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Low-Density Taqman miRNA Array Reveals miRNAs Differentially Expressed in Prostatic Stem Cells and Luminal Cells

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Abstract

BACKGROUND—miRNAs are a class of naturally occurring small RNAs that generally repress gene expression. They have been shown to actively control diverse biological processes including stem cell differentiation and lineage commitment.

METHODS—Fluorescence-activated cell sorting was utilized to isolate murine prostate stem cells and differentiated luminal cells. The expression levels of Drosha and Dicer1, the two key RNaseIII enzymes for miRNA maturation, were evaluated by quantitative RT-PCR. Low-density Taqman miRNA array analyses were also performed to identify miRNAs that are differentially expressed in individual lineages.

RESULTS—Drosha and Dicer1 are expressed at comparable transcriptional levels in murine prostate stem cells and differentiated luminal cells. Twenty-nine miRNAs were discovered to be differentially expressed in prostate stem cells and luminal cells. Many of these miRNAs are coded in clusters, suggesting a cell-specific transcriptional regulation. Some of these differentially expressed miRNAs have been reported to regulate genes relevant to the molecular and phenotypic features of each lineage.

CONCLUSIONS—miRNAs may play a potentially critical role in fine regulation of prostatic lineage identity.

Keywords

prostate stem cells; miRNA; Dicer1; Drosha

INTRODUCTION

miRNAs are a class of naturally occurring 19–25 nt single strand RNAs that generally repress gene expression at post-transcriptional levels through perfect or imperfect complementarity with mRNAs (reviewed in Ref. [1]). miRNAs were first identified in screens for genes that mediate developmental transitions in nematodes, implying their important roles in regulating developmental processes [2,3]. This has been clearly

corroborated by recent genetic studies in vertebrates [4,5]. Numerous tissue-specific and developmental stage-specific miRNAs have been identified by deep sequencing of small RNA libraries or using microarray analyses of various platforms [6–9]. Some of them have proven critical for neuronal, muscle, and lymphocyte development, such as miR-124, miR-133, miR-1, miR-181, miR-223, miR-155, etc. [10–15].

miRNAs regulate gene expression in ways very similar to those of transcription factors (TFs) [16]. Their expressions are spatio-temporally regulated, their activities are pleiotropic, and they tend to work in ways that are combinatorial and cooperative. More interestingly, recent studies have shown that both TFs and miRNAs are capable of dictating cellular identity. Stem cell-specific TFs can reprogram diverse types of differentiated cells into pluripotent cells (iPS) [17–19], while pancreatic islet-specific TFs can convert exocrine cells into islet cells [20]. Similarly, the embryonic stem cell-specific miR-302 alone has been shown to be able to confer pluripotency to human skin cancer cells [21]. Furthermore, some miRNAs like miR-145, miR-1, and miR-133 have been shown to serve as determinants of pluripotency and differentiation of human embryonic stem cells [22,23]. Therefore, it is important to study miRNA signatures in stem cells to better understand molecular mechanisms underlying diverse developmental processes, such as stem cell maintenance, lineage specification, and cell fate determination.

The expression profile of prostatic epithelial lineage-specific miRNAs has not been documented; however, several studies have demonstrated a change in miRNA expression profiles between normal and cancerous human prostate tissues [24–29]. The expression level of Dicer1 increases slightly during prostate cancer progression [29,30], which may partly account for the altered expression profiles of miRNAs in cancer. Of note, prostatic tissue specimens with stromal cells as well as cells of endothelial and hematopoietic lineages were used in these studies. Therefore, the miRNA expression profiles in those studies are not likely to exclusively represent the prostatic epithelial lineage-specific miRNA expression profiles.

Little has been known about the miRNAs specifically expressed in prostate stem cells. Prostatic epithelium is mainly composed of three major cell types: the luminal cells that directly surround the lumen and produce secretory proteins and the basal cells that align themselves between luminal cells and basement membrane [31]. Neuroendocrine cell is a third minor cell type whose function is not completely clear yet. Murine prostate tissues are capable of undergoing numerous cycles of involution and regeneration in response to fluctuating serum androgen level, implying the existence of stem cells in the prostate [32]. Stem cells are suspected to localize in the basal cell layer because basal cells are independent of androgen for their survival, while most luminal cells undergo apoptosis in the absence of androgen [33]. We and others have recently validated this hypothesis by several in vivo and in vitro functional studies. A small number of murine prostate cells that display a surface antigen expression profile of Lin(CD31, CD45, Ter-119)[−]Sca-1⁺CD49f^{high}Trop2⁺ possess stem cell activity in both an in vivo prostate regeneration system and an in vitro prostate sphere assay [34–38]. More impressively, Leong et al. [39] recently claimed successful regeneration of prostatic tissues from single murine prostate-derived cells displaying a surface antigen expression profile of Lin[−]Sca-1⁺CD44⁺CD133⁺CD127⁺. Consistently, all isolated stem cells express basal cell-specific lineage markers.

Using low density Taqman miRNA array analyses, we found that 29 miRNAs are differentially expressed in either stem cells or differentiated luminal cells while the majority of miRNAs are expressed at a comparable level in both cell populations. Many of the differentially expressed miRNAs have been shown to regulate the expression of genes

closely relevant to phenotypic features of each population. These data imply that miRNAs may act as critical fine regulators for prostate homeostasis.

MATERIALS AND METHODS

Mouse Strains

The wild-type C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All of the mice were housed and bred under the regulation of The Center for Comparative Medicine at the Baylor College of Medicine.

FACS Sorting of Murine Prostate Cells

Dissociated murine prostate cells were suspended in DMEM/10% FBS and stained with antibodies for 15 min at 4°C. The antibodies used were FITC-anti CD31, CD45, and Ter119 antibodies (eBioscience, San Diego, CA), PE-anti Sca-1 antibody (eBioscience) and Alexa 647-anti CD49f antibody (Biolegend, San Diego, CA). FACS sorting was performed by using the BD FACS Aria II (BD Biosciences, San Jose, CA).

RNA Isolation and Quantitative RT-PCR

Total RNA was isolated from cells using mirVana miRNA isolation kit (Ambion, Austin, TX). Reverse transcription was performed using the Superscript™ III first-strand synthesis system (Invitrogen, Carlsbad, CA) or the Taqman microRNA RT kit (Applied Biosystems Incorporation, Foster City, CA). Quantitative RT-PCR was performed using the Qiagen Quantitect SYBR Green RT-PCR kit (Qiagen, Valencia, CA) or the Taqman microRNA assay and universal PCR mastermix (Applied Biosystems Incorporation) on a StepOne plus real-time PCR system (Applied Biosystems Incorporation). Primer sequences are as below: *actin* forward: GACGGCCAGGTCATCACTAT; *actin* reverse: CGGATGTCAACGTCACTT; *Sca-1* forward: CCATCAATTACCTGCCCCTA; *Sca-1* reverse: GGCAGATGGGTAAGCAAAGA; *CD49f* forward: CGCTGCTGCTCAGAATATCA; *CD49f* reverse: AAGAACAGCCAGGAGGATGA; *K5* forward: ACCTTCGAAACACCAAGCAC; *K5* reverse: GTTCTGGAGGTTGGCACACT; *K8* forward: AGCTGAGGCTGAAACCATGT; *K8* reverse: TTGATGTTGCGGTTTCATCTC; *p63* forward: CTTCCGTGAGCCAGCTTATC; *p63* reverse: GAGTGGAGGAGGGAGAGCTT; *Nkx3.1* forward: CGACTGAACCCGAGTCTGAT; *Nkx3.1* reverse: ATGGCTGAACTTCCTCTCCA; *Dicer1* forward: CTTTGTGACACCTCGGCATTT; *Dicer1* reverse: CGGAACCAGGATCTGTTTGT; *Drosha* forward: CGAAGAGATGATGCCTGAGA; *Drosha* reverse: GGAGGGCTGTCTTCAAACAA.

Low-Density microRNA Taqman Array

Total RNA, including miRNAs, was isolated from FACS sorted LSC and luminal cells using mirVana miRNA isolation kit (Ambion). For miRNA cDNA synthesis, RNA was reverse transcribed using the miRNA reverse transcription kit (Applied Biosystems Incorporation) in combination with the stem-loop Megaplex primer pool (Applied Biosystems Incorporation), allowing simultaneous reverse transcription of 450 miRNAs and endogenous controls. As a limited amount of RNA (<250 ng) was procured from the LSC cells, we pre-amplified the RNA according to the manufacturer's instructions. Megaplex RT product (5 µl) was pre-amplified using Applied Biosystems' TaqMan PreAmp Master Mix (2×) and PreAmpPrimer Mix (5×) in a 25-µl PCR reaction [40]. Both LSC and luminal cells were amplified using equal amounts of RNA (30 ng) to avoid systemic variations introduced during pre-amplification.

miRNA expression profiles were acquired using the low-density miRNA Taqman array per manufacturer's instructions. For each cDNA sample, 384 small RNAs were profiled using a gene maximization PCR plate setup in a 384-well plate. The 384-well rodent array pool A plate contains 4 wells of U6 snoRNA as internal controls, 335 individual mouse miRNAs in each well, and 45 wells of two other snoRNAs and miRNAs of rat and other species. Cycling conditions were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. All PCR reactions were performed on the 7900HT RT-qPCR system (Applied Biosystems Incorporation). Raw C_q (quantification cycle: the crossing point between the baseline corrected amplification curve and threshold line) values were calculated using the SDS software v.2.3 [40]. Array experiments were performed twice using samples from two independent biological experiments.

RESULTS AND DISCUSSION

Prostatic Stem Cells and Differentiated Luminal Cells Express Comparable Levels of Drosha and Dicer1

Drosha and Dicer1 are the two key RNaseIII enzymes that are essential for the maturation of miRNAs (reviewed in Ref. [41]). Altered expression of these two enzymes in cancer tissues may partly account for the differential expression of miRNAs [29,30]. We sought to investigate whether the expression levels of these two enzymes are similar in the enriched prostatic stem cells and luminal cells by quantitative RT-PCR analyses.

We have previously shown that antigenic profiles of prostatic stem/basal cells (LSC cells) and luminal cells are $\text{Lin}^- \text{Sca-1}^+ \text{CD49}^{\text{high}}$ and $\text{Lin}^- \text{Sca-1}^- \text{CD49}^{\text{low}}$, respectively [36]. Dissociated single prostate cells were prepared as described previously [37] and stained with fluorescence-conjugated antibodies against lineage markers, Sca-1 and CD49f. LSC cells and luminal cells were FACS sorted as shown in Figure 1A. Post-sorting purity analyses confirmed the successful separation of the two populations (Fig. 1A). Total RNAs including small RNAs were extracted from sorted cells and quantitative RT-PCR analyses were performed to confirm the lineage identities of the sorted cells. The mRNA expression levels of the surface antigen Sca-1 and CD49f in the LSC and luminal cells were consistent with the protein expression levels measured by FACS analysis (Fig. 1B). LSC cells expressed high levels of the basal cell markers keratin5 and P63, while the luminal cell markers keratin 8 and Nkx3.1 were enriched in the luminal cell fraction. These data demonstrate the successful separation of the LSC stem/basal cells and the luminal cells.

Expression of Dicer1 and Drosha in LSC and luminal cells was evaluated by qRT-PCR. Figure 1C shows that the two cell populations express these two genes at similar transcriptional levels. Though the expression of Dicer in the LSC cells was slightly lower than that in the luminal cells, it was not statistically significant. This result implies that there may be no significant differential regulation in Dicer-mediated processing steps during miRNA maturation in the prostatic stem cells and differentiated luminal cells.

Low-Density Taqman miRNA Array Reveals miRNAs Differentially Expressed in Prostatic Stem Cells and Luminal Cells

Like TFs, miRNAs have been shown to regulate gene expression, dictate stem cell identity, and induce lineage differentiation [17–19]. miRNAs that are specifically or differentially expressed in individual prostatic lineages may play key roles in regulating cellular lineage commitment or status. We performed a low-density miRNA Taqman array, representing 335 individual mouse miRNAs, in order to identify these lineage specially expressed miRNAs.

We performed the array experiments twice using samples from two independent biological experiments. Raw C_q (quantification cycle: the crossing point between the baseline

corrected amplification curve and threshold line) values were calculated using the SDS software v.2.3. Relative expression level of individual miRNA in each sample was represented by $\Delta C_q(C_q^{\text{miRNA}} - C_q^{\text{U6}})$ and differential miRNA expression levels between the two samples were expressed by $\Delta\Delta C_q(\Delta C_q^{\text{luminal}} - \Delta C_q^{\text{LSC}})$. A positive value in $\Delta\Delta C_q$ indicated that a miRNA was expressed more in the stem/basal cells and vice versa for a negative value.

We found that none of the 335 miRNAs was exclusively expressed in either cell population. There were a few miRNAs that were detected in only one cell population; however, most were only detected in one of the biological experiments. For several miRNAs that were amplified in only one cell population in both biological experiments, the C_q value was usually over 30, which could be regarded as being expressed at very low levels considering that the samples have been pre-amplified for 12 cycles. In conclusion, 160 out of the 335 miRNAs were under the detection threshold in both prostatic basal/stem and luminal cells (Table I).

We then examined whether the rest of the 175 miRNAs are differentially expressed in either of the two cell populations. Statistical analysis demonstrates that the two sets of results from the two experiments were significantly correlated ($R = 0.47$, $P < 1E - 09$), indicating that the data were consistent between replicates (Fig. 2). We arbitrarily defined that miRNAs with a $\Delta\Delta C_q > 1.5$ or $\Delta\Delta C_q < -1.5$ (higher or lower, respectively, in LSC versus luminal, equal to a 2.8-fold expression difference) in both experiments as lineage differentially expressed. Based on this criterion, 146 miRNAs were expressed at comparable levels in both cell populations (Table I). Nineteen and 10 miRNAs were differentially expressed in the luminal and stem/basal cells, respectively, as tabulated in Table II.

The miRNA expression profiles of prostate basal/stem cells and luminal cells share many similarities, in accordance to the fact that they belong to the same developmental lineage. Nevertheless, a number of miRNAs differentially expressed between cell lineages may be involved in regulating their respective phenotypic features. A very interesting observation is that many of the lineage differentially expressed miRNAs are transcribed in clusters in genome. For examples, the five members of the miR-200 family (miR-200a, miR-200b, miR-200c, miR141, and miR-429) are expressed in clusters in two different chromosomes and all of them are preferentially expressed in luminal cells; another three clustered miRNAs, miR-96, miR-182, and miR-183, are all expressed at higher levels in luminal cells than in stem cells. These observations imply a cell-specific transcriptional regulation of miRNA expression. Interestingly, Shimono et al. [42] recently found that the miR-200 and miR-96 clusters are also expressed at lower levels in human breast cancer stem cells compared to non-tumor initiating cells. These results implicate that regulation of microRNA expression is conserved to a certain degree between tissues and species.

MiR-205 is preferentially expressed in the prostate basal/stem cells. Interestingly, previous in situ hybridization analysis demonstrated that miR-205 is also specifically expressed in the mammary gland myoepithelial cells that are phenotypically similar to the prostatic basal cells [43]. Mammary gland stem cells also display a lineage identity characteristic of myoepithelial cells [44]. In a separate study using a mammary epithelial cell line Comma-D β , miR-205 was found preferentially expressed in the Sca-1^{high} fraction that enriches for stem cell activities [45]. These studies demonstrate that miR-205 is expressed in both prostatic and mammary gland stem cells, which implies its potential role in regulating stem cell biology or maintaining the phenotypic features of the basal/myoepithelial cells. Studies from both the breast cancer and the prostate cancer models have demonstrated that miR-205 induces mesenchymal to epithelial transition and may act as a tumor suppressor [46,47].

MiR-203 is preferentially expressed in the luminal cells. Studies on skin stem cells have proven *P63* as a direct target of miR-203 [48]. *P63* is a TF in the P53 super-family and is specifically expressed in a portion of prostatic basal cells at both the mRNA and protein levels [49]. *P63* has been shown to regulate stem cell activity in several tissue systems and germline deletion of *P63* causes prostate agenesis [50–53]. The preferential expression of miR-203 may act as an additional layer of regulation at the post-transcriptional level to suppress the expression of *P63* from residual leaky transcription in luminal cells and tightly safeguard their differentiated status.

Finally, all the miR-200 family members (miR-200a, miR-200b, miR-200c, miR141, and miR-429) are found preferentially expressed in the luminal cells. MiR-200 family members have been shown to induce the expression of epithelial phenotypic marker E-cadherin by inhibiting the expression of epithelial–mesenchymal transition associated TFs *ZEB1* and *ZEB2* [47,54,55]. Conversely, the miR-200 family has been shown to be regulated by *ZEB1* and *ZEB2* through a negative feedback loop [56,57]. The differential expression pattern of the miR-200 family members in the prostate is consistent with a previous observation that the expression level of E-cadherin increases when undifferentiated epithelial progenitor cells undergo maturation into terminally differentiated luminal cells [58]. In summary, the cell lineage-specific differential expression of miRNAs in the prostate highlights a potentially critical role of miRNAs in fine regulation of lineage identity.

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Fig. 1.

Drosha and Dicer are expressed at comparable levels in prostate stem cells and luminal cells.

A: FACS plots show gates set up for sorting of the prostate stem/basal cells (LSC) and the differentiated luminal cells. FACS plots for post-sorting purity check confirms the successful separation. **B:** Bar graph shows the relative expression levels of the surface antigens (CD49f and Sca-1), basal cell markers (K5 and P63), and luminal cell markers (K8 and Nkx3.1) in the LSC and luminal cells measured by qRT-PCR. Beta-actin was used as the reference gene. Results were from two independent biological experiments. Expression levels in luminal cells were arbitrarily set up as 1 to generate the relative expression levels in stem cells. **C:** Bar graph shows the average relative expression levels of Drosha and Dicer by qRT-PCR from three independent biological experiments. Beta-actin was used as the reference gene.

Fig. 2.

Taqman miRNA array results from two independent experiments comparing expression of luminal with LSC. R- and P-values were by Spearman's rank. miRNAs differentially expressed over 2.8-fold (log base2 of 1.5) are highlighted. $\Delta\Delta C_q, \Delta C_q^{\text{luminal}} - \Delta C_q^{\text{LSC}}$. Positive values in $\Delta\Delta C_q$ indicate that a miRNA is expressed more in LSC versus luminal, and vice versa for negative values.

TABLE I

The miRNAs that Are Not Expressed in Prostate Epithelia or Not Differentially Expressed in Prostatic Basal/Stem and Luminal Cells

miRNAs that are not expressed in murine prostate epithelia (a total of 160 miRNAs)	105, 122, 124, 125b-3p, 129-5p, 134, 139-3p, 142-5p, 147, miR153, 154, 188-3p, 18b, 199a-5p, 201, 202-3p, 202-5p, 207, 208, 208b, 211, 215, 216a, 216b, 217, 219, 220, 23a, 224, 290-3p, 291a-3p, 291b-5p, 292-3p, 293, 294, 295, 296-3p, 297c, 297b-5p, 298, 302a, 302b, 302c, 302d, 323-3p, 324-3p, 325, 329, 330, 337-3p, 338-3p, 339-5p, 342-5p, 344, 346, 34c, 351, 363, 367, 369-5p, 369-3p, 370, 377, 379, 380-3p, 381, 382, 383, 384-3p, 409-5p, 423-5p, 431, 448, 449a, 449b, 450a-5p, 450b-5p, 452, 453, 455, 464, 465a-3p, 465a-5p, 465b-5p, 466h, 467a, 467b, 467c, 467d, 467e, 468, 469, 470, 486, 487b, 488, 489, 490, 493, 495, 496, 499, 500, 501-3p, 503, 504, 505, 509-3p, 509-5p, 511, 539, 540-3p, 540-5p, 542-5p, 542-3p, 543, 544, 546, 551b, 582-5p, 582-3p, 590-5p, 598, 615-3p, 615-5p, 654-3p, 654-5p, 665, 666-5p, 668, 669a, 670, 675-3p, 675-5p, 677, 679, 683, 684, 686, 687, 741, 742, 743b-3p, 743b-5p, 743a, 770-3p, 802, 871, 873, 874, 875-3p, 876-3p, 876-5p, 878-5p, 879, 881, 883a-3p, 883a-5p, 883b-3p, 98
miRNAs that are expressed in both basal/stem and luminal cells at comparable levels (a total of 146)	let-7b, let-7c, let-7d, let-7e, let-7f, let-7a, let-7g, let-7i, 1, 100, 101a, 103, 106a, 106b, 107, 10a, 125a-3p, 125a-5p, 125b-5p, 126-3p, 126-5p, 127, 128a, 129-3p, 130b, 132, 133a, 133b, 135a, 136, 137, 138, 139-5p, 140, 143, 146a, 146b, 148b, 150, 151-3p, 155, 15a, 15b, 16, 17, 181a, 181c, 184, 185, 186, 187, 188-5p, 190, 191, 192, 193, 193b, 194, 195, 196b, 197, 19a, 19b, 204, 20a, 20b, 210, 214, 218, 221, 23b, 24, 25, 26a, 26b, 27a, 27b, 28, 29a, 29b, 29c, 301a, 301b, 30a, 30b, 30c, 30d, 30e, 32, 320, 322, 328, 331-3p, 331-5p, 335-3p, 335-5p, 337-5p, 339-3p, 340-3p, 340-5p, 342-3p, 345-3p, 345-5p, 34a, 34b-3p, 350, 361, 362-3p, 365, 375, 376a, 376b, 376c, 380-5p, 384-5p, 409-3p, 425, 433, 434-3p, 434-5p, 451, 484, 491, 494, 497, 532-3p, 532-5p, 547, 574-3p, 652, 667, 671-3p, 672, 674, 680, 682, 708, 744, 7a, 7b, 872, 9, 92a, 93, 99a, 99b

TABLE II

The miRNAs Differentially Expressed in Prostatic Basal/Stem and Luminal Cells ($\Delta\Delta C_q$, $\Delta C_q^{\text{luminal}} - \Delta C_q^{\text{LSC}}$)

miRNA name	$\Delta\Delta C_q$ ($\pm$ SD)
Luminal cell miRNAs	
miR-10b	-3.112 ± 0.451
miR-18a	-3.104 ± 0.843
miR-21	-2.952 ± 0.260
miR-31	-1.774 ± 0.201
miR-96	-3.629 ± 2.210
miR-141	-2.635 ± 0.063
miR-142-5p	-2.617 ± 1.413
miR-148a	-3.586 ± 0.091
miR-152	-2.007 ± 0.111
miR-182	-2.793 ± 0.076
miR-183	-2.849 ± 0.246
miR-200a	-1.813 ± 0.602
miR-200b	-2.482 ± 0.506
miR-200c	-3.281 ± 0.243
miR-203	-2.494 ± 0.187
miR-223	-1.549 ± 0.184
miR-324-5p	-2.714 ± 0.060
miR-429	-2.086 ± 0.139
miR-685	-2.654 ± 0.168
Stem/basal cell miRNAs	
miR-100a-3p	3.658 ± 1.475
miR-130a	3.242 ± 0.828
miR-135b	3.685 ± 0.117
miR-145	1.648 ± 0.017
miR-205	2.533 ± 1.105
miR-222	1.891 ± 0.411
miR-296-5p	2.690 ± 0.000
miR-410	1.998 ± 0.658
miR-411	3.078 ± 1.276
miR-676	3.014 ± 0.643