Chapter 34

Sins of Fathers Through a Scientific Lens: Transgenerational Effects

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Chapter Outline

1. INTRODUCTION

"Like mother-like daughter," "like father-like son"—these and other idioms signify a long-standing fascination of humans with similarities between parents and offspring. From ancient times, numerous theories have been proposed to explain trends and mechanisms of the inheritance of phenotypic traits. Hippocrates suggested that the parent–offspring similarity and sharing of certain phenotypic characteristics may occur due to an enigmatic blending of particles or fluids of parents. The pioneering works of Gregor Mendel established the precise rules of inheritance whereby heritable factors the nature of which was unknown at his time duplicate in parents and precisely segregate to progeny. Later on, Thomas Morgan determined that chromosomes may in turns serve as vehicles of Mendelian inheritance. Finally, upon the discovery of DNA and unraveling of its structure, DNA was unequivocally recognized as a fundamental agent of inheritance. As such, the key concept of medical genetics that seeks to link genotypes to phenotypes lies in the ability to establish links between individual genetic differences and individual phenotypic differences. For decades and until the mid-2010s, this perspective has dominated our understanding of genotypic and phenotypic variation and disease risk analysis, and thus has led to several important breakthroughs in the identification of numerous genetic underpinnings of heritable diseases. It has shaped many aspects of medicine, including those of organismal biology and evolutionary biology. Genetic components of heritable cancer syndromes and other diseases have been identified, and the presence of a genetic mutation, a polymorphism or chromosomal abnormality that promote disease form the current paradigm for disease etiology. Nevertheless, heritable components of heart disease, neuro-inflammatory and neurodegenerative disorders, diabetes, obesity, cancers, autoimmune, and other conditions remain remarkably elusive and lack the defined genetic components. This apparent "missing heritability" has sparked a lot of interest and research and has led researchers to revisit concepts of gene–gene (and more importantly gene–environment) interactions. Indeed, genome–environment interactions are equally important factors in disease etiology.

Even though the mammalian genome is rather stable because cells harbor numerous elaborate and highly efficient mechanisms that repair DNA, environmental factors have the ability to damage DNA directly by inducing genome-destabilizing mutations without altering DNA sequence, thus promoting disease via mechanisms that do not always involve direct DNA-damage or -sequence changes.

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Furthermore, as it has recently been established, environmental influences on disease etiology depend upon a developmental stage of an organism upon exposure to stress. Exposures during critical periods of time of organism development can alter genome activity associated with the differentiation program of cells or organ systems.

Genome stability and the ability to have healthy offspring is of an utmost importance for each individual organism and for entire populations. Understanding the rules of inheritance and the ability of the environment to influence inheritance and affect disease predisposition have been a central focus of research for many decades. Most recently, parental exposure and origins of disease have gained a lot of attention.

The analysis of genome–environment interactions has brought forth the concept of genome instability. A phenomenon of genomic or genome instability is used to describe an increased rate of acquired alterations in the genome of an organism or its offspring; the latter is referred to as transgenerational genome instability. The field of genome instability, and especially transgenerational instability, has emerged from studies that attempted to explain an unexpectedly high frequency of mutations and chromosomal damage in the progeny of irradiated somatic and germline cells.

2. RADIATION-INDUCED GENOME INSTABILITY

It has long been thought that the main factor contributing to the negative biological effects of ionizing radiation (IR) in mammalian cells, such as chromosomal aberrations, mutations, and cell death, is the result of DNA damage in directly exposed cells; that is, residual damage that has not been eradicated by DNA-repair systems in the exposed cell [1]. This paradigm has widely been challenged since 2000, mostly originating from the results of numerous in vitro studies that demonstrated the existence of delayed effects of IR exposure [2]. These delayed effects can manifest in the unexposed progeny of irradiated cells for many cell divisions (and up to 4 years) after the initial exposure [2]. The all-encompassing term given to this phenomenon is "radiation-induced genomic instability," which is used to describe the increased rate of the acquisition of alterations in the genome. Experimentally, genomic instability is observed when a cell is irradiated, then clonally expanded, and the progeny is examined genetically. As mentioned, radiation-induced genomic instability is observed generations after the initial exposure, and a number of studies have shown that this often occurs at a high frequency [3,4].

Multiple genetic end points have been utilized to evaluate radiation-induced genomic instability in a number of in vitro systems, which include, but are not limited to, chromosomal aberrations, ploidy changes, micronucleus formation, gene mutations, and amplifications, as well as increased microsatellite/ESTR (expanded simple tandem repeat) mutation rates and delayed cell death [2,5,6]. There are a number of pathways that are implicated in the initiation and perpetuation of radiation-induced genomic instability [7]. The relative contribution of the different pathways primarily depends upon the genetic background of the irradiated cell or organism [8,9], as well as the type of radiation [4].

Various in vitro systems have demonstrated a high frequency of IR-induced genomic instability by means of examining the various end points (as described earlier) that are associated with IR-induced genomic instability [2]. As of 2016, a prevailing hypothesis is that IR exposure destabilizes the genome, thus initiating a cascade of genomic events that increases the rate of point mutations, small deletions/insertions, and large rearrangements in the progeny of irradiated cells [2].

It has long been speculated that the development of genomic instability can facilitate the process of cancer initiation and/or progression [10], and indeed the loss of genomic stability is believed to be a hallmark of many cancers, as well as an important prerequisite for cancer formation [11–13]. Therefore, the general assumption is that there is a link between the induction of IR-induced genomic instability and cancer due to an increase in the accumulation of multiple genetic events within a cell that ultimately enhances radiation-induced carcinogenesis. This assumption is also supported by the findings of epidemiological studies which suggest that some types of radiation-induced cancers may follow a relative risk model in which IR exposure enhances the rate at which cancers develop, instead of inducing a specific cohort of new tumors [14]. The demonstration of IR-induced genomic instability in somatic cell–culture systems has greatly increased interest in research on the potential long-term effects of exposure. The research area that has likely benefited the most is the potential long-term effects associated with germline exposure to IR and the transmission of adverse effects (eg, genomic instability) to future generations.

2.1 Transgenerational Effects and Transgenerational Genome Instability

Initially, in vitro data have provided overwhelming evidence for the delayed effects of IR exposure manifested in the progeny of irradiated cells (ie, genomic instability) for many cell divisions, which may ultimately enhance the carcinogenic potential of these cells. Moreover, these data suggest that genomic instability can also be induced in the irradiated germline and, therefore, may be transmitted to future generations. If this is the case, the offspring of irradiated parents become genetically unstable, which results in a plethora of transgenerational effects such as the elevated mutation rates and

a predisposition to cancer. Many publications have indeed characterized a wide variety of phenotypic traits observed in the offspring of irradiated parents, implicating the elevated mutation rates [15–19]. Such studies have been reinforced through various molecular techniques used to assess transgenerational genomic instability.

The first evidence for a *transgenerational effect* associated with IR exposure was demonstrated by Luning and colleagues, where the elevated rates of dominant lethal mutations (early and late embryonic death) were observed upon the intraperitonial injection of a plutonium salt solution to male mice [20]. Accordingly, an increase in dominant lethality was not only found in the germline of directly irradiated male mice, but also in the germline of their nonexposed first-generation progeny (F1). The offspring of irradiated male mice have also been shown to be reproductively challenged, exhibiting the reduced fertilization rates of both in vivo and in vitro fertilization [16,21] as well as the increased levels of prenatal mortality in the F2 generation [22]. An increase in teratogenic effects was also shown, since the number of malformed F2 fetuses was significantly higher in the paternally exposed group compared to a control one [22].

The elegant studies by Nomura have not only demonstrated that paternal irradiation leads to an increase in malformations in the progeny of irradiated parents, but they have also shown a significant increase in the incidence of cancer in the offspring [23,24]. Several additional transgenerational studies have also found a significant increase in cancer incidence among the offspring of paternally irradiated mice after the secondary exposure to known carcinogens [23,25,26]. The predisposition of the offspring of IR-exposed fathers to cancer has been investigated in human populations, where the data obtained have mainly been inconclusive [27,28]; however, two independent studies have shown a clustering of extremely high leukemia rates in children whose fathers had been exposed to radiation after working at a nuclear processing plant in the town of Sellafield [29,30].

Adding to the classical evidence of transgenerational impacts, the majority of data since 2000 have arisen from the application of an array of molecular techniques used to characterize genotypic alterations in unexposed offspring. Mainly, genotypic alterations found in the progeny of irradiated parents have included chromosomal aberrations, micronuclei formation, increased microsatellite/ESTR mutations, and altered gene-expression patterns, which are all key hallmarks of genomic instability seen in somatic cells [2,31,32]. Therefore, the manifestation of such alterations has collectively been termed *transgenerational genome instability*. Dubrova and colleagues have made a significant contribution to our current understanding of radiation-induced transgenerational genome instability by pioneering the investigation of transgenerational mutation rates within repetitive sequences of the genome [18,33]. These repetitive sequences were initially termed minisatellites, but now they are known as expanded simple tandem repeat (ESTR) loci because they are extended (500–16, 000 bp) stretches of relatively short (4–6 bp) repeats that are less stable than true minisatellites which generally consist of longer (6–100 bp) repeats [18]. Barber and colleagues studied mutation rates of two ESTR loci in the germline of F1 and F2 offspring of male mice exposed at either the premeiotic or postmeiotic stages of spermatogenesis [34]. They found an increased mutation rate in the germline of F1 offspring, which was similarly maintained in the germline of the F2 offspring in both pre/postmeiotic germ cell exposure groups. Furthermore, the elevated mutation rates were seen in all three of the mouse strains studied, and within each strain, male and female offspring (both F1 and F2) of irradiated fathers equally demonstrated the elevated mutation rates [34]. Further analysis of the unexposed F1 progeny showed that high ESTR mutation rates were observed along with the elevated levels of mutations in protein-coding genes in the germline as well as in somatic tissues such as spleen and bone marrow [35]. The observed transgenerational instability is not specific to one particular strain of mice; in fact, it has been observed in the F1 and F2 offspring of irradiated males from four different inbred strains of mice [32,34,36].

Furthermore, Barber and colleagues have also shown that spontaneous levels of SSBs and DSBs are significantly higher in the unexposed F1 offspring; however, the efficiency of DNA repair was not compromised [35]. Likewise, Koturbash and colleagues found that DNA DSBs were higher in the thymus of offspring of irradiated fathers [37]. They also analyzed Rad51 and Ku70 protein levels as indicators of homologous recombination (HR) and nonhomologous end joining (NHEJ) repair pathways, respectively. In contrast to the results of Barber and colleagues, they found evidence of a compromised HR-repair pathway indicated by downregulation of Rad51, while NHEJ was unaffected [37]. This may not necessarily result in a decrease in DNA-repair efficiency but may impact the accuracy and quality of DNA repair. Furthermore, changes in expression levels of Rad51, be it up or down, have been associated with genome instability and cancer [38–40].

In their study, Baulch and colleagues analyzed the F3 offspring of males irradiated at the B-type spermatogonial stage and found altered protein kinase activities and protein levels of p53 and p21 [36,41]. p21 is a target of p53 that arrests or slows cell-cycle progression [42]. Further investigation including the fourth-generation offspring revealed similar changes in the kinase signaling activity and protein levels of p53 and p21, although the magnitude and direction of changes in each end point differed between generations and within generations [43]. This finding alone highlights the phenotypic variability observed in the offspring of exposed males.

Filkowski and colleagues reported the existence of genome instability in the germline of male mice subjected to whole-body irradiation and their progeny, whereby parental irradiation led to the reactivation of long interspersed nuclear elements 1 (LINE1) and short interspersed nuclear elements B2 (SINE B2) [44].

Transgenerational radiation-induced effects seem to be paternal in nature, and up to now, the long-term genetic effects of maternal irradiation remain under-investigated. Dubrova and colleagues undertook an in-depth study to establish the effects of radiation exposure on mutation induction in the germline of radiation-exposed females and the potential of induction of radiation-induced transgenerational effects in their nonexposed offspring [45]. To address this question, adult female BALB/c and CBA/Ca mice were given 1 Gy of acute X-rays and mated with unexposed males, and the frequency of mutations at ESTR loci in the germline of directly exposed females and somatic tissues of the progeny was analyzed. Surprisingly, irradiation did not affect the frequency of ESTR mutations in the germline of exposed females and their progeny. Thus, in sharp contrast to the effect of paternal irradiation that resulted in an increase in the ESTR mutation frequency in the offspring of irradiated males, maternal irradiation did not impact genome stability of their F(1) offspring. Therefore, the transgenerational effects of maternal high-dose acute irradiation are likely to be negligible [45].

Interestingly, the study of the effects of in utero irradiation also revealed sex-based differences in the induction of transgenerational genome instability. In a large-scale study, Barber and colleagues studied the effects of in utero irradiation on mutation rates at the ESTR DNA loci in directly exposed mice and their first-generation (F(1)) offspring [46]. The analysis revealed that the ESTR mutation frequencies in the germline and somatic tissues of male and female mice irradiated at 12 days of gestation remained highly elevated during adulthood, especially due to the high frequency of singleton mutations, suggesting that fetal irradiation leads to genomic instability both in utero and during adulthood. Furthermore, the ESTR mutation frequency was significantly increased in the F(1) offspring of prenatally irradiated male mice as compared to controls, proving that fetal exposure leads to transgenerational genomic instability. Contrarily, female in utero exposure did not affect genome stability in the F(1) offspring [46]. Even though radiation-induced transgenerational instability is predominantly paternal in nature, some effects appear to be synergistic when both male and female parents are exposed [37].

Transgenerational radiation-induced effects were also observed in rainbow trout. In trout, unlike in mammals, maternal and paternal irradiation may be equally important in causing transgenerational effects [47]. Additionally, the IR-induced transgenerational effects were reported to occur in *Caenorhabditis elegans* [48], Daphnia [49], medaka fish [50], and other organisms.

Most importantly, the transgenerational effects were seen in human populations exposed to the environmental or medical irradiation, albeit data from human populations are much less clear and somewhat ambiguous. As such, the analysis of mutation rates in genomic repeat elements has also been applied to study the transgenerational IR effects in human populations, namely in individuals living in the vicinity of the Chernobyl reactor accident and nuclear test sites (Semipalatinsk, Kazakhstan) [51–53]. In all of these studies, they found an increase in mutation rates among the progeny of exposed parents. Taken together, these data support the hypothesis that exposure to IR can induce germline genomic instability that may predispose future generations to an increased risk of genetic diseases, infertility, and even cancer.

2.2 Bystander Effects

Adding to the complexity of radiation responses, several studies determined that radiation effects can be seen not only in the irradiated cells and their progeny but also in the distal naive "bystander cells" that received distress signals from the exposed cells as well as in the progeny of naive bystanders. Some initial evidence of a bystander effect has been obtained from studies performed at the beginning of the 20th century. Murphy and Morton, whose research interests were devoted to the study of lymphoid cells, showed altered morphological changes in lymphoid cells after culturing them with serum from radiation-exposed animals [54]. Additionally, in 1954, Parsons and colleagues reported the presence of soluble "clastogenic" factors in the circulating blood of patients who underwent radiotherapy [55]. These factors were found to be able to induce damage in the unexposed cultured cells [56–59]. Such clastogenic activity has also been demonstrated in the plasma from patients who received high-dose radiotherapy and from individuals accidentally exposed to radiation from the Chernobyl accident. Similar to genomic instability, bystander effects manifest themselves as the induction of gross chromosomal rearrangements, chromosome aberrations, sister chromatid exchanges, deletions, duplications, mutations, amplifications, and cell death [60]. Bystander effects occur in the whole organisms in vivo; and 2008 studies showed that localized cranial exposure causes an in vivo bystander response not only in somatic tissues but in the male germline as well [61]. Bystander damage to the germline caused by localized cranial radiation has transgenerational consequences causing profound effects in the unexposed progeny [61].

Therefore, environmental as well as diagnostic and therapeutic radiation exposures can lead to a wide array of effects in the unexposed progeny.

3. MECHANISMS OF TRANSGENERATIONAL EFFECTS: EPIGENETIC CHANGES

The aforementioned radiation-induced effects did not segregate in a Mendelian manner, and therefore they were proposed to be epigenetic in nature. Epigenetic alterations are meiotically heritable and mitotically stable alterations in gene expression that occur without changes in DNA sequence; they include DNA methylation, histone modifications, and the ncRNA-mediated regulation of gene expression [62].

3.1 DNA Methylation

DNA methylation was the first epigenetic alteration identified, and it is the most widely studied epigenetic mechanism. In mammals, DNA is methylated at the carbon 5 of cytosine residues to form 5-methyl-cytosines (5meC) which is established by de novo DNA methyltransferases (DNMT3a, DNMT3b, and DNMT3L), and is subsequently maintained by DNMT1 [63–65]. The de novo DNA methylation of transposons in the germline is dependent on DNMT3L, an isoform of DNMT3a and DNMT3b that lacks the methylation activity [66]. DNA methylation is known to be associated with inactive chromatin states and in most cases, with the repression of gene expression [67–69]. A proper regulation of DNA methylation is critically important for the normal development, cell proliferation, and the maintenance of genomic stability [62,70,71]. The global loss of DNA methylation has been linked to the activation of transposable elements, the elevated chromosome breakage, aneuploidy, the increased mutation rates, and therefore to the phenomenon of genomic instability [69,71,72]. In addition, the altered global DNA-methylation pattern is a well-known characteristic of cancer cells, and the global loss of cytosine methylation was the first epigenetic abnormality discovered in cancer cells [73–75]. The DNA methylation profile of cancer cells is frequently characterized by the global genome hypomethylation as well as by the concurrent hypermethylation of selected CpG islands within gene promoters (eg, tumor suppressors) [62,72,76,77].

Direct IR exposure has been reported to affect DNA-methylation patterns. Acute exposures to low-LET radiation, such as X-rays and/or γ-rays, have been noted to result in the global genomic DNA hypomethylation [78]. Since the early 2000s, IR exposure has been found to lead to the profound dose-dependent and sex- and tissue-specific global hypomethylation [79–82]. The loss of methylation was also associated with radiation-induced alterations in the expression of DNA methyltransferases, especially de novo methyltransferases DNMT3a and DNMT3b [80,83]. Most importantly, the radiation-induced global genomic DNA-hypomethylation patterns appear to be linked to genomic instability in exposed animals [79,80,82,83].

DNA methylation also plays a role in radiation-induced bystander effects. Kaup and colleagues lead the way in showing the importance of DNA methylation in the maintenance of radiation-induced bystander effects [84]. They have demonstrated that dysregulation of DNA-methylation patterns occurs in nonirradiated cells and can persist for 20 passages when they are treated with the medium from irradiated cells [84]. These bystander cells marked with aberrant methylation patterns exhibited numerous end points characteristic of genome instability [84]. The same pattern of genomic instability and a significant loss of nuclear DNA methylation was also observed in 3D human tissue models [85].

Much insight into the role of such epigenetic changes in bystander effects and transgenerational effects in vivo has come from the pioneering work of the Kovalchuk's and Engelward's laboratories. By demonstrating that radiation exposure limited to half of the body leads to the elevated levels of DNA strand breaks and the altered levels of key proteins involved in establishing and maintaining methylation marks in lead-shielded tissues at least 0.7 cm from the irradiated tissue, they produced the first data to clearly demonstrate that epigenetically regulated bystander effects occur in vivo [86]. Using localized cranial X-irradiation in a rat model, it was also shown that IR exposure can induce the profound global DNA hypomethylation in distant bystander tissues (the spleen) 24 h after exposure [87]. Importantly, these changes were still observed 7 months after exposure [87]. This is relevant to carcinogenesis due to the fact that epigenetic manifestations of bystander effects persisted over a long period of time (in humans, it was roughly equal to 10 years). Again, a profound and persistent reduction of methylation in the bystander spleen was paralleled by a decrease in the levels of key proteins involved in the establishment and maintenance of methylation patterns (eg, DNMT3a, DNMT1, and the methyl-binding protein 2 (MeCP2)). This was believed to contribute to the reactivation of the LINE1 retrotransposon observed in the bystander spleen [87]. Such hypomethylation was also manifested in the bystander germline of cranially exposed mice [61].

Consequently, the involvement of the same type of epigenetic effectors (the global DNA methylation and associated proteins) in transgenerational effects induced from the paternal whole body and localized exposure to IR has also been studied [37,44,61]. The paternal whole-body and cranial IR exposure were shown to result in a significant global loss of DNA methylation in the thymus, bone marrow, and the spleen of F1 offspring [37,44,61]. Whole-body exposure also resulted in a specific hypomethylation of LINE1 and SINE B2 in the germline of exposed males, which was further observed in the thymus of unexposed offspring [44]. The thymus of the progeny of paternal whole-body exposures to IR and bone marrow

of the offspring of fathers exposed to cranial IR, in which the most pronounced decrease in DNA methylation was observed, also exhibited a significant decrease in the expression of DNMT1, DNMT3a, DNMT3b, and the methyl-binding protein MeCP2 [37,44,61]. The global loss of DNA methylation and the altered levels of methyltransferases and methyl-binding proteins can lead to the activation of transposable elements, contributing to genomic instability [88–90]. Accordingly, it can also be suggested that the global loss of DNA methylation observed in the progeny of irradiated fathers may influence retrotransposons and satellite DNA, thus underlying transgenerational genome instability. If such hypothesis is corroborated, it may help elucidate the increased mutation rates in satellite DNA and ESTR loci observed in the progeny of exposed parents [32]. Even though these epigenetic alterations are the well-characterized consequences of radiation exposure, the underlying molecular mechanism that drives these alterations, especially the site-specific changes in DNA-methylation patterns, remain elusive. Such molecular mechanisms may very likely be the main contributors to IR-induced epigenetic alterations associated with germline genomic instability, and therefore they would be strongly implicated in facilitating the epigenetic inheritance of transgenerational IR effects.

3.2 Histone Modifications

Changes in DNA methylation do not occur as isolated events because they are closely connected to other components of chromatin structure, such as histones, histone variants, and histone modifications [62,72]. The main histone modifications include acetylation, methylation, phosphorylation, and ubiquitination [91]. There is a vast complexity of epigenetic control that can be exhibited from such modifications since each of these modifications has the differing transcriptional consequences compounded by further control that depends on the type of residue to be modified and the extent of modification (eg, mono-, di-, and trimethylated) [72,92,93]. Studies in 2005 indicated that the IR-induced global loss of DNA methylation may correlate with changes in histone methylation, specifically with the loss of histone H4 lysine trimethylation [83].

One of the best studied histone modifications following IR exposure is the phosphorylation of histone H2AX at serine 139 (γH2AX). γH2AX is possibly one of the earliest cellular responses to DSB and IR exposure. The formation of γH2AX is crucial for the repair of DSBs and for the maintenance of genome stability [94–96]. The involvement of H2AX phosphorylation in bystander and transgenerational IR effects has also been suggested. The elevated levels of γH2AX have been reported in somatic and notably germline bystander tissues in vivo, and this elevation has subsequently been observed in the offspring of exposed fathers [35,37,61,86,87].

3.3 Small RNA-Mediated Events

Epigenetic mechanisms also include small noncoding RNAs [97]. Among those, two types are of a particular interest: microRNAs (miRNAs) and Piwi-interacting RNAs (piRNAs). MicroRNAs are abundant, small (~21-25 nt) single-stranded noncoding RNAs that regulate gene expression primarily at the posttranscriptional level (eg, posttranscriptional gene silencing, PTGS). Initially, miRNAs are endogenously transcribed as a part of a primary transcript (pri-miRNA) that is able to form one or more hairpin structures (miRNA stem loops) formed by complementary sequences within the transcript. miRNA genes can be transcribed independently or clustered with others and transcribed as a polycistron [98]. There is also a large number of intragenic miRNAs transcribed from within introns or exons of protein-coding and noncoding genes [99]. These primary transcripts are then processed by the RNase III enzyme Drosha in the nucleus into stem-loop-structured miRNA precursors (pre-miRNA) that are about 70 nt long. They are then exported to the cytoplasm where Dicer (the RNase III enzyme) generates a characteristic dsRNA (21–25 nt in length) that is separated into two strands, one of which is incorporated into a member of the Argonaute protein family (AGO2), a central component of the microRNA ribonucleoprotein complex (miRNP) commonly known as the RNA-induced silencing complex (RISC) [100]. To control the translation of specific mRNAs, the miRNA-guided RISC complex binds to the 3′-UTR (untranslated region) of target mRNAs with a similar sequence structure, thus serving as a translational repressor that regulates protein synthesis by targeting specific mRNAs [101]. As of 2016, it is believed that miRNAs exhibiting a high degree of complementarity to their target mRNAs are able to repress translation through mRNA cleavage. However, most miRNAs have imperfections between the complementary sequences, and therefore repress translation without mRNA cleavage [102,103]. Although the precise nature of such regulation remains unclear, it is suggested that the main mechanisms include alterations of poly(A) tail length and the binding of regulatory proteins to the UTRs of target mRNAs [97,104]. One or many miRNAs can coordinate the expression of single/multiple genes, resulting in a complex mechanism for posttranscriptional gene regulation. Consequently, miRNAs can play a key role in numerous biological contexts, including cellular differentiation, proliferation, apoptosis, and even a predisposition to cancer [105–107]. The altered levels of miRNAs have been reported in a variety of cancers [108,109].

Not unexpectedly, miRNAs are also involved in IR-induced responses in vivo [44,87,110–119] and in radiation-induced germline and transgenerational effects [44,117]. Tamminga and colleagues reported that radiation exposure significantly affected miRNA expression in testes [117]. Radiation exposure caused DNA damage and led to the ATR/Rfx1-mediated increase in miR-709 expression in exposed testes. This miRNA targeted the Brother of the Regulator of Imprinted Sites (BORIS), an important regulator of DNA methylation and imprinting.

Filkowski and colleagues showed that irradiation led to the upregulation of the miR-29 family in the exposed male germline, which caused a decrease in the expression of de novo methyltransferase, DNMT3a, and a profound hypomethylation of LINE1 and SINE B2 [44]. Epigenetic changes in the male germline led to deleterious effects in the somatic thymus tissue of the progeny of exposed animals, including hypomethylation of LINE1 and SINE B2 associated with a significant decrease in the levels of lymphoid-specific helicase (LSH) that is crucial for the maintenance of methylation and the silencing of repetitive elements. Moreover, the thymus tissue of the progeny of exposed parents exhibited a significant upregulation of miR-468 that targeted LSH and led to its decreased expression in the thymus. The study suggested that miR-468-mediated suppression of LSH led to an aberrant methylation of LINE1 and SINE B2 [44].

Recently, a novel small RNA pathway has been characterized, providing evidence for yet another small RNA-mediated epigenetic effector. Known as the Piwi/piRNA pathway, it has several unique features that make it quite suitable as a mediator of epigenetic memory in germ cells. Here, key features of the piRNA pathway is introduced, followed by a further discussion in the context of spermatogenesis in the rodent germline.

3.3.1 piRNA Biogenesis and Role in Maintaining Genome Stability

Being initially characterized in *Drosophila* [120], the central component of the pathway is a large class of short, single-stranded, noncoding RNAs (~26–31 nt) and their Piwi protein partners, a subclass of the Argonaute protein family. Both Piwi-interacting RNAs (piRNAs) and Piwi proteins have expression patterns that are largely restricted to germ cells in nearly all multicellular animals studied [121]. Piwi proteins are required for the production of their piRNA partners and are essential for various stages of spermatogenesis, the self-renewal of germ stem cells, and transposon silencing [121,122]. The best studied function of the piRNA pathway is to maintain genomic integrity by the suppression of transposable elements (TEs) via transcriptional gene silencing (TGS) [121]. TGS occurs through piRNA-mediated de novo methylation of regulatory regions of retrotransposons in embryonic germ cells; methylation is believed to be subsequently maintained in germ and somatic cells throughout the life of an organism [123,124]. While mutations in the DNMT family members affected cytosine methylation, the piRNA pathway remained largely unaffected [123]. In contrast, the loss of the piRNA pathway prevents the recognition and silencing of TE by DNMT3L, thus supporting a model in which the piRNA pathway acts upstream of DNMT3L, DNMT3a, and DNMT3b to establish patterns of DNA methylation on TEs [123]. PTGS also contributes to this process because the piRNA-guided Piwi proteins also mediate the cleavage of active transposon mRNAs from which primary piRNAs are likely to be derived through a process known as the "ping-pong" amplification cycle [125,126]. However, it is important to note that the majority of mouse and rat piRNAs are not enriched in sequences derived from transposons and repeats. In mice and rats, repeats are underrepresented because only about 17% of all piRNAs map to repetitive elements, while a random distribution should yield close to 40%, which is the proportion of repetitive sequences in the genome [127,128]. In mammals, piRNAs tend to cluster within certain regions of the genome, and a large number of piRNAs are derived from intergenic regions, but are also distributed among exonic, intronic, and intergenic repeat sequences [104]. The distinguishing feature of these clusters of uniquely mapping piRNAs is the pronounced strand bias, which leads to the suggestion that the biogenesis of piRNAs involves a long, single-stranded precursor [129]. Since piRNA sequences correspond to a variety of genomic regions, the piRNA pathway may be involved in a more complex system regulating the expression of a plethora of genes other than repetitive elements.

Indeed, several studies in the mid-2000s suggest that the piRNA pathway is not limited to the repression of transposable and repetitive elements, and it plays the diverse and complex roles in regulating gene expression at all known levels of epigenetic control. Piwi proteins and piRNAs together have been associated with mRNA and mRNA cap-binding proteins in polysomes and ribonucleoproteins (RNP) which play a central role in translational control. However, the molecular mechanisms that achieve this translational regulation and the resulting outcomes remain largely unclear [104,122,130]. The biochemically purified endogenous rat piRNA complex has been shown to exhibit the RNA cleavage activity, presumably facilitated by the rat Piwi protein, Riwi [131]. On the other hand, mouse Piwi proteins may actually be responsible for the stability of a subset of mRNAs, and the positive regulation of translation [130,132]. In addition, the Piwi protein in mice (Miwi) is required not only for piRNA but also for a particular subset of miRNAs [104]. Thus, the piRNA pathway may be involved in miRNA-mediated translational control. One common feature of *Piwi* gene mutations in mice is an increase in DNA damage marked by γH2AX foci, thus suggesting a possible link to DNA-damage repair/checkpoints [133,134].

It has been proposed that such dsDNA breaks are a result of overactive transposons; however, this relationship is not fully understood as dsDNA breaks could also be the cause of transposon activity rather than a result of it [135]. The presence of RecQ1 in the rat Piwi protein complexes is consistent with a possible role of mammalian Piwi-type proteins in DNA-repair processes [131].

RecQ is a family of helicase enzymes that have highly conserved roles in dsDNA-break repair through recombination [136]. The ability of the piRNA pathway to mediate epigenetic control of gene expression at the level of histone modifications has also been described. Human cells have been transiently transfected with a human Piwi (Piwi-like4/Hiwi2) gene containing a vector construct which induces histone H3K9 methylation at the p16Ink41 locus, resulting in a significant downregulation of p16 gene expression [137]. A 2009 study has provided some intriguing evidence for the production and function of a particular subset of abundant piRNAs which are depleted in the TE content and do not engage in the ping-pong cycle [138]. They reported a substantial population of piRNAs derived from UTR of protein-coding genes. These genic piRNAs arise preferentially from 3′-UTRs produced by a piRNA biogenesis pathway that does not require the ping-pong components and are conserved across *Drosophila*, mice, and *Xenopus* [138]. This breakthrough finding and the previously discussed studies provide overwhelming evidence for an additional and much larger breadth of piRNA-mediated gene regulation, although the role of piRNAs in TGS of TEs still remains unexplained.

3.3.2 piRNAs as Mediators of the Epigenetic Memory

The piRNA/Piwi pathway has several features that make it suitable as a mediator of the epigenetic memory in germ cells. Being mainly characterized by its ability to exert TGS by driving methylation of TE, it clearly has the ability to affect genome stability in future generations. Moreover, even though this novel small RNA pathway has been shown to play a role in many epigenetic alterations observed in response to IR, no experiments have been conducted to examine a possible role and response of this pathway to IR exposure. Because this pathway is mainly restricted to the male germline in mammals, it provides a novel mechanism to facilitate the paternal epigenetic inheritance of IR-induced genomic instability. It can also provide some insight into the observed loss of LINE1 and global DNA methylation not only in the germline of exposed males, but more importantly, in the next generation [37,44,61]. The understanding of and how the piRNA pathway responds to IR exposure can also potentially corroborate and help elucidate the increased mutation rates observed in satellite DNA and ESTR loci in the somatic and germline tissue of the progeny of exposed parents [32].

Very little is known about the role of the piRNA pathway in the production/inheritance of IR-induced genomic instability. Unpublished data from our laboratory show that radiation exposure causes profound alterations in piRNA profiles, affecting several piRNA clusters. Changes in piRNA levels are associated with the altered levels of DNA methylation of the corresponding piRNA loci in the exposed germline and in the progeny of exposed animals. Therefore, piRNAs may hold the key to understanding epigenetic mechanisms of germline and radiation-induced transgenerational genomic instability.

4. TRANSGENERATIONAL EFFECTS CAUSED BY OTHER MUTAGENS

While transgenerational effects and genome instability upon IR exposure have been mostly studied, since the mid-2000s, numerous studies appeared that showed the induction of transgenerational effects by a wide array of other chemical mutagens.

Parental exposure to urethane, 4-nitroquinoline 1-oxide and 7,12-dimethylbenz(a)anthracene led to an elevated cancer risk and mutations in the offspring [139,140]. Transgenerational effects were also reported upon exposure to anticancer drugs. The F2 offspring of males exposed to cyclophosphamide were reported to exhibit genome instability and an increase in postimplantation loss and congenital malformations [141]. Cyclophosphamide or a combination of cyclophosphamide and vinblastine caused behavioral alterations in the first- and second-generation offspring of male rats [15,45]. Paternal exposure to anticancer chemotherapy altered the quality of germ cells and profoundly affected embryo development, thus causing transgenerational effects [142]. Embryos born upon paternal cyclophosphamide exposure exhibited the elevated levels of DNA damage and dramatic alterations in the levels of DNA repair and homologous recombination genes [143]. Furthermore, our unpublished data show that paternal exposure to mitomycin C and cyclophosphamide alter gene and protein expression in the frontal cortex and the whole brain of unexposed progeny.

A large number of extensive studies have focused on transgenerational effects of environmental teratogenic agents such as endocrine disruptors. Seminal studies were conducted by Skinner and colleagues. They have shown that in utero exposure of rats to vinclozolin or methoxychlor during the period of gonadal sex determination leads to an increased infertility rate and a decreased spermatogenic capacity in the F_1 male progeny. Moreover, this altered reproductive capacity was transmitted via the male germline to a majority of male offspring up to the F_4 generation [144], and manifested

as transgenerational (F1–F4) spermatogenic cell apoptosis and subfertility [145]. Moreover, Skinner and colleagues have established that exposure to endocrine disruptors leads to transgenerational reprogramming of the testis transcriptome and the development of transgenerational diseases such as spermatogenic defects, prostate disease, kidney disease, and cancer [146,147]. Transgenerational effects caused by toxicants are epigenetic in nature. Exposure to pesticides dichlorodiphenyltrichloroethane (DDT) and methoxychlor (MXC) leads to the occurrence of transgenerational sperm epimutation signatures [148] that may in turn promote genome instability [149] (also reviewed in Refs. [150–152]).

Transgenerational transmission of the effects of gestational alcohol exposure has been reported. Prenatal alcohol exposure increases the risk for alcoholism by increasing the propensity to consume alcohol and by altering a neurophysiological response to alcohol [153]. Alcohol consumption modifies the sperm epigenome and thus leads to instability and behavioral effects in the progeny [154]. In 2015, deleterious transgenerational effects were reported for alcohol, opiates, cocaine, marijuana, and nicotine [155]. Exposure to these agents causes epigenetic changes in the genome that are transmitted to the next generation [155–158]. A wide array of other life-style factors such as diet, obesity, nutritional deficiencies, and stress make their mark on the germline and cause deleterious effects in the progeny [159–163].

5. CONCLUSIONS AND OUTLOOK

In sum, parental exposure to a wide array of environmental agents affects the germline and, therefore, cause transgenerational effects in unexposed progeny. Transgenerational effects can span numerous generations and transgenerational genome instability is a key mechanism underlying transgenerational effects. Studies have reported the causes, existence, and molecular processes affected in the germline and in the progeny of exposed parents. Environmental agents were shown to cause DNA damage, altered DNA-methylation levels and deregulated gene and small RNA expression in the germline of exposed parents. In the progeny, these cause the aberrant setting of DNA-methylation marks, altered gene expression and genome instability, which result in a wide array of downstream "snowball effects," such as the accumulation of mutations, genomic rearrangements, and further genome destabilization (Fig. 34.1). While numerous studies have proposed that transgenerational effects are epigenetic in nature, the precise mechanisms of transgenerational genome instability remain to be fully elucidated. Future research in this area must rely on the use of microarray technology, next-generation sequencing, and bioinformatic approaches in order to extract the functionally relevant, causal changes influencing genetic and epigenetic reprogramming and genomic stability across generations. Such research has both practical and fundamental value, as it may offer an understating of how genotoxic factors contribute to complex diseases by altering our epigenome across generations.

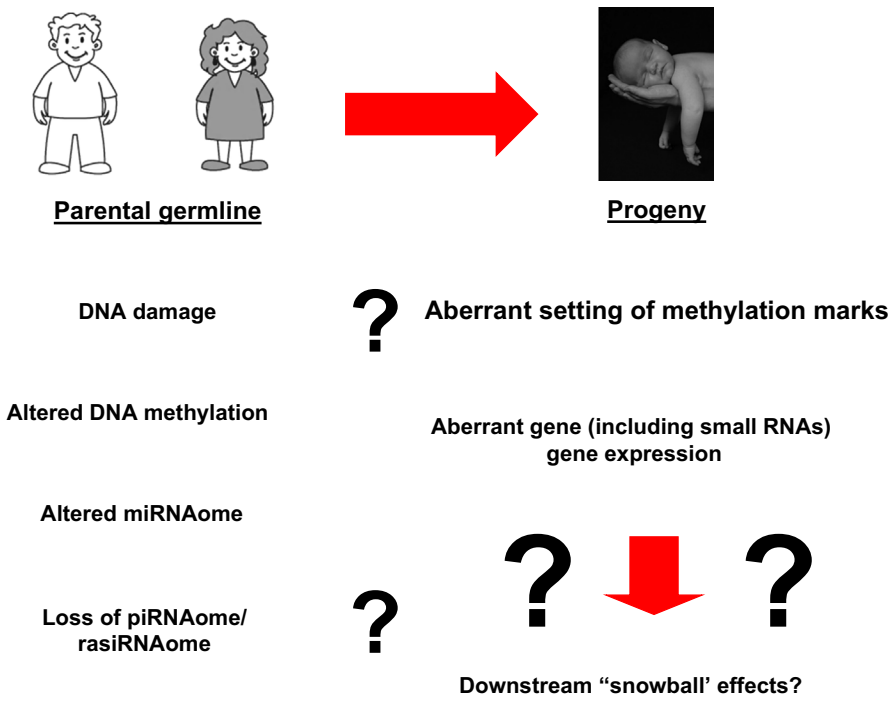

FIGURE 34.1 Transmission of stress exposure effects from the germline to progeny—potential mechanisms of transgenerational genome instability.

While this review was being written, several breakthrough articles have emerged that suggest the novel roles of small RNAs, especially TRNAs and tRNA fragments, in the germline and potentially in the transgenerational effects induced by diet deficiencies. Sperm tRNAs might mediate the transcriptional cascade effect and influence metabolic gene expression through the embryo to adulthood [164,165]. Further studies are needed to dissect the roles of small RNAs and small RNA fragments in transgenerational effects.

GLOSSARY

F1 generation The generation resulting from a cross of parental generation (the first set of parents).
Ping-pong cycle Cycle of piRNA production first identified from studies in *Drosophila*.
Transgenerational inheritance The transmittance of genetic and epigenetic information from one generation of an organism to the next ones.
Transgenerational effects A wide array of health effects that occur when environmental exposures or toxicants pass from parent to offspring.
Transgenerational genome instability Elevated frequency of mutations and genomic rearrangements transmitted from the germline of exposed parents to the progeny.

LIST OF ABBREVIATIONS

DDT Dichlorodiphenyltrichloroethane
DNMT DNA methyltransferases (3a, DNMT3b, and DNMT3L)
ESTR Expanded simple tandem repeat
LET Linear energy transfer
LINEs Long interspersed nuclear elements
miRNAs microRNAs
miRNP microRNA ribonucleoprotein complex
MIWI Mouse PIWI protein
MXC Methoxychlor
piRNA Piwi-interacting RNA
pri-miRNA Primary transcript miRNA
PTGS Posttranscriptional gene silencing
RISC RNA-induced silencing complex
RIWI Rat PIWI protein
RNP Ribonucleoproteins
SINEs Short interspersed nuclear elements
TEs Transposable elements
TGS Transcriptional gene silencing

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