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Testicular toxicity and the potential for 1, 2-ethlyene dichloride (EDC) to initiate epigenetic disruption of the paternal genome

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TESTICULAR TOXICITY AND THE POTENTIAL FOR 1, 2-Ethylenedichloride (EDC) TO INITIATE EPIGENETIC DISRUPTION OF THE PATERNAL GENOME

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Biological Sciences College of Basic Science

by

Harold J. Daigle, Jr.
B.S., Louisiana State University, 2000
December, 2009
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-aza</td>
<td>5-aza-2’-deoxycytidine</td>
</tr>
<tr>
<td>BORIS</td>
<td>Brother of the regulator of imprinted sites</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine followed by guanine</td>
</tr>
<tr>
<td>CSF-1</td>
<td>Colony stimulating factor 1</td>
</tr>
<tr>
<td>CTCF</td>
<td>CCCTC-binding factor (zinc finger protein)</td>
</tr>
<tr>
<td>dpc</td>
<td>Days postcoitum</td>
</tr>
<tr>
<td>DMR’s</td>
<td>Differentially methylated regions</td>
</tr>
<tr>
<td>EDC</td>
<td>1,2 dichloroethane</td>
</tr>
<tr>
<td>ES</td>
<td>Embryo stem cells</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell line-derived neutrophic factor</td>
</tr>
<tr>
<td>GNAT</td>
<td>N-acetyltransferase</td>
</tr>
<tr>
<td>H2A</td>
<td>Histone Protein subunit 2 A</td>
</tr>
<tr>
<td>H2B</td>
<td>Histone Protein subunit 2 B</td>
</tr>
<tr>
<td>H3</td>
<td>Histone Protein subunit 3</td>
</tr>
<tr>
<td>H4</td>
<td>Histone Protein subunit 4</td>
</tr>
<tr>
<td>H3-K4_m3</td>
<td>Histone Protein subunit 3, lysine number 4 tri-methylated</td>
</tr>
<tr>
<td>H3-K9_m1</td>
<td>Histone Protein subunit 3, lysine number 9 mono-methylated</td>
</tr>
<tr>
<td>H3-K9_m2</td>
<td>Histone Protein subunit 3, lysine number 9 di-methylated</td>
</tr>
<tr>
<td>H3-K9_m3</td>
<td>Histone Protein subunit 3, lysine number 9 tri-methylated</td>
</tr>
<tr>
<td>HAT’s</td>
<td>Histone acetyltransferase</td>
</tr>
</tbody>
</table>
HBO1  MYST histone acetyltransferase 2 [Homo sapiens]
HDAC’s  Histone deacetylase enzymes
ICRs  Imprinting Control Regions
IVF  In vitro fertilization
LIF  Leukemia Inhibitory Factor
LOI  Loss of imprinting
LLO  Listeriolysin O
MS PCR  Methylation specific PCR
MES  Mouse embryo stem cell
MYST  Histone acetyltransferases (HATs)
PESG  Primordial germ cells
RA  Retinoic acid
SSC  Spermatogonial stem cells
SCO,  Sertoli Cell-Only
UPD  Uniparental disomy
ABSTRACT

Our genetic inheritance begins with one genome copy from each parent. The presence of one or more errors in either the maternal or the paternal genome can lead to genetic disease or disruption of the embryonic program and potential loss of the offspring. Numerous chemical and physical toxicants are known to produce germ line mutagenesis based on their ability to produce DNA sequence mutations. Exposures to these cytotoxic and mutagenic agents pose a risk for human offspring. However, very little is known regarding the sensitivity of the epigenetic patterns involved in reproductive functions to adverse effects of chemical agents. The present study was undertaken to address the potential for ethylene dichloride (EDC) to disrupt the epigenetic programming of the paternal genome in mammalian (mouse) sperm.

Unfortunately, it is very difficult to study developing germ cells such as spermatogonia and their precursor spermatogonial stem cells within the in vivo mouse testis model. Thus, an in vitro mouse spermatogonial stem cell model was developed that enabled the triggering of a spermatogenesis differentiation pathway in these stem cell cultures. Since the epigenetic imprint patterns are reported to be established in the spermatogonial stem cell of prior to meiosis, this in vitro model enabled the treatment and study of the effects of EDC on DNA, 5-methylcytosine and histone modifications in the paternal gamete. Paternally imprinted genes, such as H19, Gtl2, and Rasgrf1, showed changes in histone methylation modifications in ethylene dichloride treated spermatogonia. These data demonstrate that ethylene dichloride can disrupt the genomic imprint in developing sperm, and thus perturb the embryonic programming of potential offspring in the mouse model. This work suggests that EDC may have the potential to cause genetic diseases in offspring from exposed males.
CHAPTER 1

ETHYLENE DICHLORIDE AND GENETIC IMPRINTING

Disruption of male reproductive function due to exposure to cytotoxic agents (such as environmental toxicants) and cancer therapies (such as chemotherapy and ionizing radiation) has been well documented (Gandini, Sgro et al. 2006). Alkylating agents are known to disrupt the male reproductive function by interfering with or damaging the endocrine system (Greim 2005), and Sertoli and Ledig cells (Forbes 1978; Kopf-Maier 1992), as well as the spermatogonial stem cells (Maguire, Dick et al. 1981).

Damage to spermatogonial stem cells is the most important since these are the cells that differentiate into gametes that harbor the genetic information for the next generation of. Two distinct pathways, genetic and epigenetic, transmit inherited genetic information. According to classic evolutionary theory, phenotypic variation is due to heritable genetic mutations. It is now known that other heritable mechanisms can contribute to phenotypic variation. It is these mechanisms, known as epigenetics, that give organisms a plasticity of heritable phenotypes (Rando and Verstrepen 2007). Epigenetics is defined as the study of mitotically and or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence (Russo et al. 1996).

Establishment and reprogramming of epigenetics is a process by which the expression of an organism’s genotype may be altered to produce phenotypic variations, despite an absence of change in the genetic information (Tang and Ho 2007). Heritable epigenetic information is transmitted to the next generation by three main and interlinked systems: DNA 5-methylcytosine patterns (Baylin 1997; Jones and Takai 2001), histone tail modifications (Lacoste and Cote 2003; Margueron, Trojer et al. 2005), and small-interfering RNAs (Morris 2005). Alterations in these modifications have been implicated in basic
cellular processes such as embryogenesis, differentiation, carcinogenesis, and even aging (Wilson, Smith et al. 1987).

1.1 Ethylene Dichloride (EDC)

Ethylene dichloride (EDC), also known as 1,2-dichloroethylene, is a highly flammable, colorless liquid with a sharp, harsh odor that can be detected by smell at approximately 17 ppm in air (ATSDR 2001). EDC is a high-use chemical in today's industry for the synthesis of vinyl chloride, as a chlorinated solvent, and as a constituent in paints, soaps, scouring compounds, and metal degreasers (ATSDR 2001). EDC has also been used as an extracting solvent for spices and other natural flavorings, including tobacco. The production of EDC in the United States was about 16 million metric tons in 1994 (ATSDR 2001). In Louisiana, the total EDC discharge for 1987 to 1993 was greater than 5598 metric tons (LDEQ 1995).

EDC is a known mutagen requiring metabolic activation by glutathione conjugation (Inskeep, Koga et al. 1986; Cmarik, Humphreys et al. 1992). Glutathione-dependant metabolites, such as S- (2-chloroethyl) glutathione and S- (2-chloroethyl)-L-cysteine, spontaneously rearrange to form reactive episulfonium ions that react with nucleophilic sites on cellular macromolecules, including DNA (Peterson, Harris et al. 1988; Romert, Magnusson et al. 1990). The major DNA adduct formed from EDC is reported to be N7-ethyl-S-glutathionyl deoxyguanosine (Romert, Magnusson et al. 1990; Cmarik, Humphreys et al. 1992). Interestingly, the mutation spectrum produced by EDC and other dihaloethanes appears to be limited to G → A transitions (Aafjes, van der Vijver et al. 1978; Cmarik, Humphreys et al. 1992). Since most tissues contain glutathione and glutathione-S-transferase, EDC can react and damage multiple organ systems, including testis. The US EPA has designated EDC as a potential carcinogen, hazardous substance, and priority toxic pollutant (EPA 2000). EDC is also designated as a possible human carcinogen, (Group 2B) by the International Agency for Cancer
Research (IARC 2006). Early studies with EDC have reported little or no reproductive toxicity. Inhalation studies showed only embryo toxicity at high doses and no significant reproductive toxicity (Lane, Riddle et al. 1982); (Hurtt, Valentine et al. 1993). Rats and mice exposed to this highly volatile compound in drinking water or in diet showed no effects on fertility (Alumot, Meidler et al. 1976) and lacked remarkable pathology in reproductive tissues (Daniel, Robinson et al. 1994).

These reports of limited reproductive and testicular toxicity by EDC are surprising, since tissues with significant mitotic indexes are generally sensitive to reactive compounds, such as the activated EDC-glutathione episulfonium ion. Mammalian gonadal tissues are well known to be sensitive to cytotoxic agents. The loss of fertility in males due to therapeutic ionizing radiation and chemotherapy has been well documented (Chapman 1983; Meistrich 1984; Meistrich and Samuels 1985; Meistrich 1993). Previous studies demonstrated that the closely related chemical, 2-chloroethyl methanesulfonate, is a potent premeiotic mutagen capable of inducing mutant offspring and sterility in Drosophila (Fossett, Byrne et al. 1994). In addition to induced DNA sequence mutations, EDC may also cause mutant offspring by perturbing the epigenetic imprint in the gametes.

1.2 DNA Methylation

Genomic imprinting is a genetic phenomenon by which certain genes are expressed in a parent of origin specific manner (Delaval and Feil 2004; Morison, Ramsay et al. 2005). Once referred to as the “parent of origin effect” (Crouse 1960), forms of genomic imprinting have been demonstrated in insects, mammals, and flowering plants (Sapienza 1990); (Moore and Haig 1991; (Scott, Spielman et al. 1998). Current models explain mammalian genetic imprinting (in particular for genes involved in placenta and fetal growth) by indicating that growth enhancers are expected to be imprinted and thus are transcriptionally silent on maternal alleles, just as growth suppressors are expected to be imprinted and thus are also transcriptionally silent on paternal alleles. This theory is known as the Kinship Theory of
Imprinting, and implies an evolutionary conflict between maternal and paternal alleles within an organism (Haig 2004).

In the early 1980s, researchers found that maternal and paternal chromosomes functioned differently in developing embryos (Barton, Surani et al. 1984; McGrath and Solter 1984). By 1990, DNA cytosine methylation patterns for a subset of genes were discovered to be different on the maternal versus paternal genome (Feil, Walter et al. 1994; Eden and Cedar 1995). The term *imprinting* became identified with the fact that maternal and paternal subsets could be identified in an embryo by these distinct DNA cytosine methylation patterns.

Imprinted genes are thought to play a key role in embryonic and placental development, as well as some postnatal development (Moore and Haig 1991; Tycko and Morison 2002; Constancia, Kelsey et al. 2004). Several inherited diseases are due to either genetic imprinting defects such as Prader-Willi and Angelman Syndrome, which occur either from a deletion of the imprinting region of one of the parental alleles, or due to uniparental disomy (UPD).

UPD is the presence in a diploid individual of a chromosome pair, derived from only one parent. Unfortunately, UPD results in the inheritance of only one parental imprint on all imprinted genes residing on the chromosome pair, coupled with the lack of a balanced developmental program. UPD may play a role in both the phenotypic expression of recessive autosomal diseases and imprinting errors. In 1991, the first reported clinical case of UPD involved a girl with cystic fibrosis and unusually short stature who carried two copies of maternal chromosome 7. Since 1991, 29 of 47 possible cases of UPDs have been identified among individuals ascertained for medical reasons (Cotter, Kaffe et al. 1997; Sutton and Shaffer 2000; Sutton, McAlister et al. 2003).

The first imprinted gene discovered in male germ cells was *H19/Igf2* (Davis, Yang et al. 2000; Li, Lees-Murdock et al. 2004). Of all the known imprinted genes only three are known to be
methylated in the male germ cells; \textit{H19/Igf2}, \textit{Rasgrf1/A19}, and \textit{Dlk1/Gtl2} (Shibata, Yoda et al. 1998; Takada, Paulsen et al. 2002). Sequence regions that are important in the regulation of these imprinted genes are differentially methylated regions (DMR) that closely reside near imprinted genes (Li, Beard et al. 1993; Schmidt, Matteson et al. 2000). In male germ cells, de novo methylation of the DMRs occurs during the early stages of spermatogenesis. However, these DMRs are not fully methylated until the mature sperm stage (Li, Lees-Murdock et al. 2004). Exactly when paternal genetic imprinting patterns are established in gametogenesis is still a matter of debate and the literature is confusing regarding the course of epigenetic events (Shamanski, Kimura et al. 1999; Miki, Lee et al. 2004).

It appears that in mammalian development, there are two demethylation/remethylation events. The first occurs rapidly after fertilization. In mice, the paternal pronucleus undergoes dramatic demethylation, immediately after fertilization and prior to the first cleavage (Mayer, Niveleau et al. 2000). However, imprinted genes are protected from this initial reprogramming and maintain the parental-specific DNA 5-methylcytosine patterns (Reik and Walter 2001). The second wave of demethylation/remethylation occurs during oogenesis and spermatogenesis for reprogramming and establishing the gender-specific imprint for the next generation. In spermatogenesis, the paternal imprint is established in spermatogonial stem cells, or in the early stages of differentiation commitment pre-meiotic spermatogonia (Holmes and Soloway 2006).

During spermatogenesis, paternal imprinting de novo methylation is associated with the silencing of CCCTC-binding factor zinc finger protein (CTCF) and activation of Brother of the Regulator of Imprinted Sites (BORIS) (Klenova, Morse et al. 2002). BORIS seems to be involved with early erasure of methylation along with a currently unknown demethylase(s) (Loukinov, Pugacheva et al. 2002). Expression of BORIS, as well as Dnmt3L and Dnmt3a de novo methyltransferases, were detected between the spermatogonia and spermatocyte stages just prior to meiosis (Rousseaux, Caron
et al. 2005). By the post meiotic spermatocyte stage, paternal imprints were established, suggesting that spermatogonial meiotic differentiation commitment may trigger paternal imprint establishment. Clearly, this indicates that paternal imprints are established prior to meiosis (Rousseaux, Caron et al. 2005).

One characteristic of imprinted genes is that about 80% are physically linked in clusters (Reik and Walter 2001). Clusters may contain as many as 15 genes, as in the case of chromosome 7, which contains \textit{Ins2}, \textit{Igf2}, and \textit{H19}, among others (Engemann, Strodicke et al. 2000; Paulsen, El-Maarri et al. 2000). This sort of arrangement suggests common regulation and establishment. Imprinted gene clusters are regulated by the neighboring DMRs. Imprinted genes are bi-allelically expressed in germ cells from the time that primordial germ cells (PGCs) colonize the fetal gonad until the spermatogonial stem cell stage, or possibly as late as the early stages of pre-meiotic spermatogonia in the male (Szabo and Mann 1995). This suggests that imprints are erased during germ cell development, at least for the PGCs (Shamanski, Kimura et al. 1999).

At least some imprinted genes have the correct parental establishment, even in immature mice (Miki, Lee et al. 2004). \textit{H19}/\textit{Igf2}, \textit{Meg3}, and \textit{Igf2r} showed proper parental expression in prepubescent mice when round spermatids were used to micro-inseminate female mice. In a review by Rousseaux et al., paternal imprints were thought to be established by the spermatocyte stage, before the round spermatid stage (Rousseaux, Caron et al. 2005). Of the identified DMRs, only three are known to be methylated in the paternal germ line (Delaval and Feil 2004). These are also known as imprinting control regions (ICRs). Unlike maternal methylated DMRs, paternal methylated DMRs are not located in promoter regions or within a gene (Kobayashi, Suda et al. 2006). The DMR for \textit{H19} is upstream of the \textit{H19-Igf2} locus on the distal side of chromosome 7 (Thorvaldsen, Duran et al. 1998). The DMR for \textit{Gtl2} is upstream of the \textit{Dlk1-Gtl2} domain located on chromosome 12 (Lin, Youngson et al. 2003). The DMR for \textit{Rasgrf1} is located upstream on chromosome 9 (Yoon, Herman et al. 2002). In all three of
these DMRs de novo methylation starts before birth in PGCs at 14.5-18.5 days postcoitum, while only the $H19$ is fully methylated in pachytene spermatocytes (Davis, Yang et al. 2000; Li, Lees-Murdock et al. 2004; Delaval, Govin et al. 2007).

This study focused on these DMRs rather than gene promoters, because disruption in DMRs is known to affect imprinting (Rayburn, Parker et al. 2004). However, DNA cytosine methylation patterns may represent only part of the imprinting control mechanism. Genetic imprinting may also be linked or controlled by histone modifications.

1.3 Histone Modifications and Imprinting

In eukaryotic cells, the basic repeating unit of chromatin structure is the nucleosome containing two copies of four core histone proteins H2A, H2B, H3, and H4, wrapped by ~ 147 bp of DNA (Luger, Mader et al. 1997). Modifications to histone N-terminus tails were first reported in 1964 (Allfrey, Faulkner et al. 1964). Histone subunit modifications consist of methylation (McKittrick 2004; Kondo 2004), phosphorylation (Toh, O'Shaughnessy et al. 2006), acetylation (Yamada, Mizuno et al. 2004), ubiquitinylation (Shahbazian, Zhang et al. 2005), and sumoylation (Iniguez-Lluhi 2006), commonly referred to as the histone code (Biel, Wascholowski et al. 2005). This histone code is thought to either direct and or mark the DNA (Kondo, Shen et al. 2004) for cytosine methylation and for chromosomal remodeling (Henikoff 2005; Hidenobu and Wagstaff 2005). In Drosophila and the mouse, lysine in the ninth position on the N-terminus tail of histone 3 (H3K9) methylation was linked with DNA methylation and heterochromatin, while H3K4 methylation was linked with euchromatin and active genes (McKittrick 2004; Kondo 2004). Also, histone methylation can be mono (Talasz, Lindner et al. 2005), di (Khalil and Driscoll 2006) or tri (Schneider, Wood et al. 2005) on lysine residues, which amplifies the diversity of control mechanisms intrinsic to the histone code.
A list of histone modifications and their association with gene expression is displayed in Table 1.1.

Enzymes identified in the formation and removal of histone modifications are listed in Table 1.2.

### Table 1.1 Histone Modifications and Gene Expression

<table>
<thead>
<tr>
<th>Histone Modification</th>
<th>Histone</th>
<th>Functional Effect on Gene Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylation</td>
<td>H3K4</td>
<td>On</td>
</tr>
<tr>
<td></td>
<td>H3K9</td>
<td>Off</td>
</tr>
<tr>
<td></td>
<td>H3K27</td>
<td>Off</td>
</tr>
<tr>
<td></td>
<td>H3K36</td>
<td>Off (?)</td>
</tr>
<tr>
<td></td>
<td>H1K26</td>
<td>Off (?)</td>
</tr>
<tr>
<td></td>
<td>H3K79</td>
<td>Off</td>
</tr>
<tr>
<td></td>
<td>H4K20</td>
<td>Off</td>
</tr>
<tr>
<td></td>
<td>H4R3</td>
<td>Off (?)</td>
</tr>
<tr>
<td>Acetylation</td>
<td>H2BK11</td>
<td>Off (?)</td>
</tr>
<tr>
<td></td>
<td>H3K9</td>
<td>On</td>
</tr>
<tr>
<td></td>
<td>H3K14</td>
<td>On</td>
</tr>
<tr>
<td></td>
<td>H3K56</td>
<td>Off (?)</td>
</tr>
<tr>
<td></td>
<td>H4K5</td>
<td>On</td>
</tr>
<tr>
<td></td>
<td>H4K12</td>
<td>Off</td>
</tr>
<tr>
<td></td>
<td>H4K16</td>
<td>On</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>H2BS14 (human) &amp; H2BS10 (yeast)</td>
<td>Unknown (?)</td>
</tr>
<tr>
<td></td>
<td>H3S10</td>
<td>On</td>
</tr>
<tr>
<td></td>
<td>H3S28</td>
<td>On (?)</td>
</tr>
<tr>
<td></td>
<td>H4S1</td>
<td>Unknown (?)</td>
</tr>
<tr>
<td>Ubiquitination</td>
<td>H2AK119ub1</td>
<td>On (?)</td>
</tr>
<tr>
<td></td>
<td>H2BK120ub1(human), H2BK123ub1(yeast)</td>
<td>Unknown (?)</td>
</tr>
<tr>
<td></td>
<td>H2BK123ub1</td>
<td>On</td>
</tr>
<tr>
<td>Biotinylation</td>
<td>biotinylated H4K12</td>
<td>Off</td>
</tr>
<tr>
<td>Proline isomerization</td>
<td>H3P38</td>
<td>Unknown (?)</td>
</tr>
</tbody>
</table>

1 see Bhaumik, Smith et al. 2007;  
2 see Jenuwein & Allis, 2001  
3 see Latham & Dent, 2007
<table>
<thead>
<tr>
<th>Covalent modifications</th>
<th>Enzymes ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3K4 methylation</td>
<td>Set1 (Sc), SET7/SET9 (Hs), MLL (Hs), Smyd3 (Hs), Prdm9</td>
</tr>
<tr>
<td>H3K9 methylation</td>
<td>SUV39H1 &amp; SUV39H2 (Mm, Hs), G9a (Mm, Hs), Eu-HMTase1 (Hs), ESET &amp; SETDB1 (Mm, Hs), Cdr4 (Sp), Dim5 (Nc), Kryptonite (At), Ash1 (Dm)</td>
</tr>
<tr>
<td>H3K27 methylation</td>
<td>E(z) (Dm), EZH2 (Hs, Mm)</td>
</tr>
<tr>
<td>H3K36 methylation</td>
<td>SEDT2/HYPB (Hs), NSD1 (Hs), Set2 (Sc)</td>
</tr>
<tr>
<td>H3K79 methylation</td>
<td>DOT1 (Sc), DOT1L (Hs)</td>
</tr>
<tr>
<td>H4K20 methylation</td>
<td>Pr-SET7/SET8 (Hs, Dm), SUV4-20 (Hs), SET9 (Sp)</td>
</tr>
<tr>
<td>H3R2 methylation</td>
<td>CARM1 (Mm, Hs)</td>
</tr>
<tr>
<td>H3R26 methylation</td>
<td>CARM1 (Mm, Hs)</td>
</tr>
<tr>
<td>H4R3 methylation</td>
<td>PRMT1 (Hs), RMT1 (Sc)</td>
</tr>
<tr>
<td>H3K9 acetylation</td>
<td>Gcn5 (Sc), Sre1 (Mm)</td>
</tr>
<tr>
<td>H3K14 acetylation</td>
<td>Gcn5 (Tt, Sc), Sre1 (Mm), TAF1 (Dm, Hs), CBP &amp; p300 (Hs), Sas3 (Sc), MOZ &amp; MORF (Hs), PCAF &amp; hGcn5 (Hs)</td>
</tr>
<tr>
<td>H3K18 acetylation</td>
<td>Gcn5 (Sc), CBP &amp; p300 (Hs)</td>
</tr>
<tr>
<td>H3K23 acetylation</td>
<td>Gcn5 (Sc), CBP (Hs), Sas3 (Sc)</td>
</tr>
<tr>
<td>H3K36 acetylation</td>
<td>Gcn5 (Sc)</td>
</tr>
<tr>
<td>H3K56 acetylation</td>
<td>Rtt109 (Sc)</td>
</tr>
<tr>
<td>H4K5 acetylation</td>
<td>Esa1 (Sc), Hat1 (Tt, Dm, Hs), p300 (Hs), Tip60 (Mm), HBO1 (Hs)</td>
</tr>
<tr>
<td>H4K8 acetylation</td>
<td>p300 (Hs), Esa1 (Sc), Tip60 (Mm), p300 (Hs), HBO1 (Hs)</td>
</tr>
<tr>
<td>H4K12 acetylation</td>
<td>Hat1 (Sc), Esa1 (Sc), Tip60 (Mm) and CBP &amp; p300 (Hs), HBO1 (Hs)</td>
</tr>
<tr>
<td>H4K16 acetylation</td>
<td>Mof (Dm), hMof (Hs), Sas2 (Sc), Tip60 (Mm), Esa1 (Sc)</td>
</tr>
<tr>
<td>De-acetylation³</td>
<td>HDAC’s (class I)</td>
</tr>
<tr>
<td></td>
<td>Hos3 (class II) and the sirtuins (class III)</td>
</tr>
<tr>
<td>H3S10 phosphorylation</td>
<td>Snf1 (Sc), Jil-1 (Dm), Rsk2 (Mm, Hs), Msk1 (Mm), Ip11 (Sc), Aurora B (Ce, Dm, Hs), NIMA (An)</td>
</tr>
<tr>
<td>H3S28 phosphorylation</td>
<td>Aurora B (Mm, Hs)</td>
</tr>
<tr>
<td>H4S1 phosphorylation</td>
<td>Sps1 (Sc), CKII (Sc)</td>
</tr>
<tr>
<td>H2BS phosphorylation</td>
<td>Mst1 (Hs), Ste20 (Sc)</td>
</tr>
<tr>
<td>H2BK11 acetylation</td>
<td>Gcn5 (Sc)</td>
</tr>
<tr>
<td>H2AS129 or H2AXS139 phosphorylation</td>
<td>Tel1 &amp; Mec1 (Sc), ATM &amp; ATR &amp; DNAPK (Hs)</td>
</tr>
</tbody>
</table>
Covalent modifications | Enzymes
---|---
H2AK5 acetylation | Tip60 (*Hs, Dm*)
H2AK119 ubiquitination | Ring1B (*Dm, Mm, Hs*)
H2BK ubiquitination (K120 in human, K123 in yeast) | Rad6 (*Sc*), Bre1 (*Sc*), HR6A (*Hs*), HR6B (*Hs*)
H2AZK14 acetylation | Esa1 (*Sc*), Gcn5 (*Sc*)

1 Adapted from (Bhaumik, Smith et al. 2007)
2 An, *Aspergillus nidulans*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Tt *Tetrahymena thermophila*
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In order to better understand the involvement of the histone code in imprinting and other epigenetic processes, clarification of the importance and function(s) of the individual histone modifications is needed:

1.3.1 **Histone Acetylation.** Histone acetylation is the most studied and best understood histone modification (Allfrey, Faulkner et al. 1964). Acetylation takes place on the ε amino group on lysine residues (Turner 1999). Acetylation patterns are established by highly regulated acetylation enzymes, histone acetyltransferase (HAT’s) and histone de-acetylases (HDAC’s) (Kurdistani and Grunstein 2003). HAT enzymes are grouped into different family classes, GNAT family, MYST family and CBP/p300 family (Roth, Denu et al. 2001). Some transcription factors such as yeast protein Gcn5, a putative transcriptional adaptor, are HAT enzymes (Brownell, Zhou et al. 1996). Histone acetylation modifications take place on H3 at K9, K14, and K56; on H4 at K5, K8, K12, and K16; on H2A at K5 and K9; on H2B at K5, K11, K12, K15, and K20 (Biel, Wascholowski et al. 2005) (Table 1.1).

HDACs catalyze the removal of acetyl groups from histones by activation of a water molecule through a zinc ion in the active site (Finnin, Donigian et al. 1999; Gray and Ekstrom 2001). HDACs are involved in formation of heterochromatin. In human, 18 non-redundant HDAC’s have been identified (Dokmanovic, Clarke et al. 2007). HDAC’s are divided into three classes, I, II and III (Verdin,
Dequiedt et al. 2003). Class I HDAC’s are localized within the nucleus while Class II shuttle between the nucleus and cytoplasm (Dokmanovic, Clarke et al. 2007). Regulation of HDAC’s consist of protein-protein interactions, post-translational modifications, cellular localization, and metabolic cofactors (Sengupta and Seto 2004). Inhibition of HDAC activity has been found useful in the clinical treatment of human disease processes. So far 12 different HDAC inhibitors are undergoing clinical trials (Rasheed, Johnstone et al. 2007).

1.3.2 Histone Methylation. As noted above, specific lysine residues may be mono-, di- or tri-methylated (Paik and Kim 1971; Peters, Kubicek et al. 2003). In total, five H3-lysines can be methylated; K4, K9, K27, K36 and K79 (Lachner, O'Sullivan et al. 2003) (Table 1.1). The transition from di- to tri- methylation of H3K4 and H3K9 lysines is of particular biological significance. In general, H3K4\textsubscript{me3} methylation is linked to transcriptionally active chromatin or euchromatin (Lachner and Jenuwein 2002), while H3K9\textsubscript{me3} methylation is linked to repressed transcription or heterochromatin (Lachner and Jenuwein 2002). However, recently H3K9 methylation has also been discovered in euchromatin (Vakoc, Mandat et al. 2005; Vakoc, Sachdeva et al. 2006) making the meaning and interpretation of the histone modification a difficult task. Part of this complexity may be due to different effects of mono-, di-, and tri- methylation, which were not routinely reported in journal articles. Some data shows that DNA cytosine methylation has a regulatory effect on H3 and H4 deacetylation, as well as demethylation of H3K4 and methylation of H3K9 sites (Soppe, Jasencakova et al. 2002; Hashimshony, Zhang et al. 2003).

Histone H3K9 and H3K27 were shown to work in conjunction with the formation of pericentric heterochromatin, X inactivation, polycomb-mediated gene silencing, and transcriptional repression at euchromatic positions (Lachner and Jenuwein 2002; Fischle, Wang et al. 2003). Methylation of H3K9 is generally associated with silencing of genes and is closely tied to DNA cytosine methylation and
genetic imprinting, as well as X inactivation in mammals (Peters, A.H. 2002, Heard, E. 2001). Reciprocally, H3K4 di- and tri-methylation are associated with active chromatin (Delaval, Govin et al. 2007). Histone arginine methylation, e.g., H4R3, is generally linked to gene activation, while lysine methylation (H3K4 and H3K9) is linked to both activation and repression (Kouzarides 2002; Fischle, Wang et al. 2003). Evidence shows that HP1 proteins are associated with DNA methyltransferase activity, as well as forming a link between histone and DNA cytosine methylation (Bachman, Rountree et al. 2001; Tamaru and Selker 2001) (Table 1.2). 

Histone methyltransferases Suv39h1 and Suv39h2, methylate H3K4 in mice (O'Carroll, Scherthan et al. 2000; Peters, O'Carroll et al. 2001) (Table 1.2). Although, Suv39h1 is expressed in many tissues, Suv39h2 is expressed only in the testis (Peters, O'Carroll et al. 2001). Histone methyltransferase, Prdm9, is expressed in early meiotic germ cells and is responsible for H3K4me3 formation (Hayashi, Yoshida et al. 2005) (Table 1.2). H3K4 tri-methylation is found in active chromatin. Since Meisetz methylates only di-methylated H3K4, and cannot methylate mono- or un-methylated lysine’s, H3K4 must be di-methylated before meiosis (Dawe, Sedat et al. 1994).

Although researchers assumed that histone demethylase existed, nearly 50 years passed before these enzymes were discovered. Recent research has identified two histone demethylation enzymes which can remove methyl groups from lysine and arginine residues, lysine specific demethylase 1 (LSD1) and Jumonji C (JmjC) (Cuthbert, Daujat et al. 2004; Wang, Wysocka et al. 2004). LSD1 is able to demethylate H3K4m1/2, but not H3K4m3. Since LSD1 requires a protonated nitrogen, tri-methylated histone lysine is not a suitable substrate. JmjC domain containing enzymes all function as hydroxylation enzymes (Tsukada, Fang et al. 2006). Histone demethylases are now being considered for anti-cancer therapies (Tian and Fang 2007). LSD1 is closely related to monoamine oxidases (MAO), as the MAO inhibitor, Pargyline, also inhibits LSD1 (Yang, Gocke et al. 2006).
1.3.3 **Histone Phosphorylation.** Gutierrez and Hnilica in 1967 (Gutierrez and Hnilica 1967) reported that phosphorylation of H3S10 and H3S28 are found in cell cycle chromatin condensation, active transcription, apoptosis and DNA repair (Wei, Mizzen et al. 1998; Hirota, Lipp et al. 2005). Histone H3T11 and H3S28 have been identified as mitosis-specific sites for phosphorylation (Goto, Tomono et al. 1999; Preuss, Landsberg et al. 2003).

Histone phosphorylation is also involved with activation of early response genes such as *c-fos*, *c-myc* and *c-jun*. This is a stimulant-dependent H3 phosphorylation, resulting in quick transcriptional activation by activating activation of a subset of immediate-early (IE) genes (Barratt, Hazzalin et al. 1994). Acetylation and phosphorylation are both linked to transcription activation; however, it is unknown whether the two modifications work independently, in parallel, or synergistically (Cheung, Tanner et al. 2000; Clayton, Rose et al. 2000). The involvement of histone phosphorylation in imprinting of genes is presently unknown, although the association with active transcription may suggest a potential role.

DNA cytosine methylation and histone modifications form a complex system of chromatin modification that regulates gene expression (O'Carroll, Erhardt et al. 2001; Vu, Li et al. 2004; Biel, Wascholowski et al. 2005; Morgan, Santos et al. 2005). Histone H3K9\textsubscript{m3} is linked to DNA methylation, genetic imprinting and gene repression, while H3K9 acetylation is linked to gene expression and hypomethylated promoters and DMRs (Bernstein, Kamal et al. 2005). Trimethylated marks, H3K9\textsubscript{m3} and H3K27\textsubscript{m3}, are properties of stably silenced heterochromatin (McGarvey, Fahrner et al. 2006), whereas H3K9\textsubscript{m2} is important for euchromatic gene repression (McGarvey, Fahrner et al. 2006). In mammals, H3K9 methylation may also direct 5-methyl-cytosine formation in pericentomeric heterochromatin (Lehnertz, Ueda et al. 2003). McGarvey et al. (2006) showed that when a hypermethylated gene is
demethylated and re-expressed following 5-azacytidine treatment, H3K9_m1 and H3K9_m2 are the only silencing marks on histones that are lost.

It is becoming a general view that histones are major carriers of epigenetic information. As a result, either synergistically or antagonistically, histones provide mechanisms to generate and stabilize genetic imprints. Complexes consisting of HDACs, histone methyltransferases, and 5-methylcytosine binding proteins recruit DNA cytosine methyltransferases (Fuks, Hurd et al. 2003). Finally, H3K4_m3, as a strong and stable activator of transcription was hypothesized to serve as a memory mark to enforce transcription despite subsequent histone modifications (Ng, Robert et al. 2003; Martin and Zhang 2005; Yu, Zhu et al. 2008).

Histone tail modifications are also closely linked to one another. Changes in one part of the histone code facilitate changes in other parts. For example, phosphorylation of H3S10 is reduced when H3K9 is di-methylated, while H3K9 acetylation increases H3S10 phosphorylation (Rea, Eisenhaber et al. 2000; Sims, Millhouse et al. 2007). Also, H3S10 phosphorylation activates HAT activity and acetylation of H3K14 (Lo, Trievel et al. 2000). Histone modifications and DNA 5-methylcytosine patterns function with a well regulated system of transcription factors, binding proteins, and other factors (Goeman, Otto et al. 2008). H3K4_m3, localized in the 5’ region of genes, is associated with several binding proteins, such as CHD1, BPTF, JMHD2A and the tumor suppressor family, ING. CHD1, in conjunction with H3K4_m3, is involved with pre-mRNA splicing (Sims, Millhouse et al. 2007). The ING family of tumor suppressor binding proteins acts as chromatin-regulatory proteins and is key components of HAT/HDAC complexes (Doyon, Cayrou et al. 2006). ING1 and ING2 form an HDAC co-repressor complex, while ING3, ING4 and ING5 associate with HAT complexes (Goeman, Thormeyer et al. 2005).
Maternal chromatin in the mouse zygote maintains globally methylated H3K9 and DNA cytosine methylation, but the paternal chromatin lacks H3K9 global methylation and DNA cytosine methylation (Arney, Bao et al. 2002; Lepikhov and Walter 2004; Liu, Kim et al. 2004; Yeo, Lee et al. 2005), which would suggest a dependent linkage. The theory is that H3K9_m3 in the maternal pronucleus protects the maternal DNA from demethylation, while a lack of H3K9_m3 in the paternal pronucleus allows global DNA hypomethylation to occur (Santos, Peters et al. 2005). There are high levels of H3K9_me3, in the mouse arrested maternal oocyte chromosome prior to fertilization. In this case, the paternal chromatin shows demethylation within four hours after fertilization (Arney, Bao et al. 2002). Nevertheless, only a few imprinting control centers are methylated in the male germ line (Delaval, Govin et al. 2007). Following fertilization paternal imprinting, control centers have been found to be protected from global demethylation by H4K20 and H3K9_m3, as opposed to the unmethylated allele which has H3K4_m2 and H3 acetylation (Delaval, Govin et al. 2007). In the newly fertilized zygote, the paternal pronucleus undergoes a complete remodeling as histones replace the sperm protamines. Although maternal chromatin exhibits all three H3 methylation modifications, the newly remodeled paternal chromatin receives unmodified histones from the zygote cytoplasm (Arney, Bao et al. 2002; Cowell, Aucott et al. 2002). Methylated H3K9 is found only in the maternal chromatin, and thus is completely absent in the paternal chromatin (Lepikhov and Walter 2004).

Obviously the regulation of DNA 5-methylcytosine and histone modifications are linked by complexes of binding proteins, transcription factors and transferase enzymes (Guccione, Bassi et al. 2007; Ansari, Mishra et al. 2008). What is not known is whether one modification is dominant to the other. In other words, do histone modifications direct DNA cytosine methylation, or vice versa? A well known inverse link exist between DNA methylation and histone acetylation (Fuks 2005), yet there is still controversy regarding the role of histone methylation (Gartler, Varadarajan et al. 2004; Umlauf,
Goto et al. 2004; Zhang, Fatima et al. 2005; Zhao, Soejima et al. 2005). From the known data, there are three logical possibilities for 5-methylcytosine and histone modification regulation and establishment: common regulation/dependent, common regulation/independent or independent regulation/combination. Common regulation /dependent represents a common regulator, with both 5-methyl-cytosine and histone modifications being dependent on concurrent establishment. With common regulation /independent, however, both 5-methylcytosine and histone modifications are under common regulation, but the establishment could be independent of each other. Independent regulation is separate and retains independent establishment. However, independent establishment does not rule out either of cooperation or synergistic effects; for instance, 5-methylcytosine pattern formation may increase some histone modifications.

Is DNA methylation the regulation that marks histones for modification, or is it the histones that regulate DNA methylation patterns? In vitro fertilization (IVF) studies have shown that pre-implantation embryos are sensitive to environmental manipulation (Fleming, Kwong et al. 2004). For example, embryo culture conditions and culture media may change methylation patterns of imprinted genes (Doherty, Mann et al. 2000). However, no changes in histone modifications (methylation, acetylation, or phosphorylation) between in vitro and in vivo in mouse embryos have been detected (Huang, Lei et al. 2007). In tumor suppressor gene studies, elimination of DNA methylation from \( p16^{INK4} \) a promoter resulted in marked changes in surrounding histones (Bachman, Park et al. 2003). However, this study also found that histone modifications linked to tumor suppressor gene silencing can occur independently of DNA methylation. The question still remains: Which comes first, DNA methylation or histone modifications? More precisely, are the histone modifications regulating DNA methylation and genetic imprinting, or are the 5 methylcytosine DNA modifications regulating histone modifications?
Numerous toxicants were reported such as industrial pollutants as well as naturally occurring toxins causing genetic mutations and/or as cellular damage (Nyce 1997; Edwards and Myers 2007; Hamon, Batsche et al. 2007). Chemicals such as benzene, dihalides, heavy metals, and tobacco smoke were shown to cause genetic mutations and chromosomal aberrations, as well as reproductive problems (Thier, Taylor et al. 1993; Wen, Meng et al. 2007; Xiong, Hu et al. 2007; Soares and Melo 2008; Wise, Holmes et al. 2008). In one study, low-dose benzene was linked to aberrant DNA methylation patterns such as hypermethylation in p15 and hypomethylation in MAGE-1. Low-dose airborne benzene however, was not linked to loss of imprinting (LOI) (Bollati, Baccarelli et al. 2007). Benzo (a)pyrene has been shown to alter H3K9 acetylation patterns in MCF7 breast cancer cells (Sadikovic, Andrews et al. 2008). New research now shows that even bacterial toxins may induce changes in histone modifications. L. monocytogenes modulates host gene expression by de-phosphorylation of H3-Ser\textsubscript{10} and deacetylation of H4 by its secretion of a virulence factor listeriolysis (LLO) (Hamon, Batsche et al. 2007). Dephosphorylation of H3-Ser\textsubscript{10} has also been shown to be a signature of two other toxins in the LLO family. C. perfringens perfringolysin (PFO) and S. pneumonia pneumolysin (PLY).

Pneumolysin is the most intriguing of the toxins from pneumonia, remains a common infection. The epigenetic effect on gametogenesis and embryogenesis following infection by S. pneumonia is potentially dramatic. In this study, ten genes were selected at random to test the effect of gene expression by LLO. Results showed that three were up-regulated, five were down-regulated and two remained unchanged (Hamon, Batsche et al. 2007). In mice, tobacco smoke was linked to increased mutation frequencies in spermatogonial stem cells (Yauk, Berndt et al. 2007). Research showed that nickel chloride blocked histone acetylation in yeast (Ke, Davidson et al. 2006) and other researchers showed the same affects in mammalian cells (Golebiowski and Kasprzak 2005). Changes in DNA sequences may also affect the DNA 5-methylcytosine and/or the histone modifications patterns.
The importance of studying the effects of EDC on male reproductive system is crucial to the health of both the present and future generations. Unrepaired DNA lesions or de novo mutations in spermatogonial stem cells are imparted systemically to the offspring. Even with ubiquitous human exposures to dihalides, few gametogenic and reproductive studies have been done. Alkylating agents such as EDC can disrupt heritable epigenetic information by many pathways. For example, dichloro-acids are commonly used in the disinfection of drinking water, yet were shown to be developmentally toxic in mouse embryos (Andrews, Nichols et al. 2004). While not directly disrupting epigenetics, endocrine disruptive chemicals can indirectly alter genetic imprint patterns by blocking or sending false endocrine signals as well as affecting endocrine organs (Greim 2004).

In vivo assays are the standard for reproductive and genetic toxicology assays. Because of regulations it is not only becoming an increasingly cost prohibitive burden, but is also frowned upon by the animal rights movement. Some people question if in vitro assays are reliable and efficient enough to be used for reproductive and genetic toxicological assays (Lilienblum 2008). Yet the in vitro culture of mouse embryo and spermatogonial stem cells has been well studied (Kubota, Avarbock et al. 2004; Shi, Wang et al. 2006; Shen, Zhang et al. 2008). Current research shows that long term in vitro tissue culture is a reliable way to propagate embryo and spermatogonial stem cells with certain limits due to possibility of aneuploidy (Hayflick 1965; Kubota and Brinster 2006; Sun, Tao et al. 2008). What is not yet known is whether in vitro culture of embryo and spermatogonial stem cells can be effectively used for reproductive and genetic toxicological assays. Can embryo stem cells be transformed into spermatogonial stem cells reliably and with high efficiency? Can spermatogonial stem cells be subcultured and cloned from a single cell or colony? Are embryo and spermatogonial stem cells resilient enough to survive treatments with carcinogens and toxicants? Most importantly, will treated
spermatogonial stem cells reliably and effectively trigger meiosis in order to establish the epigenetic programming?

The initial step in this dissertation research was to determine whether or not EDC adversely affects male reproductive functions. As described in Chapter 2, EDC produced dose-dependent testicular pathology and sterility in male C57/BL mice. Subsequently, the approach entailed studying the impact of EDC on the epigenetic patterns that constitute the paternal imprint. The paternal imprint is established in pre-meiotic spermatogonia. In order to study these epigenetic patterns, an in vitro culture model of spermatogonia stem cells and maturing pre-meiotic spermatogonia was required. Chapter 3 describes the development and validation of the in vitro culture model for murine spermatogonial stem cells and the induction of maturing pre-meiotic spermatogonia. It has been proposed since 2004 that stem cells are the perfect in vitro model to test for both genetic and epigenetic toxicology (Davila, Cezar et al. 2004). Finally, Chapter 4 describes this experimental approach and results of studying the impact of EDC on the paternal imprint in murine pre-meiotic spermatogonia.
CHAPTER 2
ETHYLENE DICHLORIDE DISRUPTION OF FERTILITY IN MALE MICE*

2.1 Introduction

Ethylene dichloride (EDC), also known as 1,2-dichloroethylene, is a high use chemical in today's industry, for the synthesis of vinyl chloride, as a chlorinated solvent, and as a constituent in paints, soaps, scouring compounds, and metal degreasers (ATSDR 2001). EDC has also been used as an extracting solvent for spices and other natural flavorings, including tobacco. The production of EDC in the United States was about 16 million tons in 1994 (ATSDR 2001). In Louisiana the total EDC discharge for 1987 to 1993 was greater than 15 million pounds (LDEQ 1995).

EDC is a known mutagen requiring metabolic activation by glutathione conjugation. Glutathione-dependant metabolites, such as S-(2-chloroethyl) glutathione and S-(2-chloroethyl)-L-cysteine, spontaneously rearrange to form reactive episulfonium ions that react with nucleophilic sites on cellular macromolecules including DNA (Peterson, Harris et al. 1988; Romert, Magnusson et al. 1990). The major DNA adduct formed from EDC is reported to be N7-ethyl-S-glutathionyl deoxyguanosine (Romert, Magnusson et al. 1990; Cmarik, Humphreys et al. 1992). Interestingly, the mutation spectrum produced by EDC and other dihaloethanes appears to be limited to G → A transitions (Aafjes, van der Vijver et al. 1978; Cmarik, Humphreys et al. 1992). Since most tissues contain glutathione and glutathione-S-transferase, EDC may react and damage multiple organs systems including testis. The US EPA has designated EDC as a potential carcinogen, hazardous substance, and priority toxic pollutant (EPA 2000). EDC is also designated as a possible human carcinogen (Group 2B) by the International Agency for Cancer Research (IARC 2006).

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Early studies with EDC have reported little or no reproductive toxicity. Inhalation studies showed only embryo toxicity at high doses and no significant reproductive toxicity (Lane, Riddle et al. 1982). Rats and mice exposed to this highly volatile compound in drinking water or in diet showed no effects on fertility (Alumot, Meidler et al. 1976) and lacked remarkable pathology in reproductive tissues (Daniel, Robinson et al. 1994). These reports of limited reproductive and testicular toxicity by EDC are surprising since tissues with significant mitotic indexes are generally sensitive to reactive alkylating compounds, such as the activated EDC-glutathione episulfonium ion. Mammalian gonadal tissues are well known to be sensitive to cytotoxic agents. The loss of fertility in males due to chemotherapy has been well documented (Chapman 1983; Meistrich 1984; Meistrich and Samuels 1985; Meistrich 1993). The present study was undertaken to evaluate the effects intraperitoneal EDC on the fertility and testicular tissue in male mice.

2.2 Material and Methods

Animals. Ten-week old male C57BL/6 mice were purchased from Charles River Laboratories Inc. (Wilmington, MA). The mice were housed in an animal core facility in a controlled temperature (22°C) and humidity (50%-60%) environment with greater than 10 complete exchanges of filtered air per hour. Mice were also on a 12 hr light/dark cycle, and were fed autoclaved commercial mouse chow (Purina Autoclavable Laboratory Chow 5010, Ralston-Purina, St. Louis, MO) ad libitum. Males were housed four or five to a cage during acclimatization, and then one to a cage at the beginning of the experiment. As excellent breeders with C57BL/6 males, female Balb/c mice were obtained from the LSU School of Veterinary Medicine Animal Core Facility’s breeding colony, and paired with males for fertility determinations.
EDC (CAS No. 107-06-2) (99.8%) was purchased from Sigma-Aldrich (St. Louis, MO). Each solution of EDC in corn oil was prepared fresh daily at concentrations necessary for intraperitoneal (IP) injections of 0.1 ml per 10 g mouse weight. Sexually mature (greater than 11 weeks of age) male C57BL/6 mice received five IP injections, one per day for five consecutive days (Figure 2.1). Fertility determinations utilized groups of three male mice per dose reported. Mice received a dose of 5 to 40 mg/kg of EDC or vehicle, with each IP injection, and subsequently held for 45 days to allow for complete turnover of spermatogenesis (Drost and Lee 1995) (Table 2.1). Males were then paired with Balb/c females to determine fertility. Males were determined to be permanently sterile if found to be infertile for six months or longer. Permanently sterile males and male mice that recovered to fertility were euthanized by CO\textsubscript{2} asphyxiation only after siring two consecutive litters (Figure 2.1). Upon sacrifice, the testes and epididymides were surgically excised carefully to reduce crush artifact, and immersed in formalin for subsequent pathological and histological analyses.

A separate experiment designed to determine the early testicular pathological effects of EDC was performed with the same dosing protocol above using 10 mg/kg. Controls received IP injections of the vehicle (corn oil) only. Following the five days of dosing, one to three male C57BL/6 mice were euthanized at each time interval post treatment of 8, 15, 31, and 46 days, and the testicular pathology assessed (Table 2.2).

Pathology. Hematoxylin and eosin stained sections of paraffin embedded, formalin fixed testis were analyzed for the total number of Sertoli-only (SCO) tubules, and Leydig cell counts, for each testis examined. For percent SCO determinations, between 130 and 300 separate tubules (248 tubules per testis on average) in each testis preparation were examined for the presence or absence of spermatogenic cells (spermatogonia, spermatocytes, spermatids and spermatozoa) in
addition to Sertoli cells. Leydig cells were counted in five randomly chosen high power microscopic fields (1.77 mm²) and the average cell count reported for each testis studied.

Individual testes were also given a Spermatogenesis Score. The scoring system published by Johnsen (17) is based on human testicular evaluation. Mouse spermatogenic stages are arranged in relatively linear manner compared to the helical pattern observed in humans. However, the random evaluation of twenty seminiferous tubules per histological testicular cross-section should normalize these species differences and provide an appropriate scoring system to compare changes in testicular functions. Using the criteria of Johnsen (17) as modified by Aafjes et al. (18), hematoxylin and eosin stained sections of paraffin embedded, formalin fixed testis were scored on a 10 point scale assessing the extent of spermatogenesis. Twenty randomly chosen seminiferous tubules were evaluated utilizing the testicular scoring protocol below:

10: Complete spermatogenesis
9: Many spermatozoa present, but germinal epithelium disorganized with marked sloughing or obliteration of lumen
8: All stages of spermatogenesis present, but fewer than 5-10 spermatozoa present
7: No spermatozoa, but many spermatids present
6: No spermatozoa, but fewer than 5-10 spermatids present
5: No spermatozoa, no spermatids, but many spermatocytes present
4: No spermatozoa, no spermatids, but fewer than 5 spermatocytes present
3: Only spermatogonia present
2: Sertoli cells only
1: No cells in tubular cross section.
Statistical Analysis. Statistical analysis was performed using the unpaired, two-tailed t test for differences between treated and control percentages of SCO tubules and Leydig cell counts. The linear plot for Leydig cell counts was determined using a Least Squares fit within 95% confidence interval. The criterion for significance for all tests was $P \leq 0.05$.

2.3 Results

Sexually mature male C57BL/6 mice were treated daily with IP injections of EDC in corn oil at doses of 0, 5, 10, 20 and 40 mg/kg for five days (Table 2.1). The dosing range for these experiments was chosen based on the literature. Short-term doses of 40 mg EDC/kg and lower were not anticipated to produce substantial systemic toxicity in mice; $LD_{50}$ for mice is reported to be 489 mg/kg of body weight (ATSDR 2001). In the present studies, mice treated with EDC showed no obvious toxicity even at the 40 mg/kg dose. Weight gain was consistent and matched controls throughout treatment time period and throughout the study. No loss of hair or any obvious lesions were noticed. Treated mice appeared normal with no sign of disorientation or lethargic behavior. These treated mice maintained a healthy normal appearance for the rest of the study, with the exception of one mouse (10 mg/kg dose) that died during week 13 post treatment of undetermined cause. A gross survey of organs and tissues did not discern an obvious cause of death or other notable injuries attributable to the EDC treatment.

Since spermatogenesis was active at the time treatment began, mice were held for 45 days after the end of the five day treatment to allow for complete turnover of spermatogenesis prior to testing for fertility (Drost and Lee 1995) (Figure 2.1). This experimental protocol was designed to enable the determination of the effects of EDC on the early stages of spermatogenesis without the interference of mature sperm or maturing sperm that may have been collected and retained in the caudal epididymides and vas deferens during and shortly after treatment.
Thus, 45 days post EDC treatment males were paired with female mice to determine fertility (Figure 2.1). Unexpectedly, one male from each of the 20 mg/kg and 5 mg/kg groups sired a litter of six offspring immediately after being paired with female mice and were subsequently found to be permanently sterile or sterile for a brief period, respectively (see Table 2.1). The 5 mg EDC/kg treatment group was the only dosing group where mice recovered to fertility after a brief sterile period (Table 2.1).

Male mice were euthanized by CO$_2$ asphyxiation after siring two consecutive litters or determined to be permanently sterile. For all mice, gross examination of liver, brain and kidney tissues showed no obvious effects from EDC. Testicular tissue was examined for 0, 5, and 10 mg/kg doses. The testes were atrophic and the epididymides were shrunken and deflated in the sterile mice sacrificed at 270 days post treatment, for both EDC dose levels of 5 and 10 mg/kg. Histological analyses consistently displayed hypocellular tubules lacking spermatocytes and spermatogonia, and prominent vacuolization including Sertoli cells, while Leydig cells were evident (Figure 2.2).

A more promising testicular pathology was observed from the two males treated with 5 mg EDC/kg that recovered to fertility after a 3 and 5 week sterile period, respectively, prior to sacrifice at 120 days (see Table 2.1). Histological analyses of testes from fertility-recovered males displayed active spermatogenesis among tubules with pleomorphism and hyperchromasia of spermatocytes and spermatogonia as compared to controls (Figure 2.3). Other tubules displayed disruption of spermatogenesis and significant apoptotic processes.

A short-term study was performed to determine the time course of the development of testicular pathology. Male mice were treated with 10 mg EDC/kg or vehicle under the same five-day treatment protocol above, and sacrificed at intervals of 8, 15, 31, and 46 days post treatment.
EDC induced adverse pathology was evident as early as 8 days post treatment (Figure 2.4). It appears that EDC may have induced an arrest in spermatogenesis with subsequently developing tubular germ cell aplasia within 8 days. Tubular damage, marked vacuolization of cells and loss of spermatogonia was detectable at 8 days and deteriorated with longer time periods post dose (Figures 2.2, 2.3 & 2.4). However, Leydig and Sertoli cells were consistently present in these histology sections.

As indicators of testicular pathology, histologic preparations from mouse testes were examined for Leydig cell counts, percentage of seminiferous tubules that are SCO, and scored based on spermatogenic activity (17,18). Analyses of testicular tissue from all of the mice treated with EDC displayed numerous Leydig cells in the intertubular interstitial spaces. Based on EDC dose, testes from the 5 mg/kg treated mice contained significantly more Leydig cells than controls (P < 0.041) while the 10 mg/kg treated mice were not significantly different from controls (Table 2.2). However, this lack of dose response effect may have been biased by time effects post treatment. Based on time post treatment, testicular preparations from EDC treated mice sacrificed equal to or greater than 120 days contained significantly more Leydig cells than preparations from EDC treated mice sacrificed less than 120 days (51.0 ± 9.5 (n=5) vs. 34.7 ± 4.0 (n=6); P< 0.004), and from control mice (38.5 ± 6.4 (n=8); P< 0.016). As these mouse experiments were established for clinical fertility determinations, none of the control mice were carried out to 120 days or longer. Control Leydig cell counts ranged from 30 to 45, which was similar to the less than 120 day EDC treated mice. This EDC time post treatment effect on increasing Leydig cell numbers is more clearly seen in a Least Squares curve fit analysis of a scatter plot (see Figure 2.5).

Sertoli cells also appeared to be resilient to the damaging effects of EDC as these cells were consistently present in testicular preparations from all of the treated mice. Seminiferous tubules
from each testis were analyzed for the presence of Sertoli cells and the loss of developing spermatogonia and spermatozoa, providing the recorded result of percent of the tubules that are SCO. Testicular preparations from both 5 and 10 mg/kg EDC treatment groups displayed significantly more SCO tubules than controls (P< 0.04 and P<0.0002, respectively) (Figure 2.6). Testes from mice treated with 5 mg EDC/kg ranged from 2% to 36% SCO, the 36% corresponding to the one mouse at this dose that was found to be permanently sterile, while the controls ranged from 0% to 4% SCO. Similarly, testes from mice treated with EDC at 10 mg/kg ranged from 7% to 22%, with the highest values corresponding to mice euthanized after 270 days, suggesting a time post treatment effect. Testicular preparations obtained from mice treated with EDC and euthanized 120 days or later harbored significantly more SCO tubules than EDC treated mice sacrificed less than 120 days (22.8 ± 11.9 (n=5) vs. 8.67 ± 1.63 (n=6); P< 0.017). However, both of these EDC treatment groups were significantly different than controls (1.56 ± 1.52 (n=8); P< 0.0003 and P< 0.0001, respectively), even though the controls were sacrificed in the same time frame as the latter EDC treated group, less than 120 days post treatment. The impact of 10 mg/kg of EDC treatment was observable as early as 8 days post treatment with the testis displaying 7% SCO tubules (Figures 4 & 6). Interestingly, testicular damage remained at a similar level throughout a 46-day post treatment time course, as testes obtained from EDC treated males 15, 31 and 46 days post 10 mg/kg dose displayed 7% to 11% SCO tubules.

EDC induced adverse pathology was evident within 8 days post dose and reached a plateau throughout 15 to 46 days. Using the standard methods for the evaluation of cellular activities and structural integrity, testes were given a pathology score. As early as 8 days post treatment with 10 mg/kg of EDC the testis displayed a reduced score of 8.35. Controls provided testicular pathology scores ranging from 8.85 to 9.85 (average 9.46). Testes obtained from males 15 to 46 days post 10
mg/kg treatment displayed scores ranging from 6.40 to 7.40, which were similar to the scores of sterile males (10 mg/kg dose) carried for 9 months (270 days). Comparing all testicular specimens together demonstrated a direct response to EDC and decreased spermatogenesis (Figure 2.6). The testis score decreased significantly with EDC doses of 5 and 10 mg/kg (P< 0.0001) compared to controls. The time course of the effect on testicular damage and the testis score is clearly displayed in a Spline curve of the combined 5 and 10 mg/kg treatment groups (Figure 2.7).

2.4 Discussion

Reproductive toxicity has not been previously associated with EDC, a heavily used industrial chemical (WHO 1987; ATSDR 2001). The present experimental results suggest that EDC may potentially affect the mammalian testes resulting in partial loss of germ cells and decreased fertility. Short-term IP doses of 10 mg EDC/kg, daily for five days, rendered male C57BL/6 mice sterile for at least 9 months. Previous reports on the lack of reproductive toxicity utilized drinking water and diet as a route of exposure (8 – 11). It is possible that the concentrations of EDC in the food and water were over estimated due to the high volatility of this compound. Alternatively, the intraperitoneal injection route may bias the distribution of EDC in such a way as to enhance the concentrations of EDC that reach the testes. Further studies will be necessary to clarify these issues and to determine the presence of any reproductive health risks to mammals or humans from EDC by other routes of exposure.

The sensitivity of the mammalian testis and germ cells to EDC may also be a function of the dose-rate due to the accumulation of damage produced by each daily exposure (Boekelheide and Eveleth 1988). The sensitivity of the human testis to the toxic effects of EDC as compared to the mouse is not currently known, although infertility-inducing doses of chemotherapeutic drugs are similar in man and mouse (Meistrich 1993; Seaman, Sawhney et al. 2003).
Activation of EDC occurs by glutathione conjugation, producing a highly reactive episulfonium ion (Romert, Magnusson et al. 1990; Cmarik, Humphreys et al. 1992). With the possible exception of the latter stages of maturing germ cells, most of the cells of the testes contain glutathione and express one or more glutathione-S-transferases, so that EDC is readily activated in the testes. The testicular toxic impact of EDC could result from targeting germ cells, Sertoli cells or Leydig cells. The sensitivity of germ cells to the direct actions of alkylating agents depends on the stage of maturation, such that dividing pre-meiotic spermatogonia may be much more susceptible to lethal effects of EDC than post-meiotic spermatids which no longer actively replicate DNA, are repair-deficient, and begin to condense chromatin. Alternatively, disruption of the Sertoli cell function by EDC may lead to a precipitous and persistent loss of germ cells. Diminished production of cytokines and other factors (e.g. leukemia inhibitory factor (LIF) and bone morphogenic protein-4 (BMP4)) will lead to spermatogonial stem cells losing their self-replacement character and becoming committed spermatogonia cells (Pellegrini, Grimaldi et al. 2003; Ying, Nichols et al. 2003; Lacham-Kaplan 2004). Early differentiation committed germ cells, spermatogonia A1 – A4 stages and above, require stem cell factor (SCF) and the nurturing of selective seminiferous fluid constituents produced by Sertoli cells near the basal membrane of the seminiferous tubule (Rossi, Sette et al. 2000; Sette, Dolci et al. 2000). Adhesion proteins specific for Sertoli cell-germ cell attachment may also be required for germ cell survival and maturation (Beall, Boekelheide et al. 2005). Disturbing Leydig cell testosterone production may perturb the hypothalamic-pituitary-testis axis and gonadotropic hormone balance, leading to loss of spermatogenesis and testicular atrophy (Cook, Klinefelter et al. 1999). The continued presence and health of Leydig cells in atrophic testes from sterile mice argues against this mode of action by EDC. However, Leydig cell hyperplasia, as observed in the present study, has also been reported in response to other alkylating
agents and may be the result of indirect long-term hormonal feedback due to limited or lack of spermatogenesis in the testes (30,31).

The present experimental results demonstrated the survival of both Sertoli cells and Leydig cells despite persistent and prolonged testicular damage initiated by EDC. This suggests that if either of these two cell types was targeted by EDC, the greatest impact was on Sertoli cellular function and the subsequent loss of maintenance and maturation of germ cells. Based on testicular pathology, the damage induced by EDC was complete within the first 8 days. This protracted atrophic effect on spermatogenesis was most likely due to a significant loss of required factors and the environmental niche of seminiferous fluid constituents, and/or close Sertoli cell-germ cell association. Lack of maintenance of spermatogonial stem cells as self-replacing germ stem cells would lead to an ultimate loss of germ cells, since spermatogonial stem cells would become differentiation committed spermatogonia A1 or B cells. The result is the same whether increased apoptotic pressure induced loss of germ cells during maturation, or by direct lethal alkylation damage to stem cells. However, spermatogonial stem cells have a slow cell cycle rate of 8 or more days in the testes, allowing time for significant DNA repair prior to DNA replication and division, making direct cell killing by EDC alkylation less likely (Meistrich 1984; Schoenfeld, Hall et al. 2001). It is possible that the careful determination of which stage(s) of spermatogonia are initially diminished by EDC may provide more clarification of the mechanism by which EDC disrupts spermatogenesis.

Doses of EDC at 5 mg/kg produced sterile periods of several weeks in two of three treated male mice, suggesting that recovery and repopulation of the seminiferous tubules with spermatogonial stem cells is possible at lower doses. Similar dose-dependent recovery of spermatogenesis in mice has been described for cis-platinum (Pogach, Lee et al. 1989), neutron
radiation (van Beek, Meistrich et al. 1990), 2.5-hexanedione (Boekelheide 1988), mono-2-ethylhexyl phthalate (Richburg and Boekelheide 1996), and in humans treated with chemotherapeutic agents (Meistrich 1984).

EDC is a potent mutagen and could initiate mutations in replicating germ cells. The closely related compound, 2-chloroethyl methanesulfonate, that also forms the same structural DNA adducts as EDC via an episulfonium ion following activation by glutathione, has been shown to be a potent germ cell mutagen in *Drosophila melanogaster* (Fossett, Byrne et al. 1995; Fossett, Byrne et al. 1996). Germ line mutagenesis has been demonstrated for 2-bromoethane and EDC in *Drosophila* (Kramers, Mout et al. 1991). It is also interesting to note that two offspring in the last litter sired by one of the fertility-recovered males (5 mg/kg group) were severely deformed. However, the potential risk for EDC initiated increases in germ line mutagenesis in humans is unknown and perhaps less probable (Wyrobek, Mulvihill et al. 2007).

The present study suggests that EDC administered IP is toxic to the testes and spermatogenesis in mice. Similar testicular toxicity might well be a property of the class of dihaloethanes. More research is needed to determine the mode of disruption of spermatogenesis, loss of germ stem cells and protracted testicular damage, as well as the impact of the route of exposure on this testicular toxicity. Titration of the dose of EDC used in this 5-day treatment regimen enabling fertility-recovery and expansion of surviving spermatogonial stem cells may provide an additional model for the study of germ cells at all stages of maturation and their susceptibilities to toxic actions of chemicals. With the progressive loss of spermatogonia, but not the Sertoli cells from tubules, the pathology has similarities to the testicular dysgenesis syndrome in the human clinic (Skakkebaek, Rajpert-De Meyts et al. 2001), and possibly to a nonheritable, environmentally acquired Sertoli Cell-Only Syndrome (Anniballo, Ubaldi et al. 2000).
2.5 List of Abbreviations

EDC, Ethylene dichloride

SCO, Sertoli Cell-Only

2.6 References


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2.7 Experimental Data

Table 1. Fertility Results in Mice Exposed to EDC

<table>
<thead>
<tr>
<th>Dosage a (mg/Kg)</th>
<th>Sterile Period b</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None (Fertile)</td>
</tr>
<tr>
<td>5</td>
<td>3 weeks (one mouse), 5 weeks (one mouse), Permanently Sterile (one mouse), sterile for 270 days post treatment</td>
</tr>
<tr>
<td>10</td>
<td>Permanently Sterile (2 mice c), sterile for 270 days post treatment</td>
</tr>
<tr>
<td>20</td>
<td>Permanently Sterile (all 3 mice), sterile for 180 days post treatment</td>
</tr>
<tr>
<td>40</td>
<td>Permanently Sterile (all 3 mice), sterile for 180 days post treatment</td>
</tr>
</tbody>
</table>

a Three mice per dose group, except for control (zero dose, corn oil only) which contained four mice. Each male mouse received 5 ip doses, one per day for 5 days.
b Following a 45 day post treatment holding time, male mice were housed with fertile female mice until determined to be fertile or permanently sterile (see Materials and Methods).
c One mouse died during week 13 of unknown causes (see text)

Table 2. Leydig Cell Counts in Mice Exposed to EDC

<table>
<thead>
<tr>
<th>Dosage a (mg/Kg)</th>
<th>Leydig Cells b</th>
<th>P value c</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>38.5 ± 6.4 (8)</td>
<td>--------</td>
</tr>
<tr>
<td>5</td>
<td>51.3 ± 11.7 (3)</td>
<td>P&lt; 0.041</td>
</tr>
<tr>
<td>10</td>
<td>38.6 ± 8.8 (8)</td>
<td>P= 0.975</td>
</tr>
</tbody>
</table>

a Each male mouse received 5 ip doses, one per day for 5 days.
b Average number of Leydig cells present in five randomly chosen high powered microscopic fields.
c Comparison with controls.
d Mean ± standard deviation, (number of mice analyzed)
Figure 1. Flow chart for experimental treatment and fertility testing of mice. Sexually mature male mice received 5 IP injections, one a day for 5 days, and then allowed to rest for 45 days for complete spermatogenesis turnover. Males were then housed with female mice to establish fertility. Mice were euthanized after siring 2 consecutive litters of pups or after found to be sterile for 6 months or longer.
Figure 2. Testis histological sections from a mouse determined to be sterile for 9 months following treatment with 5 mg EDC/kg. (A) Section at 100x magnification, this section displays the disruption of interstitial collagenous tissue, preservation of Leydig cells and loss of spermatogonia in many tubules, where vacuolization is prominent, signifying cellular damage. (B) At 400x magnification, this tubule shows rare spermatogonia and vacuolar change of Sertoli cells; note the intact Leydig cells adjacent to the tubule.
Figure 3. Testis histological sections from (A & C) normal control (0 mg/kg) mouse, and (B & D) 5 mg/kg treated mouse that recovered to fertility following a sterile period of 5 weeks, exhibiting noticeable differences in spermatogenesis. Note on scanning magnification the loss of cellularity, loss of adhesion amongst the tubules and interstitial tissue, and increased number of dilated seminiferous tubules in the EDC treated mouse (B) compared to the densely packed seminiferous tubules of the normal control mouse (A) (20x magnification). At 400x magnification, the normal control mouse shows complete, organized spermatogenesis with numerous spermatozoa (C); whereas, the EDC treated mouse shows disruption of spermatogenesis with loss of spermatozoa and spermatids, and scattered apoptotic bodies (D).
Figure 4. Testis histological sections from a 10 mg EDC/kg mouse euthanized only 8 days after treatment. (A) Section at 100x magnification, sections display tubular damage and disruption of testicular structure. (B) At 200x magnification, two central Sertoli Cell-Only tubules show marked vacuolization (and loss of spermatogonia).
Figure 5. Plot of testicular Leydig cell counts versus the time post EDC treatment that mice were euthanized. Both 5 and 10 mg/kg treatment groups are included. The linear curve was determined by a Least Squares fit analysis ($r = 0.74$, $P = 0.009$).
Figure 6. Dose response effects of EDC on testicular pathology. The Testes Score (light grey) decreased with EDC treatment, while the percentage of tubules that only contained Sertoli cells (dark grey) increased. The time post treatment that testes were harvested from mice following euthanization, ranged from 8 to 270 days. Two of the mice in the 5 mg/kg EDC treatment group recovered fertility prior to harvest at 120 days. The number (n) of testes (one per male mouse) in each treatment group is denoted above the bars. A single asterisk (*) indicates that the Testis Scores for the 5 and 10 mg/kg EDC treatment groups were significantly different (p< 0.0001) from controls (0 mg/kg EDC). A double asterisk (**) and triple asterisk (***)) indicates that the percent of SCO tubules were significantly elevated (P< 0.04 and P< 0.0002, respectively) from controls.
Figure 7. Spline curve demonstrating the effect of EDC on spermatogenesis over time. Spline curve was derived from the combined data of both 5 mg/kg and 10 mg/kg treated mice. This is a median Spline curve calculated by obtaining cross medians, which are used as knots to fit a cubic spline that is graphed as line plot. Insert photo shows two seminiferous tubules from a 5 mg/kg treated mouse that was sacrificed at 120 days, one (top) with sloughed necrotic material and spermatogonia only (score 3), and the other (bottom) with few spermatozoa and all stages of spermatogenesis (score 8).
CHAPTER 3

DEVELOPMENT OF THE IN VITRO MOUSE SPERMATOGONIAL STEM CELL CULTURE MODEL

In order to study the potential impact of EDC on the paternal imprint, germ cells containing the normal or perturbed DNA imprint must be isolatable to a reasonable purity. Although mature spermatozoa are easily accessible in semen specimens, the prolonged maturation time in spermatogenesis and storage prior to ejaculation leads to difficulties in studying acute effects of toxicants on the paternal imprint. Obtaining sufficient numbers of the correct stage of spermatogonia from resected testes after acute in vivo EDC exposure is complicated by the mixture of germ cells at all stages of spermatogenesis. The paternal imprint is established in spermatogonial stem cells or in early stage differentiating spermatogonia A cells prior to meiosis. The development of an in vitro model system that enables the stable culturing of spermatogonial stem cells and triggering of spermatogenesis, without the use of a ‘feeder’ layer, would provide an ability to perform acute exposure experiments, thus making available analyses of specific epigenetic patterns in DNA from a high purity of germ cells at the appropriate stage of maturation.

The value of in vitro models resides in the ability to mimic in vivo conditions while limiting the complexity of the cellular system under study. Previous reports to date have emphasized the need to use feeder cultures in order to grow and differentiate mammalian spermatogonial stem (A<sub>s</sub>) cells (Nayernia, Lee et al. 2006; Nayernia, Nolte et al. 2006). Recent reports by Nayernia et al. (2006a; 2006b) have shown that mouse embryonic stem (MES) cells can differentiate into primordial germ cells (PGC) and finally into early gametes and blastocysts. While little is known about the A<sub>s</sub> cellular niche with Sertoli Cell (De Rooij 2009), advances have been made in the use of growth factors that influence stem cell behavior and differentiation (Lee, Kanatsu-Shinohara et al. 2008). Established protocols have demonstrated
that human and mouse embryo stem cells can be maintained undifferentiated long term in the
presence of LIF (Penkov, Platonov et al. 2003; Furue, Okamoto et al. 2005), and LIF has been
shown to enhance germ cell clonal populations in mouse testis (Kanatsu-Shinohara, Inoue et al.
2007). Spermatogonial stem cells (A_s cells) however, are inhibited by LIF and require a
different set of growth factors. Glial cell line-Derived Neurotrophic Factor (GDNF), a
transforming growth factor-ß (TGFß) family member, has been shown to be a critical growth
factor for mouse A_s cell renewal and is thought to be conserved throughout all mammalian
species (Ryu, Kubota et al. 2005; Lee, Kanatsu-Shinohara et al. 2008). In vitro mouse A_s cells
cultured with GDNF up regulate transcription factors Bc16b, Erm and Lhx1 through Src Family
Kinase signaling. GDNF activates these transcription factor genes through the P13K/Akt
signaling pathway which is essential for A_s cell “general” survival and propagation in vitro
(Oatley, Avarbock et al. 2007). Over expression of GDNF in mouse testes appeared to stimulate
self-renewal of stem cells and block spermatogonial differentiation (Meng, Lindahl et al. 2000;
Kubota, Avarbock et al. 2004). After long term (over two years) in vitro culture using GDNF,
mouse A_s cells remained genetically stable (Kanatsu-Shinohara, Ogonuki et al. 2005).

Bone morphogenetic protein, Bmp 4, has been shown to be critical for differentiation of
embryo stem cells to A_s cells, along with the maintenance of spermatogenesis (Zhao, Liaw et al.
1998; Lawson, Dunn et al. 1999; Ying, Liu et al. 2000; Baughman and Geijsen 2005). Bmp4, a
member of the TGFß super family, is required for primordial germ cell renewal in mouse
(Lawson, Dunn et al. 1999). Bmp4 is a dimer that signals through heteromeric receptor
complexes and downstream SMAD pathways (Shimasaki, Moore et al. 2004). Mice that are
Bmp4 null fail to generate and propagate primordial germ cells, and heterozygous mouse
embryos for the null mutation show approximately 50% primordial germ cell populations
compared to wild type mice. The implication being that Bmp4 is not only required primordial
germ cell generation, but is dose dependent as well (Lawson, Dunn et al. 1999).

Bmp8b is a member of the Gbb-60A class of the bone morphogenetic protein BMP
family. Bmp8b has not been shown to be vital to the generation of primordial germ cells, and it
may not be required for proliferation or survival (Ying, Liu et al. 2000). Although, it has been
determined that Bmp4 and Bmp8b together influence the size of the initial populations of
primordial germ cells, the effects of Bmp4 and Bmp8b are not additive (Ying, Liu et al. 2000).

Stable, self-renewing Aₜ cells can be induced to terminally differentiate into the meiotic
spermatogenesis pathway. Previous in vitro studies have shown that retinoic acid (RA) triggers
Aₜ differentiation and allows spermatogonia to enter into and progress through meiotic prophase
I (Leid, Kastner et al. 1992; Bowles, Knight et al. 2006; Nayernia, Nolte et al. 2006). RA
appears to trigger a molecular cascade, inducing meiosis-specific genes such as Stra8, Scp3 and
Dmc1 (Koubova, Menke et al. 2006). In mature, vitamin A-deficient mice, where only
spermatogonia are found in the testis, the addition of RA has been shown to initiate the
resumption of spermatogenesis (Baleato, Aitken et al. 2005).

3.1 Mouse Germ Cell In Vitro Cultures

The present experimental work was undertaken to find a combination of selective growth
factors and culture conditions that will optimize the growth and preparation of pure cultures of
spermatogonial stem cells and meiosis-committed early stage spermatogonia, without the use of
feeder layers. Mouse C57BL/6 embryo stem cells (SCRC-1002) were purchased from ATCC
(Rockville, MD) and cultured according to the protocol for embryo stem cell self-renewal
(Kubota, Avarbock et al. 2004). MES cells are well known to freely propagate without a feeder
cell layer (Kanatsu-Shinohara, Inoue et al. 2006). MES cells were seeded at 8 ~10 x 10⁵ cells per
T25 flask and grown in StemPro-34 SFM (Invitrogen, L.A., CA) medium supplemented
with 1% FBS (Invitrogen, L., A., CA), 5% non-essential amino acids (Invitrogen), GlutaMAX (Invitrogen), 5 mM 2-mercaptoethanol (Sigma, St. Louis, MO), 2% penicillin-streptomycin (Invitrogen, L.A., CA), and 1,000 units of LIF (Chemicon Burlington, MS), in the presence of a 5% CO2 atmosphere at 37°C. Undifferentiated MES cells cultured on plastic tended to pile in clusters (fig. 3.1).

The undifferentiated status of MES cells was monitored by the use of cellular expression antigenic markers. These cultured MES cells expressed high levels of the POU transcription factor, Octamer-4 (Oct-4) (see Figure 2.2). As has been previously reported (Pesce, Gross et al. 1998) MES cells also express SSEA1, a cell surface carbohydrate antigen (Shevinsky, Knowles et al. 1982), but not CD9, SSEA3, SSEA4, the laminin receptor components Alpha 6 and Beta 1
intergrin or GFRA1 (Kanatsu-Shinohara, Inoue et al. 2004; Kanatsu-Shinohara, Toyokuni et al. 2004; Kanatsu-Shinohara, Niki et al. 2005). The in vitro differentiation of MES cells is characterized by the loss of both Oct-4 and SSEA1, and the appearance of either SSEA3 and/or SSEA4 (Solter, Shevinsky et al. 1997). Differentiated MES cells did not express CD9 (Figure 3.2).

![Figure 3.2. Photographs of Cultured Cells. Cultured MES cells, spermatogonial stem cells, and meiosis-committed spermatagonia, and fluorescent tagged antibody labeling of cell developmental markers Oct 4, CD 9, and c-Kit.](image)

3.2 Spermatogonial Stem Cells

The differentiation of MES cells and the isolation of spermatogonial stem, A5 cells, was accomplished by the removal of LIF from the growth media and the addition of 10 ng/ml GDNF (Invitrogen), and 50 ng/ml each Bmp4 and Bmp8b (see Fig. 3.2). After two weeks, these cells tested positive for CD9 and negative for both Oct 4 and c-Kit. The isolation and self-renewal maintenance of A5 cells was monitored by the loss of Oct 4 and c-Kit expression and the
presence of CD 9 expression (see Fig. 3.2). Cells expressing CD 9 were differentiated from pleuripotent MES cells to germline stem cells (Kanatsu-Shinohara, Toyokuni et al. 2004). The combination of loss of Oct-4 and SSEA-1, together with the gain of expression of CD 9, SSEA-3, SSEA-4, GFRA1, and/or alpha6 and beta1 integrins provides a positive identification of As cells (Pesce et al. 1998; Kubota, Avarbock et al. 2004; Solter and Knowles 1979; Yoshida, Takakura et al. 2004).

Mouse As cells have been reported to preferentially attach to laminin (Yoshida, Takakura et al. 2004). The present results show that mouse As cells attach and propagate well directly on plastic. However, there was an initial delay in the growth of the cells. After two weeks with very little growth (0.007 ± 0.012 PD per day (n=3)), cells showed slow growth with population doubling (PD) times of 10.4 days (or 0.096 ± 0.055 PD per day (n=3). MES cells demonstrated an average population doubling time of 2.7 days (or 0.37 ± 0.13 PD per day (n=3)).

Upon the differentiation of MES cells and monitoring using spermatogonial stem cell marker CD 9, As cells were easily subcultured into flasks and clonally expanded for study and analyses. If these As cells can be induced to differentiate into meiosis-committed spermatogonia, then this will be an excellent model for assaying the impact of toxicants such as EDC on germ cells and the establishment of the paternal imprint.

3.3 Early Stage Spermatogonia

Viable mouse As cells should be responsive to the signal to differentiate into early stage spermatogonia (A_al) and subsequently, the meiotic spermatogenesis pathway (de Rooij 2001). Following the expansion of As cells, the flasks were washed three times with PBS, and media containing RA (8µM) (Sigma-Aldrich, St. Louis, MO) was added to trigger the meiotic pathway (Farini, Scaldaverri et al. 2005). Shortly after the addition of RA, these cells appeared to slow in
growth and become quiescent. No cell division was observed during the 14 days following the addition of RA. After two weeks on RA containing media, these cells no longer expressed CD9, but now expressed c-Kit (also called CD117) (see Fig. 3.2). The expression of c-Kit demonstrated the conversion of these A8 cells into pre-meiotic Aal spermatogonia. The proto-oncogene c-Kit is expressed in maturing spermatogonia and is the best cell surface maker for initiation of meiosis in spermatogenesis (Meng, Lindahl et al. 2000). The product of c-Kit is a 150 kDa tyrosine-kinase receptor that is in the same family as α/β PDGF receptors and the CSF-1 receptor (Rossi, Sette et al. 2000). The ligand, Stem Cell Factor (SCF), is expressed by Sertoli cells in mice (Rossi, Albanesi et al. 1991; Marziali, Lazzaro et al. 1993). Expression of c-Kit is high in all stages of primordial germ cell migration, then disappears in A8 cells and returns in differentiating A1-A4 spermatogonia (Lacham-Kaplan 2004). Other markers for initiation of meiosis such as Vasa, Mil2 and Stella would have given me more confidence and will be used in future research. Point mutations in the c-Kit gene impair the SCF-mediated activation of phosphatidylinositol-3 kinase, but do not cause reduction in either primordial germ cells during embryonic development or A8 cell populations. However, males are completely sterile due to a block in DNA synthesis in differentiating Aal spermatogonia (Rossi, Sette et al. 2000).

Advances in the study of the molecular mechanisms involved in disease processes and the perturbation of heritable programming components of germ cells has been hampered by the complexity of the physiological system requiring the presence of multiple cell types. The present work demonstrates the ability to isolate and expand A8 cells prior to the induction of early stage spermatogenesis.

3.4 Materials and Methods

Mouse embryo stem cells (cell line ES-C57BL/6) were purchased from ATCC (SCRC-1002) (Rockville, MD) and sub cultured according to ATCC protocols (product information
Cryoprotectant media from ATCC. MES cells were seeded at 8 ~10 x 10^5 cells per T25 flask and grown in StemPro-34 SFM with supplements (Invitrogen, L.A., CA). Briefly, MES cells were subcultured on non-coated Falcon T-25 flask and grown on commercial media available from Invitrogen, StemPro-34 SFM with supplement (Invitrogen# 10639-011), and the published protocol for embryo stem cell self-renewal (Kubota, Avarbock et al. 2004). Non-essential amino acids 1% (Invitrogen # 11140-050), GlutaMAX 1% (Invitrogen # 35050-061), Penicillin-Streptomycin 1% (Invitrogen# 15140-148), 2-mercaptoethanol (Sigma# M7522-100ML), 1% FBS (Invitrogen# 16000077). Leukemia Inhibitory Factor (Chemicon cat# LIF2010) at 1000 units/ml (Penkov, Platonov et al. 2003). MES cells where subcultured approximately 3 days. MES cells were subcultured approximately every three days after doubling of population using protease, 1 µl/25 ml of media. Cells were washed three times with phosphorylated buffer solution then treated with protease. Cells were re-seeded at one SCC cluster per T-25 flask and fed using SCC media.

Spermatogonial stem cell differentiation media: Stem Pro-34 SFM media with the same supplements as MES cell media except with Bmp4 (Spring Bioscience #P7909) and Bmp8b at 50 ng/ml (Novus Biological #H00000656-Q01), GDNF at 100 ng/ml (Invitrogen #10907012). EDC treated cells were treated with EDC (Sigma # 03522-500ml) at 5mM/ml. SCC where subcultured on Falcon T-25 flasks. SCC were subcultured every two weeks after doubling of population using protease, 1 µl/25 ml of media. Cells were washed three times with phosphorylated buffer solution then treated with protease. Cells were re-seeded at one SCC cluster per T-25 flask and fed using SCC media. Meiosis commitment media was Stem Pro-34 SFM plus supplements except with Retinoic acid (Sigma # 951252-250mg) at 100 ng/ml in order to commit to meiosis. Meiotic committed cells were subcultured on Falcon T-25 flasks. Meiotic committed cells were not subcultured since cells where quiescent and did not expand. Cell differentiation markers: c-Kit
(CD117) antibody for meiosis committed cells (Chemicon #CBL1359F), CD9 for Primordial
Germ Cell (Millipore #000010), ES Cell Marker Sample Kit (Millipore #000010), Conjugated
fluorescent antibodies (Upstate/Chemicon #AP124F and AP124R). Cell markers were used
according to company protocols, Catalog No. SCR002. Normal goat serum was used as a
blocking solution (Chemicon # S26-100ML) at a 4% concentration. Samples were washed with
phosphorylated buffer solution. Cell marker anti-bodies were diluted according to company
protocols.
CHAPTER 4

ETHYLENE DICHLORIDE INITIATED CHANGES IN THE PATERNAL IMPRINT IN MOUSE SPERMATOGONIA

The paternal imprint is established in spermatogonial stem (A₅) cells or in differentiating meiosis committed spermatogonia (A₆₅ to A₁) prior to meiosis (Holmes, Chang et al. 2006). The progression of the in vitro tissue culture model of murine A₅ cells to A₁ cells, as discussed in the last chapter, enables the study of toxicant-induced perturbation of the paternal imprint.

Alkylating and other cytotoxic agents are known to disrupt male reproductive function, but very little information is available regarding the impact of these toxic agents on epigenetic patterns or the paternal imprint (Gandini, Sgro et al. 2006). Alkylating and arylating toxicants were shown to disrupt DNA 5-methylcytosine patterns in cultured mammalian cells (Wilson and Jones 1983). Benzo (a)pyrene has been shown to alter H3K9 acetylation patterns in MCF7 breast cancer cells (Sadikovic, Andrews et al. 2008). In yeast, nickel chloride blocked histone acetylation (Ke, Davidson et al. 2006) and Golebiowski et al. (2005) showed the same affects in mammalian cells.

Histones are major carriers of epigenetic information and either synergistically or antagonistically provide mechanisms to generate and stabilize genetic imprints. Therefore, the impact of toxicants such as EDC on histone modification patterns becomes a crucial question in the establishment of the paternal imprint. Two prominent and opposing histone methylation markers, H3K4₃ and H3K9₃ provide the optimal targets for examination of the effects of EDC on these histone modification patterns in the paternal imprint. H3K4₃, a strong and stable activator of transcription, has been hypothesized to serve as a “memory” mark to enforce transcription in spite of future histone modifications (Ng, Robert et al. 2003; Martin and Zhang 2005; Yu, Zhu et al. 2008). Only a few imprinting control centers are methylated (5-methylcytosines) in the male germ line (Delaval, Govin et al. 2007). Following fertilization,
paternal imprinting control centers have been found to be protected from global demethylation (5-methylcytosine patterns) by H4K20$_{m3}$ and H3K9$_{m3}$, as opposed to the unmethylated allele which has H3K4$_{me2}$ and H3 acetylation (Delaval, Govin et al. 2007).

Of all the imprinted DMRs in the mouse genome, only three are known to be methylated on the paternal allele: RAS guanine release factor 1 (Rasgrf1), Gtl2, and $H19$ (Delaval, Govin et al. 2007). These genes are well-studied, and proven protocols are available for methylation specific PCR techniques. Rasgrf1 has a DMR approximately 30kb upstream of the start site, which is fully methylated in mature sperm and unmethylated in oocytes (Rayburn, Parker et al. 2004). Immediately 3’ of this DMR is a 40 copy, 41 nucleotide repeat that is fully methylated in the male genome.

Gtl2 is part of a complex with Dlk. The Gtl2/Dlk1 locus has three differentially methylated regions which are methylated only on the paternal locus (Carr, Yevtodiyenko et al. 2007). Only one of the three DMR’s, 12 kb upstream of Gtl2, is methylated in the male germ line (Takada, Tevendale et al. 2000). Gtl2 DMR is completely methylated in mature sperm, but unmethylated in oocytes (Rayburn, Parker et al. 2004). Histone modifications for this locus were reported to be enriched for H3K9$_{m3}$ and H4R3$_{m2}$ in the paternal allele (Verona, Thorvaldsen et al. 2008).

$H19$, along with Igf2, was one of the first discovered imprinted genes and is the most studied (Stoger, Kubicka et al. 1993). The paternal allele is inactive in $H19$ with the 2 kb differentially methylated region (DMR) extended over 2 kb at ~ 4kb upstream from the promoter (Tremblay, Saam et al. 1995). $H19$ imprinting is present in spermatogonial stem cells before commitment to meiosis and is completely methylated in spermatogonial stem cells, type A spermatogonia, and mature sperm. However, $H19$ is unmethylated in oocytes (Davis, Trasler et al. 1999; Rayburn, Parker et al. 2004).
The non-imprinted gene, \( p16^{INK4} \), was chosen as an additional control DNA region for these experimental studies. \( P16 \) is a cyclin-dependent kinase inhibitor involved in cell cycle regulation, commonly called a tumor suppressor gene (Little and Wainwright 1995). Studies suggest that the \( p16/CDKN2 \) tumor suppressor gene may be inactivated by methylation of its promoter, but in healthy organisms CpG, promoter methylation is seldom found in tumor suppressor genes (Reamon-Buettner and Borlak 2007).

4.1 Treatment of Spermatogonial Stem Cells with EDC

Treating undifferentiated spermatogonial stem cells with EDC and allowing them to undergo expansion \textit{in vitro} should model the same DNA and chromatin impacts as \textit{in vivo} (Sun, Tao et al. 2008). Once cells have expanded, differentiation to meiotic spermatogonia can be initiated, and then the DNA 5-methylcytosine and chromatin modification patterns may be assayed. Controls include both untreated cells and cells treated with 5-aza-2’-deoxycytidine (AzadC), a DNA methyltransferase inhibitor. AzadC functions by binding to DNA Methyltransferase 1 (DNMT1), irreversibly preventing DNA methylation/imprinting (Dannenberg and Edenberg 2006). Because it was not practical for this dissertation to determine the rate of incorporation of 5-AzadC in spermatogonial stem cells, the data should not be included in the final conclusion. Although the data from this variable is very interesting, the results are not reliable.

Pure spermatogonial stem (A₃) cell cultures were prepared and grown as described in Chapter 3. Cultures were maintained with media containing 10 ng/ml GDNF, and 50 ng/ml of each Bmp4 and Bmp8, but lacking LIF. GDNF appears to stimulate self-renewal of stem cells and block spermatogonial differentiation (Meng, Lindahl et al. 2000; Kubota, Avarbock et al. 2004). After long term \textit{in vitro} studies using GDNF (over two years), mouse spermatogonial stem cells remain genetically and epigenetically stable (Kanatsu-Shinohara, Ogonuki et al.)
This research showed that after culturing for 2 years and a ~ \(10^{85}\)-fold expansion, spermatogonial stem cells retained the euploid and androgenetic imprint. Bone morphogenetic proteins, Bmp 4 and Bmp8b, are reported to be critical for both differentiation of embryo stem cells to As cells, and the stable maintenance of germ cell self-renewal (Zhao, Liaw et al. 1998; Lawson, Dunn et al. 1999; Ying, Liu et al. 2000; Baughman and Geijsen 2005). These cultures were tested for the expression of CD9, and the lack of expression of either Oct-4 or c-kit, as indicative of As cells (see Figure 3.2, Chapter 3).

It is at the stem cell stage that the genetic and epigenetic heritable information is most sensitive to external environmental and internal biological damage since damage at the spermatogonial stem cell stage would produce offspring with systemic mutations. Thus, the treatments were performed during the As stage by the addition of 5mM EDC, equal to the concentration in the in vivo experiment in Chapter 2, to the As culture media for 48 hours. For DNA methylation control, cultures were treated with 5-aza-2-deoxycytidine at 2.5 µM, but for only 24 hours. After the 24 or 48 hour treatment period, fresh media was added containing 8 nM RA, but lacking GDNF. Bmp4 and Bmp8 was added to trigger meiosis (Farini, Scaldaferrri et al. 2005). The pre-meiotic spermatogonia cells were grown for two weeks prior to harvest. Cells were harvested by trypsinization, then pelleted and washed with phosphate buffered saline (PBS).

The addition of RA triggers a meiotic differentiation of As cells (Leid, Kastner et al. 1992; Bowles, Knight et al. 2006). RA induces meiosis-specific genes such as Stra8, Scp3 and Dmc1; RA also may trigger meiosis through the expression of Stra8 (Koubova, Menke et al. 2006). Although RA may trigger the molecular cascade for meiosis, epigenetic states such as DNA methylation and histone H3K4 di-methylation patterns, set before RA, can induce meiosis...
(Kaneda, Okano et al. 2004). In vitro fertilization studies have shown that epigenetic reprogramming is completed before meiosis (Davis and Sharif 2000).

4.2 Check Treatment Groups and Doses

The present experimental work utilized the ChIP assay procedure followed by PCR, to detect gene specific histone modifications and DNA 5-methylcytosine patterns (Meissner, Mikkelsen et al. 2008; Meissner, Mikkelsen et al. 2008; Taylor, Wicks et al. 2008; Taylor, Wicks et al. 2008). In addition to in vitro germ cell cultures, mouse liver harvested from random male and female mice was obtained from the LSU Veterinary School.

Histone immunoprecipitation antibodies, such as H3K9\textsubscript{m3} (abCAM #ab8898) and H3K4\textsubscript{m3} (abCAM #ab8580) are recommended for dilutions of 2 µg per 25 µg of chromatin. Millipore Chromatin Immunoprecipitation (ChIP) Assay Kit (#17-295) was used according to the manufacturer’s protocol for immunoprecipitation and formaldehyde cross-linking. This assay kit did not include a protease inhibitor. Protease Inhibitor Cocktail (Sigma #P8340) at 1 µl per 100 µl of sample, according to Sigma protocols, to prevent protease degradation of histones during DNA purification and immunoprecipitation. Purified chromatin was then sonificated (Fisher Scientific model FS14) for 3 minutes, which was enough to produce approx 1000 bp chromatin fragments. This fragment size was estimated by gel electrophoresis analysis.

Following the ChIP procedures, PCR analyses for four specific gene-associated DMRs, or promoter regions, were performed. Precipitated chromosomes were bisulfite-treated; using the CPG WIZ\textsuperscript{®} VHL Amplification Kit (Chemicon #S7805) according to the manufacture’s protocol and MSPCR was performed on DMR sequences for three genes, \textit{Rasgrf1}, \textit{H19}, and \textit{Gtl2} and the promoter region of \textit{p16}. Disruption of genetic imprinting caused by EDC, either by hypo- or hypermethylation of DNA, should be detected by these gene-specific selection methods. Mouse control DNAs, NIH 3T3 and CpG methylated 3T3, were obtained from New
England BioLabs (Ipswich, MA) for use as standard controls. The histone methylation modification patterns have been coupled with the DNA 5-methylcytosine patterns in the final analyses.

MSPCR was performed in 25 µl containing MgCl₂ 25 mM, 10 mM dNTPs, 2.5 µl 10X buffer, 1.5 U AccuPrime Taq (Invitrogen # 12339016), 10 pM primers, and 2 µl of sonicated ChIP isolated DNA. The MSPCR amplification cycles were composed of 1 min at 94ºC, 1 min at gene specific annealing temperature, and 1 min at 72ºC. Nested PCR was performed with 40 cycles in the first PCR, and 30 cycles in the second round of amplification, obtaining gene specific primers as custom synthesis from Invitrogen (Carlsbad, CA), with the primer sequences in Table 4.1.

MSPCR products were digested with BstUI (NEB # R0518L) at 37º C for 12 hours for Rasgrf1 analyses, resulting in a 284 bp fragment if unmethylated (and uncut) or 50 bp + 160 bp fragments if methylated. Digestion products were separated by agarose gel electrophoresis in a 5% agarose gel (Amresco #0701) in TBE buffer, for 1 hour at 100V (Figure 3.1).

For Gtl2, H19 and p16 analyses, MSPCR products were digested with Taq1 (Takara Bios Inc # 1189A) 65º C for 12 hours. The expected Taq1 digestion fragments sizes for Gtl2 were 482 bp (unmethylated) and 145 bp + 337 bp (methylated) (Figure 3.2); for H19 were 422 bp (unmethylated) and 88 bp + 334 bp (methylated) (Figure 3.3); and for p16 were 492 bp (unmethylated) and 67 bp + 425 bp (methylated) (Figure 3.4).

4.3 Results of EDC Treatment on Spermatogonial Stem Cells

A total of 20 samples were harvested and tested for both DNA 5-methylcytosine imprinting status as well as histone modification (H3K4₃ and H3K9₃) status. This was done in order to determine if ethylene dichloride is able to disrupt or modify genetic imprinting and/or histone modifications. ChIP assay coupled with methylation specific PCR was used
simultaneously to show gene specific histone modifications and DNA methylation. Results of this technique showed a definite pattern of EDC induced aberrant DNA methylation and genetic imprinting patterns. Hypermethylation was expected to be found with H3K9 trimethylation in

<table>
<thead>
<tr>
<th>Table 4.1 Primer Sequence and Annealing Temperatures</th>
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<tbody>
<tr>
<td><strong>Rasgrf1 DMR</strong> (52°C annealing)</td>
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<tr>
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<tr>
<td><strong>Gtl2 DMR</strong> (52°C annealing)</td>
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<td></td>
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<tr>
<td><strong>H19 DMR</strong> (52°C annealing)</td>
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<tr>
<td><strong>P16 promoter</strong> (65°C annealing)</td>
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</table>
meiotic cells and possibly liver cells. In liver, imprinted genes are expected to keep their imprint. Paternally imprinted genes retain their preferentially expressed imprint in all tissues (Cheng, Zhang et al. 2008; Cheng, Zhang et al. 2008). This expression included other mammals, besides human and mouse. However as shown below, H3K4me3 was found to be coupled sporadically with hypermethylation in liver. This could possibly be due to random epigenetic mutations since the liver was obtained from old mice from the LSU Veterinary School.

For the three paternally imprinted genes of interest, 5-methylcytosine patterns were analyzed from both EDC treated and control spermatogonia. EDC treated meiotic cells were compared to non-treated meiotic cells, in order to determine whether EDC can disrupt this vital epigenetic and reproductive process. The results clearly show that EDC has an effect on the methylation and subsequent imprinting of the three chosen imprinted gene DMRs. However the effects seem to be limited to H3K4\textsubscript{me3} histone modification, while H3K9\textsubscript{m3} patterns show no significant changes. Twenty separate samples from each treatment and gene combination were tested for DMR methylation and histone modification.

The ChIP and DNA 5-methylcytosine pattern analysis of the DMR region specific for the Rasgrf1 gene showed perturbation by EDC and 5- AzadC treatment of spermatogonial stem cells (Figure 4.1). 5- AzadC initiated a loss of methylation, when compared with untreated meiotic spermatogonia on the H3K9\textsubscript{m3} histone modification ChIP isolate, while the lack of results for the H3K4me3 suggested that no lysine 4 trimethylation was present on the histones of this DMR region of DNA, for both untreated meiotic patterns and following the 5- AzadC treatment. EDC treated samples showed hypermethylated DMR’s on both H3K4\textsubscript{m3} and H3K9\textsubscript{m3} chromatin isolates, suggesting that the EDC treatment altered the histone modifications to include trimethylation of lysine 4 on H3. Untreated liver samples displayed the same patterns as
untreated meiotic spermatogonia, although the 50 bp and 150 bp bands for hypermethylation on the H3K9\textsubscript{m3} ChIP isolate were relatively weak (Figure 3.1).

4.4 Materials and Methods

Purified chromatin was sonificated (Fisher Scientific model FS14) for 3 minutes, which was enough to produce approx 1000 bp chromatin fragments. This fragment size was estimated by gel electrophoresis analysis. Histone immunoprecipitation antibodies, H3K9\textsubscript{m3} (abCAM #ab8898) and H3K4\textsubscript{m3} (abCAM #ab8580) where used according to company protocols with dilutions of 2\mu g per 25\mu g of chromatin. Millipore Chromatin Immunoprecipitation (ChIP) Assay Kit (#17-295) was used according to the manufacturer’s protocol for immunoprecipitation and formaldehyde cross-linking. This assay kit did not include a protease inhibitor. Protease Inhibitor Cocktail (Sigma #P8340) at 1\mu l per 100 \mu l of sample was adequate to prevent protease degradation of histones during DNA purification and immunoprecipitation.

After ChIP purification for H3K4\textsubscript{m3} and H3K9\textsubscript{m3} histone modifications, MSPCR was performed in order to determine 5-methylcytosine status of \textit{Rasgrf1}, \textit{Gtl2}, \textit{H19} DMR’s as well as promoter of \textit{P16}. ChIP purified chromatin was bisulphate treated using CPG WIZ\textsuperscript{®} VHL Amplification Kit (Chemicon #S7805) according to company protocols. MSPCR was performed using Perkin Elmer Thermocycler (Model No. 2400 ). MSPCR was performed in 25 \mu l containing MgCl2 25mM, 10mM dNTPs, 2.5 \mu l 10X buffer, 1.5U AccuPrime Taq (Invitrogen #12339016), 10 pM primers, and 2 \mu l of sonicated ChIP isolated DNA. The MSPCR amplification cycles were composed of 1 min at 94C, 1 min at gene specific annealing temperature, and 1 min at 72C. Nested PCR was performed with 40 cycles in the first PCR, and 30 cycles in the second round of amplification. Custom PCFR primers obtained from Invitrogen (Carlsbad, CA) with sequences and temperatures listed in Table 3.1.
PCR products were then treated with endonuclease to determine 5-methylcytosine status of PCR products. For Rasgrf1, PCR products were digested with *BstUI* (NEB # R0518L) at 37°C for 12 hours analyses, resulting in a 284 bp fragment if unmethylated (and uncut) or 50 bp + 160 bp fragments if methylated (Figure 4.1). For Gtl2, H19 and p16 analyses, MSPCR products were digested with *TaqI* (Takara Bios Inc # 1189A) 65°C for 12 hours. The *TaqI* digestion fragments sizes for Gtl2 were 482 bp (unmethylated) and 145 bp + 337 bp (methylated) (Figure 4.2); for H19 were 422 bp (unmethylated) and 88 bp + 334 bp (methylated) (Figure 4.3); and for p16 were 492 bp (unmethylated) and 67 bp + 425 bp (methylated) (Figure 4.4). Digestion products were separated by agarose gel electrophoresis in a 5% agarose gel (Amresco #0701) in TBE buffer, for 1 hour at 100V (Figure 4.1).

![Figure 4.1. Rasgrf1, agarose gel electrophoresis of MSPCR analysis of ChIP preparations. 284 bp is an unmethylated sample. 160bp/50bp is a methylated sample. (K4: histone H3K4_m3; K9, histone H3K9_m3) Treatments include 5-AzaC, EDC, untreated mouse liver DNA, untreated meiosis-committed spermatogonia and control methylated DNA.](image)
Table 4.2. Results of the ChIP and MSPCR analyses of 20 samples for the Rasgrf1 with methylated DMR.

<table>
<thead>
<tr>
<th>DNA Cytosine</th>
<th>K4 5-AZA</th>
<th>K9 5-AZA</th>
<th>K4 EDC</th>
<th>K9 EDC</th>
<th>K4 Liver</th>
<th>K9 Liver</th>
<th>K4 Meiotic</th>
<th>K9 Meiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypermethylated</td>
<td>0</td>
<td>7</td>
<td>15</td>
<td>18</td>
<td>3</td>
<td>16</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>13</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>11</td>
<td>18</td>
<td>18</td>
<td>4</td>
<td>17</td>
<td>7</td>
<td>18</td>
</tr>
</tbody>
</table>

The results observed in Figure 4.1 remained much the same after the ChIP and MSPCR analyses of 19 more samples for the DMR associated with the Rasgrf1 gene (Table 4.2). Apparently 7 out of 20 untreated meiotic spermatogonia samples contained H3K4m3 modifications; these samples were isolatable by the ChIP assay and displayed hypermethylation (2/7 or 29%) and an unmethylated DNA cytosine pattern (5/7 or 71%). However, none of the 18 H3K9m3 ChIP isolatable, untreated, meiotic spermatogonia samples were found to be unmethylated (Table 4.2). Liver, somatic tissue provided results very similar to that of untreated meiotic spermatogonia. The 5- AzadC treatment, which is known to diminish DNA cytosine methylation, appears to have impacted the histone modification patterns as well, since twice as many H3K4m3 ChIP isolates were obtained following 5-Aza than for untreated meiotic spermatogonia. Also, the number of H3K9m3 ChIP isolates was diminished by a third in the 5-AzadC treated cells in untreated meiotic spermatogonia. All of the 13 H3K4m3 ChIP isolates were found unmethylated, while only 3 (27%) of the 11 H3K9m3 ChIP isolates were found to be unmethylated (Table 4.2). EDC apparently enhanced the trimethylation of lysine 4, as the number of H3K4m3 ChIP isolates more than doubled (18 versus 7) following EDC treatment, compared to untreated meiotic spermatogonia. EDC also enhance DNA hypermethylation of the H3K4me3 modified chromatin, since 83% (15/18) of these ChIP isolates were hypermethylated,
compared to only 29% (2/7) for untreated meiotic spermatogonia (Table 4.2). However, EDC appears to have had no effect on either the DNA cytosine pattern or the H3K9\textsubscript{m3} pattern for the \textit{Rasgrf1} DMR.

The second DMR for the imprinted \textit{Gtl2} gene provided the same results as those described above for the \textit{Rasgrf1} gene. 5- AzadC initiated a loss of methylation when compared with untreated meiotic spermatogonia on the H3K9\textsubscript{m3} histone modification ChIP isolate, while the lack of results for the H3K4\textsubscript{m3} suggested that no lysine 4 trimethylation was present on the histones of this DMR region of DNA for both untreated meiotic spermatozoa and the following 5- AzadC treatment (Figure 4.2). EDC treated samples showed hypermethylated DMR’s on both H3K4\textsubscript{m3} and H3K9\textsubscript{m3} chromatin isolates, suggesting that EDC treatment altered the histone modifications to include trimethylation of lysine 4 on H3. Untreated liver samples displayed the same patterns as untreated meiotic spermatogonia (Figure 4.2).

The results reported in Figure 4.2 remained much the same after the ChIP and MSPCR analyses of 19 more samples for the DMR associated with the \textit{Gtl2} gene (Table 4.3). Apparently 6 out of 20 untreated meiotic spermatogonia samples contained H3K4\textsubscript{m3} modifications, as they were isolatable by the ChIP assay and displayed hypermethylation (5/6 or 83%) predominantly. However, all 16 of the H3K9\textsubscript{m3} ChIP isolates for untreated meiotic spermatogonia samples were found to be hypermethylated (Table 4.3). The liver provided results very similar to that of untreated meiotic spermatogonia.

Apparently 5- AzadC treatments had a different impact on the H3K4\textsubscript{me3} modification pattern of the DMR for the \textit{Gtl2} gene, as compared to the \textit{Rasgrf1} associated DMR (Tables 4.2 and 4.3). Only a third (2/6) as many samples were ChIP isolatable for H3K4\textsubscript{m3} modification on the \textit{Gtl2} gene DMR following the 5- AzadC treatment, as compared to untreated meiotic spermatogonia (Table 4.3). The 5- AzadC treatment initiated a loss of DNA cytosine
methylation, as both H3K4\textsubscript{m3} ChIP isolates were unmethylated and 89% (16/18) H3K9\textsubscript{m3} ChIP isolates were unmethylated, compared with 17% (1/6) and 0% (0/16) unmethylated in H3K4\textsubscript{m3} and H3K9\textsubscript{m3} ChIP isolates, respectively, for untreated meiotic spermatogonia.

Figure 4.2. *Gtl2* MSPCR. *Gtl2*, agarose gel electrophoresis of MSPCR analysis of ChIP preparations. 482 bp is an unmethylated sample. 145 bp + 337 bp is a methylated sample. (K4, histone H3K4\textsubscript{m3}; K9, histone H3K9\textsubscript{m3}) Treatments are 5- AzadC, EDC, untreated mouse liver DNA, untreated meiosis-committed spermatogonia and control methylated DNA.

Table 4.3. Results of the ChIP and MSPCR Analyses of 20 samples for the *Gtl2* DMR

<table>
<thead>
<tr>
<th>DNA Cytosine</th>
<th>K4 5-AZA</th>
<th>K9 5-AZA</th>
<th>K4 EDC</th>
<th>K9 EDC</th>
<th>K4 Liver</th>
<th>K9 Liver</th>
<th>K4 Meiotic</th>
<th>K9 Meiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypermethylated</td>
<td>0</td>
<td>2</td>
<td>8</td>
<td>14</td>
<td>2</td>
<td>14</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>2</td>
<td>16</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>18</td>
<td>12</td>
<td>14</td>
<td>4</td>
<td>14</td>
<td>6</td>
<td>16</td>
</tr>
</tbody>
</table>
EDC apparently enhanced the trimethylation of lysine 4 on *Gtl2* DMR, as the number of H3K4\textsubscript{m3} ChIP isolates was doubled (12 versus 6) following EDC treatment, compared to untreated meiotic spermatogonia (Table 4.3). However, EDC had little effect on the DNA hypermethylation of the H3K4\textsubscript{m3} modified chromatin of the *Gtl2* DMR, since 67% (8/12) of these ChIP isolates were hypermethylated, compared to only 83% (5/6) for untreated meiotic spermatogonia (Table 3.3). However, EDC appears to have had no effect on either the DNA cytosine pattern, or the H3K9\textsubscript{m3} pattern for the *Gtl2* DMR.

The ChIP and DNA 5-methylcytosine pattern analysis of the DMR region specific for the *H19* gene showed perturbation by EDC and the 5-AzadC treatment of spermatogonial stem cells (Figure 4.3). Untreated meiotic spermatogonia displayed the expected result of the lack of DNA cytosine methylation on the H3K4\textsubscript{m3} ChIP isolate and DNA hypermethylation on the H3K9\textsubscript{m3} ChIP isolate. The 5-AzadC treatment initiated a loss of methylation, when compared with untreated meiotic spermatogonia on the H3K9\textsubscript{m3} ChIP isolate.

The lack of results for the H3K4\textsubscript{m3} suggests that no lysine 4 trimethylation was present on the histones of this DMR region of DNA following the 5-AzadC treatment (Figure 4.3). Although the 334 bp and 88 bp bands are relatively weak, EDC treated samples showed hypermethylated DMRs on both of the H3K4\textsubscript{m3} and H3K9\textsubscript{m3} chromatin isolates, suggesting that EDC treatment altered the histone modifications to include trimethylation of lysine 4 on H3.

Untreated liver samples displayed the lack of DNA cytosine methylation on both the H3K4\textsubscript{m3} and H3K4\textsubscript{m3} ChIP isolates, which may suggest the loss of the DNA cytosine methylation imprint in some somatic tissues (Figure 4.3). However, this was the only sample of 15 H3K9\textsubscript{m3} ChIP isolates, found to be unmethylated.

As with the DMRs of *Rasgrf1* and *Gtl2*, the ChIP and MSPCR analyses of 20 samples for the DMR associated with the *H19* gene provided similar results (Table 4.4). Apparently 9
Figure 4.3. *H19* MS/PCR. *H19*, agarose gel electrophoresis of MSPCR analysis of ChIP preparations. 422 bp is an unmethylated sample. 88 bp + 334 bp is a methylated sample. (K4, histone H3K4m3; K9, histone H3K9m3) Treatments are 5-AzadC, EDC, untreated mouse liver DNA, untreated meiosis-committed spermatogonia and control methylated DNA.

Table 4.4. Results of the ChIP and MSPCR Analyses of 20 samples for the *H19* DMR

<table>
<thead>
<tr>
<th>DNA Cytosine</th>
<th>K4 5-AZA</th>
<th>K9 5-AZA</th>
<th>K4 EDC</th>
<th>K9 EDC</th>
<th>K4 Liver</th>
<th>K9 Liver</th>
<th>K4 Meiotic</th>
<th>K9 Meiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypermethylated</td>
<td>1</td>
<td>3</td>
<td>13</td>
<td>16</td>
<td>3</td>
<td>14</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>14</td>
<td>13</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>16</td>
<td>15</td>
<td>17</td>
<td>5</td>
<td>15</td>
<td>9</td>
<td>17</td>
</tr>
</tbody>
</table>

out of 20 untreated meiotic spermatogonia samples contained H3K4m3 modifications, as they were isolatable by the ChIP assay and displayed hypermethylation (4/9 or 44%) and an unmethylated DNA cytosine pattern (5/9 or 56%). However, all of the 17 H3K9m3 ChIP isolatable untreated meiotic spermatogonia samples were found to be methylated (Table 3.4). Liver samples provided results very similar to that of untreated meiotic spermatogonia. The 5-
AzadC treatment, which is known to diminish DNA cytosine methylation, appears to have impacted the histone modification patterns as well since more H3K4\textsubscript{m3} ChIP isolates were obtained following the 5-AzadC than for untreated meiotic spermatogonia.

All but one (14/15 or 93%) of the 15 H3K4\textsubscript{m3} ChIP isolates following 5- AzadC treatment were found to be unmethylated, while 13 (81%) of the 16 H3K9\textsubscript{m3} ChIP isolates were found to be unmethylated (Table 4.4). EDC apparently enhanced the trimethylation of lysine 4, since the number of H3K4\textsubscript{m3} ChIP isolates was increased (15 versus 9) following EDC treatment, compared to untreated meiotic spermatogonia. EDC also enhanced DNA hypermethylation of the H3K4\textsubscript{m3} modified chromatin, since 87% (13/15) of these ChIP isolates were hypermethylated, compared to only 44% (4/9) for untreated meiotic spermatogonia (Table 4.4). However, EDC appears to have had no effect on either the DNA cytosine pattern or the H3K9\textsubscript{m3} pattern for the Rasgrf1 DMR.

In order to understand the effects of EDC treatment on the epigenetic markers and the paternal imprint, the total number of ChIP isolates for each chromatin modification were tabulated (Table 4.5), and the percentage of ChIP isolates with DNA hypermethylation found coupled with each H3K4\textsubscript{m3} and H3K9\textsubscript{m3} were tabulated (Table 4.6). The EDC treatment of spermatogonial stem cells not only doubled the number of H3K4\textsubscript{m3} ChIP isolates, but also increased the proportion of those ChIP isolates, hypermethylated by more than 50% compared to untreated meiotic cells (Tables 4.5 and 4.6).

At the same time, EDC had no apparent effect on either the number of H3K9\textsubscript{m3} ChIP isolates or on the methylation status of H3K9\textsubscript{m3} modified chromatin for these three imprinted DMRs. The 5- AzadC treatment also increased the number of H3K4\textsubscript{m3} ChIP isolates, but by only half as much as EDC, compared to untreated meiotic spermatogonia (Table 4.5). However,
5- AzadC induced a 50% and a 69% loss of DNA cytosine methylation in H3K4\textsubscript{m3} and H3K9\textsubscript{m3} ChIP isolates respectively, relative to untreated meiotic spermatogonia (Table 4.6).

Table 4.5 Total ChIP Isolates for each Imprinted Gene DMR Based on Histone Modification\textsuperscript{a}

<table>
<thead>
<tr>
<th>Gene DMR</th>
<th>5-Aza K4</th>
<th>5-Aza K9</th>
<th>EDC K4</th>
<th>EDC K9</th>
<th>Liver K4</th>
<th>Liver K9</th>
<th>Meiotic K4</th>
<th>Meiotic K9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rasgrf1</td>
<td>13/20 (65%)</td>
<td>11/20 (55%)</td>
<td>18/20 (90%)</td>
<td>18/20 (90%)</td>
<td>4/20 (20%)</td>
<td>17/20 (85%)</td>
<td>7/20 (35%)</td>
<td>18/20 (90%)</td>
</tr>
<tr>
<td>Gtl2</td>
<td>2/20 (10%)</td>
<td>18/20 (90%)</td>
<td>12/20 (60%)</td>
<td>14/20 (70%)</td>
<td>4/20 (20%)</td>
<td>14/20 (70%)</td>
<td>6/20 (30%)</td>
<td>16/20 (80%)</td>
</tr>
<tr>
<td>H19</td>
<td>15/20 (75%)</td>
<td>16/20 (80%)</td>
<td>15/20 (75%)</td>
<td>17/20 (85%)</td>
<td>5/20 (25%)</td>
<td>15/20 (75%)</td>
<td>9/20 (45%)</td>
<td>15/20 (75%)</td>
</tr>
<tr>
<td>Average</td>
<td>50±35%</td>
<td>75±18%</td>
<td>75±15%</td>
<td>82±10%</td>
<td>22±3%</td>
<td>77±8%</td>
<td>37±8%</td>
<td>82±8%</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Twenty samples were subjected to the ChIP assay for each gene and histone trimethylation.

Table 4.6 Percent ChIP Isolates Hypermethylated

<table>
<thead>
<tr>
<th>Gene DMR</th>
<th>5-Aza K4</th>
<th>5-Aza K9</th>
<th>EDC K4</th>
<th>EDC K9</th>
<th>Liver K4</th>
<th>Liver K9</th>
<th>Meiotic K4</th>
<th>Meiotic K9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rasgrf1</td>
<td>0%</td>
<td>64%</td>
<td>83%</td>
<td>100%</td>
<td>75%</td>
<td>94%</td>
<td>29%</td>
<td>100%</td>
</tr>
<tr>
<td>Gtl2</td>
<td>0%</td>
<td>11%</td>
<td>67%</td>
<td>100%</td>
<td>50%</td>
<td>100%</td>
<td>83%</td>
<td>100%</td>
</tr>
<tr>
<td>H19</td>
<td>7%</td>
<td>19%</td>
<td>87%</td>
<td>94%</td>
<td>60%</td>
<td>87%</td>
<td>44%</td>
<td>100%</td>
</tr>
<tr>
<td>Average</td>
<td>2±4%</td>
<td>31±29%</td>
<td>79±11%</td>
<td>98±3%</td>
<td>62±13%</td>
<td>94±7%</td>
<td>52±28%</td>
<td>100±0%</td>
</tr>
</tbody>
</table>
An interesting gene specific phenomenon was observed following the 5-Aza treatment that was not seen following EDC. EDC induced H3K4m3 formation in all three imprinted gene DMRs, nearly doubling the number of H3K4m3 ChIP isolates for Rasgrfl and H19 and tripling the number for Gtl2 (Table 4.5). However, the 5- AzadC treatment provided similar results for Rasgrfl and H19, yet decreased the level of H3K4m3 of the Gtl2 DMR by more than 65% (Table 4.5). The reason for this anomaly is not presently known and will require further experimental work to verify and understand the mechanism(s) involved.

Untreated liver cells displayed similar patterns of DNA 5-methylcytosine and histone modification to that observed for untreated meiotic spermatogonia. The liver cells had an expectedly low number of hypermethylated DMRs on H3K4m3 modifications. However, an unexpected high number of hypermethylated DMRs were found on H3K9m3 (Table 4.6). This could be due to the fact that samples were collected from older male mice harboring age-acquired methylation (Mazin 1994). As discussed below, the p16 gene, a non-imprinted control sequence, showed some minor promoter hypermethylation in liver cells, which suggest that older mice tend to have naturally occurring age-related aberrant DNA hypermethylation as they age. The p16 gene promoter showed increases in meiotic cell promoter hypermethylation, as would be expected for condensing chromatin. Male meiotic germ cells are globally hypermethylated, in order to condense paternal DNA to fit in the sperm head.

For comparisons, a non-imprinted gene region, p16 promoter, was also included in these analyses. Interestingly, the DNA cytosine methylation patterns were very similar to the imprinted gene DMRs (Figure 4.4). The ChIP and DNA 5-methylcytosine pattern analysis of the p16 promoter region showed perturbation by EDC and the 5-Aza treatment of spermatogonial stem cells (Figure 4.4). The 5-AzadC initiated a loss of methylation when compared with untreated meiotic spermatogonia on the H3K9m3 histone modification ChIP isolate.
Blank PCR results for the H3K4\textsubscript{m3} suggests that no lysine 4 trimethylation was present on the histones of the \textit{p16} promoter region of DNA for both the untreated meiotic and following the 5- AzadC treatment. EDC treated samples showed hypermethylated \textit{p16} promoter on both H3K4\textsubscript{m3} and H3K9\textsubscript{m3} chromatin isolates, suggesting that EDC treatment altered the histone modifications to include trimethylation of lysine 4 on H3. Untreated liver samples displayed the same patterns as untreated meiotic spermatogonia, although the 67 bp and 425 bp bands for hypermethylation on the H3K9\textsubscript{m3} ChIP isolate were relatively weak (Figure 4.4).

A tabulation of 20 samples analyzed for \textit{p16} promoter DNA cytosine methylation and histone modifications yielded the following results. EDC did not significantly alter the H3K4\textsubscript{m3} pattern, but may have increased the trimethylation of lysine 9 on H3, since the number of H3K9\textsubscript{m3} ChIP isolated increased by 33\% (16 versus 12) (Table 4.7). EDC did, however, substantially reduce (by 62\%) DNA hypermethylation of H3K9\textsubscript{m3} ChIP isolates of the \textit{p16} promoter sequence (Table 4.7). The 5- AzadC treatment of spermatogonial stem cells initiated a complete loss of H3K4\textsubscript{m3} and a shift to fully (100\%) trimethylated on lysine 9 of H3, along with a complete loss (0\%) of DNA cytosine methylation of the \textit{p16} promoter region (Table 4.7). These results clearly indicate that EDC affected both the histone trimethylation and the DNA cytosine methylation pattern in the promoter sequences of the \textit{p16}, non-imprinted gene (Table 4.7).

Comparing the \textit{p16} promoter ChIP and MSPCR analyses results with those of the imprinted DMRs, there is a higher level of trimethylation of lysine 4 and decreased trimethylation on lysine 9 of H3 in the \textit{p16} promoter than in the imprinted regions in untreated meiotic spermatogonia (Tables 4.5 and 4.8). As with the imprinted DMRs, DNA hypermethylation is also associated with H3K9\textsubscript{m3} in the \textit{p16} promoter region in untreated meiotic spermatogonia (Table 4.6 and 4.8). However, unlike the imprinted DMRs, the \textit{p16} promoter displays a complete lack of DNA cytosine methylation in chromatin harboring H3K4\textsubscript{m3}. 

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Figure 4.4. *P16*, A garose gel electrophoresis of MS/PCR analysis of ChIP preparations. 492 bp is an unmethylated sample. 67 bp + 425 bp is a methylated sample. (K4, histone H3K4\textsubscript{m3}; K9, histone H3K9\textsubscript{m3}) Treatments are 5- AzadC, EDC, untreated mouse liver DNA, untreated meiosis-committed spermatogonia and control methylated DNA.

Table 4.7. Results of the ChIP and MSPCR Analyses of 20 samples for the p16 Promoter

<table>
<thead>
<tr>
<th>DNA Cytosine</th>
<th>K4 5-AZA</th>
<th>K9 5-AZA</th>
<th>K4 EDC</th>
<th>K9 EDC</th>
<th>K4 Liver</th>
<th>K9 Liver</th>
<th>K4 Meiotic</th>
<th>K9 Meiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypermethylated</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>0</td>
<td>20</td>
<td>14</td>
<td>10</td>
<td>17</td>
<td>0</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>20</td>
<td>15</td>
<td>16</td>
<td>18</td>
<td>5</td>
<td>16</td>
<td>12</td>
</tr>
</tbody>
</table>

The 5- AzadC produced a different effect on the histone modification patterns of the imprinted DMRs than on those of the *p16* promoter. The 5-AzadC initiated a dramatic increase in the trimethylation of lysine 9 and complete removal of trimethylation of lysine 4 on H3 in the *p16* promoter. Conversely, no effects were observed on H3K9\textsubscript{m3} and an increase in trimethylation of lysine 4 on H3 was found on DMRs for *Rasgrf1* and *H19* (but not *Gtl2*) (Tables 4.5 and 4.8). Unlike the imprinted DMRs, 5- AzadC also produced a complete loss of DNA cytosine methylation from the *p16* promoter regions harboring H3K9\textsubscript{m3} (Tables 4.6 and 4.8).
EDC also produced a different effect on the DNA cytosine methylation patterns of the \textit{p16} promoter, compared to the imprinted DMRs. EDC caused a loss of 62\% of the DNA cytosine methylation on the \textit{p16} promoter harboring H3K9\textsubscript{m3}, compared to no change on the imprinted DMRs (Table 4.6 and 4.8). EDC may also have increased the formation of H3K9\textsubscript{m3} on the \textit{p16} promoter, which was not seen in the imprinted DMRs (Tables 4.5 and 4.8).

Table 4.8 Total ChIP Isolates and Percent of Isolates Hypermethylated for P16 Promoter. Based on Histone Modification.\textsuperscript{a}

<table>
<thead>
<tr>
<th>P16 Promoter</th>
<th>5-Aza K4</th>
<th>5-Aza K9</th>
<th>EDC K4</th>
<th>EDC K9</th>
<th>Liver K4</th>
<th>Liver K9</th>
<th>Meiotic K4</th>
<th>Meiotic K9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of ChIP Isolates</td>
<td>0/20 (0%)</td>
<td>20/20 (100%)</td>
<td>15/20 (75%)</td>
<td>16/20 (80%)</td>
<td>18/20 (90%)</td>
<td>5/20 (25%)</td>
<td>16/20 (80%)</td>
<td>12/20 (60%)</td>
</tr>
<tr>
<td>Percent Hypermethylated</td>
<td>NA \textsuperscript{b}</td>
<td>0%</td>
<td>7%</td>
<td>38%</td>
<td>6%</td>
<td>100%</td>
<td>0%</td>
<td>100%</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Twenty samples were subjected to the ChIP assay for each gene and histone trimethylation.

\textsuperscript{b} NA, not applicable as zero ChIP isolates obtained.
CHAPTER 5
DISCUSSION

It is interesting that the \( p16 \) promoter region was found to harbor both H3K4\(_m3 \) and H3K9\(_m3 \) histone modifications in untreated meiotic-committed spermatogonia, since the H3K4\(_m3 \) is associated with gene expression and the H3K9\(_m3 \) is associated with genes that are not expressed and DNA heterochromatin. However, spermatogonial stem cells and early stage spermatogonia are still diploid, and these results most likely represent the two different imprinted alleles, one being expressed and the other shut down. The finding that DNA cytosine hypermethylation associated 100\% with H3K9\(_m3 \), while all of the H3K4\(_m3 \) chromatin contained unmethylated DNA for the \( p16 \) promoter, supports this conclusion.

Imprinted DMRs would be expected to contain only H3K9\(_m3 \) and DNA cytosine hypermethylation on both alleles, unless the paternal imprint has not been fully established in spermatogonial stem cells or early stage spermatogonia. The untreated spermatogonia DMRs provided only 82±8\% H3K9\(_m3 \) and 37±8\% H3K4\(_m3 \), suggesting that the imprint is being processed but is not yet fully established. The fact that these same DMRs were found to be totally (100\%) hypermethylated on H3K9\(_m3 \) chromatin, but mixed results (52±28) were found for DNA cytosine methylation on H3K4\(_m3 \) chromatin, supports this conclusion. However, exactly when the imprint becomes fully established is not known, except that it must take place before meiosis and these early stage spermatogonia are only a few cell cycles away from meiosis.

In meiotic cells, 5-AZA-2'-deoxycytidine was used to determine the effects of a DNA de-methylating agent on epigenetic pattern establishment. The samples treated with 5-AZA-2'-deoxycytidine showed a substantial loss of cytosine methylation from DMRs, regardless of which lysine residue was trimethylated on both imprinted DMRs and non-imprinted \( p16 \)
promoter sequences. Interestingly, 5- AzadC had opposing effects on the level of trimethylation on imprinted DMRs, than on non-imprinted sequences. 5- AzadC induced an increase in H3K9\textsubscript{m3} on the p16 promoter, and no change on the imprinted DMRs, while a complete loss of H3K4\textsubscript{m3} was found on the p16 promoter in 5-Aza treated spermatogonia.

The most interesting effect of EDC was on genes found on H3K4\textsubscript{m3} modification of imprinted DMRs. The number of samples harboring H3K4\textsubscript{m3} in the imprinted DMRs was doubled by EDC treatment, and the level of cytosine methylation was increased for this chromatin harboring H3K4\textsubscript{m3}. This was not the case for the effect of EDC on the non-imprinted p16 promoter region. EDC initiated a substantial loss of cytosine methylation on chromatin harboring H3K9\textsubscript{m3}, with little if any effect on the epigenetic patterns of H3K4\textsubscript{m3} p16 promoter chromatin.

The differences in the effects of 5-AzadC and EDC on imprinted versus non-imprinted sequences may reflect the packaging and accessibility of each of these specific DNA regions. 5- AzadC is most likely going to initiate the loss of DNA cytosine methylation throughout most, if not all of the DNA, while the selective processing of imprinted regions in the presence of 5- AzadC also results in alterations in the histone modification patterns. Since an even greater effect of 5-AzadC on the histone modifications was seen in the p16 promoter, it is possible that this DNA region is in the process of being prepared for dormancy, e.g., H3K9\textsubscript{m3} and hypermethylation, during the spermatogonial stem cell stage. The p16 gene is normally active in stem cells, but is turned off with the vast majority of genes in mature spermatozoa. EDC had different effects on the level of DNA cytosine methylation on the imprinted DMRs compared to the p16 promoter, causing a substantial increase in methylation on chromatin harboring H3K4\textsubscript{m3} in imprinted DMRs, as well as a substantial loss of methylation on chromatin harboring H3K4\textsubscript{m3} in the non-imprinted p16 promoter region.
Neither 5-Aza nor EDC altered the H3K9\textsubscript{m3} levels in the imprinted DMRs, while H3K9\textsubscript{m3} levels were decreased by 5-AzadC and increased by EDC in the non-imprinted \textit{p16} promoter. This may suggest that the H3K9\textsubscript{m3} marks in the imprinted DMRs are protected and/or no longer accessible in spermatogonial stem cells. This might be the case if these DMRs have already been imprinted, at least in what constitutes the “histone imprint” component, prior to reaching the spermatogonial stem cell stage of development. However, both 5- AzadC and EDC altered the DNA cytosine methylation patterns and the H3K4\textsubscript{m3} levels in these DMRs, indicating that the completion of the imprinting process had not taken place in spermatogonial stem cells.

Other alkylating agents have been shown to initiate global losses of DNA cytosine methylation (Wilson and Jones 1983; Wilson et al. 1987), and gene specific hypermethylation following chemical carcinogenesis (Bollati, Baccarelli et al. 2007). EDC appears to be initiating a loss of DNA cytosine methylation in the non-imprinted promoter, and a gain in the imprinted DMRs. This effect also appears to be tied to the specific lysine residue that is trimethylated. H3K4\textsubscript{m3} and H3K9\textsubscript{m3} are associated with active gene expression and repressed gene expression, possibly with heterochromatin formation. EDC increased the DNA cytosine methylation of the H3K4\textsubscript{m3} associated chromatin in imprinted regions, while decreasing the DNA cytosine methylation of the H3K9\textsubscript{m3} associated chromatin of the non-imprinted \textit{p16} promoter.

Unfortunately, there are a number of additional variables that were not studied in this experimental study. This first approach to the question of the potential impact of EDC on the paternal imprint utilized the best known of histone lysine methylation markers, H3K4\textsubscript{m3} and H3K9\textsubscript{m3}. The transition from di- to tri- methylation of H3K4 and H3K9 lysines is of particular biological significance. In general, H3K4\textsubscript{m3} methylation is linked to transcriptionally active chromatin or euchromatin (Lachner and Jenuwein 2002), while H3K9\textsubscript{m3} methylation is linked to repressed transcription or heterochromatin (Lachner and Jenuwein 2002). However, H3K9\textsubscript{m2}
methylation recently has also been discovered in euchromatin (Vakoc, Mandat et al. 2005; Vakoc, Sachdeva et al. 2006). Mono- and di-methylated lysines at H3K4 and H3K9 may prove to be play important roles in the paternal imprint, as well. Additionally, there are a number of other histone modifications, such as mono-, di- and trimethylation of lysines H3K27, H3K36, H3K79, and H4K20, arginine methylation at H4R3, and acetylation of H2B11, H3K9, H3K14, etc. The best approach most likely would entail the comparison of H3K4\textsubscript{m3} and H3K9\textsubscript{m3} patterns in mature spermatozoa first, followed by the determination of the specifics regarding the temporal processing and establishment of the paternal imprint using the in vitro spermatogonial stem cell model.

In summary, the present data suggest that EDC can disrupt 5-methylcytosine patterns and/or histone modification patterns on imprinting DMRs in the developing paternal gamete. The mechanism could involve EDC affecting CpG binding proteins, histone binding proteins, methyltransferases, or the formation of DNA base pair mutations, which may cause aberrations in 5-methylcytosine and/or histone modification patterns. DNA and epigenetic repair or maintenance mechanisms could be targeted by EDC. Further experimental work will be required to both verify the present results and clarify the histone modification patterns in the imprinted DMRs. Elucidation of the enzymatic mechanisms involved in the processing and establishment of the paternal imprint will also be important to understanding how genotoxic agents affect gamete specific imprints.
CHAPTER 6
CONCLUSIONS

The purpose of this research was to study the effects of EDC on mammalian reproductive functions, especially involving epigenetic imprinting patterns including histone modifications. This study further delineates adverse health risks to humans exposed to this high use toxicant in today’s chemical industry. As described in Chapter 2, EDC was clearly a testicular toxicant in male mice. Treatment of male mice with EDC at doses equal to or above 5 mg/kg daily for five days resulted in a dose-dependent testicular toxicity, with a precipitous loss of spermatogonia and progressing to a Sertoli cell-only state in many seminiferous tubules. Treatment of male mice to 10 mg/kg daily for five days rendered these mice permanently sterile, while male mice treated with 5 mg/kg dose displayed a short, sterile period of 3 to 5 weeks (two of three mice) and permanent sterility (one of three mice). A published testicular pathology scoring protocol for human testes was adapted for mice, and then used to clearly demonstrate a decreasing testicular score with an increasing dose of EDC. Thus, the in vivo study of male mice demonstrated that EDC interferes with the maintenance and development of spermatogonia and subsequent spermatogenesis (Daigle et al. 2009). This work provides the first evidence in the scientific literature that EDC is a testicular toxicant and potentially, a reproductive risk to humans.

The next question to be addressed in this research work was the impact of EDC on epigenetic patterns that form the paternal imprint. However, the paternal imprint is established in spermatogonial stem cells and/or during the early stages of the meiosis-committed spermatogonia prior to meiosis. This research requires the isolation of these developing gametes at specific cell stages in order to study the EDC exposure impact of epigenetic patterns specific to the paternal imprint. Also, alterations in epigenetic patterns may easily be masked by
contaminating somatic cells or by germ cells at different stages of development, therefore having different gene-specific DNA cytosine methylation and histone modification patterns. In addition, heritable deleterious changes in the paternal imprint in few spermatogonia may easily be undetectable in a million cells without some form of controlled cellular expansion. Therefore, an in vitro tissue culture model of the appropriate stages of spermatogenesis was required to advance this project.

As described in Chapter 3, an in vitro model of mouse spermatogonial stem cell cultures was developed. Using a set of three cellular antigen markers (Oct 4, CD 9, c-Kit) that provided distinct patterns for mouse embryonic stem cells, spermatogonial stem cells, and meiosis-committed spermatogonia, pure cultures of spermatogonial stem cells were prepared from embryonic stem cells. These spermatogonial stem cell cultures were stable and maintained as self-renewing stem cells for several months, prior to initiating differentiation and turning these stem cells into meiosis-committed spermatogonia. Previous reports required the use of feeder layers to maintain cultures of spermatogonial stem cells in a stable, self-renewing growth state.

By capitalizing on recent advances in stem cell growth factors, the present work developed protocols for isolating and maintaining spermatogonial stem cells on plastic without the need for feeder cells. This was important, since the presence of somatic feeder cells would interfere with the germ cell analyses of epigenetic imprint patterns. Pure cultures of spermatogonial stem cells could now be maintained, treated with EDC or other toxicants, and subsequently triggered to begin the early stages of spermatogenesis. Thus, a model for studying the establishment, as well as toxicant induced interference in the establishment, of the mammalian paternal imprint was now available.

In the interest of studying the effects of EDC on the paternal imprint, spermatogonial stem cell cultures were treated with EDC. These cultures were subsequently expanded,
following the initiation of meiosis-committed differentiation prior to harvest and analyses of gene-specific DNA cytosine methylation and histone trimethylation (H3K4\textsubscript{m3} and H3K9\textsubscript{m3}) patterns. Four genes were chosen for these analyses; three were the known paternal gamete imprinted genes (\textit{Rasgrf1}, \textit{Gtl2}, and \textit{H19}), and the fourth was a prominent, non-imprinted gene, \textit{p16}. The results of these experiments clearly demonstrated that EDC could interfere with the establishment of spermatogonial epigenetic patterns and the paternal imprint. Histone trimethylation patterns have not been previously reported for these imprinted genes, and the data from the present study suggests a few

While the 5-AzadC treated samples initiated the anticipated loss in DNA cytosine methylation in the majority of sequences studied, the results are inconclusive. 5-AzadC was included as a positive demethylating control, but cultured spermatogonial stem cells were not synchronized for optimal DNA synthesis effects and the actual level of DNA incorporation of 5-AzadC in propagating spermatogonial stem cells was not determined. Since there are no studies to establish treatment protocols for \textit{in vitro} spermatogonial stem cells and that timing and dosage are critical in order to block establishment of genetic imprinting information, more research needs to be done to determine uniform and optimal standards.

The accepted theory is that histone H3K4\textsubscript{m3} modifications are found on active chromatin, while an H3K9\textsubscript{m3} modification, along with hypermethylation (5-methylcytosine), is found on repressed chromatin. Imprinting of these gene specific DMRs entails the formation of H3K9\textsubscript{m3} and DNA cytosine hypermethylation. The present research results further support this conclusion. The majority of the imprinted DMR alleles were found to harbor H3K9\textsubscript{m3} coupled with DNA cytosine hypermethylation in meiosis-committed spermatogonia, while the promoter for the non-imprinted p16 gene was found to contain predominantly H3K4\textsubscript{m3}, coupled with DNA cytosine hypomethylation, in these same cells. Both EDC and 5-AzadC disrupted the
establishment of the imprint in treated spermatogonial stem cells. The DMRs for the imprinted genes in spermatogonia were found to harbor hypomethylated DNA (cytosine residues), coupled with histone H3K9\textsubscript{m3}, as well as increased proportion of H3K4\textsubscript{m3} chromatin. EDC initiated inappropriate hypermethylation of many of these sequences, including DMR chromatin harboring H3K4me3 in treated spermatogonia. The present work also found agent induced mismatches between the H3K9\textsubscript{me3} DNA cytosine hypomethylation patterns in these imprinted genes in meiosis-committed spermatogonia. The ramifications of mismatched lysine trimethylation modifications with DNA cytosine methylation patterns in the paternal imprint are unknown. The consequences of passing on these aberrant, epigenetic, paternal imprint patterns to a newly formed zygote can only be speculated to be deleterious to the development and survival of the embryo, but this is only speculation, based on the accepted dogma.

The paternal imprint pattern has been reported to be established in spermatogonial stem cells. The present work suggests that the process of establishing the imprint DMRs associated with \textit{Rasgrf1}, \textit{Gtl2}, and \textit{H19} is not complete until meiosis-committed spermatogonia prepare for meiosis, after the spermatogonial stem cell stage. Spermatogonia are premeiotic and therefore diploid, so that one allele may be repressed, while the second allele could be open and expressed. However, as the paternal imprint is established, both alleles should become repressed and condensed as heterochromatin, prior to meiosis, that is, the histone H3 lysine 9 residues become trimethylated, and the DNA cytosine residues become hypermethylated. Imprinting of the DMRs should entail the complete trimethylation of H3K9, loss of the H3K4\textsubscript{m3}, and DNA cytosine hypermethylation of both alleles. This was not the case in harvested, untreated, early- stage meiosis-committed spermatogonia, as approximately 30% of samples displayed the presence of H3K4\textsubscript{m3}. Since approximately 50% of these DMR alleles harboring H3K4\textsubscript{m3} were also found to be hypermethylated, it is probable that these alleles are in the midst of being imprinted and removal of
H3K4\textsubscript{m3} follows DNA cytosine hypermethylation. It is not presently known whether histone modifications guide DNA cytosine methylation, or vice versa.

The implications for the change of DNA cytosine hypermethylation on DMRs chromatin harboring H3K4\textsubscript{m3} histone modification are enormous. The EDC induced alteration in the H3K4\textsubscript{m3} pattern, observed in this study, suggests significant perturbation of gene expression, which may disrupt the imprint program for embryonic development. For example, the interaction between Inhibitor of Growth 4 (ING4), which is a histone acetyltransferase (HAT) complex and a tumor suppressor protein, and H3K4\textsubscript{m3} augments HBO1 acetylation activity on H3 tails and drives H3 acetylation at ING4 target promoters. ING4 facilitates apoptosis in response to genotoxic stress and inhibits anchorage-independent cell growth, and these functions depend on ING4 interactions with H3K4\textsubscript{m3} (Hung, Binda et al. 2009). Although this complex was not shown to be associated with Rasgrf1, Gtl2, or H19, this could be a possible mechanism for EDC’s impact on epigenetic patterns.

To correctly express a gene with this altered H3-K4\textsubscript{m3} pattern, the zygote would have to make a decision as to which epigenetic modification is correct. The conceptus would either change imprint, the DNA 5-methylcytosine pattern, to match the histone modification or change the histone modification to match the original imprint pattern. Should the epigenetic repair mechanism choose to express both copies, the developing conceptus would experience gene dosage problems (da Rocha, Charalambous et al. 2009). In studies of Dlk1/Pref1, when both copies harbored paternal imprint (repressed) or both copies harbored the maternal imprint (active), the phenotype showed growth abnormalities as well as developmental defects in muscle, cartilage/bone and placenta (Georgiades, Watkins et al. 2000; Takada, Tevendale et al. 2000; Georgiades, Watkins et al. 2001; Tevendale, Watkins et al. 2006). If the cell decides to repress both copies, this would be the same as a null gene. In Prader-Willi Syndrome (PWS), the maternally inherited copies of these genes are silent, leaving the paternal copies of the genes to be expressed. PWS results from the loss of paternal
copies of this region. If a newly formed zygote chooses to repress both alleles the phenotype may resemble PWS. However, unlike uniparental dysomy in which the conceptus has either both maternal or paternal imprints, this conceptus would just have a null at that locus in effect, a loss of expressible locus. Since no data exist concerning aberrant imprinted histone modifications, more research will be needed to determine if this epigenetic mutation has heritable consequences.

In conclusion, this research has shown that EDC targets mouse testes and spermatogenesis in vivo, which may have reproductive, embryonic, and genetic inheritance ramifications. EDC has been shown to alter H3K4m3 patterns on Rasgrf1, Gtl2 and H19 maternal imprinted gene DMRs. These disruptions most likely will have a serious, major impact on a newly formed zygote, causing embryonic death, systemic genetic disease, and/or significant birth defects.

6.1 Future Direction

The present work on the perturbation of the epigenetic patterns of the mammalian paternal imprint is not definitive. Although EDC initiated substantial changes in the histone trimethylation and DNA cytosine patterns, too many questions regarding unknowns in the epigenetic imprint patterns of the paternal (and maternal) gamete remain unanswered. Additional controls and experimental studies will be required to clarify these issues.

The final imprint histone modification patterns must be delineated in mature spermatozoa. Although doubtful, H3K4m3 and H3K9m3 could be found to play a minor role in the paternal imprint, while mono- or di-methylated lysine residues or even a different histone modification is predominantly in the mechanics of the paternal imprint. However, elucidation of the mechanisms involved in both the establishment and the functions of these epigenetic imprint patterns will require the characterization of spermatogonial stem cells, early stage meiosis-committed spermatogonia, and mature spermatozoa for mono-, di-, and trimethylation of H3K4 and H3K9 sites, as well as methylation, acetylation, and phosphorylation of additional histone sites. In fact, should EDC have
an effect on methyltransferase, then other transferases should or could be affected. Other modifications to histones, which seem to be linked to histone methylation, such as acetylation, should also be included in any future research. The paucity of published literature regarding these histone modification patterns in imprinted sites provides an open opportunity to advance this research.

The timing of the process of establishing the paternal imprint also needs further elucidation. Future experiments in this area will require finer controls and further validation of the stages of the spermatogonia, especially the early stage meiosis-committed spermatogonia. The cellular antigen markers chosen (Oct 4, CD 9, and c-Kit) were the consensus markers for each stage of cell differentiation, but additional markers might improve the resolution in the step-wise differentiation of these germ cells. Spermatogonial Stem Cell markers such as α6- and β1-integrins should also be included in future studies (Shinohara, Avarbock et al. 1999). Two monoclonal antibodies, designated BC7 and CA12, were found to identify mouse testicular germ cells during early meiotic prophase (Koshimizu, Watanabe et al. 1993). These findings should be included in future research as well. Surface and intercellular markers for negative controls would add to the confidence of these in vitro tissue culture results.

Future research would include more samplings along the time-line of treated and non-treated cells. Our results included a snapshot of DNA cytosine methylation and histone modifications. Samples taken daily (every 24 hours) after differentiation and/or chemical treatments may show not only the results of treatment, but also transitions and repair mechanisms of DNA 5-methylcytosine and histone modifications. Data on both treated and non-treated cells would clarify the relationships and linkages of DNA 5-methylcytosine and histone modifications patterns. Obviously, this approach would also establish what the normal epigenetic patterns for these germ cells.
While DMRs are thought to regulate DNA 5-methylcytosine patterns in promoter regions the actual effect of EDC treatments on gene-specific promoter regions might be worthy of investigation. The results from *P16* showed that EDC lowered the DNA cytosine methylation level in chromatin harboring H3K9m3, but the impact on the promoter regions of *Rasgrf1*, *Gil2*, and *H19* remain unknown. The effects of EDC may well be limited to the DMRs or Imprinting Control Regions. Since these regions control promoter methylation, a disruption of promoter regions may be directed from DMRs or Imprinting Control Regions.

Of course, the most definitive research would include *in vivo* experiments on mice. Unfortunately, the ability to isolate sufficient cell numbers of approximately 100% purity at each stage and time-line from spermatogonial stem cells to spermatogonia A to meiosis from live mice would be difficult at the present time. This leaves the *in vitro* approach as established and delineated in the present research work. However, as details of epigenetic patterns are reported in the literature, the present *in vitro* model approach should add substantially to the literature and advance the scientific knowledge base regarding the details of the epigenetic patterns laid down in the paternal imprint, as well as the impact of toxicants such as EDC on these patterns.
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Harold Daigle Jr started at LSU as a freshman in 1996 after being out of school for over 20 years. He was not a traditional student, three small children, cancer survivor, and divorced. In spite of all his disadvantages will graduate with a Ph.D. in Biological Sciences December 2009. He could not have done it without his family and children working with him and supporting him. Truly the most important thing in life is family.