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Embryonic stem cell–specific microRNAs promote induced pluripotency

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This report demonstrates that introduction of microRNAs (miRNAs) specific to embryonic stem cells enhances the production of mouse induced pluripotent stem (iPS) cells. The miRNAs miR-291-3p, miR-294 and miR-295 increase the efficiency of reprogramming by *Oct4*, *Sox2* and *Klf4*, but not by these factors plus *cMyc*. cMyc binds the promoter of the miRNAs, suggesting that they are downstream effectors of cMyc during reprogramming. However, unlike cMyc, the miRNAs induce a homogeneous population of iPS cell colonies.

The miR-290 cluster constitutes >70% of the entire miRNA population in mouse embryonic stem (ES) cells[1]. Its expression is rapidly downregulated upon ES cell differentiation[2]. A subset of the miR-290 cluster, called the ES cell–specific cell cycle–regulating (ESCC) miRNAs, contributes to the unique cell cycle of ES cells[3]. This subset includes miR-291-3p, miR-294 and miR-295. To test whether ESCC miRNAs promote the induction of pluripotency, we introduced these miRNAs along with retroviruses[4] expressing *Oct4*, *Sox2* and *Klf4* into mouse embryonic fibroblasts (MEFs). The MEFs carried two reporters: an *Oct4*–green fluorescent protein (*GFP*) reporter that activates GFP upon the induction of pluripotency and a β-galactosidase/neo fusion reporter ubiquitously expressed from the Rosa26 locus[5]. MiRNAs were introduced on days 0 and 6 post-infection by transfection of synthesized double-stranded RNAs that mimic their mature endogenous counterparts. This method transiently recapitulates ES cell–like levels of the miR-290 cluster miRNAs (**Supplementary Fig. 1** online).

Introduction of *Oct4*, *Sox2* and *Klf4* (ref. 6) plus miR-291-3p, miR-294 or miR-295 consistently increased the number of Oct4-GFP$^+$ colonies compared with controls transduced with *Oct4*, *Sox2* and *Klf4* plus transfection reagent (**Fig. 1a**). The miR-294 mimic showed the greatest effects, increasing efficiency from 0.01–0.05% to 0.1–0.3% of transduced MEFs. Introduction of a chemically synthesized miR-294 pre-miRNA similarly enhanced reprogramming (**Supplementary Fig. 2** online). Two other members of the miR-290 cluster that are not ESCC miRNAs, miR-292-3p and miR-293, did not increase colony number (**Fig. 1a**). The ESCC miRNAs share a conserved seed sequence, which largely specifies target mRNAs (**Fig. 1b**). MiR-302d, a member of another miRNA cluster that has the same seed sequence, also enhanced reprogramming (**Fig. 1b,c**). Mutation of the seed sequence in miR-294 blocked the increase in colony number (**Fig. 1b,c**). In summary, together with *Oct4*, *Sox2* and *Klf4*, the ESCC miRNAs and related miRNAs with a common seed sequence promoted the dedifferentiation of MEFs into Oct4-GFP$^+$ ES cell–like colonies.

Consistent with previous observations that ESCC miRNAs act redundantly[3], mixes of the different ESCC miRNAs did not further enhance reprogramming efficiency beyond miR-294 alone (**Supplementary Fig. 3** online). Therefore, subsequent studies focused on miR-294. Increasing doses of miR-294 further enhanced Oct4-GFP$^+$ colony formation and the Oct4-GFP$^+$ cellular fraction (**Fig. 1d** and **Supplementary Fig. 4** online). At the highest doses, miR-294 increased the number of colonies to ~75% of that achieved with *Oct4*, *Sox2*, *Klf4* and *cMyc* (0.4–0.7% of starting MEFs) (**Fig. 1d**). Addition of the miR-294 mimic increased the kinetics of *Oct4*, *Sox2* and *Klf4* reprogramming to rates similar to those of four-factor reprogramming (**Supplementary Fig. 5a** online). Transfection with miR-294 did not enhance the reprogramming efficiency of any other combination of three of the four factors or of all four factors (**Fig. 1c** and **Supplementary Fig. 5b**). Therefore, miR-294 substituted for, but did not enhance, *cMyc*'s contribution to reprogramming efficiency.

ES cell–like Oct4-GFP$^+$ colonies induced by *Oct4*, *Sox2*, *Klf4* and miR-294 were expanded and verified as induced pluripotent stem (iPS) cells (miR-294-iPS cells). MiR-294-iPS cell lines expressed endogenous *Oct4*, *Sox2* and *Klf4*, whereas retrovirus expression was silenced (**Fig. 1e,f**). Colonies showed an ES cell–like morphology and stained positively for the ES cell markers Nanog and SSEA-1 (**Supplementary Fig. 6a** online). The cell lines had normal karyotypes and efficiently formed teratomas containing tissues of all three germ layers (**Supplementary Figs. 6b** and **7a–c** online). Injection of miR-294-iPS cells into blastocysts resulted in high-grade chimeras, with contribution of donor iPS cells to all three germ layers and to the germ line (**Fig. 1g,h** and **Supplementary Fig. 6c**).

The mechanism by which ESCC miRNAs substitute for *cMyc* in reprogramming is not entirely clear. However, bioinformatic analysis of ES cell ChIP-seq data[7] showed that both c-Myc and n-Myc bind to the promoter region of the miR-290 cluster (**Fig. 2a**). Oct4, Sox2 and Nanog were also reported to bind this promoter[1]. Transduction of MEFs with a retrovirus expressing *Oct4*, *Sox2*, *Klf4* or *cMyc* did not induce expression of the miR-290 cluster (**Fig. 2b**). Analysis of ChIP-seq data[8] for different histone modifications (**Fig. 2c**) showed that H3K27 on the miR-290 promoter is methylated in MEFs, a modification associated with transcriptional silencing. In contrast, H3K4 is methylated in ES cells, a modification associated with transcriptional activity. Therefore, these transcription factors likely can induce expression of the miR-290 cluster only as cells replace promoter-associated H3K27 with H3K4 methylation during the reprogramming process. Indeed, with four-factor transduction, miR-294 was robustly activated

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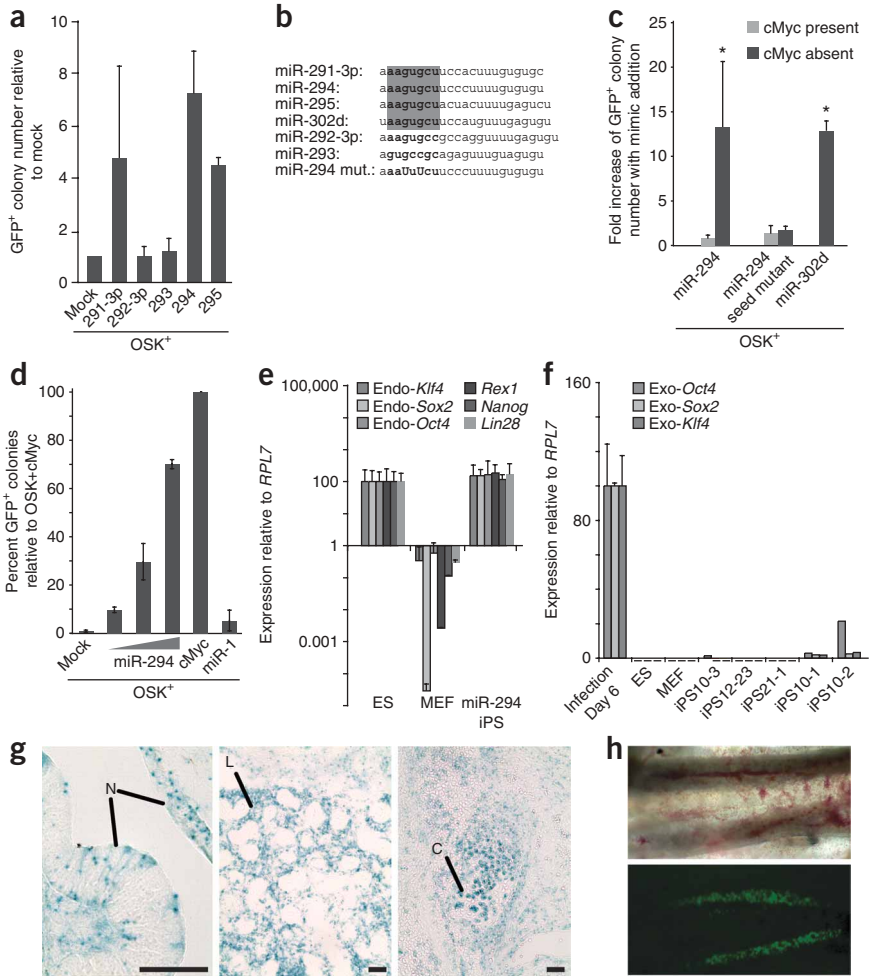

Figure 1 The ESCC miRNAs promote three-factor- but not four-factor-induced pluripotency. (**a**) Fold increase of day 10 Oct4-GFP$^+$ colonies with retroviruses expressing *Oct4*, *Sox2* and *Klf4* (OSK) together with 16 nM miRNA mimic relative to transfection reagent only (Mock). $N = 3$. Raw data in **Supplementary Table 1** online. (**b**) Sequence of miR-290 cluster, miR-302d and miR-294 seed-sequence mutant. Bold indicates seed sequence. Capitals indicate point mutations. Gray box highlights ESCC seed sequence. (**c**) Fold increase in day 10 Oct4-GFP$^+$ colonies with addition of mimic and *Oct4*, *Sox2* and *Klf4* retrovirus in the presence (light gray) or absence (dark gray) of *cMyc* retrovirus. Bars represent the number of GFP$^+$ colonies after mimic transfection divided by the number of GFP$^+$ colonies after mock transfection. $N = 6$, 26, 2, 5 and 3, left to right. Asterisk indicates $P \leq 0.0001$. Raw data for bars 1 and 2 in **Supplementary Table 2** online. (**d**) Percent day 10 of Oct4-GFP$^+$ colonies for retroviruses expressing *Oct4*, *Sox2* and *Klf4* plus 1.6, 16 and 160 nM transfected miR-294 mimic or 160 nM miR-1 relative to retroviruses expressing *Oct4*, *Sox2*, *Klf4* and *cMyc* (OSKM). (**e**) Quantitative RT-PCR for endogenous pluripotency markers in control (V6.5) ES cells, MEFs and miR-294-iPS cell lines. $N = 3$, 3 and 5. *RPL7* was used as input control. Data were normalized to ES cell expression. (**f**) Quantitative RT-PCR for exogenous *Oct4*, *Sox2* and *Klf4* in MEFs 6 d after viral infection, control (V6.5) ES cells and MEFs (each $N = 3$) and five individual miR-294-iPS cell lines. Horizontal black bars indicate cycle threshold value (Ct) > 40. *RPL7* was used as input control. Data were normalized to MEF expression 6 d after viral infection. (**g**) Staining with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) demonstrates miR-294-iPS cell chimeric contribution to ectoderm (neural tissue, N), endoderm (lung, L) and mesoderm (cartilage, C). (**h**) GFP expression in genital ridges of E12.5 chimera demonstrates *Oct4-GFP* miR-294-iPS cell contribution to the germ line. All error bars indicate s.d. Scale bars, 50 μm.

late in the reprogramming process, similar to the reported timing for expression of endogenous *Oct4* and other critical members of the core ES cell machinery (**Fig. 2d**)[9,10]. These data suggest that miR-294 is downstream of cMyc but requires epigenetic remodeling for expression.

The downstream effects of the ESCC miRNAs and *cMyc* on the reprogramming process were not identical. Unlike *cMyc*, miR-294 did not promote proliferation of MEFs early in the reprogramming process (**Fig. 2e**). Furthermore, as previously reported, ~80% of the four-factor colonies did not express GFP or have ES cell–like morphology[6] (**Fig. 2f,g**). In contrast, introduction of *Oct4*, *Sox2* and *Klf4* plus miR-294 produced a predominantly uniform population of ES cell–like GFP$^+$ colonies. The Oct4-GFP$^-$ colonies were induced by *cMyc*, and not inhibited by miR-294, as the introduction of both together produced a similar number of GFP$^-$, non-ES cell–like colonies as *cMyc* alone (**Fig. 2g**). Finally, when cells were injected into immunodeficient mice to produce teratomas, more than a third of the teratomas resulting from cMyc-iPS cells invaded the underlying body wall, whereas none of the teratomas resulting from miR-294-iPS cells did so (**Supplementary Fig. 7b,c**). These findings show that although miR-294 can substitute for *cMyc* to enhance reprogramming, its effects on the cell population are not identical to *cMyc*'s.

In summary, our data show that miRNAs can replace *cMyc* in promoting the dedifferentiation of somatic cells into iPS cells. Thus, small RNAs together with small molecules or other approaches may eventually substitute for DNA elements in the generation of iPS cells. Further analysis of the targets of the miRNAs identified here may offer insights into the reprogramming mechanism. ESCC miRNAs are highly expressed in ES cells, where they accelerate the transition through the G1/S restriction point[3]. Their expression is downregulated with ES cell differentiation as the G1 phase of the cell cycle extends[2,11]. ESCC miRNAs have also been shown to induce the expression of the *de novo* methyltransferases in ES cells, although how this may promote self-renewal is unclear[12,13]. As a target of cMyc, the miR-290 cluster likely acts downstream, but only after erasure of silencing histone modifications within its promoter. cMyc has additional targets[14], which is reflected in the differences in outcome between the introduction of *cMyc* and miR-294.

The ESCC miRNAs share a common seed sequence with a larger family of small RNAs known to promote cellular proliferation[3]. This family includes 'onco-miRs', such as members of the miR-17 cluster, miR-106 and miR-302 miRNAs[15,16]. These miRNAs, like the ESCC miRNAs, may be acting by enhancing cell-cycle progression and promoting dedifferentiation of the cells. Such parallels between induced dedifferentiation and cancer will be an exciting area of future research.

Methods are available in **Supplementary Methods** online.

Note: Supplementary information is available on the Nature Biotechnology website.

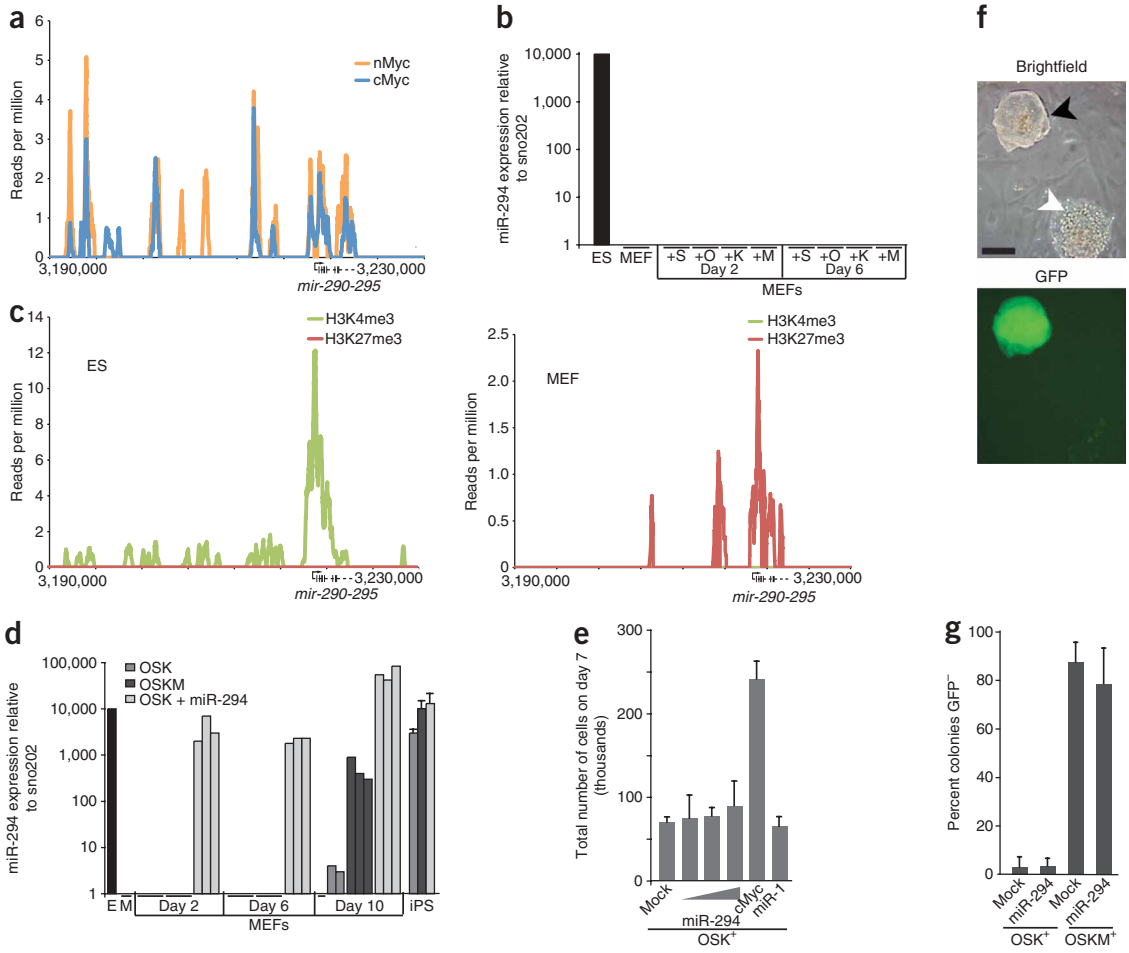

Figure 2 Characterization of the relationship between cMyc and miR-294. (**a**) cMyc (blue) and nMyc (yellow) bind the miR-290 cluster promoter. ChIP-seq data reads[7] were aligned to the mm9 assembly of the genome and peaks were generated with Findpeaks[17]. Vertical hash marks denote the positions of the miR-290 cluster miRNAs. (**b**) Quantitative RT-PCR for total mature miR-294 expression in control (V6.5) ES cells, MEFs, and MEFs infected with viruses expressing *Oct4* (O), *Sox2* (S), *Klf4* (K) or *cMyc* (M). RNA was collected on days 2 and 6. $N = 3$. Horizontal black bars indicate Ct > 40. *Sno202* was used as input control. Data were normalized to ES cells. (**c**) H3K4me3 (green) and H3K27me3 (red) surrounding the miR-290 cluster in MEFs. Chip-seq data[8] were analyzed as described in **a**. me, methylated. (**d**) Quantitative RT-PCR for total mature miR-294 expression in control (V6.5) ES cells (E), MEFs (M), MEFs infected with either *Oct4*, *Sox2*, *Klf4* (OSK); *Oct4*, *Sox2*, *Klf4* and *cMyc* (OSKM); or OSK + miR-294, and established iPS lines resulting from these conditions (iPS). RNA was collected on days 2, 6 and 10 of reprogramming. Three independent experiments are shown. Horizontal black bars indicate Ct > 40. *Sno202* was used as input control. Data were normalized to ES cells. (**e**) Total cell number during reprogramming. Cells were counted on day 7 after infection with OSKM or OSK ± miRNA mimic. Concentrations of miR-294 mimic: 1.6, 16 and 160 nM. Concentration of miR-1 mimic: 160 nM. (**f**) GFP$^-$ colonies in presence of cMyc. Oct4-GFP$^+$, ES cell–like colonies (black arrow) and GFP$^-$, non-ES cell–like colonies (white arrow). (**g**) Quantification of number of day 10 GFP$^-$ colonies after infection with OSKM or OSK ± miR-294 mimic. All error bars indicate s.d. of $N = 3$. Scale bar, 50 μm.

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AUTHOR CONTRIBUTIONS

R.L.J. performed all experiments. J.E.B. analyzed ChIP-seq data. M.V. analyzed the chimeras. R.L.J. and R.B. designed all experiments, analyzed data and wrote the manuscript.

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