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Amyloid precursor protein regulates migration and metalloproteinase gene expression in prostate cancer cells

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ABSTRACT

Amyloid precursor protein (APP) is a type I transmembrane protein, and one of its processed forms, β-amyloid, is considered to play a central role in the development of Alzheimer's disease. We previously showed that *APP* is a primary androgen-responsive gene in prostate cancer and that its increased expression is correlated with poor prognosis for patients with prostate cancer. APP has also been implicated in several human malignancies. Nevertheless, the mechanism underlying the pro-proliferative effects of APP on cancers is still not well-understood. In the present study, we explored a pathophysiological role for APP in prostate cancer cells using siRNA targeting APP (siAPP). The proliferation and migration of LNCaP and DU145 prostate cancer cells were significantly suppressed by siAPP. Differentially expressed genes in siAPP-treated cells compared to control siRNA-treated cells were identified by microarray analysis. Notably, several metalloproteinase genes, such as *ADAM10* and *ADAM17*, and epithelial–mesenchymal transition (EMT)-related genes, such as *VIM*, and *SNAI2*, were downregulated in siAPP-treated cells as compared to control cells. The expression of these genes was upregulated in LNCaP cells stably expressing APP when compared with control cells. APP-overexpressing LNCaP cells exhibited enhanced migration in comparison to control cells. These results suggest that APP may contribute to the proliferation and migration of prostate cancer cells by modulating the expression of metalloproteinase and EMT-related genes.

1. Introduction

Amyloid precursor protein (APP) is a type I transmembrane protein that is cleaved by secretases to produce various proteolytic products [1]. Among its products, β peptide, which is generated by sequential cleavage of APP by β- and γ-secretases, is implicated in the development of Alzheimer's disease. APP is also expressed in various non-neural tissues, and it may be involved in the growth of various cell types [2]. APP has been implicated in several human malignancies, including lung [3], colon [4], pancreatic [5], parathyroid [6], thyroid [7], prostate [8], and breast [9] cancers. In particular, we previously showed that APP is a primary androgen-responsive gene in prostate cancer and breast cancer, and that its high immunoreactivity is correlated with poor prognoses for patients with prostate cancer and for those with estrogen receptor-positive breast cancer [8,9]. Therefore, APP is assumed to be a promoting factor for malignant tumors. The soluble secreted form of APP (sAPP), from its N-terminal domain, is shown to exhibit a growth factor-like function [10], although the precise mechanism underlying the pro-proliferative effects of APP on tumor cells is not well understood.

In this study, we showed that APP plays a role in the proliferation and migration of prostate cancer cells. Using microarray analysis, we identified differentially expressed genes in prostate cancer cells treated with small interfering RNA (siRNA) specific to APP (siAPP) *versus* those treated with control siRNA. Among the identified genes, the expression of ADAM (a disintegrin and metalloproteinase) proteases and genes involved in epithelial–mesenchymal transition (EMT) were substantially altered by siAPP treatment. We also found that cell migration was enhanced in

Abbreviations: APP, amyloid precursor protein; EMT, epithelial–mesenchymal transition; siRNA, small interfering RNA; PVDF, polyvinylidene difluoride; DMEM, Dulbecco's modified eagle medium; ADAM, a disintegrin and metalloproteinase; ADAM10, ADAM metallopeptidase domain 10; ADAM17, ADAM metallopeptidase domain 17; CDH1, cadherin 1; VIM, vimentin; SNAI1, snail family zinc finger 1; SNAI2, snail family zinc finger 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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APP-overexpressing LNCaP prostate cancer cells. These results suggest that APP may regulate the proliferation and migration of prostate cancer cells by modulating metalloproteinase and EMT-related gene expression.

2. Materials and methods

2.1. Cell culture

LNCaP and DU145 prostate cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). LNCaP cells were cultured in RPMI and DU145 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), both supplemented with 10% fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 mg/ml). Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C. LNCaP cells stably expressing APP (APP #3 and #9) and a control vector (vector #1 and #2) were described previously [8].

2.2. Western blot analysis

The cells were lysed in cell lysis buffer (20 mM HEPES, pH 7.9, 300 mM NaCl, 1 mM EDTA, 15% glycerol, 0.5% Nonidet P-40, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride) and rocked gently for 50 min at 4 °C. The lysates were centrifuged at 14,000*g* for 5 min at 4 °C, and the supernatants were used as cell lysates. The cell lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and electrophoretically transferred onto Immobilon-P PVDF membranes (Millipore, Billerica, MA). Membranes were probed with the anti-APP (Cell Signaling Technology, Beverly, MA) or the anti-β-actin (Sigma–Aldrich, Tokyo, Japan) antibodies.

2.3. Small interfering RNA

A siRNA duplex targeting APP (siAPP) that was previously described as siAPP-B [8] was synthesized by RNAi, Co., Ltd. (Tokyo, Japan). Non-targeting siRNA (siControl) was used as a control [11].

2.4. Cell proliferation assay

Cell proliferation was examined using WST-8 (Nacalai Tesque, Kyoto, Japan) or by counting cells using Trypan blue staining. Cells were seeded in 96-well plates at a density of 5×10^3 cells/well for LNCaP cells or 1×10^3 cells/well for DU145 cells and then transfected with 1 or 10 nM siAPP or siControl using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) (day 0). At the indicated time points following transfection, 10 μl of a solution containing WST-8 was added to each well, and the cells were incubated for 2 h at 37 °C. The absorbance of the plates was read at 450 nm using a microplate reader. The results were shown as mean ± standard deviation (s.d.; $n = 4$). $^{*}P < 0.05$; $^{**}P < 0.01$ vs. siControl.

2.5. Migration assay

Cell migration was examined by using cell culture inserts with 8.0-μm pore size PET filters (Becton Dickinson Labware, Bedford, MA). Cells were seeded into the culture dish and transfected with 10 nM siAPP or siControl using Lipofectamine 2000. After transfection, 5×10^4 LNCaP cells or 3×10^4 DU145 cells were suspended in the culture medium and added to the upper chamber. For stable cell lines (APP #3 and #9, and vector #1 and #2), 5×10^4 cells were added to the upper chamber. Cells were then incubated for 20–36 h at 37 °C. The cells on the lower surface of the filter were fixed in methanol and stained with Giemsa's staining solution. After washing, the cells on the lower surface were counted in at least 5 fields under the microscope. The results were shown as mean ± s.d. ($n = 5$). $^{*}P < 0.05$; $^{**}P < 0.01$ vs. siControl.

2.6. Reverse transcription-polymerase chain reaction

Total RNA was extracted from the LNCaP or DU145 prostate cancer cells using ISOGEN reagent (Nippon Gene, Tokyo, Japan) and first-stand cDNA was generated with 1 μg of total RNA using Superscript III Reverse Transcriptase (Life Technologies) and oligo$(dT)_{20}$ primer. To measure the expression of *ADAM10*, *ADAM17*, *CDH1*, *VIM*, and *SNAI2* mRNA, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed by StepOnePlus Real-Time PCR system (Life Technologies) using specific primers for human *ADAM10* (sense 5′-CTGGCCAACCT ATTTGTGGAA-3′ and antisense 5′-GACCTTGACTTGGACTGCACTG-3′), *ADAM17* (sense 5′-GAAGGCCAGGAGGCGATTA-3′ and antisense 5′-CGGGCACTCACTGCTATTACC-3′), *CDH1* (sense 5′-GACGTTGCAC CAACCCTCA-3′ and antisense 5′-GAATCATAAGGCGGGGCTGT-3′), *Vim* (sense 5′-TGGATTCACTCCCTCTGGTTG-3′ and antisense 5′-CGT GATGCTGAGAAGTTTCGTT-3′), and *SNAI2* (sense 5′-CGGACCCACAC ATTACCTTG-3′ and antisense 5′-TGACCTGTCTGCAAATGCTCT-3′). Human *GAPDH* was used as an internal control [11]. The results were shown as mean ± s.d. Statistical analysis was performed using Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. siControl.

2.7. Microarray

Gene expression in the LNCaP and DU145 prostate cancer cells treated with siAPP was measured using the Affymetrix GeneChip (Santa Clara, CA) according to the manufacturer's protocol. The DAVID Functional Annotation Clustering Tool (http://david.abcc.ncifcrf.gov/summary.jsp) was used to perform a global analysis of gene expression and to sort differentially expressed genes into pathways and clusters of functionally related genes.

3. Results

3.1. APP knockdown reduces the proliferation and migration of LNCaP and DU145 prostate cancer cells

To investigate the role of APP in the pathophysiology of prostate cancer cells, we performed functional studies using siAPP in androgen-dependent LNCaP and androgen-independent DU145 prostate cancer cells. Consistent with our previous findings [8], siAPP significantly suppressed the proliferation of LNCaP cells (Fig. 1A). We found that siAPP also significantly repressed the proliferation of DU145 cells (Fig. 1E). We showed that siAPP significantly inhibited the migration of LNCaP and DU145 cells (Fig. 1B, C, F and G). Western blot analysis showed that both 1 and 10 nM of siAPP suppressed the expression of APP protein in LNCaP and DU145 cells (Fig. 1D and H). These results suggest that APP could contribute to the proliferation and migration of prostate cancer cells regardless of androgen sensitivity.

3.2. siAPP reduces the expression of metalloproteinase and EMT-related genes in both LNCaP and DU145 cells

Next, microarray analysis was performed to evaluate gene expression in LNCaP and DU145 cells in the presence or absence of siAPP transfection. We focused on differentially expressed genes in response to APP knockdown. We then found that metalloproteinase genes, such as *ADAM10* and *ADAM17*, were substantially downregulated in LNCaP and DU145 cells treated with siAPP (Table 1 and Supplementary Tables 1–4). qRT-PCR analysis

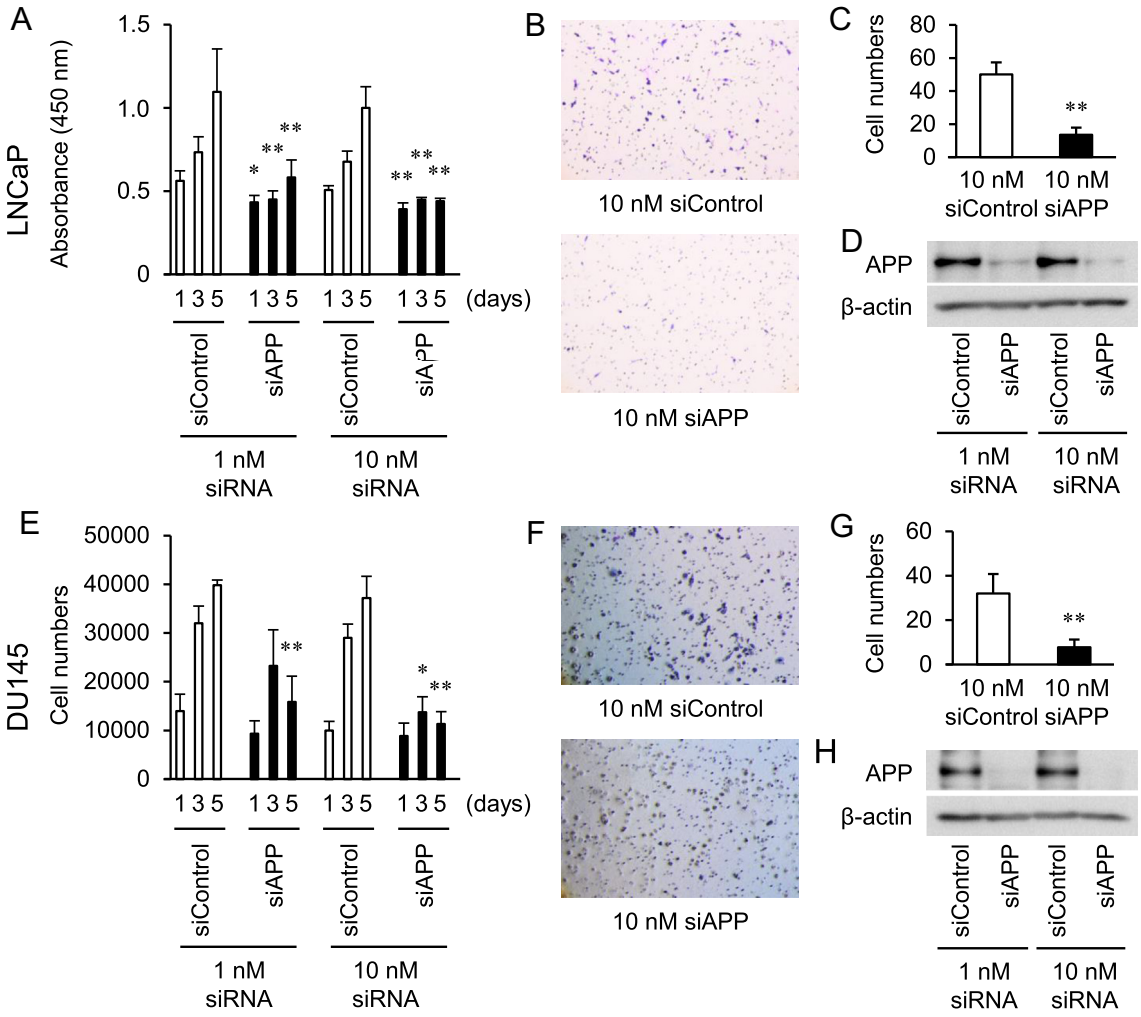

Fig. 1. Amyloid precursor protein (APP) knockdown decreases the proliferation and migration of prostate cancer cells. siAPP inhibited proliferation of LNCaP (A) and DU145 (E) prostate cancer cells. Cell proliferation was examined using WST-8 or by counting the number of cells. The absorbance of the plates was read on a microplate reader at a wavelength of 450 nm. The results are shown as mean ± s.d. (n = 4). siRNA inhibited the migration of LNCaP (B, C) and DU145 (F, G) prostate cancer cells. Cell migration was examined using cell culture inserts with 8.0-μm pore size PET filters. Cells were stained with a Giemsa's stain solution. After washing, the cells on the lower surface were counted in at least 5 fields under the microscope. The results are shown as mean ± s.d. (n = 5). siAPP decreased protein expression in LNCaP (D) and DU145 (H) prostate cancer cells. Statistical analysis was performed using Student's t-test. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. siControl.

Table 1
Pathway analysis in prostate cancer cells treated with siAPP.

Term	Count	P value	Genes
Upregulated pathway in LNCaP cells			
Hydrolase	8	1.2E−02	*SIAE, USP17L6P, USP17, NUDT14, EIF4A2, MANEA, FAHD2A, REXO1L1*
Downregulated pathway in LNCaP cells			
Notch signaling pathway	4	6.6E−04	*APP, ADAM10, ADAM17, RBPJ*
Cytoskeleton	14	2.4E−03	*ADAM10, MYO1B, MYO1B, MYO1D, PLEKHH1, EML5, FMN1, KLHL5, MYRIP, APP, PTP4A1, ADAM17, ARPM1*
Fetal hemoglobin quantitative trait locus 1	2	5.4E−03	*HBG1, HBG2*
Apical part of cell	5	5.6E−03	*APP, P2RY6, MUC20, ATP8B1, ADAM17*
Metal ion-binding site: Zinc; in inhibited form	3	8.4E−03	*ADAM10, ADAM17, MMP16*
Upregulated pathway in DU145 cells			
Zinc ion binding	3	3.2E−02	*ZNF618, RNF212*
Transition metal ion binding	3	4.6E−02	*ZNF618, RNF212*
Downregulated pathway in DU145 cells			
MAP kinase phosphatase activity	3	7.4E−04	*DUSP5, DUSP14, DUSP10*
Phosphorus metabolic process	10	1.2E−03	*DUSP5, GMFB, GMFB, APP, NCEH1, PLK2, DUSP14, DUSP10, CDK6, LPAR1, YES1*
Regulation of apoptosis	9	1.5E−03	*PPIF, APP, HTATIP2, ETS1, JUN, F3, ADAM17, TNFAIP3, BIRC3*
Anti-apoptosis	5	2.6E−03	*HTATIP2, F3, ADAM17, TNFAIP3, BIRC3*
DSPc	3	2.6E−03	*DUSP5, DUSP14, DUSP10*

confirmed that the expression of *ADAM10* (Fig. 2A and G) and *ADAM17* (Fig. 2B and H) were downregulated in LNCaP and DU145 cells with reduced expression of APP by siAPP transfection (Fig. 2F and L). It was also shown that an epithelial marker, *CDH1* (Fig. 2C) was upregulated, whereas a mesenchymal marker, *VIM* (Fig. 2D) and an EMT-related transcription factor, *SNAI2* (Fig. 2E) were downregulated in siAPP-transfected LNCaP cells. The expression of *VIM* and *SNAI2* was also reduced in DU145 cells transfected with siAPP (Fig. 2J and K). Taken together, this loss-of-function study of APP suggested that APP could modulate EMT, which could contribute to prostate cancer progression.

3.3. APP overexpression promotes migratory activity and increases the expression of metalloproteinase and EMT-related genes in LNCaP cells

To confirm the potential role of APP in the biology of prostate cancer cells as suggested by our findings, we performed cell migration assay using LNCaP cells stably expressing APP or a control vector [8]. In these APP-overexpressing cells, cell proliferation is promoted compared with control cells expressing the empty vector [8]. Here we showed that APP-overexpressing LNCaP cells exhibited enhanced migratory activity compared with control cells (Fig. 3A–C). qRT-PCR analysis confirmed that the expression of

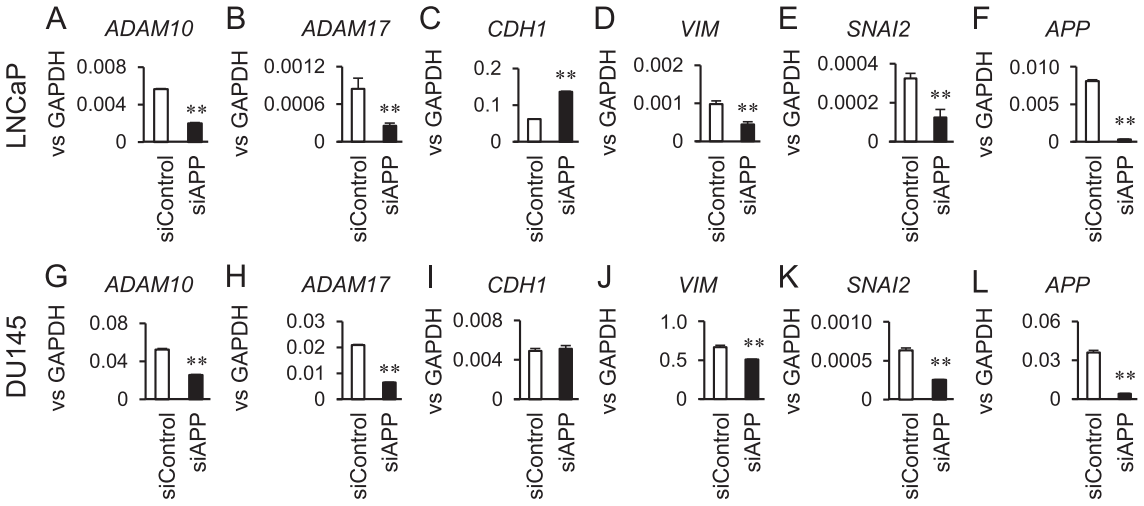

Fig. 2. Downregulation of metalloproteinase and EMT-related genes in prostate cancer cells treated with siAPP. qRT-PCR analyses of *ADAM10* (A, G) *ADAM17* (B, H), *CDH1* (C, I) *VIM* (D, J) *SNAI2* (E, K), and *APP* (F, L) expression were performed using RNAs prepared from LNCaP (A–F) and DU145 (G–L) cells treated with siAPP. The results are shown as mean ± s.d. ($n = 3$). Statistical analysis was performed using Student's *t*-test. $^{**}P < 0.01$.

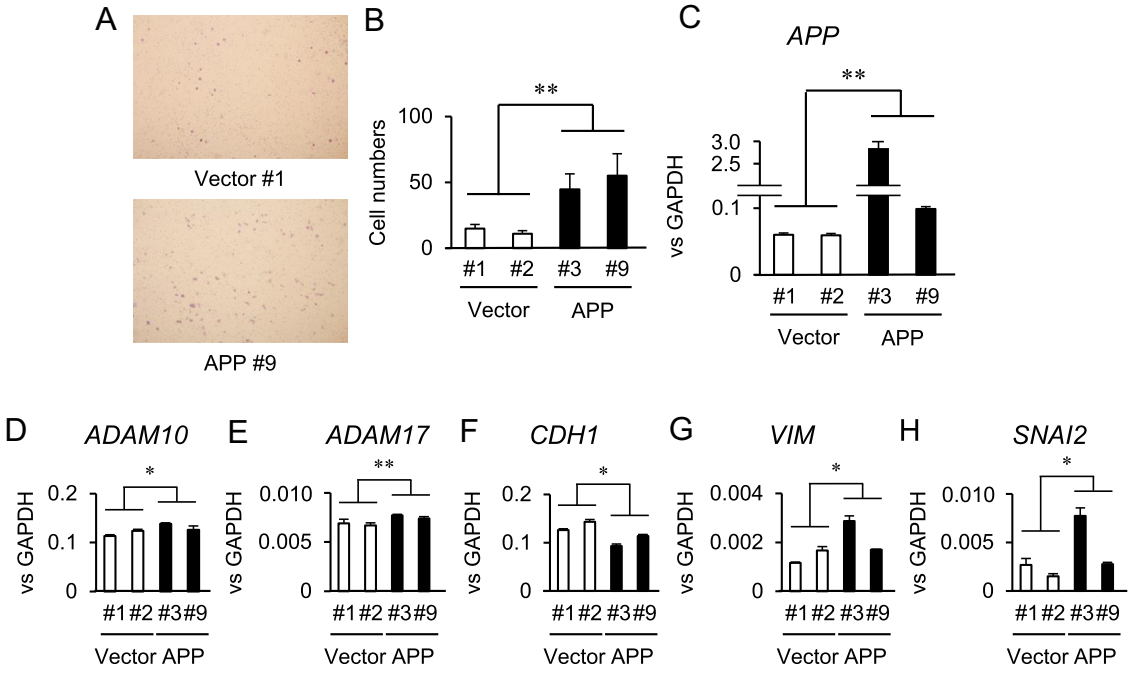

Fig. 3. Cell migration and the expression of metalloproteinase and EMT-related genes were increased in LNCaP cells stably expressing APP. APP-overexpressing LNCaP cells exhibited significantly increased cell migration as compared with control cells expressing an empty vector (A–C). Cell migration was examined using cell culture inserts with 8.0-μm pore size PET filters. Cells were stained with a Giemsa's stain solution. After washing, the cells on the lower surface were counted in at least 5 fields under the microscope. The results are shown as mean ± s.d. ($n = 5$). Metalloproteinase and EMT-related genes were upregulated in APP-overexpressing LNCaP cells. qRT-PCR analyses of *ADAM10* (D), *ADAM17* (E), *CDH1* (F), *VIM* (G) and *SNAI2* (H) were performed using RNA prepared from APP-overexpressing LNCaP cells. The results are shown as mean ± s.d. ($n = 3$). Statistical analysis was performed using the Mann–Whitney *U* test. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. siControl.

ADAM10, *ADAM17*, *VIM*, and *SNAI2* were upregulated (Fig. 3D, E, G and H), while *CDH1* expression was downregulated (Fig. 3F) in LNCaP cells stably expressing APP.

4. Discussion

In the present study, we demonstrated that APP modulates the proliferation and migration of prostate cancer cells. As determined by microarray analysis, the expression of ADAM metalloproteinases and EMT-related genes were significantly altered in both LNCaP and DU145 cells transfected with siAPP when compared to their control cells. We confirmed that APP overexpression in LNCaP cells resulted in increased migratory activity and also modulated the expression of ADAM metalloproteinases and EMT-related genes in a manner opposite that observed in siAPP-treated cells. These results suggest that APP promotes the proliferation and migration of prostate cancer cells by modulating the expression of metalloproteinase and EMT-related genes.

The ADAM family of proteins contains metalloproteinase domains [12], and some of the ADAMs function as proteinases that exert proteolytic, adhesive, and putative signaling activities [13]. ADAM metalloproteinases and matrix metalloproteinases (MMPs) control signaling events by promoting ectodomain shedding of cytokine precursors and receptors, a process that proteolytically cleaves the extracellular domain of transmembrane proteins [12]. Shed proteins are diverse in structure and function and comprise molecules such as interleukin (IL)-6 receptor, tumor necrosis factor (TNF) receptor, Fas ligand, transforming growth factor (TGF)-α, TNF-α, cadherin, and APP [14]. In addition to the ectodomain activity, the membrane-retained cleavage fragments, such as Notch [15], CD44 [16], and APP [17], can also exert signaling functions. Among ADAM proteins, ADAM10, ADAM17, and ADAM9 have been proposed to function as α-secretases for APP [18–23]. ADAM10 is overexpressed in several malignant cancers, such as ovarian [24], prostate [25], colon [26], and breast [27] cancers. ADAM17 expression is also upregulated in malignant tumors, such as prostate [28], breast [27], colon [29], ovarian [30], lung [31], and pancreatic [32] cancers. ADAM17 expression was correlated with invasive breast cancers, and anti-ADAM17 antibody could inhibit the proliferation of breast cancer cells [27]. High ADAM17 expression was also correlated with a shorter survival rate for patients with breast cancer, suggesting a pro-metastatic role for this protease [33].

Our loss- and gain-of-function studies of APP showed that *ADAM10* and *ADAM17* expression are well-correlated with APP expression, suggesting that APP could modulate the expression of its α-secretases in a feedback manner. The altered expression of ADAM metalloproteinases in prostate cancer cells could also modulate ectodomain shedding of other growth factors and cytokines, such as human epidermal growth factor receptor 3 (HER3), epidermal growth factor receptor (EGFR), and TNF-α, whose mechanisms have been reported in other cancers [29–32]. Indeed, a recent report showed that the interaction of Polo-like kinase 2 (*PLK2*) with *ADAM17* is critical for the activation of *ADAM17* via phosphorylation, which leads to ectodomain shedding of pro-TNF-α and TNF receptors [34]. In our microarray data, *PLK2* was one of the downregulated genes in DU145 cells transfected with siAPP when compared to control siRNA (Supplementary Table 4).

EMT is an essential process during embryogenesis, in which epithelium is converted into individual mesenchymal cells, so that cells lose cell–cell adhesion properties, and gain migratory and invasive properties to become mesenchymal cells [35–37]. EMT is also associated with pathological events such as cancer [38]. The downregulation of E-cadherin (*CDH1*) and upregulation of vimentin (*VIM*) expression are typical characteristics of cells undergoing EMT. Several transcription factors, such as SNAIL (*SNAI1*) and SLUG (*SNAI2*), are known to play crucial roles in EMT during embryonic development and pathological events [38]. It has also been shown that EMT plays a critical role in the initiation of metastasis for cancer progression. Transcription factors, such as SNAI1 and SNAI2, activate their target genes, leading to EMT in cancers [38]. SNAI2 functions like a metastasis regulator in tumors and its expression was associated with poor clinical outcome in several types of cancer [39]. We showed here that APP could positively contribute to EMT in both androgen-naïve and -independent prostate cancer cells. APP is originally an androgen-inducible gene in prostate cancer, and once the tumors achieve high expression of the protein, it could further promote the progression of prostate cancer regardless of androgen dependency.

In summary, with the present study, we defined a modulatory role for APP in the gene expression profiles of prostate cancer cells. It is suggested that APP could contribute to the proliferation and migration of cancer cells through the activation of metalloproteinases and the progression of EMT. Thus, our present findings indicate that APP could be a molecular target for the management of advanced prostate cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.010.

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