An androgen receptor-microRNA-29a regulatory circuitry in mouse epididymis*

Wubin Ma1, Shuanggang Hu1,2, Guangxin Yao1,2, Shengsong Xie1,2, Minjie Ni1, Qiang Liu1, Xinxing Gao3, Jun Zhang3, Xingxu Huang3#, Yonglian Zhang1,4#

1Shanghai Key Laboratory for Molecular Andrology, State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Shanghai 200031, China. 2The Graduate School, Chinese Academy of Sciences, Shanghai 200031, China. 3Model Animal Research Center, Nanjing University, Nanjing 210061, China. 4Shanghai Institute of Planned Parenthood Research, Shanghai 200032, China.

Running title: The regulatory circuitry between AR and miR-29a

#To whom correspondence should be addressed: Yonglian Zhang, PhD, 320, Yue-Yang Road, Shanghai 200031, China, Tel. 86-21-54921163, Fax. 86-21-54921011, E-mail: ylzhang@sibcb.ac.cn. Co-corresponding author, Xingxu Huang, E-mail: xingxuhuang@mail.nju.edu.cn, Tel. 86-25-58641517.

Keywords: androgen, androgen receptor, miR-29a, epididymis, IGF1, p53

Background: Abnormally up-regulated miR-29a was associated with several diseases, so miR-29a repression is important for homeostasis.

Results: MiR-29a was repressed by androgen system and inversely inhibited AR expression by targeting IGF1 and CDC42/p85α-p53 pathway in mouse epididymis.

Conclusion: There is an androgen receptor-miR-29a regulatory circuitry in mouse epididymis.

Significance: The mutual repression between miR-29a and AR may be important for epididymal development and functions.

MicroRNAs are involved in a number of cellular processes, thus their deregulation is usually apt to the occurrence of diverse diseases. Previous studies indicate the abnormally up-regulated miR-29a is associated with several diseases, such as human acute myeloid leukemia and diabet, therefore, the proper level of miR-29a is critical for homeostasis. Herein, we observed that miR-29a was repressed by androgen/androgen receptor (AR) signaling in mouse epididymis by targeting a conserved androgen response element (ARE) locating 8 kb upstream of miR-29b1a loci. It is well known that multiple regulatory programs often form a complicated network. Here, we found miR-29a reversely suppressed androgen receptor and its targeting genes by targeting IGF1 and p53 pathways. MiR-29b1a overexpression transgenic mouse displayed a hypoplasia epididymis, partially similar to the phenotype of those mice with impaired androgen-androgen receptor signal system. Taken together, the results demonstrated there was a regulatory circuitry between androgen signaling pathway and miR-29a in mouse epididymis, which may vital for epididymal development and functions.

MicroRNAs (miRNAs) are a class of endogenous, 20–23 nucleotides, small
non-coding RNAs and serve as post-transcriptional regulators of gene expression (1). miRNAs are involved in a variety of cellular processes and organ development through regulating a large number of genes (2). Consistently, the deregulation of miRNAs frequently links to distinct diseases (3-5). Therefore, the proper regulation of miRNAs in physiological state is important.

Our and other previous studies reveal miR-29a is up-regulated during postnatal development of many organs, such as heart, liver, lung, epididymis, aorta, brain, cornea, and skeletal muscle, then maintains in a high level in adult stage (6-11). However, abnormally elevated miR-29a level is associated with several diseases. For example, miR-29a increases in muscle, adipose tissue, and liver of diabetic rats, and up-regulation of miR-29a causes insulin resistance in adipocytes (12). Recently, Han et al. observe that a significant proportion of human acute myeloid leukemia exhibit enhanced miR-29a expression and suggest that miR-29a may also play a role in human myeloid leukemogenesis (13). In addition, miR-29a expression is greatly enhanced in patients with invasive breast carcinomas compared with non-invasive hyperplasias (14). All these data indicate that abnormal up-regulation of miR-29a is harmful, thus the miR-29a expression should be properly suppressed.

Indeed, previous studies reveal that miR-29a is suppressed by multiple factors, such as TGF-beta/Smad3, c-Myc, Hedgehog, and NF-kappaB signaling pathway (15-18). In male reproductive system, especially in epididymis, androgen pathway plays very important regulatory roles in their development, functions, gene expression, and even miRNA expression. For example, miR-125b, miR-99a, and miR-100 are suppressed by androgen/AR in LNCaP prostate cancer cells (19). In addition, using the analysis of chromatin immunoprecipitation followed by sequencing (ChIP-Seq), we discovered an AR binding site (ARBS) locating at 8 kb upstream of miR-29b1a loci in mouse caput epididymis (unpublished data). All these evidences suggest that miR-29b1 and miR-29a may be involved in androgen signaling pathway in male reproductive system.

Epididymis, a highly androgen-sensitive organ responsible for sperm maturation and storage, was employed here to test this possibility by using castration model. Our results showed that miR-29a was repressed by androgen/AR in mouse epididymis. Disruption of the repression by over-expressing miR-29a in transgenic mice resulted in a hypoplasia epididymis. Furthermore, elevated miR-29a expression can reversely suppress AR expression by targeting IGF1 and CDC42/p85α-p53 pathways. Taken together, our results demonstrated there was a regulatory circuitry between androgen/AR signaling pathway and miR-29a, which may be important for epididymal development and functions.

**EXPERIMENTAL PROCEDURES**

**Animals and cell culture**—Male C57BL/6 mice and male Sprague Dawley rats were purchased from the Animal Center of the Chinese Academy of Sciences (Shanghai, China). Experiments were conducted according to a protocol approved by the Institute Animal Care Committee. The protocol conforms to internationally accepted guidelines for the humane care and use of laboratory animals. The approved permit number for this study is “SYXK2007-0017”.

Immortalized mouse epididymal cell line PC-1 from proximal caput epididymis was kindly provided by Dr. Orgebin-Crist (Department of Obstetrics and Gynecology, Vanderbilt University School of Medicine, Nashville, TN). PC-1 cells were grown at 33°C with 5% CO₂ in Iscove Modified Dulbecco Medium supplemented with 10% (v/v) fetal...
bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 4 mM glutamine, penicillin-streptomycin (25000 U penicillin G sodium, 25 mg streptomycin sulfate), and 1 nM 5α-dihydrotestosterone (DHT) (20). The MCF-7 and HEK 293T cells were maintained in DMEM media; LNCaP, RM-1, U-2 OS, and T24 cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) FBS; MG-63 cells were maintained in MEM medium supplemented with 10% (v/v) FBS; RWPE-1 cells were maintained in keratinocyte SFM medium. All these cells cultured at 37°C with 5% CO₂. All the cell culture reagents were purchased from Invitrogen-Life Technologies (Carlsbad, CA). For androgen deprivation, cells were plated in the medium supplemented with 10% charcoal-stripped serum (Hyclone) 24 h before treatment with 5α-DHT. Vehicle was ethanol. The IGF1 protein was purchased from ProSpec company (Cat. No. CYT-216).  

Castration and androgen replacement—Castration and androgen replacement were conducted following a previously described protocol (21,22). Briefly, mouse castration model as following: adult 10-wk-old male C57BL/6 mice were castrated bilaterally under sodium pentobarbital anesthesia. The mice were divided into three groups (six mice per group), killed at different times after castration (0, 2 d after the initial testosterone propionate (TP, 2.5 mg/kg body weight) or sesame oil injections (at 7d after castration). For rat castration model, the 120-day-old normal male Sprague Dawley rats were divided into nine groups (4–7 rats per group) and killed on days 1, 3, 5, and 7 after castration as well as 1, 3, 5, and 7 days after the initial TP injection. Androgen supplementation began at the seventh day after castration, and the rats were injected with TP (3 mg/kg body weight) every 2 days. The epididymidis, prostate, brain were excised and used for RNA extraction.  

Northern blotting—The RNA was extracted with TRizol Reagent (Invitrogen, Carlsbad, CA). Total RNA was resolved by 15% denatured polyacrylamide gel containing 8 M urea. The RNA was then transferred to GeneScreen Plus Hybridization Transfer Membrane (PerkinElmer, Waltham, MA). After baking at 80°C for half an hour and then UV crosslinking, the membranes were stained by methylene blue to evaluate the transfer efficiency. The membranes were incubated in hybridization buffer (Toyobo, Osaka, Japan) at 45°C for 30 min, then hybridized with specific γ-32P labeled oligonucleotide probes (Sangon, Shanghai) complementary to each miRNA at 45°C for 16 h. The membranes were washed twice with washing buffer (2×SSC with 0.1% SDS) and the signal was detected by phosphorimage system scanned by FUJIFILM-9000. The hybridized membranes were then re-hybridized with specific γ-32P labeled 5S rRNA probes (Sangon, Shanghai) at 50°C for 2 h, which were used as loading control. The oligonucleotide probes for Northern blotting were listed as followings: miR-29a: 5'-TAACCGATTTCAGATGGTGCTA-3'; miR-29b: 5'-AACACTGATTTCAAATGGTGCTA-3'; 5S rRNA: 5'-CGGTATTCCCAGGCGGTCT-3'.  

ChIP, ChIP-PCR, ChIP-qPCR, Peak finding, Motif search, and Electrophoretic mobility shift assay—The performance of ChIP, ChIP-PCR, ChIP-qPCR, Peak Finding, Motif search, and Electrophoretic Mobility Shift Assay were conducted following a previously described protocol (21). The primers for ChIP-PCR and ChIP-qPCR were: forward: 5'-TTGGTTCTCATCCGTCCTTTAT-3'; reverse: 5'-AGAATGGGCTTCTCCCTCATAGA-3'.  

Generation of Transgenic Mice—A precursor of mouse miR-29b1a was amplified with the following primers:
5'-GTggeggegACGGACTTCACCTCCCT-3' (Not I) and reverse: 5'-GAgagctcGTCAGCAATAACGTAAGTCAG-3' (Sac I). The 541-bp product was digested with restriction endonuclease Not I and Sac I, and then cloned into the expression vector pUBC containing a human ubiquitin-C promoter, which was found to provide the most reliable expression across different cell types. After testing the efficacy of miR-29a and miR-29b over-expression in HEK 293T cells, the construct was linearized for pronuclear microinjection.

**Real-time RT-PCR**—Quantitative real-time RT-PCR was performed to detect mRNA expression using Toyobo SYBR Green Real-time PCR Mix (Toyobo, Osaka, Japan) according to the manufacturer’s protocol. Levels of mRNAs were normalized to GAPDH. The primers were as following: Mouse AR, 5'-CTGGGAAGGGTCTACCCAC-3' (forward), 5'-GGTGCTATGTAGCGGCCTC-3' (reverse); mouse Adam7, 5'-GGTCATTGTGCTTGTCATGG-3' (forward), 5'-ACGGAGGATTAGCCCAGTCT-3' (reverse); mouse Fkbp5, 5'-GTCCAAAGCCTCAGAGTCGTTC-3' (forward), 5'-AGCCTTTCTCATTGCCTGTCATGG-3' (reverse); mouse Gldc, 5'-AAAAGTCCGCACAGTGAAAGGA-3' (forward), 5'-CCCACGGGCAAATGTTTA-3' (reverse); mouse Gpx5, 5'-TATTGCCGCTCAGCAATCCA-3' (forward), 5'-TTAACGGGGAAGGAGGAAGA-3' (reverse); mouse Igf1, CTGGACCAGAGACCCTTTGC-3' (forward), GGACGGGGACTTCTGAGTCTT-3' (reverse); mouse Cdc42, 5'-AAAAGTGGGTGCCTGAGATAAC-3' (forward), 5'-GGCTCTTCTTCGGTCTGGAG-3' (reverse); mouse p85α, 5'-GCAGAGGCTACCAGTACAGA-3' (forward), CTGAATCCAATGGCCACTAAGG-3' (reverse).

**miRNAs, small interfering RNAs, and Transfection**—RNA oligonucleotides were transfected into cells using RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. MiR-29a and miR-29b duplex (pre-miR-29a, pre-miR-29b) or miR-29a specific inhibitor (anti-miR-29a) molecules and appropriate negative control molecules (pre-miR-negative control, pre-miR-NC; or anti-miR- negative control, anti-miR-NC) were purchased from Ambion (Austin, TX). Small interfering RNAs (siRNA) were synthesized by GenePharma (GenePharma, Shanghai, China): IGF1 siRNA-1: 5'-CAGCCUUUCAACUCUAUUATT-3', IGF1 siRNA-2: 5'-GUGACUUUCUUGAGAUAAT-3'; and p53 siRNA was: 5'-GUACUGUAAUAAGCUCCTT-3'.

**Western blotting**—Proteins were extracted from cells using RIPA buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 1% (v/v) NP-40, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS). Each protein sample (30–50 μg) was separated by 10% SDS-polyacrylamide gels (for AR and p53) or 15% SDS-polyacrylamide gels (for IGF1), and then semi-dry blotted to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Immuno-detection of proteins was carried out by standard procedures using rabbit polyclonal antibody (Ab) to AR (Santa Cruz Biotechnology, Santa Cruz, CA, 1:500 dilution), IGF1 (ABCAM, 1:500 dilution), and p53 (ABCAM, 1:500 dilution). The protein blot were probed with primary antibody and goat anti-rabbit secondary Abs conjugated to HRP (Sigma-Aldrich, St. Louis, MO) and detected using ECL (GE, Piscataway, NJ).
reagents. β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control.

**DNA constructs and mutagenesis**—792-bp AR-binding regions around the peak summit was amplified with primer-1, 5'-gaggtaccTCCATGTCTTTGACTTGATTTT C-3' (Kpn I) and primer-2, 5'-gaactcgagGCTCGCCCTTTGAGAAACATT-3' (Xho I), and subcloned into pGL3-Basic and pGL3-Promoter luciferase reporter vectors. The ARE mutated with primer 1, primer 2, primer 3 (5'-TTCAGGGCCTGAGTAGATAGATGT-3'), primer 4 (5'-TAAGAACATGGCTCAATTCTTTTTC-3'), and primer 5 (5'-TACTCAGGCCCTGAA TAAGAACATGGCTC-3'). Briefly, the upstream of ARBS fragment was amplified using primer 1 and primer 3, the downstream of ARBS fragment was amplified using primer 2 and primer 4, and then amplified the full-length mutated ARBS using primer 1, primer 2, and primer 5 with the upstream and downstream as template. Then the product was cut with Kpn I and Xho I and liganded with pGL3-Promoter luciferase reporter vectors. HEK 293T cells were grown in 96-well plates, cotransfected with 150 ng reporter constructs, 15 ng pRL-TK (Renilla luciferase), and 15 ng pcDNA3.1-AR expression vector coding for full-length human AR using X-tremeGENE HP DNA Transfection Reagent (Roche). After 12 hours transfection, cells were treated with 100 nM dihydrotestosterone or ethanol vehicle for 24 h. Luciferase activity was measured using the dual-luciferase reporter assay (Promega Madison, WI) and Mithras LB940 multimode microplate reader (Berthold, Bad Wildbad, Germany).

The three prime untranslated regions (3'UTR) of the human IGF1 were amplified using the primers 5'-GTAGgggcccCCCTTGTGTACATCTTTG GCC-3' (Not I). After cut with Xho I and Not I, the amplified products were subcloned to downstream of the Renilla luciferase gene of the psiCHECK™-2 plasmid (Promega, Madison, WI). The site-directed mutagenesis was performed using Quick-Change Site-Directed Mutagenesis kit (Stratagene, Santa Clara, CA). The primers for mutation of the target site of human IGF1 3'UTR were 5'-GTAAAACCTCGTTTTTTAGTATAATGGG TAGATTTTGTAGTTTTGTATATGAAAGAGT C-3' (forward), and 5'-GACTCTTTTCTATATAACAAACTACAAAA TCTACCCTTTACTAAAAACAGGTTT TAC-3' (reverse). Four nucleotides TGCT in “seed” sequences of miR-29a potential target site were mutated to GTAG (underlined).

**Luciferase reporter assay**—HEK 293T cells were seeded to 24-well plates 24 h before transfection. HEK 293T cells were transiently transfected with 100 ng of psiCHECK™-2 (Promega Madison, WI) vector containing the wild-type IGF1 3'UTR (named as IGF1-3'UTR-WT) or IGF1 3'UTR mutant (named as IGF1-3'UTR-Mut), and together with 30 nM pre-miR-29a or pre-miR-NC, respectively. By using dual-luciferase assay kit (Promega Madison, WI), the luciferase activities were measured 24 h after transfection. The renilla luciferase activity was normalized to firefly activity. Lipofectamine 2000 transfection reagent was used for co-transfection of RNA oligonucleotides and plasmid.

**Hematoxylin and Eosin (HE) Staining and Immunohistochemical Staining**—HE staining of the sections was carried out using standard protocols. Immunohistochemical staining was performed as described previously (23). Primary and secondary antibodies were diluted in PBS containing 10% normal goat serum. The 1:100-diluted anti-AR antiserum (Abcam, Cat. No. ab74272, Cambridge, UK) was applied to the tissues overnight at 4°C and the 1:200-diluted
biotin-conjugated goat anti-rabbit IgG was incubated for 1 h at room temperature. The expression was visualized using horseradish peroxidase (HRP) substrate. As a negative control, serial sections were subjected to the same procedure, with normal rabbit serum replacing the primary antibody. The expression was visualized using horseradish peroxidase (HRP) substrate. The sections were mounted in 80% glycerol and examined with an Olympus BX-52 microscope.

In Situ Hybridization—In situ hybridization was performed as described previously (24). Briefly, the epididymal tissues were fixed in 4% paraformaldehyde (PFA), and then embedded in paraffin. The embedded epididymidis were sectioned. The slides were dewaxed, then digested with Proteinase K (500 ng/ml), hybridized with locked nucleic acid (LNA) probes (Exiqon) special for mmu-miR-29a, U6 snRNA and a scramble oligonucleotides, which were labeled with digoxigenin (DIG). The temperature of hybridization was 65 °C. The expression of miR-29a or control small RNAs was visualized using alkaline phosphatase substrate.

TUNEL assay—The TUNEL assay was performed as the protocol of in situ apoptosis detection kit (Takara Bio Inc., Shiga, Japan). In short, after deparaffinization and rehydration, the 5 μm paraplast tissue sections were incubated with 20 μg/ml Proteinase K solution (Invitrogen/Gibco/MP, Cat. No. 25530049) for 15 min at 37 °C. Thereafter, sections were incubated with FITC-labeled terminal deoxynucleotidyl transferase. Next, sections were rinsed in PBS and observed under a fluorescence microscope.

RESULT

Androgen negatively regulates miR-29a expression in epididymis—Androgen acts through its receptor, AR, to control normal development of male reproductive organs and maintain male characteristics. Orchietomy is considered as an ideal model to investigate androgen effects, for example, its regulation on gene expression. Therefore, herein castration model was used to investigate the response of miR-29a to androgen manipulation. Firstly, we found that the level of miR-29a was higher in the mouse epididymis at 9-day after orchiectomy (after castration 7d with sesame oil injection for 2-day) than in normal epididymis. MiR-29a level in sesame oil injection group was also higher than TP injection group (Fig. 1A, left panel). These results strongly demonstrated that miR-29a level was revered with androgen level (Fig. 1A). Subsequently, similar phenomena were observed in rat epididymis by using castration model (Fig. 1B). After castration, the ratio of epididymal cell types may be altered because cell apoptosis occurred and mainly occurred in principle cells (25). In order to exclude the possibility of the change of miR-29a caused by the altered ratios of cell types in the castrated epididymis, we investigated miR-29a expression pattern in normal rat epididymis using in situ hybridization. The result showed that miR-29a was extensively expressed in epididymal epithelial cells (Fig. 1C). Thus, the change of miR-29a mainly caused the regulatory effects. Further, we tested whether miR-29a was androgen-responsive in principal cells using an in vitro system. PC-1 cell line, an immortalized epididymal principal cell line from mouse proximal caput was employed here. PC-1 cells were maintained in IMDM medium with different concentrations of DHT. Twenty four hours later, miR-29a expression was determined using Northern blotting. The expression of miR-29a in PC-1 cells cultured without DHT was higher than with DHT. Furthermore, the level of miR-29a in PC-1 cells cultured without DHT was not the consequence of the
alteration of cell type ratios, but the alteration of androgen level. Thus, miR-29a was an androgen-repressive miRNA in mouse and rat epididymis.

miR-29b1, another member of miR-29 family, is in close proximity to miR-29a on mouse chromosome 6 (26). So, we detected the response of miR-29b to androgen. Similarly, miR-29b was also suppressed by androgen (Fig. 1E). In conclusion, miR-29b1/a cluster was repressed by androgen signaling system.

Differently, Ribas et al. found that miR-29a may be one of AR-induced miRNAs in two human androgen-responsive prostate cancer cell lines LNCaP and LAPC-4 by miRNA microarray analysis (27). To validate this result, LNCaP cells were plated in medium with 0 to 10 nM of DHT, and 72 hours later, the cells were harvested to detect miR-29a level. The result showed that miR-29a was indeed induced by androgen in LNCaP cells (Fig. 2A), which differed from in epididymal cells. Hereupon, we were evoked by the question whether miR-29a was suppressed by androgen specifically in epididymis. To answer this question, we further investigated miR-29a expression in prostate and brain in castrated mouse. Consequently, the expression of miR-29a was repressed by androgen stimulation in mouse prostate tissue, while in brain there was no apparent change (Fig. 2B and C). Therefore, the repression of miR-29a by androgen was not epididymis specific.

Next, we wondered whether the responsive patterns of miR-29a were different between physiological and tumorigenic state. We then investigate the responses of miR-29a to androgen stimulation in mouse prostate cancer cell line RM-1 and normal human prostate epithelial cell line RWPE-1. As a result, miR-29a was also suppressed by androgen in RM-1 cells and RWPE-1, although the change in RWPE-1 cells is weak (Fig. 2E and F). Furthermore, we also tested the responses of miR-29a to androgen in several human AR-positive cancer cell lines, including MCF-7 cells (breast cancer), U-2 OS, and MG-63 cells (osteosarcoma), and T24 cells (bladder carcinoma). However, there are no apparent changes in these cell lines (Fig. 2G-I). RM-1 cell lines is androgen-insensitive. Why DHT also inhibit the expression of miR-29a in androgen-insensitive RM-1 cell line? Previous study revealed that DHT treatment of androgen insensitive human prostate cancer cell line PC-3 resulted in upregulation of p21CIP1 levels (28). Because the AR-mediated trans-activation could be induced in a ligand-independent manner (29,30). We deduced that miR-29a was suppressed in RM-1 cells possibly by the similar mechanism, because AR also can be upregulated by DHT stimulation in RM-1 cells (Figure 2J). These results indicated that the repression of miR-29a by androgen was context-dependent.

Androgen regulates miR-29b1a through AR binding to upstream of miR-29b1a—Then, we investigated how androgen regulated miR-29a and miR-29b expression. It is well known that the effects of androgen are mediated through AR binding to ARE DNA (31,32). Previously, by ChIP-Seq assay we identified the binding sites of AR in mouse caput epididymis. These binding sites were associated with sixty-three miRNAs, including miR-29a (21). As shown in Fig. 3A, there was an AR binding site enriched by AR at 8 kb upstream of miR-29a and miR-29b1 precursor. We first confirmed this site was a bona fide AR binding site by ChIP-PCR (Fig. 3B). Our results showed the peak was androgen-dependent (Fig. 3C). Next, we performed bioinformatics analysis and found that the binding site contained two conserved AREs (Fig. 3D). Further experiments by EMSA showed the ARE1 but not ARE2 can be bound by AR (Fig. 3E). Mutation of ARE1 (Fig. 3D) abolished the binding activity (Fig. 3F and G), indicating that androgen regulated miR-29b1a
expression through AR binding to a conserved ARE upstream of miR-29b1a.

Then the ARBS was subcloned to pGL3-Basic luciferase reporter vectors. Compared with pGL3-Basic vector, the pGL3-Basic-ARBS did not show apparent increased luciferase activity with DHT stimulation or not (data not shown), indicating the ARBS may be a enhancer/silencer. Next, the ARBS was subcloned to pGL3-Promoter vector. As Fig. 2H showed, the luciferase activity was downregulated after DHT stimulation compared to ethanol control, but the mutation of ARE1 abolished the repressive effect. These results indicated that the ARE1 was a bona fide repressive regulator element on miR-29b1a expression.

Over-expression of miR-29b1a leads to smaller epididymis—As described above, androgen signaling pathway inhibited miR-29b1a expression in mouse epididymis. To further confirm this result in vivo and explore its biological function, we generated miR-29b1a transgenic mice by over-expressing a 523-nt precursor of miR-29b1a under the control of the promoter of ubiquitin-C (UBC) gene (Fig. 4A). The genotyping of the mice was performed by PCR on genomic DNA (data not shown). MiR-29a and miR-29b expression levels were detected using Northern blotting. As anticipated, there were more miR-29a and miR-29b expression in transgenic mice epididymis compared to their littermates (Fig. 4B). Consequently, epididymis from transgenic mice displayed small size and weighty (Fig. 4C and D), demonstrating miR-29b1a over-expression disturbed epididymal development. Although the transgenic mice displayed smaller epididymis, but the ratios of cell types showed no apparent change (Fig. 4E). There was no apparent difference of cell apoptosis in wild type and transgenic mice epididymis, but more apoptotic cells were observed in transgenic mice testis (Fig. 4F).

Feedback inhibition of AR by miR-29a—It is well known that the epididymis is agenesis in AR knockout mice (33-35). We wondered whether miR-29a disturbed epididymal development by inhibiting AR expression. To test this speculation, we detected AR mRNA and protein levels in wild type and transgenic mouse epididymis by using real-time PCR and Western blotting, respectively. As expected, AR level was significantly lower in transgenic mouse epididymis than their non-transgenic littermates (Fig. 5A and B). Consistently, AR immunostaining showed a weak signal in transgenic mice epididymis compared to wild type (Fig. 4C). Next, we tested this reverse suppression in vitro. Pre-miR-29a and scramble RNA oligonucleotides were transfected into PC-1 cells. Forty-eight hours later, RNA and protein were extracted to detect the expression of AR. Compared to scrambled RNA transfection group, the mRNA and protein levels of AR was decreased in pre-miR-29a transfection group (Fig. 4D and E). Consistently, we found miR-29b can also suppress AR expression in PC-1 cells (Fig. 4F). Taken together, miR-29b1a suppresses the expression of AR in vitro and in vivo.

Such being the case, miR-29a and miR-29b should also suppress the target genes of androgen signaling pathway. Here, we detected four epididymal androgen-responsive genes, including Adam7, Fkbp5, Gldc, and Gpx5 (36,37). There was no any miR-29a and miR-29b binding sites on the 3’ UTR of these four genes using online software miRWalk (ww.umm.uni-heidelberg.de/apps/zmf/mirwalk/), indicating that they should not be direct targets of miR-29a and miR-29b. The result showed that these four genes were down-regulated in miR-29b1a transgenic mouse at mRNA level (Fig. 4G), demonstrating miR-29a and miR-29b down regulated AR expression, and thereby suppressed the activity of androgen signaling in mouse epididymis.
miR-29a represses AR expression through IGF1 and p53 pathway—Subsequently, we predicted possible miRNAs targeting sites of mouse AR at regions of 5′UTR, coding region, and 3′UTR using online software miRWalk (ww.umm.uni-heidelberg.de/apps/zmf/mirwalk/), and found miR-29a is not a candidate, thus miR-29a regulate AR expression probably in an indirect way. Then, we try to find which genes potentially mediated this regulation. Pervious study revealed that AR protein level is decreased in prostate from IGF1 knockout mice (38). Therefore, we determined whether IGF1 can also activate AR expression in mouse epididymal cells. At a concentration of 1 ng/ml, IGF1 can apparently promote AR protein level (Fig. 6A), but slightly decrease AR mRNA level (Fig. 6B). In consistent with this, after IGF1 being down-regulated by using specific siRNAs (Fig. 6C), AR protein level was also reduced (Fig. 6D), but AR mRNA was slightly increased (Fig. 6E). It is possible that IGF1 signal pathway can weakly inactivate the transcription of AR, but increase the stability of AR protein. Then, we explored whether miR-29a suppressed IGF1 expression. Over-expression of miR-29a by transfection of pre-miR-29a into PC-1 cells suppressed the expression of IGF1 at mRNA and protein levels (Fig. 6F and G). Consistently, IGF1 mRNA and protein levels were down-regulated in the epididymis of the miR-29b1a transgenic mice (Fig. 6H and I), although the IGF1 level in circulation didn’t showed apparent change (data not show). Our results, therefore, confirmed miR-29a suppressed IGF1 expression both in vivo and in vitro.

Further, to know whether IGF1 is a direct target of miR-29a, the dual luciferase reporter assay was performed. Firstly, IGF1 is predicted as a potentially conserved target of miR-29a by using three online softwares: TargetScan, MiRanda, and PicTar (Fig. 6J). Then, the wild-type or mutated 3′ UTR of IGF1 mRNA was cloned to downstream of the Renilla luciferase gene and co-transfected with pre-miR-29a or scrambled oligonucleotides into HEK 293T cells. Luciferase activities were measured 24 h after transfection. As a result, cells co-transfected with the reporter gene carrying wild-type IGF1 3′UTR and pre-miR-29a exhibited apparent reduction of the luciferase activities compared with cells transfected with the scrambled oligonucleotide (Fig. 6K, left). Then, four nucleotides (TGCT→GTAG) in “seed” sequences of miR-29a potential target site were mutated. The reduction of luciferase activity of the reporter by miR-29a was abrogated by the mutations (Fig. 6K, right). These data indicated that the IGF1 3′UTR was truly targeted by miR-29a. Consistently, Hand et al. also demonstrated recently, miR-29 a/b1 can target IGF1 in mouse liver (39). Taken together, IGF1 was a direct target of miR-29a and it mediated the suppression of AR by miR-29a.

Additionally, Alimirah et al. found p53 can negatively regulate AR expression by binding to a p53 DNA-binding consensus sequence of AR promoter in human prostate cancer cells (40). Meanwhile, p53 can be induced by miR-29 through targeting CDC42 and p85α (41). Thus, we deduced miR-29a may repress AR expression by inducing p53 expression in epididymis too. To verify this hypothesis, firstly, we wondered whether miR-29a can also promote p53 expression in epididymal cells. As expected, CDC42 and p85α, which negatively regulated p53 expression (30), were down-regulated in PC-1 cells transfected with pre-miR-29a (Fig. 7A). Consequently, over-expression of miR-29a in PC-1 cells elevated p53 protein level (Fig. 7B). Consistently, when miR-29a was inhibited, p53 protein decreased (Fig. 7C). As expected, CDC42 and p85α mRNA levels decreased (Fig. 7D), while p53 protein level increased in miR-29b1a transgenic mice epididymis (Fig. 7E). Thus, miR-29a can also promote p53 expression in epididymis too.
expression in mouse epididymis. Secondly, we want to know whether p53 can also inhibit AR expression in mouse epididymis. PC-1 cells were transfected with specific siRNA against mouse p53, and 48 h later, the AR protein level was indeed up-regulated (Fig. 7F). Therefore, p53 can also served as a mediator of the suppression of AR by miR-29a in mouse epididymis.

In summary, miR-29a and AR were mutually inhibited and formed a regulatory circuitry to regulate epididymal development (Fig. 8).

DISCUSSION

Androgen and its receptor regulate several miRNA biogenesis in different tissues or cell lines. For example, the expression of miR-21, miR-125b, miR-101, miR-148a, and miR-338 are modulated by androgen/AR in prostate cancer cells (27,42-45). Meanwhile, miR-122 and miR-216a are induced in female mouse liver and hepatocarcinogenesis by androgen (46,47). The facts that androgen is critical for epididymal development and function, and miR-29a plays vital roles in epididymal development and function (3), suggest an androgen regulation of miR-29a in epididymis. Herein, we identified a bona fide ARE upstream of miR-29b1a, and demonstrated that androgen negatively regulated miR-29a in epididymis. Our data first confirmed miR-29a was an androgen repressive miRNA in mouse and rat epididymis as well as in mouse epididymal cell line, and extended the current understanding of miRNAs regulation by androgen under different physiological and pathological states. RM-1 is an androgen-insensitive cell line. But miR-29a was also repressed in RM-1 cell by DHT. Previous study revealed the AR-mediated trans-activation could be induced in a ligand-independent manner (29,30). We deduced that miR-29a was suppressed in RM-1 cells possibly by the similar mechanism, because AR also can be upregulated by DHT stimulation in RM-1 cells (Fig. S3K).

Being the mediator of androgen functions, AR expression is well regulated by some transcriptional factors such as Wnt and NF-κB signaling pathway in prostate cancer cells (48,49). Serving as the post-transcriptional regulators, miRNAs can also regulate AR. Previous papers reported that several miRNAs, including miR-488*, let-7c and miR-331-3p down-regulated AR (50-52). Our study demonstrated miR-29a and miR-29b regulated AR expression in mouse epididymis. Furthermore, all the previous papers demonstrated that miRNAs inhibited AR at cell level. Our studies provided the first in vivo evidence that miRNAs regulated AR at organism level. Moreover, although androgen is critical for epididymal development and functions, how AR was regulated in epididymis was unclear up to now. This study, obviously, shed new light on the understanding of AR regulation in epididymis.

Androgen repressed miR-29a, and miR-29a down-regulated AR. Thus, there is a regulatory circuitry between miR-29a and AR. The regulatory circuitry fine tuned epididymal development and functions. Once the regulatory circuitry disrupted, epididymis disorders occurred. Our data showed that the expression of miR-29a level was elevated accompanying with androgen withdraw after castration. It has been reported miR-29a can induce cell apoptosis by targeting multiple genes (34-36). Thus, the castration-mediated miR-29a elevation might contribute to apoptosis of epididymal epithelial cells. Meanwhile, elevated miR-29a suppressed AR expression and deteriorated castration effects. Therefore, both castration and miR-29a over-expression mimicked the AR knockout-mediated epididymis agenesis.

Moreover, we further characterized the miR-29 and AR regulatory circuitry in epididymis by identifying IGF1 and p53 as critical mediators of the regulatory circuitry. As in the prostate (38), IGF1 enhanced AR
expression and was directly suppressed by miR-29a in mouse epididymis. Therefore, the suppression of miR-29a on AR might mediate or at least partially mediate by IGF1. Also, p53 bound to the promoter region of the AR gene to inhibit AR (40). miR-29a elevated p53 by down regulation of CDC42 and p85α (41). Herein, we demonstrated, in epididymis, miR-29a directly down-regulated CDC42 and p85α to promote p53, hence inhibited AR expression.

REFERENCE


ACKNOWLEDGEMENTS
We thank Miss Jin-Mei Chen for mouse handling.

FOOTNOTE
*This work is supported by the National Natural Science Foundation of China (31201113 and 30930053) and the Chinese Academy of Sciences (CAS) Knowledge Innovation Program (KSXC2-EW-R-07).
The abbreviations used are: miRNA, microRNA; 5α-DHT, 5α-dihydrotestosterone; TP, testosterone propionate; Pre-miR-NC, pre-miR-negative control; Anti-miR-NC, anti-miR-negative control; 3’UTR, three prime untranslated regions.

FIGURE LEGENDS
Figure. 1 Androgen negatively regulates miR-29a expression in mouse and rat epididymis. A. Use Northern blotting to detect miR-29a expression in mouse epididymis from normal, castration 7-day supplemented with sesame oil or testosterone propionate (TP, 2.5 mg/kg body weight) for an additional 2 d. Each group included three mice. The right panel showed the concentrations of serum testosterone of every mouse. B. Relative expression levels of miR-29a (Northern blotting) in the adult rat epididymis during androgen manipulation (n= 4–7). The right panel showed the average concentrations of serum testosterone of every group. C. In hybridization showed miR-29a was extensively expressed in adult rat epididymis. The probes were respectively for Scramble oligonucleotides (a, negative control), U6 snRNA (b), miR-29a (c-f). The blue signal represented positive signal. c-f represented initial segment, caput, corpus, and cauda, respectively. D. Northern blotting detection of miR-29a level in mouse PC-1 cells which were cultured without or with different concentrations (1 nM, 10 nM ) of DHT for 24 h. Fold change represents miR-29a relative to the non-stimulated state. E. Northern blotting detection of miR-29b in entire epididymis from normal, castration 7-day supplemented with sesame oil or testosterone propionate (TP, 2.5 mg/kg body weight) for an additional 2 d. 5S rRNA was used as an internal control.
Figure 2 Androgen response patterns of miR-29a in different tissues and cell lines. A. Northern blotting analysis of miR-29a dose response to DHT in human prostate cancer cell LNCaP treated with different concentrations of DHT for 72 hours. B and C. Use Northern blotting to detect miR-29a expression in mouse prostate and brain from normal, castration 7-day supplemented with sesame oil or testosterone propionate (TP, 2.5 mg/kg body weight) for an additional 2 d. In B and C, each group included three mice. The concentrations of serum testosterone of every mouse was same as Fig. 1A. D-I. Northern blotting analysis of miR-29a expression in different concentrations of DHT treated RM-1, RWPE-1, MCF-7, U-2 OS, MG-63 and T24 cells 72 hours, respectively. 5S rRNA used as a loading control. Fold change represents miR-29a relative to the non-stimulated state. J. Western blotting analysis AR expression in different concentration DHT treated RM-1 cells at 72 h. β-actin was used a loading control for western blotting.

Figure 3 Characterization of AR-binding sites associated with potential AR target gene miR-29b1a. A. Screen shots of the UCSC genome browser mm9 showing AR binding sites around miR-29b1a. The figure was generated by uploading to the UCSC genome browser one file containing unique ChIP-seq sites in the BED format. The ChIP-seq peak is shown at the upper and the AR target genes are shown at the bottom. B. Validation of the ARBS associated with miR-29b1a by ChIP-qPCR using anti-AR antibody. C. Validation of the AR-binding sites associated with miR-29b1a by ChIP-qPCR using anti-AR antibody. Enrichment folds were calculated using IgG enrichment as a control. The data are presented as the mean±SD of three replicates. ChIP samples were prepared from epididymidis of normal mice (Nor), mice castrated for 3 d (Cas), and mice castrated for 3 d but supplemented with TP for an additional 2 d. D. The ARBS included two potential conserved AREs and the wild type and mutated sequence of the two AREs were listed. E-G. AR-bound ARE1 but not ARE2 demonstrated by EMSAs. E. AR-bound ARE1 but not ARE2. ARE of C3 gene served as a positive control. Control incubation performed in the absence of protein is presented in lane 2. F and G. Competitors are indicated above and amounts of competitors used are given in molar excess. Competitors include unlabeled AREs, mutant AREs, and a consensus DNA-binding site for SP1. H. ARBS identified by ChIP-seq functions as an enhancer. The ARBS with wild type and mutated ARE1 were subcloned into the pGL3-Promoter plasmid. Plasmids were cotransfected with pcDNA3.1-AR into HEK 293T cells, and luciferase activities were measured after treatment with ethanol or 100 nM DHT. The data are presented as the mean±SD.

Figure 4 miR-29b1a over-expression transgenic mice showed smaller epididymidis. A. The vector of miR-29b1aUBC transgene, which was controlled by the promoter of UBC gene. B. The relative expression of miR-29a and miR-29b in epididymis from transgenic and littermate control at 7 weeks of age. C. Macroscopic appearance of the epididymis from transgenic mice and littermate control at 7 weeks of age. D. The ratio of testis weigh to body weight is comparable in WT and miR-29b1a transgenic mice. E. Hematoxylin and Eosin (H&E)-stained sections of epididymis from wild-type and transgenic 10-week-old mice (magnification, ×400). F. TUNEL analysis of cell death in epididymis and testis from wild type and transgenic mice 10-week-old mice. The arrows indicated apoptotic cells (magnification, ×100). WT, wild-type; TG, transgene.
Figure 5 miR-29a inhibited AR expression in mouse epididymis. A. Real-time PCR analysis of AR mRNA level in the epididymis from transgenic mice and littermate controls. Data were expressed as mean±SEM, n=3. B. Using Northern blotting and Western blotting to detect miR-29a and AR protein levels respectively in the epididymidis from three paired transgenic mice and littermate controls. tRNA staining was used as loading control for Northern blotting, and β-actin was used as loading control for Western blotting. C. AR expression in 10-week wild type (WT) and transgenic mice (TG) epididymis. AR was markedly reduced all regions of epididymis transgenic mice as shown by immunohistochemistry. IS, initial segment; Cap, Caput; Cor, Corpus; Cau, Cauda. D. Real-time PCR analysis of AR mRNA levels in PC-1 cells 48 h after miR-29a mimics transfection. Data were expressed as mean±SEM. E and F. Western blotting detection of the protein levels of AR in PC-1 cells 48 h after miR-29a and miR-29b transfection, respectively. β-actin was used a loading control for Western blotting. G. Real-time PCR analysis of AR target genes Adam7, Fkbp5, Gldc, and Gpx5 mRNA levels in the epididymidis from transgenic mice and littermate controls. Data were expressed as mean±SEM, n=3. Adam7; Disintegrin and metalloproteinase domain-containing protein 7, Fkbp5: FK506 binding protein 5, Gldc: glycine decarboxylase, Gpx5: glutathione peroxidase 5.

Figure 6 IGF1 mediated the suppression of AR by miR-29a. A and B. Western blotting and real-time PCR detection of AR protein and mRNA levels in PC-1 cells treated with DHT (10 nM) and/or IGF1 protein (1 ng/ml) 48 h later, respectively. The basic medium was IMDM without FBS. C. Real-time PCR analysis of IGF1 mRNA levels in PC-1 cells transfected with siRNAs against IGF1. D. Western blotting analysis of AR and IGF1 protein levels in PC-1 cells transfected with siRNAs against IGF1. E. Real-time PCR detected AR mRNA level in PC-1 cells transfected one mouse IGF1 siRNA 48 h later. F. Real-time PCR analysis of IGF1 mRNA levels in PC-1 cells transfected with miR-29a mimics or control mimics 48 h later. G. Western blotting analysis of AR and IGF1 protein levels in PC-1 cells transfected with miR-29a mimics or control mimics 48 h later. H and I. Real-time PCR and Western blotting analysis of IGF1 mRNA and protein levels in epididymis from transgenic mice and littermate controls, respectively. J. miR-29a target to human, mouse and rat IGF1 3’UTR. K. HEK 293T cells were transfected with reporters constructed by inserting either wild-type or mutant IGF1 3’UTR downstream of the luciferase gene. Results are normalized to an scramble RNA (mean ± SD, n = 3).

Figure 7 miR-29a suppressed AR expression by promoting p53 protein level. A. Real-time PCR analysis of CDC42 and p85α mRNA levels in PC-1 cells transfected with miR-29a mimics or control mimics. B. Western blotting analysis of AR and p53 protein levels in PC-1 cells transfected with miR-29a mimics or control mimics. C. Western blotting analysis of AR and p53 protein levels in PC-1 cells transfected with miR-29a inhibitor or control inhibitor. D. Real-time PCR analysis of CDC42 and p85α mRNA levels in epididymis from transgenic mice and littermate controls. E. Western blotting analysis of p53 protein levels in epididymis from transgenic mice and littermate controls. F. Western blotting analysis of AR and p53 protein levels in PC-1 cells transfected with siRNAs against mouse p53. The real-time PCR data were expressed as mean±SEM, n=3.

Figure 8 A model depicting androgen-AR-miR-29a regulation loop. Model depicting the role of the androgen, AR and miR-29 regulatory circuit in mouse epididymis. AR directly suppressed miR-29a expression. Conversely, on one side, miR-29 miRNAs directly suppress CDC42 and p85α, resulting
in the stabilization of p53, and then p53 inhibited the transcription of AR; on the other side, miR-29a inhibited IGF1 expression, attenuated the promotion on AR expression by IGF1 signaling pathway.
Figure 4

A  
\[ 5' \text{UBC-promoter} \quad \text{miR-29b1a} \quad \text{pA} \quad 3' \]

B  
\begin{align*}
\text{miR-29a} & : \text{WT} \quad \text{TG} \\
5S \text{ rRNA} & : \text{WT} \quad \text{TG} \\
\text{miR-29b} & : \text{WT} \quad \text{TG} \\
5S \text{ rRNA} & : \text{WT} \quad \text{TG}
\end{align*}

C  
\[ \text{WT} \quad \text{TG} \]

D  
\[ \text{Ratio of weight between epididymis and body} \]
\[ \text{WT} \quad \text{TG} \]
\[ P<0.003 \]

E  
\[ \text{WT} \quad \text{TG} \]

F  
\[ \text{Epididymis} \quad \text{Testis} \]

Downloaded from http://www.jbc.org/ by guest on October 2, 2017
Figure 5

A

B

C

D

E

F

G
Figure 6

A

DHT (10 nM) - + +
IGF-1 (1 ng/ml) - + +

AR

β-actin

1.2
1.1
1.1
1.0

B

DHT (10 nM) - + +
IGF-1 (1 ng/ml) - + +

AR mRNA/GAPDH mRNA

1.0
0.8
0.6
0.4

C

D

IGF1

Control
sICF1-1
sICF1-3

AR

1.00
0.72
0.58

β-actin

1.00
0.50
0.55

E

AR mRNA, relative level

F

IGF1 mRNA/GAPDH mRNA

G

Pre-miR-NC

Pre-miR-29a

IGF1

1.00
0.71

AR

1.00
0.62

β-actin

H

IGF1 mRNA/GAPDH mRNA

I

WT

TG

β-actin

1.00
0.76

J

Mouse IGF1 3'UTR (921-94236)-GUUUUUUAGUACAAUGU GGCUA-3'

miR-29a3'-AUUGGCUAAAGUCUACCAGAU-5'

Rat IGF1 3'UTR (921-94285)-UGUUUUAGUACAAUGUGGCUA-3'

miR-29a3'-AUUGGCUAAAGUCUACCAGAU-5'

Human IGF1 3'UTR (885-906)-5'GAAAUGUGGUUAGUAAUUGUGCUA-3'

miR-29a3'-AUUGGCUAAAGUCUACCAGAU-5'

K

Relative luciferase activity

Pre-miR-29a

Pre-miR-NC

IGF1 3'UTR-WT

IGF1 3'UTR-mut
Figure 8
An androgen receptor-microRNA-29a regulatory circuitry in mouse epididymis
Wubin Ma, Shuanggang Hu, Guangxin Yao, Shengsong Xie, Minjie Ni, Qiang Liu,
Xinxing Gao, Jun Zhang, Xingxu Huang and Yonglian Zhang

J. Biol. Chem. published online August 19, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M113.454066

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2013/08/19/jbc.M113.454066.full.html#ref-list-1