Regulation of the Timing of Puberty: Exploration of the Role of Epigenetics

by

Paulina Agnieszka Rzeczkowska

A thesis submitted in conformity with the requirements for the degree of Master of Science
Institute of Medical Science
University of Toronto

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Master of Science

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University of Toronto

2012

Abstract

Pubertal timing displays wide, normally distributed variation in a healthy population of sexually maturing adolescents. However, like many complex traits, factors contributing to the variation are not well understood. Epigenetic regulation may contribute to some of the population variation. The role that epigenetics, specifically DNA methylation and histone acetylation, may play in regulating pubertal timing was investigated in C57BL/6 female mice: investigating whether population variation in pubertal timing among inbred mice could be explained by environmental factors; whether perturbing the epigenome using a histone deacetylase inhibitor or methyl-donor would alter pubertal timing; and examining genome-wide methylation patterns in hypothalami of early versus late maturing mice. Results demonstrate that measurable micro-environmental factors have only negligible effects on pubertal timing; pubertal timing was significantly altered by administration of epigenetic modifying agents; differences in methylation patterns are subtle. This initial evidence supports the involvement of epigenetic mechanisms in regulating pubertal timing.
Acknowledgments

With an idea suitable for a “blue skies” discussion come great challenges in its realization and low expectation of success, but high hope in overcoming these challenges and making great discoveries that will pioneer great advances in the field. Such an idea was the basis of this thesis. Taking on the role as its investigator was overwhelming, but with the support and guidance of the many people that I would like to acknowledge, the challenges of this project were overcome and this “blue skies” idea was investigated with diligence, ingenuity, and excitement.

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My program advisory committee members Dr. Johanna Rommens, Dr. Rosanna Weksberg, and Dr. Stephen Matthews, thank you for your positive support, constructive feedback and thoughtful ideas. Your unique perspectives have enriched the scope of this project.

Christina Alm, you have been invaluable in advocating integrity in all experimentation, keeping everything organized, and livening up the research environment. Jonathan Ramkumar, your help in the summer was warmly welcome as you brought with you an inquisitive mind and helpful hands.

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<tbody>
<tr>
<td>AAM</td>
<td>age at menarche</td>
</tr>
<tr>
<td>Ac</td>
<td>acetyl group</td>
</tr>
<tr>
<td>AGTC</td>
<td>Analytical Genetics Technology Centre</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ARC</td>
<td>arcuate nucleus</td>
</tr>
<tr>
<td>AUP</td>
<td>animal use protocol</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6 strain of mice</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>CCAC</td>
<td>Canadian Council on Animal Care</td>
</tr>
<tr>
<td>CDGP</td>
<td>constitutional delay in growth and puberty</td>
</tr>
<tr>
<td>CG</td>
<td>cytosine guanine</td>
</tr>
<tr>
<td>CHARM</td>
<td>comprehensive high-throughput array for relative methylation</td>
</tr>
<tr>
<td>chr</td>
<td>chromosome</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CpG</td>
<td>cytosine guanine dinucleotide</td>
</tr>
<tr>
<td>CpGi</td>
<td>CpG island</td>
</tr>
<tr>
<td>Cₜ</td>
<td>cycle threshold</td>
</tr>
<tr>
<td>ctrl</td>
<td>control</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>Dyn</td>
<td>dynorphin</td>
</tr>
<tr>
<td>E ALL</td>
<td>early puberty mice</td>
</tr>
<tr>
<td>E2</td>
<td>17β-estradiol</td>
</tr>
<tr>
<td>E39</td>
<td>early puberty mouse sacrificed at postnatal day 39</td>
</tr>
<tr>
<td>EDC</td>
<td>endocrine disrupting compound</td>
</tr>
<tr>
<td>ER-α</td>
<td>estrogen receptor alpha</td>
</tr>
<tr>
<td>EVO</td>
<td>early puberty mouse sacrificed at vaginal opening</td>
</tr>
<tr>
<td>FDR</td>
<td>false discovery rate</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>G/A</td>
<td>guanine adenine</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GLU</td>
<td>glutamic acid</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin releasing hormone</td>
</tr>
<tr>
<td>GPR54</td>
<td>g-protein coupled receptor 54</td>
</tr>
<tr>
<td>GR</td>
<td>glucocoid receptor</td>
</tr>
<tr>
<td>GWAS</td>
<td>genome wide association study</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyl transferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase complex</td>
</tr>
<tr>
<td>HDACi</td>
<td>histone deacetylase inhibitor</td>
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</table>
HH  hypogonadotropic hypogonadism
HOX  homeobox domain
HPG  hypothalamic pituitary gonadal axis
HP-β-CD  hydroxypropyl-beta-cyclodextrin
ICV  intracerebroventricular
Igf2  insulin growth factor 2
IHH  isolated hypogonadotropic hypogonadism
iPSCs  induced pluripotent stem cells
IU  in utero
JAX  Jackson Laboratories
KO  knock-out
KS  Kallmann syndrome
L ALL  late puberty mice
L39  late puberty mouse sacrificed on postnatal day 39
LH  luteinizing hormone
LN₂  liquid nitrogen
LVO  late puberty mouse sacrificed at vaginal opening
MALDI-TOF  matrix-assisted laser desorption ionization time-of-flight
MB  mammillary bodies
MBD  methyl binding domain
MeCP2  methyl CpG binding protein 2
meDIP  methyl DNA immunoprecipitation
MET  L-methionine
mRNA  messenger RNA
n  number
NGS  next generation sequencing
NMDA  N-methyl-D-aspartate
ns  not statistically significant
NTC  no template control
OB  olfactory bulb
OC  optic chiasm
OD  optical density
OT  optic tract
PBS  phosphate buffered saline
PCR  polymerase chain reaction
pnd  postnatal day
PTM  post-translational modification
qPCR  quantitative real time polymerase chain reaction
qRT-PCR  quantitative reverse-transcriptase polymerase chain reaction
QTL  quantitative trait loci
r  Pearson's product-moment correlation coefficient
RNA  ribonucleic acid
RNApol  RNA polymerase
RNase  ribonuclease
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tr>
<td>RND</td>
<td>round</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse-transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SAH</td>
<td>s-adenosyl homocysteine</td>
</tr>
<tr>
<td>SAHA</td>
<td>suberoylanilide hydroxamic acid</td>
</tr>
<tr>
<td>SAM</td>
<td>s-adenosyl methionine</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sem</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interference RNA</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>TCP</td>
<td>Toronto Center for Phenogenomics</td>
</tr>
<tr>
<td>UCSC</td>
<td>University of California Santa Cruz</td>
</tr>
<tr>
<td>UHN</td>
<td>University Health Network</td>
</tr>
<tr>
<td>VO</td>
<td>vaginal opening</td>
</tr>
<tr>
<td>vs</td>
<td>versus</td>
</tr>
<tr>
<td>WGA</td>
<td>whole genome amplification</td>
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Chapter 1

1 Introduction

The onset of puberty in a healthy, adolescent girl may occur anywhere between 7 and 13 years of age (Styne et al., 2011). Her genetic make-up alone cannot fully account for the timing of her pubertal onset (Gajdos et al., 2009). Given that puberty is a complex period of development, it is not surprising that the mechanisms of its regulation have not yet fully been elucidated.

With an emerging role as a contributing mechanism to the regulation of various complex traits and disease susceptibility, epigenetic regulation surfaces as a likely contributor to the variation in pubertal timing in the population. Not yet explored in pubertal regulation, the role epigenetic mechanisms may play in the timing of puberty in females is the topic of this thesis.

The current understanding of the neuroendocrine regulation of pubertal development as well as the mechanisms of epigenetic action are reviewed in the sections that follow. The three aims set forth to address the hypothesis are outlined in section 1.4.

1.1 Puberty

Puberty is a developmental milestone for a sexually reproducing organism. Attainment of sexual maturity ensures successful propagation of a species. It is a complex period of coordinated regulation by activators and repressors, dictated by genetic predispositions, general health and environmental cues. Disorder of this regulation can prevent or severely delay maturation, which would clearly disadvantage the organism’s fitness. Variation in the timing of onset of puberty, too, has been linked to various disadvantages. In humans, for example, early onset of puberty in females is highly associated with increased risk of breast cancer (Sherman et al., 1981; Mishra et al., 2009b) and increased risk of obesity leading to diabetes, specifically Type II diabetes (Pierce et al., 2011). On the other hand, later onset of puberty in females is disadvantageous to bone, where it is associated with increased risk of osteoporosis later in life (Fox et al., 1993).

In Canada, it was recently determined in a longitudinally studied cohort from 2000 that age at menarche for 68% of the population sampled fell between 11.53 and 13.91 years of age,
encompassed fully between 9 and 16 years (Al-Sahab et al., 2010). Compared to girls born 50 years ago, the proportion of girls with pubertal onset before ages 7 and 8 has risen (Biro et al., 2010). Not only is the variation in onset wide, but puberty appears to be beginning earlier. Therefore, there must be mechanisms of regulation present that are modifiable and respond to changing conditions, shifting the timing of puberty in a population, but which also dictate individual predisposition.

Investigating the mechanisms leading to variation in pubertal regulation will enhance the understanding of this fundamental developmental process that impacts many aspects of life.

1.1.1 Neuroendocrine Regulation of Pubertal Development

Pivotal discoveries in the 1950s revealed that pubertal maturation is controlled by the neuroendocrine axis. The work by Harris and Jacobson (1952) showed that the pituitary, hypothalamus and vascular connections between the two organs were required for normal sexual maturation. By transplanting pituitaries from pre-pubertal rats into adult animals, whose pituitaries were previously surgically removed, reproductively viable functions such as estrous cyclicity in the adult rats were restored. These immature pituitaries, under the influence of mature hypothalamus, developed into mature functioning organs, indicating that the pituitaries are under hypothalamic control by signals sent through the portal vessels. Donovan and van der Werff ten Bosch (1956) further established that the control the hypothalamus exerts pre-pubertally may be that of inhibition. When they placed lesions in the hypothalamic areas of immature female rats, puberty was initiated much earlier than in those without lesions, and earlier than those with lesions in the mammillary body, hippocampus, cortex and thalamus. In humans also, disruption of hypothalamic function such as by posterior hypothalamic neoplasms, hypothalamic hamartoma or hydrocephalus in children, leads to precocious onset of puberty (reviewed in Palmert & Boepple, 2001).

1.1.1.1 Hormonal Initiation

Puberty is initiated by the pulsatile release of gonadotropin releasing hormone (GnRH) from the hypothalamus (Styne et al., 2011). In humans, GnRH pulsatility is observed during fetal development and early infancy and is suppressed until the onset of puberty during the period called the juvenile pause (Grumbach, 2002). Thus, the initiation of GnRH oscillatory secretion at
Puberty is a reactivation or release from repression. At this time, the GnRH pulses increase both in amplitude and in frequency (Knobil, 1981). GnRH is sent from the hypothalamus to stimulate the anterior pituitary to secrete the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Swerdloff and Odell, 1975). These hormones function at the gonads to facilitate their maturation (Swerdloff and Odell, 1975). In females, ovarian maturation leads to secretion of steroid hormones estrogen and progesterone (Styne et al., 2011). These gonadal hormones lead to the development of secondary sexual characteristics (Marshall and Tanner, 1969). In humans, the first characteristic is breast development or thelarche, followed by growth of pubic hair and the onset of the first menstrual cycle (Marshall and Tanner, 1969). Gonadal hormones also feed back to the hypothalamus exerting both positive and negative actions on targets such as kisspeptin, and LH and FSH secretion (Barraclough and Haller, 1970; Clarkson et al., 2009).

These components constitute the hypothalamic-pituitary-gonadal (HPG) axis through which sexual maturation occurs.

1.1.1.2 Central Nervous System Initiation

The reactivation of the collection of GnRH neurons, termed the GnRH pulse generator, is governed by a complex network operating in the central nervous system (CNS) of activating and repressing neurotransmitters, neuropeptides and neurosteroids (Figure 1-1). The complexity of this network is further demonstrated by its morphologically dynamic nature. New transynaptic and glial-neuronal connections are being formed during the time leading up to the initiation of GnRH pulsatility (Clarkson et al., 2009; Ojeda et al., 2010). As such, GnRH neurons also have more inputs, including increased dendritic and somal spines, in the post-pubertal compared to pre-pubertal brain, as evidenced in vivo in GnRH green fluorescent protein mice (Clarkson and Herbison, 2006).
**Figure 1-1 Activity of hypothalamic activators and repressors in suppression of hypothalamic pituitary gonadal axis function pre-puberty and activation during puberty**

Representative diagram of excitatory inputs from hypothalamic activators (green line ending in circle) and inhibitory inputs from hypothalamic repressors (red line ending in perpendicular line). Direction of signal transduction (arrow) of active pathways (solid lines) and inactive pathways (dotted lines) from the gonadotropin releasing hormone (GnRH) neurons of the hypothalamus to the pituitary and ovaries in female mammals during two developmental periods, pre-puberty and during puberty. LH, luteinizing hormone; FSH, follicle stimulating hormone.
1.1.1.2.1 Known Activators of GnRH

The two predominant excitatory inputs to the GnRH pulse generator are glutamatergic neurons and kisspeptin neurons. Glutamatergic neurons secrete the excitatory amino acid glutamate (GLU) which acts on N-methyl-D-aspartate (NMDA) receptors expressed by GnRH neurons (Eyigor and Jennes, 1996; Brann, 1995). GLU has been shown to evoke GnRH secretion in immature rats (Bourguignon et al., 1995) and LH release (Dyer et al., 1981).

Kisspeptin, and its action mediated by the kisspeptin receptor GPR54, was found to play a key role in pubertal regulation when gene knockout mouse models of either the ligand Kiss1 or receptor Gpr54 displayed defective sexual development (Seminara et al., 2003; de Roux et al., 2003; Lapatto et al., 2007; d'Anglemont de Tassigny et al., 2007). Kisspeptin neurons project to GnRH neurons, on which are expressed GPR54, and directly stimulate GnRH release (Clarkson and Herbison, 2006; Han et al., 2005). Kisspeptin neurons are one of the dynamic components of the network that increase connections with GnRH neurons across the pubertal transition (Clarkson and Herbison, 2006). Although having an excitatory role on GnRH neurons, kisspeptin was determined not to be the initiator of puberty. Clarkson et al. (2009) found that kisspeptin neurons depend on estrogen feedback from the ovaries to express kisspeptin. When they tested the presence of kisspeptin peptide in mice with deficient estrogen production, such as adult mice ovariectomized pre-pubertally or pre-pubertal mice with knockout of aromatase, the enzyme that converts androgens into estrogen, they found that kisspeptin peptide was absent from the kisspeptin neurons that innervate GnRH neurons in the hypothalamus but present in controls. This indicates that kisspeptin action itself relies on the initiation of puberty and the increased circulating gonadal steroids that accompany it.

Activators of GnRH are repressed pre-puberty and active during puberty (Figure 1-1).

1.1.1.2.2 Known Repressors of GnRH

The predominant inhibitory input to the GnRH pulse generator stems from GABAergic neurons (those secreting γ-aminobutyric acid [GABA]). By testing the response of the HPG axis to administration of GABA and GABA receptor antagonists in pre-pubertal and pubertal nonhuman primates, Mitsushima et al. (1994) revealed the role of GABA in maintaining pre-pubertal inhibitory tone. They found that administration of bicuculline, a GABA receptor A antagonist,
led to a significant increase in LH secretion in pre-pubertal monkeys, an indication of HPG axis activation. The antagonist had no effect during puberty, noting that GABA levels were already low at puberty, lower than during pre-puberty. Infusion of exogenous GABA during puberty, on the other hand, increased the low levels of endogenous GABA and led to decreased secretion of LH, indicating suppression of HPG activity. This was also demonstrated in a similar way in rats (Moguilevsky et al., 1991). Specifically, this inhibition occurs via GnRH neurons and the downregulation of GnRH expression (Seong et al., 1995).

Opioidergic neurons also have an inhibitory function, inhibiting GnRH release directly or indirectly through inhibition of excitatory neurons, such as kisspeptin neuron by opioid peptide dynorphin A (Dyn) (Schulz et al., 1981; Navarro et al., 2009). Kisspeptin neurons not only express the Dyn receptor (the κ opioid receptor) and can thus themselves be affected by opioid action, they also express Dyn (Navarro et al., 2009).

Repressors of GnRH are active pre-puberty and repressed at puberty (Figure 1-1).

1.1.1.2.3 Hypothalamic Distribution of the Pubertal Network

The many components involved in the network of neurons regulating pubertal development are spread throughout the hypothalamus in various nuclei. Experiments subsequent to the mid-20th century hypothalamic lesion studies mentioned earlier were interested in determining the specific regions of the hypothalamus that house the control centers of pubertal development. Following the work of Donovan and van der Werff ten Bosch (1956) that localized the control centers to the general area of the anterior hypothalamus, Gellert et al. (1960) determined that specifically the arcuate nucleus (ARC) was involved. Indeed, more recent studies using immunofluorescent labeling detect both GnRH and kisspeptin neurons in the ARC (Clarkson et al., 2006; Oka, 2009). Kisspeptin neurons are also found in the periventricular region of the anteroventral periventricular nucleus, preoptic periventricular nucleus, and dorsomedial hypothalamus in rodents (Clarkson and Herbison, 2006; Adachi et al., 2007). GnRH neurons, although sparse, as there are only about 1000 to 2000 total in the hypothalamus, are also spread across multiple nuclei including the ARC, median eminence, and pre-optic area (Zimmerman and Antunes, 1976). Glutamatergic neurons also extend up to the premammillary nucleus (Donato et al., 2011). Thus, the hypothalamus houses a heterogenous distribution of the neural components of pubertal regulation.
There is no single pathway that is solely responsible for the neuroendocrine control of pubertal development. Rather, it is a network of activators and repressors working in concert to regulate puberty (Gajdos et al., 2009; Krewson et al., 2004; Ojeda et al., 2006).

1.1.2 Genetic Regulation

Critical players and pathways in the development of the HPG axis and the regulation of pubertal timing have been identified through genetic analyses. Investigating disorders of pubertal timing and correlated genetic mutations in humans has led to the discovery of many genes and pathways. One of these disorders is Kallmann syndrome (KS), defined by reduced or lack of ability to perceive odours (anosmia or hyposmia, respectively) (Kallmann et al., 1943) along with reduced function of the gonads due to dysfunction of the hypothalamus in secreting GnRH, leading to the inability of the pituitary to secrete gonadotropins; this is referred to as hypogonadotropic hypogonadism (HH) and leads to delay or failure to enter puberty. A mutation in KAL-1 leads to the X-linked form of KS (Legouis et al., 1991). This gene encodes anosmin, a cellular matrix protein required for proper migration during embryogenesis of both olfactory and GnRH neurons from the olfactory placode to their site of function in the developed brain (Soussi-Yanicostas et al., 1998). Other early developmental genes that are involved in the migration of GnRH neurons have also been found to lead to HH if mutated, including G-protein coupled prokineticin receptor-2 (PROKR2) and ligand prokineticin-2 (PROK2) (Dode et al., 2006), fibroblast growth factor receptor 1 (FGFR1), ligand fibroblast growth factor 8 (FGF8) (Dode et al., 2003), chromodomain helicase DNA binding protein-7 (CHD7) and newly identified WD repeat domain (WDR11) (Kim et al., 2008; Kim et al., 2010). There is overlap between genes that cause KS and genes that cause isolated hypogonadotropic hypogonadism (IHH, with normal sense of smell); some genes can cause both and others cause only KS or IHH. A group of genes more commonly associated with IHH includes GnRH gene (GNRH1) and 21 different loss-of-function mutations in its receptor (GNRHR) (Bedecarrats and Kaiser, 2007), as well the kisspeptin receptor (GPR54) (Seminara et al., 2003; de Roux et al., 2003), and TAC3 and TACR3, the genes encoding neurokinin B and its receptor, respectively, which is another excitatory peptide synthesized in kisspeptin neurons (Topaloglu et al., 2009).

Other genes that lead to HH are involved in the development of the pituitary and its connection with the hypothalamus, such as orphan nuclear receptors DAX1 (dosage-sensitive sex reversal
adrenal congenital hypoplasia critical region on the X chromosome), steroidogenic factor 1 (SF-1), and pituitary transcription factors HESX-1, LHX3, and PROP-1; and obesity, such as adipocyte satiety factor leptin (LEP), its receptor (LEPR), and processor of GnRH, prohormone convertase 1 (PC1) (reviewed in Nathan & Palmert, 2005).

Identifying genes that underlie disorders of puberty has provided great insights into the developmental biology of the HPG axis. However, how much, if at all, these genes and pathways contribute to the variation of pubertal timing in healthy adolescents is unclear. Sequencing of a few of these genes in populations with normal pubertal development did not reveal any variants in these genes that correlated with timing of puberty, including GNRHR and GNRH1 (Sedlmeyer et al., 2005), and LEP and LEPR (Banerjee et al., 2006). When large-scale genome wide association (GWA) studies were performed to correlate single nucleotide polymorphisms (SNPs) with age at menarche (AAM) in the general population of sexually maturing females, they identified the strongest signals near the LIN28B gene (Ong et al., 2009; He et al., 2009; Sulem et al., 2009; Perry et al., 2009). LIN28B is a cytoplasmic protein that blocks the maturation of let-7 miRNA, which, if allowed to mature repress the expression of their target genes (Viswanathan and Daley, 2010; Reinhart et al., 2000). A meta-analysis of these GWA studies, grouping data from 87,802 women, identified 30 additional loci, none of which corresponds to KS or IHH genes (Elks et al., 2010). This is important new data, but taken together, the effect size of these genes in explaining timing of puberty in the general population is only about 5% (Elks et al., 2010).

1.1.3 Regulation by endogenous and external cues

General health and various factors in the environment during the postnatal, pre-pubertal years provide endogenous and external cues, respectively, that can impact the timing of puberty. The many physical and physiologic changes that occur during pubertal maturation have high energy demands and require optimal functioning of the body to properly develop. Suboptimal health can arrest or delay pubertal process; pubertal delay has been associated with various chronic illnesses including gastrointestinal disease, recurrent infections, renal disturbances, respiratory illnesses, eating disorders and excessive exercise, among others (reviewed in Pozo & Argente, 2002). Inflammatory bowel disease such as colitis can delay puberty in females, most likely due to systemic inflammation and upregulation of cytokines including interleukin-6 and tumour
necrosis factor alpha (DeBoer et al., 2010). Other, acute immune challenges, such as those demonstrated with the administration of endotoxin lipopolysaccharide to rats, deregulate reproductive endocrine function, specifically GnRH production (Nappi and Rivest, 1997). Stress eliciting corticosterone and corticotrophin-releasing factor also negatively impacts the HPG axis and delays puberty (Kinsey-Jones et al., 2010). Malnutrition that accompanies eating disorders such as anorexia nervosa lead to delayed puberty as well (Boyar et al., 1974). Decreased adipose tissue resulting from inadequate nutrition or excessive exercise and subsequent decreased leptin production are mediators of this delay (Chan and Mantzoros, 2005). On the other hand, early attainment of sufficient resources can advance pubertal onset as evidenced by obesity associated precocious puberty in girls (Wolff, 1955), where increased levels of leptin can accelerate pubertal maturation (Ahima et al., 1997).

The environment provides external cues and can be the source of endogenous changes mentioned earlier. Studies of migrating children report advancement or delays in pubertal timing and implicate differences in family care and food, as well as rise in stress in adapting to the change, and level of pollution from industrialization as causes of this pubertal disruption (reviewed in Parent et al., 2003). Chemicals in the environment, specifically estrogenic compounds such as phytoestrogens in soy, polychlorinated biphenyls from industry waste, bisphenol A used in plastics, and dichlorodiphenyltrichloroethane used in pesticides can act to disrupt HPG function (Diamanti-Kandarakis et al., 2009). These are referred to as endocrine disrupting chemicals or compounds (EDC). They act via estrogen receptors, inhibit aromatase, reduce circulating gondotropins or affect steriodogenesis (Dickerson and Gore, 2007). They have been implicated in leading to the recent decline in age at pubertal onset in girls which cannot be explained by an improvement in socioeconomic conditions (Mouritsen et al., 2010).

The mechanisms by which many of these environmental factors impart their influence on pubertal timing are not fully understood.

### 1.2 Epigenetic Mechanisms

Epigenetic mechanisms are a way in which the environment can impart alterations to phenotype without altering genotype (Barros and Offenbacher, 2009). They are also a way by which genetic homogeneity in all cells of the body can have heterogeneous functionality both spatially, in specific cells or tissues, and temporally, at different developmental stages (Bird, 2002).
Classically, epigenetics refer to the mitotically heritable chemical modifications found on DNA or histones that can affect gene expression. “Epi”, derived from the Greek word ἐπι meaning above or outer, in epigenetics identifies that these marks are an independent layer of transcriptional control above that of DNA that does not alter the underlying genetic sequence (Jaenisch and Bird, 2003). Epigenetic marks include methylation of cytosine residues in cytosine-guanine dinucleotides (CpGs) and modifications to histone proteins such as acetylation of their N-terminal tails (Jaenisch and Bird, 2003; Jenuwein and Allis, 2001). Patterns of epigenetic modifications are regulated by enzymes that can add or remove the marks, allowing for change or reversal of patterns affected by various exogenous and endogenous stimuli at sensitive periods (Barros and Offenbacher, 2009; Jenuwein and Allis, 2001). The traditional definition of an epigenetic mechanism requires that the epigenetic marks persist across mitosis (Bird, 2007). Contemporary views are redefining this definition to remove the constraint of mitotic and/or meiotic heritability to include marks that are transient, changing rapidly in response to an exposure and lasting only as long as the exposure, which may not be long enough to be passed on to daughter cells (Bird, 2007). Histone marks, especially, can behave in this way (Bird, 2007). Therefore, an epigenetic mechanism is a “structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states” (Bird, 2007).

1.2.1 Methylation and CpG islands

Approximately 75% of all cytosines in cytosine-guanine dinucleotides in human somatic cells are methylated (Ehrlich et al., 1982). This methylation is not uniformly distributed, as CpGs cluster in dense regions called CpG islands (CpGi) (Bird, 1986). Criteria for CpG clusters to be identified as islands include cytosine plus guanine nucleotide content of 50% or greater and an observed over expected ratio of cytosines followed by guanines of 0.6 or greater in a 200bp or longer stretch of DNA (Gardiner-Garden and Frommer, 1987). More stringent criteria have recently been proposed to exclude Alu-repetitive elements, by increasing percent content to 55% and ratio to 0.65 (Takai and Jones, 2002). The University of Santa Cruz (UCSC) genome browser with annotated genomes of various species, including mouse and humans, use the former criteria to define CpGi.

About half of all genes are associated with CpGi, and almost all of these CpGi cover one or more exons of a gene (Antequera and Bird, 1993). CpGi were thus inferred to be landmarks for
transcriptional initiation (Larsen et al., 1992). Indeed, CpGi are often associated with promoter regions and their methylation status can function to regulate transcription (Weber et al., 2007b; De Smet et al., 1999). When they function in this way, hypermethylation of CpGi at promoters leads to transcriptional repression (Stein et al., 1982; Weber et al., 2005). The strength of DNA methylation-mediated transcriptional repression is correlated with CpG methylation density (Hsieh, 1994). The majority of CpGi associated with promoters are, however, hypo- or unmethylated (Antequera and Bird, 1993). Hypermethylation of CpGs, on the other hand, occurs predominantly outside of islands and is highly correlated with transcriptional activity rather than repression (Rauch et al., 2009).

There is evidence for two different mechanisms of DNA methylation-mediated transcriptional repression. One involves the methylation-dependent repulsion of proteins that facilitate transcription and the other, the methylation-dependent attraction of proteins that facilitate repression. An example of the former mechanism controls transcription of the \textit{Igf2} (insulin growth factor 2) gene (Bell and Felsenfeld, 2000; Hark et al., 2000). Hypomethylation in the region between the promoter and downstream enhancer of \textit{Igf2} attracts a chromatin boundary element binding protein, CTCF, to bind to that region (Bell and Felsenfeld, 2000; Hark et al., 2000). The binding of CTCF itself excludes the binding of transcription factors and, effectively, represses the transcription of the \textit{Igf2} gene (Bell and Felsenfeld, 2000; Hark et al., 2000). The opposite is true when the region in hypermethylated, which leads to the repulsion of CTCF (Bell and Felsenfeld, 2000; Hark et al., 2000). The second mechanism of DNA methylation-mediated transcriptional repression is adopted by methyl-binding domain (MBD) proteins, including MBD1, MBD2, MBD3, and methyl CpG binding protein 2 (MeCP2), which are attracted to methyl-rich regions (Bird and Wolffe, 1999). As an example, it was found that mice lacking a functional Mbd2 protein (\textit{Mbd2}\textsuperscript{-/-}) had increased expression of certain hypermethylated genes, indicating that \textit{Mbd2} is required for methylation-dependent gene silencing (Hutchins et al., 2002).

\subsection*{1.2.2 Histone Acetylation}

Another way that gene transcription can be regulated is by chromatin packaging, which can physically obstruct or allow access of transcriptional machinery to DNA depending on the compactness and conformation of the chromatin structure (Strahl and Allis, 2000). The basic
elements of chromatin are nucleosomes. Nucleosomes are histone octomers of the four core histones, specifically H3/H4 tetramer and two H2A/H2B dimers, around which 146 base pairs (bp) of DNA is wound (Strahl and Allis, 2000). These histones have charged amino-terminal tails which protrude from the nucleosomes, making themselves available to post-translational covalent modifications (PTM) (Strahl and Allis, 2000). PTMs operate to remodel chromatin architecture either into euchromatin, a flexible and open state facilitating transcription, or into heterochromatin, a compact state, blocking transcription (Jenuwein and Allis, 2001). The histones tails are subject to a variety of PTMs, including acetylation, phosphorylation, methylation, ubiquitylation, sumoylation, ADP-ribosylation, proline isomerization, citrullination, butyrylation, propionylation, and glycosylation (reviewed in Gardner, Allis, & Strahl, 2011).

The first, and most well studied, PTMs on histones are acetylation and methylation discovered in 1964 by Allfrey et al. With the hypothesis that such modifications may be involved in transcriptional regulation, it was determined that they are, as evidenced by the preferential association of acetylated histones with euchromatin (Vidali et al., 1978; Sealy and Chalkley, 1978; Hebbes et al., 1988). In fact, the addition of an acetyl group facilitates the remodeling of heterochromatin into euchromatin (Strahl and Allis, 2000). It does so by neutralizing the positive charge of the histones with its negative charge, thereby weakening the attraction between the now neutral histones and negatively charged DNA (Hong et al., 1993). This alters nucleosomal conformation and stability in a way that renders the DNA accessible to interaction with transcriptional machinery (Lee et al., 1993). Histone acetylation occurs at the evolutionarily conserved lysine residues of the N-termini tails of histone H3 and H4, for example at Lys 14, 18 and 23 of histone H3 (Thorne et al., 1990).

These PTMs thus act like a language that directs transcriptional activity, and have been noted as being necessary for directing expression of genes involved in differentiation and development (Turner, 2007).

### 1.2.3 Epigenetic Machinery

DNA methylation and PTMs to histones are added and removed by various enzymes. For example, acetyl groups are added to histones by histone acetyltransferases (HATs) (Brownell et al., 1996) and removed by histone deacetylases (HDACs) (Taunton et al., 1996). Thus, hyper- or hypoacetylation of histones is determined by the relative activity of these two enzymes.
The enzymes responsible for methylation of DNA are the DNA methyltransferases (DNMTs). When the genes encoding these enzymes are deleted or mutated, in mouse models, embryonic death or severe disruption of development can result (Jaenisch and Bird, 2003). DNMTs function to either maintain DNA methylation or catalyze \textit{de novo} methylation. The mitotic heritability of DNA methylation is made possible by maintenance DNMT1, which copies the methylation pattern of the parent onto the progeny DNA (Holliday and Pugh, 1975). Maintenance DNMTs are thus responsible for maintaining patterns of cytosine methylation through replication cycles. \textit{De novo} DNMTs, DNMT3a and DNMT3b, on the other hand, establish DNA methylation patterns in the absence of a template (Okano et al., 1999). They are thus active during embryogenesis, development and carcinogenesis (Okano et al., 1999).

Figure 1-2 illustrates the involvement of the various epigenetic marks and machinery in transcriptional regulation. Transcription of a gene promoter containing a \textit{CpG}i typically requires hypomethylated \textit{CpG} dinucleotides in the island, and an open chromatin architecture consisting of widely spaced nucleosomes. Histones are typically acetylated, and the acetyl groups are added by a HAT. Flanking this transcriptionally active region may be regions of heterochromatin, where the cytosines are methylated by DNMTs and associate with MBD proteins and HDACs.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{biological_diagram.png}
\caption{Various components involved in epigenetic regulation of transcription}
\end{figure}

\textbf{Figure 1-2 Various components involved in epigenetic regulation of transcription}

Schematic diagram depicting enzymes histone deacetylase complex (HDAC), histone acetyltransferase (HAT) and DNA methyltransferase (DNMT); methyl binding domain protein (MBD); RNA polymerase (RNApol); posttranslational modification to histones, acetyl group (Ac); and methylation status of cytosines in DNA, methylated (black filled circles) and unmethylated (white filled circles).
1.2.4 Stability and Modifiability

Major changes to epigenetic marks occur during embryogenesis. At this developmental stage, DNA undergoes genome-wide demethylation followed by the establishment of a new DNA methylation pattern via *de novo* methylation (Li, 2002).

Once set during development, some marks remain unaltered throughout life and persist into adulthood. An example of this is seen in 60 year old survivors of the Dutch famine, where the specific methylation pattern of the IGF2 gene set by *in utero* exposure to suboptimal nutrition persisted unchanged into old age and was still present at age 60 (Heijmans et al., 2008). Stability of epigenetic marks is also evidenced in induced pluripotent stem cells (iPSCs), which are found to retain an epigenetic signature consistent with their cell type of origin, distinguishing between, for example, iPSCs derived from bone marrow derived granulocytes, tail tip–derived fibroblasts, splenic B cells and skeletal muscle precursors (Polo et al., 2010). Thus, epigenetic marks persist in spite of the reprogramming that occurs during induction of pluripotency.

DNA methylation is viewed as a mark that is stable across cell division. However, errors in maintenance methylation can occur. Pfeifer et al. (1990) investigated the fidelity of maintenance methylation and activity level of *de novo* methylation during mitosis in cultured mammalian cells. They found maintenance methylation to be less than 100%, with discrepancy of 0.1% per CpG site per division. Also during replication, new methylation was added at a rate as high at 5% per division. This is indicative of a stochastic origin for differential methylation, and partial stability of epigenetic regulation.

DNA methylation is also viewed as a stable mark in non-dividing somatic cells. However, methylation can change in response to endogenous or exogenous stimuli. DNA methylation or PTMs on histones can be altered by altering the activity of epigenetic machinery, such as by inhibiting HDACs or DNMTs with specific agents (Szyf, 2009). The functioning of DNMT can also be altered by altering the one-carbon metabolic pathway that supplies methyl groups for DNA methylation (Waterland, 2006). This can be achieved by changing the abundance of any of the essential nutrients, substrates or enzymes in the pathway (Waterland, 2006).

Epigenetic patterns, including both DNA methylation and histone acetylation, can also change with development by the effects of changing hormonal milieu, such as that of estrogen (Schwarz
et al., 2010; Zhao et al., 2010; Pasqualini et al., 1989) and testosterone (Auger et al., 2011). Other sources of epigenetic change include differential maternal care (Weaver et al., 2004) and variations in dietary components such as folate (Pogribny et al., 2008). Also, as animals age, progressive loss of global DNA methylation in their genome is apparent (Wilson et al., 1987) and hypermethylation of tumour suppressor genes (Teschendorff et al., 2010) can occur.

1.2.5 Epigenetic Mechanisms in Disease and Development

Epigenetic mechanisms have been identified to associate with risk of susceptibility to or as cause of a variety of diseases and developmental phenotypes. Aberrant methylation such as hypermethylation of the promoter regions of tumour suppressor genes leads to silencing of these genes and significantly increases the risk of developing cancer (Hatziapostolou and Iliopoulos, 2011). Impairment in genomic imprinting, the parent-of-origin monoallelic methylation and expression of genes (Peterson and Sapienza, 1993; Crouse, 1960), can also lead to various disorders. Loss of methylation at the putative imprinting control region of *H19/IGF2* on the maternal allele resulting in biallelic silencing of expression of these genes, leads to Beckwith–Wiedemann syndrome, a disorder characterized by overgrowth, tumor predisposition, and congenital malformations (Choufani et al., 2010).

CpGi methylation also varies and imparts functionally significant methylation-dependent silencing in normal cells and outside of imprinted regions. Its functional role in gene expression silencing is most often observed in developmentally relevant genes, including homeobox domain (HOX) genes (Futscher et al., 2002; Illingworth et al., 2008). Differential CpGi methylation is also found between tissues, and is highly correlated with expression of the CpGi associated genes in a manner relevant to the tissue function (Song et al., 2005). Tissue specificity of differentially methylated regions has been observed between the brain and kidney, colon, liver, testis and muscle (Song et al., 2005). Aberrant methylation in the brains of post-mortem individuals has been associated with major psychoses, including schizophrenia and bipolar disorder (Mill et al., 2008). Aberrant methylation in the liver can lead to hepatocarcinogenesis (Pogribny et al., 2006).

Epigenetic modifications can also reflect (or affect) stress response (Weaver et al., 2004), determine effectiveness of dieting, such as restriction of calories, on reduction of adipose tissue (Bouchard et al., 2010), and alter feeding behaviour and satiety leading to obesity and the
metabolic syndrome (Plagemann et al., 2009a) among many other complex responses. In neurons, epigenetic mechanisms are involved in a variety of functions including memory formation (Miller and Sweatt, 2007), neuronal and behavioral plasticity (Kumar et al., 2005), estrogen-induced gene expression (Schwarz et al., 2010), and sexual differentiation of the brain (McCarthey et al., 2009).

1.2.6 Puberty and Epigenetics

The role that epigenetic mechanisms may play in regulating the timing of the onset of puberty has not yet been established.

As a multi-gene trait of complex etiology whose regulation is not fully explained by either genetics or environmental factors, pubertal development shares many of the same qualities of complex traits and diseases in which roles for epigenetic mechanisms have been uncovered. Epigenetic mechanisms possess features that make them attractive candidates in explaining variation in the phenotype of complex traits. Differential epigenetic patterns can lead to the differential expression of genes relevant to the regulation of the trait, these differential patterns within an individual arise more frequently than genetic mutations (Jablonka, 1994), can arise as a response to differential environmental exposures or from stochastic events, and can persist to affect later life phenotypes. Some researchers have already speculated on the involvement of epigenetics in pubertal regulation (Pitteloud et al., 2010; Ojeda et al., 2010a; Gajdos et al., 2010; Cameron et al., 2008). This thesis aims to formally test its involvement.
1.3 Aims and Hypotheses

1.3.1 Overarching hypothesis
Epigenetic mechanisms play a role in the regulation of pubertal timing.

1.3.2 Aim 1
It is hypothesized that when variability in a phenotype is present in the absence of genetic or environmental variation, it is possible to isolate and study an epigenetic-dependent mechanism of phenotypic regulation if one exists.

Therefore, the objective of Aim 1 was to assess the suitability of using inbred C57BL/6 female mice as a model within which to investigate the contribution of epigenetic mechanisms in explaining the wide, normally distributed variability in timing of puberty in healthy mammalian populations.

1.3.3 Aim 2
If epigenetic mechanisms, such as differential DNA methylation or histone acetylation patterns, underlie the variation in pubertal timing seen among B6 female mice, then their effects can be revealed by perturbing their action.

Using a suitable in vivo model identified in Aim 1, the objective of Aim 2 was to globally alter epigenetic marks resulting in either an increase or decrease of gene function, and assess the resulting alteration in the phenotype of the timing of puberty in the population. In one condition, it is expected that epigenetic marks silencing transcription of puberty relevant genes (i.e. DNA hypermethylation and/or histone hypoacetylation) would be reverted to activating marks (i.e. with DNA hypomethylation and/or histone acetylation); and, in the second condition, the reverse would occur, where silenced genes could become activated, by employing epigenetic modifying agents with these expected actions. The timing of puberty, if affected by these alterations as anticipated, would be expected to shift in opposite directions in the two conditions.

1.3.4 Aim 3
Aim 2 provided evidence that altering epigenetic profiles could alter pubertal timing but it was not designed to identify the responsible genes. The objective of Aim 3 was to identify genes that
are affected by epigenetic mechanisms and that may modulate pubertal timing through these mechanisms.

Specifically, Aim 3 interrogated epigenome-wide DNA methylation patterns and timing of puberty by comparing these patterns in female mice with naturally disparate timings of pubertal onset. It was expected that differential methylation between early maturing and late maturing mice would be detected, and that the loci in which these differences would occur would be ones worthwhile to investigate in future studies.
Chapter 2

The ideal model in which to investigate epigenetic mechanisms in the regulation of pubertal onset would 1) be an in vivo mammalian species model translatable to human physiology from which samples from the hypothalamic-pituitary-gonadal axis can be obtained, 2) show wide, normal distribution in onset of puberty in the population, and 3) allow all other non-epigenetic factors to be held constant, including genetics and environment. Using these criteria, the females of the C57BL/6 (B6) strain of inbred mice were evaluated as a suitable model. To assess the validity of the model more fully, inherently variable micro-environmental factors, such as litter size, which may affect physiology and pubertal timing without necessarily affecting epigenetic profiles, were assessed as potential confounders/modulators of the timing of puberty.

2 Aim 1 – Evaluating Suitability of Mouse Model

2.1 Methods

All procedures involving animals are described in Animal Use Protocol (AUP) 09-08-0097, approved by the local Animal Care Committee at the Toronto Center for Phenogenomics (TCP) in accordance with their policies and guidelines, those of the Canadian Council on Animal Care (CCAC), and the Animals for Research Act, RSO 1980. Male and female wild-type B6 mice, originally purchased from Jackson Laboratories JAX®, were used for this study. Mice were housed at the TCP, and maintained at a 12/12 light-dark cycle from 7pm-7am. They were fed the 2018S Teklad Global 18% Protein Rodent Diet (Harlan 2918) and supplied with drinking water ad-libitum via a central sipper system.

2.1.1 Animal husbandry

To generate female B6 pups for phenotyping the timing of puberty, 1 to 2 females aged 6-30 weeks were put together in one cage with one male aged 5.5-45 weeks for mating. Mothers were mated for the first time or had previously given birth. To minimize exposure to male pheromones, after 7 days, the male was removed and female(s) placed in a new clean cage. After an additional 9-11 days, the females were separated to individual cages so that each litter would be housed and assessed separately. The next day onward, breeding cages were monitored for
birth, daily, 7 days per week between 10am-12pm. Date of birth postnatal day (pnd) 1 was designated as the day pups were observed. On pnd 21, female pups were separated from their mother and male siblings and weaned into clean cages with some food pellets placed at the bottom of the cage. Female mice were distributed up to 5 littermate females per cage, or divided equally if there were more than 5 females in the litter (e.g. 3+3, 3+4) to ensure that access to food and water was unfettered. Fresh bedding (strips of crinkled brown paper) was provided at each clean cage change to ensure consistent environmental enrichment across all cages. As a way to control for phenotypic drift, five rounds of 15-25 cages per round of breeding were performed over 11 months.

2.1.2  Phenotyping

At weaning, 1) each female mouse in a litter was ear-punched for identification. Ear punching was done in a single ear once or twice, in both ears or none to corresponding to an ID of either R, L, RR, LL, LR or none, respectively. 2) Mice were weighed and females weighing less than or equal to 5g or more than 12g were excluded. Exclusion criteria were calculated as the weight at pnd 21 three standard deviations above and below the mean. The mice beyond these cut-offs were considered outliers that were not representative of the general B6 mouse population. Ear-punch identification allowed for the recorded weaning weight to be later correlated with age at onset of puberty. At weaning, 3) daily phenotyping for timing of puberty was also initiated. Timing of puberty was determined by assessing age at vaginal opening (VO). Female mice were held by the tail with hind legs resting on edge of cage, for no longer than 20 seconds, and vaginal opening was viewed, with occasional use of sterile pipette tip to brush away hair near opening. Opening was determined as open or closed (Figure 2-1), mouse was then identified by ear punch, and the result recorded. Mice were also weighed at VO. Cages were rotated periodically during the phenotyping period to minimize the impact of neighbouring cages.
Figure 2-1 Distinguishing vaginal opening in a mouse

Posterior dorsal view of female mouse lifted by tail revealing genital area. Arrows point to the vaginal opening. The image on the left depicts the vaginal opening in its closed state and the image on the right in its open state. The age at which the appearance of the vaginal opening changes from closed to open is identified as the age at vaginal opening. Image has been modified from Abel et al. (2000).

Both weighing and assessment of vaginal opening occurred daily in a 4 hour window (10am-2pm) from pnd 21 to pnd 39. For a subset of mice, weight was recorded at pnd 40, 45 and every subsequent 10 days until pnd 75.

In addition, the following variables were record for each mouse: date of birth, cage ID, mother’s age at mating, mother’s first or second pregnancy, father’s age at mating, number of pups in a litter, and ratio of males to females in litter.

2.1.3 Analysis

All statistical analyses were performed using GraphPad Prism 5 software unless otherwise stated.

The D’Agostino-Pearson omnibus K2 test of normality was employed to assess the distribution of age at VO in the pooled population of all phenotyped mice. Differences between rounds of phenotyping were assessed by comparing mean age at VO using one-way analysis of variance (ANOVA) followed by Bonferroni post-test, and by assessing normality of the distributions.
Multivariate regression analysis of recorded variables against age at VO was performed using Small Stata 11.1 software using the following command: `mvreg vo = littersize mfratio agemo agefa preg wtvo wtwean growth`.

Variables were also individually correlated with age at vaginal opening in a univariate regression analysis by determining the significance of Pearson’s moment correlation coefficient at a given sample size.

Rate of growth in g/day was calculated for early (VO ≤ pnd28), average (VO at pnd31 or 32), and late (VO ≥ pnd35) onset of puberty mice as defined by results in Figure 3, at three developmental periods: pre-weaning (birth to weaning), pre-puberty (weaning to puberty), post-puberty (puberty to pnd 39).

Rate of growth from birth to weaning was calculated by dividing weight at weaning at pnd 21 by 21.

Rate of growth from weaning to puberty was calculated by subtracting weight at weaning from weight at VO and then dividing the difference by age at VO minus 21, the age at weaning.

Rate of growth from puberty to pnd 39 was calculated by subtracting weight at pnd39 from weight at VO and dividing the difference by 39 minus age at VO.

ANOVA with Bonferroni post-test was performed to assess differences in growth rate between the three groups of mice at each developmental period, and differences in growth rate between each developmental period for each group of mice.

Longitudinal growth data from pnd 21 to pnd 75 was analyzed comparing early, average and late puberty weight-age curves using repeated measures mixed model ANOVA followed by a Bonferroni post-test comparing each group to all other groups at each sampled age (24 points) according to suggestions and survey of current practice (Liu et al., 2010) addressing within experimental unit dependency and correcting for multiple comparisons.
2.2 Results

2.2.1 Distribution of Pubertal Timing

One of the first goals of this investigation was to confirm that female mice in the colony of B6 mice do show wide, normally distributed variation in the timing of puberty.

Assessment of pubertal timing of female B6 mice for this study occurred in five rounds over the course of 11 months. This is illustrated in Figure 2-2. To construct a pooled population distribution of age at VO for B6 mice, it was important to verify that no significant differences were observed between rounds and between the average of the rounds and historical laboratory data.

<table>
<thead>
<tr>
<th>Round</th>
<th>Mean (SD)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>RND I</td>
<td>32.0±2.1</td>
<td>61</td>
</tr>
<tr>
<td>RND II</td>
<td>31.0±2.1</td>
<td>52</td>
</tr>
<tr>
<td>RND III</td>
<td>31.3±2.4</td>
<td>24</td>
</tr>
<tr>
<td>RND IV</td>
<td>31.8±2.3</td>
<td>59</td>
</tr>
<tr>
<td>RND V</td>
<td>32.4±3.3</td>
<td>51</td>
</tr>
<tr>
<td>Historical</td>
<td>31.5±3.0</td>
<td>51</td>
</tr>
</tbody>
</table>

Figure 2-2 Months during which breeding and phenotyping for onset of puberty occurred for separate rounds of mice

Diagram of a timeline from March 2010 to January 2011 during which C57BL/6 female mice were phenotyped daily for age at vaginal opening starting at weaning on postnatal day 21. Coloured squares represent individual breeding cohorts for which average age at vaginal opening ±SD was calculated for n number of female offspring in each breeding cohort. There were five rounds (RNDI-RNDV) of breedings. The grey bar represents these five rounds pooled together. The black bar represents age at vaginal opening data collected on C57BL/6 female mice prior to March 2010.

Mean and standard deviation (SD) of age at VO between RND II from late spring and RND V from the early winter were found to be statistically different (p<0.01). All other variations were subtle and did not differ significantly. Importantly, the average mean age at VO across all five rounds of phenotyping did not significantly differ from laboratory data collected previously.

Also, the distributions of VO across each of the different rounds of phenotyping do not visually differ from each other and each one passed the statistical test for normality (Figure 2-3).
Figure 2-3 Distributions of pubertal timing of mice phenotyped in different months

Percent of C57BL/6 female mice with age at vaginal opening is plotted against the age (postnatal day, pnd) when the mice attained vaginal opening. Overlaid and identified by colour, weight and/or solid/dashed lines are the distributions of the following populations of mice: mice phenotyped prior to March 2010 (Historical) (n=51); mice phenotyped in round one (RND I) from March to April 2010 (n=61); round two (RND II) from April to June 2010 (n=52); round three (RND III) from June to August 2010 (n=24); round four (RND IV) from August to September 2010 (n=59); round five (RND V) from December 2010 to January 2011 (n=51).

Data from each of the five rounds of phenotyping, including historical data, were thus pooled to generate an overall distribution for age at VO within our colony (Figure 2-4).

Figure 2-4 Age at vaginal opening is normally distributed in mice

Percent of C57BL/6 female mice with age at vaginal opening plotted against the age (postnatal day, pnd) when the mice attained vaginal opening. The mean age at vaginal opening of this population is $31.7 \pm 2.6$ (n=298).
This pooled distribution also passes the statistical test for normality. The mean age at VO of this population is 31.7 days postnatally with a SD of 2.6 days. One SD above and below the mean is 34.4 and 29.1 days, respectively. Thus, for subsequent experiments, mice with VO at or younger than pnd 28 and at or older than pnd 35 are considered to have early and late puberty, respectively.

### 2.2.2 Potential Confounders

Another goal of this investigation was to determine the potential for micro-environmental variables to confound the investigation of epigenetic mechanisms in regulating puberty. Potential confounders were assessed by regression analyses using data collected from all mice.

**Table 2-1 Multivariate regression analysis of variables in micro-environment on timing of puberty**

Correlation between the dependent variable age at vaginal opening (VO) is assessed between independent variables: number of pups per litter (littersize), male to female ratio in litter (mfratio), age of mother (agemo), age of father (agefa), and mother’s first or second pregnancy (preg), weight at VO (wtvo), weight at weaning (wtwean), rate of growth (growth). n=298

<table>
<thead>
<tr>
<th></th>
<th>coefficient</th>
<th>standard error</th>
<th>t-statistic</th>
<th>p-value</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>littersize</td>
<td>-0.02</td>
<td>0.02</td>
<td>-0.93</td>
<td>0.36</td>
<td>-0.05 - 0.02</td>
</tr>
<tr>
<td>mfratio</td>
<td>-0.01</td>
<td>0.05</td>
<td>-0.17</td>
<td>0.87</td>
<td>-0.12 - 0.10</td>
</tr>
<tr>
<td>agemo</td>
<td>-0.01</td>
<td>0.01</td>
<td>-1.28</td>
<td>0.20</td>
<td>-0.03 - 0.01</td>
</tr>
<tr>
<td>agefa</td>
<td>-0.01</td>
<td>0.004</td>
<td>-1.42</td>
<td>0.16</td>
<td>-0.01 - 0.002</td>
</tr>
<tr>
<td>preg</td>
<td>0.11</td>
<td>0.13</td>
<td>0.85</td>
<td>0.40</td>
<td>-0.15 - 0.37</td>
</tr>
<tr>
<td>wtvo</td>
<td>1.52</td>
<td>0.03</td>
<td>44.85</td>
<td>0.0001</td>
<td>1.46 - 1.59</td>
</tr>
<tr>
<td>wtwean</td>
<td>-1.58</td>
<td>0.04</td>
<td>-44.28</td>
<td>0.0001</td>
<td>-1.66 - 1.51</td>
</tr>
<tr>
<td>growth</td>
<td>-15.93</td>
<td>0.33</td>
<td>-47.68</td>
<td>0.0001</td>
<td>-16.59 - 15.27</td>
</tr>
<tr>
<td>_constant</td>
<td>32.37</td>
<td>0.62</td>
<td>52.58</td>
<td>0.0001</td>
<td>31.15 - 33.59</td>
</tr>
</tbody>
</table>

Output of multivariate regression analysis in Table 2-1 shows no significant correlation between the dependent variable VO and number of pups per litter, male to female ratio in litter, age of mother, age of father, and mother’s first or second pregnancy. Weight at VO was significantly positively correlated with age at VO (p<0.0001). Weight at weaning and rate of growth were significantly negatively correlated with age at VO (p<0.0001). The equation modeling these relationships can be constructed from the values given for the coefficient (slope) and constant (intercept) in Table 2-1. For example, for every gram of lower weight at weaning, expected age at puberty is advanced by 1.58 days if all other variables are kept constant.
Univariate regression analyses between age at VO and individual variables indicated above follow in sections 2.2.2.1 through 2.2.3.4.

**2.2.2.1 Litter Size**

![Litter Size Graph](image)

*Figure 2-5 No significant effect of litter size on onset of puberty*

Age at vaginal opening of C57BL/6 female mice plotted against litter size (n=229), r=0.179, ns.

No significant correlation was found between number of mice in a litter and the age at VO (r=0.179) (Figure 2-5).

**2.2.2.2 Male to Female Ratio in Litter**

![Male:Female Ratio Graph](image)

*Figure 2-6 No significant effect of male:female in litter on onset of puberty*

Age at vaginal opening of C57BL/6 female mice plotted against male to female ratio (n=229), r=0.014, ns.
No significant correlation was found between male to female ratio in a litter and the age at VO (r=0.014) (Figure 2-6).

### 2.2.2.3 Age of Parents

![scatter plot with age of mother vs age of VO](image)

**Figure 2-7** No significant effect of mother's age on onset of puberty

Age at vaginal opening of C57BL/6 female mice plotted against mother’s age at mating (n=186), r=0.026, ns.

No significant correlation was found between mother’s age at mating and the age at VO (r=0.026) (Figure 2-7).

![scatter plot with age of father vs age of VO](image)

**Figure 2-8** No significant effect of father’s age on onset of puberty

Age at vaginal opening of C57BL/6 female mice plotted against mother’s age at mating (n=186), r=0.004, ns.
No significant correlation was found between father’s age at mating and the age at VO ($r=0.004$) (Figure 2-8).

### 2.2.2.4 Mother’s First or Second Pregnancy

Figure 2-9 No effect of being offspring from mother’s first or second pregnancy on onset of puberty

Percent of C57BL/6 female mice with age at vaginal opening plotted against the age (postnatal day, pnd) when the mice attained vaginal opening, separated into two groups: offspring from mother’s first pregnancy, mean age at vaginal opening $31.7 \pm 2.3$ (n=130); offspring from mother’s second pregnancy, mean age at vaginal opening $31.4 \pm 2.0$ (n=53).

No significant difference was found between the two distributions of female offspring’s age at VO grouped by mother’s first or second pregnancy (Figure 2-9).

### 2.2.3 Growth

Multivariate regression analysis found a significant correlation with age at VO and various aspects of weight and growth. This relationship was explored further in the following sections.

#### 2.2.3.1 Weight at Weaning

Weight at weaning is significantly inversely proportional to age at VO ($p<0.01$) (Figure 2-10). The heavier the mouse, the more likely she will attain puberty earlier. As such, weight at weaning, being a measure taken prior to the onset of puberty, has some predictive strength in predicting timing of puberty.
Figure 2-10 Weight at weaning inversely correlated with onset of puberty

Age at vaginal opening of C57BL/6 female mice plotted against weight at postnatal day (pnd) 21 (n=187), r=0.528, p<0.01.

2.2.3.2 Weight at VO

A classically held view on factors dictating the timing of puberty stated that puberty occurs when a certain weight is reached (Frisch and Revelle, 1970). If this were the case, data points for all mice, irrespective of timing of puberty, would fall in line at one given weight, as is demonstrated by the pink dots superimposed on Figure 2-11.

Figure 2-11 Weight at vaginal opening positively correlated with onset of puberty

Age at vaginal opening of C57BL/6 female mice plotted against weight at vaginal opening (green dots) (n=288), r=0.55, p<0.01. Pink dots represent hypothetical situation when age at vaginal opening always occurs when mice weight 15g.
This is not the case; weight at VO is significantly positively correlated with age at VO (p<0.01) (Figure 2-11). Mice that are younger, attain puberty earlier tend also to be lighter than older mice that attain puberty later. This relationship is, of course, confounded by age itself since on average younger mice are lighter than older mice.

### 2.2.3.3 Growth Curves

To more fully understand the correlations seen with timing of puberty and weight at two time points (weaning and VO), weight gain of early, average and late onset of puberty mice was tracked from weaning into adulthood.

![Graph showing growth curves of early, average, and late onset of puberty mice](image)

**Figure 2-12 Early, average and late onset of puberty mice follow different growth curves from weaning into adulthood**

Curves of weight to age of C57BL/6 female mice grouped by early onset of puberty (age at vaginal opening ≤ postnatal day, pnd, 28, n=7), average (age at vaginal opening on postnatal day 31 or 32, n=13) or late (age at vaginal opening ≥ postnatal day 35, n=14), followed from pnd21 to pnd75. *p<0.05, **p<0.01

The growth curves of the three groups of mice are highly significantly different (p=0.0043) (Figure 2-12). On average, mice destined to have early puberty are larger than mice destined to
have late puberty, and those that will have puberty at the average age are of an average size. This relationship is present at the time of weaning and persists through adulthood.

Statistically significant differences between groups are observed at pnd 21 through to 32 (p<0.05 or p<0.01), which corresponds to the pre-pubertal stage of development. The rise starting from pnd 21 is almost parallel between the groups, with growth of early mice plateauing earlier than average and late. By pnd 75, late mice have not caught up to the same weight as early mice and the trend of weight separation between the groups, although not statistically significant post-puberty, is maintained throughout the time-course sampled.

2.2.3.4 Growth Rate

The relationship between pre-pubertal rate of growth and timing of puberty was also addressed.

![Figure 2-13](image-url)

**Figure 2-13 Pre-pubertal rate of growth inversely correlated with onset of puberty**

Age at vaginal opening (VO) of C57BL/6 female mice plotted against weight gain between postnatal day (pnd) 21 and postnatal day of vaginal opening (n=187), r=0.59, p<0.01.

Rate of weight gain per day between pnd 21 and onset of VO is significantly negatively correlated with age at VO (p<0.01) (Figure 2-13). Mice that grow faster during the pre-pubertal stage tend to attain puberty earlier than slower growing mice. The relationship between faster growth rate from weaning to VO is true for all mice. That is, within the early, on time, and late mice, it is still the ones that are growing faster that tend to have VO sooner.
To investigate this relationship more fully, growth rate across developmental periods was assessed in mice with early, average or late onset of puberty (Figure 2-14).

**Figure 2-14 Early, average and late onset of puberty mice grow at different rates across developmental periods**

Weight gain from birth to postnatal day 21 (birth-weaning), from postnatal day 21 to age at vaginal opening (weaning-VO), and from age at vaginal opening to postnatal day 39 (VO-pnd39) of C57BL/6 female mice grouped by early onset of puberty (age at vaginal opening ≤ postnatal day 28), average (age at vaginal opening on postnatal day 31 or 32) or late (age at vaginal opening ≥ postnatal day 35). *p<0.05, **p<0.01, ***p<0.001

An examination of weight gain as a measure of growth rate across development reveals that relative rates of growth appear to be in place by weaning, and perhaps before. Growth rates increase from the weaning to VO interval compared to the birth to weaning interval. However, the percent rise from birth-weaning to weaning-VO stages is not statistically different between early, average and late onset mice (166% ±36% vs. 179% ±44% vs. 162% ±36%, respectively). The growth rate changes across developmental periods for each of the three groups follow approximately parallel trajectories. Groups are separated by absolute growth rates relative to one another, where early puberty mice during each developmental period grow faster than late
(p<0.001) and late puberty slower than average (p<0.01 and p<0.05 between VO-pnd39). The trajectory followed is a significant rise from the Birth -Weaning interval to the Weaning-VO interval, and a fall at the VO-pnd 39 interval (p<0.001 for each comparison between developmental periods in each group).
2.3 Discussion

2.3.1 Human Distribution of Pubertal Timing is Modeled by B6 Mice

The timing of puberty in the general population of healthy girls shows wide, normally distributed variation (Palmert and Boepple, 2001). A window of five years between age 7 and 13 is currently considered to be the normal range for the initiation of puberty in girls (Styne et al., 2011). Figure 2-4 illustrates that onset of puberty in a large population of B6 female mice both approximates a normal distribution and shows a wide variation, similar to the characteristics of pubertal onset observed in humans. Quantitative equivalence in spread between humans and mice is difficult to determine, but it is interesting to recognize that a range of 19 days between the earliest and latest observed age at vaginal opening (VO) in this population of mice is approximately 3% of an average 750 day lifespan of a B6 mouse (Yuan and Paigen, 2009) and 5 years is approximately 6% in an average 80 year lifespan of a human. It is possible, therefore, that the variation in onset of puberty between this strain of female mice is comparable to that of human females both qualitatively and quantitatively.

In studying timing of onset of puberty, the most common marker in humans is age at menarche (AAM). The year in which a young woman first begins menses is easily assessed and well-remembered, and thus can be documented with monthly precision in longitudinal studies or recalled with relatively high accuracy in retrospective studies (Biro et al., 2010). This marker is analogous to time to first estrous in a mouse, but in large scale studies of pubertal onset in mice, this marker is less often used because it is invasive and time consuming to recognize. Time to first estrous is assessed by vaginal smear cytology, which involves daily flushing of the vagina with saline and determining estrous cycle stage via observation of proportions of epithelial cells, cornified cells and leukocytes under a microscope (Caligioni, 2009). A reliable method that is better suited for large mouse studies is age at VO. In some respects, VO may also be a better single marker as it signifies the onset of puberty instead of marking a later maturational event (estrous). VO correlates with both the increase in gonadotropin releasing hormone (GnRH) pulsatility in the hypothalamus and the surge of estrogen from the ovaries that occurs at the initiation of puberty (Ojeda et al., 1980; Safranski et al., 1993). The estrogenic stimulation of VO coincides with apoptosis of the epithelial cells at the opening of the vagina revealing a distinct canal (as seen in Figure 2-1) (Gray and Ostby, 1995; Rodriguez et al., 1997). In humans, if
estrous is analogous to menarche, then VO may be thought of as analogous to onset of breast development, which also correlates with increased GnRH pulsatility (Styne et al., 2011). VO is a quick method of assessment, non-invasive and widely accepted by the community of mouse puberty researchers (Ojeda et al., 2006). Therefore, by using age at VO as the measure of puberty in this mouse model, the interpretation of results obtained from this model would be translatable to humans, making age at VO in female B6 mice a suitable model for the goals of this project in this respect.

Another aspect considered when determining an appropriate mouse model for studying variation in timing of pubertal onset was wideness of variation in pubertal onset. Data on age at VO of mice from the Jackson Laboratories (JAX), from which the B6 mice used in this study originate, shows that variation in age at VO varies between strains. The B6 strain of female mice appear to exhibit the widest variability in timing of puberty than all other available strains, with the highest recorded standard deviation in age at VO of 7.67 days and range of 23-49 days. Second and third most variable strains are recorded as SJL/J and CAST/EiJ at standard deviation (SD) of 7.19, range 20-44 and SD of 6.25 with range of 30-48 days, respectively (Yuan, 2009). Given that B6 has the greatest variation in a range of age at VO (that does not overlap the date of weaning yet shows the phenotype relatively early), it was chosen for this study.

Differences between strains of mice can be likened to difference in AAM between humans of different population groups. Grouped by more similar genetics, The National Longitudinal Study of Adolescent Health (Add Health) is one of the many reporting that ethnic background dictates onset of female puberty, where non-Hispanic blacks are sexually maturing significantly earlier than whites who mature earlier than Asians when socioeconomic and other factors are accounted for (Adair, 2001). Further evidence for the importance of genetic background as a modulator of the timing of puberty stems from twin studies where monozygotic twins have a higher concordance in pubertal timing than dizygotic twins (Segal and Stohs, 2007).

These differences in pubertal timing in inbred strains have been mainly attributed to genetics and, on this basis, strain differences have been used to determine quantitative trait loci that modulate the timing of puberty via generation and phenotyping of chromosomal substitution strains (Krewson et al., 2004; Nathan et al., 2006b). However in these mice studies not all the variation between strains could be explained. Similarly, monozygotic twins are not fully
concordant in age of pubertal onset. Other factors must explain some of the variation in pubertal timing, such as environmental influences and/or epigenetic factors. To study environmental factors, it is important to study mice with a uniform genetic background since (as above) genetic background has a large influence on pubertal timing. To study epigenetic factors, it is important to keep genetic background constant but also verify that variation is not explained by environmental differences that could exert their influence via non-epigenetically related mechanisms.

2.3.2 Timing of Puberty is Unaffected by Micro-Environmental Variables

When twins are reared apart vs. together, those reared apart show greater discordance in AAM (Segal and Stohs, 2007). This is attributed to greater differences in their environments including differences in aspects of parental care (Segal and Stohs, 2007), and geographic location with which may come from differences in climate and socioeconomic status, both of which factor greatly in timing of puberty (Mishra et al., 2009a; Virdis et al., 1998; Barnett and Coleman, 1959). The same strains of mice studied in different laboratories also show differences in average timing of puberty. This phenomenon was already apparent in the 1920s when the Parkes’ (1925) and Engle and Rosasco’s (1927) studies from Scotland and the US, respectively, reported a 16 day difference in median age at VO in the albino mouse. JAX reports average age of VO of B6 mice as 34.3, 2.6 days later than determined for the population of B6 mice used in this study (Figure 2-4). Some of the chromosome substitution strains that the Palmert laboratory developed and studied in Cleveland lost their early VO phenotypes when moved to Toronto (Palmert 2008, unpublished). Clearly, there is an influence beyond genetic background causing differences in VO, most likely due to environmental differences.

For this reason, during the studies carried out for this thesis, environmental variables were controlled, wherever possible. Aspects that could not be fully controlled, mainly ones that were part of the micro-environment, were assessed for their contribution to variability in age at VO in the work described in this chapter. Importantly, none of the environmental variables interrogated were found to have a significant effect on timing of puberty, as determined by both uni- and multivariate analyses (Table 2-1 and Figures 2-5 to 2-9).
Table 2-2 groups the environmental variables present in the laboratory setting that could affect timing of puberty in female mice by 1) maintaining constant conditions for some variables, and 2) by assessing their contribution to timing of puberty, where feasible.

Table 2-2 Categorization of variables present in the environment of a laboratory mouse

<table>
<thead>
<tr>
<th>Category</th>
<th>Category criteria</th>
<th>Conditions</th>
</tr>
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<tbody>
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<td>Assessed?</td>
</tr>
<tr>
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<td>Yes</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- location</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- composition of post-weaning diet</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- in utero nutrition</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- father’s pheromone exposure</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- housing enrichment</td>
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<tr>
<td>B</td>
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</tr>
<tr>
<td></td>
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<tr>
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<td>- male to female ratio in litter</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- age of parents</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- mother’s 1st or 2nd pregnancy</td>
</tr>
</tbody>
</table>

Location, as described previously, can influence age at VO substantially in either direction despite isogenic background. Mice were thus housed in the same facility and in the same room for the entire duration of the experiment. Differences in composition of diet can affect timing of puberty, as documented in studies of diets high in fat that advance puberty (Moral et al., 2011), protein restricted that delay puberty (da Silva Faria et al., 2004), and phytoestrogen richness that advances puberty (Bateman and Patisaul, 2008). Diet composition was restricted to the Tekland Global 18% Protein Rodent Diet supplied by the Toronto Center for Phenogenomics (TCP).

Female exposure to male pheromones via urine, feces and skin affects the rodent reproductive system, an effect called the Whitten effect (Whitten, 1956). Specifically, presence of males can accelerate sexual maturation in pre-pubertal females (Vandenbergh, 1969). Males were removed after 7 days of mating to minimize their exposure during conception and early in-utero development. Also, to eliminate all sources of remnant pheromones after male removal, females were changed to clean cages, and males and females were housed separately after weaning. The presence of an environmental enrichment, cage bedding (brown crinkled paper), was also maintained constant in the cages. Environmental enrichment in mouse cages has been documented to improve reproduction and reduce anxiety behaviours (Whitaker et al., 2009). For
those reasons, these Category A variables were kept constant. Despite these same environments within cages, variability in pubertal timing was seen among littermates.

Variables in Category B were not constant so their contribution to pubertal timing was assessed. The concern was that an environmental exposure contributing to the variation in pubertal timing could be doing so in an epigenetic independent manner, precluding the possibility to isolate the presence of an epigenetic mechanism. Therefore, it was a priori established that if any environmental variable significantly contributed to the variation in pubertal timing, epigenetic mechanisms could not be studied in this model unless this environmental variable could be demonstrated to have led to the variation dependent on epigenetic mechanisms. The following discussion presents possible epigenetic mechanisms through which both litter size and ratio of male to female litter mates could affect puberty. To note, though, no significant contribution of these variables to pubertal timing was found (Figure 2-5 and Figure 2-6).

Differential litter size could lead to differential mother care and/or differential competition postnatally in access to food. For example, Cameron et al. (2008) found that poor motherly care leading to advanced puberty correlated with an increase in estrogen receptor alpha (ERα) expression in the anteroventral paraventricular nucleus, which contributes to the regulation of GnRH secretion and consequently the onset of puberty. It has been suggested that these effects could derive from epigenetic modification to the gene *Esr1* coding for ERα. Moreover, it is well established in multiple studies by Weaver et al. (2004; 2005; 2006) that mother care in rats alters DNA methylation patterns in offspring leading to phenotypic differences, in this case, in stress response as a result of an epigenetic change to the gene promoter region of the glucocorticoid receptor (GR).

In the context of litter size dictating competition for food, in rats, smaller litter sizes lead to postnatal pre-weaning over-feeding, increased body weight and faster fat accumulation (Plagemann et al., 1992). This can lead to early puberty (Castellano et al., 2011). Castellano et al. (2011) investigated the neuroendocrine basis of this and found that rats from 5 times smaller litters (4 pups compared to 20) had increased expression of mRNA for the adipocyte satiety factor leptin and neuropeptide kisspeptin (*Kiss1*) in the hypothalamus. Other studies are now revealing that diets high in fat and subsequent increased fat accumulation are associated with epigenetic alterations in a variety of regulatory genes (Bouchard et al., 2010; Plagemann et al.,

No significant correlation was found between litter size and age at VO in B6 mice of this colony, perhaps because the range in litter size was more limited compared to the rat studies. Differences in number of males and females in a litter opens the possibility for differential exogenous estrogen exposure both in utero (IU) and postnatally. Male littermates IU produce estradiol (E2) (vom Saal, 1989), the developing female brain can be masculinized by E2 and lead to delayed puberty (Jahagirdar et al., 2008). Given that E2 can change methylation marks (Schwarz et al., 2010) and E2 exposure IU can change Kiss1 mRNA expression (Iwasa et al., 2010), variation in gene expression leading to variation in phenotype could be mediated by an epigenetic mechanism. Again, however, male to female ratio in a litter did not significantly correlate with age at vaginal opening and the origin of the underlying mechanism is not of concern.

Ages of female and male mice were also found to have no effect on age at vaginal opening in their female offspring (Figure 2-7 and 2-8). Likewise, mother’s first or second pregnancy was found to have no effect on the timing of puberty (Figure 2-9).

In summary, data show that timing of VO is not significantly modulated in B6 mice by litter size, by number of male siblings, mother’s first or second pregnancy or age of parents. Since no clear environmental mechanism appears to explain the variation in timing of puberty, the B6 mice remain a suitable model to investigate the existence of an epigenetic-dependent mechanism in regulating pubertal timing.

2.3.3 Relationship between Pubertal Onset and Growth

The data imply that age at VO is not necessarily linked to a critical weight but instead is associated with greater growth velocity. The critical weight hypothesis, held by the Frisch-Revelle Model (Frisch and Revelle, 1970), stated that that once a young woman reaches a critical weight of 48kg, she will begin menses. This could mechanistically be thought to correspond to leptin acting as a trigger of pubertal onset (Vogel, 1996) accompanied by further activation via increasing estrogen from the accumulation of adipose tissue (Cannady et al., 2000). Following this classical view that an ideal weight must be attained for onset of menses would dictate that for variation in timing of puberty to occur, mice would have to grow at different rates and attain the same weight at different ages. VO would then occur at a critical weight regardless of age.
The data from Figure 2-11 are contrary to this prediction and indicate that VO occurs at a variety of weights. Also, although the early mice are, on average, growing faster (Figure 2-13) they also tend to be lighter at VO (Figure 2-11).

With more investigation over the years, it is now held that instead of a trigger, leptin is a permissive factor. Leptin is not sufficient to evoke a response on its own, as evidenced by data showing that levels of leptin do not sharply rise at the onset of puberty (Blum et al., 1997) nor are high levels enough to trigger pubertal onset (Cheung et al., 2001) although high levels with increased fat mass do accelerate progression through puberty (Ahima et al., 1997). In certain disorders of hypoleptinemia, girls can attain puberty at the normal age (Musso et al., 2005) but the complete absence of leptin will delay or prevent onset (Cunningham et al., 1999; Ingalls et al., 1950). More evidence comes from realizing leptin is not involved in constitutional delay in growth and puberty (CDGP), where genome wide association studies (GWAS) found that common polymorphisms in the genes coding for leptin and the leptin receptor do not associate with this pubertal delay (Banerjee et al., 2006) and do not associate with AAM in a normal population (Rothenbuhler et al., 2009).

Therefore, the positive correlation with weight at VO and age at VO implies is that sufficient weight is related to onset of puberty but it is not the only determining factor. That is why 1) the majority of mice attain puberty when they are heavier than 12.5g, apart from some outliers, and 2) they show variation in weight at VO ranging from 12.5 and 18g. The rapid growth rate with earlier age at VO (Figure 2-13) suggests that how fast a mouse is gaining weight plays a role in determining how early she attains puberty, which is consistent with earlier mice starting out at a heavier weight at weaning (Figure 2-10) growing faster to reach puberty earlier but at a weight that is still less than that of older, later onset puberty mice.

In humans, low birth weight, faster pre-pubertal weight gain, and shorter adult stature were observed to associate with earlier menarche (Dunger et al., 2006). Figure 2-13 shows that pre-pubertal weight gain is also faster in mice with early age than later age at VO. Birth weight was not recorded for mice due to difficulty in assessing gender and tagging at birth for later identification. Instead of birth weight, measures of weight at weaning were recorded revealing that heavier weaning weight predicts earlier puberty (Figure 2-10), which is the same as found in pre-pubertal girls (Cooper et al., 1996), where heavier weight at the age of 7 years predicts
earlier AAM. The relationship between pubertal onset and growth in mice, therefore, models humans.

2.3.4 B6 Mice are a Suitable Model for Epigenetic Investigation

The B6 strain of female mice provides a suitable in vivo model in which to study the potential role of epigenetic mechanisms in the regulation of pubertal timing. The studies in this Chapter demonstrate that the timing of onset of puberty in a population of inbred mice shows wide, normally distributed variation similar to that seen in humans. Importantly, this variation could not be explained by variables in the micro-environment of these mice indicating that a mechanism other than environment or genetic variation directly, such as an epigenetic mechanism, may be contributing to the variation.

The findings of this chapter were used to inform various methodology and interpretation in the following two chapters that investigate Aims 2 and 3, respectively. The distribution determined in Figure 3 was used a reference upon which to compare age at VO of both control and perturbed mice in Aim 2. Growth data were used when interpreting growth and puberty results of perturbed mice from Aim 2. The wideness of the distribution and the set exclusion criteria for grouping mice by early and late pubertal onset informed methodology for Aim 3.
Chapter 3

It is hypothesized that epigenetic markings such as specific patterns of DNA methylation and/or histone modifications, such as acetylation, are involved in regulating the timing of pubertal onset within a population. In this chapter this hypothesis was examined by determining if modulating methylation and/or acetylation patterns would modulate the timing of puberty.

3 Aim 2 – Perturbation of Epigenetic Marks

3.1 Methods

Female B6 mice were used to evaluate the hypothesis. In brief, mice were administered epigenetic modifying agents pre-pubertally and assessed for changes in the timing of puberty. See Chapter 2, for description of general animal care, housing and husbandry.

Choice and dose of agent as well as route, mode, length and timing of administration were optimized by assessment of general health, growth, and ingestion of treated water, in two consecutive rounds of experimentation. Data from the first round of optimization informed methodological choices in optimization round II, and methodology for a full scale experiment was determined from the optimization rounds.

3.1.1 Optimization Round I

In round I of optimization, the following was tested: administration of modifying agents beginning at weaning on pnd21, via drinking water or pipette tip feeding. Each mode differed both in degree of experimenter handling (none vs. forced restraint, respectively) and in duration of exposure to agents (continuous vs. once daily bolus, respectively).

Littermates were distributed among cages, where all mice in each new cage were assigned to the same condition, allowing mice from the same litter to be exposed to different conditions. Mice were housed 4-5 per cage. Details of each condition are outlined in Table 3-1.

Pipette tip feeding involves the following steps: the mouse is loosely restrained by holding of her tail, so that the pipette tip filled one time with corn oil-containing-agents can be placed near the
mouth of the mouse. In this way, the mouse is allowed to eat the contents of the pipette as it is being slowly dispensed. A new, sterile pipette tip was used for each mouse. The corn oil-containing-agents solutions were given at volumes of 10-50μL. Solutions for drinking water were made fresh every third day, at increasing doses corresponding to increased average weight gain of the mice (determined from the age-weight curve of average puberty mice in Figure 2-12 from Chapter 2).

The agents chosen were the histone deacetylase inhibitor (HDACi) suberoylanilide hydroxamic acid (SAHA) at 100mg/kg dissolved in water with 5 molar equivalents of the vehicle hydroxypropyl-beta-cyclodextrin (HP-β-CD) for administration through drinking water (for optimal solubility as determined by Hockly et al. (2003)) or in corn oil for pipette tip feeding at 10mg/kg or 100mg/kg; L-methionine (MET) in water at 250mg/kg; and controls including the vehicle HP-β-CD in water (at the 100mg/kg SAHA dose) for drinking water, and corn oil alone for pipette tip feeding.

Table 3-1 Conditions of agent administration in optimization round I

<table>
<thead>
<tr>
<th>Mode of Administration</th>
<th>Agent</th>
<th>Dose</th>
<th>n mice</th>
<th>n litters</th>
<th>n cages</th>
</tr>
</thead>
<tbody>
<tr>
<td>pipette tip</td>
<td>corn oil</td>
<td>-</td>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>pipette tip</td>
<td>MET + corn oil</td>
<td>250mg/kg</td>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>pipette tip</td>
<td>SAHA + corn oil</td>
<td>100mg/kg</td>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>pipette tip</td>
<td>SAHA + corn oil</td>
<td>10mg/kg</td>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>drinking water</td>
<td>HP-β-CD</td>
<td>for 100mg/kg SAHA</td>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>drinking water</td>
<td>MET</td>
<td>250mg/kg</td>
<td>5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>drinking water</td>
<td>SAHA + HP-β-CD</td>
<td>100mg/kg</td>
<td>5</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

SAHA, suberoylanilide hydroxamic acid; HP-β-CD, hydroxypropyl-beta-cyclodextrin; MET, L-methionine

Agent ingestion through drinking water was ensured by the removal of sippers connected to the central water supply, leaving access to drinking water only via sippers connected to light-protected water bottles containing the agents in water. In order to minimize the number of mice used, examination of the effects of different doses of SAHA was restricted to one mode of
administration. Pipette tip feeding was chosen over water in which to test both the 10 and 100mg/kg doses of SAHA because it was initially considered that pipette tip feeding had the potential to be the better mode of administration for a more accurate dose.

Overt signs of deteriorating health, such as emaciation, hair loss, decreased activity level, etc, were monitored by experimenter and by animal care staff of the TCP in their usual daily check-up during agent administration. None were detected, and general health appeared to be unaffected by agent administration.

Mice were also weighed daily for assessment of weight gain with agent administration. Given the positive correlation seen between pre-pubertal weight gain and onset of puberty in Chapter 2, it was necessary to find a dose that did not disrupt growth rate to eliminate any potential confounding effect of agent-affected growth on timing of puberty. Therefore, differences in growth were assessed between agents and doses within each mode of administration.

![Figure 3-1 Effect of pre-pubertal agent administration on weight gain in optimization round I](image)

C57BL/6 female mice were administered either control (ctrl) vehicle, L-methionine (MET) at 250mg/kg or suberoylanilide hydroxamic acid (SAHA) at 100mg/kg via either drinking water (solid-fill bars) or pipette tip (striped bars) from postnatal day 21 to 28 and weighed daily. Weight gain, expressed as average rate in grams (g) per mouse per day over 7 days ±sem, is plotted against treatment group (n=5 per group).
No significant differences in average weight gain were observed between control mice and any of the treated mice for either mode of administration. Figure 3-1 does, however, illustrate non-significant trends after multiple hypothesis correction, where SAHA treatment at the 100mg/kg dose appears to diminish weight gain by both water and pipette tip feeding compared to control (p=0.0245 and p=0.0469, respectively). MET treatment does not appear to have an effect on weight gain.

Since an effect on growth rate with agent administration may confound the key outcome of effect of agent administration on pubertal timing, in this respect, SAHA at 10mg/kg would be the better dose to use in assessing changes specific to pubertal onset with administration of epigenetic modifying agents.

To determine if the weight data can also indicate which mode of administration may be the better option for the goals of this experiment, average rate of weight gain was graphically re-organized to be analyzed to compare growth rate between modes of administration over treatment conditions (Figure 3-2).

**Figure 3-2 Pipette tip feeding reduces weight gain irrespective of treatment in optimization round I**

C57BL/6 female mice were administered agents via either drinking water (solid-fill bars) or pipette tip (striped bars) from postnatal day 21 to 28 and weighed daily. Agents administered were control (ctrl) vehicle, L-methionine (MET) at 250mg/kg or suberoylanilide hydroxamic acid (SAHA) at 100mg/kg. Weight gain, expressed as average rate in grams (g) per mouse per day over 7 days ±sem, is plotted against treatment group (n=5 per group). *p<0.05
Results of this analysis find a statistically significant difference across treatments between the two modes of administration (p<0.0001). Specifically, pipette tip feeding significantly decreased rate of weight gain in control mice as well as in mice treated with SAHA at 100mg/kg compared to administration via drinking water (p<0.05 for both comparisons). Weight gain of MET treated mice also showed a trend toward decreased rate with pipette tip feeding (p=0.0247).

Pipette tip feeding appears to hinder growth rate irrespective of agent administered. Given that one of the main differences between pipette tip feeding and administration via drinking water is degree of experimenter handling, where the former involves forced restraint, the mice may experience stress and pain, accounting for the negative effects of pipette tip feeding. It was documented that mice fed by pipette tip were frequently defecating and urinating during administration. It is likely, therefore, that the mice were suffering stress related to administration. Stress is known to affect puberty (Kinsey-Jones et al., 2010; Cameron et al., 2008), and may even do so via an epigenetic mechanism (Mychasiuk et al., 2011). As such, stress due to mode of administration may confound or mask any epigenetic modifying effects the agents themselves may have on timing of puberty and confound the study. In these respects, administration via drinking water is better suited than pipette tip feeding for the goals of this experiment.

Administration via drinking water, however, confers the potential disadvantage of imprecision in the dose due potentially to mice in different treatment groups ingesting different amounts of water. Drinking behaviour may be affected by the presence of agents in the water and differences in taste, making it either repugnant or more appealing than regular water. To assess this, the average amount of water consumed by cage of mice was calculated by measuring water remaining in the water bottle each time the agent solution was changed. This occurred every third day, so the difference in volume was divided by 3 and further divided by the number of mice in the cage to obtain an estimate of water consumed by each mouse. Results are found in Figure 3-3.
Figure 3-3 Amount of water ingested unaffected by addition of agents to water in optimization round I

C57BL/6 female mice were administered either control (ctrl) vehicle, L-methionine (MET) at 250mg/kg or suberoylanilide hydroxamic acid (SAHA) in vehicle at 100mg/kg in drinking water from postnatal day 21 to 28. Amount of water ingested, measured per cage of 5 mice every third day, expressed as average rate in mL per mouse per day for 7 days ±sem, is plotted against treatment group (n=5 per group).

There appears to be a trend that water containing SAHA at 100mg/kg is ingested at a lower rate than regular water (p=0.4816). This suggests that a dose as great as this may be affecting the taste of the water. Testing a lower dose of SAHA is thus warranted.

However, no statistically significant differences were observed in average amount of water ingested per mouse per day by mice drinking control water with vehicle and mice drinking treated water with either MET or SAHA. Therefore, drinking water is a valid way to administer agents with adequate precision.

3.1.2 Optimization Round II

Informed by results from the first optimization, in the second round of optimization, apart from the following differences, all other procedures remained the same: SAHA and MET were administered orally via drinking water only, at 25mg/kg and 60mg/kg for SAHA and 1g/kg for MET. Details of each condition are outlined in Table 3-2. Outcomes measured were effects of agent administration on growth rate and amount of water ingested (interpretations and results follow in Figures 3-4 and 3-5).
Table 3-2 Conditions of agent administration in optimization round II

Conditions listed by column. Groups listed by rows. $n$=number, where $n$ litters per group is the number of different litters from which $n$ mice in the group originate, and total number of litters is the number of litters distributed between groups. Total number of mice and cages is the sum of all mice and cages used in each group.

<table>
<thead>
<tr>
<th>Mode of Administration</th>
<th>Agent</th>
<th>Dose</th>
<th>$n$ mice</th>
<th>$n$ litters</th>
<th>$n$ cages</th>
</tr>
</thead>
<tbody>
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<td>for 60mg/kg SAHA</td>
<td>13</td>
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<td>3</td>
</tr>
<tr>
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<td>L-methionine</td>
<td>1g/kg</td>
<td>15</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>drinking water</td>
<td>SAHA + HP-β-CD</td>
<td>60mg/kg</td>
<td>15</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>drinking water</td>
<td>SAHA + HP-β-CD</td>
<td>25mg/kg</td>
<td>12</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>TOTAL</td>
<td>4 groups</td>
<td></td>
<td>55</td>
<td>13</td>
<td>12</td>
</tr>
</tbody>
</table>

SAHA, suberoylanilide hydroxamic acid; HP-β-CD, hydroxypropyl-beta-cyclodextrin; MET, L-methionine

The dose of methionine was increased to 1g/kg from 250mg/kg. Due to the previous solubility constraints imposed by the small volumes of corn oil used in pipette tip administration, a lower dose of methionine had been administered than was originally planned. To be comparable across modes of administration, the same low dose had been administered via water. With drinking water now being the chosen mode, the dose was raised and was well above the content already in the baseline diet (as others have previously shown to work to change methylation in rodent brains (Weaver et al., 2005; Dong et al., 2005; Tremolizzo et al., 2002))

Two doses of SAHA between those that were already assessed were chosen: 25mg/kg and 60mg/kg. The goal was to determine the highest dose that would have no effect on growth or the amount of drinking water ingested.
C57BL/6 female mice were administered either control (ctrl) vehicle, L-methionine (MET) at 1g/kg or suberoylanilide hydroxamic acid (SAHA) at either 60mg/kg or 25mg/kg in vehicle via drinking water from postnatal day 21 to 32 and weighed daily. Weight gain, expressed as average rate in grams (g) per mouse per day for 11 days ±sem, is plotted against treatment group (n=12-15 per group). *p<0.05, **p<0.001

In measures of pre-pubertal weight gain, mice administered any one of the agents gained weight at a significantly slower rate than controls (MET or SAHA 25mg/kg at p<0.05; SAHA 60mg/kg at p<0.001) (Figure 3-6). SAHA at 60mg/kg affected average weight gain the most of each of the agents.
C57BL/6 female mice were administered either control (ctrl) vehicle, L-methionine (MET) at 1g/kg or suberoylanilide hydroxamic acid (SAHA) in vehicle at either 60mg/kg or 25mg/kg in drinking water from postnatal day 21 to 32. Amount of water ingested, measured per cage of 3-5 mice every third day, expressed as average rate in mL per mouse per day for 11 days ±sem, is plotted against treatment group (n=12-15 per group).

No statistically significant difference was detected between treatment groups in average amount of water ingested per mouse per day. However, there does appear to be a trend that water containing SAHA at 60mg/kg was ingested at a lower rate than regular water (p=0.056) and the 25mg/kg dose (p=0.060).

Taken together, these data suggest that SAHA at 60mg/kg, like SAHA at 100mg/kg from optimization round I, affected growth and amount of water ingested. At the lower dose of 25mg/kg, negative effects are no longer present or diminished. Therefore, the dose of 25mg/kg of SAHA appears to be optimal with respect to treatment confounders.

### 3.1.3 Method Using Optimized Conditions

The following optimized conditions were used for the full perturbation experiment: oral administration through drinking water of SAHA at 25mg/kg solubilized in HP-β-CD vehicle, vehicle control, MET at 1g/kg in water only, water control, administered from weaning to pnd39. Details of each condition are outlined in Table 3-3.
Table 3-3 Conditions of agent administration of full scale experiment

Conditions listed by column. Groups listed by rows. A) conditions of administration; B) number of mice, litters and cages used for phenotyping age at vaginal opening for the various treatment groups in optimization round two (optII) and two rounds of additional breedings (RND1, RND2); C) number of mice, litters and cages used for weighing ovaries and uteri of mice sacrificed at postnatal day 32. \( n \) = number, where \( n \) litters per group is the number of different litters from which \( n \) mice in the group originate, and total number of litters is the number of litters distributed between groups. Total number of mice and cages is the sum of all mice and cages used in each group.

### A

<table>
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<th>Mode of Administration</th>
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<th>Dose</th>
</tr>
</thead>
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<td></td>
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<td>1g/kg</td>
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### B

<table>
<thead>
<tr>
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<th>( n ) cages</th>
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</tr>
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### C

<table>
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<th>( n ) litters</th>
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<tr>
<td>HP-β-CD</td>
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<td>10</td>
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<td>MET</td>
<td>10</td>
<td>4</td>
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</tr>
<tr>
<td>SAHA</td>
<td>4</td>
<td>4</td>
<td>7</td>
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<tr>
<td>TOTAL</td>
<td>57</td>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>

SAHA, suberoylanilide hydroxamic acid; HP-β-CD, hydroxypropyl-beta-cyclodextrin; MET, L-methionine
Data presented are results from three rounds performed using these conditions. Doing this in three rounds confers the practical advantage of having manageable numbers of mice to phenotype at one time, and also allows for biological replication and for control of potential phenotypic drift.

Both water only and vehicle (water + HP-β-CD) control were included and tested separately on all parameters. No differences were found. Therefore, the two groups of controls were pooled as one control group.

Littermates were distributed between treatment groups to have littermate controls. For example, the 10 mice treated with MET in RNDII originating from 9 out of the 18 different litters used for phenotyping in that round are littermates of control mice and mice treated with SAHA (Figure 3-3 B). Littermates were also distributed in such a way as to ensure there would be no difference in average weight between groups at the start of administration on pnd21. Mice were also weighed daily and average rate of weight gain per mouse per day per group was calculated between pnd21 and pnd28.

3.1.3.1 Method of Assessment of Effect of Agent Administration on Pubertal Timing

The effect of administration of epigenetic modifying agents on pubertal timing was assessed via two methods: age at vaginal opening, and weight of ovaries and uteri at pnd32. For the latter measure, a subset of mice were sacrificed at pnd 32 that were not included in the measure of age at VO. Mice were euthanized by carbon dioxide (CO₂) followed by rapid decapitation. Ovaries and uteri were dissected under a microscope in phosphate buffered saline (PBS) and weighed on a scale with 0.0001g precision. Organs were patted dry before weighing, and weighed within 10 seconds of drying. On the basis that ovaries and uteri grow proportionally to stage of pubertal development (Styne et al., 2011), heavier weights of these organs would be indicative of earlier puberty and vice versa. To be able to compare MET and SAHA treated mice to control mice, subgroups of mice were dissected at the average age of vaginal opening for untreated mice, on pnd32.
3.1.3.2 Sample Size Calculations

Sample size calculations were performed to determine the number of mice required per group to find a minimum 1.5 day difference in mean age at VO if a difference does occur between groups. An online calculator developed by The University of British Columbia for comparing means of two independent samples was used for the calculations (http://www.stat.ubc.ca/~rollin/stats/ssize/n2.html). Parameters were set to a two-sided p-value of 0.0125 to account for the comparison of 4 groups to each other (p<0.05/4), 0.80 power, using a standard deviation of 1.87, which was the average standard deviation determined in optimization round II of the mean age of VO of the three relevant treatment groups (n=26). 35 female mice were determined to be required per group. From this information, numbers of breeding pairs for two subsequent rounds of breeding were established accordingly.

3.1.3.3 Method of Assessment of Effect of Agent Administration on Histone Acetylation

Changes to histone acetylation were assessed as a measure of determining the effectiveness of SAHA administration on globally altering epigenetic marks via Western Blot of whole brain (central nervous system) and liver (peripheral organ). Tissue samples for assessment of drug action were collected from the mice dissected for ovaries and uteri. Whole brain was removed, immediately flash-frozen in liquid nitrogen, and stored at -20°C until used. A sample of the liver was also obtained and stored in RNAlater® (Ambion, Foster City, CA, USA) at 4°C for 24 hours, followed by long-term storage at -20°C.

Histone proteins were extracted using EpiQuik™ Total Histone Extraction Kit (Epigentek, NY, USA) and protein concentration was assessed using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Canada) according to the manufacturer’s instructions. Absorbance was determined on the Ultrospec 2100 Pro Spectrophotometer. 10-20 μg of protein was loaded per sample and run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), where the same amount was used within a set of samples being compared to one another. Separated proteins were transferred to nitrocellulose membrane overnight at 25V at 4°C. Membranes were cut at the 22kDa migration of molecular mass. The membrane segment with smaller proteins was probed for acetylated histone H3 at lysine residues 9 and 18 and those above 22kDa were probed for the housekeeping gene and loading control glyceraldehyde 3-phosphate dehydrogenase (GAPDH).
simultaneously in separate solutions. Membranes were incubated with either an anti-acetyl-histone H3 Lys9/18 rabbit antibody (1:2500 dilution; Millipore, CA, USA) or anti-GAPDH rabbit antibody (3:20 000 dilution; Sigma-Aldrich, MO, USA), overnight at 4°C followed by incubation with a goat anti-rabbit IgG HPR-conjugated secondary antibody (1:10 000 dilution; Millipore, CA, USA). Proteins were detected by enhanced chemiluminescence (GE Healthcare, UK) and developed on KODAK BioMax Xar film. Optical density (OD) values were calculated by ImageJ software (National Institutes of Health, 1997-2011). Values for histone H3 acetylation were normalized over GAPDH and expressed as a percentage of controls.

3.1.4 Statistical Analysis

All statistical analyses were performed using GraphPad Prism™ 5 software (GraphPad Software, San Diego, USA) unless otherwise stated. Statistical significance was acknowledged when the p-value was <0.05.

Comparisons of growth rate, weaning weights, amount of drinking water ingested, global methylation using OD values, and age at VO, between treatments, within a mode of administration were analyzed by one way ANOVA with Dunnett’s post-test comparing treatment groups to controls.

Comparisons of growth, weaning weights and global methylation using values expressed as percent of controls, between modes of administration within treatment and across treatments were analyzed by one way repeated measures mixed model ANOVA.

Ovarian weights but not uterine weights passed the D’Agostino-Pearson omnibus K2 test of normality. Therefore, differences in ovarian weights were assessed by the parametric one way ANOVA followed by Dunnett’s post-test comparing treatment groups to controls, and differences in uterine weights were assessed by the non-parametric Kruskal-Wallis test followed by Dunn’s post-test comparing treatment groups to controls. Ovarian weight for one biological replicated was calculated as the average of the left and right ovary of one mouse.

A one-sample t-test was performed to test for differences in levels of histone acetylation in samples treated with SAHA at 25mg/kg expressed as percent of vehicle control, and in untreated brain samples expressed as percent of acetylation in liver samples, testing if the value obtained was different from 100%.
Results obtained in the full scale experiment for mice administered water only or vehicle in water for measures of age at VO, ovarian and uterine weights, weights at weaning and growth rate were compared using Student’s t-test and determined to show no statistically significant differences in any of the measures tested. Results from the two groups of control mice were pooled and used as the single control group used in comparisons between treatment groups.
3.2 Results

3.2.1 Timing of Puberty

3.2.1.1 Age at Vaginal Opening

Age at VO was assessed as a marker of pubertal timing in three populations of mice treated with either SAHA at 25mg/kg, MET at 1g/kg or control. A cumulative distribution was plotted to show earlier and later timing of puberty as lines shifted to the left or to the right, respectively.

![Figure 3-6 Average age at vaginal opening advanced with pre-pubertal administration of L-methionine](image)

C57BL/6 female mice were administered agents in drinking water from postnatal day 21 to 39 and assessed for vaginal opening (VO) daily. A cumulative distribution of percent mice with VO is plotted against age (postnatal day, pnd) for each treatment group. Control mice administered vehicle or water attained VO on average on pnd 31.6 ± 2.2 (n=51 from 32 different litters); L-methionine (MET) at 1g/kg on pnd 30.5 ± 2.2 (n=38 from 26 different litters); suberoylanilide hydroxamic acid (SAHA) at 25g/kg on pnd 32.5 ±2.1 (n=37 from 25 different litters).

*\(p<0.05\)

Average age at VO was statistically significantly earlier in MET treated mice than controls (\(p<0.05\)). Therefore, the pre-pubertal administration of MET advanced the timing of puberty. Average age at VO was later in SAHA treated mice than controls, with SAHA treatment...
appearing to lead to a delay in pubertal timing, though the difference was not statistically significant (p=0.10).

### 3.2.1.2 Ovarian Weights

As another measure of pubertal timing in which to assess the effects of agent administration, differences in ovarian weights of mice at pnd32 were assessed between treatment groups and control.

![Ovarian Weights Graph](image)

**Figure 3-7 Ovarian weights lighter in mice treated pre-pubertally with suberoylanilide hydroxamic acid**

C57BL/6 female mice were administered agents in drinking water from postnatal day 21 to 32. At pnd32, mice were sacrificed, ovaries dissected and weighed. Average ovarian weight per group, expressed as the average weight of the left and right ovary ±sem, is plotted against treatment group. Ovaries of control mice administered vehicle or water weighed on average 2.0mg ± 0.08 (n=20 from 10 different litters); L-methionine (MET) at 1g/kg weighed 2.1mg ± 0.07 (n=22 from 16 different litters); suberoylanilide hydroxamic acid (SAHA) at 25g/kg weighed 1.6mg ± 0.1 (n=15 from 12 different litters). **p<0.01

Ovaries of SAHA treated mice were found to weigh statistically significantly less than those of control mice (p<0.01). This is consistent with the trend toward a delay in age at VO with SAHA administration seen in Figure 3-6.
3.2.1.3 Uterine Weights

As a third, unique measure of pubertal timing, uteri were weighed in each group of mice to determine if treatment had an effect on uterine growth.

![Figure 3-8: Uterine weights lighter in mice treated pre-pubertally with suberoylanilide hydroxamic acid](image)

C57BL/6 female mice were administered agents in drinking water from postnatal day 21 to 32. At pnd32, mice were sacrificed, uteri dissected and weighed. Average uterine weight per group is plotted against treatment group. Uteri of control mice administered vehicle or water weighed on average 17.1mg ± 3.01 (n=20 from 10 different litters); L-methionine (MET) at 1g/kg weighed 22.2mg ± 3.59 (n=22 from 16 different litters); suberoylanilide hydroxamic acid (SAHA) at 25g/kg weighed 11.4mg ± 1.7 (n=15 from 12 different litters). **p<0.01

SAHA treated mice had the lightest uteri of all three groups analyzed, statistically significantly different from control mice (p<0.01). These findings parallel the differences found in ovarian weights and age at VO. Uteri of MET treated mice also show a trend opposite to that of SAHA treated mice by being heavier than uteri of controls, a finding that, although not statistically significant (p=0.21), is in agreement with the VO data.

Data from each of the three measures of pubertal timing show agreement in trends and differences. One or more measures are statistically different from controls for each perturbation. As such, MET treatment advanced puberty and SAHA treatment delayed puberty.
3.2.2 Growth

In the methods of the full scale experiment, it was determined that average weight of the mice in each group was to be the same at the start of agent administration. Given that this was the case, it was then assessed if subsequent weight gain had been affected by agent administration.

Figure 3-9 Growth rate is slower in mice treated pre-pubertally with suberoylanilide hydroxamic acid

C57BL/6 female mice were administered agents in drinking water from postnatal day 21 to 39 and weighed daily. Weight gain, expressed as average rate in grams (g) per mouse per day for 18 days ±sem is plotted against treatment group. Control mice administered vehicle or water grew on average 0.72g/day ± 0.01 (n=51 from 32 different litter); L-methionine (MET) at 1g/kg grew 0.71g/day ± 0.012 (n=38 from 26 different litters); suberoylanilide hydroxamic acid (SAHA) at 25g/kg grew 0.66g/day ± 0.01 (n=37 from 25 different litters). *p<0.05

Growth rate of mice administered SAHA from pnd21 onward grew significantly slower than untreated mice (p<0.05). There was no difference between MET and control mice. Given the delay in pubertal timing seen in these mice, and the relationship between slower growth and later puberty seen in untreated mice as assessed in Chapter 2, this slowed growth may be expected.
3.2.3 Drug Action

Treatment of mice with the histone deacetylase inhibitor (HDACi) SAHA led to observable phenotypic changes in pubertal timing. Whether this treatment reflected global changes in histone acetylation was determined by measuring acetylated histone levels by immune-blot.

3.2.3.1 Global Histone Acetylation

Changes to histone acetylation with administration of SAHA at 25mg/kg orally through drinking water from pnd21-32 were assessed in both whole brain and liver extracts of treated mice compared to vehicle controls.

![Western blot images](image)

**Figure 3-10 Oral suberoylanilide hydroxamic acid administration alters histone H3 acetylation in liver**

Representative Western blots of protein levels of histone H3acetylated at lysine resides 9 and 18 and housekeeping protein GAPDH in the brain (A1) (n=7) or liver (B1) (n=5) of postnatal day 32 aged C57BL/6 female mice treated with vehicle control (lane 1) or suberoylanilide hydroxamic acid (SAHA) (lane2) from weaning on postnatal day 21 onward, through drinking water. A2 and B2 are the respective graphs representing the average protein levels calculated as ratios and expressed as percent of control.
No statistically significant difference was found between levels of histone acetylation in the brain of SAHA treated mice compared to controls, but there was a trend toward increased acetylation, particularly in the liver, (p= 0.0527) (Figure 3-10).

Figure 3-11 Histone H3 acetylation is higher in brain than in liver

A1) Representative Western blots of protein levels of histone H3 acetylated at lysine resides 9 and 18 and housekeeping protein GAPDH in the liver (lane 1) and brain (lane 2) (n=3) of postnatal day 32 aged C57BL/6 female mice administered hydroxypropyl-beta-cyclodextrin through drinking water from weaning on postnatal day 21 onward. A2) Graphical representation of the average protein levels calculated as ratios and expressed as percent of control. *p<0.05

Assessment of levels of acetylation between tissues revealed that at baseline with no treatment, histone H3 acetylation in the brain is greater than in the liver (p<0.05) (Figure 3-11).
3.3 Discussion

Testing of the over-arching hypothesis that epigenetic mechanisms are involved in regulation of pubertal timing was based on a perturbation strategy. If the phenotype exists due to some pattern of modifications on DNA or histones, then by perturbing those patterns, the phenotype will change, establishing a potential role for epigenetic control in regulating this phenotype. Therefore, it was hypothesized that if pubertal timing is under epigenetic control (at least in part), then altering the pattern of control will alter the timing of onset of puberty. Alteration was performed by agents known to have epigenetic modifying actions. It was found that the administration of such agents to pre-pubertal female B6 mice altered their timing of puberty (Figures 3-6 to 3-8). These results provide initial evidence in support of the hypothesis that an epigenetic mechanism contributes to the timing of the onset of puberty.

3.3.1 Optimization

It was necessary to develop an optimized method of administration to achieve the goals of the experiment. Methodological considerations included timing and duration of treatment, route and mode of administration, and the agents administered and their doses. Conditions chosen were as follows: administration beginning at weaning and continuing until all mice in a cage have reached puberty, sustained administration through drinking water in populations of over 35 mice per treatment group with littermate controls, administering epigenetic modifying agents SAHA (HDACi) at 25mg/kg and MET (methyl-donor) at 1g/kg, assessing changes to pubertal timing via assessment of differences in age at VO and weights of ovaries and uteri at pnd32 between treatment groups.

To arrive at these choices, options were tested in optimization rounds I and II. The options chosen to be tested were determined by observations from earlier studies. These are discussed in the sections that follow.

3.3.1.1 Comparison

Studying alterations in epigenetics for pubertal onset requires a different paradigm to studying alterations to an ongoing behaviour like stress response (Weaver et al., 2005) or schizophrenia models (Dong et al., 2005), where in the latter cases of ongoing behaviour, the same mouse can be its own control for assessing effects of epigenetic modifying agents on the phenotype. The
timing of puberty for an individual mouse is unknown until it occurs. Although there are certain predictors such as weight and growth, the exact time cannot be determined. For this reason, in studying alterations to pubertal timing, the measure is the average age of a population comparing the perturbed population with the unperturbed control population.

3.3.1.2 Timing of Administration

Administration must begin at a pre-pubertal age to affect the phenotype before it occurs. Options included in utero, at birth, or at weaning. Each of these stages of life are viewed as “plastic” during which the developing brain is susceptible to changes in response to exogenous and endogenous environmental exposures, including susceptibility to epigenetic modification (Kinsey-Jones et al., 2010; Lillycrop et al., 2005; Kotsopoulos et al., 2008). In this respect, each could be considered. The start of administration was chosen to be at weaning to incorporate the use of littermate controls. Although variation in timing of puberty is seen between littermates, the average timing of puberty between litters can also differ. To avoid this confounding effect, it is possible at weaning to distribute littermates to different cages designated for different treatments.

3.3.1.3 Duration of Administration

The decision to begin administration at weaning limits the duration of treatment before onset of puberty. It was thus important to have chosen a strain, such as the B6 mice, that do not tend to attain puberty close to the day of weaning on pnd 21, in contrast to the SJL/J strain that can open at pnd 20 as discussed in Chapter 2. Using B6 mice, administration can occur for up to 10.7 days before the average mouse reaches puberty. There is evidence that transient changes to epigenetic marks like histone acetylation can occur immediately within an hour or two after administration of epigenetic modifying agents, and that changes to an observable phenotype can result after a few days of treatment (Hockly et al., 2003; Avila et al., 2007; Kilgore et al., 2010).

3.3.1.4 Choice of Agents

Agents that can modify epigenetic marks can be grouped into two categories: ones that target epigenetic machinery such as histone deacetylase complexes (HDACs) or DNA methyltransferases (DNMTs), or ones that supply substrates such as methyl groups. Two agents were chosen for this experiment, each targeting one of these categories: suberoylanilide
hydroxamic acid (SAHA) and L-methionine (MET), respectively. SAHA, also known as Vorinostat, is a histone deacetylase inhibitor that blocks substrate access by binding directly to the catalytic site of the enzyme (Marks and Breslow, 2007). With this action there is a decrease in histone deacetylation, such that acetylation levels will rise after SAHA treatment due to the decrease in active HDACs and continuing activities of the opposite acting enzymes, the histone acetyl transferases (HATs) that add acetyl groups to histones (Racey and Byvoet, 1971). L-methionine is the precursor to methyl-donor s-adenosyl methionine (SAM) (Chiang et al., 1996). By altering the availability and supply of methyl-groups to be used by DNMTs for maintenance or de novo methylation, DNA methylation is altered (Chiang et al., 1996; Cooney, 1993). Diets supplemented with or deficient in methionine and other precursor methyl-donors like folate have been documented to impart their effects on various phenotypes via epigenetic mechanisms by increasing or decreasing global levels of DNA methylation, respectively, or by changing DNA methylation patterns at specific genes, or by altering the methylation status of histone H3 and H4 lysine residues (Pogribny et al., 2008; Dong et al., 2005; Lillycrop et al., 2005; Kotsopoulos et al., 2008; Cooney et al., 2002). In following these examples, it was expected that administration of MET would increase global levels of DNA methylation.

With fundamentally different mechanisms of action, it was expected that the selected agents would alter patterns of epigenetic marks on genes relevant to pubertal regulation, and that they would likely affect different combinations of genes and result in different phenotypes.

3.3.1.5 Route and Mode of Administration

Important considerations for choosing route and mode of administration include level of invasiveness, which may dictate level of stress and pain associated with the administration technique, duration of agent exposure – bolus or sustained – and ability of the agents to reach the site of central control of puberty, the brain, via a particular route. The chosen route and mode of administration for this study was oral, via drinking water.

Implantation of intracerebroventricular (ICV) cannulae was considered as it may have more accurately administered the agents to the desired central location without passing through the periphery (Kinsey-Jones et al., 2010; Weaver et al., 2005). However, a significant rise in corticosterone has been observed in this procedure in mice (Kim et al., 1998). As determined and
discussed in the optimization rounds, any procedure may elicits a stress response may affect the pubertal phenotype and also methylation levels.

Oral administration of agents through drinking water had multiple advantages. It allowed experimenter handling during administration to be eliminated; allowed for sustained exposure to the epigenetic modifying agents, which has been noted to lead to epigenetic changes (Kelly et al., 2005); and, although oral administration requires passage through the periphery, both SAHA and MET are known to reach and exert effects in the brain when taken orally (Hockly et al., 2003; Faraco et al., 2006; Palmieri et al., 2009; Hendricks et al., 2011; McGowan et al., 2009).

3.3.2 Agent Administration Alters Timing of Puberty: Epigenetic Mechanism Implicated

Three complementary measures of pubertal timing showed that actions exerted by a known histone deacetylase inhibitor and a precursor to the donor of methyl groups for DNA methylation are successful in altering the timing of the onset of puberty in female mice, and do so in opposing directions. Likely, these epigenetic modifying agents altered the DNA methylation and/or histone acetylation patterns at loci that play a role in the regulation of puberty, altering their regulatory effects by changing levels of gene expression. Likely also, that they, SAHA and MET, had different mechanisms of action as their effects on the phenotype were of opposite nature. These data provide strong evidence that epigenetic mechanisms regulate pubertal timing.

The actions of epigenetic modifying agents, including SAHA and MET, on gene expression, however, do not confer a single gene resolution that knock-out or over-expressing transgenic mouse models confer. In the latter models, a single gene is typically altered, and it can be altered within a single tissue, at a specific point in time for a duration determined by the experimenter. No technique exists that allows for perturbation of epigenetic marks in a similarly specific manner. The level of resolution of epigenetic perturbation by modifying agents occurs on a global scale. Though SAHA inhibition is specific to class I and II HDACs, this includes HDAC 1 through 9, which have varying tissue distribution and varying gene targets (de Ruijter et al., 2003; Dokmanovic et al., 2007). Whole genome scale investigation of MET supplementation was performed and found it affects numerous genes across a wide variety of categories, the most pronounced affects being in genes involved in gene expression and transcription, organogenesis, and cellular development (Li et al., 2011). The puberty regulating genes whose epigenetic
alterations may have led to the phenotypic alterations observed in this study are likely among the genes to be affected by SAHA or MET, but remain unknown.

The complex nature of epigenetic patterns further complicates interpretation of results with epigenetic modifying agents. Epigenetic patterns are dynamic and can change over time and as a result of environmental exposures or with disease state. They can be tissue or cell specific and have varying susceptibility to alterations at varying developmental stages. For example, when fed a folate/methyl-deficient diet, DNA in rat liver was significantly hypomethylated while DNA methylation levels in the brain were globally increased (Pogribny et al., 2008; Pogribny et al., 2006). The pre-pubertal period is one during which DNA is highly susceptible to epigenetic changes (Burdge and Lillycrop, 2010a). SAHA and MET were purposefully administered during this period to direct epigenetic modifications during this critical time.

As discussed subsequently, lines of evidence that implicate epigenetic alterations to genes in the regulation of timing of puberty seen with the administration of MET and SAHA are that 1) potential alternative actions are unable to fully explain the results, 2) assessment of their molecular actions point to global or specific epigenetic alterations, and 3) they have opposite effects on the phenotype.

3.3.2.1 Potential Alternative Agent Actions Unlikely

3.3.2.1.1 Toxicity

It is acknowledged that the delay in pubertal timing accompanied by the slight reduction in growth rate with SAHA administration could be the result of a drug imparting a toxic effect. However, in deciding on use of SAHA and MET, behavioural and growth rate consequences were investigated given that adverse effects on health could in turn adversely affect pubertal development. It was concluded that SAHA and MET in the doses administered are unlikely to affect the health of the mice, and thus it was unlikely that the timing of puberty was altered by toxicity.

L-methionine, is an essential amino acid in the diet, required for maintaining proper balance of DNA methylation, and is crucial for normal brain development (Van den Veyver, 2002). Restricting the amount of MET in the diet would thus, expectedly, lead to dysfunctional development and subsequent poor health. Many studies have shown that diets deficient in
protein, including MET, can lead to symptoms related to cardio-metabolic disease including hypertension, impaired lipid and glucose homeostasis, and vascular dysfunction, among others (Reviewed by Burdge & Lillycrop, 2010). On a molecular level, methyl-group deficiency is associated with increased chromosomal aberrations and DNA single and double strand breaks (Duthie et al., 2000).

Conversely, supplementation of MET is viewed as conferring positive health effects. The widespread recommendations made by the Center for Disease Control and Prevention in 1992 to increase consumption of Vitamin B₉ or folic acid, which is converted to MET, during pregnancy are followed even if levels in the diet are normal, and thus levels are allowed to rise above baseline without any assumed risk (McNulty et al., 2011). Supporting this recent management plan, studies (including Amaral et al. 2011) find no evidence of genotoxicity or changes to methylation of tumour suppressor genes like p53 with supplementation of MET.

Given the high likelihood of serious consequences with MET deficiency and low likelihood of toxicity with MET supplementation it is unlikely that the advancement of puberty with MET administration is a result of a toxic effect. Indeed, no apparent ill effects were observed with the 1g/kg dose of MET administered. In a similar respect, green tea polyphenols with their low toxicity profile would have been a suitable option had not data appeared showing that these agents are inconsistent in their action on DNA methylation in vitro and in vivo (Morey Kinney et al., 2009; Pandey et al., 2010; Lee et al., 2005). Targeted DNMT inhibitors that effectively decrease DNA methylation could also have been selected, but these agents have high toxicity profiles and so, were not considered (Christman, 2002).

The potential toxicity of SAHA at the doses used would be minimal. SAHA has been approved for use in the treatment of cutaneous T-cell lymphoma by the US Food and Drug Administration (Mann et al., 2007; Duvic and Vu, 2007), and numerous studies have explored its therapeutic actions in other diseases. Mice modeling these diseases that were treated with SAHA at doses lower than 100mg/kg showed no signs of health abnormalities, such as hunched posture, disheveled coat, inactivity or hyperactivity, and difficulty in ambulation, emaciation, respiratory problems, solid tumour growth, and sudden death frequency (Hockly et al., 2003; Avila et al., 2007; Mann et al., 2007; Riessland et al., 2010). In these disease models, rather, SAHA has been observed to improve health by e.g. reducing occurrence of cardiac arrhythmias (Colussi et al.,
2010), improving motor deficits in a Huntington’s disease model (Hockly et al., 2003), reversing memory deficits in an Alzheimer’s disease model (Kilgore et al., 2010), and counteracting weight loss in spinal muscular atrophy (Riessland et al., 2010). In the work related to this thesis, a dose lower than 100mg/kg was utilized and no concerning side effects were observed.

The possible toxicity of SAHA administration was further reduced by the vehicle chosen for solubilization in water, obviating the requirement for dimethyl sulfoxide (DMSO) as the vehicle. DMSO is not well tolerated by rodents at high concentrations or at low concentrations for long durations (Hockly et al., 2003). Hockly et al. (2003) found DMSO can be effectively substituted with 2-hydroxypropyl-beta-cyclodextrin (HP-βCD), which is much less toxic (Gould and Scott, 2005; Hamid et al., 2009). Cyclodextrins, with their outward facing hydrophilic surface, impart aqueous solubility by complexing with hydrophobic molecules with their inward facing hydrophobic surfaces, and, consequently also reduce drug toxicity and irritation at the site of administration (Uekama et al., 1998). Importantly as well, they can mask unpleasant taste. In the administration of SAHA via drinking water, this may have be a contributing reason as to why the mice studied in optimization rounds I and II were not deterred from drinking as much SAHA treated water as untreated water (Figure 3-3 and 3-5). In this way, toxicity through possible dehydration was prevented.

3.3.2.1.2 SAHA and Estrogen

In vitro studies in breast cancer cells point to an apparent anti-estrogen action of SAHA, where SAHA treatment associates with the down regulation of ERα expression and its phosphorylation (De los Santos et al., 2007; Fiskus et al., 2007). The effects of SAHA treatment seen in vivo delaying timing of puberty cannot be explained solely by inhibitory effects on estrogen. SAHA treatment altered all three markers of puberty in female mice that were assessed, in a direction associated with delayed puberty, but not all three markers are dependent on estrogen. Vaginal opening does rely on estrogen. Ovaries grow in response to levels of luteinizing and follicle stimulating hormones secreted by the pituitary gland (Styne et al., 2011; McGee et al., 1997), and uterine weight is dependent not only on estrogen but also progesterone, growth hormone, and insulin growth factor1 (Styne et al., 2011; Murphy and Ghahary, 1990; Hull and Harvey, 2001; Wada-Hiraike et al., 2006). Thus, only VO relies solely on E2 and it was not the only marker of puberty affected.
3.3.2.2 Evidence of Epigenetic Actions

The molecular action of SAHA on histone acetylation was verified by comparing levels of acetylated histones between treatment and control groups. A global approach was taken.

3.3.2.2.1 Global Histone Acetylation

A trend towards increased global histone acetylation was observed in the liver of animals treated with SAHA at 25mg/kg for 11 days starting at weaning (Figure 3-10 B1-B2). This establishes that SAHA is exerting actions as an HDAC inhibitor when administered to mice. Importantly, the same 1) dose of SAHA, 2) timing of administration, and 3) mode of administration led to a significant delay in pubertal timing.

Although no global changes to histone acetylation were observed in the brains of the mice treated with SAHA (Figure 3-10 A1-A2), the absence of global changes in acetylation does not discount specific HDAC inhibition and subsequent increased acetylation as the mechanism of its action in the brain. It is possible that patterns of histone acetylation changed at a few but key genes that were undetected by the global assay.

Another explanation linked to isolated changes at key genes going undetected could be that changes were specific to particular hypothalamic nuclei such as the ARC and pre-optic area that house GnRH neurons (Zimmerman and Antunes, 1976). Having used whole brain samples, these changes could have been masked.

Also, the difference in untreated, baseline levels of histone acetylation in liver and brain (Figure 3-11 A1-A2) suggests that the hyperacetylation seen in the brain compared to the liver may be at a threshold where changes with perturbation are not detectable. This does not preclude the possibility that changes of small magnitude or isolated changes occurred and that they have functional significance.

When Hockly et al. (2003) assessed the action of SAHA as an HDACi in mice, they established its actions by proof of principle. They tested changes to histone acetylation in the spleen 2hours after intraperitoneal injection of a 200mg/kg dose of SAHA and found a significant increase in histone acetylation between treatment and control groups. For their study, they administered a 100mg/kg dose of SAHA via drinking water and saw differential phenotypic outcomes.
associated with the brain. Similarly in this study, the apparent increase in acetylation in the liver indicates, by proof of principle, that SAHA is exerting epigenetic modifying actions.

3.3.2.3 Opposite Actions

It was hypothesized that due to the different categories of epigenetic modifying agents, that MET and SAHA would alter pubertal timing in different ways at the molecular level and translate to different phenotypic outcomes. Indeed, administration of SAHA and MET had different effects on pubertal timing, with SAHA delaying timing and MET advancing it.

SAHA is a known HDAC inhibitor that has been show in vitro and in vivo to increase histone acetylation globally or at specific sites (Hockly et al., 2003; Kilgore et al., 2010; Kelly et al., 2005; Palmieri et al., 2009). This study also provides evidence that SAHA exerts its actions as an HADCi increasing histone global acetylation. Inhibition of HDACs by agents such as SAHA is not limited to changes to histone acetylation; it extends to DNA methylation. The relationship between histone acetylation and DNA methylation is reciprocal. On one hand, when cytosines in gene promoter regions are methylated, they interact with histone modifying enzymes such as HDACs to deacetylate the histones in that region, in effect converting the DNA to heterochromatin (Jones et al., 1998). This interaction is mediated by the recruitment of methyl CpG binding proteins (Jones et al., 1998). On the other hand, euchromatin structure can be coordinated by hyperacetylation leading to decreased levels of DNA methylation (Weaver et al., 2004; Cervoni and Szyf, 2001; Milutinovic et al., 2007).

Increasing the supply of methionine for its conversion to methyl donor S-adenosyl methionine (SAM) is expected to increase the amount of SAM mediated DNA methylation reactions and in consequence increase levels of DNA methylation (Chiang et al., 1996). SAM, after donation of its methyl group to cytosine-guanine dinucleotides, is converted to S-adenosylhomocysteine (SAH) (Chiang et al., 1996) (Figure 3-12). The ratio between SAM and SAH is considered to be an index of DNA methylation, where ratios above 1, and thus greater levels of SAM, lead to hypermethylation. Conversely, ratios below 1 and thus greater levels of SAH lead to hypomethylation (Waterland, 2006) because SAH is a potent inhibitor of SAM-directed DNMT1 activity (Deguchi and Barchas, 1971). Studies that administer MET in excess to rodents consistently report global and/or specific increases in DNA methylation (Weaver et al., 2005; Dong et al., 2005; Tremolizzo et al., 2002; Cooney et al., 2002; Detich et al., 2003).
Figure 3-12 S-adenosyl-methionine cycle

Arrows indicate direction of reaction. Arrows on both ends indicate bi-directional reactions. Factors surrounding main circular pathway interact with cycle and drive the reactions. Methioine (MET) derived from protein is converted to S-adenosyl methionine (SAM) by co-enzyme adenosine triphosphate (ATP) that releases phosphate (Pi) and pyrophosphate (PPi). DNA methyltransferase 1 (DNMT1) uses the methyl group of SAM to methylate cytosines of DNA, converting it to S-adenosylhomocysteine (SAH). Addition of a methyl group to SAH converts it back to SAM. Image modified from Chiang et al. (1996).

Weaver et al. (2005) show that HDACi vs MET supplementation can have opposite actions on DNA methylation of a single gene that leads to opposite phenotypic outcomes. In the presence of class I and II HDAC inhibitor Trichostatin A, a hydroxamic acid compound very similar to SAHA, the promoter region of the glucocorticoid receptor (GR) gene in rats was significantly demethylated. These rats were significantly less fearful in situations of high stress. In the presence of MET, the methylation status in this same region of the GR gene was reverted back to a high degree of methylation, and rats responded more to stress.

It is therefore possible that administration of SAHA and MET to pre-pubertal mice affected the same genes regulating the timing of puberty but in different ways leading to opposite outcomes. Future investigations could center on determining which activators or repressors of puberty are affected in this perturbation, and if they are silenced or activated by the specific DNA
methylation and histone acetylation patterns induced by the perturbation (See Chapter 5 for broadened discussion).

Results of this experiment provide compelling evidence in support of the involvement of an epigenetic mechanism mediated by differential DNA methylation and/or histone acetylation in regulating the timing of puberty.
Chapter 4

Having established in aim 2 that epigenetic mechanisms are likely contributing to the regulation of pubertal timing, aim 3 sought to provide further evidence that epigenetic mechanisms may underlie some of the population variation in pubertal timing. Given that timing of puberty was changed by agents that affect epigenetic marks, it is now hypothesized that naturally occurring variation in timing of pubertal onset among inbred mice occurs (at least in part) from variation in the epigenome. Therefore, differences in epigenetic marks, specifically DNA methylation in the hypothalamus, of genes and genome wide patterns between early and late puberty mice were investigated.

4  Aim 3 – Investigation of Methylation Differences between Early and Late Puberty Mice

4.1  Methods

In brief, genome wide discovery of DNA methylation differences in hypothalami of female mice with early vs. late onset of puberty was performed via methylated DNA immunoprecipitation (meDIP) hybridization to a CpG island microarray followed by validation via meDIP quantitative real time polymerase chain reaction (qPCR), bisulfite modification and pyrosequencing and/or matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF).

4.1.1  Sample collection

4.1.1.1  Groups

Early and late onset of puberty mice were designated by their timing of vaginal opening. Krewson et al. (2004) propose that although age at VO is not the only marker used to determine the status of activation of the HPG axis, it is the best one to use when investigating the genes that regulate the onset of puberty in a large number of mice. Indeed, chromosomes harboring quantitative trait loci relevant to the regulation of puberty were found via assessment of a panel of chromosome substitution strains grouped by age at VO (Krewson et al., 2004; Nathan et al., 2006a). The method used for phenotyping VO was described in Chapter 2 along with the general
animal care and husbandry that was also followed in this experiment. The cut-offs for ages at VO that were used to group mice into the early or late category were determined in Figure 2-4 from the distribution of age at VO in a population of 298 female B6 mice. Mice with VO at or earlier than pnd 28 (one standard deviation below the mean) were grouped into the early category and mice with VO at or later than pnd 35 (one standard deviation beyond the mean) were grouped into the late category.

Acknowledging the presence of two confounders, namely length of estrogen exposure and age, a four group comparison was made (see Table 4-1 and Figure 4-1). DNA methylation patterns can be affected by differing levels of hormones such as estrogen (Schwarz et al., 2010) and have been shown to change with age (Wilson et al., 1987; Teschendorff et al., 2010). By obtaining mice for comparison on their day of vaginal opening, early and late mice would have had the same degree of exposure to the increased levels of estrogen that accompany puberty, but they will be different ages. Early mice would be around pnd 28 and late mice at least 7 days older. By obtaining mice at the same age, an age by which all mice would have attained VO (pnd 39), age is no longer a confounder but the two groups of mice would have different exposures to estrogen. Early mice would have been exposed to a post-pubertal hormonal milieu for at least 11 days, and late mice 4 days or less. In making both comparisons, the effects of differential estrogen exposure and differences in age would be controlled. As such, early and late mice were collected on their day of vaginal opening or on pnd 39 and grouped by these criteria into the 4 groups outlined in Table 4-1. Samples from eight mice were collected per group.

Table 4-1 Grouping of mice by timing of puberty and time of sacrifice

<table>
<thead>
<tr>
<th>Group</th>
<th>Timing of Puberty</th>
<th>Time of Sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early (age at VO≤pnd28)</td>
<td>Late (age at VO≥pnd35)</td>
</tr>
<tr>
<td>1</td>
<td>EVO</td>
<td>X</td>
</tr>
<tr>
<td>2</td>
<td>LVO</td>
<td>X</td>
</tr>
<tr>
<td>3</td>
<td>E39</td>
<td>X</td>
</tr>
<tr>
<td>4</td>
<td>L39</td>
<td>X</td>
</tr>
</tbody>
</table>

VO, vaginal opening; pnd, postnatal day
Differences in DNA methylation were identified between early and late mice of groups 1 and 2 (both sacrificed at VO) and between early and late mice of groups 3 and 4 (both sacrificed at pnd 39). Of the differences between early and late mice in the two comparisons, those that are shared by both groups of early mice (Group 1 and 3), and those that are shared by both groups of late mice (Group 2 and 4) can be attributed to early and late puberty, respectively, independent from age or estrogen exposure (Figure 4-1).

Figure 4-1 Schematic depiction of comparison of early and late puberty mice

Venn diagram illustrating four group comparison (1-4) used to identify methylation patterns unique to early (blue) and late (orange) onset of puberty mice. Compare to detect differences in DNA methylation between group 1, early puberty mice sacrificed at vaginal opening (EVO) to group 2, late puberty mice sacrificed at vaginal opening (LVO), independent of effects of estrogen exposure; differences between group 3, early puberty mice sacrificed at postnatal day 39 (E39) to group 4, late puberty mouse sacrificed at postnatal day 39 (L39), independent of effects of age; similarities between groups 1 and 3, and similarities between groups 2 and 4.

4.1.1.2 Hypothalamic Dissection

Mice were euthanized by CO₂ followed by rapid decapitation. Whole brain was removed within 2 minutes of sacrifice. To maintain structural integrity during removal, skull was cut on the left and right sides and lifted to reveal the dorsal surface of the brain. The olfactory bulbs were cut and brain lifted from the rostral end by making successive cuts to the cranial nerves starting with the optic nerves.

The whole brain was then flash frozen. To maintain structural integrity during freezing, the brain is placed ventral side up on a flat surface of a coin wrapped in aluminum foil. This platform made from the coin is held at the surface of liquid nitrogen (LN₂) so that freezing occurs without
the tissue contacting the LN$_2$. The tissue was then placed in a tube with diameter larger than the brain and transported on dry ice for storage at -80°C.

To dissect the hypothalamus, the frozen whole brain is placed ventral side up using chilled razor blades on a chilled glass plate sitting atop dry ice. It is cut at stereotaxic coordinates -0.94mm to bregma and -2.80 mm to bregma, which correspond to a cut through the cadual optic chiasm (OC) and immediately posterior to the mammillary bodies (MB), according to Paxinos (2004) mouse brain atlas and following an accepted protocol (Plagemann et al., 2009a; Anghel et al., 2010; Sasaki et al., 2010; Vucetic et al., 2010).

![Figure 4-2 Cuts made through mouse brain to dissect hypothalamus](image)

Depiction of mouse brain ventral side up. Red dotted line represents location of razor cuts, according to coordinates in relation to bregma. OB, olfactory bulb; OC, optic chiasm; OT, optic tract; MB, mammillary body. Image modified from Baker et al. (1983).

This 2mm thick coronal section was then placed flat and cut sagitally at equidistance from midpoint and horizontally above the ventral side to obtain a specimen 3mm wide and 3mm high weighing approximately 20mg. By dissecting the hypothalamus in this way, the sample contains both hemispheres (to account for any hemispheric differences) and all hypothalamic nuclei.
4.1.2 Sample preparation for array hybridization

4.1.2.1 DNA isolation

The dissected hypothalamic region was immediately placed in 600μL of RLT Plus lysis buffer supplied by the Qiagen AllPrep DNA/RNA kit, which contains guanidine-isothiocyanate that inactivates DNases and RNases. To disrupt and homogenize the tissue, it was sonicated using SONICS Vibra Cell High Intensity Ultrasonic Processor, model VCX400, with a 33 mm stepped microtip (SONICS 630-0422) at 38% amplitude of two pulses of 5 seconds each with a 5 second pause in between. Samples were submerged in an ice bath to prevent heating during sonication. Following the manufacturer’s protocol, DNA was purified and subsequently eluted in 100μL of 10 mM Tris-Cl at pH 8.5. The 20mg starting material yielded 10-12 μg of DNA.

4.1.2.2 DNA methylation enrichment via meDIP

To prepare DNA for meDIP, 7μg of DNA was fragmented by sonication at 38% amplitude 7x pulses of 5 seconds each with a 5 second pause in between each pulse. Samples were kept on an ice bath to prevent heating during sonication. 200ng of DNA were visualized on 1.5% agarose gel electrophoresis with ethidium bromide staining. As a result of sonication, genomic DNA was fragmented to 1kb-100bp fragments (Figure 4-3), indicating successful fragmentation.

![Image of DNA fragmentation](image.png)

Figure 4-3 DNA of hypothalamic samples fragmented after sonication

Visualization of DNA on ethidium bromide stained 1.5% agarose gel. Lanes from left to right: in first and last two lanes, DNA ladders; in middle 10 lanes, representative samples of hypothalamic genomic DNA of early or late mice.
meDIP was performed as described, following a modified protocol (Mohn et al., 2009; Sorensen et al., 2009). 2μg of fragmented DNA of each sample was immunoprecipitated. The bead-antibody complex for immunoprecipitation was prepared by incubating 5μg of 5-methylcytidine monoclonal mouse IgG1 antibody (EUROGENTEC, #BI-MECY-0100) with Dynabeads® M-280 sheep anti-mouse IgG (Invitrogen, Dynal Biotech) per sample in a final volume of 200 μL IP buffer (10 mmol/L NaH₂PO₄, pH 7.0, 140 mmol/L NaCl, 0.05% Triton X-100) + 0.05% BSA at 4°C for 6hrs with rotation. DNA was denatured at 95°C for 10 min and added to the bead-antibody freshly re-suspended in 100μL IP buffer and incubated overnight at 4°C with rotation. Controls included a no antibody control containing beads and DNA pooled from all samples, as well as a no DNA control containing beads-antibody complex only. The next day, complexes with captured methylated DNA were washed 3 times with IP buffer to remove unbound fragments of DNA. The remaining fragments were treated with Proteinase K at 55°C for 2 hours with shaking to unbind the fragments, followed by purification with QIAquick PCR purification columns, and phenol-chloroform extraction and isopropanol precipitation. Methyl enriched DNA was re-suspended in 10μL Tris (10mM pH 8), and measured on a nanodrop spectrophotometer yielding approximately 100-200ng per immunoprecipitated sample.

Given that at least 1μg of DNA is required for array hybridization, DNA was whole genome amplified (WGA) using the GenomePlex® Complete WGA kit (Sigma, #WGA2) according to manufacturer’s protocol using 10ng of captured meDIP DNA or 10ng of unmethylated DNA (input). Following amplification, DNA was purified with QIAquick PCR purification columns for use in downstream applications.

4.1.2.3 Quality Control of DNA Enrichment

To assess the specificity of the meDIP assay, enrichment of two genes of known methylation status was measured by qPCR in WGA meDIP DNA compared to WGA input DNA. These were (1) the imprinted, and thus hemi-methylated, control region of gene H19 and (2) the housekeeping, unmethylated gene Gapdh. The primers used for PCR are listed in Table 4-2.

Primers were constructed using PrimerQuest online software (Integrated DNA Technologies, http://www.idtdna.com/Scitools/Applications/Primerquest/) using the sequence determined by the University of California Santa Cruz (UCSC) Mus musculus mm9 genome assembly (July 2007 NCBI build 37).
Table 4-2 *H19* and *Gapdh* primers used in meDIP qPCR testing efficiency of methylated DNA enrichment with meDIP

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Chr</th>
<th>Target Start</th>
<th>Target End</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H19</em></td>
<td>F 5'-TGG CCC GGT GGC AGC AAA AT-3'</td>
<td>7</td>
<td>149767562</td>
<td>149767762</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>R 5'-TGC AGA GAG TAA GCC GAC CTT GT-3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Gapdh</em></td>
<td>F 5'-GCG TGG GGT TGT TCC TAA TA-3'</td>
<td>6</td>
<td>125115053</td>
<td>125115228</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td>R 5'-CTG CAG TAC TGT GGG GAG GT-3'</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Mus musculus* mm9 July 2007 NCBI build 37; chr, chromosome; bp, basepairs; F, forward; R, reverse

A mixture containing 25μL of SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 5μL of 50ng of template, 900nM forward primer of *H19* and 900nM reverse primer of *H19* or 300nM forward primer of *Gapdh* and 900nM reverse primer of *Gapdh*, and water up to 50μL was assayed in duplicate on a 96-well plate.

The following conditions were used for qPCR reaction: An initial 10 minute denaturation step at 95°C was performed, followed by 40 cycles of denaturation, annealing and extension at 95°C for 15 seconds and 60°C for 1 minute, and ending with dissociation curve construction by measuring fluorescence at 95°C for 1 minute, 60°C for 30 seconds and 95°C for 30 seconds.

Dissociation curves for both primer sets showed specific amplification of the template, indicated by the single peak at 89°C and 85°C for *Gapdh* and *H19*, respectively, and absence of peaks in the no template controls (NTC) in four representative samples (Figure 4-4 A-B).
Figure 4-4 Specific amplification of Gapdh and H19

Quantitative real-time PCR dissociation curves plotting fluorescence against temperature for A) Gapdh and B) H19 of the methyl enriched (meDIP) fraction and input fraction for four representative samples, one from each group of early or late puberty mice sacrificed either at vaginal opening or on postnatal day 39. Single peak observed at 89 degrees Celsius (A) and single peak observed at 85 degrees Celsius (B). NTC, no template control.

All samples used on the array were assayed. Resulting threshold cycle (C_T) values of WGA meDIP DNA were subtracted from C_T values of WGA input DNA and averaged across all samples. High C_T values are indicative of a low level of initial template as more cycles are required to amplify enough copies of the gene to generate fluorescence above the threshold.
Given this, the negative difference observed between input and meDIP is indicative of little or no retention of fragments containing Gapdh during immunoprecipitation with 5-methylcytosine antibody (Figure 4-5). This is consistent with the expected hypomethylation or absence of methylation at Gapdh. Likewise consistent with the hemi-methylated status of H19 is the smaller CT difference observed between H19 in input and meDIP fractions indicating that there was retention of H19 fragments during immunoprecipitation (Figure 4-4). Also, in any given sample, Gapdh was always below the level of H19 as indicated by greater CT differences between input and meDIP fractions.

![Figure 4-5 Expected enrichment of DNA methylation via meDIP confirmed in H19 and Gapdh](image)

Average cycle threshold (CT) differences between input and methylated DNA immunoprecipitated (meDIP) fractions measured in hemi-methylated H19 and hypomethylated Gapdh by quantitative real-time PCR (n=14).

Differences in starting template of Gapdh between meDIP and input fractions is also apparent in the representative amplification plot shown in Figure 4-6, where the meDIP and input fractions of four samples were assayed. The first four lines that rise about the horizontal threshold level represent the amplification of Gapdh in the input fractions. The remaining four lines that rise much later after many more amplification cycles correspond to the amplification of Gapdh in the meDIP fractions.
Figure 4-6 Amplification differences between Gapdh meDIP and input fractions

Quantitative real-time PCR amplification plots of fluorescence against cycle number for Gapdh of the methyl enriched (meDIP) fraction and input fraction for four representative samples, one from each group of early or late puberty mice sacrificed either at vaginal opening or on postnatal day 39. NTC, no template control.

Corresponding to the small difference in starting template between meDIP and input for H19, its amplification plot shows lines rising above the threshold level at similar amplification cycles (Figure 4-7).

Figure 4-7 Amplification similarities between H19 meDIP and input fractions

Quantitative real-time PCR amplification plots of fluorescence against cycle number for H19 of the methyl enriched (meDIP) fraction and input fraction for four representative samples, one from each group of early or late puberty mice sacrificed either at vaginal opening or on postnatal day 39. NTC, no template control.
These results support that meDIP successfully enriched for DNA methylation. As such, samples passed the quality control for this stage of processing and were approved for assay on the microarray.

4.1.2.4 Array hybridization

Processing, labeling, hybridization and intensity acquisition were performed by the Microarray Center of the University Health Network (Toronto, ON, Canada) using standard protocols for the Agilent Mouse CpG Island 2x105K array (Agilent Technologies). The array covers 16,030 mouse CpG islands represented on 97,652 probes in or within 95bp of the CpG islands. It is sourced from the UCSC Mus musculus mm9 genome assembly (July 2007 NCBI build 37).

Briefly, 1μg of WGA meDIP was labeled with Cyanine 5 (red) and 1μg of WGA input labeled with Cyanine 3 (green) and co-hybridized to the CpGi microarray. Slides were washed and then scanned using the G2565C DNA scanner, and analyzed with Agilent Feature Extraction software (v10.7). Feature extraction software located the spots of colour, called features, representing the binding of the labelled DNA, and placed a grid (as can be seen in Figure 4-9) measuring 528 rows by 199 columns to match the corresponding signal intensity to the probe sequence. Next, linear dye normalization was applied to eliminate the bias towards lower signal intensity in the red channel. Outliers were then flagged, correction for background levels computed and Agilent Processed values for each probe were generated.

Given that the array consists of 2 individual arrays on 1 slide, an early sample was paired with a late sample in the same category of “time at sacrifice” for hybridization on each slide in all cases except on one slide where two early samples from different categories were paired (Table 4-3).
Table 4-3 Samples used in array analysis and their pairing on array slides

12 samples assayed on 6 slides (rows), paired as indicated by X under groups arranged in columns. E, early puberty mice; L, late puberty mice; VO, sacrificed at vaginal opening; 39, sacrificed at postnatal day 39.

<table>
<thead>
<tr>
<th>Slide barcode</th>
<th>EVO</th>
<th>LVO</th>
<th>E39</th>
<th>L39</th>
</tr>
</thead>
<tbody>
<tr>
<td>251527910886</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>251527910887</td>
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<td></td>
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<td>251527910888</td>
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<td>X</td>
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<td></td>
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<tr>
<td>251527910889</td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
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<tr>
<td>251527910890</td>
<td>X</td>
<td></td>
<td></td>
<td>X</td>
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<td>251527910891</td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

4.1.2.5 Pilot Samples Quality Control Metrics

Before all samples in Table 4-3 were hybridized, assay performance was evaluated and hybridization conditions were optimized using two pilot samples (EVO vs. LVO). Quality control metrics generated by the Agilent Feature Extraction software were analyzed. Spatial distribution of outliers in the green and red channel was plotted (Figure 4-8) and assessed for clustering bias resulting from technical error. Given that multiple probes spanning a single gene (thus located in same chromosomal region) are distributed in various locations across the array, clustering would not be indicative of a biologically relevant signal but rather of artifacts. Outliers appeared to be dispersed randomly across the array for both pilot samples and all samples assayed subsequently.
Next, signal intensity was evaluated and was found to be low in the pilot samples. To improve the signal, parameters of hybridization were changed for subsequent hybridizations. The amount of DNA and length of hybridization was increased from 1μg to 1.5μg and from 2 to 3 hours.

The difference these changes made to signal intensity are visible in the enlarged view of the four corners of a subsequent array (Figure 4-9). Spots or features are barely visible above the background noise in pilot samples (Figure 4-9A), and much improved with the changed parameters (Figure 4-9B), so much so that the constructed grid (white crosses) identifying features is placed with more certainty.
4.1.3 Array Analysis

To find differences in DNA methylation between early and late samples, the array signals were analyzed using two approaches: 1) finding peaks or signals that tiled across neighbouring probes or 2) finding differences in single probes.

4.1.3.1 Peak Detection

Mean pixel intensity values calculated but unprocessed by the Feature extraction software were used in the peak detection analysis. First, to reduce inter-assay variability, quantile normalization was applied. TileMap v2 software (CisGenome Project) was used as the tiling array peak detection tool. Fragment length was set to be on average 300bp. This indicated to the program that the signal should span at least 2 probes, given that probes of this array for any given CpG island sequence are on average 50bp long and spaced about 50bp. The false discovery rate (FDR) was set to <5%.

Figure 4-9 Feature intensity increased after optimization of array hybridization conditions

Four corners of Agilent CpG island microarray of A) pilot sample and B) sample used in analysis after hybridization conditions optimized. Grid (white crosses) placed where spots of colour representing probe signals expected. Green, input fraction; red, methyl enriched fraction.
Quality control of the peak detection algorithm used for the analysis was performed. The program’s ability to detect peaks was confirmed by the program being able to detect peaks that were artificially incorporated into the data set by spiking in high values across 3 or 4 probes in tandem with respect to their location on a chromosome.

A potentially biologically meaningful signal was identified when a peak was detected in early mice but not in late mice, or detected in late mice but not in early mice. These two comparisons were conducted in the following three pairs of groups: between EVO and LVO, E39 and L39, as well as between all mice with early puberty (E ALL) and all mice with late puberty (L ALL) irrespective of their day of sacrifice.

This analysis was performed by the biostatistical department of the University Health Network Microarray Center.

4.1.3.2 Single Probe Detection

Values processed by Agilent’s Feature extraction software for each probe were used in the single probe analysis. The mean of these processed probe signal intensities of the E ALL group were compared to the mean of the L ALL group.

The cut-off for significant differences was determined by applying the overlapping rule for standard error of the mean (SEM) bars (Cumming et al., 2007). The rule states that when the summed SEM of the two samples being compared is less than 2/3 of the mean difference between the two samples (SEM%diff), the difference would be found to be statistically significant in a Student’s t-test at p<0.05. This applies when the number of independent biological samples (n) is equal to or greater than 3. In the E ALL vs. L ALL comparison, 7 vs 5 samples, respectively, were compared.

The remaining probes were then sorted by smallest SEM%diff and the top 20 probes were sorted by greatest mean difference to identify the probe with the greatest and most significant difference.

Heat maps were constructed to visualize the signals identified after the SEM%diff cut-off was applied. A heat map was constructed for differences when the mean of E ALL was greater than L ALL, and when L ALL was greater than E ALL. Samples were organized to columns, grouped
by E ALL vs. L ALL, and probes into rows sorted by highest to lowest mean value for the group with the higher value.

It was acknowledged that the significance level of p<0.05 is a relaxed cut-off that is useful only for discovery as it does not account for multiple hypothesis testing. As such, detected differences could represent false positives until confirmed via other assays.

4.1.4 Array Verification

Methylation differences in the CpG islands of genes detected by peaks or singles probe signals were assessed by three different assays: 1) meDIP qPCR, 2) bisulfite pyrosequencing, 3) bisulfite modified MALDI-TOF mass spectrometry.

meDIP qPCR offers validation of the array using an aliquot of the samples hybridized directly to the array, meaning samples having undergone meDIP and WGA. The latter two assays offer both technical and biological validation using the same samples that were interrogated on the array or new samples that were not interrogated on the array, respectively.

One gene found by peak detection and one gene found by single probe detection was chosen as a candidate for validation by each of the three assays. The genes were chosen based on a combination of the following criteria:

1. The FDR of the peak or SEM%diff at a single probe was exceptionally low, indicating low variability between samples and/or great difference between groups, suggesting greater likelihood of a true positive signal.

2. The gene annotated to the probe a) possesses rare variants associated with disorders of puberty such as isolated hypogonadotropic hypogonadism (Kaminski and Palmert, 2008), b) associates with common variation such as age at menarche (AAM) in humans (Elks et al., 2010), and/or c) has been identified in various in vivo or in vitro studies to associate with the neuroendocrine control of pubertal timing (Ojeda et al., 2010b).

3. The signal for a particular probe is identified by more than one early vs. late comparison (E ALL vs L ALL, EVO vs. LVO and/or E39 vs. L39).
4. There is evidence from other studies that the gene annotated to the probe(s) may be biologically relevant to puberty and/or is susceptible to changes in its methylation, which translate to changes in transcription of the gene.

4.1.4.1 Quantification of DNA Methylation by meDIP qPCR

Quantification of DNA methylation was performed as described earlier in section 4.1.2.3 when *H19* and *Gapdh* were assayed for quality control of meDIP enrichment. Primers were constructed using PrimerQuest online software (Integrated DNA Technologies, http://www.idtdna.com/Scitools/Applications/Primerquest/) using the sequence determined by the UCSC *Mus musculus* mm9 genome assembly (July 2007 NCBI build 37).

4.1.4.2 Bisulfite Modification

To entrain the methylation code as a sequence that is identifiable by pyrosequencing and MALDI-TOF mass spectrometry, DNA underwent bisulfite modification. Under conditions of high bisulfite salt concentration, high temperatures and high acidity, unmethylated cytosines of DNA are deaminated into uracils (Clark et al., 2006). The methylated cytosines are protected and remain as cytosines after bisulfite treatment (Clark et al., 2006). In this way, a methylated cytosine is distinguished from an unmethylated one.

After extraction from the hypothalamic tissue, DNA was bisulfite modified using the EpiTect® Bisulfite Kit (Qiagen) following the manufacturer’s standard protocol for sodium bisulfite conversion of unmethylated cytosines in DNA using 1μg of starting material. The resulting bisulfite converted DNA was purified and eluted in 20μL of 10 mM Tris-Cl at pH 8.5.

4.1.4.3 Quantification of DNA Methylation by Pyrosequencing

Pyrosequencing was performed as described previously (Tost and Gut, 2007). PCR primers were designed for a sequence 50-75bp long that overlapped with the sequence of the array probe of interest using the gene sequence from the July 2007 NCBI build 37 of the *Mus musculus* mm9 genome assembly. 1 uL of bisulfite converted DNA was used for the PCR amplification of the target region. With amplification, unmethylated cytosines converted to uracils by bisulfite modification lead to incorporation of thymines. The PCR product is then isolated using the PyroMark® Q24 vacuum workstation (Qiagen). In this procedure, the amplified fragments are
biotinylated and attached to streptavidin-coated sepharose beads that can be captured by a membrane by-products are washed away. The purified fragments are then sequenced by synthesis. Individual nucleotides are dispensed into the reaction mixture and their incorporation to the synthesis of the complementary strand is monitored. When a nucleotide is incorporated, it releases pyrophosphate that is required to drive the reaction cascade that leads the conversion of luciferin into oxyluciferin generating light. The light generated is proportional to amount of nucleotides incorporated. Quantification of the relative abundance of cytosines and thymines that are incorporated thus relies on quantifying the amount of visible light that is emitted during the addition of each nucleotide.

PCR primer design, optimization and sample assay were performed by the laboratory of Dr. Rosanna Weksberg (The Hospital for Sick Children Research Institute, Toronto, Canada). Each sample was assayed in duplicate or triplicate. A technical replicate began with the PCR amplification of the target region. The average of the technical replicates was used as the value for a single biological replicate. Since pyrosequencing quantifies methylation at each CG site in a given sequence, results were analyzed both as average percent methylation across all CGs in the sequence to be able to directly compare to the array probe signal, and as methylation percent at each individual CG.

4.1.4.4 Quantification of DNA Methylation by MALDI-TOF Mass Spectrometry

Quantification of DNA methylation by MALDI-TOF mass spectrometry was performed as described previously (Ehrich et al., 2005). PCR primers were designed for a sequence 200-350 bp long that overlapped with the sequence of the array probe of interest using the gene sequence from the July 2007 NCBI build 37 of the Mus musculus mm9 genome assembly. 2 uL of bisulfite converted DNA was used for the PCR amplification of the target region. The amplified product was in vitro transcribed to RNA and cleaved at uracils by RNase A to generate CpG units. The cleavage products are described as CpG units because they can contain more than one CG dinucleotide in the sequence. When this is the case, the reading provided is an average of the methylation across all CG sites in the sequence. Fragments with a mass greater than 1500 Da and less than 7000 Da can be quantified.
Mass spectra were collected using a MassARRAY® system MALDI-TOF mass spectrometer (Sequenom, San Diego, CA). The bisulfite conversion of cytosine to thymine in the DNA was identified as a guanine/adenine variation in the RNA, or 135.13 Da/151.13 Da variation. This difference in mass was identified on the mass spectrum as a shift of 16Da, or multiples thereof, in the peak signal between methylated and unmethylated template. Percent methylation for each CpG unit was analyzed by the EpiTyper Software (Sequenom, San Diego, CA).

PCR primer design, optimization and sample assay were performed by the Analytical Genetics Technology Centre (AGTC) of the University Health Network (UHN), Toronto, Canada. Each sample was assayed in triplicate. The average of the technical replicates was used as the value for a single biological replicate. Results were analyzed both as average percent methylation across all CGs in the sequence to be able to directly compare to the array probe signal, and as methylation percent at each CG unit.

### 4.1.4.5 Statistical Analysis

A Student’s t-test was performed to compare methylation differences between early and late groups of mice. In the case of individual CGs, significance was attributed at $p<0.05$ divided by the number of CG sites in the sequence assayed, to account for multiple hypothesis testing.
4.2 Results

4.2.1 Peak Detection

The method of peak detection was used to detect differential signals on the microarray between early and late samples. When comparing EVO to LVO, differentially methylated regions were detected for Jmjd6, EG622408, Cobra1, Son, and Jph4 (Table 4-4). Signals in these 5 genes showed significant hypermethylation (FDR<5%) in early mice sacrificed at VO compared to late mice sacrificed at VO (3 vs 2) across 3-5 probes.

<table>
<thead>
<tr>
<th>gene</th>
<th>rank</th>
<th>chr</th>
<th>start</th>
<th>end</th>
<th>region length</th>
<th>n of probes spanned</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jmjd6,Ptdsr</td>
<td>1</td>
<td>11</td>
<td>116703958</td>
<td>116704276</td>
<td>319</td>
<td>4</td>
<td>0.01%</td>
</tr>
<tr>
<td>EG622408</td>
<td>2</td>
<td>13</td>
<td>113788882</td>
<td>113789103</td>
<td>222</td>
<td>3</td>
<td>0.01%</td>
</tr>
<tr>
<td>Cobra1</td>
<td>3</td>
<td>2</td>
<td>25066644</td>
<td>25067013</td>
<td>370</td>
<td>4</td>
<td>0.01%</td>
</tr>
<tr>
<td>Son</td>
<td>4</td>
<td>16</td>
<td>91660239</td>
<td>91660660</td>
<td>422</td>
<td>5</td>
<td>0.01%</td>
</tr>
<tr>
<td>Jph4</td>
<td>5</td>
<td>14</td>
<td>55733451</td>
<td>55733648</td>
<td>198</td>
<td>3</td>
<td>0.01%</td>
</tr>
</tbody>
</table>

Table 4-4 Genes detected by peak detection hypermethylated in EVO compared to LVO

†Mus musculus mm9 July 2007 NCBI build 37; chr, chromosome; n, number; FDR, false discovery rate

In the converse comparison, no significant hypermethylation was detected in LVO compared to EVO (2 vs 3).

In making the comparison between groups of mice sacrificed at pnd 39, no significant hypermethylation was detected in E39 vs L39 (4 vs 3). In the converse comparison, Hoxa11 was detected to be significantly differentially methylated at 3 neighbouring probes, specifically hypermethylated in L39 vs E39 (3 vs 4) (Table 4-5).

<table>
<thead>
<tr>
<th>gene</th>
<th>rank</th>
<th>chr</th>
<th>start</th>
<th>end</th>
<th>region length</th>
<th>n of probes spanned</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoxa11</td>
<td>1</td>
<td>6</td>
<td>52202817</td>
<td>52203047</td>
<td>231</td>
<td>3</td>
<td>0.01%</td>
</tr>
</tbody>
</table>

Table 4-5 Genes detected by peak detection hypermethylated in L39 compared to E39

†Mus musculus mm9 July 2007 NCBI build 37; chr, chromosome; n, number; FDR, false discovery rate
To identify significant differences attributable to early and late puberty without the potential confounding effects of differential estrogen exposure and differences in age, it was a priori established that a four group comparison would be made. Figure 4-10 shows that when this comparison is made, there are no genes that were detected to be differentially methylated in both groups of early onset mice or both groups of late onset mice at the FDR of <5%.

![Venn diagram illustrating number of genes detected as being hypermethylated in hypothalami of early (blue) and late (orange) onset of puberty female C57BL/6 mice. E, early puberty mice; L, late puberty mice; VO, sacrificed at vaginal opening; 39, sacrificed at postnatal day 39.](image)

**Figure 4-10 Number of peak signals detected in comparison of early and late puberty mice**

It is possible that any of the 6 genes detected in one comparison (VO or 39) could be just below threshold detection in the complementary comparison. Therefore, to increase the likelihood of detecting these differences, all early mice (EALL) and all late mice (LALL) were pooled into two groups. The difference detected in Hoxa11 in L39 vs E39 was also apparent in the pooled comparison between LALL and EALL (5 vs 7) (Table 4-6). Conversely, the 5 regions detected in EVO vs LVO were not detected in the pooled comparison. No hypermethylation was detected in EALL that was not observed in LALL (7 vs 5). This suggests that signals of these 5 regions could be differences confounded by age.
Table 4-6 Genes detected by peak detection hypermethylated in LALL compared to EALL

<table>
<thead>
<tr>
<th>inside</th>
<th>rank</th>
<th>chr</th>
<th>start</th>
<th>end</th>
<th>region length</th>
<th>n of probes spanned</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoxa11</td>
<td>1</td>
<td>6</td>
<td>52202817</td>
<td>52203047</td>
<td>231</td>
<td>3</td>
<td>0.01%</td>
</tr>
</tbody>
</table>

Mus musculus mm9 July 2007 NCBI build 37; chr, chromosome; n, number; FDR, false discovery rate.

The signal at Hoxa11 in EALL and LALL is illustrated in Figure 4-11. The green dotted line encompasses the probes that were detected to be significantly differentially methylated between the two groups. There is consistent hypermethylation in LALL in and around this region. The difference in signal between L ALL and E ALL is subtle but apparent.

![Figure 4-11 Microarray probe signal intensity detected hypermethylation in late puberty mice in Hoxa11](image)

Signal intensity values of array probes of all early puberty mice (n=7) and all late puberty mice (n=5) plotted against probe location on chromosome 6. Green rectangle surrounds probes identified with statistically significant difference between groups at a false discovery rate (FDR) of <0.01%.

Given that Hoxa11 was detected in both the ALL and 39 comparisons of early and late mice, it would be assumed that the same trend in early and late mice would be present in the VO comparison. This is the case, and it is illustrated in Figure 4-12.
Figure 4-12 Microarray probe signal intensity detected hypermethylation in LVO and L39 in Hoxa11

Signal intensity values of array probes of early puberty mice sacrificed at vaginal opening (VO) (n=3), early puberty mice sacrificed at postnatal day (pnd) 39 (n=4), late puberty mice sacrificed at VO (n=2) and late puberty mice sacrificed at pnd 39 (n=3) plotted against probe location on chromosome 6. Green rectangle surrounds probes identified with statistically significant difference between groups at a false discovery rate (FDR) of <0.01%.

The majority of probes in and around the region of significance show a consistent trend of greater signal intensity in each late group (red bars) than in the corresponding early groups (blue bars).

Interestingly, Hoxa11 may be biologically relevant to pubertal timing as it has been reported to play a role in reproductive endocrine axis and uterine development (Widschwendter et al., 2009; Dunlap et al., 2011; Kwon and Taylor, 2004). Therefore, Hoxa11 was chosen as the candidate gene from the peak detection method to be assessed by the three validation methods described earlier. Results follow in section 4.2.3.1.

4.2.2 Single Probe Detection

The second approach used to detect differences in microarray signals between early and late onset mice was detection of differences at single probes. 1000 probes out of 97,652 probes were detected to have significant differences between groups. Heat maps visualizing these differences
are presented in Figure 4-13. Differences are subtle, evidence by the small degree of colour difference between columns (grouped samples) at any one row (probes).

Figure 4-13 Subtle differences between early and late mice in signal intensities of single probes

Heat maps of probes detected by single probe detection method ordered from greatest to smallest signal intensity (rows), grouped by early and late onset of puberty mice (columns). The heat map on the left shows probes with signals greater in all late puberty mice (L ALL) than in all early puberty mice (E ALL) (n=432); heat map on the right shows probes with signals greater in E ALL than L ALL (n=468).

The top 20 probes with the most significant differences are listed in Table 4-7 and sorted by greatest absolute difference between early and late. The probe ranked at number 1 corresponds to a region in the CpGi of the gene Tox located on chromosome 4.
Table 4-7 Top 20 genes detected by single probe detection method between early and late mice

<table>
<thead>
<tr>
<th>Rank</th>
<th>Gene Name</th>
<th>Chromosome</th>
<th>Start†</th>
<th>End†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tox</td>
<td>4</td>
<td>6918151</td>
<td>6918195</td>
</tr>
<tr>
<td>2</td>
<td>BC049834-771</td>
<td>1</td>
<td>157589907</td>
<td>157589951</td>
</tr>
<tr>
<td>3</td>
<td>BC125456:271</td>
<td>7</td>
<td>25118652</td>
<td>25118696</td>
</tr>
<tr>
<td>4</td>
<td>Apbb2</td>
<td>5</td>
<td>67009116</td>
<td>67009162</td>
</tr>
<tr>
<td>5</td>
<td>Satb2</td>
<td>1</td>
<td>57027654</td>
<td>57027698</td>
</tr>
<tr>
<td>6</td>
<td>Trim36</td>
<td>18</td>
<td>46358938</td>
<td>46358983</td>
</tr>
<tr>
<td>7</td>
<td>D15Wsu169e</td>
<td>15</td>
<td>76555108</td>
<td>76555152</td>
</tr>
<tr>
<td>8</td>
<td>1110012M11Rik</td>
<td>8</td>
<td>74066770</td>
<td>74066816</td>
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<td>9</td>
<td>AK138171:68</td>
<td>1</td>
<td>54614375</td>
<td>54614424</td>
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<td>10</td>
<td>Cd2bp2</td>
<td>7</td>
<td>134339558</td>
<td>134339608</td>
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<tr>
<td>11</td>
<td>Dcun1d1</td>
<td>3</td>
<td>35831187</td>
<td>35831246</td>
</tr>
<tr>
<td>12</td>
<td>Fbxl6</td>
<td>15</td>
<td>76368790</td>
<td>76368836</td>
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<tr>
<td>13</td>
<td>Kif13a</td>
<td>13</td>
<td>47025911</td>
<td>47025956</td>
</tr>
<tr>
<td>14</td>
<td>2010200O16Rik</td>
<td>3</td>
<td>108365347</td>
<td>108365394</td>
</tr>
<tr>
<td>15</td>
<td>Zic2</td>
<td>14</td>
<td>122875100</td>
<td>122875144</td>
</tr>
<tr>
<td>16</td>
<td>Rara</td>
<td>11</td>
<td>98821317</td>
<td>98821363</td>
</tr>
<tr>
<td>17</td>
<td>Ing1</td>
<td>8</td>
<td>11558000</td>
<td>11558051</td>
</tr>
<tr>
<td>18</td>
<td>Trim14</td>
<td>4</td>
<td>46519722</td>
<td>46519767</td>
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<tr>
<td>19</td>
<td>Lhx8</td>
<td>3</td>
<td>153992726</td>
<td>153992782</td>
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<td>20</td>
<td>Zfp36l1</td>
<td>12</td>
<td>81215293</td>
<td>81215352</td>
</tr>
</tbody>
</table>

*Mus musculus* mm9 July 2007 NCBI build 37

The signals for the microarray probes annotated for the CpG of the gene *Tox* are illustrated in Figure 4-14. The bars within the green dotted line correspond to the probe found to have a significant signal between early and late onset mice, with approximately 3.2 fold greater signal intensity detected in early mice.
Figure 4-14 Microarray probe signal intensity detected hypermethylation in early puberty mice in Tox

Signal intensity values of array probes of all early puberty mice (n=7) and all late puberty mice (n=5) plotted against probe location on chromosome 4. Green rectangle surrounds probe identified with statistically significant difference between groups.

At the probe located at 691873 of chromosome 4, the signal is consistent in both VO (p=0.2977) and 39 (p=0.0028) comparisons (Figure 4-15).
Figure 4-15 Microarray probe signal intensity detected hypermethylation in LVO and L39 in \textit{Tox}

Signal intensity values of array probe of early puberty mice sacrificed at vaginal opening (VO) (n=3), early puberty mice sacrificed at postnatal day (pnd) 39 (n=4), late puberty mice sacrificed at VO (n=2) and late puberty mice sacrificed at pnd 39 (n=3) plotted against probe location on chromosome 6.

Therefore, \textit{Tox} was chosen as the candidate gene from the single probe detection method to be assessed by the three validation methods described earlier. Results follow in section 4.2.3.2.

4.2.3 Validation

4.2.3.1 \textit{Hoxa11}

Figure 4-16 summarises 1) where, on chromosome 6, methylation of \textit{Hoxa11} was assayed by each of the three validation techniques, 2) how they correspond to the regions covered by the three array probes detected to have a significant difference between early and late groups of mice, and 3) the results of the assays.
Sequences assayed by the three validation techniques each overlap the sequence covered by array probe 2. MALDI-TOF and meDIP qPCR extend beyond this region. Array probe 2, the probe of interest, contains 4 CG sites, three of which were assayed individually by pyrosequencing and two by MALDI-TOF mass spectrometry (these will be identified in the following sections).

4.2.3.1.1 meDIP qPCR

As validation of the microarray signal, meDIP qPCR was performed. By comparing $C_T$ values of the meDIP and input fractions of each sample, and comparing average $C_T$ differences between groups, relative retention of methylated fragments by meDIP and thus methylation in a given region of interest can be estimated. Primers used to amplify a region of $Hoxa11$ that overlapped the probe region of interest are listed in Table 4-8.
Table 4-8 Primer sequence of *Hoxa11* used for meDIP qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Concentration (mM)</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hoxa11</em></td>
<td>F 5’-TGG TGG AAA GAG CGA AGA ACA GGA-3’</td>
<td>300</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td>R 5’-TAG GTA GGT GGC GAA AGT CTG CTT-3’</td>
<td>300</td>
<td></td>
</tr>
</tbody>
</table>

*Mus musculus* mm9 July 2007 NCBI build 37; chr, chromosome; bp, basepairs; F, forward; R, reverse

The single melt peak for all samples and absence of a peak in the no template controls (NTC) in the dissociation curve in Figure 4-17A confirms specific amplification using this primer pair at the optimized concentrations. The amplification plot in B shows differences in C$\text{T}$ between samples. Lines of the same colour cluster and reach threshold at similar cycles. Light blue lines are further apart from dark blue lines than are pink from red lines, indicating greater differences in C$\text{T}$ values between meDIP and input fractions in early compared to late puberty mice.
Figure 4-17 Specific amplification of *Hoxa11* with amplification differences between meDIP and input fractions in early and late puberty mice

Quantitative real-time PCR A) dissociation curve plotting fluorescence against temperature and B) amplification plot of fluorescence against cycle number for *Hoxa11* input and methyl enriched (meDIP) fractions. Solid black line above 0 represents threshold line. NTC, no template control; E, early puberty mice (n=7); L, late puberty mice (n=6).

When these C<sub>T</sub> values are plotted, the results reveal that there is a smaller difference between C<sub>T</sub> values of input and meDIP in the Late group than the Early group (Figure 4-18). This indicates that there is a trend of greater methylation of *Hoxa11* in late onset mice. Average CT difference between the two groups is 0.75, which translated to a 1.7 fold difference. This trend, although larger in magnitude, is consistent with the microarray results and indicates that the methylation enrichment performed by meDIP was represented correctly by the microarray.
Figure 4-18 Hypermethylation of Hoxa11 in late puberty mice validated by meDIP qPCR

Average cycle threshold (C_T) differences between input and methylated DNA immunoprecipitated (meDIP) fractions measured in Hoxa11 of early (n=7) and late (n=6) puberty mice by quantitative real-time PCR.

4.2.3.1.2 Pyrosequencing

The pyrosequencing assay designed for a region of Hoxa11 overlapping the probe region of interest is depicted in Figure 4-19 A-B. The sequence used to design the assay was sourced from the UCSC Mus musculus mm9 genome assembly (July 2007 NCBI build 37). The PyroGram of Figure 4-19 B plotting percent methylation on the x-axis versus dispensation order of nucleotides on the y-axis shows methylation events highlighted in blue. The results of running a test sample on this assay show that the assay can measure methylation with high degree of certainty at the first three CG positions in the sequence and fails to do so at the two remaining CG positions. Therefore, quantification of methylation was restricted to the CGs in the sequence are underlined and in blue in Figure 4-19 A. These CG sites are those present in the sequence of the array probe of interest.
Figure 4-19 Pyrosequencing assay measures three CG positions in Hoxa11 sequence

CG positions labeled 1 through 5 in A) the Hoxa11 sequence assayed, sourced from mm9 July 2007 NCBI build 37 of the Mus musculus genome, and B) representative PyroGram of assay optimization. Order of nucleotide dispensation is featured on the x-axis. Blue highlights identify methylation events. CG positions in blue pass assay quality control and those in red do not.

Quantification of methylation at Hoxa11 by pyrosequencing revealed a non-significant trend in increased methylation in late onset of puberty samples, which was consistent across all CG positions measured and when the measures were averaged (Figure 4-20 A-B). This trend, although smaller in magnitude, is consistent with the microarray results.
Figure 4-20 Methylation of *Hoxa11* quantified by pyrosequencing

*Hoxa11* methylation A) at individual CG positions 1 through 3, B) average across all positions in all early (n=8) and all late (n=8) puberty mice.

### 4.2.3.1.3 MALDI-TOF mass spectrometry

The amplicon designed for a region of *Hoxa11* overlapping the probe region of interest assayed by MALDI-TOF mass spectrometry is depicted in Figure 4-21. The sequence used to design the amplicon was sourced from the UCSC *Mus musculus* mm9 genome assembly (July 2007 NCBI build 37). *In silico* fragmentation analysis of predicted RNAse A cleavage products was performed on the original sequence to determine 1) which CGs in the sequence will be assayed (yellow highlighted fragments only), 2) of those that will be assayed, for which will percent methylation be reported as an average across the CpG unit (orange CGs) and for which as an individual measure (blue CGs).
Table 4-9 reports the results of the *in silico* analysis of Figure 4-21. A 342 bp region was assayed in which 13 of the 20 CGs in the sequence were measured and reported as measurements of percent methylation for 10 separate CpG units. For example, methylation of CG 6, 7 and 8 will be reported as an average of methylation across the CpG unit while methylation of CpG unit 5 will be reported for that individual CG.

**Table 4-9 Hoxa11 amplicon properties**

<table>
<thead>
<tr>
<th></th>
<th>Length (bp)</th>
<th>Total CpG</th>
<th>CpG Units</th>
<th>CpG sites covered</th>
<th>Coverage</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoxa11</td>
<td>342</td>
<td>20</td>
<td>10</td>
<td>13</td>
<td>0.65</td>
<td>Custom</td>
</tr>
</tbody>
</table>

The EpiTYPER software presents the results of the assay in an EpiGram to aid in the visualization of methylation differences across samples (rows) and CG positions (columns) in the sequence. The EpiGram for one of three technical replicates of *Hoxa11* is depicted in Figure 4-22.
Figure 4-22 Hoxa11 EpiGram

Representation of methylation level at CG positions along amplicon. Sample IDs are listed on the left. Amplicon length is listed along the top with first base of amplicon sequence denoted as 0. Below the scale, 1 through 20 denote CG sites. Grey circles denote CG sites that could not be measured. Colour scale identifies level of methylation. EVO = 2, 3, 4, 19, 20; E39 = 5, 6, 7, 21, 22; LVO = P, 8, 9, 10, 17, 18; L39 = 11, 12, 13, 14

The EpiGram shows that methylation in all samples across all measureable CGs lies between 0% and 20% denoted as red dots. As such, no visible differences are apparent. If differences exist, they are subtle. Exact percent differences were plotted in Figure 4-23 A-B.
Figure 4-23 Methylation of Hoxa11 quantified by MALDI-TOF mass spectrometry

Hoxa11 methylation A) at individual CpG units 3, B) average across all units in all early (n=10) and all late (n=10) puberty mice.

Quantification of methylation by MALDI-TOF mass spectrometry revealed a non-significant trend of higher methylation in early onset of puberty samples than in late that was consistent across the majority of all CpG units measured and when the measures were averaged across all units (Figure 4-23 A-B). This trend is inconsistent with the microarray results that showed higher methylation in the late onset group.

CpG 14 corresponds to CG in position 2 assayed by pyrosequencing (Figure 4-20 A). Neither assay showed a significant difference in methylation at this cytosine, although there was a trend of increased methylation in late puberty mice compared to early detected by pyrosequencing that was not present at this CG in the measurement performed by mass spectrometry. The overall level of methylation detected at this region by MALDI-TOF mass spectrometry was, however, similar to that detected by pyrosequencing (on average between 10-12%).

4.2.3.1.4 Summary of Hoxa11 Validation

Methylation quantified by pyrosequencing and meDIP qPCR showed non-significant trends in the same direction as was observed in microarray results, where the late (L) group had greater methylation than the early (E) group at Hoxa11. MALDI-TOF results showed a subtle trend in the opposite direction. Therefore, the differential peak signals at Hoxa11 detected by the
microarray do not validate fully but do provide provisional positive results that are consistent with differential methylation at this gene between early and late onset of puberty mice.

4.2.3.2 Tox

Sequences assayed by the three validation techniques each overlap the sequence covered by the array probe of interest (Figure 4-24). MALDI-TOF and meDIP qPCR extend beyond this region and overlap also the region covered by the upstream array probe, which showed no signal.

The array probe of interest contains 1 CG site which was assayed by pyrosequencing along with 6 additional CG sites, and also assayed by MALDI-TOF mass spectrometry along with 7 additional sites (these will be identified in the following sections).

![Figure 4-24 Tox sequences assayed by validation techniques overlap sequence of array probe of interest](image)

Lines demarcate region of chromosome 6 for which methylation was assessed by each assay for early puberty mice (E) and late puberty mice (L). Results of validation techniques are summarized above corresponding lines.

4.2.3.2.1 meDIP qPCR

meDIP qPCR was performed at a region of Tox that overlapped the array probe region of interest. Primers used to amplify this region are listed in Table 4-10.
Table 4-10 Primer sequence of *Tox* used for meDIP qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Concentration (mM)</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tox</td>
<td>F 5’-ATT GGA GAC TCT GCC TTG CTT TGC-3’</td>
<td>50</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>R 5’-TGC AAC ACC ACA ACT CCA TCA-3’</td>
<td>300</td>
<td></td>
</tr>
</tbody>
</table>

*Mus musculus* mm9 July 2007 NCBI build 37; bp, basepairs; F, forward; R, reverse

The single melt peak for all samples and absence of a peak in the NTCs in the dissociation curve in Figure 4-25A confirms specific amplification using this primer pair at the optimized concentrations. The amplification plot in B shows differences in C_T values between samples. Lines of the same colour cluster and reach threshold at similar cycles. Pink lines are further apart from red lines than are light blue from dark blue lines, indicating greater differences in C_T values between meDIP and input fractions in late compared to early puberty mice.
Figure 4-25 Specific amplification of *Tox* with amplification differences between meDIP and input fractions in early and late puberty mice

Quantitative real-time PCR A) dissociation curve plotting fluorescence against temperature and B) amplification plot of fluorescence against cycle number for *Tox* input and methyl enriched (meDIP) fractions. Solid black line above 0 represents threshold line. NTC, no template control; E, early puberty mice (n=7); L, late puberty mice (n=6).

When plotted, the results reveal significantly greater meDIP enrichment of this region of *Tox* in the Early group compared to the Late group (Figure 4-26) (p=0.0031). Average CT difference between the two groups is 1.88, which translates to a 3.7 fold difference. The direction and magnitude of this difference is consistent with the microarray results. This indicates that the methylation enrichment performed by meDIP was represented correctly by the microarray
Figure 4-26 Hypermethylation of *Tox* in early puberty mice validated by meDIP qPCR

Average cycle threshold (C_T) differences between input and methylated DNA immunoprecipitated (meDIP) fractions measured in *Tox* of early (n=8) and late (n=7) puberty mice by quantitative real-time PCR.

4.2.3.2.2 Pyrosequencing

The pyrosequencing assay designed for a region of *Tox* overlapping the array probe of interest is depicted in Figure 4-27 A-B. The EpiGram of Figure 4-27 B shows that methylation can be quantified at all 7 CG positions in the sequence with high degree of certainty. The CGs for which methylation was quantified are underlined and in blue in A.
Figure 4-27 Pyrosequencing assay measures seven CG positions in Tox sequence

CG positions labeled 1 through 7 in A) the Tox sequence assayed, sourced from mm9 July 2007 NCBI build 37 of the Mus musculus genome, and B) representative PyroGram of assay optimization. Order of nucleotide dispensation is featured on the x-axis. Blue highlights identify methylation events. CG positions in blue pass assay quality control.

Quantification of methylation at Tox by pyrosequencing revealed subtle non-significant fluctuations in levels of methylation in late onset of puberty samples at each CG position (Figure 4-28 A), and no difference when the measures were averaged (Figure 4-28 B).
Figure 4-28 Methylation of Tox quantified by pyrosequencing

Tox methylation A) at individual CG positions 1 through 7, B) average across all positions in all early (n=4) and all late (n=4) puberty mice.

These results are inconsistent with the large differences detected by the microarray in this region of Tox.

4.2.3.2.3 MALDI-TOF

The amplicon designed for a region of Tox overlapping the probe region of interest assayed by MALDI-TOF mass spectrometry is depicted in Figure 4-29. In silico fragmentation analysis of predicted RNase A cleavage products was performed on the original sequence to determine 1) which CGs in the sequence will be assayed (yellow highlighted fragments only), 2) of those that will be assayed, for which will percent methylation be reported as an average across the CpG unit (orange CGs) and for which as an individual measure (blue CGs).

```
TTATTTGGAGACTCTGTGCTTTGAAGTTTTGTTCCA[CGTTA]GAGGCCTGGG
ATGGGGGAGTGATGCTGTGTTGAGGGAGATCAAGA[TCGGA]GGAC
ACTAGT[GCCCCGGA][CGGGCGGGGTGCCGA]CAGAGGCTGGA[GCGCGCCCAGAGG
GTACAGTTGGGGAC
```

Figure 4-29 Tox amplicon design for MALDI-TOF methylation quantification

CG individual CG in a fragment; CG multiple CGs in a fragment where methylation is reported as average; CG positions are numbered 1 through 8; yellow highlight indicates a fragment containing CGs that was assayed; sequence sourced from Mus musculus mm9 July 2007 NCBI build 37
Table 4-11 reports the results of this *in silico* analysis. A 180 bp region was assayed in which all 8 of the CGs in the sequence were measured and reported as measurements of percent methylation for 5 separate CpG units.

**Table 4-11 Tox amplicon properties**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Length (bp)</th>
<th>Total CpG</th>
<th>CpG Units</th>
<th>CpG sites covered</th>
<th>Coverage</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tox</td>
<td>180</td>
<td>8</td>
<td>5</td>
<td>8</td>
<td>1</td>
<td>Custom</td>
</tr>
</tbody>
</table>

![Figure 4-30 Tox EpiGram](image)

Representation of methylation level at CG positions along amplicon. Sample IDs are listed on the left. Amplicon length is listed along the top with first base of amplicon sequence denoted as 0. Below the scale, 1 through 8 denote CG sites. Grey circles denote CG sites that could not be measured. Colour scale identifies level of methylation. EVO = 2, 3, 4, 19, 20; E39 = 5, 6, 7, 21, 22; LVO = P, 8, 9, 10, 17, 18; L39 = 11, 12, 13, 14
The EpiGram for one of the three technical replicates of *Tox* is depicted in Figure 4-27. It shows that methylation in all samples across all measurable CGs lies between 0% and 20% denoted as red dots. As such, no visible differences are apparent. If differences exist, they are subtle. Exact percent differences were plotted in Figure 4-30 A-B.

![Graph showing methylation levels](image)

**Figure 4-31 Methylation of Tox quantified by MALDI-TOF mass spectrometry**

*Tox* methylation A) at individual CpG units, B) average across all units in all early (n=8) and all late (n=8) puberty mice.

CpG 1 corresponds to the CG in the array probe sequence. No differences in methylation, as quantification by MALDI-TOF mass spectrometry, were observed between early and late onset of puberty groups at any of the CpG units measured and when the measures were averaged across all units (Figure 4-31 A-B). This is inconsistent with the microarray results that showed significantly higher methylation in the early onset group. This is, however, consistent with the results of the pyrosequencing assay. CpG 1 through 7 correspond to CG positions 1 through 7 assayed by pyrosequencing. In both assays, no difference was detected between early and late groups, and both measured consistent relative methylation between CG sites as well as an overall level of methylation around 5%.
4.2.3.2.4 Summary Tox

Results of methylation quantification were consistent between pyrosequencing and MALDI-TOF, and consistent between meDIP qPCR and the array, but different from each other. Relative methylation between CG positions assayed by pyrosequencing and mass spectrometry were consistent. Therefore, the differential signals at the single probe for Tox detected by the microarray do not validate and instead point to a false positive result.
4.3 Discussion

With initial evidence that pubertal timing may be under epigenetic control, a genome wide examination of the methylome was undertaken to detect which genes may be involved. It was hypothesized that differential DNA methylation at key genes that play a role in the neuroendocrine axis could lead to differential transcriptional regulation of these genes leading to differential regulation of puberty and variation in the timing of pubertal onset in a population. As such, the genome wide methylation patterns in mice exhibiting differential pubertal phenotypes, early vs late onset, were compared with the goal of finding an association between a specific methylation pattern and a pubertal phenotype. The hypothalamic DNA interrogated showed few and mainly subtle differences between these two groups (section 4.2.1-4.2.3). Out of the 16,030 CpG islands (CpGis) represented on the array platform, less than 1% were detected with a significant difference in methylation levels using two different analysis methods. The heat map in Figure 4-13 further indicates that these differences were subtle. There was no instance where one group showed a signal at one end of the colour (signal intensity) spectrum e.g. red and the other group at the opposite spectrum e.g. green, at the same array probe positions.

Out of the genes detected by the two analyses, the most robust candidate was chosen from each to be validated by complementary techniques. The criteria used to choose these genes can be grouped into two categories based on 1) signal, 2) biology. The greatest weight was given to genes with the best signals in terms of greatest statistical significance and greatest fold change. These top candidates determined by an unbiased, purely mathematical analysis were then assessed on known biological relevance to puberty.

Hoxa11 was chosen from among the genes found by the peak detection method. Although each of the genes found were calculated to have the same false discovery rate, Hoxa11 was the only gene that was present in more than one comparison of early and late (LALL vs EALL and L39 vs E39). This made it the best candidate mathematically. Interestingly, Hoxa11 belongs to the Homeobox (HOX) super family of genes that function as key regulators of mammalian development (Barber and Rastegar, 2010). Specifically, Hoxa11 has been shown to play a role in female fertility, involved in endometrium receptivity to implantation (Eun Kwon and Taylor, 2004; Wang et al., 2004) and uterine development (Dunlap et al., 2011). Also, Hoxa11 shows susceptibility to epigenetic regulation, in that differential methylation at its CpGi has previously
been reported (Ronneberg et al., 2011; Shu et al., 2011) and that it can interact with polycomb repressing complexes (Luke et al., 2006). Epigenetic modifications on \textit{Hox} genes have been shown to be functionally significant, repressing and activating their transcription in a tissue and temporal specific pattern (Soshnikova and Duboule, 2009).

\textit{Tox}, thymocyte selection-associated high mobility group box, the candidate found by the probe detection analysis, has no known biologic link to the reproductive endocrine axis, but it showed a very high signal difference between early and late mice consistent in direction of difference between VO and 39 comparisons (Figure 4-13). As a member of the high mobility group box class of proteins, \textit{Tox} may be involved in the regulation of such processes as replication, transcription or DNA repair (Thomas, 2001). Its role has been specifically studied in the thymus, where it is involved in immune system development (Aliahmad et al., 2011; Aliahmad and Kaye, 2008).

Quantification of DNA methylation of these candidate genes on pyrosequencing and MALDI-TOF assays and meDIP qPCR did not fully validate the signals detected by the microarray. All assays were designed to quantify methylation of CpGs in a region that overlapped with the microarray probe region of interest. MeDIP qPCR, in both cases, reported the same direction and roughly the same magnitude of differences as the microarray. This was not always the case for the other two methods. As such, one must consider that the signals detected by the microarray may represent false positives.

4.3.1 A Whole Genome Discovery Approach

Each approach to whole genome methylation analysis has inherent limitations that could limit the detection of true positive results. Since no previous studies had investigated DNA methylation patterns of genes in the context of puberty, and as such no epigenetically regulated candidates are known, the goal was to perform an unbiased epigenome wide discovery to identify potential candidates that may not have been thought of otherwise. Such an approach is unbiased in that genes are chosen by mathematical calculations and not by the experimenter. However, as discussed in the sections that follow, the subset of genes that can be identified is biased by experimental design, sample preparation techniques, properties of the array and statistical analysis.
4.3.1.1 Experimental Design

In comparing mice with early and late onset of puberty, a four group comparison was made between early and late mice sacrificed at either VO or 39 to account for potential confounding effects of estrogen exposure or age.

This means, however, that the comparison made is conservative because it rejects the possibility that differences detected in mice at VO when the mice are different ages but not detected at pnd 39, after mice have been exposed to estrogen, could be differences underlying alterations in the timing of puberty rather than differences related to age. These differences may have played a role in dictating early or late puberty and then disappeared with estrogen exposure. The converse is true for differences detected in the 39 comparison that are not present at VO. Theoretically, the effects of estrogen on methylation of relevant puberty genes could be separated from the confound of age if differential methylation patterns could be detected prior to puberty. The caveat to this is that to collect early or late puberty samples from pre-pubertal mice, one must be able to predict pubertal timing, which cannot be done. Therefore, the four group comparison was the optimal solution.

This experimental design also dictates that epigenetic marks that persist across the pubertal transition will be the ones detected as they must be present both at VO and pnd 39. DNA methylation is the epigenetic mark that is most often found to persist from the time when it is placed, usually during embryogenesis, into adulthood (Polo et al., 2010; Heijmans et al., 2008). This does not preclude that there may be important modifications to histones that contribute to epigenetic regulation of pubertal timing, among other mechanisms, but as an initial screen, studying the methylome provides valuable insight into potential epigenetic regulation.

Importantly, epigenome wide association studies, such as this one, are powerful tools to identify novel genes, in which cause and effect can be determined with further investigation (See chapter 5 for discussion).

4.3.1.2 Sample Preparation

To assess methylation differences on an array that hybridizes to DNA sequence independent of methylation, a technique must be employed to distinguish the amount of methylation in each sample identifiable by the array. For this, meDIP was chosen as it accomplishes this by enriching
the abundance of sequences that are methylated and diminishes those that are not methylated via immunocapture with a 5-methylcytosine antibody (Weber et al., 2005). Relative enrichment occurs in a dose dependent manner, meaning that the more methylated cytosines present in a fragment, the greater the likelihood it will be captured by the antibody (Weber et al., 2005). However, it is semi-quantitative because CG content and density will bias the dose dependency (Irizarry et al., 2008). For example 3 methylated cytosines in a fragment containing 10 CpGs will be captured with the same efficiency and thus enriched by the same amount as 3 methylated cytosines in a fragment of 3 CpGs even though the methylation status of the former fragment is 30% and 100% in the latter. In interpreting the results, it is also necessary to be aware of the sensitivity of the technique, where differences in level of enrichment is indistinguishable when 0, 1 or 2 cytosines are methylated in a fragment (Keshet et al., 2006). Success of enrichment can be verified by testing genes of known methylation status, such as was performed in this study on hypomethylated Gapdh and hemi-methylated H19 (see section 4.1.2.3).

Although these limitations impose some considerations when interpreting the results, meDIP was selected because it supports high-resolution and high-throughput DNA methylation profiling. Importantly, it does not limit the regions sampled as other techniques limit, such methylation-sensitive vs. insensitive restriction enzyme digestion which is limited to enzyme specific recognition sequences (Weber et al., 2007a; Ogoshi et al., 2011).

### 4.3.1.3 Array Properties

The Agilent Mouse CpG Island 2x105K array was chosen for genome wide methylation profiling. The array covers 16,030 mouse CpG islands, which is full coverage of all estimated CpG islands in mouse genome (Antequera and Bird, 1993). The limitation of this array is that only regions that satisfy the parameters of CpGi are measured. These are: GC content greater than 50%, with an observed over expected ratio of CpG dinucleotides of 0.60, and length of region with these parameters no shorter than 200 bp (Gardiner-Garden and Frommer, 1987). Although methylation can occur in CpG scarce regions such as gene bodies, which can have functional significance (Ball et al., 2009), methylation at CpGi has been most highly linked to transcriptional regulation (De Smet et al., 1999). Indeed, the majority of gene promotors are linked to CpGi (Weber et al., 2007a). Therefore, by interrogating methylation in CpGi, it is
likely that differences found will be functionally meaningful. Chapter 5 discusses the future possibilities of exploring functionality of differential methylation patterns.

4.3.1.4 Analysis

To provide genome wide coverage, the array utilizes 97,652 probes. Interrogating tens of thousands of sequences in parallel makes the likelihood of finding significant results by chance relatively high. The establishment of valid thresholds of detection to minimize false positives while maximizing detection of true positives is a controversial issue (Sequeira and Turecki, 2006). A variety of valid statistical analyses are employed by investigators, even across the same microarray platform (Thu et al., 2010), and criteria for establishing the threshold are somewhat arbitrary. This is true even for expression arrays that have been in use much longer and for which analysis is more developed. ChIP-on-chip tiling arrays, such as is a meDIP-on-CpGi array used by this study, requires even more complex algorithms to detect signals as signals should theoretically peak across probes that neighbor each other in chromosomal location. Therefore, not only does a statistical threshold need to be set, but also a threshold for fragment size to indicate the number of probes the signal is likely to tile must be selected. As well, the program needs also to consider possibility of technical error obscuring biological significance if, for example there is a gap in the peak signal in one probe due to poor hybridization with that particular probe.

4.3.2 Biologically Significant Differences

The signals of *Hoxa11* and *Tox* detected by the microarray and quantified by pyrosequencing showed less than 5% methylation trend differences. Pyrosequencing is regarded as the gold-standard technique for quantification of methylation. It confers high resolution quantification both in terms of being able to quantify methylation at individual CpGs, with greater resolution than MALDI-TOF mass spectrometry, and detect small percent differences (Weber et al., 2005; Tost and Gut, 2007; Laird, 2010). Therefore, the differences it reported are most likely the true values. As such, the seemingly small differences do not preclude their biological significance.

Studies report of a wide range of percent methylation differences that associate with different phenotypes and/or exposures anywhere from 1% to 100% (Schwarz et al., 2010; Weaver et al., 2004; Heijmans et al., 2008). For example, Schwarz et al. (2010) studying methylation of
estrogen and progesterone receptor genes over development between genders and with the administration of estrogen, detected 2-5% differences between groups at various CpGs that changed over time. In a different study, Weaver et al. (2004) detected a change at a single CpG site in the promoter region of the glucocorticoid receptor that associated with high maternal care and low stress response when it was fully hypomethylated (~0%), and with low maternal care and high stress response when it was fully hypomethylated (~100%). This implies that a variety of magnitudes of methylation differences are biologically significant.

*Hoxa11*, specifically, has been reported to vary in methylation in breast cancers 3 fold from 15-75% (Ronneberg et al., 2011), and in extrahepatic cholangiocarcinoma human cells to be abnormally hypermethylation to above 90% (Shu et al., 2011). These large percent changes were associated with extreme states of disease. Extending this to the converse situation, small in magnitude changes could be associated with less pronounced phenotypes such as a difference in timing of puberty within a population of normal healthy B6 mice.

In summary, few and only subtle differences were detected between early and late maturing mice, raising questions regarding the biological significance of this and whether the results simply represent false positives within the array data. Determining if these data are false positives cannot be done without extensive further evaluation, as outlined in Chapter 5.

From the results of this study arise many questions that open exciting avenues of investigation. These opportunities for investigation are beyond the scope of this project but they are discussed in Chapter 5 as future directions.
Chapter 5

5 Future Directions

5.1 Epigenetic Regulation of Pubertal Timing

No published study has yet explored the role epigenetic mechanisms may play in regulating the timing of puberty. The work in this thesis takes the initial steps in this novel exploration and provides evidence in support of a contributing epigenetic mechanism. 1) Epigenetic differences emerged as a possible explanation of the differences in pubertal timing between inbred mice in a colony and especially between littermates when it was established that genetic background and micro-environmental variables could not explain the variation in this phenotype. 2) The likely existence of an underlying epigenetic mechanism was revealed by the global perturbation of epigenetic marks and resulting significant alteration in the timing of puberty. 3) Few and subtle differential methylation patterns detected at CpG island (CpGi) promoter regions between mice with different timings of pubertal onset suggest that there may be stable methylation marks at key genes that may affect pubertal regulation. Taken together, the results of these experiments provide a sufficient cause for future investigations to proceed with further exploration of epigenetics in pubertal regulation. They have opened up a novel avenue of exploration – epigenetics – that offers exciting possibilities to enhance understanding of the biology of such a fundamental and monumental developmental process. The key direction now is to explore the specific genes under epigenetic control by determining a) the marks present, b) whether these epigenetic marks confer transcriptional control, and c) how the differential expression of these genes affects puberty.

5.1.1 Identifying Differential Epigenetic Marks

The differential methylation patterns detected by Agilent’s Mouse CpG Island microarray between early and late maturing mice were not fully replicated in two candidate genes by bisulfite sequencing techniques (Chapter 4, section 4.2 Results). Given that experimentation of Chapter 3 showed significant changes in pubertal timing when the abundance of methyl-donors was increased with the administration of L-methionine (MET) and there was a resulting likely increase in DNA methylation, it indicates that differential methylation patterns can play a role in
regulating pubertal timing. It is, thus, likely that differential methylation patterns are present in mice with naturally occurring differences in pubertal timing. One goal of future experimentation could be to identify these marks.

Investigating whether the signals of the array are an accurate representation of true methylation status of hypothalamic genes in female mice with different onsets of puberty would require extensive experimentation. There is the potential that the array used may not be able to detect important differences in methylation because 1) the CpGi regions covered by the array are not the ones differentially epigenetically associated with pubertal timing, 2) the magnitude of a biologically significant difference in methylation is so small that the array is not sensitive enough to accurately detect it given the limitations of meDIP and/or number of samples used. Thus, to test these possibilities 1) the experiment could be re-done on a different array covering a random, thus unbiased, variety of different regions, such as the comprehensive high-throughput array for relative methylation (CHARM) developed by the Feinberg Lab (Irizarry et al., 2008), or using the fully comprehensive NimbleGen whole mouse set of 10 tiling arrays per sample (Roche NimbleGen, Inc); 2) apply more sensitive methods of quantifying methylation differences using next generation sequencing (NGS), which involves quantifying every nucleotide at high resolution (Metzker, 2010).

Conversely, although two genes, albeit the two with the best signals, were assessed for validation and did not fully validate, these data pertain to only 2 genes and do not preclude that true positive signals were detected by the array. One method to test this could be to bisulfite sequencing a greater number of genes. Gene options are, however, limited because very few significant differential methylation patterns were detected. Sequencing genes that showed no differences, on the other hand, could be informative as it may allow for determination of whether the array worked--if the validation method shows clear significant differences where there were none detected by the array the data would imply that the obtained array data are not reliable.

Future experiments could be performed independent of array technology. The goal of using the microarray platform was for unbiased discovery of genes and pathways. A complementary approach would be to choose candidate genes based on their known biological role in pubertal pathways without considering signal information provided by the microarray. One such gene is Lin28b, the mouse orthologue of the human LIN28B gene identified by genome wide association
studies (GWAS) in females as the gene with the greatest association with age at menarche (AAM) in the general population (Elks et al., 2010). Also, Lin28a, its functional homologue, has been shown to delay puberty in female mice when over-expressed (Zhu et al., 2010). Clearly, the control of its expression by an epigenetic mechanism, if one is present, would be functionally significant. Thus, as a putative repressor of puberty, if it were epigenetically regulated and the epigenetic control of transcription followed the typical methylation-dependent repression, Lin28b would be expected to be more highly methylated, thus less active, in early onset of puberty mice, and vice versa in late onset mice. A 1187bp CpGi with 152 CpGs is annotated at its promoter region located on chromosome 10 at position 45205488-45206674 (UCSC genome browser). Due to its length, an efficient quantification method to screen for changes across the region would be MALDI-TOF based bisulfite sequencing, which would allow for relatively large regions to be quantified in a single assay. Pyrosequencing could then be employed as both validation and more in-depth analysis of each individual CpG in a shorter stretch determined by MALDI-TOF to be of interest.

Also of interest for future investigations would be to determine which genes were altered in Chapter 3 in correlation with changes to pubertal timing when epigenetic modifying agents suberoylanilide hydroxamic acid (SAHA) and MET were administered. Due to the opposite actions of the agents, it is possible that some of the same genes were affected by the two agents. A comparison between naturally early and MET advanced puberty mice, as well as between naturally late and SAHA delayed mice would also be informative, shedding light on gene susceptibility to epigenetic differences. It may be that only a subset of genes involved in pubertal regulation are susceptible to differential epigenetic regulation and are the only ones that change regardless of the origin of the methylation differences. The converse could be true as well, where susceptibility is specific to the origin of methylation (discussed in section 5.1.4). One could suggest reasons why this experiment would or would not be successful. Given the subtleness of the differences detected in chapter 4 between mice whose timing of puberty differed by 7 or more days, differences may be even less pronounced in agent treated mice who were less different in pubertal timing, albeit significantly so, thus making detection difficult. On the other hand, these agents may work to reduce variability and thus enhance detection of differences.
5.1.2 Transcriptional Effect of Differential Marks

Identifying methylation differences associated with differential pubertal timing in a gene would not prove that the gene is under epigenetically mediated transcriptional control. In certain instances, hypermethylation does not lead to repression and conversely, hypomethylation may not correlate with increased expression (Weber et al., 2007a). In the cases when it does, however, the correlation is profound and could be exciting. Excitement stems from discovering a novel gene that has the potential to be functionally relevant in pubertal regulation and from understanding the complex and dynamic nature that could exist between its methylation status and expression across development. Tang et al. (2011) show that within a single phenotype, they found three distinct epigenetic changes that correlate with expression: permanent, concealed and nimble. Permanent methylation marks are set prenatally and persist unchanged throughout all postnatal life, with unchanged expression of the gene. Concealed marks surface only at key developmental times and act to switch gene expression on or off at that time. Nimble marks are dynamic and could be predictive of later onset phenotypes.

Gene expression of candidate genes could be measured by real-time quantification of levels of mRNA via reverse transcription polymerase chain reaction (qRT-PCR) in early and late maturing mice sacrificed at vaginal opening and postnatal day 39. Measuring expression and methylation prior to puberty as an additional time point could prove to have predictive utility.

5.1.3 Gene Function

The next step in these future discoveries would be to understand the functional significance of the epigenetic-mediated expression changes. A fundamental question to ask would be does the gene regulate puberty? The relationship between methylation and expression changes and puberty remains correlative until cause and effect can be proven. This can be achieved by employing a loss of function strategy in vivo or in vitro via gene knock out (KO) in a mouse model or introducing small interfering RNA (siRNA) to cell culture, respectively, and assessing the affect to pubertal timing.

In KO models, theoretically, the option exists to either fully or partially decrease the presence of the gene by either reducing the amount of mRNA expressed or altering the mRNA so it produces non-functional protein. This can occur in the whole body or in a specific tissue and/or throughout
life or at a specific time point for a given duration. For example, KO mouse models exist for *Hoxa11* and *Tox* (Aliahmad and Kaye, 2008; Lewis et al., 2003), the two candidate genes detected on the microarray in Chapter 4. The *Hoxa11* KO mouse has thus far been used to study male fertility (Lewis et al., 2003), finding *Hoxa11* null mice to be infertile unless they undergo orchiopexy, the surgical descension of testes into the scrotum. Thus, subsequent studies in these mice focused on understanding the mechanism of this impairment by studying the development and anatomy of the gubernaculum, a part of the testes, in these mice (Nagraj et al., 2011). By observing a general alteration to a phenotype, loss of fertility, and identifying a putative cause, impaired testicular descent, loss of function investigation can lead from answering the general question of “does this gene affect the timing of puberty?” to answering the more specific question of “how?”. The question of “how?” can be further expanded by testing gene function in *in vitro* models. If the gene is hypothesized to play a role in the hypothalamus during puberty, it may be of interest to study the mechanism of its action on GnRH neurons directly. Many avenues of functional investigation exist once a candidate gene has been identified. Epigenetic investigations hold promise of discovering novel genes important to the regulation of pubertal timing.

### 5.1.4 Expanding the Explorations

Exploration of epigenetic mechanisms could also be expanded to questions about a) the origin of the differential epigenetic mark, b) their tissue specificity, c) gender differences and d) role in humans.

A) Differential DNA methylation may arise from stochastic events (Feinberg and Irizarry, 2010; Petronis, 2006) or from differential environmental exposures. For example, the Barker hypothesis dictates that an adverse in utero environmental exposure can program (epigenetically) the trajectory of later life developmental processes (Hales and Barker, 1992; Dolinoy et al., 2007). One possibility is that pubertal development is *in utero* epigenetically programmed, and investigations could be directed to exploring the programming effects of such environmental exposures as the increased prevalence of endocrine disrupting chemicals (EDCs) in the environment and poor nutrition, such as over eating. These exposures could be a cause of *in utero* programming or could also affect the epigenome and modulate pubertal timing secondary to postnatal exposure. B) The pituitary and ovaries are also important tissues in the regulation of
pubertal timing and may house important epigenetic marks that regulate their function. C) Gender differences exist not only in gonadal anatomy and production of sex steroids – estrogen vs. testosterone – but also in hypothalamic nuclei as sexually dimorphic regions housing pubertal pathways have been identified, differing in the distribution of kisspeptin neurons and estrogen receptors (Clarkson and Herbison, 2006; Cao and Patisaul, 2011). There may also be sex specific differences in epigenetic regulation. D) Ultimately, investigating puberty in mice is used as a proxy for understanding human biology. Once important regulatory marks are identified, one could explore whether these are also present in blood samples, which would allow for investigation in human samples. As such, these marks may have diagnostic, predictive and/or preventative value (Bouchard et al., 2010; Kelly et al., 2005).

Exploration of the epigenetic mechanisms in pubertal timing is a novel idea. For the first time, it is shown that differential DNA methylation or histone acetylation patterns may be functionally involved in regulating the onset of puberty. The work of this thesis opens the doors to the multitude of avenues these explorations can take and holds promise of enhancing knowledge of pubertal biology.
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