A miR-590/Acrv2a/Rad51b Axis Regulates DNA Damage Repair during mESC Proliferation

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http://dx.doi.org/10.1016/j.stemcr.2014.10.006
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SUMMARY

Embryonic stem cells (ESCs) enable rapid proliferation that also causes DNA damage. To maintain genomic stabilization during rapid proliferation, ESCs must have an efficient system to repress genotoxic stress. Here, we show that withdrawal of leukemia inhibitory factor (LIF), which maintains the self-renewal capability of mouse ESCs (mESCs), significantly inhibits the cell proliferation and DNA damage of mESCs and upregulates the expression of miR-590. miR-590 promotes single-strand break (SSB) and double-strand break (DSB) damage repair, thus slowing proliferation of mESCs without influencing stemness. miR-590 directly targets Activin receptor type 2a (Acrv2a) to mediate Activin signaling. We identified the homologous recombination-mediated repair (HRR) gene, Rad51b, as a downstream molecule of the miR-590/Acrv2a pathway regulating the SSB and DSB damage repair and cell cycle. Our study shows that a miR-590/Acrv2a/Rad51b signaling axis ensures the stabilization of mESCs by balancing DNA damage repair and rapid proliferation during self-renewal.

INTRODUCTION

Embryonic stem (ES) cells derived from the inner cell mass of the blastocyst have been used to understand early embryonic development (Keller, 2005). The notable characteristic of ESCs is self-renewal that is critically involved in the stimulation of rapid proliferation. In fact, rapid proliferation might protect ESCs from external signals inducing differentiation (Ruiz et al., 2011). However, rapid proliferation would be harmful because it causes successive mitotic division with a long S phase in which DNA is replicating most of the time (Fluckiger et al., 2006; Savatier et al., 2002), and the successive rounds of DNA replication causes many replication errors that may lead to DNA damage (Strumberg et al., 2000; Tichy and Stambrook, 2008). In addition, there is no G1 checkpoint in ESCs (White and Dalton, 2005), which might exacerbate DNA damage during rounds of replication without enough time for repair (Hong and Stambrook, 2004). Consistent with these characteristics, there is a high level of double-strand break (DSB) damage, which is the most toxic type of DNA damage (Valerie and Povirk, 2003), and DSB damage is indicated by the γ-H2AX marker (H2AX becomes phosphorylated on serine 139) in both human ES (hES) cells and mouse ES (mES) cells (Banáth et al., 2009; Chuykin et al., 2008; Momcilovic et al., 2010). Similar to irradiated fibroblast cells, normal mESCs also contain a high frequency of single-strand break (SSB) (Chuykin et al., 2008). However, ESCs still have an integrated genome and stable pluripotency during rapid proliferation (Tichy and Stambrook, 2008; Wang et al., 2008). Additionally, the mutation frequency and mitotic recombination frequency are lower in mESCs than in adult somatic or isogenic mouse embryonic fibroblasts (Tichy and Stambrook, 2008). Thus, ESCs must have unique regulatory mechanisms that counteract DNA damage both quickly and efficiently.

Transforming growth factor β (TGF-β) signaling is closely related to DNA damage repair regulation (Mitra et al., 2013). Studies have shown that TGF-β signaling can suppress BRCA1-dependent repair of DSBs (Dubrovskva et al., 2005). Activin A, a member of the TGF-β superfamily of cytokines, interacts with Activin type I (ALK 2, ALK 4, or ALK 7) and type II (Acrv2a and Acrv2b) receptors (Bondestam et al., 1999; Donaldson et al., 1992; Robson et al., 2008), and it participates in DNA damage repair. In premalignant cells, DSB damage results in Activin A-dependent induction of Cox-2, which is associated with a high level of γ-H2AX (Carlson et al., 2013; Fordyce et al., 2012). In ductal carcinoma, DNA damage response (shorter telomeres and γ-H2AX foci) is also associated with high level of Activin A (Fordyce et al., 2012). Additionally, in hESCs, Activin A can maintain pluripotency even without feeder layers (Beattie et al., 2005). In mESCs, Activin A signaling can promote cell proliferation (Ogawa et al., 2007). However, it remains unknown whether the maintenance of a stable status in ESCs is also related to the DNA damage repair function of Activin A signaling.

DSB damage is the most toxic type of DNA damage (Valerie and Povirk, 2003). Homologous recombination-mediated repair (HRR) is thought to be used in ESCs to repair DSBs (Hasty et al., 1992; Shrivastav et al., 2008; Smith et al., 1995; Tichy et al., 2010). Rad51 family members, including Rad51, Rad51b, Rad5c, Rad51d, and so on, are evolutionarily conserved proteins that play important roles...
Figure 1. miR-590 Inhibits Rapid Proliferation and Promotes SSB and DSB Damage Repair of mESCs

(A) Analysis of the proliferation of mESCs cultured with or without LIF by MTS cell proliferation assay. (Bottom) The qRT-PCR verification of miR-590-3p and miR-590-5p levels. (Right) The morphology of mESCs cultured with (+LIF) on 24 hr or without LIF (−LIF) on 24, 48, 72 hr.

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during HHR (Baumann et al., 1996; Chun et al., 2013; Kawabata et al., 2005; Thacker, 2005). Rad51 paralogs take part in SSB and DSB damage repair (Jensen et al., 2010, 2013). RAD51B is also a protein kinase regulating the function of cell cycle-related genes (Havre et al., 2000) and is a known molecule to promote HRR by participating in the Holliday junction process (Kawabata et al., 2005; Takata et al., 2000). Previous studies have shown that overexpression of Rad51B in Chinese hamster ovary cells causes a G1 delay and UV irradiation hypersensitivity (Havre et al., 1998). TGF-β signaling has also been reported to inhibit DNA damage repair by downregulating the expression of Rad51 in Mv1Lu epithelial cells (Kanamoto et al., 2002). However, it is unknown whether Rad51 paralogs can regulate both the DNA damage repair and cell cycle or even maintain their balance in mESCs. Additionally, the upstream regulators of Rad51 paralogs in mESCs are unclear.

MicroRNAs (miRNAs) are posttranscriptional modulators of gene expression and are connected to the transcriptional regulatory circuitry of mESCs (Marson et al., 2008). Several miRNAs target DNA repair-related factors and influence DNA damage repair. Studies have shown that UV damage promotes miRNA expression in a partially ataxia telangiectasia mutated kinase (ATM)/ataxia telangiectasia and Rad3-related kinase-independent manner (Pothof et al., 2009). Overexpression of miR-24 attenuates H2AX, leading to high sensitivity to irradiation and reduced repair capacity (Lal et al., 2009). miRNAs can also regulate TGF-β signaling. miR-302/367 can modulate BMP signaling, which supports self-renewal by targeting BMP inhibitors in hESCs (Lipchina et al., 2011; Qi et al., 2004). However, there is limited understanding of miRNA modulation of these signaling pathways to regulate DNA damage repair to maintain self-renewal during ESC proliferation, which differs from differentiated cells (Tichy and Stambrook, 2008). In the present study, we found that miR-590 inhibits Activin signaling by directly targeting Acvr2a to inhibit the expression of Rad51B, balancing the DNA damage repair and rapid proliferation of ESCs.

RESULTS

miR-590 Promotes SSB and DSB Damage Repair during Rapid Proliferation of mESCs

Consistent with that, leukemia inhibitory factor (LIF) is critically needed for the self-renewal of mESCs. We found that the rapid proliferation was greatly inhibited in both the cells cultured without LIF, which detected by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay (Figure 1A) and those during the embryonic body (EB) formation, which detected by flow cytometric (FACS) analysis of 5′-bromo-deoxyuridine (BrdU) incorporation (Figure S1A available online). We further found that both of the mature miR-590, namely miR-590-3p and miR-590-5p, were upregulated in cells during the culture without LIF (Figure 1A) and EB formation (Figure S1B), indicating a possible linkage between miR-590 expression and the mESC proliferation. In order to detect that whether miR-590 is associated with the regulation of self-renewal, we transfected pre-miR-590, which can be processed to be miR-590-3p and miR-590-5p (Figure S1C), into mESCs and found that the size of mESC colonies became smaller than control cells
examined at 48 hr posttransfection (Figure 1B). We further digested mESC clones by trypsin into single cells and counted the amount of the total cells under the microscope by using blood count board and found that total cells transfected with pre-miR-590 were less than control cells (Figure S1D). Transfection of miR-590-3p or miR-590-5p mimics respectively into mESCs also resulted in a similar phenomenon of pre-miR-590 (Figure S1E). In contrast, clone size of mESCs was larger than control cells after transfected with miR-590-3p or miR-590-5p inhibitor, which can compete for the miR-590 with target mRNAs (Figure 1B). The amount of total cells was also more than control cells (Figure S1D). MTS assay and FACS of BrdU incorporation also showed that miR-590 inhibited proliferation of mESCs posttransfected for 48 hr with pre-miR-590, while cells transfected with miR-590-3p or miR-590-5p inhibitor showed faster proliferation than control cells (Figures 1C and 1D). Further, we found that the size of clones of another kind of mESC line 46C after transfected with pre-miR-590 was also smaller than control mESCs (Figure S1F).

FACS analysis of cell cycle showed that there was a significant reduction in the proportion of cells in S phase and an increase in the proportion of cells in G1 and G2/M phases in mESCs after transfected with pre-miR-590 for 48 hr (Figure S1G). In contrast, the proportion of cells in S phase was increased and in G1 and G2/M phases was reduced in mESCs transfected with miR-590-3p or miR-590-5p inhibitor (Figure S1G). miR-590 could not induce the apoptosis (Figure S1H) and showed no significant influence on the expression of stemness genes (Figure S1I).

Additionally, we found that miR-590 can regulate the SSB and DSB damage in mESCs. SSB and DSB damage was relieved in mESCs after being transfected with pre-miR-590 detected by the comet assay and the immunostaining of γ-H2AX foci, respectively (Figures 1E and 1F). In contrast, mESCs transfected with the miR-590-3p or miR-590-5p inhibitor showed increased SSB damage (Figure 1E) and DSB (Figure 1F). To investigate whether miR-590 can promote DNA damage repair directly or through regulating proliferation to free up more time to repair DNA, we used X-ray to irradiate mESCs to simulate DSB and SSB damage. We found that after treating with 8 Gy radiation, both mESCs transfected with pre-miR-590 and pre-miR-control showed violent DSB and SSB damage. Three hours later, both groups of mESCs had repaired much DNA damage (Figures 1G and 1H). However, we found that the repair capacity of mESCs transfected with pre-miR-590 was faster than that of control mESCs. The DNA damage was significantly decreased in mESCs transfected with pre-miR-590 compared with control mESCs (Figures 1G and 1H). Trypan blue staining showed that there was no significant dying cell in mESCs after transfected with pre-miR-590 or 0/3 hr postirradiated by 8 Gy X-ray (Figures S1J and S1K). Thus, we concluded that miR-590 may regulate DNA damage repair directly in mESCs. Additionally, the miR-590 regulation of proliferation led us to investigate whether miR-590 connects the cell proliferation and DNA damage repair in mESCs.

**miR-590 Regulates the Expression of Rad51b and Cell Cycle-Related Genes**

To test whether cell cycle-related, SSB damage repair-related, and DSB damage repair-related genes can be regulated by miR-590, we performed quantitative RT-PCR (qRT-PCR) and western blot analyses examined 48 hr posttransfection, and we found that Rad51b was upregulated in mESCs transfected with pre-miR-590 but that Rad51c, Rad9, and Brc1 showed no significant change (Figure 2A). Additionally, the cell cycle-related genes, Cyclin E and Cyclin B, were downregulated while P21 and Rb1 were upregulated (Figure 2A). Western blot analysis was used to confirm the change in protein levels of the cell cycle-related genes and Rad51b (Figure 2B). In contrast, the miR-590-3p or miR-590-5p inhibitor downregulated the expression of Rad51b, p21, and Rb1 and upregulated the expression of Cyclin B and Cyclin E (Figure 2C). Protein levels of the cell cycle-related genes and Rad51b were detected to confirm the function of miR-590-3p or miR-590-5p inhibitors (Figure 2D). These results showed that miR-590 may mediate downstream genes to regulate DSB damage repair, SSB damage repair, as well as the cell proliferation.

**miR-590 Directly Targets Acvr2a to Regulate Activin Signaling in mESCs**

We used Targetscan and Miranda to predict the direct target of miRNAs and found that both miR-590-3p and miR-590-5p can target Acvr2a (Figure 3A). Then we found that Acvr2a was downregulated in mESCs cultured without LIF compared with mESCs cultured with LIF (Figure 3A), which was opposite to the upregulation of miR-590 –3p/5p in the mESCs cultured in the same system (Figure 1A). These results thus suggested a negative correlation between Acvr2a and miR-590-3p/5p during the differentiation of mESCs. Furthermore, we performed luciferase reporter assay to analyze whether miR-590-3p or miR-590-5p can target the 3’UTR of Acvr2a mRNA. We generated WT and mutant 3’UTR reporters of Acvr2a and found that both miR-590-3p and miR-590-5p inhibited the luciferase activity of the WT 3’UTR reporter but not the mutant reporters (Figure 3B). Furthermore, we detected miR-590 regulation on endogenous Acvr2a in mESCs. We transfected pre-miR-590 into mESCs and found that the expression of Acvr2a on both the mRNA and protein level was decreased (Figure 3C). To investigate whether miR-590 can influence Activin signaling, we detected the level of p-SMAD2 by western blot analysis and found that mESCs transfected with
pre-miR-590 had a lower level of p-SMAD2 than the control group (Figure 3D). Transfection of the miR-590-3p or miR-590-5p inhibitor increased the level of p-SMAD2 (Figure 3E). These results demonstrated that miR-590 inhibits the Activin signaling pathway by directly targeting Acvr2a.

The miR-590/Acvr2a Pathway Balances SSB and DSB Damage Repair with Rapid Proliferation of mESCs

We next tested whether the miR-590/Acvr2a pathway can regulate mESC proliferation and DNA damage repair. We constructed the Acvr2a overexpression vector (Figure S2A). Then we found that clone size of mESCs overexpressed Acvr2a was larger than control cells (Figure 4A). The amount of total cells was also more than control cells (Figure S2B). Both the FACS analysis of BrdU incorporation and MTS assay showed the increase of proliferation of mESCs overexpressed by Acvr2a (Figures 4B and 4C). FACS analysis of cell cycle showed that there was a significant increase in the proportion of cells in S phase and a reduction in the proportion of cells in G1 and G2/M phases in mESCs with downregulation of Acvr2a (Figure S2F). The SSB and DSB damage were also decreased in mESCs with knockdown of Acvr2a (Figures S2G and S2H).

We next tested whether miR-590 can regulate the DSB, SSB damage repair, and cell proliferation of mESCs by directly targeting Acvr2a. We performed a rescue experiment. The expression level of Acvr2a was detected by qPCR in rescue experiment. We first detected the Acvr2a expression level of the rescue experiment (Figure S3A) and found that the influence on clone size (Figure 4G), cell proliferation (Figures 4H, 4I, and S3B), and cell cycle (Figure S3C) of mESCs transfected with pre-miR-590 could be restored by overexpressing Acvr2a. We also showed that overexpression of Acvr2a in mESCs transfected with pre-miR-590 could restore the state of excessive SSB and DSB DNA damage (Figures 4 and 4K) to a similar level of control mESCs. The cell cycle-related genes and Rad51b were also restored by overexpressing Acvr2a in mESCs transfected with pre-miR-590 (Figure 4L). These results showed that miR-590 regulated the DNA damage repair and cell proliferation of mESCs through directly targeting Acvr2a.

Figure 2. Effects of miR-590 on Cell Cycle and DNA Damage Repair-Related Genes

(A) qRT-PCR verification of transcript levels of the cell cycle regulation and homologous recombination damage repair genes in mESCs transfected with pre-miR-590 or control. Data shown are means ± SD of three independent experiments (n = 3). *p < 0.05 and **p < 0.01.

(B) Western blot analysis for the cell cycle regulation and homologous recombination damage repair genes in mESCs transfected with pre-miR-590 or control.

(C) qRT-PCR verification of mRNA levels of the cell cycle regulation and homologous recombination damage repair genes in mESCs transfected with miR-590-3p/5p inhibitor or control. Data shown are means ± SD of three independent experiments (n = 3). *p < 0.05 and ***p < 0.001.

(D) Western blot analysis of the cell cycle regulation and homologous recombination damage repair genes in mESCs transfected with miR-590-3p/5p inhibitor or control.
Rad51b Promotes SSB and DSB Damage Repair in mESCs to Slow the Cell Proliferation

We found that Rad51b can be regulated by the miR-590/Acvr2a pathway. To determine whether Rad51b can inhibit the mES proliferation and repair SSB and DSB damage, we downregulated the expression of Rad51b in mESCs. The colonies of mESCs with knockdown of Rad51b were larger than control cells (Figure 5A). The total number of cells was more than control cells (Figure S4A). The proliferation was increased by downregulating Rad51b in mESCs detected by MTS assay and FACS analysis of BrdU incorporation (Figures 5B and 5C). We also found that knockdown of Rad51b resulted in a reduced fraction of cells in G1 and G2/M phases as well as an increased fraction of cells in S phase (Figure S4B). The SSB and DSB damage was significantly increased after knockdown of Rad51b (Figures 5D and 5E). In mESCs with Rad51b knockdown, the expression levels of Cyclin B and Cyclin E were upregulated, and the expression levels of P21 and Rb1 were downregulated (Figure S5F). These results showed that Rad51b can regulate both the DNA damage repair and cell proliferation in mESCs to balance the rapid proliferation and DNA damage repair.

Figure 3. miR-590 Directly Targets Acvr2a to Regulate Activin Signaling in mESCs

(A) Summary of miR-590-3p and miR-590-5p target sites in the 3'UTRs of Acvr2a. In the double strands of the sequence, the upper strand is miR-590-3p or miR-590-5p, and the lower strand is the binding sites of 3'UTR mRNA. The right panel shows the level of Acvr2a expression detected by qRT-PCR. Data shown are means ± SD of three independent experiments (n = 3). *p < 0.05, **p < 0.01, and ***p < 0.001.

(B) miR-590-3p and miR-590-5p specifically repress their targets in the luciferase assay. WT-UTR indicates WT 3'UTR. Mut-UTR indicates the 3'UTR containing the mutant binding site of miR-590. Data shown are means ± SD of three independent experiments (n = 3). ***p < 0.001.

(C) Detection of Acvr2a expression on mRNA and protein levels in mESCs. Data shown are means ± SD of three independent experiments (n = 3). *p < 0.05.

(D and E) The level of p-SMAD2 was detected by western blot analysis. Glyceraldehyde-3-phosphate dehydrogenase is as an internal control.
The miR-590/Acvr2a/Rad51b Pathway Balances the Rapid Proliferation and DNA Damage Repair of mESCs

To confirm whether Rad51b is the functional component of the miR-590/Acvr2a pathway, we performed rescue experiments. We downregulated the expression of Rad51b in mESCs transfected with pre-miR-590 and found that the size of clones (Figure 6A) and amount of total cells (Figure S5A) were restored compared with mESCs transfected only with pre-miR-590. MTS assay and BrdU incorporation assay showed the rapid proliferation of mESCs was restored by downregulating Rad51b compared with mESCs transfected only with pre-miR-590 (Figures 6B and 6C). FACS analysis of cell cycle showed that the fraction of cell cycle in mESCs with exogenous pre-miR-590 transfection was restored by knockdown of Rad51b (Figure S5B). The state of SSB and DSB damage in mESCs transfected with pre-miR-590 was also restored by knockdown of Rad51b (Figures 6D and 6E). The levels of cell cycle-related genes and Rad51b in the rescue group were similar to those in the control group (Figure 6F). These results showed that Rad51b is the downstream functional factor of miR-590 and regulates both DNA damage and cell proliferation.

Furthermore, we performed a rescue experiment to test the regulation of Rad51b by Acvr2a. We found that knockdown of Rad51b restored the size of clones and the amount of total cells of mESCs with Acvr2a knockdown (Figures 6G and S5C). Additionally, the capacity of proliferation (Figures 6H and 6I) and fraction of cell cycle (Figure S5D) in Acvr2a knockdown mESCs was also restored after downregulation of Rad51b.

We also found that downregulation of Rad51b restored the DSB and SSB damage in Acvr2a knockdown mESCs, which was similar to the control mESCs (Figures 6J and 6K). The expression levels of cell cycle-related genes and Rad51B were restored to similar level of the control group (Figure 6L). These results confirmed that Rad51b is a downstream functional factor of Acvr2a and that the miR-590/Acvr2a/Rad51b pathway balances the rapid proliferation with DSB and SSB damage repair in mESCs to maintain the self-renewal and genome integrity.

DISCUSSION

Rapid proliferation can maintain the self-renewal of ESCs, but it can also result in DNA damage that leads to catastrophic mutations affecting the differentiation of organisms and causing mutations to be passed to progeny (Savatier et al., 2002; Tichy and Stambrook, 2008). In response to DNA damage stress, cells generally slow or arrest cell cycle progression to repair or prevent the transmission of damage to daughter cells (Ishikawa et al., 2006). Cell cycle and DNA damage repair can be regulated during mESC differentiation. During differentiation, mESCs accumulate in G1 phase and exhibit a cell cycle lengthened from 8–10 hr to more than 16 hr (White and Dalton, 2005). Additionally, DNA damage, especially DSB damage, is decreased during differentiation (Turinneto et al., 2012). Thus, there should be a system balancing the paradox of rapid proliferation and DNA damage repair to guarantee the normal self-renewal, pluripotency, and genomic stabilization in ESCs (Tichy, 2011). Here, we found that miR-590 balances DNA damage repair and proliferation by affecting the expression of Rad51b in mESCs. Importantly, Activin signaling regulated by Acvr2a mediates the balancing regulation of miR-590 on Rad51b. Furthermore, the miR-590/Acvr2a/Rad51b axis regulates the balance of rapid proliferation and DNA damage repair in mESCs.

The DNA damage repair system involves a complex network in which miRNAs play important roles (Chen and Rajewsky, 2007; Song et al., 2011; Wan et al., 2011). For example, miR-24 and miR-138 can downregulate H2AX, leading to high sensitivity to radiation and weak repair capacity (Lal et al., 2009). miR-182 suppresses DSB damage repair by targeting Brcal (Moskwa et al., 2011). A previous study has also shown that miR-590 participates in the regulation of cell proliferation and death. miR-590-3p regulates neuronal death in patients with Alzheimer disease and frontotemporal lobar degeneration (Villa et al., 2011). In bladder cancer, miR-590-3p inhibits cell proliferation and migration (Mo et al., 2013). In acute myeloid leukemia, miR-590-5p can be regulated by interleukin-3 to affect the growth of cells (Favreau and Sathyanarayana, 2012). These studies suggest that miR-590 widely participates in cell growth of different cell types. However, the function of miR-590 in mESCs remains unknown. We found that overexpression of miR-590 significantly inhibited the proliferation by upregulating the expression of P21 and Rb1 as well as downregulating the expression of Cyclin E and Cyclin B, but the overexpression of miR-590 did not affect the pluripotency of mESCs. This result was similar to that of a previous study where no significant induction of differentiation of mESCs occurred with overexpression of P21 and Rb (Li et al., 2012). LIF signaling is important for mESCs to maintain rapid proliferation (Furue et al., 2005). In our study, we also found that during the process of culture withdrawing LIF, the proliferation rate of mESCs was slower, and miR-590-3p/Sp were upregulated, suggesting a possible important role of miR-590 during mESC proliferation regulation. Studies have shown that ESCs have robust DNA damage repair capacity, which includes nucleotide excision repair (Saretzki et al., 2004), mismatch repair (Saretzki et al., 2004), and DSB repair (Donoho et al., 1998; Smith et al., 1995). DSB damage is considered to be the most toxic type of DNA damage (Valerie and Povirk, 2003), which may be caused by the replication fork...
Figure 4. *miR-590* Regulates the SSB Damage Repair, DSB Damage Repair, and Proliferation of mESCs by *Acvr2a*

(A) Morphology of mESCs clones overexpressing *Acvr2a*. The scale bar represents 100 μm.

(B) Analysis of proliferation of mESCs overexpressed with *Acvr2a* by MTS cell proliferation assay. Data shown are means ± SD of five independent experiments (n = 5). ***p < 0.001.

(legend continued on next page)
collapsing during rapid and continuous proliferation in ESCs (Saretzki et al., 2004). We found that overexpression of miR-590 significantly decreased DNA damage of mESCs by detecting the state of SSB and DSB damage. Our results suggested that miR-590 might be the link between rapid proliferation and DNA damage repair in mESCs. Furthermore, we found that miR-590 can accelerate the process of DNA damage repair after irradiation by X-ray but that miR-590 does not weaken the radiation sensitivity. These results showed that miR-590 not only influences the proliferation and cell cycle to assist the process of damage repair but also participates in DNA damage repair, especially for SSB and DSB repair.

Activin signaling has been reported to be crucial in maintaining ESC pluripotency and proliferation (Beattie et al., 2005; Ogawa et al., 2007). Activin performs its function through Acvr2a, which is required for binding Activin to cause downstream p-SMAD2 activation, thus initiating Activin signaling. Activin signaling has also been reported to participate in the DNA damage response. A study has also shown that Activin signaling potentiates epithelial support cell proliferation by Acvr2a and Acvr2b. Blocking Activin-Acvr2a/b signaling inhibits epithelial support cell proliferation, whereas an Activin receptor agonist increases proliferation (McCullar et al., 2010). Specific knockout of Activin βA in fetal Leydig cells represses sertoli cell proliferation (Archambeault and Yao, 2010). In our study, we found that the level of Acvr2a expression can mediate the strength of Activin signaling and regulate the efficiency of DSB damage repair, SSB damage repair, and cell proliferation in mESCs. Overexpression of Acvr2a reduced the repair of DNA damage and promoted the proliferation at a faster rate. Knockdown of Acvr2a resulted in the opposite phenomenon. We further found that the level of Acvr2a was higher in mESCs cultured in medium with LIF, which contrasted the expression of miR-590. More importantly, we determined that Acvr2a is the target gene of miR-590 in mESCs. miR-590 regulates the amount of Acvr2a on cell membranes to regulate Activin signaling in mESCs, thereby balancing mESC proliferation and DNA damage repair.

There are two major repair methods for DSB damage repair, namely HRR and nonhomologous end joining (NHEJ). HRR results in error-free repair, but NHEJ sometimes leads to errors. During HRR, DSB damage repair uses a template containing hundreds of base pairs of sequence homology, and usually the sister chromatids are available to serve as templates (Haber, 2000; Morrison et al., 2000; Takata et al., 1998). The S phase of ESCs has a long duration, so most of the ESC genomes would have sister chromatids, which assures the process of HHR (Tichy and Stambrook, 2008). Rad51b has been reported to play an important role in the HRR process to repair DSB, and Rad51b is also a protein kinase that can regulate cell cycle-related genes (Havre et al., 2000; Shrivastav et al., 2008; Stordal and Davey, 2009). In our study, we found that Rad51b is also important for mESCs. Knockdown of Rad51b results in inefficient DNA damage repair and a faster cell cycle, which creates a vicious circle of producing more DSB and SSB damage. We further found that Rad51b

(C) Analysis of proliferation of mESCs overexpressed with Acvr2a by FACS analysis of BrdU incorporation. The figure means the percentage of the population of BrdU-positive cells. Data shown are means ± SD of three independent experiments (n = 3). ***p < 0.001.

(D) Comet assay for SSB damage in mESCs. The scale bar represents 100 μm. Right panel is the quantification of average DNA tail moment. Data shown are means ± SD of three independent experiments (n = 3). ***p < 0.001.

(E) Immunofluorescence analysis of γ-H2AX (red) to indicate the state of DSB damage in mESCs. Ho.33342 (Hoechst 33342) represents nuclear staining (blue). The scale bar represents 100 μm. (Bottom) The quantification of the fluorescence of γ-H2AX immunostaining. Data shown are means ± SD of three independent experiments (n = 3). **p < 0.01.

(F) qRT-PCR verification of transcript levels of the cell cycle regulation and homologous recombination damage repair genes in mESCs. The right side shows the protein levels according to western blot analysis. Data shown are means ± SD of three independent experiments (n = 3). *p < 0.05 and **p < 0.01.

(G) Morphology of mESCs clones. The scale bar represents 100 μm.

(H) Cell proliferation assay in the rescue experiment by MTS cell proliferation assay. Data shown are means ± SD of three independent experiments (n = 3). ***p < 0.001.

(I) Cell proliferation assay in the rescue experiment by FACS of BrdU incorporation. The figure means the percentage of the population of BrdU-positive cells. Data shown are means ± SD of three independent experiments (n = 3). **p < 0.01.

(J) Comet assay shows the SSB damage state. The scale bar represents 100 μm. The right panel is the quantification of average DNA tail moment. Data shown are means ± SD of three independent experiments (n = 3). ***p < 0.001.

(K) Overexpression of ACVR2a restores DSB damage in mESCs transfected with pre-miR-590. Immunofluorescence analysis of γ-H2AX (red) indicates the state of DSB damage in mESCs with Ho. 33342 (Hoechst 33342) staining for the nucleus (blue). The scale bar represents 100 μm. (Bottom) The quantification of the fluorescence of γ-H2AX immunostaining. Data shown are means ± SD of three independent experiments (n = 3). **p < 0.01.

(L) qRT-PCR and western blot analysis of the cell cycle- and homologous recombination damage repair-related genes in mESCs. Data shown are means ± SD of three independent experiments (n = 3). *p < 0.05 and **p < 0.01.
Figure 5. **Rad51b Promotes SSB and DSB Damage Repair in mESCs and Slows the Cell Proliferation**

(A) Morphology of mESC clones with knockdown of Rad51b compared with the control group. The scale bar represents 100 μm.

(B) Cell proliferation assay by MTS cell proliferation assay. Data shown are means ± SD of three independent experiments (n = 3). ***p < 0.001.

(C) Cell proliferation assay by FACS of BrdU incorporation. The figure means the percentage of the population of BrdU-positive cells in total cells. Figure shows the percentage of the population of BrdU-positive cells. Data shown are means ± SD of three independent experiments (n = 3). ***p < 0.001.

(D) Knockdown of Rad51b increases the SSB damage in mESCs as detected by the comet assay. The scale bar represents 100 μm. Bottom panel shows the quantification of average DNA tail moment. Data shown are means ± SD of three independent experiments (n = 3). **p < 0.01.

(E) Immunofluorescence analysis of γ-H2AX (red) indicates the state of DSB damage. γ-H2AX was increased by knockdown of Rad51b in mESCs. Ho.33342 (Hoechst 33342) represents nuclear staining (blue). The scale bar represents 100 μm. (Bottom) The quantification of the fluorescence of γ-H2AX immunostaining. Data shown are means ± SD of three independent experiments (n = 3). **p < 0.01.

(F) qRT-PCR and western blot indicating the levels of the cell cycle- and homologous recombination damage repair-related genes in mESCs. Data shown are means ± SD of three independent experiments (n = 3). *p < 0.05 and **p < 0.01.
Figure 6. Knockdown of Rad51b Restores the State of Proliferation and DNA Damage in mESCs Overexpressing Pre-miR-590 or Knockdown of Acvr2a

(A) Knockdown of Rad51b can restore the size of mESC clones influenced by miR-590. The scale bar represents 100 μm.

(B) Cell proliferation assay by MTS cell proliferation assay. Data shown are means ± SD of five independent experiments (n = 5). ***p < 0.001.

(legend continued on next page)
can be regulated by the miR-590-Acvr2a pathway. Rad51b can be upregulated by miR-590 or knockdown of Acvr2a in mESCs. Moreover, knockdown of Rad51b attenuates the effects caused by knockdown of Acvr2a or overexpression of miR-590. Our results suggested that miR-590/Acvr2a/Rad51b signaling axis balances the coexistence of DNA damage repair and rapid proliferation, assuring the stabilization of mESCs. Our study provides insights into the regulation of proliferation and DNA damage repair during mESC self-renewal, and it suggests that an ESC-related signaling pathway not only maintains the stabilization of mESC self-renewal or pluripotency but also may be the regulator or responder of DNA damage repair.

EXPERIMENTAL PROCEDURES

mESC Culture
mESCs (E14) were cultured on feeder-free, gelatin-coated plates with Dulbecco’s modified Eagle’s medium (Hyclone) supplemented with LIF, 15% fetal bovine serum ( Gibco), 1% non-essential amino acids (Invitrogen, Life Technologies), 1% Gln (Invitrogen), and 0.18% β-mercaptoethanol (Sigma). mESCs were cultured at 37°C in a humidified 5% CO2 atmosphere. 46C is another kind of mESC line that cultures in the same condition as E14.

Comet Assay
A comet assay was performed according to the comet assay kit (Cwbio). All other methods can be found in the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2014.10.006.

ACKNOWLEDGMENTS

This work was supported by grants obtained from the Ministry of Science and Technology (grant numbers 2011CB965100, 2013CB967600, 2011CBA01100, 2012CB966603, and 2013CB967401), the National Natural Science Foundation of China (grant numbers 91219305, 31210103905, 31371510, 31101061, 31171432, 81170499, 31201107, 31301208, and 81201599), the Science and Technology Commission of Shanghai Municipality (grant number 12ZR1450900), IRT1168 from Ministry of Education, sponsored by Shanghai Rising-Star Program (14QA1403900), the Specialized Research Fund for the Doctoral Program of Higher Education (20110072110039), support of the “Chen Guang” project from the Shanghai Municipal Education Commission and Shanghai Education Development Foundation (12CG19), and the Fundamental Research Funds for the Central Universities (2000219099).

Received: February 2, 2014
Revised: October 16, 2014
Accepted: October 16, 2014
Published: November 20, 2014

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