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MicroRNAs in bladder outlet obstruction: relationship to growth and matrix remodeling

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Abstract

The discovery of microRNAs (miRNAs), which are ~22 nucleotide RNAs that inhibit protein synthesis in a sequence-specific manner and are present in a range of species, has born hope of new therapeutic strategies. miRNAs play important roles in development and disease, but they remain poorly studied in uropathologies beyond cancer. Here we discuss biological functions of miRNAs in the lower urogenital tract. A special focus is on miRNAs that change in bladder outlet obstruction (BOO). This is a condition that affects nearly one third of all men over 60 and that involves growth and fibrosis of the urinary bladder. Animal models of BOO, such as that in rat, have been developed and they feature a massive 6-fold bladder growth over 6 weeks. Using microarrays, we have charted the miRNAs that change during the time-course of this process and identified several with important modulatory roles. We discuss known and predicted functions of miR-1, miR-29, miR-30, miR-132/212, miR-204 and miR-221, all of which change in BOO. The majority of the miRNA-mediated influences in BOO are expected to favor growth. We also outline evidence that miR-29 represents a key effector molecule in a generic response to mechanical distension that is designed to counteract exaggerated organ deformation via effects on matrix deposition and stiffness. We conclude that miRNAs play important roles in bladder remodeling and growth and that they may be targeted pharmacologically to combat diseases of the lower urinary tract.

General introduction

The study of microRNAs (miRNAs) in urodynamic disturbances is a research field in its infancy. A Pubmed search for “microRNA AND bladder NOT cancer” retrieves only 16 titles. This is nothing compared to the thousand or so identified miRNAs in our genomes, or the wealth of data that has accumulated on important homeostatic and pathological functions of this novel class of small RNA molecules. Having identified this paucity of knowledge we set out to characterize the role of miRNAs in the urinary bladder in a series of recent papers [1-4]. As a part of this work we charted changes in bladder miRNA expression after outlet obstruction [2], a condition affecting one third of all men over 60. This uncovered a considerable number of deregulated miRNAs with tentative roles in bladder remodeling and growth. These miRNAs are the topic of the current review. Some aspects of miRNA biology in the urinary bladder have been reviewed elsewhere [5, 6].

Biogenesis of microRNAs

MicroRNAs constitute a class of short, non-coding RNAs that are thought to be involved in the posttranslational regulation of most mammalian genes [7]. MicroRNAs are transcribed by RNAPolymerase II as long primary (pri)-RNAs that contain cap structures as well as poly(A) tails [8] (Figure 1). About half of all miRNAs are intragenic and are then generally transcribed together with their host gene. The rest are intergenic microRNAs that have individual promoters [9]. Following transcription, the hairpin structure in the pri-miRNA (Figure 1) which contains the miRNA sequence is recognized and cleaved by the enzyme Drosha. Together with its essential co-factor DGCR8, Drosha forms the so called microprocessor complex in humans [10]. The activity of this complex can be regulated by acetylation, phosphorylation and interaction with other RNA binding proteins [11].

In rare cases, intronic miRNAs, called mirtrons, can form independently of Drosha cleavage by mRNA splicing [12]. After processing, the pre-miRNA is exported out of the nucleus by exportin 5 and released in the cytosol where it is cleaved by the RNase III type endonuclease, Dicer (Figure 1). Dicer is essential for the maturation of nearly all miRNAs. The only known exception is miR-451 which is instead processed by Ago2 [13]. The activity and selectivity of Dicer can be regulated by phosphorylation of the co-factors TRBP and PACT [14, 15]. Following cleavage by Dicer, the RNA duplex is bound by AGO1-4 proteins to form the RNA-induced silencing complex (RISC). Here, one of the strands, the guide strand, is selected while the other strand, the passenger strand, generally is destroyed [16, 17].

The seed sequence, consisting of nucleotides 2-7 of the mature miRNA is crucial for target recognition. Usually the mature miRNA binds to sequences present in the 3'UTR of the target mRNA via its seed. The Ago-proteins associated with the miRNA can then induce translational repression and/or mRNA degradation [18]. Although a single miRNA often have modest effects on the expression of a single gene [19], one miRNA can target hundreds of mRNAs and multiple miRNAs can in turn target a single gene resulting in prominent effects on specific cellular processes or cell function. An example of the latter is the targeting of Pim1 by the two miRNAs miR-1 and miR-204 as discussed below.

With the exception of Let-7, most microRNA are named numerically (miR-1 etc.) and the miRNAs that belong to the same family (i.e. having identical seed sequences) are indicated with lettered suffixes (miR-29a, miR-29b etc.). Since target recognition is primarily based on the seed sequence, miRNAs within a family usually act on the same, or at least overlapping, groups of mRNAs. Each miRNA locus produces a 3'- and a 5'-strand and these are referred to as miR-29a-3p and miR-29a-5p. Generally, only one of the strands is active whereas the other strand (miRNA* or passenger strand) is rapidly degraded and only expressed at a low level. Biogenesis of miRNAs and their mechanism of action is summarized graphically in Figure 1.

Conditional and tissue-specific Dicer knockout

An important starting point for our work on miRNAs in the urogenital tract was to examine whether miRNAs are functionally important in this context. To address this hypothesis we deleted the endonuclease Dicer in smooth muscle of adult mice [1]. miRNA depletion was confirmed in the detrusor at 10 weeks following Dicer deletion using PCR-based arrays and using quantitative PCR (qPCR). Many miRNAs were reduced to baseline at this time, including miR-145, miR-22, and miR-1. In parallel work we demonstrated that miRNA levels decline progressively over several weeks following deletion of Dicer, and, at earlier times (2-3 weeks), several miRNAs remain unchanged [20]. The 10 week time point was thus chosen for studying the physiological impact of miRNA depletion on bladder function.

At 10 weeks we found a disturbed micturition pattern with increased voiding in the center of the cage. We also found that the bladder developed considerably smaller pressures over a range of physiological volumes, which was accompanied by a reduced force per cross-sectional area of isolated strips on KCl stimulation. We used electrical field stimulation to activate the detrusor via its intrinsic nerves. This demonstrated impaired cholinergic activation. The purinergic component of electrically induced twitches was however increased relative to KCl, but with unchanged stress. Using electron microscopy we found an unchanged density of nerve terminals, but a conspicuous increase in the distance between smooth muscle cells. Because prior work had demonstrated reduced contractile differentiation of vascular smooth muscle following deletion of Dicer [21], we measured differentiation markers using western blotting. With exception for calponin and desmin, which were reduced, most markers were unchanged, arguing that the effects of miRNA depletion are milder in the detrusor than in vascular smooth muscle. Impairment of KCl-induced contraction was moreover associated with reduced expression of L-type Ca^{2+} channels. In all, these findings provided proof of principle that miRNAs play an important role in the urinary bladder.

Shortly after our paper on the effects of Dicer knockout in the detrusor was published [1], another paper appeared [22] using a different strategy to delete Dicer. A potential concern with the experimental protocol in that paper, however, was that mice were used already at 1 week following Dicer deletion. As outlined above, we have demonstrated, using PCR-based arrays, that many miRNAs remain unchanged at 2 weeks following Dicer deletion, and that miRNAs continue to decline over a period of at least 10 weeks [20]. It is therefore no surprise that Zhang et al. [22] failed to detect any changes in contractility at that early time point. The authors then used cyclophosphamide to induce bladder overactivity. This group of mice was analyzed 2 weeks after Dicer deletion, at which time increased purinergic activation was found in knockout bladders, in good agreement with our data in cyclophosphamide-naïve mice at later times (5 and 10 weeks [1]). The authors also reported a reduced voiding interval in this setting, with increased macrophage infiltration and increased P2X receptor expression. Taken together, this argues that different strategies to delete Dicer will eventually cause the same overall phenotype, involving an increased relative response to ATP in association with a micturition disturbance. This may be accelerated by cyclophosphamide.

MiR-143/145 knockout mice

miR-145 is the fourth most highly expressed miRNA in the rat bladder and probably the most highly expressed miRNA in the human bladder (see below). Work has demonstrated that knockout (KO) of the miR-143/145 cluster results in impaired KCl- and phenylephrine-induced arterial contraction, reduced arterial blood pressure and development of spontaneous neointimal lesions [23]. The effects of miR-143/145 KO on smooth muscle contraction thus appear similar to changes reported by us in Dicer KO bladders [1]. It was thus plausible that loss of miR-143/145 was responsible for some of the phenotypes of Dicer KO detrusors. To address this possibility we compared contractility in arterial, airway and bladder smooth muscle from miR-143/145 KO mice [3]. To our surprise, KCl-induced contraction was largely

unchanged in the urinary bladder from 9-16 week old KO mice whereas both arterial and airway contractility were severely impaired. Further work pointed to the presence of compensatory mechanisms in the bladder, including increased smooth muscle differentiation and increased ROCK1 expression. Rho-associated kinase 1 (ROCK1) is a kinase that plays a key role in smooth muscle contractility via modulation of Ca^{2+} -sensitivity [24]. We also obtained support for the hypothesis that induction of angiotensin converting enzyme (ACE) is an important pathophysiological mechanism following miR-143/145 deletion. Induction of ACE was seen in the aorta and in the airways, but not in the urinary bladder. These studies thus uncovered important differences between the detrusor and arterial smooth muscle and suggested a minor role of miR-143/145 in detrusor function. It remains somewhat difficult to imagine, however, that miR-143/145 knockout is completely without effect in the bladder. We believe that it would be worthwhile to study bladders from aging miR-143/145 KO mice, and to impose stress, such as outlet obstruction, on such bladders.

Bladder outlet obstruction – a brief background

About 30 per cent of males over 60 suffer from symptomatic benign prostatic enlargement (BPH) (see e.g. [25]). BPH results in an increased urethral resistance (bladder outlet obstruction, BOO), and an increased voiding pressure. This leads to thickening of the detrusor smooth muscle layer, trabeculation, and fibrosis [26, 27]. Clinical manifestations of BOO include increased voiding frequency and urgency (part of the overactive bladder syndrome, OAB, see [28]), hesitancy, incomplete bladder emptying, and the development of residual urine.

For ethical reasons it is difficult to obtain detrusor muscle from patients with BOO. Several animal models of partial outlet obstruction have therefore been developed, including rat [29], rabbit [30], pig [31] and mouse [32]. In the female rat a partial outlet obstruction is created by

placing a ligature (diameter 1 mm) around the proximal urethra. This leads to a rapid increase in the weight of the detrusor muscle; after 10 days the weight has increased from 80 to 240 mg, and after 6 weeks the final weight has increased to 480 mg [33]. The increase in detrusor weight is largely due to hypertrophy of the detrusor smooth muscle cells, but some hyperplasia also occurs. After 6 weeks the cross-sectional area of the individual smooth muscle cells has increased ten times [34]. There is also a considerable net synthesis of collagen in the detrusor layer [35]. Such animals void with an increased maximum micturition pressure, and develop post-void residual urine, and OAB [36]. Removal of the obstruction largely normalizes bladder weight and the size of the smooth muscle cells [37]. Obstruction leads to a reduced density of motor nerve terminals in the detrusor [4], but, due to the increased weight of the detrusor, the total number of terminals is increased. As a consequence, there is an increased size of the parasympathetic ganglion cells innervating the detrusor [38].

A survey of miRNA expression in bladder outlet obstruction

In our work on miRNAs in BOO we used the rat (rBOO) model [2]. This model is evidently of interest in uropathology, but it is moreover relevant as a model of smooth muscle pathology and growth. Indeed, and as exemplified below, rBOO has uncovered what appears to be quite general principles in miRNA biology. The design of our initial experiment is depicted schematically in Fig. 2A. We isolated bladders from sham-operated animals, and from animals that had been obstructed for 10 days and 6 weeks, respectively. We also included a group of animals that had been obstructed for 6 weeks and then operated again to relieve the obstruction. The latter bladders were excised at 10 days after de-obstruction. RNA was isolated from the whole bladder, including the mucosa, and separated in miRNA and mRNA fractions, followed by array hybridization.

63 miRNAs were differentially expressed at 10 days of rBOO, representing $\approx 5\%$ of the probes on the array. Figure 2B shows expression levels in sham versus obstructed (10 days) bladders. miRNAs that fall on the line of unity are unchanged, whereas miRNAs below this line are reduced. Those above are increased. A key point from this analysis is that the largest relative changes are seen for miRNAs with low expression levels. In fact, the two miRNAs with largest fold changes (arrows in Figure 2B) are both in the lower third of the expression range. One would assume that miRNAs with low expression levels have small biological impacts and vice versa. While this may not be true of all miRNAs, this assumption is supported by our finding that miRNAs that are significantly, and inversely, associated with predicted target mRNAs (miR-1, miR-29) are in the upper half of the expression range. A number of miRNAs that have reasonable expression levels and that change significantly are highlighted in red in Figure 2B, and the ten most highly expressed miRNAs in the rat bladder are depicted in Figure 2C. In the latter group no significant ($Q=0$) changes were seen on obstruction although both miR-26a ($Q=7$, $P=0.005$ at 10d) and miR-23a ($Q=2.4$, $P=0.0008$ at 10d) came close. These miRNAs are highlighted by arrows in Figure 2C and we believe that they are worthy of further study.

A decisive strength of the experimental design (Figure 2A) is that it allows for plotting of miRNA expression over time as shown in Figure 2D. It can be inferred from Figure 2D that the largest changes occur early after obstruction (10 days) and that miRNA expression falls back towards control levels at 6 weeks. This is also reflected in the number of differentially expressed miRNAs at these time points: 63 at 10 days and 14 at 6 weeks (not depicted). We therefore predict that an earlier time-point (3-5 days) would have revealed even more dramatic miRNA changes. Another take-home message is that most miRNAs recover after de-obstruction. There are exceptions however, miR-1 being an example, where recovery is incomplete. Such miRNAs may constitute a memory trace of obstruction.

A key question is if the changes in our array experiment are reproducible. To address this we selected eleven miRNAs for confirmation by qPCR. Eight could be confirmed (at $P < 0.05$), whereas the changes of three miRNAs failed to reach significance (those were miR-146b, miR-15b, miR-182; indicated in green in 2E). Fold change in the array experiment is plotted versus fold change in the confirmatory qPCR experiment in Figure 2E. A striking correlation between the data sets is evident (Spearman $Rho = 0.94$, $P < 0.0001$).

What do the miRNAs that are differentially expressed in rBOO do?

Having established that numerous miRNAs change in rBOO (Table 1 and Figure 2 and [2]), the next logical question is if this is biologically, or pathologically, meaningful. We reasoned that biologically relevant miRNA changes should be accompanied by changes in target mRNA levels. To approach the issue of biological relevance, we therefore first examined if differentially expressed miRNAs were associated with opposite changes in target mRNAs. We arbitrarily opted to use the 50 predicted targets with best context scores in Targetscan (<http://www.targetscan.org/>). Three miRNAs returned significant reciprocal associations with target mRNAs in this analysis: miR-1, miR-29b and miR-29c. This finding supports the notion that at least some of the miRNAs with altered expression levels leave footprints in the transcriptome. It cannot be inferred, however, that miRNAs that do not correlate with a group of predicted targets are irrelevant. This follows from the uncertainties of target predictions, and from the fact that an important mechanism of action is translational repression, in which case only protein synthesis is affected. A brief summary of established and potential functions of selected miRNAs in rBOO is given below.

miR-29b and c

The miR-29 family (miR-29a, miR-29b and miR-29c) is among the most studied miRNA families of those represented in Table 1. Excellent reviews have summarized current

knowledge on this family [39-41]. The miR-29 family has been demonstrated to repress fibrosis [42], and, accordingly, these miRNAs are reduced in many pro-fibrotic conditions. To view the miR-29 family solely as a guardian of fibrosis is an oversimplification however. Many other effects, including inhibition of cancer progression through effects on DNA methylating enzymes [43], have been demonstrated. Since two out of three of the miRNAs in our experiment that were both differentially expressed and significantly associated with opposite changes in predicted targets were from this family [2], these miRNAs clearly represented low-hanging fruits of our profiling effort. Accordingly, we focused our initial efforts on miR-29b and miR-29c which were reduced by >50% at 10 days of obstruction (Figure 2B and Table 1).

Prior work had demonstrated that miR-29 is repressed by SMAD3, c-Myc and NFκB [44], and we found evidence for activation of all of those signaling pathways; c-Myc and NFκB were activated early after obstruction whereas SMAD3 was activated in chronic obstruction. This provided a rational explanation for the repression of miR29b and miR-29c in rBOO. We also found significant correlations between miR-29bc and target genes, including collagen III and IV, fibrillin-1, laminin γ1, elastin and Sparc. Transfection of miR-29c inhibitor and mimic caused opposite changes in the corresponding proteins. Moreover, and as expected for miRNA-mediated influences, effects were uniformly larger at the protein level than at the mRNA level at 6 weeks of obstruction. Conditional deletion of Dicer in the bladder was next used to demonstrate widespread matrix changes and increased stiffness [2] similar to that seen in outlet obstruction. In all, these findings favored a model where the combined influences of SMAD3, c-Myc and NFκB in the distended bladder reduce miR-29. Alleviation of this antifibrotic influence then promotes matrix deposition and increases stiffness which in turn counteracts further distension. Because a similar scenario has been reported for the infarcted heart and in aortic aneurysms, we believe that this represents a generic response to mechanical

forces that is designed to counteract exaggerated organ deformation. Whether this signaling pathway can be targeted for therapy is of considerable interest.

miR-1

miR-1 is a muscle-enriched miRNA that gained recognition when it was demonstrated that it regulates myogenesis [45]. Its overexpression in heart causes a proliferative defect resulting in impaired cardiac expansion [46]. Work has also demonstrated a rapid drop in miR-1 in cardiac hypertrophy caused by aortic banding [47]. In that study it was demonstrated that overexpression of miR-1 reduced DNA synthesis, both in basal conditions and following serum stimulation. miR-1 may therefore constitute a break on cardiomyocyte proliferation that is relieved after cardiac outflow obstruction. A similar role in skeletal muscle is supported by the finding that functional overloading results in reduced miR-1 expression [48]. The idea that miR-1 repression favors bladder growth following rBOO is therefore appealing.

The reduction of miR-1 in our microarrays did not quite reach the predefined significance level of $Q=0$ (miR-1: $Q=0.8$ and $P=0.012$ at 10 days, table 1). However, subsequent qPCR experiments confirmed its reduction (Figure 2E). We also found a significant association with the top 50 predicted target mRNAs [2] further arguing that it is functionally relevant. Overexpression of miR-1 has been shown to inhibit smooth muscle cell proliferation [49] and the oncogenic serine threonine kinase Pim1 was identified as a target that may be responsible for this effect. In heart, on the other hand, IGF-1 and its receptor were identified as targets that could mediate the effects on proliferation [50]. No significant correlations with these particular targets were seen in our data sets (Figure 3A, B), but this is in good agreement with the original studies showing that repression was only manifest at the protein level. Taken together, the available information points to a role of miR-1 in detrusor growth, but a direct

effect on relevant proteins at a physiological miRNA concentration remains to be demonstrated in this pathological context.

miR-1 has been studied in bladder development [51]. A progressive increase in bladder capacity over the first weeks of life was found to be associated with reduced expression of smooth muscle differentiation markers. This effect was mimicked by heterozygous deletion of myocardin which also reduced miR-1 expression. miR-1 was shown to regulate the gap junction protein connexin 43 (*Gja1*). That study therefore supported a functional and growth-independent role of miR-1 in the rodent bladder.

miR-132/212

miR-132 and miR-212 are derived from a bicistronic precursor and are known to be expressed in neurons where they regulate excitability, and synaptic plasticity [52-54]. They are also expressed in non-neuronal tissue. In the heart they are involved in the development of hypertrophy [55], and in leucocytes they repress acetylcholine esterase, *Ache* [56]. In arterial smooth muscle, angiotensin-II upregulates miR-132/212 in both rats and humans [57].

10 days of partial outlet obstruction in our rat model led to a 3- to 7-fold induction of miR-132/212 in the detrusor muscle ([4], see also Figure 2 and Table 1). Strikingly, miR-132/212 induction was specific for the detrusor layer and was not seen in the mucosa. Upstream, this increase correlated with an increased nuclear translocation of the transcription factor Ahr (the dioxin receptor). Downstream, increased miR-132/212 correlated with decreases in mRNAs for the validated targets *MeCP2*, *Pnkd* and *Ache*, and a decrease of their respective proteins.

Ache, or acetylcholine esterase, is probably expressed in nerves, and not in the detrusor cells, and its decrease in outlet obstruction is therefore likely a consequence of an increased distance between nerve terminals [4], caused by the smooth muscle cell hypertrophy, rather than by the dramatic induction of miR-132/212. There are very few ganglion cell bodies in the

bladder wall and the perikarya are situated in ganglia outside the rat bladder [58]. miR-132/212-mediated repression of Ache is thus not likely involved in the defective cholinergic neurotransmission that we reported for Dicer-deficient detrusor [1]. We found however, that transfection with miR-132 and miR-212 mimics in cultured human detrusor smooth muscle cells decreased cell number, whereas miR-132 or miR-212 inhibitors had the opposite effect [4]. This argues that these miRNAs may limit the growth response of the bladder following outlet obstruction.

miR-30

Three micro-RNAs in the miR-30 family were found to be reduced at 10 days of rBOO with nominal significance (miR-30a: $Q=0.8$, $P=0.006$; miR-30d: $Q=0.9$, $P=0.006$; miR-30e: $Q=0.9$, $P=0.006$, Figure 3C). All three are highly expressed. Together these observations lend credence to the idea that the miR-30 family may have an impact in rBOO despite the fact that the individual changes just fall short of significance ($Q=0$).

The first identified target of a miR-30 family member was connective tissue growth factor (CTGF), which is inhibited by miR-30c in cardiomyocytes resulting in decreased collagen production [59]. Since then, miR-30 family members have been found to be involved in multiple biological processes including adipogenesis [60], thermogenesis [61], autophagy [62], myogenic and osteoblast differentiation [63, 64], epithelial-to mesenchymal transition [65], cellular senescence [66], and angiogenesis [67]. In smooth muscle, miR-30 family members are known to be reduced in pulmonary arterial hypertension [68], to contribute to ER stress [69] and to control vascular smooth muscle cell calcification by regulating the master osteoblast transcription factor Runx2 [70].

Interestingly, the expression of several or all miR-30 family members is down-regulated by BMP2 or TGF β stimulation, suggesting that although they are encoded by different genes,

they share common regulatory mechanisms [64, 70, 71]. In obstructed bladders we found phosphorylation of both SMAD2/3 and SMAD1/5/8, at different time points suggesting activation of both of these pathways [2]. Furthermore, several targets of the miR-30 family are upregulated during outflow obstruction, such as CTGF and B-Myb (MYBL2, [2]). There may thus be a link between SMAD-phosphorylation, miR-30 down-regulation and increased expression of effector proteins during outflow obstruction that remains to be more thoroughly investigated. In Figure 3D we have plotted the miR-30a level versus the level of Snail mRNA, a validated target that functions as a transcriptional repressor with a role in epithelial to mesenchymal transition. A tight inverse correlation is seen, supporting the overall idea that the miR-30 family plays a functional role in rBOO.

miR-221

miR-221 is increased by 60% (Q=0) at 10 days of rBOO, but it recovers rapidly, being insignificantly increased (by 26%) at 6 weeks of obstruction (Figure 2D). It then falls to control level on de-obstruction. Work by Galardi et al. [72] demonstrated that the cell cycle inhibitor p27Kip1 (Cdkn1b) is an important target of miR-221 in prostate carcinoma cells, and, consequently, overexpression of miR-221 promoted cell growth and shifted cells from G1 to S in the cell cycle. It was also shown that miR-221 promoted colony formation in soft agar. This is a hallmark of cellular transformation. Subsequent studies in smooth muscle have shown that stimulation with platelet-derived growth factor (PDGF) increases both the mature and precursor forms of miR-221, with a larger effect at the precursor level [73]. p27Kip1 was again identified as a key target. Another study demonstrated that miR-221 is dramatically induced (10-fold) 4 days after angioplasty and that it falls back toward control level over 28 days [74]. Knockdown of miR-221 inhibited proliferation and neointimal expansion, and p57Kip2 was added to the list of smooth muscle target genes. In all, these findings establish a

pro-proliferative role of miR-221 and suggest that this miRNA could be targeted for therapy in proliferative disorders.

Given the rapid dynamics of miR-221, it seems reasonable to propose that our experiment has failed to capture its peak induction in rBOO. We know that the most rapid weight gain in our experiment occurred prior to the 10 day time point [2]. We also know that peak phosphorylation of the pro-proliferative kinases Akt [4] and ERK1/2 [2] occurs at 2 and 4 days, respectively. An inference from these temporal relationships is that miR-221 is likely to peak before the 10 day time point. Since we nonetheless capture significant variation of miR-221, we can correlate the miR-221 level with the mRNA levels of validated targets. This analysis shows significant inverse correlations with both p27Kip1 (Cdkn1b) and with p57Kip2 (Cdkn1c) (Figure 3E and F). We therefore conclude that miR-221 likely plays a pro-proliferative role in bladder outlet obstruction by committing cells to mitosis via repression of p27 and p57. It is intriguing that both pro-proliferative (e.g. miR-221, miR-1, miR-204, miR-29) and anti-proliferative (miR-132/212) miRNA changes are seen following outlet obstruction. Fine tuning of growth responses by opposing influences is a recurring theme in biology, and this certainly appears to apply to miRNAs in outlet obstruction.

miR-203

The literature on miR-203 indicates that this miRNA induces apoptosis and inhibits cell invasiveness in several cancer cell types [75, 76]. In osteosarcoma miR-203 acts a tumor suppressor gene [77]. An association between reduced expression of miR-203 and poor prognosis in non-small cell lung cancer has moreover been reported [78]. Interestingly, in melanoma cells [79], miR-203 has been found to suppress caveolin-1, a protein necessary for formation of caveolae which are omega-shaped membrane organelles present at high density in the detrusor [80]. Of further interest in relation to smooth muscle regulation is the

demonstration that miR-203 contributes to estrogen receptor mediated inhibition of vascular smooth muscle cell proliferation [81].

We found that miR-203 was downregulated in the obstructed bladder (Figure 2B, D). The detrusor smooth muscle cells in those bladders are undergoing a phase characterized by rapid growth and proliferation, and one might speculate that this is promoted by the decreased miR-203. In support of this possibility, we have found increased expression of caveolin-1 and an increased number of caveolae per unit cell surface in hypertrophic detrusor cells [33]. Against this possibility speaks the complete lack of effect of Dicer deletion on the density of caveolae in the mouse detrusor [1]. A peculiar aspect of miR-203 that it shares with miR-1 is that de-obstruction does not seem to normalize its level within 10 days (Figure 2D). It is interesting to note that smooth muscle cells in de-obstructed bladders have an ultrastructural difference compared to control detrusor; the cells are more wrinkled and with an increased surface to volume ratio, as if the de-obstruction has led to a decrease in cell volume, but not in cell surface. It is not known whether the number of caveolae per unit cell area is still increased in such de-obstructed bladders.

miR-204

MiR-204, which is reduced in rBOO (Table 1), was one of the first miRNAs associated with arterial remodeling, and miR-204 is important for the onset and progression of pulmonary arterial hypertension [82, 83]. Mir-204 is suppressed by the STAT3 signaling pathway, resulting in increased proliferation and decreased apoptosis [83]. STAT3 activation is maintained by a positive feedback loop involving miR-204 and de-repression of its targets Pim1 and nuclear factor of activated T-cells (NFAT) [84]. STAT3 (GEO accession number GSE47080) and Pim-1 [85] mRNAs are increased at 10 days and at 6 weeks of rBOO which fits nicely with the reduced level of miR-204. Pim1 is also a target of miR-1 (see above).

Hence two repressive influences on Pim1 (miR-1 and miR-204) are simultaneously relieved in rBOO. In support of an increased translational efficiency for Pim1 in rBOO we have found that Pim1 protein is increased 6-fold at 6 weeks of rBOO whereas the mRNA level is increased only 1.5-fold [85]. Overexpression of Pim-1 in the heart protects mice from pressure overload-induced hypertrophy [86], and it is possible that increased expression of Pim-1 in rBOO similarly promotes functional adaptation via effects on cell survival, cell size and cell number. In our previous work [4], NFAT was not identified as an obstruction-regulated transcription factor in the rBOO model, but it is possible that NFAT is activated at earlier times than those investigated (that is before 10 days of obstruction). Recently, it was shown that STAT3 activation inhibits myocardin and down-regulates contractile, phenotype-specific, genes and promotes proliferation [87]. This may occur in rBOO, but at earlier time points than 10 days where we find no change in myocardin expression (GEO accession number GSE47080). In all, there are some compelling biological associations for miR-204 in rBOO that are worth studying further.

miR-495

miR-495 was the second most highly induced miRNA at 10 days of outlet obstruction in our array experiment (Figure 2E and Table 1). This change was readily confirmed by qPCR (Figure 2E), but, in this case, the fold induction was smaller. Ectopic expression of miR-495 has been demonstrated to reduce cell viability and to increase apoptosis with PBX3 and MEIS1 as direct target genes [88]. Another study showed the opposite, namely that miR-495 promotes proliferation in hypoxic conditions via repression of E-cadherin and REDD1 [89]. This suggests a dualistic role of this miRNA, having both pro- and antiproliferative influences, in a context-dependent manner. In our data sets, no significant correlations were observed between miR-495 and these target mRNAs (not depicted). It could be that this miRNA is exclusive for the mucosa which would dilute changes at the level of the whole

bladder, that its absolute expression level in bladder is too low to be biologically meaningful, or that we haven't considered the relevant targets.

miR-375

Identified targets for mir-375 are involved in processes such as cell proliferation, apoptosis and autophagy [90]. In rBOO, there is evidence of hypoxia-driven autophagy [85]. This process may be modulated by decreased expression of mir-375. However, examination of described targets of miR-375 [90] in our mRNA arrays indicates that none (e.g. PDK1, IGF1R, JAK2) are upregulated in rBOO when miR-375 is reduced. This may indicate that miR-375 is not expressed at a level that is biologically meaningful in rat bladder. Due to stability and ease of detection, miRNAs are increasingly considered for their potential as biomarkers. miR-375 has been evaluated in this regard. In patients with BPH, no alteration in the plasma level of miR-375 was observed [91].

miRNAs in the human bladder

A critical question is whether the miRNAs discussed herein, and that change in rat bladder outlet obstruction, have a similar function in the human bladder. It is too early to respond decisively to this question as no full study on miRNAs in the obstructed human bladder has been published. An abstract supports this notion however (Gheinani et al., 2014). Recently launched transcriptomic profiling efforts, such as the GTExPortal [92], can also be used to probe some aspects of miRNA biology in the human bladder. We have used the GTExPortal to support a cause and effect relationship between myocardin family coactivators and caveolae in numerous human tissues [93]. Here, we plotted relative miRNA expression levels in the bladder (Figure 4A) and in the prostate gland (Figure 4B). Similar to mouse and rat bladder miR-145 is highly expressed. A number of uncharacterized miRNAs also appear in these data sets. miR-6723, for example, is highly expressed in both bladder and prostate.

Since these data were generated using RNA-sequencing it is possible to test target predictions using correlation analyses. Such examples are shown in Figure 5C through F. Panels C and D show that the level of miR-145 correlates inversely with the validated target KLF5 [94] in bladder and prostate, respectively. miR-145 similarly correlates inversely with the validated target FSCN1 in bladder (Figure 5E), but not in prostate (Figure 5F). Taken together, these relationships support an important role of miRNAs in the lower urinary tract of humans.

Mir-145 has been reported to be reduced in prostate cancer, a malignancy originating from the prostate epithelium. A widely held belief in that field is that miR-145 is expressed in epithelial cells (e.g. [95]) where it plays a tumor suppressor role. Localization of miR-145 using *in situ* hybridization is a challenging task, however. MiR-145 is very cell type-specific [96], and its transcription is driven by myocardin [97], a master regulator of smooth muscle differentiation. The most straightforward interpretation of the reduced miR-145 expression in prostate cancer is therefore that the carcinoma grows relative to the stromal tissue compartment where CAV1 resides. We recently demonstrated that caveolin-1 (CAV1) is regulated by myocardin [93]. Distribution of CAV1 should therefore mirror the expression of miR-145. Figure 5G and H show that there is essentially no staining for CAV1 (green) in glandular epithelium (inside the white contour in Figure 5G) of the human prostate, whereas it is abundantly present in the stromal smooth muscle cells (outside the white contour). We consequently believe that it is timely to reinterpret the role of miR-145 in prostate cancer as has recently been done in colorectal cancer [96].

Concluding remarks

The discovery of important roles of miRNAs in physiology and pathology has opened new avenues of investigation and sparked interest in miRNA-targeted therapy in several areas. Currently, antimiRs, which are small modified oligonucleotides with slow onset of action

and long-lasting effects, have come furthest in development [98]. There are considerable obstacles however. One is their preferential accumulation in liver and kidney, which poses threats of untoward effects in these organs. Mimics are larger molecules and thus have issues with cellular penetration that need to be considered. Some of the measures required for delivery may be tolerable in life-threatening disease such as cancer, but would constitute too much of a trade-off in conditions that primarily affect quality of life. Novel delivery techniques and formulations will hopefully be associated with milder side-effects. Until these have emerged, we should make an effort to further define the roles of miRNAs in the urogenital tract, so as to be ready when this novel treatment paradigm reaches clinical application. The work discussed here and that of others [5] have provided a glimpse into the role of miRNAs in the lower urinary tract, but much remains to be discovered.

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Figure legends

Figure 1. Cartoon depicting biosynthesis of microRNAs (miRNAs). miRNA genes are present throughout the genome, including in introns of protein-coding genes, in which case they may share regulatory mechanisms with the host gene. Following transcription, a number of key processing steps are required. These are catalyzed by the endonucleases Drosha in the nucleus and by Dicer in the cytoplasm. The AGO protein is loaded with the double-stranded pre-miRNA and the complementary miRNA strand is then degraded. The mature miRNA in the RISC complex hybridizes with target mRNAs primarily via their seed regions. This leads to mRNA breakdown or translational repression. DGCR8: DiGeorge syndrome chromosomal/critical region 8, TRBP: TAR RNA-binding protein, PACT: protein activator of the interferon-induced protein kinase, AGO: argonaute, RISC: RNA-induced silencing complex.

Figure 2. Overview of array experiment measuring miRNAs and mRNAs in bladder outlet obstruction. Panel A show schematic representation of our experiment [2], which included sham operated rats, rats that had been subjected to bladder outlet obstruction for 10 days and 6 weeks, respectively, and those that been de-obstructed for 10 days following 6 weeks of obstruction (n=6-8 throughout). Bladders are drawn to reflect relative size at excision. In panel B, sham miRNA levels are plotted versus miRNA levels in obstructed bladders. The line of unity is drawn in white. miRNAs above this line are increased in obstructed (10 days) bladders, and those below are reduced. 8 miRNAs that change significantly in the upper half of the expression range are highlighted in red and their names are depicted beside the respective symbols. Panel C shows the 10 most highly expressed miRNAs in the rat bladder. Two miRNAs (miR-26a and miR-23a) which are nominally

different are highlighted with arrows. In panel D, relative miRNA levels are plotted for the different conditions on a time line. Panel E shows confirmation of miRNA changes using qPCR. The fold change (FC) in the array experiment is plotted against the fold change in the qPCR experiment. All miRNAs except three, highlighted with green symbols, were significantly different in both experiments. An excellent correlation between the different data sets is seen (Spearman rho (R) and P-value are given). Similar confirmation of differentially expressed miRNAs was made.

Table 1. Table showing fold change of expression at 10 days, at 6 weeks and after de-obstruction for selected miRNAs. Q was determined by significance analysis of microarrays and $Q=0$ was used as significance criterion. The miRNAs highlighted in red are discussed in the text.

Figure 3. Correlations between selected miRNAs and target mRNAs in our data sets. Panels A shows changes in miR-30 family members in bladder outlet obstruction. Error bars were omitted for clarity. Panel B shows a correlation analysis (Spearman) for miR-30a versus Snai1, a target that has been previously validated. Panels C and D show correlation analyses for miR-1 versus Pim1 and between miR-1 and Igf1. Panels E and F show significant inverse correlations between miR-221 and the cell cycle inhibitors Cdkn1b (p27Kip1) and Cdkn1c (p57Kip2), respectively. Official gene symbols are used. Spearman rho (R) and P-values are given in each panel.

Figure 4. The ten most highly expressed miRNAs in human bladder and prostate and miR-145 target correlations. Expression data were downloaded from the GTExPortal.org and TMM-normalized as described [93]. The 10 most highly expressed miRNAs in bladder and prostate are depicted in panel A and B. HG refers to host gene. Panels C and D show that the level of miR-145 correlates inversely with the level of KLF5 in both bladder and prostate.

Panels E and F show corresponding correlation analyses for FSCN1 in bladder and prostate, respectively. Panels G and H: human prostate, peripheral zone. The center structure, outlined in white, shows glandular tissue surrounded by stromal cells. Panel G shows staining for caveolin-1 (green). Panel H shows the same section, stained with bisbenzimidazole (1 mg/ml in PBS) to visualize nuclei in stromal (largely smooth muscle cells) and epithelial cells. There is an intense staining of caveolin-1 in the cell membranes of the stromal cells but none in the glandular epithelium. Antibodies and staining parameters are described in [80]. The width of the micrographs is 400 μm .

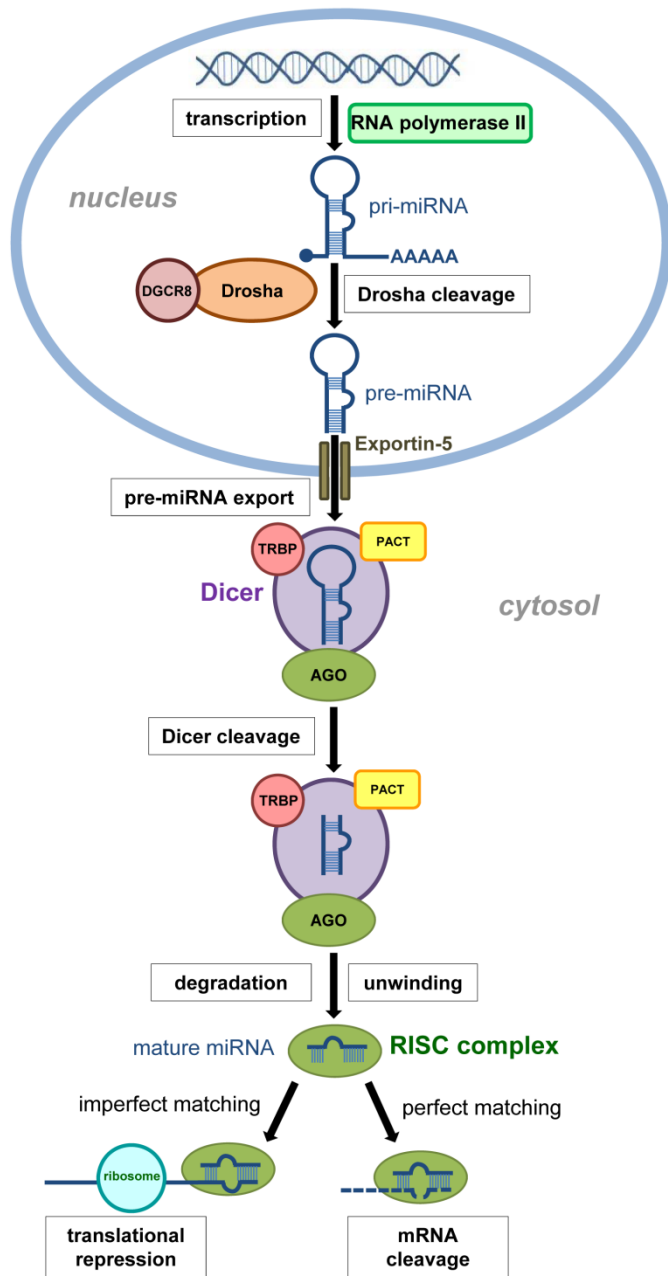


Figure 1

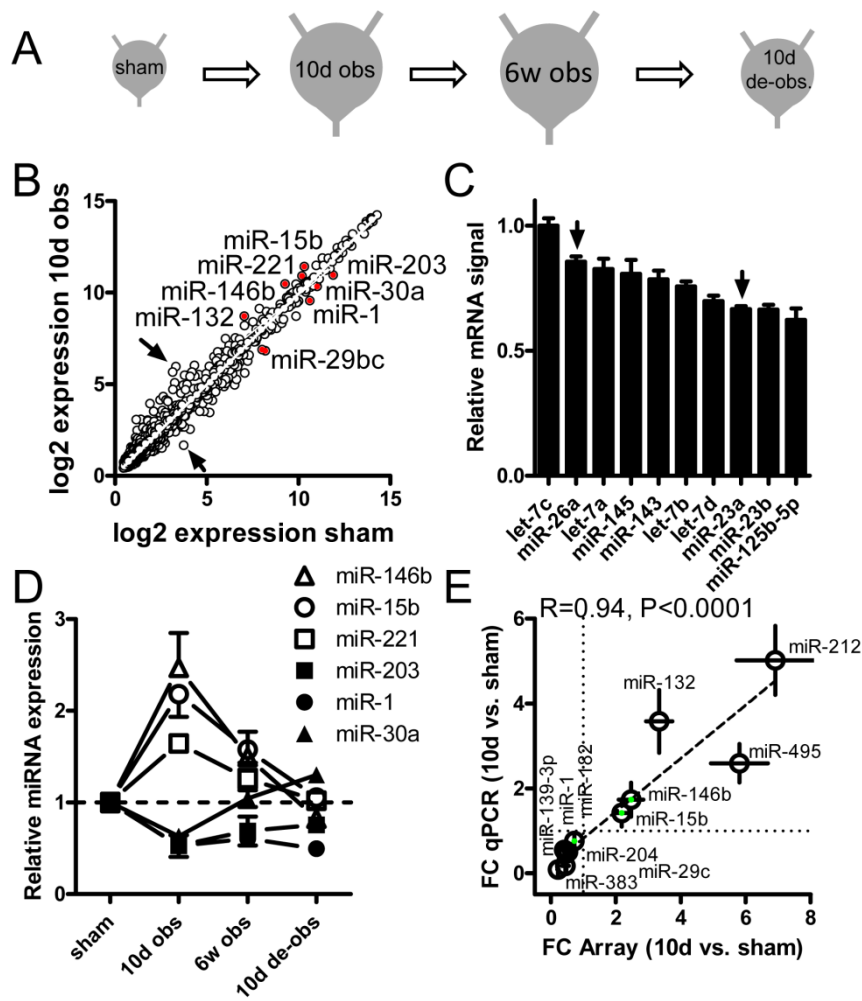


Figure 2

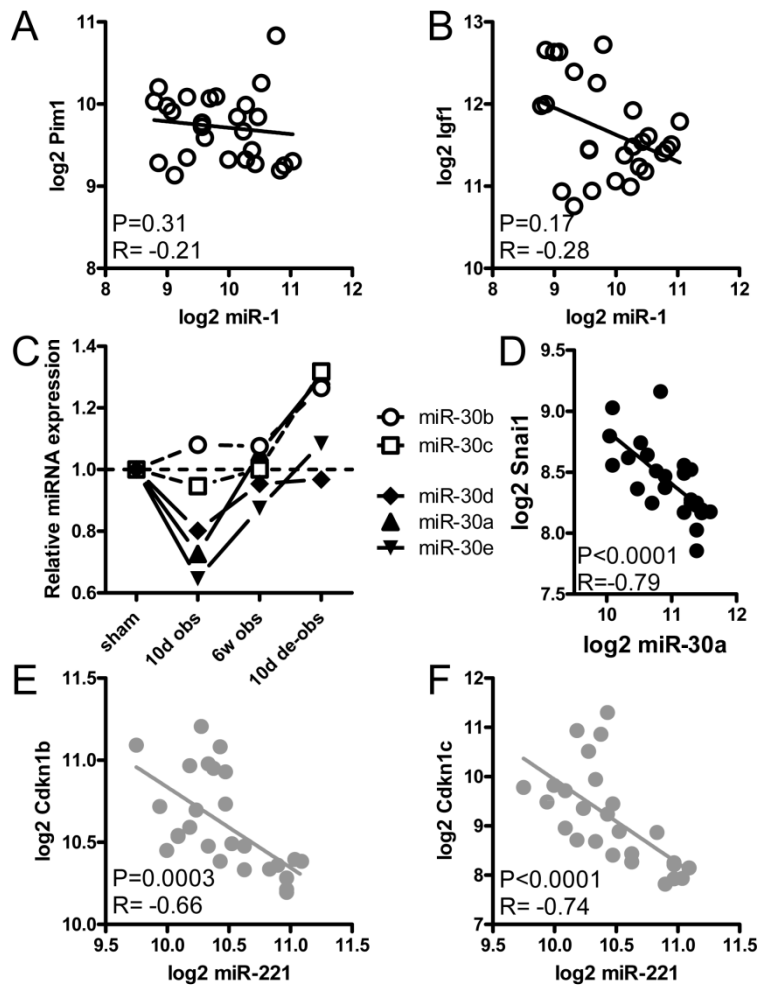


Figure 3

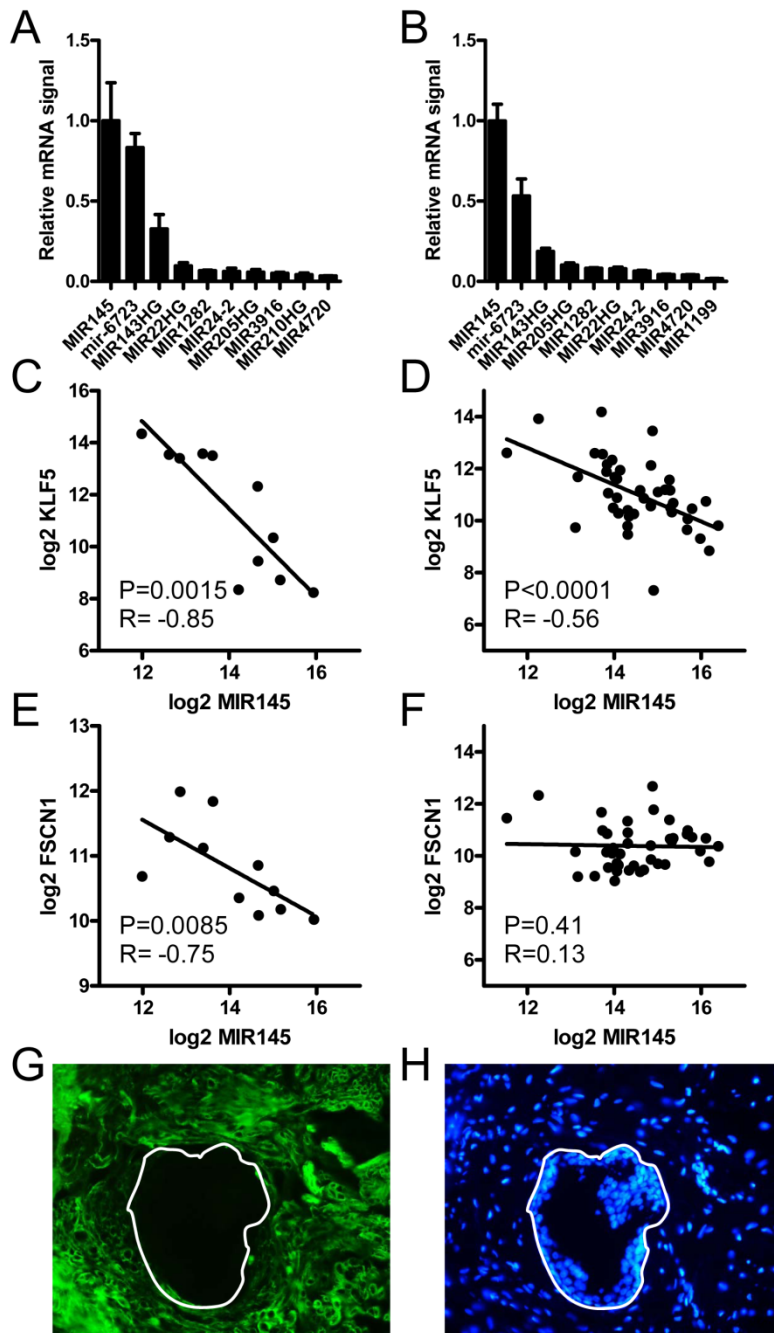


Figure 4

Probeset	10d vs. sham	Q	6w vs. sham	Q	10d de-obs vs. sham	Q
rno-miR-212_st	6,9	0,0	3,5	0,0	1,7	5,0
rno-miR-495_st	5,8	0,0	3,5	3,7	0,9	48,3
rno-miR-212-star_st	4,0	0,0	2,7	3,7	1,7	30,8
rno-miR-132_st	3,3	0,0	2,3	0,0	1,6	1,1
rno-miR-335_st	2,8	0,0	2,8	0,0	1,5	47,6
rno-miR-15b_st	2,2	0,0	1,6	3,4	1,1	37,4
rno-miR-7a-1-star_st	0,7	0,0	0,7	1,5	0,7	1,3
rno-miR-182_st	0,7	0,0	0,6	4,5	0,8	7,9
hp_rno-mir-338_st	0,7	0,0	0,7	3,8	0,7	1,3
rno-miR-141-star_st	0,6	0,0	0,5	1,5	0,6	0,8
rno-miR-339-3p_st	0,6	0,0	0,7	9,0	0,8	10,7
rno-miR-375_st	0,5	0,8	0,6	6,8	1,1	55,0
rno-miR-1_st	0,5	0,8	0,6	5,9	0,5	0,9
rno-miR-342-5p_st	0,5	0,0	0,5	1,5	0,3	0,0
rno-miR-29c-star_st	0,5	0,0	0,8	10,2	1,0	55,7
rno-miR-204_st	0,5	0,0	0,4	1,5	0,6	1,4
rno-miR-139-3p_st	0,4	0,0	0,4	2,3	0,3	1,1
rno-miR-29b-2-star_st	0,4	0,0	0,7	8,4	0,8	9,5
rno-miR-29c_st	0,4	0,0	0,6	3,5	1,0	56,4
rno-miR-708_st	0,4	0,0	0,5	1,5	0,6	0,8
rno-miR-338-star_st	0,4	0,0	0,5	0,0	0,6	0,9
rno-miR-9-star_st	0,3	0,0	0,3	1,5	0,2	0,0
rno-miR-338_st	0,3	0,0	0,3	1,5	0,4	4,1
rno-miR-383_st	0,2	0,0	0,2	0,0	0,2	0,0

Table 1