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Endogenous miRNA Sponge lincRNA-RoR Regulates Oct4, Nanog, and Sox2 in Human Embryonic Stem Cell Self-Renewal

Yue Wang,[1,4] Zhenyu Xu,[1,4] Junfeng Jiang,[1,4] Chen Xu,[1] Jiuhong Kang,[2] Lei Xiao,[3] Minjuan Wu,[1] Jun Xiong,[1] Xiaocan Guo,[1] and Houqi Liu[1,*]
[1]Department of Histology and Embryology, College of Basic Medicine, Second Military Medical University, Shanghai 200433, China
[2]School of Life Sciences and Technology, Tongji University, Shanghai 200092, China
[3]College of Animal Sciences, Zhejiang University, Hangzhou 310058, China
[4]These authors contributed equally to this work
*Correspondence: houqiliu@126.com
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SUMMARY

The embryonic stem cell (ESC) transcriptional and epigenetic networks are controlled by a multilayer regulatory circuitry, including core transcription factors (TFs), posttranscriptional modifier microRNAs (miRNAs), and some other regulators. However, the role of large intergenic noncoding RNAs (lincRNAs) in this regulatory circuitry and their underlying mechanism remains undefined. Here, we demonstrate that a lincRNA, *linc-RoR*, may function as a key competing endogenous RNA to link the network of miRNAs and core TFs, e.g., Oct4, Sox2, and Nanog. We show that *linc-RoR* shares miRNA-response elements with these core TFs and that *linc-RoR* prevents these core TFs from miRNA-mediated suppression in self-renewing human ESC. We suggest that *linc-RoR* forms a feedback loop with core TFs and miRNAs to regulate ESC maintenance and differentiation. These results may provide insights into the functional interactions of the components of genetic networks during development and may lead to new therapies for many diseases.

INTRODUCTION

Embryonic stem cells (ESCs) have an unlimited potential to be propagated in culture in an undifferentiated state (self-renewal) and the ability to generate and differentiate into most cell types (pluripotency) (Okita et al., 2007). The transcriptional and epigenetic networks controlling ESC self-renewal and pluripotency are the focus of intense interest, because of their obvious therapeutic potential as well as exceptional relevance to models of early development (Sheik Mohamed et al., 2010; Young, 2011). It has been well established that a group of core transcription factors (TFs), e.g., Oct4, Sox2, and Nanog, play a critical role in the transcriptional network by promoting the expression of ESC-specific genes and by suppressing differentiation (Boyer et al., 2005; Chen et al., 2008; Kim et al., 2008). The key role of the core TFs was highlighted by the fact that the exogenous introduction of these TFs into murine or human adult cells induced pluripotency by reprogramming these cells into induced pluripotent stem cells (iPSCs), which are functionally and phenotypically similar to ESCs (Okita et al., 2007; Yu et al., 2007).

For posttranscriptional networks, microRNAs (miRNAs), a type of small noncoding RNA that posttranscriptionally regulates gene expression, are also well known as the key posttranscriptional modifier contributing to the control of hESC self-renewal, pluripotency (Melton and Blelloch, 2010; Melton et al., 2010), and differentiation (Morin et al., 2008). A handful of miRNAs have been reported to be involved in the direct repression of these core TFs; miR-145 represses the 3′ UTR of Oct4, Sox2, and Klf4 (Xu et al., 2009). MiR-134, miR-296, and miR-470 target the coding DNA sequence (CDS) of mouse *NANOG*, *OCT4*, and *SOX2* (Tay et al., 2008). The temporal upregulation of these miRNAs may be necessary and sufficient to repress pluripotency and control ESC differentiation, which was also confirmed by the fact that ESCs deficient in miRNA-processing enzymes, such as Dicer and DCGR8, show defects in differentiation (Kanellopoulou et al., 2005; Murchison et al., 2005; Wang et al., 2007). However, in contrast to our understanding of the miRNA-mediated regulation of core TFs, it remains unclear whether components of the transcriptional and posttranscriptional networks functionally modulate the expression of miRNAs and which factors might mediate such regulation. The lack of such information may impair the balance of the current model of the regulatory circuitry in human embryonic stem cells (hESCs). We hypothesize that the characterization of these miRNA regulatory factors may be of great importance to understand the regulation of ESC self-renewal and pluripotency.

In addition to miRNA, long or large intergenic noncoding RNAs (lincRNAs) have recently been identified as novel regulators of the transcriptional and epigenetic networks (Mercer et al., 2009). A number of recent papers have revealed that lincRNA are important and powerful *cis*- and *trans*-regulators of gene activity that can function as scaffolds for chromatin-modifying complexes and nuclear bodies and as enhancers and mediators of long-range chromatin interactions (Huarte et al., 2010; Khalil et al., 2009). Interestingly, several recent reports have provided a model that suggests that lincRNA may function as competing endogenous RNA (ceRNA) in modulating the concentration and biological functions of miRNAs (Cesana et al., 2011; Tay et al.,

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2011). In previous reports, *PTENP1* has been reported to be a ceRNA that protects *PTEN* messenger RNA (mRNA) from miRNA-mediated degradation (Tay et al., 2011). *Linc-MD1* has also been identified as a ceRNA that protects MyoD transcripts (Cesana et al., 2011). These ceRNAs generally share miRNA-response elements with the transcripts of several important genes and prevent these mRNAs from being degraded. We therefore propose that some lincRNAs may also have roles as ceRNAs to link the miRNAs and transcriptional network in hESCs. However, the role of ceRNAs in the process of pluripotency regulation has not yet been elucidated.

In our current work, which seeks to determine the ceRNAs regulating hESC self-renewal and differentiation, we investigated a large intergenic noncoding RNA, *linc-RoR*, which was previously identified as a key reprogramming regulator and whose expression is linked to pluripotency under the direct regulation of core pluripotency TFs (Loewer et al., 2010). However, the functions of and mechanisms utilized by *linc-RoR* in the process of ESC self-renewal and differentiation have not been fully elucidated. In our current study, based on bioinformatic and experimental approaches, we suggest that *linc-RoR* functions as a ceRNA to regulate the expression of core TFs *OCT4*, *SOX2*, and *NANOG* and differentiation-related miRNAs in hESCs.

RESULTS

Linc-RoR Expression Is Positively Correlated with the Undifferentiated ES Cell State

To identify the mechanism of *linc-RoR*-mediated regulation in hESCs, we began by assessing the expression levels and location of *linc-RoR* in hESCs (H1 and X-01 [Wu et al., 2011] cells) growing under self-renewal conditions and various differentiated conditions, including the removal of fibroblast growth factor (bFGF), bone morphogenetic protein (BMP) 4-induction, or embryoid body (EB) formation. Employing a fluorescence in situ hybridization (FISH) assay, we found that the *linc-RoR* transcripts were abundant in the cytoplasm of self-renewing hESC cells (H1, 40 passages; X-01, 30 passages) (Figure 1A), which supports the hypothesis that *linc-RoR* interacted with miRNAs in the cytoplasm. However, we did not detect *linc-RoR* expression in differentiated hESCs in FISH assays. In a qRT-PCR analysis, a marked reduction of *linc-RoR* transcripts was observed in all differentiated hESCs, which indicated that *linc-RoR* expression is positively correlated with the undifferentiated ESC state (Figure 1B). Additionally, when we depleted Oct4 or Nanog with small interfering RNAs (siRNAs), we observed a reduction in *linc-RoR* in hESC H1 cells (Figure 1C) and X-01 cells (Figure S1A available online), compared with the control RNA-transfected cells. Furthermore, a chromatin immunoprecipitation (ChIP) assay confirmed that Oct4, Nanog, and Sox2 were present in the promoter region of *linc-RoR* during hESC H1 self-renewal but not during differentiation (Figure 1D). These data are consistent with previous reports (Loewer et al., 2010) and confirm that the *linc-RoR* gene is a direct target of core TFs.

To further reveal the dynamic changes of *linc-RoR* expression during the hESC differentiation process, we examined *linc-RoR* and core TF mRNA levels with quantitative real-time PCR at different time points. *Linc-RoR* was expected to be downregulated after Oct4, Nanog, and Sox2 reduction because its transcription is under the control of these core TFs. In our study, quantitative real-time PCR showed that the expression levels of *linc-RoR* and core TFs changed synchronously in hESC line H1 (Figure 1E) and X-01 (Figure S1B) cells. Interestingly, however, the expression of *linc-RoR* rapidly decreased (90% on the second day) under the differentiation conditions, which changed prior to the decline in levels of these core TFs (no more than 30% on the fourth day). We therefore hypothesize that *linc-RoR* may also regulate Oct4, Nanog, or Sox2 expression in hESCs, which signifies the role of *linc-RoR* in the pluripotency-regulating networks.

Linc-RoR Regulates Endogenous Oct4, Nanog, and Sox2 Expression in Self-Renewing and Differentiating hESCs

To further confirm that *linc-RoR* regulates Oct4, Nanog, or Sox2 expression in hESCs, we next investigated whether ectopic *linc-RoR* affected expression of these core TFs in hESCs under self-renewal conditions or differentiation condition. Toward this end, we constructed a *linc-RoR*-overexpressing vector and transfected it into undifferentiated hESCs to isolate the GFP-positive (GFP+) hESC population that expressed the vector-encoded GFP by fluorescence-activated cell sorting (FACS) (Figure S2A). We found that *linc-RoR* increased more than 40-fold compared with the vector-transfected hESCs. At the same time, the transient overexpression *linc-RoR* also elevated core TFs mRNA and protein expression in two hESC lines under self-renewing conditions (Figures 2A and S2C) or even under differentiation conditions (Figures 2B and S2D). We also employed a fluorescein isothiocyanate (FITC)-labeled *linc-RoR*-specific siRNA (siROR) to reduce the endogenous *linc-RoR* in hESCs with an FITC-labeled scrambled sequence with no homology to the human genome in parallel as a negative control (NC RNA). Three days after transfection, we isolated the FITC-positive hESC H1 population for quantitative real-time PCR analysis (Figure S2B). The level of *linc-RoR* RNA was decreased by more than 70% at the third day after siROR transfection, compared with NC RNA. Furthermore, we found that the mRNA and protein expression of Oct4 and Nanog was decreased in *linc-RoR*-deficient hESCs after 3 days under self-renewal conditions (Figures 2C and S2E). The mRNA expression level of Sox2, but not its protein expression level, significantly changed during this process. In differentiating hESCs, we also found that siROR resulted in a more intense reduction of the core TFs than NC RNA (Figures 2D and S2F). These data indicate that *linc-RoR* positively regulates expression of core TFs in two hESC lines. Considering the facts that core TFs also directly regulate *linc-RoR* transcription, we therefore suggested that *linc-RoR* and core TFs formed a regulatory feedback loop in hESCs.

Linc-RoR Regulated Expression of Core TFs Mainly through a MicroRNA-Dependent Mechanism in hESCs

We then evaluated the molecular mechanism for the *linc-RoR*-mediated regulation of Oct4, Nanog, and Sox2 expression. Because of the chromosome-modifying functions of many other reported lincRNAs, we first evaluated whether *linc-RoR* could also promote Oct4, Nanog, and Sox2 de novo transcription. However, using a reporter vector containing the Oct4-promoter, we found that neither ectopic *linc-RoR* nor *linc-RoR* siRNA

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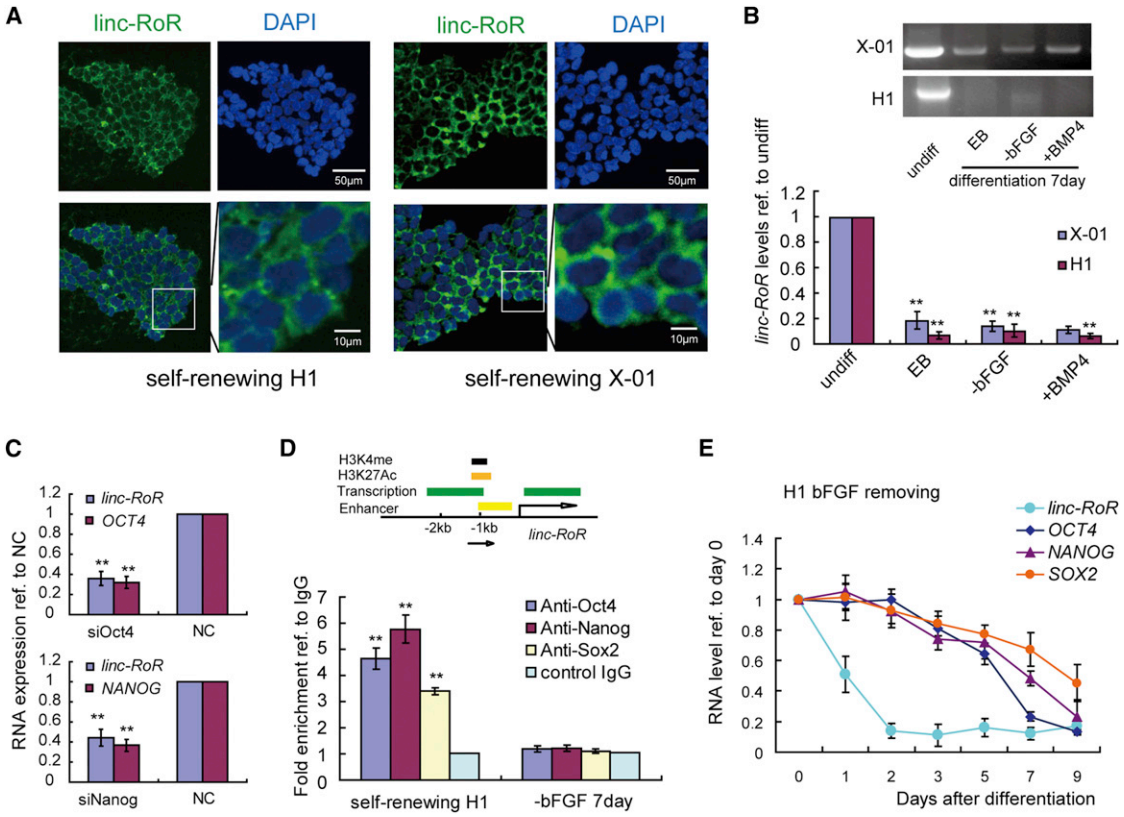

Figure 1. *Linc-RoR* Expression Is Positively Correlated with the Undifferentiated ES Cell State

(A) The in situ expression of *linc-RoR* RNA in the self-renewing hESC lines H1 and X-01. The green fluorescent signal is from the FITC-*linc-RoR* RNA probe, and the blue fluorescent signal is from nuclear DNA counterstained with DAPI. The scale bar represents 10 or 50 μm.

(B) The relative level of *linc-RoR* increased after hESC differentiation in qRT-PCR analysis. The blots from an electrophoresis assay are shown, and *GAPDH* was used as an internal normalization control. EB, embryoid bodies. Data are represented as mean ± SEM. $^{**}p < 0.01$, n = 3.

(C) The relative level of *linc-RoR* decreased in hESCs 3 days after the transfection of siRNAs (si) targeting *OCT4* or *NANOG*. The interfering efficiency was confirmed with quantitative real-time PCR. Data are represented as mean ± SEM. $^{**}p < 0.01$, n = 3.

(D) ChIP showed high Oct4, Nanog, and Sox2 enrichment at the *linc-RoR* promoter in hESCs but not in cells after bFGF removal for 7 days on. Relative enrichment is normalized to control IgG. The positions of the PCR amplicon are labeled according to information from the Web site of the University of California, Santa Cruz Genome Browser (http://genome.ucsc.edu/). Data are represented as mean ± SEM. $^{**}p < 0.01$, n = 3.

(E) The kinetic expression levels of *linc-RoR*, *NANOG*, *SOX2*, and *OCT4* in differentiated H1 cells by withdrawal of bFGF. The relative expression levels of RNA were quantified by quantitative real-time PCR and were normalized to *GAPDH*.

Data are represented as mean ± SEM. See also Figure S1.

directly influenced the luciferase activity of the Oct4-reporter in hESCs under self-renewal or differentiation conditions (Figure S2G). These results indicate that *linc-RoR* does not regulate Oct4 at the transcriptional level but may regulate them at the posttranscriptional level.

As one of the most important posttranscriptional modifiers, miRNAs have been shown to be critical regulatory factors during hESC differentiation. We therefore evaluated the expression of *linc-RoR* and core TFs in Dicer-deficient ESCs, which have impaired global miRNAs expression. The lentivirus (LV)-short hairpin (sh) RNA-mediated knockdown of Dicer mRNA and protein expression in hESCs was confirmed by comparison with LV-scrambled short hairpin RNA controls (LV-NC) (Figure 2E). Both in the self-renewing (Figure 2E) and differentiated hESCs (Figure 2F), we found that Dicer knockdown partially rescued the reduction of core TFs induced by *linc-RoR* knockdown, whereas the control lentivirus-infected cells had changes similar to uninfected cells. These results support our hypothesis that miRNAs play essential roles in the *linc-RoR*-mediated regulation of core TFs expression in hESCs.

Linc-RoR Shares Regulatory miRNAs with the Core TFs Oct4, Sox2, and Nanog

To investigate the miRNA-related functions of *linc-RoR* in ESCs, we sought to functionally characterize specific regulatory miRNAs in the maintenance of ES cell self-renewal and differentiation with a particular focus on the core TFs Oct4, Sox2, and Nanog. Thus, we mined previously published microarray data (Ivanova et al., 2006) for miRNAs elevated during hESCs differentiation and confirmed the expression levels of these miRNAs in differentiated hESCs (Figure S3A), linc-RoR-overexpressing or knockdown hESCs, and Oct4 knockdown hESCs (Figure S3C). Several miRNAs, including miR-145, miR-181a, miR-99a, and let-7a, were found greatly elevated

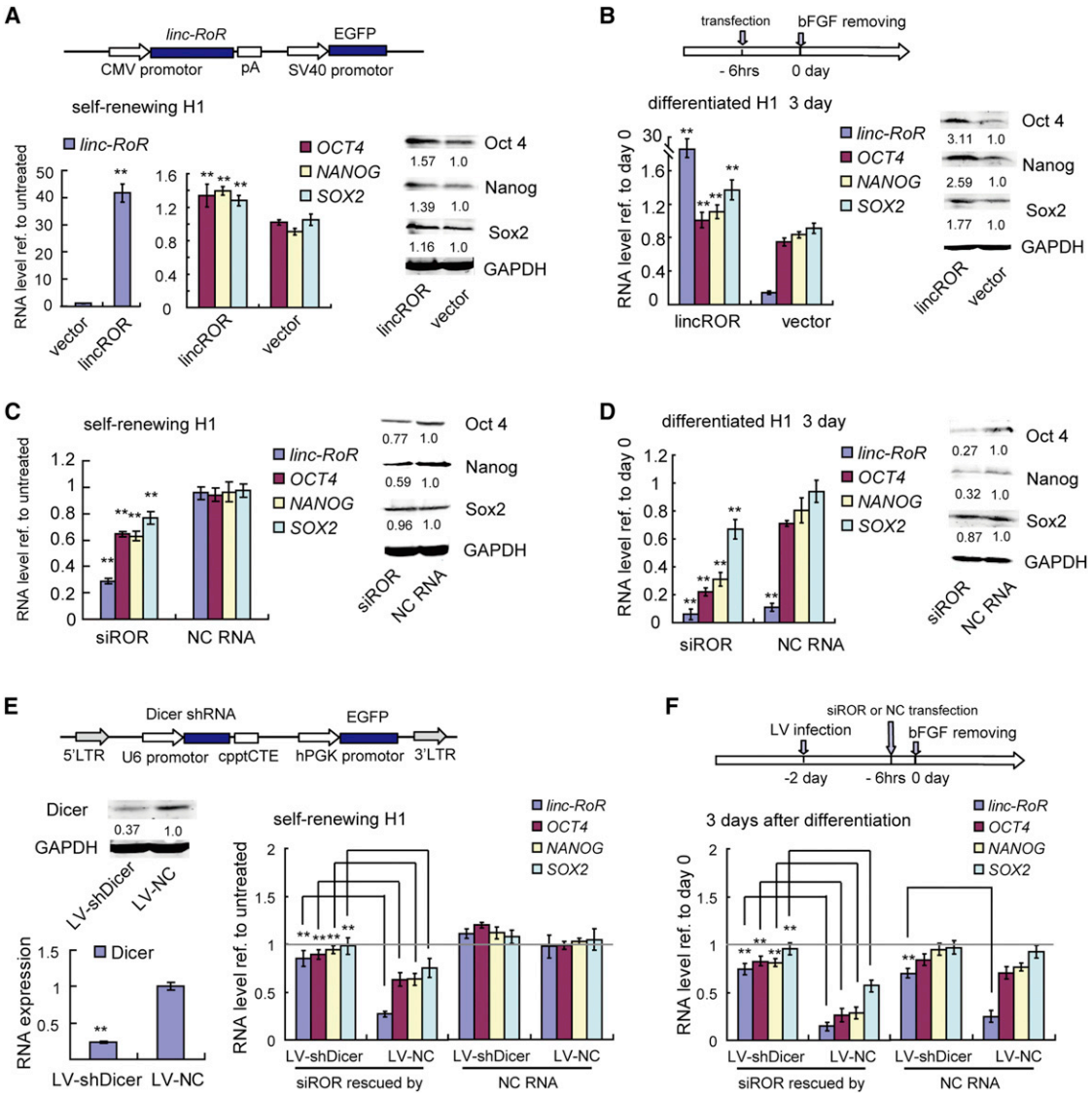

Figure 2. *Linc-RoR* Regulates Oct4, Nanog, and Sox2 Expression in Self-Renewing hESCs

(A and B) Relative mRNA and protein levels of *OCT4*, *SOX2*, and *NANOG* in hESCs under self-renewal (A) or differentiation (B) conditions that were transfected with *linc-RoR*-overexpressing vector (*linc-RoR*) or control vector (vector). The GFP-positive hESCs were isolated by FACS.

(C and D) Relative mRNA and protein levels of *OCT4*, *SOX2*, and *NANOG* in hESCs under self-renewal (C) or differentiation (D) conditions that were transfected with siRNA targeting *linc-RoR* (siROR) or negative control RNA (NC).

(E and F) Dicer deficiency rescued the siROR-mediated reduction of *linc-RoR*, *OCT4*, *SOX2*, and *NANOG* mRNA in hESCs under self-renewal (E) or differentiation (F) conditions. The interfering efficiency of a lentivirus encoding Dicer-targeting shRNA (LV-shDicer) was also confirmed comparing to negative control lentivirus (LV-NC). RNA and protein levels were assayed by quantitative real-time PCR and western blot analysis; *GAPDH* is the normalization control.

Data are represented as mean ± SEM. $^{**}p < 0.01$, n = 3. See also Figure S2.

in differentiated, linc-RoR knockdown and Oct4 knockdown hESCs. As miRNAs may also bind the elements in CDS regions besides 3′ UTR (Tay et al., 2008), we used the bioinformatics tool Miranda (Enright et al., 2003) to search for miRNAs that target the full-length transcripts of Oct4, Sox2, and Nanog and *linc-RoR* (Table S1). Of the miRNA that fit these criteria, three families of miRNAs—miR-145, miR-181, and miR-99—emerged as obvious candidates because their predicted binding sites were shared by *linc-RoR* and core TFs (Figure 3A). In addition, all three of these miRNAs have been reported to functionally regulate hESC self-renewal (Kane et al., 2012; Xu et al., 2009).

To validate the direct binding ability of the predicted miRNA-response elements on these transcripts, we next performed an RNA immunoprecipitation (RIP) analysis with MS2 binding protein (MS2bp), which specifically binds RNA containing MS2-binding sequences (MS2bs) when they are coexpressed. We constructed vectors expressing *linc-RoR*, *OCT4*, *SOX2*, or *NANOG* full-length transcripts combined with MS2bs elements and cotransfected them into HEK293 cells with an MS2bp-YFP

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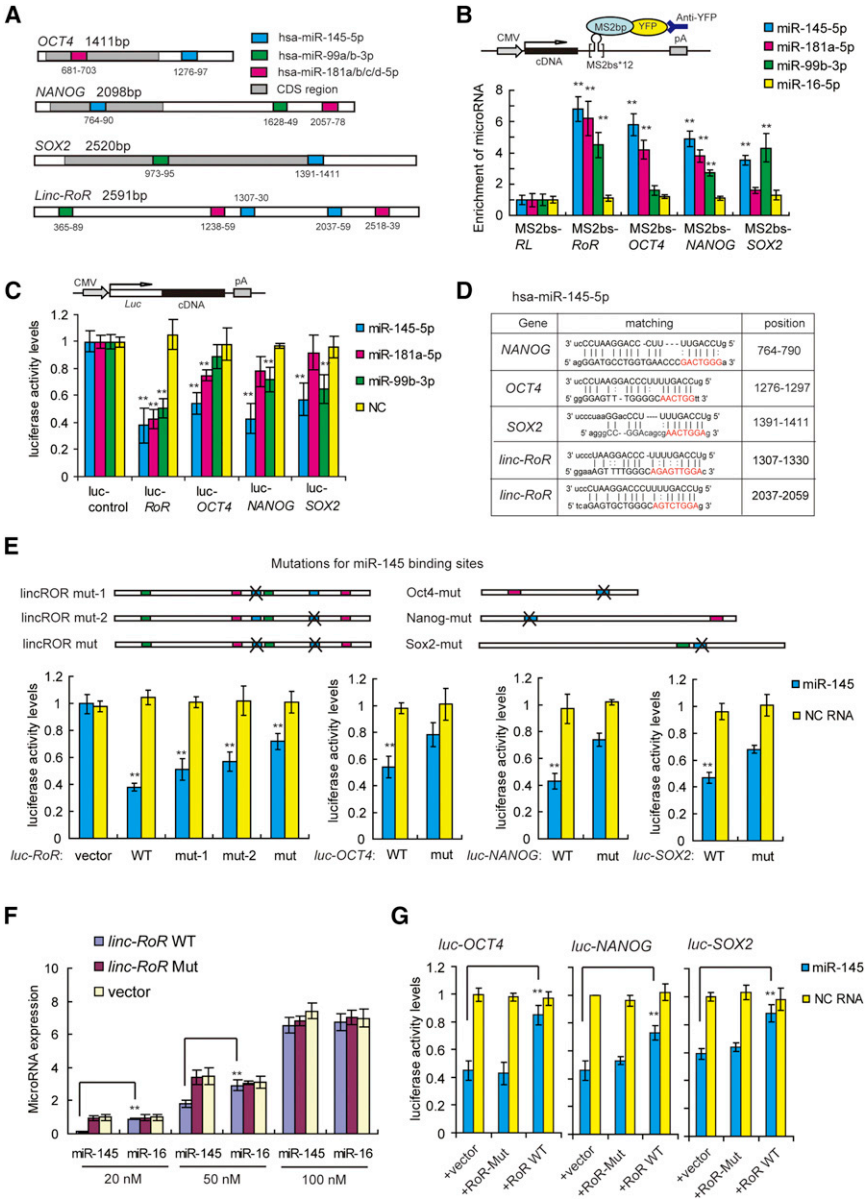

Figure 3. *Linc-RoR* Shared Regulatory MicroRNAs with the Core TFs Oct4, Sox2, and Nanog and Prevented Them from Being Suppressed

(A) The prediction for miRNA-binding elements on *linc-RoR*, *OCT4*, *NANOG*, and *SOX2* transcripts by Miranda.

(B) The binding ability of *linc-RoR*, *OCT4*, *NANOG*, and SOX2 full-length transcripts to miRNAs, which were precipitated by cDNA combined with MS2-binding sequences (MS2bs) and its binding protein MS2BP-YFP. The immunoprecipitated miRNAs were assayed by quantitative real-time PCR and normalized to U6; MS2bs-*RL* and miR-16 were used as negative controls. RL, Renilla luciferase.

(C–E) The target validation using luciferase reporters in HEK293 cells. The relative luciferase activities of luciferase reporters containing wild-type (WT) or mutant (Mut) transcripts were assayed 48 hr after cotransfection with the indicated microRNAs or scramble negative control RNA (NC). Luc, firefly luciferase; pA, polyadenylation signal; Control, the basal luciferase reporter without inserts. (D) Comparison summary of miR-145 target sites in the mRNA of *linc-RoR*, *OCT4*, *NANOG*, and *SOX2*. The red nucleotides (target sites) were deleted in the mutant constructs.

(F) *Linc-RoR* facilitated miR-145 degradation. MiRNA levels were assayed by quantitative real-time PCR in HEK293 cells cotransfected with different concentrations of miR-145 mimics and WT or mutant *linc-RoR*. MiR-16 was used as a negative control.

(G) Coexpression of wild-type *linc-RoR* rescued the relative luciferase activities of luciferase reporters containing *OCT4*, *NANOG*, and *SOX2* when cotransfected with miR-145. Blank vector (vector) and mutant *linc-RoR* were used as controls.

Data are represented as mean ± SEM. $^{**}p < 0.01$, n = 3. See also Figure S3 and Table S1.

expression vector and a mixture of the three miRNAs—miR-145, miR-181a, and miR-99b—with miR-16 as a negative control. The transcript-specific binding RNA-protein complexes were then immunoprecipitated with YFP antibody, and immunoglobulin G (IgG) was used as a negative control. We performed quantitative real-time PCR and found that miR-145-5p, miR-181a-5p, and miR-99b-3p were enriched in MS2bs-*linc-RoR*-binding RNAs and that *OCT4*, *SOX2*, and *NANOG* directly bound one or two of the three miRNAs compared with the negative control MS2BS-Renilla luciferase (RL) RNA (Figure 3B). In addition, Argonaute 2 (Ago2) enrichment was observed in the RNA binding proteins of MS2BS-linc-RoR, MS2BS-Oct4, MS2BS-Nanog, and MS2BS- Sox2, compared with MS2BS-RL RNA (Figure S3B), which indicates that *linc-RoR* is recruited to Ago2-related RNA-induced silencing complexes (RISCs) (Chi et al., 2009) and functionally interacts with miRNAs.

For further confirmation, we also constructed luciferase reporters containing *linc-RoR*, Oct4, Nanog, or Sox2 full-length transcripts for targets investigations. We found that the miR-145-5p mimics greatly reduced the luciferase activities of the reporter vectors containing *linc-RoR*, Oct4, Nanog, or Sox2, whereas the miR-181a-5p and miR-99b-3p mimics only inhibited the *linc-RoR* reporter vector but exhibited weak suppression effects (no more than 30%) on the three reporter vector genes in comparison to the negative controls both in HEK293 cells (Figure 3C) and H1 cells (Figure S3E). We therefore chose miR-145 as a model miRNA for further studies. To avoid nonspecific binding, we also constructed control transcripts with mutations (Mut) in these miR-145 binding sites (miR-145bs) (Figures 3D and 3E). We found that these mutations partially abolished the effect of miR-145 on wild-type transcripts in HEK293 cells (Figure 3E) and differentiated hESCs (Figure S3G). These data suggest that *linc-RoR* shares regulatory miRNAs with the core TFs Oct4, Sox2, and Nanog and that

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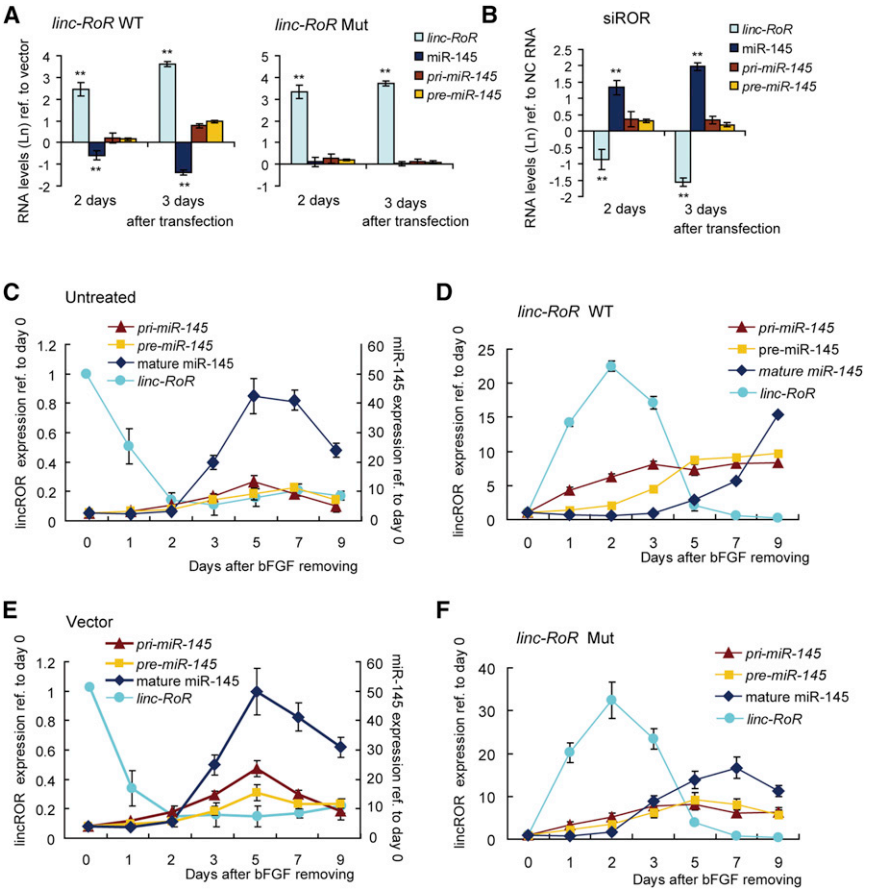

Figure 4. *linc-RoR* Functions as an Endogenous miR-145 Sponge in hESCs

(A and B) The expression levels of mature miR-145 and its primary (pri-) or premature (pre) transcripts in self-renewing hESCs transiently transfected with wild-type (WT) *linc-RoR* or mutant (Mut) *linc-RoR* overexpressing vectors or *linc-RoR*-specific siRNA (siROR). Blank vector or negative control RNA (NC) was used as controls.

(C–F) The kinetic expression levels of *linc-RoR*, mature miR-145, its primary (pri-) or premature (pre) transcripts in nontransfected differentiated hESCs transfected with WT *linc-RoR* or Mut *linc-RoR* overexpressing vectors, blank vector, or nothing. The relative expression levels of RNA were quantified by quantitative real-time PCR and normalized to *GAPDH*.

Data are represented as mean ± SEM. $^{**}p < 0.01$, n = 3. See also Figure S4.

miR-145 may be one of the critical regulatory miRNAs for these genes.

Linc-RoR Prevents Oct4, Sox2, and Nanog from miRNA-Mediated Degradation

Because *linc-RoR* functionally recruited Ago2 and RISC complexes, we next hypothesized that *linc-RoR* could have the ability to influence miR-145 expression in ES cells. We engineered *linc-RoR*-overexpressing plasmids that have either wild-type (WT) or mutant (Mut) transcripts with mutations in the two miR-145-binding sites. We found that the ectopically expressed *linc-RoR* WT reduced the concentration of miR-145 during the cotransfection of the *linc-RoR* expression vector and low concentration of miRNA mimics in HEK293 cells (Figure 3F). As expected, *linc-RoR* Mut failed to reduce miRNA expression, which indicated that the inhibition was sequence specific. However, no significant effect was observed when the ectopic *linc-RoR* was cotransfected with a high concentration of miRNA mimics, which further demonstrated that the *linc-RoR*-mediated suppression was saturable in vivo (Figure 3F). We also found that *linc-RoR* expression levels were decreased after transfection of miR-145 mimics, which indicated that both miR-145 and *linc-RoR* were cleaved and degraded during this process (Figure S3D). Taken together, these data suggest that *linc-RoR* functions as an miRNA sponge to reduce the efficient concentration of miR-145.

To further investigate whether *linc-RoR* could protect Oct4, Nanog, or Sox2 from miR-145-mediated suppression, we cotransfected HEK293 cells or hESCs with the *linc-RoR*-overexpressing vector and the luciferase reporters containing Oct4, Nanog, or Sox2 full-length transcripts in the presence of miR-145 mimics or NC RNA. We found that ectopic *linc-RoR* WT efficiently abolished the miR-145-induced reduction of luciferase activities in the Oct4, Nanog, and Sox2 reporter vector-transfected cells, whereas ectopic expression of *linc-RoR* Mut failed to protect the reporters from suppression similar to the negative control vectors (Figures 3G and S3F). These results indicate that *linc-RoR* protects the transcripts of the core TFs Oct4, Sox2, and Nanog from miRNA-mediated degradation, both in an HEK293 cell-based model and hESCs.

Linc-RoR Functions as an Endogenous miR-145 Sponge in hESCs

Next, we investigated the regulatory role of *linc-RoR* in the expression of miR-145 in hESCs. We transiently elevated *linc-RoR* expression with a *linc-RoR*-overexpressing vector or inhibited its expression by siROR in two self-renewal hESC lines. To avoid nonspecific binding and effort, *linc-RoR* Mut, with mutations on its miR-145-binding sites, was also employed as a control. As shown in Figures 4A and 4B (H1 cells) and Figures S4A and S4B (X-01 cells), the expression levels of mature miR-145 were inversely associated with the expression levels of *linc-RoR* WT, but not *linc-RoR* Mut, which indicates that *linc-RoR* negatively regulates miR-145 through specific binding sites (Figures 4A and S4A). To determine whether *linc-RoR* influenced the miRNA transcription and mature processes, we also analyzed primary (pri-) and premature (pre-) transcripts of miR-145. Interestingly, inconsistent with mature miR-145 levels, the changes of *pri-miR-145* and *pre-miR-145* were not significant after treatments, which indicated that *linc-RoR* mainly regulates

mature miR-145 expression levels through a posttranscriptional mechanism (Figures 4A, 4B, S4A, and S4B).

Because endogenous *linc-RoR* greatly decreased during hESC differentiation, we next investigated the dynamic expression levels of *linc-RoR* and miR-145 in differentiated hESCs after bFGF removal. As shown in Figure 4C, the expression levels of *pri-miR-145* and *pre-miR-145* gradually increased from the first day after ESC differentiation. However, the expression level of mature miR-145 was slightly increased in the first 2 days but greatly increased 3 days after differentiation, whereas *linc-RoR* had been greatly decreased, which indicated that mature miR-145 may not increase greatly in the presence of *linc-RoR*. We also transfected hESCs with *linc-RoR* WT or *linc-RoR*-Mut-overexpressing vectors 6 hr prior to bFGF removal (Figures 4D–4F). We found that ectopic *linc-RoR* WT suppressed the elevation of mature miR-145 in first 3 days, whereas *pri-* and *pre-miR-145* gradually increased at the same time. After 5 days, mature miR-145 started to increase when *linc-RoR* levels had significantly decreased (Figure 4D). Neither *linc-RoR* Mut (Figure 4F) nor blank vector (Figure 4E) induced such phenomenon in our assays. These results indicate that *linc-RoR* functions as an endogenous miR-145 sponge to avoid miR-145 increases in self-renewing hESCs.

To serve as a sponge, the abundance of *linc-RoR* should be comparable to or higher than miR-145. We therefore used quantitative real-time PCR to quantify the exact copy numbers of *linc-RoR* and miR-145 per cell (Figure S4C). As a result, we found that, in the self-renewal hESCs, the expression level of mature miR-145 was only about 10–20 copies per cell, whereas *linc-ROR* level was more than 100 copies per cell. We therefore suggest that *linc-ROR* may be able to function as a sponge for miR-145 in self-renewing hESCs. In differentiated hESCs, miR-145 significantly increased and was up to more than 500 copies per cell 7 days postdifferentiation, whereas *linc-ROR* decreased to no more than 20 copies per cell at the same time (Figure S4C). The sponge effort of *linc-RoR* may therefore vanish after hESCs differentiation.

Endogenous *linc-RoR* Is Essential for the Maintenance of Core TFs in Self-Renewing hESCs

To further confirm the role of the *linc-RoR*-mediated regulatory loop in hESC self-renewal, we used a *linc-RoR*-specific shRNA-expressing lentivirus (LV-shROR) to decrease the expression of *linc-RoR* in long-term hESCs culture. Three or 7 days after LV-shROR virus infection, we isolated the GFP-positive (GFP+) hESC H1 population by FACS (Figure 5A). The RNA level of *linc-RoR* was found to be decreased by more than 70% at the third and seventh days after LV-shROR infection, compared with the negative control virus vector LV-NC. Furthermore, we found that the expression levels of *OCT4*, *NANOG*, and *SOX2* mRNA were slightly decreased in *linc-RoR*-deficient hESCs after 3 days but were significantly reduced after 7 days under self-renewal conditions. The expression of mature miR-145, but not *pri-* and *pre-miR-145*, was greatly increased at the same time (Figure 5B). Similar results were also found in hES X-01 cells (Figure S5A). Oct4, Nanog, and Sox2 proteins were also found decreased in LV-shROR-infected cells in an immunofluorescence analysis (Figure 5C). These results indicated that *linc-RoR* deficiency results in obvious changes in the expression of miR-145 and core TFs in self-renewing hESCs only after long-term culture. These data are consistent with the hypothesis that *linc-RoR* does not directly regulate miR-145 and core TF transcription but instead regulates them through posttranscriptional fine-tuning.

To assess the role of miR-145 in the *linc-RoR*-mediated regulatory loop, we further employed the miR-145 inhibitor to abolish miR-145 elevation in *linc-RoR*-deficient hESCs. The single-stranded miR-145 inhibitor and negative control RNA (NC) were transfected into LV-shROR-infected cells one day after the infection (Figure 5D). After 3 days, the expression of mature miR-145 was decreased by up to 80% in miR-145 inhibitor-transfected cells, compared with negative controls. In addition, *OCT4* and *NANOG* mRNA was rescued at up to 80% of the levels in wild-type hESCs after miR-145 inhibitor transfection.

The *linc-RoR*-Mediated Regulatory Loop Is Essential for hESC Self-Renewal

For the *linc-RoR*-deficient cell phenotype, we found that the percentage of GFP+ cells in LV-shROR-infected hESCs was much lower compared with the negative control cells (Figure 5A), which indicates that *linc-RoR* knockdown may impair hESC proliferation. We next investigated, by analyzing the self-renewal marker SSEA4, whether the maintenance of self-renewal under normal culturing conditions would be affected by the loss of *linc-RoR*. In comparison to the negative control LV-NC, 7 days of LV-shROR infection caused a significant decrease in the amount of SSEA4 staining (Figure 5E) in lentiviral GFP-expressing cells, as analyzed by flow cytometry. We also employed a positive control lentivirus that expressed OCT4-shRNA (shOCT4) (Zaehres and Schöler, 2007). The addition of shOCT4 decreased the self-renewal level significantly, as expected. We also found changes in cell morphology and decreased alkaline phosphatase (AP) activity (Figure 5F for H1; Figure S5B for X-01), which are indicative of differentiation. Furthermore, LV-shROR culture exhibited a significantly higher apoptosis rate compared with the negative control LV-NC (Figure S5C), as assayed by apoptosis marker annexin V flow cytometry in lentiviral GFP-expressing cells. These results suggest that some cells resorted to apoptosis when they were unable to self-renew. Finally, a depletion of *linc-RoR* results in the elevation of the ectodermal marker *SOX1*, *VIMENTEN*, and *OTX2*, the neural progenitor gene *Nestin*, the mesodermal marker *Cdx2*, *HAND1*, *RUNX2, MIXL1*, and *NODAL* at mRNA levels. (Figures 5G, S5D, and S5E). We also confirmed the lineage specific differentiation by immunofluorescence anaslysis. Compared to normal controls, we found that the *linc-RoR*-knockdown ESCs highly expressed the ectodermal marker SOX1 and mesodermal marker Cdx2 with a weak expression of Oct4 and endodermal marker FoxA2 (Figure 5H). These data further indicate that *linc-RoR* deficiency may mainly facilitate hESCs ectodermal and mesodermal differentiation. Interestingly, the phenotypes were similar to miR-145-overexpression-induced differentiation according to previous reports (Xu et al., 2009) (Figure S5E). We therefore suggest that knocking down *linc-RoR* results in the decline of Oct4, Nanog, and Sox2 expression and the loss of hESC self-renewal.

We further investigated whether miR-145 inhibition could rescue the phenotype of *linc-RoR*-deficient cells. The Oct4- and

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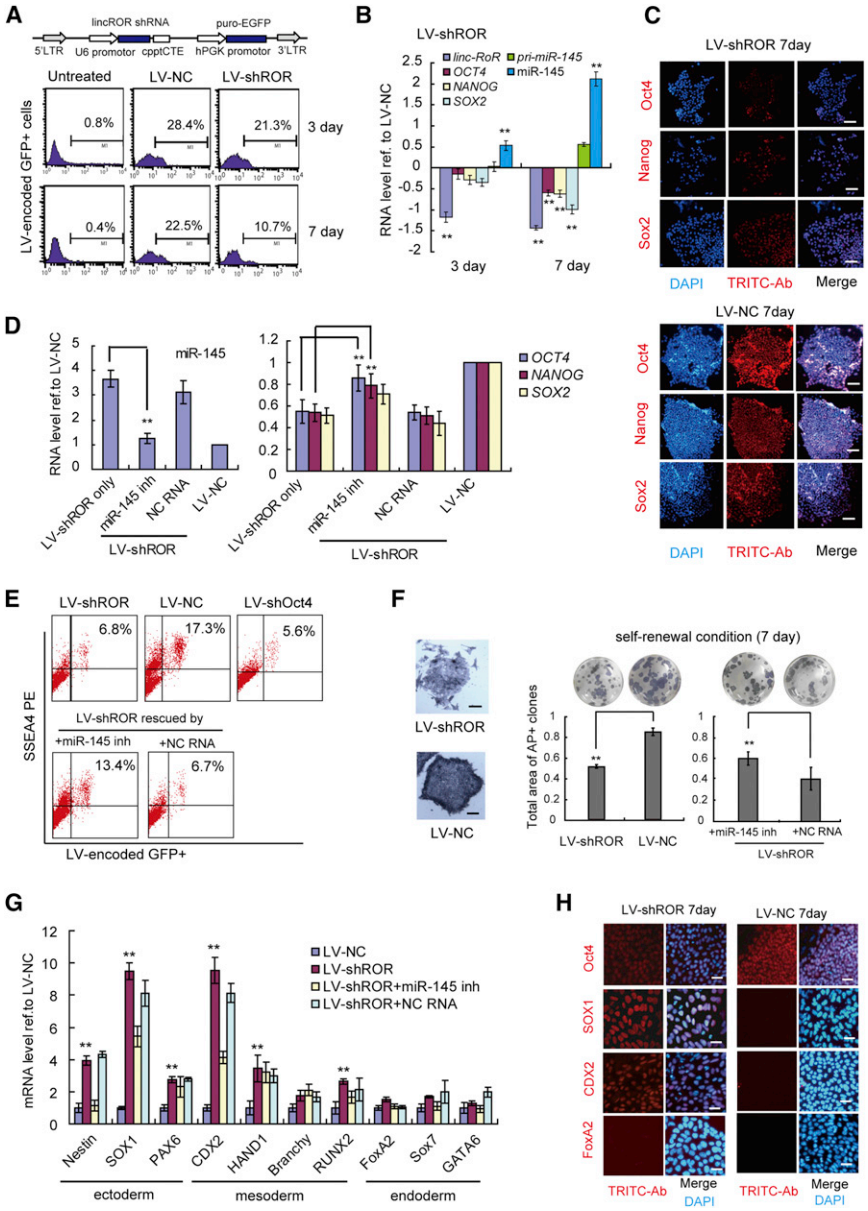

Figure 5. The *linc-RoR*-Mediated Regulatory Loop Is Essential for the Maintenance of Core TFs and hESC Self-Renewal

(A) The percent of GFP+ hESCs H1 under self-renewal conditions expressing shRNA targeting *linc-RoR* (LV-shROR) or negative control shRNA (LV-NC) by FACS.

(B) The relative mRNA or miRNA levels in LV-shROR-infected GFP+ H1 cells referring to LV-NC-infected cells.

(C) Immunofluorescence analysis to Oct4, Nanog, and Sox2 proteins. The scale bar represents 100 μm.

(D) The miR-145 inhibitor (inh) rescued the LV-shROR-mediated reduction of *OCT4*, *SOX2*, and *NANOG* mRNA in quantitative real-time PCR assays. The interfering efficiency of miR-145 inh was confirmed with quantitative real-time PCR. (B and D) GAPDH or U6 snRNA were used as the normalization controls. Data are represented as mean ± SEM. $^{**}p < 0.01$, n = 3.

(E) The expression levels of SSEA4 in LV-shROR-infected H1 cells were assayed with flow cytometry. LV-shOct4 was used as a positive control. The rescue effect of miR-145 inh was also shown. Data are represented as mean ± SEM. $^{**}p < 0.01$, n = 3.

(F) The cell morphology and alkaline phosphatase (AP) activity quantified by the total areas of AP positive (AP+) clones for LV-shROR-infected H1 cells and control cells under self-renewal conditions. The rescue effect of miR-145 inh is also shown. The scale bar represents 100 μm. Data are represented as mean ± SEM. $^{**}p < 0.01$, n = 3.

(G) The expression levels of differentiation markers for the three germinal layers in LV-shROR or LV-NC infected H1 cells and miR-145 inh rescued cells were confirmed with quantitative real-time PCR. Data are represented as mean ± SEM. $^{**}p < 0.01$, n = 3.

(H) Immunofluorescence analysis to the expression levels of differentiation markers for the three germinal layers in LV-shROR or LV-NC infected H1 cells. The scale bar represents 25 μm.

See also Figure S5.

Nanog-overexpressing vectors were also used as positive controls in this analysis. By flow cytometry analysis, we found an increase in the percentage of SSEA4+ cells (Figure 5E) and AP activity (Figure 5F) after miR-145 inhibitor or Oct4- and Nanog-overexpressing vectors transfection, compared with NC RNA and vector rescued controls. The expression of differentiation markers was also suppressed (Figure 5G). These results indicate that either miR-145 inhibition or Oct4 and Nanog overexpression partially rescued the effect of *linc-RoR* knockdown in self-renewing hESCs.

Linc-RoR Prevents Core TFs from miR-145-Mediated Degradation during hESC Differentiation

We next sought to elucidate whether *linc-RoR* plays a role as a suppressor of ES differentiation. We transfected a *linc-RoR*-encoding lentivirus vector into H1 cells and performed puromycin selection to isolate *linc-RoR*-overexpressing (*linc-RoR* OE) cells. The *linc-RoR* OE cells displayed normal cell morphology, a high level of *linc-RoR* mRNA and a low level of mature miR-145, compared with the vector-transfected cells under self-renewal conditions (Figures S6A–S6C). Under differentiation conditions, ectopic *linc-RoR* suppressed the dynamic reduction of *OCT4*, *NANOG*, and *SOX2* mRNA expression and the elevation of miR-145, both of which were found in the control hESCs during differentiation (Figure 6A). Oct4, Nanog, and Sox2 proteins were also found to maintain at high levels, compared with control hESCs (Figure 6B). We further investigated the role of miR-145 and Oct4 during the differentiation of *linc-RoR* OE hESCs. We transiently transfected miR-145 mimics or Oct4 siRNA into *linc-RoR* OE hESCs and then induced the cells to differentiate by removing bFGF (Figure 6C). After 3 days, the expression of Oct4 decreased by 60% or 80% in miR-145 or

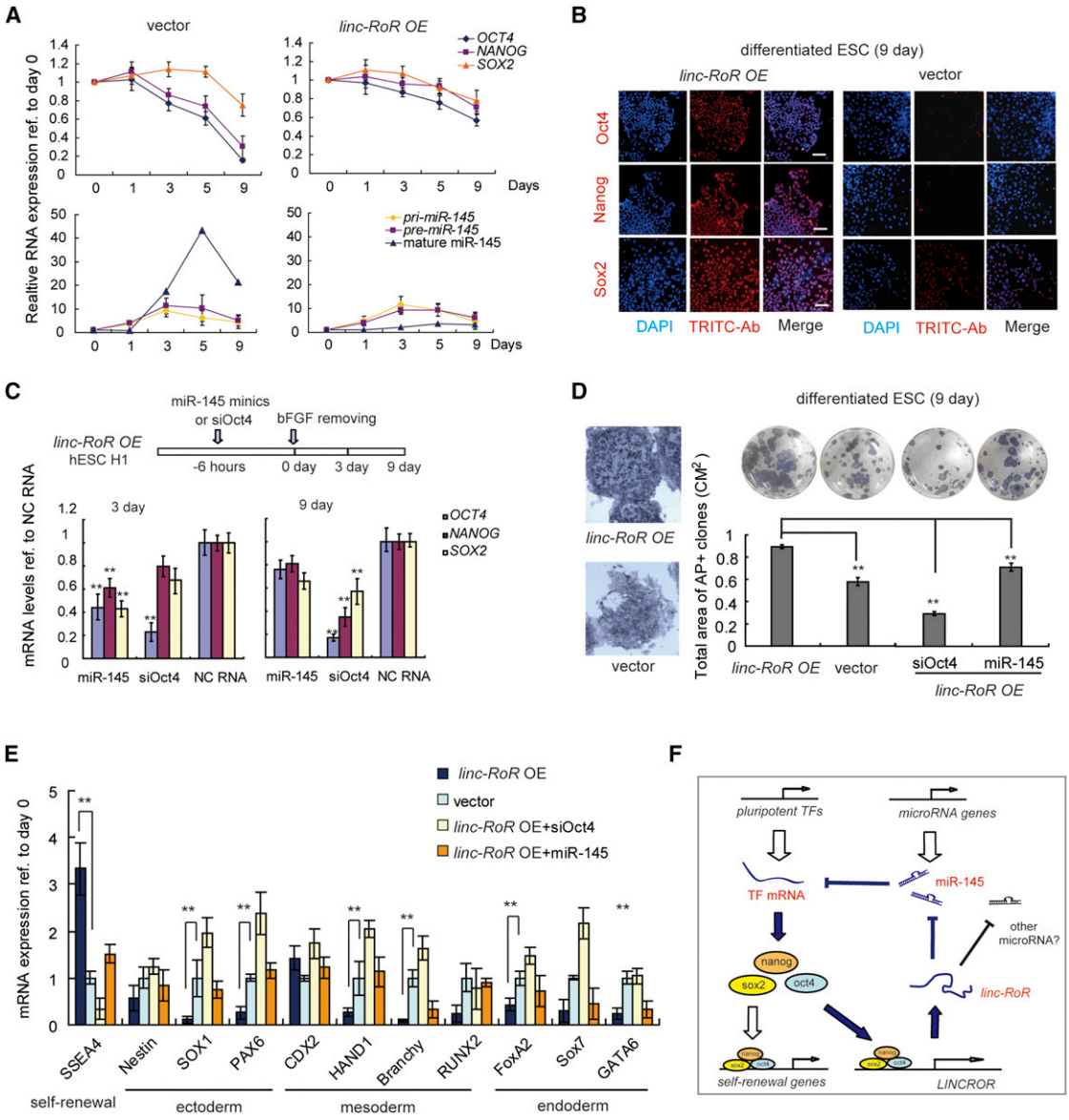

Figure 6. *Linc-RoR* Prevents Core TFs from miR-145-Mediated Degradation during hESC Differentiation

(A) The kinetic expression levels of core TFs mRNAs and microRNAs in *linc-RoR*-overexpressing (*linc-RoR* OE) hESCs or vector-transfected (vector) hESCs during differentiation by the withdrawal of bFGF from day 0.

(B) Immunofluorescence analysis to Oct4, Nanog, and Sox2 proteins. The scale bar represents 100 μm.

(C) Inhibition of miR-145 mimics and Oct4 siRNA (siOct4) for the protective efforts of *OCT4*, *SOX2*, and *NANOG* mRNA in the *linc-RoR* OE hESCs.

(D) The cell morphology and alkaline phosphatase (AP) activity quantified by the total areas of AP positive (AP+) clones for *linc-RoR* OE hESCs and control cells under differentiation conditions. The inhibitory efforts of miR-145 and siOct4 are also shown. The scale bar represents 100 μm. Data are represented as mean ± SEM. $^{**}p < 0.01$, n = 3.

(E) The mRNA expression levels of self-renewal marker SSEA4 and differentiation markers for the three germinal layers in *linc-RoR* OE hESCs and control cells after removing bFGF for 9 days. For (A), (B), and (E), the relative expression levels of RNA were quantified by quantitative real-time PCR and normalized to *GAPDH* or *U6* snRNA. Data are represented as mean ± SEM. $^{**}p < 0.01$, n = 3.

(F) Model for the *linc-RoR*-related regulatory loop in the modulation of core TFs and hESC pluripotency.

See also Figure S6.

Oct4 siRNA-transfected cells, respectively, compared with the negative control (NC RNA) (Figure 6C), which indicated that both the miR-145 mimics and Oct4 siRNA could abolish the protective effect of ectopic *linc-RoR*. For 9 days after differentiation, miR-145-induced decreases of core TFs were partially recovered, whereas siOct4 induced a further decrease of core TFs (Figure 6C). These data confirm that *linc-RoR* has a protective effect and support the hypothesis that *linc-RoR* overexpression eliminates mature miR-145 but does not directly increase Oct4 expression.

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In the phenotype analysis, we found that *linc-RoR* overexpression impaired the differentiation of ESCs, compared with the control cells, as observed by cell morphology, AP straining (Figure 6D), and the assessment of the suppression of differentiation markers (Figure 6E). However, hESCs overexpressing *linc-RoR* with the mutant miR-145 binding sites (*linc-RoR* Mut) had impaired abilities to suppress hESC differentiation, compared to hESCs overexpressing wild-type *linc-RoR* (Figure S6D). These results indicate that the ectopic expression of *linc-RoR* prevents ESCs from differentiation mainly through a miR-145-related mechanism. Furthermore, we also found that the AP-positive clones and *SSEA4* expression levels were significantly decreased after miR-145 or Oct4 siRNA transfection, whereas the levels of differentiation markers increased at the same time (Figure 6E). The Oct4 siRNA much more strongly abolished the efforts of *linc-RoR* overexpression, compared with miR-145 mimics, which also confirmed that ectopic *linc-RoR* protects core TFs through a direct suppression of miR-145 during hESC differentiation.

We also investigated the differentiation process of *linc-RoR*-deficient hESCs and found that the levels of core TF mRNA rapidly declined, whereas mature miR-145 rapidly increased in *linc-RoR*-deficient hESCs after bFGF removal, compared with LV-NC-infected hESCs, as observed by quantitative real-time PCR assays (Figure S6E). These results further confirmed that endogenous *linc-RoR* functions as a ceRNA of core TFs and that hESCs were facilitated to differentiate in the absence of *linc-RoR*.

DISCUSSION

MicroRNAs have been identified as essential posttranscriptional modulators, which facilitate the rapid clearance of core TFs transcripts during hESC differentiation (Neveu et al., 2010). However, recent developments have presented a new twist; targets can also reciprocally control the level and function of miRNAs (Pasquinelli, 2012). But such miRNA regulators remain undefined in human pluripotent cells by far. Herein, our data indicate that *linc-RoR* functions as an endogenous miRNA sponge for differentiation-related miRNAs. In self-renewing hESCs, *linc-RoR* was expressed at a high level and removed trace transcribed miRNAs when hESCs were subjected to temporary and slight differentiation agents. These observations are supported by the fact that slightly differentiated hESCs can be rescued to a self-renewing state. However, *linc-RoR* was only a competing suppressor, as it could be consumed when hESCs were under strong differentiation conditions in which abundant miRNAs were transcribed. This characteristic of *linc-RoR* is important for the reduction of core TFs and miRNAs elevation during the early phase of hESC differentiation, which permits further differentiation of hESCs.

Additionally, our current work represents a detailed characterization of any lincRNA functions as a ceRNA to protect core TFs in self-renewing hESCs. LincRNAs are emerging as key regulators in early development (Pauli et al., 2011; Ponting et al., 2009) and are required for the pluripotency of hESCs (Bertani et al., 2011; Sheik Mohamed et al., 2010) and for the reprogramming of somatic cells (Loewer et al., 2010). In the most recently established models, lincRNAs function to interact with chromatin-modifying complexes to assist in the regulation of the distinct epigenetic architecture and to impart target specificity in the control of pluripotency (Bertani et al., 2011; Mondal and Kanduri, 2012; Ponting et al., 2009). However, our data indicate a model of miRNA/lincRNA interactions in pluripotent cells. Considering the multiple targets of miRNAs, we also hypothesize that there may be many other lincRNAs that function as ceRNAs to regulate key genes expression in hESCs. The identification of these ceRNAs may promote the understanding of early development and many related diseases.

Furthermore, we put forward a regulatory feedback loop model, which integrates a transcriptional and posttranscriptional network, for the maintenance of self-renewal. Our data indicate that endogenous *linc-RoR* prevents core TFs by reduction of their suppressing miRNAs. Interestingly, as Loewer et al. (2010) previously reported and we confirmed, *linc-RoR* transcription was mainly controlled by the core TFs Oct4, Sox2, and Nanog. We therefore suggest that *linc-RoR*, miRNAs, and the core TFs form a regulatory circuit consisting of autoregulatory and dual-negative feedback loops during ESC self-renewal. This regulatory loop maintains a relative balance in self-renewing hESCs to resist slight environmental changes and to elicit a rapid response to strong differentiation signals that promote hESCs differentiation. We also hypothesize that *linc-RoR*-mediated loop contributes to somatic-cell reprogramming. Many miRNAs have been proven to serve as endogenous reprogramming barriers in somatic cells (Choi et al., 2011; Mallanna and Rizzino, 2010), for example, miR-34 (Choi et al., 2011), which was predicted under the regulation of *linc-RoR* in our works. We hypothesized that ectopic expression of core TFs strongly promotes endogenous *linc-RoR* expression, which may remove these core TF-suppressing miRNAs and facilitates endogenous core TF expression. This model may provide insights into the transcriptional regulation of stem cells and reveal how Oct4, Sox2, and Nanog maintain a stable high level during ESCs self-renewal and iPSCs formation.

Lastly, *linc-RoR* was previously identified as a ''regulator of reprogramming,'' which mainly promotes the emergence of iPSC, as its expression was elevated in iPSCs, compared with ESCs. However, despite its changes and functions in the artificial processes, we also showed that endogenous *linc-RoR* plays a key role in the ESC maintenance, which may have important physiological functions in early development and further implications in developmental biology studies and clinical applications. The expression of *linc-RoR* may also be a potential self-renewal and pluripotency marker for hESCs as its expression rapidly decreased under the differentiation conditions, even prior to the decline in levels of these core TFs. Additionally, it may also be interesting to imagine that the ectopic *linc-RoR* could be utilized to modulate the self-renewal state of in-vitro-cultured stem cells, which may facilitate related studies and cell therapies. Furthermore, considering the widespread expression of Oct4, Nanog, and Sox2, among other types of stem cells even and tumor cells (Bunaciu and Yen, 2011; Jeter et al., 2011; Pardo et al., 2010), *linc-RoR* may also contribute to the regulation of genetic networks during development and tissue regeneration and may lead to new therapies for many diseases.

EXPERIMENTAL PROCEDURES

Cell Culture

hESCs (H1 and X-01) were obtained from Prof. Xiao (Zhejiang University, Zhejiang, China) and cultured under feeder-free conditions in accordance with the protocol from the WiCell Research Institute. Differentiation by forming EB suspension was carried out in hESC medium without bFGF. An alternative differentiation method of feeder-free hESCs involved the use of nonconditioned hESC medium deprived of bFGF. BMP4 differentiation was done with a daily dose of 50 ng/ml BMP4 (R&D Systems, Minneapolis) in hESC medium without bFGF for 7 days.

Immunofluorescence and Fluorescent In Situ Hybridization

These assays were preformed according to previous reports (Xu et al., 2009). For the detection of proteins, anti-Nanog, anti-Oct4, anti-Sox2, anti-Sox1, anti-Cdx2, and anti-FoxA2 (all from Abcam, Cambridge) were used. For the detection of *lincRNA*, RNA probes were used and labeled with digoxigenin (DIG)-UTP (Roche, Indianapolis, IN, USA) using the mMESSAGE T7 Ultra In Vitro Transcription Kit (Ambion, Carlsbad, CA, USA) in accordance with the manufacturer's directions.

Lentiviral Transduction, Vectors, and RNA Oligos Transfection in hESCs

Human ESC colonies were grown on matrigel-coated 6-well plates. Lentivirus transduction for cell cluster was performed according to previously described protocols (Gropp and Reubinoff, 2006). Fugene HD reagent (Promega, Madison, WI, USA) or lipofectamine RNAiMAX (Invitrogen, Carlsbad, WI, USA) was used for vector or RNA oligos transfection, respectively, in accordance with the manufacturer's instructions.

Luciferase Reporter Transfection and Dual Luciferase Assay

Luciferase reporter transfection and dual luciferase assay was preformed according to previous reports (Xu et al., 2009). The details of construction of reporter vectors and transfections are shown in the Supplemental Experimental Procedures.

FACS and Flow Cytometry Analysis

FACS and flow cytometry analysis were performed according to previous reports (Xu et al., 2009). For self-renewal analysis, each 100 μl of cell suspension (1–5 × 10^5 cells) was incubated with the primary antibody mouse anti-SSEA4 (Cell Signaling Technology, Danvers, MA, USA) and PE-conjugated goat anti-mouse (Jackson ImmunoResearch, West Grove, PA, USA). For apoptosis analysis, 1–5 × 10^5 cells from each sample were processed with annexin V-PE (Annexin V-PE Apoptosis Detection Kit, BioVision, Milpitas, CA, USA), in accordance with the manufacturer's instructions.

RNA Isolation and Real-Time PCR Analysis

Total RNA was extracted using Trizol (Invitrogen). The miRNA levels were assayed with Taqman probes and primer sets (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. For mRNA analysis, the first-strand complementary DNA (cDNA) was generated with random primers for real-time PCR using a Power SYBR Green PCR Master Mix (Applied Biosystems) protocol in a StepOne Plus System (Applied Biosystems).

ChIP and RIP Assay

ChIP assays were performed in accordance with the manufacturer's instructions of the EZ-Magna ChIP A/G Kit (Millipore, Billerica, MA, USA). The MS2bp-MS2bs-based RIP assay was performed according to previous reports (Gong and Maquat, 2011), with modifications for using the EZ-Magna RIP Kit (Millipore) in accordance with the manufacturer's instructions.

Statistical Analysis

Data are presented as mean ± SEM. For data from quantitative real-time PCR, WB, luciferase activity, and AP+ colonies area, statistical comparisons between experimental groups were analyzed with ANOVA and Fisher's exact test or two-tailed Student's t test. $p < 0.01$ was taken to indicate statistical significance.

A more detailed version of the procedures is included in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2013.03.002.

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