OXFORD GENETICS

GENETICS, 2021, 218(2), iyab054
DOI: 10.1093/genetics/iyab054
Advance Access Publication Date: 1 April 2021
Investigation

Double-strand breaks induce short-scale DNA replication and damage amplification in the fully grown mouse oocytes

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Abstract

Break-induced replication (BIR) is essential for the repair of DNA double-strand breaks (DSBs) with single ends. DSBs-induced microhomology-mediated BIR (mmBIR) and template-switching can increase the risk of complex genome rearrangement. In addition, DSBs can also induce the multi-invasion-mediated DSB amplification. The mmBIR-induced genomic rearrangement has been identified in cancer cells and patients with rare diseases. However, when and how mmBIR is initiated have not been fully and deeply studied. Furthermore, it is not well understood about the conditions for initiation of multi-invasion-mediated DSB amplification. In the G2 phase oocyte of mouse, we identified a type of short-scale BIR (ssBIR) using the DNA replication indicator 5-ethynyl-2′-deoxyuridine (EdU). These ssBIRs could only be induced in the fully grown oocytes but not the growing oocytes. If the DSB oocytes were treated with Rad51 or Chek1/2 inhibitors, both EdU signals and DSB marker γH2A.X foci would decrease. In addition, the DNA polymerase inhibitor Aphidicolin could inhibit the ssBIR and another inhibitor ddATP could reduce the number of γH2A.X foci in the DSB oocytes. In conclusion, our results showed that DNA DSBs in the fully grown oocytes can initiate ssBIR and be amplified by Rad51 or DNA replication.

Keywords: break-induced replication; oocyte; Rad51; multi-invasion

Introduction

Nuclear DNA double-strand breaks (DSBs) could be repaired by homologous recombination (HR), nonhomologous end joining (NHEJ), single-strand annealing, or theta-mediated end joining (Ceccaldi *et al.* 2016; Schimmel *et al.* 2019). For the HR repair of DSBs, if the resected DNA end invaded into the allelic sequence, the repair process would be mediated by the synthesis-dependent strand annealing (SDSA) (Miura *et al.* 2012) or double-holiday junctions (dHJ) (Bzymek *et al.* 2010). If the resected DNA end invaded into a nonallelic sequence, it might initiate the nonallelic HR pathway and produce copy number variant (CNV) (Inoue and Lupski 2002; Gu *et al.* 2008; Liu *et al.* 2012). If the resected DNA end had invaded into a homology or a nonallelic sequence, but the new synthesized DNA single strand could not form dHJ structure or reanneal with the other broken end, it might induce break-induced replication (BIR) (Malkova and Ira 2013; Kramara *et al.* 2018).

In yeast, BIR is promoted by the structure-specific endonucleases (SSEs) such as Mus81 and Yen1 (Pardo and Aguilera 2012). SSEs-mediated DNA incisions at the displacement-loop not only promote the formation of chromosomal nonreciprocal translocations but also initiate the more error-prone replication template switching (Lee *et al.* 2007; Hastings *et al.* 2009; Pardo and Aguilera 2012; Anand *et al.* 2014; Li *et al.* 2020), which will increase the risk of complex genomic rearrangement (CGR) (Zhang *et al.* 2013; Kramara *et al.* 2018; Pellestor and Gatinois 2018). The BIR-induced template switching is promoted by the deficiency of DNA helicases such as Pif1 or Mph1 (Stafa *et al.* 2014; Sakofsky *et al.* 2015), indicating that template switching may be caused by the collapse of replication fork. Multiple rounds of Fork Stalling and Template Switching (FoSTeS) would create CGRs in genome (Lee *et al.* 2007; Li *et al.* 2020). During the template switching, new single-strand DNA end ejected from the collapsed BIR replication fork could initiate a new round of strand invasion and BIR (Smith *et al.* 2007). Sequencing results of CGRs showed that the new DNA fragments synthesized by FoSTeS are linked by microhomology sequence, which indicates that the template switching-associated BIR is a microhomology-mediated BIR (mmBIR) (Lee *et al.* 2007; Zhang *et al.* 2009). In human, FoSTeS and mmBIR-mediated CGRs could be formed in both cancer cells (Li *et al.* 2020) and germline cells (Liu *et al.* 2017). Genomic data of human family trios indicated that the specific germline CGRs might be formed at the peri-zygotic period and mediated by mmBIRs (Liu *et al.* 2017). However, it has not been widely studied about the mechanisms of how mmBIRs are formed in the peri-zygotic cells, such as oocytes, spermatocytes, and/or early embryos.

During the homology-mediated strand invasion, it has been reported that the end of the single-strand DNA is not necessary

Received: February 05, 2021. **Accepted:** March 26, 2021

for the strand exchange (Wright and Heyer 2014; Piazza *et al.* 2017, 2021; Piazza and Heyer 2018). So the large 3' overhang generated by DSB end resection might invade into multiple template DNA and form a multi-invasion joint molecule (Piazza and Heyer 2019). Notably, if the multi-invasion joint molecules are incised by SSEs, they will produce additional DSB ends and amplify the original DSB damage (Piazza and Heyer 2019). In humans, germline *de novo* mutations (DNMs) tend to gather in a cluster. These clustered DNMs had been proposed to be induced by the single DSB event in oocyte (Goldmann *et al.* 2018). Although the DNMs detected in human germlines are mostly single nucleotide variants (Goldmann *et al.* 2018), it is reasonable to deduce that DSBs in oocytes could amplify DNA damage (DSBs or nonDSBs) and produce clustered DNMs. However, it's still not well-known if and how DNA damage is amplified in oocytes.

Mammalian oocytes finish the meiotic HR at the fetal stage and arrest at the G2-like dictyate stage (it is hereafter referred as G2) before or after birth (MacLennan *et al.* 2015; Dalbies-Tran *et al.* 2020). The G2 arrest of oocytes will be maintained for weeks or even tens of years according to different species. When the females are sexually mature, their arrested oocytes will be activated for growing and maturation. For mouse, oocyte needs about two weeks for its growing, maturation, and accumulating materials for subsequent embryo development (Gosden *et al.* 1997; Li *et al.* 2010). When mouse oocytes are fully grown, their transcription activities will be silenced. Meantime their DNA will be condensed and form a ring-like Hoechst-positive staining structure surrounding the nucleolus (SN) (Dumdie *et al.* 2018). So these fully grown oocytes are termed as SN oocytes whereas the growing oocytes are termed as non-SN (NSN) oocytes (Tan *et al.* 2009). Compared with the fully grown SN oocytes, NSN oocytes are incompetent for *in vitro* development. Although about 20% of mouse NSN oocytes could be meiotic matured (Bellone *et al.* 2009), they could not bypass the 2-cell block (Goddard and Pratt 1983) or develop to the blastocyst stage after fertilized with sperms (Zuccotti *et al.* 1998; Bellone *et al.* 2009). However, NSN oocytes *in vivo* might be developmental competent. They are just smaller in size and can gradually grow to the SN stage (Xiao *et al.* 2015) to become developmental competent, as long as their corresponding follicles would not go to atresia. During the development of oocytes from NSN to SN stage, there is an intermediate stage between the NSN and SN (NSN-SN) (Xiao *et al.* 2015). These NSN-SN stage oocytes have less condensed chromatin structure and incomplete ring-like Hoechst-positive staining structure surrounding the nucleolus (Supplementary Figure S1).

For both NSN and SN oocytes, DSBs can be induced in their nuclear DNA by many factors, including endogenous factors (such as reactive oxygen species and aging) (Subramanian *et al.* 2020) and exogenous factors (such as chemotherapeutic drugs and radiotherapy treatments) (Carroll and Marangos 2013; Tubbs and Nussenzweig 2017; Winship *et al.* 2018; Stringer *et al.* 2020). Genomic data of family trios have also indicated that DSBs can form in oocytes and these DSBs might induce both single nucleotide variants and structural variants (Duyzend *et al.* 2016; Liu *et al.* 2017; Goldmann *et al.* 2018). However, it has not been fully analyzed about whether DSBs could be the contributing factor of CGR in the growing NSN and fully grown SN oocytes. Our previous works had shown that exogenous DSBs could induce the chromatin to be entangled and matted together by Rad51 in the SN oocytes (Ma *et al.* 2019a). In this study, we further investigated the features of DSB repair in mouse oocytes, which would be new clues of how CGRs are formed in germ cells and somatic cells.

Experimental procedures

Oocyte isolation and *in vitro* culture

All of the animal experiments in this study were approved by the ethics committee of Guangdong Second Provincial General Hospital. Eight to twelve weeks old ICR mice were used for oocyte collection. The large antral follicular oocytes were released directly from the ovaries. To distinguish the NSN, SN, and shifting NSN-SN oocytes, we preformed the immunofluorescence labeling firstly, and then determined the oocyte stage by observing the Hoechst staining as described in the Introduction part. To block the oocytes from meiotic resumption, all of the manipulations were in the M2 medium (Sigma, M7167) with 2.5 μM Milrinone (MCE, HY-14252).

Treatment of oocytes by molecule compounds

DSBs in oocytes were introduced with Bleomycin at different concentrations (0.1, 0.5, 1, or 10 μM) for 1 hour. Oocytes were treated with 100 μM Rad51 inhibitor IBR2 (MCE, HY-103710) to inhibit the Rad51 activity, 100 nM Chek1/2 inhibitor AZD7762 (MCE, HY-10992) to inhibit the DNA damage checkpoint, 2 μM Aphidicolin (MCE, HY-N6733) to inhibit the nuclear DNA polymerase activity, and 100 μM ddATP (Apexbio, B8136) to delay the nuclear DNA replication. The control oocytes were treated with DMSO.

Immunofluorescence labeling

To label the endogenous proteins, oocytes were fixed with 4% Paraformaldehyde Fix Solution (Sangon, E672002) at room temperature (RT) for 15–30 minutes. Then in all the following steps, the solutions were made up with PBST (0.1% Tween-20 in PBS). After fixation, oocytes were treated with 0.3% Triton X-100 at RT for 20 minutes. To unmask the antigen epitopes of specific endogenous proteins (Rad51 in this study), oocytes were treated with Quick Antigen Retrieval Solution for Frozen Sections (Beyotime, P0090) at RT for 40 minutes. Then oocytes were washed three times with PBST and blocked with 1% BSA at RT for 1 hour. After that, oocytes were incubated with primary antibodies at 4 °C overnight. After washed 5 times with PBST, oocytes were incubated with appropriate secondary antibodies at RT for 2–3 hours. Then oocytes were stained with Hoechst for 1 hour and observed by the Andor live-cell station system. The primary antibodies were: anti-Rad51 (Abcam, ab133534; and Zen Bioscience, 200514), anti-Mitofilin (Proteintech, 10179-1-AP), and anti-γH2A.X (Bioworld, BS4760). The γH2A.X foci numbers and volumes in oocytes were measured or counted by the Fiji software (https://imagej.nih.gov).

EdU labeling

To label the new synthesized DNA, the oocytes were cultured in M2 medium with 10 μM 5-ethynyl-2'-deoxyuridine (EdU) (beyotime, ST067). Then oocytes were fixed with 4% Paraformaldehyde for 15 minutes and permeated with 0.3% Triton X-100 for 15 minutes. After incubation with primary and secondary antibodies, oocytes were treated with the click reaction buffer (beyotime, C0071S) at RT for 1 hour. Then oocytes were washed with PBST for 5 times and stained with Hoechst before observation. To compare the EdU signal sizes, we measured the max length of EdU signals with the Fiji software.

Statistic methods

Students' *T*-test was used for hypothesis test. *P*-value < 0.01 was recognized as very significant and marked with **; *P*-value <0.05 and

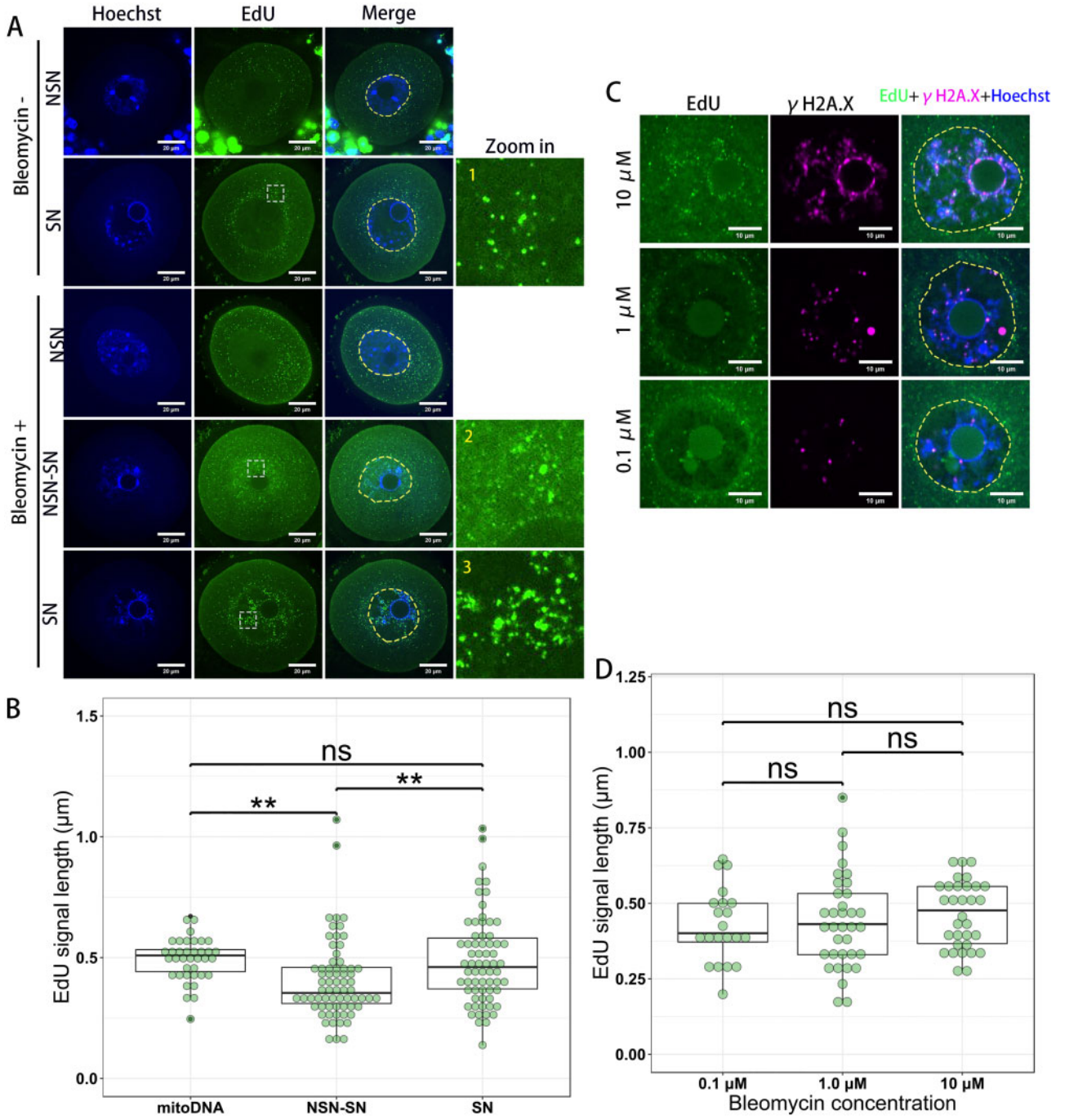

Figure 1 DNA DSBs induce the short-scale DNA replication in the NSN-SN and SN stage oocytes but not NSN oocytes. (A) Oocytes are treated with or without 10 μM Bleomycin for 1 hour and then released from Bleomycin for 15 hours. EdU signals (green) are detected in the Bleomycin-treated NSN-SN oocytes and SN oocytes. Bar = 20 μm. (B) The EdU signal sizes in the SN stage oocytes are larger than those in the NSN-SN oocytes, but are not a significant difference to mtDNA replication. (C) The γH2A.X foci (purple) can be observed beside to or overlapped with the EdU signals. Bar = 10 μm. (D) The EdU sizes induced by different Bleomycin doses have no significant difference. **Represents $P < 0.01$ and ns represents no significance.

≥0.01 were recognized as significant and marked with *. *P*-value ≥0.05 was recognized as not significant and marked with "ns."

Data availability

The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and supplemental files. Supplemental Material available at figshare: https://doi.org/10.25386/genetics.14339222.

Results

DSBs induce short-scale DNA replication in the SN oocytes

To determine whether DSBs could induce DNA replication in the growing and fully grown oocytes, EdU was used to monitor DNA replication and Bleomycin was used to induce DSBs. After treatment of 10 μM Bleomycin for 1 hour, oocytes were recovered in the Bleomycin-free media for 15 hours. After click reaction (Hein *et al.* 2008), EdU signals were found in the nuclei of NSN-SN and

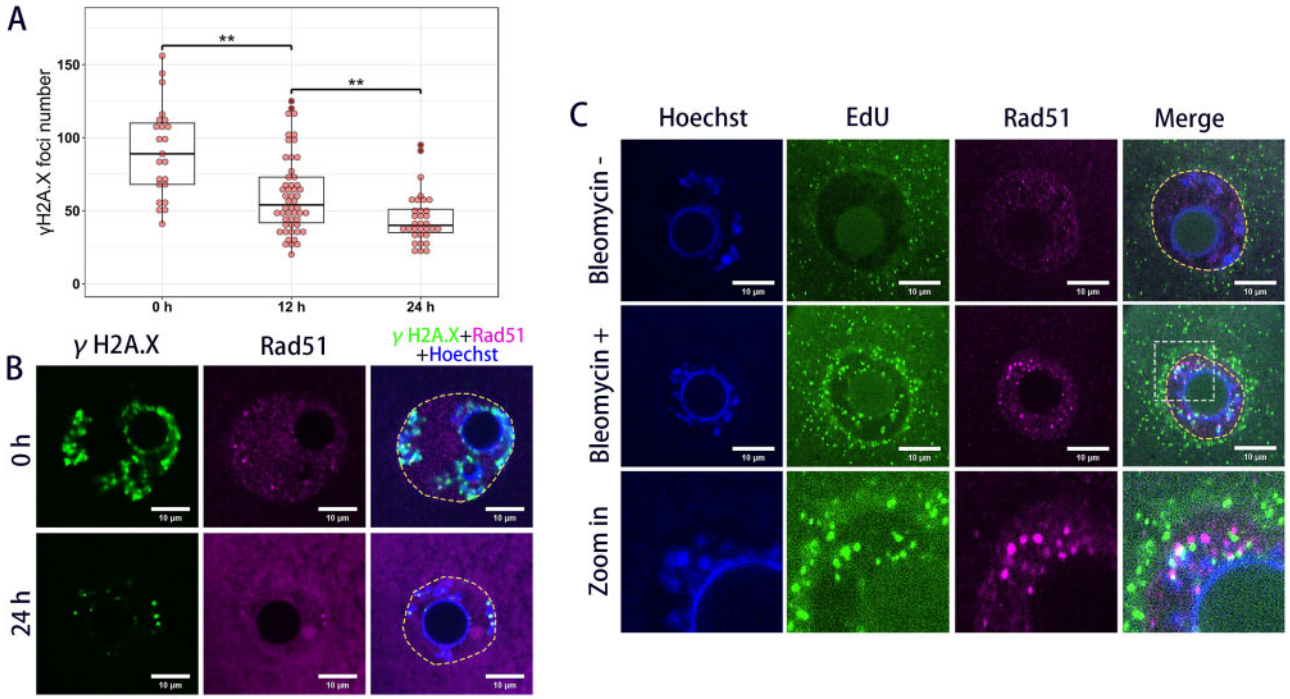

Figure 2 Rad51 participates in the DSB repair in oocytes. (A) DSBs could be repaired in the SN stage oocytes. γH2A.X foci in oocytes are counted at 0, 12, or 24 hours after 1 μM Bleomycin treatment for 1 hour. (B) Rad51 foci are overlapped with γH2A.X foci at 0 and 24 hours after Bleomycin treatment. Rad51 (purple), γH2A.X (green), and DNA (blue). (C) Rad51 foci are adjacent to the EdU signals but not overlapped with them in the Bleomycin-treated oocytes. Rad51 (purple), EdU (green), and DNA (blue). Bar = 10 μm. **Represents $P < 0.01$.

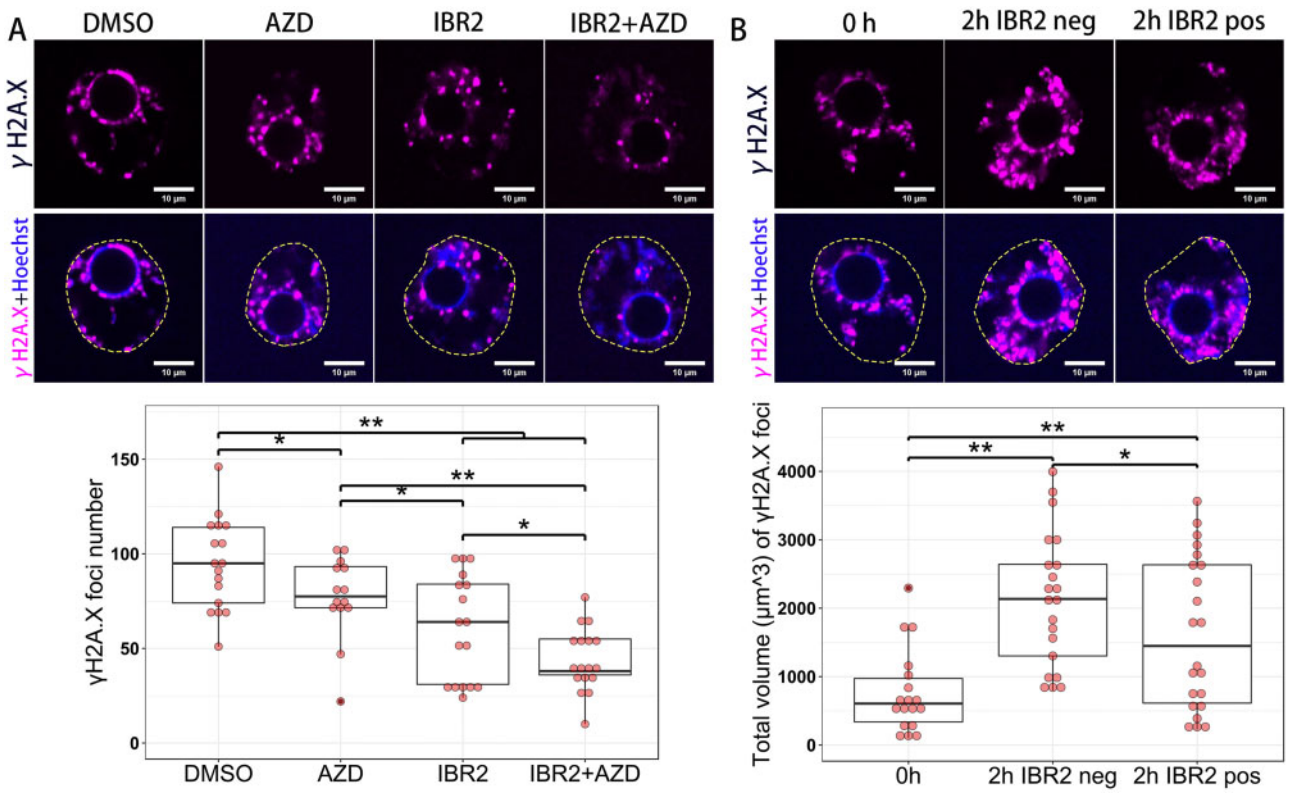

Figure 3 DNA damage is amplified in oocytes. (A) When oocytes were treated with Chek1/2 inhibitor AZD7762 (AZD), Rad51 inhibitor IBR2 or IBR2+AZD before and during DSB inducement, the numbers of γH2A.X foci are significantly decreased compared to those in the DMSO-treated oocytes. (B) The γH2A.X volume increased 2 hours after DSB inducement, and the increase of γH2A.X volume is partially inhibited by IBR2. γH2A.X (purple) and DNA (blue). Bar = 10 μm. **Represents $P < 0.01$ and *$P < 0.05$.

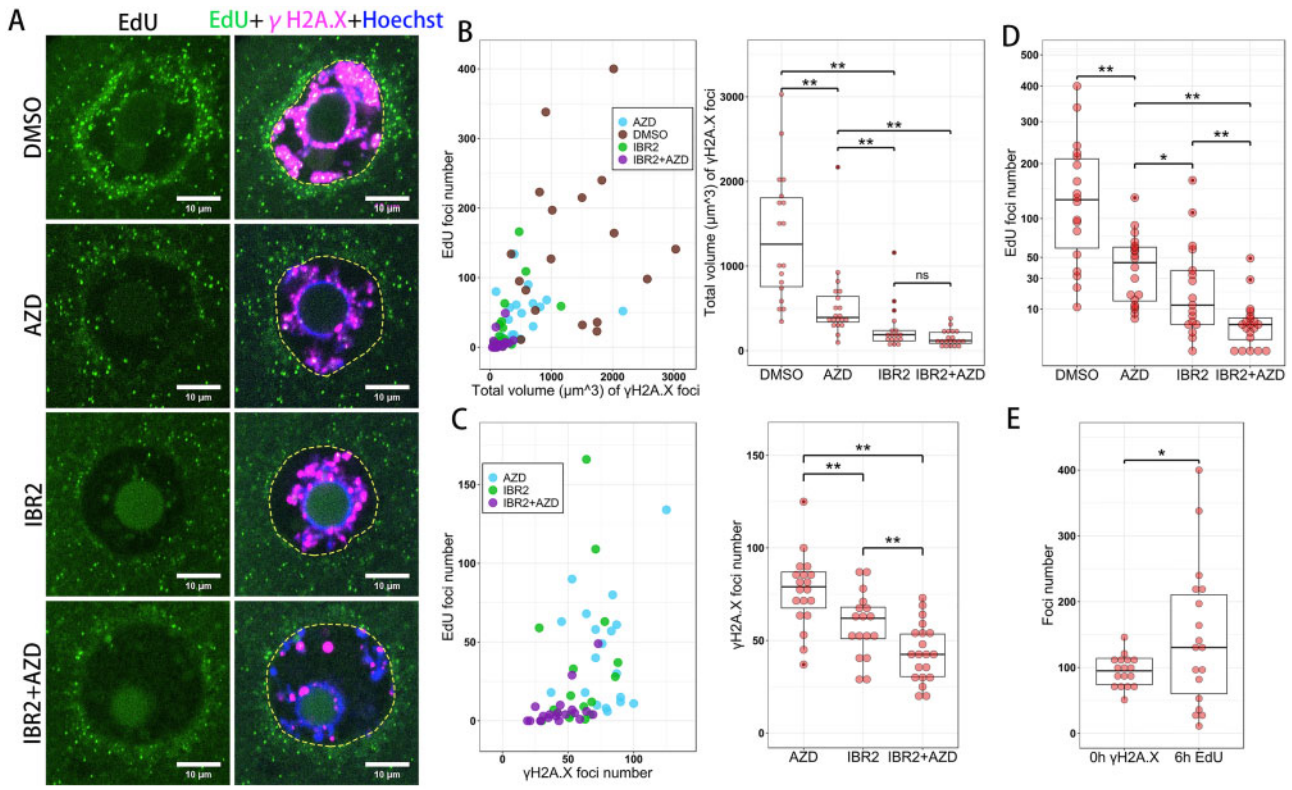

Figure 4 DSB-induced short-scale DNA replication is associated with Rad51 in oocytes. (A) Typical images of EdU signals and γH2A.X foci in the oocytes treated with DMSO, AZD7762 (AZD), IBR2, and IBR2+AZD for 6 hours after released from Bleomycin. (B) The total volume of γH2A.X foci is significantly decreased in the AZD or IBR2-treated oocytes compared with that in the DMSO-treated oocytes. (C) The numbers of γH2A.X foci in the IBR2+AZD treated oocytes are less than those in the AZD or IBR2-treated oocytes. (D) The numbers of EdU signals are significantly decreased in the AZD, IBR2 or IBR2+AZD-treated oocytes compared with those in the DMSO-treated oocytes. (E) The numbers of EdU signals at 6 hours are significantly increased than those of γH2A.X foci at 0 hour after DSB inducement. EdU (green), γH2A.X (purple) and DNA (blue). Bar = 10 μm. **Represents $P < 0.01$ and *$P < 0.05$.

SN oocytes but not NSN and control oocytes (Figure 1A), indicating that DSBs could induce DNA replication in the NSN-SN and SN oocytes.

As persistent mitochondrial DNA (mtDNA) replication exists in the NSN and SN oocytes (Supplementary Figure S2), we compared the EdU signal sizes in the nuclei of NSN-SN and SN oocytes with those in mitochondria by measuring the max lengths of EdU signals. The results showed that the EdU signal sizes in the SN oocyte nuclei and mitochondria were comparable, but the EdU signal sizes in the NSN-SN oocyte nuclei were less than those in the SN oocytes and mitochondria (Figure 1B). As these BIRs in oocytes are short, so we termed them as short-scale BIRs (ssBIRs).

By immunofluorescent labeling of the DSB marker γH2A.X, we found that most nuclear EdU signals were connected with the γH2A.X foci (Figure 1C). When DSBs were induced by Bleomycin, the numbers of γH2A.X foci and EdU signals decreased with the decreasing concentration of Bleomycin, but the nuclear EdU sizes showed no significant difference (Figure 1D).

Rad51 is involved in the oocyte DSB repair

After 1 μM Bleomycin treatment, oocytes were recovered in the Bleomycin-free media for 0, 12, or 24 hours. The numbers of γH2A.X foci significantly decreased in the recovered oocytes as time extension (Figure 2A). In the 24 hours oocyte, EdU signals were not totally held together with γH2A.X foci (Supplemental File S1), indicating that the DSBs could be repaired in the SN oocytes. Similar to our previous results (Ma *et al.* 2019a), HR repair protein Rad51 could be detected at the γH2A.X foci in the DSB oocytes (Figure 2B). Interestingly, the EdU signals were found just adjacent to the Rad51 foci (Figure 2C). All these combined data indicate that Rad51 is involved in the DSB repair in oocytes.

DSBs can be amplified in the SN oocytes

It had been proposed that the multi-invasion joint molecule is established by Rad51 and cleaved by SSEs, and the cleavage of multi-invasion joint molecule can amplify the DNA damage by producing additional DSB ends (Piazza *et al.* 2017). To test whether Rad51 could amplify the initial DSBs in oocytes, we measured the γH2A.X signal levels in the DSB oocytes treated with Rad51 inhibitor IBR2 (Zhu *et al.* 2015) (100 μM) or Chek1/2 inhibitor AZD7762 (100 nM). First, oocytes were treated with DMSO (as control), IBR2, AZD7762, or IBR2+AZD7762 for 5 hours and induced DSBs by Bleomycin (1 μM). As a result, the γH2A.X signals decreased significantly in the IBR2 or AZD7762-treated oocytes compared to those in the control oocytes (Figure 3A). To further analyze whether Rad51 had amplified the DSBs, oocytes were treated with IBR2 after DSB inducement (Figure 3B). The results showed that the γH2A.X volume increased significantly after Bleomycin removing in the control oocytes, but the increase of γH2A.X volume was suppressed by IBR2 (Figure 3B). These results demonstrated that DSBs can be amplified in the SN oocytes and the DSB amplification is dependent on Rad51.

To analyze whether the DSB-induced ssBIR is associated with Rad51, oocytes were treated with DMSO, IBR2, AZD7762, and IBR2+AZD7762 for 5 hours and then treated with Bleomycin to induce DSBs. After that the oocytes were recovered from Bleomycin for 6 hours. As a result, the γH2A.X volumes in the AZD7762 or IBR2-treated oocytes were significantly decreased ($P < 0.01$),

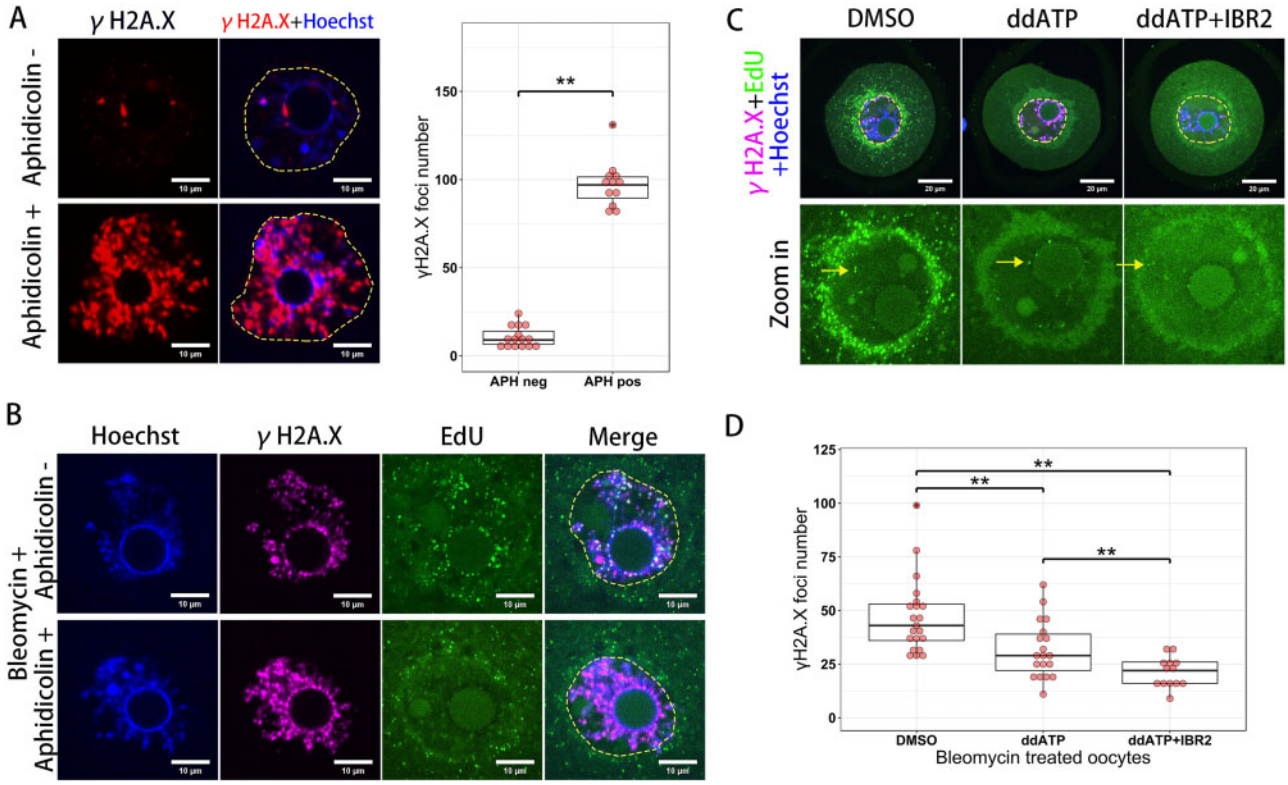

Figure 5 DNA replication participates in the DNA damage amplification in the SN oocytes. (A) Aphidicolin (2 μM for 15 hours) induces DSBs in oocytes. The numbers of γH2A.X foci are significantly increased in the Aphidicolin-treated oocytes. APH, Aphidicolin. γH2A.X (red) and DNA (blue). Bar = 10 μm. (B) Aphidicolin blocks the short-scale DNA replication induced by Bleomycin. Oocytes were cultured with or without 2 μM Aphidicolin for 12 hours after 1 μM Bleomycin treatment for 1 hour. EdU (green), γH2A.X (purple) and DNA (blue). Bar = 10 μm. (C) ddATP blocks the mtDNA replication and delay the nuclear DNA replication. Oocytes were cultured with ddATP (100 μM) or with both ddATP and IBR2 (100 μM) for 5 hours. After that, oocytes were treated with 0.5 μM Bleomycin for 1 h and then released from Bleomycin for 12 hours. EdU (green), γH2A.X (purple), and DNA (blue). Bar = 20 μm. (D) Compared with the control oocytes, the numbers of γH2A.X foci are less in the ddATP group and further less in the ddATP+IBR2 group. EdU signals in the ddATP and ddATP+IBR2 group oocytes are marked by arrows. **Represents $P < 0.01$.

compared to those in the control oocytes (Figure 4, A and B). The γH2A.X volumes in the IBR2+AZD7762-treated oocytes were significantly less than those in the AZD7762-treated oocytes ($P < 0.05$), but at a same level compared with the IBR2-treated oocytes (Figure 4B). However, the numbers of γH2A.X foci in the IBR2+AZD7762-treated oocytes were significantly less than those in the AZD7762 or IBR2-treated oocytes ($P < 0.01$) (Figure 4C). With the decreasing numbers of γH2A.X volume or foci, the EdU signals were decreased in the AZD7762, IBR2, and IBR2+AZD7762 treated oocytes compared with those in control oocytes (Figure 4D). These results indicate that the ssBIR could be suppressed by Chek1/2 inhibitor or Rad51 inhibitor.

In addition, we also compared the EdU signal numbers at 6 hours after DSB inducement with the γH2A.X focus numbers at 0 hour after DSB inducement (Figure 4E). As a result, the numbers of EdU signals were significantly more than those of γH2A.X foci ($P < 0.05$), which further indicates that the DNA damage is amplified in oocytes.

DNA replication promotes DSB amplification in oocytes

The ssBIR is associated with the DNA replication, but whether DNA replication plays functions on the DNA damage amplification in oocytes is not known. So the DNA replication was inhibited to examine whether the DSB number increased in DSB oocytes. At first, we used DNA polymerase inhibitor Aphidicolin to treat the oocytes, however, Aphidicolin not only blocked the ssBIR in DSB oocytes but also induced additional DSBs in normal oocytes (Figure 5, A and B), indicating that Aphidicolin is genotoxic to oocytes. So we next chose another DNA replication inhibitor ddATP which can block the mtDNA replication but only delay the nuclear DNA replication. Oocytes were treated with 100 μM ddATP for 5 hours and induced DSBs by 0.5 μM Bleomycin for 1 hour, and then released from Bleomycin for 12 hours. As a result, ddATP could indeed suppress the mtDNA replication but could not fully suppress the nuclear DNA replication in oocytes (Figure 5, C and D). In addition, the ddATP-treated DSB oocytes had fewer γH2A.X foci than the control oocytes ($P < 0.01$, Figure 3D), indicating that the delay of nuclear DNA replication can suppress the DSB amplification in the SN oocytes. Moreover, when the DSB oocytes were treated with both ddATP and IBR2, the numbers of γH2A.X foci were further decreased, comparing to those in the DSB oocytes only treated with ddATP (Figure 5, C and D). This result showed that nuclear DNA replication had promoted the DNA damage amplification in the SN oocytes.

Discussion

In this study, we used mouse oocytes as a model to analyze the DNA DSB repair in late G2 phase cells. The results showed that DSBs in the SN but not NSN oocytes could induce a type of short-scale DNA replication termed as ssBIR. The sizes of oocyte ssBIR-induced EdU signals were comparable with that of the mtDNA replication-induced EdU signals, and the length of mouse mtDNA

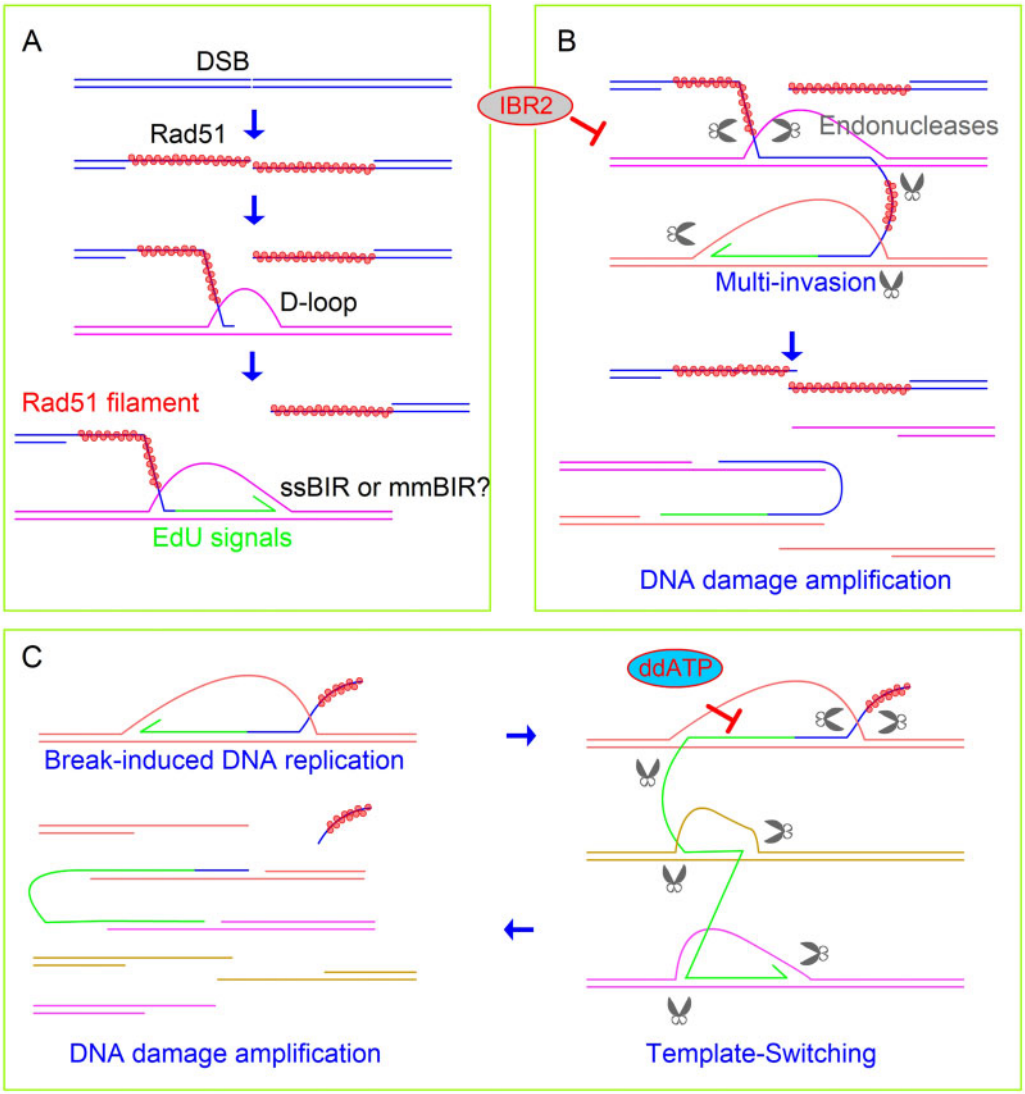

Figure 6 Supposed models of DNA DSB repair in the SN oocytes. (A) Partial strand-exchange makes Rad51 filament exist beside the newly synthesized DNA in the SN oocytes. The DNA damage in the SN oocyte can be amplified by both Rad51-mediated multi-invasion (B) and break-induced replication-induced template switching (C). The multi-invasion can be suppressed by Rad51 inhibitor IBR2 whereas the template switching can be suppressed by ddATP.

ranges from 16,299 to 16,301 bp (Bayona-Bafaluy *et al.* 2003). So the length of the ssBIR in oocytes should be approximately 10–20k bp. Evidence showed that the tract length of gene conversion is about 200–300 bp (Mansai *et al.* 2011) which is less than the length of ssBIR-mediated DNA synthesis in oocytes. On the other hand, ssBIR in the SN oocytes is also different with the classic BIR which would replicate DNA from the breakpoint to the chromosome end or replicate a large genome fragment (Mancera *et al.* 2008; Ma *et al.* 2019b).

Besides the DSB-induced DNA replication, DNA replication in G2 oocytes could also be initiated by fusing oocytes with S phase zygotes (Czołowska and Borsuk 2000). In addition, DNA replication could be initiated in unactivated *Xenopus* egg extracts (Aquiles Sanchez *et al.* 1995). In mitotic cells, replication stress-associated DNA synthesis could be induced and mediated by Rad52 and endonucleases (Bhowmick *et al.* 2016). All these results indicated that both G2 and mitotic cells have the competence for DNA synthesis. However, it is still necessary to deeply analyze which factors are essential for the DNA replication origin firing and the BIR initiation in G2/M cells, as well as how to repress these CGR-prone DNA repair.

In this study, the DSB-induced EdU signals were generally adjacent to the Rad51 foci, indicating that 3'-overhangs bound with Rad51 had not been fully exchanged with the template DNA. The partial strand exchange in the SN oocytes might be caused by two reasons: the condensed DNA configuration or the sequence difference between the template and broken DNA. As microhomology sequences had been detected in the breakpoints of most CGRs, partial strand exchange might be caused by the invasion of broken DNA ends to the microhomology regions in the SN oocytes (Figure 6A).

Rad52 has been proven to mediate BIR in the S phase of mammalian cells (Sotiriou *et al.* 2016), whereas Rad51 is essential for the BIR in yeast (Davis and Symington 2004). In this study and our previous work (Ma *et al.* 2019a), Rad51 inhibitors RI-1 and IBR2 could reduce the number of BIR events in the SN oocytes, indicating that BIR in the late G2 phase cells might be associated with Rad51. However, as the Rad51 inhibitor decreases the

numbers of not only EdU signals but also γH2A.X foci, so it is still not known whether Rad51 directly participates in the ssBIR in the SN oocytes.

It had been reported that the Aphidicolin- and Hydroxyurea-induced replication stress could lead to the formation of rare CNVs in mammalian cells (Arlt *et al.* 2009, 2011, 2012). In this study Aphidicolin could fully inhibit the ssBIR in the SN oocytes, indicating that DNA polymerase α and/or δ participate in the DSB-induced DNA replication. Aphidicolin could not only inhibit DNA replication but also induce additional DSBs. However, it is not known whether these Aphidicolin-induced DSBs are associated with DNA polymerases. One possibility is that the Aphidicolin-induced DSBs in G2 phase are also potential factors of rare CNV formation.

Although the direct evidence of Rad51-mediated multi-invasion is absent, the DSB numbers have been amplified in oocytes. The DSB amplification could be suppressed by Rad51 inhibitor, Chek1/2 inhibitor or DNA replication inhibitor ddATP in oocytes. These results might suggest that the DSB amplification is induced by not only multi-invasion (Piazza and Heyer 2019) (Figure 6B) but also DNA replication-associated template switching. Similar to the multi-invasion model, multiple rounds of template switching could also form the multi-invasion-like joint molecule which might amplify the DSBs by endonucleases (Figure 6C). All these speculations deserve a further study.

Acknowledgments

The authors thank X.Y. Fan, X.H. Sun and other members in Fertility Preservation Lab for their helps in this study.

Funding

This study was supported by the National Natural Science Foundation of China (81671425, 81971357, and 31801245), Talent Introduction Fund of Guangdong Second Provincial General Hospital (YY2018-001), Key Research & Development Program of Guangzhou Regenerative Medicine and Health Guangdong Laboratory (2019GZR110104001) and National Key Research and Development Program of China (2018YFC1003400).

Conflicts of interest: None declared

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Communicating editor: F. Cole