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A basal-enriched microRNA is required for prostate tumorigenesis in a *Pten* knockout mouse model

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Abstract

MicroRNAs (miRNAs) play important roles in prostate cancer development. However, it remains unclear how individual miRNAs contribute to the initiation and progression of prostate cancer. Here we show that a basal layer-enriched miRNA is required for prostate tumorigenesis. We identify *miR-205* as the most highly expressed miRNA and enriched in the basal cells of the prostate. Although *miR-205* is not required for normal prostate development and homeostasis, genetic deletion of *miR-205* in a *Pten* null tumor model significantly compromises tumor progression and does not promote metastasis. In *Pten* null basal cells, loss of *miR-205* attenuates pAkt levels and promotes cellular senescence. Furthermore, although overexpression of *miR-205* in prostate cancer cells with luminal phenotypes inhibits cell growth in both human and mouse, *miR-205* has a minimal effect on the growth of a normal human prostate cell line. Taken together, we have provided genetic evidence for a requirement of *miR-205* in the progression of *Pten* null-induced prostate cancer.

Keywords

miRNAs; PI(3)K pathway; prostate cancer

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AUTHOR CONTRIBUTIONS

RY and XF conceived and designed the experiments. XF carried out all miR-205 related experiments and analyzed the data with assistance from KR and LW for sequencing and bioinformatics. GAB carried out miR-21 related experiments and analyzed the data. KR and LW quantified and analyzed the miR-seq data. RY and XF wrote the manuscript and all authors edited and approved the manuscript.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

1 | INTRODUCTION

Studies using genetically engineered mouse models have provided important insights into the roles of individual genes in the initiation and progression of prostate tumorigenesis.¹ Among these models, loss of *Pten* tumor suppressor by prostate-specific deletion recapitulates many aspects of human prostate cancer development including activation of the PI(3)K pathway, development of castration resistant and metastatic tumors, as well as induction of cellular senescence.^{2–4} Supporting the relevance of these findings in mouse models, genomic profiling of human prostate cancer samples reveal altered PI(3)K signaling in 42% of primary prostate cancer and 100% of metastatic cases.⁵ Among these cases, losses of *PTEN* and *INPP4B*, negative regulators of the PI(3)K pathway,^{3,6} are the most frequently observed mutations. Furthermore, the *Pten* null model has been utilized to examine interactions among different pathways during prostate tumorigenesis when combined with other genetic mutations.^{4,7}

MicroRNAs (miRNAs) are a class of small, regulatory RNA species that control gene expression posttranscriptionally and are implicated to have diverse and important roles in cancer.⁸ In prostate cancer, miRNAs are often dysregulated in tumor cells.⁹ Genetic deletion of *Dicer1* in the *Pten* null prostate cancer model compromises tumor progression, suggesting a general requirement of the miRNA pathway for prostate tumorigenesis.¹⁰ Of note, the basal and luminal cells appear to respond differently to loss of *Dicer1*. Although loss of *Dicer1* leads to globally increased senescence and apoptosis, the apoptotic cells are largely restricted to the *Pten*-null luminal cells.¹⁰ However, because all miRNAs in both basal and luminal cells are lost in the *Dicer1* null model, it is not possible to delineate the underlying mechanisms and distinguish how individual miRNAs contribute to prostate tumorigenesis. Current knowledge about individual miRNAs in prostate cancer has been largely obtained from gain-of-function studies using cell culture systems. Genetic loss of *miR-101* was observed to promote tumor progression and metastasis through derepression of *Ezh2* in human prostate cancer.¹¹ *miR-125b* has also been shown to promote androgen-independent growth of prostate cancer cells by, at least in part, inhibiting *Bak1* expression.¹² *miR-205*, a miRNA highly enriched in *Krt5*+epithelial tissues¹³ and under transcriptional regulation of *p63*,¹⁴ inhibits the survival of metastatic prostate cancer cell lines in vitro.¹⁵ However, due to the lack of suitable mouse models, individual miRNAs have not been examined for their requirement in the initiation and progression of prostate cancer in intact tissues in vivo.

In this study, we identify *miR-205* as the most highly expressed miRNA in the *Krt5*+ basal layer, and show that it is largely absent from the *Krt8*+ luminal layer. Although genetic deletion of *miR-205* shows no discernible defects in prostate development and homeostasis, loss of *miR-205* in *Pten* null prostate significantly compromises the initiation and progression of prostate tumor and does not promote tumor invasion and metastasis. In contrast, genetic deletion of *miR-21* in *Pten* null model shows no effect on prostate tumorigenesis. Examination of each lineage suggests that loss of *miR-205* in the basal lineage attenuates *Pten* null-induced pAkt upregulation and promotes cellular senescence. Finally, we show that overexpression of *miR-205* in luminal cancer cells derived from mice and humans inhibits cell growth, whereas it has no effect on a normal human prostate cell

line. These findings provide new insights into the in vivo function of *miR-205* in prostate cancer.

2 | METHODS

2.1 | Mice

miR-205^{fl/fl} mouse strain was described previously.¹³ *miR-21^{fl/fl}* mouse strain was provided by Eric Olson.¹⁶ *Pten^{fl/fl}* mouse was obtained from Jax lab (Stock number 006440). *Probasin-Cre (Pb-Cre)* mouse was obtained from NCI (Stock Code 01XF5) and *Rosa26-LSL-tdTomato* mouse was obtained from Jackson lab (#21876). Male mice from 6-week to 1-year old were used in this study. Mouse colonies were maintained in the transgenic animal facility at the MCDB department of University of Colorado at Boulder. All animal experiments were conducted following protocols approved by the IACUC at University of Colorado.

2.2 | Isolation of prostate cell populations by fluorescence activated cell sorting

Isolation of prostate cells from adult mice was performed using the published method. Single cells were resuspended in phosphate-buffered saline (PBS) with 3% fetal bovine serum (FBS) and incubated with antibodies on ice for 45 minutes. Antibodies are: CD49f-PE (1:160; clone eBioGoH3; eBioscience; cat no 12-0495-83); Sca-1-PE-Cy7 (1:500; clone E13-161.7; BioLegend; cat no 122514); Ter119-FITC (1:250; clone TER-119; eBioscience; cat no 11-5921-85); CD31-FITC (1:250; clone 390; eBioscience; cat no 11-0311-85); CD45-FITC (1:250; clone 30-F11; eBioscience; cat no 11-0451-85). Cell isolations were performed on a Moflo XDP cell sorter (Beckman Coulter).

2.3 | miR-seq

Total RNAs from fluorescence activated cell sorting (FACS)-purified cells were isolated using TRIzol reagent (Invitrogen) and the quality was assessed by an Agilent 2100 bio-analyzer. 300 ng total RNA isolated from sorted basal and luminal cells from adult wildtype mice or 1 µg total RNA from human prostate cell lines were used for a miR-seq protocol as previously described.¹⁷

2.4 | Reverse transcription and real-time polymerase chain reaction

Reverse transcriptions were performed using miScript reverse transcription kit (Qiagen). For miRNA real-time polymerase chain reaction (PCR), SYBR Green super mix was from Bio-Rad and miScript primers for miRNAs and control Sno25 were from Qiagen. For messenger RNA (mRNA) real-time PCR, same reagents from Bio-Rad and primers were synthesized in Fisher. The primers for P16 were: 5'-ACATCGTGCGATATTTGCGTTCCG-3' and 5'-TTTAGCTC TGCTCTTGGGATTGGC-3'; the primers for Inpp11: 5'-CAGCCTGG TATCACCGTGAC-3' and 5'-GCCACGCTCTCGCTATCTC-3'; the primers for Inpp4b were: 5'-ATCGGCTGATCGAAATGTGT-3' and 5'-GAAAGCCAAGTCAACCCAAA-3'; HPRT was used for the reference gene: 5'-CGAGGAGTCCTGTTGATGTTG-3' and 5'-GTGGCCTA TAGGCTCATAGTGC-3'. Reactions were performed according to the manufacturer's manual and on a CFX384 real-time system (Bio-Rad). Differences between samples and controls were calculated using the method.

2.5 | Histology, immunofluorescence, immunoblotting, western blot analysis and in situ hybridization

Haematoxylin and Eosin (H&E) staining and immunofluorescence staining were performed using standard protocols on 10- μ m OCT sections which prefixed with 4% paraformaldehyde before embedding. Immunofluorescence and immunoblotting were performed as described previously,¹³ with the following primary antibodies/dilutions/sources: β -actin (1:1000; Cell signaling; cat no 3700P; clone 8H10D10); P16-INK4A (1:200; Proteintech; cat no 10883-1-AP); AR (1:200; Santa Cruz; cat no sc-816); Krt8 (1:2000; Abcam; cat no ab14053); smooth muscle actin (SMA; 1:200; DakoCytomation; cat no M085129-2; clone 1A4); Sox9 (1:500; Millipore; cat no AB5535); p63 (1:500; Cell Signaling; cat no 4892); Krt5 (1:2000; Covance; cat no SIG-3475); Ki67 (1:500; Abcam; cat no ab15580); pAkt (1:2000; Cell Signaling cat no 4060; clone D9E); panAkt (1:4000; Cell Signaling, cat no 4691; clone C67E7). Whole prostate tissue from adult mice were dissected and lysed in 2 $\times$ Laemmli buffer. Boil the samples at 95°C for 10 minutes, centrifuge for 10 minutes at 14 000 rpm and load the samples on 10% SDS-PAGE gel. Transfer the protein from gel to membrane at 90 V for 90 minutes at cold room. Block the membrane for 1 hour at room temperature (RT) and primary antibody incubation overnight at 4°C. Wash three times and incubate secondary antibody for 2 hours at RT. Wash three times and apply chemiluminescent substrate (Pierce ECL Western blot analysis Substrate; cat no 32106). Image the blot using X-ray film.

In situ hybridizations were performed by adapting miRNA localization protocols to skin. Signals were detected using the TSA Plus Fluorescein System (Perkin Elmer) for fluorescent signal and posthybridization staining. The miRCURY LNA miR-205 detection probe (Exiqon) was 5'-DIG and 3'-DIG double-labelled and hybridized to sections at 61°C. Alexa-Fluor-594-conjugated secondary antibody (1:2000; Invitrogen/Molecular Probes; cat no A11012 and A11007) after developing. For all of the immunofluorescence stainings, miR-205 in situ and immunoblots, at least three independent experiments were conducted. Representative images are shown in the figures. Human tissue array slides were purchased from Novus (#NBP2-30169), detailed information could be found: http://www.novusbio.com/Prostate-cancer-tissue-Tissue-Micro-Array_NBP2-30169.html#dsTab0

2.6 | Image acquisition

For immunofluorescence staining, images were acquired on a Leica DM5500B microscope with attached Hamamatsu C10600-10B camera and analyzed with a MetaMorph (version 7.7; MDS Analytical Technologies) software. For quantification of immunoblotting, exposed films were scanned by an HP scanner and quantified by the ImageJ software.

2.7 | *Pten* null cells culture and human prostate cell culture

Pten null cells were established from a 10-month *Pten* deletion mouse (cKO). Freshly collected prostate tissues were minced with blades and incubated in digestion medium at 37°C for 2 hours, and then digested with 0.05% trypsin for 5 minutes. Total single cells isolated from the prostate cancer tissue of cKO were cultured in medium containing DMEM, 10% FBS, 25 μ g/mL bovine pituitary extract, 5 μ g/mL insulin and 6 ng/mL recombinant human epidermal growth factor. After more than 10 passages, *Pten* null cells grew out.

Human prostate cell line RWPE_1 and prostate cancer cell lines DuCap, LNCap, VCap are gifts from Amy Palmer Lab at CU Boulder. RWPE_1 cells are cultured in Keratinocyte Serum Free Medium (K-SFM, purchased from K-SFM; cat no 17005-042). The human prostate cancer cell lines are cultured in DMEM with 5% FBS.

2.8 | Statistical analysis

All experiments were performed using 3 to 10 mice in independent experiments. Details for each experiment were provided in the figure legends or the manuscript checklist. Data were presented as mean $\pm$ standard deviation or standard error of the mean. Student *t* test was used to determine significance between groups. For all statistical tests, the 0.05 level of confidence was accepted for statistical significance.

2.9 | miR-seq data set

Sequencing data are deposited to NCBI/GEO database and can be accessed under the accession number: GSE75321.

3 | RESULTS

3.1 | *miR-205* is the most highly expressed miRNA in murine basal cells

Because miRNAs function in a dosage-dependent manner, and highly expressed miRNAs can regulate many targeted mRNAs, we performed quantitative miR-seq¹⁷ to examine the miRNA profile in the basal and luminal cells from wild-type (WT) animals. Basal and luminal cells were purified as *Lin^{neg}/CD49^{hi}/Sca1^{hi}* and *Lin^{neg}/CD49^{lo}/Sca1^{neg}*, respectively, by an established FACS protocol¹⁸ and validated with known lineage markers including *Krt5* and *p63* for basal cells and *Krt8* and *Nkx3.1* for luminal cells and *Vimentin* for stromal cells (Figure S1A–D). We identified *miR-205* as the most highly expressed miRNA in the basal lineage (Figure 1A), similar to the miRNA profile previously determined in murine hair follicle stem cells.¹³ *miR-205* is enriched in the basal cells with a ~22-fold higher expression over the luminal lineage (Table S1). Quantitative real-time PCR (qRT-PCR) confirmed the enrichment of *miR-205* in the basal cells (Figure 1B). To visualize the expression of *miR-205* in the prostate gland, we performed fluorescent in situ hybridization of *miR-205* together with *Krt5* and *Krt8* staining, lineage-specific markers for the basal and luminal cells, respectively. *miR-205* expression completely overlapped with *Krt5*⁺ basal cells and was not detected in *Krt8*⁺ luminal cells (Figure 1C).

To probe the expression of *miR-205* in a well-established *Pb-Cre* driven, *Pten*-null model, we performed in situ hybridization and qPCR quantification of *miR-205* in primary tumor samples obtained from 10-week-old *Pb-Cre/Pten^{fl/fl}* animals. We observed increased basal expression of *miR-205* upon *Pten* deletion (Figure 1D and 1E). Furthermore, we also detected robust expression of *miR-205* in basal cells in both normal human prostate and PCA samples with high Gleason scores (Figure 1F–H). Taken together, these data indicate that *miR-205* is highly expressed, enriched in the basal lineage of the prostate gland under both normal and *Pten* null-induced tumorigenic conditions.

3.2 | *miR-205* is dispensable for prostate development

To delete *miR-205* from the prostate, we first determined the expression of a *Pb-Cre* strain with a *Rosa26-LSL-tdT* reporter (*Pb-Cre/Rosa26-LSL-tdT*). We found that *Pb-Cre* has considerable activity in *Krt5*⁺ basal cells as well as in luminal cells (Figure S1E). Using the established cell surface markers,¹⁸ we further validated that progenies of *Pb-Cre* expressing cells (*tdTomato*⁺ cells) include basal and luminal cells (Figure S1F). These data indicated that the *Pb-Cre* strain should induce the deletion of *miR-205* in basal cells. Indeed, when we generated a *Pb-Cre/miR-205^{fl/fl}* model, we found that *miR-205* was largely depleted in basal cells in anterior prostate (AP), dorsal-lateral prostate (DLP) and ventral prostate (VP) lobes (Figure S2A). qPCR validation further confirmed the depletion of *miR-205* in the FACS purified basal cells (Figure S2B). A previous study showed a requirement of the miRNA pathway for prostate development by deleting *Dicer1* using *Pb-Cre*.¹⁹ When the entire miRNA pathway is abolished through the deletion of *Dicer1*, the prostate gland shows enhanced apoptosis mostly in the luminal lineage and compromised proliferation capacity of the basal cells.¹⁹ These observations suggest that different miRNAs may be responsible for the differential response of basal and luminal lineages to the loss of *Dicer1*. Although *miR-205* is highly expressed in the basal lineage, we did not observe any phenotypes in the *miR-205* null prostate (Figure S2C–F). Neither basal nor luminal cells showed signs of apoptosis (Figure S2D). Our previous study in the skin revealed a requirement of *miR-205* to promote pAkt levels by concurrently targeting multiple negative regulators of the PI(3)K signaling in proliferative skin stem cells during neonatal development.¹³ However, basal cells of the prostate are quiescent with little sign of cell division and we did not observe changes in rare Ki67⁺ cells in both basal and luminal lineages (Figure S2E). Furthermore, pAkt levels were undetectable during early development and normal homeostasis in the entire prostate of both control and *miR-205* cKO (Figure S2F), consistent with limited proliferation of these cells. Thus, genetic deletion of *miR-205* does not reduce basal proliferation nor promote luminal cell death.

3.3 | Loss of *miR-205* compromises tumorigenesis in a *pten* null model

Because prostate tumorigenesis is sensitive to the levels of pAkt and *miR-205* can maintain the pAkt levels, we generated a *Pb-Cre/miR-205^{fl/fl}/Pten^{fl/fl}* (dKO) model to study the function of *miR-205* in tumorigenesis of the prostate gland. We first confirmed the loss of *miR-205* in the dKO model using in situ hybridization (Figure 2A). As expected, prostate specific deletion of *Pten* by *Pb-Cre* led to early tumor development by the age of 6 weeks ($n = 3$) and robust tumor formation by the age of 10 weeks ($n = 30$) (Figure 2B and Table 1). Although the penetrance was incomplete, a majority (16 out of 24) of dKO prostate showed compromised tumorigenesis whereas all *Pten* null mice developed tumor (30 out of 30) at 10 weeks in the AP lobe ($P < .0001$, the χ^2 test) (Figure 2B and Table 1). Furthermore, when we examined *Pten* cKO and *Pten/miR-205* dKO animals at 1 year, we observed large primary tumors in all *Pten* cKO animals ($n = 8$) whereas a majority of dKO animals (4 out of 6) showed mildly enlarged prostate glands, similar to the control animals (Figure 2C and Table 1). To confirm that the compromised tumor growth observed in the *Pten/miR-205* dKO animals is caused by the loss of *miR-205* but not other nonspecific effects, we generated a *Pb-Cre/miR-21^{fl/fl}/Pten^{fl/fl}* dKO mouse model to remove *miR-21*¹⁶ together with *Pten*. Although *miR-21* has been implicated with oncogenic roles in other cancer types,¹⁶ we

found that loss of *miR-21* had no effect on the *Pten* null-induced tumorigenesis (Figure S2G–I).

Because miRNA KO mice often show incomplete penetrance, we performed our subsequent analyses in the AP, where we observed high *miR-205* deletion efficiency. By 6 weeks, *Pten* cKO prostate began to show signs of hyperproliferation and initiation of tumorigenesis, consistent with prostatic intraepithelial neoplasia (PIN) 1 or 2 (Figure 2D). In contrast, dKO prostate was largely intact with normal morphology (Figure 2D). By 10 weeks, when *Pten* cKO generally progressed to PIN 3 or 4 with apparent luminal cell expansion, dKO prostate still showed largely normal morphology with a mild expansion of the luminal lineages (Figure 2E). By 1 year, all eight *Pten* cKO animals examined developed large primary tumors with clear signs of adenocarcinoma (Figure 2F). Lung metastases were also detected in one of these eight animals. In contrast, four out of six dKO animals had largely indolent tumors at PIN 3 or 4 without overt tumor growth (Figure 2F). Previous reports indicate that loss of *miR-205* is often associated with metastatic prostate cancer cells in humans. We observed no signs of metastasis in any of our dKO mice. Thus, concurrent deletion of *miR-205* in *Pten* null primary tumors does not result in enhanced metastasis. Together, these morphological analyses indicate a requirement of *miR-205* to sustain tumor growth in the *Pten* null model.

3.4 | Loss of *miR-205* reduces cellular proliferation in both basal and luminal cells

We next examined the progression of tumorigenesis in both basal and luminal cells using immunofluorescence (IF) staining with lineage-specific markers. Basal and luminal lineages were largely distinct in dKO, with little sign of expansion of both layers, similar to the control (Figure 3A). In contrast, *Pten* null samples showed increased *Krt5/Krt8* double positive populations and an expanded luminal lineage, characteristic of *Pten* null-induced prostate tumorigenesis (Figure 3A). In addition, SMA staining that outlines the basement membrane was generally intact in dKO prostate, similar to control, whereas the basement membrane began to break down in cKO by 10 weeks (Figure 3B). Visualization and quantification of proliferative cells in basal and luminal cells by *Ki67* revealed reduced proliferation in both lineages of dKO samples, compared with *Pten* null samples (Figure 3C–F). Finally, we detected no signs of increased cell death as marked by the staining of activated caspase 3, compared with both control and *Pten* cKO prostate (Figure S3). These results suggest that deletion of *miR-205* in the *Pten* null model leads to reduced proliferation in both basal and luminal cells, consistent with macroscopically compromised tumorigenesis in dKO model.

3.5 | Hallmarks of prostate tumorigenesis are reduced by the loss of *miR-205*

To further characterize tumor initiation and progression in dKO model, we monitored the expression of androgen receptor (AR), a transcription factor which plays an important role during prostate tumorigenesis.^{20,21} In normal prostate, AR was mostly detected in the luminal cells with occasional basal expression (Figure 4A, left panel). Correlating with tumor formation in *Pten* null prostate, elevated AR signals were detected in both basal and luminal cells. In particular, elevated AR expression in *Pten* null luminal layers was evident, judged by intensity of IF staining (Figure 4A, middle panel). In contrast, although basal and

luminal expression of AR in dKO was detected, the level of AR expression in dKO samples was lower than that in *Pten* null tumors (Figure 4A, right panel). Quantification by Western blot showed that AR levels were elevated ~15-fold in *Pten* cKO sample, whereas little change in AR expression was detected in dKO samples, compared to the control (Figure 4C). Similarly, Sox9, another transcription factor whose elevated expression has been implicated in a role in prostate cancer,^{22–24} was elevated in *Pten* null prostate (Figure 4B), and the increased expression was also reduced when *miR-205* was concurrently deleted (Figure 4B and 4C). Finally, when *Pten* null tumors became invasive, we observed breakdown of the basement membrane and loss of SMA surrounding the primary tumor (Figure 4D). In dKO, SMA staining was largely intact, consistent with the lack of tumor progression in dKO samples (Figure 4D). Collectively, these results confirmed the requirement of *miR-205* for *Pten* null-induced prostate tumorigenesis.

3.6 | Loss of *miR-205* reduces pAkt levels and enhances cellular senescence

Having established the requirement of *miR-205* in *Pten* null-induced tumorigenesis, we set out to probe the underlying mechanism in a lineage-specific manner. Our previous study demonstrated that *miR-205* targets multiple negative regulators of the PI(3)K signaling pathway and, in turn, promotes pAkt levels in skin stem cells.¹³ We first confirmed the expression of several previously established targets, which are well-established negative regulators of the PI(3)K signaling including *Inpp11* and *Inpp4b*, in the FACS-purified prostate basal cells (Figure 5A). Expression of both *Inpp11* and *Inpp4b* was downregulated in *Pten* null cells and derepressed in dKO cells (Figure 5A). These data indicated that loss of *miR-205* enhances expression of these PI(3)K negative regulators in *Pten* null prostate. Consistent with these observations, although *Pten* null prostate showed strongly increased pAkt levels in both basal and luminal cells as expected, dKO prostate had reduced pAkt levels (Figure 5B and 5C). Whereas elevated pAkt levels in dKO luminal cells were still visible, the pAkt levels were largely reduced in dKO basal cells, similar to that of the control prostate (Figure S4). Because elevated pAkt is a hallmark of tumorigenesis of the prostate and the levels of pAkt are correlated with prostate tumorigenesis,²⁵ these results suggest that loss of *miR-205* compromises the induction of pAkt levels caused by the loss of *Pten*, likely through the derepression of *miR-205* targets including *Inpp11* and *Inpp4b*.

Because *Pten* null-induced tumorigenesis also causes cellular senescence, an important mechanism for tumor suppression,^{4,7} and because high pAkt levels are required to overcome cellular senescence, we examined the activity of senescence-associated beta-galactosidase (SA- β -gal) in *Pten* null and dKO prostate. As expected, *Pten* null prostate showed enhanced SA- β -gal signals throughout the entire prostate (Figure 5D). In contrast, whereas the overall SA- β -gal signals were slightly elevated in dKO prostate, we observed strong SA- β -gal activities in cells close to the basement membrane, although we were unable to confirm whether these cells are basal cells because we were unable to costain them for *Krt5* (Figure 5D). To further examine increased cellular senescence, we probed the expression of *p16*(*INK4a*), also known as *Cdkn2a*, a well-established tumor suppressor that is associated with cellular senescence. qPCR quantification of *p16* transcripts in FACS-purified basal cells from control, *Pten* cKO and dKO prostate revealed strongly elevated *p16* levels in dKO (Figure 5E). In support, IF staining confirmed elevated *p16* proteins in dKO prostate (Figure

5F). *p16* is not a target of *miR-205* based on our analysis and these data indicate that the elevated *p16* level is an indirect effect of the deletion of *miR-205* in *Pten* null. Together, these results suggest that in the absence of *miR-205*, basal cells become more sensitive to cellular senescence.

3.7 | Overexpression of *miR-205* in luminal cancer cells inhibits cell growth

The loss of *miR-205* appears to compromise tumorigenesis in both basal cells and luminal cells (Figures 2–4). However, previous studies using cultured human prostate cancer cells suggested that *miR-205* inhibits growth and invasion of luminal cell-derived cancer cells and plays a tumor suppressor role.¹⁵ To solve this discrepancy, we first cultured primary epithelial cells isolated from *Pten* null prostate. We stained these cells for *Krt5* and *Krt8* and only detected *Krt8* expression (Figure 6A). This indicated that most *Pten* null cells that grow in culture are luminal cells. We then infected these *Pten* null luminal cells with *MIGR-miR-205* and performed colony formation assays. Overexpression of *miR-205* reduced the number of colonies (Figure 6B).

We next tested the effect of *miR-205* expression in human prostate cells. First, we measured the expression of *miR-205* in normal human prostate cells (RWPE-1)²⁶ as well as several human prostate cancer cells including LNCap, Vcap, and DuCap. Normal RWPE-1 cells expressed a high level of *miR-205*, compared with all cancer cells (Figure 6C). These qPCR results were also confirmed by miR-seq, in which *miR-205* is only highly expressed in RWPE-1 cells but not in the cancer cell lines (Table S2). These data indicate that *miR-205* is not expressed in human luminal cancer cells. When we infected RWPE-1 and LNCap cells with *MIGR-miR-205*, we found that the colony forming ability of LNCap but not RWPE-1 cells was reduced by *miR-205* expression (Figure 6D and 6E). These data suggest that *miR-205* is not highly expressed in luminal cancer cells and the expression of *miR-205* in luminal cancer cells has an inhibitory effect on cancer cell growth in vitro.

4 | DISCUSSION

In this study, we have provided in vivo evidence for the requirement of *miR-205* during *Pten* null-induced tumorigenesis. Using quantitative miR-Seq and in situ hybridization, we identified *miR-205* as the most highly expressed miRNA in basal cells, and determined that *miR-205* is further elevated in *Pten* null prostate, indicative of an oncogenic role. Consistent with this hypothesis, when *miR-205* was genetically ablated together with *Pten* in the prostate, progression of tumorigenesis was significantly compromised. We previously found that *miR-205* directly targets multiple negative regulators of the PI(3)K signaling pathway, including *Inpp1* and *Inpp4b* in skin stem cells.¹³ Genetic deletion of *miR-205* in the skin leads to ~90% reduction of pAkt levels and results in reduced stem cell expansion during neonatal development. In contrast, basal cells of the prostate gland are largely quiescent and show little sign of pAkt during normal development. Genetic deletion of *miR-205* during prostate development and homeostasis had no discernible defects. However, elevated pAkt levels and dysregulated PI(3)K signaling are hallmarks of prostate cancer and a main underpinning for *Pten* null-induced tumorigenesis.^{3,5,25,27} pAkt and *Pten* levels are linked to prostate tumorigenesis in a dosage dependent manner.²⁵ Furthermore, a recent survey of

miR-205 expression in prostate cancer patients indicates an association between high *miR-205* expression in normal epithelium and relapse.²⁸ Thus, maintaining *miR-205* expression may be important to the initiation and progression of prostate cancer. Indeed, loss of *miR-205* in the *Pten* null background reduces global pAkt levels by ~50% (Figure 5B). These reduced pAkt levels are correlated with reduced proliferation and likely contribute to the elevated cellular senescence. We note that the observed defects including reduced proliferation and enhanced cellular senescence in the *Pten/miR-205* dKO model are similar to those observed in the *Pten/Dicer1* dKO model.¹⁰ Considering the high abundance of *miR-205* in these cells, it is possible that loss of *miR-205* is a main contributing factor for the observed *Dicer1* KO phenotypes in basal cells. In addition, we did not observe elevated apoptosis in either basal nor luminal cells. This suggests that other miRNAs are responsible for these defects. Using the same *Pb-Cre/Pten* model, we also deleted *miR-21*, another miRNA with well-documented oncogenic roles in other cancer types such as lung cancer.¹⁶ However, we did not observe any discernible defects in *Pten* null-induced prostate tumorigenesis. This suggests that the roles of individual miRNAs in cancer is likely specific to cellular context such as tissue types and oncogenic stimuli.

We note that both human and mouse cancer cells express a very low level of *miR-205*, in contrast to basal cell and RWPE-1 cell, a normal human prostate cell line that expresses both basal and luminal markers.²⁶ These observations were consistent with a previous report, in which *miR-205* overexpression inhibits metastatic, luminal prostate cancer cells.¹⁵ Because of selective avoidance between cell type-specific miRNAs and their targets,²⁹ it is likely that *miR-205*, the most highly expressed miRNA in basal cells, suppresses many genes in luminal cells and antagonizes the luminal fate in general. Individual miRNAs can regulate many targets and individual targets can also be regulated by multiple miRNAs. Elucidation of these complex regulatory networks will be critical for future investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AR	androgen receptor
FACS	fluorescence activated cell sorting

IF	immunofluorescence
PIN	prostatic intraepithelial neoplasia
SA-β-gal	senescence-associated beta-galactosidase
SMA	smooth muscle actin

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FIGURE 1.

miR-205 is highly expressed in the basal layer of prostate gland. A, Quantification of miR-seq data show *miR-205* is the most abundantly expressed miRNA in basal cells. Red square circles *miR-205*. B, qPCR validation of differential *miR-205* expression in basal and luminal cells. Data shown are mean $\pm$ SEM, collected from three independent experiments. *** $P < .001$. C, *miR-205* is expressed in basal but not luminal cells. Upper panel: *miR-205* is expressed in Krt5+ basal cells. Arrows point to colocalization of *miR-205* and Krt5 signals. Lower panel: *miR-205* is not expressed in Krt8+ luminal cells. D, *miR-205* expression is restricted to basal cells in both control (Ctrl) and *Pten* null (cKO) prostate samples. E, qPCR quantification shows elevated *miR-205* expression in FACS purified basal cells from *Pten* null prostate. Data shown are mean $\pm$ SEM, collected from three independent experiments. * $P < .05$. F-H, In situ hybridization for *miR-205* in normal human prostate (F) and human prostate cancer patients with Gleason score 7 (G) or 9 (H). Scale bars in all panels, 20 μ M.

FACS, fluorescence activated cell sorting; miRNA, microRNA; qPCR, quantitative polymerase chain reaction [Color figure can be viewed at wileyonlinelibrary.com]

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FIGURE 2.

Loss of *miR-205* compromises tumorigenesis in a *Pten* null model. A, *miR-205* expression is detected by in situ hybridization in all lobes of Ctrl and *Pten* null (cKO) samples, but is largely depleted in dKO (scale bars = 40 μ M). B, Dissected prostate samples show macroscopic tumor progression in Ctrl, cKO, and dKO mice at the age of 10-week old. The whisker chart shows the gross prostate weight. Data shown are mean $\pm$ SD, collected from nine Ctrl, eight cKO and six dKO mice, respectively. ** $P < .01$ and *** $P < .001$. C, Dissected prostate from 1-year-old Ctrl, cKO, and dKO mice (upper panel) and whisker chart showing the gross prostate weight (lower panel). *** $P < .001$ (scale bar = 1 cm in B and C). D-F, Morphological characterization of the prostate in Ctrl, cKO, and dKO mice at different ages. At 6 weeks, the anterior lobe of cKO prostate shows the sign of PIN1/2, while dKO prostate is largely normal (D). At 10 weeks, cKO shows features of PIN3/4 stage, while dKO still lacks signs of tumorigenesis (E). At 1 year, cKO develops adenocarcinoma,

whereas dKO shows features of PIN3/4 stages (F) (Scale bar = 20 μ M). miRNA, microRNA; qPCR, quantitative polymerase chain reaction [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 3.

Loss of *miR-205* reduces cell proliferation in both basal and luminal cells. A, Krt5/Krt8 double-positive cells, characteristic of prostate hyperproliferation, are prevalent in cKO (arrows), less frequently observed in dKO, and not observed in Ctrl. B, Basement membrane marked by *SMA* is intact in Ctrl and dKO, but compromised in cKO (arrows). C and D, Proliferative cells marked by *Ki67* signals in Krt5+ basal cells (arrows) are widespread in cKO but less frequently observed in both Ctrl and dKO. Representative images are shown in (C) and quantified in (D). Data shown are mean $\pm$ SD, collected from three sets of mice. $*P < .05$ and $**P < .01$. E and F, Proliferative cells marked by *Ki67* signals in Krt8+ luminal cells are widespread in cKO but less frequently observed in dKO (arrows). Representative images are shown in (E) and quantified in (F). Data shown are mean $\pm$ SD, collected from three sets of mice. $*P < .05$ and $**P < .01$. Scale bars in all panels = 20 μ M. SD, standard deviation [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 4.

Hallmarks for prostate tumorigenesis are reduced in the absence of *miR-205*. A, AR level is strongly increased in cKO, but less in dKO, compared with Ctrl samples. Arrows point to AR+ cells in the luminal layer and thick arrowheads point to AR+ cells in Krt5+ basal cells. B, Sox9 level strongly increases in cKO, but less in dKO, compared with Ctrl samples. C, Representative Western blot analysis using total prostate lysate shows strong upregulation of both AR and Sox9 in cKO. The elevated expression is attenuated in dKO samples. Three independent experiments were performed. D, Compromised tumor progression in dKO is shown by reduced AR expression and intact *SMA* stained basement membrane. Scale bars in all panels, 20μM. AR, androgen receptor; SMA, smooth muscle actin [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 5.

miR-205 is required to promote pAKT and bypass cellular senescence in *Pten* null prostate. A, qPCR quantification of *miR-205* targets, *Inpp11* and *Inpp4b*, in the basal cells of Ctrl, cKO, and dKO prostate. *Hprt* is used as internal control. Data shown are mean $\pm$ SD, collected from three independent experiments, $**P < .01$. B, Representative Western blot analysis quantification using total prostate lysate shows strongly upregulated pAkt levels in cKO and attenuated upregulation in dKO samples. Total Akt levels are not changed. Three independent experiments were performed. C, pAkt levels increase in both basal and luminal layer in cKO, less strongly increases in dKO and are absent in Ctrl. Basal and luminal cells are marked by Krt5 and Krt8 signals, respectively. D, SA-β-gal staining shows *Pten* null-induced senescence in cKO and more prominent increases in dKO. Arrows point to senescent cells with strong signals. E, qPCR quantification of *p16* in FACS purified basal layer isolated from Ctrl, cKO, and dKO prostate. Data shown are mean $\pm$ SD, collected from

three independent experiments, $**P < .01$. F, p16/Krt5 costaining identifies senescent cells in basal and luminal cells in both cKO and dKO samples but rarely in Ctrl. Scale bars in all panels, 20 μ M. FACS, fluorescence activated cell sorting; miRNA, microRNA; qPCR, quantitative polymerase chain reaction; SA- β -gal, senescence-associated beta-galactosidase; SD, standard deviation [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 6.

miR-205 inhibits the growth of *Pten* null luminal cells. A, Confirmation of *Krt8+ Pten* null luminal cells by immunofluorescent staining. B, Overexpression of *miR-205* in *Pten* null luminal cells inhibits cell growth. Representative images are shown on the top and quantified at the bottom ($N=3$, $*P<.05$). C, qPCR quantification of *miR-205* expression in RWPE-1, LNCap, Vcap, DuCap human prostate cells. D, Overexpression of *miR-205* in RWPE-1 cells shows no effect in colony formation ($N=5$). E, Overexpression of *miR-205* in LNCap cells reduces colony formation ($N=3$, $***P<.001$). miRNA, microRNA; ns, not significant; qPCR, quantitative polymerase chain reaction [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1

Phenotypes characterization of control, *Pten* cKO and *Pten/miR-205* dKO mice.

Age (wk)	Ctrl	<i>Pten</i> cKO	<i>Pten/miR-205</i> dKO
6	5/5 normal	3/3 PIN	3/3 normal
10	18/18 normal	30/30 carcinoma	16/24 PIN
52	7/7 normal	1/8 metastasis 2/8 died	4/6 PIN

Note: The animal number and prostate tumor characterization of control (Ctrl), cKO, and dKO mice are listed. At 6 week, five Ctrl and three dKO animals appear normal while three cKO mice progress to PIN1 or PIN2 stage. At 10 week, 16 out of 24 dKO mice show delayed tumor formation with PIN2 stage, while 30 cKO animals show invasive adenocarcinoma. At 52 week, 4 out of 6 dKO animals show compromised tumorigenesis with PIN3 or PIN4, comparing to 1 out of 8 cKO progressed to metastasis and 2 out of 8 cKO died before 1-year old.