Deep Sequencing of Small RNA Libraries from Human Prostate Epithelial and Stromal Cells Reveal Distinct Pattern of MicroRNAs Primarily Predicted to Target Growth Factors

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ABSTRACT

Complex epithelial and stromal cell interactions are required during the development and progression of Prostate cancer. Regulatory small non-coding microRNAs (miRNAs) participate in the spatiotemporal regulation of messenger RNA (mRNA) and regulation of translation affecting a large number of genes involved in prostate carcinogenesis. In this study, through deep-sequencing of size fractionated small RNA libraries we profiled the miRNAs of prostate epithelial (PrEC) and stromal (PrSC) cells. Over 50 million reads were obtained for PrEC in which 860,468 were unique sequences. Similarly, nearly 76 million reads for PrSC were obtained in which over 1 million were unique reads. Expression of many miRNAs of broadly conserved and poorly conserved miRNA families were identified. Sixteen highly expressed miRNAs with significant change in expression in PrSC than PrEC were further analyzed in silico. ConsensusPathDB showed the target genes of these miRNAs were significantly involved in adherence junction, cell adhesion, EGRF, TGF- β and androgen signaling. Let-7 family of tumor-suppressor miRNAs expression was highly pervasive in both, PrEC and PrSC cells. In addition, we have also identified several miRNAs that are unique to PrEC or PrSC cells and their predicted putative targets are a group of transcription factors. This study provides perspective on the miRNA expression in PrEC and PrSC, and reveals a global trend in miRNA interactome. We conclude that the most abundant miRNAs are potential regulators of development and differentiation of the prostate gland by targeting a set of growth factors. Additionally, high level expression of the most members of let-7 family miRNAs suggest their role in the fine tuning of the growth and proliferation of prostate epithelial and stromal cells.

1. Introduction

Prostate cancer is the most common non-cutaneous malignancy affecting men in the western world. With the highest incidence rate in the United States, it is the second leading cause of cancer-related death [1]. Prostate gland is an important accessory structure of male reproductive system [2]. Prostate gland growth, proliferation and function are androgen dependent [2; 3]. Glandular acinus is the functional unit of the prostate gland and mainly composed of both epithelial and stromal cells [4; 5; 6]. Epithelial and stromal cell communication is a key to the development and differentiation of the gland [7]. However, it has become evident that genetic alterations in epithelial and stromal cell microenvironment are also required for growth and proliferation of prostate cancer [8; 9; 10]. Epithelial-stromal interaction plays an important role in determining the development of prostate and dysfunction of these processes leads to the development of prostate carcinogenesis. Published data supports an extensive communication between the epithelial and stromal cell as well as the host microenvironment stimulates prostate cancer and facilitate its progression to advance stages [7; 8; 9; 11; 12]. Most studies have relied on use of prostate primary epithelial or stromal cell models to investigate intrinsic gene expression and compared these findings with other immortalized in vitro models of prostate cancer cells. The primary epithelial or stromal cells grown on monolayers have been used extensively to test experimental therapeutics and other molecular mechanisms to study prostate carcinogenesis [13; 14; 15; 16; 17].

A large body of data has shown that regulatory noncoding endogenous microRNAs (miRNA) play significant roles in fundamental cellular processes including differentiation, proliferation, organogenesis and apoptosis [18; 19; 20; 21]. In addition, miRNAs expression or lack thereof has been implicated in a wide variety of diseases including cancers and has been

reviewed extensively in publications [22; 23; 24; 25; 26]. It is well established that miRNAs contribute to carcinogenesis by functioning as tumor-promoting or -suppressing molecules. Numerous miRNAs participate in the regulation of expression of abundant mRNAs, thus affecting a number of genes involved in prostate tumorigenesis. Aberrant expression and dysregulation of numerous miRNAs have been implicated in many types of cancerous conditions including the carcinoma of prostate [27; 28; 29; 30; 31; 32; 33]. Majority of studies have used primary PrEC and PrSC as “normal” cell control along with Prostate cancer cell models. Furthermore, these cells have been used for studies involving miRNA expression, gene expression, drug discovery and comparison of molecular alternations associated with prostate carcinogenesis [34; 35; 36; 37]. However, a complete miRNA component of these PrEC and PrSc cells has not been determined. The major goals of this study was (1) to determine the miRNA complement of PrEC and PrSC cell lines in order to obtain comprehensive comparative differential expression of these molecule, (2) to identify PrEC and/or PrSC specific novel miRNAs, and finally (3) to develop a knowledge resource that would aid the prostate cancer investigators who could potentially compare the expression of miRNAs of immortalized prostate cancer cell culture models with the readily available miRNAs data of the primary PrEC and PrSc cells.

2. Materials and Methods

2.1 PrEC and PrSC cultures

The PrEC and PrSC (Clonetics, East Rutherford, NJ, PrEC characterized for positive cytokeratin staining and PrSC for vimentin) were used for these studies. These cell lines are derived from a 14-yr-old Caucasian male. PrEC were grown according to manufacturer’s

directions in serum-free PrEGM (Clonetics) supplemented with bovine pituitary extract (BPE) (Clonetics) and SCGM (Clonetics) with 5% FBS supplementation was used for culturing PrSC. Characterization of PrEC and PrSC in the cell culture included morphological observation throughout serial passages. For this study, PrECs were “starved” for 24 h in PrEGM media lacking BPE where no obvious cell death was observed.

2.2 RNA isolation and quality control

PrEC and PrSC cells were sub cultured for three passages after initial plating and total RNA was isolated 70% confluence, using Trizol reagent as per manufacturer’s protocol except the final wash of precipitated total RNA was performed in 80% isopropanol [38; 39; 40]. The purity and quality of RNA was determined by agarose formaldehyde gel electrophoresis. 200 µg of total RNA was fractionated on 15% urea-acrylamide gels along with 18 and 23nt long labeled oligonucleotides to select small size RNA for preparation of miRNA libraries for deep sequencing. *The small RNA libraries were sequenced in HiSeq 2000 Illumina sequencer in the Institute of Integrative Genome Biology, Genomics Core of the University of California, Riverside.*

2.3 Small RNA library construction and bioinformatics of sequences.

Computational analysis of sRNA reads obtained from human sRNA libraries were performed as reported previously [38; 39; 40; 41]. The unique sRNAs were aligned to the REPBASE (version 14, obtained from <http://www.girinst.org>), reference mRNA downloaded from the UCSC Genome Browser (<http://hgdownload.cse.ucsc.edu/goldenPath/hg19/bigZips/refMrna.fa.gz>), and known noncoding RNAs (rRNAs, tRNAs, snRNAs, snoRNAs, etc.) obtained from RFAM (<http://www.sanger.ac.uk/Software/Rfam/ftp.shtml>), noncode (<http://www.noncode.org/NONCODERv3/download.htm>), fRNAdb

117 (<http://www.ncrna.org/frnadb>), Genomic tRNA database
118 (<http://gtrnadb.ucsc.edu/download.html>), and SILVA ([http://www.arb-](http://www.arb-silva.de/no_cache/download/archive/current/Exports/)
119 [silva.de/no_cache/download/archive/current/Exports/](http://www.arb-silva.de/no_cache/download/archive/current/Exports/)) with National Center for Biotechnology
120 Information (NCBI) BLASTn [42]. Following the removal of sRNAs corresponding to repetitive
121 elements, reference mRNAs and known ncRNAs, unique sequences between 18 and 28 nt were
122 utilized to map to the genome of human (downloaded from the UCSC Genome Browser,
123 <http://hgdownload.cse.ucsc.edu/goldenPath/hg19/bigZips/chromFaMasked.tar.gz>) using
124 BLASTn searches. The unique sequences were aligned to the miRBase (version 18;
125 <http://microrna.sanger.ac.uk/>) to calculate frequencies of the known miRNA homologs in humans.
126 The potential candidate miRNAs were identified by folding the flanking genome sequence of
127 unique small RNAs using the RNAfold program [43]. Corresponding precursor sequences were
128 identified with minimal ≥ 18 matched base pairs, a single central loop, and minimum free energy
129 (MFE) < -18 kcal/mol. Novel miRNA mature sequences were designated based on the following
130 criteria: ≥ 5 total reads in the six libraries combined, ≤ 5 genomic loci, precursors identified
131 similarly as the conserved miRNAs, highest abundances compared to other reads mapped to the
132 precursors, and existence of predicted miRNA* strands. The ones without accompanying
133 miRNA* strands are considered to be novel miRNA candidates.

134 For identification of novel miRNAs, unique sRNAs with more than 10 genomic hits were
135 removed from further analysis. The flanking regions of the remaining genome-matched
136 sequences were cut out, and the fold-back structures were predicted using the RNAfold program
137 [43]. Next, we examined the resulting folding structures to choose those that had at least 18 base-
138 pairs, one central loop, and folding energy not greater than 18 Kcal/mol. We then applied the
139 MIRCHECK program [44] to choose sequences that have ≤ 6 mismatches, ≤ 2 bulged or

asymmetrically unpaired nucleotides, and ≤ 2 continuous mismatches in the regions of the sRNA reads. Subsequently, an in house developed program was used to check the existence of miRNA* sequence in the small RNAs, based on the criterion that there were 2 nt overhang(s) at the 3' end(s) of both the miRNA or miRNA*. Then, the distribution of unique small RNAs on the putative pre-miRNAs was manually examined [45].

2.4 MicroRNA target prediction and regulatory function analysis

The HitSensor algorithm [46] was used to predict potential targets of the predicted miRNAs and their miRNAs* with the default parameters. The miRNA targets with at least a seed score of 240 were chosen. Open source databases such as MiRecords, TargetScan/TargetScanS (release-6.2) were used for target prediction of differentially expressed and highly abundant miRNAs [47]. The predictions by miRecords were considered significant and taken into account, if at least 5 algorithms predicted the same target. Predictions for gene targets of each miRNA had resulted in thousands of target genes. The relative expression of each miRNA in each cell line was log2 transformed for better visualization of the data. Average linkage hierarchical clustering was performed using Euclidean distance as distance metric using cluster 3.0 and heat maps were generated using Java Tree view [48; 49; 50]. To enhance the biological interpretation of these large lists of genes, the genes are classified for functional annotation performed by ConsensusPathDB (Bioinformatics group of the Vertebrate Genomics Department at the Max-Planck-Institute for Molecular Genetics in Berlin, Germany) [51; 52].

2.5 Reverse Transcription and Quantitative Real-Time (qRT) PCR Analysis for the expression of miR-29a-3p, miR-100-5p and miR-27b-3p

The expression of the three of the highly expressed and differentially expressed microRNAs from PrEC and PrSC was further validated by qRT-PCR analysis. First strand cDNA was

synthesized from 20 ng of total RNA with the primers specific for mature miRNAs and small nucleolar RNA 66 (sno66) as the control. The miRNA-specific primers and Reverse Transcription (RT) kit for cDNA synthesis were obtained from Applied Biosystems Foster City, CA and the manufacturers protocol was followed. To quantify the miRNAs and sno66 in each cell line, the cDNAs were amplified using TaqMan MicroRNA Assays from Applied Biosystems Foster City, CA and read on a 7500 Real-time PCR System (Applied Biosystems). TaqMan assay was performed in triplicates for each RT reaction together with the 'RT negative' and 'no template' controls. The mean C_t was determined from the triplicates. Sno66 expression was used as an invariant control. The relative expression of each miRNA was calculated as $2^{-\Delta C_t}$ where $\Delta C_t = C_t$ value of each miRNA in a sample – C_t value of sno66 in that sample. All experiments were repeated twice (once on the RNA preserved from deep sequencing experiment), with three replicates each experiment.

3. Results

Sequencing small RNA population remains the most powerful method to quantitatively analyze the miRNA content of a cell [53; 54; 55; 56; 57; 58; 59]. Furthermore, sequencing of small RNAs is the only approach to identify novel miRNAs and conserved miRNA homologs among species. A total of 29,340,421 and 33,464,411 reads were obtained for PrEC and PrSC cells, respectively. From these 842,021 and 1,059,496 unique reads of 18 to 26 nucleotides were obtained after removing the adapter sequences for PrEC and PrSC cells, respectively. The mapping distribution of total and unique reads (Fig.1A & B) and the percent of unique reads for different RNA categories for both representative cell lines (Fig 1C & D). This analysis revealed equal representation of different RNA species in both PrEC and PrSC cells. The unique sequences were mapped to the human genome using BLASTn searches and to the miRBase that resulted in the identification of conserved miRNA homologs in human let-7 family miRNAs

(Table-1 Supplementary data) as described in the materials and methods. Differentially expressed miRNAs in two cell lines were clustered by Cluster 3.0 using dendrogram-based method (Figure 2). A huge proportion of miRNAs show reversal of expression from PrEC to PrSC while only a fraction demonstrate comparable expression in both the cell, as expected demonstrating the differences in miRNA population in the two cell lines.

To understand the functional implications of differential expression of miRNAs we arbitrarily selected the highly abundant (100 or higher reads) miRNAs from the two cell lines and plotted differentially expressed miRNAs (Figure 3). As shown in Figure 3a, miR-214-3p, miR-432-5p, miR-199b-3p, miR-143-3p, miR-411-3p, miR-127-3p, miR-152, miR-100-5p, miR-221-3p and miR-29a-3p exhibited 2 fold or higher upregulation in PrSC, while miR-1246, miR-200b-3p, miR-205-5p, miR-200b-3p, miR-203 and miR-200c-3p exhibited 2 fold or higher upregulation in PrEC (Figure 3b). **To validate the differential expression profiles obtained by deep sequencing of PrEC and PrSC miRNAs, we performed TaqMan quantitative RT-PCR assays on three highly expressed miRNAs using the same stock of RNA sample that was used for the construction of small RNA libraries. As shown in figure 3c, miR-29a-3p, miR-100-5p and miR-27b-3p expression profile appears to be consistent with the deep sequencing data and shows about the similar ratio as obtained in deep sequencing data (figure 3a and b). miR-29a-3p and miR-100-5p shows similar level of higher expression in PrSC than PrEC and miR-27b-3p shows higher expression levels in PrEC as compared to PrSC.** Differentiation of prostate gland requires balanced communication between epithelial and stromal cells, which is primarily driven by various growth factors secreted by the stroma. Changes in the epithelial microenvironment modulate alterations among the stromal cells which appears to facilitate promotion of tumor development [60; 61; 62]. MiRNAs play a key role in

the regulation of these processes and it appears that obtained expression profile, miRNAs of PrEC and PrSC cells predominantly regulate growth factor signaling pathways and transcription factors. As shown in [table 1](#), miR-214-3p, miR-199b-3p, miR-143-3p, miR-411-3p, miR-127-3p, miR-152, miR-100-5p, miR-221-3p and miR-29a-3p, the stromal abundant miRNAs have lower level of expression in PrEC. PrEC abundant miRNAs, miR-205-5p, miR-203 and miR-200c-3p showed downregulated expression in PrSc with the exception of miR-200b-3p. Deregulated miRNA expression in these cell types is one of the key events which influences the intrinsic cellular pathways during the development of multiple types of cancers and it has been elegantly reviewed in various articles [63; 64; 65; 66]. Therefore, we compared the expression profile of stromal and epithelium miRNAs with the published experimentally validated data in the prostate cancer literature.

We began with the lethal-7 or let-7 family of miRNAs which were originally found to modulate developmental timing in *Caenorhabditis elegans* [67]. Among numerous miRNA, let-7 family has gained a reputation for their persistent involvement and tumor suppressing activities in cancers. The let-7 family members are phylogenetically conserved from vertebrates to non-vertebrates [68]. Interestingly, the let-7 family members appear to be located in the fragile sites of the genome demonstrating their functional involvement in various human cancers including prostate cancer [69; 70; 71; 72; 73; 74]. Since, let-7 family appears to be an important miRNAs commonly altered during cancer, we compared the let-7 family members between PrEC and PrSC cells. We have detected the following 13 miRNAs belonging to let-7 family; let-7a-1, let-7a-2, let-7a-3, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7i and miR-98 ([Table 2](#)) [71]. Except let-7b-5p and let-7c, the other members of the let-7 family were more abundantly expressed in PrSC cells ([Figure 4a and b](#)). Only two miRNAs (let-7a-3p and 7f-1-3p) has

identical number of reads, however, appears to have a low level of expression in both cell types (Figure 4c and table 2). Let-7 family members have been linked with cancer inflammatory microenvironment as well as in epithelial to mesenchymal transition (EMT) in variety of tumors including prostate [73; 75; 76]. Many members of the let-7 family have been shown to target and negatively regulate a variety of cellular transformation and metastasis promoting genes including HMGA1, MHGA2, IL6, NRAS and c-Myc [68; 77; 78; 79]. Additionally, many let-7 family miRNAs were found to be downregulated in prostate cancer stem cells. Within the context of numerous reports showing tumor suppressing function of let-7, we surmise that the pervasive high level expression of many miRNAs of the let-7 family in both stromal and epithelial cells appears to maintain a controlled cellular proliferation via targeting the growth and transcription factors. We also compared the expression of let-7 family miRNAs from PrEC and PrSC to the known prostate cancer miRNA data, as shown in table 2, indicating that the let-7b and let-7d-5p exhibited significant downregulation in prostate cancer while let-7a-5p, let7c, let-7f-5p and let-7i-5p were upregulated.

Next we assessed whether altered miRNAs in these cells contribute to regulation of growth factor mediated pathways, we made predictions of miRNA target genes and integrated them using consensusPathDB which incorporates 12 interaction databases to provide a comprehensive map of different interactions regulated by a set of genes. Table 3 and figure 5A represents different pathways potentially regulated by the target genes of highly abundant PrSC miRNAs. As shown in the table 3, the target genes of PrSC abundant microRNAs were significantly involved in regulation of various growth factor regulated pathways. The predicted target genes contributed to 61% and 41% of the genes involved in regulation of EGFR1 and EGF-EGFR Signaling Pathway respectively. 32% in BDNF pathways, 37% in regulation of focal

adhesion and 38% in regulation of proteoglycan. 32% in PDGF signaling, 30% and 16% in TGF beta receptor and its signaling, 28% in focal adhesion, 21% in integrin signaling, 23 % and 16% in androgen receptor and androgen receptor signaling and 13% were found to be involved in PCa pathways. Similarly, though in a lesser percentage but the PrEC abundant microRNAs were also found to be involved in integrins, and growth factor mediated signaling pathways. As shown in table 4 and figure 4B, 7% were involved in Signaling events mediated by focal adhesion kinase and integrins in angiogenesis each. 7% were involved in HIF1 alpha transcription factor network, 15% in MAPK signaling pathways, 11% in TGF beta-receptor, 4% in VEGFR1 specific signals, 7% in IGFR1 signaling cascade and 6% in prostate cancer. In summary, a large number of highly expressed miRNAs of PrSC and PrEC cells potentially modulate the expression of a network of cellular pathways involved in epithelial-stromal communication and cell growth and developmental pathways (Figure 5A and B).

Next we sought identification of novel PrEC and PrSC miRNAs and their targets in PrEC and PrSC cells. Some of the miRNAs are specifically expressed in specific cell types or during a particular stage of the development or both. A large number of non-conserved miRNAs are specific to organisms and cell types [80]. Small RNA sequencing from the two prostate gland cell types used in this study allowed us to identify novel miRNAs that could be specific to these cells only. We have identified a total of combined 44 novel miRNAs in both PrSC and PrEC cell lines (Table 5). Among these 28 and 16 miRNAs were 5p and 3p (Or star*) species, respectively. Among 44 miRNAs, five miRNAs designated t3305116, t4312554, t3366647, t3440992, and t3318086 were unique to PrEC. In addition, six miRNAs designated t1073351, t4514899, t503747, t3320411, t3583876, and t3670590 were unique to PrSC cells (Table 5).

4. Discussion

Cellular communication between epithelial and stromal cells plays an important role in the development and differentiation of the prostate gland [9; 10; 81; 82; 83; 84; 85; 86]. It is also well established that genetic alterations in the epithelial and stromal cell promotes prostate cancer [9; 84; 87; 88]. In addition, numerous genomic alterations in prostate epithelial and stromal cells stimulates drug resistance and promotes the development of castrate-resistant prostate cancer (CRPC) post androgen ablation therapy. Experimental use of PrEC and PrSC cells is very common practice to compare differential expression of a gene or set of genes with immortalized prostate cancer cell lines. In addition, “normal” intrinsic expression of any protein factor or RNA is mostly validated in primary PrEC or PrSC cells to further define the value of comparison between a patient derived cells *versus* normal primary cells. A comparative expression profile of all miRNAs found in PrEC and PrSC was not available and this study has provided the miRNA complement of these cells. In addition, this study has revealed the expression of many novel PrEC and PrSC specific miRNAs. In total, 1919 known miRNAs and 44 novel miRNAs were detected in PrEC and PrSC cells by deep sequencing. The present study is the first to report comprehensive identification of miRNAs and their potential targets in PrEC and PrSC cells. The miRNAs of PrEC and PrSC cells belong to all three major miRNA families viz. conserved, non-conserved and poorly conserved [20].

Several novel miRNAs identified in PrEC and PrSC cells are predicted to target a plethora of transcription factors. These transcription factors include Ets-like factors [89], Elk-like factors, MYCL1 [90; 91], FOXO1, and FOXO3 [92; 93; 94]. Interestingly, many members of SMAD family proteins including SMAD1, SMAD2, SMAD3, SMAD5, (receptor-regulated SMADs), SMAD4 (common mediator) and SMAD9 (antagonistic SMAD are also predicted targets of novel miRNAs) [95; 96; 97]. Intriguingly many members of Kruppel-like family of transcription

factors including KLF3, KLF4, KLF5, KLF6, KLF8, KLF9, KLF11, KLF12, KLF13, KLF14, KLF17 [98; 99] are also potential targets of novel PrEC and PrSC miRNAs. In addition we found that many more transcription factors families including Runt related transcription factors (including RUNX1, RUNX1T1, RUNX2) [100], cAMP responsive element binding proteins (including CREB1, CREB5, CREBZF, CRABL2) [101; 102; 103], Sry-related HMG Box or SOX transcription factors (including SOX1, SOX5, SOX4, SOX6, SOX11, SOX12, SOX9, SOX14, SOX17 SOX30) [104; 105; 106; 107] as predicted targets of novel PrEC and PrSC miRNAs.

Let-7 miRNAs are widely accepted as tumor suppressor miRNAs [33; 71; 108]. Let-7 family members appears to play a significant role in cell differentiation, regulation of proliferation and developmental modulation and loss of expression of these miRNAs is associated with cancer development and progression. Data analysis revealed that the Let-7 family has very pervasive expression in both, PrEC and PrSC cells. This can be explained as multiple members of the let-7 family miRNA targets and negatively regulate metastasis promoting genes including oncogenes KRAS and MYC [70; 78], chromatin associated non-histone proteins including HMGA1, MHGA2 [74; 77; 109], cell cycle regulator CDK6 [73], and Lin28B [110; 111; 112]. Downregulation of Let-7 family members has been linked to the carcinoma of the breast [113], lung [114], melanoma [115], pancreas [116], prostate [117], Burkitt lymphoma [118], ovarian [119; 120] and mesothileoma [121]. Evidence suggests that replacement of let-7 member's expression might be a viable therapeutic option in certain types of cancer where their expression has been suppressed [122]. An overwhelming majority of let-7 family members has much higher level of expression in PrSC cells. The high level of expression of let-7 family miRNA members in both, stromal and epithelial cells appears to maintain basal

level of expression of proliferation promoting growth factors in order to govern naïve cellular microenvironment.

5. Conclusion

Noncoding miRNAs control posttranscriptional gene regulation by translational repression, deadenylation and to certain extent by mRNA degradation in animals [19; 123]. The objective of this study is to generate a comprehensive miRNA profile in the widely used cell culture models to explore their major regulatory pathways. Using bioinformatics tools, data filtering strategies and existing published data we delineated that highly expressed miRNAs in PrSC and PrEC are potential regulators of various signaling pathways involved in stromal and epithelial cell communication, growth, differentiation and proliferation. Moreover, miRNA profiling of PrEC and PrSC also demonstrates a distinct pattern of expression and its correlation with available information of miRNAs implicated in prostate cancer reflects unique molecular changes that occurs during the development of the disease. The novel miRNAs and their potential targets have potential to offer valuable insights in future studies on how miRNAs and their targets are involved during epithelial-stromal cell interactions and how dysregulation of these miRNAs may contributes to prostate carcinogenesis. In summary, sRNA analysis of PrEC and PrSC will accelerate our understanding of miRNAs which might contributes in normal prostate microenvironment and those miRNA which potentially influence prostatic diseases including cancer.

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Figure Legends

Figure 1. Distribution of annotated reads in small RNA libraries. (A &B) Histogram showing the distribution of annotated total number of reads and unique reads in PrEC and PrSC, respectively. (C and D) Percentage of number of unique reads mapped to the human genome (REPBASE) for PrEC and PrSC. These include all RNA species (premature miRNA and other ncRNAs such as snoRNA, snRNA, miscRNA, rRNA, tRNA and mRNA).

Figure 2. Cluster analysis of the fold change in expression of microRNAs of PrEC and PrSC. Heat map analysis: Differentially expressed microRNAs in PrEC and PrSC 10 or higher reads were clustered using hierarchical clustering and heat maps were generated using Tree view. Briefly, read count from 211 microRNAs was log transformed and clustered by average linkage using Cluster3.0 and heat maps for the filtered data were generated. 2A represents the heat map for miRNAs more abundant in PrSC Vs PrEC. 2B represents the microRNA more abundant in PrEC Vs PrSC. The color bar represents the scale of differential expression.

Figure 3. Expression of highly abundant microRNAs of PrEC and PrSC cells. (A) Higher PrSC and (B) PrEC differential expression (a minimum of 2 fold difference) of highly abundant

microRNA (100 or higher reads in either of the cell line). (C) Quantitative real-time PCR expression profile of miR-29a-3p and miR-100-5p and miR-27b-3p in PrEC and PrSC. The relative expression of each miRNA is plotted as $2^{-\Delta Ct}$ after normalization to sno66 expression. Each bar represents mean $\pm$ SEM of at least 3 replicates.

Figure 4. Expression of Let-7 family of microRNAs in PrEC and PrSC cells. (A) Higher PrSC and (B) PrEC differential expression (a minimum of 2 fold difference) of let-7 microRNA (100 or higher reads in either of the cell line). (C) let-7a-3p and let-7f-1-3p miRNA has same low numbers in both the cells.

Figure 5. Functional gene set overlap graphics summarizing predefined gene sets (and their pairwise overlaps) that is over-represented in an input miRNA list.

Open source databases such as MiRecords and TargetScan was used for target prediction of differentially expressed and highly abundant miRNAs. Pathways analysis of the obtained gene set was done to obtain the overlap graphs presented here. (A) Functional gene set overlap graph for a total of 1857 differentially expressed genes based on expression of highly abundant PrSC miRNA that target them. (B) Functional gene set overlap graph for a total of 468 genes differentially expressed based of expression of highly abundant PrEC miRNA that target them. Blue node in the overlap graph represents curated pathway. The node size reflects the size of the gene set and the node color—is *P*-value (deeper red means smaller *P*-value). Each edge denotes an overlap between gene sets (i.e. shared genes). The edge width reflects the size of the overlap and its color reflects the number of genes from the input list that are contained in the overlap.

The common output of most tools for gene set enrichment analysis is a table (such as Tables 3 and 4 for the gene enrichment analysis of our data) where predefined functional genes are listed, and ranked according the P-value. However, the functional sets often overlap with each other to some extent and share common key regulators. Therefore, the overlap graphics are helpful in facilitating the visual interpretation of over-representation/enrichment analysis results. This visual representation helps to quickly identify related biological processes having the common key regulators. In addition, it gives a quick overview of the relationships between the different types of functional sets.

Table 1: Experimentally determined target genes of highly abundant miRNAs of PrEC and PrSC cells: The table show the experimentally determined targeted genes of highly abundant and expressed miRNAs in PrEC and PrSC cell lines. These targets are at least once experimentally tested and reported in the publication, which is shown next to target in parenthesis. The differential expression of these miRNAs in Prostate Cancer is shown in the column 2.

Table 2: Target genes of highly expressing let-7 family miRNAs of PrEC and PrSC cells: The table show the hand-curated target genes of let-7 family of miRNAs identified in PrEC and PrSC. These targets are experimentally validated and reported in a publication, which is shown next to target in parenthesis. The differential expression of let-7 miRNAs in Prostate Cancer has also been shown in column 2 of the Table 2.

Table 3: GO functional annotation of pathways regulated by highly expressed microRNA

in PrSC: The predicted target genes of highly abundant PrSC microRNA were analyzed using over representation analysis based on the NESTs of 1-next neighbor and gene ontology level 2 categories with a cut off P value of 0.01, performed by ConsensusPathDB interaction database. The target gene associated pathways are identified using the pathways databases associated with ConsensusPathDB and are ranked in the order of P value [52; 124].

Table 4: GO functional annotation of pathways regulated by highly expressed microRNA

in PrEC: The predicted target genes of highly abundant PrSC microRNA were analyzed using over representation analysis based on the NESTs of 1-next neighbor and gene ontology level 2 categories with a cut off P value of 0.01, performed by ConsensusPathDB interaction database. The target gene associated pathways are identified using the pathways databases associated with ConsensusPathDB and are ranked in the order of P value [52; 124].

Table 5: Novel miRNAs of PrEC and PrSC cells

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FIGURE 1

C

D

FIGURE 1

A

B

FIGURE 2

FIGURE 3

Figure 3

FIGURE 4

A **B** **FIGURE 5**

Table 1

Target genes of highly abundant miRNAs of PrEC and PrSc cells and their experimentally determined expression in Prostate cancer

miRNA	Expression in Pca	Target genes (supplemental References)
hsa-miR-214-3p	Downregulated (1)	PTEN (1), ZBTB20 (2)
hsa-miR-143-3p	Downregulated (1,2)	MAPK7 (3), KRAS (4), ERK5 (5), DNMT3A (6), Bcl-2 (7)
hsa-miR-199b-3p	unknown	IKK, Hif alpha, Ets1, Mst1 (predicted targets)
hsa-miR-411-3p	Downregulated (3)	ITCH (8)
hsa-miR-127-3p	unknown	BCL6 (9), BLIMP1 (10), XBP1 (10)
hsa-miR-152	Downregulated (3)	DNMT1 (11)
hsa-miR-100-5p	Downregulated (2,4)	mTOR (12), IGF-IR (12), RPTOR (12), FGFR3 (13), PLK1 (14)
hsa-miR-221-3p	Downregulated(1,3)	SOCS3 (15) and IRF2 (15)
hsa-miR-29a-3p	Downregulated (2)	BACE1 (16), DNMT3A (17), DNMT3B (17), SPARC (18), PIK3R1 (19), CDC42 (19), COL3A1 (20), MCL1 (21), CXXC6 (22), CDK6 (22)
hsa-miR-200b	Upregulated (1)	CD276 (23), PTEN (24), col2a1 (25) ZEB1(26), ZEB2 (26), RERE (27), FOG2 (28), ERBB2IP (28) BAP1 (28), KLHL20 (28), PTPRD (28), ELMO2 (28), WDR37 (28) ERRFI1 (29), WAVE3 (30), BMI1 (31)
hsa-miR-205-5p	Downregulated (3)	ZEB2 (26, 35), ZEB1 (26), VEGFA (32), INPPL1 (33), VEGFA (34) ERBB3 (35), PRKCE (34), MED1 (36)
hsa-miR-203	Downregulated (5)	SOCS3 (37), TP63 (38), ABL1 (39)
hsa-miR-200c-3p	Upregulated (5)	ZEB1 (39,40), ERRFI1 (41), JAG1 (42)

Table 1: Experimentally determined target genes of highly abundant miRNAs of PrEc and

PrSc cells: The table show the experimentally determined targeted genes of highly abundant and expressed miRNAs in PrEc and PrSc cell lines. These targets are at least once experimentally tested and reported in the publication which is shown next to target in parenthesis. The differential expression of these miRNAs in Prostate Cancer is shown in the column 2.

Table 2

Experimentally validated target genes of highly abundant PrEC and PrSC cell line let-7 family microRNAs and their respective expression in PCa

miRNA	Expression in PCa	Target genes (Supplemental References)
hsa-let-7a-5p	Upregulated (1,5)	NF2 (43), KRAS (44), NRAS (44), TRIM71 (45), LOC561754 (46), HMGA2 (47), RAVR2 (48), LIN28 (49), CASP3 (50), DICER1 (51), PRDM1 (52), ITGB3 (53), EIF2C4 (54), ACP1 (55), DAD1 (55), EIF3S1 (55), MED28 (55), RTCD1 (55), SMOX (55), κ B-Ras2 (56), MOR (57), NIRF (58)
hsa-let-7b	Downregulated (6)	LIN28 (59), CCND1 (60), CDC25A (61), BCL7A (62), HMGA2 (63), CDK6 (61), KRT (64)
hsa-let-7c	Upregulated (7)	MYC (65), TRIM71 (45), HMGA2 (66), TGFBR1 (67), EIF3S1 (55), MED28 (55), RTCD1 (55)
hsa-let-7d-5p	Downregulated (5,6)	DICER1 (51), DAD1 (55), EIF4G2 (55), RABGAP1L (55), SMOX (55), DAD3R (68)
hsa-let-7f-5p	Upregulated (5,7)	KLK10 (69)
hsa-let-7i-5p	Upregulated (8)	TLR4, (70) BMP2K (55), DAD1 (55), EIF4G2 (55), FANCD2 (55)

Table 2: Target genes of highly expressing let-7 family miRNAs of PrEc and PrSc cells: The table show the hand-curated target genes of let-7 family of miRNAs identified in PrEc and PrSc. These targets are experimentally validated and reported in a publication which is shown next to target in parenthesis. The differentially expression of let-7 miRNAs in Prostate Cancer has also be shown in column 2 of the Table 2.

Table 3*GO functional annotation of pathways regulated by highly expressed microRNA in PrSC*

Pathway	Count	%	P-value	Pathway source
EGF-EGFR Signaling Pathway	161	41	7.32E-12	Wikipathways
BDNF signaling pathway	140	32	2.59E-08	Wikipathways
EGFR1	455	67	2.85E-07	NetPath
Focal adhesion - Homo sapiens	207	37	1.70E-06	KEGG
Proteoglycans in cancer - Homo sapiens	225	38	5.07E-06	KEGG
Signaling by PDGF	178	32	7.48E-06	Reactome
TGF_beta_Receptor	173	30	2.95E-05	NetPath
Focal Adhesion	184	28	0.000523	Wikipathways
Integrin	124	21	0.000619	INOH
Androgen Receptor	143	23	0.000738	NetPath
Androgen receptor signaling pathway	88	16	0.00123	Wikipathways
TGF-beta signaling pathway	80	14	0.00351	KEGG
Prostate Cancer	71	13	0.00322	Wikipathways

Table 3: GO functional annotation of pathways regulated by highly expressed microRNA

in PrSC: The predicted target genes of highly abundant PrSC microRNA were analyzed using over representation analysis based on the NESTs of 1-next neighbor and gene ontology level 2 categories with a cut off P value of 0.01, performed by ConsensusPathDB interaction database.

The target gene associated pathways are identified using the pathways databases associated with ConsensusPathDB and are ranked in the order of P value [52; 124].

Table 4*GO functional annotation of pathways regulated by highly expressed microRNA in PrEC*

Pathway	count	%	P value	Pathway source
Signaling events mediated by focal adhesion				
kinases	62	7	0.000942	PID
Integrins in angiogenesis	63	7	0.00104	PID
HIF-1-alpha transcription factor network	66	7	0.00137	PID
MAPK signaling pathway - Homo sapiens	257	15	0.00235	KEGG
TGF_beta_Receptor	173	11	0.00463	NetPath
VEGFR1 specific signals	29	4	0.00587	PID
IGF1R signaling cascade	86	7	0.00615	Reactome
Prostate Cancer	71	6	0.00917	Wikipathways

Table 4: GO functional annotation of pathways regulated by highly expressed microRNA

in PrEC: The predicted target genes of highly abundant PrSC microRNA were analyzed using over representation analysis based on the NESTs of 1-next neighbor and gene ontology level 2 categories with a cut off P value of 0.01, performed by ConsensusPathDB interaction database.

The target gene associated pathways are identified using the pathways databases associated with ConsensusPathDB and are ranked in the order of P value [52; 124].

Table 5

PrEc	PrSc	miR	miR_seq	miR*
1	2	1 t133266	AAAGGGUGAGAAUACCAGCCAU	t2802731
2	1	1 t3972128	UGACUACUUUGUUUGAUUUUGU	t56436
3	0	1 t1073351	AGGCGGACUGUGGAGGCUGCAG	t2936640
4	1	3 t1709332	CAUAAACUGCAUGCCUGCACAC	t3139418
5	2	1 t1887441	CCUCAGAUCUGUUGGACUAUU	t3514466
6	4	10 t1902102	CCUGGCGGCUGUGUCUUCACA	t866321
7	1	4 t1990973	CGCGCCUGCAGGAACUGGUAGA	t4423107
8	20	5 t4100209	UGCGCUCUGAAAGGCCGGGUCC	t2831915
9	0	1 t4514899	UUGAACUUGGGACUAAGCCUGU	t2444706
10	1	1 t2565575	GAUGAGGCAGAGUAAACUCACA	t4052257
11	1	1 t2621268	GCAGGAAGAGACGCAGCAGCUUG	t1035480
12	0	1 t503747	AAUAUCUCUCUUGCCUCACCCU	t2979887
13	1	1 t3427063	UAGACAGACGCAGGUACACACA	t4293560
14	2	0 t3305116	UAACAUAUUUUGAAAGGCCUUUC	t1070755
15	5	8 t3733779	UCCGUCCGCGUGUGGAAGGUCC	
16	5	3 t3545567	UAUCCGCUGGCUUACUCUCUCU	
17	1	2 t4131494	UGGAAUUAUAACUCGGUGCUCU	
18	2	1 t3138102	GUGCACGUGAGGAUGGCUCUGA	
19	1	0 t4312554	UUAAAAAUUCCCUGUUGGCAU	
20	0	1 t3320411	UAAGCAGAAGUAGUGAACUUGAU	
21	1	0 t3366647	UACAGUAGGCACAGAAUCCCA	
22	1	1 t3387461	UACCAGUGAGGCUGACAGAUGA	
23	1	0 t3440992	UAGAUAUCAUCAGAGCUGUGUAU	t626395
24	1	1 t208225	AACACCUCAGAAAAGGGACAGA	
25	1	0 t3318086	UAAGAGAGGAGACUGAAUACUG	
26	0	1 t3583876	UAUUUUUCUUAUAGGCUUCUAA	
27	1	1 t3506582	UAGGUGGAAGGACUGAGCCU	
28	0	1 t3670590	UCAGUGGGUUAUGGCGUUUGA	t1660983