MicroRNA-194 suppresses prostate cancer migration and invasion by downregulating human nuclear distribution protein

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Abstract. Human NudC nuclear distribution protein (hNUDC) is differentially expressed between normal and cancer cells. Based on its marked altered expression and its roles in modulating cell division, cytokineses and migration, a detailed understanding of the mechanisms regulating hNUDC expression in cancer cells is critical. In this study, we identified miR-194 as a downstream target of hNUDC and linked its expression to reduced metastatic capacity and tumorigenicity of prostate cancer (PCa) cells. Using miRNA target prediction programs, hNUDC mRNA was found to contain a potential binding site for miR-194 within its 3'UTR. A Reporter assay confirmed that post-transcriptional regulation of hNUDC was dependent on the miR-194 binding site. Forced expression of miR-194 in PCa cell lines, PC-3 and DU-145, led to a decrease in the mRNA and protein levels of hNUDC. Overexpression of miR-194 in these cells inhibited cell migration and invasion, and induced multinucleated cells. Our data showed that hNUDC knockdown by siRNA significantly reduced the migration and invasion in the PC-3 and DU-145 cells, phenocopying the results of miR-194 overexpression. Furthermore, lentivirus-mediated stable expression of miR-194 in PCa cells reduced the ability of colony formation as detected by a soft agar assay and exhibited significantly less tumorigenic ability *in vivo*. Our results suggest a novel role for miR-194 in effectively controlling cell metastatic processes in PCa cells via the regulation of hNUDC expression.

Introduction

Prostate cancer (PCa) continues to be the most common lethal malignancy diagnosed in men and metastatic progression of PCa is a major cause of death. During progression to metastasis, PCa cells are thought to acquire a mesenchymal phenotype, which allows them to dissociate from the primary tumor, invade, and migrate to distant organs (1,2). Cell metastasis involves the accumulation of sequential disrupted expression of genes, which in turn promotes cancer progression (3,4). MicroRNAs (miRNAs), a class of small non-coding RNAs, are single-stranded RNAs consisting of ~22 nucleotides that negatively regulate expression of their target genes at the 3'-untranslated regions (3'UTRs) (5-7). A growing number of miRNAs have been described as candidate oncogenes or tumor-suppressor genes through respective binding to target tumor-suppressor genes or oncogenic genes (8-11). Over the last two decades, distinct expression profiles of miRNAs have been linked to tumor development and progression in PCa (12-15). Nevertheless, the exact mechanism of multiple molecular events in PCa initiation, growth, invasion and metastasis remains unclear.

The miRNA-194 family is comprised of miR-215, miR-194-1, miR-192 and miR-194-2, which are clustered and expressed as two separate polycistronic pri-miRNA transcripts. The miR-192 and miR-194-2 cluster on chromosome 11q13.1 and the miR-215 and miR-194-1 cluster on chromosome 1q41. miR-194-1 and miR-194-2 have the identical mature sequence. miR-192 and miR-215 are closely related with similar seed sequence. Like many other miRNAs, miR-194 may contribute to the development and progression of different types of cancers, implicating a tumor suppressor function. Emerging evidence suggests that miR-194 is downregulated in a number of different malignancies, such as colorectal cancer (16), renal childhood neoplasms (17), liver (18) and endometrial cancer (19). Conversely, increased miR-194 expression is found in a range of cancer cellss, including those of gastric (20), colorectal (21) and endometrial cancer (22) and is associated with reduced cancer metastasis. However, the biological functions of the deregulation of miR-194 in tumor progression have not yet been completely defined.

The mammalian NudC nuclear distribution protein (NUDC) is expressed in a broad range of tissues and is increasingly being recognized as a multifunctional protein that affects various cellular responses, such as cell division (23), proliferation (24), migration (25,26), and cytokinesis (27,28). Aberrant regulation of hNUDC expression is correlated with a variety of pathologies. For example, the expression levels of hNUDC are much higher in erythroid precursor cells

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compared to other human tissues (29). In addition, hNUDC protein expression was found to be significantly upregulated in patients with acute myelogenous and acute lymphoblastic leukemia compared to aspirates from normal controls (30,31). Furthermore, hNUDC was found expressed in neuroectodermal tumors, but not in non-neoplastic brain tissue (32). There is also an inverse correlation between hNUDC expression and nodal metastasis in esophageal cancer (33). Downregulation of human hNUDC mRNA, including the use of the antisense oligonucleotides and small interfering (si)RNAs resulted in impairment of both cell proliferation in various types of cancer *in vitro* (27,34). As a consequence, the cells exhibited nuclear enlargement and multiple nuclei, possibly due to a failure to complete the inhibition of both mitosis and cytokinesis. As may be expected, a protein involved in mitotic cell division is also found to play a role in cancer. According to previous studies in the literature, adenovirus-mediated overexpression of hNUDC in LNCaP, DU-145 and PC-3 cells significantly attenuatedthe rate of cell proliferation through G2/M phase arrest. This has been interpreted as a potential requirement for hNUDC to accomplish the first steps of metastasis (35). Previous results have demonstrated that both upregulation and downregulation of hNUDC play an important role in anticancer intervention.

In the present study, the regulation of biological functions by miR-194 was identified for the first time in PCa by the targeting of the hNUDC gene. Transfection of DU-145 and PC-3 cell lines with miR-194 decreased hNUDC mRNA and protein expression and reduced cell migration and invasion. *In vitro* and *in vivo* experiments indicated that the overexpression of miR-194 suppressed colony formation and tumorigenicity of PCa cells in nude mice. These findings suggest that miR-194 may act as a tumor suppressor in PCa, which is consistent with its role in other human cancers.

Materials and methods

Cell lines and culture conditions. Human prostate cancer cell lines, PC-3 and DU-145, were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco® by Life Technologies™, Grand Island, NY, USA) supplemented with 10% v/v inactivated fetal bovine serum (FBS; Biological Industries Israel Beit Haemek, Ltd., Kibbutz Beit-Haemek, Israel) and 100 U/ml penicillin + 100 μg/ml streptomycin. Cells were maintained in a humidified incubator at 37˚C with 5% CO_2.

Transfection procedures. Cells were plated at $1x10^5$ in a 6-well plate. Twelve hours later, cells were transfected with 50 nM of scrambled control, miR-194, miR-194 inhibitor, inhibitor control or siRNA for hNUDC (Suzhou GenePharma Co., Ltd., Suzhou, China) with the use of Lipofectamine® 2000 reagent (Invitrogen by Life Technologies, Carlsbad, CA, USA). siRNA for hNUDC used here has been previously described (27). After transfection, the cells were processed for western blot analysis, quantitative real-time PCR (RT-qPCR), migration and invasion assays.

RNA isolation and RT-qPCR. Total RNA was isolated from the prostate cell lines using TRIzol reagent, and quantified spectrophotometrically. The reverse transcription of miRNAs from total RNA (1 μg) was performed with miR-194-specific stem-loop primer (5'-CTCAACTGGTGTCGTGGAGTCG GCAATTCAGTTGAGTCCACATG-3') using a Bestar™ qPCR RT kit (DBI® Bioscience, Ludwigshafen, Germany). To measure hNUDC mRNA expression levels, the first strand of cDNA was synthesized using oligo (dT) primers by Bestar™ qPCR RT kit and the reverse transcription product was amplified using Bestar™ SybrGreen qPCR Mastermix (DBI® Bioscience). The sequences of the primers specific for hNUDC were 5'-CAGTGGGGTCTTGCTGTCATCT-3' (forward) and 5'-CTAACCCTTGCCTTTCAACTCA-3' (reverse). Quantitative PCR was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems). The PCR reaction consisted in a denaturation step at 95˚C for 10 min, followed by 40 amplification cycles at 95˚C for 10 sec, and annealing at 60˚C for 35 sec and extension at 72˚C for 30 sec. Gene expression was normalized using endogenous β-actin or U6 as a control. Densitometric quantification was perfomed with the comparative cycle threshold method ($2^{-\Delta\Delta Ct}$) (36).

Western blot analysis. Total lysates from DU-145 and PC-3 cells (10 μg) were applied to 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% BSA in 1X Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature. Western blot analysis was performed by incubating the membranes with primary antibodies for hNUDC (Abcam, Cambridge, MA, USA) at a dilution of 1:3,000 in 5% BSA in 1X TBST. After overnight incubation at 4˚C, the membranes were incubated with goat anti-mouse IgG alkaline phosphatase (AP) conjugated secondary antibodies (Cell Signaling Technology, Inc.) at 1:1,000 for 1 h at room temperature. Signals were visualized using an enhanced chemiluminescence (ECL) detection system (Pierce, Rockford, IL, USA). Densitometric quantification was performed using ImageJ software.

Dual-Luciferase assay. 3'UTR mRNA sequence of hNUDC containing a miR-194 binding site was amplified by the following primers: 5'-ATTACGCGTCCCCTGTTTTTTC CTCCCTG-3' (forward) and 5'-CGGAAGCTTAGCTGGGC AAATCGTTTTAA-3' (reverse). The PCR product was then cloned downstream to the luciferase gene in plasmid pMIR-Report. A mutation construct was made by deletion of several bases within the binding site and cloned into the same vector. The control vector was used for normalization of cell number and transfection efficiency. Co-transfection of synthetic miR-194 and luciferase report constructs were transfected in HEK-293T cells by using Lipofectamine 2000 reagent. Luciferase assays were performed using the Dual-Luciferase® Reporter Assay System kit (Promega Corp., Madison, WI, USA) and luminescence was measured on a GloMax® 96 microplate luminometer (Promega).

In vitro invasion and migration assays. Cellular invasion and migration were assessed using the Cultrex® Basement Membrane Extract (BME), PathClear® (Trevigen, Gaithersburg, MD, USA) according to the manufacturer's protocol. Cells at a density of $5x10^4$ were seeded onto Transwell® Permeable

Supports (Corning Incorporated, Corning, NY, USA). For the migration assay, the plates were incubated for 12 h at 37˚C. The cells that migrated through the pores without the matrix to the lower surface of the membrane were fixed in 70% ethanol and stained with crystal violet. For the invasion assay, the plates were incubated for 48 h and the cells that invaded through the pores covered with the matrix to the lower surface of the membrane were fixed and stained. The cells were counted under a light microscope in five separate fields. Each blue point indicated an individual cell and all the images were scanned and counted at x40 magnification.

Determination of nuclear morphology. Cells were transfected for 48 h and fixed with methanol prior to being stained with May-Grünwald-Giemsa staining solution for 15 min at room temperature. The stained slides were examined by a phase contrast microscope at x40 magnification.

Generation of stable cell lines. The packaged lentivirus expressing miR-194, anti-miR-194-sponge and GFP-control were purchased directly from ViGene Biosciences (Shandong, China). Stable expression of miR-194, GFP-control and anti-miR-194-sponge in PC-3 and DU-145 cells was achieved through selection with 2 μg/ml puromycin (Amresco, Solon, OH, USA).

Clonogenic cell growth assay. The clonogenic cell growth of PC-3 and DU-145 cell lines was examined on soft agar using Cell Transformation Detection Assay (Millipore, Temecula, CA, USA). Briefly, each well of a 24-well plate was first layered with 0.8% agarose in growth medium (DMEM supplemented with 10% FBS). The cell lines to be tested were trypsinized, and $1x10^4$ cells were resuspended in a growth medium containing 0.4% agarose and then they were poured as a top layer in the 24-well plates. The plates were incubated at 37˚C for 28 days. Colony formation was observed using Cell Stain Solution overnight and the diameter of colonies was scored under a microscope at x40 magnification. Cells were counted with the Cell Quantification Solution after a 4-h incubation at 37˚C, followed by a spectrophotometer reading at OD_{490}.

Nude mouse xenograft assay. The female BALB/c nude mice (18 g, aged 6 weeks) were obtained from Guangdong Medical Laboratory Animal Center (permit no. SCXK 2013-0002). Animals were maintained and cared for in the Traditional Chinese Medicine and Marine Drugs Laboratory of Sun Yat-Sen University (permit no. SYXK 2014-0020) which conformed to the Guide for the Care and Use of Laboratory Animals. *In vivo* experiments were performed in accordance with a protocol approved by the Animal Ethics and Welfare Committee of Sun Yat-Sen University. PC-3 and DU-145 cell lines stably expressing miR-194, anti-miR-194-sponge or GFP-control were suspended in a PBS buffer at a concentration of $1x10^7$ cells/ml. Cell suspension ($2x10^6$ cells/mouse) were injected subcutaneously below the right scapula of the BALB/c-nude mice. Seven mice from each of the three groups were used. The experiment ended when the tumor volume reached 2,000 mm^3. The tumors were measured with micro-calipers three times a week and the volumes were calculated as V (mm^3) = length (mm) x (width (mm))^2 x 0.5. The mice were euthanized on day 30 and the tumors were harvested and weighed.

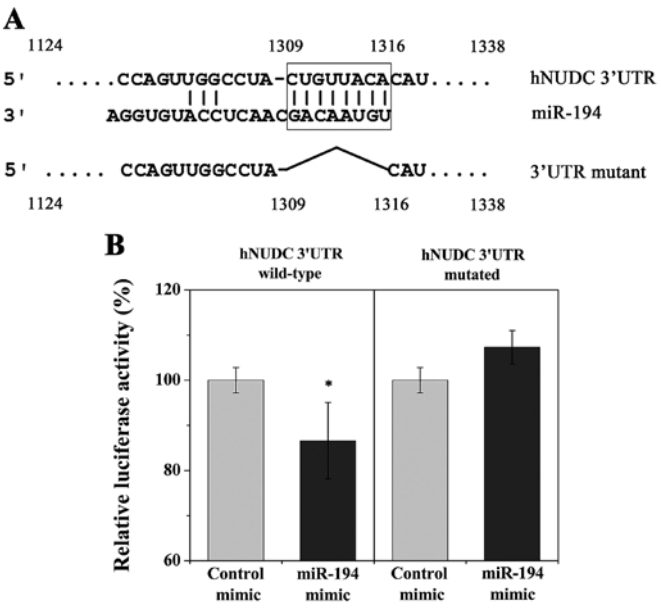

Figure 1. The 3'UTR of human NudC nuclear distribution protein (hNUDC) contains a single predicted miR-194 target site. (A) Schematic representation of firefly luciferase reporter constructs containing the 200-nt 3'UTR of hNUDC. In a mutated construct, a miR-194 target site was disrupted by deletion of eight nucleotides within the seed sequence as indicated. (B) Luciferase reporter assays using HEK-293T cells co-transfected with the reporter gene constructs described above with miR-194 or control mimic. Forty-eight hours after transfection, luciferase activity was measured. Activity was normalized to *Renilla* luciferase activity. Data for each reporter construct are expressed relative to control transfected cells. Data are expressed as the means ± SE. (n=3). *P<0.05.

Significance testing. Statistical relevant differences were assigned to a p-value of at least ≤0.05 using an unpaired Student's t-test in all functional assays and densitometric analyses of western blot analysis assays. All data represent at least three independent experiments. Results are expressed as the mean ± standard error (SE).

Results

hNUDC is a direct target of miR-194. To determine whether hNUDC can be regulated by miRNAs, we used TargetScan (37,38) (http://www.targetscan.org/) and miRanda (39-41) (http://www.microrna.org/) to predict miRNAs that could potentially target the hNUDC 3'UTR. A potential binding site for miR-194 was identified at the hNUDC 3'UTR from nucleotides 1309-1316 (Fig. 1A). To validate hNUDC as a direct target of miR-194, both wild-type and mutant hNUDC 3'UTRs were cloned downstream of the firefly luciferase ORF in a pMIR-Report vector (Fig. 1A). The wild-type and mutant hNUDC 3'UTRs luciferase expression vectors were co-transfected with scrambled control or miR-194 mimic into HEK-293T cells. Relative luciferase activity was significantly reduced for wild-type hNUDC 3'UTR, indicating that hNUDC is a potential direct target of miR-194 (Fig. 1B). Mutation in the predicted miR-194 target site abrogated

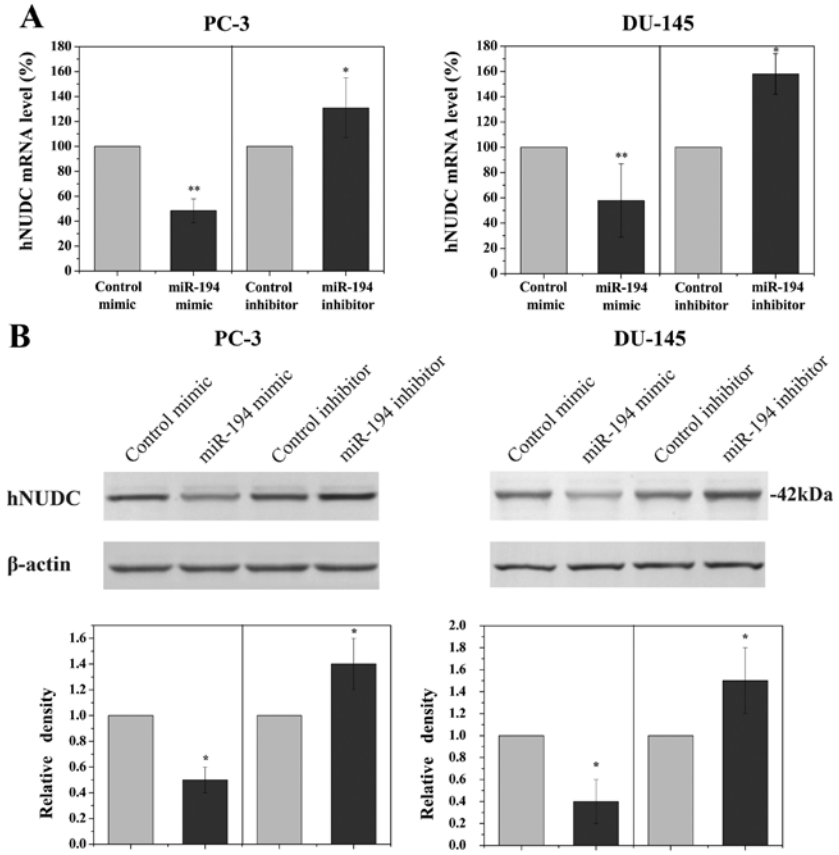

Figure 2. Validation of human NudC nuclear distribution protein (hNUDC) as a direct target of miR-194. (A) Inverse correlation between the expression of miR-194 and hNUDC at miRNA levels in the indicated cell lines. Cells were transfected with scrambled control, miR-194, miR-194 inhibitor or inhibitor control for 48 h and RT-qPCR was used to confirm the hNUDC transcript levels. (B) Inverse correlation between the expression of miR-194 and hNUDC at protein levels in the indicated cell lines. hNUDC was detected by western blot analysis; β-actin was used as a loading control. Data are expressed as the means ± SE. (n=3). *P<0.05; **p<0.001.

inhibition by miR-194 mimic, confirming the functionality of this target site (Fig. 1B).

hNUDC expression levels are regulated by miR-194 in prostate cancer cells. To determine if the hNUDC gene was a biologically relevant target of miR-194 in PCa cells, PC-3 and DU-145 cell lines were transfected with miR-194 mimic or miR-194 inhibitor. To demonstrate the specificity of miR-194 in order to evaluate the magnitude of the changes in hNUDC mRNA levels, non-targeting miRNA inhibitor and non-targeting scrambled miRNA were used as controls. qRT-PCR analyses revealed a significant reduction in the RNA expression of hNUDC following transfection with miR-194 mimic, whereas miR-194 inhibitor increased hNUDC levels (Fig. 2A). Western blot analysis was also employed to examine the hNUDC protein, showing a prominent decrease in the expression of hNUDC when PC-3 and DU-145 cells were transfected with miR-194 mimic. Conversely, inhibiting the endogenous miR-194 in PC-3 and DU-145 cells using miR-194 inhibitor increased the hNUDC protein level (Fig. 2B).

miR-194 attenuates the migration and invasion of prostate cancer cells. To date, migration and invasion are known as the key processes in many cancers. In order to investigate whether the ectopic overexpression of miR-194 is involved in these processes, we assessed the *in vitro* migratory and invasive capacities by Transwell migration assay. The ectopic expression of miR-194 decreased the invasiveness of PC-3 and DU-145 cells compared to the control mimic-transfected cells (Fig. 3A). Upon inhibition of endogenous miR-194 by miR-194 inhibitor, a visible opposite result was seen than in the control inhibitor transfected cells (Fig. 3A). Microscopy results revealed that upon transfection of miR-194 mimic, the loss of migratory capability was accompanied by a loss of cell elongation (Fig. 3B). These findings indicate that upregulation of miR-194 inhibits cell invasion and migration.

Overexpression of miR-194 induces multinucleated cells. Since hNUDC is considered an important mediator of cell proliferation and cytokinesis, thus, it is expected that the inhibition of hNUDC would result in the failure of cell growth and give rise to cells with multiple nuclei. However, our cell proliferation and cell cycle analyses revealed that transfection of the miR-194 mimics into two PC-3 and DU-145 cell lines showed no influence on cell proliferation and cell cycle compared to the scrambled control (data not shown). Morphologically, transfection of cells with the miR-194 resulted in an increase in multinucleated cells coupled with abnormally large, flattened and accumulated multiple nuclei, whereas cells transfected with the mimic and inhibitor controls had normal nuclear morphology (Fig. 4A and B).

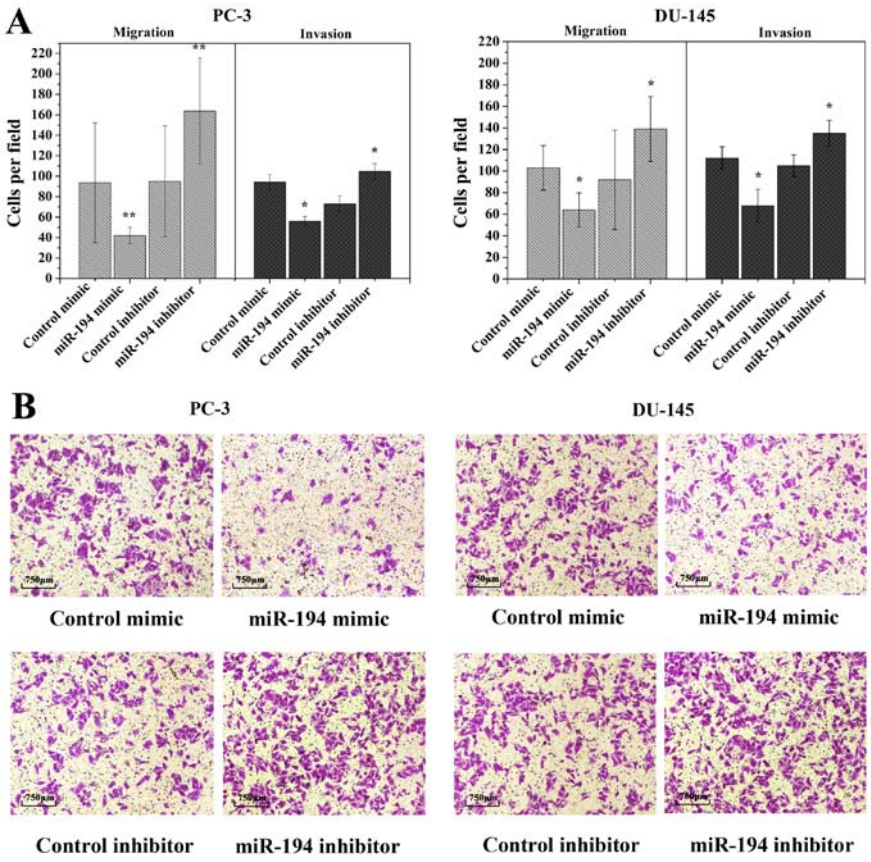

Figure 3. miR-194 affects the cell migratory and invasive potential *in vitro*. (A) Migration and invasion of PC-3 and DU-145 cell lines transfected with scrambled control, miR-194, miR-194 inhibitor or inhibitor control as depicted by a Transwell migration assay after 12 and 48 h, respectively. Data are expressed as the means ± SE. (n=3). *P<0.05; **p<0.001. (B) Morphological alterations in PC-3 and DU-145 cells upon the indicated transfection assessed by phase-contrast microsopy. Scale bars, 750 μm.

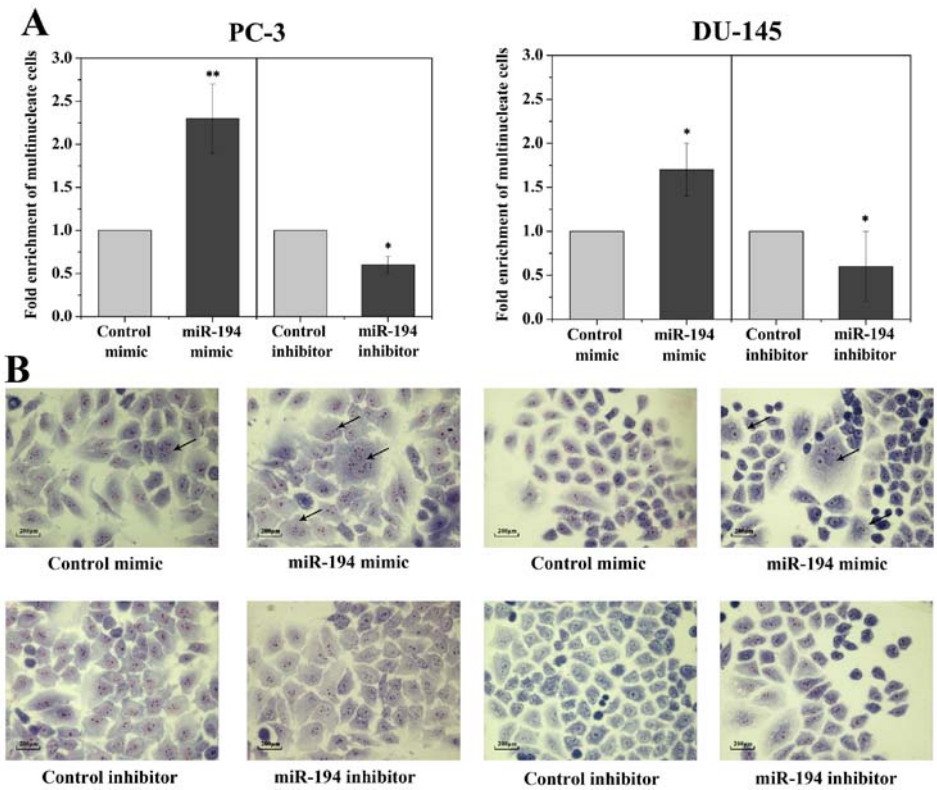

Figure 4. Overexpression of miR-194 induces cell cytokinesis. PC-3 and DU-145 cells were transfected with scrambled control, miR-194, miR-194 inhibitor or inhibitor control for 48 h. (A) The number of multinucleated cells was counted at 48 h after May-Grünwald-Giemsa staining. The columns represent the mean ± SE of three replicates. *P<0.05; **p<0.001. (B) Representative morphology of multinucleated cells as assessed by phase-contrast microsopy. Scale bars, 200 μm.

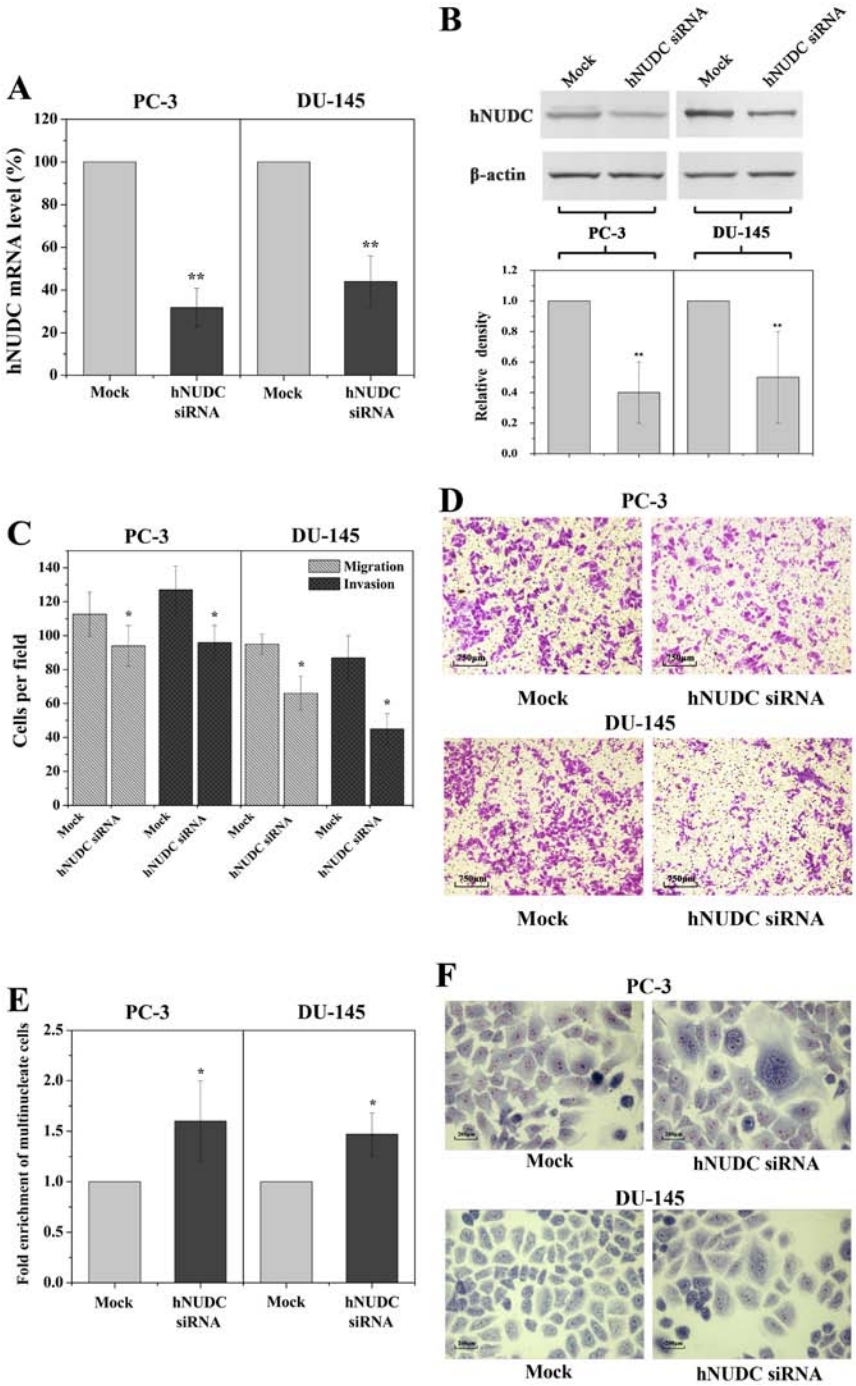

Figure 5. RNA interference-mediated functional effects of hNUDC. (A) PC-3 and DU-145 cells were transfected with hNUDC siRNA and RT-qPCR analysis demonstrated significant reductions in the expression levels of hNUDC mRNA compared to the mock control 48 h post-transfection. (B) Western blot analysis also revealed significant reduction in the protein levels of hNUDC 48 h post-transfection with hNUDC siRNA. (C) hNUDC siRNA decreased cell invasion and migration when compared with the mock control. Data are expressed as the means ± SE. (n=3). *P<0.05; **p<0.001. (D) The effect of hNUDC knockdown on the cellular morphology. Scale bars,750 μm. (E) The number of the multinucleated cells formed by the indicated transfection of PC-3 and DU-145 cells after 48 h. The columns represent the mean ± SE of three replicates. *P<0.05; **p<0.001. (F) Representative morphology of multinucleated cells as assessed by phase-contrast microsopy. Scale bars, 200 μm.

siRNA-mediated inhibition of hNUDC mimics the phenotypes induced by miR-194. The aforementioned results demonstrated that miR-194 is a potent suppressor of cell migration and invasion through the targeting of hNUDC mRNA. Thus, we reasoned that siRNA-mediated knockdown of hNUDC should result in a cell phenotype similar to that of miR-194 overexpression. Indeed, by using hNUDC siRNA we were able to obtain a decrease in the expression of the hNUDC gene, reducing mRNA levels by 3.3-fold in the PC-3 cells and by 2.5-fold in the DU-145 cells, as determined by qRT-PCR analysis 48 h post-transfection (Fig. 5A). This result was confirmed by a significant reduction in hNUDC at the protein level (Fig. 5B). Similarly, miR-194 overexpression, significantly reduced the migration and invasion potential following knockdown of hNUDC expression (Fig. 5C and D). Following transfection with hNUDC siRNA, we observed that the multinucleated

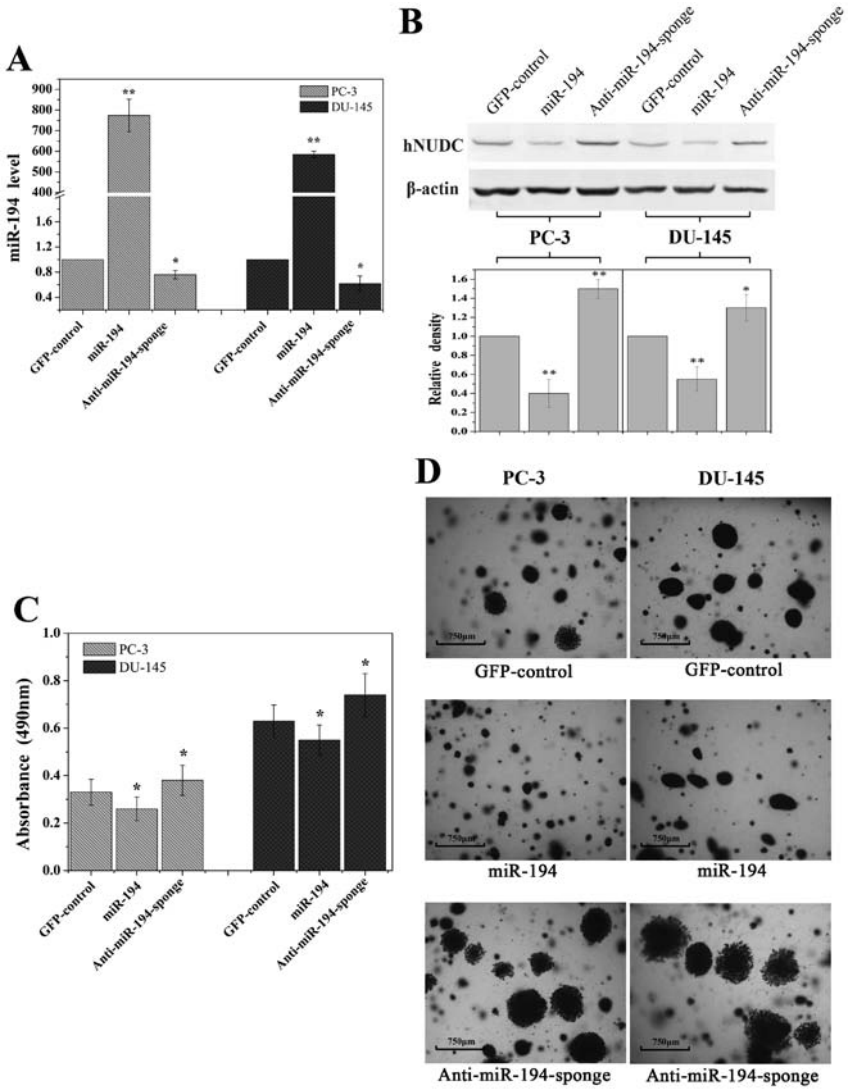

Figure 6. Overexpression of miR-194 reduces colony formation. PC-3 and DU-145 cell lines stably transfected with miR-194, anti-miR-194-sponge or the GFP-control plasmid were cultured for 30 days. (A) The expression levels of miR-194 were confirmed by RT-qPCR. (B) The protein levels of hNUDC were confirmed by western blot analysis. (C) The number of cells were detected at 30-days post-transfection. The bars represent the mean ± SE of the number of colonies from three independent experiments. *P<0.05; **p<0.001. (D) The colonies were visualized after staining with crystal violet. Scale bars, 200 μm.

cells were enhanced following inhibition of endogenous hNUDC (Fig. 5E and F). These results strongly suggest that downregulation of hNUDC expression promotes invasion and migration of prostate cancer cells.

Overexpression of miR-194 inhibits cell growth potential. To examine whether miR-194 regulates primary tumor growth, a standard colony formation assay was used for these studies. We overexpressed miR-194 in PC-3 and DU-145 cells by transduction with high-titer lentivirus expressing miR-194 or anti-miR194-sponge using an empty vector as a control. An ~700-fold increase of miR-194 expression was detected by qRT-PCR in the transduced cells, in comparison to cells that were transduced with a control vector expressing GFP (Fig. 6A). As expected, a small decrease in miR-194 in the lentiviral expressing anti-miR-194-sponge was observed (Fig. 6A). The expression level of hNUDC protein was significantly decreased in response to ectopic miR-194 expression (Fig. 6B). Overexpression of miR-194 decreased the total number of cells observed when compared with the control (Fig. 6C). In contrast, anti-miR-194-sponge did not suppress cell growth in soft agar (Fig. 6C). Moreover PC-3 and DU-145 cells transfected with miR-194 formed smaller colonies compared to larger disseminated colonies formed by the GFP-control and anti-miR-194-sponge transduced cells after 30 days (Fig. 6D). The significant reduction of colonies suggests that miR-194 exhibits a tumor-suppressive function in cancer cells.

Overexpression of miR-194 suppresses the growth of PCa xenografts. We next examined the effect of ectopic miR-194 expression on prostate cancer tumorigenesis *in vivo*. DU-145 cells stably expressing either miR-194 or anti-miR-194-sponge was subcutaneously injected into 6-week-old nude mice. The control group was injected with DU-145 stably expressing GFP. Tumor growth was monitored twice weekly for 30 days. As shown in Fig. 7, tumors derived from cells expressing anti-miR-194-sponge grew more rapidly. By the end of the study, the tumors were larger (tumor volume) and heavier (tumor weight) than the GFP-control. In contrast, tumors derived from

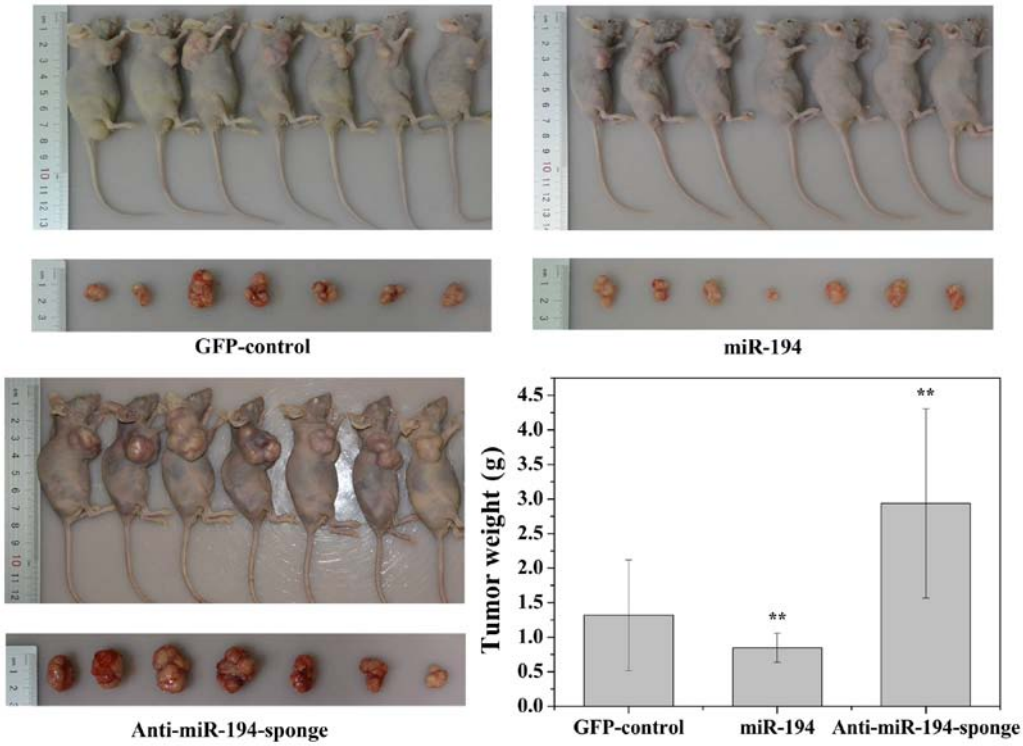

Figure 7. Overexpression of miR-194 suppresses xenograft tumor growth. DU-145 cells stably transfected with miR-194, anti-miR-194-sponge or the GFP-control plasmid were injected subcutaneously into nude mice. The tumors were grown to the indicated size and the mice were randomly divided into three groups (7 mice/group). The tumor size and body weight were measured on day-30. Data are represented as the mean ± SE of each group. $^{**}P<0.001$.

the cells with miR-194 overexpression appeared significantly smaller than the tumors formed from the cells transduced with the GFP-control.

Discussion

Little is known regarding the role of hNUDC in cancer. Previous results have demonstrated that hNUDC is ubiquitously expressed in normal human tissues. However, its protein levels are markedly overexpressed in almost all types of cancer cells, including cutaneous T-cell lymphoma and neuroectodermal tumors (29-33). Human NUDC has been identified in various molecular contexts accounting for its numerous aliases: a dynein-associated nuclear movement protein (27), chaperone protein (42), neuronal migration protein (43). Hence, hNUDC is a multifunctional protein and is involved in diverse biological events. Our laboratory has recently reported that hNUDC acts as a secondary ligand for thrombopoietin receptor (Mpl) involved in regulating proliferation and differentiation of different types of megakaryocyte cells (44-46). However, a complete understanding of all of the hNUDC functions remains unclear since this protein interacts with a number of other proteins with multi-functional effects. The present study is the second study concerning hNUDC in prostate cell lines. Our study focused on ectopic expression of miR-194 and its effects on prostate cancer by suppressing hNUDC expression. The preliminary data indicated that downregulation of hNUDC by miR-194 or si-hNUDC in DU-145 and PC-3 cells did not induce cell proliferation or cell cycle progression (data not shown) but rather resulted in morphological changes usually associated with an enhanced multinucleated phenotype. However, suppression of colony formation in the soft agar assay in the PC-3 and DU-145 cells suggests that hNUDC may play a role in the inhibition of tumor cell growth. We also observed that downregulation of endogenous hNUDC impaired prostate tumor cell migration and invasion with either miR-194 or siRNA strategies, although the mechanism by which this effect is mediated remains to be elucidated. To demonstrate that miR-194 contributes to PCa development *in vivo*, we employed a xenograft nude mouse model and found that primary tumor growth was reduced subcutaneously. Thus, it is possible that miR-194 may play a more general role in suppressing oncogenic processes in prostate cancer. This conclusion is further supported by the overexpression of miR-194 whereby it reduced protein bone morphogenetic protein 1 (BMP1) levels in PC-3 cells causing a significant decrease of invasion in Matrigel-coated Transwell chambers (47). During prostate cancer progression, most deaths from prostate cancer are not due to the primary tumor but rather to secondary metastases to distant organs. For this reason it is of fundamental importance to study the mechanisms that drive prostate cancer invasion and metastases. These data support the biological relevance of model systems implicating miR-194 and hNUDC expression as potential clinical markers.

Although it goes beyond the scope of this study, we initially tested five cell lines, RWPE-1 and WPMY-1 which are classically defined as non-tumoral, and the cancer cell lines LNCaP, DU-145 and PC-3. We found that RWPE-1, WPMY-1 and LNCaP inherently display a relative higher expression of

miR-194 compared to the DU-145 and PC-3 cells. However, the inverse relationship between the hNUDC and miR-194 expression levels was observed in all the normal and prostate tumor cell lines (data not shown). Both the DU-145 and PC-3 cell lines, derived from secondary metastatic prostate tumors, were used in our study as it remains possible that low expression of miR-194 is associated with acquisition of a more metastatic phenotype. We did not find any correlation between hNUDC expression and miR-194 in clinical parameters used to assess the poor prognosis of prostate cancer. In a future study, we will address this issue in more depth, since this issue remains to be investigated in a larger series of cases.

Recently, miR-194 expression profiles have been detected in a variety of tumor entities and it has been suggested that miRNAs are mostly downregulated in these cancer cells, although some are overexpressed, playing a critical role in tumor initiation and progression (16-19). One of the largest qRT-PCR analyses of tissue cohorts reported by Selth *et al*, indicated that miR-194 was robustly expressed in malignant prostate tissue and its expression in primary tumors was associated with a poor prognosis (48). One question that arises from this result is the mechanism that is involved in the increased upregulation in malignant prostate tissues compared to the matched poor prognosis tissues. As prostate tumors are very heterogeneous, the relative higher levels of expression of the miRNAs may be masked by the contribution of the stromal levels of miR-194 that may remain elevated. In addition, the miR-194 family consists of four members whose clusters are located on two separate chromosomes, and we believe that an explanation for this discrepancy is likely due to the fact that their spatial and temporal expression is tightly regulated by two genomic loci.

Although hNUDC is identified as a target of miR-194, it is a well accepted fact that an miRNA can target many genes. Database mining revealed that a single miR-194 may have multiple targets in tumorigenesis. One study implicated ectopic overexpression of miR-194 in the regulation of colon cancer angiogenesis *in vivo*, by suppressing its target, thrombospondin-1 (49). Moreover, upregulation of miR-194 induced downregulation of RBX1 showing significant inhibition of tumor size, invasion and tumor node metastasis (50). Similarly, BMI-1 knockdown inhibited cell proliferation and clone growth and BMI-1 was recently proposed as a biologically relevant miR-194 target in endometrial cancer cells (22). Furthermore, miR-194 was found to inhibit chondrogenic differentiation of human adipose-derived stem cells by targeting Sox5 and suppressed osteosarcoma cell proliferation and metastasis *in vitro* (51) and *in vivo* by targeting CDH2 and IGF1R (52). Therefore, we cannot exclude the possibility that these candidate targets for miR-194 other than hNUDC may be involved in tumor suppression. The activity of hNUDC as a cancer candidate target for miR-194 needs to be confirmed in future studies.

In conclusion, our study is the first to indicate that miR-194 interacts directly with hNUDC and regulates its expression and activity. More research is required to examine the effect of miR-194 on cellular growth, shape and function, especially in clinical cancerous cells, in order to determine its therapeutic potential in regulating aberrantly expressed hNUDC, especially in downregulated hNUDC-related tumors.

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