ZR Zoological Research

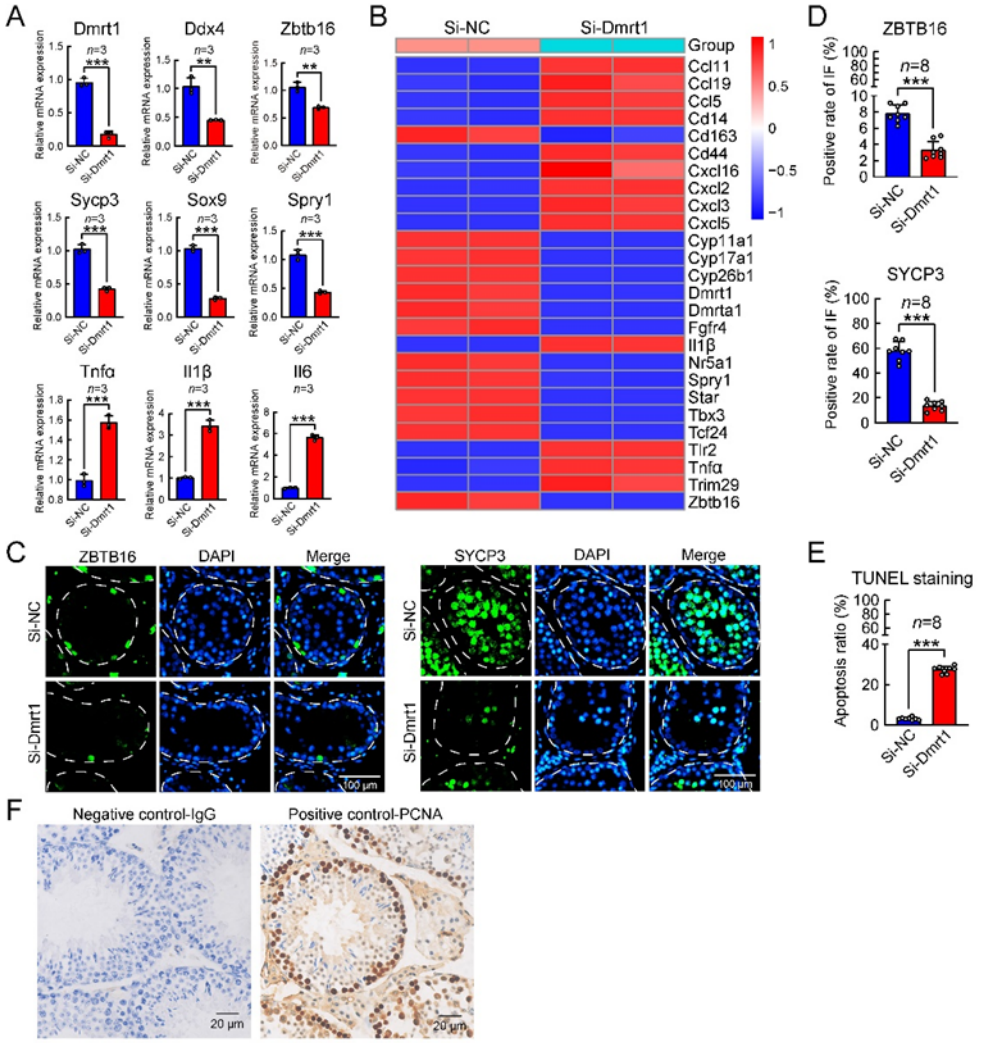
# Supplementary Materials

## Transcription factor *Dmrt1* triggers the SPRY1-NF-κB pathway to maintain testicular immune homeostasis and male fertility

Meng-Fei Zhang[1], Shi-Cheng Wan[1], Wen-Bo Chen[1], Dong-Hui Yang[1], Wen-Qing Liu[1,2], Ba-Lun Li[1], Aili Aierken[1], Xiao-Min Du[1], Yun-Xiang Li[1], Wen-Ping Wu[1], Xin-Chun Yang[1], Yu-Dong Wei[1], Na Li[1], Sha Peng[1], Xue-Ling Li[3], Guang-Peng Li[3], Jin-Lian Hua[1,4,*]

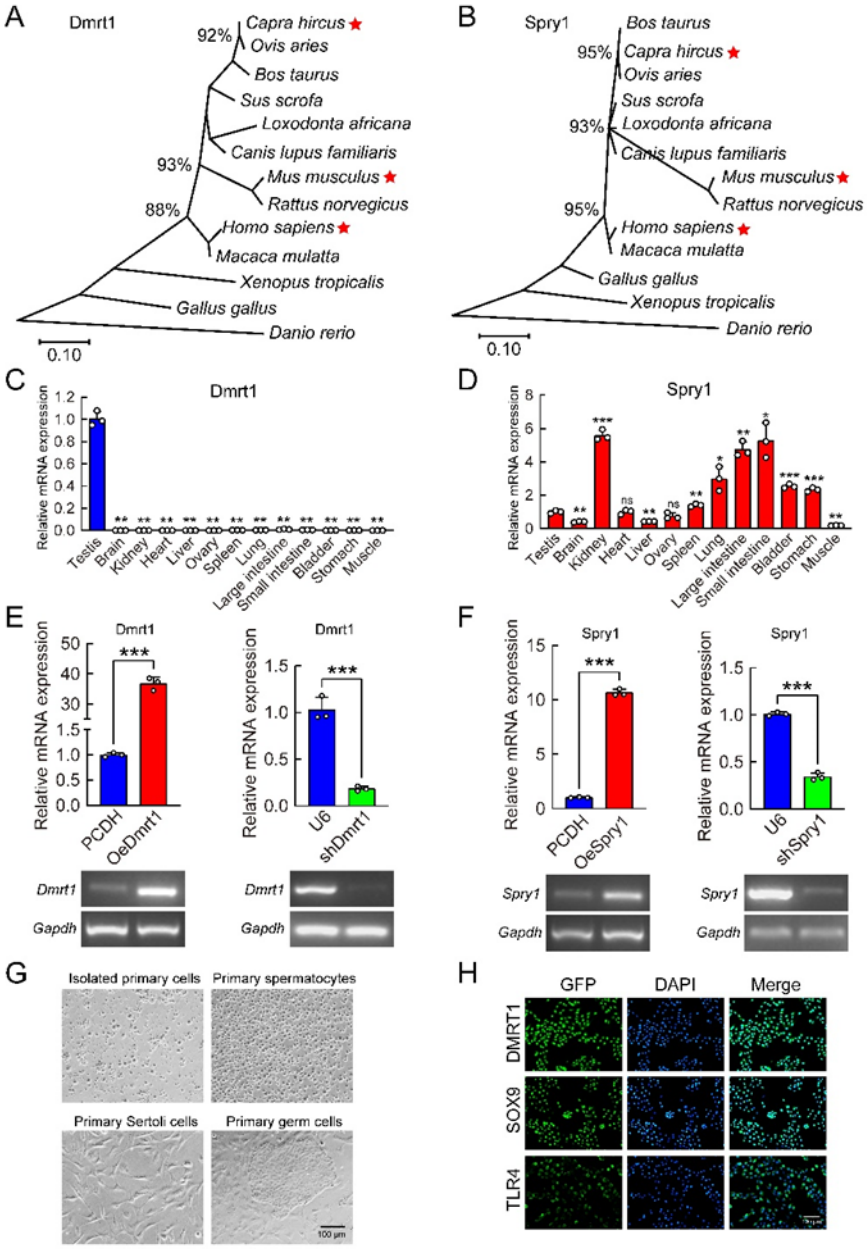
[1] College of Veterinary Medicine, Shaanxi Centre of Stem Cells Engineering & Technology, Northwest A&F University, Yangling, Shaanxi 712100, China
[2] Center of Reproductive Medicine, Amsterdam Research Institute Reproduction and Development, Academic Medical Center, University of Amsterdam 1105AZ, Amsterdam, Netherlands
[3] Key Laboratory for Mammalian Reproductive Biology and Biotechnology, Ministry of Education, Inner Mongolia University, Hohhot, Inner Mongolia 010021, China
[4] Key Laboratory of Livestock Biology, Northwest A&F University, Yangling, Shaanxi 712100, China

[*]Corresponding author, E-mail: jinlianhua@nwsuaf.edu.cn




**Supplementary Figure S1 Testicular changes in *Dmrt1* KD testes**

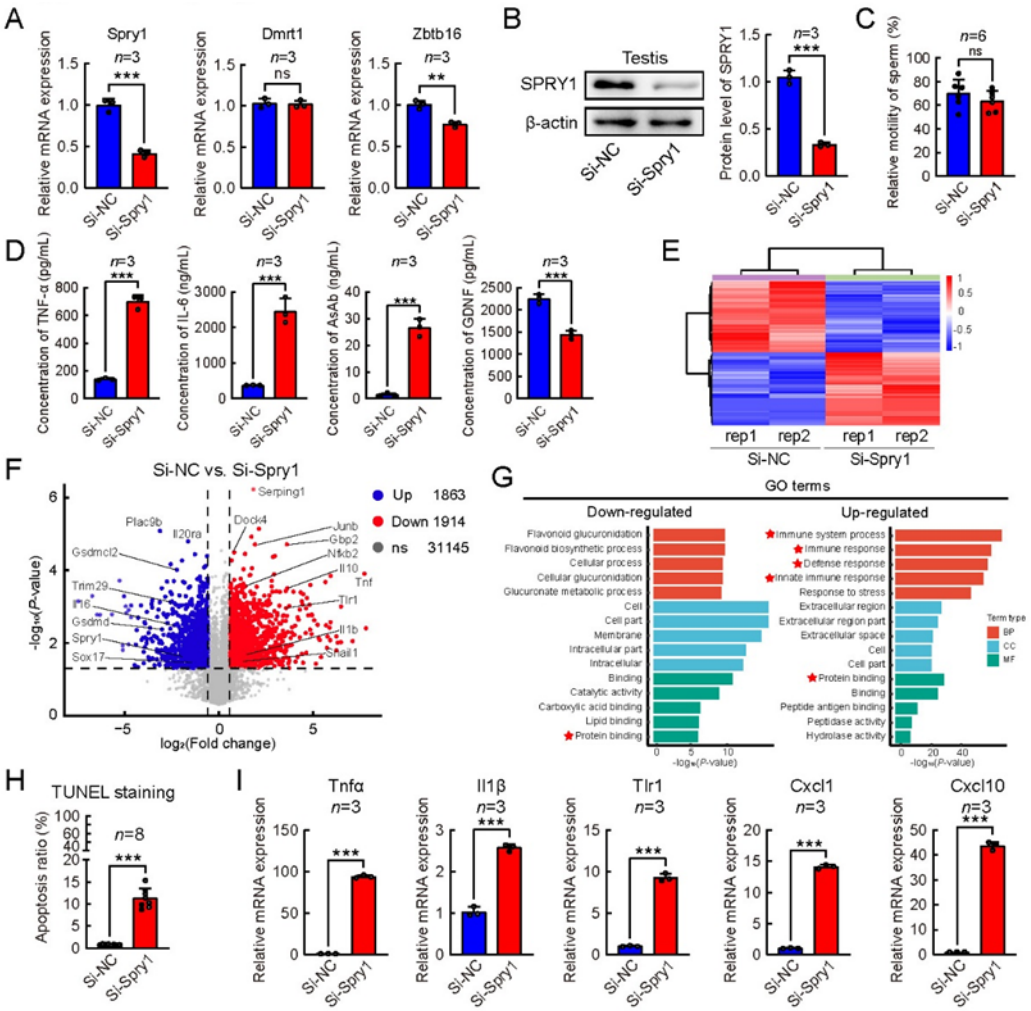
A: Relative mRNA levels of *Dmrt1*, *Ddx4*, *Zbtb16*, *Sycp3*, *Sox9*, *Spry1*, *Tnfα*, *Il1β*, and *Il6* in Si-NC and Si-Dmrt1 testes (*n*=3). B: Heatmap showing genes regulated by *Dmrt1* obtained from RNA-Seq of Si-NC and Si-Dmrt1 testes. C: IF staining of ZBTB16 (Up) and SYCP3 (Down) in Si-NC and Si-Dmrt1 testes. Nuclei were stained with DAPI (blue). Scale bar: 100 μm. D: Quantification of green fluorescence of ZBTB16 and SYCP3 in C (*n*=8). Scale bar: 100 μm. E: Apoptosis ratio of Si-NC and Si-Dmrt1 testes for quantification of TUNEL staining. F: Positive (PCNA) and negative (IgG) controls for IHC. Scale bar: 20 μm. **: $P<0.01$; ***: $P<0.001$.




**Supplementary Figure S2 Expression patterns of *Dmrt1* and *Spry1***

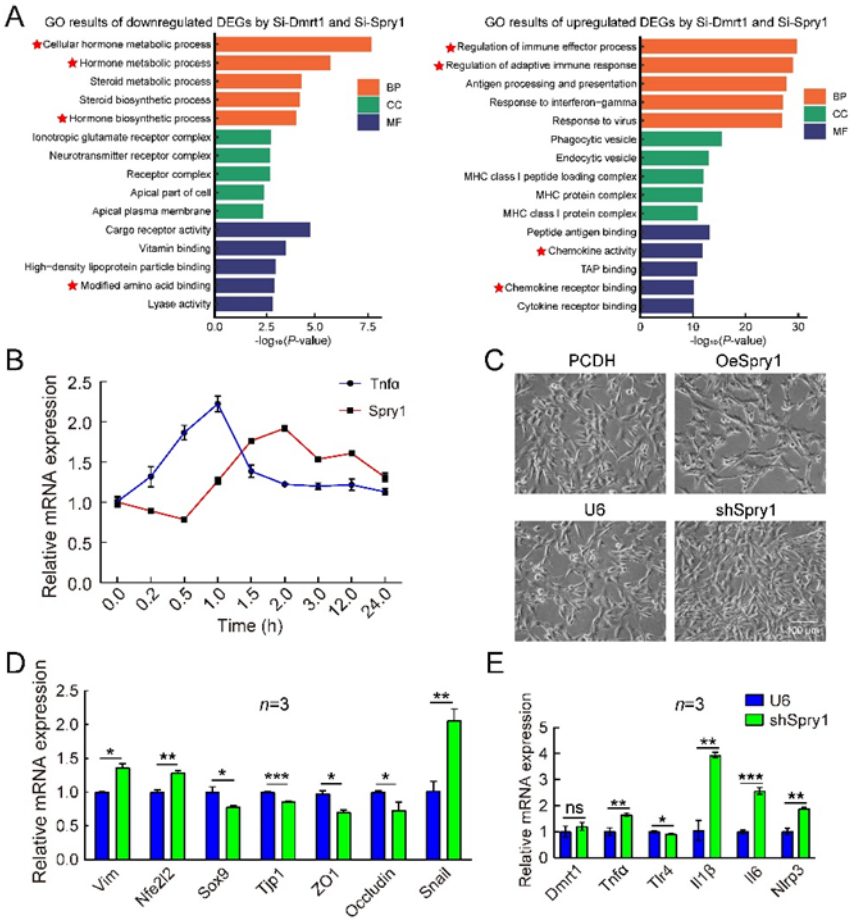
A: *Dmrt1* phylogenetic tree of different species constructed using MEGA v4. Red stars indicate species of concern. B: *Spry1* phylogenetic tree of different species constructed using MEGA v4. Red stars indicate species of concern. C: RT-PCR analysis of *Dmrt1* expression levels in different tissues of adult mice. All results were compared with testis results. D: RT-PCR analysis of *Spry1* expression levels in different tissues of adult mice. All results were compared with testis results. E: qRT-PCR analysis (top panel) and semi-quantitative RT-PCR analysis (bottom panel) of *Dmrt1* expression in PCDH, OeDmrt1, U6, and shDmrt1 cells ($n$=3). F: qRT-PCR analysis (top panel) and semi-quantitative RT-PCR analysis (bottom panel) of expression of *Spry1* in PCDH, OeSpry1, U6, and shSpry1 cells ($n$=3). G: Representative cell pictures during separation and purification of primary Sertoli cells and spermatogonial stem cells from healthy dairy goat testes. Scale bar: 200 μm. H: IF staining of Sertoli cell representative

marker SOX9, DMRT1, and TLR4 receptors. Nuclei were stained with DAPI (blue). Scale bar: 100 μm. Data are presented as means±SD and represent three independent repetitions. ns: not significant; *: $P$<0.05; **: $P$<0.01; ***: $P$<0.001.




**Supplementary Figure S3 Testicular changes in *Spry1* KD testes**

A: Relative mRNA levels of *Spry1*, *Dmrt1*, and *Zbtb16* in Si-NC and Si-Spry1 testes ($n$=3). B: Representative blots (left) and quantification (right) of SPRY1 protein levels in Si-NC and Si-Spry1 testes ($n$=3). C: Relative motility of sperm in Si-NC and Si-Spry1 testes ($n$=6). D: TNF-α, IL-6, AsAb, and GDNF levels in Si-NC and Si-Spry1 testes measured by ELISA ($n$=3). E: Heatmap depicting gene expression patterns in Si-NC vs. Si-Spry1 testes. Fold-change>2, up (red) or down (blue) with $P$<0.01. F: Volcano plot of DEGs determined by RNA-seq analysis of Si-NC vs. Si-Spry1 testes. Dotted line represents FDR cut-off 0.01. $n$=2 biologically independent samples per condition. G: Top 15 most enriched GO biological processes based on $P$-values for up-regulated and down-regulated genes in Si-NC and Si-Spry1 testes are shown. Red stars represent important signaling pathways focused on. H: Apoptosis ratio of Si-NC and Si-Spry1 testes for quantification of TUNEL staining. I: Relative mRNA levels of *Tnfα*, *Il1β*, *Tlr1*, *Cxcl1*, and *Cxcl10* in Si-NC and Si-Spry1 testes ($n$=3). *: $P$<0.05; **: $P$<0.01; ***: $P$<0.001.




**Supplementary Figure S4 *Spry1* inhibits LPS-induced immune response**

A: GO analysis of down-regulated DEGs (Left) and up-regulated DEGs (Right) based on Si-Dmrt1 and Si-Spry1 RNA-seq. B: Relative mRNA levels of *Tnfα* and *Spry1* in TM4 cells treated with LPS for different times ($n$=3). C: Typical pictures of PCDH, OeSpry1, U6, and shSpry1 TM4 cells. Scale bar: 100 μm. D: Relative mRNA levels of *Vim*, *Nfe2l2*, *Sox9*, *Tjp1*, *ZO1*, *Occludin*, and *Snail1* in U6 and shSpry1 cells ($n$=3). E: Relative mRNA levels of *Dmrt1*, *Tnfα*, *Tlr4*, *Il1β*, *Il6*, and *Nlrp3* in U6 and shSpry1 cells ($n$=3). Data are presented as means±SD and represent three independent repetitions. ns: not significant; *: $P$<0.05; **: $P$<0.01; ***: $P$<0.001.

**Supplementary Table S1 SiRNA sequences used in the study**

| Gene name | siRNA Sequence |
|---|---|
| Dmrt1-1 | GGGATTGCCAGTGCAAGAAGT |
| Dmrt1-2 | GCATGGTCATCCAGGATATTC |
| Dmrt1-3 | GCAGTCAAGATTCTGGCTTGG |
| Spry1-1 | GGCAGAGGTTAGACTATGACA |
| Spry1-2 | GCAGGTGTAGAAACTCCAACA |
| Spry1-3 | GCTACGATTCTGTCCCTAGAC |
| Negative control | TTCTCCGAACGTGTCACGT |

**Supplementary Table S2 Primers used for vector construction**

| Primer name | Sequence |
|---|---|
| U6-shDmrt1-F | GATCCGCATGGTCATCCAGGATATTCTCAAGAGGAATATCCTGGATGACCATGCTTTTTG |
| U6-shDmrt1-R | AATTCAAAAAGCATGGTCATCCAGGATATTCCTCTTGAGAATATCCTGGATGACCATGCG |
| U6-shSpry1-F | GATCCGCAGGTGTAGAAACTCCAACATCAAGAGTGTTGGAGTTTCTACACCTGCTTTTTG |
| U6-shSpry1-R | AATTCAAAAAGCAGGTGTAGAAACTCCAACACTCTTGATGTTGGAGTTTCTACACCTGCG |
| pCDH-Dmrt1-F | ACCTCCATAGAAGATTCTAGATGCCGAACGACGACACATT |
| pCDH-Dmrt1-R | GGAGCGATCGCAGATCCTTCGCTCACTCGTCCTCATCCTCTT |
| pCDH-Spry1-F | AGAATTCCACATGGATTCCCCAAGTC |
| pCDH-Spry1-R | TGGATCCAAGTCATGACAGTTTGCC |
| pGL3-Spry1-3UTR-F | GGGTACCCCTTCTGGAGGTGGGTTGGAC |
| pGL3-Spry1-3UTR-R | CAAGCTTGGAGCTATGCCAAAGGGGTGT |

| | |
|---|---|
| pGL3-Spry1-5UTR-F | GGGTACCCCGCGAGTGCTCAGCACGCAGGGG |
| pGL3-Spry1-5UTR-R | CCTCGAGGGTGGAGTGATCTCCAGTTCCAGC |
| VN173-FLAG-Spry1-FL-F | AGCTTGCGGCCGCGAATTCGATGGATTCCCCAAGTCAG CATGGCA |
| VN173-FLAG-Spry1-FL-R | ATGGATCTTCTAGAGTCGACTGACAGTTTGCCCTGAGC CCTTGAG |
| VN173-FLAG-Spry1-C-F | AGCTTGCGGCCGCGAATTCGTGCAAATGTGGAGAGTGT ACGGCCC |
| VN173-FLAG-Spry1-C-R | ATGGATCTTCTAGAGTCGACTGACAGTTTGCCCTGAGC CCTTGAG |
| VN173-FLAG-Spry1-N-F | AGCTTGCGGCCGCGAATTCGATGGATTCCCCAAGTCAG CATGGCA |
| VN173-FLAG-Spry1-N-R | ATGGATCTTCTAGAGTCGACCTTGCCACACTGTTCGCA GATGAAC |
| VC155-HA-NFκB1-FL-F | CCATGGAGGCCCGAATTCCGATGGCAGACGATGATCCC TACGGAA |
| VC155-HA-NFκB1-FL-R | CTCGAGAGATCTCGGTCGACATTTTGCCTTCAATAGGT CCTTCCT |
| VC155-HA-NFκB1-ANK-F | CCATGGAGGCCCGAATTCCGATGGGATTTCAGGATAAC CTCTTTC |
| VC155-HA-NFκB1-ANK-R | CTCGAGAGATCTCGGTCGACATTTTGCCTTCAATAGGT CCTTCCT |
| VC155-HA-NFκB1-RHD-F | CCATGGAGGCCCGAATTCCGATGGCAGACGATGATCCC TACGGAA |
| VC155-HA-NFκB1-RHD-R | CTCGAGAGATCTCGGTCGACATGTCCTGCTCCTTGTCTT CCATGG |

**Supplementary Table S3 Primers used for qRT-PCR**

| Gene | Forward sequence | Reverse sequence |
|---|---|---|
| Dmrt1 | AATGCCAGCAACCCG | GAGGAGGACTCAGCAGACA |
| Sox9 | AGTGCCCAAGCACATTTT | AACGCTGGTATTCAGGGA |
| Gdnf | CTTTGTGGCTGCTATCCC | GCGAAACGTGGGTCTTT |
| Ddx4 | CCGCATGGCTAGAAGAGA | AGAAGAAATCCCCGCTGT |
| Zbtb16 | TTTGTGCGATGTGGTCA | TTTTGGCGAGAGGAAGTC |
| Stra8 | CCTTCGCAGACCTCACC | TATGCTGGGCCTCACTCT |
| Sycp3 | GTTCAGCCAATCAGCAGAG | CACCAGGCACCATCTTTAG |
| Spry1 | GCCTACCCTGCTTGCTC | ACCCACCTCCAGAAGTCAT |
| Cldn11 | GCTTCGTGGGTTGGATT | GCCCAGTTCGTCCATTT |
| Tlr4 | CACTGGAACCTCATGCTTT | GATGCTCAACACCAAGGAA |
| Nlrp3 | CTTTATCCACTGCCGAGAG | AGCTCATCAAAGCCATCC |
| Tnfα | GACCCTCACACTCAGATCATCTTC | CACGTAGTCGGGGCAGCCTTG |
| Il1β | TGTCCTGATGAGAGCATCC | AAGGTCCACGGGAAAGAC |
| Ccl19 | GGGAAGCTCCGCAGTAT | CGCAACAGCTCCAGAAA |
| Ccl5 | CCACTCCCTGCTGCTTT | ACTTCTTCTCTGGGTTGGC |
| Cxcl1 | AACCGAAGTCATAGCCACA | GGGGACACCTTTTAGCATC |
| Cxcl2 | CCTCAACGGAAGAACCAA | AACATCTGGGCAATGGAA |
| Cxcl10 | AGCCGTGGTCACATCAG | ATCCCAGCCACTTGAGC |
| Gata4 | AGCAGGCAGAAAGCAAGG | GGGAGGGCGGACTCTATT |
| Taf4b | TCCCCAGTCCTTGTGTCT | GCTGTCGTACCTCCTGTTG |
| Sox30 | ACTTGTCCCCGAAGCAC | AGGTGGTGTCCCAAACAG |
| Adamtsl1 | CAAAGGGTCAAGGTCAGC | AGCAGCAAAGTCATTCGG |
| Tlr1 | TGCCACCCAACAGTCAG | GCATCTCCTAACACCAGCA |
| Cxcl13 | GGCATTTAGTGACAACCCA | GTCCATCTCGCAAACCTC |
| Il6 | ACAGAAGGAGTGGCTAAGGA | AGGCATAACGCACTAGGTTT |
| Occludin | CCTACTCCTCCAATGGCAAA | CTCTTGCCCTTTCCTGCTTT |
| Tjp1 | ACCATGCCTAAAGCTGTCC | GGAACTCAACACACCACCA |
| ZO1 | ACGCATCACAGCCTGGTTG | TGGCTCCTTCCTGTACACC |
| Snail1 | TTCCAGCAGCCCTACGA | ATCGGACAGCGAGGTCA |
| Nfe2l2 | GATGGACTTGGAGTTGCC | CCTTCTGGAGTTGCTCTTG |
| Vim | CTCCTACGATTCACAGCCA | GAGCCACCGAACATCCT |
| GAPDH | CGTGTCCGTTGTGGATCTGA | TGAAGTCGCAGGAGACAACC |
| Actin | CCTCACTGTCCACCTTCC | GGGTGTAAAACGCAGCTC |