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

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SUMMARY

Over the past years, microRNAs (miRNAs) have emerged as crucial factors that regulate self-renewal and differentiation of embryonic stem cells (ESCs). Although much is known about their role in maintaining ESC pluripotency, the mechanisms by which they affect cell fate decisions remain poorly understood. By performing deep sequencing to profile miRNA expression in mouse ESCs (mESCs) and differentiated embryoid bodies (EBs), we identified four differentially expressed miRNAs. Among them, miR-191 and miR-16-1 are highly expressed in ESCs and repress *Smad2*, the most essential mediator of Activin-Nodal signaling, resulting in the inhibition of mesendoderm formation. miR-23a, which is also down-regulated in the differentiated state, suppresses differentiation toward the endoderm and ectoderm lineages. We further identified miR-421 as a differentiation-associated regulator through the direct repression of the core pluripotency transcription factor *Oct4* and the bone morphogenetic protein (BMP)-signaling components, *Smad5* and *Id2*. Collectively, our findings uncover a regulatory network between the studied miRNAs and both branches of TGF-β/BMP-signaling pathways, revealing their importance for ESC lineage decisions.

INTRODUCTION

Embryonic stem cells (ESCs), derived from pre-implantation embryos, share two unique properties: the ability to grow indefinitely in culture and to differentiate into all cell types (Evans and Kaufman, 1981). ESC self-renewal is regulated by a complex network of transcription factors and signaling pathways (Ng and Surani, 2011). The transforming growth factor β (TGF-β) pathway plays a pivotal role in cell fate determination during mouse embryonic development, such as primitive streak formation (Oshimori and Fuchs, 2012). Both Smad1/5/8 and Smad2/3 branches are involved in pluripotency and differentiation of ESCs. Activin/Nodal/Smad2/3 signaling is important for proper differentiation toward the mesendoderm lineage (Fei et al., 2010), whereas bone morphogenetic protein (BMP)/Smad1/5/8 signaling promotes self-renewal in mouse ESCs (mESCs) (Ying et al., 2003).

Accumulating evidence reveals that microRNAs (miRNAs) are crucial in controlling the pluripotent stem cell state. Their important regulatory role in mouse and human ESCs has been identified using Dicer and DGCR8 knockout mice. Dicer and DGR8 deletion resulted in embryonic lethality (Bernstein et al., 2003), while DGCR8-deficient mESCs were viable but defective in proliferation and differentiation (Wang et al., 2007). Several studies reported on miRNAs maintaining the ESC state, whereas others reported miRNAs as promoting differentiation. miR-290–295 and miR-302–367 clusters include the most abundant miRNAs in mouse and human ESCs and are characterized as ES cell-specific cell cycle miRNAs (Gangaraju and Lin, 2009; Melton et al., 2010). In contrast, miR-134, miR-296, and miR-470 are related to ESC differentiation and self-renewal silencing (Tay et al., 2008). Although there is no doubt that miRNAs regulate ESC self-renewal and lineage commitment, their role in relevant signaling pathways that determine ESC function remains unclear.

In this study, we report the identification of four miRNAs as critical regulators of ESC fate. miR-16-1 (miR-16-1/15a cluster) and miR-191 (miR-191/425 cluster), which are highly expressed in mESCs, directly target *Smad2*, an Activin/Nodal signaling important mediator, leading to the inhibition of mesendoderm lineage. Another miRNA expressed in the undifferentiated state, miR-23a (miR-27/24a/23a cluster), inhibits the endodermal and ectodermal differentiation. On the contrary, miR-421 (miR-421/374b/c cluster) was identified as a differentiation regulator, by suppressing BMP signaling and the critical pluripotency factor, *Oct4*. Altogether, the mechanisms incorporating the two branches of TGF-β signaling pathway and miRNAs are highlighted, unraveling their importance to ESC lineage commitment.

RESULTS AND DISCUSSION

Global miRNA Analysis of mESCs and Day-8 Embryoid Bodies

To identify miRNAs pivotal for ESC function and biology, we performed a global miRNA analysis from mESCs and

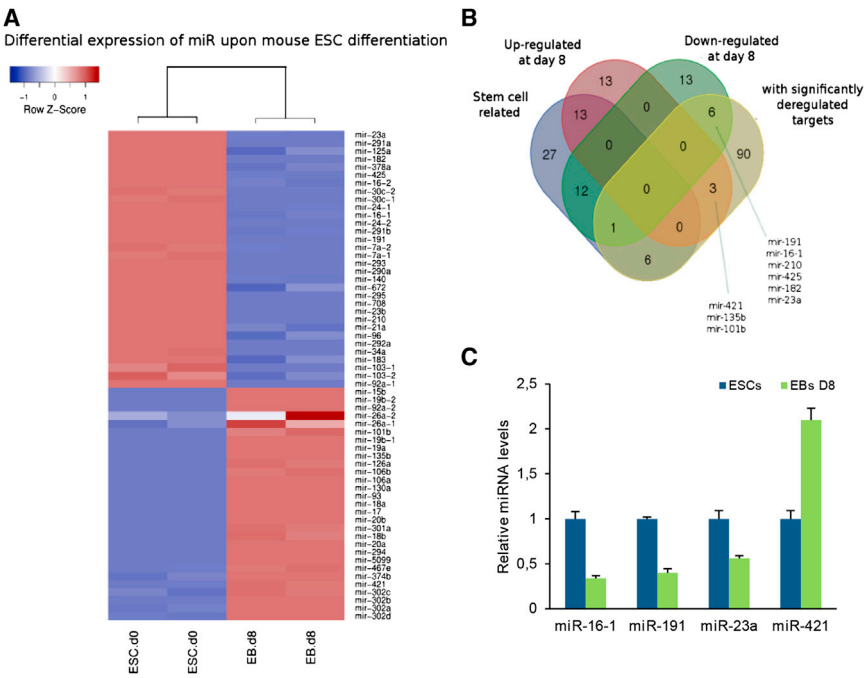

Figure 1. miRNA Profiling of mESCs and Differentiated Cells

(A) Heatmap of log2-transformed miRNA abundances in replicate samples of undifferentiated ESCs and differentiated cells (EBs D8).

(B) Venn diagram showing common miRNAs between 59 stem cell-related miRNAs based on literature compiled from miRbase, our 32 down- and 29 up-regulated miRNAs, and 107 miRNAs whose mRNA targets were found to be significantly deregulated between days 0 and 9 in an independent study. Short lists of primary miRNA candidates for down- and up-regulated species are included.

(C) The RT-PCR verification of the four selected miRNA levels. Data are shown as mean ± SD of three independent experiments.

day-8 embryoid bodies (EBs D8). Although the analysis revealed a large number (442) of differentially expressed miRNAs (Table S1), we restricted it by narrowing it down to highly abundant miRNAs. Thus, a total of 61 miRNAs with high abundance at either time point (D0 or D8) was further analyzed in terms of relative expression, relationship to the developmental process, and expression of their target genes. Of the 61 differentially expressed miRNAs, 32 were down- and 29 were up-regulated at D8, with this behavior being fairly consistent between replicates (Figure 1A). Among them, well-studied miRNAs crucial for pluripotent state were identified, such as miR-290–295 and miR-302 clusters (Gangaraju and Lin, 2009; Melton et al., 2010). In addition, those 61 miRNAs overlapped with previous published data for ESCs and EBs D5 or D7 (Table S2) (Lewis et al., 2005; Lee et al., 2011).

Screening the literature for mouse miRNAs (miRBase, Rel. 21), a list of 59 miRNAs reported to be implicated in the ESC differentiation process (Kozomara and Griffiths-Jones, 2014) was obtained (Table S3). Comparing the above list with our deregulated miRNAs, 43% of them were identical (26 of 61). However, we focused on the remaining miRNAs (57%), which have not been previously involved in ESC identity (Table S3).

Following a different approach, we performed a combination of in silico target analysis coupled with gene-expression data. Predicted miRanda (Betel et al., 2008) and TargetScan (Lewis et al., 2005) miRNA targets were gathered to form a concise table of genes targeted by our differentially expressed miRNAs. We obtained expression values of mRNA genes from a genome-wide expression profiling of mESC differentiation (Hailesellasse Sene et al., 2007). Scanning the list of all miRNA measured in our study, we obtained the mean log (fold change) of mRNA expression between D9 and D0 in the aforementioned study. By comparing this value for each of the miRNA targets with the overall mean of expression change, we pinned down 106 miRNAs whose targets were significantly deregulated during differentiation. The intersection of these 106 miRNAs with our deregulated ones, not reported to be related to stem cell differentiation, led to two short lists containing three up-regulated and six down-regulated miRNAs (Figure 1B). After further searching the literature for the predicted targets of selected miRNAs and following validation of the expression level changes, we ended up with four miRNAs. miR-16-1, miR-191, and miR-23a are down-regulated upon differentiation, whereas miR-421 is up-regulated in EBs D8 (Figure 1C).

miR-16-1 and miR-191 Inhibit Mesendoderm Differentiation by Targeting Activin/Smad2 Signaling Pathway

To evaluate the functional role of miR-16-1 and miR-191 in mESCs we used miR-16-1, miR-191 inhibitors, or miR-16-1, miR-191 mimics (Figure 2A), and examined their impact on self-renewal and pluripotency. Neither the inhibition nor the overexpression of these miRNAs caused any changes at the expression levels of *Oct4* and *Nanog* (Figure S1A). In addition, no effect on mESC morphology (data not shown) and cell cycle (Figure S1B) was observed. These data suggest

that miR-191 and miR-16-1 do not play a crucial role in mESC self-renewal.

We next examined the potential effect of miR-191 and miR-16-1 on the induction of differentiation markers in the undifferentiated state. We found that after 72 hr of their inhibition, characteristic endodermal (*Gata4*, *Gata6*) and mesodermal (*T*, *Gsc*, *Lhx1*, *Bmp4*) markers were slightly up-regulated, whereas ectodermal (*Pax6*, *Sox1*) markers did not seem to be affected (Figure S1C). Conversely, miR-16-1 and miR-191 overexpression did not exert changes on lineage markers compared with negative control mimic (data not shown).

To study the mechanism by which these miRNAs regulate mESC differentiation, we focused on their targets. *Smad2* mRNA is predicted to have binding sites for miR-16-1 and miR-191 (Figure 2B). Since it is known that Activin/Smad2 signaling is crucial for mesoderm and endoderm development in vivo (Moustakas and Heldin, 2009) and mESC differentiation in vitro (Fei et al., 2010), we hypothesized that miR-16-1 and miR-191 may compete with Activin/Smad2 signaling. To analyze whether *Smad2* is a direct target of these miRNAs, we performed luciferase reporter assays using constructs that harbor wild-type (WT) or mutant (MUT) 3′ UTR of *Smad2*. We found that either miR-191 or miR-16-1 suppressed the WT but not MUT 3′ UTR reporter activity, and a combination of both miRNAs led to higher levels of suppression (Figure 2C).

To examine whether miR-16-1 and miR-191 interfere with Activin/Smad2 signaling, we employed the Activin Response Element reporter (pARE-Lux) in mESCs and analyzed the effect of a mixture of miR-16-1/miR-191 mimics on the activity upon stimulation with 25 ng/ml activin A. Whereas activin A enhanced the reporter activity, simultaneous addition of 10 μM SB431542 (an inhibitor of activin receptors) abolished the effect. Interestingly, the combined miR-16-1/miR-191 mimics inhibited the activation of the reporter by 47% (Figure 2D). To further confirm that miR-16-1 and miR-191 influenced Activin/Smad2 signaling, we examined the effect on SMAD2 and p-SMAD2 protein levels. miR-16-1/miR-191 knockdown mESCs had higher levels of SMAD2 and p-SMAD2, while mESCs transfected with miR-16-1/miR-191 mimics exhibited lower levels compared with controls (Figure 2E). These data reinforced the hypothesis that miR-16-1 and miR-191 diminish the activity of Activin/Smad2 signaling through *Smad2* downregulation.

To examine whether the aforementioned miRNAs affect the mESC differentiation program, we transfected mESCs with a mixture of miR inhibitors or mimics and induced them to differentiate. As a control, mESCs treated with activin A or SB431542 was used. The efficiency of miR-16-1, miR-191 knockdown or overexpression (Figure S1D), as well as the expression of several lineage markers, was measured at EBs D0, D4, and D8. The induction of mesodermal (*T*, *Gsc*) and endodermal (*Gata4*, *Gata6*) markers were up-regulated upon inhibition of miR-16-1 and miR-191 (Figure 2F). Activin A caused an increase of mesodermal markers (*T*, *Gsc*) and the endodermal marker *Gata4* while *Gata6* was not affected, in line with previously published data (Lee et al., 2011). The significant increase of *Gata6* induction by the addition of miR inhibitors may be attributed to *Smad2* up-regulation (Fei et al., 2010). In contrast, the *Sox1* ectodermal marker showed no significant changes (Figure 2F). Conversely, miR mimics reduced endoderm and mesoderm induction, similarly to the activity of SB431542 (Figure 2G). Contrary to miR mimics, SB431542 increases *Gata6* induction (Lee et al., 2011). Interestingly, due to the alteration of *Smad2* expression levels, the induction of trophectoderm marker (*Cdx2*) was significantly elevated by the miR inhibitors and lowered by the miR mimics (Figures 2F and 2G), while it remained unaffected by activin A and SB431542 (Fei et al., 2010; Lee et al., 2011).

Based on the above data, we conclude that miR-191 and miR-16-1 repress mesendoderm differentiation of mESCs through direct targeting of *Smad2* and subsequent post-transcriptional control of Activin/Nodal signaling. In different settings, miR-16-1 and miR-191 are reported to regulate cell proliferation and/or cell cycle. In detail, miR-191 acts mainly as an oncomiR, but can also serve as a tumor suppressor (Nagpal and Kulshreshtha, 2014). miR-16-1 has a well-defined tumor-suppressor and cell cycle-arresting role in leukemia (Pekarsky and Croce, 2015). Our data revealed that these miRNAs did not affect the ESC cell cycle, and this difference may be attributed to the peculiar ESC cell cycle profile. It would be interesting to investigate whether Activin signaling is also involved in the tumor-regulatory functions of these miRNAs.

miR-23a Represses Ectoderm and Endoderm Differentiation of mESCs

To gain insights into the potential role of miR-23a in mESCs, we used an miR-23a inhibitor and an miR-23a mimic (Figure 3A). To assess the influence of miR-23a on mESC self-renewal, we analyzed the expression levels of stemness markers (*Oct4*, *Nanog*, *Nr0b1*) in mESCs transfected with miR-23a inhibitor or mimic, but no difference compared with the controls was observed (Figures S2A and S2B). Furthermore, miR-23a inhibition or overexpression did not cause any changes in mESC morphology (data not shown) or cell cycle (Figure S2C).

Following in silico research, we identified three differentiation markers, *Afp*, *Sox17*, and *Islet1*, that were predicted to be targets of miR-23a (Figure 3B). Indeed, compared with controls, their protein and mRNA expression levels were induced in mESCs transfected with miR-23a inhibitor

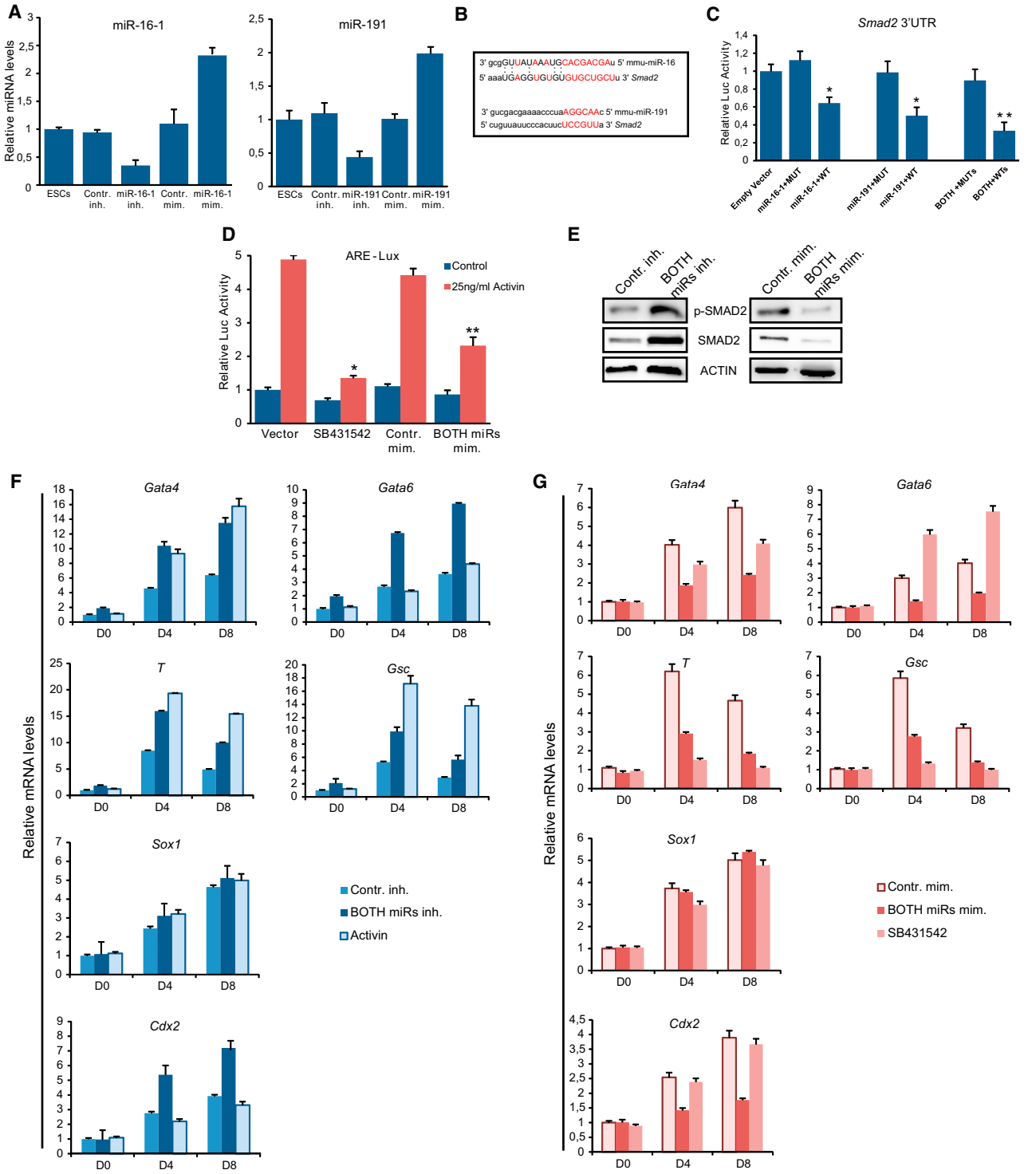

Figure 2. miR-16-1 and miR-191 Antagonize Activin/Smad2 Signaling in mESCs and Repress Mesendoderm Differentiation

(A) Measurement of miR-16-1 and miR-191 levels by RT-PCR after transient transfection with miR mimics or inhibitors. Error bars indicate SD of three independent experiments.

(B) miR-16-1 and miR-191 target sites in the 3′ UTR of *Smad2*. Red indicates complementarity between miRNA and the target gene. Error bars indicate SD of three independent experiments.

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(Figures 3C and 3D), while remained constant in overexpressing miR-23a mESCs (Figure S2D). Next, through a luciferase reporter assay, we verified the direct link between miR-23a and the three differentiation markers. To verify the specificity of miR-23a binding to *Sox17*, we used a mutated 3′ UTR (Figure 3E). In addition, two endodermal (*Gata6*, *Gata4*) and three ectodermal (*Pax6*, *Sox1*, *Fgf5*) markers were up-regulated 72 hr after miR-23a inhibition (Figure S2E), whereas no effect was detected on their levels in miR-23a mimic-transfected mESCs (Figure S2F).

Sox17 has been previously reported to drive the up-regulation of the primitive endoderm-associated program, giving rise to endodermal progenitors (Niakan et al., 2010). The suppression of *Sox17* and *Afp*, another endoderm marker gene, by miR-23a reinforces the hypothesis that miR-23a inhibits differentiation toward this lineage.

To test this assumption, we allowed mESCs transfected by miR-23a inhibitor or mimic to differentiate as EBs. miR-23a inhibition or overexpression was verified on EBs D0, D4, and D8 (Figure S2G). A significant increase in the induction of endodermal (*Afp*, *Sox17*, *Gata6*, *Gata4*) and ectodermal (*Islet1*, *Fgf5*, *Sox1*) genes was observed (Figure 3F) upon miR-23a inhibition, whereas trophectoderm and mesoderm lineage markers were not affected (Figure S2H). Interestingly, in miR-23a overexpressing mESCs the differentiation toward these lineages is suppressed, suggesting that the expression level of miR-23a is critical for pluripotency maintenance (Figure 3F).

The above results clearly show that miR-23a is an additional regulator of ESC differentiation. Recently, the miR-23a/24-2/27a cluster has been reported to be regulated by BMP4 and target *Smad5* to protect mESCs from apoptosis during the transition to epiblast stem cells (Musto et al., 2014). In addition, miR-23a inhibits the osteoblast differentiation by targeting *Runx2* (Hassan et al., 2010). In line with these observations, our results strongly support that miR-23a is a pivotal regulator of differentiation and controls ESC-specific germ-layer commitment and subsequent lineage decisions.

With respect to cancer, miR-23a has been considered either as an oncomiR (Chhabra et al., 2010) or a tumor suppressor (He et al., 2014). Apoptosis, migration, and invasion are some of its effects in cancer through regulation of molecular targets (*PTEN*, *DAPP*), while TGF-β/BMP has been implicated in the control of miR-23a expression in human cancers (Chandran et al., 2014).

In conclusion, miR-23a has a role in both tumor progression and mESC function, and the cross-regulatory relationship with TGF-β/BMP signaling awaits further investigation.

miR-421 Regulates Distinct Fate Choices of ESCs through Oct4 Repression and Competition with BMP Signaling

In contrast to the above miRNAs, miR-421 was identified as a differentiation-associated regulator, and its expression level was up-regulated during EB formation.

To study whether miR-421 is a crucial player in controlling differentiation, we ectopically expressed miR-421 in mESCs by using its mimic (Figure 4A). Compared with the control, miR-421 mimic had no effect on cell morphology (data not shown) and cell cycle progression (Figure S3A), but its addition significantly reduced the *Oct4* expression levels (Figure 4B) while other pluripotency genes remained constant. Using bioinformatics tools (Miranda, TargetScan), miR-421 was predicted to bind the *Oct4* 3′ UTR, and the direct link between the two was further confirmed by luciferase reporter assay (Figure 4C).

To test the effect of miR-421 overexpression on differentiation, we analyzed the expression levels of several lineage markers. Interestingly, the trophectoderm marker *Cdx2* was up-regulated (Figure 4D), in agreement with previous studies showing the repression of trophectoderm by *Oct4* (Strumpf et al., 2005). Moreover, miR-421 overexpression was accompanied by a slight induction of primitive endoderm markers (*Gata4*, *Gata6*, *Afp*), which is consistent with previously published data analyzing the changes of gene expression upon inhibition of *Oct4* (Hay et al., 2004; Strumpf et al., 2005). Interestingly, ectoderm-associated markers (*Pax6*, *Sox1*) were also up-regulated (Figure 4D), indicating that miR-421 might exert its action through an additional mechanism.

Due to the fact that several components of BMP signaling were predicted as candidate targets of miR-421 (*Bmpr1*, *Smad5*, *Id2*) (Figure 4E), we hypothesized that miR-421 may regulate this signaling and, thereby, lineage specification. Since the BMP pathway plays an important role in maintaining mESCs in the pluripotent state (Ying et al.,

(C) miR-16-1 and miR-191 specifically repress their target in the luciferase assay. Data are shown as mean ± SD of four independent experiments. *p < 0.05 **p < 0.01.

(D) Ectopic expression of miR-16-1/miR-191 inhibits ARE-luc activity. Data are shown as mean ± SD of four independent experiments. *p < 0.05 **p < 0.01.

(E) Total SMAD2 and p-SMAD2 protein levels detected.

(F and G) Relative mRNA levels of genes associated with the three germ layers at EBs D0, D4, and D8 in response to miR-16-1/miR-191 repression (F) or overexpression (G). Error bars indicate SD of three independent experiments.

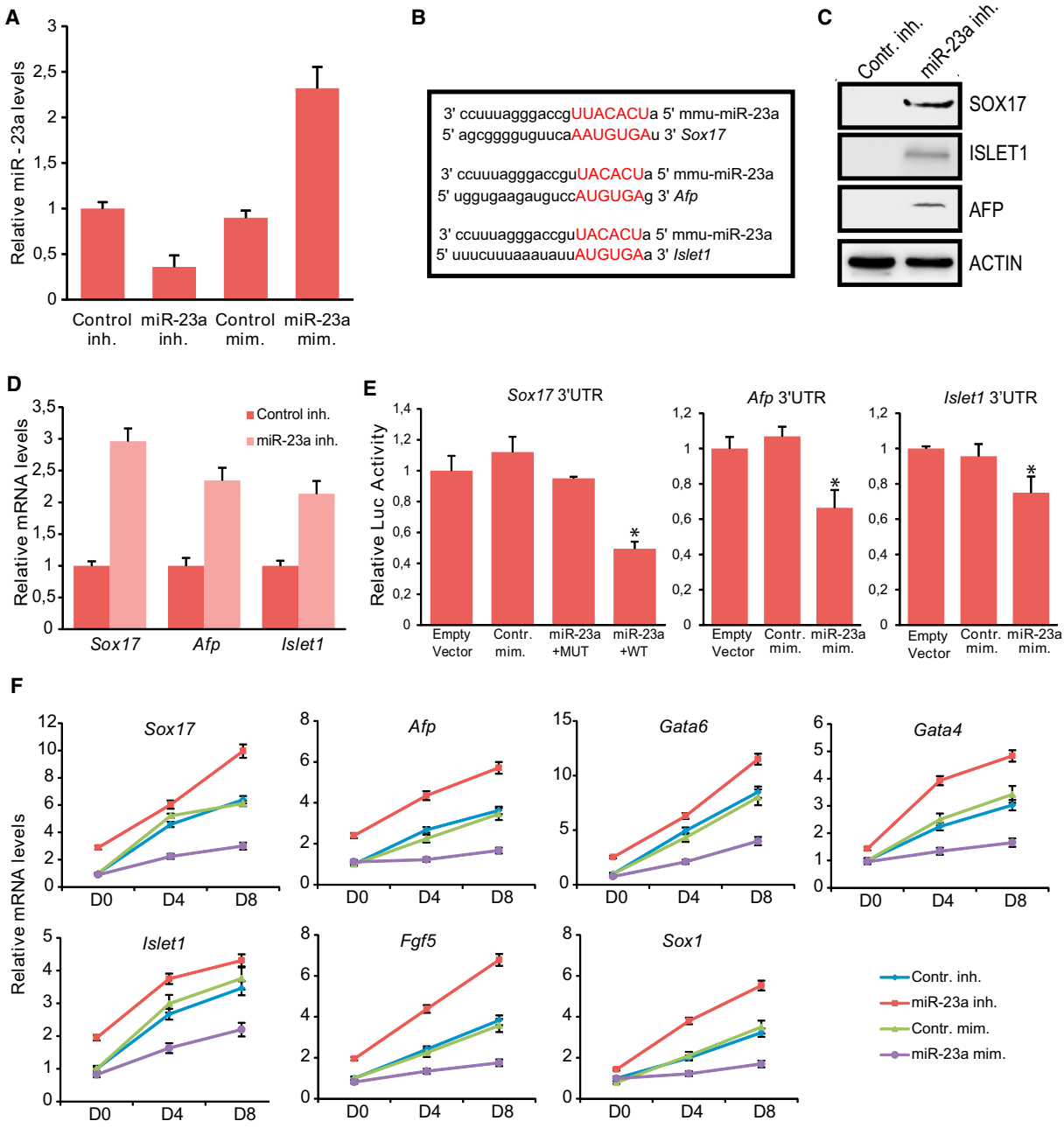


Figure 3. miR-23a Represses Endoderm and Ectoderm Differentiation

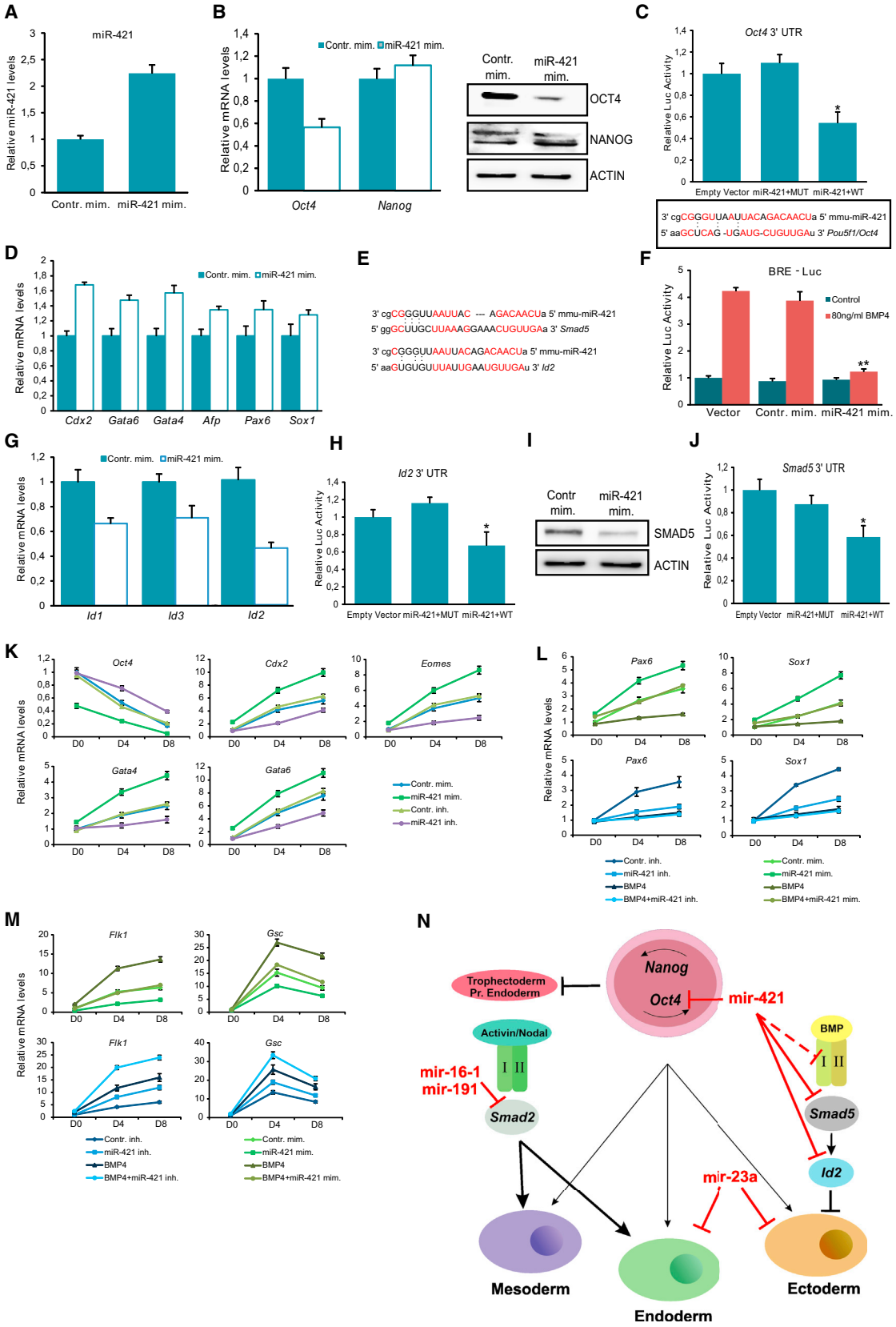
(A) miRNA expression levels in miR-23a mimic or inhibitor transfected mESCs. Error bars indicate SD of three independent experiments.

(B) Prediction of the binding sites of miR-23a on the 3′ UTR of the indicated differentiation-associated genes. Red indicates complementarity between miRNA and the target gene.

(C and D) miR-23a inhibition led to induction of SOX17, AFP, and ISLET1 protein (C) and mRNA (D) levels in mESCs.

(E) Relative luciferase activity of the WT 3′ UTR reporter co-transfected with mir-23a mimic. Data are shown as mean ± SD of four independent experiments. *$p < 0.05$.

(F) Relative mRNA levels of differentiation genes after EB formation in response to miR-23a inhibition or overexpression. Error bars indicate SD of three independent experiments.



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2003), through the activation of Id proteins acting as neuronal differentiation inhibitors (Ying et al., 2003; Zhang et al., 2010), the effect of miR-421 on BMP activity was investigated. Firstly, we confirmed that miR-421 overexpression significantly repressed the luciferase activity of the BRE-Luc reporter gene in response to BMP4 treatment compared with control (Figure 4F). Overexpression of miR-421 also reduced the mRNA expression levels of the endogenous targets of BMP signaling *Id1*, *Id2*, and *Id3* (Figure 4G). Moreover, a luciferase reporter assay confirmed that miR-421 targeted directly the *Id2* 3′ UTR (Figure 4H). Interestingly, SMAD5 protein levels were decreased in mESCs expressing miR-421 mimic (Figure 4I), while *Smad5* 3′ UTR reporter assays verified the direct regulation of *Smad5* by miR-421 (Figure 4J). To further analyze the function of miR-421 in differentiation, we differentiated mESCs transfected with miR-421 mimic or miR-421 inhibitor (Figure S3B). The overexpression of miR-421 favored the suppression of *Oct4* and at the same time enhanced the induction of trophectoderm (*Cdx2*, *Eomes*) and endoderm (*Gata4*, *Gata6*) differentiation (Figure 4K). Concerning the induction of ectodermal markers (*Pax6*, *Sox1*), miR-421 elevation caused a significant increase, whereas the addition of BMP4 did not allow differentiation toward this lineage. miR-421 inhibitor up-regulated *Oct4* expression and down-regulated the expression of trophectoderm, endoderm, and ectoderm differentiation markers (Figures 4K and 4L). Moreover, mesodermal markers (*Flk1*, *Gsc*) were not induced upon miR-421 overexpression, in contrast to miR-421 inhibition or BMP4 treatment whereby their induction was significantly raised (Figures 4M and S3C). In agreement with these data, the concurrent addition of miR-421 mimic and BMP4 did not affect the differentiation induction. It is noteworthy that ectodermal genes appeared to be decreased, while mesodermal markers were significantly increased in BMP4/miR-421 inhibitor-treated cells (Figures 4L, 4M, and S3C).

The above experimental results suggest that miR-421 is a positive regulator of mESC differentiation through two mechanisms, suppression of *Oct4* and competition with BMP signaling.

Contrary to its function in mESCs, miR-421 has been previously characterized as an oncomiR in several cancers. In neuroblastoma, miR-421 suppresses ataxia-telangiectasia mutated uncoupling DNA damage from cell cycle check points (Hu et al., 2010). In pancreatic tumor cells, miR-421 represses *Smad4*, which is critical for BMP signal transduction, and represses its target gene *Id3*, promoting cell proliferation and colony formation (Hao et al., 2011). Therefore, miR-421 regulates Smad4-mediated signaling pathways in cancer cells. In addition, miR-421 is regulated by the TGF-β and BMP4 pathway in pulmonary artery smooth muscle cells, via a conserved Smad binding element (Marchand et al., 2012).

To conclude, this study unveils an miRNA-mediated mechanism for miRNAs that regulate ESC fate decisions (Figure 4N). Regarding miR-16-1, miR-191, and miR-421, this effect is due to competition with TGF-β family signaling. Inhibition of Activin/Nodal pathway by miR-16-1 and miR-191 promotes mESC maintenance, whereas competition of miR-421 with the BMP pathway results in exit of mESCs from pluripotency and their commitment

Figure 4. miR-421 Induces Differentiation by Suppressing *Oct4* and Regulating BMP-Signaling Pathway

(A) Measurement of miRNA levels by RT-PCR after transient transfection with miR-421 mimic. Error bars indicate SD of three independent experiments.

(B) mRNA and protein levels of stemness factors (Oct4 and Nanog) after miR-421 overexpression. Error bars indicate SD of three independent experiments.

(C) mir-421 target sites in the 3′ UTR of *Oct4*. Luciferase activity of *Oct4* 3′ UTR upon miR-421 mimic supplementation. Red indicates complementarity between miRNA and the target gene. Data are shown as mean ± SD of four independent experiments. *$p < 0.05$.

(D) Relative mRNA levels of differentiation markers in miR-421-induced mESCs. Error bars indicate SD of three independent experiments.

(E) miR-421 binding sites in the 3′ UTR of *Smad5* and *Id2*. Red indicates complementarity between miRNA and the target gene.

(F) miR-421 overexpression inhibits BRE-Luc activity. Data are shown as mean ± SD of four independent experiments. **$p < 0.01$.

(G) RT-PCR analysis of BMP4 target gene (*Ids*) expression levels in miR-421 overexpressed mESCs. Error bars indicate SD of three independent experiments.

(H) Overexpression of miR-421 decreased the luciferase activity of *Id2*. Data are shown as mean ± SD of four independent experiments. *$p < 0.05$.

(I and J) SMAD5 protein levels (I) and *Smad5* 3′ UTR luciferase activity (J) were reduced by miR-421 mimic. Data are shown as mean ± SD of four independent experiments. *$p < 0.05$.

(K) Relative mRNA levels of differentiation genes at EBs D0, D4, and D8 upon miR-421 overexpression or inhibition. Error bars indicate SD of three independent experiments.

(L and M) Relative mRNA levels of ectodermal (L) and mesodermal (M) differentiation genes at EBs D0, D4, and D8 upon miR-421 overexpression or inhibition in the presence of BMP4. Error bars indicate SD of three independent experiments.

(N) Proposed mechanism for the regulation of mESC differentiation by the aforementioned miRNAs.

to ectodermal fate. Conversely, miR-23a is itself regulated by TGF-β/BMP. Taken together, our work reveals a reciprocal antagonism between the investigated miRNAs and TGF-β signaling pathways in regulating ESC differentiation (Figure 4M). Our findings link these miRNAs with TGF-β/BMP signaling and may have implications in cancer biology, as the TGF-β pathway is a critical regulator of tumor growth, invasion, and metastasis (Drabsch and ten Dijke, 2012). miRNAs that have a parallel function in cancer and stem cells may be useful candidate molecules to advance the basic knowledge and design combinatorial strategies for cancer and cell replacement therapies.

EXPERIMENTAL PROCEDURES

Cell Culture

The murine feeder-independent ESC line CGR8 was cultured in gelatin-coated flasks in Glasgow minimal essential medium (Gibco) supplemented with 500 U/ml leukaemia inhibitory factor (LIF; ESGRO-Millipore), 2 mM L-glutamine (Gibco), 100 μM β-mercaptoethanol (Gibco), and 15% heat-inactivated HyClone fetal bovine serum (FBS; GE Healthcare Life Sciences). For EB formation, cells were trypsinized and diluted in Iscove's modified Dulbecco's medium (Gibco) supplemented with the above components, to a final concentration of 1,000 cells/20 μl. EBs were cultured without LIF as hanging drops for 2 days, then collected and cultured in suspension for 6 more days.

ACCESSION NUMBERS

The accession number for small RNA-sequencing data stated in this report is GEO: GSE76375.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.01.004.

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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

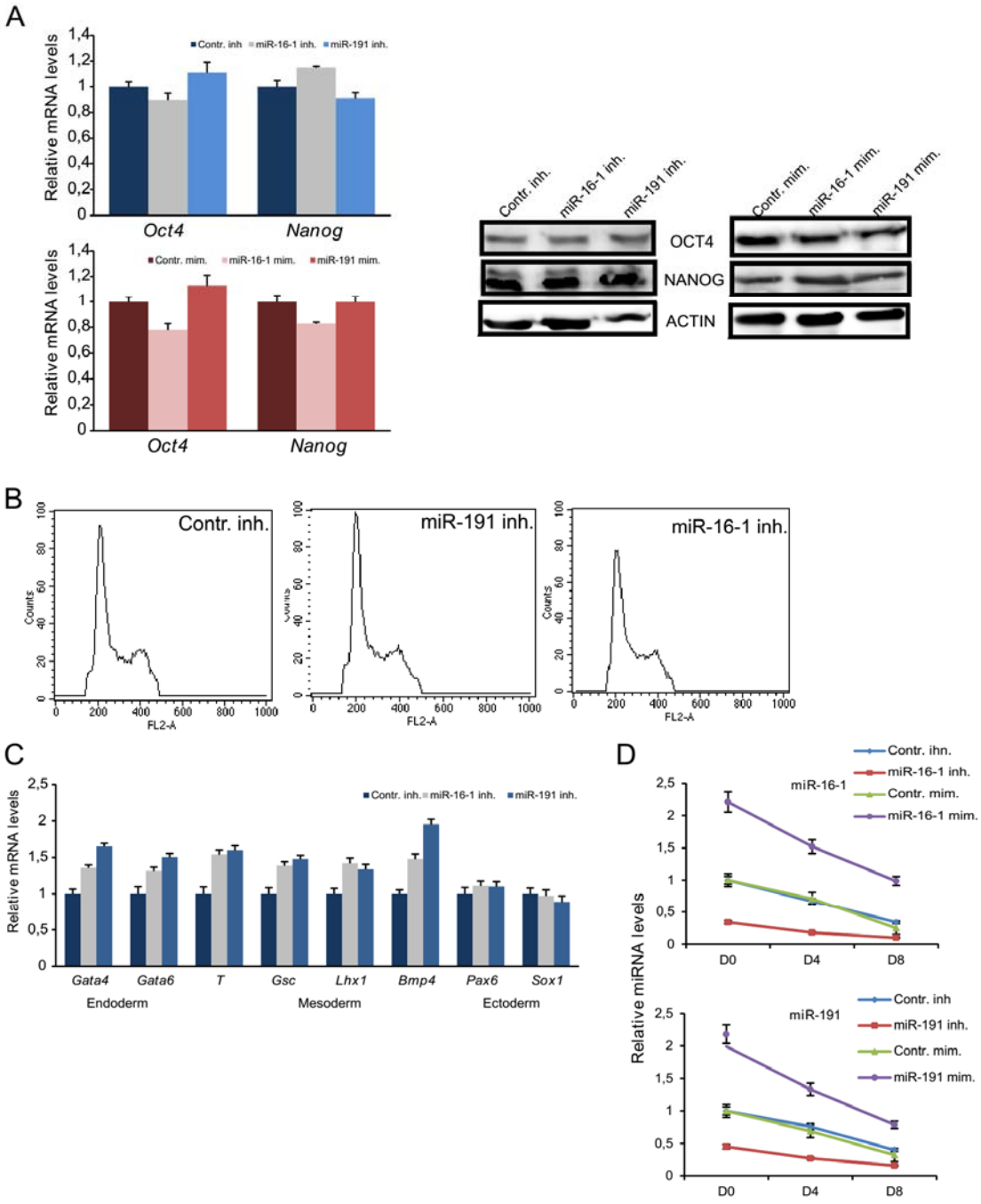

Figure S1 (related to Figure 2)

(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).

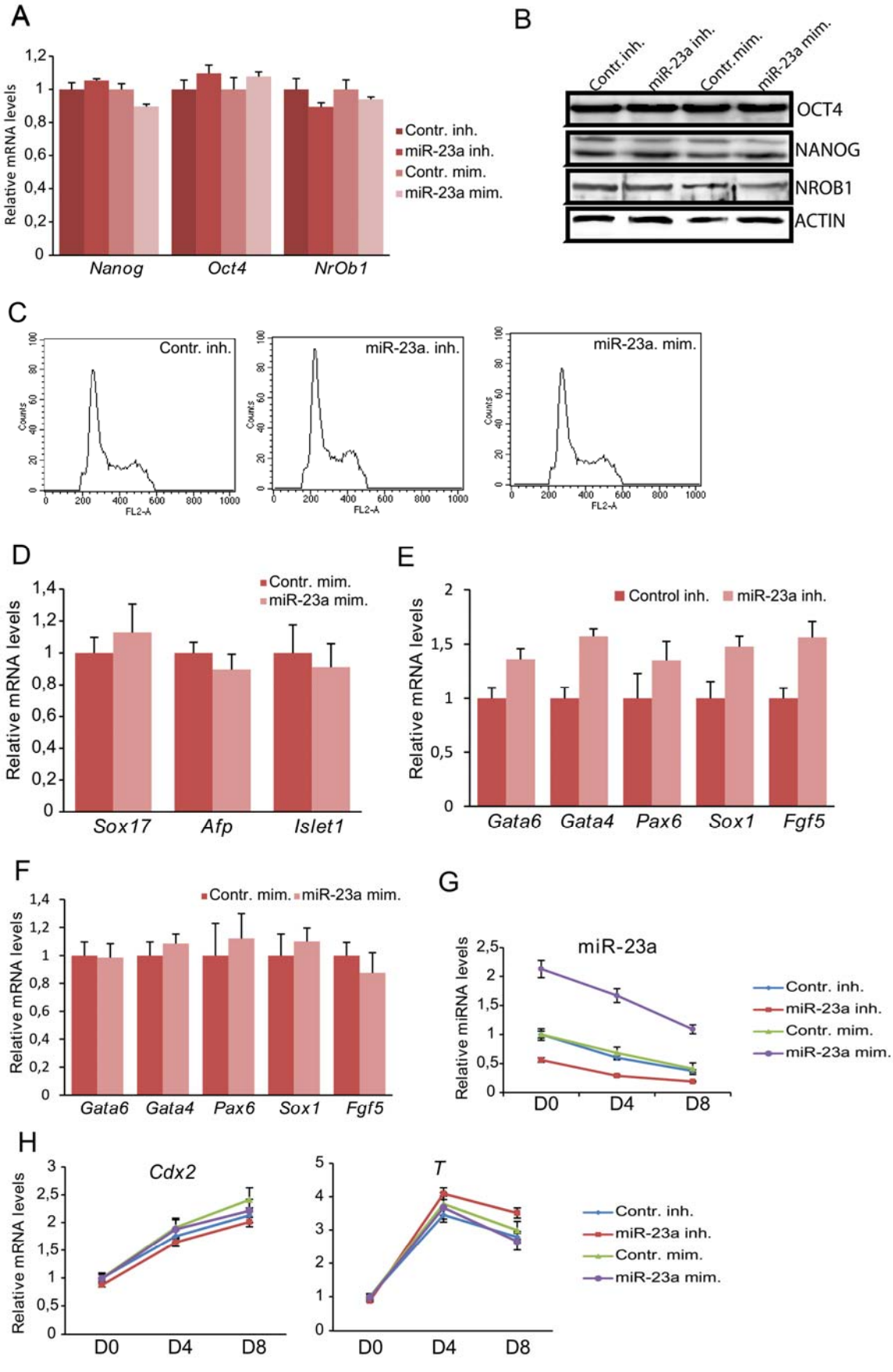
A
Relative mRNA levels
1,2
1
0,8
0,6
0,4
0,2
0
Nanog
Oct4
NrOb1
Contr. inh.
miR-23a inh.
Contr. mim.
miR-23a mim.
B
Contr. inh.
miR-23a inh.
Contr. mim.
miR-23a mim.
OCT4
NANOG
NROB1
ACTIN
C
Contr. inh.
miR-23a. inh.
miR-23a. mim.
Counts
FL2-A
D
Relative mRNA levels
Contr. mim.
miR-23a mim.
Sox17
Afp
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).

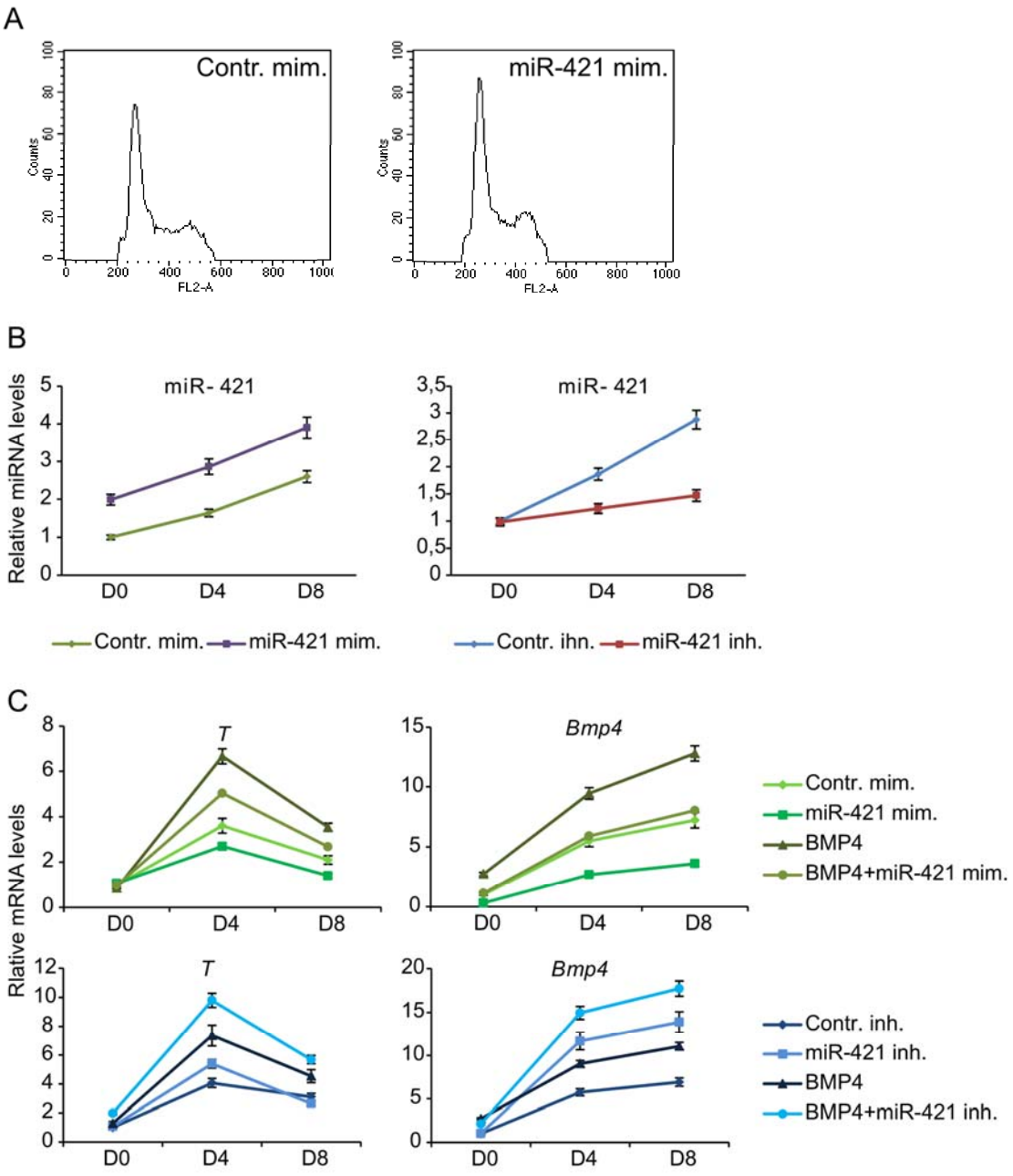

Figure S3 (related to Figure 4)

(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.