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XXXIII CYCLE

“Study of VE-cadherin processing: role of oxidative stress and ALK1 signaling”

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ABSTRACT

Cadherin superfamily is a major component of adherens junctions' (AJs) core, in epithelial and endothelial tissues. It has been shown that under apoptosis and calcium influx, metalloproteinases (MMPs) work in concert with the enzyme γ-secretase to disassemble adherens junctions. At the molecular level, MMPs and γ-secretase act by directly cleaving the classic cadherins and this cleavage leads to the formation of two different fragments, CTF1 and CTF2, as demonstrated for E-cadherin.

In this project, we investigated if MMPs and γ-secretase were involved in VE-cadherin processing under oxidative stress condition. Oxidative stress is involved in modulating endothelial functions, but the role played in AJ disassembly is still poorly understood. Moreover, the formation of CTF2 was not demonstrated before for VE-cadherin.

In order to check similarities in the proteolytic cleavage of E-cadherin and VE-cadherin, epithelial carcinoma cell line A431 that expresses both cadherins, has been initially tested. Treatment of A431 for 6-4 h with 1μM Staurosporine (STS), apoptosis inducer, or for 90-30 min with 5μM Ionomycin (Iono), calcium influx inducer, resulted in the cleavage of both E-cadherin and VE-cadherin, and led to the generation of a 28 kDa C-terminal fragment (CTF2). Thus, we demonstrated that VE-cadherin, like E-cadherin, undergoes proteolytic cleavage in response to apoptotic stimuli or calcium increase.

To be more consistent with the aim of our work we moved to a model of endothelial cells (HUVEC), and we found that the inhibition of proteasome activity was able to increase VE-cad/CTF2 level. Indeed, HUVEC exposure to 1μM Epoxomycin for 4h in the absence or presence of Staurosporin enhanced VE-cad/CTF2 accumulation proving for the first time the role of proteasome activity in the catabolism of VE-cadherin fragments.

Further, we exposed HUVEC to H_2O_2. Our data demonstrated that H_2O_2 treatment of HUVEC was able not only to induce VE-cadherin processing favoring VE-cad/CTF2 formation, but also to modify

its subcellular distribution and interaction with cytoskeleton through the activation of MMPs and γ-secretase. Indeed, pre-incubation of HUVEC with 10 μM GI254023X (metalloprotease inhibitor) or 1μM GSI (γ-secretase inhibitor) was able to block VE-cad/CTF2 formation due to oxidative stress. In addition, GI254023X enhanced VE-cadherin Full Length (FL) accumulation pointing out the upstream activity of this enzyme in the process. Thus, we proved that MMP-mediated cleavage of VE-cadherin is upstream from the γ-secretase, which only acts on VE-cad/CTF1. Furthermore, the inhibition of MMPs (ADAM10 and ADAM 17) prevented the disassembly of VE-cadherin/β-catenin/actin induced by oxidative stress.

To note, VE-cadherin's role in adherens junction disassembly and in angiogenesis has been hypothesized as a critical factor in the context of Hereditary hemorrhagic telangiectasia (HHT) pathology. The HHT is a genetic vascular disorder characterized by endothelial cell proliferation and hypervascularization that exert in multiple Arteriovenous Malformations (AVMs). Because this pathology is caused by loss-of-function mutations in bone morphogenetic protein 9 (BMP9)-ALK1-Smad1/5/8 signaling and determinates the VE-cadherin disassembly, we investigated the role of ALK1 signaling in VE-cadherin processing induced by OS. In HUVEC treated with two activators of ALK1 (BMP9 and BMP10) a reduction in VE-cad/CTF2 formation induced by OS was observed. Moreover, BMP9 and BMP10 were able to prevent AJs disassembly induced by H_2O_2.

In this project we demonstrated for the first time that VE-cadherin undergoes a MMPs and γ-secretase dependent cleavage in response to oxidative stress and that the interaction with cytoskeleton is strongly modified. This phenomenon is reduced by the activation of ALK1 pathway, shedding light on the molecular mechanism underling HHT pathology.

SUMMARY

1. INTRODUCTION

1.1 THE ENDOTHELIAL CELL JUNCTIONS – ADHERENS JUNCTIONS

Structure and selectivity of vascular barrier depend on the transmembrane molecules that are constantly and dynamically modulated in the endothelial cell junctions.

Some of the molecular complexes that form cell-cell junctions are specific for endothelial cells while others are in common with epithelial cells and have been extensively documented in two major structures: tight junctions (TJ) and adherens junctions (AJ) (Fig.1).

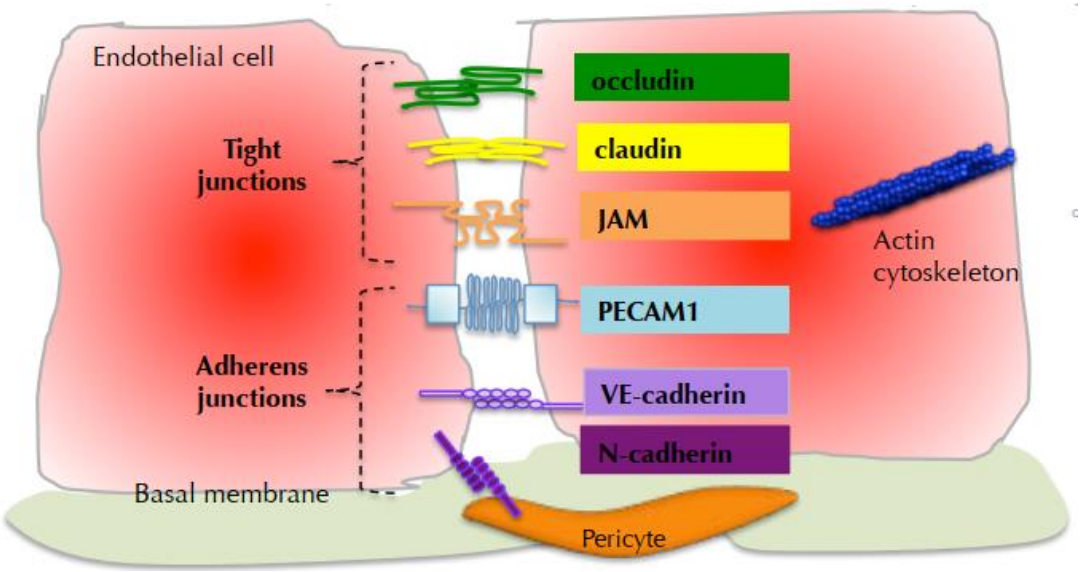


Fig.1. **Adhesive proteins within interendothelial cleft**. Two major structures: tigh junctions (TJs) and adherens junctions (AJs) form endhotelial junctions. TJs consist of claudin, occluddin, Junctional Adhesion Molecule (JAM) and Endothelial Adhesion Molecule (ESAM); AJs are composed of vascular endothelial cadherin (VE-cadherin) and Platelet Endothelial Adhesion Molecule (PECAM/CD31). TJs and Ajs are both linked to the actin cytoskeleton . Moreover, pericytes are tied to endothelial cells by the neuronal cadherin (N-cadherin).

AJs are the major ubiquitous type of cell–cell junctions and are believed to form more dynamic contacts than TJs.

The general organization of AJs presents many structural and functional similarities in different cell types, but its molecular components and biological function may vary in the different tissues.

Among the multiple functions played by AJs, initiation and stabilization of cell-cell adhesion, regulation of actin cytoskeleton, modulation of intracellular signaling and transcription, regulation of endothelial barrier properties, and angiogenesis have been described [1].

The transmembrane core of AJ consists of the cadherins superfamily described below, and the catenin family that includes p120-catenin, β-catenin, and α-catenin. Together, these proteins control the formation, maintenance, and function of AJs [2, 3].

Over the years, many tissue-specific cadherins have been identified, including epithelial (E)-cadherin, neuronal (N)-cadherin, placental (P)-cadherin, vascular endothelial (VE)-cadherin, and others. The altered expression or function of such individual cadherins results in abnormal development of the respective organs [4, 5].

1.1.1 The cadherin super-family

In 1977, following the observation made by Dr. Takeichi, that Chinese hamster lung fibroblasts treated with trypsin do not detach in the presence of calcium [6], a surface protein with approximate molecular weight of 150 kDa were identified and named cadherin.

Cadherins are a family of single-span transmembrane glycoproteins involved in Ca^{2+}-dependent homophilic cell-cell interactions [7].

The cadherin superfamily includes more than 100 members classified on the basis of their protein domain composition, genomic structure and phylogenetic analysis of protein sequence, as classic cadherins, protocadherins and atypical cadherins [8]. In particular, classical cadherins were originally named according to their pattern of expression in tissues and subdivided into type I with N (neuronal), E (epithelial), P (placental) and R (retinal) cadherin, and type II with VE-cadherin (vascular endothelial), cadherins 6-12 and cadherin 15.

Classic cadherins are constituted of three domains: the extracellular (ECD), transmembrane (TMD) and intracellular (ICD) domains, with specific functions in adhesion and signaling (Fig.2) [9, 10].

In more detail, ECD usually consists of five homologous repeated Ig-like motifs (EC) of 110 residues containing putative Ca^{2+} binding sequences required for the correct conformation [11]. The presence of a large hydrophobic region in EC1 domain differentiates type I from type II [12].

The TMD enables cadherin clustering at the plasma membrane, and the highly conserved region ICD bridges cadherins to the actin cytoskeleton through different catenins (α, β) (Fig.2).

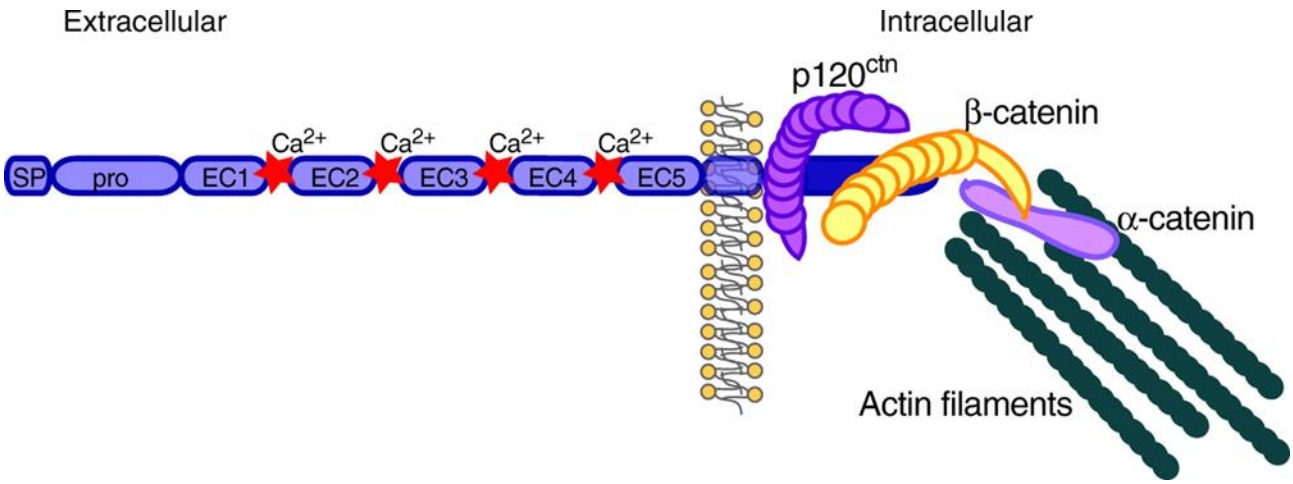


Fig.2. Cadherin domain structure. Cadherins are synthesized with a signal peptide (SP) and pro-region (pro), which are removed during protein processing. The extracellular domain comprises five homologous repeats (EC1-EC5) that are bridged by calcium ions (Ca^{2+}). The cytoplasmic domain binds to p120-catenin (p120ctn) near the plasma membrane and to β-catenin near the C-terminus. α-catenin binds to β-catenin to link the cadherin complex to the actin cytoskeleton [13].

Indeed, within the cytoplasmic domain there are two relatively well-defined catenin binding domains (CBD) encompassing a 94 amino acid juxtamembrane domain (JMD) that binds p120-catenin [14] and an extended region to the C-terminal that binds β-catenin [15].

This complex organization of cadherin-catenins and cytoskeleton is necessary not only to strengthen cell–cell adhesion but also to transfer signals between neighboring cells [16, 17] (Fig.3). In fact, there is evidence that β-catenin and plakoglobin, explained below, can act as signaling molecules when released into the cytosol from AJ to translocate to the nucleus and bind transcription factor(s) controlling gene expression [18, 19].

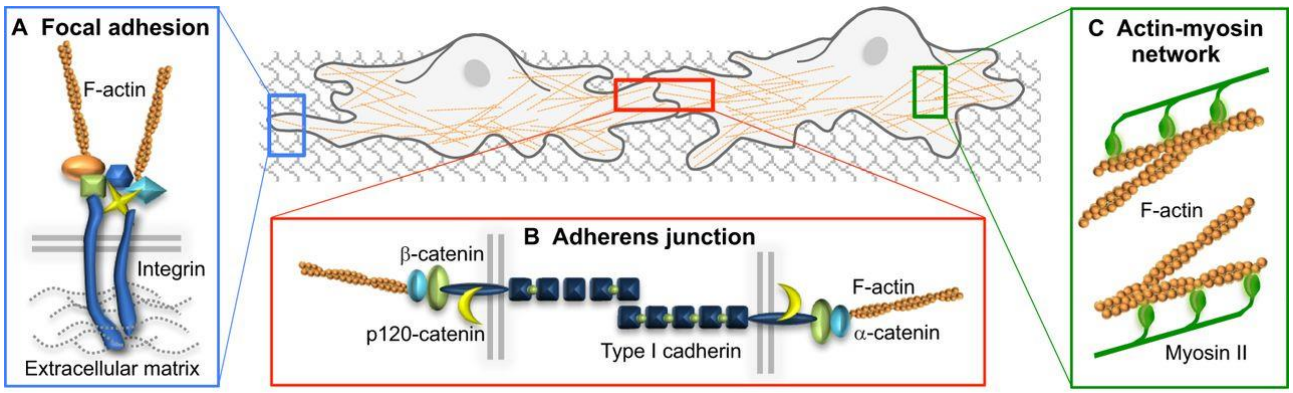


Fig. 3. Complex interaction of cadherin, catenins and cytoskeleton. Integrin-based focal adhesions (A) and cadherin-dependent adherens junctions (B) send mechanical signals through a contractile actin–myosin network (C). Focal adhesion. and adherens junctions form the link between two cells or with ECM. Integrins and cadherins are linked to the intracellular actin–myosin.

β-catenin

β-catenin originally identified in Drosophila [20, 21], contains 13 repeats of a characteristic "armadillo" domain of ~42 amino acids that form triple α-helix [22]. In the nascent junction, β-catenin binds the C-terminal cytoplasmic domain of cadherins in a phospho-regulated manner [23]. Residues and kinases involved in β-catenin phosphorylation have been identified and include: phosphorylation at Y654 by Src [24, 25], phosphorylation at Y489 by Abl kinase [26], phosphorylation of Y654 by EGF receptor [27], and phosphorylation at Y142 by Fer kinase [28].

The regulation of cytosolic β-catenin is critical as β-catenin can bind to the transcription factor Tcf/Lef and mediate the transcription of genes involved in cell proliferation as part of the signaling pathways activated by Wnt [29]. At steady state, cytosolic levels of β-catenin are low due to rapid targeting of excess β-catenin to the proteasome [30, 31].

P120-Catenin

P120-catenin was identified as a substrate for Src- tyrosine receptor kinase [32], and later defined as a member of the catenin family based on sequence homology to the armadillo domain of β-catenin [33]. There are four isoforms of p120 resulting from either post-translational modification and different internal translation starting points [34]. Mutation analysis of cadherins juxtamembrane domain has shown that this domain is necessary and sufficient for p120-catenin recruitment to AJ [35], and that this binding is crucial during the formation of cell-cell contacts and to increase adhesiveness of cells [34, 35].

Plakoglobin

Plakoglobin (also known as γ-catenin) is a paralog of β-catenin that was initially identified as an 83 kDa protein component of the desmosomal plaque [36]. Subsequently, using monoclonal antibodies, cDNA cloning, and a combination of biochemical, morphological, and molecular approaches, Cowin et al. [37] demonstrated that this 83 kDa protein was present in both desmosomes and adherens junction and was given the name plakoglobin.

As adhesive proteins, plakoglobin interacts with the cytoplasmic domain of cadherins, thereby tethering the cadherin proteins to the cytoskeleton. In addition to his cell-cell adhesive functions, it also interacts with a number of intracellular partners including signaling proteins and transcription factors, which accounts for its involvement in cellular signaling [38, 39].

α-catenin

The link between the cadherins/ β-catenin complex and the cytoskeleton is α-catenin. Whereas all other catenins (β-catenin, plakoglobin and p120 catenin) share considerable sequence similarity and belong to the armadillo family of proteins, α-catenin differs notably in both sequence and structural organization. Although it was previously considered to be solely a structural protein, new roles have begun to emerge for α-catenin in both assembling and regulating cytoskeleton dynamics at cell–cell junctions. In addition, other studies have well established that α-catenin is not only important for the cadherin/β-catenin linking to the actin cytoskeleton, but also in recruiting mainly other important proteins to developing intercellular junctions such as the actin-binding protein viniculin, vezantin, α-actin, the filamentous (F)-actin-nucleating froming proteins (Fmn) and members of the vasodilator-stimulated phosphoproteins (VASP)[40-43].

1.1.2 Cadherin Processing

It is well known that cadherins cleavage controls critical events in neurogenesis, tissue development, and tissue homeostasis. Several proteins control cadherin processing: presenilin-1, matrix metalloproteinases (MMPs) and caspase-3. The role of γ-secretase and metalloproteases in regulating intramembrane proteolysis (RIP) is explained in more details in paragraph 1.4.

Here we focused on the evidence related to Cadherin processing.

Presenilin-1 (PS1) is a transmembrane protein expressed in many tissues responsible for most cases of early-onset familial Alzheimer's disease (FAD). PS1 is cleaved to yield N-terminal (PS1/NTF) and C-terminal (PS1/CTF) fragment. Following cleavage, the resultant PS1 fragments form a stable 1:1 heterodimer [44, 45] that binds to the cytoplasmic juxtamembrane region of E-cadherin [46] and inhibits cadherin binding of p120. Furthermore, PS1 stabilizes the cadherin/catenin complex, promotes its cytoskeletal association, and stimulates Ca^{2+}-dependent cell–cell aggregation. It was shown that under conditions that favor cell-cell adhesion, PS1 stabilizes the E-cadherin-catenin adhesion complex [46].

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases specialized in the degradation of both of extracellular matrix (ECM) components (e.g. collagens and fibronectin) and basal membranes (BM) components (e.g. laminin); in the case of metastasis of epithelial tumors, it has been shown to promote cleavage and secretion of cytoplasmic E-cadherin domain [47, 48]. Marambaud et al. found that the E-cadherin proteolytic cleavages by MMP (at the extracellular side) and by PS1/γ-secretase-activity (at the membrane-cytoplasm interface) are key steps in this mechanism [49]. Indeed, E-Cad/CTF1, the C-terminal product of the MMP cleavage, remains bound to catenins and anchored to the cytoskeleton. The PS1/γ-secretase cleavage dissociates both E-Cad/CTF1 and full-length E-cadherin from the cytoskeleton and promotes solubilization of cytoskeletal β- and α-catenin, promoting disassembly of the AJs (Fig.4).

Induction of the PS1/g-secretase cleavage of E-cadherin results in the release of the E-Cad/CTF2-β-catenin complex from the cytoskeleton and in a significant increase in the soluble cytosolic β-catenin.

Free cytosolic β-catenin is a key potent regulator of the Wnt signaling pathway. It translocates to the nucleus where it binds with Lef-1/Tcf transcription factors to activate expression of target genes [50]. Thus, the production of the E-Cad/CTF2-β-catenin complex, rather than free β-catenin, may provide a large pool of complexed inactive β-catenin that could be released according to the transcriptional needs of the cell. The potential importance of E-Cad/CTF2 in signal transduction is indicated in recent studies using artificial constructs overexpressing soluble cytoplasmic cadherin sequences [51, 52].

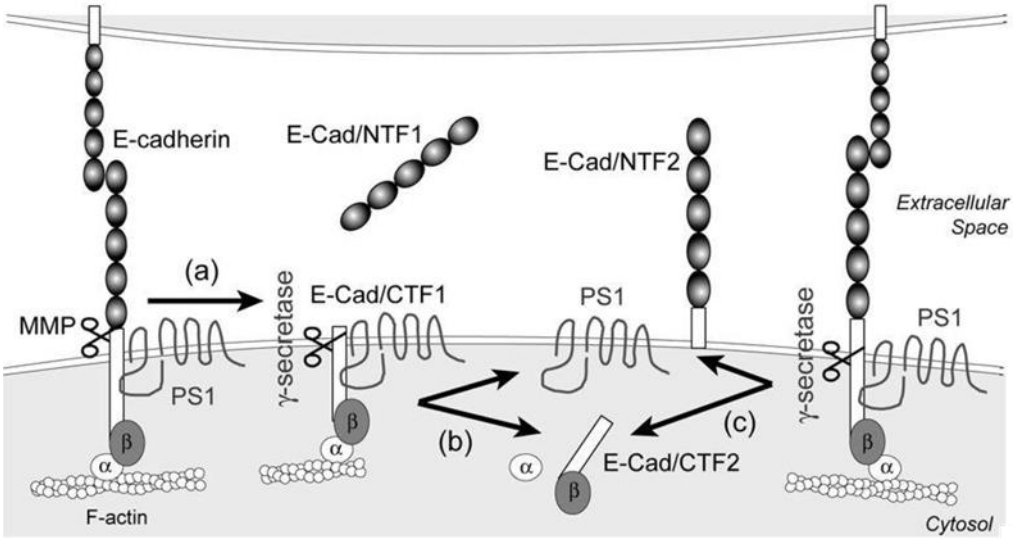


Fig.4. Schematic representation of the PS1/γ-secretase-mediated disassembly of CAJs. E-Cad/NTF1 is releases in the extracellular medium after the cleavage of E-cadherin cytoskeletal extracellular domain mediate by MMP proteolytic activity. In the E-cad/CTF1 fragment it is localized the transmembrane and cytoplasmic sequence of E-cadherins that remains bound to PS1 β-catenin and α-catenin and the actin cytoskeleton (a). At the membrane-cytosol interface PS1/γ-secretase then cleaves E-cad/CTF1 in order to produce E-cad/CTF2, which dissociates from both PS1 and F-actin and is released to the cytosol in a complex with β-catenin (b). E-cadherin full length bound to the cytoskeleton could also be cleaved by PS1/γ-secretase activity directly (c) [49].

1.1.3 VE-cadherin

Vascular endothelial cadherin (VE-cadherin) is the major adhesion molecule in endothelial AJ.

VE-cadherin was first identified in 1991 by Suzuki, Sano, and Tanihara [53], who cloned the cDNAs for eight novel cadherin family members, one of which (clone 5) was found in endothelial cells. After the analyses and comparisons of sequences, based on structural similarities to the cadherin family and because of its selective expression in endothelial cells, cadherin-5 was given the name vascular endothelial cadherin (VE-cadherin) [54].

According to its genomic structure, VE-cadherin belongs to the type II cadherins [55]. Indeed, the precursor region and the cytoplasmic tail of the atypical/type II cadherins are encoded by a single exon, whereas these regions in other cadherins contain at least one or more introns. Moreover, the type II cadherins contain two extra introns in the sequence coding for the extracellular region which are not found in other cadherin families [55]. Interestingly, although VE-cadherin is classified as a type II cadherin, it only contains 58% homology with the EC1 domain of cadherin 11, the prototypical type II cadherin [55].

Moreover, type II cadherins, like type I cadherins, have a cell adhesion recognition sequence (CAR) that is required for cell-cell adhesion [56, 57]. Since VE-cadherin does possess a tryptophan in position 2, is a cadherins lacking a central alanine in the region corresponding to the CAR site of other cadherins; for this reason it has been classified as an atypical/type II cadherin, and phylogenetic analysis further suggests the unique nature of this endothelium-specific adhesion molecule [55] (Fig.5).

VE-cadherin has been shown to play a central role in vasculogenesis, angiogenesis, and the regulation of macromolecular permeability [58].

Like other cadherins, the intracellular domain (ICD) of VE-cadherin, contains a proximal binding site for p120 and p0071 (junctional plaque component, member of the p120-subfamily of armadillo

proteins, involved in cell-cell adhesion), and a distal binding site for β-catenin and plakoglobin (Fig.6a).

Plakoglobin can be recruited to VE-cadherin in place of β-catenin. Indeed, it has been shown that VE-cadherin/plakoglobin association exists mainly in mature confluent cells, unlike β-catenin, which preferentially interacts with VE-cadherin in nascent contacts (Fig.6b) [59].

Both β-catenin and plakoglobin are linked to α-catenin, which may further interact with α-actin and vinculin (another actin-binding protein). Interestingly, α-catenin cannot simultaneously link actin and be part of the cadherin/β-catenin complex [60]. In fact, α-catenin can exist as a monomer or a dimer: the monomeric α-catenin preferentially binds to cadherin/β-catenin, whereas the α-catenin dimer can only associate with actin filaments [61] (Fig.6b).

Besides, it has been proposed that the epithelial protein eplin could act as a possible bridging molecule between VE-cadherin/β-catenin and the actin cytoskeleton [62]. Indeed, eplin is localized at inter-endothelial junctions and interacts with VE-cadherin (Fig.6c). It binds to actin independently of actomyosin contractility, unlike vinculin, which instead bridges VE-cadherin/catenin to the actin cytoskeleton only upon tension [62].

Curiously, actomyosin contractility is enhanced upon junctional remodeling under extracellular stimuli. Some works suggest the existence of dynamic focal adhesion junctions (FAJ), as opposed to stable AJ in quiescent cells [63]. FAJ are remodeled through cytoskeletal pulling forces and rely on vinculin interaction with α-catenin (Fig.6d).

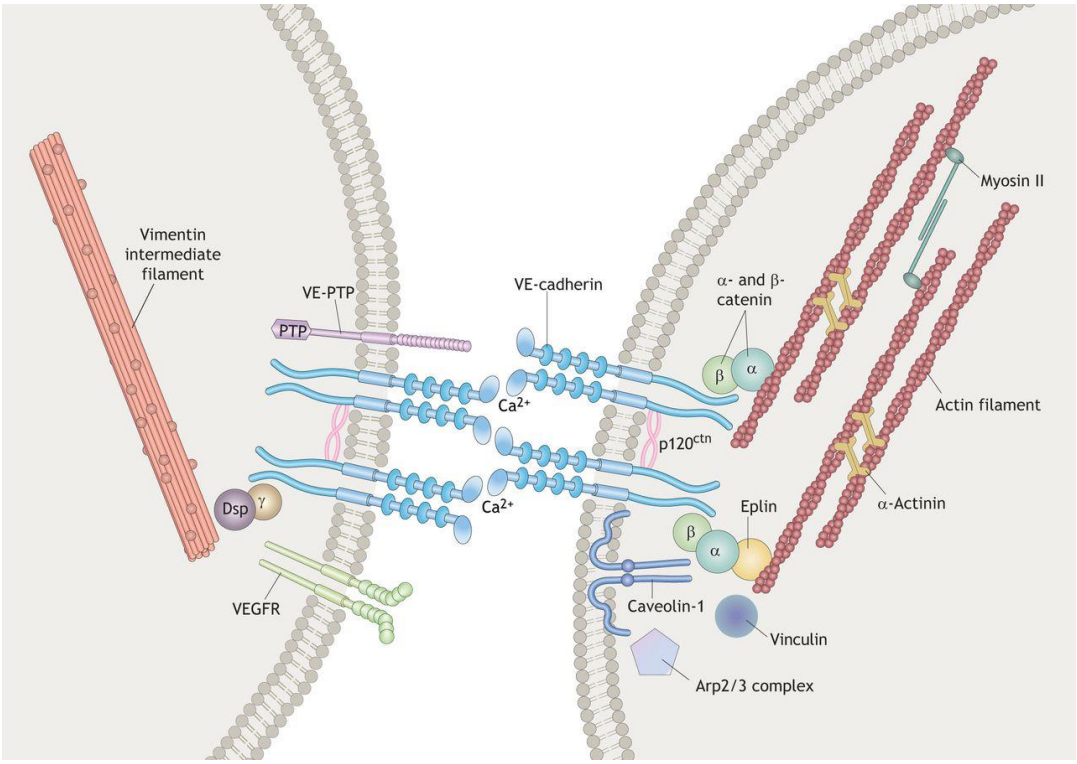


Fig. 5. VE-cadherin structure. VE-cadherin is a type II cadherin consisting of a short intracellular domain, a single-spanning transmembrane domain and a large extracellular domain that mediates homophilic cell adhesion in a Ca^{2+}-dependent manner. The cytoplasmic domain contains a sequence that binds to p120ctn (important in controlling in VE-cadherin endocytosis), β-catenin–α-catenin, as well as β-catenin–α-catenin–EPLIN (two presumable links to actin filaments) and γ-catenin–Dsp (presumably to connect to vimentin intermediate filaments). Vinculin and α-catenin are indicated to be involved in mechanotransduction. Caveolin-1 is thought to weaken the association of VE-cadherin with β-catenin upon phosphorylation. VE-cadherin clustering, stability and signaling can be modulated by VEGFR2, VE-PTP and other molecules [64].

However, in endothelial cells, the cadherin–catenin complex is remarkably dynamic, and its composition rapidly changes in relation to the functional state of the cells [54]. When the cells have weak junctions, such as at early stages of confluency, or when they are detaching and migrating VE-cadherin is heavily phosphorylated in tyrosine and mostly linked to p120 and b-catenin. In these conditions, only a very small amount of the complex is associated with plakoglobin and the actin cytoskeleton. When adherens junctions are stabilized, as in tightly confluent cells, the majority of VE-cadherin loses tyrosine phosphorylation and combines with plakoglobin and actin while both p120 and β-catenin are strongly reduced in the VE-cadherin complex [54].

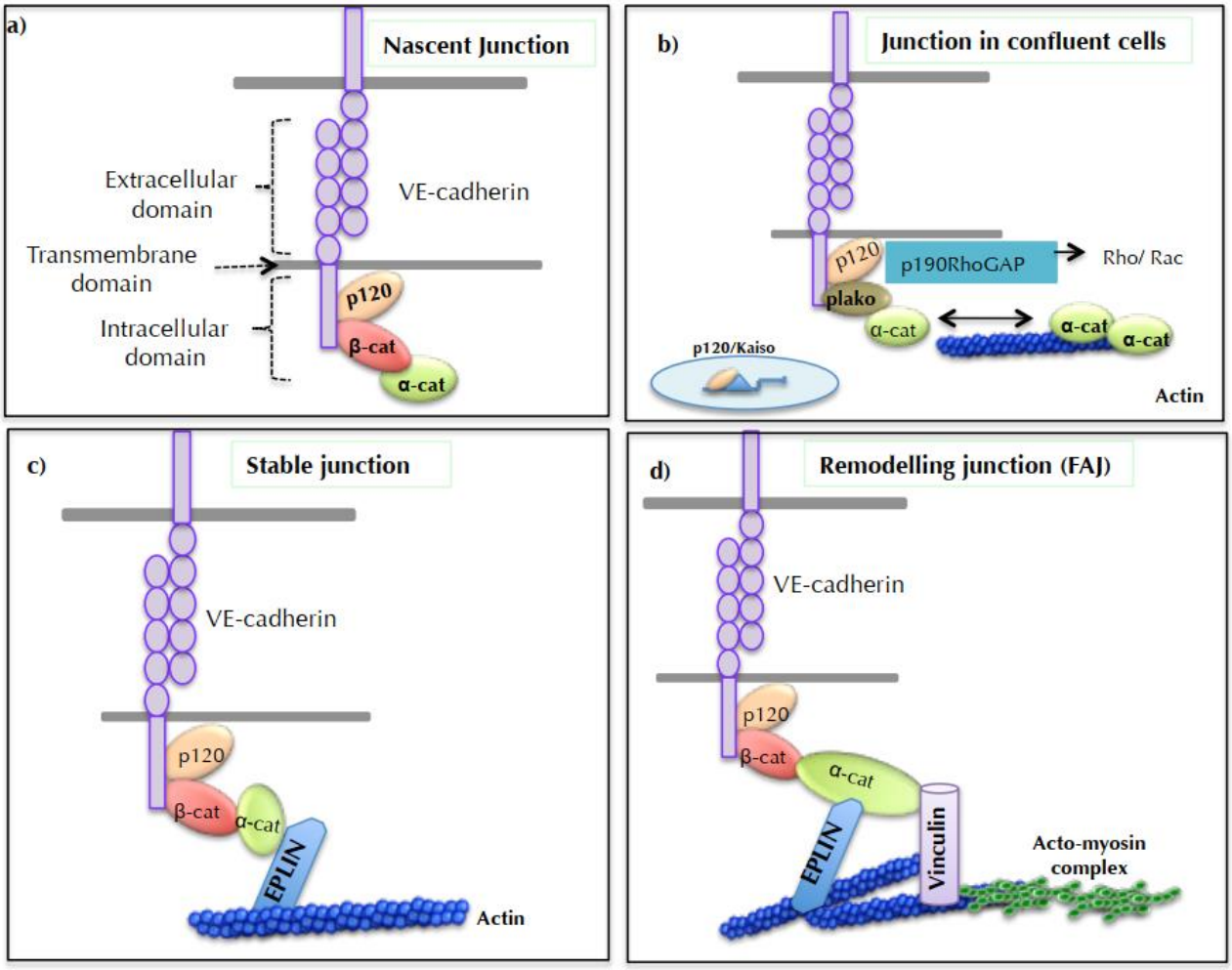
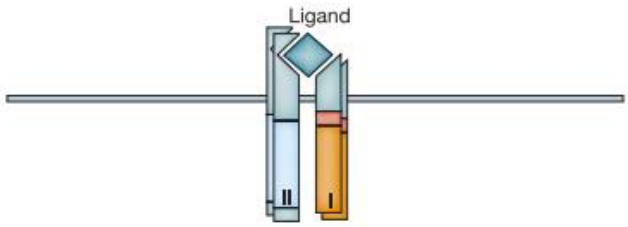


Fig. 6 VE-cadherin dynamics. (a) VE-cadherin structure consists of three domains. Extracellular domain (ECD) interacts with the ECD of VE-cadherin from adjacent cells. The transmembrane domain (TMD) enables VE-cadherin clustering at the plasma membrane. The intracellular domain (ICD) interacts with catenins such as p120-catenin, β-catenin or plakoglobin and α-catenin, which connect VE-cadherin to the actin cytoskeleton. In nascent junctions, VE-cadherin ICD is connected to β-catenin. **(b)**In confluent endothelial cells VE-cadherin ICD interacts with plakoglobin and p120-catenin. **(c)** In stable junctions, epithelial protein (EPLIN) bridges cadherin/β-catenin complex and the actin cytoskeleton. **(d)** During junctional remodeling, myosin exerts contractile tension on VE-cadherin junctions. In this scenario, vinculin is recruited and links α-catenin to actin-myosin complex.

1.2 TGF-BETA SUPERFAMILY

Transforming growth factor (TGF)-β is the prototypic member of a large family of evolutionarily conserved pleiotropic cytokines. The members of this family are involved in regulating proliferation, differentiation, migration, and adhesion of most cell types, and perturbations in their signaling pathways have been linked to different sets of developmental disorders and diseases.

At the molecular level, members of TGF-β family carry out their activities by binding a complex of type II and type I serine/threonine kinase transmembrane receptors in the intracellular juxtamembrane region; five type II receptors and seven type I receptors, also termed activin receptor-like kinases (ALKs) have been described. The receptors transduce the signals by phosphorylating carboxy-terminal serine residues of receptors-regulated (R-) Smad proteins [65, 66] (Fig.7).



Type II	Type I	R-Smad
BMPRII	ALK-2 (ActRI) ALK-3 (BMP-RIA) ALK-6 (BMP-RIB)	Smad1, Smad5, Smad8
ActRII, ActRIIB	ALK-4 (ActRIB)	Smad2
ActRIIB	ALK-7	Smad2
TβRII	ALK-5 (TβRI) ALK-1 ALK-2	Smad2, Smad3 Smad1, Smad5
AMHR	ALK-3 ALK-2 ALK-6	Smad1, Smad5

Fig.7. List of the best-documented receptor combinations and their R-Smad proteins. It is the combinatorial interactions of type II and type I receptors that define the signaling responses [67].

Depending on which type I receptor is recruited, different Smad-dependent signaling cascades are activated: ALK5 activation induces phosphorylation of Smad2 and Smad3 (TGF-β-specific R-Smads), whereas ALK1 and bone morphogenetic proteins (BMPs) lead to Smad1, Smad5, Smad8 activation (BMP-specific R-Smads) [68-71].

The activated R-Smads form hetero-oligomeric complexes with a partner Smad and the complex translocates into the nucleus where it regulates the transcription of several target genes [72] (Fig.8).

Furthermore, several non Smad-dependent signaling mechanisms have been described in mediating cellular effects of TGF-β. These include the activation of mitogen-activated protein kinases (MAPK) ERK, JNK, p38, and the phosphatidylinositol 3-kinase (PI3-kinase) [73].

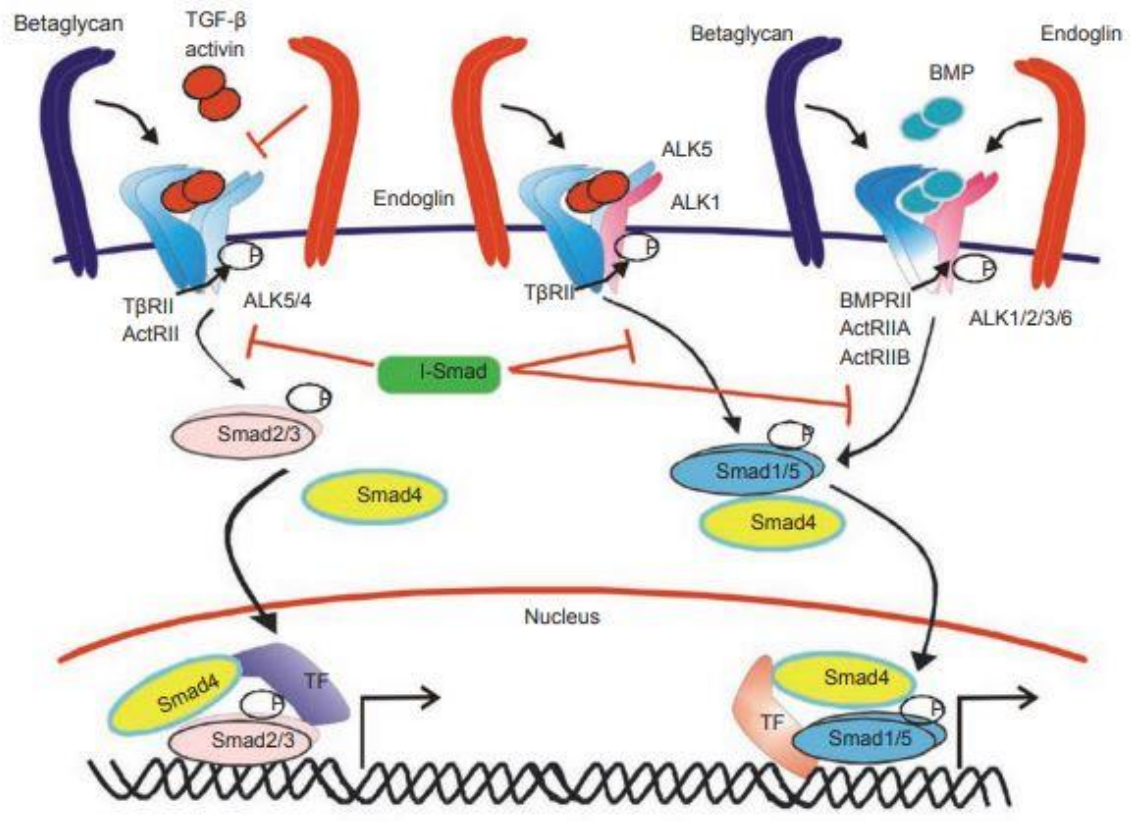


Fig. 8 TGF-β family members signal transduction. In most cells, TGF-β interacts with TβRII and ALK5, but in endothelial cells it can also signal via ALK1. BMPs interact with BMPRII, ActRIIA and ActRIIB, and type I receptors ALK1, 2, 3 and 6. Co-receptors betaglycan and endoglin can facilitate TβRII/ALK5 and TβRII/ALK1 signaling. Soluble versions of the co-receptors betaglycan and endoglin have been shown to sequester TGF-β and BMP9, respectively. Intracellular signaling can be divided into two main Smad signaling pathways: ALK5 induces phosphorylation of Smad2 and Smad3, and ALK1, 2, 3 and 6 mediate phosphorylation of Smad1, 5 and 8. Activated R-Smads form heteromeric complexes with common mediator Smad4, which accumulate in the nucleus, where they can act as transcription factor complexes and regulate the expression of specific target genes [74].

To note, Endoglin is an endothelium-expressed type I glycoprotein that mediates crosstalk between integrins and the BMP family. It is a primary vascular marker required for angiogenesis during development and increases during EC activation, inflammation, and tumor angiogenesis [75]. There is no enzymatic kinase activity associated with endoglin, but it has been hypothesized that by presenting various ligands to the receptors, endoglin specifically modulates TGF-β/ALK1 signaling [76].

1.2.1 ALK1

ALK1, also known as activin receptor-like kinase 1, is one of the seven type I receptors that mediate the proangiogenic effect of the TGF-b superfamily [77].

This receptor is expressed by endothelial cells in blood vessels during embryogenesis, adulthood and can be induced in neurovascular endothelium in response to shear stress or blood pressure during wound healing and tumor growth [78].

Biochemical studies have shown that ALK1 can interact with multiple type II receptors and their corresponding ligands, including Activin A, TGF-b1, TGF-b3, BMP-7, BMP-9, and BMP-10 [77, 79, 80], although recent studies identify BMP-9 and BMP-10 as the physiological ligands of ALK1 [80, 81].

TGF-β/ALK1 signaling has been shown to stimulate EC migration, proliferation, and tube formation [77]. However, an inhibitory effect of ALK1 signaling on EC proliferation, migration and sprouting has also been reported [81, 82].

As already mentioned, activation of ALK1 induces the phosphorylation of Smad1/5/8, whereas ALK5 promotes Smad2/3 phosphorylation. In addition, TGF-β also binds the co-receptor Endoglin; Endoglin is a type I integral membrane protein with a large extracellular domain (561 amino acids), a single hydrophobic transmembrane domain, and a short cytosolic domain [83]. The cytosolic domain of Endoglin is constitutively phosphorylated, and it can be targeted by serine and threonine kinases, including the TGF-β type I and type II receptors. Endoglin forms a protein complex with the TGF-β type I (ALK1 and ALK5) and type II receptors and the ligand. Moreover, Endoglin binds several members of the TGF-β family including TGF-β1 and TGF-β3 (but not TGF-β2), activin-A, BMP-2, BMP-7, and BMP-9 [84]. BMP-9 and BMP-10 were later demonstrated to bind endoglin with high affinity [85, 86]. Thus, BMP9 and BMP10 can bind with high affinity to TGF-β family type I receptor ALK1 while other BMPs bind with high affinity type I receptors as ALK3 and ALK6 [87].

As mentioned, ALK1 is highly expressed in the vascular structures of the embryo and markedly upregulated in response to several angiogenic stimuli [78, 88-90]. A variety of stimuli have been reported to increase endoglin or ALK1 expression in activated vessels, including hypoxia, shear stress, vascular injury, inflammation, and some related cytokines. However, BMP9 was shown to inhibit EC migration and VEGF-induced angiogenesis, and these observations suggest that the effect of ALK1 signaling on ECs is dependent on the context and specific ligand by which it is activated (Fig.9).

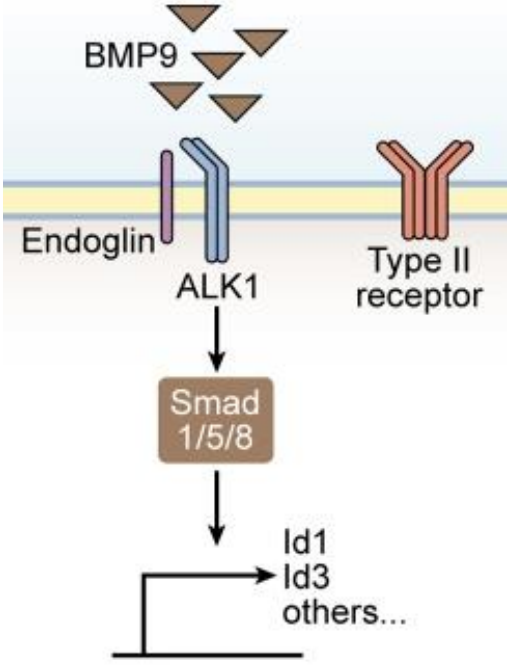


Fig. 9 Illustration of ALK1 signaling in ECs. The affinity of BMP9 for ALK1 is greater than that of TGF-β, making ALK1 will predominantly bind BMP9 when both ligands are available.

1.2.2 Bone morphogenetic proteins (BMPs)

Bone morphogenetic proteins (BMPs) were originally discovered based on their ability to induce the formation of both bone and cartilage [91].

BMPs are classified into several subgroups including the BMP-2 and -4 group; BMP-5, -6, -7, -8a, -8b group; BMP-9, -10 group; and BMP-3, -3b, -11, -12, -13, -14, -15, and -16 group [92, 93]. Among the different BMPs, BMP-2, BMP-4, BMP-6, and BMP-7 have been shown to function in vascular biology while BMP-9 and -10 have emerged as crucial factors in endothelial function and vascular diseases [92, 94-97]. To note, BMP10 regulates a similar set of genes to BMP9 except for early embryonic vascular development where BMP10 but not BMP9 works as an essential endogenous ALK1 ligand [98] (Table 1).

	Receptors			Intracellular signaling		
Ligand	**Type I**	**Type II**	**Type III**	**Pathway**	**Cellular responses**	**Refs**
BMP2	ALK6	BMPRII		Samd 1	Angiogenesis	[99]
	ALK6			Smad 3	Inflamation	[100]
BMP4	ALK6	BMPRII		Smad 1,5	Inhibition of tube formation	[101]
BMP6	ALK2	BMPRI		Samd 1,5	Vascular permeability	[102]
BMP9	ALK1	BMPRII	Endoglin	Smad 1,5	Vascular quiescence	[103]
					Proliferation, tube formation	[104] [75]
				Smad 1,5,8	Inhibition of migration, proliferation	[81]
BMP10	ALK1	BMPRII		Smad 6	Postnatal vascular development	[98]

Table 1. BMPs ligand/receptor pairs and their Smads pathway.

Once activated, the type I receptor phosphorylates and activates the receptor-associated (R)-Smads including Smads 1/2/3/5 and 8. Then, phosphorylated R-Smads bind to Smad4 and translocate to the nucleus, to regulate the transcriptional activity with coactivators and corepressors. This TGF-β-signaling pathway is regulated by a negative feedback loop via the inhibitory Smads (Smad6 and Smad7) which target R-Smads for degradation. In most cell types, the TGF-β type II receptor acts through ALK5 (TβRI) via Smad2/3, but in endothelial cells, it can also act through ALK1 (TβRI) via Smad1/5/8. Remarkably, BMP9 can specifically bind ALK1 and endoglin [105-107].

BMP9 is a key regulator of vascular quiescence [81, 103], and has been shown to protect the endothelium through the reduction of vascular permeability [102], endothelial proliferation [81], angiogenesis [108], and lymphangiogenesis [109, 110]. Although BMP9 has been more extensively characterized than BMP10, in cell culture experiments BMP10 regulates a similar set of genes as BMP9 [80] and can be a substitute for BMP9 in a mouse model of postnatal retinal vascular remodeling [108]. Moreover, like BMP9, BMP10 has been described as a mediator of flow-dependent arterial quiescence [111]. These studies suggest an overlapping role and function for BMP9 and BMP10 in the vasculature.

Optimal BMP9 and BMP10 signaling requires the type III auxiliary receptor endoglin [86]. Mutations of components of this transduction signal underline 2 important vascular diseases: hereditary hemorrhagic telangiectasia (due to mutations of ALK1, Endoglin, and MADH4/SMAD4) [112, 113] and pulmonary arterial hypertension (due to mutations of ALK1, BMPR2) [114].

1.3 HEREDITARY HEMORRHAGIC TELANGIECTASIA-HHT

Hereditary hemorrhagic telangiectasia (HHT or Rendu–Osler–Weber syndrome) is an autosomal dominant genetic disease affecting 1 in 5000 individuals [115, 116]. The clinical presentation of HHT includes potentially hemorrhagic vascular anomalies in multiple tissues and organs, including the lungs, gastrointestinal tract, liver, brain [117], in the form of arteriovenous malformations (AVMs) and mucocutaneous telangiectasias. The systemic manifestations of HHT make patient management challenging and can lead to highly debilitating and hemorrhagic events and secondary cerebral, hepatic, pulmonary, and cardiac complications [117, 118].

HHT has two most common forms HHT1 and HHT2, typically referring to the genes involved in each case [115]. Single mutations are detected in Endoglin (ENG; HHT1) (MIM # 131195) [119], Activin Receptor-Like Kinase 1 (ACVRL1/ ALK1; HHT2) (MIM # 601284) [112, 120], or MADH4/SMAD4 (JHPT, a combined syndrome of juvenile polyposis and HHT) [121-123]. There are at least two other loci for HHT, HHT-3, and HHT-4, identified by linkage analysis mapped on chromosome 5q [124] and chromosome 7p [125], respectively. A recent study has revealed that mutations in bone morphogenetic protein 9, BMP9 gene also known as GDF2 (Growth Differentiation Factor 2), cause a vascular-anomaly syndrome with phenotypic overlap with HHT [113]. 80% to 90% of HHT cases present mutations in ENG or ACVRL1 while the remaining cases are caused by mutations in SMAD4 or into other yet unknown genes [126, 127].

All the mutations responsible for HHT pathology concern proteins involved in the transforming growth factor-ß (TGF-β)-signaling pathway; these variants share common symptoms but differ from each other in the frequency of the specific vascular lesions. For example, the frequency of pulmonary AVMs in HHT2 is much lower than in HHT1 patients, whereas liver AVMs are more frequent in HHT2 than in HHT1 patients. Because of the high penetrance of the disease, the molecular genetic

testing of ENG, ACVRL1, SMAD4, and GDF2 can be highly predictive for the diagnosis of other family members in the presence of a confirmed case [128].

As describe before, BMPs acting as ligands of specific receptors, regulate different cellular functions such as cellular proliferation, differentiation, and apoptosis through the binding to a heteromeric complex of type I and type II TGF-β serine/threonine kinase receptors [129]. Downstream signaling can be mediated by a cascade of protein phosphorylation via the canonical Smad-dependent pathway, in which the gene products of the three classical HHT genes (ENG, ACVRL1, SMAD4) and GDF2 are involved. Endoglin and ALK1 are predominantly expressed in endothelial cells [88, 130-132], thus their mutations in HHT pathology exert vascular lesions.

In resting endothelial cells, endoglin is expressed at low levels, but it is highly upregulated when these cells are actively proliferating at sites of active angiogenesis and during embryogenesis [131-133]. Comparative expression studies of the endothelium in lung vessels have shown that endoglin and ALK1 have distinct expression patterns in the pulmonary vasculature and are only co-expressed in the distal (precapillary) arteries, distal veins, and capillaries, consistent with the tendency for pulmonary AVMs to form in the distal pulmonary vessels in HHT [89].

1.4 REGULATED INTRAMEMBRANE PROTEOLYSIS - GAMMA-SECRETASE

γ-secretase is a protease that cleaves peptides within the lipid bilayer in a process also called *regulated intramembrane proteolysis* (RIP) that results in the release of extracellular/luminal and/or cytoplasmic domains from transmembrane proteins.

Initially, γ-secretase was described as responsible for the cleavage at the transmembrane domain (TMD) of the amyloid precursor protein (APP) to produce the amyloid β-peptide (Aβ) that is deposited as cerebral plaques in Alzheimer's disease [134], but over the years it has been shown its ability to process several other transmembrane proteins.

It is a multimeric enzyme composed by four different integral membrane proteins: presenilin (PS), nicastrin, Aph-1, and Pen-2 [135, 136] (Fig.10).

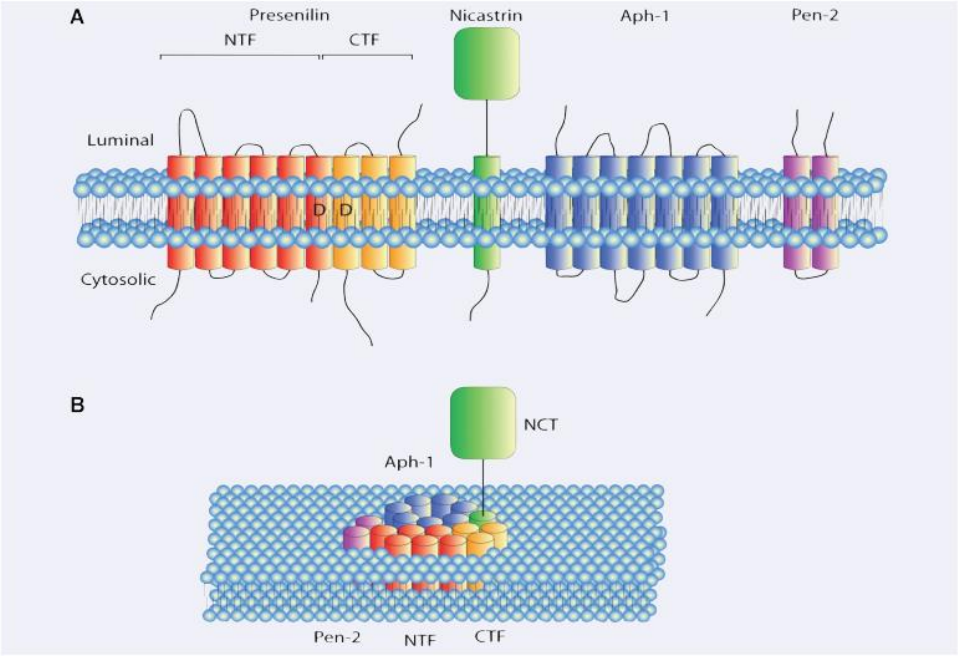


Fig.10. γ-secretase structure. a) γ-secretase is composed of four different integral membrane proteins; presenilin (PS), nicastrin, Aph-1 and Pen-2. Presenilin undergoes endoproteolysis into an N-terminal fragment (NTF) and a C-terminal fragment (CTF) that remain associated. **B)** Model of –secretase component arraignment within the active protease complex**.**

- PS is a multi-pass transmembrane protein which constitute the catalytic subunits of the gamma-secretase intramembrane protease complex. Activation of PS is dependent on its endoproteolysis into an N-terminal fragment (NTF) and C-terminal fragment (CTF) [136, 137].
- nicastrin has been described as 'the gatekeeper of the γ-secretase complex' [138]. The extracellular orientated domain of nicastrin is essential for substrate recognition by the γ-secretase complex and nicastrin binding to the substrate is required before presenilin can exert its proteolytic activity.
- Aph-1 is believed to be a scaffolding protein and Pen-2 appears to regulate PS activity.

The assembly of the γ-secretase complex begins in the endoplasmic reticulum and is concluded after translocation of the four proteins to the cell membrane [136].

Furthermore, the complex contains several different docking sites and active sites that exhibit different degrees of substrate specificity [136].

Cleavage of transmembrane proteins by presenilin leads to the generation of biologically active protein fragments that can work as signal molecules within the cell [139].

RIP of membrane receptors usually requires two sequential proteolytic cleavages, carried out by different proteases. The first cleavage occurs at the cell surface, and usually leads to the shedding of the protein's extracellular or luminal domain. This cleavage normally follows a conformational change in response to ligand binding and leads to the exposure of a cleavage site [140-142]. Notch and ErbB-4 activation have been reported to be followed by RIP involving two proteolytic steps [143, 144]. This cleavage of the ectodomain is typically carried out by proteases of the metalloprotease (ADAM) families, whose active site domain is located in the extracellular/luminal space [137, 141]. This primary cleavage shortens the ectodomain (usually to less than 30 amino acids), which allows the transmembrane cleavage to occur via the activity of γ-secretase. As a result, the cytoplasmic domain is released and translocates within the cell.

As already explained in paragraph 1.1.2, presenilin activation can result in transmembrane cleavage of E-cadherin which dissociates E-cadherin, β-catenin and α-catenin from the cytoskeleton. This leads to dissociation of the E-cadherin-catenin complex and increases the cytosolic pool of β-catenin, a key regulator of the Wnt signaling pathway [49, 145].

Around 2007 Michael E. Boulton and others identified a potential role for γ-secretase also in angiogenesis and vasculogenesis [146]. Indeed, presenilin-1 controls the growth and differentiation of endothelial progenitor cells [147]; an Ets domain, known to be a key transcriptional regulator of vasculogenesis and angiogenesis [148] is located on the PS promoter.

It has been proved that VE-cadherin can be subjected to RIP; indeed, Schulz et al. showed that fragments derived from the cleavage of VE-cadherin induced by MMPs can be generated in response to endothelial apoptosis, but they only hypothesized the subsequential involvement of γ-secretase [149].

Moreover, Georgakopoulos and colleagues have reported that presenilin-1 is a component of endothelial cell junctions and VE-Cadherin cleavage could acts as an alternative signaling pathway for angiogenesis [150].

1.5 OXIDATIVE STRESS

The use of oxygen to carry out metabolic reactions is beneficial in terms of energy balance but exposes cells and tissues to potential damage through the generation of ROS (Reactive Oxygen Species).

In fact, the mitochondrial oxygen reduction inevitably leads to the generation of unstable and reactive ROS, potentially harmful, including superoxide anion ($O_2^{-\cdot}$), hydroxyl radical ($HO^{\cdot}$) and hydrogen peroxide (H_2O_2). In addition to the mitochondrial electron transport chain, there are other enzymatic systems able to generate ROS as secondary reaction products, such as the detoxification enzymes in the smooth endoplasmic reticulum. Systems such as NADPH oxidases, on the other hand, generate ROS (superoxide anion and derivatives) as main reaction products and play a crucial role not only in the generation of toxic species needed for complete phagocytosis in specialized cells, but are also important in redox regulation of many cellular physiological processes. However, ROS generation is induced by physical (radiations) or chemical (toxics and drugs) stimuli and amplified during the inflammatory reaction.

ROS are capable to damage DNA, proteins, lipids and their production must be balanced by the presence of antioxidant defenses, in order to guarantee cell survival. The defense systems are composed of enzymatic (superoxide dismutase, catalase, peroxidase etc.) and non-enzymatic (glutathione, vitamins A, E, C) mechanisms.

Furthermore, an accurate regulation of the redox balance is guaranteed by the activation of specific "redox-sensitive" signal pathways. Various protein kinases such as AKT, PKC, MAP kinase (MAPK) and Jun kinase (JNK) are modulated by ROS and, in turns regulate the activation of redox-sensitive transcription factors such as Nrf2, NF-kB and AP-1 [151]. In this condition, different cellular responses can be observed, such as proliferation, differentiation but also the activation of apoptosis mechanism. Conversely, when the production of ROS exceeds the detoxifying capacity of antioxidant

enzymes, the cell undergoes one condition known as “oxidative stress”. The loss of redox balance occurs in various pathophysiological conditions [152-154] and has multiple effects. Irreversible damage can lead to marked denaturation of proteins, DNA mutations and membrane lipids peroxidation (Fig.11).

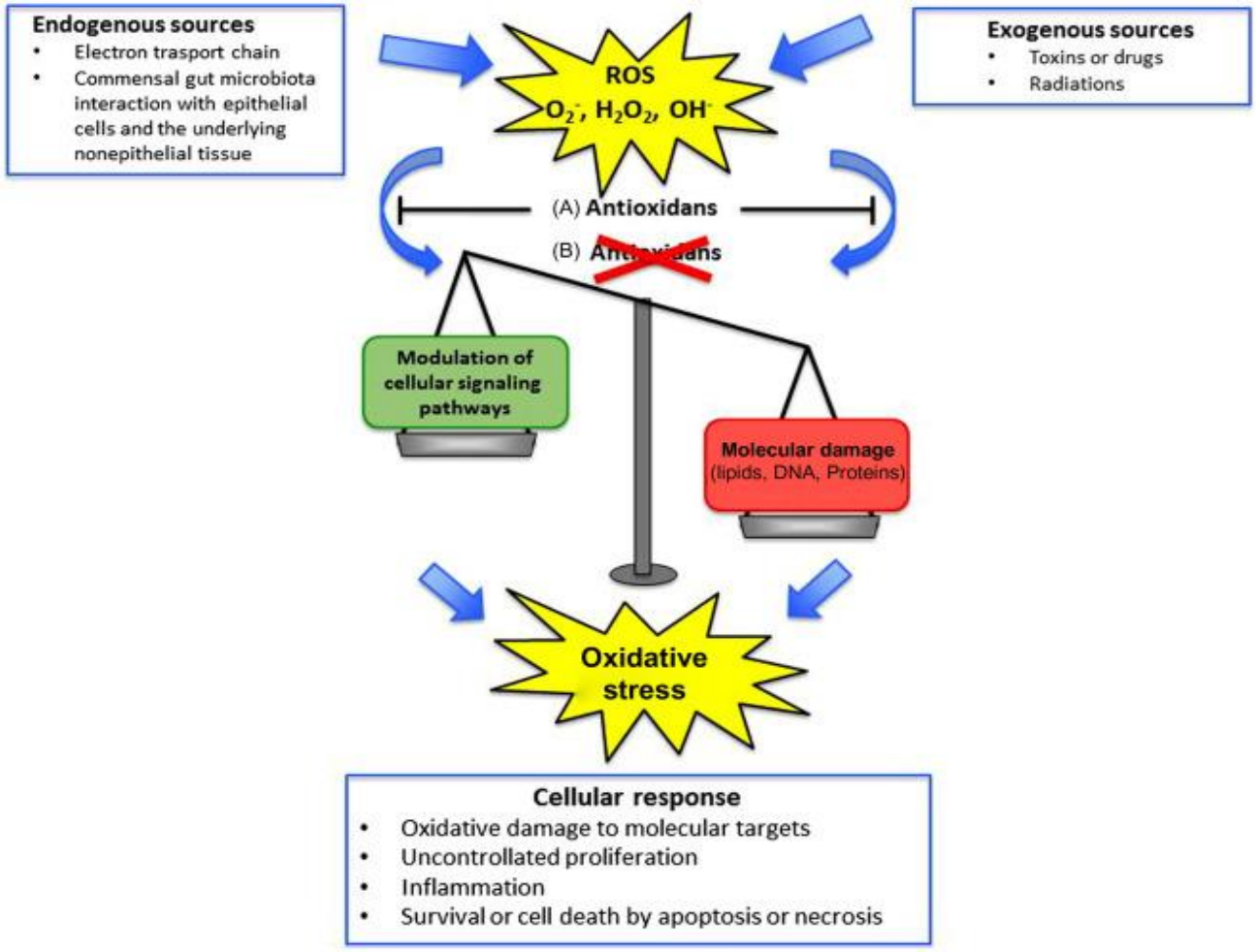


Fig.11. Factors involved in Oxidative stress and cellular response.

As a consequence, oxidative stress plays an important role in the pathogenesis of several diseases related to aging such as cardiovascular and neurodegenerative diseases, cataract, diabetes and cancer [155, 156]. In particular, oxidative stress can be both the cause and consequence of many vascular complications and is one of the biomarkers for these conditions.

In the context of endothelial cells, there is plenty of literature on the importance of ROS generation in regulating many endothelial functions as well as on their involvement in the onset and progression of different endothelial dysfunctions [157-159], but, at the same time, there are few data on the effects of oxidative stress on function and organization of AJ.

Kevil et al. have shown that endothelial cell treatment with H_2O_2 induces cadherin internalization [160]. Indeed, during oxidative stress, disruption of endothelial junctions provides the basis for increased endothelial permeability (Fig. 12b). Moreover, it has been proved that intracellular calcium concentration raises in response to oxidative stress, increasing endothelial permeability [161, 162].

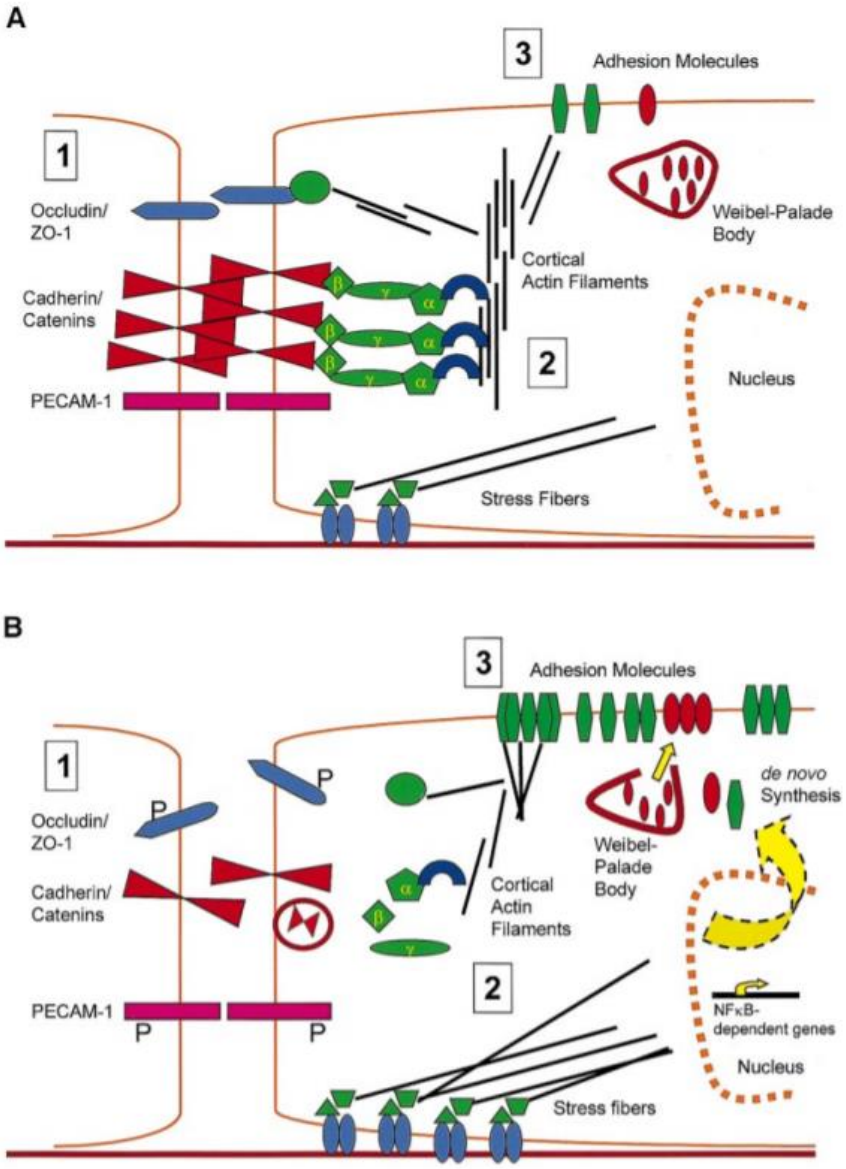


Fig. 12 structure of vascular endothelium under normal conditions (A) and oxidative stress (B).
Oxidative stress induces phosphorylation (P) and redistribution of occludin, decreases VE-cadherin level, disrupts linkage to cortical actin filaments through catenins and actin binding proteins, and induces phosphorylation of PECAM-1 (indicated in 1, in A normal condition, in B oxidative stress).
Oxidative stress reduces cortical actin band and increases formation of stress fibers, resulting in destabilization of the tight and adherens junctions, dissociation, and redistribution of the proteins (indicated in 2, in A normal condition, in B oxidative stress).
ICAM-1, ICAM-2, vascular cell adhesion molecule-1 (VCAM-1, in green) and P-selectin (red) are constitutively expressed at low level on cell surface (indicated in 3, A normal condition). Oxidative stress induces a quantitative upregulation of adhesion molecules by extrusion of preformed pools (i.e., P-selectin) and by de novo synthesis of ICAM-1, VCAM-1, and P and E-selectins. Thorugh NF-kB, nuclear factor-kB activation (indicated in 3, B oxidative stress) [157].

Furthermore, it has been proved that H_2O_2 modifies endothelial cell contraction. Indeed, myosin light chain kinase (MLC) phosphorylation is increased in response to oxidative stress [163, 164] enhancing endothelial permeability. This suggests endothelial contraction plays an important role in the oxidative stress-induced endothelial barrier dysfunction and pro-inflammatory activation.

In addition, oxidative stress may also facilitate leukocyte transmigration [160, 165]. This mechanism involves intercellular junction remodeling to enable leukocyte passage, and the remodeling involves the phosphorylation of VE-cadherin and β-catenin [166].

2 AIMS

The integrity of cell-cell junctions crucially maintains vascular functions. In particular, the complex dynamics among the different proteins in AJs are important in angiogenesis and vascular remodeling. However, the role played by VE-cadherin processing in this process is still largely unknown. In 2008 Schulz et al. [149] demonstrated that VE-cadherin undergoes MMPs-dependent proteolytic cleavage during endothelial apoptosis or calcium spikes, but the complete analysis of VE-cadherin proteolytic processing is still missing. In addition, oxidative stress is a well-recognized mediator of endothelial dysfunctions [167], being involved in the alteration of vascular permeability [165], pathological angiogenesis and endothelial damage with pro-inflammatory activation. However, the role played by oxidative stress in VE-cadherin processing or in AJ disassembly in still largely unknown. It is important to underline that more details on cadherins proteolytic cleavage came from the study of Marambaud et al. on epithelial cells [49]. Indeed, he demonstrated the involvement of both metalloprotease and γ-secretase in E-cadherin processing in cells exposed to apoptosis inducer of calcium enhancer. Thanks to the collaboration with Professor Marambaud, in this work we investigated the role of oxidative stress in VE-cadherin proteolytic processing and in AJs disassembly analyzing the involvement of MMPs and gamma-secretase. Moreover, the role of proteasome activity in VE-cad/CTFs degradation has been hypnotized accordingly with previous studies demonstrating the involvement of proteasome in the degradation of γ-secretase-derived protein fragments in other contexts [168]. Finally, considering the modifications of AJs dynamics observed in HHT pathology characterized by ALK1 and endoglin mutations [123], we analyzed the involvement of ALK1 in VE-cadherin proteolytic processing and AJs disassembly.

3 MATERIALS AND METHODS

3.1 CELL CULTURES AND TREATMENTS

Epithelial carcinoma cell line A431 (from American Type Culture Collection) was grown in Dulbecoo's Modified Eagle's medium (DMEM, Thermo Fisher Scientific) supplemented with 10% Fetal Bovine Serum (Thermo Fisher Scientific), 5% penicillin/streptomycin solution (P/S, Thermo Fisher Scientific), 2mM and Glutamine, subcultered every 2 days 1:3 and maintained in 5% CO_2 at 37° C. A431 were used between passage 2 and 15.

HUVEC (isolated from anonymous umbilical veins and from Core Facility of IRCCS AOU San Martino Genoa) were routinely cultured in Endothelial Cell Medium (ECM, Clienscience) supplemented with 5% of Fetal Bovine Serum (FBS, Clienscience), 1% of Endothelial Cell Growth Supplement (ECGS, Clienscience) and 1% of penicillin/streptomycin solution (P/S, Clienscience), subcultured every 4 days at 1:3, and maintained in 5% CO_2, humid atmosphere. HUVEC were used between passage 2 and 11.

A431 and HUVEC cells were treated on top in complete media as follows:

Sub-confluent A431 and HUVEC were exposed to 1µM Staurosporine (apoptosis inducer, from Cayman chemical), 5µM Ionomycin (calcium ionophore, from Cayman chemical) and 1µM Epoxomicin (selective and irreversible inhibitor of the 20S proteasome, from Cayman chemical).

HUVEC were also treated with 500 µm H_2O_2 (commercially available).

Pre-treatments with two different metalloprotease inhibitors, from Cayman Chemical, and γ-secretase inhibitor provided by Merck Research Labs were performed using:

- L-685,458-GSI, potent, selective, structurally novel γ-secretase inhibitor.
- GM6001, potent matrix metalloprotease (MMP) inhibitor.
- GI254023X, ADAM17 and ADAM10 inhibitor.

Pre-treatments with Recombinant Human ALK1-Fc Chimera and both Recombinant Human BMP9/10 (from R&D Systems) were performed to modulate ALK1-dependent pathway.

3.2 VIABILITY ASSAY

Viability has been measured by Trypan Blue exclusion test (from SIGMA). The blue dye enters only in non-viable cells that lost membrane integrity.

Cell viability was calculated using the ratio of total live/total cells (live and dead) and expressed as percentage values.

3.3 IMMUNOBLOTING

Protein extraction

For immunoblot analysis at the end of the experiments HUVEC were rinsed with Phosphate-Buffer Saline (PBS) and put for 2h at -80°C. After that time, Laemli buffer (1M Tris-HCl pH 6.8, 35% Glycerol, 20% SDS, 1% Blue bromophenol, 7% β-MSH) has been added and cells lysate were mechanically collected in new tubes.

Alternatively, proteins extracted with TRIZOL (Life Techonologies, USA) protocol has been used. Indeed, TRIZOL reagent allows to perform sequential precipitation of RNA and proteins.

After the separation of organic phase contains RNA, 100% ethanol was added to the aqueous phase precipitating the DNA. The samples were mix 5 times, kept at room temperature and centrifuged 2000 g for 5 min at 4°C. Phenol-ethanol supernatant was moved to a new tube with isopropanol, mixed by inversion and incubated 10 min at room temperature. After centrifugation for 10 min at 12.000 g at 4°C a protein pellet was obtained. The supernatant was discarded, and protein pellet was washed 3 times (at least) with a solution of 0.3 M guanidine hydrochloride in 95% ethanol in order to remove all TRIZOL reagent; a sequence of 20 min incubation at room temperature and centrifugation at 7000 g for 5min at 4°C was performed.

A tightly packed protein pellet was washed in 70% ethanol, allowed to dry, and resuspended in aqueous SDS 1%.

Protein assay

Protein content was measured by the BCA test (Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, USA) using, as standard, bovine serum albumin (BSA). The BCA (bicinchoninic acid) test is a colorimetric test which is based on the reduction of copper (II) to copper (I) by proteins in an alkaline environment. Two molecules of BCA are able to chelate a cuprous ion by turning the color towards intense violet, with a maximum absorbance at 570nm. The intensity of the purple color and absorbance are proportional to the concentration of proteins inside the sample.

Gel preparation

Proteins have been separated by using home-made acrylamide gels. Acrylamide and Bis-acrylamide solution (Acr/Bis) generate a molecular sieve that allows to separate proteins accordingly to their molecular weight.

Acrylamide gels are composed by two parts:

- Resolving gel: the part where proteins are separated; different percentage of Acr/Bis can be used to separate the molecular weights of interested. In our work we used 10-12 % Acr/Bis.
- Stacking gel: the part of the gel that allows to easily load the protein samples (4% Acr/Bis).

Acr/Bis is dissolved in Tris-SDS buffer and the addition of APS (Ammonium Persulfate) and TEMED (Tetramethyl Ethylenediamine), both from Bio-rad, catalyzes gel polymerization.

Electrophoresis and Western blotting

SDS-PAGE (sodium dodecyl sulphate – polyacrylamide gel electrophoresis) relies on two important features of proteins: dimension and intrinsic charge. In the electrophoresis chamber, proteins can enter the gels and can be separated into the sieve accordingly with their dimensions and their electric charge. Samples normalized on protein content, were denatured by adding of Laemli Buffer and boiled for 5 minutes. Then samples were loaded into the stacking gel.

Electrophoresis is performed in Running Buffer (50mM Tris-Base, 25mM Glycine, 1% SDS0, pH 8,5) at 100-150 V. At the end, proteins were transferred on nitrocellulose or PVDF membranes in Transfer Buffer (25mM Tris-Base, 25mM Glycine, 20% Methanol, 0,02% SDS) at 100 V.

Membranes were then incubated in a 5% milk powder solution in TBS-Tween20 (200mM Tris-Base, 1.3M NaCl, 0.1% Tween20, pH 7.5) in order to saturate free binding sites favoring subsequent recognition of the protein of interest. After 3 washes with TBS-Tween20, membranes were incubated with primary antibody overnight at 4°C. In our experiments the following antibodies were used:

- Ve-cadherin (F-8) sc-9989 mouse monoclonal IgG_1 (Santa Cruz).

- Phospho-Smad1 (Ser463/465)/ Smad5 (Ser463/465)/ Smad9 (Ser465/467) (D5B10) rabbit mAb (Cell signaling).
- Phospho-SAPK/JNK (Thr183/Tyr185) (G9) mouse mAb (Cell signaling).

After 3 washes with TBS-Tween20, membranes were incubated with specific secondary antibody conjugated with Horseradish Peroxidase (HPR) for 1h at room temperature.

The formation of complex between primary and secondary antibodies is visualized by using a chemiluminescence system ECL from Advansta. Indeed, the peroxidase on secondary antibody acts by oxidizing the luminol present in the ECL mixture, which passes to a higher state of excitation and emits luminescence. Films impressed by the luminescence were then developed revealing bands that correspond to the amount of proteins recognized by the antibody.

Densitometric analysis of the bands of interest are normalized to the result obtained, for each sample, by the analysis of the band obtained re-probing the same membrane for a housekeeping protein. In our experiments we used:

- β-actin mouse clone AC-15 (SIGMA).
- Tubulin IgG2b mouse (abcam).

For immunoblotting data, densitometric analysis was analyzed by using Image studio Lite.

3.4 CONFOCAL IMMUNOFLUORESCENCE

For confocal analysis HUVEC were seeded on 8 wells chamber slides (Nalge Nunc International) at the density of 100 x 10^3 cells per well.

At the end of the treatments cells were fixed in 4% PFA (paraformaldehyde) for 15 min, washed in PBS, permeabilized with 1% Triton X-100 + 1% BSA for 5 min, and incubated with primary antibody overnight at 4°C. In our experiments we used:

- anti-VE-cadherin antibody (mouse, Santa Cruz 1:100)
- anti-β/catenin (rabbit, Cell signaling 1:100).

Slides were washed and incubated with specific secondary ALEXA Fluor antibody (1:500 anti-mouse ALEXA Flour 546 and 1:500 anti-rabbit ALEXA 488 Thermo Fisher). For Actin staining was used Phalloidin-iFluor 633 reagent (abcam 1:1000). Nuclei were stained with To-Pro 3 iodide 1µg/ml or propidio iodide 1ug/ml. Slides were mounted, and images were acquired using a 3-channel TCS SP2 laser-scanning confocal microscope (Leica Mycrosystems).

3.5 STATISTICAL ANALYSIS

All data denote the mean ± S.E.M of *n* different experiments, as indicated. Statistical analyses were performed using Prism software package (GraphPad software, San Diego, CA). One-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests were applied when comparing more than three groups, with $p < 0.05$ considered significant.

4 RESULTS

4.1 E-CADHERIN AND VE-CADHERIN UNDERGO THE SAME PROCESSING IN EPITHELIAL CELLS

In 2002 Philippe Marambaud et al showed that apoptosis and Ca^{2+} influx stimulate a PS1/γ-secretase-dependent cleavage of E-cadherin. This cleavage results in the release of the cytoplasmic sequence of E-cadherin leading to the disassembly of cadherin-based adherens junctions [49]. Based on these previous results, it has been hypothesized that VE-cadherin could undergo the same processing in response to apoptosis and calcium spike.

Even though VE-cadherin expression characterizes ECs, they can be found in some cancer cells. Thus, the epithelial carcinoma cell line A431 that express both E-cadherin and VE-cadherin has been initially tested, in order to check similarities in the proteolytic cleavage of the two cadherins.

As shown in Fig.14, cell exposure for 6-4 h to 1μM Staurosporine (STS), apoptosis inducer, or for 90-30 min to 5μM Ionomycin (Iono), calcium influx inducer, resulted in the cleavage of both E-cadherin and VE-cadherin, and led to the generation of a 28 kDa C-terminal fragment (CTF2).

Importantly, a larger fragment of 36 kDa (CTF1) can also be observed. This band was modulated in response to cell treatments, increasing for both E- and VE-cadherin especially after the treatment with STS (4h) or Iono; to note, in the same experimental conditions, a reduction in the expression level of the full length (FL) form can be observed, evident for VE-cadherin.

Taken together, these data seem to indicate that apoptosis or calcium increase are able to activate the proteolytic cleavage of both full length (FL) cadherins, generating CTF1 and CTF2.

In addition, the inhibition of lysosomal activity obtained using 100 μM Chloroquine did not modify E-cad/CTF2 or VE-cad/CTF2 levels in A431 exposed to both STS and Iono, proving that lysosomal activity is not involved in cadherin cleavage in these experimental conditions.

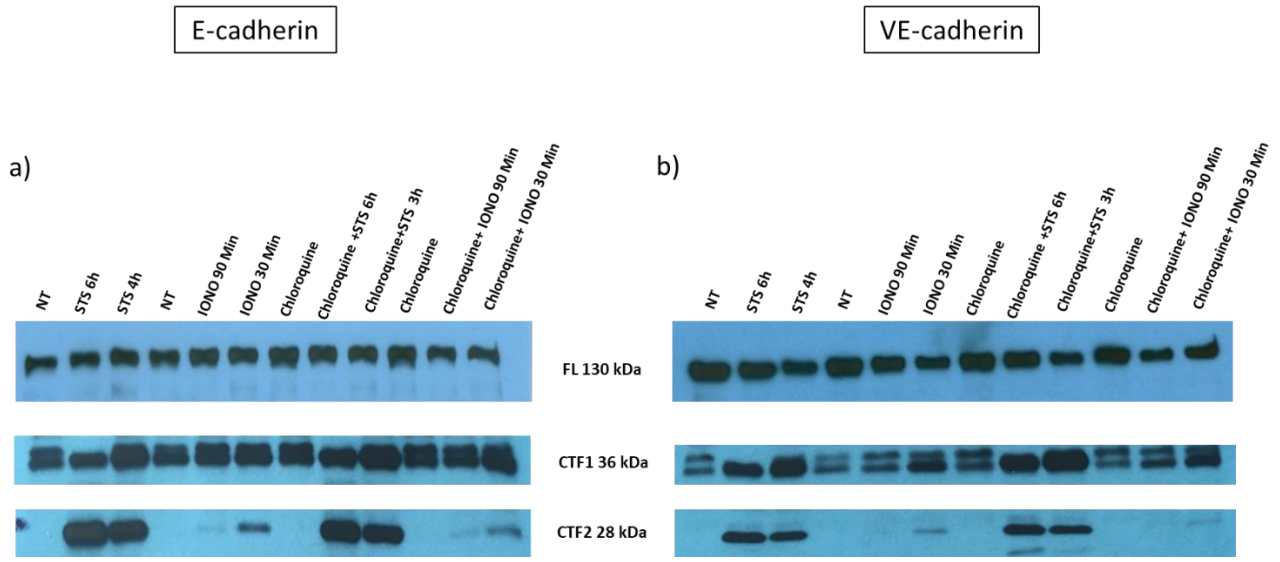


Fig. 14 Cadherins processing in epithelial cells. A431 cells were treated for the indicated times with 1µM Staurosporin (STS), 5µM Ionomycin (Iono) and 100µM Chloroquine. The blots show the expression of full length (130 kDa- upper bands), CTF1 (36 kDa- middle bands) and CTF2 (28 kDa- bottom bands) of E-cadherin (a) and VE- cadherin (b). Different blots exposures were used in order to facilitate visualization of the full length, CTF1 or CTF2 fragments.

4.2 GAMMA-SECRETASE INHIBITION PREVENTS VE-CAD/CTF2 FORMATION IN EPITHELIAL CELLS

Based on previous data showing that E-cadherin cleavage is dependent on the activity of γ-secretase [49], the involvement of this enzyme has been checked also in the context of VE-cadherin proteolysis. As shown in Fig.15A, A431 cell treatment with 1μM L-685,458 GSI, a γ-secretase Inhibitor, for 24h, was able to block VE-cad/CTF2 formation induced by STS and Iono (Fig.15A); proving that γ-secretase activity is necessary to VE-cad/CTF2 production.

Moreover, the involvement of proteasome activity in VE-cadherin cleavage has been analyzed. Indeed, proteasome can play a role in the degradation of CTFs as demonstrated in other contexts [168, 169]. By treating A431 cells with the proteasome inhibitor Epoxomicin (Epox), no significant modulations of CTFs have been observed. Indeed, 24h treatment with Epox did not induce VE-cad/CTF2 production neither modify VE-cad/CTF2 formation due to the STS and Iono treatments (Fig.15B).

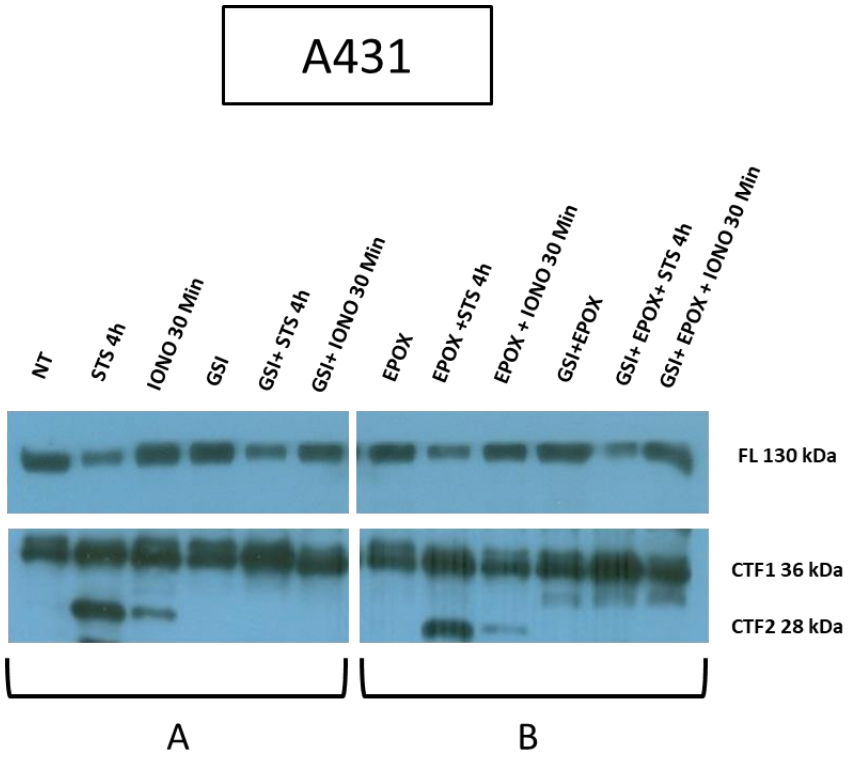


Fig. 15 VE-cad/CTF2 formation is prevented by γ-secretase inhibitor. A431 were pre-treated for 24h in absence or presence of γ-secretase inhibitor L-685,458 (GSI,1μM). Cells were then treated with STS (1μM) for 4h, Iono (5μM) for 30 min and Epoxomicin (1μM) for 6h. The blots show the three forms of VE-cadherin. Different blot exposures were used in order to facilitate visualization of the full length, CTF1 and CTF2 fragments.

4.3 PROTEASOME INHIBITION INDUCES ACCUMULATION OF THE GAMMA-SECRETASE-DERIVED VE-CAD/CTF2 FRAGMENT IN ENDOTHELIAL CELLS.

From the experiments conducted on A431 cells, we demonstrated that VE-cadherin underwent a proteolytic cleavage similar to E-cadherin after exposure to an apoptosis inducer or calcium influx enhancer, and that the generation of VE-cad/CTF2 was dependent on the activity of γ-secretase. However, given that VE-cadherin are specifically expressed in endothelial cells, we moved to Human Umbilical Vein Endothelial Cells (HUVEC), a model of endothelial cells, in order to be more consistent with the aim of our work.

The role played by proteasome in removing VE-cad/CTFs has been analyzed also in these cells. Thus, by treating HUVEC with 1μm Epox for 3-6 h the level of VE-cad/CTF2 was significantly increased (Fig. 16A), proving that proteasome activity has a role in VE-cadherin dynamics in endothelial cells. To note, it was demonstrated in other contexts that the γ-secretase products of other transmembrane proteins, such as APP, can be targeted to proteasome [168]. To the best of our knowledge, however, this is the first evidence proving that proteasome activity is involved in the catabolism of VE-cad/CTF2 fragment, and that proteasome inhibition favors VE-cad/CTF2 accumulation. Moreover, treating HUVEC with 1μM GSI for 24h both alone or in combination with Epox we demonstrated that VE-cad/CTF2 production was blocked and VE-cad/CTF1 fragment formation was increased, highlighting the role played by γ-secretase in the cleavage of CTF1 to generate CTF2 (Fig.16B).

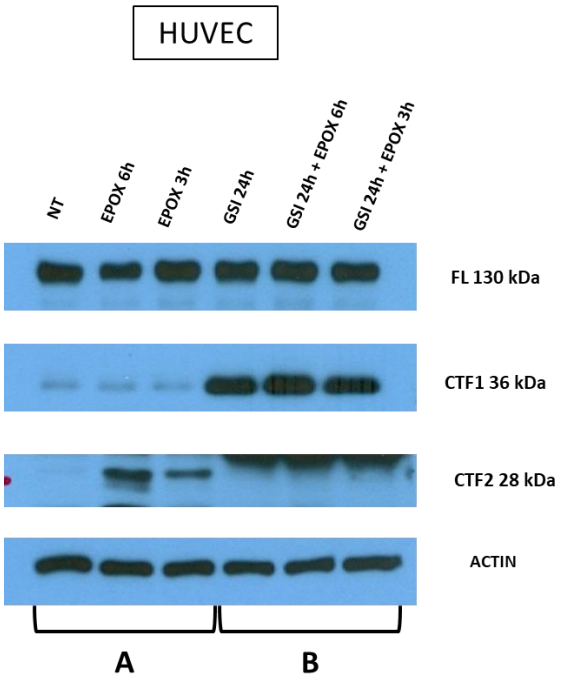


Fig. 16 Role of γ-secretase and proteasome activity in VE-cad/CTF2 formation. HUVEC were stimulated with Epoxomicin (1μM) for 3 or 6h and pre-treated with GSI (L-685,458,1μM) for 24h. The blots show the three forms of VE-cadherin and actin was used as loading control. Blots obtained by different exposure time have been used to facilitate bands visualization. The blots shown 1 representative experiment out of 4.

4.4 ALK1 PATHWAY CONTROLS VE-CADHERIN PROCESSING IN ENDOTHELIAL CELLS

It has been demonstrated that ALK1 signaling has a role in EC quiescence [170] and thus in cell-cell adhesion integrity [171]. Considering the role played by VE-cadherin in maintaining endothelial cell-cell integrity, ALK1 pathway involvement in regulating VE-cadherin cleavage has been hypothesized. Thus, in order to understand whether ALK1 signaling was involved in VE-cadherin cleavage, HUVEC have been treated with 1μg/ml ALK1-FC (ALK1 inhibitor) and 10 ng/ml BMP9 (ligand of ALK1) for 24h alone or in combination with 1μM Epox (3-6 h) that favors VE-cad/CTF2 accumulation.

As shown in Fig.17, the inhibition of ALK1 neither increased nor decreased the accumulation of VE-cad/CTF2 due to proteasome inhibition. Conversely, the activation of ALK1, provided by the treatment with its ligand BMP9, was able to decrease the VE-cad/CTF2 levels stabilized by exposure to proteasome inhibitor (Fig.17).

These findings together suggest ALK1 activity can be involved in reducing VE-cadherin disassembly.

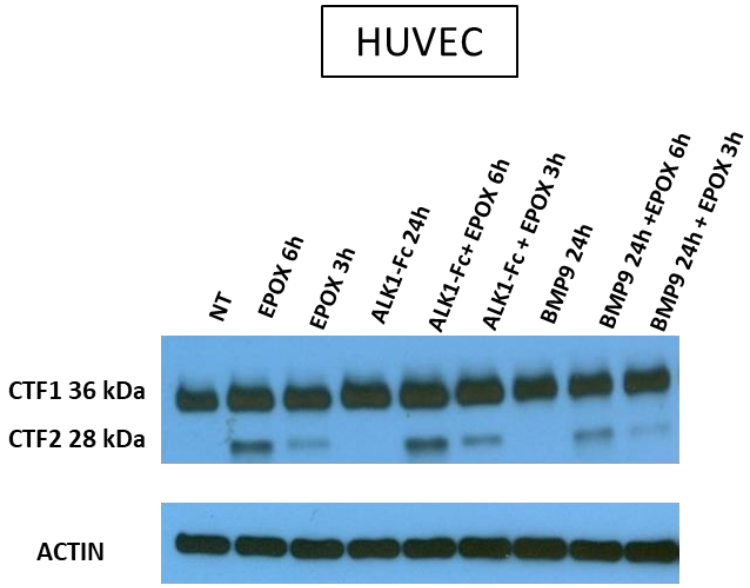


Fig. 17 ALK1 pathway in VE-cadherin processing. Representative immunoblots for VE-cadherin in HUVEC exposed to ALK1-Fc (1µg/ml) for 24h, BMP9 (10 ng/ml) for 24h with or without Epox (1µM) for 3-6h. Actin was used for normalization. Blots obtained by different exposure time have been used to facilitate band visualization.

To validate our data, HUVEC were treated not only with BMP9 but also with BMP10 that acts on the same ALK1-dependent pathway.

As shown in Fig.18A, BMP10, like BMP9, was efficient at decreasing VE-cad/CTF2 level in HUVEC exposed to Epox. Densitometric analysis shown in Fig.19 revealed that the reduction of VE-cad/CTF2 formation was about 36% and about 45% for BMP9-Epox or BMP10-Epox cotreatments (6h) compared to Epox treatment alone (6h), respectively (Fig.19).

Furthermore, cell treatment with GSI, was able to block VE-cad/CTF2 fragment formation confirming, in all the experimental conditions tested, the crucial role of γ-secretase in VE-cadherin processing (Fig.18B).

In this set of experiments, P-Smad 1,5,8 has been checked as a marker of BMP9/10-dependent signaling pathway activation (Fig.18).

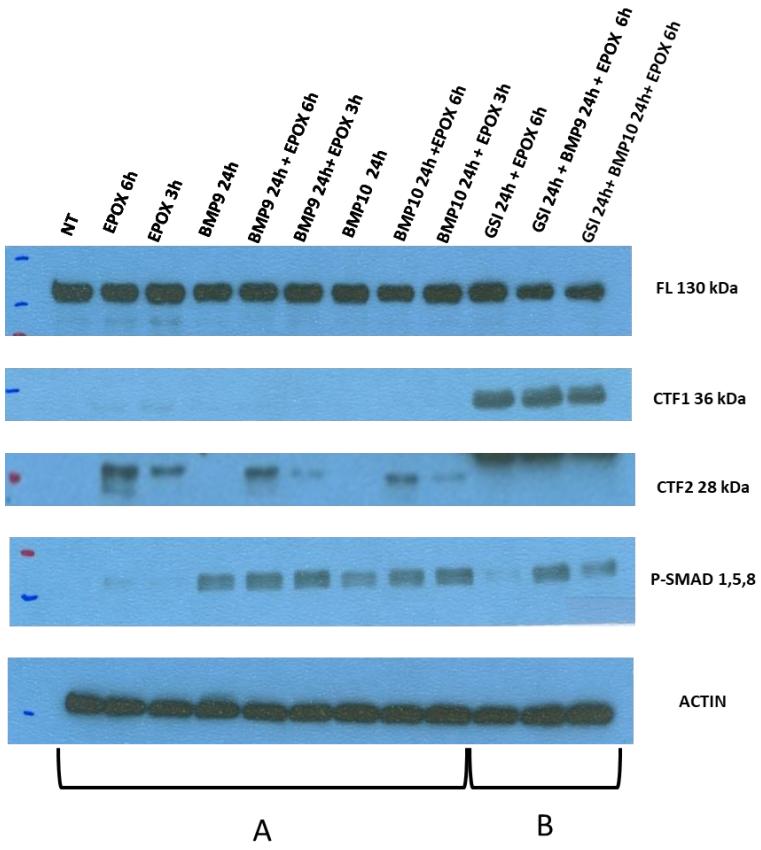


Fig. 18 BMP9 and BMP10 reduce VE-cad/CTF2 accumulation due to proteasome inhibition. HUVEC were exposed to BMP9 and BMP10 (10 ng/ml) for 24h (A) and GSI (1μM) for 24h (B) before stimulation with Epox (1μM) for 3-6h. Immunoblots for VE-cadherin, P-Smad 1,5,8 and actin were performed. Different blot exposures were used in order to facilitate the full length (FL), and CTF1 and CTF2 fragment visualization.

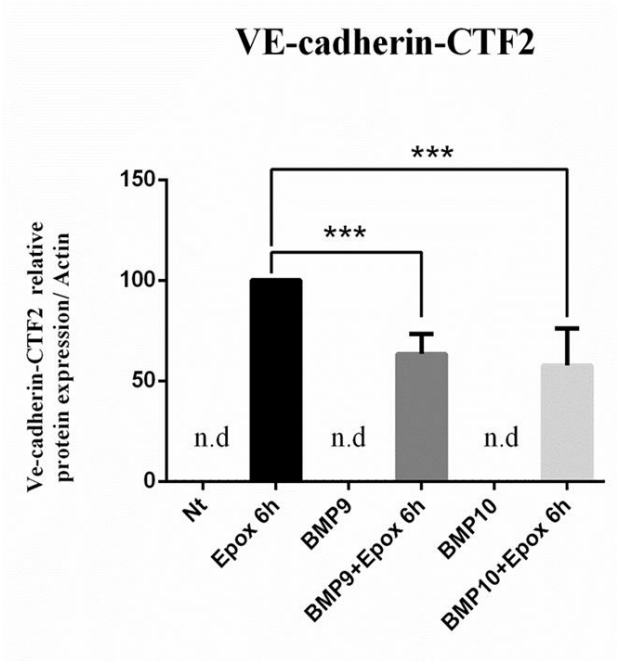


Fig. 19 BMP9/10 activity reduce VE-cad/CTF2 accumulation due to the proteasome inhibition. Densitometric analysis for VE-cad/CTF2 obtained from BMP9 and BMP10 treatments in the presence or in the absence of Epox (n = 3 for Epox and BMP9, n = 2 for BMP10). Data are mean ± s.e.m, ***P < 0.005.

4.5 OXIDATIVE STRESS PROMOTES VE-CADHERIN PROCESSING AND CTF2 PRODUCTION

Form the results shown in the previous paragraphs, it was highlighted the involvement of proteasome in the clearance of VE-cad/CTF2, the implication of γ-secretase in the generation of VE-cad/CTF2 from proteolytic degradation of VE-cad/CTF1, and the inhibitory effect of ALK1 in VE-cad/CTF2 formation.

In order to study VE-cadherin processing in the pathophysiology of endothelial cells, we decided to analyze whether the proteolytic cleavage could be triggered by oxidative stress. Oxidative stress, indeed, is a common mediator of endothelial dysfunction in different vascular pathologies [172].

As shown in Fig.20, in HUVEC exposed to 500µM Hydrogen peroxide (H_2O_2) for 6h VE-cad/CTF2 fragment was produced. In this condition, a partial reduction of cell viability was observed (Fig.20b).

To note as shown in Fig.20c, confocal microscopy showed an evident VE-cadherin redistribution in HUVEC treated with H_2O_2 compared with the untreated cells. Indeed, in non-treated HUVEC (NT), VE-cadherin was localized on plasma membrane at the endothelial cell junctions; this localization is lost when HUVEC were treated with 500µM H_2O_2, and VE-cadherin staining appeared discontinuous on the membrane with a partial redistribution inside cells. It is important to underline that for these experiments a VE-cadherin antibody mapping on C-terminal residues has been used so that the different fragments cannot be distinguished.

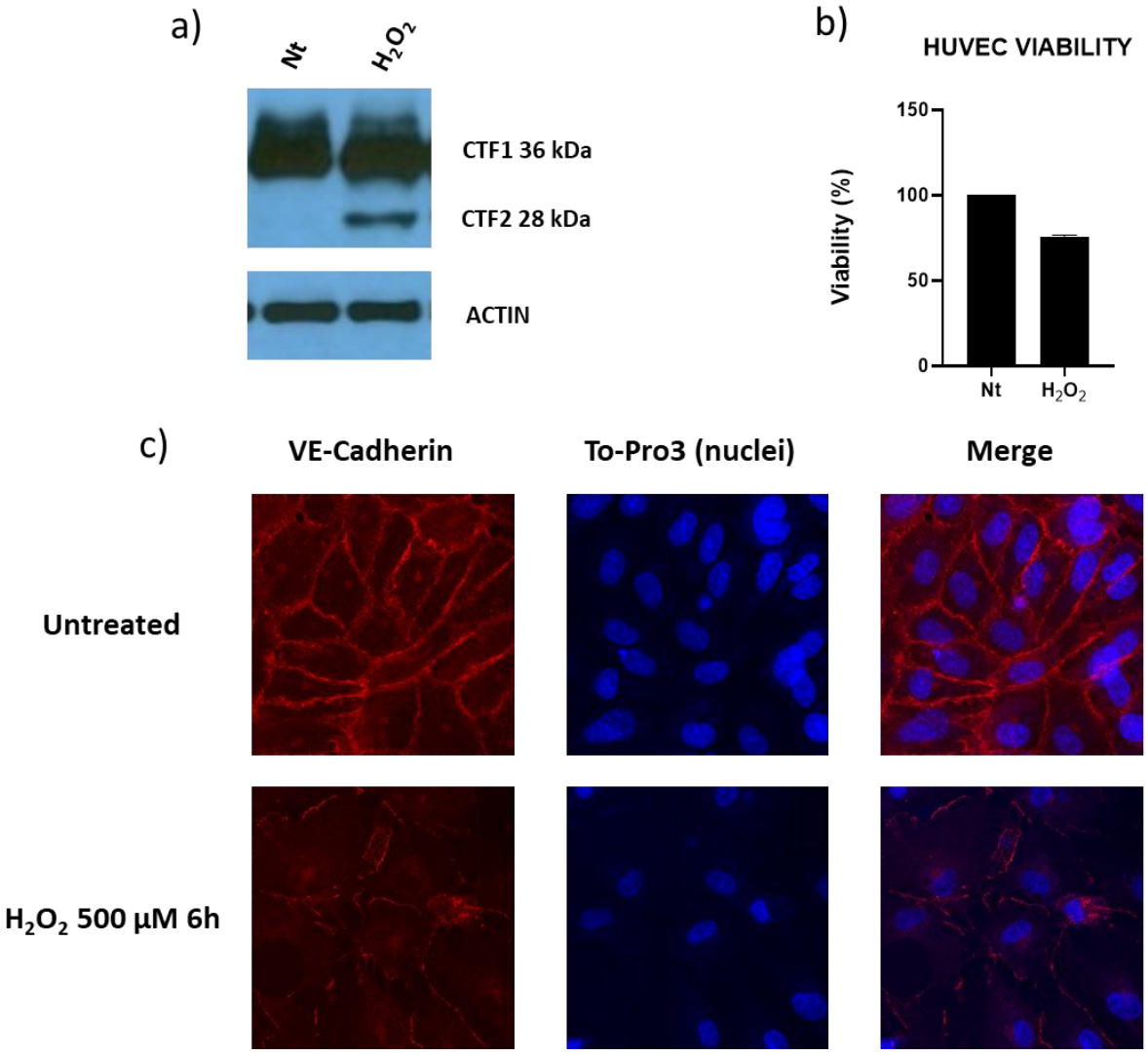


Fig. 20 H_2O_2 effects on VE-cadherin proteolytic processing and cellular localization. a) WB analysis of VE-cadherin in HUVEC cells treated with H_2O_2 (500 µM) for 6h. The expression level of actin was evaluated as internal control. One representative western blot of 4 experiments is shown. b) Trypan blue exclusion test on HUVEC exposed to H_2O_2 (500 µM) for 6h. The graph shows the mean value ± s.e.m. of 3 independent experiments. c) Confocal microscopy analysis shows the specific immunofluorescence of VE-cadherin in HUVEC cells exposed to H_2O_2 (500 µM) for 6h. Anti-VE-cadherin antibody specific for an epitope mapped between amino acids 768-784 at the C-terminus was used.

4.6 OXIDATIVE STRESS-DEPENDENT VE-CADHERIN PROCESSING IS DUE TO MMPS (ADAM10/17) AND GAMMA-SECRETASE ACTIVATION

In the 2008 Schulz et al. demonstrated that metalloproteases and γ-secretase are involved in the sequential proteolysis of VE-cadherin under apoptotic stimuli, even though they failed in the demonstration of VE-cad/CTF2 formation [149]. Based on these results, we investigated the involvement of both metalloprotease and γ-secretase in VE-cadherin processing during oxidative stress.

HUVEC have been pretreated with 1µm GSI and 10µm GI254023X (ADAM17 and ADAM10 inhibitor) and then exposed to H_2O_2. As shown in Fig.21, VE-cad/CTF2 production induced by H_2O_2 was completely prevented using MMPs and γ-secretase inhibitors.

To note, while GSI prevented VE-cad/CTF2 formation and favored the accumulation of VE-cad/CTF1, the inhibitor of MMPs was able to prevent also VE-cad/CTF1 formation highlighting that the two enzymes act on different steps, being MMPs involved in the cleavage of full length (FL) generating VE-cad/CTF1, and γ-secretase responsible for the cleavage of VE-cad/CTF1 generating VE-cad/CTF2.

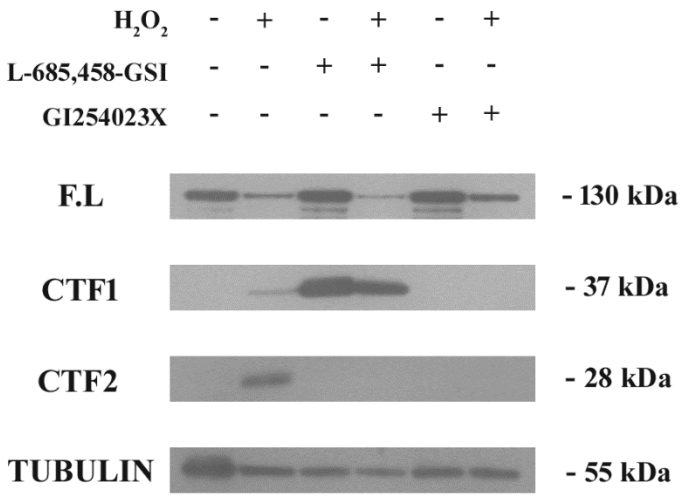


Fig. 21 γ-secretase and metalloproteases role in VE-cadherin processing under oxidative stress condition.
HUVEC were exposed to GSI (1μM) and GI254023X (10μM) for 24h before stimulation with H_2O_2 (500μM) for 6h.
Tubulin was used for normalization. Different blot exposures were used in order to facilitate the full length (FL), and CTF1 and CTF2 fragment visualization.

Moreover, pretreating HUVEC with 10 μM GM6001, a pan-metalloprotease inhibitor, VE-cad/CTF2 formation was also decreased even though not completely prevented (Fig. 22).

Based on the efficacy of GI254023X in preventing VE-cadherin cleavage, it is conceivable that the modulation of GM6001 dose or length of treatment could improve its efficacy as well. However, this has not been further investigated. Nevertheless, these experiments confirm that MMP-mediated cleavage of VE-cadherin is upstream the γ-secretase, which only acts on VE-cad/CTF1.

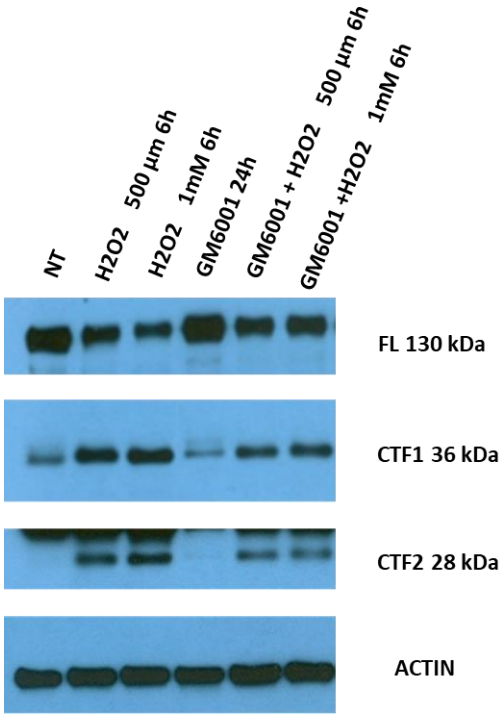


Fig. 22 Role of metalloprotease inhibitor in VE-cadherin processing under oxidative stress condition. Analysis of VE-cad/CTF2 expression levels in HUVEC treated with GM6001 (10 µM) for 24h and then exposed to H_2O_2 (500µM, 1 mM) for 6h. The expression of actin was evaluated as an internal control of the total amount of loaded proteins. Different blot exposures were used in order to facilitate the full length, and CTF1 and CTF2 fragment visualization.

4.7 ALK1 ACTIVATION REDUCES VE-CAD/CTF2 FORMATION INDUCED BY OXIDATIVE STRESS

Considering the results previously showed on the involvement of ALK1 in the reduction of VE-cad/CTF2 accumulation in HUVEC treated with proteasome inhibitor, we checked ALK1 role also in VE-cad/CTF2 formation induced by oxidative stress.

As shown in Fig.23a, pre-treatment with 10 ng/ml BMP9 or BMP10 for 24h was able to significantly reduce VE-cad/CTF2 formation induced by 500 μM H_2O_2 for 6h.

Densitometric analysis showed the 84% reduction of VE-cad/CTF2 formation in cells co-treated with BMP9 and 10 comparing to the level of VE-cad/CTF2 induced by H_2O_2 treatment alone (Fig.23b).

The level of P-JNK was monitored as marker of oxidative stress[173], and the level of P-Smad 1,5,8 as marker of BMP9 and BMP10-dependent signaling activation. Interestingly, the level of P-Smad 1,5,8 was reduced by H_2O_2. This reduction might be due to signaling cross-talks among P-Smad 1,5,8 and JNK, and future investigations will be important to clarify this aspect.

Thus, in cells exposed to oxidative stress the involvement of ALK1 signaling as negative regulator of VE-cadherin cleavage has been demonstrated.

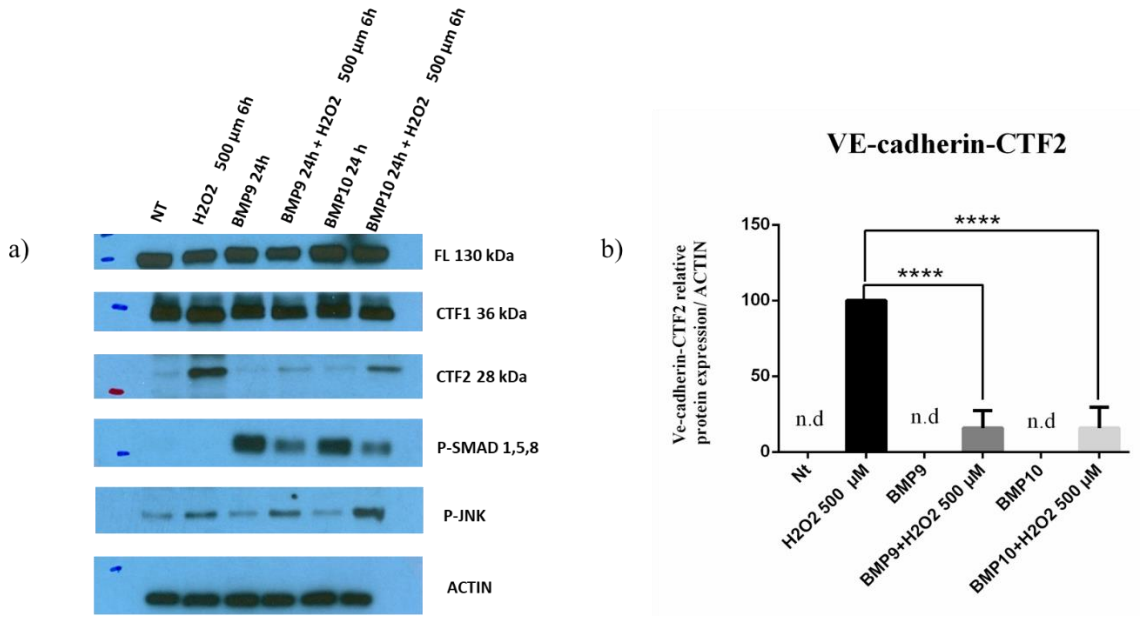


Fig. 23 BMP9 and BMP10 reduce VE-cad/CTF2 formation due to oxidative stress. a) HUVEC were exposed to BMP9 and BMP10 (10 ng/ml) for 24h before stimulation H_2O_2 (500 µM) for 6h. Immunoblots for VE-cadherin, P-Smad 1,5,8, P-JNK and actin were performed. Different blot exposures were used in order to facilitate the full length (FL), and CTF1 and CTF2 fragment visualization. b) Densitometric analyses and quantification of VE-cad/CTF2. Data are mean ± s.e.m; of 3 independent experiments. ****P<0,005.

Furthermore, VE-cadherin localization has been analysed by immunofluorescence. As shown in Fig.24, exposure to BMP9 and BMP10 strongly prevented morphological changes and VE-cadherin redistribution induced by H_2O_2. Indeed, a typical membrane localization was observed in untreated cells. The exposure to BMP9 or BMP10 alone did not modify significantly VE-cadherin membrane localization. Treatment with H_2O_2 strongly modified VE-cadherin localization that appeared discontinuous in the membrane, and also present in perinuclear zone.

Importantly, BMP9 and BMP10 were both able to reduce VE-cadherin redistribution due to H_2O_2.

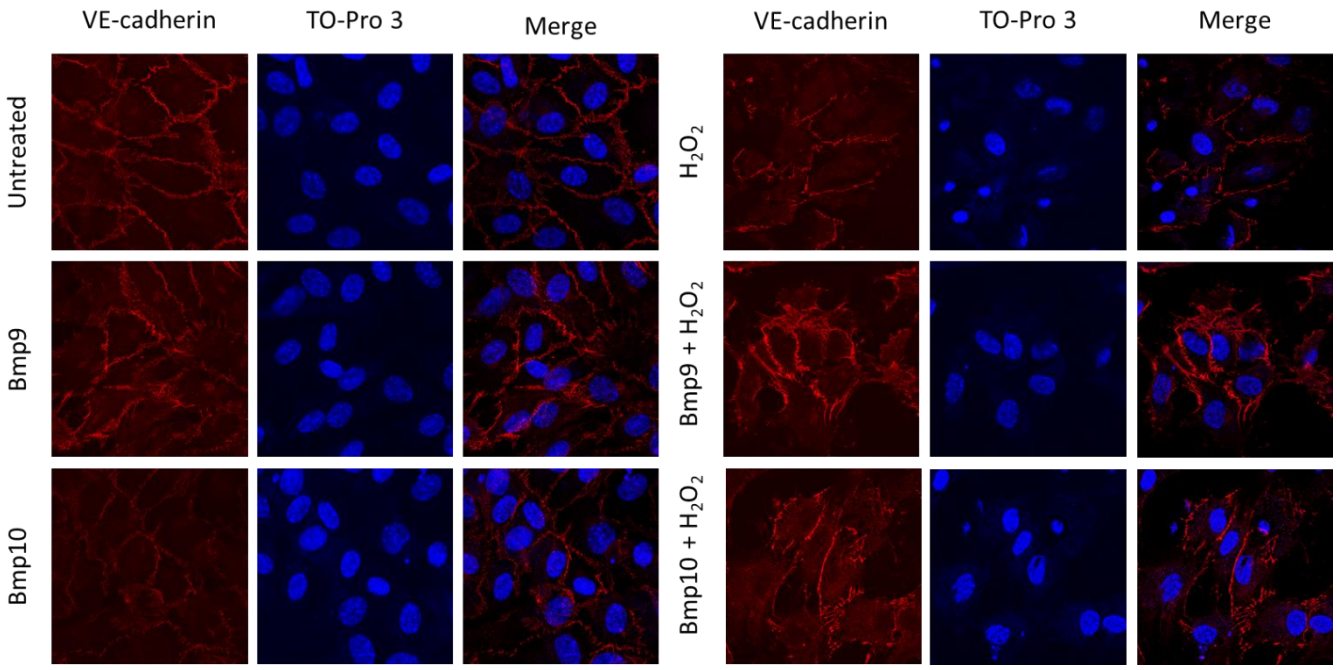


Fig.24 VE-cadherin distribution in HUVEC exposed to oxidative stress and co-treated with ALK1 activators. Immunofluorescence analysis of VE-cadherin (red) in HUVEC pre-treated with BMP9 or BMP10 (10ng/ml) for 24h and then exposed to H_2O_2 (500 μM) for 6h. Nuclei were stained with To-Pro 3 (blue).

4.8 OXIDATIVE STRESS INDUCES REDISTRIBUTION IN VE-CADHERIN/BETA-CATENIN/ACTIN COMPLEX

Based on the data obtained in the paragraph 4.5, to better understand how oxidative stress can trigger VE-cadherin processing, the organization and interaction between VE-cadherin and β-catenin was analyzed by immunofluorescence.

In Fig.25, in untreated-cells, VE-cadherin signal appeared continuously distributed along the entire cell membrane; β-catenin closely followed VE-cadherin distribution.

When HUVEC were exposed to H_2O_2, VE-cadherin and β-catenin staining on the membrane became discontinuous and some perinuclear distribution of both VE-cadherin and β-catenin was observed. Our results suggest that VE-cadherin together with β-catenin were dissociated from cell-cell junctions and re-localized inside HUVEC exposed to oxidative stress.

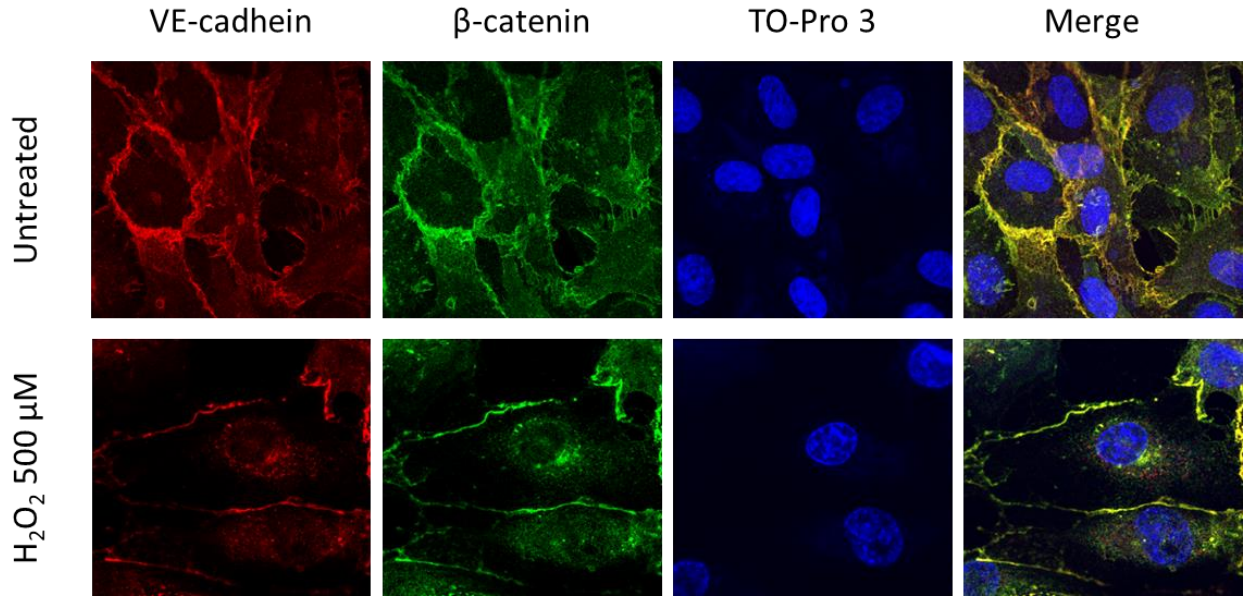


Fig.25 VE-cadherin and β-catenin localization in HUVEC under oxidative stress condition. After HUVEC treatment with H_2O_2 (500 µM) for 6h, double staining for VE-cadherin (red) and β-catenin (green) was performed. TO-Pro 3 (blu) was used as a nuclei marker.

Furthermore, the interconnection among VE-cadherin, β-catenin and actin has been analyzed in HUVEC exposed to oxidative stress and the role played by MMPs and ALK1 in AJs disassembly has been checked as well. Thus, HUVEC were pre-treated with GI254023X (ADAM10 and ADAM17 inhibitor) and BMP9 or BMP10 (ALK1 activators) and then exposed to H_2O_2. VE-cadherin, β-catenin and actin were then analyzed by immunofluorescence.

VE-cadherin/β-catenin appeared localized at cell membrane in untreated cells. Importantly, actin filaments were evident as expected. After exposure to H_2O_2 VE-cadherin/β-catenin re-localized as described before and actin appeared strongly depolarized, showing a reduced number of fibers.

Cells treated with BMP9 and BMP10 alone showed a steady state expression in VE-cadherin/β-catenin, similar to untreated cells. Indeed, they appeared as a thin line at the periphery of cells, and actin filaments appeared normal.

The combination of BMP9 and BMP10 with H_2O_2 decreased VE-cadherin/β-catenin redistribution from cell margins to the perinuclear zone and partially prevented actin depolarization, confirming the effect of BMP9 and BMP10 in reducing VE-cadherin redistribution and interaction with actin due to oxidative stress. To note, BMP9 pre-treatment seemed more efficient than BMP10 in preventing actin depolymerization.

Moreover, cell exposure to GI254023X did not modify VE-cadherin/β-catenin localization and actin distribution *per se*, but prevented VE-cadherin/β-catenin redistribution and actin depolymerization induced by H_2O_2, confirming the involvement of metalloproteases in VE-cadherin redistribution induced by oxidative stress and in the consequent AJs disassembly.

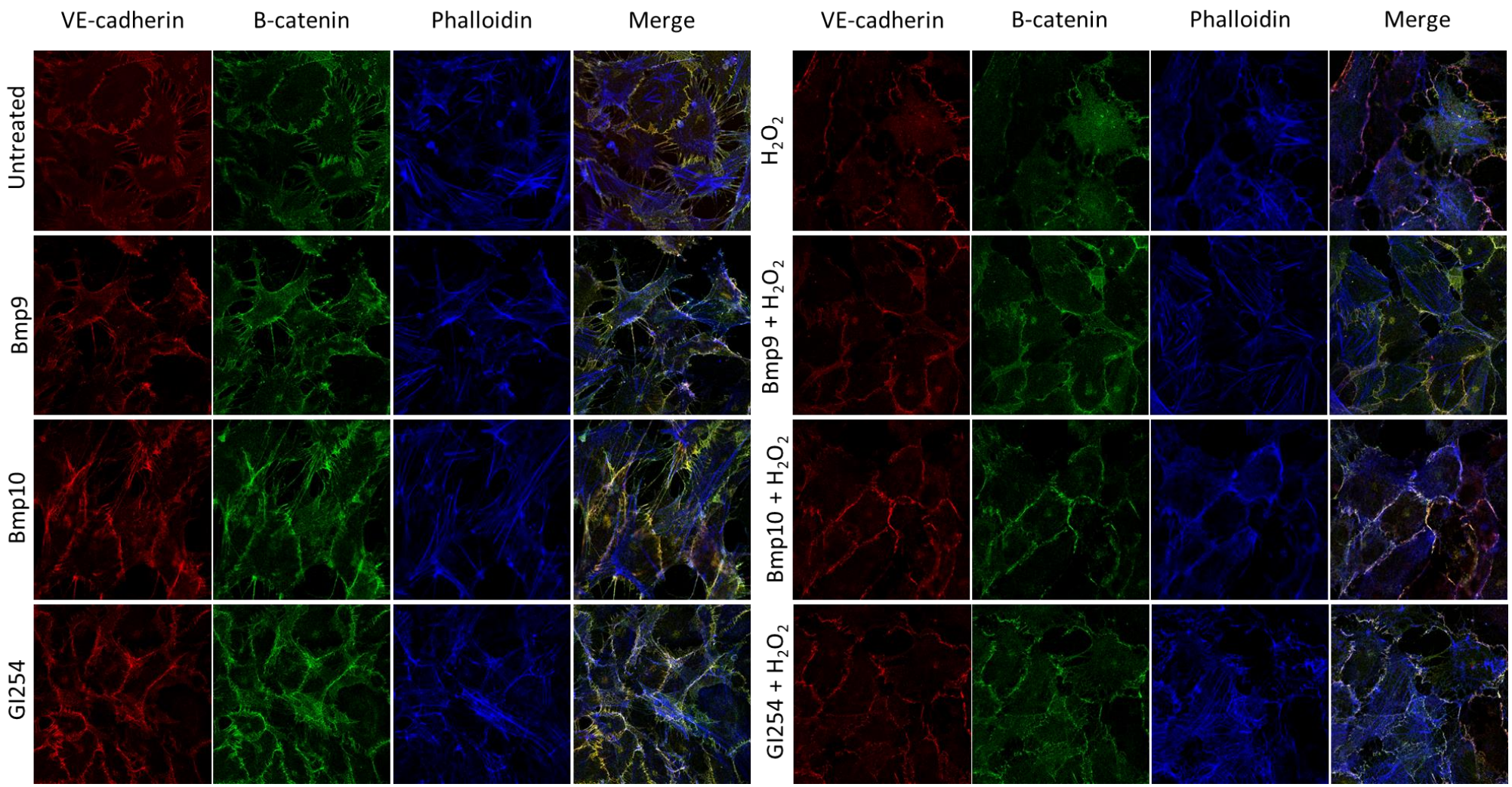


Fig.26 VE-cadherin, β-catenin and actin distribution in HUVEC exposed to oxidative stress. HUVEC were pre-treated with BMP9, BMP10 and GI254023X for 24h and then exposed to H_2O_2 for 6h. HUVEC were stained for VE-cadherin (red), β-catenin (green) and Actin (blue)

5 DISCUSSION

In this work it has been demonstrated for the first time that VE-cadherin undergoes a metalloprotease- and γ-secretase-dependent processing that can be triggered by oxidative stress and modulated by the ALK1 pathway.

Since Takeichi' observation in 1997, the family of cadherins has been recognized as primary component of cell-cell adhesion complexes. They are crucial in adherens junction formation and reorganization, which is required, for instance, in cell detachment and angiogenesis [6, 7].

Moreover, cadherin cleavage and AJs disassembly is part of cell responses to stress conditions during apoptotic death or Ca^{2+} imbalance [174, 175].

In particular, in endothelial cells, AJs play a crucial role in maintaining barrier integrity [176], in regulating permeability [177] and angiogenesis [178], and VE-cadherins are well known as mediators of survival signals in endothelial tissue [179].

Further, AJs disassembly and VE-cadherin modifications have been well demonstrated during endothelial cell damage [59, 179], and vascular pathophysiology [180] but the proteolytic pathway involved in VE-cadherin processing was largely unknown.

First of all, we demonstrated that the induction of apoptotic cell death or Ca^{2+} imbalance induce E- and VE-cadherin cleavage in epithelial cells (A431) with the generation of two C-terminal fragments of 36 and 28 kDa, named CTF1 and CTF2, respectively. Our results are in agreement with a previous study proving the proteolysis of E-cadherins induced by apoptosis and calcium and the generation of CTFs [181]. Marambaud et al. in 2002 demonstrated that under the same stimuli E-cadherin proteolytic degradation was dependent by γ-secretase [49]. The role of γ-secretase was also highlighted by other studies proving that γ-secretase is incorporated into the E-cadherin-catenin complex under conditions promoting cell adhesion [46], while under conditions of cell-cell dissociation or apoptosis the activity of γ-secretase promotes AJs dissociation [49, 150].

In this work, using A431 cell model that expresses both E- and VE-cadherin it was possible to parallel the results obtained from the analysis of E-cadherin and VE-cadherin. Thus, it has been shown that γ-secretase inhibitor (GSI) prevents VE-cad/CTF2 formation due to Ca^{2+} influx or apoptosis stimuli,

demonstrating that VE-cadherin is proteolyzed by γ-secretase as well as E-cadherin. Importantly, a massive accumulation of VE-cad/CTF1 is observed in cells treated with GSI proving the role played by γ-secretase proteolytic activity in the cleavage of VE-cad/CTF1 and in the generation of VE-cad/CTF2. This is in agreement with other studies that showed the role of γ-secretase in the proteolytic processing of VEGF receptor1 or tyrosine kinase receptors, with generation of CTFs [182, 183].

Then, in order to better understand the role of cellular degradation pathways in removing CTFs, both proteasome and lysosome activities have been considered. Indeed, it has been shown that lysosome activity is involved in the degradation of APP-CTFs [184] and that lysosomal dysfunction correlates with neurodegenerative disorders related to impaired CTFs removal [185]. Moreover, the role played by proteasome in CTFs degradation has proved for the interleukin-11 receptor that undergoes to a similar regulated intramembrane proteolysis with a generation of corresponding CTF1 and CTF2 fragments that are removed by proteasome [169]. Further, proteasome has been demonstrated to be involved in the degradation of CTFs derived by APP proteolytic cleavage [168].

Thus, co-treating A431 cells with the inhibitor of lysosomal activity chloroquine, we proved that there was no involvement of lysosomal activity in VE-cadherin processing since no modification VE-cad/CTF2 levels were observed.

Interestingly, the inhibition of proteasome activity has a strong stabilizing effect on VE-cad/CTF2 levels in HUVEC that show an accumulation of VE-cad/CTF2 when exposed to Epoxomicin. This effect was not observed in epithelial cells. However, it remains important to underline that this is the first evidence proving the involvement of proteasome activity in the degradation of VE-cad/CTF2 in primary endothelial cells.

Next, we investigated the role of oxidative stress in VE-cadherin processing. Endothelial pathophysiology is crucially driven by oxidative stress [167]. Indeed, oxidative stress is involved in modulating endothelial functions, such as in regulating permeability [165], and in many endothelial

related pathologies. However, the role played by oxidative stress in AJ disassembly is still poorly understood.

Our data demonstrate that H_2O_2 treatment, which reduces HUVEC viability of about 25%, was able not only to favor VE-cad/CTF2 formation but also to modify its subcellular localization. Indeed, under oxidative stress, the typical VE-cadherin staining on cell membrane is mainly lost with a change in junctions' conformation from zip to straight and with partial redistribution inside the cells. The straight conformation has been already observed when the cells are exposed to different flow patterns or VE-cadherin is blocked with specific antibody [176, 186] and could represent a more relaxed state of the junctions and a transition towards increased permeability [176, 186]. This interpretation will have further explored in future studies by performing permeability and migration assays in vitro. In these experimental conditions we also proved that the interconnection between VE-cadherin, β-catenin and actin are strongly modified, with redistribution of VE-cadherin and β-catenin inside the cells and actin depolymerization. Oxidative stress has been already shown to induce changes in actin cytoskeleton structure in endothelial cells [157, 187] and in myoblast [188]. Moreover, our data show that VE-cadherin and β-catenin relocate together inside the cells, suggesting the production of a cytosolic VE-cadherin/β-catenin complex. However, this aspect needs to be more investigated.

Then, we analyzed the proteolytic activities involved in VE-cadherin processing induced by H_2O_2, hypothesizing a role for MMPs and γ-secretase.

MMPs inhibition obtained using GI254023X which is high selective in the inhibition of ADAM10 and ADAM17, significantly prevents not only VE-cad/CTF2 formation, but also VE-cadherin redistribution induced by H_2O_2, as proved by immunoblot and immunofluorescence, respectively. Moreover, the analysis of the interconnection between VE-cadherin, β-catenin and actin shows the ability of metalloprotease inhibitor to prevent the complex reorganization. These results are in line with the observation that MMPs act upstream in VE-cadherin processing and their inhibition prevents the degradation of full length VE-cadherin and AJs disassembly. Our data are in agreement with other

papers in literature demonstrating the involvement of MMPs in VE-cadherin shedding and proteolytic cleavage during apoptosis [149, 174]. Besides, other studies correlate the oxidative stress with the activity of metalloprotease in the context of age-related macular degeneration (AMD) [189] and in coronary artery disease [190].

Furthermore, the activation of γ-secretase in response to oxidative stress is well proved in the context of APP processing in Alzheimer's disease [191, 192]. In our conditions, we have shown that the co-treatment with γ-secretase inhibitor and Epoxomicin (that blocks VE-cad/CTF2 degradation) or H_2O_2 (that promotes VE-cad/CTF2 formation) was able to block VE-cad/CTF2 formation leading to a dramatic accumulation of VE-cad/CTF1. To note, our data point out a two-steps proteolytic cleavage of full length VE-cadherin in response to oxidative stress that sequentially involves metalloproteases (ADAM10 and ADAM17) and γ-secretase.

VE-cadherin's role in adherens junction disassembly and in angiogenesis is a critical factor in the context of hereditary hemorrhagic telangiectasia (HHT) pathology [171]. Indeed, the formation of arteriovenous malformations (AVMs) occurs with pathological angiogenesis in ALK1-Endoglin loss-of-function mutations. ALK1 is a receptor for BMP9 and BMP10, and to investigate its role in VE-cadherin processing we first modulated ALK1-dependent signaling in HUVEC exposed to Epoxomicin. Our data show that BMP9- and BMP10-dependent ALK1 activation can reduce VE-cad/CTF2 accumulation due to Epox. In addition, ALK1 inhibition performed with a specific blocking antibody has no effect on VE-cad/CTF2 formation.

The existence of a cross-talk between VE-cadherin processing and TGF-β receptor signaling [171] or BMP/ALK signaling was demonstrated [193]. In fact, it has been demonstrated that BMP6 induces hyperpermeability by the promotion of VE-cadherin internalization and phosphorylation at Tyr685 [193]. However, our work specifically highlights the cross-talk between VE-cadherin processing and

BMP9/10-dependent ALK1 activation shedding a new light in understanding HHT pathogenetic mechanism.

Further, Jerkic et al. documented in different papers the involvement of oxidative stress in HHT pathology too. In fact, they showed that in other tissues of endoglin and ALK1 heterozygous mice, ROS levels are increased due to the formation of uncoupled eNOS [194].

Here we demonstrate that the activation of ALK1 pathway through BMP9 and BMP10 reduces VE-cad/CTF2 formation due to H_2O_2 treatment and prevents VE-cadherin cell surface disorganization. Moreover, BMP9 and BMP10 are able to reduce both VE-cadherin/β-catenin redistribution and actin depolarization induced by H_2O_2. Whether this effect is due to a reduction of VE-cadherin cleavage as to be elucidated. However, it is plausible that ALK1 pathway activation could lead to a VE-cadherin cleavage reduction preventing AJs disassembly.

6 FUTURE PERSPECTIVES

This work demonstrates the sequential involvement of MMPs and γ-secretase in VE-cadherin cleavage induced by oxidative stress. Moreover, the involvement of BMPs-dependent ALK1 activation in reducing CTFs formation and of proteasome in favoring CTFs degradation has been pointed out. Furthermore, VE-cadherin/β-catenin/actin modification due to oxidative stress is prevented by MMPs inhibition and reduced by ALK1 pathway activation.

As future perspectives of this work some important points will be deeper investigated.

Wnt/β-catenin pathway modification will be analyzed with regard to VE-cad/CTF2 production in wild type cells exposed to H_2O_2 and also in cells overexpressing VE-cadherin truncated form lacking CTFs. Moreover, the involvement of CTFs in NRF2 activity will be investigated. Indeed, it has already been demonstrated for E-cadherin that the interaction with NRF2 through β-catenin represses NRF2 nuclear translocation, which is instead favored after E-Cad/β-cat disassembly [195]. It will be interesting understand whether the VE-cadherin could have the same effects on NRF2 inhibition investigating the possible role of VE-cad/CTF2 in NRF2 nuclear translocation. Importantly, it has been demonstrated that a specific NRF2-actin complex could help NRF2 nuclear translocation under oxidative stress condition [196], and this aspect could be also analyzed in our works during VE-cadherin/β-cat/actin modification, and the F-actin/G-actin ratio will be measured as marker of depolymerization.

During the period that I spent in Professor Marambaud' lab we hypothesized to use an in vivo model to move forward this project. The use of a γ-secretase inhibitor cannot be hypothesized due to a multitude of substrates of the enzyme. However, this could be possible by identifying the exact cleavage site on VE-cadherin and generating a knocking down mouse, assuming of course that the mutation will not interfere with other properties.

Furthermore, it will be possible to create a VE-cadherin antibody to block VE-cadherin expression in mice pups affected by HHT pathology. Indeed, in another project I was involved, BL6 female mice injected with a vaccine made from ANG2-P3 peptide were used to avoid developmental defects in neonates. Indeed, all the female mice used in these experimental conditions were injected in the breast

with BMPs blocking antibody in order to create an HHT- similar pathology in the pups. Immunofluorescence on retina dissected from P5 pups from vaccinated mothers showed a decreased number of AVMs (arteriosus veins malformations) compared to the retina dissected from P5 pups from BL6 control mother. The use of VE-cadherin antibody could be also able to reduce AVMs formation.

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