

Downregulation of Sec23A Protein by miRNA-375 in Prostate Carcinoma

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Abstract

Prostate carcinoma (CaP) is a leading cause of cancer-related death in men. We have previously determined the microRNA (miRNA) profile of primary CaP in comparison with nontumor prostate tissue. miRNAs are small, noncoding RNAs that inhibit protein synthesis on a posttranscriptional level by binding to the 3'-untranslated region (3'-UTR) of their target genes. In primary CaP tissue, we have previously found by miRNA sequencing that *miR-375* and *miR-200c* were upregulated 9.1- and 4.5-fold, respectively. A computational analysis predicted the 3'-UTR of the *SEC23A* gene as a potential target for both *miR-375* and *miR-200c*. Here, we show that the 3'-UTR of *SEC23A* mRNA is indeed a target for *miR-375* and *miR-200c* and that both miRNAs downregulate Sec23A protein expression when ectopically expressed in human 293T cells. In primary samples of CaP, we found a direct correlation between reduction of *SEC23A* mRNA and overexpression of *miR-375* but not of *miR-200c*. The reduced levels of Sec23A protein were inversely correlated to the increased amount of *miR-375* in the LNCaP and DU145 CaP cell lines when compared with normal prostate fibroblasts. In primary CaP, we also detected decreased amounts of Sec23A protein when compared with corresponding normal prostate tissue. Ectopically overexpressed Sec23A in LNCaP and DU145 CaP cells significantly reduced the growth properties, indicating that Sec23A might play a role in the induction or growth of prostate carcinoma. Sec23A overexpression reduced cell growth but did not induce apoptosis, whereas inhibition of Sec23A stimulated cell proliferation. *Mol Cancer Res*; 9(6); 791–800. ©2011 AACR.

Introduction

The underlying molecular defects leading to prostate carcinoma (CaP) are still poorly understood. The deregulation of microRNAs (miRNA) has recently been described as a mechanism contributing to the induction and growth of various tumors (for review, see ref. 1). miRNAs are short noncoding RNAs of about 19 to 25 nucleotides which preferentially bind to specific sequences

in the 3'-untranslated region (3'-UTR) of their target mRNAs. They may also bind to the 5'-UTR or the open reading frame (ORF) of their targets (2, 3). Interaction of miRNA and target mRNA results in either translational repression or mRNA degradation, ultimately leading to reduced protein synthesis (for review, see ref. 4). miRNA genes are transcribed and processed in the nucleus. They are then exported to the cytoplasm where they are further processed to form the mature miRNAs. Binding to their target mRNAs is accomplished via association with the argonaute proteins within the RNA-induced silencing complex (RISC). We have recently compared miRNA profiles of CaP and normal prostate tissue by a deep sequencing approach. We have found up- or downregulation of various miRNAs, in particular an induction of *miR-375* and *miR-200c* (5). A computational analysis predicted potential binding sites for both miRNAs in the 3'-UTR of the *SEC23A* gene. Sec23A is the human counterpart of the yeast Sec23p protein and is involved in the COPII-mediated transport of proteins from the endoplasmic reticulum (ER) to the Golgi apparatus (6). The basic components of the COPII vesicle comprise the proteins Sar1, Sec23/24, and Sec12/31. Two homologues of Sec23 exist in mammalian cells. Sec23A shares 85% identity with Sec23B. Both proteins display a homology of 48% to their yeast homologue (7). A mutation of the *SEC23A* gene causes cranio-lenticulo-sutural dysplasia because of a disturbance

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of ER to Golgi trafficking (8, 9). Sec23A plays a major role in assembly and transport of COPII vesicles (reviewed in ref. 10). Here, we show that *SEC23A* is indeed a target for *miR-375* and *miR-200c* in CaP cells. Furthermore, Sec23A protein and mRNA are both downregulated in the majority of primary tumors. Ectopic overexpression of Sec23A reduces growth properties of human CaP cell lines.

Materials and Methods

Cell lines, tissue culture, and antibodies

The human CaP cell lines DU145, LNCaP, and the human 293T were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). The PNF-08 cell line has been derived from normal prostate fibroblasts and was kindly provided by Prof. Gerhard Unteregger (Department of Urology, University of Saarland Medical School). Cells were cultured as described (5). Rat monoclonal antibodies (mAb) were generated in Lou/c rats by immunization with the Sec23A-derived peptide NH₂-GLSKVPLTQATRGPVQVQPPPSNRFL-C-CO₂ coupled via the C-terminal cysteine residue to OVA as described (11, 12). For ELISA, we used a bovine serum albumin-coupled peptide variant to prevent any reactivity against OVA. Peptide conjugates were obtained from PSL Systemtechnik. A clone designated 2H4 that reacted specifically with Sec23A was subcloned and used for further analysis.

Plasmids

The pSG5-*miR-200c* expression construct was generated by PCR amplification of nucleotides 6,942,994 to 6,943,333 of chromosome 12 using primers *miR-200c-EcoRI* 5'-CTC GAATTCACCAGG AAG TGT CCC CAG G-3' and *miR-200c-BglII* 5'-CCG AAT TCA GAT CTC ACC CCT TGT GCA ACG CTC-3' and inserting into the pSG5 expression plasmid (Stratagene). To express *hsa-miR-375*, which maps on position 219,691,875 to 219,691,896 of chromosome 2, the nucleotides 219,691,677 to 219,692,128 were PCR-amplified using primers *miR-375-EcoRI* 5'-CGG AAT TCG AGG GTG GCT GGG AAA GGA G-3' and *miR-375-BamHI* 5'-CGG GAT CCG AAT GGA GCC TGG CAG CCC A-3' to obtain pSG5-*miR-375*. The dual luciferase reporter plasmid pMIR-RL has been described elsewhere (13). The 3'-UTR of *SEC23A* (accession number: NM_006364.2) was cloned via PCR amplification, using primers *Sec-SpeI* 5'-GGA CTA GTC ACT CCA GAT TTC CAA TGC-3' and *Sec-SacI* 5'-CGA GCT CGT GGC ATT TAG GCC TAG CGT A-3' from testis cDNA and ligated into the *SpeI* and *SacI* restriction sites of pMIR-RL. Mutation of the predicted target site seed sequences of pMir-*SEC23A*-3'-UTR for *miR-200c* and *miR-375* was carried out by site-directed mutagenesis according to the QuickChange Site Directed Mutagenesis Kit Manual (Stratagene). The following primers were used for site-directed mutagenesis: *mir-200c-I_for_PmlI* 5'-GCA TTA TAT CCT TAC ACG TGT TAA CAC TAT TTT GGC AG-3', *200c-I_rev_PmlI* 5'-CTG CCA AAA TAG TGT TAA CAC GTG TAA GGA

TAT AAT GC-3', *200c-II_for_SalI* 5'-GTG GTT CTT ATT GAA CGG TCG ACA TAT AAT GTT TGC-3', *200c-II_rev_SalI* 5'-GCA AAC ATT ATA TGT CGA CCG TTC AAT AAG AAC CAC-3', *375_for_SalI* 5'-GAG AGC AAT AAC GTC GAC TTT ATT TTG CTT ACC-3', and *375_rev_SalI* 5'-GGT AAG CAA AAT AAA GTC GAC GTT ATT GCT CTC-3'. For expression of Sec23A, the ORF of *SEC23A* was PCR amplified using primers *SEC23A-XhoI* 5'-CCG CTC GAG CAA TGA CAA CCT ATT TGG-3' and *SEC23A-BamHI* 5'-CGG GAT CCT CAA GCA GCA CTG GAC ACA GC-3' and inserted into pCEP4 expression vector (Invitrogen). In addition, Sec23A and Sec23B expression constructs were generated, each containing a hemagglutinin (HA)-tagged variant. The ORFs of *SEC23A* and *SEC23B* were amplified using primers *SEC23A-XhoI* 5'-CCG CTC GAG CAA TGA CAA CCT ATT TGG-3', *SEC23A-BamHI* 5'-CGG GAT CCT CAA GCA GCA CTG GAC ACA GC-3', *SEC23B-EcoRI* 5'-CGG AAT TCG ATG GCG ACA TAC CTG GAG T-3', and *SEC23B-BamHI* 5'-CGG GAT CCA CAG GCA CTG GAG ACA GCC AG-3'. Amplified PCR products were cloned into a modified pSG5 expression vector, which already contained the HA-tag sequence at the 3'-end of the multiple cloning site.

Transfections, luciferase assays, and Western blotting

293T cells were cultivated in 24-well plates and were transfected with 0.2 µg of reporter construct and 0.8 µg of miRNA expression plasmid, using Nanofectin transfection reagent (PAA). Luciferase assays were conducted 48 hours after transfection by using the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega).

For Western blotting, approximately 10⁶ 293T cells grown in 10-cm dishes were transfected with 8 µg of plasmid DNA by using Nanofectin (PAA). A total of 2 × 10⁵ LNCaP and DU145 cells grown in 6-well plates were transfected with 2 µg of plasmid DNA by using jetPRIME (Pqlab). After 48 hours, cells were lysed with 2× lysis buffer (130 mmol/L Tris-HCl, 6% SDS, 10% 3-mercapto-1,2-propanediol, 10% glycerol, 0.05% bromophenol blue). Thirty micrograms of extracted proteins was separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Whatman) by electroblotting. Primary antibodies used were SEC23A-specific mAb 2H4 and rabbit mAb anti-GAPDH 14C10 (NEB-Cell Signaling). Anti-rat (A5795) and anti-mouse (A9044) secondary antibodies were purchased from Sigma.

Extracts from primary CaP tissue were generated using TRIzol (Invitrogen). Briefly, snap-frozen tissue was macro-dissected to ensure a tumor content above 70% in tumor samples and the absence of cancer cells in normal samples. Extraction of total RNA and protein was carried out according to the TRIzol manual. Twenty micrograms of protein extracts was denatured in sample buffer [62.5 mmol/L Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, 0.2 mmol/L EDTA, 100 mmol/L dithiothreitol, 0.05% bromophenol blue, 0.05% Pyronin Y]. Protein samples were

separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Whatman) by electroblotting. Membranes were incubated with primary antibodies 2H4 and 14C10 and appropriate secondary antibodies (Dianova). Bands were visualized by enhanced chemiluminescence (Roth) in an LAS-1000 chemiluminescence detection system (Raytest).

Quantitative real-time PCR analysis of miRNA

For miRNA analysis, 10 ng of total RNA were reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit with miRNA-specific RT primers contained in the TaqMan MicroRNA Assays (Applied Biosystems). Real-time PCR (RT-PCR) was carried out with the StepOnePlus Real-Time PCR System (Applied Biosystems) by using sequence-specific primers and fluorescence-labeled probes for *miR-375* and *miR-200c* (Applied Biosystems). PCRs were carried out in triplicates in a final volume of 10 μ L containing 1 $\times$ TaqMan Universal PCR Master Mix (No Amperase UNG), 1 $\times$ TaqMan miRNA assay, and miRNA-specific primed cDNA corresponding to an input amount of 330 pg of total RNA per RT-PCR. Thermal cycling conditions were the following: 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. To quantify the miRNA expression in tumor tissues, we used the relative quantification ($\Delta\Delta C_t$) method, with RNU6b serving as an internal control. The calculated relative expression values were normalized against RNA from the LNCaP cell line. All calculations were carried out with the StepOne software (V 2.0; Applied Biosystems).

Quantitative RT-PCR analysis of mRNA expression

cDNA synthesis was carried out with the TaqMan Reverse Transcription Reagents (Applied Biosystems) by using 200 ng of total RNA and random hexamer primers. The PCR primers for *SEC23A* (forward 5'-TGC GTT CCT CTG GGG TGG CA-3'; reverse 5'-CCA GGC CCC TGA GTA GCA GGA-3') and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; forward 5'-CAT GAG AAG TAT GAC AAC AGC CT-3'; reverse 5'-AGT CCT TCC ACG ATA CCA AAG T-3') were purchased from Biomers. PCR primers were designed to amplify a cDNA fragment of 100 to 120 bp and were located on adjacent exons of the target RNA. Quantification of mRNA expression was carried out using the $\Delta\Delta C_t$ method as described, using GAPDH as the internal control mRNA. RT-PCRs were carried out in triplicates with the StepOnePlus Real-Time PCR System (Applied Biosystems) in a final volume of 10 μ L containing 1 $\times$ TaqMan Fast SYBR Green Master Mix (Applied Biosystems), 250 nmol/L forward primer, 100 nmol/L reverse primer, and 5 ng of cDNA under the following conditions: 95°C for 5 minutes, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds.

Wound-healing assay

DU145 and LNCaP cells were seeded at a density of 3.5×10^5 /mL and 4.1×10^5 /mL, respectively, into sterile cell culture inserts (Ibidi). Twenty-four hours after seeding, the

cell culture insert was carefully removed, revealing a defined 500- μ m gap in the attached cells. For ectopic expression of Sec23A, cells were transfected with 2 μ g of Sec23A expression plasmid by using jetPRIME (Pqlab). For siRNA transfection, ON-TARGETplus SMARTpool siRNA human Sec23A and ON-TARGETplus Non-targeting Pool were purchased from Thermo Scientific. Cells were transfected at a final siRNA concentration of 10 nmol/L by using jetPRIME (Pqlab). Cell culture plates were transferred to a live cell imaging system (Zeiss) and observed over a time period of 30 hours. Every 60 minutes, a photograph was taken. For evaluation of growth capabilities, the area of the defined gap was measured at the indicated time points with ImageJ software (<http://rsbweb.nih.gov/ij/index.html>).

Apoptosis assay

Apoptosis was measured with the Caspase-Glo 3/7 Assay Kit (Promega). LNCaP and DU145 cells were seeded at a density of 2×10^4 cells in 24-well plates and were transfected with 1 μ g of pCEP4-SEC23A expression plasmid or empty vector as a control by using jetPRIME (Pqlab). Forty-eight hours after transfection, cells were lysed with 100 μ L Caspase-Glo Reagent in 100 μ L medium and caspase activity was measured using 50 μ L of cell lysate in a VICTOR X multilabel plate reader (PerkinElmer). Apoptosis assays were determined in duplicates.

Cell viability assay

The MTT cell viability assay is based on the cleavage of the tetrazolium salt MTT to form a blue formazan dye by viable cells and was carried out as follows. DU145 and LNCaP cells were seeded in a density of 6,000 cells per well in a 96-well plate for 24 hours and transfected for another 24 hours with 50 ng of Sec23A expression plasmid or siRNA at a final concentration of 10 nmol/L by using jetPRIME (Pqlab). MTT was added at a final concentration of 1 mg/mL, and cells were incubated another 4 hours at 37°C in an incubator. Two hundred microliters of dimethyl sulfoxide was added and after an additional incubation at room temperature the absorbance of the colored formazan was determined using a microplate reader operating at a wavelength of 570 nm (Spectramax 190; Molecular Devices). The mean absorbance of control wells (cells transfected with either the empty expression vector or the ON-TARGETplus Non-targeting Pool) represented 100% cell viability. Viability of cells transfected with the Sec23A expression plasmid or the ON-TARGETplus SMARTpool siRNA human Sec23A was determined in triplicate and related to the absorbance of control cells.

Cell proliferation assay

Cell proliferation was measured with the bromodeoxyuridine (BrdU) cell proliferation ELISA Kit (Roche Molecular Biochemicals) according to the manufacturer's instructions, using an automated microplate reader. Cells were transfected with 50 ng of Sec23A expression plasmid or siRNA at a final concentration of 10 nmol/L by using jetPRIME (Pqlab) for 24 hours in microtiter plates. The mean

absorbance of control cells represented 100% cell proliferation, and mean absorbance of treated cells was related to control values to determine sensitivity. Cell proliferation (% of control) was determined in triplicate.

Target prediction

miRNA target prediction was carried out using TargetScan (release 5.1; <http://www.targetscan.org/>)

Statistics

Western blots were quantified by Quantity One Analysis software (Bio-Rad) and Aida Image Analyzer software (Raytest). Statistical evaluation of the luciferase assays was done with SigmaPlot 10 (Systat) by *t* test. Statistical analyses of the quantitative RT-PCR (qRT-PCR; paired *t* test) and wound-healing assay were conducted using GraphPad Prism 4.0 (GraphPad Software). For wound-healing

assays, 2-way ANOVA with the Bonferroni posttest was conducted. For proliferation, apoptosis, and viability assays, unpaired Student's *t* test was carried out. All statistical tests were 2-sided, and *P* values less than 0.05 were considered as significant.

Results

The SEC23A 3'-UTR contains binding sites for miR-200c and miR-375

We had previously established the miRNA profile of primary CaP by deep sequencing and had found that *miR-375* and *miR-200c* were upregulated 9.1- and 4.5-fold in tumor tissue. These findings had been confirmed by Northern blotting for both miRNAs (5) and by qRT-PCR analyses, using a set of 26 corresponding pairs of tumor and nontumor prostate tissue, only for *miR-375* (Fig. 1A,

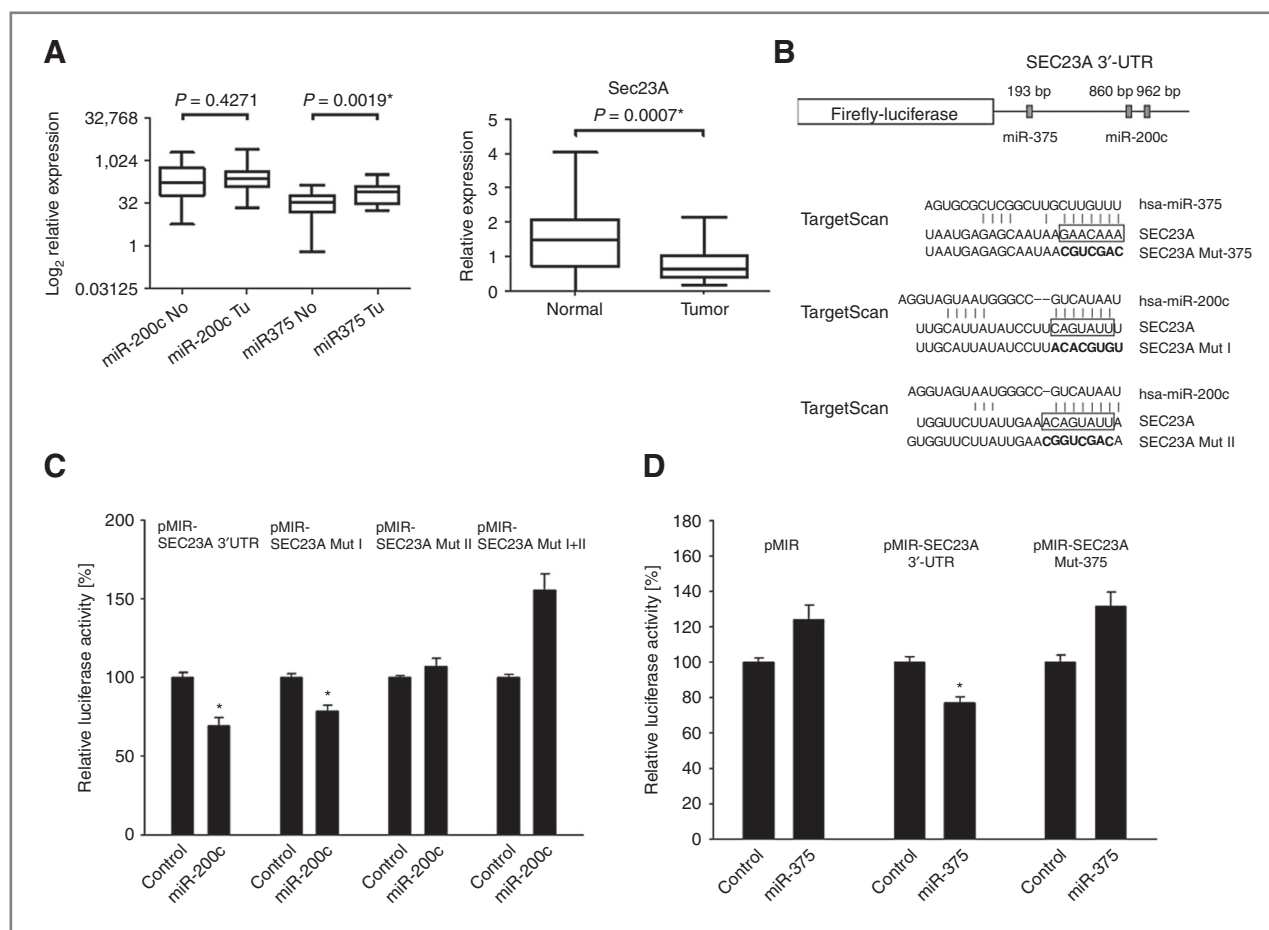


Figure 1. SEC23A mRNA is a target for miRNAs *miR-200c* and *miR-375*. A, expression of *miR-200c*, *miR-375*, and *SEC23A* mRNA in primary CaP tissue. In total RNA extracted from 26 pairs of primary CaP (Tu) and corresponding nontumor (No) prostate tissues, the relative expression of *miR-200c*, *miR-375* (left), and *SEC23A* (right) was determined by qRT-PCR. B, schematic of potential binding sites for *miR-200c* and *miR-375* in the *SEC23A* 3'-UTR. The predicted binding sites, the seed sequences, and the mutated seed sequences are shown. C, luciferase reporter gene activity with or without *miR-200c* expression. The unmodified *SEC23A* 3'-UTR reporter gene vector and vectors with mutated *miR-200c* binding sites were tested. D, luciferase reporter gene activity with or without *miR-375* expression. The unmodified *SEC23A* 3'-UTR reporter gene vector and vectors with mutated *miR-375* binding site were tested. All values represent the mean of 6 independent experiments carried out in duplicate.

left panel). As the mRNA of *SEC23A* has been predicted to be a target for both *miR-375* and *miR-200c* by a computational search, we therefore set out to determine the relative expression of *SEC23A* mRNA by using the identical set of tissue samples. *SEC23A* mRNA was significantly down-regulated in the tumor samples ($P = 0.0007$, Fig. 1A, right panel). This further supports the hypothesis of *SEC23A* being a regulative target of *miR-375* and *miR-200c*. In the *SEC23A* 3'-UTR, there are 2 potential binding sites for *miR-200c* and 1 for *miR-375*. Figure 1B shows a scheme of the *SEC23A* 3'-UTR and the location of the predicted binding sites. The *SEC23A* 3'-UTR was inserted into a luciferase reporter vector as described previously (5). This reporter gene construct was cotransfected with miRNA expression vectors into HEK293T cells. Both miRNAs significantly reduced the luciferase reporter gene activity ($P = 0.009$ for *miR-375*; $P = 0.0012$ for *miR-200c*; Supplementary Fig. S1). A reporter gene vector without the *SEC23A* 3'-UTR was irrespective toward a regulation by both miRNAs. We next removed the potential miRNA binding sites by site-directed mutagenesis. Only the mutation of *miR-200c* binding site II at position 952 ($P = 0.00005$), but not the mutation of *miR-200c* binding site at position 860 ($P = 0.21$), abolished a response to *miR-200c* (Fig. 1C). As expected, the combination of both mutations also led to a loss of responsiveness to *miR-200c*. This showed that only the predicted *miR-200c* binding site at position 952 is a target for *miR-200c*. The mutation of the single *miR-375* binding site also resulted in a loss of responsiveness to this miRNA (Fig. 1D). Taken together, these results show that the *SEC23A* 3'-UTR is an independent target for both *miR-200c* and *miR-375*.

***SEC23A* mRNA and protein are reduced in prostate cancer cell lines**

Because we showed that the miRNAs *miR-375* and *miR-200c* were upregulated in CaP and that *SEC23A* 3'-UTR was a regulative target for both miRNAs, we next analyzed the expression of Sec23A in CaP cell lines. Sec23A is highly related to Sec23B (7). Hence, we generated mAbs against a Sec23A-derived synthetic peptide divergent from Sec23B. Rats were immunized with an OVA-coupled peptide as previously described (11, 12). The HA-specific mAb 3F10 detected strong bands for both ectopically expressed Sec23A-HA and Sec23B-HA that were used for initial analysis. A mAb designated 2H4 detected a band of endogenous Sec23A in mock-transfected and Sec23B-HA-transfected cells, but the signal intensity was increased in Sec23A-HA-transfected cells (Supplementary Fig. S2). The mAb 2H4 was then stably subcloned and used to determine the protein levels of Sec23A in normal prostate fibroblasts (PNF-08) and the CaP cell lines LNCaP and DU145. In the tumor cell lines, the Sec23A levels were reduced by about 60% in LNCaP and about 80% in DU145 (Fig. 2A).

To assess the regulative capabilities of miRNAs toward endogenous proteins, we first transfected HEK293T cells with miRNA expression plasmids. Ectopic expression of

both miRNAs led to a significant reduction in Sec23A protein levels by 30% ($P = 0.005$) for *miR-200c* and by 42% ($P = 0.014$) for *miR-375* (Fig. 2B). Furthermore, overexpression of *miR-200c* and *miR-375* in LNCaP and DU145 CaP cells significantly reduced Sec23A levels in both cell lines. For LNCaP, *miR-200c* and *miR-375* yielded a 40% ($P = 0.0053$) and 50% ($P = 0.004$) reduction, respectively (Fig. 2C), and both miRNAs reduced Sec23A levels by about 50% ($P = 0.018$ for *miR-200c*; $P = 0.002$ for *miR-375*) in DU145 cells (Fig. 2D). Finally, we compared the levels of Sec23A mRNA and the 2 miRNAs and found that they were inversely correlated in CaP cells as compared with PNF-08. DU145 and LNCaP both showed a downregulation of *SEC23A* mRNA but an upregulation of *miR-375* and *miR-200c* when compared with PNF-08 normal prostate fibroblasts (Fig. 2E). Taken together, our data establish *SEC23A* as a target for both *miR-200c* and *miR-375* *in vitro*.

Sec23A protein and mRNA levels are reduced in primary CaP tissue

Nineteen pairs of primary CaP and corresponding non-tumor prostate tissue were analyzed for the expression of Sec23A by Western blotting. Protein extracts from snap-frozen, untreated tissues were assayed using 2H4 and antibodies against GAPDH. The 2H4 antibody detected a single band migrating at the expected molecular mass of about 75 kDa. A representative blot is shown in Figure 3, and a complete set of Western blots is shown in Supplementary Figure S3. After chemiluminescence detection, band intensities were quantified and normalized. GAPDH band intensities were used for normalizing signal intensities, and the band intensity derived from LNCaP protein extracts was set to 1. In 11 of 19 cases (57.9%), Sec23A expression was reduced in the tumor sample; in 2 of 19 (10.5%), no change was observed; and in 6 of 19 (31.6%), the Sec23A expression was elevated in the tumor sample. Overall, we observed a significant lower expression of Sec23A in CaP ($P = 0.038$).

Alteration of Sec23A expression has an impact on growth properties of DU145 and LNCaP cells

Because the majority of primary tumor tissue samples and also established prostate cancer cell lines displayed a reduction in Sec23A, we were interested in the functional consequences of Sec23A reduction. The CaP cell lines DU145 and LNCaP were transfected either with Sec23A expression constructs or siRNA molecules targeting *SEC23A*. To examine the growth properties of transfected cells, we established a long-term wound-healing assay. Cells were grown in tissue culture plates in a manner that they generated a defined 500- μ m linear gap. Cell growth in this defined wound was observed over a period of 30 hours and analyzed. Ectopic expression of Sec23A significantly reduced the growth properties of DU145 cells (Fig. 4A and B). Conversely, transient knockdown of Sec23A led to an increased growth potential over a time period of 30 hours (Fig. 4C and D). The ectopic overexpression of Sec23A by using pCEP4-Sec23A and the

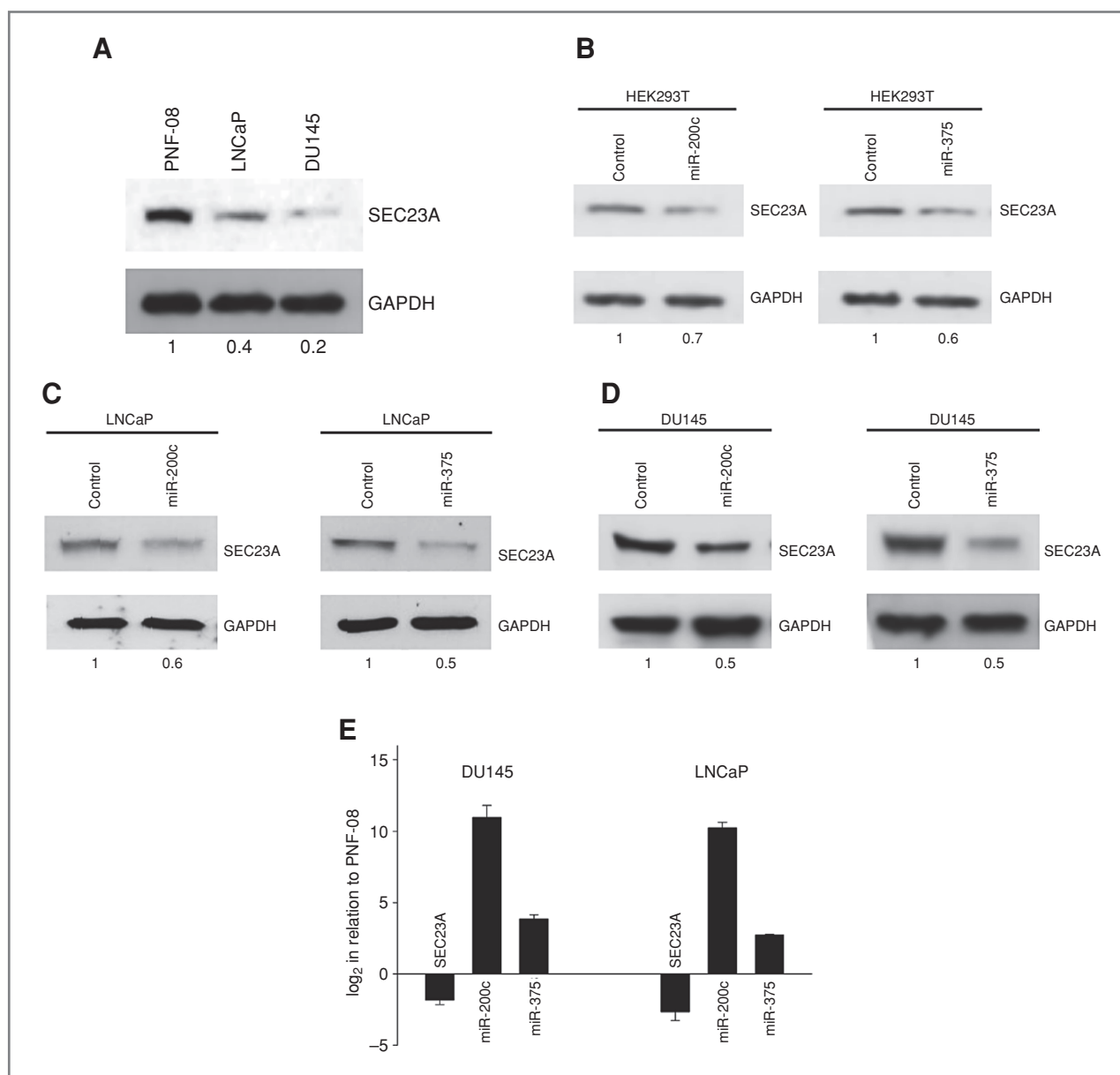


Figure 2. Expression of Sec23A in CaP cell lines. A, normal prostate fibroblast cell line PNF-08 and the CaP lines LNCaP and DU145 were analyzed for Sec23A expression by Western blotting. GAPDH served as a loading control to normalize protein signal intensity. The Sec23A signal obtained for PNF-08 was set to 1 and Sec23A expression was calculated relative to PNF-08 (shown below the lanes). B, inhibition of Sec23A expression by *miR-200c* and *miR-375*. 293T cells were transfected with miRNA expression vectors or empty control vectors as indicated above each lane. The relative downregulation was determined using the GAPDH signal as an internal standard. LNCaP (C) and DU145 (D) cells were transfected with miRNA expression vectors or empty control vectors as indicated above each lane. The relative downregulation was determined using the GAPDH signal as an internal standard. E, expression levels of *SEC23A* mRNA, *miR-200c*, and *miR-375* relative to PNF-08 cells. Total mRNA was extracted and the relative levels of *SEC23A* mRNA, *miR-200c*, and *miR-375* were determined by qRT-PCR. Levels were quantified relative to the amounts observed in PNF-08 cells.

downregulation by using siRNA were determined by qRT-PCR. After transient expression, we observed a 1×10^7 -fold increase in Sec23A mRNA, whereas knockdown with siRNA resulted in a 6.8-fold reduction in Sec23A mRNA. This is shown in Supplementary Figure S4.

In LNCaP cells, we also observed a retardation of cell growth in the wound-healing assay after ectopic

overexpression of Sec23A (Supplementary Fig. S5A) whereas siRNA-mediated knockdown of Sec23A had the opposite effect (Supplementary Fig. S5C). The graphic depiction of the results is shown in Supplementary Figure S5B and D. Here, we observed the same tendency as in DU145; however, the values did not reach a significant level.

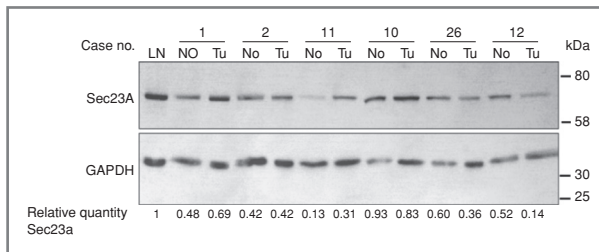


Figure 3. Expression of Sec23A in primary prostate tissue. Protein extracts from snap-frozen primary prostate cancer (Tu) and corresponding nontumor (No) tissue were analyzed for the relative Sec23A expression by Western blotting. Signal intensities of Sec23A were normalized according to the GAPDH signal. Protein extract from the LNCaP cell line (LN) served as a reference, and Sec23A signal intensities were calculated according to this reference.

Overexpression of Sec23A influences proliferation but does not cause apoptosis in CaP cells

To study the observed effects in more detail, we determined the influence of overexpression or siRNA-mediated reduction of Sec23A levels on cell viability, proliferation, and on apoptosis of the LNCaP and DU145 CaP cells. When we assayed the cell viability, we found that both overexpression or knockdown of Sec23A affected cell viability of DU145 cells whereas only knockdown of Sec23A had a significant effect on viability of LNCaP cells (Supplementary Fig. S6). We then tested cell proliferation by measuring BrdU uptake and found that inhibition of Sec23A by siRNA augmented cell growth in DU145 cells whereas overexpression reduced cell growth (Fig. 5A, left panel). In LNCaP cells, knockdown of Sec23A had no significant effect on proliferation whereas overexpression of Sec23A significantly reduced proliferation as in DU145 cells (Fig. 5A, right panel). Finally, we assayed whether the ectopic expression of Sec23A reduced growth of the CaP cells via increased apoptosis. As shown in Figure 5B, the overexpression of Sec23A did not induce apoptosis as determined by the relative levels of caspase 3 and 7 activities in those cells. As a positive control, ectopic expression of p53 in both cell lines readily induced apoptosis (not shown).

Discussion

Our data establish *SEC23A* as a novel target for *miR-375* and *miR-200c*, which are deregulated in prostate cancer. *miR-375* was found to be upregulated about 9-fold by deep sequencing and likewise *miR-200c* was upregulated 4.5-fold (5). Although this deregulation could be confirmed for both miRNAs by Northern blotting, qRT-PCR analyses exhibited only *miR-375* to be deregulated. In a follow-up study, we could show by microarray analysis that *miR-200c* and *miR-375* were upregulated in archival tissue. This upregulation could be further confirmed by qRT-PCR (14). The 3'-UTR of *SEC23A* contains predicted miRNA binding sites for both *miR-375* and *miR-200c*. We therefore tested the regulative

effects of both miRNAs on the expression of Sec23A. Both miRNAs could repress the activity of reporter genes and reduce Sec23A expression.

MiR-200c is deregulated in a variety of tumor entities (15). In some tumors, up- or downregulation of *miR-200c* was correlated with poor outcome. For instance, downregulation of *miR-200c* was correlated with relapse in stage I epithelial ovarian cancers (16) and other tumors of the female reproductive organs (17). Likewise, a reduction of this miRNA correlated with a more aggressive behavior of non-small-cell lung cancer (18) whereas lung mesotheliomas or pancreatic carcinomas in contrast exhibit increased levels of *miR-200c* (19, 20). *MiR-200c* and the transcription factor ZEB1 form a feedback loop (21). A reduced expression of *miR-200c* induces ZEB1, which, in turn, represses E-cadherin (22) and induces epithelial-to-mesenchymal transition (EMT). EMT results in invasion and metastasis of tumor cells (21). It has been recently shown that elevated levels of *miR-200c* initially induce proliferation of tumor cells in pancreatic cancer (23). Then, at later stages of tumor development, methylation of the *miR-200c* promoter, as described for breast carcinoma (24), might silence *miR-200c* to enhance metastasis of the tumor cells (19).

We discovered an overexpression of *miR-375* in CaP whereas this miRNA was downregulated in gastric carcinoma (25, 26), head and neck squamous cell carcinoma (27), esophageal carcinoma (28), and hepatocellular carcinoma (29). However, poor outcome and relapse were associated with elevated levels of *miR-375* in gastric carcinoma (30). Likewise, *miR-375* is overexpressed in breast carcinoma, where it induces cell proliferation via a positive feedback loop with the estrogen receptor (31). As the expression of the estrogen receptor is increased during CaP development, it is possible that overexpression of *miR-375* enhances the progression of this tumor. These reports showed that miRNAs are not only reproducibly deregulated in a variety of human malignancies but may also hold a prognostic value in several tumors. For instance, *miR-375* could distinguish between tissue samples of prostate cancer and nonmalignant tissue with an accuracy of almost 67% and when combined with additional miRNAs, the rate of correct classification was improved to almost 78% (14). Because it is also possible to assess miRNA expression patterns in urine (32) or blood serum (33), these miRNAs have the potential of being valuable biomarkers for the diagnosis and prognosis of prostate cancer.

A recent report described that Sec23A may play a role in the induction of prostate cancer, as Sec23A was found to interact with N-myc downstream-regulated gene 1 (*NDRG1*; ref. 34). *NDRG1* is overexpressed in tumors of the liver, kidney, or cervix but downregulated in cancers of the colon, prostate, breast, or the nervous system (reviewed in ref. 35). A concomitant downregulation of Sec23A might have an additional impact on prostate carcinogenesis. Sec23A is reduced in CaP at the mRNA and protein levels. Ectopic expression of Sec23A reduces the growth properties of CaP

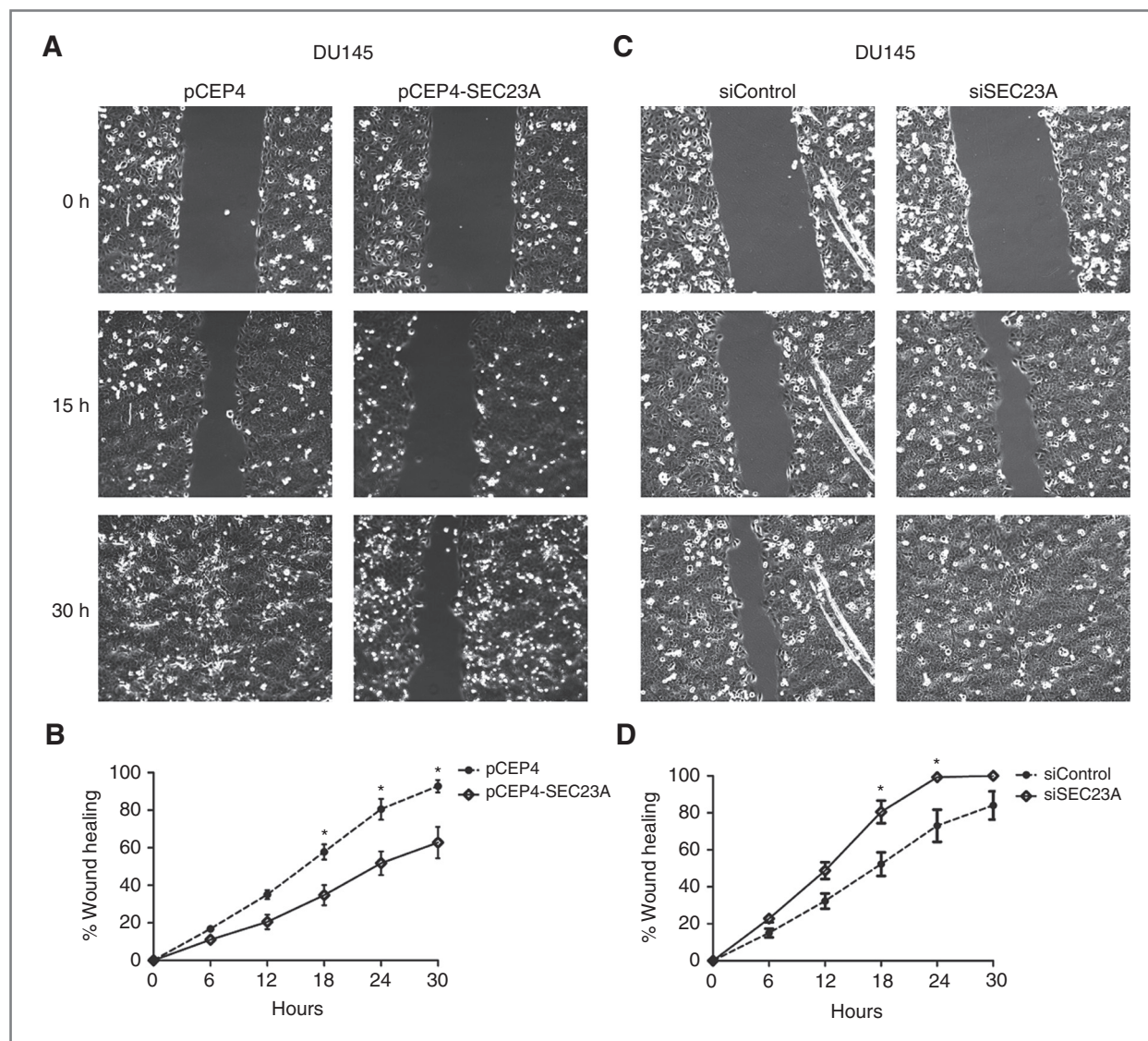


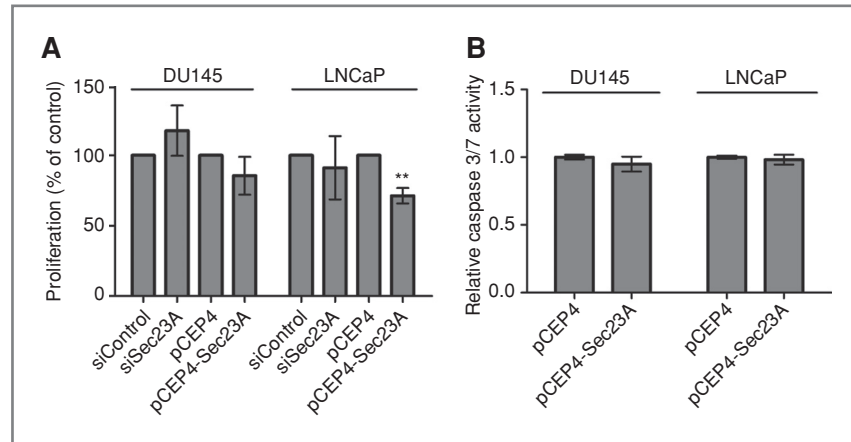
Figure 4. Wound-healing analysis. A and B, DU145 cells were transiently transfected with either control vector (pCEP4) or pCEP4 expressing Sec23A. Representative micrographs of one of the individual experiments at time points 0, 15, and 30 hours after transfection are shown. Wound-healing behavior was observed over a time period of 30 hours. Asterisks indicate significantly different wound-healing behaviors of DU145 overexpressing Sec23A compared with DU145 transfected with the control vector at the indicated time points; *, $P < 0.05$. C and D, DU145 cells were transiently transfected with either a control siRNA (siControl) or a siRNA targeting *SEC23A* (siSEC23A). Wound-healing behavior was observed over a time period of 30 hours. Asterisks indicate significantly different wound-healing behaviors of DU145 transfected with a siRNA targeting Sec23A compared with DU145 transfected with the control siRNA at the indicated time points; *, $P < 0.05$.

cell lines *in vitro* and, conversely, inhibition of the *SEC23A* gene by siRNA enhanced the growth properties of CaP cell lines. These observations suggest an inhibitory role of Sec23A in cell growth. It would be interesting to test whether Sec23A is also reduced in other tumor entities, especially those in which *miR-375* is reduced rather than elevated.

Sec23A plays a vital role in the COPII-mediated protein transport from the ER to the Golgi apparatus (36). It was found that mutations in Sec23A might cause severe craniofacial defects because of a disturbance in ER to Golgi trafficking

(8, 9). Furthermore, it has been shown that MHC-I molecules interact with the Sec23–Sec24 complex for receptor-mediated ER to Golgi trafficking (37). Several publications described a reduced expression of MHC-I in primary CaP samples (38) or a concomitant reduction in MHC-I and several components of the antigen-processing machinery in primary CaP samples and CaP cell lines (39). Despite the impact of Sec23A on growth properties of CaP cell lines, a miRNA-mediated reduction in Sec23A might contribute to a reduced immunogenicity of CaP by reducing MHC-I cell surface expression.

Figure 5. Cell proliferation and apoptosis assays. A, DU145 and LNCaP cells were transfected with control siRNA (siControl), siRNA against Sec23A (siSec23A), empty pCEP4 vector, or pCEP4-Sec23A expression vector. Cell proliferation was measured as described in Materials and Methods. B, apoptosis assay. DU145 and LNCaP cells were transfected either with empty pCEP4 vector or pCEP4-Sec23A expression vector. Activities of caspase 3/7 were measured 48 hours after transfection. Data represent 6 independent experiments, each carried out in duplicate and expressed as mean $\pm$ SEM.



Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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