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***miR-125b* promotes growth of prostate cancer xenograft tumor through targeting pro-apoptotic genes**

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Abstract

BACKGROUND—Increasing evidence demonstrates that aberrantly-regulated microRNAs (miRNAs) contribute to the initiation and progression of human cancer. We previously have demonstrated that *miR-125b* stimulated the growth of prostate cancer (CaP) cells. In this study, we further determined the influence of *miR-125b* on the pathogenesis of prostate cancer.

METHODS—To evaluate the effect of *miR-125b* on xenograft tumor growth, male athymic mice were subcutaneously injected with PC-346C-*miR-125b* cells that stably overexpressed *miR-125b*. Potential direct target transcripts of *miR-125b* were identified using a bioinformatics approach and three *miR-125b* targeted molecules were confirmed by means of biochemical analyses.

RESULTS—Enforced expression of *miR-125b* promoted tumor growth in both intact and castrated male nude mice. In an effort to define the molecular mechanism(s) mediating its tumor growth properties, we found that *miR-125b* directly targets eight transcripts, including three key pro-apoptotic genes: p53, Puma and Bak1. Increasing the abundance of *miR-125b* resulted in a dramatic decrease in the levels of these three proteins in prostate cancer cells. A direct repressive effect on each of these was supported by the ability of *miR-125b* to significantly reduce the activity of luciferase reporters containing their 3'-untranslated regions of each gene encompassing the *miR-125b*-binding sites. Additionally, we found that repression of *miR-125b* activity was able to sensitize prostate cancer cells to different therapeutic interventions.

CONCLUSION—Data obtained in this study demonstrate that *miR-125b* promotes growth of prostatic xenograft tumors by down-regulating three key pro-apoptotic genes. This suggests that *miR-125b* is oncogenic and makes it an attractive therapeutic target in prostate cancer.

Keywords

prostate cancer; microRNA; p53; pro-apoptotic gene

INTRODUCTION

Prostate cancer (CaP) is the most frequently diagnosed malignant tumor in males and the second leading cause of cancerous deaths in American men (1). Patients with metastatic CaP

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are customarily treated with androgen ablative therapy (AAT). Unfortunately, failure of AAT inevitably occurs and the patient's tumor becomes castration-resistant (CR). At present, no curative treatment is available for CR CaP (2). Although the precise mechanisms leading to castration resistance are not completely understood, considerable insight into the progression of CaP has been recently achieved. Aberrant expression of some growth/survival related signal molecules, including their modulator microRNAs (miRNAs), is thought to be one mechanism associated with the development of CR CaP (3).

Mature miRNAs are a class of 19- to 25-nucleotide RNA molecules. These endogenous small RNAs may negatively regulate up to 30% of the human genes by imperfectly binding to miRNA-binding sites located at the 3'-untranslated regions (3'-UTRs) of targeted genes, causing either degradation of mRNAs or, more commonly for mammalian miRNAs, inhibition of their translation to functional proteins. Thus, miRNAs are involved in cell proliferation, differentiation, stress response, apoptosis, immunity and transcriptional regulation in many human diseases including cancer (4). Accumulating evidence supports the potential involvement of altered expression of miRNAs in initiation and progression of a wide range of human cancers, including CaP. It was reported that a number of miRNAs exhibit distinct expression patterns in clinical prostate tumor samples compared to benign prostate tissues (5–8), suggesting that these aberrantly expressed miRNAs are likely implicated in the biology of CaP.

miR-125b is a well characterized miRNA. In humans, there are two *miR-125b* members: *miR-125b-1* and *-2*, located on chromosome 11 and chromosome 21, respectively. In CaP cells, the mature *miR-125b* is thought to be derived primarily from *miR-125b-2* (9). Dysregulation of *miR-125b* has been reported to occur in multiple human cancer types, including cancers of the stomach (10), colon (11), pancreases (12), bladder (13) and ovary (14), as well as in oligodendroglial tumors (15) and acute lymphoblastic leukemia (16). It was recently reported that highly expressed *miR-125b* confers survival advantage by suppressing apoptosis in acute lymphoblastic leukemia (17). We previously found that *miR-125b* stimulates androgen-independent growth of CaP cells *in vitro* (18). In the current study, we further explored the functional role of *miR-125b* in CaP, and found that enforced expression of *miR-125b* promoted tumor growth in intact and castrated male nude mice. We also performed a series of experiments to investigate the molecular mechanisms accounting for *miR-125b*-mediated growth of CaP cells. The data obtained in this study demonstrate that *miR-125b* has oncogenic properties through its anti-apoptotic and proliferation-promoting activities and suggest that it can be exploited as a therapeutic target in CaP.

MATERIALS AND METHODS

Reagents

Synthetic *miR-125b* mimic (miR-125bm), miRNA negative control (miR-NC), anti-*miR-125b*, anti-*miR*-negative control (anti-miR-NC) and the pMIR-REPORT Luciferase vector were purchased from Ambion. The miR-125bm is short double-stranded RNA oligonucleotide, and it has been chemically modified to increase the stability *in vivo* and the activity of *miR-125b*. The anti-*miR-125b* was single-stranded RNA oligonucleotides designed to specifically bind to endogenous *miR-125b*, inhibiting its activity but not downregulating its abundance. Genistein combined polysaccharide (GCP) is a nutritional supplement and was generously provided by Amino Up Chemical Company Ltd. (Sapporo, Japan) and stock solutions were made in 50% DMSO/50% EtOH. Antibodies to Bak1, p21, SMAC, CytC, proCas3 were purchased from Cell Signaling Technology (Danvers, MA) and antibodies to p53 and Puma from Calbiochem.

Cell lines

CaP cell lines used in this study include PC3, DU145, PC-346C, and LNCaP and its sublines LNCaP-cds2 (named as cds2) and LNCaP-R273H. Both cds2 and LNCaP-R273H were prepared previously in our laboratory (19,20). PC-346C cell line that was kindly provided by Dr. Adrie van Bokhoven (University of Colorado) was developed from a non-progressive prostate tumor (21). LNCaP, PC-346C, PC3 and DU145 were routinely maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. For LNCaP-R273H cells, G418 (200 μ g/ml) was added to the medium. cds2 cells were cultured in RPMI 1640 medium containing 5% charcoal/dextran-stripped serum (androgen-deprived medium).

Generation of stable PC-346C-*miR-125b* population

A lentiviral *miR-125b* expression vector that expresses a ~500-base pre-*miR-125b* and empty lentiviral vector were purchased from System Biosciences (SBI, Mountain View, CA). Pseudovirus production and cell transduction were performed following the manufacturer's protocol. The resulting PC-346C CaP cells, PC-346C-*miR-125b* that stably overexpress *miR-125b*, were selected through fluorescence-activated cell sorting (FACS) and were maintained in medium described above. Over-expression of *miR-125b* in infected PC-346C cells was detected using a quantitative RT-PCR approach with a *miR-125b*-specific primer (Ambion).

Tumor xenografts

Male athymic *nu/nu* mice (4- to 6-week-old, Harlan Laboratories) were injected subcutaneously with suspensions of 2×10^6 PC-346C-*miR-125b* or PC-346C-vector control cells made in 50% Matrigel basement membrane matrix (1:1 vol/vol with complete medium; Becton Dickinson). Tumor dimensions were measured once per week and volumes calculated according to the formula: $1/2$ (length \times width \times height). Castration was performed in a subpopulation of mice when tumors reached a diameter of approximately 0.6 cm. Animal studies were performed according to protocols approved by the Animal Care and Use Committee at University of California, Davis.

Annexin V binding assay

Anti-*miR-125b*-induced apoptosis was analyzed using a FACS Annexin V assay kit (Trevigen, Inc. Gaithersburg, MD) following the protocol provided by the manufacturer. Briefly, cells were transfected with anti-*miR-125b* and incubated for 72 hours. The cells were harvested, washed once with PBS, re-suspended in 100 μ l of 1X Annexin V binding buffer containing 1 μ l of Annexin V-FITC conjugate and 10 μ l of propidium iodide solution and then incubated for 30 min at room temperature in the dark. Subsequently, 400 μ l 1X Annexin V binding buffer was added and samples analyzed on a FACScan flow cytometer. Data analysis was performed using FACScan software (Becton Dickinson).

Computational identification of *miR-125b* target transcripts

In order to gain insight into the mechanism(s) through which *miR-125b* influences growth and survival of CaP cells, an integrative bioinformatics strategy was developed to identify the target transcripts specifically relevant within the context of our models. This target profiling strategy entailed four steps: 1) defining a *miR-125b*-regulated gene expression profile; 2) generating a comprehensive *miR-125b* target site database by compilation of predicted targets (~943 separate sites) sourced from various publicly-available databases (22,23); 3) using this database as an index for mining and extracting of the *miR-125b* targets present in our list of differentially-expressed genes; and 4) selecting candidate genes based upon gene ontology and pathway analyses. For the first component, the list of *miR-125b*-regulated genes in LNCaP cells was obtained by overexpression of *miR-125b* followed by

Affymetrix gene expression profiling and comparison analysis for differentially-expressed genes with DNA-Chip analyzer (dChip) software (24). Extraction of predicted *miR-125b* target transcripts from the expression profile was accomplished with an Excel add-in “parsing” tool from the Macros for Microarray (MfM) software (UC Davis Cancer Center Genomics and Expression Resource). Since miRNAs can negatively regulate the genes via accelerated decay, the resulting *miR-125b* target profile was limited to the down-regulated transcripts. Using the GO terms appended to each entity, the genes were then filtered based upon function in biological processes including apoptosis, tumor suppression and metastasis.

Western blot analysis

Cells were grown to 70–80% confluence and lysed using the cell lysis buffer (Cell Signaling Technology, Cat#: 9803). Denatured protein samples (20 to 100 µg) were resolved on 10% SDS-polyacrylamide gels, and then transferred to Immobilon PVDF membrane (Millipore). After blocking with 5% nonfat dry milk in Tris-buffered saline/0.05% Tween 20 (TBST), the membrane was incubated with a specific primary antibody, followed by the horseradixh peroxidase-conjugated secondary antibody. Proteins were visualized using ECL reagents.

Reporter constructs and luciferase assay

To construct reporter plasmids containing the 3'-DNA regions of *p53* and *PUMA*, a 0.5-kb DNA fragment of the *p53* 3'-UTR and a 0.85-kb DNA fragment of the *PUMA* 3'-UTR that contain the putative *miR-125* binding sites were prepared. Negative control plasmids containing 0.4-kb *p53* and 0.7-kb *PUMA* 3'-UTR fragments that lack the *miR-125b* binding sites were also prepared. DNA fragments were cloned into the pMIR-REPORT Luciferase vector downstream of the reporter gene. The sequences and cloning direction of these PCR products were validated by DNA sequencing. For luciferase assay, cells (4×10^4 per well) were seeded into 24-well plates and cultured for 24 hours. The cells were then transfected with reporter plasmids. The pRL-SV40 *Renilla* luciferase plasmid (Promega) was used as an internal control. Two days later, cells were harvested and lysed with passive lysis buffer (Promega). Luciferase activity was measured using a dual luciferase reporter assay (Promega). The results were expressed as relative luciferase activity (*Luc/Renilla*).

Evaluation of *miR-125b* antagonism on growth with WST-1 assay

Cells (4.5×10^3 per well) were plated in 96-well plates in androgen-deprived medium. After being cultured for 24 hours, cells were transfected with anti-*miR-125b* or anti-miR-NC at a concentration of 50 nM using lipofectamine 2000 (Invitrogen). Five hours later, cells were treated with fresh medium or, if necessary, fresh medium containing anti-CaP drug in optimized concentration. The transfection protocol was optimized using a fluorescent pEGFP-N1 vector (Clontech), and resulted in the transfection of >90% of *cds2* cells. Tetrazolium-based cell proliferation assay (WST-1; Promega) was then carried out at various days after transfection according to the manufacturer's protocol.

RESULTS

miR-125b promotes tumor growth in intact and castrated male nude mice

Our previous *in vitro* experiments demonstrated that *miR-125b* stimulated androgen-independent growth (18). In this study, we determined whether *miR-125b* was able to enhance tumorigenicity. For this purpose, we generated a stable PC-346C-*miR-125b* pool by infection with a *miR-125b*-expressing lentiviral vector. Quantitative RT-PCR analysis of *miR-125b* abundance exhibited a 19-fold increase in *miR-125b* levels in *miR-125b*-infected cells compared to that in control cells (data not shown). PC-346C cell line was selected since it expresses wild-type p53 and wild-type AR (25) and is androgen-dependent/sensitive

(26). Furthermore, this cell line has a limited ability to form tumors in intact nude mice and fails to grow in castrated mice (27). Two *in vivo* experiments were performed using the lentiviral-transduced PC-346C-*miR-125b* cells. First, we tested whether *miR-125b* determined tumorigenicity with respect to tumor formation rate and growth kinetics. Intact male nude mice (five mice per group) were injected subcutaneously with PC-346C-*miR-125b* or PC-346C-vector control cells. Tumors arose in all mice injected with PC-346C-*miR-125b* and in four of five control mice. However, PC-346C-*miR-125b* cells produced palpable tumors much faster than control cells with tumors appearing at 7 to 10 days vs. at approximately 21 days, respectively. Furthermore, PC-346C-*miR-125b* tumor volumes at 5 weeks were markedly larger than control tumors (Fig. 1A), indicating that in intact mice, elevated *miR-125b* expression accelerates tumor growth kinetics. We next tested whether *miR-125b* could support tumor growth in the absence of androgens. For this, male mice were injected with PC-346C-*miR-125b* cells. When tumors reached ~0.6 cm in the diameter, a subpopulation of mice bearing *miR-125b* tumors were castrated and tumor growth monitored. Castration resulted in a temporary growth regression of *miR-125b* tumors, followed by rapid growth (Fig. 1B). In summary, these data indicate that *miR-125b* increases the tumorigenicity of and promotes CR growth of PC-346C cells.

Inactivation of *miR-125b* induces apoptosis of CaP cells

Reduced apoptosis can figure prominently in the CR growth of CaP (28). Based upon the results above, we wanted to test the hypothesis that downregulation of *miR-125b* activity would result in increased apoptotic cell death. To address this, the activity of *miR-125b* in PC-346C cells was antagonized by transfection with anti-*miR-125b* (50 nM). Cells were then cultured in androgen-deprived medium for four days and apoptotic cells detected and quantitated by Annexin V binding assay. We observed that in the absence of androgen anti-*miR-125b* induced 30.2% of PC-346C cells to undergo apoptotic cell death (Fig. 2A). The comparative figure for the anti-miR-NC cells was 5.4% ($p < 0.01$). To provide biochemical evidence for the occurrence of apoptosis, we determined whether downregulation of *miR-125b* increases the release of mitochondrial cytochrome c, SMAC and activated caspase 3. As expected, treatment of PC-346C cells with anti-*miR-125b* induced an enhancement of cytochrome c and SMAC, and a reduction of procaspase 3 (Fig. 2B). Therefore, *miR-125b*-mediated suppression of apoptosis signaling contributes to the growth of CaP cells.

Identification of *miR-125b* targets

In order to better define the molecular mechanisms by which *miR-125b* promotes CaP cell growth and modulates apoptosis, we utilized a bioinformatics approach to identify its potential targets. For this, we compiled a database of the computationally-predicted *miR-125b* target sites (from MSKCC) and then used it as an index to mine a gene expression profile composed of transcripts that were differentially-expressed as a result of transient overexpression of *miR-125b* in CaP cells. In this manner, candidate direct targets are extracted from the entire dataset, which more than likely also contained transcripts regulated as a secondary response. The resulting putative, CaP-relevant *miR-125b* targets were then classified based upon Gene Ontology terms in order to determine the mRNAs whose suppression was most likely responsible for the alteration in one or more biological processes, which was described in Materials and Methods. In addition to Bak1 which we previously confirmed and characterized as a *miR-125b* target, seven additional genes were identified that contain *miR-125b*-binding sites in their 3'UTRs (Table I). Of these, three pro-apoptotic molecules (p53, Puma and Bak1) that are functionally inactivated either by mutation and/or dysregulation, have been reported to play important roles in the initiation and progression of CaP. The other five genes are potential tumor or metastasis suppressors in other cancer types. Myotubularin related protein 3 (*MTMR3*) induces the expression of p27 (29); BRCA1 associated protein-1 (*BAP1*) interacts with BRCA1 and suppresses cancer

cell growth (30); sphingosine-1-phosphate lyase 1 (*SGPL1*) may play a role in cancer-surveillance pathways (31); aryl-hydrocarbon receptor repressor (*AHRR*) is an angiogenic factor (32); and sialidase 1 (lysosomal sialidase) (*NEU1*) inhibits tumor metastasis by decreasing integrin $\beta 4$ signaling and down-regulating MMP-7 (33). However, their involvement in CaP remains largely unknown.

***miR-125b* down-regulates pro-apoptotic molecules**

Several approaches were then used to confirm that these three pro-apoptotic genes are *bona fide* targets of *miR-125b*. First, Western blot analysis of lentiviral-transduced PC-346C-*miR-125b* tumor cell lysates demonstrated that *miR-125b* overexpression markedly reduced the protein levels of p53, Puma and Bak1 (left panel in Fig. 3A). Similar results were observed (right panel in Fig. 3A) in *cds2* cells that express 5-fold greater *miR-125b* than parental LNCaP cells do (18). Next, we determined whether elevating *miR-125b* levels ectopically with a chemically-modified *miR-125b* mimic (*miR-125bm*) would down-regulate these pro-apoptotic molecules in untreated or irradiated LNCaP cells. As expected, irradiation significantly enhanced the cellular level of p53 and its two direct effectors, p21 and Puma. Treatment with *miR-125bm* resulted in a marked decrease in both basal and irradiation-induced p53 levels (Fig. 3B). Moreover, downregulation of p53 by *miR-125bm* also reduced the expression of its effectors p21 and Puma. Consistent with our previous observation (18), *miR-125bm* targets Bak1. Additionally, we performed immunocytochemistry using enzymatic approaches to stain both p53 and Puma in LNCaP cells that had been transfected with *miR-125bm* and then irradiated. Similar to the data obtained from Western blot analysis, ectopic expression of *miR-125bm* induced a marked downregulation of p53, Puma and Bak1 (Fig. 3C).

Mechanistic analysis of *miR-125b* as a direct regulator of Puma expression in the context of p53-expressing CaP cells is complicated by the fact that Puma is a transcriptional target of p53, which is also a target of *miR-125b*. Thus, it is difficult to discern whether the downregulation of Puma is due to a direct effect of *miR-125b* or to the decrease of p53 caused by *miR-125b*. To validate that *miR-125b* directly targets Puma, p53-null PC3 cells were treated with *miR-125bm* followed by treatment with resveratrol (100 μ M), which was previously reported to upregulate Puma and Bak1 in PC3 cells (34). Western blot analysis confirmed that transfection with *miR-125bm* resulted in obvious reductions in resveratrol-stimulated Puma and Bak1 expression, respectively (Fig. 3D). Taken together, these data strongly support that *miR-125b* down-regulates the expression of p53, Puma and Bak1 in CaP cells.

Androgen downregulates pro-apoptosis molecules

As presented in our previous reporter (18), androgen upregulates the expression of *miR-125b* and consequently resulted in a reduction of Bak1 levels, we thus tested whether androgen treatment decreases the levels of p53 and Puma as well. To address this issue, LNCaP cells were treated with the synthetic androgen R1881 and Western blot analysis was performed to determine the levels of p53 and Puma, as well as Bak1 as a positive control. Similar to the previous observation, Bak1 expression was down-regulated by androgen and up-regulated by anti-*miR-125b*. When compared to untreated and vehicle-treated cells, R1881 induced a marked reduction of basal p53 and Puma levels (Fig. 4A). LNCaP cells were also treated with anti-*miR-125b* to decrease the activity of *miR-125b*. We found that ectopic expression of anti-*miR-125b* increased the expression of p53 and Puma proteins by approximately 2.0-fold (Fig. 4A), which was quantitatively measured using a Molecular Imager. In addition, when LNCaP cells were pre-treated with R1881 for 48 hours prior to irradiation with 10 Gy to stimulate increased expression of p53, it was found that R1881 treatment resulted in a

71% and 62% reduction of irradiation-stimulated up-regulation of p53 and Puma, respectively (Fig. 4B).

miR-125b* binds to the 3'-UTRs of *p53* and *PUMA

Mature miRNAs negatively regulate gene expression by binding to the 3'-UTRs of target transcripts mediated by the complementarity of their 5'-end seed sequences (*i.e.*, nucleotides 2–8 or 2–7) with miRNA-binding site sequences (35,36). We previously reported that *miR-125b* can bind to the 3' UTR of *BAK1* transcript that contains a binding site for *miR-125b* (18). Using the TargetScan program, we identified the *miR-125b* binding sites in the 3'-UTR regions of both *p53* and *PUMA*. To determine whether these putative *miR-125b* binding sites are responsible for the negative post-transcriptional regulation of *p53* and *PUMA* by *miR-125b*, pMIR-REPORT luciferase reporter constructs containing the 3'-UTR fragments of *p53* or *PUMA* were co-transfected with miR-125bm into the *miR-125b*-null DU145 cells. As shown in Fig. 5A, cotransfection resulted in a 57% and 45% reduction of the reporter activity for *p53* and *PUMA*, respectively. As a complimentary approach, we compared the androgen-regulated expression of p53 protein in the parental LNCaP cell line and its LNCaP-R273H subline. The latter ectopically expresses a p53^{R273H} mutant allele from a cDNA expression cassette that does not contain a *miR-125b* binding site due to its lack of the *p53* 3'-UTR. Consistent with the previous observation, LNCaP-R273H cells expressed markedly increased p53 compared to the parental LNCaP (20). R1881 treatment induced an 78% reduction of p53 in the parental LNCaP cells, but only 40% reduction in LNCaP-R273H cells (Fig. 5B), suggesting that R1881-induced *miR-125b* only targeted endogenous wild-type p53 while ectopically-expressed p53^{R273H} evaded regulation. Taken together, these results provide experimental validation that the 3' UTRs of *p53* and *PUMA* are targets of *miR-125b*.

Down-regulation of *miR-125b* sensitizes CaP cells to anti-cancer drugs

Our data presented above and in previous report (18) demonstrated that downregulation of *miR-125b* abundance inhibits the growth of CaP cells. We thus asked whether this miRNA is a potential therapeutic target that would improve the efficiency of existing CaP therapy. To address this issue, we evaluated the effect of a combination of anti-*miR-125b* and an anti-CaP drug on the growth inhibition or apoptosis of CaP cells. We have previously reported that GCP, an isoflavone-enriched nutraceutical, lowers the threshold at which CaP cells undergo apoptosis; hence, it was used here. To verify that the combination would increase the growth inhibition, GCP and anti-*miR-125b* were used to treat androgen-independent cds2 cells that overexpress natural *miR-125b* (18). We observed that the combined treatment resulted in a significant inhibition ($p < 0.05$) of the CR growth compared to treatment with a single chemical (Fig. 6A). Additionally, we also tested cisplatin as it is a commonly-used chemotherapy in the treatment of solid malignancies including CaP. We used this combinational strategy to treat PC-346C cells that were cultured in medium containing 10% FBS and evaluated the extent to which they underwent apoptosis. It was found that anti-*miR-125b* plus 5 μ M cisplatin increased the number of CaP cells that entered apoptosis in the presence of androgens ($p < 0.05$, Fig 6B). The results suggest that down-regulation of *miR-125b* level can increase the sensitivity of CaP cells to different treatments.

DISCUSSION

This study extends our previous observation that *miR-125b* stimulates CR growth *in vitro* (18). In the present study, we demonstrated that *miR-125b* enhances tumorigenicity of CaP cells in intact and castrated mice. Furthermore, we found that *miR-125b* mediates a broad attenuation of the intrinsic apoptosis pathway by targeting p53, Puma, and Bak1. Although an early investigation reported that *miR-125b* was down-regulated in 5 of 9 clinical CaP

tissues related to 4 benign prostate samples (6), several later studies demonstrated that clinical CaP samples over-expressed *miR-125b*. Prueitt *et al.* (8) detected a 4.29-fold increase in *miR-125b* in locally-invasive tumors compared to those without local invasion. Mitchell *et al.* (37) observed a 6.35-fold increase in circulating *miR-125b* in metastatic CaP compared to that found in sera from healthy men. We previously reported that prostate tumors with high Gleason scores overexpressed *miR-125b* (18). Our experimental results obtained in the present study, together with a majority of previous clinical data, strongly suggest that aberrantly-expressed *miR-125b* has attributes of an oncogene by virtue of promoting the development and progression of CaP. Moreover, we found that androgen withdrawal by lowering *miR-125b* leads to an increase in p53 expression, which is consistent with Agus's observation that androgen withdrawal induced expression of p53 (38). Our finding provides a molecular rationale for why androgen withdrawal may improve the outcome for CaP patients who undergo radiation therapy. Additionally, we found that repression of *miR-125b* activity sensitizes CaP cells to undergo apoptosis after treatment with various therapies, suggesting that *miR-125b* may be exploited as a target for CaP therapy.

While *miR-125b* has been reported to be highly expressed in many types of human cancers, including CaP, it remains to be determined how *miR-125b* affects the growth of CaP cells. At the initiation of the study, like most of miRNAs, the precise physiologic targets of *miR-125b* had not been validated experimentally. We reasoned that for *miR-125b* to be a driver miRNA, it would need to satisfy the criteria of targeting relevant and potent effector molecules in the context of CaP cells. In our earlier report, Bak1 was identified as a target of *miR-125b*. In the present study, p53 and Puma were further validated to be direct targets of *miR-125b*, which is consistent with a recent finding that *miR-125b* is an important negative regulator of p53 (39). Since p53, Puma and Bak1 are pro-apoptotic molecules, our results show that *miR-125b* is involved in the p53 network and plays a role in how it regulates apoptosis. Conversely, the data provide a mechanistic explanation for why suppression of *miR-125b* can inhibit tumor growth. In addition to these three pro-apoptotic molecules, *miR-125b* also targets five additional tumor suppressor mRNAs. The precise roles of these putative targets in CaP are unknown. It is possible that decreased expression of these tumor suppressors, together with down-regulated pro-apoptotic molecules, contributes to tumorigenesis and progression of CaP by providing the cells a survival advantage in an androgen-sufficient or androgen-deprived environment. In the ongoing studies, we will conduct *in vitro* and *in vivo* experiments to evaluate the effects of individual molecules on the growth of CaP cells. Elucidation of their contribution to CaP will shed light on understanding mechanism underlying the tumorigenesis and progression of CaP, which might result in identify new molecular targets that would be used to develop potential treatments for this disease.

Data obtained from this study show that *miR-125b* is involved in the governance of proliferation and apoptosis in CaP. Under normal conditions, pro-apoptotic molecules induce mitochondrial outer membrane permeability, causing the release of apoptotic molecules (*e.g.*, cytochrome c), subsequent caspase activation, and ultimately leading to apoptotic cell death. In contrast, anti-apoptotic molecules promote survival by antagonizing apoptotic mediators at one or more levels. In CaP cells, particularly in CR tumors, growth is determined by the relative balance between cell proliferation and cell death (apoptosis). Aberrantly-expressed *miR-125b* down-regulates p53, Puma and Bak1. As critical regulators of apoptosis, these molecules have been shown to play a crucial role in suppression of the malignant phenotype of CaP cells (40–42). Therefore, aberrant *miR-125b* expression re-sets the balance of apoptosis by directly targeting these molecules and indirectly affecting other members of the Bcl-2 family, conferring a survival advantage to CaP cells. Additionally, it is well known that the AR plays a dominant role in both the initiation and progression of

CaP. Studies have demonstrated that wild-type p53 can repress AR activity (43). We observed that the AR signaling up-regulates *miR-125b* and *miR-125b* in turn down-regulates wild-type p53. We thus speculate that this is a self sustaining loop in CaP cells, which positively regulates the growth of CaP cells.

We, as well as others (39), validated that *miR-125b* is a direct, negative regulator of wild-type p53 by suppressing its basal expression and up-regulation in response to pro-apoptotic stimuli. Here, we asked how aberrant *miR-125b* expression affects apoptosis in CaP cells harboring a *p53* mutation. Addressing this issue is of clinical importance, since mutation of *p53* occurs in more than 40% of CaPs (44–46). At present, the exact answer to this question has not been determined. Data obtained from this present study provides some insight regarding repression of apoptosis by *miR-125b* in p53-inactivated CaP cells, which may involve targeting Puma and Bak1. In addition to p53 that directly regulates Puma, some of the other cell death and survival signals have been reported to upregulate Puma in a p53-independent fashion (47,48). Consistent with a previous report (34), we also observed that resveratrol induces an increase in Puma and Bak1 in p53-null PC3 CaP cells. Furthermore, increased *miR-125b* was able to significantly reduce resveratrol-stimulated Puma and Bak1. Therefore, *miR-125b*-mediated downregulation of Puma and Bak1 may play a key role in the p53-inactivated cases. Indeed, one study demonstrated that low Puma expression was associated with a rapid biochemical recurrence of CaP (41).

Given the complexity implicated in the castration resistance of CaP, treatment of this disease may rely on multimodal combination regimens. Until recently, however, chemotherapy has demonstrated only a small survival benefit in patients with CR CaP. The current strategy regarding the treatment of this disease is to prevent occurrence of castration resistance. The fact that one miRNA may target a large number of coding genes involving diverse signaling pathways make the non-encoding small RNA attractive in the design of combination therapy regimens. Indeed, recent study showed that molecular alterations elicited by targeting single miRNAs can provide growth advantages to tumor cells (17). Aberrant expression of *miR-125b* occurs in at least a subset of CaP cases. This miRNA targets various molecules related to the progression and therapy-resistance of CaP, which implicates it as an emerging alternative treatment option. In this study, we presented evidence that *miR-125b* has oncogenic function. It stimulates CaP growth in xenografts through an anti-apoptotic pathway, very importantly in both the presence and absence of androgens. Therefore, beyond CR CaP, this miRNA also is important in CaP progression. The anti-apoptotic phenotype was not an artifact of *miR-125b* overexpression in CaP cells. Its relevance to CaP cells is suggested by the sensitization to apoptosis induced by GCP after knockdown of the endogenous *miR-125b* in CaP cells. Furthermore, we validated that *miR-125b* directly targets p53, Puma and Bak1, which provide new insights into the molecular basis of CaP progression. There is little doubt that both p53 and AR play important roles in the progression of CaP. Here, we demonstrate that *miR-125b* links these two important pathways. Thus, it would appear to be an excellent target to pursue in our attempts to discover improved treatments for patients with prostate cancer.

CONCLUSION

The present results demonstrate that overexpression of *miR-125b* promoted tumor growth in intact and castrated male nude mice. A series of experiments was performed to investigate the molecular mechanisms accounting for *miR-125b*-mediated growth of CaP cells. It was found that *miR-125b* down-regulates three key pro-apoptotic molecules: p53, Puma and Bak1. Furthermore, reduction of *miR-125b* activity by using anti-*miR-125b* oligonucleotides induced apoptosis of CaP cells and sensitizes CaP cells to anti-cancer

drugs. These results suggest that *miR-125b* is oncogenic and makes it an attractive therapeutic target in prostate cancer.

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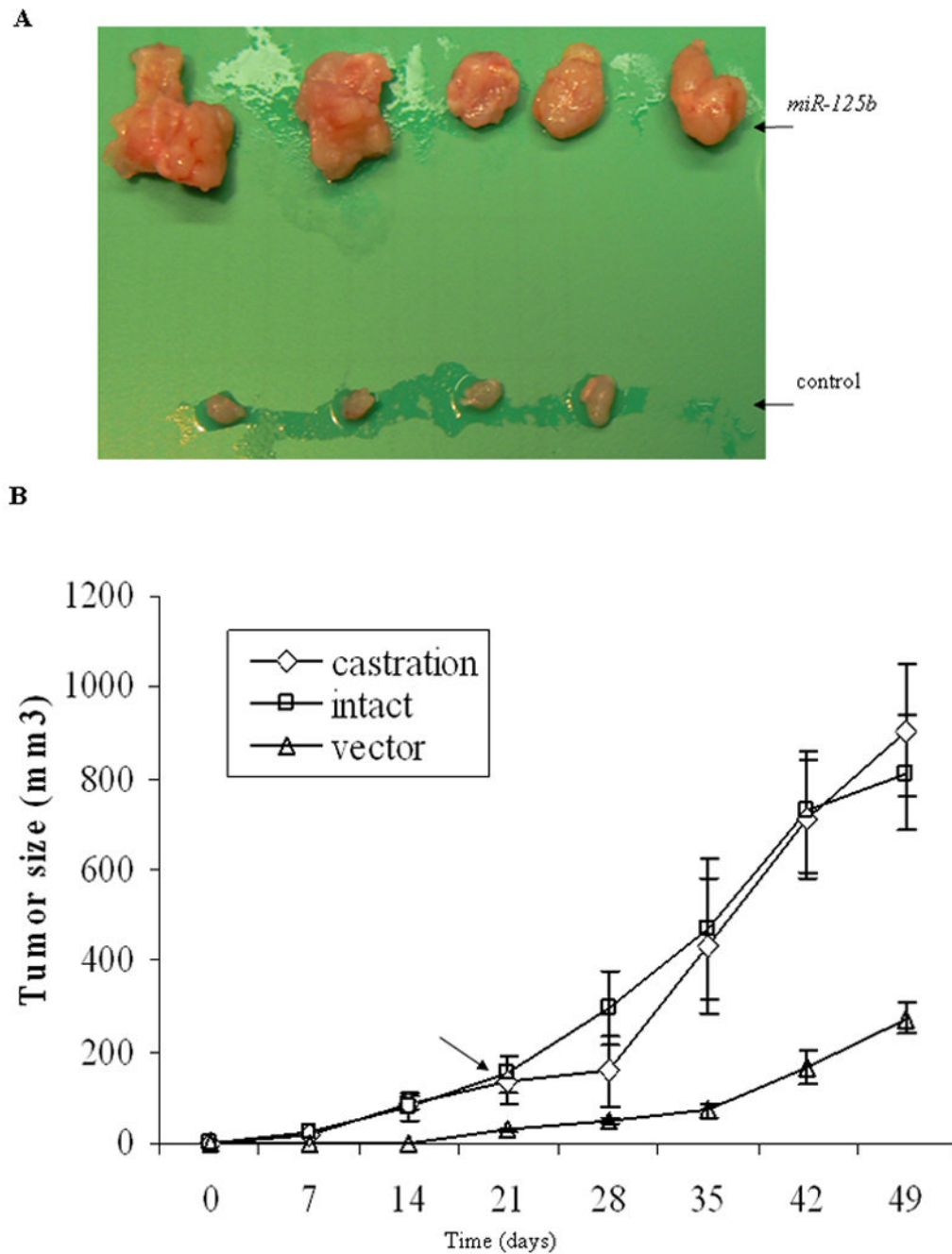
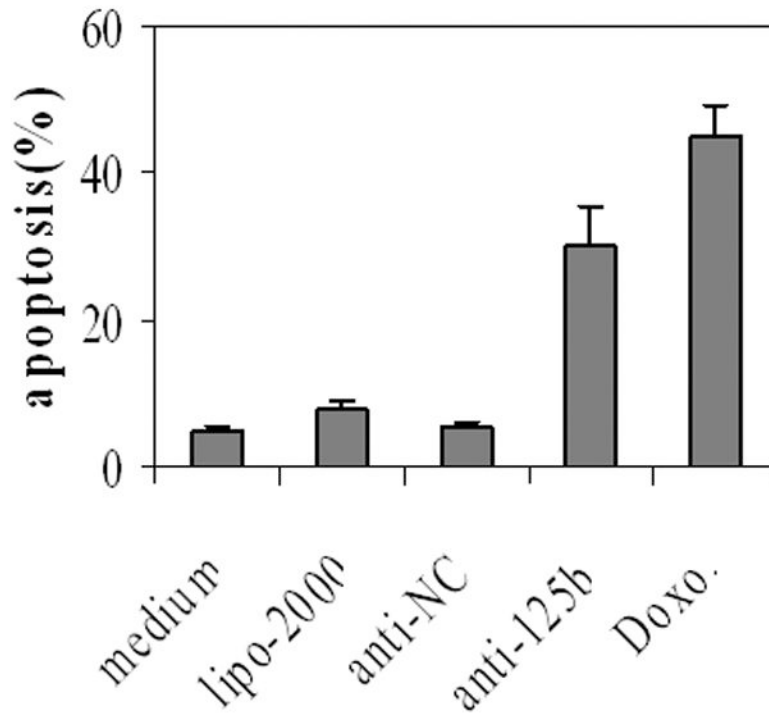
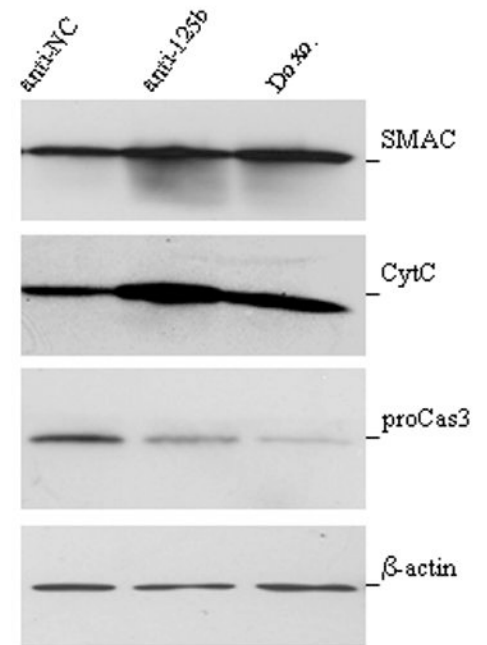


Fig. 1. *miR-125b* promotes androgen-dependent and castration-resistant growth *in vivo*. **A**) Intact male nude mice (5 per group) each were injected subcutaneously with 2×10^6 PC-346C-*miR-125b* cells or PC-346C-vector control cells. Tumors were dissected from *miR-125b* mice (top) and control mice (bottom) five weeks after inoculation. **B**) Sixteen intact nude mice each were injected subcutaneously with 2×10^6 PC-346C-*miR-125b* cells which overexpress of *miR-125b*. When their tumor size reached approximately 0.125 cm^3 ($\sim 0.6 \text{ cm}$ in the diameter), eight of these 16 mice were castrated. The arrow indicates the castration time. Eight intact nude mice were injected subcutaneously with 2×10^6 PC-346C-vector control cells (vector). Each time point represents mean \pm SD of 8 independent values.

A**B****Fig. 2.**

Downregulation of *miR-125b* activity induces apoptosis. **A**) Annexin V assay of apoptosis. PC-346C cells grown in charcoal-deprived medium were treated with 50 nM anti-*miR-125b* (anti-125b) or anti-miRNA negative control (anti-NC) for four days and stained with Annexin V and propidium iodide. Both early apoptotic cells (%) and late apoptotic/necrotic cells (%) are combined. Data are means \pm SD from 3 independent experiments. Charcoal-deprived medium (medium), transfection reagent lipofectamine 2000 (lipo-2000) and doxorubicin (Doxo) were the controls. **B**) Western blot analysis of SMAC, cytochrome c (CytC) and pro-caspases 3 (proCas3). PC-346C cells were treated with 50 nM anti-*miR-125b* (anti-125b) or anti-miRNA negative control (anti-NC) for four days. The expression levels of proteins were analyzed with specific antibodies. Significant reduction of the pro-caspase 3 band indicates the activation of caspase-3 (49).

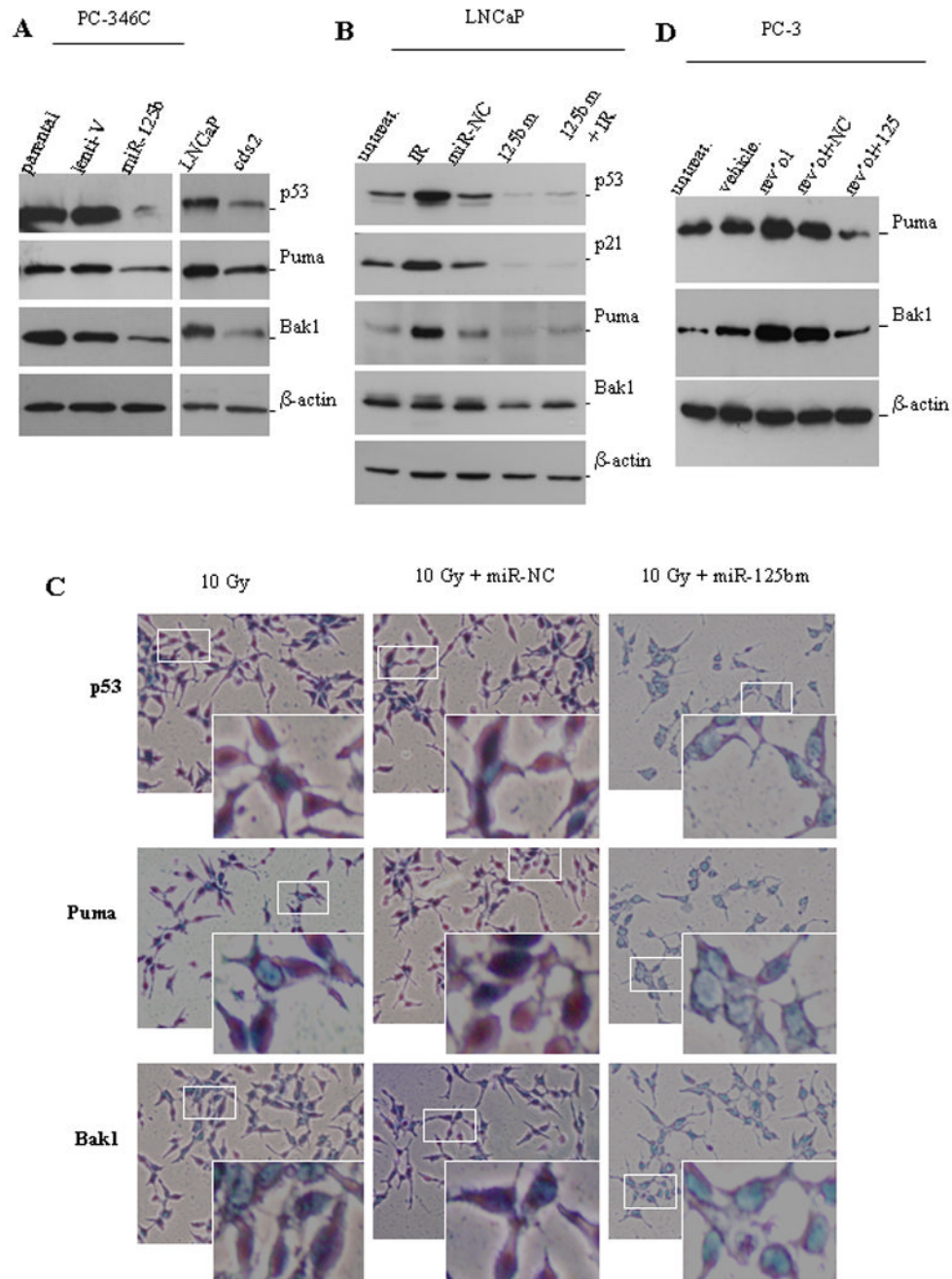


Fig. 3. *miR-125b* targets pro-apoptotic genes. **A)** Western blot analyses of the expression levels of p53, Puma and Bak1 in lenti-*miR-125b* infected PC-346C cells (left panel), and in LNCaP cells grown in 10% FBS medium and in *cds2* cells grown in androgen-deprived medium (right panel). Lent-V, lentiviral vector. **B)** Western blot analysis of the expression of p53, p21, Puma and Bak1 in miR-125bm-treated LNCaP cells. Cells were first transfected with 50 nM of chemically-modified miR-125b mimic (125bm) and 24 hours later irradiated with 10 Gys (IR). Eight hours after irradiation, cells were lysed and protein was extracted for Western blot analysis of p53, p21, Puma and Bak1. The controls include untreated cells (untreat.) and miRNA negative control (miR-NC)-treated cells. **C)** Detection of the

expression levels of p53, Puma and Bak1 in LNCaP cells by immunostaining. LNCaP cells were grown for 24 hours on sterile slides in 100-mm Petri dishes in 10% FBS medium. Cells were first transfected with 50 nM of chemically-modified miR-125bm and 24 hours later irradiated with 10 Gys. Eight hours later, cells were fixed. p53, Puma or Bak1 were stained using specific 1st antibodies followed by a HRP-labeled 2nd antibody. Then, addition of the substrate (DAB) led to generate the brown color. The enzymatic approach shows that p53 locates mainly in nuclei, and Puma and Bak1 in cytoplasm. The white squares indicate the areas of magnified images. **D)** PC3 cells were transfected with 50 nM of miR-125bm (125bm) and 24 hours later treated with 100 μ M resveratrol. Cells were lysed next day and protein was isolated for Western blot analysis of Puma and Bak1. Untreated cells (untreat.) and vehicle-treated cells are used as controls. β -actin is a loading control.

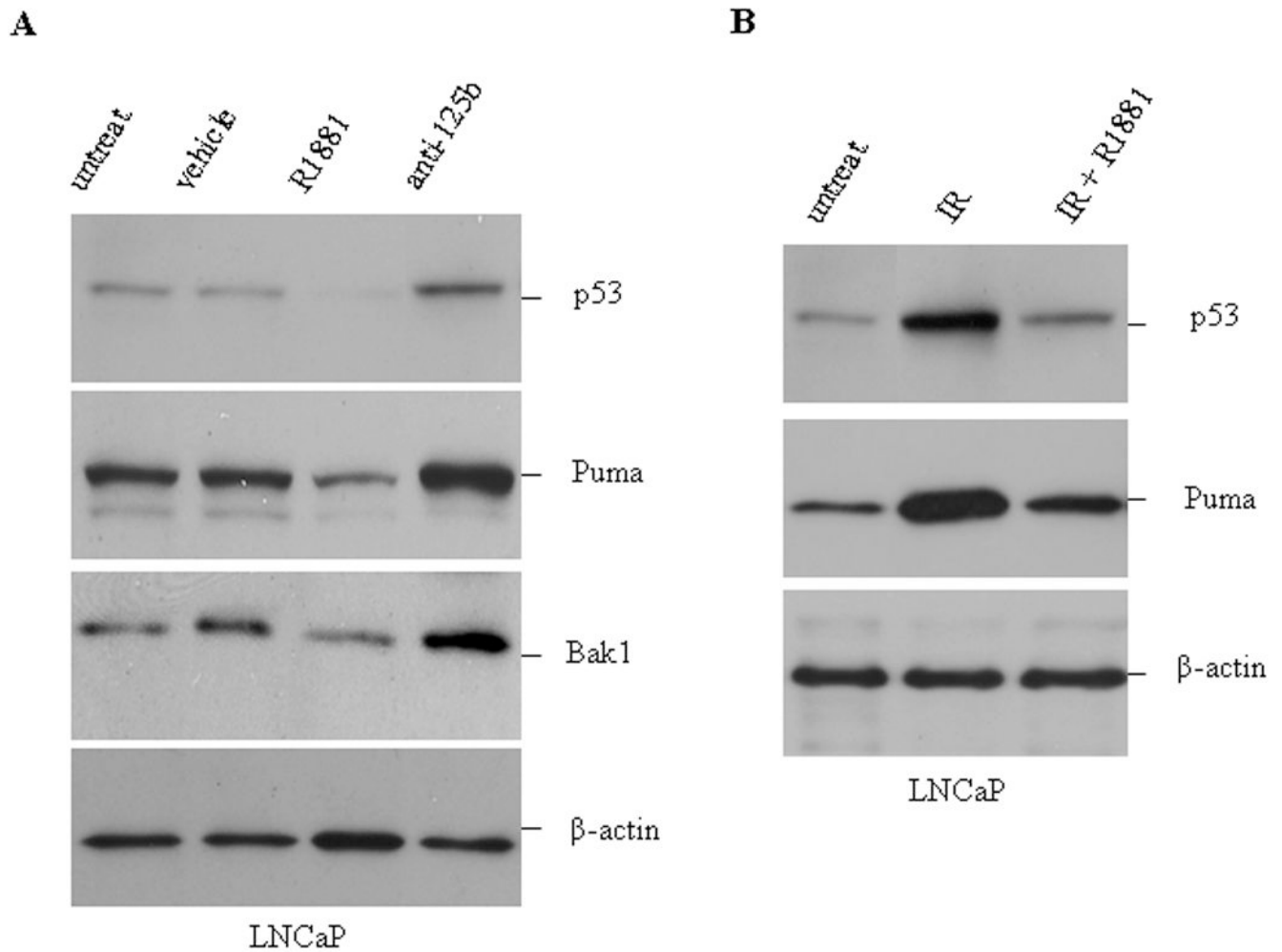


Fig. 4. Effect of androgen on the expression of p53, Puma and Bak1. **A)** LNCaP cells were treated with 5.0 nM of R1881 or 100 nM anti-miR-125b (anti-125b) for 48 hours. Subsequently cells were harvested for Western blot analysis of p53, Puma and Bak1. **B)** LNCaP cells were treated with 5.0 nM R1881 for 48 hours followed by 10 Gys of irradiation (IR). Eight hours after irradiation, the cellular levels of p53 and Puma were analyzed by Western blotting. Controls include untreated cells (untreat.) and ethanol vehicle. β -actin is a loading control.

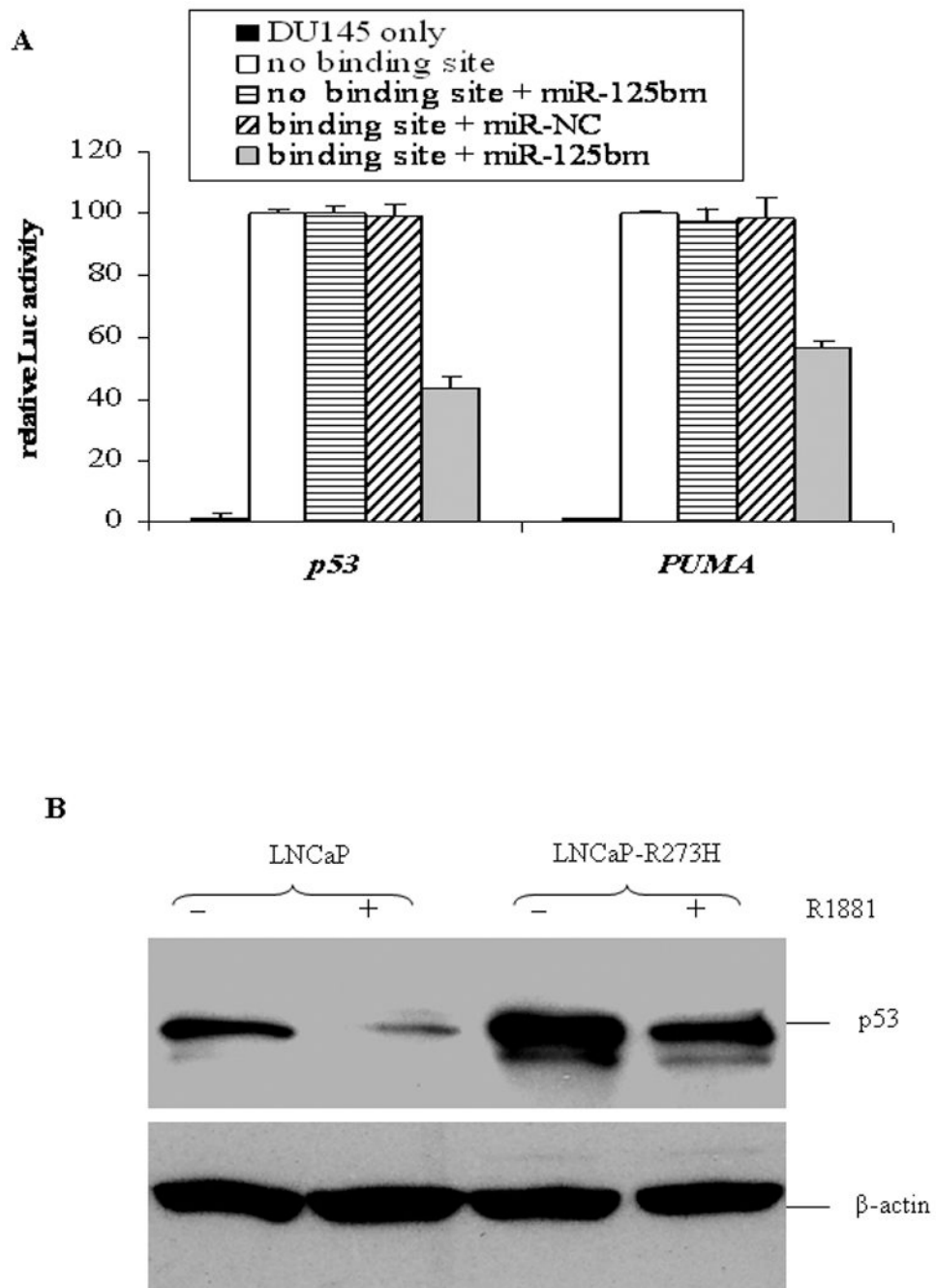


Fig. 5. *Mir-125b* binds to the 3'UTRs of *p53* and *PUMA*. **A)** Luciferase analysis of the 3'UTRs of *p53* and *PUMA* in DU145 cells. Individual truncated 3'UTRs of *p53* and *PUMA* were used as controls (no binding site) in these experiments. The assay was repeated three times with each assay being performed in three wells and similar results were obtained each time. The representative results are shown as $M \pm SD$ ($n = 3$). **B)** Western blot analysis of p53 protein in 5.0nM R1881-treated LNCaP cells and LNCaP-R273H cells.

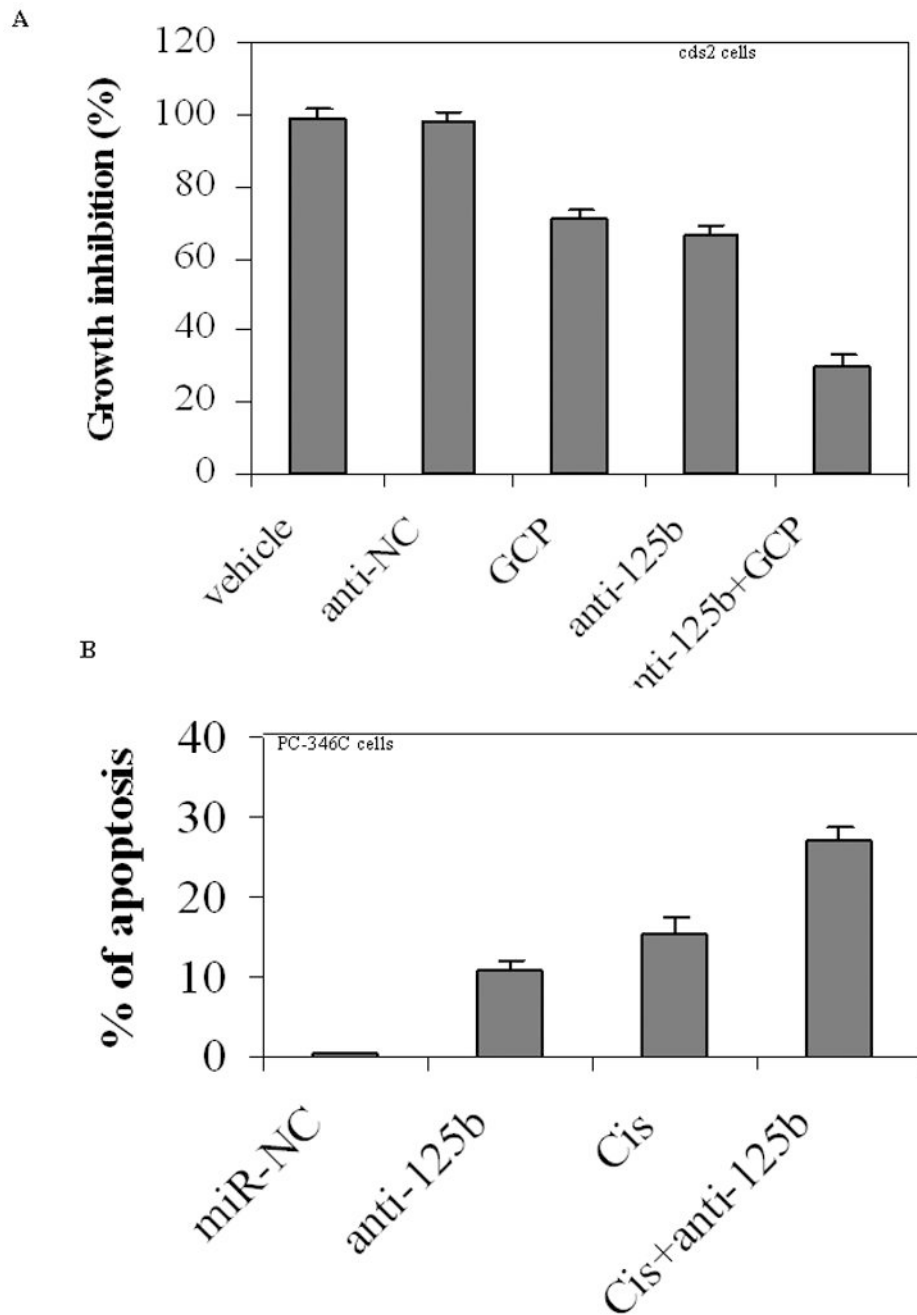


Fig. 6. Effect of down-regulation of *miR-125b* on anti-cancer drugs. **A**) WST-1 assay of growth inhibition. Castration-resistant *cds2* cells were grown in androgen-deprived medium and treated for 5 days using GCP (75 μ g/ml) and anti-*miR-125b* (anti-125b, 50 nM) in alone or in a combination. The controls include both vehicle and anti-miRNA-negative control (anti-NC). WST-1 analysis was used to measure the cell growth. The results are expressed as the growth inhibition relative to that treated with vehicle (100%), and shown as $M \pm SD$ ($n=4$). **B**) Annexin V assay of apoptosis. PC-346C cells cultured in medium containing 10% FBS were treated using Cisplatin (Cis, 5 μ M) and anti-*miR-125b* (anti-125b, 50 nM) for two days in alone or in a combination. Cells were harvested and analyzed on the FACSscan flow

cytometer. Both early apoptotic cells (%) and late apoptotic/necrotic cells (%) are combined. Data are means \pm SD from 3 independent experiments.

Table IGenes directly targeted by *miR-125b* in LNCaP cells

Name	Fold change*	Function	<i>miR-125b</i> binding site at 3'UTR
p53	-1.3	Tumor suppressor	733-739
Puma	-2.5	Pro-apoptosis	24-30
Bak1	-2.0	Pro-apoptosis	630-637
Mtmt3	-1.4	Tumor suppressor	941-947
Bap1	-1.6	Tumor suppressor	580-586
Sgpl1	-1.9	Tumor suppressor	1586-1592
Ahrr	-1.5	Tumor suppressor	1275-1281
Neu1	-1.7	Metastasis suppressor	331-337

* Fold change: signal in *miR-125* treated cells/that in miR-NC-treated cells.