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TITLE: Targeting a Novel Androgen Receptor-Repressed Pathway in Prostate Cancer Therapy

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The primary goal of this study is to investigate the potential roles of protein kinase D (PKD) in mediating therapeutic resistance to ADT and to investigate the impact of PKD small molecule inhibitor (SMI)-based combination therapies to curtail ADT-induced therapy resistance. During the past funding cycle, we examined the cross-regulation of PKD1 by androgen signaling in prostate cancer cells. Our data has identified PKD1 as a novel androgen-repressed gene at transcriptional level. Kinetic analysis indicated that the repression of PKD1 by androgen required the induction of a repressor protein, and AR was required for the suppression of PKD1 by androgen. Downstream of AR, we identified fibroblast growth factor receptor substrate 2 (FRS2) and its downstream MEK/ERK pathway as the mediators of androgen-induced PKD1 repression. Our study indicates that PKD1 is a novel androgen-suppressed gene and can be downregulated by androgen through a novel AR/FRS2/MEK/ERK pathway. The upregulation of the prosurvival PKD1 by antiandrogens may contribute to therapeutic resistance in prostate cancer treatment. The work has now been published (*Zhang et al., Oncotarget, 2017, 8:12800-12811*).

15. SUBJECT TERMS
Prostate cancer, protein kinase D, androgen, androgen receptor, therapeutic resistance, androgen-deprivation therapy.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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1. INTRODUCTION:

Prostate cancer progression to castration resistance associates with poor prognosis and high mortality. Androgen deprivation therapy (ADT) has been the standard therapy for advanced metastatic prostate cancer. However, ADT has been linked to development of therapy resistance in part through inducing prosurvival adaptive responses. Increased understandings of these adaptive mechanisms will lead to discovery of new targets/therapies for mCRPC. We will investigate the roles of protein kinase D (PKD) in therapeutic resistance to ADT and the impact of PKD inhibitor-based combination therapies to curtail ADT-induced therapy resistance.

2. KEYWORDS:

Prostate cancer, androgen, androgen receptor, Protein kinase D, androgen-deprivation therapy, therapy resistance, transcriptional regulation

3. ACCOMPLISHMENTS:

The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

We seek to test **the central hypothesis is that PKD plays a crucial role in limiting the effectiveness of ADT by increasing prostate cancer survival through upregulating AURKA expression, and PKD small molecule inhibitors may enhance the efficacy of AR antagonists in prostate cancer treatment.** Our study will provide insights to the role of PKD in ADT-induced prosurvival responses relevant to the progression to CRPC. Successful completion of this study will define the role and mechanisms of PKD in treatment (ADT)-induced prostate cancer resistance. Three Specific Aims are proposed: **Aim 1.** Determine the mechanisms through which AR represses PKD1 expression in androgen-sensitive prostate cancer cells. **Aim 2.** Test the hypothesis that androgen deprivation-induced PKD1 expression promotes prostate cancer cell survival and ADT resistance through upregulating AURKA and CENPE. **Aim 3.** Determine the functional input of PKD in ADT resistance *in vivo* and assess the efficacy of PKD SMI in combination with AR antagonists in prostate cancer tumor xenografts.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

MAJOR ACTIVITIES

The primary goal of this study is to investigate the potential roles of PKD in mediating therapeutic resistance to ADT and to investigate the impact of PKD SMI-based combination therapies to curtail ADT-induced therapy resistance. This remains our main focus during the first funding cycle. Major efforts were devoted to Aim 1. We have validated the regulation of PKD by androgen and delineated the downstream signaling pathway. The work has been concluded and published. We have also obtained data supporting a role of PKD in promoting androgen-independent growth and survival (Aim 2).

SPECIFIC OBJECTIVES

Here is a list of major tasks and milestones to be achieved:

Specific Aim 1. Determine the mechanisms through which AR represses PKD1 expression in androgen-sensitive prostate cancer cells. [This aim has been completed]

Major Task 1: Test the hypothesis that FRS2 is required for the repression of PKD1 by AR.

Major Task 2: Determine the downstream targets of FRS2 that mediate PKD1 repression by androgen

Specific Aim 2. Test the hypothesis that androgen deprivation-induced PKD1 expression promotes prostate cancer cell survival and ADT resistance through upregulating AURKA and CENPE. [Study is on-going]

Major Task 3: Test the hypothesis that androgen deprivation-induced PKD1 expression.

Major Task 4: Test the hypothesis that PKD promotes resistance to antiandrogens through modulating Aurora-A and Cenp-E expression and mitotic programing.

Specific Aim 3. Determine the functional input of PKD in ADT resistance *in vivo* and assess the efficacy of PKD SMI in combination with AR antagonists in prostate cancer tumor xenografts. [Study will be initiated soon]

Major Task 5: Determine that increased PKD1 expression confers resistance to AR antagonists in PrCa xenograft models.

Major Task 6: Determine that targeted inhibition of PKD by CRT101 enhances the efficacy of AR antagonists *in vitro* and *in vivo*.

SIGNIFICANT RESULTS (The progress is outlined along with the statement of work)

Specific Aim 1 Determine the mechanisms through which AR represses PKD1 expression in androgen-sensitive prostate cancer cells.	**Timeline**
Major Task 1: Test the hypothesis that FRS2 is required for the repression of PKD1 by AR **This task has not been partially completed. In this study, we examined the cross-regulation of PKD1 by the androgen signaling in prostate cancer cells. Our data showed that the transcription of PKD1 was repressed by androgen in androgen-sensitive prostate cancer cells. Steroid depletion caused an up-regulation of PKD1**	Months

transcript and protein, an effect that was reversed by AR agonist R1881 in a time- and concentration-dependent manner, thus identifying PKD1 as a novel androgen-repressed gene. Kinetic analysis indicated that the repression of PKD1 by androgen required the induction of a repressor protein. Further, inhibition or knockdown of AR reversed AR agonist-induced PKD1 repression, indicating that AR is required for the suppression of PKD1 expression by androgen. Downstream of AR, we identified fibroblast growth factor receptor substrate 2 (FRS2) and its downstream MEK/ERK pathway as the mediators of androgen-induced PKD1 repression. In summary, PKD1 is a novel androgen-suppressed gene and can be downregulated by androgen through a novel AR/FRS2/MEK/ERK pathway. The upregulation of the prosurvival PKD1 by antiandrogens may contribute to therapeutic resistance in prostate cancer treatment. A manuscript that describes the findings has been published (*Zhang et al., Oncotarget, 2017, 8:12800-12811*) (enclosed in appendix).	
Subtask 1: Determine the mRNA and protein levels of FGFs, FGF-BP, FGFR1-4, FRS2, and PKD1, and phosphorylation of FRS2 in prostate cancer cells upon androgen depletion +/- R1881. This subtask is still on-going.	1-4
Subtask 2: Determine the specific FGF2, FGFR, and phosphorylation of FRS2 required for androgen-induced PKD1 repression. This subtask has been partially completed. Our data have demonstrated the essential role of FGF2/FGFR and FRS2 for androgen-induced PKD1 repression. We still need to identify the specific FGF2, FGFR, and phosphorylation of FRS2. Please see Fig 4-5 in our Oncotarget paper (Enclosed in appendix).	1-6
Subtask 3: Determine if increased FGF2 is due to liberation of entrapped FGF-2 from extracellular matrix through activation of heparinase, which leads to activation of FGFR and downregulation of PKD. This subtask is on-going.	1-3
Milestone(s) Achieved: Identification of specific FGF2, FGFR, and phosphorylation sites of FRS2 involved in the repression of PKD1 by androgen. Our data demonstrated an important role of FGF2, FGFR, and phosphorylation of FRS2 in the repression of PKD1 by androgen. We have yet to identify the specific FGF2 and FGFR involved in the process. Thus, the milestones are partially achieved.	*1-6*
Major Task 2: Determine the downstream targets of FRS2 that mediate PKD1 repression by androgen **This task has been partially completed. A manuscript that describes the findings has been published (Zhang et al., Oncotarget, 2017, 8:12800-12811). The paper is included in the appendix.**	
Subtask 1: Determine the activity of MEK/ERK, PI3K/Akt, and PLCγ/PKC pathways upon androgen depletion +/- R1881in LNCaP cells. We have identified MEK/ERK as a major mediator for the suppression of PKD1 by	1-3

androgen. The results are described in Fig. 5 of our Oncotarget paper (see appendix).	
Subtask 2: Using selective inhibitors and siRNAs targeting the MEK/ERK, PI3K/Akt, and PLCγ/PKC pathways to determine their contributions to PKD1 expression upon androgen depletion. We have used the inhibitors of the MEK/ERK, PI3K/Akt, and PLCγ/PKC pathways. Our data indicated that MEK/ERK is the major mediator of the effect of androgen on PKD1 expression. The results on MEK inhibitor UO126 are described in Fig. 5E of our Oncotarget paper (see appendix).	1-2
Subtask 3: Conduct detail analysis of the pathway identified in Subtask 2 and assessing its impact on PKD1 expression. This study is on-going. We don't have results on this yet.	2-4
Milestone(s) Achieved: Identification of the specific pathway involved in the regulation of PKD1 expression by androgen. We have achieved this milestone by identify MEK/ERK as the major signaling pathway mediating the effect of androgen on PKD1.	4
Specific Aim 2 Test the hypothesis that androgen deprivation-induced PKD1 expression promotes prostate cancer cell survival and ADT resistance through upregulating AURKA and CENPE.	
Major Task 3: Test the hypothesis that androgen deprivation-induced PKD1 expression promotes tumor cell survival and confers resistance to AR antagonists in PrCa cells. **Please see description under each subtask for the progress of the studies.**	
Subtask 1: Determine the effects of knockdown of PKD1 by siRNAs on sensitivity of AR antagonists, bicalutamide and enzalutamide, in androgen-sensitivity PrCa cells. This subtask is still on-going.	1-3
Subtask 2: Determine the effects of PKD1 overexpression on sensitivity of AR antagonists, bicalutamide and enzalutamide, in androgen-sensitivity PrCa cells. We have completed this subtask. In this study, although PKD2 overexpression had little impact on LNCaP cell viability in androgen-containing medium (Fig. 1A, top), it significantly promoted cell growth/survival in androgen-free medium (Fig. 1A, bottom), indicating that PKD2 promoted androgen independence in androgen-sensitive prostate cancer cells. Further, PKD2 overexpression significantly reduced the sensitivity of LNCaP cells to AR inhibitor MDV3100 (enzalutamide) (presumably due to inhibition of AR signaling), PX-866, and docetaxel, implying that PKD may be targeted to sensitize the effect of antiandrogens and reduce chemoresistance.	1-3

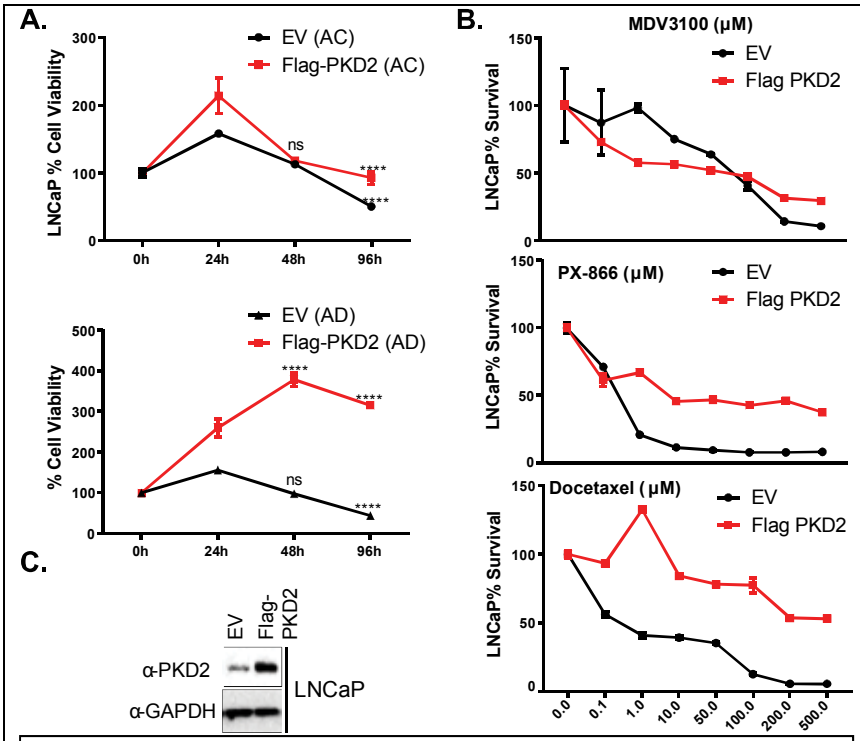

Fig. 1. PKD2 overexpression promoted androgen independence and therapy resistance. A. LNCaP cells transfected with EV and Flag-PKD2 were grown in normal androgen-containing (AC) growth medium or in androgen-depleted (AD) medium for 4 days, followed by CCK-8 proliferation assay. **B.** LNCaP cells transfected with EV and Flag-PKD2 were treated with varying concentrations of MDV3100, PX-866, and docetaxel. Cell proliferation was measured by CCK-8 assay after 3 days. **C.** PKD overexpression was confirmed by immunoblotting. *ns*, not significant; **** $p < 0.0001$.

Milestone(s) Achieved: Obtain the data that support the concept that depletion of PKD enhances sensitivity to AR antagonists. The Milestones are partially achieved. By overexpressing PKD, we showed that the increased PKD expression caused chemoresistance (Fig. 1).	3
Major Task 4: Test the hypothesis that PKD promotes resistance to antiandrogens through modulating Aurora-A and Cenp-E expression and mitotic programing. **We have made major progress on these studies. The results are described below.**	
Subtask 1: Determine the role of PKD in mitosis. Our study has demonstrated an important role of PKD in G2/M transition and mitotic entry. Our data indicated that PKD, particularly PKD2, was activated during G2 and M phase of cell cycle, and its activity is required for mitotic entry and progression. Inhibition of PKD resulted in mitotic catastrophe.	1-6
Subtask 2: Determine if Aurora-A and Cenp-E account for the effects of PKD on mitosis. We found that overexpression of Aurora A reversed the effect of PKD inhibitor on cell	2-4

cycle, thus Aurora A mediated the effects of PKD on G2/M transition and mitotic progression.	
Subtask 3: Examine a direct role of PKD in regulating Aurora-A and Cenp-E during mitosis through direct binding or phosphorylation. We found that PKD co-localized with Aurora A, but did not directly interact with Aurora A. We also did not detect changes of Aurora A phosphorylation when inhibiting or depleting PKD.	1-6
Milestone(s) Achieved: Demonstrate a causal role of Aurora-A and Cenp-E in PKD-regulated mitosis. The milestones have been partially achieved. Our study has demonstrated a causal role of Aurora-A in PKD-regulated mitotic entry. We did not conduct further study on Cenp-E because of technical difficulty (poor antibody and the large size of the protein). We decide to focus our efforts on Aurora A. We also found that PKD has a dominant role in mitotic entry. It remains to be investigated if it also regulated mitosis.	9
Specific Aim 3 Determine the functional input of PKD in ADT resistance *in vivo* and assess the efficacy of PKD SMI in combination with AR antagonists in prostate cancer tumor xenografts	
Major Task 5: Determine that increased PKD1 expression confers resistance to AR antagonists in PrCa xenograft models. **Please see description under each subtask for the progress of the studies.**	
Subtask 1: Establish stable inducible PKD1 knockdown cell lines derived from LNCaP, C4-2, and VCaP cells. It has been a challenging task to obtain stable inducible PKD1 knockdown cell lines derived from LNCaP, C4-2, and VCaP cells since LNCaP and C4-2 have unstable phenotypes and VaCaP was difficult to culture. However, we were able to successfully clone the PKD1-shRNAs into Doxycycline (Dox)-Inducible RNAi vector.	1-6
Subtask 2: Establish stable PKD1 overexpressing cell lines derived from LAPC4. This work is on-going. We are in the process of establishing the PKD1 overexpressing cell lines in LAPC4.	1-3
Subtask 2: Conduct s.c. tumor xenograft studies on the stable cell lines established above. This subtask has not been completed. The work will be initiated once we have stable cell lines established.	1-12
Milestone(s) Achieved: Obtain the stable cell lines with knockdown or overexpression of PKD. The work is on-going. We have not achieved the milestones yet.	9

Major Task 6: Determine that targeted inhibition of PKD by CRT101 enhances the efficacy of AR antagonists *in vitro* and *in vivo* **Please see description under each subtask for the progress of the studies.**	
Subtask 1: Determine the efficacy of CRT101 and enzalutamide alone and in combination on the growth of VCaP prostate tumor xenografts. We are testing CRT101 and enzalutamide in cells at the moment. Since VCaP is difficult to culture, we are also testing 22Rv1, another castration-resistance AR-positive prostate cancer cell line.	1-6
Subtask 2: Analysis of tumor tissues and biomarkers This subtask has not been completed. The study will be done along with subtask1 next year.	1-6
Milestone(s) Achieved: Demonstrate synergy between CRT1010 and enzalutamide in PrCa xenograft models. We have demonstrated the synergistic effects of CRT1010 and enzalutamide in PrCa cell lines. We will testing the combination in xenograft models in the next funding period.	18-24
OTHER ACHIECEMENTS Metastasis is a key feature of mCRPC, which directly contribute to the high mortality of advanced prostate cancer. We have developed a bone metastasis mouse model by intracardiac injection of metastatic PC3-ML cells. This model could potentially be used to examine the *in vivo* efficacy of CRT101 in combination with antiandrogens.	

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

The project provided opportunities for training postdoctoral fellows.
Since October 2011, all postdoctoral trainees in the schools of the health sciences at the University of Pittsburgh have been required to complete an annual career development plan (also known as an individual development plan - IDP) as part of our institution's Postdoctoral Career Development and Progress Assessment Process.
This process, overseen by the Center for Postdoctoral Affairs in the Health Sciences, requires that a postdoc work with his or her faculty mentor to establish an annual career development plan and to also identify two additional individuals to serve as members of the postdoc's mentoring team. The postdoc also completes an annual self-assessment relative to his or her career development plan which contributes in part to the faculty mentor's annual assessment of the postdoc's progress towards his or her career goals.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

During the next reporting period, our primary focus is to complete Specific Aim 2. A main task is to determine the roles of Aurora-A and Cenp-E in mediating the effects of PKD on androgen-independent growth, and delineating the underlying molecular mechanisms. We will also start to work on Aim 3. Although we have demonstrated that overexpression of PKD conferred resistance to AR antagonists, it is not known if it also occurs *in vivo*. We will evaluate this using both s.c. tumor xenograft model and the bone metastasis model that we have just established. It is important to examine the metastatic aspects of this regulation since tumor metastasis is a key feature of mCRPC that associates with relapse from therapies.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

This application fills an important knowledge gap in signaling mechanisms and therapeutic targeting of PKD in the context of ADT-induced therapy resistance in prostate cancer. (**1**) The successful completion of the study will provide mechanistic insights to AR-regulated prosurvival pathways centered on PKD in therapy resistance and tumor progression. (**2**) PKD may be targeted to enhance the therapeutic efficacy of antiandrogens and other chemotherapeutic agents, indicating the significant translational value of the study. (**3**) The identification of AURKA and CENPE as biomarkers of PKD SMIs may facilitate the translation of these agents to the clinic. (**4**) AURKA and CENPE may be used for identifying PKD SMI-sensitive prostate tumor subtypes and facilitate the application of PKD-targeted agents in personalized therapy.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report.

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to Report.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals.

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Zhang, L., Zhao, Z., Xu, S., Tandon, M., Lavalle, C. R., Deng, F., and *Wang, Q. J. (2017) Androgen suppresses protein kinase D1 expression through fibroblast growth factor receptor substrate 2 in prostate cancer cells. *Oncotarget*, 8:12800-12811. 2. Roy, A., Ye, J., Deng, F., Wang, Q. J. (2017) Protein Kinase D Signaling in Cancer: A Friend or Foe? *BBA-Rev Can*, 1868(1):283-294.

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report.

  Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to Report.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

Nothing to Report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report.

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *biospecimen collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *1234567*
Nearest person month worked: *5*

Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award).*

Name: Qiming Wang
Project Role: Principal Investigator
Research Identifier (e.g. ORCID ID):
Nearest Person Month Worked: 2
Contribution to the project:
Funding Support:

Name: Sahdeo Prasad
Project Role: Postdoctoral Associate
Research Identifier (e.g. ORCID ID):
Nearest Person Month Worked: 5
Contribution to the project:
Funding Support:

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Dr. Wang's previously awarded 1R01CA142580 award has closed as of 12/31/2016.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.
Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Reprint of two manuscripts:

1. Zhang, L., Zhao, Z., Xu, S., Tandon, M., Lavalle, C. R., Deng, F., and *Wang, Q. J. (2017) Androgen suppresses protein kinase D1 expression through fibroblast growth factor receptor substrate 2 in prostate cancer cells. *Oncotarget*, 8:12800-12811.
2. Roy, A., Ye, J., Deng, F., Wang, Q. J. (2017) Protein Kinase D Signaling in Cancer: A Friend or Foe? *BBA-Rev Can*, 1868(1):283-294.

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Research Paper

Androgen suppresses protein kinase D1 expression through fibroblast growth factor receptor substrate 2 in prostate cancer cells

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ABSTRACT

In prostate cancer, androgen/androgen receptor (AR) and their downstream targets play key roles in all stages of disease progression. The protein kinase D (PKD) family, particularly PKD1, has been implicated in prostate cancer biology. Here, we examined the cross-regulation of PKD1 by androgen signaling in prostate cancer cells. Our data showed that the transcription of PKD1 was repressed by androgen in androgen-sensitive prostate cancer cells. Steroid depletion caused up regulation of PKD1 transcript and protein, an effect that was reversed by the AR agonist R1881 in a time- and concentration-dependent manner, thus identifying PKD1 as a novel androgen-repressed gene. Kinetic analysis indicated that the repression of PKD1 by androgen required the induction of a repressor protein. Furthermore, inhibition or knockdown of AR reversed AR agonist-induced PKD1 repression, indicating that AR was required for the suppression of PKD1 expression by androgen. Downstream of AR, we identified fibroblast growth factor receptor substrate 2 (FRS2) and its downstream MEK/ERK pathway as mediators of androgen-induced PKD1 repression. In summary, PKD1 was identified as a novel androgen-suppressed gene and could be downregulated by androgen through a novel AR/FRS2/MEK/ERK pathway. The upregulation of prosurvival PKD1 by anti-androgens may contribute to therapeutic resistance in prostate cancer treatment.

INTRODUCTION

Prostate cancer is the most common noncutaneous malignancy and the second leading cause of cancer-related deaths among men in the United States. The initiation and progression of prostate cancer is uniquely dependent on androgen receptor (AR)-induced signaling. Although androgen deprivation therapy provides an initial favorable response in advanced prostate cancer, the more aggressive castration-resistant prostate cancer (CRPC) develops invariably in almost all patients, eventually leading to death. It has become increasingly clear that continuous activation of the AR in CRPC remains the main driving force of tumor progression and metastasis. Thus, understanding the critical events associated with the AR signaling is essential for developing novel and effective therapies to treat CRPC.

The protein kinase D (PKD) family of serine/threonine kinases belongs to the Ca^{2+}/calmodulin-dependent protein kinase (CAMK) superfamily [1, 2]. To date, three isoforms of PKD have been identified, PKD1 (formerly PKCμ) [3, 4], PKD2 [5], and PKD3 (formerly PKCν) [6]. In intact cells, PKD activation involves phosphorylation of two conserved serine residues in the activation loop by DAG-responsive PKCs [7–9], and PKD activity can be maintained independently of PKC through autophosphorylation [10, 11]. Emerging evidence supports that PKD has an important role in carcinogenesis

and tumor progression [12, 13]. A recent report suggested that a hotspot activating mutation in *PRKD1*, the gene encoding PKD1, may drive polymorphous low-grade adenocarcinoma (PLGA), the second most frequent type of malignant tumor of the minor salivary glands [14]. PKD regulates a variety of tumor-associated biological processes, including tumor cell proliferation, growth, survival, migration, invasion, secretion, and angiogenesis [12, 15–20]. Aberrant PKD activity and expression have been demonstrated in tumor cell lines and tumor tissues from the pancreas [18], skin [19, 21], breast [22], and prostate [20, 23]. In particular, PKD has been shown to play an important role in the pathogenesis of prostate cancer [20, 24–26], and targeted PKD inhibition potently blocks prostate cancer cell proliferation and survival [26, 27].

Fibroblast growth factor (FGF) signaling is a highly complex signaling network that comprises 18 ligands, which bind to and activate four highly conserved transmembrane tyrosine kinase receptors (FGFR1, FGFR2, FGFR3, and FGFR4).The FGF/FGFR pathway plays an important role in cancer development and progression by modulating a variety of biological processes, including cell proliferation, survival, and migration [28, 29]. FGFR substrate 2 (FRS2/FRS2α), also known as FGFR-signaling adaptor SNT1 (suc1-associated neurotrophic factor target 1), is regarded as the 'conning center' for intracellular signaling elicited by the activation of FGFRs at the cell surface. FRS2 forms complexes with Grb2-Sos and Grb2-Gab1 to activate the Ras/Raf/MEK/ERK and PI3K/Akt pathways [29, 30]. Although FRS2 expression is not regulated by androgen [31], androgen-sensitive prostate cancer cells express FGF2, and its expression is upregulated in response to androgen stimulation [32]. Thus, androgen regulates the activity of FGFR signaling in prostate cancer cells.

In this study, we report for the first time that PKD1 was tightly regulated by androgen at the transcriptional level in prostate cancer cells and was a novel androgen-repressed gene. Inhibition or knockdown of androgen receptor (AR) blocked androgen depletion-induced PKD1 expression, indicating that AR was required for the repression of *PRKD1* gene expression. Further analysis identified FRS2 as a novel mediator of androgen-induced PKD1 repression. The regulation of PKD1 by androgen and AR may have important implications in the therapeutic response to AR-targeted agents.

RESULTS

Androgen repressed PKD1 expression in androgen-sensitive prostate cancer cells

Androgen signaling plays a crucial role in prostate cancer initiation and progression. In this study, we sought to determine whether androgen modulated PKD1 expression and signaling. PKD1 was detected in androgen-sensitive LNCaP cells and two castration-resistant LNCaP-derivative cell lines, C4-2 (androgen-hypersensitive) and C81 (androgen-insensitive), but not in androgen-sensitive LAPC4 cells. As shown in Figure 1A, a significant increase in PKD1 expression was observed upon androgen depletion (AD) in LNCaP and C4-2 cells and to a lesser extent in C81 cells. R1881, a synthetic androgen agonist, induced remarkable concentration-dependent suppression of PKD1 expression at the transcript (Figure 1B) and protein (Figure 1C) levels in LNCaP and C4-2 cells. R1881 also suppressed PKD1 expression in VCaP cells, a castration-resistant prostate cancer cell line that expresses wild-type AR, in a concentration-dependent manner (Figure 1D). Interestingly, PKD2 expression was similarly suppressed by R1881 in a concentration-dependent manner in LNCaP and VCaP cells (Supplementary Figure 1A–1B). PKD3 was also upregulated upon androgen withdraw in LNCaP cells, despite its low endogenous expression (Supplementary Figure 1A). In contrast, androgen did not affect the expression of PKD1 and PKD2 in another castration-resistant cell line, 22Rv1, which expresses both full-length AR and truncated AR variants (Supplementary Figure 1C), suggesting that the effect of androgen may be cell context-dependent. Taken together, we concluded that PKD1 was an androgen-repressed gene.

PKD1 expression was dependent on the induction of a repressor protein

The kinetics of PKD1 regulation in response to androgen deprivation or R1881 treatment was examined. As shown in Figure 2A, androgen deprivation gradually up regulated PKD1 protein expression, which peaked at 16–24 h, while R1881 suppressed PKD1 expression with similar kinetics. The induction of PKD1 transcript and its inhibition by R1881 correlated well with the time-course of protein expression (Figure 2B).

To gain insights into the regulation of PKD1 by androgen, we first examined whether R1881 affected PKD1 mRNA stability. The half-life (t½) of PKD1 mRNA was determined in the presence of actinomycin D, an inhibitor of gene transcription. As shown in Figure 2C, the t½ of PKD1 mRNA was about 4 h, which was not significantly altered by the addition of R1881 ($p > 0.5$), indicating that R1881 did not impact the stability of PKD1 mRNA. Next, cycloheximide (CHX) was used to inhibit protein synthesis to determine whether the regulation of PKD1 gene expression by androgen involved *de novo* protein synthesis. CHX induced a nearly 2-fold increase in PKD1 expression and completely blocked R1881-induced PKD1 downregulation, indicating that the suppression of PKD1 expression likely required the induction of a repressor protein (Figure 2D). This finding was in line with the gradual onset of PKD1 regulation by androgen, further supporting the involvement of a repressor protein. Taken together, our data indicated that androgen-regulated PKD1 expression was dependent on the presence of a repressor protein.

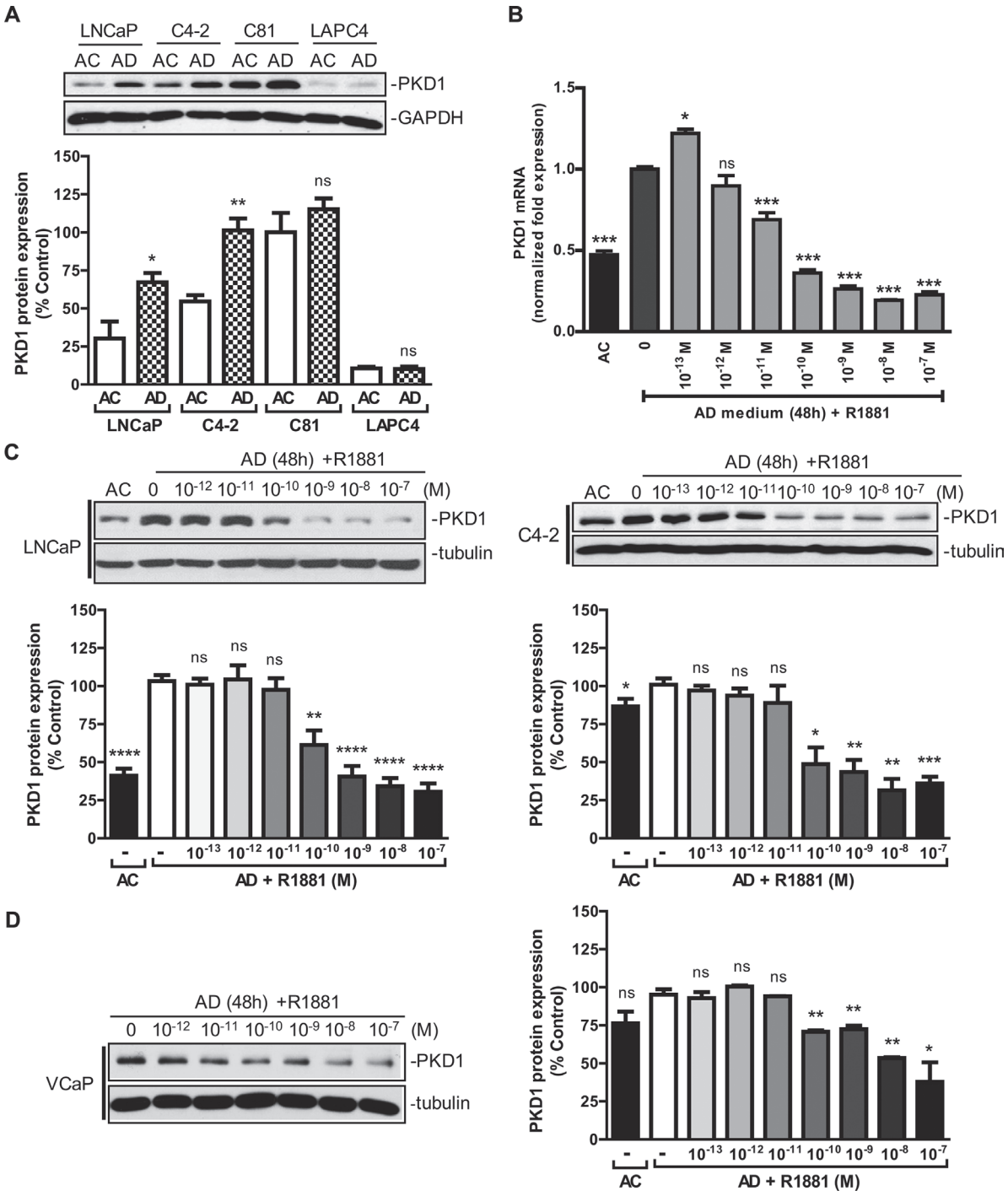


Figure 1: Androgen repressed PKD1 expression. (**A**) Effects of androgen depletion on PKD1 expression in prostate cancer cells. LNCaP, C4-2, C81, and LAPC4 cells were grown for 48 h in normal androgen-containing (AC) or androgen-depleted (AD) medium supplemented with charcoal-stripped FBS. Cells were lysed and subjected to immunoblotting for PKD1 and GAPDH (loading control). *Bottom*, quantitative measurement of band intensity by densitometry analysis. The data were expressed as % control with C81 (AC) set as 100%. Data are the mean ± SEM of four independent experiments. (**B**) Androgen inhibited PKD1 transcription. Total RNAs from LNCaP were extracted, and real-time RT-qPCR was conducted using specific PKD1 primers. *GAPDH* was used as internal control. Data are the mean ± SEM of three independent experiments. (**C**) Androgen suppressed PKD1 protein expression. LNCaP and C4-2 cells were grown in androgen-depleted medium for 48 h, following by treatment without or with increasing concentrations of androgen R1881. Cells were harvested after 24 h and subjected to immunoblotting. *Bottom*, the band intensity was quantified by densitometry analysis, and data are the mean ± SEM of ten (LNCaP) or three (C4-2) independent experiments. (**D**) Androgen suppressed PKD1 protein expression in castration-resistant VCaP cells. VCaP cells were grown in androgen-depleted medium for 48 h, followed by treatment without or with androgen R1881 for 24 h. Cells were harvested for immunoblotting. Data from one of three independent experiments are shown. *Right*, quantitative measurement of band intensity from three experiments is shown. ns, not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

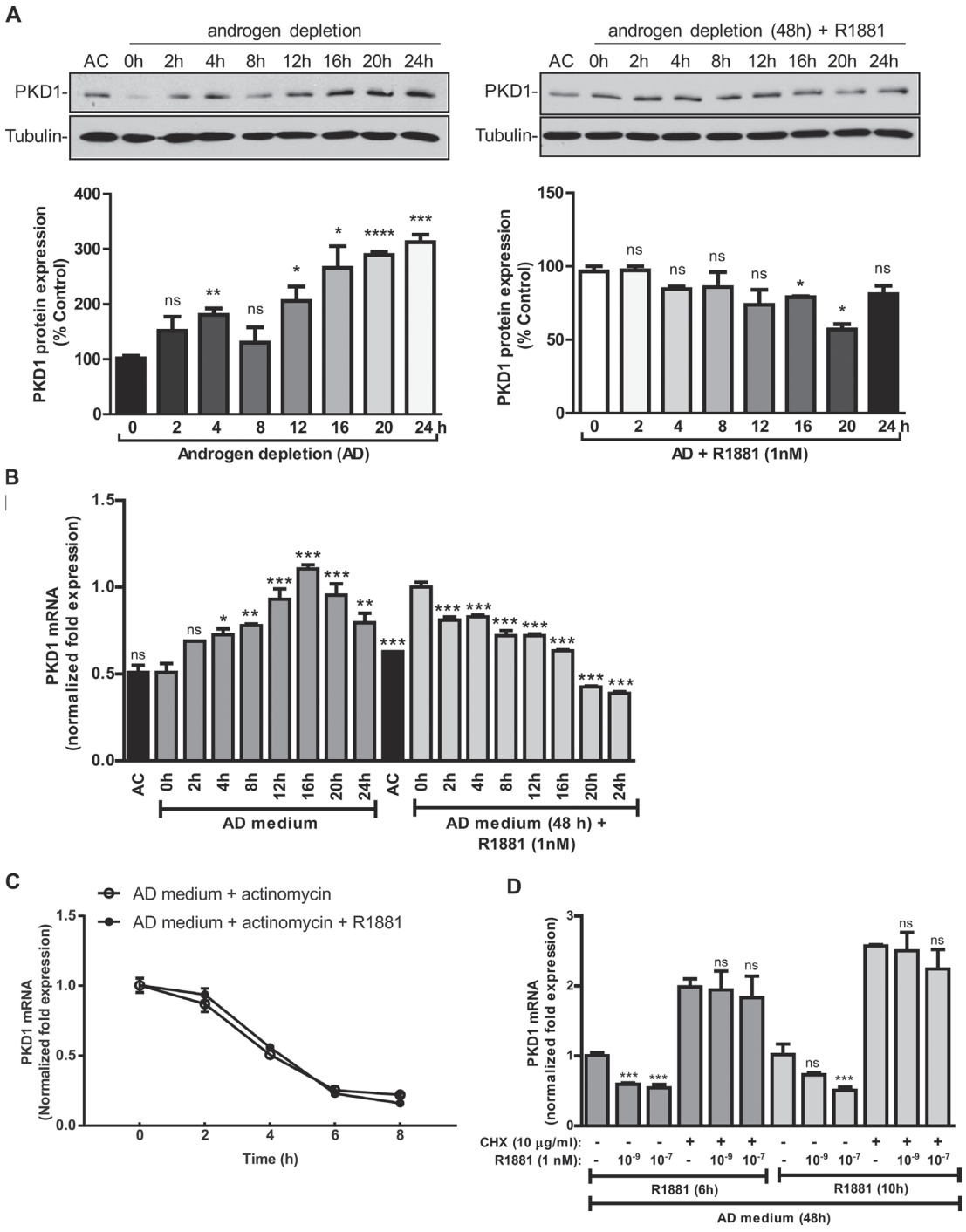


Figure 2: PKD1 expression was dependent of the induction of a repressor protein. (**A**) Kinetics of PKD1 regulation by androgen. *Left panels*, LNCaP cells were grown in AD medium for the indicated times. *Right panels*, LNCaP cells were grown in AD medium for 48 h, followed by treatment with R1881 (1 nM) for the indicated times. Cells were harvested and subjected to immunoblotting for PKD1 and tubulin (loading control). Cells grown in AC medium were used as the control. Representative data from one of four experiments are shown. *Bottom*, quantitative measurement of band intensity by densitometry analysis. Data are the mean ± SEM of three independent experiments. (**B**) Kinetics of PKD1 transcript expression. LNCaP cells were treated as above in "A". Total RNAs were extracted, and the kinetics of PKD1 mRNA induction/suppression were examined by real time RT-qPCR. (**C**) R1881 did not affect PKD1 mRNA stability. LNCaP cells were grown either in AD medium for 48 h, followed by the addition of actinomycin D (2 ng/mL) with or with R1881 (1 nM) for the indicated times. Total RNAs were extracted and subjected to real time RT-qPCR for analysis of PKD1 transcripts. Not significant by paired t test ($p > 0.5$). (**D**) PKD1 expression required the induction of a repressor protein. LNCaP cells were grown in AD medium for 48 h, followed by R1881 treatment with or without cycloheximide (CHX) for 6 or 10 h. Total RNAs were extracted, and the levels of PKD1 mRNA were measured by real-time RT-qPCR. *GAPDH* was used as a loading control. Data are the mean ± SEM of at least three independent experiments. ns, not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

AR mediated PKD1 repression by androgen

Androgens are important hormones for normal physiology and are responsible for certain disease conditions. Their actions are mediated by the AR, a ligand-dependent nuclear transcription factor. Androgens binds to AR after entering the cells to form an androgen-receptor complex, which then translocates to the nucleus where it binds to androgen response elements (AREs) in the promoter regions and regulates the transcription of its target genes. The actions of AR can be blocked by AR inhibitors, such as bicalutamide (Casodex) or enzalutamide (MDV3100). Bicalutamide is known to bind AR and leads to the formation of a transcriptionally inactive AR complex [33]. In this study, we sought to examine whether AR was required for the repression of PKD1 expression by R1881, and bicalutamide was used to determine whether the inhibition of AR activity affected PKD1 expression. After androgen deprivation, LNCaP and C4-2 cells were treated with R1881 at 1 nM in the presence or absence of bicalutamide (10 μM). As shown in Figure 3A, bicalutamide significantly reversed R1881-induced PKD1 repression in LNCaP and C4-2 cells. In LNCaP cells, inhibition of AR by bicalutamide also upregulated PKD1 protein expression in a concentration-dependent manner (Figure 3B). The specific role of AR was then examined using multiple AR-targeted siRNAs. Our data showed that knockdown of AR by three siRNAs targeting different regions of the AR transcript significantly blocked R1881-induced PKD1 suppression in LNCaP (Figure 3C) and C4-2 cells (data not shown). AR knockdown was confirmed by western blotting. Taken together, these data suggested that AR was required for the transcriptional repression of PKD1 gene expression caused by androgen stimulation.

To determine whether AR directly regulated the expression of PKD1, we analyzed the promoter region of PKD1, which led to the identification of two potential AREs upstream of the transcription start site (TSS). The human PKD1 gene spans ~45.7 kb. Analysis of up to 5000 bp of the promoter region upstream from the TSS revealed two putative AREs. (ARE1, 5′-AGTACTTTAAGCTCT-3′; ARE2, 5′-AGAACAAAATAAGCT-3′; (Supplementary Figure 2A). The regions (pm1 and pm2) that contained the AREs were separately cloned into the pTA-Luc reporter. Their activities were analyzed in LNCaP cells cultured in the presence or absence of androgen depletion, followed by treatment with or without R1881. Our data indicated that no luciferase activity was detected from both reporters in LNCaP cells (Supplementary Figure 2B), implying that the AREs in PKD1 promoter did not play an active role in regulating PKD1 transcription in response to androgen stimulation.

An AR co repressor screen revealed FRS2 as the potential mediator of androgen-induced PKD1 repression

The involvement of AR and an androgen-induced repressor protein prompted us to conduct an esiRNA screen that targeted 23 AR corepressors and other related proteins. LNCaP cells were transfected individually with 23 esiRNAs, followed by androgen depletion and treatment with or without R1881. Levels of PKD1 transcript were analyzed by real time RT-qPCR. In the controls, androgen depletion induced PKD1 expression, and treatment with R1881 caused over 2-fold reduction in PKD1 mRNA. As shown in Figure 4, similar to the non targeting siRNA, R1881-induced PKD1 repression was not affected by the depletion of all target genes, with the exception of FRS2. Knockdown of FRS2 by esiRNA completely reversed the repression of PKD1 transcription by R1881 (Figures 4C, 5A). In summary, FRS2 was identified as a potential repressor of PKD1 gene expression.

Androgen repressed PKD1 expression through a FGFR/FRS2/MEK/ERK pathway

The role of FRS2 was further validated using FRS2 siRNAs (si-FRS2-1, -2). Depletion of FRS2 abolished the R1881-induced suppression of PKD1 transcription, confirming FRS2 as a potential mediator of androgen-dependent PKD1 repression (Figure 5A). Furthermore, at the protein level, knockdown of FRS2 by two different siRNAs completely abrogated the downregulation of PKD1 by R1881 (Figure 5B). *FRS2* siRNAs caused significant knockdown of *FRS2* mRNA (Figure 5C). Thus, FRS2 mediated androgen-induced PKD1 repression.

The adaptor protein FRS2 is a major mediator of the FGFR signaling in normal and malignant cells. FGFR stimulation by FGF leads to the tyrosine phosphorylation of FRS2, which then forms a complex with Grb2 and Sos to activate the downstream Ras/Raf/MEK/ERK signaling pathway. Androgen-sensitive LNCaP cells express low levels of FGF2, and its expression is upregulated in response to androgen stimulation [32]. Here, we sought to determine whether the FRS2-mediated FGFR signaling pathway was involved in the regulation of PKD1 by androgen. As shown in Figure 5D, PD173074, an inhibitor of FGFR, significantly reversed R1881-induced PKD1 repression, indicating that FGFR activity was required for the inhibition of PKD1 by androgen/AR. Next, the role of the FGF-activated MEK/ERK MAPK signaling pathway was evaluated. Our data demonstrated that R1881-induced PKD1 suppression was abrogated in a concentration-dependent manner by UO126, a MEK inhibitor. Thus, MEK/ERK activity was also required for the suppression of PKD1 by androgen (Figure 5E). Since the suppression of PKD1 is likely associated with the secretion of a

FGFR ligand, we tested the effects of inhibiting secretory pathways on the expression of PKD1 using brefeldin A (BFA), a fungal metabolite and an inhibitor of intracellular protein transport that inhibits constitutive secretion from the *trans*-Golgi network. Our data indicated that BFA at 5 and 10 μM completely reversed androgen-induced PKD1 suppression (Figure 5F). In summary, our data implied that androgen suppressed PKD1 expression through an indirect FGFR/FRS2/MEK/ERK pathway in prostate cancer cells.

DISCUSSION

In this study, we present the novel findings demonstrating that PKD1 was repressed by androgen/AR at the mRNA and protein levels in androgen-sensitive prostate cancer cells, identifying PKD1 as a novel androgen-repressed gene. We further identified FRS2 as a novel mediator of androgen-induced repression of PKD1 expression. The cross-regulation of PKD1 by androgen/AR places PKD1 in the AR-induced signaling

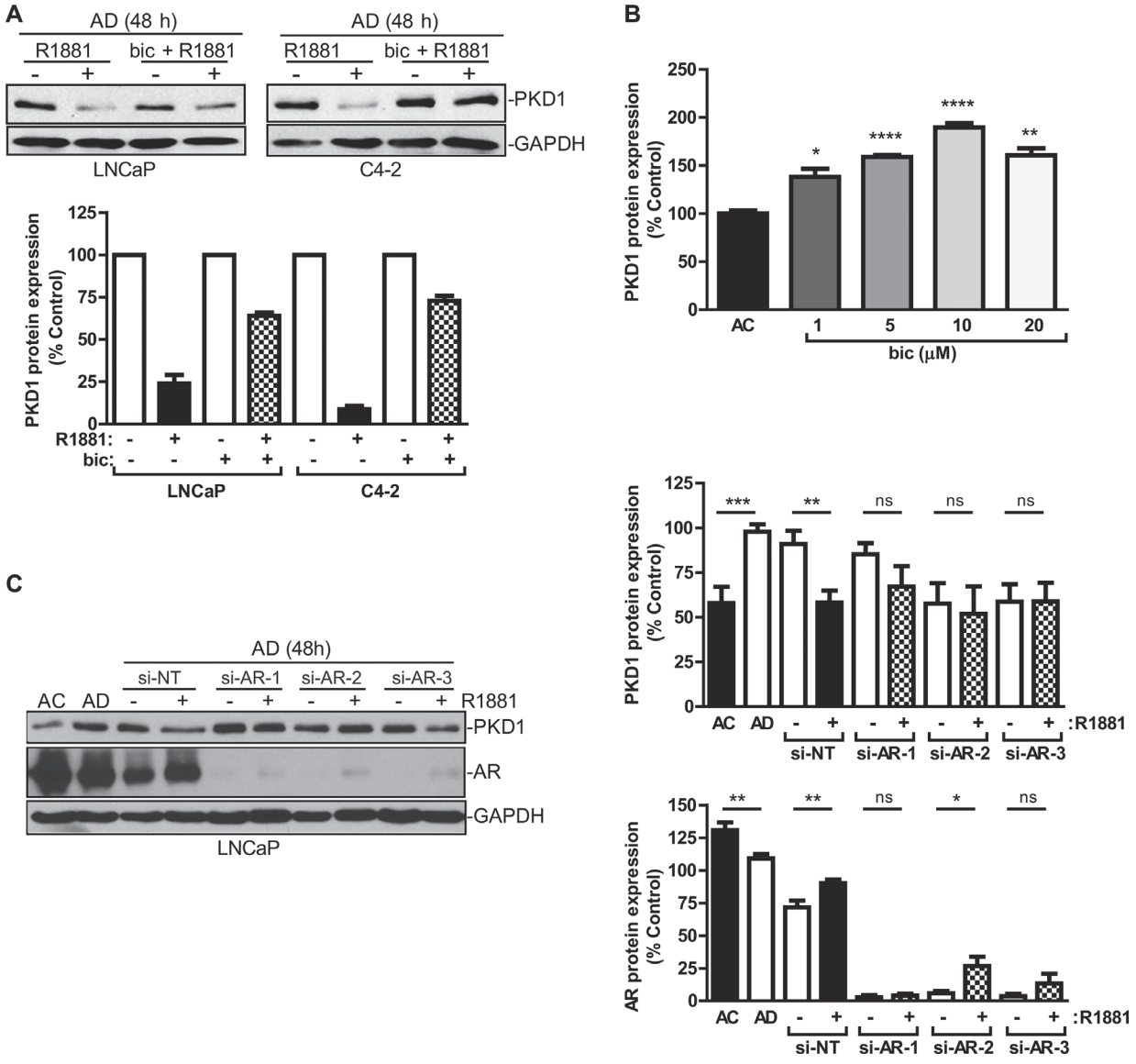


Figure 3: AR mediated PKD1 repression by androgen. (**A**) AR inhibition led to increased PKD1 expression. LNCaP and C4-2 cells cultured in AD medium for 48 h were treated with or without R1881 (1 nM) ± bicalutamide (Bic) (10 μM) for 16 h. *Bottom*, quantitative measurement of band intensity from two experiments. (**B**) Bicalutamide caused PKD1 upregulation. LNCaP cells were treated with increasing concentrations of bicalutamide for 48 h, followed by immunoblotting for PKD1. The band intensity was quantified by densitometry analysis, and data are the mean ± SEM of three independent experiments. (**C**) AR was required for transcriptional regulation of PKD1 by androgen. LNCaP cells were transfected with non targeting siRNA (si-NT) or AR siRNAs (si-AR-1, -2, -3). After 48 h, the medium was replenished with AD medium with or without R1881 (1 nM) for 16 h. Cells were collected and subjected to immunoblotting for PKD1, AR, and GAPDH. *Right*, quantitative measurement of band intensity for PKD1 (*top*) and AR (*bottom*) from three experiments is shown. ns, not significant; **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001. Data are the mean ± SEM of six independent experiments.

network, which is critical to prostate cancer progression. The transcriptional regulation of PKD isoforms has not been studied in the past. Our study provides the first mechanistic understanding of a novel androgen-induced AR/FRS2/MEK/ERK pathway that regulates the expression of PKD1. As a well-documented prosurvival signaling protein, PKD1 upregulation in response to androgen deprivation and anti-androgen treatment may have significant implications in therapy resistance and progression to CRPC.

The class I steroidal nuclear receptor AR is a critical regulator of tumor initiation and progression in both early and advanced prostate cancer. As a transcription factor, AR exerts its actions mainly through regulating the expression of a host of target genes. Among them, AR-stimulated genes have been extensively studied, with prostate-specific antigen (PSA) being the best characterized. In contrast, AR-repressed target genes have not been well characterized. These genes constitute a large portion of AR-targeted genes, and some have been shown to play essential roles in prostate cancer progression [34, 35]. Diverse mechanisms have been proposed to account for the repression of target gene expression by AR. These include both genomic mechanisms, such as active repression via the recruitment of corepressor complexes, and nongenomic mechanisms, such as regulation of signaling pathways [34]. Our data showed that inhibition or silencing of AR blocked the suppression of PKD1 by R1881, indicating that AR was required for the downregulation of PKD1. Initially, analysis of the 5′ promoter region of the PKD1 gene led to the identification of two potential AREs, which prompted us to investigate the direct role of AR in transcriptional repression of the PKD1 gene. However, analysis of the transcriptional activity of the ARE-containing PKD1 promoter failed to detect any androgen-induced transcriptional activities associated with pm1 and pm2, suggesting that the identified potential AREs may be inactive. Although less common, inactive AREs have been demonstrated, even in the presence of AR binding, and more complex mechanisms have been suggested to be involved in the regulation of genes nearby these AREs in prostate cancer cells [36, 37]. Importantly, kinetic analysis demonstrated a slow and gradual onset of PKD1 downregulation at the protein and transcript levels, which peaked at about 16–20 h in response to androgen; this finding also provides evidence against a mechanism involving active transcriptional repression through direct interaction with AREs. The involvement of an androgen-induced repressor

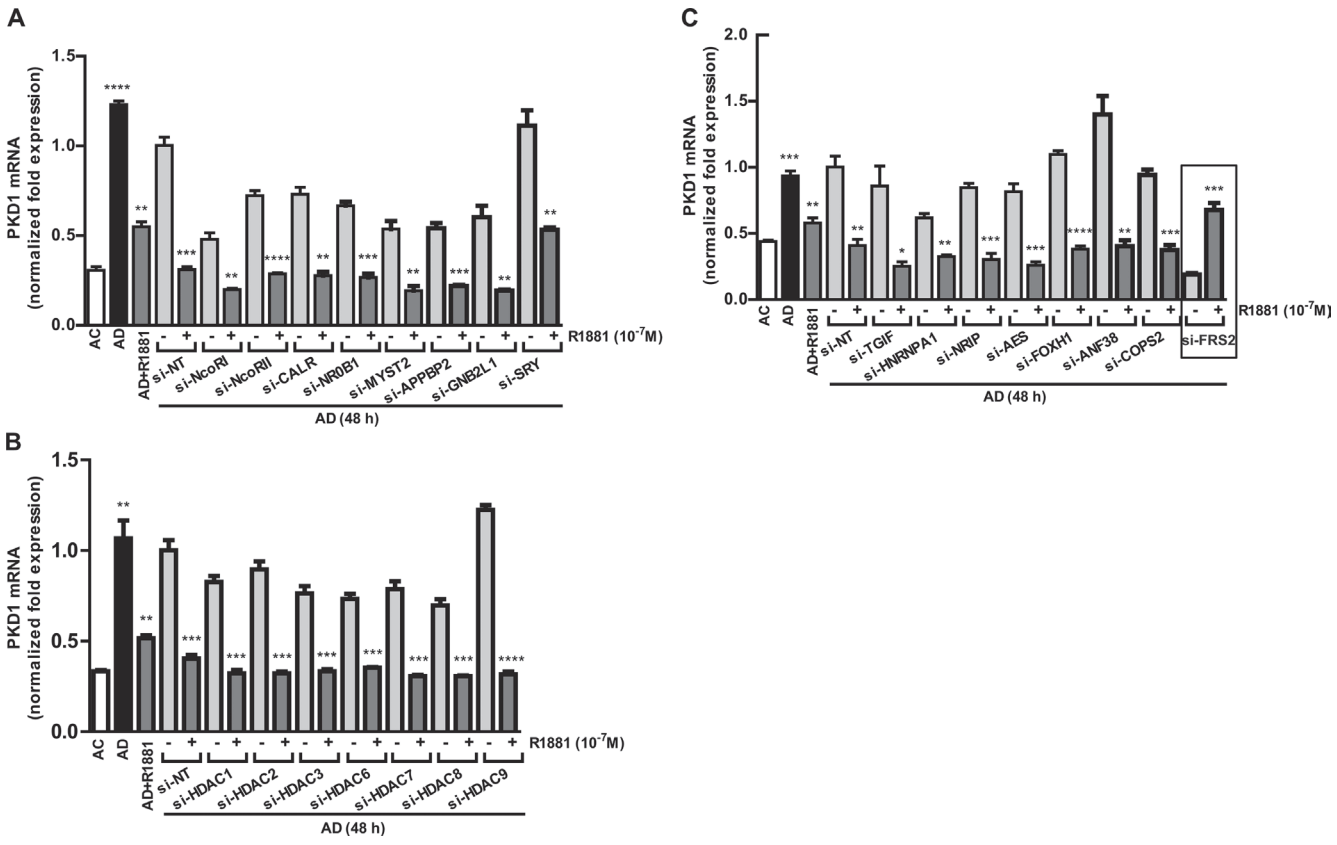
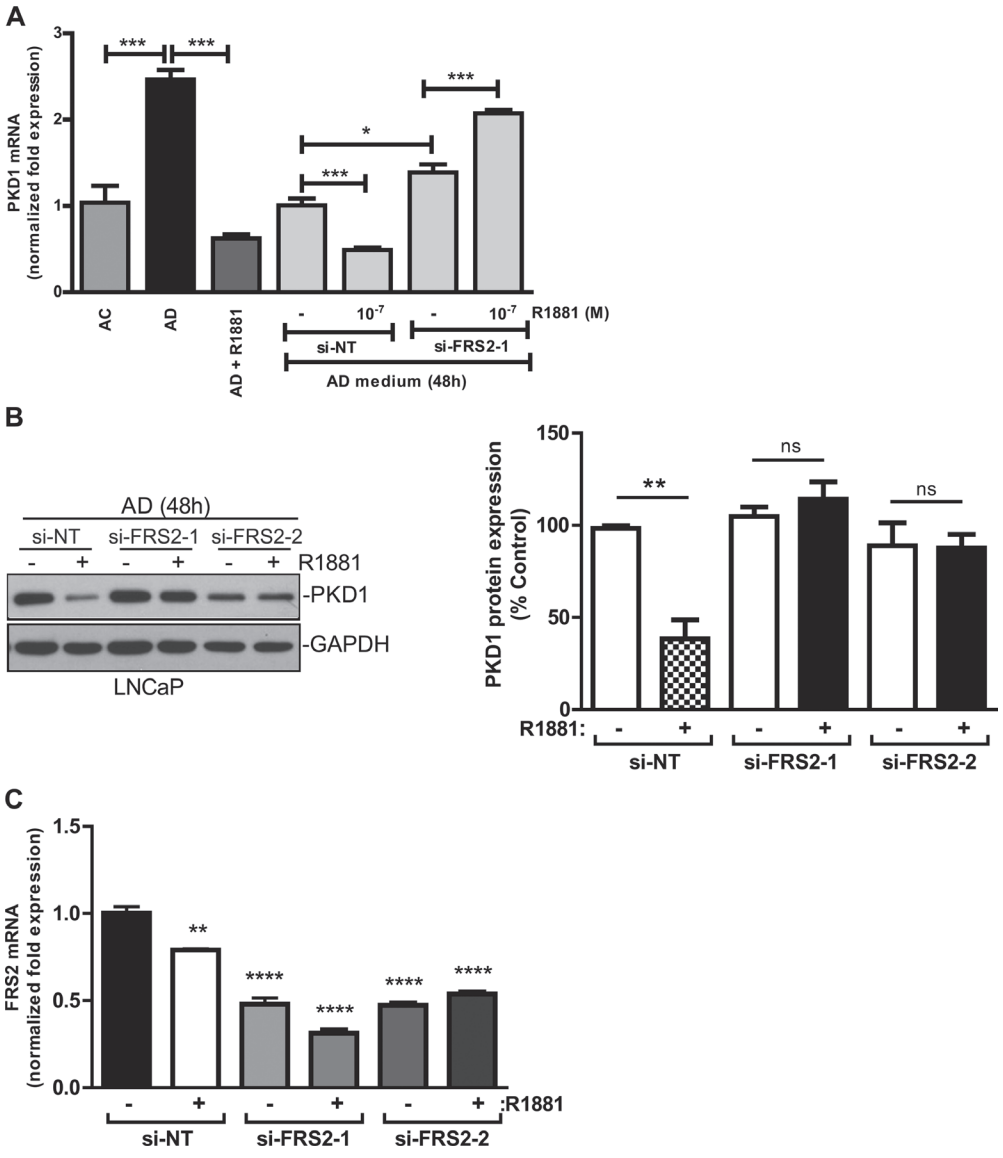


Figure 4: Screening of AR corepressors. (**A**–**C**) An esiRNA screen that targeted 23 AR corepressors and other related proteins was conducted in LNCaP cells. The cells transfected with esiRNAs were subjected to androgen depletion for 48 h, followed by treatment with or without R1881. Levels of PKD1 transcript was analyzed by real time RT-qPCR. Non targeting siRNA (si-NT) was used as the control. Student's *t*-tests were used to determine the statistical significance between the untreated and R1881-treated groups within each pair of esRNA knockdown samples. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

is supported by the data showing that CHX abolished androgen-induced PKD1 repression. Although this may also occur without the synthesis of a repressor, for example, the suppressive effects of AR could be mediated through its interaction with a pre-existing labile protein at the AR-repressed loci; CHX treatment will similarly abolish the repressive effect mediated by this labile protein. Certainly, our findings do not exclude the possibility that there may be distal ARE sites that bind to AR and contribute to AR-mediated PKD1 repression. Overall, our current data support an AR-mediated indirect mechanism involving the cell surface adaptor protein FRS2 in the repression of PKD1 by androgen. These findings were based on an unbiased RNAi screen of a library of AR corepressor proteins. Further analysis validated the role of FRS2, as well as its upstream FGFR and the downstream MEK/ERK pathway, in the regulation of PKD1 by androgen.

In androgen-sensitive prostate cancer cells, depletion of FRS2 blocked R1881-induced PKD1 suppression at both the transcriptional and protein levels. Additionally, inhibition of FGFR and MEK, as well as protein secretion, blocked R1881-induced repression of PKD1. Thus, androgen may repress PKD1 through an AR-induced FGFR/FRS2/MEK/ERK pathway to inhibit PKD1 expression in prostate cancer cells. A previous study showed that FRS2 expression is not regulated by androgen in LNCaP cells [31]. However, in androgen-sensitive LNCaP cells, low levels of FGF2 are detected, and the expression of FGF2 is upregulated in response to androgen stimulation [32]. Additionally, androgen stimulates the activity and production of FGF2 and FGF-binding protein in PC3 prostate cancer cells with stably overexpressed AR [38]. In a different study, however, Kassen et al. showed that FGF2 is not expressed, and androgen in turn acts by increasing the bioavailability of FGF2 by releasing trapped FGF2 from the extracellular matrix through activation of heparinase, which leads to activation of FGFR and stimulation of LNCaP cell proliferation



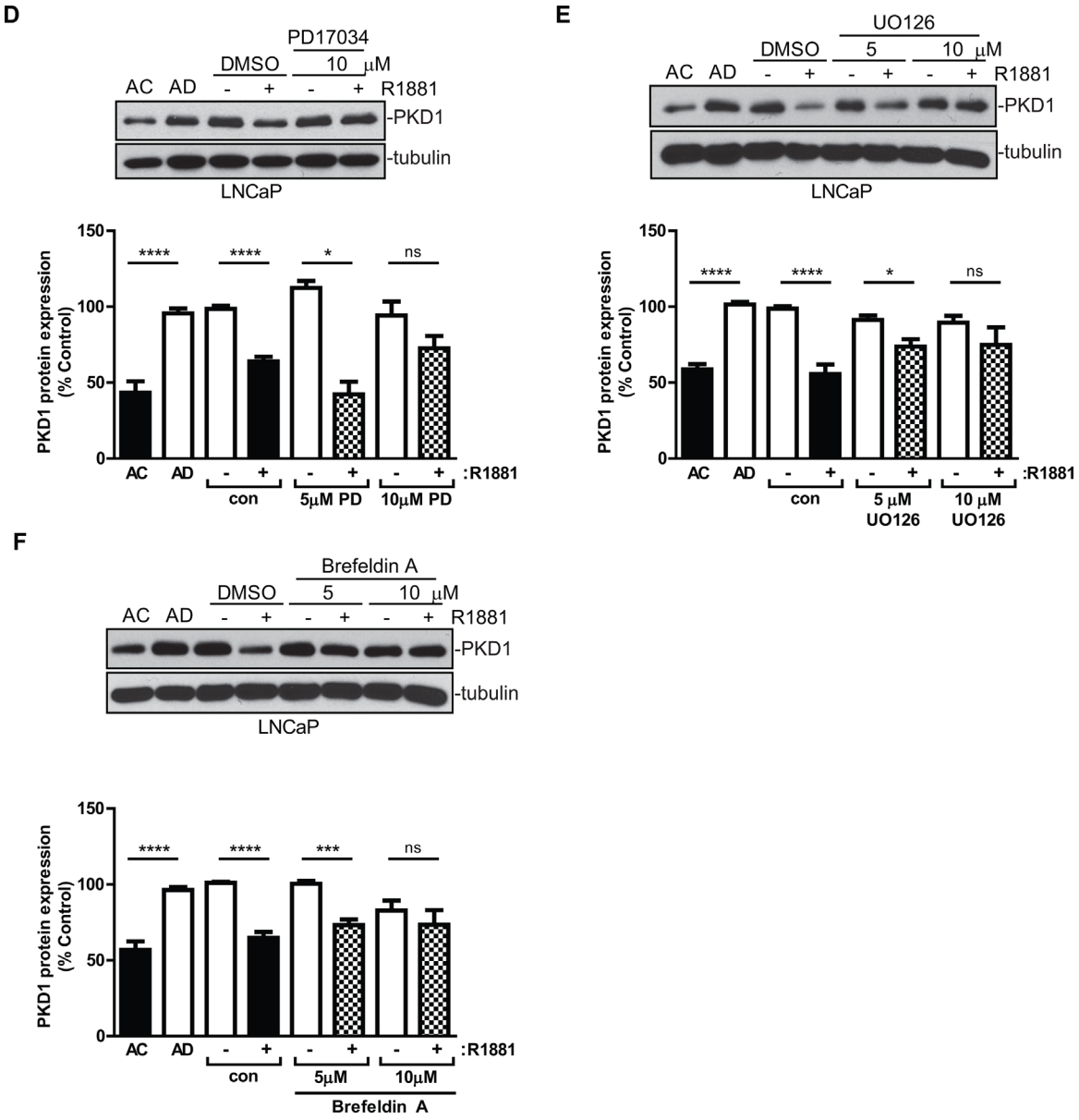


Figure 5: FRS2 was required for androgen-induced PKD1 repression. (**A**) Knockdown of FRS2 reversed androgen-induced repression of PKD1 transcription. Cells were transfected with FRS2 siRNA (si-FRS2-1) and a non targeting siRNA (si-NT), followed by treatment with or without R1881. PKD1 transcripts were analyzed by real-time RT-qPCR. Representative data from one of three independent experiments with triplicate measurements are shown. (**B**) Knockdown of FRS2 blocked the repression of PKD1 protein by androgen. LNCaP cells were transfected with two different FRS2 siRNAs (si-FRS2-1, -2), followed by treatment with R1881. *Right*, quantitative measurement of band intensity for PKD1 from three experiments is shown. (**C**) Real-time RT-PCR confirmed the knockdown of FRS2. Cells from "B" were subjected to RNA extraction, followed by real-time RT-PCR for levels of PKD1 transcript. (**D**–**F**) Androgen repression of PKD1 was dependent on a secretory pathway involving FGFR and MEK. LNCaP cells were grown in AC or AD medium for 48 h, followed by treatment with or without R1881 in the presence or absence of the FGFR inhibitor PD17034 (D), the MEK inhibitor UO0126 (E), and brefeldin A (F) for 16 h. Cell lysates were subjected to immunoblotting for PKD1. Representative images from one of at least three independent experiments are shown. *Bottom*, quantitative measurement of band intensity by densitometry analysis. Data are the mean ± SEM of five to seven independent experiments.

[39]. Regardless of these discrepancies, in all cases, AR promotes the activation of FGFR in prostate cancer cells, which results in phosphorylation of FRS2 and activation of the downstream Ras/Raf/MEK/ERK signaling pathway. By inhibiting MEK activity, we confirmed the requirement for MEK/ERK signaling activity in the suppression of PKD1 by R1881. This evidence supports the notion that PKD1 is repressed by an AR-induced FGFR/FRS2/MEK/ERK pathway in androgen-sensitive prostate cancer cells. The binding of FGF to FGFR leads to the recruitment

of multiple adaptor proteins, including FRS2, Grbs, Sos, and Gab1, and induces the activation of multiple downstream signaling pathways, including MEK/ERK, PI3K/Akt, PLCγ/PKC, and Stat3 pathways. We must state that although our data demonstrated a major role of the MEK/ERK pathway in the regulation of PKD1 expression by androgen, our data did not completely exclude the potential involvement of other pathways, which will be investigated in our future studies.

Our study identified PKD1 as an androgen/AR-repressed gene and uncovered a novel indirect mechanism through which AR regulates PKD1 expression. Although the functional implication of this regulation in prostate cancer progression is still unclear, PKD1 is an important prosurvival signaling protein in normal and cancer cells that functions by regulating multiple signaling pathways, such as stimulating NF-κB, ERK1/2, and Akt and inhibiting JNK and p38 [20, 25, 40]. This notion is further supported by our previous findings that PKD1 protects androgen-sensitive LNCaP prostate cancer cells from phorbol ester-induced apoptosis [25]. Thus, the upregulation of PKD1 as a result of inhibition or loss of AR may promote tumor cell survival and contribute to therapeutic resistance to AR-targeted agents. This further implies that PKD may represent a viable target for mitigating therapy resistance. In castration-resistant C81, 22Rv1, and VCaP cells, we observed different responses to androgen in terms of PKD1 regulation; although androgen did not affect PKD1 expression in 22Rv1 cells, VCaP cells, which express wild-type AR, did respond to androgen stimulation by downregulating PKD1 in a concentration-dependent manner, and minor effects were also observed in C81. This cell context-dependent responsiveness to androgen may be linked to the activity of the AR/FGFR/FRS2 signaling pathway and variations in the expression of its signaling components.

In summary, our study identified PKD1 as a novel androgen/AR-suppressed gene. The suppression of PKD1 was mediated through an indirect mechanism that involved FRS2, a cell surface adaptor protein that connects FGF/FGFR to the downstream MEK/ERK signaling pathway. Our findings suggested that the prosurvival function of PKD1 may have significant implications in prostate cancer progression and therapy resistance. PKD1 may be targeted to enhance the therapeutic response to anti-androgens in prostate cancer treatment.

MATERIALS AND METHODS

Reagents and antibodies

The synthetic androgen methyl trienolone (R1881) was obtained from Perkin-Elmer Life Sciences (Boston, MA), and bicalutamide was purchased from Enzo Life Sciences (Farmingdale, NY). Charcoal-treated fetal bovine serum (FBS) was from Hyclone (Logan, UT) and Sigma (St. Louis, MO). Other cell culture reagents and media were from American Type Culture Collection (ATCC; Rockville, MD). Anti-PKD1, anti-PKD2, and anti-AR antibodies were purchased from Cell Signaling Technology (Danvers, MA). Antibodies targeting GAPDH and α-tubulin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat anti-rabbit and goat anti-mouse horseradish peroxidase-conjugated secondary antibodies were from Promega (Madison, WI).

Cell culture and siRNA transfection

LNCaP, C4-2, C81, VCaP, and PC-3 cells were obtained from ATCC (Manassas, VA) and were cultured according to the manufacturer's recommendations. LNCaP cells were discarded after 12 passages. The 23 AR corepressor esiRNAs were obtained from Sigma-Aldrich (Supplementary Table 1). The non targeting siRNA and AR and FRS2 siRNAs were obtained from Integrated DNA Technologies (Coralville, IA). The esiRNAs and siRNAs were transfected into cells using DharmaFECT reagent according to the manufacturer's instructions (GE Dharmacon, Lafayette, CO).

Western blot analysis

Cells were collected and lysed in IP lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 5 mM EGTA, 20 μM leupeptin, 1 mM AEBSF, 1 mM $NaVO_3$, 10 mM NaF, and 1× protease inhibitor cocktail). Lysate protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher, Hudson, NH). Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% milk, the membranes were incubated with primary antibodies in blocking buffer at 4°C overnight. After washing, the membranes were incubated with secondary antibodies at room temperature for 1 h. Protein bands were detected using an enhanced chemiluminescence (ECL) kit. Anti-α-tubulin or anti-GAPDH antibodies were used as a loading control. Densitometry analyses were performed with ImageJ software (NIH).

Real-time RT-PCR

Total RNAs were isolated from LNCaP cells using a RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. One microgram of total RNAs was used to generate cDNA using an iScript cDNA synthesis kit. Real-time PCR was subsequently performed using SsoFast EvaGreen Supermix on a CFX96 Real-Time PCR Detection System (Bio-Rad, Richmond, CA, USA). The following primers were used: PKD1, forward primer 5′-CGCACATCATCTG CTGAACT-3′ and reverse primer 5′-CTTTCGGTGCA

CAACGTTTA-3′; FRS2, forward primer 5′-ATGG GAATGAGTTAGGTTCTGGC-3′ and reverse primer 5′-GCGGGTGTATAAAATCAGTTCTGTG-3′. Data were normalized automatically by using GAPDH as the loading control, with the following primers: forward primer 5′-GCAAATTCCATGGCACCGT-3′ and reverse primer 5′-TCGCCCCACTTGATTTTGG-3′.

Statistical analysis

All statistical analyse were carried out using GraphPad Prism IV software. A p value of less than 0.05 was considered statistically significant ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$).

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Review

Protein kinase D signaling in cancer: A friend or foe?

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ABSTRACT

Protein kinase D is a family of evolutionarily conserved serine/threonine kinases that belongs to the Ca^{++}/Calmodulin-dependent kinase superfamily. Signal transduction pathways mediated by PKD can be triggered by a variety of stimuli including G protein-coupled receptor agonists, growth factors, hormones, and cellular stresses. The regulatory mechanisms and physiological roles of PKD have been well documented including cell proliferation, survival, migration, angiogenesis, regulation of gene expression, and protein/membrane trafficking. However, its precise roles in disease progression, especially in cancer, remain elusive. A plethora of studies documented the cell- and tissue-specific expressions and functions of PKD in various cancer-associated biological processes, while the causes of the differential effects of PKD have not been thoroughly investigated. In this review, we have discussed the structural-functional properties, activation mechanisms, signaling pathways and physiological functions of PKD in the context of human cancer. Additionally, we have provided a comprehensive review of the reported tumor promoting or tumor suppressive functions of PKD in several major cancer types and discussed the discrepancies that have been raised on PKD as a major regulator of malignant transformation.

1. Introduction

Protein kinases are of utmost importance in maintaining a battery of cellular activities and the human genome encodes over 500 protein kinase genes which constitute about 2% of all human genes, collectively named as the human kinome [1]. The protein kinase D (PKD) family of serine/threonine kinases falls into the Ca^{++}/Calmodulin-dependent protein kinases (CaMKs) superfamily and consists of three isoforms in mammals, notably, PKD1, PKD2 and PKD3. PKD1 was the first member identified in human and mouse in 1994 [2,3], although initially it was categorized as a member of the protein kinase C (PKC) family and named PKCμ [2,4]. It was later reclassified into the CaMK family based on sequence homology in the catalytic domain. PKD3 and PKD2, two additional PKD isoforms, were discovered thereafter [5,6].

PKDs are evolutionarily highly conserved and homologs are found in several organisms including mice (*Mus musculus*), rats (*Rattus norvegicus*), flies (*Drosophila melanogaster*) and yeast (*Saccharomyces cerevisiae*) [7]. There is also high sequence homology among PKD isoforms, although structural and functional differences have been noted. For example, PKD3 lacks PDZ (PSD-95/Discs large/ZO-1) binding motif [8] and a Src family kinase phosphorylation motif [9]. Among other organisms, *D. melanogaster* possesses only one PKD gene [10], whereas, two orthologs termed *dfk-1* and *dfk-2* are present in *C. elegans* [11–13]. In a canonical pathway, various stimuli including hormones, phorbol esters, growth factors, cellular stress converge to the activation of PKDs through diacylglycerol (DAG) and classical or novel protein kinase C (c/nPKC) via active phospholipase C (PLC) β and γ [14,15]. Activated PKD resides in diverse subcellular locations such as cytosol, Golgi apparatus, nucleus, mitochondria to regulate a plethora of cellular functions, especially those related to malignant transformation including cell proliferation, growth, migration/invasion, apoptosis, epidermal-to-mesenchymal transition (EMT) [14,16,17].

In this review, we discuss the *status quo* of PKD isoforms in terms of their modulation of different physiological activities and mechanistic role in development and progression of human diseases focusing on cancer. In light of accumulating scientific evidence, we aim to provide an updated and comprehensive review of each of the PKD isoforms, their differential expression patterns, and how they communicate with the cellular machineries in a wide variety of cell and tissue types to coordinate its biological role in oncogenesis (Table 1). Furthermore, we discuss potential tumor promoting as well as suppressive properties of PKD in different cancer types and aim to resolve the prevailing

Abbreviations: PKC, protein kinase C; PKD, protein kinase D; PLC, phospholipase C; DAG, diacylglycerol; CRD, cysteine-rich domain; GPCR, G protein-coupled receptor; TNF, tumor necrosis factor; TGN, trans-Golgi network; EMT, epithelial-to-mesenchymal transition; MMP, matrix metalloprotease; HDAC, histone deacetylase; BMP, bone morphogenic protein; AR, androgen receptor; RUNX, Runt-related transcription factors

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Table 1
Summary of differential expression and physiological roles of PKD isoforms in major cancers.

Cancer type	PKD isoform	Function in tumorigenesis	Mode of action	References
Prostate	PKD1	Anti-tumorigenic	1. Downregulates AR.	[124]
			2. Represses AR-dependent transcription of target genes by modulating Hsp27.	[126]
			3. phosphorylates E-cadherin and regulates β-catenin activity.	[125]
		Pro-oncogenic	Wnt5 signaling pathway activation of Jun-N-terminal kinase.	[91]
	PKD2, PKD3	Pro-oncogenic	1. Modulates Akt and ERK1/2 activation.	[128,190]
			2. Activates NF-κB signaling, elevates urokinase-type plasminogen activator (uPA) and MMP-9 expression.	[90]
Breast	PKD1	Anti-tumorigenic	1. Phosphorylates and inactivates SSH1L, interacts with cortactin and paxillin.	[81,83,137,141]
			2. Suppression of MMP expression.	[81]
			3. Inhibition of EMT via inactivation of Snail function and modulation of epithelial-to-mesenchymal markers	[139]
		Pro-oncogenic	Promotes cell-cell adhesion.	[142,143]
	PKD2/PKD3	Pro-oncogenic	1. Promotes Hsp27 and HDAC4/5/7 phosphorylation.	[144]
			2. Upregulates P-glycoprotein (P-gp) expression, multidrug resistance.	[145]
Pancreas	PKD1	Pro-oncogenic	1. Activates MEK1/2 initiated by neurotensin, increases DNA replication.	[155,156]
			2. Inhibits CD95-mediated apoptosis, cell proliferation and survival.	[153]
			3. Acts downstream of TGFα/Kras and mediates formation of ductal structures through activation of the Notch pathway.	[154]
	PKD2	Pro-oncogenic	1. PKD2 activation and increase in Ser^{82} phosphorylation of Hsp27 in PANC-1 cells stimulated by neurotensin.	[157]
			2. Stimulates expression and secretion of MMP-7 and 9, induces tumor angiogenesis by releasing ECM-bound VEGF-A.	[162]
Skin	PKD1	Pro-oncogenic	1. Modulates ERK/MAPK pathway and promotes proliferation.	[166]
			2. Reduces UVB-induced apoptosis.	[167]
	PKD2	Anti-tumorigenic	Correlated with upregulation of CDK4/6 inhibitor $p15^{INK4B}$ and induction of p53-independent G1	[169]
	PKD3	Pro-oncogenic	cell cycle arrest.	[169]
Gastric	PKD1	Anti-tumorigenic	1. Epigenetically silenced.	[170]
	PKD2	Pro-oncogenic	1. Promotes AKT and ERK signaling.	[177]
			2. Inhibits apoptosis.	[177]
			3. Suppresses NF-κB signaling.	[177]

functional discrepancies it poses as a regulator of cancer.

2. PKD structural and functional relationships, phosphorylation, activation

All 3 members of the PKD family share discrete structural and functional similarities (Fig. 1). They possess an N-terminal regulatory domain which is subdivided into 2 tandem cysteine-rich Zn-finger like domains (CRD, C1a and C1b), a plekstrin homology (PH) domain and a C-terminal catalytic domain [14,16]. The regulatory domain plays a critical role by auto-inhibiting the kinase domain and maintaining the protein in an inactivated state and thus deletion or mutation of critical residues of the C1 domains results in constitutively active PKD [14]. The C1 domains bind DAG and phorbol esters, anchor PKD to membranes and modulate the localization of the protein to Golgi, nucleus as well as plasma membrane [14,18,19]. Although the C1 domains are active in PKD, there are intrinsic differences in their activity and selectivity for ligands. Our study has demonstrated isoform-specific

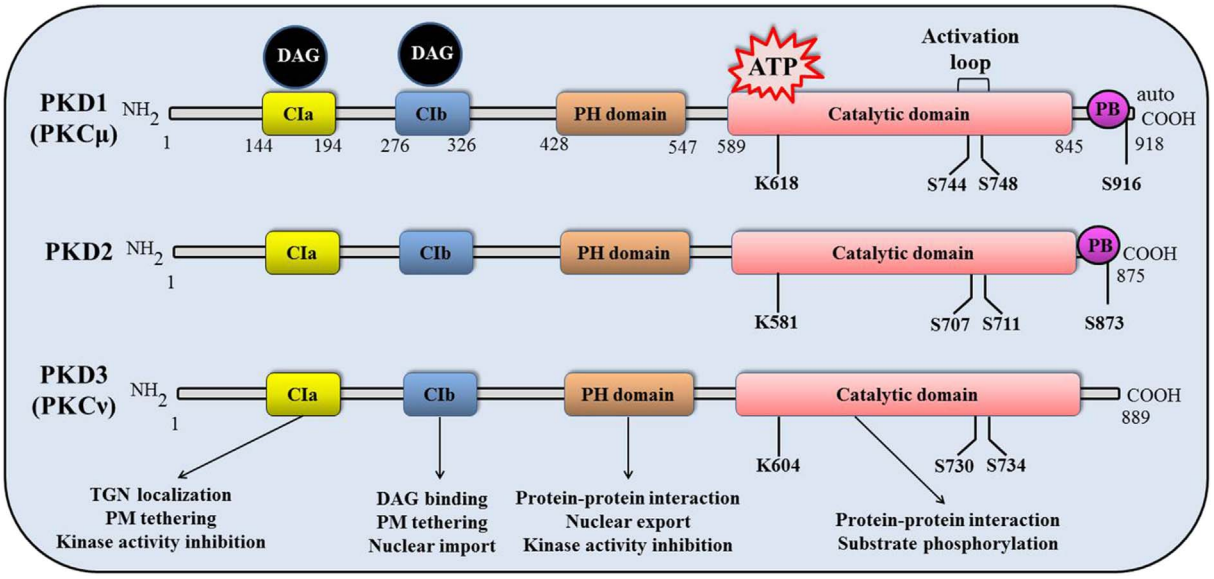

Fig. 1. Diagram of protein kinase D structure. The structure of PKD contains an N-terminal regulatory domain which consists of cysteine-rich Zn-finger like motifs (CIa and CIb), a plekstrin homology (PH) domain and a C-terminal catalytic domain, which are shared by all three isoforms. However, PKD3 lacks C-terminal PDZ binding (PB) domain as it is present in both PKD1 and PKD2. The serine residues shown in the catalytic domain represent the conserved activation loop amino acids phosphorylated by the members of the c/nPKCs, which leads to PKD activation. Note: Phosphorylation sites are numbered based on the murine PKD isoforms.

differences in the ligand binding activities of PKD isoforms with PKD3 being most sensitivity to DAG/phorbol esters [20]. As for individual C1 domains, we have shown that C1a is a high affinity receptor for DAG, while C1b is low affinity for DAG but high affinity for phorbol esters. In the context of full-length protein, C1a in several studies appears to play a more crucial role in DAG binding and membrane targeting of PKD [19–22]. Furthermore, the reduced DAG binding activity of C1b is attributed to a conserved lysine residue within this domain that impairs DAG binding [20,23]. Overall, C1 domain is central to the spatial and temporal regulation of PKD localization at different subcellular locations. In addition to the intrinsic differences of the twin C1 domains [20,24,25], their ligand binding activity, selectivity, and accessibility to ligand in a holoenyme are intricately regulated by PKD phosphorylation [22], kinase activity [19,21,22] and interactions with protein binding partners [26], and this regulation becomes more complex with embedded nuclear localization and export signals within the structure of C1 domain [27]. Whether, how, and how much they contribute to the differential biological functions of PKD isoforms remain to be determined.

The PH domain has been shown to interact with other proteins and play a role in subcellular localization as well as nuclear export of PKD [14]. The type 1 PDZ (PSD-95/Discs large/ZO-1) binding motif is found at the C-terminus of PKD1 and PKD2 which is responsible for interaction with protein substrates such as NHERF-1 and Kidins220 [8,28]. The substrate recognition motif of PKD1 (L.X.R. (Q/K/E/M).S.X.X.X.X) displays a unique preference of leucine at the −5 position [16,29,30]. There are minor structural differences in PKD2 and PKD3. For example, PKD2 contains a serine-rich region between C1a and C1b motifs and PKD3 lacks any C-terminal PDZ domain [14]. PKD can be activated by a variety of physiological factors, such as bioactive peptides [31], lipids [32], growth factors [33], tumor necrosis factor (TNF) [34], chemokines, and many of which act through binding and activating the G protein-coupled receptors (GPCR) or receptor tyrosine kinase (RTK) and further activating phospholipase Cs (PLCs) and c/nPKCs. c/nPKCs phosphorylate the conserved serine residues in the activation loop of PKDs (for example, Ser^{744} and Ser^{748} of murine PKD1, equivalent to Ser^{738} and Ser^{742} of human PKD1), leading to PKD activation [14,15]. Mutation of active site serine residues in PKD (PKD1 S744A/S748A) abolishes PKD activation [16,17]. Replacement of both serine residues with glutamic acid (PKD1 S744E/S748E) results in a constitutively active PKD implying that activation loop phosphorylation is an essential mechanism for PKD activation [14]. In further analysis of the canonical PKC-dependent activation of PKD pathway governing the functional facets of PKD in cellular physiology, emerging evidence suggests that different regulatory mechanisms control the phosphorylation at the two sites in the activation loop. For instance, Gq-coupled receptor agonists such as bombesin induce biphasic PKD activation, notably, a first rapid PKC-dependent activation through phosphorylation of PKD1 at Ser^{744}, followed by a second PKC-independent autophosphorylation at Ser^{748} to sustain PKD activity [35,36]. Several lines of evidence emerged for supporting the fact that PKD1 autophosphorylation at Ser^{748} is the major mechanism for late sustained PKD activation in cells treated with GPCR agonists [35]. Beyond the activation loop phosphorylation, there are considerable discrepancies in the understanding of an autophosphorylation site, PKD1 Ser^{916} (equivalent to human PKD1 Ser^{910}), which has been used as a marker of PKD1 activity status in many studies [37]. The lines of evidence supporting its use as a marker for PKD activation are: 1) PKD1 Ser^{916} phosphorylation increases when PKD1 is activated by growth factor receptors or phorbol esters, 2) constitutively active $PKD1^{S744E,\ S748E}$ exhibits high levels of endogenous Ser^{916} phosphorylation [37,38]. It was conceived that activation of PKD through phosphorylation of Ser^{744} and Ser^{748} residues is often followed by autophosphorylation of Ser^{916} [14,37,39,40]. However, other evidences support the contrary: 1) PKD1 activation loop phosphorylation and increase in catalytic activity by agonist stimulation do not augment Ser^{910} phosphorylation [41–43], 2) A catalytically inactive PKD1 mutant ($PKD1^{K612W}$) displays Ser^{910} phosphorylation by endogenous PKD1 and other enzymes [8,44], 3) Ser^{910} autophosphorylation can be achieved at exceedingly low concentration of ATP that does not require PKD1 phosphorylation of $Ser^{738,742}$. Additionally, the phosphorylation status on Ser^{910} does not correlate well with the inhibition of PKD by certain inhibitors, for example, Ser^{910} phosphorylation is resistant to Gö6976, an ATP competitive inhibitor [44] and another ATP competitive inhibitor, BPKDi that inhibits HDAC5 phosphorylation, does not inhibit PKD1 Ser^{910} autophosphorylation [45]. Further studies are necessary to define the precise role of PKD1 Ser^{910} autophosphorylation and its implication in the regulation of PKD1 catalytic activity.

3. Protein kinase D and somatic mutations in cancer

Benign cells acquire somatic mutations which cause dysregulation of cell proliferation, migration and invasion, key phenomenons for oncogenesis. Approaches through comprehensive genomics analysis have provided valuable cues to somatic aberrations that define individual cancers [46–48]. *PRKD* is generally thought to exhibit low frequency of somatic mutations in pan-cancer analysis. However, in several recent reports, high frequency somatic mutations in *PRKD* genes have been reported in at least two rare tumors, Polymorphus low-grade adenocarcinoma (PLGA) and angiolipomas [49–51].

Polymorphus low-grade adenocarcinoma (PLGA) is an intra-oral salivary gland malignancy which preferentially affects the minor salivary glands. Weinreb et al. subjected three consecutive PLGAs to massive parallel RNA sequencing and whole exome sequencing (WES) and identified two somatic heterozygous single-nucleotide variations (SNV), which are c.2130A > T and c.2130A > C, affecting highly conserved E710 amino acid at the catalytic loop, resulting in a mutant PKD1 (PKD1 E710D) [51]. To further validate the results, the authors analyzed 53 PLGAs by Sanger sequencing and targeted amplicon sequencing of *PRKD1* exon 15 and confirmed the presence of somatic c.2130A > T and c.2130A > C mutations in 41.5% and 30.2% of PLGAs, respectively and the total mutation frequency of 72.9%, representing a single high-frequency hotspot mutation that is indicative of a driver oncogene. Homology modelling of PKD1 suggested that this E710D mutation could alter coordination with Mg^{2+} ion, affect enzyme kinetics as well as interfere with ADP-binding [51]. Cell-free *in vitro* kinase assay showed significantly increased transphosphorylation of the substrate CREBtide by $PKD1^{E710D}$ mutant protein and its elevated catalytic activity as compared to the wild type PKD1 [51]. Further analysis indicated that the expression of $PKD1^{E710D}$ mutant protein in embryonic kidney epithelial and non-malignant breast epithelial cells caused increased phosphorylation of Ser^{738}/Ser^{742} and Ser^{910} of PKD1 as compared to the wild type PKD1. It was also demonstrated that forced expression of $PKD1^{E710D}$ mutant protein in MCF10A and MCF12A breast cells changed the hollow spheroid, acinar-like structures into large, coalescent structures filled with lumens and irregular contours, an increased invasive phenotype typically associated with the overexpression of other oncogenes in this model system [52,53]. Taken together, these results demonstrate that the somatic mutation in PKD1 encoding $PKD1^{E710D}$ is likely activating driver of PLGA and confers a neoplastic advantage to the epithelial cells [51]. There was another report which aimed to identify somatic mutations in PLGA that affect the kinase domains of *PRKD2* or *PRKD3* gene and act as a driver of neoplasia [50]. This study found PLGAs that lack *PRKD1* somatic mutations or PKD gene family rearrangement; do not harbor somatic mutations in the kinase domains of *PRKD2* or *PRKD3* genes. These findings appear to bring up an interesting concept that PKD1 is not functionally equivalent to PKD2 and PKD3 in tumorigenesis. There is a lack of evidence for the somatic mutations and their effect on the biology of adipocytic tumors, including angiolipoma as no genetic aberrations or chromosomal rearrangements/deregulations have been reported [54]. In a recent report, Hofvander et al. analyzed a cohort of

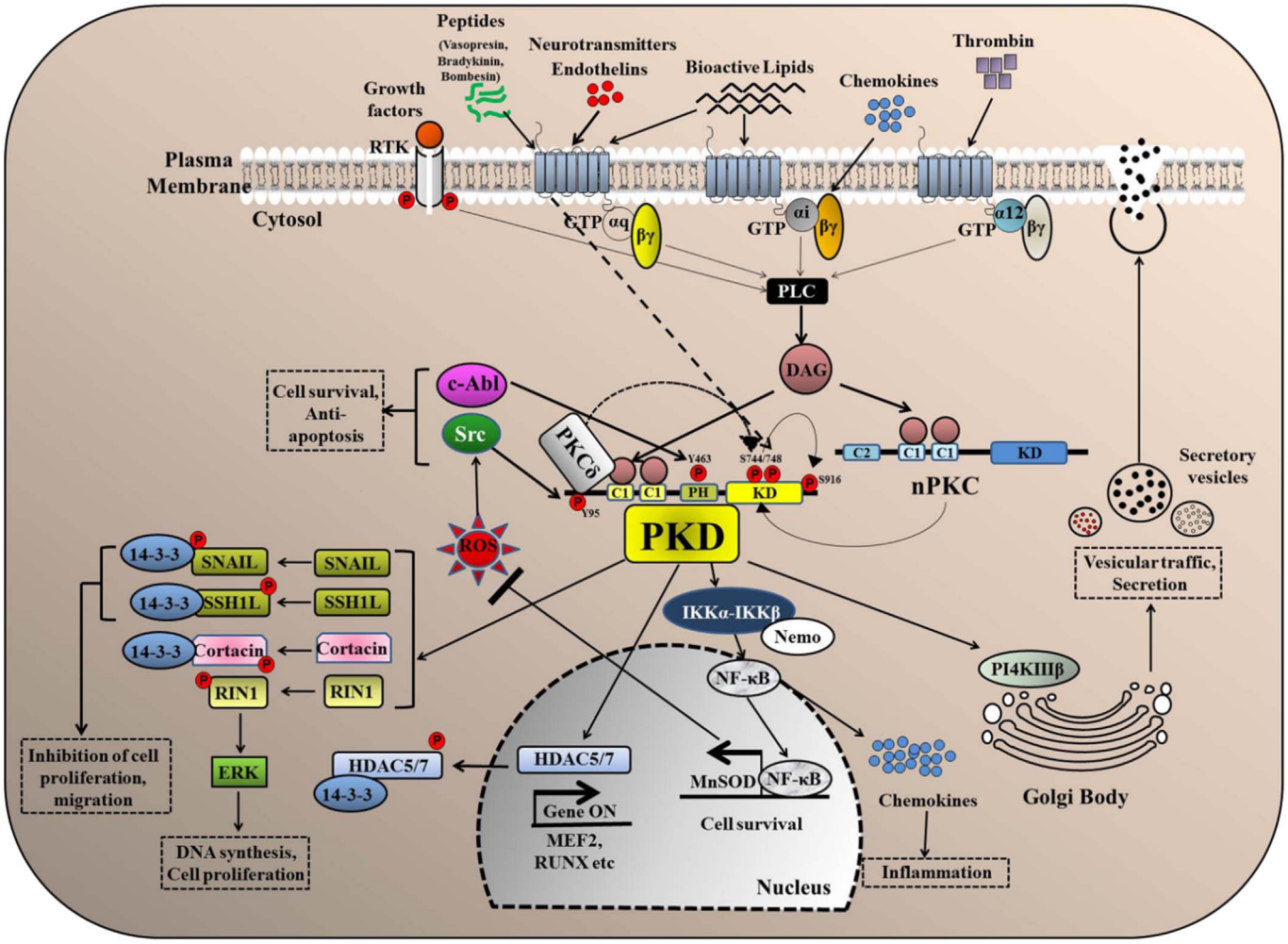

Fig. 2. Major signaling pathways and biological functions of PKD. Several extracellular stimuli activate phospholipase C (PLC) which catalyzes the formation of diacylglycerol (DAG). DAG recruits PKD and PKC to the plasma membrane inducing the activation of PKC which then further phosphorylates PKD at two serine ($Ser^{744,748}$) residues resulting in the activation of PKD. PKD can also be activated by PKC-independent pathways and the activity of PKD can be sustained through autophosphorylation at Ser^{748} residue (dashed line). Activated PKD regulates an array of cancer-associated functions including cell proliferation, migration, survival, regulation of gene transcription, protein/vesicle trafficking and secretion through several major signaling pathways.

benign adipocytic tumors including conventional lipoma, hibernoma and angiolipoma by WES and ultra-deep sequencing and demonstrated the presence of somatic mutations (18 out of 21) in the catalytic domain of PKD2 [49]. qRT-PCR confirmed that the level of *PRKD2* but not *PRKD1* or *PRKD3* was higher in angiolipoma than in lipomas. The authors argued that the finding of *PRKD2* mutations in 80% of the tumors strongly correlate with the neoplastic origin of angiolipoma. Further studies are necessary to evaluate the significance of the mutations in the catalytic domain of *PRKD2* gene. Collectively, these studies not only highlight the significance of PKD family in oncogenesis, but also reveal its potential utility as molecular diagnostic markers or therapeutic targets for certain tumors.

4. PKD regulates major biological processes that contribute to development and progression of cancer

PKD contributes to a broad spectrum of cellular processes including cell survival, proliferation, EMT, angiogenesis, gene transcription, secretion and vesicle transport through TGN and innate immunity (Fig. 2). Once activated, PKD rapidly localizes to different subcellular locations including plasma membrane, nucleus and mitochondria. In this section we discuss several major biological events and pathways regulated by PKD that directly contribute to development and progression of cancer.

4.1. Cell growth and proliferation

A major characteristic of cancer cells is uncontrolled cellular growth and proliferation. In this section, we will review the roles of PKD in modulating the biological pathways that control cell growth, proliferation and survival in context of neoplasia.

PKD is activated by many mitogenic GPCR agonists that mediate their response through $G_{q,}$ G_i and G_{12} implying a role of PKD in cell proliferation [55–58]. G_q-coupled receptor agonists including bombesin and vasopressin-induced activation of PKD1 and PKD2 and subsequent increased DNA replication and cell proliferation has been shown in Swiss 3 T3 fibroblast [36,59–61]. Extracellular-regulated protein kinase (ERK) pathway is a major mediator of GPCR agonists-induced mitogenic effects [62]. PKD contributes to the duration and intensity of MEK/ERK/RSK activation in GPCR agonist-stimulated cells leading to the induction of c-Fos that stimulates cell cycle progression [36,59].

4.2. Cell survival

Oxidative stress can be accounted as an imbalance between the systemic production of reactive oxygen species and a cell's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Abnormalities in the normal redox state of cells can cause toxic effects through the production of reactive oxygen species (ROS), such as

O_2^- (superoxide radical), OH (hydroxyl radical) and H_2O_2 (hydrogen peroxide). Oxidative stress can activate PKD via nonreceptor tyrosine kinase c-Abl and Src along with PKCδ. Reactive oxygen species (ROS) produces DAG via PLD1 and phosphatidic acid phosphatase (PAP)-mediated catalysis and further recruitment of PKD and PKCδ at the outer mitochondrial membrane [63]. A colocalized c-Abl phosphorylates Tyr^{463} in the PH domain of PKD1 which causes a conformational change, allowing the YGLY domain to be released from PH domain-mediated intramolecular autoinhibition [9]. Src-dependent phosphorylation of Tyr^{95} creates a priming site for the C1b domain of PKCδ. PKCδ efficiently activates PKD1 by phosphorylating Ser^{746}/Ser^{748}. Activated PKD in turn activates IKKα-IKKβ-Nemo complex and nuclear import of NF-κB which results in induction of antiapoptotic and, or antioxidant genes such as manganese superoxide dismutase (MnSOD) and promotes cell survival [64]. Hence, a complete understanding of the molecular mechanism underlying ROS-induced PKD-mediated cell survival and identification of PKD substrates that activate NF-κB signaling pathway will help us better decipher the mechanism behind cancer cell survival.

4.3. Epithelial-to-mesenchymal transition (EMT)

Epithelial-to-mesenchymal transition (EMT) is a prominent neoplastic characteristics, where cell-cell adhesion is disrupted leading to enhanced cell motility and invasiveness [65]. Epithelial cells undergoing EMT process show mesenchymal cell properties including expression of mesenchymal markers and ability to migrate and invade [66]. E-cadherin is a master regulator of EMT process where it binds to β-catenin to form a protein complex and maintains cell-cell adhesion by interacting with actin and microtubule cytoskeleton owing to its anti-proliferative, antimetastatic and anti-invasion properties [67,68]. Different mechanisms of E-cadherin repression in malignant tumor have been shown including mutation, transcriptional repression, epigenetic silencing [68] and many transcription factors are involved in repression of E-cadherin and induction of EMT such as Snail and Twist [4,69–71]. Regulation of Snail protein is achieved by multiple pathways. The NF-κB signaling pathway positively modulates and stabilizes Snail protein promoting cell migration [72], whereas, phosphorylation of Snail by p21-activated kinase 1 (PAK1) [73] and GSK3β [74,75] increase Snail repression activity. Snail represses E-cadherin expression in prostate and breast cancer. PKD1 phosphorylates Snail at Ser^{11} residue triggering nuclear exclusion of Snail by 14–3-3 adaptor protein. As a result, Snail-repressed genes are de-repressed and cell migration is suppressed via production of E-cadherin and other proteins that mediate cell-cell adhesion. Hence, PKD negatively regulates the function of Snail and inhibits EMT.

4.4. Cellular motility, migration and invasion

Cellular motility and invasion are coupled to remodeling of actin cytoskeleton [76] and degradation of extracellular matrix (ECM) [77,78]. Cellular movement is achieved when Cofilin slices actin filaments at the leading edge of motile cells, generating a supply of actin monomers and orchestrating the formation of WAVE-2-cortactin-ARP2/3 complex which ultimately creates an expanded, branched network of F-actin [76]. LIM kinase suppresses cellular migration by phosphorylating Ser^3 residue of cofilin [79] and motility is restored when a protein phosphatase slingshot 1 like (SSH1L) dephosphorylates cofilin [80]. The original work by the Storz group showed that PKD phosphorylates SSH1L by complexing with SSH1L and F-actin at lamellipodium [81,82]. Phosphorylated SSH1L is sequestered to cytosol by 14-3-3 adaptor protein [83], consequently, $pSer^3$-cofilin concentration rises and cell migration is inhibited. A follow-up study by the Storz group also reported PKD2/PKD3-mediated regulation of SSH1L and p21-activated kinase 4 (PAK4) resulting in phosphorylation of cofilin and decrease in cell migration [84]. PKD inhibits cytoskeleton remodeling by phosphorylating the Ras effector RIN1 [85]. Phosphorylated RIN1 activates tyrosine kinase c-Abl and the RIN1-c-Abl complex phosphorylates and brings conformational change in a scaffold protein CRK that recruits F-actin remodeling proteins [85,86], resulting in suppression of cell motility. Matrix metalloproteinases (MMPs) are a class of proteases (collagenases, e.g. MMP-1; gelatinases, e.g. MMP-2) that mediate cell migration through ECM degradation. It has been reported that PKD inhibits breast cancer cell invasion by negatively regulating the transcription of several MMPs including MMP-2, MMP-7, MMP-9, MMP-10, MMP-11, MMP-13, MMP-14 and MMP-15 [81]. Histone deacetylases (HDACs) have been shown to regulate MMP expression [87,88] and PKD1 is shown to be a negative regulator of HDACs [89]. Therefore, it is conceivable that PKD may negatively regulate MMPs via HDAC regulation. PKD has also been reported to be a positive regulator of cell migration. PKD2 and PKD3 have been shown to increase prostate cancer cell invasion and migration by promoting NF-κB and urokinase-type plasminogen activator (uPA) expression/activation [90]. Yamamoto et al. has shown that Wnt5a-JNK-PKD1 axis positively regulates cell proliferation and migration of prostate cancer [91]. It has also been shown that vascular endothelial growth factors (VEGFs) promote cell proliferation and migration via PKD-mediated phosphorylation of class IIa HDACs and subsequent expression of VEGF-responsive genes [92,93]. In summary, several PKD-regulated pathways converge on the promotion as well as inhibition of cell proliferation, invasion and migration.

4.5. Angiogenesis

Angiogenesis is a process where new blood capillaries are formed and it is essential for many physiological processes such as embryonic development, wound healing and many pathological processes including tumorigenesis [94]. VEGFs are prominent in angiogenesis [95,96]. There are two related receptor tyrosine kinases that bind VEGF, VEGFR-1 and VEGFR-2 [97,98] and induce downstream signal that activates a variety of proteins such as PLC-γ, PI3-Kinase and the Src family [99,100].

As one of the best characterized angiogenic factors, VEGF exerts different biological functions in endothelial cells, such as: 1) stimulation of endothelial cell proliferation and migration [96], 2) promotion of endothelial cell survival by inducing expression of anti-apoptotic proteins such as Bcl-2 and death antagonist A1 [101–105]. PKD1 has been shown to be activated downstream of VEGFR2-PLCγ-PKC to activate ERK1/2 pathway and stimulate endothelial cell proliferation [33]. Hao et al. found that PKD2 was a major PKD isoform that mediates endothelial cell proliferation and migration [106]. In mouse embryonic stem cells, PKD2 activity is required for angiogenesis [107]. Mechanistically, PKD-phosphorylation of class IIa HDACs enable them to be sequestered to the cytoplasm by 14-3-3 proteins leading to derepression of target genes. VEGF-stimulated, PKD-mediated phosphorylation at $Ser^{259/498}$ and concomitant nuclear export of HDAC5 induces MEF2-dependent genes and endothelial cell migration [89]. HDAC7 is involved in regulating endothelial cell morphology and migration [92]. VEGF induces PKD-mediated phosphorylation of HDAC7 at $Ser^{178/344/479}$ and its subsequent nuclear exclusion by 14-3-3 proteins. This results in the expression of VEGF-responsive genes and promotion of endothelial cell proliferation and migration, MMP expression and EMT [89,92]. Therefore, it can be concluded that PKD plays a key role in signaling pathways that regulate angiogenesis in endothelial cells.

4.6. Bone development and innate immunity

Of note, it is noteworthy to mention that PKD is also involved in bone formation and innate immunity. Bone morphogenic proteins (BMPs), a family of multifunctional growth factors belonging to the transforming growth factor β (TGFβ) superfamily maintain skeletal integrity by modulating signaling pathways that converge on Runt-

related transcription factors (RUNX), regulator of osteoblast gene transcription. It has been shown that BMP-2 induces PKD activation via PKC-independent pathway during osteoblast lineage progression [108] and PKD activation is required for the BMP-2 mediated osteoblast differentiation [42]. Our recent report using conditional PKD1-knockout mice model has shown that PKD1 positively regulates bone development and osteoblast differentiation which could be linked to the activity of the STAT3/p38 MAPK signaling pathway [109]. Cancer mortality and morbidity are mainly caused by metastasis and bone is the 3rd most common site of metastasis. Given the important role of PKD in bone homeostasis, it is conceivable that PKD may play an important role in bone metastasis of malignant tumors [110]. PKD has been shown to be involved in innate immunity in many different ways, such as: 1) functional regulator of T and B lymphocyte [14], 2) regulator of class IIa HDACs in lymphocytes [111–113], 3) modulator of β1 integrin activity in T lymphocytes [114], 4) regulator of IL-2 via TCR stimulation [115], 5) downstream target in Toll-like receptor 9 (TLR9) signaling in macrophages [116] and TLR2 in mouse bone marrow-derived mast cells [117], 7) promoter of cell proliferation in chronic myelogenous leukemia (CML) [118] and 8) regulator of neutrophil chemotaxis [119].

5. PKD: a friend or foe in cancer development and progression?

Among other hallmarks such as sustaining proliferative signaling, enabling replicative immortality and evading cell death by apoptosis are of utmost significance in cancer development [120]. Accumulating evidence shows prominent link between tumor development and diverse signal transduction pathways that are modulated by PKD. The precise role of PKD in tumor progression remains elusive as evidence suggests that PKD plays a critical role as both the potent tumor promoter and suppressor of tumor development. In this section, we discuss the role of PKD isoforms in cancer development and progression focusing on several major cancer types and attempt to discuss the prevailing discrepancies that are associated with these tumor-specific studies (Fig. 3).

5.1. PKD in prostate cancer

Prostate cancer is one of the most common malignancies in male and accounts for 13% of cancer-related deaths in the USA [121]. Although early diagnosis and screening methods have advanced, effective treatments of late-stage metastasized tumors are scarce [122]. Although elevated levels of PKD is observed in human prostate carcinoma tissue compared to normal prostate epithelium, differential expression and distribution of PKD isoforms have been reported and functional analysis of the PKD isoforms has revealed their different roles in prostate cancer progression.

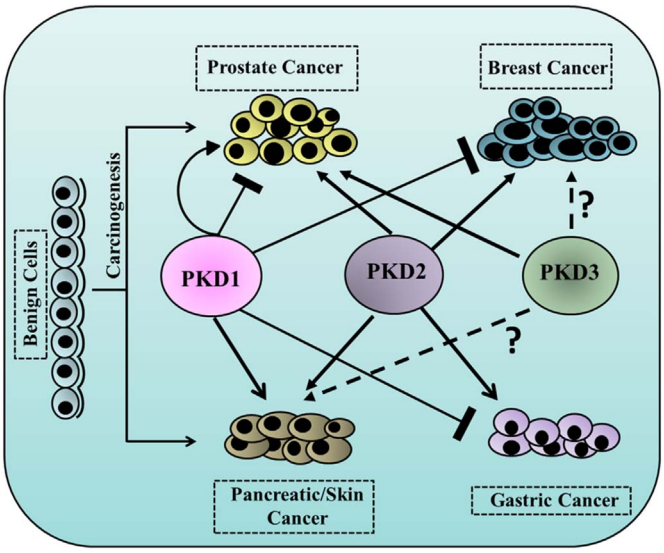

Fig. 3. Tumor-specific roles of PKD isoforms in several major cancer types. Benign cells transform into neoplasm such as carcinoma of prostate, breast, pancreas, skin and gastric. Three isoforms of PKD, namely PKD1, PKD2 and PKD3 either promote (solid arrow) or inhibit (bar-headed solid line) cancer progression in highly tumor-specific manner.

PKD1 is highly expressed in cultured androgen-sensitive, less metastatic LNCaP cell line; whereas, in contrast, PC3 and DU145, two androgen-insensitive, highly metastatic prostate cancer cell lines fail to express PKD1 but found to have high levels of PKD2 and significant PKD3 expression [123]. PKD1 has been reported to be a negative regulator of cell proliferation of prostate cancer cells [124–126]. It has been shown that PKD1 once overexpressed blocks migration through E-cadherin phosphorylation and regulation of β-catenin activity [125–127]. PKD1 has also been shown to downregulate androgen receptor (AR) function in prostate cancer cells [124]. This study demonstrated that PKD1 physically associates with AR through its kinase domain and makes a transcriptional complex. The binding of PKD1 with AR initiates transcription of target genes and this PKD1-AR interaction might be a novel signaling mechanism responsible for prostate cancer progression. In contrast to these studies showing a tumor-suppressive function of PKD1, several lines of independent studies have shown that PKD in fact can serve as an oncogenic factor in prostate cancer [91]. Its role as an enhancer of cell migration and invasion has been linked to Wnt5a overexpression. This study has demonstrated that Wnt5a activates Jun-N-terminal kinase via PKD and PKD is required for Wnt5a-mediated induction of MMP-1 expression, cell migration and invasion.

PKD3, on the other hand, has been shown to promote cell proliferation and survival of prostate cancer by increasing prolonged activation of Akt and ERK1/2 [123,128]. It is know that hyperactive Akt in PTEN-null prostate cancer has been linked to angiogenesis, invasion and metastasis [129,130]. Moreover, our study has reported that PKD2 and PKD3 positively regulate prostate cancer cell invasion by upregulating NF-κB signaling and HDAC1-mediated urokinase-type plasminogen activator (uPA) expression [90] and PKD2 and PKD3 were responsible for increased matrix metalloprotease-9 (MMP-9) expression, a key player for EMT [128] Additionally, our previous study has demonstrated that PKD3 contributes to the growth and survival of prostate cancer cells through PKCε/PKD3 pathway and downstream Akt and ERK-1/2 signaling pathways [123]. Activated PKD1 and PKD2 were shown to protect LNCaP prostate cancer cells from phorbol ester PMA-induced apoptosis by promoting downstream ERK-1/2 and NF-κB activities [131]. Using stable inducible PKD3 knockdown prostate cancer cell lines we have demonstrated that knockdown of PKD3 inhibits secretion of multiple key tumor-promoting factors including MMP-9, IL-6, IL-8, and GROα and inducible depletion of PKD3 in a subcutaneous xenograft model suppressed tumor growth and decreased levels of intratumoral GROα in mice. Furthermore, we have shown that androgen represses PKD expression in androgen-sensitive prostate cancer cells in an androgen receptor (AR)-dependent manner and the response is mediated by fibroblast growth factor receptor substrate 2 (FRS2) [132] and thus, we envision that upregulation of PKD as a result of loss or inhibition of AR may promote prostate cancer tumor cell survival.

In support of our view, using several classes of structurally distinct small molecule inhibitors of PKD discovered by our group, including CID755673 and its analogs kb-NB142–70, SD-208, 1-naphthyl PP1 (1-NA-PP1), Compound 139 [128,133–136], which are all nanomolar cell-active pan-PKD inhibitors, we demonstrated that targeted inhibition of PKD by these inhibitors led to reduced proliferation, migration and invasion of prostate cancer cells. Altogether, these studies have validated PKD as a potential therapeutic target for prostate cancer. In the future, detailed investigation should be carried out to decipher the precise role of each PKD isoform at different stages of prostate cancer development, and a PKD genetically engineered mouse model of prostate cancer will further validate the functional role of PKD in prostate carcinogenesis and tumor progression.

5.2. PKD in breast cancer

Breast cancer is the leading cause of cancer death in women and it is expected to account for 29% all new cancer diagnoses in the USA by 2017 [121]. PKD was first linked to breast cancer in a study by Bowden et al. demonstrating the association of PKD1 in a complex with cortactin and paxillin in invadopodia at sites of extracellular matrix degradation [137]. Later studies have shown that PKD1 expression, but not PKD2 or PKD3, is reduced in over 95% of invasive breast cancer samples compared to benign breast tissue [81] and thus established a tumor-suppressive role of PKD1 in breast cancer. Eiseler et al. have shown that the loss of PKD1 in breast cancer is associated with higher degree of tumor invasiveness [81]. Highly aggressive breast cancer cell lines such as MDA-MB-231 and BT-20 do not express PKD1, whereas; less invasive MCF-7 and normal mammary epithelial cells such as MCF-10A show significant PKD1 expression. It has been demonstrated that hypermethylation of the *PRKD1* promoter causes loss of PKD1 expression in invasive breast cancer [138]. In MCF-7 cell, silencing of PKD1 enhanced its migration, whereas; overexpression of constitutively active form of PKD1 in MDA-MB-231 cells led to decreased cell invasion [81]. Multicellular spheroid/3D cell culture assay using MDA-MB-231 cells demonstrated that expression of active PKD1 inhibited cell invasion compared to normal breast epithelial cells [81]. At molecular level, PKD confers its role as tumor-suppressor in breast cancer by 1) suppressing the expression of many matrix metalloproteases (MMP) such as MMP-2, MMP-7, MMP-9, MMP-10, MMP-11, MMP-13 and MMP-14 [81], 2) inhibiting EMT via inactivation of Snail function by phosphorylating Ser^{11} residue [139,140], 3) inducing the expression of epithelial-to-mesenchymal markers (vimentin, E-cadherin) [139] and 4) phosphorylating SSH1L at Ser^{937} and Ser^{978} residues and negatively regulating cofilin phosphorylation and interacting with cortactin and paxillin at lamellipodia, thereby inhibits cell motility and invasion [81,83,137,141]. In contrast, opposing effects of PKD has also been demonstrated in breast cancer. Kennett et al. and Palmantier et al. have demonstrated a tumor-promoting function of PKD1 in breast cancer cells [142,143].

Although accumulating evidence indicate PKD1 as a gross tumor-suppressor and a major contributor to the maintenance of epithelial phenotype in breast cancer [81,139–141], PKD2 and PKD3 have been assigned roles of tumor-promoters where they induce cell proliferation, invasiveness and chemoresistance [144–146]. Huck et al. have demonstrated that PKD3 is a tumor-promoter in triple-negative breast cancer (TNBC) [147]. PKD3 triggered the activation of S6 kinase 1 (S6K1) which is the main downstream target of the mammalian target of rapamycin complex 1 (mTORC1). The authors have also shown that PKD3 depletion inhibited cell proliferation of TNBC and hence, identifying PKD3 as a potent chemotherapeutic target. Borges et al. have demonstrated that PKD3 is highly upregulated in estrogen receptor (ER)-negative (ER^-) invasive ductal carcinoma (IDC) which is associated with triple-negative phenotype [148]. This study showed that ER directly binds to the *PRKD3* gene promoter and inhibits PKD3 expression. Hence, loss of ER leads to upregulation of PKD3 which eventually induce increased cell proliferation, migration and invasion. Hao et al. have shown that silencing PKD2 or PKD3 significantly inhibited proliferation of HCC1806 triple-negative breast cancer cell line and PKD3 knockdown inhibited Hsp27 and HDAC4/5/7 phosphorylation [144]. Further investigation into different PKD isoforms at cellular levels as well as using mouse genetic models will help us to better understand the biology of this family of protein kinases in the development of breast neoplasia.

5.3. PKD in pancreatic cancer

Ductal adenocarcinoma of pancreas is a very aggressive, chemotherapy-resistant type of cancer [149] and according to the American Cancer Society, pancreatic cancer will account for about 3% of all cancers in the US and about 7% of all cancer deaths by 2017. Almost 95% of all pancreatic ductal adenocarcinoma (PDAC) display somatic activating mutations of Kras [150] and increased epidermal growth factor (EGFR) signaling [151,152]. As compared to the normal pancreas, human ductal adenocarcinoma of the pancreas shows increased PKD1 expression and kinase activity [39,153]. An important study from the Storz group has demonstrated a positive role of PKD1 in the malignant transformation of PDAC. The authors have demonstrated that in a 3D explant model, using PKD inhibitors or PKD knockdown approach that PKD1 is necessary for TGFα- and Kras-mediated formation of duct-like structures originating from acinar cells, a process called acinar-to-ductal metaplasia (ADM), which converts to pancreatic intraepithelial neoplasia (PanIN), the premalignant neoplastic precursor of PDAC. Using *in vivo* mouse model ($p48^{cre}$ $Kras^{G12D}$ mice), the authors have knocked out PKD1 in acinar cells and demonstrated decreased progression of acinar-to-ductal metaplasia (ADM) to PanIN [154]. This study provided strong support for a role of PKD1 in the pathogenesis of PDAC. Additionally, other studies at cellular level support pro-proliferative and pro-survival effects of PKD1 in pancreatic cancer cells. Specifically, Trauzold et al. have shown that overexpression of PKD1 in pancreatic cancer cell decreased CD95-mediated apoptosis, increased cell proliferation rate and upregulated survivin [153]. PKD1 mediates the mitogenic effects of neurotensin in pancreatic cancer cells [155,156]. A study by Yuan et al. has shown that neurotensin increases Hsp27 phosphorylation of Ser^{82} via p38 MAPK-PKD2 signaling axis in pancreatic cancer cells [157]. Hsp27 level is markedly increased in many cancers and its elevated expression contributes to increased tumorigenicity and chemoresistance [158–161]. Another recent study has demonstrated a pro-oncogenic role of PKD2 in pancreatic cancer where PKD2 functions upstream of MMP-7 and -9 in pancreatic cancer cells and induces invasion and angiogenesis *in vivo* and *in vitro* [162]. PKD2 does so by stimulating expression and secretion of MMP-7 and -9 and induces invasion in 3D extracellular matrix (ECM) culture, and furthermore, PKD2-activated MMP9 induces tumor angiogenesis by releasing ECM-bound VEGF-A [162]. Although PKD1 and PKD2 have emerged as tumor-promoters in pancreatic cancer respectively, the role of PKD3 remains obscure and further research is needed to address the function of individual PKD isoform in pancreatic cancer.

5.4. PKD in skin cancer

Basal cell carcinoma (BCC) is the most common form of malignant skin cancer in the world. An estimated 83,000 new cases of skin cancer (6% of all cancer types) have been documented in the USA by 2016 [121]. In normal epidermis, PKD1 is primarily expressed in stratum basilis, the proliferative compartment of the skin which supports a notion that PKD1 may promote hyperproliferative disorders in skin [163] and PKD1 expression was shown to be upregulated in mouse carcinomas and human hyperplastic disorders including BCC [163–165]. PKD1 has been shown to repress keratinocyte differentiation and promote cellular proliferation through modulation of MEK/ERK1/2 pathway [166]. UVB radiation is a key risk factor for developing BCC and it has been reported that activation of PKD1 by Src family of tyrosine kinases in primary mouse keratinocytes exposed to UVB reduced UVB-induced apoptosis and this activation of PKD1 was PKC-independent [167]. Rashel et al. have demonstrated a pro-proliferative role of PKD1 in epidermal adaptive response, wound healing and skin carcinogenesis [168]. Using PKD1-conditional knockout (cKO) mouse model, the authors have presented evidence that: 1) keratinocyte cells in PKD1-cKO mice showed delayed wound healing and reduced proliferative response, 2) PKD1 is a positive regulator of epidermal hyperplasia and inflammation in response to phorbol esters and 3) PKD1-cKO mice are resistant to tumor formation when subjected to two-stage chemically-induced skin carcinogenesis [168]. In a recent study, Ryvkin et al. have demonstrated an opposing role of PKD2 and PKD3 isoforms in human keratinocyte proliferation and differentiation

[169]. The authors have shown that loss of PKD2 resulted in enhanced keratinocyte proliferation suggesting an anti-tumorigenic role of PKD2. Whereas, silencing of PKD3 showed proliferation defect, loss of clonogenicity and diminished tissue regenerative ability, implying a pro-oncogenic role of PKD3. It is to be noted that PKD1 is not expressed in human keratinocytes and thus, PKD2 and PKD3 play a key role in maintaining human epidermal homeostasis, loss of which results in BCC. Further studies are needed to decipher precise roles of PKD isoforms in skin cancer.

5.5. PKD in gastric cancer

PKD1 has been shown to be a negative regulator of gastric cancer [170]. Gastric carcinoma cells as well as patient tissue samples showed decreased PKD1 expression [170]. Gene silencing of PKD1 using siRNA increased cell invasion of gastric cancer and it was found that PKD1 was epigenetically silenced in this tissue type [170]. In an independent study, Shabelnik et al. have demonstrated opposing roles of PKD1 and PKD2 in gastric cancer where PKD1 acts as tumor-suppressor and PKD2 as tumor-promoter [171]. Using tumor samples of different histological variants of primary gastric cancer and gastric adenocarcinoma cell line AGS, the authors have shown that PKD1 and PKD2 are differentially expressed, i.e., lower and higher expression of PKD1 and PKD2 respectively, in both mRNA and protein levels. pcDNA3.1-mediated overexpression of PKD1 resulted in the inhibition of cell proliferation, migration and colony formation, whereas, that of PKD2 enhanced cell proliferation, migration and colony formation abilities in AGS cells [171]. The role of PKD3 isoform in gastric cancer development and progression remains elusive.

5.6. PKD and other cancer types

The role of PKD in other cancer types is poorly defined, although some reports link PKD to certain cancers. For example, activation of PKD by phorbol esters and bombesin via PKC has been shown in small cell lung cancer (SCLC) cell lines h69, H345 and H510 [172]. Using PKC and PKD inhibitors, Brenner et al. has shown that PKD1 is involved in renal cell carcinoma and it promotes tumor progression by positively regulating the adhesion of renal carcinoma cells to endothelial cells [173]. Studies on human malignant lymphoma cells did not conclude distinct roles of PKD as PKD1 levels were undetected and there was no change in PKD2 expression in the malignant cells as compared to benign tissues [174]. In a recent study, PKD2 has been demonstrated to be a potent mediator of glioblastoma where it promotes tumor progression by upregulating integrin α-2 and -4 (ITGA2 and -4), plasminogen activator urokinase (PLAU), plasminogen activator urokinase receptor (PLAUR), and matrix metallopeptidase 1 (MMP1) [175]. When overexpressed in SW480 colon cancer cells, PKD1 suppresses nuclear β-catenin accumulation and inhibits colon cancer [176]. On the contrary, in another study using RKO human colon cancer cell line, it has been demonstrated that targeted inhibition of PKD by small molecule inhibitor suppressed AKT/ERK signaling and NF-κB activity [177]. Hence, PKD might be a potent chemotherapeutic target for the treatment of colorectal cancer.

6. Therapeutic targeting of PKD in cancer

Accumulating evidence indicate that PKD expression is deregulated in many cancers and PKD plays a crucial role in a wide range of cancer-associated cellular processes such as cell proliferation, migration, apoptosis, EMT, and angiogenesis. This makes PKD an attractive therapeutic target for cancer and has since embarked efforts in the discovery and development of novel PKD inhibitors. Starting with the discovery of a non-ATP-competitive PKD inhibitor CID755673 [128], we have subsequently reported several first-in-class structurally distinct PKD small molecule inhibitors, including the non-ATP-competitive CID755673 and its derivatives kb-NB142-70 and KMG-NB4-23 [128,178–181], three dual PKD inhibitors (compound 139, 1-NA-PP1, and SD208), and three cell-active PKD inhibitors (CID2011756, CID5389142 and CID1893668) [134–136,181], which are all nanomolar PKD small molecule inhibitors that potently block prostate cancer cell proliferation, migration, and invasion [128,180]. CID755673, in particular, has shown *in vivo* efficacy in other disease models [182].

In addition to our efforts, several highly potent and selective PKD inhibitors have also emerged from the pharmaceutical industry [45,183–185] including an aminopyridine arene CRT0066101 (CRT101) [186] and an aminopyrimidine phenol CRT0066051 (CRT051) [187] from Cancer Research Technology Ltd. Additionally, in a recent study, Golkowski et al. have identified novel PKD inhibitors, namely compounds 1553, 1561, 1649 and 1369 using kinobead-based proteomic assay [188]. In this study, compound 1369 was found to be highly selective and potent pan-PKD inhibitor and an important tool compound to identify the roles of PKD isoforms in cellular as well as *in vivo* models. Among all available PKD SMIs, CRT101 is by far the most potent, selective, and cell-permeable PKD small molecule inhibitor with demonstrated *in vivo* antitumor activity in multiple cancer models [148,177,189]. Specifically, CRT0066101 was found to inhibit growth of pancreatic cancer tumor xenografts [189]. CRT0066101 also caused significant inhibition of tumor growth in HCT116 xenograft nude mice, supporting the therapeutic potential of this inhibitor in colon cancer [177]. CRT0066101 has been shown to be a potent anticancer agent against highly aggressive ER negative breast cancer [148]. This study showed that similar to PKD3 knockdown effect, CRT0066101 significantly reduced breast cancer cell proliferation, migration and invasion both *in vitro* and *in vivo*.

It is interesting to note that despite the differential roles of PKD isoforms in different cancer types as implicated in our discussion above, PKD inhibitors have unequivocally exhibited antitumor activities in various *in vitro* and *in vivo* cancer models. It is possible the overall selectivity profiles of these inhibitors favor anticancer activity with PKD being the primary target. Nonetheless, with the growing reports of diverse functions of PKD isoforms in different cancers, the development of isoform-selective PKD inhibitors is a well-justified direction for future studies.

7. Perspectives and concluding remarks

Growing evidence supports PKD as a key signaling molecule that orchestrates various cancer-associated biological functions such as cell proliferation, survival, EMT, migration, invasion, secretion and angiogenesis upon activation by a battery of stimuli. Despite recent advances of our knowledge about the role of PKD in various pathological conditions including cancer, cardiac hypertrophy and inflammation, the similar or opposing roles of the same PKD isoform in different cancers or of the same cancer for different PKD isoforms has raised more unanswered questions.

To summarize briefly, emerging evidence suggests that PKD1 can function as a tumor-suppressive protein in breast and gastric cancers, where PKD1 inhibits cell survival, proliferation, migration by negatively regulating several key target proteins including SSH1L, Snail and MMPs. However, in other cancers, such as pancreatic and skin cancers, PKD1 emerged as a driver of neoplasia. It does so by many mechanisms such as activating MEK1/2 pathway that increases DNA replication, inhibits apoptosis and promotes proliferation by positively regulating ERK/MAPK pathway. Compared to the opposing roles of PKD1 in cancer development and progression, majority of studies have shown that PKD2 is a tumor-promoting protein in a wide range of cancers. It activates different biological functions such as NF-κB signaling, MMP expression, induction of angiogenesis and inhibition of apoptosis and promotes some common cancers including carcinomas of prostate, breast, pancreas, stomach and also other cancers such as glioblastoma.

The actual role of PKD3 in cancer is elusive. It shows pro-oncogenic properties in case of prostate, breast and skin cancer but what it does in pancreatic and gastric cancer, is unknown.

In this review, we have discussed the major signaling pathways involved in development and progression of neoplasm that are modulated by PKD, the molecular mechanisms of regulation of cellular phenomenon orchestrated by PKD and manifestation of each type of PKD isotypes in the context of major carcinomas. Although PKD emerged as a potential target for chemotherapeutic intervention and pan-PKD inhibitors have shown potent anti-cancer activity in multiple cancer models both *in vivo* and *in vitro*. Many cellular studies have demonstrated differential effects of PKD isoforms in different biological processes. These differences seem to be isoform- and tumor type-dependent, which raise questions on whether it is appropriate and how to target PKD for cancer treatment. As developed in this review and going forward, there remain many unanswered questions. What are the molecular cues that direct precise and selective role of PKD in disease progression? Is it the isoform-specific function that enables PKD to selectively choose the cell type to exert its biological effect? What would be the best strategy to develop effective small molecule inhibitors of PKD that will preserve the tumor-suppressing capacity of PKD while eradicating those that are tumor-promoting? Further knowledge defining the precise role of PKD isoforms in different tumor models will provide a much clearer picture for targeting this family of protein kinases for cancer therapy.

Conflict of interests

The authors declare no conflict of interest.

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