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Supplemental Information

MicroRNAs for Fine-Tuning of Mouse Embryonic Stem Cell Fate Decision through Regulation of TGF-β Signaling

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Supplemental Data

I. Supplemental Figures and Figures Legends

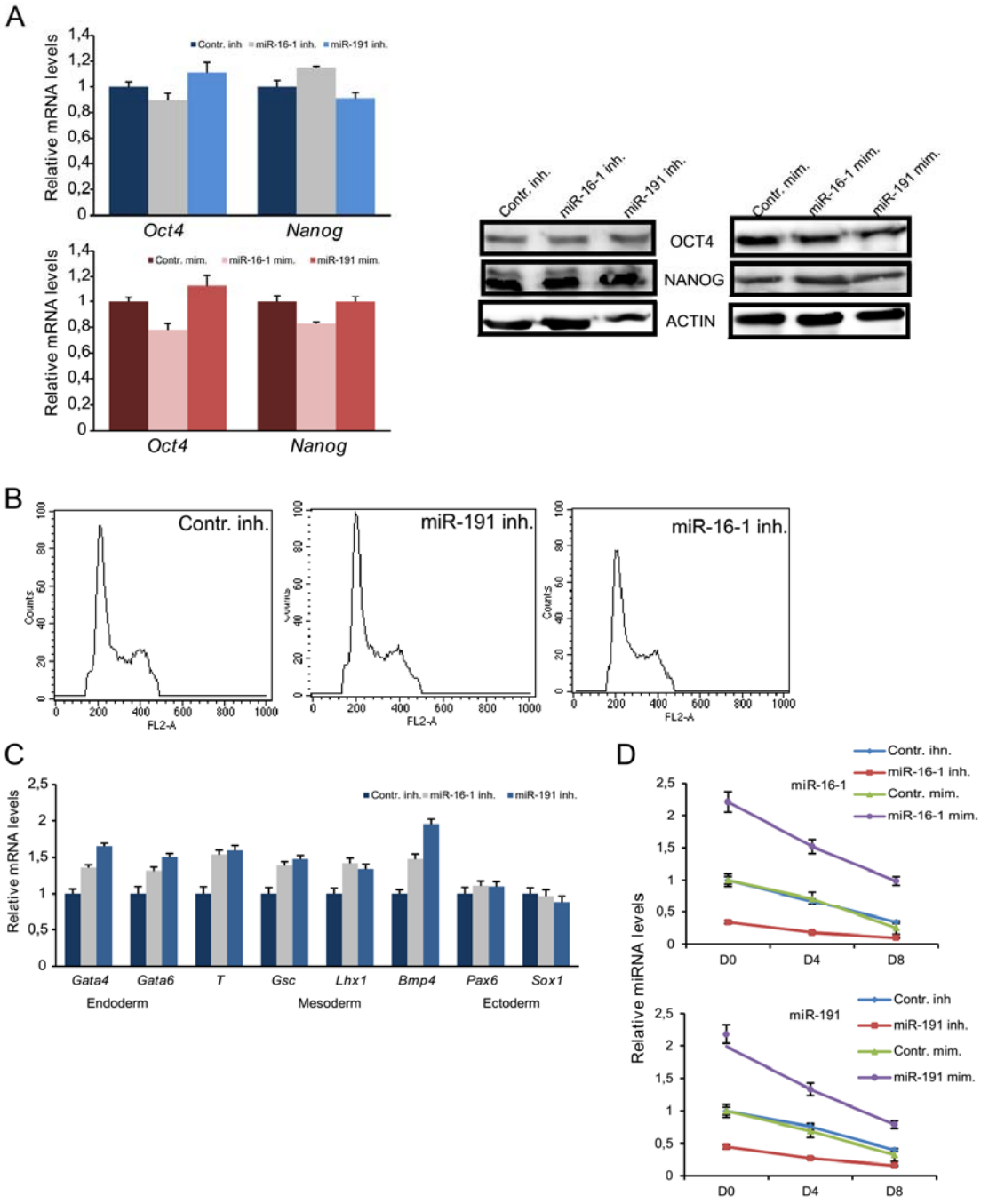

Figure S1 (related to Figure 2)

(A) mRNA and protein levels of stemness markers upon the inhibition or overexpression of miR-16-1/miR-191 in mESCs.

(B) Analysis of cell cycle distribution of mESCs transfected with miR-16-1 and miR-191 inhibitors compared to control.

(C) Relative mRNA expression levels of differentiation markers in miR-16-1 and miR-191-inhibited mESCs. Error bars indicated +SD of three independent experiments (n=3).

(D) Relative miRNA expression levels during EB differentiation of mESCs transfected with miR-16-1/miR-191 inhibitors or mimics compared to controls. Error bars indicated +/-SD of three independent experiments (n=3).

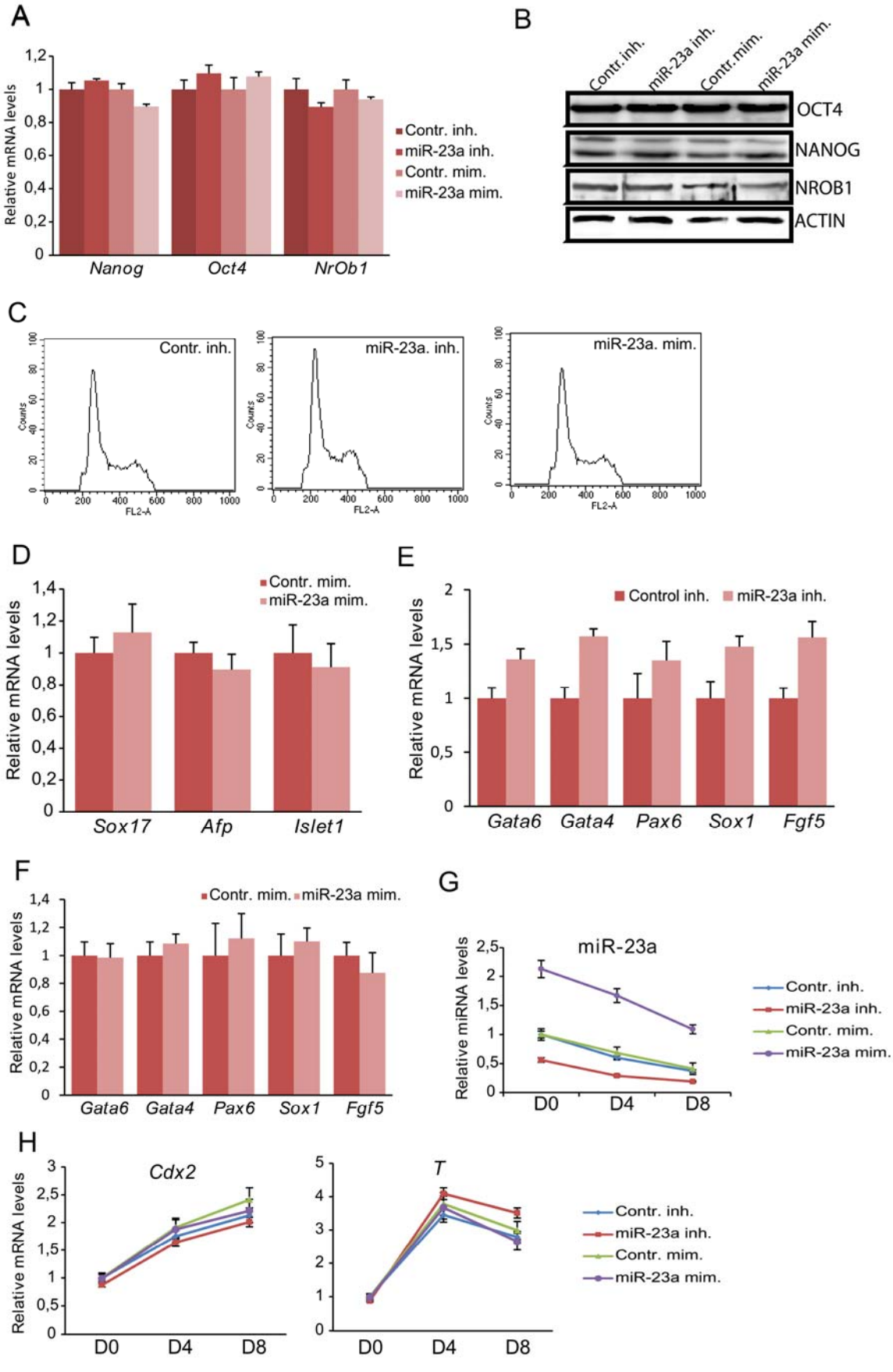
A
Relative mRNA levels
1,2
1
0,8
0,6
0,4
0,2
0
Nanog
Oct4
NrOb1
Contr. inh.
miR-23a inh.
Contr. mim.
miR-23a mim.
B
Contr. inh.
miR-23a inh.
Contr. mim.
miR-23a mim.
OCT4
NANOG
NROB1
ACTIN
C
Contr. inh.
miR-23a. inh.
miR-23a. mim.
Counts
FL2-A
D
Relative mRNA levels
Contr. mim.
miR-23a mim.
Sox17
Afp
Islet1
E
Relative mRNA levels
Control inh.
miR-23a inh.
Gata6
Gata4
Pax6
Sox1
Fgf5
F
Relative mRNA levels
Contr. mim.
miR-23a mim.
Gata6
Gata4
Pax6
Sox1
Fgf5
G
miR-23a
Relative miRNA levels
D0
D4
D8
Contr. inh.
miR-23a inh.
Contr. mim.
miR-23a mim.
H
Cdx2
T
Relative mRNA levels
D0
D4
D8
Contr. inh.
miR-23a inh.
Contr. mim.
miR-23a mim.

Figure S2 (related to Figure 3)

(A, B) Relative mRNA (A) and protein (B) levels of pluripotency factors (Oct4, Nanog, NrOb1) in response to miR-23a inhibitor or mimic. Error bars indicated +SD of three independent experiments (n=3).

(C) Cell cycle distribution of miR-23a inhibited/overexpressed mESCs compared to control.

(D) Relative mRNA expression levels of the indicated differentiation genes upon miR-23a overexpression. Error bars indicated +SD of three independent experiments (n=3).

(E, F) Relative mRNA levels of genes associated with the three germ layers in miR-23a inhibited or overexpressed mESCs. Error bars indicated +SD of three independent experiments (n=3).

(G) Relative miRNA expression levels of miR-23a inhibited or overexpressed mESCs upon differentiation compared to controls. Data shown as mean +/- SD of three independent experiments (n=3).

(H) RT-PCR indicating the levels of characteristic trophectoderm and mesoderm genes (*Cdx2*, *T*) at EBs D0, D4 and D8, upon miR-23a overexpression or inhibition. Error bars indicate +/- SD of three independent experiments (n=3).

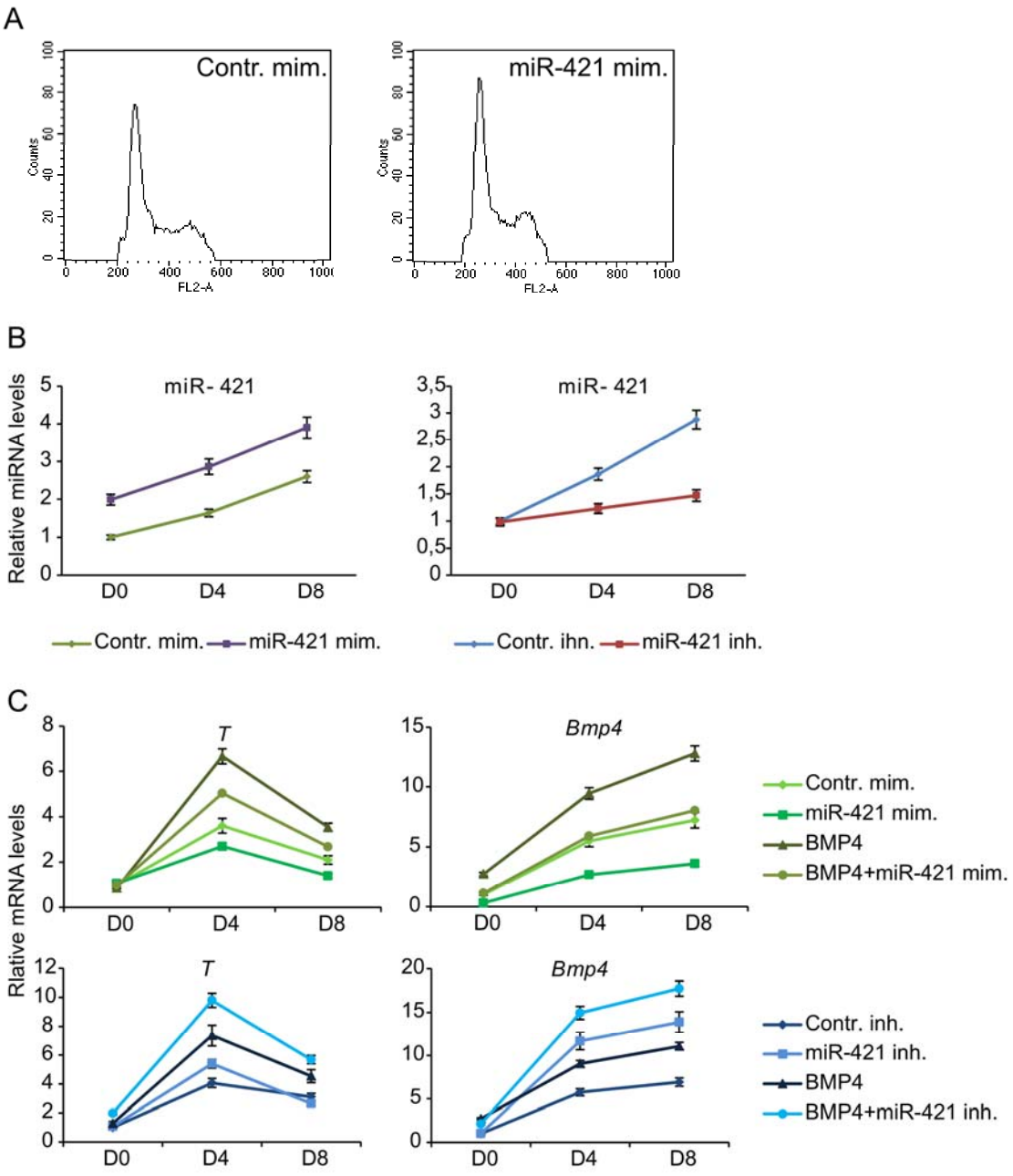

Figure S3 (related to Figure 4)

(A) Cell cycle analysis of mESCs before and after miR-421 overexpression.

(B) Relative miRNA expression level of miR-421 overexpressed and inhibited mESCs during EB differentiation compared to control. Error bars indicated +/- SD of three independent experiments (n=3).

(C) RT-PCR indicating the levels of characteristic mesoderm genes (*T, Bmp4*) at EBs D0, D4 and D8, upon miR-421 overexpression or inhibition. Error bars indicate +/-SD of three independent experiments (n=3).

II. Supplemental Experimental Procedures

Library Preparation-Sequencing

Small RNAs (smRNAs) were extracted from mESCs and EBs D8 using mirVana™ miRNA Isolation Kit (Ambion). The concentrations of smRNAs were determined using NanoDrop ND-1000 Spectrophotometer and the size and purity were assessed using the Bioanalyzer 2100 (Agilent Technologies). Approximately 100ng of miRNA was used for library constructions following the protocol for the Ion Torrent Total RNA-Seq Kit according to the manufactures' instructions (ThermoFisher Scientific). Final libraries were used for sequencing analysis on the Ion Torrent PGM instrument using 316 chips.

Analysis

Sequencing was performed for 18–30-nt small RNA libraries using the Ion Torrent PGM platform and produced a total of more than 3Mi reads after QC and annotation was performed against miRBase (Release 21). Downstream analysis of relative miRNA abundance was conducted with the use of the CLC Workbench Suite (CLC Bio, Qiagen, **http://www.clcbio.com**). Samples were normalized using quantile normalization and subsequently transformed to log2 values to normalize variation across orders of magnitude. Differential expression was calculated on the basis of a paired t-test and corrected for multiple hypotheses under an FDR of 5%.

Reverse transcription and Real-time PCR for miRNAs analysis

Total RNA was extracted from mESCs and differentiating EBs D8 with TRIzol Reagent (Invitrogen). Reverse transcription was done using the Universal cDNA synthesis kit (Exiqon) with 20 ng total RNA per 10 µl reaction and real-time PCR was performed with microRNA LNA PCR primer sets (Exiqon), according to the manufacturer's protocol. The miRNA expression was normalized against small nuclear RNA, U6.

Real-time PCR for mRNA

Total RNA was isolated using TRIzol Reagent (Invitrogen). cDNA was subsequently reverse-transcribed from mRNA by M-MLV Reverse Transcriptase (Takara). Target genes expression was normalized against *Actin*. The primers are shown below:

Actin FOR: 5' GTGTGACGTTGACATCCGTA 3'

Actin REV: 5' GTAACAGTCCGCCTAGAAGC 3'

Afp FOR: 5’ AAGCTGCGCTCTCTACCACCAGA 3’

Afp REV: 5’ ACCACAGCCGGACCATT 3’

Bmp4 FOR: 5’ TTCCTGGTAACCGAATGCT 3’

Bmp4 REV: 5’ AAGTGTCGCCTCGAAGTC 3’

Eomes FOR: 5’ GCTTCCGGGACAACTACGA 3’

Eomes REV: 5’ GAGAGGAGGCCGTTGGTCT 3’

Fgf5 FOR: 5’ GCAGAAGTAGCGCGACGTTT 3’

Fgf5 REV: 5’ TTGACTTTGCCATCCGGGTAG 3’

Flk1 FOR: 5’ GGATGGAGGCCTCTACACC 3’

Flk1 REV: 5’ TGCCGACGAGGATAATGAC 3’

Gata4 FOR: 5’ GCCAACTGCCAACTACCAC 3’

Gata4 REV: 5’ GACCTGCTGGCGTCTTAGA 3’

Gata6 FOR: 5’ GCCACTGTGGAGACGAGA 3’

Gata6 REV: 5’ CATATAGAGCCCCGAAGCA 3’

Gsc FOR: 5’ TGCTGCCCTACATGAACGTG 3’

Gsc REV: 5’ CTCCAGGGCTTCGAGCTG 3’

Id1 FOR: 5’ GACTACATCAGGGACCTGCAGC 3’

Id1 REV: 5’ GGCCGCCAAGGCACTGATCTCG 3’

Id2 FOR: 5’ ATCCCCCAGAACAACAAGGT 3’

Id2 REV: 5’ ACCTTCTTGTTCTGGGGGAT 3’

Id3 FOR: 5’ CCAGGTGGAAATCCTGCACC 3’

Id3 REV: 5’ CTCTTGTCCTTGGAGATCACAA 3’

Islet1 FOR: 5’ GCAGCAGCAACCCAACGA 3’

Islet1 REV:5’ TTTGCAAGGCGAAGTCAC 3’

Nanog FOR: 5’ CGCTGCTCCGCTCCATAACT 3’

Nanog REV: 5’ GCGCATGGCTTTCCCTAGTG 3’

Nr0b1 FOR: 5’ CTGGTGTGCAGCGTCTGA 3’

Nr0b1 REV: 5’ GTGTTGGTCTCCGGATCTC 3’

Pax6 FOR: 5’ GGTGCTGGACAATGAAAACA 3’

Pax6 REV: 5’ GGTACAGACCCCCTCGGATAA 3’

Pou5f1/Oct4 FOR: 5’ CCCTGGGCGTTCTCTTTGGA 3’

Pou5f1/Oct4 REV: 5’ ACCAGGGTCTCCGATTTGCAT 3’

Sox1 FOR: 5’ GAAGCGGCCGTTCATC 3’

Sox1 REV: 5’ TCCTTCTTGAGCAGCGTCT 3’

Sox17 FOR: 5’ CTCTGCCCTGCCGGGATGG 3’

Sox17 REV: 5’ AATGTCGGGGTAGTTGCAATA 3’

T FOR: 5’ GTTCCCGGTGCTGAAGGTAAAT 3’

T REV: 5’ GCGAGTCTGGGTGGATGTAGA 3’

Transfection

mESCs were plated to 60-70% confluence in medium one day before transfection. Mimics and inhibitors (QIAGEN) were transfected into the cells at a final concentration of 50nM, using Lipofectamine 2000 transfection reagent (Invitrogen). Negative controls (inhibitor or mimic with no homology to any known mammalian mRNA or miRNA) were also transfected. Cells were incubated at 37°C, 5% CO_2 for 72 h and harvested for protein extraction or total RNA (TRIzol; Invitrogen). For long-term experiments, cells were transfected every two days.

Luciferase Assay

For the 3’ UTR luciferase assay, at least 300bp of the 3’UTR of the target genes was inserted downstream of the firefly luciferase gene in the pGL3 vector in XhoI and BamH1 restriction sites. The following primers were used for the 3’UTR amplification:

Afp FOR: 5'ATTCTCGAGACATCTCCAGAAGGAAGAGTG 3'

Afp REV: 5' AATGGATCCAATGGAAAAAAGTATTTTTTA 3'

Id2 FOR: 5' ATTCTCGAGATAAATGGCATTTGGGGACTTT 3',

Id2 REV: 5' ATTGGATCCTTTTATTATAATCTTAATACAG 3'

Islet1 FOR: 5' AAACTCGAGGAAGAGCAGAAACAGAGA 3'

Islet1 REV: 3' AATGGATCCTGCGTTTATTTAATTATCTAT 3'

Pou5F1/Oct4 FOR: 5' ATTCTCGAGACTGAGGCACCAGCCCTCCCT 3'

Pou5F1/Oct4 REV: 5'ATTGGATCCAGCTATCTACTGTGTGTCCCA 3'

Smad2 FOR: 5' ATTCTCGAGATGATGTCTTGTTGGGCATAA 3'

Smad2 REV: 5' AATGGATCCTTTGTTTATTTTTGGTAAAAG 3'

Smad5 FOR: 5' ATTCTCGAGTGCAGAAGTATTCTTTCAACT 3'

Smad5 REV: 5' ATTGGATCCAATTAGAGTTCTTGAATTGTT 3'

Sox17 FOR: 5'ATTCTCGAGCCAGCAGTGTTACACACT 3'

Sox17 REV: 5'AATGGATCCTGACTCTAAAATTGTAGGAAT 3'

Mutation in the miRNA binding sites of targets was obtained by replacing the miRNA binding site sequence with miRNA seed sequences using the QuickChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies). HEK293T cells were transfected with the wild type or mutant 3'UTR of target genes (1µg) in the presence of miRNAs mimcs or controls. Cells were harvested 48h post-transfection and luciferase activity was measured using dual-luciferase reporter assay system (Promega). CGR8 cells were transfected with ARE-Luc and BRE-Luc reporter plasmids using Lipofectamine 2000 following the manufacture's protocol. Cells were stimulated with Activin A (25ng/ml) or SB431542 (10µM) and BMP4 (80ng/ml) respectively, 16h before harvesting for luciferase assay. pARE-lux was a gift from Joan Massague & Jeff Wrana (Addgene plasmid # 11768).

Antibodies

Proteins were detected by primary antibodies against Oct3/4 (sc-5279, Santa Cruz), Islet-1 (sc-23590, Santa Cruz), Sox17 (sc-17356, Santa Cruz), Afp (2137S, Cell Signaling), Nanog

(8600S, Cell Signaling), NrOB1 (sc-13034X, Santa Cruz), p-Smad2/3 (3101, Cell Signaling), Actin (sc-47778, Santa Cruz), Smad2 (sc-8332, Santa Cruz) and Smad5 (sc-7443, Santa Cruz).

Flow Cytometry

For cell cycle distribution, 100.000 cells from each sample were trypsinized, washed with PBS, treated with RNAse A for 20 min at 37 ^{0}C and stained with propidium iodide (PI-Sigma) according to the manufacturer's protocol. The intensity of fluorescence was measure with flow cytometer.

Statistical analyses

Student's t-test was used for all statistical analyses. Statistical significance was defined as follows: * means $p < 0.05$; ** means $p < 0.01$. Values were presented as the mean ± SD.