Characterizing the impact of maternal obesity on offspring ovarian development in rats

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By

Michael William Tsoulis, BMSc

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Author: Michael William Tsoulis, BMSc (Western University)

Supervisor: Dr. Deborah Sloboda

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Abstract

Maternal obesity predisposes offspring to non-communicable disease and reproductive dysfunction. Previous work has shown that female rat offspring born to mothers fed a high fat (HF) diet throughout pregnancy and lactation enter puberty early and display aberrant reproductive cyclicity. It is currently unknown what mechanisms are driving this reproductive phenotype, however, the ovary is likely involved. The present study shows that offspring born to HF-fed mothers have impaired ovarian function likely stemming from altered ovarian development early in life. Neonates born to HF fed mothers have reduced AMH signaling that appears to result in more primordial follicles assembled. During the prepubertal period, excess primordial follicles are removed from the primordial follicle pool likely due to increased primordial follicle demise or an increase in the number of growing follicles undergoing atresia. This study shows that as adults, offspring born to HF fed mothers also have more atretic follicles, appearing to be due to decreased FSH responsiveness that could be induced by both low expression levels of the oocyte-secreted factor, GDF9, and high expression levels in antral follicles of the granulosa-cell derived factor, AMH. Changes in ovarian follicle dynamics was not associated with increased oxidative stress or the induction of inflammation. Interestingly, the addition of a postweaning high fat diet appears to spare offspring born to HF fed mothers from an increase in follicular atresia. This study demonstrates that maternal HF diet induced obesity has long lasting effects on ovarian development and function in female offspring. Given the high rates of obesity in female populations worldwide and the growing concern over trans-generational inheritance of disease risk, future studies are needed to fully delineate the molecular mechanisms mediating ovarian dysfunction induced by a maternal obesogenic diet. This will allow the design of targeted interventions that will hopefully improve the health of future generations.

Acknowledgments

First and foremost, I'd like to sincerely thank my supervisor, Dr. Deborah Sloboda. You have given me the incredible opportunity to be part of a lab that does such interesting and important work. This experience has allowed me to develop and improve my entire scientific skill set both on and off the bench. I am extremely grateful for all the times you pushed me to think "laterally", hone poster/oral presentations until they were outstanding, and write in a clear and concise manner. These experiences have truly improved me both as a person and a scientist. Your passion for science and its dissemination is inspiring. I can only hope that one day I will be able to inspire others as much as you do. Without your expert guidance, mentorship, encouragement, critiques, and support, this thesis would not have been possible and I cannot thank you enough.

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Last, but far from least, I'd like to thank my family for all the support they've given me over the past few years. My parents, Chris and Jan, and my brother, Dave, have all been there to "lend an ear" and give me valuable perspectives that have helped me along the way.

Oh and coffee. I'd like to thank coffee. Without coffee, most research would suffer and the following is no exception.

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List of Abbreviations

1° Primary antibody
17βHSD1 17 beta-hydroxysteroid dehydrogenase type I
2° Secondary antibody
20αHSD 20α-hydroxysteroid dehydrogenase
3βHSD 3 beta-hydroxysteroid dehydrogenase/delta-5-delta-4 isomerase
4-HNE 4-hydroxynonenal
Akt Protein kinase B
AMH Anti-mullerian hormone or mullerian inhibiting substance
AMHRII Anti-mullerian hormone type II receptor
ANOVA Analysis of variance
APS Ammonium persulfate
AR Androgen receptor
ART Assisted reproductive technologies
B2M β-2-microglobulin
Bax Bcl-2 associated X protein
BCA Bicinchoninic acid
Bcl-2 B-cell lymphoma 2
BMI Body mass index
BMP15 Bone morphogenetic protein 15
bp Base pairs
BSA Bovine serum albumin
cAMP Cyclic adenosine monophosphate
CAT Catalase
cDNA Complementary DNA
ChIP Chromatin immunoprecipitation
CL Corpus luteum (singular) or corpora lutea (plural)
COCs Cumulus-oocyte complexes
CON Maternal control diet
con Postweaning control diet
Cp Crossing point
Cyp11a1 Cytochrome P450, family 11, subfamily A, polypeptide 1 (encodes P450scc)
CYP17 Cytochrome P450 17alpha hydroxylase/17,20 lyase
Cyp17a1 Cytochrome P450, family 17, subfamily A, polypeptide 1 (encodes CYP17)
CYP19 Aromatase
Cyp19a1 Cytochrome P450, family 19, subfamily A, polypeptide 1 (encodes CYP19)
DAB 3,3'-Diaminobenzidine
DHEA Dehydroepiandrosterone
DOHaD Developmental Origins of Health and Disease
DTT Dithiothreitol
E_2 17-β-estradiol
ELISA Enzyme-linked immunosorbent assay

ERE Estrogen response elements
ERα Estrogen receptor alpha
ERβ Estrogen receptor beta
FOXL2 Forkhead box protein L2
Foxo3a Forkhead transcription factor subfamily 3
FSH Follicle stimulating hormone
FSHR Follicle stimulating hormone receptor
GDF9 Growth differentiation factor 9
GnRH Gonadotropin-releasing hormone
H&E Haematoxylin and Eosin
H_2O_2 Hydrogen peroxide
HF High fat or maternal high fat diet
hf postweaning high fat diet
HPRT Hypoxanthine-guanine phosphoribosyltransferase
ICSI Intracytoplasmic sperm injection
IGF-1 Insulin-like growth factor I
IgG Immunoglobulin G
IHC Immunohistochemistry
IL6 Interleukin-6
IVF *In vitro* fertilization
KL Kit ligand
LETO Long evans tokushima
LH Luteinizing hormone
LHR Luteinizing hormone receptor
MOFs Multiple oocyte follicles
OLETF Otsuka long evans tokushima fatty
P Postnatal day
P_4 Progesterone
P450scc Cholesterol side chain cleavage enzyme
pAkt (Ser473) Phosphorylated Akt at serine 473
PBS Phosphate buffered saline
PCOS Polycystic ovary syndrome
PDK1 3-phosphoinositide-dependent kinase 1
PDK2 3-phosphoinositide-dependent kinase 2
PGCs Primordial germ cells
PI3K Phosphoinositide 3-kinase
PIP2 Phosphatidylinositol-4,5-bisphosphate
PIP3 Phosphatidylinositol-3,4,5-triphosphate
POI Primary ovarian insufficiency
PR Progesterone receptor
PTEN Phosphatase and tensin homolog deleted on chromosome 10
PVDF Polyvinylidene difluoride
qPCR Quantitative polymerase chain reaction
ROI Region of interest

ROS Reactive oxygen species
SDHA Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)
SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM Standard error of the mean
SF-1 Steroidogenic factor-1
SOD2 Manganese superoxide dismutase or superoxide dismutase 2
StAR Steroidogenic acute regulatory protein
TBST Tris buffered saline with tween
TEMED Tetramethylethylenediamine
TGFβ Transforming growth factor beta
T_m Melting temperature
TMB 3,3',5,5'-Tetramethylbenzidine
TNF Tumor necrosis factor or tumor necrosis factor alpha
Ywhag Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma
Ywhaz Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

Declaration of Academic Achievement

The present study was primarily performed by Michael William Tsoulis in the laboratory of Dr. Deborah Sloboda at McMaster University, with the following exceptions. All animal work including processing and embedding of ovaries from prepubertal (P27) and adult (P120) offspring was completed by Dr. Kristin Connor, Dr. Deborah Sloboda and colleagues at the Liggins Institute at the University of Auckland, New Zealand. All animal work from neonatal (P4) offspring was completed jointly by Dr. Deborah Sloboda, Caroline Moore, Wajiha Gohir, Kaitlyn Chan, and Michael William Tsoulis. P4 ovaries were processed, embedded, and cut by McMaster University Health Science Centre Pathology Research Services. Caroline Moore assisted with total RNA extraction, primer design and validation, qPCR and ovarian tissue protein extraction. Kaitlyn Chan assisted with immunohistochemistry. Dr. Deborah Sloboda contributed significantly to the study's design, data analysis, and interpretation of results.

1.0 Introduction

At the beginning of the 21st century, the human genome had finally been sequenced and it was thought that this advance would usher in a new era where complex diseases could be explained and treated simply based on one's genetic makeup. However, it soon became apparent that this genetic information could only explain a small proportion of variance in disease risk with the remaining bulk of unexplained variance representing heritability that was "missing", or not accounted for by individual genes (Manolio, *et al.* 2009). These events underscored the crucial role that the environment, through interactions with the genome, has in determining disease risk. Concomitant with this paradigm shift, the Developmental Origins of Health and Disease (DOHaD) theory emerged, stating that the early life environment, in particular, has distinct effects on disease risk later in life.

At the root of DOHaD is the fact that the developing conceptus is particularly susceptible to environmental influences and can modulate its developmental trajectory based on certain cues it receives from the mother's reproductive tract throughout gestation (Lane, *et al.* 2014). In this way, the developing organism can make adaptations in anticipation of a particular external environment with the aim of maximizing survival and fitness postnatally (Gluckman, *et al.* 2005, Hales & Barker. 1992, Hochberg, *et al.* 2011). Developmental programs of offspring have been shown to be highly sensitive to maternal diet. The most well studied example of how maternal diet can influence early life development has been maternal undernutrition, which can induce fetal growth

restriction and postpartum catch-up growth that increases risk of disease during adulthood (Godfrey, *et al.* 2007). On the other hand, evidence demonstrates that early life overnutrition, as a result of gestational diabetes (Ruchat, *et al.* 2013) or maternal obesity (Li, *et al.* 2011), can also change the developmental program leading to increased disease risk later in life.

Epigenetics, the study of changes in gene function without alterations in the DNA sequence that are mitotically and/or meiotically heritable (Berger, *et al.* 2009, Dupont, *et al.* 2009), offers potential molecular mechanisms by which the early life nutritional environment shapes disease risk (Vo & Hardy. 2012). Factors contributing to altered epigenetic states include histone post-translational modifications, non-coding RNAs, transcription factors, and DNA methylation (Sarkies & Sale. 2012). The latter largely occurs on cytosines at palindromic CpG dinucleotides (Bird & Wolffe. 1999) and promoter CpG methylation is generally associated with a transcriptionally silent gene (Sarkies & Sale. 2012). During early development, massive fluctuations in DNA methylation occur characterized by almost complete demethylation of both paternal (Oswald, *et al.* 2000) and maternal (Wang, *et al.* 2014a) genomes upon fertilization and remethylation of the embryonic genome just before implantation (Reik. 2007). These epigenetic "marks" established during remethylation are stably inherited in somatic daughter cells and can permanently affect gene expression into adulthood (Lane, *et al.* 2014, Reik. 2007). Animal studies have clearly demonstrated that maternal overnutrition can induce persistent methylation changes in metabolic organs, predisposing offspring to metabolic disease (Godfrey, *et al.* 2013, Hanson, *et al.* 2011). Moreover, the recent

demonstration that methylation status of genes in hair follicles and lymphocytes of human infants is associated with seasonal variation in maternal diet around the time of conception (Dominguez-Salas, *et al.* 2014) has demonstrated that similar mechanisms operating in animal models are likely at play in humans as well.

Many reports have also demonstrated that early life nutritional stress has transgenerational impacts on disease transmittance (Aiken & Ozanne. 2014, Frias & Grove. 2012). This could happen via different mechanisms and understanding of this requires an appreciation of the fact that the nutrition of a pregnant mother (F0 generation) could not only epigenetically alter her offspring's somatic cells (F1 generation), but also that F1 offspring's germ cells, which will later form the F2 generation (Skinner. 2008). Therefore, effects observed in F2 generation offspring could be due to direct alteration of F1 germ cell development or a change in the somatic cells of the reproductive system of F1 generation offspring, which aid in germ cell development.

Although the concept of epigenetic transmission of disease risk across generations through the germline has been proposed, little evidence exists regarding what epigenetic marks may be induced or eliminated in gametes by any particular early life environmental factor/insult including nutrition. Recent evidence indicates, however, that nutritional excess or deficit early in life can impair reproductive development and function in both sexes later in life (Connor, *et al.* 2012, Zambrano, *et al.* 2014) and in this way could change the epigenetic state of germ cells. This is particularly relevant to females as the majority of germ cell (oocyte) epigenetic marks that have been proposed to underlie nongenomic trans-generational inheritance of disease risk are progressively

established during oocyte growth (Pan, *et al.* 2012, Tomizawa, *et al.* 2012), unlike males (Lee, *et al.* 2014). Given the importance of the early life nutritional environment on postnatal health and disease risk, it is thus possible that disease transmittance is the result of nutritional impacts on the developing oocyte and ovarian function. As the world rapidly adopts "Westernized" diets that are high in calories, often derived from fat (Astrup, *et al.* 2008), the impact of macronutrient imbalance on developing organ systems has become a focus in perinatal research. Nutrient excess is a major contributing factor to the increased incidence of obesity worldwide that affects all demographics including women of reproductive age (Perez Rodrigo. 2013). Nearly 25% of women between 18-35 are obese in Canada (PHAC. 2011) and these rates are closer to 35% in the United States (Flegal, *et al.* 2012). Increased rates of obesity in women of childbearing age has also translated into more women entering pregnancy either overweight or obese with estimates ranging from 30-50% in developed countries (Athukorala, *et al.* 2010, Dodd, *et al.* 2011, Dummer, *et al.* 2012, LaCoursiere, *et al.* 2005). Therefore, a large proportion of female populations are exposing their offspring to an obesogenic nutritional environment before conception, throughout pregnancy, and into postnatal life. Given that major milestones in female gonadal development occur *in utero* and during early postnatal life, it is likely that the developing ovary is being shaped by maternal nutrient excess. However, our current state of knowledge in this regard is sorely lacking. Given that F1 oocytes dictate F2 embryonic development to a large extent (Lane, *et al.* 2014) and the fact that disease risk can be transmitted across generations (Aiken & Ozanne. 2014, Frias & Grove. 2012), a better understanding of the impact of an early life

obesogenic environment on ovarian development is needed. Therefore, the following thesis will aim to fill this gap in knowledge. However, before this is explored, a comprehensive overview of key aspects of the development of this highly complex organ is required.

1.1 Ovarian Dynamics

1.1.1 Primordial Follicle Assembly

1.1.1.1 Overview

Central to female fertility is the ovary, which consists of oocytes surrounded by somatic cells (follicles). Two pools of follicles exist within the ovary: the resting follicle pool and the growing follicle pool. The resting follicle pool is made up of primordial follicles, which are oocytes surrounded by a single layer of flattened (squamous) granulosa cells (Hirshfield. 1991). The majority of primordial follicles remain quiescent potentially for years (rodents) or decades (human) (Li, *et al.* 2010). However, a small subset is recruited to supply the growing follicle pool throughout reproductive life (McGee & Hsueh. 2000). Oocytes within primordial follicles originate from primordial germ cells (PGCs) that have migrated from the hindgut to the gonadal ridge during embryonic life (Mamsen, *et al.* 2012, Sánchez & Smitz. 2012a). Upon arrival to the gonadal anlagen, PGCs proliferate by mitosis to form oogonia, however, incomplete cytokinesis results in the formation of multi-nucleated syncitia, consisting of multiple oogonia connected by intercellular cytoplasmic bridges surrounded by somatic cells, also known as germ cell cysts (Haglund, *et al.* 2011, Pepling & Spradling. 1998, Pepling.

2012, Sánchez & Smitz. 2012b, Tingen, *et al.* 2009a) At approximately embryonic day 17 in rats and gestational week 13 in humans (Zambrano, *et al.* 2014), oogonia enter meiosis and become arrested in the diplotene stage of meiosis I, at which point oogonia are referred to as primary oocytes (Hunt & Hassold. 2008, Pepling. 2012). Concomitant with this oogonia to oocyte transition is cyst breakdown and the freeing of individual oocytes to form primordial follicles (Pan, *et al.* 2012). Cyst breakdown and the establishment of the primordial follicle pool occurs from postnatal day (P)1 to P4 in rodents (Pepling & Spradling. 2001, Rajah, *et al.* 1992) and from approximately 15 to 20 weeks gestation in humans (Zambrano, *et al.* 2014). During this time, there is a massive wave of germ cell death (attrition) via apoptosis and only 33% of oocytes survive to form primordial follicles (Kezele, *et al.* 2002a, Pepling & Spradling. 2001). In rodents, primordial follicles begin to form near the ovarian core (medulla) and assembly gradually shifts towards the surface (Rajah, *et al.* 1992). Similarly, human primordial follicle assembly begins in gonadal medullary regions and radiates outwards into cortical regions (Sforza, *et al.* 2003) with a wave of follicle attrition (Geber, *et al.* 2012). This process begins well before birth at approximately 13 weeks postconception and continues until birth (Forabosco & Sforza. 2007). Despite timing differences between species, the accumulated number of primordial follicles established early in life largely dictates the reproductive potential and lifespan of mammals because once this pool is depleted, reproductive life ceases. Although heavily debated, recent data has challenged the concept of a finite primordial follicle pool suggesting that the mammalian ovary may have proliferative germ cells that could replenish the pool (Johnson, *et al.* 2004, Johnson,

et al. 2005, Kerr, *et al.* 2012, Tingen, *et al.* 2009a, Woods, *et al.* 2012, Zhang, *et al.* 2012, Zhang, *et al.* 2013)(Figure 1.1).

1.2.1.2 Mechanisms Regulating Primordial Follicle Assembly

Given the importance of the primordial follicle pool in determining lifelong fertility, much work has focused on unraveling the complex process of primordial follicle assembly. Many growth factors and hormones have been identified to affect this process including members of the transforming growth factor beta (TGFβ) superfamily such as growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15), and anti-mullerian hormone (AMH) as well as sex steroids 17-β-estradiol (E_2) and progesterone (P_4) (Nilsson, *et al.* 2013, Pepling. 2012, Trombly, *et al.* 2009).

GDF9, an oocyte-secreted growth factor necessary for fertility (Dong, *et al.* 1996), has been shown to promote primordial follicle formation in the hamster (Wang & Roy. 2004). Moreover, adult mice deficient in its homolog, BMP15 (Dube, *et al.* 1998), have a greater number of multiple oocyte follicles (MOFs)(Yan, *et al.* 2001); follicles with more than one oocyte thought to be caused by incomplete germ cell cyst breakdown (Jefferson, *et al.* 2006). Therefore, it is suggested that BMP15 may also be necessary for proper primordial follicle assembly. Conversely, AMH, also known as mullerian inhibiting substance, has been shown to inhibit germ cell cyst breakdown and the formation of primordial follicles in newborn rat ovaries (Nilsson, *et al.* 2011). Mechanistically, it is unclear how AMH prevents primordial follicle assembly as it has been previously shown to be expressed only in stromal cells of neonatal rat ovaries (Nilsson, *et al.* 2011). Moreover, although its receptor, AMH type II receptor (AMHRII), is expressed at the

level of mRNA in the early postnatal period (Durlinger, *et al.* 2002), its protein localization and expression is currently unknown.

In mice, both E_2 and P_4 appear to inhibit germ cell cyst breakdown as neonates treated with either sex steroid have more MOFs during adulthood (Iguchi, *et al.* 1986). Consistent with this observation, cultured neonatal mice or rat ovaries treated with E_2 or P_4, respectively, both exhibit decreased primordial follicle formation (Chen, *et al.* 2007, Kezele & Skinner. 2003). High levels of these sex steroids in the maternal circulation during pregnancy and their decline at birth in rodents (Tsutsui. 1992, Weisz & Ward. 1980) suggests that these factors inhibit primordial follicle assembly during fetal life and their decline allows nest breakdown postnatally (Kezele & Skinner. 2003). In humans, circulating maternal E_2 and P_4 rise during gestation (Tulchinsky, *et al.* 1972) when primordial follicle assembly occurs, which is seemingly contradictory to the mechanism proposed in rodents. However, there are species-specific differences in the role that sex steroids play in primordial follicle formation (Pepling. 2012). For instance, in baboons, when maternal E_2 production is blocked mid-late gestation, less oocytes assemble into follicles and more oocytes remain in nests at term suggesting E_2 promotes primordial follicle assembly in primates (Zachos, *et al.* 2002).

Given the complexity of primordial follicle assembly, Nilsson et al. (2013) utilized a systems approach to better understand this critical process. Rat ovaries at birth were cultured for 24 hours with known extracellular signaling factors (e.g. AMH, activin) or hormones (e.g. E_2, P_4) that either promote or inhibit assembly and gene bionetwork analyses were conducted. Numerous genes were found to be differentially expressed as a

result of treatment with pro or anti-assembly factors and interestingly, these genes were associated with known genes implicated in the etiology of two ovarian diseases that negatively impact fertility later in life: primary ovarian insufficiency (POI) and polycystic ovary syndrome (PCOS). POI is characterized by depletion of the primordial follicle pool before the age of 40 and consequently early menopause (De Vos, *et al.* 2010); whereas, PCOS is defined by oligoanovulation, polycystic ovaries, and/or hyperandrogenism (Dunaif. 2011). These associations suggest that mechanisms operating early in life to regulate primordial follicle assembly may contribute to infertility later in life.

1.1.2 Follicle Development

1.1.2.1 Overview

Beginning at approximately 22 weeks gestation in humans (Maheshwari & Fowler. 2008) and postnatal day (P)3 in rodents (Rajah, *et al.* 1992), the primordial follicles established early in life are recruited to join the growing follicle pool. Once recruited, the flattened granulosa cells of the primordial follicle begin to divide and appear progressively cube-like (transitioning follicle) until reaching a stage where the oocyte is completely surrounded by a single layer of cuboidal granulosa cells (primary follicle). Subsequently, granulosa cells continue dividing forming multiple layers around the oocyte (secondary follicle). It is at this stage when the thecal layer, composed of cells thought to be derived from surrounding stromal tissue, differentiates to surround the granulosa-oocyte complex (Young & McNeilly. 2010). Across species, when the secondary follicle reaches a diameter of 200-300 μm (Monniaux, *et al.* 2014), a fluid-

filled cavity known as an antrum begins to form within the layers of granulosa cells surrounding the oocyte. Emergence of an antrum designates the antral follicle stage and it is at this stage of follicle development that many follicles are lost to atresia (Hirshfield. 1988, Hsueh, *et al.* 1994). Whether or not a follicle dies is largely dependent on gonadotropins (see below), which act as pro-survival factors from the primary follicle stage onwards (Hsueh, *et al.* 2014).

A highly orchestrated neuroendocrine negative feedback system regulates the secretion of gonadotropins. This highly interconnected pathway is characterized by hypothalamic release of gonadotropin-releasing hormone (GnRH), anterior pituitary release of gonadotropins (luteinizing hormone [LH] and follicle stimulating hormone [FSH]) and ovarian sex steroids (E_2 and P_4)(Walker & Gore. 2011). Hypothalamic GnRH stimulates the release of LH or FSH (depending on GnRH pulsatility) (Tsutsumi & Webster. 2009)) from the anterior pituitary (Popat, *et al.* 2008). These gonadotropins induce ovarian sex steroid production that not only aids in follicle development, but importantly, negatively feeds back to the hypothalamus and anterior pituitary gonadotropes to regulate production of GnRH and LH/FSH, respectively (Popat, *et al.* 2008). Once a follicle reaches a critical diameter (200 μm in rodents; 3-5 mm in humans), development and ovulation is dependent on gonadotropins (Monniaux, *et al.* 2014). Since gonadotropins are not secreted in high enough levels prepubertally, ovulation does not occur and all growing follicles are lost to atresia. However, once an organism reaches reproductive maturation and reproductive cycles begin, an increase in circulating FSH concentration “rescues” a cohort of antral follicles from atresia. The resulting output of

E_2 from these growing follicles initially suppresses anterior pituitary FSH synthesis and release. This places selective pressure on the follicular cohort resulting in survival and domination of one (humans) or a number of (rodents) antral follicles (Zeleznik. 2004). As the dominant follicle(s) grows, the size of the antrum increases and granulosa cells differentiate into those that immediately surround the oocyte (cumulus granulosa cells) and those that line the basal lamina (mural granulosa cells) separating the granulosa and thecal cell layers. At this stage (Graafian follicle), ovulation can occur because the dominant follicle(s) produces enough E_2 to trigger a surge in LH that induces ovulation (Plant. 2012). The LH surge induces the completion of oocyte meiosis I, extrusion of the first polar body, and re-arrest of the resultant secondary oocyte (i.e. mature egg) in metaphase II, a state that persists until fertilization (Brunet & Verlhac. 2011, Sun, *et al.* 2009). After ovulation of one (humans) or multiple oocytes (rodents), remnant theca and granulosa cells form the corpus luteum, a transient endocrine gland that secretes P_4 in order to maintain pregnancy if the oocyte becomes fertilized (Stocco, *et al.* 2007a).

The two cell/two gonadotropin theory of ovarian steroidogenesis (Figure 1.1) describes the interplay between granulosa and theca cells and the gonadotropin-dependent production of ovarian sex steroids (Knobile & Neill. 1994).

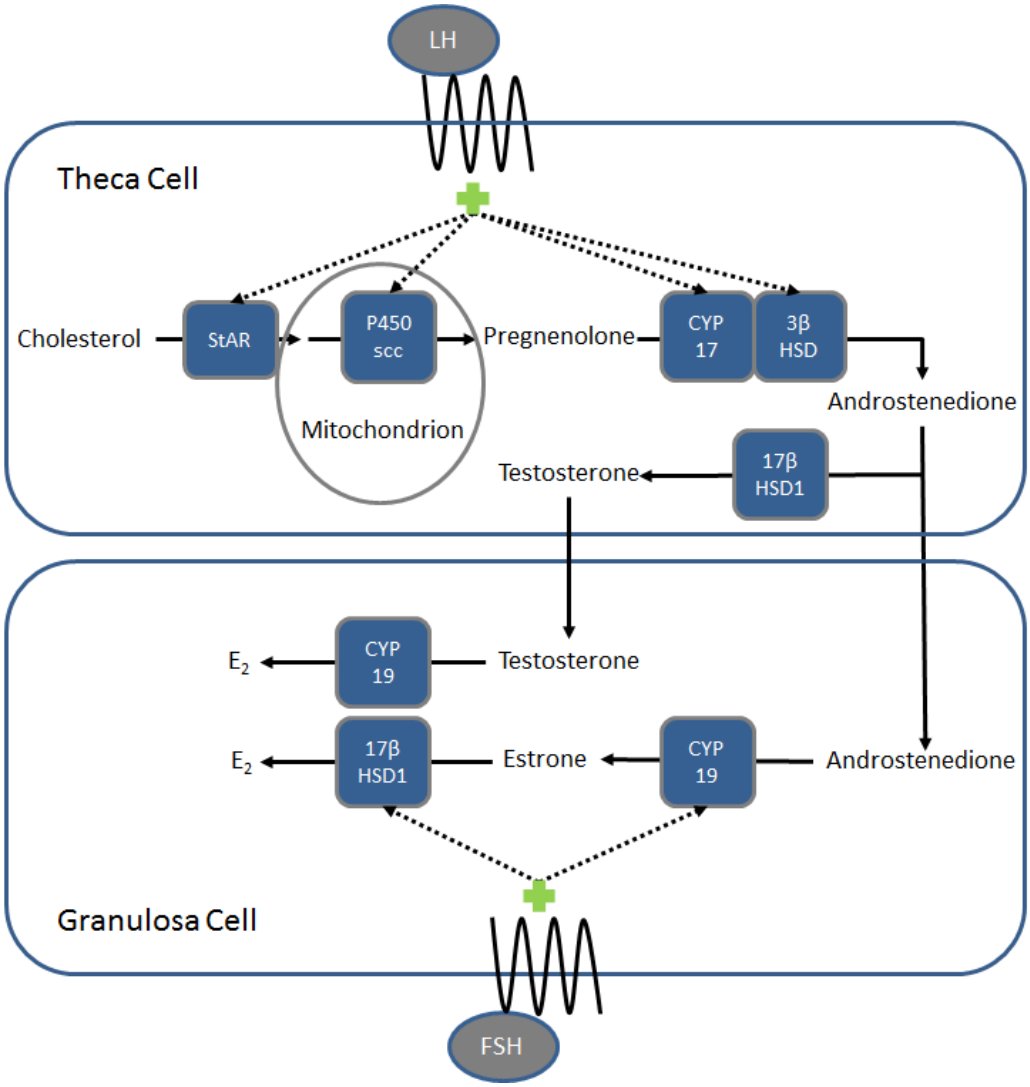

Figure 1.1 Two-cell/two gonadotropin theory of ovarian steroidogenesis in follicles: LH binding to its receptor facilitates theca cell androstenedione production and FSH binding to its receptor allows androstendione to be further processed into E_2 by granulosa cells.

The binding of LH to its receptor, LHR, on theca cells increases intracellular levels of cyclic adenosine monophosphate (cAMP) leading to upregulation of the enzymes steroidogenic acute regulatory protein (StAR), cholesterol side chain cleavage enzyme (P450scc encoded by *Cyp11a1*) cytochrome P450 17alpha hydroxylase/17,20 lyase

(CYP17 encoded by *Cyp17a1*), and 3 beta-hydroxysteroid dehydrogenase/delta-5-delta-4 isomerase (3βHSD) (Havelock, *et al.* 2004, Jamnongjit & Hammes. 2006). StAR transports cholesterol from the outer to inner mitochondrial membrane where it is converted to pregnenolone by P450scc. Outside the mitochondria, pregnenolone is converted to either P_4 or dehydroepiandrosterone (DHEA) by 3βHSD or CYP17, respectively. Both P_4 and DHEA can further be converted to androstenedione by CYP17 and 3βHSD, respectively. Androstendione either diffuses directly into granulosa cells or is converted to testosterone in theca cells by 17 beta-hydroxysteroid dehydrogenase type I (17βHSD1) before diffusion across the basal lamina into granulosa cells (Payne & Hales. 2004, Young & McNeilly. 2010). The production of these substrates and their diffusion to the granulosa cell layer is critical for E_2 production as aromatase (CYP19 encoded by *Cyp19a1*) (Sasano, *et al.* 1989) is not expressed in theca cells (Stocco. 2008). CYP19 converts either estrone, formed by conversion of androstenedione by 17βHSD1, or testosterone to E_2 (Figure 1.2). FSH binding to its receptor, FSHR, on granulosa cells increases intracellular cAMP levels and leads to upregulation of CYP19 and 17βHSD1 (Havelock, *et al.* 2004); therefore, facilitating E_2 biosynthesis. The establishment of ovarian steroidogenesis by gonadotropin signaling has been shown to be indispensable for female fertility as FSH (Kumar, *et al.* 1997), LH (Ma, *et al.* 2004), FSHR (Danilovich, *et al.* 2000) and LHR (Zhang, *et al.* 2001) knockout mice demonstrate impairments in follicle development.

1.1.2.2 Waves of Follicle Development across a Lifetime

Recently, a series of elegant tracing studies conducted by Zheng et al. (2014) has provided strong support for an idea proposed decades earlier (Hirshfield. 1992) that two waves of follicle development occur over the course of female reproductive life. In these experiments, granulosa cells of medullary primordial follicles, formed first during early postnatal life, were fluorescently labeled using Cre-lox technology in mice. These experiments demonstrated that these labeled follicles were depleted at approximately 3 months of age suggesting that these initial follicles contribute to the onset of puberty and early fertility (Zheng, *et al.* 2014). Tracing of follicles derived from primordial follicles assembled in the cortex early in postnatal life demonstrated major differences in the timing between first and second wave follicle dynamics. Second wave follicles transitioned from primordial to antral stages in twice the time as first wave follicles (47 and 23 days, respectively) with each transition (i.e. primordial-primary, primary-secondary, and secondary-antral) taking approximately twice as long as follicles formed earlier in life (Zheng, *et al.* 2014). Follicle development appears to occur in two waves in humans as well, based on evidence that follicle assembly occurs in a similar medullary-cortical manner (Sforza, *et al.* 2003) and females 3-9 years of age having significantly more medullary follicles than women 10-35 years of age (Kristensen, *et al.* 2011).

Concomitant with this initial wave of follicle development during the prepubertal period is a massive loss of primordial follicles in humans (BAKER. 1963) and mice (Bristol-Gould, *et al.* 2006a) due to atresia. This high rate of primordial follicle loss cannot be attributed solely to recruitment into the growing follicle pool (Tingen, *et al.* 2009b). Although the mechanisms regulating this atretic loss are unclear since classic

apoptotic markers are not present in primordial follicles during this period (Tingen, *et al.* 2009b), it has been speculated that non-traditional forms of cell death such as autophagy may be responsible (Tingen, *et al.* 2009b) Nonetheless, after this follicular atresia occurs prepubertally, the remaining primordial follicle pool serves as the supply for fertilizable oocytes during adulthood (Figure 1.2).

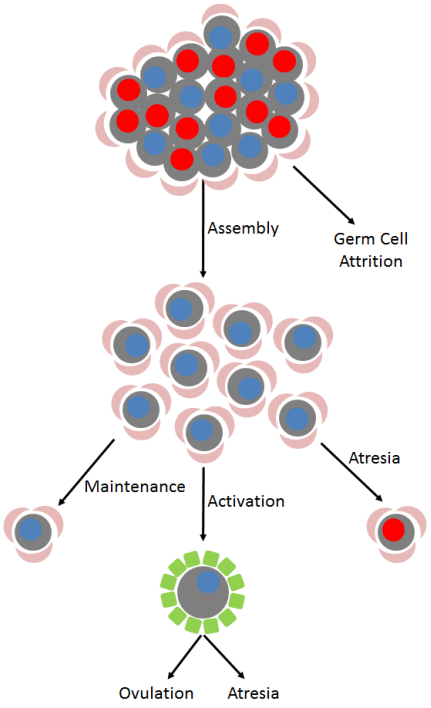

Figure 1.2 Female germ cell fate across a lifetime: Germ cells that complete the migration to the gonadal ridge either assemble into primordial follicles or undergo programmed cell death (atresia; indicated by red). Surviving primordial follicles remain dormant, become activated, or undergo atresia.

1.1.3 Primordial Follicle Recruitment

A key determinant of mammalian reproductive lifespan is the rate at which primordial follicles are recruited to join the growing follicle pool. Similar to primordial follicle assembly, primordial follicle recruitment has been shown to be regulated by numerous growth factors that influence activator and repressor pathways within the

oocyte that ultimately determine exit of primordial follicles from dormancy (Kezele, *et al.* 2005, McLaughlin & McIver. 2009, Nilsson, *et al.* 2010). A key activator pathway is the phosphoinositide 3-kinase (PI3K)-protein kinase B (Akt) cascade (Liu, *et al.* 2006). PI3K phosphorylates lipid phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3), which facilitates full activation of Akt via phosphorylation of its residues Thr308 and Ser473 by 3-phosphoinositide-dependent kinase 1 (PDK1) and PDK2, respectively (Liao & Hung. 2010). Fully activated phosphorylated Akt phosphorylates numerous target proteins including forkhead transcription factor subfamily 3 (Foxo3a) at Thr32, which results in its export from the nucleus, an event that triggers primordial follicle activation and rapid oocyte growth (John, *et al.* 2008). PTEN (phosphatase and tensin homolog deleted on chromosome 10), a lipid phosphatase that converts PIP3 to PIP2 inhibits PI3K signaling (Cantley. 2002) and therefore, prevents activation of primordial follicles (Reddy, *et al.* 2008). In mice, oocyte specific deletion of *Pten* results in activation of the entire primordial follicle pool and early depletion of the follicle reserve resulting in infertility at 12-13 weeks of age (Reddy, *et al.* 2008). Oocyte-specific PDK1 knockout mice were also found to exhibit a primary ovarian insufficiency (POI)-like phenotype, but much earlier in life (at P35) and was due to loss of primordial follicles from their dormant state, not over activation of the resting pool (Reddy, *et al.* 2009). These experiments demonstrated that not only is PI3K/Akt signaling critical for activation, but also is required to maintain the survival of the quiescent primordial follicle pool (Reddy, *et al.* 2010). Interestingly, manipulation of PI3K signaling is now being utilized in novel assisted reproductive technologies (ART).

Particularly, PTEN inhibitors and PI3K activators have been used in the culture of human ovarian cortical tissue from POI patients to drive growth of primordial follicles which, after auto-transplantation, are capable of maturing into eggs that can be retrieved and fertilized *in vitro* (Kawamura, *et al.* 2013). Following embryo transfer, this procedure has resulted in a viable birth (Kawamura, *et al.* 2013).

The PI3K/Akt pathway is activated by numerous ligands including insulin, insulin-like growth factor I (IGF-1), and kit ligand (KL) (Zheng, *et al.* 2012). KL, produced by granulosa cells, binds to its receptor protein tyrosine kinase, Kit, located on the surface of mammalian oocytes resulting in recruitment of PI3K to the cell surface and initiation of downstream phosphorylation events (Liu, *et al.* 2006). KL-Kit induced signaling has been shown to be a critical component of early follicle development, promoting the primordial to primary follicle transition both in early postnatal ovary culture systems (Packer, *et al.* 1994, Parrott & Skinner. 1999) and *in vivo* (Yoshida, *et al.* 1997). Similarly, in P4 rat ovary cultures, insulin (but not IGF-1) has been shown to promote primordial follicle development and interestingly, when combined with KL, additively increases primordial follicle growth suggesting these two factors have independent stimulatory inputs into the PI3K/Akt pathway (Kezele, *et al.* 2002b).

Similar to primordial follicle assembly, there are also factors that inhibit the primordial to primary follicle transition. To date, AMH has been the most well characterized inhibitor of primordial follicle growth. In AMH knockout mice (Durlinger, *et al.* 1999), the primordial follicle pool was significantly depleted by 4 months of age (Durlinger, *et al.* 1999). Experiments in neonatal mouse (Durlinger, *et al.* 2002) and rat

(Nilsson, *et al.* 2007) ovarian cultures demonstrate that AMH induced a decrease in the transitioning of primordial to primary follicles. Similarly, human ovarian cortical biopsies cultured with recombinant AMH have also shown inhibition of primordial follicle growth (Carlsson, *et al.* 2006). Microarray analysis of neonatal rat ovaries cultured with AMH demonstrate that 707 transcripts were changed as a result of culture with AMH including those genes associated with the TGFβ superfamily including suppression of both GDF9 and BMP15 (Nilsson, *et al.* 2007). Interestingly, AMH also downregulated Kit mRNA, which is consistent with AMH suppressing the stimulatory effects of KL on primordial follicle growth *in vitro* (Nilsson, *et al.* 2007). AMH is expressed in the granulosa cells of growing follicles of rodents (Baarends, *et al.* 1995) and humans (Weenen, *et al.* 2004b) with expression highest in healthy large secondary and small antral follicles (Durlinger, *et al.* 2002, Weenen, *et al.* 2004b). This has led to the idea that the growing follicle pool, through AMH, inhibits primordial follicle recruitment (Monniaux, *et al.* 2014). This is consistent with a slower recruitment rate in second wave follicles when the growing follicle pool is established (Zheng, *et al.* 2014).

1.1.4 Local Control of Follicle Development by Sex Steroids

In addition to their roles centrally, ovarian sex steroids act locally in the ovary to modulate antral follicle growth through signaling transduced by their respective receptors. E_2 signals primarily through its two nuclear receptors: estrogen receptor beta (ERβ) and estrogen receptor alpha (ERα). Double knockout mice lacking both ERα and ERβ are completely infertile and granulosa cells in follicles from these mice undergo conversion

to Sertoli-like, testicular cells postnatally demonstrating E_2 signaling is indispensable for maintenance of granulosa cell fate and ovarian function (Couse, *et al.* 1999, Dupont, *et al.* 2000). Each receptor subtype has distinct functions within the ovary. ERβ is located in the granulosa cells of antral and preovulatory follicles in rats (Byers, *et al.* 1997) and humans (Pelletier & El-Alfy. 2000). ERβ knockout mice have normal circulating gonadotropin levels and in the ovary, normal follicle development occurs, however ovulation rate is diminished and consequently knockout dams produce fewer pups per litter (Krege, *et al.* 1998). These results show that E_2 signaling through ERβ is necessary for normal terminal differentiation of preovulatory follicles in rodents, although the molecular mechanisms mediating this are currently poorly understood (Chaffin & Vandevoort. 2013).

ERα is not as highly expressed as ERβ within the ovary and expression is limited to the theca cell layer and interstitium in rats (Pelletier, *et al.* 2000) and humans (Pelletier & El-Alfy. 2000). ERα knockout mice have elevated levels of circulating LH owing to a lack of central negative feedback normally mediated by ERα (Couse, *et al.* 2003). In these mice, follicles develop to the antral stage, but ovulation does not occur and consequently corpora lutea are absent. Large, hemorrhagic cysts exist in the ovary similar to a PCOS phenotype, likely due to anovulation and elevated LH levels (Lubahn, *et al.* 1993). However, the dramatic ovarian phenotype of ERα knockout mice is secondary to a lack of central E_2 signaling and hence, the ovarian effect of E_2 signaling through ERα is difficult to ascertain using this model. Theca-specific deletion of ERα in mice; however, has demonstrated (Lee, *et al.* 2009) premature loss of fertility due to reduced ovulatory

capacity and the development of hemorrhagic cysts (Lee, *et al.* 2009), suggesting that local actions of E_2 on ovarian ERα are critical for normal antral follicle development.

Similar to ovarian E_2 activation of ERs, testosterone binding to its cognate androgen receptor (AR) has been shown to be critical for proper follicle development (Lebbe & Woodruff. 2013). AR is expressed in the oocyte, granulosa, and thecal layers of growing follicles with expression in granulosa cells peaking at the antral follicle stage (Sen & Hammes. 2010). Both global (Hu, *et al.* 2004, Shiina, *et al.* 2006) and granulosa-specific (Sen & Hammes. 2010) AR knockout mice are subfertile with fewer ovulated oocytes and more atretic follicles, and, in response to artificially induced ovulation, $AR^{-/-}$ mice do not upregulate expression of the P_4 receptor (PR) (Hu, *et al.* 2004), which is necessary for ovulation to occur (Conneely. 2010). Therefore, both the precursors for E_2 biosynthesis and E_2 itself, are important local regulators of follicle development in addition to their roles centrally.

1.1.5 Oocyte Control of Follicle Development

Although gonadotropic control of sex steroid production is critical for follicle growth, recently it has become established that the oocyte has an equal, if not more important role in the regulation of follicle development and ovulation rate (Eppig. 2001, Gilchrist, *et al.* 2008). Initial work by Eppig et al. (2002) demonstrated this concept of oocyte-controlled growth quite elegantly by reaggregating oocytes derived from P12 mouse secondary follicles with somatic cells from newborn mouse ovaries. Reaggregated oocytes reached the antral follicle stage in approximately half the time as normal oocytes (Eppig, *et al.* 2002) showing that the somatic cell compartment is directed by the oocyte

to develop at a rate determined by the oocyte. Since then, multiple oocyte-derived growth factors have been identified that affect growth rates with much attention being paid to the TGFβ superfamily members GDF9 and BMP15 (Gilchrist, *et al.* 2008). Understanding of the critical roles of GDF9 and BMP15 in follicle development has largely come from studies of animals deficient in these proteins. In both mice (Dong, *et al.* 1996) and sheep (Hanrahan, *et al.* 2004) lacking GDF9 and sheep lacking BMP15 (Galloway, *et al.* 2000), follicle development stops at the primary follicle stage and consequently, these animals are infertile. On the other hand, *Bmp15*$^{-/-}$ knockout mice display a relatively mild reproductive phenotype with reduced ovulation and fertilization rates (Yan, *et al.* 2001) highlighting species specific differences that may have arisen due to evolving ability of these two proteins to form homo- and heterodimers (Monestier, *et al.* 2014). Additional deletion of an allele of the *Gdf9* gene from *Bmp15*$^{-/-}$knockout mice (i.e. *Bmp15*$^{-/-}$/*Gdf9*$^{+/-}$); however, results in severe infertility (Yan, *et al.* 2001) associated with an inability of cumulus granulosa cells to expand and function properly prior to and after the LH surge (Su, *et al.* 2004). Therefore, in mice, adequate doses of both GDF9 and BMP15 are required for normal development and expansion of cumulus granulosa cells. This has been shown to be partially due to the capacity of both GDF9 and BMP15 to facilitate cumulus granulosa cell E_2 signaling (Emori, *et al.* 2013, Sugiura, *et al.* 2010) and the ability of BMP15 to reduce cumulus cell apoptosis by upregulating the anti-apoptotic factor B-cell lymphoma 2 (Bcl-2) and downregulating the pro-apopototic factor Bcl-2 associated X protein (Bax) (Hussein, *et al.* 2005).

In addition to facilitation of terminal follicle development, GDF9 has been shown to prevent granulosa cell apoptosis and enhance FSH-stimulated growth from the preantral to early antral follicle stage (Orisaka, *et al.* 2006). GDF9's role at this crucial stage of follicle development may also lie in its ability to facilitate theca cell recruitment, proliferation, and differentiation (Young & McNeilly. 2010). Evidence for this comes from mice lacking GDF9 that fail to recruit theca cell precursors to surround the granulosa-oocyte complex (Elvin, *et al.* 1999), bovine theca cells from small follicles that increase DNA synthesis and cell numbers when cultured with GDF9 (Spicer, *et al.* 2008), and GDF9 treatment that enhances androstenedione production in rat thecal-interstitial cells (Solovyeva, *et al.* 2000).

1.1.6 Corpora Lutea Formation and Regression

The LH surge is required not only to trigger ovulation, but also for the rapid differentiation of granulosa/theca cells into large and small luteal cells through a process known as luteinization (Stocco, *et al.* 2007b). In humans, large and small luteal cells are derived from granulosa and theca cells, respectively (Niswender, *et al.* 2000). These cells remain relatively distinct within the corpus luteum (CL) and retain distinct abilities to produce E_2 or androgens, respectively (Ohara, *et al.* 1987). However, in rodents, there is little evidence of derivatization of large and small luteal cells from granulosa and theca cells due to mixing between these two cell types after follicle rupture (Stocco, *et al.* 2007b) and the fact that both cell types are capable of producing E_2 and androgens (Nelson, *et al.* 1992). The post-ovulatory period in both species, however, is characterized by luteinization and the establishment of dense capillary networks that

facilitate massive production of P_4; the predominant sex steroid produced by the CL (Niswender, *et al.* 2000). Across species, increased CL output of P_4 is driven in large part by LH, which increases cholesterol uptake from the circulation and delivery to mitochondrial P450scc by StAR (Stocco, *et al.* 2007b). Moreover, the preovulatory rise in FSH upregulates LHR in mural granulosa cells (Piquette, *et al.* 1991) facilitating cAMP signaling and increased expression of StAR (Sandhoff, *et al.* 1998), P450scc (Oonk, *et al.* 1989), and 3βHSD (Kaynard, *et al.* 1992), which effectively primes follicular granulosa cells for P_4 production post ovulation (Jamnongjit & Hammes. 2006). In rodents, P_4 secretion by the CL is not only dependent on the rate of biosynthesis by StAR, P450scc, and 3βHSD, but also on the expression of 20α-hydroxysteroid dehydrogenase (20αHSD), which catabolizes P_4 into its biologically inactive 20α-dihydroprogesterone form (Stocco, *et al.* 2000). In non-fertile rodent cycles, normal functional luteal regression and the decline in circulating P_4 secreted by the CL (OECD. 2012) is partially dependent on increased expression of 20αHSD (Stocco, *et al.* 2000). Mice deficient in 20αHSD have a delay in functional luteal regression and consequently display prolonged diestrus (Ishida, *et al.* 2007); the estrous stage in rodents that occurs after ovulation when CLs are developing (Yoshida, *et al.* 2009). In rodents, functional luteal regression is necessary for the next cycle to be initiated, as P_4 negatively feeds back on the hypothalamus/anterior pituitary inhibiting gonadotropin secretion (OECD. 2012, Stocco, *et al.* 2007b). However, complete CL demise, also known as structural luteal regression, takes approximately four more estrous cycles to complete in rodents (Taketa, *et al.* 2011). Structural luteal regression is thought to be driven by luteal cell apoptosis

regulated by intrinsic and extrinsic factors, such as immune cell infiltration (Stocco, *et al.* 2007b).

Accumulation of reactive oxygen species (ROS), including superoxide anions and hydrogen peroxide (H_2O_2), is thought to be a critical component of apoptotic CL demise (Stocco, *et al.* 2007b). ROS damage DNA, lipids, and proteins ultimately causing oxidative stress that is likely to result in cell death (apoptosis) if left unchecked by antioxidant enzymes including superoxide dismutase (SOD), which catalyzes the dismutation of superoxide anions into hydrogen peroxide (H_2O_2), and catalase (CAT), which catalyzes the decomposition of H_2O_2 into water and oxygen (Agarwal, *et al.* 2012, Al-Gubory, *et al.*). Luteolysis is associated with an increase in superoxide anions (Tanaka, *et al.* 2000) and downregulation of antioxidant enzymes (Foyouzi, *et al.* 2005). For example, SOD2, the mitochondrial SOD isoform containing manganese, is downregulated during structural luteal regression; thus, facilitating ROS induced apoptosis (Agarwal, *et al.* 2012).

1.2 Obesity and Female Reproduction

As outlined in section 1.1, the establishment and growth of developmentally sound oocytes is dependent upon the spatial and temporal orchestration of numerous paracrine, autocrine, and endocrine factors across a lifetime (Amsterdam, *et al.* 2003, Edson, *et al.* 2009, Matsuda, *et al.* 2012, Oktem & Oktay. 2008). A number of environmental factors have been identified that unequivocally disrupt this synchrony and impair fertility. They include smoking (Sharpe & Franks. 2002), endocrine disrupting

chemicals (Walker & Gore. 2011), and, of relevance to the present study, obesity (defined as body mass index (BMI) >30 kg/m^2) (Cardozo, *et al.* 2011, Pasquali, *et al.* 2003, Pasquali & Gambineri. 2006). The following sections will describe the clinical and animal data that show ovarian function and fertility is impaired as a result of obesity.

1.2.1 Clinical Data

Approximately 50% of women with polycystic ovary syndrome (PCOS) are obese or overweight with increased abdominal fat usually present (Cardozo, *et al.* 2011, Gambineri, *et al.* 2002). Obese women with or without PCOS are 3 times as likely to have anovulatory infertility when compared with normal weight women (Rich-Edwards, *et al.* 1994). Reduced LH pulse amplitude and lower P_4 metabolites in eumennorheic morbidly obese women (Jain, *et al.* 2007) suggests impairment in hypothalamic/anterior pituitary control of ovarian steroidogenesis in the pathophysiology of obesity-induced anovulatory infertility. One of the hallmarks of PCOS is elevated LH pulse amplitude (Burt Solorzano, *et al.* 2012), which is seemingly paradoxical. However, body fat mass and BMI inversely correlate with LH pulse amplitude in PCOS women (Taylor, *et al.* 1997) indicating that obesity independently disrupts central neuroendocrine function.

Assisted reproductive technologies (ART) are often sought out by obese women to treat infertility and outcomes of *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) are less favourable in obese women compared with normal weight women (Kumbak, *et al.* 2012, Wu, *et al.* 2011). Obese women require larger doses of gonadotropins for ovarian stimulation (Maheshwari, *et al.* 2007) and exogenous ovarian stimulation responses are poor demonstrated by lower peak circulating E_2 levels, reduced

numbers of large follicles, and significantly fewer oocytes retrieved after high dose stimulation (Wu, *et al.* 2011). Oocytes retrieved from obese women undergoing IVF/ICSI are smaller than oocytes retrieved from normal weight women (Marquard, *et al.* 2011) and failed fertilized oocytes of severely obese women undergoing IVF have a higher prevalence of spindle abnormalities compared with those from normal BMI women (Machtinger, *et al.* 2012). This is consistent with mouse models of high fat (HF) diet induced obesity (Hirshfeld-Cytron, *et al.* 2011, Luzzo, *et al.* 2012). These lines of evidence indicate that obesity compromises ovarian function resulting in reduced oocyte quality, defined as the ability of the mature egg to undergo fertilization and healthy embryo development to term (Gilchrist, *et al.* 2008). In line with this, obese women have an increased risk of miscarriage (Metwally, *et al.* 2008) and are twice as likely to have a stillbirth (Chu, *et al.* 2007) compared with normal weight women. Although this evidence demonstrates oocyte quality is impaired as a result of obesity, the mechanisms mediating this are poorly understood in humans. In contrast, animal models of obesity have provided considerable mechanistic insight into obesity-induced reproductive dysfunction.

1.2.2 Animal Data

High fat (HF) diet (60% calories from fat) induced obesity in mice results in progressively reduced numbers of estrous cycles characterized by a reduction in the number of days in the preovulatory (proestrus) stage and more days spent in estrus, when ovulation occurs (Sharma, *et al.* 2013b). It was found that these cycle defects were due to an inability to generate the preovulatory LH and FSH surges, where reduced corpora lutea numbers were indicative of a reduction in the ovulation rate (Sharma, *et al.* 2013b).

In this model, exogenous GnRH was able to increase LH secretion in these obese mice demonstrating that the anterior pituitary was still capable of responding to GnRH leading the authors to conclude that the HF diet-induced reproductive phenotype was largely due to a hypothalamic defect (Sharma, *et al.* 2013b). Similar reproductive phenotypes have also been observed in rats fed a high-caloric, cafeteria diet (Sagae, *et al.* 2012) and rats fed a HF diet (45% calories from fat) (Balasubramanian, *et al.* 2012). HF intake also decreased proestrus E_2 levels compared to controls suggesting impaired antral follicle growth also contributes to the deficit in gonadotropin surges (Balasubramanian, *et al.* 2012). Consistent with this notion, more atretic follicles were present in HF diet-induced obese rats (Wang, *et al.* 2014b), leptin deficient obese mice (Serke, *et al.* 2012) and New Zealand obese mice (Radavelli-Bagatini, *et al.* 2011). It has also been suggested that hyperinsulinemia associated with obesity contributes to impairment in follicle growth by upregulating androgen production (Escobar-Morreale, *et al.* 2014). In line with this idea, theca cell specific deletion of the insulin receptor improves fertility in HF diet-induced obese mice by reducing androstenedione production induced by insulin (Wu, *et al.* 2014).

Evidence also indicates that excessive lipid accumulation and induction of oxidative stress and inflammation mediates ovarian dysfunction in response to obesity (Robker, *et al.* 2011). For example, protein carbonyl (marker of oxidative stress) levels are elevated in ovaries of rats fed a HF (45% calories from fat) diet (Bernal, *et al.* 2010) and gene expression levels of the pro-inflammatory cytokines tumor necrosis factor (TNF) and interleukin-6 (IL6) are elevated in ovaries of mice fed a HF (60% calories from fat) diet (Nteeba, *et al.* 2013). Functionally, these "lipotoxicity" responses induced by high

levels of lipid associated with HF diet intake have been shown to impair development in late antral follicles through increased granulosa cell apoptosis (Jungheim, *et al.* 2010, Wu, *et al.* 2010). Moreover, oocytes within cumulus-oocyte complexes (COCs) collected from mice fed a HF diet for 4 weeks had decreased mitochondrial membrane potential, which is associated with uncoupling of oxidative phosphorylation and ROS generation (Wu, *et al.* 2010). Consistent with this, COCs from mice fed a HF diet demonstrated a higher rate of superoxide radical production (Igosheva, *et al.* 2010). HF diet intake also altered mitochondrial ultrastructure in oocytes including fewer, more disarrayed cristae and increased swelling and more vacuoles (Luzzo, *et al.* 2012). Oocyte mitochondria are critical for early embryonic development (Dumollard, *et al.* 2007, Wai, *et al.* 2010). Therefore, it has been proposed that HF diet-induced mitochondrial dysfunction in oocytes causes the observed abnormal embryonic cellular differentiation (Minge, *et al.* 2008) and diminished embryonic development to the blastocyst stage (Igosheva, *et al.* 2010) following HF diet intake (Turner & Robker. 2014). This compromised embryonic development is then thought to impair fetal growth and increase disease risk later in life (Turner & Robker. 2014). Most studies have explored how metabolic disease risk increases as a result of this impaired early life development, however, evidence, mainly in animal models, indicates that the function of the female reproductive system is also compromised, which could negatively affect the development of future generations. The following section will describe said data.

1.3 Maternal Obesity and Female Offspring Reproduction

Little is known in humans about the independent impact of maternal obesity on female offspring reproduction mainly because maternal obesity strongly associates with offspring body composition and obesity (Catalano, *et al.* 2009, Drake & Reynolds. 2010), which, as outlined above, has its own deleterious effects on the female reproductive system. However, both excessive gestational weight gain (Boynton-Jarrett, *et al.* 2011, Deardorff, *et al.* 2013) and a pre-pregnancy BMI of overweight/obese (Deardorff, *et al.* 2013, Keim, *et al.* 2009) have been associated with early menarche (first menstrual cycle) even when adjusting for childhood obesity, which is strongly associated with maternal obesity (Catalano, *et al.* 2009) and early menarche (Kaplowitz. 2008). In ewes, a similar relationship has been observed. Female birthweights above 5 kg have been associated with a decrease in fecundity even after controlling for adult subcutaneous adiposity (Gardner, *et al.* 2009) suggesting that fetal macrosomia, which is associated with maternal obesity, (Mission, *et al.* 2013) independently associates with impairments in future reproductive function.

In contrast to these correlations, rodent models of maternal obesity have demonstrated causality. For example, in rats, intake of a HF diet (45% of calories derived from fat) throughout pregnancy and lactation (Connor, *et al.* 2012) or an n-6 polyunsaturated fatty acid rich diet (Hilakivi-Clarke, *et al.* 1997) throughout gestation advances pubertal onset in offspring. This effect appears to be dependent on gestational exposure to an obesogenic nutritional environment as Long Evans Tokushima (LETO)

rats fostered to obese Otsuka Long Evans Tokushima fatty (OLETF) (Schroeder, *et al.* 2013) and rats (Sanchez-Garrido, *et al.* 2013) and mice (Caron, *et al.* 2012) reared in small sized litters do not display advancement in pubertal onset. It is unclear how a gestational obesogenic maternal diet induces pubertal onset advancement in offspring. However, the observation that this major reproductive milestone in reproductive life is altered suggests that *in utero* exposure to maternal nutrient excess has long-lasting effects on hypothalamic, anterior pituitary and/or ovarian function.

In line with this notion, as adults, rat offspring born to HF-fed dams throughout pregnancy and lactation are more likely to display estrous cycles characterized by two or more days spent in estrus (Connor, *et al.* 2012) suggesting that the interplay between the hypothalamus, anterior pituitary, and ovary is disrupted. However, in contrast to pubertal onset advancement, it appears as though exposure to an obesogenic environment during lactation contributes to this phenotype as LETO rats fostered to OLETF dams also have more frequent reproductive cycles characterized by prolonged estrus during adulthood (Schroeder, *et al.* 2013). This reproductive phenotype is similar to that observed in mice fed a HF diet (Sharma, *et al.* 2013a) and may also be due to a hypothalamic defect as mice reared in small litters display significant reductions in the number of neural projections to GnRH-producing neurons of the hypothalamus into adulthood (Caron, *et al.* 2012).

Although these lines of evidence indicate that perinatal maternal nutrient excess impairs reproductive function in offspring, to date, no studies have investigated how the development and function of the ovary in offspring is impacted by an obesogenic

maternal diet. Given that this organ houses, maintains, and fosters the growth of oocytes that determine reproductive potential and lifespan, experiments are sorely needed to address this gap in knowledge.

1.4 Rationale

Maternal obesity is not only a common obstetric risk factor associated with adverse fetal, neonatal, and maternal outcomes (Gaillard, *et al.* 2013, Leddy, *et al.* 2008, Mission, *et al.* 2013), but also has long-lasting effects on female offspring health (Frias & Grove. 2012, O'Reilly & Reynolds. 2012, Rebecca, *et al.* 2013) and reproductive function (Connor, *et al.* 2012, Sloboda, *et al.* 2009, Sloboda, *et al.* 2011). Previous work has demonstrated that rat offspring born to high fat fed mothers enter puberty early and display altered estrous cyclicity as adults (Connor, *et al.* 2012). This indicates that changes in offspring reproductive function as a result of an obesogenic maternal diet are likely mediated by a defect in communication between the hypothalamus, anterior pituitary, and ovary. Given that crucial aspects of ovarian development occur perinatally, it is possible that offspring reproductive dysfunction as a result of maternal nutrient excess stems from a defect induced in the developing ovary. Consistent with this notion, mid-gestational fetal ovaries of *ad libitum* (overnourished) fed ewes have fewer primordial follicles than controls fed a standard diet (Da Silva, *et al.* 2003) indicating that maternal overnutrition can impair primordial follicle assembly, the critical ovarian process that determines reproductive lifespan. However, it remains to be determined how a maternal diet resembling that which is being increasingly consumed worldwide (i.e.

high in fat) influences offspring primordial follicle assembly. Similarly, it is not known how a maternal diet high in fat affects offspring ovarian development during the critical prepubertal period when the primordial follicle pool is reduced to a set number that will supply the growing follicle pool during adulthood (Bristol-Gould, *et al.* 2006a, Tingen, *et al.* 2009b). Furthermore, it is unknown how primordial follicle recruitment and follicle growth is impacted by a maternal obesogenic diet both before and after puberty. Given that offspring are more likely to eat a diet comparable in quality to that of their mother (Ashman, *et al.* 2014), how does feeding offspring the same high fat diet that their mother ate influence ovarian function in adulthood? If important ovarian functions are disrupted by the interaction between maternal and offspring high fat diet intake, as altered estrous cyclicity would indicate (Connor, *et al.* 2012, Sharma, *et al.* 2013a), what oocyte-derived, somatic-cell derived, and/or central factors may be mediating this disruption? Given our changing global landscape characterized by the rapid adoption of diets high in fat (Astrup, *et al.* 2008) and a concomitant rise in fertility problems (Aitken. 2013), these are questions and gaps in knowledge that sorely need be addressed.

Therefore, utilizing an established rat model of high fat diet induced maternal obesity (Connor, *et al.* 2012), the following thesis will seek to clarify how an early life obesogenic maternal diet impacts the neonatal, prepubertal, and adult ovary. Given the known effects of a postweaning high fat diet on female reproduction, the interaction between this factor and being born to an obese mother will be ascertained. Critical regulators of primordial follicle assembly, recruitment, and follicle growth will be measured to determine what aspects of these important processes change in response to

an obesogenic maternal diet. Results discussed herein surround the experimental testing of the following hypothesis:

1.5 Hypothesis

The goal of this study was to test the hypothesis that maternal high fat diet induced obesity compromises follicle formation and the development of follicles that determine lifelong fertility in offspring. Maternal high fat diet induced obesity will have independent effects on adult follicle growth when compared with the effects of a postweaning high fat diet.

1.6 Experimental Objectives

The specific aims of this study were to

1. Determine the impact of maternal obesity on the establishment of the primordial follicle pool in neonatal rat offspring
2. Ascertain how maternal high fat diet intake affects follicle recruitment and growth in the rat ovary of prepubertal offspring
3. Establish the interactive effects of maternal and postweaning high fat diet on follicle development in the adult offspring ovary

2.0 Materials and Methods

2.1 Animal Model

All animal procedures for this study were approved by the McMaster University Animal Research Ethics Board (Animal Utilization Protocol 12-10-38) in accordance with the guidelines of the Canadian Council of Animal Care.

2.1.1 Neonatal Offspring

A previously validated model of maternal HF nutrition ((Connor, *et al.* 2012, Howie, *et al.* 2009) was utilized in the present study to generate a cohort of postnatal day (P)4 offspring at McMaster University, Canada. Virgin male and female Wistar rats, aged 90 days (200-250g), were purchased from Charles River Laboratories (Willmington, MA) and housed in pairs. Rats were maintained under controlled lighting (12:12 light-dark cycle) and temperature (22°C) with *ad libitum* access to standard rat chow (CON) (14% kcal fat, 3.0 kcal/g; Diet 8604, Harlan Teklad, Madison, WI; soybean oil fat source) and water. Male and female rats were acclimatized for 3 weeks in separate cages before mating at ~P110. Stage of estrous was assessed in females using a rat estrous cycle monitor (MK-11, Muromachi Kikai Co., LTD, Tokyo, Japan) that utilizes the principle that the electrical impedance of the epithelial layer of the vaginal mucosa is significantly higher during proestrus compared to that in other stages of the estrous cycle. An impedance reading of greater than 3000 Ω is predictive of a 90% successful pregnancy; therefore, those females presenting with an impedance value of over 3000 Ω or greater were housed with a male overnight. Mating was confirmed by the presence of sperm in a vaginal smear the following morning at which time pregnant females were housed

individually. This day represented day 1 of pregnancy. On day 1, pregnant females were randomized either to the standard control chow (CON, n=8) diet or a high-fat diet (HF, n=8, 45% kcal fat, 4.73 kcal/g; D12451, Reseach Diets, New Brunswick, NJ, USA; lard and soybean oil fat sources) fed *ad libitum*. All dams were weighed and food intakes recorded every day during gestation and every 3 days during lactation. At birth, pups were weighed and sexed, and at P4 litter size was adjusted to eight pups per litter (4 males and 4 females) to ensure standardized nutrition until weaning. Remaining pups were killed by decapitation at P4. Both ovaries were removed and fixed in 10% (v/v) buffered formalin (McMaster University Health Science Centre Pathology Research Services) for later processing and embedding. At weaning (P22), dams were fasted overnight, anaesthetized with 2% isofluorane (Pharmaceutical Partners of Canada; CP0406V2; Richmond Hill, Canada), and killed by decapitation. Retroperitoneal fat pads were removed and weighed as a surrogate measure of body fat mass.

2.1.2 Prepubertal and Adult Offspring

Cohorts of P27 and P120 offspring utilized in the present study were generated by Connor et al. (2012) at the Liggins Institute in Auckland, New Zealand according to the methods outlined therein. Briefly, virgin female Wistar rats were time mated and from the beginning of pregnancy until the end of lactation fed either a control diet (CON) (6.2% kcal fat, 3.1 kcal/g; Diet 2018, Harlan Teklad, Blackthorn, Bicester, UK; soybean oil fat source) or high-fat diet (HF, 45% kcal fat, 4.73 kcal/g; D12451, Reseach Diets, New Brunswick, NJ, USA; lard and soybean oil fat sources). At birth, pups were weighed and sexed, and at P2 litter size was adjusted to eight pups per litter (4 males and 4 females) to

ensure standardized nutrition until weaning. Remaining pups were killed by decapitation at P2. At weaning (P22), remaining offspring were randomly assigned to receive either a control diet (con) or a high-fat diet (hf) *ad libitum*, with free access to water for the remainder of the study. This created four groups of offspring: CON-con, CON-hf, HF-con, and HF-hf (Figure 2.1). Bodyweights and food intake were recorded every 3 days until the end of the study (~P120). In order to determine pre-pubertal phenotypes, a subset of female offspring (fed a control diet post weaning) were fasted overnight at P27, anesthetized with sodium pentobarbitone (60 mg kg^{-1}), and killed by decapitation. Trunk blood samples were taken for later plasma analyses and organ weights were recorded. One ovary was fixed in Bouin's solution and paraffin embedded for histological analyses while the other ovary was snap frozen in liquid nitrogen for molecular analyses. At P120, rats in diestrus (determined by vaginal smearing) were fasted overnight, anesthetized with sodium pentobarbitone (60 mg kg^{-1}), and killed by decapitation. Trunk blood samples were collected in heparinized tubes, centrifuged (2,000 x g) for 10 minutes at 4°C, and plasma was collected for later analyses. Organ weights were recorded. One ovary was fixed in Bouin's solution and paraffin embedded for histological analyses while the other ovary was snap frozen in liquid nitrogen for molecular analyses.

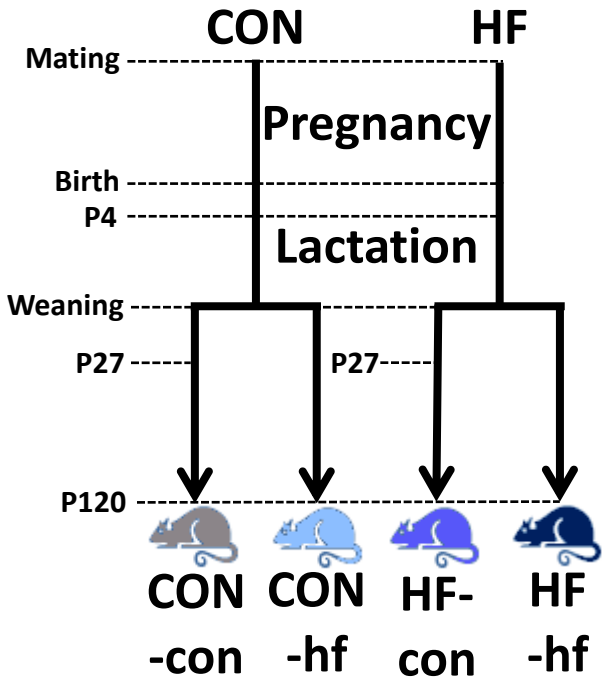

Figure 2.1 Rat model of HF diet induced maternal obesity: Abbreviations: CON, control maternal diet; HF, high fat maternal diet; con, control postweaning diet; hf, high fat postweaning diet; P, postnatal day

2.2 Plasma Analyses

Circulating plasma FSH, LH, AMH, and E_2 were measured by commercially available rat enzyme-linked immunosorbent assay (ELISA) kits (FSH, Uscn Life Science Inc., CEA830Ra; Wuhan, China; LH, CEA441Ra; AMH, CEA228Ra; E_2, Calbiotech, ES180S-100 Mouse/Rat, Spring Valley, US). For FSH and E_2 measurements, all plasma samples were run neat; for LH and AMH, all plasma samples were diluted 10-fold in phosphate buffered saline (PBS; 1.2 mM $NaH_2PO_4 \cdot H_2O$, 9.3 mM $NaHPO_4$, 154 mM NaCl, pH 7.4) as per the manufactuer's instructions. For FSH, LH, and AMH measurements, 50 µl of plasma (FSH) or diluted plasma (LH, AMH) for each sample was

aliquoted into appropriate wells; for E_2 measurements, 25 µl of plasma for each sample was aliquoted into the appropriate wells. The protocols for each ELISA kit were followed as per the manufacturer's instructions. Incubation times with 3,3',5,5'-Tetramethylbenzidine (TMB) substrate were 20, 15, 30, and 30 minutes for FSH, LH, AMH, and E_2, respectively. The sensitivity of each ELISA kit was 0.92 ng/ml, 148.3 pg/ml, 3 pg/ml, and 45.9 pg/ml, respectively. All samples were run in duplicate and the mean value determined with the dilution factor taken into account in final measurement outcome for the LH and AMH ELISA assays.

2.3 Microscopy

2.3.1 Processing and Embedding

P4 fixed ovaries were processed by a fully automated tissue processor (Leica Biosystems, TP1020, Concord, Canada) with the following incubations: 70% EtOH (2X1 hour), 90% EtOH (2X1 hour), 100% EtOH (3X1 hour), and xylene (5X1 hour). Processed ovaries were then paraffin embedded in no particular orientation using an embedding center, dispenser, and hot plate (Leica Biosystems, EG1160, Concord, Canada). P27 and P120 ovaries were processed and embedded by the Histology Laboratory at the Liggins Institute (Auckland, New Zealand).

2.3.2 Microtomy

P4, P27 and P120 paraffin-embedded ovaries were completely serially sectioned. P4 ovaries were sectioned at 4 µm (by McMaster University Health Science Centre Pathology Research Services). P27 and P120 tissues were sectioned using a Microm HM325 (Thermo Scientific) Microtome at 4 and 8 µm, respectively. All sections were

placed in a 37°C paraffin section flotation bath (Electrothermal, MH8517, Staffordshire, UK) and placed individually on Superfrost™ Plus microscope slides (Fisherbrand™, 12-550-15, Ottawa, Canada), dried overnight at 37°C, and then stored for later analyses.

2.3.3 Haematoxylin and Eosin (H&E) Staining

Every 5th (P4 and P120) or 10th (P27) ovarian section was processed for H&E for future morphometric analyses. Sections were depariffinized in xylene (3X5 minutes), rehydrated through an ethanol series (100% EtOH, 2X2 minutes; 90% EtOH, 2X2 minutes; 70% EtOH, 2X2 minutes; 50% EtOH, 2X2 minutes) followed by 5 minutes in PBS. Slides were rinsed in cold tap water for 1 minute, placed in filtered Haematoxylin Stain 2-Gill Method (Fisher Chemical, CS-401-1D, Ottawa, Canada) for 5 minutes, and rinsed again under cold tap water until water was transparent. Slides were differentiated by one dip in 1% acid alcohol (120 mM HCl in 70% EtOH) followed by rinsing in cold tap water for 1 minute and underwent bluing in Scott's Tap Water (42 mM $NaHCO_3$, 167 mM $MgSO_4$, filtered) for 1 minute. Slides were rinsed again in running cold tap water for 1 minute followed by incubation in 0.25% Eosin Y (Fisher Chemical, SE22-500D, Ottawa, Canada) in Sodium Acetate Buffer (200 mM, pH 5.5) for 5 minutes and rinsed again under cold tap water until water was transparent. Slides were dehydrated through an ethanol series (50% EtOH, 10 dips; 70% EtOH, 5 dips; 90% EtOH, 5 dips; 100% EtOH, 5 dips) followed by incubation in xylene (3X5 minutes). Sections were coversliped using Permount™ Mounting Medium (Fisher Chemical, SP15-100, Ottawa, Canada), dried overnight at room temperature, and then stored for later morphometric analyses.

2.4 Morphometric Analyses of Follicle and Corpora Lutea Populations

For all ages where appropriate, follicle and corpora lutea counting was blinded. Only follicles containing an oocyte with a visible nucleus were counted to avoid counting the same follicle twice. Atretic follicles were counted if an oocyte was present, but did not need to contain a visible nucleus because nuclear fragmentation often accompanies late stage degeneration (Osman. 1985). All follicles were classified according to Hirshfield and Midgley (1978) as follows (Figure 2.2); primordial follicles, an oocyte surrounding by one layer of flattened granulosa cells (Figure 2.2A); transitioning follicle, an oocyte surrounded by more than 2 cuboidal granulosa cells and at least one flattened granulosa cell (Figure 2.2B); primary follicles, an oocyte surrounded by one to fewer than two complete layers of cuboidal granulosa cells (Figure 2.2C); secondary follicle, an oocyte surrounded by greater than one layer of cuboidal granulosa cells, with no visible antrum (Figure 2.2D); antral follicle, an oocyte surrounded by multiple layers of cuboidal granulosa cells and containing one or more antral spaces (Figure 2.2E); type II atretic follicle (Osman. 1985), an oocyte undergoing degeneration or cytoplasmic blebbing and retraction of the granulosa cell layer with a fluid filled cavity present (Figure 2.2F). CL were classified according to previous criteria (Yoshida, *et al.* 2009) in diestrus rats: currently formed CL, basophilic luteal cells with or without angiogenesis between cells; previously formed CL, vacuolated luteal cells with or without fibrous tissue infiltration (See Figure 3.13A). The area (μm^2) of each ovarian section was measured using a Nikon DS-L3 Camera Control Unit (Figure 2.3). Total volume of each section was calculated

(area of the section X thickness of the section) and follicle counts for each animal were normalized to the total volume (μm^3) of ovarian tissue counted per animal.

2.4.1 Neonatal Offspring

At P4, every 5th H&E stained ovarian section was evaluated for follicle populations. At this age, the ovary is composed of a mix of assembled and unassembled oocytes. Therefore, oocytes were classified as either assembled or unassembled base on established criteria (Nilsson, *et al.* 2011). Oocytes were considered unassembled if they were a part of an oocyte nest with no intervening somatic cells and considered assembled if they were partially or completely surrounded by pre-granulosa cells (Nilsson, *et al.* 2011). Assembled follicles were further sub classified into one of two categories: a primordial follicle or a transitioning follicle as described above.

2.4.2 Prepubertal and Adult Offspring

At P27 and P120, H&E sections of ovaries were evaluated for follicle populations as previously described (Bernal, *et al.* 2010). In addition, the area (μm^2) of each secondary/antral follicle was measured using a Nikon DS-L3 Camera Control Unit (Figure 2.3).

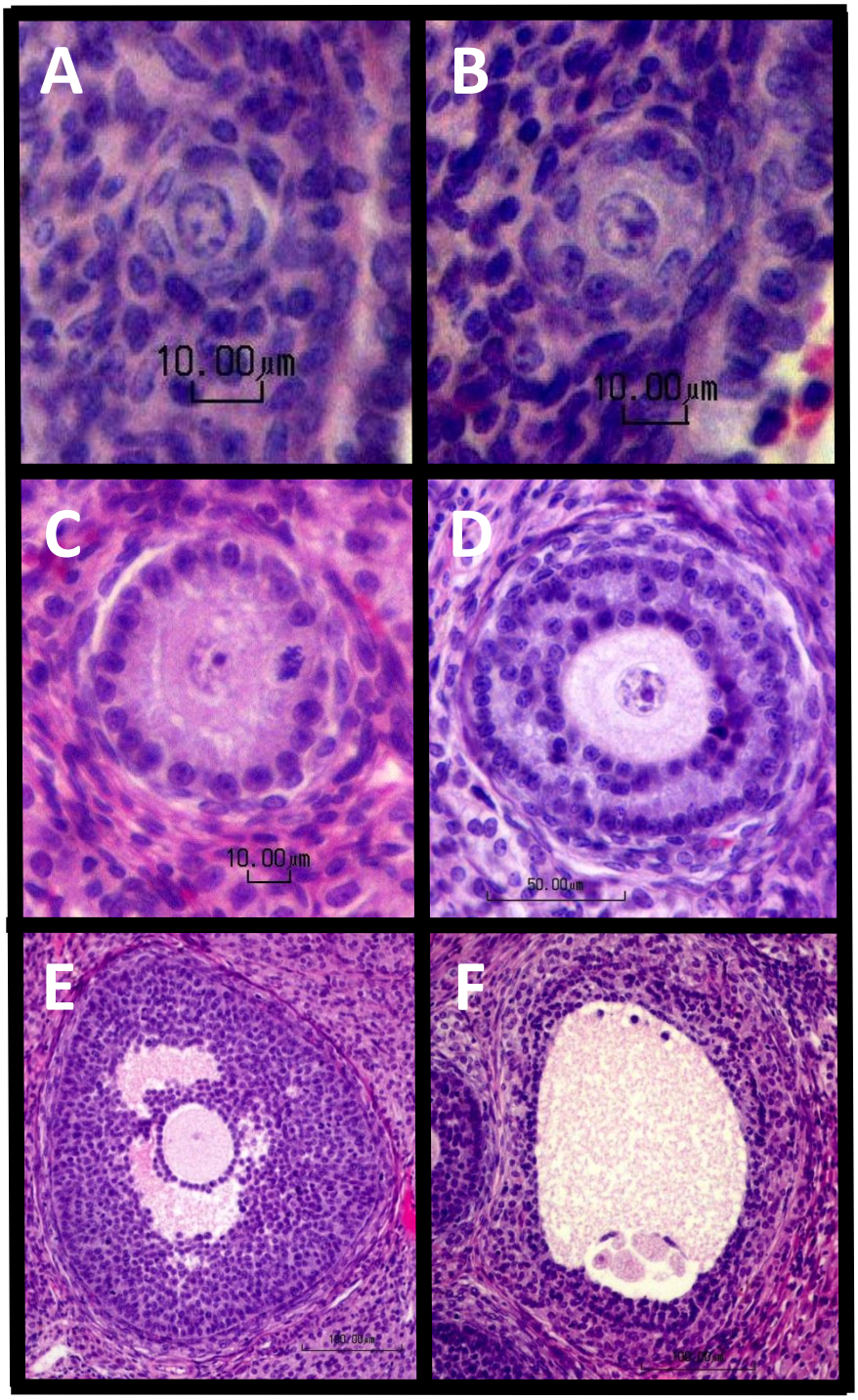

Figure 2.2 Ovarian follicle subtypes: Representative photographs of H+E stained ovarian sections demonstrating classes of follicle populations. Primordial (**A**), transitioning (**B**), primary (**C**), secondary (**D**), antral (**E**) and atretic antral (**F**) follicles. Images were captured using a Nikon Eclipse *Ci* microscope. Scale bars represent 10 (**A**,**B**,**C**), 50 (**D**), and 100 (**E**,**F**) µm.

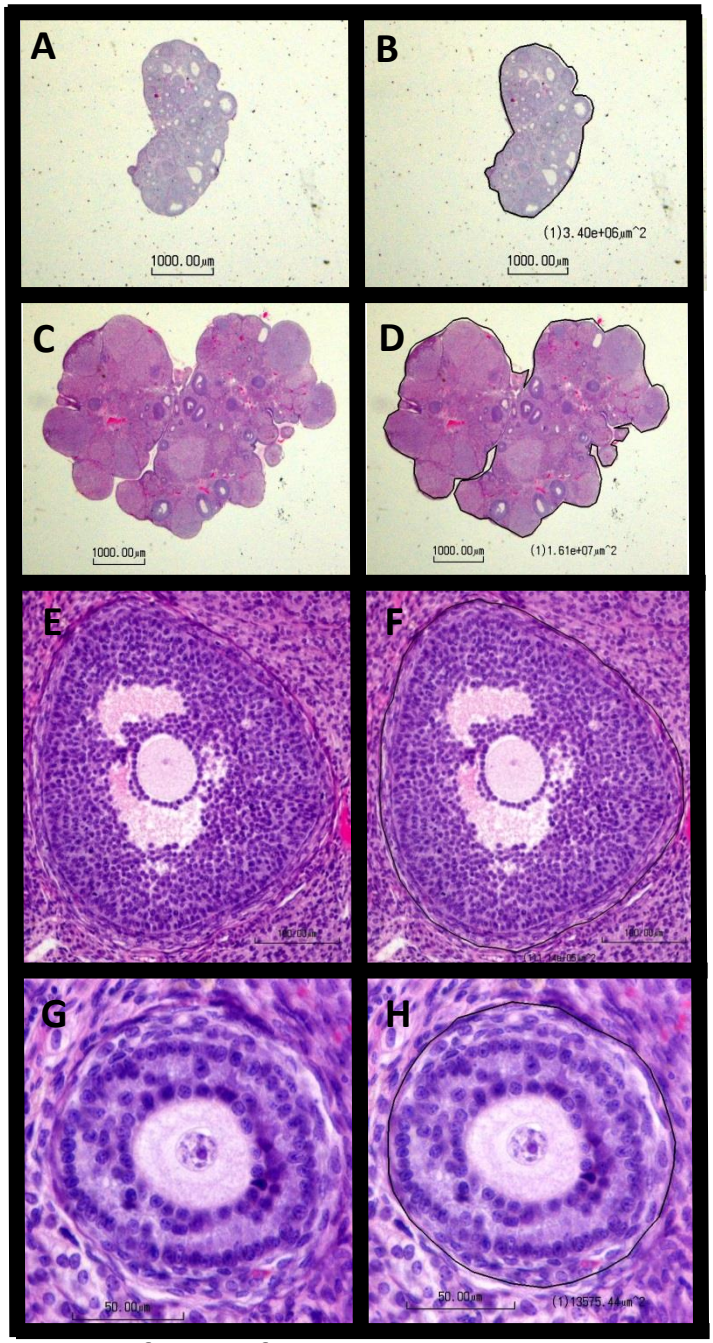

Figure 2.3 Determining of area of ovarian sections and secondary/antral follicle subtypes: Photographs of representative H&E stained P27 (**A**) and P120 (**B**) ovarian sections and those same sections with size measurement overlays (**B**,**D**). Antral (**E**) and secondary (**G**) follicles and those same follicles with size measurement overlays (**B**,**C**). Images were captured using a Nikon Eclipse *Ci* microscope and analyzed using a Nikon DS-L3 Camera Control Unit. Scale bars represent 1000 (**A**,**B**,**C**,**D**), 100 (**C**,**D**), and 50 (**G**,**H**) µm

2.5 Immunohistochemistry (IHC)

2.5.1 General Procedure

One ovarian section from each animal (5 animals per group per experiment) was depariffinized in xylene (3X5 minutes), rehydrated through an ethanol series (100% EtOH, 2X2 minutes; 90% EtOH, 2X2 minutes; 70% EtOH, 2 minutes) followed by 3 minutes in PBS and 10 minutes immersed in 1% H_2O_2 (Fisher Chemical, H325-500, Ottawa, Canada) in PBS to inhibit endogenous peroxidases. Ovarian sections were then washed in PBS (2X3 minutes) and incubated in Citrate Buffer (10 mM $Na_3C_6H_5O_7$; 0.05% Tween-20, pH 6.0) at 90°C for 12 minutes followed by 20 minutes at room temperature. Sections were then washed in PBS (2X3 minutes), blocked for 30 minutes with 5% bovine serum albumin (BSA) – fraction V (Fisher BioReagents™, BP1600-100, Ottawa, Canada) in PBS, and incubated overnight with primary antibody (Table 1) made up in 1% BSA overnight at 4°C in a humidity chamber. The next day ovarian sections were washed in PBS (2X3 minutes), incubated with a biotinylated secondary antibody (Table 1) in 1% BSA in PBS for 2 hours at room temperature in a humidity chamber. Sections were then washed in PBS (2X3 minutes), incubated with avidin/horseradish peroxidase (HRP) complex (1:200 (v:v) reagent A; 1:200 (v:v) reagent B; diluted in PBS) from VECTASTAIN® Elite ABC kit (Vector Laboratories, PK-6101, Burlington, Canada) for 1 hour at room temperature in a humidity chamber. Sections were then washed in PBS (2X3 minutes) and incubated with a 3,3'-Diaminobenzidine (DAB) (1mg/ml H_2O, 0.1% H_2O_2, DAB EASY tablets, Acros Organics, AC32800-5000, Geel, Belgium) for a set amount of time according to the primary antibody (Table 1). Following DAB incubation,

slides were washed in PBS (2X3 minutes), counterstained with Haematoxylin Stain 2-Gill Method (Fisher Chemical, CS-401-1D, Ottawa, Canada) for 12 seconds and rinsed under cold tap water until water was transparent. Sections were then dehydrated through an ethanol series (50% EtOH, 2X3 dips; 70% EtOH, 2X30 seconds; 90% EtOH, 1X1 minute; 100% EtOH, 2X3 minutes) followed by incubation in xylene (2X3 minutes) and application of a coverslip to each section on a slide using Permount™ Mounting Medium (Fisher Chemical, SP15-100, Ottawa, Canada) and dried overnight at room temperature. All experiments had one ovarian section (negative control) that underwent all steps except the primary antibody incubation (1% BSA in PBS overnight at 4°C in a humidity chamber instead).

Table 1

IHC primary/secondary antibody information, dilutions and DAB times

Age	Primary antibody (1°)	Company (Cat #)	1° dilution	Secondary antibody (2°)	Company (Cat #)	2° dilution	DAB time
P4	AMH	R&D Systems (AF1446)	1:50	Donkey anti-Goat IgG	Rockland (605-706-125)	1:1000	5 min
P4	AMHRII	R&D Systems (AF1618)	1:50	Donkey anti-Goat IgG	Rockland (605-706-125)	1:1000	5 min
P4	pAkt (Ser473)	Cell Signaling (193H12)	1:50	Goat anti-Rabbit IgG	VECTASTAIN (PK-6101)	1:200	5 min
P27	AMH	R&D Systems (AF1446)	1:300	Donkey anti-Goat IgG	Rockland (605-706-125)	1:1000	1.5 min
P27	AMHRII	R&D Systems (AF1618)	1:200	Donkey anti-Goat IgG	Rockland (605-706-125)	1:1000	5 min
P27	pAkt (Ser473)	Cell Signaling (193H12)	1:50	Goat anti-Rabbit IgG	VECTASTAIN (PK-6101)	1:200	5 min
P120	AMH	R&D Systems (AF1446)	1:200	Donkey anti-Goat IgG	Rockland (605-706-125)	1:1000	2.5 min
P120	AMHRII	R&D Systems (AF1618)	1:200	Donkey anti-Goat IgG	Rockland (605-706-125)	1:1000	3 min
P120	pAkt (Ser473)	Cell Signaling (193H12)	1:300	Goat anti-Rabbit IgG	VECTASTAIN (PK-6101)	1:200	5 min

Abbreviations: IHC, Immunohistochemistry; AMH, anti-mullerian hormone; AMHRII, type II anti-mullerian hormone receptor; pAkt (Ser473), phosphorylated Akt at Serine 473; IgG, immunoglobulin G; 1°, primary antibody; 2°, secondary antibody

2.5.2 Computer Image Analyses of AMH and AMHRII Immunostaining

2.5.2.1 Neonatal Offspring:

Ovarian section images were captured using a Nikon Eclipse *Ni* microscope at 20x magnification. Multiple images were stitched to obtain a higher quality image than a single image at lower magnification. The same microscope settings were used to capture all images. Density of oxidized DAB (brown colour) was determined by pixel classification in Nikon NIS-Elements AR 4.20.01 software. A region of interest (ROI) measurement was made around ovarian tissue in each section and the proportion of DAB immunopositive (brown) area was calculated ([# of positive pixels/(# of not positive pixels + # of positive pixels)] X 100% = % of positive immunostaining) (Figure 2.4). One ROI was made per section and there was one section per animal; therefore, there were 5 ROI density measurements per group.

2.5.2.2 Prepubertal and Adult Offspring:

In each ovarian section, the density of oxidized DAB (brown colour) in primary, secondary, and antral follicles was determined (Nikon Eclipse *Ni* microscope coupled with the pixel classifier in Nikon NIS-Elements AR 4.20.01 software). To do so, a ROI measurement was made around each follicle subtype and the proportion of DAB immunopositive (brown) area within that ROI was calculated ([# of positive pixels/(# of not positive pixels + # of positive pixels)] X 100% = % of positive immunostaining). This measurement was made for each follicle that had an oocyte within each section analysed (Figure 2.4). If multiple primary, secondary, or antral follicles were present in a

section, each follicle was measured and the mean was determined for each primary, secondary, or antral follicles for that section.

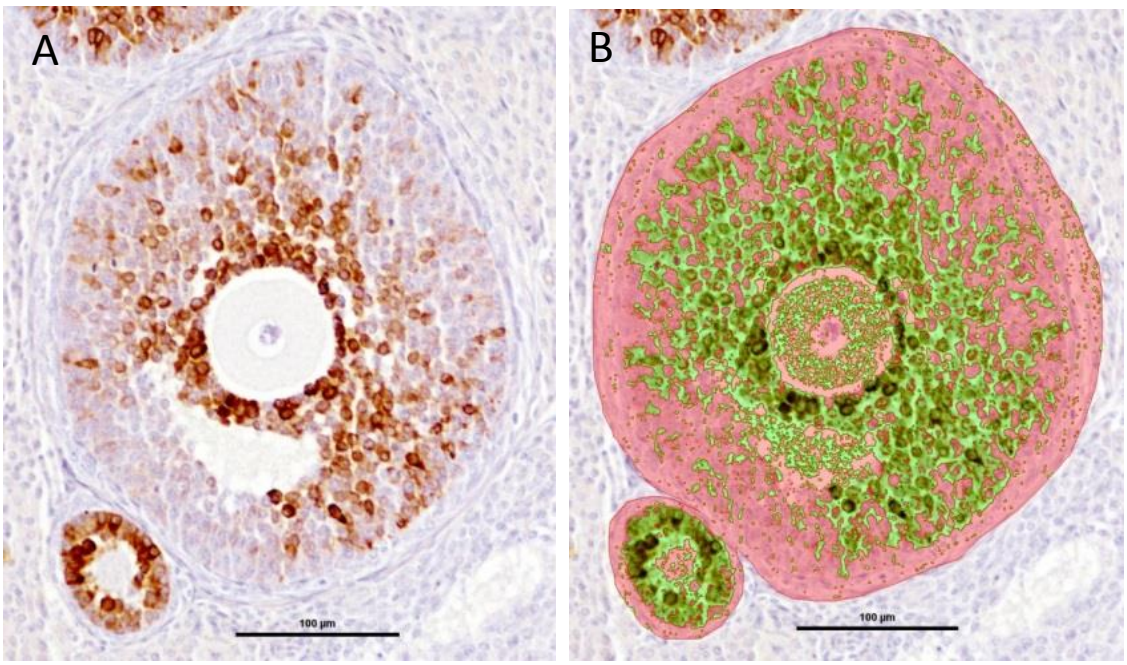

Figure 2.4 Example of density measurements of positive immunostaining within follicle subtypes: An antral and secondary follicle immunostained for AMH before (**A**) and after (**B**) pixel classification to determine proportion of positive (brown) immunostaining within each follicle. Images were captured using a Nikon Eclipse *Ni* microscope and images were classified as DAB positive (**B** – green) or not positive (**B** – red) utilizing Nikon NIS-Elements AR 4.20.01 software. Scale bars represent 100 µm.

2.5.3 Computer Image Analyses of Phosphorylated Akt and AMH/AMRII Immunostaining

2.5.3.1 Neonatal Offspring:

Primordial and transitioning follicles were classified according to Section 2.6 and counted for each section immunostained (5 per group). Each of these follicles were then scored as either having an immunopositive (brown colour) oocyte or not positive. The

percentage of positive pAkt (Ser473) oocytes within each follicle subtype was then calculated ([# of positive oocytes/(# of not positive oocytes + # of positive oocytes)] X 100% = % of oocytes positively immunostained) per group. Counts and scoring were blinded.

2.5.3.2 Prepubertal and Adult Offspring:

Primordial (P120), primary (P120), secondary (P27 and P120), and antral (P120) follicles were classified according to the methods outlined in Section 2.6 and counted for each section immunostained (5 per group). Each of these follicles were then scored as either having an immunopositive (brown colour) oocyte/follicle or not positive. The percentage of positive pAkt (Ser473) oocytes within each follicle subtype was then calculated ([# of positive oocytes/(# of not positive oocytes + # of positive oocytes)] X 100% = % of oocytes positively immunostained) per group. The percentage of positive AMH/AMHRII primordial follicles was also calculated ([# of positive follicles/(# of not positive follicles + # of positive follicles)] X 100% = % of follicles positively immunostained) per group. Counts and scoring were blinded.

2.6 Ovarian Gene Expression Analyses

2.6.1 Total RNA Extraction and Quantification

Total RNA was extracted from ~5 mg of frozen, ground ovarian tissue using the RNeasy® Mini kit (Qiagen, 74104, Hilden, Germany) with 350 µl of RLT buffer (1:100 (v:v) β-mercaptoethanol – Sigma-Aldrich, M3148, Canada) using the manufacturer's instructions. RNA quantity and purity were analyzed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Canada) using NanoDrop 2000/2000c software

(Thermo Scientific, Canada). The ratio of absorbance at 260 nm to 280 nm (A260/A280) and A260/A230 for each sample was >2.0 and >1.5, respectively. All RNA samples were stored at -80°C until required.

2.6.2 Complementary DNA (cDNA) Synthesis

Two μg of total RNA was used for first strand cDNA synthesis using the Superscript® VILO™ cDNA Synthesis Kit (Life Technologies, Canada) according to the manufacturer's instructions and using a standard thermocycler (Bio-Rad C1000 Touch).

2.6.3 Primer Design and Validation

Primer sets (Table 2) were designed using Primer-BLAST (NCBI) with the following settings changed from default (the rest being default): PCR product size – Min-50 base pairs (bp), Max-200 bp; Primer melting temperature (T_m) – Min-58°C, Opt-60°C, Max-62°C, Max T_m difference-2; Exon junction span – Primer must span an exon-exon junction. The final condition in the design of primers using Primer-BLAST ensured that genomic DNA was not amplified. Each primer set was then assessed for self and cross-dimerization by NetPrimer (Premier Biosoft, Palo Alto, US). Primers with ΔG (kcal/mol) values less than -10 for both self and cross-dimerization were excluded as potential primer sets for validation. Primer sets were then ordered from Life Technologies (Canada) with the following specifications: synthesis scale – 25 nmole; purification – Desalted. Stock primers were diluted to 100 μmole in H_2O. Primers were validated through dissociation analyses of melt curves utilizing a 10-fold serial dilution a pooled sample of ovarian cDNA created by adding 1 μl of each sample from the cDNA sysnthesis step to a

single tube. Only primer sets producing a single peak in the dissociation curves were used for quantification of unknown samples.

2.6.4 Quantitative Polymerase Chain Reaction (qPCR) assays

For the quantification of ovarian gene expression levels, quantitative PCR assays were performed using the LightCycler 480 II (Roche) in 384-well plates (LightCycler 480 Multiwell Plate 384 White – Roche, 04729749001, Canada). For every experiment, each well contained 2.5 µl of cDNA, 0.5 µl of 5 µM Forward Primer, 0.5 µl of 5 µM Reverse Primer, 1.5 µl of H_2O, and 5 µl of LightCycler 480 SYBR Green 1 Master (Roche, 04887352001, Canada). For all experiments, PCR cycling conditions were performed under the following conditions: enzyme activation at 95°C for 5 min (Stage 1); amplification of the gene product through 60 successive cycles of 95°C for 10 sec then 60°C for 10 sec then 72°C (Stage 2); followed by a melting curve starting at 65°C and ending at 95°C (Stage 3). Each plate for a gene of interest contained a standard curve (either 5 or 10-fold serial dilution) generated from a pooled sample of ovarian cDNA consisting of at least 5 standard curve points. Each standard curve point was run in triplicate and each standard curve had a negative control (H_2O). All samples were run in triplicate.

2.6.5 Analyses of qPCR Results

The crossing point (Cp) of each well was determined via the 2^{nd} derivative maximum method using LightCyler 480 Software Release 1.5.1.62 (Roche). An arbitrary concentration was assigned to each well based on the standard curve Cp values (and their corresponding arbitrary concentrations) and the geometric mean of the triplicate for each

sample was determined giving a concentration value for each sample. This value was calculated for every gene of interest in Table 2. The geometric mean of housekeeping genes Ywhaz, Ywhag, β-actin, B2M, SDHA, HPRT, and Cyclophilin for each sample was determined giving a grand reference gene value for each sample. Each sample's concentration for a particular gene of interest was then divided by it's grand reference gene value to obtain a normalized gene expression value for each sample. All values are expressed relative to the mean of the control group.

Table 2

Primer sequences and amplicon sizes for the genes of interest

Gene	Forward Primer	Reverse Primer	Amplicon length (bp)	GenBank Accession Number
Houskeepers				
Ywhaz	TTGTAGGAGCCCGTAGGTCA	AAAGAGACAGTACGTCGTTGC	152	NM_013011.3
Ywhag	TTCCTAAAGCCCTTCAAGGCA	GGCTTTCTGCACTAGTTGGTCG	101	NM_019376.2
β-actin	CACTTTCTACAATGAGCTGCGTGT	CTGGATGGCTACGTACATGGCT	148	NM_031144.3
B2M	AATTCACACCCACCGAGACC	GCTCCTTCAGAGTGACGTGT	63	NM_012512.2
SDHA	TGGGGCGACTCGTGGCTTTC	CCCCGCCTGCACCTACAAGC	134	NM_130428.1
HPRT	GCAGTACAGCCCCAAAATGG	GGTCCTTTTCACCAGCAAGCT	52	NM_012583.2
Cyclophilin	CCGCTGTCTCTTTTCGCC	GCTGTCTTTGGAACTTTGTCTGC	129	NM_017101.1
Receptors				
PR	CATGGTCCTTGGAGGTCGTAA	TGAGCAGGTTGATGAGTGGC	183	NM_022847.1
AR	TGACTCTGGGAGCTCGTAAGC	TGGAGTTTTCTCCTTCTTCCTGTAG	70	NM_012502.1
ER-α	TCCTGGACAAGATCAACGACACT	TGCAGAGTCAGGCCAGCTT	61	NM_012689.1
ER-β	CCTGCCGACTTCGCAAGT	CCACACCGTTCTCTCCTGGAT	67	NM_012754.1
FSHR	TTCAACGGAACTCAGCTAGATG	TGGTTTGGTAAGGAATGGACC	145	NM_199237.1
LHR	TATGCTCGGAGGATGGCTCT	AGCACAGATGACGACGAAGG	175	NM_012978.1
Steroidogenic enyzmes				
StAR	TATTGACCTCAAGGGGTGGC	CAAGTGGCTGGCGAACTCTA	82	NM_031558.3
Cyp11a1	TCCTCTACCAACAGTCCTCGAT	TGCCCAGCTTCTCCCTGTAAAT	166	NM_017286.2
Cyp19a1	ATTGGCATGCACGAGAATGG	TGCTGCTTGATGGATTCCAC	139	NM_017085.2
Cyp17a1	TCTGTGCTATCTGCTTCAACATCT	GCCTTTGTTGGGAAAAATCGTC	149	NM_012753.2
3βHSDI	TCATGATACTTGCGGCCCTC	AGCCAGAATATGTGCCCAGG	118	NM_001007719.3
17βHSD1	CGGGATCTGAAGTCACAGGG	GCGTCCAGCATTACAGACCAG	171	NM_012851.2
20αHSD	ATCCCTGTACTGGGCTTTGG	TTTTCTGAGATTCTCTTCGGTTGC	51	NM_138510.1
TGFB				
AMH	GACCTCAAGACCTAGCCACC	CTCCCATATCACTTCAGCCAGA	143	NM_012902.1
AMHRII	AGCTGCACAAGTATCCCCAAA	GGCATCCTTGCATCTCCACT	201	NM_030998.1
GDF9	CAACCAGATGACAGGACCCC	CACAGTGGAGGAGGAAGCAG	133	NM_021672.1
BMP15	TGATAAAGCCGTCAGCCAGT	TCTGTATATGCCAAGGACCTCT	57	NM_021670.1
Apoptotic factors				
Bax	AGAGGATGATTGCTGATGTGGAT	GCTGCCACACGGAAGAAGA	61	NM_017059.2
Bcl-2	GATGACTGAGTACCTGAACCG	CAGAGACAGCCAGGAGAAATC	124	NM_016993.1

Abbreviations: Ywhaz, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta; Ywhag, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma; B2M, β-2-microglobulin; SDHA, succinate dehydrogenase complex, subunit A, flavoprotein (Fp); HPRT, hypoxanthine-guanine phosphoribosyltransferase; PR, progesterone receptor; ER, estrogen receptor; FSHR, follicle-stimulating hormone receptor; LHR, luteinizing hormone receptor; StAR, steroidogenic acute regulatory protein; Cyp11a1, cytochrome P450, family 11, subfamily A, polypeptide 1; Cyp19a1, cytochrome P450, family 19, subfamily A, polypeptide 1; Cyp17a1, cytochrome P450, family 17, subfamily A, polypeptide 1; 3βHSDI, 3 beta-hydroxysteroid dehydrogenase/delta-5-delta-4 isomerase type I; 17βHSD1, 17β-Hydroxysteroid dehydrogenase 1; AMH, anti-mullerian hormone; AMHRII, anti-mullerian hormone receptor type II; GDF9, growth differentiation factor 9; BMP15, bone morphogenetic protein 15; Bax; Bcl-2 associated X protein; Bcl-2, B-cell lymphoma 2; bp, base pairs

2.7 Immunoblotting

2.7.1 Ovarian Tissue Protein Extraction

Total protein was extracted from ~5 mg of ground frozen ovarian sample. Tissues were homogenized using a pellet pestle (Fisherbrand™, 12-141-364, Canada) with 200 µl of ice-cold potassium phosphate homogenization buffer (50 mM KH_2PO_4/K_2HPO_4, 1 mM EDTA, 0.1 mM DTT, pH7.4) supplemented with cOmplete Mini, EDTA-free protease inhibitor cocktail (Roche, 04693159001, Canada). Lysates were passed through a 20-gauge needle and syringe 30 times and then placed on ice before being frozen at -20°C and thawed. Lysates were centrifuged at 700 *g* for 15 min at 4°C to pellet cellular debris, the supernatant was transferred to a separate tube and total protein content of samples was quantified using a commercially available bicinchoninic acid (BCA) kit (Pierce). Emission absorbance values were detected at 562 nm on a Synergy™ H4 Hypbrid Multi-Detection Microplate Reader using Gen5™ software (Bio-Tek®).

2.7.2 Western Blotting

Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (12.5%) were cast using the Mini-PROTEAN® Tetra Cell (Bio-Rad, 165-8000) handcasting system. The separating phase (12.5% (v/v) acrylamide (Bio-Rad, 161-0156); 375 mM Tris-HCl, pH 8.8; 0.1% (w/v) SDS (BioShop, SDS001, Canada); 0.1% (w/v) ammonium persulfate (APS; Bio-Rad, 161-0700); 0.08% (v/v) Tetramethylethylenediamine (TEMED; Bio-Rad, 161-0801)) was poured, covered in 100% EtOH and allowed to polymerize for 30 minutes at room temperature. The stacking phase (4% (v/v) acrylamide; 125 mM Tris-HCl, pH 6.8; 0.1% (w/v) SDS; 0.1% (w/v) APS; 0.1%

(v/v) TEMED) was poured over the separating phase and allowed to polymerase with a 15-well comb. Gels were stored at 4°C in a Ziploc bag covered in damp paper towels or used immediately.

Five µg of ovarian lysate in 1XLDS Sample Buffer (NuPAGE®) with 50 mM DTT (Bio-Rad, 161-0611, Canada) was heated to 70°C for 10 min and then resolved on 12.5% SDS-PAGE gels at 150V and transferred to polyvinylidene difluoride (PVDF; Bio-Rad, 162-0177, Canada) membrane by wet transfer at 100V for 2 hours on ice. Membranes were blocked in 5% BSA in Tris buffered saline (TBST: 150 mM NaCl, 20 mM Tris Base, pH 7.6) supplemented with 0.1% Tween-20 (SOD2 and CAT) or 1% milk in TBST (4-hydroxynonenal (4-HNE)) for 1 hour at room temperature. Membranes were then incubated with anti-4-HNE (1:1000; R&D Systems MAB3249), anti-SOD2 (1:2000; abcam ab13534), or anti-CAT (1:2000; abcam ab16731) in 5% BSA in TBST for 16 hours at 4°C followed by 3 rinses in TBST and then 4X5min TBST washes. 4-HNE membranes were then incubated with anti-mouse IgG secondary antibody (1:5000; Jackson ImmunoResearch Code # 715-035-151) for 1 hour in 1% milk/TBST and then washed with TBST. SOD2 and CAT membranes were incubated with anti-rabbit IgG secondary antibody (1:10,000; Jackson ImmunoResearch Code # 711-035-152) for 1 hour in 5% BSA/TBST and then washed with TBST. Blots were developed using Bio-Rad Clarity™ Western Enhance chemiluminescence (ECL; Bio-Rad, 170-5061), images captured using Bio-Rad ChemiDoc MP System and densitometric quantification was carried out using ImageLab Software (Bio-Rad). Following band detection, 4-HNE membranes were stripped using Restore Western Blot Stripping Buffer (Thermo

Scientific, 21059) for 10 min at room temperature on a rocker and then washed before blocking for 1 hour in 5% BSA/TBST. Membranes were then incubated with anti-β-Actin-Peroxidase (1:40,000; Sigma-Aldrich, A3854) in 5% BSA/TBST for 1 hour at room temperature and then washed in TBST prior to band detection as above.

2.8 Ovarian Levels of Protein Carbonyl, TNF, and IL6 ELISA:

Ovarian levels of protein carbonyls, TNF, and IL6 were measured by commercially available rat-ELISA kits (OxiSelect Protein Carbonyl ELISA kit. Cellbio Labs Inc., STA-310, San Diego, US; TNF; R&D Systems, DuoSet DY510, Minneapolis, US; IL-6, R&D Systems, DuoSet DY506. Minneapolis, US). Amount of protein loaded into each well was 0.1, 10, and 10 μg, respectively. Dilutions were made as per manufacturer's instructions. Incubation times with TMB substrate were 10, 20, and 20 minutes, respectively. All samples were run in duplicate and the mean value was reported.

2.9 Statistical Analyses:

For all analyses sample sizes represent litters (dams) as one litter is considered one biological replicate. Maternal gestation and lactation weight gain and food intake was analyzed via two-way repeated measures ANOVA, with maternal diet and age as factors. Bonferroni *post-hoc* analyses were used for multiple comparisons when appropriate. Dam retroperitoneal fat mass, P4/P27 follicle counts, P4/P27 AMH/AMHRII image analyses, P27 circulating factors, and P27 mRNA/protein expression were analyzed by unpaired Student's t-tests. P120 follicle/CL counts, P120 AMH/AMHRII image analyses, P120

circulating factors, and P120 mRNA/protein expression were analyzed by two-way factorial ANOVA, with maternal diet and postweaning diet as factors. Tukey's *post-hoc* analyses were made for two-way factorial ANOVA. In all cases, data that were not normally distributed were log transformed in order to achieve normality. All data are presented as box plots, where the box represents 25^{th} and 75^{th} percentiles (the lower and upper quartiles, respectively), and within which is shown the 50^{th} percentile (the median). The whiskers indicate the upper and lower values not classified as statistical outliers by Grubb's test (GraphPad QuickCalcs – free online). All data in tables and text are presented as mean ± SEM relative to the mean of the control group. Sex ratio and P4/P27 pAkt (Ser473) oocyte immunostaining were analyzed by binary logistic regression with maternal diet as the predictor variable. P120 pAkt (Ser473) oocyte immunostaining and P120 AMH/AMHRII primordial follicle immunostaining were analyzed by binary logistic regression with maternal diet and postweaning diet as predictor variables. A p-value of <0.05 was considered statistically significant. All analyses were performed using SigmaPlot 12.0 (Systat Software Inc., San Jose, CA, USA), GraphPad Prism 5.01 (GraphPad Software Inc., La Jolla, CA, USA), and PASW Statistics 18 (SPSS Inc., Quarry Bay, HK).

3.0 Results:

3.1 Maternal Outcomes

Consistent with previously validated rat models (Connor, *et al.* 2012) of maternal obesity, there was a significant main effect of maternal diet on weight gain during pregnancy and lactation ($p<0.0001$) such that HF-fed dams were heavier than controls from day 10-26 ($p<0.05$) based on Bonferroni *post-hoc* analysis (Figure 3.1A). Interestingly, there was also a significant main effect of maternal diet on energy (kcal) consumption during pregnancy and lactation ($p<0.0001$) such that HF-fed dams reduced food intake overall during pregnancy (Figure 3.1B); however, *post-hoc* analysis revealed no statistically significant differences between groups at any specific day of pregnancy or lactation. At weaning, dams fed a HF-diet throughout pregnancy and lactation had a greater retroperitoneal fat mass ($p<0.01$) compared to control dams (Figure 3.1C). Gestation length did not differ between groups (CON: 22.80±0.14 days, HF: 22.67±0.19, $p=0.57$)

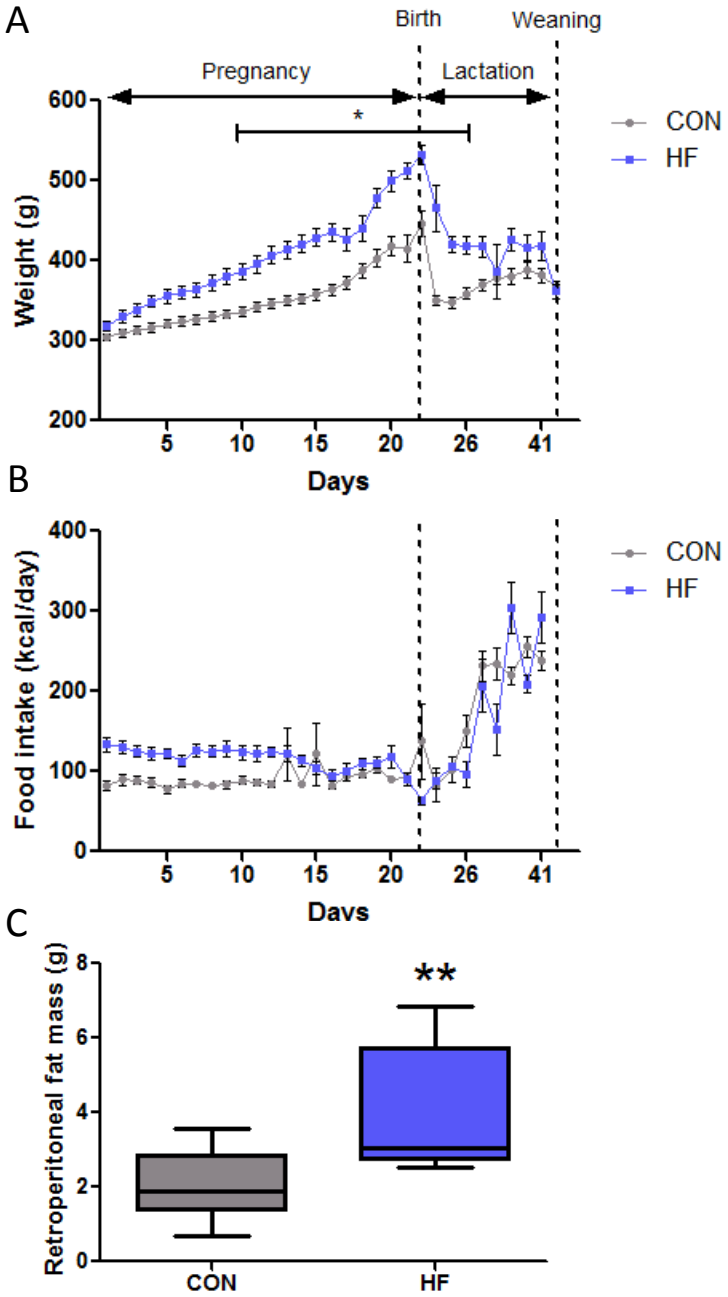

Figure 3.1 Dams fed a HF diet weighed more during pregnancy and lactation, reduced energy consumption throughout gestation, but had greater fat mass at weaning: Data (**A,B**) were analyzed using a two-way repeated measures ANOVA. Diet effect $p<0.0001$; Age (days) effect $p<0.0001$; Interaction (diet X age) $p<0.0001$ for both weight gain and food intake. Bonferroni *post-hoc* analyses revealed HF dams were heavier compared to CON dams from day 10-26, $p<0.05$. Retroperitoneal fat mass was significantly elevated in rat dams at weaning (**C**). Data were analyzed using an unpaired Student's t-test. $^{**}p<0.01$.

3.2 Offspring Outcomes

3.2.1 Neonates

The number of live pups delivered per litter did not differ statistically between groups (CON: 15.00±0.46, HF: 16.78±0.89, p=0.060). However, offspring born to HF fed mothers weighed less at birth compared to CON offspring (CON: 6.43±0.15 grams, HF: 5.91±0.09, p=0.033). Binary logistic regression analysis demonstrated that maternal diet did not influence sex ratio (B=-0.199, p=0.320, Odds Ratio=0.819).

3.2.1.1 Neonatal Oocyte/Follicle Populations

Oocytes were counted at postnatal day (P)4 in offspring to determine the primordial follicle reserve. P4 is the approximate time when the primordial follicle pool is established in rats (Kezele & Skinner. 2003, Kezele, *et al.* 2005, Nilsson, *et al.* 2011). At this time, offspring ovaries demonstrated oocytes that had not yet assembled into follicles and remained in oocyte nests as well as primordial follicles and transitioning follicles (Figure 3.2A). Although neonates born to HF fed dams had similar total numbers of oocytes compared to CON neonates, HF neonates had a significantly higher proportion of oocytes assembled into follicles compared to CON neonates (Figure 3.2B,C). Thus, not surprisingly, HF neonates had more primordial follicles present at this age (CON: 1.00±0.10, HF: 1.56±0.19, p=0.020; Figure 3.2D). The number of transitioning follicles did not statistically differ between groups (CON: 1.00±0.18, HF: 1.44±0.20, p=0.13; Figure 3.2E).

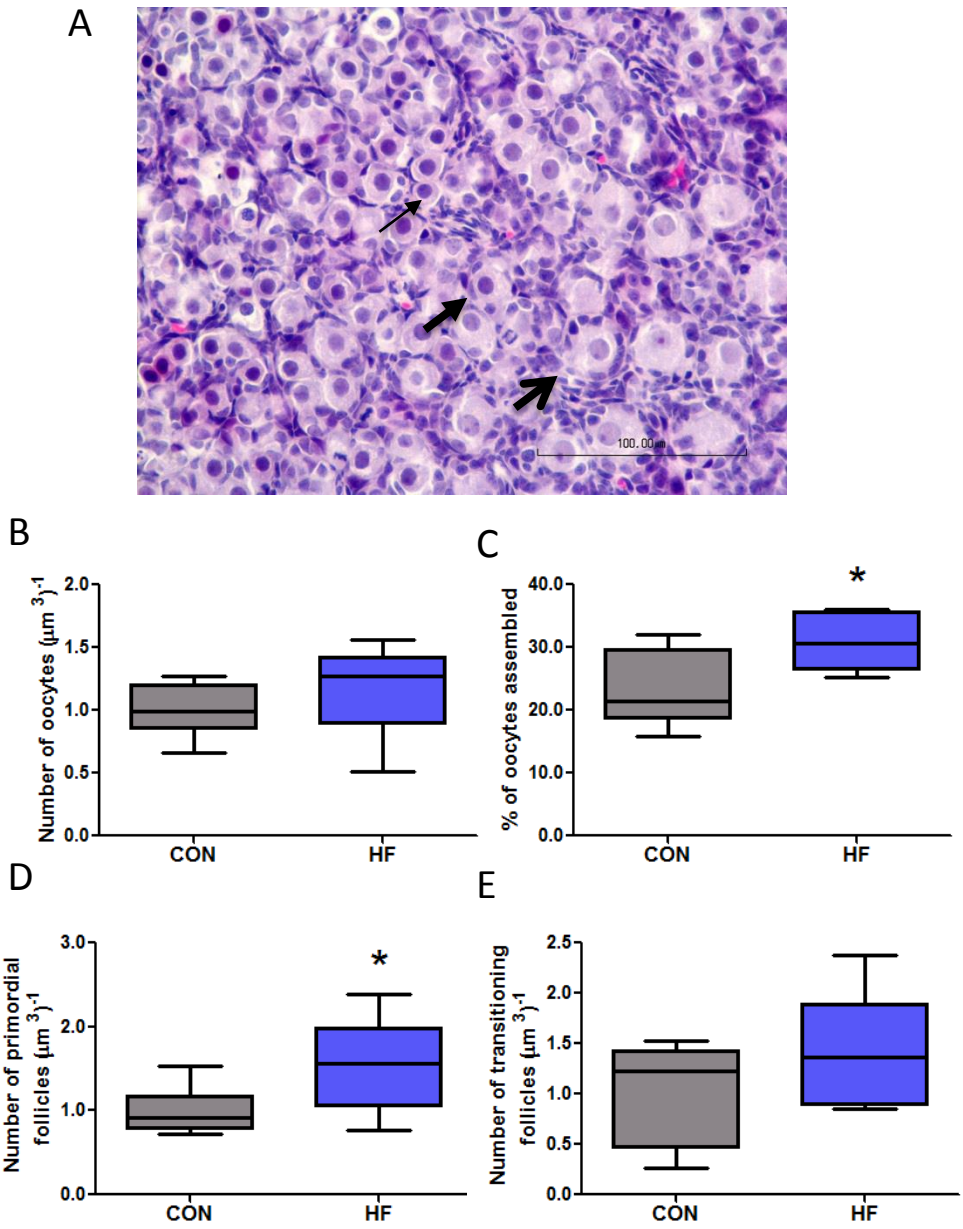

Figure 3.2 Maternal HF diet intake significantly increased the number of primordial follicles assembled at P4: A representative photomicrograph of a hematoxylin and eosin stained ovary from P4 offspring (**A**) showing unassembled oocytes (→), assembled primordial follicles (➡), and assembled transitioning follicles (➔). The total number of oocytes, primordial, and transitioning follicles were normalized to total volume (μm^3) of ovarian tissue counted per animal and expressed relative to the CON group. The total number of oocytes was not different between groups (**B**), however, the percentage of oocytes assembled into follicles (**C**). The number of primordial follicles was increased in HF neonates (**D**). There was no difference in transitioning follicles (**E**). n=8 per group, *$p<0.05$, Student's t-test. Image was captured using a Nikon Eclipse *Ci* microscope. Scale bar represents 100 µm. Abbreviations: CON, control offspring; HF, offspring of HF fed dams.

3.2.1.2 Neonatal Immunolocalization of AMH and AMH Receptor

In control offspring, AMH immunolocalized in granulosa cells of primordial and transitioning follicles. Little positive immunostaining was observed in unassembled oocytes and oocytes within primordial and transitioning follicles (Figure 3.3A). AMHRII was strongly immunolocalized to oocytes of primordial and transitioning follicles. Little positive immunostaining was observed in unassembled oocytes and granulosa cells of primordial and transitioning follicles (Figure 3.3B). The expression patterns of both AMH and AMHRII were almost completely absent in offspring born to HF-fed dams (Figure 3.3C,D). Computer image analysis revealed that the proportion of immunopositive AMH ([# of positive pixels/(# of not positive pixels + # of positive pixels)] X 100% = % of positive immunostaining) ovarian tissue was reduced in HF P4 offspring compare to CON offspring (Figure 3.3G). Similarly, the proportion of immunopositive AMHRII ovarian tissue was significantly less in HF P4 offspring compare to CON offspring (Figure 3.3H). A significantly positive correlation ($r=0.97$; $p<0.0001$) existed between immunopositive AMH and AMHRII.

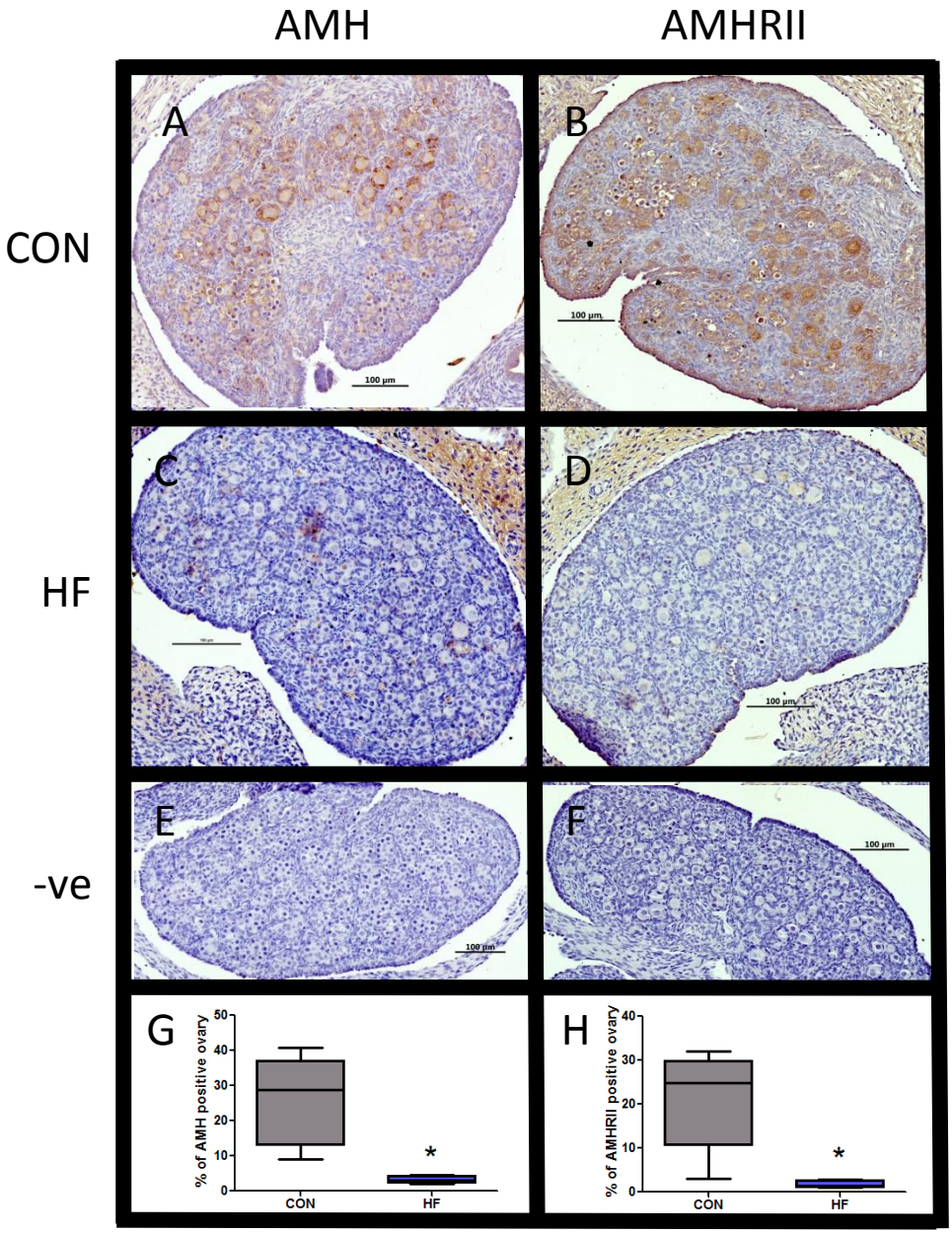

Figure 3.3 Maternal HF diet intake significantly decreased ovarian immunostaining of AMH and AMHRII in neonates at P4: Representative photomicrographs of ovarian sections from P4 offspring born to mothers fed a control (CON) (**A**,**B**) or high fat (HF) (**C**,**D**) diet with corresponding negative controls (**E**,**F**) showing immunolocalization of AMH (**A**,**C**) and AMHRII (**B**,**D**). Proportion of immunopositive staining of AMH (**G**) and AMHRII (**H**), *p<0.05, Student's t-test, n=5 per group. Images were captured using a Nikon Eclipse *Ni* microscope and pixels of 20x stitched images were classified utilizing Nikon NIS-Elements AR 4.20.01 software as outlined in Figure 2.4. Scale bars represent 100 µm.

3.2.1.3 Neonatal Immunolocalization of Phosphorylated Akt

Phosphorylated Akt at Serine 473 (pAkt(Ser473)) was localized to the oocytes of primordial and transitioning follicles with little immunostaining observed in unassembled oocytes and granulosa cells (Figure 3.4A). There was a qualitative decrease in the intensity of pAkt (Ser473) immunostaining in all oocytes in HF offspring compared to CON. Blinded scoring revealed that the proportion of primordial follicles that were positively immunostained for pAkt (Ser473) was reduced in HF ovaries compared to CON (Figure 3.4B). Immunostaining appeared similar between groups in transitioning follicles (Figure 3.4C). Binary logistic regression analysis demonstrated that maternal diet influenced whether or not neonatal ovaries had immunopositive pAkt (Ser473) in their primordial follicles ($B=-0.858$, $p<0.0001$), but not in transitioning follicles ($p=0.346$, Odds Ratio=1.475). Specifically, HF offspring were less likely to show positively immunostained pAkt (Ser473) in primordial follicles compared to CON offspring (Odds Ratio=0.424).

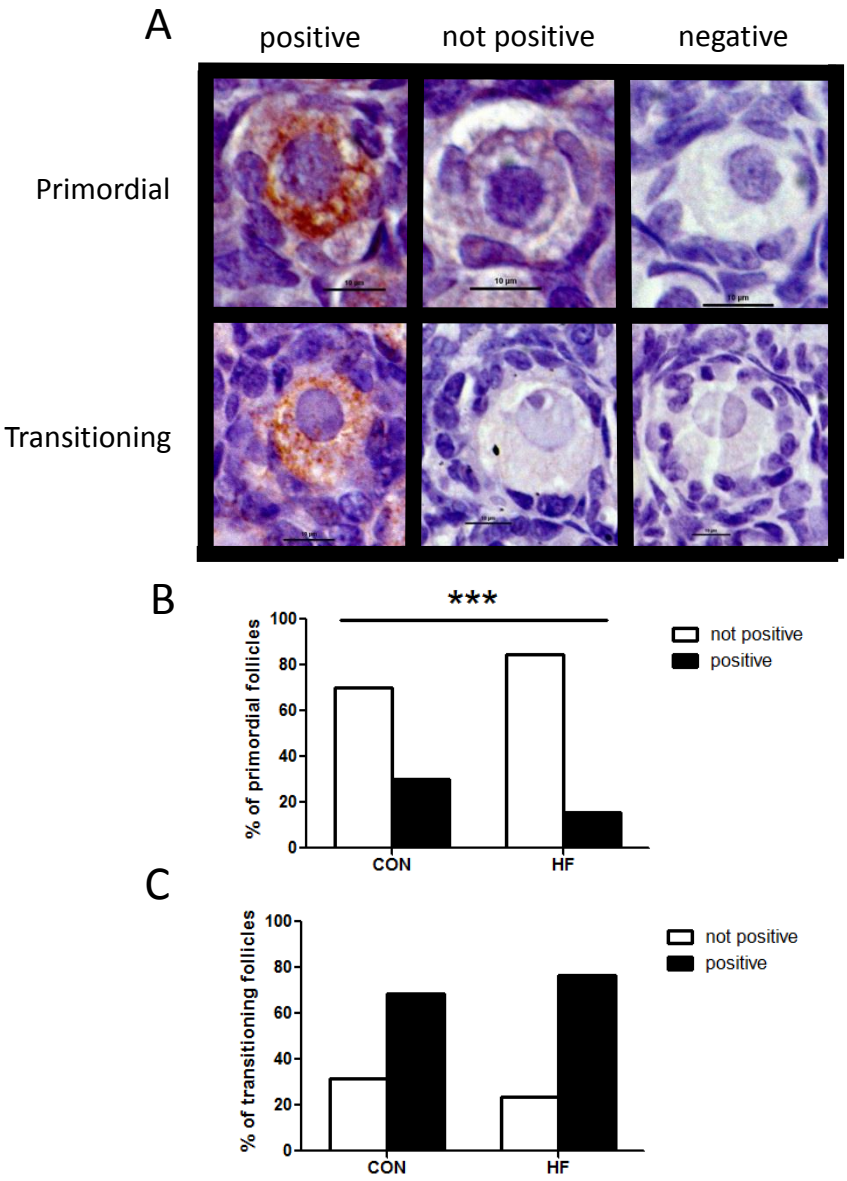

Figure 3.4 P4 offspring born to HF-fed dams are less likely to have primordial follicles positively stained for pAkt (Ser473): Representative photomicrographs of primordial and transitioning follicles immunostained for pAkt (Ser473) (**A**). Proportion of primordial (**B**) and transitioning (**C**) follicles positively immunostained for pAkt (Ser473) ***p<0.0001, Binary logistic regression. Binary logistic regression analyses demonstrated a decreased odds (OR=0.424) of positive pAkt (Ser473) immunostaining in primordial follicles of HF offspring, but not in transitioning follicles. Images were captured using a Nikon Eclipse *Ni* microscope. Scale bars represent 10 μm.

3.2.2 Prepubertal Outcomes

3.2.2.1 Prepubertal Follicle Populations

Primordial, transitioning, primary, secondary, and antral follicle numbers (Table 3) were similar between groups. However, there was a significant increase in the number of type II atretic follicles in HF offspring compared to CON (Table 3). Moreover, the size of secondary follicles was significantly smaller in offspring born to HF fed dams compared to CON (CON: 18720±717 μm^2, HF: 16150±859, p=0.041). There was no statistical difference in the size of antral follicles between groups (CON: 87140±5363 μm^2, HF: 77250±7095, p=0.28).

Table 3
Prepubertal follicle populations

	Group		
Type	CON	HF	p value
Primordial	1.00±0.11	0.88±0.11	0.46
Transitioning	1.00±0.11	0.93±0.19	0.76
Primary	1.00±0.07	0.84±0.09	0.2
Secondary	1.00±0.07	1.11±0.10	0.39
Antral	1.00±0.08	1.34±0.22	0.15
Type II Atretic	1.00±0.14	1.49±0.16	0.038

Data are presented as mean±SEM expressed as fold changes relative to CON offspring. p value is derived from Student's unpaired t-test, n=6-7 per group. Abbreviations: CON, control offspring; HF, offspring of HF-fed dams.

3.2.2.2 Prepubertal Plasma Gonadotropins, E_2, and AMH Concentrations

Although circulating concentrations of plasma FSH, LH and AMH were similar between groups, there was a significant decrease in plasma concentrations of E_2 in HF offspring compared to CON (Table 4).

Table 4
Circulating gonadotropins, E_2, and AMH

	Group		
Factor	CON	HF	p value
FSH (ng/ml)	8.70±1.13	8.37±1.49	0.86
LH (ng/ml)	22.64±1.57	18.97±1.18	0.096
E_2 (pg/ml)	8.67±1.34	4.98±0.43	0.022
AMH (ng/ml)	15.79±1.73	17.99±1.62	0.37

Data are presented as mean±SEM. p value is derived from Student's unpaired t-test, n=6-7 per group. Abbreviations: CON, control offspring; HF, offspring of HF-fed dams; FSH, follicle stimulating hormone; LH, luteinizing hormone; E_2, 17-β-Estradiol; AMH, anti-mullerian hormone.

3.2.2.3 Prepubertal Immunolocalization of AMH and AMH Receptor

AMH and AMHRII were immunolocalized to primary, secondary, and antral follicles with no detectable immunostaining observed in primordial or atretic follicles. AMH was highly expressed in the granulosa cells of primary, secondary, and antral follicles with little staining observed in oocytes (Figure 3.5).

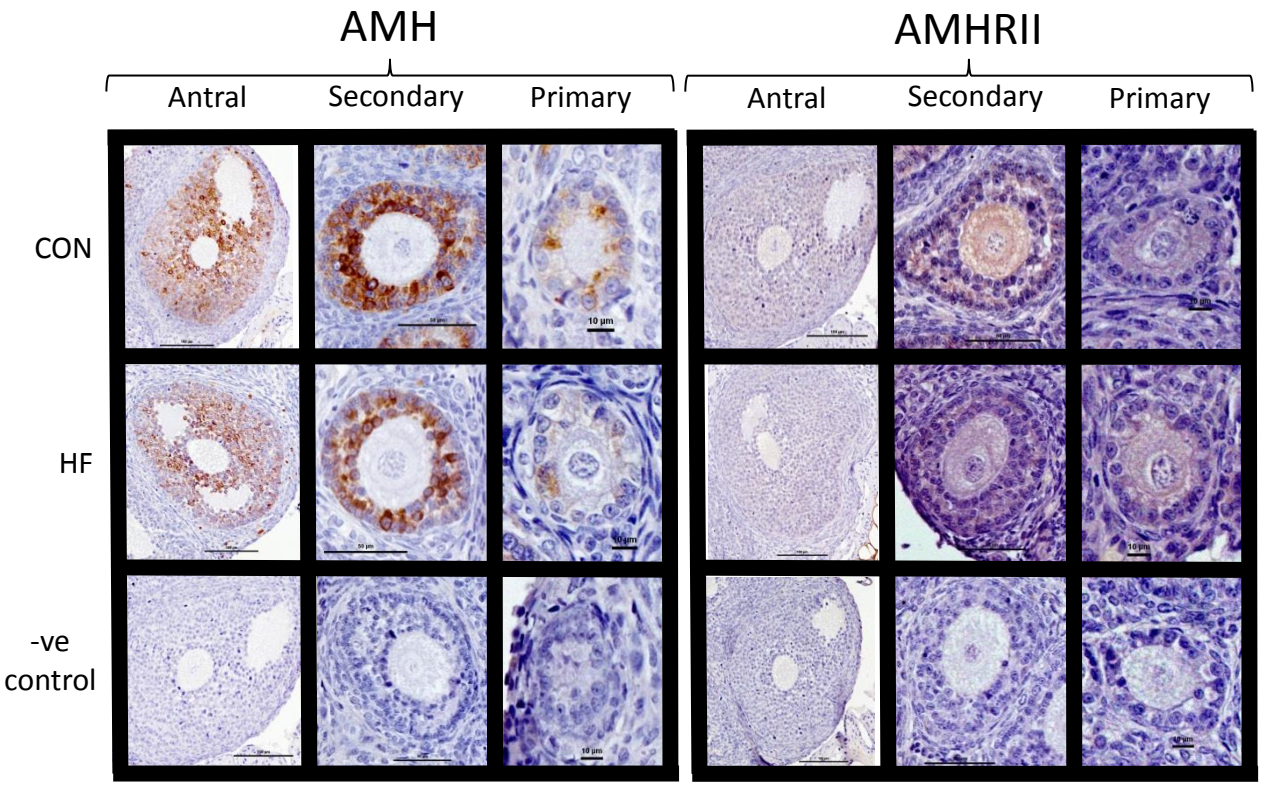

Figure 3.5 Immunolocalization of AMH and AMHRII in antral, secondary, and primary follicles in P27 offspring: Representative photomicrographs of each follicle subtype within each group. Images were captured using a Nikon Eclipse *Ni* microscope. Scale bars represent 100 µm, 50 µm, and 10 µm for antral, secondary, and primary, respectively. Abbreviations: CON, control offspring; HF, offspring of HF-fed dams; AMH, anti mullerian hormone; AMHRII, anti mullerian hormone type II receptor.

Consistent with previous reports (Durlinger, *et al.* 2002, Weenen, *et al.* 2004b), antral follicles showed diminishing immunoreactive AMH with increasing size and correlation analysis demonstrated that this relationship was significant ($r=-0.77$; $p<0.01$). Proportions of positive AMH immunostaining within antral (CON: 20.05±4.29%, HF: 28.05±2.36, $p=0.14$) and secondary (CON: 23.03±2.82%, HF: 19.70±4.53, $p=0.55$) follicles were similar between groups. However, there was a statistically significant decrease in the proportion of positive AMH immunostaining in primary follicles in HF offspring compared to CON (CON: 21.10±4.13%, HF: 8.40±3.33, $p=0.044$).

AMHRII showed a similar localization profile as AMH. AMHRII was localized within granulosa cells of primary, secondary, and antral follicles (Figure 3.5). Similar to AMH, immunopositive AMHRII declined with increasing size in antral follicles ($r=-0.67$; $p<0.05$). Proportions of positive AMHRII immunostaining within antral (CON: 6.87±3.40%, HF: 5.69±0.63, $p=0.77$), secondary (CON: 9.80±4.09%, HF: 6.55±1.44, $p=0.47$) and primary (CON: 14.39±8.12%, HF: 8.31±2.65, $p=0.46$) follicles were similar between groups.

3.2.2.4 Prepubertal Immunolocalization of Phoshorylated Akt

Immunopositive pAkt (Ser473) was only detected in oocytes of secondary follicles (Figure 3.6A) with a large proportion of oocytes in secondary follicles showing little to no detectable positive immunostaining. HF offspring showed a decreased proportion of secondary follicle oocytes positively immunostained for pAkt (Ser473) compared to CON offspring (CON: 49.47%, HF: 34.78; Figure 3.6B). Binary logistic regression analysis demonstrated that maternal diet influenced whether or not prepubertal

ovaries had positive pAkt (Ser473) in their secondary follicle oocytes (B=-0.65, p=0.030). HF offspring were less likely to have positively immunostained pAkt (Ser473) oocytes within secondary follicles than CON offspring (Odds Ratio=0.522).

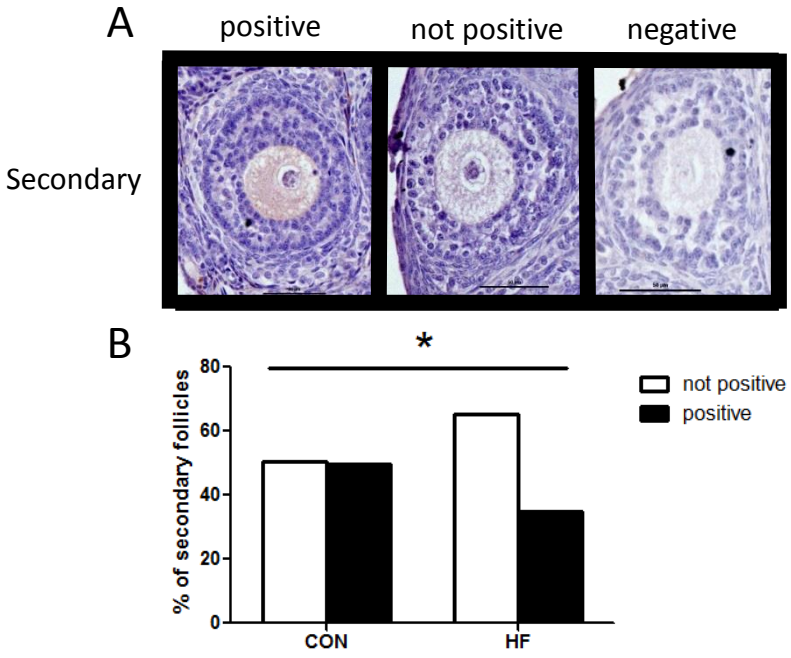

Figure 3.6 P27 offspring born to HF-fed dams are less likely to have secondary follicle oocytes positively stained for pAkt (Ser473): Representative photomicrographs (**A**) of positive, not positive, and negative secondary follicle oocytes. Proportion (**B**) of secondary follicle oocytes positively immunostained for pAkt (Ser473) *p<0.05, Binary logistic regression. Binary logistic regression analysis demonstrated a decreased odds (OR=0.522) of positive pAkt (Ser473) immunostaining in oocytes of secondary follicles from HF offspring. Images were captured using a Nikon Eclipse *Ni* microscope. Scale bars represent 50 μm. Abbreviations: CON, control offspring; HF, offspring of HF-fed dams.

3.2.2.5 Prepubertal Gene Expression Analysis

No statistically significant difference in relative mRNA levels of ProgR, AR, ER-α, ER-β, FSHR, LHR, StAR, Cyp11a1, Cyp19a1, Cyp17a1, 3βHSDII, 17βHSD1, 20αHSD, AMH, AMHRII, GDF9, BMP15, Bax, or Bcl-2 between CON and HF offspring (Table 5).

Table 5

Relative ovarian gene expression at P27

	Group		
Gene	CON	HF	p value
Receptors			
PR	1.00±0.30	0.81±0.32	0.62
AR	1.00±0.09	1.17±0.15	0.32
ERα	1.00±0.24	0.86±0.12	0.63
ERβ	1.00±0.11	0.97±0.05	0.82
FSHR	1.00±0.21	1.05±0.29	0.99
LHR	1.00±0.11	1.06±0.22	0.81
Steroidogenic enzymes			
StAR	1.00±0.37	1.77±0.46	0.1
Cyp11a1	1.00±0.29	1.45±0.26	0.28
Cyp19a1	1.00±0.26	1.71±0.37	0.18
Cyp17a1	1.00±0.15	1.34±0.18	0.16
3βHSDI	1.00±0.12	0.92±0.13	0.66
17βHSD1	1.00±0.19	0.83±0.06	0.9
20αHSD	1.00±0.18	1.18±0.22	0.54
TGFβ			
AMH	1.00±0.09	1.04±0.09	0.76
AMHRII	1.00±0.13	1.20±0.19	0.33
GDF9	1.00±0.10	1.03±0.08	0.84
BMP15	1.00±0.14	1.34±0.29	0.29
Apoptotic factors			
Bax	1.00±0.14	0.85±0.10	0.4
Bcl-2	1.00±0.14	1.21±0.22	0.39

Data are presented as mean±SEM expressed as fold changes relative to CON offspring. p value is derived from Student's unpaired t-test, n=7-8 per group.

3.2.2.6 Prepubertal Analysis of Ovarian ROS and Cytokines

Levels of ovarian 4-Hydroxynonenal (4-HNE) were not different between CON and HF offspring (Figure 3.7A,B). Ovarian protein carbonyl levels were also similar between groups (Figure 3.7F). Ovarian protein levels of catalase (CAT) (Figure 3.7C,D) and SOD2 (Figure 3.7C,E) were similar between CON and HF offspring. Ovarian protein levels of TNF (Figure 3.7G) and IL6 (CON: 1.00±0.17 pg/ml, HF: 0.72±0.08, p=0.17; Figure 3.7H) were similar between groups.

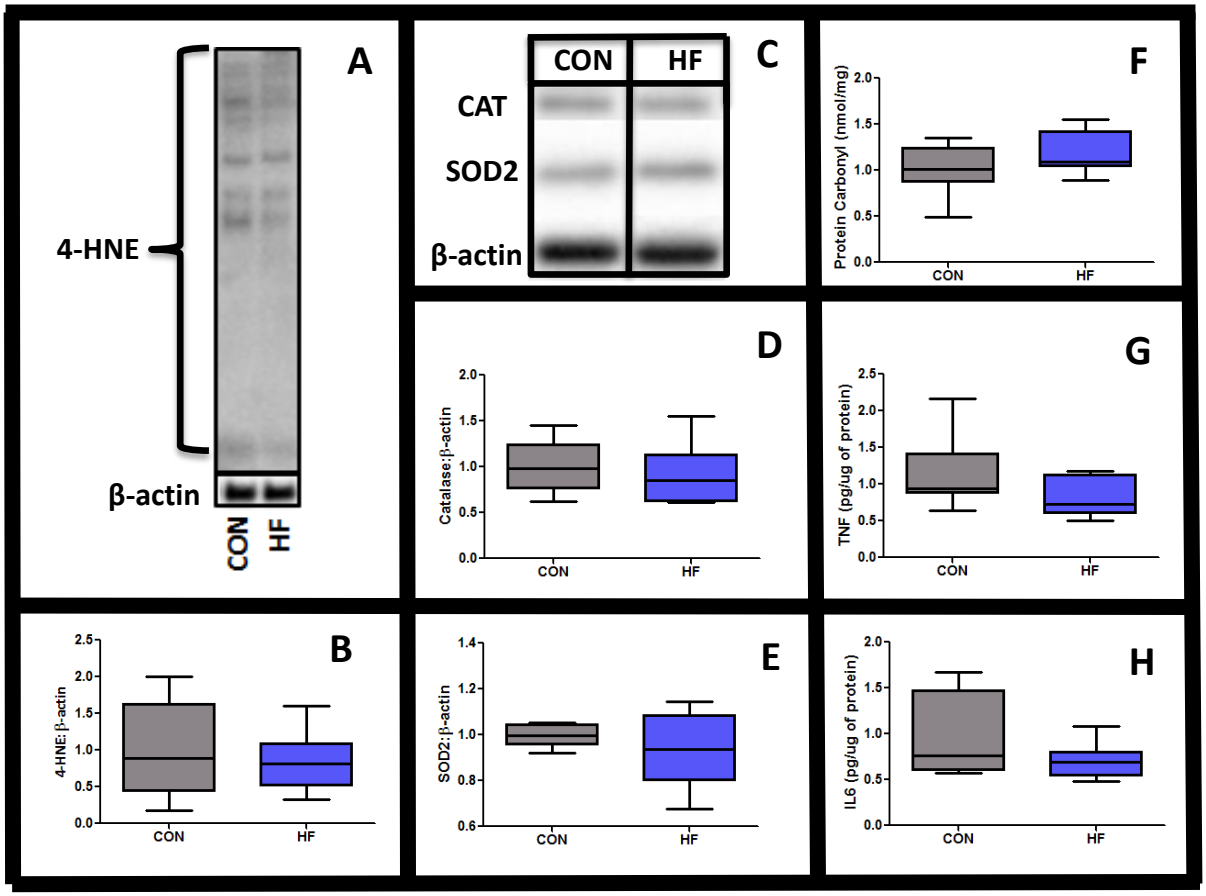

Figure 3.7 Ovarian oxidative stress markers, antioxidant defense enzymes, and pro-inflammatory cytokines in P27 offspring: Representative Western blots of 4-HNE levels (**A**) and 4-HNE relative levels (**B**). Representative Western blots of CAT and SOD2 (**C**). Relative protein levels of CAT (**D**) and SOD2 (**E**). Protein carbonyl (**F**), TNF (**G**), and IL6 (**H**) levels. n=5-7 per group. All values were expressed as a fold change in levels relative to the CON group.

3.2.3 Adult Offspring Outcomes

3.2.3.1 Adult Follicle and Corpora Lutea Populations

Primordial follicle number was significantly greater in ovaries of offspring fed a postweaning high fat diet (p=0.039), with no effect of maternal diet (p=0.573) and no interactions between maternal and postweaning diet (p=0.240; Figure 3.8A). *Post-hoc* analysis demonstrated a significant increase in the number of primordial follicles in CON-hf offspring compared to CON-con offspring (p=0.020).

There was no effect of postweaning diet (p=0.800) or maternal diet (p=0.739) on transitioning follicle number; however, there was a significant interaction between maternal and postweaning diet (p=0.016; Figure 3.8B). *Post-hoc* analysis demonstrated no significant differences between groups.

There was no effect of postweaning diet (p=0.157), maternal diet (p=0.630), and no statistically significant interactions between maternal and postweaning diet (p=0.713) on primary follicle number (Figure 3.8C).

There was no effect of postweaning diet (p=0.483), maternal diet (p=0.471), and no statistically significant interactions between maternal and postweaning diet (p=0.766) on secondary follicle number (Figure 3.8D).

There was no effect of postweaning diet (p=0.648), maternal diet (p=0.369), and no statistically significant interactions between maternal and postweaning diet (p=0.776) on antral follicle number (Figure 3.8E).

There was no effect of postweaning diet (p=0.129) or maternal diet (p=0.703) on type II atretic follicle number; however, there was a significant interaction between

maternal and postweaning diet ($p<0.001$; Figure 3.8F). *Post-hoc* analysis demonstrated a significant increase in the number of type II atretic follicles in CON-hf offspring compared to CON-con offspring ($p=0.0037$) and a significant decrease in HF-hf offspring compared to HF-con offspring ($p<0.001$).

The area of secondary follicles was greater in ovaries of offspring fed a postweaning high fat diet ($p=0.018$), with no effect of maternal diet ($p=0.305$) and no interactions between maternal and postweaning diet ($p=0.766$). *Post-hoc* analysis demonstrated no significant differences between groups.

There was no effect of postweaning diet ($p=0.981$), maternal diet ($p=0.543$), and no statistically significant interactions between maternal and postweaning diet ($p=0.987$) on the area of antral follicles.

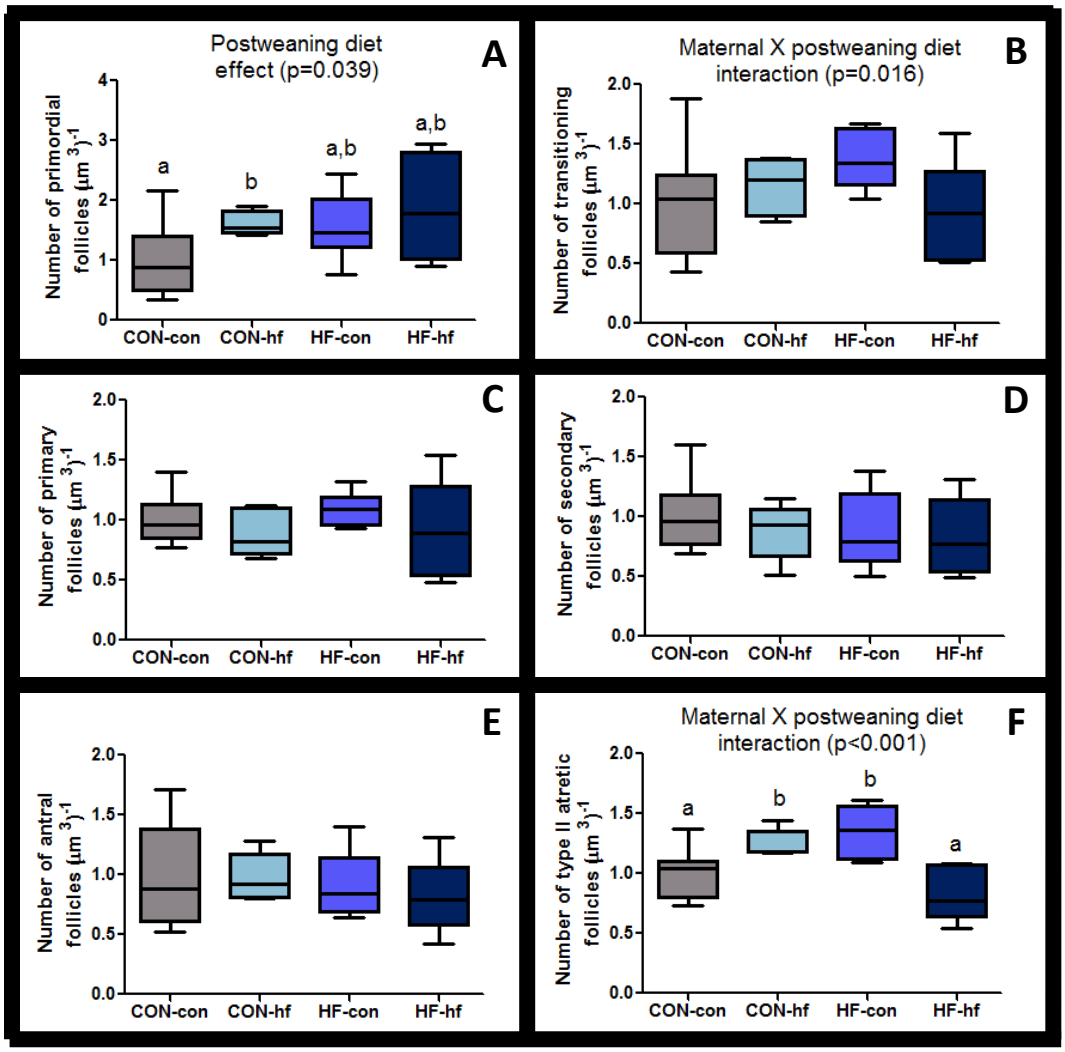

Figure 3.8 Ovarian follicle populations in P120 offspring: Primordial (**A**), transitioning (**B**), primary (**C**), secondary (**D**), antral (**E**) and type II atretic (**F**) follicle numbers normalized to total volume (μm^3) counted per animal and expressed as a fold change relative to control fed CON offspring. Offspring fed a postweaning high fat diet had a greater number of primordial follicles (**A**). The effect of a postweaning high fat diet on transitioning (**B**) and type II atretic (**F**) follicle number depended on maternal diet. Primary (**C**), secondary (**D**), and antral (**E**) follicle counts did not differ significantly between groups. Data were analyzed using a two-way ANOVA. Tukey's *post-hoc* analyses: groups with unlike letters are statistically different ($p<0.05$). n=5-9 per group. Abbreviations: CON, maternal control diet; con, postweaning control diet; HF, maternal high fat diet; hf, postweaning high fat diet.

Total CL populations were analyzed and also subdivided into currently formed and previously formed for separate analyses (Figure 3.9A)

Total CL number tended to be lower in ovaries of offspring fed a postweaning high fat diet (p=0.052), with no effect of maternal diet (p=0.271) and no interactions between maternal and postweaning diet (p=0.259; Figure 3.9B). *Post-hoc* analysis demonstrated a significant decrease in the total number of CL in HF-hf offspring compared to HF-con offspring (p=0.040).

There was no effect of postweaning diet (p=0.067), maternal diet (p=0.657), and no statistically significant interactions between maternal and postweaning diet (p=0.630) on currently formed CL number (Figure 3.9C).

There was no effect of postweaning diet (p=0.188), maternal diet (p=0.358), and no statistically significant interactions between maternal and postweaning diet (p=0.219) on previously formed CL number (Figure 3.9D).

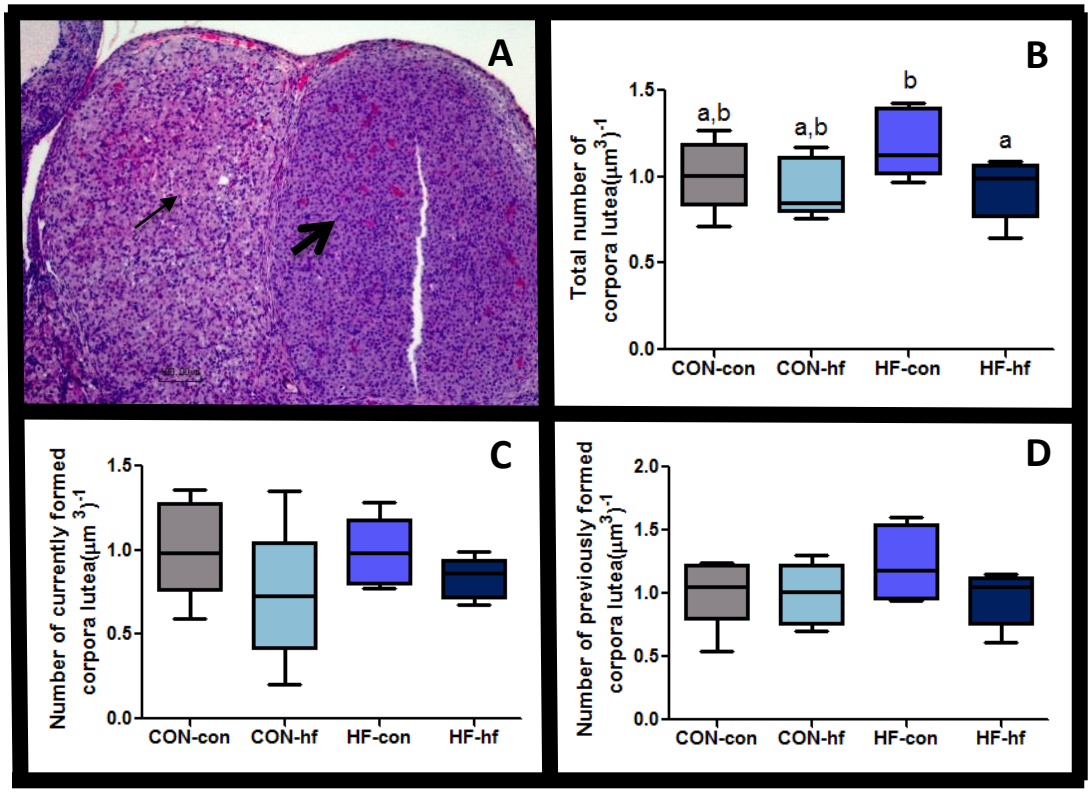

Figure 3.9 Corpora lutea populations in adult offspring: Corpora lutea (**A**) were classified as either currently formed (➔) or previously formed (⟶). Counts of corpora lutea were normalized to total volume counted (μm^3) per animal and expressed as a fold change relative to control fed CON offspring. Offspring fed a postweaning high fat diet tended (p=0.052) to have reduced total numbers of corpora lutea (**B**). Previously formed (**C**) and currently formed (**D**) corpora lutea counts did not differ significantly between groups. Data were analyzed using a two-way ANOVA. Tukey's *post-hoc* analyses: groups with unlike letters are statistically different ($p<0.05$). n=5-9 per group. Abbreviations: CON, maternal control diet; con, postweaning control diet; HF, maternal high fat diet; hf, postweaning high fat diet.

3.2.3.2 Adult Plasma Gonadotropin, E_2, and AMH Concentrations

Maternal HF diet significantly elevated plasma concentrations of FSH (p=0.028), with no effect of postweaning diet (p=0.294) and no interactions between maternal and postweaning diet (p=0.206; Figure 3.10A). *Post-hoc* analysis demonstrated a significant increase in plasma FSH in HF-con offspring compared to CON-con offspring (p=0.011).

Maternal HF diet significantly decreased plasma concentrations of E_2 in adult offspring (p=0.043), with no effect of postweaning diet (p=0.235) and no interactions between maternal and postweaning diet (p=0.572; Figure 3.10B). *Post-hoc* analysis demonstrated no significant differences between groups.

Maternal HF diet significantly increased the FSH:E_2 ratio in adult offspring (p<0.001), with no effect of postweaning diet (p=0.931) and no interactions between maternal and postweaning diet (p=0.634; Figure 3.10C). *Post-hoc* analysis demonstrated a significant increase in FSH:E_2 in HF-con compared to CON-con offspring (p=0.003) and a significant increase in HF-hf offspring compared to CON-hf offspring (p=0.034).

There was no effect of maternal diet on plasma concentrations of LH (p=0.849), however, there was a postweaning diet effect (p=0.024) and a significant interaction between maternal and postweaning diet (p=0.046; Figure 3.10D). *Post-hoc* analysis demonstrated a significant decrease in plasma LH in CON-hf offspring compared to CON-con offspring (p=0.002).

There was no effect of maternal diet (p=0.562), postweaning diet (p=0.065), and no statistically significant interactions between maternal and postweaning diet (p=0.666) on plasma concentrations of AMH.

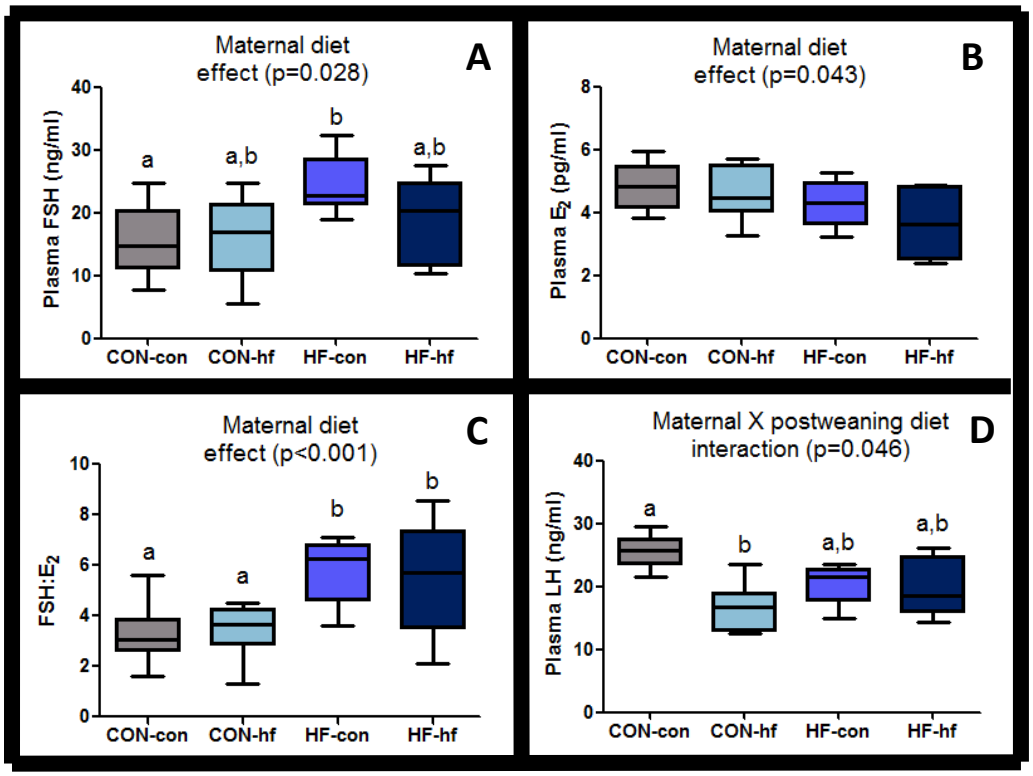

Figure 3.10 Maternal HF diet decreased plasma concentration of E_2 and LH, but increased FSH and FSH:E_2 in adult offspring: HF offspring had significantly more circulating FSH (**A**), but only when fed a control diet postweaning. HF offspring had significantly lower systemic concentrations of E_2 (**B**) and a significantly higher FSH:E_2 (**C**). Circulating LH (**D**) concentrations were significantly lower in offspring fed a postweaning high fat diet. Data were analyzed using a two-way ANOVA. Tukey's *post-hoc* analyses: groups with unlike letters are statistically different ($p<0.05$). n=5-9 per group. Abbreviations: CON, maternal control diet; con, postweaning control diet; HF, maternal high fat diet; hf, postweaning high fat diet; FSH, follicle stimulating hormone; LH, luteinizing hormone; E_2, 17-β-estradiol.

3.2.3.3 Adult Immunolocalization of AMH and AMH receptor

AMH and AMHRII were immunolocalized to primordial, primary, secondary, and antral follicles with no detectable immunostaining observed in atretic follicles. AMH was highly expressed in the granulosa cells of primary, secondary, and antral follicles (Figure 3.11).

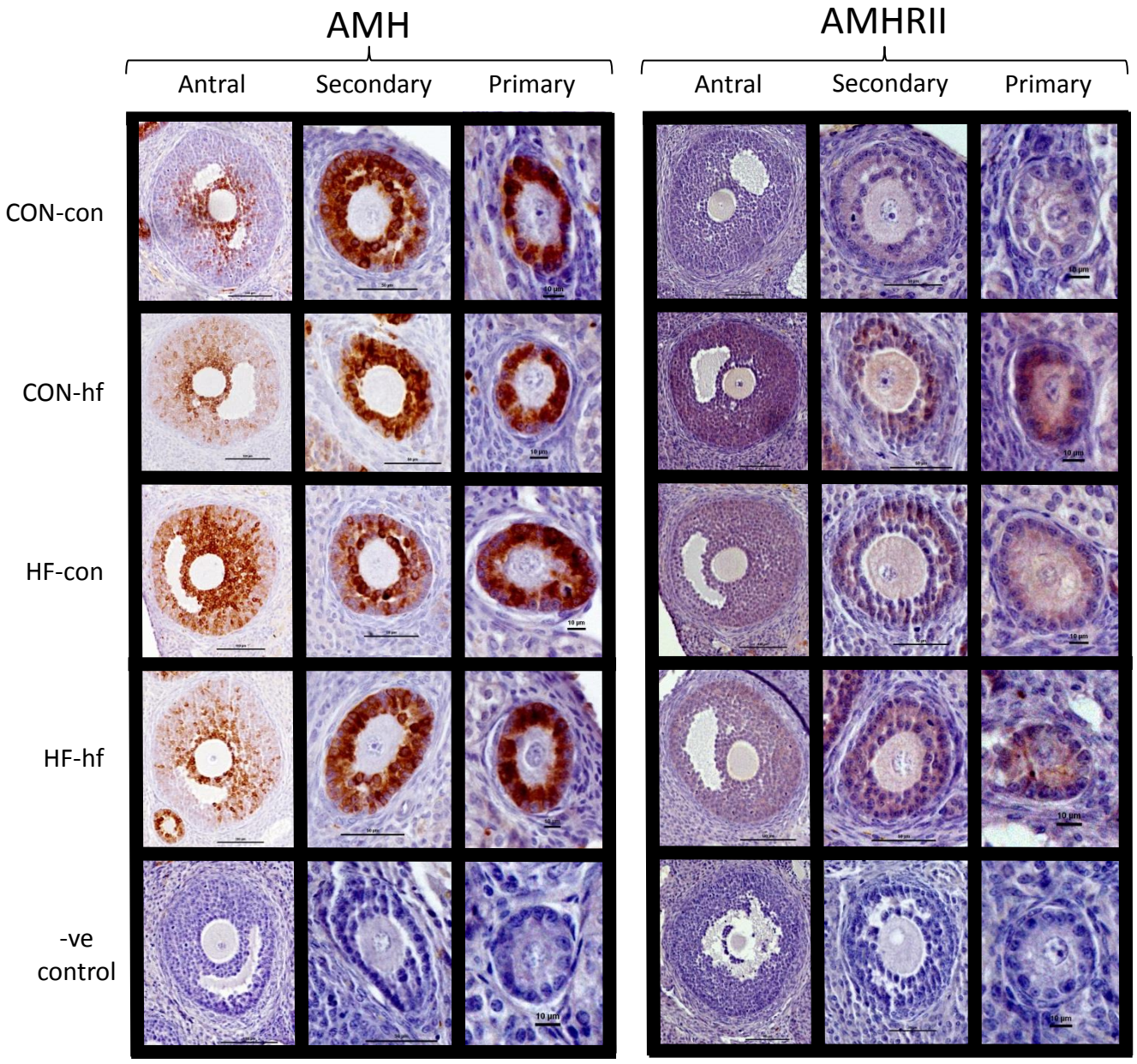

Figure 3.11 Immunolocalization of AMH and AMHRII in antral, secondary, and primary follicles in P120 offspring: Representative photomicrographs of each follicle subtype within each group. Images were captured using a Nikon Eclipse *Ni* microscope. Scale bars represent 100 μm, 50 μm, and 10 μm for antral, secondary, and primary, respectively. Abbreviations: CON, maternal control diet; con, postweaning control diet; HF, maternal high fat diet; hf, postweaning high fat diet.

Similar to prepubertal offspring, antral follicles in adult offspring showed diminishing immunoreactive AMH with increasing size and correlation analysis demonstrated that this relationship was significant ($r=-0.72$; $p<0.001$); however, in AMHRII antral follicles, this relationship was not significant ($r=-0.27$; $p=0.27$).

There was no effect of postweaning diet ($p=0.499$) or maternal diet ($p=0.147$) on the proportion of positive AMH immunostaining within antral follicles; however, there was a significant interaction between maternal and postweaning diet ($p=0.043$; Table 6). *Post-hoc* analysis demonstrated a significant increase in proportion of positive AMH immunostaining in HF-con offspring compared to CON-con offspring ($p=0.002$). Computer image analyses of AMH immunostaining in secondary and primary follicles as well as AMHRII immunostaining in antral, secondary, and primary follicles demonstrated no effect of maternal diet, postweaning diet and no statistically significant interactions between maternal and postweaning diet (Table 6).

Table 6

Computer image analysis of AMH/AMHRII in adult offspring follicles

	Group				Main Effects		
Type	CON-con	CON-hf	HF-con	HF-hf	Maternal Diet Effect	Postweaning Diet Effect	Interaction
AMH							
Antral (%)	18.51±5.72[a]	38.30±7.32[a,b]	44.01±3.23[b]	33.61±7.95[a,b]	0.147	0.499	0.043
Secondary (%)	36.97±7.86	43.06±7.94	49.00±4.19	41.07±8.44	0.502	0.901	0.351
Primary (%)	35.99±13.16	35.77±8.95	47.98±8.98	18.81±4.48	0.811	0.172	0.179
AMRII							
Antral (%)	9.98±7.16	16.15±3.62	21.70±4.62	21.48±2.99	0.083	0.526	0.496
Secondary (%)	14.38±7.61	21.91±5.93	25.93±4.03	25.73±2.62	0.155	0.486	0.463
Primary (%)	22.71±11.96	25.43±5.57	30.11±4.48	32.84±5.42	0.298	0.696	1

Proportions (%) of positive AMH and AMHRII immunostaining. Data are presented as mean±SEM. Data were analyzed using a two-way ANOVA. Tukey's *post-hoc* analyses: groups with unlike letters are statistically different ($p<0.05$). n=3-5 per group. Abbreviations: CON, maternal control diet; con, postweaning control diet; HF, maternal high fat diet; hf, postweaning high fat diet; AMH, anti mullerian hormone; AMHRII, anti mullerian hormone type II receptor.

Interestingly, there was a significant correlation (r=0.69, p<0.01) between the density of positive AMH immunostaining within antral follicles and the number of primordial follicles counted at this age (Figure 3.12A). However, there was no significant correlation (r=0.20, p=0.43) between the density of positive AMHRII immunostaining within antral follicles and the number of primordial follicles (Figure 3.12B).

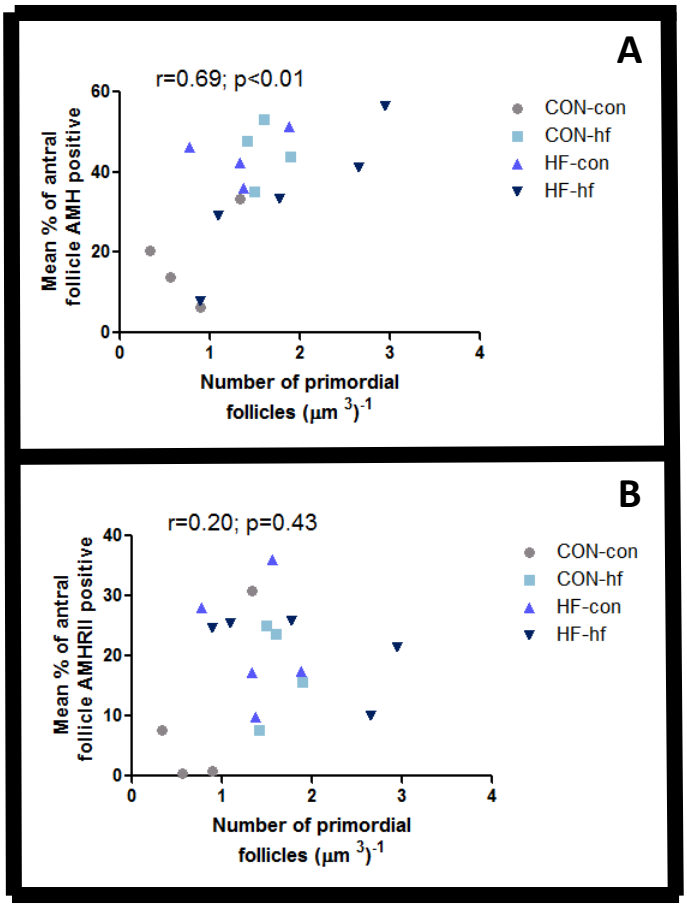

Figure 3.12: Primordial follicle number correlates with density of AMH immunostaining in antral follicles: Density of AMH (**A**) and AMHRII (**B**) positive immunostaining in antral follicles correlated with primordial follicle counts. There was a significant positive correlation (r=0.69, p<0.01) between density of positive AMH immunostaining in antral follicles and primordial follicle number, but not between AMHRII antral density and primordial follicle number. Data were analyzed using Pearson's correlation. Abbreviations: CON, maternal control diet; con, postweaning control diet; HF, maternal high fat diet; hf, postweaning high fat diet; AMH, anti mullerian hormone; AMHRII, anti mullerian hormone type II receptor.

Unlike prepubertal offspring, adult offspring showed positive AMH and AMHRII immunostaining in primordial follicles (Figure 3.13A). HF offspring showed an increased proportion of primordial follicles positively immunostained for AMH compared to CON offspring (CON-con: 26.47%, CON-hf: 25.00, HF-con: 43.75, HF-hf: 57.90; Figure 3.13B). Binary logistic regression analysis demonstrated that maternal diet influenced whether or not adult ovaries had primordial follicles with positive AMH immunostaining (B=1.032, p=0.007), but postweaning diet did not (B=0.248, p=0.521). HF offspring were more likely to have positively immunostained AMH primordial follicles than CON offspring (Odds Ratio=2.807).

There was no apparent trend in percentage of primordial follicles positively immunostained for AMHRII in offspring (CON-con: 11.77%, CON-hf: 21.62, HF-con: 24.32, HF-hf: 16.67; Figure 3.13C). Binary logistic regression analyses demonstrated that neither maternal nor postweaning diet influenced whether or not adult ovaries had primordial follicles with positive AMHRII immunostaining (B=0.178, p=0.717; B=0.008, p=0.987; respectively).

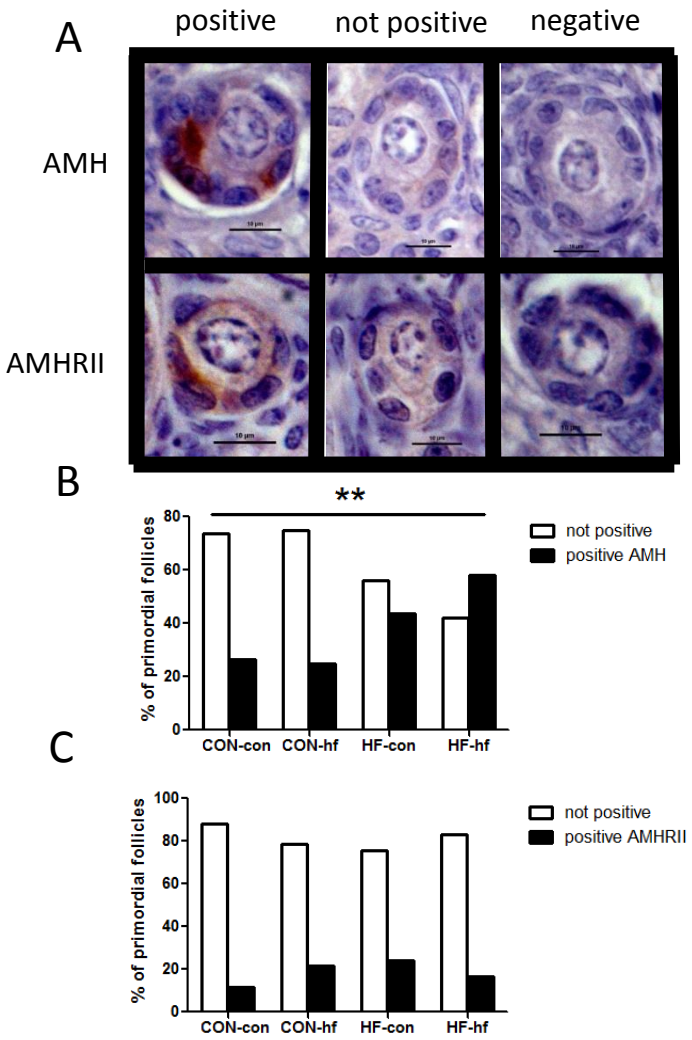

Figure 3.13 P120 offspring born to HF-fed dams are more likely to have primordial follicles positively stained for AMH, but not AMHRII: Representative photomicrographs of primordial follicles (**A**). Proportion of primordial follicles positively immunostained for AMH (**B**) or AMHRII (**C**) **p<0.01, maternal diet predictor, binary logistic regression. Binary logistic regression analysis demonstrated an increased odds (Odds Ratio=2.807) of positive AMH immunostaining in primordial follicles of HF, but not in offspring fed a postweaning high fat diet (Odds Ratio=1.282). Neither maternal nor postweaning diet significantly predicted AMHRII positive immunostaining in primordial follicles. Images were captured using a Nikon Eclipse *Ni* microscope. Scale bars represent 10 μm. Abbreviations: CON, maternal control diet; con, postweaning control diet; HF, maternal high fat diet; hf, postweaning high fat diet; AMH, anti mullerian hormone; AMHRII, anti mullerian hormone type II receptor.

3.2.3.4 Adult Immunolocalization of Phosphorylated Akt

Immunopositive pAkt (Ser473) was localized to the oocytes of primordial, primary, secondary, and antral follicles (Figure 3.14A). A large proportion of oocytes in primordial, primary, and antral follicles showed little to no detectable positive immunostaining.

There was no apparent trend in proportion of oocytes in primordial, secondary, or antral follicles positively immunostained for pAkt (Ser473) in offspring (Figure 3.14 B,D,E). However, there was a tendency towards an increase in proportion of oocytes in primary follicles positively immunostained for pAkt (Ser473) in offspring fed a postweaning high fat diet compared to control fed offspring (CON-con: 14.29%, CON-hf: 33.33, HF-con: 10.00, HF-hf: 40.00; Figure 3.14C). Binary logistic regression analysis demonstrated that postweaning diet tended to influence whether or not adult ovaries had positive pAkt (Ser473) in primary follicles; although, this was not significant (B=1.364, p=0.063). Offspring fed a high fat diet postweaning were more likely to have positively immunostained pAkt (Ser473) primary follicle oocytes than control fed offspring (Odds Ratio=3.91).

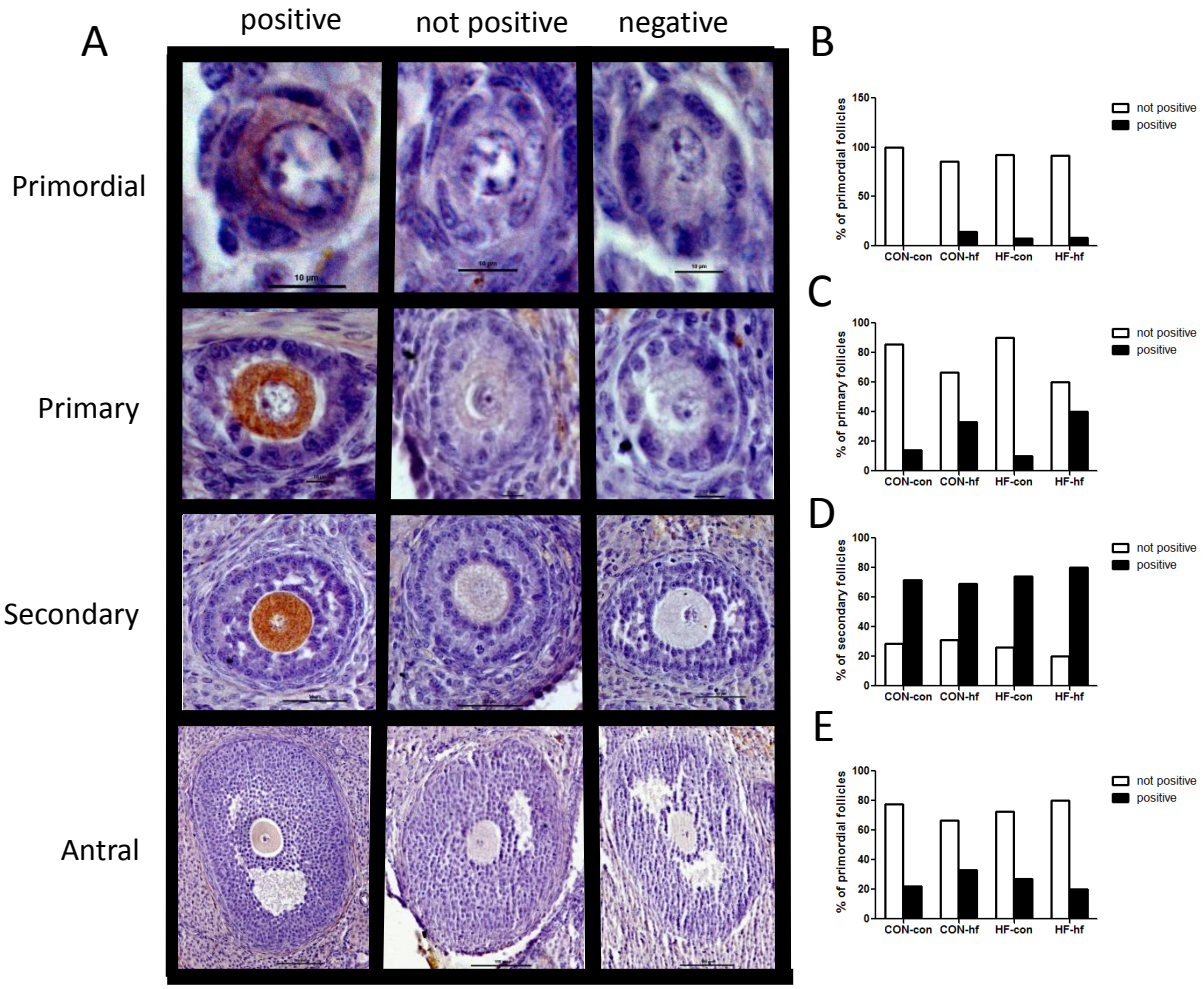

Figure 3.14 Proportions of positively stained pAkt (Ser473) in different follicle subtypes: Representative photomicrographs of oocytes within primordial, primary, secondary, and antral follicles (**A**). Proportion of oocytes in primordial (**B**), primary (**C**), secondary (**D**), and antral (**E**) follicles positively immunostained for pAkt (Ser473). Neither maternal nor postweaning diet significantly predicted positive pAkt (Ser473) immunostaining in oocytes of primordial, primary, secondary, and antral follicles. Images were captured using a Nikon Eclipse *Ni* microscope. Scale bars represent 10, 50, 50, and 100 µm for primordial, primary, secondary, and antral follicles, respectively. Abbreviations: CON, maternal control diet; con, postweaning control diet; HF, maternal high fat diet; hf, postweaning high fat diet.

3.2.3.5 Adult Ovarian Gene Expression

There was no effect of maternal diet (p=0.389) or postweaning diet (p=0.258) on relative mRNA levels of ERα; however, there was a tendency for an interaction between maternal and postweaning diet (p=0.067; Table 7). *Post-hoc* analysis demonstrated a significant decrease in ERα mRNA expression in HF-con offspring compared to CON-con offspring (p=0.025).

There was no effect of maternal diet (p=0.653) on relative mRNA levels of Cyp19a1; however, there was a tendency for a postweaning diet effect (p=0.062) and no significant interaction between maternal and postweaning diet (p=0.145; Table 7). *Post-hoc* analysis demonstrated a significant decrease in Cyp19a1 mRNA expression in HF-hf offspring compared to HF-con offspring (p=0.028).

There was tendency for a maternal diet effect (p=0.069) on relative mRNA levels of GDF9; however, there was no effect of postweaning diet (p=0.799) and no interactions between maternal and postweaning diet (p=0.183; Table 7). *Post-hoc* analysis demonstrated a significant decrease in GDF9 mRNA expression in HF-con offspring compared to CON-con offspring.

There was no effect of maternal diet, postweaning diet and no statistically significant interactions between maternal and postweaning diet on relative mRNA levels of ProgR, AR, ERβ, FSHR, LHR, StAR, Cyp11a1, Cyp17a1, 3βHSDII, 17βHSD1, 20αHSD, AMH, AMHRII, BMP15, Bax, or Bcl-2 (Table 7).

Table 7

Relative ovarian gene expression at P120

	Group				Main Effects		
Gene	CON-con	CON-hf	HF-con	HF-hf	Maternal Diet Effect	Postweaning Diet Effect	Interaction
Receptors							
PR	1.00±0.28	0.82±0.11	0.63±0.06	0.66±0.07	0.274	0.865	0.946
AR	1.00±0.06	0.92±0.10	0.89±0.12	0.97±0.06	0.713	0.998	0.382
ERα	1.00±0.13[a]	0.86±0.07[a,b]	0.60±0.11[b]	0.96±0.13[a,b]	0.258	0.389	0.067
ERβ	1.00±0.11	1.08±0.05	1.11±0.14	0.99±0.10	0.936	0.875	0.41
FSHR	1.00±0.17	1.15±0.19	1.47±0.31	0.87±0.07	0.678	0.316	0.096
LHR	1.00±0.10	0.89±0.16	0.83±0.13	0.69±0.09	0.145	0.312	0.99
Steroidogenic enzymes							
StAR	1.00±0.11	1.07±0.16	1.25±0.19	1.29±0.15	0.134	0.732	0.928
Cyp11a1	1.00±0.07	0.88±0.14	0.99±0.12	0.87±0.07	0.901	0.259	0.979
Cyp19a1	1.00±0.12[a,b]	0.91±0.15[a,b]	1.38±0.31[a]	0.70±0.14[b]	0.653	0.062	0.145
Cyp17a1	1.00±0.21	0.57±0.17	0.61±0.15	0.70±0.17	0.79	0.311	0.134
3βHSDI	1.00±0.06	0.88±0.05	1.02±0.09	1.04±0.14	0.309	0.562	0.426
17βHSD1	1.00±0.17	1.12±0.24	1.03±0.18	0.94±0.19	0.717	0.936	0.609
20αHSD	1.00±0.11	1.37±0.26	1.20±0.13	1.37±0.29	0.606	0.171	0.61
TGFβ							
AMH	1.00±0.12	0.99±0.10	0.73±0.17	0.74±0.15	0.082	0.992	0.938
AMHRII	1.00±0.14	1.09±0.16	0.95±0.22	1.19±0.21	0.784	0.275	0.59
GDF9	1.00±0.13[a]	0.85±0.05[a,b]	0.57±0.12[b]	0.78±0.15[a,b]	0.069	0.799	0.183
BMP15	1.00±0.31	1.33±0.24	0.84±0.21	0.96±0.20	0.395	0.195	0.664
Apoptotic factors							
Bax	1.00±0.07	0.95±0.12	1.01±0.15	0.98±0.15	0.865	0.745	0.921
Bcl-2	1.00±0.09	1.19±0.13	1.14±0.14	1.05±0.07	0.923	0.533	0.287

Data are presented as mean±SEM expressed as fold changes relative to control fed CON offspring. Data were analyzed using a two-way ANOVA. Tukey's *post-hoc* analyses: groups with unlike letters are statistically different ($p<0.05$). n=5-9 per group. Abbreviations: CON, maternal control diet; con, postweaning control diet; HF, maternal high fat diet; hf, postweaning high fat diet.

3.2.3.6 Adult Analysis of Ovarian ROS and Cytokines

There was no effect of maternal diet, postweaning diet and no statistically significant interactions between maternal and postweaning diet on ovarian levels of 4-HNE (Figure 3.15A,B), protein carbonyls (Figure 3.15F), CAT (Figure 3.15C,E), SOD2 (Figure 3.15C,D), and TNF (Figure 3.15G). There was no effect of maternal diet on protein levels of IL6 ($p=0.332$); however there was a significant postweaning diet effect ($p=0.031$) and no statistically significant interactions between maternal and postweaning diet ($p=0.167$; Figure 3.15H). *Post-hoc* analysis demonstrated a significant decrease in ovarian IL6 protein expression in CON-hf offspring compared to CON-con offspring ($p=0.021$).

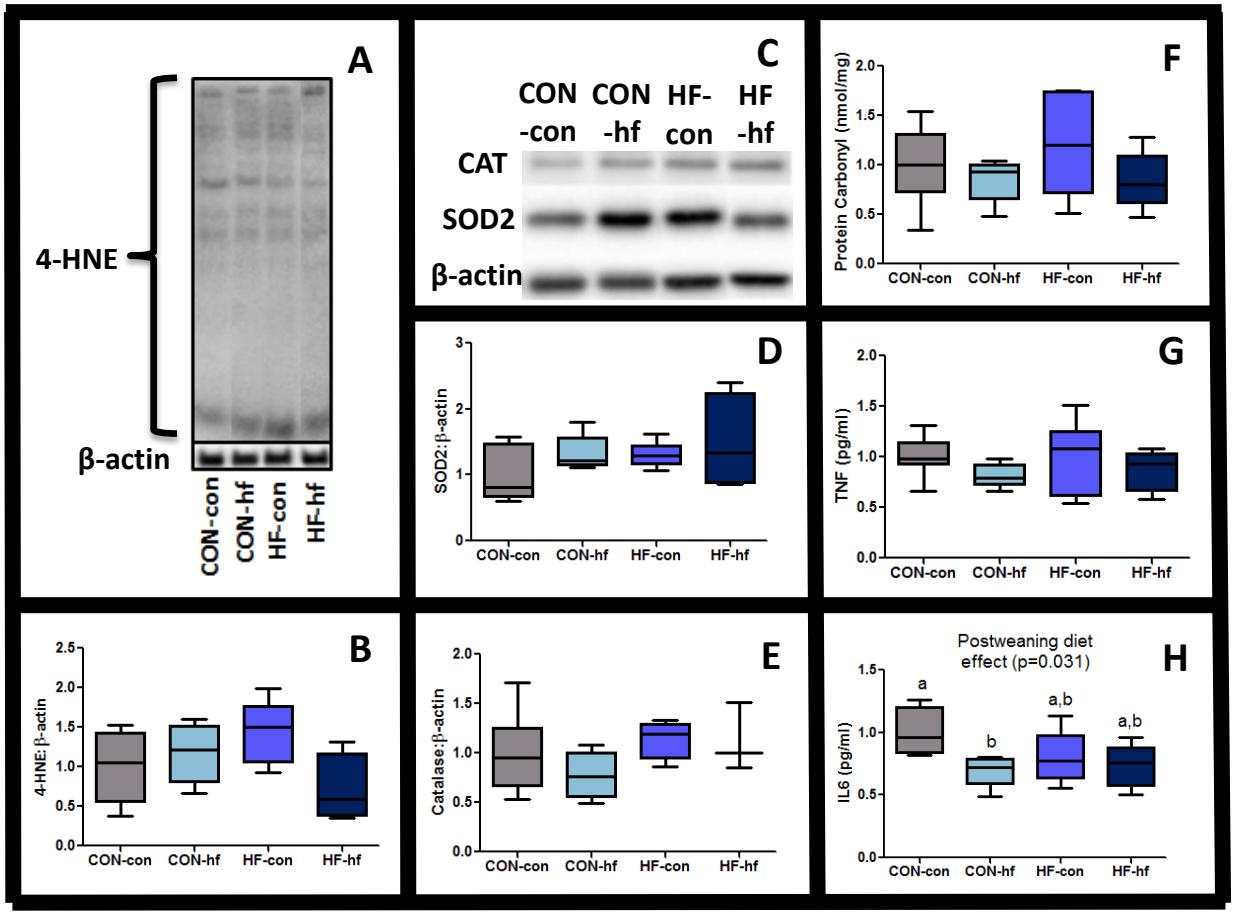

Figure 3.15 Ovarian oxidative stress markers, antioxidant defense enzymes, and pro-inflammatory cytokines in P120 offspring: Representative western blots of 4-HNE levels (**A**) and 4-HNE relative levels (**B**). Representative Western blots of CAT and SOD2 (**C**). Relative protein levels of CAT (**D**) and SOD2 (**E**). Ovarian protein carbonyl (**F**), TNF (**G**), and IL6 (**H**) levels. There was a significant decrease in ovarian IL6 in offspring fed a postweaning high fat diet Data were analyzed using a two-way ANOVA, Tukey's *post-hoc* analyses: groups with unlike letters are statistically different ($p<0.05$). n=5-9 per group. All values were expressed as a fold change in protein levels relative to the control fed CON offspring.

4.0 Discussion and Conclusions

This study has shown that maternal high fat diet induced obesity results in offspring ovarian impairments before and after puberty that stem from changes in ovarian development that are apparent during neonatal life. Neonates born to HF fed mothers have an increased number of primordial follicles at day 4 of life. This is likely due to diminished AMH signaling induced by an altered endocrine milieu, however, the exact mechanisms operating at this age have yet to be definitively determined. Before puberty, the number of primordial follicles in HF offspring appears to drop to control levels likely due to increased rates of both primordial follicle atresia and growing follicle atresia. As adults, offspring born to HF fed mothers have more atretic follicles, but only when fed a control diet postweaning. This increase in follicle death appears to be due to decreased FSH responsiveness that could be induced by both low expression levels of the oocyte-derived factor, GDF9, and high expression levels of the granulosa-cell derived factor, AMH, in antral follicles.

4.0.1 Neonatal Offspring

In neonates, it appears that maternal HF diet induced obesity results in altered expression of both AMH and AMHRII. Neonates born to mothers fed a HF diet have almost complete downregulation of AMH and AMHRII (Figure 3.3) despite having comparable numbers of transitioning follicles (Figure 3.2E). This relative deficiency in AMH signaling likely contributes to the increase in primordial follicle assembly observed at this age (Figure 3.2D) It is well documented in rat neonatal ovary culture systems that AMH treatment inhibits both primordial follicle assembly (Nilsson, *et al.* 2011) and

transition of primordial follicles to primary follicles (Durlinger, *et al.* 2002, Nilsson, *et al.* 2007). Therefore, it appears as though a lack of AMH signaling contributed to the increase in primordial follicles assembled and the tendency towards an increase in transitioning follicle number in neonates born to HF fed dams (Figure 3.2DE).

Although the effects of AMH signaling on neonatal ovarian primordial follicle assembly and transition have been documented, little is known about the factors regulating AMH and AMHRII expression. Recently, forkhead box protein L2 (FOXL2) has been shown to upregulate AMH gene expression through FOXL2 binding elements in the AMH promoter (Park, *et al.* 2014). This was demonstrated *in vitro* in a human granulosa cell line and *in vivo* in adult mouse ovaries (Park, *et al.* 2014) Therefore, it is possible that diminished FOXL2 expression or function could be mediating the observed decrease in AMH protein expression in HF neonates. Future experiments could explore this possibility by measuring expression of FOXL2 in postnatal day 4 ovaries via immunohistochemistry or assessing the binding of FOXL2 to the AMH promoter via chromatin immunoprecipitation (ChIP).

It is possible that 17-β-estradiol (E_2) may also mediate a decrease in AMH expression. E_2 binding to ER (ERα or ERβ) results in receptor dimerization, translocation to the nucleus and binding to estrogen response elements (ERE) or steroidogenic factor-1 (SF-1) elements (Klinge, *et al.* 2004). The AMH promoter contains both of these response elements (Guerrier, *et al.* 1990, Watanabe, *et al.* 2000) and AMH transcription in cultured adult granulosa cells has been shown to be differentially regulated by treatment with E_2 depending on which ER (ERα or ERβ) mediates signaling

(Grynberg, *et al.* 2012). Specifically, E_2 signaling through ERα stimulates AMH transcription; whereas, the level of AMH transcription is inhibited by signaling through ERβ (Grynberg, *et al.* 2012). Given that ERα is the predominant ER in ovarian granulosa cells perinatally (Chen, *et al.* 2009), it is likely that ovarian E_2 signaling during fetal/neonatal life upregulates AMH expression, although this has yet to be shown empirically. Thus, if ovarian E_2 signaling is reduced perinatally in offspring as a result of exposure to a maternal HF diet, AMH transcription could be dampened.

Lower levels of offspring ovarian E_2 perinatally could cause this reduction in E_2 signaling. The two sources of offspring ovarian E_2 perinatally are the fetal/neonatal circulation and endogenous ovarian production (Dutta, *et al.* 2014). Prenatally, in rodents, the main source of circulating fetal E_2 comes from E_2 produced by the maternal ovary that passes through the placenta (lipophilic nature of E_2) (Ben-Zimra, *et al.* 2002, Raunig, *et al.* 2011). Late in gestation, maternal levels of circulating E_2 surge and this initiates maternal care during lactation (Cameron, *et al.* 2008). Maternal HF diet intake throughout pregnancy and lactation has previously been shown to decrease maternal care (Connor, *et al.* 2012). This suggests that maternal circulating concentrations of E_2 could be reduced in HF fed mothers late in gestation, potentially reducing the levels of E_2 in offspring ovaries. In turn, this could reduce E_2 signaling and perhaps AMH expression, although this has yet to be tested.

Reduced E_2 signaling may also affect expression of other important regulatory factors in the neonatal ovary. For example, E_2 has been shown to suppress expression of activin (Kipp, *et al.* 2007), a TGFβ superfamily member produced by granulosa cells

(Findlay, *et al.* 2001) that promotes primordial follicle assembly in neonates (Bristol-Gould, *et al.* 2006b). Therefore, if E_2 signaling is reduced, activin may be upregulated, which could contribute to the increase in primordial follicle formation in HF neonates. However, this study did not measure circulating or ovarian E_2 levels in neonates nor did it explore the E_2 signaling pathway. Therefore, it remains to be determined if reduced E_2 signaling is mediating increased primordial follicle assembly in HF neonates. Nonetheless, future investigations delineating how a maternal HF diet increases primordial follicle assembly and suppresses AMH and AMHRII are warranted in order to better our understanding of how ovarian development is regulated during this critical window.

Neonatal levels of phosphorylated Akt at serine 473 were decreased in primordial follicle oocytes of offspring born to HF fed mothers (Figure 3.4B) suggesting that either fewer primordial follicles are being recruited to grow or more primordial follicles lack basal levels of Akt activation. The former scenario is unlikely based on the observation that the number of transitioning follicles do not differ between groups at this age (Figure 3.2E). Therefore, it is likely that more primordial follicle oocytes in the ovaries of HF neonates lack sufficient Akt activation. Adequate levels of Akt activation are required to maintain primordial follicle survival (Jin, *et al.* 2005, Reddy, *et al.* 2009) suggesting that more primordial follicles are likely to undergo demise in HF offspring. This is consistent with the observation that the number of primordial follicles in HF offspring drops to control levels near the end of the prepubertal period (Table 3). Although this study did

not measure specific regulatory factors governing this loss of Akt activation in HF neonates, insulin is a prime candidate.

Previous work has shown that neonates born to HF fed mothers are hypoinsulinemic compared to control offspring (Howie, *et al.* 2009). Insulin binding to its receptor, which is expressed in the oocytes of primordial follicles (Samoto, *et al.* 1993), results in phosphorylation of Akt at serine 473 (Guo. 2014). Therefore, decreased circulating insulin may contribute to the decreased proportion of primordial follicle oocytes with activated Akt in HF neonates, although this remains to be determined.

4.0.2 Prepubertal Offspring

This study demonstrates that primordial follicle number at postnatal day 27 is similar between groups (Table 3). Thus, it appears that in HF offspring the number of primordial follicles drops to control levels during the neonatal-to-prepubertal period. This drop could be due to either increased primordial follicle recruitment or increased primordial follicle death. Since HF offspring demonstrate an increase in atretic follicles without concomitant changes in the numbers of other follicle subtypes (Table 3), it is likely that in HF offspring, more primordial follicles are recruited into the growing follicle pool during the prepubertal period, only to undergo atresia near the end-stages of follicle development. Future studies need to verify increased loss at this age through measures of ovarian apoptosis or autophagy as both of these processes have been shown to contribute to follicular atresia (Choi, *et al.* 2013, Gannon, *et al.* 2013, Hsueh, *et al.* 1994).

This pattern of early life follicle dynamics is consistent with a hypothesis proposed first by Flaws et al. (2001) and then by Bristol-Gould et al. (2006) that the prepubertal ovary has a "census" or "quorum sensing" (respectively) mechanism to detect the number of primordial follicles present. If this number exceeds a certain threshold, excess primordial follicles are eliminated via atresia in order to meet a quota necessary for adult fertility (Bristol-Gould, *et al.* 2006b). Evidence from activin-treated neonatal mice and transgenic mice overexpressing the anti-apoptotic factor Bcl-2 support this idea. Specifically, activin treatment during primordial follicle assembly in mice initially increases the number of primordial follicles, however, at P19, these mice show comparable primordial follicle numbers compared to vehicle-treated controls (Bristol-Gould, *et al.* 2006b). Similarly, ovary specific overexpression of Bcl-2 in mice results in an increased number of primordial follicles at birth followed by a drop to wild type levels at P30 (Flaws, *et al.* 2001).

Bristol-Gould et al. (2006) proposed that this mechanism functions to eliminate poor quality oocytes. In P24 mice treated with activin during neonatal life, fewer ovulated oocytes were able to advance to metaphase II and these oocytes were smaller in diameter and had more spindle abnormalities than vehicle-treated control oocytes (Bristol-Gould, *et al.* 2006b). These observations suggest that excessive primordial follicle formation during the neonatal period results in growing follicles with poor quality oocytes. In this study, it is possible that maternal HF diet results in more follicles with reduced oocyte quality prior to puberty as secondary follicles of prepubertal HF offspring are smaller compared to control offspring and more atretic follicles are present (Table 3).

Poor oocyte quality may underlie this secondary follicle growth impairment as prepubertal offspring born to HF fed mothers have a decreased proportion of secondary follicle oocytes with activated Akt (Figure 3.6). Activation of Akt in cultured secondary follicles isolated from juvenile mice via addition of an Akt stimulating drug cocktail (PTEN inhibitor and PI3K activator) results in increased follicle diameter after 2 days in culture (Kawamura, *et al.* 2013) demonstrating that Akt activation stimulates secondary follicle growth. Therefore, in the present study, the decreased proportion of secondary follicle oocytes with activated Akt in prepubertal HF offspring likely contributes to the reduction in secondary follicle size. Furthermore, consistent with insulin-mediated changes in neonatal ovaries, a decrease in insulin may contribute to fewer secondary follicle oocytes with activated Akt. In previous work, it has been shown that these prepubertal HF offspring have decreased insulin levels compared to controls (Connor, *et al.* 2012). Therefore, it appears that future studies should investigate the effects of maternal HF diet induced obesity on insulin signaling in the ovary.

Prepubertal offspring born to HF fed mothers also show decreased circulating concentrations of E_2 despite comparable levels of both circulating FSH and LH between groups (Table 4). This may suggest that follicles have an impaired response to central gonadotropins. Responsiveness to gonadotropins is a major factor in determining follicle survival (Hsueh, *et al.* 1994, Hsueh, *et al.* 2014, Quirk, *et al.* 2004) and if growing follicles from prepubertal offspring born to HF fed mothers are less responsive to gonadotropins, more of these follicles are likely to undergo atresia. Consistent with this, prepubertal offspring born to HF fed mothers show increased numbers of atretic follicles

(Table 3). However, it is unclear how this may be occurring as mRNA expression levels of gonadotropin receptors, steroidogenic enzymes, sex steroid receptors, oocyte secreted factors, and apoptotic factors as well as oxidative stress markers and pro-inflammatory cytokine levels were similar between groups (Table 5 and Figure 3.7). Future studies should include protein measures of these regulatory factors to confirm that they are not involved in this follicle loss.

Therefore, overall in HF offspring, it appears as though "excess" oocytes assemble into primordial follicles during neonatal life and when these primordial follicles are recruited into the growing follicle pool prior to puberty, growth suffers and atresia is more likely to occur. Interestingly, it was recently shown that growth is also compromised in cultured secondary follicles derived from primordial follicles artificial activated using PTEN inhibitors (McLaughlin, *et al.* 2014) further reinforcing the fact that when primordial follicles are inappropriately recruited, follicle growth is impaired. In the present study, however, it is unknown what the driving factors are in this premature recruitment.

4.0.3 Adult Offspring

This study investigated the long term effects of maternal high fat diet induced obesity on the adult ovarian phenotype and sought to determine whether this phenotype was altered if these offspring were fed a postweaning high fat diet. Consistent with other reports, a postweaning high fat diet resulted in elevated primordial and atretic follicle numbers as well as decreased circulating levels of LH, but only in offspring born to mothers fed a control diet. Offspring born to high fat fed mothers also showed an

increased number of atretic follicles. This was likely due to a decrease in FSH responsiveness that could be mediated by a decrease in levels of GDF9 and/or an increase in expression of AMH in gonadotropin-responsive antral follicles. Interestingly, maternal and postweaning high fat diet also appeared to interact to affect the rate at which primordial follicles were recruited into the growing follicle pool.

4.0.3.1 Effects of a Postweaning High Fat Diet

A postweaning high fat diet resulted in adult offspring that displayed a reproductive phenotype similar to other models of obesity. Specifically, CON-hf offspring display an accumulation of primordial follicles (Figure 3.8A), an observation paralleled in New Zealand obese mice (Radavelli-Bagatini, *et al.* 2011). This increase in primordial follicle number could be a result of fewer ovulations as CON-hf rats have prolonged estrous cycles characterized by more time spent in estrus (Connor, *et al.* 2012). Consistent with this notion, mice fed a high fat diet also display estrous cycles characterized by prolonged estrus and this is associated with an inability to generate the LH surge, impairing ovulation (Sharma, *et al.* 2013a). Contradictory to the present study and Radavelli-Bagatini et al. (2011), Sprague-Dawley rats fed a standard chow diet supplemented with lard to induce obesity have a decrease in primordial follicle number (Wang, *et al.* 2014b). Contradictory results between these studies are likely attributable to differences in diet composition and/or primordial follicle counting methods, which are particularly prone to error when only a small number of sections are analyzed (Meredith, *et al.* 1999).

This study reports an increase in atretic follicle number as a result of a postnatal high fat diet (Figure 3.8F). This is consistent with many other reports (Radavelli-Bagatini, *et al.* 2011, Serke, *et al.* 2012, Wang, *et al.* 2014b). In the present study, a decrease in ovarian protein expression of IL6 (Figure 3.15H), a factor that can induce cumulus granulosa cell expansion (Liu, *et al.* 2009), may contribute to more follicles undergoing atresia locally. Centrally, a lack of gonadotropic support may also contribute to the observed increase in follicle death as CON-hf offspring have decreased levels of LH (Figure 3.10D) similar to obese women (Jain, *et al.* 2007). This could result in decreased binding of LH to its receptor on theca cells dampening the induction of androgen biosynthesis (Young & McNeilly. 2010), which is necessary for granulosa cell E_2 production (Figure 1.2). Local E_2 acts as an important survival factor for developing follicles (Billig, *et al.* 1993) and a decline in E_2 biosynthesis could lead to follicle atresia. However, circulating androstenedione (Connor, *et al.* 2012) and E_2 (Figure 3.10B) concentrations were not different between groups nor were the ovarian mRNA expression levels of gonadotropin receptors, steroidogenic enzymes, or sex steroid receptors indicating that the ovarian steroidogenic machinery is not impaired in these offspring. Additionally, apoptotic factors and levels of oxidative stress were similar between groups (Table 7). These observations are however limited by the fact that whole ovarian homogenates were used. Given that corpora lutea (CL) occupy the majority of the ovarian microenvironment at this age, gene expression results could be masked by CL expression. An alternate approach that could be utilized in future ovarian *in vivo* studies is laser

capture micro-dissection of specific follicle subtypes or even cell types within follicles, if possible, to eliminate the confounding effect of CL.

4.0.3.2 Effects of a Maternal High Fat Diet

Interestingly, offspring of mothers fed a HF diet display increased atretic follicle numbers as adults, despite being fed a postweaning high fat diet (Figure 3.8F). This suggests that early life exposure to a maternal HF diet has similar impacts on follicular atresia as a postweaning high fat diet (CON-hf). A contributing factor to follicular demise in HF-con offspring could be a compromised ability to respond to local levels of E_2 as the mRNA expression of ERα was significantly lower in HF-con relative to CON-con offspring (Table 7). E_2 signaling through ERα in theca cells has been shown to be necessary for antral follicle growth (Lee, *et al.* 2009) and a diminished local response to E_2 through ERα in HF-con offspring may reduce antral follicle survival. Similar to P27 offspring, impaired responsiveness to gonadotropins may also contribute to the relative increase in follicular atresia in HF-con offspring as circulating FSH concentrations were significantly higher than CON-con offspring (Figure 3.10A) without a concomitant increase in circulating E_2 concentrations (Figure 3.10BC). Interestingly, AMH immunostaining in antral follicles of HF-con offspring was higher than in antral follicles of CON-con offspring (Table 6). High levels of AMH have been shown to reduce granulosa cell responsiveness to FSH by decreasing cAMP production and aromatase and FSHR expression (Chang, *et al.* 2013, Durlinger, *et al.* 2001, Pellatt, *et al.* 2011). Therefore, reduced antral follicle FSH responsiveness as a result of increased AMH may explain why high circulating concentrations of FSH are not coupled with high levels of

circulating E_2 in HF-con offspring. Consistent with this notion, mRNA expression levels of the gonadotropin receptors and steroidogenic enzymes were similar between groups (Table 7).

It is unclear, however, the mechanism regulating an increase in AMH expression of antral follicles in HF-con offspring. Paradoxically, it has been shown that AMH expression can be stimulated by FSH through its second messenger cAMP in human granulosa cells (Taieb, *et al.* 2011). Therefore, if FSH responsiveness in HF-con offspring is decreased by AMH through a decrease in cAMP mediated signaling, AMH's own expression should also be decreased. This apparent paradox is likely attributable to the fact that FSHR signaling is more complex than simply activation of the cAMP pathway and its downstream signal could be altered by additional inputs (Landomiel, *et al.* 2014). One such input identified has been GDF9. Addition of GDF9 to cultured preantral follicles enhances FSH-induced follicle growth (Orisaka, *et al.* 2006). Conversely, injection of GDF9 antisense oligonucleotides into the oocytes of preantral follicles to block translation inhibits FSH-induced follicular growth (Orisaka, *et al.* 2006) demonstrating that levels of GDF9 influence FSH responsiveness. Interestingly, HF-con offspring have significantly lower mRNA expression levels of GDF9 than CON-con offspring (Table 7). Therefore, a decrease in GDF9 mRNA likely contributes to decreased FSH responsiveness in antral follicles of HF-con offspring.

4.0.3.3 Interactive Effects of a Maternal and Postweaning High Fat Diet on Adult Ovarian Outcome

This study shows significant interactive effects between the maternal and postweaning dietary environment on adult ovarian phenotype. Although a postweaning

high fat diet increased the number of atretic follicles in offspring born to control mothers, the reverse occurred in offspring born to high fat fed mothers (Figure 3.8F). Circulating levels of LH did not decline in HF offspring as a result of a postweaning high fat diet (Figure 3.10D). However, responsiveness to FSH still appeared to be diminished in HF-hf offspring as these offspring maintained a high circulating FSH to E_2 ratio relative to CON offspring (Figure 3.10C) and aromatase mRNA expression levels were reduced in HF-hf offspring compared to HF-con offspring (Table 7). Therefore, despite apparent decreased FSH responsiveness, fewer follicles in HF-hf offspring underwent demise. This may be a result of similar levels of antral follicle AMH immunostaining (Table 6) and mRNA expression of GDF9 and ERα (Table 7) in HF-hf offspring compared to CON offspring. Furthermore, the present study cannot exclude the possibility that a high circulating FSH to E_2 ratio is due to a central defect at the level of the hypothalamus and/or anterior pituitary rather than compromised responsiveness at the level of the ovary.

Interestingly, the interactive effect of a maternal and postweaning high fat diet on atretic follicle number was paralleled in transitioning follicle number (Figure 3.8B). This again supports the hypothesis that if a surfeit of follicles are recruited into the growing follicle pool, more growing follicles appear to undergo atresia. Conversely, this result could also mean that an increase in the number of growing follicles undergoing atresia causes a compensatory increase in the recruitment of primordial follicles. This observation is consistent with the hypothesis that a high primordial follicle recruitment rate is associated with a high rate of atresia and vice versa (Monniaux, *et al.* 2014).

One of the key factors regulating the relationship between the primordial follicle pool and the growing follicle pool is AMH. Consistent with the role of AMH in preventing depletion of the primordial follicle pool, the present study demonstrated that adult antral follicle AMH immunostaining correlated significantly with primordial follicle number (Figure 3.12A). Although growing follicle AMH production has been shown to limit primordial follicle recruitment (Durlinger, *et al.* 1999, Durlinger, *et al.* 2002), the mechanism mediating this effect is currently not well understood. Some studies have immunolocalized AMH to primordial follicles (Stubbs, *et al.* 2005), whereas, others have not (Durlinger, *et al.* 2002, Weenen, *et al.* 2004a). The present study identified positive AMH and AMHRII immunostaining in a proportion of primordial follicles in adult offspring (Figure 3.13A) demonstrating that AMH signaling is possible in a subset of these follicles. Interestingly, offspring born to mothers fed a high fat diet had a greater proportion of primordial follicles with positive AMH immunostaining (Figure 3.13B), however, it is unclear what consequences, if any, this has on recruitment rate.

Lastly, a postweaning high fat diet resulted in a decrease in total corpora lutea number only in offspring born to HF fed mothers (Figure 3.9B). This could be due to an increase in luteal regression or a decreased ovulation rate in HF-hf offspring compared to HF-con offspring. Given that estrous cycle abnormalities in HF-hf offspring (Connor, *et al.* 2012) would impede ovulation, the latter is likely. A reduction in corpora lutea number was also observed in rats that had been selectively bred to gain weight on a high fat diet (i.e. likely born to obese mothers) and when these rats were fed a high fat diet for

6 weeks (Balasubramanian, *et al.* 2012). Consistent with the results of the present study and previous work (Connor, *et al.* 2012), these rats also displayed irregular estrous cycles and failed to generate the LH surge (Balasubramanian, *et al.* 2012).

Investigations into adult ovarian function require females to be staged at one particular period of the reproductive cycle. In the present study, ovaries were collected at the diestrus stage of the cycle, representative of the nadir both in terms of follicle growth and endocrine changes. The present study uncovered effects of maternal and postweaning high fat diet on follicle growth, however, it is not known how poor early life nutrition affects terminal follicle development, endocrine status prior to ovulation and the quality of oocytes ovulated. Therefore, future studies could explore this by investigating follicle dynamics during proestrus or through artificially inducing ovulation utilizing a controlled ovarian stimulation (superovulation) protocol. However, the latter approach may mask any interaction between maternal and postweaning high fat diet as endocrine abnormalities have been observed in superovulated rats (Szoltys, *et al.* 1994). Moreover, epigenetic dysregulation has been observed in embryos conceived through superovulation and IVF (Doherty, *et al.* 2000, Stojanov & O'Neill. 2001, Waterland & Jirtle. 2004) suggesting that the epigenetic state of oocytes may be altered as a result of superovulation. Given that accurate assessment of the epigenetic state of oocytes is critical to determine how transmission of disease risk across generations through the maternal line occurs, this approach may not be ideal for future studies investigating oocyte epigenetics. Future experiments designed to measure oocyte epigenetic marks, such as methylation of imprinted genes, utilizing laser capture microdissection of oocytes during proestrus when

these marks are almost completely established (Pan, *et al.* 2012) would be useful in understanding transmission in oocytes. Another limitation of this study is that fertility of offspring was not measured. Future studies investigating fertility indices of these offspring would point towards functional impairments as a result of our observed follicular impairments.

4.0.4 Conclusions

In conclusion, this study demonstrates that maternal high fat diet induced obesity results in an increased number of oocytes assembled into primordial follicles during neonatal life, only to have this increase in number return to control levels during the prepubertal period. We speculate that these "excess" primordial follicles are of lower quality and are therefore, expunged from the pool of primordial follicles. This compensatory primordial follicle demise likely occurs via a decrease in the Akt-mediated survivability of primordial follicles or an increase in the probability of death as these follicles grow. As adults, follicle growth also appears to be impaired, but not when these offspring are fed a postweaning high fat diet. This is in line with the mismatch hypothesis of DOHaD, which states that when the early and later life environments do not match, detrimental phenotypes arise; whereas, when these two environments match, detriment is spared (Godfrey, *et al.* 2007, Uauy, *et al.* 2011). We speculate that these ovarian impairments in adulthood are partially driven by a decline in the oocyte secreted factor, GDF9, suggesting that a maternal high fat diet induces a defect in the oocytes of growing follicles later in life.

It is unclear what impact this impaired follicle growth has on oocyte quality and embryo development of the next generation. Previous work in this model has shown that offspring of mothers fed a high fat diet demonstrate significant metabolic dysfunction associated with obesity (Howie, *et al.* 2009). Given that trans-generational increases in disease risk exist (Frias & Grove. 2012), understanding how the maternal germline is impacted by poor nutrition is imperative. Future studies are needed to delineate the complex mechanisms at play so that they can be targeted with interventions to prevent the deleterious personal and societal costs of trans-generational inheritance of disease risk.

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