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miR-34 miRNAs provide a barrier for somatic cell reprogramming

Yong Jin Choi[1,*], **Chao-Po Lin**[1,*], **Jaclyn J. Ho**[1], **Xingyue He**[2], **Nobuhiro Okada**[1], **Pengcheng Bu**[1], **Yingchao Zhong**[1], **Sang Yong Kim**[3], **Margaux J. Bennett**[1], **Caifu Chen**[4], **Arzu Ozturk**[5], **Geoffrey G. Hicks**[5], **Greg J. Hannon**[6], and **Lin He**[1,#]

[1] Division of Cellular and Developmental Biology, Molecular and Cell biology Department, University of California at Berkeley, Berkeley, CA 94705.

[2]40 Landsdowne Street, Cambridge, MA, 02139

[3]Transgenetic facility, 500 Sunnyside Blvd., Woodbury, NY 11797-2924.

[4]Genomic Assays R&D, Life Technologies Corporation, 850 Lincoln Centre Dr., Foster City, CA 94404.

[5]Manitoba Institute of Cell Biology, 675 McDermot Ave. Rm. ON5029, Winnipeg, MB R3E 0V9, Canada.

[6]Cold Spring Harbor Laboratory, Watson School of Biological Sciences, 1 Bungtown Road, Cold Spring Harbor, NY 11724.

Abstract

Somatic reprogramming induced by defined transcription factors is a low efficiency process that is enhanced by p53 deficiency [1-5]. To date, p21 is the only p53 target shown to contribute to p53 repression of iPSC (induced pluripotent stem cell) generation [1, 3], suggesting additional p53 targets may regulate this process. Here, we demonstrated that *mir-34* microRNAs (miRNAs), particularly *miR-34a*, exhibit p53-dependent induction during reprogramming. *mir-34a* deficiency in mice significantly increased reprogramming efficiency and kinetics, with *miR-34a* and p21 cooperatively regulating somatic reprogramming downstream of p53. Unlike *p53* deficiency, which enhances reprogramming at the expense of iPSC pluripotency, genetic ablation of *mir-34a*

[#] Correspondence should be addressed to lhe@berkeley.edu.
[*]These authors contributed equally.

Author Contributions

Y.C., C.L. and L.H. designed all experiments, and performed majority of the experiments shown in all figures and supplementary figures. J.H. and Y.Z. performed immunofluorescence analyses and teratoma analyses to characterize the pluripotency of the iPSCs. L.H., X.H. P.B. and G.J.H generated knockout constructs for *mir-34a* and *mir-34b/c*, and identified the correctly targeted ESC clones for *mir-34a*. N.O. and P.B. identified correctly targeted ESC clone for *mir-34b/c*, validated *mir-34a* and *mir-34b/c* targeting in mice by southern, and generated *mir-34a*$^{-/-}$, *mir-34b/c*$^{-/-}$ and *mir-34* triple knockout MEFs. S.K. and G.J.H. performed the blastocyst injection for *mir-34a*$^{+/-}$ and *mir-34b/c*$^{+/-}$ ESC clones. A.Z, S.K. and G.G.H. characterized the pluripotency of iPSCs using chimera assays.

Competing financial interests

The authors declare no competing financial interests.

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promoted iPSC generation without compromising self-renewal and differentiation. Suppression of reprogramming by *miR-34a* was due, at least in part, to repression of pluripotency genes, including *Nanog, Sox2* and *Mycn* (*N-Myc*). This post-transcriptional gene repression by *miR-34a* also regulated iPSC differentiation kinetics. *miR-34b* and *c* similarly repressed reprogramming; and all three *mir-34* miRNAs acted cooperatively in this process. Taken together, our findings identified *mir-34* miRNAs as novel p53 targets that play an essential role in restraining somatic reprogramming.

Differentiated somatic cells can be induced to generate pluripotent stem cells that functionally resemble embryonic stem cells (ESCs) [6]. This reprogramming process is rooted in the remarkable cellular plasticity retained during differentiation. The process can be triggered by exogenous expression of a set of defined ESC-specific transcription factors, Pou5f1 (Oct4), Sox2, Klf4, and c-Myc [6-9], which constitute the core regulatory circuits controlling pluripotency and self-renewal. Enforced expression of these reprogramming factors generates iPSCs with low efficiency and slow kinetics, suggesting the existence of cellular and molecular barriers to the process [10].

Recent studies have revealed considerable mechanistic overlap between somatic cell reprogramming and malignant transformation [11]. Cellular mechanisms that enhance reprogramming, including cell proliferation and survival, evasion of DNA damage response, and cell immortalization, have long been known to promote tumorigenesis [3-5, 12]. Several oncogenes and tumor suppressors also serve as essential regulators for reprogramming [6, 10, 11]. Notably, the inactivation of p53, one of the most important tumor suppressors, significantly enhances iPSC generation [1-5, 13]. As a tumor suppressor, p53's transcriptional regulation converges onto multiple target genes that collectively mediate its downstream effects, including cell cycle arrest, cellular senescence, apoptosis, DNA damage response, and genomic stability [14]. Similarly, p53's role in repressing reprogramming also likely is mediated through multiple targets. To date, the cell-cycle regulator p21 is the only p53 target with a demonstrated role in repressing reprogramming. However, p21 deficiency only partially phenocopies that of p53 [1, 3, 15], suggesting that p53 represses iPSC generation through as yet unidentified mechanism(s) and target(s).

Previous studies have identified the *mir-34* miRNAs as *bona fide* p53 transcriptional targets, whose over-expression triggers cell cycle arrest or apoptosis in a cell type- and context-dependent manner [16-18]. miRNAs, a large family of small non-coding RNAs, primarily repress gene expression post-transcriptionally, by pairing with partially complementary mRNA targets [19, 20]. In response to p53 activation, induced *mir-34* miRNAs can mediate p53 downstream effects by repressing specific targets, including *cyclin D1, cyclin E2, Cdk4, Cdk6, Bcl2*, and *c-Met* [21]. Although p53 is primarily characterized for its role in transcriptional activation, it acts as a global gene regulator that both activates and represses gene expression [14]. Direct transcriptional repression, together with indirect post-transcriptional repression through miRNAs such as *mir-34*, constitute two major mechanisms for p53-mediated gene repression.

The *mir-34* miRNAs belong to an evolutionarily conserved family, with three mammalian homologues, *miR-34a, b* and *c*, localized to two distinct genomic loci, *mir-34a* and

mir-34b/c [16]. All three *mir-34* miRNAs were significantly induced when mouse embryonic fibroblast (MEF) reprogramming was triggered with Sox2, Oct4, and Klf4 in the presence or absence of c-Myc (Fig. 2a, data not shown). In both cases, *mir-34* induction, like that of *p21*, was dependent on the activation of intact p53 (Fig. 2a). We therefore investigated whether *mir-34* miRNAs are novel components of the reprogramming regulatory circuit downstream of p53. Among all *mir-34* miRNAs, *miR-34a* exhibited the highest induction level during reprogramming; while *miR-34b* was the lowest. We therefore focused initially on the role of *mir-34a* in iPSC induction.

We generated *mir-34a* knockout mice using C57BL/6 ESCs (Fig. 1a), confirmed the germ line transmission of the targeted allele (Fig. 1b), and verified the genetic ablation of *mir-34a* by expression studies (Fig. 1c).*mir-34a*$^{-/-}$ mice were born at the expected Mendelian ratio, without obvious developmental or pathological abnormalities up to 12 months. However, when we induced the *mir-34a*$^{-/-}$ MEFs for reprogramming, we observed a significant increase in the reprogramming efficiency (Fig. 2b, 2d, S1a). Three-factor-infected *mir-34a*$^{-/-}$ MEFs exhibited a ~4.5-fold increase in alkaline phosphatase (AP) -positive colonies with typical iPSC morphology (Fig. 2b), while four-factor-infected *mir-34a*$^{-/-}$ MEFs yielded a ~4-fold increase (Fig. S1a). Furthermore, when we plated infected MEFs into 96-well plates at a density of one cell per well, *mir-34a* deficiency caused a similar increase in AP-positive colonies with typical iPSC morphology (Fig. 2c).

Using MEFs carrying an *Oct4-Gfp* knockin reporter allele[22], we confirmed the effect of *miR-34a* in promoting reprogramming, scoring fully reprogrammed iPSCs based on endogenous *Oct4* expression indicated by GFP. Consistently, a greater than 4-fold increase in reprogramming efficiency was observed in *mir-34a*$^{-/-}$; *Oct4-gfp*/+ MEFs after three- or four-factor transduction (Fig. 2d). A significant increase in reprogramed *Oct4-gfp* positive colonies could also be achieved using a locked nucleic acid (LNA) inhibitor against *miR-34a* (Fig. S1b). Notably, *mir-34a* deficiency both enhanced the overall efficiency of iPSC generation and led to more rapid reprogramming kinetics. Small iPSC-like colonies first appeared 7 days post-infection in four-factor-transduced wild-type (WT) MEFs, but as early as post-infection day 5 in *mir-34a*$^{-/-}$ MEFs.

Since *miR-34b* and *c* share the *miR-34a* seed sequence and are similarly induced during reprogramming (Fig. 2a), they likely also regulate iPSC generation. We generated *mir-34b/c* knockout mice (Fig. 1a-1c). As with *miR-34a* deficiency, *mir-34b/c* knockout alone promoted somatic reprogramming, although to a lesser degree (Fig. 2e). Interestingly, MEFs deficient for all three *mir-34* miRNAs exhibited an even greater increase in iPSC generation (Fig. 2e), suggesting a cooperative effect among these genes, although the exact molecular and cellular mechanisms underlying this cooperation still remained unclear. Since the *mir-34a*$^{-/-}$; *mir-34b/c*$^{-/-}$ MEFs did not completely phenocopy *p53*$^{-/-}$ MEFs, additional mechanisms may act downstream of p53 to mediate the suppression of reprogramming.

Previous studies have identified p21 as an important mediator of p53 suppression of reprogramming [1, 12]. *mir-34* and *p21* both exhibited *p53*-dependent induction during reprogramming (Fig. 2a). *p21* induction also was observed in *mir-34a*$^{-/-}$ MEFs (Fig. 3a). While MEFs deficient for *mir-34a* alone or *p21* alone showed comparable increases in iPSC

generation, MEFs deficient for both *mir-34a* and *p21* exhibited a cooperative increase that recapitulated a significant fraction of the p53 effect (Fig. 3c). Given the functional similarities among the three *mir-34* miRNAs, the cooperative effects among *p21* and all *mir-34* miRNAs could be even greater. Thus, the *mir-34* miRNAs, together with *p21*, constitute important downstream effectors of p53 to mediate the repression of reprogramming.

p21 represses reprogramming efficiency primarily through its repression on cell proliferation[12]. Although *miR-34a* and p21 repress reprogramming efficiency similarly (Fig. 3c), their effects on cell proliferation were quite different. The increased cell proliferation in *p21*$^{-/-}$ MEFs was highly significant (Fig. 3b), yet within the time frame of our reprogramming experiments, *mir-34a*$^{-/-}$ MEFs exhibited little increase up to passage 6. We can not completely exclude the possibility that a mild increase in *mir-34a*$^{-/-}$ MEF proliferation contributed to the increased reprogramming. Yet unlike p21, whose effects on reprogramming are largely attributed to its inhibition of cell proliferation [12], *miR-34a* is likely to act mostly through a separate mechanism independent of cell proliferation. These two distinct mechanisms may underlie the cooperative regulation of reprogramming by *miR-34a* and p21.

mir-34a$^{-/-}$ iPSCs resemble WT iPSCs and ESCs, exhibiting ESC-like morphology (Fig. 4a, S1c) and expressing key molecular markers for pluripotency. A high level of Oct4, Nanog, and SSEA1 were detected (Fig. 4b, S1d). *mir-34a*$^{-/-}$ iPSCs injected into immunocompromised nude mice yielded differentiated teratomas, containing terminally differentiated cell types from all three germ layers (Fig. 4c, 4d, S1e). Furthermore, three independent lines of four-factor-induced *mir-34a*$^{-/-}$ iPSCs all yielded healthy adult chimeric mice with a high percentage of iPSC contribution (Fig. 4e, S1g, Table S1). Taken together, *mir-34a* deficiency enhances the efficiency of iPSC generation without compromising self-renewal and pluripotency.

Although *p53* deficiency induced reprogramming more efficiently than *mir-34a* deficiency, the *p53*$^{-/-}$ iPSCs exhibited compromised self-renewal and differentiation capacity [1, 4]. As also reported by the Yamanaka group [1], we have observed that four-factor-induced *p53*$^{-/-}$ iPSCs lost ESC-like morphology after 5-6 passages in culture, and failed to generate highly differentiated teratomas. In contrast, four-factor-induced *mir-34a*$^{-/-}$ iPSCs remained stable beyond passage 26, with no significant differences in self-renewal compared to WT iPSCs (Fig. S1f). Additionally, generation of healthy adult chimeras from *p53*$^{-/-}$ iPSCs was difficult. The percentage of *p53*$^{-/-}$ iPSC contribution was low, and the majority of such chimeras succumbed to tumorigenesis before 7 weeks [1]. In contrast, *mir-34a*$^{-/-}$ iPSCs exhibited functional pluripotency: all three four-factor-induced *mir-34a*$^{-/-}$ iPSC lines tested gave rise to healthy adult chimeras with a high percentage of iPSC contribution (Fig. 4e, S1g, Table S1), which remained tumor-free for 6 months (to the time of manuscript preparation).

Consistent with these differences, we observed that four-factor-induced *p53*$^{-/-}$ iPSCs, but not *mir-34a*$^{-/-}$ or WT iPSCs, failed to silence retroviral transgenes, thus exhibiting lower levels of the corresponding endogenous genes (Fig. S3a-S3c). The exogenous expression of

reprogramming factors, particularly *c-Myc* (Fig. S3e), may contribute to the impaired self-renewal and differentiation (Fig. S3d), and promote tumorigenesis [1]. In contrast, pluripotency was established easily in *mir-34a*$^{-/-}$ iPSCs by the endogenous transcription factor circuitry, leaving the exogenous transgenes dispensable for the maintenance of pluripotency.

Similar to *mir-34a*$^{-/-}$ iPSCs, *mir-34a*$^{-/-}$; *mir-34b/c*$^{-/-}$ iPSCs also exhibited robust self-renewal and differentiation capacity, with typical ESC morphology, key pluripotency markers, and the ability to generate differentiated teratomas (Fig. S2a-S2c). Yet their ability to generate chimera remains to be determined.

The increased reprogramming efficiency and compromised pluripotency of *p53*$^{-/-}$ MEFs may result from aberrant apoptosis and DNA damage response [4]. However, *mir-34a* deficiency failed to protect cells from apoptosis or DNA damage response during reprogramming (Fig. S4a-S4c). Thus, the enhanced reprogramming efficiency observed in *mir-34a*$^{-/-}$ MEFs may reflect a mechanism largely independent of apoptosis and DNA damage response. These findings contrast to the ability of *mir-34a* overexpression to trigger apoptosis in specific tumor cells [17, 18]. These *mir-34a* effects on apoptosis are likely cell-type-dependent, differing between primary fibroblasts [16] and specific cancer cell lines [17, 18]. Cells deficient for *mir-34a* may exhibit apoptotic defects under conditions other than reprogramming. It is also possible that the lack of apoptotic protection in reprogramming *mir-34a*$^{-/-}$ MEFs reflects the functional redundancy of *miR-34b* and *c*.

Enhanced reprogramming in *p53*-deficient cells has also been attributed to cell immortalization and increased cell proliferation [1-5, 12, 15]. While cell immortalization greatly promotes iPSC generation from *p53*$^{-/-}$ MEFs [5], increased cell proliferation is a key mechanism driving stochastic reprogramming of *p53*^{KD} B cells [12]. Although *mir-34* overexpression induced growth arrest and cellular senescence in primary fibroblasts [16], no defects in senescence response were observed in *mir-34a*$^{-/-}$ MEFs during reprogramming (Fig. S4d and data not shown). As described above, we did not observe significant MEF proliferation difference caused by *miR-34a* deletion within the time frame of our reprogramming experiment. Presumably, *miR-34a* regulates reprogramming efficiency largely through a mechanism independent of cell proliferation and cell senescence.

To better define the molecular mechanism of *mir-34a* in reprogramming, we initiated a search for its targets, specifically by examining genes that promotes the iPSC generation for possible *miR-34a* binding sites. RNA22 identified a number of such genes with predicted *mir-34a* sites [23] (Fig. S5d). Of all the genes tested, Nanog, Sox2, and Mycn (N-Myc) emerged as top candidates (Fig. 5a, S5a). All three exhibited *mir-34a*-dependent repression (Fig. 5b, S5c), and each had potent effects in promoting reprogramming [24, 25]. ESCs over-expressing *miR-34a*, *miR-34b*, or *miR-34c* for 48 hours had decreased Nanog, Sox2 and N-Myc protein levels (Fig. 5b), but unaltered mRNA abundance (Fig. S5b). This reduction in Nanog, Sox2, and N-Myc protein levels was not due to ESC differentiation, because the level of Oct4, another pluripotency marker, remained unaltered at 48 hours post-transfection (Fig. 5b). Consistently, levels of Sox2 and Nanog proteins were elevated in *mir-34a*$^{-/-}$ iPSCs and *mir-34a*$^{-/-}$*; mir-34b/c*$^{-/-}$ iPSCs compared to littermate- and passage-controlled WT

iPSCs (Fig. 5c). We also observed increased N-Myc levels in *mir-34a*$^{-/-}$*; mir-34b/c*$^{-/-}$ iPSCs, although this increase was moderate in *mir-34a*$^{-/-}$ iPSCs (Fig. 5c and data not shown). Real-time PCR analysis suggested effective silencing of the *Sox2* transgene in *mir-34a*$^{-/-}$ and *mir-34a*$^{-/-}$*; mir-34b/c*$^{-/-}$ iPSCs (Fig. S3a and data not shown); thus the increased Sox2 level was purely due to alterations in endogenous Sox2. Since Oct4 protein levels were largely unchanged among WT, *mir-34a*$^{-/-}$, and *mir-34a*$^{-/-}$*; mir-34b/c*$^{-/-}$ iPSCs, our data suggest that the increase in Sox2, Nanog, and N-Myc was specific for *mir-34* deficiency, and not due to differences in iPSC pluripotency (Fig. 5c). Thus, when *mir-34a*-deficient cells undergo reprogramming, the post-transcriptional derepression of multiple pluripotency genes is likely to promote and accelerate the establishment of the endogenous regulatory circuitry for pluripotency, thereby enhancing reprogramming efficiency.

N-Myc is a previously identified *miR-34a* target in neuroblastoma cell lines, whose downregulation is mediated through a *miR-34a* binding site within the 3' untranslated region (UTR) [26, 27]. To determine whether *miR-34a* directly targets Sox2 and Nanog, we constructed luciferase reporters that contained the WT 3'UTRs of these genes. Both *Sox2* and *Nanog* 3'UTRs were repressed by exogenous expression of *miR-34a* in *Dicer*-deficient HCT116 cells and in WT ESCs (Fig. S5c and data not shown); mutation of the *miR-34a* binding sites of these reporters significantly compromised *miR-34a*-dependent regulation (Fig. S5c). These results indicate that *miR-34a* directly targets *Nanog*, *Sox2*, and *N-Myc*, thereby impeding iPSC generation. Interestingly, *Nanog* was previously identified as a direct target of p53-mediated transcriptional repression in multiple stem cell systems [28, 29]. Thus, p53 mediates *Nanog* repression both by direct transcriptional silencing and by indirect post-transcriptional silencing through *mir-34*. Consistent with the role of *miR-34a* in negatively regulating multiple pluripotency genes, *mir-34a*$^{-/-}$ iPSCs exhibited delayed kinetics when triggered to differentiate by leukemia inhibitory factor (LIF) withdrawal, both in the presence or absence of retinoic acid (RA) (–LIF and –LIF+RA). Like ESCs, WT iPSCs differentiated quickly when subjected to the –LIF or –LIF+RA conditions, as evidenced by flattened cell morphology and rapid decline of *Nanog*, *Oct4*, and *Sox2* expression (Fig. 5d, 5e). In contrast, *mir-34a*$^{-/-}$ iPSCs exhibited significant kinetic delay during differentiation, evidenced by the slower alterations in morphology and pluripotency gene expression (Fig. 5d, 5e). In *mir-34a*$^{-/-}$ iPSCs, the derepression of multiple pluripotency transcription factors may reinforce the regulatory circuitry to maintain self-renewal, causing less efficient silencing of the self-renewal program during differentiation. Conversely, the derepression of pluripotency genes in *mir-34a*$^{-/-}$ MEFs during reprogramming could promote and accelerate the establishment of the endogenous regulatory circuitry for pluripotency. Our results differ from a recent study where reduced *miR-34a* function did not impact ESC differentiation [30]. This difference may reflect the limited efficacy of a nucleotide-based *miR-34a* inhibitor.

Current studies on gene regulation for pluripotency have focused primarily on transcription factor profiles. This emphasis may provide an incomplete picture, since post-transcriptional gene regulation by miRNAs could add robust and redundant controls to this process. The small size of miRNAs, combined with their imperfect target recognition, give them enormous capacity and versatility to regulate global gene expression [31]. Along with transcription factors, miRNAs have emerged as essential gene regulators in self-renewal and

differentiation of pluripotent stem cells [32, 33]. ESCs with deficient global miRNA biogenesis exhibit proliferation and differentiation defects [34]. In addition, RNA binding protein LIN28, which induces reprogramming by suppressing *let-7* biogenesis [8, 35-37], has been identified as a major reprogramming factor in humans. Beside *let-7* miRNAs, a number of miRNAs are specifically enriched or depleted in pluripotent stem cells and are demonstrated to regulate self-renewal and differentiation [30, 32, 34, 38-40]. Here, we identify the *miR-34* miRNAs as novel regulators that suppress reprogramming downstream of p53, providing direct evidence that modulation of miRNA abundance could constitute a critical step in establishing pluripotency. Since miRNA functions can be manipulated by oligonucleotide-based inhibitors or mimics, our findings suggest a paradigm for promoting reprogramming by manipulating specific miRNA functions.

Although p53 directly regulates hundreds of target genes [14], *mir-34* miRNAs and p21 are the major downstream targets that cooperatively repress iPSC generation. p53 effects on reprogramming reflect changes in cell proliferation, immortalization, apoptosis, and DNA damage response [4, 5, 12]. p21, a key downstream target of p53, represses cell proliferation. Interestingly, *miR-34a* deficiency alters MEF reprogramming not via cell proliferation but, at least in part, by posttranscriptional derepression of pluripotency genes. Thus, *mir-34* miRNAs, together with other p53 targets, including p21, collectively mediate the suppression of somatic reprogramming via multiple critical cellular pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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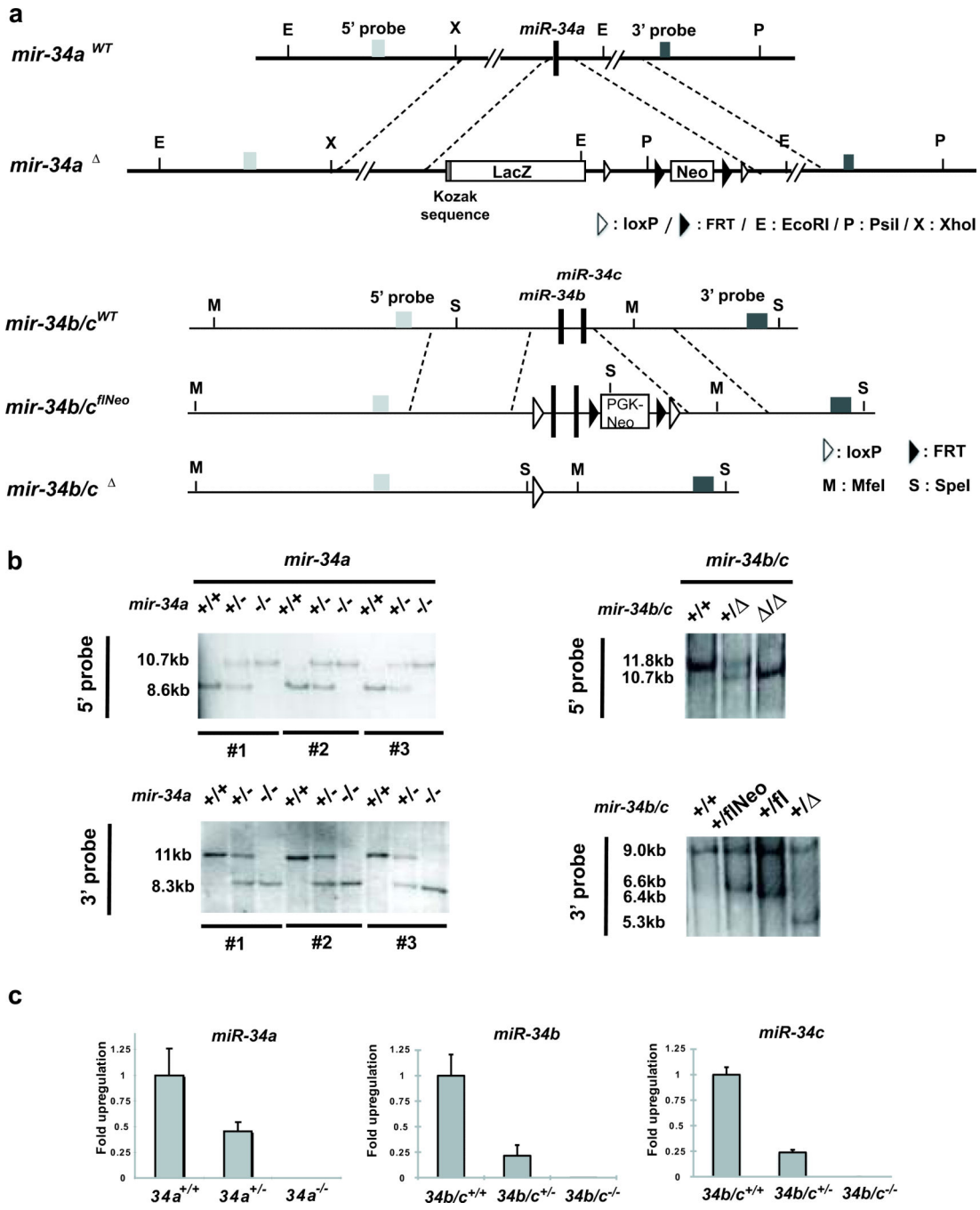

Fig. 1. Generation of *mir-34a* and *mir-34b/c* knockout MEFs

A. Diagrams of endogenous *mir-34a* and *mir-34b/c* gene structure and the knockout construct. Using recombineering, we engineered the *mir-34a* targeting vector with a ~6kb homologous arm on both 5' and 3' ends, flanking a Kozak sequence, a *lacZ* cDNA and a FRT-*neo*-FRT cassette. The *mir-34b/c* targeting vector contains a ~6kb homologous arm at each end, with the *mir-34b/c* gene and a Neo selection cassette flanked by *loxP* sites. **b.** Validating the germline transmission of the *mir-34a* and *mir-34b/c* targeted allele using Southern analysis. Putative WT, *mir-34a*$^{+/-}$ and *mir-34a*$^{-/-}$ animals derived from three

independently targeted ES line were analyzed by Southern blot using probes either 5' or 3' to the homologous arms (left). Similar validation was performed for WT, *mir-34b/c*$^{+/-}$ and *mir-34b/c*$^{-/-}$ animals (right). **c.** Confirming loss of *mir-34* expression in *mir-34a* and *mir-34b/c* knockout MEFs. Littermate-controlled WT, *mir-34a*$^{+/-}$ and *mir-34a*$^{-/-}$ MEFs were analyzed by real-time PCR to quantify the expression of *mir-34a*. While WT MEFs showed robust *miR-34a* induction upon culture stress, no *miR-34a* expression was detected in *mir-34a*$^{-/-}$ MEFs. The *miR-34a* level in *mir-34a*$^{+/-}$ MEFs was approximately half that of WT MEFs. Similar validation was performed for *mir-34b/c*$^{-/-}$ MEFs. Error bar, standard deviation, n=3.

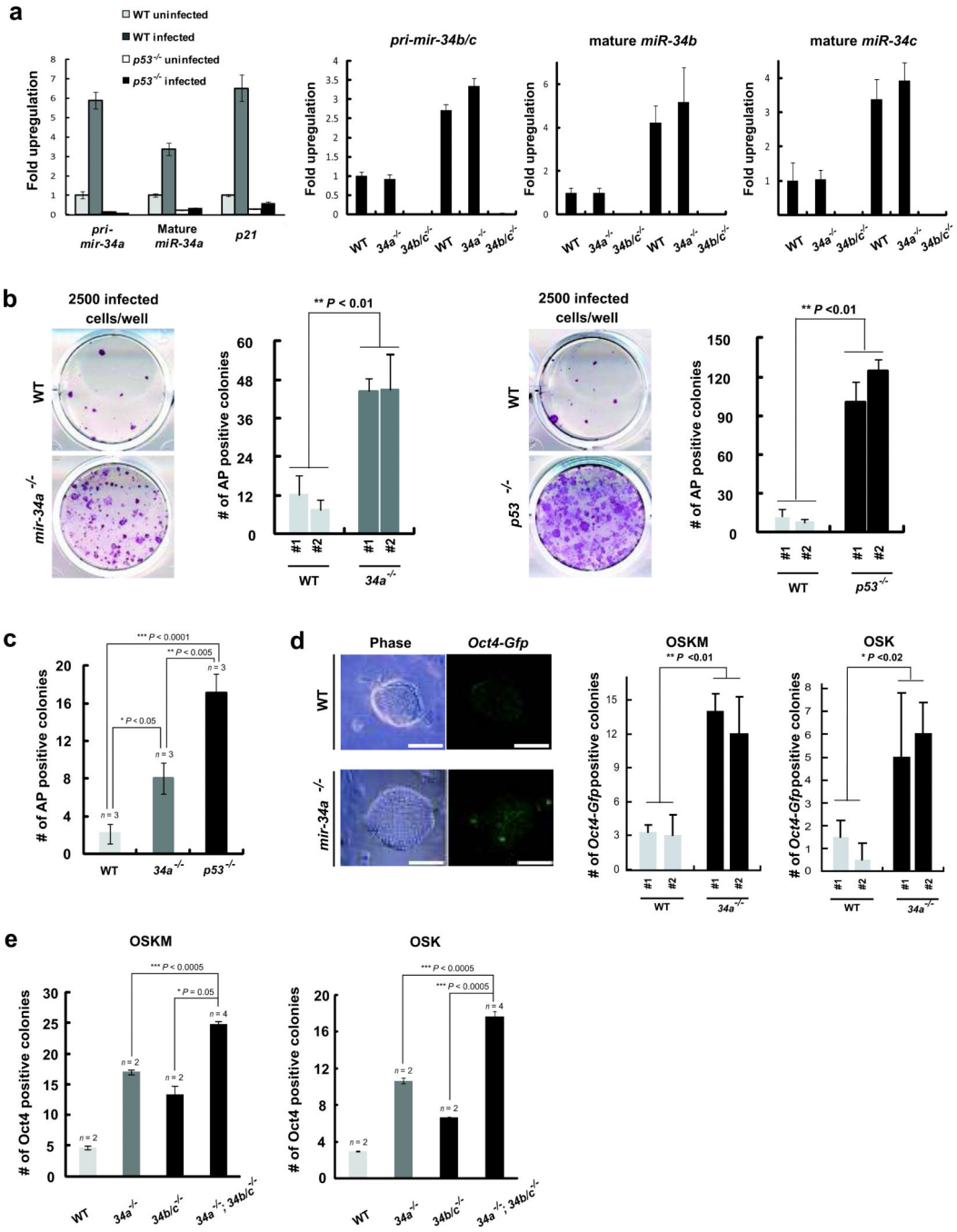

Fig. 2. Deficiency of miR-34 miRNAs increases reprogramming efficiency

a. Four reprogramming factors triggered p53-dependent induction of *miR-34* miRNAs. Three days after transduction, *pri-mir-34a*, mature *miR-34a* and *p21* were measured in uninfected and four-factor induced WT and *p53*$^{-/-}$ MEFs. Induction of *pri-mir-34a* was dependent on the intact p53 response, and was comparable to that of *p21*. Induction of *pri-mir-34b/c*, mature *miR-34b* and *c* was determined in WT, *mir-34a*$^{-/-}$ and *mir-34b/c*$^{-/-}$ MEFs. Error bar, standard deviation, *n*=3. **b.** *mir-34a* deficiency significantly enhanced three-factor induced MEF reprogramming. 2500 three-factor infected WT, *mir-34a*$^{-/-}$ or *p53*$^{-/-}$ MEFs

were plated to score reprogramming by AP-positive colonies with characteristic ESC morphology. A representative image and quantitative analysis is shown out of five independent experiments, comparing littermate-controlled WT and *mir-34a*$^{-/-}$ MEFs (left, $^{**}P < 0.01$), as well as WT and *p53*$^{-/-}$ MEFs (right, $^{**}P < 0.01$). Error bar, standard deviation, n=4. **c.** Single-sorted, four-factor infected MEFs were cultured at a density of one cell per well. Four weeks post-plating, AP-positive colonies with typical iPSC morphology were scored for WT, *mir-34a*$^{-/-}$ and *p53*$^{-/-}$ iPSCs. Four independent experiments confirmed this finding. $^{*}P < 0.05$ for comparison between WT and *mir-34a*$^{-/-}$ MEFs. Error bar, standard error, n= experiments with independent MEF lines. **d.** *mir-34a* deficiency significantly enhanced MEF reprogramming as measured by *Oct4-Gfp* reporter expression. Three- or four-factor infected WT and *mir-34a*$^{-/-}$ MEFs that carry an *Oct4-Gfp* allele were sorted at the density of 2500 cells/well and 1000 cells/well, respectively. Reprogramming efficiency was quantified by GFP positive clones. Images of *Oct4-Gfp* positive iPSCs were shown on the left. A quantitative analysis for reprogramming efficiency triggered by four factor (left, $^{**}P<0.01$) or three factor (right, $^{*}P<0.02$) was shown. Scale bar, 100μm. Error bar, standard deviation, n=3. OSKM, Oct4, Sox2, Klf4 and Myc; OSK, Oct4, Sox2 and Klf4. **e.** *miR-34a, b*, and *c* cooperatively regulate somatic reprogramming. Deficiency in *mir-34a* or *mir-34b/c* alone significantly promoted somatic reprogramming, yet deficiency in all *mir-34* miRNAs exhibited further increase. Two independent experiments confirmed this finding. Error bar, standard error, n = experiments with independent MEFs. All P-values were calculated based on two-tailed Student's *t*-test.

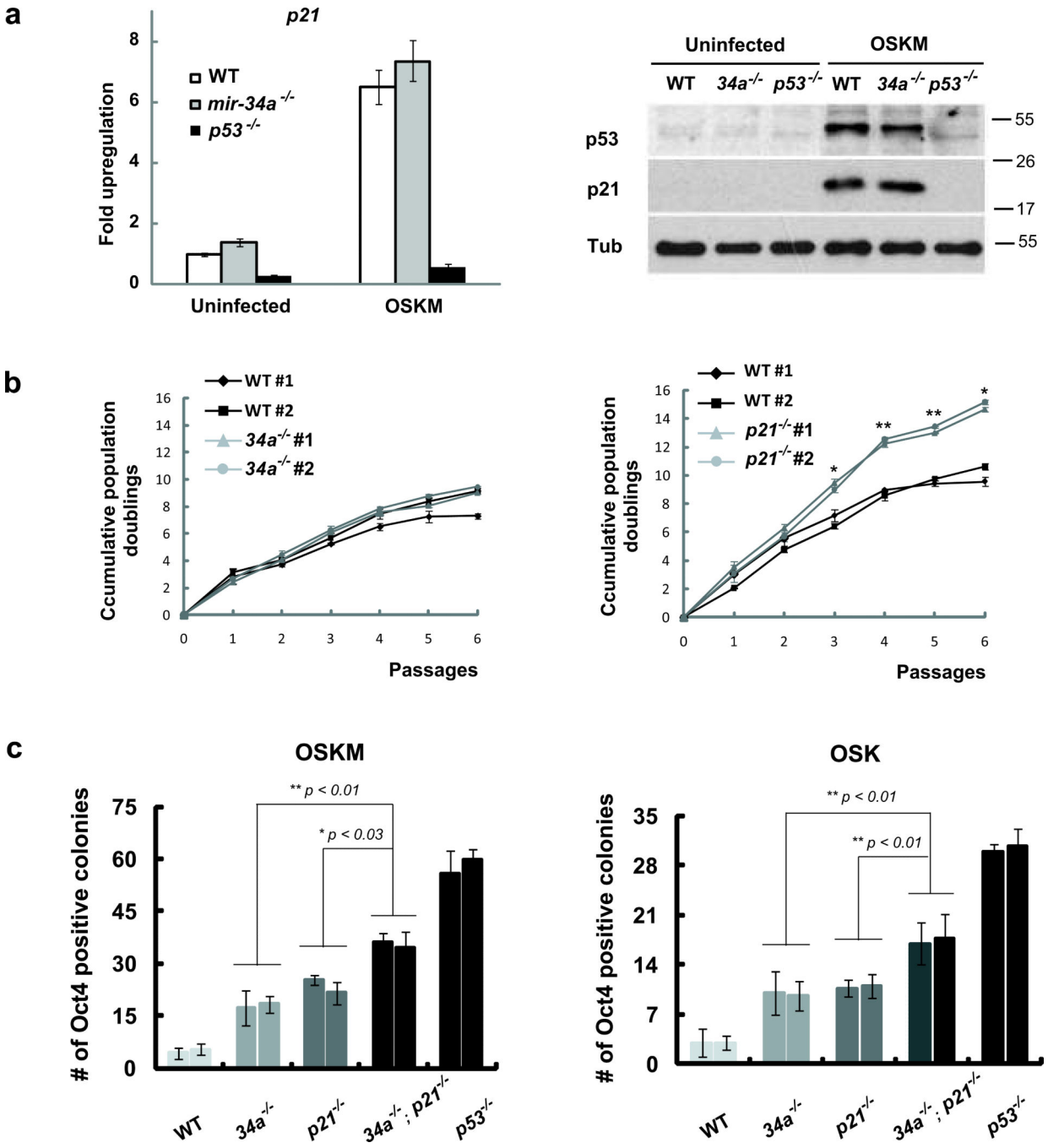

Fig. 3. *miR-34a* and *p21* cooperate to repress iPSCs generation

a. *p21* was induced in *mir-34a*$^{-/-}$ MEFs during somatic reprogramming. Three days after retroviral transduction of four reprogramming factors, both *p21* mRNA (left) and p21 protein (right) exhibited a significant increase in WT and *mir-34a*$^{-/-}$ MEFs. This increase correlated well with the elevated level of p53 proteins (right). α-Tubulin (Tub) was used as a loading control. Error bar, standard deviation, *n*=3. **b.** *p21*$^{-/-}$ MEFs proliferate more rapidly than *mir-34a*$^{-/-}$ MEFs. Cumulative population doublings were measured for 6 consecutive passages in littermate-controlled WT and *mir-34a*$^{-/-}$ MEFs (left), and in WT and *p21*$^{-/-}$

MEFs (right). Compared to the WT counterparts, *p21*$^{-/-}$ MEFs exhibited an enhanced cell proliferation rate, while *mir-34a*$^{-/-}$ MEFs showed little differences. Error bar, standard deviation, n=3 for triplicate measurements at each time point. *$P < 0.05$; **$P < 0.01$ for comparisons between two lines of MEFs for each genotype. **c.** *miR-34a* and *p21* cooperate to repress iPSCs generation. The reprogramming efficiency were compared among WT, *mir-34a*$^{-/-}$, *p21*$^{-/-}$, *mir-34a*$^{-/-}$; *p21*$^{-/-}$ and *p53*$^{-/-}$ MEFs using either three (right) or four (left) reprogramming factors. Deficiency in *mir-34a* or *p21* alone enhanced reprogramming efficiency to a comparable level. Deficiency in both *mir-34a* and *p21* gave rise to an even greater reprogramming efficiency. Quantitative analyses of Oct4-positive colonies were carried out at 2 (four-factor induced reprogramming) or 3 (three-factor induced reprogramming) weeks post-plating using immunofluorescence analyses. Error bar, standard deviation, n=3. OSKM, Oct4, Sox2, Klf4 and Myc; OSK, Oct4, Sox2 and Klf4; MW, molecular weight. All P-values were calculated based on two-tailed Student's *t*-test.

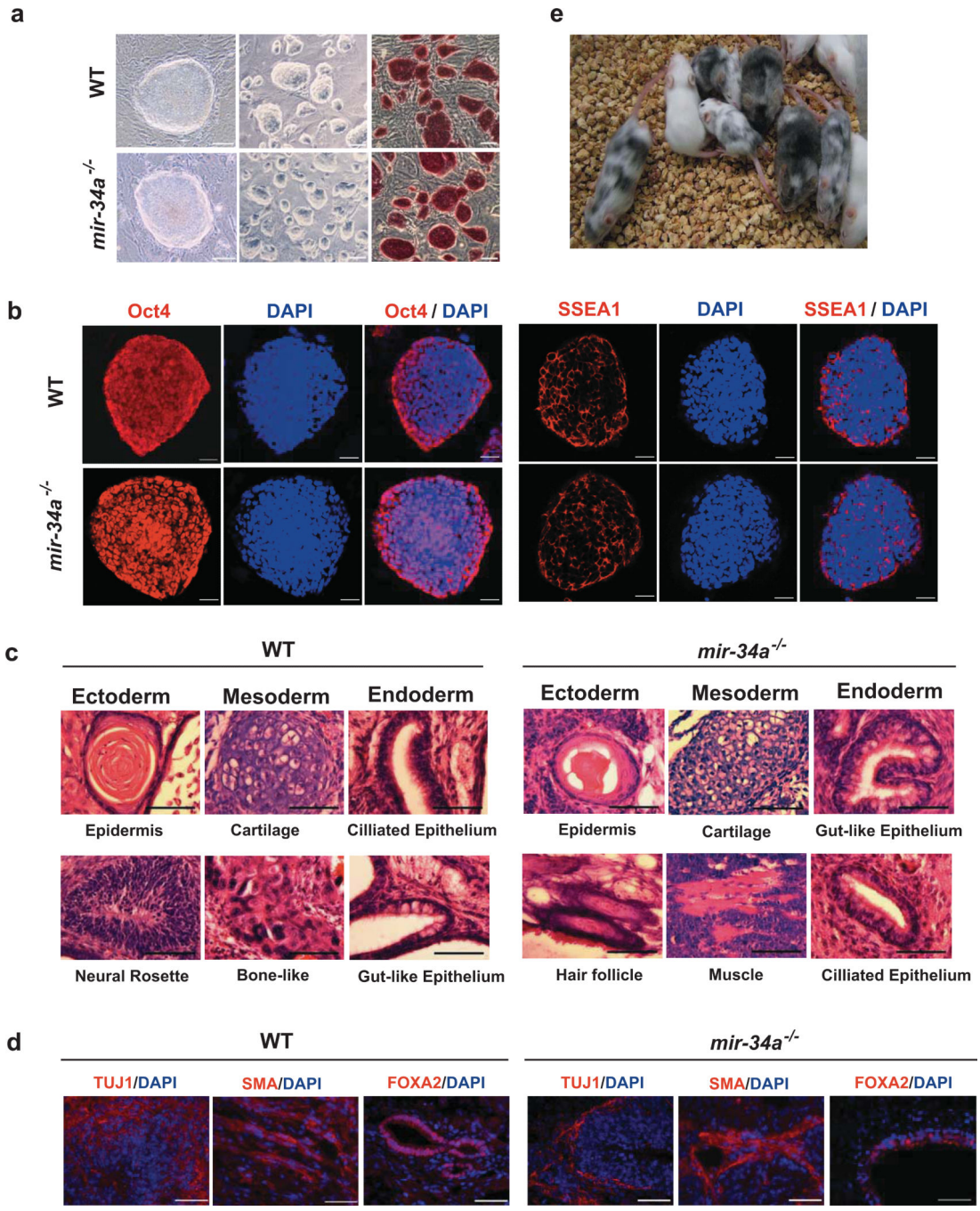

Fig. 4. *mir-34a*$^{-/-}$ iPSCs functionally resemble WT iPSCs

a. iPSCs derived from both WT and *mir-34a*$^{-/-}$ MEFs exhibited ES-like morphology in culture, with robust AP expression. Scale bar, 20μm for the left panel, 100 μm for the middle and right panels. **b.** Both WT and *mir-34a*$^{-/-}$ iPSCs expressed pluripotency markers, including nucleus-localized Oct4 and membrane-localized SSEA1. Scale bar, 20μm; **c, d.** Wildtype and *mir-34a*$^{-/-}$ iPSCs both generated differentiated teratomas. Teratomas derived from four-factor induced WT (left) and *mir-34a*$^{-/-}$ (right) iPSCs were harvested from nude mice 4-6 weeks after subcutaneous injection. H&E staining(**c**), as well as

immunofluorescence staining (**d**), revealed terminally differentiated cell types derived from all three germ layers. Scale bar in **c,** 25μm; in **d**, 50μm. **e.** Four-factor-induced *mir-34a*$^{-/-}$ iPSCs efficiently contribute to adult chimeric mice. We injected three independent lines of passage seven *Oct4-Gfp/+*, *mir-34a*$^{-/-}$ iPSCs into albino-C57BL/6/cBrd/cBrd/cr blastocysts. The iPSC contribution to adult chimeric mice was determined by coat color pigmentation.

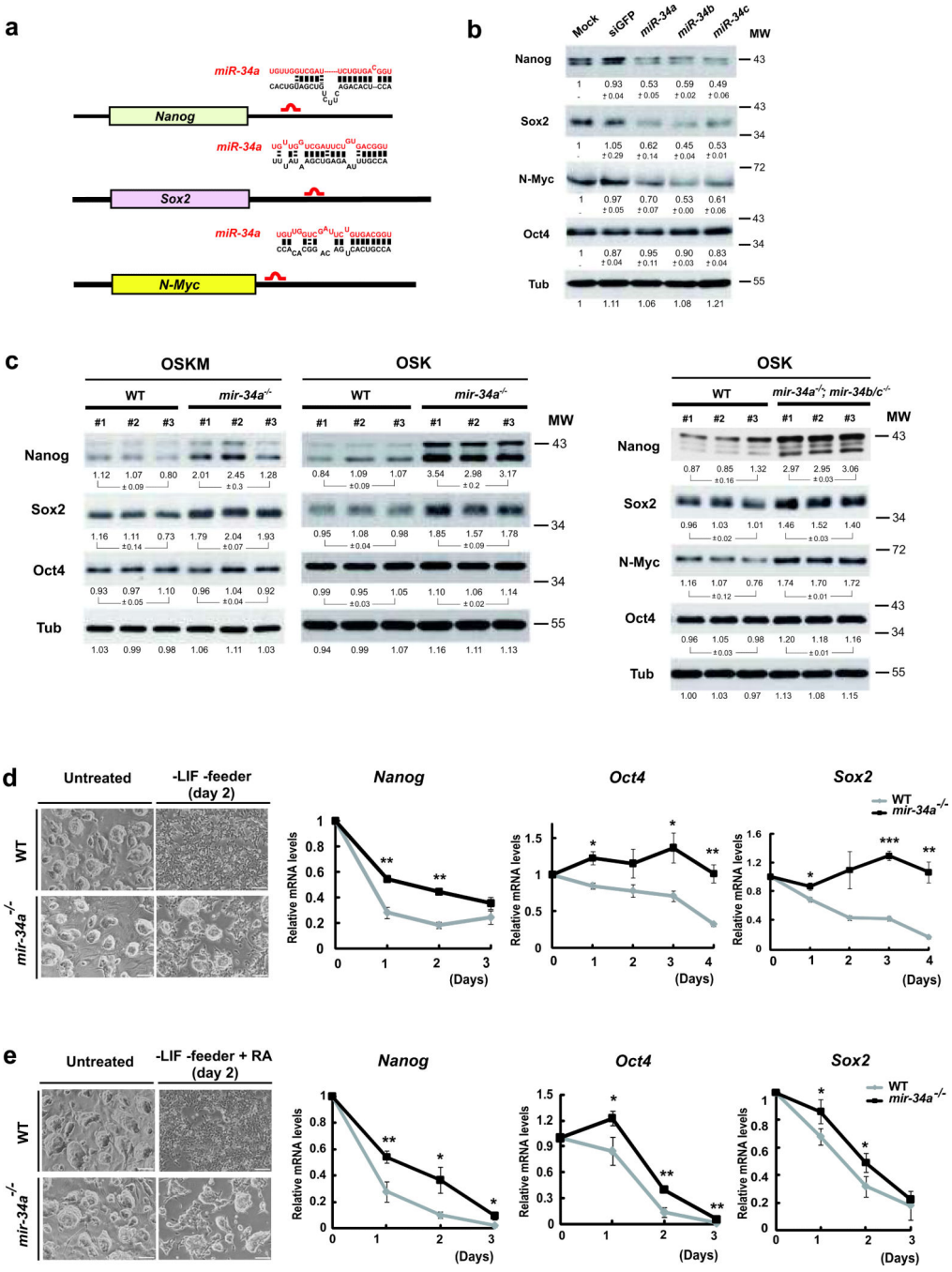

Fig. 5. *mir-34a* represses Nanog, Sox2 and N-Myc expression post-transcriptionally

a. Schematic representation of the *Nanog*, *Sox2* and *N-Myc* 3'UTR, and the predicted *mir-34* binding sites. The mouse *Nanog*, *Sox2* and *N-Myc* 3'UTR each contains one putative *miR-34a* binding site within their 3'UTRs. **b.** Enforced expression of *mir-34a, b, and c* in ESCs reduced the protein levels of Nanog, Sox2 and N-Myc, but not Oct4. Feeder-free ESCs were transfected with miRNA mimics for *miR-34a*, *miR-34b* and *miR-34c*, and a negative control, siGFP. At 48 hours post transfection, Western analysis indicated a significant reduction in the protein levels of Nanog, Sox2 and N-Myc, but not Oct4. The

value of each band indicates the relative expression level normalized by the internal control, α-tubulin, averaged among two independent experiments, and presented as mean ± s.e.m. **c.** Derepression of Nanog, Sox2 and N-Myc was observed in *mir-34* deficient iPSCs. A significant increase of Nanog and Sox2, but not Oct4, was observed in four factor induced *mir-34a*$^{-/-}$ iPSCs, when compared to passage matched, littermate controlled WT iPSCs. A similar comparison was performed for passage matched, three-factor induced WT and *mir-34a* $^{-/-}$; *mir-34b/c*$^{-/-}$ double knockout iPSCs, where derepression of Nanog, Sox2 and N-Myc was observed. For this Western analysis, the quantitation of each band was performed by Quantity One software, and was normalized against its own internal tubulin control. The standard errors of three independent iPSC lines were shown for each genotype, n=3. **d, e.** *mir-34a* deficient iPSCs exhibited slower kinetics during differentiation. Wildtype and *mir-34a*$^{-/-}$ iPSCs were both triggered to differentiate by withdrawal of LIF in the presence (**e**) or absence (**d**) of RA treatment. The image of typical iPSC culture two days after each differentiation condition were shown on the top (**d**, **e**), and the quantitative analyses on the decline of *Nanog*, *Sox2* and *Oct4* transcripts in response to these differentiating conditions were shown on the bottom (**d**, **e**). Error bar, standard error, n=3. * P<0.05, ** P<0.01. Scale bar in d and e, 100 μm. MW, molecular weight.