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WASHINGTON UNIVERISITY IN ST. LOUIS

Division of Biology and Biomedical Sciences

Computational and Systems Biology

Dissertation Examination Committee:
Patrick Jay, Chair
Sessions Cole
Donald Conrad
Kelle Moley
David Wilson

Origin of Maternal Age Effect in Congenital Heart Disease Risk for Offspring

by

Claire Elaine Schulkey

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

December 2014

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September 2014

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List of Abreviations

CHD – congenital heart disease

VSD – ventricular septal defect

C57BL/6N – C57 black six mice from Charles Rivers Laboratory line

FVB/N – FVB mice from the Charles Rivers Laboratory line

SNP – single nucleotide polymorphism

CNV – copy number variation

PCR – polymerase chain reaction

RRBS – reduced representation bisulfite sequencing

DMR – differentially methylated regions

FDR – false discovery rate

GO – gene ontology

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Financial and academic support for this project was generously supplied by a Ruth L. Kirschstein National Research Service Award from the Developmental Cardiology and Pulmonary Training Program (National Institues of Health/National Heart, Lung, and Blood Institute, T32 HL007873) and by the Lucille P. Markey Pathway in Human and Pathobiology. Additional Support from Dr. Jay's lab was supplied by the American Heart Association, Edward Mallinckrodt Jr. Foundation, Hartwell Foundation, the Child Health Research Center of Excellence in Developmental Biology at Washington University School of Medicine (NIH K12-HD001487), Children's Discovery Institute, Children's Heart Foundation, Hartwell Foundation, March of Dimes (1-FY07-453), and the National Institutes of Health (HL105857).

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Dedication

I'd like to dedicate this thesis to all the strange, wonderful, creative people who have been a part of my life during this journey. Though they sometimes might not have understood what I was doing, whether family by blood or heart they always believed I would complete the trip.

ABSTRACT OF THE DISSERTATION

Origin of Maternal Age Effect in Congenital Heart Disease Risk for Offspring

by

Claire Elaine Schulkey

Doctor of Philosophy in Biology and Biomedical Sciences (Computational and Systems Biology)

Washington University in St. Louis, 2014

Associate Professor Patrick Jay, Chairperson

Increasing maternal age is widely acknowledged to lead to greater likelihood of pregnancy complications and congenital abnormalities, but the basis of this effect has not been well studied. Often dismissed as the product of oocyte ageing, the mechanistic basis of this maternal age effect is likely more complex.

Congenital heart disease is a classic complex disease with multiple genetic and environmental modifiers, including maternal age. Maternal ageing is a known risk-factor in humans, and has been shown to exist in an *Nkx2-5* haploinsufficient mouse model for the disease. This mouse model's maternal age risk is dependent upon strain background, with C57BL/6N pure line and FVB/N x C57BL/6N F2 intercross pups being at risk due to maternal ageing, and A/J x C57BL/6N F2 intercrosses showing no maternal age risk. This indicates a maternal genetic component to maternal age risk, and implies that though ageing is inevitable, the negative effects on offspring are not. Using this model, this study examines whether the maternal age effect is due to oocyte ageing or a maternally intrinsic factor, shows a remediating treatment for maternal age risk, and defines epigenetic changes in offspring resulting from maternal ageing.

Reciprocal ovarian transplants between old and young FVB/N x C57BL/6N F1 mothers were used to localize the basis of the maternal age effect to the mother. In spite of ovulating from ovaries aged well beyond the mouse's normal reproductive life span, young mothers were at no higher risk for ventricular septal defects (VSD), while old mothers showed a persistent high risk for VSD in spite of ovulating young oocytes.

Voluntary exercise experiments where FVB/N x C57BL/6N F1 mothers were given access to running wheels over the course of their lifetime showed that exercise decreased maternal age risk to levels indistinguishable from that of young mothers. Additionally, late-onset exercise was shown to be effective at reducing maternal age risk after just three months' exposure, even with no overt changes in body mass, composition, or glucose tolerance.

To study the impact of maternal ageing on epigenetic profiles, reduced representation bisulfite sequencing was used to compare aged and young sedentary fetal hearts and aged exercise fetal heart tissue. These comparisons showed eight differentially methylated regions, altered by maternal ageing but recovered by exercise treatment.

These studies are conclusive proof that nonsyndromic maternal age risk is not due to oocyte ageing, but instead to a modifiable, maternally intrinsic risk factor. These studies also suggest the possibility of exercise as a prescription to prevent or turn back maternal age's negative impacts. Exercise as an intervention poses tempting possibilities as a safe intervention for at-risk populations. Further investigation into the mechanistic influence of epigenetics in this effect may identify risk biomarkers for testing in maternal populations, and may provide keys to the underlying genetic architecture for congenital malformations such as congenital heart disease.

Chapter 1: Introduction and Background

1.1 Introduction

Congenital heart defects (CHD) represent a source of potential life-long complications (Diller GP, 2011) but also a well-studied animal model for birth defects in humans (Kodo K, 2011). Knowledge gained from a study of CHD risk modifiers will inform this particular disease, but may also have implications in other congenital malformations such as clubfoot and various right-left patterning defects. Congenital heart disease is a classic complex disease (Nora, 1968), presenting most often with a few critical causative mutations and a wide variety of modifiers which influence the type and severity of congenital anomaly (Gruber PJ, 2004) (Wang X, 2014). Modifier loci associated with membranous ventricular septal defects (VSD) have been discovered, along with a link between advanced maternal age and heart defect incidence in susceptible offspring (Winston JB S. C., 2012). Advanced maternal age and CHD have an established correlation in humans (Miller A, 2011) which is mirrored in a mouse model. To date, several large scale epidemiology studies have been conducted in humans showing a link between advanced maternal age and a risk for non-chromosomal anomalies (Hollier LM, 2000) and CHD specifically (Materna-Kiryluk A, 2011), as well as strong correlations between certain maternal exposures to CHD risk (Patel SS, 2013). Different reporting standards for type and severity of congenital malformations in addition to the complexities of the underlying disease etiology have confounded comparison between studies (Hoffmal JI, 2002). A small subset of studies showed no effect of maternal age on birth defect incidence, and one of the studies showed a strong regional variation in the maternal age effect in humans, indicating a genetic component interacting with maternal age to increase susceptibility to congenital malformations. This interaction is mirrored in our mouse model.

The causes of these maternal age effects have not been well studied (Reefhuis J, 2004). Whether the effect is based on oocyte degradation or copy number variation, hormonal changes, increasing rates of late-life obesity and diabetes in older mothers, age related methylation imprinting changes or an unknown factor, there is little evidence for any of these as the studies have simply not been performed. Without further knowledge regarding the causes of the maternal age effect, remediating recommendations cannot be investigated.

1.2 Background and Significance

The impact of modifier loci for congenital heart disease is likely small, and identification and intervention for at-risk fetuses costly for relatively narrow gains. By understanding the basis of environmental modifiers such as maternal age, wide recommendations can be made for generally at-risk populations with the goal of preventing or significantly lowering risk. The most widely cited oocyte-intrinsic aging effect results from non-disjunction events during meiosis leading to chromosomal aneuploidy (Hollier LM, 2000). Other possible oocyte intrinsic effects could be caused by degradation of mitochondria or energy metabolism.

Often written off as a simple product of oocyte aging and chromosomal degradation, the true cause of the maternal age effect in humans and mice is more complex. An understanding of the contribution of oocyte aging as a component of maternal aging versus the effect of maternal aging alone must be reached before further research can be undertaken to identify specific modifiers and potential remediating factors. Many oocyte extrinsic factors associated with maternal aging have been shown to influence quality of offspring including maternal obesity and insulin resistance, previous number of pregnancies and various dietary deficiencies, most notably folic acid (Catalano PM, 2006).

Maternal obesity leads to a cascade of health problems for mother and fetus including insulin resistance and diabetes (Alshami HA, 2011), high birth weight, maternal and fetal hypertension (Jarvie E, 2010), nutrient imbalances and miscarriage (Kroon B, 2011) (Helgadottir LB, 2011). It has also been shown to be associated with incidence of CHD in human offspring (JL, 2010). Mirroring human aging, our mice gain weight over their lifetime with a 30 g weight gain on average in mothers from early to late reproductive life (Fig 1.1). If weight gain is to blame for the maternal age effect,

keeping mothers slimmer over the course of their reproductive lives may mitigate this effect. Conversely, early obesity may induce an old-mother-like effect.

Since little is currently known of the mechanistic basis of this ageing effect, studies of maternal metabolic change with ageing and alteration to epigenetic markers will address two major players in maternal imprinting and fetal developmental risk.

The mouse model for congenital heart defects (CHD) is a C57BL/6N inbred strain with an *Nkx2-5*$^{+/-}$ haploinsufficiency which results in elevated risk for a variety of heart defects matching those seen in humans. Ventricular septal defects (VSD) are used as a benchmark defect, as their binary diagnosis (presence/absence) is highly reliable (Fig 1.2), and they represent the most common defect diagnosed within our mouse population, and among humans. Animals identified as genetically at risk demonstrate a particularly serious risk due to maternal age (Fig 1.3). Mice are harem mated and their environment, diet, and level of genetic heterogeneity can be easily controlled. These C57BL/6N mice are crossed with both wild type FVB/N and A/J lines for the purpose of linkage mapping (Fig 1.4), but these crosses have revealed important interactions between maternal genetics and maternal age risk. These F2 intercross offspring are used for intervention studies (exercise, high fat diet) as well as providing tissue for analysis of epigenetic markers. Given that the F1 hybrid show a hybrid vigor and reduced incidence of disease (Winston JB E. J., 2010), F2 animals which have a recovered incidence are used for all experimental groups.

The neonates are euthanized, their hearts are dissected from the torso, and tissue is saved for genotyping. In the case of animals destined for methylation studies, heart, limb, and liver tissues are isolated and flash-frozen in liquid nitrogen immediately after death. For those neonates destined to be diagnosed, their hearts are fixed in formalin and EtOH, paraffin-embedded, and serially sectioned

at a thickness of 6 microns. Hearts are stained and diagnosed by two trained diagnosers for presence, type, and severity of any congenital heart defect. In the case of disagreements, a third trained diagnoser arbitrates, and the decision is entered into an Access database containing all diagnosis conducted since the formation of the lab.

NKX2-5 is a cardiac transcription factor conserved through cnidarians, and is critical for cardiac development. Complete knockouts of this cardiac transcription factor are embryonic lethal at day 10.5 due to a failure to form a heart (Schott JJ, 1998). Haploinsufficiency for this transcription factor leads to an increased incidence of all types of congenital heart defects in mice (Lyons I, 1995) and humans (McElhinney DB, 2003). The most common type of defect observed are ventricular septal defects (VSD) which appear at varying incidences in C57BL/6N pure lines, and FVB/N and A/J F2 intercross animals. Historically studies have focused on genetic modifiers of transcription factors like NKX2-5 to find possible avenues for intervention in disease development. This can be difficult, as even when modifiers are identified, they are not necessarily in a pathway targetable by pharmaceuticals or of low enough toxicity to administer to pregnant women. Our knowledge of the mouse model provides a powerful opportunity to perform highly controlled epidemiologic experiments on a well understood model organism population. Understanding the basis of the observed maternal age effect in mice will help develop interventions for at risk populations of human mothers. The maternal basis for the maternal age effect, and the influence of epigenetics and lifestyle are the focus and scope of this study.

1.3 Conclusion

It is the goal of this study to discover the basis of the maternal age effect in mice, investigate environmental interventions for this maternal age effect, and identify possible genetic or epigenetic components to the maternal age effect. Understanding the basis of the maternal age effect seen in congenital heart disease has strong implications in other congenital malformations with similar etiologies, and in the treatment recommendations of elderly pregnancy. Through careful observation of sample cohorts, and rigorous statistical methods, environmental interventions can be tested on model organisms for eventual deployment into human populations. Similarly, epigenetic markers discovered through bisulfite sequencing suggest new genetic regions which may play a part in disease risk beyond mutations. The information gathered in these studies can be used to suggest human interventions and treatments for at-risk mothers and their offspring.

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1.5 Figures:

Figure 1.1 Weekly average of non-pregnant sedentary C57BL/6N x FVB/N F1 mothers. From the initiation of breeding at three to four weeks of age to last pregnancy at >400 days, mothers gain between twenty and thirty grams of weight representing a doubling in their body mass over a breeding lifetime.

Figure 1.2 Two types of ventricular septal defects (VSD) diagnosed in our mouse population compared with a normal heart. Membranous VSDs (A) are holes directly under the aorta between the left and right ventricles. Muscular VSD (B) are holes through the muscular column of the septum which can form at any portion of the septum. Normal hearts (C) have no tunneling or thin patches in their septum, and connect fully around the base of the aorta.

Figure 1.3 Multiple logistic regression models estimate the incidence of membranous ventricular septal defect (VSD) in the FVB/N x C57BL/6N *Nkx2-5*$^{+/-}$ F2 population as a function of bearing a susceptibility genotype at 0, 1, or more of the chromosomes 6, 8, and 10 loci and maternal age. The impact of a single month of ageing is small, but when added over the course of a breeding lifespan, can be profound. OR indicates odds ratio; CI, confidence interval.

Figure 1.4 The breeding strategy for our intercross animals. The pure line F0 generation is inbred, the C57BL/6N experiencing an NKX2-5 mutation which confers a risk to all types of congenital heart disease(CHD). The FVB/N and A/J wild type animals experience no risk to CHD. The F1 cross experiences a very low risk for CHD due to hybrid vigor, while the F2 generation recovers risk of CHD in both intercross and backcross breeding, indicating the presence of homozygous recessive modifier genes.

Figure 1.1

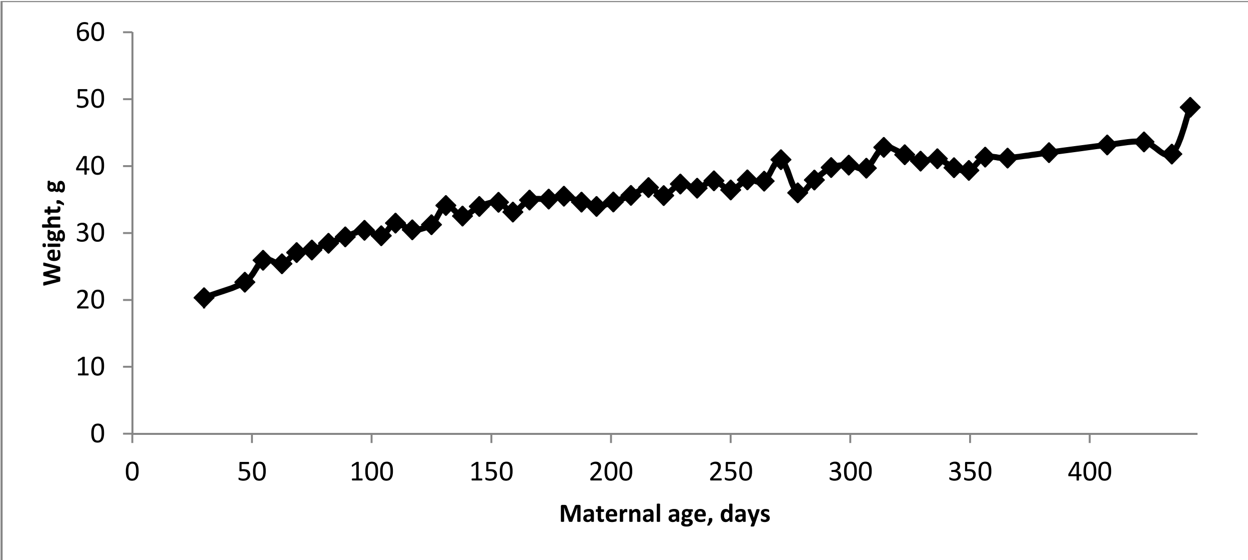
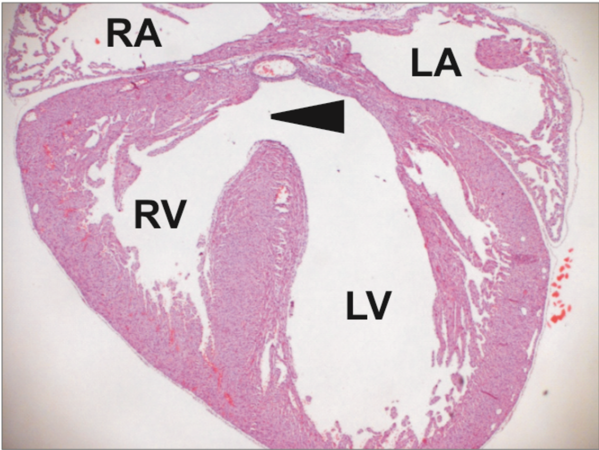
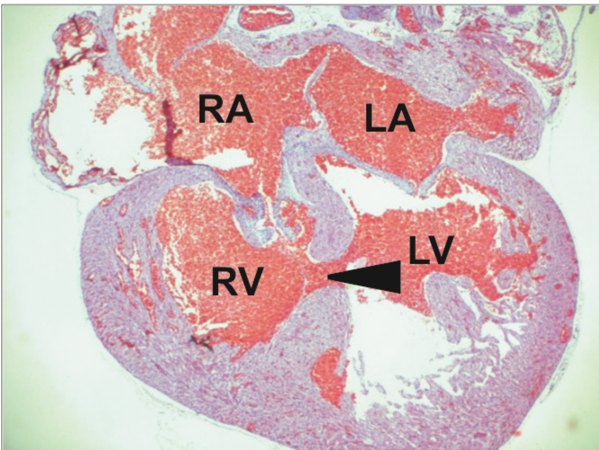


Figure 1.2





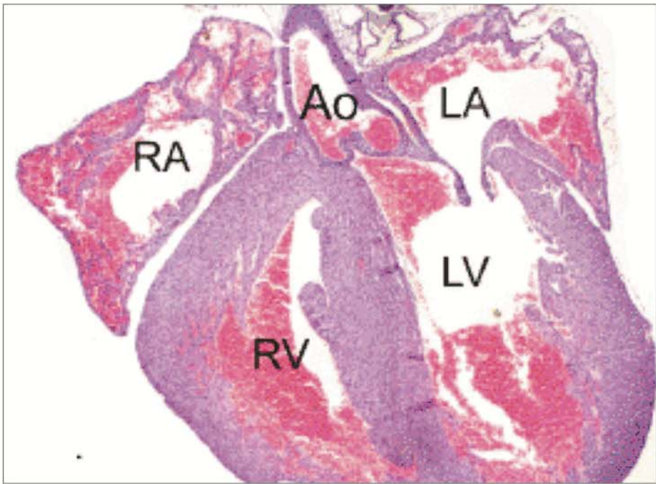
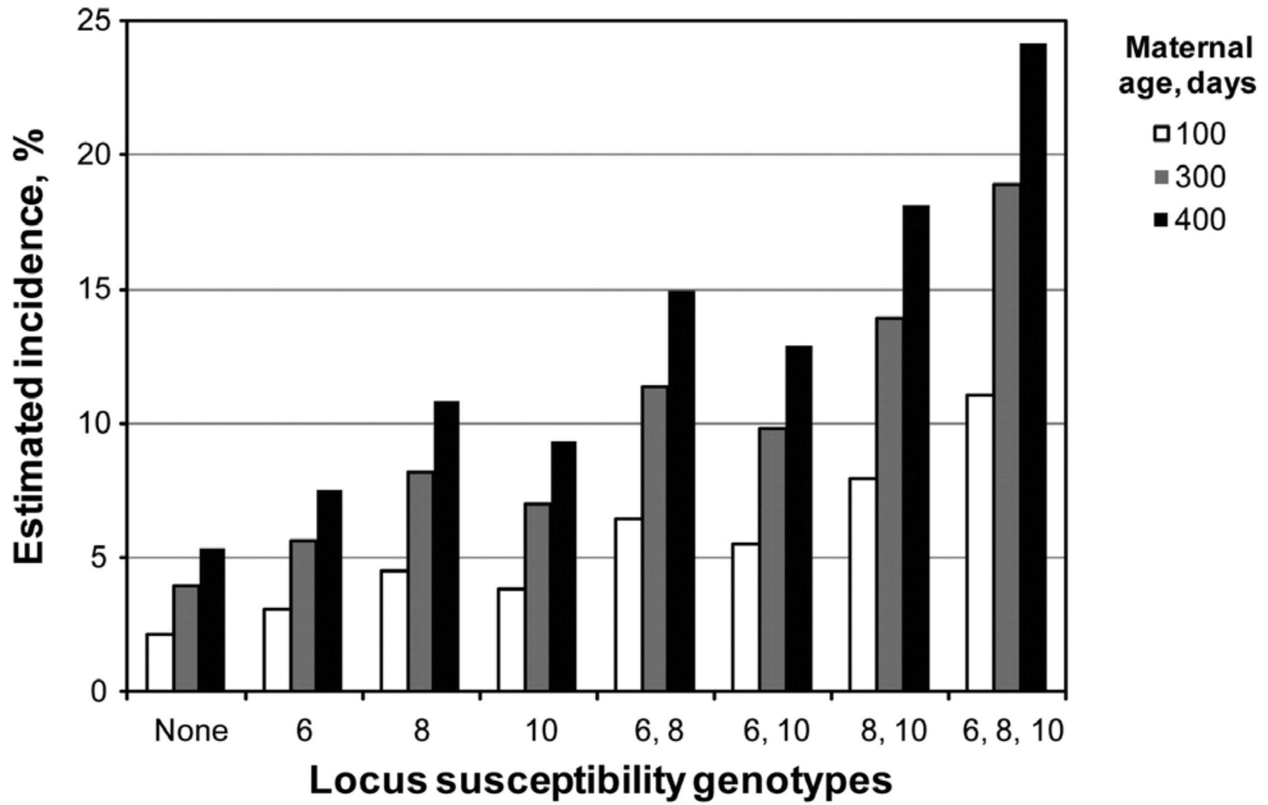


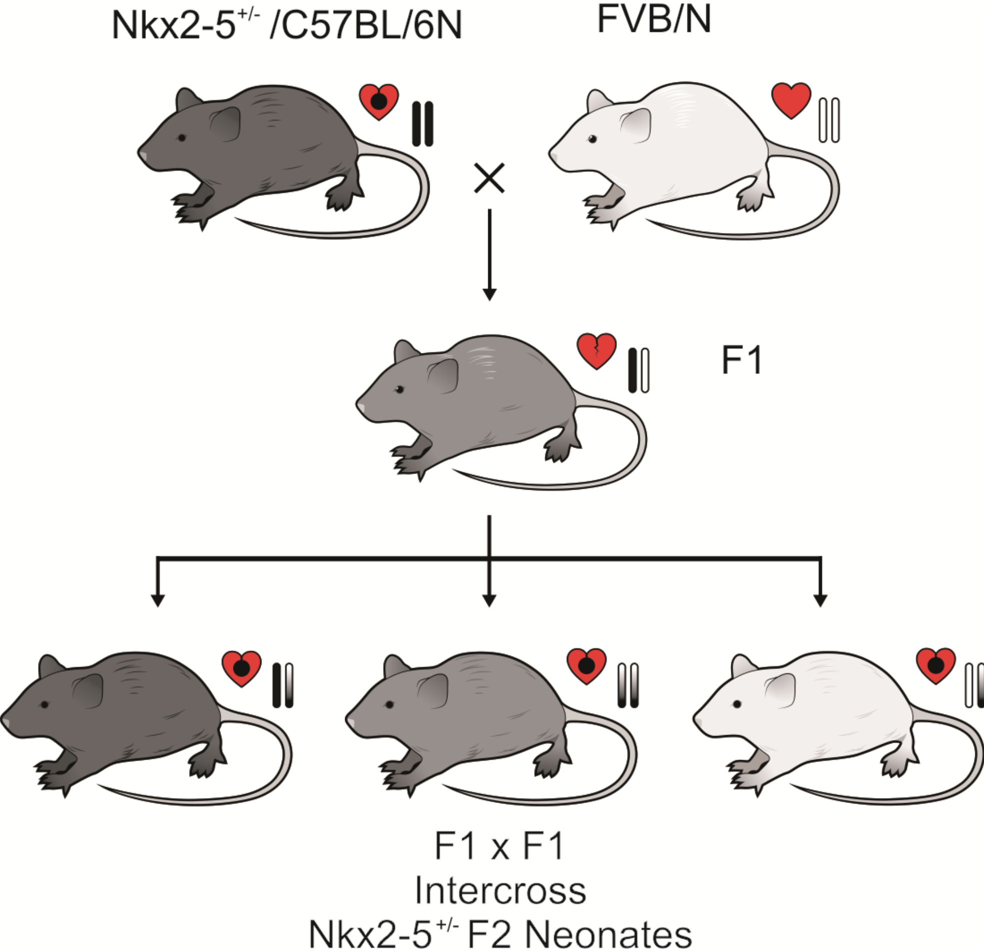
Figure 1.3



Factor	OR	95% CI
Maternal age, months	1.10	1.05-1.15
Chromosome 6	1.45	1.16-1.81
Chromosome 8	2.15	1.70-2.74
Chromosome 10	1.82	1.45-2.29

*Figure from Winston, et al 2010

Figure 1.4



Chapter 2: Localization of the maternal age effect reveals an ovarian extrinsic/maternally intrinsic origin

2.1 Abstract

Advanced maternal age is associated with a higher risk for specific types of congenital heart defects (CHD) in the newborn. This increased risk is often attributed to oocyte aging; however this research implicates a maternally intrinsic effect completely separate from oocyte aging which includes a component of maternal genetics.

Using our *Nkx2-5*$^{+/-}$ mouse model in a pure line C57BL/6N, and C57BL/6N x FVB/N F2, C57BL/6N x AJ F2 intercrosses, I showed a maternal age risk for ventricular septal defects (VSD) that was dependent upon strain background. Chromosomal aneuploidy was investigated using the R.sqnm statistical package designed to detect regions of chromosomal aneuploidy using data from genome-wide SNP genotyping. To determine if the maternal age effect was oocyte intrinsic or maternally intrinsic I performed a series of reciprocal ovarian transplants whereby old mothers received and appropriated young ovaries and vice versa.

I showed that maternal age risk was strain-dependent. No evidence for chromosomal aneuploidies of >10 cM were found in any normal or disease samples from old or young mothers. Using ovarian transfers it was found that the risk of VSD tracked with the age of the mother and not the age of the transplanted ovary ($p < 0.01$). Thus the age of the oocyte has no discernable effect the incidence of VSD.

The common assumption that advanced maternal age causes an increased risk of congenital malformations due to oocyte ageing or decreasing oocyte quality is faulty.

2.2 Introduction and lit review

The timing of pregnancy and parenthood for all species is critical. Too young and the offspring overtax the underprepared maternal systems and both parties suffer. Too old, and the offspring tend to have more congenital abnormalities and suffer from chromosomal nondisjunction events, leading to syndromic issues like Down Syndrome. The human window for ideal maternal age is thought to be between 20 and 30 years of age, with mothers >35 years classified as "elderly pregnancy" and teenage mothers suffering from a variety of poor pregnancy outcomes. Similarly, in mice, the ideal mating period is between 50 and 200 days of age, with dams often sacrificed after this window, in spite of the fact that some dams remain fertile well beyond their standard reproductive timeline. With the increase of average age of first pregnancy in humans from 21 to 25 between the years of 1970 and 2006 (Mathews TJ, 2009), the developed world is facing a rise in the age of mothers, and comorbid, a potential rise in poor pregnancy outcomes associated with advanced maternal age.

Though it is understood that the offspring of elderly pregnancy in humans, and similarly the offspring of older mothers in model organisms like mice (Hollier LM, 2000) (Winston JB, 2012), are subject to higher rates of congenital abnormalities, the mechanistic basis of this maternal ageing effect is not understood. Females are born with the majority of the eggs which they will ovulate over the course of their lifetime, and thus this maternal age effect is often thought to be a product of ovarian and oocyte ageing: in essence, the eggs 'go bad' over the course of a female lifetime. There have, however, been no known studies on the effect of oocyte ageing on non-chromosomal diseases associated with maternal ageing.

Human epidemiology studies have showed discordant maternal age effects in relation to congenital heart disease. As one of the most common congenital malformations, it affects between 1 and 2% of

live births in developed countries. Studies by Miller and Materna-Kiryluk show a significant relative risk related to maternal ageing while Baird showed no rise in risk for aged mothers. Though in some minds this might suggest poor study design on the part of one or the other group, a more valid inference would be the presence of a maternal genetic component to congenital heart disease's maternal age risk. Indeed, if some groups carry maternal age risk alleles while others do not, it would very simply explain this appearing/disappearing maternal phenotype noted in human populations.

Chromosomal abnormalities -- aneuploidies or chromosomal copy number variations -- are both associated with risk for congenital heart disease (Acevedo-Gallegos S, 2013) and at higher prevalence in aged mothers (Eichenlaub-Ritter, 2012). For more than two decades, the importance of maternal ageing in the risk of chromosomal nondisjunction events has been acknowledged as an important etiological factor (Brook JD, 1986). The mechanism of this increasing risk of chromosomal abnormalities seems to be a result of decreasing chromosomal cohesion (Duncan FE, 2012) (Eichenlaub-Ritter U. , 1998). In the past, copy number variants have been used to identify patterning defects associated with congenital heart disease (Fakhro KA, 2011), lending credence to the idea that this maternal ageing phenotype may be a result of increased risk of copy number variation with maternal age.

Transplantation of ovaries between histocompatible dams has been carried out with both fresh and cryopreserved tissue (Dawes J, 2010) (Liu LJ, 2008) leading successfully to long term fertility. Though this technique is most often used in the preservation of rare transgenic mouse lines, this technique is also a powerful tool in the study of maternal ovarian ageing. By cross-transplanting ovaries between histocompatible animals, the age of the ovary can be separated from the age of the dam for study purposes.

2.3 Methods

Copy Number Variation Analysis

SNP markers were chosen for the 19 autosomes at an average density of 15-20 cM (N = 120). The density of SNP markers was increased to ~5 cM around mapped loci of import. SNPs were also genotyped on the X (N =3) and Y (N = 1) chromosomes for mapping the X chromosome and sex assignment. Multiplexed SNP genotyping was performed on a Sequenom MassARRAY system and executed by a core facility. Chromosomal aneuploidy and copy number variation (CNV) in the *Nkx2-5*$^{+/-}$ F2 intercross progeny was evaluated using the SNP allele ratio (SAR) algorithm in the R.SQNM software application. The method plots the amounts of PCR product for the two alleles of a SNP assay against each other across the entire genotyped population. The distributions of the three genotypes, i.e., homozygous for either parental allele or heterozygous, are then determined. A genotype that falls outside the distributions is flagged as an outlier or potential CNV. Mixtures of FVB/N and C57Bl/6N genomic DNA in 1:2, 1:3, 2:1 and 3:1 ratios were used as positive controls and correctly identified at a rate > 85%.

Ovarian Transplantation

Mice were anesthetized with a cocktail (0.5-0.7 ml/kg) of 3:3:1 ketamine (100 mg/ml), xylazine (20 mg/ml) and acepromazine (10 mg/ml) via intraperitoneal injection. A small incision was made in the dorsolateral flank to access the ovarian bursa. The ovary was removed from the donor and then transplanted into the recipient. The native, contralateral ovary of the recipient was left in place, while the oviduct was ligated. Transplants were performed between C57BL/6N X FVB/N F1 females that were either old (range 244-377 days, mean 317) or young (range 30-81 days, mean 48). The

recipient females were allowed to recover for three weeks before breeding commenced. Pregnant females were noted to carry their fetuses in the uterine horn on the same side as the transplanted ovary.

Statistics

Logistic regression analyses were performed in R to determine odds ratios and 95% confidence intervals for the maternal age-based risk of a ventricular septal defect in various strains and crosses. Odds ratios were calculated per month of aging. The logistic regression analysis was used to calculate the expected incidence of defects in age-matched FVB/N x C57BL/6N F2 ovarian transfer comparisons.

Incidences were compared in chi-square tests.

Statistical significance was defined as $p < 0.05$.

2.4 Results

The maternal age effect has an important genetic component

To understand how subsets of human epidemiology studies show a conclusive maternal age effect on CHD risk in their populations while others show none, I looked at the maternal age effect in three different strain crosses in the mouse model of congenital heart disease. The pure line *Nkx2-5*$^{+/-}$ C57BL/6N mice experience the highest risk for congenital heart disease, and also the most pronounced maternal age effect. They have an odds ration of 1.10 per month of life, which can constitute a 150% rise over the course of a dam's lifetime. The FVB/N x C57BL/6N F1 and A/J x C57BL/6N F1 mothers experience similar risk for congenital heart disease in general, but their maternal age effects are different. The FVB/N cross experiences a risk similar to the C57BL/6N pure line, while the A/J cross has a negligible maternal age risk, significantly smaller than the C57BL/6N pure line. This indicates a clear genetic component in maternal age risk, similar to that experienced by genetically unique subgroups of humans (Fig 2.1).

Copy number variation (CNV) does not play a significant role in the maternal age effect

Copy number variation (CNV) is a well-documented result of chromosomal nondisjunction events in meiosis, resulting in abnormal copy number in whole or partial chromosomes. Chromosomal nondisjunction is associated with syndromic abnormalities like Down Syndrome, occurs at higher frequency in the offspring of old mothers, and is associated with fetal malformations including congenital heart disease. Though there was no indication that our mice were modeling a murine Down Syndrome, the rise in congenital heart disease as a result of a syndromic non-disjunction event with maternal age would simply explain our murine maternal age effect.

To determine if our congenital heart defects were a result of CNV, I examined the PCR product of 252 CHD and normal mice. If the ratio of PCR product was abnormal (i.e., not 1:1 at heterozygous loci) it would indicate CNV in those offspring (Fig 2.2.A). The method I used involved testing the ratios of PCR product in a Sequenom SNP array directly using the SNP allele ratio (SAR) algorithm in the R.SQNM software application. There was no evidence for copy number variation in any of the samples (Fig 2.3.B) in any of the tested SNPs.

Ovarian transplants show the maternal age effect is maternally intrinsic/ovarian extrinsic

The absence of copy number variation, and the fact that maternal genetics play a role in the maternal age effect in CHD called into question the traditional assumption that the maternal age risk was a result of aging oocytes. The question of whether the maternal age effect is maternally intrinsic or ovarian intrinsic is a tricky one to address. Though oocyte transfers and in vitro fertilization could be conducted between mothers of disparate ages, the necessary sacrifice of the donor mother made old-to-young transfers costly, and the breakdown in normal estrous cycling in older mothers makes young-to-old transfers risky. In addition, the hormonal stimulation of mothers for this method, both donor and recipient, would add another unwanted variable into the experiment.

Ovarian transplants offer the unique opportunity to perform whole organ transplant between similar mothers of disparate ages, allowing old mothers to ovulate the eggs of young ovaries, and young mothers to ovulate the eggs of old ovaries, without the intervention of hormonal stimulation. By performing reciprocal ovarian transplants on old and young mothers, I was able to disconnect maternal age from ovarian age while producing pups and litters in numbers large enough to perform statistical analysis.

Old mothers with young ovaries showed a high risk (20%) for VSD, whereas young mothers with old ovaries still maintain a low risk (9.8%) for VSD (Fig 2.3), incidences which were statistically distinct from each other, and from expected model-based risk for an oocyte intrinsic effect. This pattern indicates a maternally intrinsic, oocyte extrinsic source for the maternal age effect. Indeed, in spite of ovulating eggs well past the normal reproductive age of a mouse, the young mothers maintained a low incidence of VSD. In contrast, old mothers ovulating new, young eggs were still at a high risk for VSD. This is counter to the common belief that it is the ageing of the oocytes and ovaries which results in decreased offspring quality with maternal age.

2.5 Discussion

In spite of dramatic improvements in palliative care options for those suffering from congenital heart disease, there is still no method of disease prevention. Drug targeting for pregnant mothers and their offspring is difficult and potentially dangerous in the best case scenario, and though many critical genetic players in cardiac development have been identified, there are currently no feasible drug targets. Adding to this difficulty, there are not good ways to identify the fetuses which will develop heart defects and there is very little time between implantation and the time at which the heart develops to intervene. By studying the source of maternal age risk in congenital heart disease, potential new targets can be identified which may help both in patient stratification and treatment.

Strain-dependence in the presence and severity of the maternal age effect points to a maternally intrinsic source for this maternal ageing risk. Additionally, this strain dependence indicates the reasons for variable results and disagreements between human epidemiology studies on maternal ageing and congenital heart disease risk: human genetic variability could result in maternal age risk in some populations but not others, well explaining the seeming conflict between studies. This strain dependence also shows that though maternal ageing is inevitable, the negative effects experienced by embryos are not.

The absence of large regions of copy number variation is the first clue that embryo ageing may not be to blame for the maternal age effect. Though the screening of SNP data for evidence of CNV would not preclude small regions of chromosomal duplication, it does rule out whole chromosome duplications and other large nondisjunction events. This does not support the hypothesis that the maternal age effect is due to oocyte ageing.

Reciprocal ovarian transplants show conclusively that the maternal age effect is maternally intrinsic, and unrelated to oocyte and ovarian age. This runs counter to the prevalent assumption that the increase in risk for birth defects in the offspring of old mothers is due to her ageing eggs. The transfer of younger oocytes from a donor would not mitigate the problem leading to the higher risk for defects. This does suggest a factor which might be modified within the mother and thus encourage normal development without having to directly influence the embryo. Additionally, provided the maternal treatment had few adverse outcomes, at risk populations could be targeted for treatment without the need to identify specific embryos destined to develop congenital heart disease.

The maternally intrinsic nature of the maternal age effect informs the hypotheses developed for the following chapters. The development of an understanding of how maternal ageing alters with maternal health interventions and epigenetic marks provide important insights in the effect that the maternal environment has on embryonic cardiac development.

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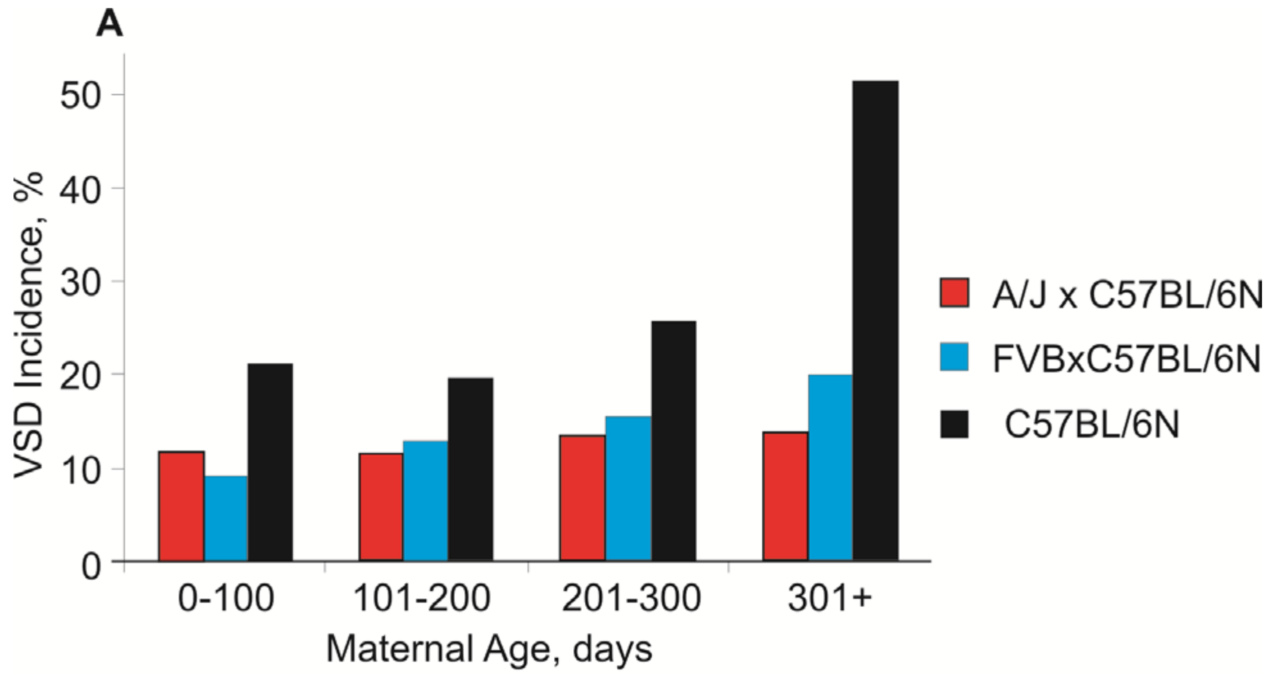
2.7 Figures:

Figure 2.1 Ventricular septal defect (VSD) incidence as a function of maternal age binned in 100-day groups (A). A/J x C57BL/6N experience little to no maternal age risk, and have similar VSD incidence in every maternal age group. The FVB/N x C57BL/6N experience an intermediary maternal age risk, the VSD incidence ranging from less than 10% to greater than 20% from the youngest to oldest groups. The C57BL/6N pure line offspring show the strongest maternal age risk with VSD incidence more than doubling over a mother's lifetime. Maternal age risk is a quantitative genetic trait (B). Odds ratios were determined using multiple logistic regression modeling, and show identical trends to the binned VSD incidence in part A, including a significant difference between C57BL/6N and A/J x C57BL/6N F2 groups. Odds ratios are shown with a 95% confidence interval, the number of pups comprising each group, and mean and standard deviation of maternal age for each group.

Figure 2.2 Copy number variation or chromosomal aneuploidy cannot account for the increase incidence of CHD in aged mothers. Representative plots of the PCR products for the 2 alleles of a SNP genotyping assay are shown. In a typical 96-well assay (A), no animal is flagged as having copy number variation in any particular SNP. By contrast (B), control samples that contain unequal mixtures of parental DNA in 2:1 or 3:1 ratios are flagged as potential copy number variations, based on a SNP allele ratio that falls below a defined clustering strength (red circles) or lies outside the statistically defined cluster (circled triangles) for heterozygotes or homozygotes.

Figure 2.3 The maternal age effect is localized to the mother by ovarian transplant experiments. The observed incidence of ventricular septal defect (VSD) is indistinguishable from the expected incidence of a maternal effect based on logistic regression models. The young mother group shows a significant deviation from an expected oocyte based effect, and the two observed groups are significantly different, indicating a maternally intrinsic, ovarian and oocyte extrinsic effect. Numbers of recipient mothers in each age group, as well as number of pups collected in each observed group are shown.

Figure 2.1



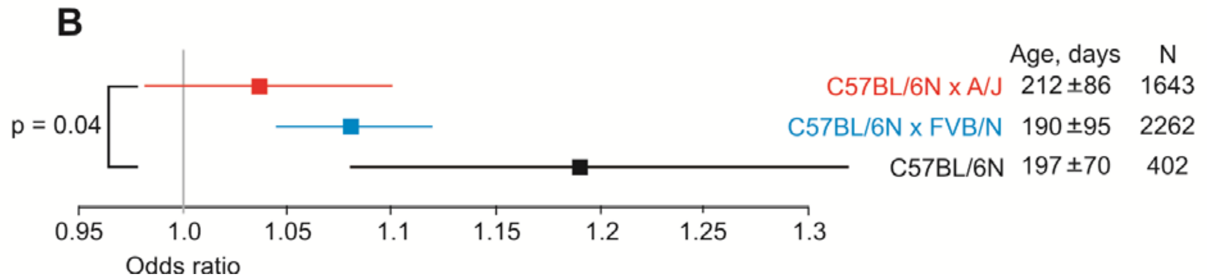


Figure 2.2

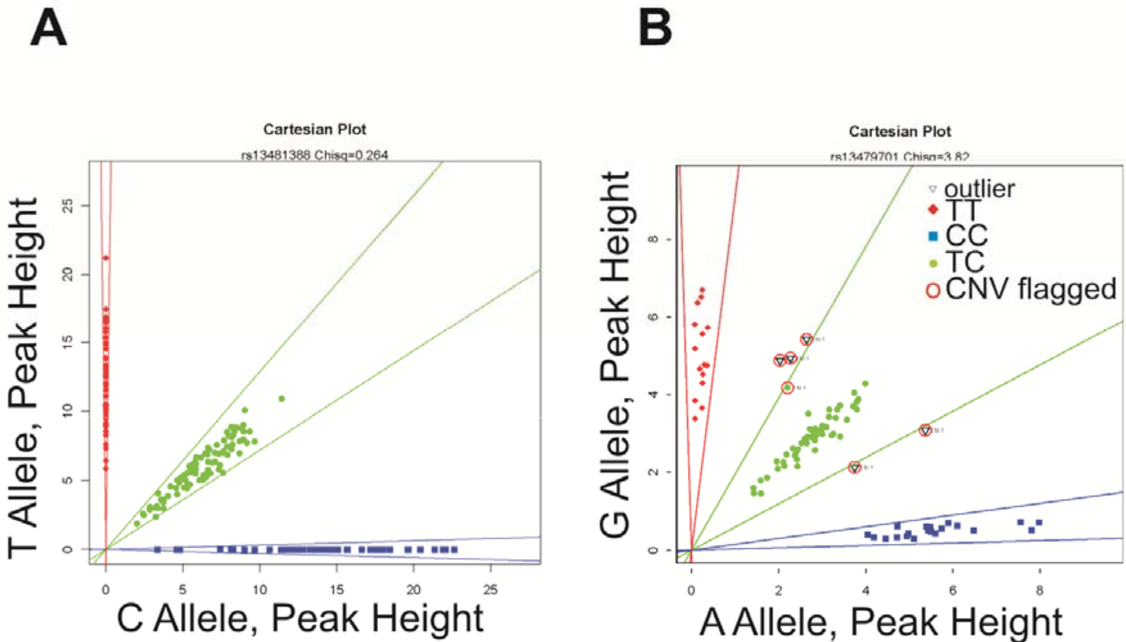
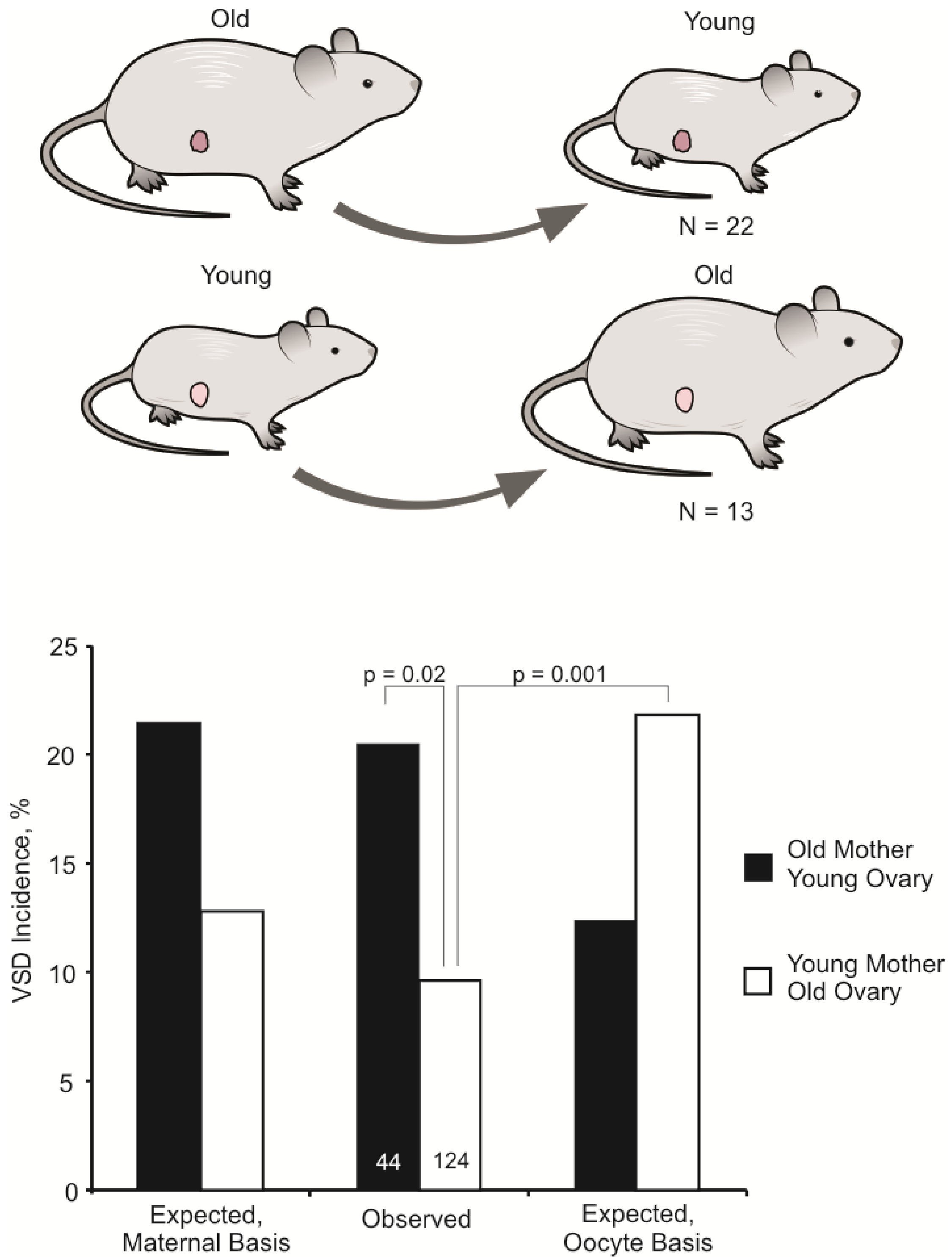


Figure 2.3



Chapter 3: Maternal exercise intervention reduces VSD risk in offspring of old mothers

3.1 Abstract

The maternal age effect in a mouse model for congenital heart disease which is haploinsufficient for *Nkx2-5* a cardiac transcription factor, is similar to that reported in epidemiologic studies. These studies consistently associate maternal age with the risk of congenital heart disease (CHD). Similar human mutations are known to cause non-syndromic CHD. Reciprocal ovarian transplant experiments show that the maternal age effect is maternally intrinsic. I hypothesized that a modifiable factor in the mother was the basis of the maternal age effect.

Insulin resistance and obesity increase as mice age. To assess the role of glucose homeostasis in the maternal age effect, dams were placed on a life-long, high-fat, high-sugar diet after weaning. Dams were glucose tolerance tested at seven weeks before mating commenced. Body composition was assessed at twelve weeks in non-gravid mothers, and the incidence of ventricular septal defects (VSD) was assessed by established methods until dams became infertile at >300 days of age.

To reduce the impact of age-related increases in insulin resistance and obesity, dams were given voluntary access to exercise wheels. Mothers exercised voluntarily, and though this did not result in significant changes to body composition or glucose tolerance, it did result in a significant reduction to the incidence of VSD.

High fat diet and resulting changes to glucose metabolism and body composition had no effect on VSD risk in offspring. The incidence of VSD in old, sedentary mothers, ~20%, falls to ~10% with exercise (chi- square test, $p < 0.05$). Of note, an exercise duration of only a few months (90-100

days) had a significant impact on VSD incidence, indicating that lifelong commitment to a healthy lifestyle is not necessary for improved outcomes in pregnancy.

Contrary to conventional wisdom, the effect of maternal aging on the risk of CHD is related to a modifiable factor in the mother. Thus, even though the embryo carries the deleterious mutation, one may target a maternal pathway to prevent disease.

3.2 Introduction and lit review

There are many markers of ageing in both human and mouse mothers, from changing hormone levels to altered bone density. One of the most significant and widely observed changes due to ageing is a tendency towards increased adiposity and glucose intolerance. Glucose homeostasis is an important factor in reproductive health (Fravola AI, 2011) and aberrant glucose homeostasis may be responsible for decreasing fecundity with age, the increased risk for congenital abnormalities, or both.

Given the absence of significant nondisjunction events to explain the increased incidence of congenital heart disease, and ovarian transfer experiments which showed the maternal age effect was present regardless of the age of ovaries and oocytes, another mechanism of age's effect must be present. Maternal adiposity and glucose intolerance is a plausible affecter, and potential target for remediation of ageing's ravages. Some systemic affecter of ageing must be acting to influence offspring development, and given the profound effects which glucose homeostasis has on the whole organism, it logically follows that the reproductive systems are also affected.

Though restoring health can often be a tricky and nebulous proposition, inducing an unhealthy state in model organisms has long been a staple of biomedical research. A high fat, high sugar diet has been shown to induce early glucose intolerance in dams, and reproductive abnormalities as early as four weeks into feeding regimen (Grindeler NM, 2013). By high fat feeding, I hypothesized that an early old-mother-like phenotype could be induced if the maternal age effect was due to decreasing glucose tolerance with age.

Maternal high fat, or 'Western' diet, has a variety of documented, negative effects. High fat diets have been shown to have similar metabolic effects in a variety of mammalian model organisms, including primates (Williams L, 2014). It is known to induce uterine vascular defects leading to altered nutrient availability for the developing embryo (Parker VJ, 2014). Even brief, timed exposures to maternal high fat diet at critical periods of gestation can lead to developmental changes in offspring (Plata Mdel M, 2014). This, or another maternally intrinsic effect of the high fat diet alters organogenesis in at risk animal models (Lin CL, 2014), and has been shown to alter cardiac development in a manner which can be offset by a drug supplementation (Elahi MM, 2014). This evidence implies that our high fat feeding model will provide a robust model for maternal ageing, if indeed the maternal age effect is due to decreasing glucose tolerance and increased adiposity with age.

Exercise is often billed as a panacea in metabolic circles. In humans, exercise improves insulin resistance in lean, obese, and type II Diabetics in just two weeks (Reyna SM, 2013) (Russell RD, 2014) . Exercise improves maximal oxygen uptake, mitochondrial content, and insulin sensitivity in healthy individuals (Larsen S, 2014), and improves cardiovascular health even in individuals already suffering from cardiovascular disease (Seals, 1985). Exercise is as effective as dietary intervention on cardiovascular markers of health, even with lower absolute weight lost in the exercise group (Maeda S, 2014). Exercise decreases fasting insulin levels and improves insulin resistance in the young (Shields, 2014), and among the elderly even a few minutes' of exercise a *week* can provide health benefits (Adamson SB, 2014). Even very low intensity exercise like standing and walking has a marked positive effect in humans (Duvivier BM, 2013).

In the ageing population specifically, short bouts of intense physical exercise (10 x 6 second sprints) can significantly improve glucose tolerance in women at the end of their reproductive lifespan

(Adamson S, 2014). Though lifelong exercise does not improve lifespan, it does stave off the onset of frailty in ageing mice (Garcia-Valles R, 2013).

The benefits of exercise during pregnancy have been debated, but in an era where obesity and pre-diabetic states are exceedingly prevalent among the fertile female population exercise interventions have been explored. Moderate exercise throughout pregnancy in humans decreases the absolute weight gained during pregnancy as well as improving the perception of maternal health (Barakat R, 2011). Limiting weight gain during pregnancy decreases the risk of pregnancy-related vascular disease, which may influence fetal development (McGiveron A, 2014).

Encouragingly, even lacking changes in adiposity or fat density, exercise improved vascular function in metabolically compromised women (Sprung VS, 2013). Looking at exercise in mouse models, voluntary exercise in aged mice has been shown to improve cognitive markers in just a few weeks of exposure (Gibbons TE, 2014) suggesting an intervention for pre-pregnant mothers requiring a relatively short duration. With this understanding of the positive effects that exercise can have on the whole organism, maternal exercise was investigated as a ‘rescue’ intervention for maternal ageing.

3.3 Methods

High-fat diet treatment

Weanling *Nkx2-5*$^{+/-}$ C57BL/6N x FVB/N F1 females were placed on a high-fat diet for 4 weeks prior to the onset of breeding. The mice remained on the diet while breeding. Calories in the diet derived from 59% fat, 25% carbohydrate, and 15% protein (AIN-76A w/58% Fat Energy/Sucrose/Red, catalog # 1810835, Test Diet). Calories in the normal, control diet derived from 13% fat, 62% carbohydrate, and 25% protein (Pico Rodent Diet 20, catalog # 0007688, Lab Diet).

Voluntary exercise treatment

Running wheels were placed in breeding cages at either 4-weeks of age (early-onset group) or 8-months of age (late-onset). The mice ran ad libitum for the remainder of their reproductive lives (Fig 3.1).

Intraperitoneal Glucose Tolerance Testing

Mice were fasted overnight (14 h) on paper bedding prior to glucose challenge (2 g/kg intraperitoneal). Blood was obtained from the tail vein for glucometry. Samples were measured with a Bayer Contour TS glucometer before and at 10, 20, 30, 60, 90, and 120 minutes after injection.

Lean and Fat Body Mass Quantification

Lean and fat body mass was measured in live mice by quantitative magnetic resonance imaging on an EchoMRI 3-in-1 instrument (Echo Medical Systems, Houston, TX). Fat body mass measurements were calibrated against canola oil standards to within 99% concordance.

Statistics

Logistic regression analyses were performed in R to determine odds ratios for the maternal age-based risk of a ventricular septal defect. Odds ratios were calculated per month of aging. The analysis of the control, sedentary group was used to calculate the expected incidence of defects in age-matched comparisons.

Incidences were compared in chi-square tests.

Two-sided t-tests were performed for blood glucose and body composition measurements. Results are reported as mean ± S.D.

Statistical significance was defined as $p < 0.05$.

3.4 Results

The simultaneous execution of experiments designed to worsen and mitigate glucose intolerance using a high fat diet and voluntary exercise provide a unique opportunity to observe exacerbated, early-onset maternal ageing phenotype, and a rescue experiment for the maternal ageing effect.

High fat diet induces early glucose intolerance

Similar to prior studies which used our high fat, high sugar diet, the FVB/N x C57BL/6N F1 mothers showed glucose intolerance after just four weeks on the diet compared with age-matched, normally fed controls (Fig 3.2). In addition to significantly altered blood glucose starting 20 minutes after glucose injection until two hours after injection, high fat fed females had significantly higher area under curve for the intraperitoneal glucose challenge, and elevated fasting blood glucose levels compared with controls (Fig 3.3). Though insulin resistance cannot be diagnosed from these indicators, they do show a deranged glucose metabolism in high fat fed dams after only four weeks' exposure compared with normally fed controls

High fat diet causes significant changes in maternal body fat composition

Though there were no detectable differences in body weight in young mothers, after eight weeks of high fat feeding females show significantly decreased lean body mass ($p = 0.04$, Fig 3.4.a), increased fat body mass ($p = 0.02$, Fig 3.4.b), and significantly increased body fat percent ($p = 0.003$, Fig 3.4.c). These effects are only exacerbated with long term high fat feeding. Mothers exposed to a high fat diet for over a year (average 55 weeks) have over 50% body fat, compared with normally fed aged matched controls who were less than half that (Fig 3.4.c). Thus we see significant alterations in body composition indicating altered metabolism, increasing with exposure time.

Glucose intolerance due to high fat diet is not sufficient to simply explain an aged mother phenotype

In spite of significant alterations to the dam's metabolism as a result of the high fat high sugar diet, this does not result in a compensatory rise in VSD risk for the offspring of young high fat fed mothers. Comparisons of young mothers' offspring (maternal age < 100 days) show identical risk of VSD (9.8%, Fig 3.5.a) between chow and high fat feeding. Even a comparison of aged mothers' (maternal age > 300 days) risk of VSD in offspring show a non-significantly different risk ($p = 0.08$, Fig 3.5.a). Though this risk might become significantly different with a much larger sample of offspring from high fat fed dams, a comparison of the odds ratios between normal and high fat fed mothers shows no significant difference in logistic regression models (Fig 3.5.b). Since logistic regression models take into account the entire continuous range of maternal ages, this indicates no significantly different maternal age risk as a result of high fat diet.

Exercise does not significantly alter maternal body composition

Dams were observed exercising daily, up to the day before giving birth, but in spite of this admirable dedication there were no appreciable differences in fasting blood glucose, area under curve (AUC) in an intraperitoneal glucose tolerance test, lean or fat body mass, or body fat percent between sedentary and early-onset exercised dams (Fig 3.6). Similarly, late-onset exercised dams show no significant difference in biomarkers when compared with sedentary age-matched controls (results not shown).

Exercise decreases maternal ageing risk to offspring

Both early and late-onset exercise show significant decreases in VSD risk in the offspring of aged mothers (maternal age > 300 days). Surprisingly the offspring of old, lifelong exercise dams, are at a

risk indistinguishable from that of young mothers (Fig 3.7.a). Logistic regression modeling shows a disappearance of the maternal age effect in the offspring of exercised dams (Fig 3.7.b). This shows that the maternal age effect is modifiable using exercise intervention.

Short-term exercise interventions is as effective as lifelong exercise in preventing the maternal age effect

To determine the minimum period of maternal exercise required to see an effect on VSD incidence the offspring were binned according to days of exercise exposure and compared with logistic regression age-matched estimates of risk. Given a 20 day gestational period in mice, three months of maternal exercise prior to conception was sufficient to induce a significant beneficial effect (Fig 3.8).

3.5 Discussion

The powerful effect of maternal exercise to reduce the VSD risk of offspring was surprising, and indicates a modifiable pathway which could in theory, be translated to at-risk human populations quickly and with low risk of side effects. The pathway influenced by maternal exercise is unknown, but it is likely something more complex or subtle than simply improving glucose tolerance or reducing obesity, as inducing those phenotypes early were insufficient to instigate an early ageing effect. This lifestyle intervention approach indicates unknown modifiers to VSD risk, though the mechanistic basis of the maternal age risk is likely separate from causative genetic mutations.

At the outset of these experiments, the most intriguing possible result was the case in which exercise was beneficial, but high fat diet was insufficient to induce an early ageing phenotype. This is because exercise and high fat diet are thought to act in opposition, exacerbating and rescuing a similar condition. In actuality, the effects of exercise are multiple and not yet exhaustively defined. Though exercise does simply improve glucose homeostasis, it also improves the uptake of nutrients into muscle tissue, alters the metabolism, and leads to changes in metabolite concentrations in blood, in addition to the psychological and hormonal benefits which have a multitude of physiological effects. It is one of these less studied pathways by which exercise is likely influencing maternal ageing.

Perhaps most interestingly, the beneficial effect of exercise is not associated with significant changes to maternal body composition. This implies that the very act of exercising provides benefits, completely aside from weight or fat loss. The improvement to offspring health is completely uncoupled with decreasing levels of obesity, challenging the supposition that obesity alone is to blame for a plethora of maladies. That exercise alone leads to improvement may change health goals in pre-pregnancy counselling, and make those health goals more attainable for a majority of the at-

risk population. Similarly, the fact that mice were in no way coerced to a certain period of exercise duration or intensity suggests that light exercise would be sufficient to induce an effect. This does highlight an area of the experimental design which was not controlled: minimum exercise duration or intensity. Indeed, the intensity of exercise undertaken by each mouse within the harem was not measured, and though mice were observed running at all points in pregnancy, and throughout the diurnal cycle, there was no measure of individual exercise exposure. This, as well as the effect of age-of-onset for exercise exposure, could be examined in future studies.

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3.7 Figures:

Figure 3.1 Sedentary mice were housed in the usual manner with no access to running wheels. Early onset exercise mice were transferred to cages with access to running wheels, at four weeks of age, while late onset exercise mice were transferred to cages with running wheels at approximately eight months of age.

Figure 3.2 Intraperitoneal glucose tolerance tests of eight week old mice show that mice which have been high fat fed for four weeks are significantly less glucose tolerant than chow fed mice of the same age. Time points for tail vein glucose measurements are at 0, 10, 20, 30, 60, 90, and 120 minutes post injection.

Figure 3.3 Fasting glucose levels (A) of eight week old mice high fat fed for four weeks are significantly elevated when compared with chow fed controls. Area under curve (AUC) for intraperitoneal glucose tolerance tests show significant elevation in both young, high fat fed mice, and old (> 300 days dietary exposure) mice which have been high fat fed from four weeks of age (B). This indicates early and persistent glucose intolerance and metabolic derangement as a result of high fat feeding.

Figure 3.4 High fat fed animals have significantly less lean body mass (A) and significantly greater fat mass (B) than age matched chow fed animals. Similarly, high fat fed animals show a significant increase in body fat percent (C) even after just four weeks on the dietary regimen. The presence of these changes in both early time points (eight weeks) and after significant ageing (> 300 days dietary exposure) indicates both early and persistent physiologic changes as a result of high fat feeding.

Figure 3.5 VSD incidence is not significantly elevated, in either young high fat fed mothers or after significant ageing and exposure, when compared to chow fed controls (A). Logistic regression modeling odds ratios are shown with 95% confidence intervals for a high fat and chow fed maternal age effect (B). There is no difference between the two models, showing that high fat diet does not alter the trajectory of the maternal age effect.

Figure 3.6 Metabolic metrics for sedentary and early onset exercise animals at > 300 days of age show no significant differences between fasting glucose (A), area under curve for intraperitoneal glucose tolerance tests (B), lean or fat mass (C), or body fat percent (D). There are no significantly different body composition metrics or glucose tolerance metrics in aged exercised mice compared with sedentary controls, in spite of lifelong exercise exposure.

Figure 3.7 The incidence of ventricular septal defect (VSD) is significantly reduced by both early and late onset exercise (A). The offspring of aged early- and late-onset exercise dams show an incidence of VSD indistinguishable from that of young mothers. Multiple logistic regression models show that the maternal age effect is reduced to near zero in the exercise cohort (B).

Figure 3.8 The incidence of VSD is shown binned according to the number of days a mother in the late-onset group had exercised on a pup's birth date. The expected incidences are calculated for age-matched, sedentary control mothers. Three months of exercise prior to conception results in a detectable reduction in the incidence of VSD.

Figure 3.1

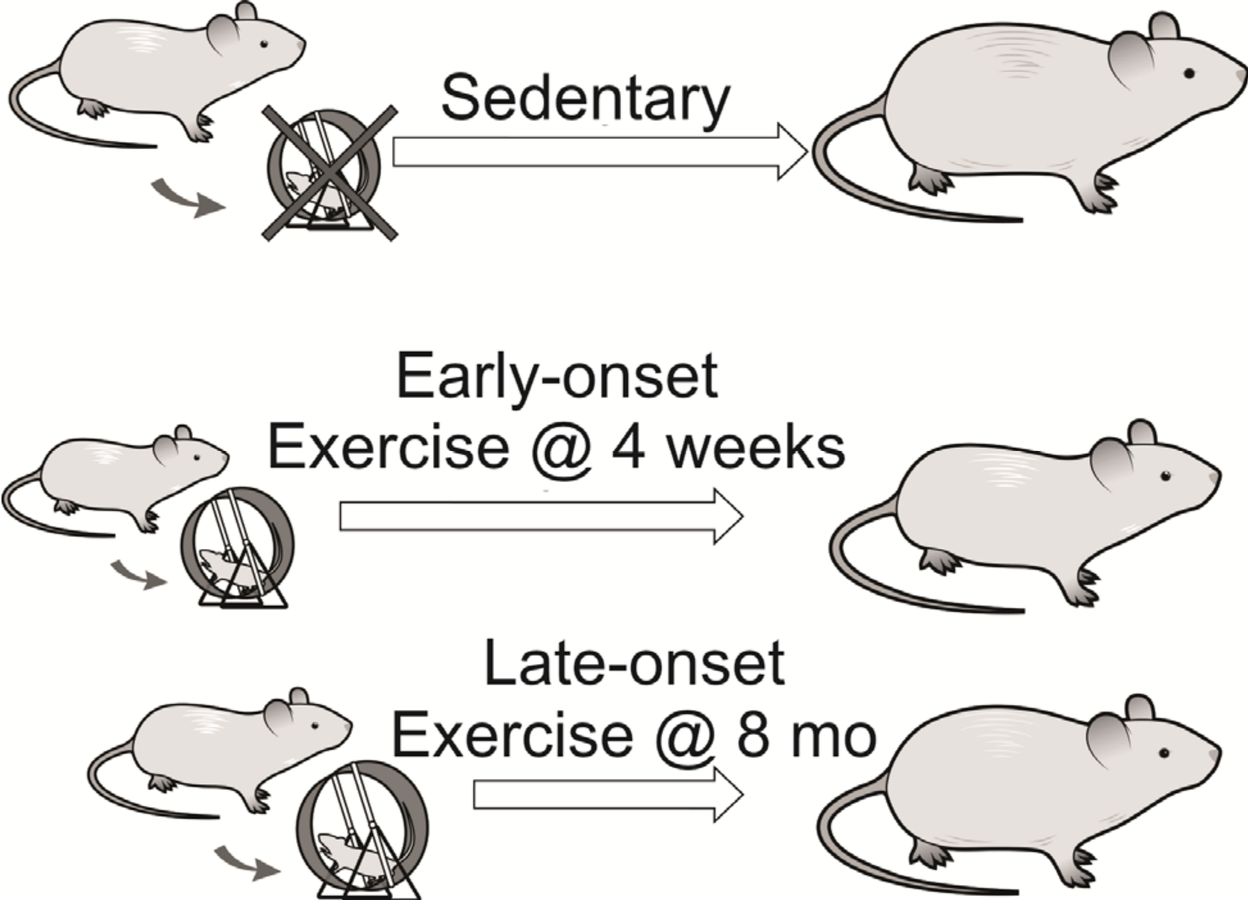


Figure 3.2

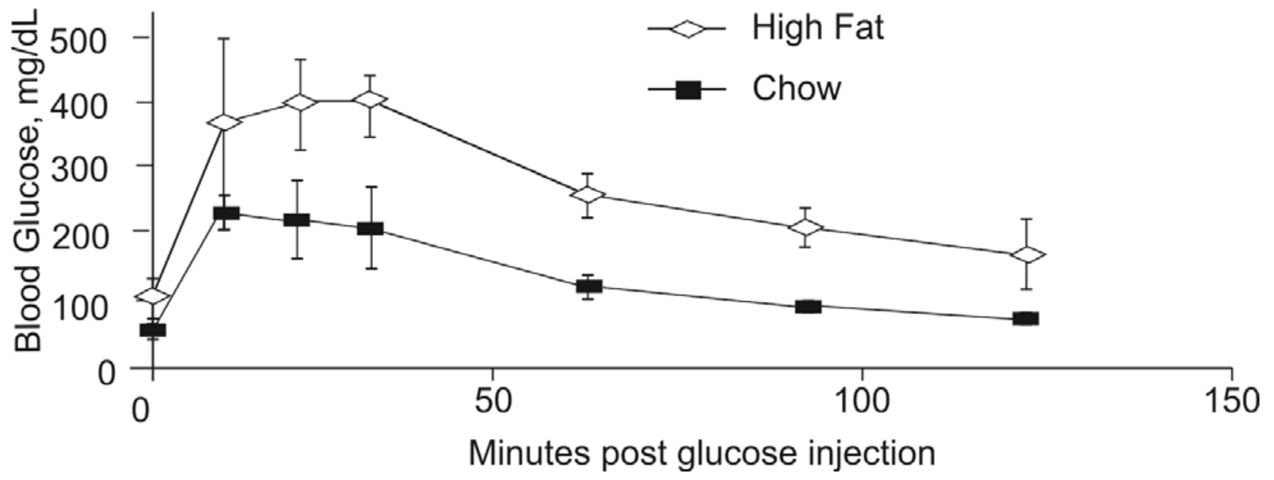


Figure 3.3

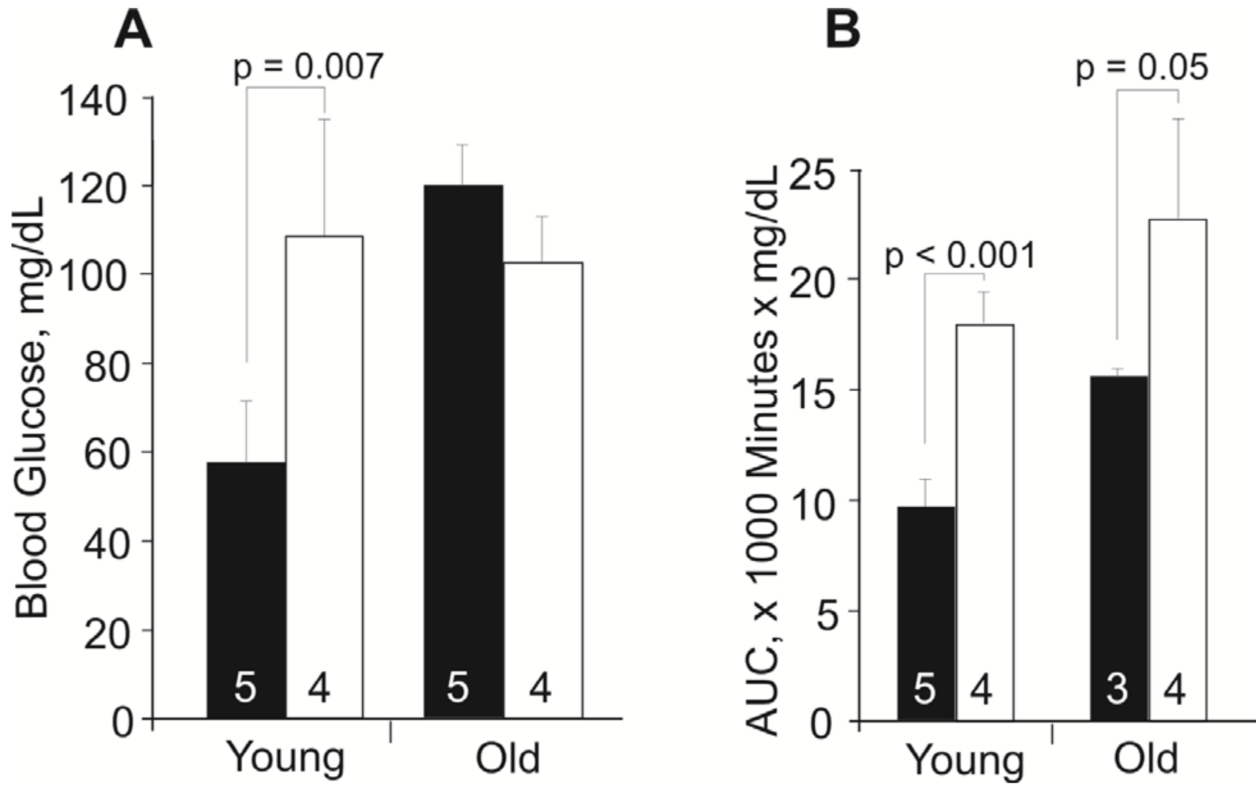


Figure 3.4

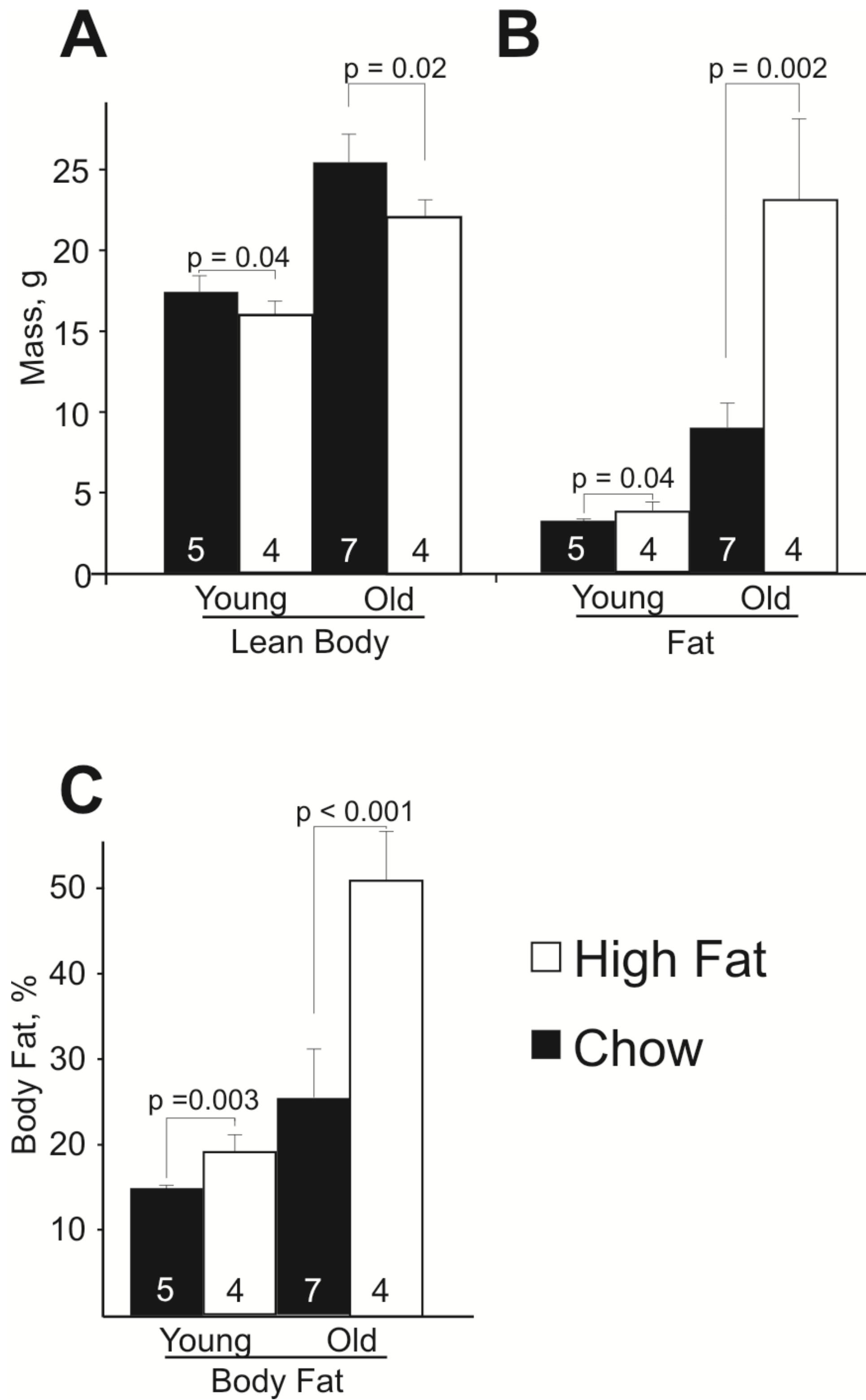


Figure 3.5

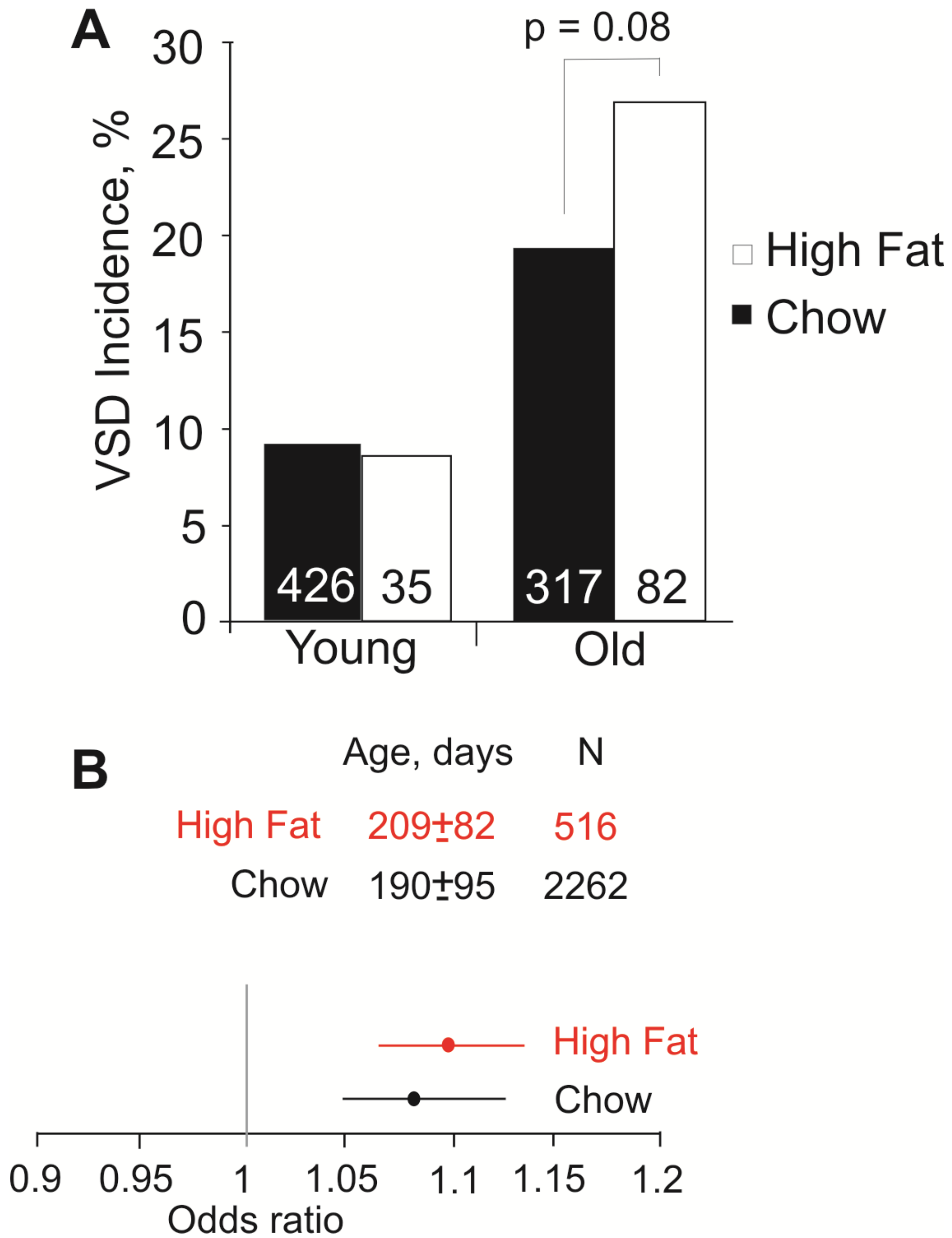


Figure 3.6

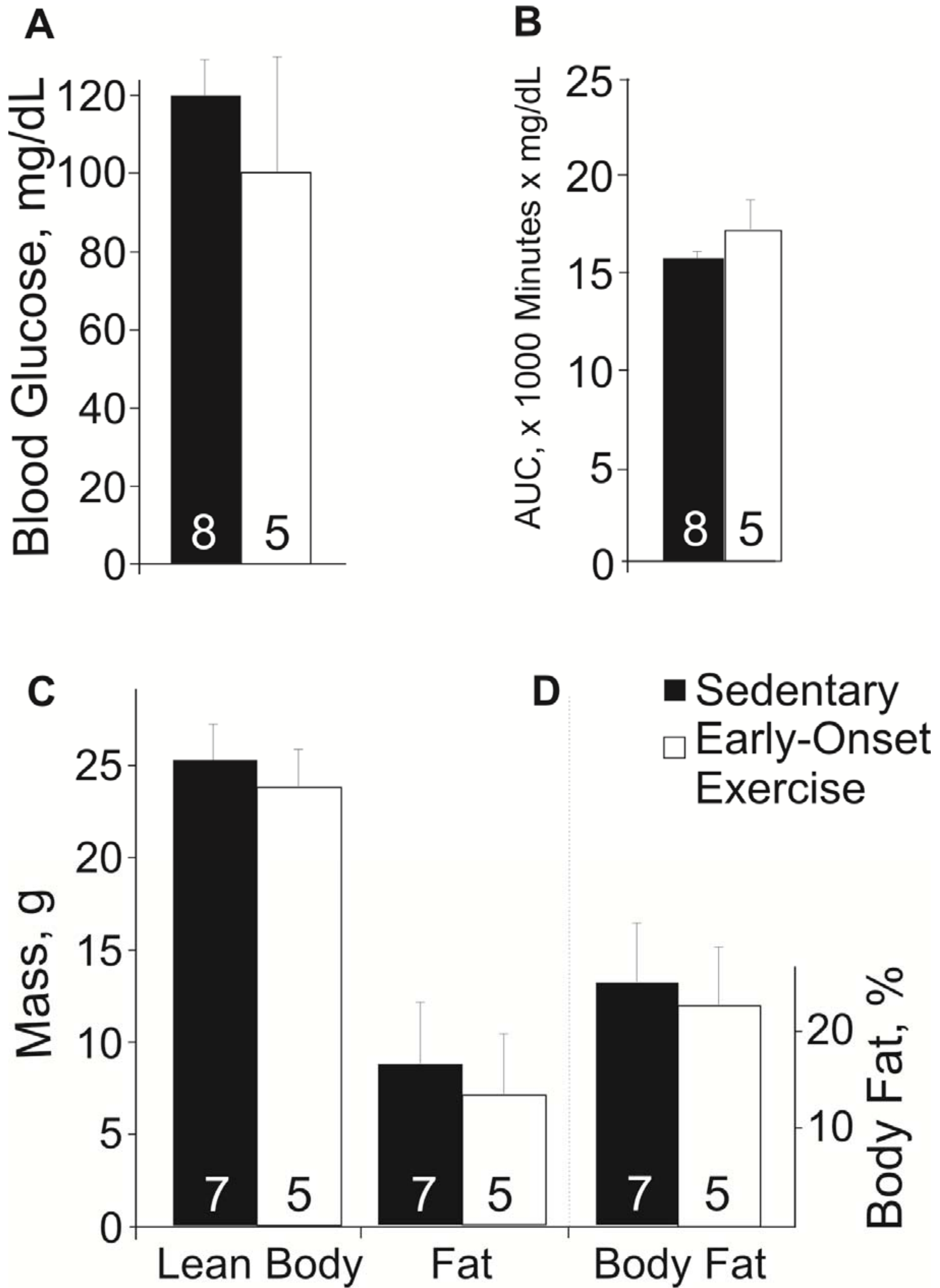
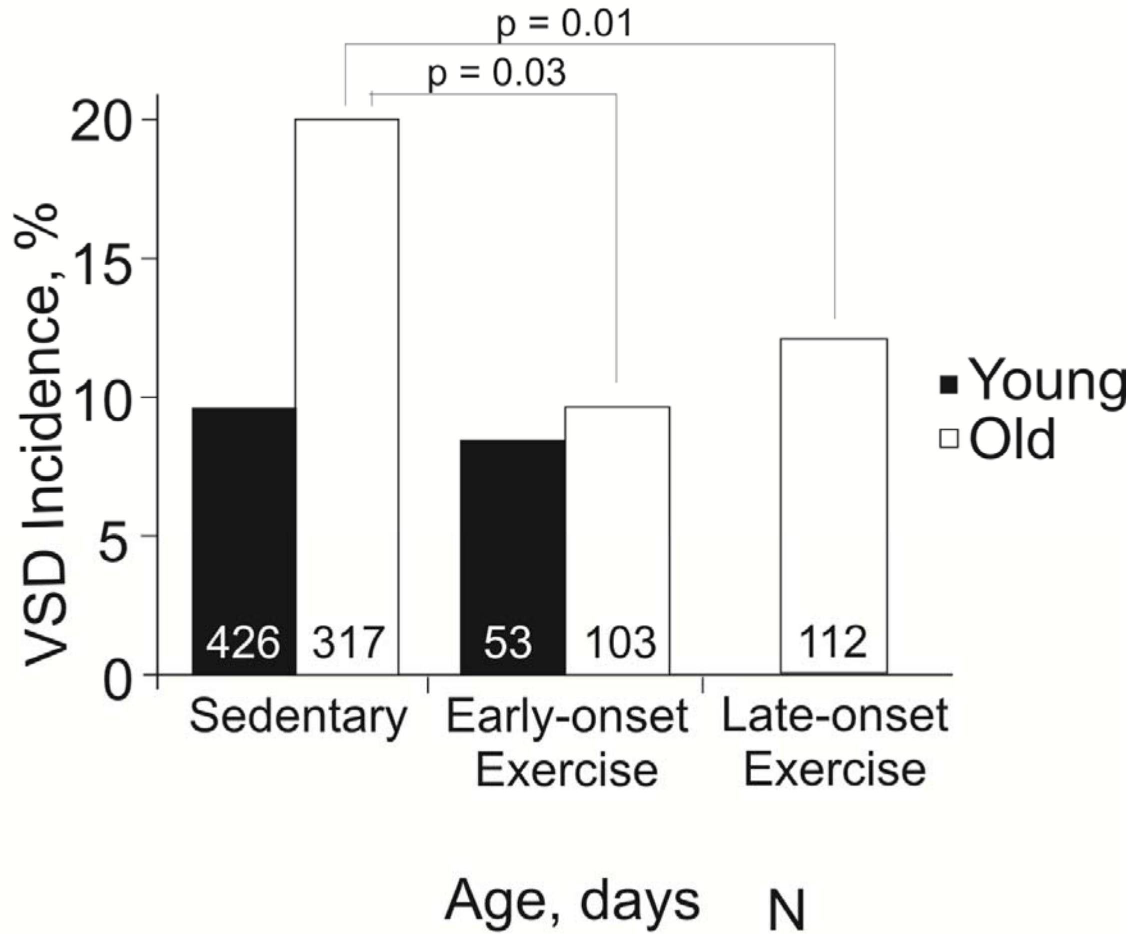


Figure 3.7



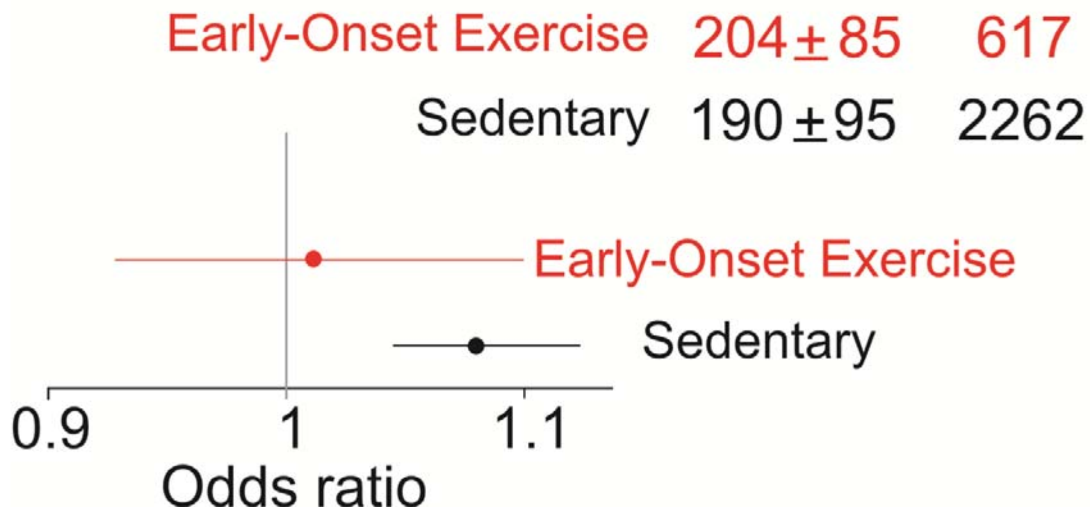
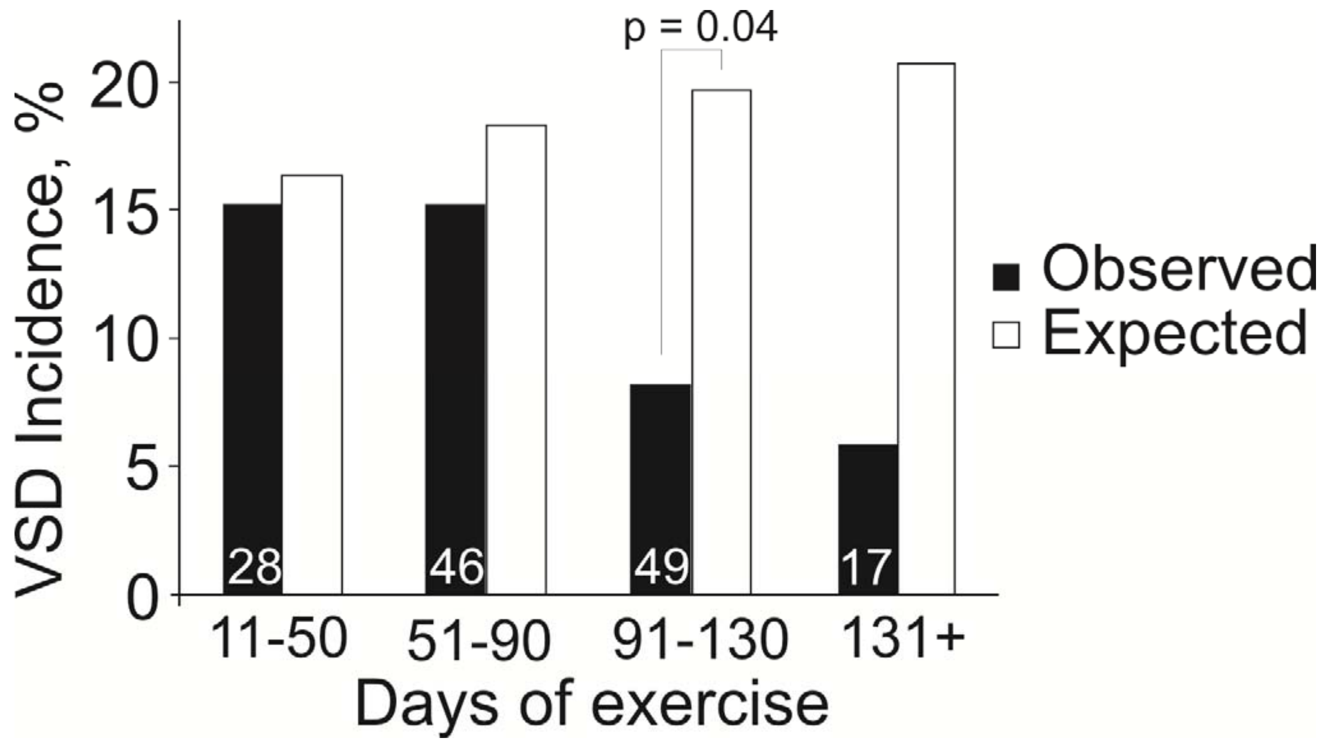


Figure 3.8



Chapter 4: Maternal ageing subtly alters methylation states in a subset of genetic regions

4.1 Abstract

Maternal ageing must alter some aspect of development to have a negative impact on fetal growth and cardiac formation. Epigenetic modifications, cytosine methylation specifically, are an excellent candidate for this maternal affecter. Here I aimed to determine the impact of maternal ageing on methylation patterning in offspring through reduced representation bisulfite sequencing (RRBS).

Heart and liver tissue from pure-line C57BL/6N dams along with A/J x C57BL/6N F2 intercross hearts, and sedentary and exercise FVB/N x C57BL/6N F2 hearts were MSP1 digested, bisulfite treated, and sequenced using a modified RRBS library preparation technique. The resulting sequencing data was aligned using Bismark, and DMRs were determined using DMAP. High-significance DMRs were identified by comparing those DMRs which overlapped between experimental comparisons to determine regions altered only in strains with the maternal age effect, and which were remediated by exercise.

Eight DMRs were identified as altered with maternal age, and returned to their 'young' methylation state with exercise. Of these eight, three are near to genes with possible cardiac implications, while the others were nearest unstudied predicted genes, and may be involved with more distant genomic regions. Looking at those DMRs which were altered in two or more experimental comparions, greater than 95% of overlapping DMRs showed identical directional changes in methylation rate.

DMRs identified in this study open the door for new biomarkers for both congenital heart disease and maternal ageing's negative outcomes.

4.2 Introduction and lit review

Congenital heart disease is a classic complex disease (Nora, 1968) that affects between 1-2% of newborn infants, making it the most common congenital abnormality in newborns (Hoffman JL, 2002) (Reller MD, 2008). Barring chromosomal and syndromic abnormalities, there are few causative mutations associated with congenital heart disease (Wessels MW, 2010) (DW, 2009), and none that have led to palliative treatment for these developing disorders. Though there is a significant heritability for the disease in humans, the absence of monogenic causes of disease indicates many modifying factors working in concert to maintain normal cardiac development. With a relative risk of 2-4 for development of a congenital heart defect (Oyen N, 2010) for an individual with an affected first degree relative there are obvious genetic components, but they are likely a constellation of small effect, low penetrance genes (Pierpont ME, 2007). Though these modifying factors which canalize angiogenesis are traditionally thought to be purely genetic, epigenetic factors may play an important role in congenital heart disease risk and risk in similar complex diseases.

The influence of parental physiology on certain disease risks aside from direct genetic mutations has been acknowledged as an important factor in adult-onset heart disease, but the role of epigenetics in cardiac development may be multiple. Though congenital heart disease is heritable, genetic risk is multifactorial and influenced by environmental factors such as maternal age which in turn influence epigenetic profiles of offspring. NKX2-5 mutations cause pleiotropic heart disease (Winston JB, 2012) in mice and humans: using an *Nkx2-5*$^{+/-}$ knockout mouse our lab showed a conclusive link between maternal ageing and congenital heart disease in multiple genetic backgrounds. Though maternal age clearly influences disease risk, increases in body weight in old age and glucose intolerance do not simply explain the mechanism, suggesting subtler means of influence on the

developing fetus. To date, no studies have been published looking at how maternal age may influence fetal methylation profiles either on a genome-wide scale or in a disease-specific manner.

Multiple studies link advanced maternal age with adverse pregnancy outcomes and infant mortality (Ludford I, 2012) (Jolly M, 2000) (Phadungkiatwattana P, 2014). There is evidence that the health of the mother is more important than her age when predicting adverse outcomes (Carolan, 2013) (Khalil A, 2013), suggesting that lifestyle interventions may increase the window in which pregnancies are unaffected by maternal age.

Methylation of cytosines is thought to alter accessibility of DNA to binding factors, usually in a manner whereby cytosine methylation is inversely proportional to gene expression. In this way genes can be silenced, or partially repressed, without altering or removing DNA sequence, and create a dynamic flux of expression which is critical for the developing animal. Rapid and time-specific methylation patterning is critical for proper organogenesis in a number of systems, and alterations in the global or specific methylation patterns could conceivably influence cardiac development.

Aberrant methylation profiles due to Dnmt1 knockouts have been shown to be embryonic lethal due to abnormal organogenesis (Li E, 1992), and similarly, knockouts of Dnmt3 are runted and die within a month, indicating systemic abnormalities resulting from changes in methylation patterning (Okano M, 1999). Dnmt knockouts are extreme examples of altered fetal methylation profiles, but demonstrate the profound effect that these epigenetic marks can have on the developing embryo.

Current studies show that the traditional methods whereby maternal ageing may affect the fetal environment (decreased glucose tolerance, increased adiposity) are insufficient to induce the increase in risk seen with maternal ageing [Schulkey et al paper in review] which indicates the presence of

some as yet unstudied factor. 5'-methylcytocine patterning, is a tempting actor in the maternal age effect as the changes can be subtle, but the results, profound.

Recent advances in high throughput sequencing and analysis techniques have made it feasible to study groups in an unbiased, genome-wide manner. RRBS provides a genome-wide approach for methylation sequencing without the accompanying cost of whole-genome shotgun sequencing. Sampling only 3-5% of the genome allows for the sequencing of larger numbers of samples than traditional bisulfite sequencing methods, preferentially selecting for CpG island and shore regions important for methylation patterning. Due to this we were able to sample twelve hearts in both old and young maternal age groups providing us with high statistical certainty. RRBS is obviously biased towards regions cut by Msp1 digestion at the appropriate length, but when compared with methylation arrays which target specific genes or regions, it is a sequencing approach, and thus does not suffer the biases of pre-selected methylation arrays. This means this sequencing approach may catch regions of interest and genes which would not have been sampled using chip methods, as well as provide a view of global methylation changes in the heart due to maternal ageing.

To understand the epigenetic changes present in offspring due to maternal age we performed reduced representation bisulfite sequencing (RRBS) on 12 *Nkx2-5*$^{+/-}$ and 12 wild type animals from aged (> 270 days) and young mothers (< 81 days). We hypothesized that maternal age would significantly alter methylation profiles in the cardiac tissue of offspring, and would indicate targets for maternal age's effect on congenital heart disease

4.3 Methods

The sample groups have been modified from those initially proposed due to methodological issues in RRBS library preparation. The scope of samples sent for sequencing has widened to include multiple strains and treatment groups, but the option of pre-selecting for animals with CHD was no longer available.

C57BL/6N pure line *Nkx2-5* wild types and heterozygote pup tissues were sent for sequencing. Males and females were sent in equal quantities using both heart and liver extracts. This allowed the determination of the influence of sex, *Nkx2-5* genotype, and tissue, on methylation profiles. In addition to pure C57BL/6N line samples, A/J x C57BL/6N F2 samples and FVB/N x C57BL/6N F2 exercise samples of from old and young mothers were also sent.

Reduced Representation Bisulfite Sequencing Library Preparation

The preparation of DNA for a sequencing library appropriate for use on a platform like an Illumina HiSeq 2500 sequencer requires multiple steps and rigorous quality control. The basic steps are outlined in figure 4.1.

Isolation of genomic DNA (gDNA) from tissue

Tissue was digested and DNA was extracted using a QIAamp DNA Minikit. Phenol chloroform extraction was tried for this method but trace quantities of phenol interfered with library preparation and resulted in poor quality product.

Msp1 digestion

Genomic DNA was digested with Msp1 overnight at 37 deg C to ensure complete digestion of samples.

Adapter annealing and end repair

Digested samples were cleaned up using a MiniElute Clean up Kit, and ends were adenylated to accept methylated primers. Specially selected methylated primers were ligated on to digested gDNA fragments with T4 DNA ligase.

Gel extraction for size selection

Samples were loaded into a 2.5% NuSieve GTG Agarose Gel with EtBr and run for 1.5 hours on 105 volts with a low MW ladder. The section corresponding to 175 to 350 bp was excised and samples were extracted using a QIAquick gel extraction kit.

Sodium bisulfite treatment for CT conversion

Bisulfite treatment leads to the conversion of unmethylated cytosines into uracils which cause them to read as T's during sequencing. Samples were sodium bisulfite treated for 2.5 hours and samples were isolated using Zymo Spin IC Columns. At this point samples underwent a test PCR to ensure bisulfite conversion.

Indexing PCR

Samples were combined with unique indexing primers and amplified using 22 cycles of PCR. Though indexes could be reused amongst library groups, they could not be duplicated in multiple samples in the same library preparation.

Ampure bead cleanup

Paramagnetic beads are used to bind DNA with carboxyl groups coating the surface of these beads. Based on the concentration of DNA compared with the concentration of beads appropriately sized DNA fragments are bound to the beads, and washed of impurities and contaminants. The DNA is unbound using an elution buffer and the beads are removed using a magnet.

Sample pooling

Sample concentration is determined using a Qubit High Sensitivity Kit for precise pooling. Samples must be diluted to similar concentrations and then combined, or proper volumes must be calculated to provide equal molar concentration of samples for sequencing.

Illumina Sequencing

Sequencing was performed by GTAC on campus at Washington University with an Illumina HiSeq Genome Analyzer. Samples are amplified using the forked-end adapters ligated early in the library preparation and bridge amplification to form clusters on the flow cell surface. The polonies are then sequenced using base-unique fluorescent labels.

Sample selection

Where available, three replicates of each combination of strain background, genotype, sex, and tissue type were sent from three unique pups. Samples were sequenced from pure-line C57BL/6N animals in heart and liver, and hearts from FVB/N x C57BL/6N F2 sedentary and exercise animals, as well

as hearts from A/J x C57BL/6N F2 animals. Due to tissue requirements for library preparation, the hearts were not diagnosed for CHD before tissue was digested for DNA.

Quality control

Fast QC (Andrews, 2014) was used to assess sample quality before and after adapter trimming and low-quality read trimming.

Sequence alignment and methylation calling

After quality trimming, sequence alignment and methylation calling was performed using Bismark, a wrapper for Bowtie designed to manage the C-T mismatches inherent in bisulfite-treated samples. Alignment was performed using standard settings (single-end reads, seed length 28) and GRCm38. Mapping efficiency was at or above 60% for all sample groups (Fig 4.2), which is the high end of expected mapping efficiency from this program (Krueger, 2014).

Fragment assembly and methylation calling using DMAP

DMAP was used for differential methylation analysis due to its focus on analysis of RRBS data specifically (Stockwell PA, 2014). DMAP identifies differentially methylated regions using a by-fragment approach. Various contiguous sequence fragments are assigned into regions for DMR testing, which comprise multiple CpG's over fragments ranging from 40 to 220 base pairs defined using an *in silico* digest of the mouse genome. Assembled regions were tested for differential methylation patterning using ANOVA tests for sample group comparisons. Because of the by-region nature of the testing, single base pair resolution is sacrificed for a wider view of region-wide

methylation patterns. Subtler methylation changes (< 50% difference between groups) can be determined using this method.

FDR determination

The FDR function in R was used to determine the point of inflection of the p-value distribution curve indicating the p-value cutoff for a FDR of 0.05 at p=0.001.

Gene mapping

Mapping of contiguous sequence fragments with differential methylation patterns was done using the identgeneloc function in DMAP, which assigns fragments to the nearest downstream gene. This method does not account for more subtle or distant gene interactions.

Gene enrichment

Gene enrichment was assessed using the GREAT (Genomic Regions Enrichment of Annotations Tool) algorithm (McLean CY, 2010) with the standard settings. Hyper- and hypo methylated regions were assessed in separate groups. Hyper methylated fragments were identified as a sample with >5% increase in methylated C's from group A to group B. Hypo methylated fragments were identified as samples with < -5% methylation change from group A to group B.

Alternate gene enrichment was assessed using DAVID, and the list of genes produced using DMAP. Standard databases were searched as well as Up_tissue and PANTHER_PATHWAY.

4.4 Results

Sex-specific comparisons show X-inactivation

When significant DMRs ($p < 0.001$) for group comparisons are binned by chromosome, DMRs are seen across the entirety of the mouse genome, except in the case of male-female comparisons (Fig 4.2). In male-female comparisons the vast majority (>90%) of the DMRs are located on the X chromosome, and are significantly more methylated in females compared with males, as would be expected from X-inactivation. Tissue specificity and maternal ageing DMRs appear on every autosome.

The similarity of test comparison correlates with the number of DMRs identified.

The number of significant DMRs identified in each experimental comparison varies based on the magnitude of difference between the samples (Fig 4.3). The heart-liver comparison has the largest percentage of DMRs compared to total methylated fragments identified with 15.5% of the total population of examined fragments being significant DMRs. The sex-specific differences are the second most populous with 0.6% of the fragments identified as DMRs. Even though greater than half of these DMR's are due to X-inactivation, the sex-specific DMRs make up roughly 0.2% of all examined fragments. The maternal age comparisons on heart tissue, including males and females as well as *Nkx2-5*$^{+/-}$ and wild type are the smallest subset, with 0.13% of the fragments identified as DMRs, only just above the 0.08% threshold identified as a false discovery rate through comparison of like groups. The relatively small subset of DMRs correlated with maternal ageing suggest that the effect is subtle, and further screening of the gene list will be helpful in the removal of false positives.

The patterns of DMR methylation levels depend upon sample comparisons.

The distribution of DMRs into heavily methylated and scantly methylated proportions can provide important insight as to the mechanistic basis of methylation changes in specific DMRs as well as wider patterns between sample groups. Drastically different density curves when comparing sample groups give confidence that there is indeed a change in methylation patterns on a genome-wide scale. The sex-specific changes segregate into two distinct curves (Fig 4.5.B) centered over 10% and 30% methylation between males and females respectively, indicating that the female samples on average have increased methylation in DMRs. Conversely, tissue specific DMRs are largely in heavily methylated fragments, with methylation rates being about 80% between heart and liver tissue (Fig 4.5.A). The difference seen due to maternal age's imprinting effects is subtle (Fig 4.5.C), but a marked change from the *Nkx2-5*$^{+/-}$ and WT comparison which shows identical curves (Fig 4.5.D) due to the non-significant and most likely falsely-discovered DMRs. These density curves reinforce the need for further screening to identify true and important DMRs in the maternal age imprinting cohort from false discoveries.

Samples from old mother offspring are more heavily methylated compared to young mother samples.

The degree of methylation and the direction of change in DMRs are both important for understanding the dynamics in methylation imprinting. Within the maternal age imprinting DMR cohort, there are similar distributions of highly methylated (>70% methylated C's) and lowly methylated (< 30% methylation) (Table 4.1.A). Though old mothers appear, on average, produce higher methylation levels than young mothers, and show more DMRs with increased methylation in old mother offspring, the difference is not stark.

Tissue-specific DMRs are related to tissue-specific processes using clustering analysis.

In order to determine if the DMRs in tissue specific comparisons were genuinely important in distinguishing tissues, the DMRs were separated into fragments which had greater methylation levels in heart tissue, and greater methylation levels in liver tissue, associated with nearby genes and subjected to GO enrichment analysis using GREAT (McLean CY, 2010). DMRs with greater than a 5% absolute methylation change were included in the analysis. The group with greater methylation in liver was expected to cluster with heart-specific genes, while the group with greater methylation in heart was expected to cluster with liver-specific mechanisms. Indeed, the first group contained GO terms such as “Cardiac myocyte formation” and “heart development” while the second group contained terms like “cholesterol trafficking” and “energy homeostasis” (Table 4.2). The successful clustering around expected GO terms implies the validity of wet lab and dry lab technique used to determine DMR in tissue specific samples as well as maternal age imprinting samples.

Overlapping DMRs show exercise reverts methylation patterning to young profile.

The discovery of exercise mediation of the maternal age effect provides a groundbreaking opportunity to identify target genes for intervention using overlapping methylation analysis between exercise and sedentary offspring. By identifying the DMRs which are changed by maternal age in sedentary animals, and which are changed in old offspring by exercise, the DMRs can be screened to identify specific high-probability DMRs for further scrutiny (Fig 4.6.A). Similar comparisons were done between these FVB/N x C57BL/6N F2 old-mother offspring, and A/J x C57BL/6N F2 old-mother offspring which do not suffer from a maternal age effect, and an exercise maternal age comparison, both of which should show no overlap (Table 4.4.).

Eight DMRs show methylation change with maternal age and as well as exercise (Fig 4.6.B. Conversely, there are no common regions between DMRs defined by strain comparisons and the set comparing exercise and sedentary ageing methylation profiles (Table 4.4).

The genes associated with DMRs related to both maternal age and exercise are a mixture of well-studied and complete unknowns (Fig 4.7.A, C). In every case, the directional change and magnitude of methylation difference between old and young samples is nearly identical to that resulting from exercise. The fact that ageing causes the inverse directional changes to exercise in methylation levels in groups suggests that these DMRs are important, and not simply chosen by chance. Three DMRs show significant changes in ageing comparisons, exercise comparisons, and strain comparisons. In all three DMRs, the direction of methylation change is the same between recovery groups. There may be unknown interactions with these DMR with transcriptional machinery for more distant genes which are not identified by DMAP's relatively simple nearest-gene method. These eight DMRs represent the most plausibly significant DMRs related to maternal ageing.

Strain, exercise, and youth, result in similar methylation profiles compared with aged unexercised samples

Looking at those DMRs which were altered in two or more experimental comparions (ageing and exercise, ageing and strain, or exercise and strain), greater than 95% of overlapping DMRs showed identical directional changes in methylation rate across young mothers, A/J x C57BL/6N mothers, and exercised old mothers even if those DMRs were not flagged as significant in all comparisons.

4.5 Discussion

The DMR identified through the above methods open avenues of inquiry into the causes of maternal ageing, the modifiers of congenital heart disease, and offer opportunities for the development of new biomarkers for these conditions for early diagnosis and treatment.

DMR as biomarkers for maternal ageing

Identifying at-risk maternal populations is still a tricky proposition depended upon genetic and environmental homogeneity and notoriously unreliable self-reporting of health metrics. The prospect of methylation levels as a testable factor for the presence or absence of a maternal age effect will provide insight on individual risk as well as enabling stratification for exercise intervention studies to identify those who will benefit most profoundly from treatment. The specificity of DMRs to affected individuals means that biomarkers which are developed as a consequence of these results will indicate not only those beginning to develop maternal age risk, but also those who have recovered through exercise from a maternal age risk. Though further investigation is needed to conclusively identify the genes associated with these DMRs, the consistent association between these regions and maternal age risk in our model organism suggests that there is a strong and reliable interaction between these regions and maternal ageing.

Identification of potential modifiers for CHD

Hoxd11, Stard13, and Hip1 all are plausible modifiers for congenital heart disease risk. Several Hoxd genes show up in the DMR list for maternal ageing, while Hoxd11 appears specifically in the overlap between maternal age risk and exercise recovery. Though the primary abnormalities associated with decreased Hoxd11 expression are axial skeletal formations, it is conceivable that changes in

expression levels would result in secondary cardiac malformation. Stard13 is known to be associated with cardiovascular malformations (MGI 5.19 - Phenotype Browser: cardiovascular system phenotype [MP:0005385], 2014) and is thought to be important in cytoskeletal organization and cell motility. Subtle changes in expression may result in profound alterations to cytoskeletal organization and lead to congenital heart disease of a variety of forms. Hip1 is most famous for its association with Huntington's disease, but its association with cytoskeletal formation and apoptosis, and expression levels in the heart which are comparable those seen in the brain indicate possible functional interactions. Interestingly, these genes were identified prior to further analysis which showed they were the only DMRs altered by ageing which were significantly different in both exercise groups, and A/J x C57BL/6N strain.

These genes represent only a handful of potential, *known* genes associated through methylation analysis with a risk of congenital heart disease induced by maternal ageing. The searchable genes are, by essence, limited to known genes and gene functions. Within this list, a quarter of the potential fragments are best associated with genes about which essentially nothing is known. As our knowledge base grows, these datasets can be reanalyzed and integrated with new experiments to provide novel insights.

Ageing DMRs do not cluster into identifiable groups

Attempts to run clustering analysis on maternal ageing DMRs were unsuccessful. GREAT, which provided excellent insights into tissue specific gene function, produced no results, and the clustered terms using alternate programs such as DAVID resulted in scattered pathway identification. The pitfall of DAVID is that the nearest-gene approach to identification of pathways means that it is likely a significant portion of gene interactions were misidentified for the DMRs, leading to scattered

and low-significance functional enrichments. GREAT likely suffered from similar issues, but with the larger problem that it does not operate well on shorter gene lists. Though the tissue specific gene lists did pull out tissue-appropriate GO terms (Table 4.2) from tens of thousands of fragment entries, from less than three hundred it found no functional enrichments. The lack of clustering of DMRs associated with maternal ageing crushes the possibility of easily identifying a 'maternal ageing functional pathway', but is not entirely surprising.

Directional changes to methylation levels are similar across all groups with low maternal age risk

Amongst DMRs identified in the overlap between two or more maternal age risk groups, the direction of change in methylation was the same across young, exercised, and A/J x C57BL/6N groups, indicating a similar mechanism of action between these groups. This indicates that strain-specific protection and exercise are acting in similar ways to maintain healthy methylation profiles.

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4.7 Figures:

Figure 4.1 The sample library preparation posed significant challenges to this project. Represented are all the important steps in sample library preparation from DNA isolation to sample pooling.

Figure 4.2 Mapping efficiency is shown to be at or above 60% in all sample groups. The mapping efficiency of Bismark is similar to the high end of efficiency described in the original release of the program.

Figure 4.3 A distribution of the absolute number of mapped fragments ($p < 0.001$) grouped by chromosome and separated into experimental comparison groups. The sex-specific test shows the vast majority of fragments on the X-chromosome, while the tissue specific and maternal age comparisons show a broad distribution over all of the autosomes.

Figure 4.4 The percent of significant DMRs relates to how similar the comparison groups are. The tissue comparisons have the largest number of DMRs (far right) while the imprinting effect of maternal age is the most subtle and hence smallest difference (far left). The empirical FDRs are calculated by comparing like-like groups for each strain cross to determine the number of DMRs falsely identified.

Figure 4.5 Different comparisons result in radically different density curves. The majority of tissue-specific DMRs are in heavily methylated regions, (top right) while the majority of sex-specific methylation changes are in low-methylation regions (top left). Changes due to maternal ageing are very subtle compared with tissue-specific and sex-specific methylation changes (middle left).

Figure 4.6 The intersection of DMRs which are changed by ageing and returned to their young state by exercise are the DMRs most probably involved in the maternal age effect (A). Part B shows that only eight DMRs are in this overlap at a p-value cutoff of 0.001. Encouragingly, there are no DMR overlaps between age comparisons of sedentary and exercise offspring, or age comparisons between the FVB/N cross and A/J cross, the latter of which does not experience a maternal ageing effect.

Figure 4.7 Each fragment is listed with its nearest gene-name, with the percent methylation across the fragment shown for old and young sedentary samples and old exercise samples (A). Young sedentary samples and old exercise samples have identical or near-identical methylation percent in each case compared with the old sedentary methylation levels. Both young sedentary and old exercise levels are significantly different than old sedentary methylation percentages (ANOVA, $p < 0.001$). In the three regions which show overlap in comparison of maternal ageing and exercise, as well as maternal ageing in strain effects (B), the direction of change is identical in the young and old groups recovered due to strain polymorphisms or exercise. Genes nearest to the overlapping DMR fragments are identified using DMAP (C), and included is information about said genes when available.

Tables:

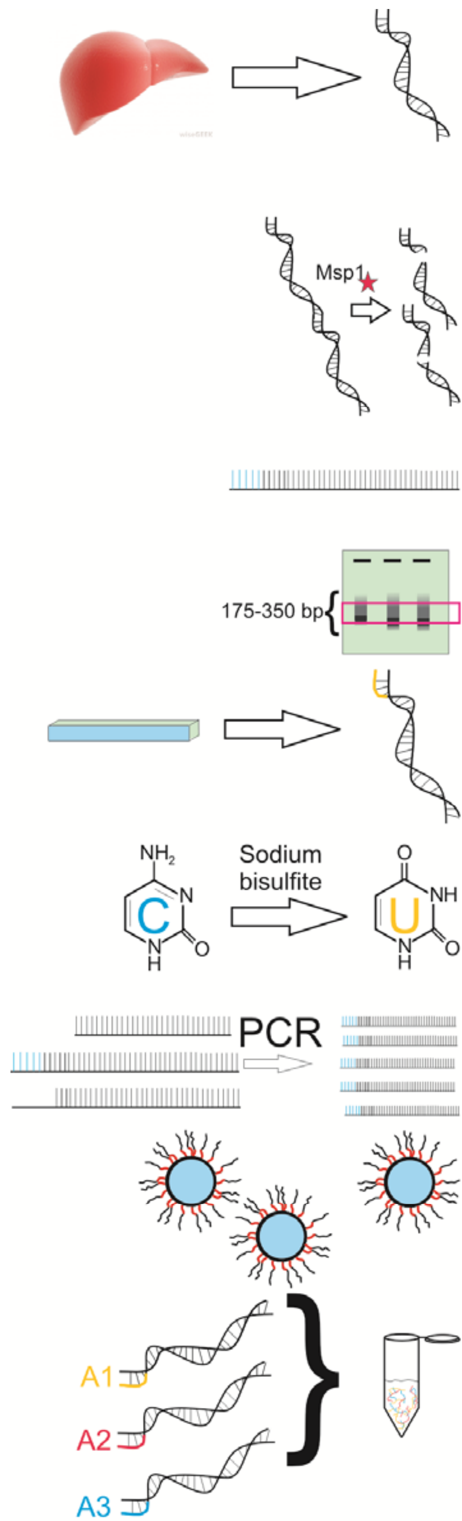
Table 4.1 The absolute level of heavily and lightly methylated DMRs in old and young mother comparisons (A), and the absolute changes in methylation between each group (B) are similar in proportion and magnitude.

Table 4.2 All of the GO terms identified in DMRs hypo methylated in heart tissue identified with GREAT. The terms represented show enrichment for heart-specific processes.

Table 4.3 All of the GO terms identified in DMRs hypo methylated in liver tissue identified with GREAT. The terms represented show an enrichment for liver-specific processes, though the specificity is less compared with heart terms.

Table 4.4 The same overlaps depicted in Fig 4.5.b, with the p-value relaxed to $p < 0.05$ to demonstrate that the overlap pattern is maintained even with a much broader cohort. The first two overlap comparisons contain a much greater proportion DMRs than expected based on a rate identified in the second two tests by a chi-square test. This reflects the fact that the second pair of overlaps do not identify a maternal age effect, while the first pair do identify maternal age effects.

Figure 4.1



Isolation of genomic DNA (gDNA)z

MSP1 digestion

Adapter annealing and end repair

Gel size-select digested sample

Gel extraction

Sodium bisulfite treat for CT conversion

Indexing PCR

Ampure bead cleanup

sample pooling

Figure 4.2

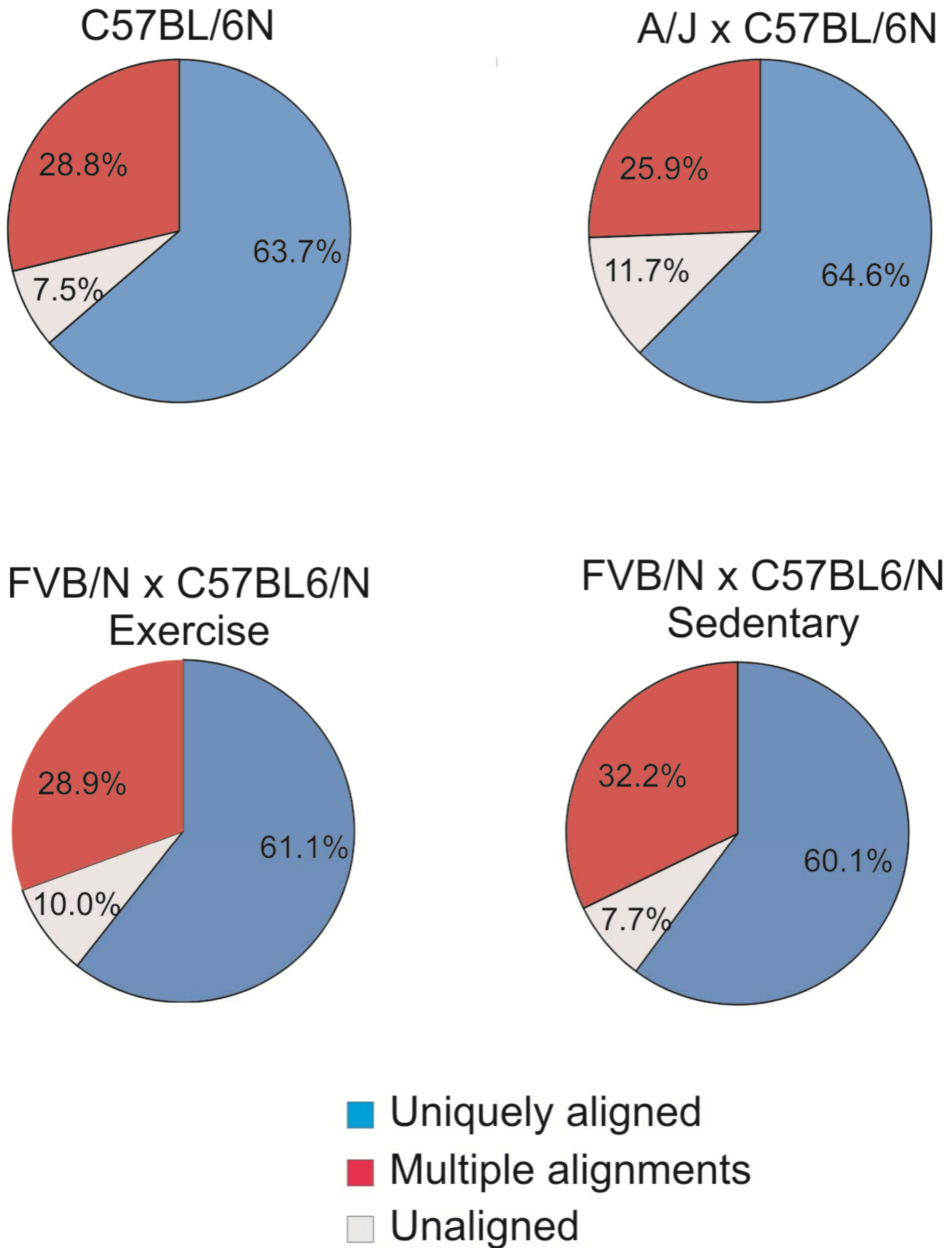


Figure 4.3

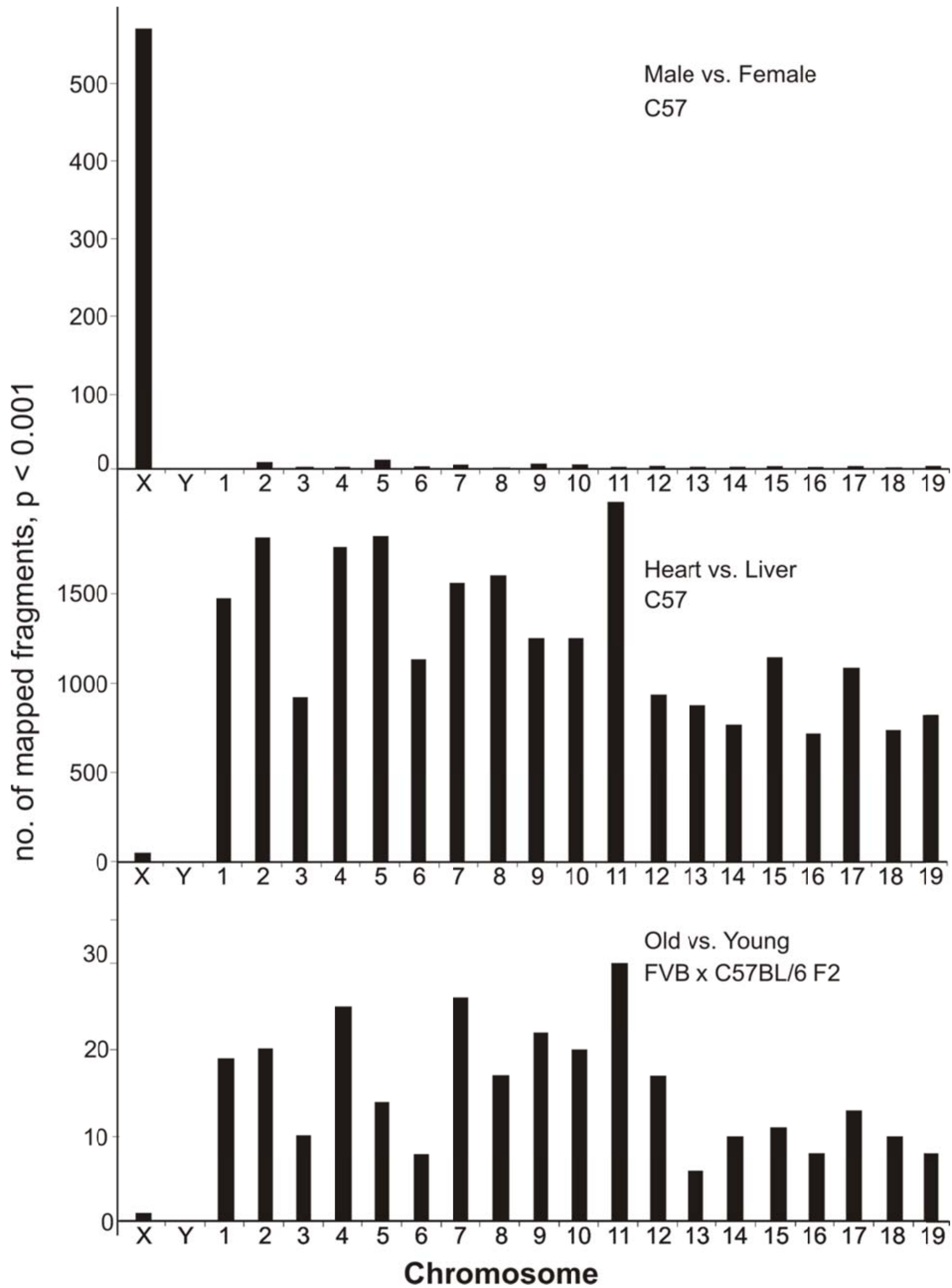


Figure 4.4

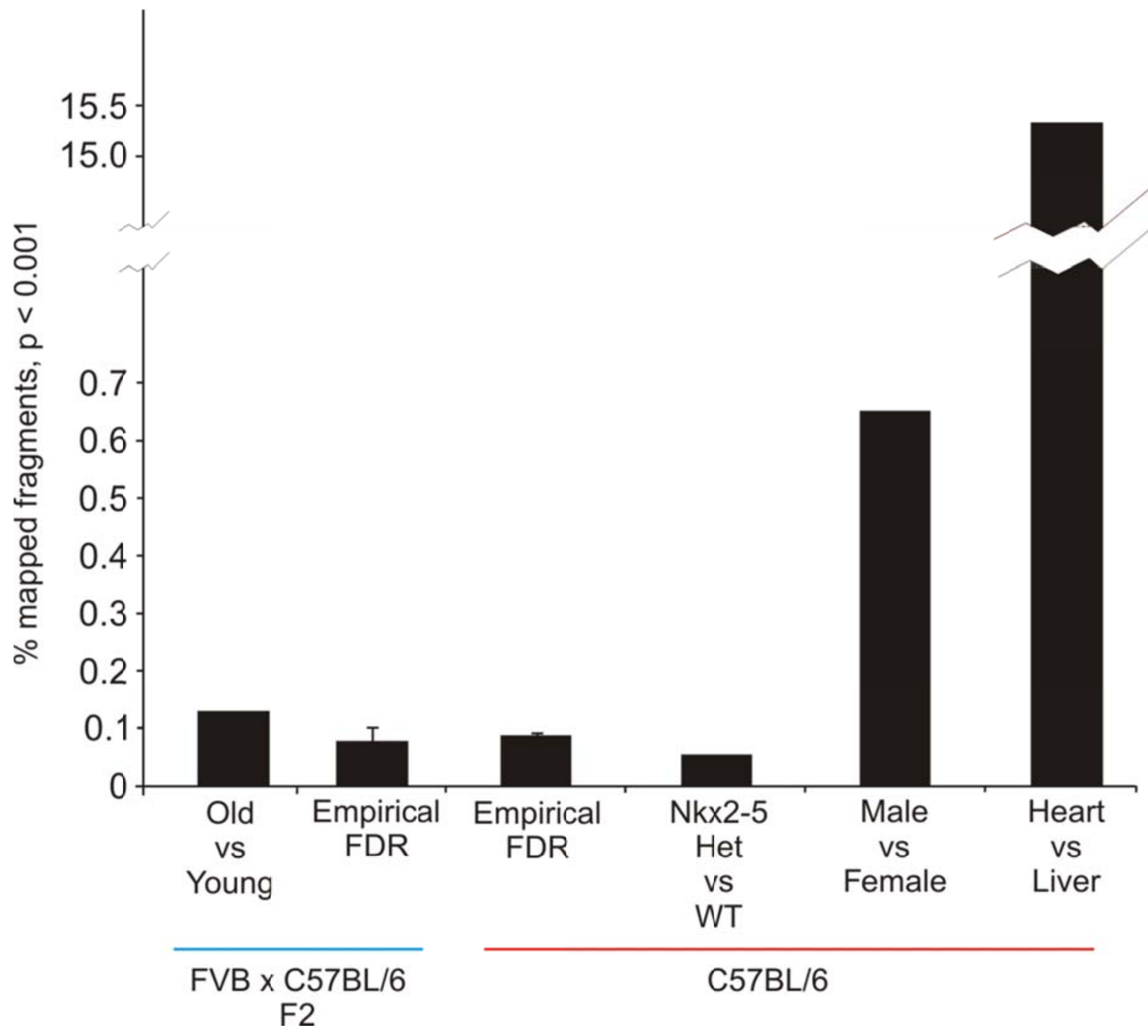


Figure 4.5

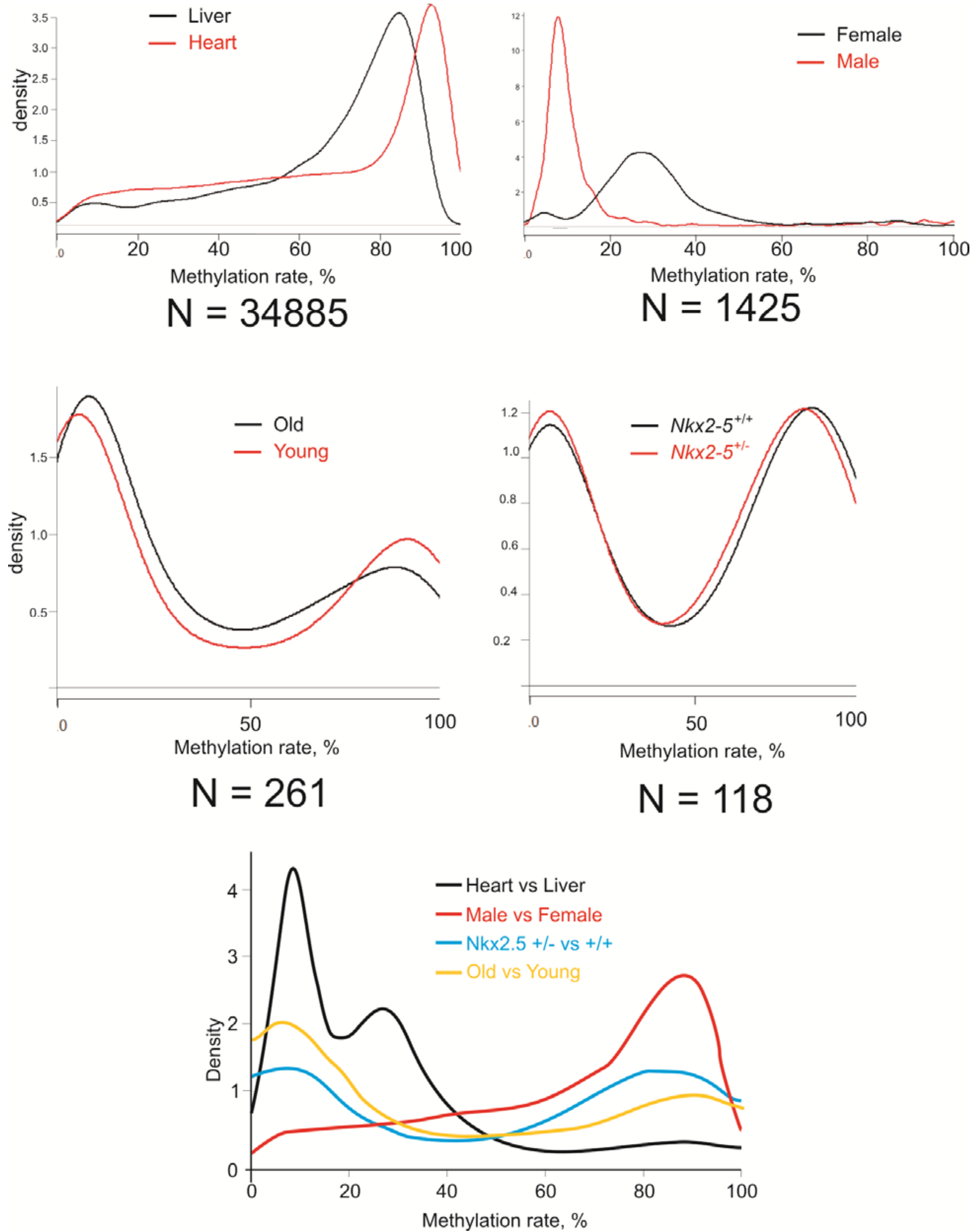


Figure 4.6

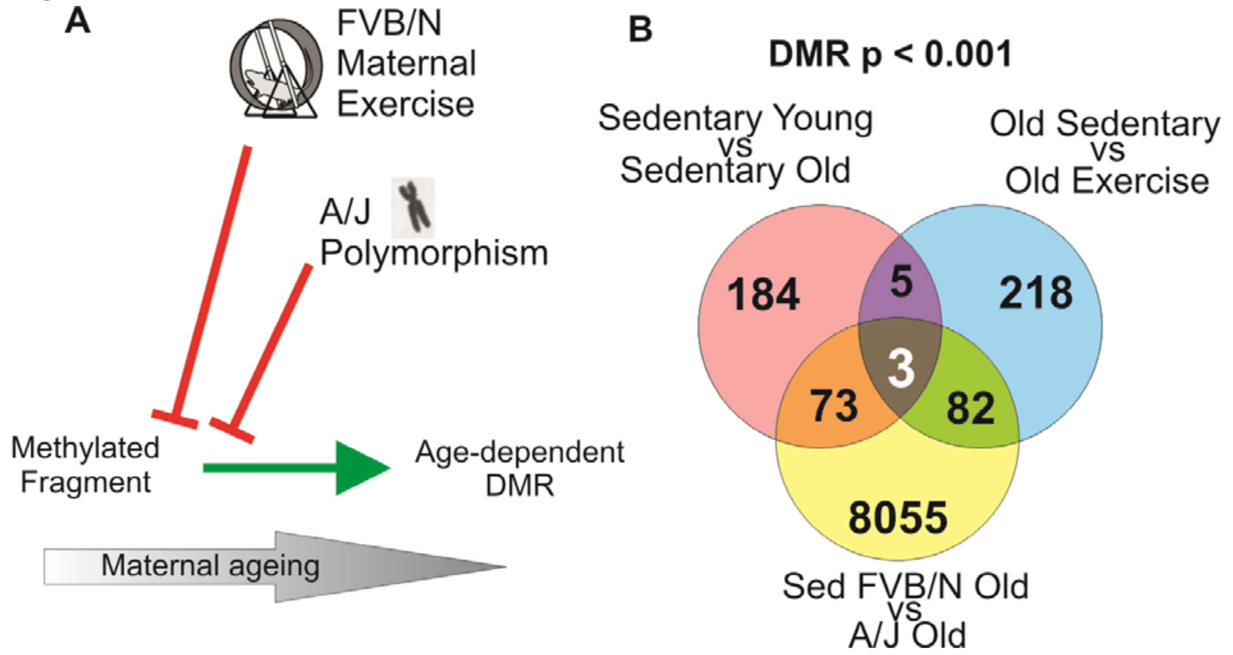
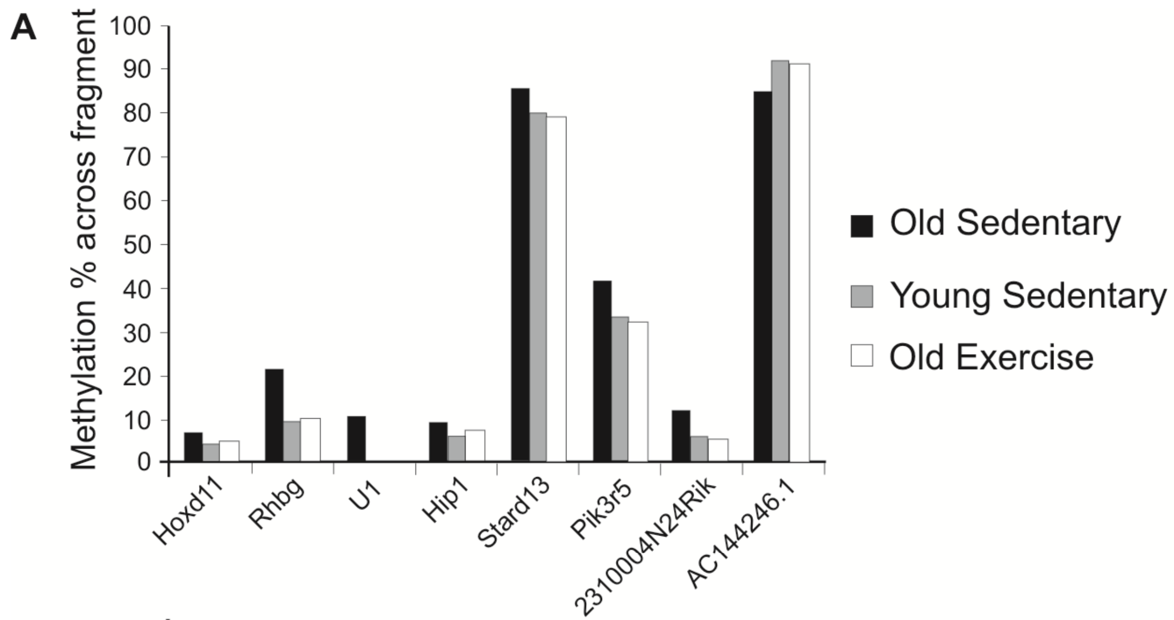
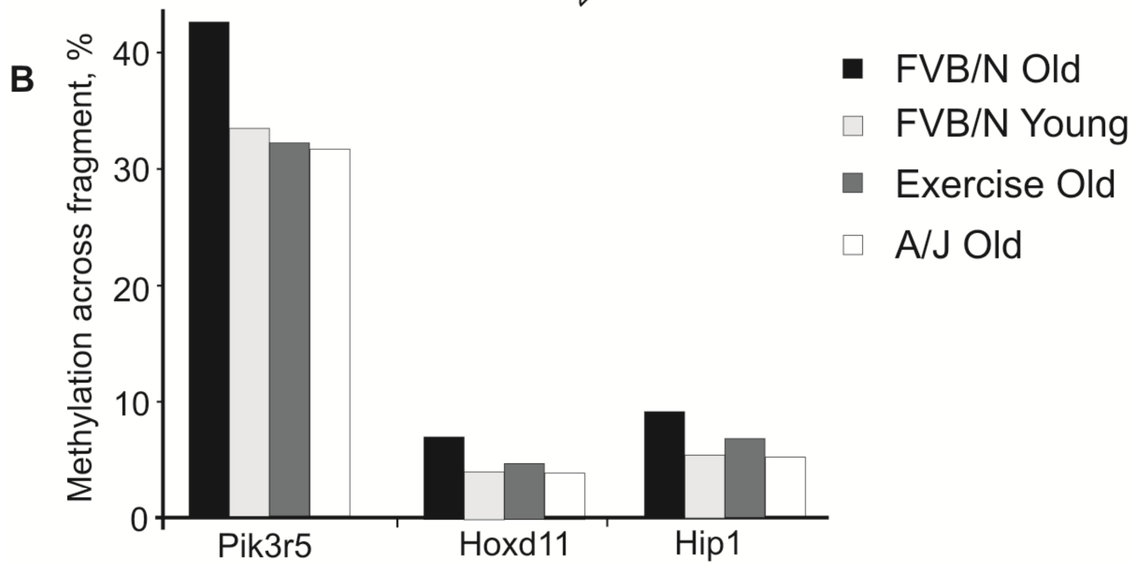


Figure 4.7





C

Nearest gene	Chromosome	Location, bp	Fragment Length	no. CpG	p-value
Hoxd11	2	74682372	70	10	0.00044581
Rhbg	3	88254687	45	4	0.000331931
U1	4	148444561	207	20	0.000493276
Hip1	5	135489038	93	7	2.24E-05
Stard13	5	151226196	119	5	0.000529567
Pik3r5	11	68432646	101	10	8.15E-05
2310004N24Rik	11	116199221	219	12	0.000928365
AC155246.1	14	73611647	111	3	0.000148492

Table 4.1

	> 70% meth	< 30% meth
Old mothers	71	151
Young Mothers	86	146

Old mothers ↑ meth	87
Young Mothers ↑ meth	70

Table 4.2

	Heart	
Ontology	**# Term Name**	**Binom Fold Enrichment**
GO Molecular Function	transforming growth factor beta receptor binding	3.2133
	protein complex scaffold	2.9590
	activin binding	2.6559
	calcium-release channel activity	2.4802
	actin-dependent ATPase activity	2.4774
	actin filament binding	2.2463
	Rho guanyl-nucleotide exchange factor activity	2.2452
	microfilament motor activity	2.2246
GO Biological Process	actin filament-based process	2.0676
	rhombomere development	5.5991
	actin-mediated cell contraction	5.3881
	actin filament-based movement	4.4046
	inositol trisphosphate metabolic process	4.2695
	negative regulation of erythrocyte differentiation	4.1563
	cardiac myofibril assembly	3.8283
	sarcomere organization	3.7374
	inositol phosphate metabolic process	3.5094
	myofibril assembly	3.4953
	regulation of myotube differentiation	3.4865
	renal absorption	3.4305
	positive regulation of focal adhesion assembly	3.4210
	actomyosin structure organization	3.2956
	immunoglobulin secretion	3.2748
	estrogen receptor signaling pathway	3.2746
	definitive hemopoiesis	3.2544
	cardiac muscle fiber development	3.1449
	plasma membrane repair	3.0732
	Golgi to plasma membrane protein transport	3.0726
	negative regulation of cytokine biosynthetic process	2.9773
	preganglionic parasympathetic nervous system development	2.9283
	Golgi to plasma membrane transport	2.8570
	thyroid gland development	2.8244
	actin filament bundle assembly	2.8077
	foregut morphogenesis	2.7477
	cardiac cell development	2.7118
	midbrain-hindbrain boundary development	2.7051
	positive regulation of isotype switching	2.6814
	heart contraction	2.6544
	cell junction organization	2.6463
	parasympathetic nervous system development	2.6391
	cardiac muscle cell development	2.6257
	cerebellar Purkinje cell-granule cell precursor cell signaling involved in regulation of granule cell precursor cell proliferation	2.5405
	cell junction assembly	2.5365
	cell-cell junction organization	2.4778
	mammary gland epithelial cell differentiation	2.4134
	protein heterotrimerization	2.3858
	heart process	2.3437
	regulation of sodium ion transport	2.3413
	positive regulation of mitotic cell cycle	2.3279
PANTHER Pathway	JAK/STAT signaling pathway	2.6767
	Notch signaling pathway	2.2888
	Alpha adrenergic receptor signaling pathway	2.2116
Pathway Commons	Signaling mediated by p38-gamma and p38-delta	3.5089
	Smooth Muscle Contraction	2.3499
	HIF-1-alpha transcription factor network	2.1275
	Hypoxic and oxygen homeostasis regulation of HIF-1-alpha	2.1217
	NOTCH	2.0582

Table 4.3

	Liver	
Ontology	**# Term Name**	**Binom Fold Enrichment**
GO Molecular Function	Rho guanyl-nucleotide exchange factor activity	2.4204
	calcium-release channel activity	2.4784
	activating transcription factor binding	2.3306
	platelet-derived growth factor binding	2.3220
	activin binding	2.2768
	carbon-nitrogen lyase activity	2.2004
GO Biological Process	establishment of monopolar cell polarity	3.8933
	reverse cholesterol transport	3.6274
	mature B cell differentiation	3.5643
	cholesterol efflux	3.1899
	embryonic retina morphogenesis in camera-type eye	3.1410
	cell differentiation involved in embryonic placenta development	2.9991
	adherens junction organization	2.8016
	embryonic foregut morphogenesis	2.8005
	foregut morphogenesis	2.7860
	negative regulation of multicellular organism growth	2.7619
	lipid digestion	2.7185
	trophectodermal cell differentiation	2.7160
	branching involved in mammary gland duct morphogenesis	2.5929
	endocytic recycling	2.5321
	mesoderm morphogenesis	2.4931
	regulation of insulin receptor signaling pathway	2.4214
	protein heterotrimerization	2.3770
	establishment or maintenance of apical/basal cell polarity	2.3437
	negative regulation of transforming growth factor beta receptor signaling pathway	2.3080
	ureter development	2.3003
	morphogenesis of a polarized epithelium	2.2942
	positive regulation of Rac GTPase activity	2.2803
	mesoderm formation	2.2783
	positive regulation of smooth muscle cell proliferation	2.2489
	regulation of striated muscle contraction	2.2220
	stem cell maintenance	2.2127
	cellular response to oxygen levels	2.2046
	mesoderm development	2.2024
PANTHER Pathway	FOXA2 and FOXA3 transcription factor networks	2.3952
Pathway Commons	Lipid digestion, mobilization, and transport	2.0773
	Beta2 integrin cell surface interactions	2.2175

Table 4.4

	Age effect	Total DMR $p < 0.05$	Overlap w/ Sed FVB Old vs Young	% overlap of total DMR
Old FVB/N Sed vs Exercise	+	13,299	2,413	18.1*
Old FVB/N vs A/J	+	57,764	6,067	10.5*
Exercise FVB/N Young vs Old	–	13,304	759	5.7
A/J Young vs Old	–	6,875	391	5.7

* $p < 10^{-10}$ chi-square

Chapter 5: Conclusion and Future Directions

5.2 Mechanistic understanding of the maternal age effect

In this study, maternal ageing was shown to be a modifiable risk factor for congenital heart defects via exercise in a murine model of the disease, and epigenetic modifications were implicated as a mechanistic basis for this effect. Through ovarian transfer experiments compared with a comprehensive database of prior murine epidemiologic data and statistical models as described in **Chapter 2** it has been shown that the maternal age effect is due to a maternally intrinsic factor. Logistic regression analysis of three mouse strains showed that maternal age risk has a strong genetic component, with some mothers being highly susceptible to maternal age risk and others being resistant. Dietary and exercise interventions have shown that the maternal age effect is modifiable, though not due simply to glucose intolerance and increased maternal adiposity (**Chapter 3**). Reduced representation bisulfite sequencing identified several candidate differentially methylated regions for further study as they relate to congenital heart disease risk and as potential biomarkers for congenital heart disease and maternal age risk (**Chapter 4**).

Future methylation studies and analysis of current data

The analysis of next generation sequencing (NGS) data, like onions, and people, is layered and complex. The analysis completed within this dissertation is but the first in the potential mining of this unique dataset. The methods enumerated herein take advantage of one of the unique properties of RRBS datasets -- the a priori approach to site sampling -- but has not yet delved into the site-specific methylation data which is available with this powerful sequencing technology, or the data

sets provided by sequencing of high fat mothers' offspring and in-depth analysis of exercise and strain effects on methylation profiles.

DMAP provided a powerful, computationally efficient method for fragment-wide methylation changes and DMR calling, but it does not currently have the power to identify site-specific methylation changes. DMRs such as those depicted in figure 5.1 would be impossible to detect with DMAP, but may have important developmental impact. Sequence regions with similar overall methylation levels but segregated methylation locations within those regions (Fig 5.1.A) would be undetectable. Similarly, strand-specific methylation changes in methods which take forward and reverse strands into account to study singular regions would be unable to detect strand-specific methylation patterning (Fig 5.1.B). Though the likely import of a single site is low, an ideal computational method would look for clusters of significant altered sites indicating a region of variable methylation with the experimental variable (sex, strain, feeding or exercise regimen, maternal age). There are as many sets of important genes, as there are subtly different methods of analyzing a dataset, but nevertheless, a method such as bsmooth (Hansen KD, 2102) which takes into account site-specific changes as opposed to averaging over large areas could provide valuable new insight.

Additionally, the effects of exercise and diet on the methylation patterning of offspring has not been explored in this project, and could provide fascinating insights in trans-generational epigenetic impact of maternal lifestyle, both by indicating genes which may be important for offspring health, and in proposing a mechanism by which maternal lifestyle may have long term health impacts on the fetus.

Metabolomics search for 'ageing metabolite'

There are many ways in which ageing may affect maternal health, and by extension, that of the fetus. One of the most obvious would be the discover of some maternal ageing metabolite in serum blood. The identification of some protein , enzyme, or hormone, which acts as a circulating factor, imbuing an individual with the maternal age effect would be invaluable both as a metric and marker of declining maternal health, and also as a potential target to the pathway beyond high-level interventions like exercise.

Though this study is still beyond us in humans, mice pose a simplified problem. Firstly, mice are composed of a genetically homogeneous population, and can be sampled at various life stages on a regimented schedule. Secondly, there is no dietary or lifestyle variation within these populations. Thirdly, some mouse strains experience the maternal age effect while others do not, providing a sample and control population on which to test for circulating factors. These benefits allow us to create a homogenous sample group to search for a metabolite responsible for maternal ageing. Mass spectronomy provides a sensitive method for detecting unique metabolites in serum samples. Currently samples from young and old mothers are being processed to determine unique markers of ageing in the blood. If a marker is found, it will provide a means of identifying at-risk mothers for additional interventions, and may help identify the age of onset of the maternal age effect in humans as well as factors relating to delay of onset.

5.2 Fine mapping of genetic modifiers

Initial goal to find genes related to congenital heart disease risk was foiled when linkage mapping indicated large regions of the mouse genome which contained no known cardiac development genes, or cardiac development genes which had been ruled out as related to CHD development. The options were twofold: choose a new method of candidate gene discovery, or narrow down the linkage maps so they consisted of a much smaller search space. The latter was feasible due to an breeding plan for an advanced intercross population. An advanced intercross interbreeds genetically heterogeneous animals as a cohort aiming for maximum genetic variation, thereby enabling much finer mapping of regions of interest in the mouse genome.

Offspring have been collected from the F_{10} generation, and informative SNPs placed at 5x the density of previous studies. These SNPs can be used to map each of the type of defects discovered in that population. Fine mapping of this population will give a nuanced view of regions of import regarding CHD development, and may provide a genetic key to the maternal ageing effect.

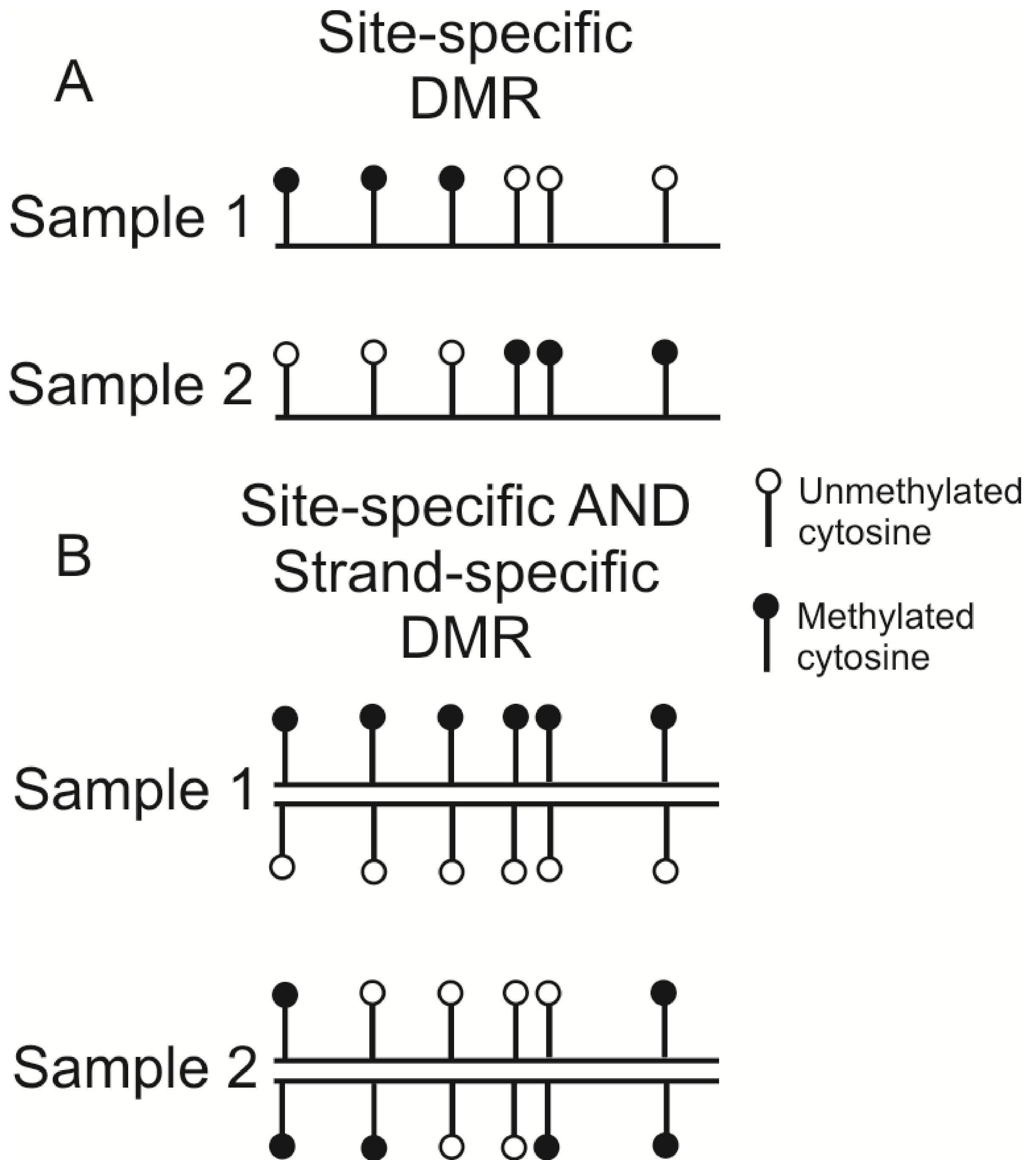
5.3 Works Cited

Hansen KD, L. B. (2102). BSmooth: from whole genome bisulfite sequencing reads to differentially methylated regions. *Genome Biology*, epub online.

5.4 Figures:

Figure 5.1 Part A depicts DMRs which are indistinguishable from one another without the aid of site-specific methylation calling, due to their overall identical methylation levels across the fragment. Part B shows two samples of forward and reverse compliment DNA which would be indistinguishable without techniques which take into account both site-specificity and strand-specificity in the DMR calling.

Figure 5.1



Claire E. Schulkey

Education

Washington University in St. Louis
PhD Computational and Systems Biology
Origin of Maternal Age Effect in Congenital Heart Disease Risk for Offspring

California State University, Los Angeles
BS Applied Mathematics

Research experience

PhD Candidate
Genetic and environmental causes of congenital heart defects in humans using statistical and mouse models
Summer Undergraduate Researcher
Southern California Bioinformatics Summer institute studying the movement of Auxins using plant cell models
Summer Undergraduate Researcher
Mapping the ternary phase diagram for the water-NaCl-Propylene Glycol system for cryobiology
Undergraduate Researcher
Statistical methods for modeling the population dynamics of cannibalistic flour beetles

Papers and Talks

Schulkey CE, Luther H, Danzo MT, Regmi SD, Hutchinson AK, Panzer AA, Grady MM, Magnan RA, Wilson DB, Jay PY, Maternal Age Is a Modifiable Risk Factor for Congenital Heart Disease (in review)

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2006 Background and Uses of Modeling in Cryobiology, California State University -- Long Beach Ca, Regional Mathematics Meeting.

2012 Advanced Maternal Age Increases the Risk of Congenital Heart Disease through an Oocyte Independent Mechanism. Poster Presentation, Society for Gynecologic Investigation.

2013 Maternal Age: A Modifiable Risk Factor for Congenital Heart Disease. Symposia Presentation, Society for Gynecologic Investigation.

2013 Complex trait analysis of ventricular septal defects caused by Nkx2-5 mutation. Plenary Session Presentation, Weinstein Cardiovascular Development Annual Meeting, Chicago Il,

2014 Maternal age as a risk factor for congenital heart disease is influenced by genes in the mother. Poster Presentation, Society for Gynecologic Investigation.

2013 Lucille P. Markey Translational Medicine Symposium, WUSM

2013, 2012, 2011 Genetics Deparment Retreat, WUSM.

2013, 2012 Developmental Biology Deparment Retreat, WUSM.

2014, 2013, 2012, 2011 Developmental Cardiology Forum, WUSM

2014, 2013, 2012, 2011 Pediatrics Research In Progress Symposium, WUSM

Grants and Awards

2013 SGI President's Presenter Award, San Diego, Ca

2012 SGI President's Presenter Award, Orlando, Fl

2012 Departmental Presenter Award, WUSM Genetics Department

2011 – 2013, Lucille P. Markey Special Emphasis Pathway in Human Pathobiology, Washington University School of

Medicine, St. Louis

2010 – 2013, NHLBI, 2 T32 HL007873 Developmental Cardiology and Pulmonary Training Program

2007 Charles Clark Scholarship for achievement in math, CSULA

2006 Academic Achievement Award, CSULA Math Department