

	Number	Suggestion
Title	1	Rno_circ_0005139 regulates apoptosis by targeting <i>Wnt5a</i> in rat anorectal malformations
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Abstract	2	<p>BACKGROUND</p> <p>The molecular mechanisms underlying anorectal malformations (ARM) are not fully established. Circular RNAs (circRNAs) are new borned non-coding RNAs, and their role in ARM is unclear. We assumed rno_circ_0005139 influences apoptosis and proliferation by acting as an miR-324-3p sponge, and downregulating <i>wingless-type MMTV integration site family, member 5a (Wnt5a)</i> in ARM.</p> <p>AIM</p> <p>RNA sequencing and RNA microarray analysis were to identify the differential expression of circRNAs and mRNAs in a rat ARM model.</p> <p>METHODS</p>
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66 pregnant Wistar rats were randomly divided into two groups: ARM group (2-imidazolidinethione (ETU)-induced) and control groups. Embryos were harvested by cesarean delivery and anorectal tissue was taken on embryonic day 16 (E16), embryonic day 17 (E17), embryonic day 19 (E19), and embryonic day 21 (E21). RNA sequencing and gene microarray analysis were used to identify differentially expressed circRNAs and mRNAs in anorectal malformation in a rat model. We selected 6 circRNAs and 3 mRNAs in the wingless-type MMTV integration site (Wnt) signal pathway from the result of the RNA sequencing and gene microarray analysis, and quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) was performed to evaluate their tissue expression. According to bioinformatics prediction, rno_circ_0005139 acted as an

miR-324-3p sponge to regulate the expression of *Wnt5a*. We chose rno_circ_0005139 and *Wnt5a* as the final candidates. We tested the function of rno_circ_0005139 and the binding sites between rno_circ_0005139 and miR-324-3p, miR-324-3p and *Wnt5a* by luciferase assays. Co-transfection of rno_circ_0005139 and miR-324-3p was to verify their functional consistency.

RESULTS

We identified 38 upregulated and 42 downregulated circRNAs on E17 ($P < 0.05$), and 301 mRNAs were upregulated and 256 downregulated in the ARM on E17 ($P < 0.05$, fold-change > 2.0). We found that rno_circ_0006880 and rno_circ_0011386 were upregulated, whereas rno_circ_0000436, rno_circ_0005139, rno_circ_0009285, rno_circ_0014367, *Wnt5a*, *wingless-type MMTV integration site family member 10b*, and *wingless-type*

were downregulated in ARM tissues. According to bioinformatics prediction, rno_circ_0005139 acted as an miR-324-3p sponge to regulate the expression of *Wnt5a*. We chose rno_circ_0005139 and *Wnt5a* as the final candidates. Because the role and molecular mechanism of rno_circ_0005139 is poorly understood, its effect on apoptosis and proliferation and was investigated by *in vitro* plasmid transfection. A luciferase experiment showed that rno_circ_0005139 could bind with miR-324-3p, which negatively regulated *Wnt5a* expression. The expression of miR-324-3p was significantly higher in ARM anorectal tissues than that in control group on E17 and E19; *Wnt5a* expression showed the opposite trend. In addition, an miR-324-3p inhibitor attenuated the

effects of rno_circ_0005139 knockdown on ARM development.

CONCLUSION

Rno_circ_0005139 influences cell proliferation and apoptosis by acting as an miR-324-3p sponge, thereby downregulating *Wnt5a* in ARM. Accordingly, rno_circ_0005139, miR-324-3p, and *Wnt5a* could be targeted therapeutic factors for ARM.

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Backgroud	3	a.1.Wong EH, Cui L, Ng CL, Tang CS, Liu XL, So MT, Yip BH, Cheng G, Zhang R, Tang WK, Yang W, Lau YL, Baum L, Kwan P, Sun LD, Zuo XB, Ren YQ, Yin XY, Miao XP, Liu J, Lui VC, Ngan ES, Yuan ZW, Zhang SW, Xia J, Wang H, Sun XB, Wang R, Chang T, Chan IH, Chung PH, Zhang XJ, Wong KK, Cherny SS, Sham PC, Tam PK, Garcia-Barcelo MM. Genome-wide copy number variation study in anorectal malformations. Hum Mol
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Suzuki K, Haraguchi R, Ogata T, Barbieri O, Alegria O, Vieux–Rochas M, Nakagata N, Ito M, Mills AA, Kurita T, Levi G, Yamada G. Abnormal urethra formation in mouse models of split-hand/split-foot malformation type 1 and type 4. Eur J Hum Genet 2008; 16: 36–44.

b. Bai Y , Chen H, Yuan Z , et al. Normal and abnormal embryonic development of the anorectum in rats[J]. Journal of Pediatric Surgery, 2004, 39(4):0–590.

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Aim	4	RNA sequencing and RNA microarray analysis were to identify the differential expression of circRNAs and mRNAs in a rat ARM model.
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Ethical statement	5	Approval was obtained from the Medical Research and New Technology Ethics Committee of Shengjing Hospital, affiliated with China Medical University (2016PS045K).
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Research design	6	a.33 pregnant rats were administered a single dose of ETU (2–imidazolidinethione; Aldrich Chemical, Penzberg, Germany) and
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33 pregnant rats were administered an equal dose of saline without ETU via oral gavage on E10

b. Pregnant rats who are pregnant at the same time are numbered and randomly selected.

c. Signal pregnant rat.

Embryos were harvested by cesarean delivery on E16, E17, E19, and E21. The embryos were divided into ARMs (short or no tail) and control (long tail) groups. The cloacae and hindguts were dissected, removed from surrounding tissues under magnification, and immediately frozen in liquid nitrogen. Then using qRT-PCR to verify the expression of rno_circ_0005139, miR-324-3p, and Wnt5a, the binding sites to test between rno_circ_0005139 and miR-324-3p, and miR-324-3p and Wnt5a by luciferase assay. IECs separately transfected with rno_circ_0005139 overexpression plasmid and siRNA,

miR-324-3p mimic and inhibitor and co-transfected rno_circ_0005139 overexpression plasmid and miR-324-3p mimic, and rno_circ_0005139 siRNA, miR-324-3p inhibitor, then tested the expression of Wnt5a and the function.

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Experimental steps	7	<p>a. The concentration of ETU was 0.01 mg/mL and the dose was 125 mg/kg via oral gavage on E10. Death from cervical spine , and embryos were harvested by cesarean delivery.</p> <p>b. Embryos were harvested by cesarean delivery E16, E17, E19, and E21.</p> <p>At animal sampling room</p> <p>Oral gavage ETU to make a animal model.</p>
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Experimental animals	8	<p>a. Female (250–280 g) and male (280–300 g) Wistar rats.</p> <p>b. Rats were provided by the Chang Sheng Biotechnology Co., Ltd. (Changchun, China).</p>
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Feeding place	9	<p>a. specific pathogen free(spf) animal laboratory, spf grade animal litter , pregnant Wistar rats are kept alone , unpregnant Wistar rats are 5 in a cage (female:male 4:1).</p> <p>b. 12h light–dark cycle, 37°C, spf feed, get water and feed anytime.</p>
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Sample	10	<p>a.Total 66 pregnant rats: 33 pregnant rats were administered a single dose of ETU and 33 pregnant rats were administered an equal dose of saline without ETU via oral gavage.</p> <p>b.</p>
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	ARM Group			Control Group		
Sequencing Sample	A1	A2	A3	N1	N	N
					2	3
The number of pregnant rats	3	3	3	3	3	3
The number of embryos on E17	33	32	32	34	3	3
					5	3

Experiment	A-	A-	A-	N-	N-	N
Sample	1	2	3	1	2	-3
The number of pregnant rats	8	8	8	8	8	8
The number of embryos on E16	26	26	25	27	2	2
					8	8
The number of embryos on E17	23	21	22	24	2	2
					3	4
The number of embryos on E19	14	13	13	17	1	1
					6	6
The number of embryos on E21	9	10	10	12	1	1
					3	2
Total	10	10	10	11	1	1
	5	3	2	4	1	1
					5	3

Group of animals	11	<p>a.The pregnant rats who were pregnant on the same day were randomly numbered, and all pregnant rats were randomly divided into two groups (the ARM group and the control group).</p> <p>b.ARM group was administered a single dose of ETU and control group was administered an equal dose of saline without ETU via oral gavage on E10.</p>
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Result	12	<p>ARM can be verified by taking tissues under the microscope in early pregnant days, and it can be seen with the naked in later pregnant days.</p>
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Statistics	13	<p>The expression of circRNA, miRNA and Wnt5a in ARM group and the control group were compared. And the functional experiment were compared between the ARM group and the control group.</p>
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Done Page15

Baseline data	14	<table border="1"> <tr> <th>Animal number</th><th>weight</th><th>status</th></tr> <tr> <td>1</td><td>271</td><td>health</td></tr> </table>	Animal number	weight	status	1	271	health
Animal number	weight	status						
1	271	health						

2	264	health
3	254	health
4	258	health
5	253	health
6	257	health
7	264	health
8	261	health
9	268	health
10	267	health
11	273	health
12	275	health
13	260	health
14	268	health
15	264	health
16	272	health
17	277	health
18	274	health
19	274	health
20	268	health
21	264	health
22	273	health
23	274	health
24	262	health
25	261	health
26	258	health
27	273	health
28	264	health
29	269	health
30	271	health
31	253	health
32	254	health
33	263	health
34	258	health
35	254	health
36	269	health
37	273	health
38	268	health
39	274	health
40	271	health
41	265	health
42	263	health
43	268	health
44	269	health

45	271	health
46	272	health
47	261	health
48	258	health
49	254	health
50	259	health
51	261	health
52	263	health
53	264	health
54	266	health
55	262	health
56	258	health
57	265	health
58	267	health
59	269	health
60	271	health
61	273	health
62	274	health
63	275	health
64	269	health
65	274	health
66	268	health

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Digital analysis 15 ARM group: total embryos 310, 218 embryos were with ARM.(218/310, 70.32%)

Control group: randomly selected the same number embryos at the same day.

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Results and 16 rno_circ rno_circ

evaluation _00004 _000513

36 9

	0.01540	0.011022
A16	1572	704
	0.04417	0.04503
N16	2366	0596
	0.05341	0.01973
A17	56	4853
	0.0277	0.04263
N17	05282	2113
	0.01618	0.05471
A19	5075	8135
	0.0595	0.00667
N19	88024	241
	0.01261	0.02200
A21	0372	75
	0.05146	0.02602
N21	1768	2788
	rno_circ	rno_circ
	_00068	_000928
	80	5
	0.0797	0.03049
A16	94507	2824

	0.0506	0.04790
N16	00607	0876
	0.0869	0.021150
A17	73254	169
	0.01789	0.03642
N17	0708	211
	0.0600	0.04243
A19	98436	1702
	0.0207	0.010177
N19	89134	665
	0.0826	0.01649
A21	85278	2332
	0.0567	0.02522
N21	33937	6978
	rno_circ	rno_circ
	_001138	_001436
	6	7
	0.0827	0.03520
A16	68518	9949
	0.0266	0.08639
N16	72118	1461

	0.04167	0.02694
A17	263	8071
	0.0386	0.02730
N17	56855	1007
	0.0638	0.013775
A19	20288	735
	0.0498	0.04608
N19	39301	0992
	0.12664	0.03545
A21	6712	5928
	0.04914	0.04718
N21	7833	9941
	Wnt2b	Wnt10b
	0.0275	0.012160
A16	62239	669
	0.01502	0.07469
N16	8134	6018
	0.02861	0.19828
A17	2173	9011
	0.03158	0.08584
N17	7484	0507

	0.03186	0.19643
A19	3999	2867
	0.0439	0.25492
N19	06335	8492
	0.0224	0.06356
A21	319	0193
	0.0229	0.117908
N21	48482	79
	miR-32	
	Wnt5a	
	4-3p	
	0.01509	0.02068
A16	684	7709
	0.01860	0.01579
N16	7302	6553
	0.0299	0.100071
A17	01747	124
	0.02142	0.03099
N17	0948	5967
	0.0223	0.04995
A19	59524	0843
N19	0.04129	0.014041

	2435	55
	0.04317	0.03267
A21	0374	9535
	0.01842	0.03807
N21	0705	4152
contr	0.0498	
ol	49461	
siR-5	0.0057	
139-1	54495	
siR-5		
	0.00173	
139-		
	1778	
2		
siR-5		
	0.00196	
139-		
	3686	
3		
	apopto	
	sis	
si-51		
	0.0025	
39-n		
	45584	
c		
si-51	0.01414	

39 2136

over-
0.02100

5139
1071

-nc

over- 0.0003

5139 88909

over-

5139

-nc+

miR- 0.00201

324- 5254

3p

mimi

c nc

miR-

324-

3p

mimi 0.0095

c+ov 81297

er-51

39-n

c

miR-

324-

3p

mimi 0.0002

c 47487

nc+o

ver-5

139

over-

5139

+miR

0.0008

-324

83883

-3p

mimi

c

si-51

39-n

c+mi

0.0033

R-32

23402

4-3p

inhibi

bor

nc

si-51

39+m

iR-3

24-3 0.0003

p 88909

inhibi

bor

nc

si-51

39-n

c+mi

0.0023

R-32

68808

4-3p

inhibi

bor

si-51

39+m

iR-3 0.0002

24-3 47487

p

inhibi

bor				
prolifer				
ation				
moke	12h	24h	36h	48h
si-51				0.022
	0.0782	0.06513	0.0312	
39-n				51734
	04232	5138	52192	
c				6
				0.025
si-51	0.01132	0.02758	0.0137	
				47593
39	6375	0317	2676	
				3
over-				0.036
	0.0274	0.02384	0.0184	
5139				25398
	55094	7565	6697	
-nc				6
over-	0.01937	0.02948	0.0183	0.028
5139	4559	5756	7466	47655
over-				
5139				
-nc+	0.01671	0.09766	0.0173	0.009
miR-	7062	9882	52566	4133
324-				
3p				

mimi				
c nc				
miR-				
324-				
3p			0.009	
mimi	0.01996	0.117966		0.006
			47491	
c+ov	1417	173		88099
			9	
er-51				
39-n				
c				
miR-				
324-				
3p			0.025	
mimi	0.0435	0.110956		0.025
			85467	
c	66705	478		8337
			2	
nc+o				
ver-5				
139				
over-				
5139	0.0405	0.01250	0.0272	0.0130
+miR	04903	9851	09253	13897
-324				

-3p

mimi

c

si-51

39-n

c+mi

R-32 0.0885 0.121586 0.0111 0.0124

4-3p 04262 184 03181 07988

inhibi

bor

nc

si-51

39+m

iR-3

24-3 0.07171 0.100169 0.0126 0.0231

p 5203 383 19623 21645

inhibi

bor

nc

si-51

0.10998 0.131068 0.0067 0.0119

39-n

0175 461 89206 59676

c+mi

R-32

4-3p

inhibi

bor

si-51

39+m

iR-3 0.058

0.0725 0.24169 0.1743

24-3 26122

68528 9148 32202

p 9

inhibi

bor

miR-32

Wnt5a

4-3p

miR-

324- 16.2891 0.02760

mimi 2236 478

c

324-

0.0803 0.071431

N-mi

50784 074

mic

miR3 0.00148 0.16293

24-in 2287 4359

		hibito
		r
		324–
		0.10550 0.02865
		N–inh
		6801 7152
		ibitor
		Done Page15–20
Adverse reactions	17	none
Interpretation	18	Our study had several potential limitations. The amount of anorectal tissue in a single embryo is too little, so the specimen is a mixture of anorectal tissues of multiple embryos. Due to individual differences in embryos, the experiment had some inaccuracies.
		Done Page20–23
Conversion	19	Circular RNAs were extensive and stable, and prenatal intervention of circular RNAs can reduce the occurrence of malformations.
		Done Page23
Funding	20	National Natural Science Foundation of China, No. 81671503.
		Done Page2