Enforced expression of miR-101 inhibits prostate cancer cell growth by modulating the cyclooxygenase-2 pathway in vivo

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Abstract:

It is commonly agreed that there is an association of chronic inflammation with tumorigenesis. Cyclooxygenase-2 (COX-2), a key regulator of inflammation producing prostaglandins, promotes cell proliferation and growth; thus, overexpression of COX-2 is often found in tumor tissues. Therefore, a better understanding of the regulatory mechanism(s) of COX-2 could lead to novel targeted cancer therapies. In this study, we investigated the mechanism of microRNA-101 (miR-101) regulated COX-2 expression and the therapeutic potential of exogenous miR-101 for COX-2-associated cancer. A stably expressing exogenous miR-101 prostate cancer cell line (BPH1CmiR101) was generated by using lentiviral transduction as a tool for in vitro and in vivo studies. We found that miR-101 inhibited COX-2 posttranscriptional expression by directly binding to the 3′-untranslated region (3′UTR) of COX-2 mRNA. The regulatory function of miR-101 was also confirmed by using antisense DNA. As a result, exogenous miR-101 is able to effectively suppress the growth of cultured prostate cancer cells and prostate tumor xenografts. The average tumor weight was significantly lower in the BPH1CmiR101 group (0.22 g) compared to the BPH1Cvec group (0.46 g). Expression levels of the cell growth regulators, such as cyclin proteins, PCNA, EGFR, were also studied. In conclusion, COX-2 is a direct target in miR-101 regulation of posttranscription. Exogenous miR-101 suppresses the proliferation and growth of prostate cancer cells in vitro and in vivo. These data suggest exogenous miR-101 may provide a new cancer therapy by directly inhibiting COX-2 expression.
Introduction:

Prostate Cancer is currently the second leading cause of cancer death for males in United States. Chronic inflammation has been revealed to contribute to the development of malignant tumors (1-4), that showed aberrant overexpression of cyclooxygenase 2 (COX-2). COX-2 is a key rate-limiting enzyme that catalyzes the biosynthesis of prostaglandins from arachidonic acid. COX-2 is an attractive target for regulating cancer cell growth and it has been found in many types of cancers including prostate cancer (5, 6). Due to the cardiovascular toxicity of selective COX-2 inhibitors in long term usage (7), such as celecoxib, it is appealing to investigate new endogenous and therapeutic targets to regulate COX-2 expression for prevention and treatment of COX-2-associated cancer.

MicroRNAs (miRNAs) are a class of approximately 22 nucleotide-long, endogenously expressed, highly conserved of noncoding RNAs with important regulatory functions in cell proliferation, apoptosis, and metastasis. MiRNAs act through imperfect base pairing with the 3'- untranslated region (3'-UTR) of the target genes, or bind their target mRNA through perfect base pairing (8, 9). Computational and experimental outcomes predict that miRNAs regulate at least 30% of protein-coding genes (10), and approximately 50% of miRNAs genes are located in cancer-related genomic regions (11). MiRNAs are thought to regulate cancer development as tumor suppressors or oncogenes (12). Therefore, miRNAs may be ideal targets of cancer prediction and treatment.
Recent studies showed that tumor suppressive miRNAs, such as miR-101, miR-126*, miR-146a, miR-330, miR-34 cluster, and the miR-200 family are usually down-regulated in prostate cancer compared with normal tissue (13). Loss of miR-101 was found to be concordant with overexpression of EZH2, a histone methyltransferase. EZH2 expression can be regulated to inhibit prostate cancer growth by means of miR-101 expression (14, 15). Moreover, it is worth mention that miR-101 has ability to target COX-2 in endometrial serous adenocarcinomas (16), colon cancer (17, 18) and gastric cancer (19). However, the role of miR-101 in COX-2-associated tumor growth in vivo is unclear.

In this study, we aim to identify the mechanism of miR-101-regulated COX-2 expression and explore the therapeutic potential of miR-101 in prostate cancer by using a stably expressed exogenous miR-101 prostate cancer cell line tested in vitro and in vivo.

**Material and Methods**

**Cell lines and culture:** A benign prostatic hyperplasia cell line (BPH1) and a tumorigenic cell line BPH\textsuperscript{CAFTD} (BPH1 transformed with carcinoma-associated fibroblasts) were kindly provided by Simon Hayward, Vanderbilt University; a transformed SV40 human prostatic epithelial cell line (PNT-1) was kindly provided by Bernard-kwabi Addo, Howard University; an androgen receptor positive prostate cancer cell line (LNCap) and a androgen negative prostate cancer cell line (PC3) were
purchased from ATCC (Manassas VA). All cells were grown in RPMI 1640 or other suitable media with 10% fetal bovine serum.

**EGFP-miR-101 expression vector:** We modified the commercial pLVX-Tight-Puro vector (Clontech, Mountain View, CA) with an expression cassette containing P<sub>CMV</sub> promoter, EGFP, miRNAs linker and pre-miR-101 by replacing the P<sub>tight</sub> promoter. The miRNA linker contained a multi cloning site (MCS). The pre-miR-101 double strand sequence was designed based on the miRBase:Sequences 12.0 data bases. An EGFP control vector was also constructed using same expression system without the miR-101 gene. The constructed vectors were verified by DNA sequencing. Lentiviral particles containing EGFP-miR-101 vectors or EGFP control vectors were produced by using the lentiphos<sup>™</sup> HT packaging system (Clontech, Mountain View, CA) following the manufacturer’s instructions.

**Stable expression miRNA-101 cell line:** BPH<sup>1CAFTD</sup> cell line was selected as a candidate cell lines for this purpose because the cell line shows a high background level of COX-2 protein. Briefly, BPH<sup>1CAFTD</sup> cells were pre-seeded in 6-well plate overnight and then infected with 200 μl of lentivirus containing EGFP-miR-101 vectors or EGFP control vectors plus 4μg/ml polybrene (Sigma), rocked the plate every 15 minutes for 2 hours followed by the adding 2 ml RPMI 1640 media with 10% FBS in each well. After 48 hours, the infected cells were selected with fresh medium containing 5 ng/ml puromycin for 4-5 passages. Two stably expressing cell lines: EGFP-miR-101 (BPH<sup>1CmiR101</sup>) or EGFP alone (BPH<sup>1Cvec</sup>) were generated and the infected cells were easily viewed under the fluorescence microscope.
mRNA 3'-UTR of COX-2 luciferase vector (3'UTR-Luci vector) and miR-101 targeting gene test: The miR-101-targeted gene was evaluated by using a luciferase reporter assay in 293T cells (ATCC, Manassas, VA). The 3'UTR-Luci vector was constructed using the phCMV-FSR luciferase reporter vector (Genlantis, San Diego, CA) with a fragment of mRNA 3'UTR of COX-2, which carries a putative miR-101 complementary site (NM_000963 3'-UTR 1735-1741). 293T cells were pre-seeded in a 24-well plate and they were 70% confluent at the time of transfection. The 3'UTR Luci vector and EGFP-miR-101 vector were transfected into 293T cells either alone or together using calcium phosphate method. Luciferase activity was detected after 48 hours transfection using bioluminescence imaging with a Xenogren IVIS instrument (Caliper Life Sciences, Hopkinton, MA) based on the manufacturer’s protocol.

MiRNA expression assay: miRNAs expression was analyzed by using quantitative RT-PCR (20). Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Germantown, MD) following the manufacturer’s protocol. MiR-101 forward primer (5' TAC-AGTACTGTGATAACTGAA 3') was synthesized at Sigma (Woodlands, TX) according to miRBase: Sequences 12.0. Total RNA was polyadenylation by E.coli polyA polymerase, and then first-strand cDNA synthesis and quantitative PCR were conducted according to High-Specificity miRNA QRT-PCR Detection kit (Stratagene, La Jolla, CA). Three controls were performed in the test: 1) a control test without the DNA template and 2) a control test without polyA polymerase to monitoring reagent contamination or false amplification, and 3) an endogenous control test to normalize variations in the amount of cDNA template across samples. The expression of miRNAs relative to U6 RNA was determined using the ∆Ct method. The average ∆Ct of each
group was calculated by the following formula: \( \Delta \text{Ct} = \text{Ct}_{\text{miRNA gene}} - \text{Ct}_{\text{u6 gene}} \). \( \Delta \Delta \text{Ct} \) was calculated by \( \Delta \Delta \text{Ct} = \Delta \text{Ct}_{\text{vector}} - \Delta \text{Ct}_{\text{miR-101}} \). The fold-change for miRNA expression level was calculated using \( 2^{-\Delta \Delta \text{Ct}} \).

**Colony formation assay:** This method was used for evaluating cell proliferation and growth (21). BPH1\(^{\text{Cvec}}\) and BPH1\(^{\text{CmiR101}}\) cells were seeded at a density of 500 cells per well in BD Falcon 6-well tissue culture plates (Palo Alto, CA). The colonies, which formed after nearly nine days were stained with 0.1% trypan blue in 50% ethanol. Any colony containing more than 50 cells was considered to represent a viable clonogenic cell. At least two independent experiments were performed with triplicate test samples.

**3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay:** The MTT method was used to estimate cell viability (21). The cells were plated at an initial density of 5000 cells per well in flat-bottom 96-well cell culture plate and allowed to grow for 48 hours. MTT solution (Sigma, St Louis, MO) was added to each well followed by a 4 hour incubation at 37 °C. After removing the media, dimethyl sulfoxide was added to each well for solubilizing the formazan formed. After 30 minutes at room temperature the plates were scanned spectrophotometrically with a microplate reader (Bio-Rad, Hercules, CA) set at 560 nm for measuring the absorbance.

**Cell cycle analysis:** Briefly, cells were collected and fixed with cold 80% fresh ethanol and stored at 4°C for 24 hours. After removing the ethanol, the cells were incubated with 1 mg/ml RNase A in PBS for 30 min at room temperature, and then the cells were incubated an additional 30 min in the dark adding 0.5 ml of 50 mg/ml propidium iodide. The cell cycle was analyzed by Becton-Dickinson FACScan.
Human tumor xenografts in athymic (nude) mice: Four-week-old, male, Balb/c athymic nude mice (Nu/Nu) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Mice were housed in temperature-controlled rooms (74 ± 2˚F) with a 12-hour alternating light-dark cycle. The mice were separated into two groups: BPH1^{CmiR101} or BPH1^{Cvec} group and each group contained 5 mice. Approximately 2 x 10^6 cells were injected subcutaneously into the left and right lower backs of mice, respectively. The mice were housed for 35 days post injection. The mice were terminated by euthanizing and the tumor tissues were removed by surgical excision. The tumor tissues were processed for RNA and protein extraction, and formalin fixed for histological studies. Tumor volume and body weight were measured once a week during the experimental period. Guidelines for the humane treatment of animals were followed as approved by the Howard University Animal Care and Use Committee.

Immunohistochemistry. The tests followed the instruction of the LSAB2 System-HRP kit (DakoCytomation, Carpinteria, CA). The tissue sections were processed, deparaffinized, and treated with hydrogen peroxide. The sections were then incubated with primary antibodies of COX-2, EGFR, and PCNA, respectively. Following which, the sections were developed with streptavidin reagent and DAB Substrate.

Statistical analysis: Quantitative data are presented as the mean ± S.D. T-test was used to determine statistical significance. Differences were considered significant at p < 0.01 and p < 0.05.
Results

Expression levels of COX-2 protein and miR-101 in human tumorigenic and non-tumorigenic prostate cell lines. COX-2, an enzyme involved in the inflammatory response of tissues is often found in tumor cells, but not in normal cells. We evaluated COX-2 expression levels among 5 prostate cell lines, including the immortalized human prostatic PNT1 cell line, the benign prostate hyperplasia (BPH) BPH1 cell line, and the tumorigenic LNCaP, BPH1<sup>CAFTD</sup> and PC3 cell lines by Western blot analysis (Fig. 1). The BPH1<sup>CAFTD</sup> cell line had the highest level of COX-2 among the cell lines tested. The expression levels of miR-101 were also investigated by a quantitative real-time RT-PCR. The miR-101 levels showed an inverse correlation with COX-2 protein expression in all five cell lines (Fig. 1B, C). The ratio of COX-2 protein/miR-101 was considered as the miR-101 contribution to regulating COX-2 expression. The BPH1<sup>CAFTD</sup> cell line had the highest ratio. Based on this result, BPH1<sup>CAFTD</sup> was chosen as a candidate cell line to stably transfect with miR-101 for further investigation of miR-101 function in the regulation of COX-2 expression under cell culture and tumor xenograft conditions.

Stably enforced expression of miRNA-101 in BPH1<sup>CAFTD</sup> cultured cells and xenografts. In order to investigate the role of miR-101 in COX-2-associated prostate cancer development in vivo, we constructed a prostate cancer cell line with stable expression of miR-101 using a lentiviral-delivery system, which contained an expression cassette of the P<sub>CMV</sub> promoter, EGFP and miR-101 precursor (Fig. 2A). miR-101 pseudotyped lentiviral particles were generated by using the Lenti-X<sup>TM</sup> HT packaging system in 293T-cells. BPH1<sup>CAFTD</sup> cells were transduced with lentiviral particles
containing EGFP-miR-101 vectors (BPH1\textsuperscript{CmiR101}) or EGFP vector (BPH1\textsuperscript{Cvec}), respectively. The lentiviral-delivered EGFP-miR-101 expression was monitored under fluorescence microscope based on EGFP expression (Fig. 2B) and also quantified by using real-time RT-PCR (Fig. 2C, D). After 100 passages in culture, the BPH1\textsuperscript{CmiR101} cells presented green fluorescence as bright as the fifth passage BPH1\textsuperscript{CmiR101} cells (Fig. 2B) as well as the miR-101 expression levels (Fig. 2C). In addition, the miR-101 level was 10 times higher in BPH1\textsuperscript{CmiR101} than BPH1\textsuperscript{Cvec} (Fig. 2C). A similar outcome was obtained from BPH1\textsuperscript{CmiR101} tumor xenografts that high levels of miR-101 were detected in BPH1\textsuperscript{CmiR101} tumor xenografts. The level of miR-101 was 5.2 fold higher in BPH1\textsuperscript{CmiR101} compared with BPH1\textsuperscript{Cvec} tumor tissues (Fig. 2D).

**MiR-101 directly inhibits COX-2 expression during posttranslation.** COX-2 as a direct target for miR-101 regulation was determined by using a luciferase reporter assay. The effect of miR-101 on inhibition of COX-2 expression was analyzed in a set of cell lines, including a pair of miR-101 transfected BPH1\textsuperscript{CmiR101} cells and vector control BPH1\textsuperscript{Cvec} cells, a pair of BPH1\textsuperscript{CmiR101} and BPH1\textsuperscript{Cvec} xenografts, and parental BPH1\textsuperscript{CAFTD} cells and COX-2 siRNA transfected BPH1\textsuperscript{CAFTD} cells as positive controls by Western blot analysis (Fig. 3A). COX-2 protein level was significantly reduced in cultured BPH1\textsuperscript{CmiR101} cells and its xenografts compared to BPH1\textsuperscript{Cvec} and BPH1\textsuperscript{CAFTD} controls. The COX-2 protein was attenuated 61% in cultured cells and 77% in xenografts by miR-101 or 78% by COX-2 siRNA, respectively (Fig. 3A). However, the COX-2 mRNA levels were not significantly different in both BPH1\textsuperscript{Cvec} and BPH1\textsuperscript{CmiR101} cell lines by real-time RT-PCR (Fig. 3B). The results indicate that miR-101 does not affect COX-2 mRNA transcription.
The Targetscan human 5.0 and Pictar Program predicted that COX-2 is one of the putative targets for miR-101. We utilized a luciferase reporter assay to further confirm the miR-101 binding site on the 3’UTR region of COX-2 mRNA. Figure 3C depicts a sequence map in which the 3’-UTR of the COX-2 mRNA fragment carries a complementary site (NM_000963 3’-UTR 1735-1741) for the seed region of miRNA-101. Luciferase activity was detected 48 hours after transfection of 3’-UTR Luci vector or EGFP-miR-101 vector alone, or both of the two vectors in 293T cells. The luciferase activity was decreased 4.5-fold in 293T cells co-transfected with 3’-UTR Luci vector and EGFP-miR-101 vector compared with 3’-UTR Luci vector alone (Fig. 3D). This data suggests that miR-101 suppresses COX-2 protein expression by directly binding to the 3’UTR of COX-2 mRNA. Interestingly, the inhibitory function of miR-101 on COX-2 expression can be reversed by introduction of the 3’UTR fragment of COX-2 (Fig. 3E). COX-2 protein levels were significant lower in BPH1^{CmiR101} cells compared with BPH1^{Cvec} cells; however, the protein level of COX-2 in BPH1^{CmiR101} cells was significantly recovered by introducing 3’-UTR of COX-2 fragments (Fig.3E). In conclusion, miR-101 negatively regulates COX-2 protein expression by specifically binding to the 3’UTR of the COX-2 mRNA region.

**MiR-101 suppresses the proliferation of prostate cancer cells in vitro and in vivo.**

The effect of miR-101 on cell proliferation was evaluated using the colony formation assay (Fig. 4A,B) and cell viability was estimated using the MTT assay (Fig. 4C). BPH1^{Cvec} and BPH1^{CmiR101} cells were separately seeded in 6-well plates and the cell growth was daily monitored under the microscope for less than twelve days. The culture was stopped when the colonies were clearly visible by the naked eye. As
expected, BPH1<sup>CmiR101</sup> cells displayed a significant proliferation delay, leading to the formation of fewer and smaller colonies even on day 9, in comparison to BPH1<sup>Cvec</sup> cells which grew more vigorously (Fig. 4A, B). The colony forming ability of BPH1<sup>CmiR101</sup> was 41% less than that of BPH1<sup>Cvec</sup> cells (Fig. 4B). The MTT assay showed that enforced expression of miR-101 in BPH1<sup>CmiR101</sup> cells led to 21% decrease in cell proliferation relative to 100% proliferation observed in the case of BPH1<sup>Cvec</sup> (Fig. 4C).

The miR-101 function was further tested in a BPH1<sup>CmiR101</sup> tumor xenograft animal model. Five weeks after subcutaneous inoculation of BPH1<sup>Cvec</sup> or BPH1<sup>CmiR101</sup> cells into Balb/c athymic nude mice, each cell inoculation developed into a solid tumor xenograft. The growth curve of tumor xenografts showed that enforced expression of miR-101 slowed tumor growth compared to control BPH1<sup>Cvec</sup> cells (Fig. 4D) since day 26. The experimental mice were euthanized on the day 36. The average tumor weight in the BPH1<sup>Cvec</sup> group was significantly higher (0.46 g) compared to the BPH1<sup>CmiR101</sup> group (0.22 g) (Fig. 4E). These findings indicated that exogenous miR-101 is able to inhibit COX-2-associated cancer cell growth.

**Exogenous miR-101 suppressed cancer cell proliferation.** To better understand the role of miR-101 in repressing cell proliferation, we analyzed a set of cell cycle markers in BPH1<sup>CmiR101</sup> and BPH1<sup>Cvec</sup> cells. Figure 5A showed that BPH1<sup>CmiR101</sup> exhibited a greater tendency for G1/S arrest (defined as an increased G1 peak with a decreased S peak) compared to BPH1<sup>Cvec</sup> cells. All levels of cell cycle related proteins, such as cyclin A, cyclin B, cyclin D1 were lower in BPH1<sup>CmiR101</sup> cells than BPH1<sup>Cvec</sup> cells determined by western blot analysis. The level of cyclin D1, which is important for the
G1 to S transition, was decreased by 28% in BPH1<sup>CmiR101</sup> cells, and cyclin A and cyclin B1 were also 25% and 13% lower in BPH1<sup>CmiR101</sup> cells compared to BPH1<sup>Cvec</sup> cells (Fig. 5B).

Cell proliferation is regulated by multiple proteins. We analyzed additional cell proliferation markers, such as Proliferating cell nuclear antigen (PCNA), epidermal growth factor receptor (EGFR), anti-apoptotic protein Bcl-2 and pro-apoptotic protein p53. The levels of PCNA and EGFR were 88% lower and 67% lower in cultured BPH1<sup>CmiR101</sup> cells than in BPH1<sup>Cvec</sup> cells, respectively (Fig. 5C). In BPH1<sup>CmiR101</sup> xenografts, the PCNA level was 31% lower and EGFR was 61% lower than in BPH1<sup>Cvec</sup> xenografts (Fig. 5C). The Bcl-2 level was 28% down-regulated, whereas P53 was 51% up-regulated in BPH1<sup>CmiR101</sup> cells than in BPH1<sup>Cvec</sup> cells (Fig. 5D).

Immunohistochemical staining of the tumor xenografts of BPH1<sup>CmiR101</sup> and BPH1<sup>Cvec</sup> further confirmed the Western blot results. We found that exogenous miR-101 resulted not only in decreased COX-2 expression, but also reduced PCNA and EGFR expression in the BPH1<sup>CmiR101</sup> xenografts compared with BPH1<sup>Cvec</sup> xenografts (Fig. 6A, B, C). The consistent expression pattern of cell proliferation and apoptotic markers in cultured cells and in tumor xenografts supported that exogenous miR-101 is able to inhibit prostate cancer growth by directly and indirectly modulating cell cycle regulators to suppress cell proliferation.

**Discussion**
MicroRNAs are predicted to regulate over 30% of all gene expression and may account for some of the aberrant gene expression in cancer cells. There is strong evidence that miR-101 plays a role as a tumor suppressor in various cancers. For instance, miR-101 inhibits Mcl-1 protein (an antiapoptotic gene of Bcl-2 family) (22), EZH2 (a histone methyltransferase) (14, 15) and DNA-PKcs (an essential factor for non-homologous end-joining repair) and ATM (an important checkpoint regulator for promoting homologous recombination repair) (23) in cancer. Although several studies have reported that miR-101 can inhibit cancer cell growth via blocking COX-2 expression, the research is still hovering at cellular level. Therefore, we are committed to such a work around exogenous miR-101 in inhibiting prostate cancer cell growth through modulation of COX-2 pathway in vivo.

There are two precursors of miR-101: pre-miR-101-1 located at chromosome 1p31.3 and pre-miR-101-2 at chromosome 9p24.2. Both of the loci are lost in 37.5% of organ confined prostate cancer cells and 66.7% of metastatic disease cells (18). This event seems to be common to a broad range of cancer types, according to public domain CGH datasets. In contrast, overexpression of miR-101 has been found to suppress cell proliferation and impair the invasive potential in prostate cancer (18), bladder carcinoma (15), and colon cancer as a tumor suppressor (17). We found that exogenous miR-101 significantly inhibited the cell proliferation (Fig. 4A-C) and tumor growth (Fig. 4D, E) in COX-2 associated prostate cancer either in cultured BPH1CmiR101 cells or BPH1CmiR101 xenografts by directly suppressing COX-2 protein expression, resulting in suppression of COX-2 associated cell growth factors (Fig. 3).
Numerous evidence has revealed that COX-2 is an inducible enzyme that is elevated and catalyzes arachidonic acid to prostaglandins during inflammatory and tumorigenic (22). Thus, COX-2 is a critical target that has implications for both prevention and treatment of prostate cancer (24). The prediction based on PicTar and TargetScan DNA analysis softwares suggest that COX-2 could be one direct target for miR-101 because miR-101 has a seed region, which is able to bind to the COX-2 mRNA 3'UTR (Fig. 3C). The observations from this present study support an inverse correlation between miR-101 and COX-2 expression in prostate cell lines (Fig. 1). This hypothesis was confirmed by following approaches: Firstly, we successfully established an enforced EGFP-miR-101 expression prostate cell line (BPH1^CmiR101^) by using stable gene transfection technology (Fig. 2). In addition, the cell was uniquely co-transfected with the EGFP gene sharing the same P_{CMV} promoter with the miR-101 gene. Therefore, we can easily monitor miR-101 expression in the cell under a fluorescent microscope (Fig. 2B). The miR-101 levels were detected over 10 times higher in the miR101 transfected cells (BPH1^CmiR101^) compared to the vector control cells (BPH1^Cvec^) (Fig. 2C, D), and the miR-101 level in BPH1^CmiR101^ cells was maintained at the same level after 100 passages (Fig. 2B, C). As a powerful tool, this enforced EGFP-miR-101 expression cell line allowed us to study the role of miR-101 in regulation of COX-2 in cultured cells and tumor xenografts.

Secondly, we examined the regulatory target of miR-101 using a luciferase report assay (fig. 3). We constructed a luciferase reporter vector (3'UTR-Luci) containing the 3'UTR of COX-2 fragment, which was carried a putative miR-101 complementary region (NM_000963 3'-UTR 1735-1741). Theoretically, luciferase expression can be blocked
and a decreased luciferase activity can be observed when the miR-101 gene binds to the complementary region of 3'UTR of COX-2 in this system. We found a significantly lower level of luciferase activity when the 293T cells were co-transfected with EGFP-miR-101 vector and 3'UTR-Luci vector (Fig. 3D) indicating a direct interaction between miR-101 and COX-2 mRNA. In contrast, the level of COX-2 protein was able to be restored in BPH1CmiR101 after transfecting the 3'UTR of COX-2 fragment (Fig. 3E). However, depending on the extent of sequence complementarity between a miRNA and its target gene, miRNA-guided posttranscriptional regulation of gene expression follows two distinct mechanisms: target mRNA cleavage and translational repression.

Thirdly, we evaluated whether the miR-101 is able to alter the mRNA expression of COX-2. We found there was no statistical significance on the levels of COX-2 mRNA between enforced expression of miR-101 BPH1CmiR101 cells and vector control BPH1Cvec cells (Fig.3B). Above all, the observations from our study clearly demonstrate that miR-101 regulates COX-2 by directly binding to 3'UTR of COX-2 mRNA and causing translational repression.

Our previous studies reported that the COX2/PGE2 pathway plays a key role in regulating cancer development along with other signaling pathways (25). Interestingly, in this study, we found that exogenous miR-101 significantly inhibits prostate cancer cell growth both in vitro and in vivo (Fig. 4). Thus, we further investigated the therapeutic potential of exogenous miR-101 for COX-2 associated prostate cancer and the mechanism(s) by which miR-101 modulated COX-2/PGE2/EGFR pathways. We found exogenous miR-101 not only is able to reduce COX-2 protein expression, but is also
able to concurrently decrease the EGFR level in cultured BPH1\textsuperscript{CmiR101} cells and xenograft tissues. EGFR exists on the cell surface and is activated by binding of ligand. Activation of EGFR in turn initiates its downstream signal transduction cascades leading to DNA synthesis and cell proliferation. Abnormally high expression of EGFR has been observed in many types of cancers, including prostate cancer. The high expression of COX-2 leads a EGFR stimulated cell proliferation (26) and COX-2 products, such as PGE2, activate EGFR signaling pathways to promote cancer growth (27, 28). Hence, COX-2/PGE2/EGFR arrest has been revealed to be a potential mechanism of miR-101 inhibition of cancer cell proliferation.

In addition, flow cytometry analysis showed miR-101 caused cell cycle arrest at the G1/S border (Fig.5 A, B). There are a series of regulatory proteins involved in cell proliferation and cell fate. Cyclin D1, which is important for G1 to S transition, increases in response to extracellular signals from a growth factor (e.g., EGFR). The cyclin D-CDK4 complex activates E2F and results in transcription of other cell cycle related genes such as cyclin A, cyclin E and PCNA. Cyclin A and cyclin E push the cell from G\textsubscript{1} to S phase (G\textsubscript{1}/S transition), and Cyclin B-cdc2 complex initiates the G\textsubscript{2}/M transition. PCNA, a member of DNA sliding clamp family, interacts with multiple partners involved in DNA repair, DNA replication, chromatin remodeling and cell cycle regulation. PCNA is essentially required to bind to CDK4-cyclin D, CDK2-cyclin E and CDK2-cyclin A complexes for progression from G1 to S phase in the cell cycle (29). We found that the levels of cyclin D1, cyclin A, and cyclin B were notably decreased in the prostate cancer cells with exogenous miR-101 (Fig. 5B). The decrease in PCNA expression was similarly found in BPH1\textsuperscript{CmiR101} contributing to the G1/S arrest (Fig. 5C). In addition, the
p53 protein is characterized as an essential mediator of cell cycle arrest that can halt progression of the cell cycle in G₁ by blocking the activity of CDK2 and senses DNA damage to apoptosis induction (30, 31) p53 showed an increased (151%) level in BPH₁<sup>CmiR101</sup> compared with BPH₁<sup>Cvec</sup>, whereas, the expression of the anti apoptotic protein Bcl-2 which negatively correlated with p53 displayed a decrease (28%) in BPH₁<sup>CmiR101</sup> compared with BPH₁<sup>Cvec</sup>. This result indicated that miR-101 decreasing cyclin D1, cyclin A, PCNA along with Bcl-2 led to the block in the G1/S transition.

In conclusion, the role of miR-101 in inhibiting COX-2 protein expression was studied in a stably enforced expression of miR-101 cell line grown in vitro and in vivo. MiR-101 is able to inhibit COX-2 protein expression through directly binding to 3'UTR region of COX-2 mRNA. Therefore, exogenous miR-101 has a high potential for treating COX-2 associated cancers through directly and indirectly modulating the COX-2/PGE2/EGFR pathways.

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**Abbreviations:** COX-2, cyclooxygenase-2; EGFP, enhanced green fluorescence protein;; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Bcl-2, B-cell lymphoma-2;; EGFR, epidermal growth factor receptor; CDK, cyclin-dependent kinase;
PBS, phosphate buffered saline; PCNA, proliferating cell nuclear antigen; PGE2, prostaglandin E2

Reference


Figure legends

Figure 1, The expression levels of COX-2 and miR-101 in prostate cell lines. (A) Comparison of COX-2 expression in various prostate cell lines. COX-2 protein levels in benign prostatic hyperplasia cell line (BPH1), androgen receptor positive prostate tumorigenic cell lines (BPH1CAF1 and LNCap), androgen receptor negative prostate tumorigenic cell line (PC3), and in non-tumorigenic human prostatic cell line (PNT1). (B) The expression levels of COX-2 and miR-101 in prostate cell lines. COX-2 levels were analyzed by western blot and semi quantified based on COX-2/β-actin relative intensity. Bio-Rad Quantity one software was used for densitometric analysis of the western blots. MiR-101 levels were analyzed by real-time RT-PCR. The fold changes of each cell line were calculated by comparing with PNT1. (C) The ratio of COX-2/miR-101 in prostate cells.

Figure 2, Stable expression of exogenous miR-101 in prostate cancer cells and xenografts. (A) Schematic representation of the design of a lentiviral mediated delivery of a miR-101 vector, which contains an expression cassette of P_{CMV} promoter, EGFP, and miR-101 precursor, and a selective cassette of P_{PGK} promoter and Puro'. (B) EGFP expression levels of BPH1^{CmiR101} cells at the 5th and 100th passages were captured by
fluorescence microscope at ×400 magnification. (C) The expression levels of miR-101 in cultured BPH1<sup>Cvec</sup> and BPH1<sup>CmiR101</sup> cells and in BPH1<sup>Cvec</sup> and BPH1<sup>CmiR101</sup> tumor xenografts (D) were revealed by quantitative RT-PCR. The p value was compared to vector control BPH1<sup>Cvec</sup> cells (*p < 0.05) and the results represented the mean ± SD of two independent tests.

**Figure 3, MiRNA-101 directly inhibits COX-2 expression at the posttranscriptional level.** (A) COX-2 protein levels in pairs of BPH1<sup>Cvec</sup>/BPH1<sup>CmiR101</sup> and BPH1<sup>CAFTD+siRNA</sup>/BPH1<sup>CAFTD</sup> cell lines were evaluated by western blot and semi quantified based on COX-2/β-actin relative intensity. The percentage indicates the relative intensity of COX-2. (B) COX-2 mRNA levels of BPH1<sup>Cvec</sup> and BPH1<sup>CmiR101</sup> cells were determined by quantitative RT-PCR and the results represented the mean ± SD of two independent experiments with triplicates. (C) A RNA sequence map to demonstrate the 3'-UTR of COX-2 mRNA carries a complementary site (NM_000963 3'-UTR 1735-1741) for the seed region of miRNA-101. (D) Luciferase report assay was applied to determine the miR-101 binding target. The luciferase activity was detected after transfection of 3'-UTR Luci vector or EGFP-miR-101 vector alone, or combination of the two vectors into 293T cells. The p value was compared to vector control BPH1<sup>Cvec</sup> cells (*p < 0.05) and the results represented the mean ± SD of two independent experiments with triplicates. (E) The COX-2 protein levels were analyzed by western blot and the amount of protein was normalized by comparing the intensity of the β-actin band.
Figure 4, Exogenous miR-101 suppresses proliferation and growth of prostate cancer cells in vitro and in vivo. Cell proliferation and cell viability of cultured BPH1_{miR101} and BPH1_{vec} cell lines were compared under light-microscope (A), colony formation assay (B) and MTT assay (C). The results from colony formation and MTT assays represented the mean ± SD of two independent experiments with triplicates. (D) Comparison of tumor xenograft growth between BPH1_{miR101} and BPH1_{vec} groups. The day of cell inoculation was the experimental start day and all mice were sacrificed on day-36. The growth of solid tumor xenografts was monitored every other day and measured using vernier calipers. (E) The tumor mass (g) was measured on the final experiment day immediately after the tumor tissue was removed from the mouse by surgical excision. The average tumor mass is indicated as a bold bar in each group. The p value was compared to vector control BPH1_{vec} group (*p < 0.05).

Figure 5, Effects of exogenous miR-101 on inducing G1/S arrest and altering cell growth-associated proteins. (A) Cell cycle profiles of BPH1_{miR101} and BPH1_{vec} cell lines. The cell distribution of G1 and S phases in each group was analyzed by flow cytometry. (B-D) The levels of cell growth-associated proteins between BPH1_{miR101} and BPH1_{vec} cell lines and xenografts were analyzed by western blot and semi quantified based on targeted protein/β-actin relative intensity. The percentage indicates relative intensity of specific protein.

Figure 6, Expression of COX-2, PCNA and EGFR in BPH1_{miR101} and BPH1_{vec} tumor xenografts. The expression levels of COX-2 (A), PCNA (B) and EGFR (C) in
the BPH1^{CmiR101} tumor xenografts were compared to the corresponding levels in BPH1^{Cvec} by immunohistochemical staining method. As described under methods, the tumor xenografts were removed at the end of the experiment, fixed in formalin and then stained with specific monoclonal antibody. Magnification, x 400.
Figure 1A

BPH1  BPH1\textsuperscript{CAF TD}  LNCap  PC-3  PNT1

COX-2

\(\beta\)-actin
Figure 2B

5th passages 100th passages
Figure 2C

Relative level of miR-101 in Cells (fold)

0 5 10 15

5th passages

100th passages

BPH1

BPH1 cvec

cmiR101

level of cells (fold)

Relative

5th passages

100th passages

BPH1
cvec

cmiR101
Relative level of miR-101 in tumor xenografts (fold)

BPH1cvec

BPH1cmir101

*
**Figure 3A**

<table>
<thead>
<tr>
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<th>Xenografts</th>
<th>BPH1\textsuperscript{CAFTD} cells</th>
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<tbody>
<tr>
<td>BPH1\textsuperscript{Cvec}</td>
<td>BPH1\textsuperscript{Cvec}</td>
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<tr>
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<tr>
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<tr>
<td>100%</td>
<td>100%</td>
<td>*COX-2</td>
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*COX-2
Figure 3B

Ratio of Cox-2 mRNA / β-actin mRNA (fold)

0 2 4 6 8 10 12

BPH1vec

BPH1cmiR101
Figure 3C

5' .CACUGACAUUUAAUG -- GUACUGUA... COX-2 3'UTR

| | | | | | | | |

3' AAGUCAAUAGUGUCAUGACAU miR-101
Figure 3D

Luciferase Activity (fold)

3'-UTR Luci  miR-101  miR-101

3'-UTR Luci
Figure 3 E

BPH1<sub>Cv</sub>  BPH1<sub>CmiR101</sub>  BPH1<sub>CmiR101</sub>

3'-UTR Luci

COX-2

β-actin
Figure 4A

BPH1Cvec

Day 2  Day 5  Day 9

BPH1CmiR101

Day 2  Day 5  Day 9
Cell viability (%)
Figure 4D

Tumor Volume (mm$^3$)

- BPH1Cvec
- BPH1CmiR101

Experiment day

12 15 19 26 33 36

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Cancer Research. Published OnlineFirst on March 23, 2011; DOI: 10.1158/1940-6207.CAPR-10-0333
Figure 4E

Tumor Weight (g)

BPH1Cvec

BPH1CmiR101

*
Figure 5A

% cells in G1 or S phase

0 10 20 30 40 50 60

G1

S

BPH1cvec
BPH1miR101
**Figure 5B**

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*Target Protein*
Figure 5C

Cultured cells

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<tr>
<td>EGFR</td>
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Xenografts

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<tr>
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Figure 5D

BPH1<sup>Cvec</sup>  BPH1<sup>miR101</sup>  BPH1<sup>Cvec</sup>  BPH1<sup>miR101</sup>

Bcl-2  p53

100%  72%  100%  151%

β -actin  β -actin
Figure 6A.

**COX-2 expression**

BPH1<sub>Cvec</sub> vs BPH1<sub>CmiR101</sub>
Figure 6B

PCNA expression

BPH1Cvec

BPH1CmIR101
Figure 6C

EGFR expression

BPH1<sup>Cvec</sup> vs BPH1<sup>CmiR101</sup>

200 µm
Enforced Expression of miR-101 inhibits prostate cancer cell growth by modulating cyclooxygenase-2 pathway in vivo

Yubin Hao, Xinbin Gu, Yuan Zhao, et al.

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