SPECIFIC FEATURES OF ENDOTHELIAL CELLS IN PRIMARY TUMORS

GERMANA ANDREIA TAIPA LEANDRO DOMINGUES
Tese para obtenção do grau de Doutor em Ciências da Vida
na Especialidade em Biomedicina
na Faculdade de Ciências Médicas

Outubro, 2014

SPECIFIC FEATURES OF ENDOTHELIAL CELLS IN PRIMARY TUMORS

Germana Andreia Taipa Leandro Domingues

Orientador: Sérgio Jerónimo Rodrigues Dias, Professor Associado

Co-orientadora: Jacinta de Fátima Rosário Serpa, Professora Auxiliar Convidada

Tese para obtenção do grau de Doutor em Ciências da Vida

na Especialidade em Biomedicina

Outubro, 2014

The result chapters presented in this thesis are manuscripts in preparation for subsequent publications. I clarify that I have participated fully in the conception and execution of the experimental work, interpretation of the results and manuscript drafting.

This work was financially supported by the fellowship SFRH/BD/69195/2010 from the Fundação para a Ciência e Tecnologia, Portugal.

INDEX

FIGURE INDEX

TABLE INDEX

AGRADECIMENTOS

Em primeiro lugar, gostaria de agradecer ao meu orientador Doutor Sérgio Dias pela oportunidade que me concedeu em realizar o meu Doutoramento no seu laboratório. Obrigada pela dedicação, empenho e paciência demonstrados ao longo destes quatro anos. Agradeço-lhe o entusiasmo com que encara a Ciência, este "modo de vida", de tal modo expressivo que contagia os seus pupilos. Obrigada pelo *input* científico que contribuiu para a elaboração da minha tese.

Quero agradecer à minha co-orientadora Doutora Jacinta Serpa pela dedicação, paciência e empenho científico que demonstrou ao longo destes anos. Tenho ainda de agradecer a amizade que construímos, porque as relações profissionais podem quebrar a barreira e tornarem-se pessoais, o meu muito obrigada pela compreensão e amizade sempre demonstrada desde os primeiros meses em que nos conhecemos. Obrigada também pelos raspanetes :)

Quero agradecer à *Animal Facility*, *Imaging and Cytometry* e *Histopathology* do IGC pelo profissionalismo e pelo apoio técnico que me deram nas minhas experiências. Obrigada também ao *Bioimaging* e *Histology and Comparative Pathology Laboratory* do IMM pelo constante apoio e persistência demonstrados, de modo a me ajudar a ter o melhor *output* possível nas minhas experiências. Gostaria também de agradecer à Doutora Sandra Casimiro e ao Mário Maia-Matos pela amabilidade com que me ajudaram a dar um maior significado clínico ao meu estudo.

Obrigada a todos os meus colegas presentes e passados do CIPM e do SDias Lab nomeadamente, Ana Magalhães, Inês Matias, Inês Martins, Filipe, Ana Clemente, Sandrina, Ana Barros, Tânia, Ana Sofia, Leonor, Catarina, Francisco, Sofia e Fernanda. Um obrigada especial às minhas colegas que se tornaram minhas amigas para além do trabalho: Magalhó, Mátias, Coisi, Tani, Nori e Fernanda. Foi tão bom partilhar com vocês estes anos! Um agradecimento muito especial à Doutora Cat, por me ter "adotado" desde o início do seu Doutoramento e por me ter permitido partilhar o seu conhecimento. Obrigada também por seres uma grande amiga, com quem posso contar sempre e a qualquer hora!

Quero agradecer às minhas amigas, porque o meu caminho teria sido muito mais difícil sem o vosso carinho e apoio. Às Crubélias, porque já lá vão 10 anos e a nossa amizade amadureceu e permanecerá para sempre. À Marta, por seres mais velha e de tão parecidas que somos, vou sempre aprendendo com os nossos erros… afinal eu sou um *upgrade* teu :) À Cat, porque desde os primeiros momentos na Faculdade que aguentámos tudo juntas e assim se fortaleceu a nossa amizade. À Pé, porque é bom desabafar e mesmo não comunicando todos os dias, sabemos que estamos lá uma para a outra. À Pá, porque quando mais precisamos estamos sempre unidas para o que der e vier. À minha amiga Rita, que passados tantos anos e a tantos quilómetros de distância continuamos a ser as mesmas e a conversar uma tarde inteira sobre tudo e mais alguma coisa, sem restrições e sem julgamentos. À Joana, que me ensinou como é possível criar laços de amizade apenas num ano e que perduram para a vida inteira (e foram tão boas aquelas aulas de Biologia Animal). À minha Natachá pelas parvoíces que passámos juntas no CIPM e porque sempre me demonstraste uma amizade leal e sincera.

Quero agradecer à minha família pelo apoio e confiança incondicional que me deram sempre, mesmo quando, às vezes, tinha dúvidas senti sempre que me empurravam para o caminho certo. Obrigada por acreditarem sempre em mim e me terem dado asas para voar. Um agradecimento infinito ao meu irmão, porque não teria conseguido superar as dificuldades sem ti, sem o teu abraço para me felicitar e sem o teu ombro para chorar. És o melhor irmão que alguém pode desejar na vida, tudo teria perdido sentido se não estivesses aqui comigo. À minha cunhada por ser o porto de abrigo do meu irmão, sem o qual tudo teria sido mais difícil. À Margarida porque apesar de ser ainda uma criança, é o símbolo da felicidade na nossa família. Foste e és tu que moves a nossa família, és tu que nos fazes acreditar que estar aqui amanhã vai valer a pena! Ao meu pai, cujo último desejo se concretiza com a defesa desta tese. Sofro por não estares aqui comigo, mas acredito que te sentirias um pai realizado e feliz com este dia! Tenho que te agradecer eternamente por sempre teres acreditado na tua "menina dos caracóis". À minha mãe, cuja perseverança e força inabalável me inspirou para chegar aqui. Tenho a certeza que cada minuto valeu a pena. Tenho que te agradecer pelas palavras sabedoras que tens sempre para mim, pelo carinho, pelo amor e pela confiança inabalável que em mim depositas. Obrigada aos meus pais e irmão por me terem

ensinado que os caminhos difíceis não são para os fracos! *What doesn't kill you makes you stronger*!

Por último, quero agradecer ao Daniel, por acreditar em mim mesmo quando eu duvidava. Por teres estado sempre ao meu lado, por aguentares todas as contrariedades que a minha vida acarretou nos últimos anos, por seres sempre compreensível e prestável. Por me teres feito sempre acreditar que o dia de amanhã iria ser melhor que o de hoje. É muito raro termos a sorte de amar e de ter alguém ao nosso lado que aguente o que tu aguentaste. Obrigada por teres estado sempre ao meu lado, pois quem aguenta isto, aguenta tudo :)

LIST OF ABBREVIATIONS

2ME	2-methoxyestradiol
3'UTR	3′ untranslated region
5'UTR	5′ untranslated region
3D	tri-dimensional
αKDR	anti-KDR
AKT1	v-akt murine thymoma viral oncogene homolog 1
ANGPT	angiopoetin
AP1	adaptor-related protein complex 1
aPKC	atypical protein kinase C
ATP	adenosine triphosphate
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
BSA	bovine serum albumin
Ca^{2+}	calcium
CAM	cell adhesion molecule
CAV1	caveolin 1
CD	cluster of differentiation
CDH5	cadherin 5 type 2
cDNA	complementary DNA
CLDN5	claudin 5
CpG	cytosine phosphate guanine
CsA	cyclosporin A
CXCL	chemokine (C-X-C motif) ligand
CXCR4	chemokine (C-X-C motif) receptor 4
DAB2	Dab mitogen-responsive phosphoprotein homolog 2
DDR	discoidin domain receptor tyrosine kinase
DLL4	delta-like 4
DMEM	Dulbecco's modified eagle medium
E	embryonic development day
E2	estradiol
EC	endothelial cell
ECM	extracellular matrix
EE2	ethinylestradiol
ELK3	ELK3 ETS-domain protein
EMA	European medicines agency

eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cell
EPH	ephrin
EPHB2	ephrin receptor 2
EPHB4	ephrin receptor 4
ER	estrogen receptor
ERG	v-ets avian erythroblastosis virus E26 oncogene homolog
ESR1	estrogen-receptor 1
ETS	v-ets avian eryhtroblastosis virus E26 oncogene
ETS2	v-ets avian erythroblastosis virus E26 oncogene homolog 2
ETV2	ets variant 2
ETV6	ets variant 6
Exe	exemestane
F	fulvestrant
FAK	focal adhesion kinase
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FLI1	Fli-1 proto-oncogene, ETS transcription factor
FLT1	fms-related tyrosine kinase1
FLT4	fms-related tyrosine kinase 4
FOSB	FBJ murine osteosarcoma viral oncogene homolog B
FOX	forkhead box
GFI1	growth factor independent 1 transcription repressor
GFP	green fluorescent protein
γH2Ax	gamma H2A family, member X
GJA	gap junction protein
GJA1P1	gap junction protein alpha 1 43KDa pseudogene1
GJA4	gap junction protein alpha 4 37KDa
GJA5	gap junction protein alpha 5 40KDa
GM-CFS	granulocyte-macrophage colony-stimulating factor
GTPase	guanosine triphosphate hydrolase
H	histone
H3K36me3	histone H3 trimethylated at lysine 36
HAT	histone acetyltransferase
HBA1	hemoglobin alpha 1
HBE1	hemoglobin epsilon 1
HDAC	histone deacetylase

HE	hematoxylin & eosin
HEK-293T	human embryonic kidney 293 T antigen
HER2	human epidermal growth factor receptor 2
HEY2	hes-related family bHLH transcription factor With YRPW motif 2
HGB2	hemoglobin gamma G
HIF1A	hypoxia-inducible factor 1, subunit alpha
HMT	histone methyltransferase
HMVEC	human dermal microvascular ECs
HOX	homeobox
HPVEC	human pulmonary valve ECs
HRP	horseradish peroxidase
HS	heparan sulfate
HSC	hematopoietic stem cell
HSM-CHLN	Hospital Santa Maria – Centro Hospitalar Lisboa Norte
HSPG	heparan sulfate proteoglycan
HUVEC	human umbilical cord vein ECs
ICAM1	intracellular adhesion molecule 1
ID	inhibitor of DNA binding
IgG	Immunoglobulin G
IHH	indian hedgehog
ILK	integrin-linked kinase
INPP5D	inositol polyphosphate-5-phosphatase 145KDa
IPOLFG	Instituto Português de Oncologia de Lisboa Francisco Gentil
IRES	internal ribosome entry site
ITAM	immunoregulatory tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitor motif
ITV	intratumoral vessel
JAG1	jagged 1
JAM	junctional adhesion molecule
JUN	jun proto-oncogene
KD	knockdown
KDM8	lysine (K)-specific demethylase 8
KDR	kinase insert domain receptor
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
KLF	Krüppel-like factor
KO	knockout
Luc	firefly luciferase
MAGUK	membrane-associated guanylate kinase

MAPK	mitogen-activated protein kinase
MBD	methyl-CpG binding domain protein
MG	mammary gland
miRNA	microRNA
MMP	matrix metalloproteinase
MYC	v-myc avian myelocytomatosis viral oncogene homolog
NAD+	nicotinamide adenine dinucleotide
N-cadherin	neuronal cadherin
ncRNA	non-coding RNA
NFATC	nuclear factor of activated T-cells
NFATC1	nuclear factor of activated T-cells cytoplasmic calcineurin-dependent
NGV	normal mammary gland vessel
NOS3	nitric oxide synthase 3
NR	nuclear receptor
NR2F2	nuclear receptor subfamily 2 group F member 2
NRP	neuropilin
OS	overall survival
ox-LDL	oxidized low density lipoprotein
PARD3	par-3 family cell polarity regulator
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PECAM1	platelet/endothelial cell adhesion molecule1
PFS	progression-free survival
PGF	placental growth factor
PgR	progesterone receptor
PIK3CA	phosphatidylinositol-4,5biphosphate 3-kinase catalytic subunit alpha
PLAU	plasminogen activator urokinase
PLC	phospholipase C
PLCG1	phospholipase C gamma 1
poly-HEMA	poly(2-hydroxylethyl methacrylate)
POU5F1	POU class 5 homeobox 1
PPARG	peroxisome proliferator-activated receptor gamma
PROX1	prospero homeobox 1
PTGS2	prostaglandin G/H synthase and cyclooxygenase
PTP	protein tyrosine phosphatase

PTPN1	protein tyrosine phosphatase non-receptor type 1
PTPN6	protein tyrosine phosphatase non-receptor type 6
PTPN11	protein tyrosine phosphatase non-receptor type 11
PTPRB	protein tyrosine phosphatase receptor type B
PTPRJ	protein tyrosine phosphatase receptor type J
PTV	peritumoral vessel
RA	retinoic acid
RAP1B	RAP1B, member of RAS oncogene family
RB1	retinoblastoma 1
ROS	reactive oxygen species
RPMI	Roswell Park memorial institute
rRNA	ribosomal RNA
RT-PCR	reverse transcription PCR
RUNX	runt-related transcription factor
SEA	S13 erythroblastosis oncogene homolog
SETD2	SET domain containing 2
sFLT1	soluble fms-related tyrosine kinase 1
SFRP2	secreted frizzled-related protein 2
shRNA	small hairpin RNA
siRNA	small interfering RNA
SIRT	sirtuin
sKDR	solube kinase insert domain receptor
SMAD2	SMAD family member 2
SP1	sp1 transcription factor
SPI1	Spi-1 proto-oncogene
SRC	SRC proto-oncogene, non-receptor tyrosine kinase
SOX	SRY(sex determining region Y) box
STAT3	signal transducer and activator of transcription 3
SUMO	small ubiquitin-like modifier
Tam	tamoxifen
TAM	tumor-associated macrophage
TEM	TIE2 expressing monocytes/macrophage
TGF	transforming growth factor
TGFB	transforming growth factor beta
TGFBR	transforming growth factor beta receptor
Th1	type 1 T helper
THBS1	thrombospondin 1
TIE	tyrosine kinase with immunoglobulin-like and EGF-like domains

TIMP	TIMP metalloproteinase inhibitor
TJP1	tight junction protein 1
TKI	tyrosine kinase inhibitor
TMCI	tumor mononuclear cell infiltrate
TNFSF11	tumor necrosis factor superfamily, member 11
TP53	tumor protein 53
VCAM1	vascular cell adhesion molecule 1
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VVO	vesiculo-vacuolar organelle
VWF	von Willebrand factor

NOTE: all the genes' names are represented accordingly to HUGO Gene Nomenclature Committee and Mouse Genome Informatics

RESUMO

As células endoteliais definem e delineiam todo o sistema vascular, sendo parte integral do lúmen dos vasos que o constituem. As células endoteliais exercem inúmeras funções na manutenção da homeostasia do organismo, nomeadamente na regulação do transporte de fluidos, nutrientes e hormonas. Para além disso, permitem também a circulação de células; modulam o tónus vascular e a corrente sanguínea; controlam a adesão das plaquetas e regulam a resposta imune e inflamatória pelo controlo das interações entre as células do sistema imunitário e a parede dos vasos sanguíneos. Qualquer perturbação neste sistema complexo pode promover o surgimento de patologia como, por exemplo, aterosclerose, doenças isquémicas, respostas inflamatórias deficitárias e várias neoplasias malignas (cancro).

As células endoteliais detêm inúmeras características e propriedades, nomeadamente: a resistência à força mecânica a que estão sujeitas devido ao fluxo da corrente sanguínea; a reduzida taxa de divisão celular; e a capacidade de regular a permeabilidade vascular por mecanismos de adesão célula a célula. Porém, a característica que diferencia as células endoteliais das demais é a sua capacidade de comandar a formação de novos vasos, um processo denominado *sprouting*.

As células endoteliais têm a capacidade de responder a determinados fatores de crescimento, nomeadamente ao fator de crescimento vascular endotelial (VEGF), através da interação com os seus recetores específicos. Esta interação desencadeia, na célula endotelial, cascatas de tradução de sinal, que podem ativar inúmeros processos celulares, desde mecanismos de sobrevivência que permitem a adaptação da célula a diferentes microambientes até aos vários processos que levam à morte celular.

A adaptação celular em resposta a diferentes estímulos poderá ser feita ao nível da regulação transcricional de genes, que é mediada pela ação de fatores de transcrição e/ou por modificações epigenéticas. Esta regulação é crucial para a diferenciação e adaptação das células endoteliais às várias condições fisiológicas e fisiopatológicas. Os fatores de transcrição ligam-se a sequências específicas ou a elementos que se encontram principalmente na região regulatória 5´ (5′ *untranslated region* - UTR), muitas

vezes designada como região promotora, promovendo o recrutamento da maquinaria de transcrição onde se inclui a RNA polimerase. O fator nuclear de células T ativadas dependente da calcineurina (NFATC1) é um fator de transcrição intimamente relacionado com a diferenciação e função das células endoteliais, dado que o ratinho *knockout* (KO) para este fator morre durante o desenvolvimento embrionário devido a defeitos vasculares. Do mecanismo de ação do NFATC1, é sabido que este é desfosforilado no citoplasma pela calcineurina, o que promove a sua translocação para o núcleo, onde irá ligar-se a outos fatores de transcrição e controlar a expressão génica. A desfosforilação do NFATC1 poderá ser bloqueada pela ciclosporina A (CsA), um imunossupressor, que bloqueia diretamente a calcineurina, impedindo assim a translocação do NFATC1 para o núcleo. O VEGF tem sido indicado em inúmeros modelos *in vitro* e *in vivo*, como responsável pelo aumento da translocação do NFATC1 para o núcleo.

Por outro lado, a transcrição é também regulada por modificações epigenéticas que poderão ser mecanismos de metilação do DNA, modificações de histonas e mecanismos modulados por moléculas de RNA não codificantes (ncRNA). Recentemente a metiltransferase associada à trimetilação da histona 3 na lisina que ocupa a posição 36 (H3K36me3), denominada domínio SET contendo 2 (SETD2), foi implicada na definição do fenótipo vascular. Ratinhos KO para esta proteína morrem devido a defeitos vasculares. Esta metiltransferase tem sido muito estudada no contexto tumoral e inclusivamente está descrita a associação de algumas mutações a vários tipos de cancro. No entanto, a nível da célula endotelial pouco se sabe sobre o seu papel.

Nesta tese procurámos explorar o papel que o ambiente tumoral (neoplasias malignas) exerce sobre as células endoteliais. Sabe-se que o endotélio neste ambiente adquire características morfológicas e fisiopatológicas distintas das que possui em condições fisiológicas. Os vasos no contexto do cancro são descritos como sendo mais tortuosos, permeáveis, dilatados e desorganizados. Já são conhecidos alguns fatores e vias de sinalização que estão envolvidos neste fenótipo, como é o caso da hipoxia e da via NOTCH.

Os fármacos anti-angiogénicos em doentes com cancro têm sido utilizados com dois objetivos: o de impedir o crescimento de vasos no tumor e o de promover a estabilização dos vasos por forma a aumentar a eficácia da quimioterapia. No entanto, os

ensaios clínicos realizados com o intuito de testar a eficácia terapêutica deste tipo de agentes demonstraram pouco ou nenhum efeito destes fármacos na sobrevida e/ou progressão livre da doença em doentes oncológicos (com diferentes tipos de tumor). Torna-se assim necessário compreender por um lado os mecanismos moleculares que expliquem a aparente "resistência" às terapias anti-angiogénicas, e por outro lado, descobrir novas estratégias para bloquear a angiogénese tumoral.

Por estas razões, a hipótese geral desta tese é que a regulação transcricional das células endoteliais, mediada especificamente pelo NFATC1 e pela SETD2, está diretamente envolvida na regulação da homeostasia das células endoteliais e na sua capacidade de diferenciação/adaptação a ambientes tumorais.

Assim, um dos primeiros objetivos desta tese foi caracterizar o papel do fator de transcrição NFATC1 no ambiente tumoral. Sendo o NFATC1 um fator de transcrição a jusante da ação do VEGF foi colocada a hipótese de este ser responsável pela modulação do ambiente tumoral por forma a explicar a falha de resposta dos doentes à terapia anti-angiogénica.

Os nossos dados, quer em modelos animais quer em doentes de cancro da mama, mostram que a expressão do NFATC1 é perdida pelas células endoteliais em ambiente tumoral. Por outro lado, a expressão deste fator está aumentada nos infiltrados de células mononucleares no tumor (TMCI), apresentando uma correlação positiva com a agressividade dos tumores em doentes com cancro da mama. Ao nível mecanístico, os nossos dados demonstram que o recetor 2 do VEGF, também conhecido como recetor do domínio de inserção da cinase (KDR), medeia a translocação nuclear do NFATC1 induzida pelo VEGF. Mostrámos também que o inibidor do KDR e a CsA cooperam na inibição desta translocação, tendo um efeito cumulativo.

Os nossos resultados indicam que a expressão e função do NFATC1 são moduladas pelo ambiente tumoral, o que poderá alterar a sinalização do VEGF e consequentemente estar na base da fraca resposta à terapia anti-angiogénica dos doentes com cancro da mama.

Seguidamente foi colocada a hipótese da SETD2 estar envolvida na integridade do fenótipo vascular, dado que o ratinho KO para esta metiltransferase morre devido a

defeitos no sistema vascular. Os nossos dados mostram que a diminuição dos níveis da SETD2 induz o aumento do número de células binucleadas, provavelmente devido à afetação do ciclo celular (ausência de citocinese) e à ativação de mecanismos de fusão entre células. Dado o nosso interesse na sinalização do VEGF e sua importância na angiogénese tumoral, também foi avaliada a regulação epigenética do NFATC1 pela assinatura H3K36me3, mediada pela SETD2. Os nossos dados demonstram que quando os níveis da SETD2 estão diminuídos, a translocação do NFATC1 para o núcleo é aumentada, de um modo independente do VEGF. Isto mostra que a SETD2 interfere com a dinâmica do NFATC1, revelando uma ligação entre os dois mecanismos de regulação transcricional, mediada por fatores de transcrição epigenética.

Nesta tese avaliamos também a capacidade anti-angiogénica de alguns derivados do estrogénio (estradiol, etinilestradiol, fulvestrant, 2-metoxiestradiol, tamoxifen e exemestane). Sabe-se que alguns destes compostos são usados como terapia adjuvante em doentes de cancro da mama. Assim, colocámos a hipótese dos derivados do estrogénio terem capacidade anti-angiogénica devido tanto ao impedimento de formação da rede endotelial como à destruição da rede previamente estabelecida.

Os nossos dados indicam que o fulvestrant, o 2-metoxiestradiol e o tamoxifen são capazes de inibir a formação da rede endotelial e de destruir a rede endotelial já estabelecida. Assim, estes resultados apontam para que estes anti-estrogénios tenham maior capacidade anti-angiogénica.

Por fim, ao longo desta tese foram desenvolvidas ferramentas importantes, quer para a obtenção dos dados que foram gerados ao longo do estudo, como para experiências futuras. Foram geradas linhas celulares proteína verde fluorescente (GFP) – luciferase (Luc) que nos permitiu acompanhar o crescimento tumoral, tanto a nível da fluorescência, como a nível da luminescência. Pelo facto do laser ter uma maior penetrância no tecido, a microscopia multifotão permitiu acompanhar a estrutura e ligação dos vasos sanguíneos com a arquitetura do tumor, sem necessidade de processar o órgão com fixadores orgânicos. Foram também geradas ferramentas *in vitro*, nomeadamente a otimização do processo de obtenção de esferóides tumorais que, por terem uma estrutura tridimensional, nos permitem estudar com mais rigor as interações

com e das células endoteliais. O ensaio de formação de tubos em Matrigel® revelou-se uma técnica útil para o teste de fármacos anti-angiogénicos.

Em suma, os nossos resultados mostram a importância de um controlo rigoroso da regulação transcricional, que se traduz em alterações da função das células endoteliais. O NFATC1 e a SETD2 são elementos importantes nesta regulação, podendo ser responsáveis, respetivamente, pela fraca resposta à terapia anti-angiogénica e pelos fenótipos anormais das células endoteliais. Os nossos resultados abrem o caminho para estudos futuros mais detalhados da função destes fatores em amostras de tumores e na resposta de doentes a fármacos com atividade anti-angiogénica.

Palavras-chave: Célula endotelial, cancro da mama, regulação transcricional, NFATC1, SETD2, derivados do estrogénio

NOTA: os textos em Português estão escritos de acordo com o novo acordo ortográfico

ABSTRACT

The vascular endothelium lines the entire vascular system, playing multiple functions namely fluid, nutrient and cell transport, modulation of the vascular tone, as well as regulation of immune and inflammatory responses. Any disturbance to this complex system may contribute towards the emergence of pathologies, such as ischemic diseases, atherosclerosis, altered immune response and even cancer.

Endothelial cells (ECs) have some specific features such as reduced cell division, the capacity to regulate vascular permeability due to cell-cell adhesion mechanisms and in response to environmental cues have the capacity to form new vascular structures. ECs respond to particular growth factors, of which one of the most important and most studied vascular endothelial growth factor (VEGF).

Transcriptional regulation controls and supervises gene expression. Transcription factors and epigenetic modifications are the main players responsible for this regulation. There are innumerous transcription factors known to be involved in EC function and differentiation. In this context nuclear factor of activated T-cells cytoplasmic calcineurin-dependent (NFATC1) is of particular relevance since it is a VEGF-responsive transcription factor. NFATC1 deficiency was shown to result in vascular defects (as observed in knockout (KO) mice). More recently, epigenetic modifications raised interest in the scientific community because it was shown that they are implicated in the regulation of EC phenotypes. In particular, SET domain containing 2 (SETD2), which is responsible for the trimethylation of H3K36, was shown to be involved in vascular remodeling. Based on this data, the general hypothesis of this thesis is that transcriptional regulation, namely NFATC1 and SETD2 are key regulators of EC homeostasis, being involved in their differentiation and adaptation to tumoral environment.

Given the lack of response to anti-VEGF therapies in breast cancer patients, one of our goals was to unravel the role of the NFATC1 transcription factor in modulation of tumor microenvironment and in VEGF signaling decrease in endothelial tumor cells. Our data shows that NFATC1 expression is lost in ECs from tumor areas both in human and mice models. This suggests that the regulation of expression and function of NFATC1 could be critical for the anti-VEGF therapy success in breast cancer patients.

In this thesis we also addressed the role of SETD2 in vascular impaired growth. With the knockdown (KD) of *SETD2*, ECs show an abnormal phenotype due to increase in cell cycle errors and cell-cell fusion. *SETD2* KD also increases NFATC1 nuclear translocation in a VEGF-independent manner. Thus, our data suggests a new VEGF-independent mechanism for the control of NFATC1 dynamics at transcriptional and trafficking level.

Our last goal was to define the role of estrogen derivatives in angiogenesis. Our data shows that fulvestrant (F), 2-methoxyestradiol (2ME) and tamoxifen (Tam) are the major anti-estrogens drugs able to inhibit EC network formation and to disrupt established networks.

In addition, this PhD thesis was important for the generation of useful tools for endothelial/tumor imaging, such as green fluorescent protein (GFP) - firefly luciferase (Luc) cell lines, multi-photon microscopy and tumor spheroids. It was also important for the generation of Matrigel® tube-formation assays, which revealed to be useful for large-scale anti-angiogenic drug tests.

Taken together, our results underline the importance of a strict control in EC transcriptional regulation, which translates into changes in EC function. NFATC1 and SETD2 are key players in this regulation, and may be responsible for the lack of response to anti-VEGF therapies and EC abnormal phenotypes respectively. Our studies pave the way for detailed analysis of the function of these factors in human cancer samples and in the response of cancer patients to anti-angiogenic drugs.

Keywords: Endothelial cell, breast cancer, transcriptional regulation, NFATC1, SETD2, estrogen derivatives

Chapter 1

GENERAL INTRODUCTION

1. The origin of endothelial cells and vascular system
2. Biology of endothelial cells : signaling pathways
3. Endothelial cell properties
4. Transcriptional regulation on endothelial cells
5. The involvement of endothelial cells in cancer
6. Novel insights and aim of this thesis

1. The origin of endothelial cells and vascular system

The "vascular" endothelium lines the entire vascular system and is composed of a monolayer that consists of approximately 6×10^{13} endothelial cells (ECs). The vascular endothelium has multiple functions including the transport of fluids, nutrients, circulation of cells, hormones, modulation of vascular tone and blood flow, platelet adherence and regulation of immune and inflammatory responses by controlling the immune cell interactions with the vessel wall. Disturbances in endothelial homeostasis may contribute towards the onset of pathologies, such as atherosclerosis, ischemic diseases, altered inflammatory and immune response and cancer.[1-7]

More than a century ago His[8] and Sabin[9] postulated that the hemangioblast is the common precursor for the hematopoietic and vascular lineages. The development of this precursor is thought to arise in some vascular beds such as the dorsal aorta, the viteline artery, the aorto-gonad-mesonephros region and the placenta. The hemangioblast is not well characterized but we now know that there are some signals involved in the differentiation of the vascular and hematopoietic lineages, such as hedgehogs transcription factors (specifically indian hedgehog – IHH), retinoic acid (RA), fibroblast growth factors (FGFs), bone morphogenetic protein (BMP) 4, vascular endothelial growth factor (VEGF) A and its receptors vascular endothelial growth factor receptors (VEGFRs), ets variant 2 (ETV2), between others.[1,2,5,10]

Although the extra-embryonic yolk sac is considered the first site of blood formation, its contribution to hematopoiesis is considered "primitive", because these cells require conditioning and the definitive hematopoietic program appears later on the initial hemangioblast stage. So, it seems that the primitive hematopoietic stem cells (HSCs) migrate to fetal liver to differentiate into definitive HSCs.[11] It has become clear over the past few years that HSCs in "definitive hematopoiesis" arise from specialized vascular ECs that acquire blood-forming potential (hemogenic endothelium).[12,13] Hirschi[14] postulated that even blood cells referred to as "hemangioblast-derived" have recently been proposed to be produced via an endothelial-intermediate. Definitive hematopoiesis cannot occur in the absence of EC development. Recently, Sandler *et al.*[15] identified four transcription factors— FBJ murine osteosarcoma viral oncogene homolog B (FOSB), growth factor independent 1 transcription repressor (GFI1), runt-related transcription factor 1 (RUNX1) and Spi-1 proto-oncogene (SPI1) capable of

reprogramming ECs into hematopoietic cells with long-term primary and secondary multilineage engraftment in immunodeficient mice. These experiments support the idea that ECs are "parent" cells from the hematopoietic lineage. Figure 1.1 summarizes the primordial ECs development into hematopoietic and vascular lineages.

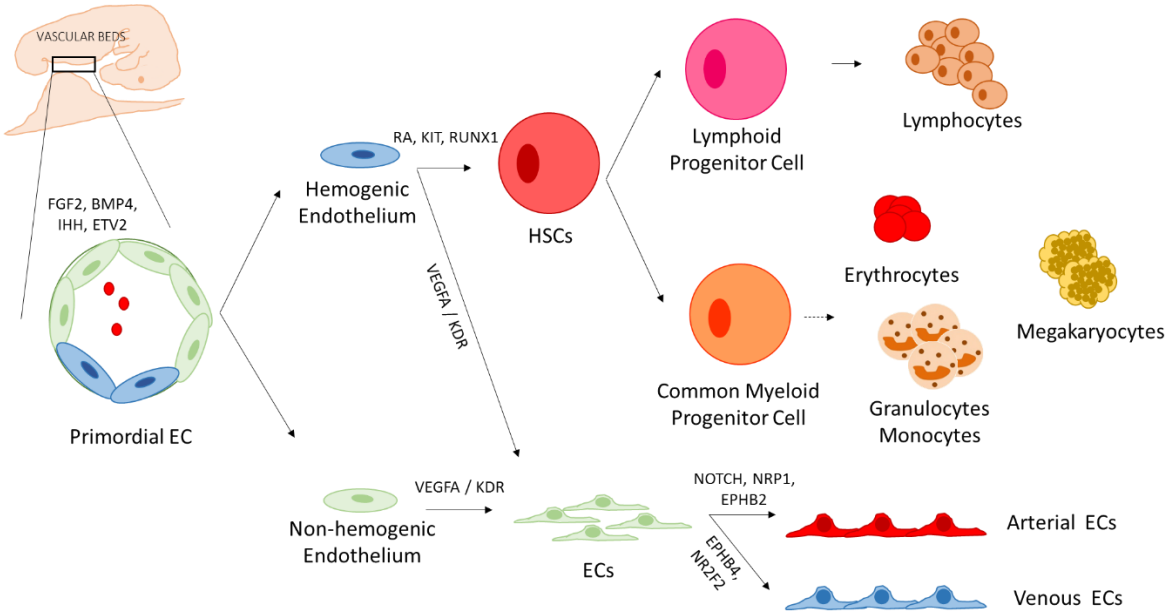


Figure 1.1 – The origin of endothelial cells. During the development in vascular beds arises the primordial ECs with primordial circulating erythrocytes. This precursor has a hemogenic endothelium which in response to some factors such as RA, v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT) and RUNX1 can differentiate into HSCs. HSCs differentiate into lymphoid and myeloid precursors, which in turn leads to mature blood cells. On the other hand, mature ECs can arise from hemogenic and non-hemogenic endothelium from the primordial EC, in response to VEGFA/ kinase insert domain receptor (KDR) signaling. Mature ECs differentiate into arterial or venous ECs in response to NOTCH, neuropilin (NRP) 1 and ephrin (EPH) stimuli: NOTCH, NRP1 and ephrin receptor 2 (EPHB2) are responsible for arterial differentiation. Ephrin receptor 4 (EPHB4) and nuclear receptor subfamily 2 group F member 2 (NR2F2) (that represses NOTCH signaling) are responsible for venous differentiation.

It is accepted that *de novo* formation of embryonic blood vessels, termed vasculogenesis, involves the differentiation, migration and coalescence of the endothelial progenitor cells (EPCs). The only exceptions are the dorsal aorta and the cardinal vein which are directly formed through the assembly of EPCs. This process is immediately followed by differentiation into arterial or venous vessels in response to a combination of hemodynamic stimuli and corresponding genetic factors.[5,6]

Arterial and venous ECs possess specific molecular identities. Some of the proteins involved are NOTCH, EPH, VEGFA, NRP and delta-like 4 (DLL4). NOTCH components are highly expressed in arteries but less in veins. Disruption of NOTCH signaling causes loss of arterial markers and re-expression of venous signature, which suggests that NOTCH promotes arterial specification by venous identity repression.[16] NOTCH also controls EPH family members: EPHB2 expression in arterial ECs increases in response to NOTCH, whereas its receptor EPHB4 in venous ECs is repressed by NOTCH. In mice, VEGFA secreted by nerves contributes to arterial differentiation of ECs and NRP1, a VEGF receptor, facilitates transduction of arterial effects of VEGFA. The transcription factors forkhead box (FOX) C1 and C2 also drive an arterial gene signature (e.g., DLL4, hes-related family bHLH transcription factor with YRPW motif 2 (HEY2), chemokine (C-X-C motif) receptor 4 (CXCR4)) by interacting with VEGFA and NOTCH signaling. The venous identity requires repression of NOTCH signaling by the vein-specific nuclear receptor NR2F2.[2,16,17]

During embryonic development and after birth the blood vessels can grow by three different mechanisms: vasculogenesis, sprouting and intussuspective angiogenesis. Usually the term angiogenesis encompasses the three mechanisms but they are quite different. While vasculogenesis involves EPCs recruitment, sprouting consists in the formation of new capillaries from the pre-existing vessels. Sprouting was first described by Clark[18] and it is a multistep process that requires the tight control and coordination of ECs behavior: a) ECs lose their cell–cell contacts in response to pro-angiogenic factors; b) degradation of the surrounding extracellular matrix (ECM) by activation of matrix metalloproteinase (MMPs); c) ECs acquire invasive and motile behavior to initiate new blood vessel sprouting.[4-6,16] This mechanism will be addressed with further detail later in the section 3.

Intussusceptive angiogenesis was first observed in postnatal remodeling of capillaries in the lung[19] and consists in the splitting of preexisting vessels in two new vessels by the formation of a transvascular tissue pillar into the lumen of the vessel. In this process ECs are remodeled by increasing in volume and becoming thinner, which does not involve the proliferation of ECs.[6,20]

After birth the normal vasculature becomes quiescent. Angiogenesis is turned on transiently in some physiological processes such as wound healing and the female reproductive cycle. In contrast, during tumor progression, angiogenesis is almost always activated, which causes continuous sprouting of vessels that help sustain tumor growth.[21]

2. Biology of endothelial cells: signaling pathways

As mentioned above, it is now well recognized that ECs have some specific features, such as their capacity to respond to particular growth factors (VEGFA, FGF, transforming growth factor (TGF), angiopoietin (ANGPT), platelet-derived growth factor (PDGF)), due to the presence of specific membrane receptors (VEGFR, NRP, fibroblast growth factor receptor (FGFR) transforming growth factor beta receptor (TGFBR), platelet-derived growth factor receptor (PDGFR), tyrosine kinase with immunoglobulin-like and EGF-like domains (TIE)) and ECM components. A scheme of all of these ECs components is presented in Figure 1.2.

2.1. Vascular endothelial growth factor and their receptors

2.1.1. Vascular endothelial growth factor

VEGFA was first described as a vascular permeability factor by Senger[22] and it is so important for EC survival that even a 50% reduction (heterozygotes) results in embryonic lethality. Interestingly, excess of VEGFA (by only 2-fold) results in embryonic lethality, which supports the significance of VEGFA dosage during development.[23-25]

Currently the VEGF family is thought to be comprised of six structurally related factors: VEGFA, VEGFB, VEGFC, VEGFD, VEGFE and placenta growth factor (PGF). The VEGF family members are homodimeric polypeptides and their ability to bind to VEGFRs, heparan sulfate (HS) and NRPs is very wide and has different affinities.[26-29]

As explained by Koch and Claesson-Welsh[26], binding of VEGFA to its VEGFRs in *cis* or *trans* geometric forms induces receptor homo- or heterodimerization. The consequent change in receptor conformation leads to exposure of the adenosine triphosphate (ATP)-binding site in the intracellular kinase domain, kinase activation and auto- or trans-phosphorylation of tyrosine residues on the receptor dimer itself as well as on downstream signal transducers. Tyrosine phosphorylation is strictly regulated by internalization and degradation or by dephosphorylation through protein tyrosine phosphatases (PTPs) such as receptor type J (PTPRJ), receptor type B (PTPRB), non-receptor type 1 (PTPN1) and non-receptor type 11 (PTPN11). Phosphorylated tyrosine residues create binding sites for intracellular signaling mediators, allowing formation of signaling chains. The activation of these cascades results in the establishment of biological responses such as cell proliferation, migration, survival and permeability.[26,27,29]

VEGFs and VEGFR members are regulated by certain transcription factors such as the v-ets avian eryhtroblastosis virus E26 oncogene (ETS) family and metabolic regulators like reactive oxygen species (ROS) and hypoxia-inducible factor 1, alpha subunit (HIF1A). Especially HIF1A is upregulated during tissue growth both in healthy conditions (wound healing and embryonic development) as well as in disease (retinopathies, arthritis and cancer).[26] This endothelial transcriptional regulation will be addressed later in section 4.

VEGFA is mostly produced by parenchymal cells and acts in a paracrine manner on adjacent ECs. However, autocrine VEGFA has been proposed to be essential for EC survival and homeostasis.[6] Lee *et al.*[30] demonstrated that paracrine VEGFA signaling is essential for the angiogenic cascade, proliferation, survival, permeability and endothelial differentiation. In contrast, autocrine VEGFA signaling only supports endothelium viability upon survival signals like irradiation, hypoxia and ROS. The paracrine and autocrine activations are mediated mainly by VEGFR2/KDR.[31]

2.1.2. Vascular endothelial growth factor receptors

VEGFR 1/2/3 are receptor tyrosine kinases that play crucial roles in blood and lymphatic vessel development and homeostasis. VEGFR1 is also known as fms-related tyrosine kinase1 (FLT1), VEGFR2 as KDR and VEGFR3 as fms-related tyrosine kinase 4 (FLT4). They are composed by seven monomeric immunoglobulin-like domains and a dimeric intracellular kinase domain. They are activated by six different VEGFs (VEGFA, B, C, D, E and PGF) with different affinities. The VEGFRs dynamically associate with coreceptors such as NRP or EPHB2 that guide receptors through the cell and promote their downstream signaling pathways. The VEGFA/KDR signaling complex is a major regulator and is required for almost every aspect of blood vessel formation and function.[6,32]

Although FLT1 is not believed to be essential for EC function, it plays an important negative role in controlling adequate KDR-mediated signaling, by binding VEGF A/B/PGF with higher affinity. It is also important in regulating monocyte migration during inflammation. FLT1 exists as a full-length form and an alternatively spliced, soluble form (sFLT1) described by Kendall *et al.*[26,33,34] It was shown that sFLT1 is expressed in a regulated manner in the placenta during gestation with the purpose of capturing VEGFA to control KDR signaling.[35] Therefore, sFLT1 has been implicated as a negative regulator

of angiogenesis – its binding to VEGFA may prevent activation of KDR. Some pathological conditions have been implicated with FLT1 defects, such as certain inflammatory conditions which may involve the recruitment of bone marrow–derived myeloid cells.[26]

KDR is expressed mainly in vascular EC and binds VEGFA/C/D/E. VEGFA binds to KDR with a 10-fold lower affinity than to FLT1.[27,34] KDR also have an alternative spliced form, soluble KDR (sKDR), which is present in various tissues such as skin, heart, spleen, kidney, ovaries and plasma. It binds to VEGFC and prevents binding to FLT4, which inhibits lymphatic EC proliferation.[26,36,37] It was shown recently that the overexpression of sKDR driven by hypoxia promoter controls angiogenesis and tumor growth.[38]

KDR can localize to the plasma membrane or in endosomal vesicles. VEGFA can induce homo- or heterodimerization of this receptor and FLT1 or FLT4, which promotes activation of these receptors through transphosphorylation, leading to the initiation of intracellular signaling pathways. After stimulation with VEGFA the homo- or heterodimers dissociate which leads to KDR internalization. A portion of the receptor localizes in membrane clusters with caveolin.[32] On binding of VEGFA, its receptor, KDR, transmits signals through several downstream molecules such as, mitogen-activated protein kinase 1 (MAPK1 or ERK1), MAPK14 (or p38), phospholipase C (PLC) and phosphatidylinositol-4,5biphosphate 3-kinase catalytic subunit alpha (PIK3CA) / v-akt murine thymoma viral oncogene homolog 1 (AKT1).[34,39]

EPHB2, a transmembrane ligand for EPH receptors and a key regulator for arterial differentiation, is required for embryogenesis, vasculogenesis, angiogenesis and lymphangiogenesis. Some recent studies highlighted that EPHB2 and its receptor EPHB4 induce KDR and FLT4 internalization without downstream signal activation.[40,41] Nakayama *et al.*[42] propose that the differential regulation of VEGFR endocytosis happens through EPHB2, Dab mitogen-responsive phosphoprotein homolog 2 (DAB2), par-3 family cell polarity regulator (PARD3) and atypical protein kinase C (aPKC) which helps to assign specific tasks to ECs. KDR can also be inactivated by dephosphorylation mediated by PTPs or degradation involving ubiquitin ligases.[32]

FLT4 binds to VEGFC/D and is a critical regulator of lymphatic ECs function. FLT4 is also expressed in embryonic vascular ECs and becomes upregulated during active angiogenesis. Recently, it was shown in a retina model by Benedito *et al.*[43] that NOTCH can upregulate FLT4 and promote angiogenesis. FLT4 forms homodimers as well as

heterodimers with KDR. Loss-of-function mutations in *FLT4* in humans cause lymphedema, whose phenotype indicates that FLT4 signal transduction is critical in regulation of lymphatic vessel function.[26,34]

2.1.3. Neuropilins

NRPs were initially identified as receptors for class-3 semaphorins involved in axon guidance, although it was found that neuropilins also interact with VEGFA.[44] Neuropilins were implicated in vascular development and in pathological angiogenesis. There are two NRPs, NRP1 which is preferentially expressed in arteries and NRP2 in veins and lymphatic vessels.[45] They are composed of an extracellular domain with binding motifs for VEGFA and semaphorins and a cytoplasmic domain of 35 amino acid residues with a PDZ-domain binding motif S13 erythroblastosis oncogene homolog (SEA). This PDZ-binding domain is critical in internalization of NRPs and have been implicated in trafficking of KDR to early endosomes. NRP1 modulates VEGFR signaling leading to enhanced migration and survival of ECs. NRP1 appears to act primarily as a coreceptor for FLT1/KDR, whereas NRP2 is a coreceptor for FLT4. Although the exact mechanism of NRPs in the signaling downstream is not yet resolved.[26,46]

It has been shown that neutralizing NRP1 could have a therapeutic potential, blocking tumor growth in mice in an anti-VEGFA additive manner. Koch *et al.*[46] have shown recently that NRP1 modulates KDR with opposite effects when the molecules are expressed in the same cell (*cis*) or in adjacent cells (*trans*), through regulation of KDR internalization and intracellular trafficking. With NRP1 being expressed in the same cell as KDR, it may promote angiogenesis. At the opposite, if NRP1 is expressed in adjacent cells of KDR, NRP1 has an anti-angiogenic effect.[46]

2.2. Angiopoietin / tyrosine kinase with immunoglobulin-like and EGF-like domains

TIE receptors and their ligands (ANGPT1–4) are master regulators of vascular maturation and quiescence. The ANGPT and the TIE receptor tyrosine kinases, together with the VEGFs and their receptors form the two signaling pathways that are almost exclusively EC specific. ANGPT1 secretion from perivascular cells is important for maintaining vascular stability and EC adhesion, while inhibiting vascular permeability. ANGPT2 is expressed by EC and stored in EC secretory granules called Weibel-Palade

bodies and is released locally in response to inflammatory stimuli. Their expression is increased in hypoxic and tumor blood vessels and is particularly high in the specialized tip cells. The TIE receptors are expressed in the blood, the lymphatic endothelium and in certain hematopoietic cells, such as the TIE2 expressing monocytes/macrophages (TEMs). TIE1 expression is regulated by blood flow and shear stress. It increases during wound healing and in tumor angiogenesis, suggesting a function during tissue neovascularization.[6,47]

ANGPT ligands assemble into unique multimeric structures, however TIE2 activation requires a tetrameric or higher order multimer. This suggests that the active TIE receptor complex is composed of several receptor units, unlike VEGFRs which are activated by dimeric growth factors. Upon binding of ANGPT1, TIE2 is constitutively phosphorylated. ANGPT1/TIE2 signaling has been shown to inhibit VEGFA-mediated vascular permeability via several downstream signaling cascades. ANGPT1 also binds to ECM of cultured ECs and promotes TIE2 localization to the basal plasma membrane, resulting in activation of the MAPK 1/2 pathway.[47]

Endothelial-derived ANGPT2 is considered to be the natural antagonist of ANGPT1 activity by inhibiting phosphorylation of TIE2. Thus, ANGPT2 sensitizes the endothelium to both growth factors and inflammatory mediators, which increase vascular destabilization. Elevated levels of ANGPT2 are associated with some diseases, such as acute liver, lung or kidney injury, sepsis, disseminated intravascular coagulation, post-cardiac arrest syndrome, sepsis and with some cancers (leukemia, multiple myeloma, breast cancer and melanoma). *TIE2* somatic mutations are associated with venous malformations, which is characterized by enlarged venous-like channels with patchy smooth muscle cell layering.[47,48] Although both ANGPT1 and ANGPT2 mediate TIE2 clustering at cell-cell contacts, their differential signaling may explain their opposing effects on vascular stability.[48]

2.3. Transforming growth factor beta / transforming growth factor beta receptor

Transforming growth factor β (TGFB) is one of the best known members of a large family of secreted pleiotrophic growth factors, composed by the BMPs and activins. Many of these cytokines have vital roles in numerous processes during development, but also in maintenance of tissue homeostasis and tissue repair in adults.[49] TGFB was shown to

promote EC proliferation and migration at low concentrations, whereas high concentrations had the opposite effect.[50]

Members of the TGFB family mediate their effects by binding specific transmembrane type I/II serine/threonine kinase receptors at the cell membrane. These receptors have a small intracellular tail and a large extracellular domain. The known co-receptor for TGFB signaling in ECs is endoglin. Upon ligand-receptor binding the type I receptor is phosphorylated by the type II receptor. The ligand-receptor binding can result in activation of the SMAD family which translocate into the nucleus where, by interacting with other transcription factors, they can regulate gene transcriptional responses (canonical SMAD signaling pathway) or can activate other non-SMAD signaling pathways.[6,49]

In ECs, some BMPs have an anti-angiogenic effect like BMP9 and BMP10 that after TGFBR1 and TGFBR2 binding induce SMAD1 phosphorylation and inhibit EC proliferation and migration. Others, like BMPs 2, 4, 6 and 7 have been shown to induce EC proliferation and angiogenesis by inducing VEGFs expression.[50]

2.4. Fibroblast growth factor / fibroblast growth factor receptor

FGF belong to a family composed of 22 elements that interact with high-affinity FGFR to regulate multiple fundamental pathways and cellular behaviors, namely cell proliferation, differentiation and survival, as well as angiogenesis and wound healing. FGFs have strong affinities for the proteoglycans from ECM, which help sequester FGFs on the surface of the secreting cell or on nearby cells, and in binding of FGF to FGFR.[51] FGFRs are transmembrane tyrosine kinases that contain 2 or 3 extracellular immunoglobulin-like domains and an extracellular heparin-binding sequence. In ECs FGFs are involved in angiogenesis because of their release by tumor and stromal cells. In particular, FGF1 may stimulate EC growth and motility through paracrine signaling. FGFs may also exert their effects through autocrine signaling in ECs, stimulating the proliferation of the cells.[6,51,52]

2.5. Platelet-derived growth factor / Platelet-derived growth factor receptor

The PDGF family consists of four different PDGF strands (A–D) that forms functional homodimers or a heterodimer PDGF-AB. These dimers bind to tyrosine kinase

receptors, PDGFRA and PDGFRB, formed by an extracellular ligand-binding domain, composed of five immunoglobulin-like subdomains, a transmembrane domain and a cytoplasmic domain with tyrosine kinase activity. This ligand-receptor binding induces receptor homo- or heterodimerization with subsequent autophosphorylation of specific tyrosine residues within the cytoplasmic domain, like VEGFRs do. PDGFRB are required for the development of the vasculature both under physiological and pathological angiogenic conditions, for example during myocardial infarction and tumor vascularization. Their expression is almost undetectable in healthy tissues. PDGFB/PDGFRB signaling contributes to maturation of the vessel wall by recruiting pericytes and smooth muscle cells to the newly formed vessel.[6,53]

It is known that VEGFA can bind PDGFRs because of its structural similarity to PDGF. Some studies reveal that VEGFA/PDGFR signaling is important for the recruitment and proliferation of mesenchymal stem cells and other positive PDGFR cells. In the meanwhile, other authors referred that VEGFA can prevent PDGFRA dimerization, activation and internalization, which attenuates PDGF-dependent signaling and cellular responses.[54,55]

2.6. Extracellular matrix and its components

As Frantz *et al.*[56] postulated, ECM is the non-cellular component present within all tissues and organs that provides physical and biochemical support for tissue homeostasis and differentiation. In general ECM is composed of water, proteins (mainly collagens, elastins, fibronectins and laminins) and polysaccharides (especially proteoglycans) but each tissue has an ECM with a unique composition and topology that is generated during tissue development. ECM is a highly dynamic structure that is constantly being remodeled and its molecular components are subjected to a post-translational modifications.[56]

The main component of the ECM in basement membrane is collagen IV that provides tensile strength, regulates cell adhesion and migration as well as direct tissue development. Each collagen IV α-chain consists of an N-terminal 7S domain, a core collagenous domain containing the characteristic Gly-X-Y repeat sequence and a C-terminal non-collagenous domain 1. Collagen associates with elastin, another major ECM fiber. The most abundant non-collagenous protein in the ECM is laminin. In humans,

there are 11 laminin genes which encode α, β, and γ laminin chains. As well as self–self-interactions, the laminins are responsible for interactions with other basement membrane components as well as with cells on either face of the membrane. Fibronectin is intimately involved in directing the organization of the interstitial ECM and has a crucial role in mediating cell attachment and function. Tensed fibronectin modulates the catch adhesion assembly of α5β1-integrin through exposure of its synergy site.[56,57]

Perlecan is the most prevalent ECM heparan sulfate proteoglycan (HSPG). Is a complex multidomain protein with a number of discrete binding partners. It is synthesized and secreted as a single molecule composed of five distinct domains. Perlecan is involved in regulating many signaling pathways, such as FGF/FGFR signaling with consequent roles in angiogenesis and tumor induction. The HS chain attachment is important for cell adhesion and basement membrane anchoring via interaction with laminin, collagen IV, and fibronectin and for growth factor and angiogenesis regulation via interaction with FGF2, PDGF and VEGFA.[57]

Tissue homeostasis is mediated by the coordinated secretion of MMPs which is counterbalanced by TIMP metalloproteinase inhibitor (TIMPs) and the controlled activity of other enzymes. In ECs, MMP1 (type I collagenase) is one of four known collagenases, capable of degrading collagens. The gelatinases (MMP2 and MMP9) degrade basement membrane collagens and elastins.[56]

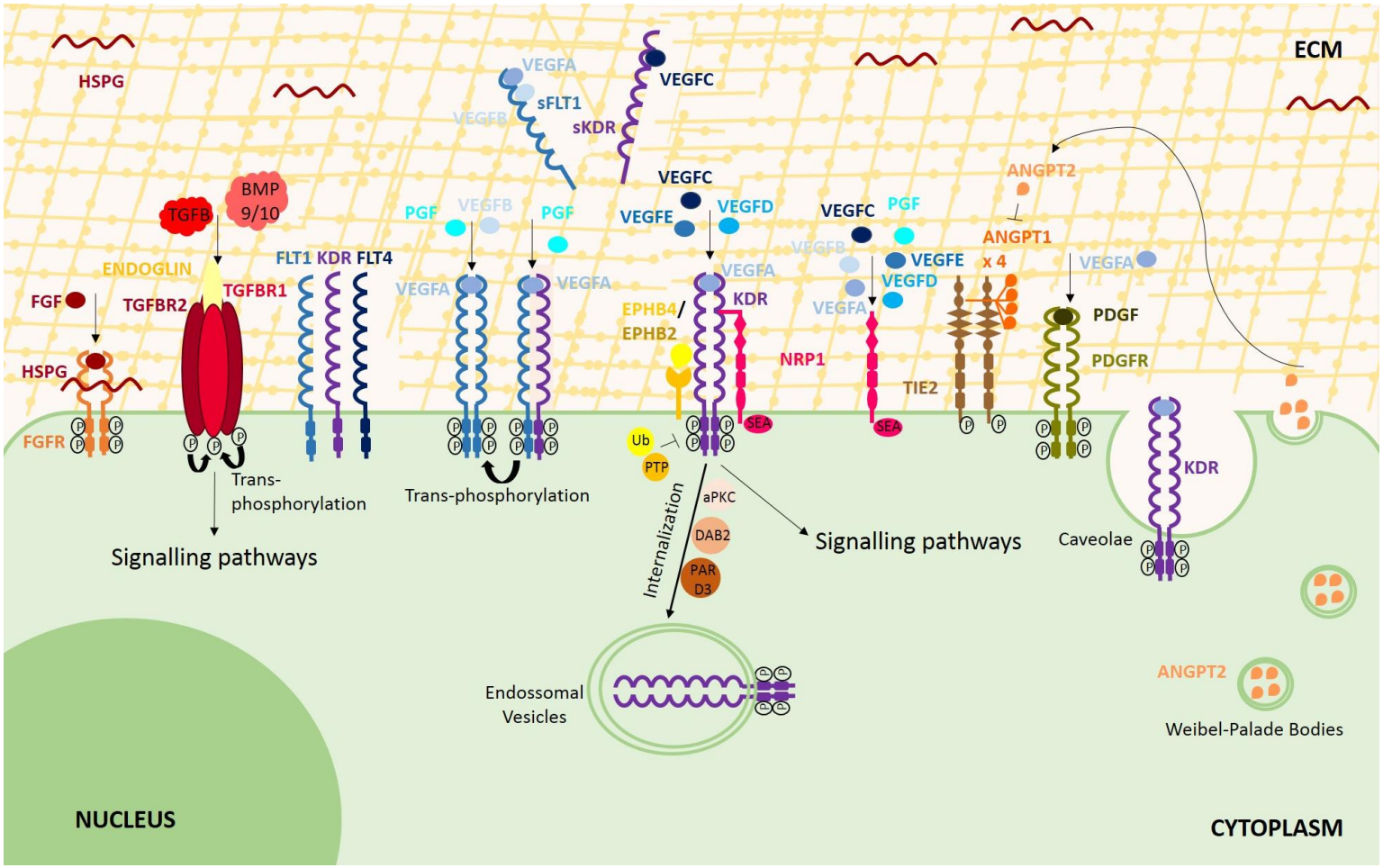


Figure 1.2 - Molecular specificities of endothelial cell. ECs sense the exterior stimuli by a number of growth factors such as FGF, TGF, BMP, VEGF, ANGPT, PDGF and their specific receptors (FGFR, TGFBR, VEGFR, NRP, TIE and PDGFR) whose tyrosine kinase domain phosphorylation promotes the activation of signaling pathways. These pathways are further explained in the text above.

3. Endothelial cell properties

Since ECs are subject not only to mechanical forces such as blood flow shear stress, but also to biochemical stimuli produced by immune cells, or due to the availability of nutrients and oxygen, they acquired some specificities in terms of permeability and adhesion. In this section I will also address the main property by which endothelium is known, that is the capacity to form novel vessels as a result of sprouting. The EC properties are depicted in Figure 1.3.

3.1. Permeability

One of the main roles of ECs is to function as a selective barrier between the blood stream and tissues. This barrier is controlled by the combination of heterotypic cell associations (inflammatory cells and mural cells), transcellular transport (across the endothelium) and adherens/tight/gap junctions (paracellular transport).[48]

3.1.1. Transcellular transport

Goddard *et al.*[48] described transcellular permeability as an energy-dependent trafficking of macromolecules from the luminal space to the interstitium by vesicular transport. This transport can occur through caveolae, vesiculo-vacuolar organelles (VVOs) and transcellular channels. Caveolae are vesicles with high levels of caveolin 1 (CAV1) that act as a scaffold protein, recruiting SRC proto-oncogene, non-receptor tyrosine kinase (SRC) and G proteins to caveolae, which lead to the internalization of cell surface macromolecular complexes. CAV1 can also affect junctional integrity through its interactions with SRC, PKC, claudin 5 (CLDN5), and actin-binding proteins, which are all involved in adherens and tight junction assembly and maintenance.[48]

VVO provides the major route of extravasation of macromolecules at sites of augmented vascular permeability, induced by VEGFA. The presence of VVOs in human ECs was associated with primary and metastatic tumors, acute and chronic inflammatory disorders and in allergic inflammation. VVOs are caveolin-positive which suggests that VVO forms from the joining of individual caveolin-positive caveolae.[58]

3.1.2. Paracellular transport

Adherens and tight junctions are essential during embryonic development and in the adult vascular system and their distribution and predominance varies between different tissues. Adherens junctions in ECs are mainly formed by vascular endothelial cadherin (VE-cadherin), whereas the main components of tight junctions are claudins 1/5/12, Occludins and junctional adhesion molecules (JAMs).

The stability of endothelial adherens junctions is mainly regulated by the plasma membrane localization of VE-cadherin. VE-cadherin establish the early EC-EC junctional contacts and myosin X (a plus-end directed actin motor) mediated by catenin, guide VE-cadherin to the tips of EC. These junctions have to be transiently disorganized (e.g. for angiogenesis or diapedesis) or they must stay tight as in the blood-brain barrier.[32] The extracellular domain of VE-cadherin contains five cadherin-type repeating units and binds to VE-cadherin molecules on neighboring ECs. VEGFs and their receptors are the main factors that promote adherens junctions disassembly in blood vessels, thus promoting vascular permeability. Binding of VEGFA to KDR leads to phosphorylation of the receptor with internalization of VE-cadherin and promotion of its degradation, which weakens adherens junctions.[32] Some studies revealed that VE-cadherin and KDR coimmunoprecipitate indicating that they cluster together in adherens junctions. ANGPT1 inhibits this VEGFA-induced VE-cadherin internalization, via inhibition of VEGFA-activated SRC kinase signaling. The endogenous PTPRB is frequently associated with the protein and prevents VE-cadherin phosphorylation, promoting an important stabilizing role of adherens junctions *in vivo*[47,48]

Major tight junction proteins include CLDN5, occludin, and JAMs. Similar to adherens junctions, phosphorylation of both tight junction proteins and their intracellular partners tight junction protein 1 (TJP1), also known as ZO1, and membrane-associated guanylate kinase (MAGUKs) regulate tight junction assembly and mediate changes in vascular permeability. Only JAM3 leads to increased permeability when expressed at the cell surface of ECs following stimulation with VEGFA or histamine.[48] Li *et al.*[59] suggest that JAM3 regulation of αvβ3 integrin localization and activation downstream of RAP1B, member of RAS oncogene family (RAP1B) signaling may account for barrier breakdown. The activated KDR also leads to the phosphorylation and ubiquitination of occludin, followed by internalization and transfer to late endosomes, which indicates that KDR also

influences tight junction formation directly. Under resting conditions, occludin and other tight junction components are constitutively internalized.[32,60]

There is emerging data indicating that adherens and tight junctions cooperate in EC permeability regulation. VE-cadherin appears as a key sensor and molecular integrator of adherens and tight junctions.[48,61,62]

Gap junctions are communication structures which allow the passage of small molecular weight solutes between neighboring cells.[63] This structure is formed by gap junction proteins (GJAs), some of these: gap junction protein alpha 1 43KDa pseudogene1 (GJA1P1), gap junction protein alpha 5 40KDa (GJA5) and gap junction protein alpha 4 37KDa (GJA4) are expressed in ECs. GJAs are organized in connexons, which act as channels for the intercellular passage of ions and small-molecular-weight molecules, like calcium (Ca^{2+}) ions and nitric oxide.[63-65]

3.2. Adhesion

Adhesion is an important cell property that allows the communication of the cell with other cells and with the surrounding ECM. There are some proteins involved in these interactions namely integrins, cadherins, selectins and members of the superfamily of Immunoglobulin-like cell adhesion molecules (CAM).

3.2.1. Integrins

The formation of blood vessels depends on EC adhesion to the ECM, a process that is mediated by ECM receptors, such as integrins and discoidin domain receptor tyrosine kinase (DDR).

Integrins are αβ heterodimeric cell surface receptors with a large extracellular domain, a single transmembrane domain and a short non-catalytic cytoplasmic tail. Integrins mediate cell-matrix adhesion by specific binding to ECM components, such as collagen, fibronectin and laminin. Integrins not only are involved in cell–cell and cell–matrix adhesion but also mediate outside and inside signaling pathways through integrin-associated signaling and adaptor molecules, such as focal adhesion kinase (FAK) and integrin-linked kinase (ILK). Therefore they are crucial during development, wound healing, and a variety of pathological conditions.[66,67]

ECs express several integrin heterodimers including αvβ3, α5β1, and αvβ5. Integrin αvβ3 is important in healthy conditions (it is expressed at low levels on quiescent ECs) and in pathological conditions (it is significantly elevated during wound angiogenesis, inflammation and tumor angiogenesis).[68] It has been shown that specific integrins have specific functions in various vascular processes, e.g. αv is believed to be the subunit primarily responsible for angiogenesis. Consistent with this finding, α5 knockout (KO) mice displayed impaired vasculogenesis during development.[69,70]

Several receptor tyrosine kinases like VEGFRs are known to associate with integrins and this association is essential for the regulation of the receptor kinase activity.[68] Integrin αvβ3 interacts with KDR and PDGFRB and it was shown by Borges *et al.*[71] that the extracellular domain of β3 is essential for interaction with KDR or PDGFRB. This study indicated that while interaction between integrin β3 and KDR is augmented by the integrin αv subunit, interaction between integrin β3 and PDGFRB did not require the integrin αv subunit.[71] Later on, it was shown by Robinson *et al.*[72] that integrin αvβ3 can also associate with NRP1 in a VEGFA-dependent manner and might contribute to the regulation of KDR signaling by sequestering NRP1. Integrins can also mediate phosphorylation of FLT4 through SRC in a VEGFC/VEGFD-independent manner. This enhances VEGFA-induced EC migration and proliferation, by promoting the receptor phosphorylation and downstream signal transduction.[26] Thrombospondin 1 (THBS1), which was one of the first discovered endogenous angiogenesis inhibitors and it is a major constituent of platelet granules is complexed with activated TGFβ. Upon release it can binds cluster of differentiation (CD)36 and some integrins on activated ECs.[73] Recent data indicate that CD36 and β1 integrins collaborate to transmit the pro- and anti-angiogenic signals that are initiated by THBS1 and THBS2, through the association with KDR.[74]

DDR2 is a unique receptor tyrosine kinase that signals in response to collagen binding and stimulates MMP production. It is implicated in malignant tumor phenotypes such as invasion and metastasis. It has been reported that DDR2 expression is upregulated in activated ECs and a recent study showed that loss of stromal DDR2 also suppresses tumor metastasis.[75]

3.2.2. Cadherins

Cadherins are transmembrane proteins which form hemophilic interactions in a Ca^{2+} dependent manner. They provide weak adhesive cell-cell forces, stabilized by catenins, which are intracellular proteins linking the cadherin cell surface molecule to the actin cytoskeleton.[76] Despite the spatially distinct locations of cell–ECM vs. cell–cell adhesions in ECs there is intimate crosstalk between integrins and cadherins.[56] As van Buul *et al.*[77] postulate, the integrin–cadherin crosstalk depends on their shared signaling pathways that control adhesion. Rho guanosine triphosphate hydrolase (GTPases) play a central role in the organization of the actin cytoskeleton that tightly associates with both cell–ECM adhesions and cell–cell junctions.[77] The specific cadherin in ECs is VE-cadherin involved in adherens junctions, whose expression and pathway have already been explained in the previous section.

Neuronal cadherin (N-cadherin) is another cadherin family member that is expressed in ECs and it is involved in rolling and attachment of breast carcinoma as well as melanoma cells to endothelium.[78]

3.2.3. Selectins

Selectins are vascular cell adhesion molecules involved in adhesive interactions of leukocytes and platelets to endothelium within the blood circulation. This family is involved in inflammation, immune response, wound repair, cancer and homeostasis. The selectin family is composed by 3 elements: P-, E-, and L-selectin. P-selectin is present in the storage granules of platelets (α-granules) and in Weibel-Palade bodies characteristic of ECs, which enables the rapid translocation on cell surfaces upon activation with thrombin and histamine for example. E-selectin is expressed in ECs but requires previous activation by cytokines, such as in inflammation conditions. Usually it is absent from resting endothelium, with the exception of the skin. E-selectins are expressed on cancer cells and contribute to rolling of various cancer cell types. L-selectin is constitutively expressed on cell surfaces of some immune subpopulations.[78,79]

3.2.4. Cell adhesion molecules

ECs can bind immune cells through cell surface adhesion molecules, being important for immune surveillance regulation and inflammation. CAMs are expressed on the apical surface of ECs and are recruited to sites of leukocyte adhesion, becoming

activated. The main CAMs on EC are intracellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1) and platelet/EC adhesion molecule (PECAM1).[80]

ICAM1 is composed by five extracellular immunoglobulin-like domains, a single transmembrane domain and a short cytoplasmic domain. The resting endothelium expresses ICAM1 at low levels, but upon activation by inflammatory stimuli the expression of ICAM1 is increased to promote integrin-mediated adhesion and transmigration of leukocytes. After leukocyte - ICAM1 binding this CAM is clustered into membrane rafts that can be stabilized to form large platforms through protein-protein interactions. This clustering induces intracellular signaling in ECs, resulting in rearrangement of the actin cytoskeleton.[80,81]

VCAM1 is a member of the immunoglobulin superfamily of proteins and is composed by seven extracellular immunoglobulin-like domains, a single transmembrane domain and a 19 amino acid carboxyl-terminus cytoplasmic domain. VCAM1 is also important for leukocyte-EC interaction. VCAM1 expression is induced by cytokines, high levels of ROS, oxidized low density lipoprotein (ox-LDL) , turbulent shear stress, high glucose and microbial stimulation of ECs.[82] VCAM1 also clusters and induces intracellular signaling, leading to ROS production and MAPK14 phosphorylation. Van Buul *et al.*[80] showed that ICAM1 and VCAM1 cooperate to facilitate the adhesion and extravasation of leukocytes, because VCAM1 is recruited to sites of ICAM1-induced adhesion independent of VCAM1-binding to its cognate receptor.

PECAM1, also known as CD31, is a 130 kDa glycoprotein belonging to the immunoglobulin superfamily of CAMs. PECAM1 expression is restricted to cells of the vascular system, namely platelets, monocytes, neutrophils, some subsets of T cells and ECs. In *in vitro* ECs, PECAM1 is localized to cell–cell borders of confluent monolayers and to lumen-facing areas of blood vessels. During development, PECAM1 is expressed early in the blastocyst, in primordial ECs and in yolk-sac blood islands. Its expression persists throughout embryonic development and in the adult. PECAM1 is highly expressed in all ECs of existing and newly-formed blood and lymphatic vessels. PECAM1 functions as an adhesive molecule, but also as a mediator for signal transduction. PECAM1 can interact with adaptor molecules, through phosphorylation of specific tyrosine residues located in an immunoregulatory tyrosine-based activation motif (ITAM) domain in the PECAM1 cytoplasmic tail.[83] Protein tyrosine phosphatase non-receptor type 6 (PTPN6), inositol

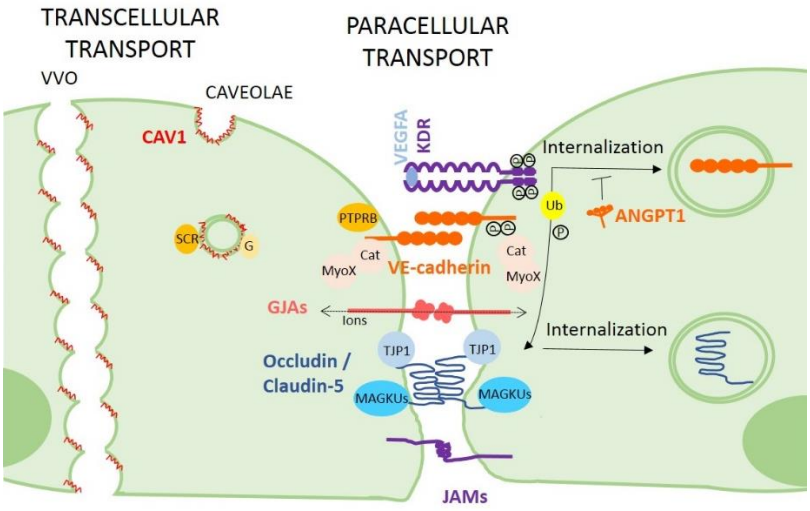
polyphosphate-5-phosphatase 145KDa (INPP5D) and phospholipase C gamma 1 (PLCG1) are proteins modulated by ITAMs.[84] PECAM1 also has immunoreceptor tyrosine-based inhibitor motif (ITIM) domains, whose signaling affect the function of various other membrane proteins such as integrin α2β3 and ICAM1. The connection between PECAM1 and ICAM1 is mediated by PTPN11 and affects ICAM1 signaling pathway, through PECAM1 mediated scavenging of ICAM1-associated PTPN11.[81] PECAM1 has also been shown to be associated with PKC-mediated serine/threonine phosphorylation instead of tyrosine-phosphorylation.[83]

3.3. Sprouting

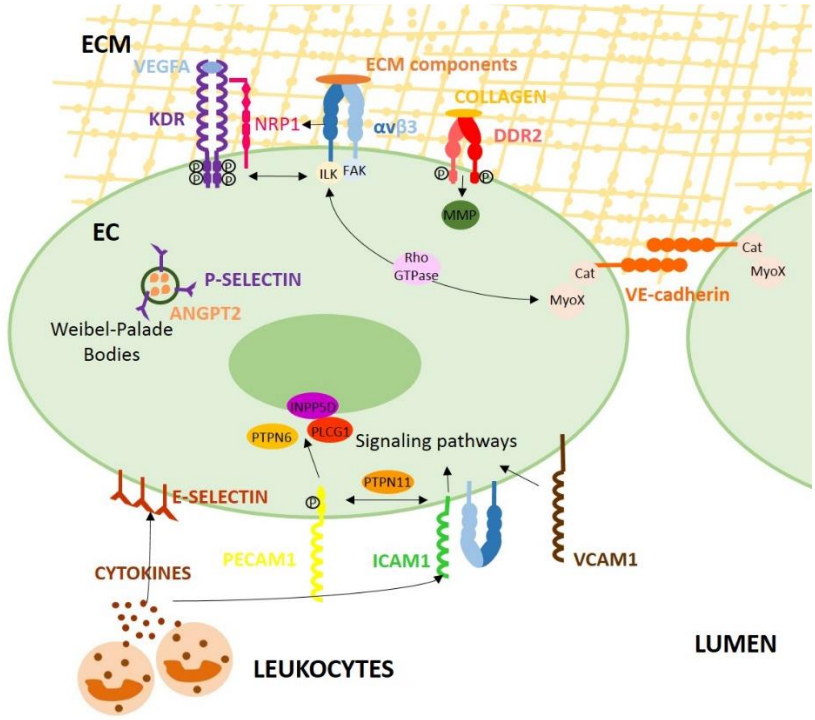
Sprouting is the most specific feature of ECs and it is the essential process of angiogenesis. Endothelial sprouting can be triggered by hypoxia, hypoglycemia and inflammatory or mechanical stimuli, such as blood pressure and is regulated by VEGFs/VEGFR and NOTCH/DLL4 signaling, as well as by other growth factors.[16,67]

The first step of sprouting begins with the breakdown of the basement membrane and detachment of mural cells. This is mediated by MMPs, such as MMP1, and ANGPT2 that are stored in Weibel-Palade bodies ready to be released. The specification of tip and stalk cells are controlled by NOTCH pathway – stalk cells have higher levels of NOTCH and low levels of DLL4, tip cells have low levels of NOTCH and high levels of DLL4. Jagged 1 (JAG1) helps to maintain differential NOTCH activity by antagonizing DLL4 in stalk cells. VEGFA through KDR also stimulates tip cell induction and filopodia. There is a connection between these two pathways, as VEGFA/KDR enhances DLL4 expression in tip cells, which inhibits tip cell behavior in neighboring ECs by downregulation of KDR and NRP1.[6,16,32]

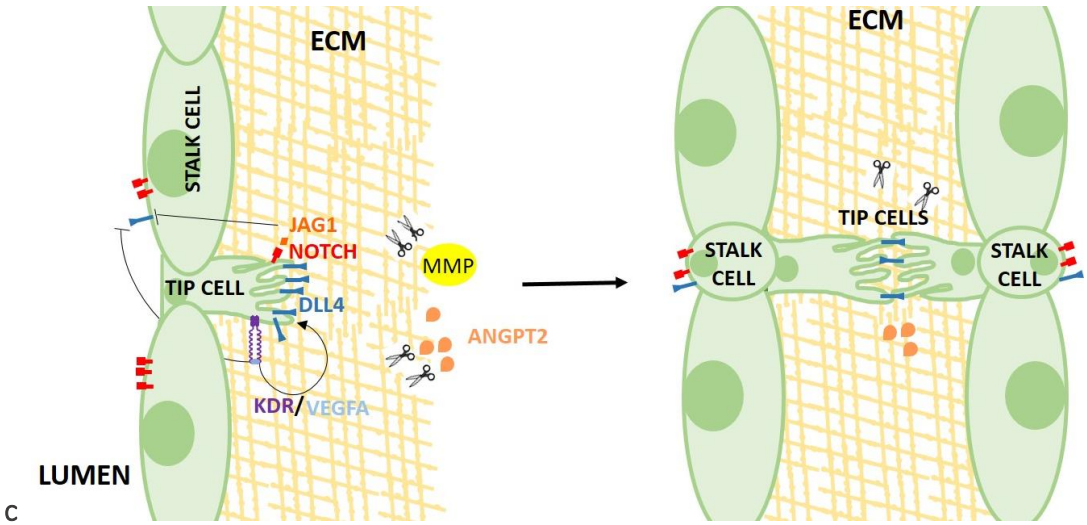
Stalk cells are more proliferative than tip cells and form a vascular lumen, establish junctions with neighbor ECs and produce ECM components. After branching, tip cells contact with filopodia of neighboring ECs and start anastomosis, to form a new functional vessel. VE-cadherin consolidate these junctions, ECM components start to deposit, PDGFB and TGFB recruit mural cells (pericytes for the arterial system and vascular smooth muscle cells for the venous system) and cell junctions start to establish. After blood flow is established the oxygen and nutrient delivery reduces VEGFA expression and inactivates endothelial oxygen sensors, which promote a shift of EC toward a quiescent phenotype.[6,16,67]


TRANSCELLULAR TRANSPORT
PARACELLULAR TRANSPORT
VVO
CAVEOLAE
CAV1
VEGFA
KDR
Internalization
Ub
ANGPT1
PTPRB
SCR
G
Cat
MyoX
VE-cadherin
GJAs
Ions
Internalization
TJP1
Occludin / Claudin-5
MAGKUs
JAMs

a


ECM
VEGFA
ECM components
COLLAGEN
KDR
NRP1
αvβ3
DDR2
ILK
FAK
MMP
EC
Rho GTPase
Cat
MyoX
VE-cadherin
P-SELECTIN
ANGPT2
Weibel-Palade Bodies
INPP5D
PTPN6
PLCG1
Signaling pathways
PTPN11
E-SELECTIN
CYTOKINES
PECAM1
ICAM1
VCAM1
LUMEN
LEUKOCYTES

b


ECM
STALK CELL
JAG1
NOTCH
MMP
TIP CELL
DLL4
ANGPT2
KDR/ VEGFA
LUMEN
ECM
TIP CELLS
STALK CELL
STALK CELL

c

Figure 1.3 – Endothelial cell properties. a) Permeability in ECs is regulated by transcellular and paracellular transport. Caveolae and Vesicular-Vacuolar Organelles mediate the transport across the endothelium. Adherens, tight and gap junctions are regulated by the crosstalk KDR/VE-cadherin. b) Adhesion: Integrin αvβ3, VE-cadherin, Selectins and CAMs are some of the specific proteins involved in the EC-surrounding environment connection. c) Sprouting is one mechanism of new blood vessel formation that involves the balance between DLL4/NOTCH and VEGFA/KDR signaling in tip and stalk cells. Others molecules like MMP, ANGPT, PDGF and TGF are also involved.

4. Transcriptional regulation on endothelial cells

Gene expression regulation can be done by transcription factors and epigenetic modifications. This regulation is the key for EC development and rearrangement in healthy and pathological conditions. As de Val and Black[85] said, new molecular targets for positively and negatively modulating vessel growth should emerge. Here will be explored these two essential mechanisms and their partners.

4.1. Transcription factors

Transcription factors bind elements in the 5' regulatory regions of genes, promoting or blocking the recruitment of RNA polymerase, so they can work as activators or repressors of transcription.[86]

Through the analysis of the promoter regions of EC specific kinase receptors such as VEGFR 1/2, TIE1 and TIE2 or other cell-specific markers such as VE-cadherin, it was possible to define conserved binding regions for different classes of transcription factors. For instance, a relatively high number of binding sites for members of the ETS family is a constant feature of endothelial-specific gene promoters, strongly implicating these transcription factors in EC determination.[87] Given the common origin of blood and ECs, it is not surprising that several of the factors important for the early development of ECs are also important for hematopoietic development.[85]

The main classes of transcription factors involved in EC development are the families ETS, GATA, basic helix-loop-helix (bHLH), FOX, SRY(sex determining region Y) box (SOX), nuclear receptor (NR), Krüppel-like factor (KLF) and homeobox (HOX). The role of the nuclear factor of activated T-cells (NFAT) family in EC phenotype will also be addressed in this chapter. Table 1.1 presents these transcription factors, their main target genes and functions.

4.1.1. v-ets avian eryhtroblastosis virus E26 oncogene

Like Meadows *et al.*[88] referred, the majority of ETS factors activate gene transcription. ETS proteins are characterized by an ETS binding consensus sequence, which is a winged helix-turn-helix that binds to the core DNA sequence 5'-GGA(A/T)- 3'.

Table 1.1 – The main transcription factors involved in endothelial cell regulation are members of ETS, GATA, bHLH, FOX, SOX, NR, KLF, HOX and NFAT superfamilies. Their functions range from being an activator of the EC transcription program in embryonic ECs to pathological angiogenesis in the adulthood.

FAMILY	MEMBERS	TARGET GENES	FUNCTIONS
ETS	ETS1	*Mmp, Cdh5, Flt1, kdr, Angpt2, Nrp1, Tie2, Vwf, Pecam1*	Angiogenesis (healthy and pathological conditions)
	FLI1	*Tgfbr*	Hematopoiesis
	ERG	*Cdh5, Cldn5, Icam2, Flt1, Vwf*	Vascular development (homeostasis, survival, differentiation ECs)
	ETV6	*Pdgfrb*	Formation of vascular network in yolk sac
	ELK3	*Vegfa*	Maintenance of vascular network
	ETV2	*Kdr, Cdh5, Tie2, Notch4, Nfatc1* (Etv2/Foxc2)	Embryonic ECs (activator of EC transcription program)
GATA	GATA2	*Kdr, Cdh5*	Embryonic ECs
bHLH	HEY1 HEY2	Notch pathway, *Nrp1, Ephb2*	Arterial-venous specification, Sprouting
	HIF1A	*Vegfa, Flt1*	Hypoxia-induced angiogenesis
	ID1/ID3	Bmp/Smad pathway	Vascular development
FOX	FOXC1 FOXC2	*Dll4, Jagg1, Nrp1*	Arterial-venous specification, lymphatics differentiation
	FOXO1	*Kdr, Cdh5, Pecam1, Tie2*	Vascular development in yolk sac
	FOXF1	*Flt1, Kdr, Pdgf, Pecam1, Tie2*	Vascular development in yolk sac
SOX	SOX7 SOX17 SOX18	*Cdh5*, *Fgf*, *Il7r*, *Mmp7*, *Cdh2*	Arterial-venous specification
NR	NR2F2	*Flt4, Fgfr, Nrp1*	Arterial-venous specification
KLF	KLF2	*Hba1, Hgb2, Hbe1*	Maintenance of vascular tone
HOX	HOXA3	*Runx1, Gata1, Gfi1*	Hemogenic endothelium regulation
	HOXD3/A3	αvβ3 and α5β1 integrins, *Plau*	Invasive angiogenesis
	HOXA9	*Nos3, Kdr, Cdh5*	Hematopoiesis, tubulogenesis
NFAT	NFATC1	*Vegfa, Cxcr7, Vcam1, Bmp2*	Cardiac development
	NFATC2	*Il2, Il4, Tnf, Csf, Wnt*	Immune system function
	NFATC3/4	*Vegfa, Ephb2*	Vascular development

All ETS factors also contain a transactivation domain, some members contain a pointed domain that mediates protein-protein interactions and oligomerization. ETS factors are involved in tumorigenesis, apoptosis, angiogenesis and hematopoiesis, but it is especially important for differentiation of hematopoietic and vascular lineages.[39]

ETS targets include many ECs proteins and it is thought that ETS proteins directly regulate the expression of most, if not all, endothelial genes. From the big ETS family, 7 ETS genes are highly expressed in embryonic ECs: *Ets1*, Fli-1 proto-oncogene, ETS transcription factor (*Fli1*), v-ets avian erythroblastosis virus E26 oncogene homolog (*Erg*), ets variant 6 (*Etv6*), ELK3 ETS-domain protein (*Elk*3), *Etv2* and v-ets avian erythroblastosis virus E26 oncogene homolog 2 (*Ets2*). Several studies have shown that ETS proteins bind to the same consensus target sequence and are capable of transactivating the same promoter, which opens the possibility of ETS proteins having redundant functions during early ECs differentiation.[85,88,89]

ETS1 expression is induced in response to hypoxia, pro-inflammatory and angiogenic stimuli. As this transcription factor can be induced by angiogenic stimuli, its expression regulates not only normal angiogenesis, but also in pathological conditions such as wound healing, tumor angiogenesis and reendothelization.[88] Watanabe *et al.*[90] showed that ETS1 in retinal vascular ECs are potentially upregulated and contribute to the formation of robust angiogenesis in ischemic retina. Accordingly, inhibition of ETS1 activity may prove to be beneficial for the treatment of ocular angioproliferative diseases. A large number of vascular genes are reported to be direct targets of ETS1 regulation including *Mmp*, cadherin 5 type 2 (*Cdh5*), *Flt1*, *Kdr*, *Angpt2*, *Nrp1*, *Tie2*, von Willebrand factor (*Vwf*) and *Pecam1*. Only double KO for *Ets1* and *Ets2* show severe vascular impairment and die around embryonic development day (E) 13, which suggests an overlapping function of ETS family members, in this case ETS1 and ETS2.[85,88]

FLI1 is expressed at high levels in primordial ECs, but its role seems to be more important in hematopoiesis than in vascular differentiation, since *Fli1* deficient mice exhibit impaired hematopoiesis.[91] Although, *Fli1* condition KO mice for ECs showed a downregulation in *Cdh5*, *Mmp9* and *Pdgfb* with a consequence in vessel maturation and stabilization.[92] ERG is constitutively expressed in all adult and embryonic ECs. ERG is a strong activator of many vascular markers *in vitro* and *in vivo*, such as *Cdh5*, *Cldn5*, *Icam2*, *Flt1* and *Vwf*. For this reason it is required for different aspects of vascular development,

including regulation of homeostasis, survival and differentiation of ECs. [93-95] ETV6 is one of the few ETS proteins that functions as a transcriptional repressor. ETV6 is able to form heterodimers *in vivo* (like ETV6-FLI1) and its repressor activity is directly linked to its ability to associate.[96] ETV6 is required for maintenance and elaboration of the complex vascular network in the yolk sac, but not for the initiation of yolk sac vasculogenesis *per se*.[88] ELK3 is expressed not only in primordial ECs (at high levels), but also during vascular development. ELK3 can function as a transcriptional repressor (under basal conditions) or as an activator in response to hypoxia signaling with downstream VEGFA activation.[97-100]

ETV2 is the most important transcriptional regulator of embryonic endothelial differentiation, being expressed in major vessels like the dorsal aorta, cardinal vein, branchial arch and intersomitic vessels. After the subsequent vascular development, the expression of ETV2 is rapidly downregulated and is practically undetectable in adult ECs. ETV2 expression is observed prior to expression of endothelial marker genes, including the crucial *Kdr*. *Etv2* KO mice is embryonic lethal at E10.5, due to complete loss of embryonic blood and primitive vascular system. Overexpression of *ETV2* in embryonic stem cells induces the generation of KDR+ cells as well as endothelial and hematopoietic cells. This data indicates that ETV2 has a conserved function as a potent activator of the endothelial gene transcription program, even before KDR.[85,88,101] It was shown also that ETV2 interacts with FOXC2, which induces the expression of endothelial genes critical for differentiation, such as *Kdr*, *Cdh5*, *Tie2*, *Notch4* and nuclear factor of activated T-cells cytoplasmic calcineurin-dependent (*Nfatc1*). These findings have revealed an important role for ETV2 in vasculogenesis and hematopoiesis.[39]

4.1.2. Other transcription factors involved in endothelial cell differentiation

Despite the fact that the ETS family seems to include the master regulators of ECs´ differentiation, there are other important transcription factors involved in this regulation.

The GATA transcription factors (GATA1-GATA6) belong to the C2H2 zinc-finger transcription factors and bind (T/A)GATA(G/A) to the genomic DNA. GATA2 is an early regulator of hematopoietic and endothelial differentiation and this transcription factor may be involved in the specification of primordial ECs from the mesoderm early in embryonic development. GATA2 is involved in the transcription of endothelial-specific

genes, such as *Kdr* and *Cdh5*.[39,85] It was postulated by Shi *et al.*[102] that the GATA2 is a cofactor for ETV2 in the regulation of primordial ECs.

The bHLH family of transcription factors possesses bipartite domains for DNA binding and protein-protein interaction. A domain of basic residues permits the binding of the referred factors to the consensus nucleotide sequence of the E-box (CANNTG) in the promoter region of target genes, whereas the helix-loop-helix domain allows it to form homo- and/or heterodimers.[89] The bHLH members play an essential role in regulation of cell differentiation.[89] In ECs, HEY1 and HEY2 (members of this family) are involved in NOTCH pathway and control arterial to venous specification and EC sprouting. KO mice for these genes developed defective vessels and died at E9.5.1.[39,85,103] HIF1A is another member of this family and plays a central role in hypoxia-regulated gene expression, like *Vegfa*. It has been very implicated in regulation of some pathologies, such as tumor angiogenesis, ischemia and neovascular eye disease.[89,104,105] The inhibitor of DNA binding (ID) transcription factors (ID1-ID4) have been implicated in embryonic and postnatal angiogenesis. They are bHLH transcription factors, but lack DNA binding domains, functioning by sequestering transcription factors that target genes, like members of SMAD/BMP pathway. Expression of ID1 and ID3 was detected in embryonic vessels and the KO mice for these genes showed abnormal vascular development.[39,106]

At least 4 FOX transcription factors are expressed in ECs: FOXC, FOXF, FOXH, and FOXO families.[85] FOXOs are characterized by the conserved DNA-binding domain, which recognizes the consensus sequence 5′-TTGTTTAC-3’. Promoters of multiple endothelial genes, such as *Kdr*, *Cdh5*, *Pecam1*, *Tie2*, *Dll4* and *Hey2* contain evolutionarily conserved FOX-ETS binding motifs.[87] FOXC1 and FOXC2 are involved in regulation of arterial vs. venous specification and lymphatic vessel differentiation.[107] In mouse embryos, FOXO1 is highly expressed in the developing vessels and KO mice embryos failed to survive beyond E10.5 because of defective vascular development in the yolk sac and embryo.[39] FOXF1 is also involved in embryonic vascular development, as *Foxf1*-deficient embryos are embryonically lethal and show growth retardation and vascular abnormalities. *Flt1*, *Kdr*, *Pdgf*, *Pecam1* and *Tie2* are direct transcriptional targets of FOXF1, so Renx *et al.*[108] concluded that this transcription factor is required for the formation of embryonic vasculature and for VEGFA signaling.

SOX family shares a conserved DNA binding domain that recognizes the consensus sequence 5′-(A/T)(A/T)CCA(A/T)G-3′ present in various genes. The SOX F group (SOX7, 17, and 18) has been found to play an important role in vascular development.[39,85] SOX18 can function redundantly with SOX7 or SOX17 in arterial-venous specification of ECs.[109] SOX18 is also involved in the regulation of permeability through *Cldn5* gene expression modulation.[110]

NR2F2 is a transcription factor that has an important role in angiogenic and neural development. Its expression is limited to venal ECs and lymphatics. KO mice is embryonic lethal, dying of abnormal development of the heart and impaired angiogenesis with missing veins or fused artery-vein structures, the same defects seen in *Nrp1* and *Notch1* KO mice. These findings suggest a regulatory loop between NR2F2 and the NOTCH pathway needed for artery–vein specification.[39,111] Moreover, a recent study showed that NR2F2 homodimerization is involved in venous differentiation and heterodimers of NR2F2/prospero homeobox 1 (PROX1) are involved in lympahtic differentiation.[112]

Members of the KLF transcription factor family act in ECs. KLF2 expression is induced by sheer stress and regulates the expression of several genes, such as hemoglobin alpha 1 (*Hba1*), hemoglobin gamma G (*Hgb2*) and hemoglobin epsilon 1 (*Hbe1*), important for maintaining vascular tone in response to blood flow.[85]

Angiogenic stimuli can induce HOX genes. Among them HOXD3 is the most studied. Transfection of ECs with HOXD3 gene resulted in enhanced expression of integrins αvβ3 and α5β1 and plasminogen activator urokinase (*Plau*). HOXD3 and HOXA3 may also regulate EC gene expression associated with the invasive stage of angiogenesis.[89,113,114] HOXA3 also regulates the hemogenic endothelium, by targeting *Runx1*, *Gata1* and *Gfi1* upstream genes.[115] HOXA9 also play an important role in hematopoiesis and in tubulogenesis of mature ECs, regulating the expression of nitric oxide synthase 3 (*Nos3*), *Kdr* and *Cdh5*.[87]

4.1.3. Nuclear factor of activated T-cells

There have been countless transcription factors first identified in other cells which revealed to be important in defining vascular phenotype. NFAT family is one of those examples, first identified by Northrop *et al.*[116], as being required for the expression of a group of proteins coordinating T-cell activation. Later on, de la Pompa *et al.*[117] and Ranger

et al.[118] showed that *Nfatc1* KO mice led to an early death of mice embryos, due to development of abnormal aortic and pulmonary valves and septa as well as circulatory failure before day E14.5. Using this model it was possible to identify the first transcription factor known to be expressed in ECs of the heart. Years after, some studies revealed the link between VEGFA and NFATC1 in cardiac malformations and the specific role of NFATC1 in the progenitor cells of coronary arterial endothelium.[119-122]

As Pan *et al.*[123] reviewed, there are four members of the NFAT gene family: NFATC1, NFATC2, NFATC3 and NFATC4. The conserved N-terminal region of this family members contains a regulatory domain, including the casein kinase 1 docking site, a transactivation domain and a calcineurin docking site. The C-terminus contains a DNA-binding v-rel avian reticuloendotheliosis viral oncogene homolog (REL) homology domain and an additional calcineurin docking site. Between those domains there are several serine-rich domains that provide phosphorylation sites for kinases targeting NFAT and two nuclear localization signal sequences in addition to the nuclear export signal sequence, which controls the cellular localization of the NFATs.[123,124]

The main characteristic of this family of transcription factors is the calcineurin-dependent activity. Calcineurin is a phosphatase that dephosphorylates NFAT proteins to expose their nuclear localization signals, triggering the transport of NFAT proteins from the cytoplasm to the nucleus. Once in the nucleus, NFAT together with other factors such as GATA, FOXP3 and adaptor-related protein complex 1 (AP1) control target gene expression, which is essential for many biological functions. Calcineurin responds to increasing intracellular Ca^{2+} levels, promoting translocation of NFAT proteins to the nucleus. This mechanism is Ca^{2+}-sensitive, meaning that when the Ca^{2+} levels drop NFAT is exported to the cytoplasm inhibiting activation of the transcriptional machinery.[123,124]

Calcineurin can be inhibited by the immunosuppressant Cyclosporin A (CsA) and FK506. As previously said, there are fourteen phosphorylation sites in the NFAT regulatory domain, which reveal the importance of phosphorylation in the control of the NFAT function through cytoplasm-nuclear translocation. This cellular localization is also regulated by other mechanisms including small ubiquitin-like modifier (SUMO)ylation, proteasome degradation and histone methylation.[123]

NFAT members have been involved in numerous pathologies such as inflammatory bowel disease, rheumatoid arthritis, systemic lupus erythematosus,

glomerulosclerosis, inflammation, oxygen-induced retinopathy and cancer.[123,125-130] Recently, a wide scale genome revealed that one third of VEGFA stimulated genes were also stimulated by the NFAT family, suggesting that the NFAT pathway is an important route for VEGFA-mediated signal transduction in ECs, which raises many unresolved questions.[131]

4.2. Epigenetic modifications

Conrad Waddington was the first to propose the concept of epigenetics in 1942.[132] Nowadays, epigenetics is referred to the study of changes in gene expression and phenotype that occur without changes in the DNA sequence itself. The known epigenetic mechanisms are DNA methylation, histone modifications and post-transcriptional gene regulation by RNA-based mechanisms, such as non-coding RNAs (ncRNAs), which in turn negatively or positively influence gene expression.[4,133]

4.2.1. DNA Methylation

As Aird mentioned, methylation is an inheritable regulation of DNA by a balance between methyltransferases and demethylases.[4]

DNA methylation is the addition of a methyl group from S-Adenyl Methionine to the fifth carbon of a cytosine residue to form 5-methylcytosine in the cytosine phosphate guanine (CpG) dinucleotides. The hypermethylation of CpG islands results in the stable silencing of gene expression, by recognition and binding of some methylation-associated proteins, methyl-CpG binding domain protein (MBD) or by preventing binding of transcription factors.[133]

In vascular ECs, the methylation of CpG islands in promoters of NOS3 and KDR were identified, which results in MBD2 binding to these methylated CpG islands and suppression of gene expression. Loss of MBD2 leads to activation of *Nos3* and *Kdr* gene expression and trigger pro-angiogenic signal pathway.[86,133] A recent study of Shirodkar *et al.*[134] showed that besides NOS3, also P-selectin is partially methylated in ECs. The function of this basal methylation remains unknown.

4.2.2. Histone modifications

Genomic DNA is packaged into chromatin, which is composed of DNA and proteins. The chromatin unit is the nucleosome, which consists of an octamer of four core histone (H) proteins (H2A, H2B, H3 and H4). Histones are subjected to transcriptional modifications mediated by histone acetyltransferases (HATs) or histone deacetylases (HDACs) at their N-terminal regions. They can also be targeted by other post-translational modifications, such as methylation (mediated by histone methyltransferases (HMTs)), ubiquination, SUMOylation of lysine residues, methylation of arginine residues and phosphorylation of serines. This influences the accessibility of the DNA to the transcriptional machinery.[133,135]

As Zhou *et al.*[136] postulated, HATs hyperacetylate histones which results in an open structure of the DNA that facilitates the binding of transcription factors and promote gene expression. On the other hand, HDACs remove acetyl groups from hyperacetylated histones, which leads to a closed chromatin structure and suppression of genes. HATs and HDACs are recruited to gene promoters by DNA binding proteins that recognize certain DNA sequences, providing specific modulation on gene expression. There are four classes of HDACs: class I and class II HDACs are considered as the "classical HDACs", whose activities can be inhibited by trichostatin A; class III HDACs represent the sirtuin (SIRT) 2 family of nicotinamide adenine dinucleotide (NAD+) dependent HDACs (SIRT1–7), and class IV HDAC is the newly discovered HDAC11 that is related to class I HDACs, but since the overall sequence similarities are low, it cannot be grouped into any of the three existing classes.[136]

HDAC7 controls EC growth through modulation of βcatenin translocation. VEGFA can induce HDAC7 degradation and partially rescue HDAC7-mediated suppression of proliferation.[137] HDAC5 suppresses EC migration and sprouting, by binding to the promoter region of FGF2.[138] HDAC3 plays a critical role in maintaining EC survival and prevents arteriosclerosis via AKT activation.[139] HDAC3 binding to VEGFA possesses a pivotal function in the differentiation of stem cells into ECs.[136,140] SIRT1 is highly expressed in the vasculature and plays a critical role in the regulation of vascular function. SIRT1 has been shown to target tumor protein 53 (TP53), FOXO3 and NOS3 for deacetylation, which functions as a negative regulator of oxidative stress. It may be

inferred that the protective role of SIRT1 in vascular diseases lies mainly in its ability to decrease oxidative stress levels.[141,142]

The promoter/repressor function of a histone methylation is influenced by the specific lysine residue where it occurs. For example, H3K4 and H3K36 methylation are associated with transcriptionally permissive chromatin, whereas H3K9 and H3K27 methylation are markers of transcriptionally silent chromatin. Single lysine residues can be methylated to mono-, di-, and trimethylated states.[143] As Yan *et al*.[143] postulated, the different histone methylation states are functionally relevant; active promoters are enriched in trimethylated H3K4, while enhancer elements are enriched in monomethylated H3K4.

Recently, the trimethylation of H3K36 mediated by the specific methyltransferase SET domain containing 2 (SETD2) has been implicated in ECs phenotype. Hu *et al.*[144] showed that *Setd2* KO mice died at E10.5-E11.5, due to severe vascular defects, such as abnormally dilated capillaries, no cohesive blood vessels networks and impaired embryonic-maternal vascular connection in placenta. The gene expression profile showed altered expression of genes involved in vascular remodeling, such as *Ang, Pdgf*, *Vegfb*, *Cav1* and *Flt1* among others.[144]

4.2.3. non-coding RNA

There are 5 classes of ncRNAs involved in epigenetic modifications: microRNAs (miRNAs), small interfering RNAs (siRNAs), piwi-interacting RNAs, small nucleolar RNAs and long non-coding RNAs. MiRNAs are the most studied ncRNA. They are single-stranded non-coding RNAs of 20-25 nucleotides in length that bind to their target genes within their 3′ untranslated regions (3′ UTRs), leading to the direct degradation of the miRNA or translational repression. In recent years it has been described the role of miRNAs in vascular development and diseases.[133] MiR-15, miR-16, miR-20a and miR-20b are anti-angiogenic miRNAs targeting VEGFA mRNA, whereas the miR-17-92 cluster, miR-27b and miR-363-5p are pro-angiogenic miRNAs.[145,146]

5. The involvement of endothelial cells in cancer

Tumor blood vessels provide nutrients and oxygen, eliminate waste from tumor tissue and act as gatekeepers for tumor cells to metastasize to distant organs.[3] In 1971, Folkman was the first to disclose the importance of angiogenesis in tumor growth.[147] Angiogenesis is determined by the balance between the pro- and anti-angiogenic molecules that are released by tumor and host cells in the microenvironment. Folkman *et al.*[148] were also the first to isolate a factor from the tumor stroma (VEGFA) and to prove that it is responsible for the formation of new capillaries. Later, Folkman and Hanahan described two distinct phases in tumor growth: a prevascular phase during early tumor development where few or no tumor cells are angiogenic and expansion of the tumor is restricted to a few mm^3; and an angiogenic phase where tumor can expand progressively and shed metastatic cells. Between these two phases there is a process called angiogenic switch, which consists in the secretion of pro-angiogenic growth factors to induce the formation of new blood vessels and to ensure the adequate diffusion of oxygen and nutrients.[149] So, as Hanahan and Weinberg[21,150] postulated, induction and sustained angiogenesis is an important key step in the expansion of a tumor.

Tumor vessels are very distinct from the normal vasculature: they are disorganized, more dilated, tortuous, fragile and defective in barrier function. Tumor vessels exhibit chaotic blood flow, increased permeability and are often leaky and hemorrhagic, due to irregular ECs connections.[3,6] As Hida *et al.*[3] postulated, abnormalities of tumor blood vessels may come from unbalanced levels of pro- and anti- angiogenic factors in the surrounding microenvironment. Indeed, ECs in the newly formed blood vessels are exposed to extracellular signals like hypoxia, variable blood flow, low pH, hypoglycemia and soluble mediators released by tumor cells and stromal cells including growth factors (e.g. VEGFA, FGF, ANGPT) and cytokines (e.g. chemokine (C-X-C motif) ligand (CXCL) 8 and CXCL1). The nature of these paracrine signals is influenced by multiple factors, including underlying activation of oncogenes and/or the inactivation of tumor suppressor genes (e.g. *TP53*, *RAS* and v-myc avian myelocytomatosis viral oncogene homolog (*MYC*)) in the tumor cells. In addition to microenvironment, epigenetic factors also play a role in mediating tumor EC differentiation.[4] In particular, miRNAs are expressed by ECs upon activation with VEGFA or hypoxic environments. Most of those stimulate angiogenesis by hijacking pro-angiogenic cascades.[6] It has been demonstrated

that some cells derived from the bone marrow, such as macrophages, neutrophils, mast cells and myeloid progenitors, play crucial roles in pathological angiogenesis. They infiltrate the tumors and help the tumor to grow, facilitate local invasion and can help with the protection of vasculature from the effects of anti-angiogenic drugs.[21]

The process of vessel formation in the tumor context not only encompasses tumor angiogenesis as the sprouting of new vessels from pre-existing vessels, but also neovasculogenesis, as the growing of blood vessels originated from EPCs, or vascular mimicry where tumor cells can be part of the vessel structure. Sprouting angiogenesis refers to the release of pro-angiogenic factors that activate ECs. At the opposite, a form of vasculogenesis occurs in adults when EPCs move to an angiogenic site and integrate into newly forming vessels.[151] Lyden *et al.*[152] proved that impairment of the recruitment of EPCs can block tumor angiogenesis and growth, providing the first evidence that blood vessel growth in cancer involved both neovasculogenesis and angiogenesis. Another process of vascular formation in tumor is vascular mimicry first described in melanoma, where tumor-derived cells differentiate to form vascular channels that augment the angiogenic response. Although a number of recent studies have proposed the involvement of cancer stem cells in this process, the existence and functional importance of vascular mimicry in cancer remains under debate.[151] There is also another process called vessel co-option, where tumors choose to grow along the pre-existing vessels and this does not involve ECs proliferation.

5.1. Anti-angiogenic therapy: successes and failures

As Folkman said, angiogenesis modulation could offer therapeutic opportunities in cancer treatment.[147] Targeting tumor ECs became more interesting than tumor cells itself, because ECs are more accessible to systemically deliver agents and are less likely to develop resistance to therapies. As Aird[4] reviewed, there are two general approaches for targeting tumor vasculature: one consists of preventing new blood vessel formation by blocking tumor-derived angiogenic signals or their receptors on the surface of tumor ECs; the other targets preexisting or established tumor blood vessels, which was termed vascular targeting and may involve vascular disrupting approaches, normalization of tumor vasculature or targeted delivery of agents with the goal of reaching the endothelium. The goal of vascular disrupting therapy is to selectively induce vascular

collapse of tumor blood vessels by targeting the cytoskeleton of tumor ECs.[4] As Potente *et al.*[16] postulated, the sustained vascular normalization concept proposes not to destroy tumor vessels but to restore their structure and function, so that improved perfusion and *oxygenation* counteract the hypoxia-driven expression of genes controlling epithelial to mesenchymal transition, invasion and intravasation. As Aird[4] reviewed, targeted delivery of drugs to tumor endothelium takes advantage of vascular markers that are preferentially expressed on tumor endothelium. The target molecule may or may not be involved in tumor pathophysiology. Targeting serves to localize the effector molecule, allowing site-specific toxicity at the level of the tumor. Several types of carrier molecules or ligands have been developed, including monoclonal antibodies, aptamers, peptides and small organic molecules.[4]

VEGFA signaling has become the prime anti-angiogenic drug target with approval by the European medicines agency (EMA) of several VEGFR-based inhibitors for clinical use. The anti-VEGFA antibody (Bevacizumab) was approved in combination with chemotherapy or cytokine therapy for several advanced metastatic cancers, including colorectal, ovarian, non-small cell lung, renal, glioblastoma, pancreatic and prostate cancer. Additionally, six multitargeted pan-VEGF receptor tyrosine kinase inhibitors (TKIs) that target multiple receptors like VEGFR and PDGFR have been approved: Sorafenib, Sunitinib, Pazopanib, Vandetanib, Axitinib and Ramucirumab. Most studies that involve these VEGFR TKIs prolong progression-free survival (PFS) (usually measured in months) but have limited overall survival (OS) in cancer patients, with the possible exception of the benefits for patients with renal-cell carcinoma.[6,16] Table 1.2 summarizes the anti-angiogenics used in the clinic and their output in terms of PFS and OS.

The clinical benefit of treatment with VEGFRs inhibitors is due to regression of pre-existing tumor vessels and sensitization of ECs for chemotherapy and irradiation by depriving them of VEGFA survival activity.[16] It is curious why monotherapy with VEGFR TKI induces benefit in some tumors but is ineffective in others or evokes side effects when combined with chemotherapy. One possible explanation is that certain tumors produce pro-angiogenic factors besides VEGFA, even prior to treatment, and are thus relatively insensitive to VEGFR inhibition. Others become unresponsive during treatment, which can be mediated by increased secretion of pro-angiogenic factors, activation of alternative angiogenic signaling pathways, recruitment of pro-angiogenic accessory cells

and other mechanisms. Angiogenic signaling is robust as well as redundant. The inhibition of individual signaling molecules and growth factors can be overcome by escape mechanisms. Depriving the tumor of its vascular supply may also select hypoxia-resistant tumor clones.[16,153]

The most effective therapies involve targeting combinations of factors, as well as improving the efficiency of drug delivery to the tumor. For example, anti-VEGF therapy leads to the upregulation of PGF, which binds to FLT1 and leads to the transphosphorylation of KDR. Aflibercept is a chimeric VEGFA/PGF neutralizing receptor that suppresses tumor growth and angiogenesis, enhancing the anti-cancer activity of VEGFR blockade in colorectal, pancreatic, non-small cell lung cancer and melanoma.[151]

Nonetheless, tumors evolve mechanisms of resistance or are refractory toward VEGFRs inhibitors and only a fraction of cancer patients show benefits. As Potente *et al.*[16] said, there is a great debate on whether anti-angiogenic treatment may trigger more invasive and metastatic tumors or the vessels normalization could offer benefits for combating metastasis.[3,16] It can be hypothesized that inhibition of sprouting angiogenesis may stimulate other mechanisms of angiogenesis, like intussusceptive angiogenesis, EPC recruitment or vessel co-option, which only involves migration of ECs but not cell proliferation, and this could be an explanation for the inefficacy of anti-angiogenic drugs. It can be also hypothesized that VEGFA blockade aggravates hypoxia, which upregulates the production of other angiogenic factors or increases tumor cell invasiveness.[6,20]

Table 1.2 - Anti-angiogenic drugs used in therapy against cancer. Their mode of action, type of cancer where it is used, Progression Free Survival (PFS) and limitations. Table taken from Domingues *et al.* (in press)[154]

DRUG	MODE OF ACTION	TYPE OF CANCERS	PFS	LIMITATIONS	REFERENCES
Bevacizumab	Monoclonal anti-VEGFA antibody	Colorectal, breast, ovarian, non-small cell lung, renal, glioblastoma, pancreatic, prostate	Most of studies with improvements	No OS improvement	155-172
Aflibercept	Chimeric VEGFA/PGF neutralizing receptor	Colorectal, pancreatic, non-small cell lung, melanoma	Improvements in colorectal, non-small cell lung and melanoma	Small or no OS improvement	160,173-175
Sorafenib	VEGFR Tyrosine Kinase Inhibitor (TKI)	Kidney, Hepatocellular, melanoma, small-cell lung	Improvements in renal and hepatocellular	No improvements in melanoma and small-cell lung	160,176-178
Sunitinib	VEGFR TKI	Kidney, stromal gastrointestinal, breast, hepatocellular, colorectal, pancreatic neuroendocrine, non-small cell lung, prostate, melanoma	Improvements in most studies	No OS improvements in the majority of studies	160,179-182
Pazopanib	VEGFR TKI	Kidney, non-small cell lung, soft tissue sarcoma	Improvements	No OS improvements	183,184
Vandetanib	VEGFR TKI	Non-small cell lung, medullary thyroid	Improvements in medullary thyroid cancer	No OS improvements	160,185
Axitinib	VEGFR TKI	Renal, pancreatic	Improvements in renal cancer	No OS improvements	186
Ramucirumab	VEGFR TKI	Gastric, non-small cell lung, melanoma	Improvements in most studies	Little or no OS improvements	187-189

6. Novel insights and aim of this thesis

As Hanahan and Weinberg[21,150] highlighted, differences in signaling, in transcriptome profiles and in vascular ‘‘ZIP codes’’ will be important for understanding the heterogeneity between normal and tumor-associated ECs.

Much is known about ECs growth factors, chemokines, receptors, signaling pathways and transcriptional regulation, but clearly much more remains to be discovered, especially in the integration of such signals in normal and in pathological contexts.

The global aim of this thesis was to identify novel putative ECs targets and to understand the molecular determinants of vessel growth in physiological and pathological conditions. We focused specifically on differences in transcriptional modulation of gene expression between normal and tumor vessels. Our main hypothesis is that NFATC1 and SETD2 are key regulators of EC homeostasis, being involved in their differentiation and adaptation to tumoral environment.

So the specific aims of this thesis were:

- To unravel the role of the NFATC1 transcription factor in ECs heterogeneity
- To discover the role and mechanisms of SETD2 in vessels impaired growth
- To define the role of estrogen derivatives in angiogenesis

Chapter 2

NFATC1 is lost in endothelial cells in tumors: could this have implications for lack of response to anti-VEGF therapies?

Germana Domingues[1], Catarina Rodrigues dos Santos[2], Tânia Carvalho[1], Lénia Rodrigues[1], Jacinta Serpa[2,3], Sérgio Dias[1]

1 Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa **2** Instituto Português de Oncologia de Lisboa Francisco Gentil **3** Centro de Estudos de Doenças Crónicas, Faculdade de Ciências Médicas da Universidade Nova de Lisboa

Manuscript in preparation

Abstract

Nuclear factor of activated T-cells cytoplasmic calcineurin-dependent (NFATC1) is a member of nuclear factor of activated T-cells (NFAT) transcription factors family which has been linked to vascular defects, as observed in the *Nfatc1* knockout (KO) mouse. NFATC1 has also been shown to act downstream of vascular endothelial growth factor (VEGF) signaling. Given the lack of response to anti-VEGF therapies in breast cancer patients, here we hypothesized that NFATC1 might be modulated in the tumor microenvironment and thus be linked to decrease VEGF signaling in tumor endothelial cells (ECs).

We characterized the expression and localization of NAFTC1 both in breast cancer mice models and in human breast cancer patients. We observed that ECs from normal mammary gland express NFATC1, but this expression is lost in tumor areas. Notably, tumor mononuclear cell infiltrates (TMCI) expressed NFATC1 and showed a positive correlation with high-grade human breast cancers. Mechanistically, we showed that kinase insert domain receptor (KDR) mediates VEGF-induced NFATC1 nuclear translocation in both human dermal microvascular ECs (HMVEC) and human umbilical cord vein ECs (HUVEC). Anti-KDR (αKDR) and cyclosporine A (CsA) (which is a specific inhibitor of calcineurin) cooperate in inhibition of NFATC1 nuclear translocation and seem to have a cumulative effect.

Together, our data suggests NFATC1 expression and function on ECs is modulated by the tumor microenvironment. This could have consequences for VEGF signaling and thereby response to anti-angiogenic therapies. Our studies pave the way for detailed studies in the regulation of NFATC1 expression and function and its importance in the context of anti-VEGF therapies in breast cancer.

Introduction

NFATC1 is a member of the NFAT transcription factor family.[116,123] Their role in vascular development was first demonstrated by de la Pompa *et al.*[117] and Ranger *et al.*[118]. *Nfatc1* KO mice have abnormal aortic as well as pulmonary valves and septa, dying before day E14.5 due to circulatory system failure. Subsequent studies demonstrated that myocardial-endocardial NFAT signaling orchestrates the molecular events required for initiation and morphogenesis of heart valves.[120,121] NFATC1 has also been involved in recruitment of bone-marrow derived macrophages, which is essential for osteoclastogenesis as well in immune adaptive response by type 1 T helper (Th1) effector cell differentiation.[190-193]

The activity of NFATC1, as well as of other members of this family, is regulated by calcineurin, a calcium (Ca^{2+})-dependent phosphatase. When the levels of intracellular Ca^{2+} remain elevated, calcineurin activity remains high, which means NFATC1 is dephosphorylated and consequently translocated to the nucleus where it binds DNA to activate gene expression.[124] CsA and FK506 are inhibitory drugs that target calcineurin directly and prevent NFATC1 translocation.[194]

Some studies have revealed the importance of NFATC1 in mediating VEGF signaling pathways.[119,131,195,196] NFATC1 is activated downstream of VEGF signaling, first studied in human pulmonary valve ECs (HPVEC) and more recently in human retinal microvascular ECs, revealing an active role in angiogenic cell behavior.[119,130] It is now postulated that NFAT inhibition could be highly efficient in the treatment of diseases, such as neovascular eye disease. Suehiro *et al.*[131] showed in a wide genome study that one third of VEGF and NFATC stimulated genes are overlapped. Some genes with angiogenic potential are regulated by NFAT signaling, such as prostaglandin G/H synthase and cyclooxygenase (*PTGS2*), hypoxia-inducible factor 1, subunit alpha (*HIF1A*), fms-related tyrosine kinase1 (*FLT1*), matrix metalloproteinase (*MMP*) 2 and *MMP9*.

NFAT members have also been associated with tumor microenvironment including, fibroblasts, infiltrating immune cells and ECs.[197] Jinnin *et al.*[198] showed that FLT1 suppressed levels and constitutive KDR levels in infantile hemangiomas are orchestrated by NFAT signaling pathways. Siamakpour-Reihani *et al.*[199] demonstrated

that a specific inhibitor of secreted frizzled-related protein 2 (SFRP2) that targets NFATC3 is localized in the breast tumor vasculature.

The NFAT family of transcription factors has a distinct role in innumerous physiological and pathological mechanisms, but it remains unclear what is the specific role of NFATC1 in the tumor vasculature.[197] In our *in vivo* studies we identified the loss of NFATC1 expression in vessels under the influence of the tumor. *In vitro* we revealed a cooperation between KDR and CsA in VEGF-driven NFATC1 nuclear localization.

Methods

Animal and experimental design

Animal experiments were performed with the approval of the Instituto Gulbenkian de Ciência's Ethical Committee.

A total of 15 Balb/c mice (6-8 weeks old) were injected with $1x10^6$ 4T1 green fluorescent protein (GFP) – firefly luciferase (Luc) cells in the mammary fat pad. Tumor mass was monitored over time and 5 animals per time point were sacrificed at 9, 13 and 17 days after tumor inoculation. 10 Balb/c *scid* mice (6-8 weeks old) were injected with $1x10^6$ MDA-MB-231 GFP-Luc cells in the mammary fat pad and 5 animals per time point were sacrificed at 58 and 78 days after tumor inoculation. Tumors, macroscopic peritumoral vessels and contralateral normal mammary glands were divided for paraffin inclusion and EC sorting.

All animals were sacrificed according to the directives of the Direcção Geral de Veterinária.

Endothelial cell sorting

Peritumoral vessels (PTV), tumors and normal mammary gland vessels (NGV) were fragmented into small pieces and then digested enzymatically by 0.02% collagenase (Sigma #C2674) in free serum Dulbecco's modified eagle medium (DMEM) media for 2h at 37ºC with agitation. After this, digested samples were passed through a filter and then washed with sterile phosphate-buffered saline (PBS) (Life Technologies #70011-044). ECs were isolated by cell sorting using a primary platelet/endothelial cell adhesion molecule1 (PECAM1) (BD Biosciences #553370) antibody incubation for 1h at 4ºC and further anti-rat immunoglobulin G (IgG) microbeads (Miltenyi Biotec #130-048-501) incubation, according to the manufacturer's instructions.

ECs RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) was further performed.

Human samples

Human sample collection was performed with the approval of Ethical Committees from Instituto Português de Oncologia de Lisboa Francisco Gentil (IPOLFG) and Hospital Santa Maria – Centro Hospitalar Lisboa Norte (HSM-CHLN) (on the scope of breast cancer biomarkers study).

Eight patients with unilateral breast cancer, with no previous chemo and radiotherapy, no familiar risk and who underwent a contralateral mammoplasty during the same surgery, were selected at IPOLFG. PTV and NGV from the same patient were collected by a surgeon from chirurgical pieces.

Vessels were dissected from the adjacent tissue, using a magnifier. Samples were collected for paraffin inclusion and for RNA extraction with TRIzol and subsequent RT-PCR analysis. Hematoxylin & eosin (HE) staining was used for confirmation that there was only minor contamination with adjacent tissues.

The two first patient vessels were used to perform a stem cell transcription factor polymerase chain reaction (PCR) array (Quiagen #PAHS-501A), according to the manufacturer's instruction. The deregulated transcription factors were validated using standard RT-PCR (check Table 2.1 for the primer list). The following samples were analyzed with RT-PCR.

Breast cancer patient slices were randomly selected from Pathology Archives of HSM-CHLN and used for NFATC1 immunohistochemistry. Some clinical information was collected, namely histologic grade, estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER2), Ki67 (marker of proliferation) and tumor protein 53 (TP53) status.

Reverse transcription polymerase chain reaction

For *in vitro* experiments, cells were washed with 1x PBS and collected in TRIzol reagent (Life Technologies #15596-026). For *in vivo* experiments, cells were directly collected in TRIzol Reagent. RNA was further extracted according to the manufacturer's instruction.

Complementary DNA (cDNA) was synthetized with SuperScript II reverse transcriptase (Life Technologies #18064-014) and primer "random" (Roche# 11034731001), according to the manufacturer's instructions.

RT-PCR was performed with fast SYBR green PCR master mix (Life Technologies #4385612) in ViiA7 real-time PCR system (Applied Biosystems). 18S ribosomal RNA (rRNA) (housekeeping gene) was used for sample normalization and as an endogenous control. Human *NFATC1*, *FLT1*, *KDR*, homeobox (*HOX*) C4, *HOXC5*, nuclear receptor subfamily 2 group F member 2 (*NR2F2*), *NOTCH2*, proliferating cell nuclear antigen (*PCNA*), POU class 5 homeobox 1 (*POU5F1*), SMAD family member 2 (*SMAD2*), sp1 transcription factor (*SP1*), signal transducer and activator of transcription 3 (*STAT3*), peroxisome proliferator-activated receptor gamma (*PPARG*) primers and mouse *Hoxc4*, *Hoxc5*, *Nfatc1*, *Nr2f2, Pcna, Pou5f1, Pparg*, *Stat3* primers were designed with Primer-BLAST. Primer sequences are listed on Table 2.1. Data were analyzed with ViiA7 software.

Histology and Immunohistochemistry

Mice tumors, mammary fat pads were formaldehyde-fixed and processed for routine histopathology. Immunohistochemistry for NFATC1 was performed on 2µm slices. Antigen retrieval was performed in a PT link pre-treatment module for tissue specimens (Dako) with target retrieval solution, high pH (Dako #K8004) for 20 minutes at 64ºC. Endogenous peroxidase was blocked with 3% hydrogen peroxide (Merck Millipore #1072981000) 30 minutes at room temperature in the dark. Unspecific bindings were blocked with 2.5% normal horse serum blocking solution (Vector Lab #S-2000) for 30 minutes at room temperature. NFATC1 primary antibody incubation was made at room temperature for 2h and EnVision + system - horseradish peroxidase (HRP) for mouse primary antibodies (Dako #K4007) was used for secondary antibody incubation and revelation, according to the manufacturer's instructions. Mayer's hematoxylin counterstain was performed afterwards.

Relative quantification of 10 hotspots (magnification of 400x) per slice was made with ImmunoRatio (Institute of Biomedical Technology, University of Tampere) in a brightfield Leica DM2500 microscope (Leica Microsystems) and statistical analysis was performed with GraphPad Prism 5 software.

Cell Culture

4T1 GFP-Luc and MDA-MB-231 GFP-Luc, mouse and human breast cancer cell lines, respectively, were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies #26140-079) and 1x antibiotic-antimycotic (Life Technologies #15240-062).

HMVEC and HUVEC were cultured in EBM-2 media (Lonza, #190860) supplemented with 2% heat-inactivated FBS and 1x antibiotic-antimycotic. HUVEC were used until the fifth passage.

For HMEC and HUVEC stimulation 50ng/mL VEGF (Sigma #V7259), 500µg/mL heparin (Sigma #H3149), 1µg/mL αKDR (IMC-1C11, ImClone Systems[200]) and 1µg/mL CsA (Sigma #30024) were used. αKDR and CsA were added to the culture 1h before the other cytokines. Cells were collected for RNA extraction 1, 4 and 16h later. Lamellas for immunofluorescence were collected 4h later.

Immunofluorescence

Cells were fixed in 2% paraformaldehyde for 20 minutes at 4ºC, blocked and permeabilized for 1h at room temperature with PBS + 1% bovine serum albumin (BSA) (Sigma #A7906) + 0.02% Triton X-100 (Sigma #X100). NFATC1 primary antibody incubation was made at room temperature for 2h and secondary antibody incubation for 1h at room temperature. F-actin staining was made 20 minutes at room temperature. Antibodies are listed on Table 2.2. One drop of Vectashield mounting medium with DAPI (Vector Laboratories #H-1200) was used for nucleus staining.

Cells were examined with a widefield fluorescent Leica DM5000B microscope (Leica Microsystems). Quantifications were made using a ImageJ software (National Institutes of Health) cell counter plugin and statistical analysis with GraphPad Prism 5 software.

Statistical Analysis

Data were analyzed with GraphPad Prism 5 Software, using an unpaired two-tailed t-test with a confidence interval of 95%. Results are expressed as mean ± standard error.

* is P-value ≤ 0.05, ** ≤ 0.01 and *** ≤ 0.001

Table 2.1 Primers list

PRIMER		SEQUENCE
h18S	F	GCCCTATCAACTTTCGATGGT
	R	CCGGAATCGAACCCTGATT
FLT1	F	ACCACTCCCTTGAACACGAG
	R	TGGGAATTGCTTTGGTCAAT
HOXC4	F	CGCCCACTCGCTGTGCCTCT
	R	GTGTTGGGGAGTCGGTGGTCCT
HOXC5	F	CGGACCCTCTAACCTCGCCCT
	R	GCCTTTGGCTGTCTAACCCGTCC
KDR	F	GTGACCAACATGGAGTCGTG
	R	CCAGAGATTCCATGCCACTT
NFATC1	F	GGCTGCGGTCTTCGGGAG
	R	GCCATAGTGTTCTTCCTCCGCTG
NOTCH2	F	CAGTTGTCGCTGCTTGCCTGG
	R	GGCACTCGTTGATGTCTCCCTCA
NR2F2	F	CGACCAGCACCATCGCAACCAGT
	R	GCCCACTTTGAGGCACTTTTTGAGG
PCNA	F	GGCGTAGCAGAGTGGTCGTTGT
	R	TCAGAGCGAGCGGGCGAACG
POU5F1	F	ACTCCTCGGTCCCTTTCCCTGA
	R	GTGGTGACGGAGACAGGGGGA
PPARG	F	TGGAGACCGCCCAGGTTTGCT
	R	TCCAGGGCTTGTAGCAGGTTGTCTT
SMAD2	F	CGCCCATTCCCCTCTTCCCCT
	R	GGGACCTTTTGTTCCTTCCTCTTCC
SP1	F	CCTTGAGCTTGTCCCTCAGC
	R	ACCACAGCTGTCATTTCATCCA
STAT3	F	GGCCATCTTGAGCACTAAGC
	R	GTCCTTCTCCACCCAAGTGA

PRIMER		SEQUENCE
m18S	F	CGCAGCTAGGAATAATGGAATAGG
	R	CCGGAATCGAACCCTGATT
Hoxc4	F	ATCGCCCACTCGCTGTGCCTC
	R	TGGGGAGTCGGTGGTCCTTCT
Hoxc5	F	CTGAAACTGGGCTAAGGGCTGCT
	R	ATCTCCCTGCCCCTCCCCAGA
Nfatc1	F	GAGTTCGATCAGAGCGGCGGGG
	R	GAAGGGGCAGGGTCGAGGTG
Nr2f2	F	ATGCCTCCTACCCAGCCTACC
	R	ACGAGTGGCAGTTGAGGGGGT
Pcna	F	AGAGGGTTGGTAGTTGTCGCT
	R	CTGCCAAGGTCCCCCGATTCA
Pou5f1	F	TAGGTGAGCCGTCTTTCCACC
	R	AGGCGAAGTCTGAAGCCAGGTGT
Pparg	F	TGCTGTTATGGGTGAAACTCTGGGA
	R	GGCAGTGCATCAGCGAAGGC
Stat3	F	ACCCCGACATTCCCAAGGAGGA
	R	GGGTCGGCTTCGGGGTGCTC

Table 2.2 – Antibodies list

ANTIGEN	BRAND	DILUTION
NFATC1	BD Biosciences #556602	1:100
PECAM1	BD Biosciences #553370	1:100
Phalloidin	Life Technologies #F432	1:200
Alexa Fluor 594 donkey anti-mouse IgG	Life Technologies #A21203	1:1000
Anti-Rat IgG Microbeads	Miltenyi Biotec #130-048-501	1:4
EnVision + System-HRP for mouse primary antibodies	Dako #K4007	ready to use

Results

NFATC1 expression is lost in endothelial cells in breast cancer mice models and human breast cancer patients

In order to characterize the NFATC1 expression profile in breast cancer mice models we inoculated MDA-MB-231 GFP-Luc and 4T1 GFP-Luc breast cell lines in the mammary fat pad of mice. We performed a microbeads EC isolation, with previous primary antibody PECAM1 incubation of PTV, tumors (to isolate intratumoral vessels (ITV)) and NGV. We further extracted RNA and analyzed the mRNA expression of *Nfatc1* and other transcription factors such as *Hoxc4*, *Hoxc5*, *Nr2f2*, *Pou5f1*, *Pparg* and *Stat3* (Supplementary data) by RT-PCR in both syngeneic and xenograft mice models.

In the 4T1 GFP-Luc syngeneic breast cancer mice model *Nfatc1* mRNA levels were upregulated in PTV after 9 days of tumor growth and soon became downregulated in the next time points. On the contrary ITV showed no difference of expression levels compared to NGV. (Figure 2.1A) In MDA-MB-231 GFP-Luc human breast cancer xenograft mice model we observed an *Nfatc1* upregulation in the last time point regardless of tumor vessels localization. (Figure 2.1B)

Given the fact that *Nfatc1* mRNA expression levels were deregulated in both syngeneic and xenograft mice models, we decided to explore the localization of this transcription factor in tumors and normal mammary glands. In normal mammary gland NFATC1 was localized in some vascular ECs and in resident inflammatory mononuclear cells. Both in syngeneic and in xenograft tumors NFATC1 was localized in TMCI, mostly in peritumoral areas and ECs from the tumor lost NFATC1 expression. MDA-MB-231 GFP-Luc xenograft mice model had a less infiltrated phenotype due to its immunocompromised Balb/c *scid* background. (Figure 2.2 A-C)

We performed a relative quantification of 10 NFATC1-positive hotspots per mice per time point, using ImmunoRatio. NFATC1-positive cells increased in both mice models along the tumor growth. (Figure 2.2D)

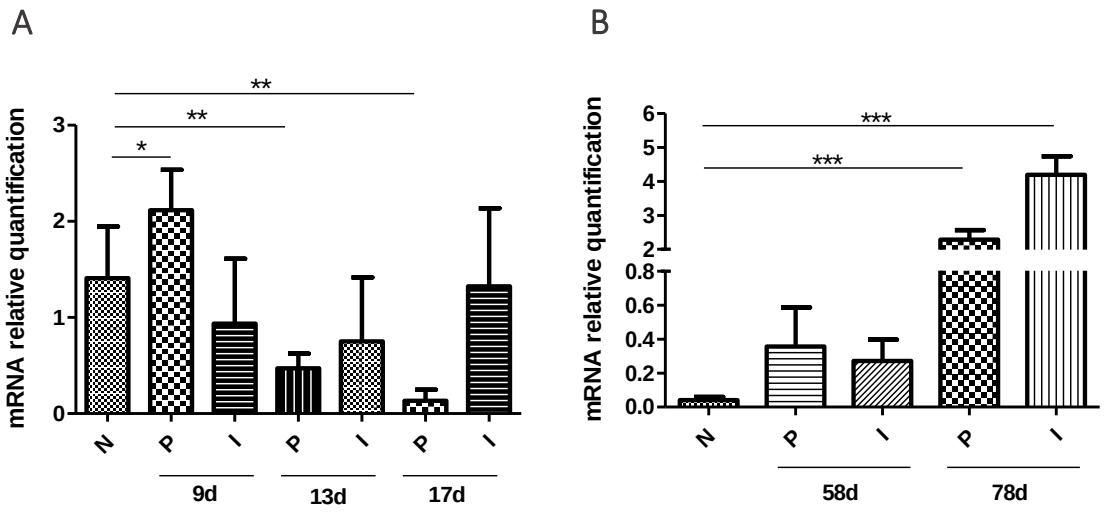
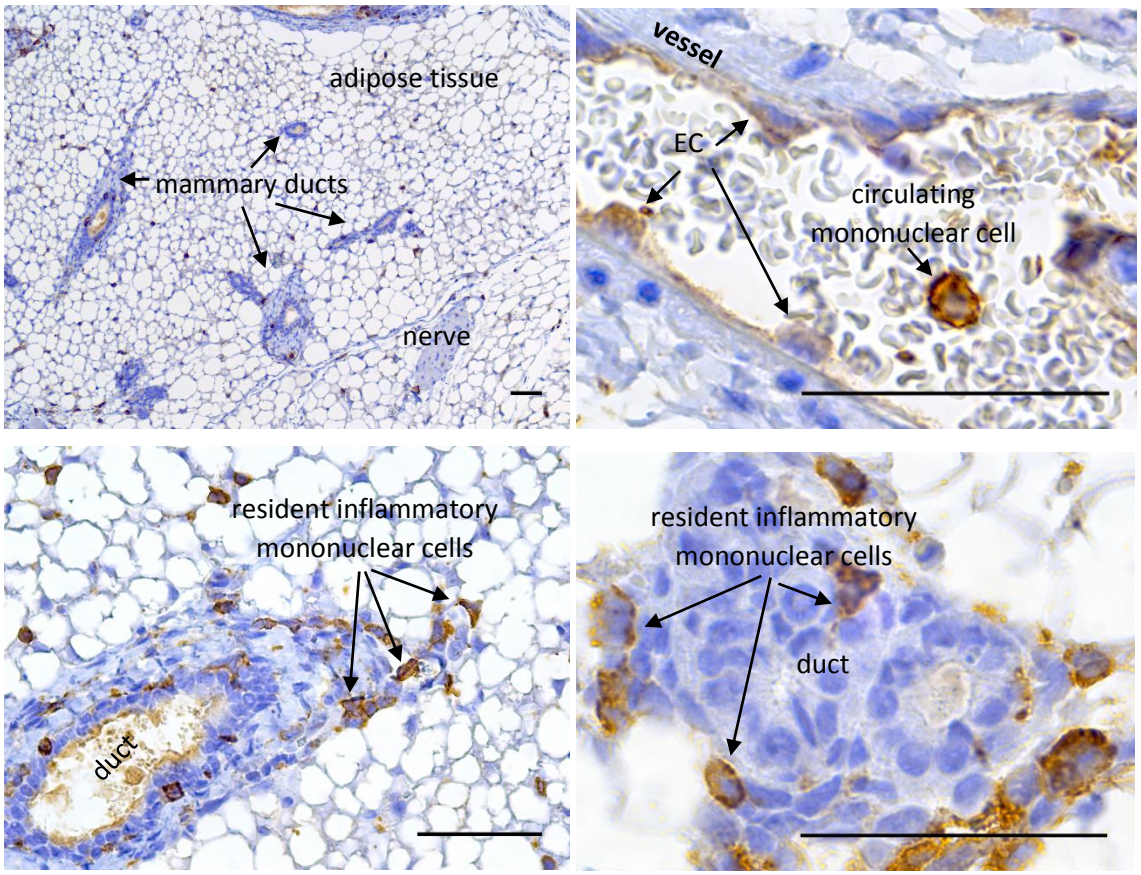


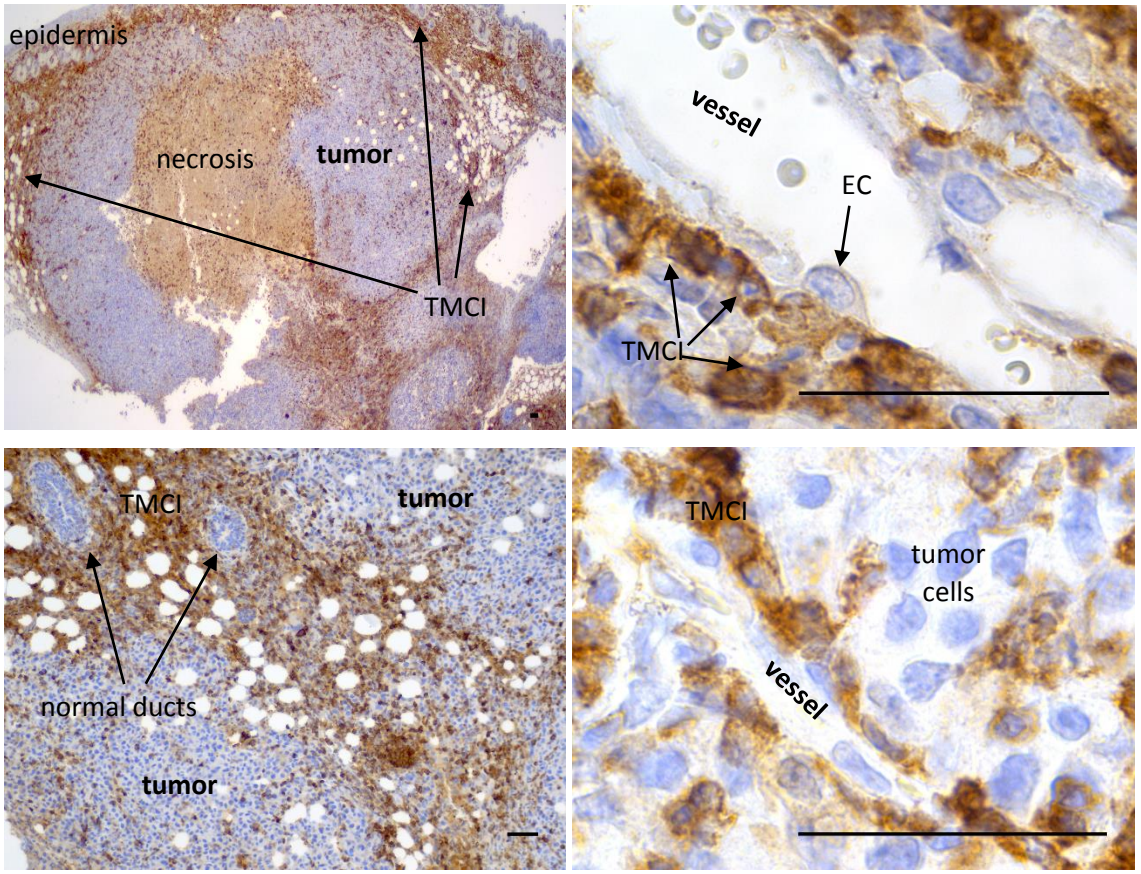
Figure 2.1 – Deregulation of transcription factors *Nfatc1* mRNA levels in PTV and ITV of breast cancer mice models

A. Relative quantification of *Nfatc1* mRNA in 4T1 GFP-Luc syngeneic breast cancer mice model at 9, 13 and 17 days after tumor inoculation; B. Relative quantification of *Nfatc1* mRNA in MDA-MB-231 GFP-Luc human breast cancer xenograft mice model at 58 and 78 days after tumor inoculation. N are NGV, P are PTV and I are ITV. Normalization to 18S rRNA expression. Data are means ± standard errors.

A


adipose tissue
mammary ducts
nerve
vessel
EC
circulating
mononuclear cell
resident inflammatory
mononuclear cells
duct
resident inflammatory
mononuclear cells
duct

B


epidermis
necrosis
tumor
TMCI
vessel
EC
TMCI
TMCI
tumor
normal ducts
tumor
TMCI
tumor
cells
vessel

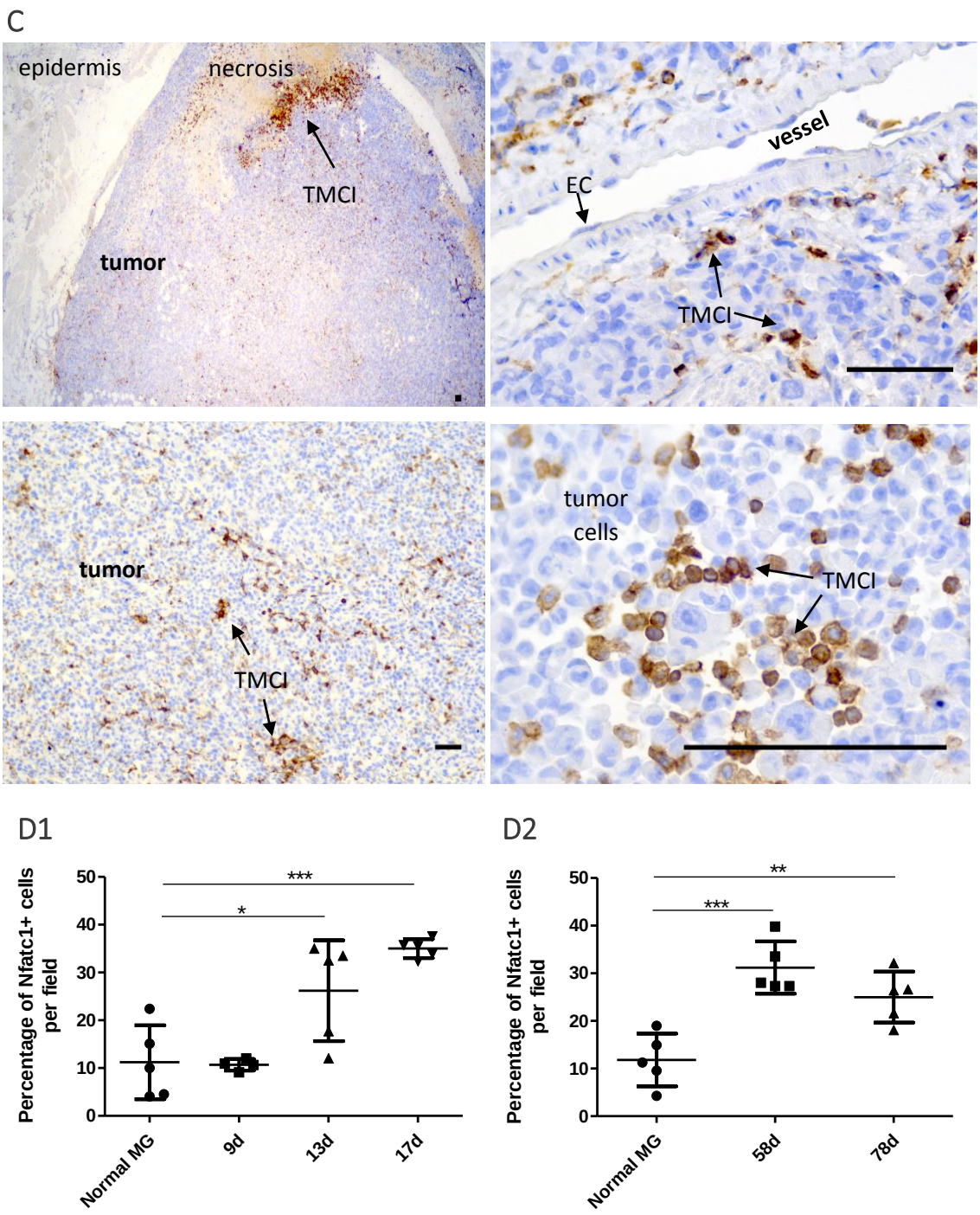


Figure 2.2 – Endothelial cells under tumor influence lose NFATC1 expression compared with endothelial cells from normal mammary gland

A. Representative image of NFATC1-positive cells in normal mammary gland (MG); B. Representative image of NFATC1-positive cells in 4T1 GFP-Luc syngeneic breast cancer mice model; C. Representative image of NFATC1-positive cells in MDA-MB-231 GFP-Luc human breast cancer xenograft mice. Magnifications of 25x, 100x, 400x and 1000x. Scale bar= 50µm; D. Quantification of NFATC1-positive cells. Representation of 10 hotspots' mean per mice per time point in 1. 4T1 GFP-Luc mice model and 2. MDA-MB-231 GFP-Luc mice model. Scored with ImmunoRatio. Data are means ± standard errors; n=5.

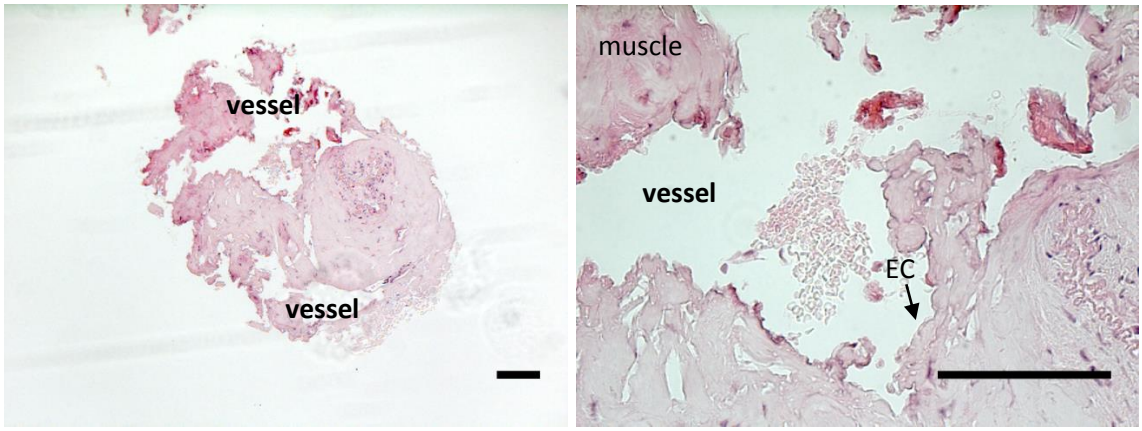
After validation of NFATC1's role in *in vivo* breast cancer mice models, we decided to look at the role of this transcription factor in vessels from breast cancer patients. We selected patients with unilateral breast cancer, with no previous chemo and radiotherapy, no familiar risk and who underwent a contralateral mammoplasty during the same surgery. Eight patients were selected at IPOLFG and PTV plus NGV were collected. An H&E from NGV and PTV were performed in order to confirm the correct macrodissection of blood vessels. No contamination with mammary gland tissue and tumor was observed. (Figure 2.3A)

We performed a stem cell transcription factor PCR array of two patients' PTV and NGV. As we can see in Figure 2.3B estrogen-receptor 1 (*ESR1*), *HOXC4*, *HOXC5*, *NR2F2*, *NOTCH2*, *PCNA*, *POU5F1*, retinoblastoma 1 (*RB1*), *SMAD2*, *SP1*, *NFATC*1 and *STAT3* mRNA levels were upregulated; *PPARG* and jun proto-oncogene (*JUN*) were downregulated in PTV when compared to NGV. Besides the great variance between the eight patients, Figure 2.3C shows that most of the identified deregulated transcription factors in the PCR arrays were further validated in standard RT-PCR analysis. The most consistent transcription factor with the same pattern of upregulation in all patients was NFATC1.

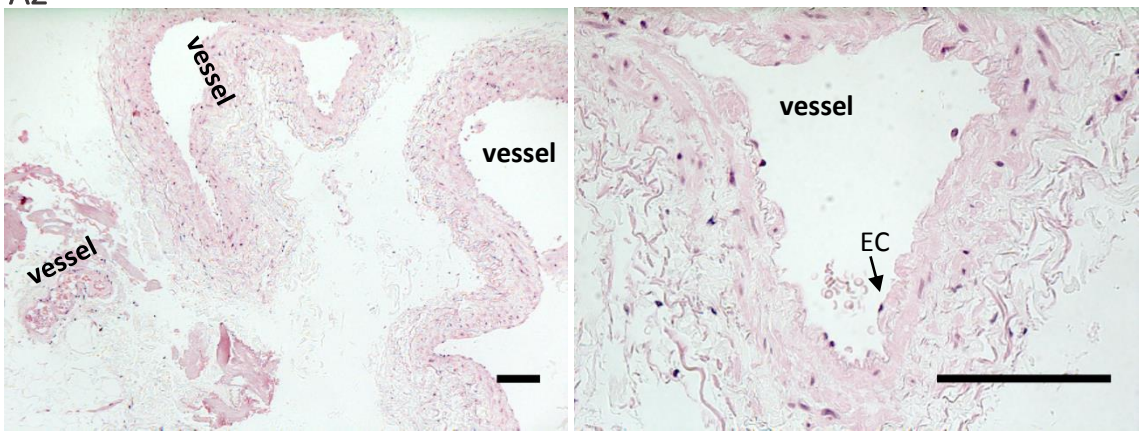
To check NFATC1 distribution in human breast tumors we performed an immunohistochemistry of fifteen breast cancer patient's slices randomly selected from the Pathology Archives of HSM-CHLN. Resident inflammatory mononuclear cells of normal mammary glands from the periphery of the tumor expressed NFATC1, as in mice models. NFATC1-positive TMCI infiltrated the tumor and were positively associated with high-grade breast tumors. Moreover, the quantification of NFATC1-positive hotspots per normal mammary gland or tumor areas showed a strong ratio between more aggressive tumors (characterized by high histologic grade, ER and PgR negative, HER2 and TP53 positive status) and the percentage of NFATC1-positive cells. (Figure 2.3E)

Together these results indicated a preponderant role for NFATC1 in vessel heterogeneity. NFATC1 was expressed in normal mammary gland ECs, in resident inflammatory mononuclear cells and in TMCI. NFATC1 expression was lost in ECs of breast cancer in both humans and mice models. NFATC1 seemed to be involved in the differentiation between a normal vessel and a vessel under the tumor influence.

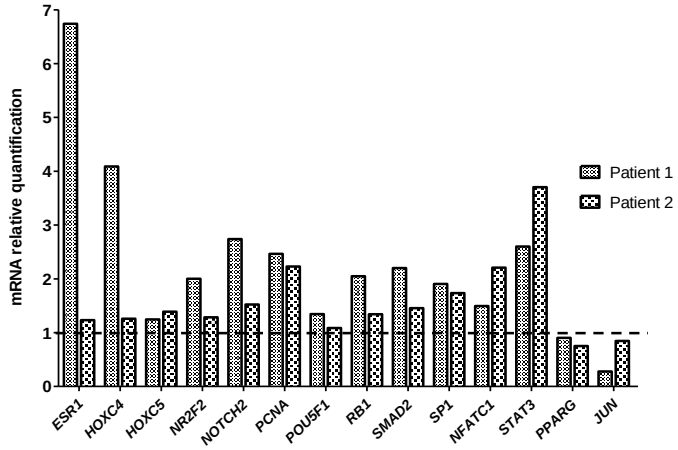
A1


vessel
vessel
muscle
vessel
EC

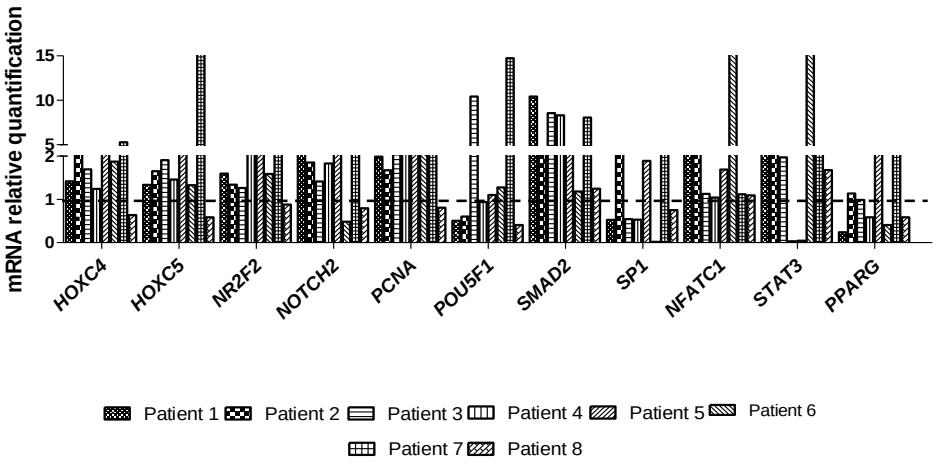
A2

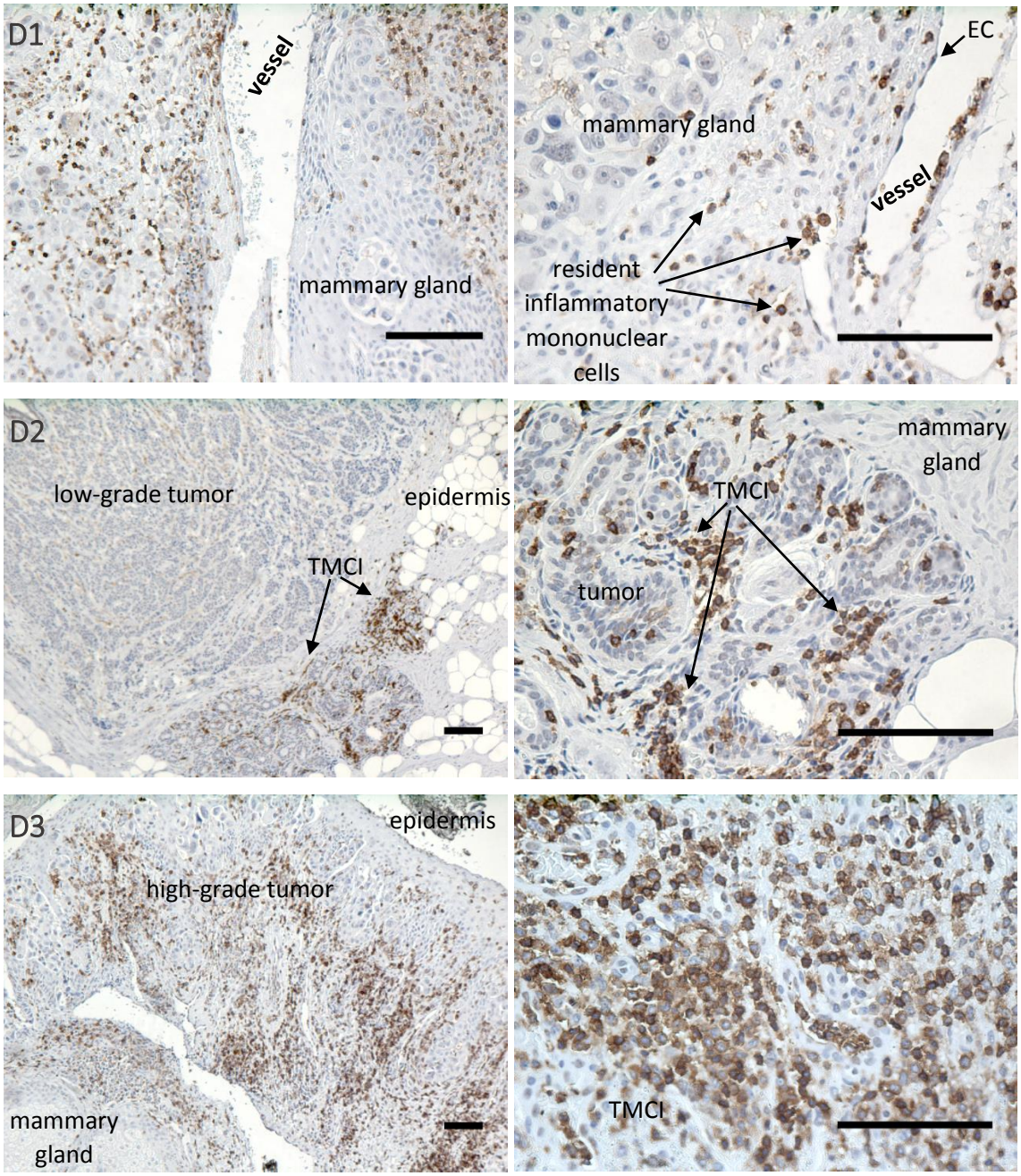

vessel
vessel
vessel
vessel
EC

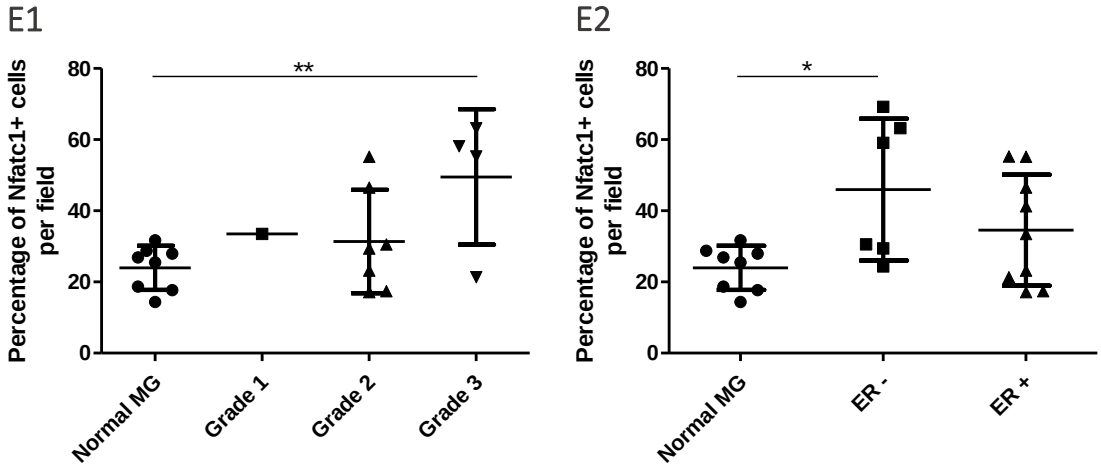
B


mRNA relative quantification
Patient 1
Patient 2
ESR1
HOXC4
HOXC5
NR2F2
NOTCH2
PCNA
POU5F1
RB1
SMAD2
SP1
NFATC1
STAT3
PPARG
JUN

C


mRNA relative quantification
HOXC4
HOXC5
NR2F2
NOTCH2
PCNA
POU5F1
SMAD2
SP1
NFATC1
STAT3
PPARG
Patient 1
Patient 2
Patient 3
Patient 4
Patient 5
Patient 6
Patient 7
Patient 8


D1
vessel
mammary gland
EC
mammary gland
vessel
resident
inflammatory
mononuclear
cells
D2
low-grade tumor
epidermis
TMCI
mammary
gland
TMCI
tumor
D3
epidermis
high-grade tumor
mammary
gland
TMCI


E1
Percentage of Nfatc1+ cells per field
80
60
40
20
0
**
Normal MG
Grade 1
Grade 2
Grade 3
E2
Percentage of Nfatc1+ cells per field
80
60
40
20
0
*
Normal MG
ER -
ER +

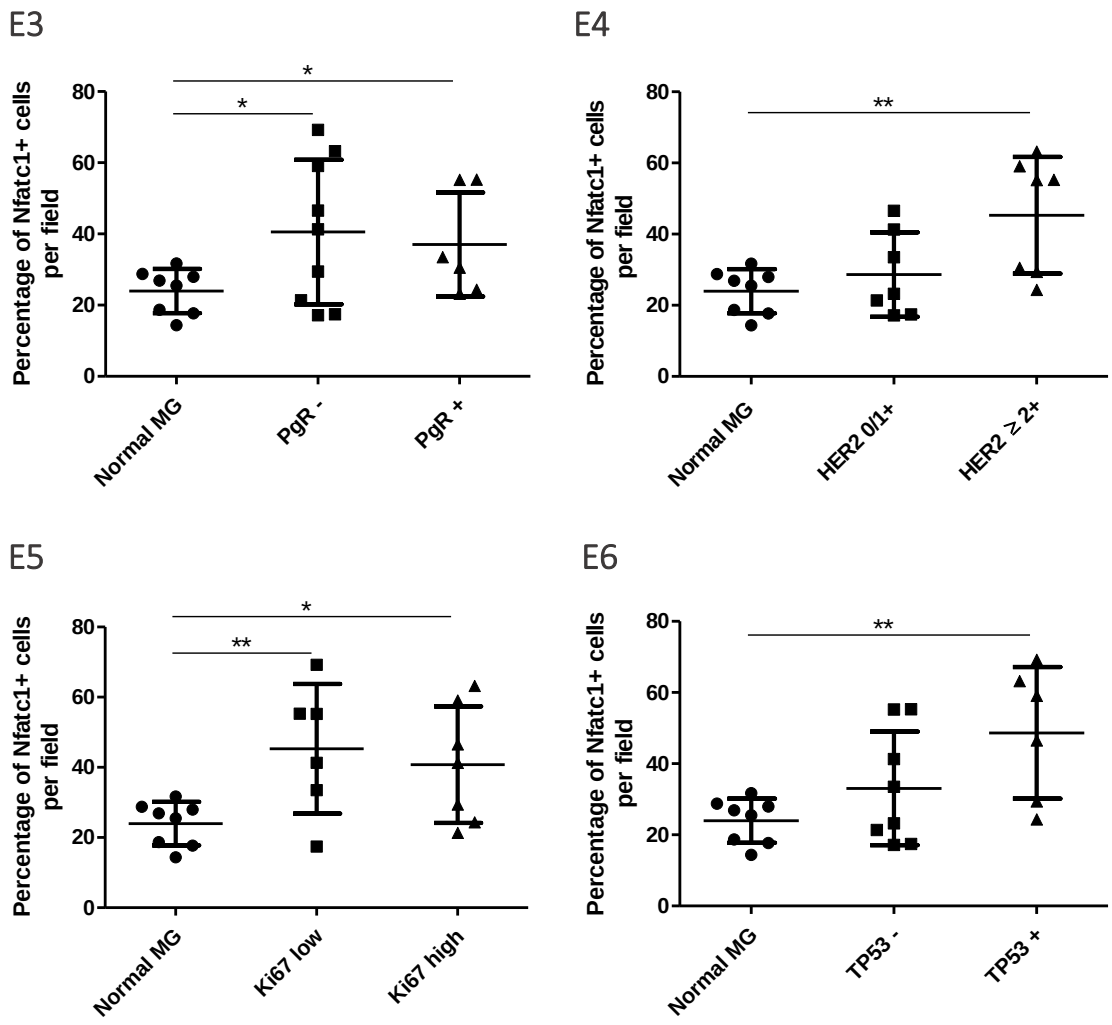


Figure 2.3 – NFATC1 expression is lost in endothelial cells of human breast cancer patients

A. H&E representative images of NGV (**1**) and PTV (**2**) isolated from breast cancer patients Magnifications of 100x and 400x. Scale bar= 50µm; **B.** Relative quantification of deregulated mRNA levels of stem transcription factors PCR array in 2 patients; **C.** Relative quantification of deregulated transcription factors in 8 patients; **D.** Representative image of NFATC1-positive cells **1** in normal mammary gland (MG), **2** low- and **3** high-grade breast tumors. Magnifications of 100x, 200x and 400x. Scale bar= 50µm; **E.** Quantification of NFATC1-positive cells. Representation of 10 hotspots' mean per normal mammary gland and tumor areas separately by **1.** Histologic grade, **2.** ER status, **3.** PgR status, **4.** HER2 status, **5.** Ki67 index and **6.** TP53 status. Scored with ImmunoRatio. Data are means ± standard errors; n=3-4 per group.

NFATC1 translocation to the nucleus in a VEGF-dependent manner: a new role for KDR

In order to investigate whether NFATC1 cellular localization in ECs responds to VEGF, we stimulated HMVEC and HUVEC with VEGF and tested different combinations with the KDR inhibitor IMC1C11[200] and calcineurin (CsA)[194]. We then collected cells for RT-PCR and performed immunofluorescence for NFATC1.

The percentage of nuclear NFATC1-positive cells clearly reveals a link between VEGF and NFATC1 signaling. When we added VEGF either HMVEC or HUVEC showed a 50% increase of nuclear NFATC1. The addition of αKDR was sufficient to lower the nuclear localization, even in the absence of VEGF stimuli. CsA inhibition restored the basal level of NFATC1 in the nucleus. Moreover, inhibition of both KDR and calcineurin in the presence of VEGF seemed to have a cumulative effect, with a reduction of nuclear NFATC1 around 50% in HMVEC and 80% in HUVEC compared to basal levels (Figure 2.4).

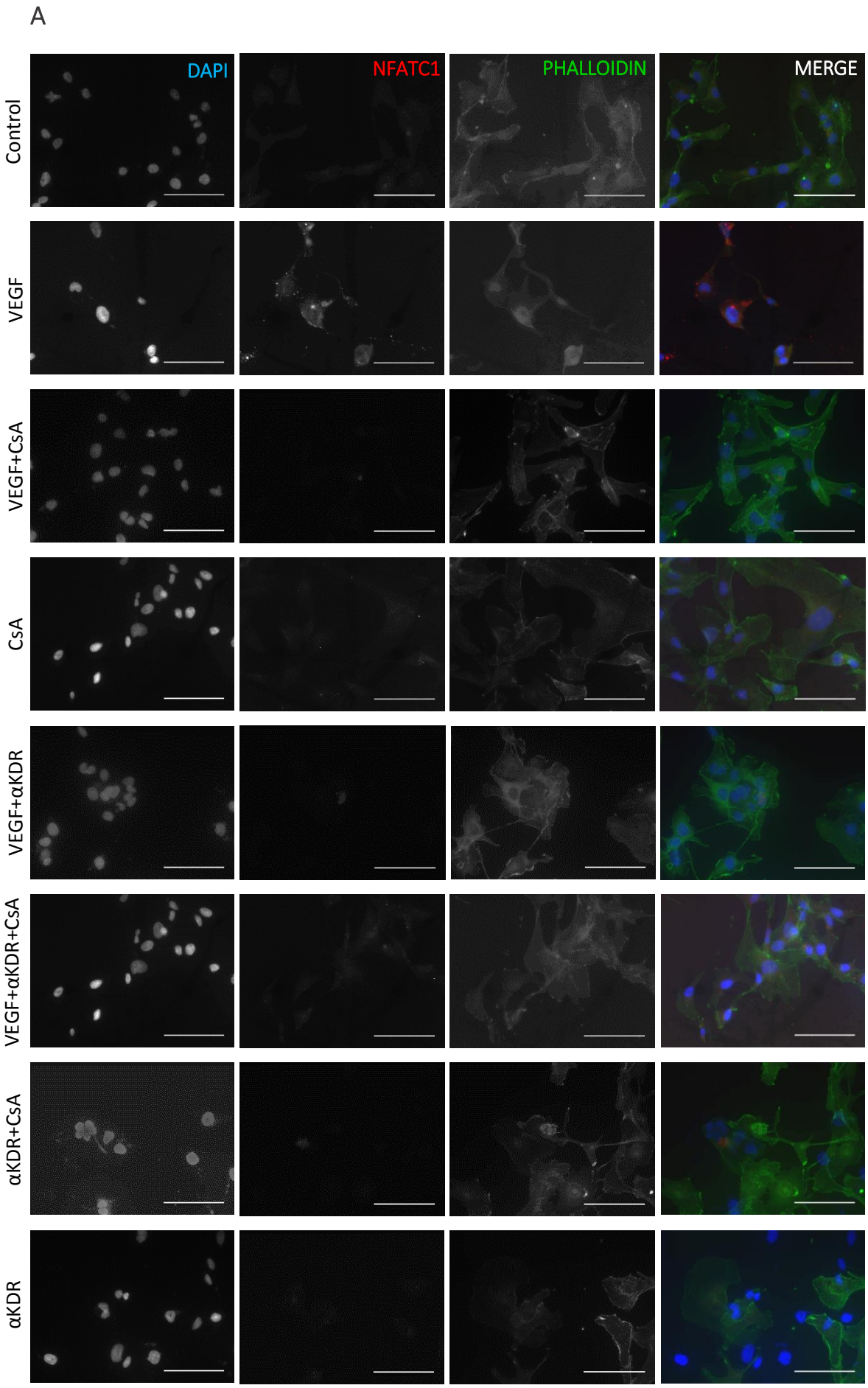
We further investigated the levels of *NFATC1*, *FLT1* and *KDR* mRNA expression in 1, 4 and 16h time points (Figure 2.5). Even though the high significance of nuclear NFATC1 localization demonstrated in immunofluorescence, NFATC1 RT-PCR analysis (Figure 2.5A) showed no relevant differences in *NFATC1* expression among all conditions. The only exceptions found were the upregulation of *NFATC1* levels in VEGF+CsA and VEGF+αKDR conditions in the 4h time point. The upregulation of *NFATC1* mRNA expression remained high in VEGF+αKDR for the 16h time point.

FLT1 mRNA levels (Figure 2.5B) were upregulated in VEGF after 1h, returned to basal levels at 4h and were downregulated at 16h. VEGF+CsA downregulated *FLT1* after 1h and 16h of incubation. CsA in the absence of VEGFA downregulated *FLT1* mRNA levels from 4h onwards. *FLT1* upregulation in VEGF+αKDR condition after 1 and 4h of incubation returned to normal levels at 16h. VEGF+αKDR+CsA upregulated *FLT1* in the 1 and 16h time point. *FLT1* levels were significantly downregulated in αKDR+CsA at the middle time point and downregulated in αKDR condition in the first and last time point.

KDR mRNA levels (Figure 2.5C) were consistently downregulated when CsA was alone or in combination with VEGF and/or αKDR in all time points. VEGF alone also diminished the *KDR* levels. VEGF+αKDR upregulated *KDR* level after 4 and 16h of incubation. αKDR alone diminished *KDR* levels only in the first time point.

Together these results suggest that NFATC1 is an EC transcriptional regulator, translocating to the nucleus in the presence of VEGF in a KDR- and calcineurin-dependent

manner. Furthermore, both inhibitors seem to have a cumulative effect on NFATC1 cytoplasm-nucleus localization.


A
DAPI
NFATC1
PHALLOIDIN
MERGE
Control
VEGF
VEGF+CsA
CsA
VEGF+αKDR
VEGF+αKDR+CsA
αKDR+CsA
αKDR

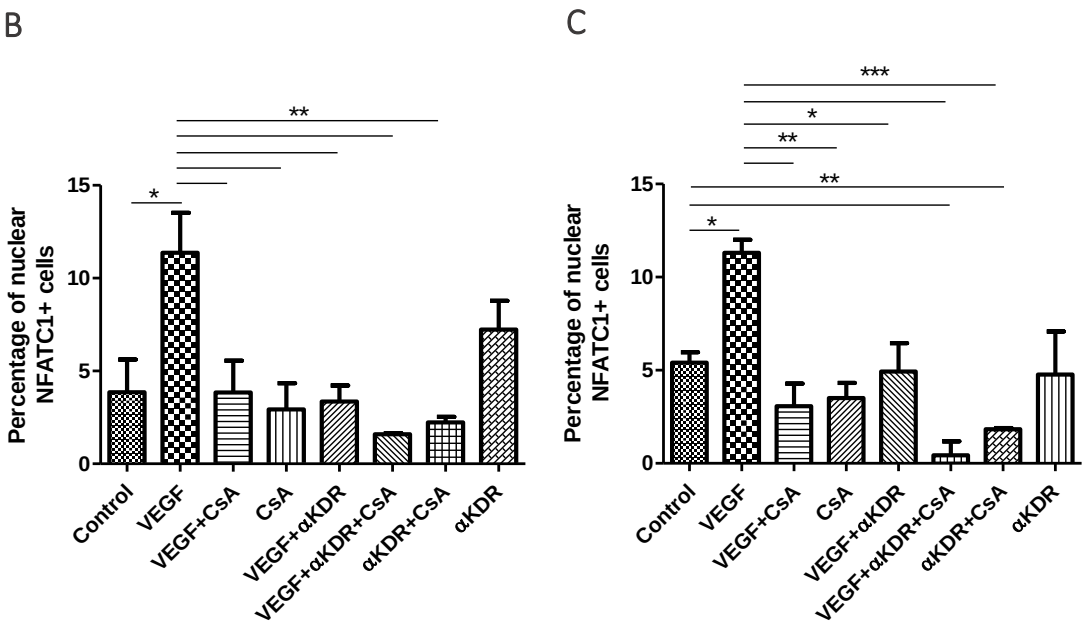
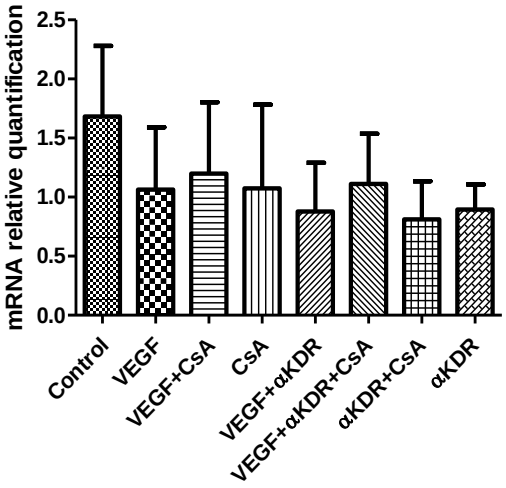


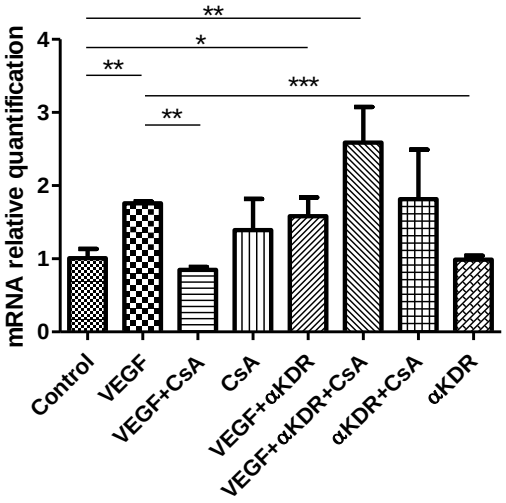
Figure 2.4 – NFATC1 nuclear localization increases with VEGF stimuli and both CsA and αKDR are sufficient to restore basal cytoplasmic levels

A. Representative images of NFATC1 immunofluorescence performed in HMVECs after 4h of cytokines and inhibitors incubation. Magnification of 400x and scale bar=100µm; B. Quantification of nuclear NFATC1-positive cells in HMVEC; C. Quantification of nuclear NFATC1-positive cells in HUVEC. Scored 100 cells per condition. Data are means ± standard errors.

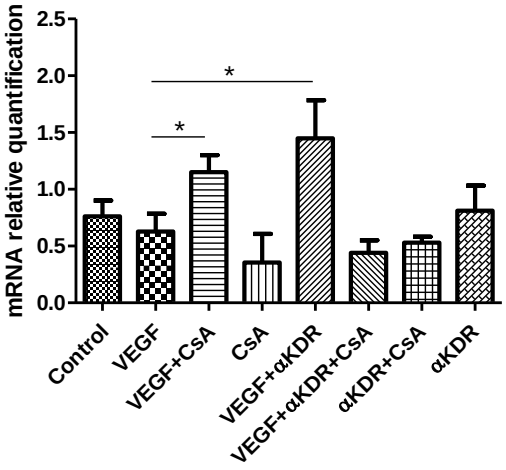
A1



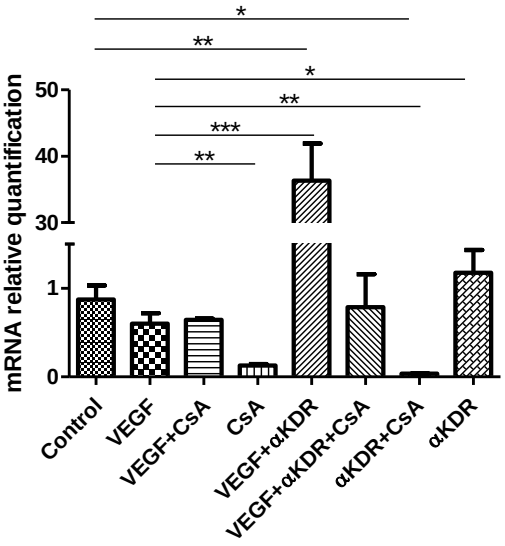
B1



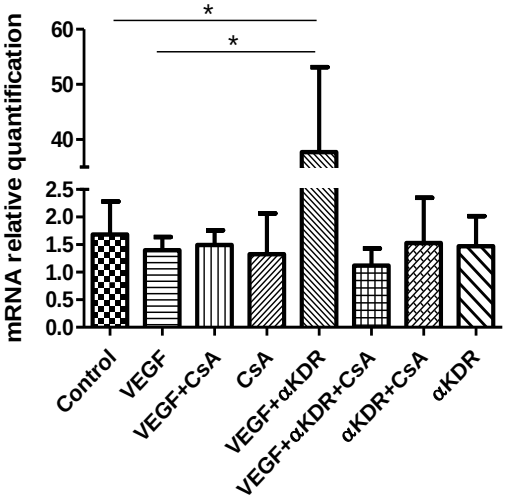
A2



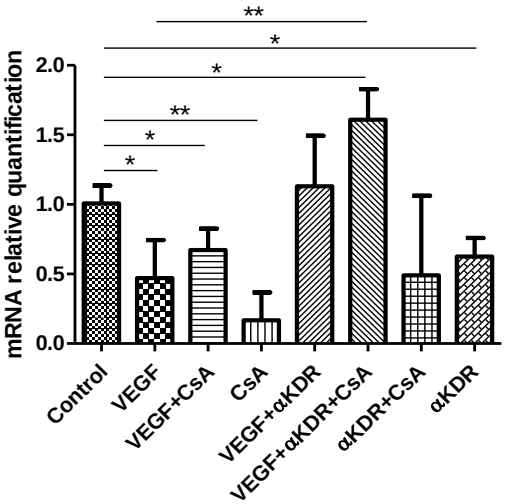
B2



A3



B3



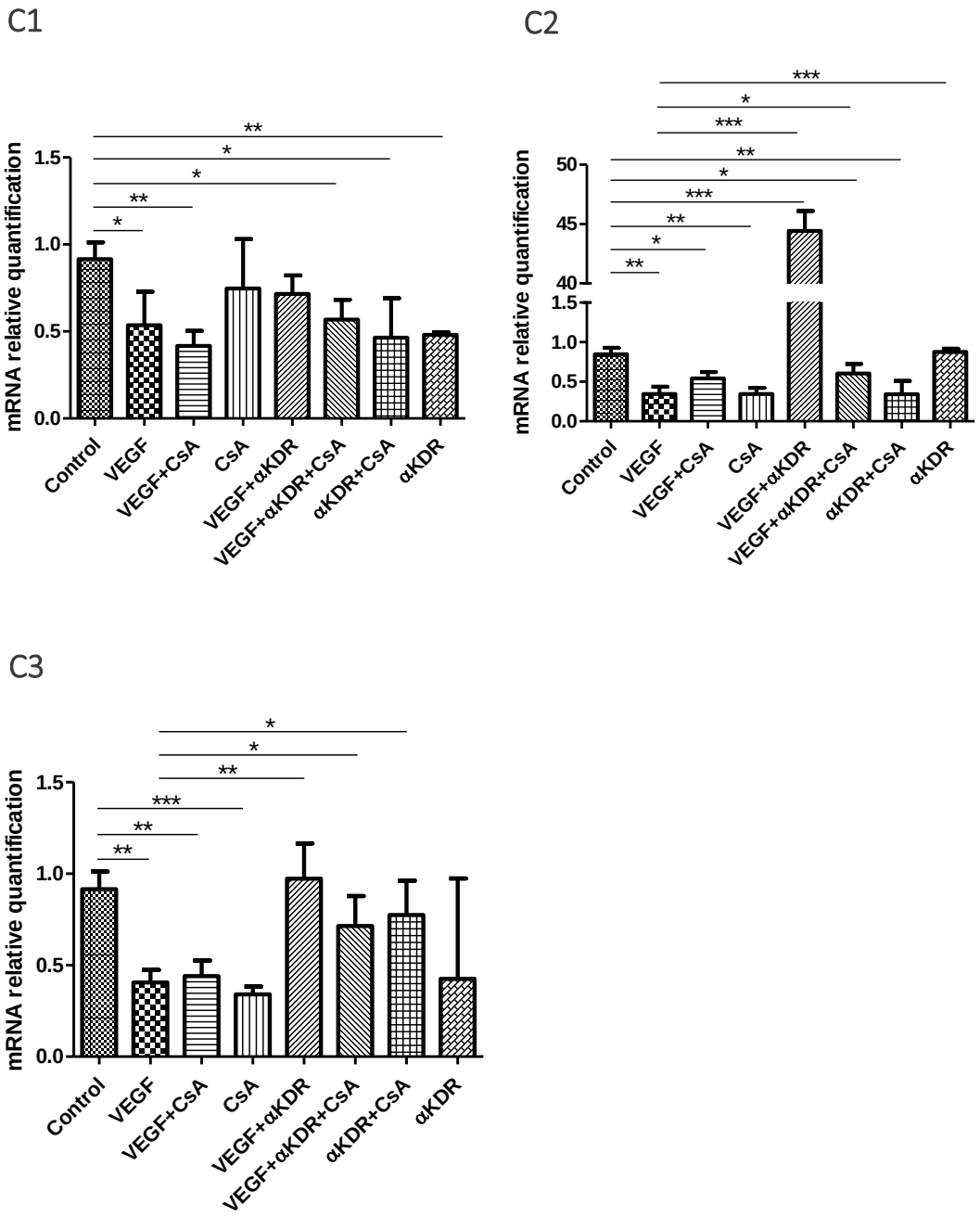


Figure 2.5 – VEGF, αKDR and CsA modify the mRNA expression levels of *NFATC1*, *FLT1* and *KDR*

A. Relative quantification of *NFATC1* mRNA; B. Relative quantification of *FLT1* mRNA; C. Relative quantification of *KDR* mRNA; Subcategory 1 refers to 1h, 2 to 4h and 3 to 16h time points. Normalization to 18S rRNA expression. Data are means ± standard errors.

Discussion

Since the NFAT family of transcription factors was first identified by Shaw *et al.*[201] as regulators of T cell activation, much more roles and mechanisms have been discovered and attributed to this family. From heart valve development to osteoclastogenesis, recruitment of bone-marrow derived macrophages, retinal vascular development and tumor microenvironment, these transcription factors are involved in many physiological and pathological conditions.[130,190-192,197,202]

Furthermore *Nfatc1* KO mice show a vascular impairment.[117,118] VEGF has also been strongly associated with NFATC1 and intracellular Ca^{2+} fluxes.[119,131,203] So, we hypothesized if in a highly VEGF-driven site as tumors, NFATC1 could have an essential role in tumor vascular heterogeneity and in the weak anti-angiogenic therapy response.

In our study we used 4T1 GFP-Luc syngeneic breast cancer and MDA-MB-231 GFP-Luc human breast cancer xenograft mice models in order to follow the tumor growth over time. Mice were sacrificed in early, middle and late tumor growth phases and PTV, ITV and NGV were isolated from different time points. The mRNA expression levels of some transcription factors were evaluated. NFATC1 has been involved in cancer, but the mechanism is still far from understood.[123,204] Minami *et al.*[205] showed that the NFAT pathway is activated specifically in lung ECs prior to the detection of tumor cells, being responsible for the support and expansion of micrometastases. In this work *Nftac1* was upregulated in PTV in the first time point of the syngeneic mice model and in ITV and PTV of the xenograft mice model, which may be correlated with the Minami study.

In our study we also observed a downregulation of *Hoxc4* mRNA levels in PTV and NGV in all time points from syngeneic model and an upregulation in the last time point of the xenograft model (Supplementary data). In spite of a lack of tumor-specific EC studies, some work in leukemia, lymphoma, skin tumors and MCF7 breast cancer mice models are concordant with this *Hoxc4* upregulation.[206-210] We verified an upregulation of *Hoxc5* levels in MDA-MB-231 GFP-Luc model, which corroborates studies in lymphomas and cervical cancers.[208,211]. Levels of *Nr2f2* were downregulated in syngeneic mice models, which contradicts the literature.[212,213] *Pcn*a mRNA levels were downregulated in our study, but some recent studies showed a positive correlation between tumor growth and its expression levels.[214-216] There has been recent evidence of *Pou5f1* upregulation in

cervical and breast cancers[217,218] and our study also revealed an upregulation in later time points of the xenograft breast cancer mice model. *Pparg* are directly related with angiogenesis in tumors, as previously demonstrated by Bishop-Bailey[219], but in our study was downregulated in the syngeneic mice model. *Stat3* transcription factor has been associated with some diseases, namely cancer[220], but in ECs of our syngeneic breast cancer mice model it was downregulated. Together, these changes in expression of transcription factor mRNA levels in ECs from normal mammary gland/tumors and the contradictory literature may be explained by the lack of tumor-specific EC studies. Most studies highlight the importance of tumor cells, instead of emphasizing the role of ECs.

Since *Nfatc*1 mRNA expression levels were deregulated in both syngeneic and xenograft mice models, we decided to verify their localization in tumors and normal mammary glands. In both models, NFATC1 localized in ECs and in resident mononuclear cells from normal mammary gland. On the contrary, tumor ECs lost their NFATC1 expression and TMCI became the major expressing cells. These differences are statistically significant and are evident even in the first time points. A revision of the literature showed NFATC1 expression in mononuclear cells associated with inflammatory conditions and osteoclastogenesis,[123,129,190,221] but as far as we know there is no association with TMCI. It has been demonstrated that NFATC induces granulocyte-macrophage colony-stimulating factor (GM-CSF) in ECs and monocytes[222], which could be an explanation for the higher percentage of infiltrated TMCI in tumor subsets.

Furthermore, we selected eight breast cancer patients in order to confirm our *in vitro* and mice data. The stem cell transcription factor PCR array and its successive RT-PCR validated some of our mice data. NFATC1 revealed to be the more consistent upregulated transcription factor in PTV compared to NGV. Furthermore random slices from breast cancer patients showed the same pattern of NFATC1 distribution as in mice models. Since ER and PgR negative breast cancers have a worse prognosis[223] and in our randomized samples these patients had more NFATC1-positive cells, we can conclude that NFATC1 is correlated with a higher aggressiveness. Moreover, HER2 status has been associated with a higher breast cancer recurrence rate.[224] Our results showed a higher percentage of NFATC1-positive cells in patients that overexpressed HER2. A recent study

of Feeley *et al.*[225] revealed that ER-positive, HER2-negative, Ki67-high and TP53-positive breast cancer patients have a worse prognosis compared to ER-positive, HER2-negative, Ki67-low and TP53-negative patients. Since the ER-negative, PgR-negative, HER2-positive and TP53-positive characterization of our patients is associated with a higher percentage of NFTAC1-positive cells, we can conclude that the quantification of NFATC1 could be a valuable biomarker for breast cancer aggressiveness. This is also supported by Pan *et al.*[123] who postulated that NFATC1 induces CSF in both monocytes and ECs, with TMCI located nearby to the tumor vasculature in order to facilitate the metastatic dissemination.

In *in vitro* studies we first described the 2-fold increase of NAFTC1 nuclear translocation with addition of VEGF. This was previously described by some authors and suggests an active role for this transcription factor.[119,121,130,226] With the inhibition of calcineurin mediated by CsA, previously described by Liu *et al.*[194], we saw a decrease of nuclear NFATC1, independently of VEGF addition. Jang *et al.*[195] showed that VEGF-driven migration of HPVEC was mediated by KDR in response to NFATC1. Jinnin *et al.*[198] demonstrated that NFATC1 suppresses the expression of FLT1 and constitutively activates KDR in infantile hemangiomas. RAF265, an inhibitor of KDR, also diminishes the expression of NFATC1 and showed a great efficacy in preventing osteoclastogenesis.[227] Together, these data indicates a strong connection between KDR and VEGF-NFATC1 signaling pathways. In our study we demonstrated that KDR inhibition in HMVEC and HUVEC is sufficient to restore NFATC1 cytoplasm localization, which suggests that this receptor blocks the entrance of VEGF and NFATC1 is no longer capable of being translocated. Furthermore the addition of αKDR and CsA has a cumulative effect in inhibition of NFATC1 nuclear localization, which supports an effective interplay of the VEGF-NFATC1 pathway mediated by KDR.

We further investigated the levels of *NFATC1*, *FLT1* and *KDR* mRNA expression in 1, 4 and 16h time points. As expected, we saw no difference in *NFATC1* expression among conditions and time points, which may indicate that NFATC1 only changes cytoplasm-nuclear localization, instead of increasing expression levels. The exception was VEGF+αKDR in the 16h time point which displayed a big increase of *NFATC1* mRNA levels. This may indicate a compensatory mechanism for lower NFATC1 nuclear localization.

FLT1 mRNA levels after addition of VEGF were downregulated in the last time point which suggests a delayed effect and is corroborated by the Jinnin *et al.*[198] study. *FLT1* upregulation in VEGF+αKDR condition in the last time points suggests the reported higher affinity of VEGF to FLT1, in this case because KDR was blocked by αKDR.[26] *KDR* mRNA levels were downregulated with VEGF addition, which may be explained by the excess of VEGF in the media and the actually low binding need of VEGF-KDR.

The interplay between FLT1, KDR and Ca^{2+} regulation (and consequently NFATC1 levels) is still far from understood. Quinn *et al.*[228] were the first to show that VEGF-KDR binding mediates the Ca^{2+} efflux and regulates homeostasis. Decades later Cudmore *et. al*[229] demonstrated that FLT1-KDR heterodimers are essential for Ca^{2+} homeostasis regulation in ECs. In sum these results suggest that NFATC1 is translocated to the nucleus in the presence of VEGF in a KDR- and calcineurin- dependent cumulative manner.

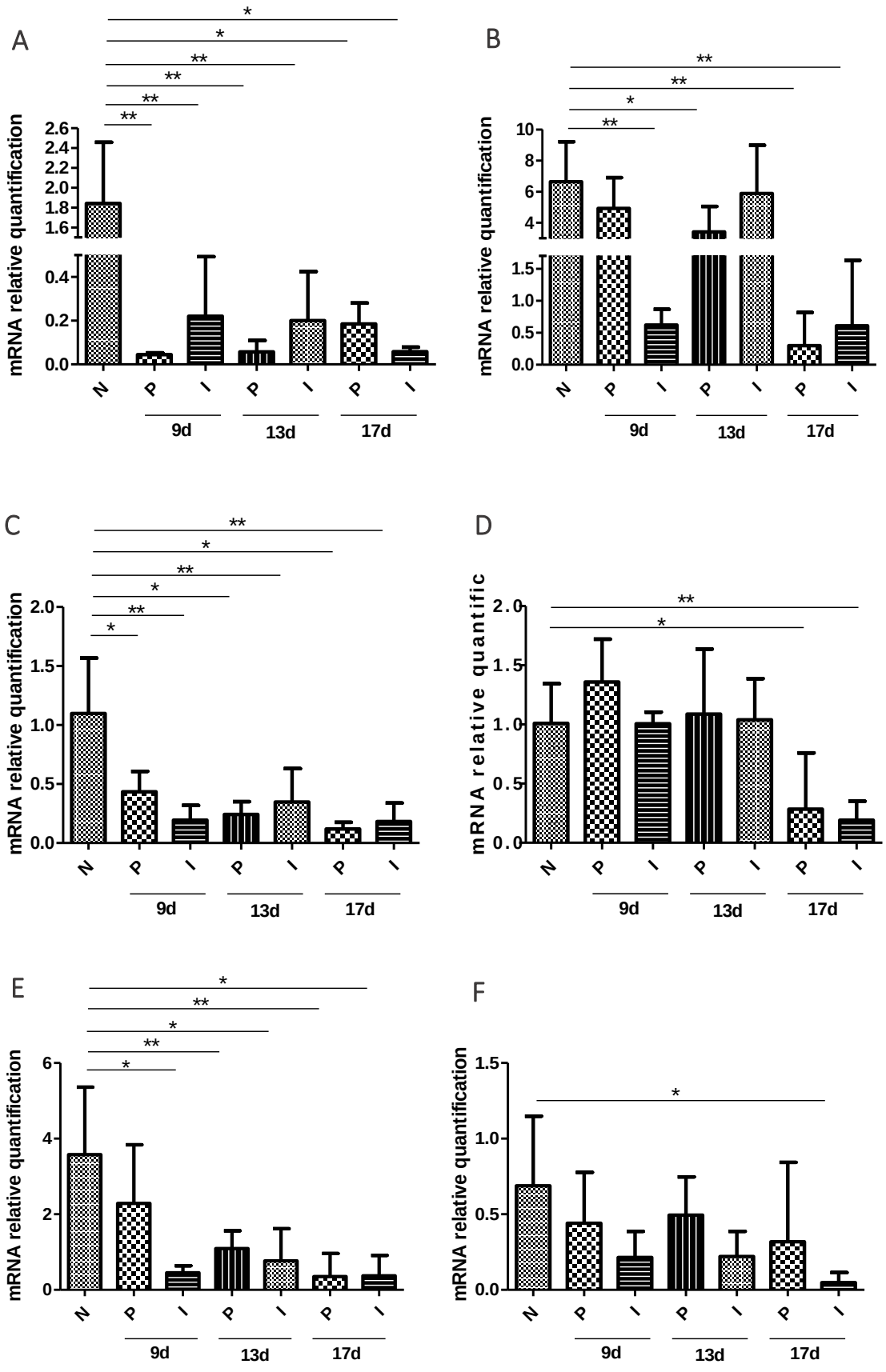
Collectively these results indicate that NFATC1 is essential for vessel heterogeneity. NFATC1 seems to be a key regulator of vessel identity, both *in vitro* and in NGV. Since anti-angiogenic therapy did not significantly improve the cancer patients' health[34,164,230,231], better strategies are needed. With our results we proved that NFATC1, a VEGF-driven transcription factor, loses expression in ECs under the tumor influence. This could be a possible explanation for the lack of response to anti-angiogenic therapies. NFATC1 is also expressed in TMCI, who is correlated with tumor aggressiveness in human breast cancer patients. In addition, we revealed a potential VEGF-KDR signaling role in NFATC1 effector function.

Supplementary data

Besides *Nfatc1*, we also identified some transcription factors in mice models that are deregulated in PTV compared to NGV, namely *Hoxc4*, *Hoxc5*, *Nr2f2*, *Pcna*, *Pou5f1*, *Pparg* and *Stat3*.

As seen in Figure S2.1 in the 4T1 GFP-Luc syngeneic breast tumor mice model we observed statistical significant downregulation of *Hoxc4* in PTV and ITV, compared to NGV in all time points. *Hoxc5* levels in PTV became downregulated after 13 days of tumor growth onwards. ITV expression was downregulated in the first and last time point. Levels of *Nr2f2* expression were consistently downregulated among time points and localization of tumor vessels. *Pcna* mRNA levels were downregulated in PTV and ITV only after 17 days of tumor growth. *Pou5f1* had a similar profile of downregulation as *Nr2f2*. mRNA levels of *Pparg* were downregulated in ITV in the last time point; *Stat3* was downregulated in ITV after 9 days and 13 days of tumor growth and in PTV in the middle time point.

In MDA-MB-231 GFP-Luc human breast tumor xenograft mice we observed an upregulation of *Hoxc4* mRNA levels in PTV and ITV in the last time point. An increase in *Hoxc5* expression in PTV after 78 days of tumor growth was observed. *Pcna* expression levels were downregulated in ITV in the 58 and 78 day time points. We observed a downregulation of *Pou5f1* in PTV and ITV at 58 days and an upregulation in PTV and ITV at 78 days of tumor growth. (Figure S2.2)


A
B
C
D
E
F
mRNA relative quantification
mRNA relative quantific
N
P
I
9d
13d
17d

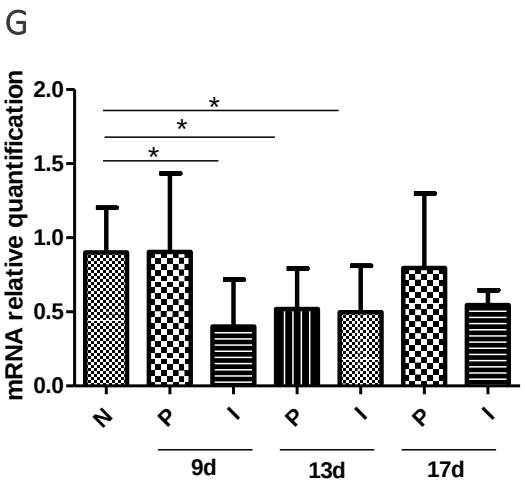


Figure S2.1 – Deregulation of transcription factor mRNA levels in PTV and ITV of 4T1 GFP-Luc syngeneic breast tumor mice model at 9, 13 and 17 days of tumor growth

A. Relative quantification of *Hoxc4* mRNA; B. Relative quantification of *Hoxc5* mRNA; C. Relative quantification of *Nr2f2* mRNA; D. Relative quantification of *Pcna* mRNA; E. Relative quantification of *Pou5f1* mRNA; F. Relative quantification of *Pparg* mRNA; G. Relative quantification of *Stat3* mRNA. N are NGV, P are PTV and I are ITV. Normalization to 18S rRNA expression. Data are means ± standard errors.

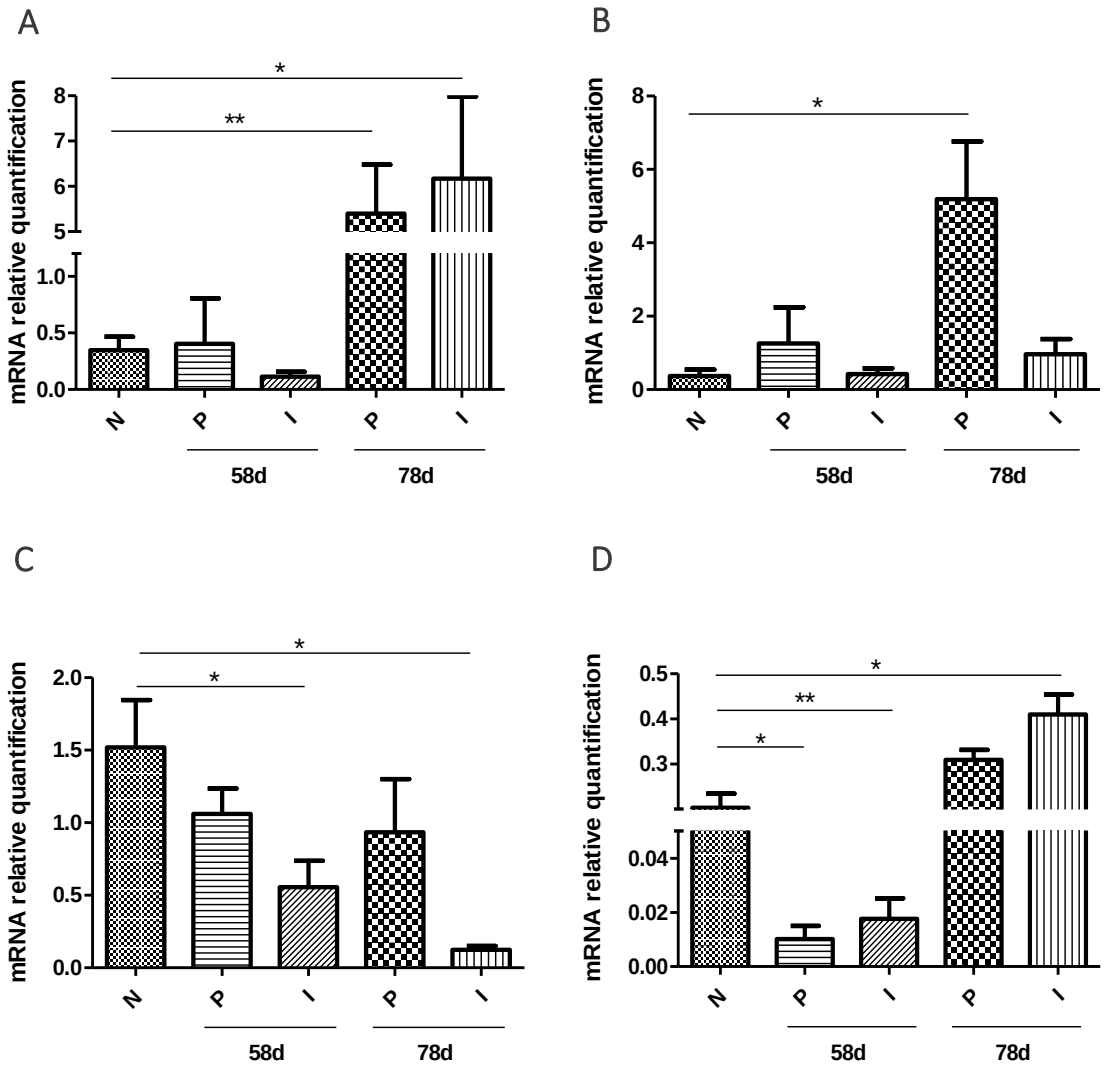


Figure S2.2 – Deregulation of transcription factor mRNA levels in PTV and ITV of MDA-MB-231 GFP-Luc human breast tumor xenograft mice at 58 and 78 days of tumor growth

A. Relative quantification of *Hoxc4* mRNA; B. Relative quantification of *Hoxc5* mRNA; C. Relative quantification of *Pcna* mRNA; D. Relative quantification of *Pou5f1* mRNA. N are NGV, P are PTV and I are ITV. Normalization to 18S rRNA expression. Data are means ± standard errors.

Chapter 3

SETD2 modulates endothelial cell biology and induces NFATC1 nuclear translocation in a VEGF-independent manner

Germana Domingues[1], Alexandra Vítor[1], Sérgio Almeida[1], Sérgio Dias[1]

1 Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa

Manuscript in preparation

Abstract

The activator/repressor function of histone methylation is influenced by the specific lysine residue where it occurs. Histone H3 trimethylated at lysine 36 (H3K36me3) is associated with transcriptional initiation, being a signature of activated genes. SET domain containing 2 (SETD2) is a methyltransferase associated with this H3K36 trimethylation. *Setd2* knockout (KO) mice are embryonic lethal due to vascular impairment, suggesting a role for SETD2 in endothelial cell (EC) biology. So, we hypothesized that SETD2 is important for the H3k36me3 signature on EC homeostasis.

We showed that *SETD2* knockdown (KD) increased the number of binucleated cell in HUVEC due to cell cycle errors, asymmetrical cell division and cell-cell fusion events. Moreover, given our interest in understanding vascular endothelial growth factor (VEGF) signaling and importance in tumor angiogenesis, we studied the regulation of a specific transcription factor (nuclear factor of activated T-cells cytoplasmic calcineurin-dependent (NFATC1)) in VEGF responsiveness and asked whether SETD2 could affect the VEGF-induced NFATC1 activation. Interestingly, we found that SETD2 is important for NFATC1 nuclear translocation (which is a measure of activation) independently of VEGF stimulation.

Together these results indicate that when SETD2 is absent ECs show an abnormal phenotype due to the increase in cell cycle errors and also the incidence of cell fusion events. *SETD2* KD also promoted NFATC1 nuclear translocation in a VEGF-independent manner, suggesting SETD2 interference with NFATC1 dynamics either at transcriptional or trafficking level.

Introduction

Transcriptional regulation is extremely important in survival, proliferation and angiogenic behavior of ECs. Histone methylation is one of the epigenetic mechanisms involved in this regulation. The specific lysine residue where mono-, di- or trimethylation occurs defines the activator/repressor function of this histone modification.[143]

H3K36me3 is associated with permissive chromatin and activated gene expression. It has also been involved in DNA repair and recombination, alternative splicing, transcriptional repression and DNA methylation.[232] SETD2 is a methyltransferase associated with H3K36 trimethylation. In yeast it is capable of all three methylations at H3K36, but in eukaryotes it is associated only with H3K36me3 methylation.[232]

SETD2 has a C-terminal domain that interacts with the largest subunit of RNA polymerase II.[233] In general, there is a shift from monomethylation to trimethylation of H3K36 in genes that underwent transcriptional initiation.[234] SETD2 is also involved in splicing regulation, for example H3K36me3 sites in fibroblast growth factor receptor (FGFR2) exon IIIb restrictly occurs in mesenchymal cells.[235] SETD2 is also associated with disease, namely cancer. Mutations in *SETD2* are one of the causes for sporadic clear renal cell carcinoma.[236] Accordingly, SETD2 has also been pointed as a tumor suppressor in some type of cancers.[232,237-239]

Surprisingly, *Setd2* KO mice die during embryonic development, due to severe vascular defects. Angiopoetin (*Angpt*), platelet-derived growth factor *(Pdgf)*, vascular endothelial growth factor *B (Vegfb)*, caveolin 1 (*Cav1*) and fms-related tyrosine kinase1 (*Flt1*) are some of the deregulated genes in this KO model.[144] So it is clear that SETD2 plays an important role in EC integrity and differentiation.

Although SETD2 has been extensively studied in tumoral context, its specific role in EC is far from being understood. The aim of this study was to characterize the changes in EC differentiation mediated by SETD2. We found that *SETD2* KD in ECs results in cytokinesis failure, asymmetrical cell division and cell-cell fusion.

The regulation of transcriptional genes by epigenetic modifications has also been explored, namely NFATC1small ubiquitin-like modifier (SUMO) modifications have been associated with T cell differentiation.[240] More recently, Martinez *et al.*[241] showed that NFATC1 immunoprecipitates with H3K36me3. Given our interest in understanding VEGF

signaling in detail, we have been studying the importance of transcription factors such as NFATC1 which are thought to act downstream of VEGF signaling, on EC biology. Therefore, in our study we investigated the connection between VEGF activation, NFATC1 and the H3K36me3 methyltransferase SETD2. We revealed that downregulation of *SETD2* leads to NFATC1 nuclear translocation in a VEGF-independent manner.

Methods

Cell Culture

Human umbilical cord vein endothelial cells (HUVEC) were cultured in EBM-2 media (Lonza, #190860) supplemented with 2% fetal bovine serum (FBS) (Life Technologies #26140-079) and 1x antibiotic-antimycotic (Life Technologies #15240-062). HUVEC were used until the fifth passage. For stimulation 50ng/mL VEGF (Sigma #V7259), 500µg/mL heparin (Sigma #H3149) and 1µg/mL cyclosporin A (CsA) (Sigma #30024) were used.

Human embryonic kidney 293 T antigen (HEK-293T) was cultured in Dulbecco's modified eagle medium (DMEM) (Life Technologies #11965-084) supplemented with 10% heat-inactivated FBS, 2mM L-Glutamine (Life Technologies #58030-081) and 1x antibiotic-antimycotic.

Transduction

SETD2 small hairpin RNA (shRNA) (Origene #TG317131) expression vectors are constructs with a specific sequence in pGFP-V-RS plasmid. For KD construct 1 the sequences GI2365733+GI365734 were used and for KD construct 2 the sequences GI365735+GI365736. shRNA cassette pGFP-V-RS plasmid and pCL-Ampho retrovirus packaging vector were used to transfect HEK-293T cell line. Transfection was made with X-tremeGENE small interfering RNAs (siRNA) transfection reagent (Roche # 04476093001), according to the manufacturer's instructions. Virions were collected 48, 60, 72 and 84h after transfection.

HUVEC were infected 4 times at 0, 12, 24 and 36h. ECs phenotype was examined with widefield fluorescent Zeiss Axiovert 200M microscope (Carl Zeiss MicroImaging) 4, 24, 48 and 72h after the first infection. Number of mononucleated, binucleated or more than binucleated cells were quantified in 10 photos per condition with ImageJ software (National Institutes of Health) and the statistical analysis was performed with GraphPad Prism 5 software.

In order to prove the KD efficacy we performed a western blot for H3K36me3, according to the Bio-rad general protocol. Supersignal west pico substrate (Thermo

Scientific #34077) was used as the chemiluminescent subtract, according to the manufacturer's instructions.

Cell staining

Cell tracker green CMFDA dye (Life Technologies #C2925) and cell tracker red CMTPX dye (Life Technologies #C34552) were used to stain HUVEC separately, according to the manufacturer's instructions. 1 day after staining, cells were seeded together in a proportion of 1:1. Transduction protocol was made on this cells.

Matrigel® tube-formation assay

BD Matrigel® basement membrane matrix (BD Biosciences #356230) was demonstrated to be effective in evaluating the angiogenic activity of ECs. ECs form tube-like structures on Matrigel® depending on the chosen conditions.[242] 200µL of Matrigel® per well was plated in a 24 well cell culture plate (Sigma #CLS3527) and incubated at 37ºC for 30 minutes. After matrix solidification, $2x10^5$ HUVEC resuspended in 100uL EMB-2 were seeded per well and incubated for 24h. After this first incubation, transduction with scramble and *SETD2* KD was performed as previously described.

EC networks were examined with widefield fluorescent Zeiss Axiovert 200M microscope (Carl Zeiss MicroImaging) 4, 24 and 48h after transduction. Number of bifurcations were quantified in 10 photos per condition with ImageJ software (National Institutes of Health) and statistical analysis was performed with GraphPad Prism 5 software.

Immunofluorescence

Cells were fixed in 2% paraformaldehyde for 20 minutes at 4ºC, blocked and permeabilized for 1h at room temperature with phosphate-buffered saline (PBS) + 1% bovine serum albumin (BSA) (Sigma #A7906) + 0.02% Triton X-100 (Sigma #X100). Primary antibodies incubation was made at room temperature for 2h and secondary antibody incubation for 1h at room temperature. Antibodies are listed on Table 3.1. One drop of Vectashield mounting medium with DAPI (Vector Laboratories #H-1200) was used for nucleus staining.

Cells were examined with a widefield fluorescent Leica DM5000B microscope (Leica Microsystems). Quantifications were made using a ImageJ software (National Institutes of Health) cell counter plugin and statistical analysis with GraphPad Prism 5 software.

Table 3.1 – Antibodies list. γH2Ax (gamma H2A family, member X)

ANTIGEN	BRAND	DILUTION
α TUBULIN	Sigma #T9026	1:100
γH2AX	Merck Millipore # 05-636	1:100
H3K36me3	Abcam #ab9050	1:100
NFATC1	BD Biosciences #556602	1:100
Alexa Fluor 594 donkey anti-mouse IgG	Life Technologies #A21203	1:1000

Statistical Analysis

Data were analyzed with GraphPad Prism 5 Software, using an unpaired two-tailed t-test with a confidence interval of 95%. Results are expressed as mean ± standard error.

* is P-value ≤ 0.05, ** ≤ 0.01 and *** ≤ 0.001.

SETD2 KD increases the occurrence of binucleated endothelial cells

In order to evaluate the effect of *SETD2* KD in HUVEC phenotype we performed a transduction with two different constructs as described in the methods section. After the fourth infection we observed the ECs phenotype and quantified a 2-fold increase in the number of binucleated cells in *SETD2* KDs (Figure 3.1). Thereafter we used KD1 construct since it showed more efficacy, both at inducing the EC binucleated phenotype and also at reducing H3K36me3 protein levels.

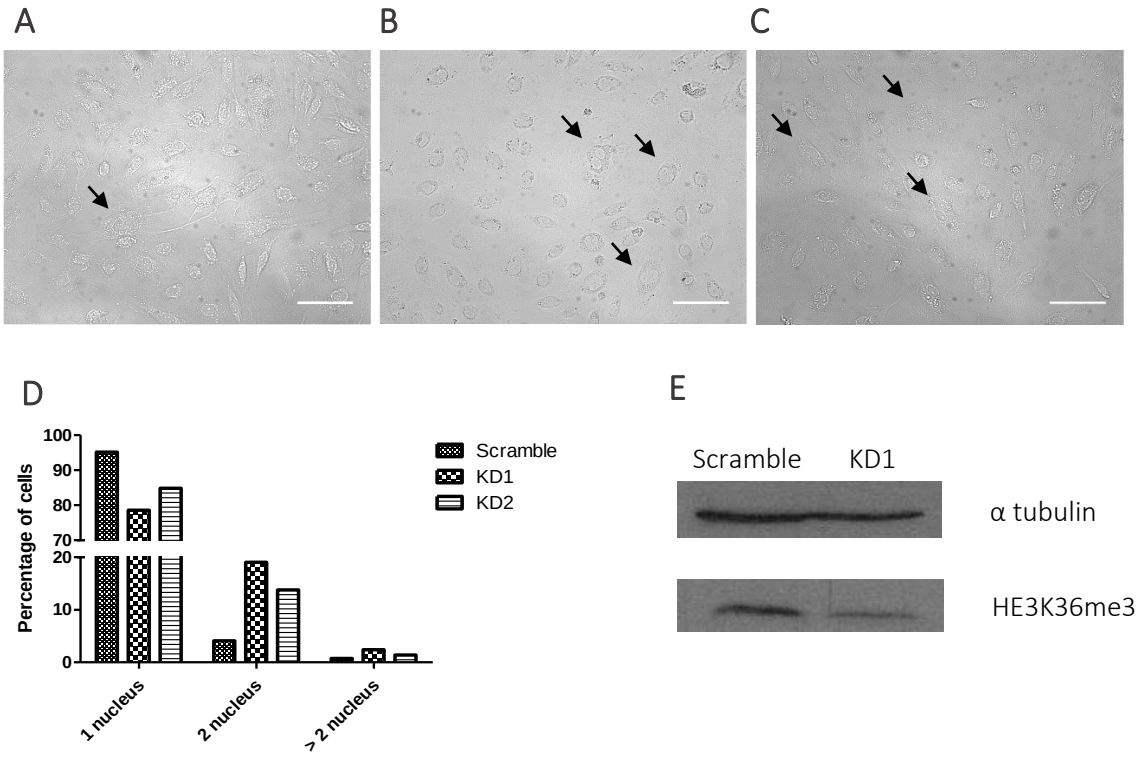


Figure 3.1 – *SETD2* KD infection results in an increase of binucleated endothelial cells

A. Representative image of scramble; **B.** Representative image of *SETD2* KD1; **C.** Representative image of *SETD2* KD2.Magnification of 200x and scale bar= 100µm; **D.** Quantification of mono-, bi and more than binucleated cells in scramble and *SETD2* KD1 and KD2 72h after the first infection. Scored of 10 photos per condition; n=1; **E.** Western blot for HE3K36me3.

Given the substantial increase in the number of binucleated cells upon *SETD2* KD, we hypothesized this could be due to cell cycle errors or cell-cell fusion. We took advantage of green and red dyes to label HUVEC and then established "mixed" cultures. As seen in Figure 3.2 we observed red-red, green-green and red-green binucleated cells. Binucleated cells labeled with different colors (red-green) can only be explained by cell-cell fusion events.

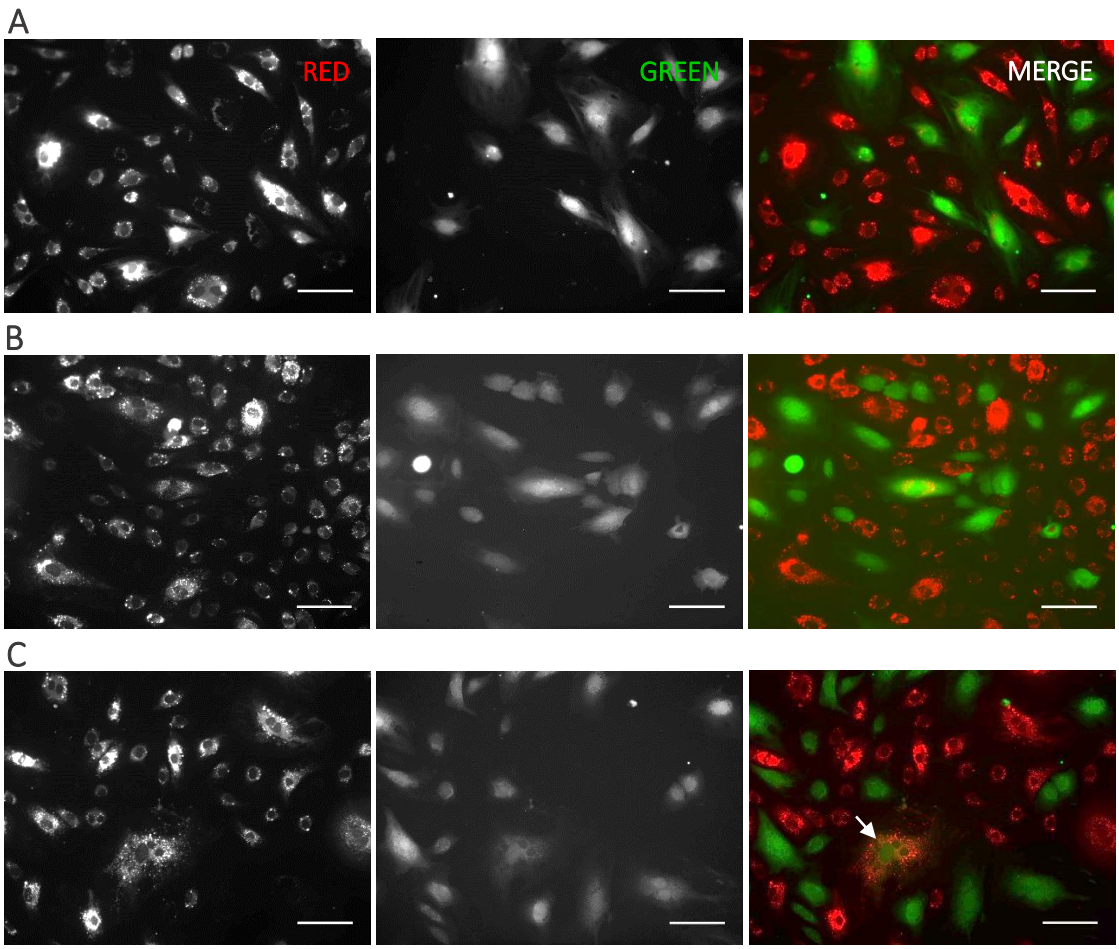


Figure 3.2 – *SETD2* KD promotes cell cycle errors and cell-cell fusion, which results in an increase of binucleated cells

A. Representative image of non-transduced cells; **B.** Representative image of scramble 48h after the first infection; **C.** Representative image of *SETD2* KD 48h after the first infection. Magnification of 200x and scale bar= 100µm

SETD2 KD binucleated cell phenotype is due to cell cycle errors and cell-cell fusion

To investigate what happened to the cells with *SETD2* KD, we performed an overnight video (please refer to the annexed videos). As can be observed in those videos, the binucleated phenotype is due to cell cycle errors, namely during cytokinesis, asymmetrical cell division and cell-cell fusion. This event occurred in a proportion of 3:3:1.

Further, we wanted to investigate if these errors could affect the HUVEC angiogenic capacity. For that we performed a Matrigel® tube-formation assay and as represented in Figure 3.3, there were no differences in the angiogenic capacity of non-transduced cells, scramble and *SETD2* KD. The next question was whether binucleated cells could lead to a more quiescent status, which could explain this increase in binucleated cells in the *SETD2* KD. We performed a γH2AX immunofluorescence in order to explore this hypothesis. As we can see in Figure 3.4, there were no significant changes in the staining of more than 5 loci-positive cells, which is considered the threshold for a quiescent status.

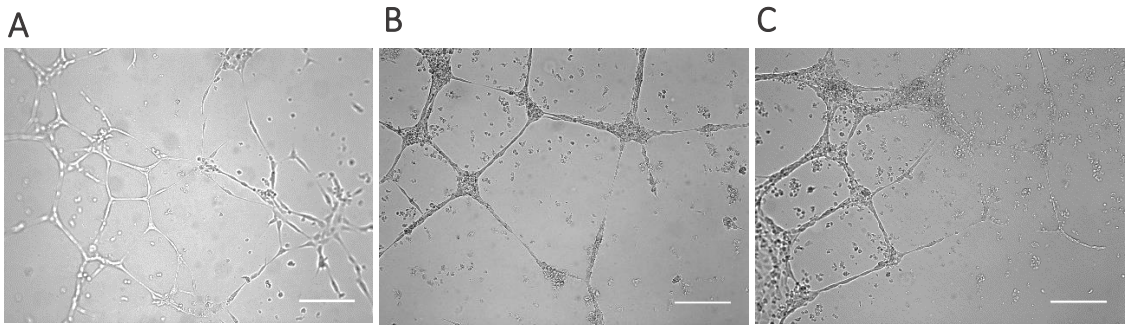


Figure 3.3 – Non-transduced, scramble and *SETD2* KD have the same ability to form endothelial cells network in a Matrigel® tube-formation assay

A. Representative image of non-transduced cells; B. Representative image of scramble; C. Representative image of *SETD2* KD. Magnification of 100x and scale bar= 100µm

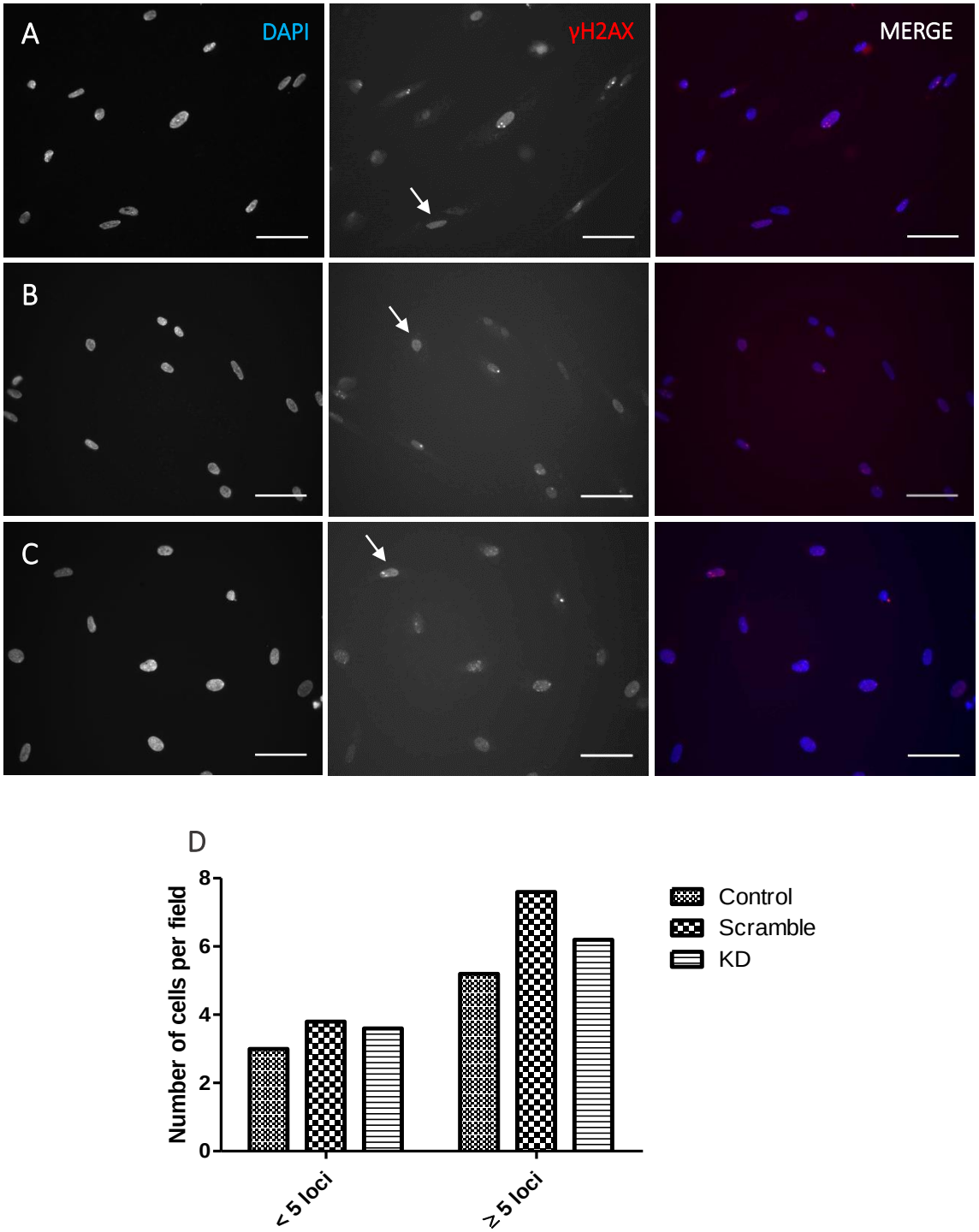


Figure 3.4 – *SETD2* KD does not change the quiescent status of endothelial cells

A. Representative image of non-transduced HUVEC; **B.** Representative image of scramble 48h after the first infection; **C.** Representative image of *SETD2* KD 48h after the first infection. Magnification of 200x and scale bar=100µm; **D.** Quantification of less or more than 5-positive loci per cell 48h after the first infection. Scored 100 cells per condition. Data represent means; n=1.

SETD2 KD promotes NAFTC1 nuclear translocation in a VEGF-independent manner

Epigenetic modifications have an essential role in transcription factor regulation. Then, we investigated a possible SETD2-mediated regulation mechanism of NFATC1, a VEGF-responsive transcription factor.

In order to assess the link between *SETD2* KD and NFATC1, we performed the same transduction protocol and immunofluorescence against NFATC1. As seen in Figure 3.5, *SETD2* KD increased the NFATC1 nuclear translocation, comparable to VEGF-positive control. NFATC1 nuclear translocation was also observed in scramble cells, which indicates that the transduction itself can affect the NFATC1 cell localization.

We further investigated the role of VEGF and CsA addition in NFATC1 nuclear translocation both in scramble and in *SETD2* KD. When we added VEGF to the scramble condition there was an increase in nuclear translocation and CsA inhibited this process as described in the literature. Interestingly, the nuclear NFTAC1 translocation in *SETD2* KD was annulled by VEGF and CsA, isolated and in combination (Figure 3.6).

To check if VEGF and CsA could change the number of binucleated cells we counted these events in the same time points as described earlier 4, 24 and 48h. As we can see in Figure 3.7, a statistically significant difference was observed between scramble and *SETD2* KD conditions. So in this KD condition, the addition of VEGF and CsA did not seem to alter the ECs phenotype.

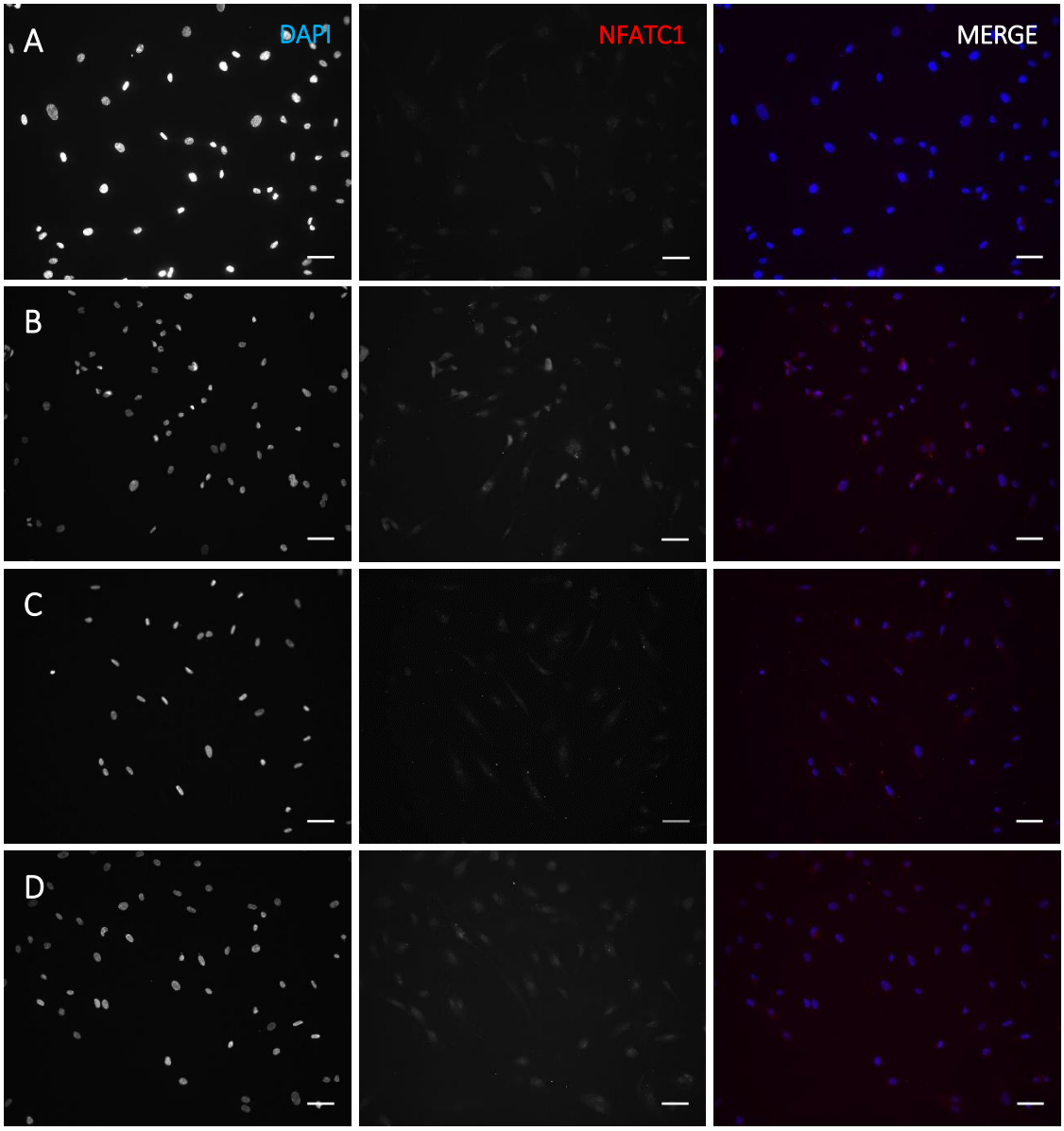
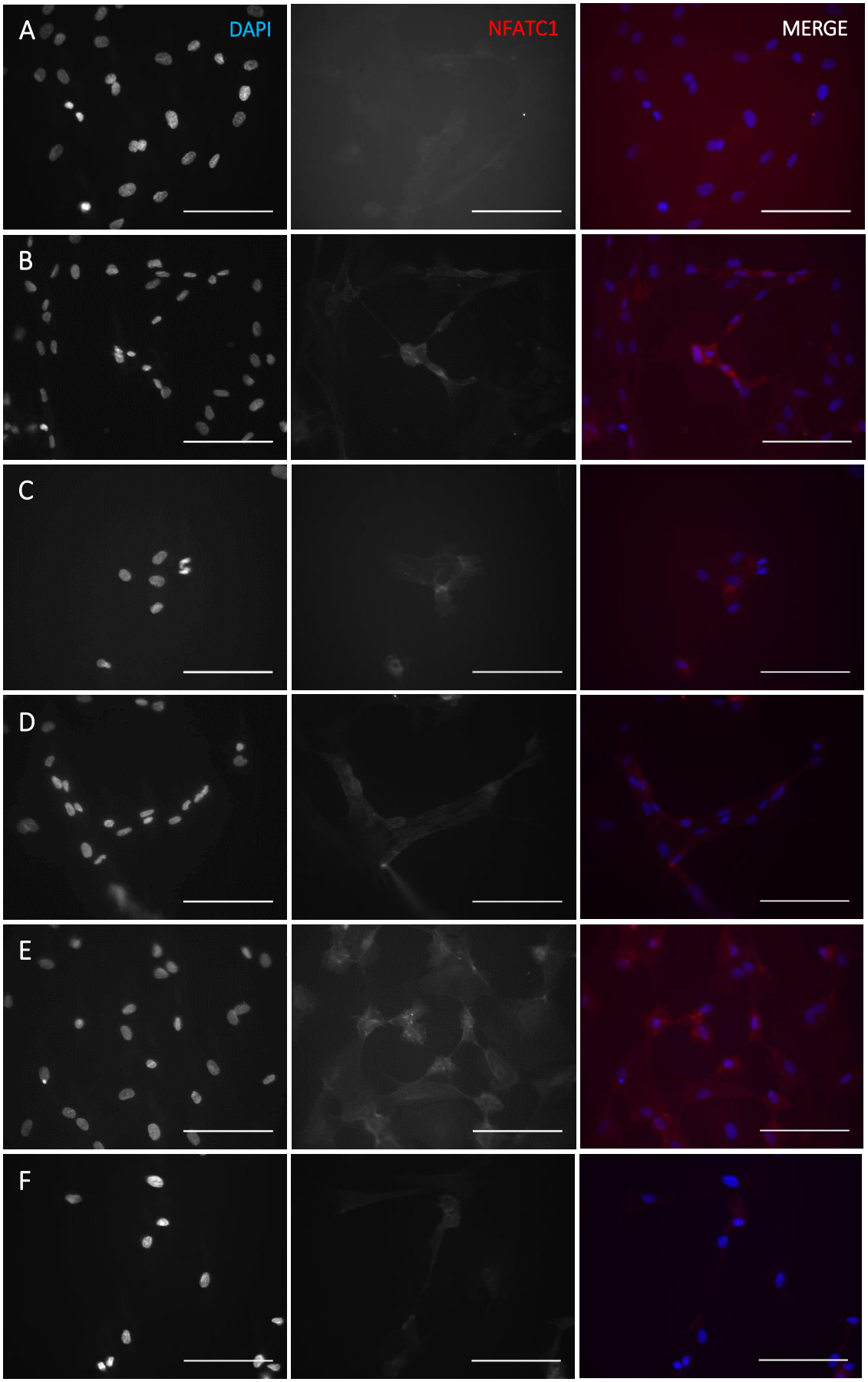


Figure 3.5 – NFATC1 nuclear localization increases with *SETD2* KD

A. Representative image of non-transduced HUVEC; **B.** Representative image of non-transduced HUVEC stimulated with VEGF; **C.** Representative image of scramble 48h after the first infection; **D.** Representative image of *SETD2* KD 48h after the first infection. Magnification of 100x and scale bar=100µm


A
DAPI
NFATC1
MERGE
B
C
D
E
F

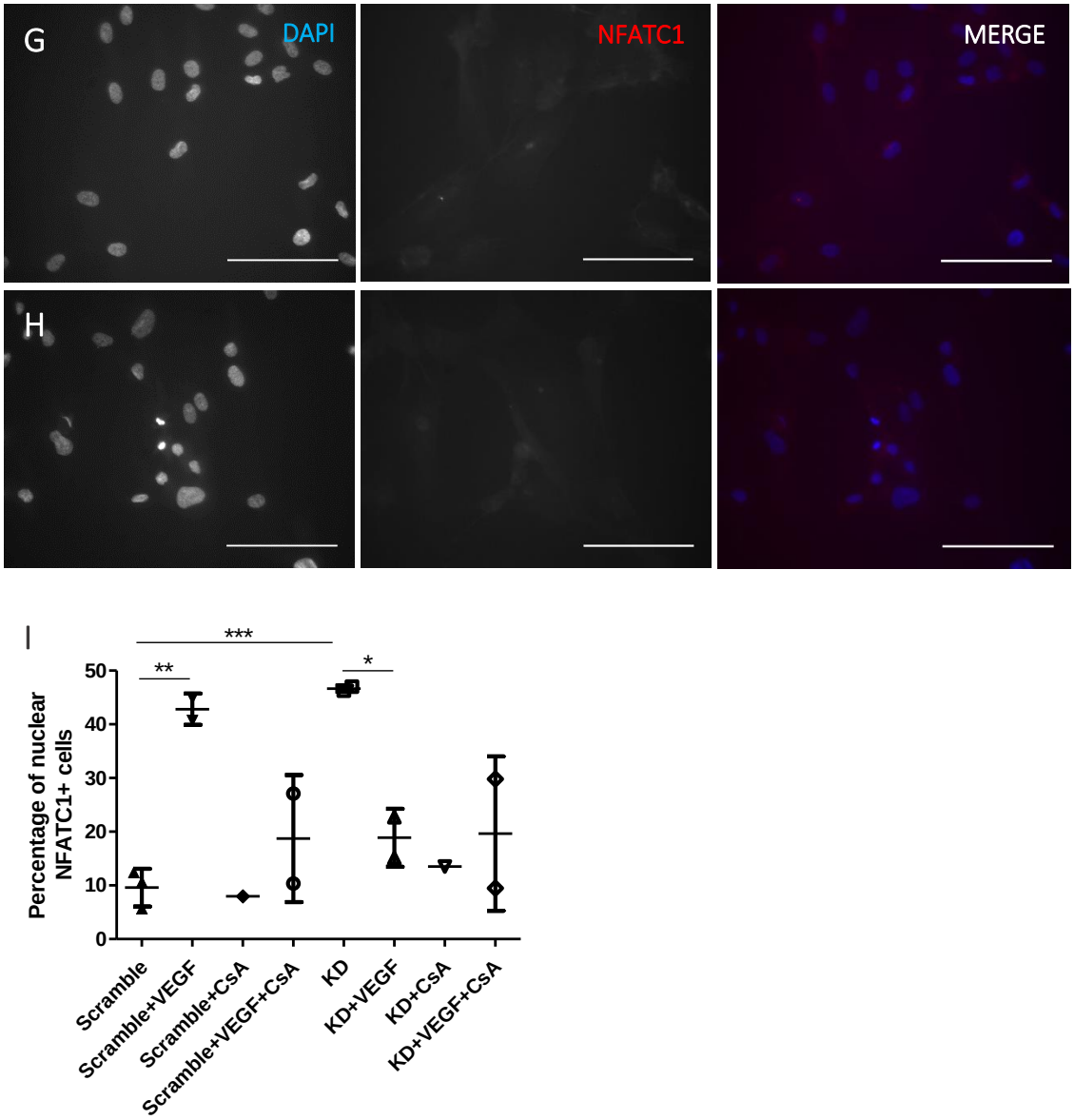


Figure 3.6 – NFATC1 nuclear localization increases in *SETD2* KD cells in a VEGF-independent manner

A. Representative image of scramble; **B.** Representative image of scramble+VEGF; **C.** Representative image of scramble+CsA; **D.** Representative image of scramble+VEGF+CsA **E.** Representative image of *SETD2* KD; **F.** Representative image of *SETD2* KD+VEGF; **G.** Representative image of *SETD2* KD+CsA; **H.** Representative image of *SETD2* KD+VEGF+CsA Magnification of 400x and scale bar=100µm; **I.** Quantification of nuclear NFATC1-positive cells. Scored 100 cells per condition. Data are means ± standard errors; n=2.

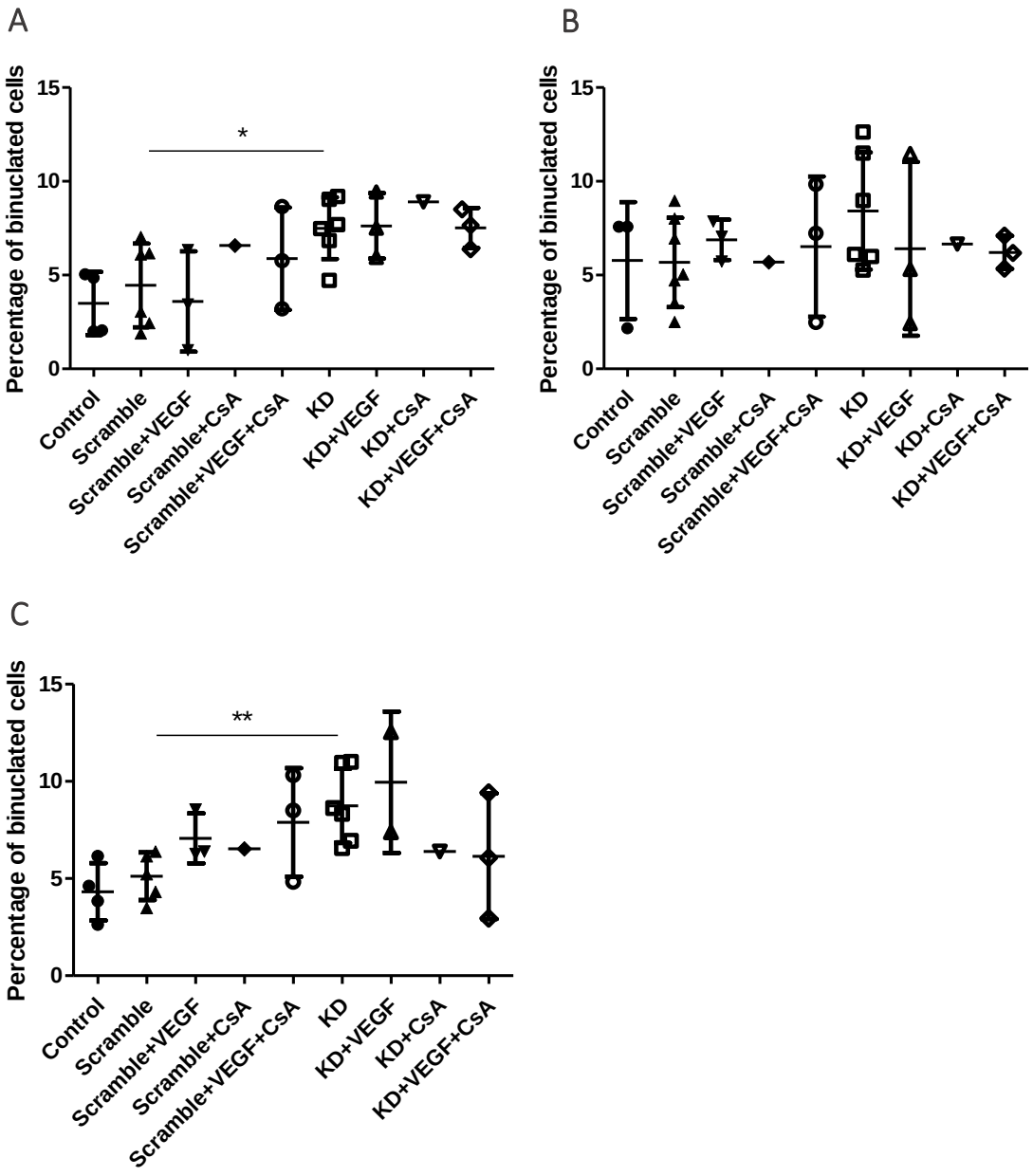


Figure 3.7 – VEGF and CsA addition does not change the number of binucleated cells induced by *SETD2* KD

Quantification of mono-, bi and more than binucleated cells **A**. 4h **B.** 24h and **C.** 48h after the first infection. Scored 10 photos per condition. Data are means ± standard errors; n=3.

Discussion

H3K36me3 is the trimethylated histone H3 at lysine 36. It is associated with the transcriptional activation. The methyltransferase associated with this signature is SETD2.[232,243] We already know that *Setd2* KO mice die due to vascular development failure.[144]

In this study we decided to investigate the role of this SETD2 in EC phenotype. We addressed this question by inducing a transient KD of *SETD2* in HUVEC with a commercially available shRNA. In the first observation 72h after the first infection it was clear that *SETD2* KD promoted an increase in the number of binucleated cells. Binucleated cells in an EC culture is not a new event, since Yamamot *et al.*[244] and Gimbrone *et al.*[245] had already shown the occurrence of this phenomena in culture. An *in vivo* correlation between binucleated ECs and vascular dysfunction was already highlighted by Tashiro *et al.*[246] This research group showed a higher number of multinucleated cells in an atherosclerosis murine model. Our observations and the previous studies indicate that *SETD2* downregulation changes H3K36me3 levels, which is important for EC homeostasis.

We further decided to assess what is the cause for this binucleated phenotype. We performed an overnight video of *SETD2* KD HUVEC and as we can see in annexed videos this phenotype is due to acytokinetic or asymmetrical cell division and cell fusion. This increased number of binucleated cells indicates that ECs underwent a transcriptional deregulation promoted by downregulated levels of SETD2, with consequent cell cycle errors. Cell fusion has been described by some authors, such as Welikson *et al.*[247], who showed that HUVEC can fuse with cardiomyocytes, starting expressing cardiomyocytes genes. As far as we know there is no literature supporting EC-EC fusion, being our study the first to prove that.

We made a Matrigel® tube-formation assay to prove that *SETD2* KD did not alter the capacity of ECs to form networks. In contrast with the published literature[144], the decrease in *SETD2* did not affect the angiogenic ability of HUVEC as assessed by using this particular *in vitro* assay. Since γH2AX downregulation is associated with a quiescent cell status[248], we also performed a γH2AX immunofluorescence and proved that the *SETD2* KD had no effect in the EC quiescent status.

Together these results suggest that SETD2 is an essential methyltransferase that maintains H3K36me3 at required levels in order to prevent DNA damage and consequent cell cycle errors or cell-cell fusion.

There is recent evidence of epigenetic modifications in the regulation of transcription factors, namely NFATC1. For instance, NFATC1 can be SUMOylated which leads to nuclear translocation and subsequent histone deacetylation, suppressing the transcription of target genes.[240] Kim *et al.*[249] showed that the genesis of osteoclasts is induced by the tumor necrosis factor superfamily, member 11 (TNFSF11)-mediated NFATC1 acetylation, being H3K27me3 signature responsible for TNFSF11-induced osteoclast differentiation.[250] Pham *et al.*[251] showed that chromatin remodeling of NFATC1 is essential for the growth of diffuse large B-lymphomas. Moreover, Youn *et al.*[252] showed that lysine (K)-specific demethylase 8 (KDM8) is a co-regulator of NFATC1, repressing osteoclastogenesis genes via its hydroxylase activity. KDM8 is mainly known as a histone demethylase for histone H3K36me2.[253] So, based on the published literature, epigenetic modifications have an essential role in NFATC1 effector function. Therefore, we hypothesized that NFATC1 can be regulated by SETD2, a methyltransferase responsible for H3K36me3 signature.

We cultured scramble and *SETD2* KD cells with VEGF, CsA and VEGF+CsA. When we added VEGF to cells in the scramble condition NFATC1 translocation increased as expected.[119,195] The addition of CsA restored the basal levels of nuclear NFATC1, as literature describes.[194] In contrast, *SETD2* KD alone promoted NFATC1 nuclear translocation which seemed to involve a VEGF-independent mechanism. This could indicate an essential role for SETD2 in NFATC1 translocation: when the levels were diminished, the H3K36me3 signature levels in HUVEC was also lower and the NFATC1 nuclear translocation was higher, with consequent transcriptional regulation. In turn, this could represent a repressor function for SETD2. H3K36me3 has been widely associated with transcriptional activation, but a study in yeast revealed a transcriptional repression function for Setd2.[232,254] We then scored the number of binucleated cells with VEGF, CsA and VEGF+CsA and confirmed that this cytokine and calcineurin inhibitor were not changing the previously described EC phenotype.

Together these results indicate that *SETD2* downregulation is essential for NFATC1 nuclear translocation, which suggests a repressor function of H3K36me3 signature in HUVEC.

As a whole, the work described in this chapter indicates two essential roles for SETD2 in ECs. First SETD2 appears to be essential to maintain a "mononucleated cell phenotype", the *SETD2* KD resulting in an increased number of binucleated cells due to acytokinetic or asymmetrical cell division and cell-cell fusion. Second, *SETD2* downregulation promotes NFATC1 nuclear translocation in a VEGF-independent manner, indicating that SETD2 interferes with NFATC1 dynamics either at transcriptional or trafficking level.

Chapter 4

Estrogen derivatives show anti-angiogenic activity

Germana Domingues[1], Manuel Bicho[1], Sérgio Dias[1], Fernanda Marques[2]

1 Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa **2** Centro de Ciências e Tecnologias Nucleares, Instituto Superior Técnico, Universidade de Lisboa

Manuscript in preparation

This chapter is part of a manuscript in preparation. It is the result of a collaboration with Fernanda Marques from Centro de Ciências e Tecnologias Nucleares. The global objective is to evaluate the cytotoxicity, genotoxicity and anti-angiogenic properties of estrogen derivatives.

Abstract

Several breast tumors are hormone responsive, being anti-estrogens a suitable and successful therapeutic strategy. Their efficacy as anti-angiogenic chemo-adjuvants was also demonstrated in breast cancer patients.[255,256] Tamoxifen is the most used drug in clinics, but fulvestrant and exemestane are also currently used.[257-259]

The aim of this study was to evaluate the anti-angiogenic potential of estrogen (as a positive control), ethinylestradiol, fulvestrant, 2-methoxyestradiol, tamoxifen and exemestane. We performed a Matrigel® tube-formation assay, aiming to analyze at first the interference of drugs in the formation of endothelial cell (EC) network and second their ability to abrogate the established network. We observed that fulvestrant, 2-methoxyestradiol and tamoxifen are the major anti-estrogen drugs that are able to inhibit EC network formation as well as to disrupt established networks. These results are in accordance with the published literature.

Together these results confirm that anti-estrogens have anti-angiogenic capacity as seen by their ability to interfere with the formation and maintenance of EC networks. Furthermore, the quantitative assay established here revealed to be a useful technique for large-scale anti-angiogenic drug tests.

Introduction

Estrogen is the most common sex hormone in women, affecting the development and function of the female reproductive system. Estrogen is also involved in the development of other tissues, including the adipose, nerve, musculoskeletal and cardiovascular system.[260] Estrogen molecules are a group of steroid compounds with 3 natural forms: estradiol (E2), estrone and estriol.[261] Concerning the therapeutic approach, conjugated and semi-synthetic estrogens were developed to be used in breast cancer patients as inhibitors of estrogen receptors (ER). Estrogen and its derivatives interacts with 3 ER subtypes: ERα and ERβ and the non-classical estrogen receptor GPR30.[261]

Estrogens are known inducers of EC migration, proliferation and survival.[256,262] Some studies in ECs showed that ERα is the most potent effector of estrogen signaling pathway and hypoxia-inducible factor 1, subunit alpha (HIF1A) mediates the activation of vascular endothelial growth factor (VEGF) pathway by estradiol.[260,263,264] Consequently, anti-estrogens have been extensively used as hormone therapy in breast cancer patients.[257,259] However, their anti-angiogenic role can be explored not only in ER-positive but also in ER-negative breast cancer cells, as Iyer *et al.*[265] demonstrated in a recent study. Circulating E2 is sufficient to recruit pro-angiogenic bone-marrow derived cells that infiltrate the tumor and promote disease progression.[265]

The estrogen derivatives used in this study were: ethinylestradiol (EE2), fulvestrant (F), 2-methoxyestradiol (2ME), tamoxifen (Tam) and exemestane (Exe).

Ethinylestradiol (EE2) is a 17β-estradiol derivative used in many formulations of oral contraceptives. Lippert *et al.*[266] showed that E2 and EE2 have the same pro-angiogenic effect in human umbilical cord vein endothelial cells (HUVEC). Although, Andozia *et al.*[267] suggested that E2 and EE2 act differently on ECs dysfunction. While E2 activates endothelial nitric oxide synthase (eNOS)-mediated vessel protection, EE2 does not. This mechanism can explain cardiovascular effects of contraceptive pills.

Fulvestrant (F) is a blocker of ER dimerization and DNA binding. It increases ER turnover and inhibits ER nuclear uptake. Tan *et al.*[268] showed that F in combination with bevacizumab is safe and tolerable, but in a phase III clinical study no statistical differences in progression-free survival were seen. Another study showed that F may enhance the

effects of a tyrosine-kinase inhibitor in non-small cell lung cancer by blocking estrogen-driven activation of the epidermal growth factor receptor (EGFR) pathway.[258]

2-methoxyestradiol (2ME) is the major biologically active metabolite of E2, being generated by any tissue that produces or uptakes E2. 2ME has no affinity to ERs, though it binds to tubulin and HIF1A, affecting their target genes, such as *VEGF*s. It has been demonstrated that 2ME inhibits the motility, migration and adhesion of circulating inflammatory cells [269] as well as proliferation in smooth muscle cells and ECs. Moreover, Fotsis *et al.*[270] demonstrated that 2ME administration inhibits angiogenesis *in vivo* and suppresses tumor growth.

Tamoxifen (Tam) is a non-steroidal molecule that binds ER with high affinity.[260] First it was used as a post coital contraceptive medication and is nowadays used as an adjuvant of breast cancer chemotherapy.[271] Tamoxifen specifically inhibits the effect of VEGF in EC proliferation, through transforming growth factor beta (TGFB) signaling pathways.[255,272]

Exemestane (Exe) is a synthetic steroidal aromatase (the enzyme that synthetizes estrogen). Such as tamoxifen, exemestane is used in adjuvant chemotherapy in breast cancer patients, however with reduced efficacy.[259]

Based on previous knowledge, in this study we aimed to characterize the anti-angiogenic efficacy of E2, EE2, F, 2ME, Tam and Exe. Using an *in vitro* Matrigel® tube-formation assay we compared the effect of the different compounds in both EC network formation and destruction. We concluded that F, 2ME and Tam are the major anti-angiogenic drugs.

Methods

Cell Culture

HUVEC were cultured in EBM-2 media (Lonza, #190860) supplemented with 2% fetal bovine serum (FBS) (Life Technologies #26140-079) and 1x antibiotic-antimycotic (Life Technologies #15240-062). HUVEC were used until the fifth passage.

Matrigel® tube-formation assay

BD Matrigel® basement membrane matrix (BD Biosciences #356230) was demonstrated to be effective in evaluating the angiogenic activity of ECs and to test anti-angiogenic drugs. ECs form tube-like structures on Matrigel® depending on the chosen conditions.[242] 200µL of Matrigel® per well was plated in a 24 well cell culture plate (Sigma #CLS3527) and incubated at 37ºC for 30 minutes. After matrix solidification, $2x10^5$ HUVEC resuspended in 100uL EMB-2 were seeded per well and incubated for 2 and for 8h. After this first incubation, media was removed and new fresh media with previously validated drugs' half of IC50 for breast cancer cell lines was replaced. (Table 4.1).

Apart from the control group, all the conditions were supplemented with 50ng/mL VEGF (Sigma #V7259), 500µg/mL heparin (Sigma #H3149) and 25ng/ml fibroblast growth factor (FGF)-basic human (Sigma #SRP3043).

EC networks were examined with widefield fluorescent Zeiss Axiovert 200M microscope (Carl Zeiss MicroImaging) 8 and 16h after drugs addition. Number of nuclei, number of bifurcations and bifurcations' length was quantified in 10 photos per condition with ImageJ software (National Institutes of Health). Statistical analysis was performed with GraphPad Prism 5 software.

Statistical Analysis

Data was analyzed with GraphPad Prism 5 Software, using unpaired two-tailed t-test with a confidence interval of 95%. Results are expressed as mean ± standard error. * is P-value ≤ 0.05, ** ≤ 0.01 and *** ≤ 0.001.

Table 4.1 – Estrogen derivatives, their solvent, IC50 for MCF7 breast cancer cell line and ½ IC50 used for endothelial cells

DRUG	SOLVENT	IC 50 for MCF7 (µM)	½ IC50 (µM)
E2	ethanol	34.7±6.5	17.35
EE2	ethanol	33.6±12	16.8
F	DMSO	15.4±3.9	7.7
2ME	DMSO	37.8±14	18.9
Tam	DMSO	30.6±8.5	15.3
Exe	DMSO	153±50	76.5

Results

Fulvestrant, 2-methoxyestradiol and tamoxifen are potent anti-angiogenic drugs

In order to evaluate the anti-angiogenic potential of estrogen derivatives, we performed a Matrigel® tube-formation assay. Drugs were added to culture media 2 and 8h after seeding ECs, in order to evaluate their effects in preventing EC network formation (2h) and in destroying the established EC network (8h).

In Figure 4.1 it is observed that F (Figure 4.1G), 2ME (Figure 4.1H) and Tam (Figure 4.1J) are able to abrogate EC network formation after 8h of incubation.

We further performed a quantification of bifurcations per condition in 10 different fields. We made the experiment in triplicates. VEGF+FGF was used as a positive control. Solvents were used as baseline control, ethanol for E2 and EE2 and DMSO for F, 2ME, Exe and Tam. The addition of F, 2ME and Tam 2h after cell seeding interfered with the network formation. (Figure 4.2A) These compounds were also capable to destroy the established EC network (Figure 4.2B). In a later time point Exe and EE2 also showed capacity to destroy EC networks.

The measured bifurcation length in 2ME, Exe and Tam was significantly shorter in both time points, as we see in Figure 4.3. 2ME and Tam showed a concordant pattern with the previous quantification of bifurcations.

To check if this effect of estrogen derivatives on the angiogenic network was due to unexpected toxicity-induced cell death, we counted the number of nuclei per field. Even being an indirect measure, there was no difference in the number of nuclei (data not shown). This leads us to believe that the observed differences are due to the anti-angiogenic capacity of the compounds and not due to toxicity-induced cell death.

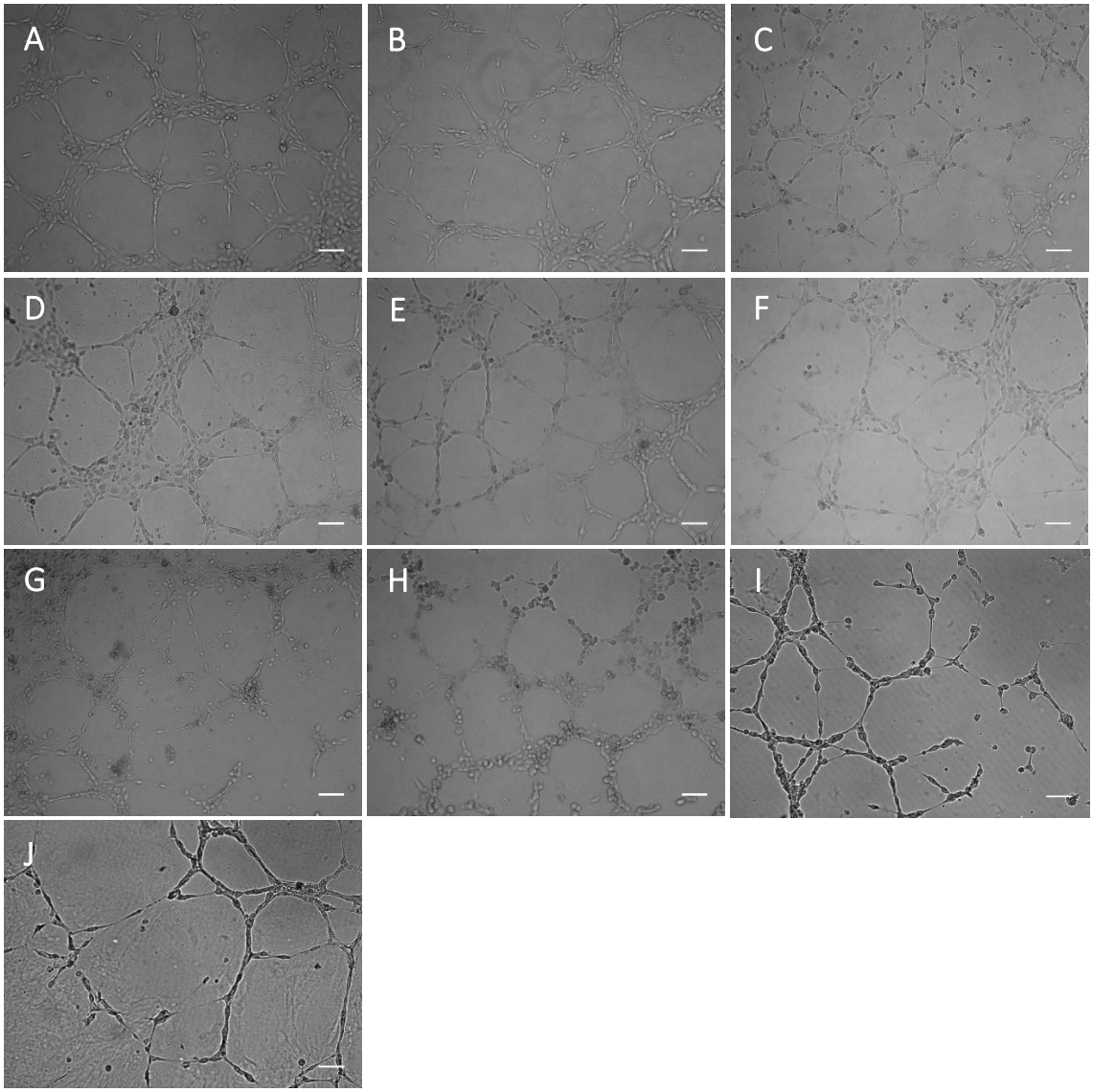


Figure 4.1 – Fulvestrant (F), 2-methoxyestradiol (2ME) and tamoxifen (Tam) prevent the endothelial cell network formation after 8h of incubation

A. Representative image of control; **B.** Representative image of VEGF+FGF; **C.** Representative image of VEGF+FGF+ethanol; **D.** Representative image of VEGF+FGF+E2; **E.** Representative image of VEGF+FGF+EE2; **F.** Representative image of VEGF+FGF+DMSO; **G.** Representative image of VEGF+FGF+F; **H.** Representative image of VEGF+FGF+2ME; **I.** Representative image of VEGF+FGF+Exe; **J.** Representative image of VEGF+FGF+Tam. Magnification of 100x and scale bar= 100µm

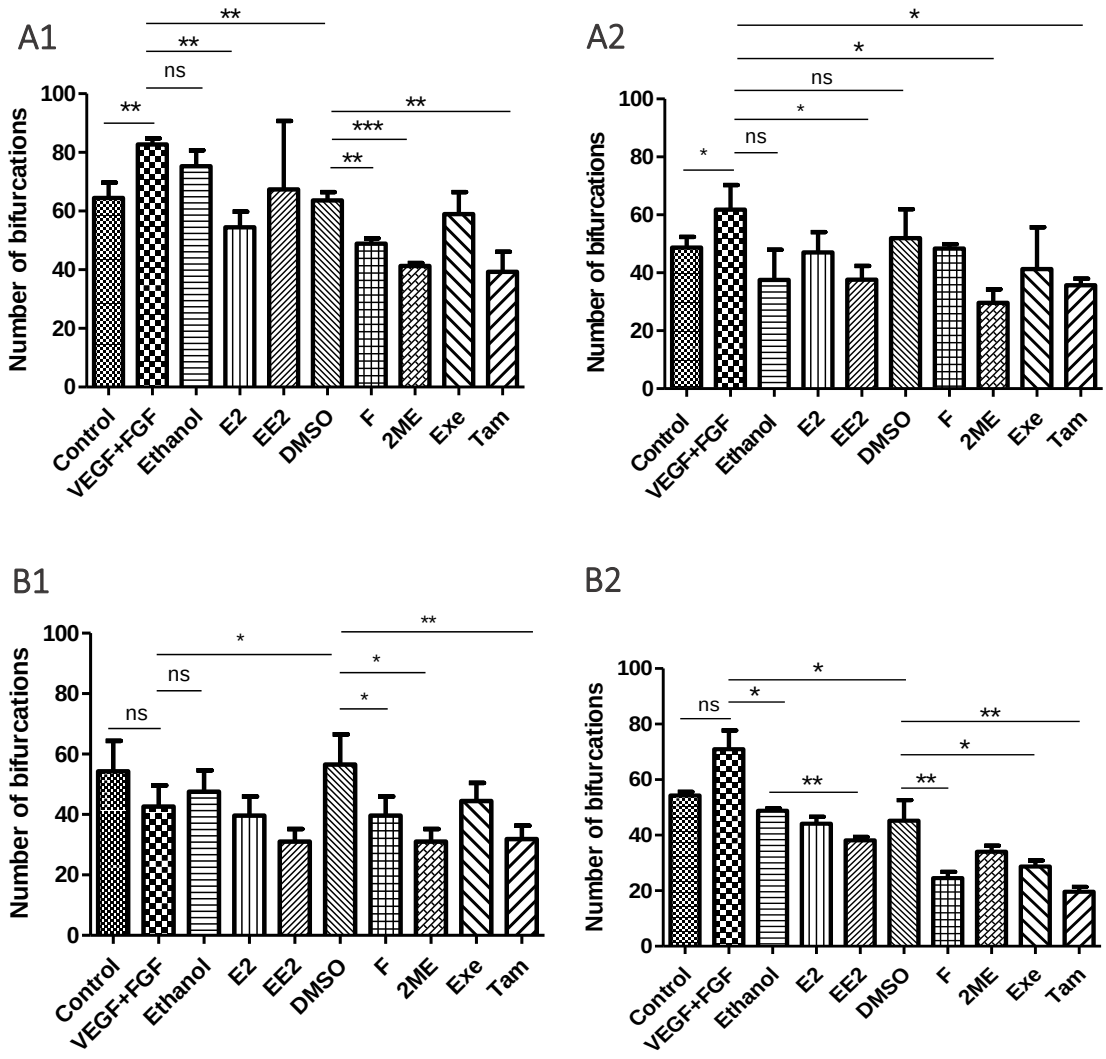


Figure 4.2 – Fulvestrant (F), 2-methoxyestradiol (2ME) and tamoxifen (Tam) have anti-angiogenic effects in prevention of endothelial cell network formation and on their destruction

A. Number of EC bifurcations in Matrigel® tube-formation assay. Addition of estrogen derivatives 2h after cell seeding. 1. Scored 8h and 2. 16h later; B. Number of EC bifurcations in Matrigel® tube-formation assay. Addition of estrogen derivatives 8h after cell seeding. 1. Scored 8h and 2. 16h later. Scored 10 photos per condition, n=3.

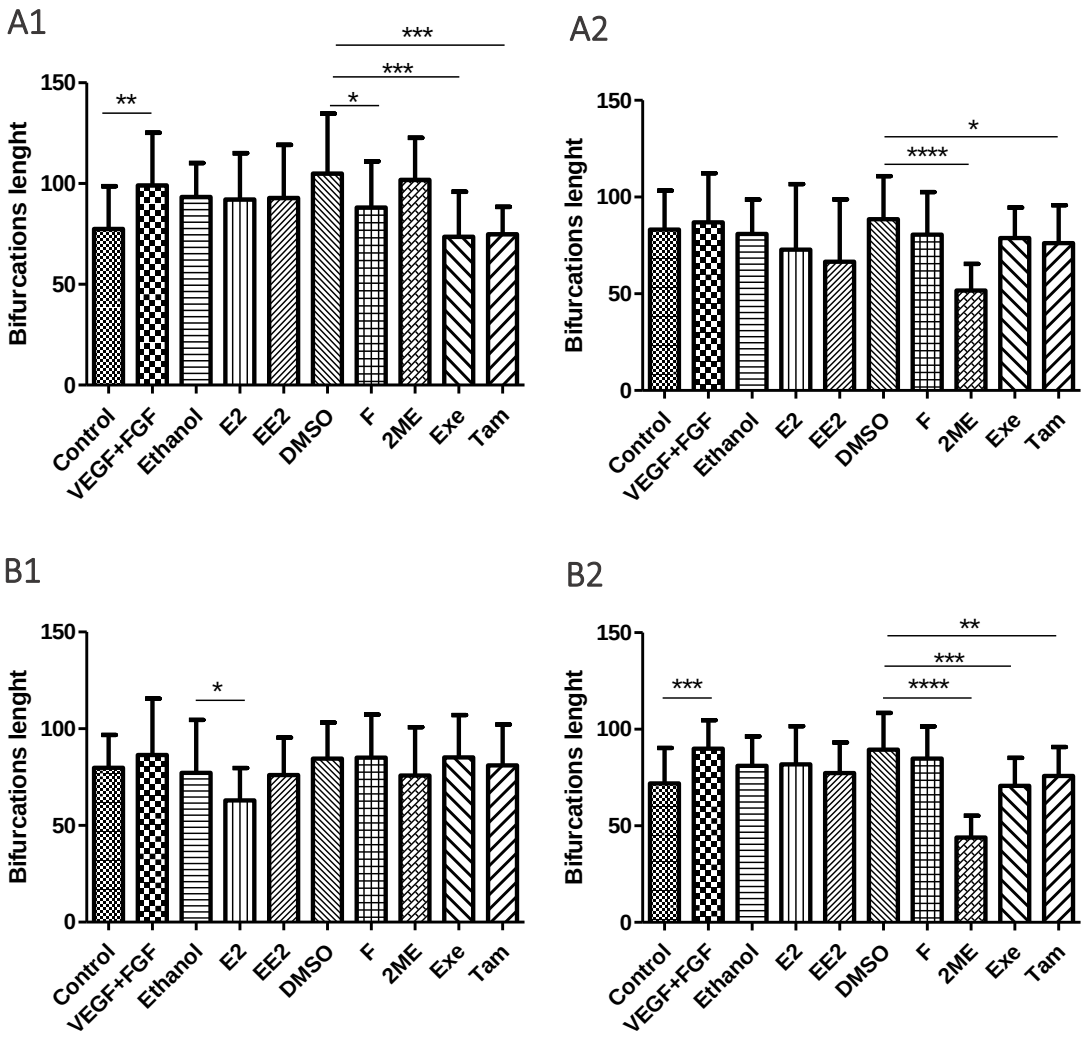


Figure 4.3 - 2-methoxyestradiol (2ME) and tamoxifen (Tam) interfere with bifurcations' length of angiogenic networks

A. Length of EC bifurcations in Matrigel® tube-formation assay. Addition of estrogen derivatives 2h after cell seeding. 1. Scored 8h and 2. 16h later; B. Length of EC bifurcations in Matrigel® tube-formation assay. Addition of estrogen derivatives 8h after cell seeding. 1. Scored 8h and 2. 16h later

Scored 10 photos per condition, n=3

Discussion

With the discovery of the pro-angiogenic potential of estradiol anti-estrogenic therapy emerged as an alternative to the anti-angiogenic therapies.[255,256,261,262] Most anti-estrogens compounds are competitors of estrogen metabolites for ER binding (such as EE2, Tam and 2ME) or estrogen/ER blockers (respectively Exe and F).

The aim of this study was to evaluate the anti-angiogenic potential of these drugs and their efficacy in interfering with the EC network formation and integrity. So we performed a Matrigel® tube-formation assay with two different strategies. The first consisted in the addition of drugs only 2h after the seeding of ECs and the second consisted in drug addition 8h after EC seeding. The first strategy allowed us to study the compounds' ability to interfere with the formation of a cohesive network. The second allowed us study the drugs' destroying capacity. We counted the number of bifurcations and the length of bifurcations, in order to analyze the drugs' anti-angiogenic effects. All the drugs were tested in combination with VEGF+FGF in order to ensure a tumor-like angiogenic microenvironment.

The global differences of this analysis revealed F, 2ME and Tam as the main anti-angiogenic drugs in the early network assembly. F, 2ME and Tam were also the main estrogen derivative compounds associated with the destruction of EC network. F anti-angiogenic pattern was concordant with the literature, since this compound is already used as an adjuvant in breast cancer chemotherapy, having an anti-vascular effect.[258,268] 2ME had been extensively associated with angiogenesis[270,273,274] and recently Solum *et al.*[275] identified that the 2ME potent inhibiting effect of angiogenesis relies on the structural position of nitrogen atom. From now on it will be possible to discover new organic modifications in order to improve the anti-angiogenic efficacy of this drug. Tam is by far the most used estrogen-derivative drug, but its use in postmenopausal women hormone therapy is still controversial.[257,271] However, its anti-angiogenic ability is well accepted in the scientific community.[272,276]

So in conclusion as Tan *et al.*[268] postulated, many studies have demonstrated that interaction between the estrogen and VEGF pathways is a very important mechanism for cancer therapy resistance. This leaves us the opportunity to investigate if the

combination of anti-angiogenic and anti-estrogenic therapies constitute an appropriate approach. Moreover, since Matrigel® tube-formation assay is easy to perform and is considered a reliable technique, this study provides us with means to analyze the anti-angiogenic capacity of other compounds.

Chapter 5

GENERATION OF TOOLS FOR IMAGING OF ENDOTHELIAL CELLS AND TUMORS

1. An *in vivo* platform to analyze vessel architecture and interactions with tumor cells
   Methods
   Results
2. A 3D *in vitro* methodology to analyze mechanisms involved in endothelial and tumor cell interactions
   Methods
   Results

1. An *in vivo* platform to analyze vessel architecture and interactions with tumor cells

Methods

Cell culture

HEK-293T was cultured in DMEM supplemented with 10% heat-inactivated FBS, 2mM L-Glutamine (Life Technologies #58030-081) and 1x antibiotic-antimycotic.

4T1 and MDA-MB-231, mouse and human breast cancer cell lines, respectively, were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1x antibiotic-antimycotic. The MCF7 human breast cancer cell line was cultured in DMEM supplemented with 10% heat-inactivated FBS, 0.01mg/mL human recombinant insulin (Sigma #91007C) and 1x antibiotic-antimycotic. The SKBR3 human breast cancer cell line was cultured in McCoy's 5a medium modified (Life Technologies #16600-082) supplemented with 10% heat-inactivated FBS and 1x antibiotic-antimycotic. E0771 mouse breast cancer cell line was cultured in Roswell Park memorial institute (RPMI) (Life Technologies #11875-093) supplemented with 10mmol/L HEPES (Life Technologies #15630049), sodium bicarbonate 0.075% (Life Technologies #25080-094), 10% heat-inactivated FBS and 1x antibiotic-antimycotic. HeLa and SiHa human cervix cancer cell lines, HCT15 and HCT116 human colon cancer cell lines were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1x antibiotic-antimycotic. HL60 human acute promyelocytic leukemia was cultured in RPMI supplemented with 10% heat-inactivated FBS and 1x antibiotic-antimycotic.

Green fluorescent protein - luciferase cell lines transduction

pCMV-Luc-IRES-EGFP which is an expression vector containing Luc protein with an inserted internal ribosome entry site (IRES)- GFP cassette, was used with pRSV-Rev, pMDLg/pRRE and pMD2.g, which encode for packaging and enveloping of viral particles, to transfect a HEK-293T cell line. Transfection was made with a calcium phosphate transfection kit (Life Technologies #K2780-01), according to the manufacturer's instruction. Virions were collected 48h after and frozen at -80ºC until it was necessary.

MDA-MB-231, 4T1, HeLa, SiHa, HCT15, HCT116, HL60, MCF7, SKBR3, E0771 cell lines were incubated with fresh media and virions in a proportion of 1:1 for 48h. GFP sorting was done with BD FACS Aria III (BS Biosciences), cells were collected in supplemented media and grown as stable GFP - Luc cell lines.

Successful sorting was confirmed using BD FACS Calibur (BS Biosciences) and a Leica DM2500B widefield fluorescent microscope for GFP assessment and IVIS Lumina XMRS (Perkin Elmer) for Luc assessment.

Animal and experimental design

Animal experiments were performed with the approval of the Instituto Gulbenkian de Ciência's Ethical Committee.

3 Balb/c mice (6-8 weeks old) were injected with $1x10^6$ 4T1 GFP-Luc cells in the mammary fat pad. 3 Balb/c *scid* mice (6-8 weeks old) were injected with $1x10^6$ MDA-MB-231 GFP-Luc cells in the mammary fat pad. 100µL of BD Matrigel® basement membrane matrix (BD Biosciences #356230) were injected in the mammary fat pad of 3 Balb/c mice (6-8 weeks old) and the same quantity enriched with 50ng/mL VEGF + 25ng/ml FGF was injected in the mammary fat pad of another 3 Balb/c mice. Animals were sacrificed 14 days after Matrigel® plug inoculation.

All animals were sacrificed according to the directives of the Direcção Geral de Veterinária.

Lumina

Animals were intraperitoneal injected with 150mg/Kg Luciferase (Sigma #L9506) at time zero. 3 minutes later animals were anesthetized with 10mg/Kg medetomidine (Virbac #Medetor) plus 50mg/Kg ketamine (Virbac #Francotar). 10 minutes later animals were analyzed in IVIS Lumina XMRS.

5mg/Kg of atipemezol (Virbac #Revertor) was used to revert the anesthesia.

Multi-photon microscopy

100µL of 20mg/mL dextran rhodamine B (Life Technologies #D-1841) were injected intravenously. 5 minutes later, animals were sacrificed.

Tumors, contralateral normal mammary glands and Matrigel® plugs were collected in DMEM media. Samples were included in a 2% low melting point agarose (Life Technologies #16520-050) and were analyzed with Prairie Ultima multi-photon microscope (Prairie). One stack per 5µm depth was taken and a video was made in ImageJ software with all stacks. Numbers of bifurcations were quantified with ImageJ software and the statistical analysis was done with GraphPad Prism 5 software.

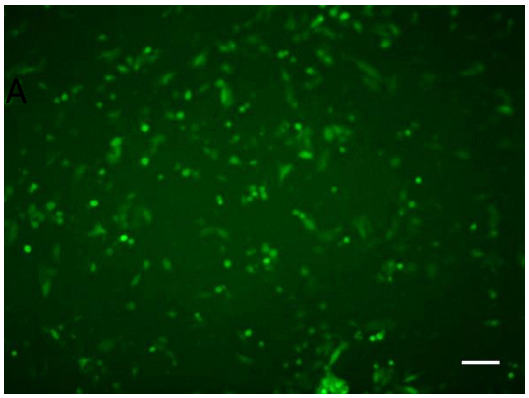
Statistical Analysis

Data was analyzed with GraphPad Prism 5 Software, using unpaired two-tailed t-test with a confidence interval of 95%. Results are expressed as mean ± standard error. * is P-value ≤ 0.05, ** ≤ 0.01 and *** ≤ 0.001.

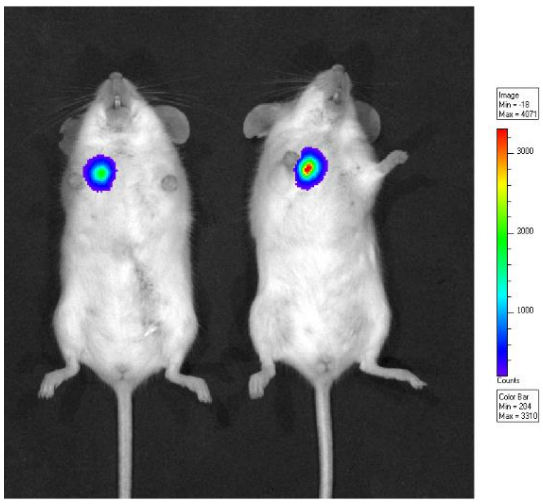
Results

We generated stable GFP-Luc tumor cell lines with a calcium phosphate transfection kit. Successful protocol was confirmed via flow cytometry and widefield fluorescent microscopy for GFP and via IVIS Lumina XMRS for Luc (Figure 5.1).

A



B



C

CELL LINE	PERCENTAGE OF GFP+
MDA-MB-231	75.31
4T1	76.54
HeLa	91.43
SiHa	80.71
HCT15	70.04
HCT116	89.26
HL60	73.82
MCF7	95.42
SKBR3	89.87
E0771	76.34

Figure 5.1 – Stable GFP-Luc tumor cell lines were generated with calcium phosphate transfection method

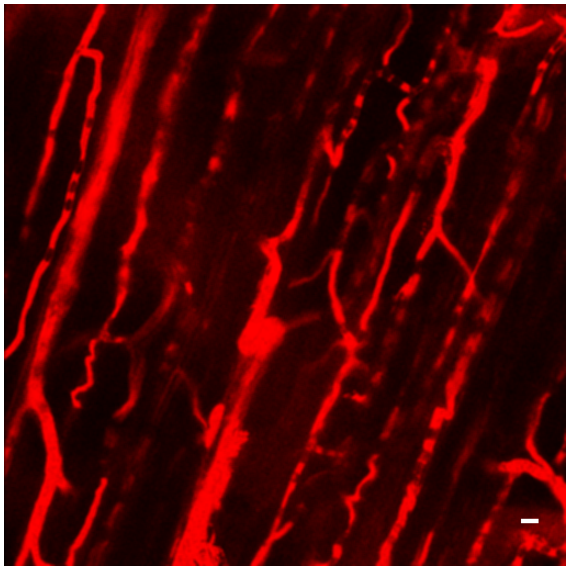
A. Image of MDA-MB-231 GFP-Luc in widefield fluorescent microscope. Magnification of 100x and scale bar= 100µm; **B.** Bioluminescence of MDA-MB-231 GFP-Luc cell line inoculate in mammary fat pad of RAG-2 KO mice. Acquired with IVIS Lumina XMRS; **C.** Percentage of GFP-Luc positive cell lines acquired in BD FACS Calibur, using non-transduced cell lines as negative control.

To better understand the tumor growth and the interplay with endothelial architecture we inoculated MDA-MB-231 GFP-Luc and 4T1 GFP-Luc breast cell lines in the mammary fat pad of mice. To have a control of new blood vessels formation we injected Matrigel® and enriched Matrigel® also in the mammary fat pad of animals. For *in vivo* imaging of blood vessels we injected high molecular weight dextran rhodamine B, which dyes vessels without tissue penetrance. Normal mammary gland, tumors and Matrigel® (enriched and non-enriched) were observed with Prairie Ultima. This multi-photon microscope was used for samples imaging because it allows a greater depth of tissue visualization. As we can see in Figure 5.2 and in the annexed videos, normal mammary gland has a highly organized network with smaller vessels on the surface and deep big vessels. Breast tumors showed rhodamine B extravasation and much less vessels than in normal mammary gland. Basal Matrigel® imaging showed a complex network with smaller capillaries and the enrichment of the basement matrix with VEGF and FGF showed an even more complex structure with higher caliber vessels.

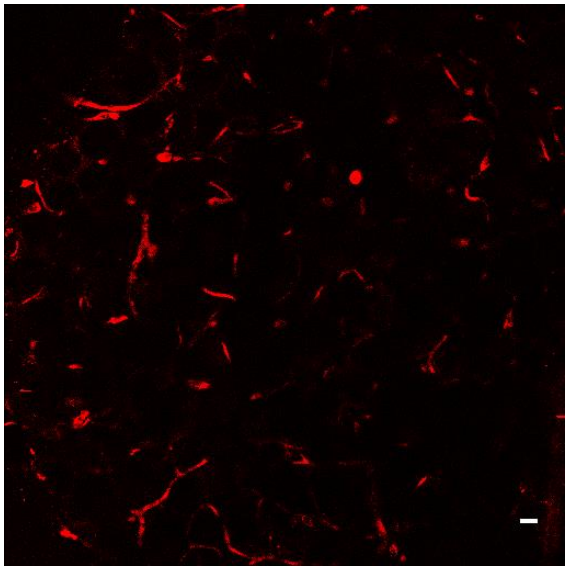
A quantification of the number of bifurcations was performed in order to better understand these difference between conditions (Figure 5.2D). As we can see, Matrigel® with growth factors show a higher number of bifurcations and breast cancer mice models have much less bifurcations compared to the control, which is expected after images visualization.

We can conclude that the generation of stable GFP-Luc tumor cell lines and multi-photon microscopy are useful tools for *in vivo* characterization of tumor growth and interactions between the tumor and ECs.

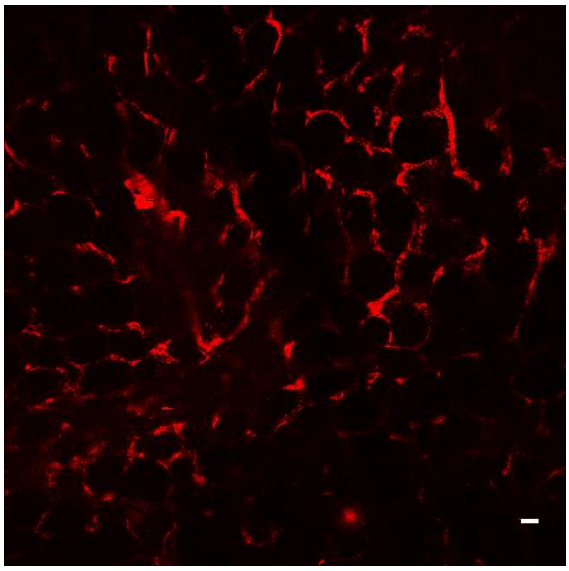
A



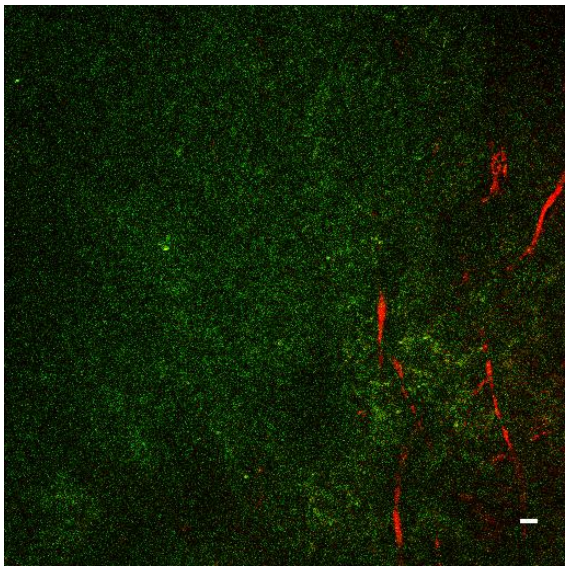
B1



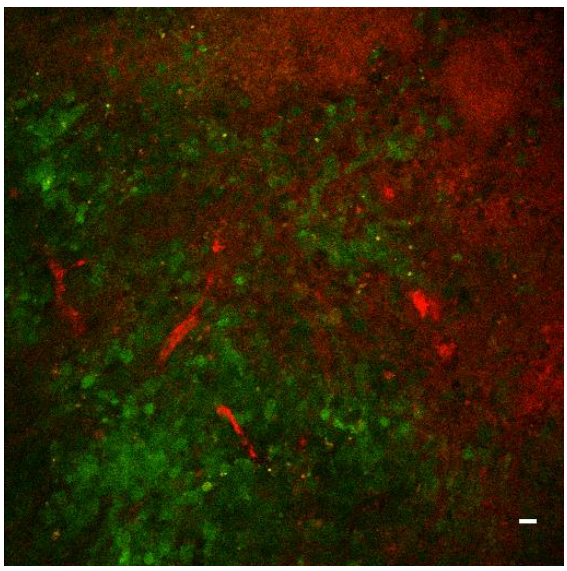
B2



C1



C2



D

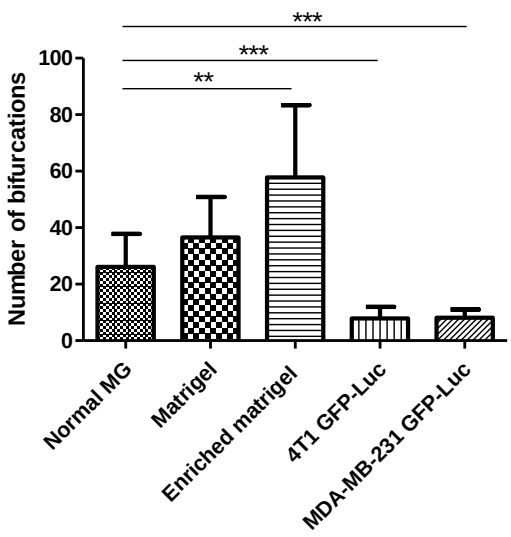

***
***
**
100
80
60
40
20
0
Number of bifurcations
Normal MG
Matrigel
Enriched matrigel
4T1 GFP-Luc
MDA-MB-231 GFP-Luc

Figure 5.2 – *In vivo* breast cancer mice models show a reduction in vascular network complexity

A. Representative image of normal mammary gland (MG); **B.** Representative image of **1.** Matrigel® and **2.** enriched Matrigel®; **C.** Representative image of breast cancer mice models **1.** 4T1 GFP-Luc and **2.** MDA-MB-231 GFP-Luc. Prairie Ultima imaging with magnification of 20x and scale bar= 100µm; **D.** Quantification the number of bifurcations umber per field. Scored all images per video per sample. Data are means ± standard errors; n=3.

2. A 3D *in vitro* methodology to analyze mechanisms involved in endothelial and tumor cell interactions

Methods

Cell culture

HUVEC were cultured in EBM-2 media (Lonza, #190860) supplemented with 2% FBS (Life Technologies #26140-079) and 1x antibiotic-antimycotic (Life Technologies #15240-062). HUVEC were used until the fifth passage.

4T1 GFP-Luc and MDA-MB-231 GFP-Luc, mouse and human breast cancer cell lines, respectively, were cultured in DMEM (Life Technologies #11965-084) supplemented with 10% heat-inactivated FBS and 1x antibiotic-antimycotic.

Tumor spheroids formation

A V-bottomed 96-well plate with 5mg/mL poly(2-hydroxylethyl methacrylate) (poly-HEMA) (Sigma #3932), 60µL per well was coated. Poly-HEMA was evaporated at room temperature, approximately 72 hours.

$2.5x10^4$ 4T1 GPC-Luc and MDA-MB-231 GFP-Luc cells were seeded per well in DMEM media with 2.5% Matrigel® basement membrane matrix (BD Biosciences #356230). Plate were centrifuged 10 minutes at 1200 rpm and incubated overnight.

Results

We generated a tri-dimensional (3D) *in vitro* model to test interactions between endothelial and tumor cells. We generated 3D 4T1 GFP-Luc and MDA-MB-231 GFP-Luc tumor spheroids and incubated them with a monolayer of HUVEC. 48h after co-culture incubation depending on the conditions, some spheroids adhered to ECs (Figure 5.3).

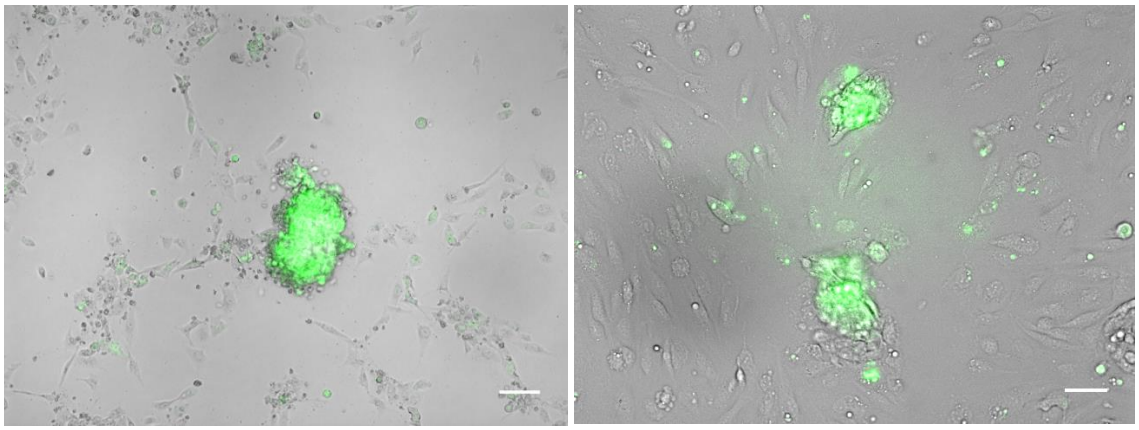


Figure 5.3 – 3D tumor spheroids adhere to a monolayer of endothelial cells

A. Representative image of MDA-MB-231 GFP-Luc spheroids adhering to HUVEC; **B.** Representative image of 4T1 GFP-Luc spheroids adhering to HUVEC. Magnification of 200x and scale bar= 100µm

As Vinci *et al.*[277] postulated, an *in vitro* 3D tumor cell culture reflects the complex *in vivo* microenvironment and it is useful for gene expression profiles, signaling pathways activity and drug sensitivity tests.

The technique applied in this study is a productive way to analyze *in vitro* interactions between endothelial and tumor cells in order to better understand the intra- and extravasation of tumor cells in metastatic processes.

Chapter 6

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

- NFATC1 as a responsible for the lack of response to anti-angiogenic therapies
- A new partnership between SETD2 and NFATC1
- Fulvestrant, 2-methoxyestradiol and tamoxifen interfere and destroy endothelial cell networks in a 3D *in vitro* model

ECs are responsible for many functions including transport, regulation of immune response and modulation of vascular tone. Any perturbation in these cells deregulates organism homeostasis and can lead to pathology, such as ischemic diseases, atherosclerosis, inflammatory diseases and cancer.[3-6] There are innumerous known growth factors associated with ECs namely VEGF, TGF, FGF, ANGPT, PDGF that together with their specific receptors orchestrate the response of EC to external stimuli.[6,26]

Given the importance of ECs in an organism homeostasis, the transcriptional regulation of the EC acquires a major relevance in coordinating healthy and pathological conditions. The major regulators of transcription are transcription factors and epigenetic modifications, which include DNA methylation, histone modifications and ncRNA.[39,85,86,278] The regulation of these proteins is so important for vascular development and homeostasis, that single KO mice are most often embryonic lethal.[85,91,103]

Given the extreme value of transcriptional regulators in EC homeostasis, this thesis aimed at addressing this issue. The role of NFATC1 transcription factor, which has been shown to be involved in EC phenotype[119,130,195,279] is not well characterized in terms of vessel heterogeneity in breast cancer. Moreover, we investigated the role of SETD2 that mediates H3K36me3 signatures[144,239] in ECs since *Setd2* KO mice are embryonic lethal and as yet no explanation for the cellular mechanisms were found. Furthermore, we wanted to clarify the anti-angiogenic role of estrogen derivatives used in clinic in order to improve their efficacy in adjuvant chemotherapy for breast cancer patients.

NFATC1 as a responsible for the lack of response to anti-angiogenic therapies

NFATC1 is a well known transcription factor involved in T cell activation and vascular development, among other functions.[130,191,192,197] We already know their response to external VEGF stimuli.[119] More recently Suehiro *et al.*[131] showed that VEGF and NFATC1 share 1/3 of target genes.

Since tumors represent a VEGF-rich environment[4,16,148] we hypothesized that NFATC1 could have a role in tumor progression, namely in ECs under tumor influence. There is much evidence of hypercalcemia and tumor correlation, namely in multiple myeloma, breast, squamous cell lung, kidney, head and prostate cancer.[280-285] Recently it was hypothesized that Ca^{2+} channels could be a potential tumor target.[286,287] It is also known that VEGF stimulates PLCG1, which leads to an increase in Ca^{2+} levels and consequently NFATC1 nuclear translocation.[123] Since ECs also respond to Ca^{2+} levels and these are very important for ECs proper function[288], we proposed a closer link between NFATC1 and VEGF/ Ca^{2+} deregulation in a tumor microenvironment context.

In our study we identified an upregulation of *Nfatc1* mRNA levels in PTV both in syngeneic and xenograft breast cancer mice models. However, histologically NFATC1 is localized in some NGVs but not in PTVs neither in ITVs. Resident mononuclear cells from mammary gland expressed NFATC1 and TMCI appeared as the most NFATC1-positive cells in tumors. This phenotype was concordant between mice models and human breast cancer samples. In addition, NFATC1 seemed to be positively correlated with tumor aggressiveness. Our study showed that ECs from normal tissues expressed this transcription factor, but ECs under the influence of breast tumor lose their expression. Here we concluded that besides the VEGF-rich environment, tumor ECs did not respond to this gradient and lost their NFATC1 expression. This could be of a great interest since most anti-angiogenic therapies show weak or no response in tumor patients[34,164,230], which means that NFATC1 can be responsible for this failure. On the other hand, TMCI became the major NFATC1-expressing cells in tumor context. This is not surprising, since NFATC1 is also expressed by mononuclear cells.[123,221,222] Our results are supported by Mancini and Toker[197], who showed that NFATC1 induces CSF in both monocytes and ECs and that tumor associated macrophages (TAM) are located nearby to the tumor vasculature, in order to facilitate the metastatic dissemination.

With our *in vitro* study we confirmed the NFATC1 nuclear translocation in HUVEC and HMVEC in response to VEGF stimuli, which is supported by previous studies.[119,121,130,226] The addition of a calcineurin inhibitor CsA,[194] diminished NFATC1 translocation to basal levels. Jang *et al.*[195] revealed the role of KDR in NFATC1's effector function in HPVEC. Garcia-Gomez *et al.*[227] proved that an inhibitor of KDR also causes lower NFATC1 levels and prevents osteoclastogenesis. In accordance with these studies, our work confirmed the importance of KDR in NFATC1 regulation, since the presence of αKDR blocked NFTAC1 nuclear localization as much as CsA. Likewise, αKDR and CsA had a cumulative effect in NFATC1 localization. It had already been proven that VEGF exerts its effect via KDR in a nephrotoxicity model, in which higher levels of VEGF protect the EC from toxic concentrations of CsA via KDR.[289,290] This indicates a common pathway between KDR-VEGF-NFATC1. Furthermore, KDR is the most active VEGFR[6,32] so it is not surprising that KDR and NFATC1 pathways are connected.

Together, our study provided new evidences of NFATC1's role in differentiation of normal ECs and those under the influence of a tumor. KDR and CsA cooperate in NFATC1 nuclear translocation inhibition. It also proved the influence of NFATC1 in tumor microenvironment, namely in TMCI, which was correlated with human breast cancer aggressiveness. Furthermore, ECs under tumor influence lost NFATC1 expression, which could be a possible explanation for the weak anti-angiogenic therapy response in cancer patients.

Further studies are needed in order to unravel the downstream genes of NFATC1 in normal ECs and upon tumor influence. Novel interesting questions were raised. Why is NFATC1 lost? What is the role of NFATC1 in TMCI? Does it helps in tumor dissemination, or it is only an immediate anti-tumoral innate response? What is the role of CsA in tumor progression? Could we give immunosuppressive drugs to cancer patients? Would an NFATC1-target therapy be efficient?

A new partnership between SETD2 and NFATC1

Recently, epigenetics became a subject of great interest in the field of EC regulation.[86,133,143,145] Since *Setd2* KO mice died due to vascular impairment[144], in this PhD thesis we aimed at characterizing the role of SETD2 in EC biology.

We already knew about the involvement of SETD2 mutations in cancer cells.[236-238] However the role of SETD2 in ECs was not well characterized. With our study we proved that SETD2 is important in EC homeostasis, since *SETD2* KD promotes an increase in the number of binucleated HUVEC. Binucleated cells are associated with tumors and with monocytes in some subsets, such as osteoclastogenesis.[190,291] Our videos proved that the binucleated phenotype was due to acytokinetic mitosis, asymmetrical cell division and cell-cell fusion events. Acytokinetic mitosis and cell-cell fusion events had previously been associated with cardiomyocytes and osteoclasts.[292,293] Binucleated ECs have been associated with some pathologies, such as atherosclerosis[246] and injury.[294] However, in our functional studies, namely in the Matrigel® tube-formation assay we saw no differences in angiogenic capacity between *SETD2* KD and scramble. Quiescent cell status was also analyzed and there were no differences between the conditions mentioned above. So we can assume that *SETD2* KD, in spite of conferring binucleated phenotype to the ECs, they still behave as normal cells. However SETD2 was essential in order to maintain H3K36me3 signatures that were crucial for avoiding multiple nuclei.

There is extensive literature to support the interplay between transcription factors and epigenetic modifications, namely histone methyltransferases at sites of target gene promoters. This communication allows an adequate biological response upon the surrounding environment.[295] Transcriptional status has to be relatively stable in order to maintain organism homeostasis. Stein *et al.*[296] demonstrated that epigenetic regulation to modulated transcription factors binding provides a strategy for targeting gene expression in transformed and tumor cells with minimal off-target consequences. Based on this literature we hypothesized that NFATC1, a VEGF-induced transcription factor, and SETD2 can cooperate in a regulatory complex relevant for EC phenotype and function.

In our work we proved that the *SETD2* KD is sufficient to promote NFATC1 nuclear translocation in a VEGF-independent manner. As far as we know this is a new regulatory

mechanism of NFATC1: with the H3K36me3 decrease NFATC1 was translocated to the nucleus without VEGF stimuli. So when SETD2 is present it prevents NFATC1 translocation and NFATC1-dependent signaling pathways are not activated in ECs. We also hypothesized that NFATC1 could be the responsible for the increased number of binucleated cells, since this transcription factor had been associated with cell fusion, especially in osteoclastogenesis.[297,298] In fact we saw a correlated increase of binucleated cells and nuclear NFATC1-positive cells in *SETD2* KD. Although, when we added VEGF to scramble cells (which also increased the nuclear NFATC1-positive cells) the number of binucleated cells did not increased. This could represent a VEGF-independent NFATC1-SETD2 cooperative mechanism in order to regulate EC homeostasis and an ability to adapt to a new environment.

Together our results may indicate that SETD2 is essential for the maintenance of H3K36me3 signature which preserves the basal cytoplasmic levels of NFATC1 – Figure 6.1. When we knock down *SETD2*, H3K36me3 levels are downregulated, NFATC1 is translocated to the nucleus and ECs became binucleated. This seems contradictory, as SETD2 maintain the EC mononuclear phenotype and also acts as a transcription and trafficking regulator of NFATC1. Although, we hypothesize that SETD2 downregulation creates a possibility for EC transcription program regulation and helps ECs responding to different microenvironments. Are multiple nuclei a mechanism to overcome adverse conditions? When SETD2 levels are low, which NFATC1 downstream genes are activated? Are cancer SETD2 mutations involved in the mechanism of NFATC1 translocation in ECs?

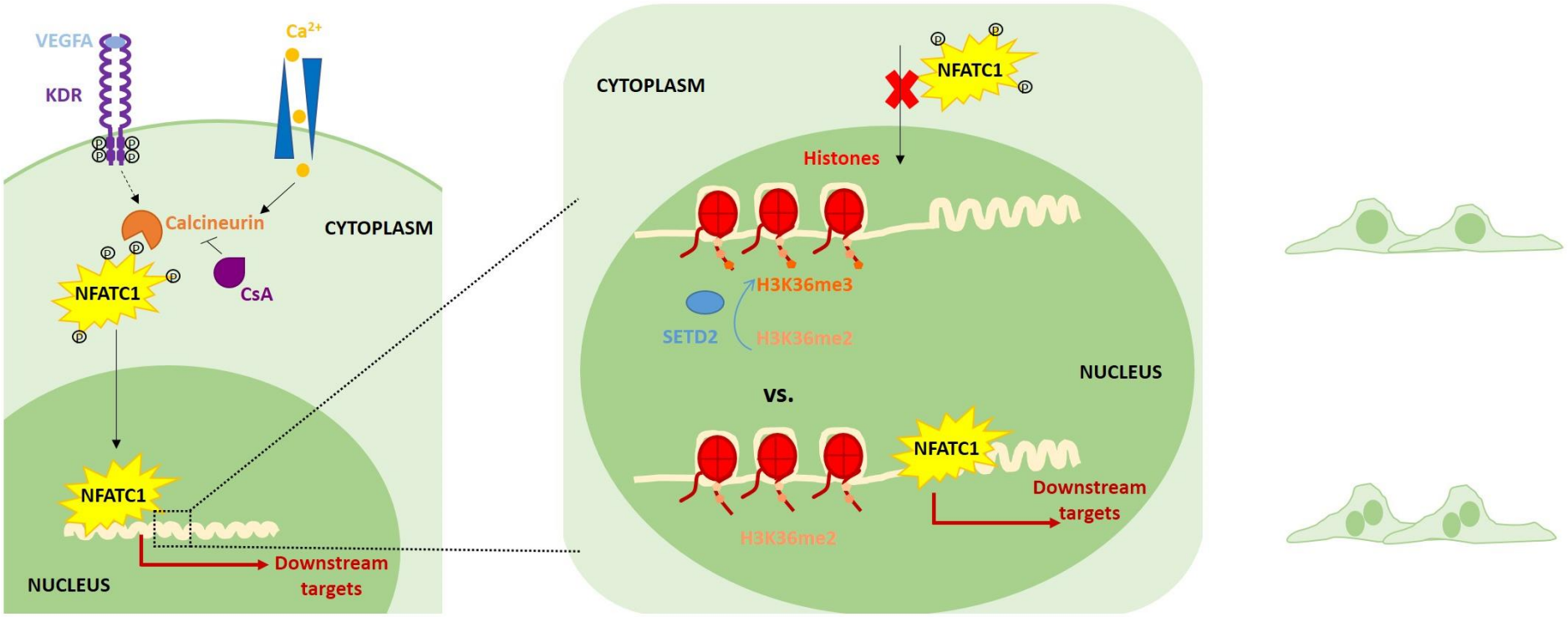


Figure 6.1 – Proposed mechanism of SETD2 and NFATC1 interaction. NFATC1 is translocated to the nucleus in response to VEGFA-KDR mediated stimuli. Calcineurin which responds also upon Ca^{2+} stimuli dephosphorylates NFATC1 and it is afterwards translocated to the nucleus. In the nucleus NFATC1 binds to promoter regions and the necessary transcriptional machinery is recruited with expression of downstream target genes. When SETD2 is present, the H3K36me3 signature prevents NFTAC1 nuclear translocation and/or DNA binding. In presence of SETD2 ECs also maintain their homeostatic mononuclear status. When SETD2 is downregulated H3K36me3 signatures are at lower levels and NFATC1 is translocated to the nucleus with consequent transcription initiation. In this context ECs become binucleated which could represent an adaptive survival mechanism.

Fulvestrant, 2-methoxyestradiol and tamoxifen interfere and destroy endothelial cell networks in a 3D *in vitro* model

The anti-angiogenic potential of estrogen derivative drugs is already known.[255] They exert their efficacy being ER antagonists.[256] 2ME and tamoxifen are potent anti-angiogenic agents, being used in clinic, mostly in breast cancer patients.[270,272,276]

The global purpose of this study was to characterize the estrogen derivatives in terms of cytotoxicity, genotoxicity and anti-angiogenic ability. We used a Matrigel® tube-forming assay in order to test the anti-angiogenic efficacy of these compounds. We verified that F, 2ME and Tam were the main estrogen derivatives with capacity to destroy the established EC network and to interfere with EC network formation. These results are in accordance to the literature, since F[258,268], 2ME[269,270,273,274,299,300] and Tam[257,272,276] were extensively studied and some are already used in clinic.

These descriptive studies are important to identify the potential of anti-angiogenic drugs in order to improve their efficacy. It was also useful to prove that Matrigel® tube-formation assay could be a reliable and easy 3D *in vitro* model to test large-scale anti-angiogenic drugs.

Chapter 7

REFERENCES

1 Sumpio, B. E., Riley, J. T. & Dardik, A. Cells in focus: endothelial cell. *Int J Bioch Cell Biol* **34**, 1508-1512 (2002).
2 Marcelo, K. L., Goldie, L. C. & Hirschi, K. K. Regulation of endothelial cell differentiation and specification. *Circulation research* **112**, 1272-1287, doi:10.1161/CIRCRESAHA.113.300506 (2013).
3 Hida, K., Ohga, N., Akiyama, K., Maishi, N. & Hida, Y. Heterogeneity of tumor endothelial cells. *Cancer science* **104**, 1391-1395, doi:10.1111/cas.12251 (2013).
4 Aird, W. C. Endothelial cell heterogeneity. *Cold Spring Harbor perspectives in medicine* **2**, a006429, doi:10.1101/cshperspect.a006429 (2012).
5 Herbert, S. P. & Stainier, D. Y. Molecular control of endothelial cell behaviour during blood vessel morphogenesis. *Nature reviews. Molecular cell biology* **12**, 551-564, doi:10.1038/nrm3176 (2011).
6 Carmeliet, P. & Jain, R. K. Molecular mechanisms and clinical applications of angiogenesis. *Nature* **473**, 298-307, doi:10.1038/nature10144 (2011).
7 Schwartz, B. G., Economides, C., Mayeda, G. S., Burstein, S. & Kloner, R. A. The endothelial cell in health and disease: its function, dysfunction, measurement and therapy. *International journal of impotence research* **22**, 77-90, doi:10.1038/ijir.2009.59 (2010).
8 His, W. Lecithoblast und angioblast der wirbelthiere. *Abhandl KS Ges Wis Math-Phys* **22** (1900).
9 Sabin, F. R. Studies on the origin of blood vessels and of red corpuscles as seen in the living blastoderm of chicks during the second day of incubation. *Contrib Embryol* **9**, 213-262 (1920).
10 Xiong, J. W. Molecular and developmental biology of the hemangioblast. *Developmental dynamics : an official publication of the American Association of Anatomists* **237**, 1218-1231, doi:10.1002/dvdy.21542 (2008).
11 Zape, J. P. & Zovein, A. C. Hemogenic endothelium: origins, regulation, and implications for vascular biology. *Seminars in cell & developmental biology* **22**, 1036-1047, doi:10.1016/j.semcdb.2011.10.003 (2011).
12 Smith, R. A. & Glomski, C. A. "Hemogenic endothelium" of the embryonic aorta: Does it exist? *Developmental and comparative immunology* **6**, 359-368 (1982).
13 Swiers, G., Rode, C., Azzoni, E. & de Bruijn, M. F. A short history of hemogenic endothelium. *Blood cells, molecules & diseases* **51**, 206-212, doi:10.1016/j.bcmd.2013.09.005 (2013).
14 Hirschi, K. H. Hemogenic endothelium during development and beyond. *Blood* **119**, 4823-4827, doi:10.1182/blood-2011-12353466 (2012).
15 Sandler, V. M. *et al.* Reprogramming human endothelial cells to haematopoietic cells requires vascular induction. *Nature* **511**, 312-318, doi:10.1038/nature13547 (2014).
16 Potente, M., Gerhardt, H. & Carmeliet, P. Basic and therapeutic aspects of angiogenesis. *Cell* **146**, 873-887, doi:10.1016/j.cell.2011.08.039 (2011).
17 Swift, M. R. & Weinstein, B. M. Arterial-venous specification during development. *Circulation research* **104**, 576-588, doi:10.1161/CIRCRESAHA.108.188805 (2009).
18 Clark, E. R. & Clark, E. L. Observations on living preformed blood vessels as seen in a transparent chamber inserted into the rabbit ear. *Am J Anat* **49**, 441-447 (1932).
19 Caduff, J. H., Fischer, L. C. & Burri, P. H. Scanning electron microscope study of the developing microvasculature in the postnatal rat lung. *The Anatomical record* **216**, 154-164, doi:10.1002/ar.1092160207 (1986).
20 Hillen, F. & Griffioen, A. W. Tumour vascularization: sprouting angiogenesis and beyond. *Cancer metastasis reviews* **26**, 489-502, doi:10.1007/s10555-007-9094-7 (2007).
21 Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674, doi:10.1016/j.cell.2011.02.013 (2011).

22 Senger, D. R. *et al.* Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* **219**, 983-985 (1983).
23 Carmeliet, P. *et al.* Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380**, 435-439 (1996).
24 Ferrara, N. *et al.* Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380**, 439-442, doi:10.1038/380439a0 (1996).
25 Miquerol, L., Langille, B. L. & Nagy, A. Embryonic development is disrupted by modest increases in vascular endothelial growth factor gene expression. *Development* **127**, 3941-3946 (2000).
26 Koch, S. & Claesson-Welsh, L. Signal transduction by vascular endothelial growth factor receptors. *Cold Spring Harbor perspectives in medicine* **2**, a006502, doi:10.1101/cshperspect.a006502 (2012).
27 Eichmann, A. & Simons, M. VEGF signaling inside vascular endothelial cells and beyond. *Curr Opin Cell Biol* **24**, 188-193, doi:10.1016/j.ceb.2012.02.002 (2012).
28 Lohela, M., Bry, M., Tammela, T. & Alitalo, K. VEGFs and receptors involved in angiogenesis versus lymphangiogenesis. *Curr Opin Cell Biol* **21**, 154-165, doi:10.1016/j.ceb.2008.12.012 (2009).
29 Grunewald, F. S., Prota, A. E., Giese, A. & Ballmer-Hofer, K. Structure-function analysis of VEGF receptor activation and the role of coreceptors in angiogenic signaling. *Biochimica et biophysica acta* **1804**, 567-580, doi:10.1016/j.bbapap.2009.09.002 (2010).
30 Lee, S. *et al.* Autocrine VEGF signaling is required for vascular homeostasis. *Cell* **130**, 691-703, doi:10.1016/j.cell.2007.06.054 (2007).
31 Goel, H. L. & Mercurio, A. M. VEGF targets the tumour cell. *Nature reviews. Cancer* **13**, 871-882, doi:10.1038/nrc3627 (2013).
32 Nakayama, M. & Berger, P. Coordination of VEGF receptor trafficking and signaling by coreceptors. *Experimental cell research* **319**, 1340-1347, doi:10.1016/j.yexcr.2013.03.008 (2013).
33 Kendall, R. L. & Thomas, K. A. Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 10705-10709 (1993).
34 Shibuya, M. Vascular Endothelial Growth Factor (VEGF) and Its Receptor (VEGFR) Signaling in Angiogenesis: A Crucial Target for Anti- and Pro-Angiogenic Therapies. *Genes & cancer* **2**, 1097-1105, doi:10.1177/1947601911423031 (2011).
35 Ahmad, S. & Ahmed, A. Antiangiogenic effect of soluble vascular endothelial growth factor receptor-1 in placental angiogenesis. *Endothelium : journal of endothelial cell research* **12**, 89-95, doi:10.1080/10623320590933888 (2005).
36 Ebos, J. M. L. *et al.* A Naturally Occurring Soluble Form of Vascular Endothelial Growth Factor Receptor 2 Detected in Mouse and Human Plasma. *Mol Cancer Res* **2**, 315-326 (2004).
37 Pavlakovic, H., Becker, J., Albuquerque, R., Wilting, J. & Ambati, J. Soluble VEGFR-2: an antilymphangiogenic variant of VEGF receptors. *Annals of the New York Academy of Sciences* **1207 Suppl 1**, E7-15, doi:10.1111/j.1749-6632.2010.05714.x (2010).
38 Collet, G. *et al.* Hypoxia-regulated overexpression of soluble VEGFR2 controls angiogenesis and inhibits tumor growth. *Molecular cancer therapeutics* **13**, 165-178, doi:10.1158/1535-7163.MCT-13-0637 (2014).
39 Park, C., Kim, T. M. & Malik, A. B. Transcriptional regulation of endothelial cell and vascular development. *Circulation research* **112**, 1380-1400, doi:10.1161/CIRCRESAHA.113.301078 (2013).
40 Wang, Y. *et al.* Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. *Nature* **465**, 483-486, doi:10.1038/nature09002 (2010).
41 Sawamiphak, S. *et al.* Ephrin-B2 regulates VEGFR2 function in developmental and tumour angiogenesis. *Nature* **465**, 487-491, doi:10.1038/nature08995 (2010).

42 Nakayama, M. *et al.* Spatial regulation of VEGF receptor endocytosis in angiogenesis. *Nature cell biology* **15**, 249-260, doi:10.1038/ncb2679 (2013).
43 Benedito, R. *et al.* Notch-dependent VEGFR3 upregulation allows angiogenesis without VEGF-VEGFR2 signalling. *Nature* **484**, 110-114, doi:10.1038/nature10908 (2012).
44 Goshima, Y. *et al.* Growth cone neuropilin-1 mediates collapsin-1/Sema III facilitation of antero- and retrograde axoplasmic transport. *Journal of neurobiology* **39**, 579-589 (1999).
45 Zachary, I. Neuropilins: role in signalling, angiogenesis and disease. *Chemical immunology and allergy* **99**, 37-70, doi:10.1159/000354169 (2014).
46 Koch, S. *et al.* NRP1 presented in trans to the endothelium arrests VEGFR2 endocytosis, preventing angiogenic signaling and tumor initiation. *Developmental cell* **28**, 633-646, doi:10.1016/j.devcel.2014.02.010 (2014).
47 Eklund, L. & Saharinen, P. Angiopoietin signaling in the vasculature. *Experimental cell research* **319**, 1271-1280, doi:10.1016/j.yexcr.2013.03.011 (2013).
48 Goddard, L. M. & Iruela-Arispe, M. L. Cellular and molecular regulation of vascular permeability. *Thrombosis and haemostasis* **109**, 407-415, doi:10.1160/TH12-09-0678 (2013).
49 van Meeteren, L. A. & ten Dijke, P. Regulation of endothelial cell plasticity by TGF-beta. *Cell and tissue research* **347**, 177-186, doi:10.1007/s00441-011-1222-6 (2012).
50 Pardali, E., Goumans, M. J. & ten Dijke, P. Signaling by members of the TGF-beta family in vascular morphogenesis and disease. *Trends in cell biology* **20**, 556-567, doi:10.1016/j.tcb.2010.06.006 (2010).
51 Lieu, C., Heymach, J., Overman, M., Tran, H. & Kopetz, S. Beyond VEGF: inhibition of the fibroblast growth factor pathway and antiangiogenesis. *Clinical cancer research : an official journal of the American Association for Cancer Research* **17**, 6130-6139, doi:10.1158/1078-0432.CCR-11-0659 (2011).
52 Murakami, M. *et al.* The FGF system has a key role in regulating vascular integrity. *The Journal of clinical investigation* **118**, 3355-3366, doi:10.1172/JCI35298 (2008).
53 Liakouli, V. *et al.* Angiogenic cytokines and growth factors in systemic sclerosis. *Autoimmunity reviews* **10**, 590-594, doi:10.1016/j.autrev.2011.04.019 (2011).
54 Ball, S. G., Shuttleworth, C. A. & Kielty, C. M. Vascular endothelial growth factor can signal through platelet-derived growth factor receptors. *The Journal of cell biology* **177**, 489-500, doi:10.1083/jcb.200608093 (2007).
55 Pennock, S. & Kazlauskas, A. Vascular endothelial growth factor A competitively inhibits platelet-derived growth factor (PDGF)-dependent activation of PDGF receptor and subsequent signaling events and cellular responses. *Molecular and cellular biology* **32**, 1955-1966, doi:10.1128/MCB.06668-11 (2012).
56 Frantz, C., Stewart, K. M. & Weaver, V. M. The extracellular matrix at a glance. *J Cell Sci* **123**, 4195-4200, doi:10.1242/jcs (2010).
57 Wiradjaja, F., DiTommaso, T. & Smyth, I. Basement membranes in development and disease. *Birth defects research. Part C, Embryo today : reviews* **90**, 8-31, doi:10.1002/bdrc.20172 (2010).
58 Dvorak, A. M. & Feng, D. The Vesiculo-Vacuolar Organelle (VVO): A New Endothelial Cell Permeability Organelle. *Journal of Histochemistry & Cytochemistry* **49**, 419-431, doi:10.1177/002215540104900401 (2001).
59 Li, X. *et al.* JAM-C induces endothelial cell permeability through its association and regulation of {beta}3 integrins. *Arteriosclerosis, thrombosis, and vascular biology* **29**, 1200-1206, doi:10.1161/ATVBAHA.109.189217 (2009).
60 Horowitz, A. & Seerapu, H. R. Regulation of VEGF signaling by membrane traffic. *Cellular signalling* **24**, 1810-1820, doi:10.1016/j.cellsig.2012.05.007 (2012).

61 Taddei, A. *et al.* Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5. *Nature cell biology* **10**, 923-934, doi:10.1038/ncb1752 (2008).
62 Walsh, T. G. *et al.* Stabilization of brain microvascular endothelial barrier function by shear stress involves VE-cadherin signaling leading to modulation of pTyr-occludin levels. *Journal of cellular physiology* **226**, 3053-3063, doi:10.1002/jcp.22655 (2011).
63 Bazzoni, G. & Dejana, E. Endothelial Cell-to-Cell Junctions: Molecular Organization and Role in Vascular Homeostasis. *Physiol Rev* **84**, 869-901 (2004).
64 Dora, K. A. & Garland, C. J. Linking hyperpolarization to endothelial cell calcium events in arterioles. *Microcirculation* **20**, 248-256, doi:10.1111/micc.12041 (2013).
65 Looft-Wilson, R. C., Billaud, M., Johnstone, S. R., Straub, A. C. & Isakson, B. E. Interaction between nitric oxide signaling and gap junctions: effects on vascular function. *Biochimica et biophysica acta* **1818**, 1895-1902, doi:10.1016/j.bbamem.2011.07.031 (2012).
66 Wang, L., Dong, Z., Zhang, Y. & Miao, J. The roles of integrin beta4 in vascular endothelial cells. *Journal of cellular physiology* **227**, 474-478, doi:10.1002/jcp.22769 (2012).
67 Niland, S. & Eble, J. A. Integrin-mediated cell-matrix interaction in physiological and pathological blood vessel formation. *Journal of oncology* **2012**, 125278, doi:10.1155/2012/125278 (2012).
68 Somanath, P. R., Malinin, N. L. & Byzova, T. V. Cooperation between integrin alphavbeta3 and VEGFR2 in angiogenesis. *Angiogenesis* **12**, 177-185, doi:10.1007/s10456-009-9141-9 (2009).
69 Yang, J. T., Rayburn, H. & Hynes, R. O. Embryonic mesodermal defects in 5 integrin-deficient mice. *Development* **119**, 1093-1105 (1993).
70 Reinhart-King, C. A. Chapter 3 Endothelial Cell Adhesion and Migration. **443**, 45-64, doi:10.1016/s0076-6879(08)02003-x (2008).
71 Borges, E., Jan, Y. & Ruoslahti, E. Platelet-derived growth factor receptor beta and vascular endothelial growth factor receptor 2 bind to the beta 3 integrin through its extracellular domain. *The Journal of biological chemistry* **275**, 39867-39873, doi:10.1074/jbc.M007040200 (2000).
72 Robinson, S. D. *et al.* Alphav beta3 integrin limits the contribution of neuropilin-1 to vascular endothelial growth factor-induced angiogenesis. *The Journal of biological chemistry* **284**, 33966-33981, doi:10.1074/jbc.M109.030700 (2009).
73 Bouis, D., Kusumanto, Y., Meijer, C., Mulder, N. H. & Hospers, G. A. A review on pro- and anti-angiogenic factors as targets of clinical intervention. *Pharmacological research : the official journal of the Italian Pharmacological Society* **53**, 89-103, doi:10.1016/j.phrs.2005.10.006 (2006).
74 Lawler, P. R. & Lawler, J. Molecular basis for the regulation of angiogenesis by thrombospondin-1 and -2. *Cold Spring Harbor perspectives in medicine* **2**, a006627, doi:10.1101/cshperspect.a006627 (2012).
75 Zhang, S. *et al.* A host deficiency of discoidin domain receptor 2 (DDR2) inhibits both tumour angiogenesis and metastasis. *The Journal of pathology* **232**, 436-448, doi:10.1002/path.4311 (2014).
76 Oakley, R. & Tharakan, B. Vascular hyperpermeability and aging. *Aging and disease* **5**, 114-125, doi:10.14336/AD.2014.0500114 (2014).
77 van Buul, J. D., Geerts, D. & Huveneers, S. Rho GAPs and GEFs: Controling switches in endothelial cell adhesion. *Cell Adhesion & Migration* **8**, 108-124, doi:10.4161/cam.27599 (2014).
78 Reymond, N., d'Agua, B. B. & Ridley, A. J. Crossing the endothelial barrier during metastasis. *Nature reviews. Cancer* **13**, 858-870, doi:10.1038/nrc3628 (2013).

79 Bendas, G. & Borsig, L. Cancer cell adhesion and metastasis: selectins, integrins, and the inhibitory potential of heparins. *International journal of cell biology* **2012**, 676731, doi:10.1155/2012/676731 (2012).
80 van Buul, J. D. *et al.* ICAM-1 clustering on endothelial cells recruits VCAM-1. *Journal of biomedicine & biotechnology* **2010**, 120328, doi:10.1155/2010/120328 (2010).
81 van Buul, J. D. & Hordijk, P. L. Endothelial signalling by Ig-like cell adhesion molecules. *Transfusion clinique et biologique : journal de la Societe francaise de transfusion sanguine* **15**, 3-6, doi:10.1016/j.tracli.2008.04.002 (2008).
82 Cook-Mills, J. M., Marchese, M. E. & Abdala-Valencia, H. Vascular Cell Adhesion Molecule-1 Expression and Signaling During Disease: Regulation by Reactive Oxygen Species and Antioxidants. *Antioxidants & redox signaling* **15**, 1607-1638 (2011).
83 Ilan, N. & Madri, J. A. PECAM-1: old friend, new partners. *Current Opinion in Cell Biology* **15**, 515-524, doi:10.1016/s0955-0674(03)00100-5 (2003).
84 Privratsky, J. R., Newman, D. K. & Newman, P. J. PECAM-1: conflicts of interest in inflammation. *Life sciences* **87**, 69-82, doi:10.1016/j.lfs.2010.06.001 (2010).
85 De Val, S. & Black, B. L. Transcriptional control of endothelial cell development. *Developmental cell* **16**, 180-195, doi:10.1016/j.devcel.2009.01.014 (2009).
86 Matouk, C. C. & Marsden, P. A. Epigenetic regulation of vascular endothelial gene expression. *Circulation research* **102**, 873-887, doi:10.1161/CIRCRESAHA.107.171025 (2008).
87 Dejana, E., Taddei, A. & Randi, A. M. Foxs and Ets in the transcriptional regulation of endothelial cell differentiation and angiogenesis. *Biochimica et biophysica acta* **1775**, 298-312, doi:10.1016/j.bbcan.2007.05.003 (2007).
88 Meadows, S. M., Myers, C. T. & Krieg, P. A. Regulation of endothelial cell development by ETS transcription factors. *Seminars in cell & developmental biology* **22**, 976-984, doi:10.1016/j.semcdb.2011.09.009 (2011).
89 Sato, Y. Molecular mechanism of angiogenesis Transcription factors and their therapeutic relevance. *Pharmacology & therapeutics* **87**, 51-60 (2000).
90 Watanabe, D. *et al.* Transcription Factor Ets-1 Mediates Ischemia- and Vascular Endothelial Growth Factor-Dependent Retinal Neovascularization. *AJP* **164**, 1827-1835 (2004).
91 Spyropoulos, D. D. *et al.* Hemorrhage, impaired hematopoiesis, and lethality in mouse embryos carrying a targeted disruption of the Fli1 transcription factor. *Molecular and cellular biology* **20**, 5643-5652 (2000).
92 Asano, Y. *et al.* Endothelial Fli1 deficiency impairs vascular homeostasis: a role in scleroderma vasculopathy. *The American journal of pathology* **176**, 1983-1998, doi:10.2353/ajpath.2010.090593 (2010).
93 Jin, E. *et al.* Differential roles for ETS, CREB, and EGR binding sites in mediating VEGF receptor 1 expression in vivo. *Blood* **114**, 5557-5566, doi:10.1182/blood-2009-05-220434 (2009).
94 Birdsey, G. M. *et al.* Transcription factor Erg regulates angiogenesis and endothelial apoptosis through VE-cadherin. *Blood* **111**, 3498-3506, doi:10.1182/blood-2007-08-105346 (2008).
95 Yuan, L. *et al.* ETS-related gene (ERG) controls endothelial cell permeability via transcriptional regulation of the claudin 5 (CLDN5) gene. *The Journal of biological chemistry* **287**, 6582-6591, doi:10.1074/jbc.M111.300236 (2012).
96 Kwiatkowski, B. A. *et al.* The ets Family Member Tel Binds to the Fli-1 Oncoprotein and Inhibits Its Transcriptional Activity. *Journal of Biological Chemistry* **273**, 17525-17530, doi:10.1074/jbc.273.28.17525 (1998).
97 Zheng, H. *et al.* The transcription factor Net regulates the angiogenic switch. *Genes & development* **17**, 2283-2297, doi:10.1101/gad.272503 (2003).

98 Gross, C., Dubois-Pot, H. & Wasylyk, B. The ternary complex factor Net/Elk-3 participates in the transcriptional response to hypoxia and regulates HIF-1 alpha. *Oncogene* **27**, 1333-1341, doi:10.1038/sj.onc.1210736 (2008).

99 Ayadi, A., Suelves, M., Dollé, P. & Wasylyk, B. Net, an Ets ternary complex transcription factor, is expressed in sites of vasculogenesis, angiogenesis, and chondrogenesis during mouse development. *Mechanisms of development* **102**, 205-208 (2001).

100 Heo, S. H. & Cho, J. Y. ELK3 suppresses angiogenesis by inhibiting the transcriptional activity of ETS-1 on MT1-MMP. *International journal of biological sciences* **10**, 438-447, doi:10.7150/ijbs.8095 (2014).

101 Wareing, S. *et al.* The Flk1-Cre-mediated deletion of ETV2 defines its narrow temporal requirement during embryonic hematopoietic development. *Stem cells* **30**, 1521-1531, doi:10.1002/stem.1115 (2012).

102 Shi, X. *et al.* Cooperative interaction of Etv2 and Gata2 regulates the development of endothelial and hematopoietic lineages. *Developmental biology* **389**, 208-218, doi:10.1016/j.ydbio.2014.02.018 (2014).

103 Fischer, A., Schumacher, N., Maier, M., Sendtner, M. & Gessler, M. The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. *Genes & development* **18**, 901-911, doi:10.1101/gad.291004 (2004).

104 Semenza, G. H. HIF-1 and human disease: one highly involved factor. *Genes Dev.* **14**, 1983-1991, doi:10.1101/gad.14.16.1983 (2000).

105 Kurihara, T., Westenskow, P. D. & Friedlander, M. Hypoxia-inducible factor (HIF)/vascular endothelial growth factor (VEGF) signaling in the retina. *Advances in experimental medicine and biology* **801**, 275-281, doi:10.1007/978-1-4614-3209-8_35 (2014).

106 Sakurai, D. *et al.* Crucial Role of Inhibitor of DNA Binding/Differentiation in the Vascular Endothelial Growth Factor-Induced Activation and Angiogenic Processes of Human Endothelial Cells. *The Journal of Immunology* **173**, 5801-5809, doi:10.4049/jimmunol.173.9.5801 (2004).

107 Seo, S. *et al.* The forkhead transcription factors, Foxc1 and Foxc2, are required for arterial specification and lymphatic sprouting during vascular development. *Developmental biology* **294**, 458-470, doi:10.1016/j.ydbio.2006.03.035 (2006).

108 Ren, X. *et al.* FOXF1 Transcription Factor Is Required for Formation of Embryonic Vasculature by Regulating VEGF Signaling in Endothelial Cells. *Circulation research*, doi:10.1161/CIRCRESAHA.115.304382 (2014).

109 Sacilotto, N. *et al.* Analysis of Dll4 regulation reveals a combinatorial role for Sox and Notch in arterial development. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 11893-11898, doi:10.1073/pnas.1300805110 (2013).

110 Fontijn, D. R. *et al.* SOX-18 controls endothelial-specific claudin-5 gene expression and barrier function. *Am J Physiol Heart Circ Physiol* **294**, H891–H900, doi:10.1152/ajpheart.01248.2007.-Members (2008).

111 You, L. R. *et al.* Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. *Nature* **435**, 98-104, doi:10.1038/nature03511 (2005).

112 Aranguren, X. L. *et al.* COUP-TFII orchestrates venous and lymphatic endothelial identity by homo- or hetero-dimerisation with PROX1. *J Cell Sci* **126**, 1164-1175, doi:10.1242/jcs.116293 (2013).

113 Boudreau, N. J. & Varner, J. A. The homeobox transcription factor Hox D3 promotes integrin alpha5beta1 expression and function during angiogenesis. *The Journal of biological chemistry* **279**, 4862-4868, doi:10.1074/jbc.M305190200 (2004).

114 Bahrami, S. B., Veiseh, M., Dunn, A. A. & Boudreau, N. J. Temporal changes in Hox gene expression accompany endothelial cell differentiation of embryonic stem cells. *Cell Adhesion & Migration* **5**, 133-141, doi:10.4161/cam.5.2.14373 (2011).

115 Iacovino, M. *et al.* HoxA3 is an apical regulator of haemogenic endothelium. *Nature cell biology* **13**, 72-78, doi:10.1038/ncb2137 (2011).
116 Northop, J. P. *et al.* NF-AT components define a family of transcription factors targeted in T-cell activation. *Nature* **369**, 497-502 (1994).
117 de la Pompa, J. S. *et al.* Roleof the NF-ATc transcription factor in morphogenesis of cardiac valves and septum. *Nature* **392**, 182-186 (1998).
118 Ranger, A. M. *et al.* The transcription factor NF-ATc is essential for cardiac valve formation. *Nature* **392**, 186-190 (1998).
119 Johnson, E. N. *et al.* NFATc1 mediates vascular endothelial growth factor-induced proliferation of human pulmonary valve endothelial cells. *The Journal of biological chemistry* **278**, 1686-1692, doi:10.1074/jbc.M210250200 (2003).
120 Chang, C. P. *et al.* A field of myocardial-endocardial NFAT signaling underlies heart valve morphogenesis. *Cell* **118**, 649-663, doi:10.1016/j.cell.2004.08.010 (2004).
121 Lambrechts, D. & Carmeliet, P. Sculpting heart valves with NFATc and VEGF. *Cell* **118**, 532-534, doi:10.1016/j.cell.2004.08.022 (2004).
122 Wu, B. *et al.* Endocardial cells form the coronary arteries by angiogenesis through myocardial-endocardial VEGF signaling. *Cell* **151**, 1083-1096, doi:10.1016/j.cell.2012.10.023 (2012).
123 Pan, M. G., Xiong, Y. & Chen, F. NFAT Gene Family in Inflammation and Cancer. *Curr Mol Med* **13**, 543–554. (2013).
124 Rao, A., Luo, C. & Hogan, P. G. Transcription factors of the NFAT family: Regulation and Function. *Ann Rev Immumol* **15**, 707-747 (1997).
125 Liu, Z. *et al.* The kinase LRRK2 is a regulator of the transcription factor NFAT that modulates the severity of inflammatory bowel disease. *Nature immunology* **12**, 1063-1070, doi:10.1038/ni.2113 (2011).
126 Danks, L. & Takayanagi, H. Immunology and bone. *Journal of biochemistry* **154**, 29-39, doi:10.1093/jb/mvt049 (2013).
127 Kyttaris, V. C., Wang, Y., Juang, Y. T., Weinstein, A. & Tsokos, G. C. Increased levels of NF-ATc2 differentially regulate CD154 and IL-2 genes in T cells from patients with systemic lupus erythematosus. *Journal of immunology* **178**, 1960-1966 (2007).
128 Wang, Y. *et al.* Activation of NFAT signaling in podocytes causes glomerulosclerosis. *J Am Soc Nephrol* **21**, 1657-1666, doi:10.1681/ASN.2009121253 (2010).
129 Aliprantis, A. O. & Glimcher, L. H. NFATc1 in inflammatory and musculoskeletal conditions. *Advances in experimental medicine and biology* **658**, 69-75, doi:10.1007/978-1-4419-1050-9_8 (2010).
130 Bretz, C. A., Savage, S., Capozzi, M. & Penn, J. S. The role of the NFAT signaling pathway in retinal neovascularization. *Investigative ophthalmology & visual science* **54**, 7020-7027, doi:10.1167/iovs.13-12183 (2013).
131 Suehiro, J. I. *et al.* Genome-wide approaches reveal functional vascular endothelial growth factor (VEGF)-inducible nuclear factor of activated T cells (NFAT) c1 binding to angiogenesis-related genes in endothelium. *The Journal of biological chemistry*, doi:10.1074/jbc.M114.555235 (2014).
132 Waddington, C. H. The Epigenotpye. *Endeavour*, 18-20 (1942).
133 Chen, L. J., Wei, S. Y. & Chiu, J. J. Mechanical regulation of epigenetics in vascular biology and pathobiology. *Journal of cellular and molecular medicine* **17**, 437-448, doi:10.1111/jcmm.12031 (2013).
134 Shirodkar, A. V. *et al.* A mechanistic role for DNA methylation in endothelial cell (EC)-enriched gene expression: relationship with DNA replication timing. *Blood* **121**, 3531-3540, doi:10.1182/blood-2013-01-479170 (2013).
135 Turunen, M. P. & Yla-Herttuala, S. Epigenetic regulation of key vascular genes and growth factors. *Cardiovasc Res* **90**, 441-446, doi:10.1093/cvr/cvr109 (2011).

136 Zhou, B., Margariti, A., Zeng, L. & Xu, Q. Role of histone deacetylases in vascular cell homeostasis and arteriosclerosis. *Cardiovasc Res* **90**, 413-420, doi:10.1093/cvr/cvr003 (2011).
137 Margariti, A. *et al.* Histone deacetylase 7 controls endothelial cell growth through modulation of beta-catenin. *Circulation research* **106**, 1202-1211, doi:10.1161/CIRCRESAHA.109.213165 (2010).
138 Urbich, C. *et al.* HDAC5 is a repressor of angiogenesis and determines the angiogenic gene expression pattern of endothelial cells. *Blood* **113**, 5669-5679, doi:10.1182/blood-2009-01-196485 (2009).
139 Zampetaki, A. *et al.* Histone deacetylase 3 is critical in endothelial survival and atherosclerosis development in response to disturbed flow. *Circulation* **121**, 132-142, doi:10.1161/CIRCULATIONAHA.109.890491 (2010).
140 Xu, S. S., Alam, S. & Margariti, A. Epigenetics in Vascular Disease – Therapeutic Potential of New Agents. *Current Vascular Pharmacology* **12**, 77-86 (2014).
141 Chen, H. Z., Wan, Y. Z. & Liu, D. P. Cross-talk between SIRT1 and p66Shc in vascular diseases. *Trends in cardiovascular medicine* **23**, 237-241, doi:10.1016/j.tcm.2013.01.001 (2013).
142 Chen, Z. *et al.* Shear stress, SIRT1, and vascular homeostasis. *PNAS* **107**, 10268–10273 (2010).
143 Shu-Ching Yan, M., Matouk, C. C. & Marsden, P. A. Epigenetics of the vascular endothelium. *J App Physiol* **109**, 916-926, doi:10.1152/japplphysiol.00131.2010.-Classical (2010).
144 Hu, M. *et al.* Histone H3 lysine 36 methyltransferase Hypb/Setd2 is required for embryonic vascular remodeling. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 2956-2961, doi:10.1073/pnas.0915033107 (2010).
145 Katoh, M. Therapeutics targeting angiogenesis: genetics and epigenetics, extracellular miRNAs and signaling networks (Review). *International journal of molecular medicine* **32**, 763-767, doi:10.3892/ijmm.2013.1444 (2013).
146 Costa, A. *et al.* miR-363-5p regulates endothelial cell properties and their communication with hematopoietic precursor cells. *Journal of hematology & oncology* **6** (2013).
147 Folkman, J. Tumor angiogenesis: therapeutic implications. *The New England journal of medicine* **285**, 1182-1186, doi:10.1056/NEJM197111182852108 (1971).
148 Folkman, J., Merler, E., Abernathy, C. & Williams, G. Isolation of a tumor factor responsible for angiogenesis *The New England journal of medicine* **133**, 275-288 (1971).
149 Folkman, J. & Hanahan, D. Switch to the angiogenic phenotype during tumorigenesis. *Princess Takamatsu symposia* **22**, 339-347 (1991).
150 Hanahan, D. & Weinberg, R. A. The Hallmarks of Cancer. *Cell* **100**, 57-70 (2000).
151 Weis, S. M. & Cheresh, D. A. Tumor angiogenesis: molecular pathways and therapeutic targets. *Nature medicine* **17**, 1359-1370, doi:10.1038/nm.2537 (2011).
152 Lyden, D. *et al.* Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nature medicine* **7**, 1194-1201, doi:10.1038/nm1101-1194 (2001).
153 Goveia, J., Stapor, P. & Carmeliet, P. Principles of targeting endothelial cell metabolism to treat angiogenesis and endothelial cell dysfunction in disease. *EMBO molecular medicine*, doi:10.15252/emmm.201404156 (2014).
154 Domingues, G., Gouveia Fernandes, S. & Serpa, J. Dynamics of VEGF-A and its receptors in cancer vascularization-an overview. *iConcept Press* Lda, Recent Cancer Research and Treatment, **in press** (2014).
155 von Minckwitz, G. *et al.* Neoadjuvant chemotherapy and bevacizumab for HER2-negative breast cancer. *The New England journal of medicine* **366**, 299-309, doi:10.1056/NEJMoa1111065 (2012).

156 Tol, J. *et al.* Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. *The New England journal of medicine* **360**, 563-572, doi:10.1056/NEJMoa0808268 (2009).
157 Allegra, C. J. *et al.* Phase III trial assessing bevacizumab in stages II and III carcinoma of the colon: results of NSABP protocol C-08. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **29**, 11-16, doi:10.1200/JCO.2010.30.0855 (2011).
158 Bennouna, J. *et al.* Continuation of bevacizumab after first progression in metastatic colorectal cancer (ML18147): a randomised phase 3 trial. *The lancet oncology* **14**, 29-37, doi:10.1016/S1470-2045(12)70477-1 (2013).
159 Perren, T. J. *et al.* A phase 3 trial of bevacizumab in ovarian cancer. *The New England journal of medicine* **365**, 2484-2496, doi:10.1056/NEJMoa1103799 (2011).
160 Rapisarda, A. & Melillo, G. Overcoming disappointing results with antiangiogenic therapy by targeting hypoxia. *Nature reviews. Clinical oncology* **9**, 378-390, doi:10.1038/nrclinonc.2012.64 (2012).
161 Miller, K. *et al.* Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. *The New England journal of medicine* **357**, 2666-2676, doi:10.1056/NEJMoa072113 (2007).
162 Miles, D. W. *et al.* Phase III study of bevacizumab plus docetaxel compared with placebo plus docetaxel for the first-line treatment of human epidermal growth factor receptor 2-negative metastatic breast cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **28**, 3239-3247, doi:10.1200/JCO.2008.21.6457 (2010).
163 Hurwitz, H. *et al.* Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *The New England journal of medicine* **350**, 2335-2342, doi:10.1056/NEJMoa032691 (2004).
164 Meadows, K. L. & Hurwitz, H. I. Anti-VEGF therapies in the clinic. *Cold Spring Harbor perspectives in medicine* **2**, doi:10.1101/cshperspect.a006577 (2012).
165 Saltz, L. *et al.* Phase III trial of cetuximab, bevacizumab, and 5-fluorouracil/leucovorin vs. FOLFOX-bevacizumab in colorectal cancer. *Clinical colorectal cancer* **11**, 101-111, doi:10.1016/j.clcc.2011.05.006 (2012).
166 Reck, M. *et al.* Overall survival with cisplatin-gemcitabine and bevacizumab or placebo as first-line therapy for nonsquamous non-small-cell lung cancer: results from a randomised phase III trial (AVAiL). *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* **21**, 1804-1809, doi:10.1093/annonc/mdq020 (2010).
167 Herbst, R. S. *et al.* Efficacy of bevacizumab plus erlotinib versus erlotinib alone in advanced non-small-cell lung cancer after failure of standard first-line chemotherapy (BeTa): a double-blind, placebo-controlled, phase 3 trial. *Lancet* **377**, 1846-1854, doi:10.1016/S0140-6736(11)60545-X (2011).
168 Rini, B. I. *et al.* Phase III trial of bevacizumab plus interferon alfa versus interferon alfa monotherapy in patients with metastatic renal cell carcinoma: final results of CALGB 90206. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **28**, 2137-2143, doi:10.1200/JCO.2009.26.5561 (2010).
169 Friedman, H. S. *et al.* Bevacizumab alone and in combination with irinotecan in recurrent glioblastoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **27**, 4733-4740, doi:10.1200/JCO.2008.19.8721 (2009).
170 Burger, R. A. *et al.* Incorporation of bevacizumab in the primary treatment of ovarian cancer. *The New England journal of medicine* **365**, 2473-2483, doi:10.1056/NEJMoa1104390 (2011).
171 Aghajanian, C. *et al.* OCEANS: a randomized, double-blind, placebo-controlled phase III trial of chemotherapy with or without bevacizumab in patients with platinum-sensitive

recurrent epithelial ovarian, primary peritoneal, or fallopian tube cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **30**, 2039-2045, doi:10.1200/JCO.2012.42.0505 (2012).

172 Kindler, H. L. *et al.* Gemcitabine plus bevacizumab compared with gemcitabine plus placebo in patients with advanced pancreatic cancer: phase III trial of the Cancer and Leukemia Group B (CALGB 80303). *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **28**, 3617-3622, doi:10.1200/JCO.2010.28.1386 (2010).

173 Van Cutsem, E. *et al.* Addition of aflibercept to fluorouracil, leucovorin, and irinotecan improves survival in a phase III randomized trial in patients with metastatic colorectal cancer previously treated with an oxaliplatin-based regimen. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **30**, 3499-3506, doi:10.1200/JCO.2012.42.8201 (2012).

174 Ramlau, R. *et al.* Aflibercept and Docetaxel versus Docetaxel alone after platinum failure in patients with advanced or metastatic non-small-cell lung cancer: a randomized, controlled phase III trial. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **30**, 3640-3647, doi:10.1200/JCO.2012.42.6932 (2012).

175 Tarhini, A. A. *et al.* Aflibercept (VEGF Trap) in inoperable stage III or stage iv melanoma of cutaneous or uveal origin. *Clinical cancer research : an official journal of the American Association for Cancer Research* **17**, 6574-6581, doi:10.1158/1078-0432.CCR-11-1463 (2011).

176 Escudier, B. *et al.* Sorafenib for treatment of renal cell carcinoma: Final efficacy and safety results of the phase III treatment approaches in renal cancer global evaluation trial. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **27**, 3312-3318, doi:10.1200/JCO.2008.19.5511 (2009).

177 Llovet, J. M. *et al.* Sorafenib in advanced hepatocellular carcinoma. *The New England journal of medicine* **359**, 378-390, doi:10.1056/NEJMoa0708857 (2008).

178 Ott, P. A. *et al.* A phase II trial of sorafenib in metastatic melanoma with tissue correlates. *PloS one* **5**, e15588, doi:10.1371/journal.pone.0015588 (2010).

179 Motzer, R. J. *et al.* Overall survival and updated results for sunitinib compared with interferon alfa in patients with metastatic renal cell carcinoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **27**, 3584-3590, doi:10.1200/JCO.2008.20.1293 (2009).

180 Goodman, V. L. *et al.* Approval summary: sunitinib for the treatment of imatinib refractory or intolerant gastrointestinal stromal tumors and advanced renal cell carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research* **13**, 1367-1373, doi:10.1158/1078-0432.CCR-06-2328 (2007).

181 Raymond, E. *et al.* Sunitinib malate for the treatment of pancreatic neuroendocrine tumors. *The New England journal of medicine* **364**, 501-513, doi:10.1056/NEJMoa1003825 (2011).

182 Decoster, L. *et al.* Activity of sunitinib in advanced malignant melanoma and its correlation with potential predictive biomarkers. *Journal of Clinical Oncology* **28**, 8518 (2010).

183 Sternberg, C. N. *et al.* A randomised, double-blind phase III study of pazopanib in patients with advanced and/or metastatic renal cell carcinoma: final overall survival results and safety update. *European journal of cancer* **49**, 1287-1296, doi:10.1016/j.ejca.2012.12.010 (2013).

184 van der Graaf, W. T. *et al.* Pazopanib for metastatic soft-tissue sarcoma (PALETTE): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet* **379**, 1879-1886, doi:10.1016/S0140-6736(12)60651-5 (2012).

185 Wells, S. A., Jr. *et al.* Vandetanib in patients with locally advanced or metastatic medullary thyroid cancer: a randomized, double-blind phase III trial. *Journal of clinical*

oncology : official journal of the American Society of Clinical Oncology **30**, 134-141, doi:10.1200/JCO.2011.35.5040 (2012).

186 Motzer, R. J. *et al.* Axitinib versus sorafenib as second-line treatment for advanced renal cell carcinoma: overall survival analysis and updated results from a randomised phase 3 trial. *The lancet oncology* **14**, 552-562, doi:10.1016/S1470-2045(13)70093-7 (2013).

187 Shah, M. A. Gastrointestinal cancer: targeted therapies in gastric cancer-the dawn of a new era. *Nature reviews. Clinical oncology* **11**, 10-11, doi:10.1038/nrclinonc.2013.231 (2014).

188 Spratlin, J. L. *et al.* Phase I pharmacologic and biologic study of ramucirumab (IMC-1121B), a fully human immunoglobulin G1 monoclonal antibody targeting the vascular endothelial growth factor receptor-2. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **28**, 780-787, doi:10.1200/JCO.2009.23.7537 (2010).

189 Garon, E. B. *et al.* A randomized, double-blind, phase III study of Docetaxel and Ramucirumab versus Docetaxel and placebo in the treatment of stage IV non-small-cell lung cancer after disease progression after 1 previous platinum-based therapy (REVEL): treatment rationale and study design. *Clinical lung cancer* **13**, 505-509, doi:10.1016/j.cllc.2012.06.007 (2012).

190 Takayanagi, H. *et al.* Induction and Activation of the Transcription Factor NFATc1 (NFAT2) Integrate RANKL Signaling in Terminal Differentiation of Osteoclasts. *Developmental cell* **3**, 889–901 (2002).

191 Feske, S., Giltnane, J., Dolmetsch, R., Staudt, L. M. & Rao, A. Gene regulation mediated by calcium signals in T lymphocytes. *Nature immunology* **2**, 316-324, doi:10.1038/86318 (2001).

192 Crabtree, G. R. & Olson, E. N. NFAT Signaling: Choreographing the Social Lives of Cells. *Cell* **2002**, S67–S79 (2002).

193 Serfling, E., Chuvpilo, S., Liu, J., Hofer, T. & Palmetshofer, A. NFATc1 autoregulation: a crucial step for cell-fate determination. *Trends in immunology* **27**, 461-469, doi:10.1016/j.it.2006.08.005 (2006).

194 Liu, J. *et al.* Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66**, 807-815 (1991).

195 Jang, G. H., Park, I. S., Yang, J. H., Bischoff, J. & Lee, Y. M. Differential functions of genes regulated by VEGF-NFATc1 signaling pathway in the migration of pulmonary valve endothelial cells. *FEBS letters* **584**, 141-146, doi:10.1016/j.febslet.2009.11.031 (2010).

196 Armesilla, A. L. *et al.* Vascular Endothelial Growth Factor Activates Nuclear Factor of Activated T Cells in Human Endothelial Cells: a Role for Tissue Factor Gene Expression. *Molecular and cellular biology* **19**, 2032–2043 (1999).

197 Mancini, M. & Toker, A. NFAT proteins: emerging roles in cancer progression. *Nature reviews. Cancer* **9**, 810-820, doi:10.1038/nrc2735 (2009).

198 Jinnin, M. *et al.* Suppressed NFAT-dependent VEGFR1 expression and constitutive VEGFR2 signaling in infantile hemangioma. *Nature medicine* **14**, 1236-1246, doi:10.1038/nm.1877 (2008).

199 Siamakpour-Reihani, S. *et al.* The role of calcineurin/NFAT in SFRP2 induced angiogenesis--a rationale for breast cancer treatment with the calcineurin inhibitor tacrolimus. *PloS one* **6**, e20412, doi:10.1371/journal.pone.0020412 (2011).

200 Hunt, S. Technology evaluation: IMC-1C11, ImClone Systems. *Current opinion in molecular therapeutics* **3**, 418-424 (2001).

201 Shaw, J. P. *et al.* Identification of a putative regulator of early T cell activation genes. *Science* **241**, 202-205 (1988).

202 Wu, B., Baldwin, H. S. & Zhou, B. Nfatc1 directs the endocardial progenitor cells to make heart valve primordium. *Trends in cardiovascular medicine* **23**, 294-300, doi:10.1016/j.tcm.2013.04.003 (2013).

203 Ferrara, N., Gerber, H. P. & LeCouter, J. The biology of VEGF and its receptors. *Nature medicine* **9**, 669-676, doi:10.1038/nm0603-669 (2003).
204 Yiu, G. K., Kaunisto, A., Chin, Y. R. & Toker, A. NFAT promotes carcinoma invasive migration through glypican-6. *The Biochemical journal* **440**, 157-166, doi:10.1042/BJ20110530 (2011).
205 Minami, T. *et al.* The calcineurin-NFAT-angiopoietin-2 signaling axis in lung endothelium is critical for the establishment of lung metastases. *Cell reports* **4**, 709-723, doi:10.1016/j.celrep.2013.07.021 (2013).
206 Kim, D. Y. *et al.* Upregulated hoxC4 induces CD14 expression during the differentiation of acute promyelocytic leukemia cells. *Leukemia & lymphoma* **46**, 1061-1066, doi:10.1080/10428190500102589 (2005).
207 Bijl, J. J. *et al.* Differentiation and cell-type-restricted expression of HOXC4, HOXC5 and HOXC6 in myeloid leukemias and normal myeloid cells. *Leukemia* **12**, 1724-1732 (1998).
208 Bijl, J. J. *et al.* HOXC4, HOXC5, and HOXC6 expression in primary cutaneous lymphoid lesions. High expression of HOXC5 in anaplastic large-cell lymphomas. *The American journal of pathology* **151**, 1067-1074 (1997).
209 Rieger, E. *et al.* Expression of the homeobox gene HOXC4 in keratinocytes of normal skin and epithelial skin tumors is correlated with differentiation. *The Journal of investigative dermatology* **103**, 341-346 (1994).
210 Mai, T. *et al.* Estrogen receptors bind to and activate the HOXC4/HoxC4 promoter to potentiate HoxC4-mediated activation-induced cytosine deaminase induction, immunoglobulin class switch DNA recombination, and somatic hypermutation. *The Journal of biological chemistry* **285**, 37797-37810, doi:10.1074/jbc.M110.169086 (2010).
211 Alami, Y., Castronovo, V., Belotti, D., Flagiello, D. & Clausse, N. HOXC5 and HOXC8 expression are selectively turned on in human cervical cancer cells compared to normal keratinocytes. *Biochemical and biophysical research communications* **257**, 738-745, doi:10.1006/bbrc.1999.0516 (1999).
212 Zhang, C., Han, Y., Huang, H., Qu, L. & Shou, C. High NR2F2 transcript level is associated with increased survival and its expression inhibits TGF-beta-dependent epithelial-mesenchymal transition in breast cancer. *Breast cancer research and treatment* **147**, 265-281, doi:10.1007/s10549-014-3095-3 (2014).
213 More, E. *et al.* Activation of the MAP kinase pathway induces chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) expression in human breast cancer cell lines. *The Journal of endocrinology* **176**, 83-94 (2003).
214 Muller, R. *et al.* Targeting proliferating cell nuclear antigen and its protein interactions induces apoptosis in multiple myeloma cells. *PloS one* **8**, e70430, doi:10.1371/journal.pone.0070430 (2013).
215 Tan, Z. *et al.* Small-molecule targeting of proliferating cell nuclear antigen chromatin association inhibits tumor cell growth. *Molecular pharmacology* **81**, 811-819, doi:10.1124/mol.112.077735 (2012).
216 Stoimenov, I. & Helleday, T. PCNA on the crossroad of cancer. *Biochemical Society transactions* **37**, 605-613, doi:10.1042/BST0370605 (2009).
217 La Porta, C. A. & Zapperi, S. Human breast and melanoma cancer stem cells biomarkers. *Cancer letters* **338**, 69-73, doi:10.1016/j.canlet.2012.03.017 (2013).
218 Yang, Y., Wang, Y., Yin, C. & Li, X. Clinical significance of the stem cell gene Oct-4 in cervical cancer. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* **35**, 5339-5345, doi:10.1007/s13277-014-1696-4 (2014).
219 Bishop-Bailey, D. PPARs and angiogenesis. *Biochemical Society transactions* **39**, 1601-1605, doi:10.1042/BST20110643 (2011).

220 Qi, Q. R. & Yang, Z. M. Regulation and function of signal transducer and activator of transcription 3. *World journal of biological chemistry* **5**, 231-239, doi:10.4331/wjbc.v5.i2.231 (2014).
221 Shih, T. C. *et al.* Aberrant activation of nuclear factor of activated T cell 2 in lamina propria mononuclear cells in ulcerative colitis. *World journal of gastroenterology : WJG* **14**, 1759-1767 (2008).
222 Cockerill, G. W. *et al.* Regulation of granulocyte-macrophage colony-stimulating factor and E-selectin expression in endothelial cells by cyclosporin A and the T-cell transcription factor NFAT. *Blood* **86**, 2689-2698 (1995).
223 Hammond, M. E. H. *et al.* American Society of ClinicalOncology/College of American Pathologists Guideline Recommendations for Immunohistochemical Testing of Estrogen and Progesterone Receptors in Breast Cancer (Unabridged Version). *Arch Pathol Lab Med* **184**, e48-e72 (2010).
224 Andrulis, I. L. *et al.* neu/erbB-2 amplification identifies a poor-prognosis group of women with node-negative breast cancer. Toronto Breast Cancer Study Group. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **16**, 1340-1349 (1998).
225 Feeley, L. P., Mulligan, A. M., Pinnaduwage, D., Bull, S. B. & Andrulis, I. L. Distinguishing luminal breast cancer subtypes by Ki67, progesterone receptor or TP53 status provides prognostic information. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* **27**, 554-561, doi:10.1038/modpathol.2013.153 (2014).
226 Mass, E., Wachten, D., Aschenbrenner, A. C., Voelzmann, A. & Hoch, M. Murine Creld1 controls cardiac development through activation of calcineurin/NFATc1 signaling. *Developmental cell* **28**, 711-726, doi:10.1016/j.devcel.2014.02.012 (2014).
227 Garcia-Gomez, A., Ocio, E. M., Pandiella, A., San Miguel, J. F. & Garayoa, M. RAF265, a dual BRAF and VEGFR2 inhibitor, prevents osteoclast formation and resorption. Therapeutic implications. *Investigational new drugs* **31**, 200-205, doi:10.1007/s10637-012-9845-3 (2013).
228 Quinn, T. P., Peters, K. G., de Vries, C., Ferrara, N. & Williams, L. Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 7533-7537 (1993).
229 Cudmore, M. J. *et al.* The role of heterodimerization between VEGFR-1 and VEGFR-2 in the regulation of endothelial cell homeostasis. *Nature communications* **3**, 972, doi:10.1038/ncomms1977 (2012).
230 Bottsford-Miller, J. N., Coleman, R. L. & Sood, A. K. Resistance and escape from antiangiogenesis therapy: clinical implications and future strategies. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **30**, 4026-4034, doi:10.1200/JCO.2012.41.9242 (2012).
231 Rapisarda, A. & Melillo, G. Role of the VEGF/VEGFR axis in cancer biology and therapy. *Advances in cancer research* **114**, 237-267, doi:10.1016/B978-0-12-386503-8.00006-5 (2012).
232 Wagner, E. J. & Carpenter, P. B. Understanding the language of Lys36 methylation at histone H3. *Nature reviews. Molecular cell biology* **13**, 115-126, doi:10.1038/nrm3274 (2012).
233 Kizer, K. O. *et al.* A novel domain in Set2 mediates RNA polymerase II interaction and couples histone H3 K36 methylation with transcript elongation. *Molecular and cellular biology* **25**, 3305-3316, doi:10.1128/MCB.25.8.3305-3316.2005 (2005).
234 Bannister, A. J. *et al.* Spatial distribution of di- and tri-methyl lysine 36 of histone H3 at active genes. *The Journal of biological chemistry* **280**, 17732-17736, doi:10.1074/jbc.M500796200 (2005).

235 Luco, R. F. *et al.* Regulation of alternative splicing by histone modifications. *Science* **327**, 996-1000, doi:10.1126/science.1184208 (2010).
236 Duns, G. *et al.* Histone methyltransferase gene SETD2 is a novel tumor suppressor gene in clear cell renal cell carcinoma. *Cancer research* **70**, 4287-4291, doi:10.1158/0008-5472.CAN-10-0120 (2010).
237 Fontebasso, A. M. *et al.* Mutations in SETD2 and genes affecting histone H3K36 methylation target hemispheric high-grade gliomas. *Acta neuropathologica* **125**, 659-669, doi:10.1007/s00401-013-1095-8 (2013).
238 Kudithipudi, S. & Jeltsch, A. Role of somatic cancer mutations in human protein lysine methyltransferases. *Biochimica et biophysica acta* **1846**, 366-379, doi:10.1016/j.bbcan.2014.08.002 (2014).
239 Schmidt, C. K. & Jackson, S. P. On your mark, get SET(D2), go! H3K36me3 primes DNA mismatch repair. *Cell* **153**, 513-515, doi:10.1016/j.cell.2013.04.018 (2013).
240 Nayak, A. *et al.* Sumoylation of the transcription factor NFATc1 leads to its subnuclear relocalization and interleukin-2 repression by histone deacetylase. *The Journal of biological chemistry* **284**, 10935-10946, doi:10.1074/jbc.M900465200 (2009).
241 Martinez, G. *et al.* Genome-wide analysis highlights the involvement of calcineurin/NFAT pathway signaling in transcriptional elongation in restimulated memory CD8 T cells (meeting abstract). *Journal of immunology* **190.8** (2013).
242 Ponce, M. L. Tube formation: an in vitro matrigel angiogenesis assay. *Methods in molecular biology* **467**, 183-188, doi:10.1007/978-1-59745-241-0_10 (2009).
243 Edmunds, J. W., Mahadevan, L. C. & Clayton, A. L. Dynamic histone H3 methylation during gene induction: HYPB/Setd2 mediates all H3K36 trimethylation. *The EMBO journal* **27**, 406-420, doi:10.1038/ (2008).
244 Yamamot, K. Human endothelial cells in culture. *Arch histol jap* **42**, 1-10 (1979).
245 Gimbrone, M. A., Cotran, R. S. & Folkman, J. Human vascular endothelial cells in culture. *The Journal of cell biology* **60**, 673-684 (1974).
246 Tashiro, K., Shimokama, T., Haraoka, S., Tokunaga, O. & Watanabe, T. Endothelial cell heterogeneity in experimentally-induced rabbit atherosclerosis. Demonstration of multinucleated giant endothelial cells by scanning electron microscopy and cell culture. *Virchows Archiv* **425**, 521-529 (1994).
247 Welikson, R. E., Kaestner, S., Reinecke, H. & Hauschka, S. D. Human umbilical vein endothelial cells fuse with cardiomyocytes but do not activate cardiac gene expression. *Journal of molecular and cellular cardiology* **40**, 520-528, doi:10.1016/j.yjmcc.2006.01.009 (2006).
248 Atsumi, Y. *et al.* Onset of quiescence following p53 mediated down-regulation of H2AX in normal cells. *PloS one* **6**, e23432, doi:10.1371/journal.pone.0023432 (2011).
249 Kim, J. H. *et al.* RANKL induces NFATc1 acetylation and stability via histone acetyltransferases during osteoclast differentiation. *The Biochemical journal* **436**, 253-262, doi:10.1042/BJ20110062 (2011).
250 Yasui, T. *et al.* Epigenetic regulation of osteoclast differentiation: possible involvement of Jmjd3 in the histone demethylation of Nfatc1. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* **26**, 2665-2671, doi:10.1002/jbmr.464 (2011).
251 Pham, L. V., Tamayo, A. T., Li, C., Bueso-Ramos, C. & Ford, R. J. An epigenetic chromatin remodeling role for NFATc1 in transcriptional regulation of growth and survival genes in diffuse large B-cell lymphomas. *Blood* **116**, 3899-3906, doi:10.1182/blood-2009-12-257378 (2010).
252 Youn, M. Y. *et al.* JMJD5, a Jumonji C (JmjC) domain-containing protein, negatively regulates osteoclastogenesis by facilitating NFATc1 protein degradation. *The Journal of biological chemistry* **287**, 12994-13004, doi:10.1074/jbc.M111.323105 (2012).

253 Hsia, D. A. *et al.* KDM8, a H3K36me2 histone demethylase that acts in the cyclin A1 coding region to regulate cancer cell proliferation. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 9671-9676, doi:10.1073/pnas.1000401107 (2010).
254 Strahl, B. D. *et al.* Set2 Is a Nucleosomal Histone H3-Selective Methyltransferase That Mediates Transcriptional Repression. *Molecular and cellular biology* **22**, 1298-1306, doi:10.1128/mcb.22.5.1298-1306.2002 (2002).
255 Gagliardi, A. & Collins, D. C. Inhibition of Angiogenesis by Antiestrogens. *Cancer research* **53**, 533-535 (1993).
256 Losordo, D. W. & Isner, J. M. Estrogen and angiogenesis: A review. *Arteriosclerosis, thrombosis, and vascular biology* **21**, 6-12 (2001).
257 Smith, G. L. The Long and Short of Tamoxifen Therapy: A Review of the ATLAS Trial. *Journal of the advanced practitioner in oncology* **5**, 57-60 (2014).
258 Siegfried, J. M., Gubish, C. T., Rothstein, M. E., Henry, C. & Stabile, L. P. Combining the multitargeted tyrosine kinase inhibitor vandetanib with the antiestrogen fulvestrant enhances its antitumor effect in non-small cell lung cancer. *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer* **7**, 485-495, doi:10.1097/JTO.0b013e31824177ea (2012).
259 Olin, J. L. & St Pierre, M. Aromatase Inhibitors in Breast Cancer Prevention. *The Annals of pharmacotherapy*, doi:10.1177/1060028014548416 (2014).
260 Smiley, D. A. & Khalil, R. A. Estrogenic Compounds, Estrogen Receptors and Vascular Cell Signaling in the Aging Blood Vessels. *Curr Med Chem.* **16**, 1863–1887 (2009).
261 Barnabas, O., Wang, H. & Gao, X. M. Role of estrogen in angiogenesis in cardiovascular diseases. *Journal of geriatric cardiology : JGC* **10**, 377-382, doi:10.3969/j.issn.1671-5411.2013.04.008 (2013).
262 Albrecht, E. D. *et al.* Effect of estrogen on angiogenesis in co-cultures of human endometrial cells and microvascular endothelial cells. *Human reproduction* **18**, 2039-2047 (2003).
263 Arany, Z. *et al.* HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1alpha. *Nature* **451**, 1008-1012, doi:10.1038/nature06613 (2008).
264 Hyder, S. M. *et al.* Regulation of vascular endothelial growth factor expression by estrogens and progestins. *Environmental Health Perspectives* **108**, 785-790 (2000).
265 Iyer, V. *et al.* Estrogen promotes ER-negative tumor growth and angiogenesis through mobilization of bone marrow-derived monocytes. *Cancer research* **72**, 2705-2713, doi:10.1158/0008-5472.CAN-11-3287 (2012).
266 Lippert, C., Seeger, H., Wallwiener, D. & Mueck, A. O. Comparison of the effects of 17alpha-ethinylestradiol and 17beta-estradiol on the proliferation of human breast cancer cells and human umbilical vein endothelial cells. *Clinical and experimental obstetrics & gynecology* **29**, 87-90 (2002).
267 Andozia, M. B. *et al.* Ethinylestradiol and estradiol have different effects on oxidative stress and nitric oxide synthesis in human endothelial cell cultures. *Fertility and sterility* **94**, 1578-1582, doi:10.1016/j.fertnstert.2009.08.052 (2010).
268 Tan, W. W. *et al.* N0539 phase II trial of fulvestrant and bevacizumab in patients with metastatic breast cancer previously treated with an aromatase inhibitor: a North Central Cancer Treatment Group (now Alliance) trial. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* **24**, 2548-2554, doi:10.1093/annonc/mdt213 (2013).
269 Dubey, R. K. & Jackson, E. K. Potential vascular actions of 2-methoxyestradiol. *Trends in endocrinology and metabolism: TEM* **20**, 374-379, doi:10.1016/j.tem.2009.04.007 (2009).

270 Fotsis, T. *et al.* The endogenous oestrogen metabolite 2-methoxyoestradiol inhibits angiogenesis and suppresses tumour growth. *Nature* **368**, 237-239, doi:10.1038/368237a0 (1994).
271 Zervoudis, S. *et al.* Main controversies in breast cancer. *World journal of clinical oncology* **5**, 359-373, doi:10.5306/wjco.v5.i3.359 (2014).
272 McNamara, D. A. *et al.* Tamoxifen inhibits endothelial cell proliferation and attenuates VEGF-mediated angiogenesis and migration in vivo. *European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology* **27**, 714-718, doi:10.1053/ejso.2001.1177 (2001).
273 Mooberry, S. L. New insights into 2-methoxyestradiol, a promising antiangiogenic and antitumor agent. *Curr Opin Oncol* **15**, 425–430 (2003).
274 Mueck, A. O. & Seeger, H. 2-Methoxyestradiol--biology and mechanism of action. *Steroids* **75**, 625-631, doi:10.1016/j.steroids.2010.02.016 (2010).
275 Solum, E. J. *et al.* Synthesis and biological evaluations of new analogs of 2-methoxyestradiol: Inhibitors of tubulin and angiogenesis. *European journal of medicinal chemistry* **85C**, 391-398, doi:10.1016/j.ejmech.2014.08.002 (2014).
276 Blackwell, K. L. *et al.* Tamoxifen Inhibits Angiogenesis in Estrogen Receptor-negative Animal Models. *Clinical cancer research : an official journal of the American Association for Cancer Research* **6**, 4359–4364 (2000).
277 Vinci, M. *et al.* Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. *BMC biology* **10**, 29, doi:10.1186/1741-7007-10-29 (2012).
278 Le Bras, A., Vijayaraj, P. & Oettgen, P. Molecular mechanisms of endothelial differentiation. *Vascular medicine* **15**, 321-331, doi:10.1177/1358863X10371685 (2010).
279 Combs, M. D. & Yutzey, K. E. VEGF and RANKL regulation of NFATc1 in heart valve development. *Circulation research* **105**, 565-574, doi:10.1161/CIRCRESAHA.109.196469 (2009).
280 Oyajobi, B. O. Multiple myeloma/hypercalcemia. *Arthritis research & therapy* **9 Suppl 1**, S4, doi:10.1186/ar2168 (2007).
281 Rao, V., Chaukar, D. & D'Cruz, A. K. Hypercalcemia and treated breast cancers: the diagnostic dilemma. *Journal of cancer research and therapeutics* **5**, 46-48 (2009).
282 Clines, G. A. Mechanisms and treatment of hypercalcemia of malignancy. *Current opinion in endocrinology, diabetes, and obesity* **18**, 339-346, doi:10.1097/MED.0b013e32834b4401 (2011).
283 Palapattu, G. S., Kristo, B. & Rajfer, J. Paraneoplastic syndromes in urologic malignancy: the many faces of renal cell carcinoma. *Reviews in urology* **4**, 163-170 (2002).
284 Alsirafy, S. A., Sroor, M. Y. & Al-Shahri, M. Z. Hypercalcemia in advanced head and neck squamous cell carcinoma: prevalence and potential impact on palliative care. *The journal of supportive oncology* **7**, 154-157 (2009).
285 Schwartz, G. G. Prostate cancer, serum parathyroid hormone, and the progression of skeletal metastases. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **17**, 478-483, doi:10.1158/1055-9965.EPI-07-2747 (2008).
286 Munaron, L., Genova, T., Avanzato, D., Antoniotti, S. & Pla, A. F. Targeting Calcium Channels to Block Tumor Vascularization. *Recent Patents on Anti-Cancer Drug Discovery* **8**, 27-37 (2013).
287 Monteith, G. R., Davis, F. M. & Roberts-Thomson, S. J. Calcium channels and pumps in cancer: changes and consequences. *The Journal of biological chemistry* **287**, 31666-31673, doi:10.1074/jbc.R112.343061 (2012).
288 Tran , Q. K., Ohashi, K. & Watanabe, H. Calcium signalling in endothelial cells. *Cardiovasc Res* **48**, 13-22 (2000).

289 Shihab, F. S., Bennett, W. M., Isaac, J., Yi, H. & Andoh, T. F. Angiotensin II regulation of vascular endothelial growth factor and receptors Flt-1 and KDR/Flk-1 in cyclosporine nephrotoxicity. *Kidney International* **66**, 422–433 (2002).
290 Caramelo, C. *et al.* Cyclosporin A toxicity, and more: vascular endothelial growth factor (VEGF) steps forward. *Nephrology Dialysis Transplantation* **19**, 285-288, doi:10.1093/ndt/gfg516 (2004).
291 Ariizumi, T. *et al.* Multinucleation followed by an acytokinetic cell division in myxofibrosarcoma with giant cell proliferation. *Journal of experimental & clinical cancer research : CR* **28**, 44, doi:10.1186/1756-9966-28-44 (2009).
292 Liu, Z., Yue, S., Chen, X., Kubin, T. & Braun, T. Regulation of cardiomyocyte polyploidy and multinucleation by CyclinG1. *Circulation research* **106**, 1498-1506, doi:10.1161/CIRCRESAHA.109.211888 (2010).
293 Bird, M. C., Garside, D. & Jones, H. B. Multinucleated giant cells in primary cultures derived from canine bone marrow--evidence for formation of putative osteoclasts. *Cell and tissue research* **268**, 17-30 (1992).
294 Gansburgskii, A. N. & Pavlov, A. V. [The mitotic origin of binucleated cells in regenerating endothelium]. *Ontogenez* **24**, 79-82 (1993).
295 Eccleston, A., Cesari, F. & Skipper, M. Transcription and epigenetics. *Nature insight* **502** (2013).
296 Stein, G. S. *et al.* Transcription factor-mediated epigenetic regulation of cell growth and phenotype for biological control and cancer. *Advances in enzyme regulation* **50**, 160-167, doi:10.1016/j.advenzreg.2009.10.026 (2010).
297 Kim, K., Lee, S. H., Ha Kim, J., Choi, Y. & Kim, N. NFATc1 induces osteoclast fusion via up-regulation of Atp6v0d2 and the dendritic cell-specific transmembrane protein (DC-STAMP). *Molecular endocrinology* **22**, 176-185, doi:10.1210/me.2007-0237 (2008).
298 Zhu, M., Van Dyke, T. E. & Gyurko, R. Resolvin E1 regulates osteoclast fusion via DC-STAMP and NFATc1. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **27**, 3344-3353, doi:10.1096/fj.12-220228 (2013).
299 Pribluda, V. S. *et al.* 2-Methoxyestradiol: An endogenous antiangiogenic and antiproliferative drug candidate. *Cancer and Metastasis Reviews* **19**, 173–179 (2000).
300 Sutherland, T. E. *et al.* 2-Methoxyestradiol--a unique blend of activities generating a new class of anti-tumour/anti-inflammatory agents. *Drug discovery today* **12**, 577-584, doi:10.1016/j.drudis.2007.05.005 (2007).

Chapter 8

ANNEXES

- **Domingues G.**, Dias S. Angiogénese tumoral. In MJGS Lda. *Melanoma 2013*. Coimbra, Portugal: Gráfica de Coimbra, 107-113, ISBN 978-989-8602-08-4 (2014).
- Rodrigues dos Santos C., **Domingues G.**, Matias I., Matos J., Fonseca I., Mendes de Almeida J., Dias S. LDL-cholesterol signaling induces breast cancer proliferation and invasion. *Lipids in health and disease* **13** (1): 16, doi: 10.1186/1476-511X-13-16 (2014).
- **Domingues G**., Gouveia Fernandes S., Serpa, J. Dynamics of VEGF-A and its receptors in cancer vascularization – an overview. In iConcept Press Lda. *Recent Cancer Research and Treatment*. In press (2014).

Domingues G., Dias S. Angiogénese tumoral. In MJGS Lda. *Melanoma 2013*. Coimbra, Portugal: Gráfica de Coimbra, 107-113, ISBN 978-989-8602-08-4 (2014).

Angiogénese no Melanoma

Germana Domingues, Sérgio Dias

Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa

1. Angiogénese tumoral

Angiogénese é o processo de formação de novos vasos sanguíneos a partir de vasos pré-existentes. Distingue-se da vasculogénese, por esta reflectir a formação de vasos durante o desenvolvimento embrionário, e por envolver a diferenciação de células endoteliais a partir de precursores endoteliais (angioblastos)[1,2].

Reconhecida há anos como uma das características intrínsecas do cancro (os *cancer hallmarks*, postulados por Hanahan e Weinberg[3]), a angiogénese é fundamental para o fornecimento de oxigénio, nutrientes, mas também para a manutenção da integridade e funcionalidade teciduais. Estudos mais recentes demonstraram inclusive que os vasos sanguíneos de diferentes tecidos desempenham um papel fundamental na recuperação tecidual após stress ou danos químicos/físicos[4]. Assim, a formação de novos vasos (angiogénese ou vasculogénese ou ambas) pode levar à "perda de identidade tecidual", gerando um ambiente mais permissivo à expansão de clones celulares malignos.

Dada a sua importância para o crescimento tumoral e eventualmente para a disseminação de células tumorais para a formação de metástases, a angiogénese tem recebido atenção por parte de clínicos e de investigadores, tendo sido uma das áreas de investigação do cancro a receber mais destaque nos últimos 15 anos.

Os mecanismos moleculares que regulam a angiogénese em contexto tumoral são amplamente conhecidos. Em tumores sólidos, foi sugerido que uma expansão celular com mais que $1mm^3$, ou caso a distância das células tumorais ao vaso sanguíneo mais próximo seja superior a 100µm, resulta no aparecimento de zonas de hipoxia, que activam um programa transcricional nas células tumorais (e outras), levando à produção de factores pró-angiogénicos. A perda de "equilíbrio" entre factores pró- e anti-angiogénicos num tecido desencadeia o processo de angiogénese, ou *angiogenic switch* (postulado por Hanahan e Folkman[5]) levando assim à expansão da vasculatura que apoia o tumor em crescimento.

1.1 Mecanismos moleculares na angiogénese: factores pró- e anti-angiogénicos

Os principais factores pró-angiogénicos identificados num contexto tumoral são o *Vascular Endothelial Growth Factor* (VEGF) o *Fibroblast Growth Factor* (FGF) e o *Transforming Growth Factor-β* (TGF-β).

O VEGF é produzido pela maioria das células tumorais como consequência do aumento dos níveis de hipoxia tecidual (o VEGF é um gene directamente regulado por hipoxia, contendo no seu promotor um "elemento que responde à hipoxia", ou *hypoxia response element*). Actua de um modo parácrino nas células endoteliais, através da ligação a *Vascular Endothelial Growth Factor Receptors* (VEGFR 1, 2 e 3) que são receptores tirosina cinase. Esta ligação VEGF e seus receptores provocam uma cascata de eventos moleculares essenciais no desencadear da angiogénese.[6]

Os FGFs estimulam a proliferação e migração das células endoteliais, assim como a produção de enzimas que degradam a matriz extracelular, sendo assim elementos essenciais à remodelação dos vasos.

O TGF-β foi originalmente caracterizado em fibroblastos tendo sido associado a diferentes processos fisiológicos como estimulação/inibição da proliferação celular, indução da diferenciação celular, regulação da produção da matriz extracelular e de inibidores de proteases e integrinas. Este papel na modelação da síntese de componentes da matriz extracelular e na estabilidade de contactos célula-célula faz com que o TGF-β tenha um papel importante na formação de novos vasos.[7]

Para além de factores que actuam de um modo parácrino existem ainda muitas proteínas que requerem o contacto célula-célula ou célula-matriz para a promoção da angiogénese, nomeadamente as integrinas. As integrinas são receptores transmembranares que permitem a adesão célula-célula e/ou à matriz extracelular. As integrinas $\alpha_v\beta_3$ e $\alpha_v\beta_5$, por exemplo, permitem que as células endoteliais de novos vasos se liguem a proteínas da matriz no tumor, sendo assim moléculas essenciais para a expansão da nova vasculatura tumoral.

Entre os factores anti-angiogénicos "endógenos" (isto é, resultantes da expansão tumoral num determinado tecido) mais importantes destacam-se a Trombospondina-1 (TSP-1), a endostatina e os inibidores teciduais das matriz-metaloproteinases (*Tissue Inhibitors of Metalloproteinases* (TIMPs)). A TSP-1 inibe a proliferação e a migração das células endoteliais através da ligação ao seu receptor de membrana CD36, levando à activação da via FAS e à consequente activação de vias de apoptose.

A endostatina é um fragmento C-terminal de 20KDa derivado da molécula de colagénio XVIII que tem um potente efeito anti-angiogénico. Foi a primeira molécula derivada da matriz extracelular com actividade anti-angiogénica, tendo sido descoberta por Judah Folkman em 1996 juntamente com a angiostatina[8].

Os TIMPs são, como o próprio nome indica, inibidores de metaloproteinases (MMPs). Exercem a sua função através da formação de complexos com as MMPs impedindo-as de exercer o seu papel na degradação da matriz extracelular, limitando assim a expansão da vasculatura.

1.2 Mecanismos moleculares na angiogénese: destabilização vascular

Para que os factores pró-angiogénicos possam desencadear o processo de angiogénese, é necessário desestabilizar os vasos pré-existentes. Os vasos sanguíneos são compostos por células endoteliais e por células que garantem a sua estabilidade, nomeadamente os pericitos e as células vasculares da musculatura lisa (*Vascular Smooth Muscle Cells* (vSMC)), presentes em vasos de maior calibre). Os pericitos encontram-se na membrana basal dos capilares individualmente ou formando uma fina camada à volta das células endoteliais, fornecendo suporte mecânico. Os pericitos são menos abundantes em tumores do que em tecidos normais devido à constante remodelação da rede vascular.[9]

A interacção pericito-célula endotelial é mediada pelo sistema *Angiopoietin* (Ang)/*Tyrosine Kinase Recepctors* Tie. A Ang-1 e Ang-2 são respectivamente agonista e antagonista dos receptores Tie. A sinalização Ang-1/Tie2 permite a interacção pericito-célula endotelial que leva à inibição da proliferação e aumento da estabilidade dos vasos recém-formados. Ang-2 é um antagonista deste processo já que compete com Ang-1 pela ligação ao receptor Tie2, levando à redução da cobertura dos vasos por pericitos e destabilização da parede vascular, mesmo na presença de VEGF. Por esta razão Ang-2 é considerado uma molécula promotora de neo-angiogénese tumoral.[10]

De forma sumária, activação da sinalização Ang-2 leva à destabilização de capilares pré-existentes, expondo receptores de factores pró-angiogénicos tais como o VEGF, permitindo assim o despoletar do mecanismo de angiogénese. A **Figura 1** resume os principais factores envolvidos na angiogénese tumoral.

1.3 Mecanismos celulares na angiogénese tumoral

A sinalização mediada pelos factores referidos anteriormente resulta na formação de novos vasos sanguíneos. Há no entanto especificidades (mecanismos) celulares que foram demonstradas a nível celular e que vieram revelar particularidades dos vasos tumorais, o porquê do seu carácter "permeável" e "pouco eficiente". Os principais mecanismos celulares envolvidos na angiogénese tumoral são a "ramificação" (*sprouting*), a "adoção" (*co-option*), a "vasculogénese" e o "mimetismo vascular" (*vascular mimicry*). O *sprouting* consiste na ramificação de neo-vasos a partir de capilares pré-existentes, que envolve a activação de receptores nas células endoteliais como resposta a factores pró-angiogénicos tais como o VEGF. Esta activação endotelial resulta na produção de MMPs que degradam a membrana basal e permite a migração de células (as *tip cells*) da parede do vaso em direcção aos estímulos pró-angiogénicos. Justapostas às *tip cells* encontram-se as *stalk cells* que alongam e formam o lúmen do novo vaso pela fusão da ramificação com o vaso pré-existente. A sinalização do receptor Notch, nomeadamente através da ligação ao ligando *Delta Like 4* (Dll4) tem um importante crucial na determinação da identidade *tip* ou *stalk*.[11]

Estudos recentes demonstraram inclusive que bloqueio da via Dll4:Notch resulta numa vasculatura tumoral extremamente "permeável" e tortuosa.[12] Finalmente, no que diz respeito a vasculogénese, estudos do nosso grupo demonstraram que o eixo Dll4/Notch está também envolvido na comunicação entre precursores endoteliais derivados da medula óssea e células endoteliais dos vasos tumorais, contribuindo para a sua destabilização e consequentemente para a angiogénese tumoral.[13,14]

2. Especificidades da angiogénese no melanoma

Os casos de melanoma correspondem apenas a 10% dos casos de cancro da pele, no entanto são responsáveis por 90% das mortes causadas. A taxa de sobrevivência a 5 anos em casos avançados com metástases a longa distância é de 5 a 10%. Apesar da ressecção cirúrgica do tumor primário oferecer uma grande possibilidade de cura, as células de melanoma disseminam-se rapidamente tanto por via hematogénea (sanguínea) como linfática, levando à formação de metástases. A sobrevivência dos doentes de melanoma depende da profundidade da invasão do tumor, segundo os critérios de Clark-Breslow.[15]

Tal como outras neoplasias da pele, o melanoma envolve processos sequenciais de transformação das células nos quais o nevus tem uma primeira fase de crescimento radial displásica, seguindo-se uma fase de crescimento vertical que requere a formação de novos

vasos, facilitando a disseminação das células do tumor primário, como demonstra a **Figura 2**. Este processo de rápida formação de novos vasos durante a fase de crescimento radial está associado à produção e sinalização por VEGF e a activação dos mecanismos de angiogénese.[16]

Outra característica que distingue o melanoma de outros tipos de tumores é a formação de novos vasos através de "mimetismo vascular" (*vascular mimicry*). Este mecanismo envolve a formação de novos vasos através da "diferenciação" de células tumorais, que adquirem marcadores e morfologia vasculares e na produção de elementos de matriz extracelular específicos da membrana basal capilar, resultando numa rede de "neo-vasos" (canais) muitas vezes acelulares que irrigam eficientemente as lesões malignas.[17]

Os mecanismos moleculares que regulam a angiogénese envolvendo "mimetismo vascular" são ainda pouco conhecidos, como são pouco conhecidos a relevância deste tipo de vasos para a disseminação metastática de células de melanoma e o eventual envolvimento de células derivadas da medula óssea no processo.

2.1 Ensaios clínicos com anti-angiogénicos e terapias associadas

Historicamente, foi Judah Folkman o primeiro a sugerir que abordagens terapêuticas dirigidas contra a angiogénese em tumores poderia ser clinicamente relevante. Estudos nas décadas subsequentes levaram ao desenvolvimento de terapias altamente específicas tendo como alvos factores pró-angiogénicos e os seus receptores tirosina cinase presentes nas células endoteliais.[18]

Um dos alvos preferenciais para a terapia anti-angiogénica é precisamente o factor pró-angiogénico VEGF. O Bevacizumab é um anticorpo monoclonal recombinante anti-VEGF que bloqueia a ligação de todas as isoformas do VEGF aos seus receptores. O efeito terapêutico (expectável) deste anticorpo será a normalização dos neo-vasos tumorais, o que potenciaria a entrega mais eficaz da quimioterapia ao tumor. O Bevacizumab foi já utilizado sozinho ou em combinação com doses baixas de interferão alfa-2b num ensaio clínico de fase II em doentes de melanoma. Foi observada uma percentagem de resposta parcial de 60% dos doentes, o que resultava num aumento da sobrevida global de 108,5 meses.[19]

A terapia conjunta de Temozolomide (agente oral de alquilação) com o Bevacizumab demonstrou num ensaio de fase II publicado em 2012 uma resposta parcial de 15%, que resultou no aumento de 9,6 meses na sobrevida global destes doentes.[20]

Alguns anticorpos estão a ser testados contra o domínio extracelular do VEGFR, prevenindo assim a ligação do VEGF. O Ramucirumab, um anticorpo monoclonal cujo alvo é o VEGFR2, despertou algum interesse como um potencial terapêutico, já que um ensaio de fase I mostrou uma resposta parcial de doentes de melanoma avançado ao longo de 5 meses.[21]

Também a proteína de fusão entre domínios extracelulares do VEGFR1 e VEGFR2, Aflibercept, mostrou ter uma maior afinidade do que os inibidores do VEGF. A sua eficácia está a ser demonstrada num ensaio de fase II, cujos primeiros resultados em melanoma foram publicados recentemente.[22]

O Sunitinib é um inibidor de tirosina cinases incluindo os receptores de VEGF, comummente utilizado para tratar casos de cancro do rim (altamente angiogénico e tendo a produção de VEGF como mecanismo fundamental na carcinogénese deste tipo de tumores) mas mostrou, num ensaio de fase II, ter um efeito mais modesto no tratamento de doentes com melanoma metastático.[23]

A tabela 1 sumariza alguns dos principais ensaios clínicos com anti-angiogénicos em combinação com outras terapias já estabelecidas no tratamento do melanoma, bem como os principais outputs clínicos tais como resposta parcial, sobrevida global, entre outros.

Conclusões e Perspetivas futuras

O melanoma apresenta uma elevada resistência às opções terapêuticas existentes e não existe terapia a nível sistémico que tenha demonstrado um impacto na sobrevida dos doentes, nomeadamente num "setting" de doença metastática. Do ponto de vista da base celular e molecular de formação de neo-vasos no melanoma (discutidos anteriormente), não é claro de qualquer dos estudos (ensaios clínicos) publicados, que as terapias anti-angiogénicas ataquem de forma eficaz os vasos resultantes do processo de "mimetismo vascular". Os ensaios clínicos que utilizaram anti-angiogénicos têm no entanto demonstrado efeitos clínicos interessantes e relevantes, podendo concluir-se que mais estudos (moleculares e clínicos) são necessários para permitir identificar os doentes que mais poderiam beneficiar deste tipo de abordagem terapêutica.

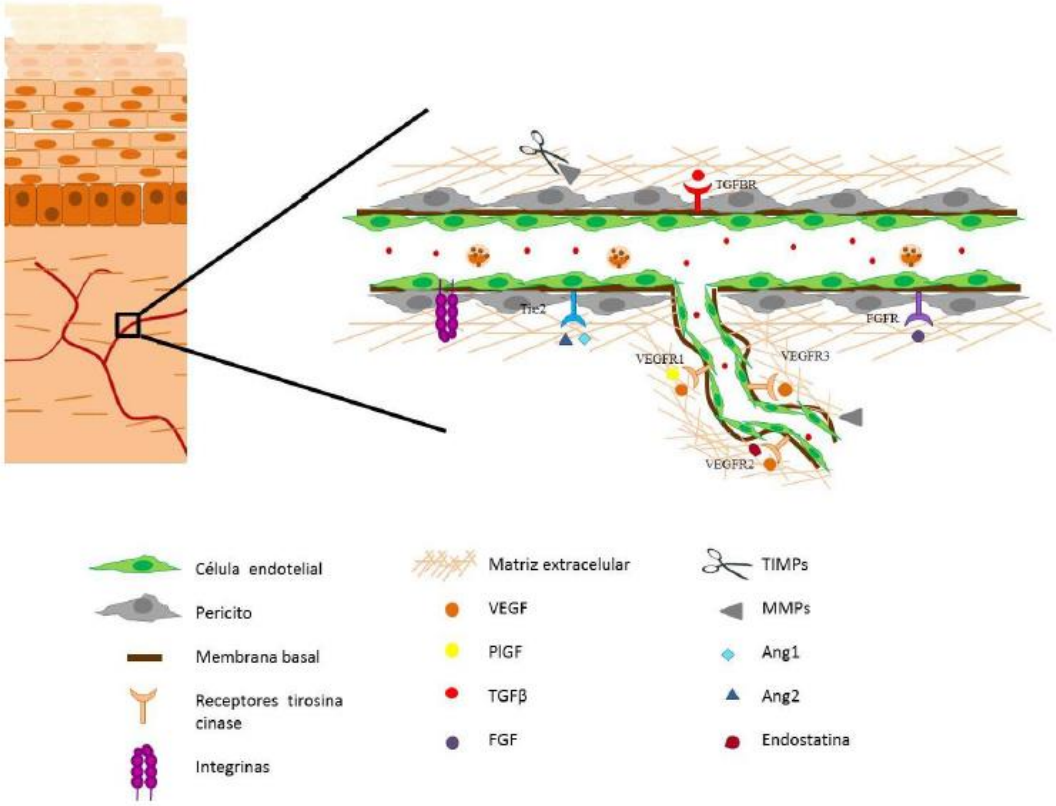


Figura 1 – Esquema representativo dos factores pró- e anti-angiogénicos envolvidos na angiogénese. VEGF (*Vascular Endothelial Growth Factor)*, PlGF (Placental Growth Factor), FGF (*Fibroblast Growth Factor*), TGF-β (*Transforming Growth Factor-β*), TIMPs (*Tissue Inhibitors of Metalloproteinases*), MMPs (metaloproteinases), Ang1 (Angiopoietina-1), Ang2 (Angiopoietina-2)

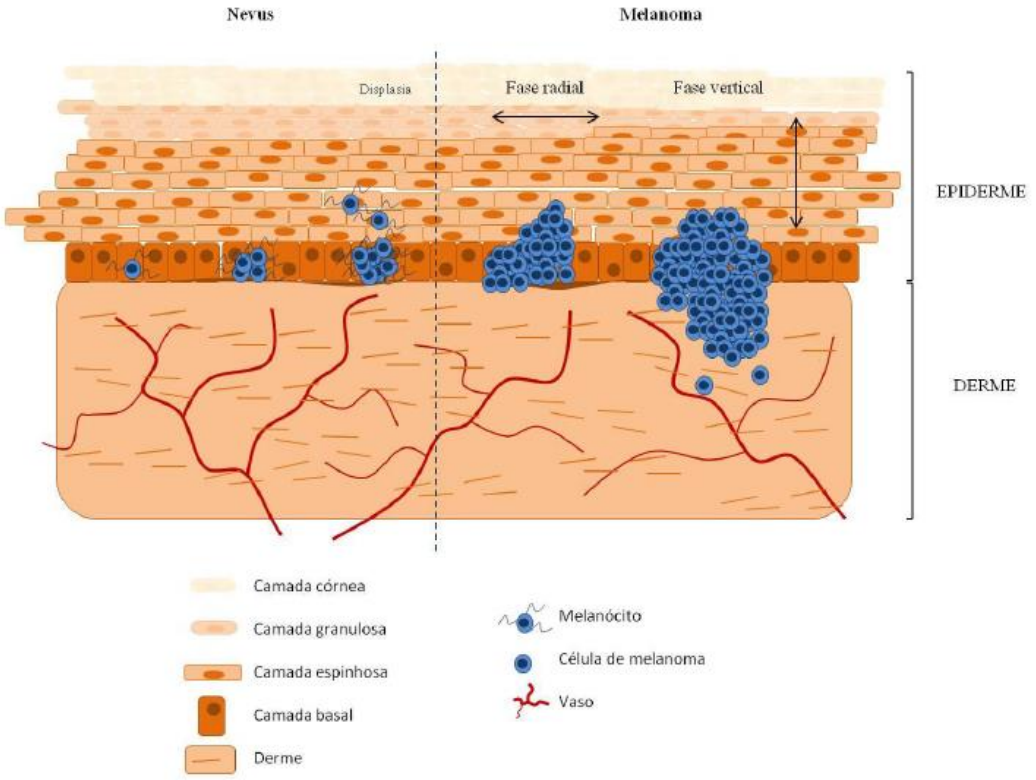


Figura 2 – Diferentes fases do desenvolvimento de melanoma metastático. Numa primeira fase os melanócitos acumulam-se na membrana basal da epiderme, formando o nevus. Após alterações que levam à malignidade da lesão, o melanoma passa primeiro por uma fase de crescimento radial e posteriormente o crescimento vertical potencia a invasão dos vasos pelas células de melanoma

ARTIGO	ANO	TERAPIA	FASE	Nº DOENTES	% RESPOSTA PARCIAL	SOBREVIDA GLOBAL (MESES)
Varker[19]	2007	Bevazimuab ± interferão α2b	II	32	60	108,5
von Moos[20]	2012	Temozolomide, bevacizumab	II	62	15	9,6
Spratlin[21]	2010	Ramucirumab	I	37	30	>5
Tarhini[22]	2011	Aflibercept	II	41	56	16,3
Decoster[23]	2010	Sunitinib	II	36	8,3	6,5
Grignol[24]	2011	Bevacizumab, high dose interferão α2b	II	25	25	17
Egberts[25]	2011	Pegylated interferão α2b, sorafenib	II	55	3,6	9,6
Hainsworth[26]	2010	Bevacizumab, everolimus	II	57	12	8.6
Del Vechio[27]	2010	Fotemustine, bevacizumab	II	20	15	20.5
Vihinen[28]	2010	Bevacizumab, dacarbazine, interferão α2b	II	26	15	11.5
Ott[29]	2010	Sorafenib	II	36	3	-

Tabela 1 – Ensaios clínicos com melhores resultados das terapias anti-angiogénicas usadas no tratamento do melanoma

REFERÊNCIAS

1. Risau W, Mechanisms of angiogenesis, Nature 386, 1997, 671-674
2. Risau W, Vasculogenesis, Annual Review of Cell and Developmental Biology 11, 1997, 73-91
3. Hanahan D, The hallmarks of cancer, Cell 100 (1), 2000, 57-70
4. Ding BS, Endothelial-derived angiocrine signals induce and sustain regenerative lung alveolarization, Cell 147 (3), 2011, 539-553
5. Folkman J, Switch to the angiogenic phenotype during tumorigenesis. Princess Takamatsu Symp.22, 1991, 339-347
6. Carmeliet P, VEGF as a key mediator of angiogenesis in cancer, Oncology 69, 2005, 4-10
7. Pardali E, Signalling by members of the TGF-β family in vascular morphogenesis and disease, Trends Cell Biology 20, 2010, 556-567
8. O'Reilly MS, Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma, Cell 79 (2), 1994, 315-328
9. Gerhardt H., Endothelial-pericyte interactions in angiogenesis. Cell Tissue Research 314, 2003, 15-23
10. Cao Y, Systemic overexpression of angiopoietin-2 promotes tumor microvessel regression and inhibits angiogenesis and tumor growth. Cancer Research 67, 2007, 3835-3844
11. Carmeliet P, Molecular mechanisms and clinical applications of angiogenesis, Nature 473, May 2011, 298-307
12. Thurston G, The Delta paradox: DLL4 blockade leads to more tumor vessels but less tumor growth, Nature Reviews Cancer 7 (5), 2007, 327-331
13. Real C, Bone Marrow-Derived Endothelial Progenitors Expressing Delta-Like 4 (Dll4) Regulate Tumor Angiogenesis, Plos One 6 Vol4, 2011
14. Caiado F, Notch Pathway Modulation on Bone Marrow-Derived Vascular Precursor Cells Regulates Their Angiogenic and Wound Healing Potential, Plos One 11 Vol 3, 2008
15. Balch CM, Final version of 2009 AJCC melanoma staging and classification. Journal of Clinical Oncology 27, 2009, 6199-6206
16. Elder DE, Neoplastic progression and prognosis in melanoma. Seminar Cutaneous Medicine Surgery15, 1996, 336–348
17. Hendrix M, Vasculogenic mimicry and tumor-cell plasticity: lessons from melanoma, Nature Reviews 3, 2003, 411-421
18. Zaki K, The role of angiogenesis inhibitors in the management of melanoma, Current Topics in Medicinal Chemistry 12, 2012, 32-49

19. Varker KA, A randomized phase 2 trial of bevacizumab with or without daily low-dose interferon alfa-2b in metastatic malignant melanoma, Annual Surgery Oncology 14 (8), 2007, 2367-2376
20. von Moos R, First-line temozolomide combined with bevacizumab in metastatic melanoma: a multicentre phase II trial (SAKK 50/07), Annuals of Oncology 23(2), 2012, 531-536
21. Spratlin J L, Phase I pharmacologic and biologic study of ramucirumab (IMC-1121B), a fully human immunoglobulin G1 monoclonal antibody targeting the vascular endothelial growth factor receptor-2, Journal of Clinical Oncology 28(5),2010, 780-787
22. Tarhini AA, Aflibercept (VEGF Trap) in inoperable stage III or stage iv melanoma of cutaneous or uveal origin, Clinical Cancer Research 17(20), 2011, 6574-81
23. Decoster L, Activity of sunitinib in advanced malignant melanoma and its correlation with potential predictive biomarkers, Journal of Clinical Oncology 28(Suppl. 15), 2010, 8518
24. Grignol VP, A phase 2 trial of bevacizumab and high-dose interferon alpha 2B in metastatic melanoma, Journal of Immunotherapy 34(6), 2011, 509-515
25. Egberts F, Sorafenib and pegylated interferon-α2b in advanced metastatic melanoma: a multicenter phase II DeCOG trial, Annuals of Oncology 22(7), 2011, 1667-1674
26. Hainsworth JD, Bevacizumab and everolimus in the treatment of patients with metastatic melanoma: a phase 2 trial of the Sarah Cannon Oncology Research Consortium, Cancer 116(17), 2010, 4122-4129
27. Del Vecchio M, Bevacizumab plus fotemustine as first-line treatment in metastatic melanoma patients: clinical activity and modulation of angiogenesis and lymphangiogenesis factors, Clinical Cancer Research 16(23), 2010, 5862-5872
28. Vihinen PP, A phase II trial of bevacizumab with dacarbazine and daily low-dose interferon-alpha2a as first line treatment in metastatic melanoma, Melanoma Research 20(4), 2010, 318-25
29. Ott PA, A Phase II Trial of Sorafenib in Metastatic Melanoma with Tissue Correlates, Plos One Vol 5 (12), 2010

Rodrigues dos Santos C., **Domingues G.**, Matias I., Matos J., Fonseca I., Mendes de Almeida J., Dias S. LDL-cholesterol signaling induces breast cancer proliferation and invasion. *Lipids in health and disease* **13** (1): 16, doi: 10.1186/1476-511X-13-16 (2014).

Rodrigues dos Santos *et al. Lipids in Health and Disease* 2014, **13**:16
http://www.lipidworld.com/content/13/1/16

LIPIDS IN HEALTH AND DISEASE

RESEARCH Open Access

LDL-cholesterol signaling induces breast cancer proliferation and invasion

Catarina Rodrigues dos Santos[1,2,5], Germana Domingues[3], Inês Matias[3], João Matos[4], Isabel Fonseca[4,5], José Mendes de Almeida[2,5] and Sérgio Dias[3,5*]

Abstract

Lipids and cholesterol in particular, have long been associated with breast cancer (BC) onset and progression. However, the causative effects of elevated lipid levels and breast cancer remain largely undisclosed and were the subject of the present study.

We took advantage of well-established *in vitro* and *in vivo* models of cholesterol enrichment to exploit the mechanism involved in LDL-cholesterol favouring BC growth and invasiveness. We analyzed its effects in models that mimic different BC subtypes and stages.

Our data show that LDL-cholesterol (but not HDL-cholesterol) promotes BC cells proliferation, migration and loss of adhesion, hallmarks of the epithelial to mesenchymal transition. In vivo studies modeling cholesterol levels showed that breast tumors are consistently larger and more proliferative in hypercholesterolemic mice, which also have more frequently lung metastases. Microarray analysis revealed an over expression of intermediates of Akt and ERK pathways suggesting a survival response induced by LDL, confirmed by WB analyses. Gene expression analysis also evidenced an activation of ErbB2 signaling pathway and decreased expression of adhesion molecules (cadherin-related family member3, CD226, Claudin 7 and Ocludin) in the cells exposed to LDL.

Together, the present work shows novel mechanistic evidence that high LDL-cholesterol levels promote BC progression. These data provide rationale for the clinical control of cholesterol levels in BC patients.

Keywords: LDL-cholesterol, Breast cancer, Tumor growth

Introduction

Cholesterol is an essential structural component of the cell membrane [1].

Proliferating cells, such as cancer cells, are believed to have increased requirements for cholesterol. To overcome its needs, tumor cells can increase lipid biosynthesis [2], but can also uptake cholesterol from the bloodstream [3,4]. Abnormal cholesterol accumulation is a characteristic of some malignancies [5,6] and the inhibition of cholesterol storage machinery, in breast cancer cell lines was associated with reduced proliferation [7].

The capacity of cancer cell to use exogenous lipids has been pointed up to explain the link between dyslipidemia and high fat diets with cancer [3,7].

Epidemiological studies tried to demonstrate a causal relationship between dyslipidemia and cancer but they have failed [8-12], or found just weak associations [13,14]. However, it should be noted that several of those studies did not account for the lipoprotein fractions, lipid lowering drugs use or the different tumor types, which could all influence the results and their interpretation. Moreover, most studies to date have sought to find a causal link between cancer incidence and lipid levels; significantly less studies tried to explore a possible link in cancer aggressiveness or progression. Thus, for now, the importance of plasma cholesterol in cancer progression remains poorly understood and was the subject of the present study.

We asked whether exposure to a host LDL-cholesterol enriched systemic environment promotes breast cancer progression by activating key signaling pathways and modulating cell behavior. To test this hypothesis we used controlled experimental environments, employing well established *in vitro* and *in vivo* models.

* Correspondence: sergiodias@f.ul.pt
[3]Instituto Medicina Molecular, Lisbon, Portugal
[5]Faculdade de Medicina Lisboa, Lisboa, Portugal
Full list of author information is available at the end of the article

Methods

Laboratory methods

Cell lines and reagents

The human breast cancer cell line HTB20 and the mouse breast cancer cell line 4 T1 were purchased from the American Type Culture Collection. The human breast cancer cell lines HTB126, MDA MB 231 were kindly provided by Instituto Português de Oncologia Porto.

The cell lines were cultured in DMEM (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco Invitrogen, Carlsbad, CA, USA) and 1% penicillin-streptomycin (Invitrogen life Technologies). Fetal bovine serum lipoprotein free (FBSLF) was purchased from Sigma-Aldrich (Germany) and human plasma low density (LDL) and high density (HDL) lipoproteins were obtained from Calbiochem (Gibbstown, NJ, USA). Mitomycin C was from Sigma-Aldrich (Germany) and Trypsin from (Gibco Invitrogen, Carlsbad, CA, USA).

Cell proliferation assay

MDA MB 231, HTB 126, HTB 20 cells (1×10^5/mL) were seeded into 24-well plate in 250 μl DMEM, 10% FBS. After overnight incubation, the medium was replaced by DMEM, 1%FBSLF, for 24 h. Then, the medium was aspirated and cells were incubated with medium containing HDL (100 μg/mL) or LDL (100 μg/mL), at 37°C in 5%CO_2, for 24 h or 48 h. The number of living cells was determined by hemocytometer counts (at least 4 counts/well, in quadruplicates), after Trypan Blue test exclusion. The number of cells is expressed as fold change over the control.

Migration assay

MDA MB 231 cells were seeded on 24-well plate and grown to confluence in DMEM, 10%FBS. Upon reaching confluence, the medium was replaced by DMEM, 1% FBSLF, for 24 h. Two-hundred-microliter tips were used to make a denuded area ("wound") in the center of the well. Each well was washed with PBS and treated with HDL (100 μg/mL) or LDL (100 μg/mL) for 24 h. Mitomycin C (0,5 μmol/L, from Sigma) was added to the medium to block cell proliferation. Serial photographs were taken at 0 h, 12 h and 24 h, and cell migration distance was determined by subtracting the values obtained at 0 h from 24 h (at least 4 measurements/well, in quadruplicates). The migration distances are expressed as percentage of the wound closure.

Adhesion assay

MDA MB 231 cells (1×10^5/mL) were seeded into 24-well plate in 250 μl DMEM, 10%FBS, overnight, and then replaced by DMEM, 1%FBSLF, for 24 h. After 24 h, cells were left untreated or exposed to LDL (100 μg/mL), overnight. Than wells were washed with PBS, and cells removed with trypsin and reseeded into 24-well plates in 250 μl DMEM, 10% FBS, as defined earlier. After 4 h, cells were washed with PBS and adherent counted. The cells in the supernatant were also counted. The results are shown as the number of adherent or supernatant cells/mL.

RNA extraction and microarray analysis

Total RNA was extracted by Trizol method from untreated or LDL treated breast cancer cells (MDA MB 231) and used to study changes in gene expression. The samples were hybridized on an Affymetrix GeneChips at Instituto Gulbenkian de Ciência core facility. The gene expression results were analyzed using Chipster 2.2.0 software. A cut-off of 1.5 fold above or below the house keeping gene expression levels was considered significant. IPA Ingenuity Systems (Ingenuity Systems, Mountain View, CA) was used to exploratory analysis of interactive networks and relevant biological interactions.

Protein extraction and Western Blot analysis

Cells were lysed in 50 mM tris, 5 mM EDTA, 2% SDS, pH6.8 buffer containing protease inhibitor cocktail. Lysates were diluted 1:1 in loading buffer (tris–glycerol, 2% SDS, 4% b-mercaptoethanol, 100 mM DTT) and 300 μg proteins were loaded on 10% tris–glycine gels. Proteins were transferred to 0.2 lM nitrocellulose membranes (Hybond-C Extra, GE Healthcare Life Sciences, Roosendaal, Netherlands) and subjected to standard immunoblotting with the antibodies: ERK, pERK, akt, pakt, pJNK, β-actin (all from Cell Signaling, Thecnology). Bands were detected with anti-species HRP conjugate. ImageJ software was used to quantify the density of the bands [15].

Statistical analysis

All results, unless otherwise indicated, are expressed as the mean ± standard error of, at least, triplicates. Data were analyzed using unpaired two-tailed Student's t test. *P* values of <0.05 were considered statistically significant.

In vivo models

All animal experiments were performed after approval from Ethics Committee of the Instituto Gulbenkian de Ciência. Animals were housed and maintained in a barrier facility at Instituto Gulbenkian de Ciência. In each experiment, 4–6 week old female mice were injected with breast cancer cells in the right axillary mammary fat pad. Than the test group was subjected to a high cholesterol diet (HD,10%fat, 1,25%cholesterol, 0,5%Na cholate diet, Ssniff, Germany) and the control group fed the standard (normal) mouse diet (ND), with no differences in energy up take values. Food and water were given *ad libitum*. Elevated cholesterol levels were confirmed by

standard dosing methods at Clinical Pathology Laboratory of the Instituto Português de Oncologia de Lisboa and parallel groups were used to control the diet effect on lipid profile. In order to test different tumor types and different host backgrounds the following trials were performed:

1) BALB SCID/MDA MB 231 (2×10^6 cells, 4–6 week old female, n = 8), 10 weeks;
2) BALB SCID/HTB 20 (2×10^6 cells, 4–6 week old female, n = 4), 20 weeks;
3) NOD SCID/4 T1 (1×10^6 cells, 4–6 week old female, n = 4), 20 days;
4) BALB C/4 T1 (1×10^6 cells, 4–6 week old female, n = 5), 20 days.

The animals were sacrificed at different times following tumor inoculation, as referred above; mammary tumors, lungs and liver were excised. Tumors were split into two parts, one frozen in liquid nitrogen and stored at -80°C and the other, and other organs, were fixed in 10% neutral buffered formalin. Photos of the tumor were taken and the large diameter measured. Blood was collected, by cardiac puncture, and serum used to determine lipid profile (total cholesterol (TC), LDL, HDL and triglycerides). Tumor sizes (large diameter, mm) are presented as fold change over the control group. Systemic metastases were searched macroscopically during organs collection and microscopically in the lungs and liver.

Immunohistochemical staining for Ki 67 was performed in a Dako Autostainer® (Dako, Glostrup, Denmark) using standard protocols, followed by counting positive cells in an automated cellular imaging system (ACIS® II, Dako, Glostrup, Denmark I), at, Department of Pathology at Instituto Português Oncologia de Lisboa.

An additional trial was done, using statins, in order to test the effect of the reduction of the systemic cholesterol on the tumor. Using as mice model NOD SCID /4 T1, the test group was subjected to a high cholesterol diet (as described above) and treated with simvastatin (5 mg/Kg in 200 μL PBS, 3 days a week, (by gastric tube). Control groups were fed with standard (normal) mouse diet and high cholesterol diet and treated with placebo (200 μL PBS, 3 days a week, (by gastric tube). The mice were injected with tumor cells 4 weeks after starting treatment and treated during 4 weeks more. Animals were sacrificed as described before.

Statistical analysis

Values are given as the mean ± standard error. Comparisons between control and test mouse samples were performed using the unpaired two-tailed Student´s t test. The number of mice used for each experiment is indicated in the Figure. *P* values of <0.05 were considered statistically significant.

Results

LDL-cholesterol stimulation induces breast cancer cell lines proliferation, migration and reduces cell adhesion

We examined the effect of LDL on breast cancer cells proliferation and found that the number of viable cells increased after LDL-cholesterol stimulation in all cell lines, reaching statistical significance in MDA MB 231 and HTB 20 at 48 h (Figure 1A). Further dissection of LDL induced phenotype was performed on MDA MB 231 cells. In detail, we also determined whether LDL could induce the migration of breast cancer cells in "wound/starch assays", in the presence of mitomycin C to inhibit cell proliferation, and we found that LDL induced migration of MDA MB 231 cells, promoting *in vitro* wound closure. Importantly, HDL was used as control and did not promote cell migration (Figure 1B and C).

Thereafter we tested if cells incubated with LDL changed their adhesive behavior. As shown in Figure 1D and E, LDL pre-treated MDA MB 231 cells, lost their adhesion to the matrix compared to control (untreated) conditions.

Together, these data suggest that LDL exposure of breast cancer cells affects their adhesive properties, favoring cell migration and proliferation.

LDL-cholesterol induced gene expression changes in breast cancer cell lines

Having shown LDL induced phenotypic changes on breast cancer cells, next we sought to demonstrate a causal mechanistic link between these changes and the activation of signaling intermediates and pathways that could explain the altered properties. For this purpose we performed Affymetrix microarray analysis of untreated versus LDL treated breast cancer cells. We found an over expression (fold change ≥1,5) of 147mapped genes and down regulation of 95 mapped genes, at 48 h. The great majority of these genes were related to cell survival and proliferation pathways (Additional file 1: Table S1). Among altered expression genes are also, the down regulation of the adhesion molecules cadherin-related family member3 (−1,53 fold change), CD226 (−1,52 fold change), Claudin7 (−1,52 fold change), Ocludin (−1,54 fold change) and integrinβ8 (−1,49 fold change). Exploratory analysis of the significant gene interactions, found an activation of akt, ERK and JNK networks, all in the dependency of the ErbB2 pathway (Figure 2).

By western blotting analysis we confirmed the raised phosphorylation of akt and ERK, but not of JNK (Figure 3).

These data evidence the LDL effect on the breast cancer cell promoting survival, proliferation and migration.

High LDL-cholesterol promotes breast cancer growth in animal models

HD fed mice showed high levels of TC, LDL and HDL (Figure 4A). However, there were no statistically

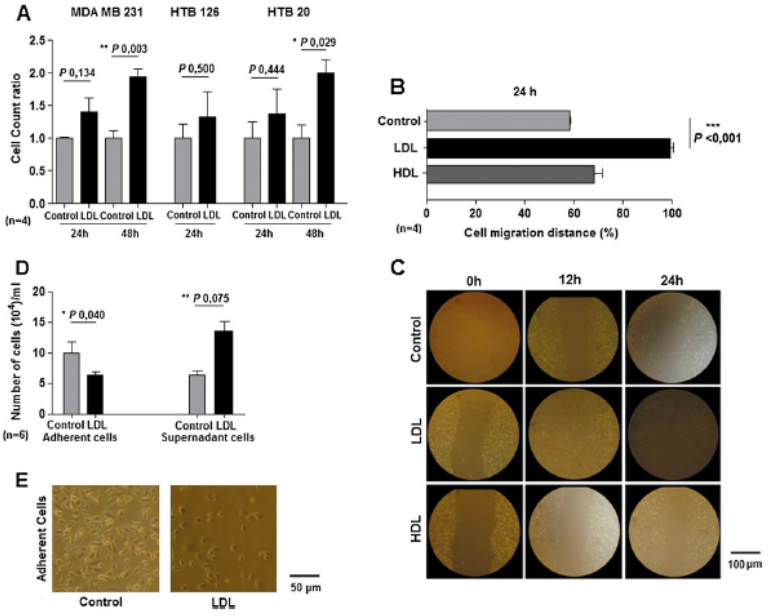


Figure 1 Proliferation, migration and loss of adhesion induced by LDL in in breast cancer cell lines. A, Number of cells (MDA MB 231, HTB 126, HTB 20) after 24 h and 48 h of LDL (100 μg/mg) exposure is consistently higher than control (non-treated) condition. **B** and **C**, Cells (MDA MB 231) were wounded and then cultured for 24 h, with LDL (100 μg/mL), HDL (100 μg/mL) or control conditions. Cell migration into the wound was examined by phase-contrast microscopy and migration distance is indicated as the percentage of the wound closure at 24 h. Representative photos are shown (original magnification 100x C). **D** and **E**, Number of cells (MDA MB 231) adherent and no adherent cells, at 4 h after being removed and reseeded on its primary conditions (control and LDL100 μg/mL) shows that LDL treated cells lose matrix adhesion compared to control. Representative photos are shown (original magnification 200x E). *P* value and the number of the experiments are represented in the figure. Columns mean; bars ± SEM.

significant differences in triglycerides levels or animal weight (Figure 4A and B). The values of the mouse lipid profile in each experiment are shown in Additional file 1: Table S2. These data validate this high fat diet model as a good model to specifically address the effects of elevated cholesterol levels.

HD promoted breast tumor growth in all models tested, with statistically significant differences in BALB SCID inoculated with MDA MB 231 and BALB C inoculated with 4 T1 cells (Figure 4C). Tumors of HD fed mice showed a proliferative ratio that was 20% higher than tumors from ND fed mice, assessed by immunohystochemistry (for Ki67 positive cells) (Figure 4F and G).

Lung metastases were observed macroscopically and microscopically in the NOD SCID and BALB C/4 T1 models. No significant differences were registered the first model, but BALB C/4 T1 hypercholesterolemic mice showed a higher frequency of lung metastasis, at the end of the trial, compared to ND fed mice (Figure 4E). No liver metastases were registered. Two mice of the longest BALB SCID/HTB 20 trial died, one of each group. No other mice deaths occurred.

Statins treatment does not reduced systemic LDL level in mice trial

The treatment with statins did not reduce cholesterol levels of HD fed mice (Additional file 2: Figure S1A). Tumor size of HD fed mice treated with statins show no significant difference from HD fed control mice, in this trial (*P* 0,104) (Additional file 2: Figure S1 B and C).

Discussion

Obesity and dyslipidemia, have long been linked with a possible increase in the likelihood of developing cancer. This possible causal relationship has gained momentum in the light of the observed obesity "epidemic" and the recognized increased incidence of cancer in western countries [16]. Moreover, the same trend in obesity, dyslipidemia and cancer incidence has been seen in Asian with the growing incorporation of western lifestyles [17,18].

However, few studies have tried to find causal and mechanistic associations between increased lipid levels and- not cancer incidence- but cancer behavior. This was tested in the present study, using well established

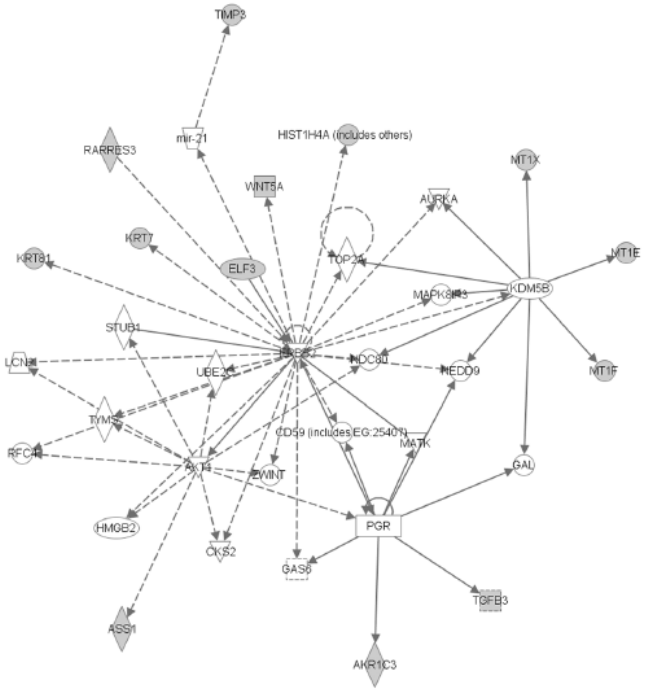


Figure 2 Activated cellular networks, at 48 h, in LDL treated breast cancer cell line. Gene expression analysis of breast cancer cells MDA MB231 exposed to LDL (100 μg/mL), for 48 h shows up regulation of molecules involved in activation of ERK, akt and ErbB2 pathways. Grey nodes are genes overexpressed, white nodes are predicted genes. Smooth lines represent direct interactions. Dashed lines represent indirect interactions.

in vitro and *in vivo* models of hypercholesterolemia and breast cancer.

In breast cancer cell lines, representative of different tumor subtypes and stages, we found that LDL (but not HDL) exposure induces cell proliferation, migration and loss of adhesion, hallmarks of the process of epithelial to mesenchymal transition [19].

Others have demonstrated that in some breast cancer cell lines, HDL induces proliferation [20,21]. We saw a discrete effect of HDL in ER negative cells proliferation

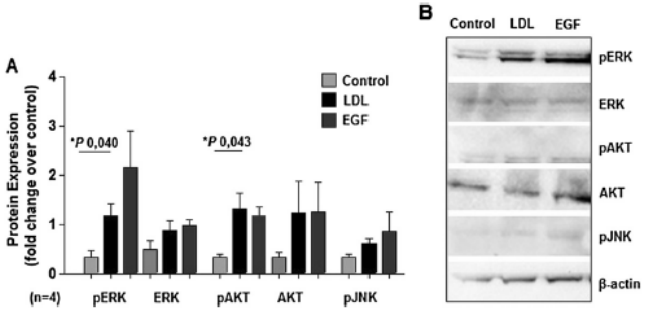


Figure 3 LDL induces ERK and akt protein phosphorylation. A. Cells (MDA MB 231) exposed to LDL show higher expression of phosphorylated ERK and akt, without significant increase in respective total protein. Phosphorilation of JNK is also higher, without reaching statistical significance. Epidermal growth factor (EGF) stimulation was used as positive control and induced, as expected, increase ERK, akt and JNK phosphorylation. **B**. Representative photos of western blot membranes are shown. *P* value and the number of the experiments are represented in the figure. Columns mean; bars ± SEM.

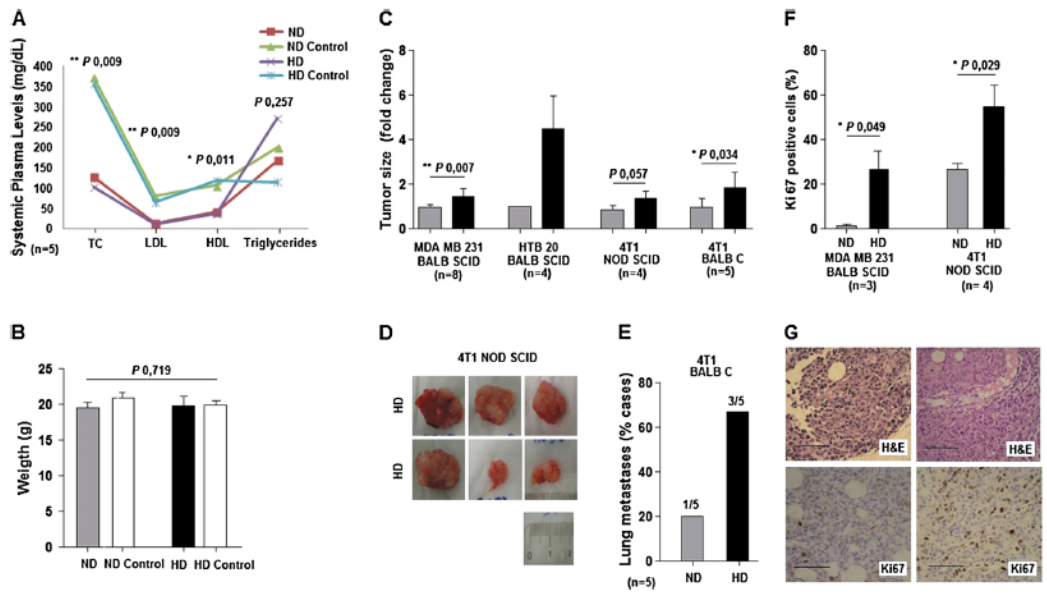


Figure 4 Hypercholesterolemic diet induces a breast cancer phenotype characterized by large and more proliferative tumors. A and **B**. HD fed mice have raised levels of total cholesterol (TC), low density lipoprotein (LDL) and high density lipoprotein (HDL). There are no statistical significant differences in triglycerides level or animal weight, as exemplified to MDA MB 231/BALB SCID trial. (Results of the other experiments are in supplementary data.) Animal in the same diet but without tumor cells inoculation were used as control to lipid profile parameters. **C** and **D**. HD fed mice show large tumors when compared to ND fed mice. The mammary tumor large diameter was measured (as exemplified to 4 T1 /NOD experiment B) and the differences are shown as the fold change over the ND fed mice. **E**. HD fed mice are more likely to have lung metastasis (100%) than ND fed mice (33%). **F** and **G**. HD fed mice have more proliferative tumors as confirmed by Ki67 immuno staining. *P* value and the number of the experiments are represented in the figure. Columns mean; bars ± SEM. Scale bar 100 μm.

(data not shown, not significant), but no influence in migration or adhesion properties.

Previous studies, also showed that LDL induces proliferation [7,20] and migration [4] of ER negative, but not ER positive breast cancer cell lines. We used an ER positive breast cancer cell line HTB 20 (BT 474) and LDL induced similar phenotype changes. HTB 20 breast cancer cells usually express ErbB2 receptor [22].

Our own unpublished data shows that there is an association between high plasma LDL level and ErbB2 positive breast cancer [23]. Also the exploratory analysis of gene expression microarrays suggested an upstream activation of an epidermal growth factor receptor (EGFR).

Human epidermal growth factor receptor –2 (Her2-neu/ ErbB2) is a membrane tyrosine kinase and oncogene that is overexpressed and gene amplified in about 20% of breast cancers [24].

Independent investigations have demonstrate that cell cholesterol depletion using Methyl-N-Cyclodextrin, reduces fluidity of the membrane and enhances phosphorilation and consequent activation of EGFR downstream cascade [25-28]. Orr et al [27] specifically demonstrated this effect in ErbB2 receptor. Since EGFR could be activated in a ligand-independent manner [28], we suggest that cholesterol mobilization across the cell membrane may be the responsible for changes in membrane equilibrium/ disorganization leading to ErbB2 activation, rather than the absolute cholesterol content itself. But, as mentioned earlier, this hypothesis remains to be fully exploited in future studies.

Gene and protein expression analysis of breast cancer cells stimulated with LDL revealed that the proliferative effect induced by LDL may be dependent on Akt and ERK pathways activation. Gene expression analysis also suggested decreased expression of adhesion molecules such as cadherin-related family member 3, CD226, Claudin 7 and Ocludin, upon breast cancer cells exposure to LDL. These findings could explain the loss of adhesion and increased migration in the functional tests, when cells were exposed to LDL and represent hallmarks of the epithelial to mesenchymal transition, characteristic of tumor progression.

To systemically test the *in vitro* results, hypercholesterolemia, controlling obesity, was induced in mice of

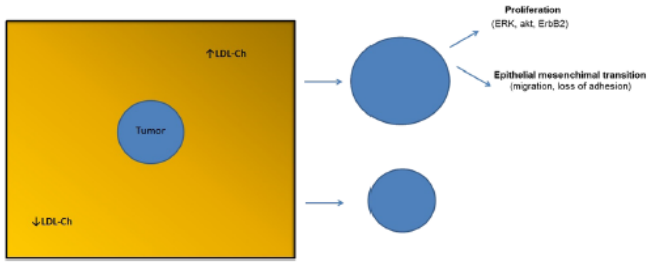


Figure 5 Phenotype of breast tumors exposed to high levels of cholesterol-Proposed Model. Breast tumors exposed to high levels of LDL-cholesterol are larger and show increased proliferation and migration while losing epithelial adhesion properties.

different background (including NOD SCID mice, which are non-obese mice) with different cells lines (to mimic different tumor subtypes) and data consistently showed greater tumor growth in the high LDL groups. This is in accordance with the results reported by Llaverias et al, in breast and prostate genetic mice models [29,30]. Our xenograft models have the advantage of being more representative of breast cancer heterogeneity.

Tumors grown in hypercholesterolemic mice were characterized by higher proliferative ratios, measured by Ki67 immunostaining, which is considered a worse prognostic marker [31]. Higher frequency of lung metastasis was also observed in HD fed mice inoculated with the 4 T1 xenogeneic breast cancer cell line. In models using human breast cancer cell lines we were not able to detect systemic metastasis, either macro or microscopically, may be because those cells do not induce metastasis in mice (reports are very scarce) or because the length of our experiments was not sufficient.

We tried to reverse the hypercholesterolemic-induced phenotype by treating mice with lipid lowering drugs. However we did not achieve systemic LDL levels reduction with statins, in our model. This is a limitation of our work but such difficulty was also found by others [32]. Rats and mice that are commonly used in experimental cancer studies are generally unresponsive to the hypocholesterolemic effects of statins [32].

In humans, the effect of statins in cancer prevention and treatment remains controversial. Two large meta-analyses from 2006 described a neutral effect of satins in cancer incidence [33,34]. A very recent cohort study crossing statins prescription/pharmacy dispense and cancer related-mortality in Denmark National databases [35], proved that statins use before diagnosis of cancer reduces cancer-related mortality. Although some limitations are found and pointed by the authors and others [36], such as lack of follow up and co-morbilities information, the association seem to be plausible, in the study population, suggesting a role for primary prevention.

While assuming that a putative effect of statins is due to plasma cholesterol levels reduction, the action of statins in cancer cells is not well known. Statins decrease intracellular cholesterol synthesis by targeting hidroxi-3-methyl-glutaril-CoA redutase 3. This may lower the products of mevalonate pathway involved in cell proliferation and by this mechanism prevent tumor progression. Some of these effects have been demonstrated in *in vitro* experiments [37]. However, there is poor evidence of direct effect of statins in cancer cells when taken orally [38]. Therefore, much remains to be learned and developed with regards to lipid control in breast cancer setting.

Taken together, our findings show that breast cancer exposed to an LDL-rich host macro environment may be in survival advantage, which will ultimately result in a more aggressive breast cancer phenotype (Figure 5). Our results are supported by functional studies in cell lines and animal models of breast cancer and are in strong accordance with clinical data.

The study exposes the importance of controlling systemic cholesterol levels in breast cancer prevention and treatment and reveals LDL as a biomarker of aggressiveness.

Additional files

Additional file 1: Table S1. Gene expression of LDL treated vs Control MDA MB 231. Table S2 . Lipid Profile in mice trails.

Additional file 2: Figure S1. Tumor size of hypercholesterolemic diet fed mice treated with statins show no significant differences from hypercholesterolemic diet fed control mice. **A** HD fed mice have raised levels of total cholesterol (TC) and low density lipoprotein (LDL) and no significant differences in high density lipoprotein (HDL) and triglycerides levels compared ND. Treatment with statins 5 mg/dL, 8 weeks, does not change lipid profile in the HD fed mice. **B** and **C** NOD SICD/ 4 T1 mice model fed with HD show no significant differences in tumor size compared to HD fed mice treated with statins 5 mg/mL. *P* value and the number of the experiments are represented in the figure. Columns mean; bars ± SEM.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
CRS designed the study, performed the functional studies, developed the breast cancer mice models, collected and analyzed data and wrote the manuscript. GD Breast cancer mice models, performed the protein extraction and Western Blot analysis, collected and analyzed data. IM cultured and expanded breast cancer cells lines, performed the RNA extraction and microarray analysis. JM performed the immunohistochemical studies. IF analyzed and validated the pathological data, revised the manuscript. JMA analyzed and validated data, revised the manuscript. SD designed and coordinated the study, analyzed data and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments
We thank to the staff of the Pathology and Clinical Laboratories of IPOLFG for assistance in data collection.

Funding
Supported by the Programme to Advanced Medical Education (Fundação Gulbenkian, Fundação Champalimaud, Fundação para a Ciência e Tecnologia, Ministério da Saúde).

Author details
[1]Gulbenkian Programme for Advanced Medical Education, Lisbon, Portugal. [2]Department of Surgical Oncology, Instituto Português de Oncologia de Lisboa, Francisco Gentil, Lisbon, Portugal. [3]Instituto Medicina Molecular, Lisbon, Portugal. [4]Department of Pathology, Instituto Português de Oncologia de Lisboa, Francisco Gentil, Lisbon, Portugal. [5]Faculdade de Medicina Lisboa, Lisboa, Portugal.

Received: 4 October 2013 Accepted: 10 January 2014
Published: 15 January 2014

References

1. Lingwood D, Simons K: **Lipid rafts as a membrane-organizing principle.** *Science* 2010, **327**(5961):46–50.
2. Menendez JA, Lupu R: **Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis.** *Nat Rev Cancer* 2007, **7**(10):763–777.
3. Nomura DK, Long JZ, Niessen S, Hoover HS, Ng SW, Cravatt BF: **Monoacylglycerol lipase regulates a fatty acid network that promotes cancer pathogenesis.** *Cell* 2010, **140**(1):49–61.
4. Antalis CJ, Uchida A, Buhman KK, Siddiqui RA: **Migration of MDA-MB-231 breast cancer cells depends on the availability of exogenous lipids and cholesterol esterification.** *Clin Exp Metastasis* 2011, **28**(8):733–741.
5. Swyer G: **The cholesterol content of normal and enlarged prostates.** *Cancer Res* 1942, **2**:372–375.
6. Clayman RV, Gonzalez R, Elliott AY, Gleason DE, Dempsey ME: **Cholesterol accumulation in heterotransplanted renal cell cancer.** *J Urol* 1983, **129**(3):621–624.
7. Antalis CJ, Arnold T, Rasool T, Lee B, Buhman KK, Siddiqui RA: **High ACAT1 expression in estrogen receptor negative basal-like breast cancer cells is associated with LDL-induced proliferation.** *Breast Cancer Res Treat* 2010, **122**(3):661–670.
8. Iso H, Ikeda A, Inoue M, Sato S, Tsugane S: **Serum cholesterol levels in relation to the incidence of cancer: the JPHC study cohorts.** *Int J Cancer* 2009, **125**(11):2679–2686.
9. Hiatt RA, Fireman BH: **Serum cholesterol and the incidence of cancer in a large cohort.** *J Chronic Dis* 1986, **39**(11):861–870.
10. Hoyer AP, Engholm G: **Serum lipids and breast cancer risk: a cohort study of 5,207 Danish women.** *Cancer Causes Control* 1992, **3**(5):403–408.
11. Eliassen AH, Colditz GA, Rosner B, Willett WC, Hankinson SE: **Serum lipids, lipid-lowering drugs, and the risk of breast cancer.** *Arch Intern Med* 2005, **165**(19):2264–2271.
12. Gaard M, Tretli S, Urdal P: **Risk of breast cancer in relation to blood lipids: a prospective study of 31,209 Norwegian women.** *Cancer Causes Control* 1994, **5**(6):501–509.
13. Kitahara CM, Berrington De Gonzalez A, Freedman ND, Huxley R, Mok Y, Jee SH, Samet JM: **Total cholesterol and cancer risk in a large prospective study in Korea.** *J Clin Oncol* 2011, **29**(12):1592–1598.
14. Kaye JA, Meier CR, Walker AM, Jick H: **Statin use, hyperlipidaemia, and the risk of breast cancer.** *Br J Cancer* 2002, **86**(9):1436–1439.
15. Schneider CA, Rasband WS, Eliceiri KW: **NIH Image to ImageJ: 25 years of image analysis.** *Nature Methods* 2012, **9**:671-675. http://www.nature.com/nmeth/journal/v9/n7/full/nmeth.2089.html.
16. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ: **Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults.** *N Engl J Med* 2003, **348**(17):1625–1638.
17. Sullivan DR: **Cardiovascular risk in the Asia-Pacific region from a nutrition and metabolic point of view: visceral obesity.** *Asia Pac J Clin Nutr* 2001, **10**(2):82–84.
18. He J, Gu D, Wu X, Reynolds K, Duan X, Yao C, Wang J, Chen CS, Chen J, Wildman RP, *et al*: **Major causes of death among men and women in China.** *N Engl J Med* 2005, **353**(11):1124–1134.
19. Kalluri R, Weinberg RA: **The basics of epithelial-mesenchymal transition.** *J Clin Invest* 2009, **119**(6):1420–1428.
20. Rotheneder M, Kostner GM: **Effects of low- and high-density lipoproteins on the proliferation of human breast cancer cells in vitro: differences between hormone-dependent and hormone-independent cell lines.** *Int J Cancer* 1989, **43**(5):875–879.
21. Gospodarowicz D, Lui GM, Gonzalez R: **High-density lipoproteins and the proliferation of human tumor cells maintained on extracellular matrix-coated dishes and exposed to defined medium.** *Cancer Res* 1982, **42**(9):3704–3713.
22. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F, *et al*: **A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes.** *Cancer Cell* 2006, **10**(6):515–527.
23. Santos CR, Mendes Almeida JC, Dias S: **Systemic LDL Promotes Breast Cancer Progression.** *Ann Surg Oncol* 2012, **19**(2 Supplement):100–101.
24. King CR, Kraus MH, Aaronson SA: **Amplification of a novel v-erbB-related gene in a human mammary carcinoma.** *Science* 1985, **229**(4717):974–976.
25. Pike LJ, Casey L: **Cholesterol levels modulate EGF receptor-mediated signaling by altering receptor function and trafficking.** *Biochemistry* 2002, **41**(32):10315–10322.
26. Ringerike T, Blystad FD, Levy FO, Madshus IH, Stang E: **Cholesterol is important in control of EGF receptor kinase activity but EGF receptors are not concentrated in caveolae.** *J Cell Sci* 2002, **115**(Pt 6):1331–1340.
27. Orr G, Hu D, Ozcelik S, Opresko LK, Wiley HS, Colson SD: **Cholesterol dictates the freedom of EGF receptors and HER2 in the plane of the membrane.** *Biophys J* 2005, **89**(2):1362–1373.
28. Chen X, Resh MD: **Cholesterol depletion from the plasma membrane triggers ligand-independent activation of the epidermal growth factor receptor.** *J Biol Chem* 2002, **277**(51):49631–49637.
29. Llaverias G, Danilo C, Mercier I, Daumer K, Capozza F, Williams TM, Sotgia F, Lisanti MP, Frank PG: **Role of cholesterol in the development and progression of breast cancer.** *Am J Pathol* 2011, **178**(1):402–412.
30. Llaverias G, Danilo C, Wang Y, Witkiewicz AK, Daumer K, Lisanti MP, Frank PG: **A Western-type diet accelerates tumor progression in an autochthonous mouse model of prostate cancer.** *Am J Pathol* 2010, **177**(6):3180–3191.
31. De Azambuja E, Cardoso F, De Castro G Jr, Colozza M, Mano MS, Durbecq V, Sotiriou C, Larsimont D, Piccart-Gebhart MJ, Paesmans M: **Ki-67 as prognostic marker in early breast cancer: a meta-analysis of published studies involving 12,155 patients.** *Br J Cancer* 2007, **96**(10):1504–1513.
32. Krause BR, Princen HM: **Lack of predictability of classical animal models for hypolipidemic activity: a good time for mice?** *Atherosclerosis* 1998, **140**(1):15–24.
33. Bonovas S, Filioussi K, Tsavaris N, Sitaras NM: **Statins and cancer risk: a literature-based meta-analysis and meta-regression analysis of 35 randomized controlled trials.** *J Clin Oncol* 2006, **24**(30):4808–4817.
34. Dale KM, Coleman CI, Henyan NN, Kluger J, White CM: **Statins and cancer risk: a meta-analysis.** *JAMA* 2006, **295**(1):74–80.
35. Nielsen SF, Nordestgaard BG, Bojesen SE: **Statin use and reduced cancer-related mortality.** *N Engl J Med* 2012, **367**(19):1792–1802.
36. Nielsen SF, Nordestgaard BG, Bojesen SE: **Correspondence. Statin Use and Reduced Cancer-Related Mortality.** *N Engl J Med* 2013, **368**:574–577. February 7, 2013. DOI: 10.1056/NEJMc1214827.
37. Clendening JW, Pandyra A, Boutros PC, El Ghamrasni S, Khosravi F, Trentin GA, Martirosyan A, Hakem A, Hakem R, Jurisica I, *et al*:

Dysregulation of the mevalonate pathway promotes transformation. *Proceedings of the National Academy of Sciences of the United States of America* 2010, **107**(34):15051–15056.

38. Bjarnadottir O, Romero Q, Bendahl PO, Jirstrom K, Ryden L, Loman N, Uhlen M, Johannesson H, Rose C, Grabau D, *et al*: **Targeting HMG-CoA reductase with statins in a window-of-opportunity breast cancer trial.** *Breast Cancer Res Treat* 2013, **138**(2):499–508.

doi:10.1186/1476-511X-13-16
Cite this article as: Rodrigues dos Santos *et al.*: **LDL-cholesterol signaling induces breast cancer proliferation and invasion.** *Lipids in Health and Disease* 2014 **13**:16.

Domingues G., Gouveia Fernandes S., Serpa, J. Dynamics of VEGF-A and its receptors in cancer vascularization – an overview. In iConcept Press Lda. *Recent Cancer Research and Treatment*. In press (2014).

DYNAMICS OF VEGF-A AND ITS RECEPTORS IN CANCER VASCULARIZATION – AN OVERVIEW

Germana Domingues[1,2,3]*, Sofia Gouveia Fernandes[1,2]* and Jacinta Serpa[1,2]

[1]CEDOC-FCM-UNL, [2]IPOLFG, [3]IMM-FM-UL ; * equal contribution

ANGIOGENESIS

In healthy adults, the vasculature is mostly quiescent (1). In a few situations, however, endothelial cells (ECs) may experience some proliferation: wound healing (1, 2), skeletal growth and the female reproductive cycle (3). Some pathophysiological processes may also encompass the *de novo* formation of new blood vessel capillaries from an existing vasculature, as result of the imbalance in the demand and supply of oxygen and nutrients (1, 4). These diseases include diabetic retinopathy (1), rheumatoid arthritis (5), pathology of the female reproductive tract and cancer (3). The formation of new capillaries from pre-existing vessels, by their sprouting or splitting, is called angiogenesis (6-8).

The switch from the normal quiescent vasculature to angiogenesis is the result of a dynamic and tightly controlled balance between angiogenic activators and inhibitors, released predominantly by surrounding pericytes and lymphocytes, and ECs themselves, which induce a number of signal transduction systems that activate ECs (5, 9-11). These angiogenic factors are increasingly receiving attention, especially in the field of malignant neoplastic vascularization (12).

Angiogenesis is a complex multi-step process, characterized by a cascade of events: firstly, vasodilatation of existing vessels and an increase of vascular permeability along with the localized degradation of the surrounding extracellular matrix (ECM), enabling further activation of ECs which proliferate and migrate to form tubes; subsequently, these cells undergo a maturation phase and remodel into capillary structures, and a new ECM is deposited (5, 11, 12).

TUMOR ANGIOGENESIS

Tumor cells initially lack angiogenic ability (13, 14). In order to sustain survival and to expand in size, inicipient neoplasias must develop angiogenic ability, which seems to be acquired in a discrete step during tumor development, via an "angiogenic switch"-Figure 1 (4, 11, 15). Tumor vessels play an essential role in supplying nutrients, oxygen and immune cells, and also in the removal of waste products, enabling tumors to grow beyond the limitations of passive diffusion. In addition, and very importantly, newly formed vessels also afford the possibility of primary tumor to invade adjacent tissues, and circulate, through bloodstream, to distant sites,

where they may form secondary tumors, known as metastases (12, 16, 17). Angiogenesis represents, therefore, a crucial step in cancer progression.

As mentioned above, angiogenic switch is induced by several factors. Such angiogenic factors (positive regulators) include fibroblast growth factors (FGFs), thymidine phosphorylase (TP), transforming growth factor β (TGF-β), tumor necrosis factor α (TNF- α), platelet-derived growth factors (PDGFs), angiopoietins, interleukin-8 (IL-8) and vascular endothelial growth factors (VEGFs) (3, 12). Among these, VEGFs, particularly VEGFA (VEGF), and their receptors assume particular relevance, VEGF being the only growth factor observed almost ubiquitously at sites of angiogenesis and representing a critical rate-limiting step in physiological angiogenesis, and fundamental in pathophysiological angiogenesis as well (3, 5, 18, 19). In the field of neoplastic neovascularization, VEGF family and its receptors have received increased attention (12).

NEOVASCULOGENESIS – ENDOTHELIAL PROGENITOR CELLS

Recent data have disclosed the occurrence of a new mechanism for formation of vessels in adult, different from angiogenesis, termed postnatal/adult vasculogenesis or neovasculogenesis. This mechanism differs from angiogenesis by comprising the *de novo* formation of a primary vascular plexus from endothelial progenitor cells (EPCs) (6, 7).

The emerging evidence seems, thus, to unsettle the dogma that, for a long time, stated EPCs would contribute to vessel growth exclusively in the embryo (vasculogenesis), whereas in the adult that growth would occur only from division of differentiated ECs (angiogenesis) (20).

EPCs are a minor population of mononuclear non-endothelial cells capable to proliferate, migrate, and differentiate into endothelial lineage cells, but have not yet acquired characteristics of mature ECs (21-23).

Asahara *et al.* (24) was the first to isolate putative EPCs for human peripheral blood on the basis of cell surface expression of CD34 and KDR markers, observing experimentally EPCs differentiation into ECs. Since then, increasing knowledge on EPCs has emerged. Although some questions persist regarding the precise panel of cell surface markers defining EPCs, the combinations of $CD133^{+}CD34^{+}KDR^{+}$, $CD34^{+}KDR^{+}$, or $CD114^{+}CD34^{low}$ are now widely used to define or select cells expressing properties attributed to EPCs (25-27).

Most circulating EPCs reside in the bone marrow in close association with hematopoietic stem cells and the stroma. EPCs circulating in the peripheral blood may correspond to cells derived from the bone marrow, not yet incorporated into the vessel wall (20). Interestingly, increasing evidence regarding EPCs sources has now introduced monocytes as putative sources of EPCs.

Peripheral blood mononuclear cells collected from humans were shown to be enriched in EPCs after addition of VEGF, FGF-2, insulin-like growth factor (IGF) and epidermal growth factor (EGF) to the culture medium for 7-10 days. Afterwards, these cells contributed to the formation of new vessels in ischemic limbs in mice (28). Another study showed that EPCs isolated from peripheral blood mononuclear cell fraction, although unable to proliferate, contributed to vascularization by secreting angiogenic growth factors (29). Monocytes cultured under angiogenic conditions also displayed an EPC phenotype with expression of specific surface markers and even formed cord-like structures (30, 31).

Tumor vascularization seems also to be supported by the mobilization and functional incorporation of EPCs. A study focusing EPCs revealed the upregulation of tumor endothelial markers on EPCs, supporting the hypothesis of the involvement of EPCs in cancer (32). Moreover, increased circulating levels of EPCs have been detected in cancer patients (33-37). A strong correlation has also been observed between EPCs number and tumor growth and progression in several neoplastic contexts (38-42). Tumor secretion of VEGF was also found to be correlated with EPC mobilization (41-43).

The recruitment of EPCs to tumors is a multistep process, involving the arrest and homing of the circulating EPCs within the vasculogenic microvasculature, transendothelial extravasation into intersticial space, extravascular formation of cellular clusters, creation of vascular sprouts and cell networks and, finally, the EPCs incorporation into a functional microvasculature (20). VEGF appears to be an inducing factor for these events (3, 20, 44, 45).

The rising data on EPCs seem to introduce EPCs as powerful tools for diagnostic and prognostic purposes, and, additionally, putative targets for interventive therapies.

VASCULAR ENDOTHELIAL GROWTH FACTORS (VEGFs)

The vascular endothelial growth factor family includes placental growth factor (PGF), VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-E (viral homologue of VEGF-A). VEGF-A, also known as vascular permeability factor (VPF) or just VEGF, was the first to be discovered and has been studied most extensively (3, 46).

VEGF is produced by a broad variety of cell types and is a key mitogen for vascular ECs (3, 47-49). Most tumor types, solid and hematological (tumor cells and stroma), overexpress VEGF, being this expression directly correlated with regions of angiogenesis and high vascular density (9, 18, 50-57).

VEGF is encoded by a gene located in chromosome 6p21.3, organized in eight exons, separated by seven introns. Through alternative splicing, this gene gives rise to several distinct VEGF

isoforms, which differ in their expression patterns and their biochemical and biological properties (5, 18, 58). Five human isoforms have been identified, having 121, 145, 165, 189 and 206 aminoacids, respectively $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$. Additionally, minor splice variants have been reported. The different isoforms vary in their ability to bind to heparin, which affects their diffusion rates and concentration gradients in relation to their secretor cell. $VEGF_{206}$ exhibits the strongest binding to heparin in contrast to $VEGF_{121}$, the most diffusible isoform (5, 18, 59, 60).

VEGF is a basic, heparin-binding, homodimeric glycoprotein of about 45 kDa. These properties correspond to those of $VEGF_{165}$, the most abundant isoform (18, 59, 60).

VEGF plays several roles, important under both physiological and pathophysiological conditions. VEGF is a potent mitogen for vascular ECs and also a critical factor for their proliferation and survival (61-63). During embryonic development, the disruption of a single VEGF allele lead to embryonic lethality due to impaired vessel formation and function (64, 65). *In vitro*, VEGF was shown to prevent apoptosis, by activation of phosphatidylinositol(PI)3 kinase(K)/Akt pathway (66) and induction of the expression of anti-apoptotic proteins Bcl-2 and A1 in ECs (67).

VEGF is also able to induce vascular permeability (hence its alternative term VPF), which appears to be mediated by the formation of vesicular-vacuolar organelles, within the ECs, that ultimately fuse with the cell membrane, creating the vascular lumen (1, 7, 8, 68, 69).

VEGF is also implicated in the degradation of ECM, required for the proliferation and migration of ECs, and finally the establishment of new tubes. That event demands the activity of matrix degrading proteases, such as matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA). VEGF acts as one of the main inducers of the expression of these proteolytic enzymes (1, 18).

The vessel formation seems to be additionally controlled by VEGF on the recruitment of bone marrow-derived cells and by increasing procoagulant activity and by recruiting pericytes (3, 45, 70). VEGF is still implicated in hematopoiesis, under both normal and neoplastic conditions (18).

Regarding VEGF role in cancer progression, some functions may be independent of angiogenesis. VEGF produced by tumor cells engages VEGF receptors on tumor cells (autocrine signaling), initiating a signaling response that promotes survival, and, thus, providing tumor cells with a degree of self-sufficiency, and, ultimately facilitating their ability to migrate and metastasize (48, 49) (see "VEGF autocrine signaling"). Studies related this self-sufficiency to the activation of PI3-kinase (PI3K) pathway (48, 71). This self-sufficiency seemed to rely, additionally, on the capacity of VEGF to mitigate apoptosis, that being mostly induced by the

expression of the anti-apoptotic protein Bcl-2 (48, 72). Moreover, migration and invasion of tumor cells have also been related to VEGF signaling, likely due to regulation of the expression of C-X-C chemokine receptor type 4 (CXCR4) by VEGF (71).

REGULATION MECHANISMS OF VEGF EXPRESSION

The molecular mechanisms governing VEGF expression in normoxia by extracellular mediators are poorly understood. A vast body of literature has demonstrated that, in hypoxia, HIF-1 is a key regulator responsible for the induction of genes that facilitate adaptation and survival of tumor cells (73). As a heterodimeric complex, HIF-1 consists of a hypoxia inducible subunit HIF-1α and a constitutively expressed subunit HIF-1β. The overexpression of HIF-1α was found in various types of human and mouse cancers (73). Song *et al.* (2009) showed, in ovarian cancer cells, that lysophosphatidic acid (LPA), a natural phospholipid that is a ligand for protein G coupled receptors, induce VEGF expression through c-Myc and Sp-1 transcription factors independently of HIF-1α (74). This mechanism can involve VEGFR-2 since regulation of VEGFR-2 signal transduction also occurs through the trimeric G proteins Gaq/Ga11, which was shown to interact with VEGFR-2 *in vitro* (75). Furthermore, very recently it was demonstrated that VEGF produced by ECs is regulated by VEGF signaling through VEGFR-2, this activation taking place mostly in the cytoplasm (76). It was previously demonstrated, in glioblastoma, the implication of IL-6 in the regulation of VEGF expression, again through Sp1 transcription factor as a partner of signal transducer and activator of transcription 3 (STAT3) (77). Sp1 and STAT3, together with HIF-1α, are also involved in the expression of VEGF when the epidermal growth factor receptor (EGFR) is stimulated by the proteoglycan decorin in mouse brain ECs (78). In mouse embryonic fibroblasts NIH3T3 cell line, it was shown that PDGF (platelet-derived growth factor) pathway was important to activate VEGF expression mediated by Sp1 and Sp3 that, in these cells, are constitutively bound to VEGF promoter (79).

The expression of a constitutively active mutated form of PI3-kinase in ovarian cancer cells results in increased expression of VEGF. PKC zeta, which is the major kinase downstream of PI3-kinase, regulates VEGF expression in a PDK-1-dependent manner. The inducible expression of Sp1 mutated in the two phosphorylation sites compromise VEGF expression in response to ERK stimulation. This finding strongly establishes that ERK phosphorylation of Sp1 is a major determinant in VEGF expression in response to RAS activation. Sp3 is also a ubiquitous factor that is highly homologous to Sp1. Under hypoxic conditions, the expression of Sp3 decreases, whereas Sp1 is unchanged. A concomitant induction of transcription is observed in the

absence of HIF-1. In this case, Sp1/Sp3 ratio represents a putative HIF-1α independent regulatory mechanism in hypoxia (80).

In gastric cancer, the overexpression of FoxM1b directly and significantly correlates with transactivation of VEGF expression and increased angiogenesis (81).

Recent studies have indicated that cells undergoing insufficient oxygen and nutrients supply experience ER (endoplasmic reticulum) stress. ER needs energy and oxygen for the protein folding process, thus nutrient depletion and hypoxia caused by insufficient blood supply lead to inefficient protein folding and ER stress in cells, especially cells that grow and spread rapidly (82-86), reported that UPR, IRE1a, PERK and ATF6a, mediate transcriptional regulation of VEGF under ER stress, during normal development of trophoblast cells in the placenta as well as in cancer cells (87). In ovary, metabolism also plays a role in the regulation of VEGF:VEGFR2 autocrine loop. Some studies showed that glucose can increase the levels of VEGF and consequently potentiate the autocrine loop, whereas glucose depletion decreases VEGF levels and increases VEGFR2 degradation with concomitant increase of VEGFR2 mRNA synthesis (88). This study suggests that initiation and/or progression of ovarian surface epithelial cells towards a neoplastic phenotype might be modulated by dietary conditions.

More recently, studies have been published revealing the importance of micro-RNAs (miRs) in the regulation of angiogenesis. Lei *et al*. (2009) and Cascio *et al*. (2010) have shown a molecular mechanism of regulation of HIF-1α and VEGF, involving miR-20b through which tumor cells are able to adapt to different oxygen concentrations (89, 90). This way, HIF-1 α is capable of regulating VEGF expression before and after transcription, since the expression of miR-20b itself is regulated by HIF-1 α. The downregulation of VEGF mRNA by miR-20b under a hypoxic environment was associated with reduced levels of nuclear HIF-1α subunit and STAT3. STAT3 is necessary for CoCl2-mediated HIF-1a nuclear accumulation and recruitment on VEGF promoter. miR-126 is also downregulated under hypoxic conditions and may halt the hypoxia-induce neovascularization by suspending cell cycle progression and inhibiting the expression of VEGF and MMP-9 (91).

It was shown that miR-126 is complementary to *VEGF* 3' UTR (untranslated region) and this interaction may inhibit the overexpression of VEGF in tumor cells both *in vitro* and *in vivo* (92). More recently, it has been shown that this miR induces angiogenesis by activating VEGF in patients with oral cancer (93).

In bone marrow ECs, miR-363-5p was the first miR identified. It regulates (directly and indirectly) the expression and availability of angiocrine and hematopoietic factors. EC with reduced miR-363-5p promote hematopoietic precursors adhesion and expansion (94).

VEGF RECEPTORS

The biological functions of VEGF are mediated upon binding to tyrosine kinase receptors (TKRs) VEGFR-1 (Flt-1), VEGFR-2 (kinase domain region (KDR)/Flk-1) (3, 19, 95).

Similar to other TKRs, signaling by VEGFRs is initiated upon binding of a covalently linked ligand dimer to the extracelular receptor domain of the TKR. This interaction promotes receptor homo-and heterodimerization followed by phosphorylation of specific tyrosine residues located in the intracellular juxtamembrane domain, the kinase insert domain, and the carboxyterminal tail of the receptor. A variety of signaling molecules is then recruited to VEGFR dimers giving rise to the assembly of large molecular complexes that activate different cellular pathways (96). Some autophosphorylation sites have also been identified in both VEGF TKRs (97).

VEGF binding sites were initially identified on the cell surface of ECs. VEGF receptors expression is now recognized to be wider, occurring also on bone marrow-derived cells (3), including hematopoietic stem cells (27) and circulating endothelial progenitor cells (98), pericytes (99), dendritic cells (100), monocytes (30, 31, 101, 102), retinal progenitor cells (103) and several types of tumor cells (47, 104-106).

VEGFR-1 and VEGFR-2 are 180 and 200 kDa glycoproteins, respectively (3, 19, 95). These TKRs comprise seven immunoglobulin-like domains in the extracellular domain, a single transmembrane region and a conserved intracellular tyrosine kinase sequence interrupted by a kinase-insert domain (3, 107, 108).

Although both are high affinity receptors for VEGF, VEGF binds with higher affinity to VEGFR-1 than to VEGFR-2 (3, 19, 95, 109). Another interesting feature of the two TKRs is their difference in tyrosine kinase activity in response to ligand binding: VEGFR-2 is a stronger kinase (109, 110). These distinct molecular features may explain their different biological activities (109).

VEGFR-2 is considered to be the predominant VEGF receptor in mediating functional VEGF signaling in ECs, including mitogenesis, cytoskeleton organization and cell migration, survival, and vascular permeability (19, 109). VEGFR-2 is also implicated in playing a distinct role in the activation of PI3K-Akt pathway, a crucial signaling pathway in the process leading to EC survival induced by VEGF (1, 66). VEGFR-2 is still involved in hematopoiesis (18). These roles were supported by experiments in which, in VEGFR-2 knockout mice, both hematopoiesis and vasculogenesis were blocked (111).

VEGFR-1 function in mediating effective biological responses in ECs is more elusive. In fact, in ECs, it may function as a negative regulator of VEGFR-2, at least during embryogenesis, serving a “decoy” purpose, by sequestering VEGF and thus rendering it less available to bind to VEGFR-

2 (3, 109), as suggested by studies in which VEGFR-1 $^{-/-}$ mice displayed overgrowth of ECs and disorganized vascular channels (112). Moreover, VEGFR-1 signaling may also be involved in hematopoiesis and EPCs recruitment (113, 114).

Furthermore, synergism between the two VEGR receptors, VEGFR-1 and VEGFR-2, enabling the modulation of a variety of VEGFR-dependent signals has been demonstrated (115).

VEGF-induced biological signaling is still influenced by the interaction with co-receptors, as neuropilins (NRPs, -1 and -2) and heparan sulphate proteoglycans (HSPGs), integrins and cadherins.

Neuropilins, broadly expressed transmembrane molecules, traditionally known to be involved in axonal guidance, have been identified as VEGF-binding proteins and part of the VEGF-VEGFR signaling complex (5, 95, 116).

These receptors recognize the exon 7-encoded domain of VEGF, binding, therefore, to $VEGF_{165}$ (mostly) but not $VEGF_{121}$, which misses that domain (95, 116, 117). The lack of this association has been hypothesized to be related to the lesser potency of this isoform in comparison to $VEGF_{165}$ (116, 118).

NRP1 and NRP2 are 120 to 130 kDa non-tyrosine kinase receptors that share an identical structure, both containing a large N-terminal extracellular domain, a short membrane spanning domain and a small cytoplasmic domain (95, 116, 117). Their wide distribution includes, besides the developing nervous system, ECs and tumor cells (19, 109, 116).

NRP1 and NRP2 role in mediating VEGF signaling seems to occur mainly through association with VEGR-2 and VEGFR-1, respectively, stimulating signaling by these TKRs (19).

HSPGs are abundant and highly conserved components in the cell surface and ECM, playing an important function in the formation and modulation of gradients of heparin-binding growth factors, as VEGF, and it has been shown to be implicated as modulators of VEGF signaling, by interaction with VEGFR-1 and VEGFR-2 (95, 119). Besides, these molecules are still involved in the restoration of function of damaged $VEGF_{165}$, prolonging its biological activity (5, 120).

Other potential co-receptors for VEGFRs include integrins, as αvβ3 and α9β1, and cell adhesion molecules, such as vascular endothelial (VE)-cadherin (19, 97, 121).

REGULATION OF VEGFR-1 EXPRESSION

The mRNA and VEGFR-1 protein are specifically expressed in most of the vascular ECs. As an exception, VEGFR1 mRNA is expressed in human peripheral blood activated monocytes, which suggests a role in the function of monocyte/macrophage *in vivo* (102).

Isotope-labeled VEGF assays detect its binding sites in most of the blood vessels from embryo to adult stages. These experiments showed the higher affinity of VEGFR1 for VEGF with more than half VEGF-binding sites being associated with VEGFR1 (122). However, the kinase activity of VEGFR1 is one-tenth lower than VEGFR2. VEGFR1 gene produces two proteins, a full length receptor and a soluble form (sVEGFR1). These facts suggest that VEGFR1 may negatively regulate angiogenesis under certain conditions (123, 124). The VEGFR2 knockout mice exhibit a lethal phenotype due to lack vasculogenesis, whereas VEGFR1 knockout mice die at the same age (E8.5-9.0) due to the overgrowth of ECs and their disorganization in blood vessels (112). This indicates that during embryogenesis VEGFR2 is a positive signal transducer and VEGFR1 is a suppressor of VEGFR2 signaling. There are two possible reasons: VEGFR1 tyrosine kinase (TK) generates a negative signal against angiogenesis or the ligand-binding site of VEGFR1 blocks VEGF activity by trapping VEGF. A VEGFR1-TK mice was generated by Shibuya et al. with a normal angiogenic growth, which demonstrates that VEGFR1-negatively regulates angiogenesis during early embryogenesis by trapping VEGF and decreasing proangiogenic signals from VEGFR2 (124).

The gene regulation of VEGFR1 is dependent on an Ets-binding motif and on an upstream CRE/ATF-binding motif (125). In addition, it was reported a hypoxia-response element which may be responsible for the upregulation of VEGFR1 under hypoxic conditions (126). Zhang *et al.* (2010) observed that VEGF induces potent activation of the JNK-c-Jun pathway and that JNK activity is associated with ubiquitination of VEGFR2 (127). At the opposite, inhibition of the ubiquitin or proteasome activity is sufficient to enhance the expression of VEGFR1 in primary ECs. These findings suggest that ECs are continuously producing VEGFR1 and that ubiquitin–proteasome activity is necessary to maintain its homeostatic levels. Interestingly, the regulation of VEGFR1 protein levels is dependent on Akt and ERK1/2 phosphorylation and since these kinases typically inhibit the degradation of proteins by the ubiquitin–proteasome system, they postulate that VEGF induces phosphorylation of Akt and ERK1/2, which in turn prevents degradation of VEGFR1 by the ubiquitin–proteasome system. Collectively, these data suggest that VEGF orchestrates an intricate process mediated by the Akt/ERK and JNK/c-Jun that protects VEGFR1 while VEGFR2 is degraded, leading to rapid reversal of the protein levels of these two receptors (127).

REGULATION OF VEGFR-2 EXPRESSION

VEGFR-2 gene promoter does not have a TATA box region, but has several DNA binding sites for general and tissue-specific transcription factors. This TATA-less gene contains four

upstream Sp1 sites and a single transcription start site that binds multi-functional transcription factor TFII-I for gene expression (128). In large vessel ECs TFII-I is a transcription factor that regulates VEGFR2 expression (129) and despite TFII-I deletions being associated with cardiovascular defects (130) its function in angiogenesis remains unknown. Sp1-dependent DNA binding within −77 and −60 region of VEGFR-2 promoter seems to be essential for the regulation of both mRNA and protein in human ECs by Rac-1 (a small Rho-GTPase) (128).

GATA2 is another transcription factor that regulates the activity of VEGFR2 promoter (131, 132) and more recently Mammoto *et al*. (2009) showed that capillary formation *in vitro* and *in vivo* is modulated by p190RhoGAP, a Rho GTPase inhibitor that controls the levels TFII-I and GATA2 antagonistic transcription factors (133).

Epigenetic modulation of transcription factors has been reported upon VEGF signaling, namely the binding of E2F1 to VEGFR-2, VEGFR-1 and angiopoetin-2 promoters is increased by VEGF stimulation (134) concomitantly with the acetylation of histones and E2F1. The promoter methylation is also described in VEGFR2 promoter in cancer cell lines from stomach, colon and liver (135).

In Human Umbilical Vein ECs (HUVECs), NP-1 si-RNA knockdown causes a marked decrease in VEGFR2 protein levels and this change is independent of VEGFR2 mRNA expression. Holmes *et al*. (2008) concluded from these findings that NP-1 is important for the stability of VEGFR2 in HUVECs, the loss of NP-1 enhances degradation of VEGFR2 both under basal conditions and following activation of VEGFR2 by VEGF (136).

Chronic hypoxia is a cause for specific downregulation of VEGFR2 in human coronary artery endothelial (HCAE) cells due to the decreasing of VEGF stimulation. The VEGF signaling seems to be affected upstream of eNOS phosphorylation (137). Notch pathway is also responsible for downregulating VEGFR2 expression in ECs mainly due to the activation of the expression of the HESR-1 that is a negative regulator of transcription that targets VEGFR2 (138).

Delta4-Notch signaling has been shown to significantly decrease the expression of VEGFR-2 thus inhibiting the proliferation of angiogenic cells. These two signals have opposite effects, meaning that if the concentration of one signal increases the other will decrease proportionally (128).

REGULATION OF NEUROPILINS EXPRESSION

Neuropilins (NRPs) are mediators of neuronal guidance and angiogenesis. They are expressed is most adult tissues, including in growing blood vessels, for example on the ECs of capillaries, arteries and veins in the postnatal mouse retina and tumor cells (139). The extracellular NRP1

domain has distinct VEGF165 and semaphoring binding domains to which these two ligands bind non-competitively. VEGF165 is the VEGF-A isoform with the strongest affinity for NRP1.

NRP1 and VEGFR2 were 50–60% colocalized even in quiescent mouse primary ECs and remained 30 min after stimulation by VEGF-A165 (140). The dependence of the NRP1-VEGFR2 association on the binding of the PDZ (Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and Zonula occludens-1 protein (Zo-1)) adaptor protein synectin suggests that the carboxy-terminus of NRP1 is required for this association. Since VEGFR2 is not known to bind synectin, it is not clear why synectin would be required for the association. Lanahan *et al*. (2010) suggests that APPL (β amyloid protein precursor-like) binds VEGFR2, so NRP1 and APPL-bound synectin dimers could cross link the two receptors, thus explaining the dependence of the NRP1-VEGFR2 association on synectin (141).

Several studies suggest that NRP1 signals are independent of VEGFRs. The contribution of NRP1 to VEGF signaling would be mediated by the cytoplasmic domain. Since the carboxy-terminus of the same domain is used by synectin to cross-link NRP1 to myosin VI, the molecular motor that drives NRP1 trafficking, it is likely that NRP 1-dependent signaling is tightly coupled to Nrp1 trafficking (142).

In vivo studies showed that neither SEMA3A (semaphoring-3A) nor semaphorin signaling through NRP1 and NRP2 are essential for embryonic angiogenesis in the mouse (143, 144). SEMA3A has been reported to also affect pathophysiological angiogenesis in mice. For example, SEMA3A prevents vascular regeneration in a mouse model of oxygen-induced retinopathy and inhibits tumor angiogenesis by eliciting EC apoptosis and normalizing the pericyte coverage of tumor vessels (145, 146). SEMA3A-induced tumor vessel normalization might be indirectly caused by SEMA3A-mediated recruitment of a subset of NRP1-expressing monocytes that secrete several factors involved in vessel maturation (147).

VEGF AUTOCRINE SIGNALING

The complex tumor microenvironment implies that cancer cells receive signals from multiple sources, which, conversely, will influence the function of other cells. This describes a paracrine signaling. However, it has become more evident the acquisition by cancer cells of a certain degree of self-sufficiency – autocrine signaling – that will favor tumor progression (14). In such autocrine signaling, the secreted growth factors may act on surrounding cells from same type of producing cells as well as on the secretor cells themselves. These autocrine loops may then provide a growth advantage to tumor cells, under limiting conditions (18).

Furthermore, there is still evident that, in certain conditions, signaling pathways, such as VEGF pathway, may be transduced even without the factor secretion – internal autocrine/intracrine signaling (18, 148).

Such profitable autocrine signaling loops have been described in both hematological and solid neoplastic contexts. Understanding the effector functions of VEGF in malignant neoplasias may be of great utility for the development of new therapeutic strategies.

In hematological malignancies, the role of VEGF/VEGFR autocrine loops is well established and coexpression of VEGF with its receptors is frequently found.

In acute myeloid primary leukemias and cell lines, previous studies have demonstrated that VEGF:VEGR-2 autocrine loop operates both internally and externally. It was demonstrated that VEGFR-2 is constitutively phosphorylated and located in the nucleus of VEGF-producing leukemia cells. Treatment with anti-VEGF antibody blocked KDR nuclear translocation and inhibited the NF_kB pathway (106). *In vivo* blocking of VEGFR-2 induced long-term remission of xenotransplanted human leukaemia (105). In B-cell non-Hodgkin's lymphomas the overexpression of VEGF and phosphorylated VEGFR-2 has been reported and correlates with expression of hypoxia inducible factor 1α (HIF1α) (149), the main known regulator of VEGF expression as it will be explored below. In infantile hemangioma, cell survival seems to be related to low levels of VEGFR-1 that promotes a constitutive VEGF-dependent activation of VEGFR-2 and downstream pathways (150). In patients with multiple myeloma it was shown that bone marrow ECs (MMEC) highly expressed VEGF and VEGFR2 at both mRNA and protein level. MMEC showed constitutive autophosphorylation in both VEGFR-2 and extracellular signal-regulated kinase 2 (ERK2). Autophosphorylation, proliferation and capillarogenesis were prevented by a neutralizing antibody against VEGF and VEGFR-2. These findings suggested the existence of an autocrine loop of VEGF in MMEC (151). In other study focusing hematological malignancies, VEGFR-1 expression was detected in the cytoplasm and nuclei of proliferating multiple myeloma cells. The inhibition of this receptor abrogated those cells proliferation and motility. The results suggested the contribution of an intracrine VEGF:VEGFR-1 signaling to multiple myeloma cells growth (152).

In solid tumours the role of VEGF:VEGFR autocrine loop is less clear.

In breast cancer, Weigand and co-workers (2005) detected VEGF levels above the range of biological activity in cell lines and primary culture media and they also verified that, in some cases, VEGFR-2 expressed on cell surface was phosphorylated, indicating its activated state . Conversely, other authors stated that in invasive breast cancer VEGFR-2 was not expressed and the VEGF receptor responsible for the autocrine loop is NRP1 (48, 49, 71). Intracrine signaling was also detected in human breast carcinoma cells through VEGF binding to VEGFR-1 (153).

In colon cancer, a concomitant overexpression of VEGF and VEGFR-1, but not VEGFR-2, was described by Bates *et al.*, (2003). The same authors showed, by disturbing VEGFR-1 function with VEGFR-1 blocking antibody and dominant-negative assays, that VEGFR-1 establishes an autocrine loop with VEGF in order to promote cell survival (47). In contrast, in another study, *in vitro* and *in vivo* colon cancer models displayed overexpression of both VEGFR-2 and VEGF in cells with an epithelial-to-mesenchymal transition (EMT) phenotype and, by blocking VEGFR-2 *in vivo*, it was observed that subcutaneous xenograft colon tumors were dramatically smaller (154). Intracrine VEGF:VEGFR-2 signaling in survival and chemoresistance of human colorectal cancer cells was also observed (155). Evidence for an autocrine VEGF mitogenic loop was also pointed out in pancreatic cancer, although in some cases the VEGF receptor involved is VEGFR-2 whereas in other situations it is VEGFR-1 (104).

In prostate cancer, Jackson *et al.* (2002) published that stimulation of LNCaP cells with $VEGF_{165}$ induces DNA synthesis and recruits quiescent cells into the S-phase of the cell cycle via signaling through VEGFR-2. These findings suggest that VEGF may regulate both angiogenesis and tumor cell growth via autocrine and/or paracrine mechanisms in prostate cancer (156). Before that, other authors described a functional autocrine VEGF loop for cell survival but in this case signaling was performed through VEGFR-1 (157). In ovarian carcinoma, an autocrine VEGF:VEGFR-2 loop was described as developing a protective role on cells from anoikis and in ascites formation (158, 159).

Tumor VEGF:VEGFR-2 autocrine loop signaling was shown to trigger angiogenesis in lung cancer (160).

VASCULAR MIMICRY

One of the new emerging paradigms is called "vascular mimicry", which describes the *de novo* formation of perfusable, matrix-rich, vasculogenic-like networks by aggressive malignant tumors. This phenomenon was first characterized in human melanoma where the tumor cells were shown to co-express endothelial and tumor markers, and formed channels, networks, and tubular structures rich in laminin, collagens IV and VI, and heparin sulfate proteoglycans containing plasma and red blood cells. This could be a way of tumor cells to escape from immune system, which allows a faster pathway for metastasis. The vascular mimicry is nowadays described in a variety of cancers, namely sarcomas (Ewing, mesothelial, synovial, osteosarcoma, alveolar rhabdomyosarcoma); carcinomas of the breast, ovary, lung, prostate, bladder and kidney; laryngeal squamous cell carcinoma, gliomas, glioblastoma, and astrocytoma (161).

Recently, EMT has been reported to contribute to the formation of vascular mimicry and the upregulation of EMT-associated transcription factors has been demonstrated in vascular mimicry-forming tumor cells (162, 163).

ANTI-ANGIOGENIC THERAPY

In 1971, Judah Folkman was the first to postulate that the anti-angiogenic therapy against tumor growth could be clinically relevant. Since then, many studies lead to the development of highly specific therapies with anti-angiogenic factors and their tyrosine kinase receptors as targets (164).

Anti-angiogenic drugs can block angiogenesis, inhibit recruitment of proangiogenic bone marrow–derived cells, induce vessel regression, and promote sensitization to radio- and chemotherapy (165). One of the most well established anti-angiogenic therapies is the use of VEGF inhibitors. Anti-VEGF, as the main anti-angiogenic molecule that limits tumor growth, has an essential role in pathophysiological and physiological angiogenesis (166). A lot of preclinical studies have demonstrated modest tumor-suppression effects in different types of cancers. VEGF and its receptors inhibitors-based therapies prolong progression-free survival and overall survival in a fraction of cancer patients – Table I.

Bevacizumab is an anti-VEGF recombinant monoclonal antibody that blocks the link between all isoforms of VEGF and its receptors and it is the most used anti-angiogenic therapy. The end result is the blocking of new blood vessels formation and the subsequent nutrients supply will stop tumor growth. Nowadays, this drug is used in colorectal, kidney, non-small cell lung cancer and certain brain tumors (50, 167). Almost at the same time, pegaptanib which is an aptamer that blocks 165 aminoacid-VEGFA isoform, was approved for the treatment of the wet form of age-related macular degeneration, with great success (168). Other good examples of success using anti-VEGF therapy include the treatment of psoriatic mice, which lead to a reduction in the severity of the disease, and the treatment of hereditary hemorrhagic telangiectasia (a disease characterized by widespread hemangiomas formation) with thalidomidem, which has shown to normalize vessels and reduce episodes of epistaxis (169).

Bevacizumab and multi-targeted tyrosine kinase inhibitors (e.g., imatinib, sorafenib, sunitinib or pazopanib), which block signaling pathways such as VEGF pathway, were approved for clinical use in various types of cancers including metastatic non-small cell lung cancer, metastatic breast cancer, recurrent glioblastoma, metastatic renal cell carcinoma and melanoma. A number of studies have reported their therapeutic efficacy – Table I.

Unfortunately, clinical trials of anti-VEGF monotherapy in patients with solid tumors have been disappointing. But the combination of anti-VEGF therapy with conventional chemotherapy has improved survival in cancer patients compared with chemotherapy alone. These opposite results could be explained by a normalization of tumor neovasculature by anti-VEGF therapy. Preclinical studies have shown that anti-VEGF therapy changes tumor vasculature towards a more mature/normal phenotype (170). So, several questions need to be answered, namely why the preclinical models showed a good efficacy of most anti-angiogenic drugs but the clinical data has a modest response, showing an increase in the overall survival just in a few months? A possible explanation could be that several proangiogenic molecules become upregulated under selective pressure by these drug inhibitors, which can explain the poor results in overall survival.

Overall, the evidence demonstrates that antiangiogenic therapy has remarkable therapeutic effects in various types of human cancers. However, the molecular bases of cancer type-dependent resistance mechanisms against VEGF blockade, especially VEGF-independent pro-angiogenic mechanisms, need to be clarified. Targeting these mechanisms would enhance the effects and minimize the required doses of VEGF blockers. There is no doubt that further efforts in this area will yield opportunities to greatly improve anti-angiogenic treatment (171). Some promising recent studies demonstrate that the combination of anti-angiogenic therapies and anti-inflammatory or immunotherapy could improve the overall response (172, 173).

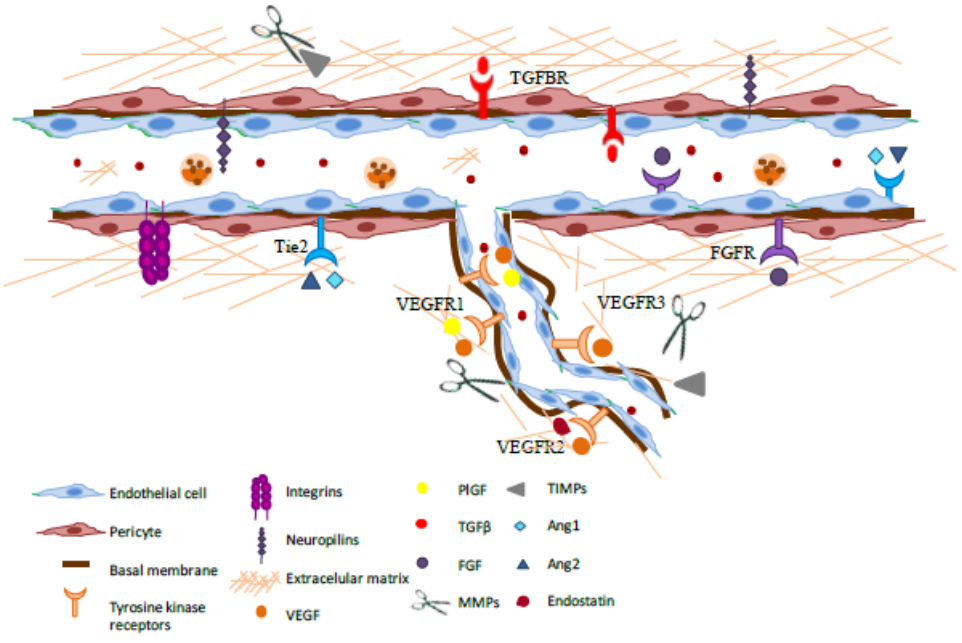


Figure 1- Normal and tumor vascularization: The main intervenients in the process of blood vessels formation.

Table I – Anti-angiogenic drugs used in therapy against cancer: mode of action, type of cancer where it is used, progression free survival (PFS) statistics and associated limitations.

	Mode of action	Type of cancers	PFS	Limitations	References
Bevacizumab	Monoclonal anti-VEGF antibody	Colorectal, breast, ovarian, non-small cell lung, renal, glioblastoma, pancreatic, prostate	Most of studies with improvements	No OS improvement	von Minckwitz, 2012; Tol, 2009; Allegra, 2011; Bennouna, 2013; Perren, 2011; Rapisarda, 2012; Miller, 2007; Miles, 2010; Hurwitz, 2004; Meadows, 2012; Saltz, 2012; Reck, 2010; Herbst, 2011; Rini, 2010; Friedman, 2009; Burger, 2011; Aghajanian, 2012; Kindler, 2010
Aflibercept	Chimeric VEGF/PlGF neutralizing receptor	Colorectal, pancreatic, non-small cell lung, melanoma	Improvements in colorectal, non-small cell lung and melanoma	Small or no OS improvement	Van Cutsem, 2012; Rapisarda, 2012; Ramlau, 2012; Tarhini, 2011
Sorafenib	VEGFR Tyrosine Kinase Inhibitor (TKI)	Renal, Hepatocellular, melanoma, small-cell lung	Improvements in renal and hepatocellular	No improvements in melanoma and small-cell lung	Rapisarda, 2012; Escudier, 2009; Llovet, 2008; Ott, 2010
Sunitinib	VEGFR TKI	Renal, stromal gastrointestinal, breast, hepatocellular, colorectal, pancreatic neuroendocrine, non-small cell lung, prostate, melanoma	Improvements in most studies	No OS improvements in the majority of studies	Rapisarda, 2012; Motzer, 2009; Goodman, 2007; Raymond, 2011; Decoster, 2010
Pazopanib	VEGFR TKI	Renal, non-small cell lung, soft tissue sarcoma	Improvements	No OS improvements	Sternberg, 2013; van der Graaf, 2012
Vandetanib	VEGFR TKI	Non-small cell lung, medullary thyroid	Improvements in medullary thyroid cancer	No OS improvements	Rapisarda, 2012; Herbst, 2010; Wells, 2012
Axitinib	VEGFR TKI	Renal, pancreatic	Improvements in renal cancer	No OS improvements	Motzer, 2013
Ramucirumab	VEGFR TKI	Gastric, non-small cell lung, melanoma	Improvements in most studies	Little or no OS improvements	Shah, 2014; Garon, 2012; Spratlin, 2010

PFS (progression free survival) OS (overall survival) **Adapted from Welti, 2013 (165)**

REFERENCES

1. Witmer AN, Vrensen GF, Van Noorden CJ, Schlingemann RO. Prog Retin Eye Res 2003;22:1-29.
2. Paavonen K, Puolakkainen P, Jussila L, Jahkola T, Alitalo K. Am J Pathol 2000;156:1499-504.
3. Ferrara N, Gerber HP, LeCouter J. Nat Med 2003;9:669-76.
4. Verbridge SS, Choi NW, Zheng Y, Brooks DJ, Stroock AD, Fischbach C. Tissue Eng Part A 2009;16:2133-41.
5. Robinson CJ, Stringer SE. J Cell Sci 2001;114:853-65.
6. Kovacic JC, Moore J, Herbert A, Ma D, Boehm M, Graham RM. Trends Cardiovasc Med 2008;18:45-51.
7. George AL, Bangalore-Prakash P, Rajoria S, et al. J Hematol Oncol 2011;4:24.
8. Risau W. Nature 1997;386:671-4.
9. Carmeliet P, Jain RK. Nature 2000;407:249-57.
10. Folkman J. EXS 1997;79:1-8.
11. Hanahan D, Folkman J. Cell 1996;86:353-64.
12. Nishida N, Yano H, Nishida T, Kamura T, Kojiro M. Vasc Health Risk Manag 2006;2:213-9.
13. Folkman J. Semin Cancer Biol 1992;3:65-71.
14. Hanahan D, Weinberg RA. Cell 2011;144:646-74.
15. Folkman J, Hanahan D. Princess Takamatsu Symp 1991;22:339-47.
16. Kawaguchi T. Curr Drug Targets Cardiovasc Haematol Disord 2005;5:39-64.
17. Sporn MB. Lancet 1996;347:1377-81.
18. Gerber HP, Ferrara N. J Mol Med (Berl) 2003;81:20-31.
19. Cebe-Suarez S, Zehnder-Fjallman A, Ballmer-Hofer K. Cell Mol Life Sci 2006;63:601-15.
20. Ribatti D, Nico B, Crivellato E, Vacca A. Histol Histopathol 2005;20:1351-8.
21. Ribatti D. An historical review. Leuk Res 2007;31:439-44.
22. Fadini GP, Baesso I, Albiero M, Sartore S, Agostini C, Avogaro A. Atherosclerosis 2008;197:496-503.
23. Medina RJ, O'Neill CL, Sweeney M, et al. BMC Med Genomics 2010;3:18.
24. Asahara T, Murohara T, Sullivan A, et al. Science 1997;275:964-7.
25. Schmidt-Lucke C, Fichtlscherer S, Aicher A, et al. PLoS One 2010;5:e13790.
26. Romagnani P, Annunziato F, Liotta F, et al. Circ Res 2005;97:314-22.
27. Peichev M, Naiyer AJ, Pereira D, et al. Blood 2000;95:952-8.
28. Kalka C, Masuda H, Takahashi T, et al. Proc Natl Acad Sci U S A 2000;97:3422-7.
29. Rehman J, Li J, Orschell CM, March KL. Circulation 2003;107:1164-9.
30. Schmeisser A, Garlichs CD, Zhang H, et al. Cardiovasc Res 2001;49:671-80.
31. Rohde E, Malischnik C, Thaler D, et al. Stem Cells 2006;24:357-67.
32. Bagley RG, Rouleau C, St Martin T, et al. Mol Cancer Ther 2008;7:2536-46.
33. Sakamori Y, Masago K, Ohmori K, et al. Cancer Sci 2012;103:1065-70.
34. Mancuso P, Burlini A, Pruneri G, Goldhirsch A, Martinelli G, Bertolini F. Blood 2001;97:3658-61.
35. Ahn JB, Rha SY, Shin SJ, et al. Cancer Lett 2010;288:124-32.
36. Pircher A, Kahler CM, Skvortsov S, et al. Oncol Rep 2008;19:345-52.
37. Richter-Ehrenstein C, Rentzsch J, Runkel S, Schneider A, Schonfelder G. Breast Cancer Res Treat 2007;106:343-9.
38. Gao D, Nolan D, McDonnell K, et al. Biochim Biophys Acta 2009;1796:33-40.
39. Yu D, Sun X, Qiu Y, et al. Clin Cancer Res 2007;13:3814-24.
40. Monestiroli S, Mancuso P, Burlini A, et al. Cancer Res 2001;61:4341-4.
41. Shaked Y, Bertolini F, Man S, et al. Cancer Cell 2005;7:101-11.
42. Real C, Remedio L, Caiado F, et al. PLoS One 2011;6:e18323.
43. Young PP, Hofling AA, Sands MS. Proc Natl Acad Sci U S A 2002;99:11951-6.
44. Aicher A, Heeschen C, Mildner-Rihm C, et al. Nat Med 2003;9:1370-6.
45. Greenberg JI, Shields DJ, Barillas SG, et al. Nature 2008;456:809-13.
46. Ferrara N. Curr Top Microbiol Immunol 1999;237:1-30.
47. Bates RC, Goldsmith JD, Bachelder RE, et al. Curr Biol 2003;13:1721-7.
48. Mercurio AM, Lipscomb EA, Bachelder RE. J Mammary Gland Biol Neoplasia 2005;10:283-90.
49. Bachelder RE, Crago A, Chung J, et al. Cancer Res 2001;61:5736-40.
50. Ferrara N. Oncology 2005;69 Suppl 3:11-6.
51. Ferrara N, Davis-Smyth T. Endocr Rev 1997;18:4-25.
52. Yuan A, Yu CJ, Chen WJ, et al. Int J Cancer 2000;89:475-83.
53. Chen J, Tang D, Wang S, et al. Tumour Biol 2014;35:2513-9.
54. Chen P, Zhu J, Liu DY, Li HY, Xu N, Hou M. Med Oncol 2014;31:775.
55. Xu XL, Ling ZQ, Chen W, Xu YP, Mao WM. Cancer Biomark 2013;13:105-13.
56. Obermair A, Kucera E, Mayerhofer K, et al. Int J Cancer 1997;74:455-8.
57. Dvorak HF. J Clin Oncol 2002;20:4368-80.
58. Vincenti V, Cassano C, Rocchi M, Persico G. Circulation 1996;93:1493-5.
59. Ferrara N, Houck K, Jakeman L, Leung DW. Endocr Rev 1992;13:18-32.
60. Grunstein J, Masbad JJ, Hickey R, Giordano F, Johnson RS. Mol Cell Biol 2000;20:7282-91.
61. Ferrara N. Am J Physiol Cell Physiol 2001;280:C1358-66.
62. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Science 1989;246:1306-9.
63. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. FASEB J 1999;13:9-22.
64. Carmeliet P, Ferreira V, Breier G, et al. Nature 1996;380:435-9.
65. Ferrara N. Eur J Cancer 1996;32A:2413-22.
66. Gerber HP, McMurtrey A, Kowalski J, et al. J Biol Chem 1998;273:30336-43.
67. Gerber HP, Dixit V, Ferrara N. J Biol Chem 1998;273:13313-6.
68. Dvorak AM, Kohn S, Morgan ES, Fox P, Nagy JA, Dvorak HF. J Leukoc Biol 1996;59:100-15.
69. Dvorak HF, Brown LF, Detmar M, Dvorak AM. Am J Pathol 1995;146:1029-39.
70. Clauss M, Gerlach M, Gerlach H, et al. J Exp Med 1990;172:1535-45.
71. Bachelder RE, Wendt MA, Mercurio AM. Cancer Res 2002;62:7203-6.
72. Pidgeon GP, Barr MP, Harmey JH, Foley DA, Bouchier-Hayes DJ. Br J Cancer 2001;85:273-8.
73. Semenza GL. Angiogenesis in ischemic and neoplastic disorders. Annu Rev Med 2003;54:17-28.
74. Song Y, Wu J, Oyesanya RA, Lee Z, Mukherjee A, Fang X. Clin Cancer Res 2009;15:492-501.
75. Zheng H, Wasylyk C, Ayadi A, et al. Genes Dev 2003;17:2283-97.
76. Lichtenberger BM, Tan PK, Niederleithner H, Ferrara N, Petzelbauer P, Sibilia M. Cell 2010;140:268-79.
77. Loeffler S, Fayard B, Weis J, Weissenberger J. Int J Cancer 2005;115:202-13.
78. Santra M, Santra S, Zhang J, Chopp M. J Neurochem 2008;105:324-37.
79. Finkenzeller G, Sparacio A, Technau A, Marme D, Siemeister G. Oncogene 1997;15:669-76.
80. Pages G, Pouyssegur J. Cardiovasc Res 2005;65:564-73.
81. Li Q, Zhang N, Jia Z, et al. Cancer Res 2009;69:3501-9.
82. Genbacev O, Zhou Y, Ludlow JW, Fisher SJ. Science 1997;277:1669-72.
83. Adelman DM, Maltepe E, Simon MC. Adv Exp Med Biol 2000;475:275-84.
84. Romero-Ramirez L, Cao H, Nelson D, et al. Cancer Res 2004;64:5943-7.
85. Koumenis C, Bi M, Ye J, Feldman D, Koong AC. Methods Enzymol 2007;435:275-93.
86. Drogat B, Auguste P, Nguyen DT, et al. Cancer Res 2007;67:6700-7.
87. Ghosh R, Lipson KL, Sargent KE, et al.. PLoS One 2010;5:e9575.
88. Adham SA, Coomber BL. Biochem Biophys Res Commun 2009;390:130-5.
89. Lei Z, Li B, Yang Z, et al. PLoS One 2009;4:e7629.
90. Cascio S, D'Andrea A, Ferla R, et al. J Cell Physiol 2010;224:242-9.
91. Ye P, Liu J, He F, Xu W, Yao K. Int J Med Sci 2014;11:17-23.
92. Liu B, Peng XC, Zheng XL, Wang J, Qin YW. Lung Cancer 2009;66:169-75.
93. Sasahira T, Kurihara M, Bhawal UK, et al. Br J Cancer 2012;107:700-6.
94. Costa A, Afonso J, Osorio C, et al. J Hematol Oncol 2013;6:87.
95. Vieira JM, Ruhrberg C, Schwarz Q. Organogenesis 2010;6:97-106.
96. Stuttfeld E, Ballmer-Hofer K. IUBMB Life 2009;61:915-22.
97. Shibuya M. J Biochem Mol Biol 2006;39:469-78.
98. Eichmann A, Corbel C, Nataf V, Vaigot P, Breant C, Le Douarin NM. Proc Natl Acad Sci U S A 1997;94:5141-6.
99. Witmer AN, Dai J, Weich HA, Vrensen GF, Schlingemann RO. J Histochem Cytochem 2002;50:767-77.
100. Gabrilovich DI, Chen HL, Girgis KR, et al. Nat Med 1996;2:1096-103.
101. Sawano A, Iwai S, Sakurai Y, et al. Blood 2001;97:785-91.
102. Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A, Marme D. Blood 1996;87:3336-43.
103. Suzuma K, Takagi H, Otani A, Suzuma I, Honda Y. Microvasc Res 1998;56:183-91.
104. von Marschall Z, Cramer T, Hocker M, et al. Gastroenterology 2000;119:1358-72.
105. Dias S, Hattori K, Heissig B, et al. Proc Natl Acad Sci U S A 2001;98:10857-62.
106. Santos SC, Dias S. Blood 2004;103:3883-9.
107. Shibuya M, Yamaguchi S, Yamane A, et al. Oncogene 1990;5:519-24.
108. Terman BI, Carrion ME, Kovacs E, Rasmussen BA, Eddy RL, Shows TB. Oncogene 1991;6:1677-83.
109. Shibuya M, Claesson-Welsh L. Exp Cell Res 2006;312:549-60.
110. Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH. J Biol Chem 1994;269:26988-95.
111. Shalaby F, Rossant J, Yamaguchi TP, et al. Nature 1995;376:62-6.
112. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Nature 1995;376:66-70.
113. Hattori K, Heissig B, Wu Y, et al. Nat Med 2002;8:841-9.

114. Clauss M, Weich H, Breier G, et al. J Biol Chem 1996;271:17629-34.
115. Carmeliet P, Moons L, Luttun A, et al. Nat Med 2001;7:575-83.
116. Ellis LM. Mol Cancer Ther 2006;5:1099-107.
117. Soker S, Fidder H, Neufeld G, Klagsbrun M. J Biol Chem 1996;271:5761-7.
118. Rollin S, Lemieux C, Maliba R, et al. Blood 2004;103:3789-97.
119. Bernfield M, Kokenyesi R, Kato M, et al. Annu Rev Cell Biol 1992;8:365-93.
120. Gitay-Goren H, Cohen T, Tessler S, et al. J Biol Chem 1996;271:5519-23.
121. Stupack DG, Cheresh DA. Curr Top Dev Biol 2004;64:207-38.
122. Jakeman LB, Winer J, Bennett GL, Altar CA, Ferrara N. J Clin Invest 1992;89:244-53.
123. Shibuya M. Int J Biochem Cell Biol 2001;33:409-20.
124. Shibuya M. J Biochem 2013;153:13-9.
125. Wakiya K, Begue A, Stehelin D, Shibuya M. A J Biol Chem 1996;271:30823-8.
126. Gerber HP, Condorelli F, Park J, Ferrara N. J Biol Chem 1997;272:23659-67.
127. Zhang Z, Neiva KG, Lingen MW, Ellis LM, Nor JE. Cell Death Differ 2010;17:499-512.
128. Guo S, Colbert LS, Fuller M, Zhang Y, Gonzalez-Perez RR.. Biochim Biophys Acta 2010;1806:108-21.
129. Jackson TA, Taylor HE, Sharma D, Desiderio S, Danoff SK. J Biol Chem 2005;280:29856-63.
130. Roy AL. Gene 2001;274:1-13.
131. Minami T, Murakami T, Horiuchi K, et al. J Biol Chem 2004;279:20626-35.
132. Minami T, Rosenberg RD, Aird WC. J Biol Chem 2001;276:5395-402.
133. Mammoto A, Connor KM, Mammoto T, et al. Nature 2009;457:1103-8.
134. Pillai S, Kovacs M, Chellappan S. Cancer Res 2010;70:4931-40.
135. Kim JY, Hwang JH, Zhou W, et al. Epigenetics 2009;4:313-21.
136. Holmes DI, Zachary IC. Cell Signal 2008;20:569-79.
137. Olszewska-Pazdrak B, Hein TW, Olszewska P, Carney DH. Am J Physiol Cell Physiol 2009;296:C1162-70.
138. Takahashi T, Shibuya M. Oncogene 1997;14:2079-89.
139. Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Cell 1998;92:735-45.
140. Salikhova A, Wang L, Lanahan AA, et al. Circ Res 2008;103:e71-9.
141. Lanahan AA, Hermans K, Claes F, et al. Dev Cell 2010;18:713-24.
142. Horowitz A, Seerapu HR. Cell Signal 2012;24:1810-20.
143. Gu C, Rodriguez ER, Reimert DV, et al. Dev Cell 2003;5:45-57.
144. Vieira JM, Schwarz Q, Ruhrberg C. Development 2007;134:1833-43.
145. Chittenden TW, Claes F, Lanahan AA, et al. Dev Cell 2006;10:783-95.
146. Maione F, Molla F, Meda C, et al. J Clin Invest 2009;119:3356-72.
147. Plein A, Fantin A, Ruhrberg C. Microcirculation 2014.
148. Gerber HP, Malik AK, Solar GP, et al. Nature 2002;417:954-8.
149. Giatromanolaki A, Koukourakis MI, Pezzella F, et al. Hematol Oncol 2008;26:219-24.
150. Jinnin M, Medici D, Park L, et al. Nat Med 2008;14:1236-46.
151. Ria R, Vacca A, Russo F, et al. Thromb Haemost 2004;92:1438-45.
152. Vincent L, Jin DK, Karajannis MA, et al. Cancer Res 2005;65:3185-92.
153. Lee TH, Seng S, Sekine M, et al. PLoS Med 2007;4:e186.
154. Serpa J, Caiado F, Carvalho T, et al. J Biol Chem 2010;285:39211-23.
155. Samuel S, Fan F, Dang LH, Xia L, Gaur P, Ellis LM. Oncogene 2011;30:1205-12.
156. Jackson MW, Roberts JS, Heckford SE, et al. Cancer Res 2002;62:854-9.
157. Soker S, Kaefer M, Johnson M, Klagsbrun M, Atala A, Freeman MR. Am J Pathol 2001;159:651-9.
158. Sher I, Adham SA, Petrik J, Coomber BL. Int J Cancer 2009;124:553-61.
159. Boocock CA, Charnock-Jones DS, Sharkey AM, et al. J Natl Cancer Inst 1995;87:506-16.
160. Chatterjee S, Heukamp LC, Siobal M, et al. J Clin Invest 2013;123:1732-40.
161. Kirschmann DA, Seftor EA, Hardy KM, Seftor RE, Hendrix MJ. Clin Cancer Res 2012;18:2726-32.
162. Liu Z, Sun B, Qi L, Li H, Gao J, Leng X. Cancer Sci 2012;103:813-20.
163. Sun T, Zhao N, Zhao XL, et al. Hepatology 2010;51:545-56.
164. Folkman J, Merler E, Abernathy C, Williams G. J Exp Med 1971;133:275-88.
165. Welti J, Loges S, Dimmeler S, Carmeliet P. J Clin Invest 2013;123:3190-200.
166. Ferrara N. Eur Cytokine Netw 2009;20:158-63.
167. Kim KJ, Li B, Winer J, et al. Nature 1993;362:841-4.
168. Gragoudas ES, Adamis AP, Cunningham ET, Jr., Feinsod M, Guyer DR, Group VISiONCT. N Engl J Med 2004;351:2805-16.
169. Schonthaler HB, Huggenberger R, Wculek SK, Detmar M, Wagner EF. Proc Natl Acad Sci U S A 2009;106:21264-9.
170. Goel S, Duda DG, Xu L, et al. Physiol Rev 2011;91:1071-121.
171. Kubota Y. Keio J Med 2012;61:47-56.
172. Wong W. Science Signalling 2013;6:ec224.
173. Shi S, Wang R, Chen Y, Song H, Chen L, Huang G. PLoS One 2013;8:e65757.
174. von Minckwitz G, Eidtmann H, Rezai M, et al. N Engl J Med 2012;366:299-309.
175. Tol J, Koopman M, Cats A, et al. N Engl J Med 2009;360:563-72.
176. Allegra CJ, Yothers G, O'Connell MJ, et al. J Clin Oncol 2011;29:11-6.
177. Bennouna J, Sastre J, Arnold D, et al. Lancet Oncol 2013;14:29-37.
178. Perren TJ, Swart AM, Pfisterer J, et al. Nat Rev Clin Oncol 2012;9:378-90.
180. Miller K, Wang M, Gralow J, et al. N Engl J Med 2007;357:2666-76.
181. Miles DW, Chan A, Dirix LY, et al. J Clin Oncol 2010;28:3239-47.
182. Hurwitz H, Fehrenbacher L, Novotny W, et al. N Engl J Med 2004;350:2335-42.
183. Meadows KL, Hurwitz HI. Cold Spring Harb Perspect Med 2012;2.
184. Saltz L, Badarinath S, Dakhil S, et al. Clin Colorectal Cancer 2012;11:101-11.
185. Reck M, von Pawel J, Zatloukal P, et al. Ann Oncol 2010;21:1804-9.
186. Herbst RS, Ansari R, Bustin F, et al. Lancet 2011;377:1846-54.
187. Rini BI, Halabi S, Rosenberg JE, et al. J Clin Oncol 2010;28:2137-43.
188. Friedman HS, Prados MD, Wen PY, et al. J Clin Oncol 2009;27:4733-40.
189. Burger RA, Brady MF, Bookman MA, et al. N Engl J Med 2011;365:2473-83.
190. Aghajanian C, Blank SV, Goff BA, et al. J Clin Oncol 2012;30:2039-45.
191. Kindler HL, Wroblewski K, Wallace JA, et al. Invest New Drugs 2012;30:382-6.
192. Van Cutsem E, Tabernero J, Lakomy R, et al. J Clin Oncol 2012;30:3499-506.
193. Ramlau R, Gorbunova V, Ciuleanu TE, et al. J Clin Oncol 2012;30:3640-7.
194. Tarhini AA, Frankel P, Margolin KA, et al. Clin Cancer Res 2011;17:6574-81.
195. Escudier B, Eisen T, Stadler WM, et al. J Clin Oncol 2009;27:3312-8.
196. Llovet JM, Ricci S, Mazzaferro V, et al. N Engl J Med 2008;359:378-90.
197. Ott PA, Hamilton A, Min C, et al. PLoS One 2010;5:e15588.
198. Motzer RJ, Hutson TE, Tomczak P, et al. J Clin Oncol 2009;27:3584-90.
199. Goodman VL, Rock EP, Dagher R, et al. Clin Cancer Res 2007;13:1367-73.
200. Raymond E, Dahan L, Raoul JL, et al. N Engl J Med 2011;364:501-13.
201. Decoster L. Journal of Clinical Oncology 2010;28:8518.
202. Sternberg CN, Hawkins RE, Wagstaff J, et al. Eur J Cancer 2013;49:1287-96.
203. van der Graaf WT, Blay JY, Chawla SP, et al. Lancet 2012;379:1879-86.
204. Herbst RS, Sun Y, Eberhardt WE, et al. Lancet Oncol 2010;11:619-26.
205. Wells SA, Jr., Robinson BG, Gagel RF, et al. J Clin Oncol 2012;30:134-41.
206. Motzer RJ, Escudier B, Tomczak P, et al. Lancet Oncol 2013;14:552-62.
207. Shah MA. Nat Rev Clin Oncol 2014;11:10-1.
208. Garon EB, Cao D, Alexandris E, John WJ, Yurasov S, Perol M. Clin Lung Cancer 2012;13:505-9.
209. Spratlin JL, Cohen RB, Eadens M, et al. J Clin Oncol 2010;28:780-7.