microRNA-mediated regulation of microRNA machinery controls cell fate decisions

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Abstract

microRNAs associate with Argonaute proteins, forming the microRNA-induced silencing complex (miRISC), to repress target gene expression post-transcriptionally. Although microRNAs are critical regulators in mammalian cell differentiation, our understanding of how microRNA machinery, such as the miRISC, are regulated during development is still limited. We previously showed that repressing the production of one Argonaute protein, Ago2, by Trim71 is important for mouse embryonic stem cells (mESC) self-renewal (Liu et al., 2021). Here we show that among the four Argonaute proteins in mammals, Ago2 is the major developmentally regulated Argonaute protein in mESCs. Moreover, in pluripotency, besides the Trim71-mediated regulation of *Ago2* (Liu *et al.*, 2021), *Mir182/Mir183* also repress *Ago2*. Specific inhibition of this microRNA-mediated repression results in stemness defects and accelerated differentiation through the let-7 microRNA pathway. These results reveal a microRNA-mediated regulatory circuit on microRNA machinery that is critical to maintaining pluripotency.

Introduction

microRNAs (miRNAs) are endogenous ~22-nucleotide(nt) RNAs with critical roles in modulating gene expression under diverse biological contexts (Bartel, 2009; 2018). Most miRNAs are produced from long primary transcripts (pri-miRNAs) through successive processing by two double-strand RNA (dsRNA) endonucleases named Drosha and Dicer, generating pre-miRNAs and ~22-nt dsRNAs, respectively. One RNA strand in the ~22-nt dsRNA, the mature miRNA, is selectively incorporated into the Argonaute (Ago) protein, forming the miRNA-induced silencing complex (miRISC) (Ha and Kim, 2014). In animals, miRISC recognizes its target mRNAs through partial base-pairings mediated by the miRNA (Bartel, 2009). The Ago protein recruits GW182 proteins to down-regulate target mRNA expression through mRNA degradation and/or translational repression (Nilsen, 2007). Although microRNAs play critical regulatory roles in mammalian cell differentiation (Ameres and Zamore, 2013; Ebert and Sharp, 2012), our understanding on how microRNA machinery, particularly the miRISC, are regulated during development is still limited.

We recently found that Ago2, a key component in the miRISC, is repressed at the mRNA translation level by an RNA-binding protein named Trim71 in mouse embryonic stem cells (mESCs) (Liu *et al.*, 2021). This repression of *Ago2* inhibits stem cell differentiation mediated by the conserved pro-differentiation let-7 miRNAs (Bussing et al., 2008; Liu *et al.*, 2021). These results suggest that *Ago2* is developmentally regulated during stem cell self-renewal and differentiation, and beg for characterization of additional regulators of *Ago2*. Moreover, besides Ago2, there are three additional Ago proteins (Ago1, Ago3, Ago4) in mammals that function redundantly in the miRNA

pathway (Meister, 2013). The relative abundance of these Ago proteins and their contribution to miRNA activities during cell differentiation, however, are still unknown.

Here, using mESC fate decisions between pluripotency and differentiation as a mammalian cell differentiation model, we determined that Ago2 is the predominant Ago protein in mESCs, and Ago2 level increases when mESCs exit pluripotency. In the pluripotent state, *Mir182* and *Mir183*, two conserved miRNAs abundantly expressed in mESCs, repress *Ago2* and control the stemness of mESCs. Specific inhibition of *Mir182*/*Mir183*-mediated repression of *Ago2* results in stemness defects and accelerated differentiation of mESCs through the let-7 microRNA pathway. Collectively, these results reveal a miRNA-mediated regulatory circuit on the miRNA machinery that is critical to maintaining pluripotency.

Results

Ago2 is the predominant developmentally regulated Argonaute protein in mESCs.

Mammals have four Ago proteins (Ago1-4) that function redundantly in miRNA-mediated regulations (Meister, 2013). Transcriptomic profiling on mESCs from different laboratories indicated that mESCs express only *Ago1* and *Ago2* (Figure 1–figure supplement 1A) (Liu *et al.*, 2021; Marks et al., 2012). To examine the relative abundance of Ago1 and Ago2 at the protein level, we generated mESCs with a Flag-tag knocked-in at the N-terminus of the Ago1 and Ago2 loci, respectively, via CRISPR/Cas9-mediated genome-editing (Figure 1–figure supplement 1B&C). These mESCs with the Flag-tag knocked-in displayed no stemness defects compared to the WT mESCs (Figure 1–figure supplement 1D) and enabled us to use the same antibody

(e.g., anti-Flag) to compare the relative abundance of Ago1 and Ago2. Western blotting via an anti-Flag antibody indicated that Ago2 is the predominant Ago protein in mESCs at the protein level (Figure 1A).

To examine whether Ago2 level is regulated during mESCs differentiation, we cultured mESCs under three different conditions that mimic three different developmental stages: ground/naive state (in 2i+Lif), primed state (in 15%FBS+Lif), and differentiating state (in 15%FBS without Lif), which resulted in decreasing stemness in mESCs, as determined by the colony formation assay (Figure 1B). Western blotting indicated that Ago2 level increased when mESCs exited pluripotency (Figure 1C). This result indicated that Ago2 is developmentally regulated in mESCs, and Ago2 level is repressed in the pluripotent state.

Mir182/Mir183 regulate _Ago2_ and maintain stemness in mESCs.

To determine how *Ago2* is regulated in mESCs, we hypothesized that miRNAs expressed in mESCs might contribute to the repression of *Ago2* because miRNAs are important negative regulators of gene expression. We identified the conserved miRNA-binding sites in the 3'UTR of *Ago2* mRNA through TargetScan (Agarwal et al., 2015) and then examined the expression level of the corresponding miRNAs in mESCs using existing small-RNA-seq datasets (Liu *et al.*, 2021) (Figure 1D). This analysis revealed that among the miRNAs that can potentially regulate *Ago2*, *Mir182* and *Mir183*, two miRNAs from the same miRNA family that are abundantly expressed in stem cells (Dambal et al., 2015), have significantly higher expression levels (Figure 1E). Interestingly, *Mir182/Mir183* decrease when mESCs transition from the ground state to

the primed and differentiating state (Hadjimichael et al., 2016; Wang et al., 2017), which negatively correlates with the Ago2 expression pattern during this transition (Figure 1C). These observations suggest that *Ago2* is repressed by *Mir182/Mir183* in mESCs. Consistent with this notion, using RNA antisense purification, we found that *Mir182* and *Mir183* specifically associated with *Ago2* mRNA in mESCs (Figure 1–figure supplement 2).

Two lines of evidence indicated that *Mir182*/*Mir183* regulate *Ago2* mRNA. First, Ago2 increased when *Mir182*, *Mir183*, or both *Mir182* and *Mir183* were knocked out in mESCs (Figure 2–figure supplement 1A & Figure 2A&B). Second, when either *Mir182* or *Mir183* was over-expressed in the wild-type (WT) mESCs (Figure 2–figure supplement 1B), the Ago2 level decreased (Figure 2–figure supplement 1C). The results from these loss-of-function and gain-of-function experiments argue that *Mir182*/*Mir183* repress *Ago2* expression in mESCs.

Interestingly, *Mir182Δ*, *Mir183Δ*, and *Mir182Δ/Mir183Δ* mESCs displayed defects in self-renewal (Figure 2C), as determined by the colony formation assay in the 15%FBS+Lif medium, where differentiation was not blocked by the two inhibitors in the 2i+lif medium. Moreover, these miRNA knockout mESCs had accelerated differentiation, as revealed by the exit pluripotency assay (Figure 2D), which evaluates the rate ESCs exit the pluripotent state (Betschinger et al., 2013), and by the measurement of pluripotency factors through Western blotting on differentiating embryonic bodies (Figure 2E). These cellular phenotypes suggest that *Mir182*/*Mir183*-mediated regulation of *Ago2* is important to mESCs.

Mir182/*Mir183*-mediated repression of *Ago2* is required for maintaining pluripotency.

A caveat in interpreting results from miRNA knockout and over-expression experiments is the pleiotropic effects. Because each miRNA can regulate hundreds of mRNAs, when a miRNA is knocked out or over-expressed, hundreds of miRNA:mRNA interactions are altered, making it difficult to determine whether a specific miRNA:mRNA interaction contributes to the phenotypical changes.

To address this issue and specifically examine the functional significance of *Mir182*/*Mir183*-mediated regulation of *Ago2* in mESCs, we mutated the *Mir182*/*Mir183* binding sites in the 3'UTR of *Ago2* mRNA via CRISPR/Cas9-mediated genome editing (Figure 3A&B). Two observations indicated that the mutations disrupted the interaction between *Ago2* mRNA and *Mir182*/*Mir183*. First, similar to the miRNA knockout mESCs (Figure 2B), Ago2 increased in the 3'UTR mutant mESCs (Figure 3C). Second, in contrast to the results in the WT mESCs (Figure 2–figure supplement 1C), over-expression of either *Mir182* or *Mir183* in the 3'UTR mutant mESCs did not decrease Ago2 (Figure 3–figure supplement 1A&B). Notably, in the *Mir182Δ/Mir183Δ* mESCs, these mutations did not increase Ago2 (Figure 3C), indicating the increased Ago2 from these mutations in the WT mESCs is dependent on *Mir182*/*Mir183*. Moreover, the 3'UTR mutations did not significantly alter the *Mir182*/*Mir183* levels in mESCs (Figure 3–figure supplement 1C). Altogether, these observations indicated that the functional significance of *Mir182*/*Mir183*-mediated repression of Ago2 could be specifically evaluated in the 3'UTR mutant mESCs.

When subject to the colony formation assay, the 3'UTR mutant mESCs displayed a defect in maintaining undifferentiated colonies (Figure 3D), indicating compromised self-renewal. When differentiation was evaluated by the exit pluripotency assay, the 3'UTR mutant mESCs had an increased differentiation rate (Figure 3E). Consistent with these findings, differentiating embryonic bodies from the 3'UTR mutant mESCs had a lower amount of pluripotency factors (Figure 3F). Collectively, these results indicate that *Mir182*/*Mir183*-mediated repression of Ago2 is important for mESC self-renewal and proper differentiation.

Mir182/_Mir183_-mediated repression of _Ago2_ in mESCs inhibits the _let-7_ miRNA-mediated differentiation pathway.

Two observations lead us to the hypothesis that *Mir182*/*Mir183*-mediated repression of *Ago2* in mESCs counteracts the differentiation pathway controlled by the *let-7* miRNAs, a conserved miRNA family that promotes stem cell differentiation (Roush and Slack, 2008). First, in *Dgcr8Δ* mESCs, where endogenous miRNAs' biogenesis is blocked, ectopic expression of *Mir183* inhibits the stem cell differentiation triggered by exogenous let-7 miRNA (Wang *et al.*, 2017). Second, our recent study indicated that increasing Ago2 levels in mESCs results in stemness defects in a *let-7*-miRNA-dependent manner. This specificity on *let-7* miRNAs is because the pro-differentiation *let-7* miRNAs are actively transcribed in mESCs, and the increased Ago2 binds and stabilizes the *let-7* miRNAs that are otherwise degraded in mESCs, thereby promoting mESCs differentiation (Liu *et al.*, 2021).

To test this hypothesis, we examined the expression of *let-7* miRNAs. The 3'UTR mutant mESCs had significantly higher *let-7* miRNAs than the WT mESCs (Figure 4A). This increase is specific to *let-7* miRNAs because non-let-7 miRNAs were not elevated (Figure 4A). Moreover, consistent with our previous observation that increased Ago2 stabilizes mature *let-7* miRNAs (Liu *et al.*, 2021), the *pri-let-7* miRNAs and the *pre-let-7* miRNAs were not significantly increased in the 3'UTR mutant mESCs (Figure 4A). To determine whether the increased *let-7* miRNAs are responsible for the stemness defects in the 3'UTR mutant mESCs, we inhibited *let-7* miRNAs using locked nucleic acid antisense oligonucleotides (LNA) targeting the conserved seed sequence of *let-7* miRNAs. When *let-7* miRNAs were inhibited, the stemness defects of the 3'UTR mutant mESCs were abolished (Figure 4B), indicating that disruption of *Mir182*/*Mir183*-mediated repression of *Ago2* in mESCs activates differentiation through the *let-7* miRNA pathway.

***Mir182/Mir183* and Trim71 function in parallel to repress *Ago2* mRNA in mESCs.**

Our previous study indicated that *Ago2* mRNA is also repressed by Trim71 in mESCs (Liu *et al.*, 2021). Interestingly, the Trim71 binding site in the 3'UTR of *Ago2* mRNA is different from the *Mir182*/*Mir183* binding sites, suggesting that *Mir182*/*Mir183* and Trim71 function in parallel to repress *Ago2* mRNA in mESCs. We performed the following experiments to test this.

At the molecular level, we observed that over-expression of Trim71 still repressed Ago2 in the 3'UTR mutant mESCs (Figure 5A), where *Mir182*/*Mir183*-mediated repression is abolished (Figure 3). Moreover, in the 3'UTR mutant mESCs,

inhibiting Trim71-mediated repression of Ago2 through deleting the Trim71-binding site in the 3'UTR of *Ago2* mRNA (CLIPΔ) (Liu *et al.*, 2021) further increased Ago2 level (Figure 5B, Figure 5–figure supplement 1). These results indicate that Trim71 and *Mir182*/*Mir183* independently repress *Ago2* mRNA in mESCs.

At the cell function level, we found that introducing the CLIPΔ in the 3'UTR mutant mESCs further decreased stem cell self-renewal, as determined by the colony formation assay (Figure 5C), and accelerated differentiation, as measured by the exit pluripotency assay (Figure 5D). These observations argue that Trim71 and *Mir182*/*Mir183* function independently in regulating stemness in mESCs through modulating *Ago2* mRNA.

Collectively, these findings indicate that *Mir182*/*Mir183* and Trim71 function in parallel to repress *Ago2* mRNA in mESCs.

Discussion

Our data reveal that the predominant Ago protein in mESCs, Ago2, is developmentally regulated, with gradually increasing levels when mESCs exit pluripotency. Two miRNAs abundantly expressed in mESCs, *Mir182*/*Mir183*, contribute to the repression of *Ago2* in the pluripotent state. This miRNA-mediated regulation of *Ago2* is critical to maintaining stemness. Our findings raise several interesting aspects of miRNAs in stem cell biology.

First, since Ago2 is the predominant Ago protein in mESCs, the Ago2 expression pattern during mESCs' transition from self-renewal to differentiation argues that although certain individual miRNAs may be required for pluripotency (e.g., *Mir182/Mir183*), the global miRNA activity is suppressed in the pluripotent state and

induced when mESCs initiate differentiation. Consistent with this notion, knocking out key components in global miRNA biogenesis, such as Dgcr8 (Wang et al., 2007), Dicer (Kanellopoulou et al., 2005; Murchison et al., 2005), or Ago2 in the miRISC (Liu *et al.*, 2021), does not negatively affect mESCs self-renewal. However, differentiation in all these mutant mESCs is severely compromised. Thus, at the global level, miRNAs may play more important roles in mESC differentiation.

Second, previous studies indicate that the two components of the miRISC, the Ago protein and its associated miRNA, mutually regulate each other. In the absence of miRNAs, the Ago protein is destabilized (Martinez and Gregory, 2013; Smibert et al., 2013), while miRNAs are also unstable if they are not associated with Ago proteins (Winter and Diederichs, 2011). Thus, the effective miRNA activity depends on the limiting component in the miRISC. Our previous studies indicated that the conserved pro-differentiation *let-7* miRNAs are sensitive to Ago2 levels because an increase of Ago2 results in specific stabilization of *let-7* miRNAs that are otherwise degraded (Liu *et al.*, 2021). Thus, for *let-7* miRISC, Ago2 is possibly the limiting component in mESCs. Repression of *Ago2* by either *Mir182*/*Mir183* as we characterized here or Trim71 as we identified previously (Liu *et al.*, 2021) likely limits the effective *let-7* miRISCs. Interestingly, the pro-differentiation *let-7* miRICSs can positively auto-regulate their own biogenesis through inhibiting Lin28a, a conserved *let-7* target, because Lin28a inhibits the biogenesis of *let-7* miRNAs through promoting their pre-miRNA degradation (Tsialikas and Romer-Seibert, 2015). Thus, the effective *let-7* miRNAs need to be tightly controlled in stem cells. The two repression mechanisms on *Ago2* mRNA contribute to limiting the amount of effective *let-7* miRISCs and maintaining pluripotency in mESCs.

We speculate that similar mechanisms of regulating miRISCs by RNA-binding proteins and microRNAs may exist in other developmental processes. Moreover, Ago2 is dysregulated under many pathological conditions, such as cancer (Adams et al., 2014). Thus, regulating miRISCs through modulating Ago2 levels may also contribute to pathogenesis.

Finally, it is noticed that the *Mir182Δ/Mir183Δ* mESCs displayed stronger defects in self-renewal and differentiation than the 3'UTR mutant mESCs did (Figure 2C&D versus Figure 3D&E). Thus, besides *Ago2* mRNA, *Mir182*/*Mir183* may regulate additional mRNAs that are important for stem cell biology.

Materials and Methods

Key Resources Table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Antibody	Mouse monoclonal anti-FLAG M2	Sigma-Aldrich	Cat# F1804	WB (1:5000)
Antibody	Mouse monoclonal anti-GAPDH (6C5)	Santa Cruz Biotechnology	Cat# sc-32233	WB (1:5000)
Antibody	Rabbit monoclonal anti-beta-Tubulin	Selleckchem	Cat# A5032	WB (1:5000)
Antibody	Rabbit monoclonal anti-Ago1 (D84G10)	Cell Signaling Technology	Cat# 5053	WB (1:1000)
Antibody	Rabbit monoclonal anti-Ago2	Bimake	Cat# A5701	WB (1:3000)
Antibody	Mouse monoclonal anti-Oct-4	BD Transduction Laboratories™	Cat# 611202	WB (1:5000)
Antibody	Rabbit monoclonal anti-Nanog (D2A3)	Cell Signaling Technology	Cat# 8822	WB (1:3000)
Antibody	Goat Anti-Rabbit IgG (H L)-HRP Conjugate	Bio-Rad	Cat# 170-6515	WB (1:5000)
Antibody	Goat Anti-Mouse IgG (H L)-HRP Conjugate	Bio-Rad	Cat# 170-6516	WB (1:5000)
Chemical compound, drug	DMEM/F-12	Gibco	Cat# 12500096	
Chemical compound, drug	FBS	Millipore	Cat# ES-009-B	
Chemical compound, drug	mLIF	Millipore	Cat# ESG1107	
Chemical compound, drug	PD0325901	APExBio	Cat# A3013	
Chemical compound, drug	CHIR99021	APExBio	Cat# A3011	
Chemical compound, drug	N2	Millipore	Cat# SCM012	
Chemical compound, drug	N27	Millipore	Cat# SCM013	
Chemical compound,	MEM NEAA	Gibco	Cat# 11140-50	

drug				
Chemical compound, drug	Penicillin-Streptomycin	Gibco	Cat# 11548876	
Chemical compound, drug	L-Glutamin	Sigma-Aldrich	Cat# G7513	
Chemical compound, drug	b-mercaptoethanol	Sigma-Aldrich	Cat# M3148	
Chemical compound, drug	Accutase	Millipore	Cat# SF006	
Chemical compound, drug	Fugene6	Promega	Cat# E2691	
Chemical compound, drug	Puromycin	Sigma-Aldrich	Cat# P9620	
Chemical compound, drug	Doxycycline	Sigma-Aldrich	Cat# D9891	
Chemical compound, drug	Protease inhibitors	Bimake	Cat# B14001	
Chemical compound, drug	Gelatin	Sigma-Aldrich	Cat# G1890	
Chemical compound, drug	One Step-RNA Reagent	Bio Basic	Cat# BS410A	
Chemical compound, drug	DNase 1	NEB	Cat# M0303L	
Chemical compound, drug	SuperScript™ II Reverse Transcriptase	Invitrogen	Cat# 18064014	
Chemical compound, drug	SsoAdvanced™ Universal SYBR® Green Supermix	Bio-Rad	Cat# 1725270	
Chemical compound, drug	Q5® High-Fidelity DNA Polymerase	NEB	Cat# M0491L	
Chemical compound, drug	Control LNA	Qiagen	Cat# 339137	
Chemical compound, drug	anti-let-7 LNA	Qiagen	Cat# YFI0450006	

Commercial assay or kit	Alkaline Phosphatase Assay Kit	System Biosciences	Cat# AP100R-1	
Commercial assay or kit	Gibson Assembly® Master Mix	NEB	Cat# E2611L	
Commercial assay or kit	Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	Cat# 23225	
Commercial assay or kit	Mir-X™ miRNA First Strand Synthesis Kit	Takara	Cat# 638313	
Cell line (*M. musculus*)	ES-E14TG2a mESC	ATCC	CRL-1821	
Cell line (*M. musculus*)	FLAG-Ago1 mESC	this paper		
Cell line (*M. musculus*)	FLAG-Ago2 mESC	PMID: 33599613		
Cell line (*M. musculus*)	*Mir182*Δ mESC	this paper		
Cell line (*M. musculus*)	*Mir183*Δ mESC	this paper		
Cell line (M. musculus)	*Mir182*Δ*/Mir183*Δ mESC	this paper		
Cell line (*M. musculus*)	3'UTR Mutant mESC	this paper		
Cell line (*M. musculus*)	*Mir182*Δ*/Mir183*Δ*/*3'UTR Mutant mESC	this paper		
Recombinant DNA reagent	PiggyBac-based dox-inducible expression vector	PMID: 33599613	pWH406	
Recombinant DNA reagent	Inducible GFP expressing vector	PMID: 33599613	pWH1055	
Recombinant DNA reagent	Inducible mouse *Mir182* expressing vector	this paper	pWH1039	
Recombinant DNA reagent	Inducible mouse *Mir183* expressing vector	this paper	pWH1040	
Recombinant DNA reagent	sgRNA and Cas9 expressing vector (pX458) pWH464	Addgene	Cat# 48138	
Recombinant DNA reagent	Super PiggyBac Transposase expressing vector (pWH252)	System Biosciences	Cat# PB210PA-1	

All the antibodies, plasmids, and oligonucleotides used in this study are listed in supplemental file 1.

Cell lines

All the cell lines from this study are based on ES-E14TG2a mESC (ATCC, CRL-1821). They are listed in the supplemental file 1. The ES-E14TG2a mESCs were authenticated through STR profiling and were negative for mycoplasma contamination determined by a PCR-based kit.

CRISPR/Cas9-mediated genome editing in mESCs

To generate the FLAG-Ago1, FLAG-Ago2 mESCs or *Ago2* 3'UTR Mutant mESCs, cells were co-transfected with 2 μg of pWH464 (pSpCas9(BB)−2A-GFP (pX458)) expressing the corresponding targeting sgRNA and 1 μg of the corresponding donor oligo or plasmid using the Fugene6 (Promega). To generate *Mir182Δ* and *Mir183Δ* mESCs, cells were transfected with 2 μg of pWH464 expressing a pair of sgRNAs targeting *pri-Mir182* or *pri-Mir183*. The transfected cells were subject to single cell sorting and the resulting clones were subject to genotyping to identify the correct clones.

qRT-PCR

For pri-miRNA quantification, reverse transcription was performed using random hexamers and Superscript2 reverse transcriptase. pre-miRNA and miRNA quantifications were using the Takara's Mir-X miRNA quantification method. qPCR was performed in triplicate for each sample using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) on a CFX96^{TM} real-time PCR detection system (Bio-Rad).

Western blotting

Proteins were harvested in RIPA buffer (10 mM Tris-HCl pH 8.0, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail) and quantified with a BCA assay kit (ThermoFisher). Equal amounts of protein samples were resolved by SDS-PAGE gel, and then transferred to

PVDF membranes. Western blotting was performed using a BlotCycler (Precision Biosystems) with the corresponding primary and secondary antibodies. The membranes were then treated with the Western ECL substrate (Bio-Rad), and the resulting signal was detected using an ImageQuant LAS 500 instrument (GE Healthcare).

Colony formation assay and exit pluripotency assay

For colony formation assay, 500 cells were plated on a 12-well plate in 2i+Lif media or Lif media (DMEM/F12 supplemented with 15% FBS, 1 × penicillin/streptomycin, 0.1 mM Non-Essential Amino Acids, 2 mM L-glutamine, and 0.1 mM 2-mercaptoethanol, and 1000 U/ml Lif). For exit from pluripotency assay, 1000 cells were plated on a gelatin-coated 6-well plate in differentiation media (DMEM/F12 supplemented with 15% FBS, 1 × penicillin/streptomycin, 0.1 mM Non-Essential Amino Acids, 2 mM L-glutamine, and 0.1 mM 2-mercaptoethanol) for 2 days, then cultured in 2i+Lif media for another 5 days. Colonies were stained using AP staining Kit and grouped by differentiation status 6-7 days after plating.

Embryoid body formation

For differentiation via embryoid body formation, 3×10^6 cells were plated per 10 cm bacterial grade Petri dish and maintained on a horizontal rotator with a rotating speed of 30 rpm in differentiation media. The resultant EBs were harvested at the indicated time points.

RNA antisense purification

mESCs were crosslinked with 0.1% formaldehyde for 5min at room temperature, and the crosslinking reaction was quenched by adding 1/20 volume of 2.5M glycine and incubating the mESCs at room temperature for 10min on a rotating platform. The cells

were then harvested and lysed in cell lysis buffer (50mM Tris-HCl pH7.4, 150mM NaCl, 5mM EDTA, 10% glycerol, 1% Tween-20, with freshly added proteinase inhibitors). The cell lysate was cleared by centrifugation at 20,000g for 10min at 4°C. The resulting supernatant was used for RNA antisense purification. 5mg lysate in 500ul lysis buffer was used for each purification. Specifically, a set of 5'-end biotinylated anti-sense DNA oligoes and 5ul RNase inhibitor (NEB) were added to the lysate, resulting in a final concentration of 0.1uM for each oligo. The lysate was incubated at room temperature for 1 hour on a rotating platform. Then 100ul Dynabeads MyOne Streptavidin C1 (Invitrogen) was added and the lysate further incubated for 30min at room temperature on a rotating platform. The magnetic beads were isolated through a magnetic stand and then subject to 4 washes, with each wash in 500ul high salt wash buffer (5XPBS, 0.5% sodium deoxycholate, 1% Triton X-100). The washed beads were resuspended in 100ul DNaseI digestion mix (1X DNase1 digestion buffer with 5ul DNase1 (NEB)) and incubated at 37°C for 20min, followed by adding 350ul LET-SDS buffer (25mM Tris-HCl pH8.0, 100mM LiCl, 20mM EDTA pH8.0, 1%SDS) and 50ul proteinase K (20mg/ml, ThermoFisher). The beads were then incubated on a thermomixer at 55°C 1000rpm for 2 hours. The RNA was isolated through phenol extraction and isopropanol precipitation with glycoblue (Ambion) as a coprecipitant.

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Competing interests

The authors declare no competing interests.

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Figure Legends

Figure 1. Ago2 is the major developmentally regulated Argonaute protein in mESCs.

A. Western blotting in the WT, Flag-Ago1, and Flag-Ago2 mESCs.

B. Colony formation assay for the mESCs. The WT mESCs were cultured under the indicated conditions, and the resultant colonies were fixed and stained for AP (alkaline phosphatase activity). The results represent the means (± SD) of four independent experiments.

C. Western blotting in the WT mESCs cultured under the indicated conditions.

D. Outline of identifying miRNAs that can potentially regulate *Ago2*.

E. Expression levels of the identified miRNAs from (D) in mESCs. CPM: counts per million reads.

The following figure supplements are available for Figure 1:

Figure 1 - figure supplement 1. Expression of Argonaute proteins in mESCs.

Figure 1 – figure supplement 2. *Mir182* and *Mir183* are associated with *Ago2* mRNA in mESCs.

Figure 1 – source data1

Tiff files of raw gel images for Figure 1A,C; Figure 1-figure supplement1C

Excel files of numbers for Figure 1B,E; Figure 1-figure supplement 1A,D, Figure 1-figure supplement 2.

Figure 2. *Mir182*/*Mir183* regulate *Ago2* and maintain stemness in mESCs.

A. qRT-PCR on *Mir182* and *Mir183*. For each miRNA, the expression level in WT cells was set as 1 for relative comparison. U6 RNA was used for normalization. The results represent the means (± SD) of three independent replicates.

B. Western blotting in the WT, *Mir182Δ*, *Mir183Δ*, and *Mir182Δ*/*Mir183Δ* mESCs. Gapdh was used for normalization in calculating the relative expression levels.

C. Colony formation assay for mESCs. The mESCs were cultured in 15%FBS + Lif for 7 days, and the resultant colonies were fixed and stained for AP.

D. Exit pluripotency assay for mESCs. The mESCs were induced to exit pluripotency in medium without Lif for 2 days and then switched to 2i+Lif medium for 5 days. The resultant colonies were fixed and stained for AP.

In C and D, the colony morphology and AP intensity were evaluated through microscopy. 100-200 colonies were examined each time to determine the percentage of undifferentiated colonies. The results represent the means (± SD) of three independent experiments.

E. Western blotting of pluripotency factors during EB formation.

The following figure supplement is available for Figure 2:

Figure 2 - figure supplement 1. *Ago2* mRNA is a target of *Mir182* and *Mir183* in mESCs.

Figure 2 – source data1

Tiff files of raw gel images for Figure 2B,E; Figure2-figure supplement1C.

Excel files of numbers for Figure 2A,C,D; Figure2-figure supplement1B.

Figure 3. *Mir182*/*Mir183*-mediated repression of *Ago2* is required for maintaining pluripotency.

A. Mutating *Mir182* and *Mir183* binding sites in *Ago2* mRNA's 3'UTR via genome editing.

B. Genotyping of the Ago2 3'UTR mutant. The PCR was performed using the oligoes (F and R) indicated in A.

C. Western blotting in the WT, *Ago2* 3'UTR mutant, *Mir182Δ/ Mir183Δ*, and *Mir182Δ/ Mir183Δ/Ago2* 3'UTR mutant.

D. Colony formation assay for mESCs.

E. Exit pluripotency assay for mESCs.

In D and E, the colony morphology and AP intensity were evaluated through microscopy. The results represent the means (± SD) of four independent experiments. *p<0.05 by the Student's t-test.

F. Western blotting of pluripotency factors in day 5 EBs.

The following figure supplement is available for Figure 3:

Figure 3 – figure supplement 1. Inhibition of *Mir182/Mir183*-mediated regulation of *Ago2* in mESCs.

Figure 3 – source data1

Tiff files of raw gel images for Figure 3C,F; Figure 3-figure supplement1B.

Excel files of numbers for Figure 3D,E; Figure 3-figure supplement1A,C.

Figure 4. The stemness defects in the 3'UTR mutant mESCs are caused by elevated *let-7* miRNAs.

A. Relative levels of miRNAs, *let-7* pri-miRNAs, and *let-7* pre-miRNAs in the WT and the *Ago2* 3'UTR mutant mESCs. For each miRNA, pri-miRNA, and pre-

miRNA, the expression level in WT cells was set as 1 for relative comparison. U6 RNA was used for normalization in miRNA and pre-miRNA quantification; 18S rRNA was used for normalization in pri-miRNA quantification. The heatmap was generated from the means of three independent replicates.

B. Colony formation assay for WT and the *Ago2* 3'UTR mutant mESCs cultured in the presence of 500 nM anti-*let-7* LNA or a control LNA. The results represent three independent experiments. *p<0.05, and n.s. not significant (p>0.05) by the Student's t-test.

Figure 4 – source data1

Excel files of numbers for Figure 4A,B.

Figure 5. *Mir182/Mir183* and Trim71 function in parallel to repress *Ago2* mRNA in mESCs.

A. Western blotting in the WT mESCs expressing either a vector or FLAG-Trim71 and in the 3'UTR mutant mESCs expressing either a vector or FLAG-Trim71.

B. Western blotting in the WT, 3'UTR mutant, and 3'UTR mutant/CLIPΔ mESCs.

In A and B, Gapdh was used for normalization in calculating the relative expression levels.

C. Colony formation assay for mESCs.

D. Exit pluripotency assay for mESCs.

The following figure supplement is available for Figure 5:

Figure 5 – figure supplement 1. Generation of the CLIPΔ in the 3'UTR mutant mESCs.

Figure 5 – source data1

463 Tiff files of raw gel images for Figure 5A,B; Figure 5–figure supplement1

464 An Excel file of numbers for Figure 5C,D.

465

466 Supplemental file 1. Antibodies, plasmids, and oligonucleotides used in this study.

microRNA-mediated regulation of microRNA machinery controls cell fate decisions

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Abstract

microRNAs associate with Argonaute proteins, forming the microRNA-induced silencing complex (miRISC), to repress target gene expression post-transcriptionally. Although microRNAs are critical regulators in mammalian cell differentiation, our understanding of how microRNA machinery, such as the miRISC, are regulated during development is still limited. We previously showed that repressing the production of one Argonaute protein, Ago2, by Trim71 is important for mouse embryonic stem cells (mESC) self-renewal (Liu et al., 2021). Here we show that among the four Argonaute proteins in mammals, Ago2 is the major developmentally regulated Argonaute protein in mESCs. Moreover, in pluripotency, besides the Trim71-mediated regulation of *Ago2* (Liu *et al.*, 2021), *Mir182/Mir183* also repress *Ago2*. Specific inhibition of this microRNA-mediated repression results in stemness defects and accelerated differentiation through the let-7 microRNA pathway. These results reveal a microRNA-mediated regulatory circuit on microRNA machinery that is critical to maintaining pluripotency.

Introduction

microRNAs (miRNAs) are endogenous ~22-nucleotide(nt) RNAs with critical roles in modulating gene expression under diverse biological contexts (Bartel, 2009; 2018). Most miRNAs are produced from long primary transcripts (pri-miRNAs) through successive processing by two double-strand RNA (dsRNA) endonucleases named Drosha and Dicer, generating pre-miRNAs and ~22-nt dsRNAs, respectively. One RNA strand in the ~22-nt dsRNA, the mature miRNA, is selectively incorporated into the Argonaute (Ago) protein, forming the miRNA-induced silencing complex (miRISC) (Ha and Kim, 2014). In animals, miRISC recognizes its target mRNAs through partial base-pairings mediated by the miRNA (Bartel, 2009). The Ago protein recruits GW182 proteins to down-regulate target mRNA expression through mRNA degradation and/or translational repression (Nilsen, 2007). Although microRNAs play critical regulatory roles in mammalian cell differentiation (Ameres and Zamore, 2013; Ebert and Sharp, 2012), our understanding on how microRNA machinery, particularly the miRISC, are regulated during development is still limited.

We recently found that Ago2, a key component in the miRISC, is repressed at the mRNA translation level by an RNA-binding protein named Trim71 in mouse embryonic stem cells (mESCs) (Liu *et al.*, 2021). This repression of *Ago2* inhibits stem cell differentiation mediated by the conserved pro-differentiation let-7 miRNAs (Bussing et al., 2008; Liu *et al.*, 2021). These results suggest that *Ago2* is developmentally regulated during stem cell self-renewal and differentiation, and beg for characterization of additional regulators of *Ago2*. Moreover, besides Ago2, there are three additional Ago proteins (Ago1, Ago3, Ago4) in mammals that function redundantly in the miRNA

pathway (Meister, 2013). The relative abundance of these Ago proteins and their contribution to miRNA activities during cell differentiation, however, are still unknown.

Here, using mESC fate decisions between pluripotency and differentiation as a mammalian cell differentiation model, we determined that Ago2 is the predominant Ago protein in mESCs, and Ago2 level increases when mESCs exit pluripotency. In the pluripotent state, *Mir182* and *Mir183*, two conserved miRNAs abundantly expressed in mESCs, repress *Ago2* and control the stemness of mESCs. Specific inhibition of *Mir182*/*Mir183*-mediated repression of *Ago2* results in stemness defects and accelerated differentiation of mESCs through the let-7 microRNA pathway. Collectively, these results reveal a miRNA-mediated regulatory circuit on the miRNA machinery that is critical to maintaining pluripotency.

Results

Ago2 is the predominant developmentally regulated Argonaute protein in mESCs.

Mammals have four Ago proteins (Ago1-4) that function redundantly in miRNA-mediated regulations (Meister, 2013). Transcriptomic profiling on mESCs from different laboratories indicated that mESCs express only *Ago1* and *Ago2* (Figure 1–figure supplement 1A) (Liu *et al.*, 2021; Marks et al., 2012). To examine the relative abundance of Ago1 and Ago2 at the protein level, we generated mESCs with a Flag-tag knocked-in at the N-terminus of the Ago1 and Ago2 loci, respectively, via CRISPR/Cas9-mediated genome-editing (Figure 1–figure supplement 1B&C). These mESCs with the Flag-tag knocked-in displayed no stemness defects compared to the WT mESCs (Figure 1–figure supplement 1D) and enabled us to use the same antibody

(e.g., anti-Flag) to compare the relative abundance of Ago1 and Ago2. Western blotting via an anti-Flag antibody indicated that Ago2 is the predominant Ago protein in mESCs at the protein level (Figure 1A).

To examine whether Ago2 level is regulated during mESCs differentiation, we cultured mESCs under three different conditions that mimic three different developmental stages: ground/naive state (in 2i+Lif), primed state (in 15%FBS+Lif), and differentiating state (in 15%FBS without Lif), which resulted in decreasing stemness in mESCs, as determined by the colony formation assay (Figure 1B). Western blotting indicated that Ago2 level increased when mESCs exited pluripotency (Figure 1C). This result indicated that Ago2 is developmentally regulated in mESCs, and Ago2 level is repressed in the pluripotent state.

Mir182/Mir183 regulate _Ago2_ and maintain stemness in mESCs.

To determine how *Ago2* is regulated in mESCs, we hypothesized that miRNAs expressed in mESCs might contribute to the repression of *Ago2* because miRNAs are important negative regulators of gene expression. We identified the conserved miRNA-binding sites in the 3'UTR of *Ago2* mRNA through TargetScan (Agarwal et al., 2015) and then examined the expression level of the corresponding miRNAs in mESCs using existing small-RNA-seq datasets (Liu *et al.*, 2021) (Figure 1D). This analysis revealed that among the miRNAs that can potentially regulate *Ago2*, *Mir182* and *Mir183*, two miRNAs from the same miRNA family that are abundantly expressed in stem cells (Dambal et al., 2015), have significantly higher expression levels (Figure 1E). Interestingly, *Mir182/Mir183* decrease when mESCs transition from the ground state to

the primed and differentiating state (Hadjimichael et al., 2016; Wang et al., 2017), which negatively correlates with the Ago2 expression pattern during this transition (Figure 1C). These observations suggest that *Ago2* is repressed by *Mir182/Mir183* in mESCs. Consistent with this notion, using RNA antisense purification, we found that *Mir182* and *Mir183* specifically associated with *Ago2* mRNA in mESCs (Figure 1–figure supplement 2).

Two lines of evidence indicated that *Mir182*/*Mir183* regulate *Ago2* mRNA. First, Ago2 increased when *Mir182*, *Mir183*, or both *Mir182* and *Mir183* were knocked out in mESCs (Figure 2–figure supplement 1A & Figure 2A&B). Second, when either *Mir182* or *Mir183* was over-expressed in the wild-type (WT) mESCs (Figure 2–figure supplement 1B), the Ago2 level decreased (Figure 2–figure supplement 1C). The results from these loss-of-function and gain-of-function experiments argue that *Mir182*/*Mir183* repress *Ago2* expression in mESCs.

Interestingly, *Mir182Δ*, *Mir183Δ*, and *Mir182Δ/Mir183Δ* mESCs displayed defects in self-renewal (Figure 2C), as determined by the colony formation assay in the 15%FBS+Lif medium, where differentiation was not blocked by the two inhibitors in the 2i+lif medium. Moreover, these miRNA knockout mESCs had accelerated differentiation, as revealed by the exit pluripotency assay (Figure 2D), which evaluates the rate ESCs exit the pluripotent state (Betschinger et al., 2013), and by the measurement of pluripotency factors through Western blotting on differentiating embryonic bodies (Figure 2E). These cellular phenotypes suggest that *Mir182*/*Mir183*-mediated regulation of *Ago2* is important to mESCs.

Mir182/*Mir183*-mediated repression of *Ago2* is required for maintaining pluripotency.

A caveat in interpreting results from miRNA knockout and over-expression experiments is the pleiotropic effects. Because each miRNA can regulate hundreds of mRNAs, when a miRNA is knocked out or over-expressed, hundreds of miRNA:mRNA interactions are altered, making it difficult to determine whether a specific miRNA:mRNA interaction contributes to the phenotypical changes.

To address this issue and specifically examine the functional significance of *Mir182*/*Mir183*-mediated regulation of *Ago2* in mESCs, we mutated the *Mir182*/*Mir183* binding sites in the 3'UTR of *Ago2* mRNA via CRISPR/Cas9-mediated genome editing (Figure 3A&B). Two observations indicated that the mutations disrupted the interaction between *Ago2* mRNA and *Mir182*/*Mir183*. First, similar to the miRNA knockout mESCs (Figure 2B), Ago2 increased in the 3'UTR mutant mESCs (Figure 3C). Second, in contrast to the results in the WT mESCs (Figure 2–figure supplement 1C), over-expression of either *Mir182* or *Mir183* in the 3'UTR mutant mESCs did not decrease Ago2 (Figure 3–figure supplement 1A&B). Notably, in the *Mir182Δ/Mir183Δ* mESCs, these mutations did not increase Ago2 (Figure 3C), indicating the increased Ago2 from these mutations in the WT mESCs is dependent on *Mir182*/*Mir183*. Moreover, the 3'UTR mutations did not significantly alter the *Mir182*/*Mir183* levels in mESCs (Figure 3–figure supplement 1C). Altogether, these observations indicated that the functional significance of *Mir182*/*Mir183*-mediated repression of Ago2 could be specifically evaluated in the 3'UTR mutant mESCs.

When subject to the colony formation assay, the 3'UTR mutant mESCs displayed a defect in maintaining undifferentiated colonies (Figure 3D), indicating compromised self-renewal. When differentiation was evaluated by the exit pluripotency assay, the 3'UTR mutant mESCs had an increased differentiation rate (Figure 3E). Consistent with these findings, differentiating embryonic bodies from the 3'UTR mutant mESCs had a lower amount of pluripotency factors (Figure 3F). Collectively, these results indicate that *Mir182*/*Mir183*-mediated repression of Ago2 is important for mESC self-renewal and proper differentiation.

Mir182/_Mir183_-mediated repression of _Ago2_ in mESCs inhibits the _let-7_ miRNA-mediated differentiation pathway.

Two observations lead us to the hypothesis that *Mir182*/*Mir183*-mediated repression of *Ago2* in mESCs counteracts the differentiation pathway controlled by the *let-7* miRNAs, a conserved miRNA family that promotes stem cell differentiation (Roush and Slack, 2008). First, in *Dgcr8Δ* mESCs, where endogenous miRNAs' biogenesis is blocked, ectopic expression of *Mir183* inhibits the stem cell differentiation triggered by exogenous let-7 miRNA (Wang *et al.*, 2017). Second, our recent study indicated that increasing Ago2 levels in mESCs results in stemness defects in a *let-7*-miRNA-dependent manner. This specificity on *let-7* miRNAs is because the pro-differentiation *let-7* miRNAs are actively transcribed in mESCs, and the increased Ago2 binds and stabilizes the *let-7* miRNAs that are otherwise degraded in mESCs, thereby promoting mESCs differentiation (Liu *et al.*, 2021).

To test this hypothesis, we examined the expression of *let-7* miRNAs. The 3'UTR mutant mESCs had significantly higher *let-7* miRNAs than the WT mESCs (Figure 4A). This increase is specific to *let-7* miRNAs because non-let-7 miRNAs were not elevated (Figure 4A). Moreover, consistent with our previous observation that increased Ago2 stabilizes mature *let-7* miRNAs (Liu *et al.*, 2021), the *pri-let-7* miRNAs and the *pre-let-7* miRNAs were not significantly increased in the 3'UTR mutant mESCs (Figure 4A). To determine whether the increased *let-7* miRNAs are responsible for the stemness defects in the 3'UTR mutant mESCs, we inhibited *let-7* miRNAs using locked nucleic acid antisense oligonucleotides (LNA) targeting the conserved seed sequence of *let-7* miRNAs. When *let-7* miRNAs were inhibited, the stemness defects of the 3'UTR mutant mESCs were abolished (Figure 4B), indicating that disruption of *Mir182*/*Mir183*-mediated repression of *Ago2* in mESCs activates differentiation through the *let-7* miRNA pathway.

***Mir182/Mir183* and Trim71 function in parallel to repress *Ago2* mRNA in mESCs.**

Our previous study indicated that *Ago2* mRNA is also repressed by Trim71 in mESCs (Liu *et al.*, 2021). Interestingly, the Trim71 binding site in the 3'UTR of *Ago2* mRNA is different from the *Mir182*/*Mir183* binding sites, suggesting that *Mir182*/*Mir183* and Trim71 function in parallel to repress *Ago2* mRNA in mESCs. We performed the following experiments to test this.

At the molecular level, we observed that over-expression of Trim71 still repressed Ago2 in the 3'UTR mutant mESCs (Figure 5A), where *Mir182*/*Mir183*-mediated repression is abolished (Figure 3). Moreover, in the 3'UTR mutant mESCs,

inhibiting Trim71-mediated repression of Ago2 through deleting the Trim71-binding site in the 3'UTR of *Ago2* mRNA (CLIPΔ) (Liu *et al.*, 2021) further increased Ago2 level (Figure 5B, Figure 5–figure supplement 1). These results indicate that Trim71 and *Mir182*/*Mir183* independently repress *Ago2* mRNA in mESCs.

At the cell function level, we found that introducing the CLIPΔ in the 3'UTR mutant mESCs further decreased stem cell self-renewal, as determined by the colony formation assay (Figure 5C), and accelerated differentiation, as measured by the exit pluripotency assay (Figure 5D). These observations argue that Trim71 and *Mir182*/*Mir183* function independently in regulating stemness in mESCs through modulating *Ago2* mRNA.

Collectively, these findings indicate that *Mir182*/*Mir183* and Trim71 function in parallel to repress *Ago2* mRNA in mESCs.

Discussion

Our data reveal that the predominant Ago protein in mESCs, Ago2, is developmentally regulated, with gradually increasing levels when mESCs exit pluripotency. Two miRNAs abundantly expressed in mESCs, *Mir182*/*Mir183*, contribute to the repression of *Ago2* in the pluripotent state. This miRNA-mediated regulation of *Ago2* is critical to maintaining stemness. Our findings raise several interesting aspects of miRNAs in stem cell biology.

First, since Ago2 is the predominant Ago protein in mESCs, the Ago2 expression pattern during mESCs' transition from self-renewal to differentiation argues that although certain individual miRNAs may be required for pluripotency (e.g., *Mir182/Mir183*), the global miRNA activity is suppressed in the pluripotent state and

induced when mESCs initiate differentiation. Consistent with this notion, knocking out key components in global miRNA biogenesis, such as Dgcr8 (Wang et al., 2007), Dicer (Kanellopoulou et al., 2005; Murchison et al., 2005), or Ago2 in the miRISC (Liu *et al.*, 2021), does not negatively affect mESCs self-renewal. However, differentiation in all these mutant mESCs is severely compromised. Thus, at the global level, miRNAs may play more important roles in mESC differentiation.

Second, previous studies indicate that the two components of the miRISC, the Ago protein and its associated miRNA, mutually regulate each other. In the absence of miRNAs, the Ago protein is destabilized (Martinez and Gregory, 2013; Smibert et al., 2013), while miRNAs are also unstable if they are not associated with Ago proteins (Winter and Diederichs, 2011). Thus, the effective miRNA activity depends on the limiting component in the miRISC. Our previous studies indicated that the conserved pro-differentiation *let-7* miRNAs are sensitive to Ago2 levels because an increase of Ago2 results in specific stabilization of *let-7* miRNAs that are otherwise degraded (Liu *et al.*, 2021). Thus, for *let-7* miRISC, Ago2 is possibly the limiting component in mESCs. Repression of *Ago2* by either *Mir182*/*Mir183* as we characterized here or Trim71 as we identified previously (Liu *et al.*, 2021) likely limits the effective *let-7* miRISCs. Interestingly, the pro-differentiation *let-7* miRICSs can positively auto-regulate their own biogenesis through inhibiting Lin28a, a conserved *let-7* target, because Lin28a inhibits the biogenesis of *let-7* miRNAs through promoting their pre-miRNA degradation (Tsialikas and Romer-Seibert, 2015). Thus, the effective *let-7* miRNAs need to be tightly controlled in stem cells. The two repression mechanisms on *Ago2* mRNA contribute to limiting the amount of effective *let-7* miRISCs and maintaining pluripotency in mESCs.

We speculate that similar mechanisms of regulating miRISCs by RNA-binding proteins and microRNAs may exist in other developmental processes. Moreover, Ago2 is dysregulated under many pathological conditions, such as cancer (Adams et al., 2014). Thus, regulating miRISCs through modulating Ago2 levels may also contribute to pathogenesis.

Finally, it is noticed that the *Mir182Δ/Mir183Δ* mESCs displayed stronger defects in self-renewal and differentiation than the 3'UTR mutant mESCs did (Figure 2C&D versus Figure 3D&E). Thus, besides *Ago2* mRNA, *Mir182*/*Mir183* may regulate additional mRNAs that are important for stem cell biology.

Materials and Methods

Key Resources Table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Antibody	(Mouse monoclonal) anti-FLAG M2	Sigma-Aldrich	Cat# F1804	WB (1:5000)
Antibody	(Mouse monoclonal) anti-GAPDH (6C5)	Santa Cruz Biotechnology	Cat# sc-32233	WB (1:5000)
Antibody	(Rabbit monoclonal) anti-beta-Tubulin	Selleckchem	Cat# A5032	WB (1:5000)
Antibody	(Rabbit monoclonal) anti-Ago1 (D84G10)	Cell Signaling Technology	Cat# 5053	WB (1:1000)
Antibody	(Rabbit monoclonal) anti-Ago2	Bimake	Cat# A5701	WB (1:3000)
Antibody	(Mouse monoclonal) anti-Oct-4	BD Transduction Laboratories™	Cat# 611202	WB (1:5000)
Antibody	(Rabbit monoclonal) anti-Nanog (D2A3)	Cell Signaling Technology	Cat# 8822	WB (1:3000)
Antibody	Goat Anti-Rabbit IgG (H L)-HRP Conjugate	Bio-Rad	Cat# 170-6515	WB (1:5000)
Antibody	Goat Anti-Mouse IgG (H L)-HRP Conjugate	Bio-Rad	Cat# 170-6516	WB (1:5000)
Chemical compound, drug	DMEM/F-12	Gibco	Cat# 12500096	
Chemical compound, drug	FBS	Millipore	Cat# ES-009-B	
Chemical compound, drug	mLIF	Millipore	Cat# ESG1107	
Chemical compound, drug	PD0325901	APExBio	Cat# A3013	
Chemical compound, drug	CHIR99021	APExBio	Cat# A3011	
Chemical compound, drug	N2	Millipore	Cat# SCM012	
Chemical compound, drug	N27	Millipore	Cat# SCM013	
Chemical compound,	MEM NEAA	Gibco	Cat# 11140-50	

drug				
Chemical compound, drug	Penicillin-Streptomycin	Gibco	Cat# 11548876	
Chemical compound, drug	L-Glutamin	Sigma-Aldrich	Cat# G7513	
Chemical compound, drug	b-mercaptoethanol	Sigma-Aldrich	Cat# M3148	
Chemical compound, drug	Accutase	Millipore	Cat# SF006	
Chemical compound, drug	Fugene6	Promega	Cat# E2691	
Chemical compound, drug	Puromycin	Sigma-Aldrich	Cat# P9620	
Chemical compound, drug	Doxycycline	Sigma-Aldrich	Cat# D9891	
Chemical compound, drug	Protease inhibitors	Bimake	Cat# B14001	
Chemical compound, drug	Gelatin	Sigma-Aldrich	Cat# G1890	
Chemical compound, drug	One Step-RNA Reagent	Bio Basic	Cat# BS410A	
Chemical compound, drug	DNase 1	NEB	Cat# M0303L	
Chemical compound, drug	SuperScript™ II Reverse Transcriptase	Invitrogen	Cat# 18064014	
Chemical compound, drug	SsoAdvanced™ Universal SYBR® Green Supermix	Bio-Rad	Cat# 1725270	
Chemical compound, drug	Q5® High-Fidelity DNA Polymerase	NEB	Cat# M0491L	
Chemical compound, drug	Control LNA	Qiagen	Cat# 339137	
Chemical compound, drug	anti-let-7 LNA	Qiagen	Cat# YFI0450006	

Commercial assay or kit	Alkaline Phosphatase Assay Kit	System Biosciences	Cat# AP100R-1	
Commercial assay or kit	Gibson Assembly® Master Mix	NEB	Cat# E2611L	
Commercial assay or kit	Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	Cat# 23225	
Commercial assay or kit	Mir-X™ miRNA First Strand Synthesis Kit	Takara	Cat# 638313	
Cell line (*M. musculus*)	ES-E14TG2a mESC	ATCC	CRL-1821	
Cell line (*M. musculus*)	FLAG-Ago1 mESC	this paper		
Cell line (*M. musculus*)	FLAG-Ago2 mESC	PMID: 33599613		
Cell line (*M. musculus*)	*Mir182*Δ mESC	this paper		
Cell line (*M. musculus*)	*Mir183*Δ mESC	this paper		
Cell line (M. musculus)	*Mir182*Δ*/Mir183*Δ mESC	this paper		
Cell line (*M. musculus*)	3'UTR Mutant mESC	this paper		
Cell line (*M. musculus*)	*Mir182*Δ*/Mir183*Δ*/*3'UTR Mutant mESC	this paper		
Recombinant DNA reagent	PiggyBac-based dox-inducible expression vector	PMID: 33599613	pWH406	
Recombinant DNA reagent	Inducible GFP expressing vector	PMID: 33599613	pWH1055	
Recombinant DNA reagent	Inducible mouse *Mir182* expressing vector	this paper	pWH1039	
Recombinant DNA reagent	Inducible mouse *Mir183* expressing vector	this paper	pWH1040	
Recombinant DNA reagent	sgRNA and Cas9 expressing vector (pX458) pWH464	Addgene	Cat# 48138	
Recombinant DNA reagent	Super PiggyBac Transposase expressing vector (pWH252)	System Biosciences	Cat# PB210PA-1	

All the antibodies, plasmids, and oligonucleotides used in this study are listed in supplemental file 1.

Cell lines

All the cell lines from this study are based on ES-E14TG2a mESC (ATCC, CRL-1821). They are listed in the supplemental file 1. The ES-E14TG2a mESCs were authenticated through STR profiling and were negative for mycoplasma contamination determined by a PCR-based kit.

CRISPR/Cas9-mediated genome editing in mESCs

To generate the FLAG-Ago1, FLAG-Ago2 mESCs or *Ago2* 3'UTR Mutant mESCs, cells were co-transfected with 2 μg of pWH464 (pSpCas9(BB)−2A-GFP (pX458)) expressing the corresponding targeting sgRNA and 1 μg of the corresponding donor oligo or plasmid using the Fugene6 (Promega). To generate *Mir182Δ* and *Mir183Δ* mESCs, cells were transfected with 2 μg of pWH464 expressing a pair of sgRNAs targeting *pri-Mir182* or *pri-Mir183*. The transfected cells were subject to single cell sorting and the resulting clones were subject to genotyping to identify the correct clones.

qRT-PCR

For pri-miRNA quantification, reverse transcription was performed using random hexamers and Superscript2 reverse transcriptase. pre-miRNA and miRNA quantifications were using the Takara's Mir-X miRNA quantification method. qPCR was performed in triplicate for each sample using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) on a CFX96^{TM} real-time PCR detection system (Bio-Rad).

Western blotting

Proteins were harvested in RIPA buffer (10 mM Tris-HCl pH 8.0, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail) and quantified with a BCA assay kit (ThermoFisher). Equal amounts of protein samples were resolved by SDS-PAGE gel, and then transferred to

PVDF membranes. Western blotting was performed using a BlotCycler (Precision Biosystems) with the corresponding primary and secondary antibodies. The membranes were then treated with the Western ECL substrate (Bio-Rad), and the resulting signal was detected using an ImageQuant LAS 500 instrument (GE Healthcare).

Colony formation assay and exit pluripotency assay

For colony formation assay, 500 cells were plated on a 12-well plate in 2i+Lif media or Lif media (DMEM/F12 supplemented with 15% FBS, 1 × penicillin/streptomycin, 0.1 mM Non-Essential Amino Acids, 2 mM L-glutamine, and 0.1 mM 2-mercaptoethanol, and 1000 U/ml Lif). For exit from pluripotency assay, 1000 cells were plated on a gelatin-coated 6-well plate in differentiation media (DMEM/F12 supplemented with 15% FBS, 1 × penicillin/streptomycin, 0.1 mM Non-Essential Amino Acids, 2 mM L-glutamine, and 0.1 mM 2-mercaptoethanol) for 2 days, then cultured in 2i+Lif media for another 5 days. Colonies were stained using AP staining Kit and grouped by differentiation status 6-7 days after plating.

Embryoid body formation

For differentiation via embryoid body formation, 3×10^6 cells were plated per 10 cm bacterial grade Petri dish and maintained on a horizontal rotator with a rotating speed of 30 rpm in differentiation media. The resultant EBs were harvested at the indicated time points.

RNA antisense purification

mESCs were crosslinked with 0.1% formaldehyde for 5min at room temperature, and the crosslinking reaction was quenched by adding 1/20 volume of 2.5M glycine and incubating the mESCs at room temperature for 10min on a rotating platform. The cells

were then harvested and lysed in cell lysis buffer (50mM Tris-HCl pH7.4, 150mM NaCl, 5mM EDTA, 10% glycerol, 1% Tween-20, with freshly added proteinase inhibitors). The cell lysate was cleared by centrifugation at 20,000g for 10min at 4°C. The resulting supernatant was used for RNA antisense purification. 5mg lysate in 500ul lysis buffer was used for each purification. Specifically, a set of 5'-end biotinylated anti-sense DNA oligoes and 5ul RNase inhibitor (NEB) were added to the lysate, resulting in a final concentration of 0.1uM for each oligo. The lysate was incubated at room temperature for 1 hour on a rotating platform. Then 100ul Dynabeads MyOne Streptavidin C1 (Invitrogen) was added and the lysate further incubated for 30min at room temperature on a rotating platform. The magnetic beads were isolated through a magnetic stand and then subject to 4 washes, with each wash in 500ul high salt wash buffer (5XPBS, 0.5% sodium deoxycholate, 1% Triton X-100). The washed beads were resuspended in 100ul DNaseI digestion mix (1X DNase1 digestion buffer with 5ul DNase1 (NEB)) and incubated at 37°C for 20min, followed by adding 350ul LET-SDS buffer (25mM Tris-HCl pH8.0, 100mM LiCl, 20mM EDTA pH8.0, 1%SDS) and 50ul proteinase K (20mg/ml, ThermoFisher). The beads were then incubated on a thermomixer at 55°C 1000rpm for 2 hours. The RNA was isolated through phenol extraction and isopropanol precipitation with glycoblue (Ambion) as a coprecipitant.

Acknowledgments

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Competing interests

The authors declare no competing interests.

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Figure Legends

Figure 1. Ago2 is the major developmentally regulated Argonaute protein in mESCs.

A. Western blotting in the WT, Flag-Ago1, and Flag-Ago2 mESCs.

B. Colony formation assay for the mESCs. The WT mESCs were cultured under the indicated conditions, and the resultant colonies were fixed and stained for AP (alkaline phosphatase activity). The results represent the means (± SD) of four independent experiments.

C. Western blotting in the WT mESCs cultured under the indicated conditions.

D. Outline of identifying miRNAs that can potentially regulate *Ago2*.

E. Expression levels of the identified miRNAs from (D) in mESCs. CPM: counts per million reads.

The following figure supplements are available for Figure 1:

Figure 1 - figure supplement 1. Expression of Argonaute proteins in mESCs.

Figure 1 – figure supplement 2. *Mir182* and *Mir183* are associated with *Ago2* mRNA in mESCs.

Figure 1 – source data1

Tiff files of raw gel images for Figure 1A,C; Figure 1-figure supplement1C

Excel files of numbers for Figure 1B,E; Figure 1-figure supplement 1A,D, Figure 1-figure supplement 2.

Figure 2. *Mir182*/*Mir183* regulate *Ago2* and maintain stemness in mESCs.

A. qRT-PCR on *Mir182* and *Mir183*. For each miRNA, the expression level in WT cells was set as 1 for relative comparison. U6 RNA was used for normalization. The results represent the means (± SD) of three independent replicates.

B. Western blotting in the WT, *Mir182Δ*, *Mir183Δ*, and *Mir182Δ*/*Mir183Δ* mESCs. Gapdh was used for normalization in calculating the relative expression levels.

C. Colony formation assay for mESCs. The mESCs were cultured in 15%FBS + Lif for 7 days, and the resultant colonies were fixed and stained for AP.

D. Exit pluripotency assay for mESCs. The mESCs were induced to exit pluripotency in medium without Lif for 2 days and then switched to 2i+Lif medium for 5 days. The resultant colonies were fixed and stained for AP.

In C and D, the colony morphology and AP intensity were evaluated through microscopy. 100-200 colonies were examined each time to determine the percentage of undifferentiated colonies. The results represent the means (± SD) of three independent experiments.

E. Western blotting of pluripotency factors during EB formation.

The following figure supplement is available for Figure 2:

Figure 2 - figure supplement 1. *Ago2* mRNA is a target of *Mir182* and *Mir183* in mESCs.

Figure 2 – source data1

Tiff files of raw gel images for Figure 2B,E; Figure2-figure supplement1C.

Excel files of numbers for Figure 2A,C,D; Figure2-figure supplement1B.

Figure 3. *Mir182*/*Mir183*-mediated repression of *Ago2* is required for maintaining pluripotency.

A. Mutating *Mir182* and *Mir183* binding sites in *Ago2* mRNA's 3'UTR via genome editing.

B. Genotyping of the Ago2 3'UTR mutant. The PCR was performed using the oligoes (F and R) indicated in A.

C. Western blotting in the WT, *Ago2* 3'UTR mutant, *Mir182Δ/ Mir183Δ*, and *Mir182Δ/ Mir183Δ/Ago2* 3'UTR mutant.

D. Colony formation assay for mESCs.

E. Exit pluripotency assay for mESCs.

In D and E, the colony morphology and AP intensity were evaluated through microscopy. The results represent the means (± SD) of four independent experiments. *p<0.05 by the Student's t-test.

F. Western blotting of pluripotency factors in day 5 EBs.

The following figure supplement is available for Figure 3:

Figure 3 – figure supplement 1. Inhibition of *Mir182/Mir183*-mediated regulation of *Ago2* in mESCs.

Figure 3 – source data1

Tiff files of raw gel images for Figure 3C,F; Figure 3-figure supplement1B.

Excel files of numbers for Figure 3D,E; Figure 3-figure supplement1A,C.

Figure 4. The stemness defects in the 3'UTR mutant mESCs are caused by elevated *let-7* miRNAs.

A. Relative levels of miRNAs, *let-7* pri-miRNAs, and *let-7* pre-miRNAs in the WT and the *Ago2* 3'UTR mutant mESCs. For each miRNA, pri-miRNA, and pre-

miRNA, the expression level in WT cells was set as 1 for relative comparison. U6 RNA was used for normalization in miRNA and pre-miRNA quantification; 18S rRNA was used for normalization in pri-miRNA quantification. The heatmap was generated from the means of three independent replicates.

B. Colony formation assay for WT and the *Ago2* 3'UTR mutant mESCs cultured in the presence of 500 nM anti-*let-7* LNA or a control LNA. The results represent three independent experiments. *p<0.05, and n.s. not significant (p>0.05) by the Student's t-test.

Figure 4 – source data1

Excel files of numbers for Figure 4A,B.

Figure 5. *Mir182/Mir183* and Trim71 function in parallel to repress *Ago2* mRNA in mESCs.

A. Western blotting in the WT mESCs expressing either a vector or FLAG-Trim71 and in the 3'UTR mutant mESCs expressing either a vector or FLAG-Trim71.

B. Western blotting in the WT, 3'UTR mutant, and 3'UTR mutant/CLIPΔ mESCs.

In A and B, Gapdh was used for normalization in calculating the relative expression levels.

C. Colony formation assay for mESCs.

D. Exit pluripotency assay for mESCs.

The following figure supplement is available for Figure 5:

Figure 5 – figure supplement 1. Generation of the CLIPΔ in the 3'UTR mutant mESCs.

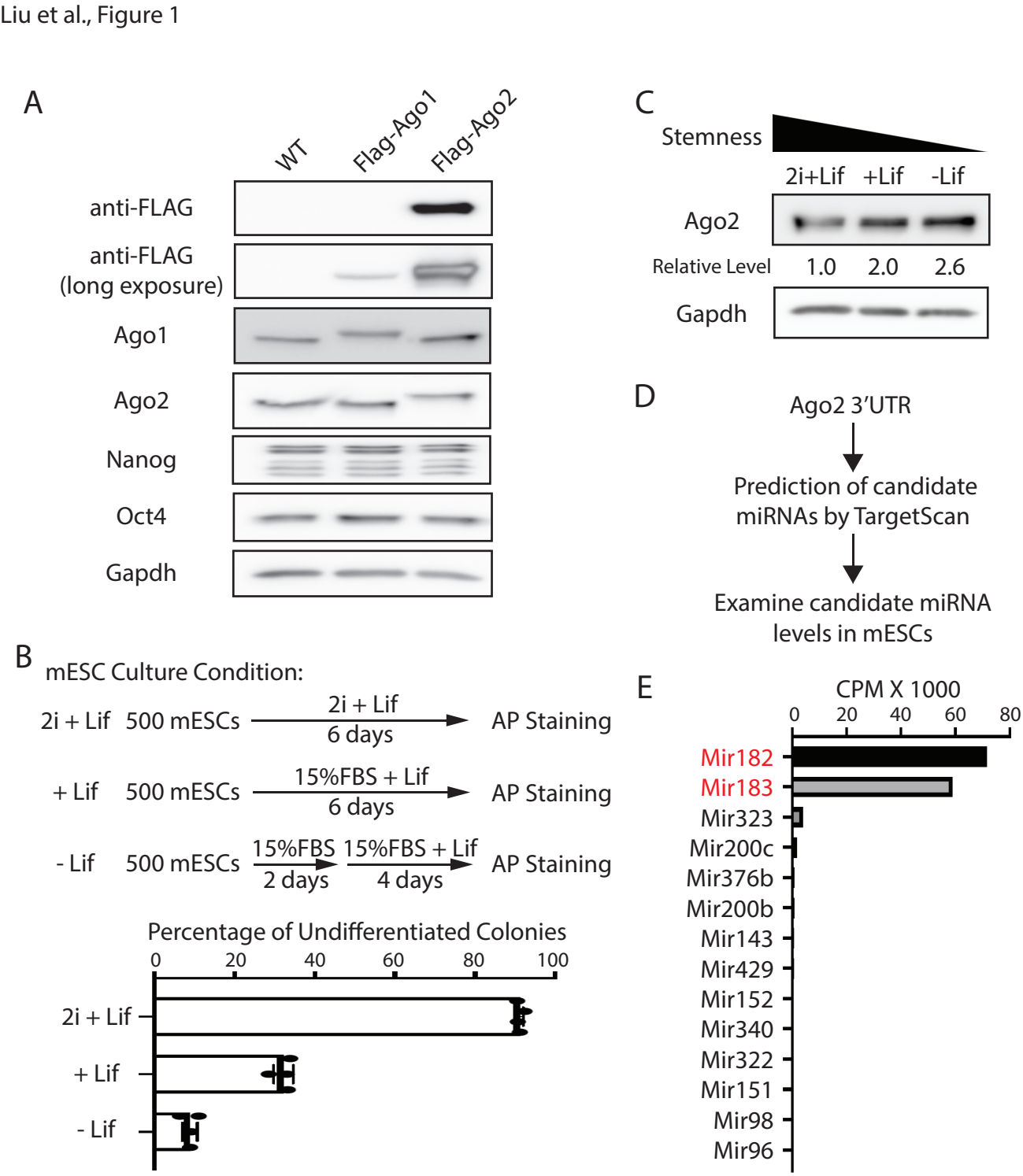
Figure 5 – source data1

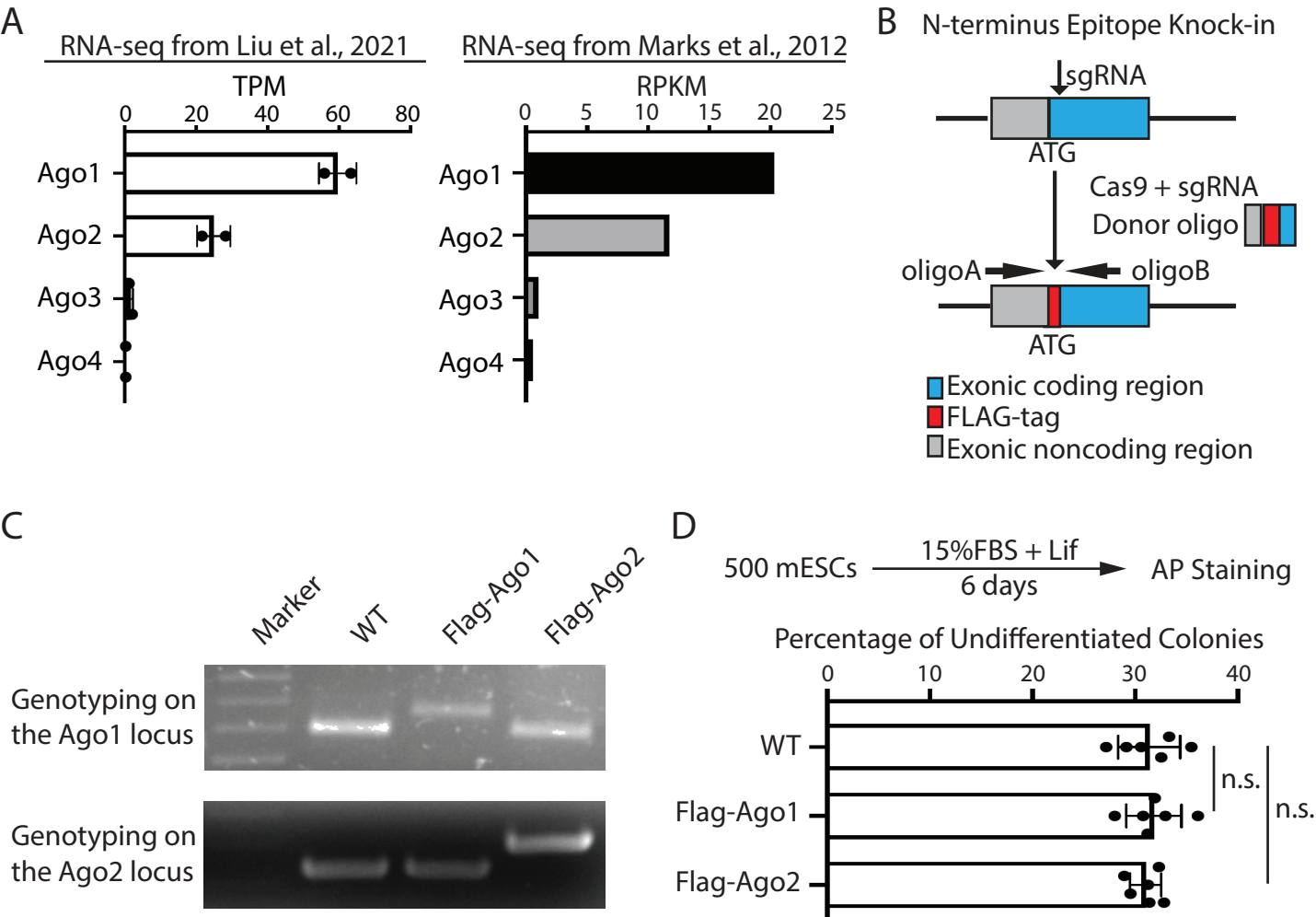
463 Tiff files of raw gel images for Figure 5A,B; Figure 5–figure supplement1

464 An Excel file of numbers for Figure 5C,D.

465

466 Supplemental file 1. Antibodies, plasmids, and oligonucleotides used in this study.


A
WT
Flag-Ago1
Flag-Ago2
anti-FLAG
anti-FLAG
(long exposure)
Ago1
Ago2
Nanog
Oct4
Gapdh
B
mESC Culture Condition:
2i + Lif 500 mESCs 2i + Lif 6 days AP Staining
+ Lif 500 mESCs 15%FBS + Lif 6 days AP Staining
- Lif 500 mESCs 15%FBS 2 days 15%FBS + Lif 4 days AP Staining
Percentage of Undifferentiated Colonies
0 20 40 60 80 100
2i + Lif
+ Lif
- Lif
C
Stemness
2i+Lif +Lif -Lif
Ago2
Relative Level 1.0 2.0 2.6
Gapdh
D
Ago2 3'UTR
Prediction of candidate miRNAs by TargetScan
Examine candidate miRNA levels in mESCs
E
CPM X 1000
0 20 40 60 80
Mir182
Mir183
Mir323
Mir200c
Mir376b
Mir200b
Mir143
Mir429
Mir152
Mir340
Mir322
Mir151
Mir98
Mir96


A
RNA-seq from Liu et al., 2021
TPM
0
20
40
60
80
Ago1
Ago2
Ago3
Ago4
RNA-seq from Marks et al., 2012
RPKM
0
5
10
15
20
25
Ago1
Ago2
Ago3
Ago4
B
N-terminus Epitope Knock-in
sgRNA
ATG
Cas9 + sgRNA
Donor oligo
oligoA
oligoB
ATG
Exonic coding region
FLAG-tag
Exonic noncoding region
C
Marker
WT
Flag-Ago1
Flag-Ago2
Genotyping on the Ago1 locus
Genotyping on the Ago2 locus
D
500 mESCs
15%FBS + Lif
6 days
AP Staining
Percentage of Undifferentiated Colonies
0
10
20
30
40
WT
Flag-Ago1
Flag-Ago2
n.s.
n.s.

Liu et al., Figure 1-Figure supplement 2

A

mESCs

↓

0.1% formaldehyde crosslinking

↓

Cell Lysis

↓

RNA antisense purification (RAP) via biotinyated probes

↓

Streptavidin-magnetic beads capture

↓

DNaseI digetstion

↓

Reverse crosslinking and Proteinase K digestion 55˚C 2hrs

↓

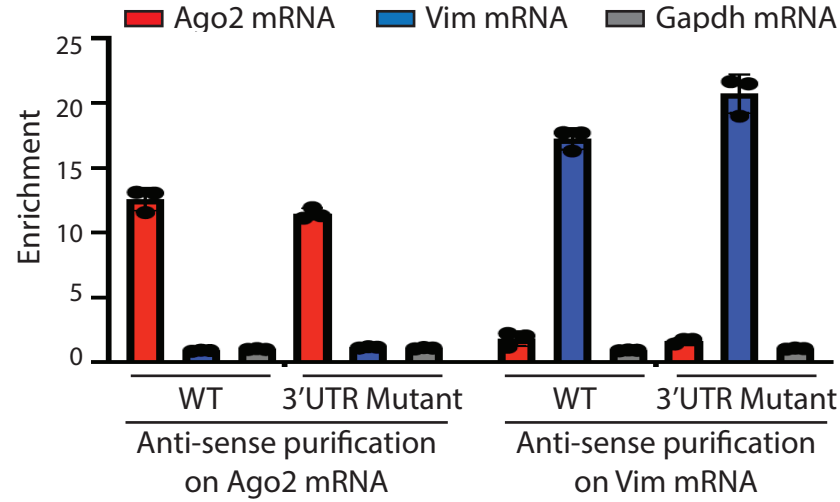
RNA isolation

↓

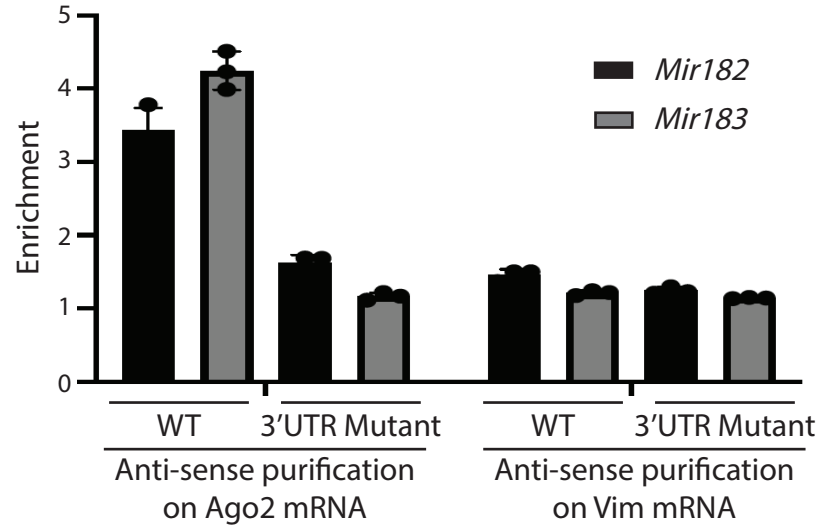
qRT-PCR analysis

$$\text{Enrichment} = \frac{\text{Signal from probes and streptavidin beads}}{\text{Signal from streptavidin beads only}}$$

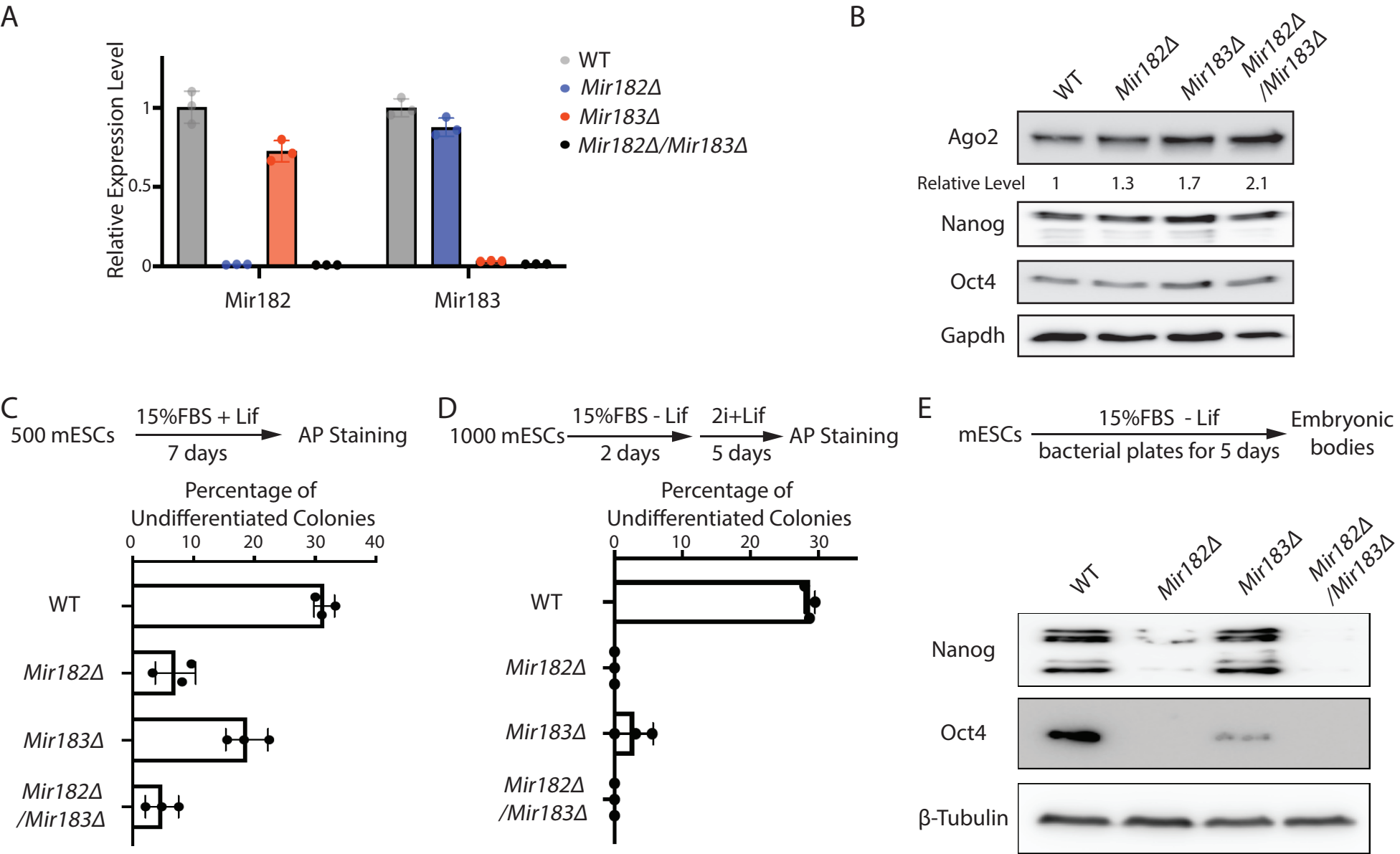
B

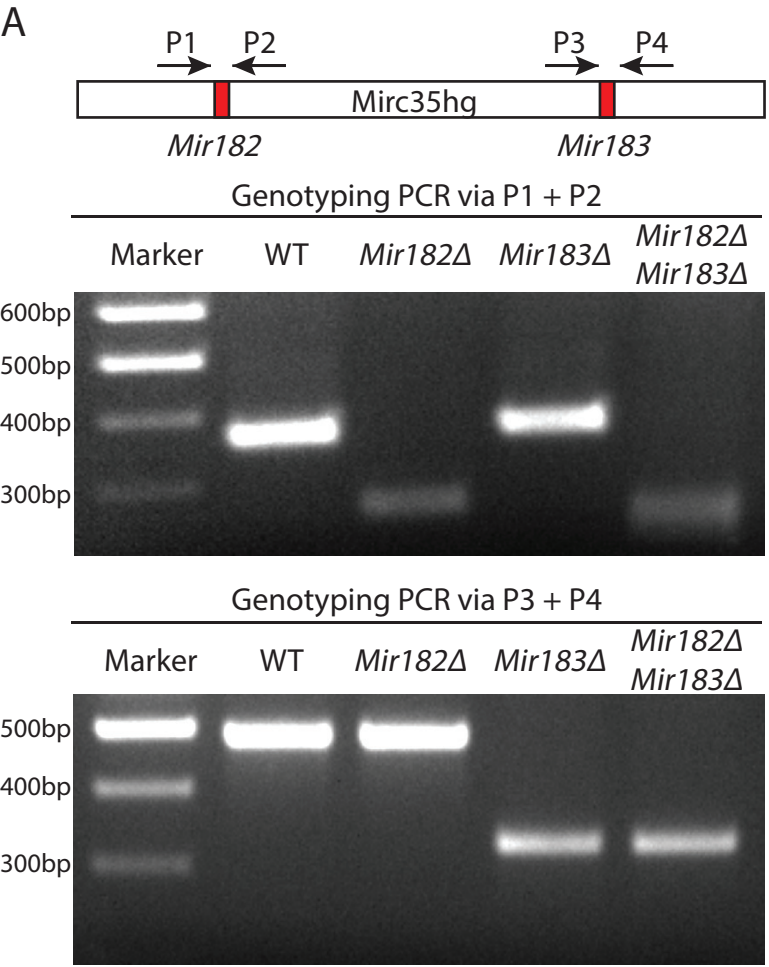


C



Liu et al., Figure 2




A
P1
P2
P3
P4
Mirc35hg
Mir182
Mir183
Genotyping PCR via P1 + P2
Marker
WT
Mir182Δ
Mir183Δ
Mir182Δ Mir183Δ
600bp
500bp
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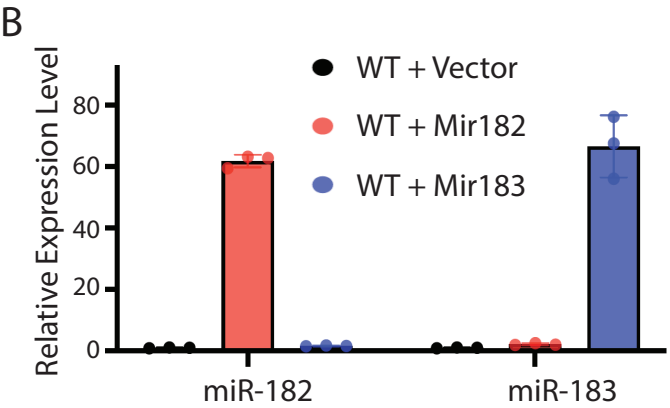
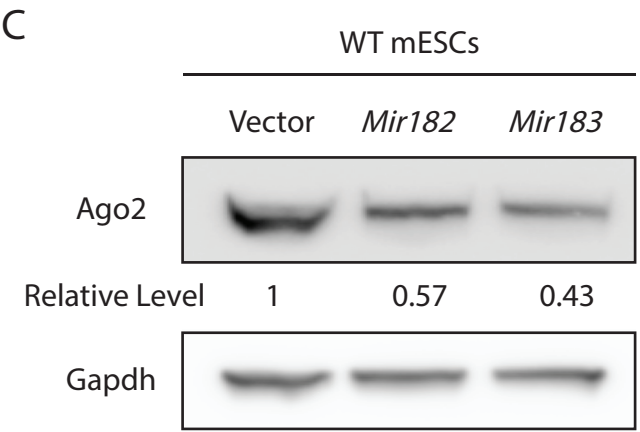
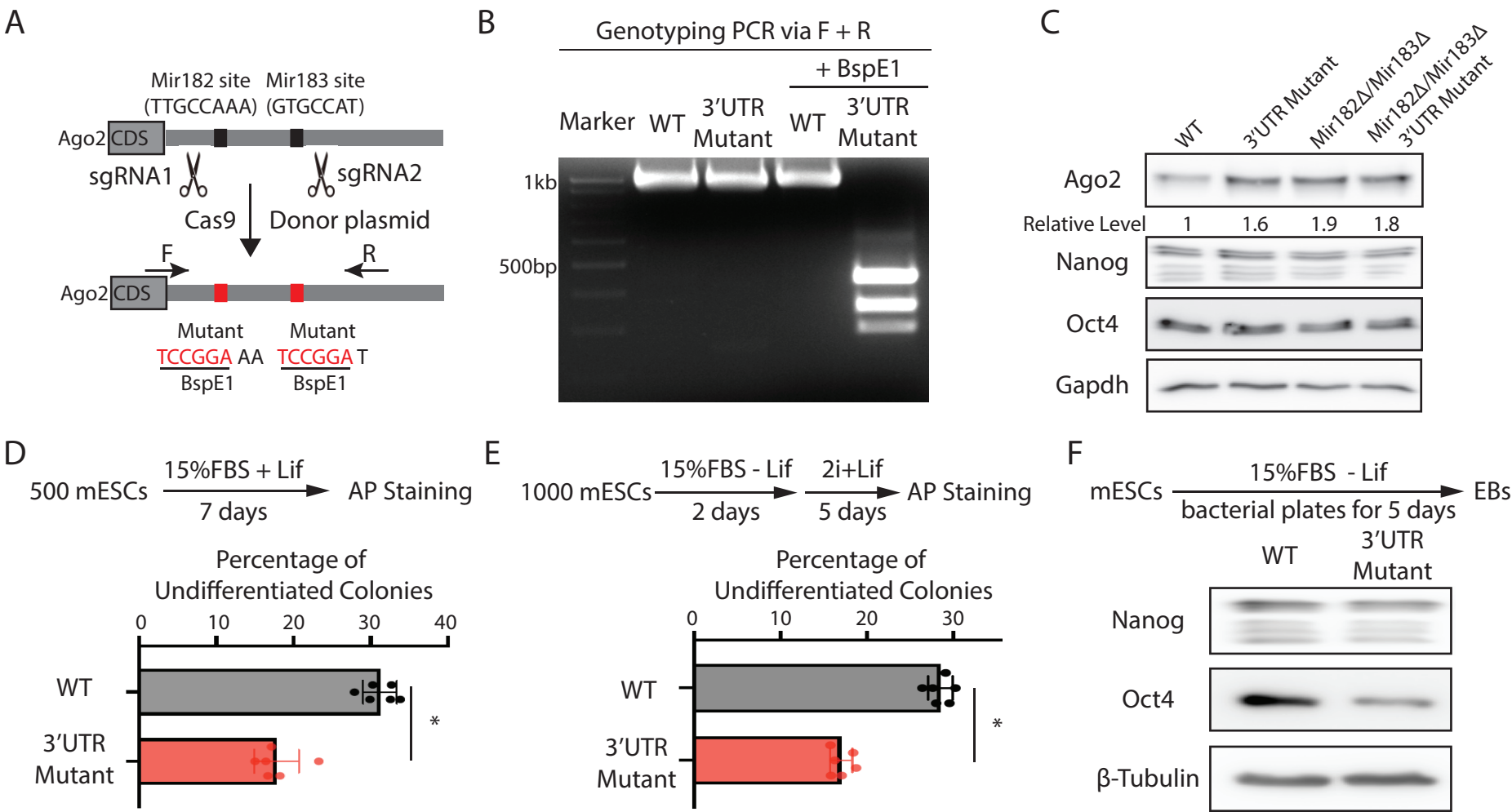
Mir182Δ
Mir183Δ
Mir182Δ Mir183Δ
500bp
400bp
300bp


B
Relative Expression Level
80
60
40
20
0
WT + Vector
WT + Mir182
WT + Mir183
miR-182
miR-183


C
WT mESCs
Vector
Mir182
Mir183
Ago2
Relative Level
1
0.57
0.43
Gapdh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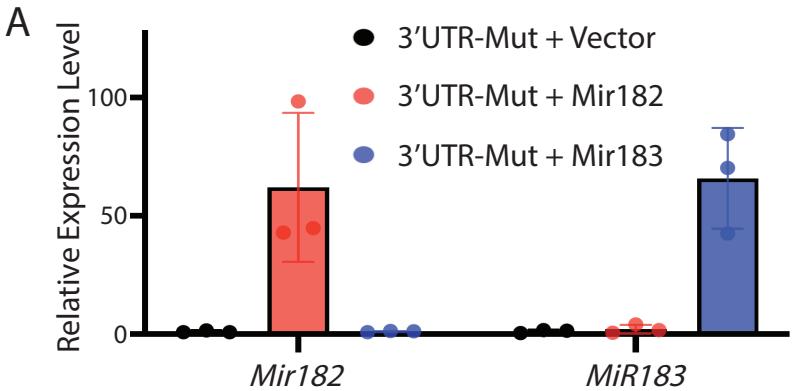
Mir182 site (TTGCCAAA)
Mir183 site (GTGCCAT)
Ago2 CDS
sgRNA1
sgRNA2
Cas9
Donor plasmid
F
R
Ago2 CDS
Mutant TCCGGA AA BspE1
Mutant TCCGGA T BspE1
B
Genotyping PCR via F + R
+ BspE1
Marker
WT
3'UTR Mutant
WT
3'UTR Mutant
1kb
500bp
C
WT
3'UTR Mutant
Mir182Δ/Mir183Δ
Mir182Δ/Mir183Δ 3'UTR Mutant
Ago2
Relative Level 1 1.6 1.9 1.8
Nanog
Oct4
Gapdh
D
500 mESCs
15%FBS + Lif
7 days
AP Staining
Percentage of Undifferentiated Colonies
0 10 20 30 40
WT
3'UTR Mutant
*
E
1000 mESCs
15%FBS - Lif
2 days
2i+Lif
5 days
AP Staining
Percentage of Undifferentiated Colonies
0 10 20 30
WT
3'UTR Mutant
*
F
mESCs
15%FBS - Lif
bacterial plates for 5 days
EBs
WT
3'UTR Mutant
Nanog
Oct4
β-Tubulin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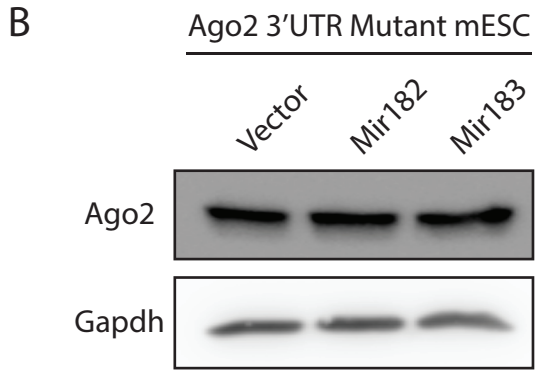
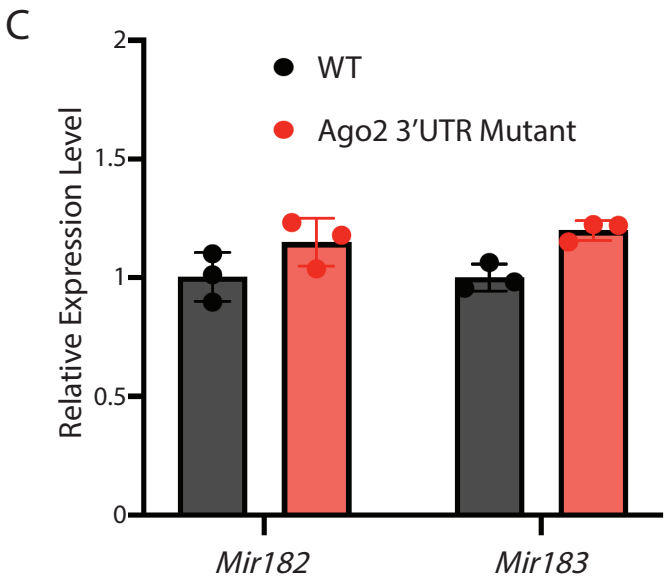
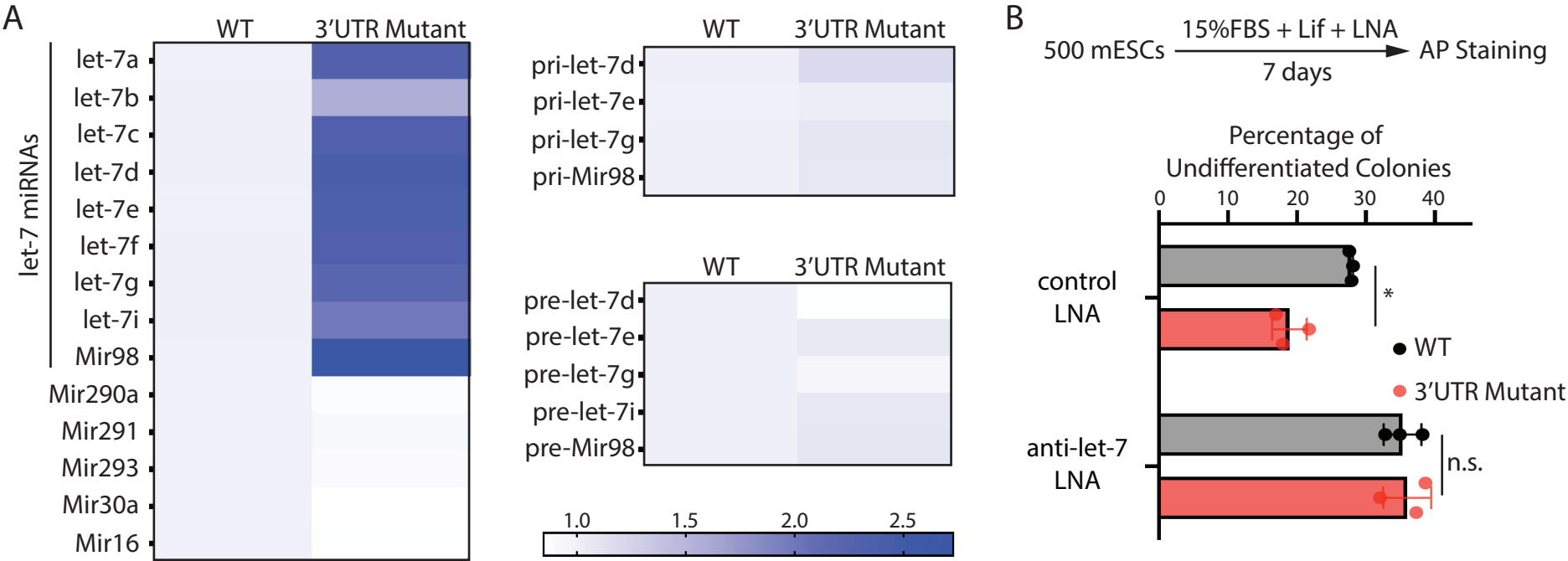
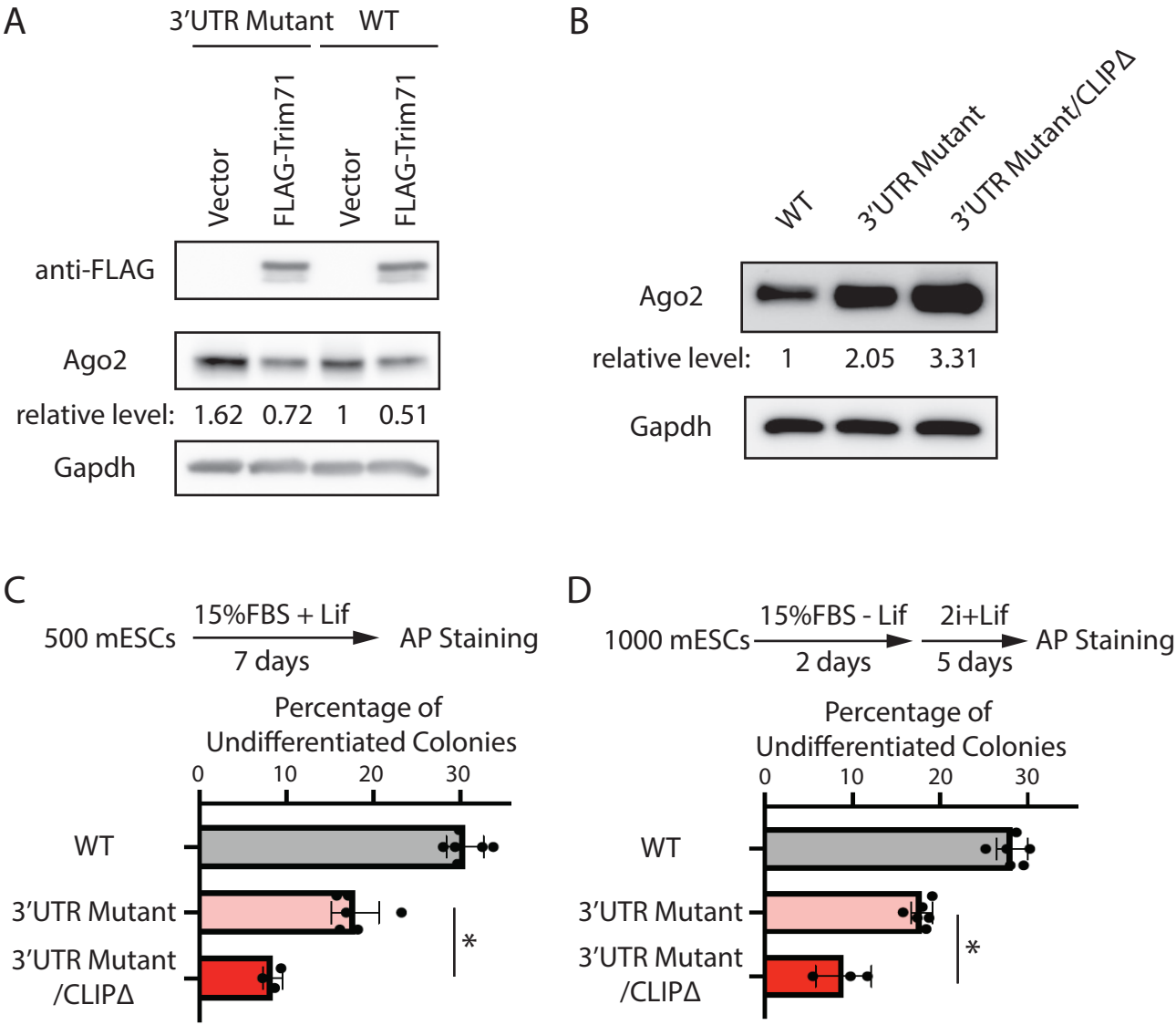
3'UTR-Mut + Mir182
3'UTR-Mut + Mir183
Relative Expression Level
100
50
0
Mir182
MiR183

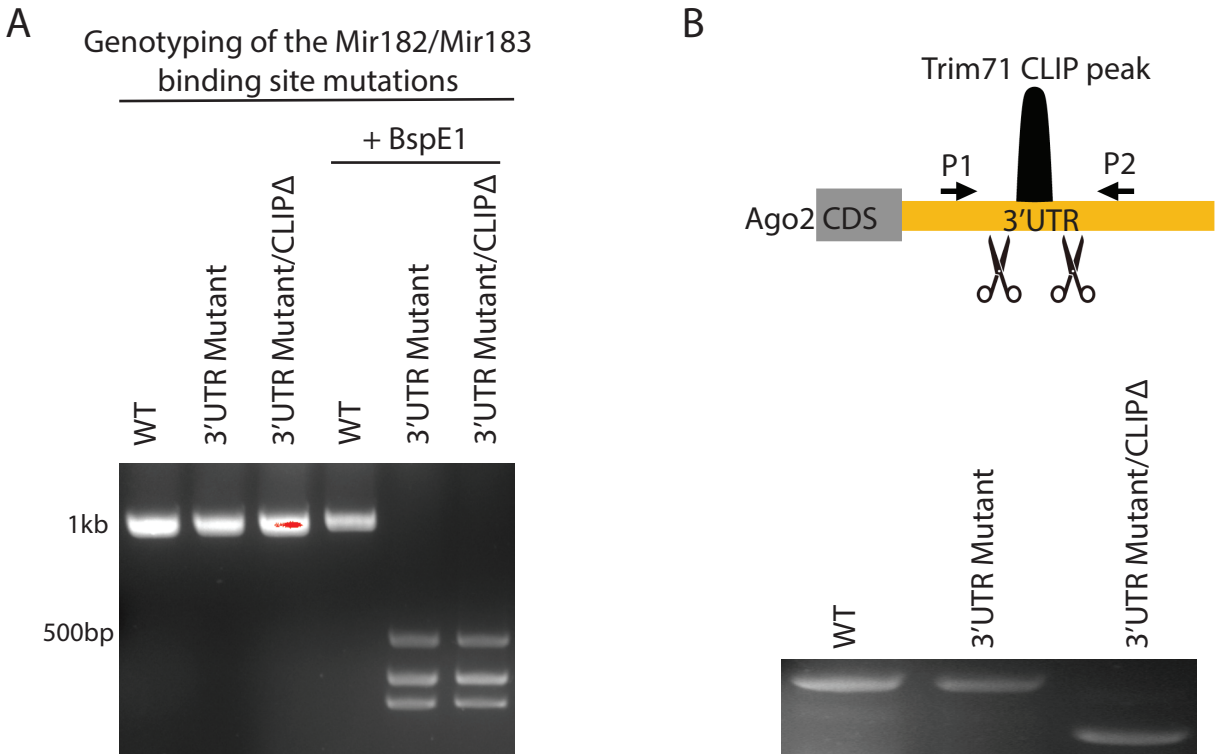

B
Ago2 3'UTR Mutant mESC
Vector
Mir182
Mir183
Ago2
Gapdh


C
WT
Ago2 3'UTR Mutant
Relative Expression Level
2
1.5
1
0.5
0
Mir182
Mir183

Liu et al., Figure 4




A
3'UTR Mutant
WT
Vector
FLAG-Trim71
Vector
FLAG-Trim71
anti-FLAG
Ago2
relative level: 1.62 0.72 1 0.51
Gapdh
B
WT
3'UTR Mutant
3'UTR Mutant/CLIPΔ
Ago2
relative level: 1 2.05 3.31
Gapdh
C
500 mESCs
15%FBS + Lif
7 days
AP Staining
Percentage of Undifferentiated Colonies
0
10
20
30
WT
3'UTR Mutant
3'UTR Mutant /CLIPΔ
*
D
1000 mESCs
15%FBS - Lif
2 days
2i+Lif
5 days
AP Staining
Percentage of Undifferentiated Colonies
0
10
20
30
WT
3'UTR Mutant
3'UTR Mutant /CLIPΔ
*


A
Genotyping of the Mir182/Mir183 binding site mutations
+ BspE1
WT
3'UTR Mutant
3'UTR Mutant/CLIPΔ
WT
3'UTR Mutant
3'UTR Mutant/CLIPΔ
1kb
500bp
B
Trim71 CLIP peak
P1
P2
Ago2 CDS
3'UTR
WT
3'UTR Mutant
3'UTR Mutant/CLIPΔ