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Plasmalemmal vacuolar H^+-ATPases in angiogenesis, diabetes and cancer

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Abstract Angiogenesis, i.e., new blood vessel formation, is required in normal and pathological states. A dysfunction in the microvascular endothelium occurs in diabetes, leading to decreased blood flow and limb amputation. In cancer, angiogenesis is increased to allow for growth, invasion, and metastasis of tumor cells. Better understanding of the molecular events that cause or are associated with either of these diseases is needed to develop therapies. The tumor and angiogenic cells micro-environment is acidic and not permissive for growth. We have shown that to survive this environment, highly metastatic and angiogenic cells employ vacuolar H^+-ATPase at their plasma membranes (pmV-ATPases) to maintain an alkaline pH^{cyt}. However, in lowly metastatic and in microvascular endothelial cells from diabetic model, the density of pmV-ATPase and the cell invasiveness are decreased. Therefore, the overexpression of the pmV-ATPase is important for cell invasion, and essential for tumor progression, angiogenesis and metastasis. Both, cancer and diabetes are heterogenous diseases that involve many different proteins and signaling pathways. Changes in pH^{cyt} have been associated with the regulation of a myriad of proteins, signaling molecules and pathways affecting many if not all cellular functions. Since changes in pH^{cyt} are pleiotropic, we hypothesize that alteration in a single protein, pmV-ATPase, that can regulate pH^{cyt} may explain the dysfunction of many proteins and cellular pathways in diabetes and cancer. Our long term goal is to determine the molecular mechanisms by which pmV-ATPase expression regulates tumor angiogenesis and metastasis. Such knowledge would be useful to identify targets for cancer therapy.

Keywords Angiogenesis · Cancer · Diabetes · Microvascular endothelial cells · Intracellular pH · V-ATPases

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Tumor angiogenesis

Angiogenesis, the recruitment of new blood vessels, is an essential component of the metastatic pathway (Carmeliet and Jain 2000; Folkman 1995; Zetter 1998). Angiogenesis is needed for the expansion of tumors beyond 1–2 mm at which point, diffusion of oxygen and nutrients become rate limiting for tumor growth (Carmeliet and Jain 2000; Folkman 1995). Tumor angiogenesis also provides a route for tumor cells to escape the primary site to enter the circulation and form secondary tumors at distant sites. Highly vascularized tumors may produce more metastasis because the vessels are immature, have high permeability, little basement membrane, and fewer intercellular junction complexes than mature vessels (Zetter 1998). However, some tumors seem to have vascularization without neoangiogenesis. In these cases, the tumor cells form extracellular matrix channels that contain blood, a process known as "vasculogenic mimicry" (Hendrix et al. 2003). Importantly, all well characterized angiogenic inhibitors such as angiostatin, endostatin, thalidomide, thrombospondin, and others, inhibit metastasis (Folkman 1995; O'Reilly et al. 1997). We have shown that highly metastatic cancer cells and angiogenic coronary microvascular endothelial cells (MEC) express V-ATPase at the cell surface, herein referred as plasmalemmal proton ATPase (pmV-ATPase; Rojas et al. 2006; Sennoune et al. 2004a). However, lowly metastatic

cells and poorly angiogenic coronary MEC from diabetic models exhibit decreased pmV-ATPase. These observations indicate that the presence of pmV-ATPase may confer the cells an "invasive phenotype" needed for tumor angiogenesis, tumor growth and metastasis.

Diabetes mellitus is a heterogeneous disease

Diabetes mellitus in humans is a genetically and clinically heterogeneous group of glucose intolerance syndromes. The decreased functional β-cell mass is caused by β-cell apoptosis and impaired proliferation consequent to hyperglycemia, hyperlipidemia and/or certain cytokines that interfere with the signaling pathways that maintain normal β cell growth and survival (Rhodes 2005). Type 1 diabetes usually has an autoimmune T cell-mediated etiology in which the prediabetic state is characterized by development of autoantibodies against proteins expressed by β cells, including insulin (Gale 2001). However, diabetes type 2 may also exhibit an autoimmune component. Type 2 diabetes is the more prevalent clinical form; in which obesity associated with progressively more severe insulin resistance are common predictors of the prediabetic state. Insulin resistance has also been observed in type 1 diabetes.

Diabetes affects neovascularization distinctly in different capillary beds

Certain organs/tissues (myocardium, lower extremities) exhibit decreased angiogenesis, whereas others (kidney, retina) exhibit increased angiogenesis (Aiello et al. 1994; Chou et al. 2002; Cooper et al. 1999; Stratton et al. 2000). The reasons underlying the differential effects of diabetes on angiogenesis in these tissues are unclear. Vascular endothelial growth factor (VEGF) is a major mediator of neovascularization. In diabetes, all major cell types in cardiac tissue produce low levels of VEGF (Chou et al. 2002). In contrast, in retina and glomeruli the VEGF levels are higher (Aiello et al. 1994; Cooper et al. 1999). This indicates that there is differential regulation of VEGF in MEC from different tissues. To test the hypothesis that angiogenesis is impaired in diabetes, we plated coronary MEC from normal and diabetic models in Matrigel (Rojas et al. 2004). Normal coronary MEC begin to extend lamellipodia-like structures within two hours; and in twenty-four hours form networks of capillary-like structures, whereas coronary MEC from diabetic do not form these structures even after extended periods of time. We also noticed that the invasive ability of coronary MEC from diabetic is severely compromised, when compared to normal (Rojas et al. 2004). Whether tumor angiogenesis is impaired in diabetes is unclear. The association between diabetes and cancer is not clear either.

The association between diabetes and cancer is dependent on the tumor site

An association between diabetes and cancer has been studied extensively. Epidemiological studies have indicated that diabetes is associated with either an increased risk, lack of association, or even a decrease of cancer, depending on the tumor site (Czyzyk and Szczepanik 2000). Specifically, both case control and cohort studies have shown a higher risk for colon cancer in diabetes type 2 (Meyerhardt et al. 2003). Women with a family history of diabetes seem to have a higher prevalence of breast cancer (Czyzyk and Szczepanik 2000). A significant association between diabetes and liver cancer has been observed (Fujino et al. 2001). There is, however, no unusual risk of pancreatic cancer associated with diabetes type 1; but the development of diabetes type 2 may be the first symptom of pancreatic cancer. In contrast, a decrease in prostate cancer has been observed in diabetes type 2 (Rosenberg et al. 2002). The reasons underlying such variable association between diabetes and cancer are unclear, but it may be due to the fact that both, diabetes and cancer are very heterogeneous diseases.

Studies on cancer in animal models suggest that diabetes can affect the incidence and size of some tumors

The hyperinsulinemia involving the insulin-like growth factor (IGF) pathway is the most prevalent hypothesis to explain the increase in cancer observed in diabetes type 2 (Giovannucci 2001). Insulin, at supra-physiological concentrations binds to IGF-1R because of its homology with the insulin receptor. IGF-1R is over expressed in human colon cancer (Giovannucci 2001). Upon binding to IGF receptor, it activates a signal cascade that triggers proliferation in several organs. Support for this pathway is provided by the observation that in *db/db* mice, the chemical induction of colon cancer is exacerbated, when compared to heterozygous *db/+* or controls (Giovannucci 2001). However, in STZ-induced diabetics nude mice, the induction of pancreatic tumors and the establishment of human pancreatic cancer implants are decreased. The growth of chemically induced pancreatic tumors was also decreased in STZ-induced diabetic hamster (Bell and Polonsky 2001). However, the growth of pancreatic human tumor cells in hamster was increased in STZ-induced diabetic hamster, suggesting that different cancer growth may be due to differences in the tumor host (Fisher et al.

1998). The reasons for such discrepancies are unclear, but may reflect differential sensitivities of the tumors or different capillary beds to the mitogenic effects of insulin or IGF. We hypothesize that the decreased tumor size in diabetes type 1 is due to decreased pmV-ATPase and tumor angiogenesis needed to support tumor growth and metastasis.

Cytosolic pH (pH^{cyt}) is regulated by three different systems

Most cells regulate pH^{cyt} using the Na^+/H^+ exchanger (NHE), HCO_3^--based H^+-transporting mechanisms (HCO_3^--transport) and H^+-ATPases (Putney et al. 2002). The NHE is ubiquitous and it is inhibited by amiloride and its analogs (Putney et al. 2002). The activity of NHE is regulated by the pH^{cyt} (Gillies et al. 1990). There are many types of HCO_3^--transporting systems that either acidify or alkalinize the cytosol (Gillies and Martínez-Zaguilán 1991). All of them are inhibited by stilbene disulfonate derivatives. Mammalian cells may also express both electrogenic vacuolar (V-type) H^+-ATPase and non-electrogenic E_1E_2 (P-type) H^+/K^+-ATPase. However, highly metastatic and other cells with an invasive phenotype only express pmV-ATPase (Blair et al. 1989; Brown and Breton 2000; Martínez-Zaguilán et al. 1993, 1996, 1998; Rojas et al. 2006; Sennoune et al. 2004a; Lu et al. 2005). V-ATPases are inhibited by bafilomycin A_1, concanamycin, and a new family of promising inhibitors (Beutler and McKee 2003; Bowman et al. 1988, 2003; Martínez-Zaguilán et al. 1993). The relevance of V-ATPases for pH^{cyt} homeostasis is not clearly understood.

V-atpases are distributed in plasma- and endo-membrane systems

V-ATPases are ubiquitously present in the membranes of vacuolar systems of animal cells (Nishi and Forgac 2002; Sennoune et al. 2004b; Fig. 1). The vacuolar system, including lysosomes, endosomes, secretory vesicles, belongs to either the endocytotic or exocytotic pathways or both, and their acidification plays a crucial role in their proper function (Mellman 1996; Nishi and Forgac 2002). Since V-ATPases are found in endo- and exocytotic vesicles, it is likely that V-ATPases are present in the plasma membrane of tumor and endothelial cells, due to enhanced membrane-recycling mechanisms (Martínez-Zaguilán 1999; Martínez-Zaguilán et al. 1999; Nishi and Forgac 2002; Raghunand et al. 1999). Indeed, in our studies the presence of V-ATPase at the plasma membrane has been corroborated in human breast cancer cells and coronary MEC by immunocytochemistry (Rojas et al. 2004; Sennoune et al. 2004a).

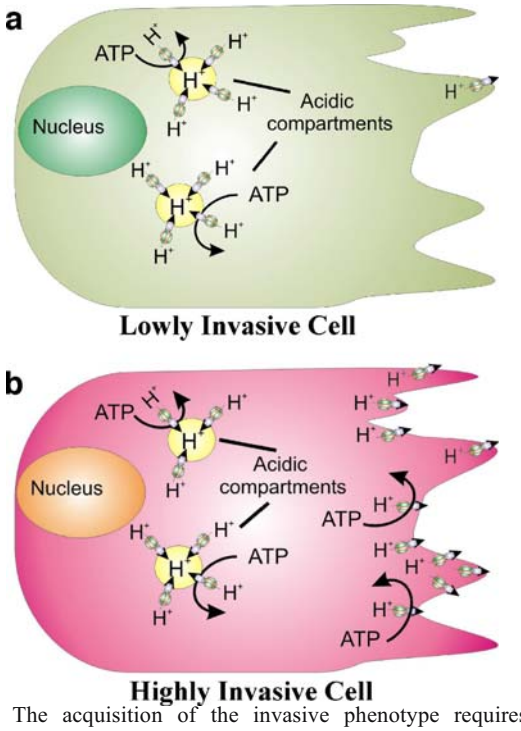

Fig. 1 The acquisition of the invasive phenotype requires the mobilization of V-ATPase from acidic compartments to the plasma membrane. V-ATPases are ubiquitously present in the membranes of vacuolar systems of animal cells. The vacuolar system belongs to either the endocytotic or exocytotic pathways or both. Since V-ATPases are found in endo- and exocytotic vesicles, it is likely that V-ATPases are present in the plasma membrane of the highly invasive cells, due to enhanced membrane-recycling mechanisms. **a** This model shows that in the lowly invasive cells, V-ATPase resides normally within acidic compartments and inconspicuous at the plasma membrane. **b** Importantly, V-ATPase is present at the plasma membrane, in addition to its localization in intracellular compartments, in the highly invasive cells. The increased density of pmV-ATPase, from acidic vesicles, in the highly invasive cells is likely due to enhanced exocytotic event

Most tumors exhibit an acidic pH^{ex} and an alkaline pH^{cyt}

It is known that the pH^{ex} of tumors is more acidic (pH 6.6–6.8) than the pH^{ex} in normal tissues (Gillies et al. 1990, 1994; Perona and Serrano 1988), due to a high rate of glycolysis and large amount of lactic acid produced in tumor areas (Racker 1972). Importantly, the cells in the tumor areas maintain a more alkaline pH^{cyt} (7.1–7.8) than normal tissue (6.9–7.1). These data suggest that tumor cells have evolved mechanisms to cope with acidic pH^{ex} environments. Further, intracellular acidosis is known to trigger apoptosis, and inhibition of V-ATPase induces apoptosis in many mammalian cell types (Tanigaki et al. 2003). This suggests that tumor and endothelial cells must exhibit unique anti-apoptotic pathways. Indeed, these cells are known to activate Raf-1 and other anti-apoptotic genes; and to suppress pro-apoptotic genes (Alavi et al. 2003). V-ATPase over expression is also a cellular anti-apoptotic

response (Torigoe et al. 2002). The presence of pmV-ATPase in endothelial and metastatic tumor cells allows them to maintain an alkaline pH^{cyt}, permissive for growth, and to invade through extra cellular matrix (Sennoune et al. 2004a). Importantly, lowly metastatic tumor cells and diabetic MEC, that are poorly angiogenic, express low pmV-ATPase (Rojas et al. 2004). Acidic pH^{ex} and hypoxia also enhance the invasiveness and migration of endothelial and tumor cells. We hypothesize that endothelial and metastatic cells express pmV-ATPase to regulate their pH^{cyt}.

PmV-ATPase has relevance to human cancers

We have characterized a number of primary human tumor cells in terms of H^+-ATPase activity, and determined that a subset of them exhibited pmV-ATPases (Martínez-Zaguilán 1999; Martínez-Zaguilán et al. 1993; Raghunand et al. 1999; Sennoune et al. 2004a). This was determined using bafilomycin, and following pH^{cyt} recovery to acid loads in the absence of Na^+ and HCO_3^-, to inhibit two major pH^{cyt} regulatory systems, i.e., Na^+/H^+ exchanger and HCO_3^--based H^+-transporting mechanisms. Thus, only cells exhibiting an alternate pH^{cyt} regulatory mechanism should recover from an acid load. Importantly, treatment of these cells with SCH28080, to block H^+/K^+-ATPase does not affect pH^{cyt} regulation. Highly metastatic human melanoma cells exhibit higher pmV-ATPase than lowly metastatic human melanoma cells (Martínez-Zaguilán 1999; Martínez-Zaguilán et al. 1993). We have made similar observation in human breast cancer cells with high and low metastatic potential (Sennoune et al. 2004a). To mimic the conditions found within a tumor, we cultured lowly invasive MCF7 human breast cancer cells at an acidic pH of 6.8, consistent with that observed in vivo (Sennoune et al. 2004a). Cells grown at pH^{ex} 6.8 exhibit faster pH^{cyt} recoveries than cells grown at pH^{ex} 7.4. This is due to pmV-ATPase expression since the pH^{cyt} recoveries are inhibited by bafilomycin. The phenotypic consequences of pmV-ATPase expression are the maintenance of a higher steady state pH^{cyt} in cells grown under acidic conditions, higher proton buffering capacity, and faster rates of H^+ extrusion when compared to cells grown at pH^{ex} 7.4. This adaptive feature may allow cells to survive and grow in a hostile, acidic environment, while cells not expressing this feature will be inhibited for growth and will undergo cell death, i.e. apoptosis. These results identify an important new pH^{cyt} homeostatic mechanism and provide an excellent model to investigate the role of pmV-ATPase expression in human tumorigenesis.

Transfection of cells with the plasmalemmal H^+-ATPase (PMA-1) gene determines a tumorigenic phenotype

To directly evaluate if the overexpression of H^+-ATPase at the plasma membrane is the cause, not a consequence, of a tumorigenic phenotype, we have transfected NIH-3T3 fibroblasts with the PMA-1 gene from yeast (Gillies et al. 1990, 1992; Gunn et al. 1994; Martinez et al. 1994; Peterson et al. 1994; Perona and Serrano 1988). NIH-3T3 cells are non-tumorigenic. To do this, we injected NIH-3T3 fibroblasts overexpressing proton ATPase (PMA-1 gene) in the flank of athymic nude mice. The parental NIH-3T3 fibroblasts do not develop tumors in mice. Importantly, overexpressing of proton ATPase renders these cells tumorigenic. Figure 2a shows human melanoma cells (C816) grown in mice for 5 weeks. The tumor shown in Fig. 2b is conspicuous at 72 h post-inoculation. The aggressiveness of NIH-3T3 cells overexpressing proton ATPase should be contrasted to the size of the tumors induced by highly metastatic C8161 expressing pmV-ATPases (evaluated at week 1 post-inoculation; Fig. 2d). We have also found that NIH-3T3 fibroblasts expressing PMA-1 exhibit J_{H+} that are significantly faster than in non-transfected counterparts (Fig. 2e). These data demonstrate that overexpression of proton ATPases have profound effects on the cell behavior and that it causes all the phenotypic characteristics found in tumors, including increased sensitivity to IGF, increase number of IGF receptors, increased fos expression, altered Ca^{2+} regulation and maintain an alkaline pH^{cyt}, ability to clone in soft agar and rapid development of tumors when injected in nude mice (Gillies and Martínez-Zaguilán 1991; Gillies et al. 1990, 1992; Gunn et al. 1994; Martinez et al. 1994; Martínez-Zaguilán et al. 1996; Perona and Serrano 1988; Peterson et al. 1994).

Localization of pmV-ATPase at the leading edge may determine cell polarity needed for cell migration

To evaluate the possibility of distinct pH^{cyt} gradients from leading to lagging edge, we used spectral imaging (Martínez-Zaguilán and Lynch 1996; Sanchez-Armass et al. 2006). In these experiments wounded monolayers of coronary MEC were loaded with SNARF-1, a pH fluoroprobe (Rojas et al. 2006). This approach allows us to monitor the full spectral output of the pH indicator. Furthermore, use of wounded monolayer experiments, to reveal the leading edge of migrating cells, indicate that pmV-ATPase is present at the leading edge. These data

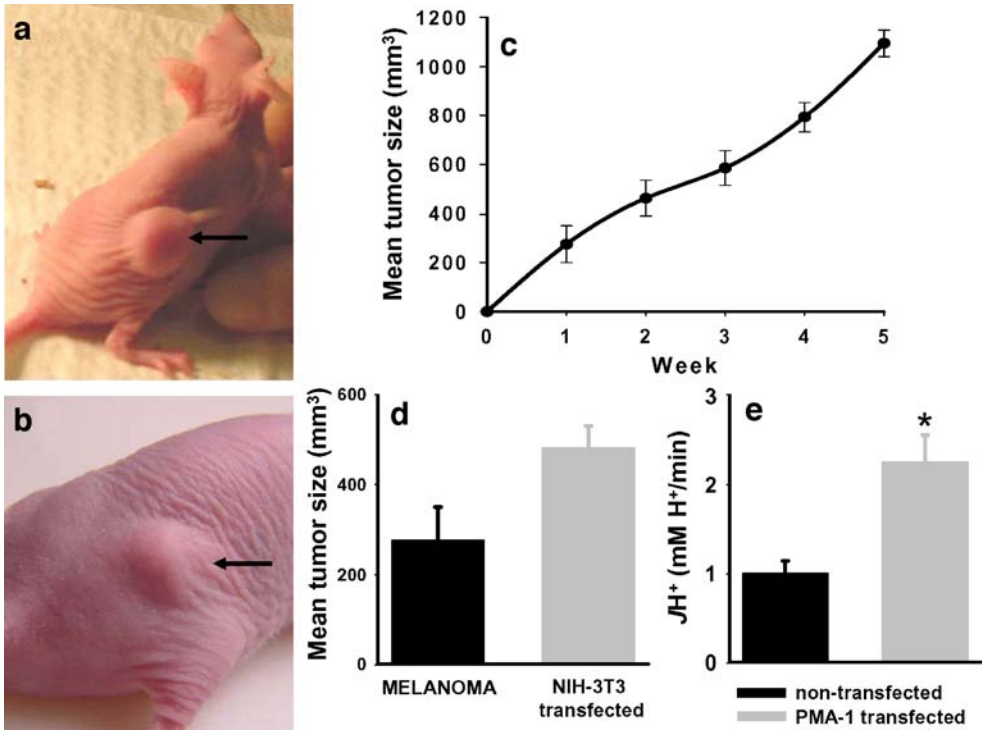

Fig. 2 Expression of H^+-ATPase at the cell surface is the cause, not the consequence, of a tumorigenic phenotype. To determine that H^+-ATPase is the cause, not the consequence, of the tumorigenic phenotype, we transfected NIH-3T3 fibroblasts cells with the gene encoding for the Plasmalemmal H^+-ATPase (PMA-1). Cells expressing this artificial construct exhibit all of the phenotypic characteristics found in tumors, including increased sensitivity to IGF, increase number of IGF receptors, increased fos expression, altered Ca^{2+} regulation and increase pH^{cyt}, ability to clone in soft agar and rapid development of tumors when injected in nude mice (Gillies and Martínez-Zaguilán 1991; Gillies et al. 1990, 1992; Gunn et al. 1994; Martinez et al. 1994; Martínez-Zaguilán et al. 1996; Perona and Serrano 1988; Peterson et al. 1994). Athymic nude mice (Balb/c nu/nu) were injected s.c. with 1×10^6 cells in the flank and tumor growth was monitored as a function of time. Tumor volume was calculated as: tumor volume=length×(width)^2/2. **a** Human melanoma cells (C8161) grown in mice for 5 weeks. **b** NIH-3T3 cells transfected with the gene encoding for PMA-1 rapidly develop tumors within 72 h. **c** Kinetic of tumor growth from melanoma cells inoculated in mice was measured every week. **d** The efficacy of cells transfected with PMA-1 is compared to the tumor growth observed with melanoma cells at 1 week post-inoculation. Notice the size of tumor at 72 h is twofold larger in cells expressing PMA-1 than in highly metastatic C8161 cells naturally express V-ATPase. **e** Transfection of NIH-3T3 cells with PMA-1 results in cells exhibiting faster proton fluxes (JH^+) than their naive counterparts. This data support our contention that the expression of H^+-ATPase at the cell surface is a necessary and sufficient mechanism to explain cancer progression

indicate that cells exhibit a more alkaline pH^{cyt} at the leading than at the lagging edge (Rojas et al. 2006).

Changes in pH^{cyt} are critical for establishing cell polarity needed for cell movement. A critical step in directed motility and migration is the asymmetric actin polymerization at the leading edge. Increase in pH^{cyt} promotes recruitment of cofilin and dynamic actin remodeling at the leading edge of migratory cells (Bernstein et al. 2000). Recently, V-ATPase has been shown to co-localize with actin at the cell cortex in activated osteoclasts and epithelial cells (Holliday et al. 2000). Our studies show that pmV-ATPase also co-localizes with actin at the leading edge (Rojas et al. 2004, 2006; Sennoune et al. 2004a). Microtubules and microfilaments regulate the transport of biosynthetic cargo of vesicles derived from the Golgi apparatus (Schmoranzer and Simon 2003). We hypothesize that pmV-ATPase activity regulates cytoskeleton proteins needed for the movement of endosomes and lysosomes to the plasma membrane that could contribute not only to surface area needed for migration, but also to the insertion of pmV-ATPase.

Mechanistic model for the acquisition of an invasive phenotype via pmV-ATPase

The presence of V-ATPase at the cell surface in cells with an invasive phenotype (i.e., MEC and highly metastatic human melanoma and breast cancer cells) and the decreased in density of pmV-ATPase in either MEC from diabetic rat or cells with low metastatic potential suggest the following model. It is possible that the increased density of pmV-ATPase in cells with an invasive phenotype may be due to enhanced vesicle recycling or targeting via specific

isoforms of V-ATPase subunits. The higher density of pmV-ATPase at the leading migratory edge in invasive cells suggests a dual role: (a) to increase pH^{cyt} in leading edge; and (b) to increase acidity at the extra cellular milieu, thus favoring protease activity and degradation of extra cellular matrix. The increase in pH^{cyt} at the leading edge creates a proton gradient from leading to lagging edge that could favor polarization. Directionality, however, needs to be provided by additional molecules such as VEGF or ATP as signaling molecules. Importantly, we hypothesize that there should be pH^{cyt} fluctuations due to dynamic insertion of V-ATPases into the plasma membrane and/or activation of pmV-ATPases by a yet unknown mechanism. The insertion of vesicles into the plasma membrane should allow not only to insert V-ATPases, but also increase the surface area needed for migration at the leading edge. The insertion of V-ATPase from acidic vesicles is likely an exocytotic event that requires increases in $[Ca^{2+}]^{cyt}$. The alkaline pH^{cyt} at the leading edge would favor a decrease in the dissociation constant (Kd) of Ca^{2+}-binding proteins and therefore decrease $[Ca^{2+}]^{cyt}$ locally to decrease fusiogenic events. This in turn could explain the pH^{cyt} fluctuations that are needed to control dynamic assembly/disassembly of microtubules, microfilaments that are known to be exquisitely regulated by pH. This in turn will allow protrusion at the leading edge and retraction at the lagging edge by changing the rigidity of the cytoskeleton structure favoring the sol–gel transition, thus decreasing the viscosity at the leading edge while increasing the viscoscity at the lagging edge to favor its retraction. Although much work remains to be done before the validity of this model is proven, the proposed model should be viewed as a framework to explain how pmV-ATPase determines the acquisition of an invasive phenotype needed for angiogenesis and metastasis (Fig. 3).

To conclude, we have evaluated the hypothesis that angiogenesis and the transition from a lowly to a highly invasive/metastatic phenotype involves the functional expression of pmV-ATPases. This was shown in human melanoma cells as well as in human breast cancer cells with low and high invasive/metastatic potential. We have also shown that MEC from normal rat exhibit increased pmV-ATPase and increase angiogenesis, whereas MEC from the diabetic rat exhibit low pmV-ATPase and decreased angiogenesis. We have also shown that MEC exhibiting pmV-ATPases exhibit a more alkaline pH^{cyt} at the leading than at the lagging edge. Both tumor and MEC exhibiting pmV-ATPase respond to V-ATPase inhibitors by decreasing cell migration and invasion. Thus, it seems that pmV-ATPase encodes information associated with the transition to a more angiogenic and metastatic phenotype. This suggests that pmV-ATPases are molecular targets to halt tumor angiogenesis and metastasis.

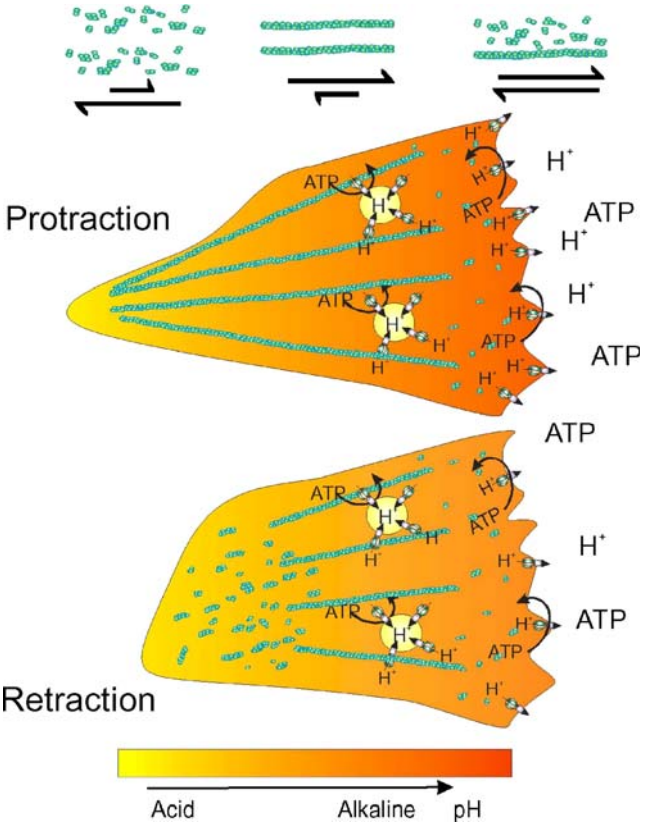

Fig. 3 Proposed mechanism by which overexpression of pmV-ATPase at the leading edge of the cell modulates cell migration/invasion. The proposed model should be viewed as a framework to explain how pmV-ATPases determine the acquisition of an invasive phenotype needed for angiogenesis and metastasis. Changes in pH^{cyt} are critical for establishing cell polarity needed for cell movement. A critical step in directed motility and migration is the asymmetric actin polymerization at the leading edge. Increase in pH^{cyt} promotes recruitment of cofilin and dynamic actin remodeling at the leading edge of migratory cells (Bernstein et al. 2000). Therefore, the pH^{cyt} fluctuations that are needed to control dynamic assembly/disassembly of microtubules/microfilaments will allow protrusion at the leading edge and retraction at the lagging edge by changing the rigidity of the cytoskeleton structure favoring the sol–gel transition. V-ATPase has been shown to co-localize with actin (Holiday et al. 2000; Rojas et al. 2004, 2006; Sennoune et al. 2004a). Therefore, we hypothesize that pmV-ATPase activity regulates cytoskeleton proteins needed for the movement of vesicles to the plasma membrane that could contribute not only to membrane-recycling needed for migration/invasion, but also to the insertion of V-ATPase. Also, the high density of pmV-ATPase at the leading migratory edge in invasive cells suggests that there is increase acidity at the extracellular milieu via pmV-ATPase. This acid release provides an optimum extracellular environment for proteases to degrade the extracellular matrix and therefore to allow cell invasion

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