FEBS Letters 584 (2010) 4426–4434

FEBS Letters

journal homepage: www.FEBSLetters.org

The small RNA expression profile of the developing murine urinary and reproductive systems

Ana Laura Gutierrez Aguilar [a], Robert Piskol [c], Michaela Beitzinger [b], Jia Yun Zhu [a], Dagmar Kruspe [d], Attila Aszodi [e], Markus Moser [e], Christoph Englert [d], Gunter Meister [a,b,*]

[a] Laboratory of RNA Biology, Max-Planck-Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany
[b] University of Regensburg, Universitätsstrasse 31, 93053 Regensburg, Germany
[c] Department of Biology II, Section of Evolutionary Biology, University of Munich, Grosshadernerstrasse 2, 82152 Planegg Martinsried, Germany
[d] Leibniz Institute for Age Research – Fritz Lipmann Institute e.V. (FLI), Beutenbergstrassse 11, 07745 Jena, Germany
[e] Department of Molecular Medicine, Max-Planck-Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany

ARTICLE INFO

Article history:
Received 30 January 2010
Revised 28 September 2010
Accepted 29 September 2010
Available online 8 October 2010

Edited by Tamas Dalmay

Keywords:
microRNA
Kidney
Testis
Ovary
Gene silencing

ABSTRACT

microRNAs (miRNAs) are small non-coding RNAs with fundamental roles in the regulation of gene expression. miRNAs assemble with Argonaute (Ago) proteins to miRNA-protein complexes (miRNPs), which interact with distinct binding sites on mRNAs and regulate gene expression. Specific miRNAs are key regulators of tissue and organ development and it has been shown in mammals that miRNAs are also involved in the pathogenesis of many diseases including cancer. Here, we have characterized the miRNA expression profile of the developing murine genitourinary system. Using a computational approach, we have identified several miRNAs that are specific for the analyzed tissues or the developmental stage. Our comprehensive miRNA expression atlas of the developing genitourinary system forms an invaluable basis for further functional in vivo studies.

1. Introduction

microRNAs (miRNAs) are small, ~22 nucleotide (nt) long, single stranded, non-coding RNAs that regulate gene expression by targeting specific sites in the 3′ UTRs of messenger RNAs (mRNAs) [1–5]. MiRNA genes are transcribed mainly by RNA polymerase II as primary transcripts (pri-miRNAs), which are processed in the nucleus by the microprocessor complex that contains the RNase III enzyme Drosha. The resulting stem-loop-structured miRNA precursors (pre-miRNAs) are transported to the cytoplasm, where another RNase III enzyme, Dicer, cleaves off the loop of the pre-miRNA and generates a double stranded intermediate. Such molecules are unwound and one strand gives rise to the mature miRNA. The other strand, referred to as miRNA*, is degraded. Mature miRNAs are actively loaded into miRNA-protein complexes (often referred to as miRNPs or miRNA-containing RNA silencing complexes – miRISCs) [4,5]. A member of the Argonaute protein family, which specifically anchors the 5′ as well as the 3′ end of the miRNA, forms the core of miRNPs. Ago proteins are the actual mediators of RNA silencing whereas miRNAs function as guides that direct miRNPs to specific functional sites on target mRNAs [6].

miRNAs repress gene expression by guiding mRNA degradation processes or by inhibiting translation without affecting mRNA stability. It has been reported that miRNAs repress translation of specific target mRNAs on the level of initiation or elongation [3,7,8]. Moreover, miRNAs have also been implicated in the activation of gene expression by inducing translation up-regulation of target mRNAs [9–11].

High throughput screenings of cell lines and animal models identified more than 700 miRNA genes in mammals, which are involved in numerous cellular processes such as cell cycle progression, cell differentiation, cell death or developmental timing. To study miRNA function in higher eukaryotes, knock out mice of different miRNAs or miRNA processing enzymes have been generated in recent years. Dicer knockout (KO) mice exhibit a lethal phenotype during early development [12]. Interestingly, conditional deletion of Dicer KOs in specific organs like retina, liver, gonads and kidney, resemble characteristics that are found in human diseases as well [13].

Despite the latest studies, the biological roles of miRNAs during earlier developmental processes remains elusive in many aspects, mainly because miRNAs have a wide spectrum of possible target

* Corresponding author at: Laboratory of RNA Biology, Max-Planck-Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany.
E-mail address: gunter.meister@vkl.uni-regensburg.de (G. Meister).

0014-5793/$36.00
doi:10.1016/j.febslet.2010.09.050

mRNAs and there is a lack of knowledge about miRNA tissue and time specific expression and regulation. During the formation of the genitourinary system, early events are finely tuned, and the disruption of this orchestrated process leads to different kinds of abnormalities.

The mesonephros or Wolffian duct (WDs) are constituted at the embryonic day E11.0 of poorly differentiated cells with mesodermal origin and mesonephric tubules within the urogenital ridge mesenchyme [14,15]. The mesonephros will form tubulis connecting the adult male gonads to the prostate and in females only inclusions will remain in the adult ovary. The metanephros starts forming at the embryonic day 10.5 when the ureteric bud triggers the WDs, stimulating the metanephric mesenchyme that surrounds it. This event promotes the differentiation of the metanephros to mature kidneys in mammals. At the embryonic day E11.5 the ureteric bud starts branching. The metanephros is formed by condensing mesenchymal cells and the first epithelial tubulis; stromal supporting tissue and mesenchymal cells surrounding the developing kidney [15]. At the embryonic days E12 and E13 the ureteric bud continues branching. At this stage the metanephros are formed of epithelial nephritic tubulis; stromal and metanephric mesenchymal zones and mesenchymal cells surrounding the developing kidney [15].

The initial stages of gonadal development (and that of the genital ducts) are identical in female and male embryos, thus generating urogenital rudiments of the ambisexual stage of sex differentiation. In males, beginning at E12.5 to E13, the mesenchymal cells within the gonadal rudiment aggregate and condense into epithelial cords, which will become the seminiferous tubules. In female at E13, the ovary has a relatively featureless internal structure in contrast to males [14,16]. Thus these embryonic days are key stages during the development of adult kidney and gonads.

To identify, which miRNAs are expressed at certain stages during early genitourinary formation, we generated 12 miRNA cDNA libraries from mouse mesonephros, metanephros and gonads from the stages during development E11, E12, E13 as well as adult gonads and kidney. We sequence the cDNA libraries by deep sequencing and validated the expression of a number of miRNAs by qPCR and Northern blotting. We performed a PAM analysis to find tissue and stage specific miRNA clusters to generate a miRNA expression profile of the developing urogenital system that sets the basis to understand their role during development and adult tissue maintenance and the differences in their expression profiles.

2. Materials and methods

2.1. Tissue collection

Tissues were collected by micro-dissection from wild type CD-1 (WT-CD-1) mouse embryos in the stages or days post coito (dpc) E11, E12, E13 with a ±0.5 days from mesonephros, kidneys (metanephros), testis and ovaries and finally adult tissues from the same organs. All the embryonic tissues were kept on ice in sterile PBS buffer. We dissected different numbers of embryos according to the embryonic stage and tissue size, to obtain at least 20 mg of tissue from each sample. The E11 embryos were identified by their telosomite number.

2.2. miRNA isolation and cDNA library construction

The miRNA libraries were generated by Vertis Biotech (Freising, Germany). Total RNA was isolated and enriched using the miRVana miRNA isolation Kit (Ambion, Texas, USA). The enriched total RNA was then run in denaturing 12.5% polyacrylamide gels to separate different RNA populations. The RNAs were precipitated with ethanol and dissolved in water. The RNAs obtained were poly-A-tailed using poly-A polymerase followed by ligation of a RNA adapter to the 5′ phosphate of the poly-A-tailed RNAs: 5′-GCCTCCCTCGC GCCATCAGCTNNNNGACCTTGGCTGTCACTCA-3′. First strand cDNA synthesis was performed using an oligo-dT linker primer and an M-MLV-RNase H reverse transcriptase. The resulting cDNAs were then PCR amplified to about 20 ng/μl using the high fidelity polymerase Phusion (Finnzymes).

Next a PCR reaction was performed for every library. Different adapters were used per library. The adapter plus the miRNA amplicons were sized between 119 and 134 bp. All the amplicons were run in a 6% PAGE gel. The concentration of each amplicon was calculated by their intensity on the gel. The band was eluted from the gel using Nucleospin Extract II (Machirey and Nagel). 15 ng/μl was delivered to 454 technologies to be sequenced.cDNA libraries were obtained corresponding to each tissue and developmental stage. Those libraries were sequenced in parallel at 454 life sciences (Brandford, USA) as previously described [17].

2.3. Validation of miRNA expression via qRT-PCR

qRT-PCR analysis of miRNA expression was performed according to the protocol described by Hurteau et al. [18]. One organ of adult origin or three E13.5 stage derived embryonic organs were pooled and used for RNA extraction using Trifast reagent (Peqlab). 400 ng of each sample were used for the polyA-tailing reaction (Ambion). Half of the polyA-tailing reaction was used for cDNA synthesis using polyA-tail specific adapter primer: 5′-AAC GAG ACG ACG ACA GAC TTT TTT TTT TTT TTT V-3′ (V represents A, G or C).

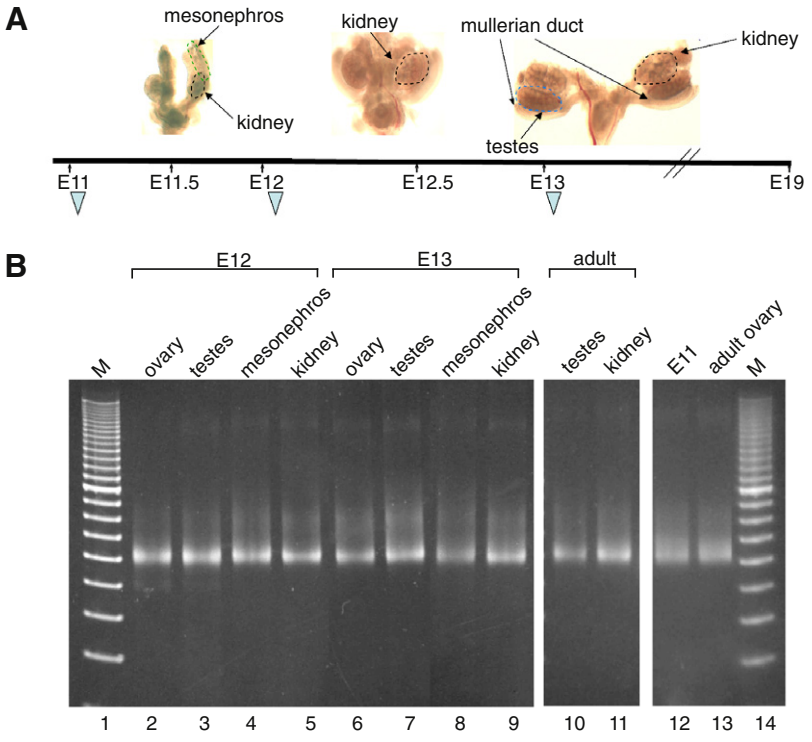
The following primers were used for qRT-PCR: PCR-reverse primer: AAC GAG ACG ACG ACA GAC TTT. Forward primer for miRNA detection: mmu-let7b: 5′-TGA GGT AGT AGG TTG TGT GGT T-3′, mmu-miR-126-5p: 5′-CAT TAT TAC TTT TGG TAC GCG-3′, mmu-miR-17: 5′-CAA AGT GCT TAC AGT GCA GGT AG-3′, mmu-miR-351: 5′-TCC CTG AGG AGC CCT TTG AGC CTG-3′, 18S-control primer: CGT AGG TGA ACC TGC GGA A-3′.

2.4. Computational identification of known ncRNAs and novel miRNA candidates

Approximately 300.000 raw sequences were obtained from 454 sequencing. To identify known non-coding RNAs in these sequences we compiled a comprehensive database of ncRNAs. This set consisted of mouse miRNA and miRNA* sequences obtained from Sangers's miRBase [19], large and small subunits of ribosomal RNAs in mouse (LSU/SSU rRNAs) from the SILVA database [20] as well as 21 mouse tRNAs. We also included other mouse non-coding RNAs (snoRNAs, snRNAs, long non-coding RNAs) from the NONCODE v2.0 database [21] and Piwi-interacting RNAs (piRNAs) from piRNABank [22]. In addition we used mRNAs of housekeeping genes Hprt1, Gapdh and ASCC3L1 to assess the confidence level in our annotation (due to possible degradation products).

All 454 reads were compared to this database using NCBI blastn [23]. A read was identified as an ncRNA only if it showed a hit against the database with a reported e-value of less than 0.01. If several hits below this threshold were found, the one with the smallest value was chosen for annotation.

To analyze possible miRNA candidates, all sequences that were not annotated by the previous procedure were mapped to the mouse genome NCBI Build 37 [24] using blastn. We only retained hits to the genome that showed an e-value smaller than 0.1 and that did neither fall into coding regions of the mouse genome nor regions annotated as repeats or transposons. Overlapping hits were grouped into consecutive hit-regions. Subsequently, the



C

	E11	E12 K	E13 K	Adult k	E12 M	E13 M	E12 T	E13 T	Adult T	E12 O	E13 O	Adult O
Number of reads	13174	30114	20289	14956	26589	37563	13488	36935	17333	20483	33654	12954
Annotated miRNAs	10895	25614	17717	11552	20530	33621	10073	27131	5258	17408	25089	11409
Annotated piRNAs	2	2	5	15	26	3	16	37	3670	5	24	4
rRNAs	262	494	369	780	1350	489	801	1333	1483	1060	1022	462
other ncRNAs	72	149	122	135	235	148	126	268	166	228	277	122
Non annotated sequences	2553	4450	2627	3678	6505	3840	3421	10422	4252	2577	9193	1421

Fig. 1. Small RNA cloning from mesonephros, ovary, testes and kidney from different developmental stages as well as from adult tissues. (A) Schematic representation of the developing genitourinary system. Dashed circles highlight the dissected tissues. (B) Small RNA cloning products from E12 tissues (lanes 2–5), E13 tissues (lanes 6–9), adult tissues (lanes 10, 11 and 13) and the tissue of the E11 stage (lane 12) were analyzed by agarose gel electrophoresis. Lanes 1 and 14 represent DNA size markers. (C) Summary of the cloned and annotated reads from the individual libraries.

genomic region of 100 nucleotides up-/downstream of each hit was evaluated for miRNA candidate genes. Therefore, we calculated the regions' minimal free energy RNA structure using rnafold from the Vienna package [25]. Only sequences that folded into a hairpin structure, that contained more than 80% pairing positions and had the original hit located in a paired region of the structure were considered for further evaluation using the miR-abela web-interface [26].

2.5. Identification of tissue- or stage-specific miRNAs

The specificity of a miRNA for a tissue t (or developmental stage) of interest was defined as the miRNA's above average occurrence in the library of that tissue. Therefore, we computed for each miRNA the difference $D_{\mathrm{miRNA}}(t)$ between its average occurrence in tissue t and its average occurrence in the libraries of all other tissues. For instance, $D_{\text{let-7}}(\text{kidney})$ was calculated as:

$$D_{\text{let-7}}(\text{kidney}) = (\text{E11}_{\text{let-7}} + \text{E12K}_{\text{let-7}} + \text{E13K}_{\text{let-7}} + \text{AdultK}_{\text{let-7}})/4 - (\text{E12T}_{\text{let-7}} + \text{E13T}_{\text{let-7}} + \text{AdultT}_{\text{let-7}} + \text{E12O}_{\text{let-7}} + \text{E13O}_{\text{let-7}} + \text{AdultO}_{\text{let-7}})/6$$

where the first term stands for the average occurrence of let-7 in the libraries of all developmental stages of kidney (i.e. E11, E12K, E13K and AdultK), while the second term quantifies the miRNA's presence in non-kidney stages (i.e. E12T, E13T, AdultT, E12O, E13O, AdultO). Values of $D_{\mathrm{miRNA}}(t) > 0$ indicate higher, $D_{\mathrm{miRNA}}(t) < 0$ lower average occurrence of the miRNA in tissue t, respectively. To detect an overproportionally high (or low) presence for a certain miRNA we compared its specific $D_{\mathrm{miRNA}}(t)$ to the overall distribution of $D(t)$ for all miRNAs in tissue t. The 0.01 and 0.99 quantiles ($Q_{0.01}$, $Q_{0.99}$) of $D(t)$ were chosen as cutoffs and miRNAs with $D_{\mathrm{miRNA}}(t) < Q_{0.01}$ and $D_{\mathrm{miRNA}}(t) > Q_{0.99}$ were described as under- or over-represented, respectively.

We calculated the distributions of $D(t)$ for kidney, testes and ovaries (Fig. 3A–C) and applied an analogous strategy for developmental stages E12, E13 and Adult (Fig. 4A–C). In each case, under- and overrepresented miRNAs were identified based on the above cutoffs.

3. Results and discussion

3.1. Small RNA profile of tissues from the mouse genitourinary system

Mesonephros, metanephros and gonads were dissected from the genitourinary system of developing mouse embryos as well as adult mice. All tissues were isolated from wild-type CD-1 mouse embryos (Fig. 1A). Small RNA libraries were generated by cloning and the amount as well as the quality of the cloning products were analyzed by agarose gel electrophoresis (Fig. 1B). As evident from the agarose gel, all libraries were comparable in terms of the

quantity as well as the quality of the small RNA products (Fig. 1B, lanes 2–13). The individual libraries were subsequently sequenced using 454 sequencing technology. The number of obtained reads ranged from 12 954 in adult ovaries to 37 563 in E13 mesonephros (Fig. 1C). Many of the reads of each library matched to known and annotated miRNAs. However, a large number of so far not annotated sequences were obtained as well. Of note, piRNA sequences were only found in significant amounts in adult testes. Interestingly, piRNAs are not present in developing E11, E12 and E13 testes suggesting that piRNA expression is restricted to adult testis.

The most abundant miRNA in the kidney libraries was identified as miR-199b (Table 1). However, read numbers for miR-199b changed during development and are reduced in adult tissues. A similar expression pattern was observed for miR-25. Reciprocally, the expression of miR-106b and miR-652 is higher in adult kidney compared to stages E12 and E13 (Table 1). In mesonephros tissues derived from the developmental stages E11, E12 and E13, the most abundant miRNAs are miR-199b, miR-652, miR-25 and miR-17 (Table 2). In the testes tissues, there were generally less miRNAs in the adult tissues compared to the embryonic tissues (Table 3). The most abundant miRNAs are miR-199b, miR-17 and miR-625. In ovaries, the expression of the let-7 family is increased during development, whereas the expression of miR-200c and miR-194 is reduced (Table 4).

Our results may reflect that different biological processes are turned on and off during individual embryonic stages of each specific tissue and therefore the expression of diverse small RNA populations is required to regulate such processes.miRNA expression profiles of kidney, gonad and testes tissues have been performed before [13,27,28]. Many of these studies, however, have analyzed adult tissues only or specific cell types of the respective organs [29–31]. Consequently, it is difficult to compare the results with our data. Differences in the miRNA profiling may derive from different technologies (qPCR, microarray, deep sequencing) as well as read numbers that have been generated and cloning protocols that have been used. Interestingly, Ho et al. have analyzed a limited number of miRNAs in adult kidney by Northern Blotting and found that let-7c, miR-23b, miR-24 and miR-26a are highly abundant in adult kidney. All these miRNAs are found in our adult kidney library as well [30].

Table 1
Top 30 miRNAs found in kidney tissues.

Top 30 known miRNAs in kidney			
Annotated miRNA[a] $e = 0{,}01$	% of total reads[b]		
	E12	E13	Adult
mmu-let-7c	0.48	0.79	0.46
mmu-miR-106b	2.10	6.24	5.19
mmu-miR-126-5p	0.28	0.78	0.62
mmu-miR-134	0.37	0.36	0.49
mmu-miR-151-5p	1.31	1.24	1.29
mmu-miR-152	0.43	1.55	0.64
mmu-miR-15b	2.89	4.46	1.97
mmu-miR-16	1.38	1.23	0.93
mmu-miR-17	8.28	14.36	7.17
mmu-miR-196a	3.68	1.75	0.37
mmu-miR-199b	21.22	8.79	6.45
mmu-miR-200b	2.06	2.42	0.47
mmu-miR-200c	1.49	1.21	0.20
mmu-miR-20a	1.94	1.16	0.64
mmu-miR-214	0.29	0.53	0.80
mmu-miR-218	1.50	1.73	1.50
mmu-miR-23a	0.73	0.99	1.11
mmu-miR-23b	3.16	5.22	2.71
mmu-miR-25	14.22	8.41	5.54
mmu-miR-26a	0.54	0.48	0.53
mmu-miR-26b	1.55	1.27	0.64
mmu-miR-27b	2.73	2.49	1.48
mmu-miR-322	0.56	0.61	2.31
mmu-miR-351	0.34	0.39	2.75
mmu-miR-361	0.36	0.32	0.45
mmu-miR-374	0.37	0.22	0.39
mmu-miR-433	0.25	0.25	0.96
mmu-miR-615-3p	0.59	0.41	0.52
mmu-miR-652	1.35	3.85	4.70
mmu-miR-92b	1.10	0.76	1.51

[a] The samples were collected at ±0.5 embryonic days.
[b] Known miRNAs from miRBase from sanger http://www.mirbase.org/.
[c] Normalized data agains the total number of reads found on each library.

Table 2
Top 30 miRNAs found in mesonephros tissues.

Top 30 known miRNAs in mesonephros			
Annotated miRNA[a] $e = 0{,}01$	% of total reads[b]		
	E11	E12	E13
mmu-miR-101b	0.57	0.29	0.13
mmu-miR-106b	6.66	3.35	4.14
mmu-miR-107	1.54	0.35	0.13
mmu-miR-125b-5p	0.71	2.07	0.24
mmu-miR-126-5p	1.65	0.62	0.42
mmu-miR-134	0.76	1.70	0.59
mmu-miR-151-5p	1.27	1.43	1.35
mmu-miR-152	0.83	1.14	1.68
mmu-miR-15b	2.03	0.68	2.04
mmu-miR-16	0.95	0.57	0.80
mmu-miR-17	9.50	3.30	14.10
mmu-miR-199a-5p	0.82	0.27	0.07
mmu-miR-199b	10.92	7.64	22.31
mmu-miR-200b	0.67	1.70	1.78
mmu-miR-20a	0.92	0.36	0.89
mmu-miR-210	1.28	0.17	0.03
mmu-miR-214	3.10	0.46	0.74
mmu-miR-218	1.19	4.04	1.34
mmu-miR-23a	0.83	0.97	2.02
mmu-miR-23b	2.40	2.12	4.33
mmu-miR-25	6.56	3.73	6.56
mmu-miR-27b	1.51	0.91	1.29
mmu-miR-320	0.60	2.28	0.24
mmu-miR-322	1.56	0.35	0.47
mmu-miR-351	1.09	3.13	0.41
mmu-miR-615-3p	0.71	1.92	0.71
mmu-miR-652	5.08	12.53	6.00
mmu-miR-92b	0.90	0.81	0.59
mmu-miR-93	0.80	0.25	0.89
mmu-miR-99b	0.65	0.85	0.36

[a] The samples were collected at ±0.5 embryonic days.
[b] Known miRNAs from miRBase from sanger http://www.mirbase.org/.
[c] Normalized data agains the total number of reads found on each library.

3.2. Validation of miRNA expression by qPCR

miRNA cloning requires the ligation of adapters to the miRNA ends, which may cause a bias towards miRNAs that are preferentially ligated to the adapters. Therefore, we validated our sequencing data by qPCR (Fig. 2). Total RNA from kidney, testes and ovary tissues derived from embryos with the developmental stage E13.5 as well as adults was isolated and further analyzed. To validate our sequencing data, miRNAs miR-17, miR-351, miR-126-5p and let-7b were chosen from the individual miRNA lists for qPCR analysis. Indeed, the most miRNAs showed a similar expression levels when analyzed by qPCR or deep sequencing. All qPCR experiments were normalized to 5S rRNA and similar results were obtained when the 7SK RNA was used for normalization. Of note, we did not succeed to validate miR-351 expression in kidney indicating that the two methods can, at least to some extend, produce different results. However, the expression levels of six out of seven miRNAs were comparable between the qPCR and deep sequencing suggesting

Table 3
Top 30 miRNAs found in testes tissues.

Top 30 known miRNAs in testes			
Annotated miRNA[a] *e* = 0,01	% of total reads[b]		
	E12	E13	Adult
mmu-miR-106b	4.46	3.68	0.23
mmu-miR-107	1.51	0.83	0.37
mmu-miR-125a-5p	0.51	0.35	0.08
mmu-miR-126-5p	0.63	0.44	0.35
mmu-miR-134	0.93	0.96	0.02
mmu-miR-151-5p	0.99	1.14	0.85
mmu-miR-152	0.74	2.52	0.33
mmu-miR-15b	2.04	1.12	1.51
mmu-miR-16	1.15	0.38	0.95
mmu-miR-17	5.29	7.91	0.59
mmu-miR-199a-5p	0.61	0.23	0.12
mmu-miR-199b	8.11	9.86	0.53
mmu-miR-20a	0.68	0.31	0.12
mmu-miR-210	0.67	0.12	0.32
mmu-miR-214	0.96	1.45	0.01
mmu-miR-218	1.59	1.71	0.17
mmu-miR-23a	0.86	0.70	0.47
mmu-miR-23b	2.25	2.15	0.66
mmu-miR-25	6.16	5.89	2.39
mmu-miR-26a	0.58	0.34	0.11
mmu-miR-26b	0.79	1.06	0.07
mmu-miR-27b	1.84	0.86	0.38
mmu-miR-320	2.45	1.51	0.38
mmu-miR-322	1.52	0.38	0.16
mmu-miR-351	2.28	1.61	0.10
mmu-miR-433	0.59	1.12	0.00
mmu-miR-652	4.09	6.85	1.20
mmu-miR-92b	1.48	0.54	0.13
mmu-miR-93	0.98	0.48	0.10
mmu-miR-99b	0.95	0.60	0.09

[a] The samples were collected at ±0.5 embryonic days.
[b] Known miRNAs from miRBase from sanger http://www.mirbase.org/.
[c] Normalized data agains the total number of reads found on each library.

Table 4
Top 30 miRNAs found in ovary tissues.

Top 30 known miRNAs in ovary			
Annotated miRNA[a] *e* = 0,01	% of total reads[b]		
	E12	E13	Adult
mmu-let-7a	0.83	1.04	2.11
mmu-let-7b	1.37	0.25	4.96
mmu-let-7c	2.01	2.72	8.47
mmu-miR-106b	1.00	2.93	1.09
mmu-miR-107	1.23	0.33	1.47
mmu-miR-125b-5p	0.79	0.55	0.93
mmu-miR-126-5p	5.60	0.25	4.49
mmu-miR-151-5p	1.95	1.45	1.54
mmu-miR-152	1.03	1.16	1.13
mmu-miR-15a	1.38	0.18	0.70
mmu-miR-16	1.61	0.52	1.20
mmu-miR-185	1.15	0.05	0.40
mmu-miR-187	1.60	0.04	0.05
mmu-miR-190	1.41	0.03	0.13
mmu-miR-192	0.91	0.01	0.02
mmu-miR-194	2.81	0.01	0.09
mmu-miR-199b	1.92	9.20	6.28
mmu-miR-200b	5.26	0.91	1.21
mmu-miR-200c	1.09	0.55	1.14
mmu-miR-218	1.21	0.83	0.19
mmu-miR-22	1.09	0.02	0.68
mmu-miR-23a	3.70	0.91	5.44
mmu-miR-23b	8.59	3.03	8.47
mmu-miR-25	1.45	5.10	1.50
mmu-miR-26b	0.81	0.68	0.46
mmu-miR-27a	1.02	0.03	1.13
mmu-miR-27b	6.85	0.82	3.64
mmu-miR-320	1.06	0.98	1.40
mmu-miR-378	1.54	0.00	0.59
mmu-miR-652	2.85	9.39	3.79

[a] The samples were collected at ±0.5 embryonic days.
[b] Known miRNAs from miRBase from sanger http://www.mirbase.org/.
[c] Normalized data agains the total number of reads found on each library.

that most of our miRNA expression data reflect real miRNA expression levels.

3.3. miRNA candidate reads

In all libraries, we found in total 1464 reads from 1117 different genomic regions that did not match any known coding or non-coding RNA and that did not fall into repetitive or coding-regions. Folding of a region of approximately 100 nt upstream and downstream of the reads revealed that all these reads derive from hairpin structures. These novel miRNA candidates are typically represented in low read numbers compared to other miRNAs present in our libraries. We further analyzed a number of the miRNA candidates and were indeed able to validate one of them as novel miRNA by Northern blotting (Table 5 and data not shown).

3.4. Clustering of the different libraries

Using a computational approach, we analyzed possible correlations between the miRNA expression patterns of the individual libraries (Fig. 3). Indeed, some libraries clustered closely together indicating similar miRNA profiles. E12 kidney (E12.K) and E13 kidney (E13.K) clustered together, whereas adult kidney (adult.k) did not cluster with E12 and E13 kidney. These data suggests that different miRNA regulatory networks are active in embryonic and adult kidney. All testes libraries clustered closer together and only some individual miRNAs show changed expression patterns. Interestingly, the miRNA expression patterns of adult (Adult.O) and E12 ovaries (E12.O) are rather unique and cluster closely together. E13 ovaries (E13.O), however, cluster with E13 testes (E13.T) suggesting that testes and ovaries may use similar cellular pathways in the E13 stage of development. All other stages, however, seem to differ significantly. No clear conclusions can be drawn for E11, the stage that contains precursors for testes, ovaries and kidney and also mesonephros.

3.5. Tissue-specific miRNAs

It was the aim of our miRNA profiling study to identify miRNAs that are specific for tissues as well as developmental stages within the developing genitourinary system. Therefore, we analyzed the distribution of individual miRNAs within the deep sequencing libraries by calculating the difference *D* between the average occurrence of a specific miRNA in a library of tissue of interest and its average occurrence in the libraries of the other tissues (see Section 2 for further details). Positive values of *D* point to an overrepresentation and negative values to an underrepresentation of the specific miRNA in that tissue compared to its occurrence in other tissues. Using this computational approach, we find that miR-23b, let-7c, miR-126-5p and miR-23a are much more abundant in the different ovary tissues than in any other tissue suggesting that the expression of these miRNAs is important for the proper function and development of mouse ovaries (Fig. 4A, right part). Furthermore, miR-25, miR-17, miR-199b and miR-106b are underrepresented in ovary-derived tissues (Fig. 4A, left part). For kidney tissues we found that miR-199b, miR-17, miR-25 and miR-106b were highly abundant (Fig. 4B, right part), whereas miR-126-5p, let-7c and let-7b were underrepresented (Fig. 4B, left part). The analysis of the miRNA profiles obtained from the different testis

Table 5
A novel miRNA candidate identified from the mouse genome.

Candidate miRNA	Stem-loop structures of putative miRNA precursors[b]	dG[b] (kcal/mol)	Genomic location[c]
Novel murine miRNA *mmu-mir-3473b*[a]	T CTGAGCCATCTCTCCAGCCC - - AAGAT A GACTCGGTAGAGAGGTCGGGAATTTTG / G	−42.2	Chr. 10B2

[a] Names of the putative new miRNA sequences are according to the submission to miRBase.
[b] RNA secondary structure prediction and free energy calculation have been done using mfold version 3.2. The miRNA sequences are underlined.
[c] The genomic location of murine miRNAs are referring to the positions in murine chromosomes according to the assembly from Ensembl (http://www.ensembl.org/).

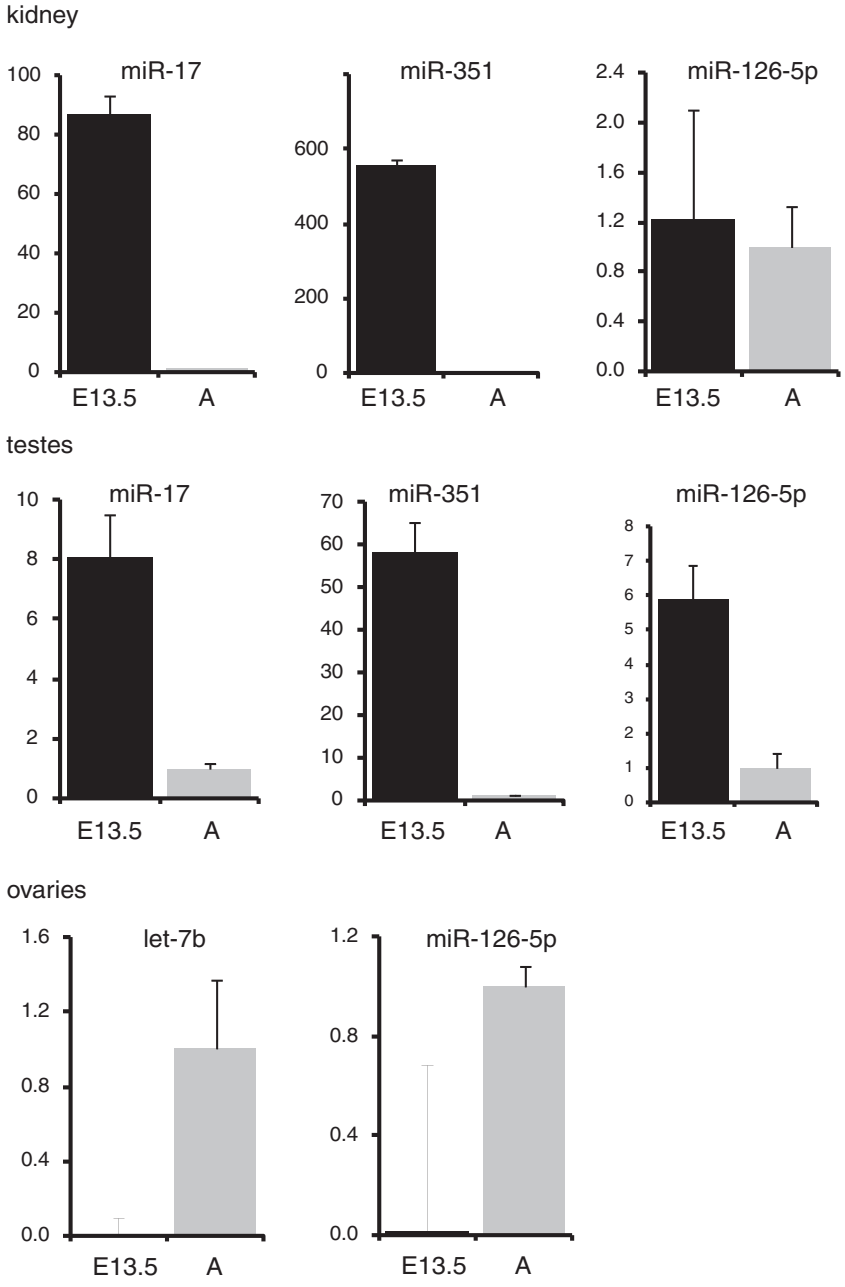


Fig. 2. MiRNA expression analysis by qRT-PCR. Fold change of miRNA expression in embryonic stage E13.5 compared to adult tissue is depicted in the graphs. Adult tissue expression levels are set to one. For each analysis one adult tissue and two different embryonic tissue pools (three embryonic tissues each) were analyzed. The mean expression value is calculated using 18S rRNA as control, according to the ΔΔCt method.

tissues revealed that miR-743a, miR-470, miR-320 and miR-351 were overrepresented (Fig. 4C, right part) whereas miR-27b, miR-17, miR-199b and miR-23b were less abundant compared to the other libraries (Fig. 4C, left part).

Interestingly, all miRNAs that are highly abundant in the libraries that are derived from kidney tissues were underrepresented in the libraries obtained from testis or ovary tissues suggesting that specific miRNA expression patterns are important

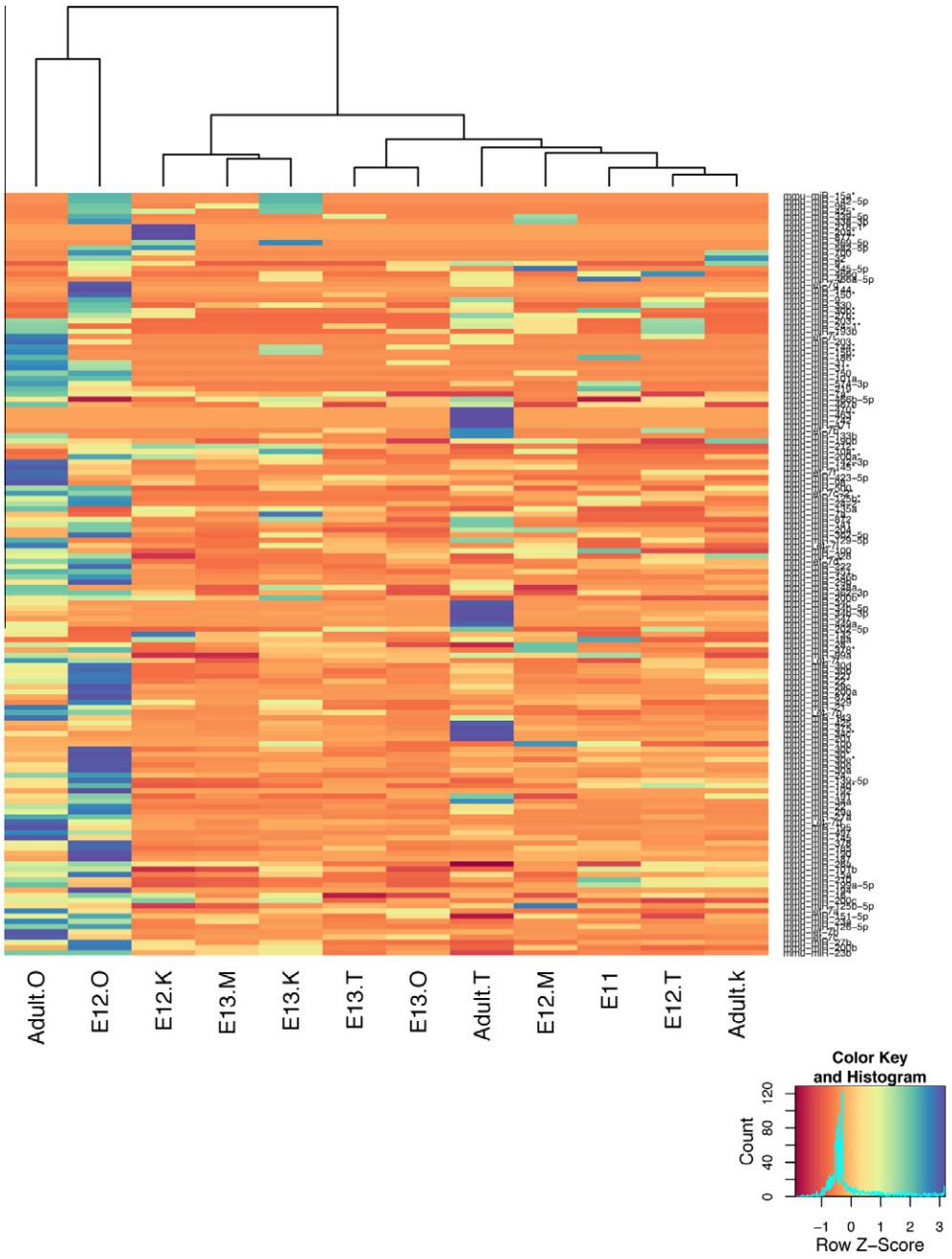


Fig. 3. Clustering of the individual libraries. The head map represents all miRNAs that have been found in the individual libraries.

for tissue identity during the development of the genitourinary system.

3.6. Developmental stage-specific miRNAs

MiRNA expression profiles that are specific for the analyzed developmental stages were analyzed next. For this purpose, we proceeded analogously to the identification of tissue-specific miRNAs (Fig. 4). We found that miR-199b, miR-25, miR-27b and miR-200b are highly abundant in all E12 and E13 libraries (Fig. 5A and B, right part). Strikingly, the same miRNAs together with miR-106b and miR-17 are underrepresented in adult tissues (Fig. 5C, left part) indicating miRNA expression specificity during embryonic development. Reciprocally, let-7a and let-7b are low abundant in the E12 and E13 tissue libraries but highly abundant in the libraries obtained from adult tissues (compare Fig. 5A–C). In adult tissues, where most cells are terminally differentiated and all organs are fully developed a different set of miRNAs is expressed that might facilitate homeostasis by fine-tuning the expression of target mRNAs.

In summary, we have analyzed miRNA expression patterns of the developing genitourinary system in mouse using miRNA cloning and deep sequencing. We report a miRNA expression atlas that can be used for further functional analyses of the individual miRNAs during kidney as well as gonad development. Using computational approaches, we further identified kidney- as well as testis- and ovary-specific miRNAs in the genitourinary system.

It has been demonstrated before that many miRNAs play important roles during animal development in many different organisms. In addition, miRNAs are also expressed in adult, fully differentiated

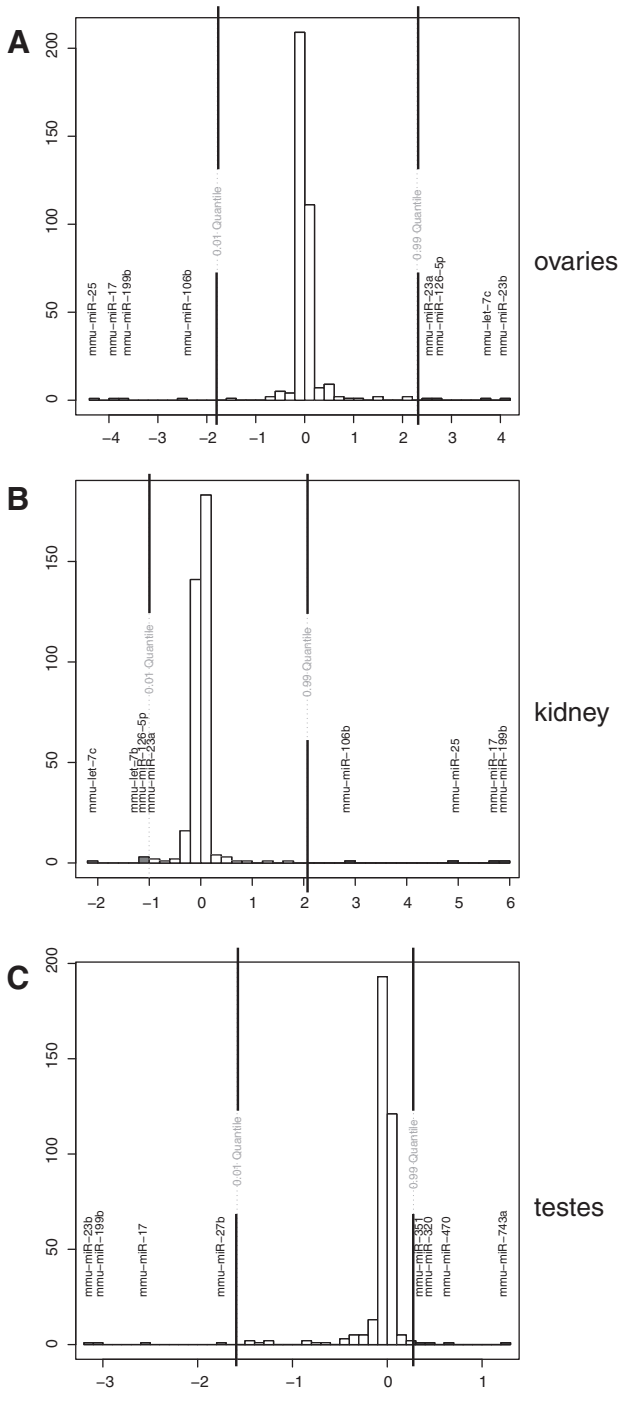


Fig. 4. Distributions of differences between average occurrence of miRNAs in tissue *t* compared to non-*t* tissues for (A) ovaries, (B) kidney and (C) testes. MiRNAs that are specific for the investigated tissues are shown to the right of the 0.99 quantile lane. MiRNAs that are underrepresented in the individual tissues are shown to the left of the 0.01 quantile lane.

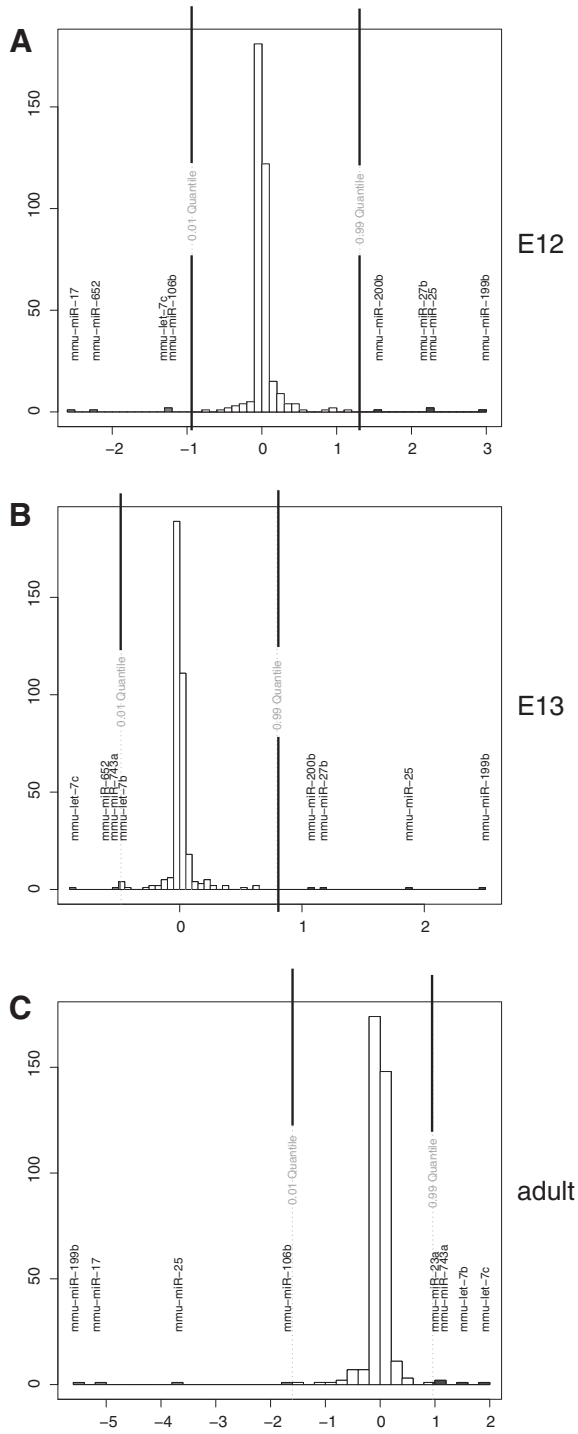


Fig. 5. Distributions of differences between average occurrence of miRNAs in tissue *t* compared to non-*t* tissues for (A) E12, (B) E13 and (C) adult. Over- and underrepresented miRNAs are shown to the right and to the left of each graph.

cells, where they contribute to cell identity by regulating the expression of various genes. Consistently, we find that the miRNA expression pattern differs significantly between the embryonic and the adult tissues. Further analysis of the individual miRNAs including the identification of their targets in different developmental stages will help to elucidate the function of miRNAs in the genitourinary system. Since various diseases including Wilm's tumour affect the genitourinary system our data also provide the basis for further analysis of such diseases in mouse models or human tissues.

Acknowledgements

We thank Sabine Rottmüller and Bernd Haas for technical support. This work was in part supported by Grants from the Deutsche Forschungsgemeinschaft (DFG; to G.M.), by the Deutsche

Krebshilfe (Grant 107166 to G.M.), the Bavarian Genome Research network (BayGene) and the Max-Planck-Society.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.09.050.

References

[1] Siomi, H. and Siomi, M.C. (2009) On the road to reading the RNA-interference code. Nature 457, 396–404.
[2] Meister, G. and Tuschl, T. (2004) Mechanisms of gene silencing by double-stranded RNA. Nature 431, 343–349.
[3] Filipowicz, W., Bhattacharyya, S.N. and Sonenberg, N. (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat. Rev. Genet. 9, 102–114.
[4] Bartel, D.P. (2009) MicroRNAs: target recognition and regulatory functions. Cell 136, 215–233.
[5] Carthew, R.W. and Sontheimer, E.J. (2009) Origins and mechanisms of miRNAs and siRNAs. Cell 136, 642–655.
[6] Peters, L. and Meister, G. (2007) Argonaute proteins: mediators of RNA silencing. Mol. Cell 26, 611–623.
[7] Meister, G. (2007) MiRNAs get an early start on translational silencing. Cell 131, 25–28.
[8] Pillai, R.S., Bhattacharyya, S.N. and Filipowicz, W. (2007) Repression of protein synthesis by miRNAs: how many mechanisms? Trends Cell Biol. 17, 118–126.
[9] Orom, U.A., Nielsen, F.C. and Lund, A.H. (2008) MicroRNA-10a binds the 5′UTR of ribosomal protein mRNAs and enhances their translation. Mol. Cell 30, 460–471.
[10] Vasudevan, S. and Steitz, J.A. (2007) AU-rich-element-mediated upregulation of translation by FXR1 and Argonaute 2. Cell 128, 1105–1118.
[11] Vasudevan, S., Tong, Y. and Steitz, J.A. (2007) Switching from repression to activation: microRNAs can up-regulate translation. Science 318, 1931–1934.
[12] Bernstein, E. et al. (2003) Dicer is essential for mouse development. Nat. Genet. 35, 215–217.
[13] Karolina, D.S., Wintour, E.M., Bertram, J. and Jeyaseelan, K. (2010) Riboregulators in kidney development and function. Biochimie 92, 217–225.
[14] Lechner, M.S. and Dressler, G.R. (1997) The molecular basis of embryonic kidney development. Mech. Dev. 62, 105–120.
[15] Vainio, S. and Lin, Y. (2002) Coordinating early kidney development: lessons from gene targeting. Nat. Rev. Genet. 3, 533–543.
[16] Kaufman, M.H. (1992) Postcranial morphological features of homozygous tetraploid mouse embryos. J. Anat. 180 (Pt 3), 521–534.
[17] Tarasov, V., Jung, P., Verdoodt, B., Lodygin, D., Epanchintsev, A., Menssen, A., Meister, G. and Hermeking, H. (2007) Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. Cell Cycle 6, 1586–1593.
[18] Hurteau, G.J., Spivack, S.D. and Brock, G.J. (2006) Potential mRNA degradation targets of hsa-miR-200c, identified using informatics and qRT-PCR. Cell Cycle 5, 1951–1956.
[19] Griffiths-Jones, S., Grocock, R.J., van Dongen, S., Bateman, A. and Enright, A.J. (2006) MiRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res. 34, D140–D144.
[20] Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J. and Glockner, F.O. (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 35, 7188–7196.
[21] Liu, C. et al. (2005) NONCODE: an integrated knowledge database of non-coding RNAs. Nucleic Acids Res. 33, D112–D115.
[22] Sai Lakshmi, S. and Agrawal, S. (2008) PiRNABank: a web resource on classified and clustered Piwi-interacting RNAs. Nucleic Acids Res. 36, D173–D177.
[23] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
[24] Waterston, R.H. et al. (2002) Initial sequencing and comparative analysis of the mouse genome. Nature 420, 520–562.
[25] Hofacker, I.L. (2009) RNA secondary structure analysis using the Vienna RNA package. Curr. Protoc. Bioinformatics, Chapter 12, Unit 12 2.
[26] Sewer, A. et al. (2005) Identification of clustered microRNAs using an ab initio prediction method. BMC Bioinf. 6, 267.
[27] Landgraf, P. et al. (2007) A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 129, 1401–1414.
[28] Baskerville, S. and Bartel, D.P. (2005) Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. RNA 11, 241–247.
[29] Chu, A.S. and Friedman, J.R. (2008) A role for microRNA in cystic liver and kidney diseases. J. Clin. Invest. 118, 3585–3587.
[30] Ho, J., Ng, K.H., Rosen, S., Dostal, A., Gregory, R.I. and Kreidberg, J.A. (2008) Podocyte-specific loss of functional microRNAs leads to rapid glomerular and tubular injury. J. Am. Soc. Nephrol. 19, 2069–2075.
[31] Ahn, H.W. et al. (2010) MicroRNA transcriptome in the newborn mouse ovaries determined by massive parallel sequencing. Mol. Hum. Reprod. 16, 463–471.