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ABNORMALITIES OF THE ENDOTHELIAL LINEAGE IN PRIMARY MYELOFIBROSIS

Benedetta Gaia Erba

IFOM, Milan

Matricola n. R09405

Supervisor: Prof.ssa Elisabetta Dejana

IFOM, Milan

Added Supervisor: Dr.ssa Monica Corada

IFOM, Milan

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LIST OF ABBREVIATIONS

Aa = Aminoacid

Ab = Antibody

ActRII = Activin receptor type II

AF-6 = Afadin-6

Ag = Antigen

AJ = Adherence Junction

ALK1 = Activin-receptor Like Kinase-1

ALK5 = Activin-receptor Like Kinase-5

AMHRII = AMH type II receptor

AMI = Acute Myocardial Infarction

Ap-1 = Activating protein-1

ASCT = Allogenic Stem Cell Transplant

αSMA = α Smooth Muscle Actin

ASO = Antisense Oligonucleotide

ASXL1 = Additional Sex Combs Like Transcriptional Regulator 1

AT = Angiotensin

AV = Atrio-Ventricular

BAC = Bacterial Artificial Chromosome

BAEC = Bovine Aortic EC

BBB = Blood Brain Barrier

BCL-ABL = Breakpoint Cluster Region-Abelson

BCL-X_L = B-cell lymphoma-extra large

BCS = Budd-Chiari Syndrome

β-gal = β-galactosidase

bFGF = basic Fibroblast Growth Factor (or FGF-2)

bHLH = basic-Helix Loop Helix

BM = Bone Marrow

BMP = Bone Morphogenetic Protein

BMPRII = BMP type II receptor

BMT = Bone Marrow Transplantation

BRE = BMP-Responsive Elements

CAF = Cancer-Associated Fibroblast

CASK/lin2 = calcium/calmodulin-dependent serine protein kinase

CCM = Cerebral Cavernous Malformations

CD = Cluster of Differentiation

CDK = Chronic Kidney Disease

CML = Chronic Myeloid Leukemia

Co-Smad = Common Smad

Cx = Connexin

DAPI = 4',6-diamino-2-phenylindole

Dep-1 = Density-enhanced phosphatase-1

DNA = Deoxyribonucleic Acid

DNMT3A = DNA-cytosine5-methyltransferase 3A

EC = Endothelial Cell

ECFC = Endothelial Colony Forming Cell

ECM = Extracellular Matrix

EGF = Epithelial Growth Factor

EGFR = Epithelial Growth Factor Receptor

EMH = Extra Medullary Hematopoiesis

EMT = Epithelial-to-Mesenchymal Transition

EndMT = Endothelial-to-Mesenchymal Transition

eNOS = endothelial Nitric Oxide Synthase

EPC = Endothelial Progenitor Cell

Epo = Erythropoietin

EpoR = Erythropoietin Receptor

ER = Endoplasmatic Reticulum

ES = Embryonic Stem cells

ET = Essential Thrombocythemia

EZH2 = Enhancer of Zeste 2

FDA = Food and Drug Administration

FDias = Fenestral Diaphragms

FGFR = Fibroblast Growth Factor Receptor

FN = Fibronectin

FOP = Fibrodysplasia Ossificans Progressiva

FoxO = Forkhead-box protein-O

FSP1 = Fibroblast Specific Protein 1

GAP = GTPase Activating Protein

GAPDH = Glyceraldehydes-3-Phosphate Dehydrogenase

GDF = Growth Differentiation Factor

GF = Growth Factor

GH = Growth Hormone

GJ = Gap Junction

G-CSF = Granulocyte-Colony Stimulating Factor

GM-CSF = Granulocyte Monocyte-Colony Stimulating Factor

GOF = Gain Of Function

HPAEC = Human Pulmonary Artery Endothelial Cell

HPC = Hematopoietic Progenitor Cell

HSC = Hematopoietic Stem Cell

HU = Hydroxyurea

HUVEC = Human Umbilical Vein Endothelial Cell

ICAM-1 = Intercellular Adhesion Molecule-1

ID1 = Inhibitor of differentiation 1

IDH1 = Isocitrate dehydrogenase 1

Ig = Immunoglubulin

IGF-1 = Insulin Growth Factor-1

IL = Interleukin

IPF = Idiopathic Pulmonary Fibrosis

IPSS = International Prognostic Scoring System

I-Smads = Inhibitory Smads

JAK = Janus Kinase

JAM = Junctional Adhesion Molecule

KO = Knock-Out

LAP = Latent-Associated Peptide

LDL = Low Density Lipoprotein

LOF = Loss Of Function

MAGUK = Membrane-Associated GUanylate Kinase homolog

MAPK = Mitogen-Activated Protein Kinase

MESEC = Mouse Embryonic Stem cell derived Endothelial Cell

MH1 = Mad-Homology-1

MIF = Mullerian Inhibitory Factors

MK = Megakaryocyte

MMP = Metalloproteinase

MPD = Myeloproliferative Disorders

MPL = Myeloproliferative Leukemia Virus Oncogene

MPN = Myeloproliferative Neoplasms

MTD = Maximum Tolerated Dose

MUPP = Multi PDZ-containing Protein

N-cadherin = Neuronal cadherin

NF-kB = Nuclear Factor-kappa-B

NO = Nitric Oxide

OS = Overall Survival

PAF = Platelet Activating Factor

PAI-1 = Plasminogen Activator Inhibitor-1

PAR3/PAR6 = Partitioning-defective protein 3/6

PCR = Polymerase Chain Reaction

PDGF = Platelet-Derived Growth Factor

PDGFR-β = Platelet-Derived Growth Factor Receptor-β

PDZ = Post synaptic density protein, Drosophila disc large tumor suppressor and Zonula occludens-1 protein

PECAM1 = Platelet Endothelial Cell Adhesive Molecule 1

PI3K = Phosphatidylinositol-3 kinase

PMF = Primary Myelofibrosis

PmT = Polyoma middle T

PRC2 = Polycomb Repressive Complex 2

PTPμ= Protein Tyrosine Phosphatase μ

PV = Polycythemia Vera

RNA = Ribonucleic Acid

R-Smad = Receptor-activated Smad

RT = Room Temperature

RTK = Receptor Tyrosine Kinase

RT-PCR = Reverse Transcriptase-Polymerase Chain Reaction

RUNX2 = Run-related transcription factor-2

SARA = Smad Anchor for Receptor Activation

SBE = Smad-Binding Element

Sca-1 = Stem Cell Antigen-1

SD = Standard Deviation

SDF-1 = Stromal cell-Derived Factor-1

SF3B1 = Splicing factor 3b, subunit 1

Shc = SH2-containing protein

siRNA = short interference Ribonucleic Acid

Smad = Small mother against decapentaplegic

SMI = Small-Molecules Inhibitor

Smurf = Smad ubiquitination regulatory factor

SN = Surnatant

Src = Rous sarcoma proto-oncogene tyrosine-protein kinase

SRSF2 = Serine/arginine-rich splicing factor 2

STAT = Signal Transducer and Activator of Transcription

STZ-induced DN = Streptozoticin-induced Diabetic Nephropathy

TβRs = Transforming Growth Factor-β Receptors

TβRI = Transforming Growth Factor-β Receptor type I

TβRII = Transforming Growth Factor-β Receptor type II

TET2 = Tet methylcytosine dioxygenase 2

TF = Transcription Factor

TGF-β = Transforming Growth Factor-β

TJ = Tight Junction

TNF-α = Tumor Necrosis Factor-a

Tpo = Thrombopoietin

TpoR = Thrombopoietin Receptor

UUO = Unilateral Ureteral Obstructive

VE-cadherin = Vascular Endothelial cadherin

VEGF = Vascular Endothelium Growth Factor

VEGFR = Vascular Endothelium Growth Factor Receptor

VE-PTP = Vascular Endothelial-Phosphotyrosine Phosphatase

vWF = von Willebrand Factor

WB = Western Blotting

WHO = World Health Organization

Wnt = Wingless-related MMTV integration site

WT = Wild-type

ZO = Zonula Occludens

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ABSTRACT

Primary Myelofibrosis (PMF) belongs to the family of Myeloproliferative neoplasms (MPNs), a heterogeneous group of related clonal malignant diseases characterized by the oncogenic transformation of the multipotent hematopoietic progenitor cell (HPC), which leads PMF patients to develop massive bone marrow (BM) fibrosis. Clinical hallmarks also include progressive splenomegaly, anemia and weakness due to ineffective hematopoiesis and excessive production of pro-inflammatory cytokines, which play a central role in mediating increased deposition of BM stromal fibers. So far, no curative treatment for this pathology exists with the exception of allogenic stem cell transplant, thus highlighting the great need to find an alternative and less risky therapy.

Although fibroblasts are directly implicated in fibrosis development, endothelial cells (ECs) can also play a role in PMF; indeed, when activated by inflammatory cytokines such as TGF-β, they undergo a process called Endothelial-to-mesenchymal transition (EndMT), leading ECs to acquire fibroblastic features.

Our results show that ECs can undergo EndMT during the development of PMF both in patients and in a MPN mouse model of the disease. This process occurs during early stages of fibrotic degeneration and is primarily mediated by the release of TGF-β by megakaryocytes (MKs) and platelets. Moreover, patients with different genetic mutations inducing PMF all undergo EndMT, thus proposing it as a common mechanism of fibrosis development.

We also demonstrate that TGF-β induces endogenous BMP4 and BMP6 up-regulation in splenic ECs, further sustaining EndMT phenotype. Moreover, our results show that the *in vitro* treatment of splenic ECs with TGF-β and BMP inhibitors can revert EndMT phenotype, thus opening the possibility to the use of specific and more targeted therapy for PMF patients to achieve fibrosis remission.

INTRODUCTION

1. VASCULAR ENDOTHELIUM

1.1 General features of endothelial cell-to-cell junctions

Endothelial cells (ECs) form a continuous monolayer that covers the inner cellular lining of the blood vessels, thus acting as gatekeepers to control and modulate the infiltration of solutes, molecules and cells into and out of the vessel wall. This fine regulation is fulfilled by a trans-cellular system of transport vesicles [1] and by cell-to-cell specialized structures called junctions, which connect ECs one to each other [2]. EC junctions are composed by transmembrane molecules that are arranged very early during the first stages of development in order to build a correct vascular network. Junction transmembrane components are linked to intracellular molecules that, in turn, mediate anchorage to actin cytoskeleton thus stabilizing the entire junction [3]. However, it is more and more clear that junctions represent not only a site of attachment between ECs aimed to maintain the integrity of the endothelium but, also, that their molecular components can act as signalling structures able to communicate cell position, restrain cell migration, inhibit cell growth and apoptosis, control permeability and maintain apical-basal polarity to finally regulate vascular homeostasis (Figure 1).

Epithelial and endothelial junctions share similar features and in both cell types they can be mainly classified in tight (TJs) and adherens junctions (AJs) [4].

Moreover, ECs lack desmosomes that are present in epithelial cells even if certain types of endothelium, as the lymphatics, are equipped with desmosomal-like structures called *complexus adhaerentes* [5]. These complexes share with epithelial desmosomes similar molecular components like plakoglobin and desmoplakin, which are then connected to vimentin and to the main component of AJs, the vascular endothelial (VE)-cadherin [6]. A third type of junction is represented by gap junctions (GJs), composed by a channel-like

structure, the connexon, which is formed by different types of connexins (Cx). In the endothelium only three types of Cxs are expressed, i.e. Cx43, Cx40 and Cx37. GJs mediate the passage of ions and small-molecular-weight molecules [7].

Junctions are very dynamic structures that follow different steps of maturation during the formation of a functional endothelial monolayer; different evidence show that, when the first contacts start to form, AJs and TJs initially organize into zipper-like structures by lateral adhesion along the cell borders [8]. This process is mediated by intracellular partners that can be interchanged depending on the type of function required [9]. Even when contacts have been stably formed, the components of the junctions are still in a dynamic equilibrium, continuously recycling between plasma membrane and cytoplasm [10].

From a topological point of view, epithelial junctions are better organized than ECs and follow a specific spatial architecture: TJs are located at the apical side of the intercellular cleft (defined *zonula occludens*) while AJs are concentrated below TJs (named *zonula adherens).* In contrast, AJs and TJs are often intermingled along the EC cleft forming a complex system with variation in depth and thickness along the submembrane plaque. Evidence support the fact that AJs and TJs are interconnected and that AJs can influence and control TJ organization both in epithelial [11] and ECs [12].

Depending on the specific functions required, AJs and TJs are also differentially expressed in the vasculature of the tissues. AJ are usually present in all type of vessels while TJ expression and organization is finely modulated. For instance, in big arteries and in the brain microvasculature junctions are well organized and rich in TJs since a strict control of permeability is required [13,14]; on the contrary, TJs are poorly represented in post-capillary venules, where a dynamic trafficking between blood and tissue molecules takes place [15].

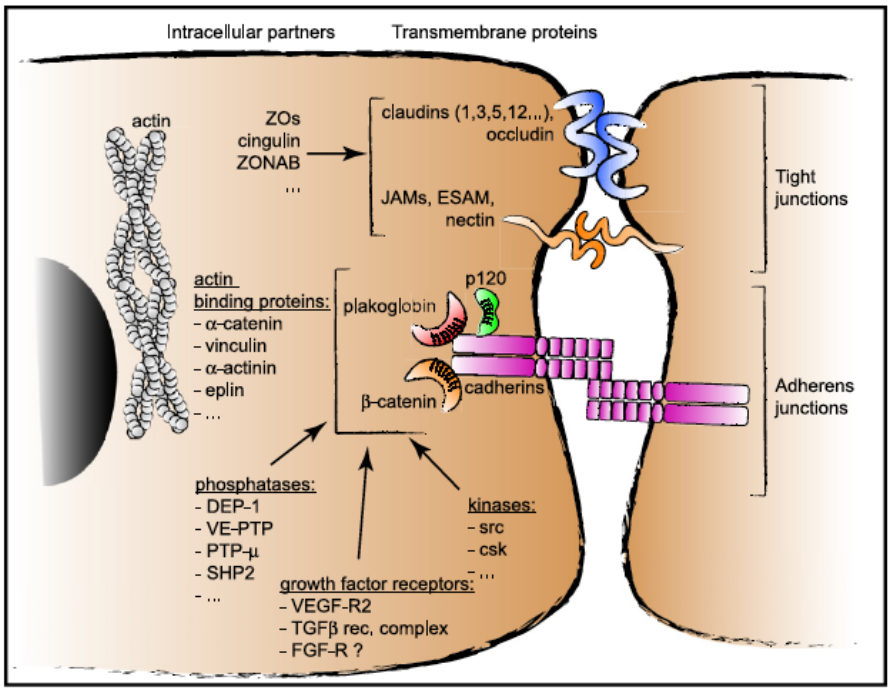

Figure 1: Schematic representation of TJs and AJs composition in ECs

Adhesion in ECs is mediated by two types of junctions: TJs and AJs. Molecular components of both structures can mediate homophilic cell-to-cell adhesion through their extracellular domains while in the cytoplasm they can interact with a plethora of intracellular structural and signalling molecules, aimed to regulate the overall hemostasis of the vessels (*from Dejana et al, 2009*).

1.1.1 AJ molecular composition and signalling functions

Homotypic cell-to-cell adhesion at AJs is principally mediated by VE-cadherin, an endothelial specific member of the cadherin family [6], expressed during development as soon as the cell is committed to the endothelial lineage. The importance of the presence of VE-cadherin in ECs is highlighted by the fact that mice knock out (KO) for VE-cadherin are embryonic lethal due to severe defect in vascular remodeling [16]. Through its five extracellular domains, VE-cadherin molecules establish Ca2+-dependent homophilic EC adhesion by a pericellular zipper-like structure along the cell border through lateral interaction *in cis* and *trans*. ECs also express another member of the classical cadherin family, called N-cadherin. Despite VE- and N-cadherin show similar structure and bind the same intracellular partners, they seem to play different roles in ECs. In physiological

condition, N-cadherin localizes at the cell membrane and one of its major roles is supposed to be the anchorage of ECs to the neighbouring cells, as N-cadherin is not EC-restricted but its expression is found in other cellular types, such as perycites, smooth muscle cells and nerve cells. However, in absence of VE-cadherin, N-cadherin expression is up-regulate, its localization concentrates at junctions [17,18] and the molecule mediates anti-proliferative and anti-apoptotic functions that compensate at least in part the loss of VE-cadherin. Of note, endothelial specific N-cadherin KO mice are embryonic lethal due to severe vascular defects [19], as well as VE-cadherin *null* mice, thus suggesting partial overplapping but non redundant role of the two cadherins in EC homeostasis.

Inside the cell, the cytoplasmic tail of VE-cadherin can bind to different intracellular partners in order to establish anchorage to cytoskeleton and transfer intracellular signals [4]. The main tasks of the VE-cadherin are related to vascular stability and control of permeability, achieved by a direct engage of signalling molecules or by limiting nuclear translocation of interactors with transcriptional activity [20,21]. In confluent condition, ECs do not usually divide and are considered in general state of quiescence. However, they continuously transfer stability signals to control and maintain a correct permeability status; thus, possible alterations in any of the pathways involved in vessel stabilization can result in pathological conditions such as chronic inflammation, atheriosclerosis, tumor angiogenesis or vascular malformations [22-24].

In AJs three main components of the Armadillo family of proteins, plakoglobin, β-catenin and p120, are connected to the intracellular domains of VE-cadherin through their Armadillo repeats. Plakoglobin and β-catenin can bind to α-catenin and VE-cadherin, forming a ternary complex [25]; in turn α-catenin can also bind actin only when it is not hired by β-catenin, thus indicating that cadherin-catenin complex indirectly influences actin cytoskeleton (Figure 2). Moreover, plakoglobin, β-catenin and p120 are also able to shuttle from the membrane to the nucleus and activate specific transcriptional programs following the EC requirements. For example, the transcriptional activity of β-catenin, a

crucial molecule of Wnt canonical pathway, is usually associated to cell proliferation and differentiation, so its recruitment at cell-to-cell contacts in complex with VE-cadherin and thereby the block of its transcriptional activity generally induces vascular stability. Recently, it was demonstrated that VE-cadherin is able to directly control TJ organization and stability, as its clustering inhibits the Forkhead-box protein-O1 (FoxO1) transcriptional activity. FoxO1 acts indeed as repressor of the main TJ component claudin5 and, therefore, its inhibition via phosphorylation induced by VE-cadherin-phosphatidylinositol-3-OH-kinase-(PI3K)-AKT axis allows the expression of claudin5 and leads to organized TJs. On the contrary, β-catenin is able to stabilize FoxO1 on the claudin5 promoter, thus mediating a repressor activity on claudin5 expression [12]. Also in this context β-catenin engagement by VE-cadherin at junctions further limits FoxO1 activity and promotes vascular stability.

On the other hand VE-cadherin can also directly activate signalling molecules, as PI3K or forming multiprotein complexes with a variety of growth factor (GF) receptors: it has been reported that the association of VE-cadherin with vascular endothelial growth factor (VEGF) receptor type 2 (VEGFR2) limits its internalization and activity in the presence of the angiogenic growth factor VEGF-A[26]. Viceversa, VE-cadherin association to other GF receptors, such as TGF-β receptor complexes [27], platelet derived growth factor receptor (PDGFR) [28] or fibroblast growth factor receptor-1 (FGFR-1) [18], controls endothelial proliferative signals and generally induces vascular stabilization.

VE-cadherin plays also an important role in regulating and controlling vascular permeability: it is generally accepted that tyrosine phosphorylation at VE-cadherin cytoplasmic tail results in weaker junctions and impaired barrier function [29,30]. Very recently it has also been demonstrated that VE-cadherin phosphorylation at specific tyrosine (Y658 and Y685) occurs also *in vivo* contributing to the regulation of vascular permeability [31]. In addition, permeability agents such as histamine [29], tumor necrosis factor-α (TNF- α) [32], platelet-activating factor (PAF) [33] and VEGF [30] are also known to induce tyrosine phosphorylation of VE-cadherin, β-catenin and p120. The tyrosine kinase

Src is directly associated with VE-cadherin/catenin complex and likely implicated in the phosphorylation of AJ components, as Src gene deletion or the use of Src inhibitors blocks VEGF-induced VE-cadherin phosphorylation [34]. VE-cadherin phosphorylation status may also be modulated by AJ-associated phosphatases, such as the vascular endothelium specific protein tyrosine phosphatase (VE-PTP) [35], density-enhanced phosphatase-1 (DEP-1) [18,36], protein tyrosine phosphatase receptor type M (PTP-μ) [37] and src-homology 2 (SH2)-containing phosphotyrosine phosphatase (SHP2) [38] that may be directly or indirectly bound to VE-cadherin to decrease its phosphorylation status and increase barrier function.

Finally, permeability can also be regulated by VE-cadherin internalization [31,39] or enzymatic proteolityc cleavage [40].

Of note, VE-cadherin is implicated in additional roles aimed at vascular homeostasis, such as protection from apoptosis [41] and maintenance of apical-basal polarity [42].

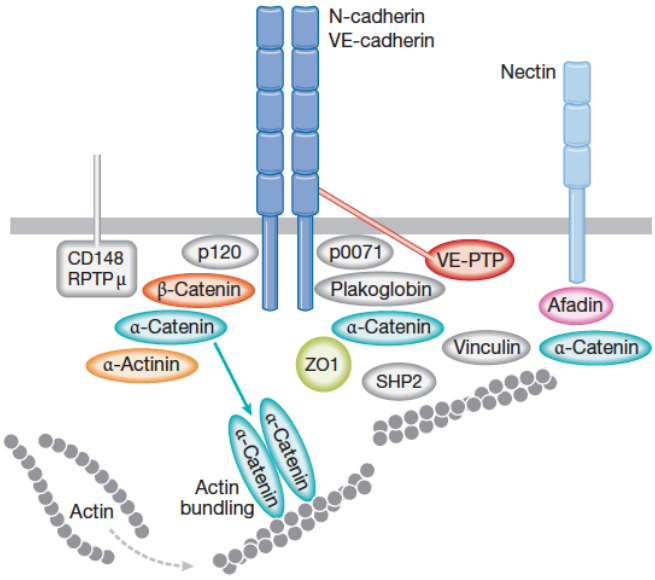

Figure 2: Schematic representation of endothelial AJs

VE-cadherin and in some cases N-cadherin mediates cell-to-cell adhesion and binds to different intracellular components aimed to anchorage at actin cyoskeleton and transfer of intracellular signalling. Among the others, plakoglobin, β-catenin and p120 play an essential role in the regulation of vascular stability while α-catenin is fundamental for the binding to actin bundling. Membrane-associated phosphatases like VE-PTP, DEP-1 (or CD148) and RPTPμ regulate VE-cadherin turnover at junction (*adapted from Nyqvist et al, 2008*).

1.1.2 TJ molecular composition and signalling functions

Cell-to-cell adhesion at the endothelial cleft is also mediated by TJs, formed by components of multiple families of transmembrane signalling proteins, which include claudins, occludin, junctional adhesion molecules (JAMs), and proteins associated to the cell membrane like Zonula Occludens ((ZO)-1, ZO-2, ZO-3) and cingulin. This complexity accounts for the specific barrier properties that TJs must exert depending on the tissues in which they are located. Indeed TJs are responsible for the controlled diffusion of molecules from vessel to tissue and they are able to restrict cell diffusion to lipids and proteins between the apical and baso-lateral plasma membrane, contributing to the cell polarity [4]. Moreover TJs are involved in the leukocyte diapedesis through the endothelium [43]. TJs are enriched in brain vessels, as they contribute to the blood-brain-barrier (BBB) formation and in arteries, while they are less organized in vascular regions requiring a high rate of transport, as in the post-capillary venules [4].

Claudins are the major trans-membrane components of TJs. The family is composed by 24 members, among which ECs express claudin-1, -3, -5 and -12 even if only the expression of claudin5 is endothelial-restricted [44]. Claudin5 deficient mice do not show gross morphological alteration of the vascular network but pups die within 10 hours of birth due to a size selective loosening of BBB against molecules smaller than 800 Dalton[45]. Together with claudins, occludin is involved in the barrier properties as its expression directly correlates with the levels of permeability required in the different vascular regions (Figure 3). For instance, occludin is more expressed in arterial than in venous ECs and its levels are even higher in the BBB [46].

Beyond their fundamental barrier properties, TJs interact with different signalling molecules like G-proteins and protein kinases involved in the regulation of cell growth and survival [4].

ZO-1, -2 and -3 belong to the family of the membrane-associated guanylate kinase homologs (MAGUKs) [4,47], a group of relate molecules that possess a Post synaptic density

protein, *Drosophila* disc large tumor suppressor and Zonula occludens-1 protein (PDZ)-binding domain at the C-terminus able to mediate the anchorage of transmembrane proteins to actin cytoskeleton. When TJs are not well developed or the cells are in sparse/migrating condition, both ZO-1 and ZO-2 lose their association at cell-to-cell contacts and migrate into the nucleus [48,49]. In tumor cells ZO-1 localization is altered and its deletion in epithelial cells causes an epithelial-to-mesenchymal-transition (EMT). Further evidence supporting that ZO proteins are involved in the regulation of contact growth inhibition come from *Drosophila Melanogaster* animal model where it was demonstrated that loss-of-function (LOF) mutation in MAGUK homologs leads to an overgrowth phenotype [50].

TJ composition includes a last family of molecules, called JAMs. At least five members are known, named JAM-A (or JAM-1 or F11R), JAM-B, JAM-C, JAM-4 and JAM-L plus a sixth molecule called ESAM that shares similarities with JAM family. JAM-A, -B and ESAM are usually concentrated at endothelial TJs even if they might be sparse along the intercellular cleft. JAM-A, -B, -C possess a consensus binding sequence for PDZ domain and JAM-A has been demonstrated to be able to interact with different PDZ-containing molecules aimed at anchorage to actin cytoskeleton, as ZO-1, Afadin-6 (AF-6), partitioning defective (PAR) 3/6/atypical PKC (aPKC) complex, calcium/calmodulin-dependent serine protein kinase CASK/lin2 and multi-PDZ domain protein (MUPP-1) [4,51]. Furthermore JAMs protein play a role in the maintenance of apical-basal cell polarity. JAM-A and JAM-C indeed bind PAR3/PAR6 polarity complex in association to the small GTPases Cdc42 and aPKC playing a central role in the induction of cell polarity and mammal TJs organization [52]. Cell polarity is finally required also for a correct angiogenesis and there are evidence showing that JAM-C is involved in tumor angiogenesis and retinal neovascularization [53].

A fundamental role for both AJs and TJs is played by the nectin-afadin system, well characterized in epithelial cells but that seems to be also present in ECs. Nectin belongs to

the Immunoglobulin (Ig) superfamily, that is linked to actin filament binding protein AF6, in turn bound to ponsin and actin [54]. Nectins and afadin colocalize with cadherins thus suggesting a role in AJ assembly and organization [54], but they also interact with TJs [55], implicating a fundamental role in both types of junction physiology.

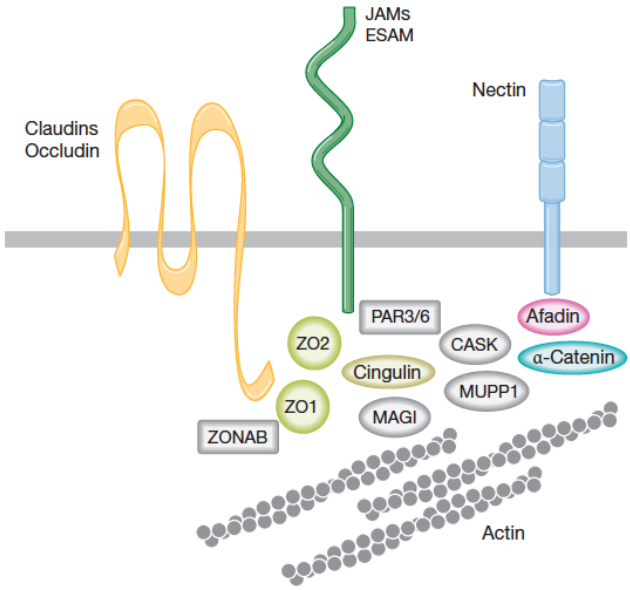

Figure 3: Schematic representation of endothelial TJs

Claudins promote cell-to-cell-adhesion at TJs with the cooperation of occludin and JAMs molecules. ZO-1, ZO-2 and cingulin mediate junctional binding to actin cytoskeleton. Nectins and their intracellualr partners such as afadin are involved in AJ and TJ organization (*adapted from Nyqvist et al, 2008*).

Outside junctional structures, ECs express other cell-specific homophilic adhesion molecules at intercellular contacts, like platelet endothelial cell adhesion molecule 1 (PECAM1 or CD31) and CD146 (or S-endo-1 or Muc18), both belonging to the Ig superfamily. PECAM1 is primarily involved in the regulation of leukocyte transendothelial migration [56] while CD146 is reported to induce heterophilic cell adhesion in both ECs and smooth muscle cells [57].

1.2 Heterogeneity of the endothelium: general features and examples of spleen and bone marrow vascular niche

According to the specificity and functions of the organ where they are expressed, EC phenotype varies not only in terms of junction composition but also in terms of cell morphology, gene expression, antigen (Ag) composition and functions. For this reason, the endothelium has been compared to a chameleon, constantly molding itself in time to the needs of the other cell types in the local tissues, but also capable to adapt to many different microenvironments in order to balance the physiological requirements of the tissues and the need of self-survival [58] .

In general, molecular mechanisms involved in EC heterogeneity are driven by environmental and epigenetic cues [59] (Figure 4). Environmental stimuli can be classified in biomechanical or biochemical: shear stress and strain forces are included in the first group, while examples of biochemical stimuli are represented by pH, oxygenation, GFs, chemokines, cytokines and components of the extra cellular matrix (ECM). All these stimuli can activate specific receptor-mediated signalling pathways in ECs that further induce post-translational modification of proteins or transcription factors (TF)-dependent induction of gene expression. These environmental cues are sufficient to induce EC heterogeneity across the vascular tree thus, if ECs are removed from the native tissue to be cultured in an *in vitro* system, they can become uncoupled from critical extracellular signals and undergo phenotypic drift. On the contrary, site-specific properties of ECs epigenetically determined can be retained also upon EC isolation. These modifications are inheritable by the nascent EC and mainly include DNA methylation, histone methylation and acetylation.

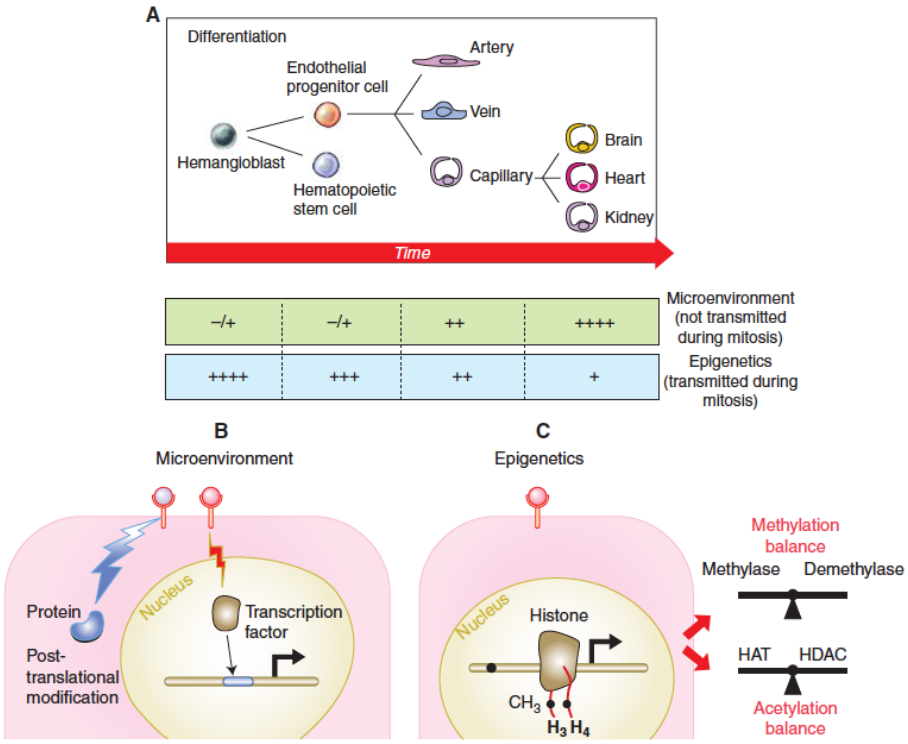

Figure 4: Mechanisms of EC heterogeneity

(A) Hemangioblast originates the endothelial progenitor cell that in turn can differentiate into EC capable to form arteries, veins, capillaries or lymphatics with specific features depending on the organ it is located in. **(B)** The role of the microenvironment in mediating changes in EC phenotype is represented by post-translational modification of protein or TF dependent induction of gene expression. **(C)** Inheritable changes are mediated by a balance of epigenetics cues including DNA methylation, histone methylation and acetylation (*from Aird et al, 2012*).

EC response to environmental and epigenetic stimuli is converted into a massive modification of the structural composition of the different branches of the vascular tree. For instance, the endothelium lining arteries and veins forms a continuous layer of cells held by an organized composition of AJs and TJs. Moreover, ECs surrounding the vessel wall of arteries are oriented along the longitudinal axis of blood flow [58] and this represents a reversible endothelial remodelling in response to hemodynamic shear stress [60]. The endothelium of the capillaries instead can be continuous, fenestrated or discontinuous, according to the tissue needs. Fenestrae are 60–80 nm diameter transcellular pores spanned

by fenestral diaphragms (FDias) [61,62] and are present in organs mostly involved in filtration or secretion, including exocrine and endocrine glands, renal glomeruli, gastric-intestinal mucosa. Discontinuous endothelium is similar to the fenestrated one with the exception that fenestrae are larger in diameter and lack a diaphragm [62]. This type of endothelium is characteristic of sinusoidal vascular beds, localized in bone marrow (BM), liver and spleen vasculature. Besides being an important secondary lymphoid organ, the spleen is one of the body largest filter of the blood, organized as a sort of tree of branching vessels, where the arterioles end in a venous sinusoidal system [63]. The spleen indeed possesses a specialized venous system in the red pulp able to filter blood and remove erythrocytes. When arterial blood comes into the red pulp it faces a unique structure, called cord, formed by fibroblasts and reticular fibres that form an open blood system without endothelial lining [64] and rich in macrophages. From the cord, blood finally enters the venous sinuses, where a discontinuous endothelial monolayer is formed by ECs connected via stress fibres to components of the ECM. Stress fibres are composed by actin-like and myosin-like filaments that run along the long axis of ECs and their contractility properties allow the formation and mediate the control of slits between ECs in order to let blood cells passing from the red pulp cord into the sinuses and back in the venous system [65]. Ageing erythrocytes with stiffening membranes that are no longer able to pass the slits, stick in the cord where they can be phagocytosed by macrophages [66] (Figure 5).

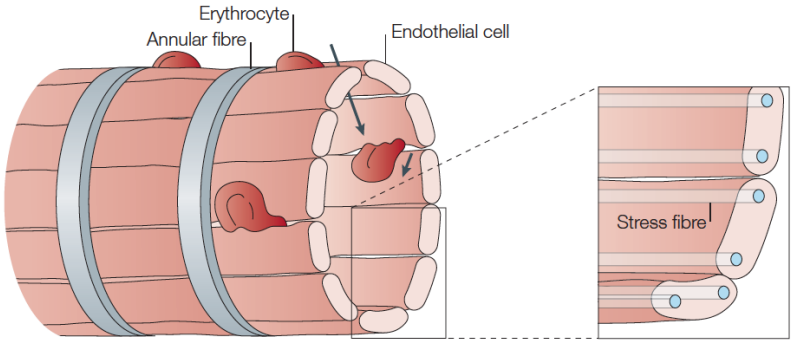

Figure 5: Venous sinuses in the red pulp of the spleen

The venous sinuses are composed by a lining of parallel-oriented ECs connected by stress fibres to anular fibres. Contractility of stress fibres mediates the formation of slits in the ECs, allowing the passage of erythrocytes from the cords to the sinuses and back to the *vena lienalis*. (*from Mebius & Kraal, 2005*).

Another example of highly specialized endothelium is represented by the vascular niche that, together with the osteoblastic (or endosteal) niche, composes the BM hematopoietic niche (Figure 6), a specific microenvironment that contains hematopoietic stem cells (HSCs) [67]. While the osteoblastic niche promotes stem cell quiescence, the vascular niche regulates proliferation, differentiation and mobilization of HSCs [68]. All these functions are achieved thanks to the unique niche composition of sinusoidal vessels supplied by a dense network of smooth muscle-covered afferent arterioles and capillaries derived from the furcation of the arterial vessels among the marrow cavity [69] . Different from the other vessels, BM sinusoidal endothelium lacks perycite coverage but it is surrounded by specialized Stromal cell-derived factor -1 (SDF-1)-producing reticular cells, that are essential for HSC colonization and maintenance [70]. A detailed definition of specific endothelial markers for BM vasculature is nowadays missing, because the capability to distinguish between arterioles and sinusoidal vessels results very problematic. Immunohistological approaches identified VEGFR3 as specific marker for sinusoids and Stem Cell Antigen -1 (Sca-1) for arterioles [71], even if the relative contribution of the two populations in the hematopoietic niche is still to be clarified.

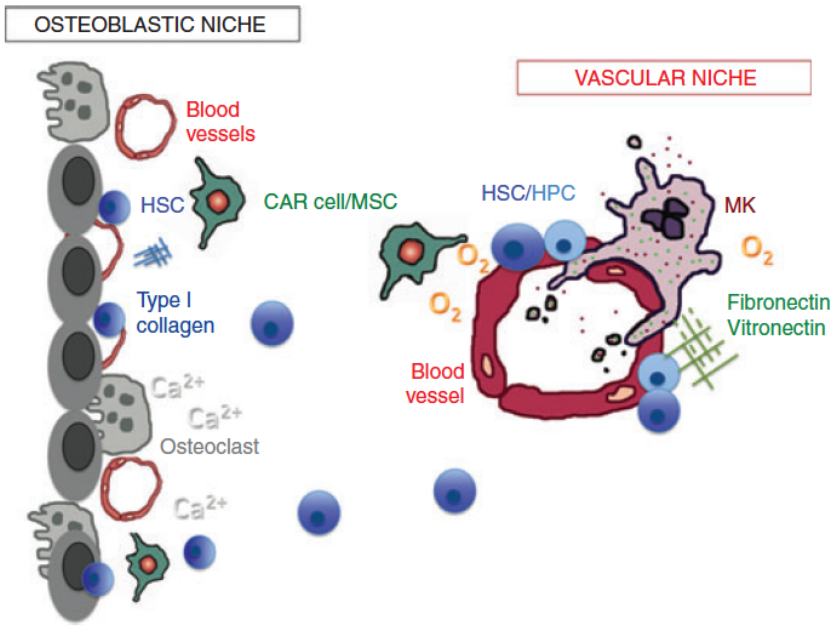

Figure 6: Schematic representation of the BM hematopoietic niche

A number of different players contribute to the homeostasis of the BM hematopoietic niche. On the left, osteoblastic niche is composed by different cell types, including osteoblasts and osteoclasts that continuously secrete factors regulating HSCs (dark blue). The endosteal surface is in strictly contact with vessels and perivascular cells, such as SDF-1 producing cells (CAR cells) and mesenchymal stem cells (MSCs) that contribute to niche maintenance. On the right, HSC/HPC (dark and light blue) are maintained in tight contact with ECs in the vascualr niche of BM and of other extramedullary sites, as in the liver and spleen. ECM components, such as fibronectin and vimentin, are found around blood vessels, ehnancing megakaryocyte (MK) development and function (*from Psaila et al, 2012*).

Finally, a recent study published last year [72] identified a novel BM compartment, namely hemosphere, present in both mouse and human long bone. Hemospheres are composed by a bulk of hematopoietic cells surrounded from one side by the inner layer of endothelium and from the other by osteoblasts in the niche. Genetic labeling identified hemospheres as a site of clonal hematopoietic cell expansion enriched in CD150-positive-CD48-negative putative HSCs. Unfortunately, it is still impossible to physically isolate cells exclusively form the hemospheres, so further investigation is ongoing to deeply understand if these structures own niche properties and can directly controll the activity of the HSC compartment. Intertestingly, murine gene targeting of VEGFR2 in ECs disrupted

the hemospheres and reduced the number of CD150-positive-CD48-negative putative HSCs [72], thus suggesting a strong interconnection between ECs and the hematopoietic compartments.

1.3 Endothelial Progenitor Cells

BM is the reservoir of different types of progenitor cells, including the hemangioblast, a common putative precursor of hematopoietic and endothelial lineages [73] that is able to give rise to hematopoietic (HPCs) and endothelial progenitors cells (EPCs). During adult life indeed mechanisms of neoangiogenesis and neovascularization are often required in case of vessel damage and regeneration, thus implicating the need of a pool of ECs always available: many evidence showed that this pool is represented not only by terminally differentiated ECs adjacent to the vascular wall that restart to proliferate but also by BM-derived EPCs. These cells usually reside in BM but can be mobilized by different peripheral stimuli to reach the tissues [74,75].

The definition of EPC and of its specific molecular markers is often misleading and not very clear mostly due to the fact that different subsets of markers have been identified. Indeed, depending on the stage of cell differentiation, the population of EPCs can express different combination of markers.

Experimentally, EPCs are obtained from culture of mononuclear cells isolated from peripheral blood. Historically, two major EPC subsets has been emerged from the *in vitro* plating of peripheral blood mononuclear cells (PBMCs): early-outgrowth EPCs and late-outgrowth EPCs [76]. The vast majority of the studies were performed with early-outgrowth EPCs, spindle shape cells that emerge from the plate after 4-7 days of culture [74,77] and possess limited proliferative capacity. Phenotypically, these cells are able to uptake acetylated low density lipoprotein (LDL), bind to Ulex europaeus agglutinin and express PECAM1, VE-cadherin, VEGFR2, von Willebrand factor (vWF) and low levels of CD34. Moreover they are also positive for the monocytic molecule CD14 and the panleucocytic

marker CD45 thus revealing the origin of early-outgrowth EPCs from hematopoietic pool. In contrast, late-outgrowth EPCs emerge after 2-3 weeks in culture, are very rare, possess cobblestone morphology and can give rise to a vascular network. They express CD34, VE-cadherin, CD146 and VEGFR2 molecules but neither CD45 nor CD14, thus indicating that late-outgrowth EPC progeny is the one which gives rise to the mature ECs.

The main inducer of EPC mobilization from the BM is eNOS-derived nitric oxide (NO)[78], mostly produced by cells of the BM microenvironment with regulatory functions, like osteoblasts and ECs. Accordingly, factors able to increase NO availability, like growth hormone (GH) and insulin growth factor-1 (IGF-1) are able to increase also EPC levels [79]. Other physiological factors positively modulating EPCs mobilization are physical exercise, that increases NO and VEGF [80,81] and oestrogens that play a direct action on α- and β-oestrogen receptors through metalloproteinase-9 (MMP-9) and eNOS mediated mechanisms [82].

In addition, several other mobilizing factors, such as granulocyte-colony stimulating factor (G-CSF), granulocyte monocyte-colony stimulating factor (GM-CSF), VEGF, SDF-1 and erythropoietin (Epo) are described to be able to induce EPC mobilization, proliferation and migration, activating AKT downstream signalling pathways[83].

On the contrary, smoking increases oxidative stress limiting NO bioavailability [84] and ageing reduces the levels of IGF-1 thus impairing the vascular repair ability of EPCs [85].

In the last years, a large portion of physiological and pathological evidence have suggested that peripheral levels of EPCs could reflect both vascular health and the potential for heart vascular repair. Indeed, the number of EPCs was demonstrated to be inversely related to the number of cardiovascular risk factors [86]. As a general rule, the greater the EPC number, the better is vasculature health. Indeed, many pathological conditions showed reduction in EPC number and mobilization; for example in arterial hypertension patients angiotensin (AT) II hastens EPC senescence by reducing telomerase activity and inducing EPC oxidative stress [87] while patients with diabetes mellitus have

impaired re-endothelization EPC-mediated capability due to an increase in NAPDH oxidase-dependent superoxide production that causes a reduction in NO bioavailability[88]. Conversely, acute myocardial infarction (AMI) seems to be the most known pathological stimulus for EPC mobilization, that induces a massive recruitment of progenitor/stem cells from the BM to the blood and subsequently into the myocardium [89].

For all these reasons, EPCs represent in the last few years intriguing target cells for the treatment of cardiovascular disease. In pre-clinical and pilot clinical studies, treatments with EPCs have shown some initial promising results. Deeper knowledge of the stimuli and the mechanisms involving EPC mobilization and proliferation will be of great help in the near future for the cure of cardiovascular pathologies, that still remain the first cause of death worldwide.

2. ENDOTHELIAL-TO-MESENCHYMAL-TRANSITION

2.1 Definition of endothelial-to-mesenchymal transition

Endothelial-to-mesenchymal-transition (EndMT) is a complex biological process in which resident ECs acquire spindle-shape morphology and gain migratory properties to delaminate from a polarized cell layer and invade the tissue. It is a behaviour of extreme plasticity in which ECs start losing their typical endothelial markers as VE-cadherin, claudin5 and CD31 (or PECAM1) to acquire mesenchymal features as the expression of α-Smooth Muscle Actin (αSMA), Fibronectin (FN), Fibroblast Specific Protein 1 (FSP1 or S100A4), N-cadherin, Vimentin, PAI-1, SOX4 or stem cell markers like CD44 and ID1. The final result of this trans-differentiation is the increase of a heterogeneous pool of fibroblastic-like cells that can contribute to fundamental physiological processes during embryogenesis[90-92]. Recent evidence suggest that this mechanism can be also pathologically activated in condition of organ fibrosis and cancer [93-98] (Figure 7).

EndMT has often been considered a specialized form of the well-known EMT, a mechanism that occurs in many epithelial cell types and of fundamental importance in different steps of embryogenesis [99].

Like EndMT, during EMT process, the epithelial cells undergo multiple biochemical changes, enhancing migratory capabilities, invasiveness, resistance to apoptosis and production of ECM components [100]. Epithelial cells undergoing EMT gradually lose their typical subset of molecular components, among which E-cadherin, ZO-1, Cytokeratin, desmoplakin in favour of the expression of mesenchymal markers. Moreover, in both EMT and EndMT the basal lamina is mainly degraded by metalloproteinase MMP-2 and MMP-9 and is replaced by FN and type I and III collagen, which activate different combinations of integrins to promote cytoskeletal reorganization [101].

In addition to its role in embryogenesis, EMT has been demonstrated to take place also in epithelial injury and be a key process for the tumoral cell invasion into the surrounding

tissue and metastatization. Both EMT and EndMT share common feature as they originate a cell with mesenchymal phenotype and are induced by similar signalling pathways, among which the most important one is the Transforming growth factor-β (TGF-β) [102].

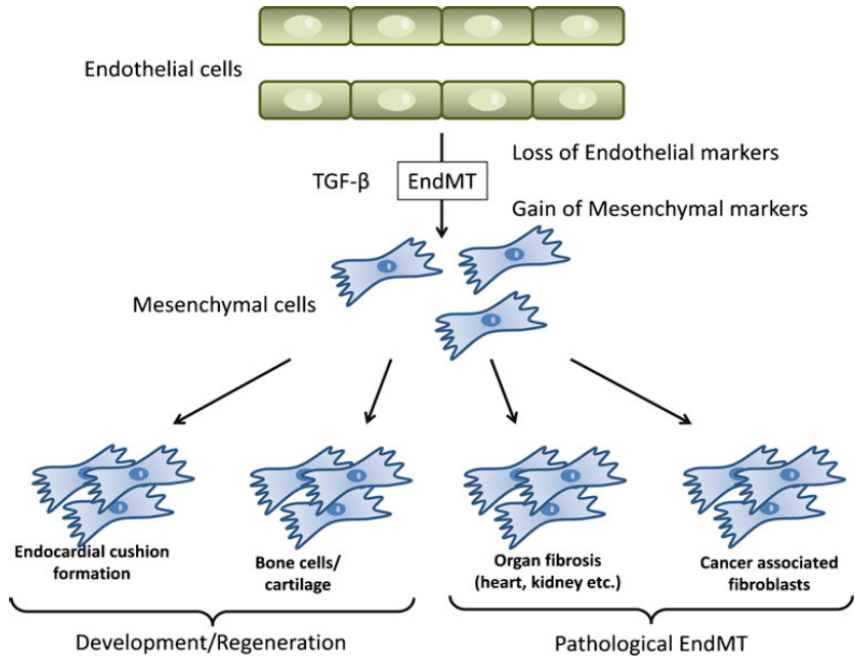

Figure 7: Schematic representation of endothelial-to-mesenchymal-transition

ECs face massive phenotypic changes while undergoing EndMT, including disruption of the monolayer and loss of endothelial markers in favour to the acquisition of mesenchymal markers and properties, like increased migration and invasiveness capability. This process is required in physiological condition during development and regeneration but can also be pathologically re-activated in the adult life contributing to fibrosis and cancer. TGF-β is one of the major EndMT inducers in both physiological and pathological conditions (*from Van Meeteren & ten Dijke, 2012*).

2.2 TGF-β signalling

TGF-β is the prototypic member of a superfamily of secreted cytokines that regulate a massive numbers of biological processes including cell proliferation, differentiation, migration, ECM production and apoptosis. Moreover, TGF-β also plays an essential role during embryonic development and vasculogenesis and alteration in its signalling pathway

results in different human diseases as cardiovascular and auto-immune disorders, wound healing, fibrosis and cancer [103]. Thirty-three members of the pleiotropic TGF-β superfamily have been identified so far in the mammalian genomes, including three distinct TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3), over twenty Bone Morphogenetic Proteins (BMPs), Growth Differentiation Factors (GDFs), Mullerian Inhibitory Factors (MIFs) and Activins or Inhibins. The members share a dimeric structure and a cysteine knot structural motif [104].

The TGF−β superfamily of ligands exerts its signalling through the binding to an heterodimeric receptor complex composed by type I and type II serine/threonine kinase receptors [105]. Type I (TβRI) and type II (TβRII) receptors are structurally similar, as they are both composed by a cytoplasmic tail that retains kinase activity, a single transmembrane spanning region and a cysteine-rich extracellular domain. So far, seven TβRI receptors, named activin receptor-like kinase (ALK1-ALK7) and five TβRII receptors, among which TGF-β type II receptor (TBRII), BMP type II receptor (BMPRII), activin receptor type IIA (ActRIIA), activin receptor type IIB (ActRIIB) and AMH type II receptor (AMHRII), have been identified. The ligands of the TGF-β superfamily can bind to different combinations of type I and type II receptors, thus mediating different signalling pathways (Table 1).

Receptor type	Ligand	R-SMAD
Type I receptor		
ALK1/ACVRL1	TGF-β, BMP9 and BMP10	SMAD1, SMAD5 and SMAD8
ALK2/ACVR1	BMPs and GDFs	SMAD1, SMAD5 and SMAD8
ALK3/BMPR1A	BMPs	SMAD1, SMAD5 and SMAD8
ALK4/ACVR1B	Activins, GDF8/myostatin and GDF11	SMAD2 and SMAD3
ALK5/TGFBR1	TGF-βs, GDF/myostatin and GDF11	SMAD2 and SMAD3
ALK6/BMPR1B	BMPs	SMAD1, SMAD5 and SMAD8
ALK7/ACVR1C	BMP16/nodal	SMAD2 and SMAD3
Type II receptor		
TGFBR2/TBRII	TGF-βs	
BMPR2/BMPRII	BMPs and GDFs	
ACVR2/ActRIIA	Activins, BMPs and GDFs	
ACVR2B/ActRIIB	Activins, BMPs, GDFs and BMP16/nodal	
AMHR2/AMHRII	MIF	
Type III receptor		
Betaglycan	TGF-β1, TGF-β2, TGF-β3, activin-A, BMP2, BMP4, BMP7 and GDF5	
Endoglin	TGF-β1, TGF-β3, activin-A, BMP2, BMP7 and BMP9	

Table 1: Complete list of the TGF-β receptors, ligands and Smad mediators

Each ligand belonging to the TGF-β superfamily can bind to a different combination of type I, II and III receptors, which in turn can take on TGF-β effectors Smad2/Smad3 or Smad1/Smad5/Smad8 thus allowing their translocation into the nucleus, where they can promote several transcriptional programs (*from Drabsch & ten Dijke, 2012*).

Commonly, BMPs and TGF-β subfamilies are synthetized as inactive monomeric pro-peptides, composed by a short N-terminal sequence, a latent-associated peptide (LAP) and a C-terminal mature domain retained in the ECM. After the synthesis, two monomeric precursors dimerize and are then processed by endoproteases of the convertase family and proteolytic enzymes, responsible for the cleavage between LAP and the release and secretion of the mature homodimer protein. This fine regulation of the protease activity is of fundamental importance since it allows the control of active TGF-β bioavailability [106].

The binding of the ligands induces the formation of the receptor complex and mediates phosphorylation of TβRI by TβRII, required to TGF-β downstream signalling. In this way

TβRI can then recruit and phosphorylate the cytoplasmic mediators Small mother against decapentaplegic (Smads) that, in turn, bind the common mediator Smad4 (Co-Smad) and translocate into the nucleus acting as TFs of specific target genes [107] (Figure 8). The TGF-β family of Smad proteins is classified in three main groups: Receptor-associated Smads (R-Smads), Co-operating Smads (Co-Smads) and Inhibitory Smads (I-Smads) [108]. R-Smads are the Smads recruited and phosphorylated by the TβR complex. Five different R-Smads have been identified in humans, namely Smad1, Smad2, Smad3, Smad5 and Smad8; their structure contains two conserved Mad Homology Domains, MH1 and MH2 that are respectively responsible for the binding to DNA and for the R-Smads oligomerization [109]. Interaction of R-Smads with the TβRI is also promoted by the presence of auxiliary proteins, such as the Smad Anchor for Receptor Activation (SARA), favouring the association of the TβR complex with Smad2/3 and subsequently their phosphorylation.

ALK4, ALK5 and ALK7 mediate Smad2 and Smad3 phosphorylation and are usually downstream of TGF-β, activins, inhibins and nodal [110]. On the contrary, ALK1, ALK2, ALK3 and ALK6 induce p-Smad1, p-Smad5 and p-Smad8 and their activation is usually mediated by BMPs and AMH, even if it has been demonstrated in ECs that TGF-β can also interact and signal through ALK1 [111].

Smad4 is the Co-Smad required for the association with phospho-R-Smads and their translocation into the nucleus, while I-Smads, that include Smad6 and Smad7, mediate a negative regulation of TGF−β signalling [112]. Among the I-Smads, Smad7 is the main involved into the switching off of the TGF-β signalling. Indeed Smad7 binds to activated TβRs and compete with Smad2 and Smad3 for the binding to ALK5, repressing its activation [113]. Moreover Smad7 recruits the E3-ubiquitin ligases Smad ubiquitination regulatory factor (Smurf)-1 and Smurf-2 to mediate ALK5 ubiquitination and degradation [114]. Smad7 is also a direct target gene of TGF-β/ALK5 dependent pathway and acts in a

negative feedback loop limiting the intensity and the duration of the signal [113]. In addition, Smad6 was shown to bind BMPR-1B inhibiting Smad1 phosphorylation, thus suggesting that also BMP signalling can be impaired by I-Smads.

In the nucleus, Smad complex binds specific sequences on the DNA called Smad-binding element (SBE) that contains the repeated 5' AGCA 3'motif [115,116]. Since the binding to SBE is relatively weak plus the fact that one of the R-Smad (Smad2) is not able to bind to DNA, the interaction with other TFs is strictly required to cooperate in order to mediate gene transcription. Among these, the best known are components of the FoxO family [117], members of the activating proteins-1 (Ap-1) family[118], run-related transcription factor 2 (RUNX2) [119] and nuclear factor-kappa B (NF-kB) [120].

While ALK5-induced p-Smad2/3 bind to DNA through SBE motif, ALK1-regulated Smads preferentially bind to BMP-responsive element (BRE), a sequence enriched in guanine and cytosine (5' GCCGNC 3' or 5' GRCGNC 3'). The term BRE is due to the fact that p-Smad1/5/8 are also the major downstream mediators of BMPs signalling [121,122].

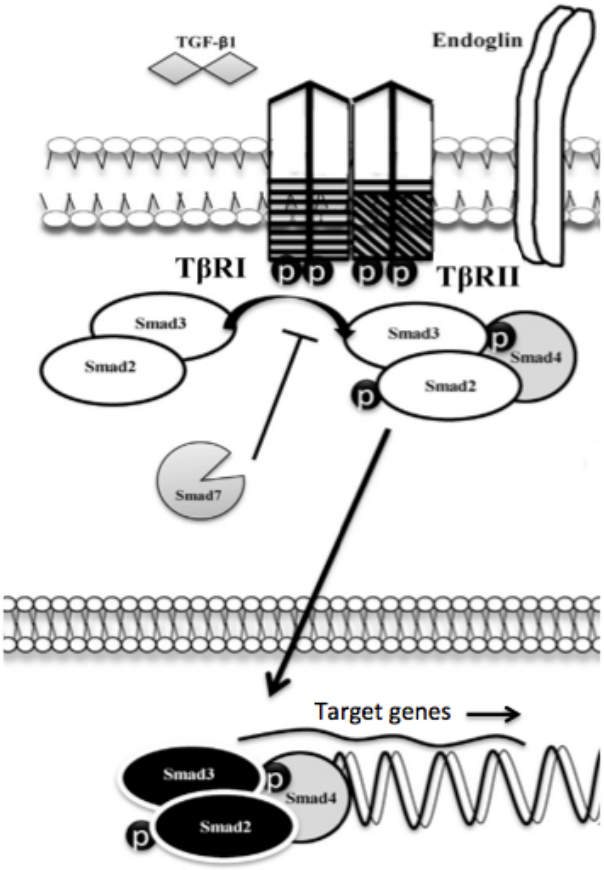

Figure 8: TGF-β canonical signalling pathway

The different members of the TGF-β subfamily bind to specific combination of heterodimeric type I (TβRI) and type II (TβRII) serine/threonine kinase receptors. When the ligand is bound, type II receptor can phosphorylate type I receptor that in turn phosphorylates Smad2/3. These leads to the recruitment of Smad4 that subsequently binds to Smad2/3 and the complex can translocate into the nucleus where it sits on specific DNA binding site to mediate the transcription of Smads target genes. Smad7 competes with Smad2/3 for the binding to type I receptor, inhibiting the signalling. The TGF-β co-receptor endoglin assists type I and type II receptors in mediating TGF-β downstream cascade (*adapted from Munoz-Felix et al, 2013*).

A further level of complexity in TGF-β signalling is added by the presence of the TGF-β co-receptors (TβRIII) betaglycan and endoglin, characterized by a large extracellular and a short intracellular domain lacking catalytic activity. Betaglycan interacts with TβRII playing a key role in the presentation of the TGF-β ligands to the receptor complex [123] since it is strictly required for the binding of TGF-β2 that has low affinity for TβRII [124].

Endoglin instead is a key modulator of the complex interplay between ALK1- and ALK5-dependent signalling; its inhibition *in vitro* decreases TGF-β/ALK1 pathway and indirectly enhances TGF-β/ALK5 [125], indicating that endoglin may be required for optimal TGF-β/ALK1 signalling pathway. In addition, endoglin and betaglycan were also reported to potentiate BMP signalling [126].

To be thorough, TGF-β superfamily can also exert its functions through a so-called non canonical non-Smad-dependent pathway, where TGF-β/TβR complex is able to directly activate different signalling pathways including MAPK, Rho-like GTPase and PI3K/AKT thus modulating a multitude of various downstream cellular responses [127].

2.2.1 TGF-β and BMP signalling in ECs

Genetic studies on mice and humans have shown the great importance of TGF-β signalling during vascular morphogenesis and angiogenesis. Mouse models and human syndromes showing either inactivation or aberrant expression of any of the components of TGF- β signalling pathway, like ligands, receptors and Smads, all undergo embryonic lethality mainly due to cardiovascular defects [106,128,129].

Moreover, in the adult, TGF-β exerts different and often divergent effects on EC homeostasis, the so-called "TGF-β paradox", and any perturbation occurring to this tuned system can lead to the onset of pathological conditions.

The effects of TGF-β signalling in ECs can be indeed finely mediated and even opposite, depending on the type I receptor involved, specifically ALK1 or ALK5 [111,130-132]. While ALK5 is broadly expressed, ALK1 is endothelium restricted. When TGF-β activates the ALK5/p-Smad2/3 axis the final outcome is the inhibition of EC migration and proliferation. The use of a specific ALK5 inhibitor, named SB-431542, has been demonstrated to facilitate EC proliferation and sheet formation in mouse embryonic stem cell-derived ECs. Furthemore SB-431542 is able to up-regulate the TJ component

claudin5, thus suggesting a role for ALK5 also in the regulation of vascular permeability [133]. One of the best-known TGF-β/ALK5 molecular targets is plasminogen activator inhibitor-1 (PAI-1), an important regulator of the plasminogen activation system [111]. PAI-1 is a proteinase inhibitor that is involved in the BM formation preventing degradation of the ECM around the emerging new vessels [134].

On the contrary, the activation of ALK1/p-Smad1/5/8-endoglin axis leads to EC migration and proliferation [135]. Indeed the activation of this pathway mediates the induction of the Inhibitor of Differentiation-1 (ID1), a negative regulator of basic-helix-loop-helix (bHLH) TF, controlling multiple TGF-β-induced biological responses. In general, ID proteins are positive regulators of cell proliferation and negative regulators of cell differentiation [136] (Figure 9).

Even if ALK1 and ALK5 induce divergent outcomes in ECs, they can also interact and modulate each other. It has been shown that ECs defective for ALK5 are not able to activate ALK1 signalling cascade because its absence does not allow the correct recruitment of ALK1 to form TβR complex [135]. Conversely, ALK1 can antagonize ALK5 signalling at the level of Smads [111,130]. Taken together, the regulation of ALK1 and ALK5 signalling and their reciprocal interaction provide a fine regulation of EC homeostasis and function.

Evidence showed that in ECs VE-cadherin can associate with TβR complexes [27]. The proper clustering of VE-cadherin at junctions is necessary for optimal TGF-β signalling to exert anti-proliferative and anti-migratory responses. Furthemore, VE-cadherin physically interacts with all the components of the TGF- β receptor complex, including TβRII, ALK1, ALK5 and endoglin, thus enhancing TβRII/ALK1 and TβRII/ALK5 complex formation and TGF-β-dependent signalling[27].

As well as TGF-β, also BMP subfamily has a prominent role in vascular development and angiogenesis, since genetic ablation or alteration in the expression of different BMP

components are embryonically lethal due to cardiovascular malformations and failure in vascular remodelling. BMP2, BMP4 and BMP6 were all reported to be able to induce angiogenesis and EC proliferation and migration [137,138]; the over-espression of these BMPs and of their target ID1 increases the ability of EC to mediate the *in vitro* assay of capillary tube formation while inhibition of ID1 expression blocks the BMP-induced migration [139]. On the contrary, BMP9 blocks either VEGF-induced angiogenesis either proliferation and migration of bovine aortic ECs (BAECs) induced by bFGF. Morevoer, BMP9 was shown to inhibit growth and migration of human dermal microvascular ECs (HDMECs). Of note, BMP9 shows different effects depending on the cellular contex and its concentration since, at low doses, BMP9 can also promote EC proliferation and promote angiogenesis in *in vitro* matrigel plug assays and *in vivo* in human pancreatic xenografts [140].

Furthermore, BMP signalling can interct with Notch signalling pathway, that fosters EC to become stalk ECs, promoting proliferation and subsequently elongation of the sprouting vessels. Data showed that inhibition of BMP downstream effector Smad1/5 in ECs both *in vivo* and *in vitro* resulted in a decrease of Notch signalling and in reduced expression of Notch target genes, overall resulting in decreasing stalk EC activity [141,142].

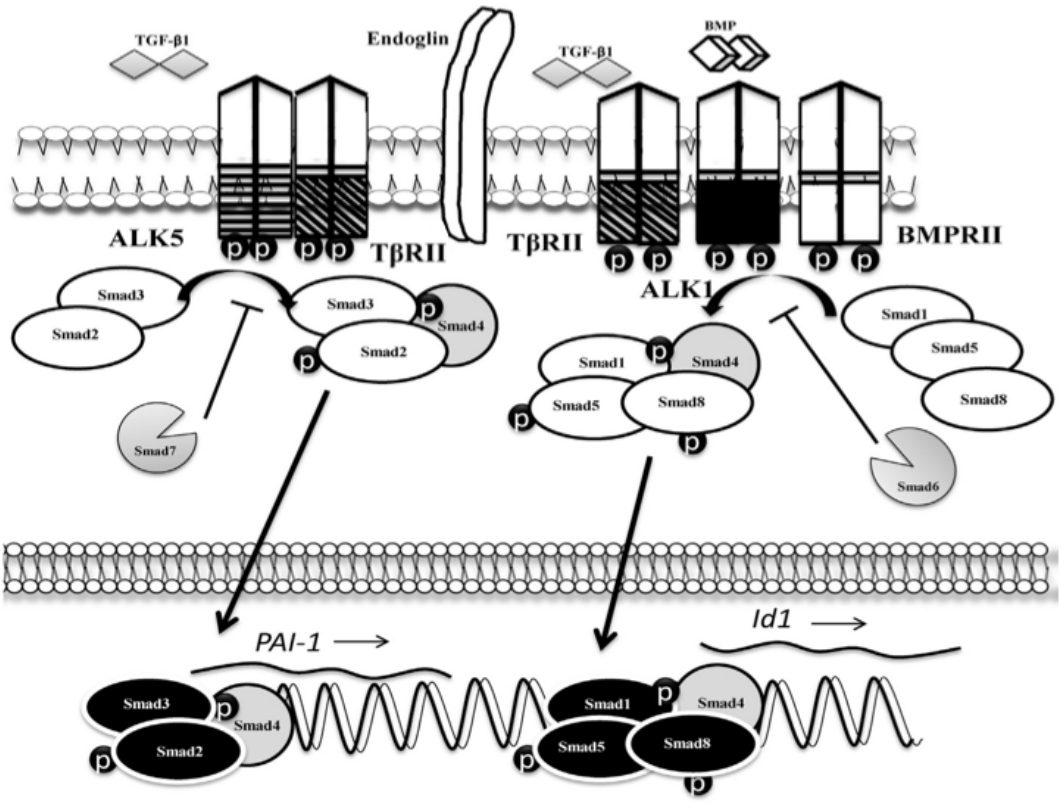

Figure 9: TGF-β signalling pathway in ECs

In ECs TGF-β can either activate ALK5/Smad2/3 resulting in the induction of the plasminogen activator inhibitor-1 (PAI-1) or ALK1/Smad1/5/8 that mediates ID1 expression. This latter pathway can also be initiated by BMPs that are recognized and interact with specific with type II BMP receptors (BMPRII). Smad7 and Smad6 compete respectively with Smad2/3 and Smad1/5/8 for the binding to ALK5 and ALK1 in order to switch off the signal, acting as negative TGF-β regulators (*adapted from Munoz-Felix et al, 2013*).

2.3 TGF-β/BMP axis controlling EndMT

Recent studies showed that ECs of different origins develop EndMT *in vivo* and *in vitro*. EndMT program can be induced by many factors, among which TGF-β plays a central role. All the three TGF-β isoforms (β1, β2 and β3) are able to induce EndMT in physiological and pathological conditions, even if TGF- β2 was shown to be the main inducer both *in vivo* and *in vitro*. Indeed, *in vivo* data demonstrated the central role of TGF- β2 during EndMT-mediated heart cushion formation both in mouse and chick

embryos and showed that TGF-β2 deficient mice could not correctly develop AV cushion [143]. Although genetic ablation of TGF-β1 and TGF-β3 in mice did not seem to affect EndMT process during development, TGF-β3 had a role in post-EndMT invasion and migration in the chick embryo but not in the mouse [144] and an involvement of TGF-β1 in inducing EndMT was also reported in a mouse model developing cardiac fibrosis[94].

In vitro data proved that TGF-β2 was able to induce EndMT in a model of human cutaneous microvascular ECs (HCMECs) both via the canonical Smad and non canonical non-Smad pathway [145] and in mouse embryonic stem cell-derived ECs (MESECs) [146]. Conversely, Moonen et al. showed that EPCs were able to give rise to smooth muscle like progeny when exposed to TGF-β1 and that the EndMT phenotype was mediated by ALK5 kinase activity [147]. Furthermore, Zeisberg and colleagues proved that also adult coronary ECs were able to undergo EndMT via TGF-β1 [94].

All these *in vitro* data turned out to be of great advantage to allow the study of the molecular mechanisms involved in EndMT. Medici and colleagues demonstrated that TGF-β2 stimulation induces the up-regulation of the TF Snail, whose expression suppresses cell adhesion and induces EndMT [145]. However, the over-expression of Snail alone is not sufficient to induce EndMT, suggesting that other players are required for the EC transdifferentiation. Effectively, other TFs like Slug, ZEB-1, Twist, SIP-1 and LEF able to suppress the transcription of genes codifying for molecules involved in the formation of TJs and AJs turned out to be up-regulated by TGF-β2 and BMP4 [148] in EndMT.

Many reports support the concept that also the BMPs family is deeply involved in the EndMT phenomenon. In particular BMP2 and BMP4 that signal through ALK2 receptor were reported to be able to mediate EndMT both in physiological [149] and pathological conditions [145]. Molecular or chemical inhibition of ALK2 was able to block EndMT transformation in ECs in culture [145] while conditional KO of BMP2, BMP4 and ALK2 all

inhibited embryonic EndMT because of the fail in coordination of the heart valve formation [149-151].

On the contrary, BMP7 can inhibit EndMT via ALK2 and p-Smad1/5/8, interestingly through the same pathway activated by BMP2 and BMP4, thus highlighting that the modulation of the EndMT switch is fine-tuned. Indeed, recent evidence reported that the combination of BMP4 and TGF-β2 is able to induce both ALK2 and ALK5 to activate Smad2/3 and Smad1/5/8 signalling, leading to a complex EndMT phenotype [145].

In addition to TGF-β, other signalling pathway have been reported to play a role in EndMT, including Fibroblast Growth Factor-2 (FGF-2) [152], Notch [153] and Wnt [154] that promote EC transformation and VEGF that, instead, inhibits EndMT [145,155].

2.4 EndMT in heart cushion formation and cardiac fibrosis

The first description of an EndMT mechanism is dated in 1977 when Markwald and colleagues, studying heart development during embryogenesis, focused their attention on endocardial cushion formation to understand the origin of the mesenchymal tissue forming the cardiac valves [90]. During heart development, the heart tube is composed by two different layers, an inner endocardium and an outer myocardium. In between the two layers there is a coat made by ECM called cardiac jelly.

The endocardium is composed by ECs expressing VE-cadherin and CD31 that, during the formation of the heart cushion, receives stimuli from the outflow tract and the atrioventricular (AV) myocardium to acquire mesenchymal characteristics in order to give rise to primordial valves and membranous septa [90-92] (Figure 10). BMP2 released from the myocardium has been suggested to be the first initiator to induce cardiac EndMT [156] because its expression triggers an autocrine production of TGFβ from endocardial cells feeding the transition of ECs to mesenchymal ones.

As previously mentioned, TGF-β2 plays a central role during cushion formation because it has been reported that its expression is required for the endocardial EndMT in the mouse [144] and TGF-β2 deficient mice show multiple defects in AV cushion formation [143].

Interestingly, during embryonic EndMT, TGF-β2 cooperates with β-catenin in the endocardial cells that mediate heart cushion formation. The transcriptional activity of β-catenin seems strictly required as TGF-β mediated EndMT is strongly inhibited in mice conditionally KO for β-catenin [154]. Moreover, in ECs KO for β-catenin TGF-β2-induced expression of αSMA is abolished, thus suggesting a possible interaction between TGF-β and Wnt signalling in mediating EndMT [154].

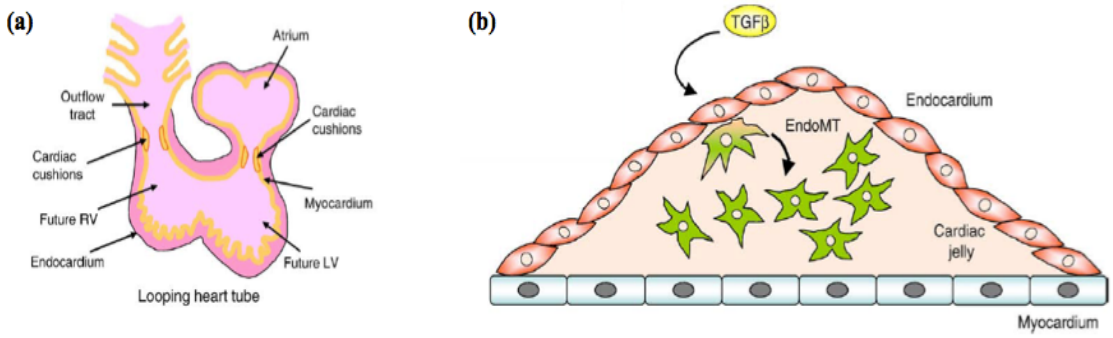

Figure 10: Heart valve development

Schematic overwiev of the anatomic formation of the nascent heart valves **(a)**. LV: left ventricle, RV: right ventricle. The developing heart tube is composed by an outer layer called myocardium and an inner one named endocardium separated by a stratum of ECM called cardiac jelly. During EndMT process, ECs from the endocardium delaminate, acquire mesenchymal properties and migrate to the cardiac jelly to develop valves and septa **(b)**. Autocrine production of TGF-β by the endocardial cells is strictly required to trigger and sustain the initial phases of the EndMT (*adapted from Goumans et al, 2008*).

The proof of concept that EndMT mechanism was taking place during heart valve formation and could also have a causative role in cardiac fibrosis was only demonstrated many years later taking advantage from the use of *Tie1Cre;R26stoplacZ* double transgenic mice that allow to follow lineage identity of the ECs [94]. *Tie1Cre*-transgenic mouse

expresses Cre recombinase under the promoter of Tie1, a tyrosine kinase receptor expressed by ECs of all organs. The crossing with the *R26stoplacZ* strain that expresses *LacZ* upon Cre-mediated excision leads to the expression of β-galactosidase (β-gal) only in Tie1 positive ECs. In this way β-gal expression will persist despite any other subsequent phenotypic cell alterations (Figure 11). Thanks to this strategy, it has been demonstrated that fibroblasts associated to the emergence of cardiac fibrosis in a mouse model of aortic banding had an endothelial origin, as well as the cells of the AV cushion in primordial heart [94]. The key regulator of the endothelial transition during cardiac fibrosis was proven to be TGF-β1 that induced EndMT in adult coronary ECs via Smad3 signalling pathway. On the contrary, administration of BMP7 reduced the overall fibrosis in the heart due to a reduction in the number of fibroblasts of endothelial origin, demonstrating its essential role in inhibiting EndMT-mediated cardiac fibrosis [94].

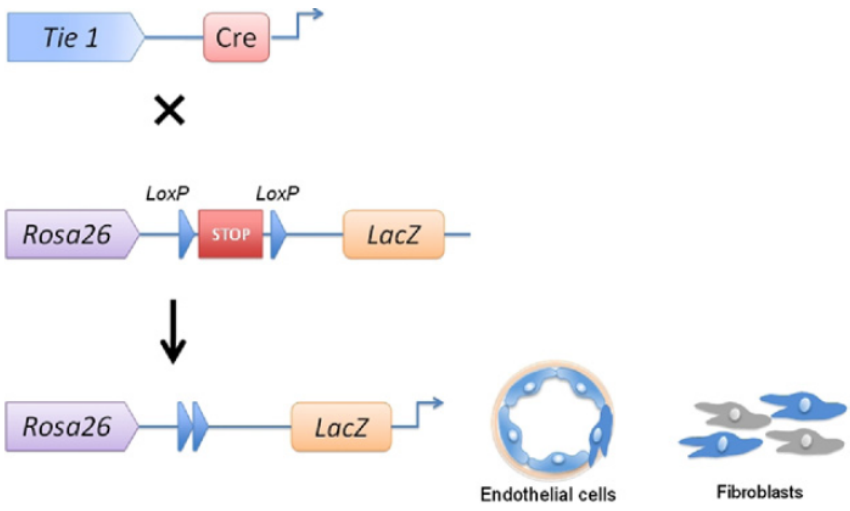

Figure 11: Experimental strategy to obtain EC lineage tracking in mouse models

In the upper vector, Cre recombinase expression is driven by the endothelial-restricted Tie1 promoter, in order to obtain Cre expression only in ECs. The Rosa26 reporter vector mediates LacZ expression and contains 2 LoxP sites flanking a stop codon. When Tie1-Cre transgenic mice are crossed with Rosa26 mice, Cre recombinase cuts the stop codon flanked by the LoxP sites allowing the irreversible expression of the LacZ marker (in blue) only in cells of endothelial origin. When ECs undergo EndMT and acquire fibroblastic phenotype, the expression of the LacZ marker clearly reveals their endothelial origin (*from Piera-Velazquez et al, 2011*).

Moreover, the TFs Slug and Snail are described to be specifically required for the initiation of cardiac EndMT [157]. Slug is directly activated by Notch to mediate VE-cadherin repression to allow endocardial cells to initiate invasion in the cardiac jelly. Furthermore, Slug deficient mice showed an up-regulation of Snail expression and double KO for Snail and Slug mice reduced heart valve development, underlying the compensatory role of the Snail family members in EndMT-mediated cardiac formation.

2.5 EndMT-associated organ fibrosis

The work by Zeisberg focused on cardiac fibrosis led to the awareness that EndMT could be inappropriately activated also in other organs. In kidney fibrosis, fibroblasts are the main mediators to develop fibrosis, but their origin is still not fully understood. Previous data showed that the transition of the renal epithelium into (myo)fibroblasts is mediated by EMT process in different experimental models and human diseases [95]. Molecular mechanisms in the kidney epithelia require a synergistical contribution of Epidermal Growth Factor (EGF) and TGF-β1, both found to be activated to induce EMT in the renal proximal tubular epithelial cells [158]. TGF-β1 requires integrin-β1 signalling to induce activation of p38 MAPK and canonical Smad3-dependent signalling to induce EMT [159]. Moreover, insulin-like growth factor II (IGF-II) induces redistribution of β-catenin from plasma membrane to nucleus, together with sequestration and degradation of E-cadherin [160] to promote mesenchymal tranformation.

Nevertheless emerging new data demonstrated that also ECs contribute to the pool of activated fibroblast to sustain kidney fibrosis through an EndMT mechanism. Two different groups of research [96,97,161] analysed the contribution of ECs to EndMT in different models of chronic kidney disease (CKD), as the unilateral ureteral obstructive (UUO) nephropathy, streptozoticin-induced diabetic nephropathy (STZ-induced DN) and Alport renal disease. Zeisberg and colleagues demonstrated that in all the three mouse models a big portion of fibroblasts (from 30% to 50%) co-expressed the CD31 and markers of

fibroblasts and myofibroblasts, such as FSP1 and αSMA [96,97]. The endothelial origin of fibroblasts expressing FSP1 and αSMA was clearly assessed by the use of *Tie1Cre;R26stopEYFP* allowing to trace EYPF positive ECs since their origin.

Li and co-workers confirmed the same results on the STZ-induced DN model; this data were in agreement with previous observation that Smad3 null mice are resistant to STZ-induced DN [162] and are protected from tubolointerstitial fibrosis induced by UUO [163].

Another organ described to develop fibrosis is the lung. The hallmark of idiopathic pulmonary fibrosis (IPF) is the presence of fibroblastic foci involved in tissue remodelling, matrix deposition and crosstalk with the alveolar epithelium. The origin of fibroblasts associated to pulmonary fibrosis is still under investigation but once again EndMT mechanism was described to be one of the most important sources [164]. Taking advantage of double-crossed transgenic mice, in which LacZ was stably expressed in ECs, lung fibrosis was induced in the mouse model by endotracheal injection of bleomycin, resulting in severe pulmonary fibrosis. Hashimoto and colleagues showed that a huge component of the fibroblasts in the fibrotic area were LacZ positive. About 16% of these fibroblasts expressing FSP1, αSMA and collagen I further cultured *in vitro* were of endothelial origin [164]. Interestingly, they also showed that the acquisition of a mesenchymal phenotype by ECs was dependent from TGF-β and Ras signalling pathways, both required to feed and maintain an EndMT phenotype.

2.6 EndMT in cancer, FOP and CCM pathologies

Tumors are composed by a plethora of different cell types where the malignant cancer cell clone requires a permissive environment to grow and colonize the surrounding tissues. Cells that compose the tumor stroma create this favourable condition and among them fibroblasts are key modifiers in mediating cancer progression. In particular a subpopulation of fibroblasts, the so called cancer-associated-fibroblasts (CAFs), are now recognized among the most important promoters of tumor growth and progression [165]. CAFs are

defined as activated and reactive fibroblasts acquiring a modified phenotype in order to provide oncogenic signals to the transformed epithelia within the tumor [166].

CAFs form a heterogeneous population with different origins: activation and proliferation of resident tissue fibroblasts mainly contribute to CAF accumulation in tumour microenvironment, but also BM and periadventitial cells are thought to be a possible origins of activated fibroblasts [166].

Evidence showed that ECs undergoing EndMT could represent one of the CAF most important source [93]. Qualitative analysis of the fibroblasts within the tumour in a mouse model of melanoma revealed them to be double positive for the endothelial marker CD31 and the mesenchymal markers FSP1 and αSMA. Approximately 40% of FSP1 positive and 10% of αSMA positive fibroblasts were co-expressing CD31, suggesting that these CAFs were of endothelial origin. In order to prove these data and to recognize possible ECs originating CAFs that had completely lost their endothelial phenotype in favour of a mesenchymal one, Zeisberg and colleagues took advantage from the *Tie1Cre;R26stoplacZ* double transgenic mice (a similar a model already described in the previous paragraphs) where the melanoma cells were implanted to trace fate lineage of ECs. As expected, a big portion of CAFs retained the endothelial lineage tracer β-gal, proving that EndMT leading to EC transdifferentiation could be an important source of CAFs. Moreover, it was also finally demonstrated that EndMT-mediated CAF origin was strongly induced by exposure of ECs to TGF-β1, supposed to act both in an autocrine and paracrine way to promote the accumulation of activated and promoting-cancer fibroblasts in the tumor stroma [93].

Fibrodysplasia Ossificans Progressiva (FOP) is a genetic disorder causing extreme heterotopic ossification (HO) mostly in muscles and ligaments, leading to paralysis. FOP patients carry an autosomal dominant germline mutation in the ALK2 gene that results in constitutive phosphorylation of ALK2 receptor and downstream Smad proteins [148]. Different studies were aimed at identifying the cellular lineage responsible of the ectopic bone formation that was recently suggested to be of endothelial origin. Indeed mouse

model for HO induced by transgenic expression of mutant ALK2 demonstrated that ectopic bone cells expressed endothelial markers VE-cadherin, Tie1, Tie2 and vWF [167]. Comparable results were obtained in pathological samples of FOP patients [148]. Lineage tracing in reporter mice further demonstrated that most of the mesenchymal cells involved in the first phases of ectopic ossification were ECs that underwent EndMT switch.

Finally, a recent paper published by our group [168] has demonstrated pathological activation of the EndMT program in Cerebral Cavernous Malformations (CCM). CCM is a vascular dysplasia affecting the brain microcirculation resulting in lesions formed by enlarged, irregular, multi-lumen blood vessels that often result in cerebral haemorrhages. CCM pathology is caused by LOF mutations in CCM1, or CCM2, or CCM3 genes and occurs in both sporadic and familial forms. We showed that EndMT contributes to the development of vascular malformations because in the CCM lesions ECs co-express different mesenchymal (FSP1, αSMA, N-cadherin, Slug, KLF4) and stem cells markers (CD44, ID1) while they lost the endothelial-specific claudin5 expression. We also demonstrated that EndMT in CCM pathology was mediated by the up-regulation of BMP6 by primary brain ECs that in turn activates TGF-β and BMP downstream pathway via canonical Smad signalling to sustain EndMT program [168].

3. PRIMARY MYELOFIBROSIS

3.1 Molecular basis

Primary Myelofibrosis (PMF) belongs to the family of Myeloproliferative Neoplasms (MPNs), a heterogeneous group of related diseases that also includes Polycythemia Vera (PV) and Essential Thrombocythemia (ET). MPNs are clonal malignant disorders characterized by the oncogenic transformation of the multipotent hematopoietic progenitor cells (HPCs), which causes abnormal proliferation from one to several myeloid lineages driven by a hypersensitivity to regulatory growth factors [169]. MPNs were first studied in 1951 by the American haematologist and founder of *Blood* journal William Dameshek (1900-1969) (Figure 12), who described a related group of haematological diseases including PV, ET, PMF and Chronic Myeloid Leukemia (CML) that he originally called myeloproliferative disorders (MPDs)[170].

The discovery of the Philadelphia chromosome and the fusion gene Breakpoint Cluster Region-Abelson (BCR-ABL) as the major molecular mechanism responsible for myeloproliferation led to the correct classification of CML in this group. On the contrary, only in 2005 the causative molecular mechanism of action of PV, ET and PMF, that are Philadelphia chromosome-negative dysfunctions, was demonstrated to be mainly dependent on a single point gain-of-function (GOF) mutation in the Janus Kinase 2 (JAK2) gene. JAK2, as well as JAK1, JAK3 and TYK2, is a member of the Janus family of cytoplasmic non-receptor tyrosine kinases that are differentially activated in the response to various cytokines [171]. JAK2 is indeed the predominant JAK activated in response to Erithropoietin (Epo), Interleukin-3 (IL-3) and GM-CSF, as well as to IL-5 and Thrombopoietin (Tpo) [171].

Figure 12: William Dameshek (1900-1969)

"Perhaps it is possible to resolve all of these dilemmas, conflicts, antagonisms and confusions by considering not that the various conditions listed are different but that they are closely inter-related. It is possible that these various conditions –"myeloproliferative disorders'" — are all somewhat variable manifestations of proliferative activity of the bone marrow cells, perhaps due to a hitherto undiscovered stimulus. This may affect the marrow cells differently or irregularly with the result that various syndromes, either clear-cut or transitional, result."

(from William Dameshek "Some Speculations on the Myeloproliferative Syndromes"; Blood, 1951; Credits: Tuft Photo Archives)

JAK2 deficiency in mice is embryonically lethal due to the absence of definitive erythropoiesis [172] and its phenotype deeply resembles that of the Epo- or Epo Receptor-deficient mouse [173], thus suggesting its essential and non redundant role in the maturation of red blood cells.

All the members of the JAK family are composed by seven homologous domains (from JH1 to JH7), including the catalytic kinase domain JH1 and a catalytically inactive pseudokinase domain JH2 that acts as an auto-regulatory subunit to switch off phosphorylation-mediated signal. JH3 and JH4 connect JH2 to JH5, JH6 and JH7, which

form the "protein four-point-one, ezrin, radixin, moesin" (FERM) domain, cointaining the sequences necessary to promote association of the kinase with specific cytokine receptors domains (Figure 13) [174].

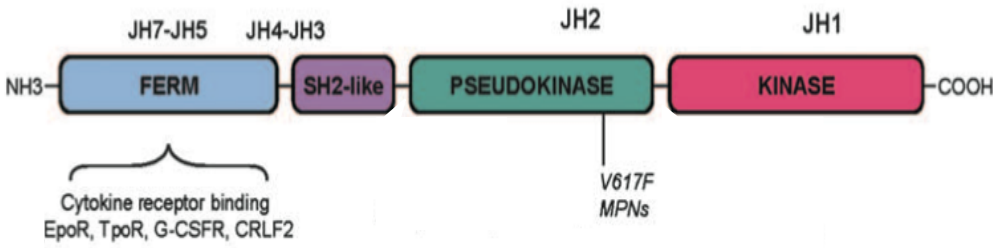

Figure 13: Domain structure of JAK2

JAK2 is composed by a tyrosine kinase domain JH1, a pseudokinase domain JH2, a SH2-like domain and a FERM-like domain (JH5-JH7) responsible for the binding to the cytosolic part of different cytokine receptors, as EpoR, TpoR (or MPL), G-CSFR and CRLF2. V617F mutation is located in the JH2 domain, thus imparing its role in preventing the activation of the kinase activity *(adapted from Vainchenker & Constantinescu 2013*).

In MPNs, JAK2 mutation results in the aminoacid change Valine-to-Phenylalanine at codon 617 (V617F), located in the JH2 domain [175]. As a consequence, the kinase loses its inhibitory frame and is constitutively active even without upstream signal activation, acting in a GOF manner. *In vitro* studies demonstrated that V617F expression is sufficient to activate different JAK2 downstream signalling molecules, like Signal Transducer and Activator of Transcription (STAT), Mitogen activated protein kinase (MAPK) and PI3K-AKT [175]. All these pathways contribute to the transcription and activation of genes involved in cell proliferation and survival, for example induce the over-expression of the anti-apoptotic gene B-cell lymphoma-extra large (BCL-X_L), a transcriptional target of STAT5 (Figure 14).

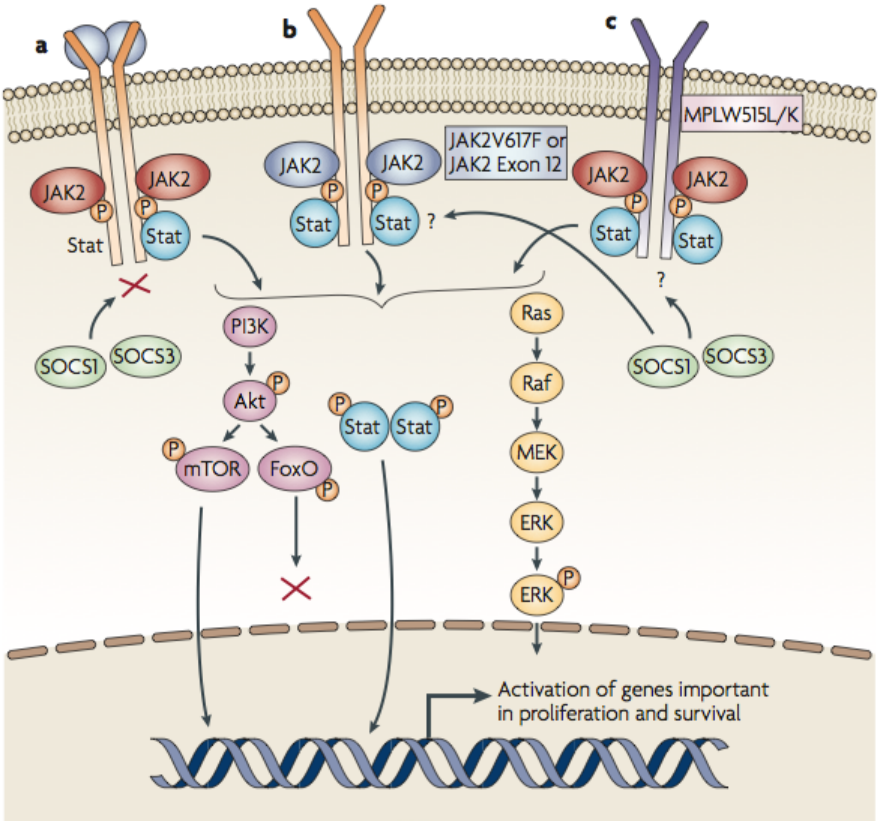

Figure 14: Activation of wild type and mutated JAK2 downstream signalling pathway

In physiological condition, the binding between ligand and cognate receptor mediate JAK2 phosphorylation that, in turn, is able to recruit STAT proteins and activate downstream signalling pathways, among which PI3K-AKT and MAPK. SOCS1 and SOCS3 protein are described to switch off JAK2 signalling **(a)**. JAK2-V617F and JAK2 Exon 12 mutated cells can activate JAK2 downstream signalling pathways even in the absence of the upstream ligand **(b)**. MPL-W515L/W mutant thrombopoietin (Tpo) receptor phosphorylates JAK2 wild type in absence of Tpo to mediate JAK2 downstream signalling activation of STATs, MAPK and PI3K-AKT **(c).** JAK2-V617F mutant was recently described to be able to escape negative regulation SOCS1 and SOCS3 mediated (*adapted from Levine et al, 2007*).

JAK2 GOF mutation is detected in 90% of PV and in 50-60% of ET and PMF patients. Till now it is not fully clear how the same single mutation can give rise to three clinically distinct pathologies but some hypotheses suggest that different inherited genetic modifiers can interplay in order to specify which type of MPNs will be developed. The presence of

JAK2-V617F homozygous subclones are for example usually associated to PV patients while they are very rare in ET [176]. Further genomic analysis highlighted also four novel somatic mutations in exon 12 of JAK2 gene [177] that are present specifically in JAK2-V617F negative PV patients with isolated erythrocytosis without leucocytosis or thrombocytosis, highlighting that also JAK2 exon 12 mutations could take a role in the pathology of MPNs. Additional genetically predisposition was hypothesized and confirmed by the discovery of hereditable alleles, among which the JAK2 46/1 haplotype, responsible for predisposition to MPL-mutated MPNs [178]. Moreover, given that a big proportion of PMF and ET patients (40-50%) is JAK2 wild type, the search of other mutation involved in the pathologies led to the discovery of another gene responsible for PMF and ET development, the dedicated TpoR encoded by the Myeloproliferative leukemia virus oncogene (MPL) gene. Somatic mutations at codon 515 located at the transmembrane–juxtamembrane junction of MPL (MPLW515L or MPLW515K) have been identified in ~10% of JAK2-V617F negative PMF and in smaller proportion of ET patients [179]. The expression of the mutated form of MPL in the hematopoietic progenitor is able to phosphorylate JAK2 wild type in absence of Tpo, thus resulting in the activation of STATs, MAPKs and AKT in a JAK2-V617F similar fashion. Last but not least, recently, two distinct groups managed to identify new somatic mutations in the CALR gene, encoding for the calreticulin protein [180,181]. Calreticulin is a multifunctional endoplasmatic reticulum (ER) protein acting mainly as a molecular chaperone but also involved in cellular calcium homeostasis. The mutations in CALR, all found in the exon 9 of the gene, have been detected in 20-25% of PMF and ET patients and appeared to be mutually exclusive with the ones in JAK2 and MPL genes. Specifically, the two more frequent mutations in CALR gene are a 52 base pair (bp) deletion (type 1) or a 5bp insertion (type 2) that both modify the C-terminal domain of the calreticulin where the KDEL motif for ER retention and the negative charged amino-acids (aa) for Ca2+ binding are located. As a result, type 1 and 2 mutations induce a frameshift in the sequence of the protein that

generate a new C-terminus where negative charged aa are replaced by neutral or positive ones and there is the loss of KDEL domain. It follows that the binding of calcium is impaired and subcellular delocalization of CALR mutated protein takes place. The first *in vitro* studies showed that if calreticulin with type 1 mutation is over-expressed in the IL3-dependent cellular line Ba/F3, the cells are able to grow also in absence of IL-3 and show hypersensibility to the cytokine [180]. Moreover JAK-STAT signalling pathway seems to be involved but further analyses are required to better understand the role of the recently discovered CALR mutations in PMF.

Through whole-genome approaches a large number of additional mutations were then identified in MPNs, belonging both to the field of epigenetics and DNA methylation, like mutations in Tet methylcytosine dioxygenase 2 (TET2), DNA-cytosine5-methyltransferase 3A (DNMT3A) and Isocitrate dehydrogenase 1/2 (IDH1/2) [182] or in the Polycomb Repressive Complex 2 (PRC2) components as in Enhancer of zeste 2 (EZH2) and in Additional sex combs like transcriptional regulator 1 (ASXL1). Mutations in splicing factor category also occurs, mostly in Serine/arginine-rich splicing factor 2 (SRSF2) and Splicing factor 3b, subunit 1 (SF3B1) genes [183]. Although these mutations are not sufficient to induce an MPN phenotype and they are also very often present in a large spectrum of haematological disease, the analysis of mutational status of a large cohort of PMF patients has been very useful to determine their individual and combinatorial prognostic relevance in terms of quality of life and survival. For example, mutational profiling for EZH2, ASXL1, SRSF2 and IDH identifies PMF patients at risk for premature death or leukemic transformation [183].

3.2 Clinical features and diagnosis

PMF specific cardinal features include massive hypercellular BM with collagen fibrosis and osteosclerosis. As a result of the lack of the space in BM, extramedullary hematopoiesis (EMH) takes place primarily in the spleen that becomes enlarged, resulting

in splenomegaly [184]. In general, first debilitating symptoms of PMF disease that patients can recognize are general malaise, exaggerated fatigue, weight loss and fever. Megakaryocytes (MKs), the progenitor cells of platelets located in BM hematopoietic niche, are the myeloid cells mostly implicated in this disease, as they are characterized by dysplastic hyperplasia and are filled with different fibrogenic factors, among which the higher expression of both latent and active TGF-β1 and MMP-9 are supposed to primarily contribute to BM fibrosis [185,186]. *In vitro* study demonstrated that PMF hematopoietic precursor CD34-positive derived cells are able to generate 24-fold greater numbers of MKs in comparison to CD34-positive normal cells. PMF-derived MKs also showed delay in the apoptosis mediated by the up-regulation of the anti-apoptotic gene BCL-X_L [185]. Furthermore PMF-derived MKs showed intrinsic defects in extending pro-platelets that resulted abnormal both in number and structure [187].

A whole-blood transcriptional profiling on PMF specimens revealed elevated levels of pro-inflammatory cytokines and proteins involved in the immune response [188,189], that participate in hematopoietic cell proliferation and mobilization, as well as in the process of fibrosis. These molecules are produced by MKs and monocytes within the BM and spleen and play a central role to mediate the increased deposition of BM stromal fibers. The hyper production of cytokines suggests an alteration in the overall microenvironment of BM niche that leads to fibrosis, which is indeed the final result of chronic inflammatory reactions. Some data suggest that progressive BM fibrosis can be fostered by the combination between fibronectin (FN) and the elevated expression of TGF-β1, IL-1 and substance P in PMF monocytes [190]. Moreover, among the pro-inflammatory cytokines found to be highly up-regulated, it has been demonstrated that also Tumor Necrosis Factor-α (TNF-α) plays a critical role in clonal evolution of MPN; indeed, its high level of expression in human MPN seems to be necessary to facilitate clonal expansion of JAK2-V617F positive cells and to simultaneously confer TNF-α resistance to a pre-MPN cell [191].

However, the conclusive mechanism leading to myelofibrosis has not clearly been elucidated yet, but it is well accepted that proliferation of fibroblasts represents one of the major reactive and non-clonal process to induce BM fibrosis [192].

PMF is a very complex and evolving disease; in the 90s' a new variant of PMF, called prefibrotic myelofibrosis (pre-MF), has been identified as a prodromic phase of PMF. This category of patients is characterized by absence of relevant reticulin fibrosis in BM with dual megakaryocytic and granulocytic myeloproliferation associated with megakaryocyte dysplasia that evolves into overt PMF [193]. Conversely, a fraction of PV and ET patients can undergo myelofibrotic transformation in time; these pathologies are defined as post-PV MF or post-ET MF and they are clinically and biologically indistinguishable from the classical PMF [194].

The diagnosis of PMF and of the other MPNs is based on the criteria established by the World Health Organization (WHO) in 2008, that has set three major criteria and four minor criteria to recognize PMF from the other MPNs [195]. Recently, haematologists proposed a revision of these criteria in order to update and introduce the newly discovered CALR mutations as one of the fundamental requirement to diagnose PMF. The new proposed criteria are summarized in Table 2.

Risk assessment of PMF is evaluated by the International Prognostic Scoring System (IPSS) that predicts inferior survival depending on the presence of five risk factors: age > 65, hemoglobin < 10 g/dl, white blood cell count > $25x10^9$/l, blasts ≥ 1% and constitutional symptoms. The Dynamic IPSS (DIPSS) calculates the same risk considering the evolution of the categories in time.

2014 Proposed revision for WHO diagnostic criteria for PMF
Major Criteria 1. MK proliferation and atypia accompanied by either reticulin and/or collagen fibrosis 2. Not meeting WHO criteria for CML, PV, ET, MDS or other myeloid neoplasm 3. Presence of JAK2, CALR or MPL mutation
Minor Criteria 1. Presence of a clonal marker (e.g. abnormal karyotype) or absence of evidence for reactive bone marrow fibrosis 2. Presence of anemia 3. Presence of palpable splenomegaly 4. Presence of leukoerythroblastosis or increased lactate dehydrogenase

Table 2: 2014 Proposed revision for WHO diagnostic criteria for PMF.

The presence of at least one of the three major criteria is required to diagnose PMF. The 2014 revised WHO criteria proposed to overhaul criteria number 3 adding the recently discovered CALR mutations. Four minor criteria are of support to establish a PMF diagnosis.

3.3 Mouse models of MPNs

In the last few years many groups of research put their effort into generating suitable animal models to better understand the molecular pathways involved in MPNs, the initiation and progression of the pathologies and to test *in vivo* new compounds for pharmacological treatment. To this aim three different approaches were followed in order to induce the expression of JAK2-V617F mutation in the hematopoietic lineage of the mouse. Initially, the first MPN mouse models were established over-expressing the mutated JAK2 gene in the murine BM with the help of a retroviral vector, a technique frequently adopted to study haematopoietic malignancies. The transplant of a BM retrovirally transduced with JAK2-V617F in recipient mice recapitulated the myeloproliferative phenotype with mainly PV characteristics, as erythrocytosis, splenomegaly, EMH and cytokine hypersensitivity [196]. Many mice underwent BM fibrosis with associated anemia and leucocytosis, with different grades and evolution of the disease depending on the strain of the transplanted mice. These first studies finally proved that

JAK2-V617F mutation was sufficient to induce a MPN phenotype in the mouse model. However, this approach had some limitations like the non-physiological and non-controlled expression of the transgene, the non-selectivity of the cell population transduced combined with the unknown integration of the retroviral vector into the genome. The next generation of mouse models was created taking advantage of the pronuclear injection strategy; murine or human JAK2-V617F cDNA under the the control of hematopoietic promoters [197,198] were injected into the pronuclei of fertilized egg at a single cell stage, creating a stable transgenic lines of mice expressing V617F mutation. Both mouse models recapitulated MPN main features, as they exhibited thrombocytosis, erythrocytosis and eventually myelofibrosis. Smart control of JAK2-V617F expression to drive variable levels of the mutated molecule was achieved through the generation of a construct carrying nine copies of the bacterial artificial chromosome (BAC) encoding for JAK2 wild type followed by the mutant form placed in the inverse orientation and flanked by antiparallel lox66 and lox77 sites [199]. When crossed with a *Cre*-expressing transgenic mouse the offspring could express different amount of the mutant JAK2 depending on the efficacy of the *Cre recombinase* cut. A similar approach was followed using mice expressing *a Mx1-Cre* promoter that allows to fine tune Cre expression depending on the amount of polyinosine-polycytosine (pIpC) arbitrary used to activate *Mx1* promoter [199]. With these *in vivo* approaches the hypothesis that higher levels of JAK2-V617F expression usually correlates with a PV phenotype more than ET and PMF was validated. The more recent knock-in mouse models recently produced were aimed to express the mutation in JAK2 at physiological levels under the control of the endogenous promoter through homologues recombination in embryonic stem (ES) cells [200,201]. Both JAK2-V617F heterozygous and homozygous mice developed a MPN-like disease with splenomegaly, leucocytosis and thrombocytosis, high levels of both haemoglobin and red blood cells count and expansion of Epo-independent erythroid colonies. Homozygous V617F mice showed a more evident PV-like phenotype and accelerated BM fibrosis in comparison to mice expressing the

mutation in heterozygous fashion [200]. Moreover, the mice showed increased megakaryopoiesis and giant platelets. Both BM and spleen were characterized by myeloid hyperplasia and an increase in erythroid and myeloid precursors was observed in the spleen [201]. All these models of the disease clearly suggested the causative role of JAK2 mutation in the initiation and progression of MPNs and added new knowledge to the weight of JAK2-V617F presence in developing PV rather than PMF or ET.

Recently, a new model of MPN, namely JAK2^{V617F} KI mouse, has been created using the FLEx switch strategy, first described by Schnutgen et al, [202]. This murine model carries a transgenic construct in one of the JAK2 alleles, which allows to turn off the expression of the wild type gene and to turn on the mutated one through a Cre-mediated recombination. In this way, the mutated allele is under the control of the endogenous promoter and it is expressed in heterozygosity at physiological levels. JAK2^{V617F} KI mouse is, thus, crossed with a *Vav-Cre* transgenic mouse, allowing the induction of the mutation only in Vav-expressing hematopoietic cells, finally mimicking the PMF patient condition. This MPN mouse model has been recently generated and characterized [203]; it shows mainly PV-like phenotype with severe thrombocytosis and leukocytosis since birth. At two to three months of age hematocrit values are strongly increased (WBCs, RBCs, Plts) and hematopoietic tissues become abnormal, showing an increase in percentage of granulocytes both in BM and spleen that, as a consequence, undergoes splenomegaly. In aged mice (from 6 months on) high-grade fibrosis in BM and spleen, as well as EMH in the liver are observed [203].

In addition to canonical mouse models bearing JAK2 mutation, the strategy of BM transplantation (BMT) was also applied to generate MPL mouse model of ET and PMF, where BM cells were transduced with retroviral vector carrying the MPL-W515L mutated TpoR. The phenotype of this mouse showed characteristics comparable to PMF and ET patients, as thrombocytosis, leucocytosis, EMH and more importantly reticulin fibrosis [179]. Hematopoietic cells bearing MPL-W515L mutation were also able to activate TpoR downstream signalling pathways, as STAT3 and STAT5, PI3K-AKT and MAPK [179].

3.4 Pharmacological treatments

PMF is a rather rare disease. The prevalence in the population is 0.5 per 100.000 people per year [204] and affects prevalently adult people over 60 years of age. Nowadays there is no resolutive therapeutic treatment for this pathology, with the exception of allogenic stem cell transplant (ASCT), even if this treatment is associated to high rate of mortality and morbidity [205]. Paliative therapy aimed to cure constitutional sympthoms and to improve the quality of life is currently administered to patients. Conventional therapies consist in the administration of cytolytic drugs as hydroxyurea (HU) that represents the treatment of choice for splenomegaly, or agents to ameliorate anemia, for example Epo or androgens. Interferon has been used in the past but with limited efficacy and heavy side effects. Moreover, none of these medical cares showed to induce fibrosis remission and ameliorated median overall survival (OS) [192].

3.4.1 Ruxolitinib and JAK2 inhibitors

Given that half of PMF patients carry JAK2-V617F mutation, in the last few years a lot of effort was spent to develop specific JAK2 inhibitors. Ruxolitinib (formerly known as INCB018424) is an oral inhibitor of JAK1 and JAK2. In preclinical models ruxolitinib showed the *in vitro* inhibition of both JAK1 and JAK2 activity as well as the inhibition of IL-6 mediated stimulation. Moreover, the drug was able to reduce cellular growth and to induce apoptosis in the hematopoietic Ba/F3 cell line carrying the JAK2-V617F mutation [206]. Mouse models of MPNs with V617F mutation in JAK2 treated with ruxolitinib resulted in reduced spleen size and increased survival [206]. Taken into consideration these encouraging results, the drug was then approved for clinical studies. Phase I/II human studies determined the maximum tolerated dose (MTD) to be administered to patients (15mg *per os* two-times a day); the treatment showed reduction in splenomegaly coupled with survival advantage, improvement of disabling symptoms and decrease in circulating cytokine concentration. Two big phase III studies namely “Controlled Myelofibrosis

Studies with Oral JAK inhibitor Treatment" (COMFORT-I and –II) confirmed results versus placebo obtained in the Phase I/II and led to the final approval of ruxolitinib by the american Food and Drug Administration (FDA) for the treatment of IPSS intermediate and high-risk PMF patients[207].

Based on the good results obtained by ruxolitinib several additional small molecules have been development in order to target JAK2 and are currently being tested in clinical trials [208]. The complete list of JAK2 inhibitors that have been testing are listed in Table 3.

JAK inhibitor	Target	Disease studied	Toxicity
Ruxolitinib	JAK1/2	PMF, post-ET/PV MF, PV, ET	Cytopenias –predominately anemia (23%), diarrhea
SAR302503 * (fedratinib)	JAK2, FLT3	PMF, PV, ET	Wernicke's encephalopathy
SB1518 (pacritinib)	JAK2, Tyk2	PMF, PV, ET	GI toxicity – diarrhea, transaminitis
CYT387 (Momelotinib)	JAK1/2, Tyk2	PMF, Post ET/PV MF, PV, ET	Hyperlipasemia (20%), thrombocytopenia
CEP701 * (lestaurtinib)	JAK 2/3, FLT 3	ET, PV, PMF	GI toxicity – diarrhea, vomiting
XL019 *	JAK2	PMF	Central/peripheral neurotoxicity
LY2784544	JAK2	PMF, ET, PV	Elevated creatinine (8%)
BMS-911453	JAK2	PMF	Cytopenias – anemia (3%)
NS-018	JAK2	PMF, post-ET/PV MF	
AZD1480	JAK1/2, Aurora-A TRKA, FGFR1, FLT4	PMF, post-ET/PV MF	
INCB039110	JAK1	MF	Anemia, thrombocytopenia, nausea

*No longer in development.

Table 3: JAK2 inhibitors in MPN development

List of all JAK2 inhibitors currently tested for the treatment of MPNs *(from Rosenthal & Mesa, 2014).*

In 2010 a study performed on MPL-W515L mouse model assessed the efficacy of INCB16562, a novel and selective small molecule inhibitor of JAK1/JAK2 whose great efficacy in shutting down cytokine-independent JAK/STAT signalling and cell proliferation was already tested on multiple myeloma [209]. INCB16562 was able to inhibit proliferation and STAT signalling in JAK and MPL mutated cells. Moreover this drug improved survival, normalized white blood cells and platelets range and strongly reduced EMH and BM fibrosis. Unfortunately, after the treatment a clear reduction in the BM malignant clone was not recorded, suggesting that anti-JAK therapy alone is not sufficient to completely eradicate the disease [210].

Although the improvement in MPN-associated splenomegaly and systemic symptoms obtained using JAK2 inhibitors, a proportion of patients are not responsive, thus suggesting that a possible mechanism of resistance to JAK inhibitors could take place. It has been hypothesized that the lack of response or clinical improvement may be due to a phenomenon of persistence more than of resistance. The JAK2 inhibitor persistence is due to reactivation of JAK-STAT pathway because of the ability of activated JAK2 to heterodimerize with JAK1 or TYK2 in cell line, murine model and PMF patients that have been previously treated with the inhibitor [211]. This data is consistent with the described activation of JAK2 *in trans* by other JAK kinases. The persistence seems however to be reversible, since the interruption of the use of ruxolitinib is for example associated with a re-sensitization of the tyrosine kinase [211].

In addition, polimorfisms able to induce resistance to ruxolitinib and cross-resistance to other JAK inhibitors have recently been described, thus implicating the need of new strategies for the design of compounds able to avoid resistance side effects.

3.5 Endothelium involvement in PMF

Despite being a hematopoietic disease, some reports show that among MPNs only PMF exhibits an endothelial phenotype very poorly characterized so far [212,213].

In condition of inflammation, accumulating evidence indicates the importance of EPC mobilization [74]. In PMF patients it has been demonstrated that the average percentage of EPCs is significantly higher than in the healthy controls and in other MPNs [213].

Moreover the number of circulating EPCs directly correlates with the phase of the disease: the higher is the mobilization, the earlier is the PMF stage [213]. So far the cause of this massive mobilization from BM to the circulation is still not known, but the presence of a strong pro-inflammatory microenvironment, enriched in TGF-β, PDGF, G-CSF in the BM of patients with myelofibrosis is supposed to promote exaggerated proliferation and

mobilization of endothelial progenitors into the bloodstream and to extramedullary sites as spleen and liver.

Another endothelial-related key feature of myelofibrotic patients is the massive neo-angiogenesis observed in BM [214,215] and spleen [212]. Angiogenesis, which consists in the formation of new vessels from pre-existing ones, has a pivotal role in tumor progression and metastasis to mainly convey nutrients to tumoral cells sustaining their growth. For this reason increased BM microvessel density has been reported in different hematological neoplasms [216]. Patients with PMF show indeed higher concentration VEGF [217] and FGF-2 [218], well-known angiogenic factors.

Interestingly, in 2009 Sozer et al. [219] reported for the first time the ECs of the lumen of hepatic venules harbored JAK2-V617F mutation in three Budd-Chiari syndrome patients, a disease characterized by the occlusion of the hepatic vein, who later on also developed PV. This finding hypothesized that if the EC shares the same identical mutation found in the fully differentiated hematopoietic cells, it is reasonable to think that both are originated from the common progenitor of the two lineages, the hemangioblast, that carries the mutation and it is the target of the malignant transformation. Later on, in 2013, a recent study [220] has further demonstrated the presence of the JAK2-V617F mutation in ECs of PMF patient spleen vasculature, both in capillaries and in large vessels. The origin of the JAK2-V617F positive ECs is still debated and different hypothesis have been raised; among them, the existence of angiogenic monocytes capable to acquire EC phenotype in condition of neo-angiogenesis or the already cited possibility of a common mutated progenitor with the myeloid lineage that transmits the V617F mutation. However, the same group reported that endothelial-colony forming cells (ECFCs) both circulating or resident in the spleen lack the mutation [221]. Conversely, Teofili and co-workers [222] demonstrated the presence of JAK-V617F mutation in ECFC only from a subset of MPN patients with thrombotic events.

All together these data highlight an involvement of the endothelial compartment in the PMF phenotype. The hypothesis that both BM and splenic ECs and their progenitors could be exposed to the pro-inflammatory stimuli and may actively participate to induce malignant transformation and possibly fibrosis is also suggested, even if the clear contribution of the endothelium has not deeply investigated yet.

MATERIALS AND METHODS

4.1 Tissue samples from PMF patients

BM, spleen tissue samples and EPCs from peripheral blood of PMF patients were collected at the IRCCS Policlinico San Matteo (Pavia, Italy) under a protocol approved by the institutional review of the institute. The diagnosis of PMF (previously also defined as myelofibrosis with myeloid metaplasia MMM or idiopathic myelofibrosis ID) was established according to World Health Organization (WHO) criteria and the Italian Consensus Conference criteria [223]. Healthy controls consisted in staff members or donors for scientific research. All donors and patients approved and signed the informative consensus.

4.2 Mice

C57B/6 mice between 8 and 12 weeks of age for MK and platelet purifications were purchased from Charles Rivers Laboratories, US.

BM and spleen sections from the following MPN mouse models were a kind gift from prof. AlessandroVannucchi (University of Florence, Italy):

- *JAK2* $^{FLEX/WT}$ *KI* mouse. A mutated exon 13 (including the GTC>TTC mutation coding for the JAK2^{V617F} mutation) was introduced in inversed direction (untranslated) into intron 13 of JAK2 murine gene through homologous recombination in embryonic stem (ES) cells. The mutated exon, upstream the WT exon 13 was surrounded with a Lox511 and a LoxP site. A further LoxP site and a Lox511 site were also introduced downstream to the WT exon 13. In absence of recombination, this mouse model expresses JAK2 WT.

- *JAK2* $^{V617F/WT}$ *KI* mouse. Due to the particular inverted or similar orientations of the LoxP and Lox511 sites in the *FLEX* construct, after crossing the *JAK2* $^{FLEX/WT}$ *KI* mice with *Cre-expressing* mice, Cre recombination results in flipping of the mutated exon, which became

translated, and flipping of the WT exon 13, which became reversed and then excised (Figure 15). *Vav-Cre expressing* mice were crossed with *JAK2* $^{FLEX/WT}$ *KI* mice to allow the expression of JAK2-V617F mutation in heterozygosity only in hematopoietic VAV-expressing cells. These mouse models were described in details in Hasan et al, 2013 and in the introduction paragraphs (see 3.3 Mouse models of MPNs).

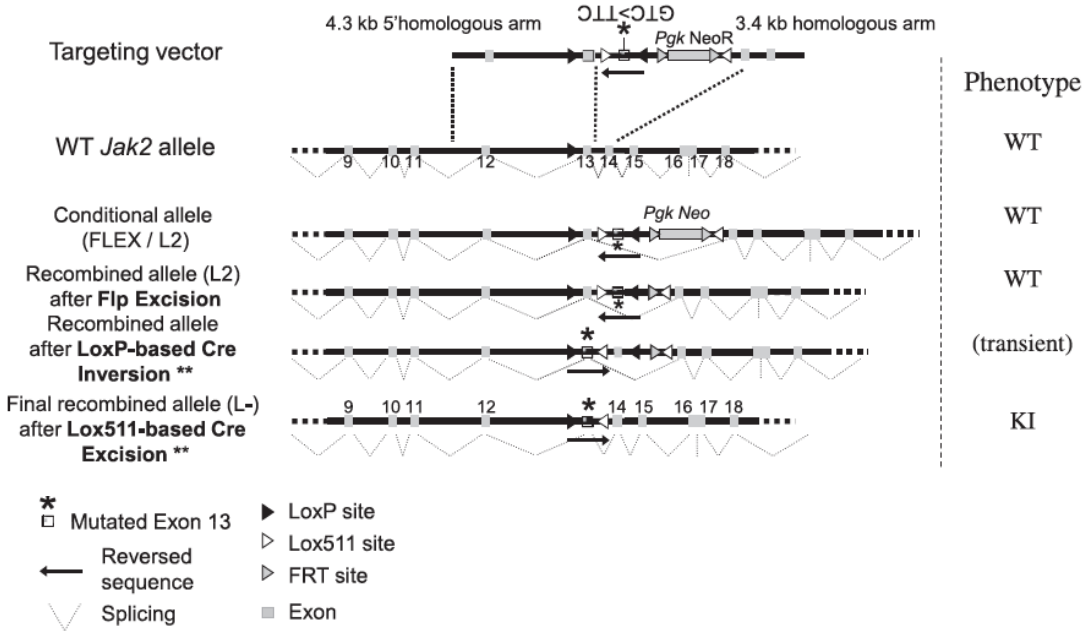

Figure 15: Schematic representation of the targeting vector and the resulting modified allele to generate the JAK2$^{V617F/WT}$ (L-) KI mouse

The targeting vector carrying the mutated exon 13 was introduced into the JAK2 WT allele of mouse ES cells through homologous recombination resulted into the FLEX/L2 conditional allele. The targeted ES clone was injected into embryos to generate L2 chimeric mice. The mice were first bred with *flipase* (FLP recombinase) transgenice mice to remove PgkNeoR cassette from the progeny and subsequently with *Cre-expressing* transgenic mice. *Cre* recombination induced LoxP site-directed inversion of a KI construct, resulting in the Lox511-directed excision of the WT exon13 and transcription of the mutated exon13 into the G1849U-mutated mRNA that results into the JAK2^{V617F} protein translation (KI phenotype, named L-) (*adapted from Hasan et al, 2013*).

4.3 Cells

Phoenix packaging cells were provided by IFOM Cell Culture Facility and cultured in DMEM medium (Gibco) supplemented with South American Fetal Bovine Serum (10%, HyClone) and L-glutamine (2 mM, Sigma Aldrich).

4.3.1 Isolation and culture of Late ECFCs

Late Endothelial Colony Forming Cells (Late ECFCs) from PMF patients and healthy donors were isolated and cultured according to protocols already described [224]. Briefly, mononuclear cells from PMF patients and healthy donors' peripheral blood were obtained by density gradient centrifugation by layering diluted blood (1:1 with PBS) onto Ficoll solution. After 30 min centrifugation at 400 g, RT, mononuclear cells were recovered at the interface between Ficoll and plasma, washed twice with PBS and resuspended in EGM-2 MV medium (Lonza) supplied with EGM-2 MV Single Quots, supplemented with Fetal Bovine Serum (5%), hydrocortisone, human fibroblast growth factor (hFGF), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), ascorbic acid, human epidermal growth factor (hEGF) gentamicin sulfate/amphotericin-B (GA-1000) onto collagen I coated plates. The morphology of ECFC-derived colony was characterized by the formation of a cobblestone-appearing colony, made of a cluster of slightly elongated cells. In addition to the morphological appearance of the colonies, the faithful belonging of an ECFC-derived colony to the endothelial lineage was usually confirmed by staining with anti-CD31 (> 90%), anti-CD105 (> 90%), anti-CD144 (> 90%), anti-CD146 (> 90%), anti-vWf (> 90%), anti-CD45 (< 1%), and anti-CD14 (<1%) monoclonal antibodies (according to Ingram et al, 2004).

4.3.2 Isolation and culture of SECs

Endothelial cells of splenic origin (SECs) from wild type C57B/6 mice were isolated and immortalized as previously described [225]. Briefly, spleens from C57/B6 mice were

collected under sterile condition and disaggregated in presence of collagenase-A (1,5 mg/ml) and DNase (25µg/ml) to facilitate tissue fragmentation and incubated at 37°C for four hours. Cell suspension was then filtered through nylon screen (70µm), centrifuged and plated in 48-multi well plates. Two days after, the heterogeneous population of splenic cells plated was infected with polyoma middle T antigen (PmT) for eight hours in order to specifically select and immortalized ECs and then complete medium was changed. After 3 months in culture we obtained a homogeneous population of ECs. Cells were cultured on 0,1% gelatin-coated flasks in MCDB131 medium (Gibco) supplemented with North American Fetal Bovine Serum (20%), ECGS (50 ug/ml, HyClone), Heparin (100 ug/ml, Sigma-Aldrich), penicillin/streptomycin (100 units/L, Sigma-Aldrich) and L-glutamine (2 mM, Sigma-Aldrich).

Starving medium consisted in MCDB131 medium containing BSA (1%, EuroClone)

4.4 Antibodies and chemicals

The following reagents were used to stimulate and treat SECs: recombinant BMP6 (100ng/ml, R&D Systems), recombinant BMP4 (10ng/ml, R&D Systems), recombinant TGF-β1 (5ng/ml, Peprotech), recombinant IL-1β (100U/ml, Peprotech), recombinant TNF-α (100U/ml, Peprotech), LY2106791 (10µM, Selleck Bio), DMH1 (2µM, Tocris, R&D). Immunofluorescence stainings and western blots were performed using the following primary antibodies (Abs): VE-cadherin (IF: 1:200, WB: 1:200 Santa Cruz Biotech), CD44 (IF: 1:100, WB: 1:500 BD), CD45 (IF: 1:100, eBioscience), CD41a (IF: 1:100, eBioscience), N-cadherin (WB: 1:2500, BD), FSP1 (IF: 1:200, WB: 1:500, Millipore), claudin5 (IF: 1:500, Abcam), Fibronectin (WB: 1:1000, Abcam), αSMA-FITC (IF: 1:200, Sigma-Aldrich), Pan-TGF-β (IF: 1:100 R&D Systems), CD31 (IF: 1:400, Abd Serotec), JAM-A (IF: 1:5, BV12 surnatant, produced in our lab), Smad1, Smad3, p-Smad1 (WB: 1:1000, Cell Signaling), p-Smad3 (WB 1:500, Epitomics, IF 1:100 Santa Cruz Biotech), STAT3, STAT5, p-STAT3 and p-STAT5 (WB: 1:1000, Cell Signaling), JAK2 and p-

JAK2 (WB: 1:1000, Cell Signaling), AKT and p-AKT (WB: 1:1000 Cell Signaling), MAPK and p-MAPK (WB: 1:1000 Cell Signaling), Tubulin (WB: 1:2000, Sigma-Aldrich), Vinculin (WB 1:5000 Sigma-Aldrich).

4.5 RNA extraction and quantitative Real Time-Polymerase Chain Reaction

Total RNA was extracted with RNEasy Kit from QIAGEN following manufacturer's instructions. 1 ug of RNA was reverse-transcribed with random examers using the High Capacity cDNA Archive Kit from Applied Biosystems. cDNA (5 ng) was amplified in triplicate with the TaqMan Gene Expression Assay from Applied Biosystems and the ABI/Prism 7900 HT Thermocycler. For any sample the expression level was first normalized to housekeeping genes 18S or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and then was determined with the comparative threshold cycle (C_t) method as previously described [41].

4.6 Retrovirus preparation and infection

Retroviral constructs MCSV-IRES(wt)/eGFP–empty, MCSV-IRES(wt)/eGFP–JAK2 and MCSV-IRES(wt)/eGFP–JAK2V617F were a kind gift from Professor Van Etten (Tufts-New England Medical Center, Boston, Massachusetts, USA). Preparation of the virus stocks was carried out in Phoenix packaging cells and SEC retroviral infection was performed as previously described [226].

4.7 Cell Sorting for GFP expression

Equal number of SECs previously infected with MCSV-IRES(wt)/eGFP–empty, MCSV-IRES(wt)/eGFP–JAK2 and MCSV-IRES(wt)/eGFP–JAK2V617F vectors were sorted according to GFP expression using MoFlo Astrios Beckman Coulter. GFP was excited at 488nm and emitted fluorescence was detected at 513/516nm. After identification of GFP-negative control cells, standard rectangle gates were then set to differentiate GFP-high and

GFP-low expressing cells for each of the three infected cell line. Specifically, 3 gates were defined: 10^2 log (GFP-A)< GFP-negative cells < 10^3 log (GFP-A); 10^3 log (GFP-A) < GFP-low expressing cells < 10^4 log (GFP-A); 10^4 log (GFP-A) < GFP-high expressing cells < 10^5 log (GFP-A).

GFP-low expressing cells were then collected and characterized for further experiments.

4.8 Western Blot

Total proteins were extracted by solubilising cells in boiling Laemli buffer. Lysates were incubated for 10 minutes at 100°C and then spinned for 1 minute at 13000 rpm to discard cell debris. The supernatants were collected and protein concentration was determined using a BCA Protein Assay Kit (Pierce) according to manufacturer's instructions. Equal amounts of proteins were loaded on acrylamide gels at different concentrations separated by SDS-PAGE, transferred to a Protran Nitrocellulose Hybridization Transfer Membrane 0,2 um pore size and blocked for 1 hour at RT in 1X TBST containing 5% BSA or 10% LFM milk. The membranes were then incubated ON at 4°C with primary Abs diluted in 1X TBST containing 5% BSA. Membranes were rinsed at least 3 times in 1X TBST and then incubated with HRP-linked secondary Abs (Cell Signaling) for 1 hour at RT with 1X TBST containing 5% BSA. After three washes, membranes were incubated with Amersham ECL WB detection reagents (GE Healthcare) for 1 minute and exposed in a ChemiDoc gel imaging system (BIORAD) for the required time.

4.9 CD34+ isolation and MK differentiation from C57B/6 mice

BM cells were flushed from femurs of ten C57B/6 mice between 8 and 12 weeks of age and lineage negative cells purified with the Lineage Cell Depletion Kit, mouse (Miltenyi Biotech). Cells were then cultured for 4 days in DMEM (Gibco) supplemented with penicillin/streptomycin (1%), L-glutamine (1%), Fetal Bovine Serum (10%) (Gibco) and added with recombinant mouse Tpo (10ng/ml, Peprotech), as previously described [187].

MKs were then resuspended in Tyrode Buffer pH 7.4 plus $CaCl_2$ 2mM to obtain a final concentration of $0,2x10^9$ MKs/ml.

4.10 Platelet purification from peripheral blood of C57B/6 mice

Blood from C57B/6 mice between 8 and 12 weeks of age was collected from the inferior vena cava into buffered citrate-dextrose solution (ACD) 3,2% pH 5.2. Blood of 10 mice was pooled and diluted with 2 parts of calcium and magnesium free Tyrode Buffer pH 6.5 prior to 2x centrifugation at 300g for 7 minutes to obtain platelet rich plasma (PRP). PRP was further diluted in 2 parts of Tyrode Buffer pH 6.5, supplemented with Apyrase 0,6U/ml (New England BioLabs) and centrifugated at 600g for 15 minutes to obtain the platelet pellet. Platelets were then resuspended in Tyrode Buffer pH 7.4 plus $CaCl_2$ 2mM to obtain a final concentration of $0,2x10^9$ platelets/ml.

4.11 *In vitro* platelet and MK release reaction and acidification

Platelets or MKs were incubated with recombinant thrombin (Sigma-Aldrich) 2U/ml for 15 minutes at RT until release reaction had taken place by the formation of a visible clot. Recombinant hirudin (Sigma-Aldrich) 2U/ml was then added to for further 15 minutes inactivate thrombin. Additional centrifugation at 600g for 15 minutes led the separation of the supernatant (SN) to the pellet clot. The pellet was then resuspended in an equal SN volume of Tyrode Buffer pH 7.4 and both of them were then supplemented with BSA 0.5% and hydrogen chloride (HCl) 70mM. After 30 minutes sodium hydroxide (NaOH) 70mM was added to neutralize acid pH environment of the solutions. Aliquots of 300µl of SN or pellet were then directly incubated with cells in culture or conserved at -80°C for later uses.

4.12 ELISA for active TGF-β1

The levels of active TGF-β1 were measured using a TGF-β1 duo-set (DY240) with a

substrate reagent pack (DY999) according to the manufacturers' instructions (both R&D Systems Europe). Active TGF-β1 levels were determined by acid activation (1M HCl for 30 minutes at RT) of the latent TGF-β1 in the samples, as already described [227].

4.13 Immunofluorescence

4.13.1 Cells

The morphology of the cells before and after treatments was evaluated by Phase Contrast analysis through transmitted light using EVOSfl AMG microscope (EuroClone).

For immunofluorescence stainings, cells were cultured in 35 mm diameter petri or 15µ-slides (Ibidi) and fixed for 15 minutes in 4% paraformaldehyde (PAF) at RT or for 5 minutes in ice cold Methanol. Cells were then blocked with PBS containing 5% Donkey Serum and 2% BSA for 1 hour at RT. Only cells fixed in PAF were permeabilized with PBS containing 0,5% TritonX-100 for 5 minutes before blocking. Cells were then incubated for 1 hour at RT with primary Abs diluted in blocking buffer. Appropriate fluorofore-conjugated secondary Abs (Molecular Probes) were applied on cells for 1 hour at RT. After washes, sections were incubated with DAPI for 10 minutes at RT, post-fixed with 2% PAF and mounted with Vectashield (Vector Laboratories). Samples were analyzed under Leica TCS SP2 Confocal Microscope. Maximum projections from Z-stack images were obtained and only adjustments of brightness and contrast were used in the preparation of the figures using Photoshop software.

4.13.2 Tissue samples

Sections (5µm in thickness) from frozen BM and spleen biopsies were obtained using a Cryostat instrument and put on Superfrost ultra plus microscope slides (ThermoScientific). Slides were then fixed for 15 minutes in 4% PAF at RT, washed 3 times with PBS,

permeabilized with PBS containing 0,1% TritonX-100 for 5 minutes and blocked with PBS containing 5% Donkey Serum and 2% BSA for 3 hours at RT.

Murine paraffin-embedded BMs and spleens were processed at the microtome and put on microscope slides (ThermoScientific). Sections (5µm in thickness) were deparaffinized by descending concentrations of ethanols and antigen unmasking was performed in EDTA (0.25mM, pH 8.0) for 50 minutes at 95°C. After three washes in TBS, sections were blocked with TBS containing 5% Donkey Serum, 0.05% TritonX-100 and 2% BSA for 1 hour at RT.

Incubation with primary Abs on both cryo- and paraffin- sections was performed in blocking solution ON at 4°C. The day after tissue sections were washed at least 5 times before incubating with appropriate fluorofore-conjugated secondary Abs (Molecular Probes) for 3 hours at RT. After washes, sections were incubated with DAPI for 10 minutes at RT, post-fixed with 2% PAF and mounted with Vectashield (Vector Laboratories). Samples were analyzed under Leica TCS SP2 Confocal Microscope. Maximum projections from Z-stack images were obtained and only adjustments of brightness and contrast were used in the preparation of the figures using Photoshop software. ImageJ (NIH) was employed for data analysis.

Hematoxylin and Eosin (H&E) staining on paraffin-embedded section was performed according to standard protocol.

4.14 Statistical analysis

Statistical significance was evaluated using Student's two-tailed non paired *t*-test by setting the significance level at $p < 0.05$ or $p < 0.01$.

RESULTS

5.1 ECs from BM and spleen of PMF patients undergo EndMT

Despite being a hematopoietic disease, some reports show that among MPNs, PMF is also characterized by a strong endothelial phenotype [212-215], poorly investigated so far. Cardinal endothelial-related features of PMF include a higher mobilization of EPCs from the BM into the peripheral blood and a massive neo-angiogenesis in BM [214,215] and spleen of PMF patients [212]. Transcriptional profiling data performed on whole-blood of PMF specimens showed elevated levels of pro-inflammatory cytokines [188] and pro-angiogenic factors [217,218], thus suggesting an alteration in the overall microenvironment of BM niche that possibly leads to fibrosis, which is indeed the final result of chronic inflammatory reactions.

Although fibroblasts are directly implicated in fibrosis development, ECs could also play a role. Indeed, when activated by inflammatory cytokines such as TGF-β, ECs undergo EndMT and acquire fibroblastic features.

To test the hypothesis that also the endothelium could contribute to fibrosis development in PMF through an EndMT mechanism, we first analysed BM and spleen sections from PMF patients recluted at San Matteo Hospital in Pavia. Granulocytes of the same PMF patients were analysed to verify the presence of JAK2-V617F mutation.

Immunofluorescence (IF) analysis on BM showed that CD44, a typical EndMT/stem cell marker, was expressed in a subpopulation of ECs, stained with the endothelial-specific marker VE-cadherin (Figure 16a). Notably, the protein was mostly localized in single ECs or in small vessels rather than in ECs of big vessels (Figure 16b), suggesting a more plastic role of these cells undergoing EndMT.

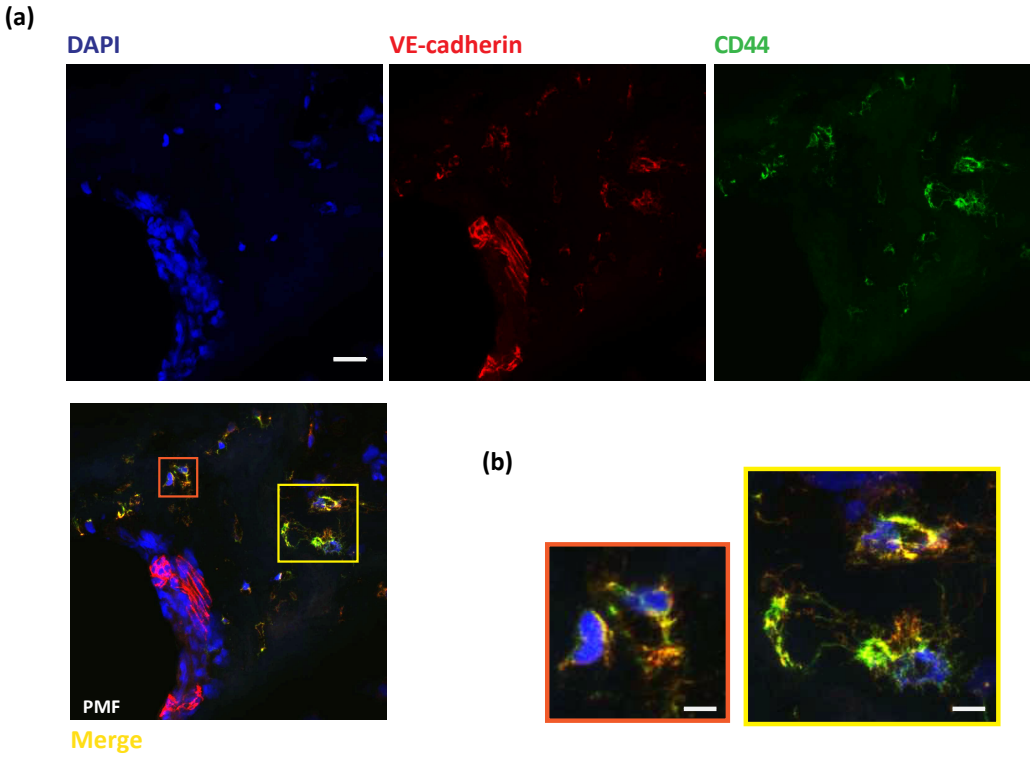

Figure 16: BM ECs of PMF patients express CD44

(a) BM sections derived from PMF patients recruited at San Matteo Hospital in Pavia co-stained with VE-cadherin (red) and the EndMT/stem cell marker CD44 (green). DAPI staining (blue) showed BM nuclei. Scale bar = 50µm. **(b)** Orange and yellow squares showed magnification of VE-cadherin/CD44 co-staining, mostly identified in small vessels or single cells. Scale bar = 10µm.

Due to the few availability of BM biopsies from both patients and healthy donors, we decided to analyze the spleen, the other organ involved in PMF where EMH takes primarily place in the disease and the BM hematopoietic niche is recreated, thus reflecting BM phenotype. Clinical features of PMF patients whose spleens were analysed are summarized in Table 4.

patient	diagnosis	JAK2-V617F	sex	therapy	HU	ruxolitinib
# 1	post PV/ET MF	yes	M	yes	✓	
# 2	PMF	no	M	no		
# 3	pre-MF	yes	F	no		
# 4	PMF	no	M	no		
# 5	pre-MF	no	M	yes	✓	
# 6	PMF	yes	F	no		
# 7	post PV/ET MF	nd	M	yes		✓
# 8	post PV/ET MF	yes	M	no		

Table 4: Clinical features of patients analysed in the study

Spleen sections from eight cases of Philadelphia chromosome MPNs with primary myelofibrosis (PMF) or prefibrotic myelofibrosis (pre-MF) or post PV/ET secondary myelofibrosis (post PV/ET MF) diagnosed at IRCCS Policlinico San Matteo (Pavia, Italy) were analysed in this study. For seven out of eight patients the presence of JAK2-V617F mutation in the hematopoietic lineage was evaluated in the granulocytes isolated from peripheral blood. Before splenectomy, three out of eight patients had been following a therapeutic treatment, including HU and ruxolitinib. In the following IF experiments JAK2 wt will refer to patients #2, #4, #5 while JAK2-V617F to patients #1, #3, #6, #8. HU= hydroxyurea, nd= not determined.

We first confirmed by IF staining a significant hypervascularization of the spleen in PMF patients compared to controls (Figure 17a), as already described in literature [212]. Quantification of vessels performed on the tissue sections from both patients and controls showed significance statistical difference ($p<0.01$) between the number of vessels in all the healthy donors and PMF patients per field analysed (Figure 17b).

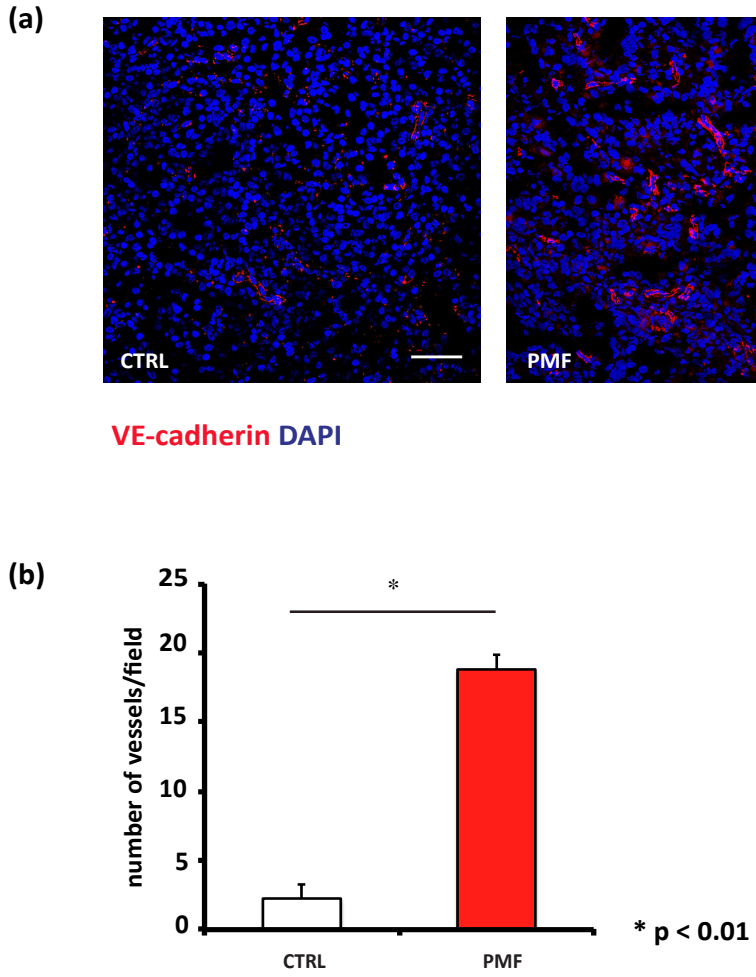

Figure 17: Spleen of PMF patients is hypervascularized

(a) IF analysis of spleen sections obtained from healthy donors (CTRL) and patients (PMF) showing splenic vessels stained with VE-cadherin (red). DAPI staining (blue) marked nuclei. Scale bar = 50µm. **(b)** Quantification of the total number of vessels per field analysed. CTRLs n=2, PMFs n =6.

To further prove the possible EndMT phenotype in the ECs of the spleen vessels of PMF patients, we checked for the expression of EndMT markers. Spleen sections showed that respectively the mesenchymal and stem cell markers FSP1 (Figure 18a; magnification in 18b) and CD44 (Figure 18c) were co-expressed with VE-cadherin in the endothelium of PMF specimens; we also osberved a diffused expression of the mesenchymal marker αSMA (Figure 18d) in PMF spleens in comparison to healthy controls.

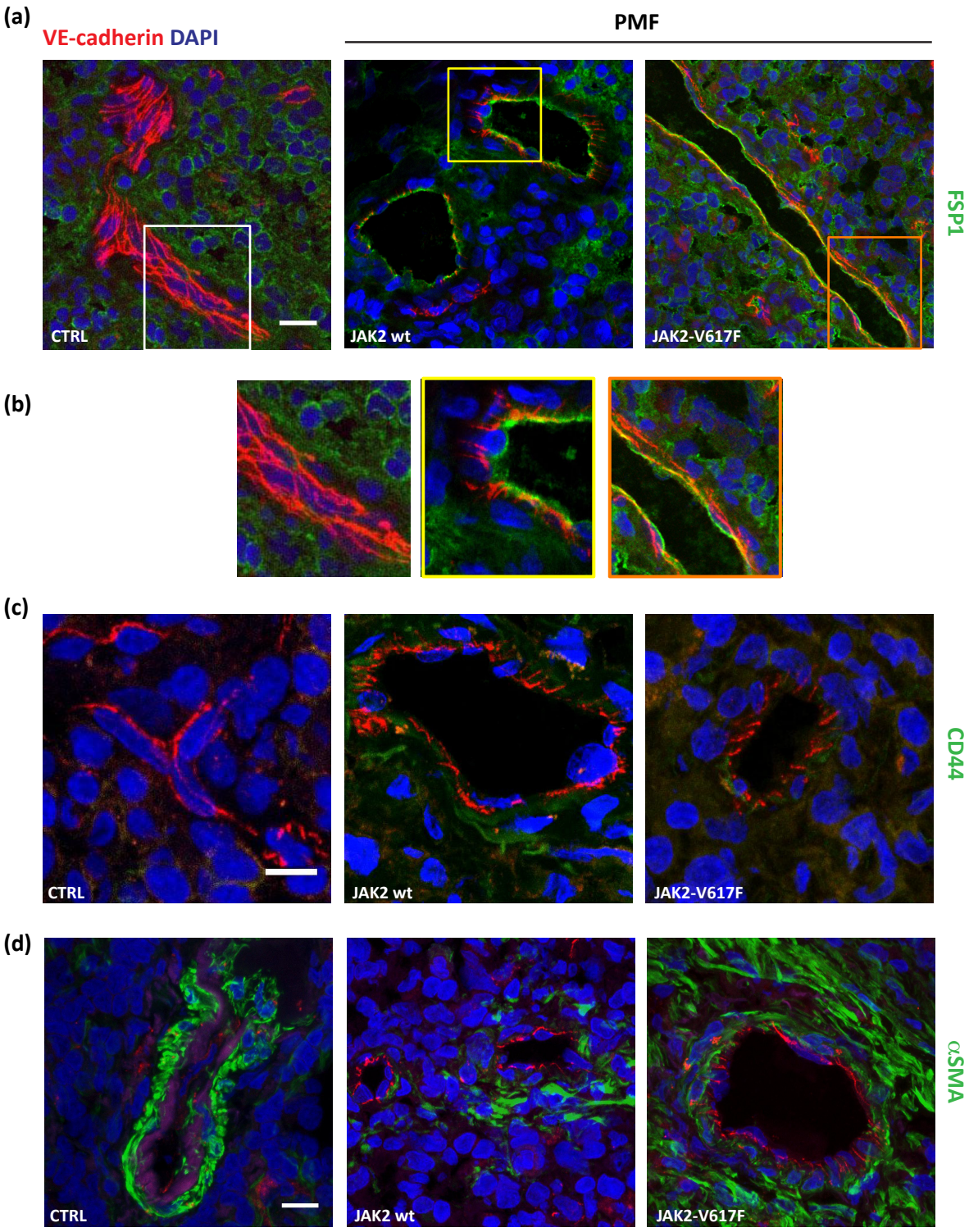

Figure 18: PMF splenic ECs exhibit an EndMT phenotype

IF staining for **(a)** FSP1 (green), magnification in **(b), (c)** CD44 (green) and **(d)** αSMA (green) detected in spleen sections of both healthy donors (CTRL) and PMF patients (PMF). ECs were stained with VE-cadherin (red). DAPI staining (blue) marked nuclei. Scale bars: (a) = 25µm, (b) = 10µm, (c) = 8µm, (d) = 20µm. CTRLs n=3, PMFs n =8.

αSMA is typically expressed by perycites and smooth muscle cells, enwrapping the endothelium to favour vessel stabilization. In healthy donors, αSMA was properly localized around the vessels while in PMF spleen it was widely expressed in the tissue, also far from the vessels, suggesting that an extended region of fibrosis was also mediated by increased number of myofibroblasts. Of note, patients carrying or not the JAK2-V617F mutation in the hematopoietic lineage were similarly able to undergo EndMT, thus suggesting that the acquisition of the phenotype was not influenced by the hematopoietic compartment but by the microenvironment.

In both physiological and pathological conditions, one of the main EndMT inducer is represented by the growth factor TGF-β, whose blood levels were shown to be increased in PMF [185].

These data were consistent with the observation that also the spleen sections from PMF patients presented higher amounts of TGF-β production detected by IF staining (Figure 19a), thus suggesting that ECs of PMF splenic vessels could undergo EndMT because of the increased local concentration of TGF-β.

Remarkably, the presence of the JAK2-V617F mutation in the hematopoietic lineage showed a trend in the increase of TGF-β production by splenic cells in comparison to patients JAK2 wt, as shown by the quantification (Figure 19b).

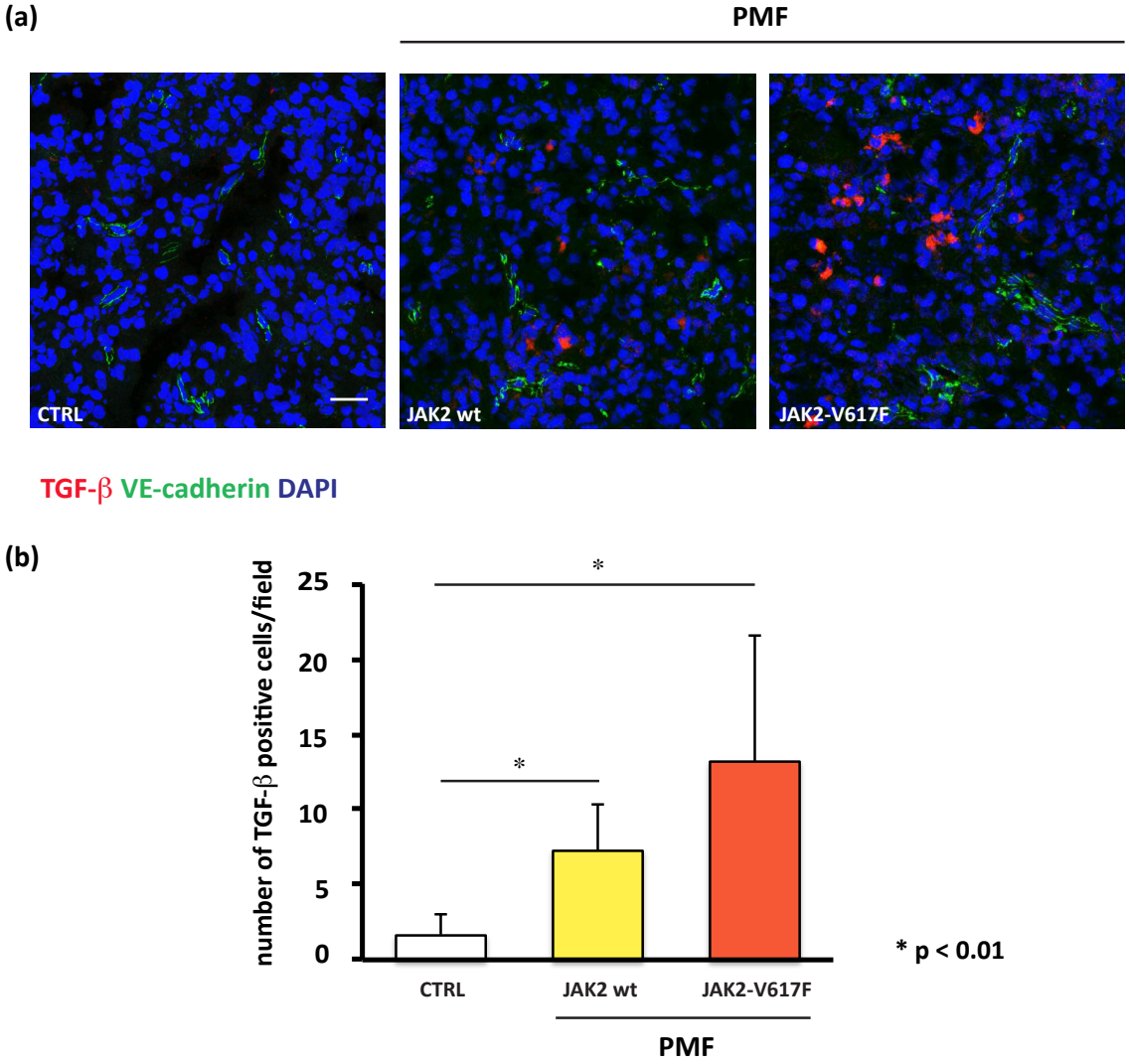

Figure 19: PMF spleens express higher amount of TGF-β

(a) IF staining of TGF- β revealed by a specific antibody (red) on spleen section of PMF patients (PMF) and healthy donors (CTRL). Vessels were stained with VE-cadherin (green) and nuclei with DAPI (blue). Scale bar = 20µm. **(b)** Quantification of the number of TGF-β positive cells per field analysed between healthy donors and PMF patients JAK2 wt ($p < 0.01$) or between healthy donors and JAK2-V617F mutated patients ($p < 0.01$). CTRLs n=3, PMFs n =8.

To confirm that the EndMT phenotype that we observed in PMF spleens was due to an increased concentration of TGF-β, that is able to activate its downstream signalling pathways in ECs, we evaluated the phosphorylation of Smad2/3 molecules in both PMF and healthy donor splenic ECs, as a read out of the activation of the TGF-β pathway. As detected by IF, an increased amount of the phosphorylated form of Smad2/3 (p-Smad2/3) molecules were detected in the EC nuclei of PMF specimens compared to the controls

(Figure 20a, magnification in 20b). This nuclear enrichment was statistically significant for either PMF patients carrying or not JAK2-V617F mutation in the hematopoietic lineage versus healthy donors, assessed by the quantification (Figure 20c). As expected, TGF-β released in the tissue induced the phosphorylation of Smad2/3 not only in ECs but also in other cell types, both in healthy donors and patients.

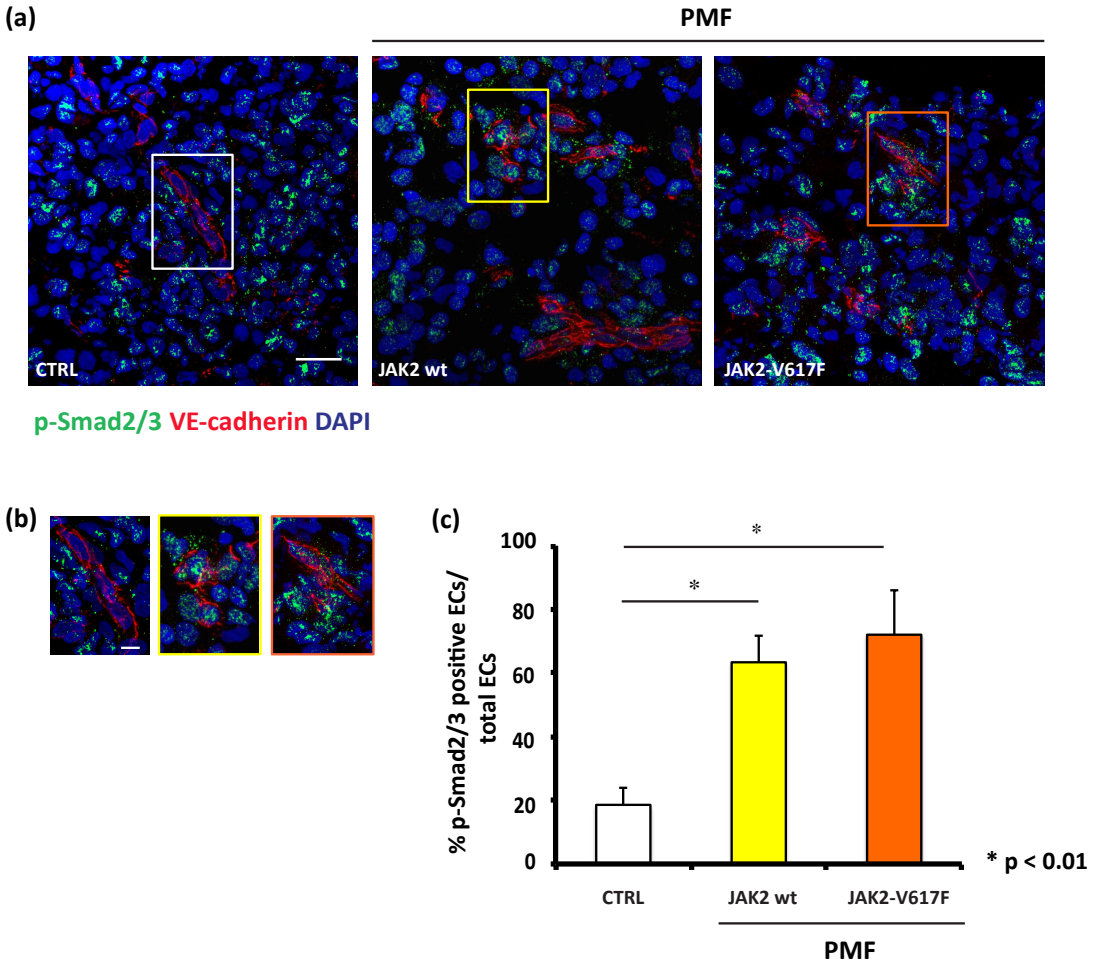

Figure 20: Smad2/3 are more phosphorylated in EC nuclei of PMF spleens than of healthy controls

(a) IF analysis for the phosphorylated form of the TGF-β downstream signalling molecules Smad2/3 (green) on splenic tissues from PMF patients (PMF) and healthy donors (CTRL). ECs were stained by VE-cadherin (red). DAPI staining (blue) marked nuclei. **(b)** Magnification of EC nuclei positive for p-Smad2/3 highlighted by coloured rectangles. Scale bars: (a) = 30µm, (b) = 10µm. **(c)** Quantification of the percentage of ECs expressing p-Smad2/3 in the nuclei for CTRL, PMF JAK2 wt or JAK2-V617F mutation. CTRLs n=3, PMFs n =8.

Moreover, to rule out a possible hematopoietic origin of the cells undergoing EndMT, we verified that ECs stained with VE-cadherin were totally negative for the pan-leukocyte marker CD45 (Figure 21) both in healthy donors and patients.

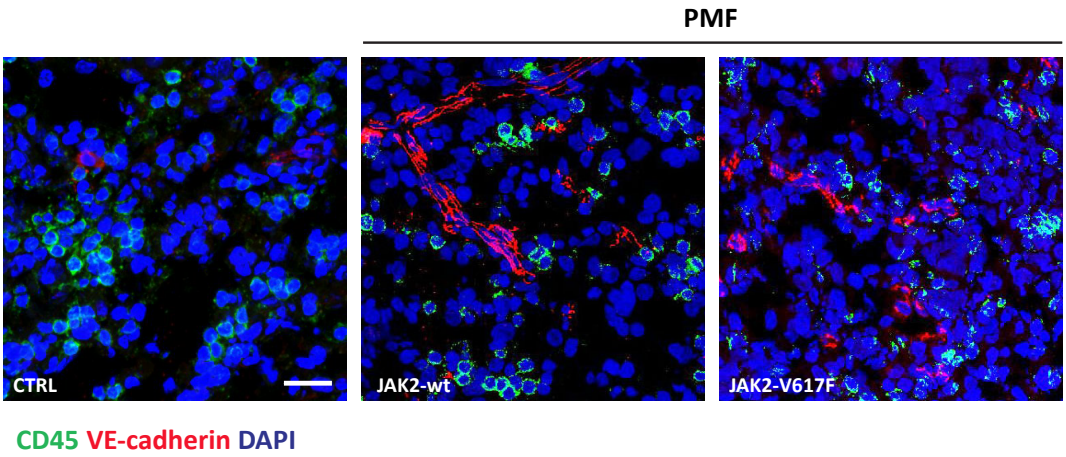

Figure 21: Splenic ECs do not express the hematopoietic marker CD45

IF staining for VE-cadherin (red) and CD45 (green) in splenic samples of both healthy donors (CTRL) and PMF patients (PMF). DAPI staining (blue) marked nuclei. Scale bar = 30µm. CTRLs n=3, PMFs n =8.

Overall, these data demonstrated that BM and spleen tissues of PMF patients can undergo EndMT switch; the higher nuclear amount of p-Smad2/3 in EC and the presence of higher concentration of TGF-β in the PMF tissue in comparison to healthy donors suggest that in PMF patients the EndMT associated to the pathology could be induced by an hyper-activation of the TGF-β signalling pathway.

5.2 EPCs and SECs develop EndMT in inflammatory environment

In order to understand the possible contribution of circulating primary EPCs to the EndMT phenotype, we isolated them from healthy donors and PMF patients. EPCs are generated in huge amount in PMF BM and are supposed to be able to reach the spleen through the circulation, so we hypothesized that they could play an important role to induce fibrosis both in BM and spleen.

Moreover, we analyzed EPCs from patients carrying or not the JAK2-V617F mutation in the hematopoietic lineage to figure out a different behaviour among them.

EPCs were isolated from the peripheral blood, amplified in culture and characterized at San Matteo Hospital in Pavia (detailed protocol in Materials & Methods). To reproduce the pro-inflammatory environment of the PMF disease, EPCs were cultured and stimulated with a cocktail of cytokines including TGF-β, IL-1β and TNF-α known to be up-regulated in PMF patients [185,190,191]. IF stainings showed that cytokine-stimulated EPCs from both healthy donors and PMF patients promptly lost the specific endothelial markers claudin5 and VE-cadherin at cell-to-cell contacts (Figure 22a). Concurrently with the decrease of endothelial molecule expression, EPCs started to express the mesenchymal markers CD44, SNAIL, N-cadherin and FSP1. These data were also confirmed by qRT-PCR (Figure 22b) and Western Blot (Figure 22c). Moreover, the switch of EPCs toward a mesenchymal phenotype induced the cells to acquire an elongated spindle shape (Figure 22d).

Notably, EPCs from PMF patients with or without the JAK2 mutation or from healthy donors were similarly responsive to undergo EndMT upon exposure to the inflammatory cytokines, suggesting a more prominent role of the microenvironment than the genetic background.

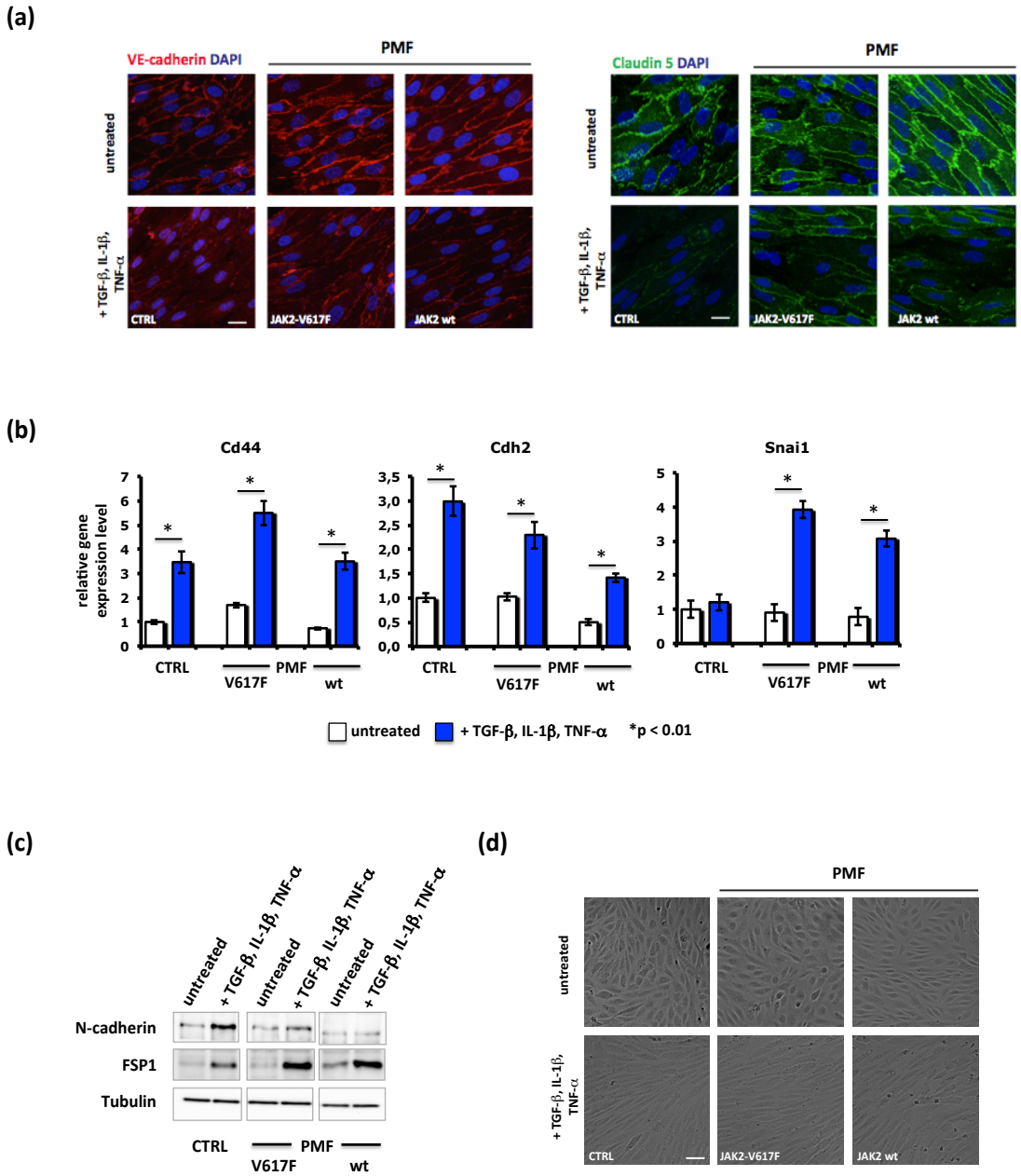

Figure 22: EPCs undergo EndMT in pro-inflammatory conditions

EPCs both from healthy donors and PMF patients treated with a cocktail of pro-inflammatory cytokines including TGF-β1 (5ng/ml), Il-1β (100U/ml) and TNF-α (100U/ml) for 72 hours. **(a)** Expression of the endothelial specific markers VE-cadherin (red) and Claudin5 (green) at cell-to-cell contacts in EPCs before and after the treatment. Scale bar =10µm. **(b)** Analysis of EPC samples for the mesenchymal markers CD44, N-cadherin (Cdh2) and SNAIL (Snai1) expression by qRT-PCR **(c)** and N-cadherin and FSP1 expression at protein levels by WB analysis. In **(b)** the levels of mRNA were normalized to GAPDH; columns are the means ± SD of triplicates from a representative experiment (n=3). In **(c)** tubulin was detected to verify equal loading of the samples. **(d)** Phase constrast analysis evaluating the morphology of EPCs before and after the cytokine treatment. CTRLs n=2, PMFs n =4.

Similar to BM, the spleen microenvironment presents high amounts of TGF-β and increased microvessel density in PMF patients. Furthermore, a recent publication has showed that splenic ECs of PMF patients could also express the JAK2 mutation [220]. This evidence prompted us to analyze ECs of splenic origin. Of note, in murine models of PMF also the spleen develops fibrosis, which is instead absent in human patients.

In order to obtain a suitable model to study the molecular mechanisms *in vitro,* we isolated and cultured ECs from the mouse spleen microvasculature and we developed an immortalized murine EC line of splenic origin, called Splenic ECs (SECs) (Figure 23).

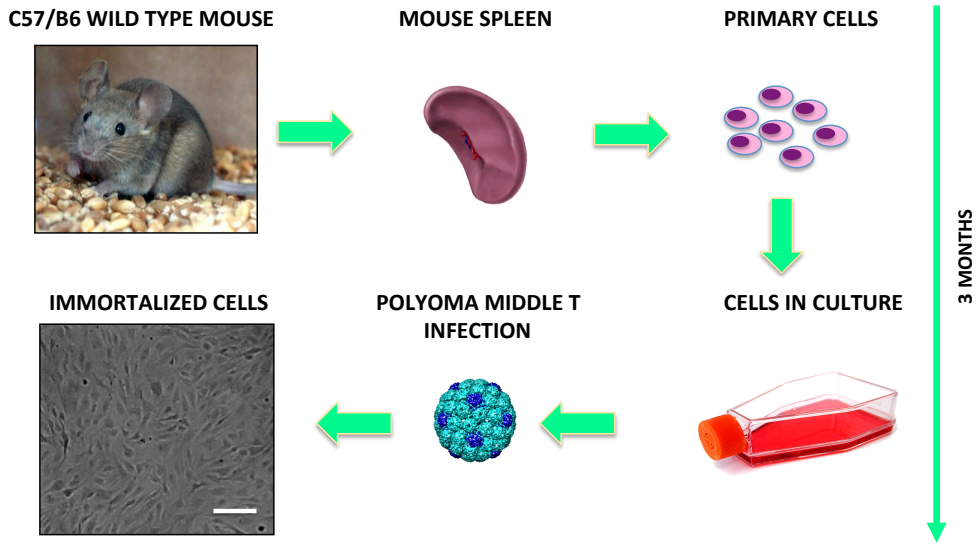

Figure 23: Experimental strategy to isolate and immortalize SECs

Five C57/B6 mice were sacrificed and their spleens collected. Crumbling of the tissues led to separate and collect primary cells, which were then pulled together and put in culture. After two days in culture, the heterogeneous population of splenic cells plated was infected with polyoma middle T antigen (PmT) for eight hours in order to specifically select and immortalized ECs. After three months in culture a homogeneous population of ECs of splenic origin was obtained and confirmed by the morphology of the cells evaluated by phase contrast analysis through transmitted light microscope. Scale bar = 30µm.

Morphological analysis (see phase contrast data in Figure 22) and characterization of the cells confirmed their endothelial origin, as they expressed the junctional molecules VE-cadherin, PECAM1 and JAM-A (Figure 24).

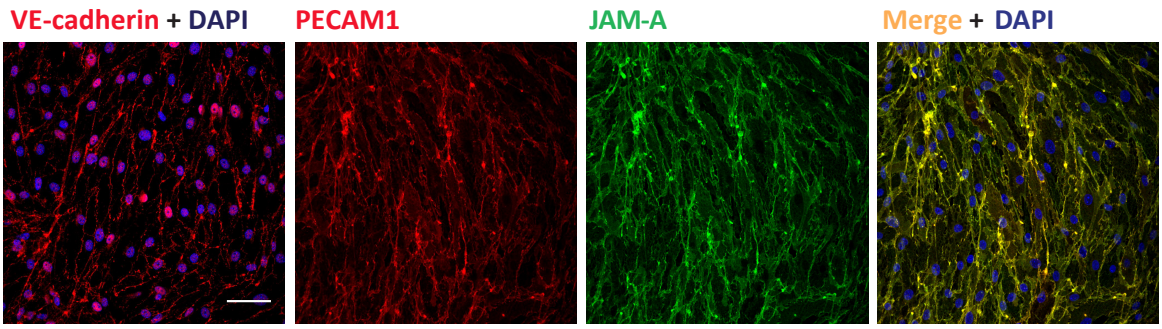

Figure 24: Immortalized SECs express endothelial junctional markers

IF analysis for the expression of junctional proteins in ECs of splenic origin (SECs) immortalized with PmT. SECs showed expression of the AJ components VE-cadherin (red, first panel on the left) and PECAM1 (red, second panel) and the TJ component JAM-A (green). DAPI staining (blue) marked nuclei. Scale bar = 30µm.

Then, we tested whether SECs were prone to undergo EndMT in a cytokine-enriched environment. As previously performed with EPCs, SECs were first stimulated with the TGF-β, IL-1β and TNF-α cocktail to mimic the pro-inflammatory environment. After the cytokine treatment, SECs acquired a spindle shape morphology (Figure 25a) and were able to undergo cadherin switch, a typical feature of EndMT, characterized by down-regulation of VE-cadherin and up-regulation of N-cadherin both at transcriptional (Figure 25b) and protein levels (Figure 25c).

Sustaining the EndMT phenotype, we found a down-regulation of endothelial markers claudin5 and PECAM1 (Fig.25d) and a up-regulation of the expression of a panel of mesenchymal markers, among which CD44, αSMA, fibronectin, vimentin, PAI-1 and the EndMT transcriptional factor SNAIL (Figure 25e). We also confirmed that the induction of these mesenchymal markers at transcriptional levels was paralleled by an increase at the protein levels as demonstrated in Figure 25f, showing an example of CD44 and fibronectin protein up-regulation.

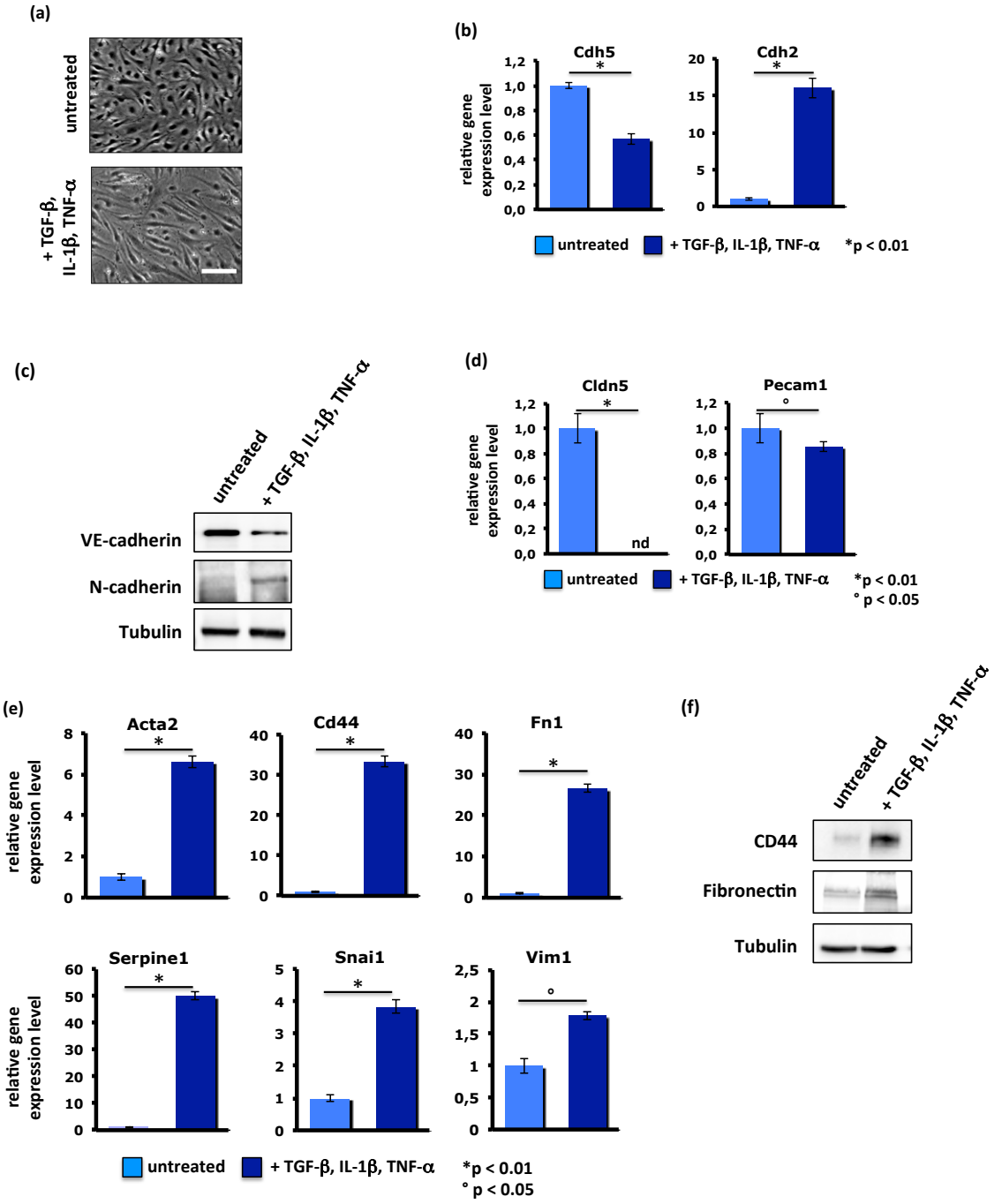

Figure 25: SECs show an EndMT phenotype when treated with a cocktail of pro-inflammatory cytokines

SECs were treated with recombinant TGF-β1 (5ng/ml), Il-1β (100U/ml) and TNF-α (100U/ml) for 120 hours in starving condition. **(a)** Morphological rearrangement before and after cytokine treatment was assessed by phase contrast analysis. Scale bar = 30µm. Evaluation of N-cadherin (Cdh2) and VE-cadherin (Cdh5) expression both at transcriptional **(b)** and protein **(c)** levels, analysed by qRT-PCR and WB respectively. mRNA levels of claudin5 (Cldn5) and PECAM1 (Pecam1) **(d)** and of αSMA (Acta2), CD44 (Cd44), fibronectin (FN1), PAI-1 (Serpine1), SNAIL (Snai1) and vimentin (Vim1) **(e)** were assessed by qRT-PCR. **(f)** WB analysis of CD44 and fibronectin protein expression in SECs treated with pro-inflammatory

cytokines. In **(b)**, **(d)** and **(e)** the levels of mRNA were normalized to 18s; columns are the means ± SD of triplicates from a representative experiment (n=3). In **(c)** and **(f)** tubulin was detected to verify equal loading of the samples; WB shown are representative of three independent experiments (n=3). In **(d)** nd = not detected.

Interestingly, among the genes up-regulated by the inflammatory cytokines, we found a significant increase in BMP4 and BMP6 transcripts (Figure 26). The BMPs are a group of heterogeneous ligands belonging to the TGF-β superfamily and their aberrant expression has been involved in different vascular disorders [228]. Specifically, BMP6 has been recently reported to be up-regulated by CCM1-null ECs to support the EndMT undergoing during the CCM vascular lesion development [168]. Furthermore, also BMP4 was demonstrated to be able to induce a conversion of vascular ECs into multipotent mesenchymal stem-like cells and push them through a mesenchymal phenotype involving an EndMT mechanism in FOP pathology [148].

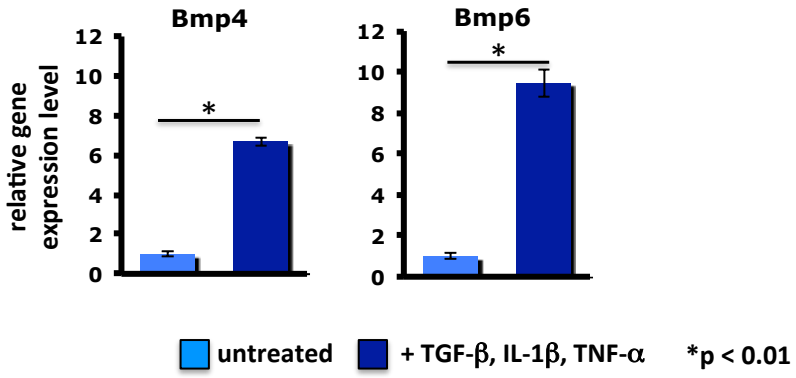

Figure 26: BMP4 and BMP6 are strongly induced by cytokine treatment

qRT-PCR analysis of BMP4 (Bmp4) and BMP6 (Bmp6) expression in SECs treated with recombinant TGF-β1 (5ng/ml), Il-1β (100U/ml) and TNF-α (100U/ml) for 120 hours in starving condition (1% BSA in MCDB131). The levels of mRNA were normalized to 18s; columns are the means ± SD of triplicates from a representative experiment (n=4).

So we hypothesized that both BMP6 and BMP4 induced by the cocktail of pro-inflammatory cytokines could sustain EndMT phenotype in our cellular model. In order to prove this hypothesis, we stimulated SECs with recombinant BMP6, which was able to trigger the phosphorylation of both Smad1 (p-Smad1) and Smad3 (p-Smad3), similarly to the cocktail of cytokines (Figure 27a).

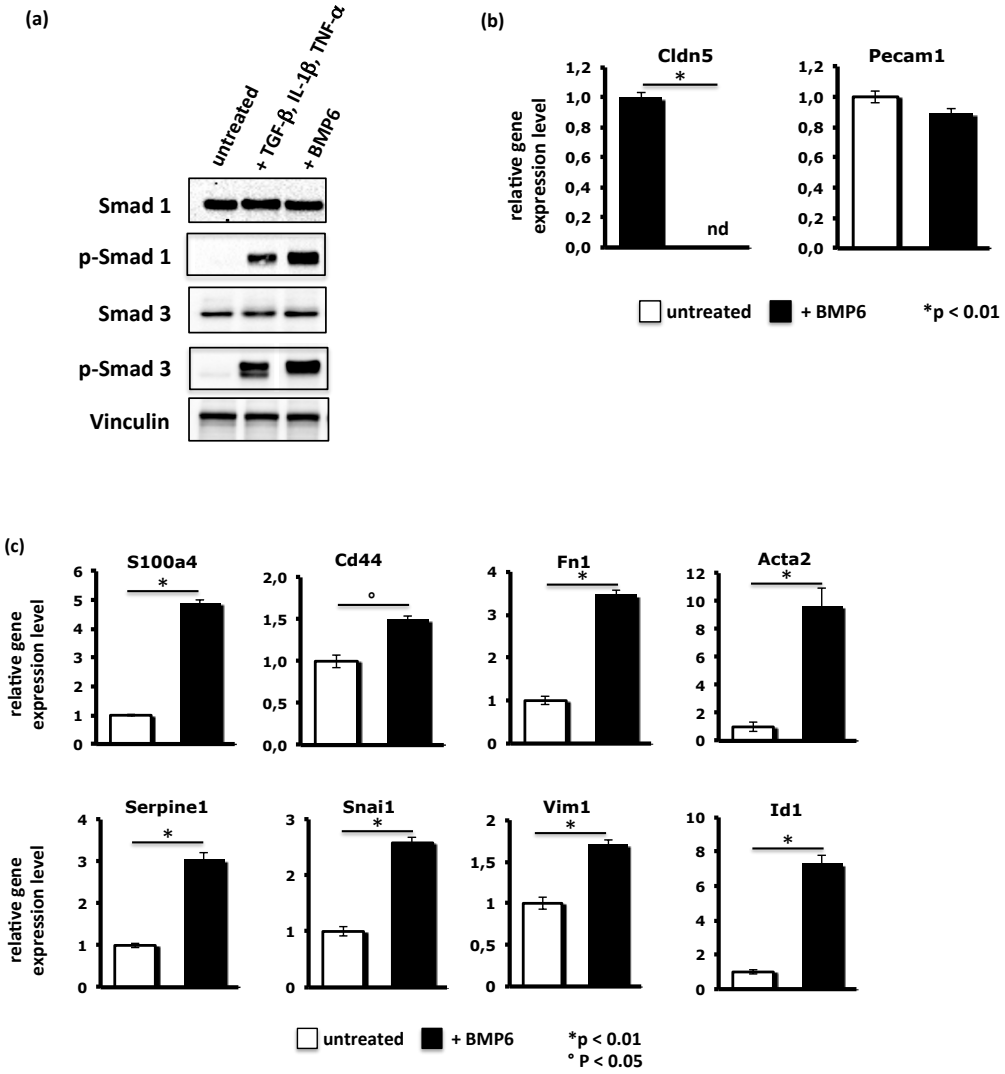

Figure 27: BMP6 treatment mediates Smad phosphorylation and EndMT markers induction in SECs

(a) WB showing the expression of total levels of Smad1 and Smad3 and their phosphorylated form (pSer463/465 and pSer423/425 respectively) in SECs lysates after the stimulation with TGF-β1 (5ng/ml), Il-1β (100U/ml) and TNF-α (100U/ml) or with BMP6 (100ng/ml) for 45 minutes. Vinculin was detected to verify equal loading of the samples. WB shown is representative of three independent experiments (n=3). **(b)** qRT-PCR analysis of endothelial claudin5 (Cldn5) and PECAM1 (Pecam1) transcripts and **(c)** EndMT markers FSP1 (S100a4), CD44 (Cd44), fibronectin (FN1), αSMA (Acta2), PAI-1 (Serpine1), SNAIL

(Snai1), vimentin (Vim1) and ID1 (Id1) transcripts in SECs after BMP6 treatment (100ng/ml) for 96 hours. In **(b)** and **(c)** the levels of mRNA were normalized to 18s; columns are the means ± SD of triplicates from a representative experiment (n=3). In **(b)** nd = not detected.

Then, we checked whether this Smad activation could induce the EndMT phenotype. We showed that SECs treated with recombinant BMP6 were able to down-regulate the expression of endothelial specific molecules, such as claudin5 and PECAM1 (Figure 27b) and concurrently up-regulate the same mesenchymal markers induced by the cocktail of pro-inflammatory cytokines, among which αSMA, CD44, fibronectin, SNAIL, PAI-1, vimentin, FSP1 (Figure 27c) together with its direct target ID1 [136].

We used the same approach with recombinant BMP4 and, even if the stimulation resulted in a mild increase in both Smad1 and Smad3 phosphorylation (Figure 28a) and ID1 expression (Figure 28b, blue bar), a significant up-regulation of αSMA and FSP1 transcripts was observed. Other EndMT features, like the down-regulation of claudin5 and the up-regulation of fibronectin, were not observed (Figure 28b, blue bars). BMP4-mediated induction of the EndMT markers was so less remarkable in comparison to BMP6-mediated phenotype (Figure 28a, Figure 28b, black bars), thus suggesting that BMP6 plays a more prominent role in the EndMT switch in SECs.

Overall, these data suggest that both the cellular model analysed (human EPCs and murine SECs) undergo EndMT in condition of pro-inflammatory environment. In particular, TGF-β, IL-1β and TNF-α induce Smad phosphorylation and consequently mediate the up-regulation of mesenchymal markers.

Moreover, TGF-β-mediated BMP6 induction could cooperate with the pro-inflammatory cytokines sustaining EndMT phenotype. On the contrary, the only presence of BMP4 was not sufficient to mediate a transition from an endothelial-to-mesenchymal phenotype at least in our cellular model, thus implying that it probably acts in a synergistic way with BMP6 and TGF-β to mediate EndMT.

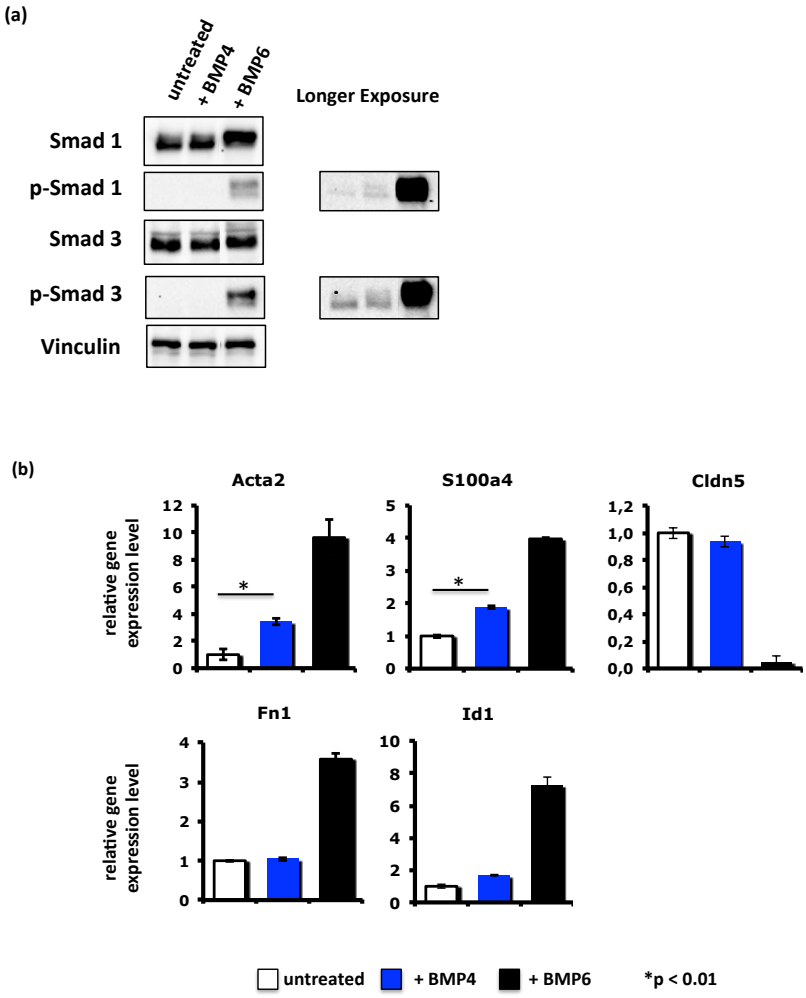

Figure 28: BMP4 does not induce the same EndMT phenotype fostered by BMP6

(a) WB showing the expression of total levels of Smad1 and Smad3 and their phosphorylated form (pSer463/465 and pSer423/425 respectively) in SECs lysates after the stimulation with BMP4 (10ng/ml) for 45 minutes. Vinculin was detected to verify equal loading of the samples. WB shown is representative of two independent experiments (n=2). **(b)** qRT-PCR analysis of αSMA (Acta2), FSP1 (S100a4), ID1 (Id1) and claudin5 (Cldn5) expression in SECs after the treatment with BMP4 (10ng/nl) (blue bars) or BMP6 (100ng/ml) (black bars) for 96 hours. The levels of mRNA were normalized to 18s; columns are the means ± SD of triplicates from a representative experiment (n=2).

5.3 MKs are physically closer to vessels undergoing EndMT in PMF spleen

ECs of BM and spleen can undergo EndMT and possibly contribute to fibrosis as showed in both *ex vivo* PMF patient sections and *in vitro* when EPCs and SECs were exposed to both TGF-β and pro-inflammatory cytokines in order to reproduce the PMF environment.

Thus, we wondered which could be the natural source of TGF-β in PMF, able to mediate the aberrant switch of ECs towards a fibroblast-like phenotype. We hypothesized that MKs could be the ideal candidates for different reasons. As already mentioned, PMF MKs are characterized by dysplastic hyperplasia and their α-granules contain higher concentration of TGF-β as compared to controls, that is supposed to primarily contribute to BM fibrosis [185,186]. Moreover, MKs were recently showed to be in close proximity to VEGFR3 positive sinusoids in the BM hematopoietic niche [229], thus supporting the fact that ECs and MKs are in close contact and they can reciprocally influence each others leading ECs to "sense" TGF-β released by MKs.

To prove this hypothesis we first searched for the presence of MKs in human PMF spleens were EMH is described to pathologically take place [184]. While spleen remains a hematopoietic organ in adult mice, in humans it is actively engaged in haematopoiesis only during foetal life. For this reason, MKs are usually located only in the BM hematopoietic niche where they originate from the HSC or in foetal liver and spleen. However, it has been recently described the presence of MKs in the spleen undergoing EMH of a patient resembling MPN phenotype [230]. Therefore, we analyzed the presence of MKs in PMF and healthy donors spleen sections by labelling the tissue with an antibody against CD41a, commonly known as GPIIb, specific for MKs [231]. We found that only splenic tissue from PMF patients showed positive staining for CD41a molecule that, on the contrary, was totally absent in the spleens of healthy donors (Figure 29), thus highlighting the presence of MKs only in the spleen of the patients where aberrant EMH takes place.

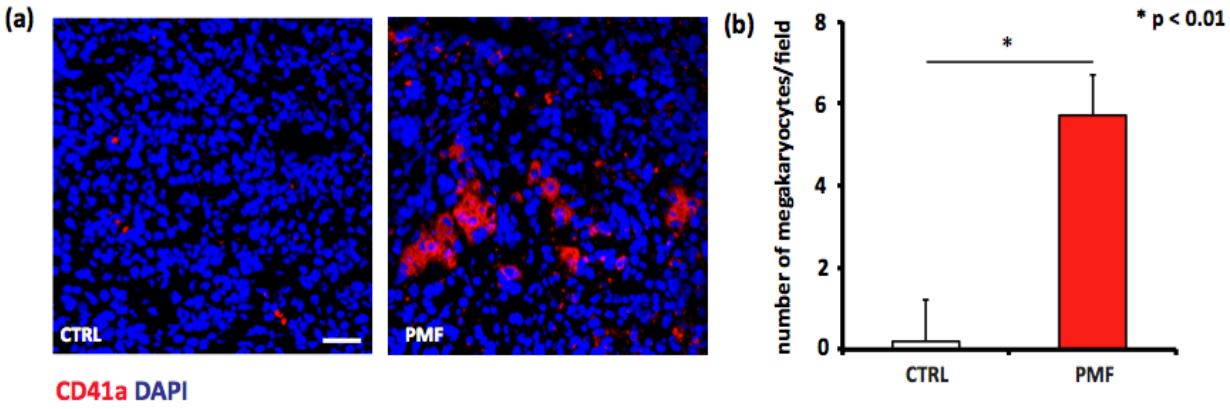

Figure 29: MKs are detected only in the spleens of PMF patients

(a) IF analysis on spleen sections from both healthy donors (CTRL) and PMF patients (PMF) for MKs stained with a specific antibody against CD41a (red). DAPI staining (blue) marked nuclei. Scale bar = 40µm. **(b)** Quantification of the total number of megakaryocytes per field analysed. CTRLs n=2, PMFs n =6.

Next, we verified whether MKs could be physically close to the vessels undergoing EndMT, thus supporting the hypothesis of a crosstalk between the platelet precursors and ECs that sense TGF-β released by MKs. As shown in Figure 30, vessels positive for both the EC marker VE-cadherin and for the EndMT marker FSP1 (Figure 30, arrow-heads) were located in proximity of MK cells stained with CD41a (Figure 30, arrows) in spleen sections of PMF patients. On the contrary, as already shown (Figure 18 and Figure 29), vessels from healthy donor tissues were completely negative for the mesenchymal marker FSP1 and the spleen did not display the presence of any MKs (Figure 30).

In conclusion, we detected the presence of MKs in PMF spleens and we demonstrated that they are closer to the vessels undergoing EndMT, thus suggesting MKs as a possible source of TGF-β.

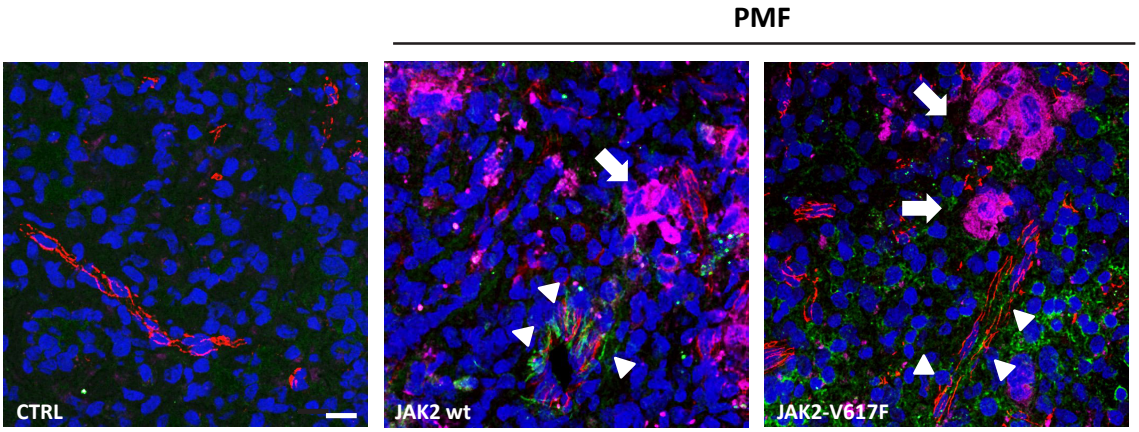

Figure 30: MKs are in proximity of vessels undergoing EndMT in PMF spleens

IF analysis on spleen sections of healthy donors (CTRL) and PMF patients (PMF) for the expression of VE-cadherin (red), FSP1 (green) and CD41a (purple). White arrow-heads highlight vessels co-expressing VE-cadherin and FSP1. MKs are indicated by white arrows. DAPI staining (blue) marked nuclei. Scale bar = 30µm. CTRLs n=2, PMFs n =6.

5.4 MK and platelet derived TGF-β is responsible for EndMT in SECs

To understand whether TGF-β released by α-granules of both MKs and platelets could induce ECs to undergo EndMT, we isolated MKs and platelets respectively from BM and peripheral blood of wild type mice.

We focused our attention not only on MKs but also on platelets since, during their development and maturation, they directly receive granules and organelles from the cell body of MKs, which represent their progenitor cells. It is likely that α-granules of platelets could be as rich in TGF-β as MKs, thus constituting an additional source of TGF–β able to mediate an EndMT switch. Supporting this hypothesis, a recent paper showed that the treatment of tumour cells with platelets induced an EMT phenotype and promoted the formation of metastasis. Platelet-released TGF-β was able to activate Smad-dependent and NF-kB-dependent pathways supporting EMT and tumor metastasis [232].

Of note, even if platelet count in PMF is very variable during the evolution of the disease, about 30% of PMF patients at the initial stage of the pathology show a higher

concentration of platelets (>400x10^9/l) in comparison to healthy subjects (150-400x10^9/l), thus theoretically increasing the chance that ECs could face higher concentration of TGF-β released in the bloodstream and aberrantly undergoing EndMT.

To this aim, CD34-positive HSC cells were isolated from femoral BM of mice and stimulated with Tpo in order to mediate their differentiation into MKs. In parallel, blood from the vena cava of the same mice was collected and platelets purified through sequential centrifugations (see Materials & Methods). Then, we induced an *in vitro* release reaction by adding thrombin to MK and platelet suspension in order to release the content of their granules. Surnatant (SN) and pellet originated from the reaction were separated and subsequently a further acidification step was introduced in order to activate latent TGF-β. The experimental procedure is described in detail in Figure 31.

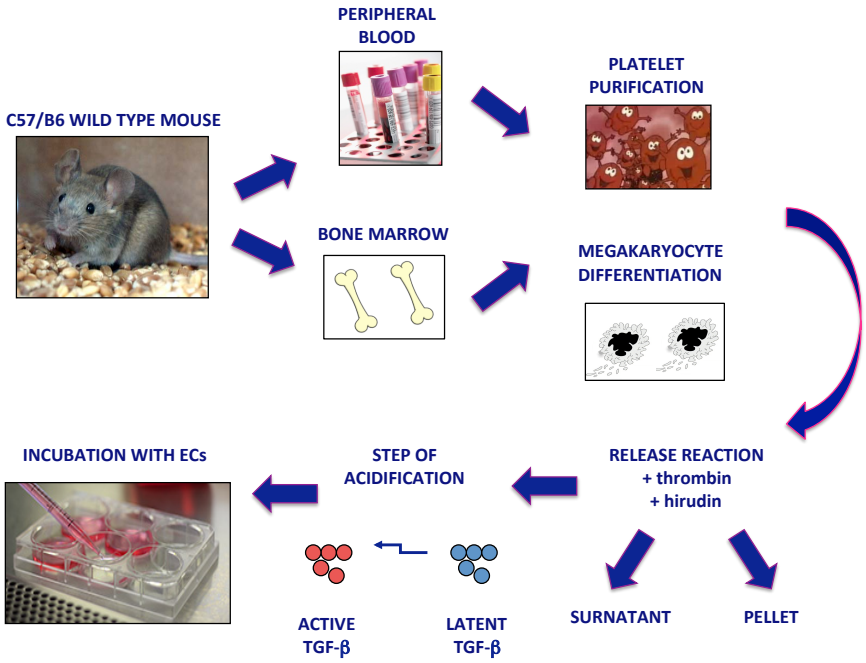

Figure 31: Experimental protocol for MK and platelet purification and *in vitro* release reaction assay

Ten C57/B6 mice were sacrificed, peripheral blood form the inferior vena cava was collected and BM cells were isolated from femurs. Peripheral blood underwent sequential centrifugation in order to obtain purify platelets content. In parallel, hematopoietic cells were plated and treated with recombinant Tpo (10ng/ml) to obtain MKs for 4 days. Platelets and MKs then went through a release reaction step where treatment with thrombin induced α-granules release. SN and pellet obtained were further subjected to an acidification step in

order to activate TGF-β possibly released by α-granules. SN and pellet derived form both MK and platelet release reaction were finally incubated with SECs.

SECs stimulated with the platelet SN or the pellet were able to activate TGF-β signalling pathway inducing both Smad1 and Smad3 phosphorylation (Figure 32a). In parallel, the incubation induced the up-regulation of several EndMT markers (αSMA, BMP4, BMP6, fibronectin, PAI-1) and the down-regulation of the endothelial specific marker claudin5 (Figure 32b), thus inducing an EndMT switch comparable to the one obtained by the stimulation of SECs with pro-inflammatory cytokines (Figure 25). Of note, the control acidificated sample shows the same behaviour of the non-acidificated sample, meaning that this step introduced to activate latent TGF-β does not alter EC physiology.

Comparable results have been obtained incubating SECs with SN and pellet derived form MK release reaction (Figure 33), as they both were able to mediate induction of Smad1 and Smad3 phosphorylation (Figure 33a) and up-regulation of EndMT markers (Figure 33b), suggesting that the products of the MK and platelet release reaction had comparable effects. For this reason, we decided to proceed with the experiments with only one of the two lineage and we choose platelets, mainly for technical and feasibility benefits; indeed every time that the *in vitro* MK differentiation is performed, it generates an heterogeneous pool of MKs at different stages of development, thus influencing the reproducibility of each experiment.

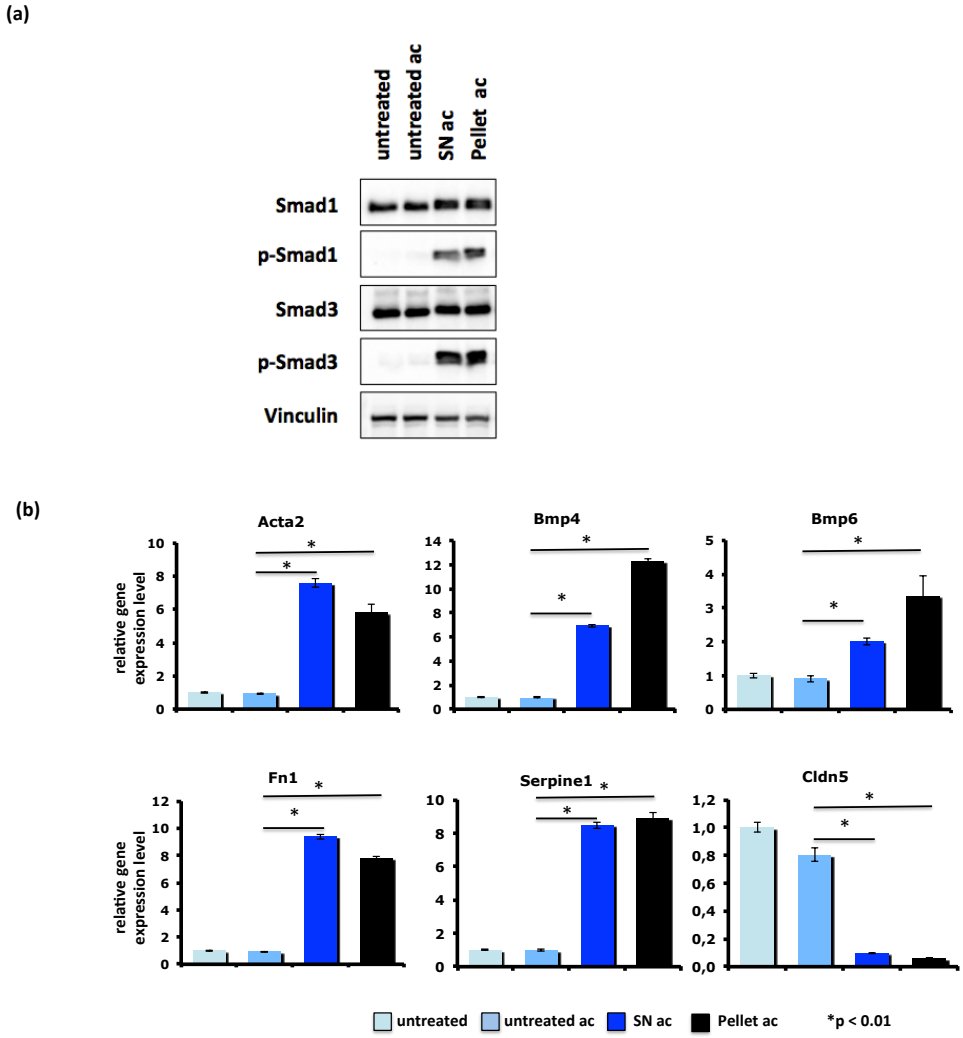

Figure 32: Platelet SN and pellet trigger Smad phosphorylation and induce EndMT markers up-regulation in SECs

(a) SN (SN ac, 300µl in 3ml of starving medium) and Pellet (pellet ac, 300µl in 3ml of starving medium) obtained by platelet *in vitro* release reaction were added to SECs for 45 minutes and phosphorylation of Smad1 (pSer463/465) and Smad3 (pSer423/425) was evaluated by WB, as well as total Smad1 and Smad3 levels. Vinculin was detected to verify equal loading of the samples. WB shown is representative of three independent experiments (n=3). **(b)** qRT-PCR analysis was performed on SECs treated with SN or pellet for 40 hours to evaluate αSMA (Acta2), BMP4 (Bmp4), BMP6 (Bmp6), fibronectin (Fn1), PAI-1 (Serpine1) and claudin5 (Cldn5) expression. The levels of mRNA were normalized to 18s; columns are the means ± SD of triplicates from a representative experiment (n=3).

In **(a)** and **(b)** ac = acidificated.

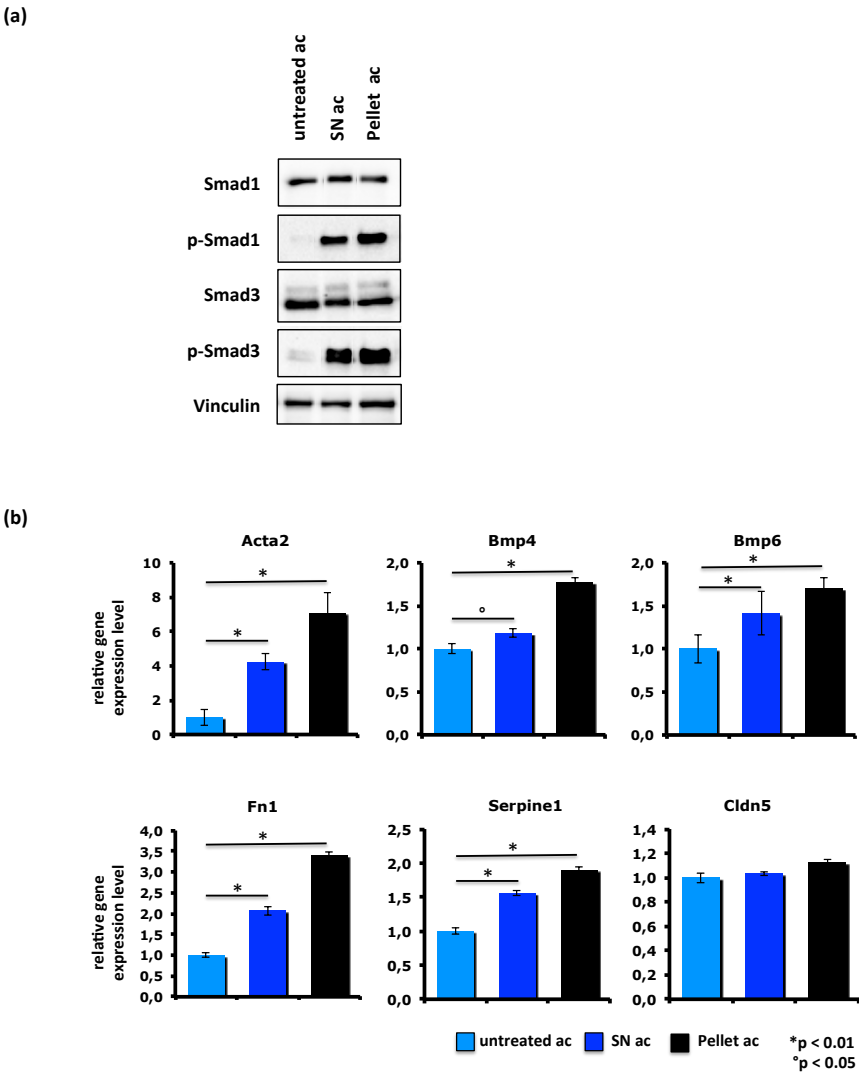

Figure 33: MK SN and pellet trigger Smad phosphorylation and induce EndMT markers up-regulation in SECs

(a) SN (SN ac, 300µl in 3ml of starving medium) and Pellet (pellet ac, 300µl in 3ml of starving medium) obtained by MK *in vitro* release reaction were added to SECs for 45 minutes and phosphorylation of Smad1 (pSer463/465) and Smad3 (pSer423/425) was evaluated by WB, as well as total Smad1 and Smad3 levels. Vinculin was detected to verify equal loading of the samples. WB shown is representative of two independent experiments (n=2). **(b)** qRT-PCR analysis was performed on SECs treated with SN or pellet for 40 hours to evaluate αSMA (Acta2), BMP4 (Bmp4), BMP6 (Bmp6), fibronectin (Fn1), PAI-1 (Serpine1) and claudin5 (Cldn5) expression. The levels of mRNA were normalized to 18s; columns are the means ± SD of triplicates from a representative experiment (n=2).

In **(a)** and **(b)** ac = acidificated.

So far we showed that the products of platelet and MK release reaction can induce SECs to undergo EndMT. To further prove if this phenotype is TGF-β dependent, we first quantified the total amount of active TGF-β present in both platelet SN and pellet by an ELISA assay (Table 5).

	Active TGFβ1 (ng/ml)
Untreated	Not detectable
Platelets SURNATANT	**13.8 +/- 0.2**
Platelets PELLET	**1.8 +/- 0.4**
Recombinant (Peprotech)	**5**

Table 5: Active TGF-β1 quantification in platelet SN and pellet samples

ELISA assay performed on SN and pellet from platelet release reaction to detect the presence of active TGF-β1 in both samples. Quantification shows the means ± SD of triplicates from a representative experiment (n=2) (Assay kindly performed by dr. Hawinkels, Leiden University Medical Center, Leiden, The Netherlands). In the last row, concentration of recombinant TGF-β1 used in the previous experiments.

ELISA quantification detected active TGF-β1 in both SN and pellet samples. The SN showed more than 7-fold higher concentration of TGF-β1 content in comparison to the pellet one but, as shown by the previous results obtained by both SN and pellet, 1.8 ng/ml of active TGF-β seems to be sufficient to induce EndMT, at least in our cellular model (Table 4).

To finally prove that the EndMT phenotype described is TGF-β-dependent, we incubated SECs with SN or pellet combined with 2 different commercially available TGF-β -signalling chemical inhibitors, named LY-2109761 (Selleck Bio) and DMH1 (Tocris, R&D) respectively, in order to possibly revert the EndMT phenotype. LY-2109761 is considered a pan-TGF-β inhibitor, as it blocks both TβRI and TβRII kinases [233] while DMH1 is a selective inhibitor of ALK2, thus acting mainly on BMP signalling [234]. We have decided to use DMH1 since we previously demonstrated that TGF-β-dependent

stimulation of SECs could induce endogenous production of BMP6 that sustained EndMT phenotype (Figure 26 and 27).

Then, we repeated the same experiments performed with platelets SN or pellet in SECs in presence or in the absence of either LY-2109761 or DMH1.

LY-2109761 added to SECs for 72 hours in presence of SN or pellet completely abolished both Smad1 and Smad3 phosphorylation, as assessed by WB analysis (Figure 34a). Smad phosphorylation was also reduced using DMH1, even if the effect mediated by the BMP inhibitor was milder in comparison to LY-2106791, as seen by the WB quantification (Figure 34b).

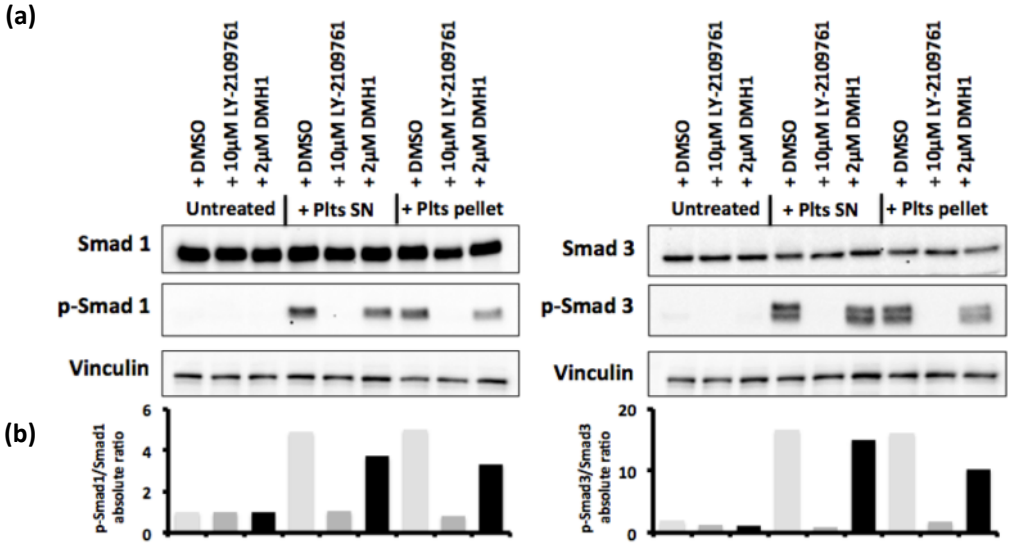

Figure 34: Smad1 and Smad3 phosphorylation is reverted by TGF-β inhibitor and partially by BMP inhibitor treatment

(a) WB analysis evaluated p-Smad1 (pSer463/465) and p-Smad3 (pSer423/425) as well as total Smad1 and Smad3 levels. SECs were incubated for 45 minutes with SN (SN ac, 300µl in 3ml of starving medium) and Pellet (pellet ac, 300µl in 3ml of starving medium) derived from platelet release reaction in presence or not of LY-2109761 (10µM) or DMH1 (2µM), added to the cells 24 hours before the stimuli. **(b)** Quantification of WB bands was calculated as the absolute ratio between p-Smad and total Smad normalized on vinculin amount. WB and quantification shown are representative of four independent experiments (n=4).

Since LY-2106791 and partially DMH1 were able to revert Smad phosphorylation induced by SN and pellet treatment in SECs, we checked for the expression of EndMT markers with the same treatments. Coherently with the results obtained on p-Smad reduction, both TGF-β/BMP inhibitors were able to down-regulate EndMT markers, previously induced by the incubation of SECs with SN and pellet released by platelets. Specifically, LY-2109761 (dark grey bar) added to SECs after the incubation for 40 hours with SN or pellet almost reverted the expression of EndMT markers PAI-1, fibronectin, Sox4, SNAIL, BMP4 and BMP6, αSMA and N-cadherin (Figure 35), thus indicating them as specifically TGF-β-up-regulated genes. DMH1 (black bar) partially reduced the same mesenchymal markers, however it was more efficient in down-regulated FSP1 and ID1 (Figure 35), possibly described as BMP target genes. Of note, BMP6 transcript up-regulation by DMH1 was probably due to a positive feedback loop mediated by the cells trying to overcome the effect of the inhibitor on the receptor.

Overall these data suggest that TGF-β present in SN and pellet released by platelets and MKs is responsible for the switch of SECs from an endothelial-to-mesenchymal state, as the treatment with the TGF-β/BMP inhibitors LY-2109761 and partially DMH1 is able to revert Smad phosphorylation and the consequent up-regulation of mesenchymal markers in SECs, with the final outcome of possible reversion of EndMT phenotype.

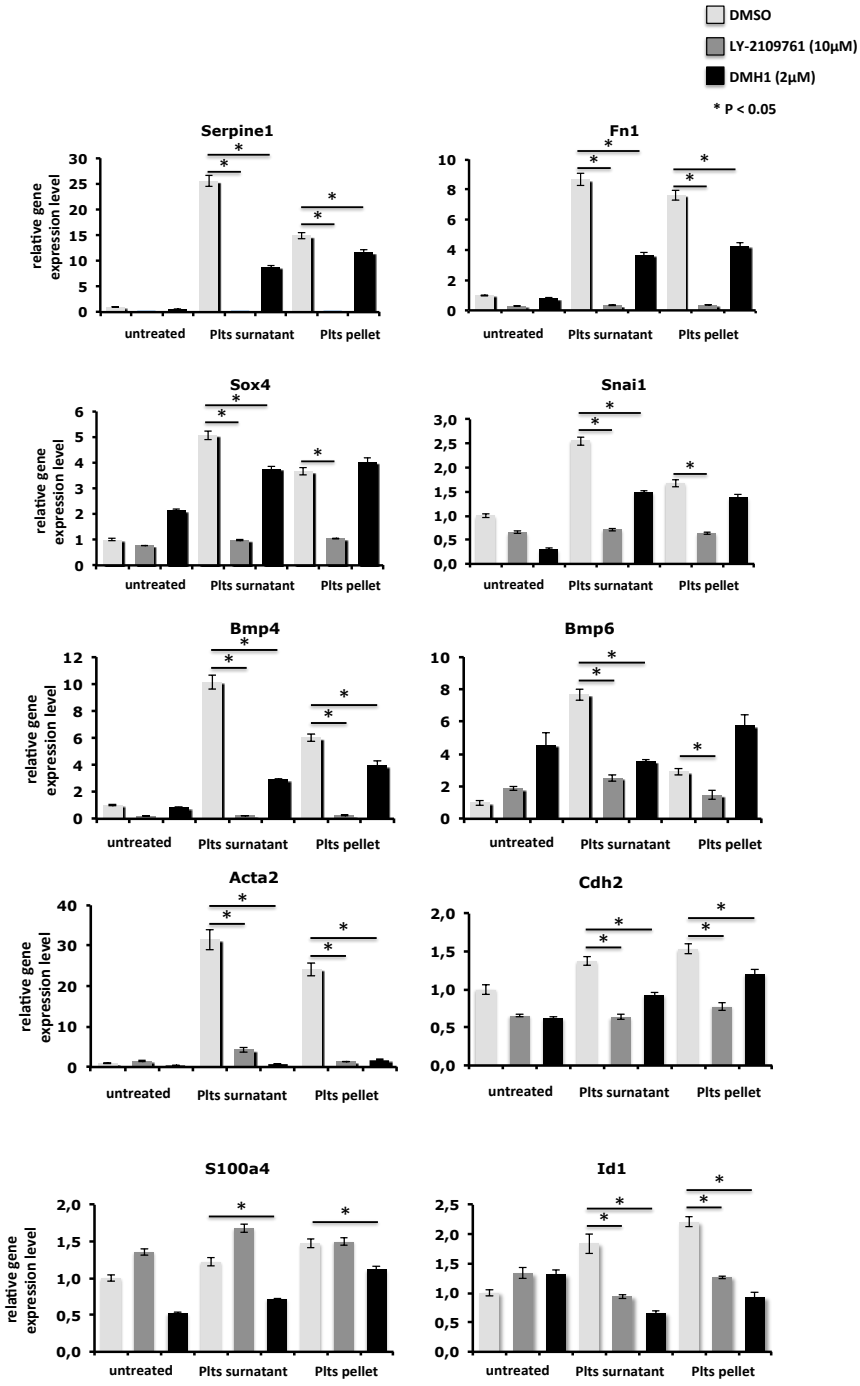

Figure 35: TGF-β/BMP inhibitor treatment down-regulates EndMT markers

qRT-PCR analysis for PAI-1 (Serpine1), fibronectin (Fn1), SOX4 (Sox4), SNAIL (Snai1), BMP4 (Bmp4), BMP6 (Bmp6), αSMA (Acta2), N-cadherin (Cdh2), FSP1 (S100a4) and ID1 (Id1) expression in SECs treated for 40 hours with SN (SN ac, 300μl in 3ml of starving medium) and Pellet (pellet ac, 300μl in 3ml of starving medium) derived from platelet release reaction in absence (light grey bars) or in presence of LY-2109761 (10μM) (dark grey bars) or DMH1 (2μM) (black bars) added to the cells 24 hours before the stimuli. The levels of mRNA were normalized to 18s; columns are the means ± SD of triplicates from a representative experiment (n=4).

5.5 JAK2-V617F mutation in the endothelial lineage specifically mediates STAT3 and STAT5 phosphorylation and induction of STAT target genes

The presence of the GOF JAK2-V617F mutation in the hematopoietic lineage induces constitutive activation of JAK2 downstream signalling pathways including phosphorylation of STATs, PI3K/AKT and MAPK effectors [235]. This specific aminoacid change in the auto-regulatory domain of the tyrosine kinase was recently identified also in splenic ECs of PMF patients [220] but its functional readout has not been elucidated yet. We then hypothesized that the expression of the mutated form of JAK2 could constitutively activate in ECs the same downstream pathways induced in hematopoietic cells, thus influencing cellular homeostasis and the response to EndMT.

To address these questions we stably transduced SECs with three different retroviral GFP-tagged constructs expressing JAK2 WT form, JAK2-V617F mutation or an empty vector as control (Figure 36).

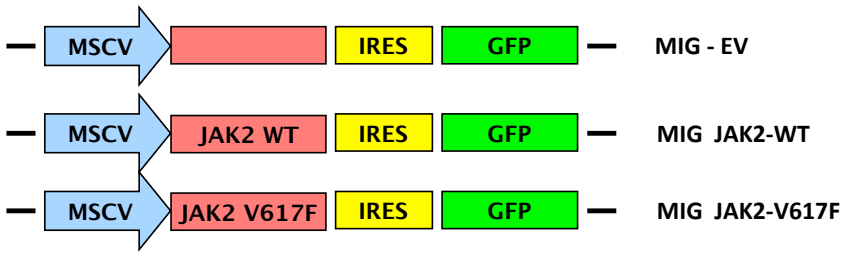

Figure 36: Scheme of the retroviral vectors suitable to over-express JAK2-WT or JAK2-V617F in SECs

Bicistronic murine stem cell virus/internal ribosome entry site/green fluorescent protein (MIG) retroviral vectors encoding JAK2-WT (MIG-JAK2 WT) or JAK2-V617F (MIG-JAK2 V617F) were cloned under the murine stem cell virus (MSCV) promoter. Vector also included GFP sequence under the control of an internal ribosome entry site (IRES) to monitor efficiency of infection. Empty vector (MIG-EV) was used as negative control.

After the infection, we sorted the cells for GFP expression to finally obtain homogeneous populations of cells, further confirmed by GFP equal expression among the three cell lines by WB (Figure 37a). JAK2 kinase was properly over-expressed in SECs (Figure 37a) infected with JAK2-WT and -V617F constructs. We also verified by phase contrast analysis that the infection did not result in any type of alterations of EC morphology (Figure 37b), thus excluding a possible toxicity due to the retroviral infection.

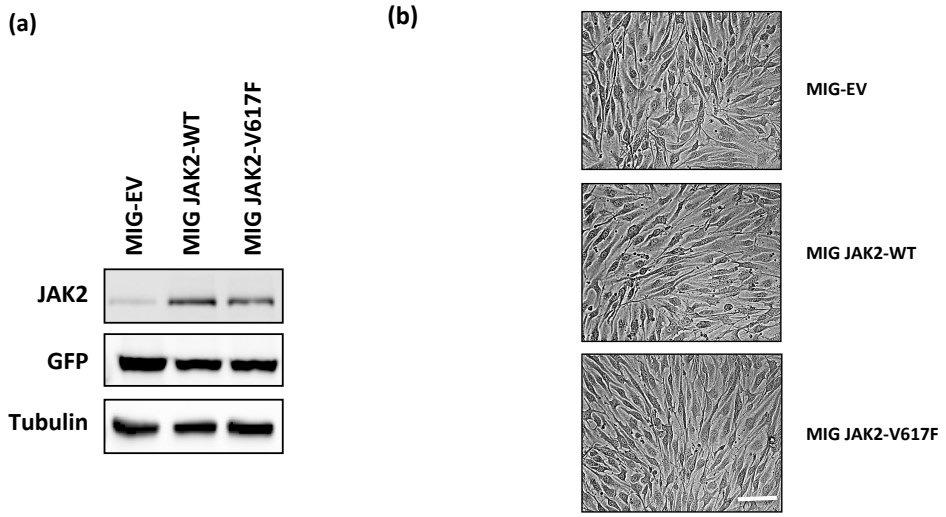

Figure 37: Infection of SECs with MIG vectors results in JAK2-WT and JAK2-V617F over-expression

(a) WB analysis for JAK2 protein expression in SECs stably infected with MIG-EV, MIG JAK2-WT and MIG JAK2-V617F vectors. The efficiency of infection was assessed by similar GFP expression in the three cell lines. Tubulin was loaded to verify equal amount of the samples. WB shown is representative of three independent experiments (n=3). **(b)** SEC morphology after MIG vector infections was evaluated by phase contrast analysis. Scale bar = 30μm.

We first tested if the JAK2-V617F GOF mutation in ECs could constitutively induce the phosphorylation of the kinase without any upstream signal stimulation, as already been described in MPN cellular models of hematopoietic origin [235].

WB analysis showed that also in SECs JAK2-V617F over-expression led to the increase of its phosphorylated form (p-JAK2) (Figure 38).

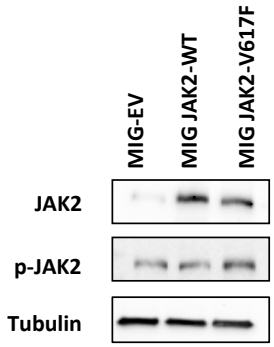

Figure 38: JAK2-V617F mutation in SECs constitutively induces JAK2 tyrosine phosphorylation

WB analysis of JAK2 and p-JAK2 (pTyr1007/1008) protein expression in SECs stably infected with MIG-EV, MIG JAK2-WT and MIG JAK2-V617F vectors. Tubulin was loaded to verify equal amount of the samples. WB shown is representative of three independent experiments (n=3).

We then verified whether constitutive phosphorylation had a functional readout in terms of activation of three main JAK2 downstream signalling pathways, i.e. STATs, PI3K/AKT and MAPK. We observed a specific increase in phosphorylation of STAT3 (p-STAT3) and STAT5 (p-STAT5) only in the cells expressing the mutated V617F form (Figure 39a). On the contrary, at least in our endothelial model, the mutation did not show any effect on the other JAK2 downstream signalling mediators, as assessed by the same expression of the phosphorylated forms of AKT (p-AKT) and MAPK (p-MAPK) (Figure 39b) among the three different retroviral-infected cell lines.

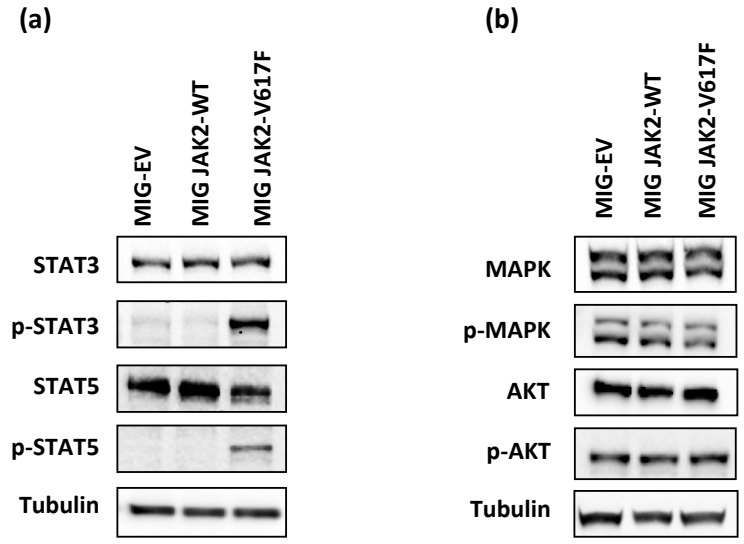

Figure 39: JAK2-V617F mutation in SECs triggers STAT3 and STAT5 phosphorylation

(a) WB showing the expression of total levels of STAT3 and STAT5 and their phosphorylated form (pTyr705 and pTyr694 respectively) in SECs stably infected with MIG-EV, MIG JAK2-WT and MIG JAK2-V617F vectors. **(b)** The same samples were also analysed for the expression of total levels of MAPK and p-MAPK (pThr202/Tyr204), as well as total levels of AKT and p-AKT (pThr308). In **(a)** and **(b)** tubulin was detected to verify equal loading of the samples. WB shown is representative of three independent experiments (n=3).

When STAT molecules are phosphorylated, they dimerize and translocate to the nucleus acting as TFs. We then analysed STAT3 and STAT5 EC-specific target genes, as endothelial nitrix oxide synthase (eNOS) [236], Intercellular Adhesion Molecule-1 (ICAM-1) [237,238] and cyclin-dependent kinase A1 (Cdc2a) [237]. Due to STAT3 and STAT5 specific phosphorylation only in ECs carrying V617F mutation, we scored a significant increase in the expression of all the three genes evaluated only in JAK2-V617F-transduced ECs but not in JAK2-WT or EV-transduced ECs (Figure 40). Interestingly, eNOS induces the synthesis of NO, the main inducer of EPC mobilization from the BM [78] and its up-regulation was detected in a model of chronic allograft nephropathy-associated EMT [239], while the adhesion molecule ICAM-1 was demonstrated to contribute to accelerate TGF-β-induced EMT in HK-2 cell line [240].

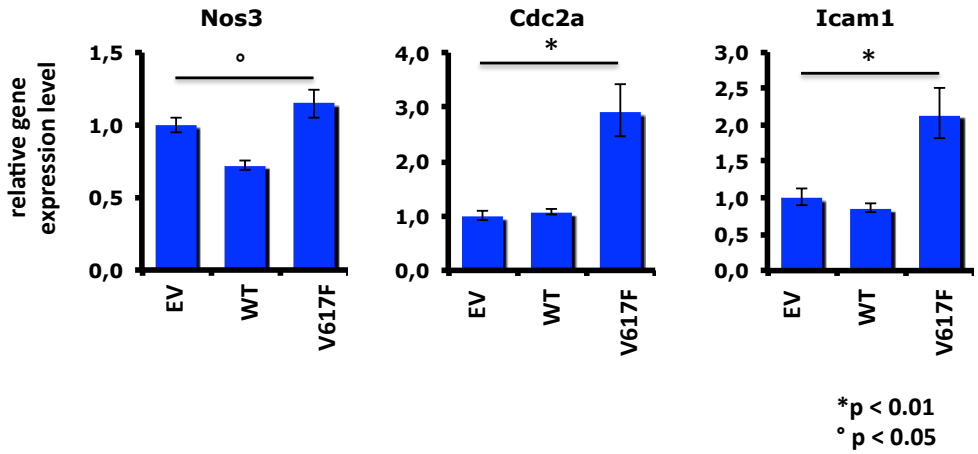

Figure 40: STAT3 and STAT5 target genes are induced in JAK2-V617F expressing SECs

qRT-PCR analysis on SECs infected with MIG-EV or over-expressing JAK2-WT or V617F mutation was performed to evaluate eNOS (Nos3), cyclin-dependent kinase A1 (Cdc2a) and ICAM-1 (Icam1) expression. The levels of mRNA were normalized to 18s; columns are the means ± SD of triplicates from a representative experiment (n=2).

Taken into consideration these results, we concluded that JAK2-V617F GOF mutation did not influence PI3K/AKT and MAPK signalling pathways but it only specifically triggered phosphorylation of STAT3 and STAT5. Moreover, STAT3 and STAT5 target genes eNOS, cyclin-dependent kinase A1 and ICAM-1 were up-regulated in the mutated line, thus indicating that the cellular system is properly working and suggesting that the mutation could actively participate in PMF endothelial phenotype and EndMT progression.

5.6 EndMT phenotype is not influenced by JAK2-V617F mutation

We next tested if the constitutive activation of JAK2-V617F mutation in ECs could make the cells more prone to develop EndMT in a pro-inflammatory environment.

Indeed, involvement of JAK/STAT pathway in mediating EMT has been already demonstrated by different reports. The crosstalk between Epidermal Growth Factor Receptor (EGFR) and IL-6 via JAK2/STAT3 axis mediates EMT in ovarian cancer[241] and that synergism between TGF-β and Raf/MAPK is required for EMT development and metastasis in tumoral epithelial cells [242]. Moreover, a recent paper also demonstrated that

JAK/STAT3 and TGF-β pathways are both necessary to de-differentiate neural stem cells into mesoderm-like cells through an EMT process [243].

So we hypothesized that JAK2-V617F mutation could synergize with the TGF-β pathway activated in the cells by the treatment with pro-inflammatory cytokines and with SN and pellet obtained from platelet release reaction in order to enhance EndMT phenotype. To this aim, we treated SECs transduced with JAK2-V617F, JAK2-WT or with MIG-EV with the cocktail of pro-inflammatory cytokines (TGF-β, IL-1β and TNF-α) and we checked for any possible difference in the expression of EndMT. WB analysis showed no major differences in the induction of Smad1 and Smad3 phosphorylation (Figure 41a) among the cells over-expressing V617F mutation and the ones over-expressing or not JAK2-WT. Consistently, no major differences in the amount of fibronectin, PAI-1, Sox4 and in the down-regulation of endothelial specific claudin5 (Figure 41b) were detected by qRT-PCR in the three cell lines. We also stimulated the same set of cells with SN and pellet released from the *in vitro* platelet release reaction to check if in this different type of stimulation the V617F mutation could preferentially promote EndMT. Also in this experimental setting, no essential variance was reported in the induction of both Smad phosphorylation (Figure 42a) and in the amount of EndMT related genes (fibronectin, SOX4, PAI-1 and claudin5) between cells carrying or not JAK2-V617F mutation (Figure 42b). Notably, SN treatment led to a stronger of induction of mesenchymal markers in comparison to the effect mediated by pellet incubation.

We conclude that the presence of JAK2-V617F mutation in ECs of splenic origin does not influence EndMT phenotype induced by the treatment of both pro-inflammatory cytokines or of the products of platelet release reaction.

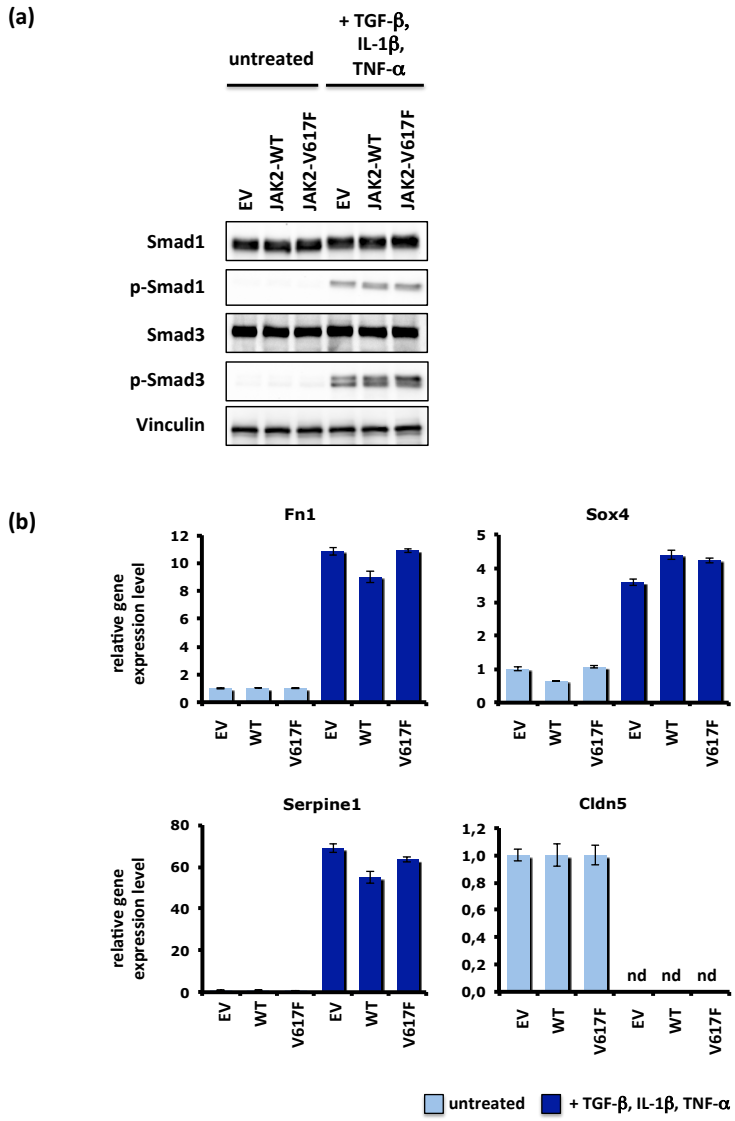

Figure 41: Endothelial JAK2-V617F mutation does not alter EndMT phenotype in response to pro-inflammatory cytokines

(a) WB showing the expression of total levels of Smad1 and Smad3 and their phosphorylated form (pSer463/465 and pSer423/425 respectively) in SECs infected with MIG-EV or MIG expressing JAK2-WT or V617F mutation after the stimulation with TGF-β1 (5ng/ml), Il-1β (100U/ml) and TNF-α (100U/ml) for 45 minutes. Vinculin was detected to verify equal loading of the samples. WB shown is representative of three independent experiments (n=3). **(b)** qRT-PCR analysis of fibronectin (FN1), SOX4 (Sox4), PAI-1 (Serpine1) claudin5 (Cldn5) transcripts in SECs infected with MIG-EV or MIG expressing JAK2-WT or V617F mutation after the stimulation with the same cytokines for 72 hours. The levels of mRNA were normalized to 18s; columns are the means ± SD of triplicates from a representative experiment (n=3). In **(b)** nd = not detected.

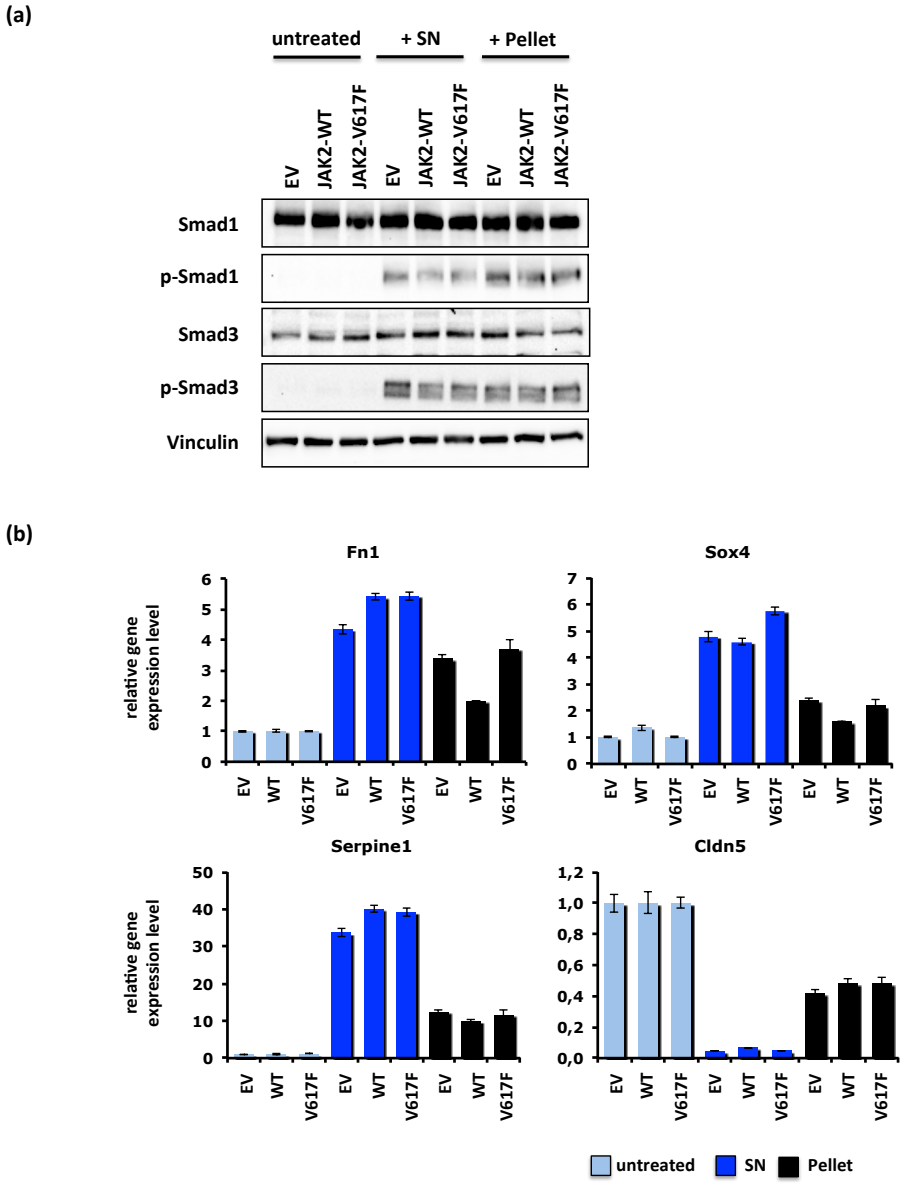

Figure 42: Endothelial JAK2-V617F mutation does not alter EndMT phenotype in response to platelet SN and pellet

(a) WB showing the expression of total levels of Smad1 and Smad3 and their phosphorylated form (pSer463/465 and pSer423/425 respectively) in SECs infected with MIG-EV or MIG expressing JAK2-WT or V617F mutation treated with SN or pellet derived from platelet release reaction for 45 minutes. Vinculin was detected to verify equal loading of the samples. WB shown is representative of three independent experiments (n=3). **(b)** qRT-PCR analysis of fibronectin (FN1), SOX4 (Sox4), PAI-1 (Serpine1) claudin5 (Cldn5) transcripts in SECs infected with MIG-EV or MIG expressing JAK2-WT or V617F mutation after the stimulation with SN or pellet for 40 hours. The levels of mRNA were normalized to 18s; columns are the means ± SD of triplicates from a representative experiment (n=3).

5.7 BM and splenic ECs express CD44 and FSP1 in the MPN mouse model before showing a fibrotic phenotype

So far we showed that ECs can undergo EndMT in the PMF characteristic pro-inflammatory environment both *in vivo* and *in vitro*. The next question we addressed was to clarify the importance of EndMT as a fundamental process in the development and progression of fibrosis. To this aim we took advantage from the $JAK2^{V617F}$ KI murine model of disease [203] described in the introduction paragraphs (see 3.3 Mouse models of MPNs), that expresses in heterozygosity the V617F mutation and develops a MPN-like phenotype.

To prove the causative role of EndMT in fibrosis progression, we analyzed BM and spleen sections from $JAK2^{V617F}$ KI murine model at initial stage of the disease, checking for the expression of mesenchymal/stem markers in ECs before the clear fibrosis stage. To this purpose, we analyzed mice two months old, when fibrosis is still not present but MPN phenotype clearly develops [203].

We first characterized the overall structure of the organs involved in PMF between $JAK2^{V617}$ mutated and wt mice. By H&E staining we clearly appreciated an increased number of MKs in BM sections of the mutated mice (Figure 43, yellow arrows), resembling PMF pathology. Both size and weight of the spleen of the mutated mice were increased as expected and a clear loss in the architecture of the organ, with no more distinction between red and white pulp, was reported (Figure 43). Conversely, morphology and structure of liver and lungs were comparable between mutated and wild type mice, given the fact that these organs are not involved in PMF pathology. All these data suggest that this mouse model faithfully recapitulates the PMF human disease and it is a good *in vivo* model for the study of the etiology of the pathology.

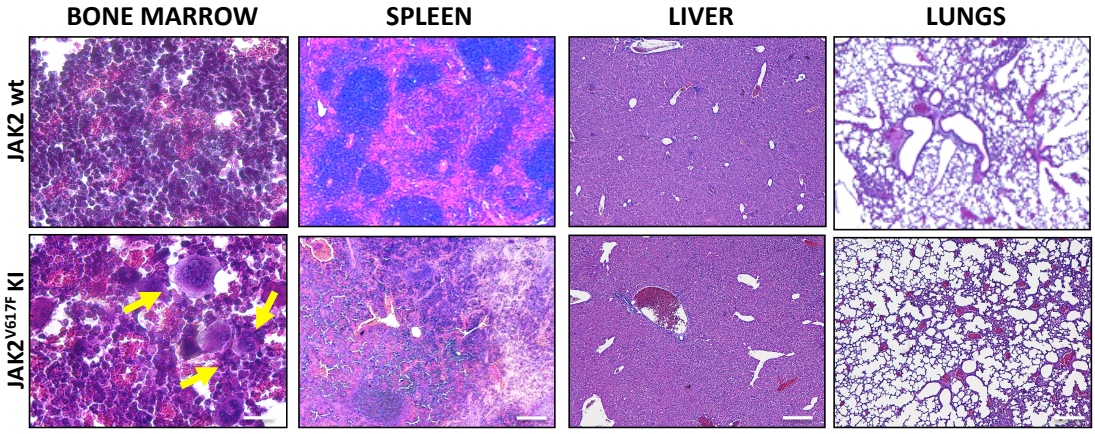

Figure 43: JAK2^{V617F} KI mouse model recapitulates main histological PMF features

H&E staining performed on BM, spleen, liver and lungs tissue sections from JAK2 wt and JAK2^{V617F} KI mouse model at two months of age. Yellow arrows in first panel on the left show MKs present in BM tissue. Scale bars: BONE MARROW= 50µm, SPLEEN= 200µm, LIVER= 500µm, LUNGS= 500µm. JAK2 wt n=4, JAK2^{V617F} KI n=3.

As the most important aim, we finally checked for early expression of EndMT markers in ECs. IF analysis showed that at two months of age, splenic- and BM-derived ECs stained with VE-cadherin co-expressed the mesenchymal markers FSP1 and CD44 only in JAK2^{V617F} KI mouse (Figure 44), that were absent in ECs of WT mice, confirming data previously obtained in spleen sections from PMF patients (Figure 18).

Notably, this co-expression was visible already at early stage, before the complete development of organ fibrosis, suggesting a direct involvement of the EndMT transition as a causative process to fibrosis. Further experiments using TGF-β/BMP inhibitors *in vivo* to revert EndMT could finally provide the proof of concept that inhibition of EndMT will prevent fibrosis development and progression.

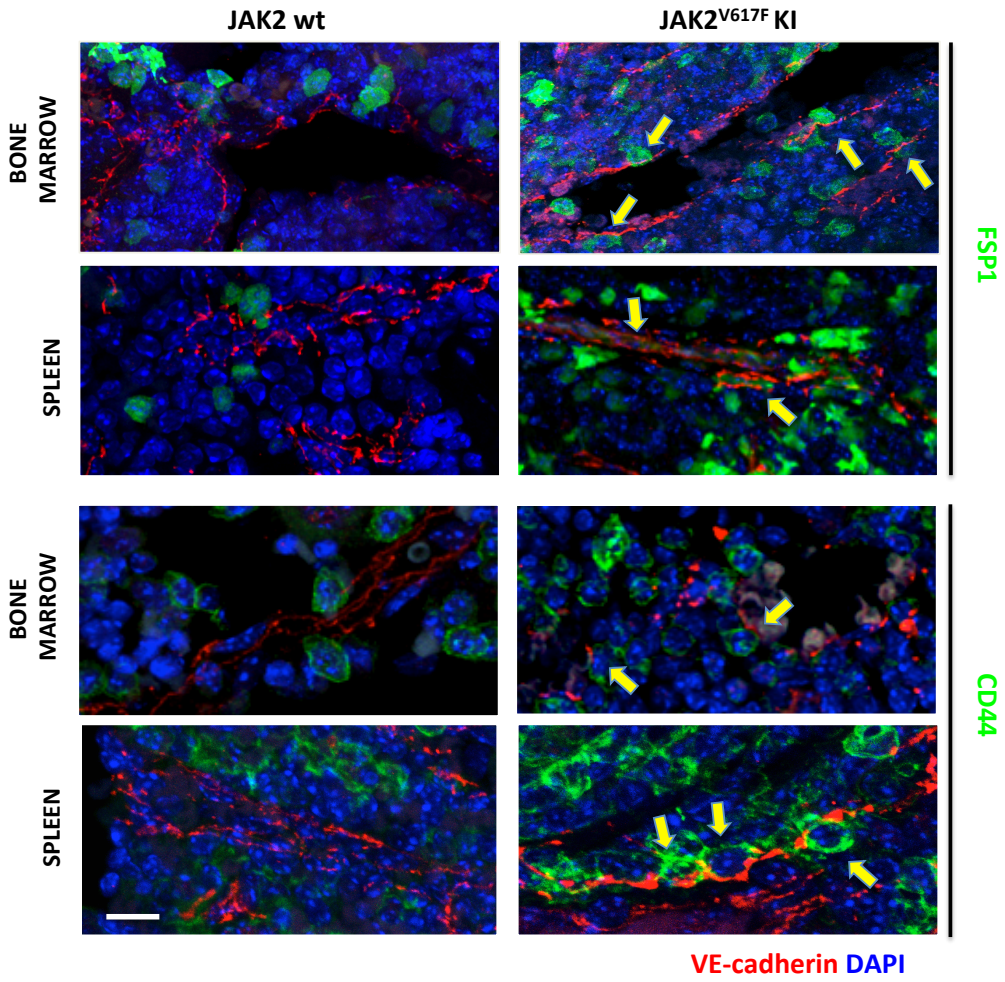

Figure 44: BM and splenic ECs of JAK2^{V617F} KI mouse model express FSP1 and CD44 before the clear fibrotic phenotype

IF staining for VE-cadherin (red) and FSP1 (green, upper panels) or CD44 (green, bottom panels) on BM and spleen sections of JAK2 wt and JAK2^{V617F} KI mice at two months of age. Yellow arrows indicate co-staining of VE-cadherin and FSP-1 or VE-cadherin and CD44. DAPI staining (blue) marked nuclei. Scale bar = 30µm. JAK2 wt n=4, JAK2^{V617F} KI n=3.

DISCUSSION

PMF is a myeloproliferative disease characterized by an abnormal proliferation of the myeloid lineages and excessive production of pro-inflammatory cytokines, leading to the replacement of the hematopoietic BM tissue by collagen fibrosis. Interestingly, PMF patients also develop massive neo-angiogenesis in BM and spleen tissues and show a significant increase in circulating EPCs, suggesting that an alteration in EC homeostasis possibly contributes to the pathological phenotype.

To this aim we investigated the role of the endothelium in PMF; our results showed that both PMF patient BM and spleen are able to undergo EndMT, thus inducing EC to acquire mesenchymal features to contribute to the fibrosis.

EndMT, similarly to the well-known EMT, is of fundamental importance during organ development; in particular ECs lining primitive heart tube acquire mesenchymal features to mediate valves and septa formation in the adult heart [90-92]. On the contrary, aberrant EndMT in the adults leads to pathological cardiac fibrosis, as first described by Zeisberg and colleagues [94] or aberrant fibrosis in other organs[93,95,96,161,164]. Both in physiological and pathological situations, EndMT is mediated by the inductive signals of TGF-β and BMPs. In PMF patients, different studies reported an exaggerate amount of TGF-β [185,186,188]; our results confirmed this data in specimens of PMF spleen sections and we further demonstrated that the TGF-β signalling pathway is hyper-activated in ECs, as proven by the increase in the phosphorylated form of Smad2/3 in EC nuclei of PMF patient spleens.

Of note, our results revealed no significant difference in TGF-β expression in the spleen, as well as in the expression of EndMT markers, between patients carrying or not the JAK2 mutation in the hematopoietic lineage. These results suggest that the pro-inflammatory microenvironment, generated as a consequence of a deregulated cytokine production, has a more prominent role for EndMT development in PMF patients rather

than their genetic background. Indeed, we propose EndMT as a common mechanism leading to fibrosis for all the patients independently from the JAK2 mutation, given that half of them is JAK2 wt.

To prove that EndMT phenotype described in PMF spleen was induced by TGF-β and fostered by the strong PMF pro-inflammatory environment, we demonstrated the capability of both EPCs form PMF patients and a splenic murine EC line to undergo EndMT in presence of a cocktail of inflammatory cytokines, including TGF-β, IL-1β and TNF-α. This cocktail was previously shown to induce EndMT phenotype in human intestinal microvascular endothelial cells (HIMEC) [244]. Similarly to what we observed in the PMF patient spleen, we obtained comparable results in terms of EndMT induction when we stimulated both EPCs from patients with or without the JAK2 mutation as well as EPCs from healthy donors, confirming the prominent role of the pro-inflammtory microenvironment in comparison to the presence of JAK2 mutation in developing EndMT.

Interestingly, our results showed that the stimulation of SECs with the cytokines induced a strong increase in the level of BMP6, which was able to trigger and sustain EndMT too; indeed the stimulation of SECs with a recombinant form of BMP6 produced a similar up-regulation of the mesenchymal markers induced by TGF-β, IL-1β and TNF-α. These data are in accordance with a recent paper published by our group [168] showing that in the CCM disease EndMT contributes to the development of vascular malformations and is mediated by the up-regulation of BMP6 by *CCM-null* ECs that, in turn, activates TGF-β/BMPs signalling pathway. Here we propose a similar mechanism of action working in other EC types, i.e. splenic and BM derived ECs, thus underlying a possible general common response of the endothelial compartment to altered TGF-β/BMP signalling. The cocktail of pro-inflammatory cytokines induced also in SECs a strong up-regulation of BMP4, previously demonstrated to be able to mediate a switch to a mesenchymal phenotype in HUVEC cells [148]; however, when added as a recombinant form, BMP4 alone did not ricapitulate the strong EndMT phenotype that we observed upon BMP6 treatment.

Different explanations for this behavior could be raised; our hypothesis is that BMP4 could act in a synergistic way with BMP6 in SECs, thus further information would be achieved through the simultaneous stimulation of the cells with both BMP4 and BMP6. Supporting this hypothesis, BMPs were also shown to be able to heterodimerize between each others and the effect of the BMP2/BMP6 heterodimers on the activation of the downstream signalling pathway turned out to be even higher than the one mediated by the equivalent homodimers.

The implications of the BMP subfamily in the development of PMF is not a new insight; high levels of BMP1, BMP6, BMP7 and BMP-Receptor 2 mRNAs have been observed in BM biopsies form PMF patients in advanced stages of the disease [245]. Moreover, mainly fibroblasts but also MKs were recorded to be the major source of BMP1. *In vitro* data showed also that human fibroblasts stimulated with TGF-β and FGF-2 were able to over-express the BMPs thus suggesting a potential induction of these TGF-β ligands promoting fibrosis progression [245]. Our data are in accordance with the proposed active role of BMPs in fostering EndMT-mediated myelofibrosis; we further identify the ECs as new player involved in BMP6 and BMP4 induction.

Surprisingly, TGF-β or BMP6 alone mediated the phosphorylation of both Smad signalling pathways, as we found a concomitant up-regulation of p-Smad1 and p-Smad3 after single cytokine stimulation of SECs. As discussed in the introduction, TGF-β superfamily-dependent signalling in ECs is quite divergent and paradoxical as TGF-β exerts opposite outcome depending on the type of receptors engaged and downstream signalling pathways that are consequently activated. Indeed, previous studies reported that activation of TGF-β/ALK5/Smad2/3 pathway leads to inhibition of EC migration and proliferation, whereas signalling through TGF-β/ALK1/Smad1/5/8 can promote cell growth and motility and the balance between the two phenotypes depended on TGF-β concentration [111,135]. Moreover, in other EC lines, VE-cadherin-mediated increased in

TGF-β signalling is coupled to both ALK1 and ALK5 receptor activation and results in the same phenotype of EC quiescience [27] .

Similarly, BMP6 that, as the classical BMPs signals through Smad1/Smad5/Smad8 axis, is able to promote Smad3 phosphorylation in our SEC model. Few cases are reported in literature underlying this "non canonical" activation of Smad2/Smad3 by BMPs; our data are in accordance with a paper published by Upton et al. [246] in 2009 where BMP9 was described to be able to activate not only the canonical Smad1/5 but also Smad2 in a model of human pulmonary artery ECs (HPAECs). In the same year, another paper reported that BMP2 mediated Smad2 phosphorylation for at least 24h after the stimulation in mouse melanoma B16 cell line [247]. In the near future, it could be very interesting to evaluate expression and phosphorylation levels of the different TβRIs and BMPRs in order to possibly clarify how the TGF-β/BMP system reacts and it is modified to a non physiological situation, such as a chronic inflammatory status characteristic of PMF pathology.

This study further introduces a new important concept, as we proposed MKs and platelets to be the main sources of TGF-β in mediating EndMT both in BM and in the spleen of PMF patients. While it is already well demonstrated that MKs born and differentiate in the BM to originate platelets [248], this is the first time that they are specifically identified in spleen of PMF patients, where EMH takes place and the hematopoietic niche is reproduced, thus reflecting BM microenvironment. Therefore we can properly consider the spleen as a mirror of the BM and thus has overcome the great limit we faced at the beginning of the study regarding the paucity of BM biopsies available both from PMF patients but especially from healthy donors.

Moreover, we identified MKs in proximity of ECs undergoing EndMT in PMF spleens, strongly suggesting their essential contribution in releasing TGF-β to develop the phenotype. Further IF analysis on spleen sections for MK specific marker CD41a and TGF-β are now ongoing to finally demonstrated that the cytokine is produced by MKs.

Afterwards we confirmed that the products of MK and platelet released reaction enriched in TGF-β as demonstrated by the ELISA assay were able to mediate an EndMT phenotype added to SECs comparable to the one induced by pro-inflammatory cytokines.

A controversial aspect is the fact that splenic fibrosis is not usually diagnosed to PMF patients, while MPN mouse models develops it. We however showed that the EndMT process clearly involved splenic ECs undergoing a transformation in order to acquire fibrotic markers plus a general up-regulation of αSMA and TGF-β that suggested a fibrotic transition in the organ. This apparent disagreement can be explained as a matter of time: in patients excessive splenomegaly due to EMH leads to the surgical removal of the spleen before the very latter stages of fibrosis, while in the case of the mouse model, splenomegaly is followed by a marked splenic fibrosis appreciable in the very elderly life of the animal when the PMF phenotype is completely displayed. So EndMT in the spleen could be proposed as a very early marker of the disease, thus pointing out the importance to find a specific therapeutical treatment aimed to revert fibrosis endothelium-mediated.

A very fascinating aspect emerging from our data is the hypothesis that the EndMT we have observed could not only contribute to the overall organ fibrosis but it could also have a functional role. Indeed, among the genes strongly up-regulated by both the stimulation with the cytokines and the SN and pellet from MK and platelet release reaction, we found FN. The hematopoietic microenvironment plays a crucial role in the differentiation of the HSC into the multiple hematopoietic lineages and, among BM ECM components, FN is one of the most abundant molecule around sinusoids, whose task is to create a pericellular matrix surrounding MKs. Even if the molecular mechanisms underlying the complex process of megakaryopoiesis have not been fully elucidated yet, FN was clearly shown to regulate MK expansion [249] via the binding to VLA-4 (α4/β1) and VLA-5 (α5/β1) receptors, MK maturation [250] and pro-platelet formation (PPF) [251]. Moreover, a very recent paper demonstrated that when BM HPCs are cultured in an *in vitro* system in presence of different ECM components, only FN is able to increase cell survival and proliferation of

BM precursors and, in presence of Tpo, FN strongly enlarges the pool of MK progenitors [229]. It is conceivable to think that in PMF the crosstalk between ECs and MKs gives rise to a pathological auto-feeding, where TGF-β produced by MKs is sensed by ECs that, as a consequence, undergo EndMT and strongly up-regulate the expression of FN, that continuously feeds MK expansion and maturation, resulting in an enhanced production of TGF-β that, in the end, fosters the myelofibrosis phenotype (Figure 45).

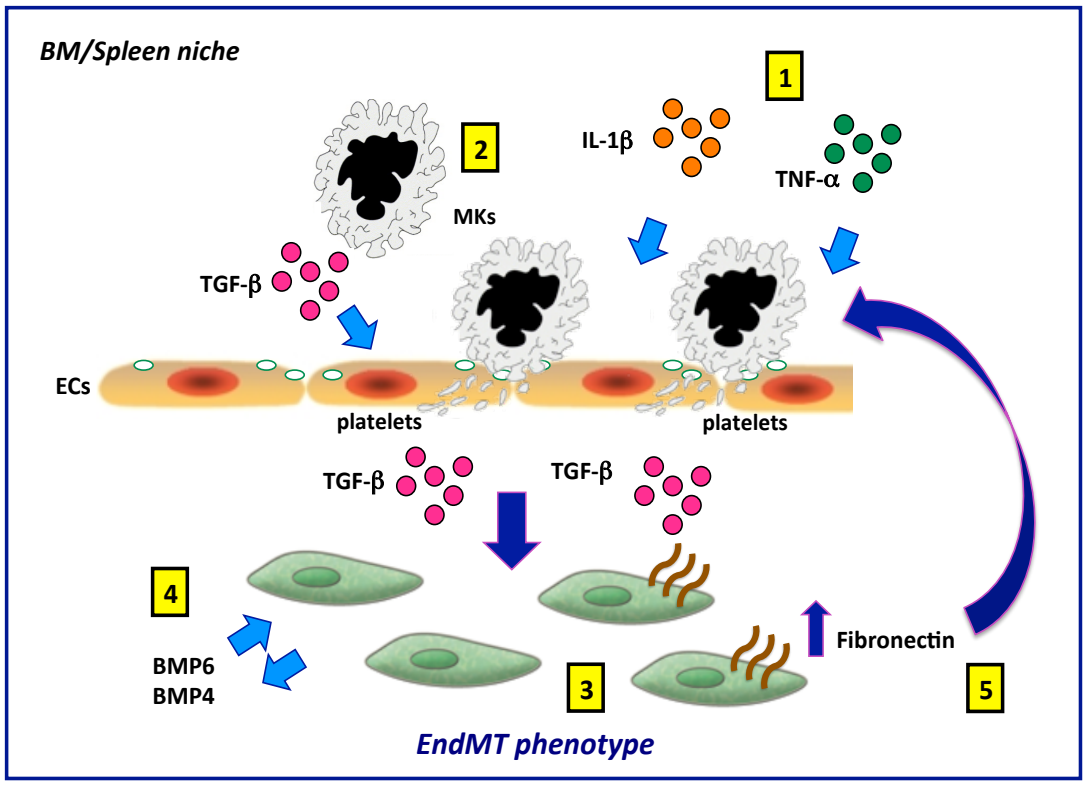

Figure 45: Proposed working model highlighting endothelial contribution to PMF phenotype

In PMF pathology, BM and spleen hematopoietic niches microenvironment are deeply altered as they are characterized by the chronic release of pro-inflammatory cytokines, as IL-1β and TNF-α, by the hematopoietic compartment [1]. MKs strongly contribute to this phenotype, representing the main source of TGF-β and of platelets releasing TGF-β [2], reported to be produced at higher concentration in PMF patients. In this context, ECs are forced to undergo EndMT, to acquire a morphological spindle shape and to express a plethora of mesenchymal markers, contributing to fibrosis development [3]. The stimulation mediated by the cocktail of pro-inflammatory cytokines induces ECs to up-regulate BMP6 and BMP4, which in turn foster EndMT phenotype [4]. Among the molecules mostly up-regulated by ECs undergoing mesenchymal transition there is fibronectin (FN) that, not only provides the fibrotic environment in BM and spleen, but it can also represent a further stimulus aimed at MK expansion and maturation, enhancing the production and release of TGF-β to support myelofibrosis phenotype [5].

Our results also demonstrated the fundamental importance of a new therapeutical approach aimed at TGF-β signalling blockade. Indeed, the *in vitro* use of TGF-β/BMP inhibitors on SECs undergoing EndMT clearly showed a strong reversion of the phenotype, as indicated by the almost complete reduction of Smad phosphorylation and of mesenchymal marker expression. Nowdays, TGF-β signalling pathway has become a hot target for drug development, since its chronic over-expression is responsible for a plethora of different diseases, including cancer, fibrosis and inflammation. The drugs that progressed in clinical trials comprise anti-ligand antisense oligonucleotides (ASOs), ligand-competitive peptides, antibodies targeting ligands, receptors and molecules involved in TGF-β pathway or small-molecules inhibitors (SMIs) against TβR kinases [252]. Some of these new compounds have been already tested in clinical trials on fibrosis disease, showing good results such as in the case of Pirfenidone (InterMune, U.S.), an inhibitor of TGF- β activity *in vitro*. In a Phase III clinical the use of Pirfenidone decreased the rate of decline in vital lung capacity and marginally increased progression-free survival in IPF patients [253]. Similarly, Fresolimumab or GC-1008 (Cambridge Antibody Technology, Genzyme, Sanofi), a pan-TGF-β neutralizing antibody, exhibited encouraging efficacy in a Phase I/II trial on patients with focal segmental glomerulosclerosis [254].

Taken into account the promising results we obtained *in vitro* with the use of the TGF-β/BMP inhibitors, such as the reduction in Smad phosphorylation and the consequent remission of EndMT markers, and considering that PMF patients have a limited expectation of life and no resolving therapies, we strongly propose TGF-β and also BMP6 as new therapeutical targets to limit EndMT in PMF patients and consequently BM and spleen fibrosis. A great challenge will be to address this new therapeutical advice *in situ,* in order to avoid side effects due to an improper systemic down-regulation of the TGF-β signalling, that is necessary, for example, to balance immune system responses. To this aim, a suitable new drug delivery system could be represented by the use of nanoparticles

that we could link from one side to TGF-β/BMP inhibitor and from the other to possible antigens specifically expressed by BM and spleen endothelium to finally target ECs that have undergone pathological EndMT.

Although PMF, PV and ET are recognized as distinct clinico-pathological entities, these disorders share specific cardinal features and it is not rare that patients with PV and ET evolve in secondary PMF, called post-PV MF or post-ET MF. Moreover, the main genetic cause of MPN is the V617F mutation in JAK2 kinase, possibly found in any of the three diseases. For this reason transgenic animal models available for MPN where the mutation in JAK2 is induced in the hematopoietic lineage are not totally specific for PMF; their phenotype usually arises as PV and evolves in PMF at late stages. Our data demonstrated an early expression of the EndMT markers FSP1 and CD44 in ECs from BM and spleen in the $JAK2^{V617F}$ KI mouse model of MPN before the clear fibrosis phenotype, when the predominant features are PV-related. Therefore this finding not only suggests EndMT as a key process in the development and progression of PMF before the clear fibrosis stage but it can also indicate a common pathological process in PV and PMF, thus potentially extending to all MPN patients a therapeutic care to reduce EndMT. These EndMT data could be further supported and confirmed by the use of lineage cell tracking strategy, using for example a transgenic mouse expressing β-galactosidase or GFP under the control of endothelial-specific VE-cadherin promoter to track lineage identity of the ECs; in this way EC undergoing EndMT and therefore expressing mesenchymal markers, such as FSP1 or CD44 and others, would be definitely recognized as cells of endothelial origin thanks to their permanent tracer expression.

So far a very conflicting point regards the presence of the JAK2 mutation in the endothelial lineage. During embryonic development hematopoietic and ECs arise from a common precursor called hemangioblast [255]. The presence of an exceeding rare hemangioblast progenitor pool was shown to be maintained also in postnatal life in a subset of CD34+VEGFR2+ cells [256] and was able to preserve both endothelial and

hematopoietic differentiation capacity. Taken into account the clear involvement of the endothelium in MPNs, it is reasonable to think that ECs derived from the common hemangioblast precursor could harbor the same JAK2-V617F mutation found in the hematopoietic progenitors. Two different papers showed opposite results studying CD34 positive cells extracted from the peripheral circulation of PMF patients and differentiated *in vitro* to endothelial progenitors. The first study pointed out that molecular alterations, including JAK2-V617F mutation, usually found in HPCs are detected also in EPCs [257], hypothesizing a common progenitor between HPCs and EPCs altered by the oncogenic hit; conversely, the second paper firmly excluded the possibility that EPCs could harbor the mutation and that these cells were clonally related to the cell giving rise to the hematopoietic malignancy [221]. These different findings could be attributed to the complexity and still debated definition of the "true endothelial-colony forming unit (ECFU)" growing *in vitro* since nowadays it is still very difficult to define a panel of markers universally identifying only EPCs. More robust *in vivo* data provide recently evidence of the presence of the mutation in mature ECs identified in splenic vessels of PMF patients [220] or in the hepatic venules of PV with BCS patients [219], thus strongly suggesting the involvement of the endothelial compartment in the process of malignant transformation. So far the origins of these mutated cells have not been fully clarified; these findings however are more likely in accordance with the theory of a common progenitor that transmits the JAK2 mutation to its progeny than to a random somatic acquisition of the JAK2-V617F mutation by the ECs that constitute the pathological vessels in PMF. Given the fact that the mutation has been detected in the splenic ECs of PMF patients, we decided to investigate its role. SECs carrying the JAK2 mutation are able to mediate its own phosphorylation (p-JAK2) and consequently modulate its downstream effectors that further mediate the expression of their EC-specific target genes, eNOS, ICAM-1 and cyclin-dependent kinase A1. Of note, EMT and oxidative stress are reported to be closely linked, as eNOS was described to be up-regulated in a model of chronic allograft nephropathy-

associated EMT [239]. Moreover, eNOS-derived nitric oxide (NO) is the main inducer of EPC mobilization from the BM to the peripheral districts in response to different stimuli. So far the mutation has been only detected in splenic ECs but, if new studies could demonstrate its expression also in BM ECs, the up-regulation of eNOS in the altered PMF hematopoietic niche and a potential increase in NO synthesis could provide at least in part a possible mechanism to explain the huge EPC mobilization from BM to peripheral blood documented in PMF patients, so far not elucidated yet. Regarding ICAM-1 over-expression, recent evidence showed its direct involvement in enhancing EMT phenotype induced by TGF-β in a cellular model of human renal epithelial cells (HK-2) [240], so we hypothized its possible contribution in our cellular model of EndMT. Nevertheless, a number of studies reported ICAM levels to be elevated in inflammation, cancer and infection [258] and the soluble form of ICAM-1 (sICAM-1) was reported to be strongly increased in the serum of Myelodisplastic Syndrome (MDS) patients and correlated with the high-risk MDS group associated with a poor prognosis [259]. If the increase in ICAM-1 levels will be confirmed also in PMF patients carrying JAK2-V617F mutation in ECs and they will be correlated with a poor prognosis, ICAM-1 up-regulation could be proposed as an additional new tool for the diagnosis and classification of PMF.

However, when we stimulated the cells both with the cocktail of cytokines or the products released by platelets or MKs, the presence of JAK2-V617F mutation did not seem to further facilitate the mesenchymal transition of SECs. To explain this unexpected result, in light of the fact that JAK2-V617F SECs up-regulate genes that were demonstrated to be able to synergize with TGF-β pathway in inducing EndMT, we can hypothesize that both the stimulations induced a maximal effect that could not let us discriminate different responses among the cells over-expressing the JAK2 mutations and the cell that did not. To this point, additional information will be added as we planned to stimulate the three cell lines with lower doses of cytokines or to perform single stimulation with each of three cytokines alone (TGF-β, IL-1β, TNF-α) in order to be able to identify a specific

contribution of the endothelial JAK2 mutation to EndMT response in a condition of minimal-dose stimulation.

For the first time we demonstrated that MKs are pathologically detected in the spleen of PMF patients still in the adult life, while they are usually identified there only during foetal life when the organ is actively engaged in haematopoiesis. This phenotype could be probably due to the fact that BM hematopoietic niche is recreated in the PMF spleens and EMH aberrantly takes place. Moreover, we found MKs located closer to vessels actively undergoing EndMT, thus supporting their direct implication as a key source of TGF-β. Indeed, TGF-β released from the SN and pellet obtained from the *in vitro* release reaction of MK and platelet granules was able to reproduce a very similar EndMT phenotype observed with stimulation with pro-inflammatory cytokines.

Finally, we demonstrated that *in vitro* treatment of SECs with TGF-β/BMP inhibitors could revert EndMT phenotype. These new findings will acquire even more importance if the use of these inhibitors *in vivo* in the MPN mouse model could finally provide the proof of concept that inhibition of EndMT will prevent fibrosis development and progression, thus opening the possibility to the use of specific and more targeted therapies for PMF patients to achieve fibrosis remission.

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