Cellular and molecular effects of mono-(2-ethylhexyl) phthalate (MEHP) in testicular cancer

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ABSTRACT

ENGLISH: Phthalates are endocrine-disrupting chemicals (EDCs) that are known testicular toxicants, used commonly as industrial plasticizers that are found in everyday items. Di-(2-ethylhexyl) phthalate (DEHP) is the most abundant phthalate in the environment, and its primary metabolite mono-(2-ethylhexyl) phthalate (MEHP) is ten-fold more potent. The purpose of this study is to examine the cellular and molecular effects of MEHP in the development of testicular cancer. Proliferation was measured for NT2 cells exposed to 10μM and 100μM MEHP at 24 and 48 hours and for cells under controlled conditions. Methylation-specific PCR (MSP) was used to determine the methylation status of the promoter region of key testicular genes post exposure to MEHP. MEHP caused a dose-dependent negative effect on proliferation and significantly altered methylation levels for key testicular genes following exposure to 10μM MEHP and 100μM, as compared to controls. This suggests that MEHP alters proliferation and methylation of testicular tumour cells in a time- and dose-dependent manner.

FRANCAIS: Les phtalates sont des produits chimiques perturbateurs endocriniens (EDC) qui sont des toxines testiculaires connues, utilisées couramment comme plastifiants industriels qui se trouvent dans les articles de tous les jours. Le phtalate de di- (2-éthylhexyl) (DEHP) est le phtalate le plus abondant dans l'environnement et son principal métabolite mono- (2-éthylhexyl) phtalate (MEHP) est dix fois plus puissant. Le but de cette étude est d'examiner les effets cellulaires et moléculaires du MEHP dans le développement du cancer du testicule. La prolifération a été mesurée pour les cellules NT2 exposées à 10 μM et 100 μM de MEHP à 24 et 48 heures et pour les cellules dans des conditions contrôlées. La PCR spécifique à la méthylation (MSP) a été utilisée pour déterminer l'état de méthylation de la région promotrice des gènes testiculaires clés après exposition au MEHP. La MEHP a provoqué un effet négatif dépendant de la dose sur la prolifération et des niveaux de méthylation significativement modifiés pour les gènes testiculaires clés suite à une exposition à 10 μM de MEHP et 100 μM, par rapport aux témoins. Cela suggère que le MEHP modifie la prolifération et la méthylation des cellules tumourales testiculaires d'une manière dépendante du temps et de la dose.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Complete name	Abbreviation
Mono-(2-carboxymethylhexyl) phthalate	2cx-MMHP
5-azacytidine	5-aza
Mono(2-ethyl-5-carboxypentyl)phthalate	5cx-MEPP
Mono(2-ethyl-5-hydroxyhexyl)phthalate	5-OH-MEHP
Mono(2-ethyl-5-oxohexyl)phthalate	5oxo-MEHP
Adenomatous polyposis coli	APC
Androgen receptor	AR
Aryl hydrocarbon receptor	AhR
Bcl2 antagonist/killer 1	BAK1
Bisphenol A	BPA
Butyl-benzyl phthalate	BBP
Carcinoma in situ	CIS
Cell index	CI
Clear cell adenocarcinoma	CCA
Cyclin-dependent kinase 4	CDK4
Cytochrome P450 3A4	CYP3A4
Deleted in azoospermia like	DAZL
Di-(2-ethylhexyl) phthalate (DEHP)	DEHP
Dichlorodiphenyldichloroethylene	DDE
Dichlorodiphenyltrichloroethane	DDT
Diethyl phthalate	DEP
Diethylstilbestrol	DES
Di-isononyl phthalate	DiNP
Dimethyl phthalate	DMP
Dimethyl sulfoxide	DMSO
Di-n-butyl phthalate	DBP
Di-n-octyl phthalate	DnOP
Electromagnetic fields	EMF
Endocrine-disrupting chemicals	EDC
Enzyme-linked immunosorbent assay	ELISA
Estrogen Receptor alpha	ERα
Estrogen Receptor beta	Erβ
Genome-wide association studies	GWAS
Glutathione S-transferase pi	GSTP1
Human development index	HDI
Hypoxanthine phosphoribosyltransferase 1	HPRT1
Hypermethylated in cancer 1	HIC1
Insulin-like growth factors	IGF

Invasive ductal carcinoma	IDC
Methylation specific restriction enzymes	MSRE
Mono-2-ethylhexyl phthalate	MEHP
Methylation specific pcr	MSP
Ntera 2/cl.D1	NT2
O6-methylguanine-DNA methyltransferase	MGMT
P16ink4	MTS1
Polychlorinated biphenyls	PCB
Polyvinyl chlorides	PVC
Protease serine 21 (Testisin)	PRSS21
Radio frequencies	RF
Ras-association domain family member 1	RASSF1A
Rna binding motif protein	RBMY1A1
Real-time cell analysis	RTCA
Retroperitoneal lymph node dissection (RPLND	RPLND
Sex hormone binding globulin	SHBG
Testicular dysgenesis syndrome	TDS
Testicular germ cell tumours	TGCT
Tetrahydrocannabinol	THC
Thyroid stimulating hormone	TSH
Tumour necrosis factor	TNF
Tolerable daily intake	TDI

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CHAPTER I – BACKGROUND

THE EPIDEMIOLOGY OF TESTICULAR CANCER

Prevalence

Testicular cancer is the most commonly diagnosed cancer among males between the ages of 15-44 years in countries that are high or very high on the Human Development Index (HDI) (Ferlay et al, 2010). The incidence rate of testicular cancer is the highest among Caucasians (6.97 per 100 000), followed by American Indian/Natives (4.66 per 100 000), Hispanics (4.11 per 100 000), Asian/Pacific Islanders (1.95 per 100 000) and black men (1.20 per 100 000) (Ghazarian et al, 2014). In Ontario, the incidence of testicular germ cell tumours (TGCT) has increased from 4.01 to 6.39 per 100 000 between 1964 and 1996 (Weir et al, 1999). The worldwide incidence has doubled over the last 40 years (Huyghe et al, 2003), leading some to believe that this is due to environmental factors rather than genetic defects (Skakkebaek et al, 2001). Testicular cancer is rare in Asian and African/African-American populations, averaging about 1/100,000 in age-standardized incidence rate, while in countries like Denmark it has risen to 9.2/100,000 in the past four decades (Parkin et al, 1997).

Testicular cancer makes up about 1% of all new male cancers globally (Znaor et al, 2013). It accounts for 1.1% of all malignant neoplasms in Canadian males (Garner et al, 2005), and since the 1970s, a 50% increase in the incidence rate of testicular cancer has been observed in Canada (Liu et al, 1999). While there are several factors for the increase in recent testicular cancer incidence, there seems to be a birth cohort effect. A study by Wanderas, Tredi & Fosta (1995) shows that males born before or after World War II had an increased risk of testicular

cancer as compared to those who were born during the war. The authors suggest that the war caused changes in lifestyle such as decreased use of polluting vehicles, increased physical activity and increased consumption of vegetables and dietary fiber, leading to an overall healthier state. This leads us to believe that, while a proper diet and exercise are essential for the prevention of almost any disease, the environment plays a substantial role in the progression of testicular cancer as well.

Pathophysiology

When examining testicular cancer, it is essential to understand the different types of tumours and their respective characteristics. Approximately 96% of all testicular cancers are testicular germ cell tumours (TGCTs), while the remaining cancers are rare stromal tumours of Leydig, Sertoli and granulose cells (Stevenson et al, 2009). TGCTs can manifest in several forms, such as seminomas, embryonal carcinomas, choriocarcinomas, teratomas and yolk sac tumours (Garner et. al, 2008). These forms are generally divided into two histological subtypes: seminomas, which are tumours of the purest form with only one type of lesion present and comprising around 52% of total tumours, and non-seminomas, which employ a combination of forms to manifest a tumour and comprise 48% of total tumours (Powles et al, 2005; Garner et al, 2008). Embryonal carcinomas are the most frequent form of TGCT, representing around 87% of all non-seminoma tumours (Bosl & Motzer, 1997). Seminomas typically peak at 30-40 years of age in men, while non-seminomas, which are far more common and aggressive, peak at 20-30 years of age (Stone et al, 1992). The incidence of testicular cancer overall tends to peak around 25-29 years (De Santis et al, 2014), and although it can affect males of all ages, testicular tumours are rare in adolescents and children. Childhood testicular cancer accounts for 1-2% of

all pediatric solid tumours (Mittal et al, 2015), with 75% of all childhood testicular cancers being malignant (Green, 1986).

TGCTs arise from their cells of origin within the testis and can therefore differentiate into whichever structures they came from. Seminomas typically differentiate into a uniform type of cell, while non-seminomas are quite varied. Embryonal carcinomas differentiate the least, making treatment more difficult while choriocarcinomas differentiate into cells mimicking placental tissue and can be quite rare with poor prognosis (Porth, Gaspard & Noble, 2011). Yolk sac tumours mimic the embryonic yolk sac and typically have a good prognosis in infants while teratomas tend to contain a combination of ectoderm, mesoderm and endoderm germline tissues and can occur at any age (Porth, Gaspard & Noble, 2011).

Mortality rates from testicular cancer are relatively low and are slowly improving due to multimodal treatment (Stevenson & Lowrance, 2015); since 1975, the 5-year survival rate has increased from 63% to more than 90% (Sheinfeld, 1994). Most cases (69%) are diagnosed at the localized stage, meaning they have not spread, and these usually consist of seminomas. The 5-year survival rate for this stage is 99.1%. Once the cancer has spread to the surrounding regions, which is the case for most non-seminomas, the survival rate decreases to 95.8%, and further metastasis to distant regions decreases the survival rate to 73.8% (DeSantis et al, 2014).

Risk Factors

Testicular dysgenesis syndrome (TDS)

Testicular cancer is a condition of testicular dysgenesis syndrome (TDS), which also includes cryptorchidism (the absence of one or both testes), hypospadias (the emergence of the urethra on the ventral surface of the penis), and impaired spermatogenesis (Bay et al, 2006). These four manifestations have been hypothesized to be interrelated due to genetic polymorphisms or environmental factors (Figure 1, Bay et al, 2006) and the implication is that the presence of TDS in any of its other three forms is usually a strong indicator of also having testicular cancer. TDS may originate in the fetus during the development of the reproductive system, when the risk of embryonic hormonal disturbances can lead to both hypospadias and cryptorchidism (Bay et al, 2006). Impaired spermatogenesis usually does not manifest until after puberty but may also have an origin in the fetus. The presence of male infertility also increases the risk of developing testicular cancer 3-fold, suggesting that this may also be a possible component of TDS (Walsh et al, 2009).

Carcinoma in situ (CIS), which is arguably the precursor of all TGCTs, is presumed to originate from primitive primordial germ cells and gonocytes which have begun neoplastic transformation by escaping typical differentiation *in utero* (Skakkebaek et al, 1987). While it has been shown that half of all patients diagnosed with CIS develop testicular cancer within 5 years (Boisen et al, 2001), the exceptions are two rare types of tumours that occur only in older men: infantile germ cell tumours and spermatocytic seminoma (Bay et al, 2006). If CIS develops into cancer at an early age, there is a higher chance that it will become a non-seminoma, whereas if it develops at a later age, it is likely the lesion will become a seminoma (Rorth et al, 2000).

Thus any hormonal disturbances occurring during embryogenesis can lead to genital malformations such as hypospadias and cryptorchidism, or impairments in germ cell differentiation and spermatogenesis. All four cases are interrelated due to their origin in the fetus and increase the risk of developing testicular cancer.

Cryptorchidism

Cryptorchidism, also known as an undescended testis, is associated with a 2-4-fold (Boyle & Zaridze, 1993), and in some cases 5-10-fold (Swerdlow et al, 1997) increased risk of developing testicular cancer. Similar genetic, environmental and lifestyle factors between cryptorchidism and testicular cancer may be associated in the shared etiology, however there may also be a cause and effect chain of events at play. The testes develop in the abdomen during fetal development and then descend into the scrotum under normal circumstances. However, the failure of this normal process leads to cryptorchidism. The development of germ cells within this undescended testis is usually compromised, leading to impaired fertility and the possibility of CIS which can eventually turn into a TGCT (Hutson & Hasthorpe, 2005). There are two hypotheses as to how cryptorchidism may contribute to the development of testicular cancer: 1) the TDS link, which roots the origins of cryptorchidism and testicular cancer in the embryo, enabling CIS to develop, and 2) that the placement of the undescended testis predisposes it to antagonistic environmental factors that promote the development of testicular cancer (Manecksha et al, 2009).

Personal and family history

A personal history of previous diagnosis of testicular cancer generally leads to a 12-fold increased risk of developing another testis tumour, with the period of greatest risk being the first 5 years after the initial diagnosis of testicular cancer (Fossa et al, 2005). A study by Fossa et al (2005) reported a 1.9% cumulative risk for developing metachronous contralateral testicular cancer (non-synchronous cancer in the previously unaffected testicle) while a study from Denmark reported a cumulative risk of 5-6% (Osterlind, Berthelsen & Abildgaard, 1991). These differences in risk assessments may be due to the geographically-based genetic makeup that is natively distinct between the two countries. Age also seems to play a role in the development of contralateral testicular tumours, with patients previously diagnosed with seminomatous TGCT aged less than 30 being 2.6 - 4.8 times more likely to develop another tumour than patients aged over 30 (Fossa et al, 2005). Meanwhile, family history of testicular cancer can be a significant factor, with estimates showing a 10-fold increased risk for brothers and sons of patients developing testicular cancer (Scardino et al, 2011).

Ethnic and geographical factors

The incidence of testicular cancer is highest among Caucasians and lowest among African and Asian populations (Purdue et al, 2005). The incidence in Northern and Western European countries are the highest worldwide. Norway has the highest incidence rate in the world, with 9.9/100,000, followed by Denmark with 9.4/100,000 (Ferlay et al, 2010). Switzerland, Germany, the UK, New Zealand and the USA are some of the top countries with high incidence rates, while the lowest rates are in countries such as Thailand, India, Japan, Brazil

and South Africa (Ferlay et al, 2010). The difference in testicular cancer incidence is striking at a geographical level. Finland has one of the lowest incidences of testicular cancer in all of Europe, while Denmark has one of the highest. These patterns also emerge in the incidences of other TDS disorders, such as cryptorchidism and hypospadias, with the prevalence of these being considerably lower in Finnish males as compared to Danish males (Boisen et al, 2004).

Impaired spermatogenesis is also affected in the same fashion, with incidences being highest in western European countries such as Norway and Denmark versus eastern European countries such as Finland and the Baltic regions (Richiardi et al, 2004). Migration studies (Ekbom et al, 2003; Hemminki & Li, 2002) have shown that Finnish men migrating to Sweden follow the Finnish incidence rate of testicular cancer, but their sons born in Sweden follow the Swedish incidence rate, suggesting that environmental factors may play a more crucial role in testicular cancer development than genetic heritability.

Testicular microlithiasis

Testicular microlithiasis is a common disorder wherein small calcifications deposit in the testes, and this disorder is prevalent in about 5.6% of the male population between the ages of 17 and 35, although it is higher (14.4%) in African Americans (Costabile, 2007). Testicular microlithiasis is present in around 50% of TGCTs (Costabile, 2007) and having microlithiasis can account for an 8-fold increased risk of developing a testicular tumour (Heller et al, 2014).

Diet

Fatty foods and dairy products consumed during childhood have been associated with an increase in the incidence of testicular cancer, especially luncheon meats and cheese but not milk. (Sigurdson et al, 1999; Garner et al, 2003). Intake of processed meats such as bacon, sausages and hotdogs are all associated with increased risk but the associations are not yet established (Garner et al, 2003).

Maternal exposure to estrogens

Maternal hormones may also play a role in the development of testicular cancer. It has been suggested that during first pregnancies, maternal endogenous estrogen levels are higher than during subsequent pregnancies, and since elevated levels of estrogen have been established to increase the risk of testicular cancer, it is posited that first born sons may be at increased risk due to the elevated circulating estrogen (Wanderas et al, 1998). However, over 90% of the maternal endogenous estrogens are bound to sex hormone binding globulin (SHBG) and are thus sequestered, protecting the fetus (Vidaeff & Sever, 2005).

Between the 1940s and 1970s, the synthetic estrogenic drug diethylstilbestrol (DES) was prescribed to over 5 million pregnant women to prevent abortions and pregnancy-related complications (Palmlund et al, 1993). It was later discovered that the female offspring of these mothers developed vaginal clear cell adenocarcinoma (CCA) (Herbst et al, 1971) and that the male offspring developed malformed testes, epididymal cysts and impaired sperm quality (Bibbo et al, 1977). DES does not bind well to SHBG, and can thus have increased potency if ingested (Vidaeff & Sever, 2005).

Environmental exposures

The testes are vulnerable to environmental toxins through both internal and external routes. They are mostly unprotected in their anatomical location of the scrotum, where they may be exposed to extreme heat exposure, electromagnetic fields and gamma radiation (Baumgardt et al, 2002). Environmental toxins must pass through the blood-testis barrier between the cords of the seminiferous tubules to reach the germ cells within, and this barrier does not appear to be specific in the size of the molecules that are allowed to pass through (Setchell, 1979). Leydig cells are the first cells encountered when passing from the blood to the testis and are therefore particularly vulnerable to environmental compounds (Meeks et al, 2012).

Organochlorines

Organochlorines were pesticides used extensively from the 1940s to the 1960s and are mostly banned in Western countries today, although they are still used extensively in developing countries. Common organochlorines include the famous dichlorodiphenyltrichloroethane (DDT) and chlordane (Meeks et al, 2012). Studies have found that DDT and chlordane are associated with a higher risk of seminoma (Guo et al, 2005), while DDT alone is associated with a higher risk of non-seminoma (McGlynn et al, 2008). While there is substantial evidence that organochlorines are carcinogenic, further studies should focus on populations of the countries where the compounds are still being used, as opposed to Western countries where organochlorines have been banned.

Marijuana

Repeated smoking of marijuana and the consumption of tetrahydrocannabinol (THC), its active chemical, has been shown to increase the risk of developing testicular cancer (Meeks et al, 2012). THC is a possible endocrine-disruptor which has been known to cause gynecomastia (enlarged breasts in men), a decreased sperm count and impotence (Watanabe et al, 2005). Incidences of only non-seminomatous TGCT are slightly higher in men who smoke marijuana than in men who do not (72.6% vs 68% respectively, but only if the men consumed it daily (Daling et al, 2009). Frequent (more than once daily) and long term (more than 10 years) users were more likely to develop non-seminomas, but not seminomas (Trabert et al, 2011). THC's mechanism of action may be via the cannabinoid receptors on Leydig and germ cells, although not enough research has been conducted on this topic.

Electromagnetic fields

Occupational exposure to extremely low frequency electromagnetic fields (EMF) or radio frequencies (RF) show increased risks for non-seminoma TGCTs, especially for men under 40 years (Floderius et al, 1999). For workers who are frequently in the range of RF sources, such as military men, seamen, fishermen and policemen, the incidence rates of testicular cancer increase slightly (Mester et al, 2010). It is hypothesized that the low frequency of EMFs and RFs are not strong enough to break covalent bonds, but even frequencies this weak can trigger cancerous effects by promoting subthermal conditions (Stevens & Davis, 1996). EMF may also inhibit melatonin production in the pineal gland, which exerts an antigonadotropic effect. This in turn leads to enhanced prolactin production in the pituitary gland, which can stimulate testosterone

secretion from Leydig cells. This can overstimulate these cells to reduce the activity of DNA repair mechanisms, thus leading to a carcinogenic effect (Mester et al, 2010).

ENDOCRINE-DISRUPTING CHEMICALS

Endocrine-disrupting chemicals, or EDCs, are chemicals present in our environment, food and consumer products that disrupt normal hormonal biosynthesis and metabolism. They are ubiquitous and heterogenous in nature, being found in industrial solvents and lubricants such as polychlorinated biphenyls (PCBs), plastics such as bisphenol A (BPA), plasticizers such as phthalates, pesticides such as dichlorodiphenyltrichloroethane (DDT), fungicides such as vinclozolin, pharmaceutical agents such as diethylstilbestrol (DES) and natural chemicals found in adult food and baby formula such as phytoestrogens (Dickerson & Gore, 2007; Diamanti-Kandarakis et al, 2009). Currently, they pose a threat to humans and wildlife, as they have a slew of toxic effects to the reproductive and endocrine systems, and are thus of great concern for public health.

The hormonal response

The target of endocrine-disrupting chemicals is the endocrine system, which has the role of secreting hormones. There are certain endocrine glands that are more traditionally described than others; these include the thyroid glands, the pituitary gland, the adrenal glands and the gonads. However, several other organs in the body contain endocrine glands or have functions similar to the endocrine system (Melmed & Williams, 2011).

The effects of each hormone secreted by this system are mediated by their interaction with specific receptors. In general, there are three main classifications of hormones: steroidal, peptide and amine (Melmed & Williams, 2011). Steroidal hormones are brought to their

receptors by carrier proteins, which travel through the bloodstream and passively diffuse into cells, however they can also interact with membrane-bound receptors (Melmed & Williams, 2011). Protein and amine hormones usually interact with membrane-bound receptors on the exterior of cells because they cannot passively diffuse through the cell membrane (Melmed & Williams, 2011). When bound, a message is transduced to the interior of the cell to perpetuate the effect of the hormone. Thyroid hormones cannot passively diffuse through the cell membrane either, but they can employ specific carrier proteins to carry them through the membrane and interact with receptors on the inside of the cell (Melmed & Williams, 2011). Steroid and thyroid hormones in particular are known for their nuclear receptors, due to their role of regulating gene transcription by binding to specific regions of DNA. This in turn regulates or alters the formation of new proteins.

Hormones work in a time- and spatially-sensitive manner. Due to the limited number of receptors, hormonal effects are highly precise and balanced. The hormone insulin has receptors throughout the body and thus its effects can be noted in most tissues, while TSH, or Thyroid Stimulating Hormone, is mainly distributed by the thyroid gland and thus its impact in the body is significantly more reduced (Charlton S. J., 2009). Many hormones are only active during certain points in human development, while others emerge only in adulthood. Hormones may also be active only in a certain type of cell at a certain time, and may also interact with multiple receptors. Estrogens interact with multiple nuclear receptors, the major two being Estrogen Receptor alpha and beta (ERα and β), while testosterone acts on a single receptor, the Androgen Receptor (AR) (Charlton, 2009).

Hormones require a very low concentration to perpetuate an effect, due to their sigmoidal dose-response relationship which generates an S-shaped curve (Li et al, 2007). Thus small modifications to the hormone concentration at the low end of the curve can produce a great effect in the body. This is extremely important, as even the smallest changes in hormonal levels caused by any disruptors can produce a large and harmful response. Due to the generally uneven distribution of receptors among different types of cells in close proximity to each other, the sigmoidal dose-response can also accordingly shift. A greater number of the same receptors in the vicinity can shift the curve to the left, requiring a lower hormone concentration to perpetuate an effect. Likewise, a low number of receptors shift the curve to the right, requiring more hormones (Li et al, 2007).

Not all hormones function with the sigmoidal dose-response relationship. Several have bi-phasic (having two phases in the curve) or non-monotonic curves (having several disjointed phases), which can show maximum hormonal response at low, intermediate, or high doses (Li et al, 2007). These responses can sometimes occur as a consequence of having very high concentrations of hormone present, which will bind to all available receptors. The cell will be incapable of replacing the occupied receptors with free receptors at a fast enough pace, causing eventual receptor down-regulation. In other cases, having very high concentrations of hormone present can sometimes cause cytotoxicity, as can be seen with certain estrogens. Receptor affinity can also cause non-monotonic responses (Li et al, 2007). Certain hormones will bind exclusively to a certain type of receptor at low concentrations, but if the hormone is present at high concentrations, it may also weakly bind with other types of receptors, leading to a non-specific response (Li et al, 2007).

Endocrine-disrupting mechanisms

Human development is controlled by genetics – the passing on of heritable traits – as well as epigenetics – heritable changes not based on genetics. Genetic development describes the process of permanently activating and deactivating certain genes to wire cells for their respective fates: a liver cell, a kidney cell, and so on (Bergman et al, 2012). But epigenetic development is a process that depends upon the environment, and thus markers such as DNA methylation and histone modifications can change those heritable traits. Thus hormones as well as endocrine disruptors affect tissue development by controlling the expression of genes, and thus change the epigenome itself (Bergman et al, 2012). Not all epigenetic mechanisms are hormone dependent, but endocrine disruptors have the ability to identify and alter the hormonally-dependent epigenetic mechanisms and therefore impact development. In most cases, these changes are permanent and may even have a chance of being inherited into the next generation, creating a transgenerational effect (Skinner, Manikkam & Guerrero-Bosagna, 2011).

The two forces that control human development are akin to the two major theories of carcinogenesis: the genetic or 'somatic mutation' theory suggests that cancer is the accumulation of mutations in the cell, while the epigenetic or 'developmental origins of adult disease' theory suggests that changes in the epigenome are what causes cancer (Hahn & Weinberg, 2002). While the theories do not agree on the methods (genetic vs. epigenetic), they do come into agreement that both cause changes within the cell, leading to abnormal cell proliferation (Ho et al, 2006). In contrast, the 'tissue organization field' theory suggests that proliferation is the default state of all cells and that cancer is an outcome of defective tissue organization, mostly occurring during early development as a result of interaction with carcinogens (Sonnenshein & Soto, 2008).

As such, there are two main methods by which hormone disruption can occur: by disrupting the hormone-receptor complex or by disrupting a protein which is critical to correct hormone delivery. This may occur during the hormone's production, during its transport to its specific destination, or to the carrier protein transporting it (Casals-Casas & Desvergne, 2011). In general, receptors have a higher affinity for natural hormones than endocrine disruptors, but affinity, or ability to bind, is not the only factor in play. Potency, or the ability to cause effects, of endocrine disruptors can sometimes be much higher than natural hormones due to their increased efficiency to activate the receptors (Casals-Casas & Desvergne, 2011). Thus endocrine disruptors can be extremely harmful if present in even low doses, as they have similar sigmoidal and non-monotonic dose-response relationships. However, endocrine disruptors are also subject to the same specificity as natural hormones in terms of cells, tissue and receptors.

Endocrine disruptors may act at any point in life, similar to hormones, but the consequences are much greater during development. If one is exposed to an endocrine disruptor during development, the changes are generally permanent, since cells and tissues are being programmed during this critical stage. By reprogramming cells and tissues during development, children can develop a predisposition to a particular disease. In contrast, exposure to the same endocrine disruptor during childhood or adulthood can have an alternate effect, and may even be transient (Alonso-Magdalena et al, 2010). The developmental stage is the most vulnerable and most frequent stage of endocrine disruptor exposure, and as such has the gravest consequences, because lower doses of endocrine disruptor are required to induce an effect than would be needed in adulthood (Alonso-Magdalena et al, 2010). Testicular cancer in particular has a notably vulnerable period during the genome-wide epigenetic reprogramming that occurs in the

early specification of embryonic cells into primordial germ cells (Kurimoto et al, 2008). Thus EDCs pose a great threat during the developmental or fœtal period, via potential epigenetic mechanisms.

The latency of EDC exposure is such that even if it occurs during the vulnerable developmental period, the consequences will not appear until childhood or adulthood. During the developmental period, extremely low levels of exposure are often even more potent than higher levels of exposure, with most EDCs employing an inverted-U or U-shaped dose-response curve (Sheehan et al, 1999). EDCs are less water soluble and very lipid soluble, causing them to accumulate in fat tissues over time, especially with repeated exposures which are commonplace with factory workers (Diamanti-Kandarakis et al, 2009).

EDCs were previously thought to primarily work through nuclear hormone receptors, such as estrogen, androgen, progesterone, retinoid and thyroid receptors (Diamanti-Kandarakis et al, 2009). Non-nuclear receptors include G protein-coupled receptors and ion channels. However, it is now widely recognized that EDCs can also act via nonnuclear steroid hormone receptors such as membrane receptors, nonsteroid receptors such as the serotonin, dopamine and norepinephrine receptors, and orphan receptors such as the aryl hydrocarbon receptor (AhR) (Diamanti-Kandarakis et al, 2009). At a molecular level, categorizing EDCs is more difficult, as the various types do not seem to share any similarities except for their small molecular mass of less than 1000 Daltons. Several of the industrial solvents and pesticides tend halogen groups such as chlorine and bromine, and it is generally believed that EDCs phenolic moiety mimics the

natural steroid hormones by acting as an analog or antagonist to the steroid hormone receptors (Diamanti-Kandarakis et al, 2009).

EDCs have the tendency to operate with multiple mechanisms at once; some can be both estrogenic and antiandrogenic. For example, DDT is an estrogen agonist but is metabolized in the body into dichlorodiphenyldichloroethylene (DDE), an androgen antagonist (Rasier et al, 2007). This dual nature of EDCs is noteworthy because of the equally dual nature of the reproductive system of both sexes and their use of androgens and estrogens. For example, cytochrome P450 3A4 (CYP3A4) is an enzyme that inactivates testosterone but also plays a role in early breast development for females (Kadlubar et al, 2003).

Modes of exposure to endocrine-disruptors

Exposure to EDCs can occur from a wide variety of sources, and often in combination with other EDCs, making it difficult to pinpoint specific effects and study them in controlled settings. Fruits, vegetables, meat, pesticides, plastics, home insulation and flame retardants are all examples of the ubiquity of endocrine disruptors in food production, pathogen control, material production and buildings (Bergman et al, 2012). Thus, it is of the utmost importance to consider studying the effects of these chemicals and their hormonal effects. Most endocrine disruptors are studied in the context of toxic chemicals, with each being individually examined. However, most endocrine disruptors are accompanied by a myriad of chemicals that combine to create a synergistic effect (Crofton et al, 2005). For example, estrogens are typically accompanied by other estradiol-like compounds that can mimic the natural hormone, and

although individually their potency is deemed to be low, together they create a deadly chemical cocktail that can have immensely toxic effects (Rajapakse, Silva & Kortenkamp, 2002).

Some EDCs are banned in certain countries while others are not, and this discrepancy makes it difficult for population-based studies to make generic conclusions regarding specific EDCs. Migration studies offer a solution to this problem, as they allow us to examine whether the move to a new location begins the onset or the end of exposure. While it is common to show direct causal relationships of toxic spills and contamination from pesticides and dioxins to reproductive dysfunctions in humans and animals, these are not sufficient to represent the cocktail of EDCs that we are exposed to commonly. Mixtures of EDCs can leak into groundwater, soil, air and sewage sludge, eventually consumed by plants and animals and then humans. Industrial workers, factory workers, and those who work in close contact with pesticides (i.e. farmers) are at increased risk for reproductive and endocrine abnormalities due to their chronic EDC exposure (Diamanti-Kandarakis et al, 2009).

EDCs were designed to be long-lasting for industrial purposes and thus they had long half-lives, however this also means they are far more damaging to humans and wildlife. Once EDCs are eventually metabolized, they are broken down into metabolites which are often far more toxic than the EDCs themselves. One prime example is mono-2-ethylhexyl phthalate (MEHP), the primary metabolite of di-2-ethylhexyl phthalate (DEHP), a common EDC found in plasticizers. MEHP is not only the most active metabolite of DEHP but also the most toxic (Pollack et al, 1985). In certain cases, EDCs that were banned for several decades have been found in trace amounts in humans and animals, and even untouched areas far from industrial

zones have been found to be contaminated due to air currents, water ways and migratory animals (Calafat & Needham, 2007).

Adverse health effects of endocrine-disruptors

The effects of EDCs extend to breast development, metabolism, obesity, male and female reproduction, neuro- and cardiovascular endocrinology, and several endocrine cancers, and the convergence of human, animal and epidemiological studies show that EDCs pose a major threat to public health. Reproductive disorders from EDC exposure differ between males and females, as male sexual differentiation is dependent upon androgens and potentially estrogens while female sexual differentiation is mainly independent of both (Diamanti-Kandarakis et al, 2009).

For males, TDS is a major disorder that suggests that more than one condition of TDS can occur at multiple points in life from exposure to EDCs, and other disorders in males include prostate hyperplasia (Maffini et al, 2006) and abnormal pubertal timing (Buck et al, 2008). In the female, it has been implied that EDCs are responsible for progressing lactation and ovulation disorders, benign breast disease, premature symptoms of puberty, breast cancer, endometriosis and uterine fibroids (McLachlan et al, 2006). Polycystic ovarian syndrome is a common disorder associated with EDCs, with studies showing the affected women having higher levels of BPA (Takeuchi, 2004). Phthalates have been associated with endometriosis, with high DEHP levels in the plasma of the affected women being found (Cobellis et al, 2003).

EDCs have been found to alter estrogen receptor alpha (ERα) expression in the hypothalamus, the epididymis and the uterus (Ceccarelli et al, 2003; Atanassova et al, 2007;

Khurana et al, 2000), but they can also alter endogenous steroid production, action, synthesis and metabolism (Diamanti-Kandarakis et al, 2009).

Endocrine-disruptors and cancer

Abnormal levels of hormones are generally thought to be essential in promoting the growth of cancer tissue. However, the mechanism by which hormones cause uncontrollable growth are yet to be determined, as most theories of carcinogenesis involve mutagens, which are not generally associated with hormones (Berman et al, 2012). Therefore, studies have begun to theorize that it is not the hormones or endocrine disrupters themselves that are causing mutagenesis, but the abnormal presence, absence, or timing of them that causes downstream changes like gene silencing, disrupted tissue organization and irregular differentiation, leading to cancer (Soto & Sonnenschein, 2010).

Breast cancer

Over the past five decades, the presence of xenoestrogens (hormones that imitate estrogen) has rapidly increased due to the industrialization of our world. While some of these cancer-inducing influences may be prescribed (anti-miscarriage drugs, etc), naturally occurring estrogens and xenoestrogens are the main influencers of breast cancers. Several periods in a woman's life are also subject to vulnerability from either naturally occurring estrogens or xenoestrogens: during puberty, during pregnancy and lactation, and during menopause.

Due to the release of estrogen throughout a woman's life, hormonal presences are a key factor in breast cancer development. Increasing estrogen secretion throughout one's life is thought to increase the overall risk of developing breast cancer (Travis & Key, 2003). Breast tumours tend to sequester estrogens for later use and growth, and thus to properly identify the effect of estrogens on breast cancer, it is critical to collect samples before a woman is diagnosed with breast cancer (Travis & Key, 2003). The cells at the end buds of the breast are generally the genesis of most breast cancers, due to the high number of estrogen receptors in these cells (Russo & Russo, 2006). The end buds are thought to have a number of cells that do not completely differentiate during development, and are thus prompted to proliferate by the high number of estrogen receptors surrounding them (Russo & Russo, 2006).

The 'tissue organization field' theory is currently the best supported in the case of breast cancer, due to the unique nature of postnatal breast development. After birth, shifting hormonal levels control the drastic development of the mammary glands, controlling their architecture in a way that is similar to organogenesis (Markey et al, 2002). It is thus during this stage that the breast is most vulnerable to endocrine disruptors, which mimic endogenous hormones and disrupt the interactions during breast tissue organization. These disrupted interactions can also cause impairment of the hormone-regulating mechanisms, eventually leading to neoplasms and abnormal cell growth (Markey et al, 2002). For example, the xenoestrogenic BPA has been found to alter the development of the fetal mouse mammary gland when exposed in environmentally relevant doses (Vandenburg et al, 2007).

Some theories of hormonal carcinogenesis in terms of breast cancer revolve around the naturally occurring cycle of estrogen levels throughout the menstrual cycle. The proliferation of ductal cells, or the cells that transport milk-producing lobules to the nipples, occurs more frequently from the late follicular phase to the early luteal phase, i.e. around ovulation (Markey et al, 2002). During this time, endogenous estrogen levels are highest, and decrease thereafter. The presence of xenoestrogens does not allow this cycle to occur, keeping the levels of estrogen in the body at a constant high. This promotes excessive proliferation of the ductal cells, sometimes leading to neoplasms, invasive ductal carcinoma (IDC) as well as other breast cancers (Markey et al, 2002).

Endometrial cancer

This mostly 'Western' cancer is the sixth most common cancer in women around the world, and has been steadily increasing over the years like most endocrine cancers (Bergman et al, 2012). Endometrial cancer has two types: one that is unrelated to estrogen and another that is dependent upon estrogen (Kellert et al, 2009). It is this type of endometriosis that has been increasing in incidence, as suggested previously by the rise of xenoestrogens over the past five decades. The main causes of endometriosis involve increased or constant levels of free estradiol, estrone and testosterone in the blood (Allen et al., 2008), and hormone replacement therapy that is commonly used during menopause - pharmaceutical estrogen and progestagen cocktails – tend to exacerbate the risks of developing endometrial cancer (Jaakola et al, 2011).

Cells in the endometrium generally grow and shed repeatedly every month with the menstrual cycle. When there are genetic modifications to cells, they lose the ability to shed and

thus form a growth in the endometrium. This can also occur from the presence of a type of stem cell progeny in the endometrium which lead to non-shedding cells (Kyo, Maida & Inoue, 2011). It has been proposed that altered ERα may be responsible for changing the expression levels for over 100 genes, creating a hyper estrogenic environment (Kim & Chapman-Davis, 2010). Another theory suggests that many phosphorylating enzymes are activated and growth factor pathways (insulin-like growth factors, or IGFs) are unregulated in endometrial cancer. These growth factors are also known to be subject to epigenetic silencing, but if the genes do not imprint from their parental genes (i.e. the DNA markers are not passed down to the progeny), IGF is overexpressed, leading to abnormal proliferation of the endometrial cells (McCampbell et al, 2006).

Prostate cancer

Prostate cancer is most common in Western countries, with the Netherlands and Austria leading the pack (Bergman et al, 2012). Benign prostatic hyperplasia is known to affect half of all men by the age of 60 (Jemal et al, 2006). Many hormonal treatment strategies involve steroids, which have been thought to induce and maintain the growth of prostate cancer. Androgens and estrogen are generally at fault here and thus treatment usually involves antiandrogens and antiestrogens (Raghow et al, 2002). Prostate cancer generally derives from the epithelial cells within the gland, where increased exposure to estrogen during fetal development can predispose one to cancer (Ellem & Risbridger, 2009).

Many organochloric pesticides, as well as cadmium and arsenic, have been shown to have estrogenic properties, mimicking the hormone and inducing prostate cancer (Soto et al,

1995; Benbrahim-Tallaa & Waalkes, 2008). Other pesticides include acetylcholine esterase inhibitors which may inhibit certain Cytochrome p450 enzymes, such as CYP1A2 and CYP3A4; incidentally, these enzymes metabolize estradiol and testosterone (Diamanti-Kandarakis et al, 2009). Prolonged exposure to these chemicals, which frequently occurs with farmers, thus often leads to disturbed metabolic conversions of steroid hormones, increasing the risks of prostate cancer (Diamanti-Kandarakis et al, 2009).

Thyroid cancer

While thyroid cancer is not the most common cancer worldwide, it is one of the most rapidly increasing, especially in industrialized countries, where it has more than doubled in the past four decades (Bergman et al, 2012). Women, children and young adults are most at risk. The three major forms of thyroid cancer are follicular, papillary and anaplastic, with anaplastic being the most fatal with a 100% mortality rate (Bergman et al, 2012). Thyroid cancer manifests itself mostly through the genetic carcinogenesis route. Well-differentiated cells called thyrocytes undergo several changes to the genome, mostly from radiation, and this can be passed onto future generations. However, due to the lack of enough evidence to prove that a succession of genomic changes lead to thyroid cancer, many other theories have been considered. These suggest that undifferentiated fetal thyroid cells are what induce thyroid cancer, as these cells have the ability to move to other regions of the body, similar to metastasis (Diamanti-Kandarakis et al, 2009). The division between the well-differentiated thyrocyte cell theory and the poorly-differentiated fetal thyroid cell theory are currently at odds and there is no consensus.

Yet another theory that has been suggested involves the pituitary gland. When the pituitary secretes excessive thyroid-stimulating hormone (TSH), the thyroid follicle cells are induced to proliferate and produce enough thyroid hormone to keep up with the demand (Bergman et al, 2012). The increased proliferation leads to hyperplasias and thyroid cancer. Another theory proposes that since the incidence of thyroid cancer is three times higher in women than men, it is possible that estrogens have some involvement. This may be attributed to an estrogenic receptor that enhances mitogenic, migratory and invasive properties of thyroid cells (Vaiman et al., 2010), and thus the presence of xenoestrogens may also have some importance here. Certain theories attribute the progression of thyroid cancer to the invasion of stem cells coming from brain marrow, which are also sensitive to signals from estrogen, explaining the higher incidence rates in women (Bergman et al, 2012).

Endocrine disruptors that disrupt the hypothalamic-pituitary-thyroid axis are considered a potential factor in progression of thyroid cancer as well. Many of these endocrine disruptors act directly or indirectly on the thyroid gland, disrupting the synthesis, secretion and metabolism of the thyroid hormones (Boas et al, 2006). Overall, the mechanisms proposed for the progression of thyroid cancer are heterogeneous in nature and can be due to genetic, epigenetic and other factors.

Testicular cancer

Testicular cancer is a mostly European and Australian disease, with incidence rates doubling in the past four decades among Caucasians (Bergman et al, 2012). Testicular

dysgenesis syndrome (TDS) is a condition that leads to diminished semen quality, male urogenital tract anomalies such as hypospadias (when the urethral opening is on the underside of the penis), cryptorchidism (undescended testes), and testicular germ cell tumours, usually beginning during fetal development (Skakkebæk et al., 2007). Cryptorchidism is not the cause of testicular cancer but is a risk factor; it is 4-6 times more likely that men with cryptorchidism will develop testicular cancer (Diamanti-Kandarakis et al, 2009). The presence of toxins in the environment seems to be the major culprit; a study found that first generation immigrants tend to have incidence rates similar to their country of origin, but that their offspring had incidence rates similar to the country of immigration (Hemminki & Li, 2002). Therefore, genetics alone cannot explain the increasing rates of testicular germ cell cancer.

Most studies testing endocrine disruptors *in vivo* show that phthalates, which are frequently found in plastics, are the main cause of hormonally-activated testicular dysgenesis syndrome (TDS) (Foster, 2005), but other endocrine disruptors such as anti-androgens (Hotchkiss et al, 2010) or prostaglandin synthesis inhibitors (Kristensen et al, 2010) can also induce testicular dysgenesis syndrome. Vinclozolin, while not an anti-androgen, produces two main metabolites which are androgen receptor antagonists (Kelce et al, 1995). DEHP and DBP are also anti-androgenic but can decrease testosterone production without affecting the androgen receptors (Wilson et al, 2008). Organochlorines have also been considered to induce TDS. Although they do not always present symptoms in the short-term, organochlorines have been found in the blood of mothers who were exposed to the chemical thirty years ago, explaining the testicular cancer present in their sons (Hardell et al, 2006).

Overall, the incidence of cancers attributed to endocrine disruptors – breast, endometrial, prostate and thyroid - are steadily increasing worldwide, and while most research has so far theorized that genetic factors are at fault, this does not seem to be the case. Epigenetic, environmental and chemical factors are clearly playing an important role, but have yet to be studied in depth. To date, the research on endocrine disruptors and their relation to hormonally-related cancers have been very specific, focusing on a single toxin or chemical and ignoring the plethora of combined effects of other toxins. A holistic method of approaching the problem should be considered, as most endocrine disruptors are present not as a singular unit, but as a family of related toxins, with multitudinous metabolites and side reactions with unrelated chemicals. The present study focuses specifically on the effects of EDCs in terms of testicular cancer.

Phthalates

One of the most commonly studied EDCs present in the environment are phthalates, which are man-made chemicals termed plasticizers that are used in several industries (Liang, Zhang, Fang, & He, 2008). Due to their extensive use in multiple consumer production industries and their ubiquity, they can spread easily through the environment thanks to their low volatility. Phthalates thus pose a great concern to the health and wellbeing of all individuals, especially vulnerable groups such as infants and expectant mothers.

Modes of exposure to phthalates

DEHP, di-isononyl phthalate (DiNP) and di-n-octyl phthalate (DnOP) are examples of high molecular weight phthalates that are used to soften PVC, which is then used to make many consumer products such as food packaging, flooring, wall coverings, children's toys and medical devices. Diethyl phthalate (DEP) and DBP are examples of low molecular weight phthalates that are used in perfumes, lotions, cosmetics, solvents, lacquers, varnishes, and the enteric coatings of pills (Chen et. al., 2012; Fromme et. al., 2002). The enteric coatings on pills allow the release of the bioactive ingredients in the small intestines or the colon, but can consist of DEP and DBP (Hauser et al, 2004). Due to these domestic uses, it is easy to be exposed to phthalates from non-occupational sources. They are present in urinary catheters, intravenous needles, foods such as cereals, biscuits, breads, oils, fats, cakes, nuts, and virtually all cheap packaging (Bajkin et al, 2014). They are not covalently bound to plastics and thus higher temperatures can increase their volatilization, causing their leakage from plastic into the surrounding environment (Yao et al, 2012; Chen et al, 2012). As a result, they are found in air, soil sediments, and water, although they are most commonly seen in sewage and sewage sludge (Chen et. al., 2012). Despite being dispersed widely throughout the environment, researchers in China have found the most common route of exposure to phthalates to be through dietary intake, accounting for 90% of all exposure; similar conclusions have been made in Canada and Germany (Chen et. al., 2012).

Phthalate exposure in human populations

Health Canada's Tolerable Daily Intake (TDI) for phthalates is set at 44 ug/kg (Chen et. al, 2012). Daily exposure to Canadians is thought to be between 5.8 ug/kg – 19.0 ug/kg for adults

and 8.9 ug/kg – 23.1 ug/kg for children due to their increased exposure to phthalates through toys (Meek & Chan, 1994). In fact, the TDI is considered to be increased in children up to 20-fold, which is highly concerning (Koch et al, 2003). Although these measures may put Canadians well under the TDI, these statistics may be misleading because they do not include exposure through foodstuffs, which, as previously discussed, are the most common route (Meek & Chan, 1994). As such, Meek and Chan believe that if statistics were truly comprehensive, Canadian exposure levels would be right around the TDI (1994). For the most widely used phthalates, different sources specialize in different exposures: dimethyl phthalate (DMP) is most frequently found in indoor air, DEP in personal care products like shampoos, and DBP and DEHP in food (more than 95%) in adults and ingestion of dust and mouthing on toys in toddlers (20-30%).

Adverse health effects of phthalates

There is a lack of research on the adverse effects on human health from low-dose, long-term phthalate exposure. Most research is short-term, *in vitro* or *in vivo*. The majority of studies have found toxic effects upon the reproductive and endocrine systems in both males and females. Research suggests that phthalates exert a possible effect on neurocognitive development and the development of allergies, asthma, insulin resistance, obesity, thyroid dysfunction and hepatic, renal and testicular carcinomas (Swan, 2008). The duration of pregnancy has also been shown to be affected by phthalates, via interleukin-1 connecting the chemical structure of phthalates to prostaglandin/thromboxane and leading to intrauterine inflammatory processes (Latini et al, 2003). Other negative impacts on females involve anovulation and premature puberty (Bajkin et al, 2014). Phthalates may also cause defects to the androgen-signaling pathway, as well as

leading to testicular dysgenesis syndrome which includes cryptorchidisms, hypospadias, impaired spermatogenesis and testicular cancer.

Among all phthalates, DEHP is the most abundant in the environment and also the most widely used phthalate in most industries (Inada et al, 2012). It is well absorbed by the body when swallowed or inhaled, and is rapidly metabolized to more than thirty metabolites by enzymes in the liver, kidneys, lungs, intestine and pancreas (Yang et al, 2015). MEHP is the primary active metabolite of DEHP, and it is formed from the hydrolysis of DEHP in the gastrointestinal tract (Koch et al, 2005). Although MEHP is the most active and toxic metabolite of DEHP (Pollack et al, 1985), being over 10-fold more potent than its parent metabolite (Hong et al, 2009), data on human exposure to MEHP is limited, with most studies focusing instead on its parent metabolite DEHP or other phthalates, and on rodents or other mammals (Guibert et al, 2013), and studies examining the epigenetic mechanisms affected by MEHP are also severely limited.

Metabolites of DEHP

Phthalates do not bioaccumulate and have a relatively short half-life (less than 24 hours), and detecting their presence in saliva, serum, semen, meconium and placental fluid has proved futile, however, they can be detected in urine, maternal milk, serum and amniotic fluid (Bajkin et al, 2014). Urine sampling in particular is highly effective for determining the concentrations of the various metabolites of phthalates within a short period of time, as well as being a non-invasive technique. The detection of monoesters of phthalates in the urine is generally easier, as the levels are higher than the level of diesters (Bajkin et al, 2014). Polar and low-molecular weight phthalates such as DBP metabolize to their monoesters, and are excreted in urine,

whereas higher-molecular weight phthalates such as DEHP are hydrolyzed first to their respective monoesters and then further metabolized to hydrophilic oxidative metabolites (Heudorf et al, 2007). Monoester phthalates have been found in *in vivo* and *in vitro* studies to be biologically more active than their diester counterparts. In the case of DEHP, MEHP can be considered an environmental contaminant of its own, even though it represents only a small portion of a DEHP dose. Koch et al (2005) examined the half-lives of all of DEHP's components, and found that 67% of the DEHP dose consisted of its major metabolites with varying half-lives: 5-OH-MEHP (23.3%, 10h), 5cx-MEPP (18.5%, 12-15h), 5oxo-MEHP (15.0%, 10h), MEHP (5.9%, 5h) and 2cx-MMHP (4.2%, 24h). Despite MEHP's short half-life and relatively small percentage in DEHP's metabolites, it has been suggested that it remains DEHP's strongest toxicant.

GENES ASSOCIATED WITH TESTICULAR CANCER

Genome-wide association studies (GWAS) have provided genetic insight into the many mechanisms that lead to the development of testicular cancer. Studies that have conducted genome-wide methylation assays on testicular cancer tissues have identified hundreds of genes that are hypermethylated in testicular cancer patients, at times more frequent in non-seminomas and sometimes in seminomas (Brait et al, 2012; Ellinger et al, 2009; Cheung et al, 2016). For example, $p16^{INK4}$(MTS1) is a tumour suppressor gene which codes for an inhibitor of cyclin-dependent kinase 4 (CDK4), a protein kinase that is important for cell cycle G1 phase progression (Chaubert et al, 1997). The $p16^{INK4}$ protein negatively regulates cell proliferation, and thus if it is silenced, CDK4 is allowed to bind to cyclin D which stimulates cell cycle progression. It has been shown that exon 1 of $p16^{INK4}$ is hypermethylated in 50% of TGCTs (Chaubert et al, 1997), suggesting that $p16^{INK4}$ inactivation can play a role in testicular cancer cell proliferation.

The KIT gene encodes a receptor tyrosine kinase, which is a protein critical for growth factor, cytokine and hormone reception. The ligand for KIT is known as KITLG, and together they form a system that regulates the survival, proliferation and migration of germ cells (Boldajipour & Raz, 2007). KIT/KITLG activate the mitogen-activate protein kinase pathway which is involved in cell proliferation, differentiation, motility, stress response, apoptosis and survival (Sasaki et al, 2003). The protein encoded by the gene SPRY8 inhibits this pathway and is therefore antagonistic to KIT/KITLG. KIT/KITLG also inhibits the expression of the protein encoded by BAK1, an apoptosis-promoting protein (Yan et al, 2000). Single nucleotide polymorphisms (SNPs) have been found KIT/KITLG, SPRY8 and BAK1 from GWAS studies of

TGCTs (Rapley et al, 2009), suggesting that this signalling pathway is critical to the development of testicular cancer progression.

The DAZL gene encodes a protein expressed solely in the testis and has been shown to play a critical role in the initial differentiation of human primordial germ cells (Kee et al, 2009). The gene is testis-specific, and mice with DAZL (-/-) are infertile and lack spermatozoa (Kee et al, 2009). PRDM14 is another such gene which encodes a transcriptional regulator that uses histone methylation in primordial germ cell specification. PRDM14 is critical for the reactivation of pluripotency and the genome-wide epigenetic reprogramming that occurs in the early specification of embryonic cells into primordial germ cells (Kurimoto et al, 2008).

The Hypermethylated In Cancer 1 (HIC1) gene is yet another tumour suppressor gene which regulates growth and is associated with Miller-Dieker syndrome, medulloblastoma, breast cancer and esophageal squamous cell carcinoma (Koul et al, 2004). It has been shown that HIC1 has a hypermethylated promoter in testicular tumours that are highly resistant (Koul et al, 2004). In non-seminoma tumours, HIC1 is 31.9% more frequently hypermethylated than in non-tumour tissues, and is 47% more frequent in resistant tumours vs. 24% in sensitive tumours (Koul et al, 2004). This suggests that the gene plays a significant role in tumour sensitivity for testicular cancer and other diseases.

The Adenomatous polyposis coli (APC) gene encodes a protein which negatively regulates beta-catenin and E-cadherin, both of which are involved in cell adhesion. The APC gene is associated with several cancers but notably testicular cancer where it suffers a loss of

heterozygosity (Peng et al, 1995) and increased hypermethylation in testicular tumours (Koul et al, 2004).

A study by Cheung et al (2016) examined the methylation profiles for six embryonal carcinomas and identified 40 genes with hypermethylated promoters, suggesting they are transcriptionally repressed. Several of these genes were sex-linked, with one gene being testis specific, RBMY1A1, binds RNA and is critical for spermatogenesis. After RT-qPCR, the RBMY1A1 was shown to be downregulated in germ cells and seminomas, as well as other sex-linked genes, suggesting that the epigenetic alterations in these genes play a role in testicular cancer progression.

Despite the growing list of genes associated with testicular cancer progression, the genes most frequently examined in genome-wide association studies are MGMT, PRSS21, RASSF1A and GSTP1 (Kawakami et al, 2003; Chen et al, 2013; Ellinger et al, 2009; Brait et al, 2011; Honorio et al, 2003; Lind et al, 2007; Koul et al, 2002). Each of these genes play a critical role in testicular cancer progression and have various roles in normal cellular function. They have also been identified as genes critical to testicular cancer progression, and thus they were focused upon in the present study.

RASSF1A

Ras-association domain family member 1 (RASSF1) is a gene family which is capable of generating eight alternative transcripts, noted as RASSF1A-H (Donninger et al, 2007). Of these, RASSF1A and RASSF1C are the two major isoforms, and both originate from separate

promoters with respective CpG island regions (Hesson et al, 2007). RASSF1A is frequently silenced by hypermethylation in lung, breast, bladder, gastric, cholangiocarcinoma and esophageal carcinomas, among others (Hesson et al, 2007). It has thus become one of the most extensively researched tumour suppressor genes. RASSF1A regulates apoptosis, cell cycle arrest, and tumour-like behavior, thus its silencing can result in a loss of cell cycle control, increased genetic instability, enhanced cell invasion and resistance to tumour necrosis factor (TNF)-induced apoptosis (Donninger et al, 2007).

Previous studies have noted that *de novo* CpG promoter methylation of RASSF1A is associated with transcriptional silencing, but expression may be restored with the use of demethylating agents (Honorio et al, 2003). Data on RASSF1A's methylation status is varied; some studies place the gene in the 30-40% methylation frequency range, while others place it near 0 or 100%. These vast differences may be due to the methods used to detect methylation as well as the type of tumour or cell line. Sodium bisulfite modification is frequently used for methylation analysis; however as Ellinger et al (2009) point out, approximately 90% of DNA is degraded during this process, limiting the detection of DNA to only a few copies.

Ellinger et al (2009) used TGCT tissues from German patients and used MSRE, or methylation-specific restriction enzymes, on 3 methylation specific sites (Bsh1236I, HpaII and HinP1I) that would amplify a sequence only if all covered restriction sites were methylated. The results yielded a 47% methylation frequency for the gene. Honorio et al (2003) meanwhile used traditional sodium bisulfite modification on paraffin-embedded archival tissues between 1 month to 10 years old, consisting of yolk sac tumours, embryonal carcinomas, mature and immature

teratomas and choriocarcinomas. Honorio et al (2003) indicate the methylation frequency of RASSF1A to be 71%, though it is higher in non-seminoma (83%) than seminoma (40%). They posit that TGCT is a multistep process that begins with the development of carcinoma in situ (CIS) and progresses to non-seminomatous TGCT, with seminomas as an intermediate stage. As such, the methylation frequency should also increase in this order, per their theory.

Koul et al (2004) identified tumour tissues obtained between 1987 and 1999 for retrospective review, used unselected TGCTs, as well as non-seminoma cell lines for their study. Like Honorio et al (2003), they too used MSP with sodium bisulfite treatment prior to PCR and obtained a methylation frequency of 35.7%. RASSF1A is known to be epigenetically inactivated in many tumour types and thus accounts for this relatively low methylation frequency. Interestingly, RASSF1A was more frequently observed in resistant tumours (52%) than sensitive tumours (28%), suggesting that RASSF1A hypermethylation is associated with the resistance phenotype.

MGMT

O^6-methylguanine-DNA methyltransferase (MGMT) is an important DNA repair enzyme encoded by a gene of the same name that repairs *O*^{6}-alkylguanine, making MGMT an important defense against the carcinogenic affects of *O*^{6}-alkylguanine adducts (Bhakat & Mitra, 2000). MGMT facilitates DNA repair by removing the alkyl groups on O^6 guanine, transferring it to a cytosine residue within its own catalytic pocket and essentially inactivating itself. Thus MGMT has been labeled as a suicide enzyme, being capable of only functioning once (Nagel et al., 2003). MGMT's promoter methylation status can be used to predict patient survival for

prediction models in clinical settings (Molenaar et al, 2014). MGMT may have a role in down-regulating many cancers, since its loss is seen in several tumour types such as glioma, lymphoma, breast and prostate (Sharma et al, 2009). This is generally thought to be due to promoter methylation. In testicular cancer, MGMT hypermethylation has been shown to be more strongly associated with non-seminomas compared to seminomas, showing as much as 44% methylation in non-seminomas (Smith-Sorensen et al, 2002). Therefore gene silencing of MGMT through hypermethylation at the promoter region may be a key event in the development of testicular cancer (Smith-Sorensen et al, 2002).

GSTP1

Glutathione S-transferase pi (GSTP1) is part of a family of powerful detoxifiers of carcinogenic electrophiles (Harries et al, 1997) that has a prominent role in oncogenesis, tumourigenesis, apoptosis, DNA repair drug resistance and cell signaling (Kraggerud et al, 2009). The gene is generally overexpressed in many neoplasms as well as in anticancer drug-resistant tumour cells, but can be inactivated by hypermethylation in cancers such as prostate (Harries et al, 1997). Hypermethylation of GSTP1 has been observed in testicular lymphoma tissues but not in TGCT tissues which may unveil a particular epigenetic phenotype between the two neoplasms (Kawakami et al, 2003).

PRSS21

PRSS21, which codes for the Testisin gene, was first identified as a serine protease in 1999 (Hooper et al., 1999). Testisin is highly expressed only in the testis, but loses its expression

in TGCT (Kempkensteffen et al, 2006), thus it is thought to be a tumour suppressor. Testisin is a member of the family of serine proteases, which are implicated in biological functions such as blood coagulation and wound healing, although they are most studied for their roles in tumourigenesis (Antalis et al, 2010, Netzel-Arnett et al, 2003). This group of proteolytic enzymes are responsible for peptide bond cleavage and make use of a catalytic triad of Ser (serine), Asp (asparagine) and His (histidine) to catalyze this hydrolysis (Di Cera, 2009). Testisin is located at chromosome 16p13.3, a region known to be susceptible to mutations, deletions and rearrangements in testicular cancers (Hooper et al, 1999). While the physiological function of Testisin is not yet known, it has been suggested that it may have a proteolytic role critical for mature germ cell migration in the seminiferous tubules, as well as releasing specific factors that coordinate spermatogenesis (Hooper et al, 1999). These events are essential for germ cell maturation, thus the loss of Testisin may lead to immature germ cells and unregulated differentiation and proliferation (Hooper et al, 1999).

CHAPTER II – RESEARCH QUESTIONS & OBJECTIVES

RATIONALE

As discussed, testicular cancer is the most commonly diagnosed male cancer worldwide, and its incidence has been rapidly increasing for the past five decades in western countries. We have also seen how EDCs can disrupt the body's normal hormonal biosynthesis and metabolism and permeate into the consumer products within our homes. Among EDCs, phthalates are well-known toxicants used as plasticizers for PVC production, and the most common route of exposure in adults is through food and food packaging. However, children are the most vulnerable population, being exposed through infant toys, baby bottles and other such products.

Among all phthalates, DEHP is the most abundant in the environment and also the most widely used phthalate in most industries (Inada et al, 2012). It is well absorbed by the body when swallowed or inhaled, and is rapidly metabolized to more than thirty metabolites by enzymes in the liver, kidneys, lungs, intestine and pancreas (Yang et al, 2015). MEHP is the primary metabolite of DEHP, and it is formed from the hydrolysis of DEHP in the gastrointestinal tract (Koch et al, 2005). It has been shown to have reproductive (Lovekamp-Swan & Davis, 2003) and hepatic toxicity, as well as causing decreased testicular weight and testicular atrophy in rodents fed high doses of MEHP (McKee et al, 2004). It can also induce spermatogenic cell apoptosis in guinea pigs (Awal et al, 2005), and when compared to DEHP, MEHP has also shown dose-dependent DNA damaging properties (Tomita et al, 1982).

Although MEHP is the most active and toxic metabolite of DEHP (Pollack et al, 1985), being over 10-fold more potent than its parent metabolite (Hong et al, 2009), data on human

exposure to MEHP is limited, with most studies focusing instead on its parent metabolite DEHP or other phthalates, and on rodents or other mammals. MEHP may even play a cytotoxic or epigenetic role in the progression of testicular cancer, but this has not been properly examined in the past.

Embryonal carcinomas represent around 87% of all non-seminoma tumours (Bosl & Motzer, 1997) and while there are several embryonal carcinoma cell lines which have been established, the Ntera-2/cl.D1 (NT2) cell line is most frequently used as a cell model to study TGCT (Cheung et al, 2010). MEHP has been shown to promote invasion and migration in these cells (Yao et al, 2012), however its proliferative and epigenetic role on NT2 cells has never before been examined. MEHP exposure may cause increased proliferation as well as epigenetic modifications such as DNA hypermethylation leading to gene expression changes in specific genes. *PRSS21, GSTP1, RASSF1A* and *MGMT* are genes that may be responsible for protecting the integrity of the testicular genome against the onslaught of exposure to phthalates, and exposures to MEHP may hypermethylate and silence the expression of these genes. Thus the objective of this study is to determine if MEHP has a proliferative and epigenetic role in the development of testicular cancer.

OBJECTIVES

Objective 1 – To examine the cell proliferation effects of MEHP

To characterize the time- and dose-dependent toxicity profile of NT2 cells from exposure to MEHP, NT2 cells were grown and analyzed both in Petri dishes and in real-time using new technologies that measure cell proliferation as a function of cell index. The cells were exposed to different doses of MEHP at different time points to examine if proliferation was affected following exposure.

Objective 2 – To examine the epigenetic alterations of genes of interest by MEHP in testicular cancer

To determine the methylation profile of the tumour suppressor genes affected by MEHP exposure in NT2 cells, methylation-specific PCR was used to determine the methylation level of the key genes. This method uses restriction enzymes to digest unmethylated DNA, thus allowing the user to differentiate between methylated and unmethylated DNA as well as calculating the methylation level of a particular gene.

CHAPTER III – METHODOLOGY

Cell culture

NTera 2/cl.D1 (NT2) pluripotent human testicular embryonal carcinoma cell line was purchased from American Type Culture Collection (Manasass, VA). The cells were grown in 58 cm2 culture flasks and maintained at 37°C in a 5% CO2 atmosphere with complete Dulbecco's modified Eagle's medium (DMEM) purchased from Wisent Bioproducts (St-Bruno, QC) and completed with 10% FBS (Sigma, Oakville, ON) and 1% Penicillin/Streptomycin (Invitrogen, Burlington, ON) until 80-90% confluency after which they were trypsinized with 0.05% Trypsin in 0.53mM EDTA (Wisent). Cell viability was determined by 0.2% Trypan blue dye exclusion in Countess I Automated Cell Counter (Invitrogen, Burlington, ON).

iCelligence

The iCELLigence-system™ (ACEA Biosciences, CA) was used to monitor cell growth of NT2 cells undergoing exposure to MEHP in real-time. NT2 cells were maintained identically to those grown in petri dishes but with a smaller seeding density to match the size of the wells. The system allows for the analysis of cells continuously over time, monitoring cell growth, proliferation, cytotoxicity, adhesion, morphology changes and barrier functions in real-time. The system involves an analyzer that can fit electronic plates with gold-plated wells and microelectrode sensors which measure impedance in each well. As more cells grow and adhere to the bottom of the wells, the ionic exchange between the cell media and the microelectrodes is blocked, producing a signal known as impedance. This is expressed as cell index (CI) which is a

measurement of proliferation. The CI can also provide information about cell viability, number, doubling time, morphology and adhesion by analyzing this data with the Real-Time Cell Analysis (RTCA) software supplied by the manufacturer.

Exposure

Mono-(2-ethyhexyl) phthalate (MEHP) in neat form (100mg) with 99.9% purity was purchased from Accustandard (New Haven, CT). DMSO is a polar, aprotic solvent that is miscible and dissolves both polar and nonpolar compounds, especially those that have extremely poor solubility in water. As such, due to MEHP's poor water solubility, MEHP was dissolved in DMSO. The final concentration of DMSO in medium was 0.05%, which has no effect on replication or differentiation of cells (Lin et al, 2010). A working solution of 50% DMSO was first made, which was then used to make a working solution of 200mM MEHP from a stock mass of 100mg.

$$c = \frac{\frac{m}{MW}}{v}$$

$$200\ mM = \frac{\frac{100\ mg}{278.15\ g/mol}}{v}$$

$$0.2\ M = \frac{\frac{0.1\ g}{278.15\ g/mol}}{v}$$

$$0.2\ M = \frac{0.00035951\ mol}{v}$$

$$v = 0.00179\ L$$

$$v = 1.79\ ml\ DMSO\ required\ to\ dilute\ 100\ mg\ MEHP\ to\ 200mM$$

This 200mM working solution was used to make a 100mM and 10mM dilution.

To make a dilution of 100mM from the working solution:

$$c_1 v_1 = c_2 v_2$$
$$(200mM)v_1 = (100mM)(100ul)$$
$$v_1 = 50ul\ of\ MEHP\ into\ 50ul\ of\ DMSO$$

To make a dilution of 10mM from the 100mM dilution:

$$c_1 v_1 = c_2 v_2$$
$$(100mM)v_1 = (10mM)(100ul)$$
$$v_1 = 10ul\ of\ MEHP\ into\ 90ul\ of\ DMSO$$

Thus, the final exposure volumes in the plates were:

$$(100mM)v_1 = (100uM)(10ml)$$
$$v_1 = 0.01ml = 10ul\ into\ culture\ medium$$

and the final concentration of DMSO in the control medium was:

$$(50\%)(10ul) = c_2(10ml)$$
$$c_2 = 0.05\%$$

NT2 cells were exposed to MEHP in a dose- and time-dependent manner at concentrations of 10µM and 100µM. Cells were additionally exposed to DMSO with a final concentration of 0.05% in medium as it is a known methylating agent. Cells were seeded at a density of 8 x 10^5 cells/ml, and exposed after 24 hours.

All exposures were conducted for 24h, 48h, 72h (not shown) and 96h (not shown). For exposures beyond 48 hours, samples were re-exposed after 48 hours. After exposure, cells were washed with PBS, then trypsinized and placed into suspension for cell counting. The suspension

was then centrifuged at 3000g for 1 minute, then the supernatant was discarded and the pellet was washed with 10ml PBS once, then resuspended. After that, the cells were centrifuged once more at 3000g for 1 minute. The supernatant was removed and the vials were kept at -20˚C for 1 hour then stored at -80˚C until further use.

Methylation analyses

Genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Toronto, ON) according to the manufacturer's protocol. Sample concentrations and purity ratios (260/230 and 260/280) were measured using a NanoDrop spectrophotometer device (Thermofisher). DNA concentrations can be found in Table 1.

Table 1. DNA concentrations following exposure

Timepoint	Exposure	Conc. (ng/µl)	260/280
24	Media	150.10	2.01
	DMSO	141.40	2.07
	100 MEHP	130.60	2.05
	10 MEHP	141.30	2.03
48	Media	714.20	2.04
	DMSO	260.00	1.94
	100 MEHP	118.80	2.22
	10 MEHP	298.00	2.19

DNA methylation quantification was conducted using the One Step qMethyl™ kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol. Methylation Specific Restriction Enzymes (MSRE) digested unmethylated CpG dinucleotides, followed by RT-PCR to selectively amplify methylation DNA. MSREs cannot cleave methylated cytosine residues and thus leave methylation DNA intact while cleaving unmethylated-cytosine residues. Primers were designed by first identifying the gene sequence in NCBI and then searching for MSRE cut sites within the

FASTA sequence. Promoter regions and CpG islands were identified, following which primers were designed using PrimerQuest (IDT DNA). At least 2-4 MSRE sites were included in the amplicon, which ranged between 150-350 bp. Primers used can be seen in Table 2. MSRE sites used are listed in Table 3.

Table 2. MSRE PCR primers

Gene	Primer Sequence
GSTP1	5'- TTCGCCACCAGTGAGTA-3' 5'- CGTTAGCGGCTTTCAGGG-3'
MGMT	5'- CCGGATATGCTGGGACAG-3' 5'- TTGGTGAGTGTCTGGGTC-3'
PRSS21	5'- GGCCTTTACTGCTCTCT-3' 5'- CAGTTCCTCTGACCATCC-3'
HPRT1	5'- CCTCAGGCGAACCTCTCGGCTTTC-3' 5'- CCGGGTTCGGCTTTACGTCACGCG-3'

Table 3. MSREs present in test reaction mix

Gene	MSRE Sequence
AccII	5'-C G | C G-3' 3'-G C | G C-5'
HpaII	5'-C | C G G-3' 3'-G G C | C-5'
HpyCH4IV	5'-A | C G T-3' 3'-T G C | A-5'

DNA was split into "test" and "reference" reactions; "test" reactions were digested with MSRE (AccII, HpaII and HpyCH4IV) while "reference" reactions were not. In unmethylated DNA, the test reaction was mock digested and resulted in limited amplification, producing largely different Ct values than the reference. In methylated DNA, the test and reference reactions both resulted in robust amplification and similar Ct values. The master mix reactions can be seen in Table 4.

Table 4. PCR Reaction mixture quantities per well

Test Reaction	Reference Reaction
10ul Test reaction (MSREs)	10ul Reference reaction (no MSREs)
2ul Gene primers	2ul Gene primers
3ul DNase/RNase-free water	3ul DNase/RNase-free water

Once these master mixes were added to the appropriate wells, 5uL (20ng) of the respective DNA samples were added. The PCR analysis was then performed using CFX96 Real-Time PCR Detection System (Bio-Rad) and accompanying software (CFX Manager Software) according to the following protocol in Table 5.

Table 5. RT-PCR Parameters

	Temperature	Time
MSRE Digestion	37˚C	2 hrs
Initial Denaturation	95˚C	10 min
Denaturation	95˚C	30 sec
Annealing	54˚C	60 sec
Extension	72˚C	60 sec
Final Extension	72˚C	7 min
Hold	4˚C	> 5min

Methylation status was determined by comparing differences between Ct values.

Statistical analysis

All statistical analyses were conducted using GraphPad (PRISM 7) software. An independent samples test (t-test) was conducted to determine the significance between MEHP exposures and controls for proliferation. For methylation, two-way analysis of variance (ANOVA) was conducted to compare the means between 1) controls and MEHP exposures, and 2) 24 and 48 hours. An association was considered statistically significant with P-value <0.05.

All P-values reported are two-sided. Data are represented as means ± standard deviation (SD) of 4 repeated measures per sample. Odds ratios (OR) were estimated with 95% confidence intervals (CI), which quantified the strength of the association and its uncertainty.

For the iCelligence data, the instructions from the RTCA manual were used. Proliferation was subjectively measured as Cell Index (CI), a unitless parameter that measures the relative change in electrical impedance to represent cell status. The CI is a relative and dimensionless value since it represents the impedance change divided by a background value. When there are no cells present in the medium, the sensor's electronic property will not be affected and the impedance will be 0, or Z_0. When there are more cells on the electrodes, the impedance will be larger. The CI calculation is based on the following formula:

$$\mathbf{CI = (Z_i - Z_0)/15\ \varsigma}$$

where Z_i is the impedance at an individual point of time during the experiment, and Z_0 is the impedance at the start of the experiment. Thus CI is a self-calibrated value derived from the ratio of measured impedances. When cells are not present or are not well-adhered to the electrodes, then the CI is about zero. Under the same physiological conditions, when more cells are attached on the electrodes, then the CI values are higher. In this case, CI is a quantitative measure of the number of cells present in a well. Additionally, a change in cell status, such as cell morphology, cell adhesion or cell viability can lead to a change in CI.

Initial methylation analyses were conducted following the One Step qMethyl kit's instructions. The methylation level for amplified regions was determined using the following equation:

$$\textbf{Percent Methylation} = 100 \times 2^{-\Delta Ct}$$

where ΔCt = the average Ct value from the Test Reaction minus the average Ct values from the Reference Reaction. For example, if the average Ct value of the test reaction was 33.82, and that of the reference reaction was 29.79, the ΔCt would be calculated by:

$$\Delta Ct = 33.82 - 29.79 = 4.03$$

following which it would be substituted into the equation $100 \times 2^{-\Delta Ct}$

$$100 \times 2^{-4.03} = 6\%$$

CHAPTER IV – RESULTS

NT2 cell viability over time

Cell viability was established using Countess™ viability information at every passage. The viability is determined by dividing the number of live cells by the number of total cells. Viability over 4 months stayed in the range of 90-100% (Figure 1).

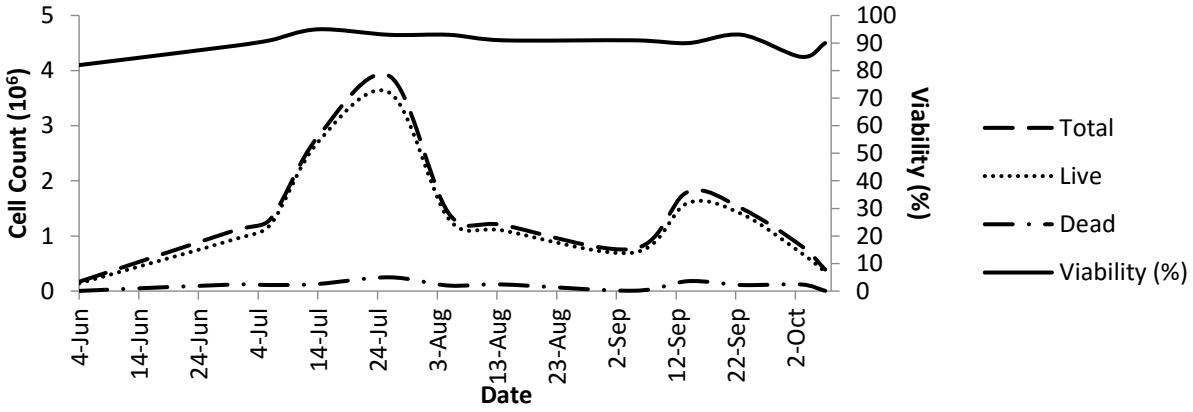


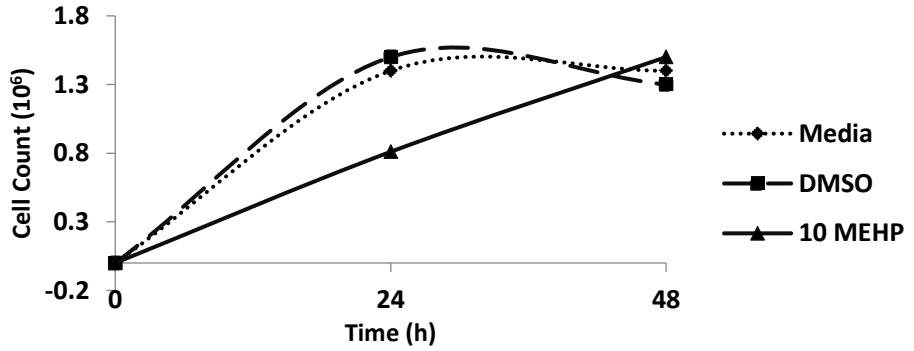
FIGURE 1. *NT2 cell viability over 4 months, shown as total cell count, as well as the proportion of live to dead cells. Viability is shown on the secondary axis. Cell numbers were measured using Countess™.*

MEHP's effect on testicular cancer cell proliferation

To assess whether MEHP had any effect on proliferation, NT2 cells were grown in both petri dishes and iCelligence. The Petri dish cultures were seeded in duplicate and exposed to 10µM and 100µM MEHP, after which they were harvested every 24 hours up to 48 hours. Cell suspensions during trypsinization were used to determine cell count using Countess, and numbers shown are an average of duplicate dishes (Figure 2). Controls used were cells in media (shown as Media) and cells in 0.05% DMSO (shown as DMSO).

MEHP was shown to decrease proliferation in the short-term at 24 hours by about half as compared to controls, but then recuperated and gained similar cell counts to controls by 48 hours. 10µM and 100µM MEHP both decreased proliferation by 58% compared to Media and 54% compared to DMSO. However, both recuperated at 48 hours with 10µM MEHP increasing to 107% of Media and 115% of DMSO, and 100µM MEHP increasing to 92% of Media and 100% of DMSO.

A) 10µM MEHP



B) 100µM MEHP

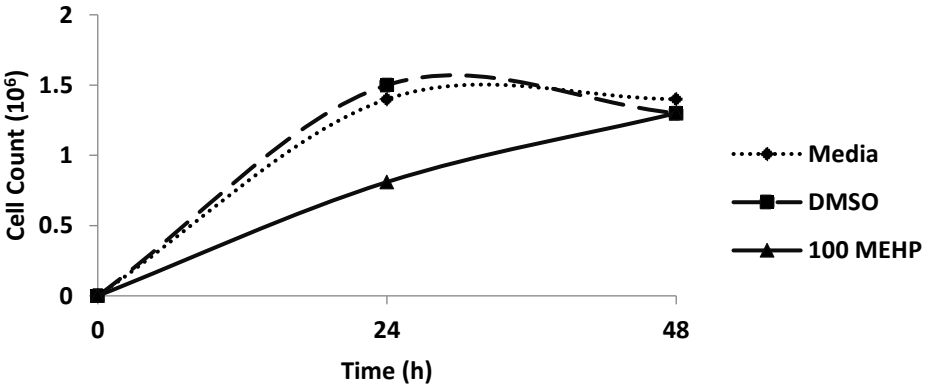


FIGURE 2. *NT2 cell proliferation over 48 hours following exposure to A) 10µM and B) 100µM MEHP, with media and DMSO as control. Cell numbers were measured using Countess™ and observed at the same time every 24 hours.*

The iCelligence cultures were grown identically to the cultures in Petri dishes but were kept for a longer period of 96 hours. Cell proliferation is shown as a function of cell index (CI), which is determined automatically by iCelligence software by measuring impedance. Cells were harvested every 24 hours up to 96 hours. There was no significant change in proliferation for cells exposed to 10µM MEHP (Figure 3A). Exposure to 100µM MEHP (Figure 3B) caused a marked decrease in proliferation, especially after 72 hours.

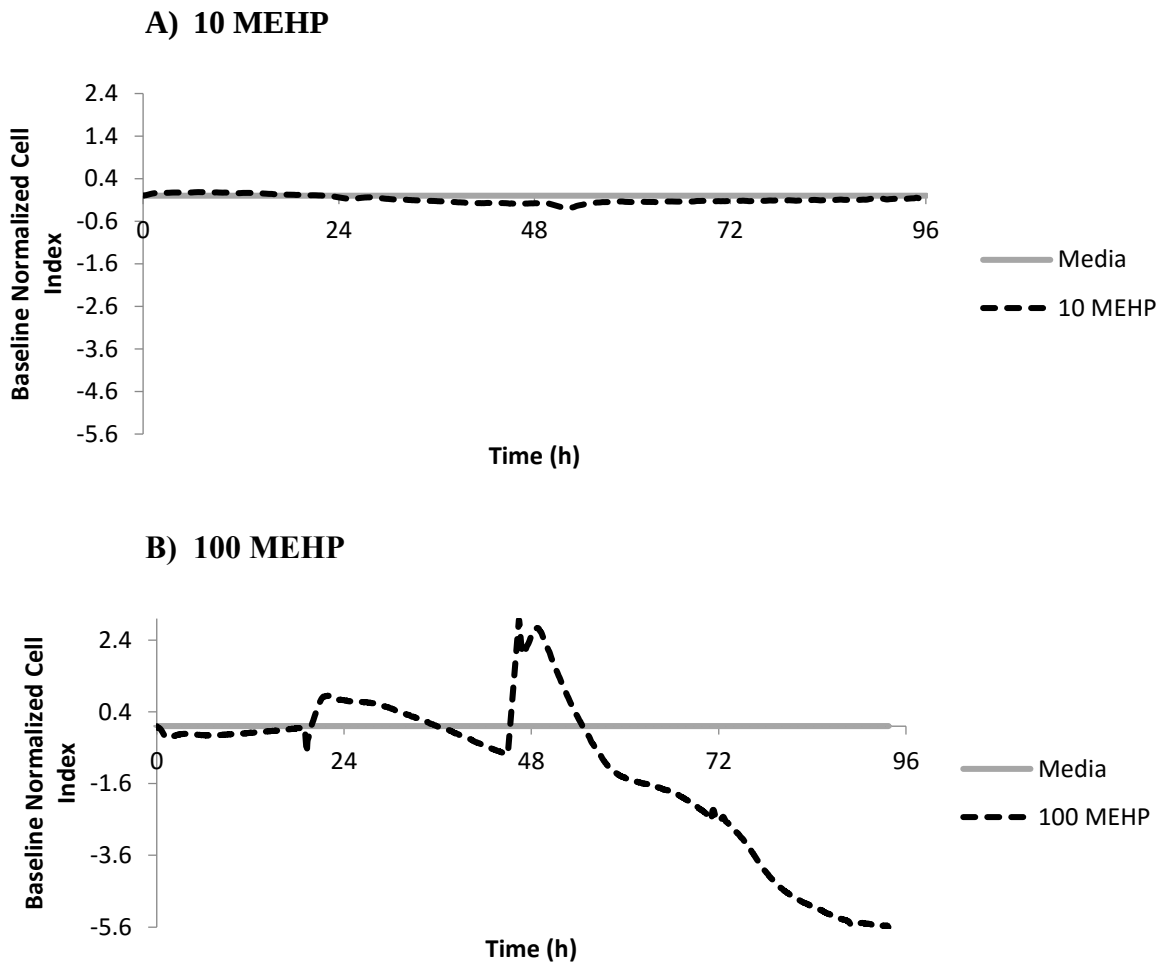


FIGURE 3. *NT2 cell index measured by iCelligence software, over 96 hours following exposure from A) 10µM, and B) 100µm MEHP, with media used as a control. The average of eight wells was taken and corrected by line fit for each exposure.*

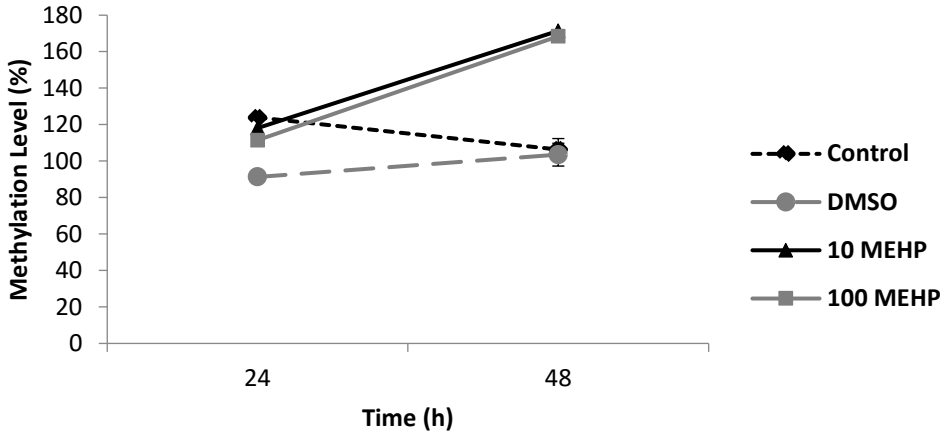
Genes used

RASSF1A did not respond to our methylation primers and was thus excluded from the rest of the study. HPRT1 was thus used as our fourth gene and also served as our housekeeping gene.

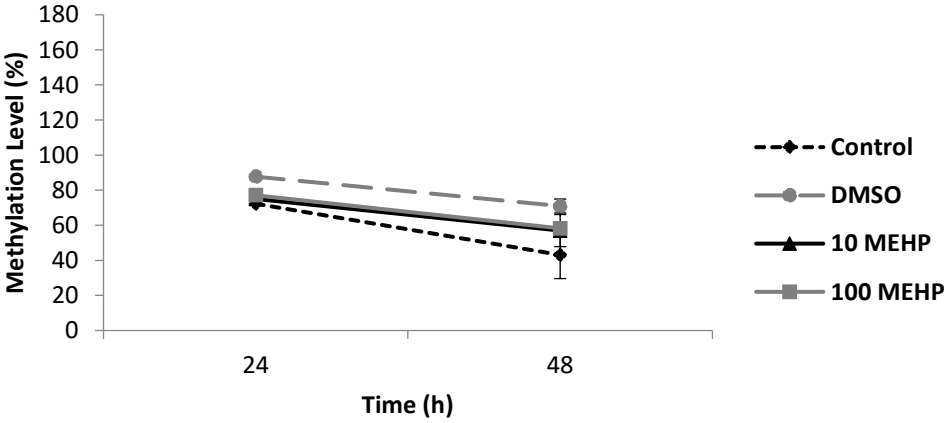
MEHP's effect on methylation level in testicular genes

To determine the effect of MEHP on key genes associated with testicular cancer, the methylation levels of *GSTP1, MGMT, PRSS21* and *HPRT1* after time- and dose-dependent exposures of MEHP were determined (Figure 4). MEHP increased the methylation level in all four genes relative to at least one of the controls, but some of these were not significant. GSTP1 (Figure 4A) showed increased methylation levels for both 10μM (171% ± 12.25) and 100μM MEHP (168% ±1.96) at 48 hours compared to Media (106% ± 9.42) and DMSO (103% ± 12.25), but not at 24 hours. MGMT did not show any significant increases in methylation (Figure 4B). PRSS21 showed increased methylation levels at 24 hours for both 10μM (125% ± 1.15) and 100μM MEHP (123% ± 0.51) compared to DMSO (71% ± 2.87), but not to Media, as well as increased levels at 48 hours for both 10μM (110% ± 3.18) and 100μM MEHP (87% ± 9.21) compared to DMSO (65% ± 2.58), but not to Media (Figure 4C). HPRT1 showed decreased methylation levels at 24 hours for both 10μM (129% ± 1.26) and 100μM MEHP (69% ± 3.39) compared to Media (138% ± 1.35), but not to DMSO, as well as decreased levels at 48 hours for both 10μM (100% ± 9.80) and 100μM MEHP (81% ± 3.19) compared to Media (150% ± 2.06), but not to DMSO (Figure 4D).

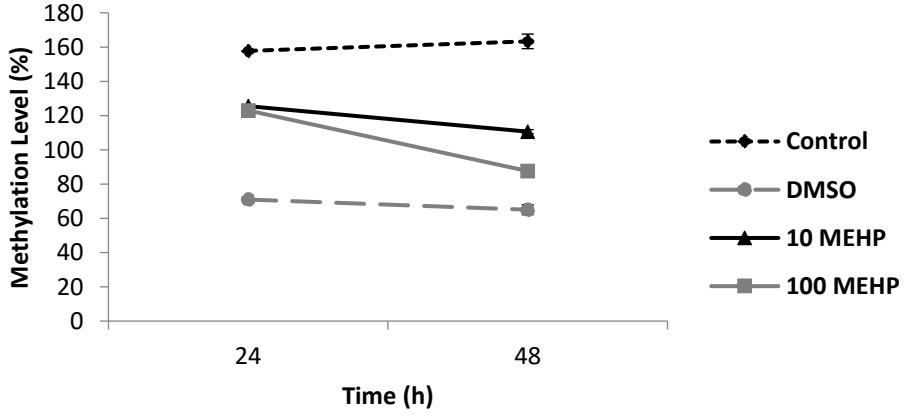
A) GSTP1



B) MGMT



C) PRSS21



D) HPRT1

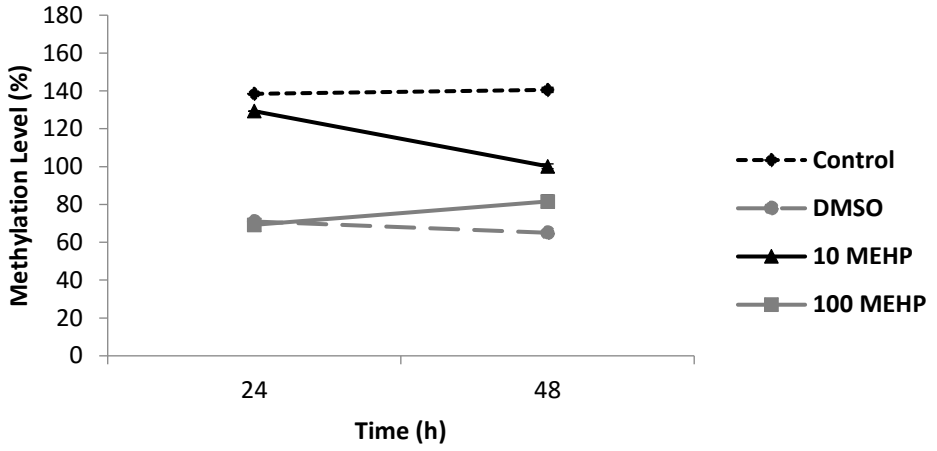


FIGURE 4. *Methylation level of A) GSTP1, B) MGMT, C) PRSS21 and D) HPRT1 for 24 and 48 hours following exposure to 10μM and 100μM MEHP. Values shown are means ± SD from 4 replicate measures.*

Dose-dependent analyses

Analysis of Variance (ANOVA) was conducted to determine the significance of MEHP dose as compared to controls. ANOVA was conducted using media and 0.05% DMSO, which was used to dissolve the MEHP, as control (Figure 5).

Media as baseline

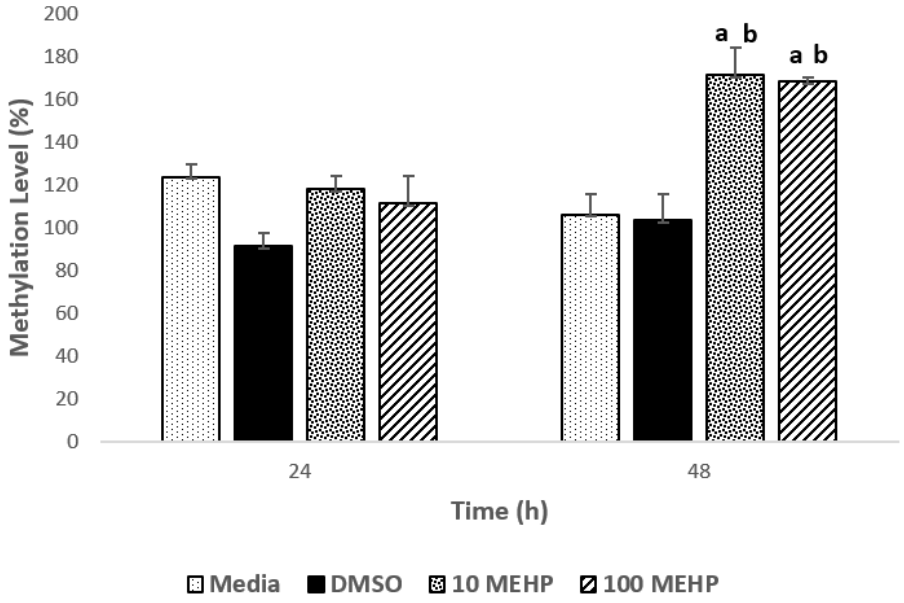
GSTP1 did not show significant differences in methylation level at 24 hours, but showed a significant increase at 48 hours for both 10μM (171% ± 12.72, $p < 0.0001$) and 100μM MEHP (168% ± 1.96, $p < 0.0001$) compared to controls (106% ± 6.03). MGMT did not show any significant differences in methylation level between exposures, but overall had the lowest methylation level among the four genes. PRSS21 showed a significant decrease in methylation at

24 hours for both 10μM (126% ± 1.15, $p < 0.0001$) and 100μM MEHP (123% ± 0.51, $p < 0.0001$) compared to controls (158% ± 4.26). PRSS21 also showed a significant decrease at 48 hours for both 10μM (110% ± 3.18, $p < 0.0001$) and 100μM MEHP (87% ± 9.21, $p < 0.0001$) compared to controls (163% ± 2.58). HPRT1 was assessed as a housekeeping gene and showed a significant decrease in methylation at 24 hours for only 100μM MEHP (69% ± 3.39, $p < 0.0001$) compared to controls (138% ± 1.35). HPRT1 also showed a significant decrease at 48 hours for both 10μM (100% ± 9.8, $p < 0.0001$) and 100μM MEHP (81% ± 3.19, $p < 0.0001$) compared to controls (140% ± 2.06).

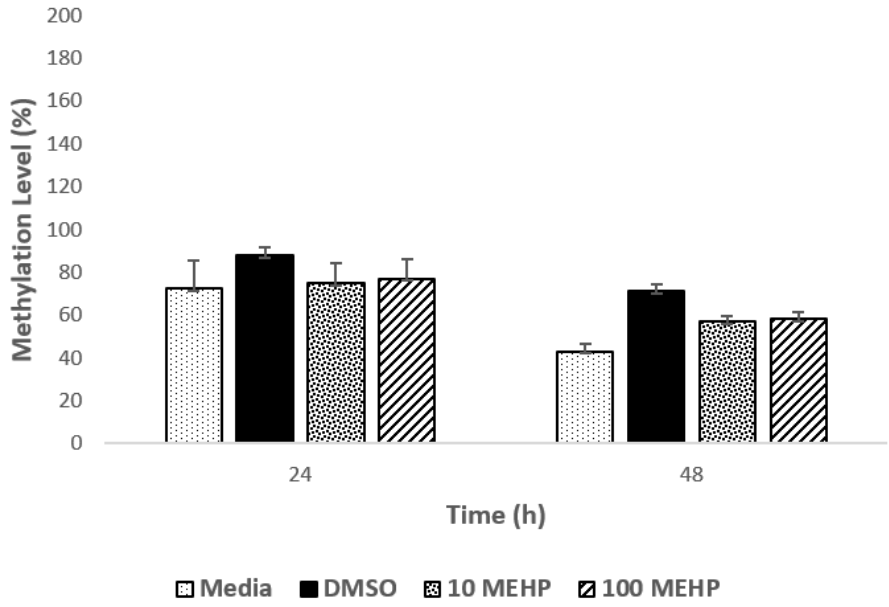
DMSO as baseline

GSTP1 showed a significant increase in methylation level at 24 hours for both 10μM (118% ± 6.42, $p < 0.0001$) and 100μM (111% ± 12.81, $p < 0.0001$) compared to DMSO (91% ± 6.24). GSTP1 also showed a significant increase in methylation level at 48 hours for both 10μM (171% ± 12.72, $p < 0.0001$) and 100μM MEHP (168% ± 1.96, $p < 0.0001$) compared to DMSO (103% ± 12.25). MGMT showed a decrease in methylation level for both exposures of MEHP at both time points, but it was not found to be significant. PRSS21 showed a significant increase in methylation at 24 hours for both 10μM (126% ± 1.15, $p < 0.0001$) and 100μM MEHP (123% ± 0.51, $p < 0.0001$) compared to DMSO (71% ± 3.47). PRSS21 also showed a significant increase at 48 hours for both 10μM (110% ± 3.18, $p < 0.0001$) and 100μM MEHP (87% ± 9.21, $p < 0.0001$) compared to DMSO (65% ± 2.58). HPRT1 was assessed as a housekeeping gene and showed a significant increase in methylation at 24 hours for 10μM MEHP (129% ± 1.26, $p < 0.0001$) and a decrease for 100μM (69% ± 3.39, $p < 0.0001$) compared to DMSO (97% ± 0.95).

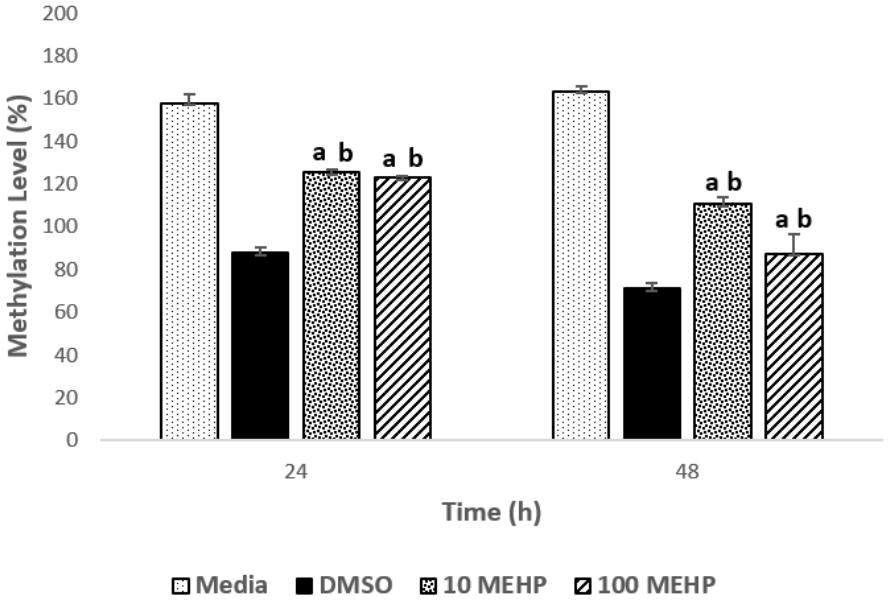
A) GSTP1



B) MGMT



C) PRSS21



D) HPRT1

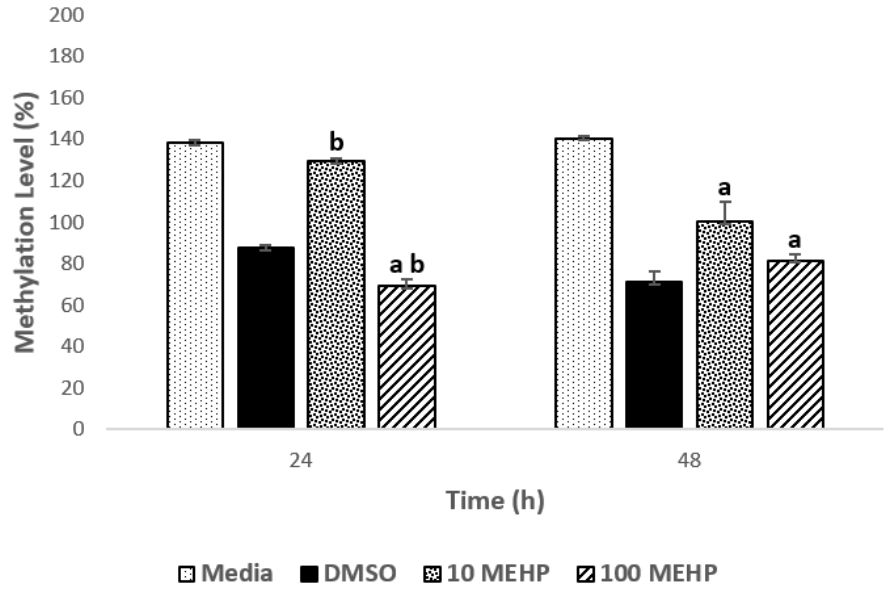


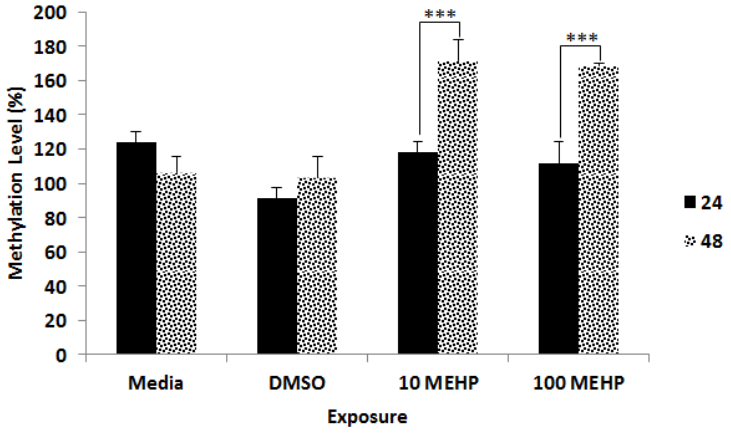
FIGURE 5*. Dose-dependent methylation level of genes associated with testicular cancer following exposure to 10µM and 100 µM MEHP for 24 and 48 hours, with media and DMSO used as control. Genes shown are A) GSTP1, B) MGMT, C) PRSS21, and D) HPRT1. Values are means ± SD. Letters denote significant differences between the treatment and control groups (a = compared to media; b = compared to DMSO, n=4, p < 0.0001; ANOVA).*

Time-dependent analyses

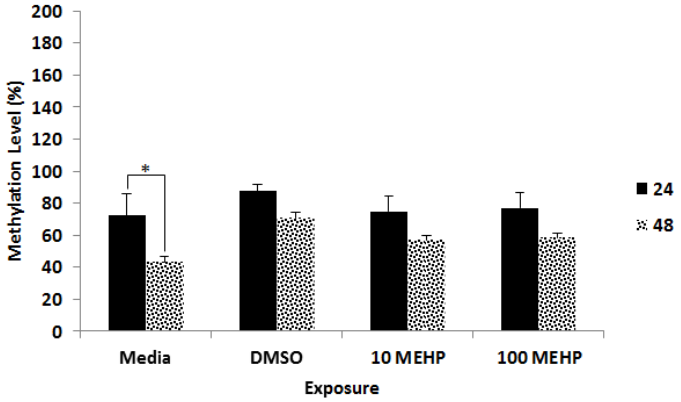
ANOVA was conducted to determine the significance of MEHP exposure over time, as compared to controls (Figure 6). ANOVA was conducted using media and with 0.05% DMSO, which was used to dissolve the MEHP, as controls.

GSTP1 showed a significant increase between 24 vs 48 hours for 10µM MEHP (118% ± 6.42 vs 171% ± 12.72) and 100µM (111% ± 12.81 vs 168% ± 1.96). MGMT did not show any significant change for MEHP, but did show a decrease in methylation for Media (72% ± 13.48 to 43%± 3.37). PRSS21 showed a significant decrease between 24 vs 48 hours for 10µM MEHP (125% ± 1.15 vs 110% ± 3.18) and 100µM (122% ± 0.51 vs 87% ± 9.21). Lastly, HPRT1 only showed a significant decrease between 24 vs 48 hours for 10µM MEHP (129% ± 1.26 vs 100% ± 9.8) but not for 100µM MEHP.

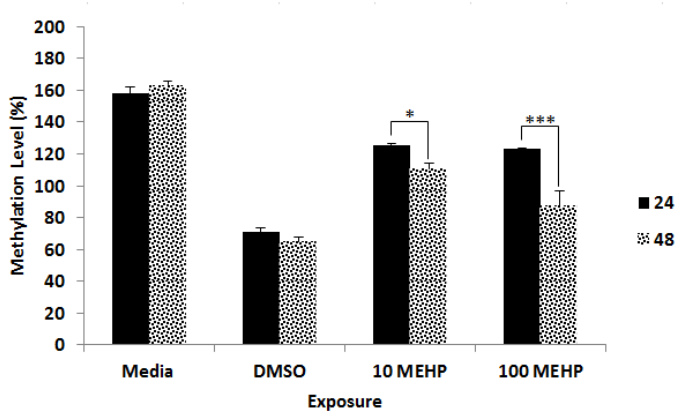
A) GSTP1



B) MGMT



C) PRSS21



D) HPRT1

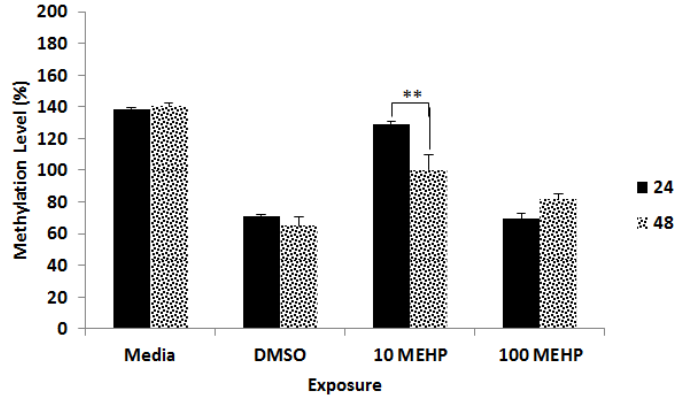


FIGURE 6. *Time-based methylation level of genes associated with testicular cancer following exposure to 10µM and 100 µM MEHP for 24 and 48 hours, with 0.05% DMSO and media as controls. Genes shown are A) GSTP1, B) MGMT, C) PRSS21, and D) HPRT1. Values are means ± SD. Asterisks denote significant differences between 24 and 48 hours (n=4, * p < 0.0001; ANOVA).*

CHAPTER V - DISCUSSION

The aim of the present study was to investigate the effects on embryonal carcinoma cells exposed *in vitro* to MEHP, with particular attention to short-term proliferation and methylation level changes. The present results suggest that MEHP decreases proliferation at 24 hours but that NT2 cells recuperate by 48 hours, suggesting that unless cells are continuously exposed to MEHP, the cells are able to metabolize and overcome any anti-proliferative effects. MEHP also was seen to significantly alter methylation levels in GSTP1, PRSS21 and HPRT1, raising the possibility that MEHP can alter expression of these genes during a crucial period of embryonal development that eventually leads to testicular cancer.

The cells used in the present study were NT2/D1 cells, which are frequently used to study testicular cancer. Embryonal carcinoma cells resemble early embryonic stem cells in terms of morphology and biochemistry, and represent an early stage of differentiation. NT2/D1 cells were derived from the metastatic lesion of a 22-year old male patient, and are multipotent, differentiating into well-developed neurons upon exposure to retinoic acid (Spinella et al, 2003). Since embryonal carcinomas are the most frequent form of TGCT, representing around 87% of all non-seminoma tumours (Bosl & Motzer, 1997), NT2 cells have been widely characterized and are frequently used to model testicular cancer.

MEHP has a short term, anti-proliferative effect on testicular cells

MEHP was shown to decrease proliferation for both doses of MEHP, but continuous exposure is required to maintain an effect. In Figure 2, the NT2 cells grown in Petri dishes show

a decrease in MEHP-exposed cells by more than 50% as compared to controls, but quickly recuperate and gain similar cell counts to controls by 48 hours (Figure 2). Given that the doubling time of NT2 cells is around 20-25 hours (Wang et al, 2013), we hypothesized that it takes approximately two cell cycles for the cells to metabolize the MEHP effectively, and thus overcome any anti-proliferative effects the MEHP might have on the cells. Based on these initial results, we grew NT2 cells in iCelligence, a cell analysis instrument which could monitor the growth of the cells in real-time. This was done to observe any acute changes in proliferation when cells were exposed to MEHP every 24 hours. Compared to the traditional method of growing cells in dishes and counting them at varying intervals, resulting in a snapshot of the cell counts at a particular moment in time, iCelligence allows a continuous exploration of cell growth over time. The instrument shows proliferation as measure of cell index, which is then analyzed using the RTCA software to normalize the data with the control as a baseline.

The readings for 10μM MEHP did not show any significant difference in proliferation compared to media, but 100μM MEHP showed a significant change (Figure 3). The wells in which the cells grow are highly sensitive to movement, and thus any disruptions to its internal or external environment are recorded by the gold plates at the bottom of each well. Delineated peaks at approximately every 24 hours are observed. These disruptions are the result of 1) fresh MEHP being administered thus causing disruption to the media and 2) any physical handling, lifting or pipetting occurring during fresh dose administration. Sharp increases in cell index, such as those occurring at 24 and 48 hours, are the result of cells being pushed down to the bottom of the well by pipetting. Overall, however, cell index appeared to decrease with time and with each subsequent MEHP dose administration, decreasing substantially by 96 hours. This indicates that

the cells lifted off the bottom of the well, i.e. they lost adherence, which indicates their death (Ishikawa et al, 2015). These results thus suggest that MEHP must be administered or exposed to cells continuously in order to see an effect in proliferation. MEHP is known to have a very short half-life, being eliminated within 3.5 ± 1.4 hours (Mittermeier et al, 2016), and therefore continuous exposure to MEHP may result in sustained changes in proliferation rather than a one-time dose.

Data on proliferative changes after exposure to MEHP is highly limited; most literature that exists focuses on DEHP, its parent metabolite. Wei et al (2017) recently conducted a study using low doses of DEHP (0.1μM, 2.5μM, 50μM) similar to the present study (10μM, 100μM) but on RKO, a colon carcinoma cell line, and HepG2, a liver carcinoma cell line, for 72 hours, and found no significant changes in proliferation. However, when exposing the cells to a much lower dose of 0.25nM DEHP over 3 months, proliferation was promoted in HepG2 cells. They concluded that long-term DEHP exposure, and not short-term exposure, promoted cell growth, which correlates with our findings. Other studies suggest that MEHP functions not as an anti-proliferative agent, but as an apoptotic one; thus, instead of the cells decreasing in number, they may be subject to programmed cell death. A study by Ferrara et al (2006) described how germ cell proliferation was decreased by 50% after in utero exposure to di-butyl phthalate (DBP) within 96 hours, and then decreased to a further 80% by day 15. This was further corroborated by Lambrot et al (2012) who showed that 36 hours after treatment, MEHP reduced the number of germ cells by 40%. The mechanisms by which MEHP may affect apoptosis have not yet been characterized, however, mechanisms regarding its anti-proliferative effects have been studied.

The mechanisms by which MEHP may serve this anti-proliferative role are varied. Several studies have reported that exposure to MEHP induces *c-myc* expression in human and murine cell lines (Yao et al, 2011; Yao et al, 2012; Zhang et al, 2014). This oncogene is responsible for regulating cellular growth and cellular metabolism, either directly or by indirectly inducing changes in expression for gene families responsible for proliferation, and its overexpression therefore leads to overall rapid cellular proliferation. This in turn increases the demand for nucleic acid, protein and lipid production in new cells. Yao et al (2011) found that 200μM MEHP upregulated *c-myc* after 12 hours and promoted invasive activity and migration capability of NT2 cells after 36 hours. Interestingly, although *c-myc*'s expression was examined and its role in cell proliferation is critical, the authors of this study did not assess proliferation in NT2 cells. Increased invasive activity and migration, however, provide an alternate facet of enhanced tumorigenicity.

MEHP has differential methylation patterns in key genes for testicular cancer

MEHP was shown to increase methylation levels to varying degrees in GSTP1, MGMT, PRSS21, and HPRT1 relative to at least one of the controls (media or 0.05% DMSO) but not relative to both. Figure 4 depicts the changes in methylation level over time, in the two doses of MEHP and the two controls. GSTP1 showed an overall increase in methylation over time, compared to both controls. MGMT only had a slight increase as compared to media, but not compared to DMSO. PRSS21 and HPRT1 meanwhile had the opposite effect, showing an overall increase in methylation as compared to DMSO but not compared to media. Interestingly, in 3 of 4 cases, 10μM MEHP had a higher methylation level than 100μM MEHP. This correlates with the low-dose theory: doses in the range of human exposure, or doses below those

traditionally tested in toxicological studies, often have a more effective response than higher doses (Vandenberg et al, 2012).

Epigenetic mechanisms have been increasingly considered as major factors in gene silencing for TGCT progression. CpG islands, which are rich in CG dinucleotides, are important regions of gene expression regulation in normal cells. However, in cancer cells, CpG islands within or surrounding the promoter regions of genes are frequent targets for DNA hypermethylation, often leading to histone deacetylation, abnormal gene silencing and the aberrant silencing of tumour suppressor genes (Manton et al, 2005).

Most tumour suppressor genes show hypermethylation in the 5' CpG island promoter region in several cancers (Esteller, 2002). Many studies have examined the frequency of hypermethylation of these genes in testicular cancer cells and tissues, as summarized by Table 6, however, the data is somewhat conflicting in certain cases. This may be a reflection of the source of the samples – TGCT cell lines, which are often undifferentiated embryonal carcinomas, will have a different methylation pattern than primary TGCT tissues, which consist of teratomas, embryonal carcinomas, yolk sac tumours and other types, each with their own methylation frequencies. Teratomas are typically the most methylated subgroup within non-seminomal TGCT, followed closely by yolk sac tumours, compared to embryonal carcinoma cell lines such as NT2 which are undifferentiated and potentially lacking hypermethylated genes. However, there are distinct frequency patterns for MGMT (40-50%), GSTP1 (15-25%) and PRSS21 (90-100%), with RASSF1A being the most inconclusive, but nevertheless indicative of methylation identities for each of the genes.

TABLE 6. *Percentage methylation for genes analyzed for promoter hypermethylation in testicular germ cell tumours (TGCT)*

Gene	Reference	Sample Size	Sample Source	Method Used	Percent methylated
MGMT	Brait et al, 2012	57	primary TGCT	bisulfite sequencing	NS: 50%
	Chen et al, 2014	25	NT2	bisulfite sequencing	Total: 40.7%
	Honorio et al, 2003	24	primary TGCT	MSP	NS: 44%
	Koul et al, 2004	70	NS cell lines	MSP	Total: 20%
	Smith-Sorensen et al, 2002	69	primary TGCT	MSP	Total: 46%, NS: 69%, S: 24%
GSTP1	Elllinger et al, 2009	73	primary TGCT	restriction endonuclease MSP	Total: 24.7%, healthy: 0%, NS: 24.3%, S: 24%
	Honorio et al, 2003	24	primary TGCT	MSP	0%-15%
PRSS21	Kempkensteffen et al, 2006	33	primary TGCT	MSP	33.5 NIM (normalized index of methylation) vs 3.9 in healthy samples
	Manton et al, 2005	-	NT2	bisulfite sequencing	100%
RASSF1A	Elllinger et al, 2009	73	primary TGCT	restriction endonuclease MSP	Total: 46.6%, NS: 51.4%, S: 41.7%
	Honorio et al, 2003	24	primary TGCT	MSP	Total: 71%, NS: 83%, S: 40%
	Kawakami et al, 2003	25	primary TGCT	MSP	0%
	Koul et al, 2004	70	NS cell lines	MSP	Total: 35.7%
	Lambrot & Kimmins, 2010	5	NT2	bisulfite sequencing	92.60%
	Lind et al, 2006	55	primary TGCT	MSP	29%

The genes listed above are given as percentages for all TGCT's (including both non-seminomas (NS) and seminomas (S)) and for non-seminomas and seminomas separately when reported. Some references come from the same research groups and may include overlapping samples.

The methylation data was analyzed using ANOVA to determine significance differences between doses of MEHP. Using media and DMSO as a control (Figure 5), comparisons were made in methylation level for both 10μM and 100μM MEHP.

GSTP1

MEHP only caused significant increases at 48 hours with media as control, and significant increases at both 24 and 48 hours with DMSO as control. GSTP1's role as a

detoxifying enzyme means that it is highly expressed in most cells. The gene is generally overexpressed in neoplasms as well as in anticancer drug-resistant tumour cells, but can be inactivated by hypermethylation in cancers such as prostate cancer (Harries et al, 1997). Time-dependent analyses showed that methylation level increases significantly with time (Figure 6).

There are very few studies examining the relationship between GSTP1 and TGCT, and methylation studies are even more limited. Ellinger et al (2009) found a relatively low level of methylation in both seminoma and non-seminoma tumours (24% and 24.7% respectively), while Honorio et al (2003) categorized GSTP1 as one of the genes with methylation less than 15% in their study. This low level of methylation in both studies correlates with its high expression in neoplasms, which is showcased by Mayer et al (2003) wherein GSTP1 was stained positive in 100% of all mature teratoma TGCT samples. Mature teratomas are generally highly resistant to chemotherapy, suggesting that GSTP1's methylation status plays a key role in drug resistance. In the present study, we show that MEHP increases the methylation of GSTP1, suggesting that MEHP can prevent the detoxification of carcinogenic electrophiles by potentially inactivating GSTP1.

MGMT

MEHP did not cause any significant changes compared to either of the controls, and overall had the lowest methylation levels of all four genes. MGMT's role as a DNA repair enzyme means that it is partially inactivated in tumours, which can be seen in our controls that have methylation levels ranging between 60 and 80%. Time-dependent analyses showed that methylation level decreases with time (Figure 6).

MGMT has been found to be hypermethylated in several epigenetic studies, demonstrated in Table 1. Smith-Sorensen et al (2002) found an overall methylation frequency of 46% in all tumours, with 69% in non-seminomas and 24% in seminomas. Methylation of the MGMT promoter was also frequently accompanied by allelic imbalance in non-seminoma samples, suggesting that MGMT hypermethylation may play a role in this imbalance. As MGMT is a repair protein, its increased activity eventually leads to lowered cell sensitivity for chemotherapy. Most chemotherapy for TGCT involves cisplatin in combination with other drugs, but its effectiveness in regards to MGMT activation has not been studied at length, and MGMT's methylation status could be an interesting marker to consider in the future for chemotherapy resistance

Nagel et al (2003) expanded further upon this idea by developing a nonradioactive MGMT enzyme-linked immunosorbent assay (ELISA) for the quantification of MGMT protein product. In this system, MGMT reacts with an O^6-benzylguanine derivative, leading to the biotinylation of MGMT and subsequent detection, thus allowing the users to quantify the amount of MGMT expression. By applying this assay to normal and testicular cancer tissues, they found that MGMT activity was limited in tumour tissues (333 ± 287 fmol/mg protein) as compared to normal tissues (428 ± 340 fmol/mg protein), suggesting that it is hypermethylated in testicular cancer.

MGMT's activity and expression can be controlled by other factors as well. A study by Chen et al (2013) showed that the changes occurring in miR-199a-3p, a micro RNA that is known to be downregulated in testicular cancer and may be involved in gene regulation, affected the activity of DNA methyltransferases, especially DNMT3A. Downstream, this caused the

downregulation of MGMT and APC. A decrease in miR-199a-3p permitted an increase in DNMT3A, which cause hypermethylation of the promoter regions of MGMT and adenomatous polyposis coli (APC) in NT2 cells. An overexpression of miR-199a-3p caused the inverse, restoring the activity of MGMT and APC, suggesting that synthetic administration of miR-199a-3p could be a potential therapeutic factor in TGCT treatment.

Koul et al (2004) found that MGMT was more frequently hypermethylated in sensitive tumours (31%) than resistant tumours (13%). This correlates well with MGMT's identity as a DNA repair enzyme, protecting cells against alkylating agents. As alkylating agents are frequently used in chemotherapy against tumours, hypermethylated (and therefore silenced) MGMT is expected to be observed in sensitive tumours, whereas high levels of MGMT gene are generally expressed in resistant tumours. Koul et al (2004) found that MGMT expression was absent in 91.7% of tumours, but that expression could be restored after exposure to demethylating agent 5-azacytidine, thus they concluded that partial methylation existed in this gene and that it resulted in down-regulation its gene expression.

PRSS21

MEHP made drastic changes to PRSS21's methylation level for both media and DMSO controls. For media, MEHP lowered the methylation level significantly for both 24 and 48 hours. For DMSO, MEHP increased the methylation level significantly for both timepoints. This suggests that a) DMSO decreases methylation level compared to media and b) MEHP's overall effect on methylation owes itself at least partially to the effects of DMSO. Time-dependent analyses showed that methylation level decreases significantly with time (Figure 6).

Kempkensteffen et al (2006) demonstrated that PRSS21 shows near complete methylation of the 5' CpG island in NT2 cells as well as reduced gene expression, while non-cancerous cell lines display no such methylation or decreased methylation. They calculated that the normalized index of methylation for PRSS21 was 33.5 in testicular tumours vs. 3.9 in normal tissues. To further this idea, Manton et al (2005) showed that PRSS21 activity suppresses testicular tumour growth *in vitro* for colony formation and *in vivo*. Using bisulfite sequencing of the 5' regulatory promoter region of PRSS21, they revealed extensive methylation up to 100%, leading to gene silencing. Both these studies correlate with the present study, where we see that the sample containing only media and cells has very high levels of methylation (Figure 5C). Knowing PRSS21's role as a serine protease, and its importance in peptide bond cleavage and germ cell migration, we would expect to see MEHP increase its methylation level further. Our findings did not correlate with those of previous studies for our analysis using media, but it did correlate for our analysis using DMSO. This may be due to our choice of solvent for MEHP, as DMSO may play a role in its methylating behavior. The only other solvent that MEHP may be dissolved in is ethanol, thus it would be interesting to see this assay repeated using ethanol in the place of DMSO.

HPRT1

Hypoxanthine phosphoribosyltransferase 1 (HPRT1) was used as a housekeeping gene, based on previous testicular studies in our lab. However, owing to HPRT1 being X-linked, it can often be a poor normalizing gene for testicular cancer as TGCTs often show a numerical increase in X chromosomes (Rajpert-De Meyts et al*,* 2007) and the gene varies greatly among murine germ cell populations (Svingen et al, 2009; van den Bergen et al, 2009). In our study, MEHP

made drastic changes to HPRT1's methylation level for both media and DMSO controls. For media, 100μM MEHP decreased the methylation level significantly at 24 hours, while both doses lowered it significantly for 48 hours. For DMSO, 10μM MEHP increased the methylation level while 100μM MEHP decreased the methylation level significantly for 24 hours. No significant changes were observed for 48 hours. Time-dependent analyses showed that methylation level increases significantly with time for 10μM MEHP (Figure 6).

Since HPRT1 is considered a housekeeping gene, it is expected to be mainly unmethylated in normal tissues (Lim & Maher, 2010). In the present study we see that it is very highly methylated for our control sample (media and cells only). HPRT1 is generally highly expressed in normal tissues but not in testicular cancer (Uhlen et al, 2015). Thus our result is expected. MEHP decreases the methylation level for HPRT1 which suggests it may reactivate the gene. HPRT1's role in maintaining the cell's purine nucleotide resources and its role in oxygen deprivation suggest that MEHP may facilitate these functions in tumour cells by reactivating HPRT1. However, more studies are needed to examine this relationship further.

CHAPTER VI – CONCLUSIONS & RECOMMENDATIONS

The present study demonstrates that MEHP decreases proliferation at 24 hours but that NT2 cells recuperate by 48 hours, suggesting that unless cells are continuously exposed to MEHP, the cells are able to metabolize and overcome any anti-proliferative effects. We also demonstrate that MEHP significantly alters methylations levels in GSTP1, PRSS21 and HPRT1, raising the possibility that MEHP can alter the expression of these genes during a crucial period of embryonal development.

We recommend that future studies focus on more genes associated with testicular cancer, such as those discussed in this paper, as well as a range of other doses of MEHP. We also recommend that gene expression be included along with methylation analyses, to conclusively determine if MEHP affects both methylation and expression of key genes. We recommend the use of *in vivo* studies, which would shed light on the effects of MEHP in an entire organism, rather than the limited approaches of *in vitro* studies. Future studies may also focus on more endocrine cancers, such as ovarian or prostate, to truly understand the potentially toxic effects of MEHP during development.

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APPENDIX
CELL PROLIFERATION

Cell counts of cells grown in Petri dishes, raw data

Timepoint	*Exposure*	*Total*	*Live*	*Dead*	*Viability*
24 HOUR	**Media**	1,400,000	1,400,000	600,000	96%
	DMSO	1,500,000	1,500,000	7,000	95%
	100 MEHP	810,000	740,000	7,000	91%
	10 MEHP	810,000	770,000	4,000	95%
48 HOUR	**Media**	1,400,000	1,300,000	160,000	89%
	DMSO	1,300,000	1,200,000	120,000	91%
	100 MEHP	1,300,000	1,300,000	5,000	97%
	10 MEHP	1,500,000	1,400,000	8,000	95%

Percent methylation of MEHP doses compared to Control

	24hr	**48hr**
10 MEHP	57.85%	107.14%
100 MEHP	57.85%	92.85%

Percent methylation of MEHP doses compared to DMSO

	24hr	**48hr**
10 MEHP	54%	115.38%
100 MEHP	54%	100%

Independent samples t-test (two-tailed)

	10 MEHP	**100 MEHP**
Media	0.6069	0.3931
DMSO	0.6796	0.5000

Significance between controls and each dose of MEHP (p-value < 0.05).

METHYLATION ANALYSES

Methylation Level, Raw Data (%)

GSTP1

	24hr	SD	48hr	SD
Media	123.9001	6.035266	106.3216	9.422914
DMSO	91.30153	6.249126	103.5445	12.25051
10 MEHP	118.0997	6.427318	171.4401	12.72147
100 MEHP	111.4734	12.81891	168.4569	1.960323

MGMT

	24hr	SD	48hr	SD
Media	72.27349	13.48188	43.07691	3.37466
DMSO	87.701	3.988856	70.97459	3.477282
10 MEHP	75.00163	9.145738	56.97702	2.791495
100 MEHP	77.07916	9.306173	58.2254	2.852653

PRSS21

	24hr	SD	48hr	SD
Media	157.7868	4.264774	163.3422	2.581989
DMSO	70.97487	2.877498	65.03356	2.581989
10 MEHP	125.4999	1.151352	110.629	3.187991
100 MEHP	122.9614	0.513642	87.60243	9.213786

HPRT1

	24hr	SD	48hr	SD
Media	138.4571	1.357221	140.5405	2.066449
DMSO	70.97487	0.954057	65.03356	5.529794
10 MEHP	129.2749	1.267206	100.1534	9.801907
100 MEHP	69.22474	3.391546	81.57054	3.197586

Two-Way ANOVA, Dose-Dependent Analysis

P-values, Control as baseline

		24hr	48hr
GSTP1	**10 MEHP**	0.0487	<0.0001
	100 MEHP	>0.9999	<0.0001
MGMT	**10 MEHP**	>0.9999	0.1654
	100 MEHP	0.7806	0.1188
PRSS21	**10 MEHP**	<0.0001	<0.0001
	100 MEHP	<0.0001	<0.0001
HPRT1	**10 MEHP**	0.4947	<0.0001
	100 MEHP	<0.0001	<0.0001

Significance between control and each of the doses of MEHP.

P-values, DMSO as baseline

		24hr	48hr
GSTP1	**10 MEHP**	0.0006	<0.0001
	100 MEHP	0.0017	<0.0001
MGMT	**10 MEHP**	0.1123	0.388
	100 MEHP	0.287	0.5503
PRSS21	**10 MEHP**	<0.0001	<0.0001
	100 MEHP	<0.0001	0.0004
HPRT1	**10 MEHP**	0.0004	0.4719
	100 MEHP	0.0021	>0.9999

Significance between DMSO and each of the doses of MEHP.

Two-Way ANOVA, Time-Dependent Analysis

	GSTP1	MGMT	PRSS21	HPRT1
Media	<0.0001	0.0021	0.9717	>0.9999
DMSO	0.2873	0.1375	0.2554	0.1700
10 MEHP	<0.0001	0.1628	0.3758	0.0118
100 MEHP	<0.0001	0.1259	0.0001	0.762

Significance between 24 and 48 hours for each dose.