Original Paper

Long Non-Coding RNA Expression Profiling in Aging Rats with Erectile Dysfunction

Lianjun Pan^{a,b} Jiehua Ma^b Feng Pan^b Dan Zhao^b Jianping Gao^a

^aDepartment of Urology, Jinling Hospital, School of Medicine, Nanjing University, Nanjing, ^bState Key Laboratory of Reproductive Medicine, Department of Reproductive Health, Nanjing Maternity and Child Health Care Hospital affiliated with Nanjing Medical University, Nanjing, China

Key Words

lncRNAs • Expression profiles • Erectile dysfunction • Gene Ontology • Pathway

Abstract

Background/Aims: Erectile dysfunction (ED) in aged people remains a topic of interest to andrological physicians. Long non-coding RNAs (lncRNAs), which form the largest group of non-coding RNAs, have been shown to regulate various biological processes. The function of lncRNAs in age-related erectile dysfunction (A-ED) pathogenesis remains poorly understood.

Methods: This study aims to assess the differential expression profiles of mRNAs and lncRNAs between A-ED and normal control (NC) samples. Using a second-generation lncRNA microarray, we detected a total of 8,744 lncRNAs and 13,585 coding transcripts. **Results:** We identified 608 up-regulated and 406 down-regulated lncRNAs in A-ED compared with NC samples, by setting a filter of fold-change >2.0. Gene Ontology and pathway analysis revealed that a muscle contraction disorder induced by abnormal ion channels might play a critical role in the pathogenesis of A-ED. **Conclusion:** Our results show significantly altered expression profiles of lncRNAs and mRNAs between A-ED and NC. This study may provide information for further research on A-ED and may be helpful for finding a new therapeutic target for A-ED.

© 2015 The Author(s)
Published by S. Karger AG, Basel

Introduction

Aging is an important risk factor for erectile dysfunction (ED) [1]. Epidemiologic studies have shown an increasing age-related prevalence and severity of ED [2, 3]. Data collected in 29 countries from 27,500 men reveals that approximately 50% of the men aged 70-80 years still have sexual intercourse and that 68% of the men were in favour of using medical treatments

L. Pan and J. Ma contributed equally to this study.

Jianping Gao

Department of Urology, Jinling Hospital, School of Medicine, Nanjing University, 305rd Zhongshan East Road, Nanjing 210002, (China)
Tel. +86 025-52226162, Fax +86 025-84726922, E-Mail drjianpinggao@163.com

to help older people enjoy sexual activity [4]. However, the value of medical treatment of ED in the older patient is limited [5]. As the number of older people increases, the needs of older people are expected to become an increasingly relevant health issue. Although age-related ED (A-ED) is attributed largely to endothelial dysfunction and increased oxidative stress in the penis, the molecular basis of this effect remains largely unknown [1]. Therefore, A-ED remains a topic of interest to andrological physicians.

The expression of non-coding RNAs (ncRNAs) appears to be important for mediating erectile function [6]. Long non-coding RNAs (lncRNAs), which are transcripts that are longer than 200 nucleotides (nt) that do not encode proteins, represent a novel class of regulatory molecules [7]. Accumulating evidence indicates that lncRNAs play significant regulatory roles in various biological processes, including genomic imprinting, cell apoptosis, cell differentiation, nuclear trafficking, heat shock response, and genome rearrangement [8, 9]. In addition, studies indicate that lncRNAs are associated with a variety of diseases such as cancer, heart disease and neurological disorders [10-12]. Although lncRNAs have been reported to have a wide range of physiological functions, their role in the progression of ED is poorly understood. Several studies have aimed at identifying and characterizing the specific roles of lncRNAs in vascular biology [13]. For example, increased expression of antisense ncRNA transcripts from the INK4 (ANRIL) locus is directly correlated with the severity of atherosclerosis [14]. These observations suggest that lncRNAs may have a functional role in A-ED, which is usually correlated with vascular factors [3].

In this study, we investigated the expression profiles of mRNAs and lncRNAs in A-ED and normal control samples to explore the molecular processes associated with A-ED.

Materials and Methods

Preparation of tissues

Samples of the corpora cavernosa in the penis were taken from 20-month old Sprague-Dawley (SD) rats. The Experimental Animal Center of Nanjing Medical University provided the rats, and the Institutional Animal Care and Use committee at Nanjing Medical University approved our study. All of the rats were treated with apomorphine to induced penile erection to evaluate erectile function. Aging rats with ED were screened using a previously published test procedure [15-17]. Then, the experimental rats were divided into two groups, aging rats with normal erectile function as the normal control (NC) group and aging rats with ED (A-ED). Thereafter, histological measurement and intracavernous pressure (ICP) test were used to confirm the groups. Finally, we carefully collected middle penile shafts and corpus cavernosa (CC) from each group. Among the 9 cases for each group, 3 and 6 cases were used for microarray analysis and quantitative real-time polymerase chain reaction (qRT-PCR), respectively. All of the samples were immediately frozen in liquid nitrogen and then stored at -80°C prior to analysis.

RNA extraction and quality control

Total RNA derived from rat penile tissue was obtained using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following a standard extraction protocol and then purified using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantification and quality of RNA samples were assessed on a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific) and an Agilent 2100 Bioanalyzer (Agilent Technologies), respectively. The OD260/280 absorbance ratios were between 1.8 and 2 for all samples, and RNA integrity was assessed using standard denaturing agarose gel electrophoresis.

Microarray analysis

For microarray analysis, the Agilent array platform was employed. Sample preparation and microarray hybridization were performed based on the manufacturer's standard protocols. Briefly, total RNA from each sample was amplified and transcribed into fluorescent cRNA using the Agilent's Quick Amp Labelling protocol (version 5.7, Agilent Technologies). The labelled cRNAs were hybridized onto the rat lncRNA Array v2.0 (4 x 44K, Arraystar). The arrays were scanned on an Agilent Scanner G2505C after having washed the slides. The analysis of acquired array images was performed using Agilent Feature Extraction software

(version 11.0.1.1). The GeneSpring GX v11.5.1 software package (Agilent Technologies) was used to carry out quantile normalization and subsequent data processing. After quantile normalization of the raw data, lncRNAs and mRNAs with present or marginal flags in at least 1 out of 2 samples ("All Targets Value") were chosen for further data analysis.

Quantitative real-time PCR (qRT-PCR)

To validate the microarray data, we investigated the expression patterns of 12 selected lncRNAs that were identified as differentially expressed in two groups using qRT-PCR. Primers were designed online using Primer3 (<http://sourceforge.net/projects/primer3/>) and evaluated using Basic Local Alignment Search Tool (BLAST) at NCBI to ensure unique amplification products. All of the primer sequences are shown in Table 1. The SYBR green method was used to perform qRT-PCR. The reactions were incubated in a 96-well optical plate at 95°C for 10 min, followed by 40 × PCR cycles of 95°C for 15 s and 60°C for 1 min. Gene expression levels were quantified based on the cycle threshold (Ct) values and values are expressed as $2^{-\Delta\Delta Ct}$. The relative levels of lncRNAs were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Gene Ontology (GO) and pathway analysis

In this study, GO and pathway analyses were performed to determine the gene and gene product attributes in GO terms or biological pathways. Fisher's exact test was used to determine whether there is more overlap between the differentially expressed (DE) list of genes and the GO annotation list than would be expected by chance. The GO categories are derived from the Gene Ontology database (<http://www.geneontology.org>), which comprise three structured networks of defined terms that describe gene product attributes. The ontology covers three domains, biological process, cellular component and molecular function. The lower the p-value, the more significant the GO terms enrichment in DE genes (p-value < 0.05 is recommended). Pathway analysis is a functional analysis that maps genes to KEGG (Kyoto Encyclopedia of Genes and Genomes, <http://www.genome.jp/kegg>) pathways. Khatri et al. reported that pathway analysis has become the first choice for learning the underlying biology of differentially expressed genes and proteins [18]. The p-value (Fisher's p-value, Hypergeometric p-value or EASE-score) denotes the significance of the pathway correlated to the conditions. The lower the p-value, the more significant is the pathway.

Statistical analysis

The SPSS 17.0 software package (SPSS, Chicago, IL, USA) was used to analyse the data. Differential expression levels of lncRNAs were compared by independent-samples t-test between two groups. Fisher's exact test was used for GO and pathway analysis. Statistical significance was defined as $P < 0.05$.

Table 1. The primer sequences used in this study

Target ID	Forward Primer	Reverse Primer	Product Length (bp)
GAPDH	F:5'GGAAAGCTGTGGCGTGAT3'	R:5'AAGGTGGAAGAATGGGAGTT3'	308
MRAK080737	F:5'CCAGTGACAGGGGAAAGCA3'	R:5'GGAGATGGGCAGGATGATG3'	95
MRUC008SUT	F:5'CCATACACGCATGACTCAATCA3'	R:5'CAGGTGCCTCCAGTCTTCTT3'	130
AB049626	F:5'AGGAAAGGGCTGACTGGA3'	R:5'CTTGGGATGTCTGTCTTTGAG3'	121
MRNR_026574	F:5'GCACGCCTTCTGTCTCT3'	R:5'GCATTGCTGATAGTCCCTCC3'	296
MRAK161159	F:5'TCTCCTGGTAATGCTGTTCT3'	R:5'GCAGTGGCTTCTCAATGTCTT3'	66
MRAK040414	F:5'GGTGCGGTCTCTGAGGTGA3'	R:5'GGTCTCAGGTTCCATTCACT3'	155
MRAK153573	F:5'GTCGGCTTGGAATCACAGTAG3'	R:5'AAGAGCATTGATGTAGGCAGTG3'	252
MRAK078930	F:5'AAACCCAGAATACCCAGTGC3'	R:5'GGAACCCAGCAGACCAACTA3'	134
BC167026	F:5'TTGTTCTCAGTTTCTCGGTTA3'	R:5'TGGTAGAGGTCAGAAGGTT3'	177
MRAK142111	F:5'GCAAGCAGATGGTGTGAGTAA3'	R:5'GGCAGGATGAGGCATTATTGTT3'	70
BC082040	F:5'TGCTGTCAGGCTGCTCAT3'	R:5'GCTTGCTCCGATTGTCCATT3'	171
MRAK084895	F:5'ACTCGCCAGTTTGAAG3'	R:5'TTTAAATGATGACATTCCTGAAG3'	185

Results

Overview of the microarray data

Global profiling of rat lncRNAs and protein-coding transcripts was performed using the rat lncRNA Array v2.0 (4 x 44K, Arraystar). The microarray data showed that a total of 8,744 lncRNAs and 13,585 coding transcripts were detected. The lncRNAs come from an authoritative database such as UCR, RefSeq_XR and misc_lncRNA (Fig. 1A) [19-22]. A scatter-plot was used for assessing the expression variation (or reproducibility) between chips. The green lines are fold change lines (The default fold change value given is 2). The lncRNAs (or mRNAs) above the top green line and below the bottom green line indicates more than 2 fold change of lncRNAs (or mRNAs) between the two samples (or samples) (Fig. 1B, C). Based on these data, the lncRNA expression levels between the A-ED group and the NC group were compared. By applying a filter of fold-change >2.0, we identified 608 up-regulated and 406 down-regulated lncRNAs. The list of the top 50 up- and down-regulated lncRNAs and mRNAs identified by microarray analysis is shown in Tables 2-5.

Validation of microarray results by qRT-PCR

To validate the microarray results, we selected 12 differentially expressed lncRNAs according to their associated mRNAs [23, 24]. 7 up-regulated lncRNAs and 5 down-regulated lncRNAs (MRAK078930, were analysed using qRT-PCR in 20 pairs of A-ED and matched NC tissues. qRT-PCR results showed that MRAK080737, MRUC008SUT, AB049626, MRNR_026574, MRAK161159, MRAK040414, MRAK153573 increased, whereas MRAK078930, BC167026, MRAK142111, BC082040 and MRAK084895 decreased in A-ED (all $P < 0.05$, Fig. 2). According to the qRT-PCR analysis, we further found that the most up-regulated lncRNAs were MRAK080737 and MRuc008sut, whereas the most down-regulated lncRNAs were MRAK078930 and BC167026. These results indicate that the expression trends of the selected lncRNAs are consistent with the microarray data, which demonstrates

Fig. 1. Profile of the microarray data. A total of 8,744 lncRNAs and 13,585 coding transcripts were detected. (A) The pie chart shows the number of lncRNAs from each authoritative database. (B) The lncRNAs above the top green line and below the bottom green line show a more than 2.0-fold change in lncRNA expression between the A-ED and NC samples. (C) The mRNAs above the top green line and below the bottom green line show a more than 2.0-fold change of mRNA expression between the A-ED and NC samples.

Table 2. Top 50 up-regulated lncRNAs in the A-ED group compared with the NC group and filtered by a fold-change >3.0

SeqID	Fold change (A-ED/NC)	Chromosome	Strand	Relationship	Associated_gene_acc	Associated_gene_name
MRAK033351	336.84	chr17	+	intergenic		
L38717	255.60	chr3	-	intergenic		
K02111	244.73	chr10	+	sense_exon_overlap	NM_001100485	
U89530	192.16	chr3	-	intergenic		
AF059344	157.25	chr3	-	intergenic		
U89531	151.39	chr3	-	intergenic		
MRAK020463	141.30	chr3	-	others		
AF525411	103.59	chr3	-	intergenic		
AF525412	86.58	chr3	-	intergenic		
X90475	43.11	chr7	-	sense_exon_overlap	NM_001100758	
EU056364	37.95	chr18	+	sense_exon_overlap	NM_012920	Camk2a
AF130879	20.03	chr1	-	others		
AY167411	18.81	chr10	-	others		
XR_007519	18.51	chr5	-	intergenic		
MRAK080737	16.78	chr4	-	bidirection	NM_022522	Casp2
AY429596	14.27	chr3	-	intergenic		
AY177417	13.87	chr16	-	sense_exon_overlap	NM_001169141	
XR_009380	11.68	chrUn	-	others		
MRuc008ytr	11.50	chrUn	-	others		
MRAK081751	10.81	chr5	+	sense_intron_overlap	NM_001106630	Jph1
AY177416	10.75	chr9	+	intergenic		
MRuc008lvx	10.39	chr3	+	sense_exon_overlap	NM_001145840	Ganc
MRuc008lvx	10.39	chr3	+	sense_exon_overlap	NM_017117	Capn3
MRNR_002452	10.02	chr1	-	others		
MRAK033229	8.12	chr4	+	others		
XR_008345	7.96	chr10	-	intergenic		
MRuc008sut	7.88	chr5	+	sense_exon_overlap	NM_031628	Nr4a3
MRAK134248	7.67	chr1	+	sense_exon_overlap	NM_030585	Rabep2
XR_009626	7.16	chr4_random	+	intergenic		
MRAK044354	6.84	chr3	-	others		
MRuc008xkb	5.85	chr14	+	others		
uc.78+	5.62	chr3	+	antisense_intron_overlap	NM_001033701	Zeb2
XR_006950	5.12	chr4	-	intergenic		
MRAK050904	5.11	chr17	+	others		
uc.346+	5.09	chr7	-	intergenic		
uc.466-	5.06	chrX	+	antisense_intron_overlap	NM_053479	
uc.432-	5.04	chr18	-	intergenic		
MRAK014008	5.01	chr2	-	sense_exon_overlap	NM_031130	Nr2f1
MRuc008czh	4.85	chr9	+	sense_intron_overlap	NM_001012129	Satb1
uc.253-	4.78	chr5	-	intergenic		
uc.162+	4.76	chr2	-	intergenic		
MRAK032315	4.69	chrX	+	intergenic		
BC063168	4.57	chr4	-	sense_exon_overlap	NM_017102	Slc2a3
MRAK021118	4.50	chr9	-	others		
AY390391	4.43	chr17	+	intergenic		
AF112256	4.37	chr1	-	intergenic		
XR_009508	4.34	chr3	+	intergenic		
XR_005466	4.26	chr10	-	intergenic		
DQ402472	4.26	chr6	-	intergenic		
XR_006612	4.24	chr4	+	others		

that the microarray data were stable and reliable. The fold-change of the selected lncRNAs, as validated by the microarray analysis and RT-PCR, are depicted in Table 6. Thus, our results show that a set of lncRNAs are consistently differentially expressed in A-ED and NC and might be related to A-ED pathogenesis.

GO and pathway analysis

GO term enrichment analysis was performed to evaluate the associated coding gene of the dysregulated lncRNAs. As shown in Fig. 3A, the top 10 GO terms for the up-regulated genes in our study included developmental process, multicellular organismal development, muscle system process, system development, muscle contraction, anatomical structure development, muscle structure development, cell differentiation, ion transport and organ development. Whereas, the top 10 GO terms on the down-regulated lncRNAs involved were

Table 3. Top 50 down-regulated lncRNAs in the A-ED group compared with the NC group and filtered by a fold-change >3.0

SeqID	Fold change (A-ED/NC)	Chromosome	Strand	Relationship	Associated_gene_acc	Associated_gene_name
XR_008965	-822.06	chr4	+	others		
XR_008714	-753.09	chr3	-	intergenic		
BC086588	-424.34	chr2	-	sense_intron_overlap	NM_001109500	Lce1 m
BC166816	-339.13	chr1	+	sense_exon_overlap	NM_001173357	
MRAK009853	-160.78	chr2	-	intergenic		
uc.150+	-105.39	chr17	+	intergenic		
MRAK004471	-58.33	chr17	+	intergenic		
XR_005499	-56.41	chr3	-	intergenic		
MRAK010032	-51.39	chr4	+	others		
XR_007003	-38.79	chrUn	-	others		
MRAK010042	-26.44	chr18	+	sense_exon_overlap	NM_001170606	
BC161881	-23.49	chr7	+	others		
BC092202	-20.21	chr10	-	others		
AY044435	-19.41	chr7	+	others		
BC087706	-15.98	chr2	-	intergenic		
XR_008731	-15.72	chr1	+	others		
BC129114	-15.67	chr1	+	intergenic		
XR_008801	-13.37	chr8	-	intergenic		
MRAK011597	-13.08	chrX	+	others		
M21759	-12.86	chr2	+	intergenic		
XR_008601	-11.57	chr9	+	intergenic		
MRAK028845	-9.56	chr10	+	antisense_exon_overlap	NM_001008759	Krt1-5
MRAK076299	-9.49	chrUn	+	intergenic		
MRuc007pas	-9.10	chr6	+	intergenic		
BC092641	-8.92	chr3	-	antisense_exon_overlap	NM_001100531	Dlx1
XR_009334	-8.80	chr1	+	others		
BC098071	-8.76	chr17	-	others		
BC160888	-8.59	chr5	+	sense_exon_overlap	NM_001135780	RGD1304693
AY321319	-8.42	chr9	-	others		
MRAK013532	-8.07	chr6	+	intergenic		
MRAK078930	-8.03	chr1	-	sense_exon_overlap	NM_013159	Ide
XR_009344	-7.67	chr7	+	others		
BC091361	-7.54	chr10	+	others		
BC167069	-7.50	chr3	-	bidirection	NM_001107762	Nusap1
BC167026	-7.49	chr15	+	sense_exon_overlap	NM_001106028	Cdkn3
MRAK048947	-7.45	chr1	+	others		
uc.176+	-7.31	chr10	-	sense_intron_overlap	NM_020088	Odz2
MRAK142484	-7.01	chr18	-	sense_exon_overlap	NM_031826	Fbn2
BC107469	-6.45	chr16	+	others		
AJ224880	-6.30	chr9	-	sense_exon_overlap	NM_053488	
MRAK009873	-6.25	chr8	+	others		
MRAK012306	-6.23	chr6	-	others		
AB011529	-6.21	chr2	-	others		
AF370442	-6.19	chr13	-	sense_exon_overlap	NM_001100827	
BC158675	-6.10	chr6	+	intergenic		
MRuc008qeu	-6.05	chr2	-	others		
MRAK132609	-5.89	chr2	+	intergenic		
MRuc008fxu	-5.66	chr1	+	others		
MRuc009gus	-5.63	chr1	-	others		
BC166461	-5.58	chr15	-	sense_exon_overlap	NM_001108379	RGD1307201

cell proliferation, epidermis development, organ development, regulation of peptidase activity, developmental process, tissue development, regulation of biological quality, anatomical structure development, negative regulation of molecular function, and regulation of cell proliferation (Fig. 3C).

Pathway analysis indicated that the top 10 pathways corresponding to the associated coding gene of up-regulated lncRNAs are involved in the following pathways: gastric acid

Table 4. The top 50 up-regulated mRNAs in the A-ED group compared with the NC group and filtered by a fold-change >3.0

Gene Symbol	Fold change (A-ED/NC)	Chromosome	Strand	txStart	txEnd
Myh4	3733.56	chr10	+	53932089	53955029
Actn3	1019.18	chr1	-	207475568	207492267
Myh1	837.57	chr10	+	53894387	53917948
Mylpf	711.12	chr1	+	186471804	186474560
Syp12	570.27	chr2	-	203777721	203792453
Apobec2	562.42	chr9	+	7947499	7961013
Myl1	421.66	chr9	-	65717097	65727679
Myl1	410.70	chr9	-	65717097	65737576
Pvalb	375.64	chr7	-	116115033	116127508
Myl3	363.98	chr8	+	115140704	115146827
Myf6	343.88	chr7	-	46199971	46201867
Art5	301.77	chr1	-	159576989	159586949
Art1	253.86	chr1	+	159586245	159590797
Trdn	250.63	chr1	-	24514751	24977515
Myoz1	177.60	chr15	+	3869804	3877019
Ampd1	169.07	chr2	+	198308511	198331019
Lmod2	148.49	chr4	+	410044	417739
Cmya3	135.65	chr3	+	49414868	49502135
Mybpc2	135.57	chr1	-	94979307	95002804
Myot	127.82	chr18	+	38003999	38023531
Tnni2	122.91	chr1	+	202687629	202690172
Cox6a2	91.67	chr1	-	187464578	187465325
Stac3	90.16	chr7	+	67478505	67485909
Ckm	89.26	chr1	+	78762201	78772681
Mypn	83.91	chr20	-	25457132	25531818
Kcnj11	83.04	chr1	-	96614959	96617993
LOC680319	79.70	chrX	+	123537651	123539744
Tpm1	66.74	chr8	-	71336842	71358063
Myod1	66.73	chr1	+	96910189	96912900
Hspb3	66.68	chr2	-	45078614	45079329
Tpm1	63.52	chr8	-	71336842	71352726
Ankrd23	59.19	chr9	-	35472714	35479314
Tpm1	57.02	chr8	-	71336842	71352726
Tnnt3	55.86	chr1	+	202748062	202761965
Mb	54.79	chr7	-	115087557	115094789
Cox8h	53.36	chr1	-	201057313	201058774
Casq1	50.21	chr13	-	88200505	88210418
Klhl31	48.72	chr8	+	82609179	82633422
Nrap	47.33	chr1	-	262591453	262672255
Hfe2	42.52	chr2	+	191329643	191333525
Myh2	40.87	chr10	+	53864776	53891711
Kbtbd5	40.70	chr8	-	127153471	127159307
Asb10	39.41	chr4	+	6010912	6019444
Ckmt2	35.43	chr2	-	22084949	22106014
Arx	34.93	chrX	+	80631374	80643987
Aqp4	29.58	chr18	-	6626312	6641850
Trim54	26.36	chr6	-	25218007	25237575
Aqp4	24.13	chr18	-	6626312	6642766
Csrp3	24.09	chr1	-	98601680	98611747
RGD1310090	24.00	chr16	-	64952302	64959604

secretion, calcium signalling, pancreatic secretion, dilated cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy (ARVC), insulin secretion, vascular smooth muscle contraction, hypertrophic cardiomyopathy (HCM), neuroactive ligand-receptor interaction, and fat digestion and absorption (Fig. 3B). The top 10 pathway corresponding to the associated coding gene of down-regulated lncRNAs are involved in protein digestion and

Table 5. The top 50 down-regulated mRNAs in the A-ED group compared with the NC group and filtered by a fold-change >3.0

Gene Symbol	Fold change (A-ED/ NC)	Chromosome	Strand	txStart	txEnd
Vcsa1	-1018.00	chr14	-	21378052	21382729
Stfa3	-949.71	chr11	-	66030826	66035282
Il1f9	-916.86	chr3	+	2432284	2438436
Serpinb12	-810.47	chr13	+	13106079	13137360
Serpinb13	-803.10	chr13	+	13175745	13209693
Kprp	-761.34	chr2	-	185839395	185843694
Cldn17	-560.59	chr11	-	28370003	28371180
Defb4	-521.12	chr16	-	75442004	75445196
Fgfbp1	-515.95	chr14	+	72242270	72243353
Aqp5	-485.23	chr7	+	138337730	138341257
Muc10	-461.11	chr14	-	21294394	21294762
Csta	-423.85	chr11	+	66456247	66467253
RGD1562234	-422.57	chr2	+	183499182	183502085
Klk12	-357.36	chr1	+	94198529	94203133
Slc15a1	-353.79	chr15	-	106550095	106592594
Stfa2	-350.44	chr11	-	66263201	66267857
Tmprss11d	-309.96	chr14	+	23474012	23536005
Il1f6	-297.95	chr3	+	2482479	2487029
RGD1562305_predicted	-265.17	chr1	+	85796214	85800682
RGD1311975	-239.31	chr9	+	56879058	56938623
Slpi	-179.45	chr3	-	155373824	155376074
RGD1561909	-165.00	chr1	+	72833802	72836472
RGD1562060	-158.11	chr5	-	17414522	17438458
Kb1	-152.57	chr7	-	140540959	140546186
Rhcg	-149.21	chr1	-	135361417	135386461
Tgm3	-147.44	chr3	+	117622726	117659103
Il1f8	-138.56	chr3	-	2510814	2515661
Slpil3	-137.05	chr3	-	155348325	155349745
Lce1f	-135.61	chr2	-	185714947	185716534
Adh6a	-129.56	chr2	+	235842524	235850491
Acpp	-125.57	chr8	-	109363250	109400296
Mt4	-119.65	chr19	-	11300056	11302153
S100vp	-117.23	chr2	-	183734038	183737388
Krt10	-116.88	chr10	-	88288619	88292615
Sprr3	-115.55	chr2	-	185436597	185437356
Klk8	-101.32	chr1	+	94227184	94234028
Krt1-12	-100.29	chr10	-	88321125	88328759
Tgm1	-99.73	chr15	-	33846366	33859615
Tnn	-98.87	chr13	-	75502878	75570266
Defb5	-96.76	chr16	-	75403110	75405271
RGD1563060	-85.47	chr18	-	37217041	37242030
S100a7a	-83.60	chr2	+	182946272	182950714
LOC290595	-81.85	chr16	-	13308804	13316412
RGD1562464	-81.81	chr13	+	88561387	88569578
LOC691259	-80.74	chr10	+	44018454	44029319
Klk10	-75.76	chr1	+	94210923	94214301
Crispld1	-71.58	chr5	-	343492	383772
MGC72615	-70.21	chr4	-	70401083	70419847
Klk11	-66.19	chr1	+	94204124	94208665
RGD1562059	-65.57	chr2	+	189315150	189321030

absorption, steroid biosynthesis, mineral absorption, amoebiasis, rheumatoid arthritis, p53 signalling, glycine, serine and threonine metabolism, MAPK signalling, cell cycle regulation, tuberculosis (Fig. 3D).

Table 6. LncRNAs are differentially expressed in A-ED vs. NC, as validated by microarray analysis and RT-PCR

SeqID	Microarray	qPCR	Chromosome	Strand	Relationship	Associated_gene_acc	Associated_gene_name
MRAK080737	16.78	2.956756	chr4	-	bidirection	NM_022522	Casp2
MRuc008sut	7.88	5.631329	chr5	+	sense_exon_overlap	NM_031628	Nr4a3
AB049626	3.34	2.488023	chr4	+	sense_intron_overlap	NM_013146	Cald1
MRNR_026574	3.12	2.359001	chr1	+	sense_exon_overlap	NM_001143847	Pde2a
MRAK161159	3.04	1.886605	chr11	+	sense_intron_overlap	NM_032062	Kalrn
MRAK040414	2.13	4.575866	chr5	-	sense_exon_overlap	NM_022383	Cap1
MRAK153573	2.05	2.485457	chr2	+	sense_exon_overlap	NM_012519	Camk2d
MRAK078930	-8.03	0.321868	chr1	-	sense_exon_overlap	NM_013159	Ide
BC167026	-7.49	0.413323	chr15	+	sense_exon_overlap	NM_001106028	Cdkn3
MRAK142111	-2.99	0.350863	chr4	+	sense_exon_overlap	NM_053356	Col1a2
BC082040	-2.83	0.424388	chr12	+	sense_intron_overlap	NM_031338	Camkk2
MRAK084895	-2.54	0.268796	chr6	-	sense_exon_overlap	NM_019153	Fbln5

Fig. 2. The differential expression of lncRNAs between A-ED and NC tissues by quantitative PCR, * p < 0.05.

Discussion

Erectile dysfunction (ED), which is defined as the inability of a man to achieve and maintain an erection sufficient for mutually satisfactory intercourse with his partner, usually causes stress and relationship problems and affects male self-confidence [25]. Little attention

Fig. 3. GO and pathway analysis. The bar plot shows the top 10 GO terms that are associated with the coding genes of up-regulated lncRNAs (A) and down-regulated lncRNAs (B). The bar plot shows the top 10 pathways that are associated with the coding genes of up-regulated lncRNAs (C) and down-regulated lncRNAs (D).

is given to sexual behaviour and to the treatment of ED in the older population, due to the general hypotheses that a loss of sexuality was natural and inevitable part of aging. However, in the current era, elderly men continue to be sexually active, and there is no upper age limit for sexual function [5, 26, 27]. Despite the huge success of oral phosphodiesterase-5 inhibitors (PDE5I), about one third of ED patients do not respond to PDE5I, which is especially true for older patients [1]. Therefore, ED remains a topic of interest to physicians treating aging and older males.

The genome is extremely intricate and can be transcribed into a spectrum of ncRNAs that are implicated in a wide range of structural, regulatory, and catalytic processes [28-31]. Some of these ncRNAs are well known, such as short hairpin RNAs (shRNAs), small interfering RNAs (siRNAs) and microRNAs (miRNAs), and the roles of these RNAs in the progression of erectile function have been studied, at least in part [6, 32, 33]. lncRNAs are one of the most abundant groups of ncRNAs, which have been reported to have in a wide range of physiological functions. However, the roles of lncRNA in the pathogenesis of ED are poorly understood.

In this study, we attempted to explore and describe lncRNAs expression signatures of A-ED using a 2nd-generation lncRNA microarray. The lncRNA microarray analysis detected a total of 8,744 lncRNAs and 13,585 coding transcripts. However, only 608 up-regulated and 406 down-regulated lncRNAs were identified in A-ED compared with NC, as determined by setting a filter of fold-change >2.0 . The results of the qRT-PCR analysis were consistent with the microarray results and demonstrated that the microarray data were reliable. Until now, few studies have been reported on these 12 randomly selected lncRNAs and little is

known about the functions of these lncRNAs in physiological and pathological processes. However, based on their significantly up-regulated or down-regulated expression levels in A-ED samples, we hypothesize that some of the lncRNAs might be involved in the regulation of the progression of A-ED which should be investigated in future studies. In addition, the unique lncRNA expression signatures of these samples prompt a potential novel way to diagnose and remedy A-ED. However, it is too premature to use these regulated lncRNAs as biomarkers of A-ED. Further studies to investigate the structure and function of these lncRNAs are necessary.

GO term enrichment analysis was performed to identify the biological processes associated with the lncRNAs that we identify as differentially expressed. We found that the lncRNAs with differential expression are most highly enriched in functions related to muscle contraction, muscle system process, muscle structure development, and ion transport. Which are closely connected to penile erection. Moreover, pathway analysis showed a significant change in the pathways for calcium signalling pathway, vascular smooth muscle contraction, mineral absorption, MAPK signalling and protein digestion and absorption during A-ED. Normal erectile function is based on the coordination of activity of the corporal smooth muscle cells. Ionic gradients play an important role in the normal physiology of the corporal smooth muscle cell [34]. The result suggests that a muscle contraction disorder induced by abnormal ion channels might play a critical role in the pathogenesis of A-ED.

In previous studies, copious collagen was found in human erectile tissues, and altered collagen content was associated with erectile dysfunction [1, 35, 36]. Immunohistochemistry analysis revealed that in the corpus cavernosum, collagen types I and IV predominate, and type III is in the minority [37]. Col1a2 is one of the collagen gene families that directly participate in collagen deposition and fibrosis [38, 39]. A recent study demonstrated that lncRNAs contributed to reduced expression of collagen [40]. Research on idiopathic pulmonary fibrosis indicated that interactions between microRNAs, lncRNAs and mRNAs might better explain the disease pathogenesis [41]. Our results revealed that MRAK142111 expression was decreased, which is associated with Col1a2. Thus, MRAK142111 may be involved in the regulation of collagen deposition and fibrosis in the corpus cavernosum.

Vascular smooth muscle contraction is an important part in erectile function. The function of smooth muscle cell in the corpus cavernosum is controlled by a balance between factors for contraction and extension [1]. Berridge et al. [42] found that smooth muscle contraction is controlled by calcium signalling pathways. Camk2d is part of the calcium signalling pathways and involved in actin cytoskeleton reorganization [43]. Other studies revealed that camk2d participates in the function of vascular smooth muscle cells by regulating cell polarization, migration and proliferation [44, 45]. MRAK153573 expression was increased during A-ED progress in our study, and its associated gene was Camk2d. Thus, MRAK153573 may be very important for the functions of vascular smooth muscle and might contribute to the pathophysiology of A-ED.

The molecular mechanisms of lncRNAs are largely unknown. Unlike protein-coding genes or microRNAs, lncRNAs sequences generally do not provide direct information to predict their function [46]. lncRNAs could be implicated in gene regulation through multifarious mechanisms such as posttranscriptional gene regulation, alternative splicing and epigenetic modifications of DNA [47-49]. Moreover, lncRNAs and other molecules such as protein complex, miRNA, and other regulatory elements form a complex interaction network [50]. Accumulating associations among lncRNAs and other molecules are being verified by experimental means or are being predicted by computational methods [51, 52]. StarBase (<http://starbase.sysu.edu.cn>) is designed for decoding the interaction networks of lncRNAs, microRNAs, competing endogenous RNAs (ceRNAs), RNA-binding proteins (RBPs) and mRNAs. To determine the role of ncRNAs in various diseases and pathway, the annotation of miRNA regulatory elements and targets can be obtained from DIANA-LncBase at www.microrna.gr/LncBase. miRWalk2.0 (<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/>) [53] is an improved version of miRWalk [54], which supplies the biggest available collection of predicted and experimentally verified miRNA-target interactions with

various novel and unique features including lncRNAs. Further research on exploring the possibility interactions of our dataset will be important and necessary to understand the molecular biology of A-ED.

In this study, we profiled the expression of lncRNAs and mRNAs in A-ED and NC animals. Our results indicate that several lncRNAs are involved in A-ED development. Further study will be necessary to investigate the molecular mechanisms of specific dysregulated lncRNAs, which may be helpful for the exploration of novel therapeutic targets in A-ED.

Conclusion

We studied the profile of differentially expressed lncRNAs between A-ED and NC rats. The data indicates that many lncRNAs are involved in the regulation of muscle contraction and relaxation through ion channel activity. We suggest that a muscle contraction disorder induced by abnormal ion channel activity, collagen deposition and fibrosis might play a critical role in the pathogenesis of A-ED. However, our study is a preliminary exploration of the lncRNA profile in A-ED. Additional research is necessary to understand the roles of differentially expressed lncRNAs.

Acknowledgements

This study was funded by the National Natural Science Foundation of China (30972995) and was supported by the Science and Technology Development Fund of the Nanjing Medical University (2013NJMU127 and 2014NJMUZD051), the Scientific Research Project of the Health Department of Jiangsu Province (Z201022) and the Science and Technology project of Zhenjiang (SH2011042).

Disclosure Statement

We have no disclosure statement.

References

- 1 Andersson KE: Mechanisms of penile erection and basis for pharmacological treatment of erectile dysfunction. *Pharmacol Rev* 2011;63:811-859.
- 2 Gareri P, Castagna A, Francomano D, Cerminara G, De Fazio P: Erectile dysfunction in the elderly: an old widespread issue with novel treatment perspectives. *Int J Endocrinol* 2014;2014:878670.
- 3 Melman A, Gingell JC: The epidemiology and pathophysiology of erectile dysfunction. *J Urol* 1999;161:5-11.
- 4 Nicolosi A, Laumann EO, Glasser DB, Moreira ED Jr, Paik A, Gingell C: Sexual behavior and sexual dysfunctions after age 40: the global study of sexual attitudes and behaviors. *Urology* 2004;64:991-997.
- 5 Albersen M, Orabi H, Lue TF: Evaluation and treatment of erectile dysfunction in the aging male: a mini-review. *Gerontology* 2012;58:3-14.
- 6 Pan F, Xu J, Zhang Q, Qiu X, Yu W, Xia J, Chen T, Pan L, Chen Y, Dai Y: Identification and Characterization of the MicroRNA Profile in Aging Rats with Erectile Dysfunction. *J Sex Med* 2014;11:1646-1656.
- 7 Kapranov P, Cheng J, Dike S, Nix DA, Duttagupta R, Willingham AT, Stadler PF, Hertel J, Hackermüller J, Hofacker IL, Bell I, Cheung E, Drenkow J, Dumais E, Patel S, Helt G, Ganesh M, Ghosh S, Piccolboni A, Sementchenko V, Tammana H, Gingeras TR: RNA maps reveal new RNA classes and a possible function for pervasive transcription. *Science (New York, N.Y.)* 2007;316:1484-1488.
- 8 Mattick JS: Long noncoding RNAs in cell and developmental biology. *Semin Cell Dev Biol* 2011;22:327.
- 9 Mattick JS: The central role of RNA in human development and cognition. *FEBS letters* 2011;585:1600-1616.

- 10 Qureshi IA, Mattick JS, Mehler MF: Long non-coding RNAs in nervous system function and disease. *Brain Res* 2010;1338:20-35.
- 11 Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Bruggmann SA, Goodnough LH, Helms JA, Farnham PJ, Segal E, Chang HY: Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* 2007;129:1311-1323.
- 12 Tahira AC, Kubrusly MS, Faria MF, Dazzani B, Fonseca RS, Maracaja-Coutinho V, Verjovski-Almeida S, Machado MC, Reis EM: Long noncoding intronic RNAs are differentially expressed in primary and metastatic pancreatic cancer. *Mol Cancer* 2011;10:141.
- 13 Aryal B, Rotllan N, Fernández-Hernando C: Noncoding RNAs and atherosclerosis. *Curr Atheroscler Rep* 2014;16:407.
- 14 Holdt LM :Alu elements in ANRIL non-coding RNA at chromosome 9p21 modulate atherogenic cell functions through trans-regulation of gene networks. *PLoS genetics* 2013;9:e1003588.
- 15 Liu G, Sun X, Bian J, Wu R, Guan X, Ouyang B, Huang Y, Xiao H, Luo D, Atala A, Zhang Y, Deng C: Correction of diabetic erectile dysfunction with adipose derived stem cells modified with the vascular endothelial growth factor gene in a rodent diabetic model. *PLoS ONE* 2013;8:e72790.
- 16 Heaton JP, Varrin SJ, Morales A: The characterization of an assay of erectile function in a rat model. *J Urol* 1991;145:1099-1102.
- 17 Chen Y, Li SX, Yao LS, Wang R, Dai YT: Valsartan treatment reverses erectile dysfunction in diabetic rats. *Int J Impot Res* 2007;19:366-370.
- 18 Khatri P, Sirota M, Butte AJ: Ten years of pathway analysis: current approaches and outstanding challenges. *PLoS Comput Biol* 2012;8:e1002375.
- 19 Nordström KJ, Mirza MA, Larsson TP, Gloriam DE, Fredriksson R, Schiöth HB: Comprehensive comparisons of the current human, mouse, and rat RefSeq, Ensembl, EST, and FANTOM3 datasets: identification of new human genes with specific tissue expression profile. *BiochemBiophys Res Commun* 2006;348:1063-1074.
- 20 Pruitt KD, Tatusova T, Maglott DR: NCBI Reference Sequence (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res* 2005;33:D501-504.
- 21 Rosenbloom KR, Armstrong J, Barber GP, Casper J, Clawson H, Diekhans M, Dreszer TR, Fujita PA, Guruvadoo L, Haeussler M, Harte RA, Heitner S, Hickey G, Hinrichs AS, Hubley R, Karolchik D, Learned K, Lee BT, Li CH, Miga KH, Nguyen N, Paten B, Raney BJ, Smit AF, Speir ML, Zweig AS, Haussler D, Kuhn RM, Kent WJ: The UCSC Genome Browser database: 2015 update. *Nucleic Acids Res* 2015;43:D670-681.
- 22 Li Y, Wang X, Li M, Pan J, Jin M, Wang J, Li X, Feng X: Long non-coding RNA expression profile in the kidneys of male, low birth weight rats exposed to maternal protein restriction at postnatal day 1 and day 10. *PLoS One* 2015;10: e0121587.
- 23 Zhang J, Cui X, Shen Y, Pang L, Zhang A, Fu Z, Chen J, Guo X, Gan W, Ji C: Distinct expression profiles of lncRNAs between brown adipose tissue and skeletal muscle. *BiochemBiophys Res Commun* 2014;443:1028-1034.
- 24 Li J, Long W, Li Q, Zhou Q, Wang Y, Wang H, Zhou B, Li J: Distinct expression profiles of lncRNAs between regressive and mature scars. *Cell Physiol Biochem* 2015;35:663-675.
- 25 Corona G, Rastrelli G, Maseroli E, Forti G, Maggi M: Sexual function of the ageing male. *Best Pract Res Clin Endocrinol Metab* 2013;27:581-601.
- 26 Lindau ST, Schumm LP, Laumann EO, Levinson W, O'Muircheartaigh CA, Waite LJ: A study of sexuality and health among older adults in the United States. *N Engl J Med* 2007;357:762-774.
- 27 Smith LJ, Mulhall JP, Deveci S, Monaghan N, Reid MC: Sex after seventy: a pilot study of sexual function in older persons. *J Sex Med* 2007;4:1247-1253.
- 28 Carninci P: The transcriptional landscape of the mammalian genome. *Science* 2005;309:1559-1563.
- 29 Cheng J: Transcriptional maps of 10 human chromosomes at 5-nucleotide resolution. *Science (New York, N.Y.)* 2005;308:1149-1154.
- 30 Kapranov P, Willingham AT, Gingeras TR: Genome-wide transcription and the implications for genomic organization. *Nat Rev Genet* 2007;8:413-423.
- 31 Mattick JS, Makunin IV: Non-coding RNA. *Hum Mol Genet* 2006;15:R17-29.
- 32 Cao ZG, Zhu YP, Sun YW, Dong XC, Qi L, Xiao J, Chen H, Ren WH, Zou L: Small interfering RNA inhibits the expression of connexin43 in the human corpus cavernosum penis smooth muscle cells. *Zhonghua Nan Ke Xue* 2007;13:440-443.

- 33 Magee TR, Kovanecz I, Davila HH, Ferrini MG, Cantini L, Vernet D, Zuniga FI, Rajfer J, Gonzalez-Cadavid NF: Antisense and short hairpin RNA (shRNA) constructs targeting PIN (Protein Inhibitor of NOS) ameliorate aging-related erectile dysfunction in the rat. *J Sex Med* 2007;4:633-643.
- 34 Noack T, Noack P: Multiple types of ion channels in cavernous smooth muscle. *World J Urol* 1997;15:45-49.
- 35 Mulhall JP: Penile rehabilitation following radical prostatectomy. *Curr Opin Urol* 2008;18:613-620.
- 36 User HM, Hairston JH, Zelner DJ, McKenna KE, McVary KT: Penile weight and cell subtype specific changes in a post-radical prostatectomy model of erectile dysfunction. *J Urol* 2003;169:1175-1179.
- 37 Luangkhot R, Rutchik S, Agarwal V, Puglia K, Bhargava G, Melman A: Collagen alterations in the corpus cavernosum of men with sexual dysfunction. *J Urol* 1992;148:467-471.
- 38 Tamaki Z, Asano Y, Kubo M, Ihn H, Tada Y, Sugaya M, Kadono T, Sato S: Effects of the immunosuppressant rapamycin on the expression of human $\alpha 2(I)$ collagen and matrix metalloproteinase 1 genes in scleroderma dermal fibroblasts. *J Dermatol Sci* 2014;74:251-259.
- 39 Dong J, Porter DW, Batteli LA, Wolfarth MG, Richardson DL, Ma Q: Pathologic and molecular profiling of rapid-onset fibrosis and inflammation induced by multi-walled carbon nanotubes. *Arch Toxicol* 2015;89:621-633.
- 40 Liu Q, Zhang X, Dai L, Hu X, Zhu J, Li L, Zhou C, Ao Y: Long noncoding RNA related to cartilage injury promotes chondrocyte extracellular matrix degradation in osteoarthritis. *Arthritis Rheumatol* 2014;66:969-978.
- 41 Huang C, Yang Y, Liu L: Interaction of long non-coding RNAs and microRNAs in the pathogenesis of idiopathic pulmonary fibrosis. *Physiol Genomics* DOI:10.1152/physiolgenomics.00064.2015.
- 42 Berridge MJ: Smooth muscle cell calcium activation mechanisms. *J Physiol* 2008;586:5047-5061.
- 43 Cai H, Liu D, Garcia JG: CaM Kinase II-dependent pathophysiological signalling in endothelial cells. *Cardiovasc Res* 2008;77:30-34.
- 44 Mercure MZ, Ginnan R, Singer HA: CaM kinase II δ 2-dependent regulation of vascular smooth muscle cell polarization and migration. *Am J Physiol Cell Physiol* 2008;294:C1465-1475.
- 45 House SJ, Ginnan RG, Armstrong SE, Singer HA: Calcium/calmodulin-dependent protein kinase II- δ isoform regulation of vascular smooth muscle cell proliferation. *Am J Physiol Cell Physiol* 2007;292:C2276-2287.
- 46 Guo X, Gao L, Wang Y, Chiu DK, Wang T, Deng Y: Advances in long noncoding RNAs: identification, structure prediction and function annotation. *Brief Funct Genomics* 2015;pii:elv022.
- 47 Mercer TR, Mattick JS: Structure and function of long noncoding RNAs in epigenetic regulation. *Nat Struct Mol Biol* 2013;20:300-307.
- 48 Tripathi V1, Ellis JD, Shen Z, Song DY, Pan Q, Watt AT, Freier SM, Bennett CF, Sharma A, Bubulya PA, Blencowe BJ, Prasanth SG, Prasanth KV: The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. *Mol Cell* 2010;39:925-938.
- 49 Yoon JH1, Abdelmohsen K, Srikantan S, Yang X, Martindale JL, De S, Huarte M, Zhan M, Becker KG, Gorospe M: lincRNA p21 suppresses target mRNA translation. *Mol Cell* 2012;47:648-655.
- 50 Guttman M, Rinn JL: Modular regulatory principles of large non-coding RNAs. *Nature* 2012;482:339-246.
- 51 Chu C, Qu K, Zhong FL, Artandi SE, Chang HY: Genomic maps of long noncoding RNA occupancy reveal principles of RNA-chromatin interactions. *Mol Cell* 2011;44:667-678.
- 52 Wang Y, Chen X, Liu ZP, Huang Q, Wang Y, Xu D, Zhang XS, Chen R, Chen L: De novo prediction of RNA-protein interactions from sequence information. *Mol Biosyst* 2013;9:133-142.
- 53 Dweep H, Gretz N: miRWalk2.0: a comprehensive atlas of microRNA-target interactions. *Nat Methods* 2015;12:697.
- 54 Dweep H, Sticht C, Pandey P, Gretz N: miRWalk-database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. *J Biomed Inform* 2011;44:839-847.