Diet-Induced Obesity Influences Oocyte Developmental Competence Via Peroxisome Proliferator-Activated Receptor Gamma (PPARG)-Mediated Mechanisms

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Abstract

Across the world more women of childbearing age are becoming overweight and obese. Although overweight women have similar co-morbidity and stigmata as men they also experience problems specific to their gender. In particular, there is significant evidence that overweight and obese women require a longer time to successfully conceive, suggesting influence of bodyweight and adipose tissue mass upon the events surrounding conception.

This thesis investigated the interaction between diet-induced obesity and female reproductive function. To achieve this, the influence of maternal obesity-induced insulin resistance on ovulation and oocyte health, as indicated by subsequent embryonic developmental competence was determined.

Obesity adversely affects many aspects of health, and rodent models of diet-induced obesity are commonly used to investigate these consequences. However the impact of strain and genetic background on phenotypic response to diet, particularly in females, has not been systematically defined. We therefore characterised female metabolic responses of five different strains of laboratory mouse (Swiss, Balb/c, C57BL/6, CBA/CaH and 129T2Sv/Ems) to a “Western” high fat diet (22% fat, 0.15% cholesterol) and matched control diet (6% fat, 0% cholesterol). After 16 weeks of diet exposure the development and extent of hyperglycaemia, hyperinsulinaemia, insulin resistance, dyslipidaemia, and markers of chronically inflamed adipose tissue depots varied profoundly across the different strains.

To then determine if a perturbed metabolic profile triggers female infertility, these female mice were mated with strain matched, non-obese males, and zygotes extracted from the reproductive tract immediately following fertilization. Despite strain-dependent variation in susceptibility to the development of obesity, dyslipidaemia and insulin resistance, all mice investigated exhibit some degree of impaired reproductive potential following exposure to a high fat diet. We documented alteration to ovulation incidence and rate, fertilization, early embryo development to the blastocyst stage, and blastomere differentiation into the inner cell mass and trophectoderm cell lineages.

The nature of obesity-induced perturbation of female reproductive processes was more closely examined using statistical modelling which identified the specific metabolic parameters that were strongly associated with reproductive defects. These associations were consistent across the range of genetic backgrounds assessed and highlighted key mediators of this interaction, in particular, insulin resistance.

To determine if ovarian gene products already implicated in other reproductive outcomes are differentially regulated under conditions of obesity, ovarian mRNA collected at the pro-estrous (pre-ovulatory) stage of the reproductive cycle was applied to microarray slides developed through Suppressive Subtractive Hybridization. Two different gene chips that were enriched for ovarian genes were used. A number of genes were minimally regulated, and there was lack of significant validation in subsequent, and larger, sample cohorts. These findings have provided substantial technical information, and new experimental designs that overcome the current limitations have been established to obtain more informative data.

The role that insulin resistance plays in folliculogenesis and the development of oocyte developmental competence was more closely investigated. Hyperinsulinemia can interfere directly with ovarian cell function or be indirectly associated with other hormonal conditions detrimental to optimal fertility. To reverse the effects of obesity/hyperinsulinemia and identify the signalling pathways responsible for disruption of pre-implantation events, obese female mice were treated for 4 days prior to mating with three different insulin-sensitizing and plasma glucose-reducing pharmaceuticals: glucose and lipid-lowering AMP Kinase activator, AICAR, 30mg/kg/day; IκK inhibitor that reverses insulin resistance, sodium salicylate, 50mg/kg/day; or Peroxisome Proliferator-Activated Receptor Gamma (PPARG) agonist rosiglitazone, 10mg/kg/day. AICAR or sodium salicylate treatment did not have significant effects on the reproductive parameters examined. However, embryonic development to the blastocyst stage was significantly improved when diet-induced obese mice were treated with rosiglitazone, effectively repairing development rates. Rosiglitazone also normalized obesity-associated abnormal blastomere allocation to the inner cell mass. Such improvements to oocyte quality were coupled with weight loss, improved glucose metabolism and changes in ovarian mRNA expression of PPARG-regulated cholesterol transporters.

Overall, this thesis has demonstrated for the first time a link between maternal obesity and the ovarian follicle can impede oocyte health and developmental potential. As a result, the oocyte released at ovulation expresses impaired developmental competence following to conception. Key cellular pathways have been identified in this relationship, specifically PPARG-directed cell responses.

Publications arising from this thesis:

- Minge CE, Bennett BD, Norman RJ, Robker RL *Peroxisome Proliferator-Activated Receptor gamma Agonist Rosiglitazone Reverses the Adverse Effects of Diet-Induced Obesity on Oocyte Quality.* Endocrinology 2008,149:2646-2656
- Minge CE, Robker RL, Norman RJ *PPAR Gamma: Coordinating Metabolic and Immune Contributions to Female Fertility.* PPAR Research 2008:243791 (in press).
- Minge CE, Bennett BD, Tsagareli V, Lane M, Owens JA, Norman RJ, Robker RL *Inflammatory and Metabolic Syndrome Phenotypes are Strain-Dependent in Female Mice with Diet-Induced Obesity* (in preparation)
- Minge CE, Bennett BD, Tsagareli V, Davies MJ, Owens JA, Norman RJ, Robker RL *Strain-specific Adverse Effects of a High Fat Diet on Ovulation and Oocyte Quality in the Mouse* (in preparation)

Abstracts arising:

2007

- Minge CE *Peroxisome Proliferator-Activated Receptor gamma (PPARγ) Agonist Rosiglitazone Reverses the Adverse Effects of Diet-Induced Obesity on Ovarian Function and Female Fertility.* Young Investigator of the Year, October 2007, Adelaide, Australia. (The Young Investigator Award is a highly successful event rewarding excellence in South Australia's young researchers in both science and their ability to communicate and 'sell' that science.)
- Minge CE, Bennett BD, Lane M, Norman RJ, Robker RL *Impaired oocyte developmental competence arises from diet-induced obesity and can be reversed by peri-ovulatory rosiglitazone treatment.* Annual Meeting of the Society for Reproductive Biology, September 2007, Christchurch, New Zealand.
- Minge CE, Bennett BD, Lane M, Norman RJ, Robker RL *Obesity-Induced Female Infertility Arises From Impaired Oocyte Developmental Competence And Can Be Reversed By Peri-*

Ovulatory Rosiglitazone Treatment. Ross Wishart Memorial Award, SA Australian Society for Medical Research (ASMR) Scientific Meeting, June 2007, Adelaide, Australia. (The Ross Wishart Memorial Award is presented to the most outstanding postgraduate presentation at the South Australian ASMR Annual Scientific Meeting.)

2006

- Minge CE, Bennett BD, Lane M, Norman RJ, Robker RL *Obesity-Induced Female Infertility Arises From Impaired Oocyte Developmental Competence And Can Be Reversed By Peri-Ovulatory Rosiglitazone Treatment.* 10th International Congress on Obesity, September 2006, Sydney, Australia.

- Minge CE PPARγ *Agonist Rosiglitazone Reverses the Adverse Effects of Diet-Induced Obesity on Ovarian Function and Female Fertility.* Young Investigator Award Semi-Finals, September 2006, Adelaide, Australia.

- Minge CE, Bennett BD, Lane M, Norman RJ, Robker RL *Peri-Ovulatory Rosiglitazone Treatment Reverses Obesity-Induced Female Infertility Arising From Impaired Oocyte Developmental Competence.* 39th Annual Meeting of the Society for the Study of Reproduction, August 2006, Omaha, USA.

- Minge CE, Bennett BD, Tsagareli V, Norman RJ, Lane M, Robker RL*. Ovulation and Oocyte Quality are Reduced in Mice with Diet-Induced Obesity.* 88th Annual Meeting of the Endocrine Society, June 2006, Boston, USA.

- Minge CE, Bennett BD, Tsagareli V, Norman RJ, Lane M, Robker RL *Ovulation and Oocyte Quality are Reduced in Mice with Diet-Induced Obesity.* "Five minutes of excitement and fame", "Womb to Tomb" Human Reproductive Health Throughout the Ages International Congress, March 2006, Adelaide Australia.

2005

- Minge CE, Bennett BD, Tsagareli V, Norman RJ, Lane M, Robker RL *Effects of Diet-Induced Obesity on Ovarian Function and Female Fertility*. Annual Meeting of the Society for Reproductive Biology, September 2005, Perth, Australia.

Abbreviations

17β-HSD	17 beta-hydroxysteroid dehydrogenase
3β-HSD	3 beta-hydroxysteroid dehydrogenase
A	androgen
aaRNA	uridine 5'-triphosphate-amino allyl ribonucleic acid
AcLDL	acetylated low density lipoprotein
AGE	advanced glycated ends
AGER	advanced glycated ends receptor
AICAR	5-aminoimidazole 4-carboxamide-riboside
AMPK	5'AMP-activated protein kinase
ANOVA	analysis of variance
ART	assisted reproductive technologies
AUC	area under the curve
BMI	body mass index
BMPs	bone morphogenic proteins
CC	clomiphene citrate
CD	control diet
cDNA	complementary DNA
CE	cholesterol ester
CETP	cholesteryl ester transfer protein
CL	corpus luteum
cm	centimetres
COC	cumulus oocyte complex
CRP	C-reactive protein
CT	threshold cycle
CYP17	cytochrome P450cyp17
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone-sulfate
DIO	diet-induced obesity
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

E/E2	estrogen
EDTA	ethylenediaminetetraacetic acid
FAI	free androgen index
FF	follicular fluid
FFA	free fatty acids
FSH	follicle stimulating hormone
g	grams
GDF9	growth differentiation factor 9
GEE	generalized estimating equation
GnRH	gonadotrophin releasing hormone
GV	germinal vesicle
GVBD	germinal vesicle breakdown
h	hours
HbA(1C)	hemaglobin A1C (glycosylated hemaglobin)
HDL	high density lipoprotein
HDL-C	HDL-cholesterol
HFD	high fat diet
HOMA	homeostasis model of assessment
HOMA-IR	HOMA-insulin resistance
ICM	inner cell mass
ICSI	intra-cytoplasmic sperm injection
IGF	insulin-like growth factor
IGFBP	IGF binding protein
IKK-b	IκB kinase beta
IL	interleukin
IPGTT	intraperitoneal glucose tolerance test
IU	international units
IVF	in vitro fertilzation
kg	kilograms
L	litres
LDL	low density lipoprotein
LDL-C	LDL-cholesterol

LH	luteinizing hormone
LMD	laser microdissection
M	metres
mg	milligrams
MJ	mega joules
ml	millilitres
mmol	milli mole
mRNA	messenger RNA
MZT	maternal-zygotic transition
NF-κB	nuclear factor-kappa B
ng	nanograms
NSAID	non-steroidal anti-inflammatory drugs
OGTT	oral glucose tolerance test
oxLDL	oxidized LDL
PBR	peripheral benzodiazepine receptor
PCOS	polycystic ovary syndrome
PCR	polymerase chain reaction
pg	picograms
pmol	pico mole
PPARG	peroxisome proliferator activated receptor–gamma
PPRE	PPAR response element
QUICKI	quantitative insulin-sensitivity check index
R2	coefficient of determination
RIA	radio-immuno assay
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	reverse transcription
RXR	retinoid X receptor
SDS	sodium dodecyl sulfate
SEM	standard error
SHBG	sex hormone binding globulin
SSH	Suppression subtractive hybridization

StAR	steroidogenic acute regulatory protein
T	testosterone
TE	trophectoderm
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
UCP	uncoupling protein
µg	micrograms
µl	microlitres
µm	micrometres
Vol	volume
WHO	World Health Organization
WHR	waist-hip ratio
Wt	weight

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Chapter 1

Literature Review

1.1 INTRODUCTION

Overweight and obesity are defined as abnormal or excessive fat accumulation that presents a risk to health, including a number of chronic diseases, such as diabetes, cardiovascular diseases and cancer. Once only considered a problem in high-income countries, overweight and obesity have risen dramatically in low- and middle-income countries, especially in urban settings.

A healthy bodyweight is considered a Body Mass Index (BMI) between 18 and 25 kg/m^2. Once a BMI exceeds 25 kg/m^2 it is defined as *overweight*, and once greater than 30 kg/m^2 it is defined as *obese*. The World Health Organization (WHO) now tracks the global incidence of overweight and obesity. Obesity rates are increasing globally, and more women of childbearing age are becoming overweight and obese (Linne 2004). With this has emerged a strong association between obesity and female reproductive disorders and infertility.

It is clear from WHO figures that overweight women are highly represented, particularly within the obese category. On a global scale, female rates of overweight (for women aged 30 years and above) are currently in excess of 75% of the population in approximately 21 counties (Figure 1.1a). Based on calculated projections of BMI for the year 2015, this level of overweight is estimated to continue increasing to include nations such as Australia, New Zealand, South Africa, and most of the North and South Americas (Figure 1.1b). This emphasises the profound scale, and relentless expansion, of the obesity crisis.

We are interested in the interaction between obesity-induced metabolic perturbations and female fertility. Female reproductive capacity contrasts with the male, in which new gametes are continuously produced throughout life. Females have a finite reservoir of oocytes, which, during storage within an ovarian follicle, are subject to the influence of the systemic nutritional and endocrine status. The quality of these oocytes contributes profoundly to the quality of subsequent embryonic development following fertilization. Furthermore, the legacy of prenatal events upon post-natal growth (Barker 1995) is now expanding to include these events occurring pre-implantation.

It is the objective of this literature review to summarize relevant background information, to review current understanding of how obesity is associated with female reproductive dysfunction, and to discuss potential metabolic mediators of this interaction.

Section 1.2 briefly summarizes the key metabolic dysfunctions that arise as a result of diet-induced obesity. Section 1.3 outlines fundamental concepts of ovarian function, and early embryo development, respectively. Section 1.4 describes current understanding of the relationship between bodyweight and female fertility based on clinical observations. Section 1.5 provides an overview of the how the ovarian follicle environment directed oocyte quality, addressesing the implications of metabolic dysfunction on the ovarian requirements for healthy folliculogenesis. Finally, section 1.6 outlines the experimental animal models available for such research, and substantiates the requirement for the well-characterised model developed in the first part of this thesis.

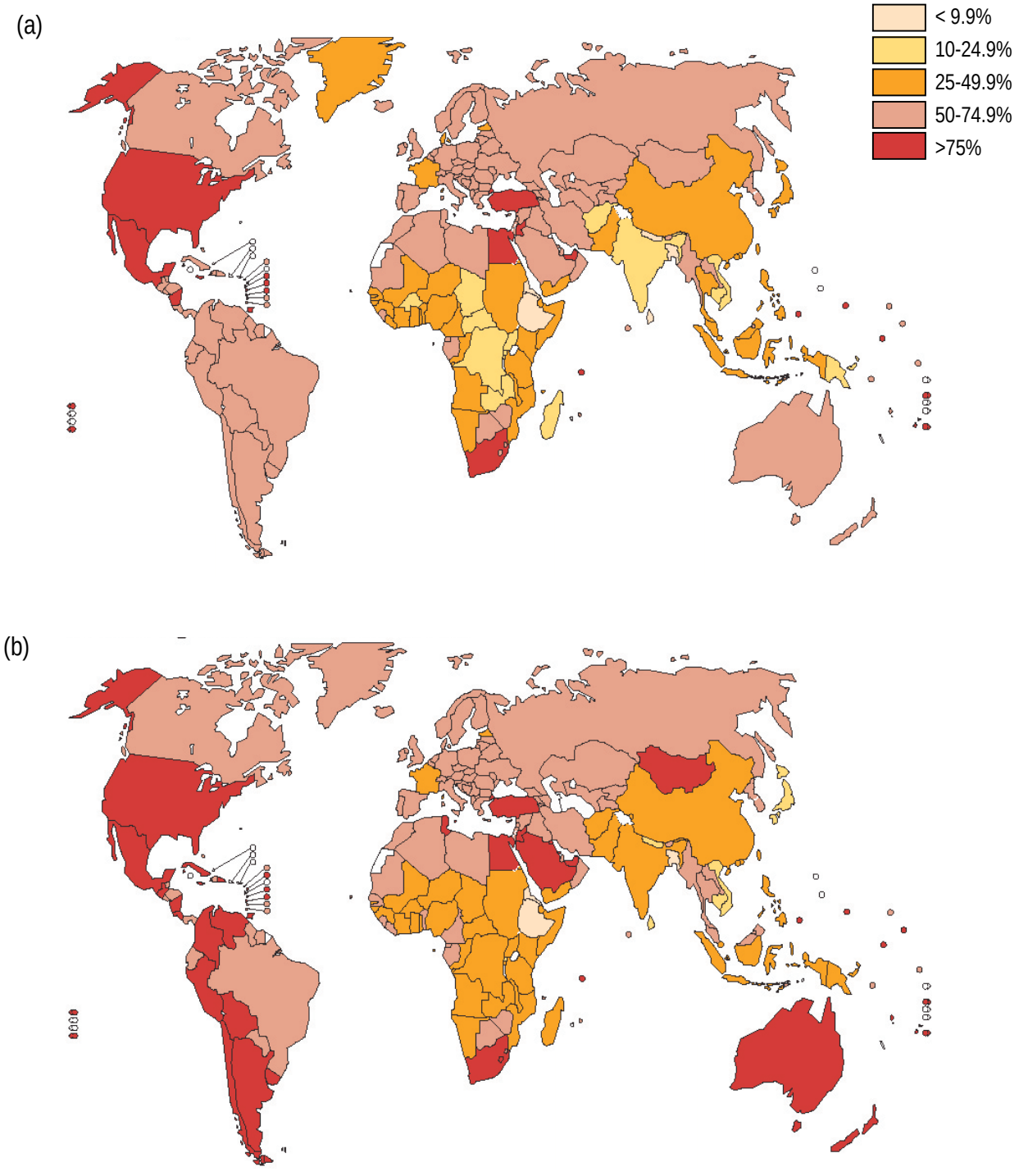


Figure 1.1 (a) Prevalence of overweight (BMI >25 kg/m^2), women aged 30 and above in 2005, and (b) projected prevalence of overweight (BMI >25 kg/m^2), women aged 30 and above in 2015 (Ono et al 2005).

1.2 OBESITY AND METABOLIC DYSFUNCTION

The accumulation of excessive deposits of adipose tissue during the development of obesity can have a multitude of consequences on a wide range of biological systems within the body (Figure 1.2). The role of adipose tissue as an endocrine organ capable of secreting a number of adipose tissue-specific or enriched hormones, known as adipokines, is gaining appreciation (Funahashi *et al.* 1999, Lazar 2005). In this way communication pathways are established between fuel reserves, the brain and other peripheral tissues that permit regulation of appetite and metabolism and in many situations can exacerbate the obese condition. Cumulatively obesity-induced changes lead to significant pathologies, particularly insulin resistance, but also type 2 diabetes, cardiovascular disease, hypertension, stroke, dyslipidemia, some cancers (Must *et al.* 1999) and, in females, reproductive disorders (Pasquali *et al.* 2003). Risks of these pathologies are especially correlated with fat localised to the visceral (or central) region (Kershaw & Flier 2004) which is usually distinguished from peripheral obesity as a waist-hip-ratio (WHR) of greater than 0.82 - 0.85. The mechanisms that account for the particularly deleterious effects of omental and mesenteric depots remain controversial, although it has been proposed that the regional differences in lipolysis, and subsequent release of free fatty acids (FFA) are involved (Jensen 1997).

There is strong evidence that obesity affects fertility rates in women within reproductive age groups (Norman & Clark 1998). However, whether the development of obesity is sufficient to disrupt fertility has not been adequately investigated. While there is strong clinical evidence that rapid weight gain, or the development of profound obesity during adulthood may worsen menstrual cyclicity and conception rates, predictive factors predisposing these outcomes are unknown. This emphasises the requirement for carefully performed investigations on this topic.

Outlined below are the mechanisms and processes accounting for the development of two key mediators of obesity-induced metabolic dysfunction: insulin resistance and dyslipidemia. In this thesis, the predictive value of such mediators, as indicators of specific reproductive disorders, is investigated, with the objective that their impact on specific ovarian processes will be revealed.

1.2.1 Insulin resistance

Insulin resistance is a condition in which normal amounts of insulin are inadequate to produce a sufficient insulin response from adipose tissue, muscle and liver cells.

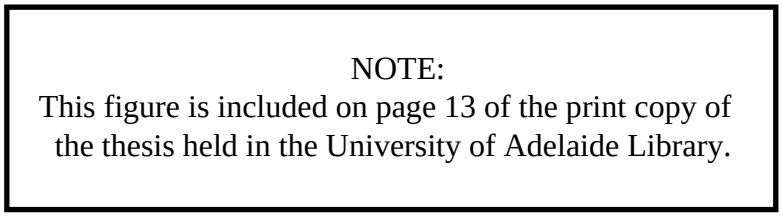


Figure 1.2 Tissues and signalling mediators involved in glucose and lipid homeostasis. Glucose derived from the diet or endogenous sources (such as gluconeogenesis) stimulates insulin secretion from the pancreas. Insulin promotes glucose uptake by skeletal muscle and adipose tissue, opposes hepatic gluconeogenesis, and inhibits fat lipolysis. Free fatty acids liberated from adipose tissue contribute to insulin resistance in skeletal muscle and liver. Additional adipose-derived signals, including TNF-α, resistin and adiponectin, modulate insulin sensitivity and fatty acid metabolism in muscle and liver. Figure adapted from Evans *et al.* 2004.

In a healthy individual with normal metabolism, a glucose challenge is met by insulin release from pancreatic beta (β) cells of the Islets of Langerhans. Post-prandial insulin signals insulin-sensitive tissues in the body (e.g. skeletal muscle and adipose tissue) to absorb glucose, thus lowering plasma glucose levels. Hepatic production of glucose via gluconeogenesis is also suppressed in response to post-prandial insulin. Glucose-responsive β cells reduce insulin output as blood glucose levels fall, with the result that blood glucose is maintained within a healthy range (approximately 5 mmol/L).

In an insulin-resistant individual, normal levels of insulin do not elicit the same signaling response from muscle and adipose cells, and as a result plasma glucose remains elevated. In the initial phases of insulin resistance, the pancreas is stimulated to respond, maintaining a state of compensatory hyperinsulinemia with gross decompensation of glucose tolerance being prevented. With increasing plasma concentrations of free fatty acids (FFAs), the obese insulin-resistant individual cannot continue to maintain this state of compensatory hyperinsulinemia, and hyperglycaemia prevails.

Insulin resistance was first recorded in association with obesity nearly 50 years ago (Rabinowitz & Zierler 1962). Since then it has been confirmed that plasma insulin concentrations are proportional to adipocyte volume (Woods *et al.* 1996). Visceral fat in particular, is a potent modulator of insulin action on hepatic glucose production. This effect is a result of 2 mechanisms:

(a) Peripheral and hepatic insulin resistance is influenced by secreted endocrine factors from visceral fat (Barzilai *et al.* 1999). In response to changes in metabolic status or specific extracellular stimuli, adipose tissue releases proteins such as tumour necrosis factor (TNF) α, leptin, adiponectin, interleukin (IL)-6 (Kershaw *et al.* 2004) and resistin (Steppan & Lazar 2004). The comprehensive functions of these adipokines are still being defined, and are occasionally controversial. It is clear however, that numerous adipose tissue-derived hormones impact insulin sensitivity (Rajala & Scherer 2003), in most cases to reduce the biological activity of insulin by interference with signalling events downstream of the insulin receptor.

(b) In addition the presence of lipids in the portal venous drainage of visceral adipose tissue which goes directly to the liver impairs hepatic glucostatic function (Bjorntorp 1990, Montague & O'Rahilly 2000). This elevated free fatty acid (FFA) flux directly into the liver particularly postprandially when it is usually suppressed by insulin, leads to inappropriate glucose production and decreased hepatic glucose utilization (impaired glucose tolerance). Reduced

hepatic clearance of insulin (Svedberg *et al.* 1990), leads to increased peripheral (systemic) insulin concentrations and to a further down-regulation of insulin receptors (Bevilacqua *et al.* 1987) and increased hepatic lipogenesis (Montague *et al.* 2000).

There is intriguing evidence that in the female, insulin actions are intrinsically different to the male. From studies associating neonatal insulin concentrations with birth weight it appears that females are at birth, and consistently throughout life, more insulin resistant than males (Wilkin & Murphy 2006). This gender-specific susceptibility to a pathologically insulin resistant state is associated with the counteractive effects of estrogen and androgens (Knopp *et al.* 2005). However, once full insulin resistance develops it is predictive of a greater cardiovascular risk in women compared to men (Pan *et al.* 1986). Such findings demonstrate the requirement for careful consideration of female obesity as distinct to the obesity-induced insulin resistance in males.

1.2.2 Dyslipidaemia

Hyperinsulinaemia and insulin resistance are both significant correlates of a dyslipidaemic state and contribute to the characteristic alterations of plasma lipid profile associated with obesity:

- Elevated fasting plasma triglyceride concentration
- Reduced plasma High Density Lipoproteins (HDL particles consist of a phospholipid monolayer studded with apolipoproteins AI, AII, CI-III, D and E encapsulating cholesterol ester and triacylglycerides)
- Increased plasma Low Density Lipoproteins (LDL phospholipid monolayer features only apolipoprotein B100)(Sniderman & Cianflone 1995).

Insulin plays a central role in the regulation of lipid metabolism, as insulin inhibits lipolysis by blocking lipoprotein lipase in adipose tissue, (Verges 1999). In the insulin resistant state, dyslipidemia is prominent, and data from the United Kingdom Prospective Diabetes Study (UKPDS) has shown that the effect of insulin resistance on plasma lipoprotein levels is more pronounced in women than in men (U.K. Prospective Diabetes Study 27. Plasma lipids and lipoproteins at diagnosis of NIDDM by age and sex 1997), with women having further decreased HDL levels, increased circulating triglycerides, and reduced clearance of LDL (Verges 1999).

In addition to these circulating lipoprotein abnormalities, insulin resistance also induces qualitative abnormalities to the lipoprotein particles themselves with pathological implications. In patients with insulin resistance with or without hypertriglyceridemia, increased activity of cholesteryl ester transfer protein (CETP) leads to an increase in LDL particles that are smaller and more dense (Okumura *et al.* 1998, Verges 1999). These small, dense LDL have a reduced affinity for apoB receptors, accumulate more rapidly within macrophages, and are more susceptible to oxidation (de Graaf *et al.* 1991), increasing further LDL uptake by atherogenic macrophages and foam cells. HDL particle modification includes abnormal enrichment with triglyceride, increasing their vulnerability to destruction by lipases (Briones *et al.* 1984).

Glycation of lipoproteins, (a process by which sugar moieties are added to proteins during hyperglycaemia), can also have profound effects on LDL and HDL metabolism. Glycated LDL decreases fibrinolysis, increases platelet aggregation, stimulates the expression of cell adhesion molecules by endothelial cells (Lopes-Virella *et al.* 1996), and exhibits an overall reduced rate of catabolism (Steinbrecher & Witztum 1984). Glycation of apolipoproteins in HDL decreases HDL receptor binding and may impair intracellular cholesterol efflux (Duell *et al.* 1990).

1.3 OVARIAN FUNCTION AND EARLY EMBRYO DEVELOPMENT

The central focus of this thesis is upon whether, and how, obesity-associated metabolic dysfunction may intersect with ovarian processes. The female ovary has two critical functions: to nurture and extrude mature oocytes capable of being fertilised by sperm in the reproductive tract; and to produce steroid hormones for both endocrine and autocrine purposes. Both of these functions are regulated through the hypothalamic-pituitary axis, and there are multiple sites at which obesity-induced metabolic dysfunction can interfere with the production of oocytes of optimal health.

Gonadotrophin releasing hormone (GnRH) from the hypothalamus stimulates the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary (Hillier 2001), which in turn direct folliculogenesis. Following follicle growth, and ovulation, a competent oocyte is ready for fertilisation, after which it is described as a zygote. The zygote then undergoes sequential mitotic divisions, (a process known as cleavage) with no substantial change in size. These embryonic cells then concurrently undergo differentiation into blastomeres as well as forming a blastocoel cavity, leading to development of a blastocyst. The ability of the oocyte to successfully fertilize and form a blastocyst is termed oocyte developmental competence- a quality which is acquired during its maturation in the ovary.

1.3.1 Follicle growth (folliculogenesis)

Folliculogenesis is the maturation of the ovarian follicle, a densely-packed shell of somatic cells that protect and provide for the developing oocyte, from small primordial follicles into large preovulatory follicles capable of ovulation (Figure 1.3). Oocyte ability to mature, be fertilized and finally to develop into a viable embryo is acquired gradually during progressive differentiation throughout folliculogenesis. In the mouse, follicular development can be categorised into 8 stages based on the number of granulosa cells and the progression of oocyte growth (Pedersen & Peters 1968). Similar patterns and classifications have been established for the development of rat and human follicles (Gougeon 1986, Gaytan *et al.* 1997). For the purposes of this review, follicle growth will be described as progressing through primordial, primary, secondary, antral and preovulatory stages, as ovulation approaches.

Follicles arise from resting primordial follicles consisting of an oocyte arrested in prophase of the first meiosis surrounded by a single layer flattened granulosa cells and a basement membrane. These are

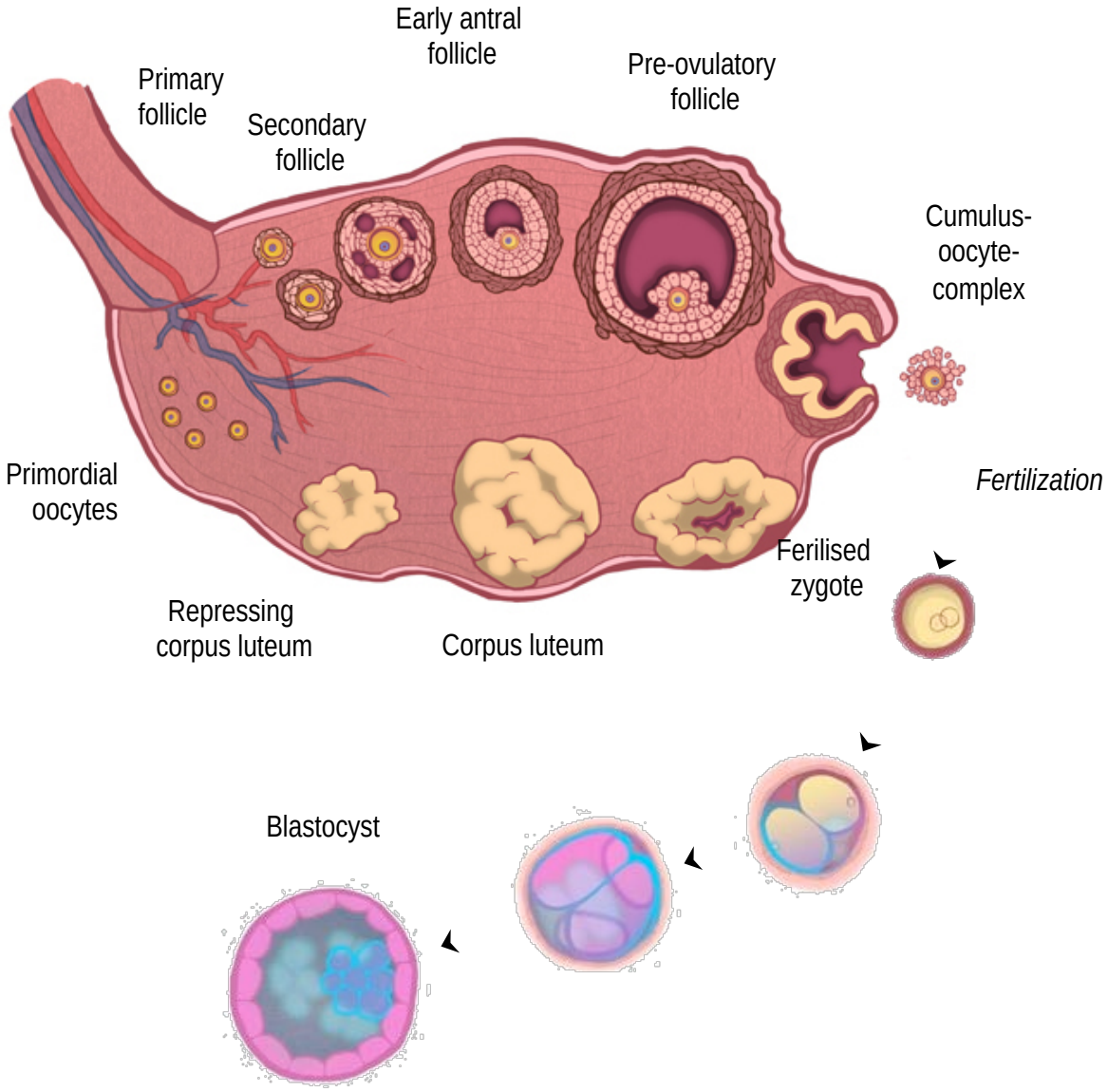


Figure 1.3 Schematic of folliculogenesis within the ovary and early embryo development. Primordial oocytes are recruited to developmentally progress until the secondary stage, whereupon hypothalamic FSH initiates cyclic recruitment. The follicular antrum forms and enlarges, and once receives the ovulatory LH surge, ruptures extruding the cumulus oocyte complex. The remaining follicular cells luteinize, whilst the ovulated oocyte, if fertilized, undergoes cleavage divisions forming a multi-celled embryo. These early embryonic cells first differentiate into either inner cell mass (ICM) or trophectoderm (TE) cells, which will be differentially stained blue or pink, respectively (original figure, artwork with assistance of D.R. Haynes).

located in the most superficial, cortical layer of the ovary. At this stage the granulosa cells become metabolically coupled with each other as well as with the oocyte via cellular processes passing through the developing zona pellucida to form gap junctions on the oolemma (Anderson & Albertini 1976). Follicular granulosa cells surrounding the oocyte not only produce growth factors and hormones but also provide the oocyte with physical support, nutrients (such as pyruvate), metabolic precursors (such as amino acids and nucleotides) and other small molecules that can be distributed between the compartments.

When primordial follicle activation is initiated, the layer of flattened granulosa cells transforms into a layer of cuboidal granulosa cells. Now called a primary follicle, the oocyte contained within also begins maturation processes, undergoing RNA synthesis and increasing in volume (Schultz *et al.* 1978). This initial recruitment through primordial follicle activation is believed to be a continuous process that starts just after follicle formation, long before pubertal onset, and appears to be independent of pituitary gonadotrophins. *In vitro* studies show that factors involved in the early stages of follicular development, include activin, transforming growth factor β (TGFβ), bone morphogenic proteins (BMPs), growth and differentiation factor 9 (GDF9), androgens, insulin and insulin-like growth factor-1 (IGF1) (Miro & Hillier 1996, Touraine *et al.* 1999, McGee & Hsueh 2000, Zeleznik 2004), although their roles *in vivo* remain to be fully established.

Granulosa-oocyte communication is essential for normal preantral follicle development. *In vitro* studies have shown that immature oocytes separated from granulosa cells do not grow, and oocytes must maintain gap junctions with granulosa cells (Tsafriri 1997). In mice, impaired oocyte-granulosa communication due to defects in connexin 37, a gap junction protein expressed at the oocyte-granulosa cell junction, leads to a block in follicle growth (Simon *et al.* 1997).

The acquisition of a second layer of granulosa cells and the formation of a primitive thecal layer of differentiated ovarian stromal cells marks the graduation of the primary follicle to the secondary follicle. Oocyte growth is a prominent feature of the growing follicle, but these oocytes remain arrested in the prophase of meiosis.

After puberty when there is sufficient FSH in circulation, cyclic recruitment begins. This phase of growth is completely gonadotrophin dependent, with LH receptors expressed on thecal cells and an increase in FSH receptors expression on granulosa cells. At the beginning of this tertiary phase, the fluid filled antrum forms, granulosa cells rapidly proliferate, and the thecal layer expands into two distinct layers

(vascular theca interna and smooth muscle-containing theca externa). During cyclic recruitment, only a limited number of follicles survive, with the majority succumbing to a process of programmed cell death or atresia. Although gonadotropins are the most important survival factors for preovulatory follicles, an elaborate intrafollicular control mechanism acting through endocrine, paracrine, autocrine, or juxtacrine mechanisms, are also required for growth of the antral follicle to the pre-ovulatory stage. Furthermore, continued communication between the follicle cells and the oocyte facilitates the transfer of both inhibitory and stimulatory meiotic signals to and from the oocyte. At this stage the oocyte is located within the follicular fluid filled antrum surrounded by a layer of differentiated granulosa cells called cumulus cells, and this cumulus-oocyte complex is secured to one side of the follicular wall (Brannstrom & Janson 1991).

It is critical to note that contrary to male spermatogenesis, which is carried out throughout life, the female follicle pool is not extensively, if at all, renewed after fetal development (Powell 2006, Liu *et al.* 2007), and the cycles of folliculogenesis are terminated when the follicle supply is exhausted (menopause). As a result, the systemic metabolic and nutritional status of the female individual, via signalling through somatic granulosa cells, can have long-lasting influence on the developmental potential of the oocytes eventually produced at ovulation.

1.3.2 Ovulation

Cyclic follicle recruitment culminates in ovulation, which is the rupture of the preovulatory follicle and expulsion of the cumulus-oocyte complex into the reproductive tract. This process can be arbitrarily separated into three phases: periovulatory, ovulatory, and postovulatory.

The large preovulatory follicle consists of many layers of granulosa cells lining the basement membrane, which is enclosed in several layers of thecal cells interspersed with blood vessels. For ovulation to be successful the connective tissue layers associated with the thecal layer and surface epithelium at the follicular apex must be degraded for the follicle to rupture.

The ovulatory phase begins through a signal transduction cascade initiated by a surge in LH released from the pituitary under the influence of LH releasing hormone and positive feedback of ovarian sex steroids (Espey & Lipner 1994). A marked influx of monocytes and neutrophils into the preovulatory ovary occurs after the LH-surge, due to expression of chemokines MCP-1 and IL-8. These leukocytes

are then activated and secrete mediators that facilitate tissue degradation and vascular changes to degrade the follicular wall at the site of the apex (Brannstrom & Enskog 2002).

The proteolytic degradation of the extracellular matrix (ECM) at the apex of ovulatory follicles prior to ovulation is facilitated by numerous proteases, including the matrix metalloproteinases (MMPs), plasminogen activator (PA)/plasmin, a disintegrin and metalloproteinase domain with thrombospondin motif (ADAMTS), cathepsin-L, pregnancy-associated plasma protein-A (PAPP-A), and bone morphogenetic protein 1/mammalian Tolloid (BMP-1/mTld). (reviewed (Ohnishi *et al.* 2005) and (Curry & Osteen 2001)). Degradation of the ECM is accompanied by increased vascular dilatation and permeability, which are also essential for follicular rupture (Murdoch *et al.* 1986, Abisogun *et al.* 1988). As this occurs, blood flow in the apex of ovulatory follicle decreases, while simultaneously increasing at the base of the follicle (Brannstrom *et al.* 1998).

Another morphological hallmark for ovulation is the period of cumulus cell proliferation and mucification known as cumulus expansion. Mucification is the secretion of hyaluronic acid that disperses and suspends the cumulus cell network in a structured matrix around the oocyte, persisting after ovulation and necessary for fertilization. At this stage an increase in antrum fluid volume causes the follicle to protrude at one site from the surface of the ovary forming a follicular apex, at which place ovulatory rupture occurs.

Under stimulation by the LH surge the fully-grown oocyte reinitiates meiosis, and now undergoes nuclear maturation as indicated by GV breakdown (GVBD; (Fan & Sun 2004)). As the microtubules become organised into a bipolar spindle and all chromosomes align at the spindle equator, the oocyte proceeds to the metaphase I (MI) stage and subsequently complete meiosis I and extrude the first polar body (the timing of these maturation events is outlined in Figure 1.4). Meiosis II follows, but the oocyte again arrests in the metaphase and remains so until fertilization. This transition from germinal vesicle stage oocyte to a metaphase II (MII) oocyte, is known as meiotic maturation, and is a 14–15 hr process (Wassarman & DePamphillis 1993). The cumulus-oocyte complex now leaves the ruptured follicle and moves out into the reproductive tract.

The postovulatory phase is also known as the luteal phase, as the remaining follicular cells luteinize and develop into the steroidogenic cells of the corpus luteum.

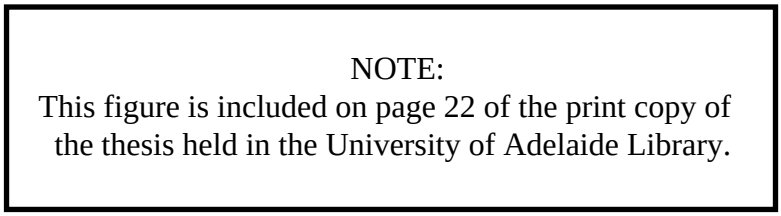


Figure 1.4 Timeline of ovulation and fertilization. Red numbers indicate hours post-LH surge, and blue numbers indicate hours post-fertilzation. This early developmental program is directed by maternally inherited proteins and transcripts, until the mid-2 cell stage when the zygotic genome is activated. Figure modified from Nagy *et al.* 2003 *Manipulating the Mouse Embryo.* 3 ed. New York: Cold Springs Harbor Laboratory Press

1.3.3 The corpus luteum

Following the LH surge and the ovulatory event, the theca interna and granulosa cells transform into a predominantly progesterone-secreting corpus luteum (CL). The CL becomes a highly vascularized structure containing epithelial cells, fibroblasts, connective tissue and leukocytes as well as two distinct types of luteal cells, denoted as small luteal cells and large luteal cells, which are derived from the thecal and granulosa cells respectively (Niswender *et al.* 2000). The primary role of the CL is to produce progesterone, which signals the endometrium to its secretory stage to prepare the uterus for implantation (Cummings & Yochim 1984). If fertilisation occurs, the CL responds to factors produced by the conceptus with further production of progesterone. This prevents initiation of another round of cyclic follicular recruitment and allows the maintenance of the pregnancy. If no signal is received directing the CL to maintain progesterone secretion, proteolytic factors from the uterus, predominately $PGF_{2\alpha}$, stimulate regression of the CL, which subsequently deteriorates over several cycles into ovarian scar tissue known as a corpus albicans (Pate 1994).

1.3.4 Ovarian steroidogenesis

A highly coordinated enzymatic cascade within the ovary converts cholesterol, a C_{27} steroid, into three types of carbon reduced steroid hormones: progestins (C_{21}), androgens (C_{19}) and estrogens (C_{18}) (Figure 1.5). Cholesterol ester (CE) can be delivered to the ovarian steroidogenic cells from HDL and/or LDL lipoprotein particles. In the rodent ovary, HDL supply the major share of the cholesterol used for progesterone production (Gwynne & Strauss 1982, Reaven *et al.* 1984). Within the human ovary, it has been less clear whether it is HDL, or LDL, responsible for supplying CE substrate. LDL is the primary circulating lipoprotein in humans, and the LDL receptor pathway for cholesterol uptake is highly developed in human cells (Richardson *et al.* 1992). However, HDL is the predominant lipoprotein within follicular fluid (Volpe *et al.* 1991), and can elicit efficient uptake, metabolism and steroidogenesis *in vitro* (Azhar *et al.* 1998).

To initiate steroidogenesis, cholesterol is transported into mitochondria via the cytoskeleton and sterol carrier proteins, where it is internalised through the action of steroid acute regulatory protein (StAR) (Miller 2007) and the peripheral benzodiazepine receptor (PBR) (Papadopoulos 1993). This cholesterol

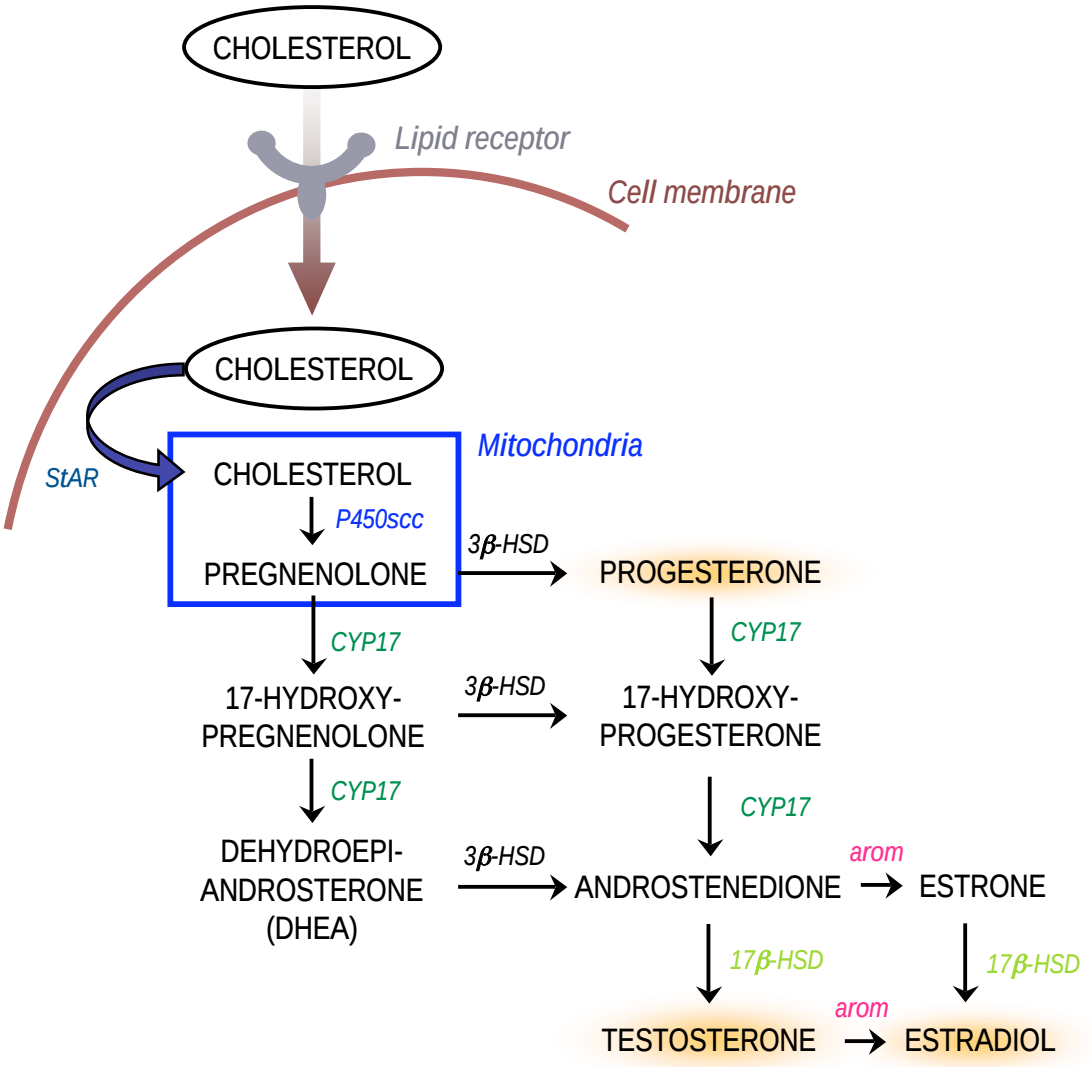


Figure 1.5 Steroidogenesis. Cholesterol is transported across the cell membrane via specific lipid regulators and entry into the mitochondria requires the activity of steroidogenic acute regulatory protein (StAR). Cholesterol is then converted to pregnenolone via the activity of cytochrome P450 sidechain cleavage enzyme (P450scc). Subsequent enzymatic conversions take place in the endoplasmic reticulum, via activity of cytochrome P450cyp17 (CYP17), hydroxysteroid dehydrogenase (HSD) and cytochrome P450aromatase (arom).

is subsequently metabolised by a progressive loss of carbon atoms in a series of hydroxylation reactions that are catalysed by three different cytochrome P450 enzymes.

Cholesterol is first converted to pregnenolone by cholesterol side chain cleavage Cytochrome P450, family 11, subfamily A (P450scc). Pregnenolone is then converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD) in the endoplasmic reticulum. Although progesterone production is predominantly restricted to cells within the CL, P450ssc and 3β-HSD are first expressed at lower levels in theca cells during the phase of follicular growth. During the luteal phase, both small and large luteal cell types contain 3β-HSD and as a result, are both capable of actively producing progesterone.

Androgens are produced from progesterone or pregnenolone in the thecal cells by steroid 17-alpha-Hydroxylase (CYP17) within ovarian theca cells (Doody *et al.* 1990). Androgen is the generic term for steroid hormones capable of binding to the androgen receptor, and includes dehydroepiandrosterone (DHEA), testosterone and androstenedione. Androstenedione is further converted to either testosterone or estrogens. Conversion of androstenedione to testosterone is catalysed by 17β-HSD. Androstenedione and testosterone are the predominant androgens in follicular fluid, remaining at fairly constant levels throughout antral follicle growth (McNatty *et al.* 1979). Subsequent conversion of androstenedione to estrogens (e.g. estrone and estradiol) requires the enzyme P450 aromatase (Simpson *et al.* 1994), and in the rodent occurs in granulosa cells using androgen precursor provided by the thecal cells. Estradiol is the major feedback mechanism from the ovary to the pituitary, reducing the amplitude or amount of LH and FSH released (Couzinet & Schaison 1993).

Stroma, theca and granulosa cells are all capable of steroidogenesis, but their specific patterns of steroidogenic enzyme expressions differ, reflecting different cyclic requirements for the secretion of sex steroids (Sasano & Suzuki 1997). In general steroidogenesis is geared prior to ovulation towards the production of estradiol by the dominant, preovulatory follicle, and in the luteal phase, towards progesterone production.

1.3.5 Fertilization

Fertilization (also known as conception) is fusion of gametes (specifically sperm and oocyte) to produce a new organism of the same species. To reach the surface of the oocyte, sperm must first penetrate the

cumulus mass, and then the zona pellucida. Upon binding to specific proteins within the zona pellucida, acrosome vacuoles within the sperm head fuse with the sperm plasma membrane, releasing various hydrolytic enzymes. The posterior part of the sperm head can now fuse with the oocyte plasma membrane, triggering the "zona reaction" which functions to prevent polyspermy. Fertilization prompts the second meiotic division and extrusion of the second polar body.

1.3.6 One-cell zygote to eight-cell uncompacted morula

From fertilisation to the first cleavage division takes about 20 hr in the mouse (Figure 1.4). This progression of the fertilized zygote from meiotic maturation through to the mid two-cell stage (27 hr post-fertilization), takes place in the absence of productive transcription (Flach *et al.* 1982, Clegg & Piko 1983). Therefore, the numerous changes to the spectrum of proteins synthesized during these two periods (Johnson *et al.* 1984) must be essentially dependent on maternal components accumulated during oocyte growth prior to germinal vesicle breakdown and ovulation.

As transcription from the zygotic genome begins, maternally inherited proteins and transcripts are degraded (reviewed in (Schultz 2002)). This transition is known as embryonic genome activation or maternal-zygotic transition (MZT), and sees maternal polyadenylated RNA rapidly decline to less than 30% of the amount present in the fertilized oocyte (Clegg *et al.* 1983).

From cleavage to the early 8-cell stage, each embryonic cell (blastomere) is roughly spherical and symmetrically organized. By the late 8-cell stage, each blastomere is transformed into a highly polarised cell, arranged axially from the centre of the embryo (baso-laterally) to the surface of the embryo (apically) yielding the first proto-epithelium of development (Fleming & Johnson 1988).

The orientation of this arrangement is directed by the asymmetric intercellular contact patterns of the blastomeres at the early 8-cell stage. At this early stage, the blastomeres have the capacity to re-orientate their polarity if their contact patterns are altered (Ziomek & Johnson 1980), although this capacity to regulate the axis of polarisation in response to cell contact is lost 4–5 h into the life of each 8-cell (Johnson & Ziomek 1981).

1.3.7 Compaction and the blastocyst

By day 3-4 the embryo consists of approximately 12-32 cells, and is referred to as a morula, from the Latin word for mulberry, reflecting its visual appearance. As cleavage proceeds to the 16-cell stage there is a gradual restriction of the equipotency demonstrated by the 8-cell blastomeres. This differentiation process starts with compaction, in which the blastomeres flatten and increase their contact with each other. Within the blastomeres there is increased calcium ion-dependent adhesiveness and establishment of gap-junction-mediated intracellular communication (Peyrieras *et al.* 1983). The subsequent position of cells within the compacted morula results in the generation of two distinct lineages: the trophectoderm (TE) and the inner cell mass (ICM). Cells located on the interior give rise to the ICM, whereas outer cells give rise to the TE which gradually develops apical, zonular tight junctions generating an impermeable outer epithelial layer. Blastocyst formation is mediated by trans-trophectoderm ion gradients, which drive the movement of water through aquaporins across the TE epithelium into the extracellular space of the blastocyst. As a result, a fluid-filled blastocoel cavity forms. The trophectoderm tight junctional permeability seal prevents the leakage of blastocoel fluid, and also assists in maintaining polarized distribution of cellular components in these mural trophectoderm cells (Watson & Barcroft 2001). During the fifth day of embryo development, the blastocyst hatches from the zona pellucida and is ready for contact with the uterine epithelium and implantation. Following implantation, the TE only gives rise to the placenta and extraembryonic membranes whereas the ICM forms all three germ layers of the fetus as well as complementary contributions to the extraembryonic membranes (Gardner & Papaioannou 1975). The division of cells into these two distinct lineages at this embryonic stage is one of the earliest markers of subsequent embryo viability. Specifically, reduced ICM numbers is indicative of poor viability (Hardy *et al.* 1989).

Early embryo development, especially prior to MZT, is critically dependent on maternal components accumulated during oocyte growth, prior to ovulation. Later embryonic indicators of these contributions include ICM/TE cell allocation.

1.4 OBESITY AND FERMALE INFERTILITY

A growing body of evidence shows that female fertility is significantly compromised under conditions of overweight and obesity (Zaadstra *et al.* 1993, Pasquali *et al.* 2003, Ku *et al.* 2006) (Summarized in Table 1.1). The first description linking obesity and altered reproductive function was by Stein and Leventhal in 1935 (Stein & Leventhal 1935) in their initial account of the cluster of characteristic symptoms that would later be defined as the Polycystic Ovarian Syndrome (PCOS). Much later, others (Rogers & Mitchell 1952) showed that 43% of women affected by various menstrual disorders, infertility and recurrent miscarriages were either overweight or obese. Since then, there have been extensive observational studies and epidemiological reports on the association of obesity, insulin resistance and infertility. A retrospective study examining body mass and the probability of pregnancy during assisted reproduction treatment revealed that an increase in BMI of 0.1-≥10 resulted in a reduction in the percentage of women achieving at least one pregnancy by 6-18% respectively (Wang *et al.* 2000). Even when pregnancy is achieved, obese or overweight women face increased risks of complications or miscarriage (Norman *et al.* 1998, Wang *et al.* 2000). The exact nature of the obesity-infertility lesions remains unclear. The review will now outline in more detail, the current state of understanding of the incidence and nature of this interaction.

1.4.1 Obesity and sex steroid imbalance

Obesity is associated with several abnormalities of sex hormone balance. Such alterations involve both androgens and estrogens and as well as their liver-derived carrier protein, sex hormone-binding globulin (SHBG) (Glass 1989). SHBG is reduced in women with increased visceral adipose tissue area and increased insulin response to oral glucose challenge (Haffner *et al.* 1988, Tchernof *et al.* 1999), and the association persists when compared to age- and weight-matched counterparts with peripheral obesity (von Schoultz & Carlstrom 1989). Testosterone unsequestered by SHBG in plasma (free and nonspecifically bound) is considered the bioavailable fraction, and thus decreased levels of SHBG available are linked with hyperandrogenism.

Obesity also affects the metabolism of androgens not bound to SHBG. Adipose tissue is crucial in controlling the balance of sex hormone availability in target non-fat tissues, and adipose tissue also functions to store various lipid soluble steroids in intravascular spaces. The steroid pool in obese

Table 1.1 Summary of studies describing the effects of high fat diet feeding on mice

Reference	Evidence of obesity-induced female reproductive dysfunction
Studies examining natural fertility and conception rates	
Rogers and Mitchell 1952	43% of women with menstrual disorders, infertility or recurrent miscarriage are either overweight or obese
Hartz *et al.* 1979	Increased incidence of anovulation, abnormal ovulation and menstrual abnormalities in obese women.
Green *et al.* 1988	Nulligravid women who are 120% or more over their “ideal” body weight are at increased risk for ovulatory infertility, although this association is not seen in women who have been previously pregnant.
Rich-Edwards *et al*. 1994	Elevated body mass index at age 18 is a risk factor for subsequent ovulatory infertility.
Lake *et al.* 1997	Overweight and obesity at age 7-23 years old increases the risk of menstrual problems, hypertension in pregnancy and subfertility
Kusakari *et al.* 1990	Obesity is related to anovulation and/or infertility, as determined by the delayed-reaction type in the LH-RH test: The rate of a delayed-reaction effect observed in a LH-RH test is 100% in anovulatory obese patients. Also, the pregnancy rate is lower in women who are obese
Balen *et al.* 1995	In women with previously identified polycystic ovaries on scan, obesity is correlated with increased rates of infertility and cycle disturbance.

Table 1.1 Continued

Reference	Evidence of obesity-induced female reproductive dysfunction
Studies examining natural fertility and conception rates cont.	
Grodstein *et al.* 1994	The risk of ovulatory infertility is highest in obese women but is also slightly increased in moderately overweight and underweight women.
Bolumar *et al.* 2000	In women who achieve a clinically detectable pregnancy, those who are obese require a longer time to conceive only if they also smoke.
Gensink Law *et al.* 2007	Obesity is associated with reduced fecundity for women regardless of menstrual cycle regularity, parity, smoking habits and age.
van er Steeg *et al.* 2008	The probability of a spontaneous pregnancy declines linearly with BMI > 29 kg/m^2 in subfertile ovulatory women.
Studies examining assisted conception	
Zaadstra *et al.* 1993	Increasing waist-hip ratio is negatively associated with the probability of conception per cycle, before and after adjustment for confounding factors. Body fat distribution in women of reproductive age seems to have more impact on fertility than age or obesity.
Lashen *et al.* 1999	Obese patients receiving exogenous hormone stimulation for ovulation induction have significantly lower peak estradiol levels. Despite this, bodyweight does not adversely affect the outcome of IVF-embryo transfer treatment.

Table 1.1 Continued

Reference	Evidence of obesity-induced female reproductive dysfunction
Studies examining assisted conception cont.	
Wittemar *et al.* 2000	Although no significant difference could be found in clinical pregnancy and miscarriage rates between normal weight, and overweight patients, obese women require more exogenous hormone stimulation, and yield fewer oocytes. Overall, overweight has negative effects on IVF parameters and outcome leading to decreased chances of pregnancy.
Loveland *et al.* 2001	Basal FSH, implantation rates, and pregnancy rates are significantly lower in overweight women, while the duration of stimulation, gonadotropin requirements, and spontaneous miscarriages are slightly higher compared to normal weight women.
Salha *et al.* 2001	Patients with a high BMI have significantly fewer oocytes aspirated, lower fertilization rate and reduced clinical pregnancy rate.
Mulders *et al.* 2003	Obese women suffering from anovulatory infertility are at an increased risk of having their IVF cycle cancelled due to insufficient response, although once oocyte retrieval is achieved, live birth rates are comparable with controls.
Fedorcsak *et al.* 2004	Obesity is associated with lower chances for live birth after IVF and ICSI and with an impaired response to ovarian stimulation.

Table 1.1 Continued

Reference	Evidence of obesity-induced female reproductive dysfunction
Studies examining assisted conception cont.	
Metwally *et al.* 2007	In women younger than 35 y/o, obesity has an adverse effect on average embryo quality, embryo utilization rate, the number of embryos discarded and cryopreserved. These effects do not persist for older women. Also, obesity does not have any significant effect on markers of oocyte quality or clinical pregnancy rates.
Maheshwari *et al.* 2007	Overweight women have a lower chance of pregnancy following IVF, require higher dose of gonadotrophins and have an increased miscarriage rate, compared to normal weight controls. Evidence of an effect of BMI on live birth, cycle cancellation, oocyte recovery and ovarian hyperstimulation syndrome was not identified however.

individuals is therefore greater than that found in normal-weight individuals (Azziz 1989). Adipose tissue is also a site of intensive sex hormone metabolism and inter-conversion, due to the presence of several steroidogenic enzymes, including 3β-HSD, 17β-HSD and P450aromatase (Azziz 1989, Gambineri *et al.* 2002). As a result, both production rates and metabolic clearance rates of dehydroepiandrostenedione and androstenedione are equally increased in obesity (Kirschner *et al.* 1990).

The effect of insulin concentration (elevated in obesity) on steroidogenic processes has been assessed. At present, there is limited knowledge about the specific effects of insulin on ovarian steroidogenic enzymes. Hyperinsulinemia potentiates ovarian hyperandrogenism by enhancing pituitary LH secretion, (potentiating ovarian CYP17 activity), and also acts in concert with FSH *in vitro* to increase ovarian granulosa cell LH-binding capacity (Adashi *et al.* 1985, Davoren *et al.* 1986, Dunaif 1999).

In vitro studies of both human and animal ovarian cells suggest a stimulatory effect of insulin on aromatase (Garzo & Dorrington 1984, Mason *et al.* 1994, Andreani *et al.* 1995, McGee *et al.* 1995). CYP17 activity also appears to be stimulated by insulin (Ehrmann *et al.* 1992, Nestler & Jakubowicz 1997), but studies of women with or without PCOS have found no correlation between insulin levels and the hormonal product of CYP17 activity, 17-hydroxyprogesterone (Sahin *et al.* 1997). Insulin increases P450scc enzyme mRNA in porcine granulosa cells (Flores *et al.* 1993), although this effect was not demonstrated in a human theca-like tumor line (McGee *et al.* 1996). In human luteinized granulosa cells, 3β-HSD expression is stimulated by insulin (McGee *et al.* 1995).

The consequence of high insulin levels *in vivo* is also unclear. In rats with experimental hyperinsulinemia, an increased estrone:androstenedione ratio was demonstrated, which is consistent with a stimulatory effect of insulin on aromatase (Poretsky *et al.* 1988). In comparisons of normoinsulinemic PCOS women to hyperinsulinemic women who also have PCOS, high insulin levels seem to increase the estrogen:androstenedione ratio following gonadotropin stimulation (Fulghesu *et al.* 1997). In women receiving insulin infusion a similar effect was seen (Dunaif & Graf 1989). Clinical situations where insulin is deficient are associated with reduced aromatase activity (Stamataki *et al.* 1996). However, circulating levels of androstenedione have been found to increase during insulin infusions in women, which in these cases suggest that insulin, in fact, inhibits aromatase function (Stuart *et al.* 1987). Thus, it remains unclear whether or how insulin regulates aromatase *in vivo*.

The effect of insulin on ovarian androgen production in women has been extensively examined in correlative studies, as well as experimental situations where circulating levels have been increased or

lowered. However, these studies are almost always carried out in women with PCOS, a disease of unclear aetiology and with complex hormonal dysfunctions. As a result it is difficult to gain clear perspective on how insulin may be influencing ovarian steroidogenesis.

Across these studies, insulin has been shown to either correlate positively with androstenedione and testosterone levels (Burghen *et al.* 1980, Pasquali *et al.* 1983, Chang *et al.* 1983, Elkind-Hirsch *et al.* 1991), or to be completely unrelated to the levels of these hormones (Anttila *et al.* 1991, Toscano *et al.* 1992, Buyalos *et al.* 1993). Also, the effect of increasing circulating insulin levels has been shown to alternatively increase androgen production (Stuart *et al.* 1987, Micic *et al.* 1988), or to not influence androgens at all (Nestler *et al.* 1987, Diamond *et al.* 1991a). Despite these inconsistent findings of how insulin may increase androgen levels, studies in which insulin levels were reduced have consistently demonstrated a decline in serum androgen levels in insulin-resistant hyperandrogenic women (Dunaif 1997). Whether insulin levels are lowered with diazoxide (Nestler *et al.* 1989, Krassas *et al.* 1998), octreotide (Najjar *et al.* 1992, Fulghesu *et al.* 1995), metformin (Velazquez *et al.* 1994, Nestler & Jakubowicz 1996, Nestler *et al.* 1997, Diamanti-Kandarakis *et al.* 1998), troglitazone (Dunaif *et al.* 1996, Ehrmann *et al.* 1997), or through weight loss (Kopelman *et al.* 1981, Bates & Whitworth 1982, Harlass *et al.* 1984, Kiddy *et al.* 1992, Clark *et al.* 1995), serum androgen levels decline and ovulatory function improves.

To date, much of the literature examining obesity, infertility and disrupted steroid hormone balanced has emerged from the study of PCOS, which is characterised by chronic hyperandrogenic anovulation (Mitwally *et al.* 1999). Although approximately 50% of PCOS women are overweight to some degree (Yen 1980, Conway *et al.* 1989, Franks 1989)), the pathogenic role of obesity in the development of PCOS is unclear. PCOS is a distinct syndrome, which derives from interactions between environment, genetic predispositions (Hickey *et al.* 2006) and likely pre-natal causes (Dumesic *et al.* 2005). This leads to specific ovarian and endocrine abnormalities that are distinct from, but exhibit some overlapping characteristics with those seen in obese women without PCOS.

1.4.2 Obesity and natural conception rates

Overweight women experience longer times to conception than women with moderate body weights (Gesink Law *et al.* 2007), an association that has been investigated by numerous population studies.

This indicates that in overweight and obese women, reproductive function is impaired pre-implantation, and that excessive body fat may lead to impaired ovulation or other defects in ovarian function.

As mentioned above, Rogers and Mitchell showed in 1952 that 43% of women affected by various menstrual disorders, infertility and recurrent miscarriages were either overweight or obese (Rogers *et al.* 1952). Following this, in 1979, a large study by Hartz et al. reported that the incidence of oligoamenorrhea or anovulatory cycles was significantly higher in obese women, compared to normal-weight controls (Hartz *et al.* 1979). Although the Oxford Family Planning Study, conducted in 1985 did not show any relationship between conception rates and weight or BMI in women who stopped taking contraceptive measures, the group selected for analysis was somewhat biased, consisting of mainly parous subjects (Howe *et al.* 1985). Others, such as Green et al. (Green *et al.* 1988) have since reported that although the fertility rate among women with more than 20% of ideal body weight is reduced, this does not apply to women who have previously been pregnant.

Since then, the Nurse Health Study of 1994 (Rich-Edwards *et al.* 1994) reported the risk of ovulatory infertility in later life increased in women with high BMI at age 18. Similarly, in a study of nearly 5800 women who were born in 1958 found that women who were overweight or obese in their early twenties were 1·32 and 1·75 times more likely, respectively, to have menstrual difficulties (Lake *et al.* 1997). Others, (Kusakari *et al.* 1990, Balen *et al.* 1995), have also reported that obese women have higher infertility rates. Grodstein and colleagues in 1994 showed that anovulatory infertility was higher in women with an overweight BMI, indicating even moderately excessive bodyweight may have an effect on fertility (Grodstein *et al.* 1994).

As part of a wider European study on infertility and subfecundity Bolúmar et al. (Bolumar *et al.* 2000) examined the effect of BMI on time to pregnancy, with consideration given to a wide range of biologic and lifestyle factors encompassing socio-demographic factors, diseases and conditions potentially related to fertility (pelvic inflammatory disease, cystic ovary, fibroids, endometriosis, appendectomy), height and weight, reproductive history, frequency of intercourse, contraceptive use, and lifestyle factors (cigarette smoking, caffeine intake, alcohol consumption). The results from this study suggested that a strong association between obesity and delayed conception in a subset of obese women who were also smokers. Interestingly, no association between obesity and delayed conception could be identified in non-smokers. A much more recent study on this topic was presented by Gensink Law et al., in which data of 7327 pregnancies collected in 1959 to 1965 (when smoking prevalence was high) was analyzed. After adjusting for age, time to pregnancy was reduced in overweight and obese women, compared with

women with an optimal BMI. In contrast to the earlier report, evidence of effect modification by smoking was not compelling (Gesink Law *et al.* 2007).

Even in women with confirmed ovulatory cycles, pregnancy rates can be impaired. Analysis of a prospectively assembled cohort of 3029 subfertile couples revealed the incidence of spontaneous pregnancy declines linearly with a BMI over 29 kg/m^2. Even corrected for possible related factors (age, duration of subfertility, previous pregnancy, semen motility and smoking status of both partners) women with a high BMI had a 4% lower pregnancy rate per kg/m^2 increase (van der Steeg *et al.* 2008).

Collectively these studies argue that although obesity disrupts menstrual cyclicity, conception rates are reduced even in cycling women. Thus, obesity leads to failure to conceive.

1.4.3 Obesity and Assisted Reproductive Technology (ART) outcomes

In ART hormones are standardized and synchronized. The influence of obesity on ART outcomes is currently unclear, but a disturbed ovarian response is consistently observed. A large percentage of women undergoing assisted reproduction technologies are overweight or obese, and currently, infertility treatments are not able to normalize pregnancy rates in these patients (reviewed Norman *et al.* 2004).

In a study of women attending a fertility clinic for artificial insemination, obese women were less likely to conceive than normal weight women, and this was found to be specifically associated with abdominal adiposity. A 0.1 unit increase in waist-hip ratio led to a 30% reduction in the probability of conception per cycle, even after adjustment for confounding factors (Zaadstra *et al.* 1993).

In contrast, a retrospective nested case-control 1999 study, Lashen et al. (Lashen *et al.* 1999) examined the effect of the extremes BMI on IVF-embryo transfer outcome. This study found there was no significant difference in clinical pregnancy rate, implantation rate or miscarriage rate when obese patients were compared to normal weight controls. In this study the only identifiable complication associated with obesity was a reduced peak estradiol concentration despite administration of similar gonadotrophin doses.

In the 2000 study by Wittemer et al. (Wittemer *et al.* 2000), how BMI related to different parameters of the IVF procedure and outcome was assessed in non-PCOS obese women. In this study the number of oocytes collected decreased significantly as BMI increased. Also overweight patients produced

significantly fewer oocytes of good quality than normally weighted women. However, no statistically significant correlation was identified between BMI and subsequent pregnancy rate in this study, although the miscarriage rate was increased in obese women. These findings were slightly different to those presented in another study (Loveland *et al.* 2001) in which a BMI>25 kg/m^2, negatively affected implantation but not oocyte number, in both initial, and follow-up cycles.

More insight into the influence of BMI on the hormonal stimulation required for successful ART was presented in 2001 by Salha and colleagues. In this study (Salha *et al.* 2001) the authors found that high BMI is detrimental to the success of IVF treatment, and that it also has influence on the metabolism of hCG. Patients who were overweight had reduced serum hCG concentration compared with controls and also required a higher dosage of gonadotrophin to stimulate follicular maturation. As in the previous study, the overweight group of patients had lower estradiol compared with controls, but here, overweight patients had significantly fewer oocytes aspirated. Further, the fertilization rate and clinical pregnancy rate per cycle were also lower in the overweight patients compared with those with normal BMI.

In 2003 a study examining IVF outcome in patients with anovulatory infertility found that elevated BMI predicted cycle cancellation due to insufficient response (Mulders *et al.* 2003). Despite this, overall live birth rates were comparable. The authors therefore concluded that once oocyte retrieval is achieved, BMI does not influence subsequent ART outcome.

In contrast, the authors of a 2004 study (Fedorcsak *et al.* 2004) reported that obesity is associated with lower chances for live birth after IVF and ICSI, and again, with an impaired response to ovarian stimulation. There was a negative correlation between BMI and the number of oocytes collected, and as a result the cumulative live birth rate within three treatment cycles was lower in obese women than in normal weight women. Obesity was further associated with an increased risk of pregnancy loss prior to 6 weeks gestation.

One of the few studies in which both oocyte and subsequent embryo quality was correlated with body weight was presented by Metwally et al. (Metwally *et al.* 2007a). The embryos were graded using a scoring system based on morphological characteristics including shape of blastomeres, texture of the cytoplasm and the degree of fragmentation. Although there was no evidence of a BMI effect on the average number of oocytes inseminated or the fertilization rate, when the results were stratified based on patient age, obese women <35 years of age had a lower embryo utilization rate, more embryos discarded, fewer embryos cryo-preserved, and a poorer mean embryo grade compared with the normal

BMI and overweight subgroups. The authors therefore suggested that the adverse effects of maternal age exceed obesity-induced effects, which as a result, only emerge in younger women.

The difficulty in conclusively establishing the effect of BMI on ART outcomes arises due to relatively small numbers of highly heterogeneous patients and major differences in technical and analytical methodologies. Also, substantial advances in embryo culture media composition, conferring vastly improved blastocyst survival rates contributes to differences between earlier and more recent studies. However, a recent meta-analysis confirms that overall BMI has a negative effect on IVF outcomes (Maheshwari *et al.* 2007).

1.5 THE FOLLICLE MEDIATES OOCYTE QUALITY

It is well accepted that oocyte–follicle communication is bidirectional and essential for both oocyte and follicular somatic cell function and development (Eppig *et al.* 1997, Eppig *et al.* 2002, Gilchrist *et al.* 2004). The timing of these critical follicular signals is still being established, but there is increasing evidence that oocyte quality is influenced by events occurring before germinal vesicle breakdown (GVBD), and that the oocyte must accumulate the appropriate information for meiotic resumption fertilization and early embryonic development prior to chromosome condensation.

Oocyte quality has been correlated with follicle size and developmental status (Pavlok *et al.* 1992, Lonergan *et al.* 1994, Blondin & Sirard 1995, Vassena *et al.* 2003), as well as other morphological indicators such as granulosa cell apoptosis (Nakahara *et al.* 1997) and follicular levels of reactive oxygen species (Seino *et al.* 2002). Follicular hormone levels have also been correlated with oocyte quality including: steroids, prolactin (Wise *et al.* 1987, Lindner *et al.* 1988, Chiu *et al.* 2002, Pena *et al.* 2002, Wunder *et al.* 2005), and IGFs and their binding proteins IGFBPs (Jimena *et al.* 1992, Artini *et al.* 1994, Kawano *et al.* 1997, Nicholas *et al.* 2005). Thus follicular events are tightly connected to the subsequent developmental competence of oocytes.

Leptin, the hormonal product of the obesity (ob) gene, has also been well studied in the ovary. It is found in oocytes, granulosa cells and follicular fluid (Karlsson *et al.* 1997, Cioffi *et al.* 1997, Ryan *et al.* 2003). Although the implications of intrafollicular leptin levels remains to be defined, some studies have suggested that leptin in follicular fluid is negatively correlated with embryo quality and pregnancy rate (Barroso *et al.* 1999, Mantzoros *et al.* 2000, Tsai *et al.* 2002). A link between follicular fluid concentrations of inflammatory mediators associated with obesity, such as TNFα and also nitric oxide, with oocyte quality has been identified (Lee *et al.* 2000, Baka & Malamitsi-Puchner 2006).

It is key to note that systemic nutritional status can influence endocrine and metabolic signals that regulate follicular growth, and influence oocyte development via changes in the components of the follicular fluid, or through direct granulosa–oocyte interactions. This has been demonstrated extensively in ruminants via short-term changes in dietary energy intake, in which nutritional status is significantly improved. Improved calorie intake is associated with an increase in the proportion of oocytes at metaphase II, and it has been proposed that this is mediated by increased IGF-I, leptin and LH concentrations. This optimization of nutritional availability can also induce significant improvements in

oocyte morphology, subsequent embryo development, and also prenatal survival (McEvoy *et al.* 1995, O'Callaghan & Boland 1999, O'Callaghan *et al.* 2000, Ferguson *et al.* 2003, Thatcher *et al.* 2006).

The current thesis is focused upon the impact that over nutrition, and subsequently perturbed metabolic parameters, may be having on the ovary, the follicle, the oocyte and the embryo in turn. Mediators of these effects are likely to be components of systems encompassing insulin-mediated glucose metabolism, and lipid metabolism (outlined in section 1.2 above). Evidence that these factors are able to adversely influence the oocyte is now summarized.

1.5.1 Impact of obesity on the oocyte: Insulin, glucose and lipids

The critical factor regulating of insulin signalling in the ovary is expression and function of the insulin receptor, and this has been well characterised in both human and animal models, with insulin receptor expression confirmed on all cell types including granulosa, thecal, and stromal cells (Poretsky *et al.* 1984, Poretsky *et al.* 1985, Poretsky & Kalin 1987, el-Roeiy *et al.* 1993, el-Roeiy *et al.* 1994) (Hernandez *et al.* 1992, Samoto *et al.* 1993). As in other tissues, insulin itself is a likely regulator of insulin receptor expression on the ovary. In rodents experimentally subjected with hyperinsulineamia, down-regulation of the ovarian insulin receptor has been observed (Poretsky *et al.* 1988). In humans however, there is additional regulation of insulin receptor expression by circulating factors such as gonadotropins, sex steroids, and locally produced IGFs and IGFBPs. It is key to note that within the ovary there is preservation of insulin receptor expression despite systemic hyperinsulineamia, and this provides explanation for the maintenance of insulin-specific ovarian effects, regardless of peripheral insulin resistance (Poretsky 1991, el-Roeiy *et al.* 1993, Samoto *et al.* 1993, el-Roeiy *et al.* 1994, Willis & Franks 1995, Willis *et al.* 1996, Nestler *et al.* 1998).

Numerous actions of insulin on the ovary have been demonstrated *in vitro* and *in vivo* through both pathways operating via the insulin receptor, and alternative receptors, with few differences between humans and other species (Poretsky *et al.* 1987):

- In a rat model, the growth- and cyst-promoting effects of insulin are directly exerted on the ovary during experimentally induced hyperinsulinemia. A synergistic interaction between LH/hCG and insulin in the ovary enhances hCG-induced ovarian growth and cyst formation

(Poretsky *et al.* 1992, Kuscu *et al.* 2002). In humans this effect can be observed in women with PCOS.

- Insulin can inhibit IGFBP1 expression in granulosa cells (Poretsky *et al.* 1996), which can in turn have negative implications for appropriately controlled follicle growth and atresia (Poretsky *et al.* 1999).

As outlined in Table 1.2, circulating levels of insulin can be greatly elevated under conditions of even normoglycemic obesity (Poretsky 1991, Dunaif 1992). Insulin reaches the follicular fluid (FF) from the circulation, most likely via transudation (Poretsky *et al.* 1999). FF insulin concentrations range from less than 2 μU/ml to 65 μU/ml, with a mean value of approximately 16 μU/ml (Diamond *et al.* 1985). This study reported no correlation with plasma insulin of FF estradiol or androstenedione concentrations, but a positive correlation directly with progesterone concentration.

A variety of clinical and experimental observations in states of either hyper- or hypo-insulinemia suggest that insulin may be involved, either directly or indirectly, in the process of ovulation. Insulin deficiency in type 1 diabetes (in which insulin is absent, but can be delivered exogenously to elicit normal signalling) has been associated with disordered ovulation (Poretsky *et al.* 1987). In rodent models of type 1 diabetes, the loss of insulin production is associated with cessation or reduction of ovulatory cycles, which can be restored with insulin treatment (Rogers *et al.* 1990, Powers *et al.* 1996). Hyperandrogenism and polycystic ovaries or ovarian hyperthecosis are common in states of extreme insulin resistance (Poretsky 1991). However, hyperinsulinemia under conditions of normal insulin sensitivity does not lead to the development of hyperandrogenism, but rather hypothalamic-pituitary-ovarian axis hypofunction (Griffin *et al.* 1994), which also impairs hormonal signalling essential for ovulation.

In animal studies of *in vitro* oocyte maturation, it has been reported that addition of insulin to media also containing FSH resulted in a dose-dependent reduction in oocyte competence to undergo fertilization, cleavage to the 2-cell stage, as well as the transition from the 2-cell stage to blastocyst (Eppig *et al.* 1998). This was subsequently found to be associated with significant alterations in protein synthesis pattern of the oocyte and its surrounding somatic cells (Latham *et al.* 1999).

There are conflicting reports regarding the effect of high insulin FF concentrations on human reproductive outcomes. In their 1992 study Jimena et al. (Jimena *et al.* 1992) revealed that in healthy

Table 1.2 Level of circulating insulin in women categorised as normal weight, obese, diagnosed PCOS or women with extreme insulin resistance (such as type 2 diabetes)

	FASTING	GLUCOSE LOAD
Normal	10 μU/ml	50 μU/ml
Obese	15 μU/ml	60 μU/ml
PCOS (or early Type 2 diabetes)	20-35 μU/ml	120-180 μU/ml
Extreme insulin resistance (Type 2 diabetes)	200 μU/ml	1400-2000 μU/ml

women there does not appear to be a difference in the concentration of insulin between follicular fluids from which mature oocytes that fertilized and cleaved were obtained, and follicular fluid associated with mature oocytes that did not fertilize. This agreed with others (Diamond *et al.* 1985). In contrast, Ng et al. 1993 found the concentrations of insulin in FF were inversely proportional to the health and developmental potential of the oocyte it surrounds (Ng *et al.* 1993).

The central function of insulin is to facilitate glucose uptake, a process that is disrupted under conditions of insulin resistance. The consequences of poor glucose utilization on oocyte maturation have been investigated using mouse models of Type 1 diabetes. In these animals, high glucose produced deficits in the regulation of meiotic resumption, ovulation, and the completion of meiotic maturation both *in vivo* and *in vitro*. (Colton *et al.* 2002, Colton *et al.* 2003). In these studies the authors conclude that cell-cell communication between cumulus cells and the oocyte is disturbed, and that such conditions may contribute to later post-fertilization developmental abnormalities. In both chemically induced and spontaneous Type 1 diabetic models there are significant delays in preimplantation embryo development, as well as a high incidence of degenerate and fragmented embryos observed. Furthermore, blastocysts retrieved from hyperglycemic animals contain fewer cells than those from normoglycemic control animals (Vercheval *et al.* 1990, Pampfer *et al.* 1990, Moley *et al.* 1991) (Beebe & Kaye 1991, Lea *et al.* 1996). This effect is also observed *in vitro*, where elevated glucose concentration in embryo culture medium also leads to impaired embryo development (Diamond *et al.* 1990, Diamond *et al.* 1991b).

At the ovarian level, there is an essential requirement for lipid substrate for steroid hormone synthesis, and as was outlined in Section 1.2.2, this cholesterol is obtained from binding of HDL and LDL particles. These lipoprotein particles are recognised by membrane-bound scavenger receptors such as SCARB1 and CD36. Both SCARB1 and CD36 engage in a selective pathway of cholesterol ester uptake. This differs from the classical apolipoprotein B/E receptor pathway of holoparticle uptake in that intact lipoproteins are not internalised by the cells, but, rather, cholesterol esters are extracted from the lipoproteins at the cell surface and are directly internalised (Reaven *et al.* 1988, Reaven *et al.* 1989). Movement of hydrophobic lipid molecules within the cytoplasm of the cell is then facilitated by a variety of binding proteins, such as fatty acid binding protein 4 (FABP4) (Gillilan *et al.* 2007), which can ultimately deliver the substrate for downstream conversion or signalling requirements.

Intracellular, cytoplasmic lipid is a key component of oocytes, although the relative concentration of this lipid varies widely across species. A pig oocyte contain high amounts of lipid, approximately 156 ng

(McEvoy *et al.* 2000), a value considerably higher than that contained within murine or bovine oocytes, which typically contain 4 (Loewenstein & Cohen 1964) and 58 (Ferguson & Leese 1999) respectively. Although cytoplasmic oocyte lipids also include cholesterol, phospholipid and non-esterified fatty acids, triglyceride is the major component of intracellular lipid (Homa *et al.* 1986) and provides a large intracellular energy store. The amount of triglyceride in immature oocytes decreases after *in vitro* maturation in both the pig and bovine (Ferguson *et al.* 1999, Sturmey & Leese 2003), supporting a metabolic role for triglyceride in the *in vitro* maturation of oocytes, and potentially also in preimplantation embryonic development (Ferguson & Leese 2006).

Mice with targeted homozygous null mutations in the SCARB1 gene exhibit skewed ratios of plasma lipids, specifically, elevated plasma cholesterol carried in both normal size, and abnormally large HDL particles (Rigotti *et al.* 1997, Braun *et al.* 2003). As a result, although SCARB1$^{-/-}$ females exhibit a normal estrous cycle and a normal post-mating increase in plasma progesterone, they ovulate oocytes that are dead or defective, and which cannot be fertilized or undergo embryogenesis (Trigatti *et al.* 1999). Furthermore, pre-ovulatory oocytes within the ovary are rescued when SCARB1-null ovaries are transplanted into wild type recipients. This suggests that although SCARB1 plays a critical role in mediating the delivery of HDL cholesterol, providing steroidogenic substrate, ovarian steroidogenic dysfunction alone is not responsible for the impairment of oocyte developmental potential in SCARB1$^{-/-}$ females (Miettinen *et al.* 2001). Instead, abnormal circulating lipoproteins, and abnormal lipid metabolism, when recognised by the oocyte somehow results in this defect.

The development of a good quality follicle is essential for oocyte health, later embryo survival and the maintenance of pregnancy. This supports the idea that embryo viability originates during oocyte development. There is substantial evidence that metabolic components of obesity, such as abnormal insulin levels, high glucose, and dyslipidemia can call induce strong effects on the oocyte (summarized in Figure 1.6). However, the cumulative effects of all the metabolic disturbances that accompany obesity are currently unknown.

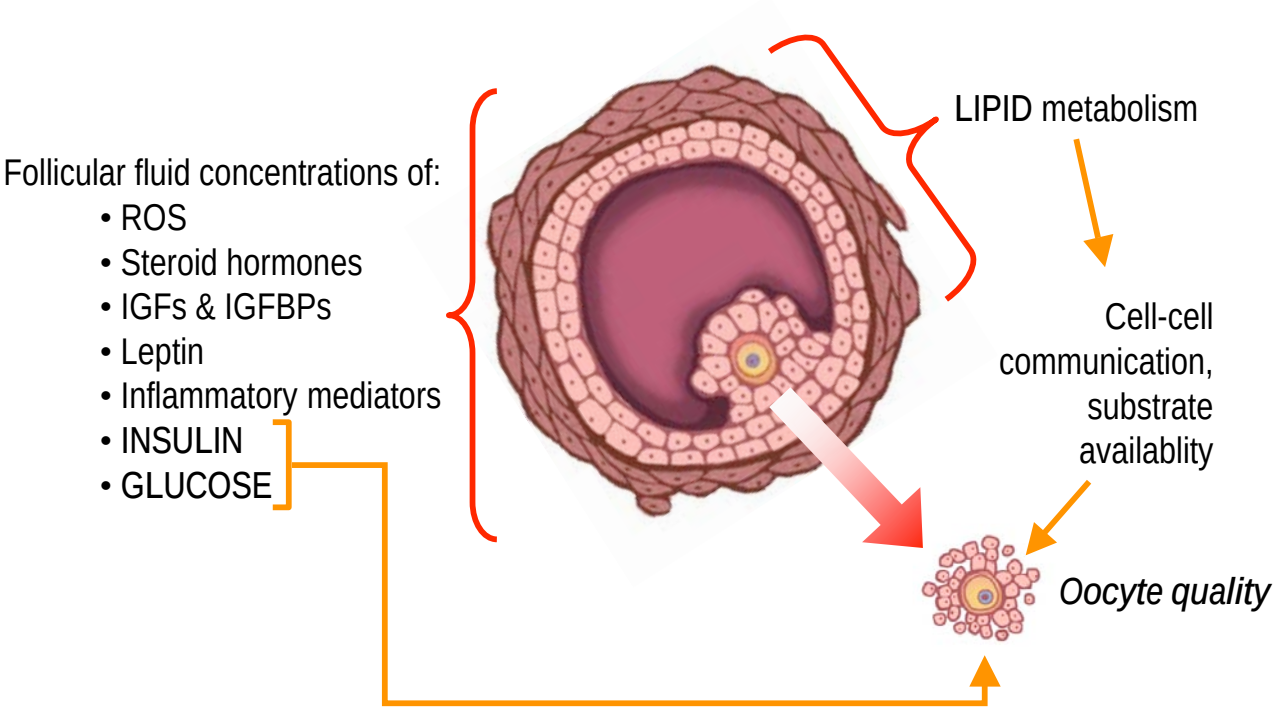


Figure 1.6 Metabolic factors influencing oocyte quality via the follicle. Numerous follicle factors have been shown to adversely affect the function of the follicle and the oocyte released. In particular, abnormal lipid metabolism, insulin and glucose have been demonstrated to do so both *in vitro*, and in genetic models of metabolic dysfunction.

1.6 ANIMAL MODELS FOR OBESITY RESEARCH

Animals with shorter life spans that can be maintained in conditions that carefully control for genetic and environmental influences are fundamental and indispensable tools for many types of scientific research. Animal models of diseases with relevance to human conditions are especially valuable, and establishment of well-characterised models is key, particularly in situations where human tissue specimens are not easily available, or experimental research on human subjects is considered unethical. For instance, much of the progress made in understanding the pathogenesis of PCOS and improvements in the treatment of PCOS symptoms, are the result of studies on experimental animals. PCOS-like symptoms can be induced in female rats pre-pubertally administered with testosterone (Beloosesky *et al.* 2004), although to date, one of the most successful animal models for PCOS is the prenatally androgenized fetal rhesus monkey, programmed to develop a permanent PCOS-like phenotype (Dumesic *et al.* 2005). These studies implicate critical times during fetal development when the hormonal, nutritional or metabolic status of the mother permanently alters the physiology of the fetus and modifies its genetic susceptibility to disease after birth. Similar finding are also reported that like-wise relate maternal insulin resistance and type 2 diabetes with offspring predisposition to the disease or related pathologies (Gerber *et al.* 1999, Pratley & Weyer 2001, Khan *et al.* 2003).

In the project described in this thesis, the mouse has been selected as the model. This is due to the comparatively minimal financial burden of rodent housing and feeding requirements, their rapid development to adulthood, and the comprehensive understanding of their reproductive biology. Many studies that have also used the mouse, both in the context of genetic models of obesity or dietary induction of obesity, have reflected upon associated conditions of female reproductive dysfunction.

1.6.1 Genetic models of obesity in the mouse

Identifying obesity genes is an attractive tool for elucidating the full impact of genetic profiles upon bodyweight. Numerous transgenic mouse models characterised by the development of obesity have been described. The most well known are mutations in the genes of the satiety hormone leptin and its receptor, ob and db, respectively.

The obese (ob) mutation was first described in the mouse in 1950 (Ingalls *et al.* 1950), as a recessive obesity phenotype in which mutants exhibit excessive obesity due to uncontrolled appetite and food

intake. In 1994 the adipose-derived, secreted protein leptin was determined to be the product of this gene (Zhang *et al.* 1994). The homozygous knockout of the leptin receptor, db/db (Tartaglia *et al.* 1995), also results in profound and early onset obesity (Bray & York 1979), nearly identical to the phenotype of ob/ob mice (Coleman 1978).

To date, studies investigating ovarian function in response to obesity have commonly utilized these genetically mutant ob/ob and db/db mouse strains. Infertility in these models is characterised by normal pre-pubertal development, but estrous cycles and ovulatory processes fail to initiate and the mice remain pre-pubertal indefinitely.

However, as with all genetic models it is difficult to determine whether the observed phenotypes are due to systemic or ovary intrinsic effects. For instance, there is an extensive, and very interesting literature clearly demonstrating that, at the ovarian level, the ob/ob mutants have perturbed folliculogenesis (Barash *et al.* 1996) including increased granulosa cell apoptosis (Hamm *et al.* 2004) and hypercytolipidemia (Garris *et al.* 1985, Garris & Garris 2003). However, the ob/ob genetic obesity model is complicated by the lack of hormonally controlled leptin signalling, which is known to be an important factor for ovarian cell function Leptin receptors are present in the ovary (Spicer & Francisco 1997, Karlsson *et al.* 1997), and have been shown to be cyclically regulated (Ryan *et al.* 2003), and influence ovarian gonadotropin and steroid secretion (Zachow & Magoffin 1997, Spicer *et al.* 1997, Karlsson *et al.* 1997). Indeed, ob/ob mice exhibit impaired fertility even when caloric restriction prevents excessive weight gain (Chehab *et al.* 1996) and exhibit altered folliculogenesis even when gonadotropin levels are restored exogenously (Olatinwo *et al.* 2005).

Other non-leptin targeted genetic models of mouse obesity also report associated infertility. One such example is the recently developed fat aussie (foz/foz) mouse, a model for Alström Syndrome, a rare autosomal-recessive condition (Arsov *et al.* 2006). The foz/foz mouse has a spontaneous 11-bp deletion (foz) in the mouse Alms1 gene. This mutation leads to significantly increased body fat, increased food intake at age > 60 days, initial hyperinsulinemia and eventual glucose intolerance and hyperglycemia. Although young female Alms1 foz/foz mice are fertile, their litter sizes are reduced compared to corresponding heterozygotes and wild type mice. Interestingly, following the development of obesity, female mutant mice are infertile. Their ovaries are found to contain only primary and secondary follicles and are devoid of corpora lutea, indicating an anovulatory state. Despite these interesting observation, no more is known about the mechanisms of this example of obesity-induced ovarian failure.

These examples of genetically determined obesity are not reflective of the majority of human obesity. Although much insight can be gained into the progressive influence of metabolic disturbance in reproductive capacity, for the most relevant application to clinical investigations, a model of diet-induced obesity must be applied.

1.6.2 Diet-induced obesity in the mouse

Many research groups have utilized high fat diet (HFD) feeding and the easy handling, rapid breeding cycle and well-characterized genetics of the mouse to investigate the pathophysiological consequences of obesity, insulin resistance and the Metabolic Syndrome. Overall these studies have established that the rodent diet-induced obesity (DIO) model is applicable to human obesity, such that the phenotype is associated with diminished sensitivity to the physiological effects of circulating leptin and insulin with concomitant increases in their circulating concentrations (Van Heek *et al.* 1997, Wang *et al.* 2001).

The beginning of the doctoral project was marked with careful analyses of the available literature to identify protocols and strains for such research. Appendix I provides the full details of diet composition, strain, age at feeding start, experimental duration and main findings of those studies in which relevant information could be identified.

These publications addressed divergent areas of research, and included

- 8 studies demonstrating the development of insulin resistance
- 4 studies indicating a link between HFD-feeding and diseased conditions
- 3 studies investigating HFD-feeding in transgenic strains
- 5 studies showing strain differences in susceptibility to obese phenotypes
- 1 study showing impaired fertility.

Further, differing percentages of fat, differing composition of lipids, differing amounts of cholesterol, and differing ratios of carbohydrate have been addressed. Percent fat content in the experimental HFD reported varied from 11.3-72%.

Collectively, these studies indicate strain-specific susceptibilities to these various, non-reproductive conditions, and reported on C57BL/6J, Swiss, DBA/2, 129, MSM, JF1, LG/J, SM/J, FVB/NJ, A/J ,Balb/c, AKR/J, C57L/J, C3H/HeJ, SJL/J, I/STN, and SWR/J. In addition, a number of transgenic lines were also investigated. The general consensus that emerges is that the C57BL/6 mouse represents an obesity-susceptible strain compared to others such as the Balb/c and 129 lines.

Further variability exists within the parameters of high fat diet feeding. Animal age at the start of the feed period varied from 3 weeks to 11 months of age, and the period of experimental during spaned 5 weeks to 9 months of feeding.

In addition to the studies highlighted in Appendix I, many other investigations have reported similar and other effects of HFD feeding on the pathogenesis of various diseases in the mouse: (Sinha *et al.* 1977, Ho & Chin 1988, Surwit *et al.* 1995, Parekh *et al.* 1998, Scrocchi & Drucker 1998, Murray *et al.* 2000, El-Haschimi *et al.* 2000, Surwit *et al.* 2000, Watson *et al.* 2000, Lambert *et al.* 2001, Le Lay *et al.* 2001, Ludwig *et al.* 2001, Masuzaki *et al.* 2001, Hildebrandt *et al.* 2002, Hileman *et al.* 2002, Bachman *et al.* 2002, Kumar *et al.* 2002, Joseph *et al.* 2002, Moon *et al.* 2002, Razani *et al.* 2002, Pierroz *et al.* 2002, Dube *et al.* 2002, Ishii *et al.* 2003, Felipe *et al.* 2003, Gavrilova *et al.* 2003, Brunengraber *et al.* 2003, Hildebrandt *et al.* 2003, Hancock *et al.* 2004, Bluher *et al.* 2004, Dhar *et al.* 2004).

Despite this large body of work, at the time this doctoral research project was initiated, only the work of Tortoriello et al. in 2004 (Tortoriello *et al.* 2004), had reported any correlation between HFD feeding and impaired fertility in the mouse. Here, HFD fed DBA/2J female mice weighing at least 20% more than those fed the control diet, displayed a 60% reduction in natural pregnancy rates. Quantification of hypothalamic cDNA revealed a 50% suppression of GnRH expression accompanied by a 95% attenuation of leptin receptor type B expression in obese female DBA/2J mice. No such effect was observed on C57BL/6 mouse, nor was weight gain or female infertility, despite these HFD-fed mice also developing insulin resistance. The authors therefore attributed the DBA/2J fertility effect to central leptin resistance and hypothalamic hypogonadism rather than insulin resistance. Interestingly, the experimental diet selected by this group to bring about DIO in the mice contained only trace amounts of cholesterol (0.02%). A diet consisting of more cholesterol may constitute a feed more comparable to the

"Western Style" diet consumed by humans (eg. ~ 0.15% cholesterol), and could possibly provide different weight gain and fertility results.

Collectively, the evidence that DIO in the mouse is a reliable and robust model, as well as the evidence presented by Tortoriello and colleagues, lays the foundations for this doctoral research investigation to interrogate the precise nature of the lesion in ovarian performance under conditions of obesity.

1.7 SUMMARY AND HYPOTHESIS

The association between excessive, centrally distributed body fat and impaired female fertility has been recognised for many years. However, the mechanisms that contribute to this association remain to be definitively determined. They likely encompass multiple and overlapping interactions, and may incorporate components of the obese phenotype with the development of oocyte containing follicles of the ovary, ovarian and extra-ovarian steroidogenesis, ovulatory signals and events, implantation and gestation.

This thesis focuses upon a critical aspect of the obesity-infertility relationship: the potential interaction between systemic nutritional status and the oocyte prior to ovulation (Figure 1.7), specifically, the effect of maternal obesity on oocyte developmental competence, that can consequently impact maternal capacity to produce a healthy embryo for implantation.

Hypothesis

Systemic metabolic signals are received by the ovarian follicle, which negatively influences oocyte quality. This will be evidenced in mice fed an obesogenic, high fat diet, which will develop a perturbed metabolic profile that consequently disturbs the ovarian follicular environment and resultant oocyte developmental potential.

Aim 1

To determine the metabolic consequence of high fat diet feeding compared to control diet, in a variety of laboratory mouse strains (Swiss, Balb/c, C57BL/6, CBA/CaH and 129T2Sv/Ems). A strain that consistently develops the metabolic dysfunctions that characterise human adiposity – weight gain, insulin resistance and dyslipidemia - will be sought.

Aim 2

To analyse indicators of ovarian function – ovulation, fertilisation, progesterone production and oocyte developmental competence, in mice fed a high fat diet. These analyses will be conducted in the strains listed in Aim 1, to determine whether strains with the most profound metabolic defects also have the greatest loss of reproductive capacity.

Aim 3

To identify specific metabolic parameters for statistical prediction of the incidence of indicators of impaired ovarian function.

Aim 4

To identify ovarian cellular pathways perturbed by high fat diet-induced obesity, utilising pre-optimised, custom-designed gene expression microarray analyses.

Aim 5

To elucidate critical pathways mediating obesity-induced ovarian dysfunction, by administering insulin-sensitizers that target distinct cellular pathways. Comparing the effects of a PPARG agonist (rosiglitazone), and AMP kinase activator (AICAR) and an NFκB inhibitor (sodium salicylate) will elucidate whether these specific pathways, known to be altered by insulin resistance, play a role in the manifestation of ovarian defects.

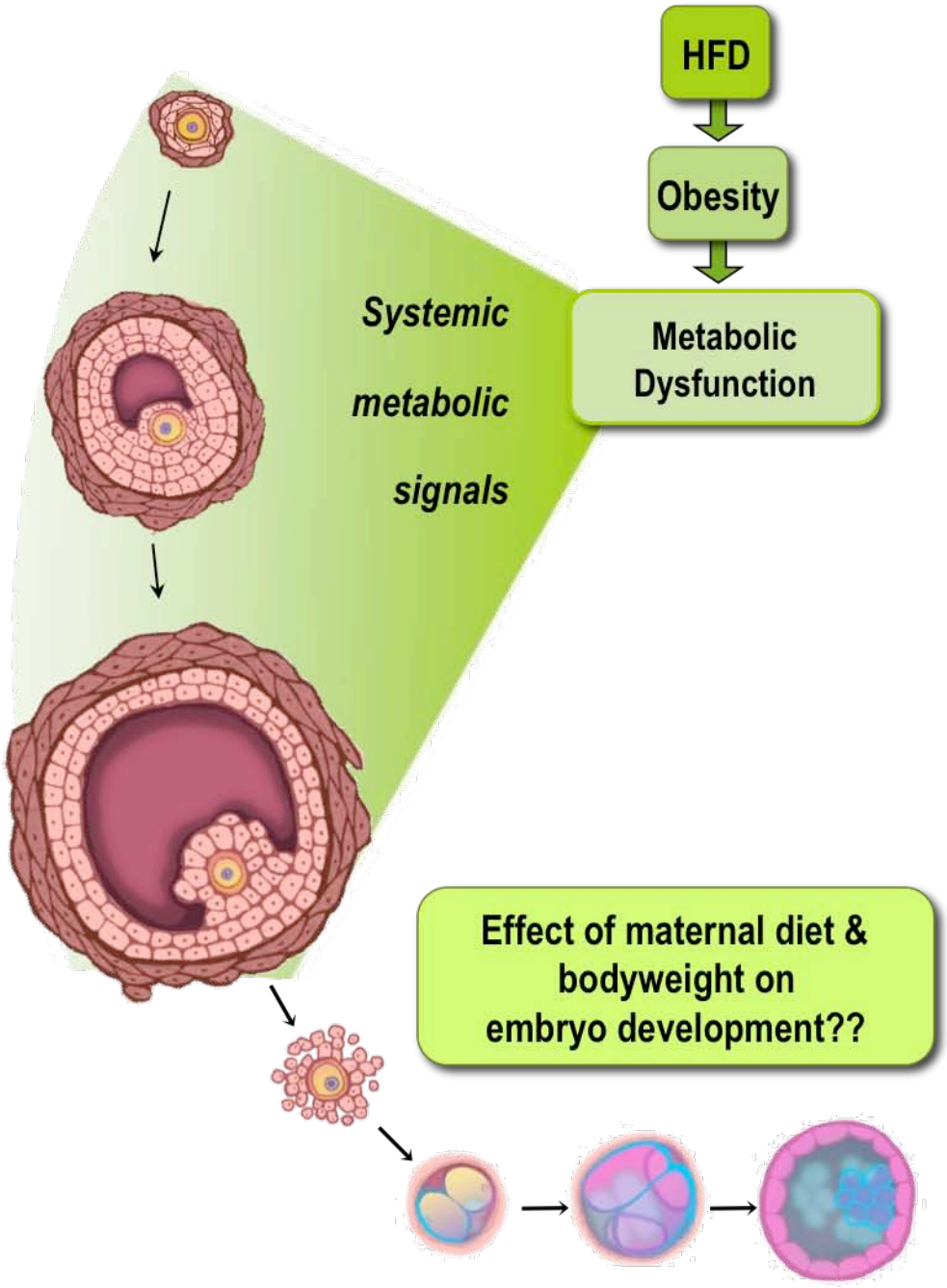


Figure 1.7 Experimental hypothesis. Systemic metabolic signals are received by the ovary and negatively influence oocyte quality.

Chapter 2

Materials and Methods

2.1 INTRODUCTION

This chapter describes methods utilized in the majority of subsequent chapters. All other methods used are outlined in the MATERIALS AND METHODS sections of the relevant chapters.

2.2 MATERIALS AND METHODS

2.2.1 Animals (background and source)

All mice were obtained from the University of Adelaide Laboratory Animal Services (LAS), Adelaide, Australia. Prior to breeding at LAS, all animals were sourced from the Animal Resource Centre (ARC), Western Australia in 1996-1999.

2.2.1.1 Swiss

The only outbred strain, in 1991 ARC obtained stock from Charles River Breeding Laboratories; these were acquired from Hauschka and Mirand Roswell Park. Swiss are considered a robust strain, and in reproductive biology Swiss females are commonly used as recipients receiving in vitro derived embryos. Mean litter size is 10.15 pups (Weatherly 1971).

2.2.1.2 Balb/c

ARC obtained the colony at F34 in 1970 from the Laboratory Animal Centre, England; these were sourced from Jackson Laboratory USA in 1955. MacDowell originally developed Balb/c's in 1923, and inbred them from Bagg Albino. Snell then distributed them worldwide in 1932 at F26. Balb/c are described as atherosclerosis-resistant (Paigen *et al.* 1987), but have high systolic blood pressure (Schlager & Weibust 1967), and high plasma cholesterol levels (Jiao *et al.* 1990). Mean litter size is 4.8 ± 0.06 pups (Verley *et al.* 1967).

2.2.1.3 C57BL/6

ARC acquired in 1981 from the Institute of Medical and Veterinary Science Adelaide at F108+. CC Little originally developed them in 1921 from a mating of Miss Abbie Lathrop's stock, Jackson then received them in 1937 and split them into 6J and 10J. C57BL/6 are susceptible to the development of key complications arising from the consumption of a high fat diet, including atherosclerosis, insulin resistance and glucose intolerance (Roberts & Thompson 1976, Mills *et al.* 1993). Mean litter size is 6.23 ± 0.06 pups (Verley *et al.* 1967).

2.2.1.4 CBA/CaH

ARC received the CBA/CaH strain from Combined Universities Laboratory Animal Services. Strong developed them in 1920 from Bagg albino x DBA, the offspring was then split into a low mammary

tumour incidence stain (CBA) and a high incidence strain (C3H). No reports of susceptibility to obesity or complications, but reported to have high systolic blood pressure (Schlager *et al.* 1967). Mean litter size is 5.8 pups (Festing & Blackmore 1971).

2.2.1.5 129T2Sv/Ems

The 29 parental strain was developed by Dunn in 1928 from coat colour stock from English fanciers and a chinchilla stock from Castle. The 129 T2Sv/Ems sub-strain is derived from the 129 congenic, which originally carried the teratoma mutation. No reports of susceptibility to obesity or complications, but reported to have high plasma cholesterol (Weibust 1973) and low plasma triglyceride levels (Jiao *et al.* 1990) when maintained on normal feed. Mean litter size is 4.5 pups (Festing *et al.* 1971).

A comprehensive discussion of the genetic origins of each strain can be found on the Jackson laboratory Mouse Genome Informatics website:

http://www.informatics.jax.org/external/festing/mouse/STRAINS.shtml

2.2.2 Animals (experimental protocols)

Upon arrival at 5 weeks of age, female mice were randomly allocated to either the control group (CD) or the high fat diet group (HFD) for 16 weeks. The control group was fed a diet containing 6% fat, 19% protein and 64.7% carbohydrate (SF04-057 Specialty Feeds, Glen Forrest, Australia), and the high fat diet group was fed a diet containing 22% fat (0.15% cholesterol), 19% protein and 49.5% carbohydrate (SF00-219 Specialty Feeds, Glen Forrest, Australia). The compositions of these two diets are shown in Table 2.1.

Males of all strains were 10-16 wks old at time of mating, were proven fertile, and had been maintained on normal rodent chow. All mice had free access to feed and water and were maintained at The Queen Elizabeth Hospital animal house at 24˚C on a 14L:10D illumination cycle.

The Animal Ethics Committees of both The Queen Elizabeth Hospital and The University of Adelaide approved all experiments and the animals were handled in accordance with the Australian Code of Practices for the Care and Use of Animals for Scientific Purposes.

Table 2.1 Composition of control diet and high fat diet

Ingredient	Control Diet	High Fat Diet
	g/kg	
Casein	195	195
DL Methionine	3	3
Sucrose	341	341
Wheat Starch	306	154
Cellulose	50	50
Canola oil	60	-
Clarified butter (Ghee)	-	210
USP Cholesterol	-	15
Calcium carbonate	17.1	17.1
Sodium chloride	2.6	2.6
Potassium citrate	2.5	2.5
Potassium dihydrogen phosphate	6.9	6.9
Potassium sulfate	1.6	1.6
AIN93G trace minerals	1.4	1.4
Choline chloride (65%)	2.5	2.5
SF00-219 vitamins	10	10
	Calculated Values	
Protein (%)	19	19
Carbohydrate (%)	64.7	49.5
Total Fibre (%)	9.4	9.4
Fat (%)	6	22
Digestible Energy (MJ/kg)	16.1	19.4

2.2.3 Measurement of glucose metabolism and insulin

After 15 weeks feeding, animals were subjected to an intraperitoneal glucose tolerance test (IPGTT). Mice were intraperitoneally injected with 1.5 g D-glucose/kg bodyweight after an overnight fast. Tail blood was sampled and blood glucose analyzed by an Accu-Chek® Advantage glucometer at 0, 15, 30, 60, 90 and 120 minutes post-glucose injection. Glucose tolerance was assessed by the area under the glucose curve (AUC). Lower values indicate a more robust response to glucose, and better glucose tolerance.

Samples for analysis of plasma insulin levels were analyzed by a Sensitive Rat Insulin RIA Kit (Linco Research, Inc. Missouri USA), with a sensitivity of 0.02 ng/ml, and an intraassay coefficient of 3.13%.

Insulin sensitivity was assessed by the homeostasis model assessment-insulin resistance (HOMA-IR) (Radziuk 2000), which incorporates fasting plasma insulin levels and fasting blood glucose levels (HOMA = [fasting insulin concentration (mIU/L) x fasting blood glucose level (mmol/L)] ÷ 22.5).

2.2.4 Measurement of plasma lipids

The circulating lipid profile of each mouse was determined from samples of serum utilising a Roche Cobas Mira automated sample system. Cholesterol levels were measured using the Cholesterol (CHOL-PAP) assay kit (Roche), and the mean coefficient of variation was less than 2.7%. Triglycerides were measured using the triglycerides (TRIG) assay kit (Roche) and the mean coefficient of variation was less than 2.6%. Each of these also used the Calibrator for Automated Systems and the Precinorm U and Precipath U Quality Controls (Roche). Free fatty acids were measured using the NEFA-C Free Fatty Acid assay kit (NovoChem) and quality controls: QCS 1 and 2 (Bio-Rad, Australia). The mean coefficient of variation was less than 4.6%. All assays have been validated for use in the mouse.

2.2.5 mRNA collection and preparation

Tissues to be used for gene expression analysis were snap-frozen in liquid nitrogen, and total cellular mRNA isolated using a Tri Reagent (Sigma Aldrich, St Louis, MO) protocol, with overnight precipitation at -20ºC.

2.2.6 Real-time RT-PCR

mRNA concentration and purity were determined using NanoDrop Spectrophotometer (ND-100, Biolab). 500ng mRNA was reversed transcribed using random primers (Roche, Castle Hill, Australia) and a SuperScript™ II RNase H- Reverse Transcriptase (Invitrogen, Carlsbrand, CA), RNaseOUT™ modified preamplification system for first strand cDNA synthesis according to the manufacturer's instructions. For each reverse transcription, a control was performed in which all incubations and buffers were identical, but no Superscript RT enzyme was added, verifying the absence of contaminating genomic DNA in PCR reactions. Complementary DNA templates were then subjected to fluorometric semi-quantitative real time PCR in triplicate. In Chapter 3 this was performed using the ABI PRISM® 5700 Sequence Detection System (Applied Biosystems), and in Chapters 5 and 6 using the Corbett Rotor-Gene™ 6000 (Corbett Life Sciences) real-time rotary analyser. In all chapters, real-time PCR was performed with SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA).

Chapter 3

Metabolic phenotypes in female mice with diet-induced obesity

3.1 INTRODUCTION

The number of reproductive-aged women becoming overweight and obese is increasing, as is the incidence of reproductive pathologies linked to female obesity. However, the onset of obesity and its ensuing complications has been much more extensively studied in men than in women, and indeed even in mouse models the majority of studies are undertaken using male mice. A suitable model of female diet induced obesity (DIO) is now urgently required, as obesity and its complications (the metabolic syndrome, Type II diabetes) are increasing in prevalence among women of reproductive age, and are associated with female infertility. Of particular concern is Polycystic Ovary Syndrome (PCOS), a disorder that shares many of the same diagnostic criteria as the Metabolic Syndrome (Balen & Rajkowha 2003) and is exacerbated by obesity, is now one of the leading causes of female factor infertility requiring assisted reproduction intervention (Norman *et al.* 2007).

Thus, it was the objective of this study to thoroughly characterise the metabolic perturbations that develop in response to a high fat diet in 5 different strains of female laboratory mouse. Mouse strains that developed a spectrum of disorders mirroring the Metabolic Syndrome in humans, including obesity, elevated circulating lipids, and insulin resistance and symptoms of chronic inflammation were sought. The overall hypothesis of this thesis; that such metabolic disturbances contribute directly to female infertility, could then be addressed.

Identification of mouse strains that develop a similar diet-induced obese phenotype to humans would permit useful application to the clinical situation. However, many rodent models used for obesity research are established through genetic mutation-induced obese phenotypes, and human obesity results primarily from excessive caloric intake and inadequate physical exertion, and is not, in the general case, due to genetic abnormality. In addition analysis of reproductive function in many of the genetic mutants used for obesity research, ie the ob/ob mouse which lacks leptin, are confounded by the fact that defects due to lack of gene function within the tissue of interest versus defects due to obesity can not be discriminated (Ryan *et al.* 2002, Garris 2004). For these reasons we designed our experiments using a mouse model of obesity induced in response to a high fat diet.

To date, the effects of high fat diet-induced obesity on general or specific physiological systems in rodent models have mostly been defined in only two or three strains, only males, and only for limited aspects of various physiological systems. For example, it is reported that obese male C57BL/6 mice are more likely to develop insulin resistance upon exposure to a HFD than male 129 mice (Almind & Kahn

2004), are less susceptible to islet dysfunction than DBA/2 (Kooptiwut *et al.* 2005), but are more inclined to develop excessive hepatic intracellular lipid droplets than C3H mice (Park *et al.* 2004). However, this does not comprehensively indicate which strain of these 4 would be most suitable for a specific investigation into diet-induced obesity (DIO)-associated insulin resistance, and significant gaps in knowledge remain.

Because the majority of obesity studies have utilized only male mice, little is known about the gender-specific responses and complications arising in female rodents. From humans we know that females have a higher amount of body fat and a more gluteal/femoral pattern of fat distribution, and it is evident that sex steroid hormones have an action on the metabolism, distribution and accretion of adipose tissues (Mayes & Watson 2004). Consistent with gender-specific fat deposition, subcutaneous adipose tissue has a higher concentration of estrogen and progesterone receptors (Price & O'Brien 1993, Mizutani *et al.* 1994, O'Brien *et al.* 1998), and intra-abdominal adipose tissue has a higher concentration of androgen receptor (Dieudonne *et al.* 1998). Directly influencing adipose tissue via both genomic and nongenomic mechanisms, sex steroid hormones can elicit significantly different effects in females, compared to males.

With this background knowledge, we set about cataloguing a range of metabolic parameters accompanying the development of obesity in outbred Swiss as well as inbred Balb/c, C57BL6, CBA/CaH and 129T2Sv/Ems female mice. We assessed daily caloric intake and established weight gain dynamics. Body composition of each animal was determined at post mortem, and we could observe the strain-specific influence of high fat diet upon distinct tissues.

Metabolic status, indicated by plasma lipids and glucose tolerance was assessed. In particular we focused on the incidence of elevated triglycerides, cholesterol and free fatty acids, each typically disordered in human obesity, and also incorporated into the criteria of the Metabolic Syndrome in women with PCOS (Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS) 2004). Critically important also were our measurements of glucose intolerance through resistance to the actions of insulin. Insulin has multiple targets within the ovary (Adashi *et al.* 1985, Davoren *et al.* 1986), and can contribute to hormonal imbalances, as well as general reproductive pathologies, with obesity (Pasquali *et al.* 2003).

Lastly, adipo-inflammation, as revealed by increased infiltation of macrophages to adipose tissue depots was assessed. This has been reported in both humans and male animal models of DIO (Weisberg *et al.*

2003, Xu *et al.* 2003, Cancello & Clement 2006) and low-grade inflammation is thought to contribute to the burden of insulin resistance and associated pathophysiology (Neels & Olefsky 2006). Based on work suggesting gender-specific differences in the ratios of cells that constitute adipose tissue (Robker *et al.* 2004), it is unclear if such a close relationship exists between bodyweight and adipose macrophage accumulation in female animals. We therefore aimed to examine the female response, evaluating the relative expression of two macrophage-specific genes *Emr1* (F4/80) (Hume *et al.* 2002) and *Cd68* (macrosialin) (Holness *et al.* 1993) in abdominal adipose tissue.

The results of this study establish the occurrence of excessive weight gain, insulin resistance, and dyslipidemia in female mice following diet-induced obesity. Furthermore, we report dramatic strain-dependent differences in susceptibility to the development of these Metabolic Syndrome-like symptoms.

3.2 MATERIALS AND METHODS

3.2.1 Animals and diets

Twenty-four female mice of each strain (Swiss, Balb/c, C57BL/6, CBA/CaH and 129T2Sv/Ems) were utilised for these experiments. Details on animal sourcing, handling and feeding are contained in Chapter 2.

3.2.2 Metabolic and endocrine measurements

Mice were housed in groups of 12 or 13 and body weights were determined weekly for 16 weeks. Daily food intake, presented as energy intake (MJ) normalised to number of animals housed together, was established by weighing food remaining in food tray of each cage each morning for 5-7 days, and dividing by the total weight of animals housed in each cage (see Appendix II for details regarding the conversion of energy in MJ to calories). Cumulative weight gain was determined for each mouse as the difference in bodyweight at week 0 and week 16 of diet exposure. Body length was measured as the number of centimetres from nose tip to anus. Body mass index (BMI) was calculated as $(\text{weight})/(\text{length})^2$, and presented in g/cm^2 .

Please refer to Chapter 2 for details on assessment of glucose tolerance, plasma insulin and lipids.

3.2.3 mRNA collection and preparation

After 16 weeks exposure to experimental or control diets mice were sacrificed and tissues collected. Abdominal (perigonadal) adipose tissue, retroperitoneal adipose tissue, liver, kidney, and spleen weights were determined. Total cellular mRNA was isolated as described in Chapter 2.

3.2.4 Real-time RT-PCR

Determination of mRNA concentration and purity, reversed transcription and semi-quantitative real time PCR (using the ABI PRISM® 5700 Sequence Detection System (Applied Biosystems)) was performed as described in Chapter 2. Ribosomal protein S3 (*Rps3*) was used as an internal control for every sample. For analysis of *Emr1* mRNA primers were designed using Primer Express™ software and synthesized by GeneWorks (Thebarton, South Australia, Australia), and *Cd68* primers described in Weisberg *et al.* 2003 (Table 3.1).

3.2.5 Statistical analysis

To evaluate differences between groups, data was subjected to one-way ANOVA with Tukey's post hoc analysis. For specific comparisons between HFD and CD outcomes, values were compared with a Student's t-test. In all cases differences were considered significant at $P < 0.05$. All statistical evaluation was performed using the software packages GraphPad InStat Tm version 2.04a (GraphPad Software Inc., San Diego, CA), and SigmaStat for Windows version 2.03 (Jandel Corp., San Ramon, CA). For genes of interest, mRNA content was calculated for each sample relative to the housekeeping gene, Rps3. Rps3 mRNA was unaffected by the treatment administered within this experiment, as the critical threshold (CT) value per microgram of RNA did not vary statistically across treatment groups. Before collation and analysis of data, samples with a CT value > 36 were considered negative and therefore not detectable. All real time RT-PCR data was analyzed using the equation $\Delta\Delta CT$, where ΔCT is the difference between the gene of interest and the housekeeping gene. Data were subsequently normalized to become the fold change compared to a designated group, which was 129T2Sv/Ems CD for both genes.

Table 3.1 Primer sequences

Gene	Accession #	Sequence
Emr1	NM_010130.2	F: 5'-CACATCCAGCCAAACAGAA-3' R: 5'-CTCGGATGCTTCCACAATCTC-3'
Cd68	NM_009853.1	F: 5'-CTTCCCACAGGCAGCACAG-3' R: 5'-AATGATGAGAGGCAGCAAGAGG-3'
Rps3	NM_012052.2	F: 5'-ATCAGAGAGTTGACCGCAGTT-3' R: 5'-AATGAACCGAAGCACACCATAG-3'

3.3 RESULTS

3.3.1 Energy consumption, bodyweight and length

Intake of energy from feed was influenced by an interaction between strain and diet available at both experimental timepoints (week 1 P=0.036; week 16 P=0.001). In the first week of diet exposure, energy consumption (normalised to bodyweight) was significantly increased when HFD was available to Swiss, Balb/c, CBA/CaH and 129T2Sv/Ems mice (Figure 3.1a). After 16 weeks of diet exposure Swiss and CBA/CaH HFD animals were still consuming more energy relative to their bodyweight, compared to CD controls (Figure 3.1b). However, daily intake of 129T2Sv/Ems was no longer significantly different between CD and HFD groups.

The dynamics of weight gain for mice of each strain over 16 weeks of exposure to control or high fat diet are illustrated in Figure 3.2a-e. The initial bodyweights of female mice of each strain at 5 weeks of age were similar, but responsiveness to the effects of the HFD was subsequently determined by strain. C57BL/6 HFD female mice had gained significantly more weight than C57BL/6 CD mice after only 7 days of diet exposure, whereas Swiss, Balb/c, CBA/CaH and 129T2Sv/Ems mice required 2, 10, 6 and 6 weeks respectively. Cumulative weight gain of Swiss mice maintained on HFD or CD did not differ consistently at any point after 3 weeks of diet exposure. At the conclusion of the feeding protocol the cumulative weight gain of C57BL/6, CBA/CaH HFD groups was dramatically greater than CD groups whilst weight gain was more modestly increased by HFD in Balb/c and 129T2Sv/Ems groups.

At the conclusion of the feeding period, C57BL/6, CBA/CaH and 129T2Sv/Ems female mice were significantly heavier (P=0.0002, P=0.005 and P=0.007 respectively, Figure 3.3a). Weight of Balb/c HFD females tended to be higher as well (P=0.08). At this time animal body length was also significantly increased in Balb/c, C57BL/6 and 129T2Sv/Ems HFD mice (Figure 3.3b). The rodent body mass index, calculated to incorporate both body size and weight was only higher than CD in C57BL/6 mice fed the HFD (P=0.0035, Figure 3.3c).

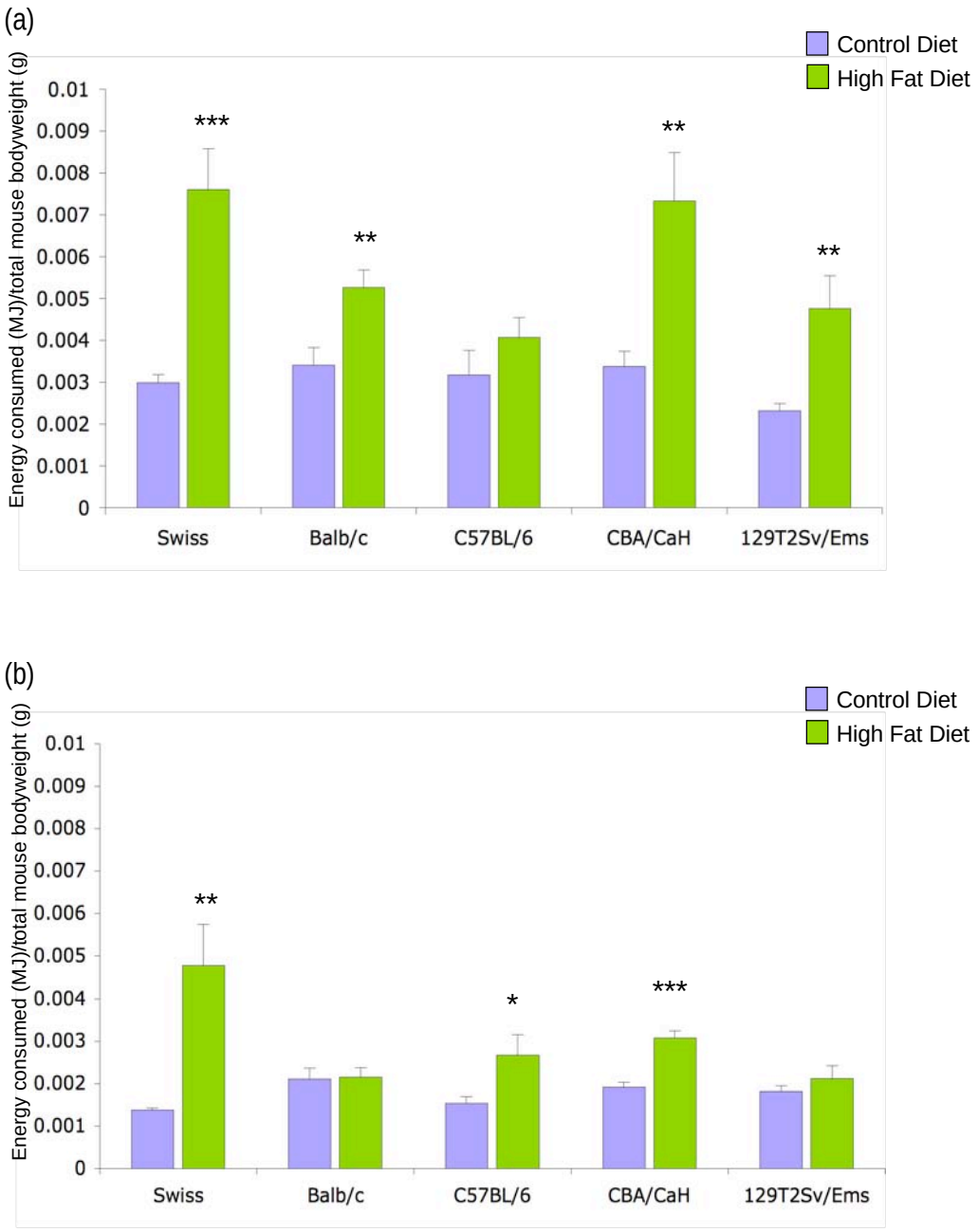
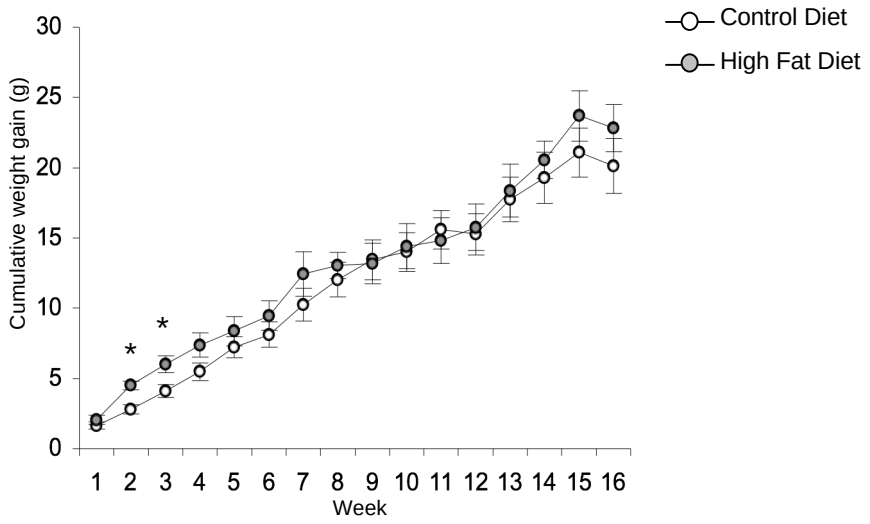
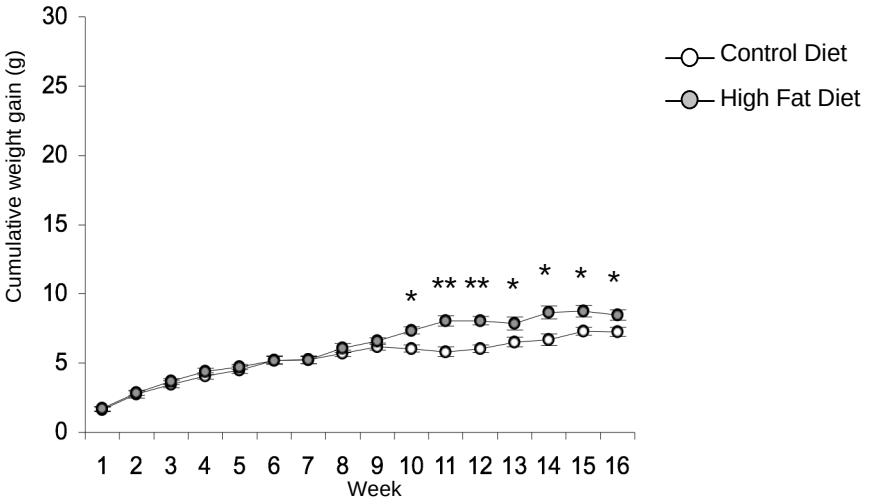


Figure 3.1 Daily energy intake (MJ/g). Intake normalised to cohort bodyweight (g) over the first (a) and final (b) week of exposure to control diet (blue bars) or high fat diet (green bars). Results are expressed as mean ± SEM (n=5-7 days). * P<0.05, ** P<0.01, *** P<0.001.

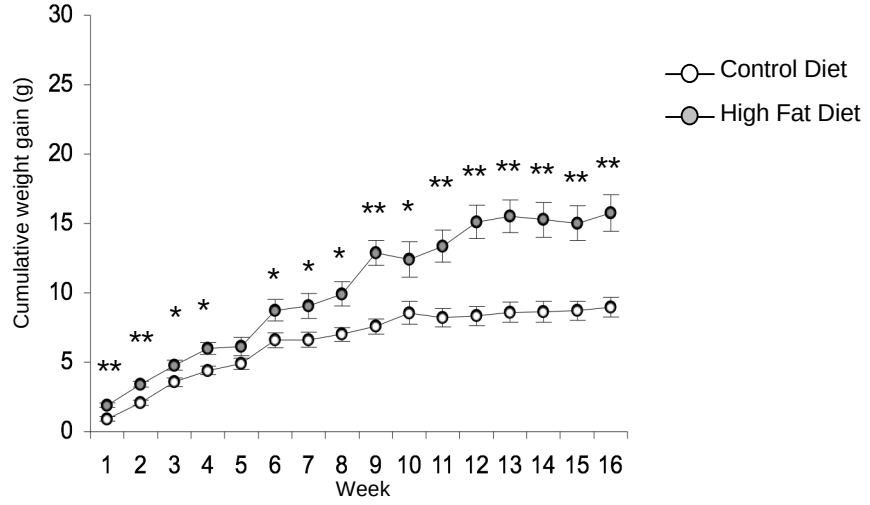
(a)


Control Diet
High Fat Diet
Cumulative weight gain (g)
Week

(b)


Control Diet
High Fat Diet
Cumulative weight gain (g)
Week

(c)


Control Diet
High Fat Diet
Cumulative weight gain (g)
Week

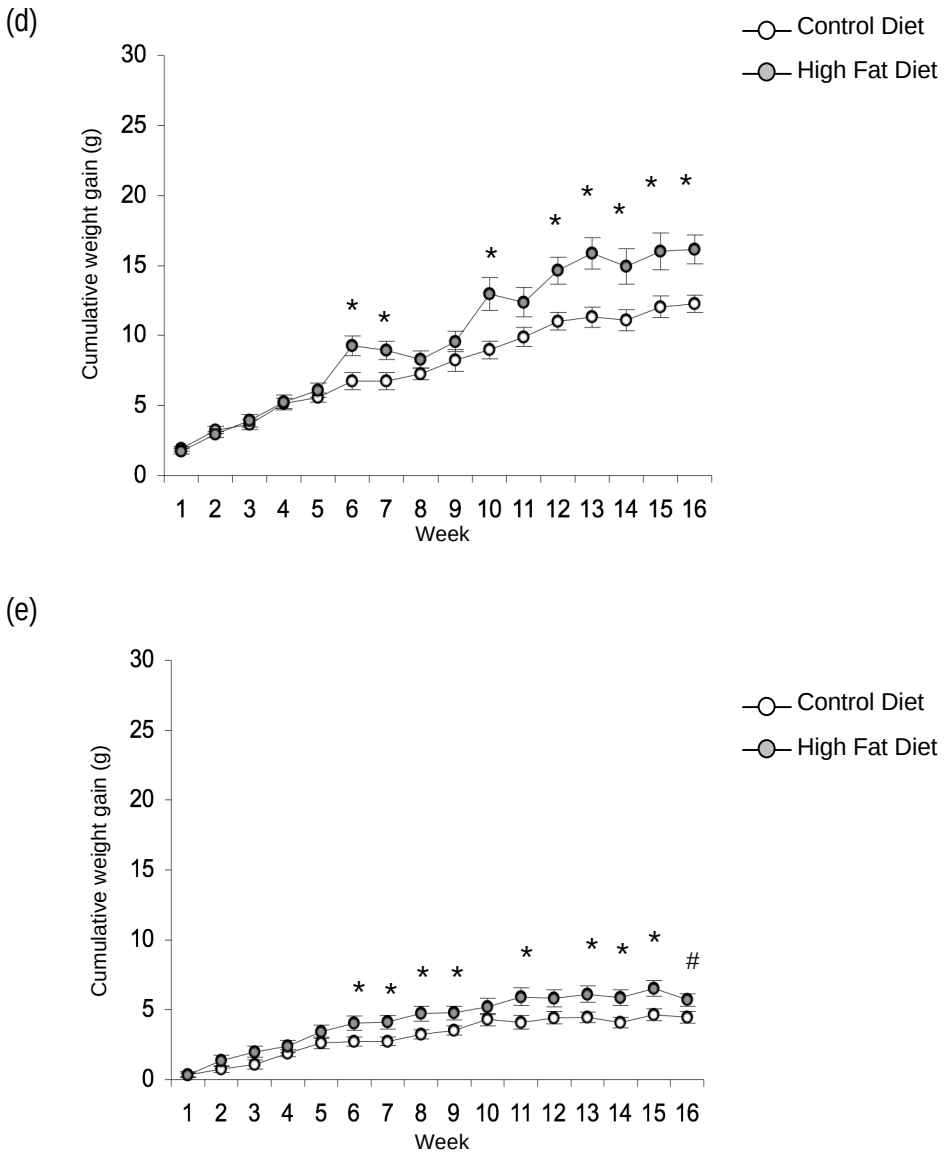


Figure 3.2 Cumulative weight gain. Female (a) Swiss, (b) Balb/c, (c) C57BL/6, (d) CBA/CaH and (e) 129T2Sv/Ems mice fed control diet (white dots) or high fat diet (grey dots). Cumulative weight gain established as difference between each weekly bodyweight and initial bodyweight at commencement of feeding regime. Results are expressed as mean ± SEM (n=12 mice). * $P<0.05$, ** $P\leq0.001$.

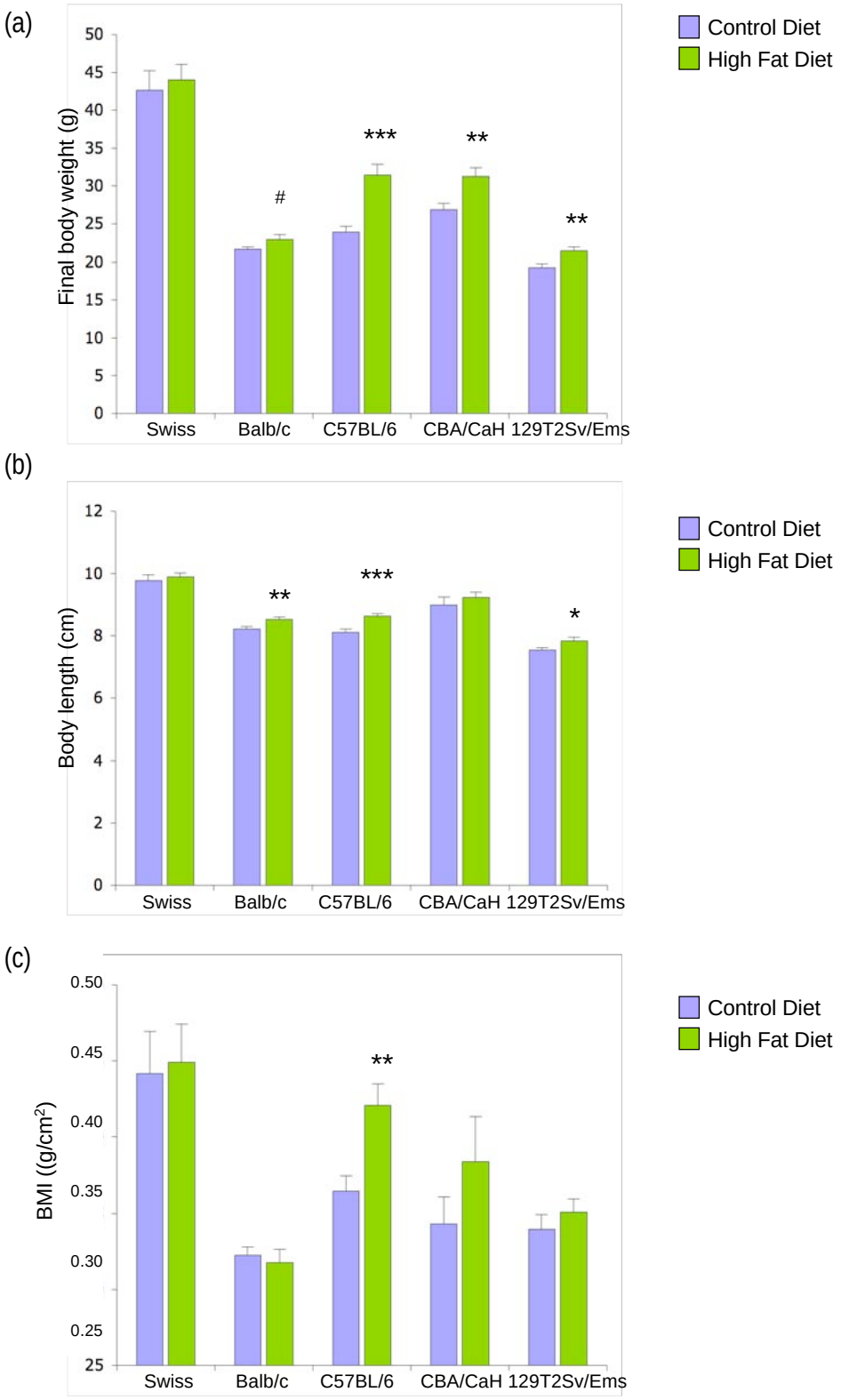


Figure 3.3 Body proportions. (a) Final body weight, (b) body length and (c) body mass index (BMI) after 16 weeks of diet exposure. Results are expressed as mean ± SEM (n=12 mice in all groups except CBA HFD where n=8 mice). # P=0.080, * P<0.05, ** P<0.01, *** P<0.001.

3.3.2 Tissue weights

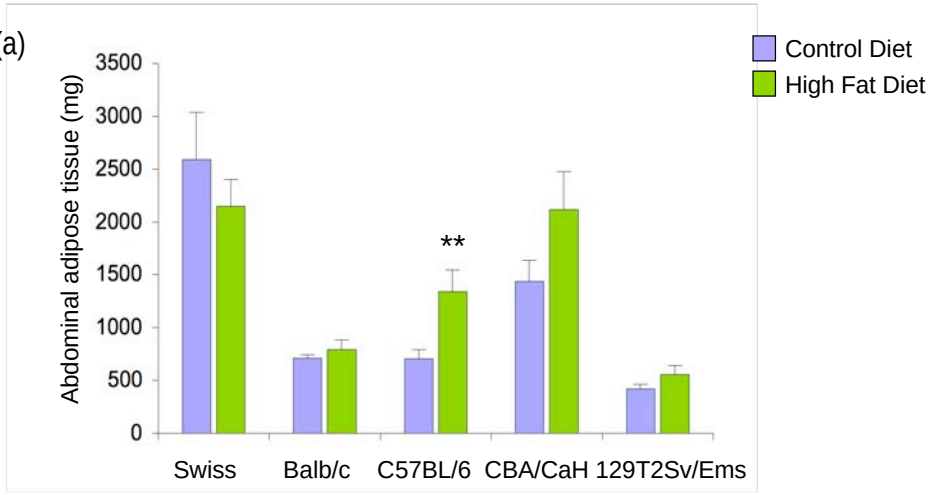
There was a significant interaction of mouse strain with diet on both abdominal (gonadal) and retroperitoneal adipose tissue (P=0.046 and P=0.039 respectively). Weight of abdominal adipose tissue was increased in response to HFD feeding in C57BL/6 female mice only (Figure 3.4a), although retroperitoneal depots were also significantly greater in 129T2Sv/Ems mice (Figure 3.4b). Liver weight was increased in C57BL/6 mice, with a mass approximately 22% greater than CD (Figure 3.4c), likely due to development of fatty liver as documented in C57BL/6 substrains (Colombo *et al.* 2003, Inoue *et al.* 2005). It has also been reported that C57BL/6 mice develop significantly enlarged kidney weights when fed HFD (Jiang *et al.* 2005). Although we report a trend for increased kidney weights in C57BL/6 HFD mice, this only approached significance in the CBA/CaH HFD group (P=0.059) (Figure 3.4d). Spleen weights were not affected by the HFD in any strain of mouse examined (Figure 3.4e).

3.3.3 Dyslipidemia

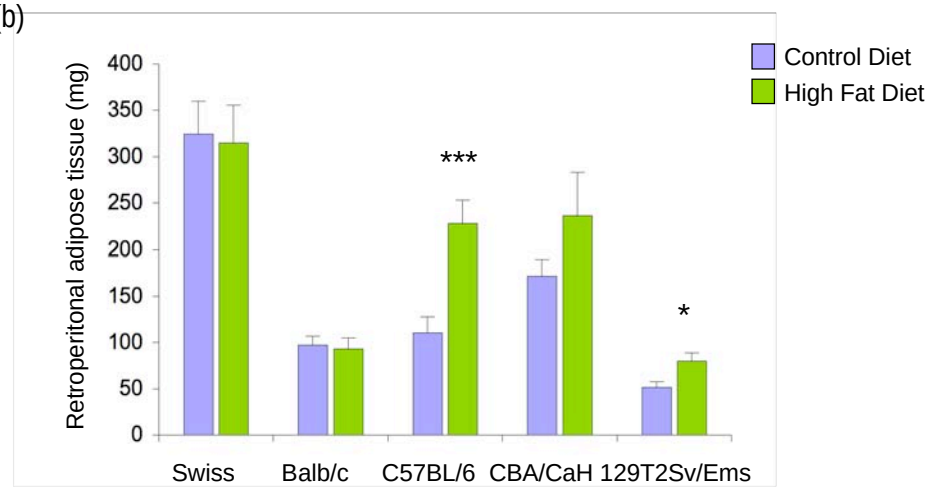
Consumption of the obesogenic diet altered circulating lipids in all mouse strains (Figure 3.5). A strain and diet interaction was observed in measurements of plasma cholesterol only (P<0.001). Plasma cholesterol was increased in HFD Swiss, C57BL/6 and CBA/CaH mice (P=0.014, P=0.034 and P=0.049 respectively), but reduced in HFD Balb/c and 129T2Sv/Ems mice (P=0.042, P=0.004 respectively, Figure 3.5a). This result may be due to limitations of measurement, which did not distinguish between relative HDL and LDL cholesterol levels. It is possible that while the obesogenic diet elevated LDL levels, the concurrent drop in HDL cholesterol was most pronounced in Balb/c and 129T2Sv/Ems mice.

Free fatty acids (FFA) were uniformly increased in all strains, except for CBA/CaH (Figure 3.5b). Circulating triglycerides were mostly unaffected by diet, and were significantly elevated in only 129T2Sv/Ems HFD mice (Figure 3.5c).

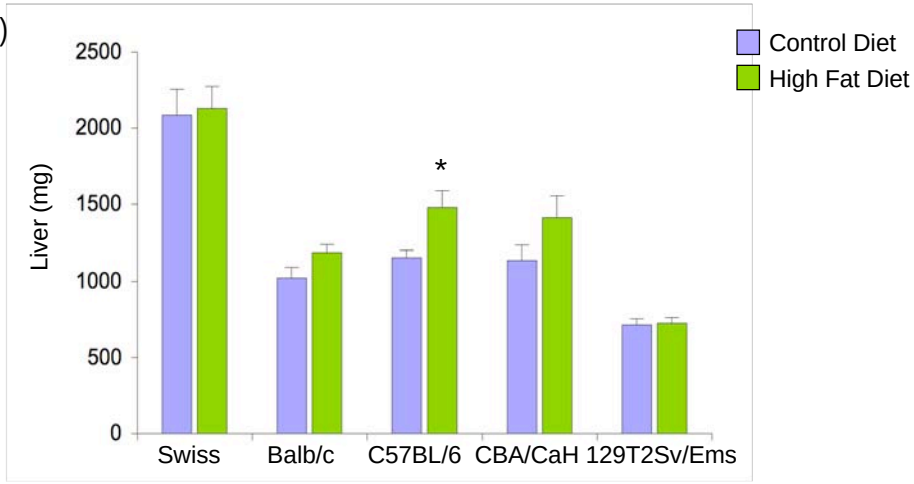
(a)


Abdominal adipose tissue (mg)
3500
3000
2500
2000
1500
1000
500
0
**
Swiss
Balb/c
C57BL/6
CBA/CaH
129T2Sv/Ems
Control Diet
High Fat Diet

(b)


Retroperitonal adipose tissue (mg)
400
350
300
250
200
150
100
50
0
***
*
Swiss
Balb/c
C57BL/6
CBA/CaH
129T2Sv/Ems
Control Diet
High Fat Diet

(c)


Liver (mg)
2500
2000
1500
1000
500
0
*
Swiss
Balb/c
C57BL/6
CBA/CaH
129T2Sv/Ems
Control Diet
High Fat Diet

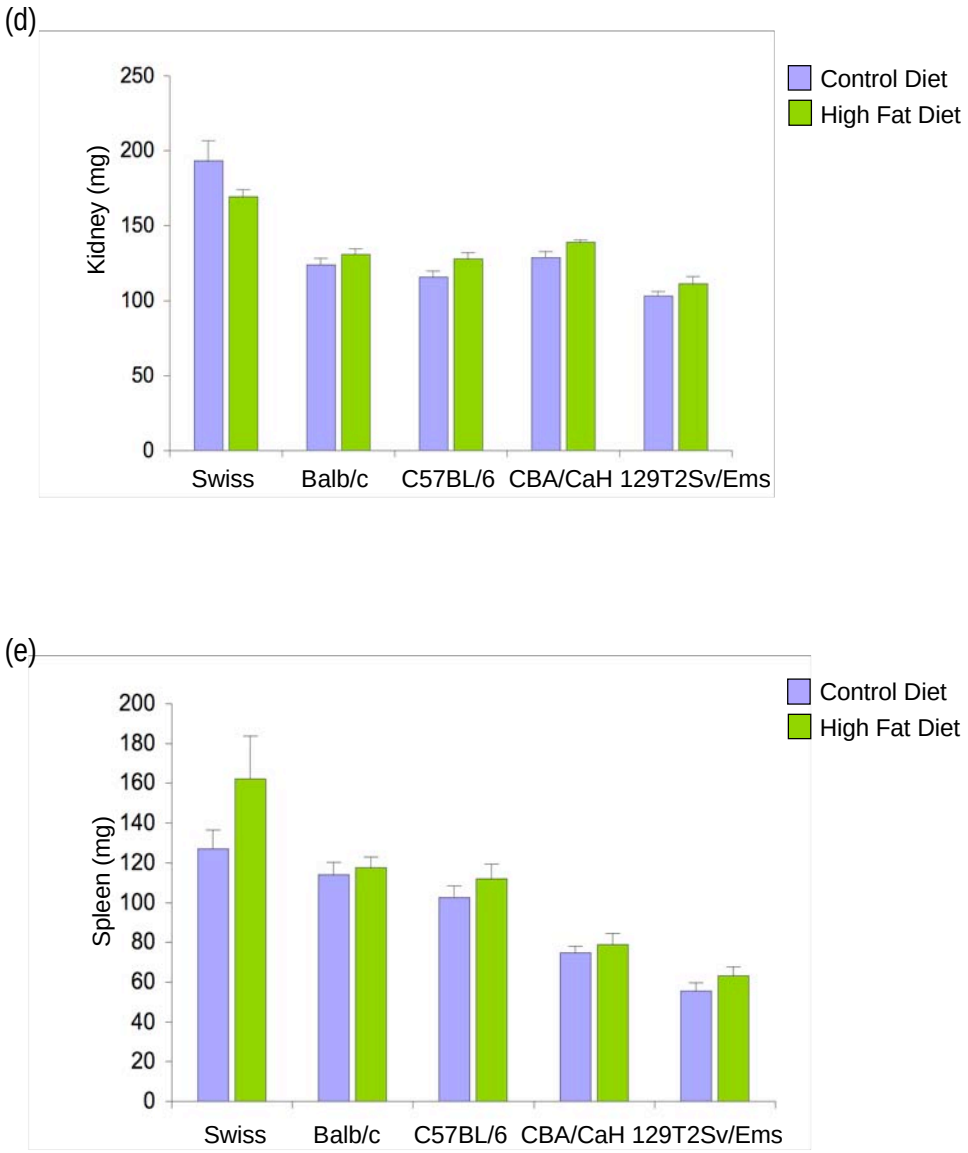


Figure 3.4 Tissue weights. (a) Abdominal adipose tissue, (b) Retroperitoneal adipose tissue, (c) Liver, (d) Kidney, (e) Spleen weight after 16 weeks on the control diet (blue bars) or high fat diet (green bars).. Bars indicate mean ± SEM, (n=12 mice in all groups except CBA HFD where n=8 mice). * P<0.05, ** P<0.01, *** P<0.001.

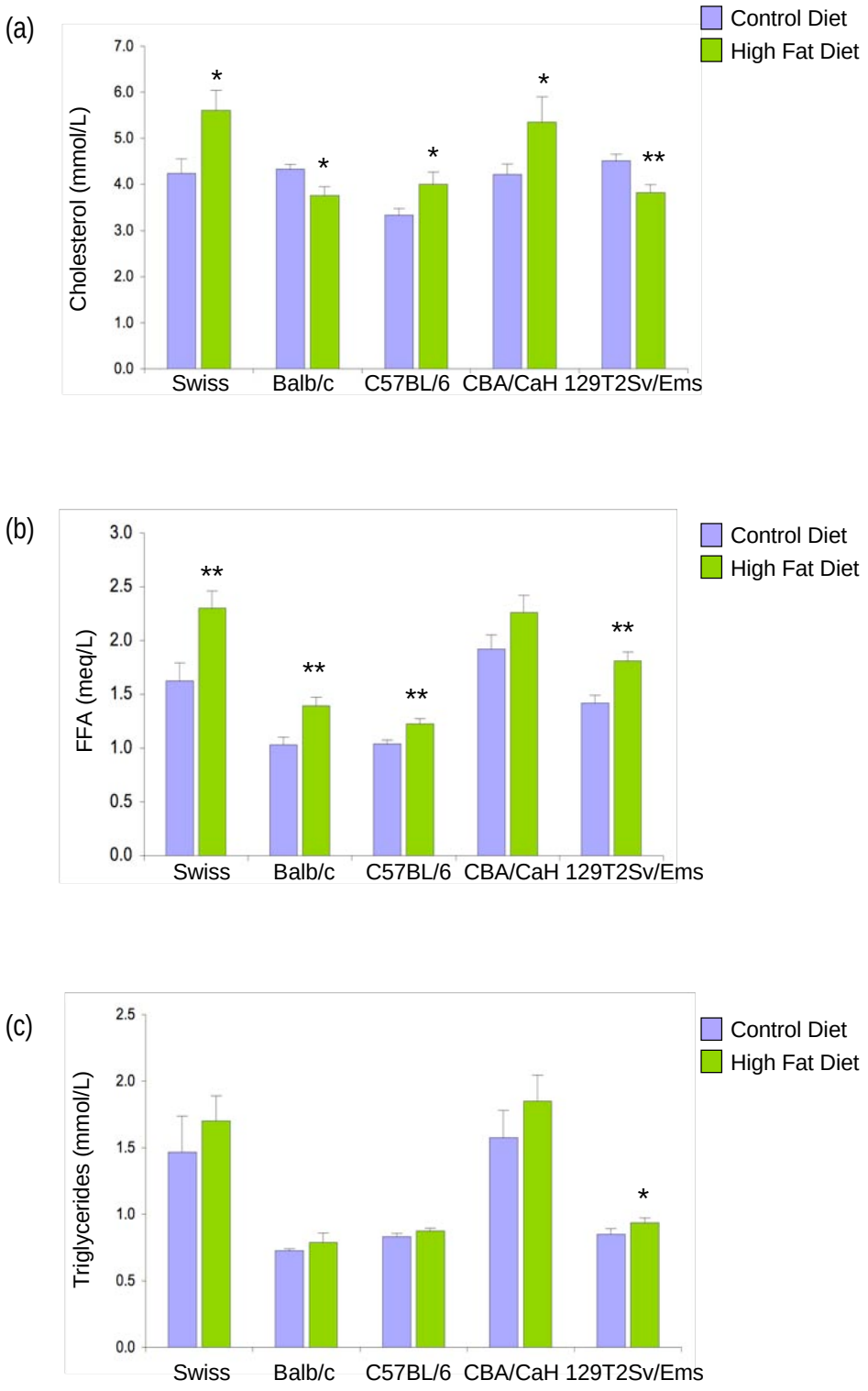


Figure 3.5 Circulating lipid profile. (a) Total cholesterol, (b) Free Fatty Acids (FFA) and (c) triglycerides in female mice fed control diet (blue bars) or high fat diet (green bars) for 16 weeks. Bars indicate mean ± SEM (n=12 mice in all groups except CBA HFD where n=8 mice). * P<0.05, ** P<0.01.

3.3.4 Glucose tolerance and insulin resistance

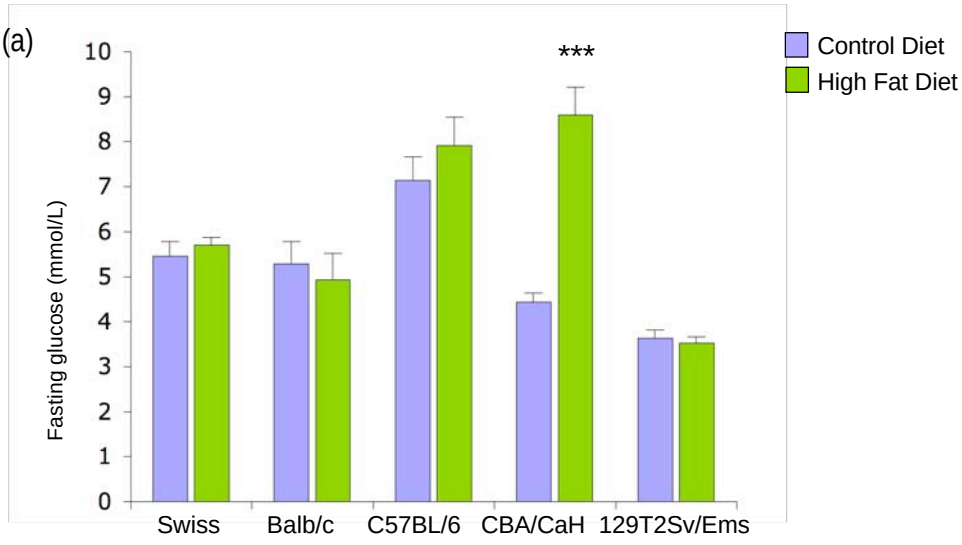
Fasting glucose was the only indicator of glucose metabolism that exhibited strain by diet interaction ($P<0.001$), and only CBA/CaH mice developed elevated fasting blood glucose in response to the HFD (Figure 3.6a).

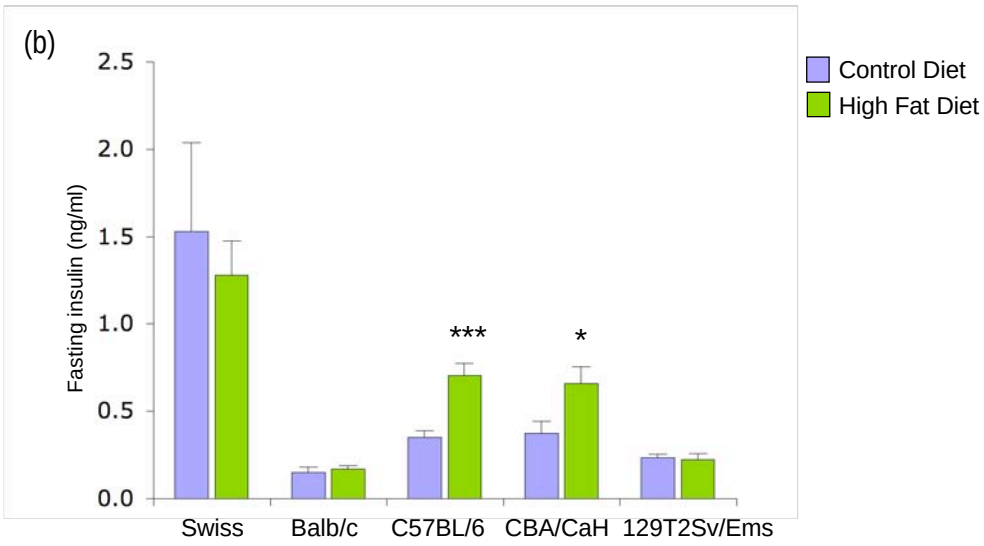
Insulin levels were also assessed in a fasting state (Figure 3.6b). Only C57BL/6 and CBA/CaH mice exhibited fasting hyperinsulinemia in response to the obesogenic diet. ($P=0.0003$, $P=0.019$ respectively). In C57BL/6 animals fed HFD, fasting plasma insulin correlated with percent body fat ($R^2= 0.465$, $P=0.015$), emphasising the importance of adipose tissue volume relative to total body mass, and supporting the concept of specific adipose tissue-derived modulators of insulin function (Wellen & Hotamisligil 2005). Interestingly, Swiss mice exhibited much higher fasting insulin levels than other strains ($P<0.01$, compared to all other strains, CD only).

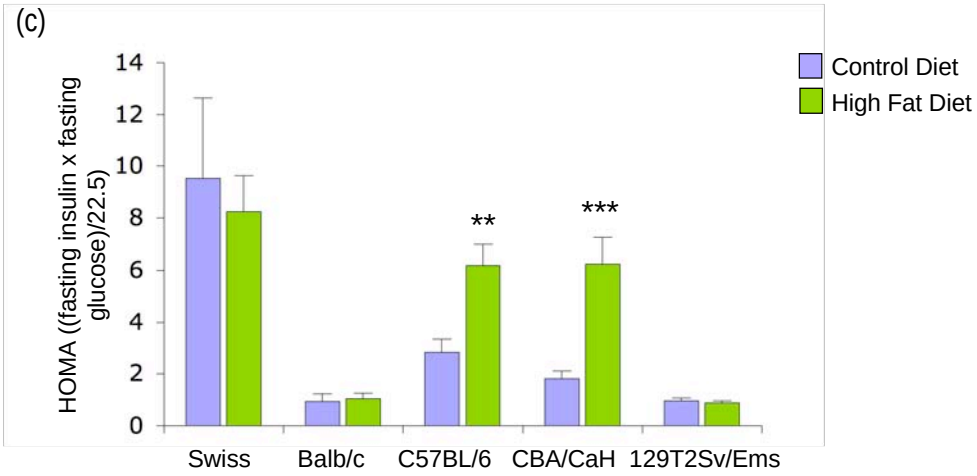
HFD consumption significantly increased indices of insulin resistance (HOMA score) in C57BL/6 and CBA/CaH female mice (C57BL/6 $P=0.003$, CBA/CaH $P=0.0008$), but no such effect was observed in Swiss, Balb/c or 129T2Sv/Ems mice (Figure 3.6c). Due to high fasting insulin levels, the average HOMA score for CD Swiss mice is approximately 9 fold higher than that observed in all other CD mice. HFD did not alter the maximum blood glucose concentration attained following glucose administration in any strain (data not shown). Similarly, glucose tolerance as indicated by Area Under the Curve (AUC) of glucose clearance, was not increased by the HFD, indicating that the mice were not yet overtly diabetic (Figure 3.6d). CBA/CaH HFD mice had an unexpectedly lower AUC value than CD mice ($P=0.034$). Comparison of AUC values across the four different strains showed that glucose clearance over 2h was poorest in C57BL/6 (AUC>1600 units^2), and most rapid in 129T2Sv/Ems (AUC<1180 units^2), regardless of diet (One-Way ANOVA $P<0.0001$ C57BL/6 vs. 129T2Sv/Ems).

3.3.5 Adipo-inflammation

The development of increased infiltration of macrophages into adipose tissue depots has been reported in both human patients and male animal models of DIO (Weisberg *et al.* 2003, Xu *et al.* 2003, Cancello *et al.* 2006), and there are potential gender-specific differences in the ratio of adipocyte to leukocyte


(a)
Fasting glucose (mmol/L)
10
9
8
7
6
5
4
3
2
1
0
***
Swiss
Balb/c
C57BL/6
CBA/CaH
129T2Sv/Ems
Control Diet
High Fat Diet


(b)
Fasting insulin (ng/ml)
2.5
2.0
1.5
1.0
0.5
0.0
***
*
Swiss
Balb/c
C57BL/6
CBA/CaH
129T2Sv/Ems
Control Diet
High Fat Diet



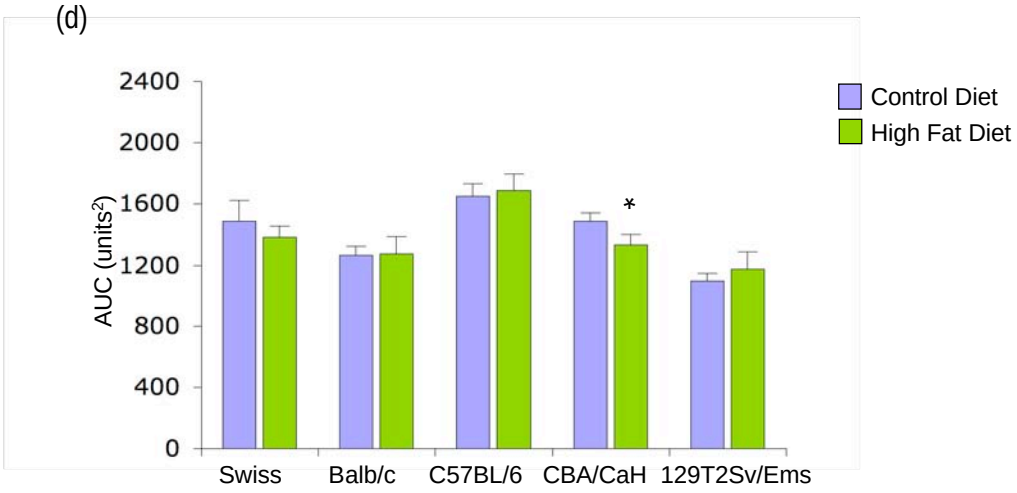


Figure 3.6 Glucose metabolism. Fasting blood glucose level (a), fasting plasma insulin level (b), HOMA score (c) and area under the curve (AUC) following 2h glucose tolerance test (d) in mice fed control diet (blue bars) or high fat diet. (green bars) Bars indicate mean ± SEM (n=12 mice in all groups). * P<0.05, ** P<0.01, *** P<0.001.

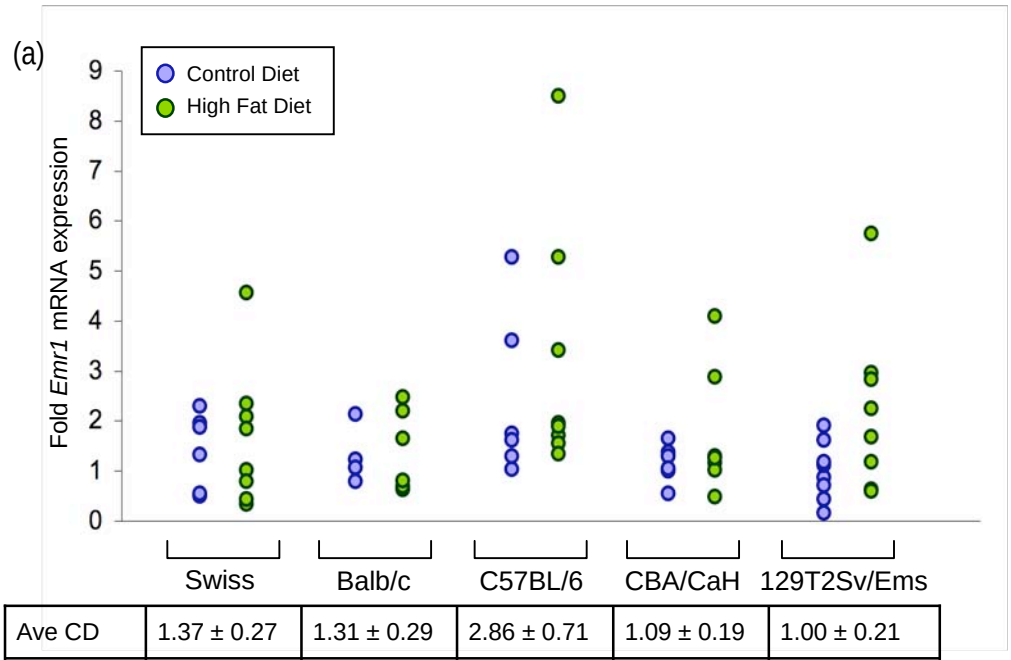
cells that comprise adipose tissue (Robker *et al.* 2004). We therefore analyzed the relative expression of *Emr1* and *Cd68* macrophage-specific genes in abdominal adipose tissue.

Emr1 expression was not significantly elevated in adipose tissue of any strain of mouse in response to the high fat diet (Figure 3.7a). However a trend towards higher expression was noted in all groups, and approached statistical significance in the 129T2Sv/Ems mice fed the HFD (increased approximately 55.4%, P=0.071).

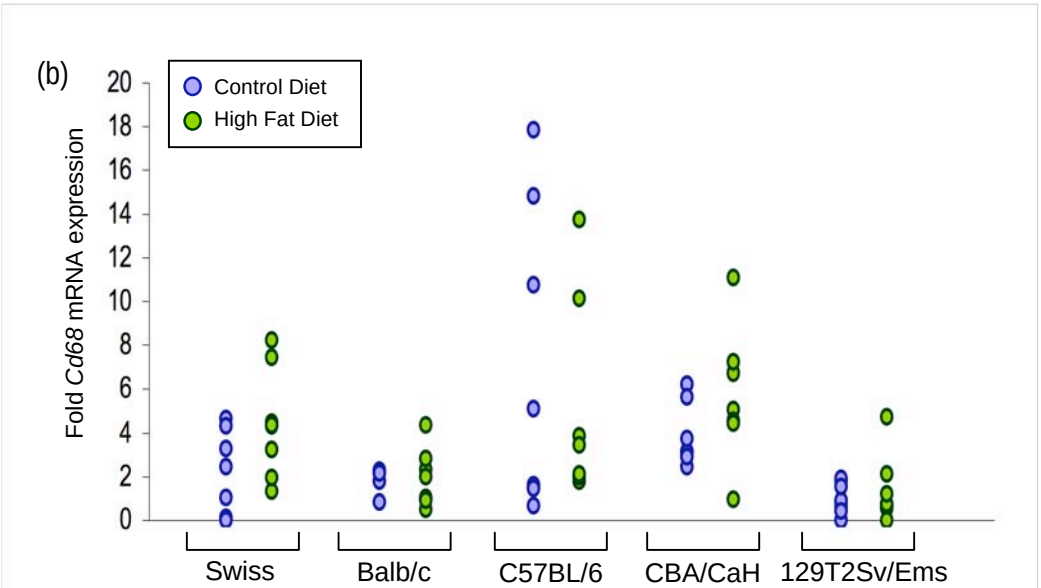
Similarly, *Cd68* expression was also statistically unaffected by diet, although most strains, with the exception of C57BL/6, suggested a trend towards higher expression in the HFD groups (Figure 3.7b). This was most pronounced in the Swiss strain (increased approximately 49.0%, P=0.079).

The regulation of these markers of adipo-inflammation was consistent; animals presenting with high adipose *Emr1* expression were also found to exhibit elevated adipose *Cd68* (R^2=0.382, P≤0.001).

In addition, elevated values of *Emr1* in adipose tissue correlated with HOMA score of insulin resistance in CBA/CaH mice (R^2=0.415, P=0.047). Evidence that elevated expression of macrophage markers is correlated with markers of insulin resistance is consistent with the emerging concept that macrophages are an important, and potentially initiating, cell type in the process of inflammation-induced insulin resistance.



Ave CD	1.37 ± 0.27	1.31 ± 0.29	2.86 ± 0.71	1.09 ± 0.19	1.00 ± 0.21
Ave HFD	1.68 ± 0.49	1.42 ± 0.33	3.21 ± 1.25	1.74 ± 0.64	2.24 ± 0.60 #



Ave CD	2.26 ± 0.72	1.75 ± 0.33	7.47 ± 2.65	3.89 ± 0.73	1.00 ± 0.24
Ave HFD	4.43 ± 0.85 #	1.99 ± 0.54	5.29 ± 2.36	5.72 ± 1.56	1.33 ± 0.53

Figure 3.7 Expression of macrophage markers (a) *Emr1* and (b) *Cd68* in abdominal adipose tissue. All data are normalised to *Rps3*, and are expressed as the mean fold change ± SEM from the group 129T2Sv/Ems CD, which is given the arbitrary value 1 (n=4-8 mice per treatment group). #P<0.08.

3.4 DISCUSSION

Gender-specific problems experienced by obese women including amenorrhoea, anovulation, and increased risk of PCOS, start in puberty and continue until after menopause with higher incidence of endometrial cancer and breast cancer (Bongain *et al.* 1998, Ramsay *et al.* 2002). An understanding of health complications experienced by overweight and obese women, in particular the link between insulin resistance and female reproductive function, necessitates well-described animal models. The current study provides new information from controlled comparisons of frequently used mouse strains, and illustrates the profile of female-specific complications arising from diet-induced obesity across genetically diverse strains.

The objective was to determine the influence of post-pubertal over-nutrition on parameters likely to influence female fertility, specifically, body composition, glucose metabolism, insulin sensitivity, circulating lipids and adipo-inflammation.

Five different strains were investigated, all used routinely worldwide as rodent models. All of the animals used here were recently derived from the Animal Resource Centre in Western Australia. Observations regarding each strain's susceptibility to diet-induced obesity and associated complications are summarised in Table 3.2. Our results clearly demonstrate that each mouse strain studied (both inbred and outbred) is characterized by its own metabolic profile, which can be attributed to genetic differences resulting in variable phenotypes.

As expected, two distinct groups of mice emerged. Swiss, Balb/c and 129T2Sv/Ems (non-affected) did not develop obvious perturbations, or were only minimally affected, whilst C57BL/6 and CBA/CaH (affected) both experienced the development of classical Metabolic Syndrome-like complications.

Amongst the non-affected group, calorie consumption was significantly higher by all animals when the high fat variety was available, although this did not translate to significant differences in body composition. It is possible that factors not measured within this protocol, such as different levels of physical activity, may be responsible for this. Alternatively, it is likely there is differential expression of genes controlling metabolism and fat deposition in the different strains of mice. Indeed, it has been shown that mice of a 129 lineage have higher rates of energy expenditure than obesity-prone mice, associated with high expression of skeletal muscle uncoupling protein-1 (UCP1) (Almind *et al.* 2007). Consistent with previous investigations in male mice, these non-affected female mice also maintained

Table 3.2 Summary of metabolic effects seen in mice from 5 strains fed the high fat diet, compared to those fed the control diet.

	Swiss	Balb/c	C57BL/6	CBA/CaH	129T2Sv/Ems
Energy intake	+++	-	++	++	+
Body weight	-	+ (minimal)	+++	++	+
Body length	-	++	+++	-	+
Adiposity	-	-	+++	+ (minimal)	+
Liver weight	-	+ (minimal)	+	-	-
Dyslipidemia	+	+	+	+	++
Glucose intolerance	-	-	- *	++	-
Insulin resistance	-	-	++	++	-
Adipoinflammation	+ (minimal)	-	-	-	+ (minimal)

"-" indicates no statistically significant difference
"**+(minimal)**" indicates P<0.1
"**+**" indicates P<0.05
"**++**" indicates P<0.01
"**+++**" indicates P<0.001
* Uncharacteristic result, see Discussion

normal glucose tolerance and insulin sensitivity. However, all animals did develop some degree of dyslipidemia, in particular, significantly elevated circulating FFA. Also, although Swiss HFD mice were hypercholesterolemic, HFD Balb/c and 129T2Sv/Ems females were found to have lower levels of circulating cholesterol, which is currently unexplained. Despite the potential systemic impact of these dysregulated parameters, our findings support the application of these strains, particularly the outbred Swiss, as models for identification of genes or pathways that confer resistance to diet-induced obesity.

In contrast C57 and CBA strains were markedly affected. Our results that describe DIO, and its associated metabolic disturbances in C57BL/6 mice are mostly supported by the considerable body of literature that indicates these mice are particularly susceptible to the development of this obese disease manifestation (Surwit *et al.* 1995, Watson *et al.* 2000, Collins *et al.* 2004, Alexander *et al.* 2006).

In the two affected strains, calorie intake from the HFD was higher, and CBA/CaH were found to consistently consume significantly more energy than their counterparts maintained on the control diet from the initiation of the experimental timeframe. Subsequent CBA/CaH weight gain, although moderate, was eventually significant, and it is interesting to note that body length and composition were not considerably influenced. However, while C57BL/6 mice did not initially consume increased energy from the high fat feed, they did respond dramatically to it, with rapid acquisition of bodyweight. This was coupled with the accumulation of excessive adipose tissue. It has been proposed previously that this is controlled by catecholamines binding to β-adrenergic receptors, increasing lipolysis and decreasing triglyceride-rich lipoprotein accumulation in white adipose tissue (Rothwell & Stock 1979, Bukowiecki *et al.* 1981, Lafontan *et al.* 1985). It has been reported that in C57BL/6 mice raised on a HFD, expression of β-adrenergic receptors is severely reduced, and this down-regulation is not as dramatic in less-severely affected strains (Collins *et al.* 1997).

Circulating blood lipids were abnormal in CBA/CaH and C57BL/6 female mice fed HFD, in particular total circulating cholesterol was significantly elevated. It would be of interest to identify the influence of this high fat feed on the relative levels of lipoproteins contributing to the total cholesterol measurement, as it is known that C57BL/6 mice in particular, whilst becoming hypercholesterolemic, typically reduce circulating HDL (Stewart-Phillips *et al.* 1988, Park *et al.* 2004). Close examination of hepatic cholesterol metabolism between murine strains is important in determining their different susceptibilities to diet-induced atherosclerosis, and even insulin sensitivity, and as of yet has not been conducted in female mice.

Although both CBA/CaH and C57BL/6 female mice developed hyperinsulinemia, neither was found to have any signs of abnormal glucose metabolism, as evidenced by the glucose tolerance test AUC. This is particularly surprising for the C57BL/6 mice, as assessment of glucose tolerance in an identical cohort of C57BL/6 females 8 months later, showed significant HFD induced hyperglycemia and impaired glucose clearance (Chapter 5). This suggests there are considerable seasonal influences, and we encourage careful consideration of this in the future.

Despite the maintenance of glucose tolerance, CBA/CaH and C57BL/6 mice in this experimental context were found to be insulin resistant with elevated HOMA scores. In these mice, fasting insulin correlated very strongly with percent body fat (R^2= 0.465, P=0.015), rather than total bodyweight, emphasising the importance of adipose tissue volume relative to total body mass. This also supported the concept of specific adipose tissue-derived modulators of insulin function (Wellen *et al.* 2005). In particular, proinflammatory factors secreted by infiltrated macrophages can directly influence adipocyte biology and systemic insulin resistance. Evidence indicates the causal role of macrophage populations in leading to insulin resistance in mice (Arkan *et al.* 2005), and for these reasons, it was hypothesized that C57BL/6 mice would have the most marked adipose recruitment of macrophages, but this was not the case, and contrasted with literature that reports significant infiltration of macrophages into adipose tissue in male mice (Xu *et al.* 2003, Weisberg *et al.* 2003).

It is becoming clear that there exist significant gender differences in the degree and nature of the immune cell response to diet-induced obesity (Robker *et al.* 2004, Brake *et al.* 2006). Coupled with this, the location of adipose tissue deposits appears be important. New evidence has found that unlike in the male, only mesenteric, but not peri-uterine adipose tissue of HFD female mice becomes infiltrated with inflammatory leukocytes (Zhang *et al.* 2007). Our results support this, and underscore the importance of greater consideration of female-specific responses to obesity.

We also found that the affected strains exhibited a greater degree of variability than the non-affected strains, particularly in weight gain and insulin resistance scores indicating the development of subpopulations, even within inbred genetically homologous mice, based on their degree of affectedness. This result is supported by preceding studies of divergence of a random C57BL/6 population into obese and diabetic, lean and diabetic, or lean and non-diabetic subpopulations, as well as differential expression of metabolic genes in the liver and muscle in response to a HFD (Burcelin *et al.* 2002, de Fourmestraux *et al.* 2004). Such observations are thought to indicate differentially distributed epigenetic control of metabolically responsive genes.

Despite overweight women being at risk for similar co-morbidity and stigmata as men (Ford *et al.* 2002) relatively little is known about female complications that result from a diet-induced obesity. Reports such as the present study demonstrate there are female-specific responses to a high fat diet. In particular we have shown that female mice exhibit strain-dependent weight gain and obesity-related complications. Secondly we have also shown that these complications are less severe than those typically reported for male mice, specifically we did not observe greatly impaired glucose tolerance or increased adipo-inflammation in HFD females compared to those on CD. Cumulatively these observations form our baseline measurements enabling us to next examine the effects of such metabolic perturbations on ovarian function.

Chapter 4

Ovarian phenotypes in female mice with diet-induced obesity

4.1 INTRODUCTION

In the previous chapter we demonstrated that female mice fed a "Western Style" high fat diet develop a gender-specific profile of metabolic perturbations. In addition, the nature and severity of these perturbations are strongly influenced by strain. We now address the next two aims of the project (Aim 2 & Aim 3), and examine the consequences of a high fat diet on ovarian function, and female reproductive capacity. We will incorporate the effects of strain and various diet-induced metabolic perturbations into the reproductive function of diet-induced obese mice. In this way we hope to identify measurable parameters strongly influencing ovarian functions such as ovulation, steroid production and/or oocyte developmental competence.

A range of reproductive parameters were measured in female mice fed the high fat diet, each providing information on how a high fat diet and obesity influences the pre- and peri-ovulatory environment of the ovarian follicle, and subsequent indicators of oocyte quality. Follicles contained within high fat diet ovaries were histologically observed; to crudely detect any impact obesity may be having upon the growth or maturation of the pre-ovulatory follicle and/or oocyte. The capacity of these follicles to undergo the processes of ovulation (which requires an appropriate surge of gonadotropins, extracellular matrix breakdown, follicle rupture and expulsion of the cumulus-oocyte-complex) was determined. Subsequently the fertilisation of ovulated oocytes under natural mating conditions was assessed. Subsequent embryonic cell division and differentiation events, until the blastocyst stage, were used as additional measures of initial oocyte health. This included morphological assessment of on-time development as well as differential staining of blastocyst inner cell mass (ICM) and trophectoderm (TE) cell lineages. By using a well-established *in vitro* culture system, the oocyte contribution to this early embryo development was the key variable between high fat and control diet comparisons.

It is known that strain can substantially influence background reproductive performance, in particular, the number of primordial follicles, oocyte maturation rates and litter size (Polanski 1986, Canning *et al.* 2003). In the current study we conducted all analyses in four different strains of laboratory mice; Balb/c, C57BL/6, CBA/CaH and 129T2Sv/Ems. Having already documented the strain-dependent susceptibility to the development of obesity, dyslipidemia and insulin resistance (Chapter 3), the extent of impaired reproductive potential following exposure to a high fat diet was then integrated into statistical correlations to reveal specific metabolic perturbations that universally target specific reproductive events, ie ovulation, blastocyst development, or embryonic cell allocation, in all strains of mouse.

4.2 MATERIALS AND METHODS

4.2.1 Animals and diets

The animals used for these analyses are the same individuals assessed for metabolic phenotypes in Chapter 3. They consisted of twenty-four female mice of each (Balb/c, C57BL/6, CBA/CaH and 129T2Sv/Ems) strain. In addition 12 males of each strain were obtained to use for mating. Details of the source and background of each strain, as well as housing and diet information is found in Chapter 2.

4.2.2 Metabolic and endocrine measurements

All metabolic and endocrine measurements were made as described in Chapter 2. For details on metabolic outcomes, please refer to Chapter 3.

Circulating progesterone was assayed on day 1 of pregnancy using an RIA kit (Diagnostic System Laboratories, Webster, TX) in accordance to the manufacturer's instructions. This kit has a sensitivity of 0.25 pmol/ml, an intraassay coefficient of 8.4%, and an interassay coefficient of 12%.

4.2.3 Mating and tissue collection

After 16 weeks exposure to experimental or control diets, two female mice (i.e. one from each diet group) were caged with a non-obese strain-matched proven fertile male mouse. Mice remained housed together for a maximum of 9 days, a time course that permitted at least 2 normal estrous cycles. All female mice were checked daily at 0800 for post-coital seminal plugs. On day of presence of vaginal plug, females were deemed to be at day 1 of pregnancy and sacrificed at 1300 that day by cervical dislocation. Ovary and uterus weights were determined. Ovaries were fixed immediately in 4% paraformaldehyde (wt/vol) (BDH Laboratory Supplies, Poole, UK) at 4°C for approximately 20h, and then processed for paraffin embedding. Ovulated oocytes/1-cell zygotes were collected immediately from the oviduct ampulla into G-MOPS media.

4.2.4 Ovarian histological assessment

Each ovary contained within paraffin wax blocks was serially sectioned at 6μm thickness on a Microm rotary microtome (HM 325), and every 5th section was stained with haematoxylin and eosin. Sections were analyzed microscopically using NDP NanoZoomer Digital Pathology equipment (Hamamatsu Photonics K.K.). Follicles were categorised as either pre-antral (Type 3-5b), or antral (Type 6-8) based on the criteria of Pedersen and Peters 1968 (Pedersen *et al.* 1968). Follicle counts were normalized to total area of assessable ovarian tissue, to control for differences in tissue area and integrity. The number of sections assessed per animal ranged from 8 – 43. Oocytes contained within these follicles were measured in μm^2 using the NDP.view software (version 2.0.30, Hamamatsu Photonics K.K.), in sections in which a germinal vesicle was visible, to ensure consistent measurements in all sections examined. The germinal vesicle was only visible in sections where the oocyte was greater than 90% of maximum size (Figure 4.1). It was also confirmed that the germinal vesicle is visible across 18-24μm, and would therefore be observed only once in sections which are 30μm apart.

4.2.5 In vitro embryo culture

In vitro culture of embryos was conducted in overlapping cohorts, under identical culture conditions for all zygotes regardless of diet or strain. Cumulus cells were removed by treatment with hyaluronidase (0.5mg/ml, Sigma, bovine testes, type IV) and zygotes were maintained for 48h in G1.2 media (Gardner 1994, Gardner *et al.* 2003), with assessment for fertilization indicated by first cleavage division occurring after 24h. 2-cell embryos were transferred to EDTA-free G2 media (Barnes *et al.* 1995, Gardner *et al.* 2003), shown previously to provide an optimum environment for growth of the post-compaction embryo. Development of fertilized oocytes was assessed at 09:00 day 3, 16:00 day 4, 9:00 day 5 by assessors blinded to maternal treatment group. Embryos were indicated as “on-time” if normal morphology was observed, (ie: day 3: 4-8 cells, day 4: morula-blastocyst, day 5: expanded or hatching blastocyst), fragmentation was less than 10%, and the zona pellucida was intact (until day 5). Any developmentally impaired (indicating cellular arrest) or overtly accelerated (indicating insufficient or incomplete processing of cellular division) embryos were duly noted as such and consequently categorised as not “on-time” for the remainder of the culturing period, even if their developmental progress later normalised.

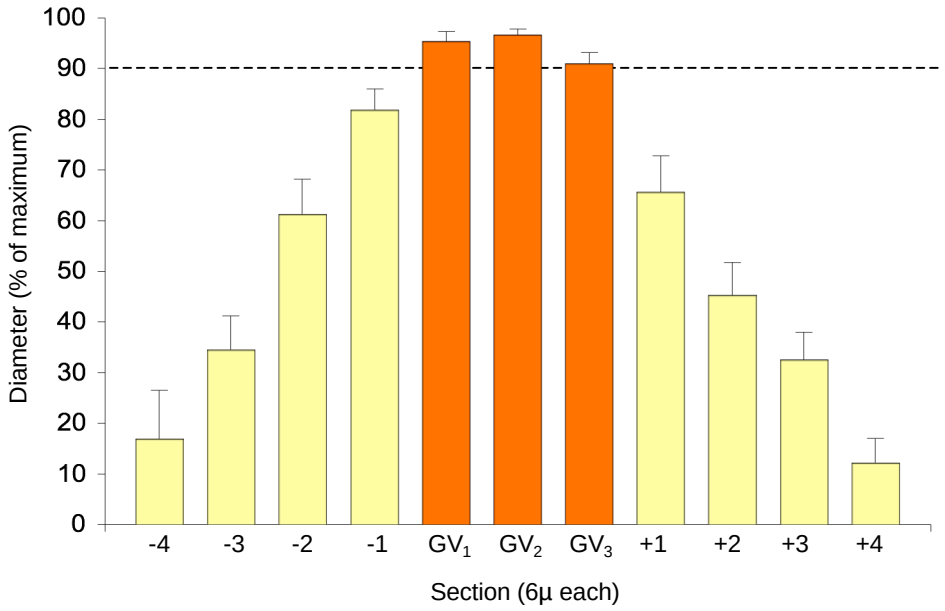


Figure 4.1 Diameter (as % of maximum) of oocytes in 6mm serial sections. Orange bars indicate sections in which germinal vesicle is visible. Germinal vesicle is only present within a maximum of 3 serial sections (GV_1, GV_2, GV_3) and these have the largest diameter. Info about number of sections analyzed from number of mice. Bars indicate mean+/- SEM.

4.2.6 Differential nuclear staining

Expanded and hatching blastocysts surviving at day 5 of culture were subjected to a differential staining protocol for counting of cells within the fetal precursor-inner cell mass (ICM) and placental and extra-embryonic tissue precursor-trophectoderm (TE) layer following the methods of Hardy et al (Hardy *et al.* 1989). A blinded assessor then counted red (TE) and blue (ICM) fluorescent cells on an Olympus VANOX AHBT-3 photomicroscope (Faulding Imaging, Mulgrave North, Victoria, Australia).

4.2.7 Statistical analyses

Values are reported as mean ± SEM, and P-value < 0.05 was considered significant. Statistical differences were determined by ANOVA and Chi-squared analysis using SPSS 13.0 for Windows (SPSS Inc. Chicago, IL). Two-way ANOVA was used to determine the effects of strain and diet, and any interactions. Post-hoc analyses of significance were made by Tukey's test. The Student's t-test was used to compare independent means. Pearson correlation coefficients were used to assess relationships between fasting plasma insulin, total bodyweight, percent body fat, and adipose tissue mass, using SigmaStat for Windows version 2.03 (Jandel Corp., San Ramon, CA). To determine differences in population percentages between treatments, a z-test comparison of proportions was used.

Statistical analysis of data to identify predictors of reproductive outcomes (ovulation success, number of oocytes ovulated, zygote cleavage, zygote survival to blastocyst stage, relative TE and ICM cell number) was performed using the statistical software SAS 9.1 (Cary, NC, USA), and controlled for multiple observations per animal. Predictors identified as being of significance P < 0.2 in an initial univariate analysis were fitted to the most appropriate linear or log binomial model. Risk and rate ratios were calculated for any categorical or continuous variables that were significant (P < 0.05) in the log binomial model.

4.3 RESULTS

4.3.1 Follicle number and oocyte size

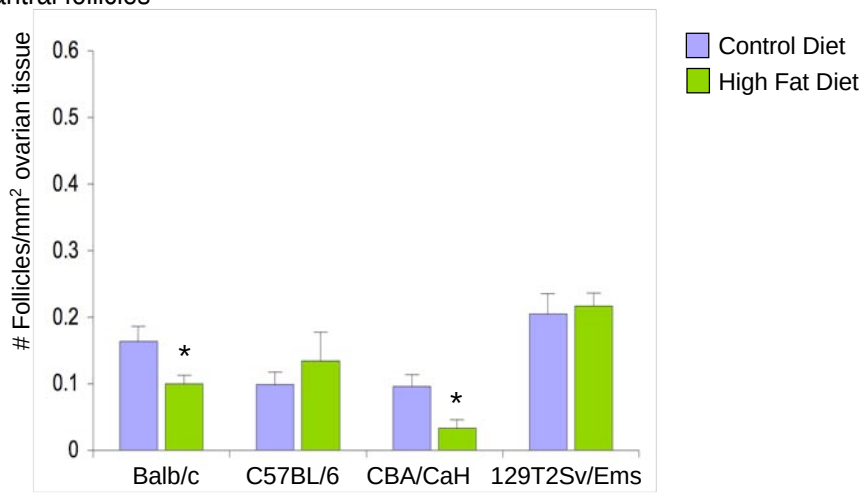
The number of pre-antral growing follicles (type 3-5b) was significantly reduced in ovaries from Balb/c and CBA/CaH mice fed the HFD (P=0.023 and 0.045 respectively, Figure 4.2a). The high fat diet did not influence the number of larger, antral follicles (type 6-8) within the ovaries of any strain (Figure 4.2b). However, there was a pronounced strain effect (P<0.00001), as Balb/c and 129T2Sv/Ems ovarian sections contained many more growing follicles. The effect of high fat diet on oocyte size was consistent in all strains except the C57BL/6 (Figure 4.2c), where there was no diet effect. The high fat diet tended to increase the size of oocytes contained in pre-antral follicles in remaining strains, and in 129T2Sv/Ems female mice the effect was significant (P=0.006). The size of fully-grown oocytes within antral follicles was greatly increased by HFD in Balb/c, CBA/CaH and 129T2Sv/Ems mice (all P<0.00001), suggesting that in many strains, maternal diet composition is capable of inducing alterations to oocyte cytoplasmic contents.

4.3.2 Reproductive tissue morphology, weight and steroidogenesis

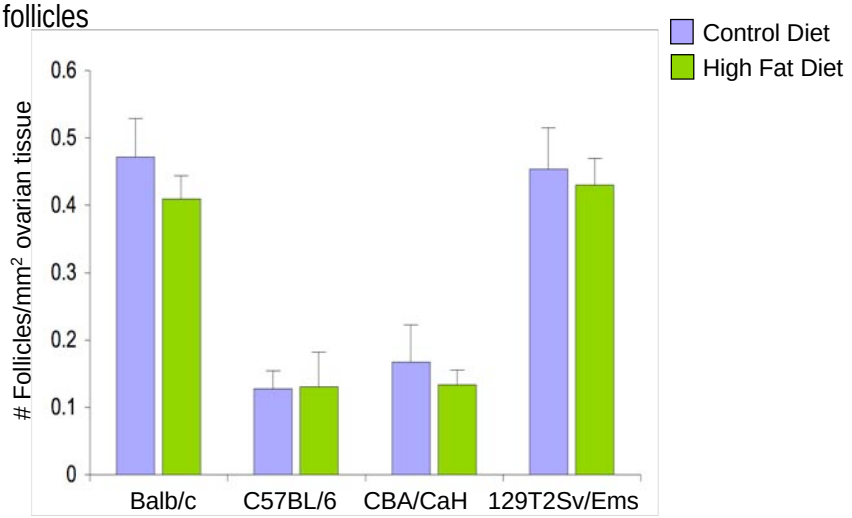
Observations that, independent of diet, Balb/c and 129T2Sv/Ems ovarian sections contained many growing follicles, whilst CBA/CaH and C57BL/6 ovaries consisted of mainly of luteal structures in various regressive stages, is supported by gross morphological assessment of sections (Figure 4.3). 129T2Sv/Ems ovarian sections of both diets frequently contained follicles encapsulating multiple oocytes (arrowhead), which is thought to arise from failure of primordial oocyte nest breakdown (Chen *et al.* 2007). Coupled with this, ovarian tissue weights, although not influenced by diet in any strain, were subjected to a significant strain effect (P<0.00001, Figure 4.4a). Also, uterine weight was not influenced by diet, but was by strain (P<0.00001, Figure 4.4b). Ovarian function indicated by progesterone synthesis and secretion at this time was found to unaffected by diet (Table 4.1). Although progesterone production is dependent on capacity of steroidogenic luteal cells, the lack of a diet effect persisted when circulating progesterone was evaluated relative to corpora lutea number (data not shown).

Figure 4.2 Number of pre-antral (a) and antral (b) follicles observed in ovaries from female mice consuming control diet (blue bars), or high fat diet (green bars). Size (area, mm^2) of oocytes contained within pre-antral (PA) and antral (A) follicles (c). Follicle counts are normalised to area of ovary (mm^2). Results expressed as mean ± SEM (a & b, n=6-12 animals/diet group; c, n=18-256 oocytes/diet group). *P<0.05, **P<0.01, ***P<0.001.

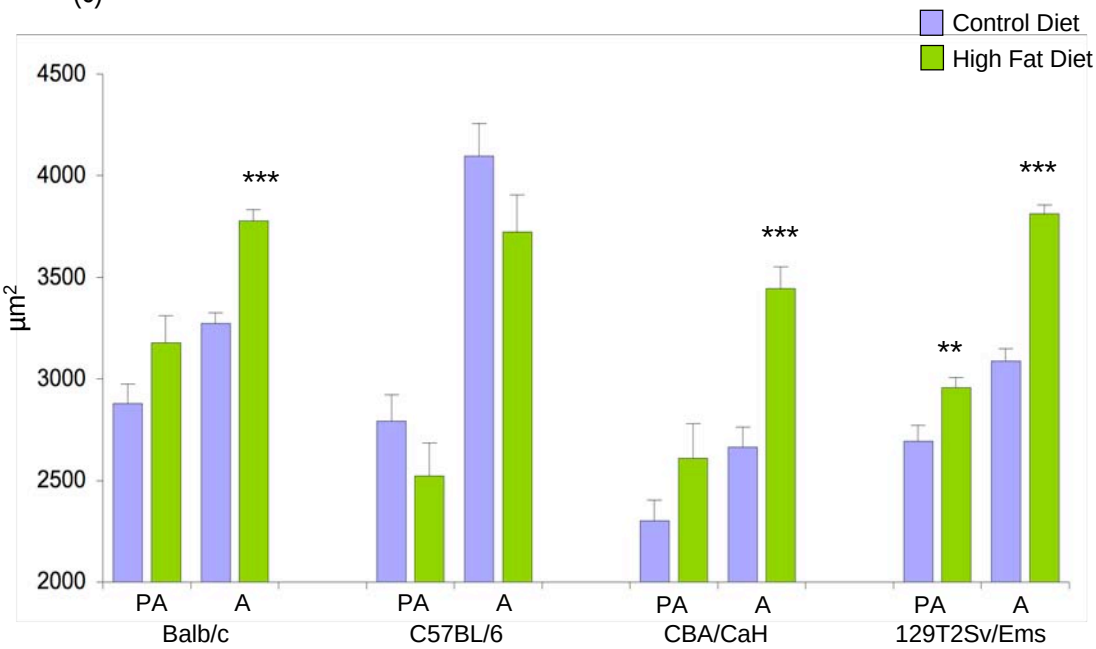
(a) Pre-antral follicles



(b) Antral follicles



(c)



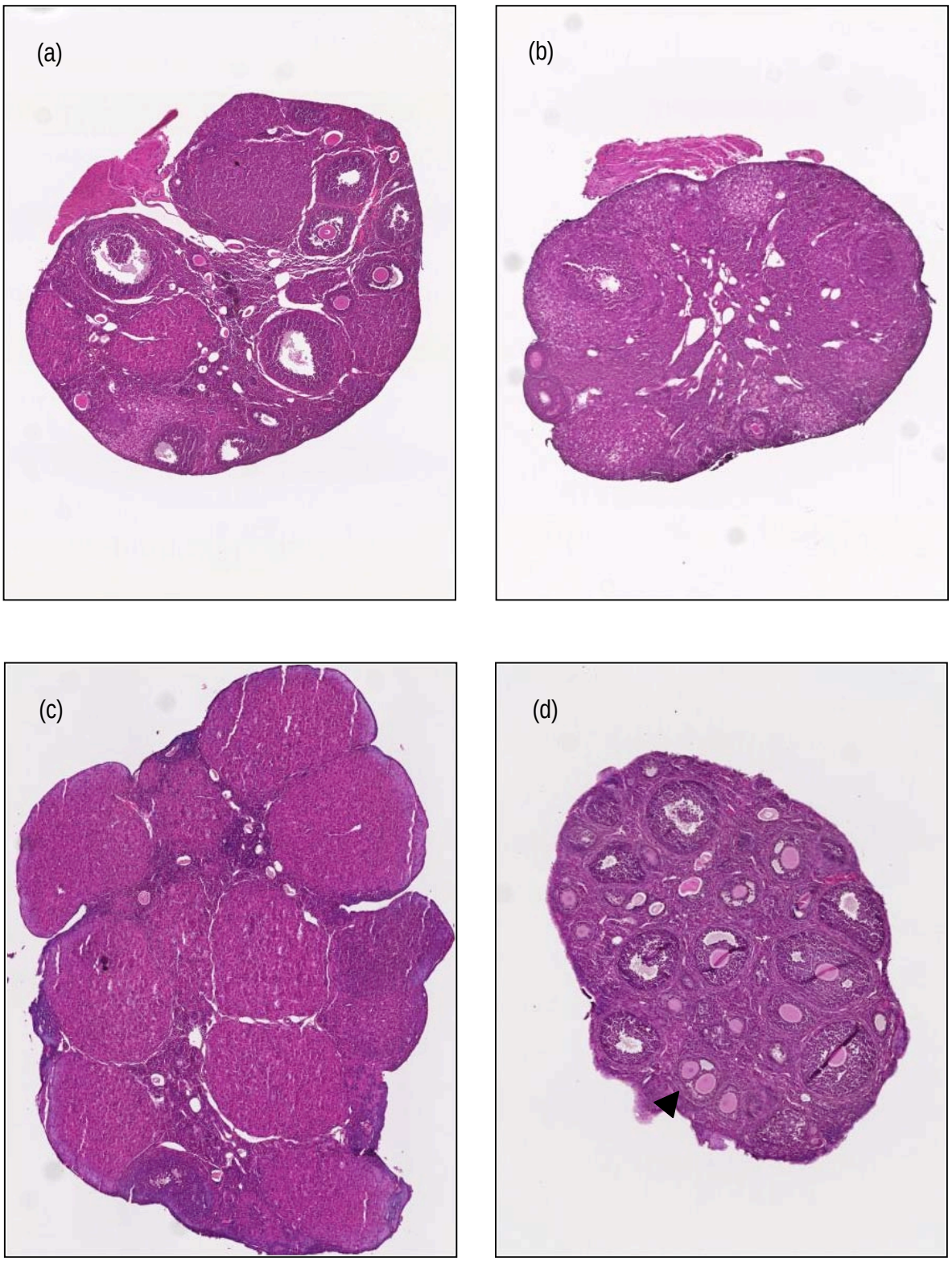


Figure 4.3 Representative histological sections of Balb/c (a), C57BL/6 (b), CBA/CaH (c) and 129T2Sv/Ems (d) ovary. All images taken at 2.5X from control diet fed animals. Arrowhead indicates multiple oocyte follicle, characteristic of both CD and HFD 129T2Sv/Ems ovaries.

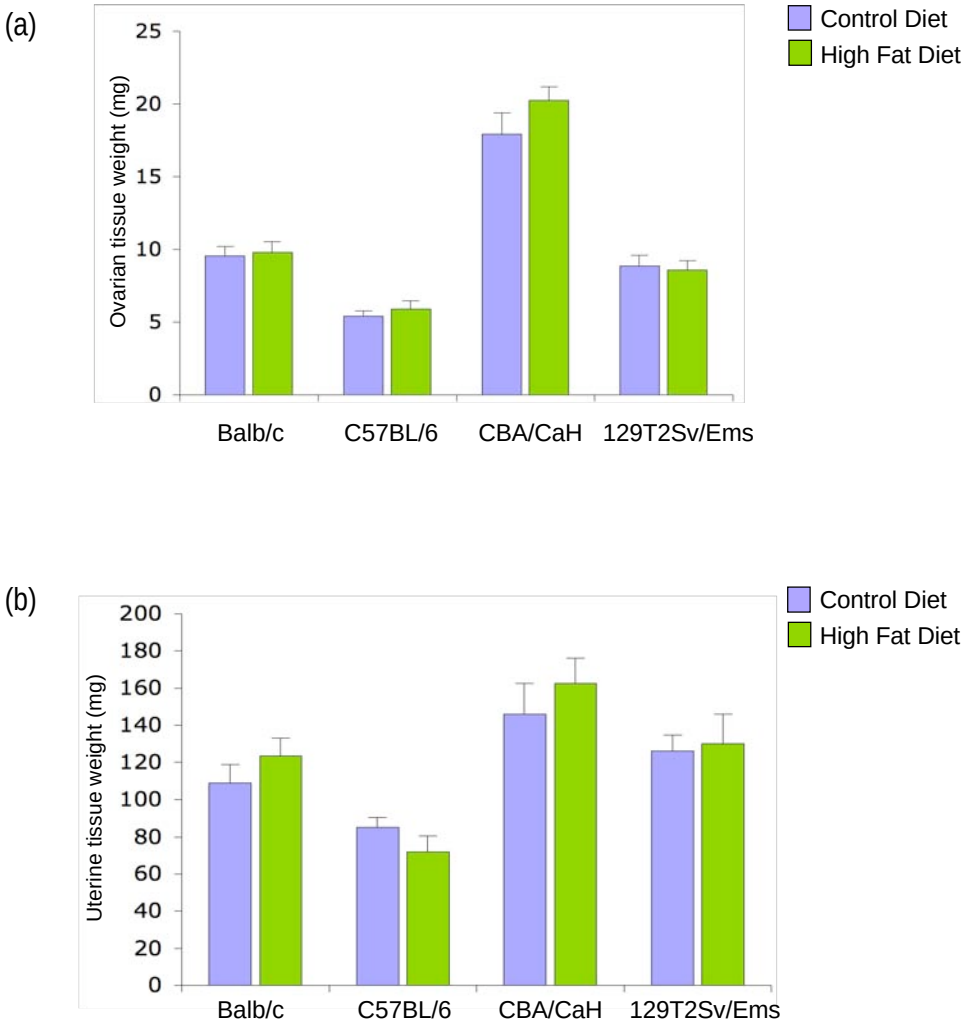


Figure 4.4 (a) Ovarian and (b) uterine tissue weights from mice fed control diet (blue bars) or high fat diet (green bars), measured on day 1 of pregnancy. Results expressed as mean ± SEM (n=9-12).

Table 4.1 Ovulation incidence and rate in response to HFD across all strains

		Days to Mate	Circulating Progesterone (nmol/L ± SEM)	Incidence of Ovulation	Oocytes/ ovulatory mouse
Balb/c	CD	1.9 ± 0.31	5.70 ± 1.20	11/12 (92%)	8.1 ± 0.49
	HFD	1.9 ± 0.26	5.21 ± 0.66	8/12 (67%) ***	8.0 ± 0.89
C57BL/6	CD	2.1 ± 0.26	8.36 ± 2.73	11/12 (92%)	5.5 ± 0.89
	HFD	2.2 ± 0.32	10.03 ± 2.02	7/12 (58%) ***	8.7 ± 1.13 *
CBA/CaH	CD	2.4 ± 0.34	16.30 ± 2.74	9/12 (75%)	6.7 ± 0.82
	HFD	3.9 ± 0.74	15.83 ± 2.43	7/11 (64%) *	7.6 ± 1.17
129T2Sv/ Ems	CD	1.8 ± 0.32	9.86 ± 2.45	10/12 (83)	6.9 ± 0.38
	HFD	1.7 ± 0.29	7.78 ± 1.92	11/13 (85%)	5.9 ± 0.77

* P < 0.05, *** P < 0.001 vs. CD

4.3.3 Ovulation incidence and rate

As shown in Table 4.1, the number of days between pairing with the male and the mating event was not influenced by diet in any strain. Ovulation was assessed by the presence of oocytes within the oviduct on the day of observation of the mating plug. The incidence of ovulation was reduced within Balb/c, C57BL/6 and CBA/CaH strains ($P<0.001$, $P<0.001$ and $P=0.011$, respectively). 129T2Sv/Ems female mice were unaffected. Post-hoc analyses using a log binomial model indicated failed ovulation was predicted across all strains by elevated circulating cholesterol ($P=0.005$, Table 4.2) and elevated bodyweight ($P=0.021$).

In females that did ovulate (i.e. had at least 1 oocyte), the net number of oocytes collected from both oviducts was not influenced by HFD in Balb/C, CBA/CaH or 129T2Sv/Ems strains. However, in C57BL/6 mice fed the HFD, ovulation rate was significantly elevated ($P=0.042$). Further analysis using a normal linear model indicated that the number of oocytes ovulated was positively correlated with the total amount of body weight gained during the 16 weeks of treatment ($P=0.043$, Table 4.2).

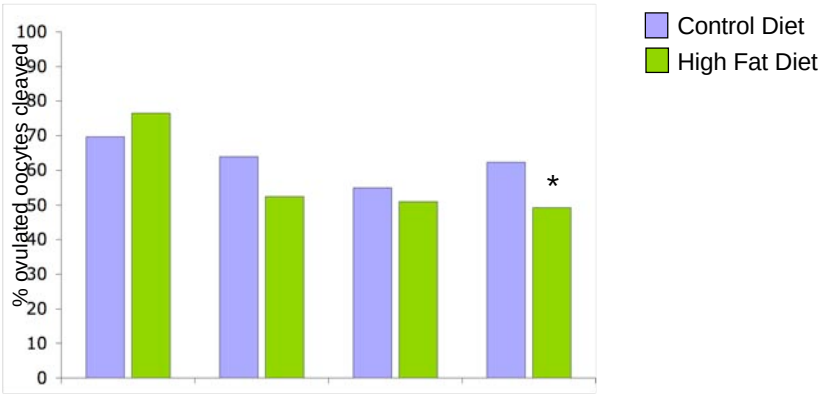
4.3.4 Zygote cleavage and blastocyst development

The number of oocytes that completed the first cleavage division was reduced in the 129SvT2/Ems HFD group ($P=0.028$, Figure 4.5). Other strains were not affected, and none of the metabolic parameters assessed were associated with this outcome.

Subsequent embryonic development in vitro was assessed by blinded, daily evaluation and scoring for correct morphology. The percent of cleaved zygotes to reach the 4-8 cell stage (on day 3 of in vitro culture), morula/blastocyst stage (on day 4 of in vitro culture), or expanded/hatching blastocyst stage (on day 5 of in vitro culture) was most severely affected by high fat diet consumption in CBA/CaH mice, where only 7.4% (2/27) of cleaved zygotes developed on-time to the expanded/hatching blastocyst stage ($P=0.0047$, Figure 4.6c). High fat diet consumption also tended to effect oocyte developmental ability of embryos collected from C57BL/6 mice ($P=0.059$, Figure 4.6b). On-time development rate of blastocysts from CBA/CaH mothers receiving either diet was significantly lower compared to those of other the strains ($P<0.003$), which should be considered if the CBA/CaH strain is used in future reproductive investigations. Analysis of impaired on-time embryo development to blastocyst stage using

Table 4.2 Metabolic parameters found to predict abnormal reproductive functions arising from HFD

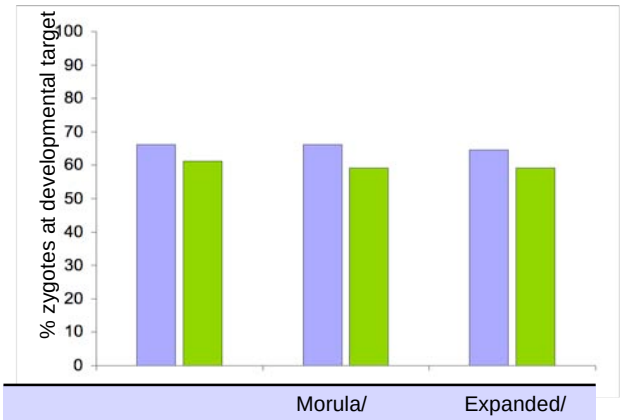
Event	Metabolic Predictor	P-value	Estimates	95% Confidence Limits
Anovulation (binary outcome)	Final bodyweight	0.005	Risk ratio = 0.8759	0.8085 - 0.9490
	Blood cholesterol	0.0213	Risk ratio = 0.7607	0.5954 - 0.9718
Increased # of oocytes (continuous variable)	Bodyweight gained	0.0429	Linear trend = 0.2635	0.0120 - 0.5149
Impaired blastocyst development (binary outcome)	Maternal fasting glucose	0.0505	Risk ratio = 0.9723	0.9445 – 1.0008
Decreased % ICM (continuous variable)	Maternal fasting insulin	0.0285	Rate ratio = 1.1851	1.0550 – 1.3312
	Maternal progesterone	0.0379	Rate ratio = 1.0034	1.0014 – 1.0055



	Balb/c	C57BL/6	CBA/CaH	129T2Sv/Ems
CD	62 / 89	39 / 61	33 / 60	43 / 69
HFD	49 / 64	32 / 61	27 / 53	32 / 65 *

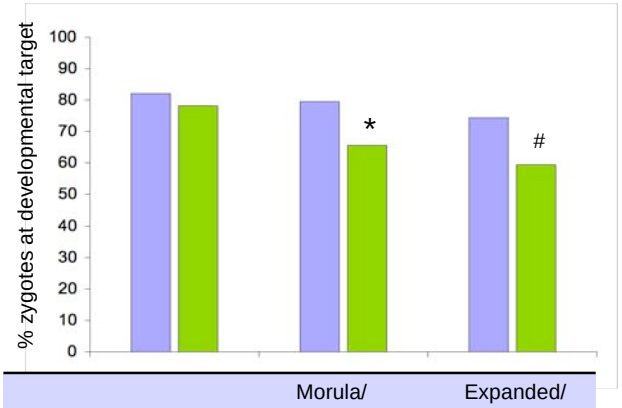
Figure 4.5 Percentage ovulated oocytes that cleaved by day 3 of in vitro culture from mice fed control diet (blue bars) or high fat diet (green bars). The number of cleaved zygotes per ovulated oocytes is indicated in the table below the graph. *P<0.05.

(a)



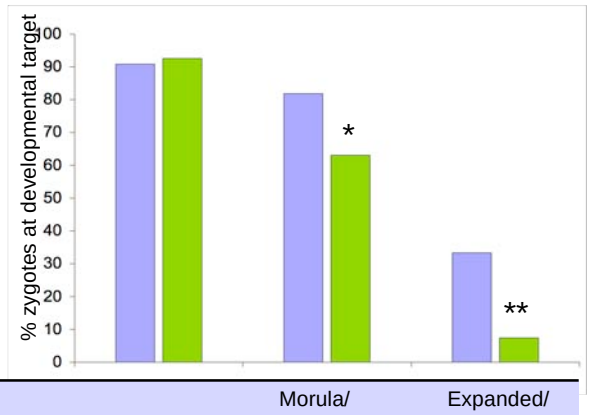
	4-8 cell	Morula/ compacted blastocyst	Expanded/ hatching blastocyst
CD	41 / 62	41 / 62	40 / 62
HFD	30 / 49	29 / 49	29 / 49

(b)



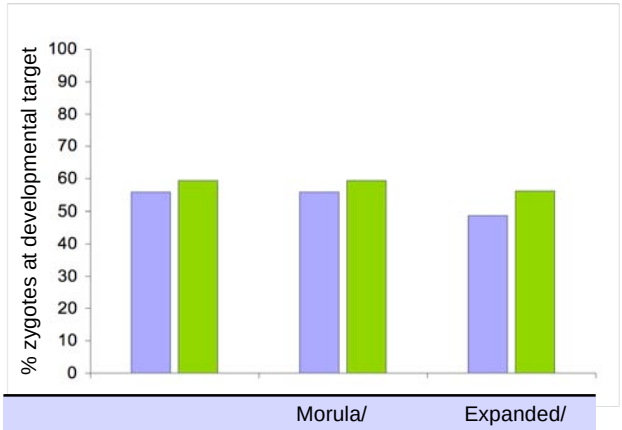
	4-8 cell	Morula/ compacted blastocyst	Expanded/ hatching blastocyst
CD	32 / 39	31 / 39	29 / 39
HFD	25 / 32	21 / 32 *	19 / 32 #

(c)



	4-8 cell	Morula/ compacted blastocyst	Expanded/ hatching blastocyst
CD	30 / 33	27 / 33	11 / 33
HFD	25 / 27	17 / 27 *	2 / 27 **

(d)



	4-8 cell	Morula/ compacted blastocyst	Expanded/ hatching blastocyst
CD	24 / 43	24 / 43	21 / 43
HFD	19 / 32	19 / 32	18 / 32

Figure 4.6 Percentage of cleaved oocytes that reached on-time the 4-8 cell, morula/blastocyst and expanded blastocyst/hatching blastocyst stages. (a) Balb/c, (b) C57BL/6, (c) CBA/CaH and (d) 129T2Sv/Ems female mice fed control diet (blue bars) or high fat diet (green bars). Tables below each graph indicate the numbers of embryos constituting percentage at each stage. # P=0.059, *P<0.05, **P<0.01.

a log binomial Generalized Estimating Equation (GEE) was predicted by elevated maternal fasting plasma glucose (P=0.05).

4.3.5 Blastomere differentiation

All embryos surviving to day 5 of culture were subjected to a differential staining protocol to permit counting of cells constituting the trophectoderm layer (TE) and the inner cell mass (ICM). The lack of CBA/CaH embryo survival precluded their inclusion in these analyses. The average number of cells in TE and ICM compartments was reduced in 129T2Sv/Ems-derived embryos (TE P=0.0017, ICM P=0.0075 Figure 4.7a, b). Interestingly, C57BL/6 HFD embryos did not demonstrate such a uniform reduction in cell number, and instead exhibited a diversion of cells into the trophectoderm in preference to the inner cell mass, resulting in blastocysts possessing a reduction in the percent of cells comprising the inner cell mass (P=0.016 Figure 4.7c). The reduction in the number of cells in the ICM relative to the TE was analyzed using a log poisson GEE. This was correlated with both increased fasting insulin (P=0.028) and increased progesterone (P=0.037) in maternal circulation.

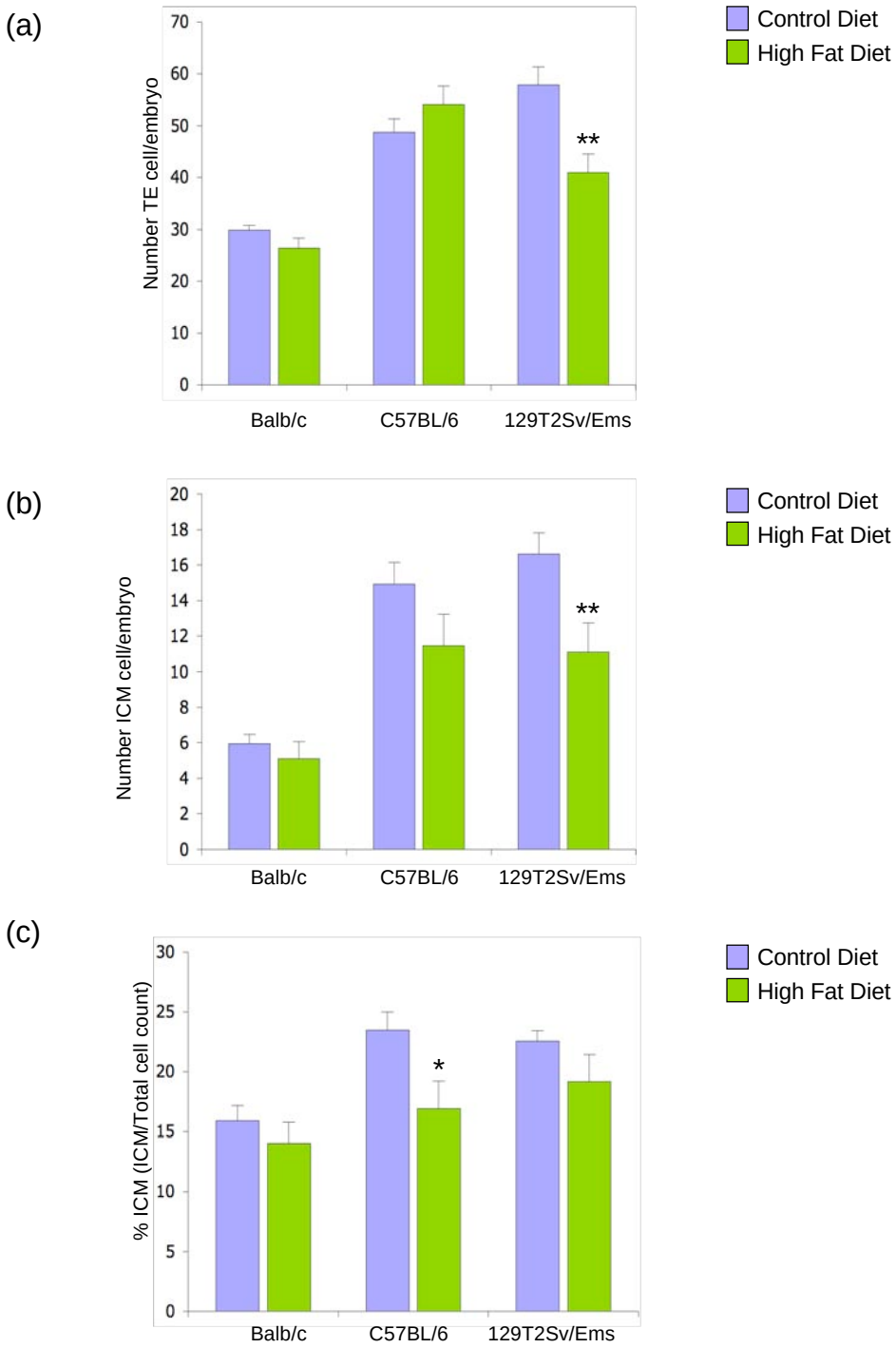


Figure 4.7 Average number of cells comprising the (a) trophectoderm (TE), (b) inner cell mass (ICM), and (c) ICM as % of total cells of day 5 embryos. Zygotes were derived from Balb/c, C57BL/6 and 129T2Sv/Ems mice fed control diet (blue bars) or high fat diet (green bars). Bars indicate mean ± SEM (n=18-40 blastocysts/group). *P<0.05, **P<0.01.

4.4 DISCUSSION

We report here that multiple reproductive processes including successful ovulation, blastocyst development and embryonic cellular differentiation are negatively influenced by maternal consumption of a diet high in fat. The HFD used in these studies mimics a “Western diet” and led to the onset of obesity, as well as hyperinsulinemia and hyperlipidemia (Chapter 3). In the current study oocytes were removed from the maternal environment, and maintained within a standardized in vitro culture milieu. As a result, each of these post-fertilization outcomes reflects the health and competence of the peri-ovulatory oocyte. This provides insight into the contribution of initial oocyte health to embryonic outcomes that might otherwise appear far removed from the ovarian follicle. Our analysis of multiple strains of laboratory mice also demonstrates the subtle influence of genetic background (summarized in Table 4.3). We have documented marked strain differences in ovarian tissue weight, progesterone production, and antral follicle number in mice of different strains irrespective of diet. In addition, we report strongly heterogeneous responses of the four strains to the systemic and localised reproductive effects of a high fat diet, particularly dramatic differences in blastocyst development and embryonic cell allocation. The parallel investigation of multiple strains is indeed a primary strength of this study, and permits identification of statistical correlations concurrent with consideration of both genetic and epigenetic influences.

When the ovaries of these animals were histologically examined, two specific defects were observed in tissues collected from HFD females. Firstly, the number of healthy follicles observed within the ovary is influenced by both strain and maternal diet. In a previous study, pre-pubertal 129/Sv mice were found to have significantly more follicles of all developmental stages compared to C57BL/6 females (Canning *et al.* 2003). In our study Balb/c and 129T2Sv/Ems ovaries also contained many more large follicles than either C57BL/6 or CBA/CaH ovaries. Further to this, the effect of diet on the number of follicles observed, and hence the progression of atretic processes, was also influenced by strain. In this respect, Balb/c and CBA/CaH ovarian tissues were affected, and had significantly fewer small, pre-antral follicles. However, this did not persist as the follicles continued to develop, and at this stage it is unclear whether early stage abnormal follicle numbers is a reflection of oocyte recruitment into the growing pool, or functions of follicle atresia. Despite these histological analyses suggesting that HFD follicle numbers are atypical at some stages, it did not influence the number of oocytes ultimately ovulated in the Balb/c CBA/CaH or 129T2Sv/Ems strains.

Table 4.3 Summary of reproductive defects observed in mice fed high fat diet, compared to those fed control diet.

	Balb/c	C57BL/6	CBA/CaH	129T2Sv/Ems
Follicle number	↓	-	↓	-
Oocyte size	↑	-	↑	↑
Anovulation	↑	↑	↑	-
Ovulation rate	-	↑	-	-
Zygote cleavage	-	-	-	↓
Blastocyst development	-	↓	↓	-
Inner cell mass composition	-	↓	ND	↓

ND: not determined

More interesting is the observation that healthy oocytes contained within large, antral follicles from most HFD females were oversized. The oocyte expands and undergoes a considerable increase in volume after recruitment into the growing pool, accumulating water, ions and lipids and increasing both the rate of protein synthesis and total cellular protein content (Picton *et al.* 1998). Our findings suggest that in many strains of mouse, dietary composition can significantly influence these processes, and result in oocytes with discordant growth and maturation. It is surprising therefore, when later outcomes such as blastomere differentiation are considered, that C57BL/6 ovarian tissue did not display any defects in follicle number or oocyte size that were observed in the other strains.

The HFD lowered the chance of a successful ovulatory event in most strains investigated here, which was associated with excessive bodyweight and hypercholesterolemia. This finding is supportive of correlations between elevated bodyweight and natural pregnancy rates in mice (Tortoriello *et al.* 2004) as well as well-documented ovulatory infertility in women with increased BMI (Pasquali *et al.* 2007). Our data suggests circulating cholesterol levels should also be considered in such populations, and actions of this metabolite on ovarian steroid production and the hypothalamic-pituitary-ovarian axis may be involved (Azhar *et al.* 1990, Robins *et al.* 1994, Tortoriello *et al.* 2004).

Interestingly, amongst animals able to successfully release oocytes at the appropriate stage of the estrous cycle, there appeared to be an additional perturbation. Although no circulating metabolites correlated with the number of oocytes ovulated, we did observe a positive correlation between final bodyweight and oocyte number, such that as maternal bodyweight increased, the number of oocytes ovulated also increased. The Linear Trend estimate for the relationship between the number of oocytes ovulated and bodyweight is 0.2635, such that as mouse bodyweight increases by 1g, the number of oocytes released increases by 0.2635. A bodyweight increase of 3-4 g above the population average therefore results in the ovulation of 1 additional oocyte above the population average for that strain. Thus, while the most severely diet-affected animals lose all ovulatory potential, we hypothesize that those that can overcome this barrier are afflicted with incorrectly timed follicle growth and maturation that inappropriately releases an excessive number of sub-standard oocytes. This may contribute to the reduced chance of embryonic survival, and discordant cellular allocation seen in later stage of blastocyst growth.

We report that only 129T2Sv/Ems HFD mice have reduced cleavage of fertilized oocytes in this experimental protocol. Expedient cleavage of the oocyte relative to insemination has been shown in previous settings to be an important indicator of embryonic developmental potential and can be used to

distinguish between oocytes of different quality (Lonergan *et al.* 1999, Gutierrez-Adan *et al.* 2004). In the current protocol zygotes were not disturbed for approximately 44 hours after the beginning of culture, which equates to approximately 56 hours post-fertilization. Consequently we cannot comment on the effect of diet on the timing of cleavage, although it would certainly offer an interesting perspective as we try to characterise the complete spectrum of oocyte defects induced by this high fat diet.

Early embryonic development was assessed as fertilised zygotes progressed to the expanded or hatching blastocyst stage. When fertilised zygotes from HFD females were assessed in comparison to those produced by CD mice, two strains produced embryos that were slower to develop, or more likely to developmentally arrest altogether. Although C57BL/6 HFD embryos appeared affected to some degree, CBA/CaH HFD embryos exhibited a profound inhibition of growth and development in response to maternal HFD consumption. High maternal blood glucose levels were associated with this embryonic defect across all strains. Previous studies have reported that both chronic and acute hyperglycemia impacts the developing preovulatory oocyte by delaying growth and maturation, increasing apoptosis of the surrounding granulosa cells via up-regulating TRAIL-KILLER apoptotic pathways, and diminishing levels of the gap junction protein connexin-43 (Chang *et al.* 2005). It is possible impaired blastocyst development reflects such perturbed follicular events.

Blastomere differentiation into TE and ICM cells occurs at cavitation, when the compacted embryo undergoes cell polarization and the onset of cellular differentiation (Pratt *et al.* 1982). The ICM cells contribute to all embryonic tissues and are part of the extraembryonic membranes, whereas the TE cells form the outer layer of the placenta (Gardner 1989). An appropriate ratio of both cell lineages is crucial for optimal embryonic and fetal development. Even though zygotes from the 129T2Sv/Ems strain were not developmentally impaired by HFD exposure in a temporal manner, they were still negatively impacted by this diet such this early cellular differentiation was abnormal. A general shift towards reduced numbers of ICM cells in otherwise morphologically normal HFD embryos was even further highlighted in C57BL/6 embryos that compensated with increased TE cell proliferation. Similar changes toward TE allocation in embryos from mothers fed diet with adequate to excessive nutrients compared to insufficient nutrient supply has been described in the sheep (Kakar *et al.* 2005) and rat (Kwong *et al.* 2000). Furthermore, although not directly analyzed here, these early perturbations have been documented by previous studies to result in reduced fetal survival and/or reduced birth weight (Lane & Gardner 1997, Kwong *et al.* 2000). This embryonic outcome was strongly predicted by both elevated maternal fasting insulin and progesterone, although it seems likely that the association with high

progesterone is secondary to the insulin effect: it has been reported that human luteinized granulosa cells treated with insulin increased production of 3β-HSD protein, and when treated with insulin and FSH these cells up-regulated production of progesterone (McGee *et al.* 1995). Confirmation of the genuine effect of insulin in vivo has not yet been presented, and it appears that insulin may have either stimulatory or inhibitory effects on ovarian steroidogenic enzymes differentially regulated by cell type and across species.

The four strains of laboratory mouse interrogated in the current study responded heterogeneously to the systemic effects of HFD feeding. An understanding of the genetic origins of each of these colonies of mouse may assist in explaining the dramatically adverse effects on CBA/CaH embryos. The work of Tortoriello and colleagues (Tortoriello *et al.* 2004) has described, albeit subtle, diet-induced infertility utilising DBA/2J female mice. DBA and CBA mice share some common ancestry, with CBA being derived from a cross involving DBA (Taylor 1972). It is possible that both CBA and DBA strains have retained genetic remnants leaving the reproductive axis of these females sensitive to environmental stressors. We therefore suggest these strains do not provide an ideal choice for studies that require a reproductively robust model. It is our opinion that the C57BL/6 strain still presents the most applicable model for rodent research interested in the implications of diet on reproductive function, despite their genetically atypical profile when compared to other laboratory strains (Taylor 1972). They are particularly attractive to metabolic/reproductive research, as they develop a comparative spectrum of metabolic disorders to humans when fed a high fat diet, and also sustain moderate, yet wide ranging defects at the ovarian level. This ultimately results in the production of embryos with a legacy of adverse outcomes.

It is well established that oocyte competence is essential for normal development. Such competence is progressively acquired during late folliculogenesis, through a range of cellular and molecular attributes that allow the oocyte to complete meiosis, ensure monospermic fertilization, decondense the sperm head, proceed through maternal-zygote transition, and undergo pre-implantation development (Coticchio *et al.* 2004). Previously, these were considerations primarily for situations in which immature oocytes are removed from follicles and must then undergo not only nuclear and cytoplasmic, but also molecular maturation in vitro (Sirard 2001). Here we report that such defects are also displayed by naturally ovulated oocytes, when the ovarian environment is in the context of an overweight, insulin resistant and hyperlipidemic individual. These disturbed follicular dynamics result in oocytes that do not have the capacity to support normal embryo development. The implications for human reproductive

biology are enormous. In most developed societies, as the incidence of obesity in reproductive-aged women increases, so does the number of women requiring assisted reproductive technologies to aid their fertility. The results of this study once again place the coordinated growth and development of the oocyte at the controls of early embryo development and emphasise appropriate preovulatory follicular events as crucial to the eventuation of a healthy pregnancy.

In particular, we show that the peri-ovulatory environment controls the establishment of oocyte maturation and developmental competence prior to conception. In addition we have demonstrated that maternal diet and metabolic status influence these essential processes.

Chapter 5

Preliminary microarray analyses of diet regulated preovulatory gene expression in ovaries from C57BL/6 mice

5.1 INTRODUCTION

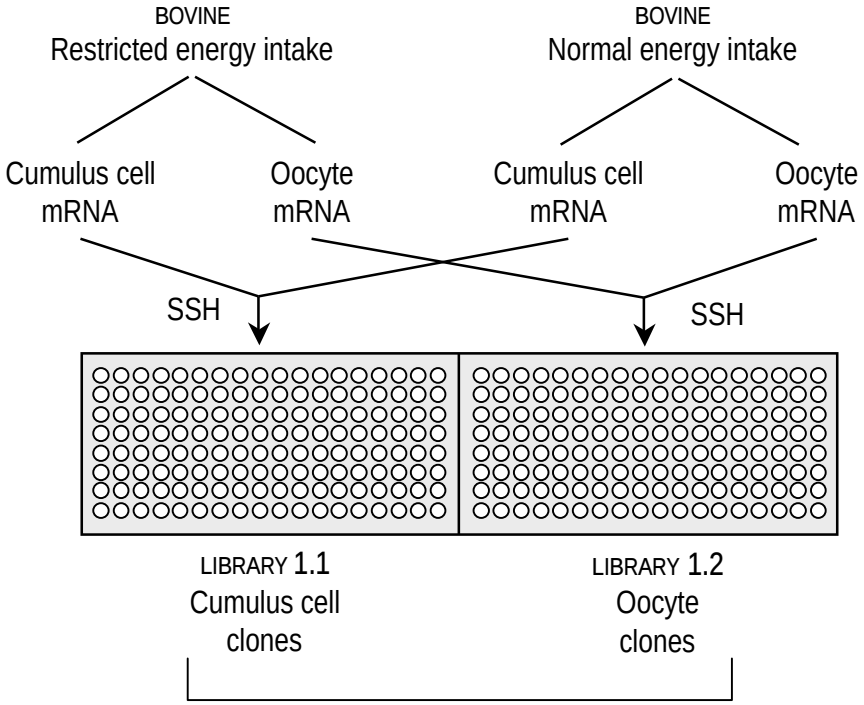
The ovary, which responds to cyclic pituitary gonadotrophin secretion to produce fertilisable oocytes, is a dynamic and cyclically morphogenic tissue. The various follicular compartments (theca cells, mural granulosa and cumulus cells) interact in a highly integrated manner, and participate in bi-directional communications between the somatic follicular cells and the oocyte itself (Eppig *et al.* 2002, Gilchrist *et al.* 2004); processes essential for the acquisition of oocyte developmental competence, and subsequent healthy embryonic development.

Our preceding findings, which show the capacity for normal embryonic development is severely impaired when the oocyte is produced by a high fat diet fed and obese mother, alludes to substantial alteration in these communications and functions. It was the aim of this study to conduct large-scale analyses of gene expression changes in the ovarian tissue prior to ovulation and oocyte release in samples collected from both control diet and high fat diet animals.

The laboratory of Prof Marc-André Sirard has established a method of microarray analysis directed against ovarian gene expression using Suppressive Subtractive Hybridization (SSH). This technique enriches for transcripts important to specific cell types or treatments. The SSH method is based on a suppression PCR effect and combines normalization and subtraction; the normalization step equalizes the abundance of DNA fragments within the target population, and the subtraction step excludes sequences that are common to the populations being compared (Diatchenko *et al.* 1996). There are substantial advantages of using this system, enriched for oocyte/cumulus/granulosa cell genes, over commercial slides: it increases dramatically the likelihood of obtaining low-abundance, differentially expressed cDNA, and simplifies analysis of the subtracted library. In this case, six distinct subtracted libraries were used, and printed on 2 slides. Slide 1 was printed with 2 SSH libraries; bovine oocyte and cumulus cells genes differentially expressed in ovaries from cows under energy restricted versus normally fed conditions (Figure 5.1a). Slide 2 was printed with 4 SSH libraries; human and bovine granulosa and cumulus cells from follicles that gave rise to embryos that did, versus did not, implant (Figure 5.1b). This approach required cross-species hybridization, and several studies have previously performed different analyses to demonstrate the reliability of results obtained through microarray cross-species hybridization (Chismar *et al.* 2002, Renn *et al.* 2004, Ji *et al.* 2004, Bar-Or *et al.* 2006).

In the preceding chapter we demonstrated that C57BL/6 mice develop profound metabolic perturbations in response to a high fat diet, and that this strain also exhibits significant deficits to oocyte

(a)



Genes differentially expressed in energy restricted vs. normal energy intake animals

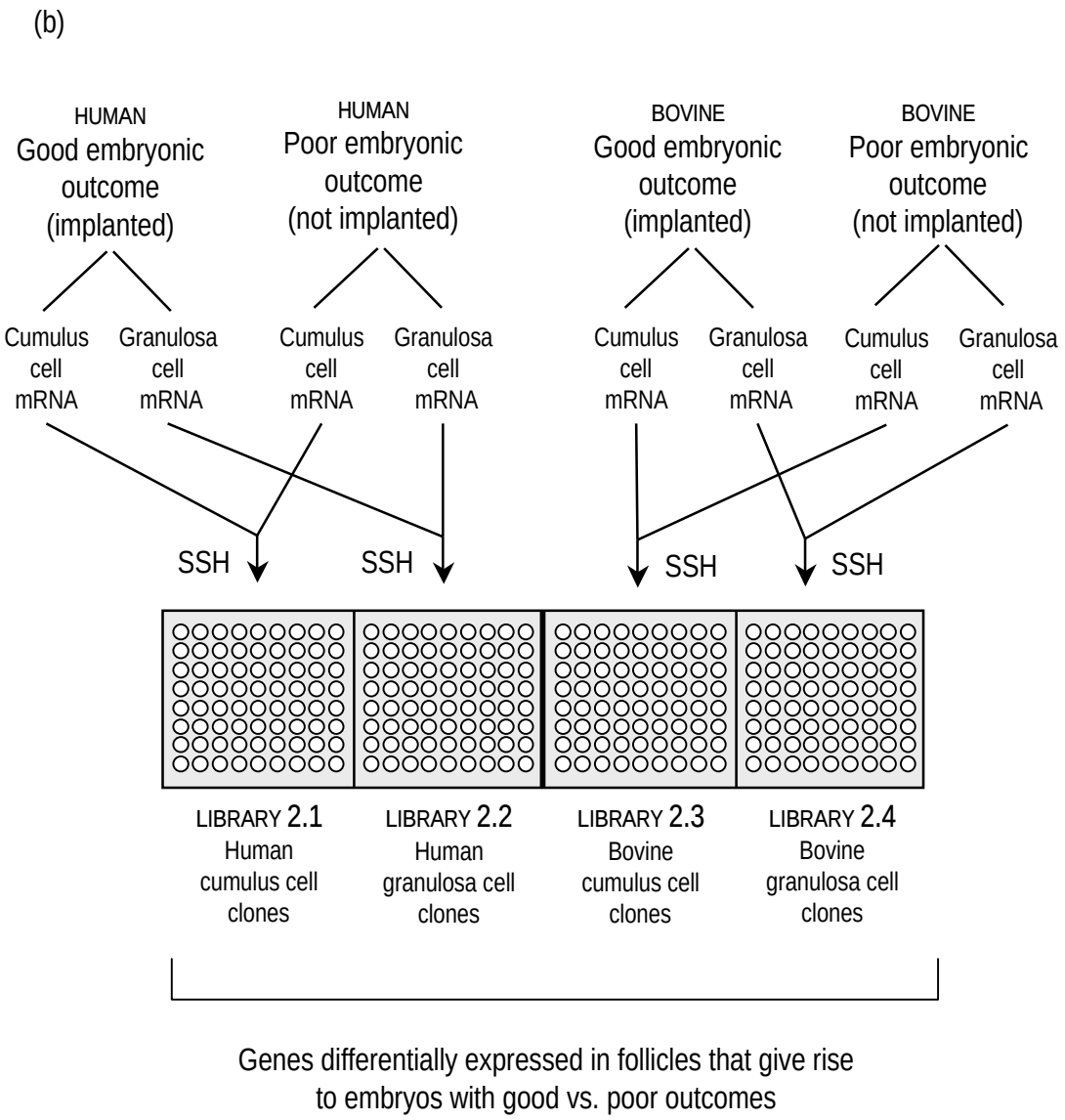


Figure 5.1. Schematic of Slide design. Slide 1 (a), printed with 2 suppressive subtractive hybridization (SSH) libraries. Slide 2 (b), printed with 4 suppressive subtractive hybridization (SSH) libraries.

developmental potential. Our next objective was thus to determine differences in ovarian mRNA expression associated with the observed alterations in oocyte competence. In collaboration with the Sirard group, we were able to further focus our investigations on genes known to be expressed in ovarian cells and altered by nutritional status or associated with differential embryo outcomes by screening the SSH libraries.

Female mice were fed the control diet or high fat diet, and representatives selected for pooling and microarray analysis (see Figure 5.2). Genes exhibiting potential differential expression patterns could then be validated in the original, larger cohort. As our previous findings were observed in zygotes arising from natural matings, and not hormonally stimulated ovulations, the current experiment was also conducted without exogenous hormonal stimulations. This was to mirror normal ovarian events as closely as possible, without inducing the additional, and potentially misleading effects of superovulation on oocyte quality, known to contribute to delayed embryo development, decreased implantation rates, and abnormal imprinting of important oocyte genes (Fossum *et al.* 1989, Ertzeid & Storeng 1992, Van der Auwera & D'Hooghe 2001, Ertzeid & Storeng 2001) (Fortier *et al.* 2008). All ovarian samples were collected at pro-estrous, characterized by the final growth of large, antral follicles ready for ovulation. By capturing these follicles before ovulatory release, insight would be gained into the gene transcription profile present at the final stages of oocyte maturation, which we hypothesized would be altered by maternal obesity.

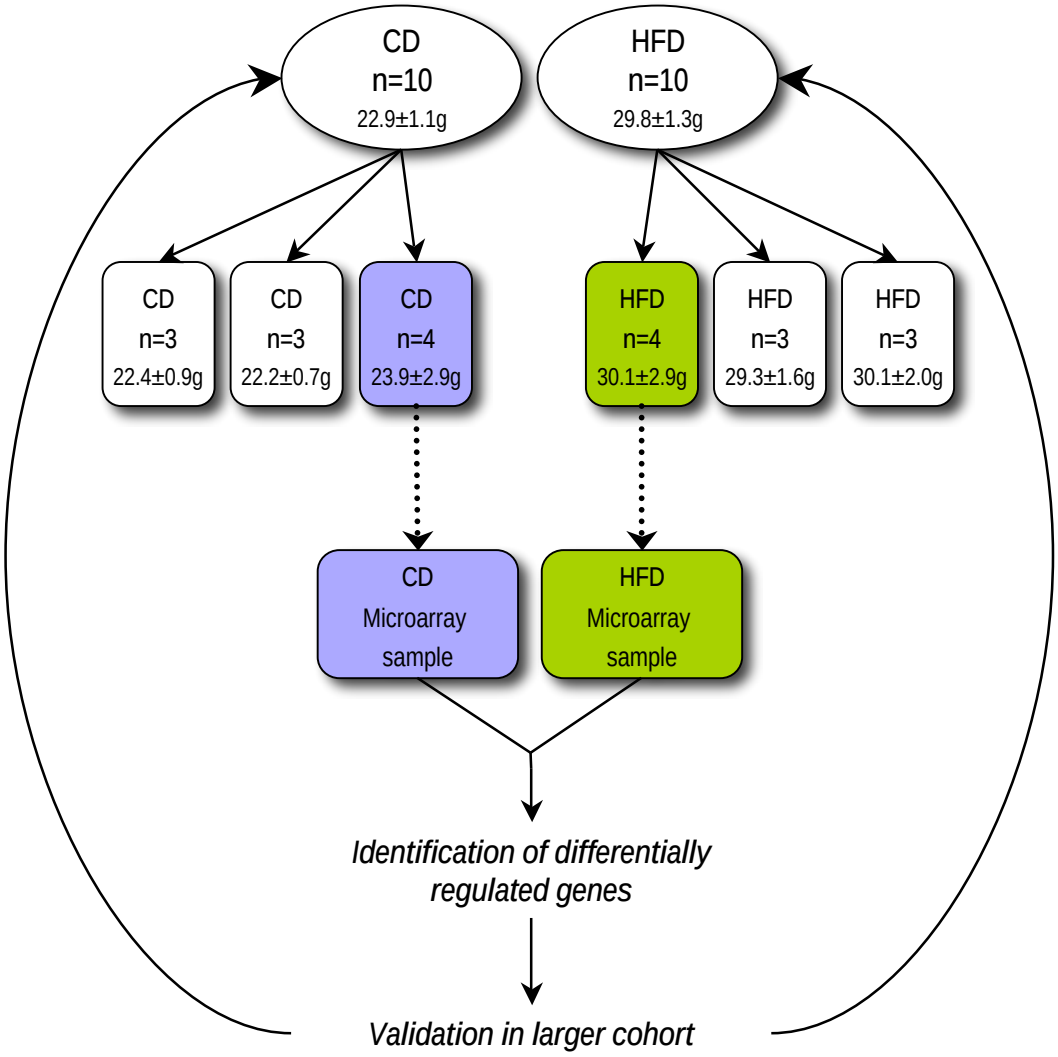


Figure 5.2. Schematic of experimental design. Female mice fed control diet (CD) or high fat diet (HFD) (n=10) were allocated into 3 subgroups (n=3 or 4, by bodyweight), one of which was selected as the sample for application to the microarray slides. Genes identified as differentially regulated were then validated in individual samples from the larger, original cohort.

5.2 MATERIALS AND METHODS

5.2.1 Animals and diets

Twenty C57BL/6 5-week-old female mice were utilised for these experiments. Details on animal sourcing, handling and feeding are contained in Chapter 2.

5.2.2 Glucose tolerance

Please refer to Chapter 2 for details on assessment of glucose tolerance.

5.2.3 Tissue collection and RNA preparation

Vaginal smears were obtained daily at 0800h, using the modified method of Nelson et al. (Nelson *et al.* 1982) to classify the phases of the estrous cycle (Appendix III). Briefly, the vagina was flushed with 20μl sterile saline using a pipette, and the sample was examined as a wet, unstained preparation on a microscope slide under a coverslip on an inverted light microscope (DMIRB, Leica). Smears were read by one individual (RLR) to minimise variance, and mice were considered to be in pro-estrous when smears showed a medium density of clumped nucleated epithelial cells. Mice were weighed, then sacrificed by cervical dislocation at 1300h of the day of pro-estrous. Both ovaries were dissected and snap frozen in liquid nitrogen.

Total cellular RNA was isolated from both pooled ovaries as described in Chapter 2. 10 samples from each diet group were divided into 3 subgroups containing an equal distribution of bodyweights. 1μg of ovarian mRNA from each animal in the microarray subgroup was pooled to total 4μg/diet group. This material was then air-shipped on dry ice to the Centre de Recherche en Biologie de la Reproduction, Departement des Sciences Animales, Universite Laval, Quebec, Canada. Upon arrival, total RNA content, quality and integrity was confirmed using an Agilent Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, USA). mRNA was purified using poly-A extraction with a DINAL extraction kit (DYNABEADS® mRNA DIRECT™, Lake Success, USA), followed by amplification using the RiboAmp™ RNA Amplification kit (Molecular Devices, Mountain View, USA) according to the manufacturer's instructions. Briefly, purified RNA was reverse transcribed with a primer incorporating a

T7 RNA polymerase promoter sequence. Double-stranded cDNA was synthesized, column purified (Qiagen, Mississauga, Canada) and used as a template to drive in vitro transcription using the T7 polymerase. This global amplification was linearly amplified by one round and the resulting UTP-amino allyl RNA (aaRNA) was column purified and the quantity of aaRNA was established by spectrophotometry at 260 nm using a NanoDrop Spectrophotometer (NO-100, Biolab).

5.2.4 Custom-made cDNA microarray hybridization

Probes were made by labelling aaRNA with Alexa Fluor 555 and 647 reactive dye packs (Invitrogen) according to the protocol from Molecular Probes. Labelled probes were purified from excess, unbound fluorophore using a PicoPure™ RNA Isolation Kit (Arctus Bioscience, Mountain View, USA). Labelled purified probes were hybridized to microarray slides in SlideHyb #1 buffer (Ambion, Austin, USA) overnight at 50°C. Hybridisations were performed in an ArrayBooster using the AdvacardAC3C (The Gel Company, San Francisco, USA). Slides were then washed twice with 2 x standard saline citrate (SSC)/0.5% sodium dodecyl sulphate (SDS) for 15 min at 50°C and twice with 0.5 x SCC/0.5% SDS for 15 min at 50°C.

A dye swap hybridisation for the two sets of probes (CD and HFD pro-estrous ovary) was also performed for each microarray slide.

5.2.5 Analysis of hybridized microarray slides

Slides were scanned using the VersArray ChipReader System (Bio-Rad) and analyzed using the ChipReader and ArrayPro Analyzer software (Media Cybernetics, Bethesda, USA). Fluorescence signal intensities for each replicate were log2 transformed and normalised by the Loess method, and corrected for background. The determination of the background signal threshold was performed with the SpotReport cDNA controls (Stratagene), which determine the background (t=m+2 x sd, where “t” is the calculated threshold, “m” is the mean of the negative control raw data and “sd” is the standard deviation of the same negative control raw data, n=58). Transcripts above the threshold were considered as present in the ovarian cells, whereas other transcripts were eliminated from further analysis. If one replicate was lower than the background, or if the direction of regulation was not consistent, the clone

was completely eliminated from the analysis. Only the spots with the highest ratio were selected for un-blinding and of those (fold change ±0.05), only 2 were high enough to test further with real-time PCR.

5.2.6 Real-time PCR

Reversed transcription and semi-quantitative real time PCR using the Corbett Rotor-Gene™ 6000 (Corbett Life Sciences) real-time rotary analyser was performed as described in Chapter 2. Ribosomal protein L19 (Rpl19) was selected as internal control for Secreted Acidic Cysteine Rich Glycoprotein (Sparc), and ribosomal protein S3 (Rps3) was selected as internal control for High Mobility Group Box 1 (Hmgb1). All primers were designed using Primer Express™ software except for Rps3 (Weisberg *et al.* 2003), and synthesized by GeneWorks (Thebarton, South Australia, Australia) (Table 5.1).

5.2.7 Data analysis

For genes of interest, mRNA content was calculated relative to its housekeeping gene. All real-time PCR data was analyzed using the $\Delta\Delta C_T$ method (Livak & Schmittgen 2001) where C_T is the difference between the gene of interest and the housekeeping gene. HFD data were subsequently normalized to fold change compared to CD. Differences were considered significant at $P < 0.05$, determined by Student's t-test using SPSS 13.0 for Windows (SPSS Inc. Chicago, IL).

Table 5.1. Primer sequences

Gene	Accession #	Sequences
Sparc	NM_009242	F: 5'-CACCTGGACTACATCGGACCAT-3' R: 5'-CAGGACATTTTTGAGCCAGTCA-3'
Hmgb1	NM_010439	F: 5'-GGTCAAGGCTGAAAAGAG-3' R: 5'-ACCACCAGGACAGGGCTA-3'
Rpl19	NM_009078	F: 5'-TTCCCGAGTACAGCACCTTTGAC-3' R: 5'-CACGGCTTTGGCTTCATTTTAAC-3'
Rps3	NM_012052.2	F: 5'-ATCAGAGAGTTGACCGCAGTT-3' R: 5'-AATGAACCGAAGCACACCATAG-3'

5.3 RESULTS

5.3.1 Metabolic disturbance induced by high fat diet

As previously described, the consumption of the high fat diet induced weight gain in the C57BL/6 female mice. After 16 weeks of high fat feeding, bodyweight was significantly increased in HFD animals compared to those on CD (P=0.0007, Figure 5.3a). Mice selected for the microarray analysis of ovarian gene expression were also significantly heavier than their CD counterparts (P=0.033, Figure 5.3b).

The glycemic status of these animals was determined with an IPGTT, as a relatively non-invasive method to demonstrate metabolic dysfunction. This cohort of female mice fed HFD had impaired glucose tolerance, with significantly elevated blood glucose level at 60, 90 and 120 min post-glucose challenge (Figure 5.4a). This resulted in an increased AUC for the IPGTT (P=0.016). Previous cohorts of C57BL/6 female mice fed the HFD (Chapter 3, Figure 3.6d) had not exhibited such glucose intolerance when tested in this way, suggesting the influence of seasonal factors.

In the smaller microarray group, blood glucose levels peaked at similar levels (approx. 25 mmol/L), at 30 and 60 min post-glucose challenge in the CD and HFD animals respectively (Figure 5.4b). However, hyperglycemia persisted in the HFD animals, with elevated blood glucose levels at 90 and 120 min post-glucose challenge (P=0.004, P=0.022 respectively). Overall, the AUC value approached significance (P=0.08).

5.3.2 Differential gene expression: Microarray Slide 1- bovine cumulus cell and oocyte genes from cows on restricted energy intake versus normal energy intake

Following hybridisation with ovarian mRNA from the control diet and high fat diet fed animals there were only minimal differences in the intensity of clone spots on the microarray slides. Table 5.2 illustrates the cumulus/oocyte genes potentially regulated by diet, although as the fold change column indicates, each is consistently less than a 0.1 fold change. We selected Hmgb1 for closer examination. HMGB1 (also known as amphoterin) is a nuclear protein that functions as a signal transduction ligand following AGER activation by binding to advanced glycation end products (AGEs). HMGB1 is capable of up-regulating the transcriptional activity of steroid hormone receptors such as the estrogen receptor (Das *et al.* 2004), but of particular relevance here, is thought to be involved in the maternal- zygotic transition (MZT), as

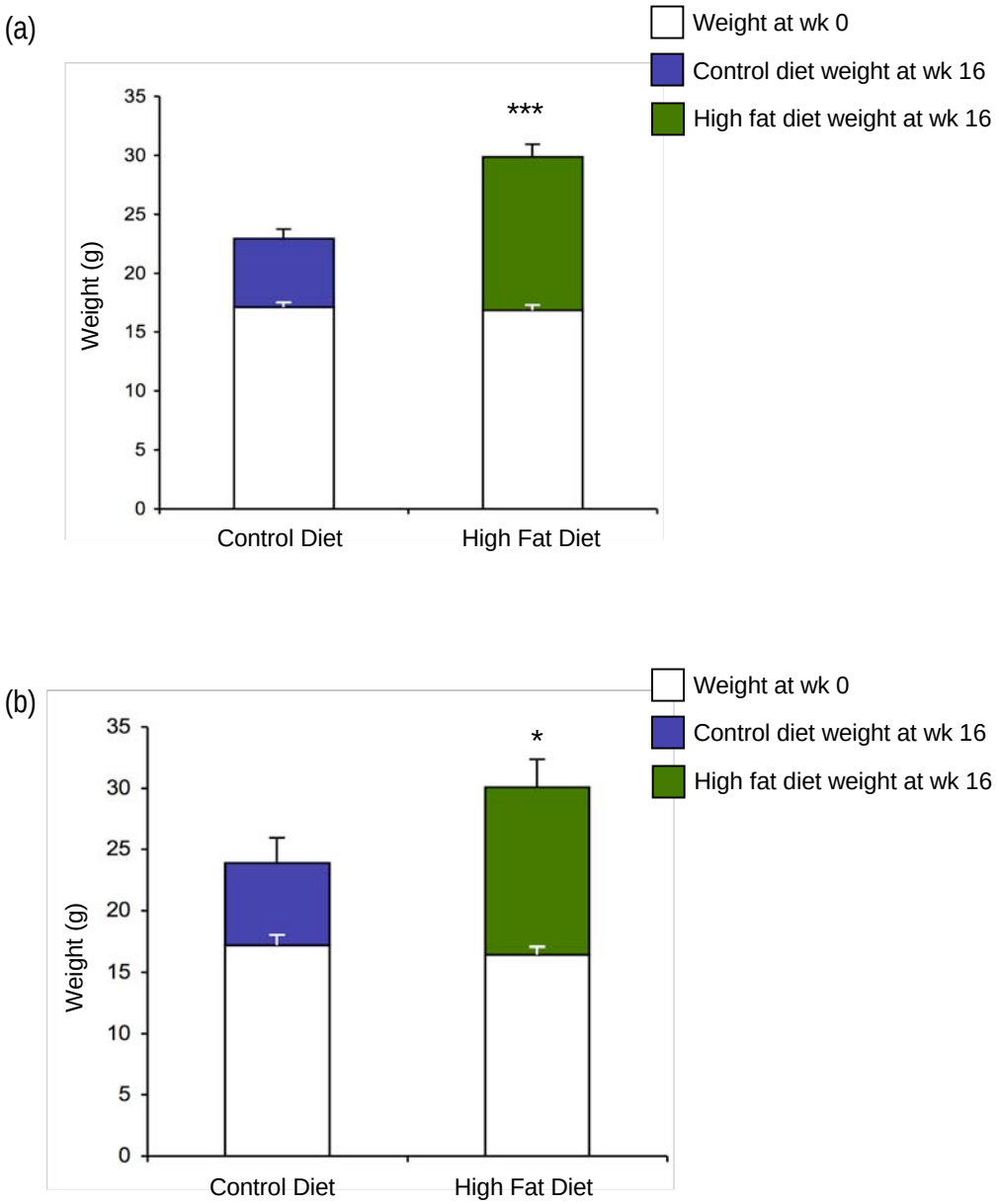
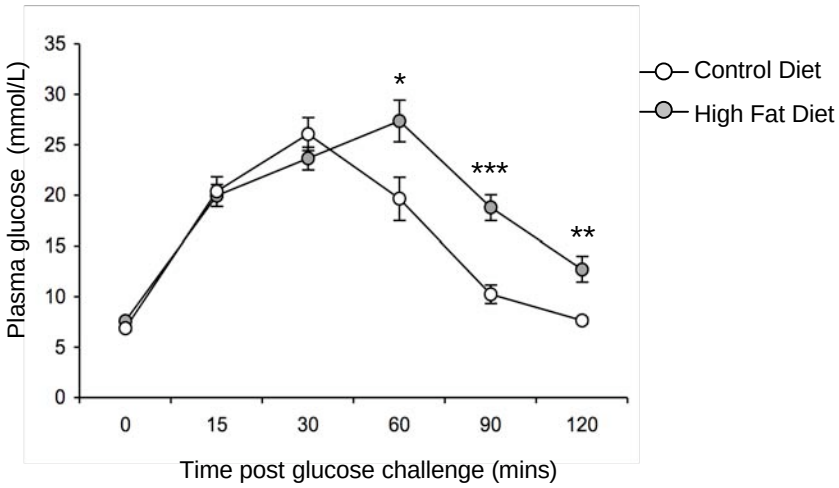


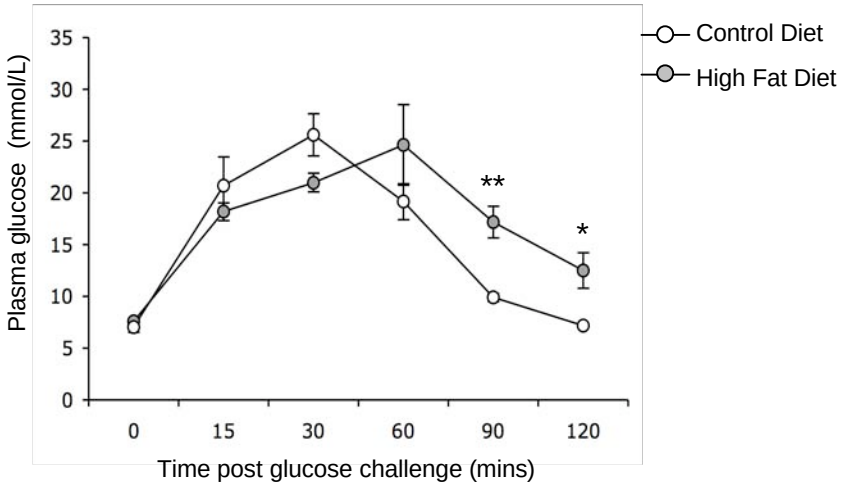
Figure 5.3. Bodyweight of mice fed control diet or high fat diet at week 0 (white bars) and at week 16 (dark blue or green bars). Mice in the initial cohort (n=10)(a), and the representative microarray pool (n=4) (b). Bars indicate mean ± SEM, * P<0.05, *** P<0.001.

(a)



	AUC
Control Diet	1955.8 ± 143.0
High Fat Diet	2463.8 ± 125.6 *

(b)



	AUC
Control Diet	1919.2 ± 118.1
High Fat Diet	2243.1 ± 171.7 #

Figure 5.4. Plasma glucose levels following IPGTT in mice fed control diet (white dots) and high fat diet (grey dots). Mice in the initial cohort (n=10)(a), and the representative microarray pool (n=4) (b). Table below graph indicates the area under the curve (AUC). All data presented as mean ± SEM (n=10), # P=0.08, * P<0.05, **P<0.01, ***P<0.001.

Table 5.2 Differentially expressed oocyte genes from Slide 1

Gene name	Fold change in HFD
Up-regulated	
High-mobility group box 1 (HMGB1)	1.0535
Polymerase (RNA) III (DNA directed) polypeptide D	1.0535
Histone deacetylase 2 (HDAC2)	1.0563
Vacuolar protein sorting protein (VPS18)	1.0503
2-oxoglutarate/malate carrier protein	1.0578
Down-regulated	
Ribosomal protein L23 (RPL23)	0.9472
Mitochondrial RNA, similar to 12S rRNA	0.8968

embryonic transcription begins and maternal mRNA is replaced by embryonic mRNA (Vigneault *et al.* 2004). When real time RT-PCR was conducted on the microarray cohort mRNA samples, evidence of minimal up-regulation was confirmed (~1.15 fold induction by high fat diet, Figure 5.5a). However, validation of differential expression was not demonstrated when the larger cohort was analyzed (Figure 5.5b).

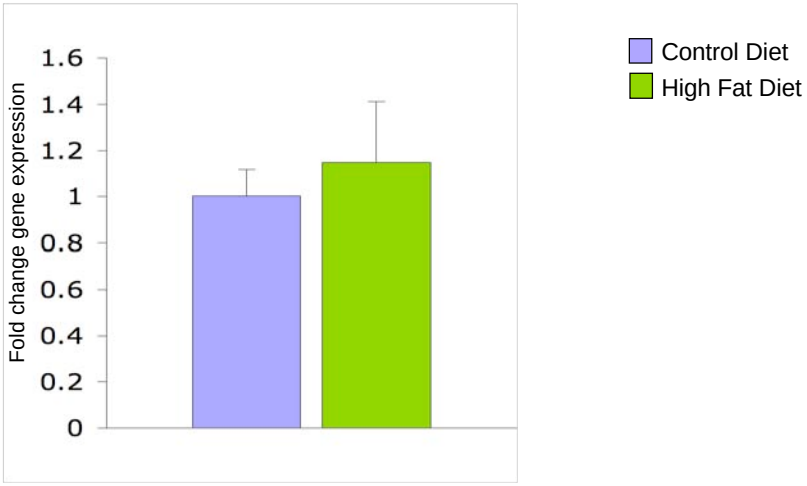
Table 5.3 lists the cumulus cell gene spots that exhibited differential hybridisation between the two diet groups. A striking result here was the identification differential binding of multiple spots for the same gene: Secreted protein, acidic, cysteine-rich (SPARC), although once again, fold change was limited. Sparc was investigated with real time RT-PCR, as it is known to be involved in the development of an adipogenic and hyperleptinemic phenotype (Bradshaw *et al.* 2003). Also, as a mediator of cell-to-matrix interactions, SPARC is hormonally regulated within the ovary (reviewed (Irving-Rodgers & Rodgers 2005)).

As expected, in the microarray cohort of animals ovarian Sparc was confirmed to be up-regulated, but not to a statistically significant extent (~1.1 fold induction, Figure 5.6a). However, validation of differential expression was not demonstrated when the larger cohort was analyzed (Figure 5.6b).

5.3.3 Differential gene expression: Microarray Slide 2- human and bovine cumulus and granulosa cell genes associated with good versus poor embryo outcomes

When control diet and high fat diet mRNA was applied to the microarray slide enriched for ovarian somatic cell genes differentially regulated in follicles giving rise to embryos that did versus did not implant after transfer, a much wider array of gene clones were flagged as potentially influenced by also high fat diet (Table 5.4). However, as the expression differences were exceedingly small and such minimal differences in other genes (ie HMGB1 and SPARC) were not maintained in the larger validation cohorts these candidates were not pursued further.

(a)



(b)

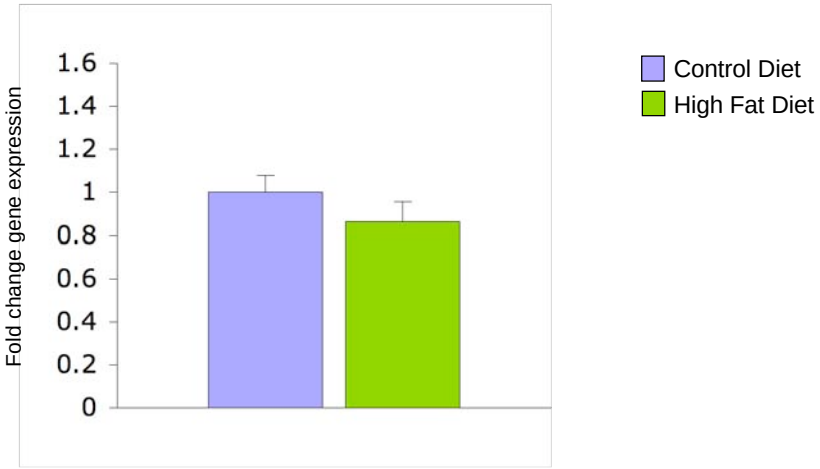


Figure 5.5 *Hmgb1* mRNA expression in ovarian samples from mice fed control diet (blue bars) and high fat diet (green bars). Expression pattern from microarray pool (n=4) (a), and larger sample cohort (n=10) (b). Bars indicate fold expression relative to CD group, normalised to *Rps3* mRNA content, expressed as mean ± SEM.

Table 5.3 Differentially expressed cumulus genes from Slide 1

Gene name	Fold change in HFD
Up-regulated	
Secreted protein, acidic, cysteine-rich (SPARC) *clone 1*	1.0873
Secreted protein, acidic, cysteine-rich (SPARC) *clone 2*	1.0825
Secreted protein, acidic, cysteine-rich (SPARC) *clone 3*	1.0816
Secreted protein, acidic, cysteine-rich (SPARC) *clone 4*	1.0696
Secreted protein, acidic, cysteine-rich (SPARC) *clone 5*	1.0650
Secreted protein, acidic, cysteine-rich (SPARC) *clone 6*	1.0585
Down-regulated	
Ubiquitin-S27a fusion protein	0.9510
Ribosomal protein S27a	0.9455

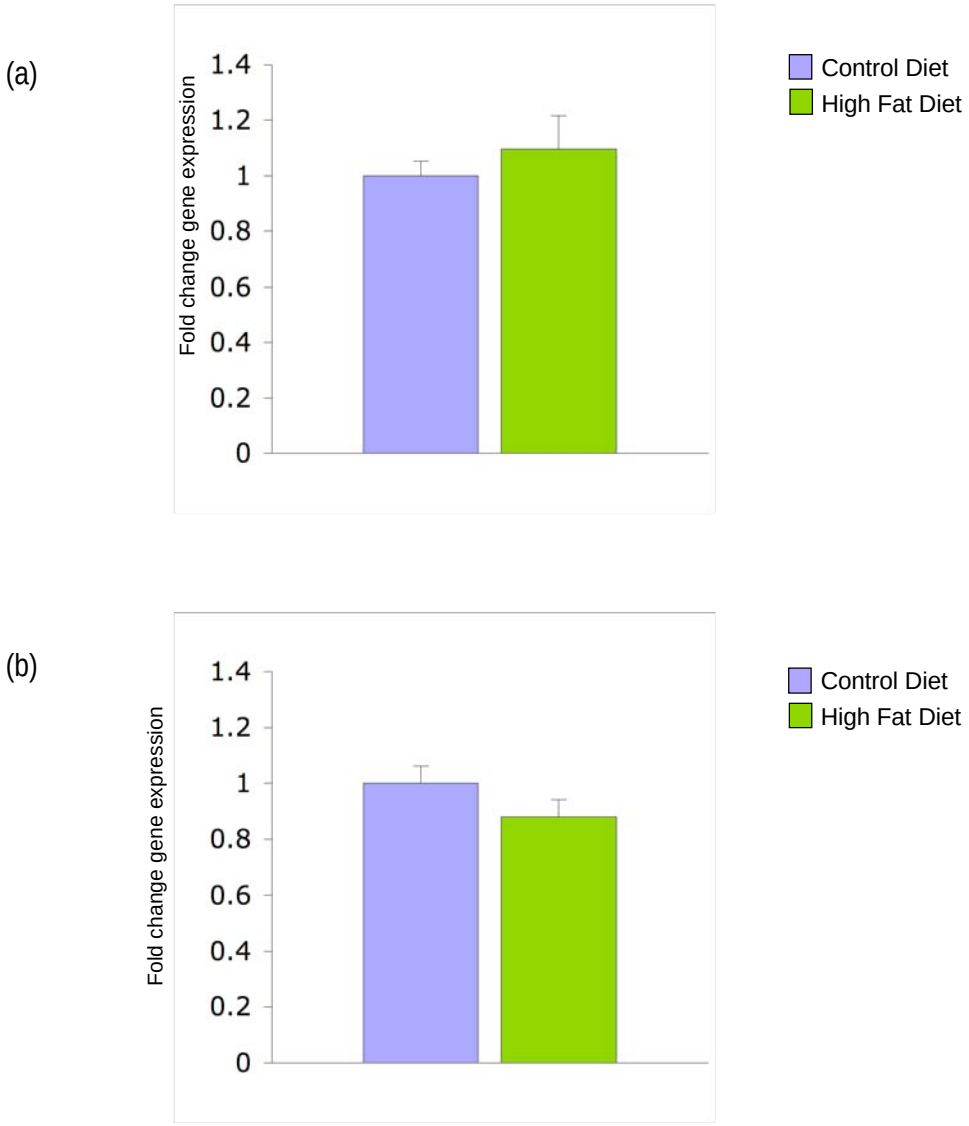


Figure 5.6 *Sparc* mRNA expression in ovarian samples from mice fed control diet (blue bars) and high fat diet (green bars). Expression pattern from microarray pool (n=4) (a), and larger sample cohort (n=10) (b). Bars indicate fold expression relative to CD group, normalised to *Rpl19* mRNA content, expressed as mean ± SEM.

Table 5.4 Differentially expressed genes from Slide 2

Gene name	Fold change in HFD
Up-regulated	
GNAS (GNAS complex locus, variant 2)	1.0541
IFITM3 (Interferon induced transmembrane protein 3)	1.0495
PKD1-like (Polycystic kidney disease 1-like) *	1.0421
PSAP (Prosaposin)	1.0436
STK17B (Serine/threonine kinase 17b - apoptosis-inducing)	1.0411
MARCKS (Myristoylated alanine-rich protein kinase C substrate)*	1.0494
YWHA (Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein - e and z polypeptides))	1.0422
Down-regulated	
INHBA (Inhibinb A)	0.9442
GTF2A2 (General transcription factor IIA, 2)	0.9591
MALAT1 (Metastasis associated lung adenocarcinoma transcript 1)	0.9083

* Genes with PPREs identified within proximal promoter region permitting potential regulation by PPARG

5.4 DISCUSSION

This investigation failed to identify any substantially regulated genes by custom-designed microarray hybridisation. When the criteria for unblinding of clone spots was expanded to encompass any with binding ratios greater than ±0.05 fold change, a reasonable list of genes was revealed, but as might be expected, none withstood further validation by real time RT-PCR.

We believe the disappointing outcome of these experiments is due in many respects to the application of whole ovarian extracts to the microarray slides. Purified COC and granulosa cell genetic material could have been obtained if animals were hormonally stimulated to induce superovulation. However, in all previous experiments of this thesis, ovaries have been obtained under non-hormonally stimulated conditions, and it is known that superovulation alters the follicular events supporting oocyte maturation, and results in the production of an increased number of reduced quality oocytes (Fossum *et al.* 1989, Ertzeid *et al.* 1992, Van der Auwera *et al.* 2001, Ertzeid *et al.* 2001, Fortier *et al.* 2008). Our desire to obtain information about the events occurring within follicles approaching ovulation under normal physiological conditions precluded the use of such hormonal stimulating protocols. Although it was hoped that the use of subtracted libraries enriched for granulosa, oocyte and cumulus genes would overcome the limitations of using heterogenous samples, ultimately it appears any potential signals were swamped by the excessive presence of other ovarian populations, such as stromal cells, theca cells and leukocytes.

We must therefore conclude that it is absolutely necessary to obtain purified cell-specific samples for such analyses in the future. Newer techniques, in particular, laser microdissection (LMD) would facilitate this, and avoid the requirement for hormonal stimulations normally used. LMD enables visual, microscopic identification of the ovarian sub-structure to be targeted for extraction from the surrounding ovarian tissue. In addition, multiple populations can be collected simultaneously, which would allow us to obtain samples of COC, mural granulosa and thecal cells for microarray investigation.

Secondly, applying this purified material to new, commercially available microarray libraries, such as that used for the Affymetrix GeneChip®, would be beneficial. As a single-labelled protocol, this system facilitates the comparison of more than 2 groups. Within our experimental paradigms, it would also allow the inclusion of an additional group, for instance HFD + intervention, which would allow us to examine the effectiveness of obesity/insulin resistance treatments on reversing any changes in ovarian gene expression.

In conclusion, these experiments have forced careful consideration of the techniques and systems best able to provide meaningful information about the global gene expression changes induced in the HFD ovary prior to ovulation. With this knowledge, we continue the development of improved microarray analyses that will provide insight to the key influences of HFD on ovarian events contributing to oocyte development potential.

Chapter 6

Effect of insulin-sensitizing agents on the adverse effects of diet-induced obesity on oocyte quality

6.1 INTRODUCTION

Chapter 4 demonstrated that a high fat diet leading to obesity induces reproductive defects that are initiated within the ovarian environment and manifest as impaired development of the early embryo. Such perturbations of blastocyst development are increasingly understood to result in sub-optimal fetal growth as well as to contribute to the fetal origins of adult disease (Lane *et al.* 1997, Kwong *et al.* 2000). Our findings support evidence of altered granulosa cell phenotype and ovarian dysfunction under conditions of gene mutation-induced obesity (Garris *et al.* 1985, Garris *et al.* 2003, Hamm *et al.* 2004, Olatinwo *et al.* 2005), but expand our understanding to show it persists in wild-type populations in which caloric intake is excessive.

The previous investigations endorsed the use of C57BL/6 mice, in which high-fat diet causes excessive weight gain and hyperinsulinemia (Chapter 3). Specifically, these mice develop the metabolic perturbations predictive of reproductive dysfunction; obesity, and elevated cholesterol, glucose and insulin (Chapter 4). It is evident that insulin resistance arising from obesity is key in the development of female reproductive dysfunction, and hyperinsulinemia can interfere directly with ovarian cell function or be indirectly associated with other hormonal conditions detrimental to optimal fertility (Poretsky *et al.* 1987, Poretsky *et al.* 1992, Poretsky *et al.* 1999, Kuscu *et al.* 2002). To demonstrate whether insulin resistance is responsible for the observed defects in oocyte developmental competence in mice fed high fat diet, we investigated whether oocyte health could be restored by in vivo treatment with insulin sensitising pharmaceuticals. Obese mice were treated with specific insulin-sensitizing and plasma glucose-reducing pharmaceuticals in order to reverse the effects of obesity/hyperinsulinemia and identify the signalling pathways responsible for disruption of pre-implantation events. The agents selected for this study, AICAR, sodium salicylate and rosiglitazone, are all expected to provide improved insulin sensitivity and glucose tolerance through distinct cellular pathways, thus providing insight as to the key processes disturbed by obesity.

AICAR (5-aminoimidazole 4-carboxamide-riboside) is an adenosine analogue that acts through stimulation of AMP kinase (AMPK) activity (Corton *et al.* 1995). In this effect, AICAR is similar in mechanism to metformin, although it is recognised as more specific in action (Musi *et al.* 2002b, Musi & Goodyear 2002a). AMPK itself phosphorylates and inactivates a number of key biosynthetic enzymes (Carling & Hardie 1989, Davies *et al.* 1990, Clarke & Hardie 1990, Davies *et al.* 1992) consequently inhibiting glycogen synthesis, fatty acid synthesis and isoprenoid/sterol synthesis. AICAR administration

to rats increases the activity of the insulin receptor signaling (Jessen *et al.* 2003), and increases glucose uptake (Merrill *et al.* 1997), via up-regulated translocation of glucose transporter GLUT-4 to the plasma membrane (Russell *et al.* 1999, Kurth-Kraczek *et al.* 1999).

Closely related to aspirin, sodium salicylate is a non-steroidal anti-inflammatory drug (NSAID) with two distinct molecular modes of action; at low doses sodium salicylate inhibits the classical NSAID targets, cyclooxygenase-1 (Patrignani *et al.* 1997, Giuliano & Warner 1999, Warner *et al.* 1999) and –2 (Mitchell *et al.* 1997, Patrignani *et al.* 1997), thus blocking prostaglandin formation, but at high doses sodium salicylate blocks the action of nuclear factor kappa B (NF-κB) (Kopp & Ghosh 1994) and its upstream activator IκB kinase β (IKK-β) (Yin *et al.* 1998). Via reduced signalling through the IKK-β pathway, sodium salicylate treatment lowers blood glucose concentrations (Baron 1982), and improves insulin resistance (Shoelson *et al.* 2003), and restores normal insulin sensitivity in mice with diet-induced obesity (Cai *et al.* 2005).

Rosiglitazone (Avandia, GlaxoSmithKline) is an insulin-sensitizing agent of the thiazolidinedione class of drugs that also includes pioglitazone (Actos, Takeda/Eli Lilly) (Mudaliar & Henry 2001). The thiazolidinediones are highly selective and potent agonists for the nuclear receptor peroxisome proliferator activated receptor–gamma (PPARG) (Lehmann *et al.* 1995), strongly implicated in female reproduction (Minge *et al.* 2008). Following rosiglitazone activation of PPARG, a heterodimeric complex with the retinoid X receptor (RXR) forms and binds to PPAR response elements (PPREs) located in promoter regions of target genes (Schoonjans *et al.* 1997), thus altering transcription. Recent investigation using genome-wide screening has produced a comprehensive list of genes containing PPREs that are potentially regulated by the PPARG/rosiglitazone complex (Lemay & Hwang 2006). Among these genes are many related to lipid metabolism, including those involved in fatty acid transport and lipid clearance from the circulation (apolipoproteins and lipoprotein lipase), fatty acid transport through plasma membranes (CD36 and SCARB1), fatty acid oxidation (acyl-CoA oxidase), mitochondrial uncoupling (uncoupling proteins (UCP)-1, UCP-2, and UCP-3), lipogenesis (acetyl-CoA carboxylase, fatty acid synthase), transcription factors involved in lipid metabolism control (sterol-regulatory element-binding protein 1) (reviewed in (Semple *et al.* 2006)), as well as other genes related to glucose metabolism (Hamm *et al.* 1999). A number of studies have reported on the effects of rosiglitazone treatment in obese rodents, frequently reporting changes in bodyweight, plasma lipid profile, blood glucose levels, and circulating insulin levels (Wilson-Fritch *et al.* 2004, Carmona *et al.* 2005).

In the present study, the effect of high fat diet, as well as exposure to each of these insulin-sensitizing agents on ovarian gene expression was examined, and compared to the gene effects in hepatic control tissue. Focus was directed to three specific genes involved in lipid uptake and utilisation: CD36, SCARB1 and Fatty Acid Binding Protein 4 (FABP4).

CD36 is a highly glycosylated 88kDa protein that binds to fatty acids, thrombospondin, collagen, anionic phospholipids and OxLDL (Febbraio *et al.* 2001). CD36 has two main functions: as an OxLDL receptor in macrophages (Endemann *et al.* 1993), and as a fatty acid transporter in adipocytes (Abumrad *et al.* 1993). It was first observed in 1991, in a study investigating the recognition of apoptotic cells by mononuclear phagocytes (Savill *et al.* 1991). In this process CD36 interacts with plasma membrane $\alpha v\beta 3$ integrin and recognises anionic phospholipid phosphotidylserine, modified lipids and thrombospondin-1 on the surface of apoptotic cells (Fadok *et al.* 1998, Moodley *et al.* 2003). Evidence would suggest that scavenger function related to innate immunity is the most archaic role for CD36, but recently emphasis has rested on new roles relating to fatty acid transport. Up-regulation of adipocyte CD36 mRNA levels occurs early in rat models of genetic obesity (Berk *et al.* 1997), and in a study of monozygotic twins, CD36 expression levels were positively correlated with measures of obesity, insulin concentrations and the HOMA index (Gertow *et al.* 2004). Within the ovary the CD36 antigen is highly expressed by granulosa cells of preantral and early antral follicles, with moderate expression also evident in the vascular thecal layers (Petrik *et al.* 2002). Following ovulation, there is some CD36 expression in luteal cells, although this disappears by the late luteal phase (Petrik *et al.* 2002). CD36 has also been implicated in immune-like functions of cumulus and granulosa cells during ovulation, suggesting this cell surface receptor may regulate surveillance and cell survival during the ovulation process (Shimada *et al.* 2006).

Closely related to CD36, SCARB1 is a receptor for native and modified lipoproteins and anionic phospholipids, including AcLDL, OxLDL, LDL and HDL. It is expressed by the ovary, adrenal gland, liver, testes, heart, mammary glands, intestines (Landschulz *et al.* 1996, Acton *et al.* 1996, Cao *et al.* 1997) and macrophages (Buechler *et al.* 1999), brain astrocytes (Husemann *et al.* 2001), endothelial cells (Uittenbogaard *et al.* 2000, Goti *et al.* 2001) and intestinal cells (Cai *et al.* 2001, Lobo *et al.* 2001). Many studies have demonstrated SCARB1-driven macrophage cholesterol metabolism as it relates to the vasculature (Krieger & Kozarsky 1999, Trigatti *et al.* 2004, Van Eck *et al.* 2004), and its proposed protective effect against atherosclerosis (Zhang *et al.* 2003). Rodent steroidogenic cells obtain much of their cholesterol for cholesteryl ester storage and steroid production via selective uptake from HDL-

cholesterol ester using SCARB1. Within the ovary, thecal cells consistently express high levels of SCARB1 at all stages of both healthy and atretic follicle development based on studies in the rat (Svensson *et al.* 1999), and high expression is also found within luteal structures (Reaven *et al.* 1998). This reflects HDL-cholesterol ester requirement for theca interna production of androgen for aromatase-mediated conversion by the granulosa cells, and progesterone synthesis by luteal cells. Immunolocalisation at the electron microscopic level indicate that most SCARB1 is associated with microvilli and microvilli channels (Reaven *et al.* 1998), which in steroidogenic cells are areas for selective uptake of lipoprotein cholesterol (Reaven *et al.* 1988, Reaven *et al.* 1989). In the rat, ovarian expression of SCRB1 responds to treatment with estrogen, and is increased in granulosa-derived luteal cells, where progesterone is synthesised, and decreased in thecal (non-luteal) cells (Landschulz *et al.* 1996). The requirement of functional SCARB1 for normal female fertility has been demonstrated with SCARB1-deficient mice (Miettinen *et al.* 2001), which display exclusively female infertility. This study reported that the oocytes of these mice were not viable, although this disorder was found to not be intrinsically related to the lack of ovarian SCARB1, but rather to manifest abnormal circulating lipid profile.

FABPs bind and solubilize lipophilic fatty acids facilitating their intracellular transport to different cellular organelles or proteins (Gillilan *et al.* 2007). These key functions of FABPs are also thought to regulate gene expression by binding to and shuttling FFA to target cellular compartments (Hotamisligil *et al.* 1996). FABP4 is known to, in particular, position lipid ligands of PPARG in close proximity to this nuclear receptor allowing selective enhancement of PPARG transcriptional activity (Tan *et al.* 2002). FABP4 is of interest to obesity research, as obesity and insulin resistance are associated with disturbed fatty acid metabolism and handling. In obese humans FABP4 adipocyte expression increases, and is released into the circulation, regardless of insulin sensitivity or secretion (Stejskal & Karpisek 2006). Circulating FABP4 levels correlate positively with BMI, adiponectin, triglycerides and CRP i(Cabre *et al.* 2007). The link between FABP4 and mechanisms of fatty acid metabolism is strengthened by examination of rodent model of disturbed expression. FABP4 null mice fed a high fat diet have increased fat mass, but decreased lipolysis, increased muscle glucose oxidation and normalised insulin sensitivity (Hotamisligil *et al.* 1996, Hertzel *et al.* 2006). FABP4 has been identified in apoptotic granulosa cells (Nourani *et al.* 2005) of the rodent ovary, which suggests that FABP4 is involved in the apoptosis of ovarian granulosa cells, possibly through an interaction with PPARG. As with SCARB1 and CD36, there is no data on how an obese phenotype influences the expression, or function, of FABP4 within the ovary, or how this may impact on the production and health of ovulated oocytes.

In this chapter, the effects of diet-induced obesity on aspects of female reproductive function (ovulation, fertilization and embryonic development to the blastocyst stage) were again assessed. Obese animals were also treated with one of the three insulin sensitizers. Comparing the effects of each drug on metabolic status and ovarian gene expression, as well as upon later reproductive outcomes, identified the pathway most important for these processes. In order to focus on the peri-conception effects of these drugs, the treatment timeframe was limited to 4 days immediately prior to ovulation and mating (Figure 6.1), thereby restricting systemic effects of persistent treatment yet elucidating acute effects on the ovarian follicle and oocyte. Following indication of mating, all oocytes were isolated from the oviducts and monitored in vitro, enabling evaluation of oocyte health and precise temporal assessments of developmental competence.

By comparing the effects of each drug on ovarian gene expression, oocyte health and early embryonic developmental competence in DIO mice, we have identified cellular pathways affected by insulin resistance that are important in regulating oocyte potential.

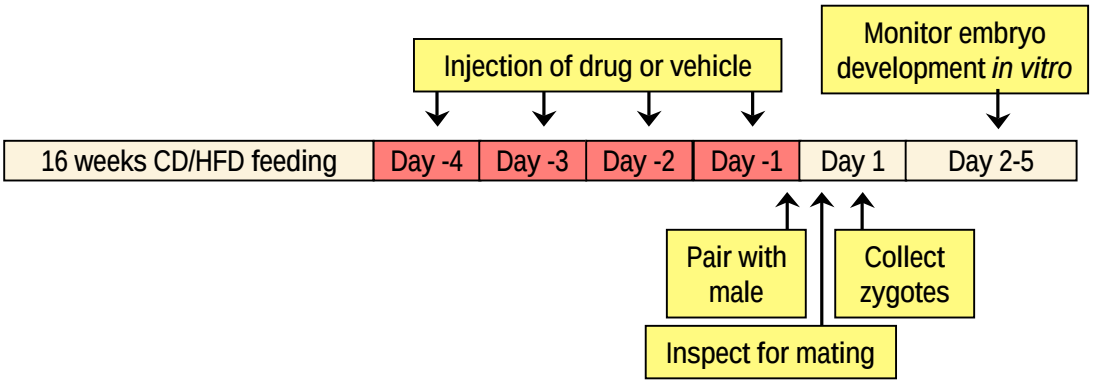


Figure 6.1 The experimental treatment protocol. Female mice were fed control diet (CD) or high fat diet (HFD) for 16 weeks starting at 5 week of age. 4 days prior to pairing with male, mice fed HFD were treated with AICAR, sodium salicylate, rosiglitazone or vehicle via intraperitoneal injection once daily. Mice were inspected for evidence of mating (vaginal plug) at 0800 hr each morning, and deemed to be at day 1 of pregnancy. Zygotes were removed from the oviduct at 13:00 and maintained in *in vitro* culture until day 5, when differential stain was performed on blastocysts.

6.2 MATERIAL AND METHODS

6.2.1 Animals and Diet

Seventy-five female and eleven male C57BL/6 mice were utilised for these experiments. Details on animal sourcing, handling and feeding are contained in Chapter 2.

6.2.2 Insulin sensitizer treatment

Female mice fed the HFD were randomly allocated to either the HFD + vehicle group, the HFD + AICAR group, the HFD + sodium salicylate group or the HFD + rosiglitazone group (all groups n=15). Four days before the conclusion of the 16-week feeding period, they were injected intraperitoneally once daily with 30mg/kg bodyweight/day AICAR (Toronto Research Chemicals, Canada, in 0.9% saline), 50mg/kg bodyweight/day sodium salicylate (Sigma Aldrich, St Louis MO, in sterile water (Gepdiremen & Suleyman 2003)), 10mg/kg bodyweight/day rosiglitazone (Avandia, GlaxoSmithKline, in 10% DMSO (Cuzzocrea *et al.* 2004)) or vehicle (10% DMSO). Bodyweight was recorded immediately prior to the first dosage of drug or vehicle (day -4) and immediately prior to the final dose of drug or vehicle (day -1) to indicate bodyweight flux resulting from drug administration.

6.2.3 Tissue and zygote collection

After 16 weeks of control diet, or 16 weeks of high fat diet, including 4 days of drug vehicle treatment, two female mice (mixed experimental groups) were caged with one male mouse for a maximum of 8 nights. Females were checked daily at 0800 for the presence of a post-coital vaginal plug. On day of presence of vaginal plug, females were deemed to be at day 1 of pregnancy, and tissues collected at 1300 that day. Any females in which a plug was not observed over the 8 day period of cohabitation (10/75 mice), were housed individually for a further 7 days to allow conceptions that may have occurred to progress to a developmental stage where visual inspection of the uterine horns could confirm pregnancy. From these, 1 female was found to have implantation sites, and 1 was found to have implantation and resorption sites, indicating 2 plugs had been missed out of 75 animals. The number of days post-coitus was estimated.

Prior to killing by cervical dislocation, blood was collected, allowed to clot at room temperature and centrifuged at 4000 rpm for 10 minutes and serum removed. Ovulated oocytes were collected immediately from the oviductal ampulla into G-MOPS media and treated with hyaluronidase (0.5mg/ml, Sigma, bovine testes, type IV) to facilitate removal of the surrounding cumulus cells. Abdominal and retroperitoneal adipose tissue, ovary and liver were dissected, weighed and snap frozen in liquid nitrogen.

6.2.4 Metabolite and endocrine measurements

All analyses on serum were conducted on samples collected from a fed state. Mice were not fasted to avoid detrimental physiological impact of short-term starvation that would be compounded by potentially hypoglycemic insulin sensitising agents. For details of measurement protocols, please refer to Chapter 2.

6.2.5 mRNA preparation and real-time RT-PCR

Total cellular RNA was isolated from liver and ovary. Please refer to Chapter 2 for details describing RNA isolation, assessment of concentration and purity, and subsequent reverse transcription. Semi-quantitative real time PCR was performed using the Corbett Rotor-Gene™ 6000 (Corbett Life Sciences) real-time rotary analyser with SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA). Ribosomal protein L19 (Rpl19) was used as an internal control for every sample. All primers were designed using Primer Express™ software and synthesized by GeneWorks (Thebarton, South Australia, Australia) (Table 6.1).

6.2.6 In vitro embryo culture

Ovulated and denuded oocytes were maintained for 48h in G1 media (Gardner 1994, Gardner *et al.* 2003), with assessment for fertilization indicated by first cleavage division occurring after 24h. 2-cell embryos were transferred to EDTA-free G2 media (Barnes *et al.* 1995, Gardner *et al.* 2003), shown

Table 6.1 Primer sequences

Gene	Accession #	Sequences
Pparg	NM_011146	F: 5'-CCACTATGGAGTTCATGCTTGTG-3' R: 5'-TTTGTGGATCCGGCAGTTAAG-3'
Cd36	NM_007643	F: 5'-TCATGCCAGTCGGAGACATG-3' R: 5'-TGGTGCCTGTTTTAACCCAGTT-3'
Scab1	NM_016741	F: 5'-GGTCCTCAACGGCCAGAAG-3' R: 5'-CACGGTGTCGTTGTCATTGAA-3'
Fabp4	NM_024406	F: 5'-TGATGCCTTTGTGGGAACCT-3' R: 5'-ATCCTGCCACTTTCCTTGT-3'
Nos2	NM_010927	F: 5'-CATCAGGTCGGCCATCACT-3' R: 5'-CGTACCGGATGAGCTGTGAA-3'
Ager	NM_007425	F: 5'-AGGGAAGGAGGTCAAGTCCAA-3' R: 5'-ATTAGGGACACTGGCTGTGAGTTC-3
Rpl19	NM_009078	F: 5'-TTCCCGAGTACAGCACCTTTGAC-3' R: 5'-CACGGCTTTGGCTTCATTTTAAC-3'

previously to provide an optimum environment for growth of the postcompaction embryo. Development of fertilized oocytes was assessed at 9:00 day 3, 16:00 day 4, 9:00 day 5 by assessors blinded to maternal treatment group. Embryos were indicated as “on-time” when normal cell numbers and morphology was observed, (ie: day 3: 4-8 cells, day 4: morula-blastocyst, day 5: expanded or hatching blastocyst), fragmentation was less than 10%, and the zona pellucida was intact (until day 5). Embryos were categorised as not “on-time” if any of these developmental targets were not achieved. Also, embryos exhibiting slow/ceased cell division events (indicating cellular arrest) or overtly accelerated divisions (indicating insufficient or incomplete processing of cellular division) were duly noted as such and consequently categorised as not “on-time” for the remainder of the culturing period, even if their developmental progress later appeared normal.

6.2.7 Differential nuclear staining

Expanded and hatching blastocysts surviving at day 5 of culture were subjected to a differential staining protocol for identification of cells within the fetal precursor-inner cell mass (ICM) and placental precursor-trophectoderm (TE) layer following the methods of Hardy et al (Hardy *et al.* 1989). A blinded assessor then counted red (TE) and blue (ICM) fluorescent cells on an Olympus VANOX AHBT-3 photomicroscope (Faulding Imaging, Mulgrave North, Victoria, Australia).

6.2.8 Statistical analysis

Values are reported as mean ± SEM. Statistical differences were determined by ANOVA and Chi-squared analysis using SPSS 13.0 for Windows (SPSS Inc. Chicago, IL). One-way ANOVA across control diet and high fat diet ± drug treatments was used, with post-hoc analyses of significance made by Tukey’s test. A Student’s t-test was used in the case of comparison of normalized gene expression between control diet or high fat diet and specific drug treatments. For all analyses, $P<0.05$ was defined as statistically significant.

6.3 RESULTS

6.3.1 Insulin sensitizer influences on metabolic and endocrine measurements

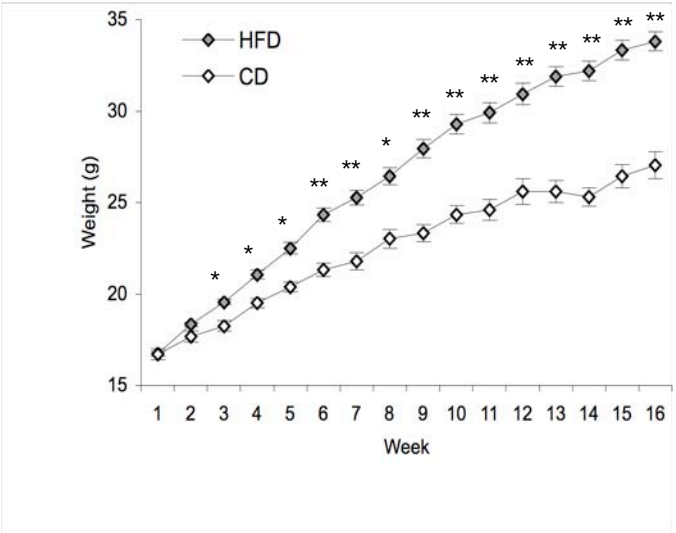
Mice fed a high fat diet gained significantly more weight than those on control diet over the course of 16 weeks (Figure 6.2a). Animals on the high fat diet were assigned to 4 different groups, and at the initiation of treatment regimes bodyweight was not significantly different between HFD experimental groups (Figure 6.2b). Over the 4 treatment days bodyweight was maintained in both CD animals (Δweight = 0.36 ± 0.35g), as well as in the HFD + vehicle group (Δweight = -0.26 ± 0.26g) (Figure 6.2c). Treatment with AICAR or sodium salicylate had no effect on bodyweight compared to vehicle treated animals, however, treatment with rosiglitazone caused significant weight loss (Δweight = -3.27 ± 0.21g, $P<0.001$ compared to all other groups).

None of the treatments reduced adipose tissue mass, otherwise elevated by consumption of HFD (Figure 6.3a). Rosiglitazone treatment reduced liver mass (Figure 6.3b $P=0.048$ compared to HFD + vehicle), indicating that the significant weight loss observed with this treatment is likely due to fat mobilization from the liver.

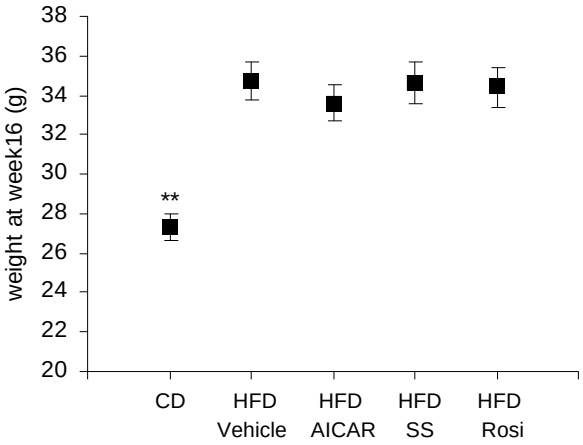
Blood collected at post-mortem was analyzed for circulating lipids, glucose and insulin. Circulating total cholesterol was not significantly different in HFD + vehicle mice (Figure 6.4a) nor affected by treatment with insulin sensitizers. Circulating Free Fatty Acids were significantly increased by high fat diet feeding, but were not altered in mice treated with insulin sensitizers compared to vehicle (Figure 6.4b). Triglyceride levels (Figure 6.4c) were not affected by high fat diet or insulin sensitizers, however, rosiglitazone treatment resulted in the lowest levels ($P=0.047$, compared to HFD + AICAR). Non-fasting blood glucose and insulin levels were not significantly elevated in response to HFD feeding. Blood glucose levels in high fat diet fed mice were not significantly altered by treatment with AICAR or sodium salicylate (Figure 6.5a). Rosiglitazone however significantly lowered blood glucose levels compared to vehicle treated mice ($P=0.028$ vs. HFD + vehicle). Circulating insulin was significantly reduced by both sodium salicylate and rosiglitazone delivery (Figure 6.5b), compared to vehicle treatment (HFD + vehicle vs. HFD + sodium salicylate or rosiglitazone, $P=0.021$ and $P=0.032$ respectively). All blood samples were obtained in the non-fasted state to avoid acute effects of fasting on the fertilized oocytes. Previous cohorts of mice, which were fasted, showed significantly elevated cholesterol, free fatty acids

Figure 6.2 Body weights of mice following feeding with a control diet or high fat diet and in response to treatment with insulin sensitizers or vehicle. (a) Weight gain of mice fed control diet (CD) or high fat diet (HFD) for the 16 weeks preceding drug administration, (b) bodyweight of the CD and 4 HFD groups immediately prior to drug administration, (c) change in bodyweight after 3 days of drug or vehicle treatment. Bars and data points indicate mean ± SEM (n=15). CD = control diet, HFD = high fat diet, SS = sodium salicylate, Rosi = rosiglitazone. * $P<0.05$, **$P<0.001$, Different letters indicate statistically different means, $p<0.05$.

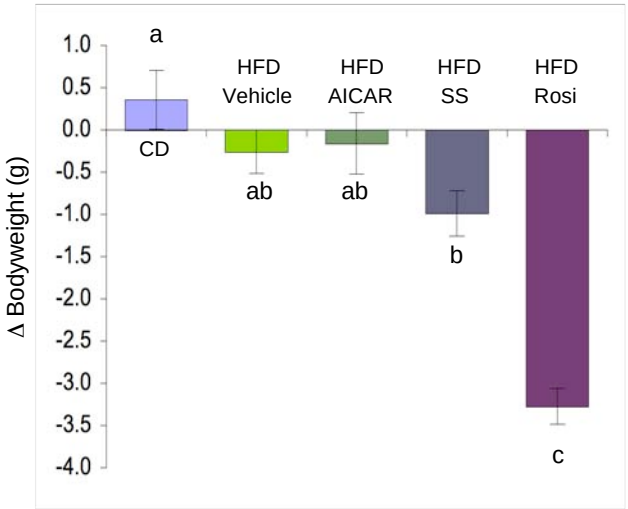
(a)


HFD
CD
Weight (g)
Week
35
30
25
20
15
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
* * * * ** ** * ** ** ** ** ** ** ** **

(b)


weight at week16 (g)
38
36
34
32
30
28
26
24
22
20
**
CD
HFD Vehicle
HFD AICAR
HFD SS
HFD Rosi

(c)


Δ Bodyweight (g)
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
a
ab
ab
b
c
CD
HFD Vehicle
HFD AICAR
HFD SS
HFD Rosi

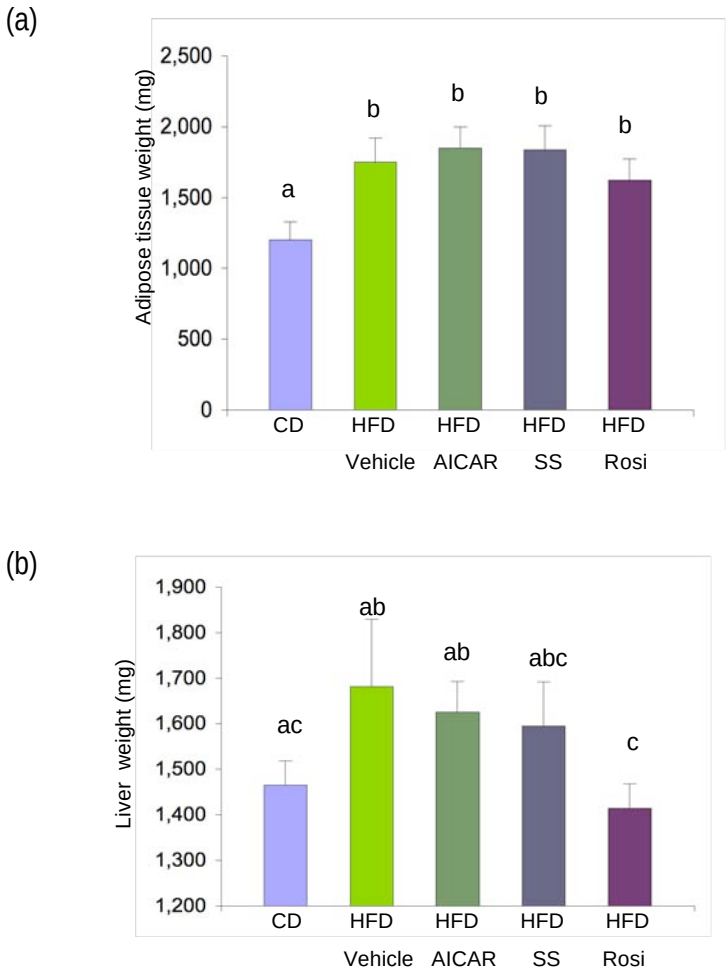


Figure 6.3 Tissue weights of mice fed control diet or high fat diet and in response to treatment with insulin sensitizers or vehicle. (a) Adipose tissue (abdominal + retroperitoneal) weight, and (b) liver weight. Bars indicate mean ± SEM (n=15). CD = control diet, HFD = high fat diet, SS = sodium salicylate, Rosi = rosiglitazone. Different letters indicate statistically different means, $p<0.05$.

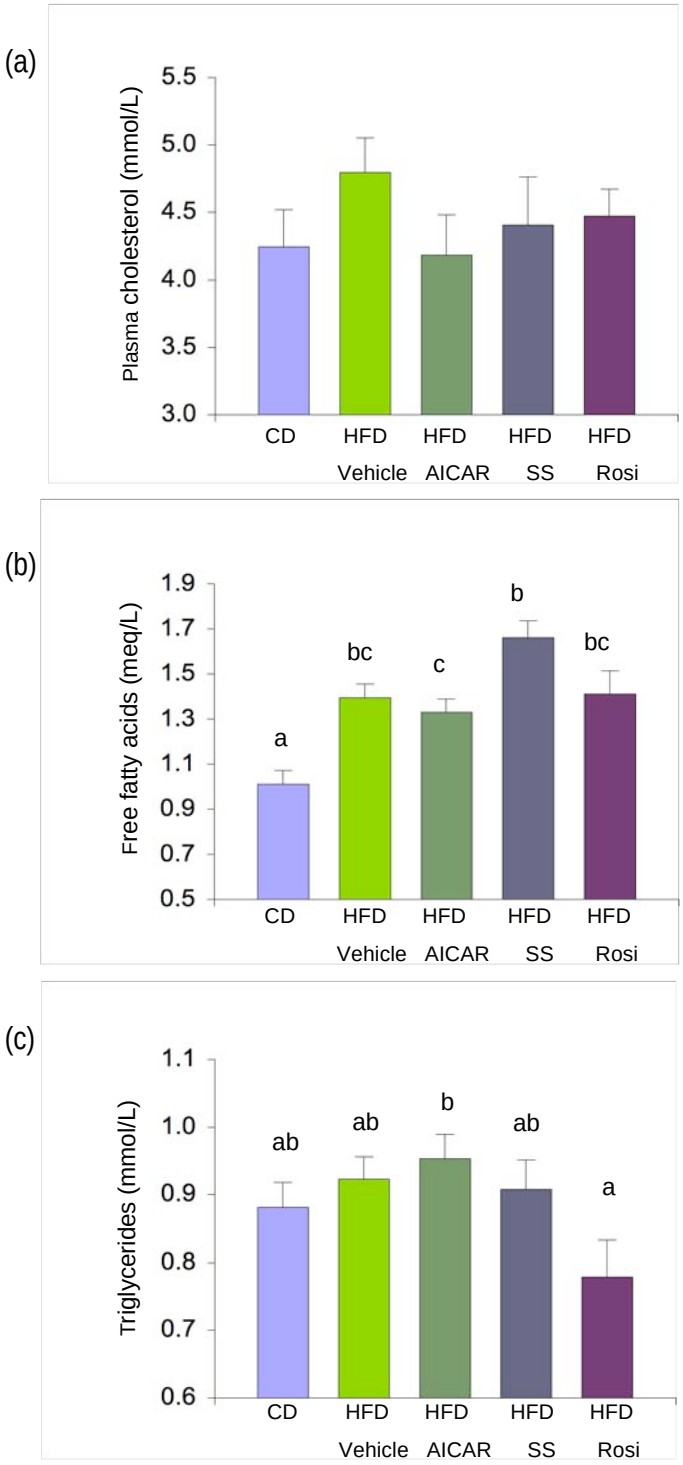


Figure 6.4 Circulating lipids in mice fed control diet or high fat diet and in response to treatment with insulin sensitizers or vehicle. (a) Total cholesterol, (b) free fatty acids, and (c) triglycerides. Bars indicate mean ± SEM (n=15). CD = control diet, HFD = high fat diet, SS = sodium salicylate, Rosi = rosiglitazone. Different letters indicate statistically different means, $p<0.05$.

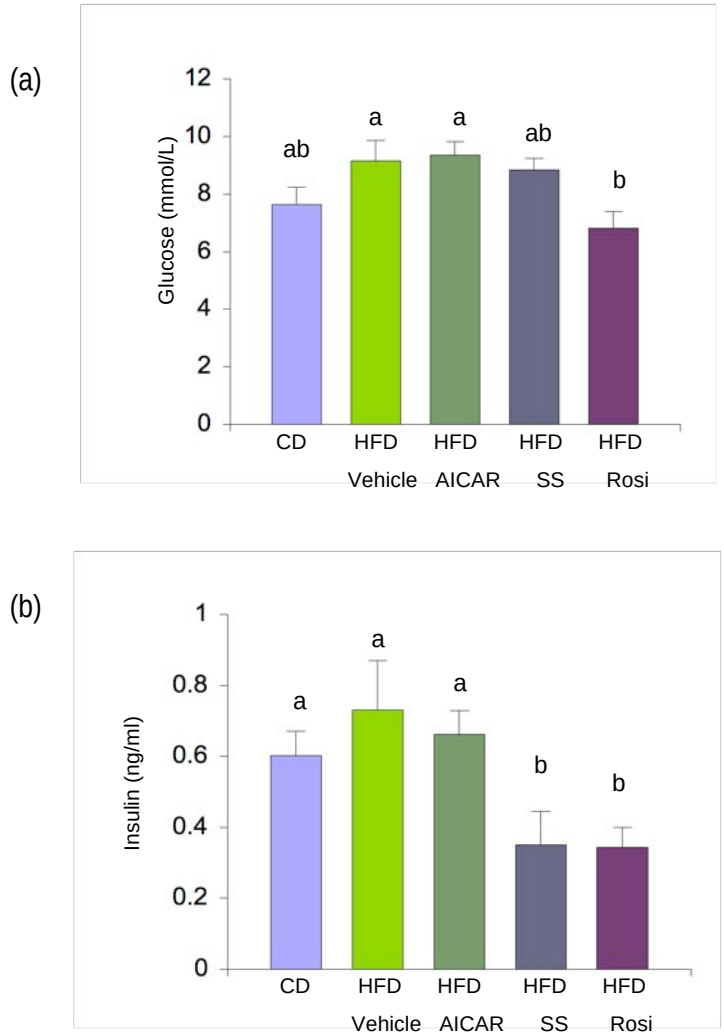


Figure 6.5 Circulating glucose (a) and insulin (b) in mice fed control diet or high fat diet and in response to treatment with insulin sensitizers or vehicle. Measurements were obtained under non-fasted conditions. Bars indicate mean ± SEM (n=15). CD = control diet, HFD = high fat diet, SS = sodium salicylate, Rosi = rosiglitazone. Different letters indicate statistically different means, $p<0.05$.

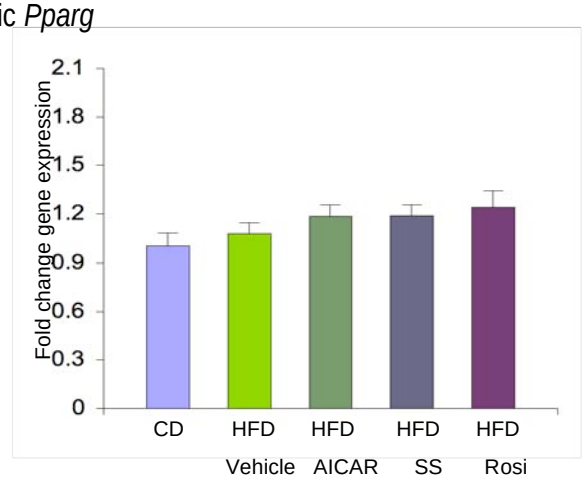
as well as significant hyperinsulinemia (Chapter 3) in high fat diet compared to control diet treatments.

6.3.2 Modulation of ovarian gene expression

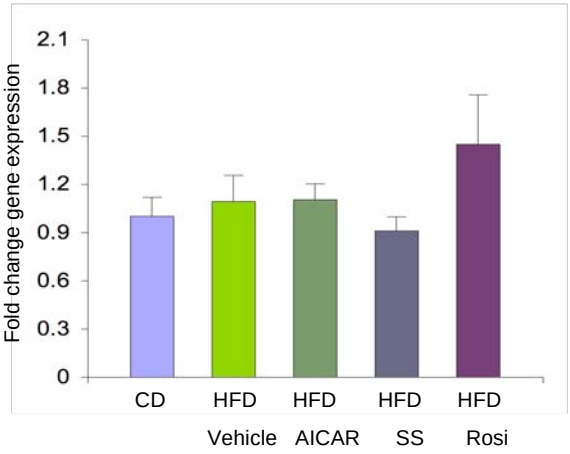
To determine if drug treatment was affecting ovarian gene expression, real time RT-PCR was performed on ovarian RNA collected from day 1 pregnant animals (Figure 6.6). Specifically, the transcription of genes with previously documented nutritional and hormonal regulation was investigated. Hepatic RNA was used for comparison as a control tissue in which the effects of each insulin sensitizer have been better characterized. Expression of the rosiglitazone receptor *Pparg* was not found to be different between control diet and high fat diet fed mice, in either hepatic or ovarian samples (Figure 6.6a, b), although expression tended to be higher in ovaries from HFD + rosiglitazone animals. Rosiglitazone treatment increased expression of scavenger receptor *Cd36* mRNA within the liver of HFD fed mice (Figure 6.6c). HFD tended to increase ovarian expression of *Cd36*, which was moderately lower in both HFD + AICAR and HFD + sodium salicylate ovaries (Figure 6.6d). Treatment with rosiglitazone increased expression of *Cd36* when compared to control diet ovaries (P=0.037). Consistent with previous reports (Srivastava 2003) hepatic expression of scavenger receptor, *Scarb1*, was not influenced by diet (Figure 6.6e). It was also unaffected by administration of any insulin-sensitizing drug within the liver. However, rosiglitazone lowered ovarian expression of *Scarb1* by 61% compared to HFD + vehicle treated ovaries (P=0.003, Figure 6.6f). Hepatic expression of intracellular lipid transporter *Fabp4* (also known as adipocyte P2) was affected by rosiglitazone in a similar way to *Cd36*, with an up-regulation of transcription (P=0.0005, Figure 6.6g). This effect was mirrored within the ovary, although to a greater extent (7.6-fold increase, compared to 3.4-fold increase in the liver, Figure 6.6h). In general, ovarian expression of each gene reflected the same pattern of changes as those observed in the liver.

Ovarian expression of inducible nitric oxide synthase (*Nos2*) and receptor for advanced glycation end products (*Ager*) mRNA was also assessed. Although our previous investigations had indicated *Nos2* expression changes in ovarian macrophages following in vitro treatment with PPARG agonist troglitazone (Minge *et al.* 2006), in the current in vivo whole ovary samples, no gene modulation was observed (Figure 6.7a). Gene expression of *Ager* has been previously shown by others to be inhibited in response to PPARG activation (Wang *et al.* 2006). This effect was not exhibited within the ovary,

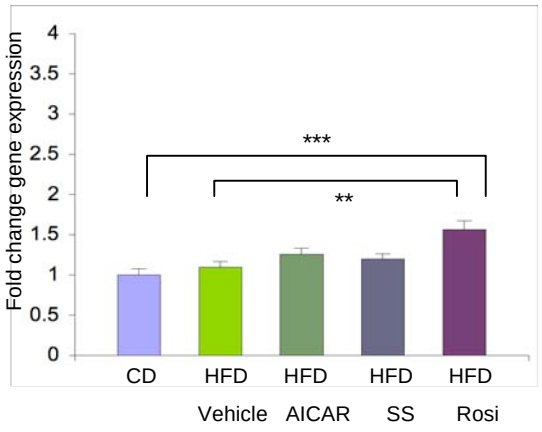
(a) Hepatic *Pparg*



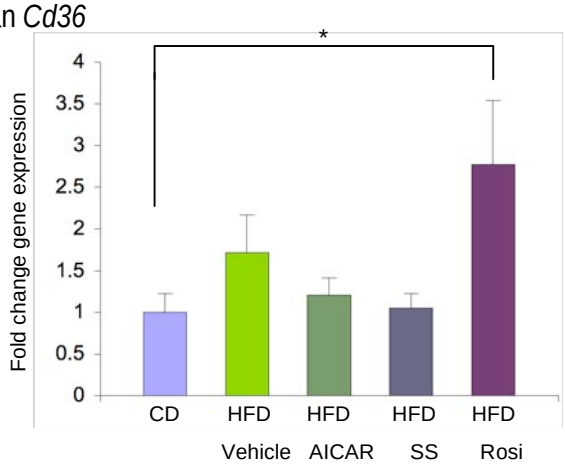
(b) Ovarian *Pparg*



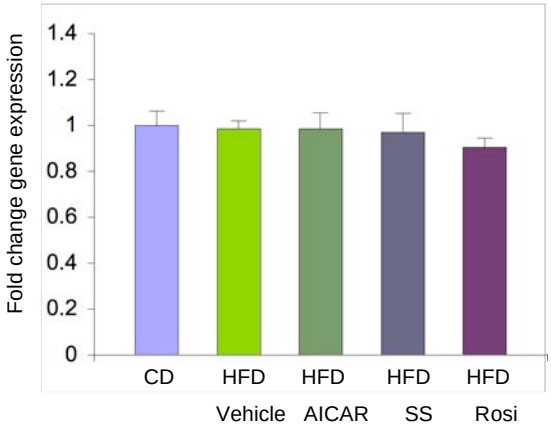
(c) Hepatic *Cd36*



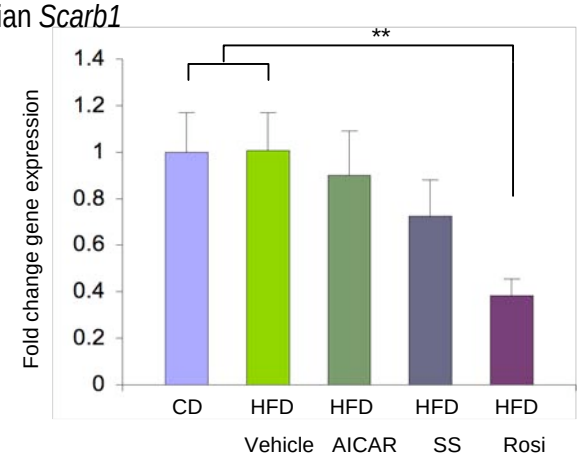
(d) Ovarian *Cd36*



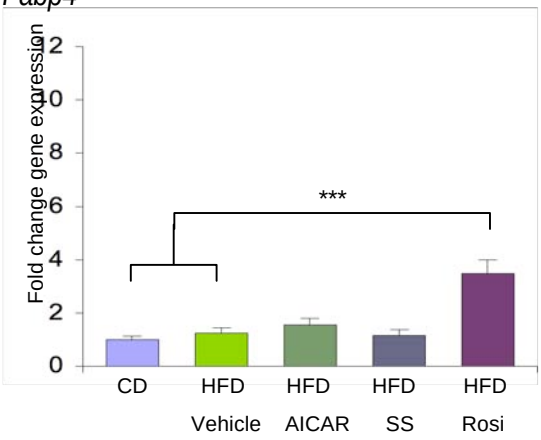
(e) Hepatic *Scarb1*



(f) Ovarian *Scarb1*



(g) Hepatic *Fabp4*



(h) Ovarian *Fabp4*

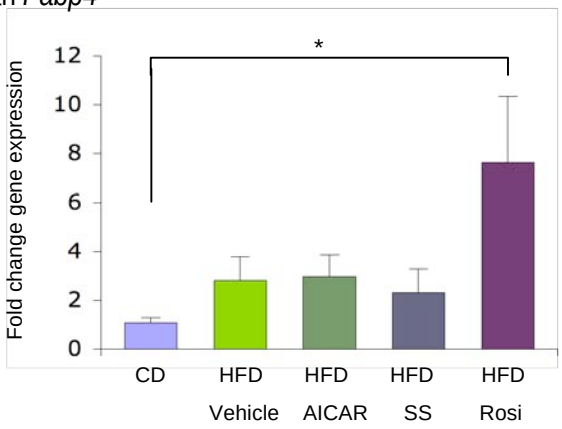
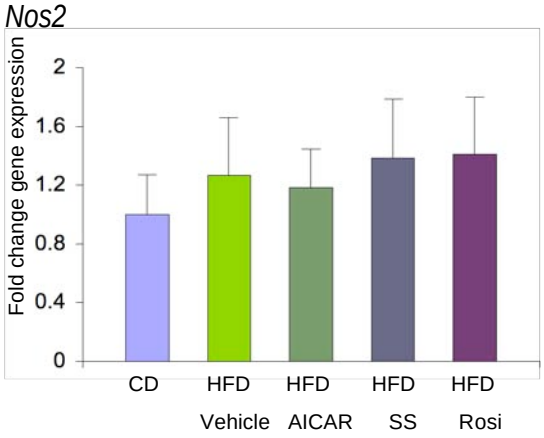


Figure 6.6 Gene expression in liver (a,c,e,g) and ovary (b, d, f, h) from mice fed control diet or high fat diet and in response to treatment with insulin sensitizers or vehicle. Genes analyzed were *Pparg* (a, b), *Cd36* (c, d), *Scarb1* (e,f) and *Fabp4* (g, h). Each is normalized to ribosomal L19 and expressed as fold change from the control diet group. CD= control diet, HFD = high fat diet, SS = sodium salicylate, Rosi = rosiglitazone. Bars indicate mean ± SEM (n=15). *P<0.05, **P<0.01, ***P<0.001.

(a) Ovarian *Nos2*



(b) Ovarian *Ager*

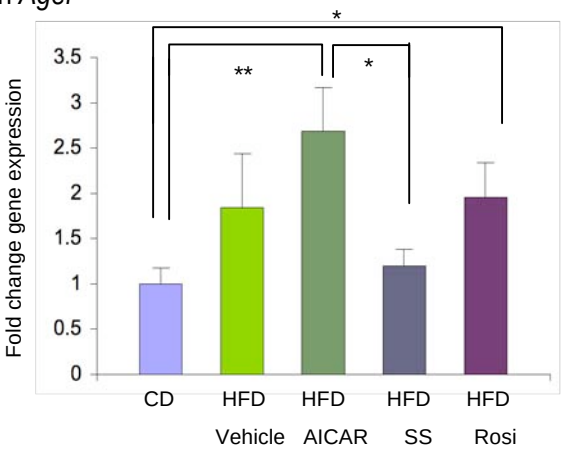


Figure 6.7 Gene expression in ovary of mice fed control die or high fat diet and in response to treatment with insulin sensitizers or vehicle. Genes analyzed were *Nos2* (a) and *Ager* (b). Each is normalized to ribosomal L19 and expressed as fold change from the control diet group. CD= control diet, HFD = high fat diet, SS = sodium salicylate, Rosi = rosiglitazone. Bars indicate mean ± SEM (n=15). *P<0.05, **P<0.01.

although there was some evidence that sodium salicylate could suppress *Ager* mRNA expression (Figure 6.7b).

6.3.3 Incidence and rate of ovulation

Reproductive parameters were next assessed; onset to mating event, ovarian weight, incidence of anovulation and ovulation rate (Table 6.2). Number of days to plug tended to be longer in the HFD group, with HFD + vehicle females requiring 3.00 ± 0.59 days compared to CD females requiring 1.93 ± 0.4. Ovarian tissue weight was also increased by HFD (P=0.049 vs. CD). Surprisingly, in several mice zero oocytes were present in both oviducts, with this incidence of anovulation highest (6/15 animals) in response to HFD. In mice that did ovulate, HFD increased the ovulation rate (7.0 ± 0.6 oocytes) compared to CD (4.9 ± 0.9 oocytes, P = 0.036). Serum progesterone, although not analyzed in this series of experiments, was measured in previous cohorts of mice and was not influenced by HFD (Chapter 4).

None of the outcomes listed in Table 6.2 were significantly affected by treatment with the insulin sensitizers with the exception that HFD mice treated with sodium salicylate required an extended period of time before mating, 3.92 ± 0.40 days (P < 0.044 vs. CD, HFD + AICAR and HFD + rosiglitazone groups). Sodium salicylate actions associated with the inhibition of cyclooxygenases required for ovulation may be responsible for this observation. Interestingly, with each parameter rosiglitazone treatment resulted in outcomes most similar to those exhibited by CD mice.

6.3.4 Early embryo development

Embryonic development in vitro was assessed by blinded, daily evaluation and scoring for correct morphology of the fertilized oocytes. HFD fed mice produced embryos with reduced on-time progression to all developmental milestones assessed (Figure 6.8); the 4-8 cell stage (P = 0.0001), the morula/early blastocyst stage (P=0.002) and the expanded/ hatching blastocyst stage (P=0.002), compared to embryos produced by CD fed females. Neither AICAR nor sodium salicylate administration affected embryo on-time development. However fertilized oocytes obtained from HFD+ rosiglitazone animals demonstrated significantly improved developmental potential compared to HFD + vehicle oocytes at the

Table 6.2 Ovulation incidence and rate in response to HFD ± insulin sensitizers

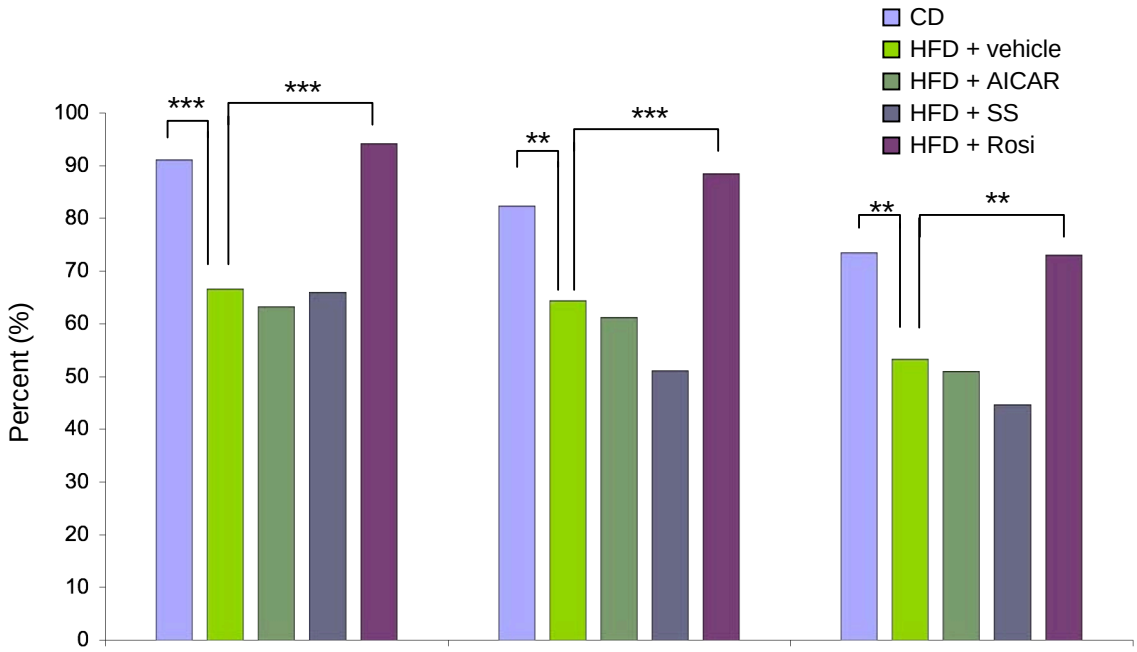
	Days to mate	Ovarian weight (mg)	Oocytes/ ovulatory mouse	Incidence of anovulation
CD	1.93 ± 0.47	5.59 ± 0.53	4.9 ± 0.9 (n=11)	4/15 (27%)
HFD + vehicle	3.00 ± 0.60	7.03 ± 0.60 *	7 ± 0.6 * (n=9)	6/15 (40%)
HFD + AICAR	2.60 ± 0.45	5.94 ± 0.47	6 ± 0.8 (n=9)	6/15 (40%)
HFD + SS	3.92 ± 0.40 *	6.15 ± 0.28	6.3 ± 0.9 (n=10)	5/15 (33%)
HFD + Rosi	1.92 ± 0.27	6.14 ± 0.35	5.6 ± 0.7 (n=11)	4/15 (27%)

CD = control diet, HFD = high fat diet, SS = sodium salicylate, Rosi = rosglitazone, * $P < 0.05$ vs. CD.

4-8 cell stage (P = 0.001), at the morula/compacted blastocyst stage (P = 0.0003), and at the expanded/hatching blastocyst stage (P=0.004). Overall, there was no discernable difference in developmental dynamics of oocytes obtained from CD animals, and oocytes obtained from obese, HFD animals that had been treated with pre-ovulatory rosiglitazone.

6.3.5 Blastomere differentiation

All embryos surviving to day 5 of culture were subjected to a differential staining protocol to permit counting of cells constituting the trophectoderm layer and the inner cell mass. Embryos derived from HFD fed mice had higher numbers of cells allocated into the trophectoderm layer, and slightly reduced cell numbers within the inner cell mass. Consequently the proportion of inner cell mass, as a percentage of the total embryonic cell number, is smaller (Figure 6.9). Both AICAR and rosiglitazone administration significantly reduced the number of cells within the trophectoderm layer (P=0.047 HFD + vehicle vs. HFD + AICAR; P=0.007 HFD + vehicle vs. HFD + rosiglitazone, Figure 6.9a). None of the drug treatments had a dramatic influence on the number of cells within the inner cell mass (Figure 6.9b). However, there was a statistically significant increase in the percent of cells contained within the inner cell mass in response to rosiglitazone treatment (P=0.033 HFD + vehicle vs. HFD + rosiglitazone, Figure 6.9c).



	4-8 cell	Morula/compacted blastocyst	Expanded blastocyst/ hatching blastocyst
CD	31/34	28/34	25/34
HFD + vehicle	30/45	29/45	24/45
HFD + AICAR	31/49	30/49	25/49
HFD + SS	31/47	24/47	21/47
HFD + Rosi	49/52	46/52	38/52

Figure 6.8 Percentage of cleaved oocytes that reached the 4-8 cell, morula/blastocyst and expanded blastocyst/hatching blastocyst stages on-time. Data shows the % of fertilized zygotes to pass each developmental stage with the actual number of zygotes indicated in the table below. Each female contributed between 1 and 11 oocytes. CD= control diet, HFD = high fat diet, SS = sodium salicylate, Rosi = rosiglitazone. *P<0.05, **P<0.01, ***P<0.001

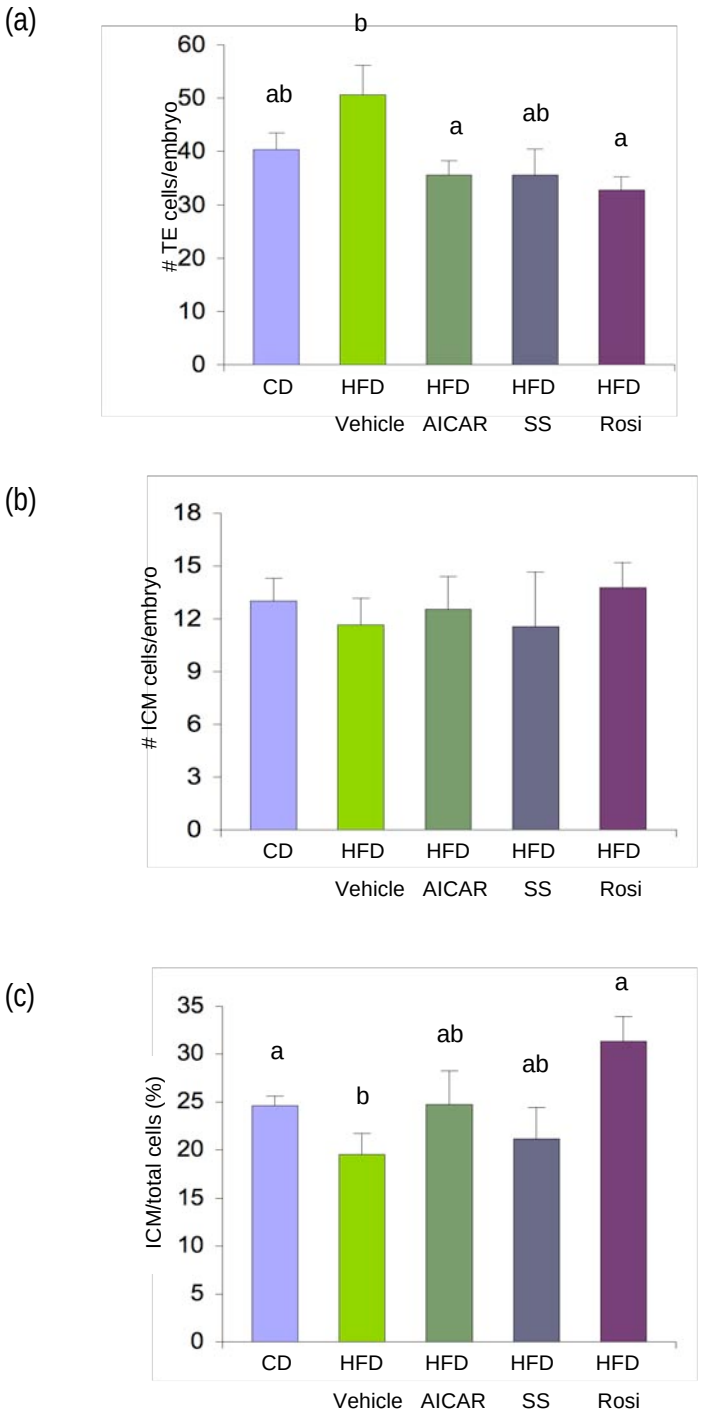


Figure 6.9 Cellular composition of embryos. Number of (a) trophectoderm (TE), and (b) inner cell mass (ICM) cells per embryo. (c) Percentage of inner cell mass per embryo. Bars indicate mean ± SEM (n=21-38 blastocysts/group). CD = control diet, HFD = high fat diet, SS = sodium salicylate, Rosi = rosiglitazone. Different letters indicate statistically different means, $p<0.05$.

6.4 DISCUSSION

In the current chapter we have confirmed that consumption of a diet high in fat, leading to obesity and insulin resistance, severely impairs reproductive health and oocyte developmental competence. We have further demonstrated that the PPARG pathway, potentially operating at the ovarian level, is intrinsically involved in this interaction. Rosiglitazone is uniquely able to overcome the negative influence of high fat diet consumption on embryonic on-time development. Rosiglitazone treatment also increased the percentage of inner mass cells per embryo, a measurement previously confirmed as indicative of improved fetal outcomes (Kwong *et al.* 2000, Kakar *et al.* 2005). Neither AICAR nor sodium salicylate were able to induce such profound effects, even though they exhibited some expected systemic effects, such as lowering the level of circulating insulin. This suggests that either; a) the metabolic consequences of rosiglitazone (including reducing plasma triglycerides, and lowering elevated blood glucose) or; b) the direct molecular targets of rosiglitazone within ovarian cells are responsible for the observed effects.

The molecular action of rosiglitazone is well established; upon entry into the cell via transmembrane diffusion (as a small and lipophilic molecule) rosiglitazone binds to PPARG, stimulating formation of regulatory complexes that either up- or down-regulate transcription of target genes. Among the comprehensive list of PPARG-regulated genes are many genes principally involved in lipid uptake and metabolism, glucose uptake and metabolism, and immune cell responses (Lemay *et al.* 2006). Systemically insulin resistance in peripheral tissues is ameliorated and circulating levels of lipids are lowered in response to treatment with PPARG agonists (Ehrmann *et al.* 1997, Picard & Auwerx 2002). Within ovarian tissue, PPARG is most highly expressed in the granulosa cells and luteal cells, in the ovaries of rodents and ruminants (Gasic *et al.* 1998, Komar *et al.* 2001, Froment *et al.* 2003, Minge *et al.* 2006). In addition, ovarian macrophages, which surround ovarian follicles and release pro-inflammatory cytokines, have high levels of transcript and protein expression (Minge *et al.* 2006). Within the oocyte itself, PPARG expression seems to be dependent upon species. Although moderate expression has been reported in ruminants (Mohan *et al.* 2002), oocyte PPARG expression is low within rodents (Komar *et al.* 2001, Komar & Curry 2002, Minge *et al.* 2006). It is therefore likely that modulation of somatic ovarian cell functions are mediating the improved outcomes observed when mice are treated with rosiglitazone.

In support of this, we identified significant, and specific, modulation of ovarian gene expression; namely, up-regulation of *Cd36* and *Fabp4*, and suppression of *Scarb1*, genes known to possess PPARG Response Elements (PPREs) in proximal promoter regions (Graves *et al.* 1992, Teboul *et al.* 2001, Malerod *et al.* 2003) and to be regulated in response to PPARG activation (Tontonoz *et al.* 1994, Tontonoz *et al.* 1998, Chinetti *et al.* 2000, Cabre *et al.* 2007). Within the ovary, high levels of CD36 protein is found in granulosa cells of preantral and early antral follicles, and also within the vascular thecal layer; SCARB1 expression is strongly associated with the HDL-cholesterol ester requirement for production of steroid hormones such as androgen (estradiol precursor) and progesterone; and FABP4 is found predominately within granulosa cells of follicles undergoing atresia (Nourani *et al.* 2005). FABP4 is known to solubilize lipophilic fatty acids facilitating their intracellular transport, in particular, positioning lipid ligands of PPARG in close proximity to this nuclear receptor allowing selective enhancement of PPARG transcriptional activity (Tan *et al.* 2002). Both CD36 and SCARB1 are involved in selective cholesterol ester uptake from HDL lipoproteins, and SCARB1 is additionally able to bind and uptake unmodified LDL. By up-regulating *Cd36* expression, and down-regulating *Scarb1* expression, rosiglitazone may specifically increase HDL-uptake potential, and minimize the possibility of native LDL cholesterol ester uptake within the ovary. At the time of sample collection (day 1 of pregnancy) ovarian function comprises establishment of luteal activity, which includes increased cholesterol uptake. Rosiglitazone-induced modifications to transport protein, and subsequently cholesterol, availability would dramatically influence such functions as progesterone production initiates. Further, activity and expression of key molecular regulators of steroidogenesis, including Steroidogenic Acute Regulatory Protein (StAR) and 3β-hydroxysteroid dehydrogenase (3β-HSD) are also both regulated by PPARG activation (Gasic *et al.* 2001, Seto-Young *et al.* 2007). Precisely how these functions would benefit ovarian follicular function to improve oocyte quality remains to be determined, and close inspection of hormonal synthesis in response to this treatment, and implications for oocyte developmental competence is required.

Overall, PPARG activation may present a promising option in the IVF setting, if pharmaceutical alternatives are required to overcome the influence of a sub-optimal maternal metabolic profile on oocyte health. Although currently listed as a Pregnancy Category C drug (not tested for use during pregnancy), recent studies have reported no adverse effects of maternal treatment with rosiglitazone during the peri-ovulatory period on embryonic or fetal outcomes (Chan *et al.* 2005, Klinkner *et al.* 2006). In addition, alternative activators of PPARG, including many of the naturally occurring, fatty acid-based ligands may prove useful in circumventing the limitations of rosiglitazone.

In conclusion, this study has demonstrated that rosiglitazone, either through systemic improvements to specific metabolic parameters, or by directly modulating PPARG-regulated gene expression in ovarian cells, is able to reverse deficits in oocyte quality brought about by diet-induced obesity, such that early embryonic developmental competence is greatly improved. It emphasises the important contribution of PPARG-controlled genes in optimal ovarian biology that are consequently key mediators of female reproductive potential.

Chapter 7

Final Discussion

7.1 SUMMARY OF FINDINGS

This thesis documents a sequence of key findings that expand our understanding of how diet-induced obesity impacts upon female reproductive function. The reliable, well-characterized rodent model of diet-induced obesity (DIO) established here will also now facilitate continued investigation of how the human obesity epidemic may manifest compromised health and wellbeing for women. The key findings are now outlined below.

7.1.1 Female mice exhibit metabolic phenotypes in response to diet-induced obesity that are strain-specific

Through a direct, controlled comparison of a range of murine strains, the development of female-specific, metabolic perturbations were identified. Our results clearly demonstrate that each of the analysed mouse strains is characterized by a specific metabolic profile that is attributable to genetic differences and that results in variable phenotypes.

In the current investigation, the CBA/CaH and C57BL/6 strains were the most profoundly affected by high fat diet, and Swiss and Balb/c the most resistant. However, the seasonal variability in glucose tolerance tests observed here, as well as factors that may be associated with the genetic isolation of mouse breeding facilities, highlight the merits of consistent and comprehensive characterization of mouse populations used for DIO studies.

Future studies examining DIO utilizing the mouse, as well as studies in humans, should more carefully consider the gender-dependent facets of the obesity-induced metabolic syndrome. In particular adipoinflammation, which although characteristic of obesity phenotypes in male mice, was not exhibited by the female mice we examined. We are now understanding more about how adipose depot location can influence macrophage infiltration in females (Zhang *et al.* 2007), and the implications this may have for the development of further metabolic perturbations. Specifically, it has been demonstrated that there are significant differences in the patterns of leukocyte recruitment into adipose tissue surrounding the deep viseral depot surrounding the mesenteric vessels compared to the more superficial adipose tissue associated with the uterus and peri-uterine area. This suggests distinct endocrine influences, which can contribute to the different functional capacities of these deposits. Future examination of the effects of

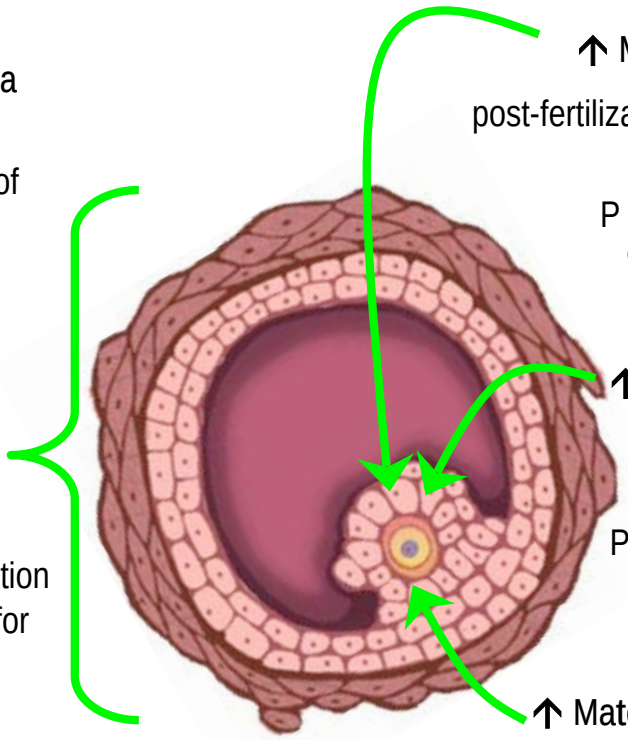
HFD on female metabolic status must therefore take care in the dissection of these deposits and the interrogation of their gene expression.

7.1.2 Diet-induced obesity results in altered ovarian function and impairs blastocyst development

Novel evidence was provided demonstrating that ovarian function and oocyte developmental competence is reduced in response to increased maternal intake of dietary fat. Specifically, ovarian follicle counts, oocyte morphology, ovulation, fertilization, blastocyst development rates, and blastocyst cell distribution were each impaired in a strain-specific manner, with those displaying the most extensive metabolic defects also developing severe reproductive abnormalities.

This finding, of dietary influence upon the quality and competence of oocytes, is of considerable significance, firstly, for future animal studies. Many diverse research investigations require robust rodent breeding capacities, and although commercial maintenance or control diets are usually less than 10-15% fat (Appendix I), it may be worth reflecting upon how lipid constituents of rodent feed may be influencing the ovary, and subsequently reproductive function. Other, recently released evidence from our colleagues supports our conclusion that fatty acid content of maternal diet can affect oocytes within the ovary prior to ovulation, as they described how high maternal dietary omega-3 polyunsaturated fatty acids reduce normal embryo development, and is associated with perturbed oocyte mitochondrial metabolism in the mouse (Wakefield *et al.* 2008). Although the impact of high dietary fat intake on mitochondrial parameters (associated with oxidative stress and the metabolism of energy-containing compounds) was not addressed within the current study, the pertinent report of Wakefield et al. encourages us to consider this contribution in future assessments.

More importantly, this evidence of a link between diet, obesity and the oocyte, is of great significance when the human obesity crisis is considered. These results provide some insight into the increased time to conception experienced by overweight and obese women. Although unconfirmed, it has been proposed for many years that female adiposity increases the likelihood of sub-optimal oocyte quality, particularly in the context of complex models of hormonally stimulated assisted reproduction. The current evidence, documented in a well-controlled, non-hormonally stimulated model, strongly affirms this hypothesis.



↑ Maternal plasma cholesterol:

impaired ovulation of ovarian follicles

P = 0.028
Risk ratio = 0.7607
95% CI = 0.5954 - 0.9718

Maternal obesity:

inappropriate selection of ovarian follicles for ovulation

P = 0.001
Risk ratio = 0.8759
95% CI = 0.8085 - 0.9490

↑ Maternal plasma glucose:

post-fertilization defects in embryonic survival and development

P = 0.05, risk ratio = 0.1422
95% CI = 0.0211 - 0.9604

↑ Maternal plasma insulin:

impaired embryonic blastomere differentiation

P = 0.028, rate ratio = 0.4431
95% CI = 0.2609 – 0.7525

↑ Maternal day 1 progesterone:

impaired embryonic blastomere differentiation

P = 0.038, rate ratio = 1.0034
95% CI = 1.0014 - 1.0055

Figure 7.1 Conclusion I. Female mice fed a high fat diet develop metabolic perturbations which are statistically predictive of specific ovarian dysfunctions, thus demonstrating that ovarian function is negatively influenced by obesity

When the metabolic and reproductive outcomes of data collected from these experiments were statistically correlated across mouse strains, specific metabolic perturbations were found that predicted the incidence or extent of female reproductive disruptions. These correlations were maintained across the diverse genetic backgrounds included in this study, and highlight mechanisms that may be critical for healthy somatic cell contributions to oocyte maturation. Specifically, circulating cholesterol, glucose and insulin levels at the time of conception predicted failure to ovulate, to produce blastocysts capable of normal growth and development and to produce blastocysts with appropriate ICM and TE cell distribution, respectively (Figure 7.1).

It is now essential to confirm that these associations persist in other species, and to elucidate whether the interaction is occurring directly within the ovary. This can be established by studies in which follicle growth is supported in vitro, to allow the temporal and dose-dependent effects of glucose, insulin and cholesterol to be elucidated. This technique, developed to exploit a new source of developmentally competent oocytes to facilitate the development of novel assisted reproductive technologies and cell-replacement therapies, has advanced significantly in recently months (Kim *et al.* 2008), and these studies have confirmed the feasibility of follicle culture technology. Importantly, the targeted modulation of specific metabolic parameters will allow further description and refinement of the dose-effects of each, with profound relevance for human extrapolation.

7.1.3 The insulin sensitizer, and PPARG agonist, rosiglitazone reverses the adverse effects of diet-induced obesity on oocyte quality.

Improved reproductive function can be conferred by short-term amelioration of insulin resistance associated with DIO. Although multiple agents were assessed, only one specific insulin-sensitizing agent, rosiglitazone, elicited this effect, indicating that its target, PPARG, contributes essential functions required for ovarian improvements (Figure 7.2).

One of the most striking aspects of these experiments is that DIO-induced defects in oocyte quality can be reversed very rapidly. The developmental phase immediately prior to ovulation therefore offers a particularly efficacious window for influencing oocyte quality, and this may be of relevance for clinical procedures in which optimal oocyte quality is sought. The dynamics of this effect, and oocyte receptivity to improvement, can also be more closely examined using the in vitro follicle system described above.

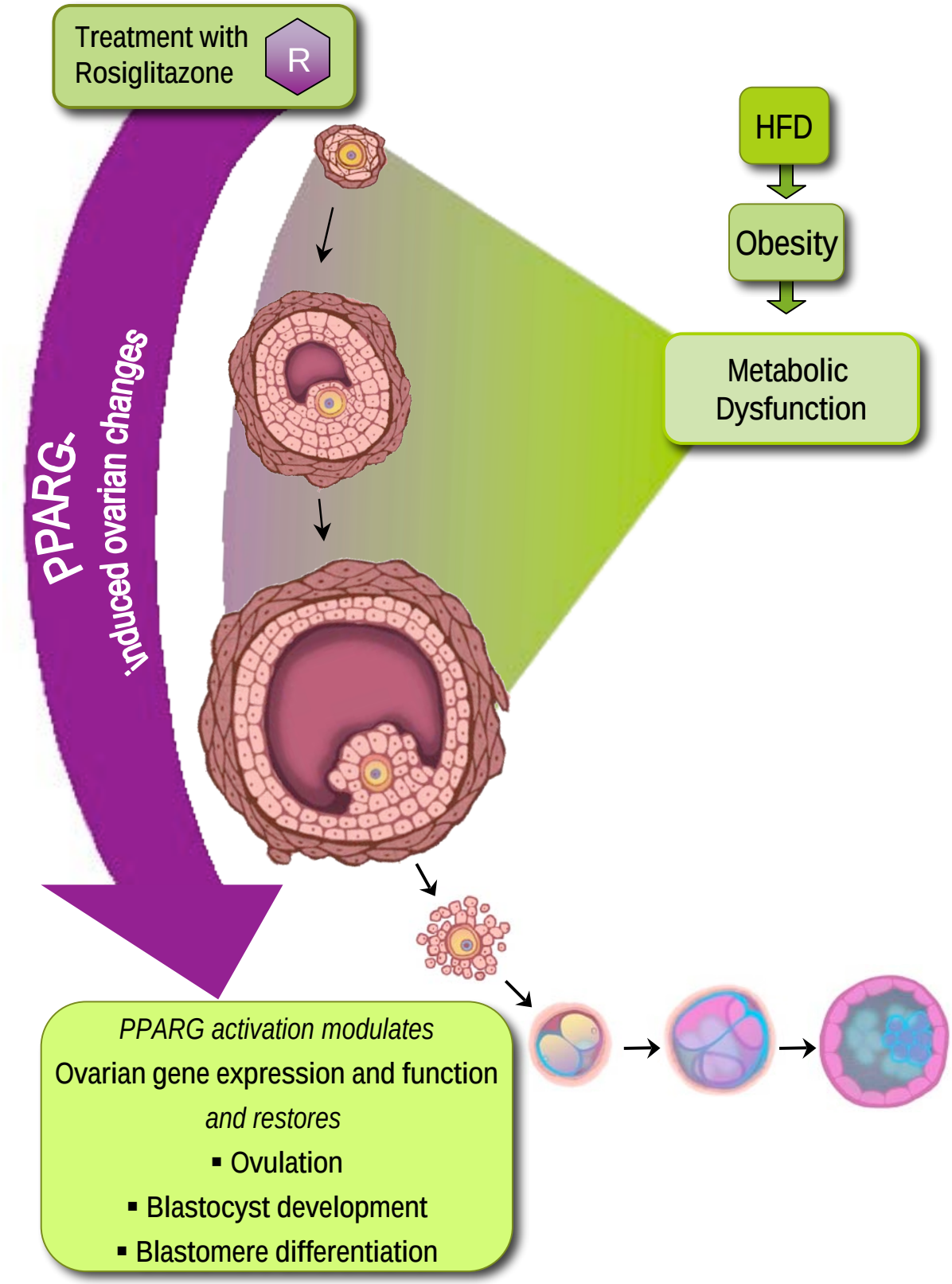


Figure 7.2 Conclusion II. Activation of ovarian PPARG reverses the obesity-induced defects to female fertility. Other insulin-sensitizing agents, which improve insulin sensitivity, but do not activate PPARG pathways, did not confer this effect.

PPARG activation by rosiglitazone successfully induced transcriptional changes in specific ovarian genes that are involved in various, and important lipid handling pathways. It is a present aim to investigate this more closely. In new microarray experiments currently underway, a HFD + rosiglitazone treatment group is to be included. It is hoped this will reveal a more complete spectrum of transcriptional changes induced by rosiglitazone, and the precise ovarian cell compartments in which these changes are occurring.

As outlined in the discussion of Chapter 6, PPARG activation may present a promising option in the IVF setting, to overcome the influence of a sub-optimal maternal metabolic profile on oocyte health. Rosiglitazone is currently listed as a Pregnancy Category C drug (not tested for use during pregnancy), although from animal studies there is no evidence that maternal treatment with rosiglitazone during the peri-ovulatory period induces any adverse effects on embryonic or fetal outcomes (Chan *et al.* 2005, Klinkner *et al.* 2006). However, alternative, potentially endogenous, activators of PPARG may prove useful in circumventing the limitations of rosiglitazone. A number of naturally occurring, fatty acid-based compounds act as ligands for PPARG, and it would of interest to investigate whether dietary supplementation with examples of these agents can induce any of the effects observed with rosiglitazone. Of course, the lipid-signalling actions of these agents (potentially eliciting the reproductively-disruptive effects seen in Chapter 3) must also be carefully considered. Alternatively, if improvements to oocyte developmental capacity using rosiglitazone can be elicited in vitro, novel options regarding the in vitro culture conditions for oocytes during the IVF process could be developed.

7.1.4 Future directions

A number of further experiments conducted in this model would provide even more insight into the reproductive consequences of DIO. In particular, examination of whether, and/or how, the composition of follicular fluid is altered by obesity would reveal additional information about how the obese phenotype is perceived by the oocyte in vivo. Such analyses were not conducted within the current study, as the mouse model limits the volume of follicular fluid available for aspiration and examination. However, it would be very interesting to determine if triglyceride and lipoprotein components of follicular fluid are altered by HFD, and how this might contribute to the oocyte morphological abnormalities described in this thesis. Experimental strategies are currently being established to enable this, and to also expand the serum and follicular fluid steroid analyses to include estradiol and testosterone.

The experiments described in Chapter 5, in which whole, homogenised ovarian extract was applied to custom-designed, enriched microarray slides, yielded no obviously significant results, but did indicate new strategies to offer improved experimental outcomes. Key to these is the application of purified, cell type-specific extracts to an array, as the heterogenous collection cells within the whole ovary appears to inhibit the clear identification of diet-induced effects. With respect to this, the future direction of this investigation includes the use of laser microdissection (LMD) to selectively isolate mural granulosa, cumulus-oocyte complexes and thecal cells from ovarian follicles of mice fed either control or high fat diet. This technique has been shown to yield samples of cell-specific RNA with minimal contamination, and is invaluable in permitting the simultaneous analysis of the biochemical and morphological status of each follicle (Sakurada *et al.* 2006). Such approaches will provide clearer answers to questions of how obesity influences the expression of genes throughout folliculogenesis, as well as how obesity, and additional therapeutic treatments (such as rosiglitazone), are impacting upon discrete ovarian cellular compartments.

The key question that arises from this thesis is whether the oocyte and embryo defects observed persist throughout pre-natal development to eventually impact upon the survival and health of live offspring.

To conduct such experiments, a number of complicating variables must be carefully considered. It has been suggested that obesity can contribute to endometrial disturbances, which impair the receptive endometrial status required for implantation (Wang *et al.* 2000). Post-natally, maternal influences associated with altered composition of breast milk (Rocquelin *et al.* 1998), may also conceal or even exacerbate, the effect of maternal DIO on early embryo development. In order to elucidate the specific contribution of oocyte and embryo quality to pregnancy success, and postnatal outcomes it would be necessary to establish a model of embryo transfer, and post-natal cross fostering. By observing the post natal outcomes when (a) fertilized oocytes from an obese mother are transferred into non-obese recipients; and when (b) fertilized oocytes from non-obese mother are transferred into obese recipients, the specific influences of ovarian versus extra-ovarian (i.e. uterine) factors on offspring health may be revealed.

If the early embryonic defects demonstrated in this thesis persist into gestation and result in offspring born with reduced birth weight, there would be profound implications for the inter-generational influence of dietary behaviours. Associations between small body size at birth and later biological risk factors such as insulin resistance have been found consistently. It is proposed that an intra-uterine lesion limiting gestational growth can influence the regulatory mechanisms that establish metabolic

homeostasis. When under-nutrition during early development is followed by improved nutrition after birth, accelerated or 'compensatory' growth can occur (Metcalfe & Monaghan 2001). Such individuals are at an increased risk of developing their own obesity and insulin resistance, and associated diseases such as hypertension, stroke, type 2 diabetes, and cardiovascular disease (Barker *et al.* 2002) (McMillen & Robinson 2005). Indeed, human maternal obesity has recently been shown to influence insulin sensitivity and secretion in the offspring (Mingrone *et al.* 2008). In this way, maternal obesity becomes self-perpetuating cycle.

7.1.5 Final conclusions

The project described in this thesis was established in response to concerns articulated by ART clinics about an emerging link between female infertility and obesity. In the face of a mounting obesity crisis in industrialised societies, such an association will have profound implications for the reproductive health of millions of women. This thesis demonstrates that Western-style dietary imbalances directly impact the ovary, and result in negative consequences for oocytes eventually released. Since these experiments were conducted, more evidence of an effect of increased body mass index on oocyte and embryo quality in IVF patients (Metwally *et al.* 2007a) and miscarriage rates in obesity (Metwally *et al.* 2008), has been released. Both of these studies report an association between obesity and assisted reproduction success, specifically on oocyte and embryo quality, as well as miscarriage. In addition, both of these studies are to be commended for considering the influence of patient age, in an effort to minimize the known confounding effect of age on these parameters, allowing reasonable confidence that any observed differences would most probably be due to the effect of the BMI alone. It is most likely that similar data will continue to emerge. It is hoped that increased consideration will therefore be given to the consequences that poor diet and lifestyle may have on the capacity of the female ovary to produce healthy and viable oocytes.

This thesis has also demonstrated that rosiglitazone, either through systemic improvements to specific metabolic parameters, or by directly modulating PPARG-regulated gene expression in ovarian cells, is able to rapidly reverse deficits in oocyte quality brought about by diet-induced obesity. As a result, early embryonic developmental competence is greatly improved. It emphasises the important contribution of PPARG-controlled genes in ovarian function that are consequently also key mediators of female

reproductive potential. In addition, it highlights the peri-ovulatory window of follicular development as a key time in which ovarian influences on oocyte developmental competence are established.

Chapter 8

Appendices

Appendix I. Summary of studies describing the effects of high fat diet feeding on mice

Prior to initiating this doctoral investigation, the literature regarding the use of mice in studies of diet-induced obesity was reviewed. The following table summarizes the studies that appropriately reflect the model sought for the current investigation. In particular, the strain and age of the animals used is outlined, as are the compositions of the high fat, and control diets fed and experimental duration.

Appendix I. Summary of studies describing the effects of high fat diet feeding on mice

Reference	Strain	Sex	Age	Control Diet			High Fat Diet			Experimental duration	Main findings
				% Fat	*% Carbs*	*Kcal/g*	*% Fat*	*% Carbs*	*Kcal/g*		
Studies demonstrating development of insulin resistance											
de Fourmestraux *et al*. 2004	C57BL/6J	male	5 weeks	12	60	n/a	72	<1	N/a	9 months	Different responses to HFD in B6 mice is related to the expression of genes in liver and muscle
Munzberg *et al.* 2004	C57BL/6J	male	3-4 weeks	6	N/a	3.3 (8664, Harlan Teklad)	35.8	17.5	5.6 (D12331, Research Diets)	16 weeks	Defects in leptin action within the brain may have a role in leptin-resistant obesity
Rajala *et al.* 2004	C57BL/6J	male	4 weeks	4	50	4 (5001, LabDiet)	45	35	4.7 (D12451, Research Diets)	20 weeks	Resistin expression is nutritionally regulated in response to insulin and glucose
Petro *et al*. 2004	C57BL/6J	male	4 weeks	4.8	74.3	4.06 (D1232, Research Diets)	35.8	35.5	5.6 (D12330, Research Diets)	11 weeks	In B6 mice restricting HFD caloric intake to that of CD does not prevent obesity or diabetes
Noonan *et al.* 2000	C57BL/6J	male & female	5 weeks	5	54	4.1 (LM-485, Teklad)	35	35	5.4 (F2685, Bioserv)	12 weeks	The C57BL/6J strain of mouse is a good model for studying diet-induced obesity, but they did not display hypertension or kidney dysfunction

Appendix I. Continued

Reference	Strain	Sex	Age	Control Diet			High Fat Diet			Experimental duration	Main findings
				% Fat	*% Carbs*	*Kcal/g*	*% Fat*	*% Carbs*	*Kcal/g*		
Studies demonstrating development of insulin resistance (cont)											
Tsunoda *et al.* 1998	C57BL/6J	female	7 weeks	4	60	3.4	32	17.6	4.9	17 weeks	Previously recommended high-monounsaturated fat diet might induce obesity and diabetes, compared to high-carbohydrate diet.
Ahren *et al.* 1997	C57BL/6J	female	4 weeks	4.8	74.3	4.06 (Lactamin)	35.8	35.5	5.6 (D12330, Research Diets)	12 weeks	Used B6 mice to study islet function following IR. Found an effect after 1 week on HFD
Van Heek *et al.* 1997	C57BL/6J	male	4 weeks	4	67	3.8 (D12450Research Diets)	24	41	4.7 (D12451, Research Diets)	7 weeks	Diet-induced obese mice develop peripheral but not central resistance to leptin

Appendix I. Continued

Reference	Strain	Sex	Age	Control Diet			High Fat Diet			Experimental duration	Main findings
				% Fat	*% Carbs*	*Kcal/g*	*% Fat*	*% Carbs*	*Kcal/g*		
Studies indicating a link between high fat diet feeding and diseased conditions											
Kim *et al.* 2004	C57BL/6J	male	3 weeks	7	62.94	N/a	18	51.95	N/a	12 weeks	97 hepatic genes differentially expressed on HFD
Moraes *et al.* 2003	C57BL/6J	male	4 weeks	3.1	60	3.3 (UAR, Harlan Tecklad)	36.1	35	5.4 (DIO Harlan Tecklad)	8 weeks	472 genes are differentially regulated when on HFD, including those regulating lipid metabolism and inflammation
Xu *et al.* 2003	C57BL/6J	male	4-5 weeks	4	67	3.8 (D12450, Research Diets)	24	41	4.7 (D12492, Research Diets)	16 weeks	Inflammatory macrophages in fat respond to total adiposity
Bell *et al.* 1997	Swiss	female	6 weeks	5	65	3.9 (AIN 1977 and 1980)	20.5	43.4	N/A	8 weeks	Voluntary exercise decreases body fat in all mice and prevents diet-induced obesity in mice fed high fat diets

Appendix I. Continued

Reference	Strain	Sex	Age	Control Diet			High Fat Diet			Experimental duration	Main findings
				% Fat	*% Carbs*	*Kcal/g*	*% Fat*	*% Carbs*	*Kcal/g*		
Studies investigating high fat diet feeding in transgenic strains											
Di Gregorio *et al.* 2004	C57BL/6J (WT & IL-6$^{-/-}$)	male & female	11 months	6	63	6.3 (2019, Harlan Teklad)	59	N/a	N/a (Bio-serve)	14 weeks	The absence of IL-6 prevents obesity, insulin resistance, hyperglycaemia or abnormal lipid metabolism
Wang *et al.* 2004	C57BL/6J (GDF3 transgenic)	male	12 weeks	4	67	3.8 (D12450B, Research Diets)	34.9	26.3	5.24 (D12492, Research Diets)	7 weeks	The adipogenic factor GDF-3 is only active under high lipid load, and not responsive to insulin or hyperglycaemia
Febbraio *et al.* 2000	C57BL/6J (CD36$^{-/-}$ & Apo E$^{-/-}$)	male & female	N/A	4	50	4 (5001 LabDiet)	21	49.1	4.5 (TD88137, Harlan Teklad)	12 weeks	Double KO mice show a 76.5% decrease in aortic tree lesion area when exposed to HFD, compared to apo-E null mice, suggesting that blockade of CD36 can be protective even in more extreme proatherogenic circumstances.

Appendix I. Continued

Reference	Strain	Sex	Age	Control Diet			High Fat Diet			Experimental duration	Main findings
				% Fat	*% Carbs*	*Kcal/g*	*% Fat*	*% Carbs*	*Kcal/g*		
Studies showing strain difference											
Kokkotou *et al.* 2005	C57BL/6 & 129	male	8 (B6) /12 weeks (129)	6.5	37.82	4.15 (5008, Purina Formulab)	24	41	4.7 (D12451, Research Diets)	12 weeks	C57BL/6 develop obesity, and 129 do not, despite having lower locomotor activity levels. The effect of MCH ablation if distinct in each strain
Funkat *et al.* 2004	C57BL/6, DBA/2 & 129T2	male	10 weeks	6.9	70	3.8 (Glen Forrest Stockfeeders)	65	15.2	6.7 (Glen Forrest Stockfeeders)	5-6 weeks	DBA/2 mice gained the most weight and adipose tissue mass. Glucose and fat oxidation did not differ. Plasma glucose concentrations in food-deprived mice were higher and insulin concentrations lower in 129T2 compared with C57BL/6 mice, but not affected by the HFD.
Kobayashi *et al.* 2004	MSM, JF1 & C57BL/6J	male	6 weeks	4.4	62.5	3.47 (CE-2)	33.5	36.9	N/A	11 weeks	JF1 mice are similar to B6 in their susceptibility to DIO, but MSM appear resistant

Appendix I. Continued

Reference	Strain	Sex	Age	Control Diet			High Fat Diet			Experimental duration	Main findings
				% Fat	*% Carbs*	*Kcal/g*	*% Fat*	*% Carbs*	*Kcal/g*		
Studies showing strain difference (cont)											
Cheverud *et al.* 2004	LG/J with SM/J (LGXSM lines)	male & female	3 weeks	6.5	62	4.1 (D12284, Research Diets)	21.2	49.1	4.5 (TD88137, Harlan Teklad)	20 weeks	Genetic correlations in dietary response followed a pattern similar to that found for the traits themselves. Several strains manifested discordant responses for obesity, glucose, and insulin, consistent with the presence of genotypes protective for diabetes in the presence of obesity.
Weisberg *et al.* 2003	C57BL/6J & FVB/NJ *Csf1op/op*	male & female	6-8 weeks	4.5	36.8	3.08 (PicoLab Rodent Diet 20)	24	41	4.7 (D12451; Research Diets)	12 weeks	Adipose tissue macrophage numbers increase in obesity and participate in inflammatory pathways that are activated in adipose tissues of obese individuals.

Appendix I. Continued

Reference	Strain	Sex	Age	Control Diet			High Fat Diet			Experimental duration	Main findings
				% Fat	*% Carbs*	*Kcal/g*	*% Fat*	*% Carbs*	*Kcal/g*		
Studies showing strain difference (cont)											
Black *et al.* 1998	C57BL/6J & A/J	male	4 weeks	4.8	74.3	4.06 (D12328, Research Diets)	35.8	35.5	5.6 (D12330, Research Diets)	16 weeks	B6 more sensitive to HFD than A/J, and exhibit severe obesity, hyperglycemia, hyperinsulinemia and altered body composition. Conclude that factors relative to fat metabolism rather than sucrose metabolism are responsible for obesity.
Schreyer *et al.* 1998	C57BL/6 & Balb/c	female	6-8 weeks	4	72	Not avail. (Wayne Rodent BLOX 8604, Teklad)	35.5	36.6	Not avail. (No. F1850, Bioserve)	6-14 weeks	14 weeks of feeding induced obesity and diabetes and 2-fold increases in plasma lipoprotein concentrations in B6 mice. Balb/c mice were resistant
West *et al.* 1992	AKR/J, C57L/J, A/J, C3H/HeJ, DBA/2J, C57BL/6J, SJL/J, I/STN, & SWR/J	male	6-10 weeks	5.5	37.78	4 (no. 5001, Purina Rodent Chow)	16.3	59	4.5 (C11024, Research Diets)	7 weeks	Increased carcass lipid content in AKR/J, C57L/J, A/J, C3H/HeJ, DBA/2J, and B6, but no/marginal effect on adiposity in SJL/J, I/STN, and SWR/J. Only AKR/J increased adiposity and consumed more energy.

Appendix I. Continued

Referece	Strain	Sex	Age	Control Diet			High Fat Diet			Experimental duration	Main findings
				% Fat	*% Carbs*	*Kcal/g*	*% Fat*	*% Carbs*	*Kcal/g*		
Studies demonstrating impaired fertility											
Tortoriello *et al.* 2004	DBA/2J & C57BL/6J	male & female	3-5 weeks	4	67	3.8 (D12450B-I, Research Diets)	24	41	4.7 (D12451-I, Research Diets)	15 weeks	Both strains on HFD developed glucose intolerance and insulin resistance, but only the DBA/2J developed dietary-induced obesity and hyperleptinemia. Male DBA/2J mice did not manifest diminished fertility. HFD was associated with > 60% decrease in natural pregnancy rates of female DBA/2J mice, but fertility of female C57BL/6J mice was unaffected. Obese female DBA/2J mice achieved normal ovulatory responses and pregnancy rates after exogenous gonadotropin stimulation, suggesting fertility defect to be central in origin.
Proposed protocol for doctoral research project											
	Swiss, Balb/c, C57BL/6, CBA/CaH 129T2Sv/Ems	female	5 weeks	6	64.7	3.8 (SF04-057, Specialty Feeds)	22	49.5	4.6 (SF00-219, Specialty Feeds)	16 weeks	To be determined

Appendix II.

1 joule (J) is the amount of mechanical energy required to displace a mass of 1 kg through a distance of 1 m with an acceleration of 1 m per second (1 J = 1 kg × 1 m2 × 1 sec-2). Multiples of 1 000 (kilojoules, kJ) or 1 million (megajoules, MJ) are usually used in human nutrition.

For reference, conversion factors between joules and calories are:

1 kcal = 4.184 kJ

or conversely,

1 kJ = 0.239 kcal

Appendix III. Classification of stages of the estrous cycle by cell morphology in vaginal smears. Modelled on criteria of Allen (1922), Boot *et al.* (1956) and Bronson *et al.* (1966).

Stage of cycle	Cell type			Smear density	Ovary
	Nucleated epithelia	Cornified epithelia	Leukocytes		
Proestrous	+++ (predominant)	-/+	-/+	Medium	Large, antral follicles
Estrous	-	+++ (predominant)	-	Medium-heavy	Ovulation occurs
Metestrous 1	-	++ (clumped)	+	Medium-heavy	Early CL present
Metestrous 2	+	+	++	Medium-heavy	Growing CL
Diestrous	+	-	+++ (predominant)	Thin, often with mucous	Quiescence, then rapid follicle growth prior to proestrous

Appendix IV.

Publications arising from this thesis.

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Peroxisome Proliferator-Activated Receptor-γ Agonist Rosiglitazone Reverses the Adverse Effects of Diet-Induced Obesity on Oocyte Quality

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PPAR Research
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Review Article

PPAR Gamma: Coordinating Metabolic and Immune Contributions to Female Fertility

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Peroxisome proliferator-activated receptor gamma (PPARG) regulates cellular functions such as adipogenesis and immune cell activation. However, new information has indicated additional roles of PPARG directing the cyclic changes that occur within ovarian tissue of female mammals, including those that facilitate the release of oocytes each estrous cycle. In addition to ovarian PPARG expression and function, many PPARG actions within adipocytes and macrophages have additional direct and indirect implications for ovarian function and female fertility. This encompasses the regulation of lipid uptake and transport, insulin sensitivity, glucose metabolism, and the regulation of inflammatory mediator synthesis and release. This review discusses the developing links between PPARG activity and female reproductive function, and highlights several mechanisms that may facilitate such a relationship.

1. INTRODUCTION

Since its initial identification in the early 1990's, peroxisome proliferator-activated receptor gamma (PPARG) has been primarily recognised as a regulator of cellular functions such as adipogenesis and immune cell activation. However, some recent reviews have discussed additional roles of PPARG directing the cyclic changes that occur within ovarian tissue of female mammals, including those that facilitate the release of oocytes each estrous cycle [1–4]. In addition to ovarian PPARG expression and function, many PPARG actions within adipocytes and macrophages have additional direct and indirect implications for ovarian function and female fertility. For instance, PPARG, through activation by thiazolidinediones (TZDs), is known to regulate the metabolism of lipids, providing both self-regulatory PPARG transcriptional mechanisms, and stimulating an increase in adipogenesis. Whilst the net volume of adipose tissue carried within an individual can influence reproductive potential, genes associated with lipid metabolism are also important for ovarian cells directly. As a result, PPARG has the potential to influence the cellular operations of follicles containing oocytes and, consequently, the health of those oocytes released. Likewise, the PPARG regulation of insulin sensitivity, downstream signalling pathways, and ultimately glucose uptake are likely to be also vitally important for normal ovarian function and overall female fertility.

Similarly, PPARG regulation of macrophage function has been addressed in vitro and within the context of the adipose tissue for many years; but appropriate activity of resident immune cells is also a prerequisite for normal ovarian function, as they are required for tissue remodelling facilitating ovulation, luteinization, and luteolysis [5]. Therefore, not only are adipose/circulating macrophage-sourced inflammatory mediators sensed by ovarian cells, but these mediators, when produced locally by the ovary, may influence the ovarian function in an autocrine fashion.

This review aims to provide evidence for how PPARG-regulated pathways influence the female's ability to produce healthy, developmentally competent oocytes. This is impacted by cellular function operating primarily at the local ovarian level, either directly acting upon the oocyte itself, or influencing the supporting ovarian cells that supply the oocyte with hormonal signals and nutrients. In addition,

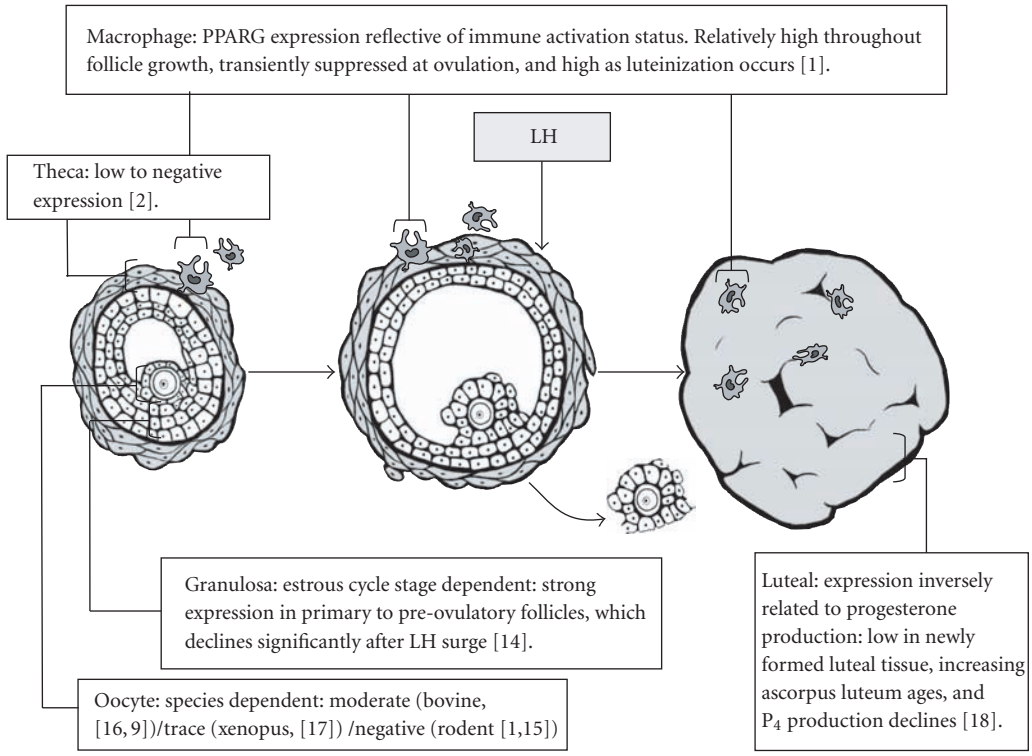

Figure 1: Overview of PPARG expression by specific ovarian cell types, as follicular development progresses from early antral and preovulatory follicle to postovulatory corpus luteum.

signals from extraovarian tissues, in particular adipose tissue and the circulating and/or resident immune cells, also exert powerful influences over the normal function of the ovary.

These concepts of overlapping influence on female fertility are particularly important when we consider conditions of reduced and impaired fertility such as polycystic ovary syndrome (PCOS), as well as reduction of reproductive function associated with excessive bodyweight and insulin resistance. In these situations, profound dysregulation of both metabolic and immune signalling pathways exacerbate ovarian perturbations, which are often successfully treated with administration of PPARG-activating pharmaceuticals.

2. PPARG GENE EXPRESSION

Successful mammalian reproduction requires a female body adequately, but not excessively, nourished, equipped to produce healthy eggs and to supply a growing fetus with sufficient energy. In this way, many tissues within the female body are able to influence the level of fertility. The extent of PPARG expression and its temporal regulation within these tissues can provide an interesting insight into the role of PPARG in female fertility.

2.1. Ovarian PPARG

Within the ovary, processes that are modulated by the PPAR superfamily, particularly PPARG, are among the most critical to normal ovarian function (Figure 1). Steroidogenesis, tissue remodelling, angiogenesis, lipid metabolism, immune cell activation, and production of proinflammatory mediators are all, to some extent, controlled by the presence and activity of the PPAR nuclear receptors. All three PPAR isotypes have been identified in the ovary of many species including the rat [2, 6], mouse [7], pig [8], sheep [9], cow [10, 11], and human [12, 13]. Localisation of these nuclear receptors has been established by both in situ hybridisation and immunohistochemistry [6]. Transcripts for PPAR alpha (PPARA) have been identified in immune cells and cells in the theca and stroma, whilst PPAR delta (PPARD) is found across all ovarian compartments [2]. Ovarian expression of both PPARA and PPARD is relatively stable across the ovulatory cycle, which suggests these isotypes are likely involved in regulating basal ovarian functions. PPARG is expressed strongly in the granulosa cells (primarily responsible for both estradiol production and the regulation of follicular fluid content), and lessstrongly in the thecalregion (site of androgen precursor production for granulosa estradiol synthesis) and luteal cells (postovulatory progesterone production) in the ovaries of rodents and ruminants [1, 2, 9, 14]. PPARG is detected earlyin folliculogenesis, and in contrast to PPARA and PPARD isotypes, its expression is dynamic, increasing until the large folliclestage [9], followed by downregulation in response to the LH surge [2].

Within the oocyte itself, PPARG expression seems to be dependent upon species, as moderate expression has been reported in ruminants [16], trace levels in Xenopus oocytes [17], and undetectable expression in rodents [1, 18]. It has not yet been investigated within the human oocyte.

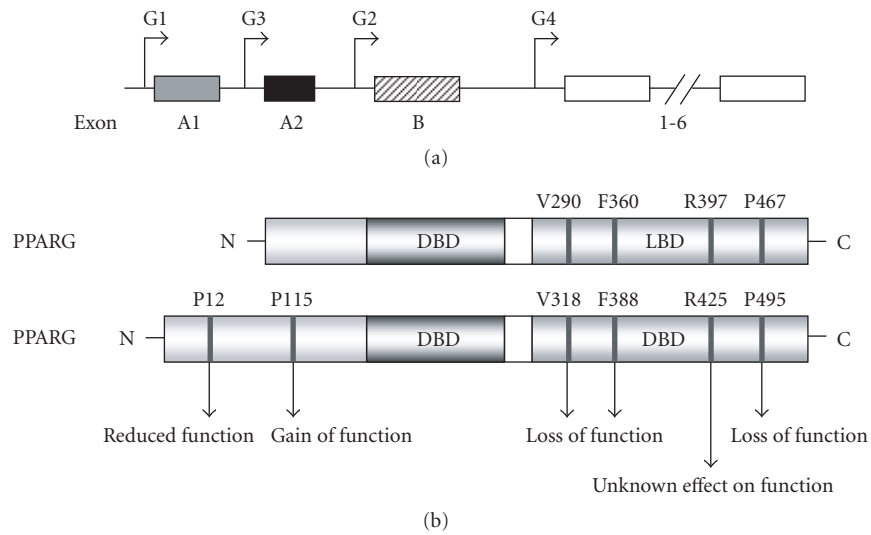

Figure 2: (a) The genomic structure of the 5′ end of the human PPARG gene. Exons 1-6 are common. Exons A1 and A2 are untranslated, and exon B is translated, giving rise to two different proteins corresponding to the G1 or G2 transcripts. (b) The domain structure of PPARG1 and G2 isoforms with the positioning of mutations or polymorphisms resulting in substituted amino acid residues, and altered protein functions. DBD, DNA-binding domain; LBD, Ligand-binding domain. (Figure adapted from Sundvold and Lien[33], Tsai and Maeda [37], and Stumvoll and Häring [38]).

2.2. Extraovarian PPARG

The highest level of mammalian PPARG expression is found within adipose tissue [19, 20], and activation of this adipose PPARG is sufficient [21] and essential to induce adipogenesis [22, 23]. Adiposity is also a key regulator of female fertility, affecting multiple aspects of the reproductive axis in women [24, 25]. Many of the adipocyte-sourced factors that are under PPARG control, such as the production of non-esterified free fatty acids, have widespread effects including ovarian targets [26–28]. In addition, any activation of adipose PPARG that may influence the amount and activity of adipocytes/adipokines can subsequently impact upon reproductive potential [29].

Both the ovary and adipose tissue are comprised of a considerable proportion of immune cells, in particular macrophages. Macrophages recruited into tissues are an important source of many inflammatory mediators that have functions both locally and systemically. Within the ovary, macrophage contribution to the pool of functional PPARG has been assessed [1]. TZD treatment has also been found to affect adipose-recruited macrophages, by increasing the rate of apoptosis, providing a subsequent reduction in the number of proinflammatory cytokine-producing cells [30]. Improvements to the chronically inflamed profile of women with PCOS may well go some way in explaining the beneficial systemic effects of PPARG activation in such patients (see Sections 3.2 and 4.4).

2.3. Mutations in PPARG negatively influence female fertility

The PPARG gene contains 9 exons, and spans more than 100 kb [31] (Figure 2(a)). There are at least 4 isoforms of PPARG, resulting from the use of different initiator methionines [31–33], which are believed to be involved in regulated gene expression in specific cells and tissues. PPARG1, expressed utilizing the untranslated exons A1 and A2, is 477 amino acids long, and is expressed at low levels in many tissues [34]. PPARG2 contains the translated exon B, and as a result is 28 amino acids longer than PPARG1 [35]. This isotype is expressed selectively in white adipose tissue, colonic epithelium, and macrophages [36]. PPARG3, which contains only exon A2, is found only in the large intestine and macrophages [31]. PPARG4 is limited to exon 1-6 common to all isotypes [33]. There have been numerous studies into the effects of genetic variability of PPARG gene sequence and expression, in both rodent models and human patients (Figure 2(b), Table 1).

Work initiated in rodent knockout models revealed that total PPARG$^{-/-}$ mutants display two independent lethal phases [23]. Firstly, PPARG deficiency interferes with terminal differentiation of the trophoblast cells and with placental vascularization, leading to myocardial thinning, and death by embryonic day 10. When PPARG null embryos are provided with a wild-type placenta, this cardiac defect was corrected permitting delivery, although postnatal pathologies (including multiple haemorrhages and lipodystrophy) resulted in lethality. To circumvent such restrictions, the Cre-loxP system can be applied, where Cre recombinase was under the control of the whey acidic protein (WAP) or mouse mammary tumour virus (MMTV) promoters. This causes PPARG gene deletions specific to secretory and hematopoietic tissues (alveolar epithelial cells of mammary tissue, salivary gland cells, oocytes, granulosa cells, megakaryocytes, and B- and T-cells) [50]. The results of this study revealed an important PPARG role in fertility: although the mutant mice appeared to ovulate normally, they exhibited reduced

Table 1: *Phenotypes and reproductive effects associated with PPARG mutations in mice and humans.* Abbreviations used: ART: artificial reproductive technology; BAT: brown adipose tissue; BMI: body mass index; HbA(1C): haemoglobin A1C; KO: knock-out; PCOS: polycystic ovary syndrome; T2DM: Type 2 Diabetes Mellitus; TG: Triglycerides; WAT: white adipose tissue.

Species	Genetic Abberation	Outcome	Effect on female fertility	Reference
Mouse	Global PPARG$^{-/-}$	Neonatal death	—	[23]
	Global PPARG$^{-/+}$	Improved insulin sensitivity	Fertile	[39]
	Mammary, epithelium, ovary, B- and T-cell null	Ovarian dysfunction and abrogated mammary development	30% of animals completely infertile, remainder had delayed conception, reduced litter size	[7]
	PPARG$^{hyp/hyp}$: WAT BAT, liver, and muscle null.	Normal birthweight but subsequent growth retardation, lipodystrophy, hyperlipideamia, and mild glucose intolerance	Heterozygote matings produce normal sized litters, but homozygote matings result in reduced litter size.	[40]
Human	Pro12Ala (34C > G), PPARG2 only.	Ala allele ↓ PPREs affinity, ↓ PPARG transactivation. ↑ Insulin sensitivity in some studies, conflicting reports on association with BMI.	Possible relationship with PCOS. In wider, non-PCOS population Ala allele associated with ↓ testosterone production	[41–43]
	Pro115Gln (344G > T), PPARG2 only.	Constitutively activated PPARG, ↑ adipocyte differentiation. Severe obesity, 3/4 subjects T2DM.	Fertility not assessed.	[44]
	His447His (1431C > T)	T allele may increase adipocyte differentiation. Presence of T allele associated with ↑ BMI, and insulin sensitivity.	T allele more common in PCOS compared to BMI-matched controls. T allele associated with ↓ testosterone.	[43, 45]
	Pro467Leu (1647C > T)	Mutation in LBD, ↓ coactivator recruitment and downstream transactivation. ↓ Basal gene activity. Lipodystrophy but normal BMI, severe insulin resistance and hypertension. One carrier (from 4) responsive to rosiglitazone therapy.	Oligomenorrhoea and hirtsutism, required ART for 1st pregnancy, complicated by pre-eclampsia and induced labour. 2nd pregnancy spontaneously conceived, with pre-eclampia, preterm emergency caesarean, and neonatal infant death.	[46, 47]
	Val290Met (1115G > A)	Mutation affects LBD, profound blockage of transcriptional activation. Similar phenotype to P467L. Unresponive to rosiglitazone therapy.	Primary amenorrhoea, hirsutism, acanthosis nigricans, and hypertension.	[46, 47]
	Phe388Leu (1164T > A)	↓ PPARG-ligand binding, ↓ basal transcriptional activity. Lipodystrophic and hypertensive with ↑ TG. Hyperinsulinemic, later T2DM.	Irregular menses, and bilateral polycystic ovaries treated with salpingo-oopherectomy. Prior to this carried two pregnancies.	[48]
	Arg397Cys (1273C > T)	Mutation in LBD, unknown effect on PPARG function. Lipodystrophic, ↑ TG and T2DM.	Hirsutism but no other indications of hyperandrogenism. Delayed menarche, but regular menses.	[49]

progesterone secretion as well as impaired implantation. Interestingly, fertility is affected even when the lesion in PPARG expression is restricted to extraovarian sites, as homozygote matings of $PPAR^{hyp/hyp}$ mutants, lacking PPARG expression in white and brown adipose tissue, liver, and muscle, had reduced litter size [40].

Examinations of naturally occurring human polymorphisms have focussed on susceptibility to Type II diabetes, insulin sensitivity, and obesity, and to date at least seven polymorphisms within the PPARG gene have been described.

The Pro12Ala polymorphism is located in exon2, and is only translated within the adipose tissue-, macrophage-, and colonic epithelium-specific PPARG2 isotype. The Pro12 allele is carried by approximately 85% of certain regional populations [51], and a single nucleotide mutation (C→G) leads to the substitution of an Ala amino acid [41]. PPARG protein produced by the Ala12 allele shows reduced in vitro affinity for PPAR response elements (PPREs) in target gene proximal promoters, and subsequently has reduced PPARG transactivation [41]. This PPARG SNP was extensively studied, following initial reports that it was strongly associated with bodyweight and insulin sensitivity [41], and the effect of the Ala12 mutation on PCOS symptoms has been closely studied, although some specific conclusions are difficult to reach. There are conflicting reports regarding the effect of this allele on BMI: either linked with increased BMI [45, 52–55], lower BMI [41, 56–59], or not associated at all [45, 60–64]. Current assumptions are that differential environmentalinteractions between populations can modify the function of this polymorphism. However, the relationship between Pro12Ala and insulin sensitivity appears more conclusive. Populations of women positive for Ala12 and PCOS have lower fasting insulin, reduced measures of systemic insulin resistance, lower insulin secretion, and lower hirsutism scores than women without the allele [54, 65, 66]. Consequently, the frequency of this allele is much lower in groups categorised as PCOS [54, 65, 67]. It appears that the Pro12Ala polymorphism of the PPARG gene may be a modifier of insulin resistance in women with PCOS, which can have a profound influence on fertility (see Section 4.1).

Another PPARG2-specific polymorphism is the rare Pro115Gln substitution in exon 3 that results in permanent, ligand independent activation [44]. This induces excessive adipocyte differentiation, and as a result the 4 individuals known to carry this (nonfamilial) SNP suffer extreme obesity [44], although present with only moderate metabolic complications including Type 2 Diabetes. The reproductive implications of hyperactive PPARG2 have not been addressed in these subjects.

All other reported polymorphisms are located in regions common to both PPARG1 and PPARG2. The His447His polymorphisms resulting from a C to T substitution at nucleotide 1431 in exon 6 is a silent polymorphism that encodes histidine with either allele [55]. Also referred to as the C161T polymorphism, it is proposed that this substitution may modulate expression of PPARG by altering mRNA processing or translation, leading to increased adipocyte differentiation. Subsequently, carriers of the T allele have elevated BMI. The T allele is also more common in women with PCOS compared to non-PCOS BMI-matched controls [45], and therefore has suspected involvement in the high incidence of obesity in PCOS population. However, both PCOS subjects and controls with T allele appear to be protected from other complicating symptoms of obesity, having better insulin sensitivity in addition to lower circulating testosterone.

The remaining polymorphisms are all extremely rare and restricted to single families.

Reported by Barroso et al. [46] and Savage et al. [47], there is a PPARG1 Pro467Leu substitution in the region required for ligand-dependent transactivation (PPARG2 residue 495) which results in impaired coactivator recruitment and downstream transactivation. This mutation also inhibits basal gene activity and has been found within 4 members of a single family spanning 3 generations. Medical histories reveal that in addition to lipodystrophy and hypertension (both frequently associated with PPARG mutations), the female carrier also experienced oligomenorrhea and hirsutism, and required ART intervention to conceive. This, and a subsequent spontaneously conceived pregnancy were both complicated with severe pre-eclampsia. Treatment of the male carrier with rosiglitazone (8 mg/day) was found to normalise chronic hyperglycaemia after 6 months, suggesting that the mutant PPARG protein is still able to be activated by exogenously sourced ligands, indicating the phenotypic profile of these subjects results from abnormal basal and endogenously activated PPARG activity.

Also identified by this study was a similarly positioned PPARG1 Val290Met mutation (PPARG2 residue 318) in a single female individual. This mutation results in a profound loss of PPARG function evidenced by both in vitro reporter gene activity, and in vivo response to rosiglitazone. Experiencing comparable metabolic complications to subjects with the Pro467Leu substitution, this individual also reported primary amenorrhoea, hirsutism, and acanthosis nigricans. Implications of these gynaecological and endocrine aberrations relating to conception and pregnancy have not been reported.

Another loss-of-function mutation is the phenylalanine to leucine substitution at position 388 (reported with respect to PPARG2, the substitution corresponds to residue 360 in PPARG1) found in 4 individuals from 3 generation of a single family [48]. Despite the reduction in normal PPARG function, concurrent treatment of one individual with both metformin and rosiglitazone (8 mg daily) provided effective glycemic control. Two of the affected individuals were female (46-year-old mother and her 22-year-old daughter), with the older individual experiencing a history of irregular menses and polycystic ovarian disease, eventually treated with bilateral salpingo-oophorectomy. At the time of study, the daughter did not have any significant medical problems (other than diet-controlled hyperinsulinemia and mild type IV hyperlipoproteinia), with regular menses and no polycystic ovarian pathology observed.

A heterozygous arginine to cysteine mutation at position 397 in PPARG1 (corresponding to residue 425 in PPARG2) was identified in a 64-year-old woman in 2002 by Argarwal and Garg [49]. Although the effect on PPARG functionality

has not been explicitly described, but the mutation lies in a region of the protein that forms a salt-bridge, and as a result, the mutated form may lack proper protein configuration. The Arg397Cys substitution was associated with lipodystrophy, elevated triglycerides, and early-onset Type 2 Diabetes. In addition, although pregnancy was never sought, moderate hirsutism as well as a history of delayed menarche (age 18) and subsequently irregular menstrual cycles were reported.

Overall, these studies demonstrate that PPARG precisely controls various aspect of systemic metabolism in humans. As female fertility is also disrupted in a significant number of these patients, it is likely that PPARG regulates female reproduction either directly, by intrinsic actions within reproductive organs such as the ovary, or indirectly via the myriad effects on metabolic tissues such as adipose and liver. To better define links between the metabolic and reproductive consequences observed in so many of these PPARG mutations, it would be interesting to recapitulate, in a tissue-specific manner, some of these PPARG genetic aberrations in mice.

3. LIGANDS

Together with expression of PPARG itself, availability of ligands is a primary regulating factor determining the ability of PPARG to influence target gene expression. Ligands can be produced endogenously, providing physiological significance, or sourced exogenously, as therapeutic factors given to target specific metabolic and reproductive symptoms.

3.1. Endogenous ligands: physiological function of PPARG

All PPARs bind and are activated by naturally occurring fatty acids and their metabolites [68], thus acting as fatty acid-activated receptors that function as key regulators of glucose and cholesterol metabolism. The precise nature of endogenous PPARG ligand binding and activation remains poorly defined and more research is needed in this area. However, the potential for important physiological ovarian PPARG activation is considerable, as many natural ligands have been shown to be present within the ovary, and produced locally by ovarian cells. Included in this list are ω3- and ω6-polyunsaturated fatty acids (PUFAs) such as the essential fatty acids linoleic acid, linolenic acid, arachidonic acid, and eicosapentanoic acid ([69] and reviewed [34]). Additional PPARG agonists such as prostaglandin metabolites of these substances and immunologically-derived eicosanoids are also produced within the ovarian environment in a hormonally regulated manner, with elevated production as ovulation progresses [70–73]. It is possible that PPARG may have a role in the feed-forward production of eicosanoid ligands, based on identification of a PPRE in the prostaglandin-endoperoxide synthase 2 (a.k.a. COX-2) promoter [74], which would facilitate amplified production of pro-ovulatory prostaglandins.

3.2. Exogenous ligands: therapeutic application of PPARG activation

As information emerges regarding the endogenous roles for naturally activated PPARG within the ovarian follicular environment, other evidence of PPARG involvement with ovarian function comes from reports utilising synthetic PPARG ligands, specifically, administration of TZDs to women diagnosed with PCOS (Table 2).

PCOS is the leading cause of infertility and menstrual irregularities in women of reproductive age and is characterised by chronic hyperandrogenic anovulation [90]. This is thought to be due, in general, to hypothalamic-pituitary axis dysregulation causing elevated basal LH levels that overstimulate cells of the theca interna [91]. Insulin resistance also appears to contribute to the syndrome in many instances [92], as the pituitary responds to elevated plasma levels of insulin to augment LH release [91].

The potential merits of applying TZDs to improve reproductive outcomes in infertile PCOS women was first demonstrated by Azziz et al. [93]. Since then, treatment of PCOS patients with the TZDs rosiglitazone or pioglitazone have been shown to not only improve insulin action in peripheral tissues, attenuate hyperinsulinemia, and lower circulating levels of lipids [92, 94], but also to improve a range of reproductive outcomes particularly circulating sex hormone levels, and ovulation rate [77, 88, 90, 95–97] (see recent reports summarised in Table 2).

The beneficial effects of TZDs on ovarian PCOS symptoms were first attributed to improvements in defective insulin action and secretion [92]. However, actions upon various ovarian cells directly illustrated both in vitro [1, 9, 14, 98, 99] and in vivo [100, 101] confirms a direct interaction between these compounds and ovarian PPARG.

Particular focus has been directed upon the effect of PPARG activation on the synthesis of ovarian steroid hormones and the expression of many rate-limiting steroidogenic enzymes has been investigated.

(1) Steroidogenic acute regulatory protein (StAR): facilitates that rapid mobilization of cholesterol for initial catalysis to pregnenolone by the P450-side chain cleavage enzyme located within the mitochondria [102]. It has been recently reported that both rosiglitazone and pioglitazone significantly up regulate StAR protein synthesis by human granulosa cells in vitro [103].

(2) 3β-hydroxysteroid dehydrogenase (3β-HSD): catalyses the conversion of pregnenolone to progesterone by luteal cells [104]. Work on porcine granulosa cells has found that troglitazone competitively inhibits 3β-HSD enzyme activity within these cells [99].

(3) Steroid 17-alpha-hydroxylase (P450c17): converts progesterone to androgen within ovarian theca cells [105]. Conflicting reports have arisen regarding the effect of TZDs on the expression and activity of this enzyme, many of which may be artefacts of various culture conditions. P450c17 mRNA production has been found to increase following porcine thecal cell exposure to TZDs [8], whilst other reports indicate suppression

Table 2: *Summary of reports published within the past 2 years on the use of PPARG activating agents for reproductive symptoms.* Abbreviations used: AUC, area under the curve; BMI body mass index; CC clomiphene citrate; DHEA-S dehydroepiandrosterone sulfate; E2, estradiol; FAI, free androgen index; FSH, follicle-stimulating hormone; GnRH, gonadotropin releasing hormone; HbA(1C), haemoglobin A1C; HDL-C, high density lipoprotein-cholesterol; HOMA, homeostasis model of assessment for insulin sensitivity; IGF1 insulin-like growth factor 1; IGFBP-1/3, insulin-like growth factor binding protein 1 or 3; LDL-C, low density lipoprotein-cholesterol; LH, luteinizing hormone; OGTT, oral glucose tolerance test; PCOS, polycystic ovary syndrome; QUICKI, quantitative insulin-sensitivity check index; SHBG, sex hormone binding globulin; T, testosterone; WHR, waist to hip ratio.

Reference:	Rautio et al. [75] and Rautio et al. [76]
Patient profile:	Overweight but not obese PCOS ($n = 30$)
PPAR agonist:	Rosiglitazone (4 mg once daily for 2 weeks then 4 mg twice daily for 16 weeks)
Metabolic outcomes:	Serum C-reactive protein levels, leukocyte count, and alanine aminotransferase enzyme activity decreased, but lipid and blood pressure did not change. Glucose tolerance and peripheral insulin response normalized in the rosiglitazone group.
Reproductive outcomes:	Rosiglitazone improved menstrual cyclicity, SHBG levels; and decreased serum levels of androstenedione, 17-hydroxyprogesterone (17-OHP), DHEA and DHEA-S.
Reference:	Rouzi and Ardawi [77]
Patient profile:	Obese PCOS ($n = 12$)
PPAR agonist:	Rosiglitazone (4 mg twice daily for 3 cycles, CC administered for 5 days starting 3 days after rosiglitazone initiated)
Metabolic outcomes:	No changes in fasting plasma glucose or HbA(1C) or IGFBP-3 values. Fasting serum insulin, DHEA-S, androstenedione, and IGF-1 levels decreased significantly and IGFBP-1 exhibited significant increases.
Reproductive outcomes:	Total-T, free-T, LH, and SHBG decreased. Follicular development and ovulation rate increased, trend for increased pregnancy rate in group receiving short-term administration of rosiglitazone compared to matched control receiving metformin.
Reference:	Mitkov et al. [78]
Patient profile:	Obese, insulin resistant PCOS ($n = 15$)
PPAR agonist:	Rosiglitazone (4 mg/day for 12 weeks)
Metabolic outcomes:	Hyperinsulinemia and insulin resistance normalized.
Reproductive outcomes:	Total-T and FAI profile tended to normalise. Number of women with oligomenorrhea was reduced by 67%
Reference:	Cataldo et al. [79]
Patient profile:	Insulin resistant PCOS ($n = 11$–16/group)
PPAR agonist:	Rosiglitazone (2, 4 or 8 mg/day for 12 weeks)
Metabolic outcomes:	Steady state plasma glucose declined and hyperinsulinemia fell in a dose-dependent manner.
Reproductive outcomes:	Serum LH, total-T, and free-T were unchanged; SHBG increased. Ovulation occurred in 55%, without significant dose dependence. Before and during treatment, ovulators on rosiglitazone had lower circulating insulin and free-T and higher SHBG than nonovulators.
Reference:	Lemay et al. [80]
Patient profile:	Overweight, insulin resistant PCOS ($n = 15$)
PPAR agonist:	Rosiglitazone (4 mg/day for 6 months)
Metabolic outcomes:	Plasma insulin, insulin resistance indices and insulin AUC in response to OGTT all decreased compared to controls receiving antiandrogenic estrogen-progestin. Effect on lipids was limited.
Reproductive outcomes:	No significant effect on androgens or hirsutism.
Reference:	Garmes et al. [81]
Patient profile:	Obese insulin resistant PCOS ($n = 15$)
PPAR agonist:	Pioglitazone (30 mg/day for 8 weeks)
Metabolic outcomes:	Insulin response to OGTT significantly decreased.
Reproductive outcomes:	Total-T and free-T levels decreased, SHBG increased, and LH response to GnRH stimulation decreased.
Reference:	Yilmaz et al. [82–84]
Patient profile:	Obese or lean PCOS ($n = 20$ obese, $n = 20$ lean)
PPAR agonist:	Rosiglitazone (4 mg/day for 12 weeks)
Metabolic outcomes:	Indices of oxidative stress improved. HOMA, insulin AUC, fasting insulin and C-peptide levels decreased significantly. Glucose/insulin ratio and BMI increased

Table 2: Continued.

Reference:	Rautio et al. [75] and Rautio et al. [76]
Patient profile:	Overweight but not obese PCOS ($n = 30$)
PPAR agonist:	Rosiglitazone (4 mg once daily for 2 weeks then 4 mg twice daily for 16 weeks)
Metabolic outcomes:	Serum C-reactive protein levels, leukocyte count, and alanine aminotransferase enzyme activity decreased, but lipid and blood pressure did not change. Glucose tolerance and peripheral insulin response normalized in the rosiglitazone group.
Reproductive outcomes:	Rosiglitazone improved menstrual cyclicity, SHBG levels; and decreased serum levels of androstenedione, 17-hydroxyprogesterone (17-OHP), DHEA and DHEA-S.
Reproductive outcomes:	Seurm levels of free-T, androstenedione, and DHEA-S decreased significantly. Menstrual disturbances improved in 61.5% of lean and 53.8% of obese patients. In a second cohort of patients, menstrual cycles became regular in 87.8%.
Reference:	Tarkun et al. [85]
Patient profile:	Young, lean PCOS ($n = 31$)
PPAR agonist:	Rosiglitazone (4 mg/day for 12 months)
Metabolic outcomes:	Fasting insulin and insulin resistance indices significantly improved. No changes in BMI, waist circumference, serum total cholesterol, or LDL-C. Serum C-reactive protein levels decreased; and endothelium-dependent vascular responses improved.
Reproductive outcomes:	Significant decreases in serum T, although no change in FSH and LH levels. Hirsutism score decreased significantly after treatment. 77.4% of women reverted to regular menstrual cycles. Levels of SHBG increased significantly after treatment.
Reference:	Dereli et al. [86]
Patient profile:	Nonobese PCOS ($n = 20$/group)
PPAR agonist:	Rosiglitazone (2 mg/day or 4 mg/day for 8 months)
Metabolic outcomes:	75% of women in the 2 mg group and 95% in the 4 mg group achieved normal glucose tolerance. Improved insulin resistance in a dose-related fashion, without adverse events or liver enzyme elevations.
Reproductive outcomes:	Decreased free-T levels were better in the 4 mg group than the 2 mg group, and 70% of women in the 2 mg group and 85% of women in the 4 mg group achieved ovulatory menses.
Reference:	Mehta et al. [87]
Patient profile:	Obese PCOS ($n = 9$)
PPAR agonist:	Pioglitazone (45 mg/day for 20 weeks)
Metabolic outcomes:	Significant improvement in insulin sensitivity
Reproductive outcomes:	LH levels, LH pulse frequency and amplitude, as well as gonadotropin responses to GnRH were not influenced.
Reference:	Ortega-González et al. [88]
Patient profile:	Obese, insulin resistant PCOS ($n = 25$)
PPAR agonist:	Pioglitazone (30 mg/day for 6 months)
Metabolic outcomes:	Body weight, BMI, and WHR increased significantly. Fasting insulin and insulin AUC during a 2-h OGTT decreased. Insulin resistance decreased and insulin sensitivity increased after treatment with either pioglitazone or metformin received by control group.
Reproductive outcomes:	Hirsutism, free-T and androstenedione declined to a similar extent after treatment with either drug. Treatment with both drugs was associated with the occurrence of pregnancy
Reference:	Sepilian and Nagamani [89]
Patient profile:	Obese insulin resistant PCOS ($n = 12$)
PPAR agonist:	Rosiglitazone (4 mg/day for 6 months)
Metabolic outcomes:	Fasting insulin, insulin AUC, fasting glucose, and glucose AUC significantly decreased. No significant change in BMI
Reproductive outcomes:	Both total-T, free-T and DHEA-S levels decreased significantly. No significant change in LH levels. Levels of SHBG increased significantly after treatment, 91.7% of women reverted to regular ovulatory cycles during the treatment period

of enzymatic expression and/or activity in primary porcine thecal cells or human cell lines [106–108].

(4) P450 aromatase (P450arom): aromatises androgen precursor to estradiol, and is expressed by ovarian granulosa [109] and luteal [110] cells. Although there is no correlation between the expression of the P450arom enzyme and PPARG itself during folliculogenesis, many reports have described the down-regulation of P450arom following TZD exposure in human ovarian cell cultures [13, 111–113].

Taken together, these findings provide strong evidence for the direct effect of TZD administration and PPARG activation on ovarian hormonal synthesis and secretion. Specifically the following.

(1) Androgen: TZDs found to inhibit LH- and insulin-stimulated androgen biosynthesis by purified porcine thecal [108], and mixed human ovarian [98] cells. They have also been found to reduce plasma testosterone levels in women with PCOS [76, 77, 81–84, 86, 89, 91, 97, 114, 115].

(2) Estrogen: While it is accepted that TZDs indeed influence estrogen secretion, estrogenic responses to TZDs appear to be dependent on confounding factors such as species, age, and endocrine setting. For instance, TZDs have been found to increase estradiol secretion [2], and decrease estradiol production [116]. PPARG activation by TZDs and phthalate toxins are believed to mediate the antiestrogenic effects of these agents in cultured rat granulosa cells [116], and TZDs have also been found to suppress stimulated estradiol secretion in human granulosa cell cultures [98].

(3) Progesterone: As for estrogen, progesterone responses to PPARG activation via natural or endogenous ligands are unclear, and are probably regulated by species, and stage of folliculogenesis. Most publications investigating a range of species, including primary bovine, ovine, porcine, or rodent cell cultures, report increases in progesterone secretion following administration of PPARG activators in vitro [2, 8–10], whilst some others suggest inhibition of stimulated progesterone secretion by porcine granulosa cells [14].

The net influence of TZD treatment on ovarian PPARG activation and subsequent steroidogenesis in vivo remains poorly defined across all species investigated. The most conclusive evidence for an advantageous outcome on hormonal (specifically androgen) profile following treatment is observed in women with PCOS, as overviewed in Table 2. As a result, increasing attention may be paid towards the application of these drugs in such conditions of significant hormonal perturbations.

Rosiglitazone and pioglitazone are currently listed as a Pregnancy Category C drug (i.e., not tested for use during human pregnancy), and some side effects of TZD administration, such as weight gain, fluid retention (reviewed in [117]), and possible bone demineralisation [118], preclude their widespread use during pregnancy. However, in vitro treatment of 2 cell mouse embryos, or in vivo treatment of pregnant mice with rosiglitazone was not found to impact upon normal blastocyst development, or litter rates and sizes [119]. In situations where conception has occurred following TZD treatment for PCOS, no adverse fetal outcomes have been observed [88, 96, 120]. Also in a recent study, examining tissue obtained from women with scheduled pregnancy terminations, it was found that placental transfer of maternally administered rosiglitazone to fetal tissues is minimal in the first 10 weeks of pregnancy [121].

4. MECHANISMS: PPARG REGULATION OF METABOLIC AND IMMUNE FACTORS INFLUENCING FEMALE FERTILITY

PPARG is known to regulate many pathways involving insulin sensitivity, glucose metabolism, adipokine signalling, lipid uptake and metabolism, and secretion of inflammatory mediators. As a result, PPARG is being revealed as a key mediator of the fundamental metabolic and immune contributions that are required for normal female fertility.

4.1. Insulin sensitivity

Normal insulin sensitivity and subsequently efficient metabolism of glucose are essential for healthy reproduction in the female. Conditions of hyperinsulinemia can interfere with normal ovarian cell function or be indirectly associated with other hormonal conditions detrimental to optimal fertility [122–124]. Also, exposure to high levels of glucose can have a deleterious effect on the oocyte [125, 126]. By normalising peripheral insulin signalling, PPARG activation can circumvent many of these adverse effects of hyperinsulinemia, as well as those detrimental outcome associated with persistently elevated blood glucose levels.

The genetic studies detailed above, and the pharmacokinetics of TZD treatment improving insulin sensitivity are both consistent with a direct role for PPARG in the regulation of cellular insulin utilization. Despite this, it remains to be determined exactly how TZD treatment and subsequent PPARG activation impacts gene expression directly related to insulin signalling and glucose uptake (through genes such as the insulin receptor (IR), IR-substrates, and glucose transporters), as a range of conflicting results have emerged. Suggested mechanisms include increases in glucosetransport protein 4 (GLUT4), stimulation of phosphatidyl-3-kinase andmodified phosphorylation of insulin receptor substrates [127–133]. In addition, it is well accepted that activation of PPARG does improve not only basal hepatic glucose secretion, but also peripheral insulin-stimulated glucose uptake, potentially indirectly via reduction of FFA, TNFα, plasminogen activator inhibitor-1, and other autocrine/endocrine signalling molecules which otherwise interfere with efficient insulin signalling (reviewed in [134]). In this way, PPARG activation may improve female infertility exacerbated by obesity and insulin resistance [25, 135–141].

New reports are also describing some of the first investigations into the ovarian-specific responses to TZD that facilitate insulin sensitivity in this tissue. The work of Seto-Young et al. [103] has shown that ovarian cells directly respond to

TZDs to increase transcription of insulin signalling components including IR alpha and beta subunits and IRS-1, which would subsequently provide more efficient signal transduction and cellular response to insulin.

4.2. Adipokines: leptin and adiponectin

Produced primarily by adipose tissue, leptin and adiponectin are "adipokines" with contrasting actions on insulin sensitivity. Whilst other adipokines such as visfatin and retinol-binding protein 4 (RBP-4) are also linked with insulin sensitivity [142, 143] and the incidence of PCOS [144, 145], leptin and adiponectin are of particular interest to those investigating female reproduction as it is known their presence can be detected by ovarian cells which express leptin and adiponectin receptors. In addition, although only the adiponectin promoter has been shown to contain a PPRE [146], transcriptional activity of both leptin and adiponectin genes is known to be decreased and increased, respectively, in the presence of PPARG-activating ligands [147–151]. In this way, they can operate as secondary messengers of signals initiated by PPARG activation.

Leptin receptors are present in the granulosa and thecal layers of the ovary [152, 153], and have been shown to be cyclically regulated [154]. Leptin appears to influence ovarian gonadotropin and steroid secretion [152, 153, 155], and affect oocyte quality and developmental potential [156, 157].

Adiponectin receptors AdipoR1 and AdipoR2 are also both expressed by ovarian tissue [158] and adiponectin itself has been identified within the follicular fluid of developed follicles in similar concentrations to that observed in the serum [159]. Adiponectin appears to be involved in many processes including those essential for ovulation, such as induction of COX-2 and prostaglandin E synthase expression in ovarian granulosa cells [159].

As the entire range of leptin and adiponectin effects on ovarian cellular functions, including the outcomes of PPARG activation (including enhancement of insulin sensitivity), are gradually established, it is likely we will find that the improvements to reproductive profiles and ovarian function of sub-fertile or infertile women treated with TZDs are mediated, at least in part, through modulation of these two adipokines.

4.3. Lipid uptake: CD36 and SCARB1

PPARG has a critical role in the regulation of adipocyte differentiation [94]. Among the best characterised PPARG target genes are those involved in lipid metabolism, including phosphoenolpyruvate carboxykinase [160], lipoprotein lipase [161], fatty acid binding protein [162, 163], and CD36 and SCARB1 [164, 165]. CD36 and SCARB1, class B scavenger receptors that mediate the endocytosis or selective cholesterol uptake from oxLDL and HDL lipoproteins, are also both strongly expressed by the ovary. The CD36 antigenis highly expressed by granulosa cells of preantral and earlyantral follicles, with moderatestaining also evident in the vascular thecal layers [166].In this context, CD36 has been reported as a facilitator of thrombospondins-1 and -2 activities [166, 167], influencing cell adhesion, wound healing, and angiogenesis [168, 169]; important components of the tissue and cellular changes that occur during the ovarian cycle. CD36 is upregulated following activation of PPARG in macrophages [164, 170], and a summary of PPARG control of gene expression [171] suggested this might act as a positive feedback mechanism, such that more potential PPARG ligands can be imported, enhancing expression of both PPARG and CD36.

Ovarian SCARB1 expression appears to be strongly associated with HDL-cholesterol ester requirement for production of androgen for aromatase-mediated conversion to estradiol by the granulosa cells, and progesterone synthesis by luteal cells. Thecal cells consistently express high levels of SCARB1 at all stages of both healthy and atretic follicle development [172], and high expression is also found within luteal structures [173].

In these respects, PPARG activation may have profound influence on ovarian function through the regulation of these genes or others regulating lipid metabolism, by affecting availability of substrate for hormone synthesis, and the remodelling of tissue structures required for oocyte release, luteinization, and luteolysis.

4.4. Suppression of chronic inflammation

An important role for PPARG is the suppression of immune cell synthesis and secretion of proinflammatory mediators [174–182] (reviewed [183–185]). The role of the immune system in female fertility is critical, both systemically, and locally at the ovarian level.

In addition, there are also interesting correlations between the development of adiposity, insulin resistance and, chronic inflammation. Increased serum concentrations of TNF, NO, and IL-6 are strongly associated with obesity [186, 187], and proinflammatory cytokines sourced from adipose tissue including TNF, and IL-6 are among several important factors that participate in the development of insulin resistance and type 2 diabetes mellitus [188–191]. Interestingly, together with central adiposity and insulin resistance, we also find aspects of systemic inflammation independently associated with impaired female fertility and PCOS [192, 193]. PPARG is implicated in improvements to the systemic inflammation observed in obese and insulin resistant individuals treated with TZDs. These studies describe reductions in serum C-reactive protein, IL-6, and soluble TNF receptor 2 [194–198]. Other studies investigating the chronically inflamed profile of PCOS patients support these findings, reporting that in addition to restoring menstrual cyclicity and improving markers of hyperandrogensism, TZD treatment is able to lower circulating C-reactive protein levels and the number of circulating leukocytes [75, 85].

4.5. Ovarian macrophages

Macrophages, dendritic cells, lymphocytes, and neutrophils have unique roles in the context of ovarian physiology, and are essential for the normal regulation of ovulation and control of the reproductive cycle [5, 199–201]. Macrophage

distribution and numbers within the ovary varies across the cycle, influenced by gonadotrophins and ovarian steroidogenic hormones. Resident macrophages are present in the theca and stroma of the ovary during the late stages of folliculogenesis [202]. Once the LH surge commences prior to ovulation, there is a massive recruitment of new leukocytes from the circulation into the theca of the preovulatory follicle [202, 203], where they function to release proinflammatory cytokines and mediators assisting the breakdown of the ovarian epithelium at ovulation. Their presence persists until after ovulation, further increasing in number in the developing and regressing corpus luteum [204].

Ovarian macrophages maintain high levels of PPARG transcript expression until a significant reduction in response to the proovulatory LH surge [1]. Immediately following ovulation, expression is restored to high preovulatory levels [1]. In vitro treatment of purified ovarian macrophages with the TZD troglitazone has been shown to significantly alter proinflammatory gene expression [1]. Specifically, these cells respond to TZD exposure by significantly suppressing mRNA production of NOS2 (or inducible Nitric Oxide Synthase, iNOS), the enzyme that catalyses the reaction producing the potent vasodilator, nitric oxide (NO). In the human, NO seems to direct follicular selection and maturation [205], and application of this NO property to IVF patients, deemed "poor responders", has been found to increase the number of oocytes retrieved [206]. This is an indication that recruited and specialized ovarian macrophages can potentially respond directly to TZDs administered systemically, and can regulate the availability of ovulatory mediators. Such responses parallel the anti-inflammatory effects of PPARG activation in nonovarian-activated macrophages [171], but was here found to be specific to macrophages closely associated with the ovarian environment (distinct to those located in the peritoneal cavity for instance). This illustrates the unique influence of the ovarian milieu on normal PPARG function and effects.

5. CONCLUSIONS

Many diverse endocrine and metabolic components profoundly influence female fertility, including hormone production as well as the development and ovulation of healthy oocytes. The role of PPARG in these events is two-fold. PPARG activation of transcription has outcomes operating both directly within the ovarian structure itself, and also indirectly through influences on other tissue systems such as the adipose tissue and immune cells (Figure 3). In this way, PPARG controls key signals regulating the capacity for normal reproduction. As PPARG is able, and required, to regulate many of these actions, it is important that the roles of PPARG be carefully considered as new concepts develop regarding the effects of dietary supplements such as PUFAs, which are PPARG ligands, and the consequences of increased immunological activation, such as occurs during obesity. As the health crisis surrounding the obesity epidemic widens to include the damaging effects on female fertility, it is important to remember the systemic implications of metabolism and immune regulation on female fertility, and to consider the role of PPARG in coordinating these contributions. Tremendous opportunity exists for those interested in elucidating further the exciting interactions between PPARG and female fertility. Publication of the most extensive list to date of all genes containing potential PPREs in their promoter regions [207] will provide a valuable tool for such research, as many identified genes have known functions within the context of ovarian physiology and pathology, in addition to characterized roles in other tissues, including macrophages and adipose tissue.

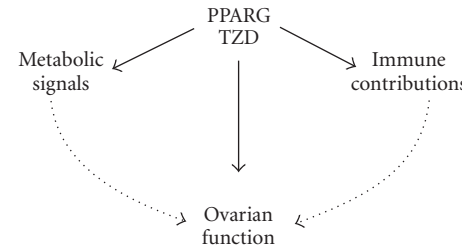

Figure 3: Schematic summarising the developing concept of PPARG influence on ovarian function and female fertility. PPARG is able to strongly influence the activity of ovarian cells directly, in particular steroidogenesis and tissue remodelling. In addition, PPARG can further influence ovarian function via regulation of external metabolic signals and immune cell contributions.

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Chapter 9

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