Running title: Podoplanin and EMT

NEW INSIGHTS INTO THE ROLE OF PODOPLANIN IN THE EPITHELIAL TO MESENCHYMAL
TRANSITION

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ABSTRACT

Podoplanin is a small mucin-like transmembrane protein expressed in several adult tissues and with an important role during embryogenesis. It is needed for proper development of kidneys and lungs as well as accurate formation of the lymphatic vascular system. In addition, it is involved in the physiology of the immune system. A wide variety of tumors express podoplanin, both in the malignant cells and in the stroma. Although there are exceptions, the presence of podoplanin results in poor prognosis. The main consequence of forced podoplanin expression in established and tumor-derived cell lines is an increase in cell migration and, eventually, the triggering of an epithelial to mesenchymal transition, whereby cells acquire a fibroblastoid phenotype and increased motility. We will examine the current status of the role of podoplanin in the induction of epithelial to mesenchymal transition as well as the different interactions that lead to this program.

KEYWORDS

Cell motility; cancer; protein-protein interactions; cell signaling; actin cytoskeleton; CLEC-2; ERM; CD44

ABREVIATIONS

BMP Bone Morphogenetic Protein

CT cytoplasmic domain

ECM extracellular matrix

EMT epithelial to mesenchymal transition

ERM Ezrin, radixin and moesin

FRC Fibroblastic Reticular cells

HA hyaluronan

LEC lymphatic endothelial cell

MDCK Madin-Darby Canine Kidney

MET mesenchimal to epithelial transition

Pdpn podoplanin

SCC squamous cell carcinoma

TF transcription factor

TGF- β Transforming Growth Factor β

1. Introduction

Podoplanin (pdpn) is a transmembrane protein that has been identified independently by several groups in different biological contexts and, therefore, has been christened several times. It was first described by Nose et al (1990) while searching for genes induced after long-term TPA-treatment in mouse osteoblastic cells. The isolated clone (OTS-8) was shown to be present in lung tissue. Farr et al (1992) identified a glycoprotein (gp38) in mouse peripheral lymphoid tissue, which was found to be homologous to the OTS-8 protein. Rishi et al (1995) described a highly expressed gene, T1 α in rat type I lung alveolar cells. Again, T1 α was found to be homologous to the OTS-8 gene. While trying looking for influenza C virus receptors in type I Madin Darby canine kidney (MDCK) cells, Zimmer et al (1995; 1997) discovered a cell surface sialoglycoprotein, which they named gp40, that was later determined to be podoplanin; gp40 was absent from type II MDCK cells. Wetterwald et al (1996) reported E11 antigen (homologous to OST-8/gp38) in rat osteoblast and newly formed osteocytes.

Our laboratory identified PA2.26 antigen in mouse skin keratinocytes after wound healing and chemical carcinogenesis (Gandarillas *et al.*, 1997). We also detected this antigen in stromal fibroblasts. Subsequently, we characterized this antigen (Scholl *et al.*, 1999), and cloned and identified the human homolog (Martin-Villar *et al.*, 2005).

In 1997, Breiteneder-Geleff *et al.* identified a 43 kDa protein in the surface of rat podocytes, which was down-regulated during puromycin nephrosis. They named this protein “podoplanin”, due to its association with the flattening of arborized processes

present in podocytes, and this is the name that has been adopted by the HUGO Gene Nomenclature Committee.

Although other groups have continued to identify podoplanin with different names in different settings [Aggrus/gp44 as a platelet-aggregation factor (Kato *et al.*, 2003) that reacts with the monoclonal antibody 8F11 (Toyoshima *et al.*, 1995)], M2A as an antigen present in germ cell neoplasia (Sonne *et al.*, 2006), RANDAM-2 in retinoic acid-differentiated mouse P19 cells (Kotani *et al.*, 2002; 2003)], the name podoplanin is now being used routinely. However pathologists, who use immunostaining, more often revert to the designation "D2-40", that is, that of the antibody rather than that of the protein (Huse *et al.*, 2007).

Expression of podoplanin in some epithelial cells triggers the phenotypic conversion known as epithelial to mesenchymal transition, EMT (Martin-Villar *et al.*, 2005; Scholl *et al.*, 1999). Furthermore, podoplanin is expressed in a variety of tumors, being its expression associated with poor prognosis. As the malignant cells expressing podoplanin often show a mesenchymal phenotype reminiscent of an EMT (Thiery *et al.*, 2009), podoplanin could be considered as an EMT inducer. In this review, we will address this point after discussing in more detail podoplanin biochemistry and physiology, and the features of the epithelial to mesenchymal transition.

2. Podoplanin

Podoplanin is a type I transmembrane protein that can be classified as a mucin-like protein, given the fact that it is heavily O-glycosylated (approximately one third of its mass is due to carbohydrate). The podoplanin precursor is 162 amino acids long in humans (Fig. 1). Most of the variability in podoplanin size observed in different species

is due to the ectodomain (ED), which in some species (particularly in fishes) contains several small repeats, and in other groups may contain insertions or deletions of several amino acids. Three platelet activating domains, PLAG1-3 are detectable in most mammals and, perhaps, birds, although there is no experimental evidence for the latter. The dimerization motif GXXXG in the transmembrane α -helix is strictly conserved in all species, as well as the size and overall structure of the CT domain.

2.1. Protein structure

2.1.1. The ectodomain

The ectodomain of podoplanin is responsible for one of its main functions, namely the interaction with CLEC-2 (C-type lectin-like receptor 2) (Suzuki-Inoue *et al.*, 2007). These authors have studied this process in detail, identifying the residues involved. The PLAG3 motif (EDDVVTPG), glycosylated at T52 in humans, is essential for this interaction (Kaneko *et al.*, 2007; Kato *et al.*, 2003; Nagae *et al.*, 2014).

O-glycosylation of podoplanin has been studied by Scholl *et al.* (1999), Kaneko *et al.* (2007), Zimmer *et al.* (1997) and Kato and Kaneko (2014). In this later publication, in which human podoplanin was expressed in human glioblastoma LN229 cells, the authors have identified 12 O-glycosylation sites. In all the studies mentioned, the same glycan has been identified that corresponds to what is known as the Thomsen-Friedenreich (T) epitope, Gal- β -1 $\rightarrow$ 3-GalNAc-O-S/T with one or two sialic acid moieties in position 3 of galactose and 6 of galactosamine (in both cases the bond is with C 2' of sialic acid) (Springer, 1997) (Fig. 2).

2.1.2. The transmembrane domain

The transmembrane domain of podoplanin contains a strictly conserved motif, GXXXG, that has been shown to be involved in transmembrane protein homodimerization by Russ and Engelman (2000). We have shown that this motif is necessary for podoplanin homodimerization and localization in plasma membrane lipid rafts microdomains that are essential for podoplanin function (Fernández-Muñoz *et al.*, 2011). Furthermore, proteolysis by β -secretase (see below) also takes place in this domain (Yurrita *et al.*, 2014).

2.1.3. The cytoplasmic domain

Although the cytoplasmic domain (CT) is rather small, it is also essential for podoplanin activity. Our group has shown that CT mediates EMT (Martín-Villar *et al.*, 2006) through recruitment of members of the ERM family of proteins (ezrin, radixin and moesin) and activation of RhoA GTPase and its effector kinase ROCK.

Curiously, the sequence first described by Nose *et al.* (1990) has a single nucleotide deletion which results in an extended C-terminal region, from 9 to 41 amino acids. As this sequence was obtained experimentally, it would be interesting to find out whether the osteoblastic cell line MC3T3-E1 used in these experiments had the mentioned deletion or it was an experimental artifact.

2.2. Gene structure

In humans, the PDPN gene is located in chromosome 1p36.21, from 13782933 to 13815334 bp, and is 32.4 kb in length (Fig. 1, lower part). The *PDPN* gene has 6 exons; the first one contains almost exclusively the signal sequence (plus the 5'-UTR). The extracellular domain is coded by the exons 2-4. Exon 5 codes the trans-membrane domain and the 8 first amino acids of the intracellular domain. The sixth exon codes for the last amino acid, the stop codon and approximately 2 kb of 3'-UTR. This general structure is found in all organisms studied, except in lower vertebrates [chondrichthyes (cartilaginous fishes), actinopterygii (bonny fishes) and choelacanthimorpha], in which the extracellular domain is coded by a single exon. In the 3'-UTR sequence there are two poly(A) signals that account for the two mRNAs (0.9 and 2.7 kb) found in humans (Martin-Villar et al., 2005; 2009). The long mRNA has a canonical poly(A) signal, AAUAAA. However, the smaller mRNA has a non-canonical poly(A) signal, AUUAAA. Another feature of the human PDPN gene is the presence of an AluSc insertion in reverse orientation into the 3'-UTR.

In several organisms, including mammals, there is an in-frame open reading frame (76 amino acids long) upstream of the initiator AUG codon. Although this larger podoplanin cDNA could be transcribed from the mRNAs just described, it is very unlikely to be physiologically relevant: the mature polypeptide would be the one starting at Ala-23, just after the only signal sequence present in the podoplanin cDNA. Actually, transfection of this "large" cDNA in mammalian cells resulted in the normal mature polypeptide (unpublished results).

The donor splice junction between human exon 5 and intron 5 contains an alternative splice site that results in the appearance of a variant protein lacking two amino acids, Tyr-160 and Ser-161 at the end of the CT domain (see Fig. 1); about 30%

of human ESTs contain the alternative-spliced form (PDPN- Δ YS). Transcripts for both forms are co-expressed (around 66% the WT form and 33% the Δ YS form) together in different normal tissues and tumors (seminomas and SCC, unpublished results). PDPN- Δ YS is not active, both in the promotion of EMT and activation of ERM proteins (Fernández-Muñoz et al., 2011); however, its presence *in vivo* has not been proved yet.

Care must be taken with *ab initio* identification of podoplanin polypeptides from genomic assemblies of non-model organisms: the same splice that produces the PDPN- Δ YS form site contains an in-frame stop codon in the intron, so the software tends to show podoplanin ending at Ser-161 (or equivalent) instead of Pro-162.

2.3. Expression of podoplanin

2.3.1. Tissue distribution

Podoplanin is expressed in a wide variety of tissues, originating from the three primary germ layers (Table I). Besides being considered now one of the main lymphatic endothelial markers (Breiteneder-Geleff *et al.*, 1999), its importance in the physiology of the immune system has recently gained attention (Acton *et al.*, 2014; Astarita *et al.*, 2014).

In a recent global analysis of the human proteome (<https://www.proteomicsdb.org>, accessed July 2014), podoplanin is detected in lung and at low levels in the oral cavity, myometrium, ovaries, and prostate. There are numerous reports on the expression of podoplanin in tumors (see section 2.7.1).

Kotani et al. (2003) have studied podoplanin during mouse neurogenesis, finding positive cells from day E6.5 and in adult brain in glutamatergic (but not GABAergic) neurons. Other reports describe podoplanin expression in other tissues: , podoplanin has been found in the choroid plexus by Williams *et al.* (1996) and Scholl et al. (1999); Song *et al.* (2014) reported its presence in cortical neurons and its up-regulation during inflammation; it has been also described in cells of the ciliary epithelium of the eye (Williams et al., 1996); Sonne et al (2006) have identified podoplanin with the onco-fetal antigen M2A, which is expressed in developing testis and in carcinoma in situ. Interestingly, whereas M2A monoclonal antibody depends on sialic acid for recognition, D2-40 (another monoclonal antibody to the same epitope) does not (Marks *et al.*, 1999). Subpopulations of chondrocytes have also found to express podoplanin (Smith and Melrose, 2011).

2.3.2. Transcriptional regulation

Given the wide variety of tissues where podoplanin is detected, it could be expected that the *PDPN* promoter would have abundant transcription factor binding sites. However there is very scarce information on the subject. The 5'-UTR sequence from the human *PDPN* gene has been cloned by two different groups (Hantusch *et al.*, 2007; Pan *et al.*, 2014). In the former article, the general transcription factors Sp1 and Sp3 were studied in two osteoblast-like osteosarcoma cell lines MG63 and Saos-2. They also showed that there are two GpC-rich islands in the proximal part of the promoter and no TATA box. This situation is typical of GpC-rich promoters (Antequera, 2003). On the other hand, Pan et al (2014) have looked for Prox1 sites in the *PDPN* promoter, since Prox1 is a master regulator of lymphangiogenesis (Hong *et al.*, 2002;

Mäkinen *et al.*, 2007; Tammela and Alitalo, 2010; Wigle *et al.*, 2002). They found four binding sites for Prox1. However, the presence of Prox1 is not sufficient for podoplanin expression, as a recent report on the development and structure of the Schlemm's canal (a drainage tube for fluid from the anterior chamber of the eye) shows that endothelial cells of the canal, which express Prox1, do not express podoplanin (Kizhatil *et al.*, 2014). The results of Boumahdi *et al.* (2014) on cancer stem-cells in SCCs, show that Sox2 also binds directly to the *PDPN* promoter.

The rat *pdpn* promoter has been studied by Ramirez *et al.* (1997) and Vanderbilt and Dobbs (1998), who have found also sites for Sp1, Thyroid transcription factor (TTF1) and FoxM1 (previously known as TGT3). Durchdewald *et al.* (2008) and Peterziel *et al.* (2012) have demonstrated that the AP-1 transcription factor is another modulator of podoplanin. The latter authors showed that podoplanin and PTEN levels were inversely correlated in human glioblastoma cells; furthermore, down-regulation of AKT and AP-1 levels reduced those of podoplanin.

Besides these *in vitro* studies trying to identify specific transcription factors acting on the *PDPN* promoter, it is important to look for signals that result in podoplanin up-regulation. Honma *et al.* (2012) studied neonatal human keratinocytes and found that TGF- β induces podoplanin expression by the canonical TGF- β signaling pathway via Smads. They also showed induction of podoplanin expression by INF- γ , via the JAK-STAT pathway, and by IL-6 and IL-22 via STAT3 phosphorylation. IL-3 has been shown to be necessary for lymphatic endothelial cell (LEC) maintenance and, therefore, for the expression of the LEC markers Prox1 and podoplanin. Furthermore, blood endothelial cells seem to undergo trans-differentiation to LECs if cultured with IL-3 (Groger *et al.*, 2004). These authors showed induction of podoplanin by treatment

of LECs with the inflammatory cytokines: TNF α plus IFN- γ , likely by an indirect mechanism involving the induction of the IL-3 receptor. In an interesting report, Ekwall et al (2011) showed that podoplanin is up-regulated in patients with rheumatoid arthritis, but not in patients with osteoarthritis. In another report, IL-7, IL-1 α and IL-12 were shown to induce podoplanin expression; however only IL-7 was able to induce also Prox1, and to stimulate endothelial cell growth (Al-Rawi *et al.*, 2005).

Contact normalization is a phenomenon by which tumor cells surrounded by normal cells of the same isotype revert to normal (Rubin, 2006; 2008). Shen *et al.* (2010) studied genes affected by contact normalization of Src-transformed cells. Src has been shown to up-regulate podoplanin and promote cell migration by phosphorylation of the focal adhesion adaptor protein Cas (Crk-associated substrate), likely through AP-1. Conversely, podoplanin was suppressed by contact normalization.

In the human mammary adenocarcinoma MCF7 cell line, Wicki et al (2006) have shown that podoplanin expression is induced by epidermal growth factor (and to a lesser extent by FGF 2 and TGF β).

A recent report by Mei et al (2014) suggests that the cell density is another mechanism for transcriptional regulation of podoplanin. These authors have shown that podoplanin promotes tumorigenesis in oral squamous cell carcinomas, and that this process is mediated by transcriptional up-regulation of podoplanin due to the nuclear import of ErbB3-binding protein 1 (Ebp1) under conditions of high cell density. Although antibodies against Ebp1 are able to immunoprecipitate a specific DNA fragment around the Sp1 binding sites, these antibodies failed to make a super-shift in an EMSA experiment, so Ebp1 does not bind directly the podoplanin promoter.

All this information is summarized in Fig. 3, which shows the binding sites for transcription factors detected in the proximal podoplanin promoter and the signals involved in the regulation of podoplanin expression.

2.3.3. Post-translational regulation

2.3.3.1. miRNAs

As mentioned above, podoplanin mRNA has approximately 2 kb of 3'-UTR; therefore, it is not surprising to find that its expression is down-regulated by miRNAs. Two reports have addressed this issue. Cortez et al (2010) have found that miR-29b and miR-125a are deregulated in glioblastomas. These two miRNAs target *PDPN* 3'-UTR, down-regulating podoplanin expression. Interestingly, miR-29b effectively induced apoptosis and inhibited invasiveness and proliferation in glioblastomas, suggesting a link between podoplanin expression and glioblastoma aggressiveness. In another report, Sun et al (2013) have shown that miR-363 is down regulated in head and neck SCC. As this miRNA down-regulates podoplanin, the net effect in head and neck SCC is the up-regulation of podoplanin, contributing to invasiveness and metastasis of these tumors.

Contrary to the mechanism of miRNAs, a recent report shows stabilization of podoplanin mRNA by direct interaction of its 3'-UTR with the insulin-like growth factor-II mRNA-binding protein-3 (Hwang *et al.*, 2012)

2.3.3.2. Glycosylation

We have already mentioned that the ectodomain of podoplanin is heavily O-glycosylated. Several lines of evidence indicate that this modification is required for podoplanin physiological activity. First, O-glycosylation at T52 (in the human protein) is necessary for the interaction of podoplanin with CLEC-2. Animal models in which the pathway involved in podoplanin glycosylation is abrogated have the same phenotype as podoplanin null mice (Fu *et al.*, 2008). Second, a mutation in the cosmc chaperone, needed for proper folding of Core 1 β 1,3-galactosyltransferase, causes podoplanin to transform into a tumor antigen (Schietinger *et al.*, 2006).

In a recent report, Kato and Kaneko (2014) have found a new podoplanin form in cancer cells due to aberrant glycosylation. The authors observed the overexpression of the keratan sulfate biosynthetic enzymes in LN229 glioblastoma cells (WHO Grade IV); concomitantly, podoplanin expressed in these cells was found aberrantly glycosylated and sialylated, containing polylactosamine chains, a signature of keratan sulfate. An antibody recognizing this type of modification reacts with mesotheliomas, glioblastomas, lung carcinomas, and SCC cell lines (some of them transfected with human podoplanin), but not with HEK293T, lymphatic endothelial and mesothelial cells. On the other hand, a single N-Ac-galactosamine bound to serines or threonines constitutes the Tn antigen, present in numerous types of cancer (Springer, 1997). As mentioned above (Schietinger *et al.*, 2006), podoplanin with Tn antigen is the immunogen present in the aggressive mouse fibrosarcoma Ag104A (Ward *et al.*, 1989).

2.3.3.3. Proteolytic processing

Podoplanin undergoes several proteolytic processing events. Degradation by calpain might explain the absence of podoplanin in some tumor cell lines that express significant levels of podoplanin mRNA (Martin-Villar et al., 2009).

In addition, we have described that the ectodomain of podoplanin is first cleaved by a metalloprotease and, thereafter, the membrane-bound C-terminal fragment is further proteolyzed by γ 1-secretase, between Val150 and Val151, in the cytoplasmic side of the TM domain, thus releasing the intracellular domain (Yurrita et al., 2014). This processing is analog to that of CD44 (Nagano and Saya, 2004); in this case the cytosolic released fragment has been shown to translocate to the nucleus and act as a transactivation factor (Okamoto *et al.*, 2001). This regulatory pathway, however, has not been detected in the case of podoplanin.

2.3.3.4. Phosphorylation

The cytoplasmic domain of podoplanin contains two serine residues, Ser-157 and Ser-161 (Ser-167 and Ser-171 in the mouse) that are potential phosphorylation sites. In particular, Ser-157(167) is predicted to be phosphorylated by PKA and PKC. Using the mouse protein, Krishnan *et al.* (2013) observed *in vitro* phosphorylation of the CT peptide by PKA. By using cells transfected with WT, double Ser-167 and Ser-171 non-phosphorylatable (SS $\rightarrow$ AA) and phosphomimetic (SS $\rightarrow$ DD) mutants, it was shown that the AA mutant enhanced cell migration, whereas the DD mutants decreased it. The same behavior was observed in *in vitro* invasion experiments. The authors have also shown that melanoma cells in co-culture with podoplanin-expressing fibroblasts have increased motility and viability.

2.4. Podoplanin partners

Podoplanin interacts with several proteins in different cell types. Fig. 4 shows a general view of these interactions, which are described below.

2.4.1. CLEC-2

Podoplanin was characterized as a platelet aggregating factor (Kato *et al.*, 2003; Watanabe *et al.*, 1988; 1990), and C-type lectin domain family 1 member B (CLEC-1B, CLEC-2) protein was identified as its partner in the platelet membrane (Suzuki-Inoue *et al.*, 2007). These authors had previously unraveled the mechanism of platelet activation induced by CLEC-2 after binding the snake venom rhodocytin (Suzuki-Inoue *et al.*, 2006). Podoplanin and rhodocytin are the only known ligands for this receptor. Upon ligand binding CLEC-2 dimerizes and is phosphorylated in a YXXL motif in the cytoplasmic domain by the non-receptor protein kinase Syk. Further binding of Syk (and/or Src) to the CLEC-2 phospho-tyrosine via the SH2 binding domain-containing proteins LAT and SLP76 results in activation of PLC β 2 (Navarro-Nunez *et al.*, 2013). The CLEC-2 receptor is also present in dendritic cells (Colonna *et al.*, 2000); its binding with podoplanin present in LECs and in fibroblastic reticular cells plays an important role in the immune response (Acton *et al.*, 2012).

The presence of soluble CLEC-2, produced by a shedding process dependent on PKC, has been described by Xie *et al.* (2008) and Fei *et al.* (2012). This fact could increase the complexity of CLEC-2/podoplanin signaling. Two recent reports, explain in detail the functional consequences of CLEC-2/podoplanin interactions for the immune system (Acton *et al.*, 2014; Astarita *et al.*, 2014).

The interaction between CLEC-2 and podoplanin has been studied in great detail. Kaneko *et al.* (2006) described three tandem repeats (PLAG1-3), conserved in several species, responsible for CLEC-2 binding. The O-glycans present in the PLAG domains were also found necessary for CLEC-2 binding. In a subsequent work, Kaneko *et al.* (2007) observed that only PLAG3 domain was O-glycosylated, in Thr-52. The same group identified all O-glycosylation sites, besides Thr-52 (Kato and Kaneko, 2014) (see Fig. 1). Very recently Nagae *et al.* (2014) reported the crystal structure of CLEC-2 bound to a peptide containing the PLAG2 and PLAG3 domains, and O-glycosylated in Thr-52. The structure shows that the interaction takes place with the two first amino acids of PLAG3 (Glu-Asp) and the sialic acid residue linked to the GalNAc residue (actually, PLAG2 and the sialic acid bound to Gal were exposed to the solvent in the crystal and did not give any electron density). These acidic residues interact with a series of Arg residues in CLEC-2. These data are consistent with the results of Bianchi *et al.* (2014), which showed that in mouse podoplanin, O-glycosylation of Thr-34 (localized in PLAG1 and not O-glycosylated) may not be necessary for *in vivo* podoplanin function in lymphangiogenesis.

2.4.2. Tetraspanin CD9

Tetraspanins are a family of integral membrane proteins, with four transmembrane domains, that have been linked to a variety of functions in different tissues (Hemler, 2005; 2013; Yanez-Mo *et al.*, 2009). Nakazawa *et al.* (2008) have studied the interaction between the tetraspanin CD9 and podoplanin in the fibrosarcoma cell line HT1080. Consistent with the suppressor role of CD9 and the metastasis-enhancing activity of podoplanin (see below, section 2.7.2), these authors

showed that CD9-podoplanin interaction suppresses platelet aggregation and pulmonary metastasis. The interaction between the two proteins is dependent on TM1 and TM2 transmembrane domains of CD9, in agreement with previous structural studies on CD9 interactions (Kovalenko *et al.*, 2005). The fact that interaction with CD9 does not inhibit podoplanin binding to CLEC-2 suggests that CD9 affects CLEC-2 activation independently of podoplanin, probably by preventing multimerization of the protein. Interestingly, Iwasaki et al (2013) have found that CD9 is abundantly expressed in LECs and is necessary for lymphangiogenesis. These data clearly poses interesting questions in relation with the control of podoplanin-mediated interactions within the lymphatic vasculature and with platelets.

2.4.3. Galectin 8

Galectins (β -galactoside lectins) are ubiquitous proteins in mammals, with more than 15 families described. Although galectin structure indicates an intracellular localization, they can be exported to the extracellular milieu by a non-canonical mechanism in the absence of a signal sequence (Hughes, 1999). Galectin-8 has been identified as a podoplanin-binding protein, enriched in lymphatic endothelial cells. It is hypothesized that galectin-8 and podoplanin are involved in connecting the lymphatic endothelium with the extracellular matrix (Cueni and Detmar, 2009).

2.4.4. Heat shock protein A9

Tsuneki et al (2013) have looked for extracellular matrix (ECM) proteins that could interact with podoplanin. By means of immunoprecipitation and liquid

chromatography-tandem mass spectrometry analysis, they identified heat shock protein A9 (Grp75) as a ligand for podoplanin. HSPA9 has been found in multiple subcellular locations, including the plasma membrane (Wadhwa *et al.*, 2002).

2.4.5. CD44

CD44 is the main receptor for hyaluronan (HA), one of the major components of the ECM. Therefore, it is found in a wide variety of cells and tissues (Bourguignon, 2008; Toole, 2009). Due to a complex differential splicing, CD44 can be found in different isoforms; all of them have the hyaluronan binding site, a transmembrane domain and a cytoplasmic domain (the so called standard form, CD44s). Besides the hyaluronan binding site, the extracellular domain can have several additional domains that confer cell specificity, which are coded by combination of different exons (variable forms, CD44v). CD44 also interact with different proteins, such as members of the ERM protein family, ankirin or Src protein kinase.

CD44 binds podoplanin, both in tumor vascular endothelial cells (Ohizumi *et al.*, 2000) and in SCC cells (Martin-Villar *et al.*, 2010). Podoplanin and the CD44s isoform are up-regulated together during malignant progression in mouse skin chemical carcinogenesis (Martin-Villar *et al.*, 2010).

2.4.6. ERM proteins

The ERM protein family comprises three related proteins, ezrin, radixin and moesin; they belong to a larger family, the band 4.1 superfamily, whose other components are erythrocyte band 4.1 and merlin (Bretscher *et al.*, 2002). Although

located in different chromosomes, ERM proteins are highly similar: they have an N-terminal FERM/N-ERMAD domain, responsible for the interaction with numerous proteins, and a C-terminal domain (C-ERMAD) that binds the actin cytoskeleton. Both domains are linked by a flexible domain rich in α -helices that can form a coiled coil structure that allows inter- or intra-molecular interactions between N- and C-ERMADs. In the closed conformation, ERM proteins are inactive. A threonine residue (Thr-558 in moesin, Thr-576 in ezrin and Thr-564 in radixin) in the C-ERMAD domain is the key for activation: if phosphorylated, the interaction between N- and C-ERMAD domains is abolished, and both domains are able to interact with cognate proteins and F-actin respectively. In addition, ERM protein activation requires the binding of phosphatidylinositol 4,5-bisphosphate (Fehon *et al.*, 2010), although the crystallization of the *S. fugiperda* moesin cast some doubts about this mechanism (Arpin *et al.*, 2011).

Several kinases have been shown to phosphorylate the regulatory threonine, including Rho kinase, protein kinase α and θ , NF- κ B-inducing kinase (NIK; also known as MAP3K14), MST4 and lymphocyte-oriented kinase (LOK; also known as STK10). Once activated, ERM proteins interact with a variety of transmembrane proteins (see the reviews mentioned above).

The ERM binding motif of transmembrane proteins was first studied by Yonemura *et al.* (1998) in CD44 and other cell surface receptors. These authors defined a cluster of basic amino acids located in the juxta-membrane CT domain. Although these clusters are clearly involved in binding, more refined X-ray crystallographic studies (Hamada *et al.*, 2003; Pearson *et al.*, 2000; Terawaki *et al.*, 2007) have shown that the binding motif is a non-polar region, [R/K/Q]-x-x-T-[Y/L]-x-[V/L]-x-x-[A/G] (being x any amino acid), flanked by N- or C-terminal basic regions. The detailed study by

(Hamada et al., 2003) shows this motif interacts with a hydrophobic binding groove in subdomain C of the FERM domain. The C-terminal basic region of the interacting protein is disordered, but coincides with an acidic surface in the FERM subdomain C. The N-terminal basic region of the interacting protein (present in ICAM 1-3 proteins, CD43, and CD44) must stabilize the binding, although no direct interaction was detected in this study. EBP50 (ERM-binding phosphoprotein 50, also known as NHERF) binds to a different surface of the C subdomain of FERM, with a sequence M-D-W-x-x-x-x-x-(L/I)-F-x-x-(L/F) (Terawaki *et al.*, 2006). Although similar in structure and function, there must be differences between the three ERM proteins; ezrin is found mainly in the apical side of epithelial cells, moesin in endothelial cells and radixin in hepatocytes.

Our group has studied extensively the binding of podoplanin to ERM proteins (Martín-Villar et al., 2006; Scholl et al., 1999). Podoplanin CT domain, 9 amino acids long, contains a cluster of three basic amino acids responsible of this interaction (RKMSGRYSP, see Fig. 1). Site-directed mutagenesis of these basic amino acids (mainly the two juxta-membrane ones, RK) disrupts the podoplanin-ezrin interaction.

2.4.7. CCL21

Chemokines play an important role in the proper functioning of the immune system; CCL19/CCL21 interacts with the CCR7 receptor and are important to drive cell migratory events in the adaptive immune response (Comerford *et al.*, 2013). Podoplanin has been shown to interact with high affinity with CCL21, helping to create the CCL21 gradient needed for directed migration of CCR7-positive cells (Kerjaschki *et al.*, 2004). This interaction involves the re-localization of podoplanin from the apical to the basal membrane of LECs and its eventual shedding.

2.4.8. Others

The interaction between podoplanin and the product of the gene TOM1L1 [Target Of Myb1 (Chicken)-Like 1] has been detected in a global study of human protein-protein interactions, using an automatic yeast two-hybrid procedure (Stelzl *et al.*, 2005). The corresponding database (<http://www.mdc-berlin.de/neuroprot/database.htm>) was accessed in October 2014. This is a putative interaction, as there is no other reference published.

2.5. Signaling and molecular mechanisms

Although we have described a plethora of podoplanin-interacting proteins in the previous section, functional and mechanistic studies are scarce.

Podoplanin is an endogenous ligand for the platelet CLEC-2 receptor. The fact that CLEC-2 can undergo shedding may result in complex interactions between platelets and podoplanin-expressing LECs. One more added complexity is the presence of other podoplanin-interacting proteins, like CD9 (present in LECs and tumors) or galectin-8 (highly expressed in LECs).

The CT domain of podoplanin plays an important roles in podoplanin-expressing cells through its interaction with ERM proteins, as discussed above. Our results show that recruitment by podoplanin of ezrin/moesin to the plasma membrane trigger activation of the small GTPase RhoA and its downstream effector ROCK. The activation of this pathway is required for the induction of EMT by podoplanin (see section 4).

In a recent article (Martín-Villar *et al.*, 2014), we also report that podoplanin is a novel component of invadopodia, actin-rich cell membrane protrusions with proteolytic activity, used by tumor cells to invade through the basement membrane. Podoplanin increases invadopodia stability and promotes efficient degradation of the ECM by modulating a pathway that involves the activation of ROCK and LIMK kinases and inactivation by phosphorylation of the F-actin severing protein cofilin.

We and others (Barth *et al.*, 2010; Fernández-Muñoz *et al.*, 2011) have found that podoplanin is located in plasma membrane detergent-resistant-domains (lipid rafts). Our work demonstrates that the TM and CT domains are necessary for detergent insolubility of podoplanin. The PDPN-ΔCT mutant, which lacks the cytoplasmic tail, does not localize in lipid rafts. The change of the podoplanin TM domain for that of non-lipid raft proteins abolishes completely the detergent insolubility of podoplanin. The mutant form PDPN-G137L, which destroys the dimerization motif GXXXG, is detergent-soluble. Finally, we found that the presence of podoplanin in lipid rafts is needed for podoplanin-mediated recruitment and activation of ERM proteins, induction of EMT and invadopodia functionality.

Two recent articles (Acton *et al.*, 2014; Astarita *et al.*, 2014) have reported a podoplanin-mediated signaling pathway in the interaction of Dendritic and Fibroblastic Reticular cells (FRC) in the mouse lymph node in mouse model systems. Podoplanin would maintain a contracted state in FRC by interaction with ERM proteins and activation of RhoA. The authors hypothesize that upon engagement of CLEC-2, podoplanin would be phosphorylated in Ser-167 releasing ERM proteins and therefore the RhoA-dependent contractivity would disappear. The interaction between podoplanin and CLEC-2 would maintain podoplanin in lipid rafts bound to CD44, which

could act as an inhibitor of phosphorylation to retain podoplanin in an inactive state.

The mechanism proposed by the authors about the role of Ser-167 must be taken with caution, as it is based on the results of Krishnan et al. (2013) who used double mutants of Ser-167 and Ser-171 in *in vitro* systems.

2.6. Podoplanin in development

The use of podoplanin null mice has allowed the identification of developmental programs affected by this protein. Ramirez et al (2003) generated *pdpn*^(-/-) mice that die immediately after birth, due to a failure in the development of lung alveolar type I cells.

Another group has studied heart development in a new podoplanin null mouse (Douglas *et al.*, 2009; Gittenberger-de Groot *et al.*, 2007; Mahtab *et al.*, 2009; Mahtab *et al.*, 2008); the KO mice show hypoplasia of the pulmonary vein, left atrium dorsal wall and the atrial septum. These authors hypothesize that in the developing heart podoplanin is involved in EMT (detailed in Section 4). Zavery et al (2014), in a different approach, have studied deletions in chromosome 1p36 responsible of cardiovascular malformations and cardiomyopathies. Genes that may cause cardiovascular malformations include *PDPN*.

The best studied developmental role of podoplanin is in lymphangiogenesis. The first data from Schacht et al (2003) showed that podoplanin null mice (but *prox1*⁺) have a disrupted lymphatic vasculature. Further work (Abtahian *et al.*, 2003; Sebzda *et al.*, 2006; Suzuki-Inoue *et al.*, 2010; Uhrin *et al.*, 2010) demonstrated that this disruption was due to a defect of platelet activation with concomitant failure in the separation of budding lymphatic vessels from the developing blood vessels. The same

phenotype was obtained in mice deficient in one of the enzymes of the O-glycan synthesis (Fu et al., 2008).

2.7. Podoplanin in cancer

2.7.1. Expression in tumors

As shown in Table II, podoplanin is expressed in a wide variety of tumors. An interesting case has been recently described in benign fibrous histiocytoma, in which it has been found in-frame fusions between the whole podoplanin molecule and the catalytic domain of PKC ζ (Płaszczyc *et al.*, 2014). Two other proteins, CD63 and LAMTOR1 (late endosomal/lysosomal adaptor, MAPK and MTOR activator 1), both located in endosomal membranes, have been found fused with the catalytic domain of PKC ζ . All three fusions have been detected in the genome and also as transcribed RNA. To our knowledge, this is the first time that a gene fusion with podoplanin is described. Most of the studies on the presence of podoplanin in tumors have been done by immunostaining, with antibodies directed against the EC domain. The results of Płaszczyc and coworkers, therefore, poss the possibility of other podoplanin fusions in other types of tumors, and offers interesting new avenues in the role of podoplanin in tumorigenesis and signaling.

The relevance of podoplanin expression in tumors with regard to its prognostic value is not completely defined; In general, podoplanin expression correlates with increased tumor progression and metastasis; however, several reports have shown that podoplanin may be a favorable prognosis factor in SCC of uterine cervix (Carvalho

et al., 2010; Dumoff *et al.*, 2005; Dumoff *et al.*, 2006) and SCC of the lung (Ito *et al.*, 2009; Shimada *et al.*, 2009).

Atsumi *et al.* (2008) have found that podoplanin expression in the A431 SCC cell line fulfills some characteristics that make it a candidate for a marker of tumor initiating SCC cells.

Our group has studied the presence of podoplanin in human cell lines (Martin-Villar *et al.*, 2009; 2014). Table III shows the tumor cell lines that express podoplanin. In this study, we found that some lines did express podoplanin mRNA, but not the protein. One possible mechanism for this fact could be calpain mediated degradation of the protein (Martin-Villar *et al.*, 2009)

2.7.2. Role in cell migration, invasion and tumor progression

We have shown that expression of podoplanin in MDCK cells increases their motility (and induces EMT, see section 4.1), switching from a relatively slow collective pattern of cell migration to a faster, individualized cell locomotion during wound healing, and increased invasiveness through matrigel (Martín-Villar *et al.*, 2006). Podoplanin expression in a human immortalized keratinocyte cell line, HaCaT, also promoted cell scattering and ECM degradation (Martin-Villar *et al.*, 2005). Other cell lines, however behaved differently; Wicki *et al.* (2006) have studied the expression of podoplanin in human MCF7 breast carcinoma cells. In this system, podoplanin induced collective migratory properties, without the induction of EMT.

In a series of 189 tumors (histological sections of squamous cell carcinomas from esophagus, skin, larynx, cervix, and lung and adenocarcinomas from lobular breast cancer, prostate, and colon) it was found that 80% of SCC did express podoplanin,

whereas none of the adenocarcinomas did. Podoplanin staining in SCC was always in the invasive front of the tumor (Wicki et al., 2006).

Podoplanin has also been involved in the migration of dendritic cells due to its interaction with the CLEC-2 receptor present in these cells. This could be a more general mechanism, as it has been shown that the A375 melanoma cell line over-expresses CLEC-2 that could interact with podoplanin present in LECs and fibroblastic reticular cells (Acton et al., 2012; Acton et al., 2014).

Studies carried out using the mouse skin chemical carcinogenesis model and cell lines derived from these tumors (Martin-Villar et al., 2010) have shown up-regulation of podoplanin in highly undifferentiated SCCs, also called spindle cell carcinomas, where it is localized in the invasive front and in surface protrusions (Martin-Villar et al., 2005). Experiments of gain-of- or loss-of-function carried out in cell lines suggest that podoplanin regulates the motility of tumor cells.

The report of Krishnan et al (2013) indicates that phosphorylation of podoplanin in the CT domain diminishes cell migration. Furthermore, in co-cultures with podoplanin-expressing fibroblasts, melanoma cells have increased motility and viability.

2.7.3. Presence in the tumor stroma

The interplay between tumor cells and the surrounding stroma is an accepted fact in today's cancer research taking his modern formulation in the work of Bissell et al (1982), and reviewed recently (Allen and Jones, 2011; Kalluri and Zeisberg, 2006; Pula *et al.*, 2013). The tumor microenvironment contains mainly ECM, fibroblasts, immune cells (macrophages, B and T lymphocytes) and blood and lymphatic

vasculature. In normal tissues, fibroblasts are responsible of the synthesis and deposition of the ECM. In the tumor stroma, however, the fibroblasts (cancer-associated fibroblasts, CAF) exhibit an activated profile, which differs from the fibroblasts found in normal tissue. Since the findings of Orimo et al (2005) on the capability of invasive breast cancer-derived CAFs to induce tumor growth and angiogenesis, numerous reports have confirmed this fact in different types of tumors, including its role in the recruitment of inflammatory cells into the tumor (Erez *et al.*, 2010).

During the last years, podoplanin has been found in CAFs from different tumors, as shown in Table IV. Although there are exceptions, in general, the presence of podoplanin in the tumor stroma correlates with bad prognosis. For cancers other than those shown in Table IV, the reader is referred to the work of Kitano et al (2010).

In a recent report, Yoshida *et al.* (2014) showed that podoplanin expressing CAFs induce primary resistance to EGFR tyrosine kinase inhibitors in EGFR-mutated lung adenocarcinomas. This effect is dependent on the CT domain of podoplanin but independent of RhoA activity. Riganti *et al.* (2013) have shown that Ras (and RhoA) can induce Multi Drug Resistance proteins overexpression via HIF-1 α , and that this induction can be abolished by mevalonate pathway inhibitors.

3. Epithelial to mesenchymal transition

Epithelial to mesenchymal transition (EMT) refers to the biological process by which an epithelial cell undergoes multiple biochemical alterations that result in the change of its phenotype to a mesenchymal one. Phenotypic changes include loss of apico-basal polarization and junctions with neighbor cells resulting in a more individual

cell with enhanced migratory capacities and greater production of ECM products.

Publications on the EMT field have had an explosive increment during the last years [tenfold from 2000 to 2010, Nieto (2011)], with the appearance of numerous reviews emphasizing different aspects of the process (Acloque *et al.*, 2009; Diaz-Lopez *et al.*, 2014; Kalluri and Weinberg, 2009; Lamouille *et al.*, 2014; Moreno-Bueno *et al.*, 2008; Nieto, 2011, 2013; Peinado *et al.*, 2007; Thiery *et al.*, 2009). The reader is referred to these (and other) reviews for detailed description of the field. Here we will only discuss basic notions that may be related with the topic of this chapter.

According to a stablished nomenclature, EMT can be classified in three types (Kalluri and Weinberg, 2009; Zeisberg and Neilson, 2009). Type 1 refers to the EMT that takes place in embryo formation and organ development; in this situation, epithelial cells acquire a mesenchymal phenotype allowing them to migrate to other places where, by the reverse process, called mesenchymal to epithelial transition (MET), they form a secondary epithelia with different properties and responsiveness. A classic example is the migration of cells from the neural crest that give rise to craniofacial structures, most of the peripheral nervous system, some endocrine cells and melanocytes.

Type 2 EMT takes place in the context of wound healing and tissue regeneration, in which fibroblasts are needed for the restoration of healthy tissue. This EMT ceases once inflammatory signals disappear. However, if these signals are maintained in time, fibrosis and eventually organ destruction may be the outcome. Fibrosis in the kidney, liver, lung and intestine may be caused by this type of EMT (Bucala, 2012; Insel *et al.*, 2012; Lee and Nelson, 2012). A special case is the endothelial to mesenchymal transition (EndMT); this can be a normal process in embryogenesis that produces the

heart valves, but it can take place after ischemic injury of the heart, giving rise to cardiac fibrosis, involving the endocardium and microvascular endothelium of the heart.

Type 3 EMT is involved in cancer progression and metastasis, and will be considered in detail in the following sections.

3.1. Molecular mechanisms of EMT

As mentioned above, the most important property of EMT is the loss of cell-cell junctions that, in epithelial tissues, is carried out by loss of E-cadherin. E-cadherin is a calcium-dependent cell adhesion protein present in epithelia, localized mainly in the adherens junctions, which participates in homotypic interactions between neighbor cells. E-cadherin binds β - and γ -catenin, which links it to the cytoskeleton.

The down-regulation of E-cadherin was demonstrated to take place through transcriptional repression by the TF Snail1 (Batlle *et al.*, 2000; Cano *et al.*, 2000). Other TFs have been identified as repressors of the E-cadherin gene and also induce EMT: Snail2, ZEB1 and ZEB2, Goosecoid, FoxC2, KLF8 and some members of the bHLH TF family (E12 and E47, E2-2A AND E2-2B and TWIST1 and TWIST2). From this rather long list, only Snail 1/2, ZEB1/2, E47 and KLF8 repress the E-cadherin promoter directly (Thiery *et al.*, 2009). Given the prevalence of these TFs in EMT, they are normally referred as “core TFs”.

An interesting case of EMT without the apparent involvement of the core TFs has been reported in colorectal cancer (Lu *et al.*, 2012). In this system, prox1 TF induces miR9-2, which in turn down-regulates E-cadherin by degradation of its mRNA. Prox1 is an important regulator during development (Tammela and Alitalo, 2010) and in cancer

progression Ragusa *et al.* (2014), although it can have tumor suppressor activity (Elsir *et al.*, 2012).

3.1.1. Properties of the core Transcription factors

The Snail TFs (Snail1 and Snail2) are zinc-finger proteins, with 4 and 5 zinc-fingers, respectively, located in the C-terminus region of the protein. They have a conserved domain in the N-terminus, SNAG, and other less well conserved domains in the central region. Zinc-fingers are responsible for the DNA-binding activity, but the SNAG domain is also needed for repression, via recruitment of histone deacetylases (Peinado *et al.*, 2004). Interestingly, E-cadherin expression is also modulated by DNA methyltransferase 1, independently of its DNA methyltransferase activity, by interactions with the SNAG domain of Snail1 (Espada *et al.*, 2011).

ZEB TFs (ZEB1 and ZEB2) have two zinc-finger clusters at both ends of the molecule, surrounding a central homeodomain. Although both TFs can replace one another in some settings, the *zeb2* null mouse has defects in neural crest cells migration and is embryonic lethal.

The HLH TFs family has a large number of members. The main feature of the family is a helix-loop-helix structural motif (that gives their name) for DNA binding. The presence of other domains modulates the dimerization capacities and DNA binding specificities. The members mentioned above are known to repress the E-cadherin gene.

In addition to E-cadherin, the core TFs repress or modulate other genes, related or not to EMT. Besides the core TFs, some others have been described to induce EMT (Nieto, 2011), although they act upstream of Snail or Zeb factors. An interesting

exception is Brachyury, a mesodermal inducing TF that has been shown to induce Snail2 and at the same time interact directly with the E-cadherin promoter (Fernando *et al.*, 2010).

The EMT pathway in tumorigenesis needs not only to disrupt cell-cell interactions, but to degrade the basement membranes that would allow carcinoma cells to reach the connective tissue and the tumor vasculature. Whether this is a secondary aspect of EMT has been addressed by Ota *et al.* (2009), who have found that Snail1 by itself is able to induce the expression of membrane-anchored metalloproteinases MT1-MMP and MT2-MMP, which by themselves mimic Snail1 effects. Furthermore, these metalloproteinases cannot be substituted by secreted metalloproteinases. Interestingly, MT1-MMP AND MT2-MMP are also increased in invadopodia (Martín-Villar *et al.*, 2014; Paz *et al.*, 2014). A recent report (Hsu *et al.*, 2014) has unraveled the mechanisms by which Snail1 acts as a trans-activator factor: Snail1 has been shown to interact with the CREB binding protein (CBP) and p300 coactivators in promoters of induced genes (ERCC1 and IL8). This interaction results in acetylation of two lysines in the zinc fingers of Snail1, that further prevents the assembly of repressor complexes.

The core TFs are also involved in the disruption of the apical-basal polarity, by down-regulating the expression and/or function of several components of the apical-basal core complexes responsible for tight junction formation. These complexes establish the biochemical differences between the apical and the baso-lateral membranes, (Moreno-Bueno *et al.*, 2008).

3.1.2. Signaling pathways that converge on the core transcription factors

Although the above-mentioned TFs are able to trigger directly the EMT process *in vitro*, EMT is regulated by a large number of signaling pathways both during development and in pathological conditions (Gonzalez and Medici, 2014; Thiery et al., 2009).

The most potent inducers are the Transforming Growth Factor β (TGF- β)/Bone Morphogenetic Protein (BMP) family. The role of the different family members in embryonic, type 1, EMT is well established (Yang and Weinberg, 2008). TGF- β has an important role in cancer (Massague, 2008), and it has been shown to induce Snail1 in several tumor cell lines (Horiguchi *et al.*, 2009) and in MDCK cells (Peinado *et al.*, 2003).

An important mediator in TGF β -induced EMT is the myocardin-related transcription factor A (MRTF-A). MRTF-A is a sensor of free cytoplasmic actin; which sequesters MRTF-A in the cytoplasm. If the equilibrium between free and polymerized actin changes, MRTF-A is transported to the nucleus where it acts as a cofactor of Serum Response Factor. In epithelial cells, TGF- β triggers the nuclear translocation of MRTF-A inducing EMT, and down-regulation of MRTF-A prevents it. Furthermore, MRTF-A and Serum Response Factor increase the expression of actin, helping to reorganize the cytoskeleton. Finally, MRTF-A binds Smad3 and this complex induces the transcription of Snail2 (Morita *et al.*, 2007).

Besides TGF- β signaling, other pathways involving Tyrosine kinase receptors, Wnt, Notch, Hedgehog, hypoxia and inflammation, can induce EMT (Lamouille et al., 2014). Chanrion *et al.* (2014) have shown that the synergistic action of Notch activation and p53 deletion triggers EMT in the gut epithelium.

An interesting signal inducing EMT is that carried out by mTOR (mammalian target of rapamycin) (Chen *et al.*, 2014; Gulhati *et al.*, 2011) through RhoA and Rac1 small GTPases. This is an interesting mediator, because mTOR is a master integrator of the cellular metabolic status (Huang and Fingar, 2014).

Nuclear Factor (NF)- κ B responds to a wide variety of signals and control genes involved in inflammation, angiogenesis, cell proliferation, cell survival, immunity and metastasis (Karin, 2009). It has been shown that NF- κ B induces Snail1 and also YingYang1, which is an activator of Snail1; Snail1 represses the Raf-kinase inhibitor protein (RKIP), a metastasis suppressor, and also the tumor suppressor protein phosphatase PTEN (Bonavida and Baritaki, 2011).

Another level of regulation has recently been added to the EMT repertoire: switching from epithelial-specific to mesenchymal-specific proteins isoforms. This is carried out by down-regulating the epithelial splicing regulatory proteins, ESRP. In EMT, epithelial-specific forms of CD44, p120-catenin and FGFRIII are replaced by the mesenchymal ones (Warzecha *et al.*, 2010).

It should be noticed that in most of the pathways described above epigenetics mechanisms also take place (Wu *et al.*, 2012). (Tan *et al.*, 2014) have recently shown that High Mobility Group protein A2 (HMGA2) remodels the chromatin of mammary epithelial NMuMG cells, and favours binding of DNMT3A to the E-cadherin promoter. This results in hypermethylation of the promoter and effective epigenetic silencing of the E-cadherin gene.

3.1.3. Post-transcriptional controls of EMT

EMT can be regarded as a half-way pathway; it allows cells, in normal or pathological conditions, to become motile and invasive. However, once the cells have reached a new niche suitable for continuing their particular developmental/differentiation program, they must lose their mesenchymal properties in order to regain growth. This process, called the mesenchymal to epithelial transition, starts a series of control loops, mediated by miRNAs, which results in the repression and down-regulation of the core TFs. Among them, the most relevant are the miR-200 and miR-34 families (Nieto, 2013; Zavadil *et al.*, 2007).

Xu *et al.* (2014) have recently shown that the core TFs are regulated by ubiquitination and degradation by the proteasome using an atypical E3 ubiquitin ligase, SPF^{Fbxo45}. Furthermore, this E3-ligase is transcriptionally repressed by miR-27a*.

3.2. The epithelial phenotype

In order to maintain epithelial homeostasis, the induction of EMT must be a strictly regulated process. Two recent reports (Lee *et al.*, 2014; Watanabe *et al.*, 2014), however, show that EMT could be a default pathway that needs to be actively repressed during epithelia differentiation. The two groups characterize two TFs of the Ovol family as actively safeguarding epithelial cells against a mesenchymal program, both in skin and in the mammary gland. Furthermore, Watanabe *et al.* (2014) show that Ovol2 is a master suppressor of almost all core TFs in both systems.

An interesting work using *Drosophila melanogaster*, which has only one ERM protein, moesin, demonstrated that moesin functions in maintaining epithelial integrity, promoting cortical actin assembly and preserving apical-basal polarity (Speck *et al.*, 2003) by antagonizing the activity of Rho small GTPase.

3.3. EMT and cancer

During the last years the role of EMT in cancer dissemination has become widely accepted, in which a significant correlation between the activation of the core TFs and the loss of E-cadherin during the metastatic cascade has been established. This correlation also exists with invasiveness, poor prognosis and appearance of the most aggressive types of tumors (Sarrió *et al.*, 2008).

EMT implies disruption of cell-cell interactions, individual cell motility, intravasation, diffusion by the vascular and/or lymphatic systems, extravasation, and colonization of distant niches. However, an alternative pathway has been described (Wicki *et al.*, 2006) in which collective invasion by SCC cells takes place in the absence of EMT. It is not known yet whether these two pathways are completely equivalent in terms of invasiveness. Giampieri *et al.* (2009) have shown that tumors developed by injection of rat mammary carcinoma cells MTLn3E in the mammary fat pad can switch from collective to individual motility behavior by TGF- β signaling. Furthermore, cells migrating individually can invade both lymphatic and blood vessels, but collectively migrating cells can do so only in lymphatic vessels. It is not known, nevertheless, if TGF- β signals from the tumor stroma could allow collectively migrating cells to become invasive by transient pulses of EMT (Nieto, 2011).

Another possibility (Christiansen and Rajasekaran, 2006) is the existence of partial or incomplete EMT. In this situation, tumor cells could have a mixed phenotype with epithelial (cell adhesion) and mesenchymal (motility) traits. Lu *et al.* (2014) have studied this incomplete EMT from a gene network perspective. They describe two highly interconnected modules, the miR34/SNAIL and the miR200/ZEB, which create

mutual inhibition feedback circuits. This network regulates the coexistence and transition between three different phenotypes: epithelial (MET), mixed (partial EMT), and mesenchymal (full EMT). The network is regulated by external signals influencing the concentration of the different components which, in turn, can control other signals, like TGF- β , p53, RhoA/Rac1 small GTPases or hypoxia and oxidative stress. The same group has studied how the interactions and inter-regulation of the RhoA/Rac1 small GTPases can work like a three-way switch that controls amoeboid-hybrid-mesenchymal transition (Huang *et al.*, 2014).

Jiang *et al* (2014) have studied the metabolic connections of EMT. Using A459 SCC cells and TGF- β as an EMT inducer, they found that treated cells repressed pathways leading from glucose to fatty acid synthesis and enhanced respiration. Furthermore, over-expression of Snail1 repressed the carbohydrate-responsive element-binding protein and its effector fatty acid synthetase. In agreement with these findings, silencing of fatty acid synthetase induced EMT, migration, extravasation and lung metastasis in animal models. If EMT has to be considered as an early step in tumorigenesis, we should think in a two-step model: first, cells enhance respiration and repress fatty acid synthesis, and second, induction of aerobic glycolysis with impairment of mitochondrial respiration and induction of fatty acid synthesis (Lunt and Vander Heiden, 2011). In this regard, Hamabe *et al* (2014) report that upon induction of EMT by treatment of SW480 colon cancer cells with TGF- β and EGF, pyruvate kinase M2 (responsible of the Warburg effect) translocates to the nucleus to interact with the TGF- β -induced factor homeobox 2 (TGIF2), which results in the recruitment of histone deacetylase 3 to the E-cadherin promoter and suppression of E-cadherin transcription. The data reported by Tran *et al.* (2011) could help to understand this situation, as they

showed that Snail 1 is only needed at the onset of EMT, while Twist 1 is needed for its maintenance; in the absence of Snail 1, lipogenesis would start again, while E-cadherin repression would continue by means of pyruvate kinase M2.

Mechanisms for suppression of cancer cell invasion (through abrogation of the migratory and invasive properties of tumor cells) have also been found. The tumor suppressor p53 (lost or mutated in 50% of cancers) is responsible of this activity. In a recent report, Kim et al (2014) have shown that p53, together with p21^{WAF1} and Mdm2, binds to Snail 2, resulting in its ubiquitination and posterior degradation by the proteasome.

3.4. EMT and cellular reprogramming

Since the classical results of Yamanaka and coworkers on the generation of induced pluripotent stem cells by ectopically expressing four TFs in somatic cells (Takahashi and Yamanaka, 2006), the relationship between EMT and cellular reprogramming was sought. Upon transfection with TFs, fibroblasts seem to start a MET program as they transform into tightly packed clusters of rounded cells. It was found that Sox2 and Oct4 suppress Snail 1, c-Myc down regulates TGF- β 1 and TGF- β receptor 2, and, finally, Klf4 induces epithelial genes like E-cadherin (Li *et al.*, 2010).

In a recent report, however, Liu et al (2013) have dissected this process further. Using sequential introduction of TF (first Oct4–Klf4, then c-Myc and finally Sox2) the authors show an initial EMT (with Snail 2 and N-cadherin up regulation), followed by MET. In the same direction, Unternaehrer et al (2014) have found the need of Snail1 in the initial phases of fibroblast reprogramming, by directly repressing let-7 miRNA

promoter. These findings (and the fact that reprogramming can be induced by small-molecule inhibitors) suggest a complex interplay between cell fates (Li *et al.*, 2014). In accordance with these data, Gingold et al (2014) have found that during the Nanog-dependent phases of reprogramming Snail 2 is active and antagonizes Snail 1; in subsequent phases, Snail1 acts as an effector (and partner) of Nanog, resulting in down-regulation miR290, thus allowing reprogramming.

Taking together this plethora of information, EMT can be seen as an extremely controlled process that evolved during embryogenesis and tissue repair. EMT then, was coopted by neoplastic cells to develop tumors and metastasis. In both normal and pathological situations, this process must be reversible once the motile cells have reached a new niche suitable for continuing their particular developmental/differentiation program. We should think, therefore, in a tightly-controlled continuum epithelial plasticity between epithelial and mesenchymal states (Nieto, 2013; Nieto and Cano, 2012).

4. Podoplanin and EMT

Our group was the first one in describing EMT induced by podoplanin in the mouse keratinocyte MCA3D cell line (Scholl *et al.*, 1999; 2000). Several other studies have been done, sometimes with contradictory results. In this section we will address the available evidence.

Given that podoplanin is clearly involved in cell motility, we will also look for the pathways involved in motility, and ask for their interactions with podoplanin.

4.1. *In vitro* studies

When the immortalized mouse keratinocyte cell line MCA3D was transfected with podoplanin, a clear change in phenotype (compare panels A and B in figure 5) was observed. Non-transfected cells had the typical cobblestone appearance of epidermal keratinocytes. On the contrary, podoplanin-transfected cells showed fibroblastoid morphology, with membrane protrusions and were unable to pack together, even at high density. Moreover, transfected cells lost the cortical actin bundles and stress fibers present in non-transfected cells. In podoplanin-expressing cells, podoplanin co-localized with ezrin in filopodia and membrane ruffles (Scholl et al., 1999). In another set of experiments (Scholl et al., 2000) it was found that podoplanin-transfected cells down-regulated the expression of keratin K14 and had distorted adherens junctions (with loss of E- and P-cadherins), whereas the expression of keratin K8 and vimentin was enhanced. Keratin K8 is typical of simple epithelia, but is induced in poorly differentiated squamous cell carcinomas (Moll *et al.*, 2008). MCA3D cells are non-tumorigenic, but podoplanin-expressing MCA3D cells were able to induce tumors in athymic nude mice that metastasized to lymph nodes. The molecular changes observed in these podoplanin-expressing keratinocytes are consistent with EMT: fibroblast-like morphology, loss of epithelial markers and expression of mesenchymal ones.

We have studied the effect of podoplanin expression in MDCK cells (Martín-Villar et al., 2006). In this model, podoplanin induced a full EMT (figure 5, panels C and D), associated with individual motility and increased invasiveness, loss of E-cadherin and keratin K8, down-regulation of β -catenin and p120-catenin, and up-regulation of N-cadherin, fibronectin and vimentin. By using different mutant forms of podoplanin

fused to the Enhanced Green Fluorescent Protein (EGFP), it has been shown that EMT is strictly dependent on the CT domain: point mutations of the basic amino acids present in the juxta-membrane region of the CT domain (triple mutation R154Q, K155N, R159N and double mutation R154Q, K155N) abrogated EMT (the R159N single mutation resulted in a partial EMT). The behavior of these mutants suggests the involvement of ERM proteins in the process; furthermore, it is also dependent on the activation of RhoA GTPase and its effector ROCK, as confirmed by the use of dominant-negative forms of ezrin and RhoA.

One intriguing fact in EMT the induced by podoplanin in MDCK cells is the absence of induction of Snail1/2 expression. Although no other core TFs were studied, there are other possible explanations. First is the temporal regulation of Snail TFs. As mentioned in section 3.3, Snail 1 is not needed for the maintenance of EMT, but only for the initial steps (Tran et al., 2011). This is relevant as *in vitro* models for inducing EMT have a delay period in which stable cell lines are selected. Second, the levels of E-cadherin protein in the plasma membrane can be also regulated independently of the core TFs; Pujuguet *et al.* (2003) have shown that transfection of an active form of ezrin (the phosphomimetic mutant T567D) in MDCK cells results in formation of lamellipodia, as well as altered cell contacts. This effect was mediated by Rac1, but not by RhoA or Cdc42. Furthermore, E-cadherin levels decreased in the plasma membrane and the protein accumulated in intracellular compartments. In this regard, it is interesting to note that E-cadherin levels are also controlled by ubiquitination mediated by Mdm2, endocytosis and degradation by the proteasome without the up-regulation of core TFs (Yang *et al.*, 2006). As mentioned above (Section 3.3), E-cadherin expression can also be repressed by pyruvate kinase M2 (Hamabe et al., 2014).

In contrast with these findings, studies with the human breast adenocarcinoma cell line MCF7 (Wicki et al., 2006) show that although podoplanin does enhance spreading, migration, and invasion, it does not down-regulate E-cadherin expression; actually, migration of podoplanin-expressing MCF7 cells is collective (figure 5, panels E and F), with no signs of EMT. However, like in the MDCK model, podoplanin co-localizes with ezrin in membrane protrusions. In both MDCK and MCF7 cells, ezrin is phosphorylated in podoplanin-expressing cells.

It is remarkable that podoplanin induces a decrease in the activity of the three Rho GTPases in these cells, and it may be another example of the complex interplay between Rho GTPases and their GAP and GEF partners. In this regard, it is interesting to note that a poorly studied RhoA-GAP, ARHGAP18, suppresses RhoA activity and stress fibers (Maeda *et al.*, 2011). In another study in *D. melanogaster*, Neisch et al (2013) have found that Conundrum, the orthologue of ARHGAP18, directly interacts with moesin (the unique ERM protein in *D. melanogaster*).

Although RhoA is the most studied and common Rho GTPase, other members of the family may be also involved. Rif (Rho in filopodia, the product of gene *RHOF* in humans) has been shown to induce filopodia independent of Cdc42 (Ellis and Mellor, 2000). Rif is distantly related to other GTPases (32-49% identity), but retains the common switch 1 and 2 regions that allows the function of small GTPases. The mechanism of Rif induced filopodia has been studied by Goh *et al.* (2011), who showed a direct interaction of Rif with mDia1 without involving the Cdc42 effectors N-WASP and IRSp53, the IRSp53 binding partner Mena, or the Rac effectors WAVE1 and

WAVE2. ARGGAP18 and Rif are ubiquitous proteins and have not been studied in MCF7 cells, so their role (if any) must be considered speculative.

In a recent study (Ziegler *et al.*, 2014), MCF7 cells with increased invasiveness (“aggressive” MCF7) were selected, but these cells did not express podoplanin. In another study with MCF7 cells, Yang and Kim (2014) showed that inhibition of ROCK activity leads to MCF7 cells activation, with loss of epithelial characteristics (loss of cell junctions, loss of E-cadherin and β -catenin in the membrane and increased proliferation, migration and invasion). In this case, however, inhibition of ROCK leads to Rac1 activation and no up-regulation of EMT markers (Snail 1/2, vimentin) was observed. This study stresses the possibility (discussed in section 3.3) of mixed epithelial-mesenchymal phenotypes.

The human immortalized keratinocyte HaCaT cell line has also been used as a model to study podoplanin function (Martin-Villar *et al.*, 2005). Expression of podoplanin-EGFP in these cells decreased their intercellular cohesiveness and resulted in a disorganized pattern of E-cadherin and β -catenin distribution. Expression of E-cadherin showed a slight decrease that did not correlate with podoplanin levels, and the cellular morphology, although altered, remained epithelial (figure 5, G and H).

4.2. *In vivo* studies

The group of Gittenberger-de Groot has studied the role of podoplanin in heart development (Mahtab *et al.*, 2008). Using *Pdpn*-null mice, they observed the need of podoplanin for the preepicardial organ formation and migration, which precedes the

spreading and migration of epicardium derived cells. EMT is a prerequisite for both processes, arguing strongly in favor of a specific role for podoplanin in EMT.

Witty et al (2014) have reported the presence of podoplanin as a marker of epicardial lineage cells generated from human pluripotent stem cells by stage-specific activation of the BMP and Wnt signaling pathway.

Sailer et al (2013) have studied the properties of rat neural stem cells, normally non-invasive. However, in the presence of FGF2 and BMP4 they acquire a migratory and invasive phenotype. These cells expressed podoplanin, plus other makers, *Msx1*, *Snail1*, *Snail2*, *Ngfr* (p75 nerve growth factor receptor). Cell migration from explants containing these motile cells was of the amoeboid type. The migration characteristics and markers in these cells support an EMT process.

The study of Wicki et al (2006) with the mouse model of pancreatic β cell carcinogenesis engineered to express podoplanin shows a different outcome. First, podoplanin-expressing mice had a higher incidence of carcinomas, indicative of a role of podoplanin in tumor progression. However, immunohistochemical analysis showed that the podoplanin-expressing tumors contained also E-cadherin, which co-localized with podoplanin at the plasma membrane of most cells, whereas N-cadherin was absent. These results indicate that podoplanin is able to induce tumor invasion without launching the EMT program, as was shown also in podoplanin-expressing MCF7 cells.

In a study of human papillomavirus (HPV)-related oropharyngeal carcinoma (Wakisaka *et al.*, 2014), it was found that HPV-positive tumors had an EMT-associated phenotype. However, these tumors did not express podoplanin as opposed to HPV-negative tumors, which did express the protein. The latter tumors had worse

prognosis. A possible mechanism to explain these data could be the up-regulation by HPV infection of miR-363 (Lajer *et al.*, 2012), a miRNA known to regulate podoplanin expression (Sun *et al.*, 2013).

4.3. Rho GTPases and ERM proteins

The master regulators of cell motility are the Rho family of small GTPases, RhoA, Rac and Cdc42. These three protein families are involved in different aspects of cell motility. Rac and Cdc42 are involved in the branching of actin cytoskeleton by activation of the Arp2/3 actin nucleation complex, whereas RhoA activates formins allowing parallel actin fibers to grow. In a steady-state condition, the actomyosin cytoskeleton is in equilibrium with ERM proteins and transmembrane proteins like receptors, cell adhesion molecules, etc. (figure 6A). ERM proteins are in equilibrium between their inactive closed state and the active, phosphorylated forms, which permits their interaction with actin cytoskeleton on one side and transmembrane proteins on the other (equilibrium that is controlled in part by binding to PIP2). On their side, Rho GTPases are in equilibrium between active (GTP bound), inactive (GDP bound) or sequestered (GDP bound) in the cytoplasm by Rho guanine nucleotide dissociation inhibitors (GDI). Equilibrium between active and inactive forms is maintained and displaced by guanine nucleotide exchange factors (GEF) and GTPase-activating proteins (GAP) (Murali and Rajalingam, 2014). On top of this network, different Rho proteins can regulate positively or negatively each other via GAP, GEF, GDI and downstream effectors (Guilluy *et al.*, 2011).

The expression of podoplanin in these conditions would alter the equilibrium between the different partners by its binding to active ERM proteins (figure 6B). The increase in active ERM proteins would allow their interaction with Rho-GDI, making more RhoA-GDP available for Rho-GEF, thus increasing the levels of active RhoA and activation, in turn, of its effectors, like ROCK, which could phosphorylate more ERM proteins, closing a positive feed-back loop. This new cellular state would increase ERM proteins interaction with the actin cytoskeleton, promoting cell movement and polarization. Other substrate of ROCK is the myosin light chain (MLC) phosphatase, therefore promoting the actin filament cross-linking activity of myosin II. All these interactions would then promote the formation of lamellipodia, filopodia and actin-myosin filaments, needed for cell motility (Jaffe and Hall, 2005). Besides these protein networks, the status of actin polymerization (driven by Rho GTPases) also triggers a transcriptional network centered in Serum Response Factor (SRF) and its cofactor myocardin-related transcription factor A (Olson and Nordheim, 2010).

It has been shown recently that TIPE3 (TNFAIP8L3, tumor necrosis factor- α -induced protein 8 type 3) acts as a phosphatidylinositol phosphate-carrier to the plasma membrane which, then, could increase active ERM proteins in the membrane, reinforcing the loop (Fayngerts *et al.*, 2014).

4.4. Role of podoplanin in EMT

We have seen that cell motility is regulated by a complex network of interactions between the Rho small GTPases, their regulators GAPs, GEFs and GDIs, and ERM proteins. These players orchestrate the organization of the actin-myosin II

cytoskeleton in different manners to give rise to lamellipodia, filopodia, stress fibers, or lobopodia (Petrie and Yamada, 2012). These structures are ultimately responsible of different forms of movement: collective or mesenchymal in 2D matrices, or the different 3D alternatives.

As we discussed previously, the phenotypic hallmark of EMT is E-cadherin loss (and expression of mesenchymal intermediate filament proteins like N-cadherin or vimentin). The most studied mechanism for loss of E-cadherin is transcriptional repression by the core TFs. But, as mentioned above, other mechanisms besides transcriptional repression can be involved, like temporal variations in expression of the core TFs (Tran et al., 2011), E-cadherin promoter methylation, which has been observed in a wide variety of mammary tumors (Lombaerts *et al.*, 2006), and post-translational regulation by endocytosis and degradation by the proteasome (Yang et al., 2006; Dong *et al.*, 2014; Hartsock and Nelson, 2012)

We propose the existence of two superimposed mechanisms (figure 7). One is the increase in cellular motility driven by podoplanin interacting with the ERM proteins-Rho GTPases axis; the second is what we call “effective cohesiveness” in the cells. Effective cohesiveness designates a sensor mechanism or metabolite that would integrate the overall adhesive properties of a cell, and could be regulated by transcriptional and epigenetic mechanisms, protein stability, presence in the plasma membrane and context-specific mechanisms (like, for instance, the properties of the extracellular matrix) (Adhikary *et al.*, 2014; Boulter *et al.*, 2012; Engl *et al.*, 2014; Friedl *et al.*, 2014; Gueron *et al.*, 2014; Marjoram *et al.*, 2014; McGrail *et al.*, 2014; Murali and Rajalingam, 2014; Orgaz *et al.*, 2014; Sadok and Marshall, 2014; Thiery *et al.*, 2012;

Zegers and Friedl, 2014). If podoplanin is expressed in a cellular context with low effective cohesiveness, it will promote mesenchymal motility that will end up in EMT. If, on the contrary, the cellular context is one of high effective cohesiveness, podoplanin will continue to promote increased motility but in a collective fashion. The relationship between these two superimposed mechanisms does not need to be strictly causal. Podoplanin does not necessarily alter the effective cohesiveness at the beginning of the process (Brown *et al.*, 2014; de Toledo *et al.*, 2012; Ewald *et al.*, 2012; Hartsock and Nelson, 2012; Priya *et al.*, 2013; Ratheesh *et al.*, 2013; Smith *et al.*, 2012).

Gotzmann *et al* (2004) have proposed a two-hit model for epithelial cell plasticity, so that a given cell line could be “pre-activated” and need only one signal or two signals to trigger EMT; for example, TGF- β and active Ras, or TGF- β and EGF. MDCK cells would be already pre-activated and therefore podoplanin would be sufficient for triggering EMT. Curiously, MCF7 cells seem to be also pre-activated, as over-expression of High Mobility Group Protein HMG-Y is able to induce EMT and metastatic progression through integrin signaling (Reeves *et al.*, 2001). Therefore, in this two-hit model, signals inducing EMT could have different strength, as a given cell line could undergo EMT or not depending of the signal.

Although it is intriguing that in human cell lines (MCF7, HaCaT) the expression of podoplanin did not result in EMT, whereas in MDCK (dog) and MCA3D (mouse) cells did, there are not enough cases yet to substantiate this observation. According to the model in figure 7, MCF7 cells could be on the high effective cohesiveness part, and HaCaT cells would be somewhat in between, whereas the other two systems in which podoplanin expression results in EMT should be in the low effective cohesiveness part.

5. Concluding remarks

There are two clear functions described for podoplanin; one is its role in immune cells interactions (Acton et al., 2014; Astarita et al., 2014; Pollitt *et al.*, 2014). The other is the rearrangement of the cytoskeleton and the promotion of cell motility by its interactions with ERM proteins and Rho GTPases. This can have profound effects on the cell shape, function and responses to external stimuli. From the experimental evidence reviewed here, the involvement of podoplanin in EMT could be considered as a secondary aspect related with the effects that alterations of the cytoskeleton have on the specific cell where podoplanin is expressed.

One important question that remains to be solved is the primordial activity of podoplanin, which has to have an evolutionary answer. Podoplanin is a relatively modern protein, first detected in cartilaginous fishes that appeared around 523-435 My ago. The transmembrane and cytoplasmic domains are strictly conserved, with the GXXXG motif at the same position of the TM, and the 8-9 amino acid-long CT containing three basic amino acids in the same position, and having proline as the C-terminal amino acid coded in a different exon with a large 3'-UTR. Podoplanin ectodomain, however, is poorly conserved between the different vertebrate classes. CLEC-2 is present in mammals has been detected in birds, with thrombocyte (equivalent to mammalian platelets)-activation activity (Fuller et al., 2007; Neulen and Göbel, 2012), although podoplanin involvement was not studied. Although databases report CLEC-2 orthologs in zebrafish and other fishes (and even in *Caenorhabditis elegans*), it is not possible to ascribe any function to these molecules without a deep phylogenetic study.

With the available information, we think that the TM+CT domains are the primordial functional ones and that the thrombocyte/platelet-aggregating activity of podoplanin was co-opted later during evolution

Future research in podoplanin biology is clearly a promising and fascinating field, with specific and wide reaching challenges.

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LEGENDS TO THE FIGURES

Figure 1. Structure of human podoplanin. SP, signal sequence; ED, ectodomain; TM, transmembrane domain; CT, C-terminal domain. PLAG motifs (green, residues 30-37, 39-45 and 47-53, respectively), involved in CLEC-2 binding. GXXXG (red, residues 133-137), involved in homodimerization; the CT sequence (red), 9 amino acids long, is also shown. The black line above YS residues in the CT sequence may be spliced out (see text). Blue labels show the sites of O-glycosylation according to Kato and Kaneko (2014). Black regions mark the exon boundaries. The lower part shows a scheme of the *PDPN* gene. The figure has been prepared with the DOG2.0 software (Ren *et al.*, 2009).

Figure 2. O-glycans found in podoplanin. Taken from Ju *et al.* (2011).

Figure 3. The *PDPN* promoter. Black boxes represent known binding sites for the transcription factors shown below the line; the white box indicates that STAT should bind the promoter, but has been not studied. Signaling molecules known to modulate podoplanin expression are shown in the upper part, with indication of the transcription factor involved, if known. The drawing is not in scale and transcription factor binding sites are not in order. See the text for more details.

Figure 4. Known interactions of podoplanin. The molecules that have been described to directly interact with podoplanin are shown in this cartoon. Interactions are indicated by bold, double-headed arrows; black, intercellular; red, intracellular; green, intramembrane; blue, extracellular; orange, not defined. No attempts have

been made to scale the proteins or define specific cellular locations, except clearly known.

Figure 5. Effect of podoplanin expression in selected cell lines. The upper row shows control transfection, and the lower row, podoplanin transfections. A and B taken from Scholl et al. (1999); C and D, from Martín-Villar et al. (2006); E and F, from Wicki et al. (2006); G and H, from Martin-Villar et al. (2005)

Figure 6. The Rho/ERM proteins cycle. In a steady state cell (A), the different forms of Rho and ERM proteins are in dynamic equilibrium which allows actin to polymerize into stress fibers in the cell cortex. A generic adhesion molecule is shown, but more proteins are known to interact with ERM, like EBP50, PDZK1, CD43, CD44, ICAM, etc. When podoplanin is expressed (B), it interacts with ERM, driving the equilibrium towards activated Rho and ROCK, which allows the polymerization of actomyosin bundles facilitating the formation of lamellipodia and consequent motility. A dashed arrow indicates the involvement of Rho in other pathways not described here.

Figure 7. Proposed model for podoplanin involvement in EMT. The main function of podoplanin is to drive motility by interacting with ERM proteins and Rho-GTPases. Depending on the effective cohesiveness of the cell (which is affected by context specific, transcriptional, and epigenetic mechanisms, as well as by stability and localization) this motility will be of the mesenchymal type, facilitating EMT, or will be collective, without EMT. See text for details.

Table I. Expression of podoplanin in normal tissues

Organ/Tissue/Cell type	reference
kidney Kidney podocytes	Zimmer et al. (1995; 1997); Breiteneder-Geleff et al. (1997); Scholl et al. (1999)
Lung Lung alveolar type I cells	Zimmer et al. (1995; 1997); Ramirez et al. (2003); Rishi et al. (1995); Scholl et al. (1999)
Immune system Lymphatic endothelium Fibroblastic reticular cells Peripheral lymphoid tissue	Breiteneder-Geleff et al. (1999); Farr et al. (1992); Scholl et al. (1999) (1)
Placenta	Martin-Villar et al. (2005); Scholl et al. (1999)
Myometrium	(2)
Brain Choroid plexus Neurons Eye ciliary epithelium	(Zimmer et al. (1995; 1997); Scholl et al. (1999); Kotani et al. (2003); Song et al. (2014) (3), (4)

Muscle	
Skeletal	Martin-Villar et al. (2005)
Heart	Mahtab et al. (2008; 2009); Martin-Villar et al. (2005); Zimmer et al. (1995; 1997)
Intestine	Zimmer et al. (1995; 1997)
Osteoblasts	Nose et al. (1990); Wetterwald et al. (1996)
Mesothelia	Ordenez (2006)
Chondrocytes	(5)
Developing testis	Marks et al. (1999); Sonne et al. (2006)
Prostate	(2)
Ovaries	(2)
Epidermis basal layer	Honma et al. (2012); Gandarillas et al. (1997)

(1) Peduto et al. 2009 J Immunol 182 (9), 5789-5799; (2) Wilhelm et al. 2014. Nature 509, 582-587; (3) Tomooka et al. 2013. Acta Histochemica et Cytochemica 46, 171-177; (4) Williams et al. 1996. Am J Respir Cell Mol Biol 14, 577-585; (5) Smith et al. 2011. Tissue and Cell. 43, 39-44

Table II^a. Expression of podoplanin in tumors

Tumor	Reference
Squamous cell carcinoma Oral cavity, larynx, cervix, oesophagus, skin,	(1), (2), (3), (4), (5), (6), (7), (8), Martin-Villar et al. (2005), Wicki et al. (2006)
Central Nervous System Ependymal tumors, choroid plexus papillomas, meningiomas, pilocytic astrocytomas,	Peterziel et al. (2012) (9), (10), (11), (12), (13)
Germ cell tumors Dysgerminomas, granulosa cell tumors, seminomas, embryonic carcinomas, teratomas, yolk sac tumors, testicular germ-cell tumors	(8), (14), Sonne et al. (2006)
Malignant mesotheliomas	(15), (16), (17), Ordonez (2006)
Vascular tumors Angiosarcomas, Kaposi's sarcomas, lymphangiomas, epithelioid hemangioendotheliomas	(18), (19), Breiteneder-Geleff et al. (1999)
Bone tumors Chondrosarcomas, chondroblastomas, small cell osteosarcomas	(20), Huse et al. (2007)
Pleomorphic carcinoma of the lung	(21)
Parotid gland tumors	(22)

^aAdapted from Fernández-Muñoz (2010). (1) Cueni et al. 2010. *Am J Pathol* 177, 1004-1016. (2) Dumoff et al. 2005. *Mod Pathol* 18, 97-104. (3) Dumoff et al. 2006. *Mod Pathol* 19, 708-716. (4) Kato et al. 2005. *Tumour Biol* 26, 195-200. (5) Rodrigo et al. 2010. *Mol Cancer* 9, 48, doi:10.1186/1476-4598-9-48. (6) Yang et al. 2008. *Am J Dermatopathol* 30, 549-554. (7) Yuan et al. 2006. *Cancer* 107, 563-569. (8) Schacht et al. 2005. *Am J Pathol* 166, 913-921. (9) Mishima et al. 2006. *Acta Neuropathol* 111, 563-568. (10) Mishima et al. 2006. *Acta Neuropathol* 111, 483-488. (11) Shibahara et al. 2006. *Virchows Arch* 448, 493-499. (12) Shintaku et al. 2010. *Brain Tumor Pathol* 27, 23-27. (13) Wicki and Christofori., 2007. *Br J Cancer* 96, 1-5. (14) Kato et al. 2004. *Oncogene* 23, 8552-8556. (15) Chu et al. 2005. *Mod Pathol* 18, 105-110. (16) Hu et al. 2010. *Appl Immunohistochem Mol Morphol* 18, 411-413. (17) Kimura and Kimura. 2005. *Pathol Int* 55, 83-86. (18) Fukunaga. 2005. *Histopathology* 46, 396-402. (19) Naqvi et al. 2008. *Head Neck Pathol* 2, 25-30. (20) Ariizumi et al. 2010. *Pathol Int* 60, 193-202. (21) Kenmotsu et al. 2008. *Pathol Int* 58, 771-774. (22) Hansen and Kirkpatrick. 2010. *Oral and Maxillofacial Surgery* 14, 223-226.

Table III^a. Expression of podoplanin mRNA and protein in human tumor cell lines.

Cell line	Origin	mRNA	protein
KLE	Endometrial carcinoma	+	+
SK-UT1	Endometrial carcinosarcoma	+	±
SK-UT1B	Endometrial carcinosarcoma	+	±
SCC13	Skin SCC	+	±
HN19	Oral cavity SCC	+	±
HN30	Oral cavity SCC	+	+
SCC9	Oral cavity SCC	+	+
FaDu	Oral cavity SCC	+	+
DU-145	Prostate carcinoma (brain metástasis)	+	±
NCCIT	Embryonal carcinoma	+	++
NTERA-2	Embryonal carcinoma	+	+
HT1080	Fibrosarcoma	+	±
SAOS-2	Osteosarcoma	+	-
HN-5	Oral cavity SCC	+	+++
A253	Submandibular SCC	+	++

^aData taken from Martin-Villar et al. (2009; 2014)

Table IV^a. Presence of podoplanin in different tumor CAFs

Tumor type	Prognosis	reference
Skin cancer	-	Gandarillas et al. (1997)
Lung adenocarcinoma	Poor	(1) Kawase <i>et al.</i> (2008)
Squamous lung carcinoma	-	(2), Kitano et al. (2010)
Breast cancer	Poor	(3)Pula <i>et al.</i> (2011)
Intrahepatic cholangiocarcinoma	Poor	(4)
Colorectal carcinoma	Favorable	(5)
Uterine cervical carcinoma	+/-	(6)
Esophagus adenocarcinoma	Poor	(7)

^aData taken from Pula et al. (2013). (1) Kawase et al. 2008. Int J Cancer 123, 1053-

1059. (2) Kadota et al. 2010. Lung Cancer 70, 88-93. (3) Pula et al. 2011.

Histopathology 59, 1249-1260. (4) Aishima et al. 2008. Mod Pathol 21, 256-264. (5)

Yamanashi et al. 2009. Oncology 77, 53-62. (6) Carvalho et al. 2010. Clinics (Sao Paulo)

65, 1279-1283. (7) Schoppmann et al. 2013. Clin Exp Metastasis 30, 441-446.

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T antigen
(TF antigen)
Core 1 O-glycan
(CD176)

Sialyl-T antigen
(Sia3Core 1)

Sia6Core 1

Disialyl-T
antigen

Renart_et_al_Fig4

MCA3D

MDCK

MCF7

HaCaT

∅

+ PDPN

A

B

Rho GTP

Rho GDP

ERM

PIP2

Adhesion molecule

PDPN

Actin bundle

Myosin