SIZE-DEPENDENT ACQUISITION OF GLOBAL DNA METHYLATION IN OOCYTES IS ALTERED BY HORMONAL STIMULATION

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The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

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NOMENCLATURE

ART	Assisted Reproductive Technology
AS	Angelman Syndrome
AU	Arbitrary Unit
BMP-15	Bone morphogenetic protein 15
BWS	Beckwith-Wiedemann Syndrome
cDNA	Complementary DNA
ct	Cycle Threshold
DMR	Differentially Methylated Region
DNA	Deoxyribonucleic Acid
DNAm	DNA methylation
Dnmt 3a	DNA methyltransferases 3a
Dnmt 3b	DNA methyltransferases 3b
Dnmt 3L	DNA methyltransferases 3L
Dnmt 1	DNA methyltransferases 1
eCG	Equine Chorionic Gonadotropin
ERα	Estrogen Receptor α

ERβ	Estrogen Receptor β
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
GDF-9	Growth Differentiation Factor-9
GV	Germinal Vesicle
HAT	Histone Acetyltransferase
hCG	Human Chorionic Gonadotropin
HDAC	Histone deacetylase
IAP	Intracisternal A Particle
ICSI	Intracytoplasmic Sperm Injection
IU	International Unit
IUI	Intra Uterine Insemination
IVC	*In Vitro* Cultured
IVF	*In Vitro* Fertilization
LOI	Loss-of-imprinting
LH	Luteinizing Hormone
MII	Metaphase II

NCOA1	Nuclear Receptor Coactivator-1
NO	Natural Ovulation
NSN	Non Surrounded Nucleolus
PCR	Polymerase Chain Reaction
PMSG	Pregnant Mare Serum Gonadotropin
RNA	Ribonucleic Acid
RTPCR	Real-Time Polymerase Chain Reaction
SN	Surrounded Nucleolus
SO	Superovulation
ZP	Zona Pellucida
5MeC	5-Methyl Cytosine

Chapter I

Literature Review

Introduction

Assisted Reproductive Technologies

The overall worldwide prevalence of infertility in women is nine percent with 56% of couples seeking medical care (Boivin *et. al.,* 2007). Assisted reproductive technologies (ART) are commonly used for the treatment of infertility problems, and children born from ART procedures can account for as high as 3.9% of all children in developed countries (Andersen *et. al.,* 2009). In humans, the most common ART procedures used are ovarian hyperstimulation, intrauterine insemination (IUI), *in vitro* fertilization (IVF), *in vitro* culture (IVC) and embryo transfer. Ovarian hyperstimulation (superovulation [SO]) by administration of exogenous gonadotropins is an ART procedure used to increase the number of mature oocytes in order to improve the success rate of achieving a pregnancy per reproductive (menstrual) cycle.

Adverse Outcome of Assisted Reproductive Technologies

Superovulation in mice has been documented to increase the proportion of abnormal preimplantation embryos, to result in higher postimplantation

mortality rates, to delay embryonic development, and to cause a reduction in fetal weight when compared to embryos produced after natural ovulation (NO) (Ertzeid and Storeng 1992). The abnormalities observed in pre- and post-implantation development have been concluded to be due to impaired oocyte competency as well as to an altered uterine milieu (Ertzeid and Storeng 2001; Van der Auwera and D'Hooghe 2001; Fortier *et. al.,* 2008).

Superovulation may cause alterations in oocyte quality and uterine environment as a consequence of the increased level of circulating steroid hormones which result after gonadotropin administration (Ertzeid and Storeng 2001). Even though the uterine milieu can be altered by exogenous hormone administration (Ertzeid and Storeng 2001), this does not account for all of the reported abnormalities. For example, in a reciprocal embryo transfer study, the rate of implantation was significantly lower and the mortality of embryos greatly increased in SO mice when compared to controls after transferring both types of embryos to separate uterine horns of individual pseudopregnant recipients (Ertzeid and Storeng 2001).

In humans, a number of studies have shown an increased incidence of the loss-of-imprinting (LOI) syndromes Beckwith-Wiedemann (BWS; Maher *et. al.,* 2003; DeBaun *et. al.,* 2003) and Angelman (AS; Cox *et. al.,* 2002; Ørstavik *et. al.,* 2003) in children conceived with ART. A retrospective study found that ovarian hyperstimulation was the only common feature in ART-related BWS (Chang *et. al.,* 2005). Another study (Olson *et. al.,* 2005) found that children conceived by the use of IVF had increased probabilities of major birth defects as compared

with the control group (*i.e.* children conceived after natural ovulation). Furthermore, another observation made in the Olson retrospective study is that infants conceived by the use of IUI exhibited higher incidence of musculoskeletal defects (Olson *et. al.,* 2005). In this procedure, the only ART procedures used were hormonal stimulation and the insemination of sperm into the uterus.

Superovulation recruits a large number of ovarian follicles containing oocytes < 70 µm diameter found in gonadotropin responsive secondary follicles. This in turn may affect the normal acquisition pattern of imprinted methylation that occurs during oocyte growth (discussed further in methylation and genomic imprinting sections). Published works have found inconsistent results regarding the effect of superovulation on the acquisition of DNA methylation (DNAm). Sato *et. al.* (2007) examined the effects of superovulation on the establishment of DNAm marks in germinal vesicle (GV) and meiosis I (MI) oocytes collected from humans and mice. The authors found that superovulation caused an abnormal gain of DNAm at the *H19/IGF2* imprinting control region (ICR) in both stages of oocytes examined. The ICR for the *H19/IGF2* gene cluster is normally unmethylated on the maternal chromosome. In human oocytes, there was also loss of methylation of *PEG1*, a normally methylated maternal imprint, whereas the study could not find any significant differences in the level of DNAm at *Peg1* or *Zac1* differentially methylated regions (DMRs) in the oocytes from superovulated mice. Another study used individual blastocyst-stage mouse embryos to determine the effect of superovulation on several gametic imprints (Market-Velker *et. al.,* 2010). This study utilized low (6.25 international units) and

high (10.0 international units) doses of the hormone pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) to evaluate whether the previously reported aberrant DNAm at imprinted loci was dose dependent. It was found that superovulation causes loss of methylation at the *Snrpn* and *Peg3* DMRs, and gain of methylation at the *H19/IGF2* ICR in the maternal alleles. The perturbation of DNAm was more pronounced in the high hormone dose group.

Imprinted genes are expressed from one of the parental alleles in a parent-specific manner. Fortier and coworkers (2008) showed that superovulation treatment in mice causes biallelic expression of the imprinted genes *Snrpn* and *H19* in placentae of concepti at 9.5 days of gestation. However, this was not observed in the fetus where both *Snrpn* and *H19* were expressed in the normal monoallelic fashion (Fortier *et. al.,* 2008). Interestingly, they found no connection between biallelic expression of those genes and the level of methylation of their regulatory regions.

CpG methylation at several ICRs was recently evaluated in order to determine the transgenerational effects of superovulation on genomic imprinting (Stouder *et. al.,* 2009). These authors set out to determine if transgenerational effects of superovulation were detectable in two month old male offspring of females that underwent SO regimens. The imprinted loci studied included paternally methylated *H19/IGF2* and *Gtl2* regions as well as the maternally methylated *Peg1, Snrpn,* and *Peg3* imprinted regions. They observed a significant decrease (6%) in methylation levels at the *H19/IGF* ICR, and an

increase in methylation at the *Peg1* and *Snrpn* loci (2.8 and 7.0 fold, respectively) in sperm of two month old males born from SO mothers when compared to the offspring conceived after natural ovulation. This study could not find any differences in methylation status in somatic tissues (tail, liver, skeletal muscle) of male and female offspring of SO and control mothers. In addition this study also examined the methylation status in the sperm of second generation male offspring. They found that methylation was decreased by 3.9% at the *H19/IGF2* ICR while methylation on *Snrpn* was increased by 3.1 fold in those males.

In summary, superovulation affects oocyte competence and uterine environment which results in inappropriate pre- and post implantation development as well as cause fetal growth retardation. The effects on oocyte competence may be due in part to improper levels of DNAm as some studies have observed a negative association between DNAm at imprinted loci and the superovulation procedure.

Folliculogenesis and Oogenesis

The fertilized egg and its pre- and postimplantation development depend on the acquisition of meiotic and developmental competencies during oogenesis. Meiotic competency is the ability of an oocyte to complete the first meiotic division with the extrusion of the first polar body whereas the ability of an oocyte to fertilize and produced viable offspring is referred to as a developmental competence (Armstrong 2001).

Murine oogonia within the germ cell nest, enter a premeiotic state around 12.5 days post coitum (dpc; reviewed in Tingen *et. al.,* 2009) and meiotic arrest occurs at 16.5 dpc. Oocytes within the nest remain connected to each other by cytoplasmic bridges and are arrested at diplotene stage of meiotic prophase I. The germ cell nests are surrounded by pre-granulosa cells and stromal mesenchymal cells. These nests start to break down immediately after birth to form individual follicles. One-third to two-thirds of the oocytes die during the process of germ cell nest break down due to genetic defects or failure of the germ cells to generate all the essential cytoplasmic organelles. Abnormal breakdown of germ cell nests can augment the loss of germ cells or cause the development of multi-oocyte follicles (Tingen *et. al.,* 2009). A number of mechanisms including apoptosis, local factors, and steroids as well as circulating hormones regulate the nest breakdown and assembly of primordial follicles where an oocyte is surrounded by a single layer of flattened pre-granulosa cells (McGee and Hsueh 2000). Molecular factors such as Kit ligand, basic fibroblast growth factor, and the members of transforming growth factor beta (TGF-β) family initiate the transition from primordial to primary follicles. A primary follicle refers to the stage of follicle in which the oocyte is surrounded by a single layer of cuboidal granulosa cells.

Once recruited, primordial follicles continue to grow until ovulation or they undergo atresia. Only a few of the follicles achieve full maturation and ovulate while the majority of the follicles are destined to undergo atresia once they start to differentiate (Fortune 1994). The preantral follicle is responsive to follicle

stimulating hormone (FSH) and its growth is regulated by the interactions between the oocyte and granulosa cells, and the local production of the growth factors, growth differentiation factor-9 (GDF-9) and bone morphogenetic protein 15 (BMP-15) by the oocyte (Hutt and Albertini 2007). GDF-9 is required for the proliferation of granulosa cell as well as for the assembly of theca cells around the follicle. BMP-15 is a potential stimulator of granulosa cells proliferation (Otsuka *et al.,* 2000). Cytoplasmic processes of granulosa cells known as trans-zonal projection (TZP) are established at the onset of folliculogenesis and maintain oocyte-granulosa cell communication.

On the other hand, antral and preovulatory follicular growth depend on the cyclical secretion of the pituitary gonadotropins FSH and luteinizing hormone (LH), and the coordinated actions of the oocyte, granulosa cells, and theca cells. The oocyte acquires developmental competence during this time. It is likely that exogenous gonadotropin treatment may stimulate the growth of both large antral and smaller preantral follicles as the latter follicles (secondary) are gonadotropin responsive (McGee and Hsueh, 2000). A secondary follicle has two or more layers of granulosa cells without formation of an antrum. The secondary follicle continues to grow becoming a tertiary follicle which contains a fluid filled cavity (or antrum)**.** FSH regulates the interaction between the oocyte and its surrounding cumulus cells during antral follicle development and controls the organization of TZP networks.

Theca cells are closely associated with the basal lamina of the ovarian follicles. Theca cells produce androgens as the ovarian follicle starts growing.

These androgens are taken up by the granulosa cells of late preantral follicles and get converted to estrogen under the influence of FSH (reviewed in Drummond, 2006). Estrogen exerts negative feedback on the anterior pituitary and controls the secretion of FSH. Estrogen receptors are present in the cytoplasm and nucleus and play role in the activation of transcription after receptor binding. In addition, estrogen enhances follicular responsiveness to gonadotropin by maintaining FSH and LH receptors on the follicular cells (Couse and Korach, 1999). Estrogen is also essential for the acquisition and maintenance of cumulus cell expansion (Sugiura *et. al.,* 2010).

In mammals, oocytes are arrested at the dictyate stage of prophase I and start growing shortly after birth. The maternal genome gradually acquires developmental competence in oocytes with the increase in size of oocytes. This has been demonstrated by a nuclear transfer experiment (Bao *et. al.,* 2000), in which there was no difference in blastocyst formation of embryos produced with the chromatin from 50-59 µm, 60-69 µm size of oocytes. However, the former produced only 5% while the later resulted in 15% live offspring. The maternal chromatin from full-grown oocytes (75-80 µm) produced 30% live offspring. In the mouse, the diameter of oocytes ranges from ~10 µm (non-growing) to a full-grown size of 70 – 80 µm.

The LH surge stimulates oocytes to undergo nuclear maturation. The resumption of meiosis and first meiotic division is completed in the full-grown oocyte immediately before ovulation (Schultz and Wassarman, 1977). This results in the extrusion of the first polar body. The polar body contains a set of

homologous chromosomes with a small amount of cytoplasm. The other set of chromosomes remain in the oocyte and meiosis becomes arrested at metaphase of meiosis II (MII) during its release from the follicle (*i.e.* ovulation) (Nagy *et. al.,* 2003). Fertilization of the ovulated oocyte takes place at the ampulla of the oviduct and this induces the resumption of meiosis causing the extrusion of the second polar body.

Oocytes within antral follicles are classified as either surrounded nucleolus (SN) or non-surrounded nucleolus (NSN). This nomenclature refers to the conformation of the chromatin. In the NSN, the chromatin is less condensed while SN is highly condensed and concentrated around the nucleolus. Oocytes with SN have increased meiotic competence when compared to NSN oocytes (Christians *et. al.,* 1999). In addition, SN oocytes have greater developmental competence than NSN oocytes. Epigenetic modifications, like histone modifications, may be responsible for the changes in chromatin configuration as a study has shown that the levels of histone acetylation and methylation were higher in SN oocytes than in NSN oocytes (Kageyama *et. al.,* 2007).

Superovulation/Ovarian hyperstimulation

A large number of high quality oocytes with meiotic and developmental competence are vital for the success of ART procedures, and for the study of the basic physiological and cellular mechanisms of oocytes. FSH regulates the proliferation as well as the differentiation of granulosa cells and stimulates the

expression of LH receptors. In turn LH stimulates nuclear maturation, follicle rupture and release of the oocyte from the follicle. In mice, superovulation regimen involves the use of an exogenous follicle stimulating hormone (e.g. PMSG) to induce follicular growth and an exogenous luteinizing hormone (e.g. hCG) for the induction of ovulation. A combination of PMSG/hCG is most commonly used for the induction of superovulation in laboratory mice. PMSG is used to mimic the endogenous FSH, and hCG is used to mimic the endogenous LH (Nagy *et. al.,* 2003).

In humans, superovulation involves a more invasive approach. Generally the hypothalamic/pituitary axis is shut down by a gonadotropin releasing hormone (GnRH) analogue to inhibit the production of endogenous FSH and LH. Exogenous gonadotropins (human menopausal gonadotropins, purified urinary FSH or recombinant FSH) are administered for 7-12 days; hCG is then administered when the dominant follicle becomes 17-20 mm diameter to induce ovulation. Oocytes are surgically removed at ~ 34-36 hours after administration of hCG. Clomiphene citrate is also used for the indirect stimulation of pituitary gonadotropins. Clomiphene citrate binds to estrogen receptors of the hypothalamus and pituitary without exerting the biological functions of estrogen, thus inhibiting the endogenous estrogen’s negative feedback on the pituitary. As a result, the pituitary secretes gonadotropins and stimulates folliculogenesis. Clomiphene citrate is administered orally and started on the days two to five of the menstrual cycle and continued for five days. The LH surge is monitored by

measuring serum and/or urinary LH levels (at ~ 14 days) and oocytes are collected at 38 hours after LH surge (Davis and Rosenwaks 2001).

Epigenetic modifications

DNA methylation

DNA methylation is a reversible and heritable epigenetic modification where a methyl group is added to a cytosine base at the carbon-5 position in CpG dinucleotide pairs (Razin and Riggs, 1980). Methylated DNA controls the expression of genes as methylated cytosines (5Me-C) attract methyl-DNA binding proteins (MeCP2, MBD1, MBD2, MBD3) and promotes chromatin condensation into a transcriptionally repressive conformation (Berger 2007; Newell-Price *et. al.,* 2000; Bird 1992). DNAm is essential for genomic imprinting, X-chromosome inactivation and the silencing of endogenous retroviruses (Bestor 2000; Reik and Walter 2001; Walsh *et. al.,* 1998).

DNA methyltransferases (Dnmts) are required for the establishment and maintenance of DNAm. Dnmt 3a, Dnmt3b and Dnmt3L are responsible for *de novo* DNAm whereas Dnmt1 is responsible for the maintenance of methylation. A study has shown that disruption of global DNAm by introducing a mutation in the Dnmt1 gene caused abnormal development, and embryonic death in mice (Li *et. al.,* 1992). Dnmt3L mediates the binding of Dnmt3a to its target sequences (Kato

et. al., 2007). Homozygous Dnmt3a mutant mice survive until around four weeks after birth while Dnmt3b mutant embryos are unable to survive past midgestation (Okano M. *et. al.,* 1999). DNA methylation is globally erased in primordial germ cells during migration from the yolk sac to the genital ridge and are hypomethylated in male and female embryos at E13.5 (Popp *et. al.*, 2010; Hajkova *et. al.,* 2002). Remethylation of the genome occurs in the male germ line before birth (~ E18.5) and in the female after birth during oocyte growth (Reik and Walter 2001). The expression of Dnmt3a, Dnmt3b, Dnmt3L and the oocyte specific Dnmt1 (Dnmt1o) increase with the increased size of the oocyte starting from 10-20 μm diameter to a fully grown GV stage oocyte and then decreases in MII oocytes (Lucifero *et. al.,* 2007).

Transposable elements are abundant in the mammalian genome and they are positioned around and within most genes. These elements are normally transcriptionally silenced by DNAm (Walsh *et. al.,* 1998). Intracisternal A particles (IAP) are one type of transposable element and methylation on IAPs occurs during oocytes growth in a pattern similar to imprinted loci (Lucifero *et. al.,* 2004). IAP in the primordial germ cells of E13.5 of both male and female embryos is unmethylated whereas IAP is highly methylated in unfertilized oocytes (Walsh *et. al.,* 1998). Their activation by deletion of Dnmt1 can cause misregulation of nearby genes which can translate into new products with harmful effects (Yoder *et al.,* 1997).

Genomic Imprinting

Genomic imprinting is an epigenetic form of gene regulation in mammals that leads to the parent specific expression of approximately 100 genes. These genes are found throughout the genome and are often found in clusters. The allele-specific monoallelic expression of clustered imprinted genes is directed by differential DNAm of a region referred to as the ICR. The correct allelic expression of imprinted genes is required for normal embryonic growth, placental function, and neurobehavioral processes (Fowden *et. al.,* 2006; Plagge *et. al.,* 2004).

Methylation imprints are erased in primordial germ cells early in fetal life and re-established during gametogenesis in a sex-specific manner (Reik and Walter 2001; reviewed in John and Lefebvre 2011). The methylation imprints acquired during gametogenesis are referred to as gametic or primary imprints. There are 21 identified differentially methylated regions that direct parent-specific gene expression in mammalian gametes; 17 on the maternal genome and four on the paternal genome (reviewed in Chatolia *et al*., 2009; reviewed in John and Lefebvre 2011). In mice, DNAm on imprinted genes (monoallelic expressed gene) is acquired asynchronously during oocyte growth in a gene-specific manner (Hiura *et. al.,* 2006; Lucifero *et. al.,* 2004). The imprinted mark must be maintained during preimplantation development to establish the correct parental expression pattern of these genes in somatic tissues.

Histone modifications

The nucleosome is the basic unit of chromatin, which is composed of 146/147 bp of DNA wrapped around an octamer of histone proteins. The amino terminal tail of histones provides sites for several posttranslational modifications. These covalent modifications include acetylation, methylation, phosphorylation, ubiquitylation and sumoylation. Histone modifications control chromatin structure and have both transcriptional activation and repression effects depending on the type of modification and the amino acid residues involved. The most common histone modifications are histone acetylation and histone methylation. These modifications are catalyzed by histone acetyltransferases (HATs) and histone methyltransferases, respectively. Histone modifications are extensively changed during oogenesis. For example, histone acetylation, a mark associated with transcriptional activation (Shogren-Knaak *et. al.,* 2006), is increased with the growth of the oocyte (Kageyama *et. al.,* 2007). The expression of HATs is also increased with the increase size of oocytes (Kageyama *et. al.,* 2007). Histone acetylation neutralizes the positive charge of histone, thus decreasing the binding affinity of histone for the negatively charged DNA resulting in a permissive chromatin structure for the binding of transcription factors. Other modifications such as methylation on lysine 4 of histone 3 (an activating mark) and methylation on lysine 9 of histone 3 (repressive mark) increased with the increased age of mice and the growth of oocytes (Kageyama *et. al.,* 2007). Histone deacetylases (HDAC) catalyze the removal of acetyl groups from histone tails (Li, 2002). HDACs often form complexes with methyl-DNA binding proteins and together

they create a closed chromatin conformation which is refractory to transcriptional activity (Li, 2002). A balanced level of histone acetylation is preserved by the opposing activities of HATs and HDACs (reviewed in Ruijter *et. al.,* 2003).

Synopsis- Rationale for thesis

The knowledge we have thus far about the acquisition of DNA methylation (DNAm) in mouse oocytes comes from imprinting studies in which the acquisition of gametic imprints were determined. From those studies we have learned that DNAm on imprinted genes is acquired asynchronously during a oocyte growth in a gene-specific manner. In most studies associated with the identification of epigenetic marks during oogenesis, ovaries of new-born and juvenile mice were used. The main reason being that a cohort of oocytes are recruited around the time of birth and grow concomitantly with the animal, therefore making the collection of a large number of oocytes of similar size more manageable. In addition, neonatal ovaries have less connective tissue than adult ovaries allowing the follicles to dissociate more easily from the ovary. A confounding factor in those studies is that oocytes of different sizes are collected into a single group because an array of different oocyte sizes is present at any given postnatal age. Furthermore, the oocytes collected from newborn and juvenile females have not attained reproductive age. It is possible to have an age effect on the acquisition of DNAm in oocytes. Lastly, infertility is an adult condition and the oocytes that will provide the most accurate data about the effects of ovarian stimulation on the epigenetic program of oocytes are those of an adult animal model.

In a previous study we found a 50% reduction in global DNAm in the maternal pronucleus of one-cell embryos produced from SO females when

compared to NO females. The effect of exogenous gonadotropin on the progression of global DNAm during oocyte growth in adult female has not been studied. In fact, no work has been done to characterize the acquisition of global DNAm during oocyte growth in mice. We hypothesize that the global DNAm of the maternal genome is acquired during oocyte growth in adult animals and that exogenous gonadotropins treatment will alter the normal progression of methylation acquisition.

Chapter II

Size-dependent acquisition of global DNA methylation in oocytes is altered by hormonal stimulation

Abstract

Works from several laboratories have demonstrated a lower developmental competence for embryos produced after superovulation (SO). In addition, we recently demonstrated that the level of global DNA methylation (DNAm) is 50% lower in the maternal pronucleus of one-cell mouse embryos produced after SO when compared to their naturally-ovulated (NO) counterparts. In this study, we determined the acquisition of global DNAm in growing and full-grown oocytes from NO and SO females. Briefly, a group of seven diestrus-stage females were selected for the study. The ovaries of one female were collected at this time while the remaining six females were divided into NO and SO groups. Ovaries were collected 24, 44, and 54 hours after diestrus or gonadotropin injection. The experiment was repeated three times. In the NO group, methylation is acquired in a linear manner from the time the oocyte starts growing (~10 μm diameter) until it reaches 40-50 μm. An exponential increase in DNAm was observed thereafter reaching a maximum at the 70-80 μm diameter (fully-grown stage). DNAm continued to increase in >70 μm oocytes until the approximate time of the LH surge. Similar to the NO groups, DNAm increased in SO oocytes up to the 60 μm size. However, in stark contrast to the NO group, no further acquisition of DNAm was observed in oocytes >60 μm in the SO groups.

We propose that the reduced level of DNAm observed in oocytes of SO females might in part be responsible for the lower developmental competence observed by others.

Introduction

The overall worldwide prevalence of infertility in women is nine percent with 56% of couples seeking medical care (Boivin *et. al.,* 2007). Assisted reproductive technologies (ART) are commonly used for the treatment of infertility/subfertility. The percent of children born from ART ranges from 0.1 to 3.9 of all children born in developed countries (Andersen *et. al.,* 2009; Centers for Disease Control and Prevention. (2007)). The most commonly used ART procedures are ovarian hyperstimulation (superovulation), intrauterine insemination (IUI), intracytoplasmic sperm injection, *in vitro* fertilization, *in vitro* culture, and embryo transfer.

Superovulation by administration of exogenous gonadotropins is used to increase the number of mature oocytes to circumvent the inefficiencies of the subsequent procedures. However, some evidence points at this seemingly harmless procedure as a causative factor in the etiology of some epigenetic syndromes as well as abnormal embryonic and fetal growth in humans and mice. For example, a retrospective study by Olson *et. al.* (2005) concluded that children conceived after IUI (343 infants) had a higher incidence of musculoskeletal defects when compared with naturally conceived children (8,422

infants). This outcome can be attributed to the superovulation procedure as 95% of the IUI cases received ovulation-inducing medications. Similarly, studies in mice showed that superovulation increased the proportion of abnormal preimplantation embryos, increased the rate of postimplantation mortality, and caused pronounced fetal growth retardation when compared to naturally ovulated counterparts (Ertzeid and Storeng, 1992; Auwera and D'Hooghe 2001; Fortier *et. al.,* 2008). Even though the uterine milieu can be altered as a result of the superovulation regimen, a reciprocal embryo transfer study demonstrated that embryos produced from hormonally stimulated oocytes had lower implantation rates and higher mortality rates when transferred to a pseudopregnant recipient uterus compared to embryos produced without hormonal stimulation (Ertzeid and Storeng 2001).

DNA methylation (DNAm) is a reversible and heritable epigenetic modification where a methyl group is added to the carbon-5 position of cytosine in CpG dinucleotide pairs (Razin and Riggs 1980). DNA methyltransferases (Dnmts) catalyze this reaction using S-adenosyl methionine as a methyl donor, and these enzymes are required for the establishment (*i.e.* Dnmt 3a, Dnmt3b and Dnmt3L) and maintenance (*i.e.* Dnmt1) of DNAm. Methylated DNA influences the expression of genes as methylated cytosines (5Me-C) attract binding proteins and promotes chromatin condensation creating a transcriptionally repressive conformation (Berger 2007; Newell-Price *et. al.,* 2000; Bird 1992). In the mouse, global DNAm is erased or greatly reduced in primordial germ cells during early fetal life (Hajkova *et. al.,* 2002) and re-established during gametogenesis in a sex

specific manner (Reik and Walter 2001). In mouse oocytes, it is well documented that DNAm at imprinted loci begins when the oocyte reaches around 40 µm diameter (Hiura *et. al.,* 2006), is acquired asynchronously in a gene specific manner (Song *et. al.,* 2009; Hiura *et. al.,* 2006; Lucifero *et. al.,* 2004), and is completed when the oocyte is ~65 µm diameter (Hiura *et. al.,* 2006). However, no information exists as to how global DNAm is acquired in mammalian oocytes.

A number of studies have demonstrated that hormonal stimulation induces aberrant DNAm at several imprinted loci in mice. Market-Velker *et. al.,* (2010) showed that superovulation perturbed DNAm at four differentially methylated regions (DMR). In their study, they found that blastocyst stage embryos produced after superovulation exhibited loss of methylation at the *Kcnq1ot1, Peg3* and *Snrpn* DMRs and gain of methylation at the *H19* ICR. Intriguingly, another study failed to observe changes in DNAm at Snrpn and *H19* DMRs even though these genes were biallelically expressed in the placenta of day 9.5 concepti produced after superovulation (Fortier *et. al.,* 2008). Ovarian hyperstimulation may also be playing a role in the apparent increase the incidence of the loss-of-imprinting disorder Beckwith-Wiedemann Syndrome (BWS; Maher *et. al.,* 2003; DeBaun *et. al.,* 2003; Halliday *et. al.,* 2004; Gicquel *et. al.,* 2003; Sutcliffe *et. al.,* 2006) in children conceived with the assistance of ART. To this effect, a retrospective study found that the cause of infertility, ART procedures utilized, type of culture medium used, number of embryos transferred, as well as the timing of embryo transfer were different in all cases of

ART-related BWS with the only common procedure being ovarian stimulation (Chang *et. al.,* 2005).

In a previous study we observed an increased level of gene expression in *in vivo* produced superovulated (SO) mouse blastocysts when compared to their natural ovulated (NO) counterparts (unpublished observation). As mentioned previously, DNAm is an epigenetic modification that is associated with a repressive transcriptional state. Therefore, we reasoned that this modification might be inappropriately acquired in the SO egg thus causing an improper transcriptionally permissive state at the blastocyst stage. Indeed, we found a 50% reduction in global DNAm in the maternal pronucleus of SO when compared to NO 1-cell embryos (unpublished observation). From these results we hypothesized that the exogenous gonadotropins, eCG and/or hCG were altering the deposition of methyl marks on the oocyte's DNA during their growth. Even though it is well established that methylation imprints are acquired asynchronously during oocyte growth, knowledge about the acquisition of global DNAm and the effect of hormonal stimulation during oogenesis is not known.

In our study, we determined the progression of global DNAm acquisition during oocyte growth in naturally cycling and hormonally-stimulated adult mice. We found that in adult mice the global DNAm is acquired in an oocyte size-specific manner. Further, we observed that administration of exogenous gonadotropins changed the level of mettylated DNA in growing oocytes. Acquisition of DNAm continues in full-grown oocytes until the presumed LH surge and we speculate that this is necessary to attain full developmental competence

(*i.e.* the ability of an oocyte to produce an embryo capable of full term development). On the other hand, SO full-grown oocytes do not attain complete levels of methylation before ovulation and this may in part be responsible for the abnormalities observed in embryos produced from SO oocytes.

Materials and Methods

Hormonal stimulation and Collection of ovaries

All animal experiments were approved by the University of Missouri Institutional Animal Care and Use Committee.

A group of 7-8 week old CF1 female mice (Harlan) were kept in an environmentally-controlled room at 20-23ºC under a 12 h light:12 h dark regimen (light on at 7:00 AM) with access to *ad libitum* food and water. The stages of estrous cycle were determined by observing changes in the appearance of the vulva as described by Champlin *et. al.* (1973). Following one estrous cycle, a group of females exhibiting diestrus (Hour 0; H0) were selected (**Figure 1**). At H0, at least one female was randomly chosen and its ovaries were collected for subsequent immunohistochemical processing. Half of the remaining females were left undisturbed to continue cycling normally (natural ovulation group; NO) while the other half received an intraperitoneal (IP) injection of 5 international units (IU) of equine chorionic gonadotropin (eCG, Calbiochem, La Jolla, CA) at 5:00 PM (superovulation group; SO). Twenty four (H24) and 44 hours (H44) after eCG injection, ovaries were collected from both the NO and SO. At H44, the

remaining SO group mice received 5 IU of human chorionic gonadotropin (hCG; Sigma, St. Louis, MO) intraperitoneally. The last set of NO and SO ovaries were collected 54 hours (H54) from the initiation of the experiment (*i.e.* H10 post-hCG in SO group). Ovulation in mice occurs approximately at the midpoint of the dark cycle (Nagy A et. al. 2003). In addition, mice ovulate approximately 12 h after hCG injection. Therefore, both NO and SO groups were expected to ovulate at approximately 1:00 AM. Hence, the ovaries from the H54 groups were collected two hours before presumed ovulation. Animals were sacrificed and ovaries were collected from the NO and SO groups at H0, H24, H44, and H54. Ovaries were immediately fixed in 10% buffered formalin for 24 hours and then preserved in 70% alcohol until ovarian sections were made by the Veterinary Medical Diagnostic laboratory at the University of Missouri-Columbia. The experiment was repeated three times on different days.

Immunohistochemistry

All reagents were purchased from Vector Laboratories (Burlingame, CA), unless otherwise specified. Thirty six to 42 continuous 6 µm ovarian sections (equivalent to five to six slides) were processed for immunohistochemical localization of 5MeC by using a commercially available kit (VECTOR M.O.M Immunodetection kit). Paraffin embedded ovarian sections were deparaffinized with Shandon xylene substitute (Thermo Scientific, Kalamazoo, MI), and then rehydrated through a series of graded ethyl alcohol (100%, 95%, and 80% of

ethyl alcohol). Rehydrated ovarian sections were microwaved for 15 minutes at full potency with a citrate-based unmasking solution, for antigen retrieval. Endogenous peroxidase activity was blocked by incubating the sections with 3% hydrogen peroxide for 5 minutes. Endogenous biotin was blocked by using a commercially available kit (Avidin/Biotin blocking kit), while endogenous IgGs were blocked by incubating the sections for 1 hour with mouse IgG blocking reagent. Ovarian sections were then incubated overnight at 4.0°C with an antibody against methylated cytosines (6 µg/ml; Anti-5-methylcytosine mouse mAb, Calbiochem, EMD Biosciences, Inc., La Jolla, CA). Biotinylated anti-mouse IgG was used as the secondary antibody (10 minutes). Tissue sections were stained and visualized by the ABC method. Stained ovarian sections were dehydrated through a series of graded alcohol (70%, 95%, and 100%) and xylene substitute before mounting on glass slides. Negative control consisted of sections processed as described above except they were incubated in diluent without primary antibody. Oocyte sections containing the germinal vesicle (GV) were photographed with a Leica DFC480 camera mounted on a Leica microscope. Images were taken at 1000x and saved as TIF files for subsequent image analyses. All pictures were acquired using the same microscopic and computer settings (**Figure 2**).

Image Analysis

An image of a stage micrometer was acquired with the same microscopic and computer settings used on the ovarian sections. The size of all GVs and oocytes were measured on a computer screen using the above mentioned micrometer image. Each series of ovarian sections was visually scanned to identify the largest cross section for each oocyte and this section was used to determine oocyte size. The largest cross section contains the nucleolus (Lintern-Moore and Moore, 1979). The average of two perpendicular measurements was taken across the oocyte (excluding the zona pellucida) to get the diameter. In addition, the largest cross section was used to measure GV area and 5MeC staining intensity (in arbitrary units; AU). The staining integrated density (sum of the values of the pixels in the image selection) was measured by using the background-corrected density macro of Image J program (NIH). Normalization between treatments and immunolocalization procedures were achieved by dividing the staining intensity of the GV with the average 5MeC staining measurement of five granulosa cells located immediately adjacent to the zona pellucida.

Statistical analysis

Differences in global DNAm between different oocyte sizes within and between treatment groups were analyzed using SAS 9.2. The independent variables used were: GV size, GV area, oocyte size, and number of granulosa

cell layers while the dependent variable was intensity of 5MeC (i.e. levels of methylation). We used the PROC GLM procedure of SAS 9.2 to determine least-squares means analysis of variance. The pdiff option of SAS was used to detect differences in methylation levels of growing oocytes within and between treatments and time points.

Results

In all, 620 NO and 379 SO oocytes were used for data analysis (Table 1). For each experimental group, oocytes were classified according to size into 10-20 µm, >20-30 µm, >30-40 µm, >40-50 µm, >50-60 µm, >60-70 µm, and >70 µm (fully grown). Further, the type of follicle (i.e. primordial, primary, secondary, antral) and the number of layers of granulosa cells surrounding each oocyte analyzed was determined and used to make comparisons between follicle stage and acquisition of DNAm in the oocyte.

The average size of NO oocytes contained in primordial and primary follicles was 15 and 23 µm, respectively (**Figure 3**). During the formation of the second layer of granulosa cells the oocyte increased in diameter from 45 µm to 57 µm and reached ~60 µm with the completion of the second layer. The formation of the third layer of granulosa cells was not accompanied by further oocyte growth, however with the addition of the fourth layer, most of the oocytes could be considered as full grown based on diameter.

Acquisition of DNA methylation during oogenesis in unstimulated control mice

Once recruited, an oocyte will grow from 10 µm diameter to >70 µm diameter in approximately 21 days (i.e. 3 µm per day; Eppig *et. al.* 2002). Therefore, in this study, oocytes classified as having a specific size at H0 will be expected to remain within that same size category or increase by one category at H54. Acquisition of global DNAm occurred in a size-specific manner **(Figure 4A and Table 2A**). While levels of methylation doubled from 10 µm (~2 arbitrary units) to 40 µm (~ 4 arbitrary units) in diameter, methylation levels quadrupled from >40 µm to 70 µm (~17.5 arbitrary units). The level of methylation further increased in full grown oocytes (> 70 µm; $P < 0.001$) from diestrus (H0) until the approximate time of the LH surge (H44). This acquisition occurred in a stepwise manner with respect to approaching ovulation with a 16% increase from H0 to H24 ($P < 0.04$) and a 12% increase from H24 to H44 ($P < 0.04$). Levels of methylation precipitously declined (31%; $P < 0.001$) from H44 to H54 coincident with the expected LH surge.

Acquisition of DNAm was positively correlated with progression of follicular stage ($P < 0.001$; **Figure 3**). The most dramatic increase in methylation (150%) occurred during the transition from primary to secondary follicular stage.

Comparison of global DNA methylation during oocyte growth between unstimulated and hormonally-stimulated mice

Acquisition of DNAm was similar between NO and SO oocytes up to 50 µm in size (**Figure 4**). The progression of methylation was altered at H24 and H44 of eCG injection in >50-60 µm oocytes (**Figure 5 and Table 2B**). At this oocyte size, exogenous hormonal stimulation caused an increase in the amount of methylation when compared to similar size oocytes in the control group at H24 (11.38 ± .66 vs. 13.66 ± .90 for unstimulated and hormonally-stimulated, respectively $P < 0.05$). A further increase in levels of methylation was observed in the SO group by H44 (12.30 ± .80 vs. 15.72 ± .1.19; $P < 0.02$). The increase in methylation levels observed as a result of eCG injection was not maintained at subsequent oocyte sizes but rather started to decline at >60-70 µm ($P = 0.09$; **Figure 5B**) and the decline was most pronounced at the full grown stage ($P < 0.001$). As opposed to the NO group, in which the LH surge caused a drastic decrease in the levels of DNAm by H54 (**Figure 4A**), the LH surge in the SO group (hCG) did not cause a further decrease by H54 (**Figure 4B**). This result was probably due to the lower level of methylation already observed at H44 in SO full grown oocytes.

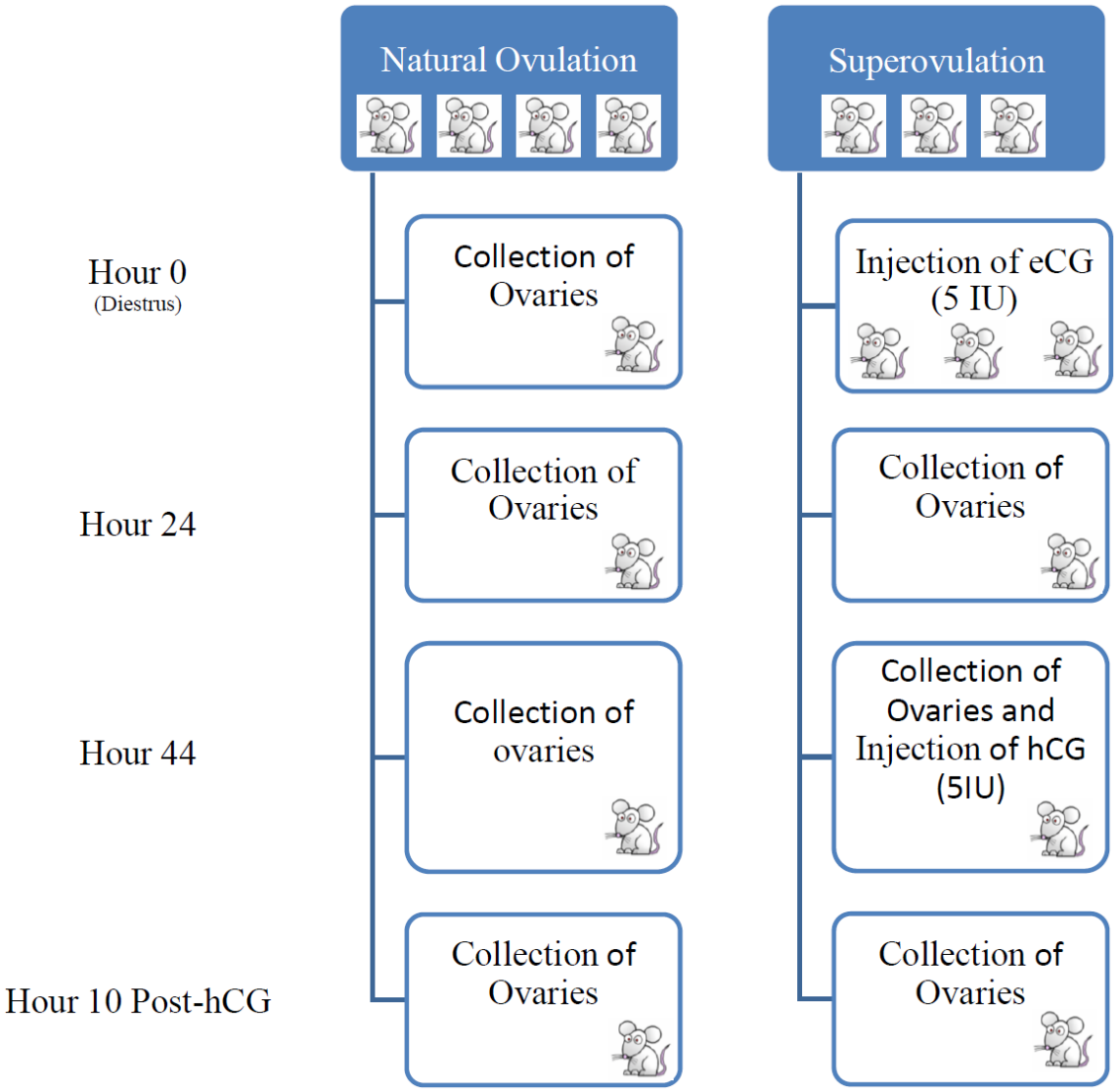


Figure 1: Experimental Design. A group of seven females at diestrus were randomly separated into two experimental groups: natural ovulation (control) and superovulation. Labels on the left of the figure denote time from diestrus. Experiment was repeated for three times.

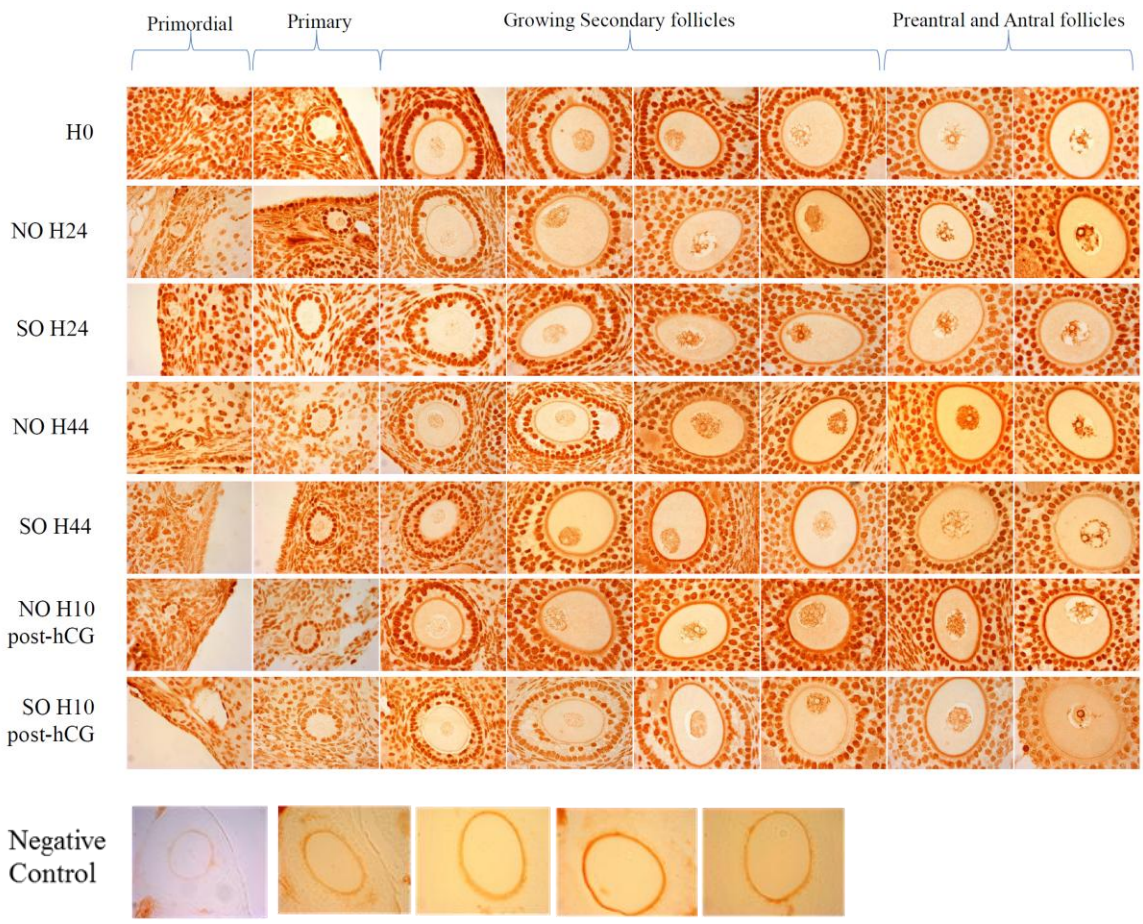


Figure 2: Immunohistochemical localization of 5MeC in mouse ovarian sections. Shown are representative pictures of growing oocytes in unstimulated and hormonally-stimulated cycles. Global DNA methylation was determined at several time-points from diestrus (H0). NO=natural ovulation, SO=superovulation. No specific staining was observed in the oocyte or the surrounding granulosa cells in the negative control.

Group	Oocyte size (µm)	10-20	>20-30	>30-40	>40-50	>50-60	>60-70	>70
	Number of oocytes from 3 Replications							
NO	H0	47	36	11	10	19	18	10
	H24	26	29	18	15	35	35	31
	H44	53	21	8	10	21	30	19
	H54	15	25	9	8	16	27	18
SO	H24	52	28	10	17	20	22	23
	H44	19	16	5	7	12	13	17
	H54	26	29	9	14	14	10	16

Table 1: Number of oocytes analyzed. NO = naturally ovulated. SO = superovulated. H0, H24, H44, H54 = time from diestrus.

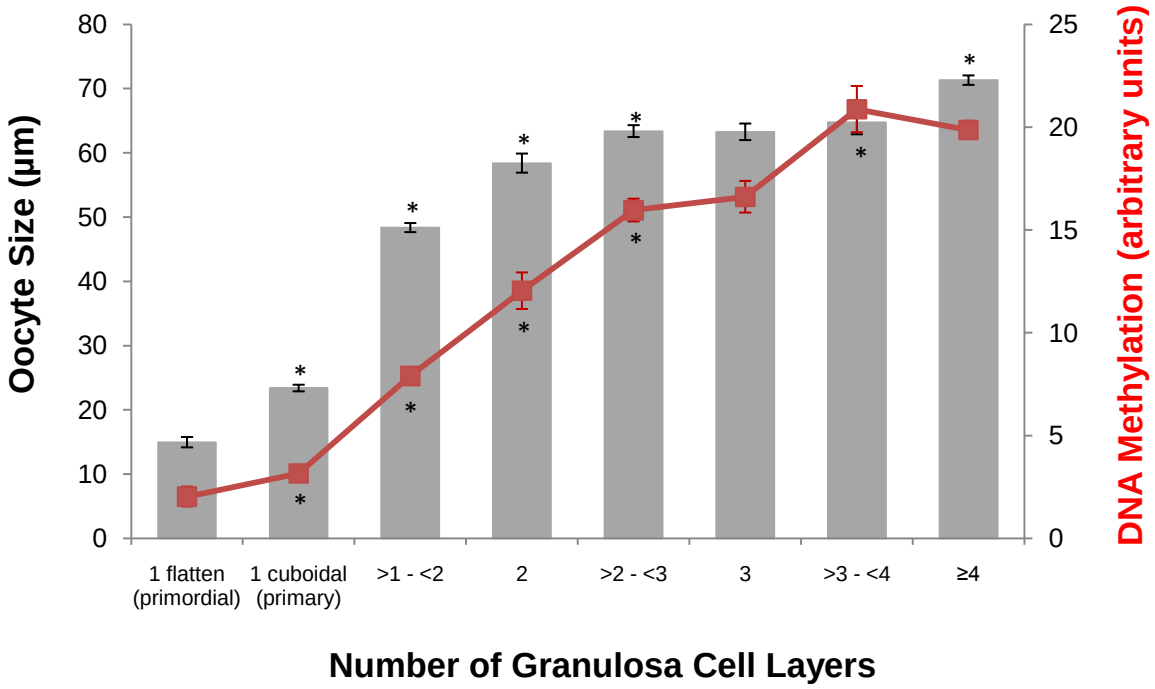


Figure 3: Follicular growth and DNA methylation in naturally cycling mice. Formation of a secondary stage follicle is correlated with the acquisition of DNA methylation in mouse oocytes. Values are mean ± SEM. *= denotes statistically significant difference from previous follicular stage (*P*< 0.05).

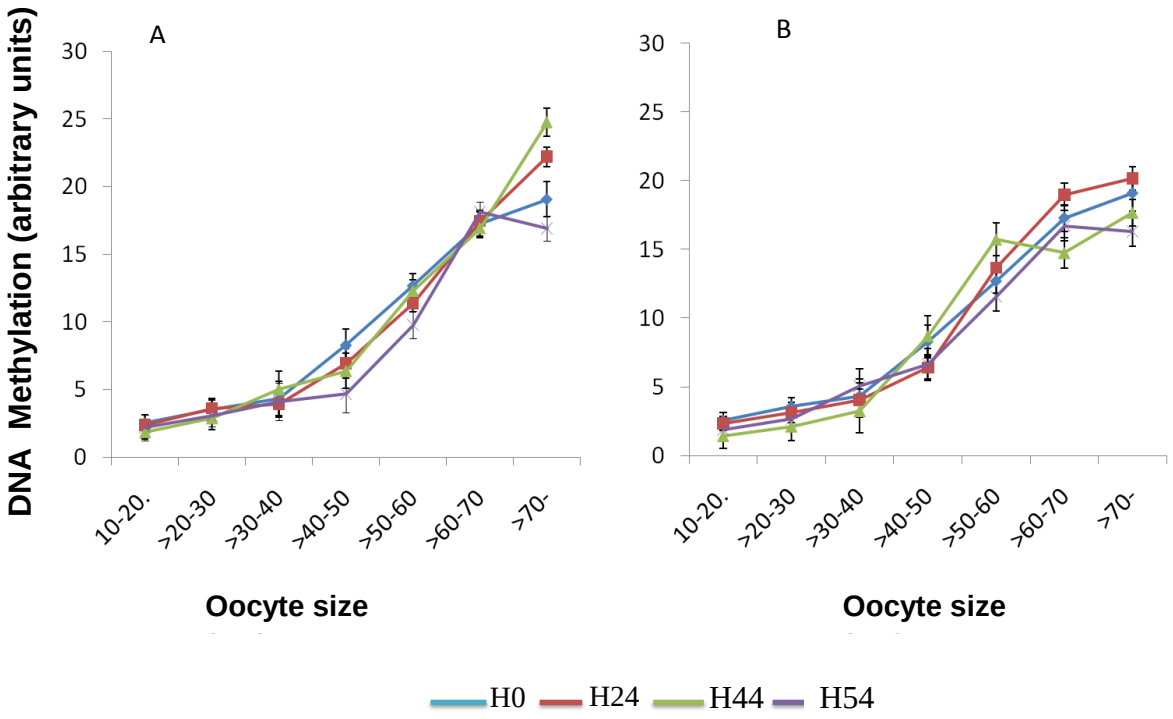
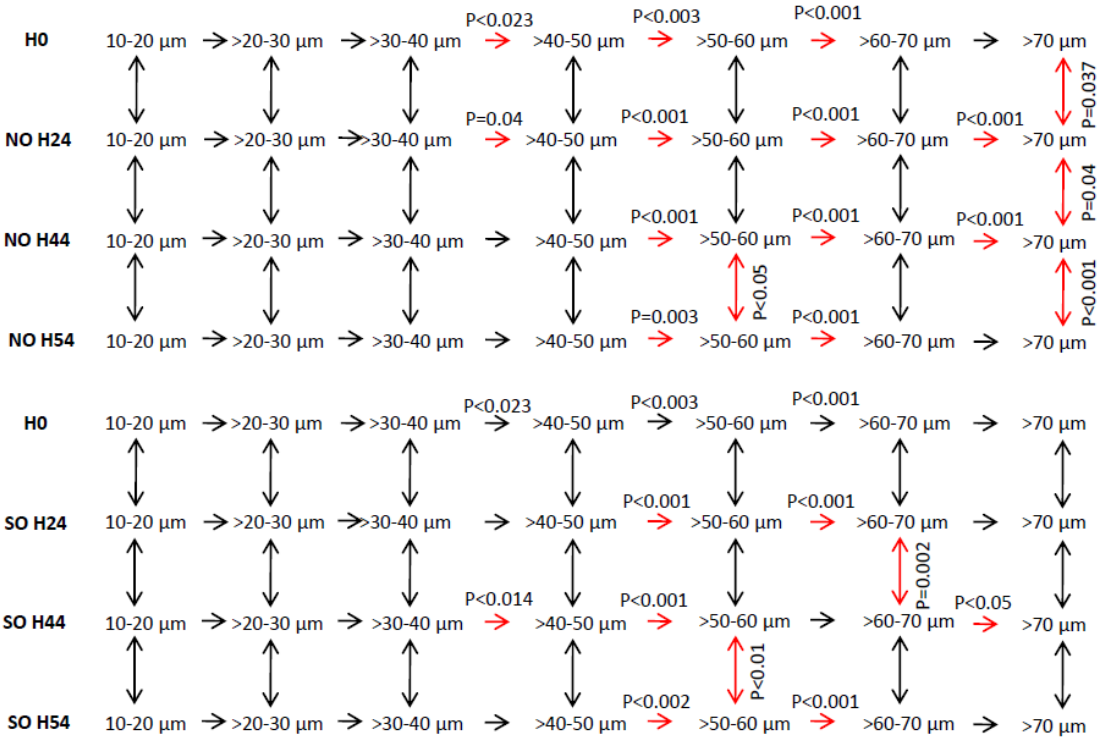


Figure 4: Pattern of acquisition of global DNA methylation in mouse oocytes. Level of DNA methylation in natural (A) and hormonally-stimulated (B) mice during the two days preceding estrus. The average values of three biological replications were determined for each oocyte size group. Values are mean ± SEM. Statistical differences among and between groups are shown in Table 2A and 2B.

A.



B.

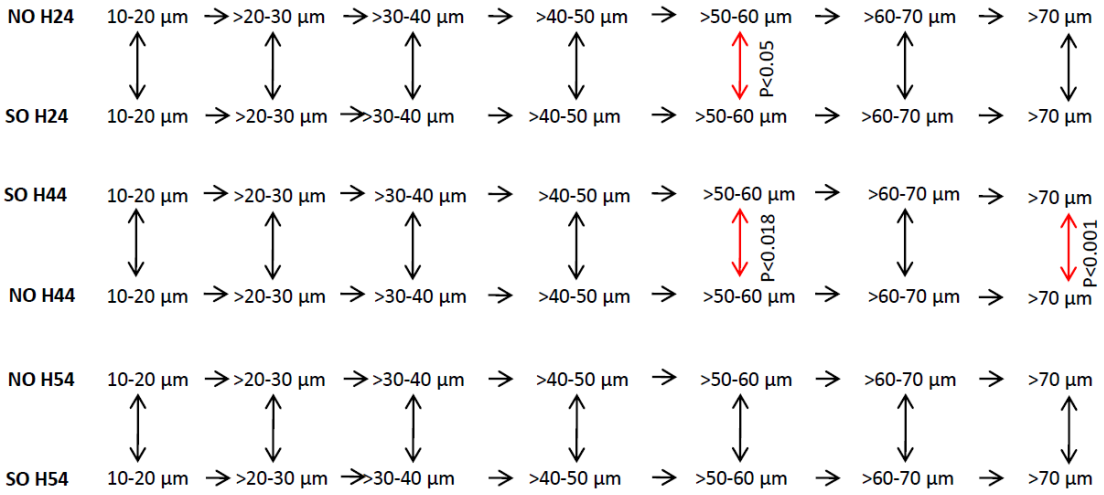


Table 2. Statistical analysis. Red arrows = significant differences within (**A**) and between (**B**) oocyte sizes of different treatments groups

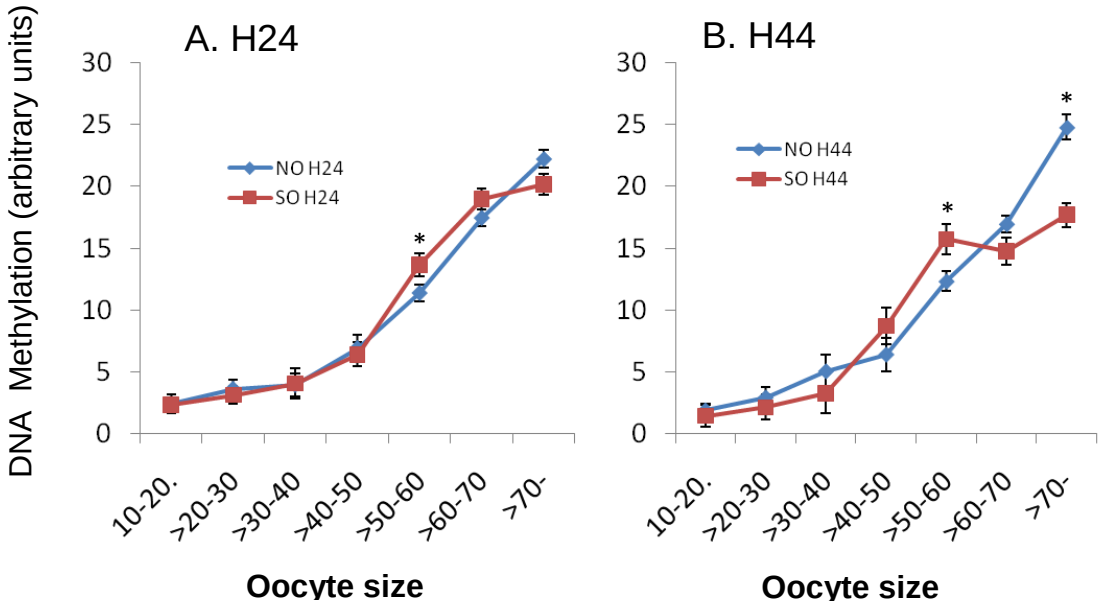


Figure 5: Effects of hormonal hyperstimulation on the acquisition of DNA methylation in mouse oocytes. A = Hour 24 and B = Hour 44. Values are mean ± SEM. *= denotes statistically significant difference *(P*< 0.05).

Discussion

In the present study we examined the pattern of acquisition of global DNAm during oocyte growth in adult CF1 females. We observed that oocyte growth is accompanied by a concomitant increase in the levels of DNAm. The increase in DNAm is most likely the result of the accumulating levels of the DNA methyltransferases seen during oocyte growth (Lucifero *et. al.,* 2007).

In mammals, DNAm occurs primarily at the carbon-5 position of the cytosine pyrimidine ring when in a CpG context. The key role of DNAm is to control gene expression as methylated sequences undergo transcriptional repression (Chomet 1991). Oocytes are transcriptionally active during their growth (Oakberg 1968; Moore *et al.,* 1974; Rodman and Bachvarova 1976; Sternlicht and Schultz 1981) but become transcriptionally quiescent once they reach their full-grown size (~70 um), an event coincident with antrum formation (Ericson and Sorensen 1974). It may be speculated that the acquisition of DNAm in oocytes may be in part responsible for transcriptional silencing. Conversely, the transcriptionally silent state may bring about *de novo* DNAm in preparation for chromatin remodeling. To this effect, Dnmt1 (the maintenance methyltansferase) has been demonstrated to bind directly to the heterochromatin associated proteins G9 and SUV39H1 in mammalian cells (Estève 2006).

During the majority of its growth, the oocyte's chromatin is in what is known as the non-surrounded nucleolus (NSN) configuration. This is microscopically visible as "naked" nucleoli due to a decondensed chromatin

state. However, the chromatin undergoes a restructuring event while approaching the final stages of growth by forming a heterochromatin rim around the nucleolus. This configuration is known as surrounded nucleolus (SN; De la fuente 2006). SN is a chromatin conformation which is found in competent oocytes immediately prior to ovulation. In our study we also observed that DNAm continues to be acquired even after the oocytes have reached their full-grown size and does not subside until after the expected time of the LH surge. Even though the majority of the transcriptional activity of the oocyte has halted when they reach their full grown size (Abe *et. al*. 2010; Ericson and Sorensen 1974), low level of transcriptional activity is still detectable up to two hours prior to ovulation (measured as ^{3}H-uridine incorporation; Rodman and Bacharova 1976). We propose that the increased level of DNAm observed prior to ovulation may be involved in silencing the remaining transcriptional activity and/or in assisting the oocyte's chromatin establish the SN conformation.

The timing of acquisition of global DNAm in the adult female mouse has not been previously reported. The knowledge we have to date about DNAm in mouse oocytes comes from studies looking at locus-specific methylation of imprinted loci (Hiura *et. al.,* 2006) and repetitive elements (*i.e.* IAP; Lucifero *et. al.* 2004). The literature suggests that methylation imprints are acquired asynchronously during oocyte growth, mostly when they are between 40 µm and 65 µm in diameter (Hiura *et. al.,* 2006). Repetitive elements, on the other hand, begin to get marked by methylation from the time of follicular recruitment. By using an immunohistochemical approach, we were able to determine the

dynamics of methylation acquisition during oocyte growth. We show that in the unstimulated (i.e. without the use of exogenous hormones) cycle, global methylation begins in oocytes as soon as they start their growth. The pattern of methylation transforms from linear to exponential around 50 µm in diameter which is nearing the size when oocytes become meiotically competent (i.e. ~60 um; Sorensen and Wasserman 1976; Eppig *et. al.,* 1994). Meiotic competence is defined as the ability of an oocyte to spontaneously resume meiosis beginning with the breakdown of the germinal vesicle and culminating with arrest at metaphase of the second meiosis. Full developmental competence of oocytes requires additional events such as nuclear and cytoplasmic modifications which continue well past the time they have reached their full grown size and into the last stages of follicular growth. We observed that an exponential progression of 5MeC levels in oocytes starts approximately at 50 µm and continues for the last one third of their growth. Oocytes reach their full grown size in a secondary follicle around the time of antrum formation (Brambel 1928). Most of the follicles at this time will undergo atresia (McGEE and Hsueh 2000) but those that are destined to ovulate will continue to grow for another two weeks before they are ready for ovulation (Chouinard 1975). We also observed that methylation of full grown-sized oocytes increases by ~30% from diestrus (i.e. two days before ovulation) until the time of the LH surge. We did not measure time points in between the approximate time of the LH surge and the subsequent 10 hours; therefore, we are unable to make further conclusions about the dynamics of DNAm immediately after the LH surge. We did however, measure DNAm in

oocytes approximately two hours prior to ovulation and obtained some puzzling results. We found that global DNAm decreased in full-grown oocytes as the time of ovulation approaches. Upon closer examination of the immunohistochemical sections, we observed that the ovarian sections from the H54 group appeared to have less staining that those of previous time points. In order to make sure that this was not due to technical error, we performed the 5MeC localization simultaneously of all seven groups (one slide/group) on two different occasions with sections from different mice obtained on different days. Yet, we still observed that the sections that belonged to ovaries of mice that were close to ovulation stained lighter than all other sections. At this time, we are unable to explain this observation.

Ovarian hyperstimulation had a profound effect on the total levels of DNAm in mid-sized and full-grown oocytes. Levels of DNAm were for the most part lower in the SO group when compared to the NO group. This is in accordance to our previous observation that the maternal pronucleus of zygotes produced after SO contain ~50% less methylated DNA than their NO counterparts (Unpublished observation).

An interesting observation is that DNAm might be directed by the granulosa cells as we observed a dramatic increase in the total amount of DNAm when the second layer of granulosa cells began forming around the oocyte. It is established that bidirectional communication between the oocyte and granulosa cells is needed for proper growth and differentiation of the oocyte (Eppig *et. al.,* 1997). The follicular cells that are in close proximity to the oocyte develop

projections (transzonal projections; TZP) that reach the oocyte and develop a communication channel which is the site of exchange of paracrine factors (Plancha *et al.,* 2005). Several studies have shown that FSH is involved in retracting the TZP (Combelles *et. al.,* 2004) and this in turn causes the oocyte to go from a NSN to a SN chromatin configuration (Combelles *et. al.,* 2004; De la Fuente and Eppig 2001). As mentioned earlier, the pattern of global DNAm shifts from linear to exponential around the 50 µm in diameter in NO oocytes. However, this pattern was disrupted by the administration of eCG since the methylation level of the oocyte's DNA from SO group plateaus at 50-60 µm and undergoes no further increase thereafter. Our observation that the granulosa cells may be directly involved in methylating the chromatin would be in accordance with this result. The follicle becomes responsive to the gonadotropins (FSH and LH) at the secondary stage when the granulosa cells express functional FSH receptors (McGee and Hsueh 2000). The binding of eCG to the FSH receptors may affect the ability of the granulosa cells to communicate with the oocyte through the TZP thus affecting the methylation program.

By administering exogenous gonadotropins we could potentially be recruiting oocytes that have not completed their developmental program and that have lower developmental competence. To that effect, superovulation in mice results in slowed preimplantation development (Ertzeid and Storeng 2001; Auwera and D'Hooghe 2001) and decreased the proportion of morphologically normal preimplantation embryos (Ertzeid and Storeng 1992). Postimplantation results showed that the weight of the fetuses (Malket-Velker *et. al.,* 2010; Ertzeid

and Storeng 1992; Auwera and D'Hooghe 2001) and pups (Ertzeid and Storeng 2001) was lower in SO oocytes when compare to NO controls. Those authors observed the main malformation of the SO fetuses to be related to skeletal malformations. This is intriguing since in a large retrospective human study by Olson et al. (2005) noted an increased incidence of musculoskeletal birth defects in children conceived by intrauterine insemination after ovarian hyperstimulation (i.e. no *in vitro* procedures, control = 8,422 children, IUI - 343 children).

The decreased level of global DNAm in superovulated oocytes may cause activation of the parasitic sequence elements like intracisternal A particle (IAP) retroviruses (repetitive retroviral-like sequence) that are normally transcriptionally silent by cytosine methylation (Walsh *et. al.,* 1998). The activation of parasitic particles can produce new forms of protein with adverse effects by misregulation of nearby genes (Yoder *et al.,* 1997). It has been reported that methylation on IAP occurs during oocyte growth (Lucifero *et. al.,* 2004). While repetitive elements are undermethylated in the dictyate stage oocyte, they are completely methylated in the ovulated egg. Therefore, it may be possible that SO recruits oocytes without the proper silencing of transposon elements before ovulation. This hypothesis remains to be tested.

In conclusion, acquisition of global DNAm occurs in an oocyte size specific manner in adult mice. Initiation of global DNAm started in non-growing oocytes and continued in full-grown oocytes until ovulation. Furthermore, superovulation affects the normal progression pattern of global DNAm and we put forth the

hypothesis that the lower developmental competence observed in superovulated oocytes may be in part due to improper DNAm during oocyte growth.

Chapter III

Long term effects of hormonal hyperstimulation on gene expression in mouse oocytes

Abstract

The mouse oocyte synthesizes and store mRNA during its growth and any intervention in the accumulation of transcripts during oocyte growth may affect fertilization as well as subsequent embryo development. Our previous study showed that global DNA methylation (DNAm) on the maternal genome is established during oocyte growth in an oocyte size-specific manner. Further we observed that superovulation affected the acquisition pattern of global DNAm in medium (50- 60 μm) and large (> 70 μm) size oocytes. DNAm is associated with transcriptional silencing. Therefore, we hypothesize that superovulation causes differential expression of genes involved in epigenetic control of the genome in ovulated mouse eggs and these deviant expression levels may be responsible for the observed aberrant DNAm and the lower developmental competence that other studies have reported. We determined the mRNA levels of the DNA methyltransferases (Dnmt3a, Dnmt3b, Dnmt3l and Dnmt1), the histone acetyltransferase Ncoa1and Cited1, and the histone deacetylases HDAC1 and HDAC2. In addition we determined if the lower level of methylation of the oocyte genome correlated with increased level of expression of the transposon IAP by quantitative RT-PCR. For our analyses we used naturally ovulated eggs (control) and eggs collected from the superovulated cycle as well as the three subsequent

cycles after hormonal hyperstimulation of ovulation. The mRNA level of *Dnmt3a* was 78% higher than *Dnmt3b* whereas the expression level of *Dnmt3l* was similar to *Dnmt3a*. The *Dnmt1* (maintenance DNA methyltransferases) is the most abundant transcript in ovulated eggs. The expression levels of all candidate genes analyzed in this study did not significantly differ among naturally ovulated and superovulated mice.

Introduction

Mammalian preimplantation embryos produced *in vivo* after superovulation have been used as controls for the study of gene expression and developmental competency in many research works. Recently, a microarray study showed that hormonal stimulation cause misregulation on the expression of 92 genes (76 down regulated and 16 up regulated) in blastocyst stage mouse embryos when compared to naturally ovulated counterparts (Zhang *et. al.,* 2010). In addition, a study from our laboratory (unpublished data) shows that gene expression at the blastocyst stage of development is different between *in vivo*-produced embryos generated after superovulation when compared to those produced after natural ovulation (*i.e.* control).

Any intrusion during the growth phase of oocytes could potentially affect the expression of genes required for oocyte maturation, fertilization, and successive embryo development. A number of studies in mice show that superovulation increases the proportion of abnormal preimplantation embryos,

increases the rate of postimplantation mortality, and causes pronounced fetal growth retardation when compared to naturally ovulated counterparts (Ertzeid and Storeng 1992; Ertzeid and Storeng 2001; Auwera and D'Hooghe 2001; Fortier *et. al.,* 2008). The abnormalities observed in pre- and post-implantation development have been concluded to be due in part to impaired oocyte competence (Ertzeid and Storeng 2001). In humans, a retrospective study by Olson *et. al.* (2005) concluded that children conceived after IUI (343 infants) had a higher incidence of musculoskeletal defects when compared with naturally conceived children (8,422 infants). This outcome can be attributed to the ovarian hyperstimulation procedure since 95% of the IUI cases received ovulation-inducing medications.

Methylation of CpG dinucleotides silence gene expression as the methylated cytosine attracts binding proteins and undergo a transcriptionally repressive conformation (Berger 2007; Newell-Price *et. al.,* 2000; Bird 1992; Chomet 1991). DNA methyltransferases (Dnmts) are required for the establishment and maintenance of DNAm. DNAm is required for genomic imprinting and X-chromosome inactivation (Bestor 2000; Reik and Walter 2001). The members of Dnmt3 (Dnmt3a, Dnmt3b, Dnmt3l) family of enzymes cause de novo DNAm in germ cells and the developing embryo/fetus (Okano *et. al.,*1999), whereas Dnmt1 is responsible for the maintenance of DNAm after DNA replication. Dnmt3a together with its cofactor Dnmt3L are required to establish methylation imprints during oocyte growth (Hata *et. al.,* 2002). The expression of Dnmt3a, Dnmt3b, Dnmt3L and Dnmt1o (the oocyte specific Dnmt1 isoform)

transcripts increase with the increased size of oocytes starting from 10-20 µm diameter to the full grown stage and then decrease in MII oocytes (Lucifero *et. al.,* 2007). Oocytes accumulate Dnmts as maternal transcripts and take part in *de novo* and/or maintenance of methylation during preimplantation development.

DNA methylation is also required for the silencing of transposable elements. Transposons are positioned around and inside most genes. The activation of retrotransposons causes mis-regulation of nearby genes and can produce new translations with negative effects (Yoder *et. al.,* 1997). One of the most abundant transposable elements in the mouse is intracisternal A particle (IAP). Methylation of IAP occurs during oocyte growth in a pattern similar to imprinted loci (Lucifero *et. al.,* 2004) and it has been shown that IAP is highly methylated in unfertilized oocytes. This methylation has been demonstrated to be necessary to maintain this transposable element in a silent state as lack of Dnmt1 results in transcription of IAP in mouse embryos (Walsh *et. al.,* 1998).

The amino terminal tails of histones provide sites for several posttranslational modifications namely acetylation, methylation, phosphorylation, ubiquitylation and sumoylation. Histone modifications control higher order chromatin structures and have both transcriptional activation and repression effects. Acetylation of histones is catalyzed by histone acetyltransferases (HATs). Acetylation is associated with decondensed chromatin structure and affects the interaction with chromatin associated proteins, and contributes to the establishment of transcriptionally active euchromatic regions (Shogren-Knaak *et. al.,* 2006). The expression level of HATs is relatively low in oocytes from five day

old mice, slightly increases in day-10 old oocytes, and is highest in oocytes of 15 day old mice (Kageyama *et. al.,* 2007). Nuclear receptor coactivator 1 (Ncoa1) also know as steroid receptor coactivator (SRC1) is a transcriptional coregulatory protein with HAT activity in its carboxy-terminal region and is specific for histone H3 and H4 (Spencer *et.al.,* 1997). Cbp/p300 interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1 (Cited1) also has histone acetrytransferase activity. Histone deacetylases (HDAC) remove acetyl groups from histone tails and direct a more closed chromatin configuration resulting in decreased transcriptional activity. A balanced level of histone acetylation is maintained by the opposing activities of HAT and HDAC (reviewed in Ruijter *et. al.,* 2003).

In our previous study (Chapter II), we determined the effect of exogenous hormonal stimulation on the acquisition of global DNAm in different sized of oocyte in adult mice (7-9 week old CF1 mice). We noticed that the total levels of DNAm were 35% lower in full grown oocytes from superovulated females when compared to control. We also observed that superovulation affected the level of global DNAm in medium sized oocytes (50-60 μm diameter). In addition, another study from our laboratory (unpublished), showed an effect of hormonal stimulation on the levels of histone acetylation in one-cell stage mouse embryos. Superovulation caused a 50% increase in the acetylation of lysines 9 and 14 on histone 3. From these observations, we were interested in determining whether the difference in the expression level of epigenetic modifiers is the underlying mechanism for the observed decrease in DNAm and increase in histone

acetylation in oocytes and embryos from superovulated mice. In this present study we determined the expression profile of Dnmt3a, Dnmt3b, Dnmt3l, Dnmt1, Ncoa1, Cited1, HDAC1, HDAC2 as well as the expression profiles of IAP in eggs from naturally ovulated and superovulated females in four consecutive cycles.

Materials and Methods

All animal experiments were approved by the University of Missouri Institutional Animal Care and Use Committee.

A group of 7-8 week old CF1 female mice (Harlan) were monitored for the detection of the various stages of the estrous cycle. Mice were kept in an environmentally-controlled room at 20-23ºC under a 12 h light:12 h dark regimen (light on at 7:00 AM) with access to *ad libitum* food and water. The stages of the estrous cycle were determined by observing changes in the appearance of the vulva as described by Champlin *et. al.* (1973). All reagents were obtained from Sigma unless otherwise specified.

Hormonal Stimulation and Oocyte collection

Naturally ovulated group

Estrus-stage mice were cocaged with a vasectomized male at 5:00 PM. Ovulation is expected to occur at the middle of dark phase (~ 1:00 AM, based on

a 7:00 PM to 7:00 AM light cycle) and ovulated eggs were collected the following morning at 8 AM from females that had a copulatory plug.

Superovulated group

Diestrus-stage mice were injected intraperitoneally with 5 IU of PMSG (at 5:00 PM) followed 44 hours later by an injection of 5 IU of hCG and then mated to vasectomized males. Ovulation is expected to occur 12 hours later (~1:00 AM). Ovulated eggs were collected the following morning at 8:00 am from females which exhibited a copulatory plug. This group was designated as superovulation cycle zero (SOC0). To study the long term effects of superovulation on gene expression, naturally ovulated eggs were collected at one (SOC1), two (SOC2), and three (SOC3) cycles after the superovulation treatment.

Ovulated egg collection

The cumulus-oocyte complexes were isolated from the oviduct by nicking the ampulla with a 30 gauge needle. The oocytes were collected into a drop of minimum essential medium (MEM) containing 0.3 mg/ml of Type IV-S hyaluronidase (from bovine testis) as described by Nagy *et. al.* (2003). Cumulus cells were completely removed from the egg by washing the oocytes several times in MEM. A group of 60 ovulated eggs collected from approximately two to five females were pooled and added to 100 µl lysis buffer (Dynadeads). Lysed oocytes were stored at – 80°C until RNA isolation. The number of eggs collected

per mouse varies in different treatment groups (**Table 2**) therefore; several mice were required to complete each individual biological replicate.

RNA extraction

A full-grown mouse oocyte is approximately 80 µm in diameter and contains about 80 pg of RNA (Duncan and Schultz 2010). For our experiment, total RNA was isolated from a pool of 60 ovulated eggs by using commercially available kit (Absolutely RNA Microprep Kit; Stratagene). Briefly, 60 ovulated eggs in a 1.5 ml microcentrifuge tube containing 100 µl lysis buffer were mixed with 0.7 µl of β- Mercaptoethanol and one microliter of exogenous *GFP* RNA. GFP RNA was added to all groups prior to RNA isolation to serve as a normalizer for RNA recovery efficiency. An equal volume (101.7 µl) of freshly prepared 70% ethanol was added to the tube. The manufacturer's instructions were followed as specified. RNA was eluted in 60 µl of prewarmed (60°C) elution buffer. RNA was precipitated overnight at – 20°C, followed by centrifugation at 4°C for 30 minutes. The RNA pellet was washed with 100 µl of freshly prepared cold 70% ethanol and then air dried and the pellet resuspended in 10 µl of RNAse/DNAse free water. The RNA was immediately used for reverse transcription.

Reverse transcription

Extracted RNA was converted to cDNA using 0.5 µg random primers (Invitrogen), 1 µl of 10 mM dNTP (Fisher Scientific), 2 µl of 0.1M DTT, 1X First Strand Buffer, 40 units of Optizyme RNase Inhibitor (Fisher Bioreagents), 200

units of Superscript II (Invitrogen) in a final volume of 20 µl. The reverse transcription reaction was performed at 42°C for 50 minutes, followed by heat-inactivation at 70°C for 15 minutes. Total volume was adjusted to 30 µl so that each µl of sample contained amount of cDNA equivalent to two ovulated eggs. The synthesized cDNA was stored at – 20 °C until use for RT-PCR.

Quantitative RT-PCR

qRT-PCR quantifies the PCR products in real time during each PCR cycle and yields a quantitative measurement of the PCR products accumulated during the course of the reaction (VanGuilder *et. al.,* 2008). qRT-PCR was performed in a 20 µl reaction volume, containing 1 µl of cDNA (equivalent to two ovulated eggs) for 50 cycles by using commercially available TaqMan Gene Expression Assays (**Table 1**) and TaqMan universal PCR master mix (Applied Biosystems). The genes analyzed were; Dnmt3a, Dnmt3b, Dnmt3l, Dnmt1, Ncoa1, Cited1, HDAC1, HDAC2, IAP and MLV. The candidate genes and probe (labeled with FAM) sequences are presented in **Table 2**.

For each treatment group (i.e. NO, SOC0, SOC1, SOC2, SOC3) we analyzed three to four biological replicates. Due to the paucity of material that we had for the analysis (i.e. 60 eggs) we were unable to include a minus reverse transcriptase group to ascertain any DNA contamination in our samples. This does not create a problem with the analysis of the expression of all the epigenetic modifiers since our primers were intron-spanning. However, IAP and

MLV are intronless. In order to circumvent this problem, we generated primers for a region of DNA within the *H19/IGF2* ICR. These primers amplified genomic DNA from mouse somatic tissue but there was no amplification with cDNA isolated from ovulated eggs. No DNA contamination was observed for any of the egg cDNA samples used for analysis.

Statistical Analysis

The level of mRNA encoding *Dnmt3a, Dnmt3b, Dnmt3l, Dnmt1, Ncoa1, Cited, HDAC1, HDAC2, IAP* and *MLV* for each group was normalized to the level of *GFP* mRNA of the same replicate. The difference in cycle threshold (ΔC_T) from *GFP* was used for statistical analysis. The difference in C_T of the genes in each SO group was compared to the C_T of the genes in the NO group using the PROC GLM procedure of SAS 9.2 to determine least-squares means analysis of variance. For data representation in fold difference from NO, we used the comparative C_T method ($\Delta\Delta C_T$) to determine the difference C_T between groups. Lastly, we determined fold difference from control by using the $2^{-\Delta\Delta C_T}$ formula. Additionally, we compared the mRNA levels of the DNA methyltransferases by ANOVA.

Results

Levels of expression differed among the Dnmts in naturally ovulated eggs ($P < 0.0001$; **Figure 1**). The amount of *Dnmt3b* transcript was 78% lower than that *of Dnmt3a* while the expression level of *Dnmt3l* was similar to *Dnmt3a*. The most abundant Dnmt transcript in ovulated eggs belongs to the maintenance Dnmt (*i.e.* Dnmt1; **Figure 1**).

Even though numerical differences are apparent between the naturally ovulated and the superovulated groups for several of the epigenetic modifiers studied (**Figure 2 and 3**) as well as for the transposon IAP (**Figure 4**), these differences did not reach statistical significance. Lastly, the mRNA levels were undetectable for the histone acetyltranferase *Cited1* and the retrosnposon *MLV*.

Table 1. List of Candidate genes and probe used for RT-PCR gene expression study.

Nuclear receptor coactivator 1 (Ncoa1)	NM_010881.2	77	gtcccagagccagtttacagctgac	Mm00447958_m1*
Cbp/p300 interacting transactivator with Glu/Asp-rich carboxy terminal domain 1 (Cited 1)	NM_007709.3	81	tgggaaggaggatgccaaccaggag	Mm01235642_g1*
Intracisternal A particle (IAP)	M17551	62	acaagactggctcaggg	AIN1ELT_F
Moloney murine leukemia virus	NC_001501.1	72	caaaacccaagagtgctg	AIPACR1_F
DNA methyltransferases 3A (Dnmt3a)	NM_153743.3	80	cttcctggcatgaacaggcctttgg	Mm00432884_m1
DNA methyltransferases 3B (Dnmt3b)	NM_001003960.3	83	ttcctggcatgtaacccagtgatga	Mm01240113_m1
DNA methyltransferases 3-like (Dnmt3l)	NM_019448.3	77	agggctgaagagcaagcatgcgccc	Mm00457635_m1
DNA methyltransferases 1 (Dnmt1)	NM_010066.3	62	tggcttctccactgcatttgctgaa	Mm01151050_m1
Histone deacetylase 1 (HDAC1)	AK087725.1	84	gtgccaggctgtttgtgctgtggat	Mm01351187_m1
Histone deacetylase 2 (HDAC2)	NM_008229.2	62	gtggttcagttgctggggctgtgaa	Mm00515108_m1
H19	U19619	98	tgcattttctaggctgg	AIQJAX9_F
GFP			CAGCACGACTTCTTC	GFP-4EX

Table 2. Number of eggs collected per mouse

	NO	SOC0	SOC1	SOC2	SOC3
Number of ovulated eggs/mouse	12.10	43.67	10.46	12.31	12.64

Natural Ovulation

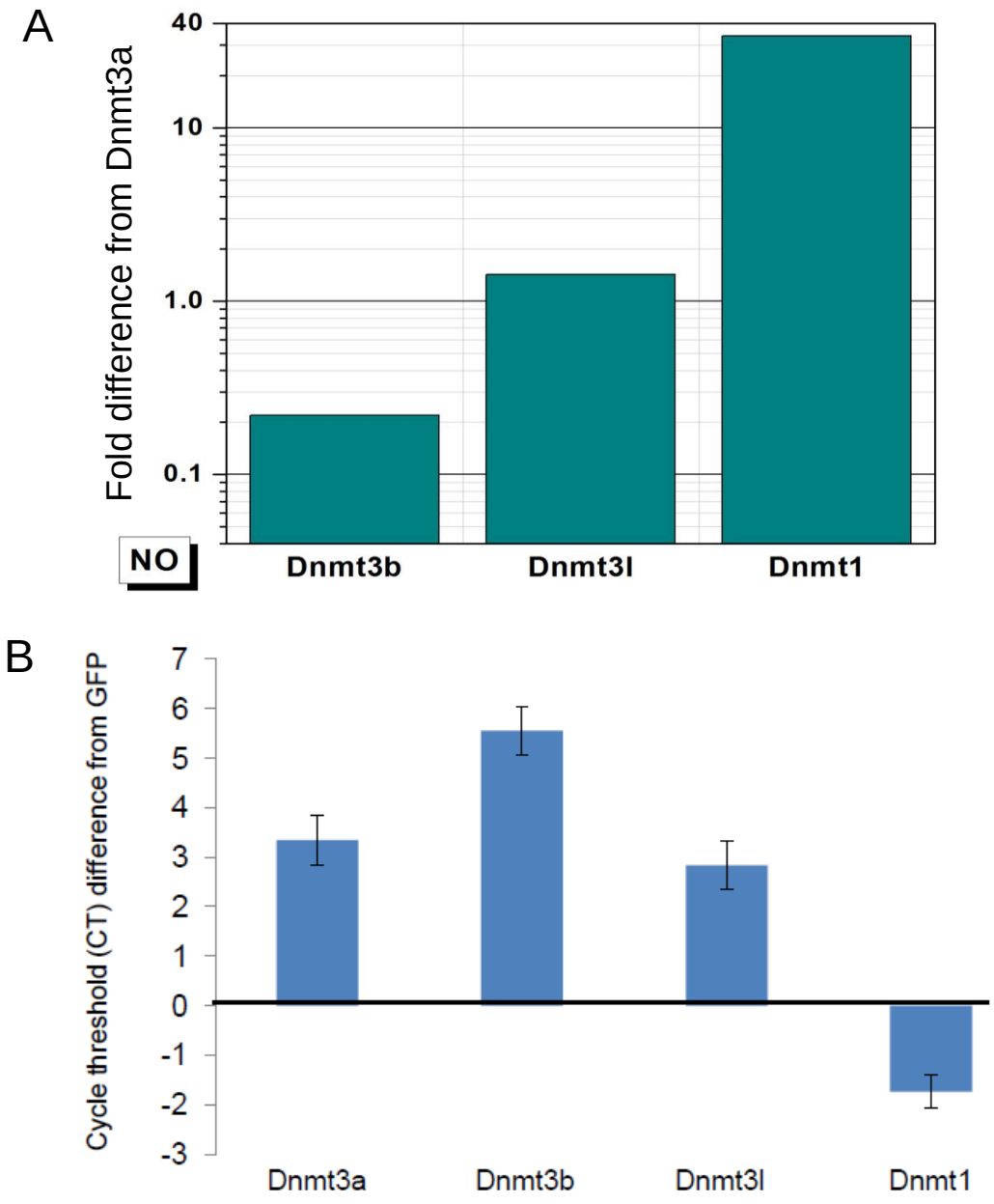
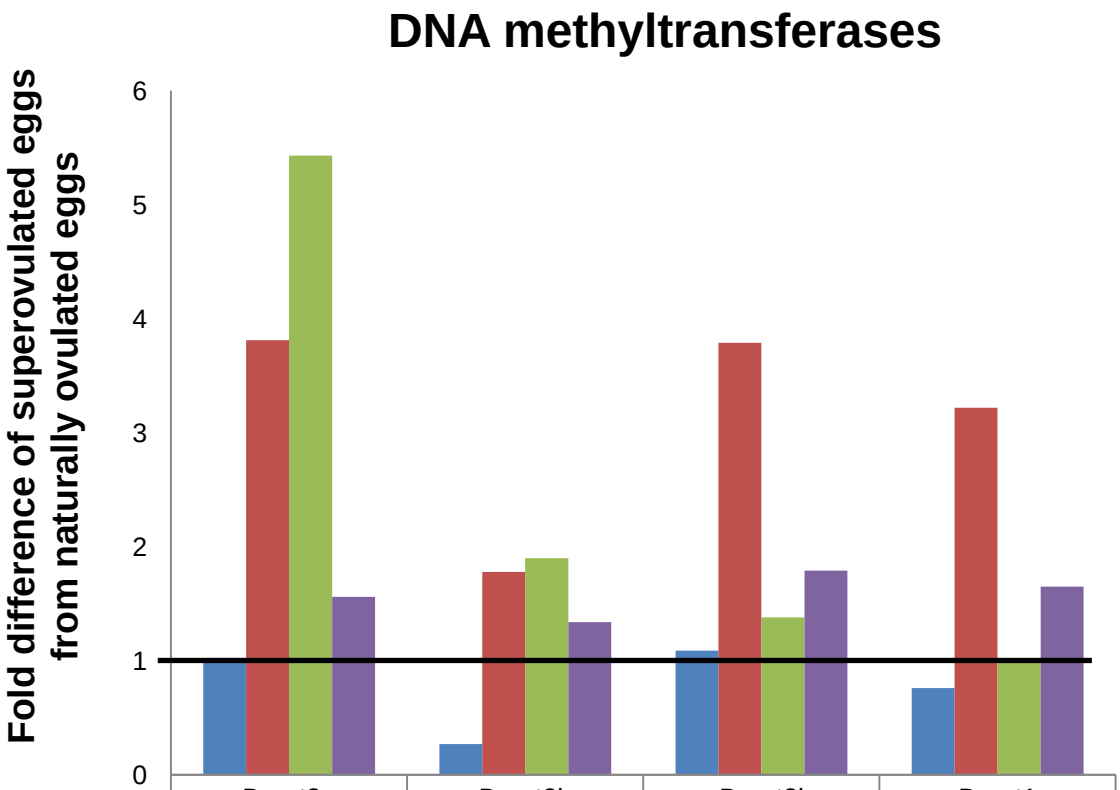
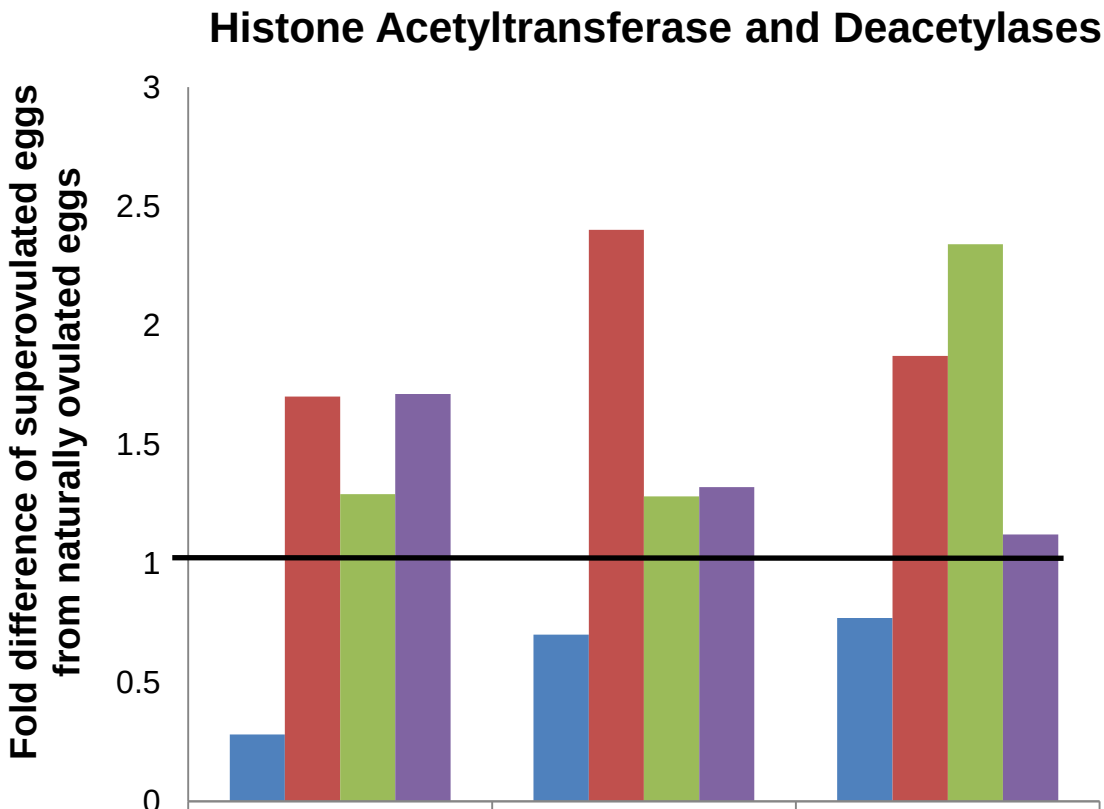


Figure 1. Expression levels of all DNA methyltranferases in ovulated eggs from naturally ovulated mice. A= Fold difference (in log scale) of *Dnmt3b*, *Dnmt3l* and *Dnmt1* as compared to the expression level of *Dnmt3a*. B= Difference in cycle threshold of all Dnmts from the exogenous normalizer *GFP*. Data for B are expressed as mean ± SEM.



	Dnmt3a	Dnmt3b	Dnmt3l	Dnmt1
Cycle 0	1	0.27	1.09	0.76
Cycle 1	3.81	1.78	3.79	3.22
Cycle 2	5.43	1.9	1.38	1
Cycle 3	1.56	1.34	1.79	1.65

Figure 2. Expression levels of all DNA methyltransferases in naturally ovulated and superovulated mouse oocytes. Data is expressed as fold difference from control. SOC0 = (superovulation cycle 0), SOC1, SOC2, SOC3. No statistical differences in gene expression were detected for any of the genes analyzed between the naturally and the superovulated groups.



	Ncoa1	HDAC1	HDAC2
Cycle 0	0.28	0.7	0.77
Cycle 1	1.7	2.4	1.87
Cycle 2	1.29	1.28	2.34
Cycle 3	1.71	1.32	1.12

Figure 3. Expression levels of a histone acetyltranferase and two class I histone deacetylases in naturally ovulated and superovulated mouse oocytes. Data is expressed as fold difference from control. SOC0 = (superovulation cycle 0), SOC1, SOC2, SOC3. No statistical differences in gene expression were detected for any of the genes analyzed between the naturally and the superovulated groups.

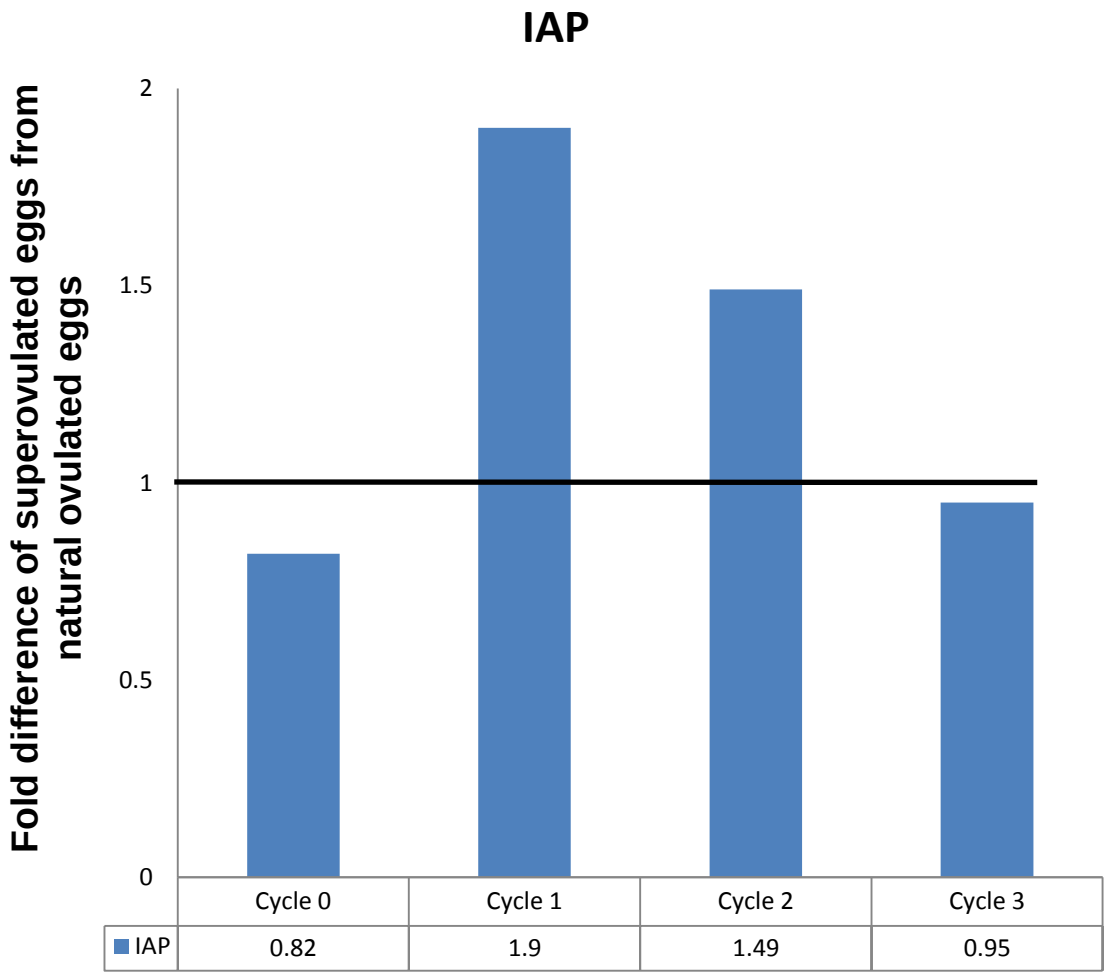


Figure 4. Expression levels of the retrotransposon IAP in naturally ovulated and superovulated mouse oocytes. Data is expressed as fold difference from control. Cycle 0 = superovulation cycle. No statistical differences in gene expression were detected between the naturally and the superovulated groups.

Discussion

We determined the level of expression of several epigenetic modifiers (*i.e.* DNA methyltransferases and histone acetyltranferases and deacetylases) in ovulated mouse eggs from unstimulated and hormonally stimulated females.

We observed that *Dnmt3a* and *Dnmt3l* are expressed in equal amounts while *Dnmt3b* is expressed at 20% of the level of the other two *de novo* methyltransferases. This is in accordance to previously published work (Lucifero *et. al.,* 2007). DNAm is established and maintained by the DNA methyltransferase (Dnmts) enzymes. Dnmt3a together with it cofactor Dnmt3l are required to establish methylation imprints during oocyte growth (Hata *et. al.,* 2002; Kato *et. al.,* 2007). In addition, we observed that the highest number of transcripts for a DNA methyltansferase belonged to *Dnmt1*. Published work has also shown that the mRNA expression of *Dnmt1o* (the oocyte specific Dnmt1 isoform) increase with the increased size of oocytes. DNA methyltransferase activity is required for proper developmental competence. Dnmt1 is required for proper preimplantation development (Li *et. al.,* 1992) and for the maintenance of methylation imprints. For example, developmental abnormalities and embryonic death were observed in DNA methyltransferase (Dnmt) gene mutant mice (Li *et. al.,* 1992; Okano *et. al.,* 1999).

In this study, our main interest was to determine if the effects of superovulation previously observed by us (Chapter II) at the DNAm levels in the full grown and medium-sized oocyte would translate into improper level of gene

expression of proteins required to set, maintain, and erase the epigenetic program of oocytes. The data presented here, although negative, provide the levels of the Dnmts (Dnmt3a, Dnmt3b, Dnmt3l and Dnmt1), Ncoa1, HDAC1, HDAC2 and IAP mRNA in ovulated eggs from naturally ovulated females as well as the levels of mRNA of eggs from superovulated females immediately after the superovulation procedure and during the next three subsequent unstimulated cycles. Even though we observed several differences in the pattern of expression of the DNA methyltransferases *Dnmt3a*, *Dnmt3b* and *Dnmt1* between the superovulated and naturally ovulated groups those differences did not reach statistical significance. The paucity of biological material used (i.e. two eggs equivalent = ~160 pg; Duncan and Schultz 2010) in this study may have contributed to the variation within treatment, thus making it difficult to detect differences between treatments. Further work will have to be done in order to determine if the numerical differences observed in the expression of some genes are authentic, and may contribute to the lowered developmental competence observed by others (Ertzeid and Storeng 1992; Ertzeid and Storeng 2001; Auwera and D'Hooghe 2001; Fortier *et. al.,* 2008).

General Summary and Conclusion

In our study, we determined the progression of global DNAm during oocyte growth in naturally cycling and hormonally stimulated mice at different time points from diestrus by immunohistochemical localization using an antibody specific for the methylated state of cytosines. We found that global DNAm is acquired in a size-specific manner in the adult animal. Our data also revealed that the acquisition of DNAm continues in full grown oocytes until the presumed LH surge and we speculate that this is necessary to attain full developmental competence. On the other hand, full-grown oocytes from superovulated mice do not attain an optimal methylation level before ovulation and this may in part account for the lower developmental competence observed in embryos produced from superovulated females. We also observed that the levels of global DNAm in medium-sized oocytes were different between the naturally ovulated and the superovulated groups. These medium sized oocytes could potentially ovulate in the subsequent estrous cycles and the inadequate levels of methylation could translate into inappropriate levels of gene expression in those oocytes when they continue to grow and ovulate.

For our second study we set to determine the levels of gene expression in ovulated eggs superovulated (SOC0) as well as one (SOC1), two (SOC2), and three (SOC3) cycles after the superovulation treatment and then compared those expression levels to ovulated eggs from unstimulated mice. We failed to detect differences in gene expression among naturally ovulated and all superovulated groups.

In conclusion, we found that global DNAm is acquired during oocyte growth in a size-dependent fashion and superovulation affects the normal acquisition patterns of DNAm in medium and large size oocytes. Further experiments are required to determine the underlying mechanism responsible for the decrease level of DNAm in oocytes from superovulated mice.

BIBLIOGRAPHY

Abe K, Inoue A, Suzuki MG, Aoki F. Global gene silencing is caused by the dissociation of RNA polymerase II from DNA in mouse oocytes. Journal of Reproduction and Development 2010; 56:502-507.

Andersen AN, Goossens V, Bhattacharya S, Ferraretti AP, Kupka MS, Mouzon J, Nygren KG. Assisted reproductive technology and intrauterine inseminations in Europe, 2005: results generated from European registers by ESHRE. Human Reproduction 2009; 24(6):1267–1287.

Armstrong DT. 2001 Effects of Maternal age on oocyte developmental competence. Theriogenology 55:1303-1322.

Auwera IVD, and D'Hooghe T. Superovulation of female mice delays embryonic and fetal development. Hum Reprod 2001; 16(6):1237-1243.

Bao S, Obata Y, Carroll J, Domeki I, and Kono T. Epigenetic modification necessary for normal development are established during oocyte growth in mice. Biol. Reprod. 2000; 62:616-621.

Bachvarova R and Leon VD. 1980. Polyadenylated RNA of Mouse Ova and Loss of Maternal RNA in Early Development. Developmental Biology 74:1-8.

Berger LS. The complex language of chromatin regulation during transcription. Nature 2007; 447:407-412.

Berker B, Kaya C, Aytac R, Satiroglu H. Homocysteine concentrations in follicular fluid are associated with poor oocyte and embryo qualities in polycystic ovary syndrome patients undergoing assisted reproduction. Hum Reproduction 2009; 24(9): 2293-2302.

Bird A. The Essentials of DNA Methylation. Cell 1992; 70: 5-8.

Boivin J, Bunting L, Collins JA, and Nygren KG. International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. Hum Reprod 2007; 22(6): 1506–1512.

Brambell FWR. The development and morphology of the gonads of the mouse. Part III.—The growth of the follicles. Proceedings of the Royal Society of London. Series B, containing papers of a Biological Character 1928; 103:258-272.

Centers for Disease Control and Prevention, American Society for Reproductive Medicine, Society for Assisted Reproductive Technology. 2005 Assisted Reproductive Technology success Rates: National Summary and Fertility Clinic Reports, Atlanta: Centers for Disease Control and Prevention. (2007).

Champlin AK, Dorr DJ, Gates AH. Determining the stage of the estrous cycle in the mouse by the appearance of the vagina. Biology of Reproduction 1973; 8:491-494.

Chang SA, Moley HK, Wangler M, Feinberg PA, DeBaun RM. Association between Beckwith-Wiedemann syndrome and assisted reproductive technology: a case series of 19 patients. Fertility and Sterility 2005; 83(2):349-354.

Chomet PS. Cytosine methylation in gene-silencing mechanisms. Curr Opin Cell Biol. 1991; 3:438-443.

Chotalia M, Smallwood SS, Ruf N, Dawson C, Lucifero D, Frontera M, James K, Dean W, Kelsey G. Transcription is required for establishment of germline methylation marks at imprinted genes. Genes and Development 2009; 23:105-117.

Chouinard LA. A light- and electron-microscope study of the oocyte nucleus during development of the antral follicle in the prepubertal mouse. J Cell Sci 1975; 17:589-615.

Christians E, Boiani M, Garagna S, Dessy C, Redi CA, Renard JP, Zuccitti M. Gene expression and chromatin organization during mouse oocyte growth. Developmental Biology 1999; 207:76-85.

Combelles CM, Carabatsos MJ, Kumar TR, Matzuk MM, Albertini DF. Hormonal control of somatic cell oocyte interactions during ovarian follicle development. Mol Reprod Dev 2004; 69(3):347-355.

Couse JF and Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? Endocr. Rev. 1999; 20:358-417.

Cox GF, Bürger J, Lip V, Mau UA, Sperling K, Wu BL, Horsthemke B. Intracytoplasmic Sperm Injection May Increase the Risk of Imprinting Defects. Am. J. Hum. Genet. 2002; 71:162–164.

Davis OK and Rosenwaks Z. Superovulation strategies for assisted reproductive technologies. Seminars in Reproductive Medicine 2001; 19(19):207-212.

DeBaun MR,Niemitz EL, Feinberg AP. Association of In Vitro Fertilization with Beckwith-Wiedemann Syndrome and Epigenetic Alterations of LIT1 and H19. Am. J. Hum. Genet. 2003; 72:156–160.

De la Fuente R. Chromatin modifications in the germinal vesicle (GV) of mammalian oocytes. Dev Biol. 2006; 292(1):1-12.

De la Fuente R and Eppig JJ. Transcriptional activity of the mouse oocyte genome: companion granulosa cells modulate transcription and chromatin remodeling. Dev Biol 2001; 229(1):224-236.

Drummond AE. The role of steroids in follicular growth. Reproductive biology and endocrinology 2006; 4:16 doi:10.1186/1477-7827-4-16.

Duncan FE and Schultz RM. 2010. Gene expression profiling of mouse oocytes and preimplantation embryos. Methods in Enzymology 477:458-479. (Chapter Twenty-Three).

Eppig JJ, Chesnel F, Hirao Y, O'Brien MJ, Pendola FL, Watanabe S, Wigglesworth K. Oocyte control of granulosa cell development: how and why. Hum Reprod 1997; 12(11): 127-132.

Eppig JJ, Schultz RM, O'Brien M, Chesnel F. Relationship between the developmental programs controlling nuclear and cytoplasmic maturation of mouse oocytes. Dev Biol 1994; 164(1):1-9.

Eppig JJ, Wigglesworth K, Pendola FL. The mammalian oocyte orchestrates the rate of ovarian follicular development. PNAS 2002; 99(5): 2890-2894.

Ericson GF and Sorensen RA. In vitro maturation of mouse oocytes isolated from late, middle, and preantral graafian follicles. J Exp Zool 1974; 190(1):123-127.

Ertzeid G and Storeng R. The impact of ovarian stimulation on implantation and fetal development in mice. Human Reproduction 2001; 16(2):221-225.

Ertzeid G and Storeng R. Adverse effects of gonadotrophin treatment on pre- and postimplantation development in mice. *J. Reprod. Fert.* 1992; 96:649-655.

Estève P, Chin HG, Smallwood A, Feehery GR, Gangisetty O, Karpf AR, Carey MF, and Pradhan S. Direct interaction between DNMT1 and G9a coordinates DNA and histone methylation during replication. Genes & Development 2006; 20:3089-3103.

Fauque P, Jouannet P, Lesaffre C, Ripoche MA, Dandolo L, Vaiman D, Jammes H. Assisted reproductive technology affects developmental kinetics, H19 imprinting control region methylation and H19 gene expression in individual mouse embryos. BMC Developmental Biology 2007; 7:116.

Fortier AL, Lopes FL, Darricarrere N, Martel J, Trasler JM. Superovulation alters the expression of imprinted genes in the midgestation mouse placenta. Human Molecular Genetics 2008; 17(11):1653-1665.

Fortune JE. Ovarian follicular growth and development in mammals. Biology of Reproduction 1994; 50:225-232.

Fowden AL, Sibley C, Reik W, Constancia M. Imprinted Genes, Placental Development and Fetal Growth. Horm Res 2006; 65(suppl 3):50-58.

Gicquel C, Gaston V, Mandelbaum J, Siffroi J, Flahault A, Bouc YL. In vitro fertilization may increase the risk of Beckwith-Wiedemann syndrome related to the abnormal imprinting of the KCN1OT gene. Am. J. Hum. Genet. 2003; 72:1338-1341.

Hajkova P, Erhardt S, Lane N, Haaf T, El-Maarri O, Reik W, Walter J, Surani MA. Epigenetic reprogramming in mouse primordial germ cells. Mechanisms of Development 2002; 117:15-23.

Halliday J, Oke K, Breheny S, Algar E, Amor DJ. Beckwith-Wiedemann syndrome and IVF: a case-control study. Am. J. Hum. Genet. 2004; 75: 526-528.

Hata K, Okano M, Lei H, Li E. Dnmt3L cooperates with the Dnmt3 family of de novo methyltransferase to establish maternal imprinting. Development 2002; 129:1983-1993.

Hiura H, Obata Y, Komiyama J, Shirai M, Kono T. Oocyte growth-dependent progression of maternal imprinting in mice. Genes to Cells 2006; 11:353-361.

Hutt KJ, and Albertini DF. An oocentric view of folliculogenesis and embryogenesis. Reproductive BioMedicine 2007; 14(6): 758-764.

John RM and Lefebvre L. Developmental regulation of somatic imprints. Differentiation 2011. doi:10.1016/j.diff.2011.01.007.

Kageyama S, Liu H, Kaneko N, Ooga M, Nagata M. Alterations in epigenetic modifications during oocyte growth in mice. Reproduction 2007;133:85-94.

Kato Y, Kaneda M, Hata K, Kumaki K, Hisano M, Kohara Y, Okano M, Li E, Nozaki M, Sasaki H. Role of the Dnmt3 family in de novo methylation of imprinted and repetitive sequences during male germ cell development in the mouse. Hum. Mol. Genet. 2007; 16:2272-2280.

Kota SK and Feil R. Epigenetic transitions in germ cell development and meiosis. Developmental Cell 2010; 19: 675-686.

Li E. Chromatin modification and epigenetic reprogramming in mammalian development. Nature 2002; 3:662-673.

Li E, Bestor TH, Jaenisch R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 1992; 69:915-926.

Lintern-Moore S and Moore GPM. The initiation of follicle and oocyte growth in the mouse ovary. Biology of Reproduction 1979; 20:773-778.

Lucifero D, Salle SL, Bourchis D, Martel J, Bestor TH, Trasler JM. Coordinate regulation of DNA methyltransferases expression during oogenesis. BMC Developmental Biology 2007; 7(36).

Lucifero D, Mann MRW, Bartolomei MS, Trasler JM. Gene-specific timing and epigenetic memory in oocytes imprinting. Hum. Mol. Genet. 2004; 13(8):839-849.

Maher ER, Brueton LA, Bowdin SC, Luharia A, Cooper W, Cole TR, Macdonald F, Sampson JR, Barratt CL, Reik W, Hawkins MM. Beckwith-Wiedemann

syndrome and assisted reproduction technology (ART). J Med Genet 2003; 40:62–64.

Market-Velker AB, Zhang L, Magri SL, Bonvissuto CA, Mann RWM. Dual effects of superovulation: loss of maternal and paternal imprinted methylation in a dose-dependent manner. Human Molecular Genetics 2010; 19(1):36-51.

McGEE EA, and Hsueh AJW. Initial and cyclic recruitment of ovarian follicles. Endocrine Reviews 2000; 21(2): 200-214.

Menezo Y, Elder K, Benkhalifa M, Dale B. DNA methylation and gene expression in IVF. Reproductive BioMedicine online 2010; 20: 709-710.

Moore GPM, Lintern-Moore S, Peters H, Faber M. RNA synthesis in the mouse oocyte. The Journal of Cell Biology 1974; 60:416-422.

Nagy A, Gertsenstein M, Vintersten K, Behringer R. Manipulating the Mouse Embryo, A laboratory manual, third edition. Cold Spring Harbor Laboratory Press; 2003.

Newell-Price J, Clark AJL, King P. 2000 DNA Methylation and Silencing of Gene Expression. Trends Endocrinol Metab. 2000;11(4):142-148.

Oakberg EF. Relationship between stage of follicular development and RNA synthesis in the mouse oocyte.Mutation Research 1968; 6:155-165.

Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 1999; 99:247-257.

Olson KC, Keppler-Noreuil MK, Romitti AP, Budelier TW, Ryan G, Sparks TEA., Voorhis VJB. In vitro fertilization is associated with an increase in major birth defects.Fertility and Sterility 2005; 84(5):1308-1315.

Ørstavik KH, Eiklid K, Van der Hagen CB, Spetalen S, Kierulf K, Skjeldal O, Butting K. Another Case of Imprinting Defect in a Girl with Angelman Syndrome Who Was Conceived by Intracytoplasmic Sperm Injection. Am. J. Hum. Genet. 2003; 72:218–219.

Otsuka F, Yao Z, Lee T, Yamamoto S, Erickson GF, Shimasaki S. Bone morphogenetic protein-15. Identification of target cells and biological functions. Journal of Biological Chemistry (J Biol Chem) 2000; 275(50): 39523-39528.

Plagge A, Gordon E, Dean W, Boiani R, Cinti S, Peters J, Kelsey G. The imprinted signaling protein XLαs is required for postnatal adaptation to feeding. Nature Genetice 2004; 36 (8): 818-826.

Plancha CE, Sanfins A, Rodrigues P, Albertini D. Cell polarity during folliculogenesis and oogenesis. Reprod Biomed Online 2005; 10(4):478-484.

Popp C, Dean W, Feng S, Cokus SJ, Andrews S, Pellegrini M, Jacobsen SE, Reik W. Genome-wide erasure of DNA methylation in mouse primordial germ cells is affected by AID deficiency. Nature 2010; 463(7284): 1101-1105.

Razin A and Riggs AD. DNA methylation and Gene Function. Science 1980;210(4470):604-610.

Reik W and Walter J. 2001 Genomic imprinting: Parental influence on the genome. Nat Rev Genet 2001; 2(1):21-32.

Rodman TC and Bachvarova R. RNA synthesis in preovulatory mouse oocytes. The Journal of Cell Biology 1976; 70:251-257.

Ruijter AJMD, Gennip AHV, Carson HN, Kemp S, Kuilenburg ABPV. Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem. J. 2003; 370:737-749.

Sato A, Otsu E, Negishi H, Utsunomiya T, Arima T. Aberrant DNA methylation of imprinted loci in superovulated oocytes. Human Reproduction 2007; 22(1):26-35.

Santos F, Hendrich B, Reik W, Dean W. Dynamic reprogramming of DNA methylation in the early mouse embryo. Developmental Biology 2002; 241:172-182.

Schultz RM and Wassarman PM. Biochemical studies of mammalian oogenesis: protein synthesis during oocyte growth and meiotic maturation in the mouse. J. Cell Sci. 1977; 24:167-194.

Shogren-Knaak M, Ishii H, Sun J, Pazin MJ, Davie JR, Peterson CL. Histone H4-K16 acetylation controls chromatin structure and protein interactions. Science 2006; 311: 844-847.

Song Z, Min L, Pan Q, Shi Q, Shen W. Maternal imprinting during mouse oocyte growth in vivo and in vitro. Biochemical and Biophysical Research Communications 2009; 387:800-805.

Sorensen RA and Wassarman PM. Relationship between growth and meiotic maturation of the mouse oocyte. Dev Biol. 1976; 50(2): 531-536.

Sternlicht AL and Schultz RM. Biochemical studies of mammalian oogenesis: kinetics of accumulation of total and poly(A)-containing RNA during growth of the mouse oocyte. J Exp Zool 1981; 215(2):191-200.

Spencer TE, Jenster G, Burcin MM, Allis CD, Zhou J, Mizzen CA, McKenna NJ, Onate SA, Tsai SY, Tsai M, O'Malley BW. Steroid receptor coactivator-1 is a histone acetyltransferase. Nature 1997; 389:194-198.

Stouder C, Deutsch S, Paoloni-Giacobino A. Superovulation in mice alters the methylation pattern of imprinted genes in the sperm of the offspring. Reproductive Toxicology 2009; 28:536-541.

Sugiura K, Su Y, Li Q, Wigglesworth K, Matzuk MM, Eppig JJ. Estrogen promotes the development of mouse cumulus cells in coordination with oocyte-derived GDF9 and BMP15. Mol Endocrinol 2010; 24(12):2303-2314.

Sutcliffe AG, Peters CJ, Bowdin S, Temple K, Reardon W, Wilson L, Clayton-Smith J, Brueton LA, Bannister W, Maher ER. Assisted reproductive therapies and imprinting disorders--a preliminary British survey. Hum. Reprod. 2006; 21(4):1009-1011.

Tingen C, Kim A, Woodruff TK. The primordial pool of follicles and nest breakdown in mammalian ovaries. Molecular Human Reproduction 2009; 15:795-803.

VanGuilder HD, Vrana KE, Freeman WM. Twenty-five years of quantitative PCR for gene expression analysis. Biotechniques 2008; 44: 619-626.

Walsh CP, Chaillet JR, Bestor TH. Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. Nature Genet. 1998; 20:116-117.

Wetzels AMM, Artz MT, Goverde HJM, Bastiaans BA, Hamilton CJCM, and Rolland R. Gonadotropin hyperstimulation influences the ^{35}S-methionine metabolism of mouse preimplantation embryos. Journal of Assisted Reproduction and Genetics1995; 12(10):744-746.

Yoder JA, Walsh CP, Bestor TH. Cytosine methylation and the ecology of intragenomic parasites. Trends Genet. 1997; 13:335-340.

Zhang X, Wang L, Li X, Fang J, Yao Y.. Ovarian stimulation retards postimplantation development and alters global gene expression profile of blastocysts in mouse. Fertility and Sterility 2010; 93:2770-2773.

Zhang Y., Cui Y., Zhou Z., Sha J., Li Y., Liu J. Altered global gene expressions of human placentae subjected to assisted reproductive technology treatments. Placenta 2010; 31:251-258.

VITA

Md Almamun was born September 01, 1980 in Mymensingh, Bangladesh. He is the son of Abdul Aziz and Khosh Nahar Begum. Mamun (as he is known) started his college education in Veterinary Medicine in 1998 at the Bangladesh Agricultural University in Mymensingh, Bangladesh. After receiving his Doctor of Veterinary Medicine (DVM) degree in 2003, he joined a Pharmaceutical company and worked as a product executive. In the year 2007, he moved to the USA and worked as a Research Assistant with Dr. OJ Ginther. There he studied on reproductive physiology of cattle and horse. In April of 2009, Dr. Almamun moved to the Division of Animal Sciences at the University of Missouri to work on a Master of Science degree in developmental epigenetics under the supervision of Dr. Rocío M. Rivera. After graduating with an M.S., Mamun will return to Bangladesh, after four years, for some well-deserved rest and relaxation before commencing work towards his Doctor of Philosophy degree in Cancer Epigenetic under the supervision of Dr. Charles Caldwell in the Pathobiology Area program at the University of Missouri.