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"hMENA splicing program in tumor progression: functional role of isoforms in pancreatic cancer cell adhesion and migration"

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Chapter 2 - Introduction

Normal glandular epithelial tissues are composed of a simple layer of epithelial cells that adopt apical–basal polarity, where the basal side contacts the basement membrane (BM) and the apical side is opposite the fluid-filled lumen. In some glandular epithelium there is a basal or myoepithelial cell layer that separates the luminal epithelium from the interstitial extracellular matrix (ECM) (Barsky and Karlin, 2005). Epithelial tissue homeostasis depends on the maintenance of tissue organization and a dynamic dialogue with a surrounding stroma composed primarily of non-activated fibroblasts and adipocytes, and a steady-state population of transiting, non-stimulated leukocytes (Ronnov-Jessen, 1996). Thus, non-activated tissue fibroblasts secrete and organize type I and III collagens, elastin, fibronectin, tenascin and a repertoire of proteoglycans (hyaluronic acid and decorin), which all maintain the structural and functional integrity of the interstitial ECM. Most glandular epithelial tissues including breast, saliva gland, lung, pancreas and prostate are in a state of tensional homeostasis so that their normal state is highly mechanically compliant (Paszek and Weaver, 2004).

Tumor microenvironment

Cancer is the loss of tissue organization and aberrant behaviour of the cellular components. Tumor microenvironment is enriched of multiple non-malignant, albeit altered, cell types – fibroblasts, endothelial cells and leukocytes – all of which interact, either physically or via the secretion of paracrine signaling molecules with tumor epithelial cells (Fig 1) (reviewed in Xu, 2009). There is an intricate mutually sustaining synergy between the tumor epithelial cells and their surrounding stroma. The

relationship of these cells with tumorigenesis is complex; they can possess tumour suppressing properties but are frequently polarised into invasion and metastasis promoting roles as tumours progress. The structural support for these various cell types is provided by the ECM. These components do not function in isolation but communicate through a variety of mechanisms.

Compelling evidence has emerged in recent years for activated stromal cells playing an important role in tumor cell invasion into surrounding normal tissues, proliferation and survival, and metastasis to local and distant sites (Paraic, 2010). Activated stromal cells act either releasing chemotactic factors, modifying interstitial tissue around tumor cells or promoting actively tumor cell migration. Many studies have demonstrated a prominent role for soluble factors in communication between different cell types in the tumour environment. The production of chemokines, such as CCL2, CCL5, CCL9, CXCL12 (SDF-1a), and cytokines, such as TNFa, CSF-1, IL-1 and IL-6, by cancer cells recruits a range of leukocytes including macrophages, myeloid derived suppressor cells (MDSCs), T-cells and neutrophils (Mantovani, 2008). On the other hand, stromal cells release autocrine and paracrine signals which mediate pro-migratory, anti-apoptotic and inflammatory stimuli in cancer cells. For example, resident fibroblasts receive activation signals through growth factors (TGFβ, Il-1) developing into cancer associated fibroblasts (CAFs). They are able to modify the properties of the ECM through altered expression of structural components such as collagen, fibronectin and tenascin-C isoforms and ‘matricellular’ proteins (Egeblad, 2010). ECM deposition and remodelling by CAFs increased cancer cell migration, as observed in murine squamous cancer cells (Caggioli, 2007).

Tumor tissue remodelling is required for a cancer cell to degrade physical barriers during local expansion and intravasation at nearby blood vessels, extravasation and invasion at a distant location, through multiple protease systems which are up-regulated in tumor cells. These systems include matrix-metalloproteinases (MMPs), ADAMs, cathepsins, the serine protease urokinase plasminogen activator (uPA) (Mason and Joyce, 2011). Upregulated proteases contribute to tumor invasion and progression through at least three mechanisms (Wolf and Friedl, 2011). First, they execute proteolysis of structural ECM proteins, including fibrillar and nonfibrillar collagens, fibronectin, and laminin (Wolf, 2007). Second, they process other proteases or surface receptors, including adhesion and growth factor receptors (Overall and Blobel, 2007). Finally, they regulate the repertoire of available extracellular growth factors (Kessenbrock, 2010). In fact, MMPs and ADAMs can release ECM-bound factory which then form diffusing gradients toward neighbour cells (Shiao and Coussens, 2010).

The molecular and physical characteristics of the ECM strongly modulate tumor cell activities and invading cancer cells often simultaneously integrate signals from soluble factors and ECM molecules (laminins, collagens, fibronectin). The density of collagen fibers is often increased (Franz, 2010), promoting integrin activation and consequently cancer cell migration (Even-Ram, 2005). Most ECM proteins undergo enzymatic postprocessing; for example collagens, when glycosilated, increase their stiffness and resistance to proteolytic degradation (Levental, 2009). This increased stiffness enhances the clustering of integrins and focal adhesion formation augmenting both cell contractility and mesenchymal functions (Levental, 2009).

The physical tissue properties determine consequences for cell growth and differentiation (Discher 2005). As a central downstream signaling pathway that connect mechanotransduction to gene expression, cell proliferation in response to substrate stiffness is regulated by Yap1, a transcription factor downstream of the hippo pathway (Dupont, 2011). Yap1 supports also epithelial stem cell growth and proliferation (Schlegelmilch 2011).

The inflammation is often associated with tumors and may contribute to the so-called reactive stroma, a stroma with an increased number of fibroblasts, enhanced capillary density and type I collagen. The presence of leukocytes in tumors in the past was generally thought to be a consequence of a failed attempt at cancer cell destruction. However tumors are not only effective in escaping from immune-mediated rejection, but they also modify certain inflammatory cell types to render them tumor promoting rather than tumor suppressive. Depending on the tissue context, $CD4^+$ T cells, macrophages and NKT cells have either tumor-suppressive or tumor-promoting properties (De Visser 2006).

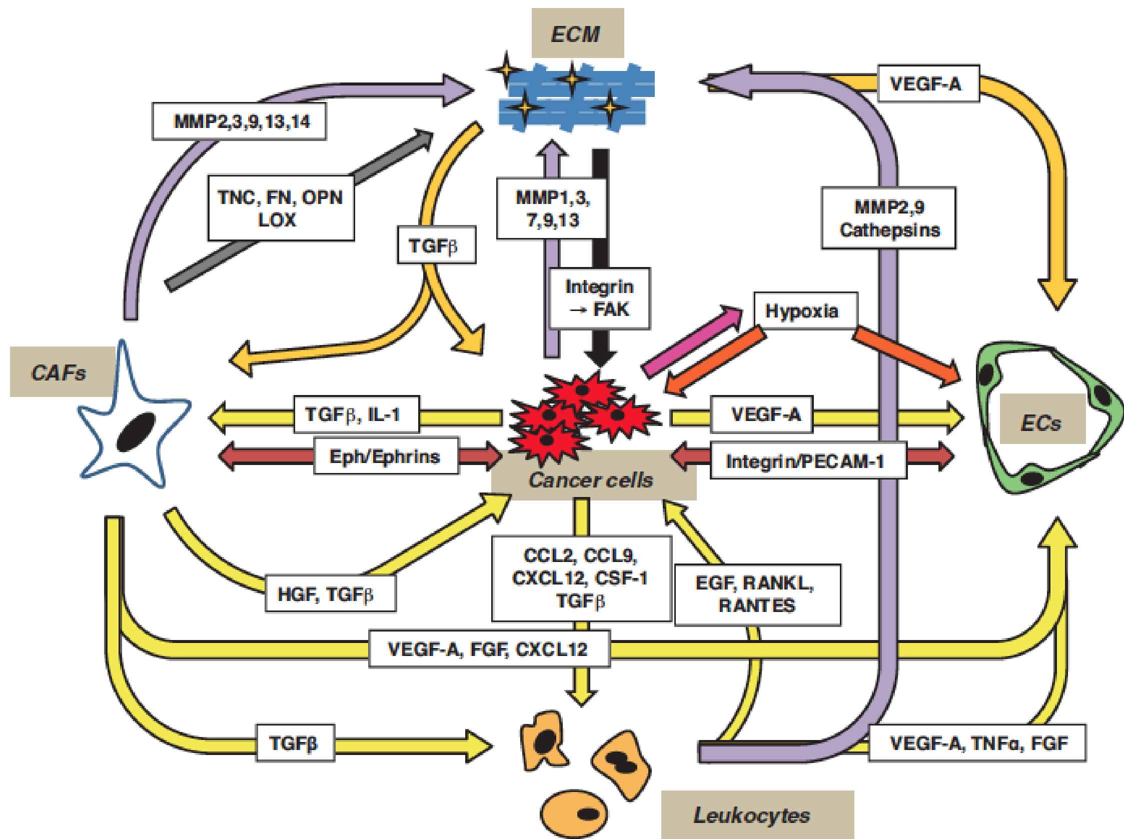

Figure 1. Communication networks in cancer invasion. Components of the network are shown in grey boxes with italics. Mechanisms of communication are indicated with coloured arrows and specific mediators are in white text boxes. Cancer cells sit at the centre of the network modulating the recruitment and activation of cellular and non-cellular environmental components. In turn, these components may regulate the invasive behaviour of the cancer cell by diverse mechanisms (depicted in different colours). Yellow, soluble factors; violet, proteases; grey, ECM remodelling proteins; orange, ECM-released GFs; black, mechanical signalling; dark red, cell–cell contacts; and red, oxygen tension. TNC, tenascin-C; FN, Fibronectin; OPN, Osteopontin. Adapted from " Calvo F et al (2011) Cell communication networks in cancer invasion. Current Opinion Cell Biology".

Epithelial-to-Mesenchymal Transition

About 90% of human malignancies are carcinomas, tumors of epithelial origin. The early steps in carcinoma metastasis often bear a striking resemblance to developmental programs involving Epithelial-to-Mesenchymal Transition (EMT), a process that converts organized epithelial cells into isolated, migratory cells with a mesenchymal morphology. EMT plays a critical role in early developmental processes, such as

gastrulation, leading to formation of mesoderm and during neural crest formation determining the release of mesenchymal cells that migrate through the body, generating the vertebrate head and awide variety of tissue types, including glial and neuronal cells, adrenal glandular tissues, melanocytes, and skeletal and connective tissues (reviewed in Nisticò, 2012). Several evidences implicate EMT-like mechanisms in tumor cell invasion and in experimental systems and, recently, in human cancer. Normal epithelia are comprised of cells with aligned apical-basal polarity that are interconnected laterally by several types of junctions, which play important roles in establishing and regulating cell-cell adhesion. During EMT, apico-basolateral polarity is lost, cell-cell junctions dissolve and the actin cytoskeleton is remodeled to endow cells with mesenchymal characteristics, including an elongated, migratory and invasive phenotype. Importantly, as a consequence of EMT cells may escape tumors, invade the surrounding tissue and migrate towards blood or lymphatic vessels guided by the cells and extracellular matrix present in their microenvironment (Fig 2).

EMT can be prompted by various intrinsic signals (e.g. gene mutations) as well extrinsic signals (e.g. growth factor signaling). Among the growth factors known to induce EMT are TGFβ, HGF, members of the EGF family, IGF, and FGF (Yilmaz, 2009). Notch signaling has been implicated in EMT in human breast cancer cells by activating the transcription factor Slug, a potent repressor of E-cadherin gene expression (Leong, 2007). Changes in the composition of the ECM are also able to induce EMT, as shown for collagen I (Shintani, 2006). These signals are able to mediate the transcriptional repression of E-cadherin by a list of transcription factors, among them intensely studied factors like Snail1 (Snail), Snail2 (Slug), ZEB1 (δEF1), ZEB2 (Sip1)

and Twist (Peinado, 2007). Engagement of these transcriptional repressors at the E-cadherin gene promoter eventually leads to epigenetic silencing of the gene by histone modifications (acetylation, methylation, phosphorylation, ubiquitinylation, sumoylation) and subsequently by DNA hypermethylation (Kouzarides, 2007). EMT is often driven by repressing expression of E-cadherin but recent reports demonstrate that EMT occurs also in presence of E-cadherin. In *Drosophila*, E-cadherin mRNA expression is maintained throughout *Serpent*-induced EMT, which does not involve Snail or Zeb orthologs (Campbell, 2011). The authors also find this *Serpent*-regulated EMT in a mammalian cell line. Specifically, GATA-4 and GATA-6, two mammalian orthologs of *Serpent*, induce EMT in MDCK cells. Furthermore, GATA-6 induces actin cytoskeletal remodeling, lamellipodia formation, and subsequent cell motility and invasion.

Most studies of EMT in the context of cancer biology have been conducted in vitro, and thus the relevance of EMT to carcinogenesis continues to be debated (Ledford, 2011). If EMT does play a crucial role in cancer cell spread in vivo, then detection methods that rely on cellular expression of epithelial markers alone are likely to provide an incomplete picture of metastasis.

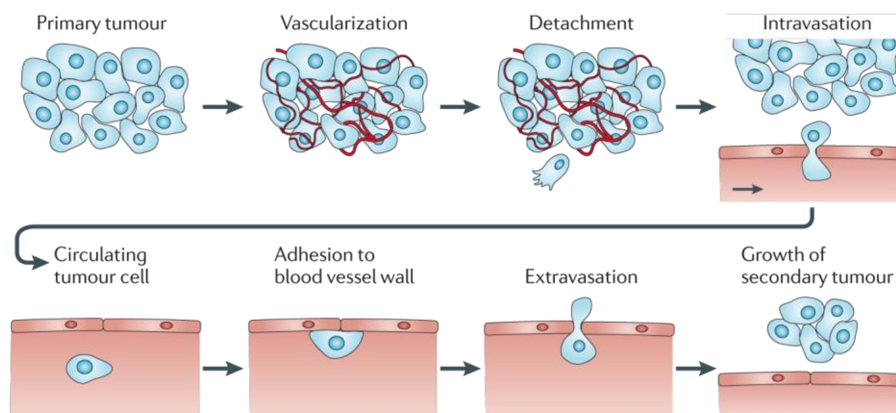

Figure 2. The epithelial-to-mesenchymal transition (EMT) is associated with a loss of adhesion through downregulation of E-cadherin (E-cad) and a change in morphology. Invasion by tumour cells of the surrounding tissue and subsequent motion is dictated by the physicochemical properties of the extracellular matrix (ECM). By squeezing between blood vessel endothelial cells, tumour cells can enter the vascular system. All of these steps involve physicochemical processes, such as adhesion and deformation, that are dependent on the local environment. Adapted from " Wirtz et al (2011) The physics of cancer: the role of physical interactions and mechanical forces in metastasis. Nat Rev Cancer "

Actin cytoskeleton dynamics role in Cell adhesion and Cell migration

The actin cytoskeleton is a highly dynamic structure, which is constantly remodeled in a living cell. This dynamics are based on a well-balanced and highly controlled equilibrium of local assembly and disassembly of actin filaments. Obviously, such regulation is a prerequisite for processes like endocytosis, cell morphology, cell adhesion, cell motility, and cancer cell invasion (Yilmaz, 2009). Cell adhesion and cell migration coordination required interaction of many different molecules and signaling pathways, but the actin cytoskeleton and regulators of actin dynamics held a determinant role in all processes. Each actin regulators, in turn, is controlled by several signaling molecules, usually including Rho GTPases, membrane phospholipids and protein phosphorylation. Understanding adhesion turnover in migrating cells and the

mechanisms underlying this process is now a critical area of emerging interest.

Cell adhesion in normal epithelium

A normal epithelium is able to either maintain tissue architecture or facilitate cell movement through dynamic changes in cell adhesion in order to resolve and establish new cell contacts during developmental cell movements, tissue renewal and wound repair. Intercellular adhesions are essential for compartmentalization and integrity of tissues in an organism, cell-cell communication and morphogenesis. Critical in mediating cell-cell interaction, adherens junctions (AJs) are formed primarily by cadherin family adhesion receptors and are strengthened by the actin cytoskeleton, which interacts with cadherins through additional proteins. The cytoplasmic tail of classic cadherins binds to β-catenin and once at the plasma membrane, the cadherin-β-catenin complexes recruits α-catenin. The latter is essential for AJs formation and function and operates at the interface of the cadherin-catenin complex and the actin cytoskeleton (reviewed in Harris & Tepass, 2010).

AJs are especially important for epithelial and endothelial cells that line the tissue surfaces and therefore should form cohesive sheets to resist mechanical challenges and maintain tissue integrity. In epithelial cells, AJs exist in two forms, as stable linear zonular adherens forming circumferential rings around the apical cell surface in polarized cells, and as dynamic punctate discontinuous junctions characteristic for tissues undergoing remodeling or neoplastic transformation (Ayollo, 2009; Taguchi, 2011). E-cadherin is the prototype family member of classical cadherins, single-span

transmembrane glycoproteins that interact in a calcium-dependent, homophilic manner with E-cadherins on neighboring cells.

Observations of cell-cell contact initiation in various epithelial cells suggest that the initial contact between cells is made through activity of actin-rich protrusions, lamellipodia and filopodia (Fig 2) (Mattila and Lappalainen, 2008). Within the lamellipodium, the actin filaments form a branched network with barbed ends facing the leading edge (Svitkina, 1997; Svitkina and Borisy, 1999). Filopodia, often originating from lamellipodia (Svitkina, 2003), are thin bundles of long actin filaments also oriented with their barbed ends toward the filopodium tip. Studies of cell-cell junction formation in primary keratinocytes suggested that the initial contact commences with interdigitating filopodia that establish a series of point contacts, which subsequently zipper into a continuous cell-cell junction (Raich, 1999; Vasioukhin, 2000; Vasioukhin and Fuchs, 2001).

As AJs assemble, cadherin-catenin clusters actively transform the actin cytoskeleton, initially co-opting cell protusions to expand cell contacts and ultimately reconfiguring actin into a circumferential belt to support cell-cell adhesion. Inhibition of the actin remodelling proteins, such as Arp 2/3, Ena/VASP or formins, distrupts AJ organization (Verma, 2004; Vasioukhin, 2000). Since these proteins are active at cell protusions before cell-cell contact is made and are also involved to cell migration pathways, cadherin-catenin clusters must co-opt these factors from cell migration signals for use in AJs assembly.

The small GTPases Rac1, RhoA and Cdc42 are downstream to cadherin-catenin clusters and a fine balance between their activity is fundamental for AJs assembly and the

maintenance of an epithelial structure (Braga, 2000). During de-novo contacts between stationary epithelial cells, Rac1 and RhoA have different roles in initiating adhesive contacts (Rac1) and expanding and completing the contact (RhoA) (Yamada, 2011). GTPases coordination can be mediated by upstream signals, such as Abelson Kinase activation (Zandy, 2007) or by the recruitment of many GTPase activating proteins (GAP) or guanine nucletodice exchange factors (GEF), that are key regulators of small GTPases, with GEFs promoting activation to the GTP bound state and GAPs promoting inactivation by stimulating GTP hydrolysis (Bos, 2007).

Cell migration in normal epithelium

Cell migration underlies tissue formation, maintenance and regeneration as well as pathological conditions such as cancer invasion. For most cells, including epithelial, stromal or neuronal cells, migration phases are confined to morphogenesis and cease with terminal differentiation toward intact tissue to become reactived only for tissue regeneration or neoplastic processes. For other cell types, such as leukocytes, migration maintained throughout their life span.

Cell migration is a coordinated event involving polarization, protrusion, adhesion to the ECM, tissue remodelling through proteolysis of ECM, myosin II-driven contraction of the cell body, and adhesion disassembly at the cell rear. The first step of cell migration is a morphological polarization that cell must acquire in order to generate intracellular forces that produce a net body translocation. A master regulator of cell polarity in eukariotic organisms ranging from yeast to humans, is Cdc42 (Heasman and Ridley,

2008). Cdc42 together with Rac and RhoG, are the main activators of the WASP/WAVE-Arp2/3 complex pathway, leading to formation of a branched actin network and extension of lamellipodia. In order to move, cells must extend their plasma membrane foward at the front, or “leading edge”, of the cell, through specialized actin structures such as lamellipodia, filopodia, blebs and invadopodia (reviewed in Ridley 2011). A protrusion must form and then stabilize by attaching to the surrounding matrix. Different kind of cell-matrix adhesions are described, based primarily on morphology or method of formation, including focal adhesions, fibrillar adhesions, focal complexes and podosomes. For example, fibrillar adhesions are central structures that contain α5β1 integrin, tensin and actopaxin (Zamir, 2000), whereas focal complexes are small adhesions induced by Rac activation at the periphery of the cell (Rottner, 1999). It is the small adhesions at the cell front, undetectable in some cell types, that drive rapid cell migration (Svitkina, 1997). Under tension, these small adhesions can mature into larger, more organized adhesions, such as focal adhesions (Balaban, 2001). Focal adhesions, which have been intensively studied, are relatively stable structures and tend to inhibit cell migration. They seem to be involved in the generation of contractile forces that function in processes unrelated to migration, such as the formation and remodeling of extracellular matrices (Geiger, 2001).

Integrins are the major family of migration-promoting receptors and may act as “feet” of a migrating cell supporting adhesion on ECM. Integrins link ECM to the actin cytoskeleton and may activate signaling cascades, so Integrins are molecules that allow an “inside-out signaling”. Integrins are heterodimeric receptors with large ligand-binding extracellular domains and short cytoplasmic domains. The binding of ligands

confers conformational changes in the receptor that leads to receptor clustering, that initiate intracellular signaling such as protein phosphorylation, activation of small GTPases, change in phospholipids biosynthesis. This is linked to formation and strenghting of adhesion sites and organization and dynamics of the actin cytoskeleton (Geiger, 2001). The mechanism by which adhesions assemble in migrating cells is still not well understood. It may starts with little focal contacts, dependent on Cdc42 and Rac that stabilize the lamellipodium by mediating attachments to ECM.

A migrating cell must be able to protrude and then detach from the older adhesions in order to move. By connecting the ECM to the intracellular cytoskeleton, integrins serve as both traction sites over which the cell moves and as mechanosensors, transmitting information about the physical state of the ECM into the cell and altering cytoskeletal dynamics (Galbraith, 2002). The force transmitted to sites of adhesion derives from the interaction of myosin II with actin filaments that attach to these sites. Myosin II activity is regulated by myosin light-chain (MLC) phosphorylation. MLC phosphorylation activates myosin, resulting in increased contractility and transmission of tension to sites of adhesion.

At the rear of migrating cells, adhesion must also disassemble. The cell rear is a region where Integrin-cytoskeletal linkages tend not to form and the membrane is not well supported by the cytoskeleton network. The Rho/ROCK/MLC/Myosin II pathway is implicated in the high tension exerted on the rear adhesions and contributes to the detachments (Riento and Ridley 2003).

Cell adhesion and Cell migration in tumor cells

Cancer cells recaputilate the types and mechanisms of migration used by normal cells. They activate the same machineries for changing shape, generating force and remodelling ECM (Friedl, 2004), as normal cells, but neoplastic cells lack phisiological “stop signals” (Cox, 2001). Since cell migration, cell adhesion and cell shape depend on the actin cytoskeleton and actomyosin contractility there has been much interest how they are controlled by cell signalling mechanisms.

To detach from the primary tumor and to invade into the surrounding tissue, tumor cells have to break down cell-cell contacts, remodel cell-matrix adhesion sites, and follow a chemoattractive path through the extracellular matrix, mined by secreted proteinases (reviewed in Ridley, 2011). These processes are commonly observed in various non-pathological conditions, such as in developmental processes like gastrulation or neural crest cell migration, where differentiated, epithelial cells dedifferentiate, move to a distant site, and then re-differentiate to form a new structure.

Each of these steps requires a distinct molecular program in which the modulation of the adhesive and migratory and, thus, the cytoskeletal properties of the disseminating tumor cells play essential roles.

Physical and Molecular Determinants of Invasion

Abnormal cell migration and tissue invasion occurs frequently in cancer and is an essential component of metastasis, the major clinical problem in cancer. Depending on the cell type and tissue environment, cells can migrate in two major ways: individually,

when cell-cell junctions are absent, or collectively as multicellular groups, when cell-cell adhesions are retained (Friedl and Wolf, 2010). Invasive single-cell migration in turn divides into ameboid/rounded and mesenchymal migration, depending on cell morphology, actomyosin-mediated contractility and proteolytic activity. The molecular mechanisms underlying each migration mode depend on a set of connected mechanical and signaling pathways, which vary in their coordination and strenght depending on the particular migration mode (Sanz-Moreno and Marshall, 2010).

Actin cytoskeleton is involved in the generation of localised structures such as protrusions and adhesions between cells and to the matrix (Fig 3). The latter linked to the actin cytoskeleton through integrins. In particular, after associating with matrix-derived ligands, the cytoplasmic tails of integrins connect to cytoskeletal adaptor proteins and activate protein kinases, including focal adhesion kinase (FAK) and Src (Geiger, 2009). They might regulate migration by modulating the phosphorylation and dephosphorylation of key adaptor molecules in adhesions. For example, the FAK–Src complex mediates the phosphorylation of two well-known scaffolding molecules, paxillin and p130 Crk-associated substrate (CAS) that can recruit other molecules to adhesions and regulate the organization of the actin cytoskeleton (Bellis 1995, Cary 1998).

It has fully demonstrated that the integrins play important role in tumor progression. The shifting profile of integrin receptor expression may promote tumor cell survival and migration in multiple tissues with different matrix compositions (Guo and Giancotti, 2004; Wilhelmsen, 2006). Increased expression of the integrin b1 subunit correlates with decreased breast cancer survival, whereas other integrin receptors such as avb3 and

a6b4 are induced in highly metastatic melanoma cells and pancreatic adenocarcinoma progression, respectively (Desgrosellier, 2010). Changes in the expression of the integrin-associated proteins FAK, paxillin, a-actinin, and vinculin are also observed during tumor progression. Tumor cells appear to take advantage of the ability of FAK to regulate pathways important for cell proliferation, cell migration, gene expression, and survival (Mitra, 2005; Slack-Davis, 2007). For instance, FAK expression and activity are enhanced in metastatic tumors of the oral cavity, colon, rectum, thyroid, prostate, and cervix. In ovarian cancer, increased FAK expression correlates with decreased patient survival (Brunton, 2008). In breast carcinoma, FAK activity is important for VEGF expression and tumor angiogenesis (McLean, 2005). Similar to FAK, increased expression of paxillin is observed in breast carcinoma, and a-actinin expression levels are increased in melanomas and in tumor cell lines with faster migration rates (Vadlamudi, 1999).

Downstream integrin effectors further include the small GTPases Rho, Rac, and Cdc42, have all been linked to cell movement (Ridley, 2003) and are deregulated in some tumors and correlates with progression of disease (Sahai and Marshall, 2002). Rac1 drives motility by promoting lamellipodia formation (Ridley, 1992), whereas RhoA signals to the kinases ROCKI and II, promoting the formation of actin stress fibers and generation of the actomyosin contractile force required for cell movement (Kimura, 1996). Melanoma cell movement analysis demonstrated that rounded/amoeboid forms of movement are driven by high actomyosin contractility through Rho and Cdc42 signalling while Rac signalling is required for actin assembly in elongated-protrusive movement (Sanz-Moreno, 2008).

Receptors that transmit cell-cell adhesion forces toward the actin cytoskeleton support single-cell and collective movement along the surfaces of other tissue-resident cells. Different sets of cadherins are expressed depending on the type of tumor and they show duality in delivering both migration-inhibiting and migration-promoting signaling in a context-dependent manner. In fact, in polarized resting epithelium, E-cadherin suppresses migration signaling by inhibiting Rac1 activation (Kitt and Nelson, 2011), whereas in activated and neoplastic cells E-cadherin and other cadherins coordinate collective movement (Friedl and Glimour, 2009).

Another cell adhesion molecules involved to cancer invasion belong to the Immunoglobulin superfamily of cell adhesion molecules (CAM). Among them, L1CAM is up-regulated in the leading edge of collectively invading epithelial tumors (Bergmann, 2010). Similar to leukocytes, tumor cells develop heterophilic cell-cell interactions with endothelial cells or platelets and these interactions occur between endothelial cells expressing CAMs and neoplastic cells expressing specific integrins, allowing intravasation and extravasation of tumor cells (Zecchini, 2011).

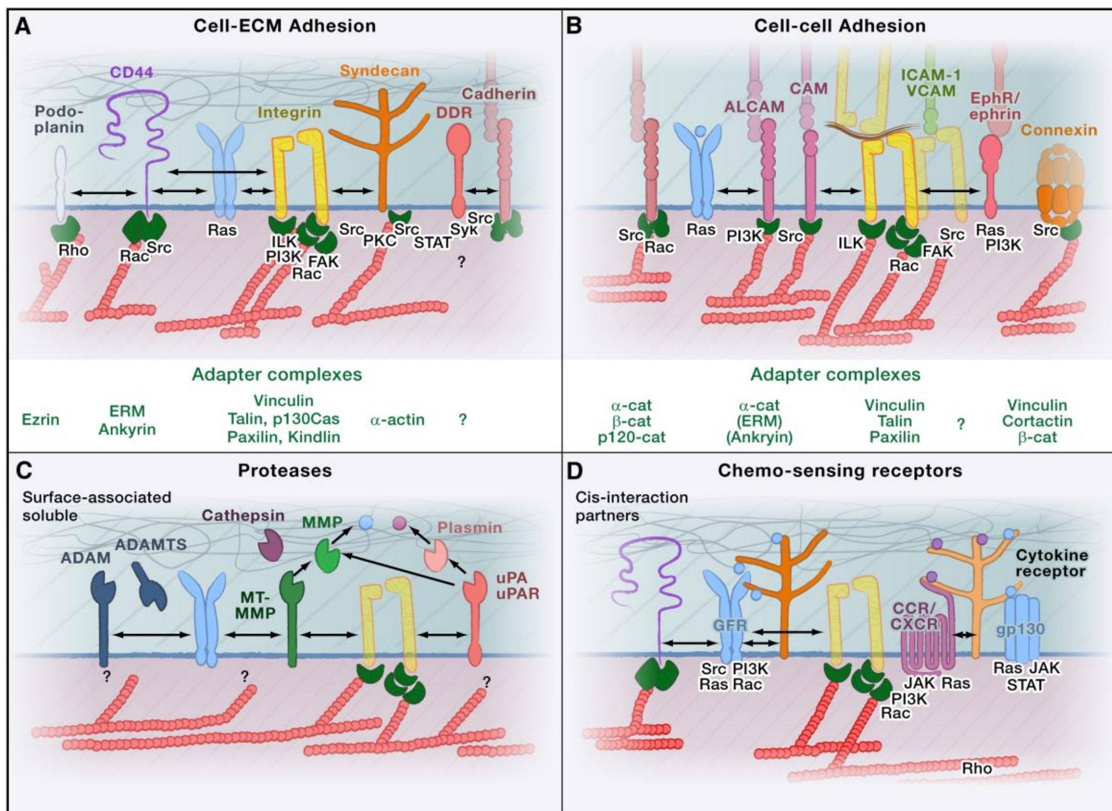

Figure 3. Molecular Determinants of Cell MigrationSimplified view of molecules mediating adhesion and migration signaling.(A) Cell surface receptors and adaptors that mediate the dynamic interface between the actin cytoskeleton and promigratory signaling and the extracellular matrix (ECM).(B) Cell surface proteins that mediate and regulate interactions between cells. Similar adhesion mechanisms may mediate homotypic cell-cell cohesion during collective invasion and transient and more dynamic heterophilic interaction to resident tissue cells encountered during tissue invasion.(C) Protease systems upregulated in cancer progression, invasion, and metastasis.(D) Receptors for chemokines, cytokines, and growth factors, which sense soluble, ECM-, or proteoglycan-bound factors and interaction partners. Green symbols represent selected intracellular adapters to the actin cytoskeleton, as specified below the drawing (A and B); shaded labels represent major signaling molecules regulating actin organization and cell migration. Adapted from " Friedl P et al (2011) Cancer invasion and the Microenvironment: plasticity and reciprocity. Cell".

Ena/VASP

The Ena/VASP family consists of Drosophila Ena, C. elegans Unc-34, Dictyostelium DdVASP, and the three mammalian family members VASP, Mena, and EVL (Ena-VASP-like). All Ena/VASP family members share a conserved domain structure. An amino-terminal Ena/VASP homology 1 (EVH1) domain followed by a proline-rich

central region and a carboxy-terminal Ena/VASP homology 2 (EVH2) domain (Fig 4).

Domain and functional organization of the Ena/VASP protein family

The EVH1 domain belongs to the PH domain superfamily (Ball, 2000; Barzik, 2001). Unlike PH domains, EVH1 domains do not appear to bind to phosphatidylinositol lipids, although only PI(4,5)P2 has been tested (Volkman, 2002). Instead, the EVH1 domain, similar to PTB domains (another branch of the PH domain superfamily), binds to peptide ligands with high affinity. EVH1 domains are also found in the WASP family, the Homer/Vesl family, and two other proteins, Spred and SMIF (Brakeman, 1997; Callebaut, 2002; Callebaut, 1998; Gertler, 1996; Kato, 1997). Similar to WW and SH3 domains, the characterized EVH1 domains appear to bind to peptides containing a poly-proline II helix (PPII helix), a left-handed helix with three residues per turn (Ball, 2000; Carl, 1999). Flanking residues in the vicinity of the PPII helix confer specificity of binding to SH3, WW, and EVH1 domains. The other EVH1 domains found in the Homer/Vesl and WASP protein families have binding specificities that differ from those of the Ena/VASP family (Barzik, 2001; Volkman, 2002). Functional binding sites for EVH1 domains are found in Zyxin, LPP, Vinculin, Fyb/SLAP, the *Drosophila* and *C. elegans* axon guidance receptor Robo/Sax-3, and in the ActA protein of *Listeria monocytogenes* (Bashawm, 2000). EVH1 domains recruit Ena/VASP proteins to specific sites within the cell.

Following the EVH1 domain, Mena contains a long insertion not found in other Ena/VASP family members. This insertion in Mena is a striking five-amino acid long

stretch of highly charged basic and acidic amino acids (LERER) that are repeated 14 times. This repeat might adopt an extended helical structure that function as a protein-protein-binding interface (Gertler, 1996). Mena's *Drosophila* ortholog Ena contains a glutamine-rich insertion in the same relative part of the molecule with no known function.

The central proline-rich region harbors binding sites for SH3 and WW domain–containing proteins and the actin monomer-binding protein Profilin. This region is the most divergent within the family and therefore may have different binding partners and mechanisms of regulation. Ena binds to the SH3 domains of Abl, Src, and the carboxy-terminal SH3 domain of Drk (Ahern-Djamali, 1999; Comer, 1998; Gertler, 1995). Similarly, EVL binds to the SH3 domains of Lyn, N-Src, Abl, and the WW domain of FE-65 (Lambrechts, 2000). In contrast, Mena does not bind to the SH3 domain of N-Src, but is bound by the SH3 domain of IRSp53, Abl, Arg, Src, and the WW domain of FE65 (Ermekova, 1997; Gertler, 1996; Krugmann, 2001). It is not clear which of these interactions is physiologically significant and shared among all members of the Ena/VASP family. All Ena/VASP family members contain proline-rich-binding sites for the small G-actin-binding protein Profilin, and this binding is independent of their phosphorylation status (Ahern-Djamali, 1999; Gertler, 1996; Lambrechts, 2000). Profilin II, the major Profilin isoform expressed in brain tissues, binds as a dimer with high affinity to VASP but with low affinity to PI(4,5)P2. In contrast, Profilin I displays the opposite preferences (Jonckheere, 1999).

The EVH2 domain of the Ena/VASP family harbors three conserved blocks: starting from the amino terminus, a G-actin-binding site, an F-actin-binding site, and a coiled-

coil motif required for oligomerization (Bachmann, 1999; Gertler, 1996). The G-actin-binding site resembles a motif in the small actin-monomerbinding protein ¯-thymosin (Gertler, 1996) and is required for the in vitro actin nucleation activity of VASP (Walders-Harbeck, 2002). The second conserved block in the EVH2 domain of VASP (259–276 aa in human VASP) can co-sediment and bundle F-actin at low salt conditions (Bachmann, 1999). The third conserved block (336–380 aa in human VASP) consists of a coiled-coil structure that mediates oligomerization of Ena/VASP proteins (Bachmann, 1999; Zimmermann, 2002). VASP behaves as a tetramer, and the coiled-coil motif is sufficient to mediate tetramer formation (Bachmann, 1999; Zimmermann, 2002)

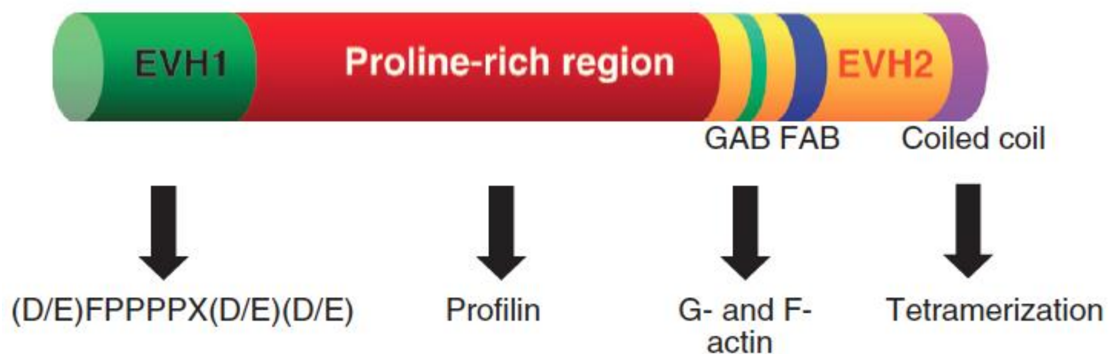

Figure 4. Domain structure of Ena/VASP proteins. The EVH1 and EVH2 domains and proline-rich region are indicated. EVH1 mediates protein:protein interactions; most, but not all, EVH1 ligands bind to a motif that has the consensus sequence (D/E)FPPPPX(D/E)(D/E). The proline-rich region harbors binding sites for profilin,including a high-affinity site adjacent to the EVH2 domain. The EVH2 domain contains a G-actin-binding site (GAB), an F-actinbinding site (FAB) and a coiled-coil at the very C-terminus that mediates tetramerization. Adapted from "Bear J et al (2009) Ena/VASP: towards resolving a pointed controversy at the barbed end. J Cell Sci".

Function of Ena/VASP in cells and tissues

Ena/VASP proteins are localized at actin cytoskeleton hot spots in the cell (the leading edge, filopodia tips, cell-substrate contacts, cell–cell contacts), and are additionally associated with the movement of several viral and bacterial pathogens, as well as objects mimicking these pathogens (reviewed in Krause, 2003).

Filopodia, actin-rich, finger-like structures that protrude from the cell membrane of a variety of cell types, play important roles in cell migration, neurite outgrowth and wound healing (Mattila, 2008). Filopodia are characterized by a small number of long and parallel actin filaments that deform the cell membrane, giving rise to protrusions. In order for filaments to grow to the characteristic length observed in filopodia, capping proteins, specialized molecules that inhibit actin polymerization, need to be locally inhibited or sequestered and nucleation of new filaments needs to be favored. Furthermore, individual actin filaments are not sufficiently stiff to deform the cell membrane (Mogilner et al 2005). Proteins, such as VASP-family proteins are thought to be required to promote the initial transient association of actin filaments as they directly (Lebrand, 2004) or indirectly antagonize capping proteins (Breitsprecher, 2008), capture barbed ends (Pasic, 2008) and cross-link actin filament. Furthermore, they can act as processive filament elongators especially upon high-density clustering, at least in vitro (Breitsprecher, 2011). Actin filaments are then further stabilized by other crosslinkers, such as fascin, thus permitting the formation of bundles of sufficient stiffness to overcome buckling and membrane resilience (Vignjevic, 2006).

Nevertheless, there is considerable controversy concerning the mode of action of Ena/VASP proteins and there is a partial convergence concerning only two points:

Ena/VASP proteins increase the protrusion of the leading edge, and the speed of propulsion of the bacteria *Listeria monocytogenes* and of objects like beads and droplets; and Ena/VASP decreases the frequency of actin filament branches formed by the Arp2/3 complex in actin networks in cells and on moving surfaces. Concerning the first point, the only note of caution appears to be associated with the behavior of whole cells: Moeller et al. (2004) see decreased overall cell motility when VASP is depleted from the leading edge of cells, whereas Bear et al. (2002) see the opposite. This is not particularly surprising given the complexity of processes at work for whole cell movement, and the fact that VASP is involved not only in leading edge dynamics, but in the formation of stress fi bers and cell-substrate adhesions. What is more important is that, in both cases, kymographs of protruding edge movement show more jagged profiles when VASP is present, supporting the general thesis that VASP promotes protrusion and propulsion. A similar result is observed in very different experimental conditions during repulsion from ephrin ligands (Evans, 2007).

The knock-out mouse lacking individual members of Ena/VASP results in relatively minor phenotypes in mouse models (Aszodi, 1999), whereas the generation of triple null Ena/VASP mice reveal that Ena/VASP proteins have crucial roles in neuritogenesis and neural-tube closure (Kwiatkowski, 2007). Furman and collaborators (2007) demonstate also that mice lacking Ena/VASP proteins develop edema and hemorrhage, both resulting from disruption in endothelial barrier function, which lead to embryonic lethality, probably through a defect in actomiosin contraction in regions of cell-cell junction.

Ena/VASP role in endothelia/epithelia

In addition to its role in actin remodeling for cell motility, Ena/VASP is being increasingly implicated in cell–cell adhesion processes. Ena/VASP proteins localize with E-cadherin/catenin enriched puncta between primary keratinocytes (Vasioukhin, 2000), and disruption of Ena/VASP function perturbs cadherin-associated actin bundles in CHO cells (Scott, 2006). In the developing *Drosophila* embryo, Ena is implicated in actin filament formation at apical adherens junctions between cells of the egg chamber (Baum and Perrimon, 2001). A study in *Drosophila* shows that Ena/VASP proteins are essential for epithelial zippering during dorsal closure (Gates, 2007), and the Ena/VASP-like protein in *Xenopus* seems to promote cell–cell adhesion during morphogenesis of the otic vesicle epithelium (Wanner and Miller, 2007). Along the same lines, Ena/VASP has been shown to be required for endothelial barrier function and correct embryogenesis in mice (Furman, 2007), and has been shown to stabilize cell – cell adhesion for a decrease in endothelial permeability in endothelial cells in culture (Benz, 2008).

Endothelial cells line blood vessels and provide both a barrier function between blood and other tissue while still allowing immune cells access to the surrounding tissue. This barrier function is maintained through tightly controlled cell-cell adhesions and is positively regulated by PKA. Interestingly, PKA phosphorylates VASP at serine 157 (Howe, 2002) following that phospho-VASP accumulate to cell-cell junctions and co-immunoprecipitate with the tight-junction marker zonula-occludens-1 (ZO-1) protein from endothelial cells (Comerford, 2002).

Similarly, by adhering to each other, epithelial cells form a physical boundary capable

of regulating the transfer of molecules across the epithelial sheet. This permits selective exchange of molecules between the organism and its environment, exemplified in the waste removal function of the kidney or nutrient uptake in the gut. In these systems Ena/VASP proteins localize to adherens junctions, and disruption of their function affects epithelial dynamics (Lawrence, 2002; Vasioukhin & Fuchs, 2001). Studies in *Drosophila* have demonstrated that reduction of either D-Abl or Ena affects epithelial morphology and epithelial sheet migration. Genetic interactions suggest that Ena functions in concert with components of the cadherin-catenin complex (which mediates cell-cell adhesion) and D-Abl in epithelial sheets at the adherens junction (Grevengoed, 2001). In mammals, Ena/VASP proteins are found in epithelial contacts that are revealed as a double row of dot-like structures also containing F-actin, Cadherin, Zyxin, and Vinculin. These cell-cell contacts have been suggested to represent an early stage in cell-cell contact formation and have been termed the adhesion zipper (Vasioukhin, 2000). It has been proposed that cell-cell contacts are generated after first contact of lamellipodia and/or filopodia of opposing cells (Ehrlich, 2002). Overexpression of a fragment of Ena/VASP responsible for oligomerization disrupts epithelial sheet formation (Vasioukhin, 2000). However, the specificity of this approach for Ena/VASP proteins has not been demonstrated. Although it seems likely that Ena/VASP proteins play an important role in epithelial dynamics, further work will be required to clarify their role in such processes.

Isoforms and spliced derived isoforms

The Mena mRNA undergoes extensive alternative splicing to give rise to multiple protein isoforms that are expressed in specific tissues and cell-types (Gertler, 1996). By contrast, the EVL gene contains one alternatively spliced exon and VASP has none. There are 14 constitutively included exons in Mena mRNA and five alternatively spliced exons that can all encode protein sequence in frame. There has not been a comprehensive analysis of which of the possible combinations of alternatively included exons are actually produced as mRNA, nor do we know all of the cell types which produce the various Mena isoforms.

At least four different isoforms of MENA has been described on Western blots of tissue extracts. The larger isoform of MENA, named +, presents a longer exon 6 which includes an extra 246 additional amino acids after the LERER repeats (Gertler, 1996; Urbanelli, 2006), and is expressed in developing and adult brain tissues (Lanier, 1999) and is tyrosine phosphorylated in vivo (Gertler, 1996). Some cDNAs containing the + exon may also contain two other small exons that introduce 4 and 19 aminoacids, right after the EVH1 domain, named ++ or +++ (or INV) (Gertler, 1996). Tani and colleagues described another isoform, named MENA-s, expressed in spleen and in lymphoid cell lines (Tani, 2003). Nisticò's group described another isoform named hMENA^{11a} expressed in human epithelial cancer cell lines (Di Modugno, 2007; Pino, 2008) and belonged to an epithelial splicing program driven in part by the activity of the splicing regulators ESRPs proteins (Warzhecha, 2009).

The alternatively spliced 11a exon encodes 21 amino acids that are inserted into the EVH2 domain between the FAB sequence and the coiled-coil tetramerization domain.

The MENA paralog EVL also has an alternatively spliced 21 amino acid insertion (EVL-I) in a relative location identical to that of the 11a insertion site, but the sequences share no similarity (Lambrechts, 2000).

Regulation of Ena/VASP

Ena/VASP family members share each other for serine/threonine phosphorylation sites. VASP harbors three phosphorylation sites (Ser157, Ser239, Thr278), whereas Mena contains the first two, and EVL only the first site (Butt, 1994; Gertler, 1996; Lambrechts, 2000). Ser157 is located N-terminally of the central Proline rich region. Ser239 and Thr278 are within the EVH2 domain, adjacent to the G- and F-actin binding sites, respectively (Kwiatkowski, 2003).

The mammalian Ena/VASP proteins are known substrates of cAMP and cGMP-dependent serine and threonine kinases PKA, PKG, AMPK (Gertler, 1996; Lambrechts, 2000). In vivo, Ser157 is preferentially phosphorylated by PKA, whereas Ser239 and Thr278 are targeted by PKG and AMPK respectively (Blume, 2007).

These is a divergence between Ena protein in invetebrates and vertebrates: *Drosophila* Ena lacks a clear equivalent of VASP Ser157 (Gertler, 1996). When expressed in Ena/VASP-deficient cells, Ena localizes properly but fails to complement the hypermotility phenotype, possibly because it cannot be phosphorylated by PKA (Loureiro, 2002), suggesting that the basic molecular functions of the family have been conserved across evolution but that the vertebrate proteins may require additional regulation by PKA/PKG.

Ena/VASP can be phosphorylated in tyrosine as well. In fact, Ena was identified as target of Abl kinase in *Drosophila* (Gertler, 1995). Another difference between vertebrate and invertebrate Ena/VASP proteins is that the tyrosine phosphorylation sites in Ena are not conserved in the mammalian Ena/VASP proteins.

VASP phosphorylation effects

Originally VASP was identified in platelets as a substrate for cyclic-nucleotide-dependent protein kinases (Halbrugge, 1990). Its strategic position in important kinase-driven signaling cascades established phospho-VASP as a marker for the integrity of cyclic-nucleotide-dependent pathways.

Successively, it has been demonstrated a key role for this protein in many cellular processes such as actin dynamics, chemiotaxis, motility, cell-cell and cell-matrix adhesion (Howe, 2002; Lee and Chung, 2009; Worth, 2010). Despite its importance, the cellular function and complexity of VASP phosphorylation has remained uncertain.

VASP phosphorylation is able to drive its subcellular localization; VASP Ser157 accumulates at sites of high actin dynamics, at focal adhesion and at the cell periphery (Benz, 2008). Phosphorylation of VASP Ser239 and Thr378 has minor effect on the subcellular distribution of VASP. However, cell-type specific regulation of Ena/VASP proteins might exist because phosphorylation status of Ser239 modulates VASP localization in renal epithelial cells (Linsday, 2007).

VASP goes to phosphorylation in response to microenviroment signals such as matrix associated protein (Collagen Type I, Fibronectin) or soluble factors (NO, IL-8) (Neel,

2009; Pula, 2006) .VASP is associated with actin filaments and focal adhesions, which form the interface between the cytoskeleton and the extracellular matrix. During cell detachment, VASP becomes heavily phosphorylated by PKA on Ser157, and is dephosphorylated transiently during reattachment to fibronectin (Howe, 2002). At focal adhesion VASP phosphorylation status regulates β1 integrin – RIAM/Talin complexes modulating cell speed migration (Worth, 2010). A study has shown that dephosphorylation of VASP at Ser157 may be required for the growth of adhesion strength during membrane retraction in microglia, which supports a role for VASP in regulation of integrin function (Lee and Chung, 2009).

VASP phosphorylation is critical for its role in the architecture of the actin cytoskeleton during cell-cell junction formation in epithelial (Vasiouckin, 2000; Quinlan, 2004; Scott, 2006) and endothelial cells (Benz, 2008; Hoelzle 2011). VASP Ser157 is enriched at focal adhesion and phosphorylation level decreased time-dependently when cells established cell–cell contacts (Benz, 2008). This fine regulation through phosphorylation can influence its protein-protein interaction and actin regulatory properties. It is known that binding of proteins to the EVH1 domain occurs independently of phosphorylation (Harbeck, 2000). In contrast, binding of certain SH3-domain containing proteins to the proline-rich central region of Ena/VASP proteins is regulated by serine/threonine phosphorylation. VASP, when is not phosphorylated, forms complexes with different focal adhesion proteins such as nSrc (Lambrechts, 2000), c-Abl in murine fibroblasts (Howe, 2002), α-II-Spectrin in human endothelial cells (Benz, 2008), Integrin αvβ3 in osteoclasts (Yaroslawkiy, 2005). On the other side, VASP phosphorylation is able to induce other interactions, such as PKA

phosphorylation of Ser157 is able to regulate integrin β1-Talin complexes in murine fibroblasts (Worth, 2010), or PKG phosphorylation of Ser239 is able to induce VASP-CXCR2 interaction in neutrophils (Neel, 2009).

The role of VASP in actin dynamics is in part influenced by its phosphorylation status. In fact, phosphorylation of VASP by PKA in vitro abolished VASP anti capping activity in the presence and absence of profilin and bundling formation (Barzik 2005), probably because of an negative charge over phosphates that can interfere with F-actin binding. The phosphorylation reduces the actin nucleating activity and F-actin-binding of VASP (Harbeck, 2000). In the EVH2 domain, the binding to G-actin is greatly reduced by phosphorylation of Ser239 (Walders-Harbeck, 2002). This phosphorylation site is conserved in Mena but absent in EVL, which points to differences among the family members in regard to their regulation (Gertler, 1996).

Studies of mutant mice revealed that VASP is required for the PKA-mediated inhibition of platelet aggregation (Aszodi, 1999) suggesting that VASP is the critical PKA substrate required for this process, and that Ser157 may be the key site of regulation.

MENA phosphorylation effects

Most studies of MENA phosphorylation effects are been concetrated on tyrosine. First evidences in *Drosophila* demonstrated that Abl phosphorylates Ena during development. However, Ena may be phosphorylated by other tyrosine kinases (Gertler, 1995). The phosphorylated tyrosine residues are clustered in the proline-rich region and it has been shown that the binding of the SH3 domain of Abl and Src to Ena is

abrogated when it is phosphorylated by D-Abl in vitro (Comer, 1998). One difference between vertebrate and invertebrate Ena/VASP proteins is that the tyrosine phosphorylation sites in Ena are not conserved in the mammalian Ena/VASP proteins, however human MENA is phosphorylated in Tyrosin 296 by c-Abl (Gertler, 1996; Tani, 2003).

MENA Tyr-296 is not conserved in VASP or EVL, raising the possibility that only MENA, but not EVL or VASP, is regulated by c-Abl. Tyr-296 in MENA is localized close to the proline-rich central region, as it is in Ena, suggesting that this phosphorylation could regulate binding of ligands to MENA.

Few studies are been concentrated on serine phosphorylation effects. In random fibroblast migration, phosphorylation of MENA at its first phosphorylation site (Ser236, the equivalent of VASP Ser157) is essential for this function and MENA phospho-mutant studies demonstrate that phosphorylation is not essential for its subcellular localization (Louireiro, 2002).

The epithelial isoform $hMENA^{11a}$ is phosphorylated in response to EGF and NRG1 stimulation and cooperates with the mitogenic HER2 activity in breast cancer cell lines (Di Modugno, 2007, 2010). The site of the 11a insertion is adjacent to the F- and G-actin binding sites, and the 11a insertion can be phosphorylated, potentially disrupting actin binding. Therefore, it is possible that the 11a inclusion affects the way in which MENA interacts with barbed ends and adds an extra site for phosphoregulation of MENA function.

Potential Role of Ena/VASP during Tumor Formation and Metastasis

MENA has several unique features not found in the other Ena/VASP proteins that endow it with the ability to potentiate carcinoma metastasis dramatically (Gertler & Condeelis 2011). Analysis of the invasion signature of murine mammary carcinoma cells revealed that MENA expression is upregulated in invasive cells compared to average primary tumor cells (Wang, 2004; Wyckoff, 2004). Robert Weinberg's group found MENA upregulated in metastatic cells that also upregulated the transcription factor Twist (Yang, 2004). Increased MENA levels were also observed in invasive human breast cancers compared to normal mammary tissue (Di Modugno, 2004). In a cohort of breast cancer tissue samples a significant correlation among hMENA, epidermal growth factor receptor 2 (HER2) overexpression, the proliferation index (high Ki67), and phosphorylated Mitogen Associated Protein Kinase (MAPK) and AKT was found. Furthermore, the overexpression of hMENA/hMENA^{11a} cooperated with Epidermal growth factor receptors family and downstream pathways to increase cell proliferation in breast cancer cell lines (Di Modugno 2007, 2010). In addition to breast cancer, MENA upregulation has been observed in advanced pancreatic, colon, gastric and cervical carcinomas (Pino, 2008; Gurtzu, 2009; Toyoda, 2009; Junilla 2010).

It has been demonstrated that MENA alternative splicing generate isoforms with different biological function. In murine model, the comparison between stationary and invasive carcinoma cells revealed that the 11a exon is expressed in tumor cells making up the bulk of the primary tumor, but this exon is essentially undetectable in the mRNA from invasive tumor cells (Goswami, 2009). Consistent with this finding, in human the 11a exon is specific to MENA isoforms expressed in tumor epithelial cell lines and is

not found in mesenchymal cells (Di Modugno, 2007; Pino, 2008). In fact, the 11a exon becomes excluded in human mammary epithelial cells that are driven to undergo EMT by expression of the EMT inducing transcription factor Twist (Warzecha, 2009). $hMENA^{11a}$ is also expressed in normal ovarian tissue where its inclusion is promoted by the Fox2 splicing factor (Venables, 2009). Interestingly, analysis of 21 aggressive ovarian tumors revealed a reduction in Fox2 levels compared to normal tissue and a concomitant loss of 11a inclusion in MENA (Venables, 2009). Therefore, $MENA^{11a}$ appears to be included in epithelial cell and primary carcinoma mRNA but is excluded in mesenchymal cells as well as in invasive/aggressive tumor cells.

Recent findings suggest that expression of MENA classic, and MENA+++ (or MENA INV) in particular, promotes carcinoma cell invasion in 3D collagen gels and increases carcinoma cell motility in vivo (Philippar, 2008). MENA classic and MENA INV localize to and stabilize invadopodia, actin-rich protrusions required for degradation and movement through the extracellular matrix and possibly for invasion across basement membranes, thereby increasing the invasive and metastatic potential of tumor cells. MENA-INV expressing migratory carcinoma cells are highly sensitive to EGF in their protrusion and chemotaxis activities, leading to significantly enhanced in vivo invasion, whereas $MENA^{11a}$ expression is correlated with decreased EGF-induced in vivo invasion (Roussos, 2011). Altogether, these findings demonstrate that hMENA may be a crucial axis of “druggable” signaling pathways leading to tumor cell invasion.

Different from MENA, the contribution of VASP in tumor progression is still unknown. While its engagement in migration and membrane protrusion dynamics in normal cells is well-established, a similar role for VASP in cancer cells has not emerged. Dertsiz and

collagues in 2005 demonstrated lung adenocarcinomas had significantly greater VASP expression than normal epithelium and VASP expression increased significantly with more advanced tumour stage (Dertsiz, 2005). Recently in the same tumor model, it has been demonstrated that ribosomal S6 kinase 1 (RSK1) was reduced in metastatic versus primary lung cancer lesions and RSK1 is able to interact and phosphorylate VASP on Thr278, suggesting an anti-invasive role for phophorylated VASP (Lara, 2011). Another recent paper indicated a possible alteration of VASP phosphorylation and function in colorectal cancer, since Ser157 and Ser239 VASP phosphorylation were significantly reduced in colorectal adenocarcinomas compared to their normal counterparts (Zuzga, 2011). Furthermore, they demonstrated that VASP Ser239 phosphorylation mediates arrest of invadopodia mediated proteolysis (Zuzga, 2011).

Pancreatic ductal adenocarcinoma: an overview

The pancreas is an organ that has endocrine and exocrine compartments. The endocrine compartment consists of α, β, δ, e, and pancreatic polypeptide cells organized into islets, whereas the exocrine compartment consists of acinar, ductal, and centroacinar cells. Acinar cells synthesize and secrete digestive enzymes, which are concentrated into a bicarbonate rich fluid that traverses through a complex network of ducts. The terminal, or intercalated, ducts are lined by flat, almost squamous-like epithelia. Terminal end duct cells that interface with acini are called centroacinar cells. Intercalated ducts merge to form intralobular ducts (lined by cuboidal epithelia), and these in turn merge to form interlobular ducts, which finally merge to form into the main duct (lined by simple

columnar epithelia) that traverses the pancreas to the duodenum, delivering fluid laden with digestive enzymes.

Pancreatic cancer is one of the most dreaded of all cancers, having the highest case-fatality rate of any major cancer. It is difficult to detect, early to metastasize, resistant to treatment and causes death with relatively little tumor burden. The best hope for curing pancreatic cancer is early detection and surgery, although specialists focus on early detection because discovering pancreatic cancer at a stage when surgery can be performed only happens in a minority of all cases. Chemotherapy has been shown to be of some help in treating pancreatic cancer, while radiation therapy is more controversial (reviewed in Hidalgo, 2010). The most common subtype of human pancreatic malignancy is Pancreatic Ductal AdenoCarcinoma (PDAC). This cancer originates in the ductal epithelium and evolves from premalignant lesions to fully invasive cancer. It has been established the most common neoplastic precursor to invasive adenocarcinoma of the pancreas is known as PanIN (pancreatic intraepithelial neoplasia) (Hruban, 2001; Maitra, 2006). There is a clear accumulation of genetic alterations associated with the histological progression from low-grade PanINs (PanIN-1) to intermediate grade PanINs (PanIN-2), to high-grade PanIN (PanIN-3), to invasive carcinoma.

The molecular basis underlying the development of PDAC involves a number of diverse pathways and molecules. Data suggest that pancreatic cancer results from the successive accumulation of gene mutations (Li, 2004; Maitra, 2006). The four hallmark mutations implicated in PDAC (KRAS [>90%], p16INK4A [>90%], TP53 [70%], and SMAD4 [55%]) have all been confirmed by genetically engineered mouse models in which targeted activation of *Kras* with concomitant inactivation of *Trp53* or *Ink4A* results in

the development of pancreatic cancer that is identical to the cognate human disease (Hingorani, 2005; Bardeesy, 2006). Almost all patients with fully established pancreatic cancer carry one or more of four genetic defects (Maitra, 2008).Oncogenic KRAS is known to stimulate cellular migration and permit survival in limiting nutrients, effects that may be accentuated when the wild-type Kras allele is lost as observed in metastases (Qiu, 2011). Deletion of the Ink4a locus in Kras mutant pancreatic cells promotes Notch and NF-kB signaling and metastasis in mouse models (reviewed in Mazur and Siveke, 2011); likewise, point mutant TrP53 alleles possess novel properties that promote tissue invasion and metastasis by stimulating integrin/EGFR signaling (Muller, 2009). Finally, Smad4 loss promotes PDAC metastasis in mice (Mazur and Siveke, 2011), and SMAD4 loss correlates with high metastatic burden clinically (Iacobuzio-Donahue, 2009).

Characteristic of pancreatic cancer is the formation of a dense stroma termed a desmoplastic reaction (Chu, 2007; Mahadevan, 2007). The stroma is not just a mechanical barrier; rather, it constitutes a dynamic compartment that is critically involved in the process of tumor formation, progression, invasion, and metastasis (Chu 2007; Mahadevan 2007). The pancreatic stellate cells (also known as myofibroblasts) play a critical role in the formation and turnover of the stroma. On activation by growth factors such as TGFβ1, platelet-derived growth factor (PDGF), and fibroblast growth factor, these cells secrete collagen and other components of the extracellular matrix; stellate cells also appear to be responsible for the poor vascularization that is characteristic of pancreatic cancer (Masamune, 2009). Furthermore, stellate cells regulate the reabsorption and turnover of the stroma, mainly through the production of matrix metalloproteinases (Zhang, 2007) and comigrate with PDAC cells to promote

metastasis in a transplantation model system (Xu, 2010).

The role of angiogenesis in pancreatic cancer remains controversial. Although early data suggested that pancreatic cancer is angiogenesis dependent, as are most solid tumors, treatment with angiogenesis inhibitors has failed in patients with pancreatic cancer. A recent study in a mouse model showed that targeting the stromal hedgehog pathway increases tumor vascularization, resulting in increased delivery of chemotherapeutic agents to pancreatic tumors and greater efficacy (Olive, 2009).

Inflammation has a well-established role in promoting tumor progression (Grivennikov, 2010) and genetically engineered mouse models using mutant Kras support the notion that inflammation accelerates pancreatic carcinogenesis (Guerra 2007, 2011). In humans, chronic pancreatitis represents one of the highest risk factors for developing PDAC (Lowenfels, 1993). However, most PDAC patients do not have a history of chronic pancreatitis. PanIN lesions is associated with the appearance of an inflammatory stroma characterized by activated fibroblasts and myeloid-derived cells (Clark, 2007). Also, Inflammation is able to induce EMT even in low-grade PanINs that harbor only oncogenic Kras mutations, driving cell delamination from the glandular preneoplasm into the surrounding tissue and circulatory system (Rhim, 2012), suggesting that inflammation may promote cancer progression through two independent mechanisms: by facilitating changes in the microenvironment at the primary site of neoplasia and by facilitating invasion and dissemination by increasing cellular access to the circulation.

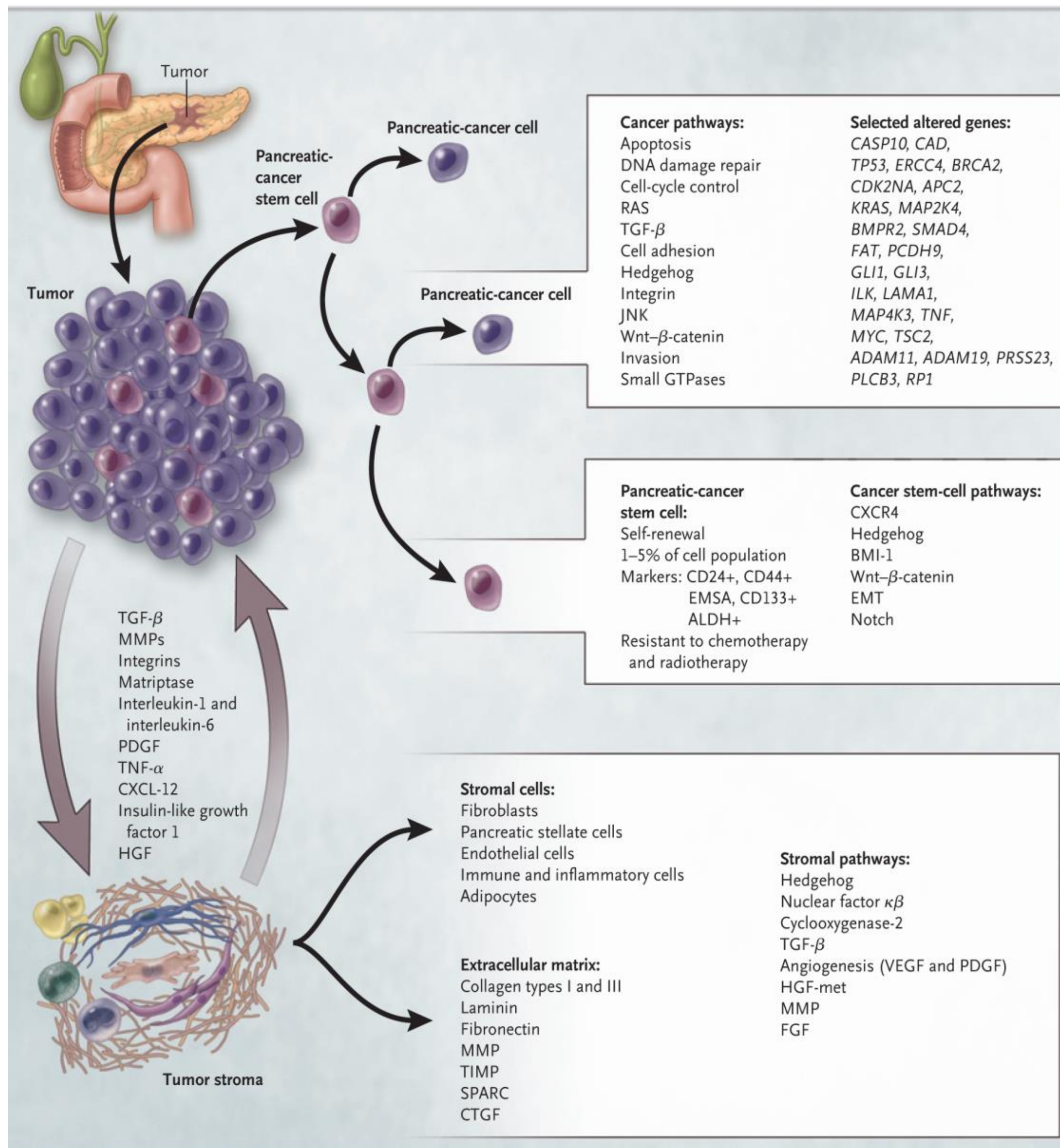

Figure 5. *Components of Pancreatic Cancer. Pancreatic cancers are composed of several distinct elements, including pancreatic-cancer cells, pancreatic cancer stem cells and the tumor stroma. Pancreatic cancer is characterized by a dense, poorly vascularized stroma; this microenvironment contains a mixture of interacting cellular and noncellular elements. Autocrine and paracrine secretion of growth factors such as plateletderived growth factor (PDGF) and transforming growth factor β (TGF-β) and cytokines results in continuous interaction between the stromal and cancer cells. Pancreatic stellate cells are a key cellular element in the stroma. They are characterized by the expression of desmin, glial fibrillary acidic protein, and intracellular fat droplets. ALDH+ denotes aldehyde dehydrogenase, CTGF connective-tissue growth factor, CXCL-12 chemokine 12 ligand, EMSA electrophoretic mobilityshift assay, EMT epithelial-to-mesenchymal transition, FGF fibroblast growth factor, GTPase guanosine triphosphatase, HGF hepatocyte growth factor, HGF-met hepatocyte growth factor mesenchymal–epithelial transition factor, JNK Jun N-terminal kinase, MMP matrix metalloproteinase, SPARC secreted protein, acidic, cysteine-rich, TIMP tissue inhibitor of MMP, TNF-α tumor necrosis factor α, and VEGF vascular endothelial growth factor. Adapted from "Hidalgo M (2010) Pancreatic cancer; NEJM".*

Alternative splicing in cancer

Alternative splicing is a key molecular event in the gene expression process, consisting in the recognition of exons and introns through sequences at the boundaries between them as well as on splicing regulatory sequences located within exons and introns. Splicing is a post-transcriptional process involved in the maturation of mRNAs and contributes to proteomic diversity. In fact, almost all human genes are susceptible to alternative splicing generating at least 10 times more mRNAs than genes (Blencowe BJ 2006; Trapnell, 2010). This process is tightly regulated in a tissue- and cell-type dependent fashion (Matlin, 2005) and partecipates in different cellular program, including apoptosis, cell growth, angiogenesis, cell motility and EMT (Blencowe, 2006). Aberration of splicing machinery results from mutations in splicing sites or dysfunction of splicing regulatory proteins (Licatalosi and Darnell, 2010) and this could occur during tumor initiation and progression, producing splicing variants expressed in cancer tissue compared to the normal counterpart and viceversa (Dutertre, 2010). A variety of cancer-associated genes express alternatively spliced isoforms (Srebrow, 2006), indicating that regulation at the level of splicing may play important roles in cancer onset and progression.

EMT is also likely regulated by post-transcriptional mechanisms including alternative pre-mRNA splicing. In human breast cancer, changes of fox-1 homologue (RBFOX) and epithelial splicing regulatory proteins (ESRPs) families of splicing regulator are found (Shapiro, 2011; Horiguchi, 2011). Warzecha and collaborators demonstrated that ESRPs are components of an epithelial genes signature and demonstrated a downregulation of these proteins in cells that undergo EMT (Warzecha, 2009). ESRP

splicing program regulates epithelial and mesenchymal variants of FGF receptors 2 (FGFR2), CD44, ENAH, P120-catenin and other many genese with functions in cell-cell adhesion, migration and cytoskeletal dynamics, all processes involved to phenotypic changes that occur during EMT. Horiguchi and collaborators demonstrated also that ESRPs proteins are downregulated during TGFβ mediated EMT in breast and pancreatic cancer cell lines, linking alternative splicing with EMT (Horiguchi, 2011).

Alternative splicing permits the generation of protein isoforms having different biological activities, for example, the splicing of fgfr-2 genes generated spliced variants with distinct FGF-binding specifities and ESRPs proteins activate the splicing of exon IIIb and silence the splicing of exon IIIc leading to alternatively variants expression in epithelial and mesenchymal cells (Warzecha 2009,2010).

Outline of the PhD thesys

My PhD thesis aims to shed light on *hMENA* splicing program deregulated in cancer, focusing on pancreatic adenocarcinoma. In particular, my work links *hMENA* splicing program altered in PDAC with the well known role that Ena/VASP proteins have in cell adhesion and migration, processes frequently altered during cancer progression and metastatic events.

Previous and recent results shown that splicing regulatory factors, which process numerous genes regulating biological processes such as cell morphology, adhesion, migration, and proliferation, are altered in ovarian, prostatic, breast and pancreatic cancer (Venables, 2009; Warzecha, 2010; Shapiro, 2011). A variety of cancer-associated genes, among them *hMENA,* express alternatively spliced isoforms, indicating that regulation at the level of splicing may play important roles in cancer onset and progression.

Based on these findings, this thesis aims to evaluate whether hMENA alternative isoform expression observed in breast cancer, occurs also in PDAC. Futhermore, since Ena/VASP proteins play an important role in epithelial dynamics, cell – cell junction formation and cell invasion (Vasiouckin, 2000; Ehrlich, 2002; Loureiro, 2002), this thesis focuses on hMENA alternative isoform expression potential role in cell-matrix adhesion and cell invasion.

Our group has characterized three *hMENA* isoforms, one 88-kDa unspliced isoform defined as “classic” and two splice variants, named hMENA^{11a} and hMENAΔv6. The first (90 kDa) characterizes epithelial cancer cell lines expressing E-Cadherin, whereas the latter (80 kDa) is expressed in breast and cervix tumor cell lines displaying the

phenotypic features of EMT with migratory behaviour.

Differently from the breast model, where hMENA is not expressed in normal epithelium but overexpressed following transformation and tumor progression, hMENA and hMENA^{11a} are co-expressed in normal pancreas whereas primary tumors lack the expression of the epithelial hMENA^{11a} isoform, in line with recent results indicating that *hMENA* splicing may be altered in pancreatic tumor progression (Horiguchi, 2011). Western Blot analysis of pancreatic cell lines evidence an alternative expression of hMENA isoforms. hMENA^{11a} is expressed in the normal human pancreatic ductal epithelial cell line (HPDE) besides epithelial pancreatic tumor cell lines (CFPAC, T3M4, and PACA44), whereas hMENAΔv6 is expressed in PANC1 cells that lack the epithelial isoform hMENA^{11a} and express VIMENTIN.

Epithelial cells express one or more of the Ena/VASP proteins, and these in turn localize to the leading edges of lamellipodia, the tips of filopodia, focal adhesions, cell–cell junctions, suggesting that Ena/VASP proteins may be important regulators of actin dynamics in epithelia.

Starting from the knowledge that the phospho-VASP is absent in resting monolayers, we tested all cell lines grown at sub-confluence after 48h of reattachament and we observe that VASP is more phosphorylated at Ser-157 either in normal or pancreatic cancer cell lines with an epithelial phenotype and expressing hMENA^{11a}, whereas we don't observe an appreciate level of P-Ser157 in the hMENA^{11a} negative/hMENAΔv6 positive PANC1 cell line.

Further evidences indicate that Ser157 VASP phosphorylation is mediated by PKA, observed in hMENA^{11a} expressing cells respect than hMENA^{11a} negative cells when

cell-cell contacts are yet stabilized. Specific silencing of hMENA^{11a} isoform in HPDE cells determines a significant reduction of P-Ser157 VASP level. Furthermore, confocal analysis of HPDE cells demonstrate P-Ser157 VASP and hMENA^{11a} co-localize at focal adhesion and the co-localization is maximum when PKA is activated. On the contrary, in PANC1 (hMENA^{11a} negative) cells, Ser157 VASP is not phosphorylated when cell-cell contacts are stabilized. The transfection of hMENA^{11a} isoform in PANC1 cells rescues either P-Ser157 VASP level or P-Ser157 VASP co-localization with hMENA^{11a} at focal adhesion.

We also investigated ESRPs proteins which are a component of an epithelial gene signature and the downregulation of the ESRPs in cells that undergo EMT corresponded with switches in splicing of several ESRP-regulated exons, among them *hMENA*. We focused on *hMENA* splicing impaired in PANC1 cells, transfecting ESRP1 cDNA in these cells. The resulting expression of hMENA^{11a} determine an increase of P-Ser157 VASP signal, an increase of adhesive properties to different matrix-derived components and a reduction of cell invasive capabilities.

Altogheter, our results demonstrate that *hMENA* splicing is a critical step in cancer and the lost of epithelial hMENA^{11a} isoform strongly contributes to the aggressivness of this incurable neoplasia.

Chapter 3- Material and Methods

Reagents and antibodies production

The mouse monoclonal antibody (IgG1) specific for the hMENA^{11a} isoform was developed against a 18-aa peptide (RDSPRKNQIVFDNRSYDS) belonging to the 11a exon, purified and employed for WB, immunohistochemistry and confocal analysis. To obtain antibodies specific to the hMENAΔv6 isoform, rabbits were immunized with a peptide coded by a region covering the 5 and 7 exon junction (NH2-ERRISSAGIVLG-COOH). The rabbit antiserum was then purified by two-step affinity chromatography using peptides coded by the 5 and 6 exon junction or by the 6 and 7 exon junction (EWERERRISSAAA and SQQGIVLGPLAPP) in order to deplete the serum from the antibodies recognizing regions common to all the hMENA isoforms. The specific IgGs were then purified using the immunogenic peptide (ERRISSAGIVLG) by affinity chromatography (Di Modugno, paper submitted).

H89 and Forskolin were purchased from Sigma.

Cell lines

The following cell lines were purchased from the American Type Culture Collection: CFPAC, PANC1. The T3M4, PACA44, and PT45 cell lines were kindly provided by Dr. F. Velotti (Tuscia University, Viterbo, Italy). The normal human pancreatic ductal cell line (HPDE) was kindly provided by Dr. P. Allavena (Humanitas Fundation, Milano, Italy). All cell lines were maintained in RPMI 1640 (Life Technologies, Inc.). The medium was supplemented with 10% fetal bovine serum (Life Technologies) and L-glutamine (Bio Whittaker).

To correlate VASP phosphorylation with cell density, all cell lines tested were detached

with trypsin-EDTA and collected into culture medium. Cells were seeded on tissue culture plates for 48h before lysis and analysis by Western blotting.

RT-PCR

hMENA splice variants were detected by RT-PCR using MTC1*f* and MTC4*r* primers as reported (Di Modugno, 2007). PCR products were analyzed on a 1% agarose gel electrophoresis and ethidium bromide staining.

In vitro Transcription-coupled Translation

The *in vitro* translation of the $hMENA^{11a}$, *hMENA* and *hMENAΔv6* cDNA (inserted into the pcDNA3.1 vector) was examined in an *in vitro* transcription-coupled translation system following the manufacture's instruction (TNT, rabbit reticulocyte lysate system, Promega Corporation).

3D cultures

HMT-3522 cell lines (S1, S2, T4-2) were cultured in conventional tissue culture polystyrene and in 3D assays using growth factor reduced reconstituted basement membrane (Matrigel; BD), according to previously described methods (3, 4). Briefly, S2 and T4-2 cells were maintained on flasks coated with acid extracted collagen type I (PureCol; Advanced BioMatrix), while for 3D assays, cells were cultured on a thin layer of polymerized Matrigel for 4 days in the presence of growth media containing 5% lrECM or pharmacological inhibitors. For growth factor depletion, EGF was removed from S1 cultures 24h prior to extraction from lrECM. Each lot of

pharmacological inhibitors and lrECM used in the study were carefully validated prior to use in experiments

Transfections, Small-interfering RNA treatment and hMENA^{11a}/ESRP1 transduction

The *hMENA^{11a}*–pMSCV, pMXs-IRES-blast2 Esrp1-FF and pMXs-IRES-blast2 retroviral transductions were performed using the 293T packaging cell line. Cells were transduced in T25 flasks with 5μg of the retroviral expression vector, 1,25 μg of pCMV-VSV-G and 3,25 μg of Gag-Pol using LipofectAMINE 2000. After 5 hr, the media was replaced with fresh DMEM with 5% FBS, and virus was harvested after an additional 24 hr. Target cells in exponential growth phase were plated in 6-well plates at a density of $3x10^5$ cells/well and were transduced with a 50/50 mix of viral supernatant and growth media. Selection was carried out using 10 μg/ml blasticidin (for *ESRP* transduction) or 500 μg/ml of G418 (for *hMENA^{11a}* transduction) in complete culture medium.

Specific 11a exon silencing was achieved by transfecting cells with a mix of three siRNAs, each matching 21 nucleotides within the 11a exon sequence; highly functional siRNA sequences were selected using siDirect 2.0 web server, that provides efficient and target-specific siRNA design for mammalian RNAi.

Mutants of *hMENA^{11a}* were obtained by the use of QuikChange II Kit (Qiagen), following the manufacturer instruction. The primers used were designed to present the substitution of the codons corresponding to two Ser (TCT and TCA) of the 11a exon, with codons (**G**CT and **G**CA) encoding Alanine. PCR products were cloned into pcDNA3.1 vectors and sequenced.

Western blot (WB) analysis and Immunoprecipitation (IP)

Cells were lysed for 30 min at 4°C in 10% glycerol, 0.1% SDS, 0.5% DOC, 1% NP-40 in PBS containing protease and phosphatase-inhibitors. Lysates were centrifuged and protein quantification of supernatants was determined using BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Lysates (50 µg) were resolved on 10% polyacrylamide gel and transferred to nitrocellulose membrane (GE-Healthcare, Little Chalfont, UK). Blots were blocked for 1 h with 3% skimmed milk in TBST and probed in 3% skimmed milk/TBST overnight at 4°C with the following antibodies: 10 µg/ml anti-hMENA rabbit CKLK1 (pan-hMENA) antibody; 1 µg/ml anti-hMENA^{11a} mouse monoclonal antibody; anti-hMENAΔv6 (0,6µg/ml); mouse anti-E-CADHERIN from BD Biosciences (San Jose, CA); mouse anti-N-CADHERIN from DakoCytomation (Glostrup, Denmark) VASP Ab from BD bioscience (USA); P-Ser157 VASP and P-Ser239 VASP Abs from Nanotools (Germany).

After 3 washes of 15 min each, blots were incubated with the appropriate secondary antibody conjugated with HRP for 1 h and then washed again three times. The protein signals were detected by ECL kit (GE-Healthcare). For actin signal, blots were reprobed with 1 µg/ml monoclonal anti-actin, mouse-ascites fluid clone AC-40 (Sigma Aldrich, Poole, UK). X-ray films were scanned by HP Scanjet 5470 and processed by Corel Photo Paint 12.

Immunoprecipitation experiments were carried out using Pierce Crosslink IP kit (Thermoscientific, Rockford, USA), following the manufacturer Instruction, with Pan-hMENA CKLK1 rabbit Ab (Di Modugno et al. 2007); hMENA11a rabbit Ab and pre-immune rabbit IgG as negative control.

Two-Dimensional Electrophoresis (2DE)

Cells were washed extensively with PBS, pellets lyophilized and solubilized with 2DE solubilization buffer (9 M Urea, 10 mM Tris, 4% CHAPS, 65 mM DTT, 2% IPG buffer ampholine pH 3-10, protease inhibitor cocktail). Protein samples (200 µg) were applied to 7 cm IPGstrips pH 3-10NL (GE-Healthcare) by in-gel rehydration. Iso-electro Focusing (IEF) was performed with an IPGphor system (GE-Healthcare) following a standard protocol as described (Conti, 2005). Strips were equilibrated in 50 mM pH 8.8 Tris-HCl buffer containing 6 M urea, 30% glycerol, 2% SDS and 2% DTT, followed by an incubation in the same buffer replacing DTT with 2.5% iodoacetamide. The strips were loaded on the top of 10% acrylamide SDS-PAGE gels for the second dimension separation. Proteins were electrontransferred onto nitrocellulose membranes and Western blot performed as described above. Images were acquired at high resolution and 2D immunoreactivity patterns analyzed using Progenesis PG240 v2005 software (Nonlinear dynamics, Newcastle, UK). Relative molecular mass (Mr) were estimated by comparison with Mr reference markers (Precision, Bio-Rad, Hercules, CA, USA) and isoelectric point (pI) values assigned to detected spots by calibration as described in the GE-Healthcare guidelines.

Confocal microscopy and image analysis

Cells were cultured in individual wells of a 24-well plate. Cells were fixed in modified paraformaldehyde fixative (60mM PIPES, 25mM HEPES, 10mM EGTA, 2mM $MgCl_2$, 120mM sucrose, 4% paraformaldeyde; final pH 7.4) for 15 minutes at room

temperature. Cells were incubated with P-Ser157 VASP (Cell Signaling) at dilution 1:100, or with hMENA^{11a} mouse monoclonal antibody at dilution 1:50 or pan-hMENA antibody (gently provided from Gertler's Lab) at dilution 1:1000, and were stained using AlexaFluor594-conjugated, AlexaFluor488-conjugated antibody, and DAPI (Invitrogen).

Fixed and stained cells were imaged using a Zeiss 710 laser scanning microscope under 63x Plan Apochromat (1.4 numeric aperture) oil immersion lens with Zen 2008 imaging software (Zeiss).

Adhesion Assays

Adhesion assays were performed using plates (96 well) pre-coated with collagen I, collagen IV, fibronectin (BD, Biosciences) or with 1% bovine serum albumin as the control. Cells were harvested with trypsin/EDTA and resuspended in 2% FCS medium. 0,1 ml cells suspension were allowed to attach at 37 °C for 1,5 or 3 h. Unbound cells were removed by washing twice with phosphate-buffered saline. Attached cells were fixed in 96% ethanol at room temperature for 10', stained with 0,1 % crystal violet for 30', then resuspended in 0,2% TritonX in dH20 to lyse cells and read absorbance at 570 nm. Each experiment was reproduced three times.

Cell invasion assay

Cells were counted and equal numbers (50.000) were seeded in Matrigel invasion chamber (24 wells; BD Biocoat Matrigel invasion chamber, BD Biosciences) in

duplicate following the manufacturer's instruction. Cells were allowed to invade for 24h, then were stained, photographed and at least 10 fields were counted. Each experiment was performed three times.

Patients and tissue specimens

A series of 26 patients (median age, 62 y; range 39-78 y) who underwent pancreatic resection or biopsy at the Regina Elena National Cancer Institute between 2002 and 2005 with a diagnosis of pancreatic adenocarcinoma were retrospectively collected for immunohistochemical studies. This series included 12 primary (9 stage II, 1 stage III, and 2 stage IV) and 14 metastatic carcinomas (11 liver metastasis and 3 other abdominal sites). Tumors were staged according to the Unione Internationale Contre le Cancer TNM System 2002 and collected according to the Internal Ethic Committee guidelines.

Immunohistochemistry

Pancreatic cancer specimens were fixed for 18 to 24 h in buffered formaldehyde and then processed through to paraffin wax. hMENA expression was evaluated by immunohistochemistry using the monoclonal antibody clone 21 (BD Transduction; 2.5 μg/mL) that recognizes all the hMENA isoforms and does not crossreact with other members of Ena/VASP family proteins (Di Modugno, 2006). $hMENA^{11a}$ was revealed using the anti-$hMENA^{11a}$ specific mAb described above. Dewaxing, antigen retrieval,

incubation with the primary antibody, chromogenic reaction with 3,3′-diaminobenzidine, and counterstaining with Mayer Haematoxylin were done with an automatic autostainer (Vysion Biosystems Bond; Menarini). Sections were mounted in aqueous mounting medium (Glycergel; DakoCytomation). The intensity of hMENA staining, detected in the cytoplasm, was scored from 0 to 3+ according to the following criteria: no staining, score 0; weak cytoplasmic staining of neoplastic cells, score 1+; moderate cytoplasmic staining, score 2+; and strong cytoplasmic staining, score 3+. Evaluation of the immunohistochemical data was done independently and in blinded manner by two investigators.

Statistical analysis

All experiments were repeated at least twice. Statistical significance was determined by Student's t test (two tailed) comparison between two groups of data. Asterisks indicate significant differences of experimental groups compared with the corresponding control condition. Statistical analysis was done using GraphPad Prism 4, V4.03 software (GraphPad, Inc.). Change in the phosphorylation status by 2DE was evaluated, using Progenesis v.2004 software (Nonlinear Dynamics), by absorbance indicated as normalized spot volume. Normalization was done by multiplying the total spot volume by the constant factor 100, which produces spot percentage volume. Densitometric quantitation of hMENA immunoreactivity was determined by ImageJ and normalized in comparison with the actin immunoreactivity.

Chapter 4 - Results

hMENA splicing program generates isoforms differently expressed in epithelial and mesenchymal cells

To characterize *hMENA* splicing variants relevant to breast cancer progression, we performed *hMENA* transcript synthesis and cDNA sequencing using hMENA-specific primers in the breast cancer cell line MDA-MB-231. Using this method, in addition to the sequence of *hMENA* (1713nt), we were able to identify a new sequence of 1602 nucleotides from the ATG to the stop codon. We named this *hMEN*A isoform *hMENAΔv6* (GenBank, Accession: EU255274) since it lacks the internal exon 6 of 111 nucleotides (Fig 6 A-B). *hMENAΔv6* cDNA encodes a protein of 533aa lacking a 37aa internal peptide located between the LERER and the Proline-rich region of hMENA (Fig 6 A). The absence of this peptide brings the LERER domain and PKA Ser phosphorylation site (Ser 236 in mice) with the proline-rich domain closer together (Fig 6 B). The WB analysis of $hMENA^{11a}$- (*$hMENA^{11a}$* variant includes an exon absent in *hMENA*), hMENA-, and hMENAΔv6- *in vitro* translated proteins, using an antibody, which recognizes all hMENA isoforms (pan-hMENA), showed that the proteins migrate with apparent molecular weights of 90kDa ($hMENA^{11a}$), 88kDa (hMENA) and 80kDa (hMENAΔv6) (Fig 6 C). The two *in vitro* translated hMENA and hMENAΔv6 isoforms appear to correspond to the bands revealed by WB analysis in MDA-MB-231 tumour cell protein extracts (Fig 6 C). RT-PCR experiments performed on SBT and MDA-MB-231, the breast cancer cell lines we used to clone *$hMENA^{11a}$* and *hMENAΔv6* respectively, revealed that, *hMENA* was expressed in both. Differently, *$hMENA^{11a}$* was expressed only in the luminal breast cancer cell line SBT, whereas *hMENAΔv6* only in

the basal MDA-MB-231 (Fig 6 D).

We previously showed that hMENA is absent in normal breast tissue, but becomes expressed in high-risk benign lesions and invasive tumors, suggesting that hMENA expression could be an early marker of breast tumorigenesis (Di Modugno, 2006). We therefore turned to the HMT-3522 isogenic cell strain series, a novel model of human breast cancer progression where no oncogenes were used for transformation (Rizki, 2008), to ask whether hMENA alternative splicing is linked to malignant progression. In three-dimensional laminin-rich gel assays (3D lrECM) (Petersen, 1992), we found that non-malignant HMT-3522-S1 mammary epithelial cells do not express hMENA^{11a} unless treated with EGF, whereas the premalignant HMT-3522-S2 cells, which have autocrine EGFR signalling (Madsen, 1992), express hMENA^{11a} (Fig 7 A). The tumorigenic and invasive, but not metastatic, HMT-3522-T4-2 cells express both the hMENA^{11a} and hMENAΔv6 isoforms. Targeting the EGFR, MAPK, or PI3K signalling in HMT-3522-T4-2 cells using small molecule inhibitors, (AG1478, PD98059, or LY294002, respectively) or using an anti-EGFR function blocking antibody, mAb-225 'reverts' the phenotype of these cells from malignant to non-malignant by restoring apicobasal polarity and inducing growth arrest in 3D lrECM. We found that reversion leads to concurrent down-regulation of two hMENA isoforms (Fig 7 A). These data suggest that polarized and growth-arrested cellular architecture correlates inversely with hMENA expression.

We asked whether expression of hMENA^{11a} and hMENAΔv6 isoforms correlates with either epithelial or mesenchymal markers in a panel of breast cancer cell lines. With the exception of DAL cells, we found that all E-CADHERIN positive cell lines express

hMENA^{11a} (Fig. 7 B). However, hMENA^{11a} expression was independent of functional E-CADHERIN since SKBr3 cells, which have a mutation in the *E-CADHERIN* gene, retained hMENA^{11a} expression. On the other hand, absence of hMENA^{11a} expression correlated with the expression of hMENAΔv6, absence of E-CADHERIN and the presence of N-CADHERIN. This pattern of hMENA isoform expression is found also in cervix cancer cell lines (C33A and SiHa) (Fig. 7B)

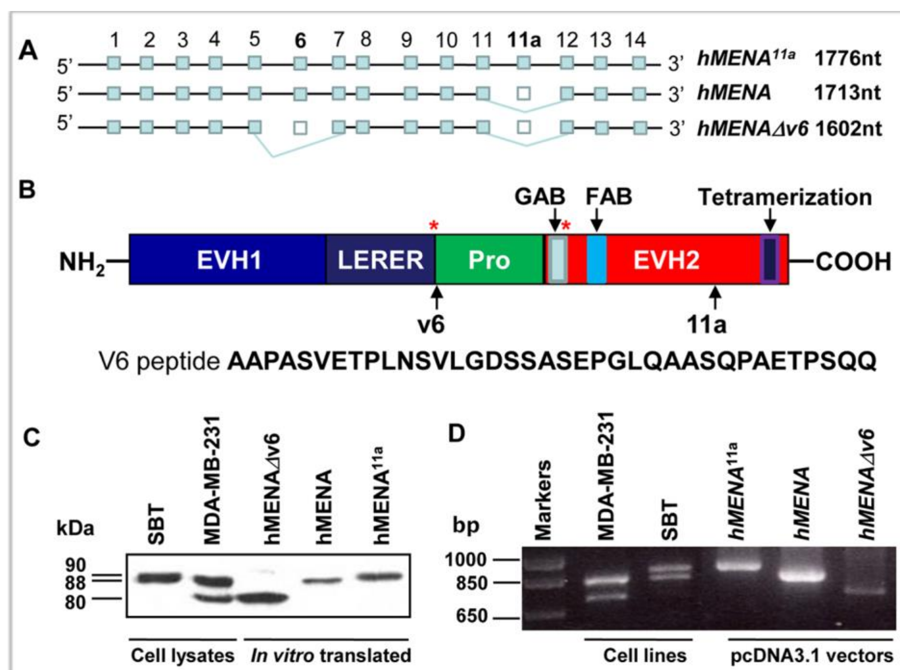

Figure 6. (A) Diagrammatic representation of hMENA splice variants. (B) hMENA protein domains. Asterisks indicate Ser phosphorylation sites. GAB and FAB correspond to the G actin and F-actin binding sites, respectively. Sites of V6 and 11a peptides are indicated by arrows. Sequence of peptide V6, absent in hMENAΔv6 isoform, is reported. (C) In vitro translated hMENA^{11a}, hMENA and hMENAΔv6 analyzed by WB analysis using the pan-hMENA Ab (10μg/ml). Cell lysates (30μg) of SBT and MDA-MB-231 breast cancer cell lines (used to obtain hMENA^{11a} and hMENAΔv6 cDNAs respectively) were also tested by WB to identify the corresponding in vitro translated protein bands. (D) RT-PCR performed with primers (MTC1f and MTC4r) flanking the region of alternative splicing on MDA-MB-231 and SBT RNA. PCR experiment was also done on the pcDNA3.1 vectors containing the three hMENA variants. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

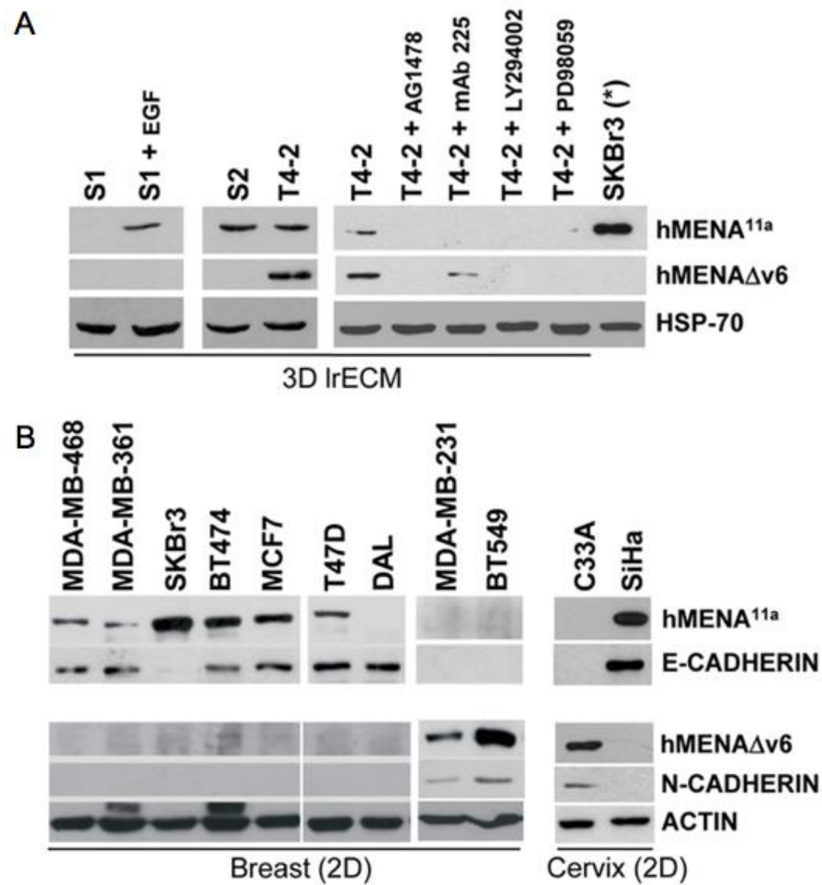

Figure 7. (A) hMENA isoform expression in a 3D model of breast cancer progression. The WB analysis of lysates of 3D grown HMT-3522 S1, S2 and T4-2 progression series cells with hMENA isoform specific antibodies is shown. S1 cells grown for 24h in growth medium depleted of EGF (S1) do not express hMENA^{11a} isoform, which is expressed in the presence of EGF. WB analysis of T4-2 cells and T4-2 cells reverted with AG1478, mAb225, LY294002 and PD98059 treatments, showing a strong reduction of hMENA isoform expression following the reversion of the transformed phenotype. 2D grown SKBr3 (*) cell lysate is reported as a control of hMENA^{11a} expression. (B) WB analysis of lysates of breast and cervix tumor cell lines with hMENA isoform specific antibodies, anti E-CADHERIN and N-CADHERIN antibodies, indicating a strong correlation between hMENA^{11a} and E-CADHERIN as well as between hMENAΔv6 and N-CADHERIN expression.

hMENA^{11a} and hMENAΔv6 spliced isoforms correlates with an epithelial and a mesenchymal phenotype in normal and pancreatic cancer cells

We previously found that hMENA^{11a} is associated with pancreatic cancer cell lines with an epithelial phenotype and identifies EGFR-dependent cell lines that are sensitive to the EGFR inhibitor erlotinib (Pino, 2008). To investigate whether hMENA^{11a} and hMENAΔv6 expression correlates with epithelial and mesenchymal markers, we analysed by WB five pancreatic tumor cell lines and one normal cell line. hMENAΔv6 is expressed together with hMENA in PANC1 cells that lack hMENA^{11a} and express VIMENTIN (Fig 8). hMENA^{11a}, together with E-CADHERIN, is expressed in epithelial pancreatic cell lines (CFPAC, T3M4, and PACA44) and in the normal human pancreatic ductal epithelial cell line (HPDE), whereas it was undetectable in mesenchymal PT45 and PANC1 pancreatic cancer cell lines, as previously reported (Pino, 2008).

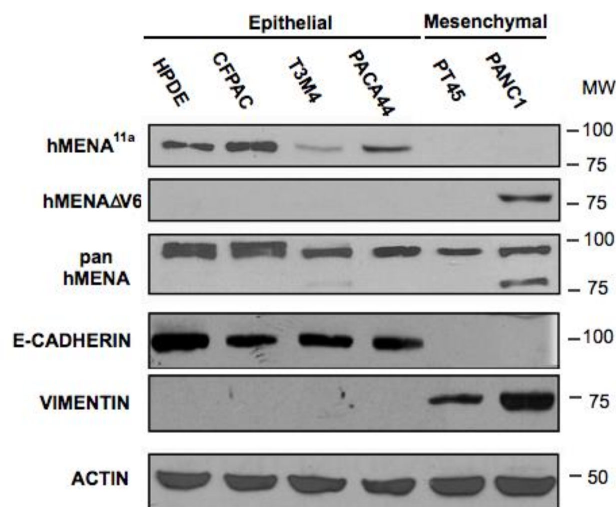

Figure 8. WB analysis of lysates of normal and tumor pancreatic cell lines. Cells were cultured at sub-confluence for 48h before scraped and lysed. Lysates were analyzed with antibodies against hMENA^{11a}, hMENAΔv6, pan-hMENA, E-CADHERIN, VIMENTIN and ACTIN.

hMENA^{11a} correlates with an epithelial phenotype and VASP phosphorylation at Ser157 in normal and pancreatic cancer cells

hMENA belongs to Ena/VASP protein family, which members are able to tetramerize and act sinergistically (Gertler, 1996; Krause, 2003). VASP, the other member of the family, undergoes phosphorylation in different serine/threonine residues, which are critical for the role exerted by this protein in the architecture of the actin cytoskeleton during cell-cell adhesion in epithelial cells (Vasioukhin, 2000; Quinlan, 2004; Scott, 2006). Thus we investigated whether VASP phosphorylation status may be related to the *hMENA* splicing program. The results of WB analysis evidence that the Ser157 is more phosphorylated in normal and pancreatic cancer cells with an epithelial phenotype, hMENA^{11a} positive and hMENAΔv6 negative with respect to the cells hMENAΔv6 positive and hMENA^{11a} negative (Fig 9). The phosphorylation of VASP at Ser157 induces a shift in the electrophoretic mobility of VASP protein (Howe, 2002). In line with the data obtained with the phospho-VASP Ab, the analysis with VASP Ab reveals that phosphorylated VASP (upper band) is more expressed in hMENA^{11a} positive cells, whereas the not-phosphorylated VASP (lower band) shows a comparable expression among the cell lines (Fig 9, lower panel). The phosphorylation status of Ser239 is unchanged among the cell lines evaluated.

Since after the formation of cell–cell contacts, VASP is strongly de-phosphorylated (Howe, 2002; Benz, 2008), the biochemical experiments have been perfomed in cells grown at sub-confluence and scraped after 48h of reattachment before analysis.

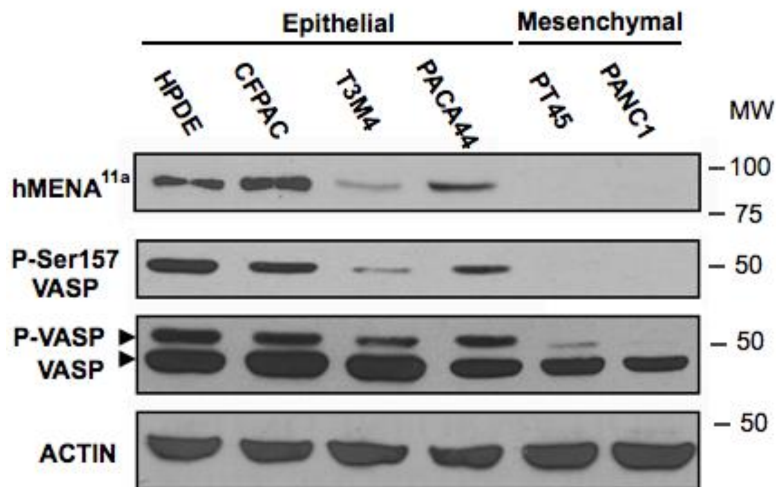

Figure 9. WB analysis of lysates of normal and tumor pancreatic cell lines. Cells were cultured at sub-confluence for 48h before scraped and lysed. Lysates were analyzed with antibodies against hMENA^{11a}, VASP or the phosphorylated form of ser157 VASP and ACTIN.

hMENA^{11a} enhances VASP phosphorylation at Ser 157

Reasoning that the invasive PANC1 cancer cell line does express neither hMENA^{11a} nor P-Ser157 VASP, whereas the normal HPDE express both the proteins, we performed experiments of gain and loss of function to identify the influence of hMENA^{11a} isoform on VASP phosphorylation. A specific knock-down of *hMENA^{11a}* isoform by si-RNA evidences that the down-regulation of *hMENA^{11a}* resulted in a reduction of phosphorylation of VASP at Ser157 in HPDE cells (Fig. 10 A), thus suggesting a link between hMENA^{11a} expression and P-Ser157 VASP.

Then, we studied the influence of hMENA^{11a} on VASP phosphorylation status in invasive PANC1 hMENA^{11a} negative cells. We stably transduced *hMENA^{11a}* isoform in PANC1 cells and By sub-cloning we produced and characterize PANC1- hMENA^{11a} clones. Three representative clones with different levels of hMENA^{11a} and hMENAΔv6

are shown in Figure 10 panel B. Clone 13 shows the lowest level of hMENAΔv6 and the highest level of $hMENA^{11a}$ and P-Ser157 VASP. A strong correlation between $hMENA^{11a}$ and P-Ser157 VASP is evident suggesting that the presence of $hMENA^{11a}$ enhances VASP phosphorylation at Ser157. This is confirmed by experiments of silencing of $\textit{hMENA}^{11a}$ in PANC1-$hMENA^{11a}$ (clone 13) where a strong reduction of P-Ser157 VASP is observed in $\textit{hMENA}^{11a}$ silenced cells (Fig 10 C).

To confirm the data obtained by WB analysis, we studied the pattern of VASP post-translational modifications in pancreatic cells by 2D-electrophoresis analysis. We found that VASP signal in PANC1-$hMENA^{11a}$ protein lysates, consists of four spots, two of them also revelead by the P-Ser157 VASP Ab (Fig 10 D). In HPDE and PANC1-$hMENA^{11a}$ protein lysates the spots revealed by VASP Ab show a more pronounced shift toward lower pH with respect to control PANC1 cells, suggesting a higher level of phosphorylated VASP (Fig 10E).

Previous data from our group demonstrated that $hMENA^{11a}$ undergoes phosphorylation following growth factors cell stimulation, and the 21 aa of the 11a peptide contains two putative serine sites of phosphorylation (Fig 11 A; Di Modugno, 2007). To explore the relevance of $hMENA^{11a}$ phosphorylation in modulating the phosphorylation of VASP, we have mutated the two serine, present in the 11a peptide, to alanine. Mutant $\textit{hMENA}^{11a}$ transduction in PANC1 cells does not induce Ser157 VASP phosphorylation differently from wild type $\textit{hMENA}^{11a}$ transduction (Fig 11 B), suggesting that $hMENA^{11a}$ should be phosphorylated to exert its effect on VASP phosphorylation.

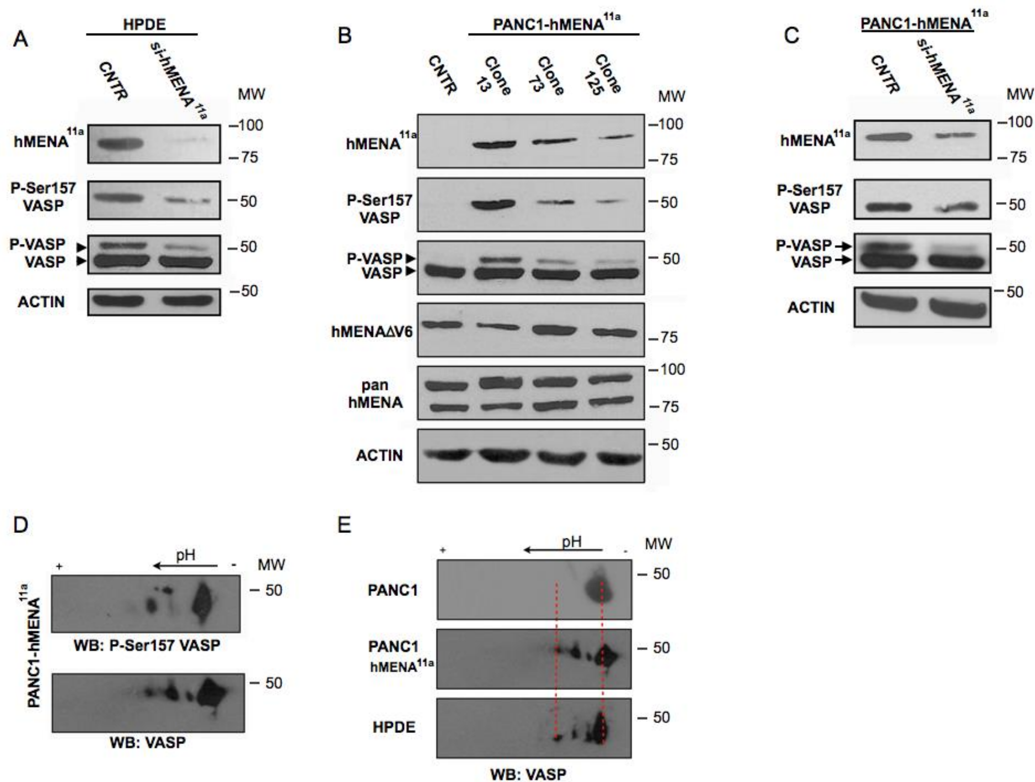

Figure 10. (A) WB analysis of HPDE cells transduced with the empty vector (CNTR) or with specific siRNA targeting hMENA^{11a}, using antibodies against VASP, the phosphorylated form of ser157 VASP, hMENA^{11a} or ACTIN.

(B) WB analysis of lysates of PANC1 cells transduced with empty vector (CNTR) or with hMENA^{11a}. After transduction and selection with antibiotic geneticin, stably transduced cells are sub-cloned and three reprensentative clones are analysed. Lysates were analyzed with antibodies against hMENA^{11a}, hMENAΔv6, pan-hMENA,VASP or the phosphorylated form of ser157 VASP or ACTIN.

(C) WB analysis of PANC1- hMENA^{11a} cells transduced with the empty vector (CNTR) or with specific siRNA targeting hMENA^{11a}, using antibodies against VASP, the phosphorylated form of ser157 VASP, hMENA^{11a} or ACTIN.

(D) 2D-page analysis of PANC1- hMENA^{11a} cells using antibodies against VASP or the phosphorylated form of ser157 VASP.

(E) 2D-page analysis of PANC1, PANC1- hMENA^{11a} or HPDE cells using antibody against VASP.

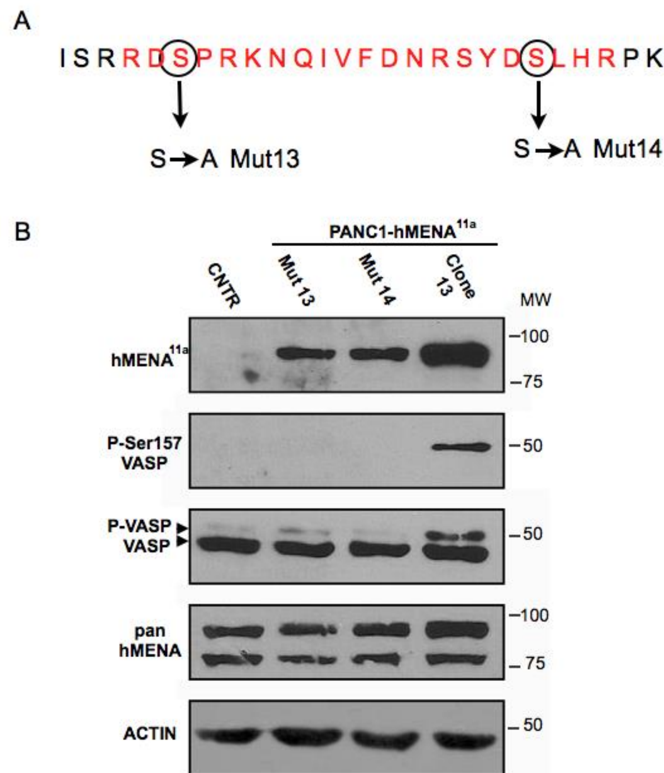

Figure 11. (A) Representative scheme of 21 aminoacids of 11a exon (in red) pinpointing the two putative serines susceptible to phosphorylation. Two different hMENA^{11a} constructs were generated switching the two serines to alanine in order to impede phosphorylation.
(B) WB analysis of PANC1 cells transduced with the empty vector (CNTR) or with specific mutant hMENA^{11a} cDNAs (named mut13 and mut14) using antibodies against VASP, the phosphorylated form of ser157 VASP or hMENA isoforms.

hMENA^{11a} interacts with VASP when Ser 157 is phosphorylated by PKA in normal pancreatic cells

In order to analyze whether hMENA^{11a} directly interact with VASP, we have immunoprecipitated protein extracts from HPDE cells with hMENA^{11a} and pan-hMENA Abs. WB analysis of hMENA^{11a} immunoprecipitates with VASP and P-Ser157 VASP Abs demontrates that hMENA^{11a} co-immunoprecipitates either with phosphorylated VASP or with unphosphorylated VASP (lower band stained by VASP

Ab). Immunoprecipitates with Pan-hMENA Ab, which binds both hMENA^{11a} and hMENA isoforms in HPDE cells, also evidences a coimmunoprecipitation of VASP, but with a lesser amount of the phosphorylated form, indicating a preferential interaction between hMENA^{11a} and phosphorylated VASP (Fig. 12A).

Mena and VASP proteins play an important role in epithelial dynamics (Vasioukhin, 2000) and VASP Ser157 phosphorylation level is dinamically regulated by PKA during cell adhesion (Howe, 2002; Quinlan, 2004; Scott, 2006). VASP phosphorylation patterns impact on subcellular protein localization (Benz, 2009), whereas MENA phophorylation by PKA in the Ser236 (corresponding to Ser157 in VASP), does not affect the protein localization (Loureiro, 2002). The impact of Ser157 phosphorylation on the localization of endogenous VASP is investigated in HPDE cells treated with the PKA activator forskolin (Forskolin, 25 μM, 20’), buffer solution (DMSO) or PKA inhibitor H89 (H89, 50 μM, 60’). WB analysis confirms that HPDE cells express P-Ser157 VASP (DMSO), whereas Forskolin increased and H89 inhibited phosphorylation of Ser157 and Ser239 (data not shown). VASP forms homo- and heterotetramers with MENA and EVL (Bachmann, 1999). To understand whether hMENA^{11a} and P-Ser157 VASP co-localize, we analyze by immunofluorescence and confocal analysis. In untreated HPDE cells, P-Ser157 VASP localizes at focal adhesions, while hMENA^{11a} is distributed in cytoplasm and focal adhesion with few area of co-localization with P-Ser157 VASP (Fig 12 B). PKA-stimulation strongly increases P-Ser157 signal and consistently modifies the subcellular VASP and hMENA^{11a} distribution. In fact, hMENA^{11a} disappears from cytoplasmatic sites and localizes to focal adhesions and the plasma membrane, together with P-Ser157 VASP

(Fig 12 C). On the contrary, the treatment with the PKA inhibitor H89 abrogates P-Ser157 VASP signal, as expected, along with the traslocation of hMENA^{11a} from focal adhesion to cytoplasm (Fig. 12 D). Similar results are obtained by staining the cells with a pan-hMENA Ab (data not shown).

These results confirm an interaction between P-Ser157 VASP and hMENA^{11a} in HPDE cells, and suggest that the binding of hMENA^{11a} with VASP favour its phosphorylation of Ser157 and their localization at focal adhesion is regulated by PKA.

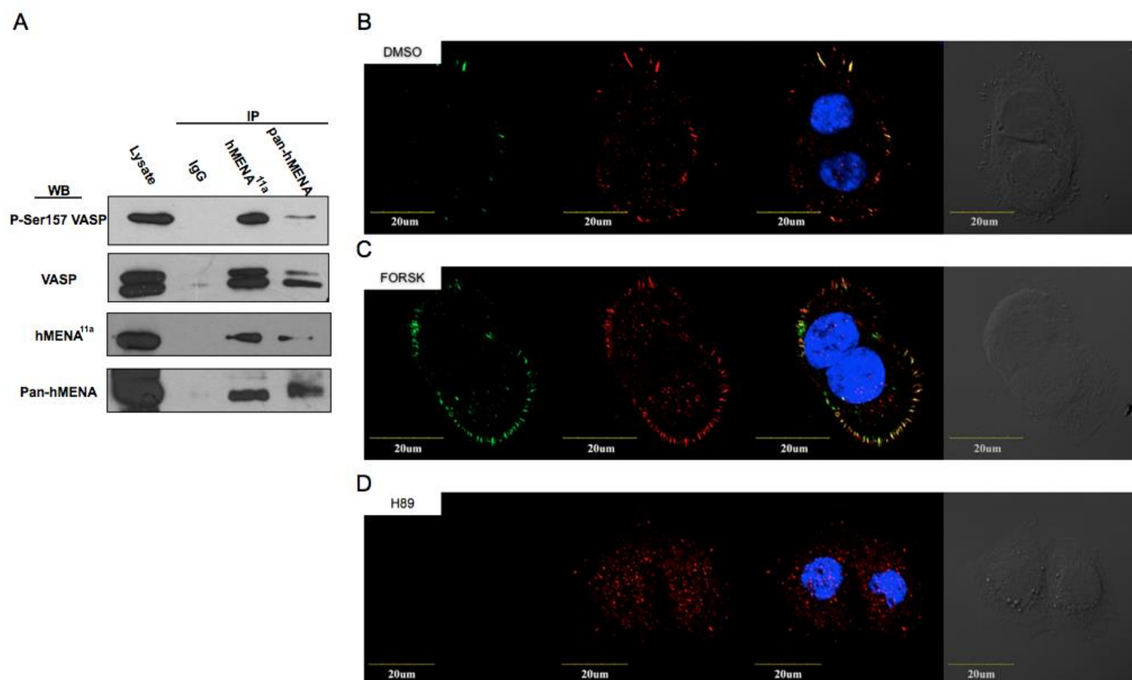

Figure 12. (A) WB analysis of HPDE cell lysate (lane 1) or proteins immunoprecipitated by pre-immune rabbit IgG (lane 2), hMENA^{11a} Ab (lane 3) and pan-hMENA Ab (lane 4) with the antibodies reported on the left.
Confocal and phase-contrast analysis of HPDE cells treated with DMSO (B), Forskolin (20’, 25 μM) (C) or H89 (60’, 50 μM) (D), using hMENA^{11a} mAb, (red), P-Ser157 VASP (green) and DAPI (blue). Cells were imaged using a Zeiss 710 laser scanning microscope. Magnification 63x.

hMENA^{11a} transduction increases Ser157 VASP localization at focal adhesions in pancreatic tumor cells

We hypothesized that hMENA^{11a} presence is necessary for a correct VASP localization at focal adhesion. Thus, we compared the P-Ser157 VASP localization in PANC1 and PANC1-hMENA^{11a} cells. Confocal analysis shows that hMENA^{11a} ectopic expression in PANC1 cells increases P-Ser157 VASP localization at focal adhesion (Fig 13 A), although the co-localization of P-Ser157 VASP and hMENA^{11a} is less appreciated respect to that observed in HPDE cells. Forskolin treatment increases the co-localization between hMENA^{11a} and P-Ser157 VASP at focal adhesion (Fig 13 B), but the treatment with the PKA inhibitor does not induce hMENA^{11a} translocation from focal adhesions to the cytoplasm (Fig 13 C), differently from HPDE cells. These results suggest that hMENA^{11a} localization at focal adhesion may be indipendent by PKA in PANC1 cells. WB analysis of PANC1-hMENA^{11a} cells treated with PKA inhibitor shows a reduction of P-Ser157 VASP level, confirming that VASP phosphorylation is mediated by PKA in these cells (data not shown).

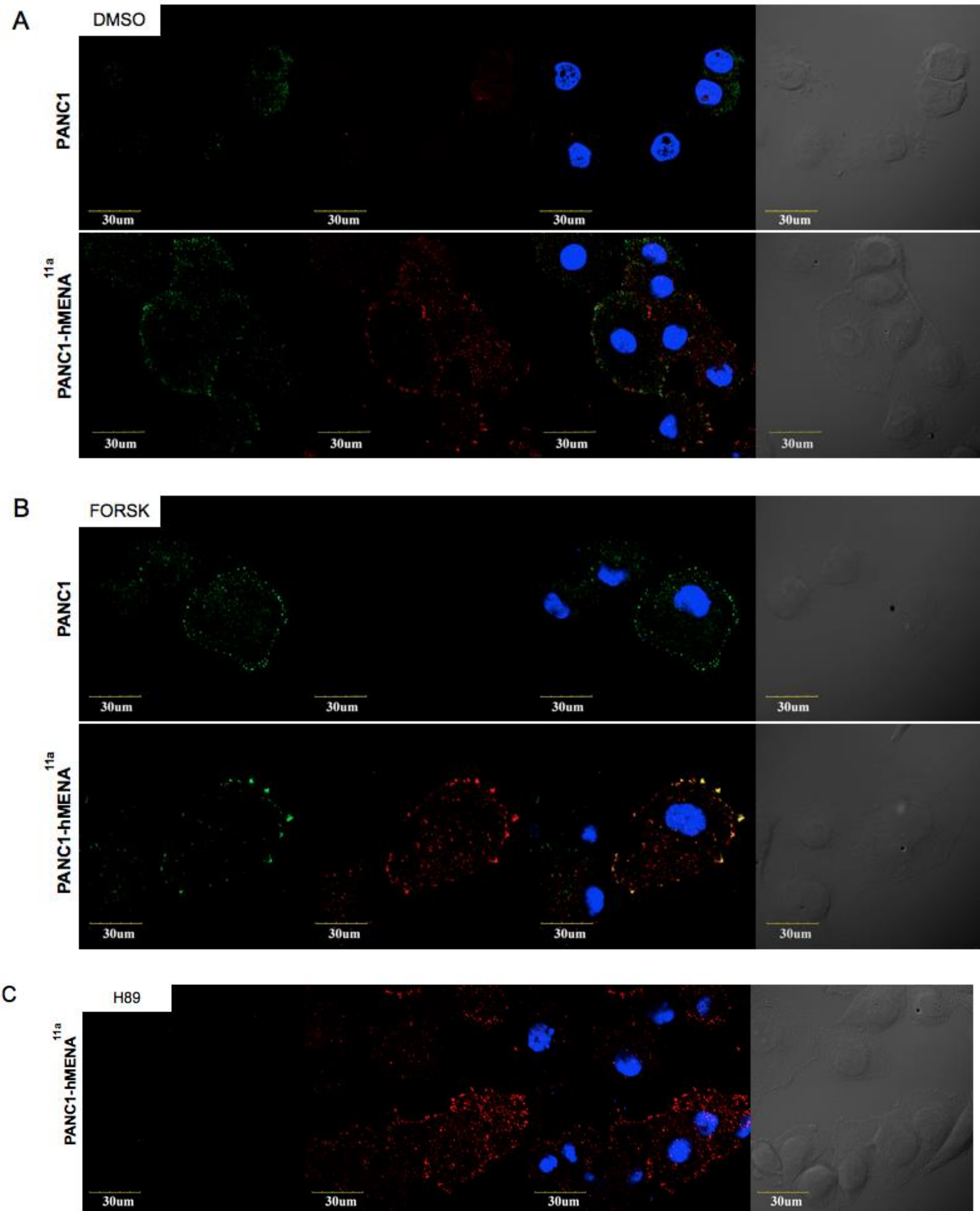

Figure 13. Confocal and phase-contrast analysis of PANC1 and PANC1-hMENA^{11a} cells treated with DMSO (A) or Forskolin (B) or H89 (C), using hMENA^{11a} mAb, (red), ser157 VASP (green) and DAPI (blue). Cells were imaged using a Zeiss 710 laser scanning microscope. Magnification 63x.

hMENA^{11a} transduction increases cell-matrix adhesion and inhibits cell invasion

P-Ser157 VASP levels are dynamically regulated during cell-matrix adhesion (Pula, 2006; Lee and Chung, 2009; Worth, 2010). Thus, we tested the role of *hMENA^{11a}* may have in cell-matrix adhesion.

After 2h of adhesion on collagen I, PANC1 cells exhibit a low signal of P-Ser157 VASP, whereas the transduction of hMENA^{11a} induces a higher level of P-Ser157 VASP (Fig 14 A). Phase-contrast microscopy analysis shows that, after 2h of adhesion on collagen I, either PANC1 or PANC1-hMENA^{11a} cells exhibit multiple lamellipodia and extensive membrane ruffling, but PANC1-hMENA^{11a} cells extend large lamellae with ruffles 30% more than control cells (Fig 14 B). Therefore, we extended analysis of cell adhesion at different substrates (collagen type I, collagen type IV, fibronectin) and we observed that hMENA^{11a} transduced cells adhere to all the substrates evaluated more rapidly than control cells (Fig 14 C); the number of hMENA^{11a} transduced cells double than control after 1,5 h of adhesion. These data evidence that hMENA^{11a} expression induces the phosphorylation of VASP at Ser157 with an increased lamellipodial extensions and further increased cell-matrix adhesion.

MENA and VASP proteins are known to play a critical role on cell migration in mouse and human (Loureiro, 2002; Wang, 2004) and in mouse model MENA^{11a} mRNA dissapeared in disseminated cancer cells (Goswami, 2008). To more directly assess the role of hMENA^{11a} in the regulation of tumorigenic activity, we examined the effects of hMENA^{11a} expression on pancreatic cancer cell migration and invasion. Our results demonstrate a dramatic reduction of migratory (data not shown) invasive capability in hMENA^{11a} clones respect than control cells (Fig 14 D). Interestingly, the clone which

express the highest level of hMENA^{11a} is the less invasive.

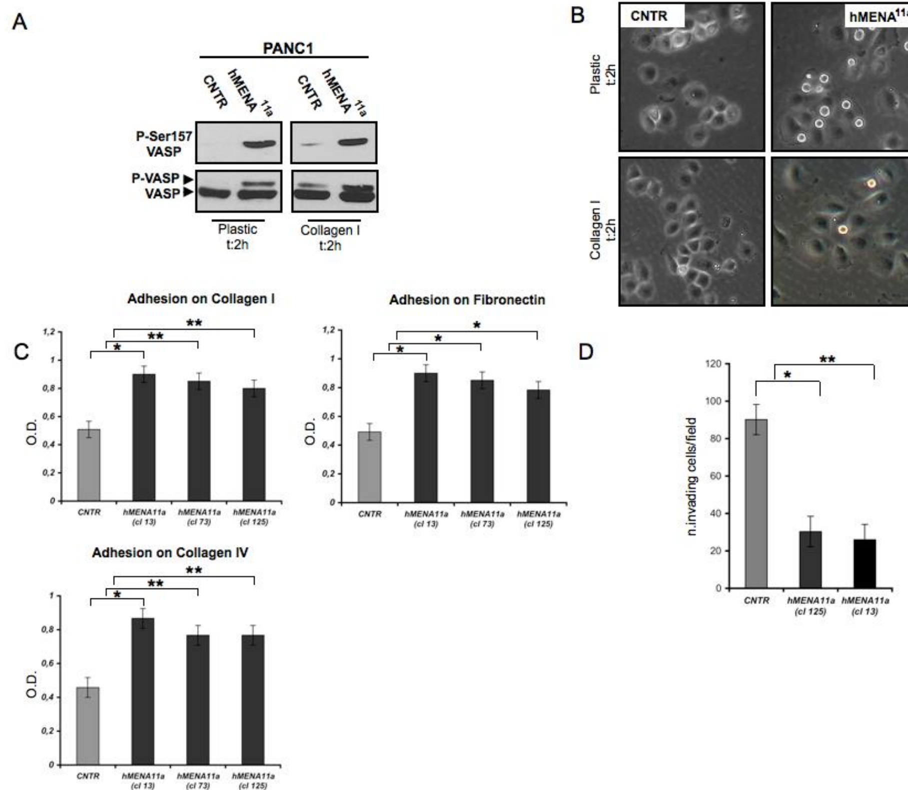

Figure 14. (A) PANC1 and PANC1- hMENA^{11a} cells were plated for 2h on tissue culture plates, collagen I or Fibronectin prior to lysis and WB analysis with VASP and the phosphorylated form of ser157 Abs.
(B) Phase-contrast images of PANC1 and PANC1- hMENA^{11a} (clone 13) cells plated for 2h on tissue culture plates or collagen I. PANC1- hMENA^{11a} (clone 13) cells exhibit large lamellae with ruffles 30% more than control cells (CNTR).
(C) Graphs depict cell capabilities to adhere on different substrates (Collagen I, IV and Fibronectin).
** P<0,001; **P=0,001*
*(D) Matrigel invasion assays of PANC1 cells stably transduced with the empty vector (CNTR) or hMENA^{11a} (50.000 cells; 24h of invasion). * P=0,002; ** P=0,001*

ESRP1 transduction resembles hMENA^{11a} effects on VASP phosphorylation, cell-matrix adhesion and cell invasion

ESRP1 and ESRP2, the recently discovered splicing regulatory proteins, abrogate EMT

program and regulate *hMENA* splicing in cancer cell lines (Warzecha 2009, 2010; Di Modugno, paper submitted). Furthermore, Horiguchi et al demonstrate that TGFβ1 mediated EMT induces down-regulation of ESRPs splicing program in PANC1 cells (Horiguchi, 2011). Herein, we tested whether *ESRP1* transduction into PANC1 cells could shift the *hMENA* splicing program from hMENAΔv6 to $hMENA^{11a}$ isoform. We found that *ESRP1* transduction increased $hMENA^{11a}$ and decreased the hMENAΔv6 protein levels (Fig 15 A). The additional band recognized either by the anti-$hMENA^{11a}$ mAb or the anti-hMENAΔv6 Ab in PANC1 ESRP1 cells, corresponds to the 11a inclusion in hMENAΔv6 sequence (Fig 15 A).

WB analysis of PANC1-ESRP1 evidence that the Ser157 VASP is more phosphorylated than control cells, as we observed in *$hMENA^{11a}$* transduced cells (Fig 15 A). In parallel to an increase of P-Ser157 VASP, *ESRP1* transduction induces an increase of adhesive properties to different matrix-derived components (Fig 15 B) and a reduction of cell invasive capabilities (Fig 15 C), comparable to that observed in PANC1-$hMENA^{11a}$ cells.

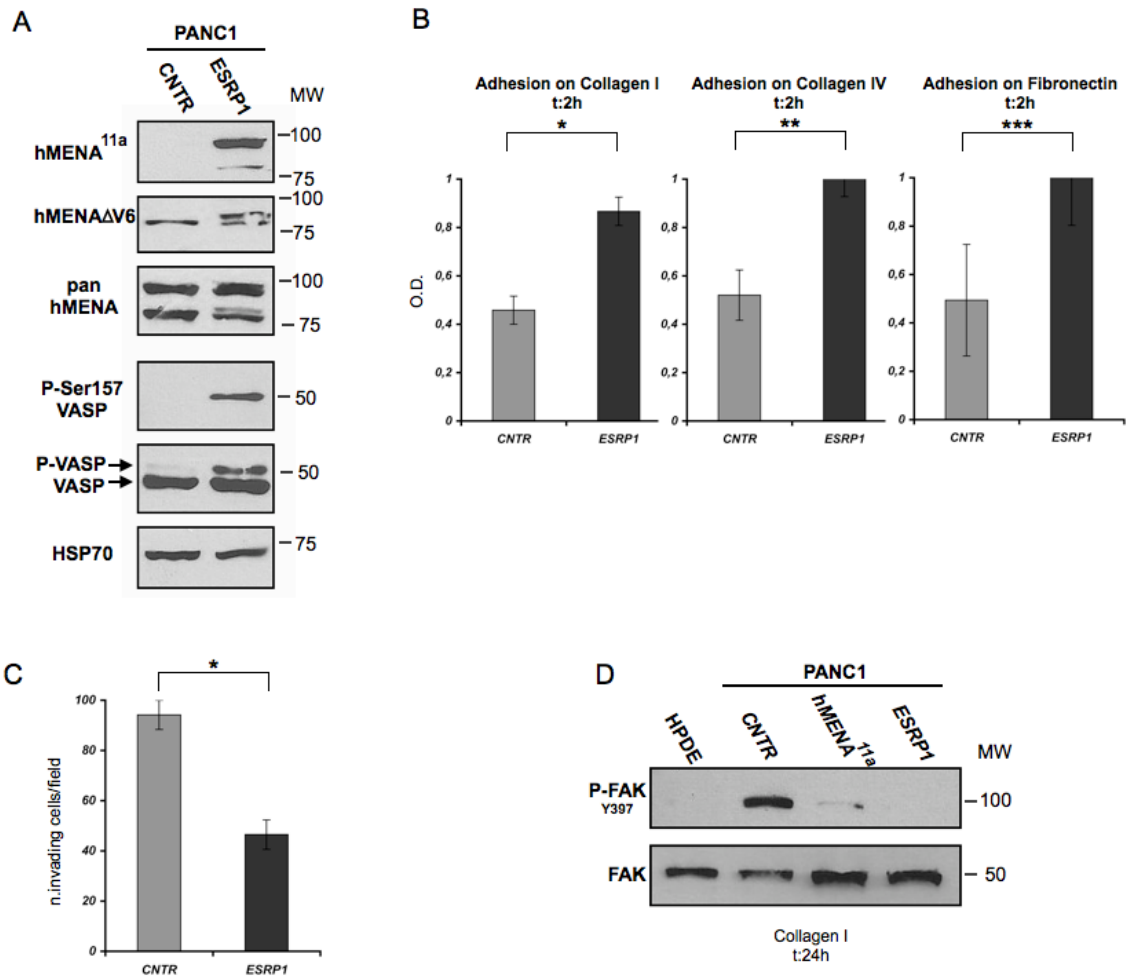

Figure 15. (A) WB analysis of lysates of PANC1 cells transfected with empty vector (CNTR) or with ESRP1. After transduction and selection with antibiotic geneticin, stably transduced cells are sub-cloned and one reprensentative clone is analysed. Lysates were analyzed with antibodies against hMENA^{11a}, hMENAΔv6, pan-hMENA, VASP or the phosphorylated form of ser157.
*(B) Adhesion assay performed on PANC1 and PANC1-ESRP1 cells plated for 2h on different matrix-enriched plates. Graphs depict cell capabilities to adhere on different substrates (Collagen I, IV and Fibronectin). * P=0,0015; **P=0,0015; *** P=0,0016*
*(C) Matrigel invasion assays of PANC1 cells stably transduced with the empty vector (CNTR) or ESRP1 (50.000 cells; 24h of invasion). * P=0,002.*
(D) WB analysis of HPDE, PANC1 cells stably transduced with the empty vector (CNTR), hMENA^{11a} or ESRP1. Cells were grown on collagen for 24 hours before lysis. Lysates were analyzed with antibodies against Tyr-396 FAK and total FAK.

ESRP1/hMENA^{11a} transduction induces FAK tyr397 dephosphorylation

There is accumulating evidence that supports an important role of FAK pathway in sustaining cell adhesion and invasion of cancer cell (Schaller, 2010). The overexpression and phosphorylation of FAK correlates with the increase of cell motility and invasion (Crowe, 2004). Based on these results, considerable evidence points to a critical role of FAK in cancer. Thus, we compared phosphorylation of Tyr397 FAK in parental PANC1 cells plated on collagen I to that of PANC1 cells transduced with *ESRP1* or *hMENA^{11a}*. Tyr397 FAK phosphorylation is evident in parental cells and it is significant down-regulated *ESRP1* or *hMENA^{11a}* cells, comparable to the "normal" HPDE cells (Fig 15 D).

hMENA^{11a} is down-regulated in pancreatic primary tumor

hMENA isoform expression was evaluated by immunohistochemistry using both a pan-hMENA antibody that recognizes all the hMENA isoforms, and an antibody specific for hMENA^{11a}. The anti-hMENAΔv6 antibody was unsuitable for immunohistochemical evaluation in paraffin-embedded tissues, and we did not succeed to either raise suitable antibodies or find other sources. We tested 26 human pancreatic tumor tissues demonstrating that hMENA^{11a} expression is expressed only in 4 cases, whereas pan-hMENA Ab stain the majority of the cases (table 1). One section from the same primitive pancreatic tumor are illustrated in Figure 16, where is evident that hMENA^{11a} Ab stains normal ducts (Fig 16 A) whereas cancer cells are negative for hMENA^{11a} (Fig 16 B).

Considering the pattern of expression identified in cancer cell lines (Fig 7-8), and the reactivity of antibodies employed, the group of tumors that are pan-hMENA positive/hMENA^{11a} negative would include the tumors that express the hMENA and hMENAΔv6 isoforms, with a mesenchymal-like phenotype. In support for this hypothesis, immunohistochemical analysis of two cases reported in Figure 17, evidences a concomitant expression of hMENA^{11a} and E-CADHERIN in tumor negative for VIMENTIN, whereas the hMENA^{11a} and E-CADHERIN negative tumor expresses VIMENTIN.

Human Pancreatic tumor lesion

	Positive	Negative
hMENA	23	3
hMENA^{11a}	4	22

Table 1. hMENA^{11a} and pan-hMENA protein expression in human pancreatic carcinoma by immunohistochemistry.

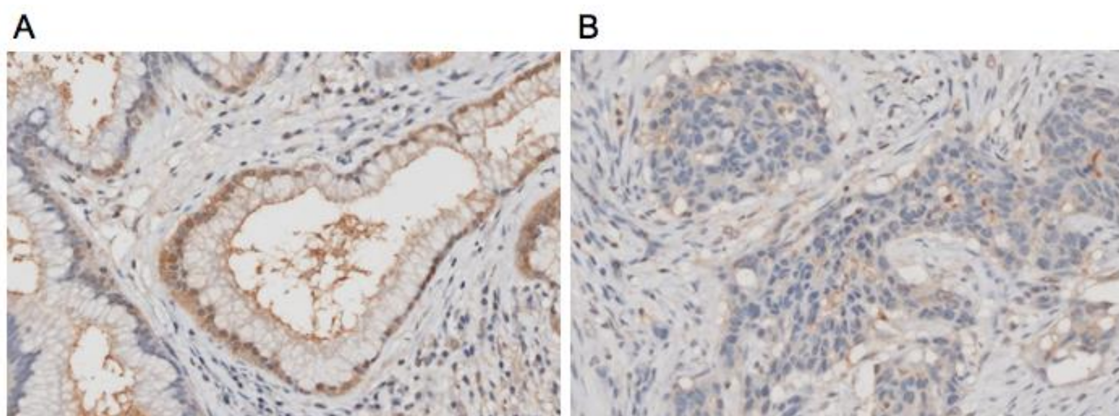

Figure 16. One section of a primary pancreatic carcinoma, stained with hMENA^{11a}.(A) normal ducts; (B) tumor cells. Magnifications,40x

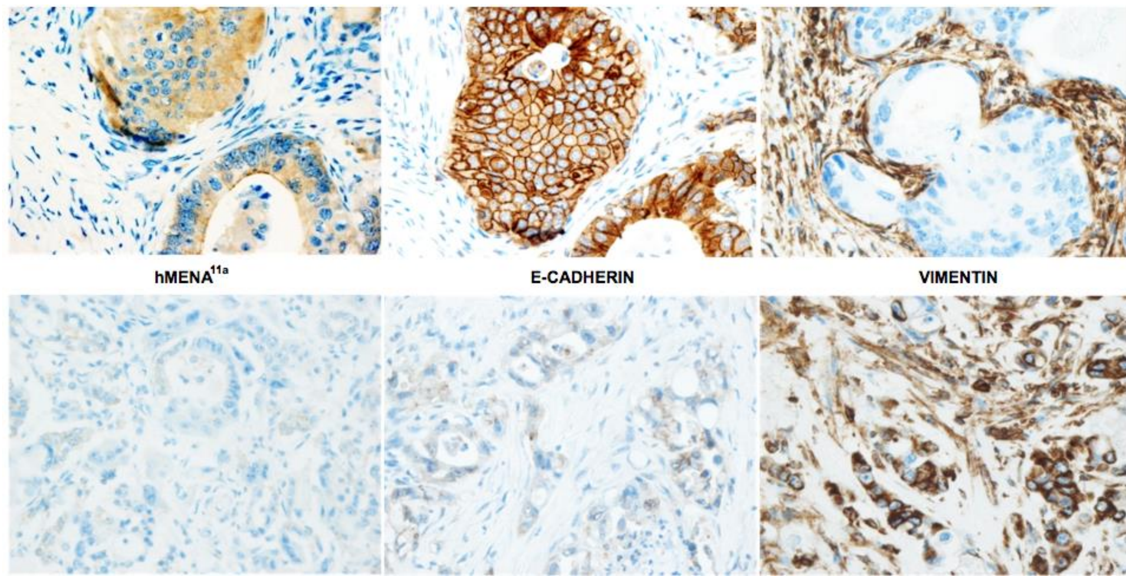

Figure 17. Two representative cases of primary pancreatic carcinoma, stained with hMENA^{11a}, E-CADHERIN or VIMENTIN. Magnifications,40x

Chapter 5 - Discussion

This PhD work is focused on the study of hMENA splicing program in cancer progression focusing on pancreatic cancer as a tumor model in which the disruption of tissue homeostasis is an early event along with an early metastatic propensity.

hMENA along with VASP and EVL comprise the Ena/VASP family of actin regulatory proteins, which, acting as tetramers, modulate cell adhesion and migration by antagonizing actin capping proteins, bundling actin filaments, and nucleating and extending filopodia, underlying important physio-pathological processes, from neural development to inflammation, endothelial barrier formation, cancer cell spreading and dissemination (Gertler, 1996; Krause, 2003; Applewhite, 2007). Several isoforms have been identified for *MENA* message generating multiple protein isoforms that are expressed in specific tissues and cell-types (Gertler, 1996; Tani, 2003; Urbanelli 2006; Di Modugno, 2007). By contrast, the *EVL* gene contains one alternatively spliced exon (Lambrechts, 2000) and *VASP* has none.

We show here that hMENA alternative splicing is related to EMT process and this is in line with recent data demonstrating that *hMENA* belongs to a cluster of genes whose alternative splicing is related to EMT (Warzecha, 2009). Nisticò's group has previously characterized two *hMENA* isoforms, one 88-kDa isoform defined as "classic" and one splice variants, named $hMENA^{11a}$ (90 kDa). Here we report (Di Modugno, paper submitted) a novel splice variant of human *MENA*, named *hMENAΔv6* (80 kDa) and we suggest that the exon 6 deletion influences protein function and regulation by bringing two functional regions closer together, with one site containing a PKA Ser phosphorylation residue, and the other site containing the regulatory proline-rich region.

Firstly, we focused our study on an isogenic model of human breast cancer progression (Petersen, 1992), and we show that hMENAΔv6 is expressed only in the HMT3522-T4-2 invasive, but not metastatic tumor cells. Conversely, the premalignant HMT3522-S2 cells, which are self-sufficient for EGFR signalling, express the epithelial-associated hMENA^{11a} isoform. We find that 'phenotypic reversion' of malignant HMT3522-T4-2 cells using different pharmacological inhibitors of the EGFR, which restores apicobasal polarity and induces growth arrest, leads to concurrent down-regulation of all the hMENA isoforms. On the other hand, hMENA isoforms are not expressed by non-malignant HMT3522-S1 cells, in agreement with previous results that hMENA is not expressed in normal breast tissues or in low risk benign breast lesions as detected by a pan-hMENA antibody, recognizing all the hMENA isoforms (Di Modugno 2004).

Furthermore, a wide analysis on 2D cancer cell lines shows that hMENA^{11a} expression is usually associated with breast and cervix cancer cell lines that express E-CADHERIN, whereas hMENAΔv6 is expressed in those cancer cells displaying EMT features and migratory behaviour. These data suggest that hMENA^{11a} and hMENAΔv6 can identify cancer cells with non-invasive and invasive phenotypes, respectively.

We then focused the study on pancreatic cancer, one of the most lethal cancer. Pancreatic ductal adenocarcinoma has dismal prognosis and is highly chemoresistant and only 5% of the patients can be surgically resected and long-term survival is directly correlated with tumor size. Early detection is the only means to substantially impact long-term survival. The human pancreatic malignancy is characterized by invasiveness and early metastasis (Hidalgo, 2010). A hallmark in pancreatic ductal adenocarcinoma is the development of desmoplasia, a three-dimensional structure supporting epithelial

carcinoma cells through an altered ECM, maintained by diffusible paracrine growth factors and cytokines, tumor-associated vasculature, inflammatory cells, and stromal fibroblasts. The interaction of tumor cells with their microenvironment has been suggested to play a pivotal role in tumor progression and acquisition of metastatic phenotype (Liotta, 2001). Autocrine and paracrine signals derived from tumor stroma may induce EMT (Shintani, 2006), an early event in pancreatic cancer cells (Rhim, 2012). The molecular mechanisms underlying EMT have been studied extensively in the last decade and many studies have been focused on the contribution of changes in gene expression to EMT, whereas much less is known regarding the role of alternative splicing of mRNA during EMT. Alternative splicing processes provide an additional layer of gene regulation that is critical in shaping the EMT process, particularly in cancer progression (Nisticò, 2012). A number of genes involved in functions, such as cell adhesion, cell motility, and cytoskeletal remodeling, are spliced and play a major role in EMT and tumor progression (Venables, 2009; Shapiro, 2011).

Our study in pancreatic cancer shows that, similar to the results obtained in breast cancer cell lines, hMENA "classic" is detected in all the pancreatic tumor cell lines tested and in a human pancreatic ductal epithelial cell line (HPDE). The expression of hMENA^{11a}, is evident in the 'normal' HPDE cell line and to the three cancer cell lines positive for expression of E-CADHERIN and negative for expression of VIMENTIN as previously reported by Pino, 2008. Herein we show that hMENAΔv6 is expressed in pancreatic cancer cell line negative for hMENA^{11a} and positive for VIMENTIN expression, thus providing evidence that also in pancreatic cancer, hMENA^{11a} and

hMENAΔv6 can identify cancer cells with non-invasive and invasive phenotypes, respectively.

MENA and VASP proteins localize to the leading edges of lamellipodia, the tips of filopodia, focal adhesions, cell–cell junctions. Localization of these proteins at actin-rich sites coupled with their ability to promote actin nucleation, suggests that MENA and VASP proteins are regulators of actin dynamics in epithelia (Vasioukhin, 2000; Linsday, 2007). On the basis of this knowledge, we hypothesized that MENA and or its splicing variants may influences the function of the other member of the family VASP in the actin-mediated processes such as cell-matrix adhesion and cell invasion. Many evidences demonstrate a key role for phospho-VASP mediated regulation of actin dynamics in endothelial cells (Benz, 2008), platelets (Pula, 2006), fibroblasts (Worth, 2010) and epithelial cells (Linsday, 2007) and Ena/VASP family members are substrates of nucleotide-dependent protein Kinases PKA, PKG, PKC and AMPK (Howe, 2002; Krause, 2003).

We observed that the Ser157 of VASP is more phosphorylated either in normal or pancreatic cancer cell lines with an epithelial phenotype and expressing hMENA^{11a}, whereas the hMENA^{11a} negative/hMENAΔv6 positive PANC1 cell line did not show appreciate level of P-Ser157 VASP. By a gain and loss of function, we demonstrate that hMENA^{11a} espression is able to induce VASP phosphorylation at Ser157 and that *hMENA*^{11a} transduction in PANC1 cells resembles the pattern of VASP phosphorylation observed in HPDE cells. Studies are in progress to elucidate the mechanism underlying the influence of hMENA^{11a} expression on VASP phosphorylation. However, results of co-IP experiments suggest that the presence of hMENA^{11a} along with VASP renders

this protein more susceptible to PKA mediated phosphorylation. One hypothesis may be attributable to conformational changes, induced by the presence of the additional 21aa peptide of the 11a exon in the tetramer complex. Furthermore, hMENA^{11a}, unlike hMENA ‘classic’, is phosphorylated following activation of the EGFR or its other family members, suggesting that exon 11a may represent a site for regulation of hMENA^{11a} (Di Modugno, 2007, 2010). Thus, we analyzed the effects of hMENA^{11a} mutant in PANC1 cells by the substitution of the two putative sites of phosphorylation present in 11a peptide from serine to alanine. Biochemical results evidenced that only wild type *hMENA*^{11a} transduction is able to induce VASP phosphorylation at Ser157, whereas the two mutants did not affect VASP phosphorylation, thus suggesting that the phosphorylation of hMENA^{11a} is relevant for the functional activity of VASP in epithelial cells.

Ser157 is responsible of subcellular VASP localization in spreading endothelial cells (Benz, 2009), where is enriched at lamellipodia and focal adhesions in spreading cells and the activation of PKA has been shown to increase Ser157 phosphorylation and to regulate VASP accumulation at the cell periphery in endothelial cells (Benz, 2008). In other cell types it has been observed that Ser157-phosphorylated protein is enriched at focal adhesions where it could interact with vinculin, zyxin, and migfilin at these sites (Drees, 2000; Hoffman, 2006). On the contrary, the localization of MENA appeared to be independent of their pseudophosphorylation status (Loureiro, 2002). The strictly connection between hMENA^{11a} and P-Ser157 VASP has been investigated in normal and pancreatic cancer cells. Our results demonstrate that P-Ser157 VASP interacts with hMENA^{11a} and both proteins localize within focal adhesions in HPDE cells and the

PKA activation increases the pool of P-Ser157 VASP and hMENA^{11a} at the cell periphery, whereas both proteins disappear from the cell periphery when PKA is inhibited. A different picture emerged from our study in PANC1 cancer cell line, where P-Ser157 VASP and hMENA^{11a} signals are almost undetectable in unstimulated cells. When treated with forskolin, PANC1 cells show an increase of VASP phosphorylation, whereas only few cells, respect to HPDE ‘normal’ cells show P-Ser157 VASP localized at the cell periphery. *hMENA*^{11a} transduction rescues the level of P-Ser 157, although only in PKA stimulated cells there is an appreciate co-localization between P-Ser157 VASP and hMENA^{11a}, whereas the treatment with the PKA inhibitor does not induce hMENA^{11a} translocation from focal adhesions to the cytoplasm, differently from HPDE cells, suggesting that hMENA^{11a} localization at focal adhesion may be indipendent by PKA in transformed cells.

hMENA message is spliced by ESRP1/2 that regulate splicing of a subset of genes involved in actin cytoskeleton organization, cell adhesion and cell motility contributing to the epithelial phenotype in breast and prostatic cancer cells (Warzecha, 2009, 2010)

Recent results showed that TGFβ1 mediated EMT induced down-regulation of ESRPs splicing program in pancreatic cancer cells (Horiguchi, 2011), suggest us to investigate *hMENA* splicing program in the PANC1 cell line. PANC1 cells transduced with *ESRP1* express hMENA^{11a} and this regulation of hMENA splicing program determines an increase of P-Ser157 VASP level.

At functional level, we analyze whether the hMENA splicing program differently affect the ability of PANC1 cells to adhere and invade ECM. *hMENA*^{11a} *or ESRP1* transduction increases P-Ser157 level in cells plated on collagen type I or fibronectin,

together with an increase of cell-matrix adhesion. Phase-contrast images analysis of *hMENA*^{11a} *or ESRP1* transductant cells shows that during collagen I-reattachment, cells exhibit multiple lamellipodia and extensive membrane ruffling and *hMENA*^{11a} *or ESRP1* transduced cells spread and extended large lamellae more than control cells. Furthermore, Ena/VASP proteins are known to play a critical role on cell migration in mouse and human (Loureiro, 2002; Wang, 2004) and in mouse model *Mena*^{11a} mRNA dissapeared in disseminated cancer cells (Goswami, 2008). To more directly assess the role of hMENA^{11a} in the regulation of tumorigenic activity, we examined the effects of hMENA^{11a} expression on pancreatic cancer cell migration and invasion. *hMENA*^{11a} *or ESRP1* transduction determines a reduction of cancer cell invasion in a BM-coated Boyden chamber.

Intracellular VASP redistribution and removal from cell periphery in pancreatic cancer cells, destabilizes actin-mediated processes such as cell-matrix adhesion and cell invasion, which are tightly regulated by integrin signaling and downstream effectors, such as FAK (Schaller, 2010). Adhesion of cells on a variety of ECM proteins, leads to an increase in tyrosine phosphorylation and activation of FAK (Schaller, 2010). Furthermore, suppression of adhesion induced tyrosine phosphorylation of FAK may interrupt cancer cell-ECM interactions and affect the invasive and metastatic potential of cancer cells. In addition, overexpression and phosphorylation of FAK correlates with the increase of cell motility and invasion (Crowe, 2004). We examined the activation of FAK in *ESRP1* or *hMENA*^{11a} transduced cells, plated on Collagen I and from WB analysis evinces that the level of FAK phosphorylation goes down in *ESRP1* or *hMENA*^{11a} cells, as well as in HPDE cells, respect than parental PANC1 cells.

In summary, we have shown how hMENA^{11a} regulates VASP phosphorylation at Ser157, the favourite PKA site and the phosphorylation at this site favors VASP and hMENA^{11a} localization to focal adhesions and lamellipodia as cells spread. Therefore, this study evidences an additional layer of regulation of these processes, since *hMENA* splicing perturbation in cancer cells may promote effects either in VASP phosphorylation or in FAK signaling. As splicing studies evolve, this study paves the way for defining *hMENA* splicing and its regulation as promising new biomarkers of invasiveness. Combined with other clinico-pathological markers, hMENA and its spliced forms may improve the early diagnosis of pancreatic and breast cancer and efficacious clinical decision for patient treatment.

Chapter 6 - References

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