Chapter

Mouse Models to Understand Mutagenic Outcomes and Illegitimate Repair of DNA Damage

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Abstract

Maintenance of genome integrity is critical to prevent cell death or disease. Illegitimate repair of chromosomal DNA breaks can lead to mutations and genome rearrangements which are a well-known hallmark of multiple cancers and disorders. Endogenous causes of DNA double-strand breaks (DSBs) include reactive oxygen species (ROS) and replication errors while exogenous causes of DNA breaks include ionizing radiation, UV radiation, alkylating agents, and inhibitors of topoisomerase II (Top2). Recent evidence suggests that a growing list of environmental agents or toxins and natural dietary compounds also cause DNA breaks. Understanding the consequences of exposure to a broad spectrum of DSB-inducing agents has significant implications for understanding mutagenicity, genome stability and human health. This chapter will review *in vivo* mouse models designed to measure DNA damage and mutagenicity, and illegitimate repair of DNA DSBs caused by exposure to environmental agents.

Keywords: mutagenicity, double-strand breaks, illegitimate repair, genome rearrangement, transgenic mouse model, genome instability

1. Introduction

The faithful repair of DNA lesions is central to the maintenance of genomic integrity [1]. Illegitimate repair of chromosomal DNA breaks can lead to mutations and genome rearrangements which are a well-known hallmark of multiple cancers, aging, and disease [2, 3]. DSBs can occur in a programmed manner during a metabolic process such as DNA replication, during meiosis, and the development of the immune system during V(D)J recombination and immunoglobulin class switch recombination [4] or endogenous agents such as ROS and replication errors [5]. DSBs also occur as a result of exposure to exogenous agents such as ionizing radiation, UV radiation, alkylating agents, topoisomerase inhibitors, and chemotherapeutic drugs [6–8]. Evidence shows that a growing list of natural compounds in the human diet or the environment also causes DNA breaks [9].

Mammalian cells have evolved sophisticated mechanisms to detect the damage via the DNA damage response (DDR) and signaling pathway which then activates repair pathways to maintain genome integrity [10]. Major mammalian processes to detect

and repair DNA DSBs include homologous recombination (HR) and non-homologous end joining (NHEJ) (**Figure 1**). Both of these repair pathways are cell cycle-specific and differ based on their requirement for a donor DNA template with significant DNA sequence similarity. Studies suggest NHEJ is most prevalent in non-cycling somatic cells during the G1 stage, while HR is particularly active during the S, G2, and M stages due to its requirement for a homologous sequence as a donor template [11, 12]. In NHEJ the broken ends are processed and ligated together without

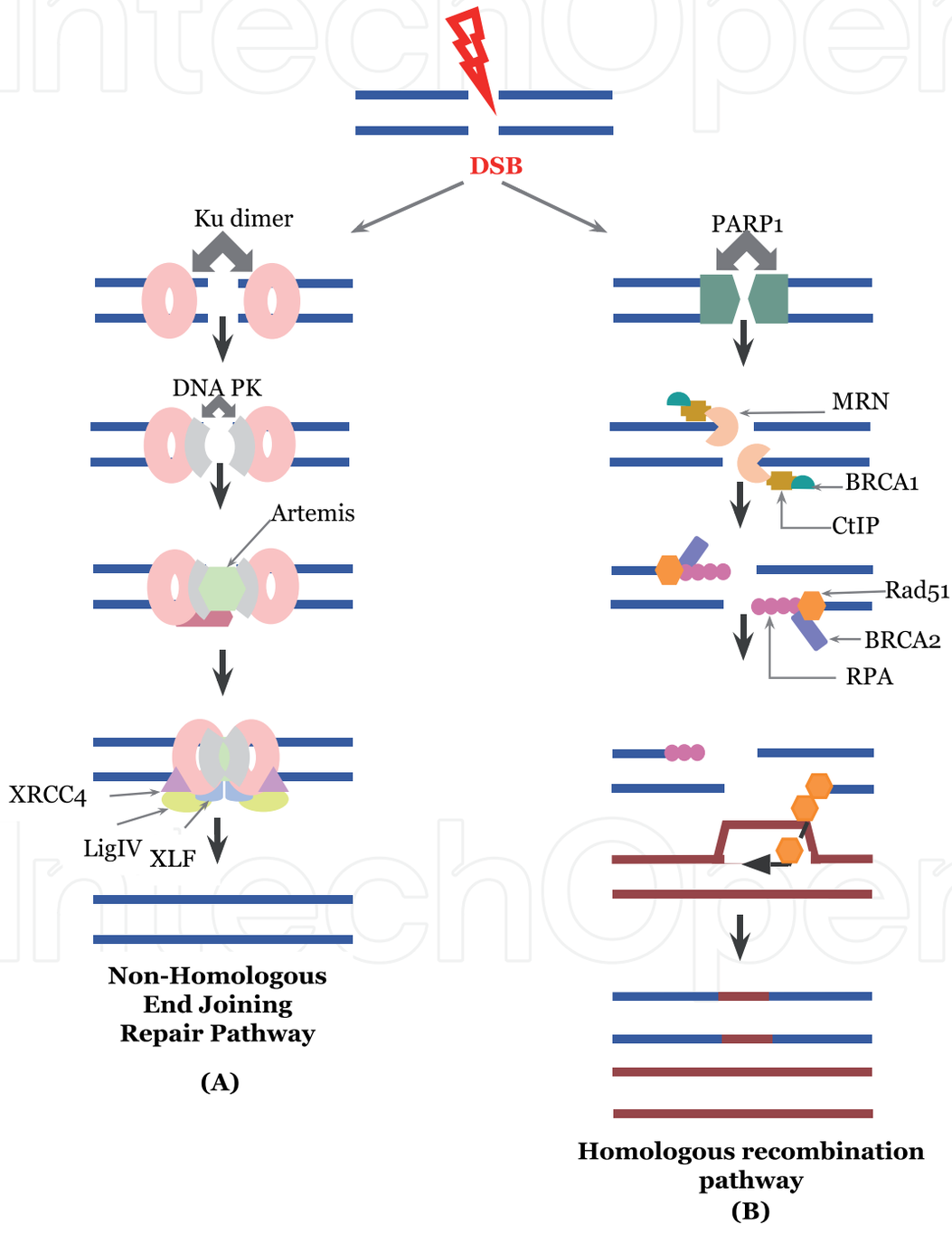

Figure 1.
The DNA double-strand breaks (DSB) are repaired by the two pathways; These are—(A) non-homologous end joining (C-NHEJ) which modifies the ends and allows ligation of the broken ends to repair the DSB; (B) homologous recombination (HR) that uses a homologous sequence from sister chromatid or homologous chromosome or a homologous sequence within the genome.

requiring homology. By contrast, HR uses an undamaged homologous sequence from a sister chromatid, allelic locus, or an ectopically located sequence from a heterologous chromosome as a template to initiate HR or break-induced replication repair at the broken site [13].

Laboratory mice (*Mus musculus*) have been key to most *in vivo* studies on DNA damage and mutagenicity or illegitimate repair that take into account the complex environment of the mammalian system including tissue architecture, cellular differentiation programs, chromatin landscape patterns, and aging [14–18]. Multiple *in vivo* models have been developed to examine the potential for the repair of DNA DSBs [19, 20]. Furthermore, specific cell types within tissues and organs encounter a diverse set of DNA damaging insults that produce distinct types of DNA damage. Individual cells differ in their capacity for sensing, responding, and repairing specific DNA lesions [17].

2. Induction and assessment of mutagenicity by endogenous sources

2.1 Programmed DNA DSBs

Endogenous DNA DSBs can occur as deliberate, cell-required mechanisms. DNA DSBs drive the non-sister chromatid HR events responsible for genetic diversity in meiotic cells [21]. These events can lead to rearrangements including deletions, tandem duplications, inversions, and translocation of chromosomes which are not always favorable for the cell [22]. Analogous to topoisomerase II (Top2), the Spo11 enzyme initiates DSBs during prophase I of meiosis. The locations Spo11-mediated DSBs are not random and are referred to as DNA hotspots expected to occur somewhere between 10,000 and 40,000 times within the mammalian genome [23]. These DSBs initiate meiotic HR via gene conversion and crossover events. $Spo11^{-/-}$ knockout mice have errors in normal meiotic chromosome synapsis formation [24]. PRDM9 methyltransferase and its associated binding specificity determine the DSB hotspot locations in mice by generating nucleosome-depleted regions, allowing for the programmed DSBs to occur via Spo11 cleavage [25].

2.2 Reactive oxygen species and replication stress

DNA DSBs can occur due to the accumulation of ROS-induced oxidative stress or as the result of replication or transcription stress. ROS are often linked to neurological diseases and cancer, although they result from endogenous cellular metabolism. Some examples of endogenous ROS include the superoxide radical anion ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), peroxynitrite ($ONOO^-$), and hypochlorous acid (HOCl) [26]. ROS cause DNA damage through their ability to alter the overall reduction-oxidation (redox) cell conditions to cause oxidative stress. By changing redox conditions of the cell, important cellular processes including signal transduction and proliferation may not be able to occur. The failure of these processes can be lethal for the cell or promote mutagenesis through GC to TA changes [27]. Guanine lesions that lead to a miscoding error change the structural integrity of the DNA by weakening hydrogen bonding between bases [26]. These guanine mutations are associated with ROS-related oxidative stress and can promote cancer development [27]. 8-Oxo-7,8-dihydroguanine (8-oxoG) is a common output of guanine oxidation. It is an

important compound because of its susceptibility to further oxidation and overall genotoxicity [28].

The base excision repair (BER) pathway is a mechanism deployed to resolve DNA lesions, as the presence of 8-oxoG, and has three major steps: (1) recognition of the lesion by DNA glycosylases, (2) base excision, (3) resynthesis and replacement of the removed base [29]. DNA glycosylases initiate BER through cleaving the *N*-glycosidic bond between the damaged base and sugar. DNA glycosylases can be either monofunctional or bi-functional whereas bi-functional DNA glycosylases include a β-elimination or β,δ-elimination step after *N*-glycosidic bond cleavage [30]. Defects in the BER pathway's mechanism can lead to the accumulation of BER intermediates, unrepaired lesions, point mutations, and DNA DSBs. DNA polymerase β (Pol β) is one of the most active DNA polymerases involved in BER. A single nucleotide polymorphism (SNP) on the gene coding for Pol β results in proline residue 242 becoming arginine (P242R). This mutation is suggested to cause chromosomal aberrations, and therefore, genome instability. P242R was associated with an increase in SSBs and DSBs compared to wild-type cells, and cellular transformation in mouse and human cells. An observed increase in cellular proliferation with the expression of the P242R suggested this mutation may induce a carcinogenic phenotype [31].

Replication stress is any event causing changes to the replication rate and can include halting replication. Unrepaired DNA lesions contribute to replication stress by acting as a physical block of the replication fork and its motion [32]. Single strand breaks (SSBs) generated by replication stress can further generate DSBs by nucleases, deamination, or spontaneous hydrolysis [22]. These DSBs, as well as meiotic-related DSBs, will use NHEJ or HR for repair. Errors in HR, which are less common than in NHEJ, can lead to mutagenesis and overall genome instability [33]. Phosphorylation of target proteins by ATM also triggers DDR. Chk2 has protein kinase activity allowing it to phosphorylate several effector proteins in the cell cycle checkpoint including p53 which can be modified by either ATM or Chk2 (or ATR or Chk1). ARF protein (p14) seems to stabilize TIP60 interactions with ATM for better activation and is associated with maintaining genome stability [2].

2.3 Spontaneous DNA breaks

A reporter fluorescent yellow direct repeat (FYDR) mouse model was developed to assess DSB-induced intra-chromosomal recombination events in multiple tissues including skin [34, 35]. In this model, spontaneous DSBs or DSBs induced by replication fork collapse can lead to unequal sister chromatid exchange between tandem truncated enhanced yellow fluorescent protein (EYFP) sequences resulting in gene conversion and expression of EYFP quantifiable by flow cytometry. This model showed the *in vivo* frequency of spontaneous intra-chromosomal HR in multiple tissues calculated at approximately 10^{-5} to 10^{-6} per base pair per cell division. A modification of the model using a direct repeat-GFP (RaDR-GFP) inserted in the Rosa*26* locus contains two truncated EGFP sequences in tandem [14, 34–36]. This model detected spontaneous and DNA damage agent-induced intra-chromosomal HR in most gastrointestinal organs and respiratory organs. Cell-type-specific immunohistochemistry staining of the lung [36] and pancreata [35] demonstrated cell-type and tissue-type specificity of intra-chromosomal HR recombinant populations. This model also demonstrated that older mice show an order of magnitude increase in the accumulation of recombinant cells.

3. Induction and assessment of mutagenicity by exogenous agents

Exposure of mice to nonspecific agents such as IR, Top2 inhibitors and chemotherapeutic drugs induce DSBs more broadly across the genome and in physiologically relevant contexts (**Figure 2**).

3.1 Ionizing and non-ionizing radiation

Ionizing radiations such as X-rays and gamma rays can cause direct damage by depositing energy or indirect damage by ionization of water molecules to produce free radicals that influence SSBs or DSBs [37–40]. The complexities of the damage vary according to the linear energy transfer of the radiation [37]. Alpha particles are high LET radiation and directly cause breaks [41] while non-ionizing radiations such as UVA and UVB create indirect DSBs and SSBs [7]. Several DSB repair pathway-specific proteins have been examined on bases of the IR sensitivity such as MRE1 resection protein [42], BRCA1 [43], Ku 70 [44], and Pol θ [45–47]. Exposure of mice to irradiation can cause a variety of DNA lesions including base damage, SSBs and DSBs. However, DSBs have been deduced to be amongst the toxic lesions and contribute to cell death [37]. Erroneous repair of the DSBs causes chromosomal aberrations and influences carcinogenesis [38].

The earliest methods for detection of DSBs induced by irradiation included physical separation of the broken DNA from undamaged DNA by pulse-field gel electrophoresis and comet assays [47, 48]. However, these methods were not efficient for mouse studies because of their low reproducibility and limited approximation of DSB levels [50]. More recently, micronuclei scoring is more commonly used as these cytogenetic biomarkers are easily detectable through microscopy. Micronuclei are cytoplasmic chromatin masses resulting from damaging agents such as IR [49, 50]. Another prominent and widely used method for the detection of DSBs include the identification of DSB downstream biomarkers such as γH2AX that binds to DNA at sites flanking DSBs [51, 52]. This protein is a variant of H2AX histone and forms a focus at the sites of DSBs which further signals DDR and repair response [50, 51]. The γH2AX foci can be analyzed by immunohistochemical staining and visualization under fluorescent or confocal microscopy.

To determine the repair pathway choice of DSB repair association of DSBs with proteins specific for one pathway or another is typically employed. For example, HR requires resection of the broken DNA ends from ssDNAs that are recognized and covered by replication protein A (RPA) which can be detected through immunofluorescence. To monitor the length and speed of resection per DNA molecule, BrdU antibody is used which binds to the ssDNA and forms fibers visible under a fluorescent microscope. To increase the resolution of DNA fibers, Single-Molecule Analysis of Resection Tracks (SMART) can be used [53, 54].

3.2 Radiomimetic drugs

Commonly used chemotherapeutic drugs are categorized into 5 different types based upon their chemical composition and mode of action. Widely used anti-cancer drugs for DNA damage include alkylating agents such as temozolomide (TMZ) melphalan, and cyclophosphamide [55, 56]. These agents act by attaching the alkyl groups onto the DNA and interfering with the cell cycle and transcription process.

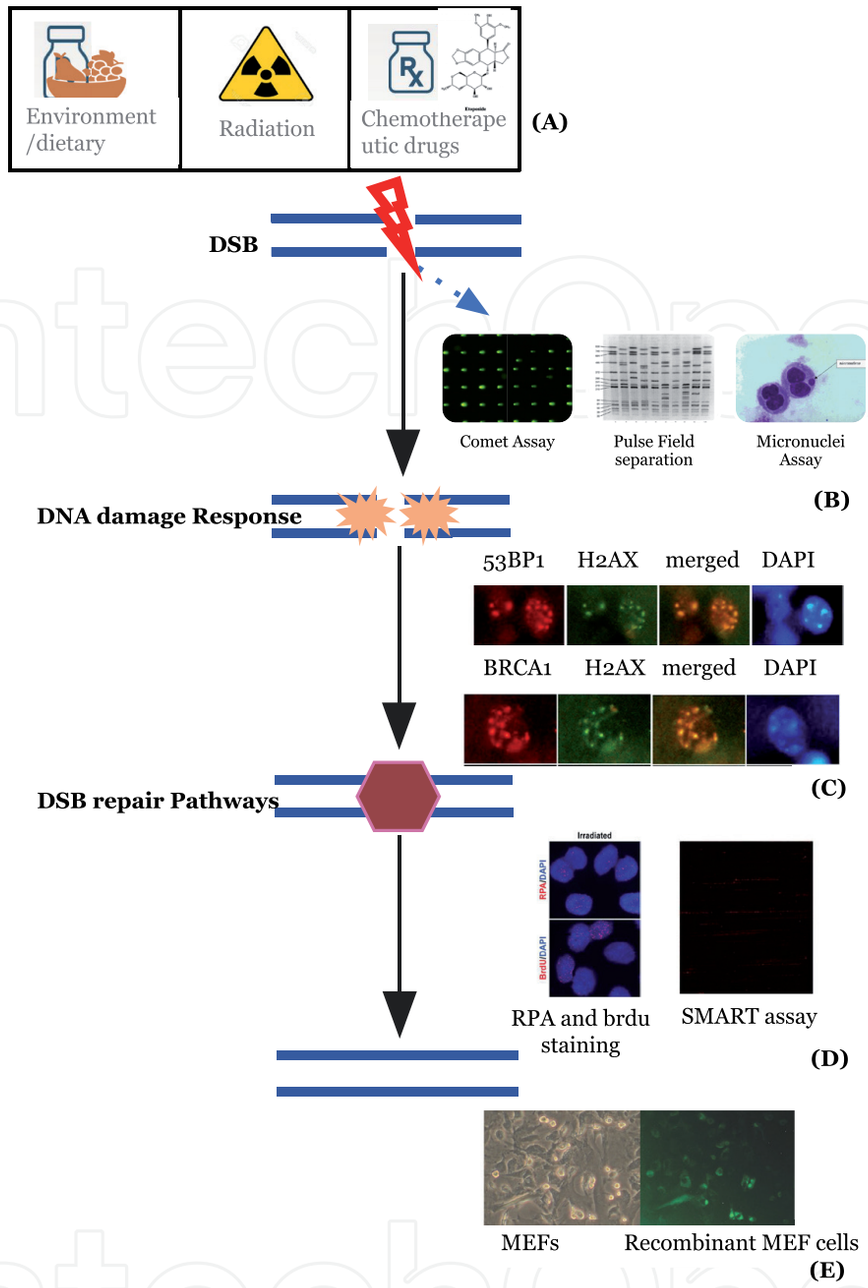

Figure 2.
Exogenous exposure DNA double strand break induction, damage response pathway and repair. (A) The schematic figure shows induction of DNA damage via chemotherapeutics, radiation and environmental compounds. (B) The preliminary assessment of the DSB can be done by techniques such as comet assay, pulse electrophoresis and micronuclei staining. (C) Detection DNA damage response proteins such gamma H2AX, 53BP1 and BRCA1 foci using immunofluorescence staining. (D) Hr specific techniques such as SMART assay and brdu staining. (E) To determine repair frequencies several reporters are developed. For example, GFP recombinant cells shown in bottom right.

They can also cross-link two double-strand DNA molecules creating inter-strand cross-links (ICLs). ICLs are dangerous lesions if not repaired. Alkylating agents can also add mismatched nucleotides which can cause genome instability [56]. Studies targeting DDR and DSB repair proteins that can alter the sensitivity of chemotherapeutic drugs are used for cancer treatment modalities. Recent research proposed that deficiency of the NHEJ protein DNA ligase4 significantly enhanced the sensitivity of cells to TMZ [57]. Mouse embryonic fibroblasts (MEFs) of DNA ligase 4 knockout mice treated with a D50 dose of TMZ have higher numbers γH2AX foci

and significantly reduced cell survival when compared to wild-type suggesting that Ligase4 protects the cells against lesions from TMZ [57].

ICL-inducing agents, such as mitomycin C (MMC), nitrogen mustards, and platinum can create cross-links that hinder DNA replication, thus preferentially targeting highly proliferative cells. Thus, these agents are widely used in the treatment of cancers and several skin conditions [55, 58]. The repair of ICLs involves both translesion break repair and HR proteins, and mutation of HR genes leads to sensitivity to ICL agents [59, 60]. Brca1 mutant mice ear fibroblasts and MEFs treated with MMC showed significantly reduced HR frequency and increased sensitivity to MMC. Interestingly, ATM mutant mice did not have a significant change in HR frequency even with higher MMC doses suggesting that ATM is dispensable for HR [59].

Molecular studies indicate the necessity of Top2 in the maintenance of genome integrity. The ability to halt Top2 function and generate enzyme-mediated DNA damage is a key reason why it is used in secondary cancer chemotherapy such as therapy-related acute myeloid leukemia (t-AML) [61, 62]. Top2 enzyme acts by catalyzing the interconversion of topological DNA isomers through the generation of a transient DSB on one DNA helix ("gate" strand) while remaining covalently linked to the 5′ end of the DNA, followed by passage of a second DNA helix ("transfer" strand) through the DSB, and then religation of the DSB [63]. Mammals have two isoforms of Top2—α and β [61]. Chemotherapeutic drugs doxorubicin and etoposide inhibit the catalytic activity of Top2 after generating the DSB resulting in high levels of trapped Top2:DNA complexes and unrepaired DSBs [9, 61]. Such agents are also referred to as Top2 "poisons" [61]. A novel insight into secondary malignancies induced by these Top2 targeting drugs has come from studies using a transgenic mouse model with a skin-specific ablation of Top2β [63]. These skin-specific *top2*β-knockout mice were exposed to etoposide to evaluate the role of the two isozymes of DNA, Top2α and Top2β. The results demonstrated that in the absence of Top2β, there was a reduction in NHEJ induced by etoposide, suggesting a potential role of NHEJ repair in promoting the malignancies created by improper repair of these lesions [64, 65].

3.3 Pollutants and environmental compounds

Chemical compounds including air and water pollutants, pesticides and some dietary compounds are genotoxic and linked to carcinogenesis. Air pollutants such as benzene and sulfur oxide are released by the combustion of fossil fuels are often linked with leukemias [65, 66]. An *in vivo* study demonstrated how benzoquinone (BQ) environmental agent-induced recombination in fetal hematopoietic cells in pKZ1 transgenic mice [67]. BQ potentially induced ROS measured by a significant increase in the ROS product 8-OH-2′-dG. This was followed by DSB induction that was detected by a significant increase in γH2AX foci in the BQ treated cells. The widely used pesticide endosulfan is speculated to cause chromosomal abnormalities in humans [68, 69]. Adult wild-type BALB/c mice fed endosulfan and analyzed for DSBs and ROS-mediated damage showed an increase in γH2AX foci and a significant increase in the levels of the NHEJ-associated protein 53BP1 in lungs and testes. Furthermore, elevations of several other proteins involved in the alternative end joining (Alt-EJ) pathway were evaluated by Western blot. This study provided compelling insight on the mechanism of action of endosulfan pesticide [69].

Bisphenol A (BPA) is a hormonally active environmental xenoestrogen widely found in food products. It is an epigenetic toxicant that can alter the DNA by the generation of ROS [70]. Bioflavonoids are polyphenolic compounds found in various

dietary products such as soy, coffee, fruits, and vegetables [71]. These compounds have been characterized to be mechanistically and biochemically similar to the Top2 inhibitor and chemotherapeutic drug etoposide [72, 73]. In addition, bioflavonoids have been shown to cross the placental barrier and can induce *MLL* breakpoint cluster region cleavage suggesting an association with the initiation of infant leukemia [74]. A study reported prenatal exposure to flavonoids genistein or quercetin can increase the risk for leukemia onset, as assessed by the frequency of *MLL* translocations in an ATM mutant mouse model prone to develop cancer [75]. Prenatally exposed fetuses were examined at gestation day 14.5 by inverse-PCR to detect *MLL* translocations and their frequency in the fetal liver. Additionally, mice prenatally exposed to flavonoids genistein or quercetin were euthanized at 12-weeks and inverse PCR was performed to determine the presence of *MLL* translocations. These prenatally exposed mice developed leukemia albeit at later ages [75]. These results are further supported by an embryonic stem cell GFP-NHEJ model to identify chromosomal translocations between *MLL* and *AF9* breakpoint cluster regions analogous to those observed in infant leukemia [76]. Upon damage induced by etoposide or a large panel of flavonoids, DSBs in the two loci and repair by NHEJ produced a chromosomal translocation resulting in a functional full-length GFP at least partly dependent on Top2 [76, 77]. Another study examined epigenetic effects of genistein on hematopoiesis in mice; mice prenatally exposed to genistein showed the significant increase in erythropoiesis. Furthermore, transcriptional microarray analysis suggested that genistein exposure was associated with hypermethylation of certain repetitive elements which coincided with a significant down-regulation of genes involved in hematopoiesis in bone marrow cells and estrogen-responsive genes of genistein-exposed mice [78].

Another reporter system assesses mutagenic events through the *Escherichia coli*-derived LacZ gene, which codes for the production of β-galactosidase. β-Galactosidase cleaves lactose forming galactose and glucose, but is receptive to substrate 4-bromo-5-chloro-3-indolyl β-D-galactopyraniside (X-Gal) and produces blue precipitate when bound to β-galactosidase. The blue precipitate is observable through light microscopy [79]. Shuttle vectors carrying the bacterial reporter gene include micro-injection of bacteriophages and electroporation of plasmids for the development of transgenic mice for mutagenetic assay. Transgenic $LacZ^+$ mice have been dosed with different mutagenic chemical compounds, like ethyl nitrosourea, chlorambucil, and benzo[α]pyrene, to observe changes in the production of X-Gal's blue precipitate as an indicator of mutagenicity [80]. The Mutamouse and Big Blue transgenic mouse models were developed via bacteriophages. Mutamouse utilizes bacteriophage λ DNA (λgt10) as a vector for LacZ insertion at an *Eco*RI restriction site. Excision of the LacZ gene for analysis and a positive agar selection system is used with scoring of the clear plaques to identify mutants. Big Blue also has a λ bacteriophage shuttle vector for LacZ, but a non-selectable color screening assay to provide a ratio of blue plaques to white plaques and consequently a mutation frequency [81]. In the 35.5 transgenic mouse system, the LacZ transgene concameter is in a particularly unstable chromosomal region near the pseudo-autosomal region on the X-chromosome resulting in an increased potential for germinal and somatic mutations [80].

4. Induction and assessment of mutagenicity by site-directed cleavage

Nonspecific DNA damaging agents including chemotherapeutic drugs, environmental agents and radiation provide a global understanding of cell function during

the response to DNA damage and DSBs. Molecular analysis of specific repair is difficult as spontaneously occurring DNA breaks occur in unknown locations. Off-target effects on the genome can be limited by using tools such as endonucleases and retroviruses. In addition to site-specific damage induced by specific endonucleases, a defective selectable marker or a defective fluorescent protein such as green fluorescent protein (GFP) can be added to develop a reporter system [82]. The endonuclease induces DSBs, and repair can result in a fluorescent or selectable active marker that was previously defective (**Figure 3**).

4.1 Recombinase cleavage and repair reporters

Development of conditional and inducible *in vivo* reporter assays allows for manipulation of gene expression, and molecular identification of deletion or addition of DNA sequence at specific loci. Generally, a DNA recombinase enzyme is involved in the development of conditional reporter systems. Recombinase enzymes such as Cre and FRT catalyze a concerted recombination reaction between two target sequences (loxP for Cre and FRT for FLP). Depending on the relative orientation of the target sites, catalysis results in the excision of the DNA gene sequences between the target sequences [83, 84]. A conditional Nbs1 null mouse MEF system developed with cre-lox recombinase provided insight regarding the role of the MRE11, RAD50 and NBS1 (MRN) complex with other repair proteins in DSB processing and HR. Nbs1 null MEFs treated with MMC or IR followed by Western blotting and immunohistochemistry of Brca1 and Rad51 indicated that loss of Nbs1 affected single-strand annealing via Rad51 suggesting its role in promoting HR. In support of this, PCR and Southern blotting suggested that loss of Nbs1 in an embryonic stem cell line promoted NHEJ repair [85].

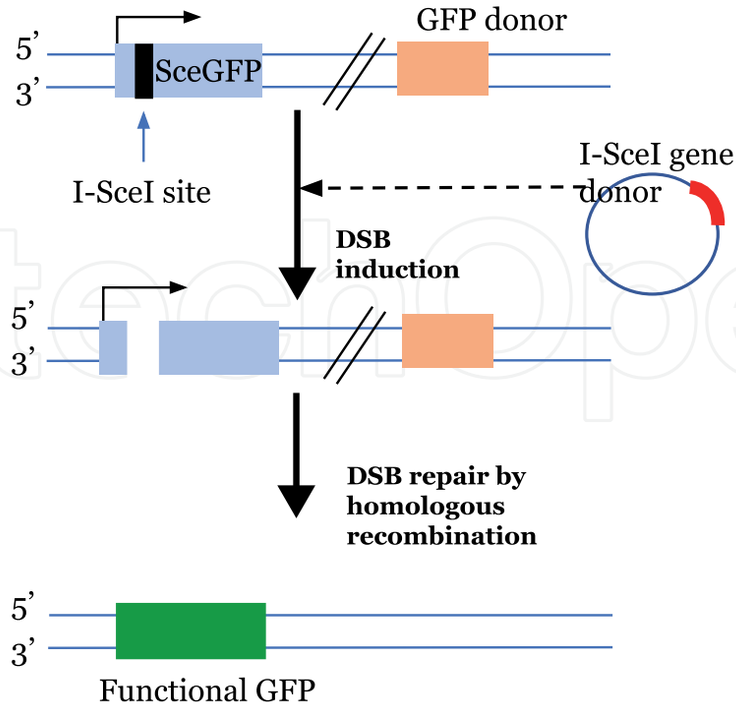

Figure 3.
Schematic of a GFP reporter system. This cassette consists of a ISceI-GFP is a modified GFP gene, which contains an 18 bp long ISceI recognition site and in-frame termination codons and a downstream a GFP donor fragment. Addition of an ISceI gene donor to this system induces DNA DSB at the ISceI site. Homologous recombination by gene conversion results in a functional GFP gene.

4.2 Endonuclease cleavage and repair reporters

Restriction enzymes can induce site-specific DSBs with their sequence specificity to target DNA. Intron specific encoded endonuclease 1 (ISceI) derived from *Saccharomyces cerevisiae* is one of the first endonucleases used for the study of HR in mammalian cells and, subsequently, *in vivo* [82, 86].

The G2S mouse model was developed to determine the potential for DSB-induced inter-chromosomal HR repair *in vivo* [84]. This G2S mouse model was genetically engineered to contain three distinct transgenes—two non-functional green fluorescent protein (GFP) reporter transgenes and a bi-cistronic doxycycline (Dox)-inducible ISceI transgene. Each GFP reporter construct contains an ISceI recognition site that renders it non-functional and provides for the induction of specific DSBs. Repair of the ISceI-induced DSBs by inter-chromosomal HR generates a functional GFP gene. While no GFP+ cells were detected without Dox ($<1 \times 10^{-8}$), following the addition of Dox to mouse chow or drinking water, fluorescent GFP+ cells were detected in a large spectrum of tissue types and hematopoietic progenitor cell populations visualized by fluorescent microscopy and quantitated by flow cytometry. Similar to results with RaDR mice and intra-chromosomal HR, aged G2S mice showed reduced numbers of inter-chromosomal HR cell populations [87].

Another study examined the genetic interactions between ATM, BRCA1, and 53BP1 in mice using a hypomorphic mutant, $Brca1^{S1598F}$ ($Brca1^{SF}$) [88]. To study the role of these proteins in intra-chromosomal HR, primary fibroblasts from $Brca1^{SF/SF}$ mice and $Atm^{-/-}$ mice were integrated with a direct repeat GFP (DR-GFP) reporter and a Dox-inducible ISceI endonuclease. The DR-GFP contains a full-length nonfunctional GFP gene containing an ISceI endonuclease site followed by a downstream GFP homologous donor sequence; DSBs induced by ISceI cleavage can promote intra-chromosomal HR repair to result in GFP+ cells. While spontaneous GFP+ cells were minimal (<0.01%), Dox addition to wild-type cells resulted in detection of GFP+ cells indicating HR repair (3–4%). Both $Brca1^{SF}$ and $Atm^{-/-}$ models showed a 3- and 2-fold reduction in GFP+ cells, respectively. ATM inhibition in wild-type cells only reduced HR by 1.6-fold, while ATM inhibitor exacerbated the generation of GFP+ cells in $Brca1^{SF/SF}$ fibroblasts as compared to wild-type and $Atm^{-/-}$ fibroblasts. PCR-based assay with the DR-GFP reporter was used to quantify the SSA pathway which suggested significant reduction. Interaction of Atm, Brca and 53 bp1 in HR, was demonstrated by the appearance of RAD51 foci from ear fibroblasts. Examination of triple mutants indicated the plausible role of ATM in generating end-resected intermediates for RAD51 filament formation in cells with compromised BRCA1 and 53BP [88].

A recent age-dependent study developed a knock-in R26BHEJ model to determine the efficiency of frequency of intrachromosomal NHEJ for repair. R26BNHEJ knock-in is a GFP-based NHEJ reporter inserted into the ROSA26A locus. The DSBs are created using ISceI and repair by NHEJ was analyzed in several tissues using flow cytometry. This model demonstrated that there was a 1.8 to 3.8-fold decline of NHEJ efficiency with increased age [89].

In the past two decades, new approaches of gene editing have enormously expanded mutagenesis studies. Use of artificial nuclease like zinc-finger (ZFN), transcription activator-like effector (TALEN) nuclease, and the latest clustered regularly interspaced short palindromic repeat (CRISPR)/associated (Cas9) system has enhanced precision of gene editing [90, 91]. ZFN and TALEN nucleases consist of sequence-specific DNA-binding domains that are fused to a nonspecific DNA cleavage module such as FokI endonuclease. These systems readily search for sequence

homology and the endonuclease cleaves at the recognition site, removing the target gene. Several development studies use ZFN and TALEN for gene editing [90]. A powerful approach for gene alteration is the CRISPR-Cas9 system. This system was initially observed in bacteria as an immune response against viruses. It consists of a single-guide RNA (sgRNA), that targets a palindromic region in the specific location of the genome, which is recognized by Cas9 nuclease generating a DNA DSB that subsequently activates the cellular DNA repair machinery. HR or NHEJ repair would result in alteration of the target gene by indel mutations [92, 93].

5. Induction and assessment of mutagenicity *in utero*

In utero studies can provide valuable insight into the physiological processes that make mammalian models unique. Although, the single-cell *Saccharomyces cerevisiae* has a large number of genes with homologs in mammals that are involved in DNA damage, signaling and repair [94], it is important to consider the mouse model's advantage to understanding DNA damage and repair in multiple organ systems that a single-cell model cannot provide. Oogenesis, embryogenesis, and spermatogenesis are processes that give valuable insight to mutagenicity because of their roles in development and meiotic recombination and their potential to lead to trans-generational mutational consequences.

5.1 Gametocyte-based assays

Understanding the mammalian recombination pathway is useful for developing mouse models that can be used to appropriately study meiotic recombination stress and DSB repair. Because knockout of MRN complex components causes embryonic lethality, conditional disruption of NBS1 has been utilized in germ cells to assess how the MRN complex is functioning during meiotic DSB repair in mice [95]. A germ cell-specific transgenic mouse model inactivates targeted gene expression utilizing *Vasa-cre* [95, 96]. In *Nbs1*$^{flox/-}$*;Vasa-Cre (Nbs1 vKO)* transgenic mice, NSB1 was conditionally knocked out preceding the time in meiotic development when Spo11-mediates DSBs. In this system, male mice were infertile. Zhang *et al.* observed improper chromosome synapsis using SYCP3 and γH2AX immunostaining of spermatocytes. Immunostaining also showed nuclear localization of MRE11 in the spermatocytes was disrupted by the depleted NBS1. Development of the Nbs1 vKO transgenic mouse model allowed for the assay of NBS1 as an indicator of MRN function, and in turn, meiotic recombination stress [95].

5.2 Applications

As modern healthcare concerns center around fetal development, mouse models can be used to understand how meiotic recombination is affected by compounds in our environment. Oogenesis is particularly important because the events of meiotic prophase I are highly influential on fetal survival. An *in utero* model has been used to assess fetal exposure to supplemented estrogen and how meiotic prophase I progression is altered in response. 17-ß-estradiol (E_2) was administered to pregnant mice. The meiotic outcomes were analyzed through γH2AX staining and examination by super-resolution structured illumination microscope where γH2AX presence would signify whether meiotic recombination occurred via the initiation of a DSB.

Quantifying γH2AX *in utero* is a valuable tool for assessing meiotic mutagenicity and then later influences fetal development and success [97].

In utero exposure to other environmental agents that cause DNA damage can be valuable for understanding carcinogenesis. The absence of the P53 tumor-suppressor gene is linked to spontaneous tumorigenesis [27, 98]. P53 knockout mice can be used as a model for assessing tumor development when exposed to cancer-causing agents. An *in utero* study evaluated the effects of high-dose vitamin E, hypothesized to have antioxidative properties, on tumorigenesis. Pregnant P53 knockout mice were fed high-dose vitamin E until gestation day 13 or gestation day 19. The addition of vitamin E altered the redox state of the *in utero* environment. Furthermore, the oxidative stress on the ROS-dependent embryonic and fetal pathways was evaluated. DNA isolation was performed for the fetal and embryonic tissues and high-performance liquid chromatography was used to quantify the formation of 8-oxo-dG which would be used as an oxidation marker. Vitamin E dosing was associated with an increase in tumorigenesis in the p53 knockout mice; however, further studies are needed to explore the relationship between vitamin E and the tumorigenesis pathway [27]. Assessing vitamins sold commercially is valuable to improving our understanding of what supplements are safe during pregnancy and how ROS may influence *in utero* cancer development.

Oxidative stress has important connections to ovarian aging because these ROS lesions in ovarian follicles increase with age. Oocytes remain dormant in the diplotene stage until they are released for fertilization providing time for ROS-induced oxidative damage lesions to accumulate, and an increase of these lesions in ovarian follicles with age [99]. Pol β and BER, a pathway for repairing DNA lesions caused by ROS and oxidative damage, have been associated with the aging process. As rats age, Pol β levels decline, and BER becomes less efficient [100]. Injection of small interfering RNA (siRNA) targeting Pol β into young murine oocytes resulted in decreased numbers of normal oocytes, reduced oocyte survival, and an increase in detectable 8-oxoG levels, as compared to controls. In a complementary study, injection of Pol β complementary DNA (cDNA) into aged murine oocytes resulted in overexpression of Pol β, increased oocyte survival, and a decrease in detectable 8-oxoG levels, as compared to controls. These studies suggest that Pol β function is important for oocyte survival and aging. There is a potential to apply the overexpression of Pol β in clinical settings to improve oocyte survival and potentially slow the damaging effects of DNA lesions on aging oocytes. This is a potentially important finding for improving fertility and pregnancy outcomes as aging signs of progress [99].

6. Conclusion

Genomic instability plays a prominent role in the initiation of pathologies such as aging, immunodeficiencies and carcinogenesis. To combat the lethal effect of DNA damage and strand breaks, cells have evolved multiple, often overlapping DNA repair pathways efficiently and accurately repair DNA. Induction and assessment of genotoxicant DNA damage are particularly important *in vivo*. Further, these mouse models to assess DNA damage and repair can be combined with traditional mouse genetics to determine the impact of genetic modifications or polymorphisms with a focus on molecular analysis of DNA damage repair. As the number of designed and widely used synthetic environmental agents increases, understanding their impact on DNA integrity and downstream potential to promote mutagenicity is increasingly significant.

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Conflict of interest

The authors indicate no conflict of interest.

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