MicroRNA-320 suppresses the stem cell-like characteristics of prostate cancer cells by downregulating the Wnt/beta-catenin signaling pathway

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Prostate cancer (PCa) is a leading cause of mortality and morbidity in men worldwide, and emerging evidence suggests that the CD44high subpopulation of prostate cancer cells contains CSCs that exclusively target CSCs.

Materials and methods

Clinical tissues and cell lines

Human prostate samples were obtained via a research protocol that was approved by the Institutional Review Board of National Cheng Kung University Hospital, Tainan, Taiwan and Taipei Medical University Hospital, Taipei, Taiwan. The human prostate specimens were taken from the operating room and immediately frozen in liquid nitrogen and stored at –80°C for quantitative reverse transcription–PCR (RT–PCR). Supplementary Table S1, available at Carcinogenesis Online summarizes the characteristics of the clinical specimens used in the study. The human non-tumorigenic prostate epithelial cell lines PZ-HPV-7, PWR-1E and RWPE-1 were cultured in keratinocyte-serum-free medium supplemented with 5 ng/ml rEGF and 30 μg/ml bovine pituitary extract (Invitrogen, Carlsbad, CA). The human PCA cell lines PC3, LNCaP and 22Rv1 were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics. The DU145 cell line was cultured in modified Eagle’s medium supplemented with 10% FBS and 2 mM L-glutamine with antibiotics. All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Recombinant human Wnt3a (R&D Systems) was added to the cells at a concentration of 100 ng/ml for 1 h before performing functional studies.

Quantitative RT–PCR

Total RNA was isolated from clinical tissue or cell lines using TRIzol Reagent (Invitrogen) and reverse transcribed to complementary DNA (cDNA) using a well-known oncogenes or tumor-suppressor genes (4). Recent studies have demonstrated that the presence of miRNAs in body fluids may contain numerous non-invasive biomarkers for early cancer detection (7). These findings provide a firm rationale for the usefulness of miRNAs in the detection, diagnosis, prognosis and possible treatment of human cancer.

Prostate cancer (PCa) is the second most common cause of cancer-related deaths in men worldwide. Numerous studies in recent years have described the presence of stem cells in PCa, termed cancer stem cells (CSCs) or tumor-initiating cells (TICs) (8,9) and suggested that the proportion of CSCs correlates with a poor prognosis (10). Prostate CSCs are highly tumorigenic cell type to drive cancer growth and might be a promising therapeutic target due to their resistance to chemotherapy and irradiation (8). However, CSCs are rare and current methods to identify and characterize prostate CSCs are limited because CSCs quickly differentiate into other cell types. Increasing evidence suggests that CD44high subpopulations contained CSC populations in prostate cancer (11–13). In addition, a recent study showed that breast cancer cells experimentally induced into an epithelial–mesenchymal transition (EMT) condition lost breast CSC characteristics and exhibit increased drug resistance (14). Therefore, the enrichment of CSCs in PCa will be helpful in the screening of therapeutic agents that exclusively target CSCs.

Until recently, only a few studies have assessed the effects of miRNAs in human prostate cancer tumor-initiating cells (15). Our previous miRNA microarray analysis revealed that miR-320 is downregulated in PCa (0.32-fold of the non-tumor miR-320) (16). However, the functional role and mechanism of action of miR-320 in PCa have yet to be clarified. Recent studies also show that miR-320 is downregulated in breast cancer (17), colon cancer (18) and acute myelogenous leukemia (19), suggesting that miR-320 may act as a tumor suppressor in cancer progression. In this study, we demonstrate that miR-320 exhibits a tumor-suppressive role in PCa by targeting the Wnt/beta-catenin pathway. Inhibition of miR-320 enriches the proportion of prostate stem-like TICs, increases PCa cell self-renewal capacity, increases anticancer drug resistance and promotes tumorigenic capabilities. Increases of miR-320 effectively reverse the EMT-induced tumorigenic capabilities in PCa cells. These findings provide evidence that miR-320 is a suppressor of tumor initiation and suggest that miR-320 might be a potential therapeutic target for prostate CSCs.

Introduction

MicroRNAs (miRNAs) are a class of small non-coding RNAs that negatively regulate their targets by binding to the 3'-untranslated regions (UTRs) of the mRNA targets (1). miRNAs regulate numerous physiological and pathological processes, such as cell proliferation, development, cancer progression, stem cell self-renewal and differentiation (2,3). Increasing evidence suggests an important role for miRNAs in cancer progression (4); miRNA genes are frequently located at fragile chromosomal sites that have been specifically linked to human cancers (5), the aberrant expression of miRNAs is significantly correlated with various human cancers (6) and miRNAs can function as tumor suppressors or oncogenes by directly regulating key-negative regulatory pathways such as the Wnt/beta-catenin signaling pathway.
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**Cell transfection and western blotting**

Cells were seeded (1.5 x 10^5) into 6-well plates and transfected with 30 nM of microRNA precursors, negative control miRNA, antisense oligonucleotides (Applied Biosystems/Ambion) and pcDNA3 or pcDNA3-S33Y-β-catenin plasmids (Addgene plasmid 19286, kindly provided by Kolligs et al.) (23) using Lipofectamine 2000 at 24 h. The cells were harvested at 48 h, and RNA and protein extractions were carried out. The protein lysate was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Protein expression was analyzed by western blotting using primary antibodies against β-catenin (BD Biosciences), E-cadherin (Clone 36B5, NeoMarkers), Vimentin (C-20, Santa Cruz Biotechnology) or β-actin (Sigma-Aldrich), followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Specific proteins were detected by chemiluminescence using ECL Plus Western Blotting Detection Reagents (GE Healthcare Biosciences).

**Cell proliferation and soft-agar colony formation assays**

For viable cell quantification, fresh medium containing 20% of CellTiter MTS cell proliferation reagent (Promega) was added to each well, the plates were incubated at 37°C for 1–2 h, and the absorbance at 490 nm was measured in an ELISA assay reader. For the soft-agar colony formation assay, 2 × 10^4 of miR-320-transfected or control cells were plated in 3 ml of Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS and 0.36% agar was plated on a layer of 0.5 ml of DMEM/10% FBS. Two weeks after plating, the colonies were stained with 0.005% crystal violet, and photographs of the stained colonies were taken.

**Microarray analysis and pathway analysis**

Total RNA from PC3 cells treated with 30 nM of miR-320 precursors or negative control was isolated after 48 h of treatment. The microarray hybridization was carried out at the Phalanx Biotech Service Center (Hsinchu, Taiwan). The cDNA microarray consisted of 39,068 gene-specific oligonucleotides, with 1082 experimental control probes in a one-block array format. For each probe, a 60mer oligonucleotide was designed in the sense direction. The filters data were log2 transformed and corrected using quantile normalization before their average ratios and significance values were calculated. Genes that showed a significant (P < 0.01) difference in expression between miR-320-transfected PC3 cells and control cells underwent pathway analysis (GeneGo Map Folders of the MetaCore software suite; GeneGo, St. Joseph, MI).

**MicroRNA reporter assays**

Luciferase reporter constructs were made by ligating the 3′UTR of β-catenin to a pMiR-REPORT plasmid (Applied Biosystems/Ambion). The construction of the mutated 3′ UTR was performed in the same manner, but DNA oligonucleotides carrying the mutations were used instead. Reporter plasmids plus hTERT or pcDNA3 (β-gal) reporter plasmids as internal controls were transfected alone or in combination with miRNA precursors or antisense miRNA oligoribonucleotides by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Luciferase assays were performed 24 h after transfection using the Dual-Light® Luciferase and β-Galactosidase Reporter Gene Assay System (Applied Biosystems). Firefly luciferase activity was normalized to β-gal activity for each transfected well.

**In situ hybridization and immunohistochemistry**

The human prostate specimens were fixed for 24 h in 4% paraformaldehyde. Endogenous miR-320 was detected by using a LNA probe against miR-320 (Exiqon, Denmark) on fresh-frozen prostate tissue. The samples were placed in a humidified chamber and incubated in an incubator at 55°C overnight. The signals were amplified with an NBT/BCIP chromogen at 37°C using the IsHyb In situ Hybridization Kit (Biochain). For immunohistochemistry, the sections were treated with 3% H2O2 and incubated with anti-β-catenin (610153; BD Biosciences), anti-Ki-67 (550699; BD Biosciences) or anti-CD3 (ab9498; Abcam) antibodies for 4°C overnight after washing with phosphate-buffered saline (PBS). The sections were then mounted to react with the horseradish peroxidase polymer-conjugated secondary antibodies, incubated with aminoethyl carbazole (AEC) chromogen, and then counterstained with hematoxylin.

**Immunofluorescence staining**

For immunofluorescence staining, 5 x 10^5 cells were seeded onto poly-l-lysine-coated glass coverslips in 24-well dishes. After 24 h, the cells were fixed with 4% paraformaldehyde in PBS, permeabilized in 0.1% Triton X-100 and blocked with 1% bovine serum albumin in PBS. The cells were incubated with an anti-human β-catenin or anti-CD44 (ab112178; Abcam) antibody diluted 1:100 in blocking solution. The cells were washed and incubated with fluorescein isothiocyanate (FITC)- or TRITC-conjugated anti-mouse antibody (Jackson ImmunoResearch).

**TOP/FOP flash Wnt reporter assay**

The cells were transfected with TOP flash or FOP flash Wnt reporter plasmids (Millipore Corporation) containing wild-type or mutant T cell factor (TCF) DNA binding sites by using Lipofectamine 2000. The cells were also cotransfected with β-gal reporter plasmids. To determine Wnt activity in response to stimulation, TOP/FOP flash-transfected cells were cotransfected with miRNA precursors or negative control. The reporter activity was analyzed as described above.

**Lentivirus production and infection**

Retroviral plasmids were obtained from the National RNAI Core Facility located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica. The shRNAs targeting E-cadherin, β-catenin and luciferase were expressed from the pLKO.1 hairpin vector, which harbors an expression cassette for a puromycin resistance gene driven by the human phosphoglycerate kinase promoter. The shRNA sequences used in this study are shown in Supplementary Table S2, available at Carcinogenesis Online. MicroRNA-320-expressing lentivirus (lenti-miR-320) was initially generated by amplification of pre-miR-320 from human genomic DNA, digestion with Nhel and BglII and cloning into pLKO-AS2.neo to generate lenti-miR-320. To stably knockdown the expression of endogenous miR-320, anti-microRNA lentivector containing the GFP reporter miRZIP-320 was purchased from System Biosciences (SB). All lentiviruses were generated from HEK293T packaging cells in the RNAi service laboratory, National Cheng Kung University Hospital. The cell culture supernatant containing lentivirus was harvested every 24 h until 72 h after transfection. Viral titer were determined by transmuting HEK293T cells using diluted culture supernatants and tested by Giemsa staining. The number of viable cells after 2 days of culture in the presence or absence of antibiotics. Viral supernatants were stored at −80°C. The cells were infected with lentiviruses in the presence of 8 μg/ml of polybrene (Sigma-Aldrich). After 48 h of infection, the cells were treated with puromycin or G418 for selection. Antibiotic-resistant cells were pooled for subsequent analysis.

**Sphere formation assay**

To assay sphere formation, 1 x 10^3 cells were plated onto a 6-well ultra-low attachment plate (Corning Glass) in serum-free DMEM/F-12 supplemented with 10% FBS and 10 ng/ml EGF and 10 ng/ml bFGF and cultured for 6 days. The number of spheres formed was counted using an inverted microscope.

**Colony formation assay**

For the colony formation assay, 1 x 10^3 cells were plated onto a 6-well plate in serum-free DMEM/F-12 supplemented with N-2 supplement, 10 ng/ml EGF and 10 ng/ml bFGF (Invitrogen). After 14 days of culture, the number of tumor spheres formed was counted using an inverted microscope.

**Cytotoxicity assay**

To assay cytotoxicity, 3 x 10^5 cells were plated onto 96-well microtiter plates. After adhering overnight, the cells were treated with cisplatin (25 μM), carboplatin (60 μM) and taxol (15 μM) for 72 h. After treatment, cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenoxy)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay.

**Tumorigenicity assays in NOD/SCID mice**

All experiments were carried out using male 6-week-old NOD−SCID mice. After lentiviral infection and antibiotic selection, PC3 or DU145 cells were resuspended in PBS. The cells were mixed with Matrigel (BD Biosciences) at a 1:1 ratio and subcutaneously injected into the right flanks of NOD/SCID mice. On day 30, the primary tumors were excised and weighed. The tissue specimens were fixed in 10% buffered formalin and embedded in paraffin and sectioned. Sections were stained with hematoxylin and eosin or used for immunohistochemical analysis.

**Flow cytometric analysis**

The cells were suspended in 100 μl of PBS containing 1% FBS and incubated at 4°C for 30 min with FITC-conjugated mouse anti-human CD44 (1:100 dilution, Southern Biotech) or isotype control antibodies. After extensive washing with PBS, the labeled cells were analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences).
Magnetic cell sorting
For the separation of CD44<sup>high</sup> and CD44<sup>low</sup> cells, single cell suspensions obtained from prostate cancer cells were preincubated for 10 min with species-specific FcR blocking antibody to block non-specific binding of antibodies to Fcγ receptors (StemCell Technologies). The cells were then incubated with FITC-conjugated mouse antihuman CD44 antibody (Southern Biotech) in 100 μl of PBS containing 1% FBS for 15 min at room temperature. Briefly, the cells were incubated in EasySep FITC selection cocktail for 15 min followed by incubation with EasySep magnetic nanoparticles (StemCell Technologies) for 10 min. PBS containing 1% FBS was added to the cells at a total volume of 2.5 ml, and the labeled cells in the tube were placed into the magnet for 5 min. Unlabeled and labeled cells were collected, and the purity of separated cells was confirmed by flow cytometric analysis.

Results
MicroRNA-320 is downregulated in PCa and prostate cancer cell lines to promote tumor formation
To investigate whether miR-320 is a tumor suppressor in PCa, we first examined the expression levels of miR-320 in 10 PCa tissues and 13 non-tumor prostate tissues by qRT–PCR. Expression of miR-320 is significantly downregulated in human PCa tissue (P = 0.01; Figure 1A). Because clinical PCa tissues might be contaminated with adjacent benign tissue and/or stromal cells, we performed in situ hybridization (ISH) to visualize the intact miRNA expression patterns. The ISH analyses showed that miR-320 is strongly expressed in non-cancerous prostatic epithelium but is greatly diminished in most advanced prostate carcinomas (Figure 1B). Furthermore, we confirmed that miR-320 expression was much lower in the four PCa cell lines, PC3, LNCaP, 22Rv1 and DU145, than in the three immortalized prostatic epithelial cell lines, PZ-HPV-7, PWR-1E and RWPE-1 (Figure 1C, Supplementary Figure S1, available at Carcinogenesis Online). The significantly decreased expression of miR-320 in PCa tissues and PCa tumor tissues and multiple cancer cell lines prompted us to assess the biological role of miR-320 in prostate cancer progression, we examined the effects of miR-320 on cell proliferation and soft agar colony-forming activities of PC3 and DU145 cells. MTS assays showed that miR-320 significantly inhibited the proliferation of
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PC3 and DU145 cells (Figure 1D, Supplementary Figure S2A, available at Carcinogenesis Online). Anchorage-independent clonogenic assays showed that most of the colonies in the miR-320-transfected PC3 and DU145 cells were much smaller than those in the negative control groups after 14 days of culture. The average number of colonies (colony was defined as >20 μm) in the miR-320-transfected DU145 and PC3 cells was decreased by 57 and 78%, respectively (Figure 1E, Supplementary Figure S2B, available at Carcinogenesis Online). To further confirm these findings in vivo, we evaluated the role of miR-320 in tumor formation using a xenograft model. PC3 cells infected with lentivirus containing pre-miR-320 or empty control genes were subcutaneously implanted into 6-week-old male NOD/SCID mice. As shown in Figure 1F, PC3 cells expressing miR-320 exhibited nearly a 20-fold reduction in tumor size and effectively suppressed tumor growth compared with cells with control lentivirus (Supplementary Figure S2C, available at Carcinogenesis Online). Ki67 and CD31 immunohistochemical staining indicated that proliferation and vascularization were reduced with high expression of miR-320 (Supplementary Figure S2D, available at Carcinogenesis Online). Taken together, these findings from in vitro and in vivo assays demonstrate that miR-320 potently represses the tumorigenic abilities of PCa cells.

cDNA microarray revealed that Wnt pathway and stem cell markers could be regulated by miR-320

To determine cellular pathways globally regulated by miR-320, we performed microarray analysis on total RNA from miR-320 overexpressing PC3 cells and control cells. We identified ~959 genes (680 upregulated and 279 downregulated) with significant differential expression. A MetaCore software analysis of global genes revealed that Wnt/β-catenin and transforming growth factor pathway were the two main pathways that were significantly altered by miR-320 expression in PC3 cells (Figure 2A). A qRT–PCR further confirmed that mRNA transcripts of multiple target genes of the Wnt/β-catenin pathway were substantially downregulated in miR-320 expressing PC3 and DU145 cells, including the c-MYC, LEF-1, CD44, SOX9, Oct-4 and CCND1 (Figure 2B). β-Catenin signaling plays an important role not only in cancer development but also in regulating CSC self-renewal (20, 21). Interestingly, some CSC-associated transcripts, such as Oct-4, CD44, CD133, CD117, CXCR4 and ABCG2, were also downregulated by miR-320 (Figure 2C).
MicroRNA-320 directly targets β-catenin and regulates its function

We used bioinformatics searches to identify relevant targets of mir-320 and found that mir-320 may target nt229-246 of the 3′UTR of β-catenin; therefore, we proposed that β-catenin is an important functional target for miR-320. A luciferase reporter assay with a vector containing the putative β-catenin 3′UTR target site downstream of a luciferase reporter gene was performed to test this hypothesis. Base pairing between miR-320 and the wild-type (wt-CTNNB1) or mutant (mut-CTNNB1) putative target site in the 3′UTR of β-catenin mRNA is shown in Figure 3A. Transfection with miR-320 precursors (pre-miR-320) significantly inhibited 40% of the luciferase activity of the wt-CTNNB1 3′UTR reporter (P < 0.05; Figure 3B). In contrast, the luciferase activity of cells cotransfected with pre-miR-320 and mut-CTNNB1 was not measurably different (Figure 3B). We then investigated whether miR-320 affects β-catenin protein or mRNA expression by transient transfection of pre-miR-320 or antisense oligonucleotides into PC3 and DU145 cells. We found that β-catenin protein expression decreased in both PC3 and DU145 cells overexpressing miR-320, whereas the mRNA expression level of β-catenin in the miR-320-overexpressing cells did not change. Conversely, the introduction of antisense miR-320 (320-AS) blocked miR-320 expression and markedly enhanced β-catenin protein levels (Figure 3C). Similar results were obtained in the immunofluorescence microscopy analysis; miR-320 inhibits endogenous β-catenin expression and nuclear localization (Figure 3D). Previous studies have shown that β-catenin activates TCF or lymphoid enhancer factor (LEF) proteins, stimulates the transcription of TCF/LEF target genes, and hence promotes neoplastic transformation (22). To explore whether the β-catenin/TCF/LEF signaling pathway is also inhibited in the miR-320-overexpressing cells, we analyzed β-catenin/TCF/LEF transcriptional activities in cells transfected with miR-320 using TCF and mutant TCF luciferase reporter plasmids (TOPflash and FOPflash, respectively). As shown in Figure 3E, the relative transcriptional activity of the β-catenin/TCF complex in miR-320-overexpressing PC3 cells decreased by ~65% compared with cells transfected with a control miRNA (P < 0.01). A reporter plasmid with mutated TCF binding sites (FOP) showed minimal change, confirming the specificity of this effect. To further confirm that the downregulation of these CSC markers was mediated by miR-320 direct targeting to the 3′UTR of β-catenin, miR-320 and cancer-derived mutant β-catenin without 3′UTR (pcDNA3-S33Y-β-catenin, a codon 33 substitution of tyrosine for serine 23) were coexpressed in PC3 and DU145 cells. Western blotting showed that miR-320 was unable to decrease the amount of the mutant β-catenin lacking the 3′UTR (Supplementary Figure S3A, available at Carcinogenesis Online). We found that constitutively active β-catenin remarkably increased CSC markers expression and PC3 tumorsphere formation. Additionally, miR-320 no longer inhibited CSC-associated gene expression and tumorsphere induced by the 3′UTR null β-catenin (Supplementary Figure S3B and C, available at Carcinogenesis Online). This finding implies that miR-320 modulates β-catenin activation to regulate PCa stem cell-like characteristics. β-Catenin is a dual function protein involved in cell–cell adhesion and Wnt signaling (24). To evaluate whether Wnt/β-catenin signaling plays a major role in the miR-320-repressed tumorigenic abilities of PCa cells, we first analyzed β-catenin expression levels from PC3 and DU145 cells treated with Wnt3a. Western blot analyses showed that treatment of both PC3 and DU145 cells with Wnt3a increased the level of β-catenin, which can be blocked by miR-320 (Supplementary Figure S4A, available at Carcinogenesis Online). In addition, we observed that miR-320 significantly suppressed the Wnt3a-driven anchorage-independent growth of PC3 and DU145 cells in soft agar (Supplementary Figure S4B, available at Carcinogenesis Online). These results suggest that the inhibitory effects of miR-320 on prostate cancer cells may primarily involve the suppression of Wnt-dependent transcriptional activity.

Fig. 3. MiR-320 represses β-catenin translation. (A) Schematic diagram of the β-catenin-3′UTR reporter constructs. Sequences were compared between mature miR-320 and the wild-type (wt) or mutant (mut) putative target sites in the 3′UTR of β-catenin mRNA. (B) Luciferase assay of PC3 cells transfected with luciferase constructs containing the wild-type (Wt) or mutant (Mut) target site of the β-catenin 3′UTR plus miR-320 precursor (miR-320) or negative control miRNAs (C) for 48h. The luciferase activity was normalized to the β-galactosidase activity as an internal transfection control. Values represent the mean ± SD of three independent experiments. *P < 0.05. (C) Western blotting and RT–PCR analysis of β-catenin in PC3 and DU145 cells transfected with the control miRNA, control antisense-miR (C-AS), pre-miR-320 or antisense-miR-320 (320-AS). β-Actin was used as the loading control. The level of β-catenin expression relative to β-actin was quantified using ImageJ software and is shown below the western blots (fold). (D) β-Catenin immunofluorescence of PC3 and DU145 cells treated with the control miRNA or pre-miR-320 for 48h. β-Catenin is shown in green, and the nuclei are counterstained with 4',6-diamidino-2-phenylindole show in blue. Scale bar, 10 μm. (E) Luciferase reporter assay of β-catenin-Tcf3/lef1 activity in PC3 cells transfected with the Wnt signaling reporter (TOPflash or the control FOPflash) together with the control miRNA or pre-miR-320 for 48h. SV40-β-gal is an internal control.
MicroRNA-320 is downregulated in cancer stem-like cells and overexpression of miR-320 inhibits stem cell-like properties

We next sought to determine whether miR-320 could affect stem cell-like properties by regulating the β-catenin signaling pathway. PCa cells with a CD44+ phenotype have been suggested by others to have tumor-initiating properties (11–13). Flow cytometry analysis indicated that CD44 high cells represented ~1 and 8% of LNCaP and DU145 cells, respectively (Supplementary Figure S5A, available at Carcinogenesis Online). We purified CD44 high and CD44 low prostate cancer cells from LNCaP and DU145 cells using magnetic beads (Supplementary Figure S5B, available at Carcinogenesis Online) and confirmed the expression of stem cell-like markers, including CD44, Nanog and Nestin (Figure 4A). Importantly, we found that CD44 high LNCaP and DU145 cells express significantly less miR-320 but high levels of β-catenin compared with CD44 low LNCaP and DU145 cells (Figure 4B and C). Previous studies have shown that PCa cells with an EMT phenotype could increase the CSC population (25). We used two lentiviruses, designated shEcad#1 and shEcad#2, that contain a potent shRNA targeting E-cadherin or a scrambled shRNA control (shLuc) to stably knockdown E-cadherin in PC3 and DU145 cells. After infection, shEcad-infected cells had a significant reduction in E-cadherin expression and increased vimentin expression compared with shLuc-infected cells (Supplementary Figure S6A, available at Carcinogenesis Online). Notably, shEcad-infected PC3 and DU145 cells had a substantial increase in stem cell markers, such as Nestin, Nanog and Oct-4 (Supplementary Figure S6A, available at Carcinogenesis Online). We further used sphere formation assays to confirm that E-cadherin-depleted cells have stem cell-like properties. We observed an increase in the sphere-forming ability of the E-cadherin knockdown DU145 and PC3 cells compared with control cells (Supplementary Figure S6B, available at Carcinogenesis Online). Interestingly, we found that miR-320 was decreased in the E-cadherin knockdown DU145 and PC3 cells compared with control cells (Supplementary Figure S6C, available at Carcinogenesis Online). To further establish the role of miR-320 in prostate stem-like TICs, we restored miR-320 expression in the E-cadherin knockdown PC3 and DU145 cells. Re-expression of miR-320 significantly decreases the stem cell markers, Nestin, Nanog and Oct-4, compared with control shEcad-cells (Supplementary Figure S6D, available at Carcinogenesis Online). These results suggest that stem-like TICs within PCa cell populations could be enriched by miR-320 inhibition. In addition, PCa cells probably lost the EMT-associated self-renewal after miR-320 re-expression.

Downregulation of miR-320 promotes cancer stem cell-like properties

Moreover, we were interested to examine whether decreasing miR-320 expression in PCa cells could enrich the population of prostate stem-like TICs and induce stem cell-like properties in PCa cells. We used the miRZip antisense microRNA lentiviral system to reduce miR-320 in PC3 and DU145 cells. The downregulation of miR-320 (miR-320 low) after infection with miRZip-320 induced upregulation of β-catenin and CD44 protein expression (Figure 5A and B) and increased tumor spheres formation and clonogenic capacity (Figure 5C, Supplementary Figure S7A, available at Carcinogenesis Online). Additionally, we also found decreased E-cadherin expression and increased vimentin expression in miR-320 low DU145 cells (Supplementary Figure S7B, available at Carcinogenesis Online). Because the low expression of miR-320 significantly affected the maintenance of stem-like TICs, we were interested in whether miR-320 low PC3 cells had increased drug resistance. The miR-320 low PC3 cells had a significant increase in chemotherapeutic drug resistance toward cisplatin, carboplatin and...
We also assessed the functional presence of stem-like TICs by depleting miR-320 in vivo. The miRZip-C-transduced DU145 cells were subcutaneously injected in serial limiting dilutions into NOD/SCID mice. The miRZip-C-transduced DU145 cells were injected as controls. Consistent with the in vitro observation, the inhibition of miR-320 also increased the tumor initiation rate and tumor growth rate (Figure 5E, Supplementary Figure S7C, available at Carcinogenesis Online). To confirm miR-320 specificity and β-catenin’s involvement in miR-320-regulated cancer stem cell-like properties, we used lentiviruses, designated shCTNNB, to stably knockdown β-catenin in miR-320 low DU145 cells (Supplementary Figure S7D, available at Carcinogenesis Online). After infection, the CSC markers expression and tumorsphere formation increased by miR-320 inhibition could be significantly decreased by depletion of CTNNB (Figure 5F and G). Cumulatively, these findings strongly suggest that miR-320 is important for the prostate stem-like TICs maintenance pathway by modulating β-catenin.

Finally, we investigated whether there was an inverse correlation between miR-320 and β-catenin protein in PCa tissues. We found that
prostate tumors and non-tumor prostate tissues with high endogenous miR-320 expression showed relatively low expression of β-catenin protein, whereas the clinical specimens with low miR-320 expression showed relatively high levels of β-catenin protein (Pearson correlation coefficient $r = -0.855$; Supplementary Figure S8A, available at Carcinogenesis Online). The inverse correlation between miR-320 and β-catenin expression was confirmed in clinical specimens by ISH and immunohistochemistry (Supplementary Figure S8B, available at Carcinogenesis Online) and in prostate cell lines (Supplementary Figure S8C and S9, available at Carcinogenesis Online). These results indicate that high miR-320 levels in normal prostate epithelial cells may play a tumor-suppressive role by negatively regulating β-catenin expression and that the downregulation of miR-320 may be involved in prostate tumorigenesis and progression.

Discussion

MicroRNAs are crucially important in maintaining stemness in normal stem cells and are frequently dysregulated in most cancers. This study highlights a novel role of miR-320 in malignant transformation and cancer initiation by inhibiting the canonical Wnt/β-catenin pathway in PCA (Supplementary Figure S10, available at Carcinogenesis Online). Four lines of evidence provide support for the conclusion. First, the significantly decreased expression of miR-320 in the resected tissues of PCA patients compared with non-cancerous prostate specimens is verified. Second, overexpression of miR-320 in PCa cells decreases β-catenin as well as downstream target genes, self-renewal ability and tumorigenic activity. Third, targeting miR-320 by antisense oligonucleotides dramatically augments chemotherapeutic drug resistance, stem cell-like properties and tumor-initiating capability. Fourth, expression of miR-320 and β-catenin were found to be significantly inversely correlated in PCA clinical specimens. To the best of our knowledge, this study is the first to report that miR-320 is a significant suppressor of tumor initiation, as evidenced by studies using cell lines, animal models and clinical sample analyses.

Our clinical studies indicate that miR-320 is decreased in resected tissues of PCA patients. Recent studies also show that miR-320 is downregulated in breast cancer, colon cancer and acute myelogenous leukemia (17–19). The expression levels of miR-320 correlate with the probability of recurrence-free survival in colon cancer (18). However, a conflicting report indicated that miR-320 is upregulated in PCa (26). In this study, we performed independent real-time RT–PCR and ISH assays to validate the tumor-suppressive role of miR-320 in PCa to visualize the intact miR-320 expression patterns. This discrepancy may have been due to variable contamination of PCa tissues with adjacent benign tissue and/or stromal cells, including fibroblasts, smooth muscle cells and endothelial cells. Whether the levels of miR-320 are correlated with recurrence in PCa patients and could be a predictor of clinical outcome in PCa is worth studying further.

We also successfully verified that β-catenin is negatively regulated by miR-320 at the posttranscriptional level via a specific target site (nt 229–246) within the 3′UTR of β-catenin. MiRanda for the prediction of miRNA targets indicated that miR-320 could target the 3′UTR of β-catenin possibly involved in promoting cancer cell invasion and growth (30,31). Therefore, we cannot rule out the possibility that miR-320 may play a crucial role in maintaining cancer stem cell-like characteristics through other target genes. But when we transfected PC3 cells with miR-320 plus pcDNA5-S33Y-β-catenin, which lacks the 3′UTR containing the miR-320 binding sites, and found that miR-320 decreased the expression of CSC markers and sphere-forming activity could be completely rescued (Supplementary Figure S3, available at Carcinogenesis Online). Further, knockdown of β-catenin reversed CSC markers expression and tumorsphere formation in miR-320low DU145 cells, indicating that β-catenin is a critical and functional target of miR-320 in modulating cancer stem cell-like features.

Recent studies in prostate cancer cell lines have suggested that CD44 is a prostate cancer TIC marker (11–13). Elevated levels of β-catenin were observed in CD44high PCa cells (11). To further validate that downregulation of miR-320 correlates with prostate cancer stem cell-like properties, we purified CD44high PCa cells and found a significant correlation between miR-320 downregulation and β-catenin upregulation, with enhanced clonogenic and tumor-initiating capacities (11,12). Accumulating evidence has demonstrated that cells with an EMT phenotype, induced by different factors, can turn into cancer stem-like cells (32). During the EMT process, E-cadherin is downregulated and β-catenin accumulates in the nucleus where it binds to TCF/LEF transcription factors to activate the expression of Wnt target genes. In this study, we observed that with knockdown of E-cadherin by shRNA, there was a significant increase in the cancer stem-like population in PC3 and DU145 cells, which accompany a decrease of miR-320 expression. Interestingly, we established stably suppressing miR-320 in PC3 and DU145 cells could successfully enrich stem-like TIC population and enhance sphere formation, drug resistance, EMT marks in vitro and tumorigenicity in vivo. Thus, miR-320 downregulating approaches in cancer cells might overcome the rarity of stem-like TIC and relative instability in culture to facilitate the identification of agents that kill stem-like TIC in the future.

The mechanisms responsible for regulating miR-320 expression are still unclear. MiR-320 is located at chromosome 8p21.3, a region frequently reported to undergo a loss of heterozygosity during the progression of PCa (33). Also, miR-320 was reported to be downregulated in squamous cell carcinoma cells under hypoxic conditions that increase invasiveness and drug resistance (34). It has been reported that miR-320 expression is significantly decreased in hearts during regulation of ischemia/reperfusion-induced cardiac injury (35). In human pancreatic cancer cell lines, miR-320 is upregulated following exposure to chromosome demethylating agents and histone deacetylase inhibitors suggesting the epigenetic inactivation of this miRNA in pancreatic cancer (36). A recent study found that miR-320 is a key mediator in the PTEN tumor-suppressive pathway of stromal fibroblasts (37). PTEN maintains expression of miR-320 in stromal fibroblasts by repressing JNK1 and JNK2, and consequently increased miR-320 expression. Loss of PTEN downregulates miR-320 expression to unleash the tumor-promoting potential of its targets, such as ETS2 and MMP2, and thereby contributes to tumor progression (37). Loss of PTEN can increase the development of prostate cancer stem cells (38,39). In PTEN deletion, intestinal epithelium models have been shown to be increased in β-catenin activation (40). These results implied that PTEN deficient might regulate miR-320 in prostate cancer to promote the development of prostate stem-like TICs. Furthermore, the mechanism by which miR-320 is silenced during tumor development and progression will need to be explored in the future.

Taken together, this is the first study to demonstrate that miR-320 is a novel tumor suppressor and a negative regulator of the key stem cell initiator β-catenin. Decreased miR-320 participates in the development and progression of human PCa by promoting the therapeutic outcome (29). Although a single miRNA could potentially target multiple genes, in silico analyses using TargetScanHuman and MiRanda for the prediction of miRNA targets indicated that miR-320 could regulate the expression of LEF1 and SOX9 via the 3′UTR. LEF1 is known to promote cancer cell invasiveness and growth (30,31). Therefore, we cannot rule out the possibility that miR-320 may play a crucial role in maintaining cancer stem cell-like characteristics through other target genes. But when we transfected PC3 cells with miR-320 plus pcDNA5-S33Y-β-catenin, which lacks the 3′UTR containing the miR-320 binding sites, and found that miR-320 decreased the expression of CSC markers and sphere-forming activity could be completely rescued (Supplementary Figure S3, available at Carcinogenesis Online). Further, knockdown of β-catenin reversed CSC markers expression and tumorsphere formation in miR-320low DU145 cells, indicating that β-catenin is a critical and functional target of miR-320 in modulating cancer stem cell-like features.

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β-catenin-mediated Wnt signaling pathway that regulates cancer initiation. This study suggests that targeting the miR-320/β-catenin interaction or perturbing miR-320 expression may prove to be a new therapeutic strategy in the treatment of PCa patients.

Supplementary material

Supplementary Tables 1 and 2 and Figures 1–10 can be found at http://carcin.oxfordjournals.org/

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References


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