Supplementary Experimental Procedures

Ethics Statement

All animal work was conducted under the institutional guidelines of Jiangsu Province, which were developed according to the principles expressed in the Declaration of Helsinki. All animal protocols were approved by the Animal Care and Use Committee of the Model Animal Research Center (permit number: XH1), the host for the National Resource Center for Mutant Mice in China, Nanjing University. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Plasmids

For the over-expression of mmu-ca-65, one pair of primers were used to amplify 300-nt precursors with mouse genomic DNA as template (from 5' to 3'): ***GGATCC***GCCAGGAAGCAGCTATTC and ***CTCGAG***TCAAGGCTAAGTTGTCGC. The products were digested with *BamH I* and *Xho I* (bold italic) and then inserted into the lentiviral vector to obtain constructs termed lenti-65.

Transfection

RAW264.7 or PC1 cells were transfected with 50 or 100 nM mmu-ca-65 mimic (Ambion, Austin, TX), locked-nucleic-acid (LNA) inhibitor (Exiqon, Vedbaek, Denmark), siRNAs targeting *Egr1* (GenePharma, Shanghai) or their corresponding controls using Fugene HD Transfection Reagent according to the manufacturer's instructions. Two days after transfection, the cells were washed three times and then underwent the various treatments. The sequences of the *Egr1* siRNAs were as follows (from 5' to 3'): si-1: sense GGUGGUUUCCAGGUUCCCATT, antisense UGGGAACCUGGAAACCACCTT; si-2: sense GGACUUAAAGGCUCUUAAUTT, antisense AUUAAGAGCCUUUAAGUCCTG; si-3: sense CGCAAGAGGCAUACCAAATT, antisense UUUGGUAUGCCUCUUGCGTT. For expressing mmu-ca-65 precursor, we compared different methods and reagents. At the beginning, we sought to pack lentivirus to express mmu-ca-65 by infecting RAW264.7 cells. However, by unknown reasons the expression of mmu-ca-65 in RAW264.7 cells infected by lentivirus was not high even after selection. So we tried transfection of the lenti vector containing mmu-ca-65 precursor later by using a series of different transfection reagents, like Fugene HD, Lipofectamine 2000, Nucleofector kits, etc. Finally, we found that electronica transfection of the lentiviral vector by using Cell Line Nucleofector kit V (Lonza, Basel, Switzerland) in an Amaxa Nucleofector gave rise to the highest efficiency for expressing mmu-ca-65 in cells (data not shown).

Immunoblotting

Proteins were extracted from cells as described before (1), separated by electrophoresis on 12% SDS-polyacrylamide gels and semi-dry-blotted to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Proteins were immuno-detected by standard procedures using anti-EGR1 antibody. Labeling of the first Ab was detected using goat anti-rabbit secondary antibodies conjugated to HRP (Sigma-Aldrich, St. Louis, MO) and detected using ECL reagents (GE, Piscataway, NJ). GAPDH or β-actin (Kangcheng, Shanghai) was used as a loading control.

Real-time RT PCR

Quantitative RT-PCR was performed as previously described (2). Briefly, cDNA was synthesized using a Tiangen Quant cDNA synthesis kit (Tiangen, Beijing). Real-time PCR was performed using a Rotor-Gene 3000 real-time PCR machine (Corbett, Australia). The reagent used was QuantiTect SYBR Green PCR reagent (Qiagen, Valencia, CA). The primers (from 5' to 3') synthesized by Sangon (Shanghai) were as follows: *Brd3*: TGAAAAGCGACAACTTAGCCTT and GGCTCCCGAGACTGAATGAT; *Egr1*: CCAACATCAGTTCTCCAGCTC and TTGCTCAGCAGCATCATCTC; and *Gapdh*: TCAACAGCAACTCCCACTCTTCC and GGTGGTCCAGGGTTTCTTACTCC. Quantitative analysis of miRNAs by stem-loop RT-PCR were performed as previously described (3). The stem-loop RT primers were as follows (from 5' to 3'): rno-co-606, GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTACAC; rno-ca-179, GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGGGTG; rno-ca-2, GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGCTT; rno-ca-786, GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACATGT; and rno-ca-715, GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCGAAG; The forward primers: rno-co-606, GCCTGAACAGCGCCTTTCTG; rno-ca-179, GGCGTCACTTCAGGATGTACCA; rno-ca-2, TGCGTCAACATCAGTCTGATAAGC; rno-ca-786, GCGCTGTGTTTTGTGTGTGTAC; and rno-ca-715, TTCCACACACAGTCGCCATCT; The universal reverse primer was GTGCAGGGTCCGAGGT. The products were sequenced after ligation into the T-easy vector (Promega, Madison, WI) to confirm the accuracy of the PCR results.

Cytokine ELISA

Mouse TNFα and IL6 concentrations in the culture supernatant or blood plasma were quantified using a commercially available ELISA Duoset kit (R&D systems, Minneapolis, MN) according to the manufacturer's instructions.

Chromatin Immunoprecipitation (ChIP) Assays

The ChIP assays were performed as described before with minor modifications (4). Briefly, after experimental treatments, RAW264.7 cells were fixed with 1% formaldehyde for 10 min. The cells were sonicated to yield 200- to 800-bp DNA fragments. One percent of the cell extract was taken out as input, and the rest of the extract was incubated with 4 μg rabbit anti-p65 polyclonal antibody or normal rabbit IgG (Santa Cruz) overnight. The NF-κB-responsive elements upstream of mmu-ca-65 or the promoter region of TNFα was amplified by PCR using the precipitated genomic DNA. The primer sequences (from 5' to 3') for mmu-ca-65 were sense AGAAAATCGTGTGTGTGTGCA and antisense AAACCCTAGTTCAATCCCCAG. The primer sequences for the TNFα promoter were sense ACCGCCTGGAGTTCTGGAA and antisense CGAGGACAGCAAGGGACTAG. Non-target refers to the amplification of a region without the potential NF-κB binding site, which was approximately 10 kb downstream of mmu-ca-65. Those primers were sense CTAGCCCAGCTGAGCTATTTGA and antisense TGGATGGTTGTGAACCACCAT.

Northern Blot

Small RNAs (< 200-nt), extracted with the miRcute miRNA isolation kit (Tiangen, Beijing), were resolved by 15% denatured polyacrylamide gel containing 8 M urea. The RNA was then transferred to a GeneScreen Plus Hybridization Transfer Membrane (PerkinElmer, Waltham, MA). After UV crosslinking, the membrane was incubated at 80 °C for 30 min and then stained by methylene blue to view transfer efficiency. The membrane was incubated in hybridization buffer (Toyobo, Osaka, Japan) at 37 °C for 30 min and then hybridized with specific γ-^{32}P-labled probe (Sangon, Shanghai) at 37 °C for 24 h. The membrane was washed for 15 min three times with washing buffer (0.1% SDS 2×SSC), and the signal was detected by a phosphorimager system (Fuji FA-9000). The hybridized membrane was striped with 1% SDS at 65 °C for 1 h and re-hybridized with specific γ-^{32}P-labeled U6 probe (Sangon, Shanghai) as loading control. The probes for detecting mmu-ca-65, rno-ca-65, miR-155, snRNA U6 and the hypothetical mmu-ca-65* were (from 5' to 3'): TCTGAGGCAGAAGACAGAGCCATG, TCTGAGGCAGAAGACGAGCCACG, ACCCCTATCACAATTAGCATTAA, GCTTCACGAATTTGCGTGTCAT and CCTAGCCCCTTTCTCCTTCACTAAAGA, respectively.

Reporter Gene Assay

To generate constructs for luciferase assays, the 3' UTR of *Egr1* was amplified from mouse genomic DNA by primers containing restriction enzyme sites (bold italic): ***TCTAGA***GGGAATGAAAGAGAGCAAA and ***GCGGCCGCC***AGTTAGTTTCTTTATTTTCCT. The PCR products were digested with *Xba I* and *Not I* and then inserted into pRL-TK. The resulting plasmid was termed pUTR-fl. The UTR lacking the three mmu-ca-65 binding sites a, b and c was obtained by two-round PCR amplifications according to the protocol described previously (5). pUTR-fl was used as template for the following primers (from 5' to 3'): MRE a: TACTGACTAGAAGGATGAGATCTTCCATCT and AGATGGAAGATCTCATCCTTCTAGTCAGTA; MRE b: GGTGAAGAACTTGGATTTCAGGCAGCTGAA and TTCAGCTGCCTGAAATCCAAGTTCTTCACC; and MRE c: ATCACCATTGTTTGTTGCTCCCCCTCCCC and GGGGAGGGGGAGCAACAAACCAATGGTGAG. Three deletion sites were obtained by one-by-one amplification using the above products as templates. After amplification, four mutated constructs with the a, b or c MRE sites individually or simultaneously deleted were obtained and termed pUTRΔa, pUTRΔb, pUTRΔc and pUTRΔabc, respectively. Various luciferase reporter constructs were co-transfected with mmu-ca-65 LNA inhibitor or over-expression vector lenti-65 into PC1 cells. pGL3 was co-transfected as the internal transfection control. The cells were lysed and the luciferase activities (pRL-TK) were measured and normalized to the co-transfected pGL3 activities.

Statistics

Statistical analysis was performed with the software SPSS 10.0 (SPSS Inc., USA). Student's t-test was used to compare two independent groups. A least significant difference (LSD) test of one-way analysis of variance (ANOVA) was performed for multiple-group comparisons (e.g., the luciferase assays). All graphs presented herein were produced using the software SigmaPlot 10.0 (SPSS Inc., USA). Differences were considered significant only at $P < 0.05$. The "x, y, yz, etc" above each bar was used to represent the significance of the difference among more than 2 groups by ANOVA analysis. For example, the difference between "x" and "z" is much more than that between "x" and "y", and "yz" means no significant difference to either "y" or "z". The relative intensity of signals in Northern blot and western blot results was analyzed with Multi Gauge Version 3.0 (Fujifilm, Tokyo).

Reference

1. Zhu, C. F., Liu, Q., Zhang, L., Yuan, H. X., Zhen, W., Zhang, J. S., Chen, Z. J., Hall, S. H., French, F. S., and Zhang, Y. L. (2007) *Biology of reproduction* **76**, 63-73
2. Zhang, J. S., Liu, Q., Li, Y. M., Hall, S. H., French, F. S., and Zhang, Y. L. (2006) *Molecular and cellular endocrinology* **250**, 169-177
3. Chen, C., Ridzon, D. A., Broomer, A. J., Zhou, Z., Lee, D. H., Nguyen, J. T., Barbisin, M., Xu, N. L., Mahuvakar, V. R., Andersen, M. R., Lao, K. Q., Livak, K. J., and Guegler, K. J. (2005) *Nucleic acids research* **33**, e179
4. Shang, Y., Myers, M., and Brown, M. (2002) *Molecular cell* **9**, 601-610
5. Calin, G. A., and Croce, C. M. (2006) *Nat Rev Cancer* **6**, 857-866

Supplementary Figures

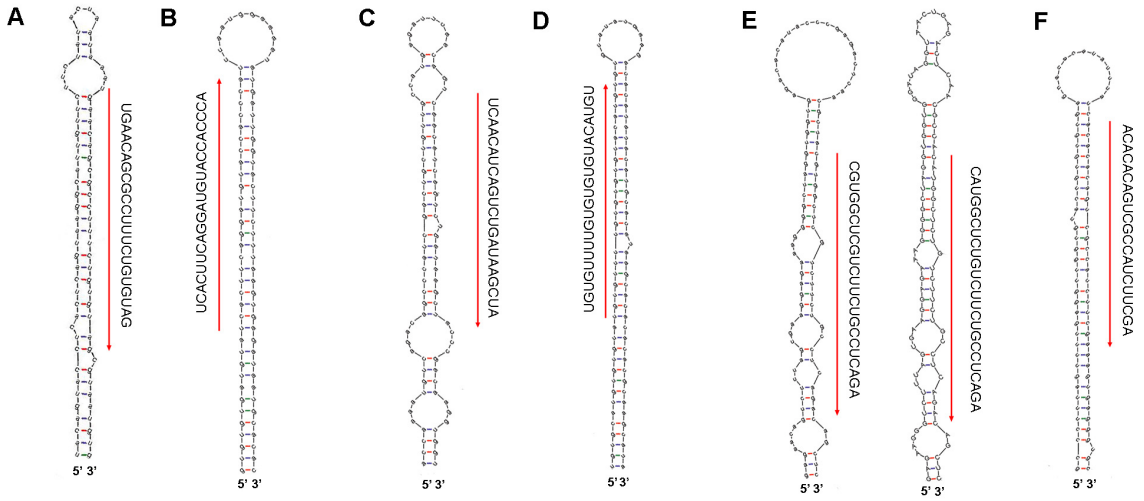

Figure S1 Predicted hairpin structure for novel miRNA candidates.

(A) rno-co-606. (B) rno-ca-179. (C) rno-ca-2. (D) rno-ca-786. (E) miR-ca-65. Left: rat; right: mouse. (F) rno-ca-715. The mature sequences were highlighted by red arrows.

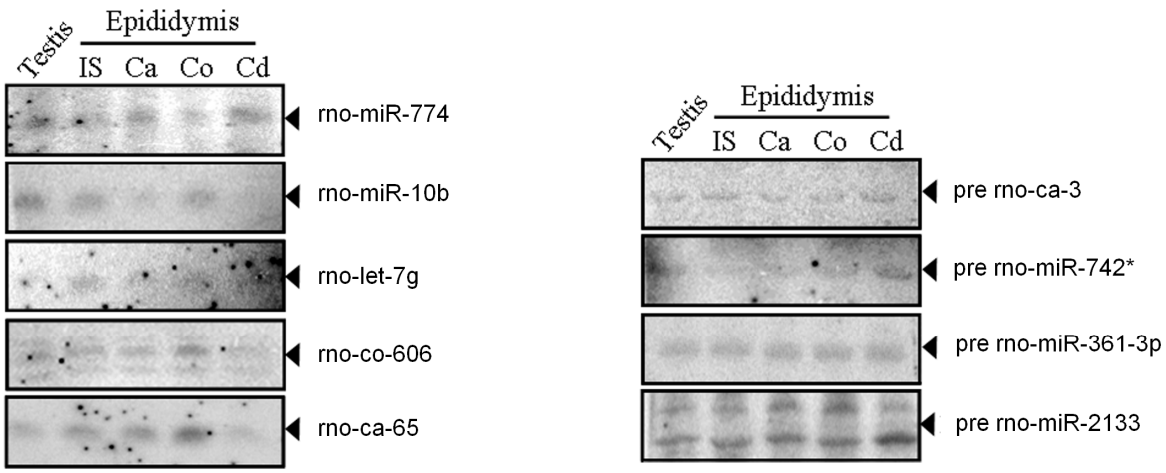

Figure S2. Confirmation of novel miRNA candidates in testis as well as four parts of epididymis.

Northern blots were performed on tissues from testis and four epididymal regions. 20 μg small RNA was loaded in each well. IS, initial segment, Ca, caput, Co, corpus and Cd, cauda, pre, precursor. The data shown represented at least two independent experiments.

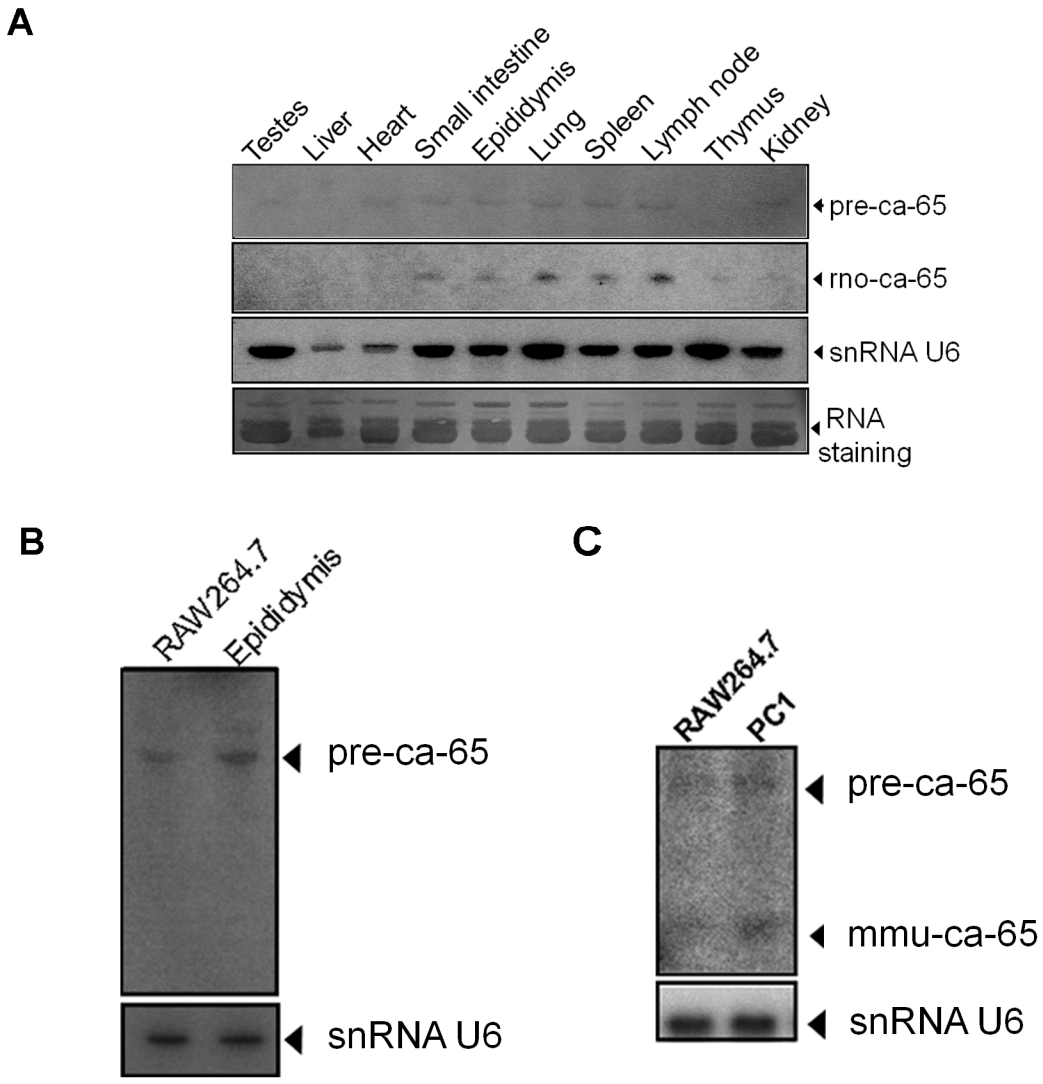

Figure S3. Expression of miR-ca-65 in rat tissues and mouse cells.

(A) Tissue distribution of rno-ca-65 expression in different tissues in rats. (B) Detection of hypothetical murine mmu-ca-65* in RAW264.7 cells and epididymis. (C) Expression of mmu-ca-65 in murine epididymal PC1 cells. snRNA U6 served as loading control and the data shown represented at least two independent experiments.

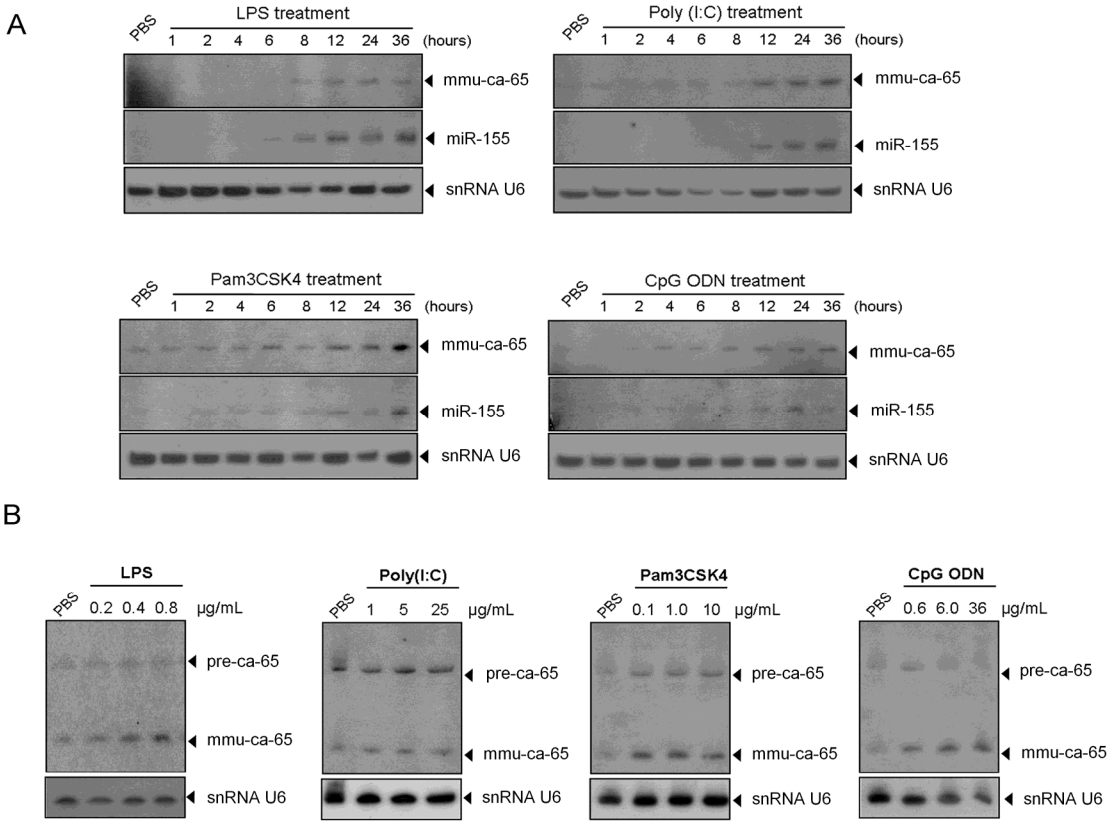

Figure S4. Induction of mmu-ca-65 by multiple ligands was time- and dose-dependent.

(A) Time-dependent induction of mmu-ca-65 by LPS, Pam3CSK4, poly (I:C) and CpG ODN. The cells were treated with 1 μg/ml LPS, 0.1 μg/ml Pam3CSK4, 5 μg/ml poly (I:C) or 6 μg/ml CpG ODN for the indicated time. After hybridization with the mmu-ca-65 probe, membranes were stripped and rehybridized using the miR-155 probe. (B) Dose-dependent induction of mmu-ca-65 by LPS, Pam3CSK4, poly (I:C) or CpG. These ligands of different concentrations were used to stimulate RAW264.7 cells for 12 h and mmu-ca-65 expression was detected by Northern blotting. SnRNA U6 was used as internal control. Data shown represented at least two independent experiments.

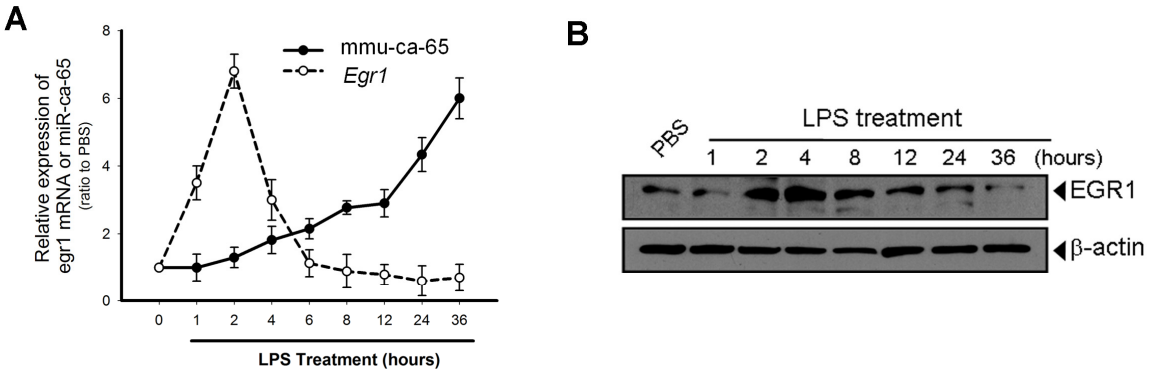

Figure S5. Expression profiles of EGR1 and mmu-ca-65 in LPS-exposed cells.

Gradual up-regulation of mmu-ca-65 was negatively correlated with down-regulation of *Egr1* mRNA (A) and protein (B) upon LPS stimulation. RAW264.7 cells were treated by LPS for the indicated time and the mature mmu-ca-65 expression was detected by Northern blot. *Egr1* mRNA or protein was measured by real-time PCR or immunoblotting. The relative expression level of mmu-ca-65 was calculated based on Northern blot intensity. The expression level of mmu-ca-65, *Egr1* of 0 h was set as 1. Values was presented as mean±SD from three independent experiments in *A*, at least two independent experiments were performed in B.

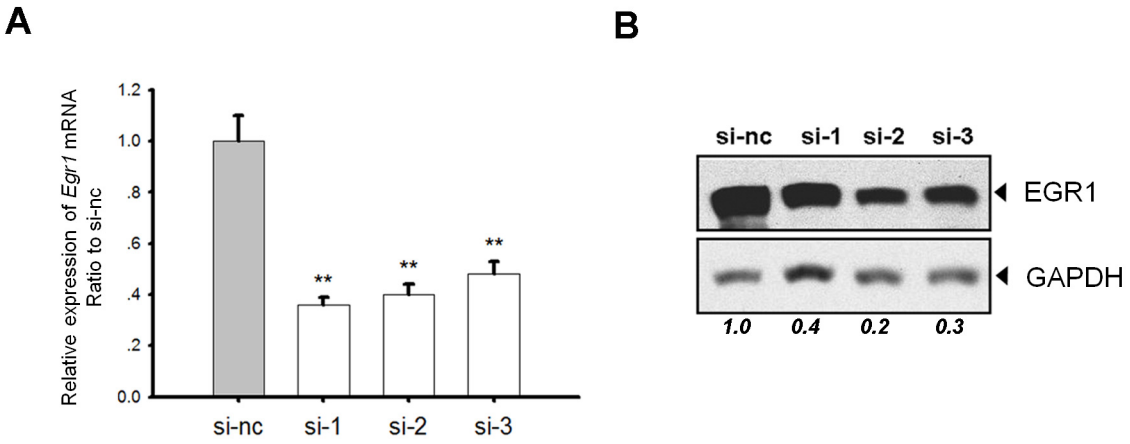

Figure S6. Silencing efficacy of siRNAs on *Egr1*.

Egr1 mRNA (A) or protein (B) silenced by siRNAs targeting *egr1*. siRNAs (si-1, si-2 or si-3) were transfected into RAW264.7 cells for 48 h. Real-time PCR and immunoblotting were performed to determine *Egr1* mRNA or protein. Si-nc transfected cells served as negative control and the expression level of *Egr1* of si-nc transfected cells was set as 1. GAPDH was used as an internal control for immunoblotting and real-time PCR. Values were presented as mean±SD from three independent experiments. For immunoblotting, data shown represented at least two experiments.

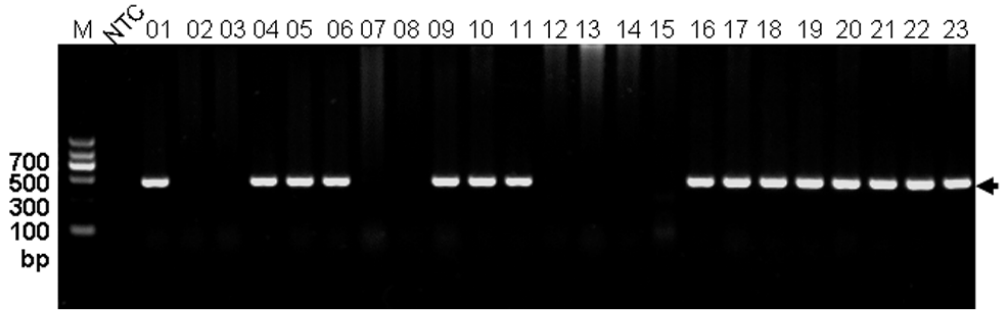

Figure S7. Genotyping of transgenic mice by RT-PCR. Genotyping PCR was carried out using genomic DNAs of transgenic mice as templates. Primers used for the analysis were described in the section of Materials and Methods. NTC, no template control.

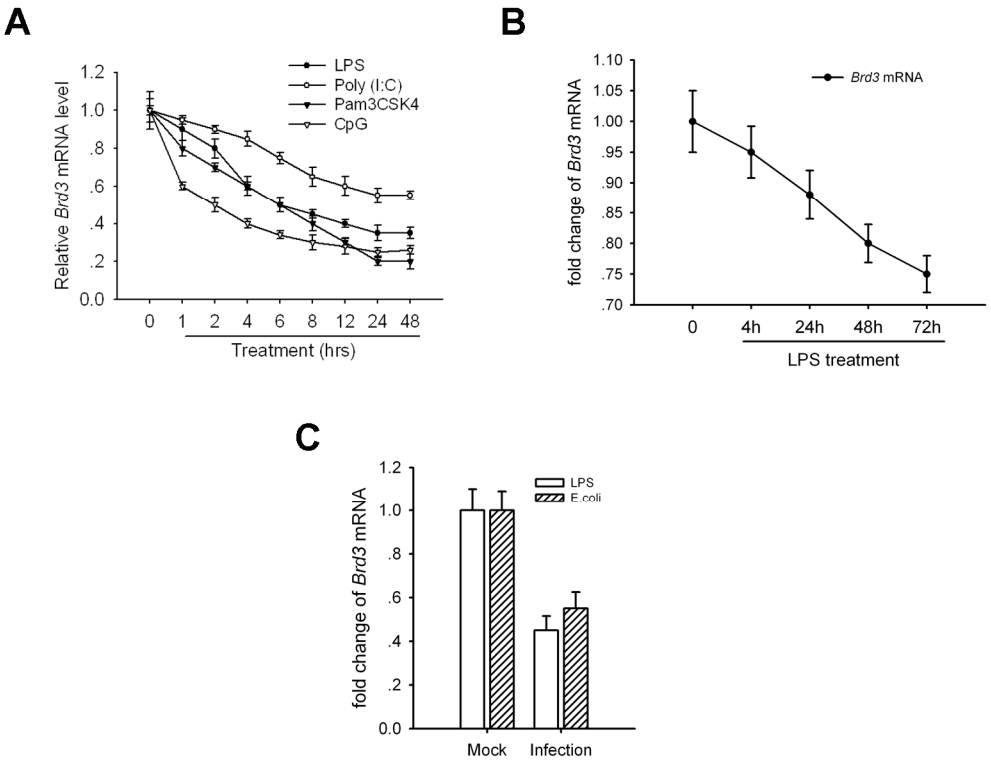

Figure S8. *Brd3* mRNA gradually decreased after various ligand treatments.

(A) *Brd3* mRNA in the various ligands treated cells for the indicated time was measured by real-time PCR. *Brd3* expression of PBS treated cells was set as 1. (B) *Brd3* mRNA in the lung infected by LPS administration. (C) *Brd3* mRNA in the epididymis infected by LPS administration or *E.coli*. *Gapdh* was used as an internal control for real-time PCR. Values were presented as mean±SD from three independent experiments.

Supplementary Tables

Table S1 Novel miRNA homologues cloned from the rat epididymis

Species name	Sequence 5'->3'	Size (nt)	Chro.	Arm	Strand	Northern blot	Existed homologue
rno-miR-742*	UACUCACAUGGUUGCUAAUCACG	20, 21, 23	X	5'	–	+[a]	*mmu-miR-742**
rno-miR-490-5p	CCAUGGAUCUCCAGGUGGGUAG	22	4	5'	+	–	*hsa-miR-490-5p*
rno-miR-425*	CAUCGGGAAUAUCGUGUCCGC	21	8	3'	+	–	*mmu/hsa-miR-425**
rno-miR-361-3p	CCCCCAGGUGUGAUUCUGAUUCGU	24	X	3'	+	+[a]	*has-miR-361-3p*
rno-miR-362-5p	AAUCCUUGGAACCUAGGUGUGAAUG	22, 25	X	5'	+	–	*mmu-miR-362-5p*
rno-miR-744	UGCGGGGCUAGGGCUAACAGCA	21, 22	10	5'	–	+	*mmu-miR-744*
rno-miR-741	AAAGAUGCCACGCUAUGUAGAU	21, 22	X	3'	–	NA	*mmu-miR-741*
rno-miR-149	UCUGGCUCCGUGUCUUCACUCCC	23	9	5'	+	NA	*mmu-miR-149*
rno-miR-141*	UCCAUCUUCCAGUGCAGUGUUGGA	24	4	5'	–	NA	*mmu-miR-141**
rno-miR-743b-5p	UGUUCAGACUGGUGUCCAUCA	21-23	X	5'	–	NA	*mmu-743b-5p*
rno-miR-463*	UACCUAAUUUGUUGUCCAUCAU	22	X	3'	–	NA	*mmu-miR-463**
rno-miR-31*	UGCUAUGCCAACAUAUUGCCAUC	23	5	3'	–	NA	*mmu-miR-31**
rno-miR-702	UGCCCACCCUUUACCCCACUCCAG	24	12	3'	+	NA	*mmu-miR-702*
rno-let-7g	UGAGGUAGUAGUUUGUACAGUU	21-23	8	5'	+	+	*mmu-let-7g*
rno-miR-103-2*	AGCUUCUUUACAGUGCUGCCUUGU	24	1, 3, 10	5'	+	–	*hsa-miR-103-2**
rno-miR-676	CCGUCCUGAGCUUGUCGAGCU	21	X	3'	+	–	*mmu-miR-676*
rno-miR-503*	GGAGUAUUGUUUCCGCUGCCUGG	23	X	3'	–	–	*mmu-miR-503**
rno-miR-196b*	UCGACAGCACGACACUGCCUUCA	23	4	3'	–	–	*hsa-miR-196b*
rno-miR-125b-2*	AUCACAAGUCAGGCUCUUGGG	21	11	3'	+	–	*hsa-miR-125b-2**
rno-miR-2133	UCCCGCGGGGCCCGAAGCGUU	21	Un	5'	+	+	*mmu-miR-2133*

[a] Only precursor form of miRNA was detected by Northern Blot.

Table S2 Novel miRNAs cloned from the rat epididymis

Species name	Hairpin dG	Paring rate	Sequence 5' →3'	Chro.	Arm	Length (nt)	Strand	qPCR	Northern blot	Genomic position	Location
rno-co-606	-42.3	18/22	UGAACAGCGCCUUUCUGUGUAG	X 1	3'	22	–	+	+	X: 13051059-13051080 1: 2369947-2369968	Intergenic
rno-ca-179	-51.8	18/22	UCACUUCAGGAUGUACCACCCA	X 1	5'	22,25	– +	+	–	X: 13051394-13941415 1: 2369612-2369633	Intergenic
rno-ca-2	-38.3	16/22	UCAACAUCAGUCUGAUAAGCUA	10	3'	22	+	+	–	10: 74864553-74864574	Intergenic
rno-ca-786	-56.2	21/22	UGUGUUUUGUGUGUGUACAUGU	17	5'	22	–	+	–	17: 32606568-32306589 (18 copies)	Exonic /intronic
rno-ca-65	-50.6	17/23	CGUGGCUCGUCUUCUGCCUCAGA	3	3'	23	–	+	+	3: 6372721-6372743	Intronic /exonic
rno-ca-715	-51.1	20/21	ACACACAGUCGCCAUCUUCGA	X	3'	21	–	+	–	X: 92107919- 92107939	Intergenic