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Phthalates, Embryo Development, and Sperm DNA Methylation

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Phthalates, Embryo Development, and Sperm DNA Methylation

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Phthalates, Embryo Development, and Sperm DNA Methylation

A Dissertation Proposal

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To anyone and everyone who contributed to my education

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ABSTRACT

PHTHALATES, EMBRYO DEVELOPMENT, AND SPERM DNA METHYLATION

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Infertility affects 15% of all couples and presents a major public health issue. Animal and human data indicate that epigenetic dysregulation in sperm is associated with poor male reproductive health and extensive epigenetic reprogramming during spermatogenesis presents a window of vulnerability for environmentally-induced epigenetic dysregulation.

Phthalates is a class of ubiquitous environmental contaminant and global health concern. Phthalate exposure in humans has been associated with diminished male fertility, adverse birth outcomes, and altered offspring development. In order to facilitate additional research on this topic, we aimed to developing a higher throughput method of sperm nucleic acid extraction. In addition, this dissertation also examined the associations of paternal phthalate exposure with sperm methylation patterns and blastocyst development.

In order to maximize efficiency of the limited and difficult to acquire samples while increasing throughput for epidemiologic studies, we developed a novel method of sperm nucleic acid extraction that improved ease and efficiency. Compared to previously published methods, our new method has 1) increased yield of DNA per sperm cell, 2) reduced time required for DNA processing, and 3) eliminated the need for odorous sulfur-based reducing compounds.

As part of the Sperm Environmental Epigenetics and Development Study (SEEDS), we recruited 50 couples from an in vitro fertilization (IVF) clinic. We observed that concentrations of select paternal urinary metabolites of phthalates or phthalate alternatives were associated with a marked decrease in blastocyst quality. With respect to sperm DNA methylation, we found that the male urinary anti-androgenic phthalate metabolite concentrations were associated with 131 differentially methylated regions in sperm DNA. Gene ontology analyses revealed that these differentially methylated regions were enriched in genes related to growth and development as well as cellular function and maintenance.

Overall, the evidence presented by this dissertation show that paternal adult exposure to select phthalates and phthalate alternatives may influence both embryo development and sperm DNA methylation patterns. Additional work is required to replicate our findings as well as determine whether the observed aberrant sperm DNA methylation patterns associated with urinary metabolites of phthalates and phthalate alternatives are true biological mediators of the concurrent decrease in embryo quality.

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CHAPTER 1

INTRODUCTION

Summary

Infertility affects 15% of all couples and studies have associated phthalates, a class of endocrine disrupting compounds with widespread exposure, with adverse male reproductive outcomes. However, research in this area has been hampered by both methodological challenges and lack of understanding of sperm biology. This dissertation addressed both issues as well as examines the influence of adult human preconception phthalate exposure on both embryo development and sperm DNA methylation.

Infertility and Its Implications for Public Health

Infertility, commonly defined as the inability for a couple to conceive within one calendar year, affects 15% of all heterosexual couples [1-3]. Male factor infertility is estimated to be present in 40-50% of diagnosed couples [1, 4] and affects 2.5% to 12.5% of all men [5]. In addition to the inability to conceive, poor male reproductive health has several adverse health implications. First, male infertility may be an indicator of general wellbeing as it has been reported to be associated with increased mortality rate [6] and poorer overall health [7]. Second, despite advances in assisted reproductive technology (ART), offspring conceived via ART are reported to have lower birthweights, increased adiposity, higher blood pressure and fasting glucose concentration, and changes in the bone and thyroid

during childhood [8, 9]. Lastly, infertility has mental and social consequences [10] on the affected families, resulting in higher rates of divorce or end of cohabitation [11]. Together, it is clear that male fertility has broad public health implications beyond the diagnosed individual.

In a follow up to previous analyses [12, 13], a recent meta-analysis showed that after accounting for semen collection methods and other relevant factors, sperm counts in men from North America, Europe Australia and New Zealand have declined by 59.3% between 1973 and 2011 [14], indicative of a possible decline in male fertility. Other studies around the world reported similar observations of declining semen parameters [15-20]. Despite this alarming observation, the extent and causes of the decline in male fecundity are unknown. In addition, while many causes of male factor infertility are known, nearly half of all cases of male infertility have no known causes [21]. Thus, there is a need to better understand the biological biomarkers and mediators of male fertility as such investigations may help elucidate underlying causes to male infertility and clarify the role of male infertility as a general health indicator.

Sperm Epigenetics and Its Implications for Public Health

Epigenetics is the study of semi-heritable changes in gene expression that do not involve changes in the DNA sequence. Epigenetic reprogramming refer to non-permanent changes to epigenetic features leading to differential gene expression. Of the many known mechanisms of epigenetics, DNA methylation, the addition of a methyl group on nucleobases of DNA, is the best characterized. In

mammals, DNA methylation primarily occurs at the 5 position of the pyrimidine ring in CpG dinucleotides and is one means of gene expression control, with the degree of methylation generally inversely correlated with degree of gene expression. DNA methylation can be found in ~1% of the total nucleotides in human somatic tissue [22] and it is currently believed that DNA methylation regulates gene expression by inhibiting transcription factors from binding or by recruiting repressor complexes [23]. The latter results in chromatin changes that ultimately lead to suppression of transcription [23]. Imprinting is a parent-specific, germline inherited, form of DNA methylation based gene regulation. Imprinted genes will have either >90% or <10% methylation, depending on the parent of origin. For example, a paternally imprinted gene will have >90% methylation on the paternal allele and <10% on the maternal allele. Because imprinting "defects" are known to cause several disease phenotypes, it is hypothesized that methylation at these genes may act as targets for environmental agents [24].

Traditionally, spermatozoa have been considered solely as a delivery vehicle for the paternal genome to oocytes. However, this paradigm is challenged by the accumulation of evidence that sperm epigenetics is also a critical determinant of fertility. Spermatogenesis involves extensive epigenetic reprogramming [25, 26] and there is a growing body of compelling animal and epidemiologic data that suggest the epigenome of male germ cells is central to reproductive health due to its potential to affect offspring phenotype and lifelong health trajectory [27-29]. It is believed that environmental exposures at critical windows such as spermatogenesis may introduce alterations in the sperm

epigenome and lead to adverse male reproductive outcomes [25, 30]. Not surprisingly, sperm epigenetic dysregulation, particularly DNA methylation, has been associated with male infertility [26, 31]. Similarly, aberrant sperm DNA methylation are associated with reduced IVF success [32], poor sperm parameters [33-36], and adverse offspring outcome [37-39].

Given the public health importance, there is a need to identify biological determinants and prognostic markers of male fertility. Traditionally, male fertility status is determined on the basis of WHO guidelines on semen analysis, a battery of tests to evaluate features of the semen such as sperm count and concentration, semen volume, sperm morphology, etc. [40]. However, it is recognized that semen analysis is a poor predictor of pregnancy and more generally, male fecundity [1]. Given its role in normal sperm function, the sperm epigenome is thought to be a candidate diagnostic and prognostic marker in the context of male infertility [27]. A better understanding of the environmental influences on sperm DNA methylation may clarify the mechanisms by which the male environment affects fertility and offspring health.

Phthalates, Phthalate Alternatives, and Their Implications On Fertility

Phthalates are one class of endocrine disrupting compounds used in plastics, medical devices, toys, paints, and personal care products. Phthalates are not covalently bound to the plastics in which they are found and thus easily leach into the environment. Combined with the ubiquitous nature of products that contain phthalates, there is a constant and unavoidable exposure through ingestion,

inhalation, and dermal contact. Not surprisingly, recent and historical data show extensive phthalate exposure worldwide [41, 42].

Though phthalates are a family of compounds, the absorption, distribution, metabolism, and excretion properties of its members share similar features. Phthalate esters are lipophilic and typically enters the human body via dermal absorption, inhalation, or ingestion [43, 44]. It is also possible to have direct human exposure to phthalate esters via plastic medical products [43, 44]. Once the phthalates have entered the blood stream, it is usually protein bound [43]. Generally, the gastrointestinal tract and execratory organs are initial sites of storage, but phthalates do not accumulate in any tissue. The majority of the internal dose is excreted within 24 hours and none is left after a few days [43]. Phthalate metabolism involves a hydrolysis and conjugation step. In the hydrolysis step, the phthalate esters are hydrolyzed by lipases and esterases in the intestine and parenchyma. Short-branched phthalates are excreted in the urine in the hydrolyzed form while long-branched phthalates undergo several hydroxylation and oxidation steps before excretion via urine and feces [45].

The potential of phthalates to adversely influence reproductive health is well documented [46-59]. Certain phthalates display anti-androgenic activities [60-62] and have been associated with adverse reproductive outcomes. In men, phthalates exposure have been associated with reduced serum testosterone [63-65], reduced sperm counts and concentrations [53, 66], sperm morphological changes [66], sperm aneuploidy [64], and increased sperm DNA damage [54, 55, 63-65, 67]. Similarly, in women, lower oocyte yield [68], and increased risk of

endometriosis [69-72] and leiomyomata [72] have been associated with phthalates exposure.

Preconception phthalate exposures, in particular, have been associated with adverse pregnancy and birth outcomes. A series of studies from the Longitudinal Investigation of Fertility and Environment (LIFE) cohort reported that male preconception exposure to select phthalates are associated with a 20% reduction in fecundity as measured by increased time-to-pregnancy [52], smaller birth size [58], increased gestation time [58], and skewed secondary sex ratio [73]. Another group reported that male preconception urinary concentrations of select phthalate metabolites were associated with decreased odds of implantation and live birth among couples seeking fertility treatment [57]. However, no association was observed with the embryo quality at the cleavage stage [57]. Given the controlled nature of IVF, if the observed association between male preconception urinary concentration and decreased odds of implantation is true, then the results suggest that paternal contributions to early-life development occur after the cleavage stage of embryo development. Equally important, both maternal and paternal preconception exposures to phthalates are associated with specific behavioural changes in the offspring, even after adjustment for prenatal exposures [74].

Given the accumulating evidence that phthalates exposure may be harmful for reproductive health, certain traditional phthalates are being replaced by chemical alternatives such as 1,2-Cyclohexane dicarboxylic acid diisononyl ester (DINCH). Little is known regarding the health impacts of DINCH, although recently

published toxicologic data from screening assays and animal models suggest that it also has adverse effects on male reproductive health [75-78]. The investigation of these replacement compounds is important to public health as moving forward, it is likely that the general public will experience increased exposure to these replacement compounds.

Despite the accumulating evidence, the mechanism and pathogenesis by which preconception exposure to phthalates and phthalate alternatives contribute to the observed adverse reproductive effects have not been elucidated. There is a need to identify potential critical windows of susceptibility as well as investigate the mechanism by which phthalates and their alternatives affect reproductive health.

Research Gaps

Despite advancing and accumulating evidence in the field of sperm biology and sperm epigenetics, research into potential environmental influences on sperm epigenetics is still lacking. We have identified the following four specific research gaps:

1. To our knowledge, there were no reviews that comprehensively integrated all available toxicologic and epidemiologic data on the environmental influences of sperm epigenetics. In addition, no one has comprehensively described and characterized the known and suspected windows of reprogramming and environmental susceptibility in germ cell development.
2. Sperm DNA is resistant to somatic tissue DNA isolation techniques. Historically, the isolation of high quality sperm DNA required slow and labor

intensive methods. Such techniques are low throughput and present a challenge for sperm genetic and epigenetic research. Thus, there is a need for a simple and fast method of sperm DNA extraction.

3. To date, no study has examined the associations of paternal preconception urinary phthalate and phthalate alternative metabolite concentrations with embryo development through day 5 post fertilization.
4. To date, no study has examined the associations of paternal preconception urinary phthalate and phthalate alternative metabolite concentrations on sperm DNA methylation.

This dissertation addresses all four of the identified research gaps in chapters 2-5.

CHAPTER 2

ENVIRONMENTAL SUSCEPTIBILITY OF THE SPERM EPIGNOME DURING WINDOWS OF MALE GERM CELL DEVELOPMENT

Summary

This chapter presents a review from 2015 of available evidence of environmental influences on sperm epigenetics. There are compelling data that suggest environmental exposures, both chemical and dietary, can influence the epigenome of male germ cells, which in turn can affect offspring phenotype. It is likely that each of the numerous epigenetic reprogramming events throughout the life-course of the male germ cell represents a discrete window of susceptibility to environmental exposures and human research into this area is lacking.

Abstract

Male germ cells require multiple epigenetic reprogramming events during their lifespan to achieve reproductive capacity. An emerging body of compelling data demonstrates that environmental exposures can be embodied within the developing male germ cell as epigenetic marks. In turn, these epigenetic marks can impart information at fertilization to affect the trajectory of offspring health and development. While it is recognized that *in utero* epigenetic reprogramming of male germ cells is a particularly susceptible window to environmental exposures, other such windows exist during germ cell development. The objective of this review is to discuss epigenetic reprogramming events during male germ cell

development and to provide supporting evidence from animal and human studies that during specific periods of development, germ cells are susceptible to environmentally-induced epigenetic errors. Moving forward, the nascent field of sperm epigenetics research is likely to advance our understanding of paternal environmental determinants of offspring health and development.

Introduction

Spermatozoa have been traditionally considered vehicles for the sole delivery of the paternal genome to oocytes upon fertilization. In this context, paternal contributions to offspring phenotype are strictly limited to germline genetic information without the ability to impart environmental information that is encountered during the life-course. However, a growing body of compelling data demonstrates that certain environmental exposures can be embodied within the developing male germ cell without altering the germline genetic information and in turn can affect the offspring phenotype.

Epigenetics is the study of semi-permanent, mitotically-heritable and in germ cells meiotically-heritable changes in gene expression that primarily result from modifications of chromatin structure, rather than changes in the underlying DNA sequence [79]. The three major mechanisms of epigenetics are DNA methylation primarily within CpG dinucleotides [80], a host of modifications to histone tails [81], and noncoding RNAs (e.g. microRNAs and long noncoding RNAs) [82]. In concert, these epigenetic mechanisms control chromatin structure to confer cell-specific gene expression.

In humans, male germs cells do not attain reproductive capacity until the second decade of life. Despite this long latency period, male germ cells begin development early in fetal life, and upon sex determination, embark on a remarkable journey of cellular differentiation and morphological changes to prepare for its sole purpose – the propagation of its genome. During development, male germ cells progress from primordial germ cells (PGCs), diploid spermatogonia to haploid spermatozoa that involves stage- and testis-specific gene expression, mitotic and meiotic divisions, and chromatin remodeling that is unique only to sperm [83, 84]. To undergo these transformations, stage-specific epigenetic reprogramming is required in addition to more modest, but still significant, epigenetic changes that gradually progress germ cell phenotype towards reproductive capacity. As the epigenome allows considerable cellular plasticity, epigenetic changes across the many stages of male germ cell development represent windows of susceptibility by which environmental exposures can sculpt the epigenetic landscape.

In this review, we identify and discuss multiple windows of susceptibility during mammalian male germ cell developmental in which dietary and toxicant exposures have been shown to influence sperm epigenetics as well as offspring phenotype in animal models and humans.

Windows of Male Germ Cell Development

In utero period and primordial germ cells

Primordial germ cells (PGCs) arise from the proximal epiblast with a population of < 50 cells and undergo clonal expansion as they migrate and colonize

the genital ridge, the precursor to the gonads [85] (Figure 1). As PGCs are derived from cells of the epiblast, which have begun on a course of somatic fate, epigenetic reprogramming is essential to re-establish totipotency for sex-specific epigenetic programming of germ cells. The loss of genome-wide methylation occurs passively during the rapid proliferation of PGCs. Although the maintenance DNA methyltransferase 1, DNMT1, is readily expressed in PGCs, its essential cofactor, Uhrf1, is not, resulting in the loss of maintenance of methylation during cell divisions [86]. Imprinted-specific differential methylated regions (iDMRs), which are methylated in a parent-of-origin manner and have escaped epigenetic reprogramming shortly after fertilization, follow slower kinetics requiring active demethylation via Tet proteins in mice [87]. In humans, a second wave of reprogramming in PGCs occurs several weeks later to erase imprinted marks via histone remodeling, most notable depletion of H3K27me3 and removal of the histone variant, H2A.Z [88] . At the end of methylation erasure, global levels of methylation of male PGCs are estimated at 16.3% compared to the 70% methylation in the embryo [89]. The lack of complete erasure is mostly due to the resistant nature of intracisternal A particles (IAPs), a class of retrovirus-like transposons, and their proximal genes, to demethylation, which bestows a potential mechanism for epigenetic inheritance [90]. The majority of methylation is re-established in mitotically-arrested type A spermatogonia prior to birth and is fully resolved postnatally during spermatogenesis [91].

Nutritional manipulation. Given the extensive reprogramming that occurs in PGCs to redefine their epigenetic landscape in a sex-specific manner,

environmental exposures in animal models during this period have been shown to induce inter- and transgenerational effects through the sperm epigenome. Severe *in utero* caloric restriction during the window of re-acquisition of DNA methylation in mouse (E16.5) led to differential methylated regions (DMRs) in F1 sperm mainly at intergenic regions and CpG islands, which were also reported to associate with regions of histone retention [92]. While both F1 and F2 male mice exhibited metabolic-related disorders, DMRs of F1 sperm did not persist in somatic tissue of F2 males [92]. Interestingly, the expression of nearby metabolic genes were altered in F2 males, indicating that although sperm DMRs were lost, other epigenetic mechanisms, not measured in this study, could persist to influence F2 gene expression [92]. These results are in contrast to another study in which *in utero* caloric restriction resulted in the transmission of altered DNA methylation of a lipogenic gene, Lxra, in F1 sperm to F2 somatic tissues [93]. Additionally, streptozotocin-induced gestational diabetes altered the expression of imprinted genes, IGF2 and H19, in F1 sperm and F2 pancreatic islets [94] and increased Peg3 DNA methylation in F1 sperm [95].

In an intriguing study in male mice, life-long (i.e., *in utero* and adult) deficiency in folate, a key component of one-carbon metabolism that facilitates the transfer of methyl groups for DNA and histone methylation reactions, resulted in craniofacial and musculoskeletal birth defects in their offspring [96]. Genome-wide analyses of sperm from folate-deficient animals in adulthood identified 57 DMRs, none of which were associated with iDMRs, but rather, they were associated with genes related to cancer, diabetes, and neurological diseases. Moreover, global

mono- and tri- methylation at H3K4 and H3K9 were also reduced in folate-deficient F1 sperm [96]. While in the placenta, over 300 genes were differentially expressed; however, only two associated with sperm DMRs, suggesting that other epigenetic modifiers such as sperm H3 methylation were involved. It must be noted however, that since exposure was life-long, it is difficult to discern the timing of germ cell development (e.g., PGCs or spermatogenesis) in which folate deficiency induced these observed epigenetic effects.

Environmental toxicants. Skinner and colleagues have repeatedly demonstrated environmental toxicant-induced transgenerational effects through the paternal germ line in outbred rats. *In utero* exposure to chemicals exhibiting endocrine disrupting characteristics, such as vinclozolin [97-100], DDT [101], 2,3,7,8-tetrachlorodibenzo[p]dioxin (TCDD) [102, 103], the jet propellant, JP8 [102, 104], pesticide mixture of permethrin and DEET [102, 105], and plastic mixture of bisphenol-A, (BPA), bis(2-ethylhexyl)phthalate (DEHP) and dibutyl phthalate (DBP) [102, 106] all elicited DMRs in F3 sperm without any additional exposures in subsequent generations. Interestingly, the DMRs, which were mostly intergenic, displayed little overlap between exposures [101, 102], indicating the lack of specificity of environmentally-induced DMRs in male germ cells. Moreover, vinclozolin exposure at a similar dose and timing produced no overlapping DMRs in sperm of F3 rats [99] and mice [98], demonstrating again that the sperm epigenome may be programmed by environmental toxicants in a stochastic fashion.

In contrast to the faithful inheritance of the transgenerational effects reported above, other studies have reported that *in utero* exposure to pesticides, vinclozolin and methoxychlor, modified methylation of iDMRs in F1 sperm, but a trend towards recovery was observed starting in the F2 sperm and continued through the F3 [107, 108]. Similarily, *in utero* exposure to the endocrine disruptors, vinclozolin, BPA or DEHP, in mice resulted in DNA methylation changes in F1 prospermatogonia but these changes did not persist into the F2 germline [109]. Most recently, *in utero* exposure to vinclozolin was found to alter the expression of miRNAs, miR-23b, miR-21, and *let-7*, in F1-F3 PGCs; however, no prominent changes in DNA methylation were observed in either F1 PGCs or mature sperm [110].

It is currently unclear from the above studies whether the observed environmentally-induced DMRs in sperm are direct effectors of offspring programming or they are themselves biological intermediates for other epigenetic modifiers [92, 96], such as unmeasured histone modifications and/or altered non-coding RNA expression. Alternatively, these DMRs may act as non-causal markers of exposures, such that environmental exposures may operate through other pathways to induce adverse offspring health. As a consequence of the difficulty of conducting life course studies in humans, there is currently no data we are aware of on the associations between in utero exposures and adult sperm epigenetic endpoints.

Infancy and Prepubertal Periods

The timing of postnatal testicular development varies considerably among mammalian species with a marked distinction between rodents and higher primates [111] (figure 1). In laboratory rodents, testicular development begins a few days after birth in which mitotically-arrested prospermatogonia resume clonal expansion resulting in an estimated 30-fold increase in spermatogonia prior to puberty [112]. In contrast, humans have a long latency period between birth and puberty, whereby after the first few months of postnatal life, referred to as mini-puberty, steroidogenic activity and testicular development is thought to remain quiescent until the onset of puberty [113].

This notion of inactive testicular development in childhood was largely driven by palpation and Prader's orchidometer measures that detected no change in testicular volume until the onset of puberty [114]. However, employing more sensitive methods, such as stereological measures from testes obtained after autopsy, data indicate that testes, despite displaying no outwardly changes in size, are actively developing organs during infancy [115, 116]. For example, during the first ten years of life, stereological measures revealed that testicular volume tripled with increases in seminiferous tubule length and the number of spermatogonia and Sertoli cells [115]. Another study reported that germ cell proliferation is not linear with age but may occur in waves, such that during periods from 3-8 years and at 10 years to the onset of puberty experienced marked proliferative activity [116]. This proliferation has been proposed to be related to transient awakening of the hypothalamus-pituitary-gonadal (HPG) axis during childhood [117] and to a more pronounced awakening around two years before puberty onset, also known as the

slow growth period [118-120]. Thus, the HPG axis during infancy, and especially prior to puberty, may be activated to “prime” spermatogonia proliferation prior to full activation at puberty.

This “priming” of spermatogonia proliferation provides a biological explanation for the epidemiologic data associating prepubertal environmental exposures with male germ line effects [121]. In Seveso, Italy, acute high TCDD exposure from a chemical plan accident during infancy/prepuberty was associated with reduced sperm concentration and motility, while the opposite was observed with exposure around puberty [122]. Moreover, a reduction in estradiol and an increase in FSH was observed in both groups; however, no changes in hormone levels or sperm quality were observed among TCDD-exposed adults [122]. In support of the observed time-dependent effects of TCDD, using population data in Överkalix, Sweden, studies reported that the grandchild experienced shorter survival and greater risk of diabetes mortality if the paternal grandfather experienced at least one “good” harvest during the ages of 9 to 12 and longer survival and decreased risk of diabetes if the paternal grandfather experienced at least one “poor” harvest during the same age period [123-125]. More recently, male, but not female, offspring of men who smoking before the age of 11 were found to have an increased BMI at age 7 and increased waist circumference and fat mass by age 13 [124], which persisted through the latest follow-up at age 17 [126]. Offspring of mothers who reported smoking before the age of 11 showed no increase in BMI up to age 17 [126]. Together, these studies, while they lack sperm epigenetic data, provide compelling data indicating that the prepubertal period, a

time in which the HPG axis begins to awaken to drive spermatogonia proliferation, is a sensitive period in which environmental exposures may target the epigenetic programming of germ cells. Epidemiologic studies are needed to confirm these observational studies by demonstrating that environmental exposures during the prepubertal period are associated with sperm epigenetics across generations.

Spermatogenesis in Adulthood

To date, the majority of experimental research in animals has focused on environmental exposures during *in utero* epigenetic reprogramming of PGCs with little regard to other susceptible periods occurring in the adult. Spermatogenesis, the final process of germ cell development that entails the progression from diploid spermatogonia to haploid spermatozoa, requires dynamic epigenetic reprogramming for the production of viable sperm for fertilization (figure 1). In humans, spermatogenesis is estimated to take around 74 days (around 35 days in mice) to produce mature spermatozoa from undifferentiated spermatogonia, and it can be divided into two sequential processes: spermatocytogenesis, which includes spermatogonial proliferation and differentiation through mitosis to produce spermatocytes and meiosis I and II to produce round spermatids; and spermiogenesis, in which differentiation and maturation of spermatids occurs without further cellular division (Figure 1). In the end, 32 spermatozoa are produced from one type B spermatogonium in humans, which is in great contrast to rodents where premeiotic cell divisions are intense, such that one spermatogonium has the potential to produce 4,096 spermatids [111]. This dramatic difference in clonal expansion of male germ cells among man and rodents

may beget caution in the interpretation of rodent data. For example, if an epigenetic error such as DNA methylation occurs in the first few cell divisions in humans, this error, if not corrected would propagate to affect only a few spermatozoa in a large pool, compared to the same scenario in mice where this error is likely to be more pronounced.

Acquisition and loss of methylation have also been reported during spermatocytogenesis [91, 127]. In adult mice, passive demethylation, likely occurring during spermatogonial cell divisions, was found to be enriched in interspersed repeat sequences, while methylation acquisition was observed in the pachytene stage of primary spermatocytes and was enriched in non-repeat sequences located within or flanking gene bodies as well as in paternal iDMRs [91]. The mechanism of this targeted resetting of DNA methylation during spermatocytogenesis may be linked with the expression of BORIS [127], a testis-specific protein paralogous to the insulator protein of imprinted marks, CTCF. Interestingly, BORIS and CTCF were expressed in a mutually-exclusive manner during spermatogenesis in mice and humans [127]. The proposed model suggests that BORIS is upregulated in primary spermatocytes and associates with demethylases that erase methylation marks and once CTCF is reactivated (and BORIS removed), targeted *de novo* methylation of paternal imprints and other regions is initiated in postmeiotic cells [128], which contradicts previous findings [91], likely due to methodological differences in methylation detection. Furthermore, age-dependent intra-individual alterations in sperm DNA methylation have been reported, indicating that sperm methylation can be modified throughout

the adulthood [39]. Taken together, these data signify that spermatocytogenesis is an important developmental period that shapes DNA methylation profiles of mature spermatozoa.

After acquisition of final DNA methylation profiles, spermatids enter spermiogenesis, a multi-step developmental window of global reorganization of chromatin [129]. Starting during meiosis, the canonical histones, H1, H2A, H2B and H3, are replaced by testis-specific variants, which decrease the stability of nucleosomes [130, 131]. Next, hyperacetylation of histone tails occurs, most notably at H4K5 and H4K8, which "relaxes" nucleosomes to further enhance histone destabilization[129]. Brdt, a testis-specific protein harboring two bromodomains capable of specifically recognizing acetylated histones, is then recruited to H4K5 and H4K8 acetylation to facilitate histone removal [132, 133]. Transition nuclear proteins, TNP1 and TNP2, then displace histones and are themselves replaced with the protamine proteins, protamines 1 and protamine 2 (PRM1 and PRM2), which are typically found in equal proportions [134]. Protamine packaging of DNA restricts transcriptional activity and therefore has been proposed as a nontraditional form of epigenetic regulation unique to sperm cells [26]. It also is critical to enhance motility and safeguard the paternal genome from the harsh environment soon to be encountered in the epididymis and female reproductive tract [135].

This histone-protamine exchange; however, is not complete, such that an estimated 10% and 1% of histones in humans and mice, respectively, are retained in mature sperm [136, 137]. Histone retention is also not randomly distributed

throughout the genome, suggesting that they may play a form of postfertilization epigenetic regulation. Several studies using human and mouse sperm report that histone retention is enriched in regulatory regions of developmental and imprinted genes [136, 138-141]. However, two recent studies contradict these findings showing that nucleosomes were generally not located in promoter regions including developmental promoters but rather in gene-poor regions [142, 143]. Additional work is needed to resolve these opposing findings before a definitive role for sperm nucleosomes, as well as their histone modifications, is assigned to embryo development.

Upon exiting the testes, spermatozoa are morphologically transformed but are immotile and lack fertilization potential. Sperm maturation occurs through sequential modifications within distinct microenvironments during the 1–2 week transit through of the epididymis, which is estimated to be 6-7 meters long in humans [144, 145]. Additionally, epididymal-specific exosomes ("epididymosomes") are reported to act as carriers of somatic proteins and RNAs to sperm [146-148]. Interestingly, epididymal secretions are regulated by androgens [149, 150], indicating that environmental factors that disrupt endocrine signaling may impact sperm procurement of exosomal proteins and RNAs [151]. Thus, while epididymal sperm maturation is directed at the acquisition of fertilization potential, exosomal shuttling may also provide the final opportunity for sperm to "epigenetically match" their current environment prior to fertilization. To our knowledge, no study has examined the direct effect of environmental

exposures in the epididymis on the sperm epigenome; however, recently developed model systems may provide future insights [147].

Nutritional manipulations. Along with *in utero* environmental exposures, emerging data indicate that the epigenome during the dramatic transformation of male germ cells that occurs in spermatogenesis is also susceptible to environmentally-induced epigenetic programming. Nutritional manipulation, such as low-protein diet [152] and pre-diabetic conditions [153], in adult rodents induce metabolic disorders in offspring through changes in sperm epigenetics of founder male mice. For example, a low protein diet in adult mice resulted in the down-regulation of transcriptional factors and chromatin regulators as well as a decrease in H3K27me3 of specific loci in sperm; however, genome-wide DNA methylation was largely unresponsive to the diet [152]. This latter finding is in contrast to other studies, such that streptozotocin-induced prediabetes conferred widespread alterations to sperm DNA methylation patterns [153]. The susceptibility of chromatin to nutritional manipulation during spermatogenesis is most recently highlighted in work in *Drosophila*, where high sugar diet in adult males altered methylation of H3K9/K27me3 within chromatin-bound regions of mature sperm that conferred metabolic programming of offspring [154]. Similarly, high fat diets in adult mice resulted in altered miRNA content [155] and increased the acetylation of H3K9 in late round spermatids to early elongating spermatids, possibly mediated by a corresponding decreased expression of SIRT6, a stress-response deacetylase [156]. The effect of high-fat diet on global DNA methylation of sperm, however, is inconsistent [155, 157]

Toxicant exposure. Exposure of adult mice to particulate air pollution obtained from Hamilton, Ontario increased global methylation of spermatogonia, which persisted through spermatogenesis and remained elevated in mature sperm [158]. Interestingly, these effects were observed after 10 weeks, but not after 3 weeks, of exposure, and persisted for 6 weeks after exposure removal, indicating that the epigenetic modifications occurred in early stages of spermatogenesis (e.g., premeiotic germ cells) [158]. Using a gene-candidate approach, chromium (III) chloride exposure to adult mice for two weeks decreased sperm DNA methylation of the 45S ribosomal RNA gene [159, 160].

In regard to iDMRs, adult exposure to methoxychlor, an endocrine disrupting compound, decreased sperm DNA methylation of the paternal iDMR of Meg3 and increased methylation of the maternal iDMRs of Mest, Snrpn and Peg3 [108]. Similarily, acrylamide exposure for two weeks in adult rats decreased sperm DNA methylation of IGF2 iDMR after 35 days, but not after 19 days, indicating that imprinted regions of spermatogonia and primary spermatocytes are susceptible to environmental exposures [161]. These studies demonstrate that the loss and gain of methylation in iDMRs during spermatocytogenesis as previously described [91, 127] can be modified by environmental exposures.

In humans, eight cross-sectional studies in adults to date have documented that chemical exposures, mostly cigarette smoking, are linked with alterations to the sperm epigenome (Table 1). Sperm from adult male smokers exhibited altered miRNA expression [162], higher LINE-1 methylation [163], elevated histone-to-protamine ratios [164, 165], and increased global acetylation of H4K8 and H4K12

[166], compared to sperm from non-smokers, suggesting that chronic smoking exposure may lead to a host of epigenetic changes in the sperm, though imprinted genes H19 and IGF2 were unchanged [167]. In regard to endocrine disrupting compounds, exposure to perflouroalkyl substances among a general population study in Europe did not find consistent associations between exposures and global as well as repetitive sequence DNA methylation [168]. Miao et al. (2013) found that urinary BPA exposures were inversely associated with LINE-1 methylation in occupationally-exposed workers as well as in non-exposed workers with low exposures. Interestingly, no significant associations were found between urinary BPA and LINE-1 methylation of leukocyte DNA [169]. This observed decrease in LINE-1 methylation in sperm may have strong public health implications as the occupationally non-exposed workers in the study had lower BPA levels than what has been reported for the US general population [170].

Sperm epigenetics and offspring development

After fertilization, parental-specific epigenetic marks of gametes undergo reprogramming to establish totipotency in the developing embryo. The kinetics of demethylation differs between parental genomes, whereas the paternal genome is actively and the female genome is passively demethylated [171] (figure 1). While it has been widely recognized that parental-specific iDMRs and certain classes of repetitive sequences, such as IAPs, escape this reprogramming event [172], sperm DNA methylation in other genomic loci may also be resistant to reprogramming and also contribute to this non-Mendelian form of inheritance, as demonstrated in the numerous studies discussed in the previous sections.

Technical advances in next-generation bisulfite sequencing of small quantities of cells have recently allowed for the resolution of genome-wide methylation maps of mouse gametes and through post-implantation embryogenesis to better understand gamete-specific heritable DMRs. In addition to known iDMRs, one study identified over 1,600 CpG island germline DMRs between oocytes and sperm and over half of these were found to be at least partially resistant to demethylation of which 34 were sperm-methylated germline DMRs [173]. Moreover, Meissner and colleagues identified over 4,894 sperm-derived DMRs that were enriched in intergenic regions and retained intermediate methylation values during demethylation [174]. Similarly, 34 sperm-derived DMRs identified within CpG islands were also partially resistant to demethylation [173]. However, in both of these studies, these DMRs appear to be targets for *de novo* methylation after implantation [173, 174]. The relevance of these sperm-derived DMRs in regard to environmental exposures and epigenetic inheritance remains unclear.

Furthermore, the epigenetic inheritance via sperm is not confined to DNA methylation, as other epigenetic factors such as histone retention and ncRNA are likely to act, in concert, to elicit paternal epigenetic inheritance. Since sperm protamines are quickly replaced, within 1 hour, by oocyte-derived histones in the zygote [175, 176], the location and modifications of retained histones in the sperm genome likely provide a structural framework to govern reprogramming events within the paternal genome. Similarly, sperm-derived RNAs, including ncRNAs, are proposed to influence embryo development and transgenerational inheritance by providing a window into the environmental history of sperm [177]. For example,

paternal stress in adult mice altered sperm miRNA content as well as offspring stress responsivity [178]. Recent data also indicate that sperm-derived factors may not be the only paternal component for proper embryo development, such that ablation of the seminal plasma by surgical excision in mice impaired conception, and among surviving offspring, altered growth trajectory and metabolic parameters [179]. Recently, human seminal exosomes were found to harbor unique profiles of small ncRNAs, including miRNAs, Y RNAs and tRNAs [180]. These results indicate that the seminal plasma is not only a transport medium for sperm but contains important non-genetic constituents, such as hormones and exosomes, that act to regulate the female tract environment to support embryo development [181].

Conclusions

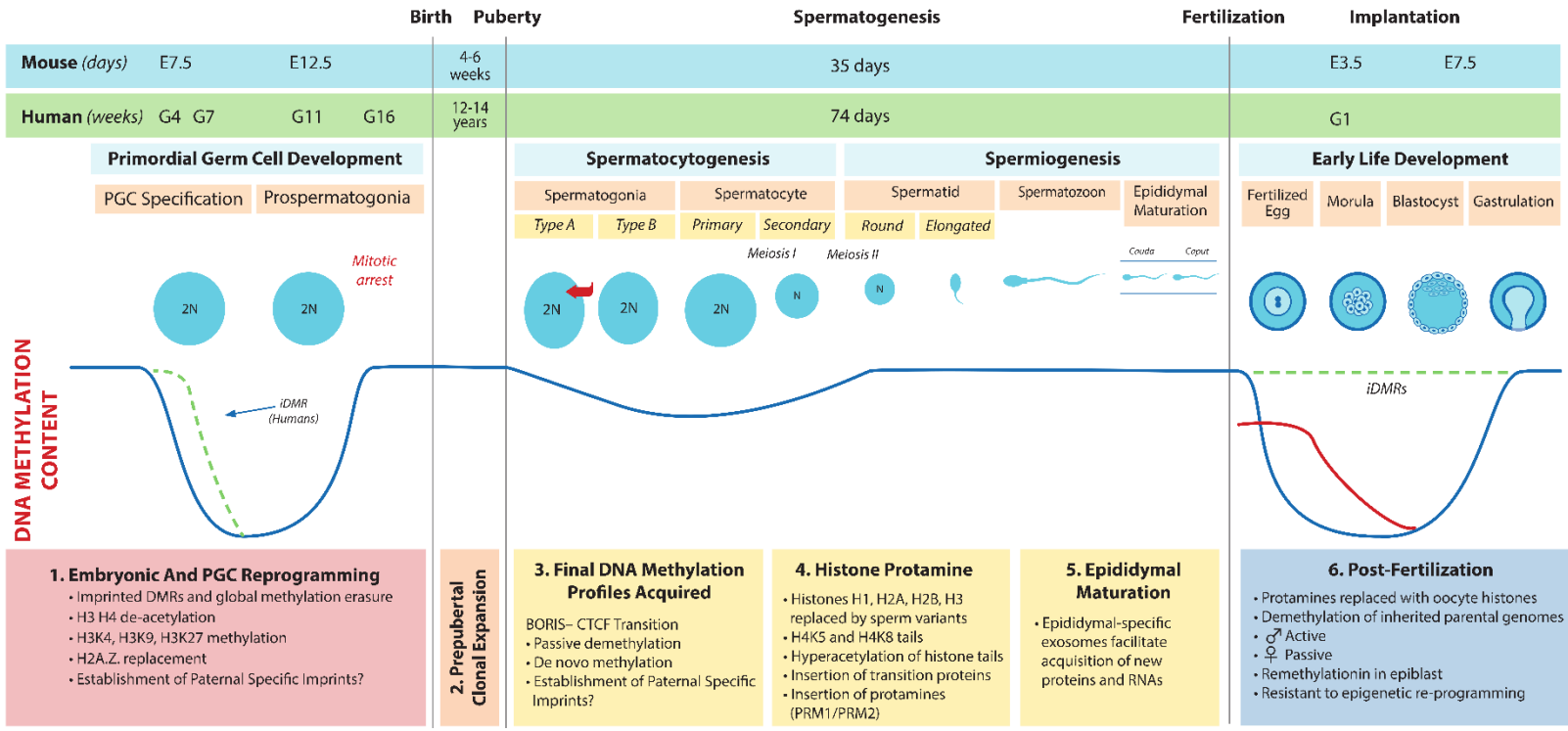
There are numerous epigenetic reprogramming events throughout the life-course of the male germ cell and each may represent a unique window of susceptibility to environmental exposures. Data demonstrate that such inputs from the environment are embodied within the epigenome of sperm, and in turn, are acquired during embryo development. Future animal research needs to expand on these findings by characterizing the full spectrum of sperm epigenetic changes induced by environmental exposures at each window of germ cell development. Additionally, prospective cohort studies are necessary to determine the response of sperm epigenetics in relation to early life environmental exposures. Understanding sperm epigenetics is critical to advance our understanding of paternal environmental determinants of offspring health and development. Such

research may result in a paradigm shift in the way reproductive success is viewed, such that the burden of environmental health may not be restricted to expectant mothers but rather is shared with male partners. In this manner, males may need to monitor their environmental health months prior to conception in order to optimize their sperm epigenome for fertilization.

Table 2.1. Summary of the epidemiologic studies of environmental influences on sperm epigenetics in adulthood.

Life Period	Design	Exposure	Main Results	Reference
Adulthood	Cross-Sectional	Smoking	25 unique miRNA showed different expression levels between smokers and non-smokers	Marczylo et al. 2012
Adulthood	Cross-Sectional	Smoking	Before swim up, acetylation of H4K8 and H4K12 sperm cells were statistically significantly increased in smokers compared to non-smokers while no significant changes were observed in the global 5-mC%, or acetylation of H3K9, H3K14, H4K5, and H4K16. The sperm cells isolated after swim up revealed no differences in acetylation of any histone or global 5-mC%	Kim et al. 2015
Adulthood	Cross-Sectional	Smoking	Heavy smokers showed significantly higher percentage of sperm cells with elevated histone-to-protamine ratios compared to never smokers	Yu et al. 2014
Adulthood	Cross-Sectional	BPA	BPA exposure is significantly correlated with lower sperm Line-1 methylation among Chinese factory workers, including those exposed to BPA levels equal or lower than reported in the U.S. general population	Miao et al. 2014
Adulthood	Cross-Sectional	Smoking	Smokers showed more abnormal histone to protamine transition compared to non-smokers	Hammadeh et al. 2010
Adulthood	Cross-Sectional	Smoking	Smoking is associated with elevated methylation of Line-1, but not Alu and Sata.	Consales et al. 2014
Adulthood	Cross-Sectional	Smoking	H19 and IGF2 methylation were not different between smokers and non-smokers	Ouko et al. 2009
Adulthood	Cross-Sectional	Perfluoroalkyl	No consistent associations between exposure to perfluooralkyl substances (perfluorooctane sulfonate, perfluorooctanoic acid, perfluorohexane sulfonic acid, perfluorononanoic acid) and global or repetitive sequence (Line-1, Alu, Satα) DNA methylation	Leter et al. 2014

Figure 2.1. Windows of susceptibility during male germ cell development.



(1) Primordial germ cells (PGCs) arise from proximal epiblast (E7.5 in mouse and G4 in humans) and undergo clonal expansion as they migrate and colonize the genital ridge. Epigenetic remodeling of histone and DNA methylation marks of PGCs are essential to achieve totipotency for sex-specific epigenetic programming. In mice, comprehensive loss of methylation in PGCs occurs (around E13.5) passively via Uhrf1 silencing and actively via Tet proteins to remove imprinted marks; while in humans, the first wave occurs around G7 with the second wave,

via loss of H3K27me3, to erase imprinted marks at G11. Afterward, de novo methylation occurs via Dnmt3a, Dnmt3b, and the non-catalytic Dnmt3l. Histone modifications after PGC specification include hypoacetylation of H3 and H4; hypermethylation of H3K4, H3K9, and H3K27; and replacement of the histone variant, H2A.Z. (2) After birth, rapid expansion of spermatogonia occurs in mice; however, after an initial clonal expansion, germ cells remain most dormant with intermittent expansion, most notable a few years before puberty upon awakening of the HPG axis. This prepubertal clonal expansion may be susceptible to environmental exposures as indicated by epidemiologic evidence. (3) Initiated at the onset of puberty by the activation of HPG axis, spermatogenesis occurs in the seminiferous epithelium and is supported by mitotically inactive Sertoli cells. Final DNA methylation patterns, including imprinted domains, are acquired possibly via CTCF–BORIS switch during spermatocytogenesis. Also, histone variants begin to be incorporated. (4) During the first stage of spermiogenesis, extensive chromatin remodeling occurs via the histone-protamine exchange, with acetylation of histone, insertion and removal of transition proteins, and then insertion of protamines 1 and 2. Approximately 90 and 99 % of histones are replaced with protamines in humans and mice, respectively. (5) During epididymal maturation, the last stage of spermiogenesis, germ cells become motile and exosomes shuttle proteins and ncRNA to mature spermatozoa. (6) Shortly after fertilization, the two parental genomes are demethylated in an asymmetrical manner: the paternal genome is actively depleted of DNA methylation, while the maternal genome (shown in read), which harbors

substantially lessDNAmethylation than sperm, undergoes a passive loss ofDNA methylation that is characterized by a dilution effect as a result of the lack of maintenance of DNA methylation over multiple cleavage divisions. Demethylation is not complete as imprinted genes intra-cisternal A particles (IAPs) and heterochromatin regions around centromeres largely escape this demethylation event. Sperm protamines are replaced with oocyte histones with hours of fertilization.

CHAPTER 3

RAPID METHOD FOR THE ISOLATION OF MAMMALIAN SPERM DNA

Summary

This chapter presents a novel method for isolation of high quality sperm DNA for genetic and epigenetic studies. Previously published methods are slow, labor intensive, and use chemicals ill-tolerated in *in vitro* fertilization (IVF) settings. The new protocol is simple, reduces the workflow from 24 hours to 20 minutes, and eliminates the need for unstable and odorous chemicals.

Abstract

There is a growing interest in elucidating the role of sperm genetics and epigenetics on reproductive success and offspring health. The unique DNA packaging renders spermatozoa resistant to isolation techniques used for somatic cells and existing protocols use slow and labor intensive methods. Our objective was to develop a rapid method for isolating high quality sperm DNA. Isolated human sperm cells were homogenized with 0.2mm steel beads for 5 minutes at room temperature in the presence of guanidine thiocyanate lysis buffer supplemented with 50mM tris(2-carboxyethyl)phosphine (TCEP). Our method resulted in yields >90% of high quality DNA using three different commercially available silica-based spin columns. DNA yields did not differ between immediate isolation (2.84±0.04 pg/cell) and after two weeks of homogenate storage at room temperature (2.91±0.13 pg/cell). DNA methylation analyses revealed similar methylation levels at both time points for three imprinted loci. Our protocol has

many advantages: it is conducted at room-temperature; lengthy proteinase K digestions are eliminated; the reducing agent, TCEP, is odorless and stable at room-temperature; nucleic acids are stabilized to allow storage of homogenate; and it is adaptable for other mammalian species. Together, our improved method has important implications for settings where sample processing constraints may exist.

Methods Summary

Our optimized protocol utilizes bead-based homogenization to facilitate sperm cell lysis in concert with an odorless reducing agent, tris(2-carboxyethyl)phosphine (TCEP), to dissociate disulfide bonds without the use of proteinase K. After homogenization, DNA can be extracted by user-preferred silica-based spin columns for a total processing time of 15-20 minutes. Our protocol has many advantages: it is conducted at room-temperature; lengthy proteinase K digestions are eliminated; the reducing agent, TCEP, is odorless and stable at room-temperature in aqueous solutions; nucleic acids are stabilized to allow storage of homogenate for future DNA isolation; and it is adaptable for other mammalian species.

Introduction

There is a growing interest in elucidating the role of sperm genetics and epigenetics on reproductive success and the life-course trajectory of health outcomes of subsequent generations. Recent genetic studies have shown a positive association between germline *de novo* mutations and paternal age [182-185]. Aberrations in sperm DNA methylation of imprinted genes [35, 186, 187] and

epigenome-wide dysregulation [138, 188, 189] have also been reported among men with infertility issues such as low sperm count and sperm quality. Moreover, compelling animal data indicate that the epigenome of sperm harbors a legacy of environmental exposures that can influence offspring phenotype [92, 96, 153].

Spermatogenesis requires extensive epigenetic reprogramming during the progression from diploid spermatogonia to haploid spermatozoa and involves stage- and testis-specific gene expression and mitotic and meiotic divisions [83, 84]. Extensive reorganization of chromatin structure occurs where 90% and 99% of histones are replaced by protamines in humans and mice, respectively [136, 137]. During this protamine-histone transition, tight compaction of the sperm nucleus is achieved by the oxidation of cysteine-rich residues of protamines and the subsequent formation of disulfide bridges that link protamines together [190]. This nuclear compaction is necessary for sperm motility and protection of the genome from oxidation within the female reproductive tract [135]. Furthermore, the protamine bound packaging of DNA precludes transcriptional activity and has been considered a nontraditional form of epigenetic regulation unique to sperm cells [26].

The unique DNA packaging renders spermatozoa resistant to DNA isolation techniques used for somatic cells [191, 192]. The development of efficient methods for isolating DNA from mammalian sperm has been a gradual process. All existing protocols use a combination of three components to gain access to sperm DNA: 1) detergents and/or chaotropic salts to facilitate cell lysis; 2) proteinase K (ProK) to digest nuclear proteins; and 3) reducing agents to break disulfide bonds

between protamines. One such widely adopted method for the isolation of mammalian sperm DNA uses an ionic detergent, sodium dodecyl sulfate (SDS), ProK, and either dithiothreitol (DTT) or 2-mercaptoethanol (βME). After overnight incubations at 55°C, DNA is isolated by ethanol precipitation [188] or silica-based spin columns [92]. Other popular approaches utilize guanidine salts, such as guanidinium thiocyanate (GTC), as the cell lysis reagent. GTC is a chaotropic agent that disrupts cell membrane and organelles by solubilizing individual molecules or cellular structures, including separating nucleic acids from associated proteins [193]. In addition, it is able to denature proteins, inactivate nucleases, and enhance activity of ProK [193, 194]. Bahnak et al. first reported a protocol incorporating GTC in a lysis buffer along with the ionic detergent, Sarkosyl, and βME [191]. However, this protocol required overnight incubations and time consuming CsCl ultracentrifugation for DNA isolation. More recently, this protocol has been modified to include ProK in the lysis buffer, which significantly reduced incubation time to 2 hours and replaced lengthy CsCl ultracentrifugation with isopropanol precipitation of DNA, resulting in 80% yield of sperm DNA [194, 195].

While the previous methods for sperm DNA isolation have progressed over time, they still have drawbacks. First the limited stability of DTT, βME, and ProK in aqueous solutions at room temperature requires fresh preparation of lysis buffers and involves long incubations ranging from 2 hours to overnight at 56°C [188, 194-196]. In addition, DTT and βME possess odors that may not be tolerated, especially in clinical settings. Finally, most protocols recover DNA from sperm lysate through ethanol precipitation, which increases processing time and may

result in co-precipitation of proteins and/or ethanol carryover that may affect downstream applications.

Given the need for a simple and rapid protocol for sperm DNA isolation, we developed a novel approach for isolating high quality sperm DNA. Our protocol incorporates a five minute mechanical homogenization step in the presence of a guanidine-based lysis buffer and a thiol-free reducing agent, TCEP, to facilitate sperm cell lysis and dissociation of disulfide bonds without the use of ProK. Sperm lysate is then applied to silica-based columns for the isolation of high-quality DNA with > 90% yield. To further streamline our protocol, we use commercially-available reagents that are stable at room temperature. This method is likely to expedite genetic and epigenetic research of sperm in clinical settings as well as in other mammalian species.

Methods

Isolation of sperm cells**:** This study was approved by the Institutional Review Board at the University of Massachusetts Amherst (#2014-2337). All participants gave written informed consent and were required to have at least 48 hours of abstinence prior to each donation. Five healthy male participants each donated multiple whole ejaculate samples throughout the course of the study. To remove somatic cell contamination, sperm cells were isolated using a continuous one-step 90% gradient (Sage, Beverly, MA: ART-2100 and ART-1006) as per the manufacturer's protocol. Sperm pellets were washed once, re-suspended, counted on a hemocytometer using the average of eight grid areas, and visually inspected

for somatic cell contamination. Isolated sperm from individuals ranged from 20 to 109 million cells.

Cell Lysis**:** Reducing agents, TCEP (Pierce, Rockford, IL, Catalog: 77720, final concentration: 10-50mM), DTT (Promega, Madison, WI: V3151 final concentration: 150 mM), or βME (final concentration: 2%), were added to Buffer RLT (Qiagen, Limburg, Netherlands Catalog: 79216) to a final volume of 500 µl. Three different homogenization techniques were also evaluated: 1) sperm were pulse-vortexed in lysis buffer for 5 minutes, diluted 1:1 in nuclease free water, and then incubated with ProK (final concentration: 200 µg/mL) at 56°C for 2 hours; 2) sperm were pulse-vortexed in lysis buffer for 5 minutes and lysates were loaded onto Qiashredder columns (Qiagen: 79656) and centrifuged for 2 minutes at max speed (≥17,000 x g); 3) sperm cells in homogenized in the presence of lysis buffer and 0.1g of 0.2mm stainless steel beads (Next Advance, Averill Park, NY, Catalog: SSB02) for 5 minutes on a Disruptor Genie (Scientific Industries, Bohemia, NY, Catalog: SI-238). To ensure equal aliquots of sperm, resuspended sperm cells after gradient isolation were vortexed for 10 seconds between each aliquot as previously described [197].

DNA Isolation**:** Sperm DNA was extracted with three different commercially-available kits using modified protocols:

AllPrep DNA/RNA Mini Kit (Qiagen: 80204). Lysates were added to spin columns and centrifuged at 10,000 x g for 30 seconds to bind DNA. Subsequent washing steps followed manufacturer's protocol. To elute, 50 uL of Buffer EB (preheated to 70°C) was added to the columns, incubated at room temperature for

3 minutes, and centrifuged for 1 minute at max speed. This was repeated twice for a total elution volume of 150uL.

QIAamp DNA Mini Kit (Qiagen: 51304). Lysates were combined with equal volumes of Buffer AL and 100% ethanol, loaded onto the spin columns, and centrifuged at 6,000 x g for 1 minute to bind DNA. Wash and elution steps followed manufacturer's protocol, including three separate 200 µl elutions to maximize yield.

Quick-gDNA MiniPrep (Zymo, Irvine, CA*,* Catalog*: D3025).* DNA/RNA Shield (Zymo: R1100) and Quick gDNA Genomic Lysis Buffer (included in kit), were used for sperm lysis instead of Buffer RLT. Samples in the Genomic Lysis Buffer were loaded onto the columns while samples in DNA/RNA Shield were combined with 3 volumes of Genomic Lysis Buffer before loaded onto spin columns. Samples were centrifuged at 10,000 x g for 1 minute to bind. Wash and elution steps followed manufacturer's protocol for a final elution volume of 100 uL.

DNA yields and quality were determined using the Nanodrop 2000 Spectrophotometer (Thermo Scientific, Somerset, NJ: E112352). A total of 350 ng of genomic DNA was resolved on a 0.7% agarose gel at 100V for 45 minutes, stained with 0.5 µg/mL ethidium bromide solution and visualized on a BioDoc-It Imaging System (UVP: M-26X). Provided that DNA quantity of a haploid cells is expected at 3 pg/cell, DNA yields were calculated by observed yield/expected yield based on cell count. The full protocol for DNA isolation is provided in Appendix A.

RNA isolation: Sperm cell lysate may be partitioned for the isolation of sperm RNA by adding 1:1 ratio of Qiazol and following the protocol of Goodrich et al. [198] starting at step 18 under section 3.1.

DNA Methylation Analysis: DNA methylation analyses of imprinted genes were performed on Sequenom's MassARRAY platform, which uses RNA base-specific cleavage (MassCLEAVE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for quantitative DNA methylation analyses of PCR-amplified bisulfite-converted DNA [199]. Briefly, 7.5 ng of bisulfite converted DNA (EZ DNA Methylation Kit, Zymo Research: D5002) was amplified with reverse primers containing a T7-promoter tag. Primers and PCR conditions are provided in Appendix B. After treatment with shrimp alkaline phosphatase to remove unincorporated dNTPs, amplification products were subjected to *in vitro* transcription and T-specific cleavage and were then analyzed by MALDI-TOF MS. The C/T changes introduced by bisulfite treatment are reflected as G/A changes on the T7-directed RNA transcript and result in a mass difference of 16 Da for each CpG dinucleotide. The level of methylation for each CpG unit was quantified using EpiTYPER software and methylation across loci was calculated as the average methylation of individual CpG units.

Results and Discussion

To improve processing time and work flow, we exploited several areas in current protocols where considerable improvements could be achieved. Because recently published methods for sperm DNA isolation relied on user-prepared GTC-based lysis buffer and ethanol precipitation, we reasoned that commercially-available GTC lysis buffers could offer an effective alternative to user-prepared lysis buffers as well as provide optimal DNA binding conditions for silica-based spin columns, thereby avoiding ethanol precipitation. Similar to the reported yield

of 80% in a recent GTC-based method with ethanol precipitation [194], treatment of sperm cells with diluted Buffer RLT, 150 mM DTT, and 200 µg/mL ProK for two hours and DNA isolation via AllPrep DNA columns resulted in a 79% yield (Figure 2.1, Panel A; 2.37 ± 10 pg/cell). As an alternative, we examined the utility of TCEP, an odorless, room-temperature stable, thiol-free reducing agent primarily used for protein biochemistry. We found that 50 mM TCEP (Figure 2.1, Panel A; 2.32 ± 0.09 pg/cell) resulted no appreciable differences in DNA yields compared to 150 mM DTT, indicating that TCEP is a viable alternative to thiol-based reducing agents for the isolation of sperm DNA.

With Buffer RLT's high concentration of GTC (2.8-4M), we also reasoned that lengthy ProK digests could be circumvented if sperm cells are efficiently lysed. Therefore, we examined the utility of different mechanical homogenization techniques (Figure 2.1, Panel B). QIAshredder, a column based mechanical homogenization system offered by Qiagen for the rapid homogenization of cells and tissues, resulted in better yield (Figure 2.1, Panel B; 1.51 ± 0.23 pg/cell) compared to no homogenization (Figure 2.1, Panel B; 0.42 ± 0.01 pg/cell), but was less effective compared to the 80% yields achieved by ProK digestions.

We next incorporated the novel homogenization method used for sperm RNA isolation developed by Krawetz and colleagues that utilizes 0.2mm stainless steel beads [198]. In the presence of Buffer RLT and 50 mM TCEP, homogenization with 0.2mm stainless steel beads resulted in >90% yields (Figure 2.1, Panel B; 2.78 ± 0.23 pg/cell). Given the interest in obtaining sperm DNA from abnormal sperm parameters (e.g., low count), we determined the efficiency of our

method was >80% (e.g. 2.40 pg/cell) with 5 x 10^5 or more cells. Moreover, we tested our protocol down to 1 x 10^4 cells and were able to isolated sufficient DNA for bisulfite PCR of imprinted genes (data not shown).

Next, we evaluated the compatibility of the bead-based homogenization procedure with other reducing agents as well as other commercially-available DNA column kits. As compared to 50mM TCEP, we found no appreciable differences in DNA yields for 150 mM DTT and 2% βME using the same beads-based homogenization (data not shown). Furthermore, ProK digestion after homogenization did not increase DNA yield (data not shown), indicating that lengthy ProK digestions are unnecessary. Therefore, this homogenization procedure circumvented the need for time-consuming ProK incubations. As compared to the AllPrep DNA columns, QIAamp DNA and and Zymo's Quick-gDNA columns resulted in slightly higher overall yields (Figure 2.1, Panel C; 2.70 ± 0.09 pg/cell vs. 2.95 ± 0.13 pg/cell and 3.01 ± 0.28 pg/cell, respectively. Use of Zymo's Quick-gDNA kit by replacing Buffer RLT with their genomic lysis buffer (contains 4M GTC) resulted in lower 260/280 and 260/230 ratios compared to the other kits (data not shown).

We then aimed to test the stability and integrity of DNA in Buffer RLT and 50 mM TCEP after bead homogenization. Compared to immediate processing (T0), two week storage (T2) at room temperature showed no decrease in DNA yield (Figure 2.1, Panel D: T0; 2.84 ± 0.05 pg/cell vs T2; 2.91 ± 0.13 pg/cell, respectively). Sperm genomic DNA resolved on 0.7% agarose gel revealed high molecular weight DNA that displayed no differences in integrity between samples

processed immediately (T0) or after two week storage (T2) at room temperature (Figure 2.2). Finally, to verify the feasibility of our protocol with downstream applications, we conducted DNA methylation analyses of three imprinted loci (SNURF, PEG10, H19). As expected for sperm DNA [200], differentially methylated regions (DMRs) of maternally-expressed, H19, displayed high levels of methylation, while DMRs of paternally- expressed genes, SNURF and PEG10, showed very low level of methylation (Figure 2.3). Furthermore, we found no differences between samples that were processed immediately (T0) and after two week storage (T2) at room-temperature for DNA methylation levels of H19 (T0: 94.1% ± 0.1% and T2: 92.2% ± 1.9%), SNURF (T0: 3.5% ± 0.7% and T2: 2.6% ± 1%), and PEG10 (T0: 3.7% ± 1.8% and T2: 6.9% ± 3.2%).

The use of the commercially-available GTC lysis buffers streamlined our protocol by eliminating the preparation of lysis buffers and allowing for optimal binding conditions for standard silica column-based DNA kits. We recommend Qiagen's RLT lysis buffer and columns because 1) DNA yields and 260/280 ratios were consistently greater than 90% and 1.80, respectively and 2) sperm cell lysates can be partitioned for sperm RNA purification using a previously published protocol [198]. In addition to the DNA columns from the AllPrep DNA/RNA kit, we found that the QIAamp DNA Mini kit and Zymo's Quick-gDNA MiniPrep kits can also be adapted to use the RLT-based lysate with similar total yields. However, there are drawbacks to both. The QIAamp DNA Mini Kit achieves similar total yield compared to AllPrep DNA/RNA kit, but it requires a higher elution volume for maximum yield (600 µL), thereby lowering final concentrations. This may not be

desirable when there is limited starting material. While Zymo's Quick gDNA kit had the highest yield, it must be noted that the 260/280 and 260/230 ratios were consistently low, < 1.8 and < 1.0, respectively, suggesting low purity of DNA due to protein and/or GTC carryover.

Clinical-based research can present its own set of unique challenges. In studies where semen samples are processed at an in vitro fertilization laboratory, organic thiols may not be permissible due to their strong sulfur odor. TCEP irreversibly reduces disulfides and is reported to be more effective at reducing disulfides than DTT below pH 8.0 [201], which represents the typical pH of GTC lysis buffers (e.g. Qiagen RLT is pH 7.0). Moreover, while DTT and βME readily undergo atmospheric oxidation, TCEP is resistant to oxidation allowing for room temperature storage in aqueous solutions. Our results show no appreciable difference in DNA yield between TCEP and thiol reductants and supports previous data that TCEP is equally effective as DTT in lysing sperm cells and may provide an effective substitute for organic thiols [197]. Together, TCEP offers an odor-free and room-temperature stable alternative for the reduction of disulfide bonds, thereby making it our preferred reducing agent for DNA isolation of sperm cells.

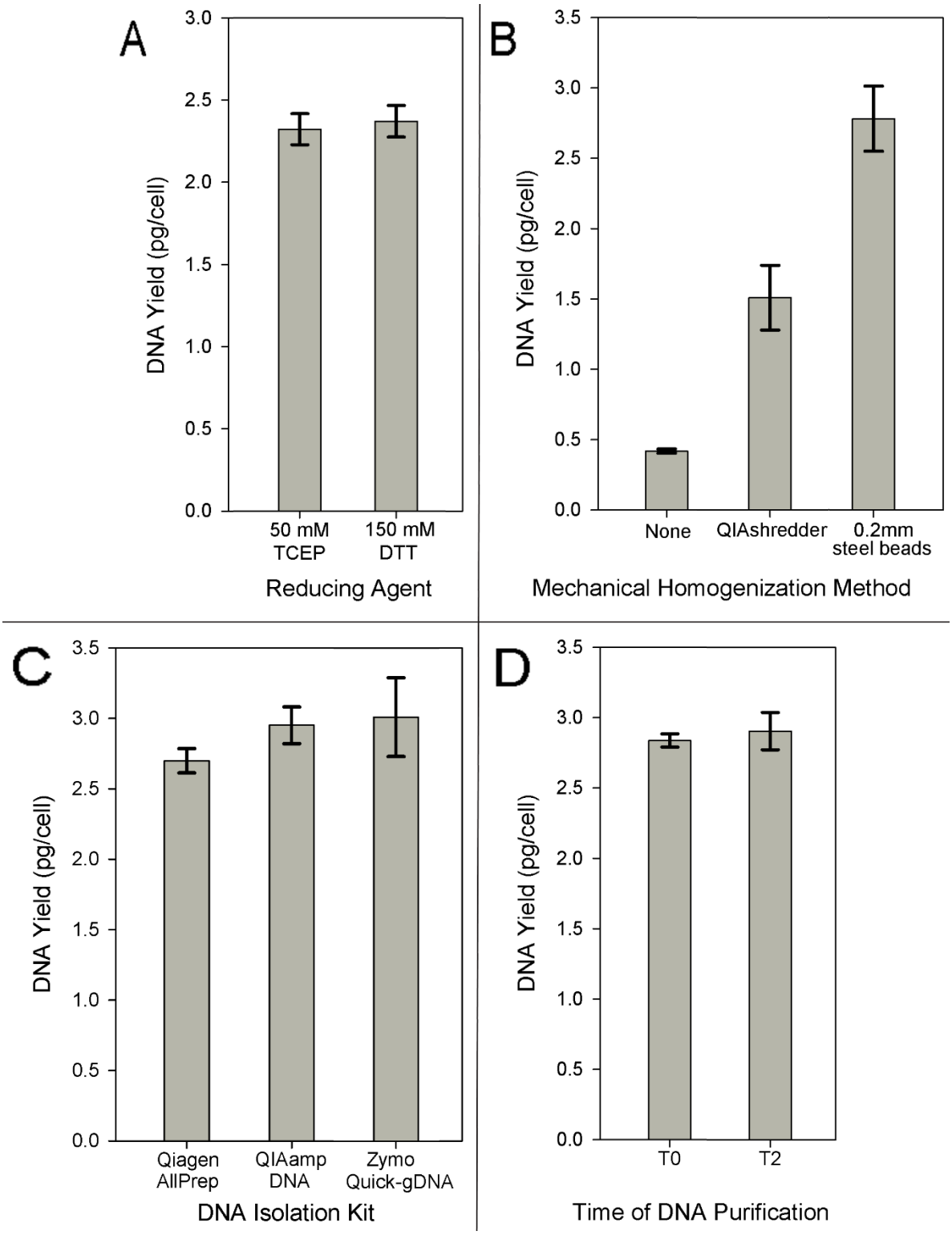
The workflow of our protocol presents several practical advantages when isolating sperm DNA. First, our protocol provides flexibility, such that the stability of DNA after homogenization allows for the convenient storage of lysates for future downstream DNA isolation. Secondly, our protocol is streamlined by incorporating commercially available reagents that are stable at room temperature and eliminating the need for lengthy ProK digestions. These first two points have

important implications, especially in regard to research in clinical settings when technician time may be limited. Moreover, the potential for lysate storage prior to DNA isolation and the use of commercially available reagents minimizes potential batch effects when conducting large epidemiologic studies. Our protocol is also amenable to partitioning lysates for RNA purification from the same sample. For example, given a 500 µl of lysate that contains 3 x 10^7 sperm, 100 µl of lysate is expected to provide, at 90% yield, approximately 16 µg of DNA with our protocol. The remaining 400 µl of lysate can then be used for the isolation of RNA, which is in extremely low quantities in sperm (50 fg/cell) [198]. By incorporating the sperm RNA isolation method of Goodrich et al [198], our theoretical example is estimated to provide 1.2 µg of RNA. These yields provide ample nucleic acids for many downstream applications, including next generation sequencing techniques. Additionally, our protocol is optimized for silica-based spin columns, thereby avoiding ethanol precipitation procedures. Lastly, our protocol can be extended for isolation of sperm DNA in other mammalian species. For example, our method is equally effective in isolating sperm DNA from mice (data not shown).

In conclusion, our optimized 5 minute room-temperature homogenization protocol results in > 90% yield of high quality sperm DNA by utilizing steel beads to facilitate sperm cell lysis in concert with an odorless reducing agent, TCEP, to dissociate disulfide bonds without the use of ProK. After homogenization, in lieu of lengthy ethanol precipitation, DNA can be extracted by user-preferred silica-based spin columns for a total processing time of 15-20 minutes. Our protocol also stabilizes nucleic acids to allow for optional storage of homogenate for DNA

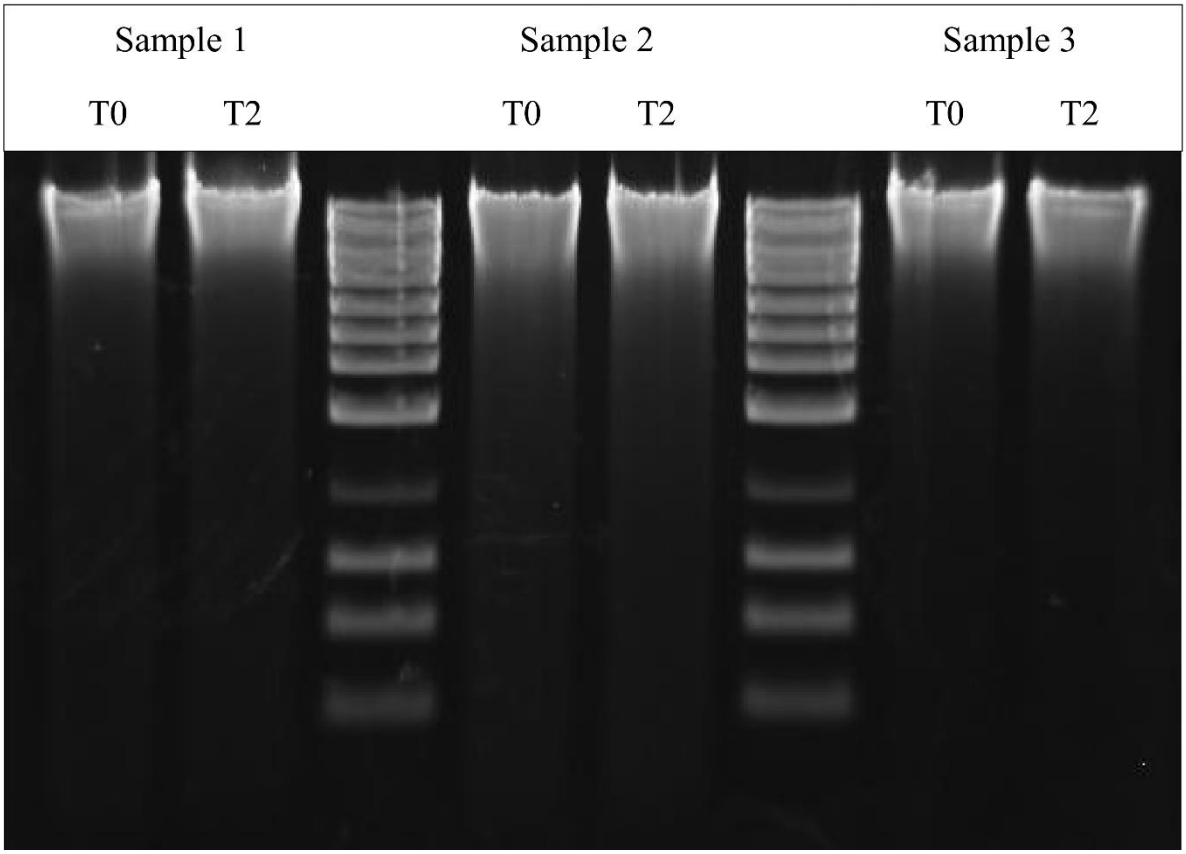
isolation at a later date. A schematic and full protocol of the DNA isolation method is found in Figure 2.4 and Appendix A, respectively. Together, our improved method has important practical advantages for research in clinical settings where sample processing constraints likely exist.

Figure 3.1 Utility of tris(2-carboxyethyl)phosphine (TCEP) as a reducing agent for the isolation of sperm DNA



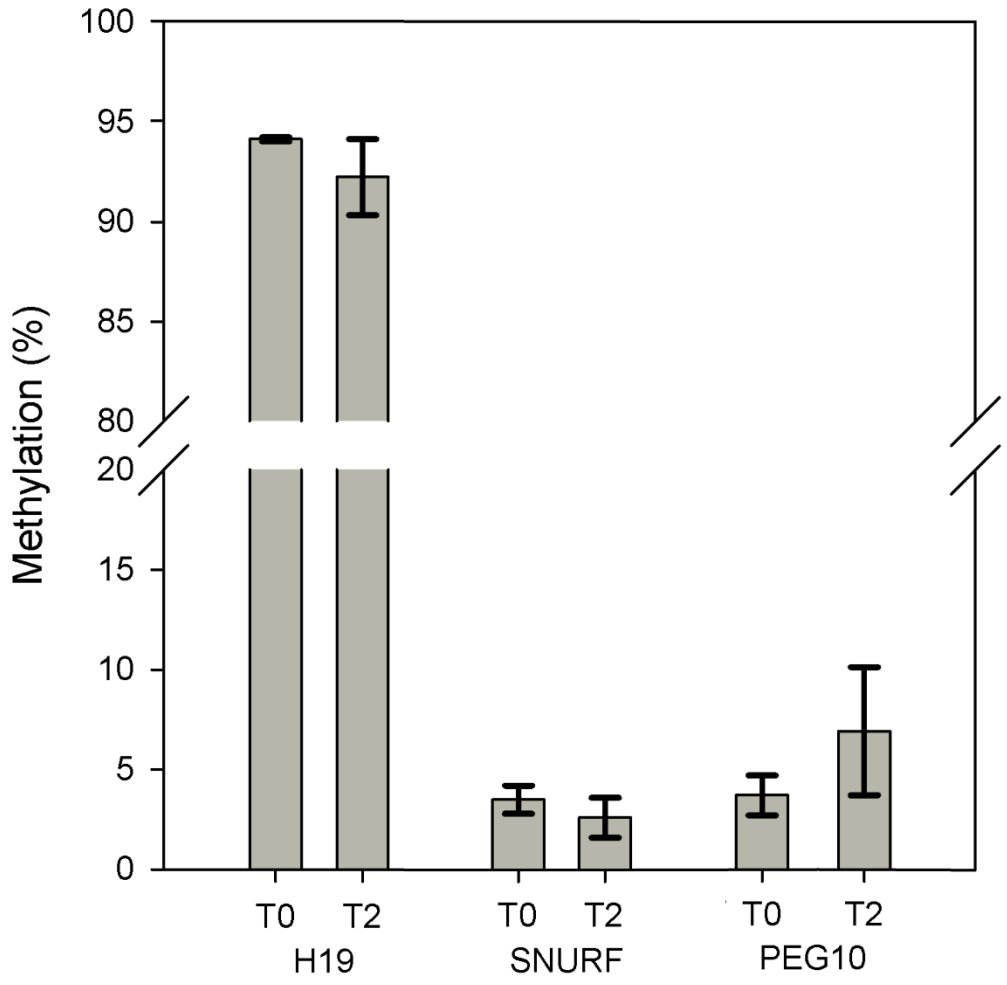
(A) Mean (±SD) DNA yield (pg/cell) of sperm cells treated with Buffer RLT, either 50 mM TCEP or 150 mM DTT, and proteinase K (ProK) for two hours at 56°C. DNA was isolated from sperm cell lysate via AllPrep DNA columns. (B) Mean (±SD) DNA yield (pg/cell) of sperm cells treated with Buffer RLT (50 mM TCEP) and the following homogenization methods: none; Qiashredder (as directed); or 0.1 g of 0.2 mm stainless steel beads. DNA was isolated from sperm cell lysate via AllPrep DNA columns. (C) Mean (±SD) DNA yield (pg/cell) of sperm cells purified with Qiagen AllPrep, QIAmp Mini, or Zymo Quick-gDNA spin columns. Sperm cells were homogenized by 0.2 mm stainless steel beads in either Buffer RLT (AllPrep and DNA Mini) or Zymo gDNA lysis buffer (Quick gDNA), both supplemented with 50 mM TCEP. (D) Mean (±SD) DNA yield (pg/cell) of sperm cells homogenized by 0.2 mm stainless steel beads in the presence of Buffer RLT and 50 mM TCEP. DNA was isolated from sperm cell lysate via AllPrep spin columns processed immediately (T0) and after 2 weeks storage (T2) at room temperature (22°C).

Figure 3.2 Electrophoresis of sperm DNA



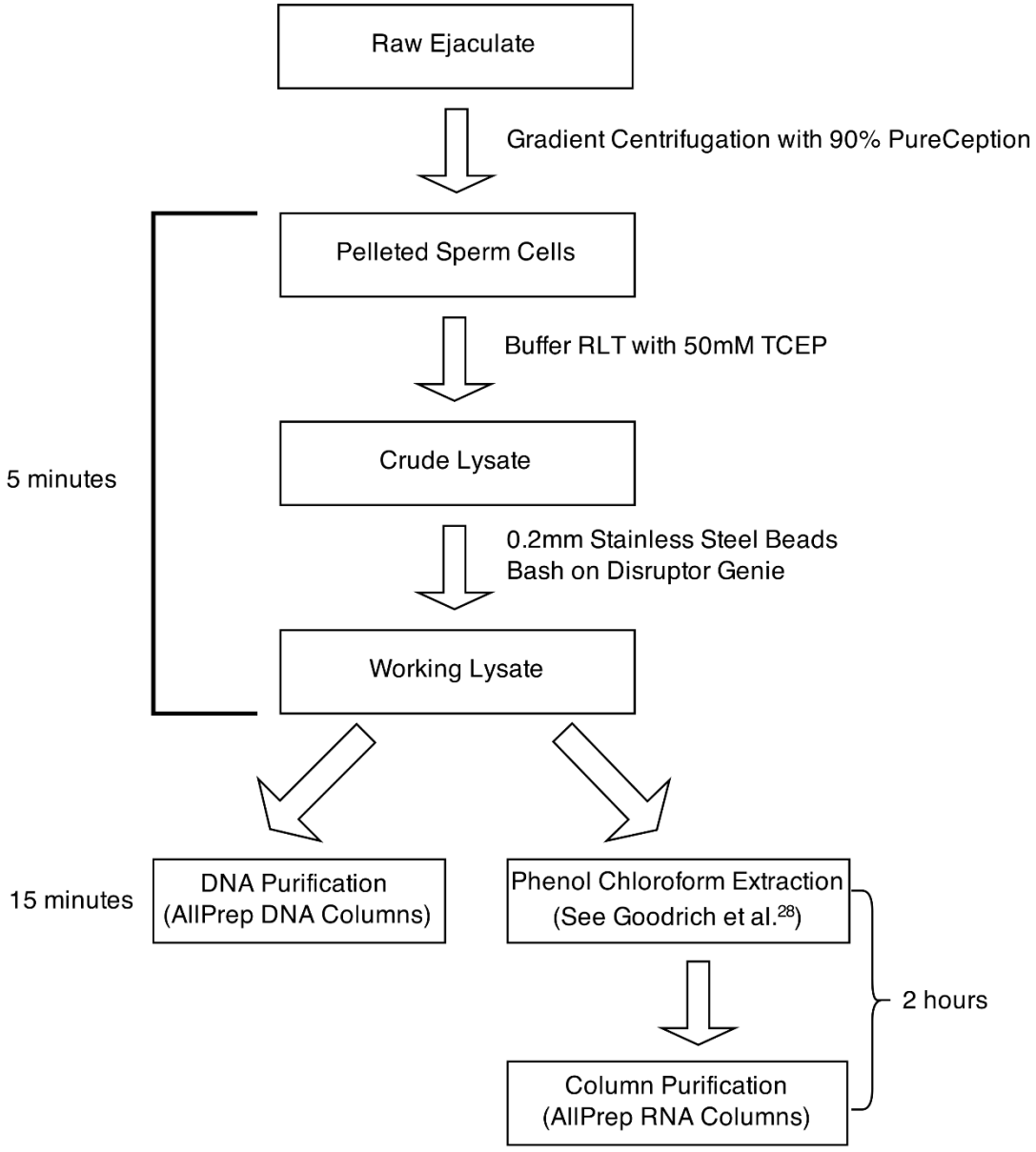
Sperm DNA isolated from 3 individuals immediately (T0) and after 2 weeks of storage at room temperature (T2) on a 0.7% agarose gel.

Figure 3.3 DNA methylation of three imprinted loci.



Mean (±SD) percentage DNA methylation of CpG sites within imprinted regions of *SNURF*, *PEG10*, and *H19* using NA isolated from sperm lysate immediately (T0) and after 2 weeks of storage at room temperature (T2).

Figure 3.4 Schematic of sperm DNA isolation workflow.



Sperm cells are isolated from ejaculate via gradient centrifugation with 90% PureCeption. Pelleted sperm cells are then homogenized with Buffer RLT, 50 mM tris(2-carboxyethyl)phosphine (TCEP), and 0.1 g of 0.2 mm stainless steel beads on a Disruptor Genie for 5 min. This produces a working lysate that is ready for DNA and RNA isolation.

CHAPTER 4

PARENTAL CONTRIBUTIONS TO EARLY EMBRYO DEVELOPMENT: INFLUENCES OF URINARY PHTHALATE AND PHTHALATE ALTERNATIVES AMONG COUPLES UNDERGOING IVF TREATMENT

Summary

This chapter evaluates the associations of parental urinary phthalate and phthalate alternative exposures with embryo quality through day 5 post-fertilization. Overall, we found that paternal, but not maternal urinary metabolite levels of select phthalate and phthalate alternatives were associated with a marked decrease in blastocyst quality.

Abstract

STUDY QUESTION: Are preconception urinary concentrations of phthalates and phthalate alternatives associated with diminished early stage embryo quality in couples undergoing *in vitro* fertilization (IVF)?

SUMMARY ANSWER: Male, but not female, urinary concentrations of select metabolites of phthalates and phthalate alternatives are associated with diminished blastocyst quality.

WHAT IS KNOWN ALREADY: Phthalates, endocrine disrupting compounds with widespread exposure worldwide, are associated with adverse reproductive health. Male and female preconception exposures to select phthalates have been

previously associated adverse reproductive outcomes in both the general population and in those undergoing IVF.

STUDY DESIGN, SIZE, AND DURATION: This prospective cohort included 50 subfertile couples undergoing IVF in Western Massachusetts.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The current study includes the first 50 couples recruited from the Baystate Medical Center's Fertility Center in Springfield, Massachusetts as part of the Sperm Environmental Epigenetics and Development Study (SEEDS). Relevant data from both partners, including embryo quality at the cleavage (day 3) and blastocyst (day 5) stages, were collected by clinic personnel during the normal course of an IVF cycle. A spot urine sample was collected from both male and female partners on the same day as semen sample procurement and oocyte retrieval. Seventeen urinary metabolite concentrations were quantified by liquid chromatography mass spectrometry (LCMS) and normalized via specific gravity. Generalized Estimating Equations (GEE) were used to estimate odds ratios (OR) and confidence intervals (95% CI), with urinary phthalates and phthalate alternatives fitted as continuous variables and embryo quality as a binary variable.

MAIN RESULTS AND THE ROLE OF CHANCE: The 50 couples contributed 761 oocytes, of which 423 progressed to the cleavage stage, 261 were high quality cleavage stage embryos, 137 were transferrable quality blastocysts, and 47 were high quality blastocysts. Adjusting for age of both partners, urinary phthalate concentrations of female partners, and male infertility status, male urinary monoethyl phthalate (MEP) concentrations were positively associated with high

quality cleavage stage embryos (OR=1.20, 95% CI 1.01-1.43., p=0.04); no other significant associations were observed at this stage. At the blastocyst stage, male urinary concentrations of monobenzyl phthalate (MBzP) (OR=0.55, 95% CI 0.36-0.84, p=0.01), mono-3-hydroxybutyl phthalate (MHBP) (OR=0.37, 95% CI 0.18-0.76, p=0.01), mono-n-butyl phthalate (MBP) (OR=0.55, 95% CI 0.42-0.73, p<0.01), and monomethyl phthalate (MMP) (OR=0.39, 95% CI 0.26-0.60, p<0.01) were inversely associated with high quality embryos. A borderline statistically significant relationship was observed for male concentrations of mono(2-ethylhexyl) phthalate (MEHP) (OR=0.52, 95% CI 0.27-1.00, p=0.05) and cyclohexane-1,2-dicarboxylic acid-monocarboxy isooctyl ester (MCOCH) (OR=0.21, 95% CI 0.04-1.03, p=0.05) at the blastocyst stage. Similar inverse associations were observed between male urinary phthalate metabolite concentrations and likelihood of being transferrable quality blastocysts. For female partners, select metabolites were positively associated with odds of high or transferrable blastocyst quality, but the observed associations were not consistent across blastocyst quality measures or between sex-specific and couples-level models.

LIMITATIONS, REASONS FOR CAUTION: Our modest sample size only included 50 couples contributing one cycle each. In addition, non-differential misclassification of exposure remains a concern given the single spot urine collection and the short half-life of phthalates.

WIDER IMPLICATIONS OF THE FINDINGS: Our results suggest that an inverse association between male preconception concentrations of select phthalate

metabolites and blastocyst quality, likely occurring after genomic activation. If corroborated with other studies, such findings will have public health and clinical significance to both the general population and those undergoing IVF.

Introduction

Endocrine disrupting compounds (EDCs) have received considerable attention due to their potential to disrupt hormonal function and their association with a range of adverse reproductive health consequences in humans [202, 203]. Phthalates, one class of EDCs, are used in a large variety of commercial products such as medical products, food packaging, personal care products, and solvents [204]. Because they are not chemically bound to products, phthalates are easily released into the environment resulting in chronic and widespread human exposure in the United States [41] and globally [205-208]. The potential of phthalates to adversely influence reproductive health has been well documented [209]. Similar to other EDCs, phthalates display anti-androgenic properties [60-62]. In addition, both male and female preconception phthalate exposure has been associated with a range of adverse pregnancy and birth outcomes [52, 58, 59, 210]. The recent rise in use of replacement phthalates and phthalate alternative compounds is a developing public health concern. Human exposure to these compounds has increased over the past decade [41]; however, there is limited research on their role in male and female reproductive health.

To our knowledge, only one cohort previously investigated the association of preconception phthalate concentrations with embryo development. Studies from

the Environmental and Reproductive Health (EARTH) have reported that male preconception urinary concentrations of select phthalate metabolites were associated with decreased odds of implantation and live birth among couples seeking fertility treatment while female phthalate metabolite concentrations were associated with lower mature and overall oocyte yield as well as decreased probability of clinical pregnancy and live birth; however, null associations were observed with embryo quality at the cleavage stage (day 3) [57, 68]. These results suggest that parental contributions to early-life development may appear after day 3, the cleavage stage, when the embryo transitions away from maternal control and zygotic genome activation occurs.

In light of the compelling evidence from these previous studies, we conducted a prospective study to examine the relationship of preconception concentrations of phthalates and phthalate alternatives on *in vitro* embryo quality through the blastocyst stage of development.

Methods

Study Population. The study population comprised 761 oocytes from the first 50 couples recruited as part of the Sperm Environmental Epigenetics and Development Study (SEEDS), a prospective cohort designed to investigate parental preconception exposures to EDCs and early life development. All couples were recruited from the Baystate Medical Center Fertility Center in Springfield, Massachusetts, which primarily serves western Massachusetts and northern Connecticut. Couples were included if the male partners were 18-55 years old without vasectomy, female partners were ≤ 40 years old with expected delivery at

Baystate Medical Center, and fresh ejaculate sperm were used for IVF, including intracytoplasmic sperm injection (ICSI). Relevant data on demographics, lifestyle factors, and medical history were collected by clinic personnel from both partners during the course of an IVF cycle. Prior to sample collection, each partner completed an intake questionnaire regarding lifestyle factors. Each couple contributed only one cycle to the study.

Written consent was obtained by attending physicians from eligible males and females interested in study participation. This study was approved by the institutional review boards at Baystate Medical Center and at the University of Massachusetts Amherst.

Urinary Biomarkers Measurement. A spot urine sample was collected from both male and female partners in a sterile polypropylene collection cup on the same day as semen sample procurement and oocyte retrieval. Urine samples were vortexed, divided into several aliquots, and stored at -80°C before being shipped overnight on dry ice to the CDC's National Center for Environmental Health, where the urinary biomarkers were quantified via enzymatic deconjugation, solid-phase extraction, separation, and analyses using LCMS [211].

In total, seventeen urinary metabolites were quantified: mono(2-ethylhexyl) phthalate (MEHP); mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP); mono(2-ethyl-5-oxohexyl) phthalate (MEOHP); mono(2-ethyl-5-carboxypentyl) phthalate (MECPP); mono-carboxy-isooctyl phthalate (MCOP); mono-isononyl phthalate (MNP); monobenzyl phthalate (MBzP); mono (3-carboxypropyl) phthalate (MCPP); monocarboxy-isononyl phthalate (MCNP); mono-n-butyl phthalate (MBP); mono-

3-hydroxybutyl phthalate (MHBP); mono-isobutyl phthalate (MiBP); mono-hydroxyisobutyl phthalate (MHiBP); monoethyl phthalate (MEP); monomethyl phthalate (MMP); cyclohexane-1,2-dicarboxylic acid-monocarboxy isooctyl ester (MCOCH); and cyclohexane-1,2-dicarboxylic acid-mono(hydroxy-isononyl) ester (MHINCH). The limits of detection (LODs) ranged from 0.2 to 0.6 ng/mL, depending on the metabolite. Specific gravity (SG) was measured at room temperature using a digital handheld refractometer (Atago Co., Ltd., Tokyo, Japan).

Embryo Quality Assessment. Embryos were evaluated on a five-point scale (1 being the best and 5 being the worst quality) on days 3 and 5 post-insemination, during the cleavage stage and blastocyst stage, respectively. A separate category was reserved for all arrested embryos at both time points. At the cleavage stage, embryos were evaluated morphologically for the presence or absence of blastomere multinucleation, symmetry, cell number, and amount of fragmentation using the Veeck system [212]. For example, embryos with 8 cells, no multinucleated blastomeres, and no fragmentation were considered grade 1, while embryos with 7 cells, no multinucleated blastomeres, and <10% fragmentation were considered grade 2, and embryos with 6 cells, no multimucleated blastomeres, and 20% fragmentation are grade 4. At the blastocyst stage, embryos were evaluated for the developmental stage including the expansion of blastocoel, and quality of trophectoderm and inner cell mass. For example, embryos at blastocyst stage with full expansion, hatching or hatched with large compact inner cell mass and well-defined cohesive trophectoderm with many cells

are considered grade 1 blastocyst. An expanded blastocyst with a large, mostly compacted inner cell mass and medium number of trophectoderm cells is classified as grade 2. Blastocysts lacking full expansion or having an inner cell mass comprised of a small number of cells or a small number of non-uniform trophectoderm cells are grade 4 quality. At both time points, grades 1-2 were classified as high quality embryos, similar to that of a previously published study [57], while at day 5 grades 1-4 were additionally classified as transferrable quality.

Statistical Analysis. Urinary biomarker concentrations below the limit of detection (LOD) were assigned a value of the LOD divided by the square root of 2. All urinary phthalate measurements were corrected for SG and log transformed. SG-correction followed the formula Pc=P[(SGm-1)/(SG-1)] where Pc is the SG-corrected urinary metabolite concentration (ng/mL), P is the measured metabolite concentration, SGm was the sex-specific median SG, and SG is the specific gravity of the urine sample. Geometric means and selected percentiles were calculated for both males and females to describe the distributions of SG-adjusted urinary phthalate and phthalate alternative metabolites. Wilcoxon Paired-Signed Rank test and Fisher's exact test were used to test for differences in distribution of demographics, where applicable.

For embryo level outcomes, general estimating equations (GEE), using a binomial distribution and exchangeable correlation structure, were used to account for the correlated structure of the embryo data within couples and to estimate the associations between partner urinary concentrations of phthalates and phthalate alternatives and embryo development measures through 5 days post insemination.

Log transformed and SG corrected urinary biomarker concentrations were fitted as continuous values when the majority of the population had levels above the LOD and dichotomous values based on the LOD when majority of the population had levels below the LOD. Fertilization, high quality, and transferable quality statuses for each embryo were fitted as a dichotomous outcome. All ORs represent the impact of a one unit increase in log transformed paternal urinary metabolite concentration, except for MCOCH and MHINCH, where the ORs represent above or below the limit of detection (0.4 and 0.5 ng/mL, respectively). Two types of models were fitted: sex-specific and couple-level. The sex-specific models included only the covariates of specific partners, male or female, but not both, whereas couples-level models adjusted for covariates and metabolite concentrations from both partners. Inclusion of covariates in multivariable models was based on biological plausibility and statistical significance ($p<0.1$) in bivariate models of embryo quality. Covariates considered for inclusion were age, body mass index (BMI), race, infertility status, alcohol use, and smoking status.

For additional sensitivity analyses, we restricted the embryos under analysis to mature oocytes, fertilized oocytes (for outcomes at days 3 and 5), embryos of sufficient quality to be cultured past cleavage stage (for blastocyst stage embryos), or oocytes from non-smokers. Lastly, to cross-validate our GEE model, we fitted 1) Poisson models at the couple level, aggregating the number of embryos and using the total number of embryos at day 3 as an offset, and 2) two-level mixed models specifying random intercepts and binomial distribution.

Analysis was performed with R (v3.3.0) using the “gee” package (v4.13.19) for GEE models. Statistical significance was defined as p-values < 0.05.

Results

Select demographics for the current study population of 50 heterosexual couples seeking fertility treatment are shown in Table 2.1. Compared to female partners, the male partners were 1.7 years older on average and more likely to be over the age of 40 whereas female partners were more likely to be between 30 and 40 years of age. The majority of the participants in our sample identified as non-Hispanic white, with only 4 males and 2 females identified as non-white. The mean body mass index (BMI) for males and females were 28.8 and 28.9, respectively, with 78% of males and 66% of females classified as overweight (BMI 25-29.9) or obese (BMI ≥30). Race and BMI were not found to be associated with embryo quality and thus were not included in the multivariable models.

The majority of the couples at the fertility clinic had at least one partner diagnosed with subfertility or infertility (Table 2.1). In our sample, 20 couples had a diagnosis of female factor alone (40%), 6 couples with male factor alone (12%), 8 couples with both (16%), and 16 couples with unexplained infertility (32%). Fourteen males were considered as infertile based on semen quality according to the World Health Organization references values (Cooper 2010). Females were diagnosed infertile based on a variety of known, non-exclusive, conditions. Of the 28 females classified as infertile, 10 had polycystic ovary syndrome, 9 were anovulatory, 7 had diminished ovarian reserve, 5 had tubal factor, 1 had endometriosis, and 6 had other diagnoses.

Descriptive statistics of couple-specific oocyte and embryo data are also presented in Table 2.1. A total of 761 oocytes were retrieved, yielding an average of 15.2 oocytes per couple per cycle. Of the 761 oocytes, 599 were mature at the time of insemination, 449 were fertilized, and 423 progressed to the cleavage stage. Three days after conventional IVF or ICSI, 261 of the oocytes developed into high quality cleavage stage embryos while 391 embryos were of sufficient quality to be cultured until day 5. Of those 391 embryos, 47 developed to high quality blastocysts at day 5; however, 137 were considered to be of transferrable quality.

The distributions of SG-adjusted urinary metabolite concentrations for male and female partners are reported in Table 2.2. For both male and female partners, 13 of the 15 of the measured urinary phthalate metabolite concentrations (MEHP, MEHHP, MEOHP, MECPP, MBP, MBzP, MiBP, MHiBP, MEP, MCPP, MCNP, MCOP, and MMP) had detection frequencies ranging from 75% - 100%. Urinary MHBP was detected in 74% of male samples and 65% of female samples, while urinary MNP was detected in 62% of male samples and 57% of female samples. Concentrations of MHINCH and MCOCH, metabolites of the phthalate alternative DINCH, were only detected in 43% and 16% of couples' samples, respectively, and were fitted as dichotomous predictor variables, above and below the LOD, in subsequent regression analyses. Compared to male partners, female partners had higher urinary concentrations for the four metabolites of DEHP (MEHP, MEHHP, MEOHP, and MECPP) and MCOP, MCNP, MHiBP, MHBP, and MEP ($p<0.05$).

In sex-specific and couple-level multivariate models, male or female urinary concentrations of phthalate and DINCH metabolites were not found to be associated with fertilization (Appendix B1). In sex-specific models, there were no significant associations between urinary biomarkers and embryo quality at the cleavage stage (Table 2.3). However, after the inclusion of partners' metabolite concentrations and age, there was a statistically significant positive association between male urinary MEP concentrations and high quality cleavage stage embryos (OR=1.20, 95% CI: 1.01-1.43).

Table 2.4 presents the associations between urinary biomarkers and high quality embryos at the blastocyst stage in sex-specific and couple-level multivariate analyses. Among male partners, there were statistically significant inverse associations between urinary concentrations of MBzP (OR=0.64, 95% CI: 0.44-0.94), MBP (OR=0.73, 95% CI: 0.57-0.94), MHBP (OR=0.38, 95% CI: 0.20-0.73), MMP (OR=0.62, 95% CI: 0.45-0.85), and MCOCH (OR=0.34, 95% CI: 0.13-0.90) and high blastocyst quality. In couple-level models with adjustment for female age and female metabolite concentrations, the significant inverse associations between male urinary metabolite concentrations and high blastocyst quality largely persisted: MBzP (OR=0.55, 95% CI: 0.36-0.84), MBP (OR=0.55, 95% CI: 0.42-0.73), MHBP (OR=0.37, 95% CI: 0.18-0.76), and MMP (OR=0.39, 95% CI: 0.26-0.60); however, the estimates for MCOCH became borderline significant (OR=0.21, 95% CI: 0.04-1.03; Table 2.4). In both models, male urinary MEHP concentrations were associated with a > 40% decrease in odds of high blastocyst quality, but it did not reach statistical significance in either model (p=0.08 and 0.05

in sex-specific and couple-level models, respectively). In sex-specific models, female urinary concentrations of MHiBP were positively associated with high blastocyst quality (OR=1.86, 95% CI: 1.01-3.4); however, this association did not reach statistical significance in couple-level models after adjusting for male age and male urinary MHiBP concentrations (OR=2.00, 95% CI: 0.95-4.17). Conversely, female urinary concentrations of MMP (OR=2.43, 95% CI: 1.23-4.79) and MCOCH (OR=2.51, 95% CI: 1.02-6.14) were found to be positively associated with high blastocyst quality in the couple-level models with adjustment for male age and urinary metabolite concentrations, but not in the sex-specific models.

When considering transferrable blastocyst quality (Table 2.5), the couple-level model estimates for male urinary concentrations of MBzP (OR=0.70, 95% CI: 0.48-1.01), MBP (OR=0.67, 95% CI:0.43-1.03), MHBP (OR=0.41, 95% CI: 0.21-0.80), MMP (OR=0.72, 95% CI: 0.52-1.00), and MCOCH (OR=0.41, 95% CI: 0.19-0.88) were all slightly attenuated compared to estimates from models of high quality blastocysts, but all remained either statistically significant or borderline significant ($p < 0.07$). However, male urinary concentrations of MEHP were not associated with transferrable blastocyst quality in either sex-specific or couples-level models. In contrast to the results for fertilization and cleavage stage embryos, male urinary concentrations of MEP were not associated with increased high (OR=0.69, 95% CI: 0.44-1.08) or transferrable (OR=1.06, 95% CI: 0.89-1.26) blastocyst quality. Female urinary concentrations of MHiBP (OR=1.56, 95% CI: 1.00-2.43) and MNP (OR=1.54, 95% CI: 1.14-2.09) were positively associated with transferrable blastocyst quality in the sex-specific models; however, in couple-level

models accounting for male age and metabolite concentrations, only MNP remained statistically significant (OR=1.74, 95% CI: 1.25-2.42).

A series of sensitivity analyses were conducted to validate the observed associations between male urinary metabolites and blastocyst quality. Sensitivity analyses were not performed on the DINCH metabolites due to small number of partners with detectable urinary concentrations. Eleven of the 15 phthalate metabolites were correlated between couples, three inversely and eight positively (Appendix C2). Of the four phthalate metabolites (MBzP, MBP, MHBP, and MMP) associated with diminished blastocyst quality, only MBzP ($r=0.50$, $p<0.01$) and MMP ($r=0.47$, $p<0.01$) were correlated between couples. Furthermore, stratification of the results for these four metabolites based on whether female partner's exposure was above or below the median (Appendix C3) showed similar effect estimates within the two strata of female urinary metabolite concentrations, further indicating that the associations of male phthalates on decreased blastocyst quality are independent of female concentrations.

Quartile analyses adjusting for male and female age, male infertility status, embryo grade at cleavage stage, and female urinary metabolite concentrations, revealed that male urinary MMP displayed an inverse dose-response relationship with high blastocyst quality. Moreover, most quartiles of male urinary MEHP, MBP, MHBP, and MBzP concentrations were negatively associated high blastocyst quality (Figure 1). Restriction of analyses to mature oocytes, fertilized oocytes, embryos of sufficient quality to be cultured past the cleavage stage, or oocytes from non-smokers did not appreciably alter the model estimates (Appendices C4-

C7). Similarly, inclusion of other variables that did not meet the criteria for covariate selection, such as BMI, race, smoking, and alcohol use, did not appreciably alter the effect estimates (data not shown). Lastly, to cross-validate our model fit, we used Poisson regression and mixed models logistic regression approaches, which produced similar estimates and trends compared to our GEE models (data not shown).

Discussion

In our prospective study of 761 oocytes from 50 couples undergoing IVF, we observed that select male urinary concentrations of phthalate and phthalate replacement metabolites were inversely associated with high embryo quality. Specifically, male urinary concentrations of MBzP, MBP, MHBP, MMP, and MCOCH were associated with a pronounced decrease in blastocyst quality; however, no such associations were observed during the cleavage stage for either male or female metabolites. Such results suggest that the negative influences of paternal phthalates on embryo quality arose after day 3 - during the transition from cleavage to blastocyst stage of embryo development. It is noteworthy to mention that zygotic genome activation occurs during this transition. Select female metabolites were associated with increased odds of high blastocyst quality, but the lack of consistency hinders the reliability and interpretability of these results. Overall, our observed inverse association of male preconception phthalate and phthalate alternative metabolites with blastocyst quality supports the growing evidence that preconception paternal environmental health may contribute to reproductive potential.

The findings from our study are supported by previous work from the LIFE Study [213], which reported that preconception male urinary concentrations of MMP, MBP, and MBzP were negatively associated with couples' fecundity, as measured by time to pregnancy (TTP) [52]. The association between male preconception phthalates and increased TTP is a broad observation and is likely facilitated by unobserved intermediate clinical endpoints throughout pregnancy such as altered embryo development, decreased chances of implantation, and increased chances of pregnancy loss. While the LIFE Study could not observe some of these early life endpoints, the EARTH study of 211 couples totalling 406 treatment cycles recently reported that higher male preconception urinary concentrations of MCPP and MCOP were associated with decreased odds of implantation and live birth following IVF; while MBP was associated with decreased odds of live birth following IUI, but not IVF [57]. In agreement with our findings, male urinary phthalate metabolite concentrations in the EARTH study were not associated with fertilization rate or embryo quality at day 3; however, it must be noted that blastocyst quality data were unavailable because the majority of EARTH participants received embryo transfers on day 3.

One advantage of our SEEDS cohort of couples undergoing fertility treatment was the investigation of early embryo development through blastocyst development. We found that five male concentrations of phthalates or phthalate alternatives metabolites, including all three aforementioned metabolites found to be associated with decreased fecundity in the LIFE Study, were associated with decreased blastocyst quality, resulting in a lower odds of high quality transferrable

embryos. However, in contrast to the findings that male urinary MCNP and MCOP concentrations were associated with decreased odds of implantation and live birth in the EARTH cohort, we found no such associations between urinary concentrations of these metabolites and embryo development. Such discrepancies in findings may be due to a smaller sample in our study and require further investigation. Lastly, male urinary MEP concentrations were associated with increased embryo quality at the cleavage stage in our study, but this association was not observed at the blastocyst stage. This particular observation may be either spurious or transient. Together with previous reports from the LIFE and EARTH cohorts, our results add to the growing body of literature suggesting that preconception male phthalate exposures are likely contributors to adverse reproductive health outcomes.

IVF laboratory protocols bypass direct exposure of female partners to seminal fluid and other paternal inputs; therefore, the only avenue for male environmental contributions to embryo development is through acquired characteristics of sperm. It follows that our observed negative association between male urinary metabolite concentrations of phthalates and blastocyst morphology imply a sperm-mediated effect. Though our results do not show altered embryo development associated with urinary metabolites at day 3, it is possible that the molecular changes associated with phthalates and phthalate alternatives were too subtle to be detected morphologically during these early cleavage stages and that such early molecular changes manifest at the morphological level during later stages of development. Alternatively, it is possible that urinary phthalates and

phthalate alternatives are not associated with embryo development until after day 3. Our observed associations with blastocysts, but not cleavage stage embryos, coincides with the timing of the embryonic genome activation (EGA). At the 4- to 8- cell stage, corresponding to day 3 of human embryo development [214], the human embryos are in a stage of relative transcriptional silence, relying on maternally-derived products for the first cell divisions [215, 216]. EGA facilitates a transition from maternal to embryonic control, which includes the degradation of maternal mRNAs and major changes in expression of histone isoforms, histone modifications, chromatin structure [217], and DNA methylation [218]. In this scenario, that the timing of observed morphological changes may be due to altered features of the paternal genome or factors that would otherwise govern the paternal genome. It has been hypothesized that paternal epigenetic inheritance of factors governing DNA expression is important for embryogenesis [219] and is modifiable by the male environment [30]. Furthermore, studies have reported that select phthalates, including those that are inversely associated with embryo quality in our study, have been associated with sperm DNA damage and abnormalities in humans [54, 55, 63, 64].

In recent years, certain phthalates have been substituted for newer phthalate compounds and phthalate alternative compounds due to their potential negative impacts on human health [220]. The detection frequency and concentrations of urinary metabolites of DiNP and DiDP, two relatively new phthalate compounds, have increased dramatically over the last decade. For example, the creatinine-corrected mean urinary concentration of MCOP, a

metabolite of DiNP, has risen from 5.26 µg/g creatinine in 2005-2006 to 22.4 µg/ng creatinine in 2011-2012 according to NHANES data [41]. Similarly, DINCH was commercialized in 2002 and has been detected in increasingly higher number of the general US population since 2007 [41, 211]. While most of the metabolites of the new phthalates and phthalate alternatives were not found to be associated with embryo quality, we observed that having detectable levels of MCOCH in the male partner's urine was associated with nearly 80% decreased odds of high blastocyst quality and 60% decreased odds of transferable blastocyst quality. However, we must note only 16% of male partners, contributing to 129 oocytes out of 761 , had detectable levels of MCOCH; therefore these estimates need to be interpreted cautiously and replicated in other studies.

To our knowledge, our study was the first prospective study to assess the associations between paternal exposure to phthalates and phthalate alternatives and embryo quality in humans. The urine samples were collected on the day of sperm donation and embryo retrieval and is representative of parental phthalate exposures prior to conception, eliminating any potential temporal bias. Unlike previous studies using couple level data, our study employed embryo level data, which allowed us to account for inter-individual differences that likely exist when considering the potential of oocytes and sperm to form high quality embryos. We also recognize some limitations of our study. First, our modest sample size only included 50 couples contributing one cycle each. However, data was collected on over 700 individual oocytes, including over 400 embryos. In addition, non-differential misclassification of exposure remains a concern given the single spot

urine collection and the short half-life of phthalates, though such non-differential misclassification of exposures will likely bias the results towards the null. Furthermore, previous studies suggest that a single spot urine is sufficient to represent phthalate exposure over several months [221, 222].

CONCLUSION

Results from our prospective study of 50 couples undergoing IVF show that male urinary metabolite concentrations of select phthalates and phthalate alternatives are inversely associated with high and transferrable quality blastocysts. Our study provides the first data demonstrating associations between phthalate and phthalate alternatives and embryo development, a critical step towards our understanding of the paternal preconception contributions to reproductive success. Future studies are needed to investigate the long term effects of altered embryo development and elucidate the mechanism by which paternal preconception exposure to phthalates and phthalate alternatives affects embryo development.

Table 4.1 Demographic and clinical characteristics of SEEDS couples (N=50).

Individual Characteristics	**Males**	**Females**
Age	35.8 ± 5.3	34.1 ± 4.2
<30	10 (20%)	9 (18%)
30-40	27 (54%)	38 (76%)
40+	13 (26%)	3 (6%)
Race		
White	39 (78%)	47 (94%)
Non-White	4 (8%)	2 (4%)
Unknown/Refused	7 (14%)	1 (2%)
BMI†	28.8 ± 5.0	28.9 ± 6.8
<25	11 (22.4%)	17 (34%)
25-30	19 (38.8%)	15 (30%)
30+	19 (38.8%)	18 (36%)
Couple/Cycle-Specific Characteristics	**Couples**	
Primary Infertility Diagnoses		
Male Factor Alone	6 (12%)	
Female Factor Alone	20 (40%)	
Both	8 (16%)	
Unexplained	16 (32%)	
Oocytes Retrieved	15.2 ± 7.9	
Mature Oocytes	12 ± 7.1	
Fertilized Embryos	9.0 ± 6.5	
Embryos at Day 3	8.5 ± 6.1	
High Quality Embryos at Day 3	5.2 ± 4.1	
Embryos Cultured Past Day 3*	8.1 ± 5.8	
Embryos at Day 5*	6.2 ± 5.5	
High Quality Embryos at Day 5*	0.94 ± 1.6	
Transferrable Embryos at Day 5*	2.7 ± 3.0	

Values are mean ± SD or N (%)
† Male BMI (N = 49)
*Excluded 2 couples with embryo transfers on day 3

Table 4.2. Distribution of specific gravity-adjusted urinary metabolite concentrations (ng/mL) of SEEDS couples (N = 50).

Parent Esters	Metabolite	Sex	% > LOD	GM†	Percentiles			
					25th	50th	75th	95th
HMW Phthalates								
DEHP	MEHP	Males	78	1.1	0.7	1.1	1.7	3.7
		Females	88	8.0	2.4	10.2	22.6	64.8
	MEHHP	Males	100	6.0	4.2	6.2	8.7	13.6
		Females	100	19.7	10	19.7	40.9	86.4
	MEOHP	Males	100	4.0	3	4	5.2	8.3
		Females	100	13.8	6.7	14.5	25.6	48.7
	MECPP	Males	100	8.8	6.5	9.2	12.7	18.4
		Females	100	27.8	15	27.9	49.3	118.2
DiNP	MCOP	Males	100	24.5	10.4	21.5	47.4	179.3
		Females	100	34.9	12.7	37.5	94.6	214
	MNP	Males	62	0.9	<LOD	0.7	1.6	6.4
		Females	57	1.0	<LOD	1	2.1	5.6
BBzP	MBzP	Males	98	3.7	2	3.7	7.8	20.8
		Females	98	4.4	1.8	4.6	9.2	49.3
DOP	MCPP	Males	98	2.1	1	1.8	3.8	13.5
		Females	92	2.4	1.2	2.1	4.2	10.8
DiDP	MCNP	Males	100	2.9	1.8	2.6	4.4	10.1
		Females	100	3.8	2.1	3.8	5.8	14.4
LMW Phthalates								
DBP	MBP	Males	96	6.9	5.6	7.4	11.2	21.9
		Females	90	6.7	4.3	7.9	13.9	23.4
	MHBP	Males	74	0.6	<LOD	0.6	0.9	1.5
		Females	65	1.0	<LOD	0.6	0.9	3.1
DiBP	MiBP	Males	100	6.2	4.1	6.6	10.2	15.3
		Females	100	5.8	3.1	6.4	10.3	18.9
	MHiBP	Males	98	1.9	1.4	1.9	2.7	5.4
		Females	94	2.8	1.8	2.8	4	9.9
DMP	MMP	Males	94	2.1	1.3	1.9	3.9	7
		Females	88	2.2	1.3	2.3	3.3	12.6
DEP	MEP	Males	100	25.1	9.2	19.5	49.9	246.7
		Females	100	43.1	17.2	38.7	85.1	327.2
Phthalate Alternative								
DiNCH	MCOCH	Males	18	*	<LOD	<LOD	<LOD	1.8
		Females	14	*	<LOD	<LOD	<LOD	1.7
	MHINCH	Males	44	*	<LOD	<LOD	0.9	2.4
		Females	43	*	<LOD	<LOD	0.5	1.9

LOD, limit of detection; GM, geometric mean
† For those samples < LOD, a value is assigned by taking LOD divided by the square root of 2.
* GM not calculated due to large number of samples < LOD.

HMW, high molecular weight; LMW, low molecular weight.

Abbreviations: Bis(2-ethylhexyl) phthalate (DEHP); diisononyl phthalate (DiNP); benzyl butyl phthalate (BBzP); dioctyl phthalate (DOP); diisodecyl phthalate (DiDP); dibutyl phthalate (DBP); diisobutyl phthalate (DiBP); diethyl phthalate (DEP); dimethyl phthalate (DMP); 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH); mono(2-ethylhexyl) phthalate (MEHP); mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP); mono(2-ethyl-5-oxohexyl) phthalate (MEOHP); mono(2-ethyl-5-carboxypentyl) phthalate (MECPP); monocarboxy-isooctyl phthalate (MCOP); mono-isononyl phthalate (MNP); monobenzyl phthalate (MBzP); mono (3-carboxypropyl) phthalate (MCPP); monocarboxy-isononyl phthalate (MCNP); mono-n-butyl phthalate (MBP); mono-3-hydroxybutyl phthalate (MHBP); mono-isobutyl phthalate (MiBP); mono-hydroxyisobutyl phthalate (MHiBP); monoethyl phthalate (MEP); monomethyl phthalate (MMP); cyclohexane-1,2-dicarboxylic acid-monocarboxy isooctyl ester (MCOCH); and cyclohexane-1,2-dicarboxylic acid-mono(hydroxy-isononyl) ester (MHINCH).

Table 4.3 Adjusted odds ratios (95% CIs) for high quality embryos at cleavage stage (day 3) and couples urinary metabolite concentrations of phthalates and phthalate alternatives.

Parent Esters	**Metabolite**	Males		Females		Males		Females	
		OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
HMW Phthalates[3]									
DEHP	MEHP	0.87	0.65-1.17	1.02	0.87-1.19	0.88	0.66-1.18	1.03	0.87-1.21
	MEOHP	0.89	0.56-1.41	1.07	0.90-1.28	0.93	0.57-1.51	1.12	0.91-1.39
	MEHHP	0.96	0.61-1.50	1.07	0.91-1.25	1.03	0.64-1.66	1.12	0.93-1.35
	MECPP	1.21	0.77-1.92	1.06	0.89-1.26	1.26	0.77-2.06	1.12	0.93-1.35
DiNP	MCOP	1.08	0.91-1.28	1.01	0.87-1.16	1.08	0.87-1.32	1.00	0.85-1.17
	MNP	0.93	0.76-1.14	0.96	0.82-1.14	0.92	0.71-1.21	1.02	0.81-1.27
BBzP	MBzP	1.01	0.84-1.23	1.08	0.94-1.25	0.96	0.77-1.21	1.08	0.91-1.29
DOP	MCPP	0.96	0.77-1.20	0.97	0.84-1.13	0.94	0.71-1.25	1.00	0.84-1.20
DiDP	MCNP	1.18	0.84-1.66	0.94	0.72-1.22	1.23	0.87-1.73	0.93	0.69-1.24
LMW Phthalates[3]									
DiBP	MBP	1.12	0.86-1.45	1.13	0.88-1.46	1.12	0.86-1.44	1.09	0.83-1.43
	MHBP	0.97	0.68-1.38	1.26	0.90-1.77	0.98	0.68-1.42	1.18	0.82-1.69
DBP	MiBP	0.89	0.67-1.17	1.06	0.82-1.38	0.84	0.61-1.15	1.11	0.82-1.51
	MHiBP	0.78	0.59-1.03	1.01	0.75-1.37	0.76	0.56-1.03	1.08	0.75-1.56
DMP	MMP	1.00	0.81-1.25	0.98	0.80-1.22	1.01	0.75-1.35	0.99	0.72-1.37
DEP	MEP	1.18	0.99-1.41	0.94	0.80-1.10	**1.20**	**1.01-1.43***	0.94	0.81-.109
Phthalate Alternative[4]									
DiNCH	MCOCH	1.08	0.65-1.81	1.06	0.44-2.55	1.01	0.49-2.07	1.27	0.48-3.37
	MHiNCH	1.12	0.69-1.81	0.79	0.52-1.20	1.17	0.71-1.93	0.84	0.54-1.31

[1]Each model adjusted for age and infertility status of the specific partner (i.e. not both)
[2]A single model adjusted for age of both partners and infertility status of male partner
[3]Log transformed and specific gravity-adjusted
[4]Dichotomous variable based on above/below LOD
* p-value < 0.05
** p-value <0.01
*** p-value <0.001
HMW, high molecular weight; LMW, low molecular weight. Other abbreviations are detailed in table 2.2.

Table 4.4 Adjusted odds ratios (95% CIs) for high quality embryos at blastocyst stage (day 5) and couples urinary metabolite concentrations of phthalates and phthalate alternatives.

Parent Esters	Metabolite	Sex-Specific Models[1]				Couple-Level Models[2]			
		Males		Females		Males		Females	
		OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
HMW Phthalates[3]									
DEHP	MEHP	0.57	0.30-1.06	1.11	0.84-1.45	0.52	0.27-1.00	0.99	0.82-1.19
	MEOHP	0.94	0.42-2.10	1.00	0.69-1.46	0.92	0.41-2.07	1.05	0.78-1.42
	MEHHP	0.75	0.37-1.53	1.00	0.71-1.40	0.72	0.35-1.51	1.00	0.77-1.30
	MECPP	0.77	0.39-1.51	0.90	0.58-1.38	0.76	0.38-1.51	1.03	0.74-1.44
DiNP	MCOP	1.01	0.60-1.70	1.02	0.63-1.66	1.03	0.63-1.67	0.99	0.60-1.62
	MNP	1.17	0.75-1.81	1.29	0.82-2.02	1.10	0.69-1.77	1.11	0.66-1.84
BBzP	MBzP	**0.58**	**0.42-0.82****	1.04	0.73-1.48	**0.55**	**0.36-0.84****	1.09	0.70-1.68
DOP	MCPP	1.10	0.60-2.01	1.05	0.56-1.99	1.11	0.63-1.96	1.07	0.56-2.07
DiDP	MCNP	0.96	0.46-2.01	0.94	0.39-2.27	1.01	0.45-2.27	1.08	0.41-2.88
LMW Phthalates[3]									
DiBP	MBP	**0.66**	**0.53-0.82*****	1.29	0.86-1.95	**0.55**	**0.42-0.73*****	1.31	0.87-1.98
	MHBP	**0.37**	**0.19-0.75****	1.16	0.52-2.58	**0.37**	**0.18-0.76****	0.88	0.50-1.56
DBP	MiBP	1.34	0.70-2.56	1.50	0.83-2.69	1.08	0.51-2.28	1.34	0.68-2.65
	MHiBP	1.16	0.53-2.54	**1.86**	**1.01-3.41***	0.86	0.41-1.78	2.00	0.95-4.17
DMP	MMP	**0.63**	**0.46-0.87****	1.17	0.62-2.20	**0.39**	**0.26-0.60*****	**2.43**	**1.23-4.79***
DEP	MEP	0.78	0.56-1.09	1.31	0.85-2.02	0.69	0.44-1.08	1.37	0.96-1.94
Phthalate Alternative[4]									
DiNCH	MCOCH	**0.34**	**0.13-0.90***	0.20	0.04-1.103	0.21	0.04-1.03	0.73	0.20-2.71
	MHiNCH	0.75	0.30-1.85	1.03	0.42-2.51	0.48	0.18-1.41	**2.51**	**1.02-6.14***

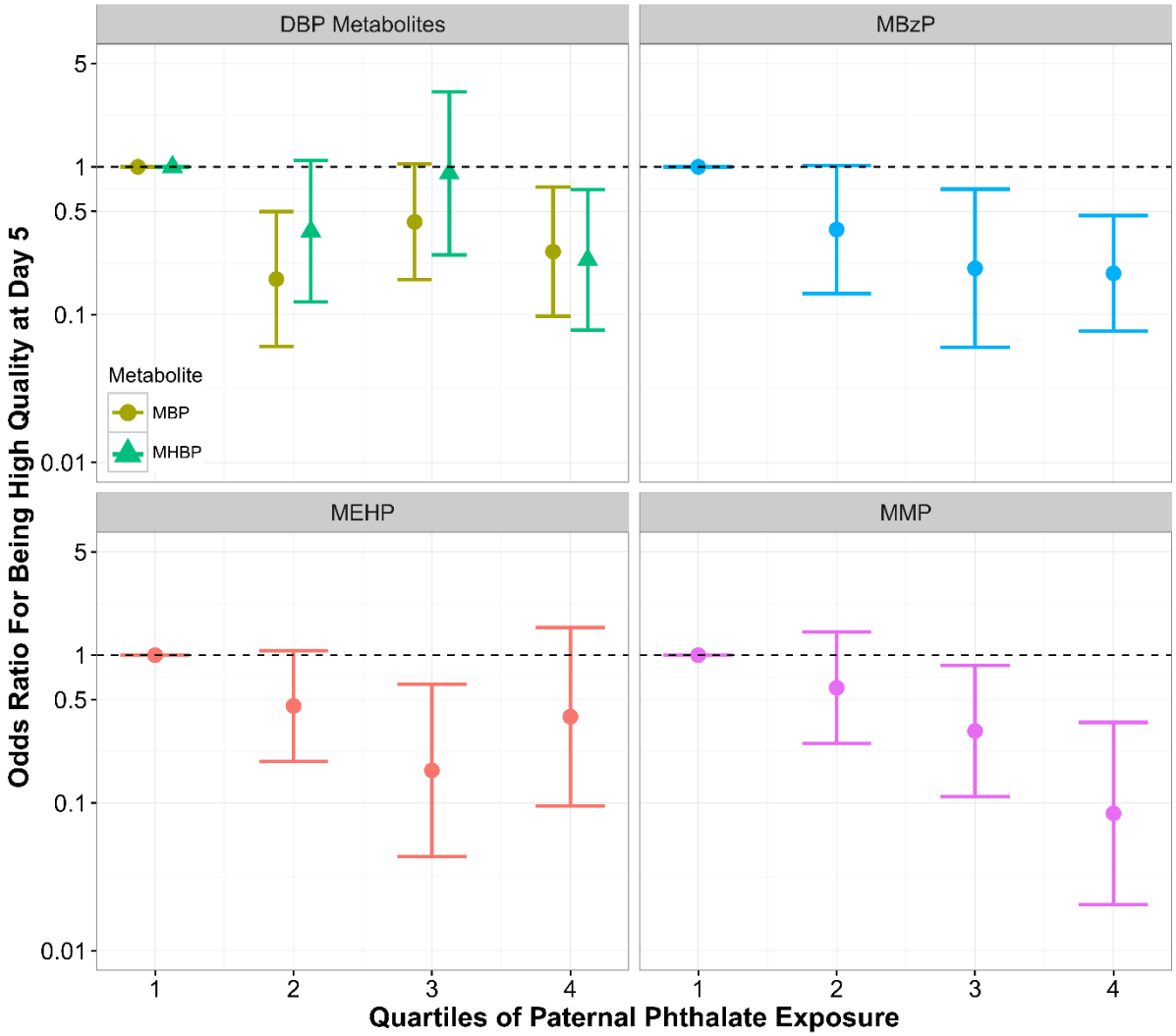
[1]Each model adjusted for embryo grade at cleavage stage and the age and infertility status of the specific parent (i.e. not both)
[2]A single model adjusted for embryo grade at cleavage stage, age of both parents, and infertility status of male parent
[3]Log transformed and specific gravity corrected
[4]Dichotomous variable based on LOD
* p-value < 0.05
** p-value <0.01
*** p-value <0.001
HMW, high molecular weight; LMW, low molecular weight. Other abbreviations are detailed in table 2.2.

Table 4.5 Adjusted odds ratios (95% CIs) for transferrable quality embryos at blastocyst stage (day 5) and couples urinary metabolite concentrations of phthalates and phthalate alternatives.

Parent Esters	Metabolite	Sex-Specific Models[1]				Couple-Level Models[2]			
		Males		Females		Males		Females	
		OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
HMW Phthalates[3]									
DEHP	MEHP	1.06	0.76-1.48	1.10	0.95-1.27	1.05	0.76-1.45	1.09	0.93-1.27
	MEOHP	1.30	0.81-2.07	1.09	0.86-1.39	1.40	0.89-2.21	1.14	0.89-1.47
	MEHHP	1.08	0.64-1.84	1.09	0.88-1.34	1.20	0.71-2.03	1.13	0.90-1.42
	MECPP	1.11	0.64-1.93	1.12	0.84-1.50	1.22	0.71-2.10	1.17	0.88-1.56
DiNP	MCOP	1.01	0.73-1.39	1.21	0.88-1.67	0.83	0.62-1.10	1.33	0.96-1.84
	MNP	1.16	0.87-1.56	**1.54**	**1.14-2.09****	0.83	0.63-1.10	**1.74**	**1.25-2.42****
BBzP	MBzP	**0.71**	**0.53-0.96***	0.91	0.72-1.13	0.70	0.48-1.01	1.03	0.78-1.34
DOP	MCPP	1.10	0.77-1.57	1.29	0.85-1.94	0.96	0.69-1.35	1.33	0.90-1.97
DiDP	MCNP	0.75	0.40-1.41	1.28	0.71-2.29	0.59	0.33-1.06	1.66	0.93-2.96
LMW Phthalates[3]									
DiBP	MBP	0.71	0.47-1.08	1.09	0.82-1.45	0.67	0.43-1.03	1.13	0.84-1.52
	MHBP	**0.42**	**0.21-0.83***	0.89	0.47-1.69	**0.41**	**0.21-0.80****	0.82	0.51-1.33
DBP	MiBP	1.54	0.92-2.55	1.40	0.94-2.06	1.29	0.66-2.55	1.23	0.77-1.96
	MHiBP	1.32	0.73-2.40	**1.56**	**1.01-2.43***	1.06	0.54-2.06	1.49	0.93-2.41
DMP	MMP	0.86	0.64-1.16	1.16	0.73-1.85	**0.72**	**0.52-1.00***	1.45	0.91-2.32
DEP	MEP	1.07	0.92-1.24	1.05	0.80-1.39	1.06	0.89-1.26	1.01	0.78-1.31
Phthalate Alternative[4]									
DiNCH	MCOCH	0.48	0.22-1.03	0.57	0.22-1.48	**0.41**	**0.19-0.88***	1.07	0.43-2.65
	MHiNCH	0.78	0.45-1.38	1.07	0.58-1.97	0.65	0.34-1.27	1.59	0.77-3.28

[1]Each model adjusted for embryo grade at cleavage stage and the age and infertility status of the specific parent (i.e. not both)
[2]A single model adjusted for embryo grade at cleavage stage, age of both parents, and infertility status of male parent
[3]Log transformed and specific gravity corrected
[4]Dichotomous variable based on LOD
* p-value < 0.05
** p-value <0.01
*** p-value <0.001
HMW, high molecular weight; LMW, low molecular weight. Other abbreviations are detailed in table 2.2.

Figure 4.1 Odds ratios for high-quality blastocysts status by quartiles of male urinary phthalate concentrations.



The results shown here were products of general estimating equations with quartile exposures and binary embryo quality outcomes. The models also included age of male and female partners, male infertility status, embryo grade at cleavage stage, and female urinary metabolite concentrations.

CHAPTER 5

PRECONCEPTION URINARY PHTHALATE CONCENTRATIONS AND SPERM DNA METHYLATION PROFILES AMONG MEN UNDERGOING IVF TREATMENT: A CROSS-SECTIONAL STUDY

Summary

This chapter evaluates the associations of male urinary phthalate and phthalate alternative concentrations with sperm DNA methylation. Numerous male urinary anti-androgenic phthalate metabolite concentrations were associated with differentially methylated regions in sperm DNA. Functional analyses revealed enrichment of genes related to growth and development as well as cellular function and maintenance.

Abstract

STUDY QUESTION: Are preconception phthalate and phthalate replacements associated with sperm differentially methylated regions (DMRs) among men undergoing in-vitro fertilization?

SUMMARY ANSWER: Ten phthalate metabolites were associated with 131 sperm DMRs that were enriched in genes related to growth and development, cell movement, and cytoskeleton structure.

WHAT IS KNOWN ALREADY: Several phthalate compounds and their metabolites are known endocrine disrupting compounds and are pervasive environmental contaminants. Rodent studies report that prenatal phthalate

exposures induce sperm DMRs, but the influence of preconception phthalate exposure on sperm DNA methylation in humans is unknown.

STUDY DESIGN, SIZE, AND DURATION: An exploratory cross-sectional study with 48 male participants from the Sperm Environmental Epigenetics and Development Study (SEEDS).

PARTICIPANTS/MATERIALS, SETTING, METHODS: The first 48 couples provided a spot urine sample on the same day as semen sample procurement. Sperm DNA methylation was assessed with the HumanMethylation 450K array. Seventeen urinary phthalate and 1,2-Cyclohexane dicarboxylic acid diisononyl ester (DINCH) metabolite concentrations were measured from spot urine samples. The A-clust algorithm was employed to identify co-regulated regions. DMRs associated with urinary metabolite concentrations were identified via linear models, corrected for false-discovery rate.

MAIN RESULTS AND ROLE OF CHANCE: Adjusting for age, BMI, and current smoking, 131 DMRs were associated with at least one urinary metabolite. Most sperm DMRs were associated with anti-androgenic metabolites, including mono(2-ethylhexyl) phthalate (MEHP, n=83), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP, n=16), mono-n-butyl phthalate (MBP, n=22), and cyclohexane-1,2-dicarboxylic acid-monocarboxy isooctyl (MCOCH, n=7). The DMRs were enriched in lincRNAs as well as in regions near coding regions. Functional analyses of DMRs revealed enrichment of genes related to growth and development as well as cellular function and maintenance. Finally, 13% of sperm DMRs were inversely associated with high quality blastocyst-stage embryos after IVF.

LIMITATIONS, REASONS FOR CAUTION: Our modest sample size only included 48 males and additional larger studies are necessary to confirm our observed results. Non-differential misclassification of exposure is also a concern given the single spot urine collection.

WIDER IMPLICATIONS OF THE FINDINGS: To our knowledge, this is the first study to report that preconception urinary phthalate metabolite concentrations are associated with sperm DNA methylation in humans. These results suggest that paternal adult environmental conditions may influence epigenetic reprogramming during spermatogenesis, and in turn, influence early-life development.

Introduction

Human spermatogenesis, a 72 day process by which diploid spermatogonia progress to haploid spermatozoa, requires several epigenetic reprogramming events, which may provide a final opportunity for sperm to epigenetically respond to their current environment prior to fertilization [30]. Indeed, two intriguing rodent studies found that sperm DNA methylation can be influenced by adult exposures to pre-diabetic conditions [153] and fear conditioning [223], which subsequently produced phenotypic changes in the offspring of affected fathers. In humans, adult sperm DNA methylation patterns were associated with body weight and was modifiable within individuals after gastric bypass surgery [224]. Similarly, sperm DNA methylation was responsive to high dose folic acid supplementation (5 mg/day) [225], but not to low dose supplementation (400 µg/day) or food fortification [226]. With respect to environmental toxicants, adult exposures to particulate air pollution [158], Chromium III chloride [160], and methoxychlor [108]

altered sperm DNA methylation patterns in rodents. Together, these studies provide compelling data that the sperm epigenome can respond to environmental conditions experienced in adulthood.

Phthalates are a ubiquitous class of compounds found in many commercial products such as medical equipment, food packaging, and personal care products. Select phthalate metabolites have been associated with decreased sperm concentration and motility, increased sperm DNA damage, and increased sperm apoptosis [54, 55, 63, 67, 227-230]. In a cohort of the US general population, select male phthalate metabolites were associated with decreased fecundity as evidenced by a 20% increase in time to pregnancy [52]. Similarly, among couples undergoing fertility treatment, we have previously shown that male, and not female, phthalate metabolites were associated with diminished blastocyst quality [231], while others have shown decreased odds of implantation and live birth [57]. Such results suggest a sperm-derived effect because IVF protocols eliminate all other paternal inputs such as seminal plasma. However, the mechanism by which male preconception phthalates affect these early-life outcomes has not been fully resolved.

Several animal studies report that *in utero* phthalate exposure alters sperm DNA methylation. For example, maternal exposure to bis(2-ethylhexyl)phthalate (DEHP) during gestation altered sperm DNA methylation in subsequent generations of offspring [106, 109, 232], although it is unclear if the effects persist for more than one generation [106, 109].

Given the compelling data from animal studies showing that the sperm epigenome is responsive to adult environmental conditions, we conducted a cross-sectional study to explore the relationship of preconception urinary phthalate and 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH) metabolite concentrations with sperm DNA methylation in men undergoing fertility treatment.

Methods

Study Population. Forty-eight couples were recruited from the Baystate Reproductive Medicine in Springfield, Massachusetts, as part of the Sperm Environmental Epigenetics and Development Study (SEEDS). Inclusion criteria were: male partners were 18-55 years old without vasectomy, female partners were ≤ 40 years old with expected delivery at Baystate Medical Center, and fresh ejaculate sperm was used for in-vitro fertilization (IVF) treatment. Relevant demographics (race, age, height, weight), lifestyle factors (current and past alcohol and cigarette use), medical history (diagnoses of infertility) data were collected by clinic personnel during the IVF cycle for both partners. Written consent from eligible males and females who were interested in participating was obtained by attending physicians. This study was approved by the institutional review boards at Baystate Medical Center and at the University of Massachusetts Amherst.

Urinary Exposure Biomarker Measurements. A spot urine sample was collected from couples in a sterile polypropylene collection cup on the same day as semen sample procurement and oocyte retrieval. Urine samples were vortexed, divided into several aliquots, and stored at -80°C before being shipped overnight on dry ice to the National Center for Environmental Health of the Center for

Disease Control (CDC), where the urinary biomarkers were quantified via published methods [211, 233]. Analytical standards, quality control (QC) materials (prepared from spiked pooled urine), and reagent blank samples were included in each batch along with study samples. The QC concentrations—averaged to obtain one measurement of high-concentration QC and one of low-concentration QC for each batch—were evaluated by using standard statistical probability rules [234].

In total, seventeen urinary metabolites were quantified: mono(2-ethylhexyl) phthalate (MEHP); mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP); mono(2-ethyl-5-oxohexyl) phthalate (MEOHP); mono(2-ethyl-5-carboxypentyl) phthalate (MECPP); monocarboxy-isooctyl phthalate (MCOP); mono-isononyl phthalate (MNP); monobenzyl phthalate (MBzP); mono (3-carboxypropyl) phthalate (MCPP); monocarboxy-isononyl phthalate (MCNP); mono-n-butyl phthalate (MBP); mono-3-hydroxybutyl phthalate (MHBP); mono-isobutyl phthalate (MiBP); mono-hydroxyisobutyl phthalate (MHiBP); monoethyl phthalate (MEP); monomethyl phthalate (MMP); cyclohexane-1,2-dicarboxylic acid-monocarboxy isooctyl ester (MCOCH); and cyclohexane-1,2-dicarboxylic acid-mono(hydroxy-isononyl) ester (MHINCH). The limits of detection (LODs) ranged from 0.2 to 0.6 ng/mL, depending on the metabolite. Specific gravity (SG) was measured at room temperature using a digital handheld refractometer (Atago Co., Ltd., Tokyo, Japan).

Sperm Collection and DNA Isolation. Semen samples were collected in a sterile plastic specimen cup after a recommended 2-3 day abstinence period, per

standard IVF protocol. Motile sperm cells were isolated using a two-step gradient fractionation and DNA was isolated using our previously published protocol [235].

450K Beadchip Analysis. Genomic sperm DNA (400 ng) was bisfulfite converted and employed on the 450K Infinium Methylation Beadchip Array (Illumina) at Wayne State University's genomic core. The 450K array provides genome-wide coverage of 485,577 methylation sites. Samples were randomized within and across beadchip to minimize any potential batch effects.

450K Data Analyses. The minfi package in R was used to correct for technical variation in background signals [236], to remove probes below the background fluorescence level, and to adjust for differences in Type I and Type II probes [237]. The ComBat function in the sva package [238] was used to correct for batch effects. Cross-hybridizing probes and sex chromosome probes were also removed using the DMRcate package [239].

Statistical Analyses. We used the A-clustering algorithm [240] to identify co-regulated regions by generating clusters of ≥2 CpG sites ≤1000 base pairs apart. These CpG clusters formed the unit of our analyses. To balance both validity and interpretability, we conducted statistical analyses using both M-values and β-values. First, to identify differentially methylated regions (DMRs) associated with urinary exposure concentrations, we used M-values due to their better adherence to homoscedasticity in linear models [241]. Next, we used β-values, which are the ratio of the methylated probe intensity to the overall intensity (sum of methylated and unmethylated probe intensities), to generate CpG methylation values between 0% and 100% to facilitate the biological interpretation of our results.

General estimating equations (GEE) were used to identify DMRs from CpG clusters associated with paternal exposure concentrations. For each model, we specified Gaussian distribution and an exchangeable correlation structure. The 15 phthalate metabolites were fitted as continuous variables (after SG-correction and log-transformation), while the two DINCH metabolites were fitted as dichotomous variables (above or below the LOD) due to their low detection rates. To account for multiple comparisons, we used the Benjamini-Hochberg method, which corrects the p-values generated from linear GEE models using the total number of comparisons and the rank order of the p-values [242]. Statistical significance was set at a false discovery rate (FDR) q-value < 0.05.

Inclusion of covariates in multivariable models was based on biological plausibility and statistical significance in bivariate models ($p<0.1$). Covariates considered for inclusion were age, body mass index (BMI), race (white vs. non-white), alcohol use, cigarette smoking, and season of biological collections. Bivariate analyses showed that age, BMI, and current smoking status were associated with ≥1 metabolite and ≥1 DMR and were included in all models. In contrast, race and seasonality did not fulfil this criteria and were not included in the models. Alcohol was not included in the model as 92% of those who had available data reported current alcohol use (data not shown).

Analysis was performed with R (v3.3.0, R Foundation for Statistical Computing, Vienna, Austria) using “aclust” package (v2.0.1) the ‘gee’ package (v4.13.19).

Bioinformatics Analyses. Prior to gene set enrichment analysis (GSEA) and Ingenuity Pathway Analysis (IPA), each cluster was assigned the closest gene using GRCh37 assembly data from ENSEMBL via the ChIPpeakAnno R package (version 3.6.5). Similarly, we determined genic (exons, introns, promoters, enhancers, intergenic regions), CpG (island, shelves, shores), functional (protein coding, pseudogene, lincRNA), and protamine (protamine vs. nucleosome) features of clusters via annotations from Ensembl. Binding site locations for transcription factors EZH2 and CTCF were retrieved from ENCODE. Fisher's exact test was used to test for significant enrichment or depletion of each feature in the DMRs versus the entire set of clusters.

We used IPA (www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/) and GSEA (www.broadinstitute.org/gsea) [243] to determine whether the observed exposure-associated DMR was affiliated with sets of genes with similar biological function, chromosomal location, or regulation. For IPA, we restricted results to include only networks with score ≥20 or functional groups with $p<0.05$ and an available activation Z-score. For GSEA, we set the cut-off to be normalized enrichment score ≥1.8 or ≤-1.8 and q-value <0.20. IPA and GSEA analyses were restricted to clusters within 1500 bp of a gene.

Sequenom Validation. To validate the results from the 450K array, eight CpG sites from five clusters were assayed on the Sequenom MassARRAY system (van den Boom and Ehrich, 2009). The sites were randomly selected from all clusters.

Embryo Quality Assessment. A full description of embryo quality assessment methods can be found elsewhere [231]. In brief, blastocysts were graded by trained embryologists at the IVF clinic five days post-fertilization based on standard morphological characteristics. For analysis, blastocysts were classified as high vs. low quality embryos.

Results

Table 3.1 presents the demographic and semen parameter data. The majority of the men were non-Hispanic white, over the age of 30, and overweight. Four of the 48 participants were current smokers. Although all men were seeking fertility treatment with their female partners, only 12 (25%) were diagnosed by attending physicians with male factor infertility based on WHO semen reference levels [40]. Of the remaining 36 men, 20 sought fertility treatment due to female factor infertility and 16 couples were diagnosed with unexplained infertility. In our study, 42.2% and 18.2% of participants had values below the WHO semen reference levels for percent morphologically normal sperm and semen volume, respectively. In contrast, less than 10% of our population were below the WHO semen reference levels for percent motile sperm, total sperm count, and sperm concentration.

The distributions of SG-corrected urinary phthalate and DINCH metabolite concentrations for male partners are summarized in Table 3.2. Fourteen of the 15 of the measured urinary phthalate metabolite concentrations (MEHP, MEHHP, MEOHP, MECPP, MBP, MHBP, MBzP, MiBP, MHiBP, MEP, MCPP, MCNP, MCOP, and MMP) had detection frequencies above 75%, while urinary MNP

concentrations were detected in 67% of the samples. Concentrations of MHINCH and MCOCH, metabolites of the phthalate alternative DINCH, were only detected in 50% and 17% of the samples, respectively.

DMR Identification. Of the original 485,577 interrogated CpGs, 74,193 were removed, leaving 411,384 available for analysis. The A-clust algorithm identified 6,479 clusters spanning 22,420 CpG sites, with a range of 2 - 46 sites per cluster over a length of 3 - 4,456 bp. Adjusted for age, BMI, and current smoking status, we observed that urinary phthalate metabolite concentrations were associated with 138 sperm DMRs ($q<0.05$), comprising 131 unique DMRs (Table 3.3). Seven overlapping DMRs were identified between individual metabolites (Appendix D1), all of which had identical direction of effects across metabolites.

To aid in the interpretations of the magnitude of associations, we also report effect estimates based on models built from beta-values, which directly correspond to percent methylation of DMRs. Figure 4.1 shows the minimum, median, and maximum percent difference associated with one interquartile range (IQR) increase in urinary phthalate metabolite concentrations for all 131 DMRs. For example, for DMRs ($q<0.05$) positively associated with MEHP concentrations, the median beta-value difference per IQR was 2.9% and ranged from 1% - 7.4%. Results of the GEE models for all DMRs, including annotations, are included in Appendix D2. Furthermore, Figure 4.2 provides a graphical representation of the mean methylation of two significant sperm DMRs by tertiles of urinary phthalate metabolite concentrations.

To validate our DMR findings from the 450k array, we chose eight CpGs from five randomly selected clusters. Seven of the eight CpGs showed a high concordance of methylation between the two platforms (Appendices D3-D4). Moreover, GEE models using MassARRAY data provided similar estimated effects compared to those statistical models using 450K data (data not shown).

Imprinted Genes. To further validate our results, we examined the potential for somatic cell contamination by analyzing methylation levels (e.g, beta-values) of 203 probes across 18 imprinted loci identified previously [244]. Our analyses showed that all maternal and paternal imprinted regions were <10% or >85%, respectively, indicating negligible somatic cell contamination in the sperm samples.

Given that imprinted genes are known to escape the re-programming event in pre-implantation embryos, we examined them independently from the main statistical analysis described above. Using the average M-values across the 18 imprinted loci, we found that MCNP was associated with increased methylation at eight (PLAGL1/HYMAI, KCNQ1/KCNQ1OT1, PEG3/ZIM2, MESTIT1/MEST, GNAS-AS1/GNAS, NAP1L5, PEG10/SGCE, L3MBTL) of the 14 maternally imprinted loci and with decreased methylation at one (H19) of the four paternally imprinted loci, adjusting for age, BMI, current smoking status, and multiple comparisons (Appendix D5). No statistically significant results were observed for any of the other urinary phthalate metabolites.

Enrichment Analysis. To determine the functional significance of the significant DMRs, we conducted enrichment analyses (Table 3.4). To investigate

if our sperm DMRs were enriched in DNA regions known to retain nucleosomes, we used previously published mnase-seq data (Donkin *et al.*, 2016). Although there was a 7% increase in nucleosome retention in our significant DMRs, compared to all DNA methylation clusters, it was not statistically significant ($p=0.13$). The proportion of lincRNA was twice as high in DMRs as compared to all clusters (8% vs. 4%, $p=0.03$) while no differences were observed for the proportion of pseudogenes ($p=0.60$) and protein coding genes ($p=0.24$). DMRs were enriched in CpG islands ($p=0.08$), shelves ($p=0.01$), and shores ($p<0.01$) at the expense of open sea regions ($p<0.01$). This was also reflected by the fact that DMRs were enriched in exons ($p=0.03$) and introns ($p=0.02$) whereas intergenic regions were depleted ($p=0.10$). We observed no significant enrichment of DMRs for predicted binding sites for transcription factors CTCF ($p=0.74$) and EZH2 ($p=0.25$).

Pathway Analysis. Network and functional analyses via IPA examined three sets of DMRs – 1) those associated with DEHP, 2) DBP, or 3) all known or suspected anti-androgenic parent compounds (DEHP, BBzP, DiNP, DBP, DiBP, and DINCH). For DEHP-alone and anti-androgenic compounds, most of the DMRs were associated with one of three general pathway categories – “cancer”, “cellular function and maintenance”, and “growth and development” (Figure 4.3, Appendix D6). For “cellular function and maintenance” and “growth and development”, more pathways were associated with an increase in methylation compared to loss of methylation, as determined by the Activation Z-score (Figure III). In contrast, the number of DMRs within the “cancer” pathway was more balanced with respect to

gain or loss of methylation. Network analysis also revealed alterations in genes related to overall themes of “development” and “cellular function” (Figure III). DBP-associated DMRs were not statistically related to specific diseases or functional groups, but were found to be related to genes involved in the cell cycle. Consistent with the results from IPA, GSEA showed that all four tested metabolites (MEHP, MEOHP, MBP, MCOCH) were associated with gene sets related to early development (Appendix D7).

Blastocyst Quality. We previously reported that paternal anti-androgenic phthalate metabolite concentrations (MEHP, MHBP, MBP, MMP, and MCOCH) were associated with diminished blastocyst quality [231]. Here, we find that three of these anti-androgenic metabolites (MEHP, MBP, MCOCH) are associated with the majority of sperm DMRs. Of the 57 DMRs inversely associated with urinary phthalate metabolite concentrations, two were positively associated with high blastocyst quality while one was negatively associated. Conversely, of the 74 DMRs positively associated with urinary phthalate metabolite concentrations, 16 were inversely associated with high blastocyst quality and none were positively associated (Appendix D8). All GEE models were adjusted for age, BMI, and current smoking. Four of these 19 DMRs associated with both urinary metabolite concentrations and embryo quality were located in nucleosomes and 17 were located on exonic, intronic, or promoter regions.

Discussion

In our investigation of 48 males undergoing fertility treatment, 131 sperm DMRs were associated with at least one urinary phthalate and DINCH metabolite

concentration. Functional analyses revealed that sperm DMRs were enriched in pathways related to development and general cell function and maintenance. In particular, urinary concentrations of MEHP, MEOHP, MBP, and MCOCH were associated with the greatest number of sperm DMRs. Interestingly, the parent compounds of these metabolites, DEHP, DBP, and DINCH all have known or suspected effects on androgens [245-247]. Our previous findings have shown that these same metabolites in the male, but not the female partner, were also associated with diminished blastocyst quality [231]. These results suggest that phthalates may be associated with sperm DNA methylation in or near genes relevant to early embryogenesis, providing a pathway linking the observed inverse associations between anti-androgenic phthalates and blastocyst quality. More broadly, our results support the growing evidence that preconception paternal environmental health may contribute to both male reproductive potential and offspring development.

Functional enrichment analyses showed that many DMRs associated with both DEHP alone and all anti-androgenic compounds as a whole are within or near genes related to “growth and development” and “cellular function and maintenance”. It is generally believed that proper sperm DNA methylation is important for embryogenesis and one recent study reported that DNA methylation patterns are predictive of embryo quality in an IVF setting [248]. Alterations in methylation of genes associated with growth/development and cellular function/maintenance in sperm may have potential implications for embryo development. Aside from cell death and survival, most of the genes within the

cellular function and maintenance group are related to cytoskeleton structure and cell migration. Cytoskeleton structure is known to play an important role during fertilization and pre-implantation embryo development [249] while many genes related to cell migration are similarly important for early life development. For example, DMRs associated with DEHP (and thus also anti-androgenic compounds as a whole) were found on or near genes C-C motif chemokine 11 (CCL11) [250] and Wnt Family Member 7b (Wnt7b) [251], known regulators of trophoblast development and migration. The potential for phthalate and DINCH metabolites associated DMRs to influence embryogenesis is further supported by the observation that lincRNAs were enriched in our DMRs compared to the background of all clusters. LincRNAs are a class of non-coding RNAs with diverse functions that include gene regulation [252] and roles in early development, including myogenesis, haematopoiesis, adipogenesis, and neurogenesis [253].

The observation that 19 of the 131 DMRs were associated with phthalates and DINCH as well as with poor blastocyst quality extend our previous findings that paternal MEHP, MHBP, MBP, MMP, and MCOCH were associated with diminished blastocyst quality [231] by suggesting that sperm DNA methylation is a pathway linking paternal exposures with embryo quality. These observed associations with blastocyst quality coincide with the timing of embryonic genome activation, which marks the earliest time in the developing embryo when the paternal genome and its methylation patterns may be relevant.

Despite the global epigenetic reprogramming that occurs shortly post-fertilization, imprinted sites and certain other regions may escape reprogramming

[254]. In our study, we observed that methylation at 9 imprinted genes are associated with urinary concentrations of MCNP, implying that these imprinted DMRs may have the potential to escape the epigenetic reprogramming event and thus be inheritable. Furthermore, we observed that the 131 sperm DMRs associated with metabolites of phthalates and DINCH were enriched in exons and introns, regions previously reported to be preferentially maintained during the transient hypomethylation of pre-implantation embryos [255]. It is unknown if our sperm DMRs at non-imprinted regions can escape, at least in part, reprogramming in the developing embryo. However, compelling animal data supports the notion that some sperm DMRs may be resistant to this reprogramming event [153, 223].

In regard to the interpretation of sperm DMRs, we need to consider that each sperm carries a haploid genome with a binary option (methylated or unmethylated) at each CpG site and that differences in percent methylation (beta-values) represent differences in the frequency of sperm with methylation at those specific CpG sites and/or regions. For example, a 5% increase in beta-values is interpreted as a 5% increase in the frequency of sperm containing methylation at that particular CpG site and/or region. Given that only a single sperm is needed for fertilization, even modest changes in frequency of methylation in motile sperm may be important for early-life development. Thus, while ≤10% methylation changes may not be biologically relevant in somatic cells such as leukocytes, such changes in frequency could be biologically significant in the “winner takes all” scenario of fertilization.

We recognize that there are some limitations to our study. First, our modest sample size only included 48 males and additional larger studies are necessary to confirm our observed results. Second, as our population was recruited from the IVF clinic, our findings may not be generalizable to the broader population. Third, non-differential misclassification of exposure remains a concern given the single spot urine collection and the short half-life of phthalates. Though studies have reported temporal variations in urinary concentrations of phthalate metabolites [221, 256-259], most have concluded that spot urine samples are comparable to the more intensive 24-hour sample. Most importantly, a study using a population very similar to our study population showed that a single urine sample adequately represents exposure over 3 months, which spans over one spermatogenesis cycle [221]. The non-differential misclassification of exposure due to within-individual temporal variability in urinary phthalate metabolite concentrations likely biased our results toward the null and could have led to fewer DMRs being detected. Additionally, while we did collect information on lifestyle factors such as smoking and alcohol use, we do not have information on socioeconomic status and other related variables. Lastly, due to confidentiality considerations, we do not have information on refusal rates or characteristics of non-participants, and thus cannot rule out potential selection bias related to factors associated with both phthalates and sperm DNA methylation.

Despite these limitations, our study has notable strengths. First, our study utilized state-of-the-art exposure analytical methods for profiling urinary metabolite concentrations of phthalates and new emerging non-phthalate replacements.

Second, we measured DNA methylation from the motile fraction of sperm, which represents those sperm with the highest fertilization potential and may better approximate relevant phthalate-related impacts on sperm DNA methylation in IVF and non-IVF populations. Third, the same cohort of sperm in our DNA methylation analyses was used for IVF; thus, our study design allowed us to connect sperm DMRs directly with embryo quality to facilitate our understanding of the influence of sperm DMRs on early-life development, which otherwise would not be possible among couples from the general population.

Conclusion

Our study is the first, to our knowledge, to examine the associations between preconception phthalates and sperm DNA methylation profiles in humans. Overall, we found that select preconception anti-androgenic phthalate metabolites are associated with 131 sperm DMRs, which are enriched in genes related to growth and development and basic cellular functions such as cell movement and cytoskeleton structure. This provides a critical step towards our understanding of the paternal preconception contributions to reproductive success and offspring development. Future studies are needed to replicate such findings and further clarify the role of phthalate-associated sperm DNA methylation on subsequent offspring health and development.

Table 5.1 Demographics and Semen Parameters of SEEDS Participants (n=48)

	n	%
Age		
<30	10	21%
30-40	25	52%
40+	13	27%
BMI		
<25	10	21%
25-30	19	40%
30+	18	38%
Missing	1	2%
Current Smoking		
Yes	4	8%
No	44	92%
Diagnosed Infertility		
Male Factor	6	13%
Female Factor	20	42%
Both	6	13%
Unexplained	16	33%
Race		
White (non-Hispanic)	37	77%
All Others	4	8%
Missing	7	15%

	Median	% < WHO Reference
Normal Morphology (%)	5.5	42.2
% Motile	59.0	9.1
Sperm Count (10^6)	90.8	4.6
Semen Volume (mL)	3.1	18.2
Sperm Concentration (10^6/mL)	47.8	9.1

Table 5.2 Distribution of specific gravity-adjusted urinary phthalate metabolite concentrations (ng/mL) among SEEDS participants (N=48).

Parent Metabolite	Metabolite	Detection Rate	Geometric Mean	Percentiles		
				25th	50th	75th
HMW						
DEHP	MEHP	77%	1.15	0.72	1.14	1.86
	MEOHP	100%	4.3	3.23	4.41	5.62
	MEHHP	100%	6.53	4.52	6.84	9.27
	MECPP	100%	9.51	7.12	9.87	13.83
DiNP	MCOP	100%	26.39	11.17	22.21	51.53
	MNP	67%	0.99	0.44	0.75	1.74
BBzP	MBzP	100%	4.06	2.15	3.94	8.69
DOP	MCPP	100%	2.38	1.22	1.98	4.09
DiDP	MCNP	100%	3.1	1.94	2.83	4.42
LMW						
DBP	MBP	98%	7.42	6.04	7.99	11.76
	MHBP	77%	0.65	0.46	0.64	1.00
DiBP	MHiBP	98%	2.09	1.58	2.01	2.79
	MiBP	100%	6.69	4.53	7.11	10.98
DEP	MEP	100%	26.72	9.84	19.28	54.46
DMP	MMP	96%	2.28	1.43	2.07	3.80
Phthalate Alternatives						
DINCH	MHiNCH	50%	0.48	0.30	0.39	0.65
	MCoCH	17%	0.61	0.34	0.53	0.89

Abbreviations: Bis(2-ethylhexyl) phthalate (DEHP); diisononyl phthalate (DiNP); benzyl butyl phthalate (BBzP); dioctyl phthalate (DOP); diisodecyl phthalate (DiDP); dibutyl phthalate (DBP); diisobutyl phthalate (DiBP); diethyl phthalate (DEP); dimethyl phthalate (DMP); 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH); mono(2-ethylhexyl) phthalate (MEHP); mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP); mono(2-ethyl-5-oxohexyl) phthalate (MEOHP); mono(2-ethyl-5-carboxypentyl) phthalate (MECPP); monocarboxy-isooctyl phthalate (MCOP); mono-isononyl phthalate (MNP); monobenzyl phthalate (MBzP); mono (3-carboxypropyl) phthalate (MCPP); monocarboxy-isononyl phthalate (MCNP); mono-n-butyl phthalate (MBP); mono-3-hydroxybutyl phthalate (MHBP); mono-isobutyl phthalate (MiBP); mono-hydroxyisobutyl phthalate (MHiBP); monoethyl phthalate (MEP); monomethyl phthalate (MMP); cyclohexane-1,2-dicarboxylic acid-monocarboxy isooctyl ester (MCOCH); and cyclohexane-1,2-dicarboxylic acid-mono(hydroxy-isononyl) ester (MHINCH).

Table 5.3 Number of Differentially Methylated Regions (DMRs) Associated with Individual Phthalate Metabolites.

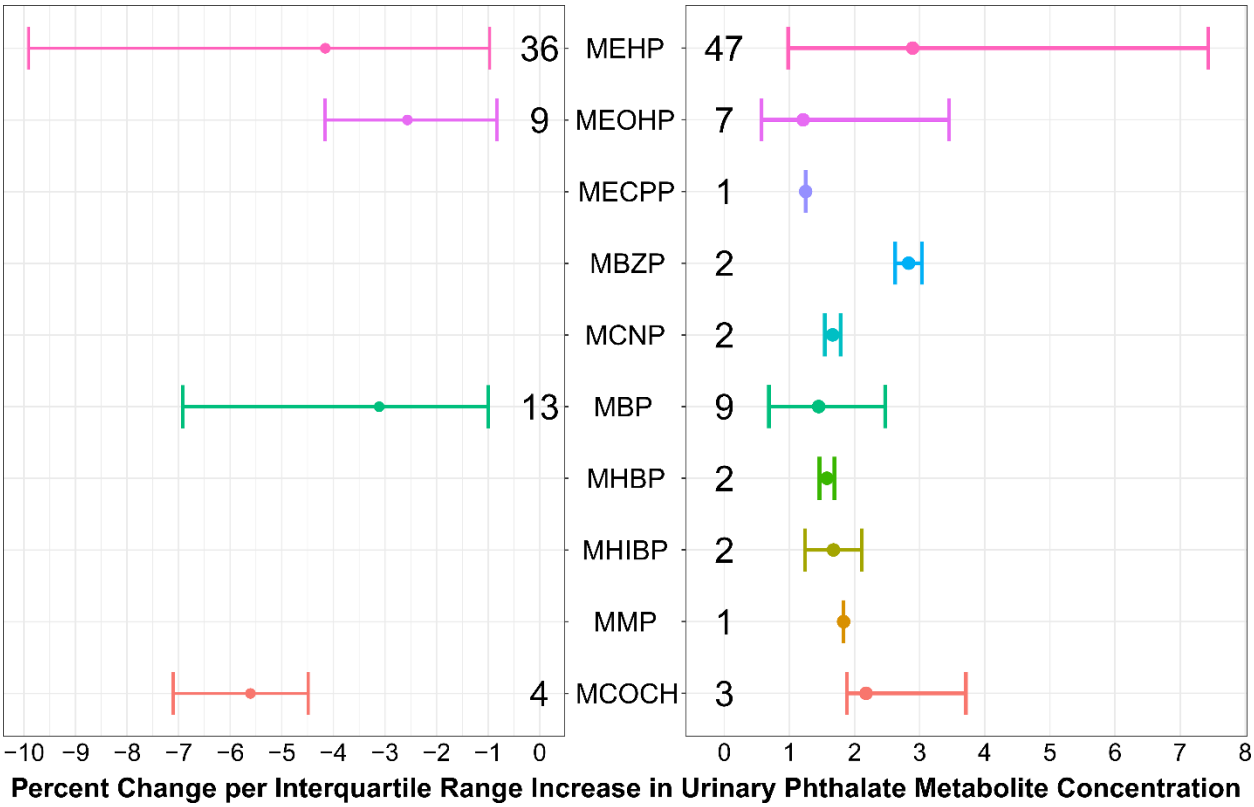
Parent Metabolite	Metabolite	DMRs (q<0.05)	Shared DMRs
High Molecular Weight Phthalates (HMW)			
DEHP	MEHP	83	1
	MEOHP	16	
	MEHHP	0	
	MECPP	1	
DiNP	MCOP	0	
	MNP	0	4
BBzP	MBzP	2	
DOP	MCPP	0	
DiDP	MCNP	2	2
Low Molecular Weight Phthalates (LMW)			
DBP	MBP	22	
	MHBP	2	
DiBP	MHiBP	2	
	MiBP	0	
DEP	MEP	0	
DMP	MMP	1	
Phthalate Alternatives			
DINCH	MHiNCH	0	
	MCoCH	7	

Table 5.4 Enrichment Analysis of Differentially Methylated Regions (DMRs) Compared to All Clusters

	DMRs (n=131*)		All (n=6479*)		
	n	**%**	**n**	**%**	**p-value****
Chromatin Features					
Nucleosome	35	27%	1356	21%	0.1287
Protamine	96	73%	5123	79%	
Gene Features					
lincRNA	11	8%	277	4%	0.0300
Pseudogene	5	4%	196	3%	0.6009
Protein Coding	86	66%	3904	60%	0.2409
CpG Features					
Island	39	30%	1496	23%	0.0760
Shelves	28	21%	860	13%	0.0131
Shores	75	57%	2744	42%	0.0009
Open Sea	38	29%	2901	45%	0.0003
Genic Regions					
Exons	49	37%	1728	27%	0.0408
Introns	47	36%	1872	29%	0.0218
Promoters	32	24%	1404	22%	0.4540
Enhancers[#]	6	5%	192	3%	0.2905
Intergenic	30	23%	1933	30%	0.1002
Transcription Factors					
CCCTC-binding factor *(CTCF)*	29	22%	1344	21%	0.744
Histone-lysine N-methyltransferase *(EZH2)*	15	11%	509	8%	0.249

*Categories are not exclusive, the numbers may not all add to 100%
**calculated from Fisher's exact test
[#]Taken from FANTOM5 project

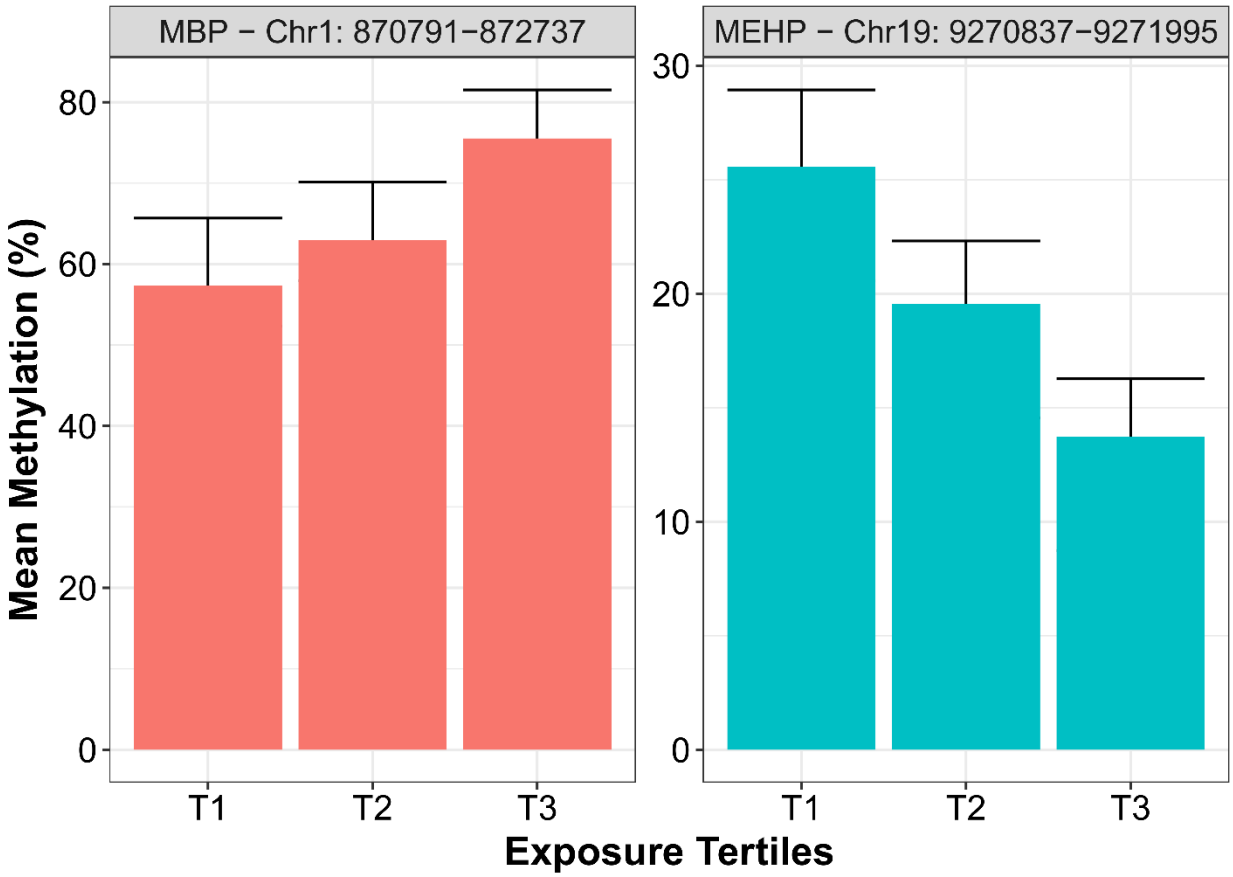
Figure 5.1 Associations of urinary phthalate and phthalate-alternative metabolite concentrations with sperm differentially methylated regions.



Sperm DNA methylation clusters (n = 6479) were generated by A-clust and modeled with phthalate metabolite concentrations as continuous variables using general estimation equation models adjusting for age, BMI, and current smoking and were corrected for false discovery rate (FDR). The dots represent the median percent difference in sperm DNA methylation associated with interquartile range (IQR) increase in phthalate metabolite concentrations, except for MCOCH, where the analysis was binary (those with detectable levels versus those below limits of detection). The error bars represent the minimum and maximum percent methylation change per IQR, and the numbers above/below

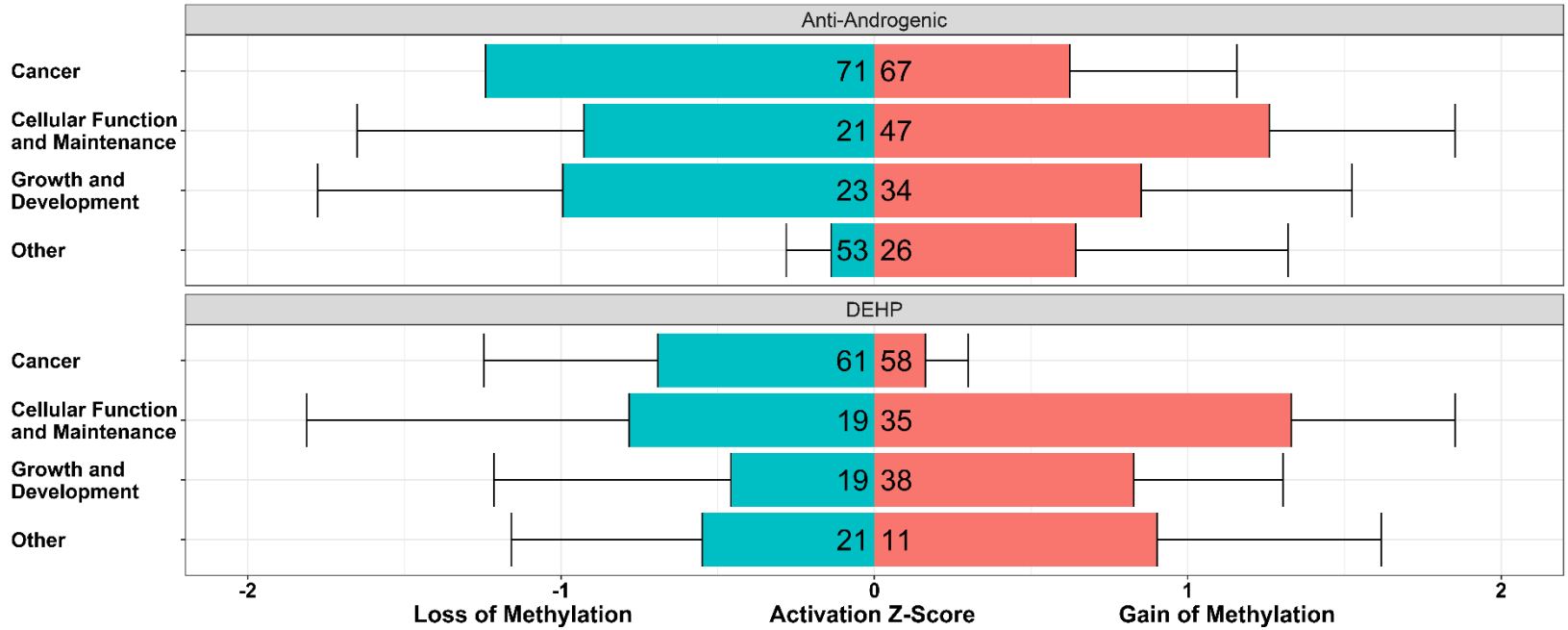
metabolites represent the number of differentially methylated regions (DMRs) in each group. MEHP (mono(2-ethylhexyl) phthalate); MEOHP (mono(2-ethyl-5-oxohexyl) phthalate); MECPP (mono(2-ethyl-5-carboxypentyl) phthalate); MBzP (monobenzyl phthalate); MCNP (monocarboxy-isononyl phthalate); MBP (mono-n-butyl phthalate); MHBP (mono-3-hydroxybutyl phthalate); MHIBP (mono-ydroxyisobutyl phthalate); MMP (monomethyl phthalate); MCOCH (cyclohexane-1,2-dicarboxylic acid-monocarboxy isooctyl ester).

Figure 5.2 Mean percentage methylation of two DMRs by tertiles of urinary MBP and MEHP concentrations.



Using GRCh37 annotations, the region Chr1:870791–872737 is located within the gene body of SAMD11 while the region Chr19:9270837–9271995 is located within the promoter region of ZNF317 gene. Error bars are standard deviations.

Figure 5.3 Ingenuity pathway analysis of DMRs associated with urinary concentrations of DEHP metabolites and all anti-androgenic metabolites (MEHP, MEOHP, MBzP, MBP, MHBP, MHiBP, MCOCH).



The general categories of disease and functional groups are shown by their calculated activation Z-score, which is an overall measure of loss or gain of methylation at genes associated with this functional category. The bars represent the mean activation Z-score in each category while the error bar represents the absolute maximum. The number in each bar represents the number of DMRs in each group. DEHP, bis(2-ethylhexyl)phthalate.

CHAPTER 6

SYNTHESIS AND FUTURE DIRECTION

Summary

Paternal adult environmental conditions may influence epigenetic reprogramming during spermatogenesis and influence early-life development. In our research, we found that paternal urinary anti-androgenic phthalate metabolite concentrations are associated with diminished blastocyst quality and DMRs in the sperm DNA.

The Hypothesized Relationships Between Phthalates, Sperm DNA Methylation, and Embryo Development

As described by the literature review in chapter 2, additional human data are necessary to understand the response of sperm epigenetics to preconception environmental exposures. To this point, a major focus of this dissertation was to examine the association between phthalates and select reproductive health outcomes. In chapter 4, we used the data from the first 50 couples in the SEEDS study to examine the associations of urinary metabolite concentrations of phthalates and phthalate alternatives with embryo development measures through the blastocyst stage. We observed no associations between any urinary concentrations of metabolites of phthalates or phthalate alternatives and embryo quality through the cleavage stage, which is consistent with the results published

from a similar study [57]. However, interestingly, select paternal urinary metabolite concentrations (MBzP, MBP, MHBP, MMP, and MCOCH, with MEHP as borderline statistically significant) were associated with poor blastocyst quality, suggesting that negative influences of paternal exposure to phthalates or phthalate alternatives manifest in the embryo during the transition from cleavage stage to blastocyst stage. This timing coincides with zygotic genome activation, which marks the activation of the paternal genome. Given the controlled setting of the IVF, our results suggest that paternal preconception exposure to phthalates and phthalate alternatives results in poor embryo quality via features related to paternal genome.

Stemming from the findings in chapter 4, the associations of urinary concentrations of phthalates and phthalate alternative metabolites with sperm DNA methylation were presented in chapter 5. Not only were select phthalate metabolites (MEHP, MEOHP, MECPP, MBzP, MCNP, MBP, MHBP, MHiBP, MMP, MCOCH) associated with ≥1 sperm DMRs, many of these metabolites (MEHP, MBzP, MMP, MHBP, MBP, MCOCH) were also the metabolites found to be associated with diminished embryo quality in chapter 4. It is also interesting to note that MMP, MBP, and MBzP were previously reported to be positively associated with time-to-pregnancy in a cohort of 501 couples from the general population [52]. Of the 131 total unique DMRs associated with urinary phthalate metabolite concentrations, 19 were also associated with blastocyst quality. Eighteen of the 19 followed the expected direction where if the urinary metabolite concentrations were positively associated with methylation at that region, then the

methylation at the region is inversely associated with odds of being a high quality blastocyst; and vice-versa.

Given the evidence from chapters 4 and 5, we hypothesize that exposure to phthalates and phthalate alternatives adversely impacts embryo development via altered sperm DNA methylation patterns. Despite the global “erasure” of the paternal genome in early embryogenesis described in chapter 2, the demethylation process is not complete [260] and provides biologic plausibility for the hypothesis. In support, as described in chapter 5, functional analyses of the 131 DMRs associated with urinary metabolites of phthalates and phthalate alternatives revealed enrichment of genes related to growth and development as well as cellular function and maintenance. For example, two DMRs were found on or near genes CCL11 and Wnt7b, known regulators of trophoblast development and migration.

Research Gaps and Future Directions

Despite the evidence presented in this dissertation, there are numerous gaps remain in our understanding of the mechanisms by which phthalates affect reproductive health.

First, It is important to note that the results presented in chapters 4 and 5 were based on 50 couples recruited from an IVF clinic. On one hand, the IVF setting provides advantages such as a controlled environment where any observed differences in embryogenesis and development are only dependent on the characteristics of sperm and oocyte. On the other hand, such results may not be

generalizable to the broader population. Ultimately, our results need to be replicated not only in a larger study, but also in the general population.

Alternative explanations exist for the apparent lack of associations observed between phthalates and phthalate alternatives and embryo quality prior to the blastocyst stage. It is possible that the molecular changes associated with phthalates and phthalate alternatives were too subtle to be detected morphologically during these early stages, and such changes only manifest at the morphological level at the later stages of development. For example, such early paternal effects on embryo development may be mediated by sperm-borne RNA. It has recently been reported that levels of sperm miRNA-34c expression were associated with rates of high quality embryos at day 3, implantation, pregnancy, and live birth among 162 ICSI cycles [261] though it must be noted that mouse data on the role of miR-34c on embryo development have been conflicting [262, 263]. Alternatively, phthalates-associated sperm DNA damage [54, 55, 63-65, 67] may be another contributor. However, these alternative explanations do not necessarily contradict current results as numerous mechanisms may mediate the association between phthalates and phthalate alternatives exposure and poor embryo quality. These mechanisms could be working in concert or independently.

Sperm DNA methylation may also be mediating the association between urinary metabolite concentrations of phthalates and phthalate alternatives and poor semen parameters. As described in chapter 5, when examining the DMRs associated with phthalates, there was an enrichment of genes related to growth and development and cellular function and maintenance, but these are not specific

to embryos. A closer examination of the ontological analyses suggests that many DMRs are on or near genes relevant to cell movement and cell assembly and organization, which indicates a potential to affect sperm function. For example, it appears that all six DMRs on or near genes related to transport of Ca2+ (AVP, CCL11, CDK5R1, TRPM4, COL18A1, TMBIM6), a key regulator of sperm motility [264, 265], have increased methylation associated with phthalates. Similarly, in general, other functional groups related to cell movement, which comprised 24 other genes, also have increased methylation associated with phthalates. With respect to cell assembly and organization, 22 DMRs were found on or near genes related to formation and organization of cytoskeleton and protrusions.

In addition to replication in larger cohorts of general population, future work should clarify whether phthalates affect other sperm characteristics such as chromatin features and RNA species. Such work will better our understanding of the environmental influences on sperm epigenetics; whether these influence male fecundity; and if these influences have effects on embryogenesis and development at the molecular level before the blastocyst stage. Furthermore, it is of both biologic and public health relevance to address whether sperm DNA methylation can influence pregnancy health and fetal development beyond the blastocyst stage. Lastly, given that inter- and transgenerational effects have been observed as a result of exposure to environmental toxins and famine, additional long term observational studies are necessary to examine whether adult exposure to phthalates is associated with health of the subsequent generations via epigenetic mechanisms.

It is necessary to recognize that logistical and ethical concerns will likely limit the scope of human research. However, in areas where human research is not possible, animal models may address unanswered questions. Experimental evidence may be necessary to validate the observational data. For example, model organisms such as rodents would be exposed to various doses of phthalates and phthalate replacements to observe potential changes in sperm DNA methylation and other epigenetic features. Another interesting line of investigation would utilize transgenic animal models where the parental allele or origin can be tracked. This will allow us the ability to track the contribution of each parent's environmental influence on embryogenesis, fetal development, and offspring health.

APPENDIX A

PROTOCOL – SPERM DNA ISOLATION

Equipment
Disruptor Genie (Scientific Industries, Bohemia, NY, # SI-238)
0.2mm stainless steel beads (Next Advance, Averill Park, NY, # SSB02)

Reagents
100% PureCeption Isotonic Solution (Sage, Beverly, #: ART-2100)
Quinn's Sperm Wash Solution (Sage #: ART-1006)
Bond-Breaker TCEP Solution, Neutral pH (Pierce, Rockford, IL, # 77720)
Buffer RLT (Qiagen, Limburg, Netherlands, # 79216)
AllPrep DNA/RNA Mini Kit (Qiagen # 80204)

A. Sperm Cells Isolation from Whole Ejaculate
(If sperm is already pelleted, skip to step 12)

1. Make 90% PureCeption solution by combining 9mL 100% PureCeption with 1mL Quinn's sperm washing solution
2. Warm up 90% PureCeption solution and Quinn's sperm washing solution to 37°C
3. Add 1 mL 90% PureCeption solution to 15 mL conical tube
4. Carefully layer 1-2 mL clear ejaculate on top of PureCeption solution
5. Spin at 500 x g for 25 minutes
6. Transfer the pelleted sperm cells to a new 15 mL conical tube
    - Use 1mL pipette to transfer 500 µL from the bottom, including the pelleted cells
7. Add 3-5 mL Quinn's Sperm Washing Media
8. Spin at 500 x g for 5 minutes
9. Transfer 1mL of the cells and solution to a microcentrifuge tube (1.5 mL or 2.0 mL)
    - Better to transfer 2 x 500 µL in order to capture the entire pellet
    - Take out 10 µL for cell counting, make to vortex vigorously before
10. Spin at 4,000g for 1 minute
11. Carefully decant supernatant

B. Sperm Cell Lysate

12. Add 450 µL Buffer RLT and 50 µL TCEP-HCl to sperm pellet

- Final concentration of TCEP should be 50 mM

13. Add 0.1g stainless bashing beads
14. Bash on Disruptor Genie for 5 minutes
    - *Rest: Store lysate short-term at RT or long-term (< -20°C) or continue on to step 15*

C. DNA Isolation from Sperm Cell Lysate (Qiagen AllPrep Kit)

Before starting: heat Buffer EB (from the kit) to 70°C

15. Add the sperm lysate from step #14 to one DNA column from the AllPrep kit
16. Centrifuge at 10,000 x g for 30 seconds, discard the flowthrough
    - Repeat steps 14-15 if the lysate is >700 µL
17. Add 500 µL Buffer AW1
18. Centrifuge at 10,000 x g for 15 seconds, discard the flowthrough
19. Add 500 µL Buffer AW2
20. Centrifuge at 10,000 x g for 2 minutes, move the column to a new 1.5 mL microcentrifuge tube
21. Apply 50 µL Buffer EB directly onto the silica column, incubate for 3 minutes
22. Centrifuge at max speed for 1 minute to elute DNA
23. Repeat steps 21-22 twice with fresh 70°C Buffer EB, for a total of 150 µL
    a. *Helpful hint: dilution volume can be adjusted when starting material is <2 x 10^6 cells*

APPENDIX B

PRIMERS USED TO AMPLIFY *SNURF*, *PEG10*, AND *H19* LOCI BY PCR[1] FROM BISULFITE CONVERTED DNA EXTRACTED FROM HUMAN SPERM SAMPLES.

Gene	Chromosome	Start[2]	End	Primer	Sequence (5' – 3')
SNURF	15	25,201,077	25,201,305	F[3]	AGGGGGTATTAGAAGGGGTAGTAGT
				R[4]	ACAAATTCTACACATCATTCCAATCT
PEG10	7	94,285,836	94,286,063	F[3]	GTTTGGTTTAGGTGTGGGATTTTAT
				R[4]	CCCAAACCTTTAAAACTTAATTTCC
H19	11	2,020,925	2,021,200	F[3]	GGAAAATGTAAGATTTTGGTGGAATAT
				R[4]	CAATACAAACTCACACATCACAACC

[1]PCR conditions: 1x (94°C, 2 minutes); 40x (94°C, 15 sec; 58°C, 30 sec; 72°C, 30 sec).

[2]Feb 2009, GRC37/hg19 build, UCSC Genome Browser

[3]5' tag for all forward primers: aggaagagag

[4]5' tag (T7) for all reverse primers: cagtaatacgactcactatagggagaaggct

APPENDIX C1

ADJUSTED ODDS RATIOS FOR FERTILIZABILITY AND PARENT URINARY METABOLITE CONCENTRATIONS OF PHTHALATES AND PHTHALATE ALTERNATIVES

Parent Esters	Metabolite	Sex Specific Models*						Couples Level Model**					
		Males			Females			Males			Females		
		OR	95% CI	p-value	OR	95% CI	p-value	OR	95% CI	p-value	OR	95% CI	p-value
High Molecular Weight Phthalates													
DEHP	MEHP	0.83	0.64-1.08	0.17	1.04	0.89-1.21	0.65	0.88	0.68-1.16	0.37	1.07	0.92-1.25	0.38
	MEOHP	1.03	0.57-1.81	0.92	1.07	0.88-1.30	0.50	1.15	0.64-2.06	0.65	1.18	0.96-1.44	0.11
	MEHHP	1.02	0.62-1.66	0.95	1.06	0.88-1.27	0.53	1.13	0.68-1.86	0.64	1.16	0.96-1.39	0.12
	MECPP	1.10	0.66-1.85	0.72	1.12	0.89-1.42	0.33	1.18	0.69-2.04	0.55	1.23	0.95-1.59	0.12
	MCOP	1.15	0.90-1.46	0.27	0.94	0.77-1.16	0.59	1.20	0.92-1.57	0.19	0.90	0.71-1.12	0.34
DiNP	MNP	1.05	0.81-1.36	0.70	0.95	0.77-1.18	0.65	1.08	0.80-1.46	0.60	0.93	0.73-1.19	0.58
BBzP	MBzP	1.11	0.88-1.40	0.38	1.09	0.89-1.35	0.40	1.08	0.83-1.41	0.56	1.05	0.83-1.32	0.69
DOP	MCPP	0.95	0.74-1.23	0.72	0.85	0.69-1.05	0.13	0.97	0.72-1.29	0.82	0.86	0.70-1.06	0.16
DiDP	MCNP	1.10	0.69-1.76	0.69	0.78	0.51-1.20	0.26	1.16	0.75-1.78	0.50	0.78	0.49-1.23	0.29
Low Molecular Weight Phthalates													
DiBP	MBP	0.94	0.75-1.18	0.60	1.05	0.79-1.41	0.72	0.95	0.73-1.24	0.73	1.04	0.78-.139	0.80
	MHBP	0.88	0.61-1.28	0.51	1.13	0.78-1.65	0.51	0.88	0.59-1.31	0.52	1.03	0.71-1.49	0.89
DBP	MiBP	1.09	0.79-1.50	0.59	1.05	0.79-1.40	0.74	1.13	0.78-1.63	0.51	0.97	0.69-1.37	0.87
	MHiBP	0.85	0.58-1.24	0.39	1.02	0.73-1.43	0.91	0.83	0.54-1.28	0.39	1.04	0.71-1.52	0.84
DMP	MMP	1.13	0.89-1.45	0.31	1.21	0.89-1.65	0.23	1.05	0.82-1.35	0.70	1.20	0.84-1.72	0.30
DEP	MEP	1.15	0.96-1.37	0.13	0.88	0.72-1.07	0.20	1.21	0.98-1.50	0.08	0.86	0.70-1.05	0.14
Phthalate Alternatives													
DiNCH	MCOCH†	1.65	0.89-3.05	0.11	1.73	0.67-4.48	0.26	1.54	0.74-3.2	0.25	1.63	0.65-4.11	0.30
	MHiNCH†	1.14	0.64-2.03	0.67	1.00	0.60-1.67	0.99	1.05	0.52-2.11	0.90	1.10	0.60-2.01	0.76

*Each model adjusted for embryo grade at cleavage stage and the age and infertility status of the specific parent (i.e. not both)
**One model with both parental exposures, adjusted for cleavage stage embryo grade, age of both parents, and infertility status of male parent
‡Log transformed and specific gravity corrected
†Dichotomous variable based on LOD

APPENDIX C2

SPEARMAN CORRELATIONS COEFFICIENTS (R) OF URINARY METABOLITE CONCENTRATIONS BETWEEN SEEDS COUPLES (N = 49).

Parent Ester	Metabolite	r	p-value
HMW Metabolite			
DEHP	MEHP	-0.18	0.23
	MEHHP	-0.41	<0.01
	MEOHP	-0.34	0.02
	MECPP	-0.20	0.18
DiNP	MCOP	0.50	<0.01
	MNP	0.47	<0.01
BBzP	MBzP	0.50	<0.01
DOP	MCPP	0.31	0.03
DiDP	MCNP	0.40	<0.01
HMW Metabolite			
DiBP	MBP	0.01	0.93
	MHBP	-0.11	0.43
DBP	MiBP	0.36	0.01
	MHiBP	0.30	0.03
DMP	MMP	0.47	<0.01
DEP	MEP	0.32	0.03
HMW, high molecular weight; LMW, low molecular weight			

APPENDIX C3

STRATIFIED ODDS RATIOS (95% CIS) FOR HIGH QUALITY EMBRYOS AT BLASTOCYST STAGE AND SELECT URINARY METABOLITE CONCENTRATIONS OF PHTHALATES

Metabolite concentrations[1]			
Male	Female	OR[2]	95% CI
MEHP	<Median	1.03	0.54-1.97
	>Median	0.28	0.14-0.55
MBzP	<Median	0.52	0.10-2.82
	>Median	0.20	0.07-0.58
MBP	<Median	0.74	0.60-0.90
	>Median	0.51	0.10-2.47
MHBP	<Median	0.39	0.18-0.86
	>Median	0.34	0.09-1.32
MMP	<Median	0.34	0.22-0.52
	>Median	0.31	0.12-0.80

[1] Log transformed and specific gravity corrected

[2]A single model adjusted for embryo grade at cleavage stage, age and infertility status of male parent, and the age and metabolite concentration of the female parent

APPENDIX C4

ADJUSTED ODDS RATIOS FOR HIGH QUALITY AT BLASTOCYST STAGE AND PARENT URINARY METABOLITE CONCENTRATIONS OF PHTHALATES AND PHTHALATE ALTERNATIVES, RESTRICTED TO MATURE OOCYTES

		Sex Specific Models*								**Couple-Level Models****							
Parent Esters	Metabolite	Males				Females				Males				Females			
		OR	Lower CI	Upper CI	p-value	OR	Lower CI	Upper CI	p-value	OR	Lower CI	Upper CI	p-value	OR	Lower CI	Upper CI	p-value
DEHP	MEHP	0.58	0.31	1.08	0.08	1.10	0.84	1.44	0.49	0.53	0.28	1.02	0.06	0.98	0.81	1.19	0.87
	MEOHP	0.97	0.43	2.21	0.95	0.99	0.69	1.44	0.98	0.96	0.43	2.16	0.92	1.05	0.78	1.41	0.75
	MEHHP	0.77	0.38	1.58	0.48	0.99	0.71	1.39	0.96	0.74	0.36	1.55	0.43	1.00	0.77	1.30	0.98
	MECPP	0.79	0.41	1.54	0.49	0.89	0.58	1.36	0.59	0.78	0.39	1.54	0.47	1.03	0.74	1.44	0.87
	MCOP	1.00	0.59	1.68	0.99	1.02	0.63	1.65	0.93	1.01	0.62	1.65	0.95	1.00	0.61	1.61	0.99
DiNP	MNP	1.16	0.75	1.80	0.51	1.28	0.81	2.01	0.29	1.09	0.68	1.75	0.72	1.11	0.67	1.84	0.69
BBzP	MBzP	0.59	0.42	0.82	0.00	1.04	0.73	1.48	0.82	0.56	0.37	0.85	0.01	1.09	0.71	1.67	0.71
DOP	MCPP	1.10	0.61	1.99	0.76	1.06	0.57	1.97	0.86	1.10	0.63	1.95	0.73	1.08	0.57	2.05	0.82
DiDP	MCNP	0.95	0.46	1.98	0.89	0.94	0.40	2.24	0.89	1.01	0.46	2.22	0.98	1.08	0.41	2.83	0.87
DiBP	MBP	0.67	0.53	0.84	0.00	1.30	0.87	1.95	0.21	0.56	0.42	0.75	0.00	1.31	0.87	1.98	0.20
	MHBP	0.38	0.19	0.77	0.01	1.19	0.55	2.57	0.66	0.38	0.18	0.78	0.01	0.89	0.51	1.57	0.70
DBP	MiBP	1.33	0.71	2.50	0.37	1.49	0.84	2.63	0.17	1.07	0.52	2.23	0.85	1.34	0.69	2.62	0.39
	MHiBP	1.15	0.53	2.49	0.72	1.84	1.01	3.35	0.05	0.85	0.42	1.76	0.67	1.99	0.96	4.13	0.06
DMP	MMP	0.64	0.46	0.88	0.01	1.18	0.63	2.20	0.60	0.40	0.26	0.61	0.00	2.38	1.21	4.69	0.01
DEP	MEP	0.78	0.56	1.10	0.16	1.30	0.85	2.00	0.22	0.69	0.44	1.09	0.11	1.37	0.96	1.93	0.08
DiNCH	MCOCH	0.35	0.13	0.92	0.03	0.20	0.04	1.02	0.05	0.21	0.04	1.06	0.06	0.71	0.19	2.71	0.62
	MHiNCH	0.77	0.31	1.90	0.56	0.99	0.40	2.41	0.98	0.50	0.18	1.39	0.18	2.42	0.98	5.95	0.05

*Each model adjusted for embryo grade at cleavage stage and the age and infertility status of the specific parent (i.e. not both)
**A single model adjusted for embryo grade at cleavage stage, age of both parents, and infertility status of male parent
‡Log transformed and specific gravity corrected
†Dichotomous variable based on LOD

APPENDIX C5

ADJUSTED ODDS RATIOS FOR HIGH QUALITY AT BLASTOCYST STAGE AND PARENT URINARY METABOLITE CONCENTRATIONS OF PHTHALATES AND PHTHALATE ALTERNATIVES, RESTRICTED TO FERTILIZED EMBRYOS

		Sex Specific Models*								**Couple-Level Models****							
Parent Esters	Metabolite	Males				Females				Males				Females			
		OR	Lower CI	Upper CI	p-value	OR	Lower CI	Upper CI	p-value	OR	Lower CI	Upper CI	p-value	OR	Lower CI	Upper CI	p-value
DEHP	MEHP	0.60	0.32	1.10	0.10	1.08	0.84	1.40	0.55	0.54	0.29	1.03	0.06	0.98	0.81	1.18	0.83
	MEOHP	1.06	0.46	2.43	0.90	0.98	0.69	1.39	0.90	1.03	0.45	2.37	0.94	1.05	0.78	1.40	0.76
	MEHHP	0.82	0.40	1.68	0.59	0.98	0.71	1.35	0.89	0.79	0.38	1.65	0.52	1.00	0.78	1.29	0.99
	MECPP	0.84	0.44	1.63	0.61	0.88	0.58	1.34	0.56	0.83	0.42	1.63	0.58	1.03	0.74	1.43	0.87
	MCOP	0.97	0.58	1.63	0.92	1.03	0.64	1.63	0.91	0.98	0.61	1.58	0.93	1.01	0.64	1.62	0.95
DiNP	MNP	1.13	0.73	1.75	0.58	1.25	0.79	1.97	0.33	1.06	0.67	1.69	0.79	1.11	0.68	1.83	0.68
BBzP	MBzP	0.60	0.43	0.83	0.00	1.03	0.73	1.45	0.87	0.57	0.37	0.86	0.01	1.08	0.71	1.66	0.71
DOP	MCPP	1.08	0.60	1.92	0.81	1.07	0.59	1.95	0.82	1.08	0.62	1.87	0.80	1.09	0.59	2.02	0.77
DiDP	MCNP	0.94	0.46	1.91	0.86	0.96	0.41	2.23	0.92	0.99	0.47	2.08	0.97	1.10	0.44	2.76	0.84
DiBP	MBP	0.70	0.56	0.88	0.00	1.30	0.87	1.93	0.20	0.59	0.44	0.78	0.00	1.31	0.87	1.99	0.19
	MHBP	0.39	0.20	0.79	0.01	1.21	0.57	2.56	0.62	0.39	0.19	0.81	0.01	0.93	0.54	1.59	0.78
DBP	MiBP	1.30	0.72	2.35	0.38	1.47	0.85	2.54	0.17	1.05	0.52	2.12	0.89	1.34	0.70	2.57	0.38
	MHiBP	1.14	0.55	2.38	0.72	1.85	1.03	3.33	0.04	0.85	0.42	1.71	0.65	1.99	0.98	4.04	0.06
DMP	MMP	0.65	0.47	0.90	0.01	1.17	0.64	2.14	0.60	0.40	0.27	0.62	0.00	2.32	1.19	4.51	0.01
DEP	MEP	0.79	0.57	1.11	0.17	1.31	0.87	1.97	0.19	0.70	0.45	1.09	0.12	1.37	0.98	1.92	0.07
DiNCH	MCOCH	0.37	0.14	0.95	0.04	0.20	0.04	1.04	0.06	0.23	0.05	1.11	0.07	0.69	0.17	2.75	0.60
	MHiNCH	0.80	0.33	1.97	0.63	0.96	0.40	2.28	0.92	0.54	0.19	1.51	0.24	2.25	0.91	5.55	0.08

*Each model adjusted for embryo grade at cleavage stage and the age and infertility status of the specific parent (i.e. not both)
**A single model adjusted for embryo grade at cleavage stage, age of both parents, and infertility status of male parent
‡Log transformed and specific gravity corrected
†Dichotomous variable based on LOD

APPENDIX C6

ADJUSTED ODDS RATIOS FOR HIGH QUALITY AT BLASTOCYST STAGE AND PARENT URINARY METABOLITE CONCENTRATIONS OF PHTHALATES AND PHTHALATE ALTERNATIVES, RESTRICTED TO EMBRYOS CULTURED PAST DAY 3

		Sex Specific Models*								**Couple-Level Models****							
Parent Esters	Metabolite	Males				Females				Males				Females			
		OR	Lower CI	Upper CI	p-value	OR	Lower CI	Upper CI	p-value	OR	Lower CI	Upper CI	p-value	OR	Lower CI	Upper CI	p-value
	MEHP	0.60	0.33	1.11	0.10	1.08	0.84	1.40	0.55	0.55	0.29	1.04	0.06	0.97	0.80	1.18	0.77
DEHP	MEOHP	1.09	0.46	2.59	0.84	0.98	0.69	1.38	0.90	1.07	0.46	2.45	0.88	1.04	0.78	1.39	0.77
	MEHHP	0.83	0.40	1.74	0.62	0.98	0.72	1.34	0.89	0.80	0.38	1.66	0.54	1.00	0.77	1.29	0.97
	MECPP	0.85	0.43	1.70	0.65	0.89	0.59	1.34	0.57	0.85	0.43	1.67	0.63	1.03	0.74	1.43	0.88
	MCOP	0.92	0.57	1.50	0.74	1.03	0.66	1.63	0.88	0.96	0.60	1.56	0.88	1.02	0.65	1.62	0.92
DiNP	MNP	1.04	0.65	1.67	0.86	1.25	0.79	1.96	0.34	1.06	0.67	1.68	0.81	1.11	0.68	1.81	0.67
BBzP	MBzP	0.59	0.40	0.88	0.01	1.03	0.73	1.43	0.88	0.58	0.38	0.88	0.01	1.08	0.70	1.65	0.74
DOP	MCPP	1.02	0.58	1.80	0.94	1.08	0.60	1.94	0.79	1.06	0.61	1.85	0.83	1.10	0.61	2.00	0.75
DiDP	MCNP	0.87	0.47	1.62	0.67	0.97	0.43	2.21	0.94	0.97	0.47	2.01	0.93	1.11	0.45	2.73	0.81
DiBP	MBP	0.66	0.51	0.84	0.00	1.29	0.87	1.91	0.21	0.59	0.44	0.79	0.00	1.31	0.87	1.98	0.20
	MHBP	0.39	0.19	0.77	0.01	1.21	0.58	2.53	0.62	0.39	0.19	0.81	0.01	0.93	0.54	1.58	0.78
DBP	MiBP	1.14	0.60	2.17	0.70	1.46	0.85	2.50	0.17	1.05	0.53	2.11	0.88	1.33	0.70	2.53	0.39
	MHiBP	0.90	0.46	1.77	0.75	1.85	1.03	3.32	0.04	0.85	0.43	1.71	0.65	1.97	0.97	3.99	0.06
DMP	MMP	0.43	0.29	0.65	0.00	1.17	0.65	2.12	0.60	0.40	0.26	0.61	0.00	2.29	1.18	4.43	0.01
DEP	MEP	0.75	0.52	1.10	0.14	1.31	0.88	1.95	0.19	0.71	0.45	1.10	0.12	1.37	0.98	1.92	0.07
DiNCH	MCOCH	0.42	0.16	1.09	0.08	0.19	0.04	1.04	0.06	0.23	0.05	1.14	0.07	0.67	0.16	2.75	0.58
	MHiNCH	0.58	0.19	1.74	0.33	0.92	0.39	2.17	0.85	0.56	0.20	1.56	0.27	2.18	0.89	5.37	0.09

*Each model adjusted for embryo grade at cleavage stage and the age and infertility status of the specific parent (i.e. not both)
**A single model adjusted for embryo grade at cleavage stage, age of both parents, and infertility status of male parent
‡Log transformed and specific gravity corrected
†Dichotomous variable based on LOD

APPENDIX C7

ADJUSTED ODDS RATIOS FOR HIGH QUALITY AT BLASTOCYST STAGE AND PARENT URINARY METABOLITE CONCENTRATIONS OF PHTHALATES AND PHTHALATE ALTERNATIVES, RESTRICTED TO NON-SMOKERS

		Sex Specific Models*								**Couple-Level Models****							
Parent Esters	Metabolite	Males				Females				Males				Females			
		OR	Lower CI	Upper CI	p-value	OR	Lower CI	Upper CI	p-value	OR	Lower CI	Upper CI	p-value	OR	Lower CI	Upper CI	p-value
DEHP	MEHP	0.57	0.29	1.13	0.11	1.11	0.81	1.51	0.52	0.46	0.24	0.88	0.02	1.05	0.86	1.29	0.62
	MEOHP	0.99	0.40	2.47	0.98	1.07	0.70	1.62	0.76	0.96	0.39	2.37	0.94	1.12	0.79	1.58	0.53
	MEHHP	0.79	0.37	1.70	0.54	1.05	0.71	1.55	0.81	0.81	0.37	1.81	0.61	1.07	0.79	1.44	0.65
	MECPP	0.80	0.38	1.65	0.54	0.99	0.59	1.67	0.97	0.75	0.36	1.60	0.46	1.06	0.73	1.55	0.75
	MCOP	0.97	0.57	1.65	0.92	1.00	0.58	1.72	1.00	0.86	0.50	1.48	0.60	1.12	0.65	1.96	0.68
DiNP	MNP	1.15	0.75	1.77	0.52	1.26	0.77	2.07	0.35	1.03	0.63	1.68	0.91	1.18	0.69	2.00	0.55
BBzP	MBzP	0.55	0.38	0.79	0.00	0.98	0.69	1.40	0.92	0.52	0.32	0.85	0.01	1.12	0.72	1.74	0.61
DOP	MCPP	1.08	0.60	1.95	0.79	1.06	0.54	2.10	0.87	1.07	0.63	1.82	0.80	1.12	0.61	2.05	0.72
DiDP	MCNP	0.80	0.33	1.96	0.63	0.98	0.39	2.47	0.96	0.68	0.30	1.53	0.35	1.29	0.53	3.11	0.58
DiBP	MBP	0.67	0.50	0.89	0.01	1.34	0.87	2.08	0.18	0.56	0.40	0.77	0.00	1.28	0.82	2.01	0.28
	MHBP	0.33	0.15	0.73	0.01	1.15	0.50	2.64	0.73	0.36	0.16	0.84	0.02	0.87	0.48	1.56	0.63
DBP	MiBP	1.38	0.77	2.50	0.28	1.62	0.83	3.16	0.15	1.23	0.59	2.56	0.57	1.23	0.55	2.72	0.62
	MHiBP	1.17	0.57	2.40	0.68	1.97	1.01	3.81	0.05	0.98	0.49	1.94	0.94	1.90	0.87	4.17	0.11
DMP	MMP	0.62	0.41	0.94	0.02	1.21	0.62	2.34	0.57	0.44	0.26	0.74	0.00	2.38	1.23	4.61	0.01
DEP	MEP	0.79	0.56	1.13	0.19	1.36	0.85	2.17	0.20	0.73	0.45	1.17	0.18	1.31	0.92	1.86	0.14
DiNCH	MCOCH	0.27	0.07	1.03	0.06	0.21	0.04	1.12	0.07	0.26	0.06	1.22	0.09	0.81	0.21	3.12	0.76
	MHiNCH	0.78	0.31	1.99	0.61	1.19	0.46	3.06	0.73	0.56	0.20	1.54	0.26	2.56	1.05	6.22	0.04

*Each model adjusted for embryo grade at cleavage stage and the age and infertility status of the specific parent (i.e. not both)
**A single model adjusted for embryo grade at cleavage stage, age of both parents, and infertility status of male parent
‡Log transformed and specific gravity corrected
†Dichotomous variable based on LOD

APPENDIX D1

OVERLAPS FOUND IN DIFFERENTIALLY METHYLATED REGIONS (DMRS) ASSOCIATED WITH URINARY METABOLITES OF PHTHALATES AND PHTHALATE ALTERNATIVES

Gene	Alternative Names	Associated Metabolites	Cluster Numbers	Function (Gene Cards)
ESPN	Espin Autosomal Recessive Deafness Type 36 Protein Deafness, Autosomal Recessive 36	MEOHP/MBP	91	Multifunctional actin-bundling protein. It plays a major role in regulating the organization, dimensions, dynamics, and signaling capacities of the actin filament-rich, microvillus-type specializations that mediate sensory transduction in various mechanosensory and chemosensory cells. Mutations in this gene are associated with autosomal recessive neurosensory deafness, and autosomal dominant sensorineural deafness without vestibular involvement.
HERPUD2	Homocysteine-Inducible, Endoplasmic Reticulum Stress-Inducible, Ubiquitin-Like Domain Member 2	MEOHP/MBP	5719	Could be involved in the unfolded protein response (UPR) pathway
MTHFSD	Methenyltetrahydrofolate Synthetase Domain Containing	MEOHP/MBP	2567	nucleic acid binding and nucleotide binding.
MX1	MX Dynamin Like GTPase 1 Interferon-Regulated Resistance GTP-Binding Protein MxA Myxovirus (Influenza) Resistance 1, Homolog Of Murine (Interferon-Inducible Protein P78) IFI78	MBP/MBZP	4083	The encoded protein is induced by type I and type II interferons and antagonizes the replication process of several different RNA and DNA viruses. There is a related gene located adjacent to this gene on chromosome 21, and there are multiple pseudogenes located in a cluster on chromosome 4. GO annotations related to this gene include GTP binding and GTPase activity.

PLEKHG5	Pleckstrin Homology And RhoGEF Domain Containing G5	MEHP/MEOHP	93/94	activates the nuclear factor kappa B (NFKB1) signaling pathway. Mutations in this gene are associated with autosomal recessive distal spinal muscular atrophy.
SAMD11	Sterile Alpha Motif Domain Containing 11	MEHP/MBP	5/7	Transcription coactivator activity and PH domain binding; May play a role in photoreceptor development. From UCSC: GO annotations 0003713 (transcription coactivator activity) and 1903506 (regulation of nucleic acid tempalted transcription)
STK11	Serine/Threonine Kinase 11 LKB1	MEHP/MEOHP	3110	Regulates cell polarity and functions as a tumor suppressor. Mutations in this gene have been associated with Peutz-Jeghers syndrome, an autosomal dominant disorder characterized by the growth of polyps in the gastrointestinal tract, pigmented macules on the skin and mouth, and other neoplasms Isoform 2: Has a role in spermiogenesis.
TIMM8B	Translocase Of Inner Mitochondrial Membrane 8 Homolog B DDP2 DDPL	MEOHP/MBP	1318	Acts as a chaperone-like protein that protects the hydrophobic precursors from aggregation and guide them through the mitochondrial intermembrane space
RP11-81N13.1		MBZP/MBP	4337	

APPENDIX D2

LOCATIONS AND CPGS WITHIN DIFFERENTIALLY METHYLATED REGIONS (DMRS) ASSOCIATED WITH URINARY METABOLITES OF PHTHALATE AND PHTHALATE ALTERATIVES (BH ADJUSTED P-VALUE <0.05)

Metabolite	cluster_sites	chr	start	end
MBP	;cg04269747;cg19537591;cg07540593;cg03594819;cg18268988	chr11	111956647	111956844
MBP	;cg09604167;cg21070740	chr12	50134882	50134915
MBP	;cg19846096;cg10095011	chr12	133049188	133049413
MBP	;cg26872968;cg10448831;cg26434653	chr13	43930362	43930423
MBP	;cg03489965;cg10619342;cg23058194;cg18376860;cg01569660	chr15	65368982	65369213
MBP	;cg26813301;cg04737286;cg08981282;cg02512202;cg01195053;cg03314158;cg06035616;cg25000382;cg01124843;cg00433159	chr16	2653222	2653839
MBP	;cg05633380;cg04733911	chr16	78082579	78082701
MBP	;cg05981033;cg05225549;cg06093811;cg16540921;cg00608860;cg08086906;cg04531202;cg26436829;cg06874952;cg12403956;cg16359547;cg10756578;cg06599274	chr16	86588144	86589076
MBP	;cg12134570;cg15013019	chr19	13213428	13213451
MBP	;cg06262280;cg14512870;cg02670123	chr2	173330296	173330395
MBP	;cg16785077;cg20451722;cg03215005;cg12605796;cg14166395;cg16733866;cg13507964	chr21	42791867	42792703
MBP	;cg19433066;cg00837649;cg11197418	chr3	67705222	67705285
MBP	;cg04672903;cg23576358	chr4	6675309	6675513
MBP	;cg24069286;cg02970919	chr4	186300477	186300945
MBP	;cg06531475;cg06624358;cg14156792;cg24997886;cg01727431;cg10146935;cg02439789;cg24362661;cg05819296;cg00582671;cg22485363;cg06036236	chr1	870791	872737
MBP	;cg24092907;cg07302938	chr7	12729367	12729527
MBP	;cg15476479;cg06466797;cg23759826;cg11075346	chr7	35734978	35735308
MBP	;cg04906462;cg02875185	chr7	130788305	130788584
MBP	;cg05828992;cg23430295	chr7	140732689	140732738
MBP	;cg11248957;cg23332005	chr10	15963599	15963738
MBP	;cg22150335;cg14391247;cg12354377;cg26607103	chr10	62148959	62149642
MBP	;cg13284574;cg12406391	chr1	6519923	6520194
MBZP	;cg16785077;cg20451722;cg03215005;cg12605796;cg14166395;cg16733866;cg13507964	chr21	42791867	42792703
MBZP	;cg19433066;cg00837649;cg11197418	chr3	67705222	67705285

MCNP	;cg10360323;cg23758822	chr17	41437877	41437982
MCNP	;cg25072359;cg07153921	chr17	41440525	41440717
MCOCH	;cg24778776;cg14485103	chr11	69017394	69017579
MCOCH	;cg19359983;cg27181005;cg05491608;cg04057861	chr13	103346900	103347391
MCOCH	;cg13686919;cg19004608;cg13671412;cg20379170	chr19	5335045	5335260
MCOCH	;cg16407924;cg26605809;cg19368625	chr2	1452260	1452665
MCOCH	;cg09154639;cg07931024	chr20	55925570	55925586
MCOCH	;cg09009074;cg02130040;cg08935301	chr3	8543508	8543732
MCOCH	;cg12513994;cg17922326;cg20455197;cg16957758;cg19942454;cg12581741	chr10	1558762	1559249
MECPP	;cg11912315;cg05986168;cg19441908	chr8	41528946	41529140
MEHP	;cg17255148;cg26884837	chr11	2563802	2563897
MEHP	;cg12882392;cg01822289;cg01748193;cg09337943;cg09035529;cg25165501	chr11	64335229	64335895
MEHP	;cg18855356;cg06093070	chr1	16957483	16957489
MEHP	;cg17004104;cg14520913;cg18367578;cg14209518	chr11	114165730	114166636
MEHP	;cg18645647;cg13686847;cg19253743	chr12	109678885	109678973
MEHP	;cg09788586;cg15008124;cg05738240	chr12	114337676	114337927
MEHP	;cg24691910;cg13031611;cg06530563;cg26471674;cg11302945;cg04528829;cg25033380;cg08078751	chr12	115132460	115133735
MEHP	;cg21415724;cg04770364;cg22078988;cg01273565	chr12	120127920	120128116
MEHP	;cg22295211;cg04415616;cg27608806;cg10940545;cg01446612	chr12	125509610	125510168
MEHP	;cg10052597;cg24461952	chr12	132903921	132904014
MEHP	;cg02477305;cg15439717;cg10560368;cg01282150;cg04691180	chr13	23993013	23993527
MEHP	;cg02166450;cg01330096;cg02058624;cg10621576	chr13	112712795	112714526
MEHP	;cg23181831;cg25928208;cg10300728;cg07364729;cg16578825	chr13	112785388	112786727
MEHP	;cg01318265;cg13981078;cg13052453	chr14	105884721	105885138
MEHP	;cg11763800;cg02395812	chr14	105955745	105955879
MEHP	;cg18146152;cg16133244;cg01522083	chr16	1400772	1400861
MEHP	;cg08142918;cg03697918;cg24908603;cg07056644;cg27453745;cg09338251	chr16	86546374	86546979
MEHP	;cg06706029;cg26879339	chr16	89981752	89981919
MEHP	;cg01881265;cg17298275;cg20699780	chr17	934534	935017
MEHP	;cg02089963;cg12121983;cg02061130	chr17	1387495	1387922
MEHP	;cg19986200;cg04740558	chr17	7660449	7660494
MEHP	;cg07437263;cg21855910	chr17	30817129	30817397
MEHP	;cg24870391;cg05999628;cg27224069;cg11155489	chr17	32612675	32613298
MEHP	;cg02186277;cg23632849	chr17	72208213	72208322

MEHP	;cg08470991;cg01246266;cg03190661;cg20943461;cg19056418	chr17	76126449	76127217
MEHP	;cg22357164;cg22749810;cg14175304	chr17	78237117	78237397
MEHP	;cg06444575;cg00702126;cg14667273;cg27391934	chr1	1369772	1369948
MEHP	;cg01133401;cg25232541	chr19	409679	409888
MEHP	;cg22467470;cg20637199;cg16192413;cg06909547;cg07536072	chr19	1226523	1227141
MEHP	;cg09487101;cg14796563	chr19	1287428	1287576
MEHP	;cg16889945;cg03404027	chr19	1523070	1523442
MEHP	;cg04154502;cg15062725	chr19	3831293	3831364
MEHP	;cg03088725;cg01914743;cg11277220;cg10002088;cg15894653;cg14737370	chr19	9270837	9271995
MEHP	;cg08177015;cg22865824;cg03767475	chr19	18902527	18902727
MEHP	;cg18627816;cg10092957;cg12574296	chr19	39686695	39687098
MEHP	;cg03716852;cg17583504	chr19	49669281	49669542
MEHP	;cg19309676;cg00711496;cg17417856	chr19	50191439	50191637
MEHP	;cg22511368;cg13728299;cg01387220	chr19	55281057	55281274
MEHP	;cg27247510;cg05014291	chr2	11780349	11780446
MEHP	;cg09836979;cg10266648;cg20890180	chr2	106560496	106560771
MEHP	;cg03850256;cg11272491	chr2	176974592	176974934
MEHP	;cg15039182;cg02301319;cg04365609;cg02960938;cg00993677	chr2	239930734	239931250
MEHP	;cg06541968;cg06531129	chr2	240431144	240431270
MEHP	;cg05116145;cg17500202;cg01662942	chr2	241721922	241722113
MEHP	;cg14684854;cg14059464	chr2	241901865	241901988
MEHP	;cg17465173;cg19506677;cg13388615;cg12203072;cg00551733	chr2	242878496	242879153
MEHP	;cg25551168;cg16536918;cg24257309;cg05136169;cg02187522	chr20	3065343	3065488
MEHP	;cg20975074;cg24776480	chr1	2066430	2066446
MEHP	;cg01753209;cg02983759;cg20206277;cg12792526;cg12656497;cg27263741;cg03364381	chr21	43098516	43099460
MEHP	;cg22978940;cg11113589;cg03705621	chr21	46898610	46898789
MEHP	;cg27324619;cg26373518;cg18053607	chr22	31518860	31518963
MEHP	;cg20539366;cg05475172	chr22	46370644	46371152
MEHP	;cg19808085;cg08173919;cg10341513	chr22	51040520	51041242
MEHP	;cg12870811;cg03652676;cg01427909	chr3	62361449	62362324
MEHP	;cg27550618;cg17577431	chr3	175696286	175696388
MEHP	;cg03538934;cg18030372;cg16417840	chr4	2069683	2069925
MEHP	;cg08796692;cg13847226	chr4	7192699	7193237
MEHP	;cg20245328;cg13701738	chr4	186799820	186799847
MEHP	;cg06572420;cg02208657	chr5	175621395	175621409
MEHP	;cg11981639;cg08124209	chr6	1622383	1622941
MEHP	;cg25786333;cg23584176;cg09081266	chr6	29364799	29365287

MEHP	;cg19813135;cg11812071;cg25934495;cg01981433;cg19862242;cg18287768;cg12200164	chr6	138200071	138200679
MEHP	;cg14714009;cg26698664;cg11783014;cg06259339;cg25697490;cg19757131;cg15408737	chr6	149285724	149286375
MEHP	;cg20067575;cg04076264	chr6	150437072	150437118
MEHP	;cg15246238;cg12600030;cg24239690	chr7	5635134	5635953
MEHP	;cg04107994;cg13337615	chr7	6209931	6210236
MEHP	;cg19781251;cg07278332;cg09265417;cg06999014	chr7	94294278	94294905
MEHP	;cg09377301;cg13696531;cg19128364;cg01262413;cg09193751	chr7	157691177	157691591
MEHP	;cg11966432;cg21819129;cg17638856;cg08575860;cg06040034	chr8	1617616	1618448
MEHP	;cg19913626;cg09405635;cg23402467	chr8	49533357	49533485
MEHP	;cg19526685;cg12619165	chr8	126963507	126964345
MEHP	;cg17493839;cg00111503;cg22424020;cg04066400	chr8	140630830	140631269
MEHP	;cg00664093;cg00316921;cg07295586;cg00187981	chr8	142984321	142984613
MEHP	;cg00118808;cg13693256	chr9	132097931	132098311
MEHP	;cg10827460;cg15742605;cg09756115;cg23731742	chr1	879375	879958
MEHP	;cg10588310;cg14841011	chr1	3332000	3332125
MEHP	;cg15605704;cg15580684	chr1	4770676	4770711
MEHP	;cg06620993;cg04388244	chr10	105420831	105421005
MEHP	;cg01799460;cg16146806	chr1	6529710	6530205
MEHP	;cg19478951;cg23976652;cg00595223;cg15281331;cg04766005	chr10	133879084	133879763
MEHP	;cg09033333;cg24324584;cg17348201	chr10	133892406	133892567
MEHP	;cg09205751;cg22331032;cg18016138;cg23437420;cg15451020;cg03009397;cg06328831	chr11	278394	278912
MEHP	;cg08596817;cg06046490	chr11	320929	320940
MEOHP	;cg04269747;cg19537591;cg07540593;cg03594819;cg18268988	chr11	111956647	111956844
MEOHP	;cg05981033;cg05225549;cg06093811;cg16540921;cg00608860;cg08086906;cg04531202;cg26436829;cg06874952;cg12403956;cg16359547;cg10756578;cg06599274	chr16	86588144	86589076
MEOHP	;cg03125909;cg07509252;cg06534221	chr16	88725584	88725763
MEOHP	;cg16275118;cg18775736;cg09323570;cg24530271	chr18	74153342	74154260
MEOHP	;cg23260026;cg16825133;cg05304405;cg24341615;cg22619412;cg17710576;cg10503234	chr19	675390	676739
MEOHP	;cg22467470;cg20637199;cg16192413;cg06909547;cg07536072	chr19	1226523	1227141
MEOHP	;cg02512596;cg15274684	chr21	48054563	48054897

MEOHP	;cg12960467;cg23304085	chr5	133703730	133703889
MEOHP	;cg10578681;cg13962681	chr1	213223453	213223461
MEOHP	;cg15476479;cg06466797;cg23759826;cg11075346	chr7	35734978	35735308
MEOHP	;cg05307234;cg26292018	chr8	133786975	133787071
MEOHP	;cg20017995;cg01605984	chr9	136216076	136216154
MEOHP	;cg06202426;cg07670566;cg13994338;cg05656770	chr9	139836994	139838089
MEOHP	;cg19158553;cg24305579	chr1	5356471	5356578
MEOHP	;cg13284574;cg12406391	chr1	6519923	6520194
MEOHP	;cg00918541;cg17329304;cg24535622;cg18125265	chr1	6546027	6546646
MHBP	;cg09646593;cg19898448	chr4	125631168	125631415
MHBP	;cg11046421;cg04713108	chr5	44806923	44807541
MHIBP	;cg04321566;cg03921396;cg26268843	chr7	64466846	64467056
MHIBP	;cg25645879;cg05151824;cg26702985;cg19180542;cg14271085;cg17830980;cg08197448;cg02949969;cg17003301;cg07876289;cg14058825	chr10	43047255	43048734
MMP	;cg19039481;cg10717610;cg23982445;cg23399257	chr2	394115	394481

APPENDIX D3

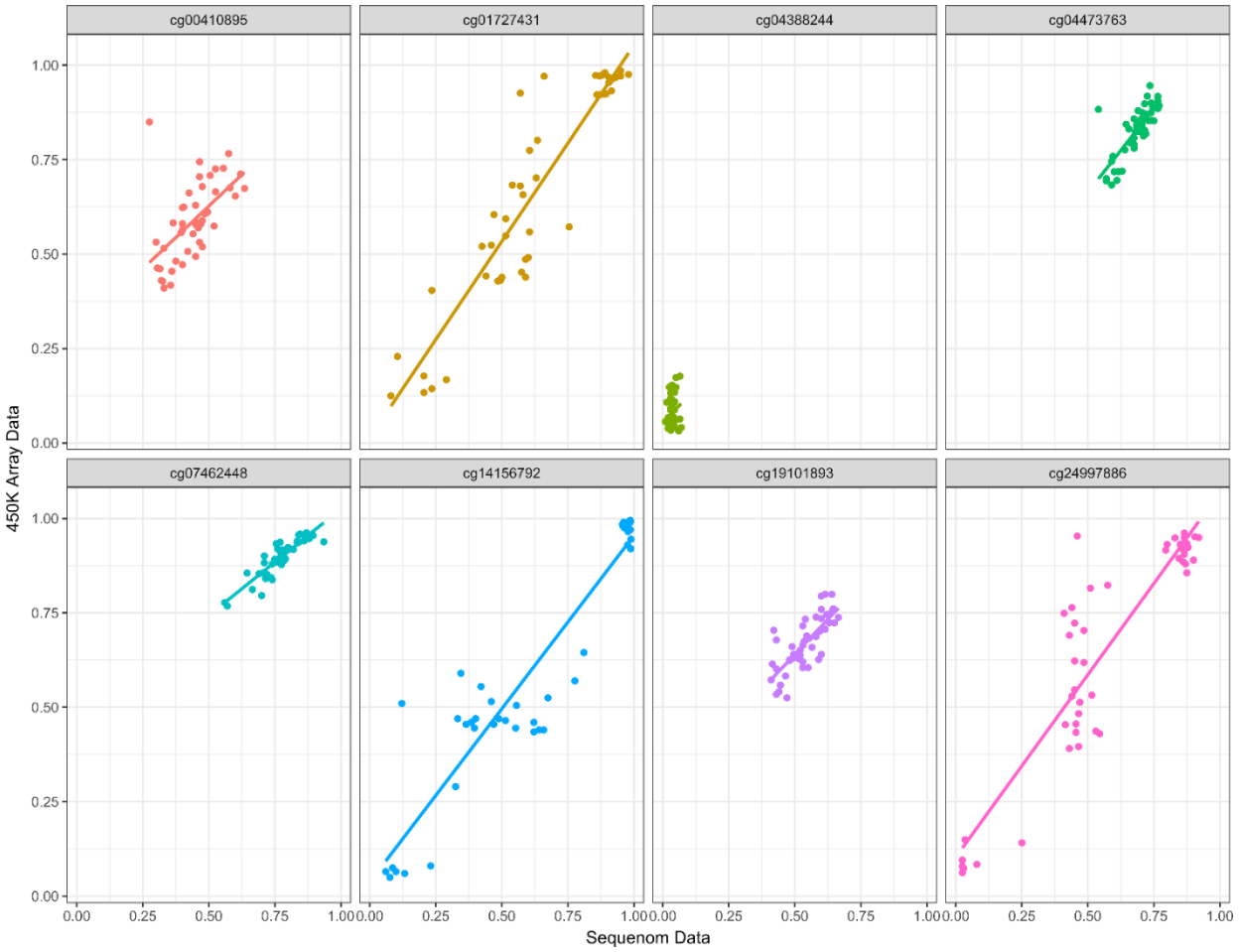
COMPARISON OF BETA-VALUES DERIVED FROM 450K VS. METHYLATION VALUES FROM SEQUENOM (N=47)

CpG ID	Method	Mean	Min	Max	SD	Spearman Rho	p-value
cg04388244	sequenom	0.034	0.010	0.070	0.014	0.233	0.1155*
	450K	0.079	0.032	0.177	0.040		
cg14156792	sequenom	0.615	0.050	0.995	0.316	0.939	<2.2E-16
	450K	0.636	0.060	0.989	0.323		
cg24997886	sequenom	0.560	0.025	0.920	0.281	0.911	<2.2E-16
	450K	0.649	0.062	0.961	0.296		
cg01727431	sequenom	0.629	0.080	0.980	0.253	0.925	<2.2E-16
	450K	0.673	0.125	0.985	0.283		
cg00410895	sequenom	0.442	0.275	0.635	0.088	0.660	4.55E-07
	450K	0.587	0.410	0.850	0.101		
cg19101893	sequenom	0.540	0.410	0.665	0.073	0.785	6.25E-11
	450K	0.668	0.525	0.799	0.071		
cg07462448	sequenom	0.775	0.560	0.935	0.077	0.894	<2.2E-16
	450K	0.898	0.768	0.962	0.048		
cg04473763	sequenom	0.683	0.540	0.770	0.058	0.770	2.43E-10
	450K	0.823	0.683	0.946	0.069		

*Removal of one influential point reduces Spearman p-value to 0.04318

APPENDIX D4

SCATTERPLOT AND TREND LINE COMPARING THE EIGHT VALIDATED CPG SITES USING SEQUENOM MASSARRAY (X-AXIS) AND THE 450 K BETA-VALUES (Y-AXIS).



APPENDIX D5

GENERALIZED LINEAR MODEL (GLM) RESULTS OF LOG TRANSFORMED MCNP AND AVERAGED METHYLATION OF 21 IMPRINTED GENES*

Gene	Beta_Value_Betas^	Mval_Betas	Mval_LCI	Mval_UCI	Mval_p_value	Mval_padjusted
mean_PLAGL1_HYMAI	0.011	0.345	0.203	0.487	0.000	0.000
mean_KCNQ1_KCNQ1OT1	0.005	0.188	0.090	0.286	0.001	0.004
mean_PEG3_ZIM2	0.004	0.138	0.064	0.212	0.001	0.004
mean_MESTIT1_MEST	0.005	0.170	0.073	0.268	0.001	0.006
mean_GNAS_GNASAS1	0.003	0.103	0.042	0.165	0.002	0.007
mean_H19	-0.015	-0.218	-0.357	-0.079	0.004	0.009
mean_NAP1L5	0.008	0.226	0.082	0.370	0.004	0.009
mean_SGCE_PEG10	0.003	0.117	0.043	0.192	0.004	0.009
mean_L3MBTL	0.004	0.130	0.041	0.219	0.006	0.013
mean_MAGEL2	0.004	0.141	0.001	0.281	0.055	0.076
mean_MEG3	0.003	0.109	0.001	0.217	0.055	0.076
mean_MKRN3	0.006	0.136	0.003	0.268	0.051	0.076
mean_SNRPN	0.003	0.094	0.005	0.183	0.044	0.076
mean_GRB10	0.001	0.081	-0.005	0.166	0.071	0.092
mean_KCNK9	-0.003	-0.114	-0.249	0.020	0.102	0.122
mean_ANKRD11	0.003	0.097	-0.038	0.232	0.165	0.186
mean_DIRAS3	0.001	0.050	-0.045	0.144	0.310	0.328
mean_DLGAP2	-0.001	-0.055	-0.169	0.060	0.353	0.353

*Identified from PMC3348513
^GLM beta coefficients using the beta-values

APPENDIX D6

CURATED INGENUITY PATHWAY ANALYSIS (IPA) AND GENE SET ENRICHMENT ANALYSIS (GSEA) RESULTS

Networks related to Differentially Methylated Regions (DMRs) associated with anti-androgenic phthalates, restricted to those with Ingenuity Pathway Analysis (IPA) score >20

Molecules in Network	**Score**	**Focus Molecules**	**Top Diseases and Functions**
ABR, Actin, AMPK, ANK3, AVP, CCL11, CDK5R1, CIT, COL18A1, Collagen type IV, Creb, EFNA2, ERK1/2, ESPN, F Actin, FOXF1, FSCN1, FSH, FSTL3, Gsk3, GTPase, ITGA6, Laminin, Lh, MAPK8IP2, Mek, PI3K (family), Pld, PRKCZ, PTPRN2, Rac, SH3PXD2A, Tgf beta, TMBIM6, TNFAIP3	41	19	Nervous System Development and Function, Organ Morphology, Organismal Development
ACACB, ADCY, Akt, ANK1, caspase, ERK, FBXW5, Focal adhesion kinase, Histone h3, HTR1B, IFITM3, IgG, IL1, Insulin, Jnk, KIF1A, Mapk, MC1R, MUC8, MVD, MYO1C, NFkB (complex), P38 MAPK, PI3K (complex), Pkc(s), PLEKHG5, PRDM16, PRMT1, PRMT2, PRMT1/PRMT2, RAE1, Ras, SETD1B, TIMM8B, TRPM4	33	16	Cancer, Gastrointestinal Disease, Organismal Injury and Abnormalities
AGT, CYTH3, DLGAP2, DNAH2, DNAH3, EEF1A1, EEF2K, EIF1AD, ELAVL1, ELK1, ENOX1, ESR1, FRAT2, GREB1, HERPUD2, HOXD11, IGF2BP3, JMY, KCNK3, KCNK9, Krt10, LSM14A, MDM2, miR-22-3p (miRNAs w/seed AGCUGCC), MTHFSD, NCCRP1, PEG10, phosphatidylinositol 4, 5-diphosphate, SAMD11, TMC6, TMC8, TP53, TUBA8, ZNF282, ZNF516	28	14	Cell Cycle, Dermatological Diseases and Conditions, Infectious Diseases
AAK1, ADGRB2, ANKRD50, APP, BCKDK, C21orf59, CCDC115, CDKL3, DDX49, DNAH1, ELAVL4, HNF4A, HSPA5, KANSL2, KIR2DL1/KIR2DL3, LGR5, LRP2BP, METTL21A, METTL21C, MRPS18A, MSL3, NNMT, PHF20L1, PIH1D3, PNPO, RASL12, REL, RNF213, RPS6KC1, SF3A3, SIX2, TNIP2, TSR3, TUBA8, ZNF317	23	12	Post-Translational Modification, Protein Degradation, Protein Synthesis
1, 3, 4, 5-IP4, AJAP1, ARL4A, butyric acid, C2CD4C, Ca2+, CALB1, CALCOCO1, CCND1, CDH5, CDH13, COL18A1, COMP, CRIP1, CTNNB1, Fascin, HNF1A, IFITM1, INPP5J, LGR5, LMCD1, LYL1,	23	12	Endocrine System Disorders, Organismal Injury and Abnormalities,

MIR17HG, PAH, PCDH11Y, RHOU, RNF14, RTKN, SAA1, SLC22A11, Sphk, TRPC5, WNT3, WNT7B, ZNF117			Cell-To-Cell Signaling and Interaction

Networks related to Differentially Methylated Regions (DMRs) associated with anti-androgenic phthalates, restricted to those with Ingenuity Pathway Analysis (IPA) score and p<0.05

Categories	**Diseases or Functions Annotation**	**Predicted Activation State**	**Activation z-score**	**Assigned Category***
Cellular Development	differentiation of cells		0.185	Growth and Development
Cell Morphology, Cellular Assembly and Organization, Cellular Function and Maintenance	formation of cellular protrusions		-0.316	Cellular Function and Maintenance
Cancer, Organismal Injury and Abnormalities	epithelial cancer		0.246	Cancer
Cancer, Organismal Injury and Abnormalities	malignant solid tumor		-1.241	Cancer
Cellular Assembly and Organization, Cellular Function and Maintenance	organization of cytoskeleton		-0.867	Cellular Function and Maintenance
Cell Morphology, Cellular Assembly and Organization, Cellular Development, Cellular Function and Maintenance, Cellular Growth and Proliferation, Nervous System Development and Function, Tissue Development	neuritogenesis		-0.651	Growth and Development
Cellular Assembly and Organization, Cellular Function and Maintenance	microtubule dynamics		-0.564	Cellular Function and Maintenance
Cellular Development, Connective Tissue Development and Function, Tissue Development	differentiation of connective tissue		0.555	Growth and Development
Behavior	anxiety		1.408	Other
Cellular Growth and Proliferation, Tissue Development	generation of cells		0.086	Growth and Development
Cellular Development, Cellular Growth and Proliferation, Nervous System Development and Function, Tissue Development	development of neurons		-0.524	Growth and Development

Cellular Movement	cell movement		0.94	Cellular Function and Maintenance
Cancer, Organismal Injury and Abnormalities	metastasis		1	Cancer
Cell Morphology, Cellular Assembly and Organization, Cellular Development, Cellular Function and Maintenance, Cellular Growth and Proliferation, Nervous System Development and Function, Tissue Development	morphogenesis of neurites		-0.152	Growth and Development
Cellular Development, Connective Tissue Development and Function, Tissue Development	differentiation of connective tissue cells		0	Growth and Development
Gastrointestinal Disease, Hepatic System Disease, Organismal Injury and Abnormalities	liver lesion		-0.068	Other
Cell Death and Survival, Organismal Injury and Abnormalities	necrosis of epithelial tissue		1.768	Cellular Function and Maintenance
Cellular Assembly and Organization	formation of cytoskeleton		1.406	Cellular Function and Maintenance
Cellular Assembly and Organization, Cellular Function and Maintenance, Tissue Development	formation of actin filaments		1.951	Growth and Development
Cellular Assembly and Organization, Tissue Development	formation of filaments		1.406	Growth and Development
Cellular Movement	migration of cells		1.454	Cellular Function and Maintenance
Cellular Growth and Proliferation	proliferation of cells		1.908	Cellular Function and Maintenance
Cell Death and Survival	cell death		0.319	Cellular Function and Maintenance
Gene Expression	expression of RNA		0.124	Other
Cellular Movement	invasion of cells		1.878	Cellular Function and Maintenance
Cell Morphology, Cellular Assembly and Organization, Cellular Function and Maintenance	formation of filopodia		1.109	Cellular Function and Maintenance
Tissue Development	growth of epithelial tissue		-1.944	Growth and Development

Cellular Movement	invasion of tumor cell lines	Increased	2.421	Cellular Function and Maintenance
Cell Signaling, Post-Translational Modification	tyrosine phosphorylation of protein		1.199	Cellular Function and Maintenance
Cell Signaling, Cellular Function and Maintenance, Molecular Transport, Vitamin and Mineral Metabolism	influx of Ca2+		0.862	Cellular Function and Maintenance
Cell Signaling, Molecular Transport, Small Molecule Biochemistry, Vitamin and Mineral Metabolism	release of Ca2+		0.254	Cellular Function and Maintenance
Developmental Disorder, Skeletal and Muscular Disorders	muscular hypertrophy		1.091	Growth and Development
Cellular Movement	migration of tumor cell lines		0.987	Cellular Function and Maintenance
Behavior	behavior		0.396	Other
Connective Tissue Development and Function, Tissue Development	development of connective tissue		0.686	Growth and Development
Inflammatory Response	inflammatory response		-0.117	Other
Cell Death and Survival, Cellular Compromise	cytotoxicity of cells	Increased	2	Cellular Function and Maintenance
Cellular Movement	cell movement of tumor cell lines		0.904	Cellular Function and Maintenance
Embryonic Development, Organismal Development	development of body axis		-1.698	Growth and Development
Organismal Survival	organismal death		-0.344	Other
Cell Death and Survival, Neurological Disease, Organismal Injury and Abnormalities	cell death of cerebral cortex cells		-1.958	Cellular Function and Maintenance
Gene Expression	transcription		-0.024	Other
Cell Death and Survival	cell survival		1.212	Cellular Function and Maintenance
Behavior	cognition		0	Other

Cell Death and Survival	necrosis		0.8	Cellular Function and Maintenance
*Assigned by authors				

Networks related to Differentially Methylated Regions (DMRs) associated with Bis(2-ethylhexyl) phthalate metabolites, restricted to those with score >20

Molecules in Network	**Score**	**Focus Molecules**	**Top Diseases and Functions**
ACACB, ADCY, AMPK, AVP, CCL11, CDK5R1, CDKL3, COL18A1, Collagen type IV, Creb, DLGAP2, ERK, ERK1/2, FOXF1, FSCN1, FSH, FSTL3, Gsk3, Hsp90, IFITM3, IgG, IL1, Insulin, Lh, Mapk, MAPK8IP2, Mek, MVD, Pka, Pld, PRKCZ, PTPRN2, STK11, Tgf beta, TNFAIP3	37	17	Amino Acid Metabolism, Small Molecule Biochemistry, Cell Morphology
ABR, Actin, AFAP1L2, Akt, ANK1, CIT, ELAVL4, ESPN, F Actin, Fascin, FBXW5, Focal adhesion kinase, Histone h3, HTR1B, Jnk, KIF1A, MC1R, MYO1C, NFkB (complex), P38 MAPK, PI3K (complex), Pkc(s), PLEKHG5, PRDM16, PRMT1, PRMT2, PRMT1/PRMT2, Rac, RHBDD3, SETD1B, SH3PXD2A, SPAG8, TIMM8B, TRPM4, TYRO3	31	15	Neurological Disease, Cardiovascular Disease, Cell Morphology
AJAP1, beta-estradiol, C11orf57, C2CD4C, CCND1, CD14, COMP, COQ10B, CREBL2, CRIP1, DEPDC1, ELAVL1, GREB1, HERPUD2, HNF1A, HNF4A, INPP5J, JUN, L-dopa, MTHFSD, NPTX2, NXF1, PCNP, PEG10, PHF20L1, PNPO, RPS6KC1, SLC22A11, STARD10, TGFB1, TM4SF4, TMC6, TMC8, WNT7B, ZNF317	31	15	Dermatological Diseases and Conditions, Infectious Diseases, Organismal Injury and Abnormalities
ADAMTS7, AGT, ARRB2, ARRDC3, CYP11B1, CYTH3, DCBLD2, DNAH2, DNAH3, EFNA2, ESR1, FRMD4B, GPR18, HCAR3, HOXD11, HTR1B, KCNK3, KCNK9, KIR2DL1/KIR2DL3, MLANA, MMP17, NCCRP1, NNMT, PTHLH, REL, RNF213, SAMD11, SLC14A1, SLC16A3, TAC1, TNF, TSR3, TUBA8, VIPR2, ZNF516	24	12	Endocrine System Development and Function, Molecular Transport, Protein Synthesis

Networks related to Differentially Methylated Regions (DMRs) associated Bis(2-ethylhexyl) phthalate metabolites, restricted to those with Ingenuity Pathway Analysis (IPA) score and p<0.05

Categories	Diseases or Functions Annotation	Predicted Activation State	Activation z-score	Assigned Category*
Cancer, Organismal Injury and Abnormalities	malignant solid tumor		-1.083	Cancer
Cancer, Organismal Injury and Abnormalities	epithelial cancer		0.067	Cancer
Cell Morphology, Cellular Assembly and Organization, Cellular Development, Cellular Function and Maintenance, Cellular Growth and Proliferation, Nervous System Development and Function, Tissue Development	neuritogenesis		-0.022	Growth and Development
Cellular Development	differentiation of cells		0.859	Growth and Development
Cell Morphology, Cellular Assembly and Organization, Cellular Function and Maintenance	formation of cellular protrusions		0.553	Growth and Development
Cell Morphology, Cellular Assembly and Organization, Cellular Development, Cellular Function and Maintenance, Cellular Growth and Proliferation, Nervous System Development and Function, Tissue Development	morphogenesis of neurites		0.44	Growth and Development
Cellular Development, Cellular Growth and Proliferation, Nervous System Development and Function, Tissue Development	development of neurons		-0.019	Growth and Development
Behavior	anxiety		1.408	Other
Cellular Assembly and Organization, Cellular Function and Maintenance	organization of cytoskeleton		-0.35	Cellular Function and Maintenance
Cellular Movement	cell movement		1.43	Cellular Function and Maintenance
Cellular Growth and Proliferation, Tissue Development	generation of cells		0.437	Growth and Development
Cellular Assembly and Organization, Cellular Function and Maintenance	microtubule dynamics		-0.041	Cellular Function and Maintenance

Cellular Assembly and Organization	formation of cytoskeleton		1.673	Cellular Function and Maintenance
Cellular Development, Connective Tissue Development and Function, Tissue Development	differentiation of connective tissue		0.896	Growth and Development
Cellular Movement	migration of cells		1.947	Cellular Function and Maintenance
Cellular Assembly and Organization, Tissue Development	formation of filaments		1.673	Cellular Function and Maintenance
Cellular Assembly and Organization, Cellular Function and Maintenance, Tissue Development	formation of actin filaments	Increased	2.186	Cellular Function and Maintenance
Cellular Development, Connective Tissue Development and Function, Tissue Development	differentiation of connective tissue cells		0.447	Growth and Development
Gastrointestinal Disease, Hepatic System Disease, Organismal Injury and Abnormalities	liver lesion		-0.298	Cancer
Tissue Development	growth of epithelial tissue		-1.331	Growth and Development
Cellular Growth and Proliferation	proliferation of cells		1.575	Growth and Development
Cell Death and Survival	cell survival		1.472	Cellular Function and Maintenance
Cellular Movement	invasion of tumor cell lines		1.689	Cellular Function and Maintenance
Cell Death and Survival, Organismal Injury and Abnormalities	necrosis of epithelial tissue		1.544	Cellular Function and Maintenance
Cancer, Organismal Injury and Abnormalities	metastasis		0.259	Cancer
Cell Death and Survival	cell viability		1.47	Cellular Function and Maintenance
Behavior	behavior		0.396	Other
Cellular Movement	invasion of cells		1.604	Cellular Function and Maintenance
Cellular Development	branching of cells		0.059	Growth and Development

Cell Death and Survival, Organismal Injury and Abnormalities	apoptosis of endothelial cells		0.414	Cellular Function and Maintenance
Cellular Movement	cell movement of tumor cells		0.152	Cellular Function and Maintenance
Inflammatory Response	inflammatory response		-0.117	Other
Cell Signaling, Cellular Function and Maintenance, Molecular Transport, Vitamin and Mineral Metabolism	influx of Ca2+		0.862	Cellular Function and Maintenance
Connective Tissue Development and Function, Tissue Development	development of connective tissue		0.686	Growth and Development
Cell Death and Survival	cell death		0.595	Cellular Function and Maintenance
Cellular Movement	invasion of carcinoma cell lines		0.895	Cellular Function and Maintenance
Cellular Development, Cellular Growth and Proliferation	cell proliferation of tumor cell lines		1.364	Growth and Development
Cell Death and Survival, Neurological Disease, Organismal Injury and Abnormalities	cell death of cerebral cortex cells		-1.958	Cellular Function and Maintenance
Organismal Survival	organismal death		-0.98	Other
Cellular Movement	migration of tumor cell lines		1.364	Cellular Function and Maintenance
Cardiovascular System Development and Function, Cellular Movement	cell movement of endothelial cells		1.184	Growth and Development
Cellular Movement	cell movement of tumor cell lines		1.291	Cellular Function and Maintenance
Cell Death and Survival	necrosis		1.638	Cellular Function and Maintenance
Cardiovascular System Development and Function, Organismal Development	angiogenesis		1.439	Growth and Development
Cell Death and Survival	cell viability of tumor cell lines		1.369	Cellular Function and Maintenance

APPENDIX D7

Gene Set Enrichment Analyses (GSEA) results for all gene sets with normalized enrichment score> 1.8 and false discovery rate (FDR) corrected p-value < 0.2

Metabolite	Gene Set	NES	FDR p-value	Effect
MBP	IL2_STAT5_SIGNALING	-1.96	0.005	Negative
	MEISSNER_BRAIN_HCP_WITH_H3K27ME3	2.07	0.049	Positive
	CHICAS_RB1_TARGETS_GROWING	-2.06	0.063	Negative
	SENESE_HDAC3_TARGETS_UP	-2.00	0.084	Negative
	ZHANG_RESPONSE_TO_IKK_INHIBITOR_AND_TNF_DN	-1.98	0.073	Negative
	SENESE_HDAC1_TARGETS_UP	-1.91	0.139	Negative
	FOSTER_TOLERANT_MACROPHAGE_UP	-1.88	0.147	Negative
	BURTON_ADIPOGENESIS_6	-1.80	0.197	Negative
	V$CREBP1_01	-1.89	0.166	Negative
	TTCYNRGAA_V$STAT5B_01	-1.84	0.197	Negative
MCOCH	CCANNAGRKGGC_UNKNOWN	-1.85	0.050	Negative
	GO_ANTERIOR_POSTERIOR_PATTERN_SPECIFICATION	-1.98	0.012	Negative
	GO_REGIONALIZATION	-1.95	0.013	Negative
	GO_DIGESTIVE_SYSTEM_DEVELOPMENT	-1.90	0.030	Negative
	GO_PATTERN_SPECIFICATION_PROCESS	-1.87	0.041	Negative
	GO_CARBOHYDRATE_HOMEOSTASIS	-1.83	0.068	Negative
	GO_NEGATIVE_REGULATION_OF_CELL_DEVELOPMENT	-1.83	0.061	Negative
	GO_FORMATION_OF_PRIMARY_GERM_LAYER	-1.82	0.064	Negative
	GO_GLUCOSE_HOMEOSTASIS	-1.81	0.062	Negative
	GO_ENDOCRINE_SYSTEM_DEVELOPMENT	-1.80	0.062	Negative
	E2F3_UP.V1_DN	-1.93	0.007	Negative
MEHP	MEISSNER_BRAIN_HCP_WITH_H3K27ME3	2.32	<0.001	Positive

NIKOLSKY_BREAST_CANCER_16P13_AMPLICON	2.12	0.005	Positive
PENG_LEUCINE_DEPRIVATION_DN	2.04	0.018	Positive
SMIRNOV_RESPONSE_TO_IR_6HR_DN	2.00	0.026	Positive
MEISSNER_NPC_HCP_WITH_H3K27ME3	1.90	0.100	Positive
WAKABAYASHI_ADIPOGENESIS_PPARG_RXRA_BOUND_36HR	1.89	0.092	Positive
KEGG_WNT_SIGNALING_PATHWAY	1.87	0.101	Positive
BENPORATH_ES_WITH_H3K27ME3	1.85	0.105	Positive
V$ARNT_02	1.94	0.116	Positive
GO_ANTERIOR_POSTERIOR_PATTERN_SPECIFICATION	2.25	0.001	Positive
GO_TRANSCRIPTION_FACTOR_COMPLEX	2.24	0.001	Positive
GO_REGIONALIZATION	2.15	0.005	Positive
GO_TRANSFERASE_ACTIVITY_TRANSFERRING_HEXOSYL_GROUPS	2.09	0.010	Positive
GO_CARDIAC_CHAMBER_MORPHOGENESIS	2.08	0.010	Positive
GO_UDP_GLYCOSYLTRANSFERASE_ACTIVITY	2.02	0.021	Positive
GO_MESENCHYME_DEVELOPMENT	1.99	0.028	Positive
GO_PATTERN_SPECIFICATION_PROCESS	1.95	0.041	Positive
GO_CELLULAR_CARBOHYDRATE_METABOLIC_PROCESS	1.93	0.049	Positive
GO_EMBRYO_DEVELOPMENT_ENDING_IN_BIRTH_OR_EGG_HATCHING	1.92	0.052	Positive
GO_CANONICAL_WNT_SIGNALING_PATHWAY	1.91	0.052	Positive
GO_NUCLEAR_EXPORT	1.90	0.053	Positive
GO_NEGATIVE_REGULATION_OF_CELL_DEVELOPMENT	1.88	0.067	Positive
GO_LIMB_DEVELOPMENT	1.88	0.063	Positive
GO_CENTRAL_NERVOUS_SYSTEM_NEURON_DIFFERENTIATION	1.88	0.061	Positive
GO_EMBRYONIC_SKELETAL_SYSTEM_DEVELOPMENT	1.86	0.072	Positive
GO_CELLULAR_RESPONSE_TO_LIGHT_STIMULUS	1.85	0.076	Positive
GO_ORGAN_MORPHOGENESIS	1.85	0.075	Positive
GO_MESENCHYMAL_CELL_DIFFERENTIATION	1.83	0.084	Positive
GO_APPENDAGE_DEVELOPMENT	1.83	0.086	Positive
GO_CELL_FATE_COMMITMENT	1.81	0.097	Positive

	GO_REGULATION_OF_CELL_MORPHOGENESIS_INVOLVED_IN_DIFFERENTIATION	1.81	0.095	Positive
	GO_MUSCLE_ORGAN_MORPHOGENESIS	1.81	0.093	Positive
	PDGF_ERK_DN.V1_DN	1.90	0.063	Positive
	GO_MICROTUBULE_ASSOCIATED_COMPLEX	-1.96	0.159	Negative
MEOHP	IL2_STAT5_SIGNALING	-1.82	0.034	Negative
	LENAOUR_DENDRITIC_CELL_MATURATION_UP	-2.02	0.110	Negative
	GCAAAAA,MIR-129	-1.99	0.126	Negative
	V$CREBP1_01	-1.95	0.093	Negative
	V$E4BP4_01	-1.84	0.172	Negative
	ATGCAGT,MIR-217	-1.81	0.176	Negative
	KRAS.KIDNEY_UP.V1_UP	-1.81	0.149	Negative
	TBK1.DF_UP	-1.80	0.085	Negative

APPENDIX D8

Locations and CpGs of All Models from GEE Analyses of Methylation Clusters and Blastocyst Quality

Cluster ID	p-value	Chr	start	end	distance	closest gene
3240	0.001	chr19	13213428	13213451	73	LYL1
6224	0.003	chr8	41528946	41529140	865	ANK1
1641	0.004	chr12	115132460	115133735	-11805	TBX3
5	0.007	chr1	870791	872737	602	SAMD11
4083	0.007	chr21	42791867	42792703	-308	MX1
4332	0.010	chr3	62361449	62362324	-1900	FEZF2
986	0.011	chr11	320929	320940	-126	IFITM3
4204	0.013	chr22	46370644	46371152	766	WNT7B
2133	0.015	chr14	105884721	105885138	156	RP11-521B24.3
2907	0.019	chr17	76126449	76127217	-77	TMC8
3123	0.021	chr19	1523070	1523442	-903	PLK5
1913	0.021	chr13	112712795	112714526	7238	SNORD44
5763	0.023	chr7	64466846	64467056	39	ZNF117
3411	0.025	chr19	50191439	50191637	-24	PRMT1
3116	0.026	chr19	1287428	1287576	1272	EFNA2
94	0.032	chr1	6546027	6546646	-381	PLEKHG5
4879	0.037	chr5	44806923	44807541	1524	RP11-53O19.1
848	0.043	chr10	105420831	105421005	-112	SH3PXD2A
4337	0.048	chr3	67705222	67705285	-70	RP11-81N13.1
2626	0.052	chr16	89981752	89981919	1149	MC1R
91	0.055	chr1	6519923	6520194	-56	ESPN
4656	0.055	chr4	125631168	125631415	500	ANKRD50
6315	0.056	chr8	126963507	126964345	406	SOD1P3
3082	0.060	chr19	409679	409888	-712	C2CD4C
30	0.062	chr1	1369772	1369948	-100	RP4-758J18.10
3702	0.064	chr2	106560496	106560771	15018	AC009505.4
2566	0.064	chr16	86546374	86546979	-628	FOXF1
5881	0.076	chr7	130788305	130788584	2896	LINC-PINT
3325	0.078	chr19	39686695	39687098	-884	NCCRP1
3908	0.078	chr2	242878496	242879153	2531	AC131097.3
7	0.086	chr1	879375	879958	44	SAMD11
3283	0.087	chr19	18902527	18902727	-565	COMP
3487	0.095	chr2	394115	394481	5794	AC105393.1
4713	0.096	chr4	186300477	186300945	-811	LRP2BP
4122	0.106	chr21	48054563	48054897	-647	PRMT2
709	0.111	chr10	15963599	15963738	35258	snoU13

1694	0.116	chr12	125509610	125510168	-2768	BRI3BP
2878	0.121	chr17	72208213	72208322	-545	CTD-2514K5.2
2524	0.124	chr16	78082579	78082701	447	KRT8P22
3775	0.144	chr2	176974592	176974934	585	HOXD11
3876	0.144	chr2	240431144	240431270	-69099	AC079612.1
4557	0.150	chr4	7192699	7193237	-1591	SORCS2
2227	0.163	chr15	65368982	65369213	-150	RASL12
2748	0.169	chr17	32612675	32613298	191	CCL11
6332	0.173	chr8	140630830	140631269	-128	KCNK9
128	0.176	chr1	16957483	16957489	8	CROCCP2
3448	0.178	chr19	55281057	55281274	-134	KIR2DL1
5798	0.181	chr7	94294278	94294905	375	PEG10
5673	0.195	chr7	6209931	6210236	138	CYTH3
4551	0.201	chr4	6675309	6675513	69	RP11-539L10.3
3929	0.202	chr20	3065343	3065488	-130	AVP
5682	0.204	chr7	12729367	12729527	272	ARL4A
4428	0.206	chr3	175696286	175696388	-515	RP11-809F4.2
1524	0.213	chr12	50134882	50134915	-150	TMBIM6
3040	0.217	chr18	74153342	74154260	383	ZNF516
1204	0.244	chr11	64335229	64335895	384	SLC22A11
4163	0.253	chr22	31518860	31518963	-95	INPP5J
3891	0.254	chr2	241721922	241722113	-2	KIF1A
5062	0.254	chr6	1622383	1622941	1592	GMDS
1760	0.266	chr12	133049188	133049413	101	MUC8
3160	0.268	chr19	3831293	3831364	100	ZFR2
4084	0.270	chr21	43098516	43099460	-518	LINC00111
619	0.277	chr10	1558762	1559249	-9957	ADARB2-AS1
5719	0.300	chr7	35734978	35735308	-37	HERPUD2
93	0.313	chr1	6529710	6530205	-288	PLEKHG5
2744	0.318	chr17	30817129	30817397	879	CDK5R1
1323	0.319	chr11	114165730	114166636	-807	NNMT
2607	0.323	chr16	88725584	88725763	-116	MVD
1786	0.333	chr13	23993013	23993527	-5	SACS-AS1
3402	0.339	chr19	49669281	49669542	27	TRPM4
5669	0.355	chr7	5635134	5635953	1160	FSCN1
1043	0.370	chr11	2563802	2563897	14647	KCNQ1
5902	0.374	chr7	140732689	140732738	-17752	MRPS33
6110	0.381	chr8	1617616	1618448	102	DLGAP2
1658	0.387	chr12	120127920	120128116	90	CIT
2567	0.397	chr16	86588144	86589076	-171	MTHFSD
942	0.398	chr10	133879084	133879763	-38887	JAKMIP3

6323	0.418	chr8	133786975	133787071	-731	PHF20L1
4523	0.418	chr4	2069683	2069925	1652	NAT8L
3182	0.427	chr19	5335045	5335260	5592	PTPRS
2795	0.436	chr17	41437877	41437982	9880	LINC00910
3588	0.443	chr2	11780349	11780446	-1266	GREB1
81	0.451	chr1	4770676	4770711	-536	AJAP1
1318	0.453	chr11	111956647	111956844	116	TIMM8B
5432	0.458	chr6	149285724	149286375	-292	RP11-162J8.2
3088	0.469	chr19	675390	676739	-395	FSTL3
4940	0.472	chr5	133703730	133703889	-1094	CDKL3
6256	0.480	chr8	49533357	49533485	32	RP11-567J20.1
3513	0.507	chr2	1452260	1452665	-5020	TPO
2649	0.513	chr17	1387495	1387922	210	MYO1C
40	0.523	chr1	2066430	2066446	-39	PRKCZ
6009	0.537	chr7	157691177	157691591	13	PTPRN2
2640	0.538	chr17	934534	935017	164	ABR
3893	0.547	chr2	241901865	241901988	996	AC104809.4
1638	0.552	chr12	114337676	114337927	15030	RBM19
1261	0.554	chr11	69017394	69017579	-44338	MYEOV
1817	0.568	chr13	43930362	43930423	-220	ENOX1
3110	0.570	chr19	1226523	1227141	272	STK11
4234	0.577	chr22	51040520	51041242	611	MAPK8IP2
982	0.589	chr11	278394	278912	171	NLRP6
4722	0.591	chr4	186799820	186799847	15655	SORBS2
502	0.593	chr1	213223453	213223461	-1263	RPS6KC1
3208	0.598	chr19	9270837	9271995	-34	ZNF317
3770	0.651	chr2	173330296	173330395	17	ITGA6
3870	0.656	chr2	239930734	239931250	-1062	RP11-648F7.1
5020	0.661	chr5	175621395	175621409	4866	RP11-844P9.2
83	0.682	chr1	5356471	5356578	-197362	RP1-58B11.1
2134	0.703	chr14	105955745	105955879	33	CRIP1
6346	0.707	chr8	142984321	142984613	-42456	AC104417.1
4111	0.726	chr21	46898610	46898789	446	COL18A1
2694	0.733	chr17	7660449	7660494	-10	DNAH2
71	0.769	chr1	3332000	3332125	893	PRDM16
5134	0.774	chr6	29364799	29365287	111	OR12D2
2796	0.783	chr17	41440525	41440717	7189	LINC00910
4248	0.793	chr3	8543508	8543732	32	LMCD1
2366	0.799	chr16	2653222	2653839	-10	AC141586.5
1914	0.811	chr13	112785388	112786727	23880	LINC00403
734	0.823	chr10	43047255	43048734	-411	ZNF37BP

6451	0.832	chr9	136216076	136216154	-173	SNORD24
6464	0.857	chr9	139836994	139838089	-454	FBXW5
943	0.861	chr10	133892406	133892567	-25825	JAKMIP3
1622	0.882	chr12	109678885	109678973	-55	ACACB
6438	0.891	chr9	132097931	132098311	-1091	RP11-65J3.1
5435	0.904	chr6	150437072	150437118	-27328	PPP1R14C
2344	0.915	chr16	1400772	1400861	88	TSR3
1863	0.917	chr13	103346900	103347391	-360	METTL21C
1755	0.927	chr12	132903921	132904014	1702	GALNT9
759	0.949	chr10	62148959	62149642	36	ANK3
5421	0.967	chr6	138200071	138200679	347	TNFAIP3
4013	0.970	chr20	55925570	55925586	-694	RAE1
2938	0.993	chr17	78237117	78237397	-218	RNF213

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