Received: 21 June 2019 | Accepted: 22 November 2019
DOI: 10.1002/stem.3136

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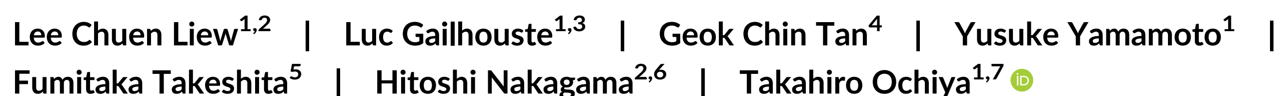
MicroRNA-124a inhibits endoderm lineage commitment by targeting Sox17 and Gata6 in mouse embryonic stem cells



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Funding information
Japan Agency for Medical Research and Development, Grant/Award Numbers: 16fk0310512h0005, 17fk0310101h0001

Abstract

The role of microRNAs (miRNAs) during mouse early development, especially in endoderm germ layer formation, is largely unknown. Here, via miRNA profiling during endoderm differentiation, we discovered that miR-124a negatively regulates endoderm lineage commitment in mouse embryonic stem cells (mESCs). To further investigate the functional role of miR-124a in early stages of differentiation, transfection of embryoid bodies with miR-124a mimic was performed. We showed that overexpression of miR-124a inhibits endoderm differentiation in vitro through targeting the 3′-untranslated region (UTR) of *Sox17* and *Gata6*, revealing the existence of interplay between miR-124a and the *Sox17/Gata6* transcription factors in hepato-specific gene regulation. In addition, we presented a feasible in vivo system that utilizes teratoma and gene expression profiling from microarray to quantitatively evaluate the functional role of miRNA in lineage specification. We demonstrated that ectopic expression of miR-124a in teratomas by intratumor delivery of miR-124a mimic and Atelocollagen, significantly suppressed endoderm and mesoderm lineage differentiation while augmenting the differentiation into ectoderm lineage. Collectively, our findings suggest that miR-124a plays a significant role in mESCs lineage commitment.

KEYWORDS
embryonic stem cells, endoderm, lineage specification, miR-124, teratoma

1 | INTRODUCTION

The distinctive feature of embryonic stem cell (ESC) being able to self-renew, coupled with its potential to differentiate into any adult cell type in the body, has made it a promising candidate for use in tissue regeneration. Although a growing number of studies have demonstrated in vitro derivation of multiple cell types from ESCs,[1-3] their application in cell replacement therapy for human disease remains challenging. One of the factors appears to be the scarcity of methods that can generate fully functional mature cell types. Therefore, an in-depth understanding of the molecular mechanism governing cell fate decisions is crucial to ensure success in regenerative medicine.

MicroRNAs (miRNAs) are a group of small, endogenous, noncoding RNAs that are approximately 18-25 nucleotides in length. These small RNAs are known to regulate gene expression through

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complementary base pairing to the 3′-untranslated region (UTR) of target messenger RNAs (mRNAs) and are important regulators of fundamental cellular processes, including proliferation, differentiation, and apoptosis.[4-7] Growing evidence has shown that miRNA plays a critical role in fine-tuning the expression levels of genes involved in stem cell self-renewal and differentiation. For instance, the miR-290-295 cluster and miR-302/367 cluster have been designated as ESC-specific cell cycle-regulating miRNAs, owing to their functions in repressing key cell cycle regulators.[8,9] In addition, miRNAs have been reported to be involved in lineage specification. Although many lineage-specific miRNAs have been uncovered for ectodermal- and mesodermal-derived cells,[10,11] only a handful of miRNAs have been revealed to be associated with the formation of definitive endoderm (DE).[12-16] Considerable weight should be given to study the basic molecular mechanism that governs the formation of endoderm progenitors, because they are important intermediates that ensure proper generation of fully functional hepatic and pancreatic cells, which in turn paves the way for development of effective regenerative medicinal therapies for chronic liver disease and type I diabetes.

The present work aimed to study the miRNA profile during the course of differentiation from mouse embryonic stem cells (mESCs) to DE. We identified miR-124a as a key repressor of endoderm lineage commitment in mESCs. We showed that in embryoid bodies (EBs), overexpression of miR-124a significantly inhibits the expression of *Sox17* and *Gata6*, which are indispensable transcription factors for endoderm germ layer formation. In agreement with the in vitro results, forced-expression of miR-124a in teratomas led to significant downregulation of endodermal and mesodermal gene expression levels. Importantly, we proposed a feasible method to evaluate the in vivo effect of miRNA on lineage determination using a teratoma model, thus providing an alternative approach to study the complex dynamic processes in early development.

Significance statement

An additional role of miR-124a in the early development of mouse embryonic stem cells (mESCs) was discovered, on top of its widely reported role in neuronal fate determination. The present study demonstrated that miR-124a negatively regulates endoderm lineage commitment in mESCs. Most of the studies reported on the effect of microRNA (miRNA) in stem cell differentiation were limited to in vitro analysis. In the current study, a feasible strategy by utilizing teratoma as an in vivo system to quantitatively assess the functional role of miRNA in lineage specification through gene expression profiling was presented. This approach would be a useful addition to the currently available methods in the field of miRNA analysis in stem cell differentiation and lineage specification.

2 | MATERIALS AND METHODS

2.1 | Cell culture

mESC, EmbryoMax Embryonic Stem Cell Line derived from murine strain 129/SVEV, was purchased from Merck Millipore (cat# CMTI-1, RRID:CVCL_GS41). mESCs were cultured under feeder-free conditions in 2i medium as previously described.[17] Mouse Hepa 1-6 cells were purchased from Riken Cell Bank (RCB) (cat# RCB1638, RRID: CVCL_0327) and maintained in Dulbecco's Modified Eagle's medium (DMEM; Gibco) supplemented with 1% penicillin-streptomycin and 10% heat-inactivated FBS (Gibco).

2.2 | Endoderm differentiation

The induction of Endoderm differentiation from mESCs was performed using Activin A, Noggin, and GSK3β inhibitor (CHIR99021) as previously reported,[18,19] with a lower initial cell seeding density (10 000 cells/cm^2). Endodermal cells derived from this method were validated with positive staining of the DE markers *Sox17* and *FoxA2*, as well as gene expression analysis. The yield of the induced DE cells was quantified based on coexpression of Cxcr4 and c-Kit determined via FACS analysis. Immunofluorescence staining, mRNA expression analysis and flow cytometry were performed as described in the Supplementary Experimental Procedures.

2.3 | Total RNA isolation and qPCR

Cells were lysed using QIAzol Lysis Reagent (Qiagen). Total RNA was extracted using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's protocol, followed by DNase treatment using TURBO DNA-free kit (Ambion). Total RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific), and the integrity of the RNA was evaluated with an Agilent 2100 Bioanalyzer (Agilent Technologies). Reverse transcription of mRNA and miRNA was performed using SuperScript III Reverse Transcriptase (Invitrogen) and TaqMan miRNA Reverse Transcription Kit (Applied Biosystems), respectively, following the manufacturer's instructions. qPCR was carried out using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) or TaqMan MicroRNA Assays, and the results were analyzed as described in the Supplementary Experimental Procedures.

2.4 | MicroRNA array and data analysis

Total RNA samples were harvested at six different time points (days 0, 2, 3, 4, 5, and 6) from two independent experiments during differentiation of mESCs into DE. A miRNA microarray was performed as described in the Supplementary Experimental Procedures. Raw and normalized microRNA array data are available in the Gene Expression Omnibus (GEO) database (accession number GSE131337).

2.5 | miRNA transfection

Reverse transfection of 1×10^6 mESCs was performed in a 60-mm gelatin-coated dish with miRNA mimic candidates or miRNA Negative Control mimic at a final concentration of 100 nM using Lipofectamine RNAiMAX Reagent according to the manufacturer's instructions. At 24 hours post-transfection, EBs were formed via the hanging drop method. EBs were re-transfected with miRNA mimics on days 3, 5, and 7. At day 10, EBs were harvested. See Supplementary Experimental Procedures for details.

2.6 | 3′-UTR luciferase reporter assay

Hepa 1-6 cells were cotransfected with 2 μg firefly luciferase reporter vector pEZX containing a 3′-UTR sequence of *Sox17* or *Gata6* and miR-124a mimics at a concentration of 100 nM using TransFectin lipid reagent (Bio-Rad). Mutated 3′-UTR reporter vector and control miRNA mimics served as negative controls. The firefly-Renilla luciferase signals were assayed using the Dual-Glo Luciferase Assay System (Promega) with a Synergy H4 Hybrid Microplate Reader (BioTek).

2.7 | Teratoma formation and in vivo delivery of miR-124a

Generation of teratomas from mESCs was described in the Supplementary Experiment Procedures. Mice were randomly divided into two groups (miR-124a vs Control) for the in vivo miRNA transfection experiment (n = 3 in each group). in vivo miRNA transfection was performed via intratumoral administration of miRNAs/Atelogen QG complexes at a final concentration of 15 μg/100 μL. in vivo delivery of miRNAs was performed once every 3 days until day 28. At the end of the experiment, teratomas were harvested for histological and microarray analyses. Animal handling and the experiments were conducted in accordance with the guidelines for animal experiments of the National Cancer Center Research Institute, Japan.

2.8 | Statistical analysis

All the in vitro experimental data are presented as the means ± SD. Student's *t* test and ANOVA were performed as appropriate to estimate the statistical significance of the data, except for the correlation analysis data. The equality of the variances was tested using an *F*-test. All *P*-values are two-tailed. Correlation between the expression of *Sox17* and miR-124-3p (qRT-PCR) was assessed through calculation of Spearman's rank coefficient. The statistical significance of the differences in specific-lineage gene expression between teratomas treated with miR-124-3p mimic and miRNA Negative Control mimic was assessed using Mann-Whitney *U* test. All statistical analyses were performed using GraphPad Prism 8 and MedCalc software. The experimental data represent at least three independent experiments and were considered statistically significant at $P < .05$.

3 | RESULTS

3.1 | ActA-NG-CHIR promotes DE differentiation at high efficiency

To study the miRNA expression profiles during endoderm differentiation in mice, we first induced DE from mESCs with Activin A, Noggin, and the GSK-3 inhibitor CHIR99021 (ActA-NG-CHIR). Induction of endoderm for 4 days resulted in the formation of epithelial-like cells, a typical DE morphology (Figure 1A). Using this method, an average of 52.4% ± 0.42% of DE cells (from two independent experiments), indicated by coexpression of the DE-associated surface markers Cxcr4 and c-Kit,[20,21] was obtained (Figure 1B). The remaining cells (Cxcr4+ and c-Kit-) were mesodermal cells, which differentiated within the mixture of the induced population during the DE induction. In addition, exogenous addition of ActA-NG-CHIR efficiently induced DE from mESCs was also evidenced by the significant upregulation of endodermal-specific genes (*Sox17*, *FoxA2*, *Cxcr4*, and *Gsc*) expression, downregulation of ectoderm gene (*Sox1*) expression, and insignificant change in mesodermal gene (*Meox1*) expression (Figure 1C) in differentiated DE samples. In particular, the expression levels of both *Sox17* and *FoxA2* were induced more than 1000-fold in day 6 DE as compared to mESCs. As shown in Figure 1D, the generation of induced endodermal cells was also confirmed by positive staining of Sox17 and FoxA2 (top panel), whereas the cells cultured without ActA-NG-CHIR (CDM D2) (bottom panel) showed negative staining for both markers.

3.2 | miRNA expression profile during endoderm differentiation from mESCs

To analyze the expression profile of miRNAs during endoderm differentiation, a miRNA microarray was performed with RNA samples harvested at six different time points (days 0, 2, 3, 4, 5, and 6). microRNA microarray analysis revealed a large number of differentially expressed miRNAs throughout the process of endoderm differentiation from mESCs. A total of 155 common miRNAs were differentially expressed (fold change >2) between mESCs and differentiated endodermal cells (day 6 DE). Among these miRNAs, 59 were downregulated and 96 were upregulated (Figure 2A). Notably, we observed decreased expression of the miR-290-295 cluster in our differentiated endodermal samples, which was consistent with its well-reported role in mESC self-renewal.[22] In addition, we observed upregulation of miR-21 expression, which was previously reported to regulate cell cycle progression by targeting the pluripotency factors Nanog and Sox2.[23]

To identify miRNA candidates involved in endoderm development, we focused on downregulated miRNAs that target the transcription factor *Sox17*.[24] Using miRanda, we identified a list of 29 miRNAs that were predicted to target *Sox17*. Among these miRNAs, six were found to be downregulated in the induced DE samples (Figure 2B). These six miRNA candidates were then validated by

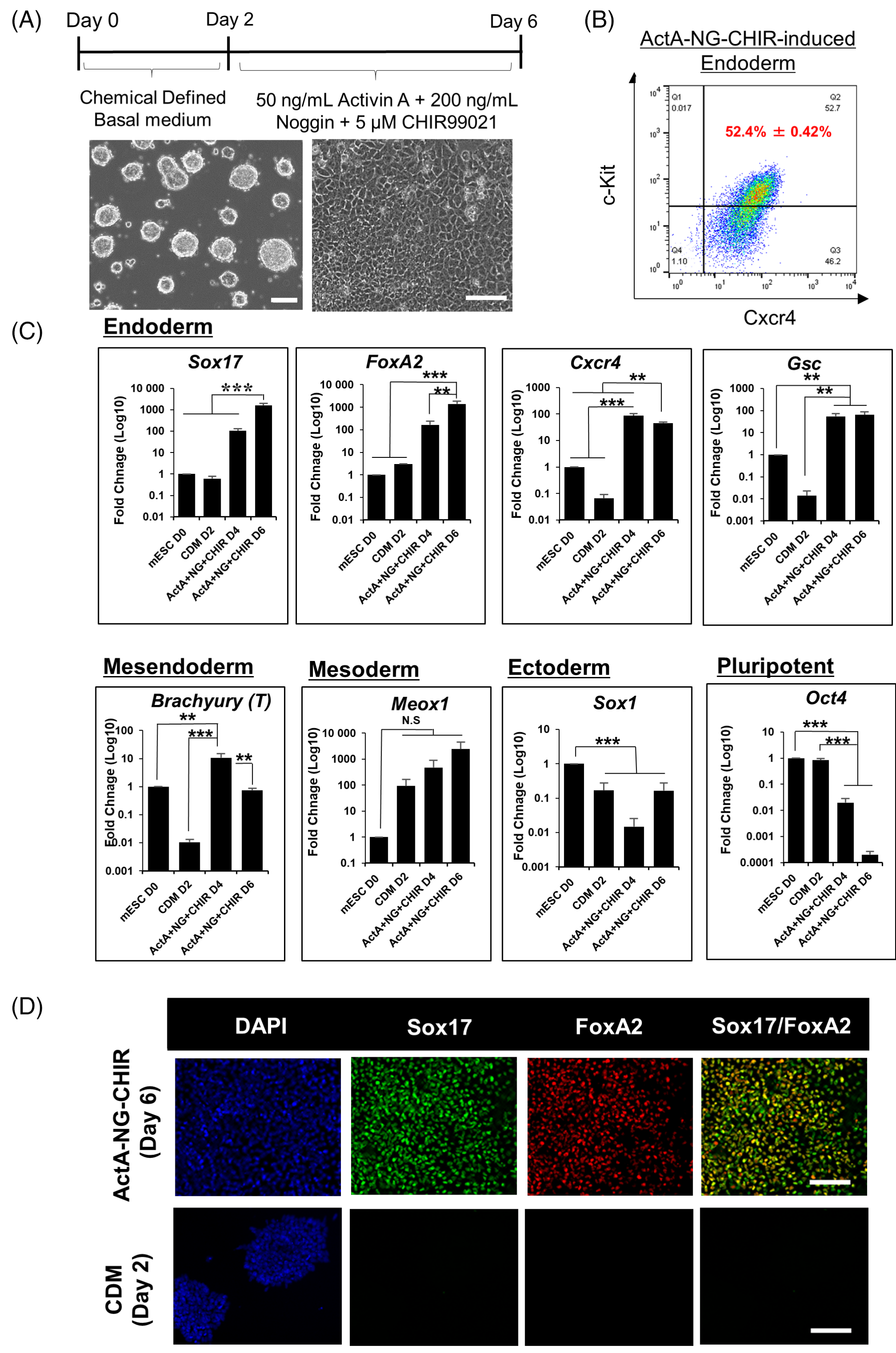

FIGURE 1 Induction of DE from mESCs with Activin A, Noggin, and CHIR99021. A, Morphological analysis of mESCs (left) differentiated into DE (right), an epithelial-like cell structure, by day 6. B, Flow cytometry analysis of DE (Cxcr4 + and c-Kit+) after 4 days of induction. Data are representative of two independent experiments performed. Number indicating the percentage of mean ± SD of induced-DE. C, Gene expression analysis via qPCR during DE induction from mESCs. The data represent the mean ± SD of three independent experiments. Fold change is relative to mESC day 0. $^{**}P < .01$; $^{***}P < .001$ (one-way ANOVA, Tukey's multiple comparison post hoc test). D, Detection of Foxa2 and Sox17 coexpression in ActA-NG-CHIR-induced endoderm at day 6 (top panel). Cells cultured for 2 days in the differentiation medium without the induction factors were negative for the staining (bottom panel). Scale bar represents 100 μm. ActA, Activin; NG, Noggin; CHIR, CHIR99021

qPCR analysis. Consistent with the microarray results, except for miR-101a-3p, all the miRNA candidates were dramatically downregulated on day 4, and their expression levels remained low until day 6 (Figure 2C; Figure S1A). In contrast, the expression level of *Sox17* was observed to gradually increase along the course of endoderm differentiation. Among the six miRNA candidates, only miR-101a-3p showed no correlation with *Sox17* expression, whereas the rest (Figure 2D; Figure S1B) were negatively associated with the expression of *Sox17* (Figure S2 showed the correlation analysis from the mean of two independent experiments). Since the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) has been previously reported to be associated with endoderm differentiation,[25-27] the miRNA candidates were therefore narrowed down to miR-7a-5p and miR-124a-3p.

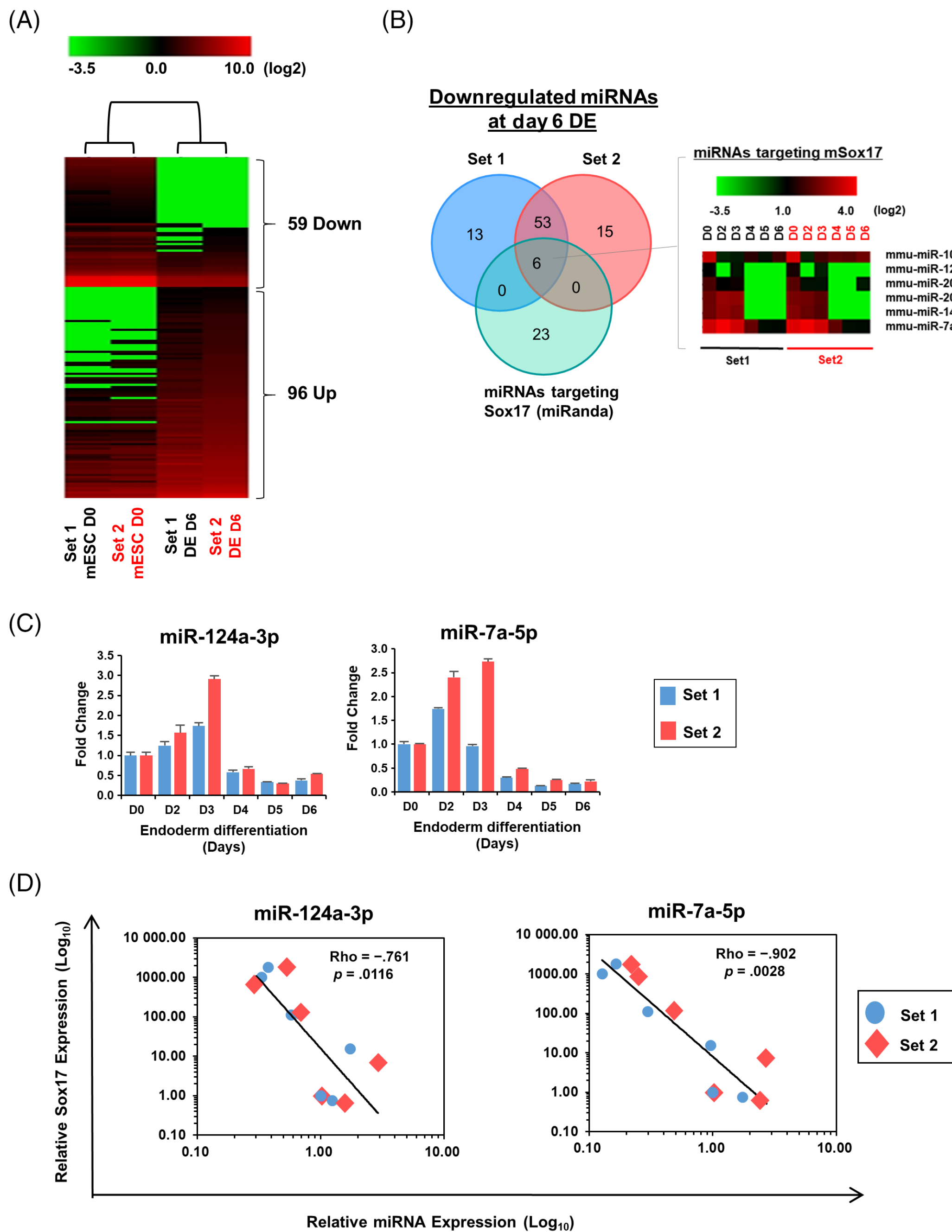

FIGURE 2 Downregulation of miR-124a expression during endoderm differentiation in mESCs. A, Heat map generated from miRNA microarray showing differentially expressed miRNAs in mESCs and differentiated endodermal cells (day 6 DE). Analysis was performed based on data from two independent experiments (designated as set 1 and set 2). A twofold threshold was set to identify miRNAs with significant differential expression, and the scale bar represents the logarithm of the relative miRNA expression level. B, Venn diagram showing six miRNA candidates targeting *Sox17* (identified via miRanda) that appeared in both samples. Heat map generated from miRNA microarray shows the expression of the six miRNA candidates during endoderm differentiation from days 0 to 6. A twofold threshold was set to identify miRNAs with significant differential expression. C, qRT-PCR validation of the expression level of two selected candidates, miR-124a and miR-7a, during endoderm differentiation from mESCs. The data represent the mean ± SD of two independent experiments with three technical replicates. D, Correlation analysis between miR-124a and miR-7a vs *Sox17* expression during endoderm differentiation from mESCs using Spearman's correlation

3.3 | Forced expression of miR-124a-3p inhibits *Sox17* expression in EBs through 3′-UTR targeting

To validate whether the selected miRNAs were involved in regulating *Sox17* expression, EBs were transfected with miR-7a-5p and miR-124a-3p mimics as shown in Figure 3A. Efficient overexpression of miRNA was confirmed by the increased expression of the respective miRNAs in day 10 EBs, which was 3 days after the last transfection. Representative data showed that miR-124a (Figure 3B, left) and miR-7a (Figure S3, left) expressions were increased about 23-fold and

55-fold in day 10 transfected EBs, respectively. However, remarkable inhibition of *Sox17* expression (81.74 ± 18.25%; $P < .01$; *t* test) was only observed in day 10 EBs transfected with miR-124a-3p mimics (Figure 3B, right) but not in those transfected with miR-7a-5p mimics (Figure S3, right). Therefore, miR-124a was identified as a potential miRNA candidate targeting *Sox17*. In line with the gene expression data, similar inhibition was observed at the protein level. Figure 3C shows the decrease of Sox17 protein level in the nuclear fraction of EBs transfected with miR-124a-3p mimics compared with control EBs (n = 2). Interestingly, we occasionally observed Sox17 inhibition in the cytoplasmic fraction (n = 2) (Figure S4). To confirm *Sox17* as a direct target of miR-124a, a 3′-UTR luciferase reporter assay was performed in Hepa1-6 cells. In contrast to the miRNA Negative Control mimic, cotransfection of Hepa1-6 cells with the miR-124a-3p mimic and firefly luciferase reporter vector significantly downregulated the 3′-UTR luciferase activity of *Sox17* by 19.69% ± 6.04% ($P < .05$; *t* test). No inhibition of luciferase activity was observed when the cells were cotransfected with the mutated 3′-UTR vector (Figure 3D).

3.4 | Overexpression of miR-124a-3p suppresses endodermal gene expression in EBs

Next, we further examined the effect of ectopic miR-124a expression on lineage commitment in vitro by analyzing the lineage-specific gene expression in miR-124a-overexpressed EBs. As a result, we found that on top of *Sox17*, overexpression of miR-124a also significantly inhibited *Gata6* expression (64.25% ± 29.75%; $P < .05$; *t* test), another

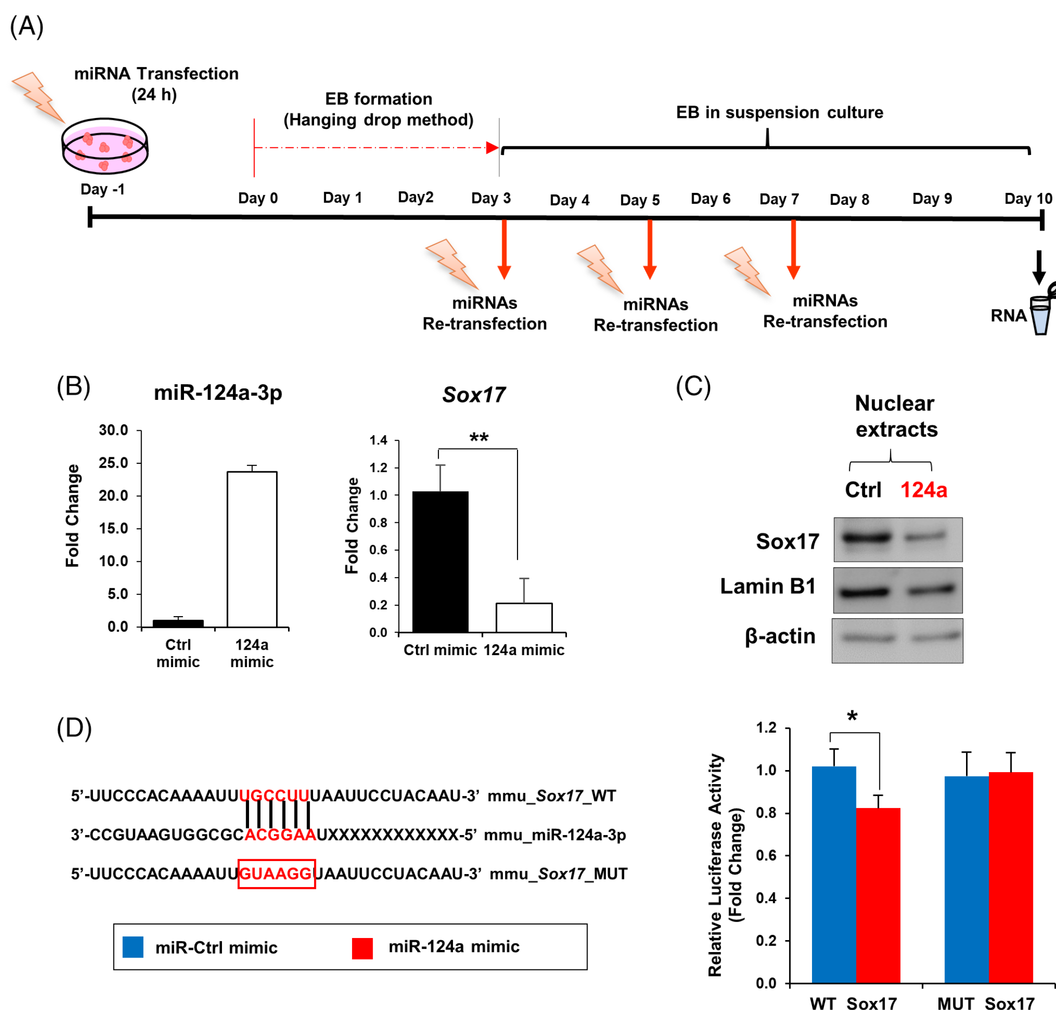

FIGURE 3 miR-124a inhibited *Sox17* expression through 3′-UTR targeting. A, Schematic diagram illustrating the protocol for transfection of EBs with miRNA mimic. B, Representative data from an individual experiment with three technical replicates showing the relative level of miR-124a expression in transfected EBs (left). The relative level of *Sox17* mRNA expression in transfected EBs (right). The data represent the mean ± SD of three independent experiments. C, The Sox17 protein level was detected in day 10 EBs via Western blotting (n = 2); β-actin was used as the internal control, and Lamin B1 was used as a technical control to assess the efficiency of subcellular fractioning. D, Alignment of miR-124a-3p with predicted binding sites in the 3′-UTR of *Sox17*. Dual luciferase assay of Hepa1-6 cells cotransfected with miR-124a mimic and the firefly/Renilla luciferase construct containing the *Sox17* 3′-UTR. Mutated 3′-UTR sequences were used as negative controls, and the ratios of firefly/Renilla luciferase activities were determined. The data represent the mean ± SD of three independent experiments. $^*P < .05$; $^{**}P < .01$ (*t* test)

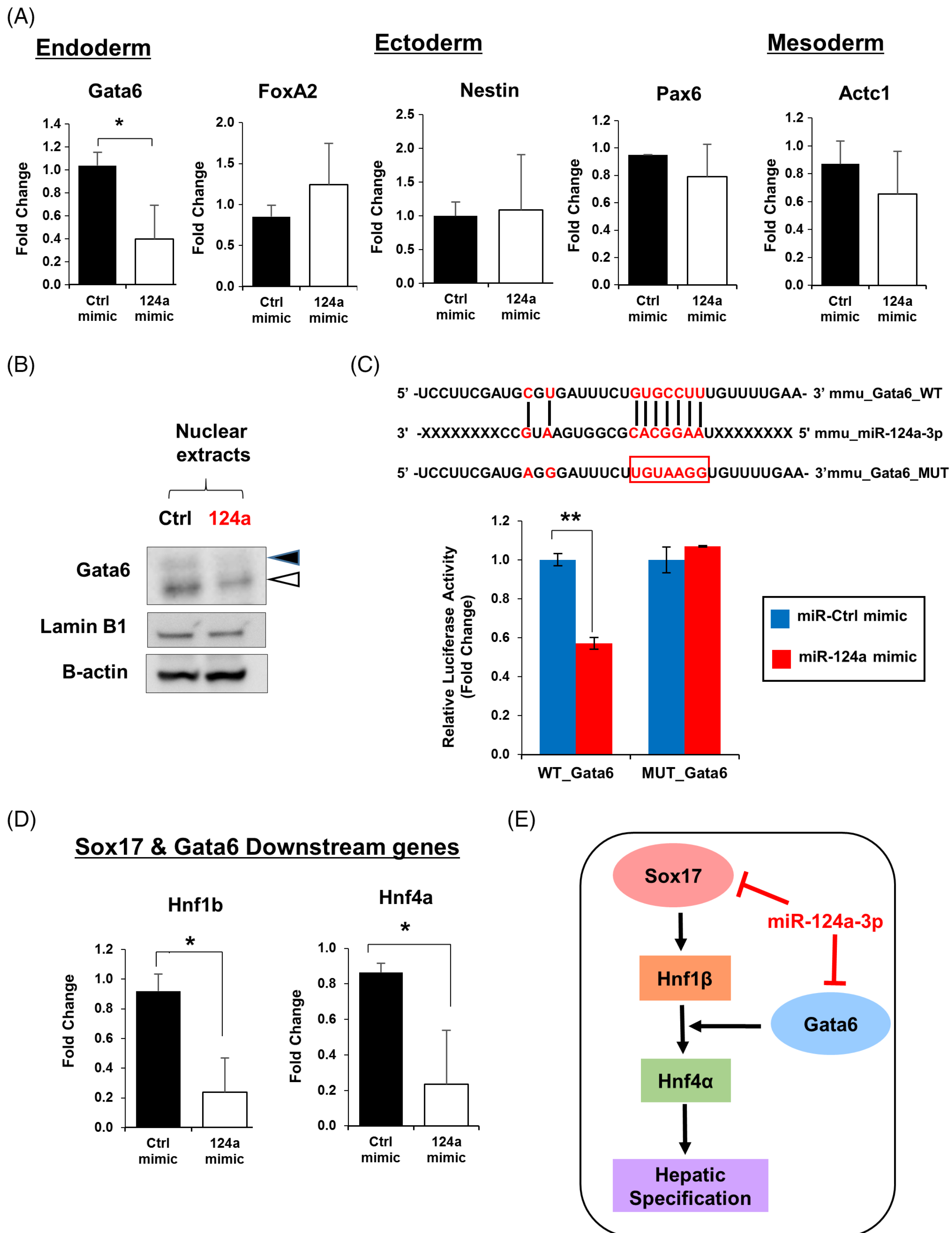

FIGURE 4 miR-124a suppressed endoderm lineage commitment and hepatic specification in EBs. A, The relative expression of lineage-specific genes in miR-124a-overexpressed EBs compared with control EBs. B, The Gata6 protein level was detected in day 10 EBs via Western blotting (n = 3). Black and white arrowheads indicate the long and short isoforms of Gata6, respectively; β-actin was used as the internal control, and Lamin B1 was used as a technical control to assess the efficiency of subcellular fractioning. C, Dual luciferase assay of Hepa1-6 cells cotransfected with miR-124a mimic and the firefly/Renilla luciferase construct containing the *Gata6* 3′-UTR. Mutated 3′-UTR sequences were used as negative controls, and the ratios of firefly/Renilla luciferase activities were determined. D, The relative expression levels of the hepato-specific genes *Hnf1β* and *Hnf4α* in miR-124a-overexpressed EBs compared with control EBs. E, Schematic outlining the proposed regulatory network between miR-124a-3p, *Sox17*, *Gata6* and their downstream targets in liver specification. All the data represent the mean ± SD of three independent experiments. $^*P < .05$; $^{**}P < .01$ (*t* test)

important transcription factor for endoderm differentiation (Figure 4A). In contrast, gene expression specific to other lineages, such as ectodermal (*Nestin and Pax6*) and mesodermal gene (*Actc1*) expression, was not affected. Similarly, inhibition of Gata6 protein expression was observed in miR-124a-overexpressed EBs (Figure 4B). On the other hand, cotransfection of Hepa1-6 cells with miR-124a-3p mimic and the firefly luciferase reporter vector significantly downregulated the 3′-UTR luciferase activity of *Gata6* by 50.48% ± 8.35% (P < .01) compared with that observed after cotransfection with the miRNA Negative Control mimic (Figure 4C), demonstrating that *Gata6* is another direct target of miR-124a.

Interestingly, overexpression of miR-124a also significantly suppressed *Hnf1β* (68.08% ± 23.12%; P < .05) and *Hnf4α* (62.94% ± 30.34%; P < .05) expression levels (Figure 4D). Both *Hnf1β* and *Hnf4α* are downstream targets of *Sox17* and *Gata6*, and are known as the master regulators of hepatic differentiation.[28,29] *Hnf1β* and *GATA6* act in a synergistic manner to strongly induce the expression of *Hnf4α* in the HNF regulatory network.[30] Collectively, we revealed a regulatory network of miR-124a in endoderm lineage development. In this network, miR-124a inhibited the expression of *Sox17* and *Gata6*, which in turn downregulated the expression of *Hnf1β* and *Hnf4α*, thereby possibly affecting hepatic specification (Figure 4E). The function of miR-124a in endoderm lineage commitment was also confirmed by transfection of EBs with miRNA inhibitor (Figure S5).

3.5 | Expression of miR-124a during endoderm differentiation is retinal noncoding RNA 3 (*Rncr3*) dependent

In the mouse genome, miR-124a is encoded by three different genes, *miR-124-1* (also known as *Rncr3*), *miR-124-2*, and *miR-124-3*, which are located on chromosomes 14, 3, and 2, respectively.[31-33] Although derived from three different host genes, each precursor miRNA generates the same mature miR-124a sequence. To investigate from which host gene miR-124a is generated, we analyzed and compared the expression level of all three primary miR-124 between mESCs and induced-endoderm via qPCR. Interestingly, of the three primary miRNAs, pri-miR-124-1 was the most abundantly expressed in undifferentiated mESCs, accounting for 97.60% ± 1.53% (Figure 5A). In addition, we observed significant downregulation of pri-miR-124-1 expression in induced-endoderm samples as compared to mESCs (Figure 5B), whereas the expression level of pri-miR-124-2 and pri-miR-124-3 remained largely unchanged after differentiation.

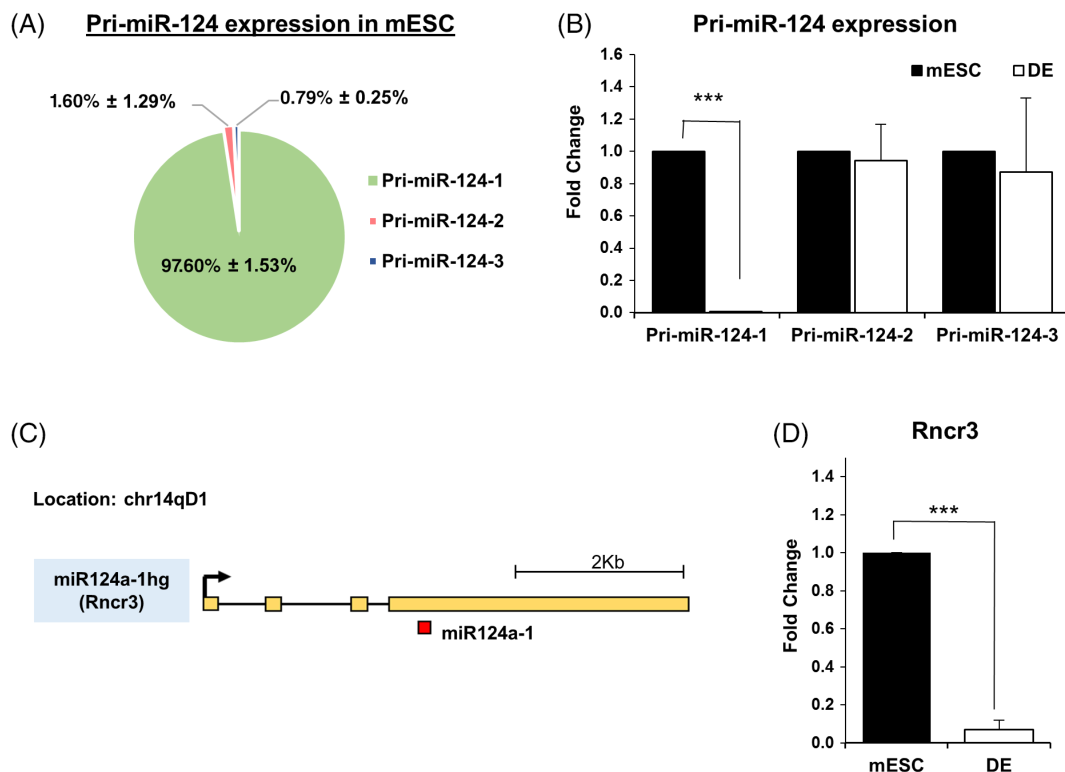

FIGURE 5 miR-124a expression during endoderm differentiation is retinal noncoding RNA 3 (*Rncr3*) dependent. A, Pie chart representing the percentage of each primary miR-124 variant expressed in mESCs. The percentage of each variant was calculated from the mean ddCT value of the respective variant divided by the mean ddCT value of all three variants in mESC samples. The data represent the mean ± SD (in percentage) of three independent experiments. B, The expression of primary miR-124a variants in mESCs vs induced endoderm determined by qPCR. C, Schematic representation of *Rncr3* gene. Yellow boxes represent exons, horizontal lines represent introns, and the red box represents the coding region for miR-124a-1. D, The expression level of *Rncr3*, the host gene of pri-miR-124-1, in mESCs vs induced endoderm assessed via qPCR. The data represent the mean ± SD of three independent experiments. ***P < .001 (t test)

Therefore, we decided to focus on pri-miR-124-1. Since *Rncr3* encodes for pri-miR-124-1 (Figure 5C), we then examined its expression level in mESCs and induced-endoderm samples to further validate whether its expression is correlated with the expression of mature miR-124a during mouse endoderm differentiation. As shown in Figure 5D, *Rncr3* expression was significantly downregulated in differentiated endodermal cells as compared to mESCs, which was consistent with the expression of the encoded pri-miR-124-1. These findings suggest that the expression of mature miR-124a during endoderm development is likely to be *Rncr3* dependent.

3.6 | miR-124a-3p overexpression interferes with the endoderm lineage commitment in vivo

To further validate the in vitro results, the effect of miR-124a on lineage commitment in vivo was assessed via intratumoral injection of miRNA mimics in teratomas, followed by microarray analysis (Figure 6A). Teratomas treated with miRNA Negative Control mimic or miR-124a mimic showed no significant difference in tumor size (data not shown). The trilineage differentiation potential of the teratomas was confirmed by H&E staining. Both teratomas treated with

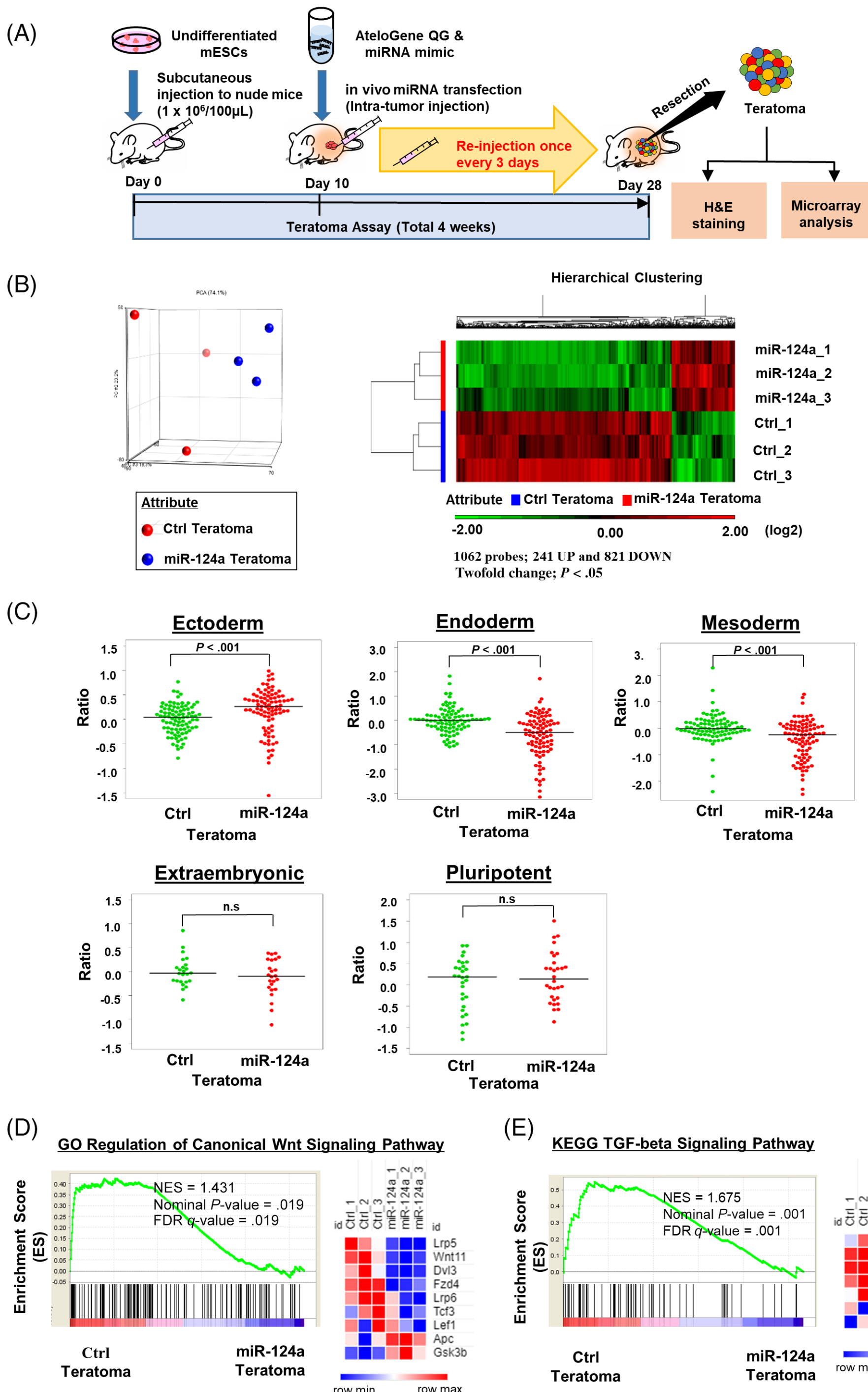

FIGURE 6 miR-124a-3p overexpression affected the tendency of lineage-specific commitment in vivo. A, Schematic representation of the protocol for in vivo delivery of miRNA mimics into teratoma. B, PCA plot (left) showing the differential gene expression patterns of miRNA-treated teratomas. The heat map (right) represents the differential gene expression profiles between the two groups of teratomas. A twofold threshold and *P*-value <.05 were used to identify genes with significant differential expression. C, Dot plots showing genes expressions in the three germ layers in miRNA-treated teratomas. Each bar represents the median expression level of all the genes representing the respective lineages. D, GSEA plot for GO regulation of the canonical Wnt signaling pathway (left) and (E) KEGG TGF-beta signaling pathway (left) in control teratomas vs miR-124a-treated teratomas (FDR < 0.1, *P* < .05). The heat maps generated from microarray data showing the expression of downregulated genes involved in the respective pathways (right)

miR-124a or miRNA Negative Control mimics contained tissues derived from the three germ layers. Routinely identified structures that were found in the harvested teratomas are shown in Figure S6.

Next, we applied principal component analysis (PCA) to assess the variation between the samples. It showed that teratomas from the same miRNA-treated group were clustered together (Figure 6B, left). The heat map in Figure 6B (right) shows the different expression profiles between the two groups of miRNA-treated teratomas. A total of 1062 genes were differentially expressed (fold change >2; $P < .05$), with 241 upregulated and 821 downregulated genes. The inhibitory effect of miR-124a on lineage commitment was determined by comparing the lineage-specific differentiation tendency between miRNA Negative Control-treated and miR-124a-3p mimic-treated teratomas. To quantitatively measure the composition in the teratomas, we adopted the gene set of Teratoscore analysis, which contains 100 genes representing genes from all three germ layers and extraembryonic membranes.[34] Since the gene set was originally established for human samples, we annotated the genes to their mouse counterpart. We then generated the lineage gene expression profile and subsequently compared the profiles between miR-124a-3p-treated and control teratomas. As shown in Figure 6C, there was a shift in the differentiation tendency for all three germ layers in the miR-124a-treated group as compared to the control group. Ectopic expression of miR-124a via intratumor delivery into the teratomas suppressed endoderm ($P < .0001$; Mann-Whitney test) and mesoderm ($P = .001$; Mann-Whitney test) lineage commitment but augmented ectoderm lineage differentiation ($P < .0001$; Mann-Whitney test).

Similarly, via Gene Set Enrichment Analysis (GSEA), we discovered that the top 20 gene sets (gene ontology biological processes) enriched in miR-124a-overexpressed teratomas were related to neuroectoderm development (Table S1). On the other hand, gene sets involving the Wnt/β-catenin (Figure 6D, left) and TGF-β pathways (Figure 6E, left), which are essential for differentiation along mesodermal and endodermal lineages,[35-38] were downregulated in miR-124a mimic-treated teratomas ($P < .05$, FDR < 0.1). Expression of the downregulated regulatory genes involved in Wnt/β-catenin pathway (*Lrp5*, *Wnt11*, *Fzd4*, *Dvl3*, and etc), as well as TGF-β pathway (*Smad3*, *Smad4*, *Tgfb1*, *Tgfb2*, and etc) were shown in the heat maps (Figure 6D,E). These findings provide additional evidence that miR-124a plays critical role in mESC lineage commitment.

4 | DISCUSSION

Mouse embryonic development is tightly regulated by temporal and spatial expression of genes at multiple stages. Growing evidence has shown that miRNAs regulate gene expression at the post-transcriptional level and thus act as important modulators in cell fate determination.[11] For instance, miR-124 and miR-9 have been reported to be neuron-specific miRNAs, whereas miR-1 and miR-133 are classified as cardiomyocyte-specific miRNAs.[39,40] Although considerable efforts have been focused on the development of mature cell types, the role of miRNAs in orchestrating early embryonic development remains elusive.

In the present work, we found that miR-124a plays a prominent role in mouse early development. Our findings suggest that mature miR-124a, whose expression was observed in undifferentiated mESCs and subsequently downregulated during endoderm differentiation, is processed from the pri-miR-124-1 transcript in an *Rncr3*-dependent manner. In addition, we revealed the existence of an interplay between miR-124a and *Sox17/Gata6* transcription factors in hepato-specific gene regulation, which could be considered a new clue providing insight into the transcriptional machinery in mouse early development. The discrepancy between the significant *Sox17* inhibition at the mRNA level and the moderate reduction in luciferase activity (approximately 20%) observed in the *Sox17* 3′-UTR reporter assay prompted us to investigate the presence of an interaction between miR-124a and *Sox17* that is possibly beyond the 3′-UTR region of mRNAs. Although in most cases, miRNA induces mRNA degradation and translational repression through targeting the 3′-UTR of the transcript, it has been reported that miRNAs can also target the amino acid coding sequence (CDS).[41] Indeed, using Basic Local Alignment Search Tool (BLAST), we found a complementary sequence for miR-124a located within the coding region of *Sox17*, suggesting the possibility that miR-124a regulates *Sox17* gene expression through simultaneously targeting both its 3′-UTR and coding regions. Interestingly, Fang and Rajewsky also reported that miRNA targeting of the CDS and the 3′-UTR has a synergistic effect on the expression of corresponding mRNA.[42] Hence, this may explain the difference in the degree of reduction between *Sox17* mRNA expression and luciferase activity of the 3′-UTR reporter assay.

To date, most studies that have reported the effect of miRNA in lineage specification were largely limited to in vitro analysis, in particular using EB as a model. Despite their cellular self-organization and ability to form three germ layers, the differentiation events in EBs are restricted to certain cell types in the early stages of development, but not the subsequent development. This makes it challenging to further analyze the long term effects of miRNA in cell specification of later developmental stages. Unlike EBs, teratomas are heterogeneous tumors comprised of terminally differentiated cells from all germ layers.[43] In the current study, we presented a feasible strategy of utilizing teratoma as an in vivo system to evaluate the functional role of miRNA in lineage commitment. By comparing lineage gene expression, we managed to distinguish tissue-specific signatures in teratomas treated with miR-124a vs those treated with miRNA Negative Control. We successfully demonstrated that forced expression of miR-124a in teratomas via intratumor delivery interfered with the formation of the three germ layers in vivo. Additionally, the gene expression data generated from teratomas enabled us to perform GSEA, which is undoubtedly useful to further understand the underlying biological processes that are affected by miRNA. Nevertheless, we acknowledge that the in vivo instability of miRNA is a potential technical challenge in this method. Therefore, we addressed this issue by performing repeated miRNA injections and using a delivery system that limits miRNA degradation (ie, Atelogen).

MicroRNA-124a has been widely reported to play a role in inducing neurogenesis.[44-46] Among these studies, Burkholder et al showed

that miR-124a suppressed the expression of small C-terminal domain phosphatase 1 (*SCP1*) by targeting its 3′-UTR region. Interestingly, *SCP1*, on the other hand, dephosphorylates RE1-silencing transcription factor (*REST*) and protects it from degradation.[47] *REST*, in turn, is a neuronal repressor that negatively regulates miR-124a expression. Therefore, when miR-124a is overexpressed, a double negative cascade exists, which suppresses the expression of *SCP1*, followed by degradation of *REST*, consequently making it possible to maintain a high level of miR-124a and ultimately leading to neuronal differentiation. In addition to the induction of ectodermal differentiation, it was also found that the development of endo- and meso-lineages in *REST* knockout ESC lines were disrupted. Loss of *REST* decreased or delayed the expression of both endodermal markers (*Sox17*, *Gata6*, and *Gata4*) and mesodermal markers (*T-bra*, *Mixl1*, *Kdr*, *Mesp1*, *Hand1*, and *Mef2c*).[48] Collectively, these studies support our results that showed the suppression of both endoderm and mesoderm lineage commitment but increase in ectoderm lineage development when miR-124a was overexpressed in the teratomas. In addition to the reported targets, our finding of *Sox17* and *Gata6* as new direct targets of miR-124a suggests that miR-124a could act at different hierarchical levels to regulate genes that need to act in concert to drive normal development of germ layers.

5 | CONCLUSION AND SUMMARY

In conclusion, we discovered that miR-124a negatively regulates endoderm differentiation in mESCs by targeting *Sox17* and *Gata6*. To the best of our knowledge, this report is the first to utilize teratoma as an in vivo system to quantitatively assess the functional role of miRNA in lineage specification using gene expression profiling. Since miRNAs function in concert to preserve the balance in the differentiation of the three lineages, miRNAs that promote one lineage can at the same time act as inhibitors for other lineages. Therefore, further study should focus on the upregulated miRNAs that were observed in our miRNA microarray profile because they could possibly function as inhibitors to block ectoderm and mesoderm lineage commitment. It would be interesting to use these miRNAs mimics together with miR-124a inhibitor as a cocktail to maximize endoderm lineage specification. Such further studies would likely advance research that aims to generate fully functional mature cells for the use in regenerative medicine.

ACKNOWLEDGMENTS

We would like to thank A. Inoue (National Cancer Center Research Institute, Japan) for her technical assistance, and R. Takahashi (Hiroshima University) for his contribution in flow cytometry analysis. We are also thankful to T. Katsuda and A. Sanchez Calle (National Cancer Center Research Institute, Japan) for the constructive comments on the study. This work was supported in part by Grants-in-Aid from the Research Program on Hepatitis from Japan Agency for Medical Research and Development (AMED: 16fk0310512h0005 and 17fk0310101h0001).

CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

T.O.: conceived the original idea, supervised and obtained funding for the project; L.C.L.: designed the study, performed the majority of the experimental work, analyzed the data, wrote the manuscript with input from all authors; L.G.: assisted in the experimental design, experimental work, data analysis; T.G.C.: provided expertise for histological analysis; Y.Y.: helped in microarray analysis; F.T.: assisted in animal experiment; H.N.: co-supervised the project, advised with regard to the experimental design and manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are accessible through GEO Series accession numbers GSE131337 and GSE131346.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Liew LC, Gailhouste L, Tan GC, et al. MicroRNA-124a inhibits endoderm lineage commitment by targeting Sox17 and Gata6 in mouse embryonic stem cells. *Stem Cells*. 2020;38:504–515. https://doi.org/10.1002/stem.3136