

Prostate-Derived ETS Factor (PDEF) Regulates Yes Associated Protein 1 (YAP1) in Prostate Cancer Cells: A Potential Cross-Talk between PDEF and Hippo Signaling

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Abstract:

PDEF is expressed in luminal epithelial cells of the prostate gland and associates with luminal phenotype. Hippo pathway regulates cell growth/proliferation, cellular homeostasis, and organ development by modulating phosphorylation of its downstream effectors. In previous studies, we observed decreased levels of PDEF during prostate cancer progression. In the present studies, we evaluated the effects of the expression of PDEF on total/phosphoprotein levels of YAP1 (a downstream effector of the Hippo pathway). We observed that the expression of PDEF in PC3 cells resulted in increased YAP1 levels and increased phospho-YAP1 (Ser127) protein. Our immunofluorescence analysis for YAP1 revealed an increased cytoplasmic/nuclear ratio of YAP1 in PDEF-PC3 cells as compared to VC-PC3 cells, suggesting PDEF may play a critical role in modulating YAP1, and by extension in the regulation of the Hippo pathway. We also observed a decrease in YAP1 protein levels in prostate cancer tissues as compared to normal prostate tissues. Our analysis of multiple publicly available clinical cohorts revealed a gradual decrease in YAP1 mRNA expression during prostate cancer progression and metastasis. This decrease was similar to the decrease in PDEF levels which we had reported earlier, and we observed a direct correlation between PDEF and YAP1 expression. To the best of our knowledge, these results provide the first demonstration of modulation of YAP1 by PDEF in any system and suggest a cross-talk between PDEF and the Hippo pathway.

Keywords: YAP1, PDEF, Prostate cancer

1. Introduction:

Prostate Cancer (PCa) is the second most common cause of cancer deaths in men in the USA. Despite advancements in the early diagnosis and treatment of localized PCa, about 31,620 men will die of PCa in 2019, mostly due to mCRPC. The progression of PCa initially depends on androgen receptor (AR) signaling. Androgen deprivation therapy (ADT) is the primary treatment option for PCa¹. However, androgen deprivation therapy fails and leads to the development of castrate resistance prostate cancer (CRPC) which is a continuum of an advanced/aggressive stage of PCa². Patients with CRPC phenotype are poor responders to available current therapy including the second-generation drugs e.g. Enzalutamide (ENZ)³.

PCa is associated with dysregulation of many signaling pathways. One of the important signaling pathways that control cell growth/proliferation, cellular homeostasis, and organ development, is the hippo pathway⁴. This tumor suppressor pathway was first identified in *Drosophila melanogaster*⁵ and is highly conserved across species including humans⁶. The downstream effector of the hippo pathway is YAP (Yes-associated protein). YAP lacks a DNA-binding domain and interacts with other transcription factors such as TEAD to bind DNA and regulates gene expression⁷. Multiple signaling events such as cell-cell contact, cell density/polarization, mechnao-transduction, G-protein-coupled receptors mediated signaling regulate hippo pathway activation⁸.

Altered expression of YAP1 has been associated with many solid tumors including PCa⁹⁻¹⁷. The role of PDEF in PCa remains highly debated¹⁸⁻²⁵. We observed that PDEF suppresses EMT transition and metastasis in part by driving the expression of epithelial/luminal differentiation-related genes^{20, 24}. Present studies investigated the relationship between PDEF expression and the Hippo pathway in PCa.

We observed that the expression of PDEF in PC3 cells resulted in increased YAP1 and phospho-YAP1 protein levels. We observed a gradual decrease in YAP1 mRNA expression during prostate cancer progression (low to high Gleason grade and during metastasis). Analysis of YAP1 and PDEF in NEPC/CRPC datasets showed a further decrease in YAP1 as well as PDEF mRNA levels in NEPC as compared to CRPC. A direct correlation was observed between PDEF and YAP1 expression in the clinical data set. These exciting results show for the first time potential cross-talk between PDEF and Hippo pathway.

2. Results:

Expression of PDEF in PC3 cells results in increased YAP1 and phospho-YAP1 protein:

To investigate the relationship between PDEF and YAP1, levels of YAP1 total and phosphorylated protein in PDEF-PC3 cells²⁰ and VC-PC3 cells were analyzed by western blots. We observed PDEF-PC3 cells have a higher amount of YAP1 protein (total and phosphoprotein levels) compared to VC-PC3 cells (Figure 1 A). Analysis by immunofluorescence (IMF)²⁴ images for YAP1 showed more cytoplasmic/nuclear ratio of YAP1 in PDEF-PC3 cells as compared to VC-PC3 cells (Figure 1 B). These results are the first direct demonstration of regulation of YAP1 by PDEF in any system.

YAP1 protein levels are decreased in high-grade PCa:

To elucidate the status of YAP1 protein levels in PCa patient's sample, we analyzed the IHC images for YAP1 protein. For these studies, we analyzed data for IHC images for YAP1 protein in PCa patients with low and high-grade tumors from the free publicly available The Human Protein Atlas website²⁶. We observed decreased levels of YAP1 protein in patients with high-grade PCa tumors as compared to low-grade PCa tumors patients (Figure 1 C).

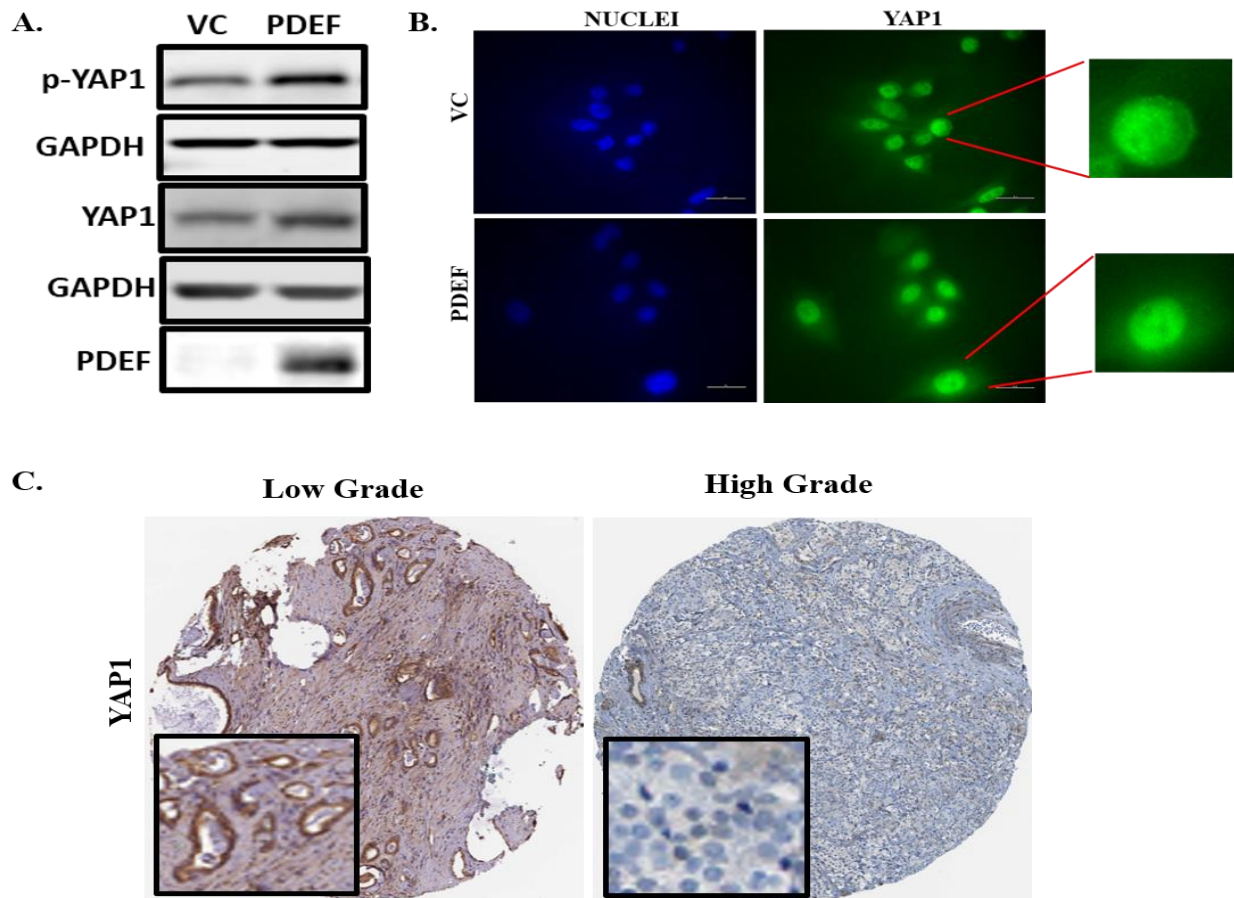


Figure 1: YAP1 protein levels in prostate cancer cells in culture and in clinical specimens: A. PDEF-PC3 cells have a higher amount of phospho-YAP1 protein and total YAP1 protein as compared to VC-PC3 cells. **B.** PDEF-PC3 cells showed more cytoplasmic/nuclear ratio distribution of YAP1 protein, while VC-PC3 cells showed more nuclear localization of YAP1 protein. **C.** Representative Image from The Human Protein Atlas showed a decreased level of YAP1 protein in high-grade PCa tumor samples as compared to low-grade PCa tumor samples.

YAP1 mRNA expression is decreased in PCa patients from different clinical cohorts:

We analyzed the multiple clinical cohorts of PCa for YAP1 mRNA expression using UALCAN²⁷ and c-Bioportal^{28, 29}. The results revealed a significant decrease in YAP1 mRNA levels in patients with PCa (n=497) as compared to normal control (n=52) ($p = 3.81E-10$) (Figure 2 A) in The Cancer

Genome Atlas (TCGA) data set³⁰. We also analyzed YAP1 mRNA expression data for patients with different Gleason scores (GS). Compared to normal control, we found a significant decrease in YAP1 mRNA levels in all grades of PCa (GS6; $p=6.12E-10$, GS7; $p=9.20E-10$, GS8; $p=6.24E-10$ and GS9; $p=1.75E-10$) (Figure 2 B). Moreover, YAP1 mRNA levels were significantly decreased in PCa patients irrespective of lymph node metastasis (N0, $p=7.45E-10$; $n=345$ and N1, $p=1.36E-9$; $n=79$) as compared to normal controls ($n=52$; Figure 2 C). Further analysis of YAP1 mRNA data in Prostate Adenocarcinoma MSKCC dataset³¹ ($n=216$) revealed that 51% of patients have decreased YAP1 mRNA levels (Figure 2 D). These data suggest that a decrease in YAP1 mRNA expression might be an early event in prostate cancer.

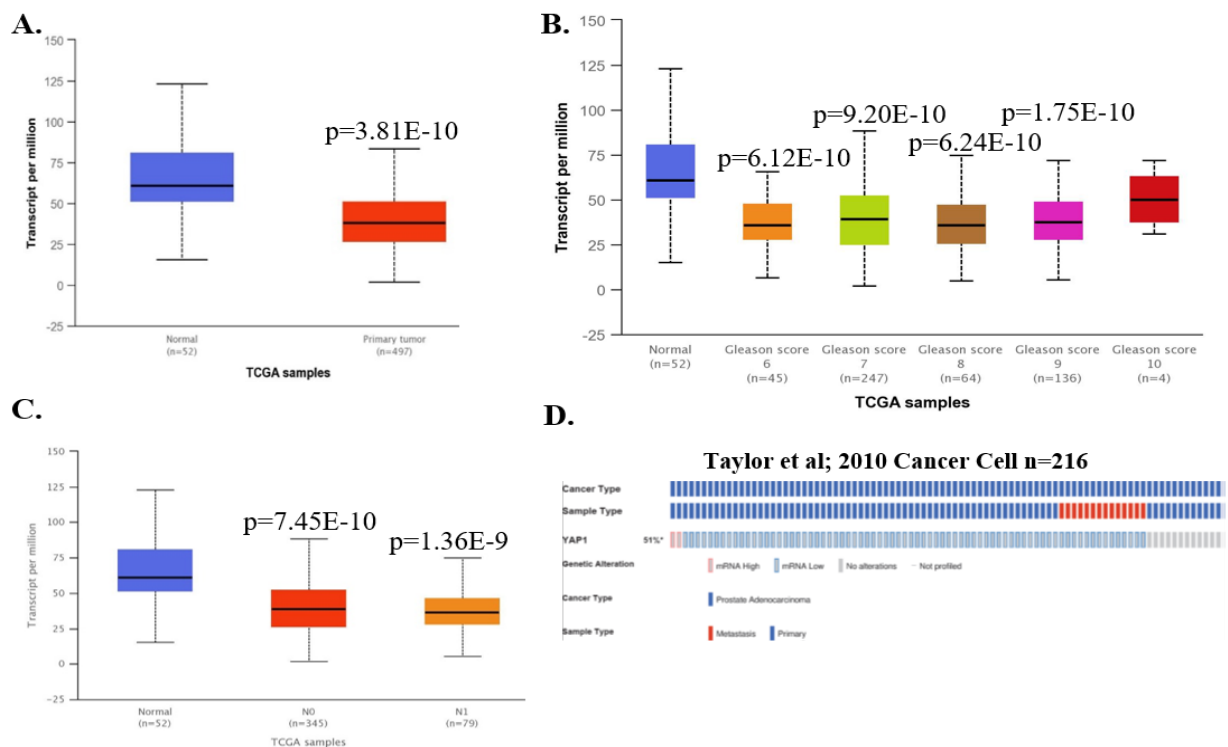


Figure 2: YAP1 mRNA expression in multiple PCa clinical cohorts: YAP1 mRNA data were analyzed from TCGA datasets through UALCAN and the c-Bioportal web server. **A.** mRNA level of YAP1 was significantly decreased in primary prostate tumors as compared to normal prostate

tissues. **B.** A decrease in YAP1 mRNA levels was observed in patients with a higher Gleason score (TCGA datasets) as compared to normal controls. **C.** YAP1 mRNA levels were significantly decreased in PCa patients irrespective of lymph node metastasis as compared to normal controls (TCGA datasets). **D.** 51% of patients showed a

genetic alteration in YAP1 mRNA levels in the Prostate Adenocarcinoma MSKCC dataset.

YAP1 and PDEF mRNA expression is lost in NEPC patients:

We analyzed the Neuroendocrine/Castration-Resistant Prostate Cancer dataset (NEPC/CRPC)³² for expression of YAP1 and PDEF mRNA levels. Our analysis of NEPC/CRPC dataset revealed that expression (mRNA levels) of YAP1 and SPDEF was decreased in NEPC patients as compared to CRPC patients (Figure 3 A; Table 1). Furthermore, we observed a significant positive correlation (spearman $p = 3.065E-4$, Pearson $p = 0.0227$) between YAP1 and PDEF mRNA with a Spearman correlation (r) of 0.49 (Figure 3 B). These results show for the first time that transition to NEPC is associated with complete loss of PDEF expression and suggests the plausible role of PDEF and YAP1 in the NEPC/CRPC phenotype.

Table 1: mRNA expression of YAP1 and PDEF in NEPC/CRPC patients:

Gene Name	Median Expression in NEPC	Median Expression in CRPC	P-Value
YAP1	-0.3623018	1.1336036	3.22E-05
PDEF	0.4419626	224.12931905	6.47E-07

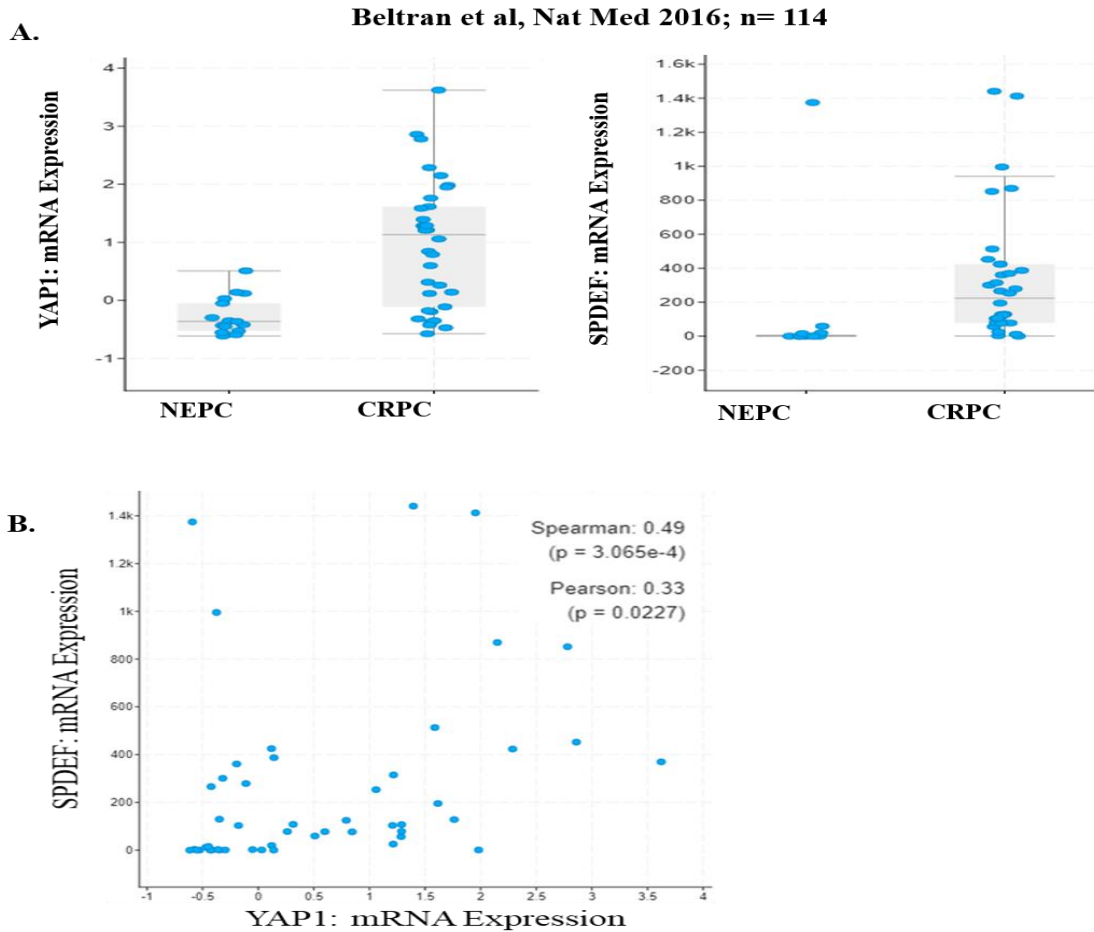


Figure 3: PDEF and YAP1 mRNA levels in CRPC/NEPC patients:

A. YAP1 and PDEF mRNA levels were significantly decreased in NEPC patients as compared to CRPC patients (CRPC/NEPC dataset). **B.** A significant positive correlation was observed between YAP1 and PDEF mRNA in CRPC/NEPC dataset.

3. Discussion:

In the present studies, we observed that the expression of PDEF in prostate cancer cells resulted in increased protein levels of YAP1 and phospho (Ser127) YAP1. ETS transcription factors have been associated with tumor progression as well as therapy resistance in several cancers including prostate cancer. However, the role of PDEF in Prostate cancer remains debated¹⁸⁻²⁵. Others and we

have demonstrated that PDEF limits PCa cell migration invasion and clonogenic activity, but the mechanisms by which PDEF regulates these diverse functions are not completely understood. YAP1 is a downstream effector molecule in the Hippo signaling pathway. Hippo signaling modulates cellular functions by regulating the phosphorylation of YAP1. Thus, decreased expression of YAP1 expression in prostate cancer cells might render prostate cancer cells resistant to modulation of the hippo pathway. In light of the above discussion, our observation of increased expression of YAP1 by PDEF in prostate cancer cells, suggests that PDEF by regulating expression of YAP1 could sensitize prostate cancer to modulation by the hippo pathway. Indeed, we also observed increased phospho (Ser127)-YAP1 following PDEF expression demonstrating reestablishment of an active Hippo signaling cascade. However, the mechanism by which PDEF regulates YAP1 expression/ phosphorylation and the consequences of these effects need additional studies. It has been proposed that the activated Hippo signaling pathway activates MST 1/2 kinase that activates and phosphorylate of LATS 1/2 kinase^{33, 34}. Activated LATS 1/2 kinase then phosphorylate YAP at Serine 127 that triggers interaction of phospho-YAP with 14-3-3 protein and that leads to cytoplasmic retention and ubiquitin-mediated degradation of YAP protein. Inactivation of the hippo pathway results in decreased phosphorylation of YAP and nuclear localization^{35, 36}. Further studies are warranted to understand the molecular mechanisms by which PDEF expression modulates Hippo signaling. Based on our results to date, we propose a working model (Figure 4) with respect to the potential mechanism by which PDEF regulates Hippo signaling.

Several studies have explored the role of YAP1 in PCa and castration-resistant prostate cancer (CRPC)¹⁴⁻¹⁷. YAP1–AR axis appears to play a role in prostate cancer progression¹⁵. YAP regulates cell motility, invasion and castration-resistant growth of prostate cancer cells¹⁷. However, the present study investigated the relationship between PDEF expression and the Hippo pathway in

PCa for the first time. Interestingly, PDEF is an AR co-activator and it is conceivable that PDEF plays an important role in the AR-mediated YAP1 axis. We also observed a decrease in YAP1 protein, and mRNA levels in high-grade tumors as compared to low-grade tumor samples, which parallels that of PDEF.

With the advent of next-generation AR targeted therapies (Abiraterone acetate and Enzalutamide) there is an increasing burden of lethal therapy-resistant NEPC prostate cancer for which current treatments are ineffective^{2,3,32}. There is an unmet need at present for the identification of molecular markers that can be exploited for diagnostic and therapeutic intervention in NEPC. Our exciting observations, that PDEF expression is completely lost in clinical specimens of NEPC patients and this parallels the loss of YAP1 expression, are tempting to speculate the role of PDEF/YAP-1 in reversing the NEPC phenotype to luminal phenotype. However, additional studies are warranted to support the reversal of the NEPC phenotype to epithelial/luminal phenotype by reactivation of the Hippo signaling.

Loss of YAP1 may lead to distorted hippo signaling and may render advanced prostate cancer impervious to the modulators of the Hippo signaling. We found that PDEF-PC3 cells have more YAP1 protein levels as compared to VC-PC3 cells, suggesting that YAP1 levels can be restored by PDEF, which may, as such, re-sensitize the advanced prostate cancers to regulators of the Hippo signaling pathway. This possibility became apparent as we also observed increased phospho-YAP1 levels, and increased cytoplasmic/nuclear ratio in PDEF-PC3 cells as compared to VC-PC3 cells, pointing to the restoration of the hippo signaling pathway in these cells upon PDEF expression. This is significant as we have demonstrated previously that PDEF promotes Epithelial/Luminal Phenotype in Prostate Cancer Cells²⁴. Additional studies are warranted to evaluate the effects of such a cross-talk between PDEF and YAP-1 in modulating phenotypic

changes in aggressive PCa. At present, even in the absence of such studies, our data at least point to the use of decrease levels of YAP1 and PDEF as potential biomarkers to distinguish lethal PCa from otherwise indolent disease.

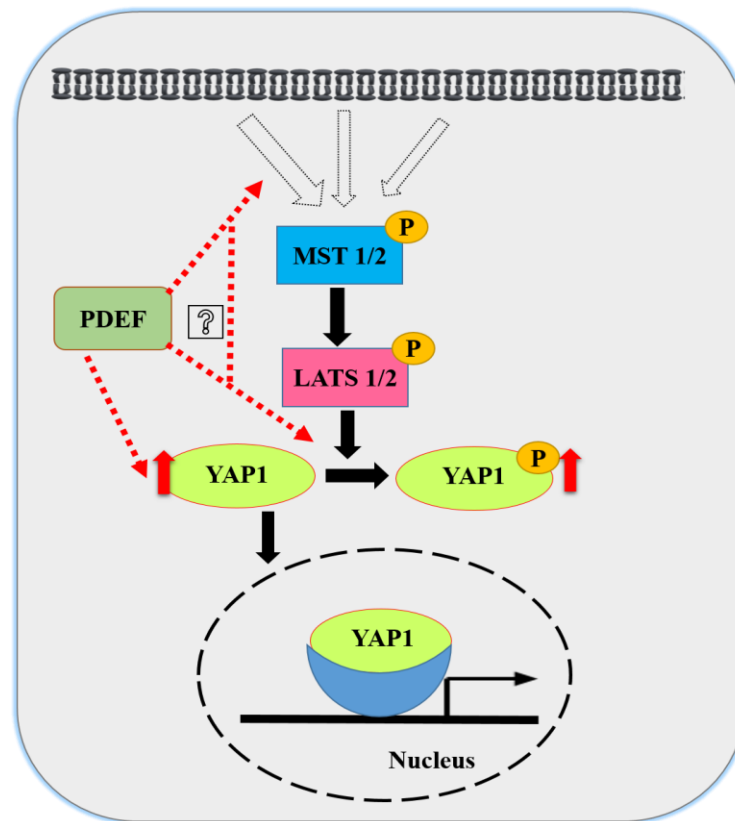


Figure 4: Proposed model for hippo pathway modulations by PDEF: Our results show that PDEF increases expression and phosphorylation (Ser127) of YAP1 in prostate cancer cells. We hypothesize that PDEF might regulate YAP1 phosphorylation indirectly by modulating expression and or activities of various components of the Hippo pathway as shown.

4. Material & Methods:

Materials:

Antibodies PDEF (sc-166846, 1:1000 dilution), GAPDH (Sigma G8795, 1:3000 dilution), YAP1 (sc-101199, 1:1000 dilution for western blots, 1:50 dilution for Immunofluorescence) and, phospho-YAP1 (cst-13008, 1:1000 dilution) were purchased from respective vendors.

Cell Lines and Culture:

We used prostate cancer cell line PC3 obtained from American Type Culture Collection (ATCC). PC3 cell line was stably transfected with PDEF²⁴ and cultured as described previously²⁴.

Data mining from multiple clinical cohorts:

We analyzed the YAP1 mRNA expression data from PCa TCGA³⁰ (The Cancer Genome Atlas) data sets through UALCAN²⁷ (<http://ualcan.path.uab.edu/>) and c-BioPortal^{28, 29} (<http://www.cbioportal.org>) web-server. Further, we analyzed YAP1 mRNA expression in Prostate Adenocarcinoma MSKCC dataset³¹. YAP1 and SPDEF mRNA expression data were also analyzed in NEPC/CRPC dataset³² through c-BioPortal.

Immunohistochemistry (IHC):

We procured IHC images for YAP1 protein in PCa patients with low and high-grade tumors from The Human Protein Atlas website²⁶ (<https://www.proteinatlas.org/ENSG00000137693-YAP1/pathology/prostate+cancer#img>).

Western blot:

Western blots for PDEF, YAP1, and Ser127-phospho-YAP1 protein were performed as described³⁷. Blots were scanned by LI-COR Odyssey CLx (LI-COR, Lincoln, USA) system by using IRDye 680 goat anti-mouse/IRDye 800 goat anti-rabbit secondary antibodies as described³⁸.

Immunofluorescence (IMF):

Immunofluorescence for YAP1 protein was done as described²⁴.

5. Conclusions:

Our finding show for the first time potential regulatory role of PDEF in activation of Hippo pathway in prostate cancer. To the best of our knowledge, these results provide the first demonstration of the regulation of the Hippo pathway by PDEF in any system.

Authors contributions: Conceived the project: H.K.K. Designed the experiments: H.K.K, and P.K.J. Performed the experiments: P.K.J., S.M., S.K. Analyzed data: P.K.J., S.K., F.W., R.S., H.K.K. Wrote the manuscript: P.K.J., H.K.K., S.K, S.M.

Funding: This work was supported in part by NCI RO1-CA242839 (H.K.K). P.K.J was supported in part by Urology Care Foundation (American Urological Association) Research Scholar Award 2019.

Acknowledgments: IMF imaging was performed at the Microscopy core facility at LSUHSC-Shreveport.

Conflicts of Interest: The authors declare no conflicts of interest.

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