Abstract

Prostate cancer is a leading cause of tumor mortality. To characterize the underlying molecular mechanisms, we have compared the microRNA (miRNA) profile of primary prostate cancers and noncancer prostate tissues using deep sequencing. MiRNAs are small noncoding RNAs of 21 to 25 nucleotides that regulate gene expression through the inhibition of protein synthesis. We find that 33 miRNAs were upregulated or downregulated >1.5-fold. The deregulation of selected miRNAs was confirmed by both Northern blotting and quantitative reverse transcription-PCR in established prostate cancer cell lines and clinical tissue samples. A computational search indicated the 3′-untranslated region (UTR) of the miRNA for myosin VI (MYO6) as a potential target for both miR-143 and miR-145, the expression of which was reduced in the tumor tissues. Upregulation of myosin VI in prostate cancer was previously shown by immunohistochemistry. The level of MYO6 mRNA was significantly induced in all primary tumor tissues compared with the nontumor tissue from the same patient. This finding was matched to the upregulation of myosin VI in established prostate cancer cell lines. In luciferase reporter analysis, we find a significant negative regulatory effect on the MYO6 3′UTR by both miR-143 and miR-145. Mutation of the potential binding sites for miR-143 and miR-145 in the MYO6 3′UTR resulted in a loss of responsiveness to the corresponding miRNA. Our data indicate that miR-143 and miR-145 are involved in the regulation of MYO6 expression and possibly in the development of prostate cancer.

Introduction

MicroRNAs (miRNAs) are small, noncoding RNAs of about 21 to 25 nucleotides in length that usually bind to partially complementary sites in the 3′-untranslated region (UTR) of their mRNA targets (1, 2). By this, they either repress translation or induce the sequence-specific degradation of their targets (3-5). MiRNA-encoding genes are transcribed in the nucleus by RNA polymerases II and III and are then processed to mature miRNA in the cytoplasm by the RNase III Dicer (6). Some but not all miRNAs with complete complementarity to their mRNAs use the RNase eukaryotic translation initiation factor 2C2 (eIF2C2, AGO2) to direct the cleavage of their targets (7, 8). Presumably, miRNAs regulate multiple targets and are involved in a variety of cellular processes such as cell differentiation, cell cycle control, cell growth, and immune response (for review, see ref. 9).

Recently, the potential role of miRNAs in human oncogenesis has been indicated by several studies, all of them confirming the widespread deregulation of miRNAs in cancer tissue. Regarding prostate cancer, the most frequently diagnosed malignant tumor in American men, several miRNA expression profiles have been reported (10-15). Although all studies agree in the detection of aberrantly expressed miRNAs in prostate cancer cell lines, xenografts, and primary prostate cancer samples, the findings are yet inconsistent potentially due to differences in sample acquisition or microarray platforms.

Here, we compare the miRNA profiles of normal versus neoplastic prostate tissues by deep sequencing. We find that 16 miRNAs are upregulated and 17 miRNAs are downregulated >1.5-fold in prostate cancer tissues. Furthermore, we show that myosin VI (MYO6) is a target for both miR-143 and miR-145.

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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Materials and Methods

Clinical Samples
This study was approved by the local ethical review board and was performed according to the Declaration of Helsinki. After informed consent, prostate cancer tissues were obtained after radical prostatectomy from thus far nontreated prostate cancer patients. Following prostatectomy, the specimens were dissected by a uropathologist, snap frozen within 1 h of surgical excision, and stored at −80°C until extraction of RNA. Only samples containing >70% tumor cells were included in the study. Gleason score, pathologic stage, histologic diagnosis, and tumor-node-metastasis classification was done according to the guidelines of the Union International Contre Cancer 2002. None of the patients had detectable distant metastases at the time of surgery. As a control, RNA was prepared from prostate tissue samples obtained from patients undergoing radical cystectomy for bladder cancer. In each case, the absence of cancer cells in the prostate tissue was verified. To identify miRNA transcripts that are differentially expressed in tumor specimens versus nonmalignant prostate tissue, we pooled tumor and normal prostate samples in four subsets. Pool 1 and 2 contained five normal prostate tissue samples each, whereas pool 3 and pool 4 were generated from five prostate carcinomas each with a Gleason score of 6 or 7. Patients’ clinicopathologic characteristics are summarized in Supplementary Table S1.

For the validation of sequencing results, a second series of tumor and normal RNAs both prepared from the same prostate glands was examined by real-time quantitative reverse transcription-PCR (qRT-PCR). A total of 26 cryoconserved tumor tissue samples were available for miRNA and mRNA expression analysis. Median age at the time of diagnosis was 67.5 y (46–75 y). Gleason scores of these tumors ranged from 5 to 9. Detailed patients’ clinicopathologic characteristics are summarized in Supplementary Table S1.

Cell Culture
Normal human prostate fibroblast cell line PNF08 was generated from nonmalignant prostate tissue and was cultivated in DMEM (Invitrogen) supplemented with 10% heat-inactivated FCS, 1-glutamine (1 mmol/L final concentration), Penicillin (100 U/mL), Streptomycin (100 μg/mL; all purchased from PAN Biotech), and 1% nonessential amino acids (Sigma). All other cell lines were purchased from the German collection of microorganisms and cell cultures (DSMZ). DU145 and the subline DU145-MN1 were maintained in DMEM (Invitrogen) supplemented with 10% FCS (Sigma). DU 145-MN1 was generated from a s.c. reimplanted lymph node metastasis after orthotopic implantation of DU145 (16) LNCaP was cultured in RPMI 1640 without phenol red and supplemented with 10% FCS (PAN biotech). 293T cells were cultivated in DMEM (Invitrogen) supplemented with 10% FCS (Sigma).

Small RNA Cloning
The small RNA fraction of each of the 10 prostate cancer and normal prostate tissues was isolated and four libraries each containing a pool of five cancer or normal tissue samples were generated. Small RNA cloning and sequencing was carried out by an external company (Vertis Biotechnologie AG) as described (17). Briefly, total RNA was isolated from the tissue samples using standard methods and separated by PAGE. The region of the gel containing RNA molecules of 15 to 30 nucleotides was excised. RNA was eluted and further used for the construction of a cDNA library. Libraries containing cDNA of five pooled tumor or normal tissue samples were subsequently sequenced by the 454-pyrosequencing method on a Roche/454 GS FLX sequencing system.

Northern Blotting
Northern blotting analysis was carried out as described (18, 19). The membranes were analyzes with a PhosphorImager (Amersham-Biosciences). For reprobing, the nylon membranes were stripped with 5 mmol/L Tris (pH 8), 0.2 mmol/L EDTA, 0.05% NaPP, and 0.1% Denhardt’s solution for 2 h at 80°C. The antisense probes used were as follows: mirR-375, 5′-tcacgagcggagaagaaacaa-3′; mirR-200c, 5′-uccauaucuaccaggauaaua-3′; mirR-143, 5′-gagcuacagugcuuacucua-3′; and mirR-145, 5′-aggauuuucuggaaucaac-3′.

Plasmids
Hsa-mir-143 was PCR amplified using primers mirR-143-EcoRI 5′-CGGATTCCTGCTCAATGTTGAGCAGCAAGCACACCG-3′ and mirR-143-BamH1 5′-CGGATTCCTGCTGAAAGGATCTGGTGCACCA-3′ (MWG-Eurofin) and inserted into pSG5 (Stratagene) to yield pSG5-mir143. Similarly, the pSG5-miR-145 expression construct was generated using primers mirR-145-EcoRI 5′-GGAATTCCGAGGCCTAAAGGATCTGGTGCACCA-3′ and mirR-145-BamH1 5′-CGGATTCCTGCTGAAAGGATCTGGTGCACCA-3′. The pSG5-miR-23a/27a expression construct was generated with primers EcoRI-mir23a 5′-CGGATTCCTGCTGAAAGGATCTGGTGCACCA-3′ and miR-23a/miR-27a 5′-GGAATTCCGAGGCCTAAAGGATCTGGTGCACCA-3′. The pSG5-miR-23a/27a expression construct was generated using primers EcoRI-mir23a 5′-CGGATTCCTGCTGAAAGGATCTGGTGCACCA-3′ and miR-23a/miR-27a 5′-GGAATTCCGAGGCCTAAAGGATCTGGTGCACCA-3′.

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(Stratagene) using the following primers: miR-145-1_for_EcoRV 5′GCACAGTACCATGCGATATCTC-TAATAAAAATGAGC-3′, miR-145-1_rev_EcoRV 5′GCTCATGTTTTATTAGAGATATCGCATGG-TACTCGTG-3′, miR-145-2_for_SacII 5′-GGCA-TAGTGGCTAGCCGCGGAGGAATTCAAATATTC-3′, miR-145-2_rev_SacII 5′-GAATATTTGAATTC-CTCCGGGCTAGCCTACTATGCC-3′, miR-143_for_EcoRV 5′-CAAATATTCTTTCAATCGA-TATCGTATAGTGATTTTTG-3′, and miR-143_rev_EcoRV 5′-CAAAAATCACTATACGATATCGATT-GAAAGAATATTTG-3′.

Transfections and Luciferase Assays

293T cells were cultivated in 24-well tissue culture plates and were transfected with 0.2 μg pEGFP (Clontech), 0.2 μg reporter construct, and 0.8 μg miRNA expression plasmid using Nanofectin transfection reagent (PAA). Transfection efficiency was determined by fluorescence-activated cell sorting analysis and luciferase reporter assays were done 48 h after transfection as described (17, 19).

Western Blotting

LNCaP cells grown in six-well tissue culture plates were transfected with 4 μg of miRNA expression vector using Lipofectamine 2000 (Invitrogen). After 48 h, cells were lysed with 2× lysis buffer (130 mmol/L Tris-HCl, 6% SDS, 10% 3-Mercapto-1,2-propandiol, 10% glycerol, and 0.05% Bromophenol blue). Thirty micrograms of extracted proteins were separated by 6% SDS-PAGE and transferred to a nitrocellulose membrane (Whatman, GE health care) by electroblotting. The primary antibodies used were anti-myosin VI (KA-15) and anti–β-actin (both from Sigma), and anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling).

Statistical Analyses

Northern blot quantification was done with the ImageQuant 5.1 software (Molecular Dynamics, GE Health Care) and Western blots were quantified by Quantity One analysis software (Bio-Rad). Statistical evaluation of the luciferase assays was done with SigmaPlot 10 (Systat). Statistical analyses of the real-time quantitative RT-PCR (qRT-PCR) were done using GraphPad Prism 4.0 (GraphPad Software).

Results

MiRNA Gene Expression in Prostate Cancer

Two pooled miRNA cDNA libraries of each normal prostate and primary prostate cancer samples were generated with each library containing five pooled samples from normal and tumor tissue, respectively. Analysis was done as described (17). The comparison of the two libraries from normal prostate tissue showed a high degree of homology in the miRNA expression pattern, whereas the two tumor libraries exhibited a slight variation in the miRNA profile (Supplementary Table S3-5). Nevertheless, the 10 most prevalent miRNAs were the same in the tumor-derived libraries.

We then compared the combined tumor versus normal tissue libraries and found 16 miRNAs that were at least 1.5-fold upregulated and 17 miRNAs that were at least 1.5-fold downregulated in Table 1.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Rel. expression (%)</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upregulated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>has-miR-375</td>
<td>1.35</td>
<td>9.12</td>
</tr>
<tr>
<td>has-miR-148a</td>
<td>0.74</td>
<td>4.93</td>
</tr>
<tr>
<td>has-miR-200c</td>
<td>9.93</td>
<td>4.54</td>
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<td>has-miR-106a</td>
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</tr>
<tr>
<td>hsa-let-7c</td>
<td>0.49</td>
<td>2.74</td>
</tr>
<tr>
<td>hsa-let-7f</td>
<td>1.61</td>
<td>2.71</td>
</tr>
<tr>
<td>has-miR-106b</td>
<td>0.57</td>
<td>2.33</td>
</tr>
<tr>
<td>has-miR-126*</td>
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<td>1.87</td>
</tr>
<tr>
<td>has-miR-30b</td>
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</tr>
<tr>
<td>has-miR-25</td>
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<td>has-miR-21</td>
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<tr>
<td>has-miR-26b</td>
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<td>has-miR-20a</td>
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<tr>
<td>has-miR-15b</td>
<td>1.59</td>
<td>1.51</td>
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<table>
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<tr>
<th>Downregulated</th>
<th>Rel. expression (%)</th>
<th>Fold change</th>
</tr>
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<tbody>
<tr>
<td>has-miR-143</td>
<td>0.63</td>
<td>0.24</td>
</tr>
<tr>
<td>has-miR-145</td>
<td>1.06</td>
<td>0.26</td>
</tr>
<tr>
<td>hsa-miR-223</td>
<td>0.11</td>
<td>0.33</td>
</tr>
<tr>
<td>has-miR-30e</td>
<td>0.15</td>
<td>0.41</td>
</tr>
<tr>
<td>has-miR-29a</td>
<td>0.57</td>
<td>0.42</td>
</tr>
<tr>
<td>hsa-miR-27b</td>
<td>4.16</td>
<td>0.46</td>
</tr>
<tr>
<td>has-miR-152</td>
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<td>has-miR-125a-5p</td>
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<td>has-miR-221</td>
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<td>has-miR-199a-5p</td>
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<td>has-miR-101</td>
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</tr>
<tr>
<td>has-miR-22</td>
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<td>has-miR-424</td>
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<td>0.58</td>
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<tr>
<td>has-miR-27a</td>
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<td>0.58</td>
</tr>
<tr>
<td>has-miR-320</td>
<td>0.29</td>
<td>0.63</td>
</tr>
<tr>
<td>has-miR-24</td>
<td>1.76</td>
<td>0.65</td>
</tr>
<tr>
<td>has-miR-125b</td>
<td>1.10</td>
<td>0.65</td>
</tr>
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</table>
the tumor tissue. Only miRNAs with a representation of at least 0.1% of the total miRNA reads were included. The results are summarized in Table 1. The strongest upregulation was \( \sim 9 \)-fold for \( \text{miR}-375 \), 5-fold for \( \text{miR}-148a \), and 4.5-fold for \( \text{miR}-200c \). Of note, \( \text{miR}-200c \) represented \( \sim 10\% \) of all miRNAs present in the tumor tissue. Conversely, \( \text{miR}-143 \) and \( \text{miR}-145 \) were downregulated \( \sim 4 \)-fold and \( \text{miR}-223 \) showed a 3-fold reduction. To confirm the sequencing data, total RNA from matched pairs of normal and tumor prostate tissues from the same patients were prepared and analyzed by Northern blotting. Representative Northern blots are depicted in Fig. 1. As shown in Fig. 1A, we could confirm the upregulation of \( \text{miR}-375 \) in 6 of 9 (66.7%) cases analyzed, whereas the upregulation of \( \text{miR}-200c \) was found in 10 of 14 (71.4%) cases (Fig. 1B, bottom). The quantitative analysis of the Northern blots using a PhosphoImager is shown for \( \text{miR}-375 \) in Supplementary Fig. S1A and for \( \text{miR}-200c \) in Supplementary Fig. S1B. The 26 pairs of tumor and normal prostate tissue samples were further analyzed by real-time qRT-PCR for the relative levels of these miRNAs and we could also confirm the upregulation of \( \text{miR}-375 \) in 21 (81%) cases and of \( \text{miR}-200c \) in 17 (65%) cases. The results of all qRT-PCR analyses are given in Supplementary Table S2. The comparative analysis of the qRT-PCR results for \( \text{miR}-375 \) and \( \text{miR}-200c \) is shown in Fig. 1C and D, respectively. We found a significant upregulation of \( \text{miR}-375 \) in the tumors tested \((P = 0.0019, \text{paired Student’s } t \text{ test})\). As \( \text{miR}-200c \) and \( \text{mir}-141 \) are located on the same transcript and are repressed by zinc finger E-box binding homeobox 1 (20), we next assayed the levels of \( \text{miR}-141 \) in the primary tissues and also found an upregulation of this miRNA in the tumor tissues (see Supplementary Information; Fig. 2). The expression levels of \( \text{miR}-200c \) and \( \text{mir}-141 \) were significantly correlated \((r = 0.436; 95\% \text{ confidence interval}, 0.05848-0.7045; P = 0.026, \text{Pearson correlation})\). Nevertheless, no statistically significant upregulation in the Cancer samples could be observed for \( \text{miR}-200c \) when regarding the whole series. This may be due to the fact that the subgroup of prostate cancers exhibiting \( \text{miR}-200c \) upregulation is too small.

We next assayed the expression of \( \text{miR}-143 \) and \( \text{miR}-145 \), the two miRNAs most strongly downregulated in tumor tissue in a similar way as described above for \( \text{miR}-375 \) and \( \text{miR}-200c \). The corresponding Northern blot analyses and qRT-PCR data for \( \text{miR}-143 \) and \( \text{miR}-145 \) are shown in Fig. 2. Again, the sequencing analysis was confirmed in individual tissues both by Northern blot and qRT-PCR.

![Figure 1](https://mcr.aacrjournals.org/content/8/4/532){#fig1}

**Figure 1.** Analysis of \( \text{miR}-375 \) and \( \text{miR}-200c \) expression in prostate versus normal tissue by Northern blotting and qPCR. RNA was extracted from primary, snap-frozen prostate tumor and corresponding normal tissue, and analyzed by Northern blot analysis using radioactive labeled probes for \( \text{miR}-375 \) (A) or \( \text{miR}-200c \) (B). tRNA served as a loading control. The relative expression of \( \text{miR}-375 \) and \( \text{miR}-200c \) was determined by qRT-PCR and the ratio between tumor and corresponding normal tissue was calculated (C and D).
analysis. We found a reduction of miR-143 in 8 of 11 (72%) and of miR-145 in 11 of 14 (78%) cases analyzed by Northern blotting (the quantification is shown in supplementary Fig. 1C and D, respectively) and in 23 of 26 (88%) for both miRNAs by qRT-PCR (Fig. 2C and D, respectively). Both miRNAs showed a significantly reduced expression in the tumor tissues compared with normal control tissues ($P = 0.0022$ and $P = 0.0017$, respectively, paired Student’s $t$ test).

We then compared the miRNA levels of miR-375, miR-200c, miR-143, and miR-145 in the established prostate cancer lines DU145, DU145-MN1, and LNCaP to the levels of these miRNAs in nontransformed PNF08 prostate cells. As shown in Fig. 3, miR-200c and miR-375 were almost undetectable in PNF08 cells. For miR-200c, we found strongly upregulated amounts in DU145, DU145-MN1, and LNCaP cells. MiR-375 was consistently upregulated in DU145 and DU145-MN1. In LNCaP, miR-375 was undetectable by Northern blotting, whereas qRT-PCR analyses showed an upregulation of this miRNA. Conversely, PNF08 cells contained high levels of miR-143 and miR-145 but these miRNAs were virtually absent in the cancer cell lines confirming the downregulation in the tumor tissues. The relative expression of miR-375, miR-200c, miR-143, and miR-145 in the cell lines LNCaP, DU145, and PNF08 as determined by qRT-PCR is shown in Table 2.

Association of MiRNA Gene Signature with Clinicopathologic Features of Prostate Cancer

We analyzed 26 primary prostate cancer samples of different stage and grade and corresponding nonmalignant prostate tissue for differences in the expression of miR-200c, miR-375, miR-143, and miR-145 by qRT-PCR. No association neither to tumor stage nor to the Gleason grading could be identified for miR-200c, miR-375, or miR-145. For miR-143, a tendency toward a lower expression in high-grade tumors was found, although the association did not reach statistical significance (Gleason sum: $\geq 4 + 3 = 7$ versus $<4 + 3 = 7$; $P = 0.054$, unpaired Student’s $t$ test). Regarding the primary Gleason pattern, we also observed a tendency toward a lower expression of miR-143 in poorly differentiated tumors (Pearson $r = -0.26$; 95% confidence interval, $-0.588$ to $0.142$; $P = 0.199$, Pearson correlation).

**MYO6 Is a Direct Target of miR-143 and miR-145**

We next searched for potential targets for the differentially regulated miRNAs using Targetscan (http://www.targetscan.org/). For both miR-143 and miR-145, we found that the 3’UTR of the MYO6 gene contains potential binding sites (three sites each for every miRNA...
in the 4.5-kb 3′UTR). It was previously described that myosin VI levels were increased in prostate cancer (21, 22).

Two potential binding sites for miR-145 with a high predictive score are located about 400 and 800 bp downstream of the MYO6 stop codon, which we denote miR-145 site I and site II, respectively. The first predicted binding site for miR-143 is located ∼850 bp downstream of the stop codon. We therefore inserted the 1,350 bp of the MYO6 3′UTR adjacent to the stop codon behind the 3′-end of the luciferase reporter gene in the pGL3 vector and assayed the effect of the two miRNAs on its luciferase activity in cell lines cotransfected with miRNA expression plasmids. As shown in Fig. 4A, both miRNAs significantly decreased the activity of the luciferase reporter gene by 25% to 30%, whereas a construct expressing miR-23a and miR-27a had no effect. We used the miRNAs miR-23a and miR-27a as a control because these were also downregulated in prostate cancer tissue (Table 1) but were not predicted to bind to the MYO6 3′UTR. We note that miR-23a and miR-27a are encoded in one cistron and were thus expressed from the same vector construct. None of the miRNA expression plasmids had a regulatory effect on the pGL3 reporter vector without the MYO6 3′UTR (Fig. 4A).

The seed sequences in the potential binding sites for miR-145 and miR-143 in the 3′UTR of MYO6 were altered by site-directed mutagenesis and subsequently assayed for their regulative capabilities. As shown in Fig. 4B, the mutation of the miR-145 and miR-143 binding sites resulted in a loss of responsiveness to their cognate miRNA but were still downregulated by the miRNA targeting the unmodified binding site. Interestingly, when either miR-145 site I or site II were mutated, we still observed a downregulation by miR-145 of 33% and 40%, respectively, whereas the mutation of both sites resulted in a statistically not significant reduction of 10%. Although both sites are located ∼400 bp apart from each other, this result indicates that there is no additive effect of these two miRNA binding sites. The mutation of the binding site for miR-143 abrogated the repression by miR-143 but not miR-145. These data show that the MYO6 mRNA is an independent target for both miR-145 and miR-143.

Inhibition of Myosin VI Expression by miR-143 and miR-145

To analyze whether overexpression of miR-143 and miR-145 indeed decreases the level of myosin VI protein, we first measured the amounts of myosin VI in normal PNF08 cells and in LNCaP and DU145 prostate cancer cells by Western blot analysis. As shown in

Table 2. Relative expression level of miRNAs in prostate normal fibroblasts and the prostate cancer lines LNCaP and DU145 as determined by qRT-PCR

<table>
<thead>
<tr>
<th>Cell line</th>
<th>hsa-miR-375</th>
<th>hsa-miR-200c</th>
<th>hsa-miR-143</th>
<th>hsa-miR-145</th>
</tr>
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<tbody>
<tr>
<td>PNF08</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>LNCaP</td>
<td>7.810</td>
<td>1,419.630</td>
<td>0.002</td>
<td>0.001</td>
</tr>
<tr>
<td>DU145</td>
<td>20.480</td>
<td>635.870</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

NOTE: The value obtained for the PNF cells was set to 1. Expression values of 0.000 denote miRNAs undetectable by qRT-PCR. Abbreviation: PNF, prostate normal fibroblast.
Fig. 5A, myosin VI was undetectable in PNF08 while we obtained a strong signal in both LNCaP and DU145 in accordance with the absence of miR-143 and miR-145 in the latter two cell lines. Preliminary studies using various transfection methods had indicated that LNCaP cells could be transiently transfected with an efficiency of ~40% using the Lipofectamine 2000 reagent. Because we observed an efficiency of only 10% for DU145 cells using this or any other transfection reagent tested, we therefore chose to analyze the myosin VI levels in LNCaP cells transiently transfected with expression vectors for each of the miRNAs. As shown in Fig. 5B, expression of miR-145 resulted in ~48% decrease of myosin VI. We also detected a downregulation of β-actin by miR-145 in line with a previous demonstration that β-actin is a target of miR-145 (data not shown; ref. 23). Therefore, we used the GAPDH

![Graph A](image1.png)

**FIGURE 4.** Reporter gene analysis of the miRNA responsiveness of the MYO6-3′-UTR. The first 1,350 nucleotides of the MYO6 3′ UTR adjacent to the stop codon of the MYO6 mRNA were inserted into the pGL3 luciferase reporter vector. Then, miRNAs expressed from the pSG5-vector and the pGL3-MYO6-3′-UTR reporter vector were coexpressed in the indicated combinations in the 293T cell line. Luciferase activity of the reporter vector without miR expression was set to 100% (A). Reporter gene vectors containing mutated miRNA binding sites in the Myo6 3′ UTR were coexpressed with the miRNAs indicated. Luciferase activity of the reporter vector without miR expression was set to 100% (B). Columns, mean values of four independent experiments carried out in duplicate; bars, SEM.
protein levels as an internal control and could not detect any miRNA-dependent regulation of GAPDH expression as shown in Fig. 5B. Furthermore, the expression of miR-143 in the LNCaP cell line resulted in a 20% decrease of myosin VI protein, whereas the amount of β-actin remained unchanged.

Discussion

The miRNA profiles of prostate carcinoma had been determined in previous efforts either by microarray analysis (11-15) or a liquid hybridization technique (mirMASA; ref. 24). Although the data obtained by these methods are dependable, a drawback is that the expression of a given miRNA cannot be quantified exactly vis à vis the total amount of miRNAs present in a given sample and that previously unknown miRNAs will not be present on any microarray. Here, we describe the results of a deep sequencing analysis in an effort to get an unbiased account of the miRNAs present in normal prostate tissue and to compare the relative levels of the miRNAs in normal versus tumor tissue. The study design used included the sequencing of two independent pools of RNA for tumor and normal control tissues. This study design with an independent replication of the deep sequencing reaction was intended not only to reduce the possibility of a false-positive discovery but also enabled us to assess the reproducibility of RNA preparation procedures and cDNA library synthesis.

We found a total of 260 of the presently >1,000 known human miRNAs in either the normal or tumor-derived cDNA libraries. The two cDNA libraries from normal prostate tissues showed the same distribution of miRNAs proving that the procedure to generate the libraries yielded reproducible results. In the two libraries from the normal tissues, the 20 most abundant miRNAs were the same. In the tumor-derived libraries, only the 10 most abundant miRNAs were consistent indicating that the tumors were less homogeneous.

The comparison of our data with the published miRNA profiles revealed discrepancies about upregulated or downregulated miRNAs. Ozen et al. (13) already noted that their microarray-based analysis, when compared with the microarray analysis reported by Volinia et al. (12), showed discordant results, whereas their data were consistent with the data reported by Lu et al. (15). Our data were most consistent with those reported by Tong et al. (24) with the notable exception of miR-375 where we found a 9-fold induction, whereas they did not notice any deregulation of that particular miRNA gene. In addition, we found a 1.6-fold induction of miR-let-7f, which was unchanged in their analysis. We assume that these differences in expression data at least in part are due to the technical characteristics of the detection systems used.

It was recently published that the ratio of miR-221 to miR-375 was predictive for the outcome of head and neck squamous cell carcinoma (25). In our analysis, we found a consistent downregulation of miR-221 and an upregulation of miR-375, whereas the situation was reversed in the head and neck squamous cell carcinoma study. Schäfer

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**FIGURE 5.** Western blot analysis of myosin VI. Protein extracts were prepared from the cell lines PNF08, DU145, and LNCaP with or without ectopic expression of miR-143 or miR-145 and analyzed by Western blotting. The Western transfer membranes were incubated with antibodies against myosin VI, GAPDH, or β-actin, suitable secondary antibodies and visualized by enhanced chemiluminescence. A, myosin VI expression in prostate normal fibroblasts and the established prostate carcinoma cell lines LNCaP and DU145. β-Actin served as a loading control. B, expression of myosin VI protein in LNCaP cells with (miR-145) or without (pSG5) ectopic expression of miR-145. GAPDH served as a loading control. C, expression of myosin VI protein in LNCaP cells with (miR-143) or without (pSG5) ectopic expression of miR-143. β-Actin served as a loading control.
et al. (26) also recently showed by qRT-PCR analysis that miR-375 was upregulated whereas miR-221 was downregulated in prostate carcinoma. This phenomenon has been noticed in several occasions. Although it has been shown that miR-200c is consistently upregulated in prostate cancer (20), the opposite has been reported for bladder cancer (27). We conclude that the role of an individual miRNA in tumor initiation or growth is organ or cell type specific so that differences in its deregulation between different tumor entities are not a contradiction, but rather reflect different roles of its target gene or genes in different tumor types.

Avissar et al. (25) also noted a highly inconsistent upregulation or downregulation of most of the analyzed miRNAs in established head and neck squamous cell carcinoma cell lines compared with primary tumors. This raises the question about the usefulness of established tumor cell lines in analyzing tumor-specific miRNA expression patterns. In the cell lines we used for this study, we did not notice any difference between the miRNA expression profiles of the cell lines and primary tumors for miR-143, miR-145, and miR-200c. MiR-375 was upregulated in the DU145 cell line as observed in the tumor tissues but was undetectable in LNCaP by Northern blotting. Using qRT-PCR analyses, an upregulation of miR-375, however, was also documented in LNCaP.

Moreover, Ladeiro et al. (28) reported that the reduction of miR-375 expression was inversely correlated to the expression of β-catenin. It is still unclear whether there is a direct regulative link between miR-375 and β-catenin. Because β-catenin levels have been found to be increased in prostate cancer, this possibility seems rather unlikely for this tumor entity.

We also found upregulation of miR-200c, which targets the transcription factors ZEB1 and ZEB2. Both factors are capable to negatively regulate miR-200c expression, thus generating a double-negative feed-back loop, which has been shown to increase E-cadherin expression (29). An increase of E-cadherin through miR-200c seems counter-intuitive; however, it has been discussed that the downregulation of epithelial surface molecules such as E-cadherin may only occur at the invasive front of solid tumors, thus preventing a detailed analysis of these regulative processes in homogenized tumor tissue samples (30). Although the increase in miR-200c representation from ∼2.4% of all miRNA transcripts in the normal tissue to 9.6% in tumor tissue is impressive, it could be possible that an increase in miR-200c does not further affect an already existing feedback loop between ZEB and miR-200c.

The decrease of miR-145 and miR-143 in prostate tumors was also noted in the aforementioned publications: Ambs et al. (14) found a 20% reduction of miR-145; Tong et al. (24) reported a 20% reduction of miR-143; and Porkka et al. (11) described a nonquantified reduction of both miR-143 and miR-145. We find that miR-143 and miR-145 negatively regulate MYO6 and could prove this effect at the mRNA as well as at the protein level. Myosin VI in contrast to all other myosins is the only identified member of this protein family that is capable of moving to-ward the minus end of the actin filament (31) and is involved in cancer-related cell migration (32). The dramatic loss of both miR-143 and miR-145 in prostate cancer tissue establishes one molecular mechanism for the described increase in myosin VI protein levels in prostate cancer (22, 33). The fact that a downregulation of miR-143 and miR145 has been reported in several studies including patients with different tumors and different ethnic backgrounds points to the possibility that myosin VI overexpression may be an important tumorigenic event in several tumors, including breast, ovarian, and prostate cancer. In Drosophila melanogaster, it has been shown that myosin VI is essential for E-cadherin-mediated border cell migration (34) and specific inhibition of myosin VI by small interfering RNA transfection of LNCaP cells leads to a decreased in vitro cell migration and anchorage-dependent cell growth (33). This points to the possibility that myosin VI overexpression might be able to promote invasive growth in the respective tumors.

A major problem in studies addressing a comparison between tumor and normal tissue specimens is the heterogeneous cellular composition of the samples analyzed, e.g., a higher proportion of stromal cells in the normal prostate tissue samples compared with the tumor samples. Immunohistochemical studies have shown a strong expression of myosin VI in prostate cancer cells but only a weak expression in the normal epithelial and surrounding stromal cells (33). In conjunction with our results that MYO6 is a target gene of miR-143 and miR145, these data support our conclusion that the described deregulation of specific miRNAs is in fact tumor-associated and not an artifact due to a different content of stromal cells in the normal prostate tissue.

Because it is possible to assess miRNA expression patterns using urine (35) or blood serum (36), the identified deregulated miRNAs pose the potential of being valuable tumor markers for the diagnosis of prostate cancer. It is known that the myosin VI expression level is even more predictive for the aggressiveness of prostate cancer (33). The fact that we did not observe any statistically significant correlations of the expression levels of miR-143 or miR-145 to tumor stage or grade could be due to the limited number of cases analyzed. At least the tendency toward an association between miR-143 and Gleason grading in our series is encouraging to perform further analyses in larger series.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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