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SHORT COMMUNICATION

Widespread deregulation of microRNA expression in human prostate cancer

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MicroRNAs (miRNAs) are small regulatory RNAs that can regulate gene expression by binding to mRNA sequences and repressing target-gene expression post-transcriptionally, either by inhibiting translation or promoting RNA degradation. We have analysed expression of 328 known and 152 novel human miRNAs in 10 benign peripheral zone tissues and 16 prostate cancer tissues using microarrays and found widespread, but not universal, downregulation of miRNAs in clinically localized prostate cancer relative to benign peripheral zone tissue. These findings have been verified by real-time RT–PCR assays on select miRNAs, including miR-125b, miR-145 and let-7c. The downregulated miRNAs include several with proven target mRNAs whose proteins have been previously shown to be increased in prostate cancer by immunohistochemistry, including RAS, E2F3, BCL-2 and MCL-1. Using a bioinformatics approach, we have identified additional potential mRNA targets of one of the miRNAs, (miR-125b) that are upregulated in prostate cancer and confirmed increased expression of one of these targets, EIF4EBP1, in prostate cancer tissues. Our findings indicate that changes in miRNA expression may have an important role in the biology of human prostate cancer.
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MicroRNAs (miRNAs) are small (20–22 nucleotide) regulatory RNAs. Initially, primary-miRNAs (pri-miRNAs), which are several hundred to thousand-nucleotide-long sequences, are produced by RNA polymerase II enzyme, which are further processed to a shorter miRNA–miRNA duplex and finally to a single-stranded mature miRNA. Mature miRNAs regulate gene expression by either perfect or nearly perfect complementary binding to mRNA sequences and repressing target-gene expression post-transcriptionally, either by inhibiting translation or promoting RNA degradation (Voorhoeve *et al*., 2006; Wu *et al*., 2007).

There is emerging evidence that miRNAs are involved in cancer pathogenesis. A number of studies have detected frequent alterations of miRNA expression in a variety of human malignancies (Lu *et al*., 2005; Volinia *et al*., 2006). miRNAs might function biologically as either tumor suppressors or oncogenes. For example, it has been shown that miRNAs of the let-7 family negatively regulate RAS (Johnson *et al*., 2005). Thus these miRNAs function biologically as tumor suppressors and loss of expression would be predicted to promote transformation and tumor progression. The picture is complicated by the fact that a given miRNA may have many mRNA targets, so that the biological effects of changes in miRNA expression are likely to be dependent on the cellular context.

There are a limited number of studies in the literature on the role of miRNAs in prostate cancer. Lu *et al*. (2005) reported the use of miRNA expression profiling to classify human cancers. They analysed 217 mammalian miRNAs from multiple human cancers including 8 normal prostate and 6 prostate cancer samples. Prostate tumor samples were part of the study by Volinia *et al*. (2006) in which they described a large-scale analysis of the miRNA expression profiles in 540 samples from six types of solid tumors. To determine if there are changes in miRNA expression in a well characterized set of human prostate cancers we analysed RNAs from 10 benign peripheral zone tissues and 16 prostate cancer tissues (consisting of 70–90% cancer) from radical prostatectomies using miRNA microarrays containing probes targeting 328 known and 152 novel miRNA targets. We used equal numbers of cancers from men with no PSA recurrence within 5 years of surgery and from men with PSA recurrence within 1 year of surgery in order to examine samples with a range of clinical aggressiveness. A total of 85 of the 480 miRNAs were detectable in normal and/or neoplastic prostate tissues (Figure 1, left panel). Of the miRNAs detected, almost all were downregulated in the majority of the prostate cancer samples. Overall 76 of the 85 miRNAs were significantly downregulated

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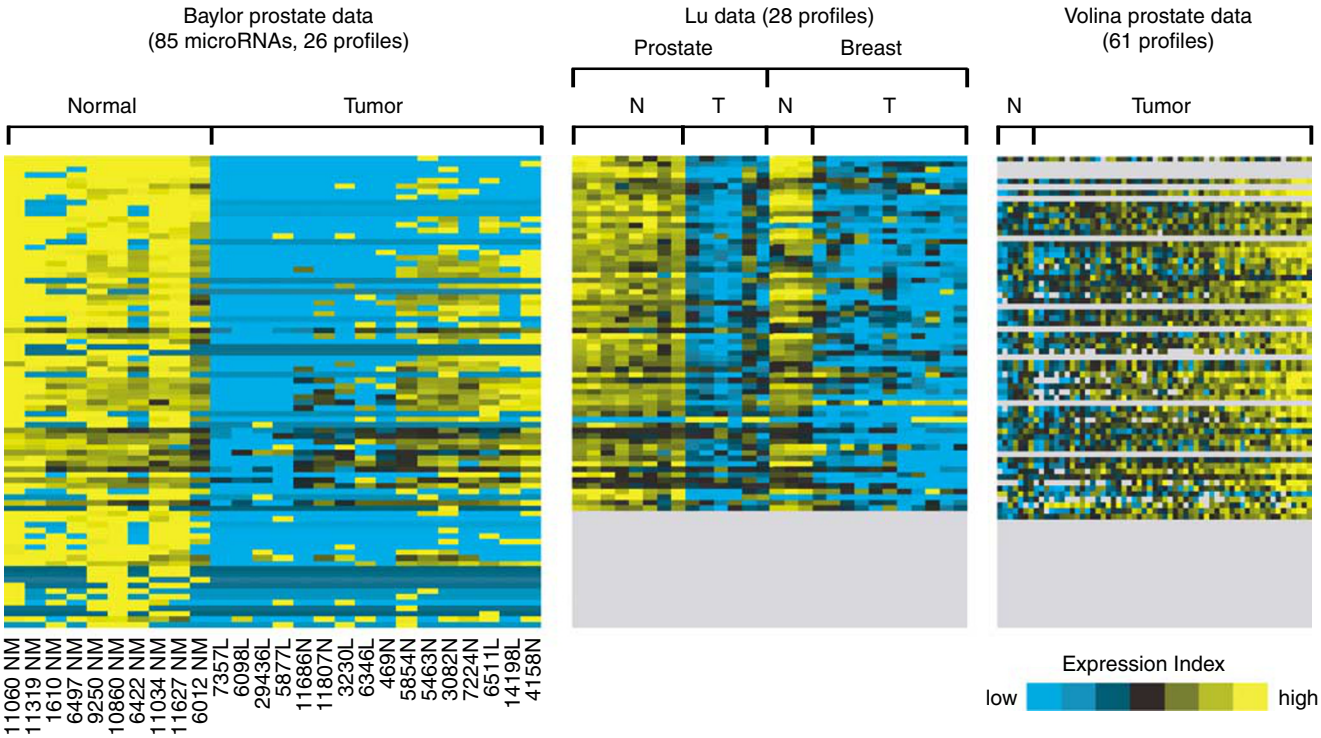


Figure 1 Global miRNA expression patterns in human prostate cancer. Heat map representation of expression patterns for the set of 85 miRNAs in 10 normal prostate and 16 prostate cancer samples from our group ('Baylor,' left panel). The corresponding expression patterns from Lu *et al*. (2005) (middle panel) and Volinia *et al*. (2006) (right panel) are also shown. Rows of the heat map represent miRNAs; columns, profiled tumors. Relative expression is represented as colorgram (yellow: high expression). The order of the miRNAs is the same in the Baylor and the previously published datasets. For Baylor data, sample labels are given ('NM,' normal benign peripheral zone tissue; 'N,' no PSA recurrence (follow-up >5 year); 'L,' early PSA recurrence (<1 year)). Total RNA was isolated using Trizol reagent from snap frozen tissues from radical prostatectomy specimens as described previously (Wang *et al*., 2006). Purified miRNAs were hybridized to miRvana miRNA bioarrays targeting a comprehensive selection of known human (328 miRNAs), as well as novel human (152) miRNAs as described in Supplementary Methods. This microarray technology has been described previously (Shingara *et al*., 2005). Expression values were visualized as heat maps (color maps) using the Cluster and Java TreeView software (Eisen *et al*., 1998; Saldanha (2004)) with expression values first centered on the mean centroid. Treatment of the miRNA profile dataset from Lu *et al*. (2005) and Volinia *et al*. (2006) was similar to that for our dataset.

in the cancer samples ($P<0.05$, *t*-test). It should be noted that while overall the expression of these miRNAs was decreased, there was significant variability between cancers, with some cancers showing downregulation of almost all miRNAs (for example, cases 7357, 6098) and others (for example, case 4158) had only modest overall downregulation, while the majority had mainly downregulation, with variable retention of expression. One possible explanation for widespread downregulation of miRNAs in prostate cancer tissues would be if normal stromal tissues express higher levels of miRNA and thus, since cancers have less stroma than normal tissues, the relative expression of miRNAs would appear to be decreased. However, the variable pattern seen argues strongly against this possibility since all of the cancer tissues have a relatively uniform, high percentage of cancer confirmed by both histopathology and quantitative RT–PCR of prostate cancer specific markers (Wang *et al*., 2006) and thus have little variability in the percentage of stroma. However, this possibility can ultimately only be excluded by analysis of miRNA expression in laser captured stromal tissues. Several miRNA paralogous groups, consisting of miRNAs with high levels of sequence similarity, had multiple group members significantly downregulated in the prostate cancers, including the let-7 family (7b–g, 7i), miR-26a-b, miR-29a-c, miR-30a-e, miR-99a-b, miR-125a-b and miR-200a-b. Since these miRNAs have very similar sequences (Griffiths-Jones *et al*., 2006) and may potentially target similar mRNAs, such coordinate regulation may be biologically significant. Of note, we have identified a number of downregulated miRNAs with known targets that are upregulated in prostate cancer including the oncogenic proteins RAS and E2F3 and the anti-apoptotic proteins BCL-2 and MCL-1 (see, Supplementary Table 1). There was a tendency for the cases with early PSA recurrence to have more global downregulation on miRNAs in that six of the eight cases with downregulation of most miRNAs have early PSA recurrence, but no single miRNA was useful as a predictor of recurrence.

In addition to the 85 miRNAs that had expression in the majority of samples, we noted a group of 60 miRNAs that had undetectable expression in 85–96% of the samples analysed (data not shown). However, there was detectable expression in 1 or 2 normal and/or 1 or 2 cancer tissues. Of these, 40 miRNAs showed no

detectable expression in normal tissues but were expressed in 1 or 2 cancer tissues. The significance of these observations is unclear.

We next compared our data to the miRNA expression reported by Lu *et al*. (2005) using a bead-based detection system, in contrast to our microarray-based analysis. As seen in Figure 1 (middle panel), their results are very similar to ours, with widespread downregulation of miRNA expression in prostate cancer. Results in breast cancer show a similar widespread downregulation of miRNA. Mattie *et al*. (2006) also found widespread downregulation of miRNA on two biopsies of cancer versus pooled normal tissue using a microarray-based approach. In contrast, the data of Volinia *et al*. (2006), which are also based on microarray technology, show upregulation of many miRNAs relative to non-cancerous prostate tissue which are downregulated in our analysis and that of Lu *et al*. (2005), as seen in Figure 1 (left panel). There are a number of potential explanations for this difference. Volinia *et al*. (2006) used total mRNA, which would include precursor miRNAs, for labeling and spotted oligonucleotides that detect both precursor and mature miRNAs (Liu *et al*., 2004). In contrast, our protocol, and that of Lu *et al*. (2005) and Mattie *et al*. (2006) included purification of small RNAs, including mature miRNAs, before analysis, so precursor miRNAs would not be detected. If there is a significant block in precursor miRNA processing in prostate cancer without a corresponding decrease in transcription, this could explain this discrepancy. A second potential explanation is differences in the tissues analysed. In our studies we have used benign peripheral zone tissue from radical prostatectomy specimens as our normal reference while the normal tissues of Volinia *et al*. (2006) are described by these authors as being from non-cancer individuals. The exact source of these tissues is not stated but such tissues could be hyperplastic transition zone tissue removed surgically or tissue from either peripheral zone or transition zone from organ donors or rapid autopsy protocols. Similarly, our cancer cases are all from men with clinically localized disease. The clinical and pathological stage of the prostate cancers analysed by Volinia *et al*. (2006) is not stated and this could potentially have a major impact on miRNA expression. Finally, it is possible the methodology of tissue harvesting could impact miRNA expression. It has been shown that hypoxia (Kulshreshtha *et al*., 2007) and other cell stresses (Marsit *et al*., 2006) can impact miRNA expression. Our tissues are harvested within minutes of removal so that hypoxia is not significant in our tissues and several hypoxia inducible miRNAs such as mi26a, mir 27a, mir103 are actually downregulated in our cancer tissues (Kulshreshtha *et al*., 2007). In a similar manner, adjuvant treatments might potentially impact miRNA expression. In our cases, there were no treatments other than surgery. Obviously, further studies using multiple analytical methodologies are needed to clarify the range of miRNA expression in all types of prostate tissue and in cancers representing the full spectrum of disease.

To validate our miRNA microarray data we carried out quantitative RT–PCR using RNAs from 10 benign

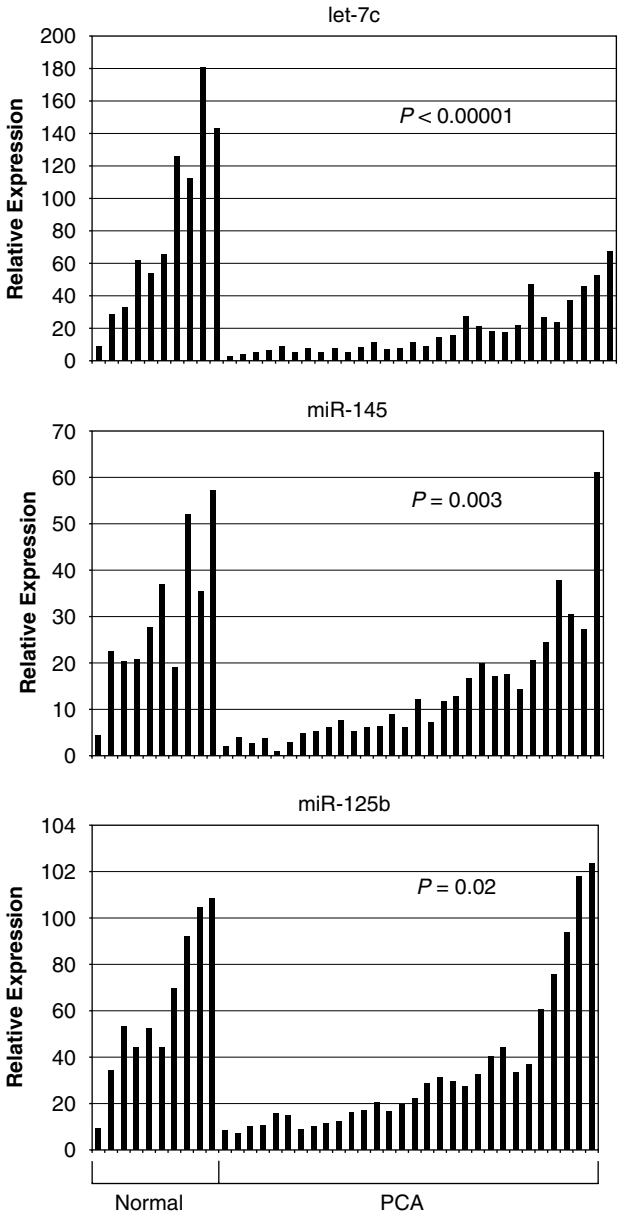


Figure 2 Validation of select miRNAs predicted to be downregulated in prostate cancer. Real-time RT–PCR assays on miRNAs let-7c (top panel), miR-145 (middle panel) and miR-125b (bottom panel) in normal prostate and 30 prostate cancer tissues. The order of the tissue samples is the same for all three plots. *P*-values by two-sided *t*-test. TaqMan microRNA assays for individual miRNAs have been used following manufacturer's protocol (Applied Biosciences, Foster City, CA). Ten nanograms of total RNA from each sample was subjected to reverse transcription reaction in a 15 μl volume at 42° C for 30 min and 1.33 μl used for quantitative PCR in duplicate as follows: 95° C for 10 min, and then 95° C for 15 s and 60° C for 1 min for 40 cycles in an Applied Biosystems 7000 Real-Time PCR system. Two different small nuclear RNAs, RNU43 and RNU6B, were used as internal controls. Primer pairs for these controls and for miR-125b, miR-145 and let-7c were obtained from Applied Biosciences. Each assay included a negative control, and the experiment was done in duplicate. The threshold cycle (C_t) of each sample was recorded as a quantitative measure of the amount of PCR product in the sample. The signals were normalized against the relative quantity of control miRNA and expressed as $\Delta C_t = (C_{tsample} - C_{tcontrol})$. Relative changes in expression were then calculated as $2^{[\Delta C_t]}$.

peripheral zone tissues and 30 cancer tissues, including 10 with no recurrence, 10 with late PSA recurrence (>1 year but <5 years) and 10 with early PSA recurrence. We chose to analyse let-7c due to its established biological function (Johnson *et al*., 2005) and miR-125b and miR-145 because they had previously been shown to be downregulated in breast cancer (Iorio *et al*., 2005), and all three were significantly downregulated in prostate cancer as determined by miRNA microarrays. All of these miRNAs had variable expression in both the normal and cancer tissues (Figure 2) but overall, each was significantly downregulated in prostate cancer samples when compared to benign peripheral zone tissue ($P \leqslant 0.02$). Again, some samples tended to have higher expression of multiple miRNA species while other cancers had marked downregulation of all three miRNAs. Since there is no established standard for normalization of quantitative RT–PCR expression data of miRNA we used two different small nuclear RNAs for normalization. Both gave similar results. We did not detect any significant correlation of expression levels of any single miRNA with clinical or pathological variables related to patient outcome in either the microarray or quantitative-PCR analysis.

To determine if miRNA may modulate mRNA levels in prostate cancer we examined putative gene targets of miR-125b induced degradation in human prostate cancer versus normal prostate. Since there is a significant error in assessment of potential miRNA targets using any one algorithm we adopted the approach of Iorio *et al*. (2005). As described by these authors, if a given target was identified using at least two of three different algorithms it was considered likely to be a genuine miRNA target. We used the gene list developed by these investigators for potential targets of miR-125b (286 potential targets) based on this criterion. We then queried a microarray expression database from Lapointe *et al*. (2004) to determine if any of the potential targets were significantly upregulated in prostate cancer compared to normal prostate, as would be predicted if there was decreased degradation due to loss of the miRNA targeting them. Results of this analysis are shown in Table 1. A total of 26 (of 286) possible miR-125b target genes were significantly increased in prostate cancer. Some of these genes may have a significant impact on the biology and clinical behavior of prostate cancer. SEL1L, a protein that may play a role in breast and pancreatic cancer aggressiveness (Biunno *et al*., 2006), has been shown to be upregulated in prostate cancer by immunohistochemistry (Barberis *et al*., 2006). Of note, several genes that are involved in translation and the control of translation are upregulated including EIF4EBP1, RPL29, MGC16063 and PAPB. EIF4EBP1 is a key regulator of translation, so we sought to confirm its upregulation in prostate cancer by quantitative RT–PCR. As shown in Figure 3, EIF4EBP1 mRNA is significantly increased in prostate cancer compared to benign peripheral zone tissue, validating the microarray results of LaPointe *et al*. (2004) in an independent sample set. EIF4EBP1, a protein that can inhibit translation initiation of capped mRNAs is a key downstream target of the AKT/mTOR pathway and when EIF4EBP1 is phosphorylated its inhibitory effects are decreased, allowing increased translation initiation.

Table 1 Potential miR-125b mRNA targets* that are upregulated in prostate cancer**

Genebank access #	Gene symbol	Entrez ID	Gene description
NM_001755	CBFB	865	Core-binding factor, β-subunit
AB044548	EIF4EBP1	1978	Eukaryotic translation initiation factor 4E binding protein 1
NM_017572	MKNK2	2872	MAP kinase-interacting serine/threonine kinase 2
NM_002252	KCNS3	3790	Potassium voltage-gated channel, delayed-rectifier, subfamily S, member 3
AI457120	PPAT	5471	Phosphoribosyl pyrophosphate amidotransferase
AF116627	RPL29	6159	Ribosomal protein L29
AI139252	MGC16063	6165	Ribosomal protein L35a
NM_000332	SCA1	6310	Spinocerebellar ataxia 1 (olivopontocerebellar ataxia 1, autosomal dominant, ataxin 1)
NM_005065	SEL1L	6400	Sel-1 suppressor of lin-12-like (*C. elegans*)
NM_003045	SLC7A1	6541	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 1
AF113019	SMARCD2	6603	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily D, member 2
NM_003983	SLC7A6	9057	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 6
NM_014746	RNF144	9781	Likely ortholog of mouse ubiquitin conjugating enzyme 7 interacting protein 4
AI948503	ABCC4	10 257	ATP-binding cassette, subfamily C (CFTR/MRP), member 4
NM_007260	LYPLA2	11 313	Lysophospholipase II
NM_007359	MLN51	22 794	MLN51 protein
AL117482	DKFZP434C131	25 989	DKFZP434C131 protein
U64661	PABP	26 986	Polyadenylate-binding protein 1
AF263462	CGN	57 530	Cingulin
AF308300	FAPP2	84 725	Phosphoinositol 4-phosphate adaptor protein 2
AW341649	TP53INP1	94 241	Tumor protein p53 inducible nuclear protein 1
BE618656	MGC2541	113 829	Similar to RIKEN cDNA 2610030J16 gene
BE892574	LACTB	114 294	Lactamase, β
AA025877	C20orf136	140 700	Chromosome 20 open reading frame 136
BE781961	DKFZp762A2013	169 714	Hypothetical protein DKFZp762A2013

*Based on the computational analysis by Iorio *et al*. (2005). **Based on RNA expression profile dataset from Lapointe *et al*. (2004); $P<0.001$.

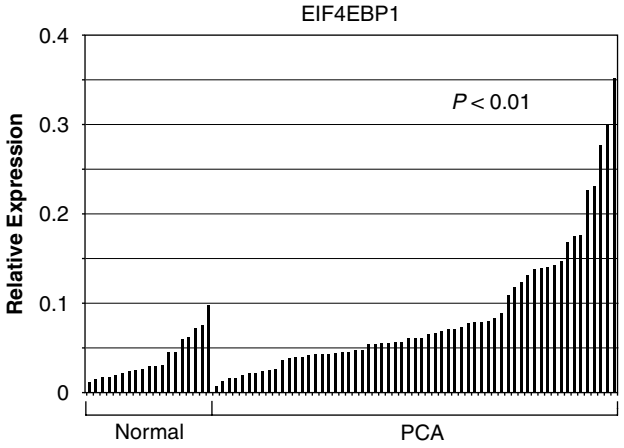


Figure 3 Upregulation of putative miR-125b gene target EIF4EBP1 in prostate cancer. Real-time RT–PCR assay results in normal (19 tissues) and prostate cancer (PCA, 61). *P*-value by two-sided *t*-test comparing PCA to normal peripheral zone. Oligonucleotide primers for EIF4EBP1 (NM_004095.2) were forward, 5′-CTGATGGAGTGTCGGAAC-3′, and reverse, 5′-ATGGCTGGTGCTTTAAATG-3′; for β-actin were forward, 5′-AGCACGGCATCGTCACCAACT-3′, and reverse 5′-TGGCTGGGGTGTTGAAGGTCT-3′. Real-time PCR was carried as described previously (Wang *et al*., 2006) with the following conditions: 3 min at 95° C; followed by 40 cycles at 95° C for 15 s, 60.5° C for 30 s and a final 72° C extension for 30 s. RT–PCR of β-actin was performed as described previously (Wang *et al*., 2006). The threshold cycle (C_t) values in log linear range representing the detection threshold values were used for quantitation and expressed as copy numbers based on a standard curve generated using plasmid DNA.

The increase in EIF4EBP1 mRNA is somewhat surprising since such an increase would inhibit translation of capped mRNAs that can promote tumor progression. On the other hand, Poly-A binding protein-1 (PAPB-1) known to promote translation of capped mRNAs (Gorgoni and Gray, 2004) may also be targeted by miR-125b. Thus in cells with decreased miR-125b, PABP-1 could potentially increase translation but this is held in check by the inhibitory activity of EIF4EBP1, which can be relieved by activation of the AKT/mTOR pathway. Recent studies in breast cancer, which also show loss of miR-125b, indicate that there is indeed an increase in EIF4EBP1 protein levels in poorly differentiated breast cancers (Rojo *et al*., 2007), but in such cases the protein was almost all phosphorylated, primarily due to activation of the AKT pathway, consistent with this hypothesis. Thus loss of miR-125b may potentiate the translational response to activation of the AKT pathway. Obviously, this hypothesis requires direct experimental validation, but does illustrate the potential pleotropic effects of miRNA downregulation.

In summary, we have demonstrated widespread, statistically significant downregulation of miRNAs in clinically localized prostate cancer when compared to benign peripheral zone tissue. We have also shown upregulation of potential mRNA targets of one of the miRNAs in prostate cancer tissues and correlated loss of several miRNAs with the published literature regarding protein expression of the validated targets of these miRNAs in prostate cancer tissues. Our findings are consistent with recently published studies showing that global repression of miRNA expression enhances tumorigenesis (Kumar *et al*., 2007). Validation of other potential targets of the downregulated miRNAs experimentally is needed to confirm the role of miRNA downregulation in prostate carcinogenesis. In addition, further investigations are needed to determine if there is upregulation of selected miRNAs during progression of prostate cancer to the stage of metastatic disease. In addition, studies of the mechanism of the observed widespread dysregulation of miRNA expression in prostate (and other cancers) are clearly needed. Our data certainly support the concept that alterations of miRNA expression can play an important role in prostate carcinogenesis and as such may be a critical therapeutic target.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).