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Pluripotency-associated miR-290/302 family of microRNAs promote the dismantling of naive pluripotency

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The molecular mechanism controlling the dismantling of naive pluripotency is poorly understood. Here we show that microRNAs (miRNAs) have important roles during naive to primed pluripotency transition. *Dgcr8*$^{-/-}$ embryonic stem cells (ESCs) failed to completely silence the naive pluripotency program, as well as to establish the primed pluripotency program during differentiation. miRNA profiling revealed that expression levels of a large number of miRNAs changed dynamically and rapidly during naive to primed pluripotency transition. Furthermore, a miRNA screen identified numerous miRNAs promoting naive to primed pluripotency transition. Unexpectedly, multiple miRNAs from miR-290 and miR-302 clusters, previously shown as pluripotency-promoting miRNAs, demonstrated the strongest effects in silencing naive pluripotency. Knockout of both miR-290 and miR-302 clusters but not either alone blocked the silencing of naive pluripotency program. Mechanistically, the miR-290/302 family of miRNAs may facilitate the exit of naive pluripotency in part by promoting the activity of MEK pathway and through directly repressing Akt1. Our study reveals miRNAs as an important class of regulators potentiating ESCs to transition from naive to primed pluripotency, and uncovers context-dependent functions of the miR-290/302 family of miRNAs at different developmental stages.

Keywords**:** microRNAs; naive pluripotency; primed pluripotency; embryonic stem cells; MEK pathway; AKT pathway
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Introduction

Multiple types of pluripotent stem cells (PSCs) have been derived from early mouse embryos of different developmental stages [1, 2]. These PSCs provide invaluable *in vitro* cell models to understand early mammalian development and hold great potential for regenerative medicine. Mouse embryonic stem cells (ESCs), derived from the inner cell mass and cultured in chemically defined media with inhibitors to MEK and GSK3 and leukemia inhibitory factor (2i/Lif), are thought to be akin to naive epiblast cells (E4.5) and postulated to be in an uncommitted ground pluripotent state [3-5]. Alternatively, when post-implantation embryos (E5.5-7.5) or preimplantation embryos are cultured in basal media supplemented with bFGF on feeders, mouse epiblast stem cells (EpiSCs) can be derived [6-8]. Later it was shown that EpiSCs can also be derived from ESCs through laborious *in vitro* differentiation procedures [9]. EpiSCs are similar to later gastrulation stage epiblast and can be differentiated into somatic cells or form chimeras when injected into post-implantation embryos [10, 11]. Remarkably, in contrast to protracted and inefficient process in deriving EpiSCs from ESCs, epiblast-like cells (EpiLCs), which represent an intermediate state between ESCs and EpiSCs, can be efficiently obtained by culturing ESCs in media containing bFGF and knockout serum replacement (KSR) with or without Activin A in just 2 days [12-14]. EpiLCs are similar to early post-implantation epiblast

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cells (E5.5-6.5) and are transient in nature. As a functional equivalent of the pre-gastrulation epiblast, EpiLCs can be efficiently differentiated to primordia germ cell-like cells, which eventually generate functional gametes in mice [12, 15-17]. Due to its high efficiency, ESC to EpiLC conversion has become a feasible system to study transitions between different pluripotent states. Investigating molecular mechanisms that control the transition from ESCs to EpiLCs is important for understanding early mammalian development and the eventual utilization of PSCs in regenerative medicine.

Transcriptome analysis has revealed that thousands of genes are differentially expressed between ESCs and EpiLCs [12-14]. Among them, naive markers such as Rex1 (also known as Zfp42), Klf2, Esrrb and Nanog are exclusively expressed in naive state of ESCs, whereas EpiLCs express an unique set of genes including Fgf5, Otx2 and Oct6 (also known as Pou3f1). Intriguingly, general pluripotency transcription factors Oct4 (also known as Pou5f1) and Sox2 are expressed in both cell types. The transition between two states is at least partially driven through the cooperation of Oct4 and Otx2 [13, 18]. Oct4 interacts with different sets of transcription factors and chromatin remodeling factors in ESCs and EpiLCs. Among them, Otx2 has been shown to be sufficient to redirect the binding of Oct4 to differentiation-associated enhancers. However, other transcription factors are also likely to be important for the dismantling of naive pluripotency program and the establishment of EpiLC program, as knocking out *Otx2* only blocks the upregulation of a small set of post-implantation epiblast genes. In addition, screens have identified hundreds of genes including transcription factors, RNA-binding proteins, signal transduction proteins and epigenetic modifiers as potential molecular drivers or facilitators for ESCs to exit the naive state [19-21]. Together, these studies support a model in which the early differentiation of ESCs is dependent on multiple layers of regulation including chromatin modification, transcription and post-transcriptional regulation.

To date, whether noncoding RNAs, especially microRNAs (miRNAs), have any important roles during ESC to EpiLC transition is still not clear. miRNAs are a class of short noncoding RNAs (~22 nucleotides) that post-transcriptionally regulate gene expression by inhibiting protein translation and/or destabilizing mRNAs [22]. Knocking out *Dgcr8*, a key gene for miRNA biogenesis, results in the loss of all canonical miRNAs in mouse ESCs [23]. In current study, we investigated the potential role of miRNAs in regulating the transition from naive state ESCs to post-implantation EpiLCs using *Dgcr8*$^{-/-}$ ESC model.

Results

miRNAs are required for efficient naive to primed pluripotency transition

To test whether miRNAs are important for the ESC to EpiLC transition, we differentiated 2i/Lif-cultured wild-type and *Dgcr8*$^{-/-}$ ESCs for 48 h in the basal media supplemented with 12 ng/ml bFGF and 1% KSR [12-14]. We then performed quantitative RT-PCR (qRT-PCR) on RNA samples from 2i/Lif cultured and differentiated cells. As expected, naive markers including Nanog, Rex1, Esrrb and Klf2 were all drastically downregulated in wild-type cells, whereas post-implantation epiblast markers Fgf5 and Dnmt3b were significantly upregulated (Figure 1A). Interestingly, the changing pattern for these genes was similar in *Dgcr8*$^{-/-}$ cells; however, the magnitude of change was markedly diminished in *Dgcr8*$^{-/-}$ cells. For example, the expression level of naive marker Rex1 decreased ~94% in wild-type cells, but only ~40% in *Dgcr8*$^{-/-}$ cells during differentiation; and the expression level of epiblast marker Fgf5 was 10-fold higher in wild-type than *Dgcr8*$^{-/-}$ EpiLCs. Consistently, immunofluorescence (IF) analysis for Nanog and Oct6 (also known as Pou3f1) also confirmed that *Dgcr8*$^{-/-}$ ESCs had defects in naive to primed pluripotency transition (Supplementary information, Figure S1A and S1B). More importantly, we found that the naive to primed pluripotency transition in *Dgcr8*$^{-/-}$ cells was not as complete as in wild-type cells even after being differentiated for 6 days (Supplementary information, Figure S1C). These data indicate that miRNAs are at least important for the transcriptional changes of several key naive and epiblast markers during ESC to EpiLC transition.

To understand how miRNAs globally impact the transcriptional landscape during ESC to EpiLC transition, we performed high-throughput transcriptome sequencing. During wild-type ESC to EpiLC transition, 2 047 genes were upregulated and 1 305 genes were dowregulated for more than twofold (Figure 1B and Supplementary information, Table S1). Among them, only 889 upregulated and 394 downregulated genes were expressed at similar levels in differentiated *Dgcr8*$^{-/-}$ cells as in wild-type EpiLCs. In other words, around 57% and 70% genes were not appropriately upregulated and downregulated during differentiation of *Dgcr8*$^{-/-}$ cells, respectively. Confirming these results, quantitative analysis revealed considerably less profound changes for genes normally upregulated or downregulated during ESC to EpiLC transition in *Dgcr8*$^{-/-}$ cells than wild-type cells (Figure 1C). Consistent with quantitative analysis, gene set enrichment analysis (GSEA) [24, 25] demonstrated that genes upregulated during normal transition were strong-

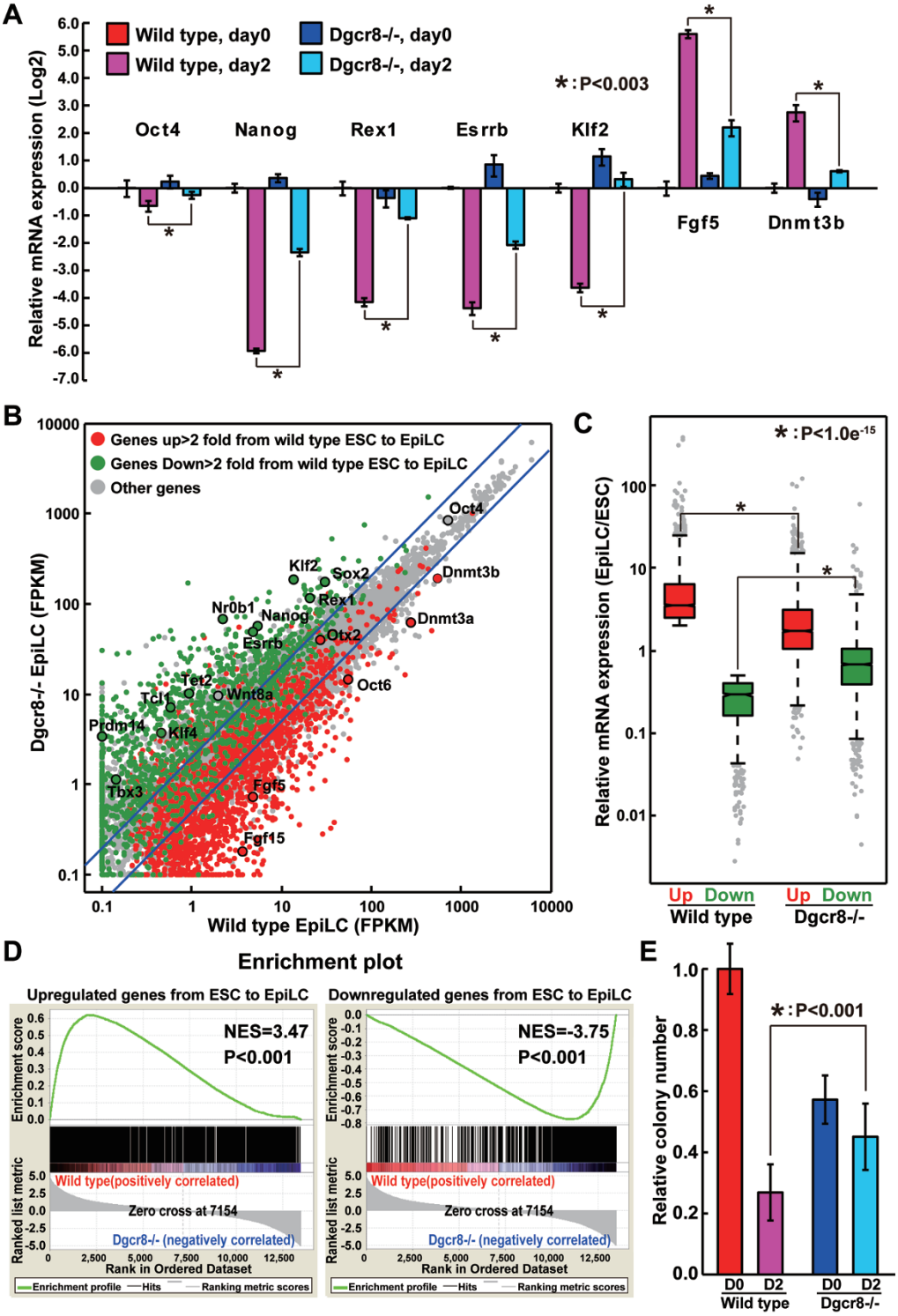


Figure 1 miRNAs are required for efficient ESC to EpiLC transition. **(A)** qRT-PCR analysis of naive and early post-implantation markers. β-actin is a control. For each gene, data are normalized to mRNA levels of wild-type ESCs. Data are shown as mean ± SD, *n* = 3. **(B)** RNA-Seq analysis for wild-type and *Dgcr8*$^{-/-}$ EpiLCs. Averages of two biological replicates for each cell type are shown. **(C)** Quantitative analysis of expression changes for genes that are upregulated or downregulated more than twofold during wild-type ESC to EpiLC transition. *P* values are calculated based on Wilcoxon signed-rank test. **(D)** GSEA for genes normally upregulated (left) and downregulated (right) during ESC to EpiLC transition in wild-type and *Dgcr8*$^{-/-}$ EpiLCs. For *x* axis, genes are ranked based on the ratio of wild-type vs *Dgcr8*$^{-/-}$ EpiLCs. **(E)** Colony formation assay for wild-type and *Dgcr8*$^{-/-}$ cells. Data are normalized to wild-type day 0 ESCs and are shown as mean ± SD, *n* = 9.

ly associated with wild-type EpiLCs, whereas genes downregulated during normal transition were strongly associated with *Dgcr8*$^{-/-}$ EpiLCs (Figure 1D). More importantly, naive pluripotency associated genes including Rex1, Nanog, Esrrb, Klf2/4, Tbx3 and Prdm14 were not completely silenced, and early post-implantation epiblast-associated genes including Fgf5, Fgf15, Dnmt3a/b and Oct6 were not appropriately upregulated in *Dgcr8*$^{-/-}$ EpiLCs (Figure 1B). In addition, we noticed that a small subset of genes (169 and 32 for upregulated and downregulated genes, respectively; Wnt8a as an example) showed more profound changes during differentiation in *Dgcr8*$^{-/-}$ cells than wild-type cells (Figure 1B and Supplementary information, Table S1), suggesting that miRNAs may constrain transcriptional changes of certain genes to modulate the transcriptome of EpiLCs. Collectively, these data support that miRNAs are important for the transcriptome change during ESC to EpiLC transition.

Encouraged by the transcriptome analysis, we performed colony formation assay for differentiated cells in which they were plated back into 2i/Lif culture media. This assay selects naive stem cells against differentiated cells, as differentiated cells usually die in the absence of Erk signaling. After differentiation, the colony formation ability was significantly inhibited in wild-type EpiLCs. Interestingly, although the colony formation ability of *Dgcr8*$^{-/-}$ ESCs was lower than wild-type ESCs, the *Dgcr8*$^{-/-}$ EpiLCs retained high colony formation ability (Figure 1E). Together, these data suggest that miRNAs are important for the exit of naive state and the establishment of post-implantation epiblast state.

miRNA expression changes dynamically during ESC to EpiLC transition

To identify miRNAs that facilitate ESC to EpiLC transition, we performed miRNA sequencing for wild-type cells at 0, 12, 24 and 48 h time points during differentiation. We then focused our analysis on miRNAs with estimated average expression level at more than 10 copies per cell. Unsupervised hierarchical clustering [26] and principal component analysis showed that two biological replicates of the same time point clustered closely together and could be clearly discriminated from samples at different time points on the basis of their miRNA signatures (Figure 2A and Supplementary information, Figure S2A). Of the 298 mature miRNAs analyzed, 4, 8 and 50 miRNAs were upregulated and 0, 8 and 45 miRNAs were downregulated by at least 2-fold at 12, 24 and 48 h time points (Figure 2B and Supplementary information, Figure S2B and S2C, Table S2), respectively. Previously, we and others identified many pluripotency-associated miRNAs and differentiation-inducing miRNAs that regulate the self-renewal of ESCs and/or the reprograming of somatic cells into induced PSCs [27-37]. Intriguingly, most members of pluripotency-associated miRNA clusters including miR-290 and miR-17-20-106 families remained at similar levels during differentiation with one exception for miR-302 cluster, of which all members were significantly upregulated during differentiation (Figure 2C). This is consistent with previous findings that miR-302 cluster is highly expressed during early differentiation of ESCs and in EpiSCs [37-40]. Unexpectedly, most members of differentiation-inducing miRNAs including those from let-7 families, miR-26, miR-199a and miR-99b [31] were considerably downregulated during differentiation (Figure 2D). However, miR-21 and miR-34 were significantly upregulated at all time points during differentiation. In addition, several members of miR-23-24-27 families were slightly upregulated at 12 h and then downregulated at later time points. These data demonstrate that miRNA profiles change dynamically and markedly during ESC to EpiLC transition.

Multiple miRNAs facilitate ESC to EpiLC transition

On the basis of miRNA-Seq data, we picked 21 miRNAs that were either highly expressed at all time points during differentiation or significantly upregulated at any time point of differentiation to test their function during ESC to EpiLC transition. We transfected miRNA mimics of these miRNAs into *Dgcr8*$^{-/-}$ ESCs and then evaluated their effects by qRT-PCR analysis of naive markers Rex1 and Klf2 and post-implantation epiblast marker Fgf5 (Figure 2E). Surprisingly, pluripotency-associated miRNAs with a seed sequence "AAGUGCU" from miR-290 and miR-302 clusters showed the most marked effects on silencing Rex1 and Klf2 and inducing Fgf5. In addition, miR-467a-5p with a similar seed sequence "AAGUGCC", and miR-17-5p and miR-20a-5p, which have a similar seed sequence "AAAGUGC" also showed some degree of differentiation-promoting function. Furthermore, miR-21-5p markedly induced the expression of Fgf5, whereas miR-24-5p and miR-494-3p significantly downregulated Rex1 and Klf2. These data indicate that multiple miRNAs act redundantly to facilitate ESC to EpiLC transition with miR-290/302 family of miRNAs showing the most dramatic effects.

miR-290/302 family of miRNAs facilitate ESC to EpiLC transition

On the basis of screening results, we then focused on miR-290/302 family of miRNAs. Because these miRNAs shared the same seed sequence and our previous studies have shown no functional differences between them [27,

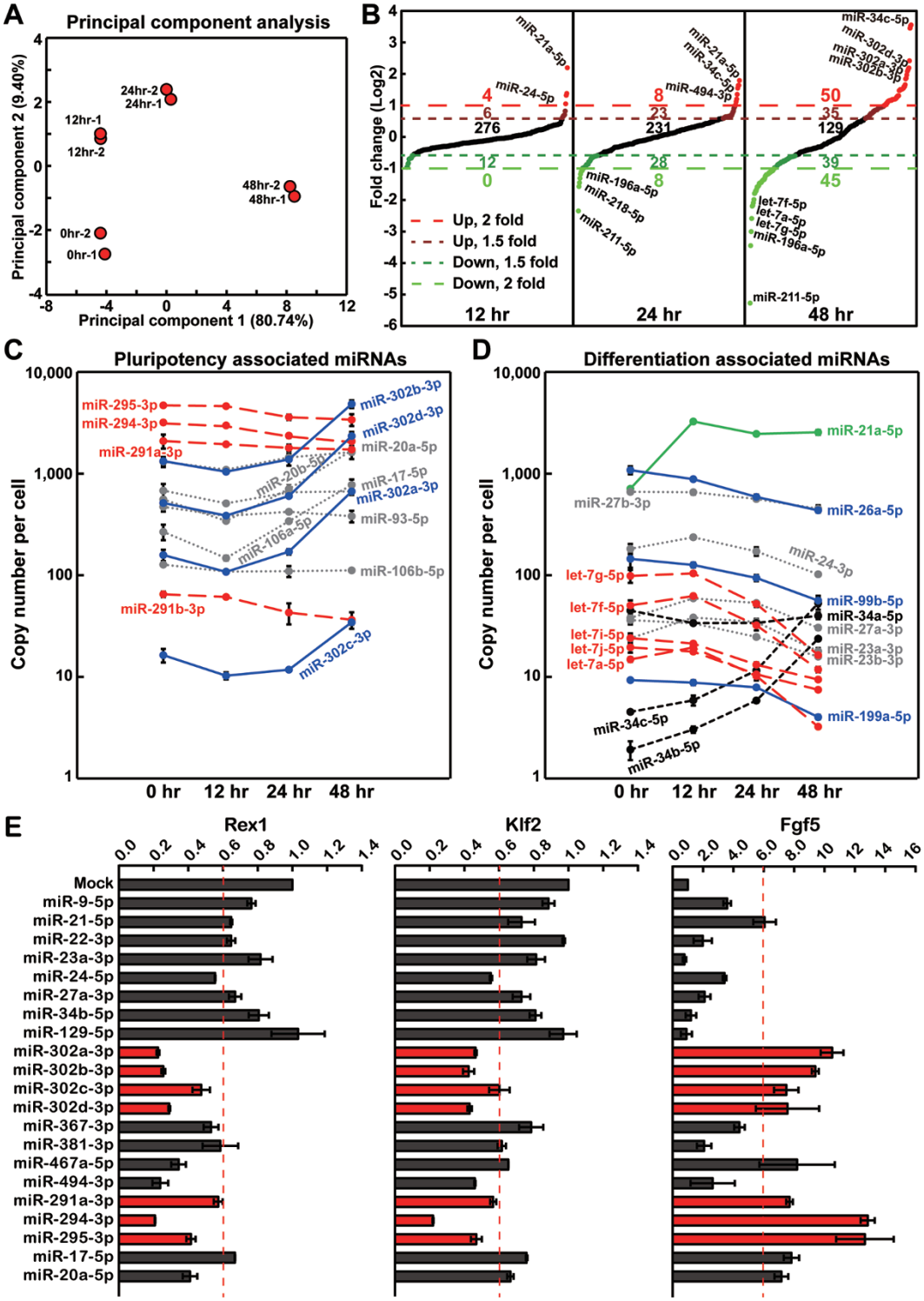


Figure 2 miRNA expression profiles change dynamically during ESC to EpiLC transition. **(A)** Principal component analysis of miRNA expression profiles at 0, 12, 24 and 48 h during ESC to EpiLC transition. **(B)** Distribution of fold changes of mature miRNAs at 12, 24 and 48 h during differentiation versus undifferentiated ESCs (0 h). Data are shown as the average of two biological replicates. **(C)** Expression levels of pluripotency-associated miRNAs during ESC to EpiLC transition. **(D)** Expression levels of differentiation-inducing miRNAs during ESC to EpiLC transition. **(E)** qRT-PCR analysis of Rex1, Klf2 and Fgf5 in differentiated *Dgcr8*$^{-/-}$ cells transfected with selected miRNAs. Cutoffs (red dotted lines) are arbitrarily set at 0.6 for Rex1 and Klf2 and 6.0 for Fgf5. β-actin is used as a control. For each gene, data are normalized to mRNA levels of mock-transfected *Dgcr8*$^{-/-}$ ESCs. Average of two biological replicates per treatment is shown. Error bars indicate SD.

28, 31, 41, 42], we used miR-294-3p (later shown as miR-294) and miR-302a-3p (later shown as miR-302a) as two representative members to carry out further characterization. We resynthesized miRNA mimics for miR-294 and miR-302a, and tested their function in facilitating ESC to EpiLC transition by qRT-PCR. The results showed that these miRNAs markedly downregulated naive genes including Rex1, Nanog, Esrrb and Klf2, and upregulated post-implantation markers Fgf5 and Dnmt3b in *Dgcr8*$^{-/-}$ cells (Figure 3A and Supplementary information, Figure S3A). IF analysis of Nanog and Oct6 in miR-294-tranfected cells were consistent with its function in facilitating ESC to EpiLC transition (Supplementary information, Figure S3B and S3C). Importantly, by transfecting various concentrations of miR-294 mimics, we demonstrated that physiological level of miR-294 was sufficient to promote the ESC to EpiLC transition (Supplementary information, Figure S3D). In addition, transfection of miR-294 mimics did not accelerate ESC to EpiLC transition in wild-type cells (Supplementary information, Figure S3E), consistent with saturated level of miR-290/302 cluster of miRNAs in wild-type cells.

To understand how these miRNAs globally impact the transcriptome during ESC to EpiLC transition, we performed RNA-Seq analysis for miR-294- or miR-302a-transfected cells. Principal component analysis for 84 selected genes that are related to pluripotency and differentiation [43] demonstrated that miR-294- or miR-302a-transfected cells clustered closely with wild-type cells and were clearly separated from *Dgcr8*$^{-/-}$ cells (Figure 3B). In addition, principal component analysis based on the whole transcriptome showed similar results (Supplementary information, Figure S4A). There were 2 226 and 1 873 genes abnormally upregulated and downregulated more than twofold in *Dgcr8*$^{-/-}$ versus wild-type EpiLCs (Figure 1B and Supplementary information, Table S1), respectively. Among them, 907 upregulated genes (41%) and 926 downregulated genes (49%) were rescued by the transfection of miR-302a (Figure 3C). Similarly, 889 upregulated genes (40%) and 962 downregulated genes (51%) were rescued by the transfection of miR-294 (Supplementary information, Figure S4B). In addition, for genes that were upregulated or downregulated more than twofold during normal differentiation of wild-type cells, quantitative analysis showed substantially more profound changes in miR-302a- or miR-294-transfected cells than mock-transfected *Dgcr8*$^{-/-}$ cells (Figure 3D and Supplementary information, Figure S4C), supporting that the transfection of miR-294/302a essentially promoted *Dgcr8*$^{-/-}$ cells to transition into EpiLC state. Consistent with the quantitative analysis, GSEA showed that genes upregulated and downregulated in *Dgcr8*$^{-/-}$ versus wild-type EpiLCs were strongly associated with mock-transfected and miR-294/302a-tranfected *Dgcr8*$^{-/-}$ cells (Figure 3E and Supplementary information, Figure S4D), respectively. Notably, rescued genes by miR-294/302a included multiple key naive markers such as Rex1, Nanog, Esrrb, Klf2/4, Tbx3, and Prdm14, and epiblast-associated genes including Fgf5, Fgf15, Dnmt3a/b and Oct6 (Figure 3C and Supplementary information, Figure S4B). A more careful analysis revealed that miR-294/302a did not completely rescue the expression of these genes to the level observed in wild-type EpiLCs (Figure 3A and Supplementary information, Figures S3D and S4E), consistent with multiple other miRNAs playing roles during ESC to EpiLC transition. Importantly, colony formation assay showed that miR-294 and miR-302a significantly reduced the colony forming ability of *Dgcr8*$^{-/-}$ EpiLCs (Figure 3F and Supplementary information, Figure S4F). Interestingly, miR-294/302 transfection also caused a slight but significant increase in colony formation ability of *Dgcr8*$^{-/-}$ ESCs in 2i/Lif condition, suggesting context-dependent function of these miRNAs. These data support that miR-290/302 family of miRNAs facilitate ESC to EpiLC transition and regulate the expression of hundreds of genes during the transition.

miR-290/302 family of miRNAs are required for the appropriate silencing of naive genes

From the above data, we concluded that miR-290/302 family of miRNAs are sufficient to drive ESC to EpiLC transition in *Dgcr8*$^{-/-}$ cells. However, whether they are required for ESC to EpiLC transition remains to be tested. To answer this question, we constructed miR-290$^{-/-}$, miR-302$^{-/-}$ and miR-290/302 double-knockout (DKO) ESCs using CRISPR/Cas9 (Figure 4A and 4B). Knocking out miR-290 and miR-302 culsters individually or in combination had little effects on the expression of pluripotency genes in ESCs (Supplementary information, Figure S5A). In addition, consistent with decreased colony formation ability in *Dgcr8*$^{-/-}$ ESCs, knocking out these miRNA clusters had negative impact on colony formation ability of ESCs in 2i/Lif condition, suggesting that these miRNAs have protective function at 2i/Lif condition (Figures 1E and 4E, Supplementary information, Figure S5B). Importantly, when compared with wild-type EpiLCs, most naive markers were strongly upregulated in DKO EpiLCs but not in single knockout EpiLCs, although only a few early post-implantation epiblast markers such as Fgf5 and Dnmt3b were slightly downregulated in DKO EpiLCs (Figure 4C and Supplementary information, Figure S5C and S5D). Furthermore, these differences were effectively rescued by putting

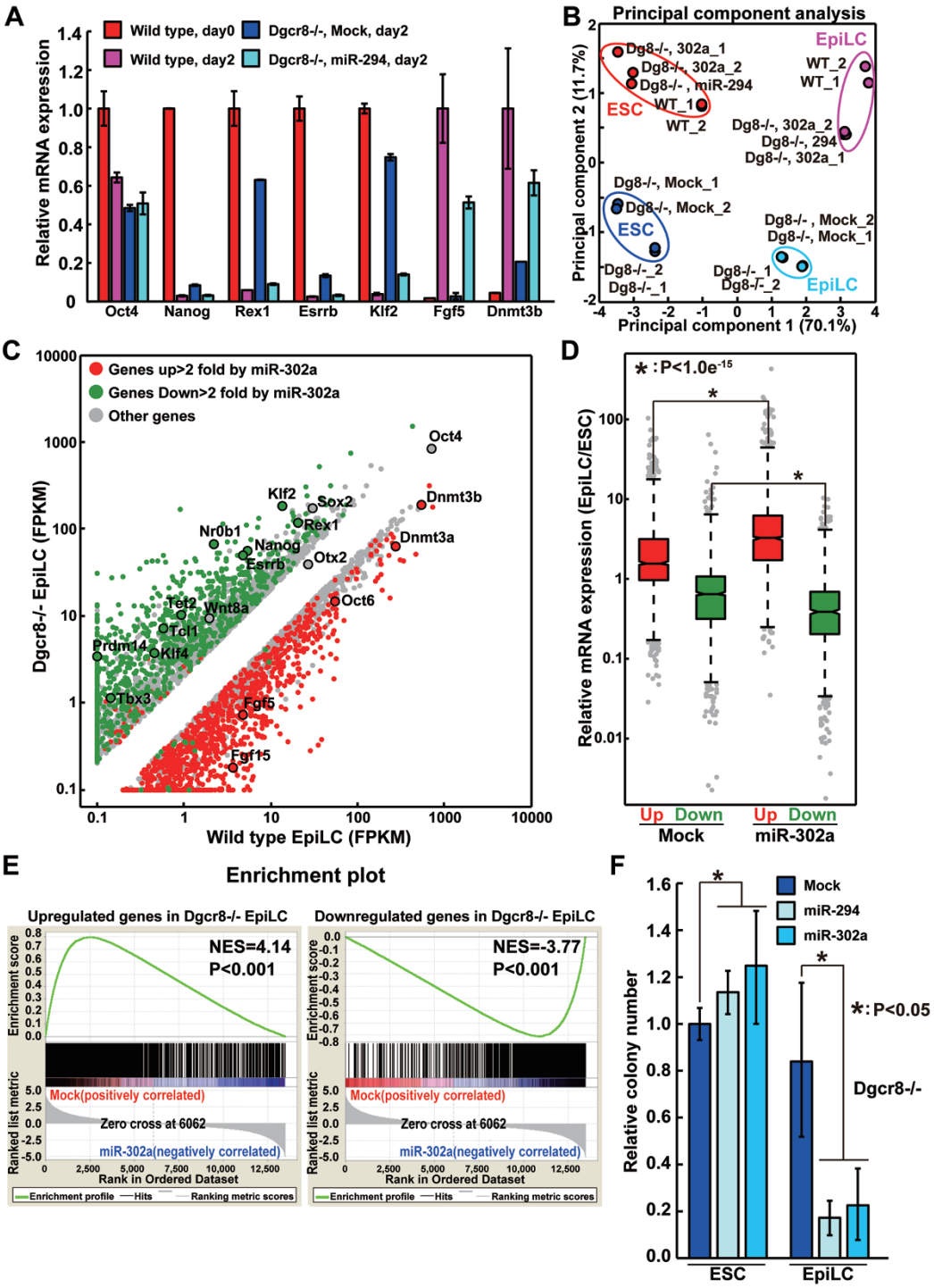


Figure 3 miR-294/302 family of miRNAs facilitate naive to primed pluripotency transition. **(A)** qRT-PCR analysis of naive and early post-implantation markers. β-actin is used as a control. For each gene, data are normalized to mRNA levels of wild-type ESCs and are shown as mean ± SD, *n* = 2. **(B)** Principal component analysis of 84 selected pluripotency-related genes. Dg8$^{-/-}$, *Dgcr8*$^{-/-}$; WT, wild-type. **(C)** RNA-Seq analysis for mock- and miR-302a-transfected *Dgcr8*$^{-/-}$ EpiLCs. The average of two biological replicates for each cell type is shown. **(D)** Quantitative analysis of gene expression changes for genes that are upregulated or downregulated more than twofold during normal ESC to EpiLC transition. *P* values are calculated based on Wilcoxon signed-rank test. **(E)** GSEA for genes upregulated (left) or downregulated (right) in *Dgcr8*$^{-/-}$ vs wild-type EpiLCs in mock- and miR-302a-transfected *Dgcr8*$^{-/-}$ EpiLCs. For x axis, genes are ranked based on the ratio of mock/miR-302 vs *Dgcr8*$^{-/-}$ EpiLCs. **(F)** Colony formation assay for mock-, miR-294- or miR-302a-transfected *Dgcr8*$^{-/-}$ cells. Data are normalized to mock-transfected *Dgcr8*$^{-/-}$ ESCs and are shown as mean ± SD, *n* = 6.

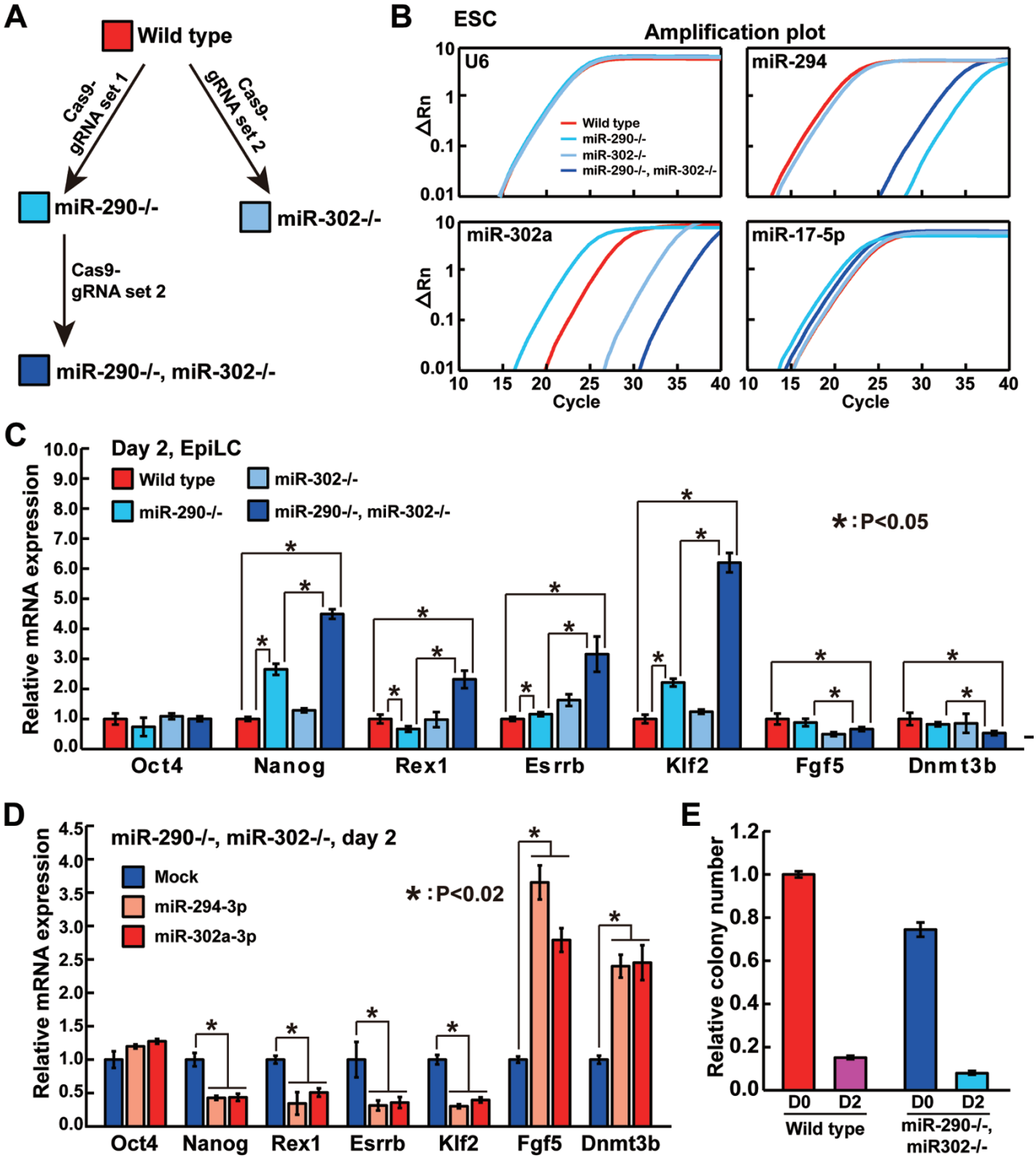


Figure 4 miR-290/302 family of miRNAs are required for proper ESC to EpiLC transition. **(A)** Schematic design for knocking out miR-290 cluster, miR-302 cluster and double knockout in ESCs by CRISPR/Cas9. **(B)** Amplification plot for miRNA qPCR. Representative miRNAs of miR-294/302 families are shown. U6 and miR-17-5p are loading controls. **(C)** qRT-PCR analysis of naive and early post-implantation markers in miRNA knockout cells. β-actin is used as a control. Data are normalized to mRNA levels of wild-type EpiLCs and are shown as mean ± SD, *n* = 3. **(D)** qRT-PCR analysis of naive and early post-implantation markers in miR-294- and miR-302a-rescued DKO cells. β-actin is a control. Data are normalized to mRNA levels of mock-transfected DKO EpiLCs and are shown as mean ± SD, *n* = 3. **(E)** Colony formation assay for miR-290/302 DKO cells. Data are normalized to wild-type day 0 ESCs and are shown as mean ± SD, *n* = 3.

back miR-294 or miR-302a into DKO cells (Figure 4D), suggesting that the phenotype was indeed due to the lack of miR-294/302 miRNAs in DKO cells. In addition, we noticed that the difference in gene expression between DKO and wild-type EpiLCs were much less profound than that between *Dgcr8*$^{-/-}$ and wild-type EpiLCs (Supplementary information, Figure S5E). Colony formation assay showed no increase in colony formation ability in DKO versus wild-type EpiLCs (Figure 4E). Together, these data show that miR-290/302 clusters are required for efficient silencing of naive pluripotency genes, but not the colony formation ability. The phenotypic difference between DKO and *Dgcr8*$^{-/-}$ cells suggests that other miRNAs act redundantly with miR-290/302 clusters to facilitate ESC to EpiLC transition. This is in concert with screening results showing that other miRNAs such as miR-17, miR-20, miR-21, miR-24, miR-467a and miR-494 could also silence the expression of Rex1 and Klf2 or induce the expression of Fgf5 (Figure 2E). Interestingly, qPCR analysis showed that miR-21 and miR-24 were markedly upregulated in miR-290/302 DKO versus wild-type ESCs (Supplementary information, Figure S5F), which may compensate for the function of miR-290/302 clusters during ESC to EpiLC transition. Together, these data indicate that miR-290/302 family of miRNAs are required for appropriate silencing of multiple naive markers and induction of a few early post-implantation epiblast markers, and a high degree of functional redundancy between miR-290/302 family of miRNAs and other miRNAs likely exists.

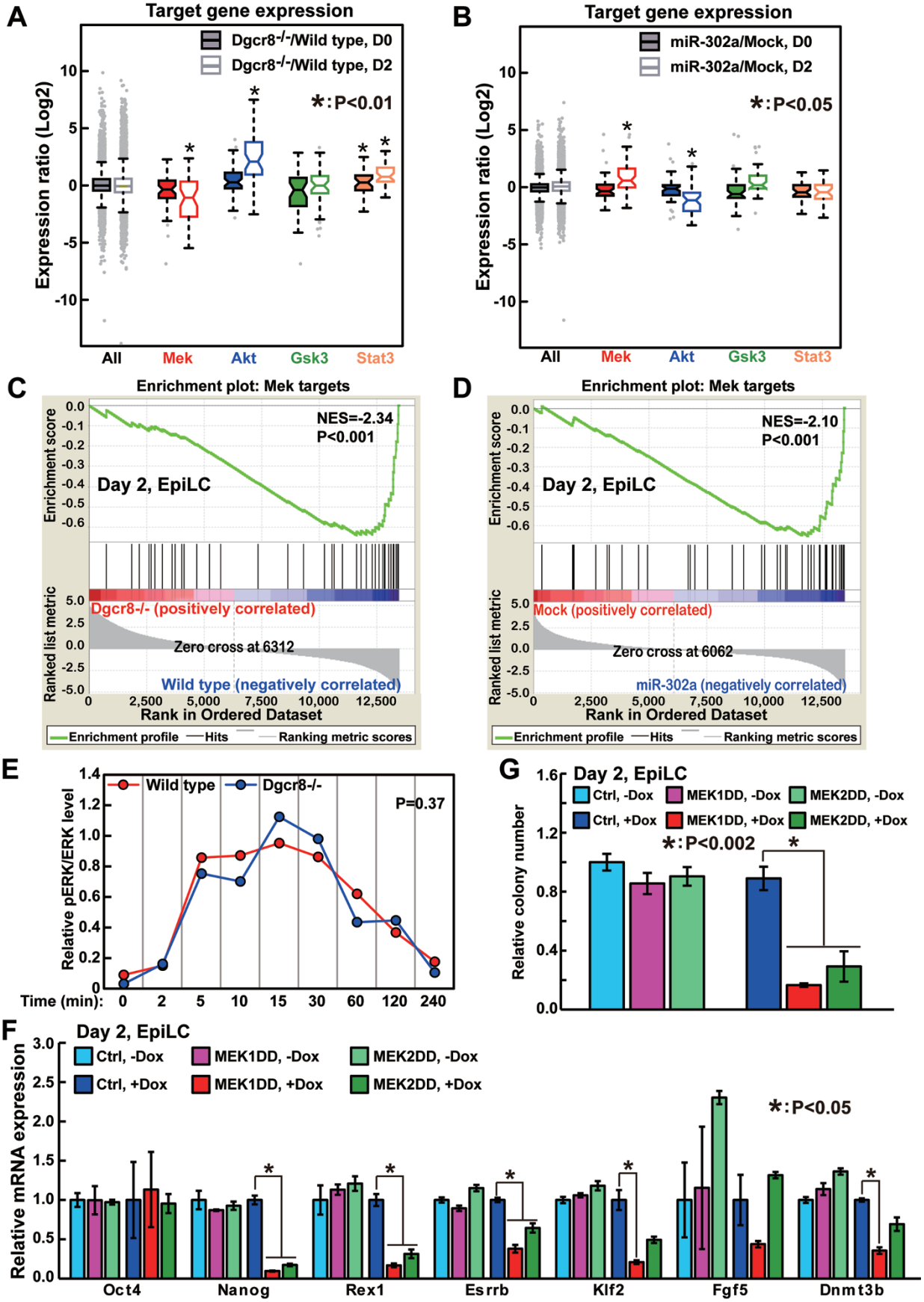
miR-290/302 family of miRNAs modulate MEK and AKT pathways during ESC to EpiLC transition

To understand how miR-290/302 family of miRNAs facilitate ESC to EpiLC transition, we analyzed the expression of genes from four pluripotency- or differentiation-related signaling pathways [14], including MEK, AKT, GSK3 and STAT3. The activation of MEK and GSK3 pathways is associated with differentiation, whereas the activation of AKT and STAT3 pathways is associated with pluripotency. In all cases, target gene expression were not profoundly different between wild-type and *Dgcr8*$^{-/-}$ ESCs in 2i/Lif culture conditions (Figure 5A); however, in differentiation conditions, MEK targets were significantly reduced (~1.8-fold) and targets of AKT and STAT3 pathways were significantly upregulated (~5.9-fold and ~1.8-fold, respectively) in *Dgcr8*$^{-/-}$ versus wild-type EpiLCs (Figure 5A). Interestingly, the MEK targets were evidently upregulated (~1.4-fold), whereas the AKT targets were markedly downregulated (~1.8-fold) by the transfection of miR-294 or miR-302a in *Dgcr8*$^{-/-}$ cells (Figure 5B and Supplementary information, Figure S7A). GSEA confirmed these findings (Figures 5C and 5D, 6A and 6B, Supplementary information, Figures S7B and S8A). These data suggest that miR-290/302 family of miRNAs modulate MEK and AKT signaling pathways during ESC to EpiLC transition.

Forced activation of MEK pathway facilitates Dgcr8$^{-/-}$ *cells to exit naive pluripotency state*

We then checked whether miR-290/302 activate MEK pathways by directly affecting the phosphorylation status of ERK. Western analysis at a series of timepoints after adding bFGF/KSR differentiation media showed no significant difference in the phosphorylation status of ERK between wild-type and *Dgcr8*$^{-/-}$ cells (Figure 5E and Supplementary information, Figure S7C). However, we cannot exclude the possibility that there are subtle differences in the level of phosphorylated ERK between these cells due to the sensitivity of western blot analysis. To exclude the possibility that the differential expression of MEK targets in wild-type and *Dgcr8*$^{-/-}$ EpiLCs is secondary due to differentiation defects, we compared MEK targets in wild-type and *Dgcr8*$^{-/-}$ ESCs cultured in CHIR/Lif condition, which maintains ESCs at the pluripotent state (Supplementary information, Figure S6A). We found that the expression of most MEK targets was also significantly inhibited in *Dgcr8*$^{-/-}$ ESCs in this condition (Supplementary information, Figure S6B). Furthermore, in the presence of MEK inhibitor PD,

Figure 5 miR-290/302 family of miRNAs silence the naive pluripotency program possibly through promoting the activation of MEK pathway. **(A)** Pathway analysis for wild-type and *Dgcr8*$^{-/-}$ cells in 2i/L (filled boxes) or differentiation (open boxes) conditions. Boxes indicate the 25th to 75th percentiles and the central bar represents the median. **(B)** Pathway analysis for mock- and miR-302a-transfected *Dgcr8*$^{-/-}$ cells in 2i/L (filled boxes) or differentiation (open boxes) conditions. **(C)** GSEA analysis for MEK targets in wild-type and *Dgcr8*$^{-/-}$ EpiLCs. **(D)** GSEA analysis for MEK targets in mock- and miR-302a-transfected *Dgcr8*$^{-/-}$ EpiLCs. **(E)** Protein level analysis for pERK/ERK at various time points after induction of differentiation with bFGF and KSR in wild-type and *Dgcr8*$^{-/-}$ cells. **(F)** qRT-PCR analysis of naive and early post-implantation markers in *Dgcr8*$^{-/-}$ cells overexpressing constitutively active MEK. β-actin serves as the control. For each gene, data are normalized to control cells with or without doxycycline treatment accordingly, and are shown as mean ± range, n = 2. **(G)** Colony formation assay for *Dgcr8*$^{-/-}$ cells overexpressing constitutively active MEK. Data are normalized to control cells without doxycycline treatment and are shown as mean ± SD, n = 3.


A
Target gene expression
Dgcr8-/-/Wild type, D0
Dgcr8-/-/Wild type, D2
*:P<0.01
Expression ratio (Log2)
All
Mek
Akt
Gsk3
Stat3
B
Target gene expression
miR-302a/Mock, D0
miR-302a/Mock, D2
*:P<0.05
C
Enrichment plot: Mek targets
NES=-2.34
P<0.001
Day 2, EpiLC
Enrichment score
Ranked list metric
Dgcr8-/- (positively correlated)
Zero cross at 6312
Wild type (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
D
Enrichment plot: Mek targets
NES=-2.10
P<0.001
Day 2, EpiLC
Mock (positively correlated)
Zero cross at 6062
miR-302a (negatively correlated)
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Wild type
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G
Day 2, EpiLC
Ctrl, -Dox
MEK1DD, -Dox
MEK2DD, -Dox
Ctrl, +Dox
MEK1DD, +Dox
MEK2DD, +Dox
*:P<0.002
Relative colony number
F
Day 2, EpiLC
*:P<0.05
Relative mRNA expression
Oct4
Nanog
Rex1
Esrrb
Klf2
Fgf5
Dnmt3b

transfection of miR-294 still caused significant upregulation of MEK targets (Supplementary information, Figure S6C and S6D). These data suggest that miR-290/302 might cooperate with MEK pathway to upregulate some key genes in promoting ESC to EpiLC transition. On the basis of this, we reasoned that overexpression of MEK may partially substitute for miR-290/302 in promoting the exit of naive pluripotency. To test this hypothesis, we constructed *Dgcr8*$^{-/-}$ cell lines that ectopically expressed constitutively active human MEK1 (S218D and S222D mutation) [44] and MEK2 (S222D and S226D mutation) [45] in a doxycycline-inducible manner (Supplementary information, Figure S7D and S7E). Expression of constitutively active human MEK1 and MEK2 drastically downregulated the expression of naive genes including Nanog, Rex1, Esrrb and Klf2 (Figure 5F and Supplementary information, Figure S7F). A caveat is that most of the post-implantation epiblast markers except Oct6 were also downregulated, suggesting that miR-290/302 family of miRNAs upregulate epiblast markers through other pathways. Nonetheless, the colony formation assay showed that forced activation of MEK pathway indeed drove *Dgcr8*$^{-/-}$ cells out of naive pluripotency state (Figure 5G). Together, these data demonstrate that miR-290/302 family of miRNAs may silence the expression of naive genes in part through upregulating MEK pathway targets, but promote the expression of post-implantation epiblast genes through other targets.

miR-290/302 family of miRNAs facilitate ESC to EpiLC transition in part by repressing Akt1

Ectopic activation of AKT pathway has been previously shown to block differentiation of ESCs [46, 47], raising a possibility that miR-290/302 might function through the inhibition of AKT pathway during differentiation. Mouse has three homologs of Akt genes: Akt1, Akt2 and Akt3. To our surprise, all three genes were predicted as potential targets of miR-290/302 family of miRNAs by Targetscan [48]. qPCR analysis showed that mRNA levels of Akt2 and Akt3 but not Akt1 were suppressed in wild-type ESCs (Supplementary information, Figure S8B). However, luciferase reporter assay for 3′UTRs demonstrated that all three Akt genes were repressed through their 3′UTRs in wild-type ESCs and by miR-290/302 family of miRNAs (Supplementary information, Figure S8C). To test functional roles of Akt proteins, we synthesized two sets of siRNAs for each Akt gene and transfected them into *Dgcr8*$^{-/-}$ cells. qPCR results showed that siRNAs against Akt1 but not those against Akt2 or Akt3 inhibited the expression of naive markers (Figure 6C and Supplementary information, Figure S8D-S8F). Combination of siRNAs against three Akt genes together showed no greater effects than siRNAs against Akt1 alone (Supplementary information, Figure S8G), suggesting Akt1 as a major functional target of miR-290/302 family of miRNAs. Consistent with this, results from colony formation assay further supported that knocking down Akt1 promoted *Dgcr8*$^{-/-}$ ESCs to exit naive pluripotency state (Figure 6D). More importantly, the effect of Akt1 siRNAs in promoting the exit of naive pluripotency is specific for *Dgcr8*$^{-/-}$ cells under differentiation conditions (Figure 6E). Furthermore, overexpression of Akt1 or a constitutively active Akt1 partially blocked the exit of naive pluripotency in wild-type cells under differentiation conditions (Figure 6F). These data indicate that ectopic activation of AKT pathway is partially responsible for differentiation defect in *Dgcr8*$^{-/-}$ cells.

To further prove that Akt1 is a bona fide target of miR-290/302 family of miRNAs, we performed western analysis and found that protein level of Akt1 was markedly downregualted in wild-type ESCs or miR-294/302-transfected *Dgcr8*$^{-/-}$ ESCs comparing to *Dgcr8*$^{-/-}$ ESCs despite that the mRNA level of Akt1 was not affected (Figure 6G and Supplementary information, Figure S8B), suggesting that the regulation is at the translational level. More importantly, luciferase reporter assay showed that although miR-294 repressed the luciferase reporter bearing the 3′UTR of Akt1, mutating the predicted target site noticeably rescued the activity of the reporter in wild-type ESCs or miR-294/302-transfected *Dgcr8*$^{-/-}$ ESCs (Figure 6H). In addition, there was minimum influence of knocking down Akt1 on the expression of MEK targets (Supplementary information, Figure S8H), suggesting that the repression of Akt1 by miR-290/302 family of miRNAs functions independently of the MEK pathway to silence the naive pluripotency program. Together, these data demonstrate that miR-290/302 family of miRNAs facilitate ESC to EpiLC transition partially through the translational repression of Akt1.

Discussion

Molecular mechanisms underlying the dismantling of naive pluripotency state and the establishment of primed pluripotency state are just beginning to be revealed. In this study, we show that miRNAs are essential for silencing the expression of naive genes and inducing the expression of epiblast genes. We further identify miR-290 cluster and miR-302 cluster of miRNAs, as well as several other miRNAs that function redundantly to facilitate ESC to EpiLC transition (Figure 7). The miR-290 cluster of miRNAs are highly expressed in naive state and only slightly downregulated during ESC to EpiLC transition,

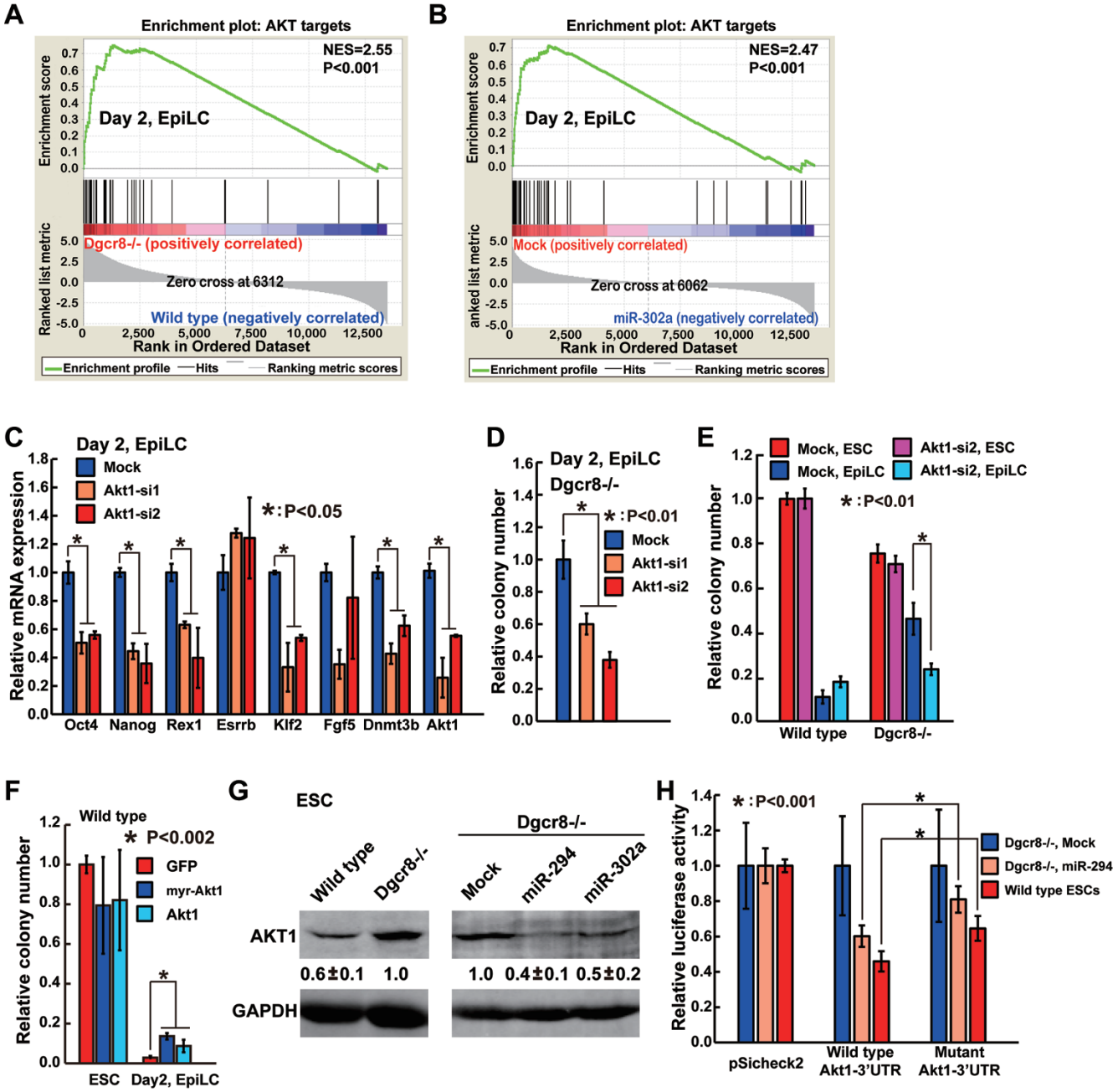

Figure 6 miR-290/302 family of miRNAs silence the naive pluripotency program in part through directly repressing Akt1. **(A)** GSEA for AKT targets in wild-type and *Dgcr8*$^{-/-}$ EpiLCs. **(B)** GSEA analysis for AKT targets in mock- and miR-302a-transfected *Dgcr8*$^{-/-}$ EpiLCs. **(C)** qRT-PCR analysis of naive and early post-implantation markers in *Dgcr8*$^{-/-}$ cells treated with siRNAs against Akt1. β-actin gene is a control. For each gene, data are normalized to mock-transfected cells and are shown as mean ± SD, n = 3. **(D)** Colony formation assay for *Dgcr8*$^{-/-}$ cells treated with siRNAs against Akt1. Data are normalized to mock-transfected cells and shown as mean ± SD, n = 3. **(E)** Colony formation assay for wild-type and *Dgcr8*$^{-/-}$ ESCs and EpiLCs treated with siRNAs against Akt1. Data are normalized to mock-transfected wild-type ESCs and shown as mean ± SD, n = 3. **(F)** Colony formation assay for wild-type overexpressing GFP, myr-Akt1 or Akt1. Data are normalized to wild-type ESCs overexpressing GFP and shown as mean ± SD, n = 6. **(G)** Western blotting analysis of AKT1. Left: AKT1 level in wild-type and *Dgcr8*$^{-/-}$ ESCs. Data are normalized to *Dgcr8*$^{-/-}$ ESCs and shown as mean ± SD, n = 3; Right: AKT1 level in mock-, miR-294- and miR-302a-transfected *Dgcr8*$^{-/-}$ ESCs. Data are normalized to mock-transfected *Dgcr8*$^{-/-}$ ESCs and shown as mean ± range, n = 2. **(H)** Luciferase reporter assay for wild-type or mutant Akt1 3′UTR in wild-type, mock- or miR-294-transfected *Dgcr8*$^{-/-}$ ESCs. Data are first normalized to empty vector then to mock-transfected *Dgcr8*$^{-/-}$ ESCs, and shown as mean ± SD, n = 6.

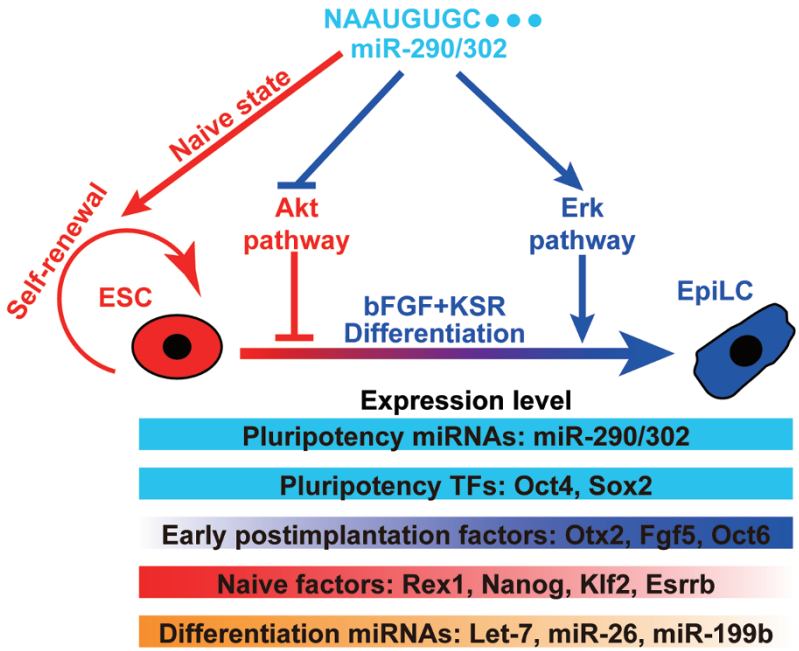

Figure 7 Graphic model showing the role of miR-290/302 family of miRNAs promoting naive to primed pluripotency transition. The expression changes for important protein-coding genes and miRNAs during the transition are shown. The expression levels of miR-290/302 family of miRNAs are constantly high in ESCs, EpiLCs and during ESC to EpiLC transition, which is similar to the expression pattern of general pluripotency factors Oct4 and Sox2.

whereas the miR-302 cluster of miRNAs are expressed in naive state at a low level but upregulated to a level similar to that of miR-290 cluster during differentiation. Therefore, the total level of miR-290/302 family of miRNAs is constantly high in ESCs, EpiLCs and during the transition. Mechanistically, we demonstrate that miR-290/302 family of miRNAs repress the expression of AKT targets and upregulate the expression of MEK targets to facilitate the dismantling of naive pluripotency program. Importantly, we identify Akt1 as a direct target of miR-290/302 family of miRNAs; the repression of Akt1 silences the expression of naive genes and reduces colony forming ability of *Dgcr8*$^{-/-}$ cells upon differentiation. Our study reveals important roles of miRNAs in naive to primed pluripotency transition, and uncovers the surprising context-dependent function of miR-290/302 family of miRNAs in different developmental stages.

It is remarkable that mature miRNAs from miR-290/302 clusters are highly expressed in both naive state and early post-implantation epiblast state, yet they only silence the naive pluripotency program potently. The expression pattern suggests that these miRNAs poise the ground PSCs for developmental progression. This is in concert with screening results for factors that are required for the exit of naive pluripotency state, in which a majority of identified factors are actually expressed in naive PSCs [2, 19-21]. Hence, this study reveals non-coding RNAs as another class of important regulators facilitating the rapid transition during early embryonic development by poising naive cells for differentiation. More interestingly, miR-290/302 family of miRNAs are also highly expressed at late developmental stages such as late post-implantation epiblast and EpiSCs [39, 40, 49]. Future studies are warranted to investigate whether miR-290/302 family of miRNAs are also crucial regulators in late developmental transitions. Evidence supporting this notion comes from the studies that a single miRNA, miR-430, which shares the same seed sequence with miR-290/302 family of miRNAs, can rescue most developmental defects in maternal/zygotic *Dicer* knockout zebrafish embryos [50, 51]. Also consistent with an important function of these miRNAs during development is a recent study showing a severe early lethal phenotype after miR-290/302 DKO [52].

This study reveals that miR-290/302 family of miRNAs promote the dismantling of naive pluripotency program. However, the same miRNAs have also been shown to promote pluripotency in different circumstances by several previous studies including our own [27-37]. How the same miRNAs achieve two apparently opposing func-

tions remains unresolved. Distinct cellular contexts may play a role in determining the outcome of the activity of a molecule or signaling pathway. As demonstrated in this study, knocking down Akt1 suppresses naive pluripotency only in differentiation but not in 2i/Lif condition. However, other functional targets must exist, as Akt1 alone obviously cannot explain the marked effects caused by miR-290/302 in both dismantling of naive pluripotency and establishing of primed pluripotency, especially the latter. The miR-290/302 may also serve as a buffer to restrict the expression of key pluripotency genes such as Klf2, which is already highly upregulated in *Dgcr8*$^{-/-}$ ESCs in 2i/Lif condition. Although in our initial analysis knocking down Klf2 in *Dgcr8*$^{-/-}$ cells did not rescue the differentiation defect (data not shown), it remains to be tested systematically whether other key pluripotent genes are accountable. Finally, a more interesting hypothesis is that these miRNAs may have different repertoire of preferred targets in 2i/Lif and differentiation conditions. The identification of these targets requires the combination of unbiased screening strategy and the elucidation of miRNA targets by global approaches like Ago CLIP-Seq or CLASH [53, 54]. In addition, general pluripotency factors such as Oct4 and Sox2 are redirected to bind new enhancers with help from other transcription factors such as Otx2 during naive ESC to EpiLC transition [13, 18]. It would be fascinating to demonstrate that a similar paradigm exists for miR-290/302 family of miRNAs, that is, these miRNAs are redirected to bind new mRNA targets assisted by other RNA-binding proteins during the transition. Understanding the pleiotropic function of miR-290/302 family of miRNAs will not only uncover new mechanisms controlling early mammalian development and pluripotent state transitions, but also provide novel insights into the derivation of naive ESCs in human and other species.

Materials and Methods

ESC culture

Mouse ESCs were grown on gelatin-coated plates in N2B27 supplemented with 2i/Lif: PD0325901 (1.0 μM), CHIR99021 (3.0 μM) and leukemia inhibitory factor (1 000 unit per ml). For differentiation, cells were grown in N2B27 supplemented with 12 ng/ml bFGF and 1% KSR (Gibco). For colony formation assay, 300 cells were plated on a 12-well plate with irradiated mouse embryonic fibroblasts as feeders in 2i/Lif media. Colonies were counted 5-8 days after plating.

Transfection of miRNA mimics and siRNAs

miRNA mimics or siRNAs (Dharmacon, ThermoFisher; or GenePharma, Shanghai) were transfected at 25 or 50 nM using the DharmaFECT1 transfection reagent (Dharmacon, ThermoFisher) following the manufacturer's protocol. Sequences of siRNAs to Akt genes are listed in Supplementary information, Table S3. For miRNA screening, not all miRNAs fulfilling the criteria "expressed throughout differentiation or upregulated during differentiation" were tested. To control the cost, we picked miRNAs known to have functions in cellular differentiation, e.g., miR-23-24-27 clusters, miR-21 and miR-34. miR-290/302 clusters were selected for their extremely high expression level.

RNA extraction, qRT-PCR and miRNA RT-PCR

Total RNA was extracted following standard Trizol protocol (Invitrogen). Samples were centrifuged at > 12 000× *g* during precipitation and washing steps to preserve small RNAs. qPCR and miRNA RT-PCR were performed using Sybr Green mix (Applied Biosystems). For miRNA qPCR, RNA samples were first polyadenylated with polyA polymerase and then reverse transcribed using a modified oligodT primer as described previously [55]. Sequences for qPCR primers are listed in Supplementary information, Table S4.

RNA-seq and bioinformatics analysis

Total RNA was subjected to two rounds of purification using poly-T oligo-attached magnetic beads before the synthesis of double-stranded (ds) cDNA. The ds cDNA was ligated to adaptors and sequenced using Illumina Genome Analyzer (High-Throughput Sequencing Facility, Peking University). GSEA [24] was used to test for the enrichment of selected gene sets by java GSEA Desktop Application. R 3.1.1 and Matlab were used for the generation of scatter plot and boxplot.

Small RNA cloning and high-throughput sequencing

Total RNA was extracted using Trizol (Invitrogen) as described above and submitted to Bioanalyser (Agilent 2100) for quality assessment. Small RNAs were purified, ligated to 3′ and 5′ adapters, reverse transcribed and amplified using corresponding primers using the Small RNA Sample Prep v1.5 Kit (Illumina). Libraries were sequenced using the Illumina HiSeq2000 (High-Throughput Sequencing Facility, Peking University).

miRNA expression profile and normalization

After trimming the adapter sequence, sequence reads were matched to the miRBase r19 miRNA stem-loop sequences by Bowtie. The miRNA expression profile was obtained according to the criterion described previously [39]. In brief, to assign read counts to either the 5p or 3p miRNAs, each miRNA stem-loop sequence was split sliding between −20 and +20 nt relative to the center of a miRNA stem-loop sequence. For each split position, reads mapping across the split position were discarded. The split position with the maximum sum of the upstream and downstream reads was retained and the upstream and downstream read counts were assigned to the 5p and 3p miRNAs, respectively. Expression values for each miRNAs were normalized and converted to copy number per cell based on the previous estimation that ESCs have total population of ~110 000 molecules of miRNAs per cell [56]. Two-dimensional hierarchical clustering analysis was performed on selected miRNA genes with average expression level of more than 10 copy per cell by Cluster 3.0 and visualized by Java Treeview. Expression values were first log transformed, then mean centered for each gene before clustering.

miRNA cluster knockout strategy

Knockout of miR-290 cluster was previously reported [42]. To knockout miR-302 cluster, a pair of guide RNA sequences, which target the miR-302 cluster genomic locus, was designed by http://crispr.mit.edu/. Knockout of miR-302 cluster was verified by genomic PCR and miRNA RT-PCR. Guide RNA sequences are listed in Supplementary information, Table S5.

Western blot analysis

For western analysis of AKT1, cells were plated in 2i/Lif media 24 h before transfection of 50 nM miR-294 or miR-302a. Proteins were extracted 32 h after transfection. Similar results were obtained for proteins extracted 46 h after transfection. Antibodies against ERK (#9102), pERK (#9101) and AKT1 (#2938) were from Cell Signaling Technology (Massachusetts, USA), against GAPDH (MB001) was from Bioworld Technology (Nanjing, China).

Luciferase reporter assay

Luciferase constructs were produced as follows. 3′UTRs for candidate genes were amplified from ESC cDNA or genomic DNA and cloned into the *Not*I and *Xho*I sites in psiCheck-2 vector (Promega). Sequences of cloning primers are listed in Supplementary information, Table S6. For Akt1 mutant 3′UTR reporter construct, the sequence of predicted miRNA-binding site 5′-GAGCCCTTCTCCTAGCACTT-3′ was mutated to 5′-GAGCCCTTCTCCTCGACCGT-3′. Approximately 24 h before transfection of miRNAs, 8 000 *Dgcr8*$^{-/-}$ or 4 000 wild-type ESCs were plated in 2i/Lif media in a 96-well plate pretreated with 0.2% gelatin. For the cotransfection of miRNAs and reporter plasmids, miRNA mimics were first transfected using DharmaFECT1 (Dharmacon, Thermo Fisher), and ~10 h later 40 ng luciferase reporter plasmid was transfected. Cells were lysed 36 h later and processed for luciferase assay using Dual-Luciferase Reporter Assay System (Promega).

Statistical analysis

The data are presented as mean ± SD except where indicated otherwise. We performed two-tailed unpaired Student's *t*-test to determine statistical significance except for analysis shown in the boxplot graph, for which we performed two-tailed Wilcoxon signed-rank test. P value < 0.05 is considered as statistically significant.

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Author Contributions

YW conceived and planned the study and wrote the paper. KLG, QZ, YY and FFD performed the experiments with the help from other authors. TTL, KLG and JH performed bioinformatic analysis for data from RNA-Seq and small RNA-Seq experiments. All authors participated in data analysis and figure preparation.

Competing Financial Interests

The authors declare no competing financial interests.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)