Examination of generational impacts of adolescent chemotherapy: Ifosfamide and potential for epigenetic transgenerational inheritance

Highlights
- Chemotherapy-induced sperm DNA methylation facilitates epigenetic inheritance
- Chemotherapy promotes epigenetic transgenerational inheritance of pathology
- Pubertal exposure to chemotherapy impacts later life sperm epigenetics and genetics
- Cancer chemotherapy treatment needs to consider later life and generational impacts
Examination of generational impacts of adolescent chemotherapy: Ifosfamide and potential for epigenetic transgenerational inheritance

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SUMMARY
The current study was designed to use a rodent model to determine if exposure to the chemotherapy drug ifosfamide during puberty can induce altered phenotypes and disease in the grand-offspring of exposed individuals through epigenetic transgenerational inheritance. Pathologies such as delayed pubertal onset, kidney disease, and multiple pathologies were observed to be significantly more frequent in the F1 generation offspring of ifosfamide lineage females. The F2 generation grand-offspring ifosfamide lineage males had transgenerational pathology phenotypes of early pubertal onset and reduced testis pathology. Reduced levels of anxiety were observed in both males and females in the transgenerational F2 generation grand-offspring. Differential DNA methylated regions (DMRs) in chemotherapy lineage sperm in the F1 and F2 generations were identified. Therefore, chemotherapy exposure impacts pathology susceptibility in subsequent generations. Observations highlight the importance that prior to chemotherapy, individuals need to consider cryopreservation of germ cells as a precautionary measure if they have children.

INTRODUCTION
Epigenetics is defined as “molecular factors and processes around the DNA that alter genome activity, independent of DNA sequence, and are mitotically stable”.1,2 The epigenetic molecular factors and processes include: DNA methylation,3 histone modifications,4 non-coding RNAs,5,6 chromatin structure,7 and RNA methylation.2,8 The term “epigenome” is used to describe the collective sum of these modifications and how they affect gene expression.7 Considering these modifications, DNA methylation is the most investigated and understood.9,10 DNA methylation is used to describe the addition of a methyl group to the DNA base cytosine in a CpG sequence.10 Addition of a cytosine methyl group does not alter the DNA sequence itself,10 but does affect protein binding, genome structure, and gene expression to subsequently impact phenotype.9 Differential DNA methylated regions (DMRs) refer to a particular site of the genome in which two or more individuals have methylation patterns that are distinct.11

Epigenetic modifications typically arise during development in an individual in response to an environmental exposure.2,6 These epigenetic modifications can be correlated with higher rates of disease later in life.2 When these epigenetic modifications develop in the sperm or egg, they can be passed down through the germline to affect somatic cell gene expression in generations removed from the direct exposure.2,6 Figure 1. This is termed epigenetic transgenerational inheritance and is described as “germline-mediated inheritance of epigenetic information between multiple generations in the absence of a continued environmental exposure”.1,2,12 Epigenetic transgenerational modifications typically occur during key windows of development.2,13,14 These include two DNA methylation erasure periods which take place during development.13–15 The first of these erasure events takes place post-fertilization at the time of preimplantation development.13–15 The second of these epigenome CpG island erasure events happens in the primordial germ cell, germline pluripotent stem cells.13–15 Studies have indicated that some epigenetic marks (imprinted genes) are not entirely erased at these windows of germ cell and embryonic reprogramming.13–15 These findings demonstrate that the germline (sperm or egg) has the potential to pass on epigenetic modifications even in the absence of a direct exposure.13–15 In epigenetic inheritance, it is important to differentiate between multigenerational and transgenerational studies (Figure 1).1,2 Multigenerational

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studies are useful for understanding the direct exposure and effects of environmental factors. For example, a father’s smoking in adolescence, periconceptional period, and other environmental exposures have been observed to alter epigenetics in offspring. This involves a multigenerational exposure of the F0 generation individual and the germline of the subsequent F1 generation, Figure 1. Transgenerational studies are required for understanding epigenetic inheritance not involving direct effects of environmental exposures, Figure 1. These types of studies are often times difficult to find in models which have long generation times due to the cost and time required to conduct them. Many environmental exposures including dioxin, bisphenol A (BPA), and atrazine have been observed to induce epigenetic transgenerational inheritance following ancestral exposure. Each exposure has unique epigenetic modifications, but can promote similar phenotypes and disease.

Cancer and epigenetics

According to the National Cancer Institute, cancer is a broad term used to describe any type of disease in which abnormal cell growth occurs without normal control. It is estimated that worldwide, 19.3 million new cancer cases and nearly 10 million cancer deaths occurred in 2020. In 2021, 1,898,160 new cancer cases and 608,570 cancer deaths were projected in the United States alone. Chemotherapy is often used as part of the cancer treatment regimen, yet the long-term effects both in treated individuals, as well as offspring and grand-offspring, are not fully understood. The potential long-term and generational effects of chemotherapy exposure require analysis of correlated adverse effects and alterations in epigenetics. A previous study detected alterations in DNA methylation in the sperm of rats following a combination chemotherapy regimen commonly used to treat testicular cancer, suggesting that treatment can induce epigenetic alterations in sperm. A separate study observed peripubertal paternal exposure to the common chemotherapeutic drug cisplatin was linked to adverse effects in both male and female offspring reproductive development. While a rodent model is useful, understanding the effects in humans is important.

Childhood cancer survivors have increased risk of serious morbidity, premature mortality, and diminished health status. Four common problems noted in survivors include: neurocognitive dysfunction, cardiovascular disease, infertility and gonadal dysfunction, and psychosocial problems. A human study observed that both male and female children born to women exposed to chemotherapy had fewer children in comparison to controls. This effect was not observed in the children of men exposed to chemotherapy. It has also been observed that the children of men exposed to chemotherapy have a higher incidence of bipolar disease. The children of women exposed to chemotherapy do not appear to have a higher incidence of somatic diseases, with the exception of potential infertility. It is unclear if these findings are correlated with epigenetic alterations, so further investigation is required.

Chemotherapy generational impacts

Literature examining generational effects resulting from chemotherapy is limited. However, two studies suggest that effects persist in generations removed from the direct exposure. One study used a single
intraperitoneal injection of the common chemotherapeutic agent doxorubicin to investigate the long-term effects in a female-treated mouse model.32,33 The study determined that the effects were in fact transgenerational and were the most prominent in the fourth and sixth generations.33 Specifically, there were heightened rates of neonatal death, physical malformations, and chromosomal abnormalities in mice from the treated lineage.33 These doxorubicin-induced effects in oocytes were transmitted by both the males and females born to an exposed mother.33

Recently, a separate study used a mouse model to examine the effects that stem from preconceptional maternal exposure to cyclophosphamide, an agent commonly used in cases of breast cancer.34 Adult female mice from the treated lineage produced F1 generation offspring with delayed growth and altered methylation of three imprinted genes (H19, Igf2r, and Peg 3) in their oocytes.34 These same observations were discovered in the F2 generation as well, which indicates that preconceptional maternal exposure to a chemotherapeutic agent can result in long-term effects on the health of subsequent generations.34 The persistence of these effects in individuals not directly exposed to the compound supports the hypothesis that chemotherapy results in heritable alterations to the germline. Although these studies come from rodent models, it is suggested their findings could be applied to a human model due to physiological similarities.35

Childhood cancer
Childhood cancer is rare in comparison to cancer in adults.36 Yet each year there are an estimated 429,000 children and adolescents between the ages of 0 and 19 expected to develop cancer.37 Treatments for these individuals are typically very specialized and carried out at pediatric cancer centers, since childhood cancers are different from adult cancers.36–40 Osteosarcoma is the most common primary solid malignancy of bone.41 Worldwide incidence of osteosarcoma is approximately 3.4 cases per million people annually.42 In 2014, it was estimated that 450 children and 370 adolescents would be diagnosed with bone tumors in the United States, with osteosarcoma being responsible for 56% of pediatric tumors.36 Males are 1.4 times more likely to be affected when compared to females.41,43,44 Osteosarcoma is defined by the presence of malignant mesenchymal cells which generate osteoid and/or immature bone.41 If left untreated, the prognosis is poor with local and often metastatic progression of the disease.41 Throughout much of the twentieth century, five-year survival rates for conventional osteosarcoma were approximately 20%.42 However, since adjuvant chemotherapy was introduced to the treatment protocol in the 1970s, in combination with limb salvage, survival rates have increased to above 65%.42,45–48 Standard care of patients with osteosarcoma currently includes neoadjuvant chemotherapy, surgery, and adjuvant chemotherapy.42 Common therapeutic agents used in treatment of osteosarcoma include methotrexate with leucovorin rescue, doxorubicin, cisplatin, and ifosfamide.42 Etoposide may be used in cases where the patient has metastatic disease.42

The current study was based upon a previous collaboration between the Seattle Children’s Research Institute and Washington State University to obtain sperm samples from survivors of osteosarcoma between the ages of 19 and 30 years old.28 These patients had undergone cisplatin-based chemotherapy, with two of the cases requiring both cisplatin and ifosfamide when they were between the ages of 14 and 20.28 Analysis of the sperm samples demonstrated statistically significant differentially methylated regions of the genome, ten years after receiving treatment when compared to control samples.28,49,50 This indicates chemotherapy can permanently modify the spermatogenic stem cell (i.e., spermatogonia) of the sperm.

Based upon the previous study, the current study was designed to determine the potential impacts the chemotherapy drug ifosfamide may have in offspring (F1 generation) and the transgenerational grand-offspring (F2 generation) in a rodent model. It was hypothesized that the chemotherapy drug ifosfamide will induce germline epimutations that are transgenerational and persist through the F2 generation. Ifosfamide was selected for this experiment as it is commonly used in the treatment of cancers in male adolescents. These findings contribute to our understanding of chemotherapeutic agents and if treatment during adolescence can promote epigenetic modifications of the sperm that through epigenetic transgenerational inheritance impacts subsequent generations’ pathology and disease.

RESULTS
Pathology analysis
The transgenerational actions of control vehicle phosphate buffered saline (PBS) and ifosfamide (8.5 mg/kg of body weight) treatments administered to pubertal male rats (F0 generation) once every three days for
three treatments, starting at 26 days of age were investigated. Therefore, the F0 generation pubertal male was exposed to chemotherapy and as an adult bred to generate the F1 generation and F1 generation adult bred to generate the F2 generation for a transgenerational assessment, Figure 1, left example. F0 generation males were used for each control and ifosfamide exposure lineages and then pathology assessed at 1 year of age. Pathology was assessed for testis, prostate, or kidney disease in the F0 generation males that had aged to 1 year following pubertal exposure. No behavioral analysis was performed in the F0 generation males due to the low numbers. In the control lineage, (0/5) testis disease, (1/5) prostate disease, and (2/5) kidney disease was observed. The 1-year chemotherapy lineage (1/5) testis disease, (0/5) prostate disease, and (1/5) kidney disease was observed. No obesity or tumors were observed in any of the F0 generation animals at 1 year of age. Therefore, no statistically significant disease was observed in the F0 generation when control and chemotherapy were compared. The F1 generation (direct germline exposure) and F2 generation (transgenerational) rats of control and ifosfamide lineages were aged to 1 year and euthanized for analysis. No inbreeding (sibling or cousin crosses) was performed to maintain the outbred nature of the lines of rats.51 Testis, prostate, kidney, ovary, and adipose were collected and examined for histopathologies as described in the STAR methods (Figures S1–S5). The male and female pathologies are summarized in Figures 2 and 3, respectively, with the diseased individuals per total number of individuals presented for each generation and lineage, Tables S1–S4. An animal was considered to have a diseased tissue if the number of histological abnormalities was significantly increased (i.e., greater than 1.5 standard deviations) when compared to the control group for that tissue, as described in the STAR methods.

In testis, there was no significant increase in the frequency of pathology observed in the F1 generation, (Figure 2A). However, the F2 generation control group was found to have a significantly higher frequency of testis pathology than the F2 generation ifosfamide lineage animals (p<0.05), indicating a decrease in testis pathology frequency in F2 generation ifosfamide males. Ifosfamide treatment did not have a significant effect on the frequency of prostate pathology, (Figure 2B). Ifosfamide treatment also did not have a significant effect on the frequency of male kidney pathology, (Figure 2C).

In females, no significant differences were found in regard to the frequency of ovarian pathologies (Figure 3A). In female kidney, there was a significant increase in pathology frequency in the F1 generation ifosfamide lineage animals relative to the controls (p < 0.05) (Figure 3B).

Tumor frequency in males was not found to be significantly different between chemotherapy lineage and control animals (Figure 2D). The frequency across all treatment groups and generations was below 5%. The type of tumor observed with the highest frequency in males was pilomatricoma. No significant differences were observed in regard to the frequency of tumors in females as well (Figure 3C). The types of tumor observed with the highest frequency in females were mammary fibroadenomas and mammary adenocarcinomas.

Obese and lean phenotypes were analyzed as described in the STAR methods. Males had no significant differences observed in the F2 generation lean phenotype (Figure 2E), but there was a trend for an increased lean phenotype frequency in the F1 generation ifosfamide lineage males that was not significant, p = 0.068. There was a trend for a decrease in obesity phenotype frequency in F2 generation ifosfamide lineage males, but was not significant p = 0.062 (Figure 2F). In females, there were no significant differences observed across treatment and generation upon analysis for the lean phenotype (Figure 3D). There were no significant differences found in the frequency of the obese phenotype in females across treatment or generation (Figure 3E).

Puberty was analyzed for both early and late onset puberty in males and females. In males, a significant increase in the frequency of early pubertal onset was observed in F2 generation ifosfamide lineage animals (p<0.05) (Figure 2G). There were no significant differences found in the frequency of late onset puberty of males across treatment and generation. In females, no significant differences in the frequency of early onset puberty were observed (Figure 3F). However, there was a significant increase in the frequency of the late pubertal onset phenotype in the F1 generation ifosfamide lineage females when compared to the controls (p < 0.05) (Figure 3G).

Behavioral analysis of the F2 generation ifosfamide and control lineage consisted of an elevated plus maze (EPM), as well as a light and dark box (LDB) to measure anxiety-related behavior as described in the STAR methods.
methods. In F2 generation ifosfamide lineage males, a significant decrease in the frequency of anxiety behavior was observed using the EPM, measured as an increase in the proportion of animals that spent more time in the exposed arms of the maze (p < 0.05) (Figure 2H). No significant change in anxiety-related behavior was observed in F2 generation ifosfamide lineage males when tested using the LDB. In F2
generation ifosfamide lineage females, a significant decrease in anxiety behavior was observed using the EPM (p < 0.05) (Figure 3H). No significant change in anxiety-related behavior was observed in F2 generation ifosfamide females when using the LDB.

Multiple pathology analysis was performed by combining the results of other analyses. In the males, there were no significant differences in frequency of multiple pathologies when F1 and F2 generation ifosfamide lineage females, a significant decrease in anxiety behavior was observed using the EPM (p < 0.05) (Figure 3H). No significant change in anxiety-related behavior was observed in F2 generation ifosfamide females when using the LDB.

Multiple pathology analysis was performed by combining the results of other analyses. In the males, there were no significant differences in frequency of multiple pathologies when F1 and F2 generation ifosfamide lineage females, a significant decrease in anxiety behavior was observed using the EPM (p < 0.05) (Figure 3H). No significant change in anxiety-related behavior was observed in F2 generation ifosfamide females when using the LDB.
lineage animals were compared to their respective control groups (Figure 2). In females, there was a significant increase in the frequency of multiple pathologies in the F1 generation ifosfamide lineage females when compared to the F1 generation controls (p < 0.05) (Figure 3). A summary of the pathological findings has been provided (Table 1).

Seizures were reported in two F1 generation controls males from separate litters, one of which had to be euthanized due to health concerns. The WSU Veterinary School, Washington Animal Disease Diagnostic Laboratory (WADDL) performed a necropsy on that rat and discovered bronchioalveolar adenoma and glomerulosclerosis. Seizures were reported in a F1 generation ifosfamide lineage female as well. Upon dissection, no additional abnormal pathologies were noted in these rats.

**Sperm epigenetic analysis**

Analysis of sperm differential DNA methylation regions (DMRs) between F1 generation ifosfamide and control lineage animals was assessed with methylated DNA immunoprecipitation (MeDIP) followed by next-generation sequencing (MeDIP-Seq), as described in the STAR methods. The sperm DMRs at various p-values are presented with a p-value cutoff of p<1e-05 yielding a total of 201 sperm DMRs, with eleven of them having multiple adjacent 1000 bp windows (Figure 4A). A complete list of the F1 generation DMR can be found in Table S5. The same analysis was performed again, but between the F2 generation ifosfamide lineage animals and F1 generation control lineage animals (Figure 4B). A complete list of the F2 generation sperm DMR can be found in Table S6. This analysis identified at p < 1e-05 a total of 182 DMRs, with five of them having multiple adjacent windows. All DMRs identified at a raw edgeR p < 1e-05 also met the FDR adjusted p-value threshold of 0.05. To clarify the magnitude of DNA methylation change at each DMR, an analysis of MeDIP-Seq read depth for control and exposure for each DMR is listed in Figure 4C for F1 generation and Figure 4D for F2 generation. The F1 generation sperm generally had a decrease in DNA methylation of DMRs, Figure 4C, while the F2 generation sperm had a mixture of increase and decrease in DMR methylation in ifosfamide lineage males, Figure 4D. The DMR minimally had a >40% change in control (% Cont), and typically 200% change, that correlated with the log fold change in Tables S5 and S6.

Chromosomal locations of the DMRs for both the F1 and F2 generation are shown in (Figures 5A and 5B). At least one DMR was identified on nearly every chromosome, with exception of the Y chromosome in the F2 generation. Analysis of DMR genomic features determined that there were generally between one and two CpG sites per 100 bp and a DMR length of 1 kb in both the F1 and F2 generation (Figures 6A–6D). Principal component analysis of methylation at DMR sites revealed separation between control and ifosfamide lineage animals for both the F1 and F2 generation (Figures 7A and 7B). An overlap diagram comparing F1 and

| Table 1. Summary of pathological findings across the F1 and F2 generation ifosfamide lineage rats |
|---------------------------------|---------|---------------------------------|
| Generation                      | Sex     | Pathological findings            |
| F1 Ifosfamide lineage           | Males   | - None *(Non-significant trend for increase in lean phenotype, p=0.068) |
|                                 | Females | - Increase of kidney pathology   |
|                                 |         | - Increase of late pubertal onset phenotype |
|                                 |         | - Increase of multiple pathologies *(Non-significant trend for reduction in obesity phenotype, p=0.061) |
| F2 Ifosfamide lineage           | Males   | - Increase of early pubertal onset phenotype |
|                                 |         | - Reduction of anxiety-like behavior |
|                                 |         | - Reduction of testis pathology *(Non-significant trend for reduction in obesity phenotype, p=0.062) |
|                                 | Females | - Reduction of anxiety-like behavior |
F2 generation DMR at $p<1\times10^{-5}$ indicates that there were 194 distinct DMR for the F1 generation, 176 distinct DMR for the F2 generation, and seven shared DMR between the F1 and F2 generation (Figure 8A). An expanded overlap analysis shows that of the 201 DMRs below the $p$ value cutoff of $1<e^{-05}$ in the F1 generation analysis, 34 (17%) have a $p$ value of <0.05 in the F2 generation analysis. Of the 182 DMRs below the $p$ value cutoff of $1<e^{-05}$ in the F2 generation analysis, 45 (25%) have a $p$ value of <0.05 in the F1 generation analysis (Figure 8B).

A summary of DMR-associated gene categories indicates that signaling, receptor, and metabolism are the most affected (Figure 9A). The top five DMR-associated gene pathways for both the F1 and F2 generation are identified, along with the number of DMR-associated genes linked to the pathway (Figure 9B). The F1 generation DMR-associated gene network identified cytotoxic T cell selection, immune system function, and transmembrane as the cellular processes (Figure 10A). Phenotypes of disease these DMRs are associated with include insulin resistance, pancytopenia, and carcinogenesis (Figure 10A). The full F1 generation DMR-associated gene network list can be found in Table S7. The F2 generation DMR-associated gene network identified activated B cell fate, endothelial cell recognition, trophoblast migration, and adherens
junction organization as the cellular processes (Figure 10B). Phenotypes of disease these F2 generation DMRs are associated with include experimental metastasis, metabolic syndrome X, and cholesterol gallstone (Figure 10B). The full F2 generation DMR-associated gene network list can be found in Table S8.

**DISCUSSION**

**Ifosfamide exposure and generational pathology**

Prior to this experiment, few studies had examined the potential transgenerational effects of chemotherapy. Although chemotherapy is understood to have direct long-term effects on treated individuals, it was previously unclear what the effects may be on offspring (F1 generation) and grand-offspring (F2 generation). Observations suggest that direct F1 generation effects appear to be limited to the female offspring of exposed fathers. Increased frequency of both kidney and multiple pathologies in F1 generation ifosfamide lineage females suggests that the effects are more pronounced in females than males initially. An increased incidence of the late onset puberty phenotype observed in F1 generation ifosfamide lineage females also suggests that chemotherapy may have impacts on reproductive endocrine development in offspring. It is proposed that epigenetic alterations in the germline induce an endocrine effect responsible for this phenotype. There did not appear to be any impact in those females being able to successfully reproduce later in life. Similar findings were observed in a human study, in which children born to chemotherapy-exposed fathers did not have less offspring.31

In the male F1 generation ifosfamide lineage animals, there were surprisingly no significant increases in the frequency of diseases examined in this study, although there was a trend toward the lean phenotype. In the male F2 generation ifosfamide lineage, there was a significant increase in the early onset puberty phenotype. This is the opposite of the effect than what was observed in F1 generation ifosfamide lineage females where there was a late onset puberty phenotype. This is not surprising since often effects from a direct exposure and transgenerational impacts can result in different pathologies. Based upon these findings, it is proposed that the transgenerational epimutations are promoting these effects on puberty. Interestingly, ifosfamide lineage F2 generation males were observed to have a significant reduction in testis pathology.
A separate rodent study examined peripubertal paternal exposure to the chemotherapy drug cisplatin and observed adverse effects in both male and female offspring. Findings in the F1 generation females appear to be in alignment with these findings; however, findings in the F1 generation males differ as no significant pathologies were observed. The observations in the current study in regard to pathology induced by ifosfamide are distinct.

As mentioned in the Results, there was increased frequency of abnormal (i.e., anxiety) behavior in both the males and females of the F2 generation ifosfamide lineage when using an elevated plus maze. While no significant effect on anxiety-related behaviors was observed when using the light/dark box, this is speculated to be a result of the setup, such as room lighting. Another potential explanation for this result is that although both apparatuses may be used to measure anxiety, they may test different aspects of anxiety-related behavior. A separate study using an elevated plus maze observed transgenerational effects on anxiety-like behavior in F2 generation females following an adolescent morphine exposure in the F0 generation. While that study was carried out using an exposed mother instead of a father, both studies were focused on adolescent exposures, indicating that an adolescent exposure to common medical treatments can have transgenerational effects in regard to anxiety-like behavior.
As mentioned in the Methods, the F1 generation controls were used as the control group for both the F1 and F2 generation DMR analysis. Use of F1 generation controls for both comparisons allowed for better comparisons between the DMR numbers for the F1 and the F2 generations. The expanded overlap analysis shows only a small increase in the DMR overlap comparison, indicating that there is little overlap between the two generations. The presence of DMRs in both the F1 and F2 generations suggests that ifosfamide induces epimutations in generations removed from the initial direct exposure. This provides support for the previous human study which suggested that pubertal direct exposure could modify the germline 10 years removed from their treatment.28 A previous study in rats had also observed alterations to the methylation of spermatozoa following exposure to the chemotherapy drug cisplatin.26 The DMRs from this study appear to be located genome-wide as previously mentioned with the exception of the Y chromosome in the F2 generation DMRs. This indicates that the effects are not limited to specific genomic sites, meaning there is greater potential for alterations resulting in phenotypic change. Although the F1 generation is considered to have had a direct exposure due to alteration of the germline of the father (F0) generation, the F2 generation has no exposure. Therefore, the F2 generation is the first

Figure 7. DMR Principal Component Analysis
PCA analysis for F1 generation (A) and F2 generation (B) DMRs from control and ifosfamide lineage rats.
transgenerational generation, indicating the effects must be passed through the germline and are therefore transgenerational.

Gene-associated DMRs are within 10 kb of the gene, so they can include both proximal and distal promoter regions. As mentioned in the results, a large number of the associated genes are involved in categories of signaling, receptor, and metabolism. All of these pathways are involved in processes which can impact phenotype such as disease. In the F1 generation, a large number of the genes are involved in phenotypes of disease such as insulin resistance, pancytopenia, and carcinogenesis. In the F2 generation, a large number of the affected genes are involved in phenotypes of disease such as experimental metastasis, metabolic syndrome X, and cholesterol gallstone. Functional links need to be assessed in the future in order to better understand the effects of DMRs and their contribution to the listed phenotypes of disease.

As previously mentioned, chemotherapy has drastically improved survival rates for childhood cancers including osteosarcoma. This is encouraging, but it also indicates the importance of more studies that examine the generational impacts of chemotherapy exposures. As survival rates increase and treatment options are improved to reduce the impacts of chemotherapy on fertility, it is possible that a number of these individuals may father children. From these findings, one can better understand potential phenotypes we may see in children and grandchildren, as well as better understand the mechanism of origin behind them. The current study indicates that there are effects present in both the offspring and grand-offspring, highlighting the importance of understanding the generational effects of common chemotherapy drugs such as ifosfamide.

Epigenetics is as equally important to our understanding of disease etiology as genetics. Epigenetic modifications regulate gene expression, so alterations can alter phenotypes and disease without any changes to the genetic code itself. The current study identified pathology and epimutation DMRs that appear to be correlated with an ancestral chemotherapy exposure around the time of puberty. These findings suggest the importance of proper precautionary measures which should be taken prior to chemotherapy. Cryopreservation of sperm prior to undergoing chemotherapy would allow these patients to have children later in life, without any concern of the potential effects of chemotherapy on their children and later generations. Trials need to be expanded in the future, since it is possible that different chemotherapy drugs may induce different phenotypes or pathologies. This would be similar to the observation

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**Figure 8. DMR Overlaps**

(A) Venn diagram of DMRs between the F1 and F2 generations sperm.

(B) Expanded DMR overlap analysis between the F1 and F2 generations at p<1e-05 versus p<0.05 as indicated in the axis legend. The number of DMR and percent (%) of total DMRs is presented.
that different toxicants promote distinct transgenerational epimutations. Although rodent studies are useful in investigating the effects of environmental exposures, studies in humans would be the most beneficial to understanding effects of chemotherapy that go beyond just the children of exposed individuals. A sample bank of sperm samples from chemotherapy exposed individuals and subsequent generations should be created to better understand potential impacts of germline epimutations. Until such a sample bank exists, these findings offer insight into potential phenotypes of disease which may be observed in the future as we begin to better understand the long-term effects of chemotherapy exposure. Therefore, precautionary steps to collect and store sperm and eggs from individuals undergoing chemotherapy need to be considered to avoid these generational impacts.

Treatment with the chemotherapy drug ifosfamide around the time of puberty in males appears to promote higher incidence of disease in F1 generation females. In addition to these direct germline exposure-induced phenotypes, there were a greater number of pathology phenotypes in the male and female F2 generation grand-offspring as well. A summary of the significant pathological findings is provided in Table 1. These findings combined with the presence of DMRs in both the F1 and F2 generation suggest that chemotherapy promotes the epigenetic transgenerational inheritance of disease and pathology through modifications to the germline. The effects in grand-offspring of treated individuals appear to be linked to altered development that impacts physiology and behavior. It is not recommended that individuals avoid chemotherapy, as it has been proven to be an effective treatment in various types of cancers. Rather, these observations support taking proper precautionary measures prior to undergoing treatment, including cryopreservation of sperm and eggs to minimize potential effects if that individual decides to have children later in life. Precautionary measures would also reduce the potential of fertility issues such
as azoospermia in patients exposed to chemotherapy. Future studies are necessary to investigate if other chemotherapy drugs have similar effects, but the current study and previous reports suggest general chemotherapy agents will act like other environmental toxicants and all promote the epigenetic transgenerational inheritance of disease and pathology.

Limitations of the study
The current study used a rodent model to identify the ability of chemotherapy to induce the epigenetic transgenerational inheritance of pathology in offspring and grand-offspring. Although a previous study

Figure 10. DMR-associated gene networks
(A) F1 generation DMR-associated gene network with the most common associated cellular processes and phenotypes. (B) F2 generation DMR-associated gene network with the most common associated cellular processes and phenotypes.
has been shown in humans that chemotherapy can promote permanent epigenetic alterations in male sperm over ten years following treatment, generational studies in humans are now needed from cancer survivors. The current study does not suggest chemotherapy should not be used, but cryopreservation of germ cells prior to chemotherapy should be considered.

Ethics
All experimental protocols for procedures using the rats were pre-approved by the Washington State University Animal Care and Use Committee (protocol IACUC # 6252). All methods were performed in accordance with the relevant guidelines and regulations.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105570.

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AUTHOR CONTRIBUTIONS
R.P.T. performed study, animal studies, data analysis, wrote and edited manuscript; D.B. performed bioinformatic analysis, data analysis, edited manuscript; E.N. performed animal studies, cell isolations, data analysis, edited manuscript; M.B.M. performed molecular, analysis, data analysis, edited manuscript; M.S. conceived, obtained funding, edited manuscript; M.K.S. conceived, oversight, obtained funding, wrote, and edited manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.
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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Monoclonal mouse anti 5-methyl cytidine | Diagenode | C15200006-500 |
| Magnetic beads (Dynabeads M---280) | Life technologies | 11202D |
| Sheep anti-Mouse IgG (11201D) | Life technologies | 11201D |
| Bacterial and virus strains | | |
| Female and male rats of an outbred strain | Harlan/Envigo (Indianapolis, IN) | Hsd:Sprague Dawley SD |
| Aspen Sani chips | Harlan | |
| 8640 Teklad 22/5 Rodent Diet | Harlan | 8640 |
| Chemicals, peptides, and recombinant proteins | | |
| Protein precipitation solution | Promega | A7953 |
| Bovine serum albumen (BSA) | Sigma | A2153 |
| Collagenase (Sigma C1639) | Sigma | C1639 |
| F-12 culture medium | Gibco-Life Technologies, USA. | Ref 11765-054 |
| Glycoblue | Life Technologies | AM 9516 |
| Protease K | Bio Basic | P80451 |
| Buffered Phenol-Chloroform-Isoamylalcohol solution | Fisher Scientific | 327111000 |
| Dimethyl sulfoxide (DMSO) | J. T. Baker | 9224-01 |
| Critical commercial assays | | |
| Qubit ssDNA kit (Molecular Probes Q10212) | Life Technologies | Q10212 |
| NEBNext Ultra II RNA Library Prep Kit for Illumina | NEB, San Diego, CA | E7770L |
| Deposited data | | |
| The public database at NCBI GEO | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE117995 | GSE117995 |
| The public database at NCBI GEO | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE151458 | GSE151458 |
| The public database at NCBI GEO | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE121585 | GSE121585 |
| The public database at NCBI GEO | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE217141 | GSE217141 |
| Oligonucleotides | | |
| NEBNext Multiplex Oligos for Illumina | NEB, San Diego, CA | E7335L, E7500L |
| Software and algorithms | | |
| FastQC program | http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ | N/A |
| Trimmomatic | http://www.usadelab.org/cms/?page=trimmomatic | PMID: 24695404 |
| Bowtie2 | http://bowtie-bio.sourceforge.net/bowtie2/index.shtml | PMID: 22388286 |
| MEDIPS | https://bioc conductor.org/packages/release/biocl/html/MEDIPS.html | PMID: 24227674 |
| BiomaRt R package | https://bioc conductor.org/packages/release/biocl/html/biomaRt.html | PMID: 19617889 |
| Ensembl database | https://www.ensembl.org/index.html | PMID: 16082012 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by
the lead contact, Dr. Michael K. Skinner (skinner@wsu.edu).

Materials availability
This study did not generate new unique reagents.

Data and code availability

- All molecular data has been deposited into the public database at NCBI (GEO # GSE217141).
- R code computational tools available at GitHub (https://github.com/skinnerlab/MeDIP-seq) and www.
skinner.wsu.edu.
- Any additional information required to reanalyze the data reported in this paper is available from the
lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal studies and breeding
As described in a previous study, female and male rats of an outbred strain Hsd:Sprague Dawley SD (Har-
lan) were fed ad lib using a standard rat diet and ad lib tap water. Male rats were treated with 8.5 mg/kg of
the chemotherapeutic agent ifosfamide (intraperitoneally), once every three days for three treatments,
starting when they were 26 days old (days 26, 29, and 32) of rat pubertal development. Ifosfamide injection
solution was 7.65 mg/mL ifosfamide in 1x PBS (phosphate buffered saline), filter sterilized. Control rats were
treated with 1.1 mL/kg 1X PBS.

The treated male rats were designated as the F0 generation. The F0 generation consisted of three male
controls and five ifosfamide treated males. The F1 generation consisted of: ifosfamide females n=24,
control females n=34, ifosfamide males n=18, and control males n=25. The F2 generation consisted of:
ifosfamide females n=51, control females n=47, ifosfamide males n=43, and control males n=51. F1 and
F2 generation control and ifosfamide lineages were housed in the same room and racks with lighting,
food and water as described in previous experiments. Non-littermate females and males aged
80–90 days from the F1 generation of ifosfamide or control lineages were bred to other unrelated F1 gen-
eration individuals within their lineage group to obtain F2 generation offspring. Efforts were made to cull
litters to 10 rats per litter, however in the F1 generation controls there were two litters of 12 rats and one
litter of 15 rats. Only F0 generation individuals received ifosfamide treatments and they exhibited no gross
pathology upon dissection.

Puberty data was collected from the F1 and F2 generations. Onset of puberty was assessed in females start-
ing at 30 days of age and individuals were determined mature after there was a clear vaginal opening
observed. Onset of puberty was assessed in males starting at 39 days of age and individuals were deter-
mined to be mature after balano-prepuptial separation.

All experimental protocols for procedures using the rats were pre-approved by the Washington State Uni-
versity Animal Care and Use Committee (protocol IACUC # 6252). All methods were performed in accor-
dance with the relevant guidelines and regulations.
METHOD DETAILS

Tissue harvest and histology processing
As described,55 rats were euthanized at 12 months of age by method of CO2 inhalation and cervical dislocation for tissue harvest. Testis, prostate, kidney, ovary and adipose tissue were fixed in Bouin’s solution (Sigma) followed by 70% ethanol, then processed for paraffin embedding and hematoxylin and eosin (H&E) staining by standard procedures for histopathological examination. Paraffin five-micron sections were processed, stained, and provided by Nation-wide Histology, Missoula, MT, USA.

Histopathology and disease classification
Stained testis, prostate, kidney, and ovary slides were imaged through a microscope using 4x objective lenses for testis and prostate and 10x objective lenses for kidney and ovary. Tiled images were captured using a digital camera and then photo-merged into a single image using Adobe Photoshop. Images were evaluated and pathology features were digitally marked using Photoshop software. The Washington State Animal Disease Diagnostic Laboratory (WADDL) at the Washington State University College of Veterinary Medicine has board certified veterinary pathologists, who assisted in initially establishing criteria for pathology analyses and identifying parameters to assess.59 WADDL also performed full necropsies as required on animals that died prior to the time of scheduled sacrifice at one year and performed tumor classifications in the current study. The tissues evaluated histologically were selected from previous literature showing them to have pathology in transgenerational models,19,21,51,59–64 with an emphasis on reproductive organs. Histopathology readers were trained to recognize the specific abnormalities evaluated for this study in rat testes, ventral prostate, kidney, and ovary. Two individuals blinded to the exposure evaluated each tissue section for abnormalities. If there was disagreement regarding the disease status, then a third individual blinded to the exposure evaluated the tissue. A set of quality control (QC) slides were generated for each tissue and were read by each reader prior to evaluating any set of experimental slides. These QC slide results were monitored for reader accuracy and concordance.

As described,2 testis histopathology criteria included the presence of vacuoles in the seminiferous tubules, azoospermic atretic seminiferous tubules, and ‘other’ abnormalities including sloughed spermatogenic cells in the center of the tubule and lack of a tubule lumen (Figure S1). As previously described,65,66 prostate histopathology criteria included the presence of vacuoles in the glandular epithelium, atrophic glandular epithelium and hyperplasia of prostatic gland epithelium (Figure S2). Kidney histopathology criteria included reduced size of glomerulus, thickened Bowman’s capsule, and the presence of proteinaceous fluid-filled cysts >50 μm in diameter (Figure S3). Ovary histopathology criteria consisted of evaluation of one stained section through the central portion of the ovary. This largest cross-section was evaluated microscopically for number of primordial follicles, small cystic structures and large cysts67 (Figure S4). Follicles had to be non-atretic and have the oocyte nucleus visible in the section in order to be counted. Primordial follicles are in an arrested state and have an oocyte surrounded by a single layer of either squamous or both squamous and cuboidal granulosa cells.69 Cysts were defined as fluid-filled structures of a specified size that were not filled with red blood cells and which were not follicular antra. A single layer of cells may line cysts. Small cysts were 50–250 μm in diameter measured from the inner cellular boundary across the longest axis. Large cysts were greater than 250 μm in diameter. A cutoff was established to declare a tissue ‘diseased’ based on the mean number of histopathological abnormalities plus 1.5 standard deviations from the mean of the control group tissues, as assessed by each of the three individual observers blinded to the treatment groups. This number (i.e., greater than 1.5 standard deviations) was used to classify rats into those with and without testis, prostate, kidney, or ovary disease in each lineage. A rat tissue section was finally declared ‘diseased’ only when at least two of the three observers marked the same tissue section ‘diseased’. The statistical analyses for pathology results were expressed as the proportion of affected animals that exceeded a pre-determined threshold (testis, prostate, kidney or ovary disease frequency, tumor frequency, obese frequency). Groups were analyzed using Fisher exact test.

Obesity classification
As described,69 obese and lean phenotypes were assessed by an increase or decrease in adipocyte size (area) within the gonadal fat pad, body mass index (BMI), and observed abdominal adiposity of visceral fat pads at dissection. Animals were initially assessed for an obese or lean phenotype at dissection based on observed excess or lack of abdominal adiposity of the visceral fat pads and body mass index (BMI). BMI was calculated with weight (g)/length (cm).2 Gonadal fat pad slides were imaged using a Nikon Eclipse 55i.
microscope (10x) with a Zeiss Axiocam 105 color camera. Five field of view image captures were taken per slide in varying parts of the fat pad. The gonadal fat pad was utilized as it is the most easily accessible visceral fat pad and visceral fat depot size is the most relevant fat deposition site to metabolic pathology in obesity. Adipocyte size was measured by converting pixels into microns utilizing Adiposoft. Measurements of the 20 largest cells from each image for a total of 100 cells per animal were averaged as hypertrophic cells are an indicator of a pro-apoptotic phenotype strongly associated with metabolic pathology. A cutoff was established to declare an individual rat ‘obese’ or ‘lean’ based on the mean adipocyte area plus 1.5 standard deviations from the mean of the control group adipose tissue (Figure S5). Males and females were assessed for this cutoff separately. Adipocyte area was prioritized to make obese and lean calls for the purpose of phenotypic consistency as adipocyte hypertrophic and hyperplastic obesity have differential epigenomic profiles. Rats with adipocyte areas above 1.5 standard deviation from the mean of the control group adipose tissue were considered to have an obesity (adipocyte hypertrophy) phenotype. Rats with adipocyte areas below 1.5 standard deviation from the mean were considered to have a lean (adipocyte hypotrophy) phenotype.

Behavioral analysis
As described, behavioral analysis was performed to evaluate general anxiety with both an elevated plus maze and Light/Dark box, as described. F2 generation male and female rats from control and ifosfamide lineages were used for the behavioral studies at 11 months of age. The elevated plus-maze consisted of a “plus”-shaped platform made of black opaque Plexiglas, with each platform 10 cm in width and 50 cm in length, creating a 10 x 10 cm neutral zone in the center. Two of the arms were enclosed with black Plexiglas walls 40 cm high, with no ceiling. For this task, rats were placed individually into the center (neutral) zone of the maze, facing an open arm. Rats were allowed to explore for a 5 min period, and the number of open and closed arm entries and time spent on the open and closed arms were recorded. The F2 generation animals tested on the elevated plus maze consisted of: ifosfamide females n= 30, Control females n=31, ifosfamide males n=24, and control males n=30. Individuals that fell off of the maze were excluded from the analysis. The Light/Dark box consists of a small dark compartment that made up one third of the apparatus with the other two thirds being an illuminated compartment. The rats were placed individually in the light zone, facing the dark zone, as recommended. Similarly, the number light and dark compartment entries and time spent in the light and dark compartments were recorded. The F2 generation animals tested with the Light/Dark Box consisted of: ifosfamide females n= 30, Control females n=30, ifosfamide males n=22, and control males n=23. A cutoff was established to declare an animal ‘abnormal’ based on the mean plus or minus 1.5 standard deviations from the mean of the control group, as assessed by each of the two individual observers. Behavioral analysis was excluded from the category of multiple disease. The light/dark box procedure appears to have been compromised due to having excess lighting in the room, so data was not presented as described in the results section.

Epididymal sperm collection and DNA isolation
The protocol is described in detail in reference. Briefly, the epididymis was dissected free of fat and connective tissues, then, after cutting open the cauda, placed into 6 mL of phosphate buffer saline (PBS) for 20 min at room temperature. Further incubation at 4°C will immobilize the sperm. The tissue was then minced, the released sperm was pelleted at 4°C 3000 x g for 10 min, then resuspended in NIM buffer and stored at -80°C for further processing. An appropriate amount of rat sperm suspension was used for DNA extraction. Previous studies have shown mammalian sperm heads are resistant to sonication, unlike somatic cells. Somatic cells and debris were therefore removed by brief sonication (Fisher Sonic Dismembrator, model 300, power 25), then centrifugation and washing 1–2 times in 1X PBS. The resulting pellet was resuspended in 820 μL DNA extraction buffer and 80 μL 0.1 M DTT added, then incubated at 65°C for 15 min. Eighty μL proteinase K (20 mg/mL) was added and the sample was incubated at 55°C for 2–3 h under constant rotation. Protein was removed by addition of protein precipitation solution (300 μL, Promega A795A), incubation for 15 min on ice, then centrifugation at 13,500 x g for 30 min at 4°C. One mL of the supernatant was precipitated with 2 μL of GlycoBlue (Invitrogen, AM9516) and 1 mL of cold 100% isopropanol. After incubation, the sample was spun at 13,500 x g for 30 min at 4°C, then washed with 70% cold ethanol. The pellet was air-dried for about 5 min then resuspended in 100 μL of nuclease free water.

Methylated DNA immunoprecipitation (MeDIP)
Sperm samples were prepared as described. F1 generation samples were pooled, with three to four samples per pool and six pools each for both the ifosfamide and control lineage. F2 generation samples were pooled as well, with seven samples per pool for the control lineage, and eight or nine samples per pool for
the ifosfamide lineage. Genomic DNA was sonicated and run on 1.5 % agarose gel for fragment size verification. The sonicated DNA was then diluted with 1X TE buffer to 400 µL, then heat-denatured for 10 min at 95°C, and immediately cooled on ice for 10 min to create single-stranded DNA fragments. Then 100 µL of 5X IP buffer and 5 µg of antibody (monoclonal mouse anti 5-methyl cytidine; Diagenode #C15200006) were added, and the mixture was incubated overnight on a rotator at 4°C. The following day magnetic beads (Dynabeads M280 Sheep anti-Mouse IgG; Life Technologies 11201D) were pre-washed per manufacturer’s instructions, and 50 µL of beads were added to the 500 µL of DNA-antibody mixture from the overnight incubation, then incubated for 2 h on a rotator at 4°C. After this incubation, the samples were washed three times with 1X IP buffer using a magnetic rack. The washed samples were then resuspended in 250 µL digestion buffer (5 mM Tris PH 8, 10 mM EDTA, 0.5 % SDS) with 3.5 µL Proteinase K (20 mg/mL), and incubated for 2–3 h on a rotator at 55°C. DNA clean-up was performed using a Phenol-Chloroform-Isoamyl-Alcohol extraction, and the supernatant precipitated with 2 µL of GlycoBlue (20 mg/mL), 20 µL of 5 M NaCl and 500 µL ethanol in –20°C freezer for one to several hours. The DNA precipitate was pelleted, washed with 70% ethanol, then dried and resuspended in 20 µL H2O or 1X TE. DNA concentration was measured in Qubit (Life Technologies) with the ssDNA kit (Molecular Probes Q10212).

MeDIP-sequencing analysis
As previously described,81 MeDIP DNA was used to create libraries for next generation sequencing (NGS) using the NEBNext Ultra RNA Library Prep Kit for Illumina (San Diego, CA) starting at step 1.4 of the manufacturer’s protocol to generate double stranded DNA from the single-stranded DNA resulting from MeDIP. After this step, the manufacturer’s protocol was followed indexing each sample individually with NEBNext Multiplex Oligos for Illumina. The WSU Spokane Genomics Core sequenced the samples on the Illumina HiSeq 2500 at PE50, with a read size of approximately 50 bp and approximately 40 million reads per pool.

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistics and bioinformatics
The DMR identification and annotation methods follow those presented in previous published papers.21,81 F1 generation controls were used in both the F1 and F2 DMR analysis to ensure consistency. Although the F2 generation control was obtained, a background issue from MeDIP on the analysis developed, such that the F1 generation control was used to allow a better comparison of the two generations. Data quality was assessed using the FastQC program (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and reads were cleaned and filtered to remove adapters and low quality bases using Trimmomatic.82 The reads for each MeDIP sample were mapped to the Rnor 6.0 rat genome using Bowtie283 with default parameter options. The mapped read files were then converted to sorted BAM files using SAMTools.84 The MEDIPS R package85 was used to calculate differential coverage between control and exposure sample groups. The edgeR p-value86 was used to determine the relative difference between the two groups for each genomic window. Windows with an edgeR p-value less than an arbitrarily selected threshold were considered DMR. The site edges were extended until no genomic window with an edgeR p-value less than 0.1 remained within 1000 bp of the DMR. The edgeR p-value was used to assess the significance of the DMR identified. Differential epimutation sites were annotated using the biomart R package97 to access the Ensembl database. The DMR associated genes were then automatically sorted into functional groups using information provided by the DAVID97 and Panther90 databases incorporated into an internal curated database (www. skinner.wsu.edu under genomic data). A Pathway Studio, Elsevier, database and network tool was used to assess physiological and disease process gene correlations. All molecular data has been deposited into the public database at NCBI (GEO # GSE217141) and R code computational tools available at GitHub (https://github.com/skinnerlab/MeDIP-seq) and www.skinner.wsu.edu.