PDEF downregulates stathmin expression in prostate cancer

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Received December 14, 2011; Accepted January 30, 2012

DOI: 10.3892/ijo.2012.1392

Abstract. The Ets proteins are a family of transcription factors characterized by an evolutionarily conserved DNA binding domain that controls key cellular processes. Prostate-derived Ets transcription factor (PDEF), a member of the Ets family, is reported to be present in tissues with high epithelial content, notably breast and prostate. However, the role of PDEF in cancer development is not fully understood. To gain insight into the molecular mechanisms associated with prostate cancer progression, we employed iTRAQ labeling followed by mass spectrometric (MS) analysis to identify candidate proteins that are differentially expressed in prostate cancer cells with or without PDEF. To this end, we overexpressed PDEF in PC3 human prostate cells using a tetracycline inducible system (Tet-On). Many differentially expressed proteins which play important roles in various cellular and biological processes were identified. Among them, stathmin (STMN), which is a microtubule (MT)-destabilizing protein, was found to be downregulated in multiple analyses. We demonstrated that re-expression of STMN reversed the antitumor properties of PDEF in PDEF-overexpressing PC3 cells. Using *in vitro* functional assays, we showed that STMN overexpression counteracted PDEF's effects against cell proliferation, colony formation and tumor migration. Similar results were further confirmed with the prostate cancer cell line CWR22rv1. In conclusion, many differentially expressed proteins were identified and STMN was found to be downregulated by PDEF. These results suggest that PDEF may inhibit prostate cancer progression by transcriptional downregulation of oncogenic STMN expression. Analyzing the association among differentially expressed proteins may provide a basis to better understand the molecular mechanisms underlying the process of cancer progression and development and further aid in designing therapeutics in the future.

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Abbreviations: iTRAQ, isobaric tags of relative and absolute quantitation; STMN, stathmin; MT, microtubule; PDEF, prostate-derived Ets transcription factor; MS, mass spectrometry; Tet-On, tetracycline inducible system

Key words: prostate cancer, prostate-derived Ets transcription factor, iTRAQ labeling, mass spectrometry, stathmin

Introduction

Prostate cancer is the most common cancer diagnosed among men and the second leading cause of death in American men, behind only lung cancer. The American Cancer Society (ACS) estimates about 1 man in 36 will die of prostate cancer (1). Although screening for prostate cancer, based on prostate specific antigen (PSA) has revolutionized early detection and diagnosis of the disease, the challenge that clinicians face are to determine which patients progress to aggressive disease (2,3). In order to determine the possibility of disease progression and ability to manage patient outcomes, it is necessary to better understand the molecular processes underlying the disease development and progression. Gene regulation is an important process in maintaining the integrity of cells for their proper growth and survival. Improper regulation of genes can lead to various diseases like cancer. The Ets family of transcription factors has been long investigated for their role in genetic loss of cellular homeostasis which results in development of various cancers. So far, 25 human and 26 murine Ets family members have been reported (4). Ets family of proteins share a conserved winged helix-turn-helix DNA binding domain called the Ets domain which recognizes unique DNA sequences containing GGAA/T. Eleven Ets proteins have a pointed (PNT) domain, important in protein-protein association. Ets proteins control many important cellular processes, including proliferation, differentiation, angiogenesis and apoptosis (4-11). Prostate-derived Ets transcription factor (PDEF), a member of the Ets family has been widely investigated for its role in cancer development and progression. Originally identified from the prostate epithelium, PDEF controls the expression of PSA, a specific marker for prostate cancer (12). Immunohistochemical analysis has shown the presence of PDEF in all high-epithelial content tissues such as breast and

prostate (13,14). Early reports showed that PDEF was a tumor promoter (13,14). However, recent studies support its role as a tumor suppressor based on differing model systems and varying experimental conditions (14,15-18).

In order to gain insight into the function of PDEF, in the present study we used isobaric tags for relative and absolute quantitation (iTRAQ)-labeling coupled with mass spectrometry (MS) to identify differentially expressed proteins upon PDEF induction using a Tet-On system in prostate cancer cells (19). This study is the first to report the use of iTRAQ labeling MS to identify differentially expressed proteins upon PDEF induction and validate the results with *in vitro* functional assays (20,21). iTRAQ coupled with MS has significant advantages over other methods due to its capability of multiplexing samples in one experimental setup (20,22). Another positive aspect includes unbiased peptide labeling, since iTRAQ tags label lysine side groups and all free amino-terminal groups of the peptides present in the sample (23). Keeping this background in mind, we thought it would be worth exploring the PDEF proteome using iTRAQ labeling with MS and validate the results with *in vitro* assays to study the role of PDEF in prostate cancer progression. To date only one study has been done in MDA-MB-231 human breast cancer cells analyzing interacting protein complexes to overexpressed PDEF (24). Cho *et al* used adenoviral-mediated gene delivery system to overexpress PDEF and LC-MS/MS analysis to identify protein complexes. They identified 121 proteins and their findings suggested PDEF may be regulated by ERBB2 or EGFR-activated signaling pathways in breast cancer (24). No proteomics research work has been done coupled with *in vitro* functional assays with PDEF in prostate cells. Our research is the first to utilize iTRAQ labeling to identify differentially expressed proteins upon PDEF induction in prostate cancer cells and verify the results with *in vitro* assays to study the role of PDEF. We identified 115 proteins, of which 35 were upregulated and 80 were downregulated in the two sets of prostate cancer cells. Stathmin (STMN), a microtubule (MT)-destabilizing protein, was found downregulated mulitple times in the presence of PDEF in the analyzed sample sets and therefore took our attention. Further, the *in vitro* functional assays such as proliferation, migration and colony formation used to verify the results confirmed correlation of PDEF with the downregulation of STMN in PDEF overexpressing prostate cancer cells (PC3 and CWR22rv1).

Materials and methods

Chemicals and reagents. The following primary antibodies were used: hPDEF rabbit polyclonal antibody (PDEF N-terminal construct obtained from the Laboratory of Dennis K. Watson from the Medical University of South Carolina antibody made by Proteintech, IL), STMN antibody (EMD Chemicals, Inc.) and anti-actin antibody (Santa Cruz Biotechnology, Inc.); secondary antibodies used for western blots were: horseradish peroxidase-conjugated anti-goat (Roche Applied Science), anti-mouse (Bio-Rad) and anti-rabbit Santa Cruz Biotechnology, Inc.); and G418 Sulfate/Neomycin (Invitrogen, Inc.) and Puromycin (Sigma, Inc.) were used for selection of stable clones.

Cell culture. The human prostate cancer cell lines PC3 and CWR22rv1 and stable transfectants (ATCC) were maintained in RPMI-1640 (Invitrogen, Inc.) supplemented with 5% fetal bovine serum (FBS; Invitrogen, Inc.) and antibiotic liquid (penicillin and streptomycin; Invitrogen, Inc.) at 37˚C under 5% CO_2.

A Tet-On prostate cancer cell line was used (19) and PDEF expression was routinely induced by using doxycycline (DOX) (RPI, Inc.) at a concentration of 1 μg for 24 h in PC3 cells. This system can induce activation of target gene (i.e., PDEF in our study) in a stringent, reversible, specific manner to study underlying biological and pathological processes (25).

RT-PCR analysis. Total cellular RNA was isolated from cell lines using the TRIzol Reagent (Invitrogen, Inc.) according to the manufacturer's instructions. A two-step RT-PCR was used to analyze mRNA expression of STMN and GAPDH genes. A cDNA was created using oligo(dT) primer and the Moloney murine leukemia virus reverse transcriptase enzyme according to manufacturer's instructions (Invitrogen, Inc.). Standard PCR techniques were then conducted with gene-specific primers. The primer set used for PDEF amplification was as follows: forward primer 5'-GGT GAA AGA ACT GGA GAA GCG-3' and reverse primer 5'-GTG CTT ATC CTT CTC TCG C-3'.

Transfection. PDEF cDNA coding sequence was amplified by RT-PCR and cloned into a eukaryotic expression vector pcDNA3.1. PC3 and CWR22rv1 cells were transfected with this recombinant plasmid pcDNA3.1 hPDEF or control vector alone by Lipofectamine 2000 (Invitrogen, Inc.). The stable transfectants were selected with G418 medium. PDEF protein expression was detected with western blot analysis in the transfected cell lines. STMN cDNA bacterial stock was obtained from Thermo Scientific, Inc. and the STMN cDNA was cloned into pEF puromycin expression vector. PC3 pcDNA3.1 hPDEF and CWR22rv1 pcDNA3.1 hPDEF cells were transfected with pEF-STMN vector alone by Lipofectamine 2000. The stable transfectants were selected with puromycin medium. From now on the respective stable transfectants will be referred to as follows: PC3/CWR22rv1 pcDNA3.1 control, PC3/CWR22rv1 control; PC3/CWR22rv1 pcDNA3.1 hPDEF, PC3/CWR22rv1 hPDEF; PC3/CWR22rv1 pcDNA3.1 hPDEF+pEF STMN, PC3/CWR22rv1 hPDEF+STMN.

iTRAQ protein sample preparation. Protein extraction was performed according to Applied Biosystems (now AB-SCIEX) protocol available from their website (http://www.absciex.com/). Briefly, adherent cells were detached using trypsin then washed twice with phosphate-buffered saline (PBS). Pelleted cells were homogenized in radioimmunoprecipitation assay buffer (RIPA) buffer containing a cocktail of HALT protease inhibitors (Thermo Scientific, Inc.). The cells were vortexed and kept on ice for 30 min and then centrifuged at 10000 x g for 20 min at 40˚C. The protein concentration of the supernatants was determined by the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Inc.) and stored in aliquots at -20˚C until use.

Isobaric labeling. One hundred micrograms of protein from the [PC3 tetracycline (Tet-On) control-uninduced and PC3 Tet-On PDEF-induced] cells were subjected to trichloroacetic acid (TCA) precipitation followed by several washes in acetone

to remove Tris and detergents. The pellet was reconstituted by resuspending into 20 μl of 8 M urea/500 mM triethylammonium bicarbonate (TEAB). Subsequently, the resuspended proteins were reduced, alkylated with iodoacetamide and digested with trypsin (Promega, Madison, WI) according to manufacturer's protocol (AB SCIEX, Framingham, MA, USA). Samples were iTRAQ labeled as follows: PC3 Tet-On control-uninduced was labeled with the 114 reagent and PC3 Tet-On PDEF-induced was labeled with the 117 reagent, samples were pooled and a preparative desalting was done using an mRP C18 reverse phase column (Agilent Technologies). iTRAQ labeling experiment was repeated for reproducibility. Peptide fractions were collected (~15 ml each) and concentrated in a vacuum concentrator to ~0.3 ml prior to fractionation.

OFFGEL fractionation. Peptides were separated using an Agilent 3100 OFFGEL Fractionator (Agilent, G3100AA). The OFFGEL isoelectric fractionation was run with commercially available IPG Dry strips pI 3-10 (GE Healthcare) in dilute glycerol only (no amphophiles added) with the standard 12-well peptide fractionation program until 20 kVh was accumulated (~24 h), then each of the 12 fractions was either injected directly onto the LTQ (Thermo Fisher) linear ion trap mass spectrometer or fractions were desalted using C18 Porous R2 (Applied Biosystems) prior to loading onto the LTQ (26).

Mass spectrometric analysis and data processing. Individual OFFGEL fractions were infused separately onto a Thermo Electron Finnigan linear ion trap mass spectrometer (LTQ) operated in positive ion mode via a Dionex U-3000 Ultimate nano LC system running. Peptides were separated on a C18 column (Agilent Zorbax 300SB-C18, 3.5 μm, 75 μm x 150 mm) at a flow rate of 250 nl/min eluted with a linear gradient of 10-60% solution B (95% ACN, 0.1% formic acid) over 80 min. A survey full scan (m/z = 400-2000) and the five most intense ions were selected for a zoom scan to determine the charge state, after which MS/MS was triggered in Pulsed-Q Dissociation mode (PQD) with minimum signal required (1000), isolation width 2.0, normalized collision energy 31.0, activation Q 0.600 and activation time of 0.400. PQD mode is critical in order to visualize the low m/z reporter ions from the iTRAQ reagents used for relative quantitation. Mass spectrometry data were acquired with Xcalibur software version 2.0 SR2. Each resulting RAW file was extracted using Readw.exe (version 4.0.2, Institute for Systems Biology) to produce mzXML files and with DTASuperCharge (University of Southern Denmark) to give MGF files. Individual MGF files were merged using a text editor for searching. Protein identification was performed using a Sorcerer search appliance (SageN) running the SEQUEST algorithm using the IPI Human database v3.50 using iTRAQ (K and N-terminal) as fixed modifications and oxidation of methionine and carbamidomethylation of cysteine as variable modifications using a peptide tolerance of 2.0 Da and fragment tolerance of 0.6 Da. These results were visualized using Scaffold 2.1 (Proteome Software). Proteins were also identified by searching the merged MGF file against Mascot 2.2 search engine (MatrixScience) using the IPI Human database v3.50, using iTRAQ 4-plex quantitation (quantitation methods were modified for carbamidomethylation instead of methylthio). The resulting .DAT file was extracted to generate the .CSV result file for further analysis.

The Mascot search engine returns the relative ratio to 114, so 117/114 <1 corresponds to downregulation and 117/114 >1 corresponds to upregulation. The searches were also run using a decoy database to give us an estimate of false positive rate against the IPI human database. The 114 and 117 ratios were compensated during the analysis.

MTT in vitro cell proliferation assay. Cells were seeded at 1000 cells/well in 96-well dishes and allowed to grow at 37˚C with 5% CO_2. At each time point MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] reagent (5 mg/ml) was added in a volume of 10 μl per well and incubated at 37˚C with 5% CO_2 for 3 h. The media were aspirated and 100 μl of DMSO was added and mixed until a uniform purple color formed. The cell samples were measured using a plate reader at 570 nm. Assays were performed in triplicates.

Soft agar colony formation assay. As described previously (16), 6-well dishes were plated with bottom agar (1.3 ml 1.8% agarose and 0.3 ml of 2X DMEM) for 20-30 min. Cells were mixed with the top agar (0.3 ml 1.8% agarose and 0.7 ml DMEM) at a concentration of $5x10^4$ cells per ml and plated in 6-well dishes and allowed to solidify. The cells were grown at 37˚C for 2 weeks, cells stained for 1 h with 0.05% crystal violet and the number of purple colored colonies were tabulated. Three independent experiments (in duplicates) were averaged for graphical representation.

Wound healing migration assay. Cells were seeded in 6-well plates in duplicates so that the cells were 90% confluent the next day. Cells were seeded in RPMI-1640 medium containing 5% FBS overnight. The next day, monolayer of cells was washed with PBS and a wound was created with a pipette tip. Cells were washed again with PBS to remove any leftover cells after the wound was created. The wound was allowed to close in serum-free medium for 24 h. Images were taken at a magnification of x100 and at time points of 0 and 24 h at the same position the wound was created. Cells which migrated towards the wound for closure were counted in duplicate wells and averaged: results represented as percent migrated cells.

Results

Selection criteria of peptides for quantitation of iTRAQ analysis. In order to obtain reliable analytical measurements, the experiments were repeated more than once. Based on the selection criteria pre-set, all peptides were used for quantitation with: i) a significance threshold $p<0.05$, ii) require bold red, as per Mascot's search criteria (http://www.matrixscience.com) and iii) minimum 2 peptides/protein. A total of 115 proteins were identified and quantified (≥95% confidence; ≥2 peptides/protein), 35 proteins were upregulated (>1.0) and 80 downregulated (<1.0) in uninduced cells relative to induced PC3 Tet-On cells.

Identification of differentially expressed proteins upon PDEF induction. To identify proteins differentially expressed upon PDEF induction, PC3 Tet-On system was used as described previously (25). The western blot analysis of the induced sample (Fig. 1A) showed a 3-fold higher expression of PDEF

Table I. Differentially expressed proteins upon prostate-derived Ets transcription factor induction.

iTRAQ ratio	Protein name
Upregulated proteins	
1.229	HNRNPU heterogeneous nuclear ribonucleoprotein U isoform a
1.049	TUBA1A tubulin α-1A chain
1.049	TUBA1C α-1C chain
1.049	TUBA1C 29 kDa protein
1.626	TUBB3 tubulin, β, 4
1.626	cDNA FLJ56903, highly similar to tubulin β-7 chain
1.307	TUBB tubulin β chain
1.307	TUBB2A tubulin β-2A chain
1.050	PHB prohibitin
1.395	CTTN protein
1.395	CTTN Src substrate cortactin
1.395	CTTN putative uncharacterized protein CTTN
1.141	CAST calpastatin isoform e
1.141	CAST calpastatin isoform a
1.088	TAGLN2 24 kDa protein
1.344	21 kDa protein
1.344	CDV3 5 kDa protein
1.344	CDV3 isoform 2 of protein CDV3 homolog
1.071	FAU 40S ribosomal protein S30
1.071	FAU ubiquitin-like protein fubi and ribosomal protein S30 precursor
1.599	ATP5A1 ATP synthase subunit α, mitochondrial
1.309	AK2 isoform 1 of adenylate kinase isoenzyme 2, mitochondrial
1.750	LDHA isoform 2 of L-lactate dehydrogenase A chain
1.150	RBM3 putative RNA-binding protein 3
1.150	RBM3 putative uncharacterized protein RBM3
1.045	MRCL3 myosin regulatory light chain MRCL3 variant
1.045	MRLC2 myosin regulatory light chain MRLC2
1.336	CSRP1 cysteine and glycine-rich protein 1
1.198	SCAMP3 isoform 1 of secretory carrier-associated membrane protein 3
1.148	RPS21 40S ribosomal protein S21
1.046	PPP1R12A isoform 1 of protein phosphatase 1 regulatory subunit 12A
1.082	LOC653665 similar to mCG4465, partial
1.082	LOC641293 similar to 60S ribosomal protein L21
1.082	LOC653156 similar to hCG1782414 isoform 2
1.078	EIF4A1 eukaryotic initiation factor 4A-I
Downregulated proteins	
0.667	ALB isoform 1 of serum albumin
0.812	HSPD1 60 kDa heat shock protein, mitochondrial
0.764	PPIA peptidyl-prolyl cis-trans isomerase A
0.829	STMN1 stathmin
0.829	STMN1 stathmin 1 variant
0.916	NPM1 isoform 1 of nucleophosmin
0.970	cDNA FLJ52243, highly similar to heat-shock protein β-1
0.574	TUBB2C tubulin β-2C chain
0.569	cDNA FLJ11352 fis, clone HEMBA1000020, highly similar to tubulin β-2C
0.835	NCL nucleolin
0.666	ACTA1 actin α 1 skeletal muscle protein
0.666	ACTA2 actin, aortic smooth muscle
0.666	ACTB cDNA FLJ52842, highly similar to actin, cytoplasmic 1

Table I. Continued.

iTRAQ ratio	Protein name
0.666	ACTB 11 kDa protein
0.782	PRSS1 trypsin-1
0.670	ACTA2 ACTA2 protein (fragment)
0.670	A26C1B ANKRD26-like family C members 1B
0.670	LOC653269 similar to protein expressed in prostate, ovary, testis and placenta
0.670	A26C1A isoform 1 of ANKRD26-like family C member 1A
0.568	TUBB4Q putative tubulin β-4q chain
0.568	TUBB4Q tubulin, β polypeptide 4, member Q
0.568	RP11-631M21.2 tubulin β-8 chain
0.630	TUBB4 tubulin β-4 chain
0.774	Mitochondrial heat shock 60 kDa protein 1 variant 1
0.813	AHNAK neuroblast differentiation-associated protein AHNAK
0.935	TPM4 isoform 1 of tropomyosin α-4 chain
0.935	TPM4 cDNA FLJ52936, weakly similar to tropomyosin α-4 chain
0.846	TPM3 isoform 2 of tropomyosin α-3 chain
0.912	HNRNPA1 isoform A1-B of heterogeneous nuclear ribonucleoprotein A1
0.912	LOC645691 similar to heterogeneous nuclear ribonucleoprotein A1
0.912	LOC645691 similar to heterogeneous nuclear ribonucleoprotein A1
0.912	HNRNPA1 isoform 2 of heterogeneous nuclear ribonucleoprotein A1
0.912	cDNA FLJ51587, highly similar to heterogeneous nuclear ribonucleoprotein
0.960	MIF; LOC284889 macrophage migration inhibitory factor
0.857	TPM3 tropomyosin 3
0.964	RPS10 40S ribosomal protein S10
0.964	LOC388885 similar to ribosomal protein S10
0.871	PDIA3 protein disulfide-isomerase A3
0.821	HNRNPM isoform 2 of heterogeneous nuclear ribonucleoprotein M
0.985	TPM2 isoform 2 of tropomyosin β chain
0.985	TPM2 isoform 3 of tropomyosin β chain
0.985	TPM1 tropomyosin 1 α variant 6
0.985	TPM1 tropomyosin 1 α chain isoform 7
0.985	TPM3 tropomyosin 3 isoform 1
0.985	TPM1 isoform 4 of tropomyosin α-1 chain
0.985	TPM1 tropomyosin isoform
0.983	KHSRP ιsoform 2 of Far upstream element-binding protein 2
0.983	cDNA FLJ51330, highly similar to Far upstream element-binding protein 2
0.900	RPL23A protein
0.765	LOC654188 similar to peptidylprolyl isomerase A-like
0.765	PPIAP19 similar to peptidylprolyl isomerase A-like
0.900	LOC130773 similar to ribosomal protein L23a
0.744	EIF4B cDNA FLJ59405, highly similar to eukaryotic translation initiation
0.744	EIF4B cDNA FLJ54492, highly similar to eukaryotic translation initiation
0.744	cDNA FLJ59206, highly similar to eukaryotic translation initiation factor
0.903	YAP1 65 kDa Yes-associated protein
0.903	YAP1 Yes-associated protein 1, 65 kDa isoform 1
0.850	cDNA FLJ56081, highly similar to lamin-A/C
0.850	LMNA progerin
0.850	LMNA isoform A of lamin-A/C
0.914	AHNAK putative uncharacterized protein AHNAK
0.629	HIST1H1C histone H1.2
0.629	HIST1H1D histone H1.3

Table I. Continued.

iTRAQ ratio	Protein name
0.629	HIST1H1E histone H1.4
0.852	TAGLN2 21 kDa protein
0.738	C1QBP complement component 1 Q subcomponent-binding protein, mitochondrial
0.889	SF3B2 splicing factor 3B subunit 2
0.975	HSPA9 stress-70 protein, mitochondrial
0.911	SF3B2 putative uncharacterized protein DKFZp781L0540 (fragment)
0.269	HIST2H2BE histone H2B type 2-E
0.269	HIST2H3PS2 histone H2B
0.862	HSPA5 HSPA5 protein
0.844	MKI67 isoform long of antigen KI-67
0.850	PRKCSH cDNA FLJ59211, highly similar to glucosidase 2 subunit β
0.456	TPM3 tropomyosin 3
0.456	LOC147804 tropomyosin 3 pseudogene
0.783	STMN1 stathmin 1/oncoprotein 18
0.565	ACTG1 actin, cytoplasmic 2
0.948	NCL cDNA FLJ10452 fis, clone NT2RP1000966, highly similar to nucleolin
0.432	PRDX1 peroxiredoxin-1

Ratios are relative to 114 (uniduced) and reagent 117 (induced) >1.0 upregulated and <1.0 downregulated upon PDEF induction.

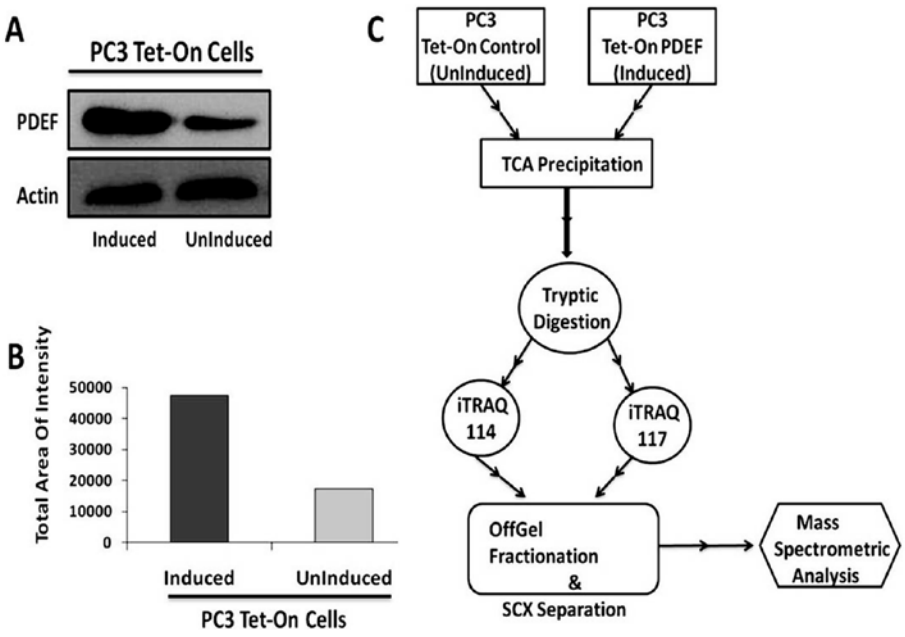

Figure 1. Western blot analysis of PDEF overexpression in PC3 cells using a tetracycline-inducible system (Tet-On). (A) PC3 Tet-On cells were induced with 1 μg/ml doxycycline (DOX) for 24 h. Blot was probed with polyclonal antibody specific to PDEF and actin (as loading control). (B) ~2.7-fold increase in area of intensity was observed upon quantitation of induced and uninduced PC3 Tet-On cells. (C) Overview of the iTRAQ labeling process from sample preparation to fractionation and protein identification.

in comparison to uninduced sample control (Fig. 1B). After confirming the protein expression, uninduced and induced cells were iTRAQ labeled separately as represented schematically in Fig. 1C. We identified 115 proteins from our analysis of the iTRAQ labeling experiment (Table I). All 115 proteins fell into the preset selection criteria set by us and only these proteins were used for any further analysis. Of the 115 proteins identified as differentially expressed, 80 proteins were downregulated and 35 proteins were upregulated upon PDEF induction (Table I). As mascot search returns its ratios relative to 114, therefore all those proteins >1.0 were found upregulated and all the proteins <1.0 were found downregulated upon PDEF induction.

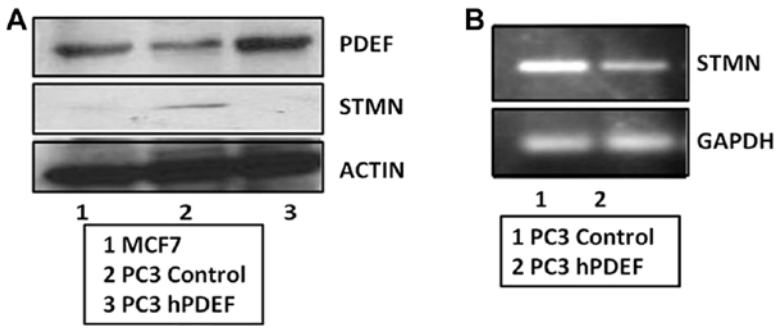

Figure 2. Validation of expression of microtubule (MT)-destabilizing protein STMN upon PDEF induction. (A) Western blot analysis of PC3 pcDNA3.1 hPDEF cells for PDEF and STMN protein expression levels with specific antibodies. MCF7 cell lysate was used as positive control. Actin was used as loading control. (B) RT-PCR analysis of PC3 pcDNA3.1 hPDEF cells for mRNA expression analysis with specific STMN primers. GAPDH was used as the loading control.

Criteria for selection of target protein for further analysis. Our study was based on 3 basic criteria for the selection of target protein for further study in relation to PDEF in the process of prostate cancer progression: i) proteins with the iTRAQ ratio (>1.0 or <1.0), ii) number of times the protein showed up in the analyzed sample sets (>2 times) and iii) role in the prostate cancer process (based on the literature).

Based on the above criteria, we first analyzed the differentially expressed proteins with the iTRAQ ratios >1.0 and <1.0. 80 proteins were downregulated with a ratio below 1.0 and 35 were upregulated with a ratio above 1.0 (Table I). Most of the proteins were found downregulated (<1.0) upon PDEF induction in PC3 prostate cancer cells.

Based on our second selection criteria, 11 out of the 115 proteins showed differential expression three times or more (>3 times) in the analyzed sample sets (Table I). The proteins were heterogeneous nuclear ribonucleoprotein (HNRNPU), tubulin, heat shock proteins (HSP), peptidyl propyl isomerase, stathmin, actin, tropomyosin (TPM), cortactin (CTTN), ribosomal protein, eukaryotic translation initiation factor (EIF) and histones. In order to further narrow down our search, we decided to exclude those proteins which were associated to any isoforms or were highly similar to the identified proteins. Also proteins that were identified as chains (α or β), types (type 2E and B), subunits such as S21, S10 or proteins that came as forms of the identified protein (such as mitochondrial and cytoplasmic) were all excluded from our study. Based on our exclusion criteria we found only one protein STMN. STMN was found differentially expressed multiple times and was taken into consideration for further analysis.

STMN was found extensively studied in relation to the cancer progression process (27,28) and in many studies directly linked to prostate cancer (29,30). STMN in our study was found downregulated multiple times (0.829, 0.829 and 0.783) as seen in Table I. Keeping all these considerations in mind, we focused our research primarily on the association of STMN and PDEF in the process of prostate cancer progression.

Stathmin, a differentially expressed protein. Stathmin (STMN) has been extensively studied in cancer progression, specifically in relation to prostate cancer (29,30). STMN, also known as p17, p18, p19, 19k, metablastin, oncoprotein 18, LAP18, Op18, is a 19-kDa cytosolic protein. It was the first discovered member of family of MT-destabilizing phosphoproteins

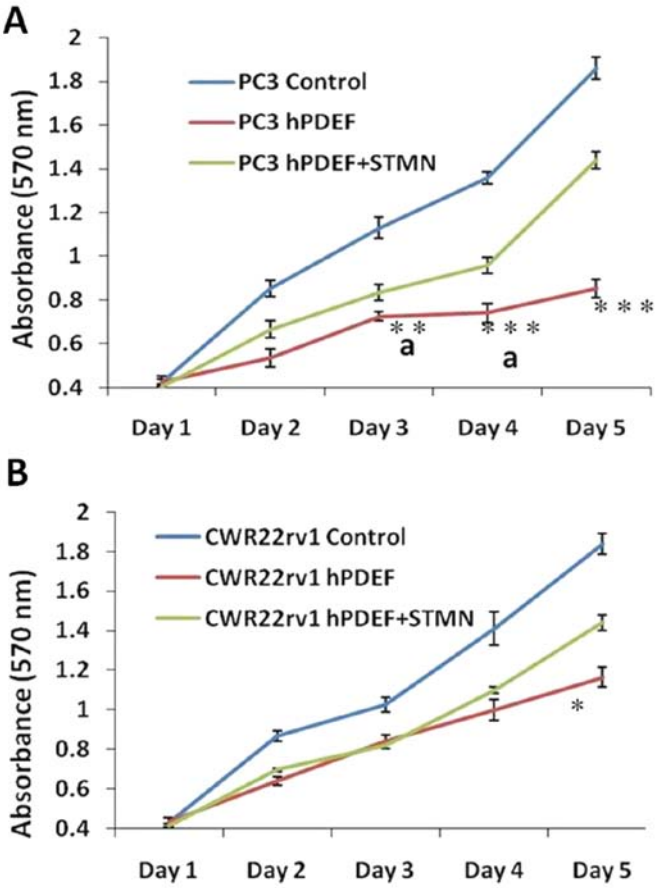

Figure 3. Expression of STMN reverses *in vitro* cell growth of PC3 and CWR22rv1 hPDEF cells. *In vitro* cell growth of various PC3 cell lines (A) and CWR22rv1 cell lines (B) a, PC3/CWR22rv1 hPDEF vs. PC3/CWR22rv1 hPDEF+STMN on respective days. $^{**}p<0.01$; $^{***}p<0.001$.

(31,32). This protein was critically involved in the assembly and disassembly of the mitotic spindle involved in the progression through the cell cycle (29,33). Western blot analysis (Fig. 2A) and mRNA expression level (Fig. 2B) of PC3 control and hPDEF cell lysates confirmed the downregulation of STMN in PC3 hPDEF cells.

Relevance of STMN to the prostate cancer progression process. To further evaluate the relevance of STMN and PDEF associ-

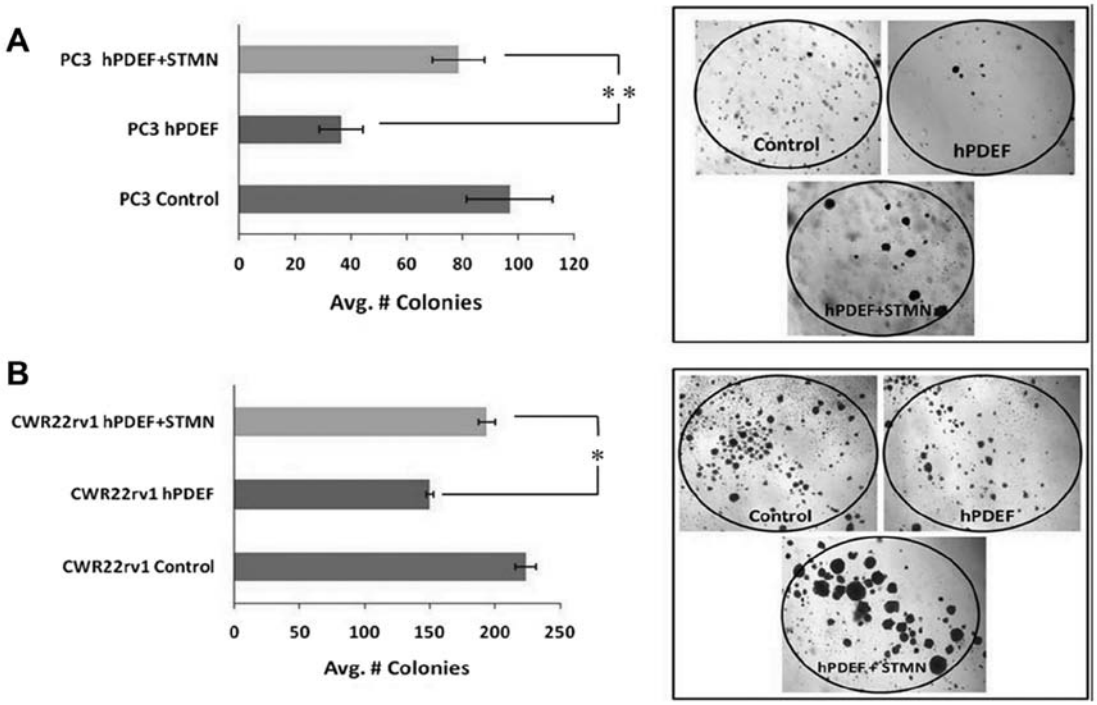

Figure 4. STMN re-expression increases *in vitro* three-dimensional soft agar colony formation in PC3 and CWR22rv1 cell lines. *In vitro* anchorage independent growth assay of various PC3 cell lines (A) and various CWR22rv1 cell lines (B). It is worth noting that the hPDEF+STMN colonies are larger in comparison to others in both PC3 and CWR22rv1 cells. PC3/CWR22rv1 hPDEF vs. PC3/CWR22rv1 hPDEF+STMN $^{**}p<0.01$, $^{*}p<0.05$.

ation during the process of prostate cancer progression. The proliferative, migratory and colony forming potential of prostate cancer cells were evaluated in PC3 cells as well as CWR22rv1, another prostate cell line. Both PC3 and CWR22rv1 stable transfectants showed a significant change in cells overexpressing hPDEF+STMN in comparison to hPDEF cells alone. PC3 and CWR22rv1 hPDEF cells display a tumor suppressing effect, which is reversed in the presence of STMN. Our previous *in vitro* and *in vivo* data prove that PDEF is a tumor suppressor in breast cancer cells. We showed that PDEF-expressing cells block the cell growth rate, and this retardation was reversed when PDEF expression was silenced with PDEF-specific small interfering RNA (16). Upon STMN expression PDEF overexpressing cells show increased growth potential in an MTT proliferation assay in both PC3 (Fig. 3A) and CWR22rv1 (Fig. 3B) prostate cells. The increase in growth rate was ~2 times more than that of PC3 hPDEF cells, but was lower than the PC3 prostate cancer cells. CWR22rv1 prostate cancer cells also showed similar reversal effect in CWR22rv1 hPDEF+STMN cells but was not as significant as PC3 prostate cancer cells. This difference in both the prostate cancer cell lines could be attributed to the aggressiveness of the cell lines. Soft agar colony formation potential of hPDEF cells in both prostate cancer cell types was significantly increased (PC3, $p<0.015$; CWR22rv1, $p<0.036$) in the presence of STMN in hPDEF cells (Fig. 4). The colonies formed were distinctly larger in the presence of STMN in comparison to all other cell types. This result clearly demonstrates the anchorage-independent growth potential. In the presence of STMN, the colonies formed faster and were significantly larger in both the PC3 and CWR22rv1 hPDEF+STMN cells than in the PC3/CWR22rv1 hPDEF cells alone. Clearly showing the importance of the association of STMN and PDEF in the cancer progression process, as anchorage-independence is one of the key characteristics of cancer cells. Anchorage-independence helps cancer cells to grow and distribute themselves throughout the body. The migratory potential was significantly increased in the presence of STMN in PC3 ($p<0.0055$) and CWR222rv1 ($p<0.023$) cells (Fig.5). The migratory potential of PC3 hPDEF+STMN cells increased by ~50% in comparison to PC3 hPDEF cells. In CWR22rv1 hPDEF+STMN cells the migratory potential was increased by ~30-35%. STMN in the presence of hPDEF showed a significant change in the key steps of the cancer progression process such as proliferation, colony formation and migration. The above results from the *in vitro* functional assays clearly demonstrate the change due to the introduction of STMN in hPDEF in PC3/CWR22rv1 cells. It also shows the importance of the association of STMN and PDEF in the cancer progression process. The results from the above described *in vitro* functional assays have been summarized graphically in Fig. 6. The data clearly point to the importance of PDEF and STMN association in the process of prostate cancer progression.

Discussion

The Ets family of proteins consists of a large number of evolutionarily conserved transcription factors. Many of Ets factors have been implicated in key steps of the tumor progression process (6,11). Extensive studies have been done with Ets family proteins focusing on their biochemical properties and cellular functions (4,34). Following the discovery of PDEF in

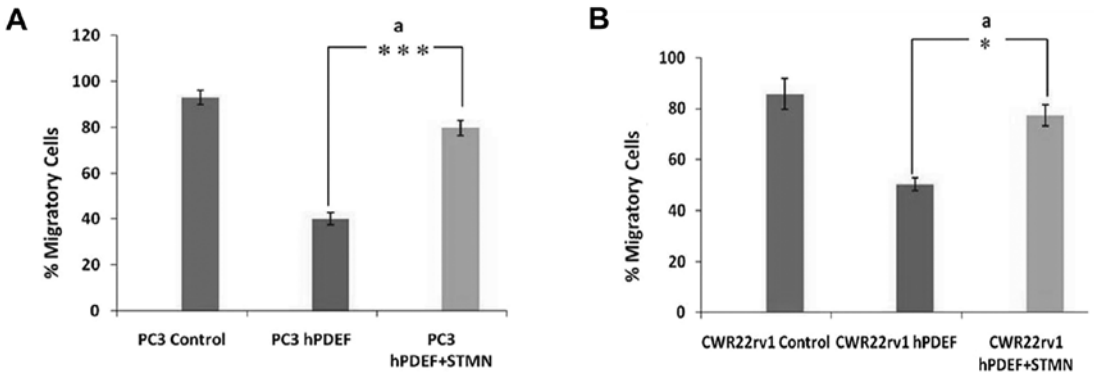

Figure 5. STMN expression increased the migratory potential of PC3 and CWR22rv1 hPDEF cell lines. *In vitro* wound healing assay, analyzing the migratory potential of various PC3 cell lines (A) and CWR22rv1 cell lines (B). STMN re-expression significantly increases *in vitro* migration. a, PC3/CWR22rv1 hPDEF vs. PC3/CWR22rv1 hPDEF+STMN $^{***}p<0.001$, $^{*}p<0.05$.

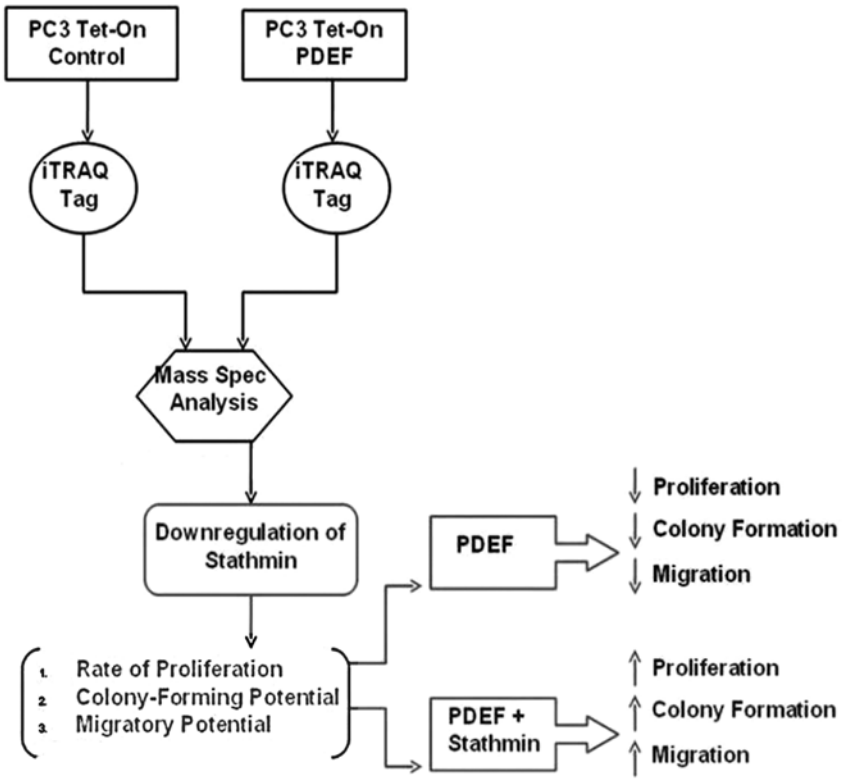

Figure 6. Graphical abstract: overview of the role of stathmin in PDEF overexpressing prostate cells.

year 2000 (35), much work has been done to understand the role of PDEF in the process of normal growth and development and in cancer progression. PDEF, a key Ets transcription factor has been studied by various groups involved in the process of tumor progression (17,36,37).

In the present study, we used iTRAQ labeling and proteomic approaches to analyze the differentially expressed proteins in prostate cancer cells upon PDEF induction and confirmed their association using *in vitro* functional assays. We have not only used a different approach to identify PDEF regulated proteins but also analyzed the effect of PDEF and STMN association on prostate tumor progression. We are the first to use iTRAQ technology to explore the PDEF proteome in cancer cells and verify our results with *in vitro* assays. We used iTRAQ technology as it has significant advantages over other methods coupled with mass spectrometric analysis. The major advantage of iTRAQ labeling is the capability of multiplexing different samples at the same time to avoid experimental bias. Another positive aspect includes unbiased peptide labeling, as the peptide reactive group is able to bind with all free amino groups to the peptide in the sample (20,38,39).

We identified various proteins as differentially expressed from the analyzed experimental sets. Many of the proteins identified were downregulated upon PDEF induction. STMN was selected as the candidate protein for further analysis based on three set selection criteria. Firstly, STMN was identified

with an iTRAQ ratio (<1.0), secondly STMN showed up multiple times (3 times) in all analyzed sample sets and lastly extensive research was done with STMN in prostate cancer, including designing therapeutic targets. Finally one other reason in consideration was the fact that STMN gene reveals multiple transcription factor recognition sequences: two AP-2 sites, five GC boxes and four E2F sites (40,41). The presence of multiple transcription factor recognition sequences gives us more confidence of the possibility of a site for PDEF binding on STMN.

STMN is overexpressed in many human malignancies including leukemia (42), lymphoma (43), neuroblastoma (44); ovarian (45), prostatic (46), breast (47,48) and lung cancer (49). In many of these cancers, high STMN expression correlates with bad prognosis. It is upregulated in normal proliferating cell lines and also rarely upregulated in non-proliferating cell lines with the exception of neurons (50). STMN expression is highest when the prostate is undergoing morphogenesis or tumorigenesis and these processes may be regulated through differential phosphorylation (30). One of key function of STMN is to alter microtubule dynamics based on the need of the cells. STMN plays a role in both the overexpression and inhibition of expression, which could result in mitotic arrest due to microtubule altering effects providing an attractive therapeutic target in cancer therapy (29,51,52).

STMN has been extensively studied in relation to prostate cancer, exerting a profound influence on cell proliferation, differentiation and cell motility. Mistry *et al* has shown a dramatic dose-dependent growth inhibition in LNCaP prostate cancer cells transduced with anti-STMN adenovirus (53). STMN has provided an attractive molecule to target for cancer therapies that can disrupt the mitotic apparatus (29). Research showed that the anti-STMN ribozyme and low non-inhibitory concentrations of Taxol and etoposide had a profound synergistic inhibitory effect on proliferation, clonogenicity and induction of apoptosis. This was found to be very relevant for the treatment of prostate cancer as both Taxol and etoposide are agents that have been used earlier to treat this disease (53).

Our *in vitro* functional data showed that STMN was not seen in PC3 hPDEF cells in comparison to control cells with an antibody specific to STMN (Fig. 2A). PC3 hPDEF cells had a protein expression level 3-fold greater than the PC3 control cells (Fig. 1A and B). RT-PCR analysis showed that the mRNA expression level of STMN using specific primers was decreased ~1.5-fold in PC3 hPDEF cells in comparison to controls (Fig. 2B). This result was in consensus with our iTRAQ data that showed decreased expression of STMN upon PDEF induction.

We were able to further analyze the effect of STMN in the presence of PDEF induction by *in vitro* functional assays. *In vitro* MTT proliferation assay, anchorage-independent soft agar colony formation and wound healing migration assay clearly showed that PC3 and CWR22rv1 hPDEF cells were able to inhibit proliferation (Fig. 3), colony formation (Fig. 4) and migration of prostate cancer cells (Fig. 5). Upon STMN expression in the hPDEF cells, all important steps of the cancer progression process were almost completely reversed back to that of the control (Fig. 6). STMN expression significantly increased proliferation, colony forming potential and migratory effects of PC3/CWR22rv1 hPDEF cells. Interestingly our data clearly suggested an association of STMN and PDEF with prostate cancer cells. Though we did see an almost complete reversal of prostate cells upon STMN expression, it was not 100% and this difference could be attributed to other PDEF-associated proteins that may play role in the process either along with STMN or alone.

STMN has proven to be an interesting protein that was identified multiple times as downregulated upon PDEF induction. The data obtained from functional assays further confirm the findings and provide new insights into the role of PDEF in regulating the prostate cancer process associated with STMN. PDEF has been studied in our laboratory as a tumor suppressor (16) and with the use of iTRAQ labeling and mass spectrometric analysis techniques we were able to shed light on a possible role of PDEF in prostate cancer cells.

Because of the interesting *in vitro* functional data that support our iTRAQ-MS research it would be worth studying the in-depth association of STMN with PDEF at a molecular level to better understand the disease process. Validating other identified significantly expressed proteins in the presence or absence of STMN upon PDEF induction could explain why there was not a complete reversal upon STMN expression seen in our *in vitro* studies.

Compelling evidence from the literature has shown that STMN is a target for anti-cancer therapeutics. Our findings suggest that PDEF may be used to suppress STMN oncogene expression and be used for the development of effective therapeutic strategies for the treatment of prostate cancer. The therapeutic strategies designed can also be used in other cancers.

Acknowledgements

The authors would like to thank Dr George P. Tuszynski for his helpful discussion. This study was supported by an NIH P50 CA090386 pilot project fund and a PCF fund to M.Z.

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