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Vacuolar H^+-ATPase is Down-regulated by the Angiogenesis-Inhibitory Pigment Epithelium-Derived Factor in Metastatic Prostate Cancer Cells

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Abstract

The Vacuolar H^+-ATPases (V-ATPases), a multi-subunits nanomotor present in all eukaryotic cells resides in the endomembranes of exocytotic and endocytotic pathways. Plasmalemmal V-ATPases have been shown to be involved in tumor cell metastasis. Pigment epithelium-derived factor (PEDF), a potent endogenous inhibitor of angiogenesis, is down-regulated in prostate cancer cells. We hypothesized that the transduction of PEDF in prostate cancer cells will down-regulate V-ATPase function; that in turn will decrease the expression of the V-ATPase accessory protein ATP6ap2 and a-subunit isoforms that target V-ATPase to the cell surface. To test these hypotheses, we used the human androgen-sensitive prostate cancer cells LNCaP, and its castration-refractory-derivative CL1 that were engineered to stably co-express the DsRed Express Fluorescent Protein with or without PEDF. To determine if PEDF down-regulates the function of V-ATPase, we measured the rate of proton fluxes (J_{H+}) of the cytosolic and endosome/lysosome compartments. The mRNA levels for subunit-a isoforms and the ATP6ap2 were measured using quantitative reverse transcription-PCR. The results showed that PEDF expression decreased the rate of J_{H+} in metastatic CL1 cells without affecting J_{H+} in non-metastatic LNCaP cells, when studying pH^{cyt}. Interestingly, PEDF did not affect JH^+ in endosomes/lysosomes either in metastatic cells or in non-metastatic cells. We also showed that PEDF significantly decreases the levels of a4 isoform and ATP6ap2 in metastatic CL1 cells, without affecting the levels of a4 isoform in the non-metastatic LNCaP cells. These data identify PEDF as a novel regulator of V-ATPase suggesting a new way by which PEDF may inhibit prostate tumor growth.

Key words: LNCaP and LNCaP-derivative CL1 cells, pH regulation, ATP6ap2, subunit a-isoforms.

Introduction

The Vacuolar H^+-ATPase (V-ATPase) belongs to a family of ATP-dependent proton pumps that are responsible of acidification of endosomes and lysosomes (1-3). V-ATPase regulates the cytosolic pH, mediates intraluminal acidification and regulates vesicular trafficking and fusion. The V-ATPases are responsible for tumor microenvironment acidification and have been implicated in tumor cell metastasis (4-7). The V-ATPase is composed of two reversibly-associated multi-subunit domains called V1 and V0. The cytosolic V1 domain contains the catalytic site for ATP hydrolysis and the membranous V0 domain translocates protons. There are eight different subunits (A to H) in the catalytic domain. In mammals, the trans-membrane V0 domain contains six hydrophobic subunits (five c and one c"), and single copies of a, d, and e subunits (8,9). In addition to the core subunits, two accessory proteins were found to be associated with V-ATPase: ATP6ap1 and ATP6ap2/(Pro)Renin Receptor/(P)RR (10,11).

The a-subunits are present in four isoforms (a1, a2, a3 and a4) that contain information to target VATPases to different membranous locations, and are expressed in a tissue-specific manner (12-15). The a1-, a2- and a3-isoforms are ubiquitously expressed in mammalian cells, albeit their levels of expression are tissue-specific (16-18), while a4-isoform is expressed in renal intercalated cells and epididymal clear cells, where it is localized in the apical membrane (13). It has been demonstrated that more than one a-isoform can be expressed in the plasma membrane, as for rat vas deferens and epididymal cells (13). In our previous studies, we have shown that although all four isoforms are detectable in both highly (MB231) and lowly (MCF7) metastatic human breast cells, the levels of a3 and a4 are much higher in MB231 than in MCF7 cells (19). The knockdown of either a3- or a4-isoforms significantly inhibited invasion of highly metastatic MB231 cells. We have also found that the isoform a1 is down-regulated in highly metastatic cells compared to the lowly metastatic cells. Altogether, these data emphasize the significance of a1, a3 and a4 for the acquisition of a more metastatic phenotype.

The biogenesis of V-ATPase complex requires the coordinated association of V1 with V0 domains. Studies in yeast have shown that several genes Vma12p, Vma21p, and Vma22p are required for V-ATPase assembly (20). This suggests that mammalian cells may have similar assembly mechanism. However, the assembly chaperone(s) of mammalian V-ATPase is (are) unclear. A co-localization between PRR/ATP6ap2 and V-ATPase has been shown in renal intercalated cells that may depend on the V-ATPase activity, since V-ATPase is primarily responsible for final urinary acidification (21). Recently, Kinouchi et *al*., suggested that PRR/ATP6ap2 is essential for V-ATPase assembly in murine cardiomyocytes (22). These studies demonstrated for

the first time that ATP6ap2 might be an essential assembly chaperone of mammalian V-ATPase; and that genetic ablation of ATP6ap2 created a loss-of-function model for V-ATPase representing a function that is unique to mammalian cells.

Increasing evidence suggests that V-ATPase is required for signaling pathways regulating cell growth and survival. Prominent in the signaling pathways leading to cell growth and differentiation is the Wnt pathway (23-25). This signaling pathway involves proteins that directly participate in both gene transcription and cell adhesion. Defects in this pathway are strongly associated with tumorigenesis (26,27). Cruciat et *al*., characterized V-ATPase and (P)RR/ATP6ap2 as essential components of Wnt signaling in Xenopus embryos, which is crucial for several processes in embryonic development (28). It has been shown that phosphorylation of the Wnt co-receptor LRP6, and thereby activation of intracellular Wnt signaling, is dependent on its sequestration in vesicles, which are acidified by the V-ATPases. Thus, it appears that the crucial link between LRP6 and V-ATPase is formed by (P)RR/ATP6ap2 complexing with V-ATPase. We hypothesized that (P)RR/ATP6ap2 is the pH sensor, and accordingly assembles V-ATPase and targets it via the a-subunit isoforms to specific compartments to regulate the pH gradient and/or initiates signaling pathways. Whether this is a direct effect of the (P)RR/ATP6ap2, or an indirect effect due to interactions with a-subunit, a putative pH sensor in V-ATPase (29) is unclear.

Pigment epithelium derived factor (PEDF) is a secreted glycoprotein which belongs to the serine protease inhibitor (serpin-like peptide) family and has been described as one of the most potent anti-angiogenic factors (30,31). PEDF was first identified in the conditioned media of cultured pigment epithelial cells (32); and was also found to be highly expressed in different tissues including brain, prostate, kidney, heart, testis (33). In contrast to normal tissues, PEDF expression has been shown to be down-regulated in most human solid tumors including prostate cancer. As well, PEDF down-regulation has been associated with poor prognosis in human prostate specimens. In previous studies, we have demonstrated that the re-expression of PEDF in human castration-refractory prostate cancer cells inhibits the growth of primary tumors in vivo, delays their dissemination to secondary sites and, as a corollary, prolongs the survival of tumor-bearing mice (34-37). While PEDF anti-tumor properties have been attributed to its angio-inhibitory, anti-proliferative and differentiation properties, the key effectors involved still need to be identified. Previous studies have shown that PEDF binds to several cell surface proteins including the phospholipase A2ξ, F1/F0 ATP synthase, and the non-integrin laminin receptor (38-41).

A recent study identified PEDF as a novel inhibitor of the Wnt pathway via its co-receptor LRP6 (42). PEDF was found bound to LRP6 with high affinity and blocked Wnt ligand-induced LRP6-Frizzled receptor dimerization, an essential step in Wnt signaling. These data suggest that PEDF is an endogenous antagonist of LRP6. Since the crucial link between LRP6 and the V-ATPase is formed by its accessory protein 2 (PRR/ATP6ap2), we hypothesized that the re-introduction of PEDF in metastatic prostate cancer cells will down-regulate ATP6ap2 and therefore V-ATPase function. We also hypothesize that down-regulation of the function of V-ATPase will also alter the expression of a isoforms. This mechanism could explain how PEDF inhibits tumor growth and metastasis via down-regulation of V-ATPase.

Materials and methods

Cell Culture

The prostate cancer cell lines LNCaP and LNCaP-derivative CL1 were used in this study (43). LNCaP cells are androgen-sensitive, tumorigenic and non-metastatic. CL1 cells were derived from LNCaP by growing the LNCaP cells in medium depleted from androgen. Androgen deprivation treatment of LNCaP cells was carried out by replacing FBS by charcoal/Dextran-stripped serum. CL1 cells have been continually maintained in androgen-depleted medium. It has been shown that CL1 cells expressed the androgen receptors but lost the transcriptional regulation of prostate-specific genes. In contrast to the parental LNCaP cells, CL1 cells are androgen-refractory/Castration-Refractory Prostate Cancer, highly tumorigenic and metastatic. LNCaP cells were grown in RPMI 1640 media (Corning Cellgro, Mediatech Inc., VA) supplemented with 10% FBS and antibiotics. CL1 cells were grown in RPMI 1640 media supplemented with 5% charcoal-treated serum, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 1 mM Non-Essential Amino Acids. All the cells were maintained at 5% CO_2 at 37°C.

Generation of PEDF expression

To evaluate the significance of PEDF in our studies, we used LNCaP and LNCaP-derivative CL1 cell lines that stably co-express the fluorescent DsRed Express protein with or without PEDF as we previously described (37).

The human PEDF cDNA was cloned into the retroviral bicistronic pRetroX-IRES-DsRedExpress plasmid (Clontech). The PT67 cells were co-transfected with 1 µg pPUR (Clontech) and 10 µg of either pRetroX-IRES-DsRedExpress or pRetroX-PEDF-IRES-DsRedExpress using the Lipofectamine LTX kit (Invitrogen, Carlsbad, CA). Transfectant single clones were selected in 3 µg/ml puromycin. The day before the infection, LNCaP and LNCaP-derivative CL1 cells were seeded in 6-well plate. At the time of the infection, viral supernatants were collected, filtered, combined to 4 µg/µl polybrene and incubated with these cells for two cycles of 12 hours. Transduced prostate cancer cells were selected by fluorescence-activated cell sorting (BD FACS Aria flow cytometer; BD Biosciences) to reach a purity of 96-99%. PEDF secretion was analyzed in conditioned media by western blotting (figure 2) using anti-PEDF antibody (Millipore, Temecula, CA).

Cytosolic and Endosomal/lysosomal H^+ flux (J_{H+}) measurements

Cells growing on rectangular coverslips (8 x 22 mm) were loaded with SNARF-1 (5-[and-6] carboxy-SNARF-1-AM) and pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt) (Life Science Molecular

Probes, Eugene, OR) to simultaneously measure cytosolic and endosome/lysosome H^+ fluxes over the whole cell, respectively (19). Cells were transferred to the cell perfusion system and were continuously perfused at 3.0 ml/min at 37°C with Cell Superfusion Buffer (CSB) containing: 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 5.4 mM KCl, 0.44 mM KH_2PO_4, 110 mM NaCl, 0.35 mM NaH_2PO_4, 5 mM glucose, 2 mM glutamine, and 20 mM HEPES at pH^{ex} 7.4. After steady state cytosolic pH (pH^{cyt}) was reached (5 min), the perfusate was exchanged for one containing 50 mM KOAc in a Na^+-free buffer, where Na^+ was substituted with 60 mM N-methylglucamine. The excitation spectra of pyranine (450/405 nm) in endosomes/lysosomes were acquired using an emission wavelength of 514 nm; and the emission spectra of SNARF-1 (644/584 nm) were acquired using 534 nm as excitation wavelength. The conversion of ratio values to cytosolic pH (pH^{cyt}) and endosomal/lysosomal pH ($pH^{E/L}$) were performed as described previously (19). The proton fluxes (J_{H+}) were estimated within the first 3 min of the KOAc-induced acidification in both the cytosol and the endosome/lysosome compartments. The fluorescence was monitored with a SLM-8100/DMX spectrofluorometer (Spectronics Instruments, Rochester, NY) equipped for sample perfusion. The sample temperature was maintained at 37°C by keeping both the water jacket and perfusion media at 37°C using an iso-temperature immersion circulator water bath (Lauda model RM20, Brinkmann Instruments, Westbury, NY). All measurements were performed using 4 nm-bandpass slits and an external rhodamine standard as a reference. Fluorescence data were converted to ASCII format for analyses.

In situ calibration

In situ calibration curves were generated as described previously (44). Briefly, the cells attached to cover slips were perfused with High K^+ buffer (NaCl 10 mM, KCl 146 mM, HEPES 10 mM, MES 10 mM, Bicine 10 mM, 2 μM valinomycin and 6.8 μM nigericin, Glucose 5 mM, Glutamine 2 mM, pH 5.5 to 8.0 adjusted with NaOH). The buffer contains high K^+ to approximate intracellular K^+ concentration. Nigericin is an ionophore which exchanges H^+ and K^+ across the membrane rendering the pH^{cyt} equal to the extracellular pH (pH^{ex}). Valinomycin is an ionophore which moves K^+ across the plasma membrane and helps to equilibrate $pH^{cyt} = pH^{ex}$. The pH of the buffers was determined using a Beckman pH meter with a glass electrode (Corning Inc., Horseheads, NY) calibrated at 37°C with commercially available standard solutions (VWR Scientific, San Francisco, CA). The ratios (R=644/584) of SNARF-1 and (R=450/405) of pyranine were converted to pH using a modified Henderson-Hasselbalch equation. The equation was solved using nonlinear least squares analysis with Sigma Plot to obtain the values of pK_a, R_{min}, and R_{max} for SNARF-1 and pyranine in these cells.

Data analysis

The initial rate of pH_{cyt} recovery from an acid load induced by K^+-acetate was measured as the dpH/dt which is the slope of the linear regression curve relating time and pH_{cyt}. Briefly, the cells loaded with SNARF-1 were first perfused with the CSB until the steady-state pH_{cyt} was reached. Then, the cells were perfused with 50 mM K^+-acetate in HCO_3^- and Na^+-free CSB to eliminate the contribution of potential HCO_3^- transporters and Na^+/H^+ exchanger. The perfusion with K^+-acetate will cause a rapid intracellular acidification followed by a subsequent pH^{cyt} recovery. We then evaluated the pH^{cyt} recovery from this acidification within the first 3 minutes. The individual pH^{cyt} data points were then used to plot a linear regression curve relating time and ΔpH^{cyt}. To quantify the pH^{cyt} recovery, we expressed the recoveries as proton flux (J_{H+}) which is given by the apparent H^+ buffering (B_{H+}) capacity multiplied by the dpH/dt (45).

Real Time Reverse Transcription PCR

Cells were harvested and lysed, and the RNA was isolated using RNeasy® mini kit (Qiagen). After RNA isolation, mRNA was isolated with the MicroPoly(A) PuristTM kit from Ambion®. The verso cDNA kit (Thermo Scientific) was used to make cDNA from the RNA sample previously isolated. One-step quantitative RT-PCR was performed in a 96-well format on a Bio-Rad MyIQ thermal cycler system using Brilliant® SYBR® Green QRT-PCR master mix (Applied Biosystem). The PCR cycling sequence consisted of 60 min at 40° C to allow for reverse transcription followed by 40 cycles of 60 seconds at 62° C and 60 seconds at 92° C. To quantitate the results, cDNA clones of the a1, a2, a3, a4 isoforms and ATP6ap2 accessory protein (Open Biosystems) were purchased. Plasmid DNA for each isoform and accessory protein isolated from Escherichia coli was quantitated by measuring the absorbance at 260 nm; and these DNA standards were used during the quantitative RT-PCRs to facilitate quantification of the initial mRNA levels for each experimental sample by use of a standard curve. The primers were purchased from Thermo Scientific. As a control, the levels of mRNA for the ribosomal protein S15 in all the cell lines were measured. Amplication reactions were loaded on a 2% agarose gel to verify the proper size of the amplicons.

Statistical analysis

All results are expressed as mean ± SEM. The significant differences were determined by using a t-test or an ANOVA with Holm-Sidak test for multiple comparisons of normal distributions. The Mann-Whitney test or the Kruskal-Wallis ANOVA with Dunn's test for multiple comparisons was used for nonparametric distributions (SigmaStat v.3.5; Statistical Software, Jandel Scientific). All statistical tests were considered significant at $p < 0.05$.

Results

Proton fluxes (J_{H+}) activity via V-ATPase

To study the significance of V-ATPase for the regulation of proton fluxes in metastatic and non-metastatic cells, we monitored the rate of pH^{cyt} recovery following an acute acid load induced by K^+-acetate (KOAc). To monitor pH^{cyt}, cells loaded with SNARF-1 were superfused with cell superfusion buffer (CSB) in the absence of HCO_3^-. Once steady-state pH^{cyt} was reached, cells were superfused with Na^+-free KOAc CSB to induce an acid load. In the absence of both Na^+ and HCO_3^- we

eliminate the contribution of HCO_3^- transporters and Na^+/H^+ exchangers for pH^{cyt} recoveries. As shown in figure 1, the rate of H^+ extrusion (J_{H+}), as evaluated by SNARF-1 (cytosol), were significantly faster in metastatic than in non-metastatic cells. To determine whether the pH^{cyt} recovery in response to the acid load in the absence of HCO_3^- and Na^+ is mediated via V-ATPase, we evaluated the effect of bafilomycin A1, an inhibitor of V-ATPase (44,46). These experiments indicated that bafilomycin A1 significantly inhibited the pH^{cyt} recoveries in metastatic cells (Figure 1).

To evaluate the relationship between PEDF and V-ATPase in terms of J_{H+}, we used cells that stably express PEDF. PEDF secretion in the cell media was verified by western blotting (figure 2). As shown in figure 3, PEDF re-expression significantly decreased the rate of J_{H+} in metastatic CL1 cells without affecting J_{H+} in non-metastatic cells, when studying pH^{cyt} (figure 3A). Simultaneous measurements of pH^{cyt} and $pH^{E/L}$ with SNARF-1 and pyranine, respectively, allow us to study J_{H+} in both cytosol and endosomes/lysosomes. These studies indicated that the J_{H+} in the endosome/lysosome are ca. 5 fold faster in metastatic (CL1) than in non-metastatic (LNCaP) cells. Interestingly, PEDF did not significantly affect J_{H+} in endosomes/lysosomes of either metastatic cells or non-metastatic cells (Figure 3B).

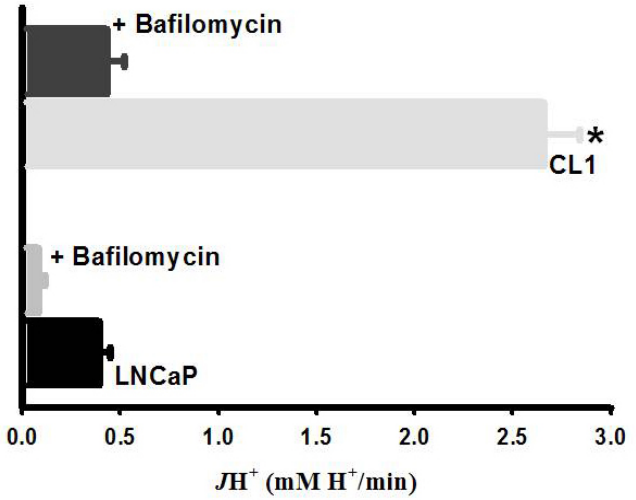

Figure 1. Proton fluxes (J_{H+}) via V-ATPase are greater in metastatic LNCaP-derivative CL1 cells than in non-metastatic LNCaP cells. Cells were loaded with 7 µM of the carboxyseminaphthorhodafluor-1 (SNARF-1-AM) to study pH^{cyt}. Acid loading experiments were performed as described in "Materials and Methods" to determine JH^+ at extracellular pH 7.4 in the absence of Na^+ and HCO_3^-. For bafilomycin experiments, cells were treated with 1 µM bafilomycin A1 throughout the experiment. Values are means ± SEM (n =4 in triplicate for the controls; n=3 in duplicate for bafilomycin treatment). *p< 0.001: CL1 cells without treatment versus treated with bafilomycin A1. **p < 0.001: CL1 versus LNCaP.

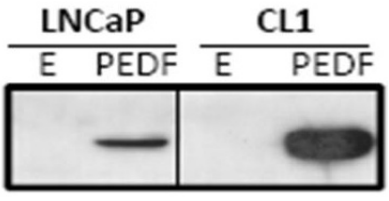

Figure 2. LNCaP-derivative CL1 and LNCaP cell lines stably express the fluorescent DsRed Express protein in combination with PEDF. The human PEDF cDNA sequence was cloned into the retroviral bicistronic pRetroX-DsRed expression vector. After establishment of stable packaging PT67 cells that express the DsRed protein with or without PEDF, we isolated single clones that were used to transduce LNCaP and LNCaP-derivative CL1 cells. These different clones were sorted by flow cytometry. The resulting cell lines express and secrete PEDF at various levels in the cell media. E: empty vector; PEDF: Pigment epithelium-derived factor.

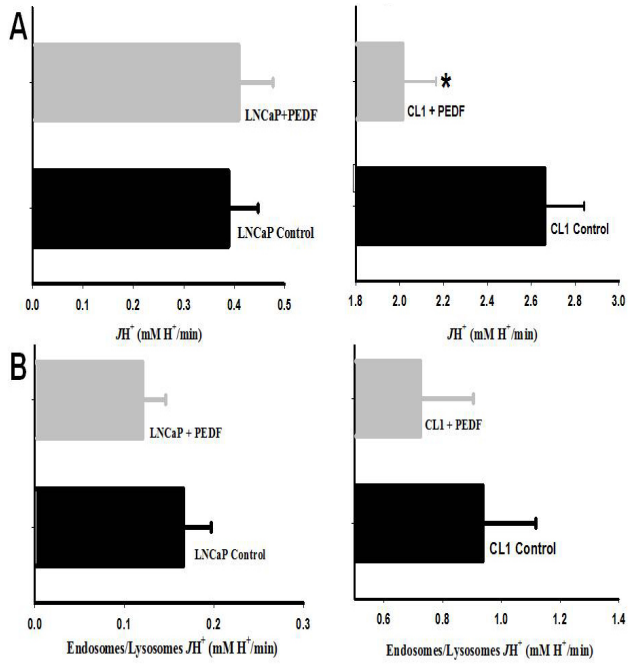

Figure 3. A. PEDF decreased the proton fluxes (J_{H+}) via V-ATPase in metastatic LNCaP-derivative cells when studying pH^{cyt}. Cells were loaded with 7 µM of the carboxyseminaphthorhodafluor-1 (SNARF-1) to study pH^{cyt}. Acid loading experiments were performed as described in "Materials and Methods" to determine JH^+ at extracellular pH 7.4, in the absence of Na^+ and HCO_3^-. **A.** proton fluxes in non-metastatic LNCaP cells. **B.** proton fluxes in metastatic LNCaP-derivative CL1 cells. Values are means ± SEM (n = 3 in duplicate). *p< 0.001: CL1 vs CL1+PEDF. **B. The J_{H+} in endosomes/lysosomes via V-ATPase are not affected by PEDF.** Cells were loaded with 1 µM 8-hydroxypyrene-1,3,6-trisulfonic acid (pyranine), to label endosomes/lysosomes. Acid loading experiments were performed as described in "Materials and Methods" to determine J_{H+} at extracellular pH 7.4, in the absence of Na^+ and HCO_3^-. **A.** proton fluxes in non-metastatic LNCaP cells. **B.** proton fluxes in metastatic LNCaP-derivative CL1 cells. Values are means ± SEM (n = 3 in duplicate). Data were not statistically different on either LNCaP or CL1 cells.

mRNA levels of a-isoforms and the accessory protein ATP6ap2

Quantitative RT-PCR was performed with isoform-specific primers on mRNA isolated from each cell line, using plasmid-borne cDNAs encoding each isoform as standards. As shown in Figure 4A, all four a-subunit isoforms can be detected in both cells. Importantly, the relative abundance of a-subunit isoforms differs between the metastatic and non-metastatic cells. Notice that the levels of a2 isoform in metastatic and non-metastatic cells are not statistically different (Figure 4A). Interestingly, the levels of a3 and a4 isoforms are significantly greater in metastatic than in non-metastatic cells (Figure 4B), consistent with greater J_{H+} via V-ATPase activity

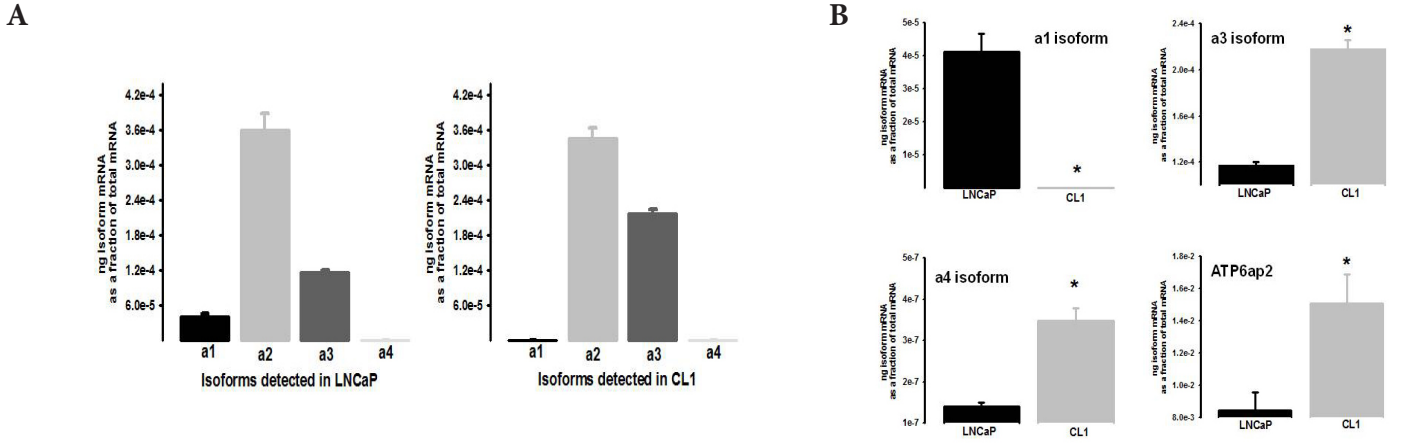

Figure 4. A. mRNA levels of subunit a-isoforms detected in LNCaP and LNCaP-derivative CL1 cells. Quantitative RT-PCR was used to determine the mRNA levels of the different subunit a isoforms. Plasmids expressing the cDNA for each isoform were used to construct a standard curve. All values are normalized to total mRNA loaded. The values reported are the ratio of nanograms of isoform-specific mRNA to the total nanograms of mRNA. All four a subunit isoforms are detected in both cell lines. Values are mean ± SEM of 4 experiments each. **B. The mRNA levels of the a3-, a4-isoforms and the accessory protein ATP6ap2 are predominant in metastatic LNCaP-derivative CL1 cells, whereas a1-isoform is the predominant isoform in the non-metastatic LNCaP cells.** Quantitative RT-PCR was performed with isoform-specific primers on mRNA isolated from each cell line, using cDNAs encoding each isoform as standards. Values are mean ± SEM of 4 experiments each. *p< 0.05 CL1 vs LNCaP.

in metastatic cells (cf. figure 1). It is also of interest to note that the mRNA level of a1 isoform is decreased in metastatic (CL1) cells compared to their parental non-metastatic (LNCaP) cells (Figure 4B). Associated with greater levels of expression of a3 and a4 isoforms in CL1 than in LNCaP, the mRNA levels of ATP6ap2 (Figure 4B) are also more elevated in the metastatic (CL1) than in non-metastatic (LNCaP) cells.

To further evaluate the significance of PEDF in regulating V-ATPase, we evaluated the mRNA levels of subunit-a isoforms in cells stably expressing PEDF. As shown in figure 5A, PEDF re-expression in metastatic (CL1) and non-metastatic (LNCaP) cells did not affect a1 isoform. Importantly, PEDF significantly decreased the levels of a4 isoform in metastatic CL1 cells, without affecting the levels of a4 isoform in the non-metastatic LNCaP cells (Figure 5B). PEDF did not change the levels of a3 isoform in both cell lines (data not shown). Furthermore, PEDF also significantly decreased the levels of expression of ATP6ap2 in metastatic but not in non-metastatic cells (Figure 5C). These data indicate

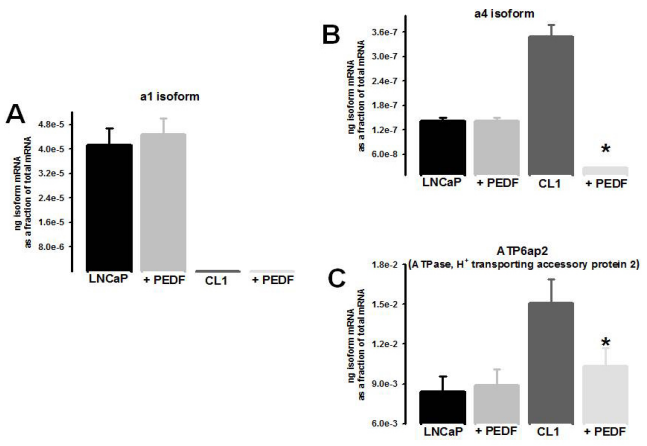

Figure 5. PEDF decreased the levels of a4-isoform and ATP6ap2 accessory protein only in metastatic LNCaP-derivative CL1 cells. Quantitative RT-PCR was performed with isoform-specific primers on mRNA isolated from each cell line. We used cDNAs encoding each isoform as standards. Values are mean ± SEM of 4 experiments each. *p< 0.05 CL1 control versus CL1+PEDF.

that PEDF is an important regulator of V-ATPase and highlights its significance in prostate cancer growth and progression.

Discussion

Our findings identify Pigment Epithelium-Derived factor (PEDF) as a novel regulator of V-ATPase which belongs to a family of ATP-dependent proton pumps that were found to be involved in angiogenesis and tumor metastasis (4-7). V-ATPase maintains the cytocolic pH, mediates intraluminal acidification thereby regulating vesicular trafficking and fusion and is responsible for tumor microenvironment acidification (7). V-ATPase is a multi-subunits nanomotor present in all eukaryotic cells and resides in the endomembranes of exocytotic and endocytotic pathways.

PEDF has been identified as a novel inhibitor of the Wnt pathway via LRP6 (42). Wnt pathway mediates multiple physiological and pathological processes including angiogenesis and cancer (26,27). Its regulation in these processes has not been fully elucidated. There are recent studies suggesting a role for the V-ATPase, via its subunits, in Wnt signaling pathways (47,48). V-ATPases has been shown to interact physically with Wnt receptor complex Fz and LRP6 and characterized as an essential component of Wnt signaling in Xenopus embryos (28). Thus, it appears to be functional overlap between V-ATPase and PEDF expression. We hypothesize that the re-introduction of PEDF in prostate cancer cells inhibits tumor growth by down-regulating V-ATPase. The down-regulation of V-ATPase will in turn decrease the expression of the V-ATPase accessory protein ATP6ap2 and subunit-a isoforms.

We have previously shown that PEDF expression limited the proliferation of androgen-sensitive LNCaP and CRPC CL1 prostatic cell lines (37). PEDF also reduced the number and size of 3D-tumor spheroids *in vitro*. Similarly, PEDF inhibited the migration of CRPC cells suggesting both anti-proliferative and anti-migratory functions. *In vivo*, PEDF decreased by 85% the growth of subcutaneous CL1 tumors. In the CL1 ortho-

topic model, tumor intake with lethal metastases was found in all animals; nevertheless, PEDF prolonged the median survival of tumor-bearing mice (95% CI-53±0.001 to 57±1 days). Accordingly, PEDF delayed the emergence of skeletal-related event in intra-tibial CL1 xenografts (37).

Since PEDF expression in prostate cancer cells, we have re-expressed PEDF gene in order to determine if its expression will affect the function of V-ATPase.

We first studied the significance of V-ATPase for the regulation of proton fluxes in metastatic and non-metastatic cells. To address this, we evaluated whether there were differences in the response to acid loads in LNCaP and LNCaP-derivative CL1 cell lines. The results showed that the metastatic CL1 cells exhibited a pH^{cyt} recovery that was faster than in non-metastatic cells LNCaP. Importantly, inhibition of V-ATPase with bafilomycin significantly decreased the J_{H+}. These data indicate that V-ATPase is responsible for the increased J_{H+} observed in metastatic CL1 cells. These data support our contention that V-ATPase is positioned at the cell surface to extrude H^+ across the plasma membrane to regulate pH^{cyt} in prostate metastatic cells. These data are consistent with our early studies in human melanoma and breast cancer cells with distinct metastatic potential, where the J_{H+} were faster in highly compared to the lowly metastatic cells (44,49,50). Altogether, these data suggest that the expression of V-ATPase at the cell membrane increase the J_{H+} to regulate pH^{cyt} and is responsible for a more metastatic phenotype in several types of cancer.

In order to evaluate the relationship between PEDF and V-ATPase in terms of J_{H+}, we used both cell lines that do not express and secrete PEDF. These cells were engineered to stably express and secrete PEDF. We simultaneously monitored the J_{H+} in both cytosol and endosomes/lysosomes. These studies indicated that PEDF significantly decreased the rate of J_{H+} in metastatic CL1 cells without affecting J_{H+} in non-metastatic cells, when studying pH^{cyt}. The results on the endosomes/lysosomes pH ($pH^{E/L}$) indicated that the JH^+ are faster in metastatic (CL1) than in non-metastatic (LNCaP) cells. Interestingly, PEDF did not significantly affect J_{H+} in $pH^{E/L}$ either in metastatic cells or in non-metastatic cells. These data indicate that PEDF regulates the V-ATPases that are important for acid extrusion across the plasma membrane, but not the V-ATPases involved in endomembranous recycling pathways.

The targeting of V-ATPases to different membranous locations is controlled by the subunit a-isoforms that are present in four isoforms (a1, a2, a3, and a4) and are expressed in a tissue-specific manner (12-15). Our previous studies in breast cancer cells have shown that the levels of a3- and a4-isoforms are greater in highly metastatic breast cancer cells (MB231) than in lowly metastatic breast cancer cells (MCF7) (19). The knockdown of either a3- or a4-isoforms significantly inhibited invasion of highly metastatic MB231 cells. Also, the isoform a1 is down-regulated in highly metastatic breast cancer cells compared to the lowly metastatic cells. In order to understand the role of a-isoforms of V-ATPases in prostate cancer metastasis, we compared their expression profile in both human prostate cancer cell lines LNCaP and CL1. The results using quantitative RT-PCR showed that all four a subunit isoforms are detected in both cell types. However, the relative abundance of these isoforms is not the same between both cells. Importantly, the levels of a3 and a4 isoforms are significantly greater in metastatic than in non-metastatic cells, consistent with greater J_{H+} via V-ATPase activity in metastatic cells. It is also of interest to note that the mRNA level of a1 isoform is decreased in metastatic cells CL1 compared to their parental non-metastatic cells LNCaP. Our present data in human prostate cancer cell with high metastatic potential is consistent with our prior observations in human breast cancer cells, further emphasizing that either a3 or a4 isoform is responsible for the acquisition of a more invasive and metastatic phenotype.

In addition to the V-ATPase core subunits, two accessory proteins have been found to be associated with V-ATPase including ATP6ap2 (also called (P) RR/ATP6ap2 or M-89) (10,11,51). An early study has shown a co-localization between PRR/ATP6ap2 and V-ATPase in renal intercalated cells that may depend on the V-ATPase activity (21). Another study, in cardiomyocytes, suggested that ATP6ap2 is essential for V-ATPase assembly (22). The authors generated a mouse with a cardiac-specific deficiency in ATP6ap2. The ATP6ap2-disrupted cardiomyocytes showed extensive vacuolization, a phenotype that could be reproduced by pharmacological inhibition of intracellular acidification with bafilomycin A1. These studies demonstrated that ATP6ap2 might be an essential assembly chaperone of mammalian V-ATPase; and that genetic ablation of ATP6ap2 created a loss-of-function model for VATPase representing a function that is unique to mammalian cells. To establish the significance of ATP6ap2 and its relationship to PEDF anti-tumor properties, we determined the levels of expression of ATP6ap2 in cells stably expressing PEDF. We found greater levels of expression of ATP6ap2 in CRPC than androgen-sensitive prostate cancer cells.

To further evaluate the significance of PEDF in regulating V-ATPase, we evaluated the effect of PEDF on the mRNA levels in cells stably expressing and secreting PEDF. a1 and a3 isoforms mRNA levels were not affected. Importantly, PEDF significantly decreased the levels of a4 isoform and ATP6ap2 in metastatic CL1 cells, without affecting them in the non-metastatic LNCaP cells. In a previous work, we have demonstrated that the re-expression of PEDF inhibits the proliferation and migration of CL1 cells, and the formation of 3D tumor spheroids *in vitro* (37). In another study, we showed that the knockdown of a4 isoform decreased cell invasion in highly metastatic breast cancer cells MB231 (19). Our present study indicates that PEDF could decrease the invasive phenotype of human prostate cancer cells via the plasmalemmal V-ATPase.

To conclude, we showed that PEDF expression decreased the rate of J_{H+} in metastatic CL1 cells without affecting the cytosolic J_{H+} in non-metastatic LNCaP cells and the endosomes /lysosomes J_{H+} in either cells. We also showed that PEDF significantly decreases the levels of a4 isoform and ATP6ap2 in metastatic CL1 cells, without affecting the levels of a4 isoform in the non-metastatic LNCaP cells. Altogether, these data indicate that PEDF might be a novel regulator of the plas-

malemmal V-ATPase and highlights its significance in prostate cancer growth and progression.

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