model) to extravasate where the metastatic niche had been prepared. Tracking these cells was allowed by marking LLC cells with GFP. The lung vasculature was characterized, in order to rule out possible effects of endothelial *Dll4* loss-of-function in this capillary bed that could signal to tumor cells. Chapter IV described the effect of endothelial Dll4 overexpression in different mouse tumor models, in terms of tumor vasculature and growth, and how it affected tumor drug delivery and the frequency of metastasis. Full elucidation of the consequences of endothelial *Dll4* overexpression in each step of the metastasis process was not investigated.

In particular, we are the first to demonstrate the crucial effect of endothelial-specific *Dll4* loss and gain-of-function in tumor growth dynamics. Both approaches produced a reduction in tumor burden, by regulating the vasculature maturity and functionality. If endothelial *Dll4* loss-of-function compromises the endothelium coverage with less perivascular cells (smooth muscle cell layer and pericytes), endothelial *Dll4* overexpression does precisely the opposite. In the first case, although there is an increase in vessel density, the vasculature is extremely leaky and not functional, suffering regression in some areas. Downregulation of PDGF-B/ PDGFRβ pathway reflects this phenotype, already presented in past studies (Ana-Rita Pedrosa, Trindade, Carvalho, et al., 2015). In the second case, vessel density is reduced but the vessels are more mature as evidenced by larger branches, displaying increased mural cell recruitment. Although a higher percentage of blood vessels were perfused, tumor growth was still arrested, likely due to the marked decrease in vascular density. Here transcription analysis suggests that Dll4/Notch signaling amplification induces EphrinB2/EpB4 and PDGF-B/ PDGFRβ signaling that is determinant in vessel stabilization (Adams & Alitalo, 2007; Djokovic et al., 2010). Most importantly, the novelty is the demonstration that tumor growth arrest through a non-productive and aberrant angiogenesis when Dll4 signaling inhibition is endothelial-mediated, as documented by the downregulation of *Dll4*, *Notch1* and *Hey1* genes in LLC-eDll4cKO mice (chapter I). Additionally, tumor growth arrest could also be achieved under endothelial Dll4 overexpressing through a reduction in vessel proliferation and

increased vessel maturation. Despite producing different vascular phenotypes both present an increase in hypoxia that may explain the reduction in tumor burden. Meaning that tumor growth dynamics is more affected by vascular function than morphology, like in wound healing process, as we previously reported (Trindade et al., 2012).

So, when looking to the relevance of the primary tumor angiogenesis in metastization we can say that endothelial *Dll4* loss-of-function creates an immature vasculature with multiple points of access to the bloodstream. Contrarily, endothelial *Dll4* overexpression enhances vessel wall coverage with smooth muscle cell, that is a barrier for tumor cells intravasation to the bloodstream. By consequence we could assume that the disorganized vasculature in endothelial *Dll4* loss-of-function mice facilitates metastasis. Surprisingly, we found a substantial reduction in CTCs and distant metastases foci count and growth. One possible explanation may be that tumor cells easily escape through tumor neovessels but fail to achieve the bloodstream due to the dysfunctional state of these vessels. Another reason is the effect of endothelial *Dll4* loss-of-function over two early metastasis mechanisms: EMT and CSC. Evidence of the inhibition of these events after the administration of an anti-DLL4 antibody (Xu et al., 2016) prompted us to explore if it was endothelial-mediated. In fact, immunostaining and gene expression analysis of LLC-eDll4cKO mice revealed a downregulation of the EMT markers *Snail-1*, *Twist*, *Slug*, *TGF-β* and *E-cadherin* upregulation. Additionally, we observed a reduction of two CSC markers (CD49f and p63) in the tumor immunostaining. Endothelial Dll4/Notch signaling blockade was confirmed by the reduction of Dll4 and N1ICD positive endothelium, supported by transcription analysis. The reverse phenotype is observed in endothelial *Dll4* overexpression mice with an increase in EMT markers. Nonetheless, metastases formation was reduced which indicates that, in this case, metastasis is impaired by the highly smooth muscle cell covered neovasculature of the primary tumor that blocks intravasation, with tumor cells becoming trapped.

The tumor neovasculature is therefore determinant in the subsequent step of the metastatic cascade, the intravasation. Tumor immunostainings of endothelial *Dll4* loss-of-function mice revealed an important reduction in the number of GFP-tagged tumor cells inside the blood vessels. Whether this effect is mainly directed by the reduction in EMT and CSC, *per se*, needs clarification. In agreement, there is a reduction in CTCs. Again, this effect may simply result from less tumor cells intravasated and/or result from the blood flow restriction experienced in these vessels.

Apart from angiogenesis we must consider vessel co-option as another way for metastatic cells to resist and evade (William P J Leenders et al., 2002). It involves hijacking the blood vessels in surrounding normal tissue along with the invasion of a solid tumor, and has now been implicated as a mechanism of resistance to anti‑angiogenic drugs in glioblastoma (de Groot et al., 2010; di Tomaso et al., 2011; Rubenstein et al., 2000), hepatocellular carcinoma (Kuczynski et al., 2016), lymph node metastases (Jeong et al., 2015), liver metastases

(Frentzas et al., 2016), brain metastases (Küsters et al., 2002; W. P. J. Leenders et al., 2004) and lung metastases (Bridgeman et al., 2017). Interestingly, multiple layers of pericytes from outside of the vessels support the co-opted vessels (C.-N. Qian, Tan, Yang, & Cao, 2016). Although we did not assess this vessel pattern in our endothelial *Dll4* mouse models, the substantial reduction in perivascular cell coverage in the endothelial *Dll4* loss-of-function mutants may predict the same phenotype in the co-opted vessels, since vessel remodeling usually accompanies vessel co-option (Kim et al., 2002).

Furthermore, the metastatic niche has a role in signaling to these tumor-evading cells. This niche preparation requires factors known as tumor-derived secreted factors (TDSFs) (Sceneay et al., 2013), such as *TGF-β*, that is downregulated in endothelial *Dll4* loss-of-function. They induce the mobilization and recruitment of BMDCs like the myeloid Cd11c/VEFR-1 positive cells and fibronectin deposition (Kaplan et al., 2005; Petty & Yang, 2017; B.-Z. Qian & Pollard, 2010; B. Qian et al., 2009) that are reduced in endothelial *Dll4* loss-of-function, thus creating a microenvironment less suitable for tumor cells extravasation and engraftment. Local cells and their interplay with BMDCs are also important, with recent demonstration that lung-resident dendritic cells (Cd11c positive/VEFR-1 negative) inhibit metastasis of B16 melanoma (Headley et al., 2016). Plus, these BMDCs secrete a multitude of factors (chemokines/ cytokines and exosomes), facilitating the subsequent recruitment and colonization of disseminated tumor cells (Peinado, Lavotshkin, & Lyden, 2011; Smith & Kang, 2013).

Despite lung vasculature being in a quiescent state as demonstrated by similar pattern of vessel density and maturation between groups in immunostainings, we cannot exclude the possibility of its activation at a later endpoint, when the angiogenic switch occurs and micro-metastasis grow to macro-metastasis. We can only assume that apparently there isn't a crosstalk between lung-endothelial and metastatic cells as a part of the metastatic niche preparation.

In summary, endothelial *Dll4* loss-of-function reduces metastasis by interfering with EMT, CSCs and CTCs numbers, tumor angiogenesis plus intravasation/extravasation, and the pre-metastatic niche or the signaling to bone marrow for metastatic niche preparation. Apparently this involves the downregulation of Dll4/Notch1/TGF-β pathway. A recent report reinforces that hyperactivation of endothelial-Notch1 in the primary tumor and metastatic niche facilitates metastasis through a senescence-like endothelium that allows intravasation and extravasation. Moreover, they demonstrate that this endothelium secretes VCAM-1, responsible for neutrophil infiltration that contributes to metastasis (Wieland et al., 2017).

Lung metastases can occur in virtually any type of cancer but are more frequently found in breast and colorectal (CRC) cancers. Translating to the clinical setting, these may be the two situations where we could propose testing the endothelial *Dll4* loss and gain-of-function induction-based therapies. Although distinct phenotypes, both lead to the same result, a

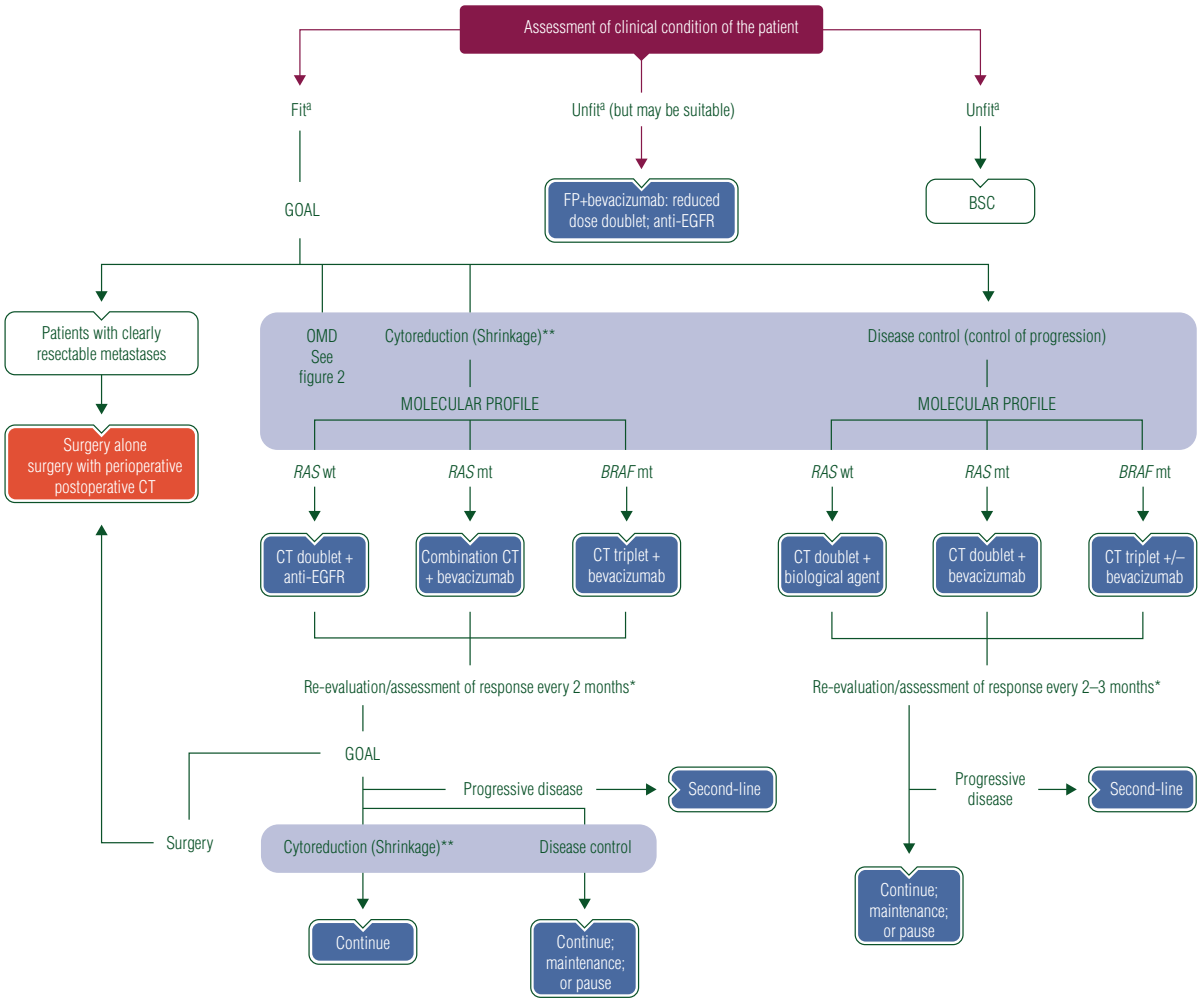
substantial reduction in metastization, which is in line with the concept of *Dll4* dosage effect (Trindade et al., 2012). But the option between one or another requires more pre-clinical investigation.

Advanced Breast Cancer (ABC) comprises both locally advanced (LABC) and metastatic breast cancer (MBC) (Cardoso et al., 2014). Despite treatable, MBC remains an incurable disease with a median overall survival (OS) of 2–3 years and a 5-year survival of only 25% (Giuliani & Bonetti, 2015). ESMO consensus guidelines (Cardoso et al., 2016, 2017) for ABC treatment choice should take in account at least the following factors: hormone receptor (HR) and epidermal growth factor receptor 2 (HER) status, previous therapies and toxicities, tumor burden (defined as number and site of metastases), biological age, performance status, co-morbidities (including organ dysfunctions), menopausal status, need for a rapid disease/ symptom control, socio-economic and psychological factors, available therapies in the patient's country and patient preference.

Anti-angiogenic agents like bevacizumab, in combination with chemotherapy, as 1st or 2nd line therapy for MBC provides only a moderate benefit in progression-free survival (PFS) and no benefit in OS. The absence of known predictive factors for bevacizumab efficacy renders recommendations on its use difficult. Bevacizumab can only therefore be considered as an option in selected cases in these settings and is not recommended after 1st / 2nd line (Cardoso et al., 2016, 2017). Theoretically, the same would apply to endothelial *Dll4* loss and gain-of-function induction-based therapies. But another opportunity for those therapies could be the treatment of triple-negative breast cancer (TN-ABC), with only recent efforts made in the field of immunotherapy-chemotherapy combination.

Over the last decade in particular, the clinical outcome for patients with metastatic CRC (mCRC) has improved. Today, the median OS for patients with mCRC being treated both in phase III trials and in large observational series or registries is ~30 months and more than double that of 20 years ago (Van Cutsem et al., 2016). The ESMO consensus recommends that initial categorization of patients with mCRC for treatment should be made according to whether they were clinically 'fit' or 'unfit' and subsequently according to treatment goal, outlined in Zurich's treatment algorithm (Fig. 10).

Figure 10 - Zurich treatment algorithm. *In* (Van Cutsem et al., Annals of Oncology, 2016).



BSC, best supportive care; CT, chemotherapy; EGFR, epidermal growth factor receptor; FP, fluoropyrmidine; mt, mutant, NED, no evidence of disease; OMD, oligometastatic disease; wt, wild-type. [a]Patients assessed as fit or unfit according to medical condition not due to malignant disease. *After two re-evaluations, consider maintenance. **(A) Includes two subgroups: (1) those for whom intensive treatment is appropriate with the goal of cytoreduction (tumour shrinkage) and conversion to resectable disease; (2) those who need an intensive treatment, although they will never make it to resection or LAT, since they need a rapid reduction of tumour burden because of impending clinical threat, impending organ dysfunction, severe symptoms.

There are several recommendations, with specifications relative to use of anti-angiogenic agents, depending on the patient metastatic disease stage (Van Cutsem et al., 2016). For example, the 1st line systemic therapy combinations state that the VEGF antibody bevacizumab should be used in combination with:

- The cytotoxic doublets FOLFOX (5-fluorouracil (5-FU), leucovorin, oxaliplatin)/ CAPOX (capecitabine, oxaliplatin)/ FOLFIRI (5-FU, leucovorin, irinotecan)
- The cytotoxic triplet FOLFOXIRI (5-FU, leucovorin, oxaliplatin, irinotecan) in selected

fit and motivated patients where cytoreduction (tumor shrinkage) is the goal – and potentially also in fit patients with tumor BRAF mutations.

In terms of maintenance therapy, bevacizumab is usually continued in combination with any cytotoxic agent or any combination of cytotoxic agents until disease progression or unacceptable toxicity (Van Cutsem, Cervantes, Nordlinger, Arnold, & ESMO Guidelines Working Group, 2014). Currently, there is no validated predictive marker for bevacizumab. For this reason, the cumulative toxicity and resistance, novel anti-angiogenic agents like endothelial *Dll4* loss and gain-of-function induction-based therapies may be worth further testing. In practice, these therapies will consist in *Dll4* antagonists or agonists and given that metastasis process is an early event, it will even more interesting to test them in a neo-adjuvant/ prevention context.

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ANNEX I

GENES	Forward Sequence (5' – 3')	Reverse Sequence (5' – 3')
Notch primers		
Dll4	GGAACCTTCTCACTCAACATCC	CTCGTCTGTTCGCCAAATCT
Notch1	ACAGTAACCCCTGCATCCAC	GGTTGGACTCACACTCGTTG
Hey1	GGTACCCAGTGCCTTTGAGA	GTGTGCAGCATTTTCAGGTG
Hey2	CTGAATTGAGAAGACTAGTGCCA	AGCATCTTCAAATGATCCACTGT
Nrarp	AGTCGCTGCTGCAGAACAT	AACAGCTTCACCAGCTCCAG
VEGF primers		
VEGFA	GGAGAGCAGAAGTCCCATGA	ACACAGGACGGCTTGAAGAT
VEGFC	CCTGAATCCTGGGAAATGTG	TCGCACACGGTCTTCTGTAA
Vegfr1	GACCCTCTTTTGGCTCCTTC	CAGTCTCTCCCGTGCAAACT
Vegfr2	GGACTCTCCCTGCCTACCTC	CGGCTCTTTCGCTTACTGTT
Vegfr3	CGAAGCAGACGCTGATGATA	CCCAGGAAAGGACACACAGT
Angiocrine primers		
PDGFRβ	TGATGAAGGTCTCCCAGAGG	AGGAGATGGTGGAGGAAGTG
Pdgfb	CCTCGGCCTGTGACTAGAAG	TTTCGGTGCTTGCCTTTG
Ephrinβ2	TCCCTTTGTGAAGCCAAATC	TACTTGAGCAGCAGCAGCACCAC
Tek	CCCCTGAACTGTGATGATGA	CTGGGCAAATGATGGTCTCT
Tie2	CCCCTGAACTGTGATGATGA	CTGGGCAAATGATGGTCTCT
EMT primers		
E-cad	CCAAAGTGACGCTGAAGTCC	TACACGCTGGGAAACATGAG
Snail-1	CTTGTGTCTGCACGACCTGT	GGAGAATGGCTTCTCACCAG
Slug	GGCTGCTTCAAGGACACATT	TGCCCTCAGGTTTGATCTGT

Hif1α	GCCTTAACCTGTCTGCCACT	GGAGCCATCATGTTCCATTT
Tgf-β	TGGAGCAACATGTGGAACTC	CGTCAAAAGACAGCCACTCA
Control primers		
β-Actin	TGTTACCAACTGGGACGACA	GGGGTGTTGAAGGTCTCAAA
Pecam	CAAGCAAAGCAGTGAAGCTG	TCTAACTTCGGCTTGGGAAA