Deregulation of miRNAs Contributed to Development and Progression of Prostate Cancer.

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Deregulation of miRNAs Contributes to Development and Progression of Prostate Cancer.

The overall hypothesis of our proposal is that miR-125b acts as an oncogene, contributing to the development and progression of prostate cancer. In this grant, we proposed to test the ability of aberrantly-expressed miR-125b to promote tumorigenesis and to induce AI growth in three specific aims. In the first year, our focus was on Aim 1: to validate targets of miR-125b in prostate cancer. We have found that i) miR-125b directly targets three key pro-apoptotic molecules: p53, Puma and Bak1, and ii) AR signaling down-regulates p53, Puma and Bak1 via miR-125b in CaP cells. Our results obtained in the first year have provided new insight into the molecular mechanisms related to tumorigenesis and CR growth of CaP.

miRNA, tumorigenesis, oncogene, prostate cancer, Bak1, Puma p53
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The focus of this research is on the Deregulation of miRNAs and how it contributes to the development and progression of prostate cancer. Our overall hypothesis is that miR-125b acts as an oncogene, contributing to the development and progression of prostate cancer. In this study, we proposed to test the ability of aberrantly-expressed miR-125b to promote tumorigenesis and to induce AI growth in three specific aims. The first aim is to elucidate the mechanism by which miR-125b contributes to pathogenesis of prostate cancer. We proposed to validate targets of miR-125b in prostate cancer (CaP). The second aim is to evaluate the effects of miR-125b on prostatic tumorigenesis and AI growth. We will determine the influence of miR-125b on CaP in nude mice. The third aim is to determine the application of miR-125b as a biomarker for prostate cancer. We will detect the abundance of miR-125b in clinical prostate samples and determine whether the expression of miR-125b correlates to the outcome of patients with CaP. Here, we report the completion during our first year.

Research and results (body)

In the first year, our focus was on Aim 1: to validate targets of miR-125b in prostate cancer. The following studies have been performed and these results have been obtained.

1. Identification of miR-125b targets. In preliminary study, we observed that miR-125b is upregulated in CaP and stimulates proliferation of CaP cells in the absence of androgens. In order to gain insight into the mechanism(s) through which miR-125b promotes CaP cell growth, 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. A total of eight genes were identified that contain miR-125b-binding sites in their 3′UTRs (Table I in Appendices), including Bak1 that was previously validated as a miR-125b target by us (1) and others (2), and was used as a control in this study. Of these, p53, Puma and Bak1, which 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, including myotubularin related protein 3 (MTMR3) (3), BRCA1 associated protein-1 (BAP1) (4), sphingosine-1-phosphate lyase 1 (SGPL1) (5), aryl-hydrocarbon receptor repressor (AHRR) (6) and sialidase 1 (lysosomal sialidase) (NEU1) (7). Their involvement in CaP remains to be defined.

2. 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. To validate that miR-125b negatively regulates p53 and PUMA, we determined whether the putative miR-125b binding sites in their 3′-UTRs are responsible for regulation by miR-125b. These 3′-UTR fragments containing the binding site were separately cloned into the pRIM reporter-luciferase vector (Ambion). The 3′-UTR fragments without the binding site were used as negative controls. These reporter vectors were co-transfected with synthetic miR-125b mimic (miR-125bm) into the miR-125b-null DU145 cells for measuring luciferase (luc) activity. As shown in Fig. 1 in Appendices, cotransfection resulted in a 57% and 45% reduction of the reporter activity for p53 and PUMA, respectively. These results provide experimental evidence that the 3′ UTRs of p53 and PUMA are targets of miR-125b.

3. miR-125b downregulates the expression of p53 and Puma. The effect of miR-125b on the expression of p53 and Puma was further evaluated by the following experiments. We first determined whether miR-125b downregulates the expression of p53 and Puma. LNCaP cells were transfected with 50 nM of miR-125bm and 24 hours later received irradiation (IR) of 10 Gys. Eight hours after IR, cells were harvested for Western blot analysis and immunostaining. Western blotting exhibited that IR significantly enhanced the cellular level of p53
and its two effectors, p21 and Puma. Treatment with synthetic miR-125bm dramatically decreased the basal p53 level and IR-stimulated p53 expression (Fig. 2A in Appendices). As expected, miR-125bm-induced p53 downregulation resulted in a marked decrease in the p21 and Puma levels. miR-125bm also represses the expression of Bak1 (Fig. 2A in Appendices). Additionally, we performed an immunostaining of p53 and Puma. Similar to Western blot assay, IHC demonstrated that ectopic expression of miR-125bm induced significant reduction of these proteins, and the representative results are shown in Fig. 2B in Appendices.

The analysis of the regulation of Puma by miR-125b in LNCaP cells is complicated by the expression of p53 that itself is an inducer of Puma. Thus, it is difficult to discern whether the downregulation of Puma is due to direct effect of miR-125b or to the decrease of p53 caused by miR-125b. We therefore utilized the p53-negative PC3 cells. Cells were treated with miR-125bm and then with 100 µM resveratrol that was reported to upregulate Puma and Bak1 in PC3 cells (8). Western blot analysis shows that transfection with miR-125bm resulted in a significant reduction of resveratrol-stimulated Puma and Bak1 (Fig. 2C in Appendices), which validates the direct regulation of Puma by miR-125b. Taken together, these results indicate that miR-125b targets p53 and Puma in CaP cells, and miR-125b targets Puma in both p53 positive and null CaP cells.

4. Androgen downregulates p53 and Puma. We preliminarily found that androgen upregulates miR-125b that downregulates Bak1 (1), and thus asked whether androgen can downregulate p53 and Puma. To address this issue, LNCaP cells were treated with R1881 and anti-miR-125b. Western blot analysis was performed to determine the expression levels of p53 and Puma. When compared with untreated and vehicle-treated cells, R1881 induced a significant reduction of basal p53 and Puma, as well as Bak1 as control, while ectopic expression of anti-miR-125 decreased these proteins (Fig. 3A in Appendices). In addition, LNCaP cells were treated with R1881 for 48 hours followed by irradiation with 10 Gy. It was found that R1881 treatment resulted in a reduction of irradiation-stimulated upregulation of p53 and Puma (Fig. 3B in Appendices). These results further support our hypothesis that androgen signaling regulates p53, Puma and Bak1.

5. Casodex stimulates the expression of miR-125b in androgen-independent CaP cell lines. The AR antagonist Casodex (CDX, or bicalutamide) is clinically used for treatment of castration-resistant (CR) CaPs. This compound inhibited the androgen-induced expression of miR-125b in LNCaP cells (data not shown). Since Casodex stimulated the CR growth (9, 10) and induced the expression of PSA in AI LNCaP subline (11, 12), we tested the role of Casodex in regulating miR-125b in androgen-independent CaP cell lines cds1 and 22Rv1. In situ hybridization (ISH) assays showed that treatment of both cell lines with 5.0 µM Casodex stimulated miR-125b expression to a level similar to that in R1881-treated cells. The representative results obtained in cds1 cells are shown in Fig. 4A in Appendices. We previously found that the p160 coactivator TIF2 modulated the function of a casodex-liganded AR, and knockdown of TIF2 using a shRNA to TIF2 (shTIF2) decreased Casodex’s agonistic effect (11). We thus determined that shTIF2 inhibits the Casodex-stimulated expression of miR-125b. It was found that infection with 5 MOIs (multiplicities of infection) of adeno-shTIF2 decreased miR-125b expression in cds1 (data not shown). Infection with only shTIF2 did not alter the basal level of miR-125b and the adeno-shGFP control did not reduce Casodex-stimulated expression of miR-125b (data not shown). To quantitate the miR-125b signal, we used a fluorescence microscope to capture mean fluorescent intensity from >50 cells. Both R1881 and CDX induced a >2-fold increase in the fluorescence signal in cds1 and 22Rv1. shTIF2 was able to block the CDX-stimulated increase in miR-125b (Fig. 4B in Appendices). These data suggest that aberrantly activated AR regulates the expression of miR-125b and that p160 coactivators are likely involved in regulation of miR-125b expression.

6. Upregulation of miR-125b by EGF. Studies have revealed that in addition to androgens some protein growth factors (GFs) binding to specific membrane receptors (GFRs) activate multiple downstream signaling pathways, resulting in AR activation (13, 14). We are very interested in determining whether protein factors are able to upregulate the expression of miR-125b in an androgen-depleted environment. Since epidermal growth factor (EGF) and its membrane receptor HER2 are frequently upregulated in advanced stages of CaP (15), we thus evaluated the effect of EGF on the expression of miR-125b in androgen-dependent LNCaP cells and
androgen-independent cd51 cells. Cells were treated with 20 ng/ml EGF for 2 days and RNA isolated. The level of miR-125b was measured by qRT-PCR. It was found that EGF stimulated the level of miR-125b in both LNCaP and cd51 cells (Fig 5 in Appendices).

**KEY RESEARCH ACCOMPLISHMENTS**

In our first year, we have obtained exciting results. The key accomplishments are that 1) oncogenic miR-125b directly targets three key pro-apoptotic molecules; 2) some factors (like Casodex and EGF) that abnormally activate androgen receptor (AR) in the absence of androgens can stimulate the expression of miR-125b in CaP cells; and 3) our data strongly suggest that there an AR-miR-125b signaling pathway in CaP cells that may mediate CR growth of CaP cells.

**REPORTABLE OUTCOMES**

*Published abstract*

- Xu-Bao Shi, Lingru Xue, Clifford Tepper, Ralph W. deVere White. Suppression of key apoptosis-related molecules by miR-125b contributes to androgen-independent growth of prostate cancer cells. AACR 101st Annual Meeting 2010, April 17-21, 2010 Washington, DC.

**CONCLUSION**

Our results obtained in the first year demonstrated that miR-125b directly targets three key pro-apoptotic molecules: p53, Puma and Bak1. These data also provide strong evidence showing that androgen- or non-androgen-stimulated AR signaling down-regulates p53, Puma and Bak1 via miR-125b in CaP cells. Therefore, this study sheds new insight into the molecular mechanisms related to tumorigenesis and CR growth of CaP.

In the next year, we will focus on addressing the ability of aberrantly-expressed miR-125b to promote prostatic tumorigenesis and to induce AI growth.

**REFERENCES**


Appendices

Table I. Genes directly targeted by miR-125b in LNCaP cells

<table>
<thead>
<tr>
<th>Name</th>
<th>Fold change*</th>
<th>Function</th>
<th>miR-125b binding site at 3’UTR</th>
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<tbody>
<tr>
<td>p53</td>
<td>-1.3</td>
<td>Tumor suppressor</td>
<td>733-739</td>
</tr>
<tr>
<td>Puma</td>
<td>-2.5</td>
<td>Pro-apoptosis</td>
<td>24-30</td>
</tr>
<tr>
<td>Bak1</td>
<td>-2.0</td>
<td>Pro-apoptosis</td>
<td>630-637</td>
</tr>
<tr>
<td>Mtmr3</td>
<td>-1.4</td>
<td>Tumor suppressor</td>
<td>941-947</td>
</tr>
<tr>
<td>Bap1</td>
<td>-1.6</td>
<td>Tumor suppressor</td>
<td>580-586</td>
</tr>
<tr>
<td>Sgpl1</td>
<td>-1.9</td>
<td>Tumor suppressor</td>
<td>1586-1592</td>
</tr>
<tr>
<td>Ahrr</td>
<td>-1.5</td>
<td>Tumor suppressor</td>
<td>1275-1281</td>
</tr>
<tr>
<td>Neu1</td>
<td>-1.7</td>
<td>Metastasis suppressor</td>
<td>331-337</td>
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*Fold change: signal in miR-125 treated cells/than in miR-NC-treated cells.

Fig. 1. Luciferase analysis of miR-125b binding to 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 ± SD (n = 3).
**Fig. 2.** miR-125b targets pro-apoptotic genes. A) 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. B) 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. C) PC3 cells were transfected with 50 nM of miR-125bm (125bm) and 24 hours later treated with 100 μM 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. 3.** 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 4.** ISH assays of casodex-stimulated expression of *miR-125b*. 

**A** *miR-125b* in cds1 that were treated with vehicle, 1.0 nM R1881, 5.0 μ M Casodex (CDX) or CDX+shTIF2. The squares indicate areas where the images are magnified. 

**B** Quantitation of *miR-125b* signal in cds1 and 22Rv1.

**Fig 5.** qRT-PCR analysis of *miR-125b* level in LNCaP and cds1 cells treated with 20 ng/ml EGF for 2 days. R1881 is positive control and U6 small RNA is negative control.