The miR-590/Acyr2a/Terf1 Axis Regulates Telomere Elongation and Pluripotency of Mouse iPSCs

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SUMMARY

During reprogramming, telomere re-elongation is important for pluripotency acquisition and ensures the high quality of induced pluripotent stem cells (iPSCs), but the regulatory mechanism remains largely unknown. Our study showed that fully reprogrammed mature iPSCs or mouse embryonic stem cells expressed higher levels of miR-590-3p and miR-590-5p than pre-iPSCs. Ectopic expression of either miR-590-3p or miR-590-5p in pre-iPSCs improved telomere elongation and pluripotency. Activin receptor II A (Acyr2a) is the downstream target and mediates the function of miR-590. Downregulation of Acyr2a promoted telomere elongation and pluripotency. Overexpression of miR-590 or inhibition of ACTIVIN signaling increased telomeric repeat binding factor 1 (Terf1) expression. The p-SMAD2 showed increased binding to the Terf1 promoter in pre-iPSCs compared with mature iPSCs. Downregulation of Terf1 blocked miR-590- or shAcyr2a-mediated promotion of telomere elongation and pluripotency in pre-iPSCs. This study elucidated the role of the miR-590/Acyr2a/Terf1 signaling pathway in modulating telomere elongation and pluripotency in pre-iPSCs.

INTRODUCTION

During reprogramming, the inefficient induction and low quality of induced pluripotent stem cells (iPSCs) with abnormal gene expression and chromosome integrity limit potential applications for future clinical therapy (Jiang et al., 2013; Pera, 2011). The telomere is re-elongated after sperm-oocyte binding (Liu et al., 2007). Telomerase activity is high in iPSCs (Takahashi and Yamanaka, 2006). The efficiency of chimera generation of iPSCs is higher in cells with longer telomeres than those with short telomeres. Moreover, embryonic stem cells (ESCs) with short telomeres also show reduced teratoma formation and chimera production (Huang et al., 2011). Previous study showed that the reprogramming efficiency of iPSCs derived from mouse embryonic fibroblasts (MEFs) of telomerase gene (Terc)−/− mice was decreased (Marion et al., 2009). Mouse iPSCs with short telomeres show a lower capacity for teratoma formation or chimera production, failing to generate complete pups (Zhao et al., 2009). During reprogramming, the re-elongation of telomeres is thus critically important for pluripotency acquisition.

Lengthening of telomeres in iPSCs induced with four factors (Oct4, Sox2, c-Myc, and Klf4) is dependent on telomerase activity and is a very slow process, involving several passages after reprogramming to reach similar lengths to ESC telomeres (Marion et al., 2009). By contrast, telomere lengths quickly and extensively increase during somatic cell nuclear transfer (SCNT) reprogramming (Lanza et al., 2000; Wakayama et al., 2000). Oocytes contain sufficient factors for efficient high-quality somatic reprogramming using SCNT compared with the limited factors in the induction of iPSCs (Brambrink et al., 2006; Wakayama et al., 2006; Yang et al., 2007). The overexpression of Zscan4, an oocyte-related factor, during iPSC induction significantly improved telomere elongation and increased reprogramming efficiency and pluripotency through the telomerase-independent alternative lengthening of telomeres (ALT) (Jiang et al., 2013; Lee and Gollahon, 2015). In addition, the protein complex Shelterin, which contains TERF1, TERF2, POT1, RAP1, TIN2, and TPP1 (Xin et al., 2011), was also important for telomere length and stabilization. TERF1 has been reported to play a key role in both telomerase-dependent telomere maintenance and the ALT process (Ho et al., 2016). TERF1 co-localizes and interacts indirectly with ZSCAN4 in the cell nucleus (Lee and Gollahon, 2015). TERF1, along with TERF2, normally prevents telomerase from adding more telomere units to telomeres to balance the telomere length (Diotti and Loayza, 2011). However, when telomere lengthening is required, TERF1 recruits helicases to facilitate the process (Steir, 2012). In addition, TERF1 is highly expressed in ESCs and iPSCs as a stemness marker (Boue et al., 2010; Schneider et al., 2013). However, whether TERF1 participates in the regulation of reprogramming telomere re-elongation remains unknown.
Pre-iPSCs, the partially reprogrammed cells, provide a useful model for studying the regulatory mechanism of reprogramming. Pre-iPSCs exhibit ESC-like morphology but show low pluripotency and cannot form chimeras (Silva et al., 2008; Wei et al., 2015). Pre-iPSCs could be activated to naïve pluripotency cells by inhibitor of mitogen-activated protein kinase (MAPK, PD0325901) and inhibitor of glycogen synthase kinase 3 beta (GSK3B, CHIR99021) with the leukemia inhibitory factor (LIF) culture (Theunissen et al., 2011). Knockdown of Hdac2 promotes the four factors that induce iPSC generation and also converts pre-iPSCs into the fully reprogrammed state (Wei et al., 2015). The pre-iPSCs can also be a valuable resource for investigating telomere regulation during iPSC induction.

Recently, a group of microRNAs (miRNAs) were connected to the transcriptional regulatory circuitry of telomere length modulation and pluripotency during the reprogramming of iPSCs. The overexpression of miR-302/367 inhibited the expression of Hdac2 to promote efficient iPSC induction (Anokye-Danso et al., 2011). The miR-17~92, miR-106b~25, and miR-106a~363 clusters were highly expressed and inhibited the expression of transforming growth factor beta receptor 2 (Tgfbr2) to promote reprogramming (Li et al., 2011). MiR-23a repressed the expression of Terf2, resulting in abnormal telomere function (Luo et al., 2015). MiR-155 directly targeted Terf1 to induce telomere fragility (Dinami et al., 2014). However, whether miRNAs can regulate telomere-related genes and increase pluripotency during iPSC induction remains largely unknown.

Transforming growth factor beta (TGFβ) family genes were reported to inhibit Tert expression via the regulation of SMAD3 phosphorylation (Cassar et al., 2010). BMP7 induced telomere shortening in breast cancer cells (Cassar et al., 2009). The inhibition of signaling substituted for Oct4 during iPSC induction and maintained pluripotency (Tan et al., 2015). The inhibition of TGFβ also promoted the expression of Nanog (Ichida et al., 2009). In addition, the repression of NODAL/ACTIVIN signaling mediated the function of Polycomb in increasing the reprogramming efficiency (Dahle and Kuehn, 2013). However, whether TGFβ family-related signaling influences telomere elongation and pluripotency of iPSCs during cell reprogramming remains unclear.

Our study showed that miR-590-3p and miR-590-5p in mature iPSCs and ESCs had increased expression compared with pre-iPSCs. The ectopic expression of miR-590-3p and miR-590-5p promoted the telomere elongation and pluripotency of pre-iPSCs. We further found that both miR-590-3p and miR-590-5p upregulated Terf1 expression by targeting Activin receptor II A (Acvr2a). This work elucidated the role of the miR-590/Acyr2a/Terf1 signaling pathway in modulating pluripotency and telomere elongation in pre-iPSCs.

RESULTS

miR-590 Is Highly Expressed in Fully Pluripotent Cells and Promotes Telomere Elongation and Pluripotency in Pre-iPSCs

To observe the difference in quality among pre-iPSCs, mature iPSCs, and ESCs, we detected stemness marker expression levels and differentiated these cells into three germ layers. Compared with ESCs and mature iPSCs, pre-iPSCs showed lower expression levels of stemness markers Oct4, Sox2, Nanog, and Esrrb (Figure 1A). In addition, the pre-iPSC cell line used for this research was established in our laboratory for previous study and failed at maturation process (Wei et al., 2015). The immunofluorescence staining indicated that SOX2 and SSEA1 were significantly lower in pre-iPSCs than in mature iPSCs and ESCs (Figure S1A). The expression level of Nanog, Esrrb, Rex1, Cripto, and Sall4 (Figures 1A and S1B) also confirmed the maturation of iPSCs (Wei et al., 2015). The differentiation potentials to the three germ layers were also decreased in pre-iPSCs (Figures 1B and S1C). The telomere length of pre-iPSCs cells was significantly shorter than that of the ESCs and mature iPSCs (Figures 1C and 1D). The expression levels of miR-590-3p and miR-590-5p were higher in mature iPSCs and ESCs than in pre-iPSCs (Figure 1E). Then, we enhanced the pre-iPSC maturation by adding the 2i compounds (1 μM PD0325901 and 3 μM CHIR99021) into the medium and found that the stemness markers were upregulated (Figure 1F) with the telomere elongation (Figure 1G) during 1 week in pre-iPSCs. We transfected the miR-590-3p and miR-590-5p inhibitors in the pre-iPSCs and found that inhibition of miR-590 repressed the pluripotency acquisition of pre-iPSCs treated by 2i (Figure S1D). However, the expression of Gsk3b related to the CHIR99021 and Map2k1 related to the PD0325901 were not significantly changed in the pre-iPSCs transfected with miR-590-5p and miR-590-3p inhibitors (Figure S1E). Overexpression of Oct4 could increase the expression level of miR-590-3p and miR-590-5p (Figure S1F). Our previous study indicated the inhibition of miR-590 could increase the proliferation of ESCs (Liu et al., 2014). We also confirmed the inhibition of miR-590 promoted the proliferation of mature iPSCs (Figure S1G). Knockdown of Rad51b, the downstream target of miR-590 (Liu et al., 2014), could also increase the proliferation of mature iPSCs (Figure S1H) but had no significant effect on pluripotency (Figure S1I). During the maturation process of pre-iPSCs, the expression level of miR-590 was also upregulated (Figure 1H). Overexpression of miR-590-3p or miR-590-5p in pre-iPSCs promoted the re-elongation of
telomere length compared with the control group (Figures 1J–1K). Pluripotency was also increased by overexpressing miR-590 (Figures 1L and 1M).

**Inhibition of Acvr2a Improves Telomere Re-elongation to Promote Pluripotency in Pre-iPSCs**

Our previous study identified Acvr2a as a downstream target of miR-590-3p and miR-590-5p (Liu et al., 2014). Here, we found that Acvr2a expression was downregulated in pre-iPSCs overexpressing miR-590 (Figure 2A). Then, knockdown of Acvr2a in pre-iPSCs (Figure 2B) showed that the telomeres could be elongated (Figures 2C and 2D). The expression level of stemness markers (Figures 2E and S2A) and the differentiation potentials to the three germ layers were both increased after knockdown of Acvr2a (Figures 2F and S2B).

**The miR-590/Acvr2a Pathway Modulates Telomere Length and the Pluripotency of Pre-iPSCs**

To determine whether Acvr2a mediated the function of miR-590 in regulating telomeres and pluripotency in pre-iPSCs, we performed rescue experiments and found that the overexpression of Acvr2a significantly blocked the promotion of telomere re-elongation by miR-590-3p overexpression (Figures 3A and 3B). The expression levels of stemness markers (Figures 3C and S3A) and three germ layer markers (Figures 3D and S3B) were blocked by Acvr2a overexpression. Furthermore, we co-overexpressed Acvr2a with miR-590-5p and found that Acvr2a could block the telomere re-elongation promoted by miR-590-5p overexpression (Figures 3E and 3F). The expression levels of stemness markers (Figures 3G and S3A) and three germ layer markers (Figures 3H and S3C) were also blocked by Acvr2a overexpression.

**Terf1 Is Critically Involved in Modulating Pre-iPSC Telomere Length and Pluripotency**

We performed differential expression analysis of telomere-related genes between mature iPSCs and pre-iPSCs and found that Terf1 was highly expressed in mature iPSCs and was much higher than in pre-iPSCs (Figure 4A). So we hypothesized that Terf1 might play critical role in regulating pre-iPSCs telomere. Overexpression of either miR-590-3p or miR-590-5p upregulated Terf1 in pre-iPSCs (Figure 4B). Furthermore, we found that Terf1 expression was upregulated during the induction process of iPSCs (Figure 4C). The overexpression of Terf1 increased telomere length (Figures 4D and 4E). In addition, Terf1 promoted pluripotency acquisition in pre-iPSCs (Figures 4F, 4G, S4A, and S4B).

**Terf1 Mediates the Acvr2a Function in Regulating Telomere Elongation and Pluripotency**

After the addition of SB431542 (50 μM), an ACTIVIN signaling inhibitor of p-SMAD2 signaling, into the pre-iPSC culture medium, we found that Terf1 was upregulated.

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**Figure 1. High Expression of miR-590 in Fully Pluripotent Cells Promotes Telomere Elongation and Pluripotency in Pre-iPSCs**

(A) qRT-PCR showed the low expression of stemness markers Oct4, Sox2, Nanog, and Esrrb in mouse pre-iPSCs compared with ESCs and iPSCs. Data shown are the mean ± SD (n = 5).

(B) Detecting markers of three germ layers, including endoderm (Mixl1, Gata6, and Afp), mesoderm (Gata4, T, and Snail), and ectoderm (Oct6, Sox1, and Nestin), derived from mouse ESCs, iPSCs, and pre-iPSCs by qRT-PCR. Data shown are the mean ± SD (n = 3).

(C) Fluorescence in situ hybridization (FISH) detection of telomere staining in ESCs, iPSCs, and pre-iPSCs showed the shorter length of telomere in pre-iPSCs. The bottom panel shows the statistics of relative telomere length (relative telomere fluorescence intensity) by histogram measured by FISH. Scale bar indicates 10 μm.

(D) Relative length of telomeres in ESCs, iPSCs, and pre-iPSCs detected by qPCR. Data shown are the mean ± SD (n = 3).

(E) Lower expression of miR-590-3p and miR-590-5p in pre-iPSCs than in ESCs and iPSCs detected by qRT-PCR. Data shown are the mean ± SD (n = 4).

(F) qRT-PCR detection showed the gradual upregulation of stemness markers by 2i induction in pre-iPSCs on 4 days (4d) and 8 days (8d). Data shown are the mean ± SD (n = 3).

(G) Telomere could elongate in the 2i-treated pre-iPSCs detected by qPCR. Data shown are the mean ± SD (n = 4).

(H) Upregulation of miR-590-3p and miR-590-5p during the maturation process by 2i treatment. Data shown are the mean ± SD (n = 3).

(I) Both miR-590-3p and miR-590-5p promoted telomere elongation. Data shown are the mean ± SD (n = 3).

(J) FISH detection of telomere staining showed improvement in telomere elongation by overexpression of miR-590-3p or miR-590-5p. Scale bar indicates 10 μm.

(K) The statistics of (J).

(L) miR-590-3p or miR-590-5p overexpression increased the expression level of stemness markers in pre-iPSCs. Data shown are the mean ± SD (n = 3).

(M) Three germ layer markers were upregulated in the cells derived from the miR-590-3p or miR-590-5p-overexpressing pre-iPSCs. Data shown are the mean ± SD (n = 3).

For all data, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. In (A), (B), and (D), the asterisk (*) means the significance in the comparison of ESCs group with the pre-iPSCs group; hash (#) means the significance of the statistics of the iPSCs and pre-iPSCs group. In other panels, the asterisk means the significance of the comparison of treated cell group with the control (ctrl) group.
Then, we performed chromatin immunoprecipitation (ChIP) assays to assess the binding of p-SMAD2 to the Terf1 promoter in pre-iPSCs and found that it was increased compared with binding in mature iPSCs (Figure 5A). Inhibition of p-SMAD2 by SB431542 also showed more binding of POL II on Terf1 promoter (Figure S5A). Knockdown of Acvr2a also increased the expression of Terf1 (Figure 5C). Knockdown of Terf1 blocked the effect on telomere elongation caused by the downregulation of Acvr2a (Figures 5D and 5E). In addition, knockdown of Terf1 blocked the promoting effect of Acvr2a downregulation on pluripotency (Figures 5F, 5G, S5B, and S5C).

**Terf1 Is the Functional Downstream Mediator of miR-590 in Regulating Telomere Elongation and Pluripotency**

We performed rescue experiments and found that the downregulation of Terf1 blocked the miR-590-3p-mediated increase in telomere elongation (Figures 6A and 6B). The expression levels of pluripotent genes (Figures 6C and S6A) and three germ layer markers (Figures 6D and S6B) were also blocked by knockdown of Terf1 in miR-590-3p overexpressing pre-iPSCs. Similarly, downregulation of Terf1 blocked the reelongation of telomeres promoted by miR-590-5p overexpression (Figures 6E and 6F). Moreover, the expression levels of pluripotent genes (Figures 6G and
Figure 3. The miR-590/Acvr2a Pathway Modulates Telomere Length and the Pluripotency of Pre-iPSCs

(A) Telomere FISH staining and histogram statistics (bottom) showed that the overexpression of Acvr2a blocked the function of miR-590-3p on regulating telomere elongation in the rescue experiments. Scale bar indicates 10 μm.

(B) Detection of the telomeres by qPCR in the rescue experiment. Data shown are the mean ± SD (n = 3).

(C) Acvr2a restored the expression of stemness markers upregulated by miR-590 to be similar with the control group in the rescue experiment. Data shown are the mean ± SD (n = 3).

(D) Acvr2a blocked the increase of pluripotency caused by overexpression of miR-590-3p in pre-iPSCs. Data shown are the mean ± SD (n = 3).

(E and F) (E) FISH staining of telomeres and histogram statistics (right) and (F) qPCR detection both showed that Acvr2a blocked the miR-590-5p function in promoting telomere elongation. Scale bar indicates 10 μm. Data shown are the mean ± SD (n = 3).

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S6A) and three germ layer markers (Figures 6H and S6C) were similar to the control groups following the downregulation of Terf1 with the overexpression of miR-590-5p in pre-iPSCs.

DISCUSSION

iPSC induction remains an inefficient process (Stadtfeld and Hochedlinger, 2010). Although studies have suggested (G and H) Detection of the Acvr2a function blocking the promotion of stemness marker expression and (H) the pluripotency of differentiation capacity regulated by miR-590-5p. Data shown are the mean ± SD (n = 3). For all data, *p < 0.05, **p < 0.01, ***p < 0.001. Asterisk (*) means the statistical significance of the miR-590-5p and miR-590-3p group compared with the control group; hash (#) means the statistical significance of the miR-590-5p + Acvr2a and miR-590-3p + Acvr2a group compared with the corresponding miR-590-5p and miR-590-3p group.
that chemical compounds (Chen et al., 2010; Li and Ding, 2010) that regulate some gene expression signaling pathways (Guo et al., 2013; Ye et al., 2012) or that promote epigenetic alteration with reprogramming factors (Huangfu et al., 2008; Shi et al., 2008) increase the quality and efficiency of iPSCs, how cells re-elongate their telomeres remains largely unknown. Our study showed that miR-590 can target Acvr2a to upregulate the expression of Terf1 and finally promote the elongation and pluripotency of pre-iPSCs.

**Figure 5. Terf1 Mediates the Acvr2a Function in Regulating Telomere Elongation and Pluripotency**

(A) Inhibition of ACTIVIN signaling by SB431542 increased Terf1 expression. Data shown are the mean ± SD (n = 3).

(B) ChIP assays indicated that p-SMAD2 shows increased binding to the Terf1 promoter in pre-iPSCs compared with mature iPSCs. "Ctrl region" means the sequence without p-SMAD2 binding, while "Pr region" means the prediction region of p-SMAD2 in promoter. Data shown are the mean ± SD (n = 3).

(C) Downregulation of Acvr2a upregulated the expression of Terf1. Data shown are the mean ± SD (n = 3).

(D and E) Pre-iPSCs with Terf1 knockdown blocked the increase in telomere elongation promoted by downregulation of Acvr2a, which was detected by both qPCR and (E) FISH assays. Scale bar indicates 10 μm. Data shown are the mean ± SD (n = 3).

(F and G) (F) Downregulation of Terf1 prevented the function of Acvr2a knockdown in promoting stemness marker expression and (G) pluripotency in pre-iPSCs. Data shown are the mean ± SD (n = 3). For all data, * and #p < 0.05, ** and ##p < 0.01. Asterisk (*) means the statistical significance of the shR-Acvr2a-1 and shR-Acvr2a-2 group compared with the control group; hash (#) means the statistical significance of the shR-Acvr2a-2+siTerf1 group compared with the shR-Acvr2a-2 group.

Efficient telomere elongation is critically involved in the gain of pluripotency and is the key factor ensuring the higher quality of iPSCs (Marion et al., 2009). For ESCs, in addition to higher expression of telomerase genes and
activity, Zscan4, which participates in the ALT process, increases the frequency of sister chromatid exchange to promote recombination (Zalzman et al., 2010). A previous study showed that Zscan4 combined with the reprogramming factors promoted iPSC induction, quality, and telomere elongation with decreased DNA damage (Jiang et al., 2013). However, compared with the ALT process of efficient telomere elongation by utilizing SCNT (Wakayama et al., 2000), the induction of iPSCs by telomerase-dependent telomere elongation using four factors is inefficient (Le et al., 2014). In this study, we found that pre-iPSCs showed much shorter telomere lengths and lower pluripotency than ESCs or mature iPSCs. Coincidentally, miR-590 showed higher expression in ESCs and mature iPSCs. Previous studies showed that miR-590 increased both calcium deposition and the osteoblast differentiation marker genes’ expression in mouse mesenchymal stem cells (Vishal et al., 2017). MiR-590 could prevent the progression of aortic atherosclerosis in apolipoprotein E knockout mice (He et al., 2015). In addition, miR-590 was also reported to be the epithelial-mesenchymal transition suppressor and downregulated in unilateral ureteral obstruction kidney mouse model (Liu et al., 2015). These studies suggested the critical roles of miR-590 on many kinds of physiology process. Further ectopic expression of miR-590-3p or miR-590-5p in pre-iPSCs significantly promoted telomere elongation and the pluripotency of pre-iPSCs. In addition, cell reprogramming may result in abnormal chromosomes (Gore et al., 2011; Hussein et al., 2011). Our previous study indicated the role of miR-590 in promoting DNA damage repair in mouse ESCs (mESCs) (Liu et al., 2014). These results suggested the critical function of miR-590 during cell reprogramming by improving telomere elongation, genome stability, and cell pluripotency.

MiRNAs have been reported to be key regulators in ESCs and iPSCs and to participate in many molecular signaling pathways (Li et al., 2011; Liao et al., 2011; Samavarchi-Tehrani et al., 2010). Our previous study reported that miR-590 directly targets Acvr2a, which belongs to the TGFB superfamily to enhance ESC self-renewal and DNA damage repair (Liu et al., 2014). TGFB family genes that play important roles in ESCs and iPSCs can also be regulated by miRNAs (Tan et al., 2015). It has been reported that miR-590 directly targets Acvr2a to enhance ESC self-renewal and DNA damage repair (Liu et al., 2014). The miR-302/367 cluster regulates the TGFB/R2/E-CADHERIN pathway to accelerate the mesenchymal-epithelial transition process during reprogramming (Liao et al., 2011). Our study indicated that downregulation of Acvr2a promoted telomere elongation and pluripotency in pre-iPSCs. Rescue experiments confirmed that Acvr2a mediated the function of miR-590. These results identified the function of the TGFB family receptor gene Acvr2a and the miR-590/Acvr2a signaling pathway in modulating telomere elongation and reprogramming.

Telomere-related genes have been reported to be regulated by TGFB family members. Telomerase TERT can be repressed by the TGFB/p-SMAD3 pathway (Cassar et al., 2010; Wang et al., 2016). SMAD3 is recruited by OCT4 to restrict Rif1 expression by binding to its promoter to maintain the stability of mESCs. Deletion of Terf1 in iPSCs induced DNA damage with an increasing number of γH2AX-positive cells (Schneider et al., 2013). In MEFs, downregulation of Terf1 inhibited cell proliferation (Martinez et al., 2009). Terf1 is important to recruit helicases to facilitate telomere lengthening (Steir, 2012). Previous study of the impairment of generation of iPSCs derived from the MEFs of second- and third-generation telomerase-deficient mice found that that telomeres no longer elongated and were shortening continuously during the reprogramming. In addition, although the induction efficiency of Terc−/− iPSCs derived from first-generation MEFs of telomerase-deficient mice was similar to wild-type controls, the generation of chimeric mice failed (Marion et al., 2009). Here we found that inhibition of p-SMAD2 increased the expression

Figure 6. Terf1 Is the Functional Downstream Mediator of miR-590 in Regulating Telomere Elongation and Pluripotency
(A) FISH assays showed that downregulation of Terf1 blocked the improvement of telomere elongation caused by the miR-590-3p overexpression in the rescue experiment. Scale bar indicates 10 μm.
(B) qPCR detection of telomere length in the rescue experiment. Data shown are the mean ± SD (n = 4).
(C) Increased expression of stemness markers by miR-590-3p was prevented by Terf1 knockdown. Data shown are the mean ± SD (n = 4).
(D) Detection of three germ layer markers by qPCR showed that Terf1 downregulation blocked the improvement of pluripotency acquisition through miR-590-3p. Data shown are the mean ± SD (n = 3).
(E and F) (E) Downregulation of Terf1 also blocked the miR-590-5p function of promoting telomere elongation as detected by both FISH and (F) qPCR. Scale bar indicates 10 μm. Data shown are the mean ± SD (n = 3).
(G and H) (G) Downregulation of Terf1 prevented miR-590-5p-mediated promotion of stemness marker expression and (H) pluripotency in pre-iPSCs. Data shown are the mean ± SD (n = 3).
For all data, * and p < 0.05, ** and #p < 0.01, *** and ##p < 0.001. Asterisk (*) means the statistical significance of the miR-590-5p and miR-590-3p group compared with the control group; hash (#) means the statistical significance of the miR-590-5p + siTerf1 and miR-590-3p + siTerf1 group compared with the corresponding miR-590-5p and miR-590-3p group.
of Terf1 in pre-iPSCs, elongated the telomere, and led to the maturation of pre-iPSCs. Overexpression of Terf1 itself also promoted the telomere elongation and the maturation of pre-iPSCs. These results collectively indicated the importance of the re-elongation of short telomeres for normal iPSC generation and maturation.

Further, we determined that inhibition of ACTIVIN signaling by SB431542 or the downregulation of Acvr2a increased Terf1 expression. These results suggested the function of ACTIVIN signaling in regulating Terf1 expression. Our rescue experiments found that Terf1 mediated the function of Acvr2a in regulating telomere elongation and pluripotency in pre-iPSCs. We further determined that Terf1 is a functional downstream mediator of the miR-590/Acvr2a pathway and forms the miR-590/Acvr2a/Terf1 axis. In addition, a previous study indicated that Zscav4 participates in the ALT process to improve telomere elongation and the quality of iPSCs (Jiang et al., 2013). TERF1 not only interacts with ZSCAN4 but also regulates the ALT process (Lee and Gollahon, 2015). These results suggested the critical function of Terf1 and the miR-590/Acvr2a/Terf1 signaling pathway in improving the quality and pluripotency of iPSCs. Our previous study showed that miR-590 was upregulated during the differentiation of mESCs after LIF withdrawal. MiR-590 could regulate the expression of Rad51b to balance the DNA damage and cell cycle after but could not influence the pluripotency of mESCs (Liu et al., 2014). Interestingly, here we found that miR-590 was highly expressed in ESCs and iPSCs compared with pre-iPSCs. The expression of miR-590 was also upregulated during the pre-iPSCs’ maturation promoted by 2i (CHIR99021 and PD0325901). We found that inhibition of miR-590 repressed the pluripotency acquisition of pre-iPSCs treated by 2i. This result indicated the important role of miR-590 during the reprogramming, and the aberrant inhibition of miR-590 expression during reprogramming might arrest the reprogramming cells in the pre-iPSC status. These findings also indicated that the regulatory mechanism of miR-590 expression might be different during iPSC reprogramming and mESC differentiation. Our study elucidated the role of the miR-590/Acvr2a/Terf1 signaling axis in improving telomere elongation and pluripotency of pre-iPSCs, establishing a foundation for the clinical application of iPSCs in the future.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

Mouse ESCs were cultured in 15% fetal bovine serum (FBS) (Gibco), while iPSCs and pre-iPSCs were cultured in 20% knockout serum replacement (KOSR) (Gibco) of knockout-DMEM (Gibco) medium. The medium also contained 1% nonessential amino acids (NEAAs) (Thermo), 1% mM L-glutamine (Thermo), 55 μM β-mercaptoethanol (Gibco), and LIF (Millipore). Cells were maintained on feeders at 37°C in a humidified 5% CO₂ atmosphere. The pre-iPSCs have been reported in our previous study (Wei et al., 2015).

**qRT-PCR**

Total RNA was isolated using RNAiso Plus (TaKaRa). cDNA synthesis from mRNA and miRNAs was performed using the cDNA Synthesis Kit (TaKaRa) and the TIANScript RT Kit (TIANGEN), respectively. The Bulge-Loop miRNA qPCR Primer Set was purchased from the Ribobio Company. Primers for the qRT-PCR analysis of mRNA are shown in Table S1.

**Immunofluorescence Staining of Three Germ Layers**

For the differentiation of three germ layers, cells were cultured in the medium for 48 hr, followed by withdrawing the LIF for another 7-day culture process. Medium should be changed every day. Then the differentiation cells were used for staining. First, cells were fixed with 4% paraformaldehyde (PFA) for 20 min and permeabilized with 0.2% Triton X-100 for 8 min. Cells were washed with PBS and blocked with 10% FBS (Gibco) for 1 hr at room temperature. Then, cells were stained with primary antibodies against SOX2 (Abcam, ab59776), SSEA1 (Santa Cruz, sc-101462), GATA4 (Santa Cruz, sc-9053), GATA6 (R&D Systems, AF1700), and REST (Abcam, ab105389) for 12 hr at 4°C. After washing with PBS, cells were incubated with the corresponding secondary antibodies. Finally, cells were counterstained with Hoechst33342 (Sigma, 14533) to show the entire cell nucleus.

**Fluorescence In Situ Hybridization Assay**

Chromosomes were separated onto glass slides and dried overnight. Chromosomes with telomeres were denatured at 80°C for 10 min to hybridize with the red Cy3-labeled telomere peptide nucleic acid probe. Whole chromosomes were stained with 0.5 μg/ml Hoechst33342 (blue). Fluorescence of the chromosomes and telomeres was digitally imaged on a Nikon confocal laser scanning microscope with fluorescein isothiocyanate/DAPI filters. Relative telomere length was presented as telomere fluorescence intensity detected using the TFL-TELO software program.

**qPCR Analysis of Relative Telomere Length**

DNA of cells was isolated using a DNA isolation kit (TIANGEN). Measurements were performed as described in previous studies (Behrens et al., 2017; O’Callaghan and Fenech, 2011).

**ChIP Assay**

ChIP assays were performed with rabbit IgG (Cell Signaling Technology, 27295), p-SMAD2 (Cell Signaling Technology, 3101L), POL II (Millipore, 05-263) antibodies using the ChIP assay kit (Millipore) according to the instructions. Both immunoprecipitated DNA and whole-cell DNA were used for qPCR assays of Terf1 with the following primers: for the binding region, 5'-GGA GGGGAAGAGGAGTGAAG-3' (forward) and 5'-TGTTCGGCAC CGTTCTCAG-3' (reverse); for the control region, 5'-TCAGGAA GTCCCCTGAGAT-3' (forward) and 5'-GCATTCCCTTCGGGTA TTTT-3' (reverse).
**RNAi Assay**

For Terf1, the siRNA was used for RNAi by targeting the sequence GGAAGUUACUUAAGAUAAUC (siTerf1) in the rescue experiments. Details of the shRNA-Acvt2a vectors have been reported in our previous study (Liu et al., 2014).

**Statistical Analysis**

The error bars represent the SD of at least three independent experiments. The statistical significance was analyzed by one-way ANOVA or Student's t test. * and #p < 0.05, ** and ##p < 0.01, ###p < 0.001.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures and one table and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.05.008.

**AUTHOR CONTRIBUTIONS**

All authors discussed the experiments and contributed to the text of the manuscript. Q.L. and G.W. designed the experiments, acquired the data, and performed the analysis. Y.L., M.B., and Z.J. contributed to plasmid construction. T.H., R.W., and Y. Yu provided materials and contributed to technical assistance. W.J. and Y. Yang discussed the project conception and design. Q.L., G.W., and J.K. conceived the project, analyzed the data, and wrote the manuscript.

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**REFERENCES**

Anokye-Danso, F., Trivedi, C.M., Juhr, D., Gupta, M., Cui, Z., Tian, Y., Zhang, Y., Yang, W., Gruber, P.J., Epstein, J.A., et al. (2011). Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. Cell Stem Cell 8, 376–388.

Behrens, Y.L., Thomay, K., Hagedorn, M., Ebersold, J., Henrich, L., Nustede, R., Schlegelberger, B., and Gohring, G. (2017). Comparison of different methods for telomere length measurement in whole blood and blood cell subsets: recommendations for telomere length measurement in hematological diseases. Genes Chromosomes Cancer 56, 700–708.

Boue, S., Paramonov, I., Barrero, M.J., and Izpisua Belmonte, J.C. (2010). Analysis of human and mouse reprogramming of somatic cells to induced pluripotent stem cells. What is in the plate? PLoS One 5, e12664.

Brambrink, T., Hochdelinger, K., Bell, G., and Jaenisch, R. (2006). ES cells derived from cloned and fertilized blastocysts are transcriptionally and functionally indistinguishable. Proc. Natl. Acad. Sci. USA 103, 933–938.

Cassar, L., Nicholls, C., Pinto, A.R., Li, H., and Liu, J.P. (2009). Bone morphogenetic protein-7 induces telomerase inhibition, telomere shortening, breast cancer cell senescence, and death via Smad3. FASEB J. 23, 1880–1892.

Dahle, O., and Kuehn, M.R. (2013). Polycomb determines responses to smad2/3 signaling in embryonic stem cell differentiation and in reprogramming. Stem Cells 31, 1488–1497.

Dinami, R., Ercolani, C., Petti, E., Piazza, S., Cian, Y., Sestito, R., Sacconi, A., Biagnion, F., Le Sage, C., Agami, R., et al. (2014). miR-155 drives telomere fragility in human breast cancer by targeting TRFI. Cancer Res. 74, 4145–4156.

Dietzi, R., and Loayza, D. (2011). Shelterin complex and associated factors at human telomeres. Nucleus 2, 119–135.

Gore, A., Li, Z., Fung, H.L., Young, J.E., Agarwal, S., Antosiewicz-Bourget, J., Canto, I., Giorgetti, A., Israel, M.A., Kiskinis, E., et al. (2011). Somatic coding mutations in human induced pluripotent stem cells. Nature 471, 63–67.

Guo, X., Liu, Q., Wang, G., Zhu, S., Gao, L., Hong, W., Chen, Y., Wu, M., Liu, H., Jiang, C., et al. (2013). microRNA-29b is a novel mediator of Sox2 function in the regulation of somatic cell reprogramming. Cell Res. 23, 142–156.

He, P.P., OuYang, X.P., Li, Y., Lv, Y.C., Wang, Z.B., Yao, E., Xie, W., Tan, Y.L., Li, L., Zhang, M., et al. (2015). MicroRNA-590 inhibits lipoprotein lipase expression and prevents atherosclerosis in apoE knockout mice. PLoS One 10, e0138788.

Ho, A., Wilson, F.R., Peragine, S.L., Jeyanthan, K., Mitchel, T.R., and Zhu, X.D. (2016). TRF1 phosphorylation on T271 modulates telomere length and in reprogramming. Stem Cells Dev. 25, 1488–1497.
A small-molecule inhibitor of tgf-beta signaling replaces sox2 in reprogramming by inducing nanog. Cell Stem Cell 5, 491–503.

Ichida, J.K., Blanchard, J., Lam, K., Son, E.Y., Chung, J.E., Egli, D., Loh, K.M., Carter, A.C., Di Giorgio, F.P., Koszka, K., et al. (2009). A small-molecule inhibitor of tgf-beta signaling replaces sox2 in reprogramming by inducing nanog. Cell Stem Cell 5, 491–503.

Jiang, J., Lv, W., Ye, X., Wang, L., Zhang, M., Yang, H., Okuka, M., Zhou, C., Zhang, X., Liu, L., et al. (2013). Zscan4 promotes genomic stability during reprogramming and dramatically improves the quality of iPS cells as demonstrated by tetraploid complementation. Cell Res. 23, 92–106.

Lanza, R.P., Cicelli, J.B., Diaz, F., Moraes, C.T., Farin, P.W., Farin, C.E., Hammer, C.J., West, M.D., and Damiani, P. (2000). Cloning of an endangered species (Bos gaurus) using interspecies nuclear transfer. Cloning 2, 79–90.

Le, R., Kou, Z., Jiang, Y., Li, M., Huang, B., Liu, W., Li, H., Kou, X., He, W., Rudolph, K.L., et al. (2014). Enhanced telomere rejuvenation in pluripotent cells reprogrammed via nuclear transfer relative to induced pluripotent stem cells. Cell Stem Cell 14, 27–39.

Lee, K., and Gollahon, L.S. (2015). ZSCAN4 and TRF1: a functionally indirect interaction in cancer cells independent of telomerase activity. Biochem. Biophys. Res. Commun. 466, 644–649.

Li, W., and Ding, S. (2010). Small molecules that modulate embryonic stem cell fate and somatic cell reprogramming. Trends Pharmacol. Sci. 31, 36–45.

Li, Z., Yang, C.S., Nakashima, K., and Rana, T.M. (2011). Small RNA-mediated regulation of iPS cell generation. EMBO J. 30, 823–834.

Liao, B., Tao, X., Liu, L., Feng, S., Zovoilis, A., Liu, W., Xue, Y., Cai, J., Guo, X., Qin, J., et al. (2011). MicroRNA cluster 302-367 enhances somatic cell reprogramming by accelerating a mesenchymal-to-epithelial transition. J. Biol. Chem. 286, 17359–17364.

Liu, L., Bailey, S.M., Okuka, M., Munoz, P., Li, C., Zhou, L., Wu, C., Czerwiec, E., Sandler, L., Seyfang, A., et al. (2007). Telomere lengthening early in development. Nat. Cell Biol. 9, 1436–1441.

Liu, Q., Wang, G., Chen, Y., Li, G., Yang, D., and Kang, J. (2014). A miR-590/Acrv2a/Rad51b axis regulates DNA damage repair during mESC proliferation. Stem Cell Rep. 3, 1103–1117.

Liu, T., Nie, F., Yang, X., Wang, X., Yuan, Y., Lv, Z., Zhou, L., Peng, R., Ni, D., Gu, Y., et al. (2015). MicroRNA-590 is an EMT-suppressive microRNA involved in the TGFbeta signaling pathway. Mol. Med. Rep. 12, 7403–7411.

Luo, Z., Feng, X., Wang, H., Xu, W., Zhao, Y., Ma, W., Jiang, S., Liu, D., Huang, J., and Songyang, Z. (2015). MIR-23a induces telomere dysfunction and cellular senescence by inhibiting TRF2 expression. Aging Cell 14, 391–399.

Marion, R.M., Stratt, K., Li, H., Tejera, A., Schoeftner, S., Ortega, S., Serrano, M., and Blasco, M.A. (2009). Telomeres acquire embryonic stem cell characteristics in induced pluripotent stem cells. Cell Stem Cell 4, 141–154.

Martinez, P., Thanasoula, M., Munoz, P., Liao, C., Tejera, A., McNees, C., Flores, J.M., Fernandez-Capetillo, O., Tarsounas, M., and Blasco, M.A. (2009). Increased telomere fragility and fusions resulting from TRF1 deficiency lead to degenerative pathologies and increased cancer in mice. Genes Dev. 23, 2060–2075.

O’Callaghan, N.J., and Fenech, M. (2011). A quantitative PCR method for measuring absolute telomere length. Biol. Proced. Online 13, 3.

Pera, M.F. (2011). Stem cells: the dark side of induced pluripotency. Nature 471, 46–47.

Samavarchi-Tehrani, P., Golipour, A., David, L., Sung, H.K., Beyer, T.A., Datti, A., Woltjen, K., Nagy, A., and Wrana, J.L. (2010). Functional genomics reveals a BMP-driven mesenchymal-to-epithelial transition in the initiation of somatic cell reprogramming. Cell Stem Cell 7, 64–77.

Schneider, R.P., Garrobo, I., Foronda, M., Palacios, J.A., Marion, R.M., Flores, L., Ortega, S., and Blasco, M.A. (2013). TRF1 is a stem cell marker and is essential for the generation of induced pluripotent stem cells. Nat. Commun. 4, 1946.

Shi, Y., Do, J.T., Desponts, C., Hahn, H.S., Scholer, H.R., and Ding, S. (2008). A combined chemical and genetic approach for the generation of induced pluripotent stem cells. Cell Stem Cell 2, 525–528.

Silva, J., Barrandon, O., Nichols, J., Kawaguchi, J., Theunissen, T.W., and Smith, A. (2008). Promotion of reprogramming to ground state pluripotency by signal inhibition. PLoS Biol. 6, e253.

Stadtfeld, M., and Hochedlinger, K. (2010). Induced pluripotency: history, mechanisms, and applications. Genes Dev. 24, 2239–2263.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblasts by defined factors. Cell 126, 663–676.

Tan, F., Qian, C., Tang, K., Abd-Allah, S.M., and Jing, N. (2015). Inhibition of transforming growth factor beta (TGF-beta) signaling can substitute for Oct4 protein in reprogramming and maintain pluripotency. J. Biol. Chem. 290, 4500–4511.

Theunissen, T.W., van Oosten, A.L., Castelo-Branco, G., Hall, J., Smith, A., and Silva, J.C. (2011). Nanog overcomes reprogramming barriers and induces pluripotency in minimal conditions. Curr. Biol. 21, 65–71.

Vishal, M., Vimalraj, S., Ajeejha, R., Gokulnath, M., Keerthana, R., He, Z., Partridge, N.C., and Selvamurugan, N. (2017). MicroRNA-590-5p stabilizes Runx2 by targeting Smad7 during osteoblast differentiation. J. Cell. Physiol. 232, 371–380.

Wakayama, T., Tateno, H., Mombaerts, P., and Yanagimachi, R. (2000). Nuclear transfer into mouse zygotes. Nat. Genet. 24, 108–109.

Wakayama, S., Jakt, M.L., Suzuki, M., Araki, R., Hikichi, T., Kishigami, S., Ohta, H., Van Thuan, N., Mızutani, E., Sakaide, Y., et al. (2006). Equivalency of nuclear transfer-derived embryonic stem cells to those derived from fertilized mouse blastocysts. Stem Cells 24, 2023–2033.

Wang, X.H., Liu, M.N., Sun, X., Xu, C.H., Liu, J., Chen, J., Xu, R.L., and Li, B.X. (2016). TGF-beta1 pathway affects the protein expression of many signaling pathways, markers of liver cancer stem cells, cytokeratins, and TERT in liver cancer HepG2 cells. Tumour Biol. 37, 3675–3681.

Wei, T., Chen, W., Wang, X., Zhang, M., Chen, J., Zhu, S., Chen, L., Yang, D., Wang, G., Jia, W., et al. (2015). An HDAC2-TET1 switch at
distinct chromatin regions significantly promotes the maturation of pre-iPS to iPS cells. Nucleic Acids Res. 43, 5409–5422.
Xin, H., Liu, D., and Songyang, Z. (2008). The telosome/shelterin complex and its functions. Genome Biol. 9, 232.
Yang, X., Smith, S.L., Tian, X.C., Lewin, H.A., Renard, J.P., and Wakayama, T. (2007). Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. Nat. Genet. 39, 295–302.
Ye, D., Wang, G., Liu, Y., Huang, W., Wu, M., Zhu, S., Jia, W., Deng, A.M., Liu, H., and Kang, J. (2012). MiR-138 promotes induced pluripotent stem cell generation through the regulation of the p53 signaling. Stem Cells 30, 1645–1654.
Zalzman, M., Falco, G., Sharova, L.V., Nishiyama, A., Thomas, M., Lee, S.L., Stagg, C.A., Hoang, H.G., Yang, H.T., Indig, F.E., et al. (2010). Zscan4 regulates telomere elongation and genomic stability in ES cells. Nature 464, 858–863.
Zhao, X.Y., Li, W., Lv, Z., Liu, L., Tong, M., Hai, T., Hao, J., Guo, C.L., Ma, Q.W., Wang, L., et al. (2009). iPS cells produce viable mice through tetraploid complementation. Nature 461, 86–90.