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Review

Review

Current understanding of genomic stability maintenance in pluripotent stem cells

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Abstract

Pluripotent stem cells (PSCs) are able to generate all cell types in the body and have wide applications in basic research and cell-based regenerative medicine. Maintaining stable genome in culture is the first priority for stem cell application in clinics. In addition, genomic instability in PSCs can cause developmental failure or abnormalities. Understanding how PSCs maintain genome stability is of critical importance. Due to their fundamental role in organism development, PSCs must maintain superior stable genome than differentiated cells. However, the underlying mechanisms are far from clear. Very limited studies suggest that PSCs utilize specific strategies and regulators to robustly improve genome stability. In this review, we summarize the current understandings of the unique properties of genome stability maintenance in PSCs.

Key words pluripotent stem cell, genomic stability, DNA damage response

Introduction

Pluripotent stem cells (PSCs) including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are able to undergo unlimited proliferation and differentiation into all cell types in the body. PSCs have been widely used in basic research in developmental biology, disease modeling, and drug screening. In particular, PSCs hold great promise in cell-based regenerative medicine [1].

Due to their fundamental functions in organism development, PSCs possess higher level of genome integrity than differentiated somatic cells. For example, the genome mutation rate in mouse ESCs is 100-time lower than in isogenic mouse embryonic fibroblasts (MEFs) [2]. The super stable genome is critical for stem cell identity maintenance. Its perturbation would not only impair the differentiation potential of PSCs, but also cause tumorigenicity, which is the biggest hurdle to the full applications of PSCs. Therefore, understanding how PSCs maintain super stable genome is of fundamental importance. However, the majority of studies on PSCs focused on pluripotency regulations. Much less attention has been paid to the genomic stability regulations of PSCs. Limited studies show that PSCs express higher levels of many genes involved in DNA damage response and repair [3]. Moreover, PSCs can employ unique strategies and regulators to efficiently maintain genome stability. In this review, we will summarize the current understanding regarding the specific properties and regulators of genome stability maintenance in PSCs. Regulations that are common to both PSCs and differentiated cells will not be discussed. It should also be mentioned that PSCs can be maintained *in vitro* in naïve, primed and formative states [4]. The different pluripotent states are related not only to distinct *in vivo* developmental potential, but also to different properties of genomic stability. For example, a recent study compared the genomic stability properties of mouse naïve and primed ESCs, and uncovered that primed ESCs have lower capacity to maintain genome stability manifested by reduced DNA recombination and repair, telomere lengthening as well as aberrant transposable elements activation [5]. In addition, human and mouse PSCs in the same pluripotent state (naïve state for instance) can be different in the regulation of genomic stability. Compared to the mouse naïve ESCs which have very stable genome, human naïve ESCs cultured in medium supplemented with MEK inhibitors showed overall lower level of genome stability [6]. Thus, to avoid the complexity of genomic stability regulation associated with different species and pluripotent states, we mainly focus on the studies of mouse naïve ESCs.

Here, we summarize the current knowledge on the specific strategies and regulators that ESCs utilize to (1) alleviate the accumulation of endogenous DNA damages, (2) repair the damaged DNA, (3) maintain the telomere homeostasis, and (4) rapidly eliminate cell populations with un-repairable DNA damages (Table 1). We also describe the developmental defects caused by the dysfunction

Table 1. ESC-specific strategies in genome stability maintenance

Regulatory layers	Unique strategies in ESCs	ESC-specific regulators
Endogenous DNA damage prevention	Elevated DNA replication stress responses	Filia, Floped, *Discn*
	Glycolysis to reduce oxidative DNA damage	
DNA damage repair	Strengthened DNA damage responses	Filia, Sall4
	HR repair preference	
Telomere maintenance	HR-mediated alternative telomere lengthening	Zscan4, Dcaf11
	TRF2-independent telomere protection	
Elimination of damaged cells	High mitochondria priming	*Lnc956*
	Constitutive Bax activation	
	Alternative p53 function	
	Sequestration of KLF4	

of the specific regulators.

DNA Replication Stress Responses in PSCs

During DNA replication, the replication forks are frequently slowed or stopped by various physical obstacles including high-order DNA structures, DNA-protein complex, DNA-RNA complex, or DNA damages, leading to DNA replication stress. If stalled forks are not properly repaired, they could be converted to DNA double strand breaks (DSBs), which can further induce chromosome translocation or nonrecurrent copy number variations (CNVs) [7]. Thus, DNA replication stress represents a major source of endogenous DNA damage and genome instability [8]. To prevent the replication-associated DNA damage, cells develop a conserved replication stress responses which elicit cascades of local and global reactions via ATR-ChK1-dependent and ATR-ChK1-independent pathways to repair stalled forks and preserve genomic stability [9].

With mouse ESCs as models, previous studies showed that compared to differentiated somatic cells, ESCs encounter higher replication stress predominantly due to frequent DNA replication, especially the short of G1 phase [10]. However, ESCs are able to efficiently resolve replication stress [11]. Several unique strategies have recently been uncovered to play important parts in promoting replication stress responses in ESCs. Isolation proteins on nascent DNA (iPOND) combined with mass-spectrometry analysis identified an ESC-specific protein complex (Filia-Floped complex) on replication forks. This protein complex is recruited by ATR signaling onto stalled forks to form functional scaffold, which then regulates two independent downstream events. On one hand, the scaffold enhances ATR activation by an unknown mechanism. On the other hand, it recruits Bloom syndrome (BLM) protein, a key regulator of fork reversal and restart [12], onto stalled replication forks to facilitate fork restart [11]. Ectopic expression of the Filia and Floped proteins in differentiated cells can significantly improve the ability to resolve replication stress [11]. Besides proteins, long non-coding RNAs (lncRNAs) can also reside on replication forks of ESCs to promote the stalled replication fork restart and ensure stem cell genomic stability (bioRxiv doi: https://doi.org/10.1101/2022.03.13.484185). LncRNA NONMMUT028956 (*Lnc956* for short) is predominantly expressed in mESCs and is recruited to stalled replication forks where it drives the assembly of a ribonucleoprotein (RNP) complex comprising of *Lnc956*-TRIM28-HSP90B1. This RNP complex physically associates with MCM2-7 hexamer and directly regulates the CMG helicase retention on chromatin to promote fork restart (bioRxiv doi: https://doi.org/10.1101/2022.03.13.484185).

In addition to the fork-resided local players, a mouse ESC-specific lncRNA *Discn* (DNA damage-induced stem cell specific noncoding RNA) functions outside of stalling forks to regulate replication stress response by targeting replication protein A (RPA) complex [13], which is a master regulator of DNA metabolism and plays crucial roles in DNA replication, recombination and repair [14]. During DNA replication stress, single-stranded DNA (ssDNA) is persistently generated and rapidly coated by RPA. RPA binding to ssDNA not only protects the naked ssDNA from nucleolytic degradation and prevents the secondary structure formation in ssDNA, but also serves as a platform to launch downstream events including the ATR activation. RPA haploinsufficiency, depletion, or exhaustion can result in the conversion of ssDNA into DNA breaks, leading to replication catastrophe characterized by massive generation of DNA DSBs. Thus, the RPA availability plays a central role in DNA replication stress response and acts as a common denominator in avoiding replication catastrophe [15,16]. Cells usually express more RPA than required under the normal conditions. However, excessive ssDNA induced by replication stress can rapidly exhaust RPA reservoir [16]. Efficient sustaining the free RPA pool is therefore critical to survive the replication stress. To this end, ESCs express higher level of RPA proteins than differentiated somatic cells [13]. In addition, ESCs robustly evoke the expression of *Discn*, which can preserve the free RPA reservoir to protect genomic stability in response to genotoxic stress [13]. *Discn* localizes in nucleolus and interacts with neucleolin (NCL), which is a negative regulator of the free RPA pool. *Discn*-NCL association prevents NCL from translocation into nucleoplasm and avoids undesirable NCL-mediated RPA sequestration. Of interest, the *Discn* expression is adjusted by the strength of stress in stem cells, thereby achieving fine-tuned regulation [13]. These limited studies suggested that PSCs could promote the efficiency of DNA replication stress response at different regulatory layers.

Energy Metabolism in PSCs

Reactive oxygen species (ROS) is the by-product of energy production through oxidative phosphorylation, and can cause DNA oxida-

tive lesions [17]. Compared to differentiated cells, PSCs produce less ROS by utilizing glycolysis over oxidative phosphorylation to generate ATP [18]. Concordantly, mitochondria in ESCs are immature, having a globular shape and poorly developed cristae [19]. Several mechanisms are implicated in glycolysis regulation in ESCs, including the decouple of glycolysis from oxidative phosphorylation by UCP2 in human ESCs [20], higher expression of glycolytic enzyme hexokinase II and lower expression of pyruvate dehydrogenase [21]. In addition, a recent study reported that the glycolysis and oxidative damages in mouse ESCs are minimized by Cops5, a COP9 signalosome subunit, to ensure genomic stability [22].

DNA Damage Response and Repair in PSCs

Different types of DNA damages require distinct repair pathways. Among all types of DNA damage, DSB is most deleterious and threatens the viability of a cell if not properly repaired. Upon DSBs, many proteins are recruited to the DSB sites, and the central kinase ATM is activated, which then phosphorylates numerous downstream effectors to generate the signaling cascades. These signals initiate multiple DNA damage responses (DDR) including cell cycle arrest and DNA damage repair processes [23]. Compared to differentiated cells, PSCs display higher efficiency of DDR in which PSC-specific regulators are involved [24,25]. Sall4, which regulates the ESC stemness, is recruited to DSB sites where it interacts with RAD50 and stabilizes the Mre11-Rad50-Nbs1 (MRN) complex to enhance the ATM activation in mouse ESCs [26]. PARP-catalyzed PARylation promotes many DDR processes including ATM activation, DSB repair pathway selection, and repair efficiency [27]. Upon DNA DSBs, ESCs display much higher PAR level than differentiated cells. A mouse ESC-specific protein Filia can physically interact with PARP1 and stimulate PARP1 enzymatic activity, thereby robustly promoting DDR and repair efficiency [28]. The roles of Filia in DDR are conserved between mouse and human. Its human ortholog KHDC3L similarly binds to PARP1 and stimulates PARP1 activation [29].

DNA DSBs can be repaired by several pathways including homologous recombination (HR) pathway, classic non-homologous end joining (cNHEJ) pathway, and microhomology-mediated end joining (MMEJ; also called alternative end joining, altEJ) pathway. HR pathway has high fidelity but low efficiency, whereas the end joining pathways are mutagenic. In particular, MMEJ pathway is the most mutagenic and prone to generating insertions or deletions at the sites of repair [30]. There are two sequential key events which determine the choice of DSB repair pathway. The first one is the initiation of DSB end resection, which blocks the cNHEJ pathway but commits the break to repair via HR or MMEJ pathway. Both HR and MMEJ pathways share the step of initial end resection of DSBs, this makes them potentially in competition. Data have shown that the DNA polymerase theta (Polθ) which is essential for MMEJ repair can directly interact with RAD51 to suppress HR repair [31]. Inhibition of Polθ expression significantly increases HR [32]. Thus, Polθ directly determines the repair pathway choice between HR and MMEJ after the end resection is initiated. Compared to differentiated somatic cells, mouse ESCs prefer to employ HR pathway to repair DNA DSBs [33]. How the ESCs prefer to initiate the end resection of DSBs is currently unknown. However, our unpublished work showed that mouse ESCs substantially suppressed the Polθ expression via alternative splicing (AS) of *Polq* (gene encoding Polθ) transcripts. ESCs predominantly express a longer *Polq* isoform in which a cryptic exon is included between exons 3 and 4. The presence of this cryptic extra exon generates a premature stop codon which blocks the translation of functional Polθ protein. Intriguingly, an ESC-specific RNA binding protein Dppa5 is involved in regulating the *Polq* AS event. Depletion of Dppa5 in ESCs leads to somatic cell-type *Polq* AS pattern. Conversely, ectopic expression of Dppa5 in NIH3T3 cells induces stem cell-type *Polq* AS pattern. Concordantly, depletion of Dppa5 significantly reduces the HR repair in ESCs.

Telomere Maintenance in ESCs

Telomeres of the chromosomes have specific structure and are difficult to replicate [34]. In differentiated cells, telomeres are replicated via telomerase, which adds nucleotides at a relatively slow pace (50–150 bp per cell cycle) [35]. The slow pace of telomere lengthening via telomerase may not fit the fast rates of proliferation and DNA replication in ESCs. Indeed, the telomerase plays a minor role in telomere lengthening in ESCs, and a telomerase-independent mechanism known as alternative lengthening of telomeres (ALT) is operated to lengthen the telomere [36]. ALT relies on telomere recombination, which is more robust and requires the participation of ESC-specific protein Zscan4. Zscan4 locates at telomere to regulate the event of telomere sister chromatid exchange (T-SCE). Concordantly, mouse ESCs depleted of telomerase activity can proliferate for more than 450 population doublings [37], whereas ESCs depleted of Zscan4 suffer from rapid telomere attrition, genomic instability and culture crisis [36]. Although Zscan4 is essential for telomere elongation in ESCs, it is transiently expressed and the persistent expression is harmful to ESCs [36]. Therefore, ESCs need to precisely control the expression of Zscan4 in order to maintain telomere homeostasis. *Zscan4* gene is located at the subtelomeric heterochromatin region where histone undergoes suppressive H3K9 methylation. Factors involved in modulating the H3K9 methylation in this region can regulate Zscan4 expression. Two such factors, Rif1 and Dcaf11, have been identified to modulate H3K9 methylation and Zscan4 expression. Rif1 is essential to prevent the persistent expression of Zscan4 in mouse ESCs [38]. At telomere and subtelomeric heterochromatin regions, Rif1 interacts with and stabilizes the H3K9 methylation machinery to facilitate epigenetic silencing. Removal of Rif1 de-represses the expressions of genes located in subtelomeric regions including Zscan4. The elevated expression of Zscan4 in turn promotes the telomere recombination and compromises telomere length homeostasis. On the contrary, Dcaf11 acts as an activator of Zscan4 expression. It is specifically expressed in ESCs and modulates the H3K9 modification at distal enhancer of *Zscan4* gene. As an E3 ligase substrate receptor, Dcaf11 interacts with KAP1 (also known as TRIM28), an essential regulator of H3K9 modification, and recruits the ubiquitylation machinery to degrade KAP1. By this means, Dcaf11 erases the H3K9 modification and activates the Zscan4 expression [39].

Telomere is protected by protein complex called shelterin complex, which binds with telomeric repeats and prevents end-to-end chromosome fusions [40]. TRF2 is a core component of shelterin complex and plays an essential role in protecting telomere by promoting the formation of the t-loop structure [41,42]. t-loop structure sequesters the telomeric ends and prevents the ATM activation as well as the NHEJ-mediated end-end fusion. The indispensable role of TRF2 in t-loop formation and telomere protection has been validated in more than 700 cell lines tested so far (https://depmap. org) [43].

However, two recent studies revealed that TRF2 is not necessary for telomere protection in mouse ESCs [43,44] . In TRF2-depleted ESCs, the t-loop structure is normally formed, indicating that ESCs utilize distinct mechanism to regulate the t-loop formation [43]. Intriguingly, the expressions of Zscan4 family members and other totipotent state-associated factors are increased and the telomeres are lengthened by these factors in TRF2-depleted ESCs [43]. The telomeric lengthening by homology recombination may provide alternative mechanism to protect telomere in the absence of TRF2 in ESCs. Future efforts are required to elucidate the mechanism of t-loop formation in ESCs.

Cell Fate Determination after DNA Damages in PSCs

In DDR, when the DNA damage is too heavy to be repaired, cells initiate apoptosis or senescence to prevent the mutations from passing to descendent cells. This genome quality control mechanism is conserved in somatic cells as well as in PSCs. However, compared to differentiated somatic cells, PSCs show particularly higher sensitivity to DNA damage-induced apoptosis in order to protect the organism from propagating harmful mutations at the earliest stage of embryonic development [45]. Several unique mechanisms are operated in PSCs to ensure superior DNA damage sensitivity. For example, PSCs express lower level of the anti-apoptotic protein Bcl-2 and higher level of the proapoptotic protein PUMA, which enables the PSCs having intrinsically lower threshold for apoptosis (also called high mitochondria priming) [45]. In addition, pro-apoptotic factor Bax is differentially regulated in PSCs when compared to that in differentiated cells. In differentiated somatic cells, Bax remains inactive and resides in the cytosol until stress stimuli trigger its activation and translocation to the mitochondrial outer membrane. In contrast, Bax is constitutively activated and localizes on the trans-Golgi networks in PSCs. Following DNA damage, active Bax rapidly relocates to the mitochondrion in a p53-dependent fashion, thereby generating a robust apoptotic response [46].

Except for undergoing apoptosis, PSCs also initiate differentiation to eliminate the cell populations with unrepaired DNA damage [47]. The well-known factor responsible for genome quality surveillance is the tumor suppressor protein p53, which plays distinct roles in PSCs compared to that in differentiated cells [48]. In mouse ESCs, p53 acts as a transcription factor to directly activate and repress the expressions of more than 3600 genes (about 55% are activated and 45% are repressed) after activation by ATM in response to DNA damage. Genes associated with ESC differentiation are activated by p53, whereas most of the ESC core transcription factors (e.g. Oct4, Nanog, Sox2, Zic3, Jmjd1c, Esrrb, Tcfcp2l1, Utf1, n-Myc) are suppressed by p53 [49]. Thus, p53 controls the ESC differentiation in response to DNA damage through the dual functions.

Depletion of p53 does not completely block the cell death or differentiation of stem cells, suggesting that other p53-independent mechanism(s) might operate to determine the cell fate. Our recent study identified a novel p53-independent quality control pathway mediated by *Lnc956* in mouse ESCs. *Lnc956* acts in parallel with p53 to regulate the differentiation of ESCs after DNA damage. Mechanistically, damage-driven ATM signaling phosphorylates and activates RNA methyltransferase METTL3, which induces m^6A methylation of *Lnc956*. This modification promotes the interaction of *Lnc956* with KLF4, which in turn sequestrates the KLF4 protein and prevents KLF4's transcriptional regulation on pluripotency. This post-translational mechanism favors the rapid shut-down of the regulatory circuitry of pluripotency (unpublished data). Thus, ATM in ESCs activates two parallel pathways mediated by p53 and *Lnc956*, respectively. These two pathways act in concert to ensure robust differentiation and apoptosis in response to unrepaired DNA damage.

Developmental Defects Associated with Genomic Instability of Stem Cells

Pluripotent stem cells exist *in vivo* as epiblast cells in peri-implantation embryos. Epiblast cells differentiate into all three germ layers and act as building blocks for organism development. Previous studies have provided a line of evidence supporting that DNA damage and genomic instability in epiblast compromise the quality and quantity of epiblast cells, potentially leading to the impairment of embryogenesis. For example, low dose of ionizing radiation of mouse embryos at gastrulation stage (embryonic day (E) 6.5–7.5) induce apoptosis of epiblast cells and embryonic lethality [50]. Depletion of ESC-specific regulators of genomic stability could also compromise embryogenesis. Our previous work showed that germline depletion of Filia, which is prevalently expressed in epiblast cells, can cause embryonic death at post-implantation stage in mice [11]. Similarly, genetic mutations of its human ortholog KHDC3L, which impair the regulatory functions of KHDC3L on ESC genomic stability, were reproducibly detected in female patients with recurrent pregnancy loss [29]. Knockout of lncRNA *Lnc956* in mice also caused embryonic lethality (bioRxiv doi: https://doi.org/10.1101/2022.03.13.484185). It is intriguing to note that knockout of *Discn* in mice did not cause embryo death [13]. The distinct outcomes could be due to the different extent of the resulting DNA damages.

We observed that ESC-prevalent genomic stability regulators are often highly expressed in neural stem/progenitor cells (NSPCs) and play critical role in neurogenesis by safeguarding NSPC's genomic stability [13,51]. Their dysfunctions induce DNA damages in NSPCs, which in turn impairs the stem cell proliferation and screws the stem cell differentiation. Moreover, cytoplasmic micronuclei are often generated along with DNA DSBs. Accumulation of cytosolic DNA fragments can further evoke severe inflammatory reactions by the cGAS-STING pathway. These effects can together generate damaging outcomes in neurogenesis and brain functions, and even threaten life [13]. For example, Filia is specifically expressed in mouse hippocampal NSPCs. It maintains genomic stability of hippocampal NSPCs by preventing replication-associated DSBs and promoting HR-mediated DSB repair. Filia loss impairs the hippocampal neurogenesis and functions [51]. Similarly, lncRNA *Discn* is highly expressed in NSPCs and ensures their genome integrity. *Discn* knockout leads to newborn death with high penetrance due to severe inflammation induced by DNA damage in the brain. Survived adults display abnormal brain functions characterized by impaired emotions, learning, spatial memory, and sport performance [13]. In the future, identifying more regulators of ESC-specific genomic stability could provide new clues to understand the etiology of developmental failure or diseases.

Perspectives

Genomic integrity is crucial for stem cells to maintain the self-renewal and differentiation abilities. Genomic instability represents the number one obstacle that impedes the clinical applications of ESCs in regenerative medicine. Moreover, genomic instability of stem cells often induces developmental abnormalities or failure. Understanding the relative mechanisms is in urgent need to develop strategies to

overcome this hurdle. Future studies are required to clarify the following key questions in this area: (1) what are the mutation hotspots in ESCs? (2) how does the genomic instability affect stem cell self-renewal and differentiation, and induce tumorigenesis? (3) what are the PSC-specific regulatory networks of genomic stability? and (4) how to modulate the regulatory networks in order to improve the genomic stability of PSCs under long-term culture?

Conflict of Interest

The authors declare that they have no conflict of interest.

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