University of Southampton

Faculty of Natural and Environmental Sciences

School of Biological Sciences

The Effect of Folic Acid Supplementation in the Ovary and Upon Embryo Development

by

Reyna Stephanie Penailillo Escarate

Thesis for the degree of Doctor of Philosophy

March 2019

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Abstract

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Altered maternal nutrition around conception can affect oocyte and embryo development which influences later-life health outcomes. Low folate is related to poor reproductive outcomes. Folic acid (FA) supplementation and fortification have been effective strategies to avoid NTD. On the other hand, the FA levels in women of reproductive age are increased more than recommended, with unknown consequences. This project aims to determine the effect of high FA diet on the ovary and embryo development.

C57BL/6 female mice at PND74 were fed with control (1mg FA/kg food) or high (5mg FA/kg food) FA diet for four weeks and culled at diestrus stage (PND102). In parallel, a group of animals were maintained on the control diet for another four weeks (PND130) or either mated and culled at 3.5 days post coitum (dpc). Ovaries and embryos were collected, RNA extracted and analysed by qPCR. Morphological and immunostaining analyses were also performed to determine the effect of FA in the ovary and blastocyst.

High FA diet reduced expression of follicle developmental control genes at PND102 such as *Fshr* and *Oct4*, but also epigenetic writers like *Ezh2* and *Bmi1*. In contrast, four weeks after FA diet release, the same genes were upregulated in the ovary. Females with a preconceptional high FA diet showed an increased mating period compared to the control group. The embryos of these mice showed reduced TE cells and lower expression of CDX2. In parallel, embryos exposed to high FA diet exclusively during preimplantation showed delayed development with decreased total cell number and lower expression of lineage markers (*Oct4*, NANOG and *Gata6*).

High FA diet not only altered follicle growth factors during and after supplementation in a different pattern but also affected blastocyst biogenesis. This could impact on later-life health outcomes of the offspring

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Research Thesis: Declaration of Authorship

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I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

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Acknowledgements

I want to express my most profound appreciation to my supervisors, in especial to two brilliant scientist women who enlightened and encouraged me every day. Prof Karen Lillycrop, thank for your constant support and the confidence that you placed in me, to let me dream and perform this project. In the same way, I would like to thank Dr Judith Eckert, who taught me and show me everything that I know about the embryo. Both strong women challenged and guided me to become the researcher and the person that I am today.

I would want to say thanks to Prof Graham Burdge and Em Prof Tom Fleming for their constant feedback and confidence in my work. I also would like to extend my gratitude to my both research group Developmental Epigenetics and Tom Fleming group.

I am indebted to many of my friends at IDS to support me every day during this PhD Journey, special thanks to Eduardo, my partner in and outside of the lab, Ale, Carina, Camelia, Yurani and Paola. Thanks for all the meals and coffees shared and for your effort trying to do the world a better place.

Thanks to my family; Mom, Dad and my brothers, without you guys, this would not be possible. I am grateful for the great parents and brothers who even in the distance were always there to hold me and cheer me up. Thanks for always believing in me.

I cannot finish my acknowledgements, without dedicating a few words to my beloved partner, Felix. I feel very grateful for your constant support, advice and energy to overcome these four years, your words every day made me achieve what I am finishing today.

Abbreviations

ACTB	Actin Beta
ART	Artificial Reproductive Technology
ATM	Ataxia-telangiectasia-mutated
BMI1	B Lymphoma Mo-MLV insertion region 1 homolog
BRCA	Breast cancer gene
BSA	Bovine Serum albumin
CDX2	Caudal type homeobox 2
CL	Corpus luteum
CYC	Cyclophilin A
DAPI	4’,6-Diamidine-2’-phenylindole dihydrochloride
DOHaD	Developmental Origins of Health and Diseases
DMR	Differentially methylated region
DNMT	DNA methyltransferase
DNP	Dinitrophenol
DSB	Double-strand breaks
E2	Oestradiol
EED	Embryonic ectoderm development
EGF	Epidermal growth factor
EOC	Epithelial ovarian cancer
EOMES	Eomesodermin
EPC	Ectoplacental cone
Exe	Extra-embryonic ectoderm
EZH2	Enhancer of zeste homolog 2

Abbreviations

EPI	Epiblast
ER	Oestradiol receptor
FA	Folic acid
FGF	Fibroblast growth factor
FR	Folate receptor
FSH	Follicle stimuli hormone
FSHR	Follicle stimuli hormone receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCs	Granulosa Cells
GPCR	G protein-coupled receptor
GR	Glucocorticoid receptor
hCG	human choriogonadotropin
H2afz	H2A Histone Family Member Z
ICM	Inner cell mass
IF	Immunofluorescence
IGF	Insulin-like growth factor
IVF	*In-vitro* fertilisation
LH	Luteinizing hormone
LHCGR	Lutropin-choriogonadotropin hormone receptor
LPD	Low protein diet
mES	Mouse embryonic stem
M-MLV	Moloney murine leukemia Virus
mPRs	Membrane progesterone receptors
MTX	Methotrexate

NANOG	Homeobox transcription factor
OCT	Octamer-binding transcription factor
PBS	Phosphate buffered saline
P.C	Periconceptional
PCO	Polycystic ovary syndrome
PCFT	Proton-coupled folate transporter
PE	Primitive Endoderm
PGCs	Primordial germ cells
PGK1	Phosphoglycerate Kinase 1
PGRMCs	Progesterone receptor membrane components
PI	Propidium iodide
P.I	Preimplantation
PLL	Poly-L-lysine hydrobromide
PND	Post-natal day
PPARα	Peroxisome proliferator-activated receptor alpha
PPIB	Peptidylprolyl Isomerase B
POI	Premature ovarian insufficiency
PR	Progesterone receptor
PVP	Polyvinil-pyrrolidone
P4	Progesterone
RFC	Reduced-folate carrier
rRNA	Ribosomal RNA
SAM	S-adenosylmethionine
SDHA	Succinate dehydrogenase complex flavoprotein subunit A

SOX17	Sex determining region Y-Box 17
SOX2	Sex determining region Y-box 2
SPTBN1	Spectrin beta non-erythrocytic 1
StAR	Steroidogenic acute regulator protein
SUZ12	Suppressor of Zeste 12 homolog
TBP	TATA-Box-Binding protein
TE	Trophectoderm
TEAD4	TEA-domain family member 4
TNBS	Trinitrobenzene sulphonic acid
TS	Trophoblast stem
TUBA	Tubulin Alpha
UBC	Ubiquitin C
YAP1	Yes-associated protein 1
18S	RNA, 18S Ribosomal 5

Chapter 1 Introduction

1.1 Developmental origin of health and diseases (DOHaD)

The DOHaD hypothesis proposes that during critical periods of the development, nutrition and other environmental stimuli alter developmental pathways and shape the long-term health of an individual (Barker, 1997; Waterland and Michels, 2007). Prenatal and early postnatal life have been widely described as critical periods of plasticity acting as a decisive time to alter the pattern of future development. In the early '90s, Hales and Baker defined the Thrifty Phenotype Hypothesis as the adaptive response from the foetus to an adverse environment producing permanent changes in the physiology and metabolism to increase its survival postnatally. This adaptation only becomes detrimental when nutrition is more abundant in the postnatal environment than it had been in the prenatal period (Hales and Barker, 2001; Gluckman and Hanson, 2004).

Birth outcomes like weight, birthweight-placenta ratio and length have been associated with cardiovascular disease in adult life (Barker *et al.*, 1989, 1990; Barker, 1995). For example in the Helsinki Birth Cohort, where more than 13,000 men and women born in Finland were studied, low weight and low ponderal index (birth weight/length^3) at birth were associated with increased risk of coronary heart disease. Moreover, among the 1 year old boys with low birth weight, rapid weight gain was associated with a further increase in risk (Eriksson *et al.*, 2001).

Another example of how the in-utero environment can have consequences in later-life have come from studies of the Dutch Hunger Winter, a famine that occurred in 1944. Adults exposed to prenatal famine had decreased glucose tolerance compared with adults born before or after the famine (Ravelli *et al.*, 1998). Moreover, the prenatal undernutrition consequences were depending on the gender and gestational timing of the insult. Individuals born to mothers exposed to famine during the first trimester had a higher risk of cardiovascular disease and lowered cognitive function later in life; whereas individuals born to mothers exposed during the mid to late pregnancy developed with impaired kidney and lung function or an impaired glucose tolerance (Roseboom, de Rooij and Painter, 2006). Six decades after the famine, studies have shown that individuals who were prenatally exposed to this deprivation had reduced levels of DNA methylation of the imprinted insulin-like growth factor 2 (IGF2) gene when they were compared with their unexposed, same-sex sibling (Heijmans *et al.*, 2008).

Although initial studies of the DOHaD hypothesis indicated undernutrition as the main detrimental factor during early development, later studies in both human and animal models reported that either deficiency or excess of nutrients led to an increased risk of obesity, type-2 diabetes and cardiovascular diseases in adulthood (Tarry-Adkins and Ozanne, 2017). The impact of maternal diet during gestation has been widely studied about its impact on the embryonic development and modulation of the phenotype of offspring through epigenetic programming. However, the pre-conception status of the parents is also an important factor that can influence the gamete status and then the future embryo and foetus development. The periconceptional period covers the completion of meiotic maturation of the oocytes, differentiation of spermatozoa, fertilisation, and resumption of mitotic cell cycles in the zygote and the further development until implantation in the wall of the uterus (Fleming *et al.*, 2018). Undernutrition (e.g. low protein and low folate) and overnutrition (e.g. obesity and high fat) models have been used to explain the substantial consequences that maternal diet can have on the future health of the offspring. Therefore, nutritional disruption around conception could have later consequences in the ovary and then the embryo development.

1.2 Ovary role in the developmental origin of health and disease

Environment, nutrition and maternal disease can affect foetal development and predisposition to some diseases. As the gametes will give rise to the foetus, it is possible that the link between early-life adversity and postnatal disease susceptibility lies within the developing ovary, involving the developing germ cells and their function (Chan, Tsoulis and Sloboda, 2015). Ovaries are the essential part of the female reproductive system which are responsible for the production (oogenesis), and release of gametes (ovulation); both processes are necessary for fertilisation and successful reproduction (Richards and Pangas, 2010). The germ cells (oocytes) in the developing foetal ovary are vulnerable to prenatal events such as variations in maternal nutrition and health; any modifications in foetal gonadal development will contribute to trans-generational disease risk (Dumesic *et al.*, 2015).

Follicles are defined as the basic functional unit of the ovary, and each follicle consists of an oocyte enclosed by somatic cells (Richards and Pangas, 2010). Follicle growth is regulated by a neuroendocrine feedback system, the hypothalamic-pituitary-ovarian axis. In this process, hormones and proteins function to permit the perfect synchronisation between oogenesis and ovulation. The quality of the ovarian follicle and female fertility can be modulated by the disruption of key factors of follicle development as will be described later.

1.2.1 Development of the follicle

Oogenesis takes place in the foetus when primordial germ cells (PGCs) progress through mitotic divisions generating an excess of interconnected oogonia. Mitotic divisions then cease, and germ cells start meiosis I to become a diploid primary oocyte, progressing through the first stage of prophase I before arrest (Grive and Freiman, 2015). The primary oocyte does not complete meiosis I, but stops at the first meiotic prophase division; this stage is referred to as the germinal vesicle. However, it is not until puberty when primary follicles will continue to grow and be recruited during each cycle (a term known as primordial follicle recruitment), and only one oocyte (in human) from the dominant follicle matures and will be ovulated (Virant-Klun, 2015).

In puberty, growing follicles can be triggered by a surge of luteinizing hormone (LH), which results in a breakdown of the germinal vesicle, nuclear maturation and completion of meiosis I, and the formation of the first polar body. The oocyte is then arrested in metaphase II (Grive and Freiman, 2015). The second meiotic division is only completed after fertilisation by a sperm cell and leads to the formation of the second polar body.

The follicle development comprises the development of primordial follicles into primary, secondary, antral and finally to the pre-ovulatory or Graafian follicles (Figure 1.1). Follicle classification may vary among studies, according to the number of granulosa cells surrounding the oocyte or according to the size and morphological appearance (Pedersen and Peters, 1968; Myers *et al.*, 2004). Follicles are classified depending upon the number and proportion of granulosa cells surrounded the oocyte can be defined as follows (Figure 1.1):

- **Primordial follicles** are defined as a partial or complete single layer of flattened (squamous) granulosa cells surrounding the oocyte; these are formed during foetal development and are commonly located in the peripheral cortex. When sexual maturity is achieved, and meiosis is resumed, some primordial follicles will start to develop into primary follicles during each menstrual cycle.

- **Primary follicles** are characterising by one layer of granulosa cells with a cuboidal shape. During the transition between primordial and primary, follicles were classified as primary when cuboidal granulosa cells are more than the 50% of the surrounding cells, otherwise were classified as primordial. Following development, a proliferation of the cuboidal cell monolayer results in the formation of a multi-layered zone of granulosa cells called zona granulosa. This occurs together with the formation of a thick glycoprotein and acid proteoglycan coat, denominated zona pellucida, located between the oocyte and the zona granulosa.

- **Secondary follicles** are described as an oocyte with more than one layer of cuboidal granulosa cells surrounded by theca cells.

- **Pre-antral follicle** will appear when the secondary follicle continues growing, and fluids fill spaces on the zona granulosa (follicular antrum).

- **Antral follicle** is a follicle with a bigger follicular antrum within the zona granulosa coalesce and the oocyte.

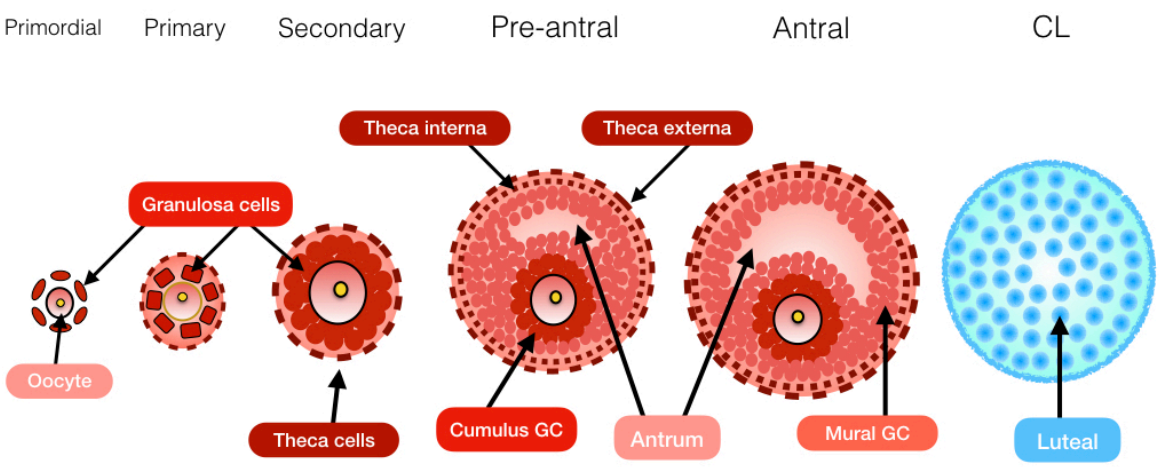

Figure 1.1 Stages of follicle development

Stages of follicle development from primordial follicle until corpus luteum formation.

The theca cells can be divided into two zones: an internal and external. The internal theca consists of polygonal cells with vacuolated cytoplasm and open-faced, vesicular nuclei. These cells are the main sites of synthesis of androstenedione under LH stimulus. In contrast, the cells of the external theca are spindle-shaped and merge with the surrounding ovarian stroma, with apparently no endocrine function. In the case of granulosa cells (GCs), there are two types: cumulus cells surrounding the oocyte, and mural cells that surround the antrum. The growth of the follicle and its antral cavity will induce the separation of both cumulus and mural GCs to allow detachment of the oocyte from the zona granulosa and float freely within the follicular antrum (Graafian follicle, or pre-ovulatory follicle) until it is discarded into the fallopian tube during ovulation (Richards and Pangas, 2010).

After the extrusion of the oocyte from the Graafian follicle at ovulation, the granulosa and theca cell remnants undergo hypertrophy and hyperplasia. This process called luteinisation occurs under the presence of LH and prolactin and will give rise to the corpus luteum (CL). This postovulatory follicle includes the degeneration of separation between granulosa and theca cells and infiltration of blood vessels. The CL contains steroidogenic cells, which produce progesterone to prepare the uterus for the pregnancy. However, if there was no implantation, the degenerating CL progressively shrinks in size with an increase in the fibrous tissue, in this stage, the follicle is now called the corpus albicans (Tomac, Cekinović and Arapović, 2011).

In women, 99% of the primordial follicles finish in atresia. Follicles undergo apoptosis constantly, which reaches a threshold in the fifth decade of life. According to that, the follicles can be

classified by two types: atretic (degenerated) and non-atretic (healthy follicles) (Johnson *et al.*, 2004). Granulosa cells can also undergo apoptosis in early atretic follicles, these apoptotic cells are mainly located in the inner surface, but not in the cumulus or theca cells, indicating a role of granulosa cells in early stages of atresia. The cause of apoptosis is mainly related to the downregulation of vital survival-promoting factors or stimulation by death ligands (Matsuda *et al.*, 2012). Some factors that participate in the prevention of follicle atresia are Insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and cytokine interleukin-1β (Cheng *et al.*, 2002).

1.2.2 Regulation of follicle development

The primordial follicle activation is regulated by several factors involved in the communication between oocyte and GCs. KITL (c-kit ligand) produced by the GCs, regulated by FGF2, LIF or FGF7 and oocyte-specific factors such as SOHLH1, SOHLH2, LHX8 and NOBOX are transcription factors necessary to trigger the recruitment of primordial follicles into the growing pool (Georges *et al.*, 2013). NOBOX (newborn ovary homeobox) is an oocyte-specific transcription factor that regulates differentiation. Knockouts of NOBOX in the ovary affect oogenesis, and the primordial transition to a primary follicle fail. NOBOX regulates GDF9/BMP15/DNMT1 and OCT4 binding to their promoter regions (See Figure 1.2) (Rajkovic *et al.*, 2004; Choi and Rajkovic, 2006; Bayne *et al.*, 2015). OCT4 is a pluripotency factor expressed in the oocyte of small and large follicles. Lack of OCT4 in mice at 3 weeks old reduces the number of primordial possibly due to increased recruitment of primordial follicles to the pool of maturing follicles. Moreover, at 6 weeks old mice also showed very few large follicles which directed to infertility (Kehler *et al.*, 2004).

Anti-Mullerian hormone (AMH) is a factor safeguarding the ovarian reserve, which is expressed in the GCs and downregulates primordial follicle recruitment (Durlinger *et al.*, 1999). AMH expression has been described to be regulated by TGFβ family members GDF9/BMP15 promoting histone acetylation of H3K27, increasing *Amh* in mouse GCs; meanwhile, this effect is inhibited by follicle-stimulating hormone (FSH) (Roy *et al.*, 2018).

After primary follicle formation, the next steps in the follicle development are regulated by the TGFβ family stimulating GC proliferation. GDF9 is an oocyte-secreted factor important in the regulation of follicle and oocyte growth, GCs proliferation of small and large follicles, and the prevention of early differentiation inhibiting steroidogenic synthesis (Gilchrist *et al.*, 2004; Spicer *et al.*, 2006; Cook-Andersen *et al.*, 2016).

At the antral stage, development is under the control of the gonadotropin hormones (FSH and LH) through the hypothalamic-pituitary-ovarian axis. FSH plays a role in the oestradiol and inhibin secretion from the large dominant follicle. GCs produce inhibin, whereas theca cells produce androgens that are used by GCs to synthesise estradiol-17β. Oestradiol and inhibin act on the hypothalamic-pituitary system as negative feedback and decrease FSH secretion. By that time, the follicles acquire LH receptors on GCs resulting in ovulation induced by an LH surge (Matsuda *et al.*, 2012). In the oocyte, meanwhile, the LH surge starts the expansion of the cumulus cells. These cells are essential not only during ovulation allowing the release of the oocyte but also during the capture of the oocyte by the oviduct, sperm penetration and fertilisation (Dragovic *et al.*, 2005).

Luteinisation is the process were the LH surge induces GCs of mature follicles to develop into CL cells (luteal cells) and to the production of oestrogen and progesterone. Following the expulsion of the ovum, GCs layer is thrown into the follicular antrum, and a remodelling of the follicle takes place. This process is characterised by greater cell proliferation and angiogenesis, a decrease in FSH receptor and an increase in LH receptor in luteal cells.

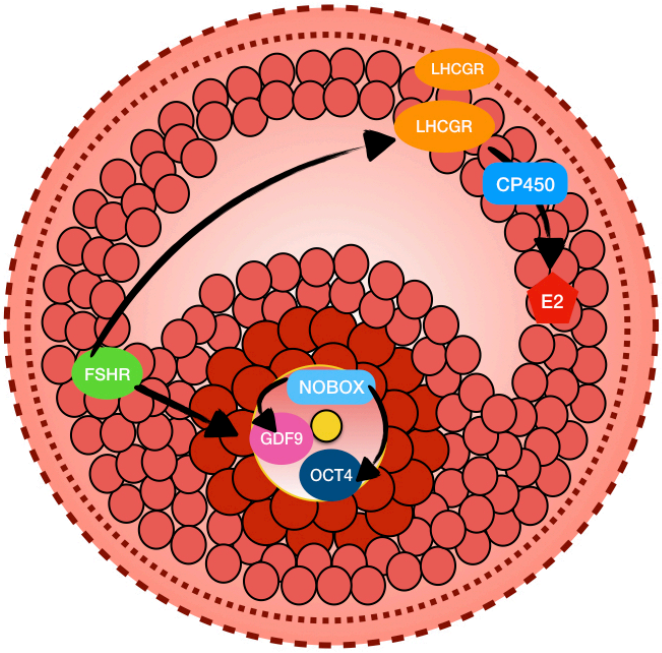

Figure 1.2 Regulation of follicle development

NOBOX, oocyte-growth factor regulate OCT4 and GDF9 in the oocyte. Meanwhile, FSHR present in the GCs regulate the expression of LH receptor (LHCGR) in granulosa and theca cells and therefore oestrogen production (E2).

1.2.2.1 Gonadotropin role during follicle development

The hippocampus releases the gonadotropin-realising hormone (GnRH), which stimulates the anterior pituitary gland to secrete the FSH and LH, both known as pituitary gonadotropins. Low-frequency GnRH pulses stimulate a rise of FSH levels early in the menstrual cycle controlling the follicular growth. Meanwhile, high-frequency GnRH pulses produce to a sharp increase of LH just before mid-cycle (also known as LH surge), triggering ovulation and formation of the CL (Richards and Pangas, 2010).

1.2.2.1.1 Follicle stimulating hormone (FSH)

FSH is crucial for maintenance of the normal function of the ovaries to produce oocytes and hormones. FSH is composed by an α-subunit it shares with LH, hCG and TSH and a β-subunit that is specific for FSH. Once in the circulation, hormonal activity is directed through specific cell-surface receptors. Four isoforms have been described (FSHR1, R2, R3 and R4), where R1 and R3 have known biological functions (Sairam and Babu, 2007). FSHR1 is expressed on GCs of growing follicles; meanwhile, FSHR3 is predominant in ovarian surface epithelial (OSE) cells and cancer cells compared to GCs (Hermann and Heckert, 2007; Y. Li *et al.*, 2007).

FSH, besides inducing FSHR, also stimulates the expression of genes such as aromatase cytochrome P450 (CYP19A1), cholesterol side-chain cleavage cytochrome P450 (CYP11A1) and LH receptor (LHCGR) through FSHR (See Figure 1.2). Its effect on cells involves activation of protein kinase A (PKA). FSH attaches to a ligand-specific G protein-coupled membrane receptor in GCs, activates adenylyl cyclase, then this leads to cAMP production and activation of cAMP-dependent protein kinases. Activated PKA is known to phosphorylate specific transcription factors, thereby modulating their activity and regulating gene expression (Alliston *et al.*, 1997).

KO mice for FSH show low levels of FSH and aromatase expression, higher FSHR, lack of oestrus cycling and sterility. The ovarian follicles shown to be arrested in the earlier stages, showing mostly primordial and primary follicles and no preovulatory follicle or CL (Kumar *et al.*, 1997; Burns *et al.*, 2001). These results confirm that the early follicle development is mostly independent of FSH. In the case of transgenic mice that ectopically express high FSH, an increase in oestrogen, progesterone and haemorrhagic cystic ovaries were observed and mice were infertile. Impairment of FSHR also shows alteration of the follicular growth similar to the FSH KO mice, reduction of the steroidogenic hormone production and absence of mature follicles (Dierich *et al.*, 1998; Danilovich *et al.*, 2000).

1.2.2.1.2 Luteinizing Hormone (LH)

LH participates in the development of small to large and pre-ovulatory follicles during the follicular phase and promotes the steroidogenesis in granulosa and theca cells. The LH surge triggers ovulation by promoting the rupture of the pre-ovulatory follicle and the release of the ovum (Dufau, 1998). After ovulation, LH participates in the regulation of CL stimulating the secretion of progesterone.

Lutropin-choriogonadotropin hormone receptor (LHCGR) or LHR is a member of the subfamily of glycoprotein hormone receptors within the superfamily of G protein-coupled receptor (GPCR). Its expression is stimulated by FSH, LH and hCG (human choriogonadotropin) (Casarini *et al.*, 2018). LHCGR has a high affinity for LH and hCG, when LH receptor binds its ligand androgen and progesterone production result, a significant step in the follicle maturation, ovulation and CL formation. Moreover, LHR has been localised in GCs and theca cells of early antral follicles and low expressed in cumulus cells in women ovary (Yung *et al.*, 2014).

1.2.2.2 Steroid hormones role during follicle development

Steroidogenesis is crucial for the synchronisation of follicle growth and oocyte development. Oestradiol and progesterone, both hormones regulated by the gonadotropin hormones, are fundamental for cell proliferation during follicle development.

1.2.2.2.1 Oestradiol (17β-oestradiol, E2)

Oestrogens play a significant role in the growth, differentiation and functioning of the female reproductive system. They not only act in the ovary, uterus and mammary gland but also participate in bone maintenance and cardiovascular system. Oestrogens are mainly produced in the ovaries. They diffuse in and out of all cells but are retained with high affinity and specificity in the target cell by an intranuclear binding protein, the E2 receptor (ER). Once bound by oestrogens, the ER undergoes a conformational change, allowing the receptor to bind with high affinity to chromatin and to modulate transcription of target genes. There are two types of E2 receptors described ERα and ERβ. They produce different biological effects, regulating different genes in response to oestradiol. Both receptors are required for the normal function of the ovary. ERα promotes the expression of genes related to cell survival and proliferation, and it is expressed in theca and interstitial cells in mice. In contrast, ERβ acts as anti-proliferative and pro-apoptotic, and is expressed in GCs of growing follicles (Matsuda *et al.*, 2012; Ciucci *et al.*, 2014).

Oestradiol is the most potent and prevalent endogenous oestrogen. E2 is synthesised by conversion of cholesterol to pregnenolone in the mitochondria. In the last step, the conversion of testosterone into E2 is catalysed by the enzyme aromatase encoded by the CYP19A1 gene. Aromatase catalyses the three precursors (androstenedione, testosterone and 16α-hydroxydehydroepiandrosterone sulphate), into oestrone, oestradiol and estriol, respectively. In humans, the CYP19A1 gene is expressed in the ovary, testis, placenta, adipose tissue, skin and the brain. Oestrogen levels in the blood are primarily maintained by aromatase activity in the ovarian GCs of ovulatory women and adipose tissue of men and postmenopausal women. Mutations in the CYP19A1 in females have given rise to congenital genital ambiguity, pubertal failure and multicystic ovaries (Bulun, 2014).

E2 plays a role in the control of the cyclic pattern of ovarian follicular development, where GCs are the primary source and target of E2. Among the functions of E2 are the promotion of the follicle development causing an increase in the expression of gonadotropin receptors and inhibition of cell apoptosis (Matsuda *et al.*, 2012). Consequently, atretic follicles are characterised by reduced activity of aromatase, leading to a decrease of E2.

1.2.2.2.2 Progesterone

Progesterone (P4) is a steroid hormone, primarily secreted by the ovary giving rise to cyclical fluctuation with the oestradiol. P4 has an important role in the ovulation, implantation and the maintenance of pregnancy. After ovulation, GCs generate the CL and secrete P4 and E2 in the luteal phase of the cycle being modulated by the hormones like FSH, LH, prostaglandins and β-adrenergic (Graham and Clarke, 1997). Cholesterol is the biosynthetic precursor of P4. When required, cholesterol is bound by Steroidogenic acute regulatory protein (StAR) and transported into the mitochondria where the cytochrome P450 side-chain cleavage enzyme (encoded by CYP11A1 gene) converts cholesterol to pregnenolone, the first step for steroid hormone synthesis.

The physiological effect of P4 is mediated by the progesterone receptor (PR). There are two PRs (PRA and PRB) that are encoded by the same gene but generated from two distinct promoters. PR expression is driven by oestrogen bound ER; therefore, ER is permissive for the actions of PR and progesterone. PRA and PRB have different functions, both are crucial for the follicle, but only PRA is essential for ovulation. In mice with a PRA knockout, there was a decrease in oocytes available for ovulation due to a cessation in the development of mature follicles and most mice consequently were infertile. Meanwhile, mice lacking PRB showed a normal function of the ovaries and a regular littler size (Mulac-Jericevic, 2000; Mulac-Jericevic *et al.*, 2003).

Gava et al. analysed the expression of PRA and PRB in mouse ovary and found a different distribution of these receptors through the oestrus cycle. Moreover, they described that PRA was restricted to theca cells of preantral and antral follicles, but not in GCs. However, before ovulation (proestrus stage) PRA was detected in both granulosa and theca cells of the pre-ovulatory follicles, and during CL formation, but not detectable when CL was formed. In contrast, PRB was detected in both granulosa and theca cells throughout follicle growth, including CL formation (Gava *et al.*, 2004).

Besides the nuclear progesterone receptors (PRA and PRB), P4 can interact with non-classical hormone receptors localised in the membrane and cytoplasm: membrane progesterone receptors (mPRs) and the progesterone receptor membrane components (PGRMCs). These receptors have a rapid effect on cell-signalling pathways independently of transcription. There are three types of mPRs encoded by different genes and belonging to the PAQR (Progestin and AdipoQ-Receptor) family: mPRα, mPRβ and mPRγ which are expressed in reproductive, neural and gastrointestinal system respectively (Fernandes *et al.*, 2005). PGRMCs are situated in the cell membrane and participate in processes like the oestrous cycle, pregnancy and cell cycle progression in GCs, breast and ovarian cancer (Valadez-Cosmes *et al.*, 2016).

1.2.3 Alteration of the ovarian function

The ovarian function can be affected by diverse factors which include genetic mutations and diseases (e.g. polycystic ovary syndrome PCO and premature ovarian insufficiency POI) but also the diet. *In-vitro* fertilisation (IVF) studies are a good source of evidence where environmental perturbations and health status can alter the reproduction rate and the probabilities of a successful pregnancy. In these studies, the follicular fluid provides an interesting microenvironment analysis for the development of the follicle. This fluid is a product of both the transfer of blood plasma constituents that cross the blood follicular barrier and of the secretory activity of granulosa and theca cells (Dumesic *et al.*, 2015). Therefore, the analysis of the follicular fluid provides information about changes in the blood associated to the diet.

Alterations of diet and the dysregulation of the tumour suppressor BRCA1 are two examples discussed below that propose the sensitivity of follicle and the oocyte and its consequences in the reproduction outcomes.

1.2.3.1 Alteration of the ovarian function related to diet

Body mass has significant implications on the reproduction status. Women who are overweight or obese have significantly lower live birth rates and higher miscarriage rates compared with healthy weight women (Rittenberg *et al.*, 2011). In animal models, rodents fed with high fat diet have a reduced ovarian reserve (fewer primordial follicles) and subfertility. Moreover, these animals showed an altered oestrus cycle, with a longer diestrus stage (Hussain *et al.*, 2016; Hohos *et al.*, 2017). Other dietary factors that have shown to modulate the ovarian function are the metabolites and cofactors of one-carbon metabolism. Disruption of this cycle through unbalanced levels of folate, homocysteine, vitamin B12 and B6 is associated with reproductive failure (Steegers-Theunissen *et al.*, 2013).

PCO women show high levels of homocysteine in the plasma and follicular fluid (Yaral *et al.*, 2001; Berker *et al.*, 2009). In IVF studies, homocysteine levels in plasma and the follicular fluid have been negatively associated with embryo quality and pregnancy outcome (Boxmeer, Brouns, *et al.*, 2008; Boxmeer, Steegers-Theunissen, *et al.*, 2008; Boxmeer *et al.*, 2009). For that reason, folic acid supplementation has been suggested as a beneficial factor for PCO treatment and IVF patients. In some of the cases of IVF, the intake of folate was associated with positive pregnancy outcomes, higher birth weight and lower risk of abortion (Relton, Pearce and Parker, 2005; Gaskins, Afeiche, *et al.*, 2014; Gaskins, Rich-Edwards, *et al.*, 2014). However, in others, there were no significant changes (Murto *et al.*, 2014).

1.2.3.2 Mutations in BRCA1/2

Among the most common mutations in high-grade ovarian cancer are TP53, BRCA1 and BRCA2 (Bell *et al.*, 2011). Women with BRCA1 mutations reported a lifetime risk of ovarian cancer between 16 to 44% (Cannistra, 2004) and in sporadic ovarian cancer, the BRCA1 expression is downregulated in 90% of the cases (Russell *et al.*, 2000). BRCA1 and BRCA2 are tumour suppressor genes, which encode proteins expressed during the cell cycle, specifically during S and G2 phases. Additionality, both belong to the family of ataxia-telangiectasia-mutated (ATM)-mediated DNA double-strand breaks (DSB) repair genes with an essential role in the safeguarding of DNA integrity (Cortez, 1999). In the ovary, the decrease in expression of the DNA repair genes like BRCA1, ATM and H2AX are associated with the ageing oocyte (Govindaraj, Keralapura Basavaraju and Rao, 2015). H2AX is a variant of H2A protein component of the histone in nucleosomes, which is phosphorylated like BRCA1 for ATM kinases and participates in the preservation of the genome integrity (Burma *et al.*, 2001). A decrease in BRCA1 in the oocyte and GCs of primordial and primary follicles of ageing monkey ovaries was reported together with an increase of DSBs and telomere damage, suggesting that DSBs in GCs increase with age (D. Zhang *et al.*, 2015).

In the healthy adult ovary, BRCA1 is highly expressed in GCs of developing follicles but decreases in large antral or pre-ovulatory follicles. Moreover, the BRCA1 expression is decreased in mural GCs and expression is restricted to cumulus cells (Hu *et al.*, 2005). Studies have shown a negative relationship between BRCA1 expression and oestradiol synthesis through aromatase P450 (CYP19A1), suggesting an additional role for BRCA1 in GCs, besides DNA repair. Consistent with this, mutation of BRCA1 in human and mice have shown increased levels of oestradiol and increased endometrial stromal cell proliferation (Chand, Simpson and Clyne, 2009; Yen *et al.*, 2012). Hong et al. showed that adult mice that do not express BRCA1 in their GCs have a more extended proestrus phase, increasing the time of exposition to high levels of oestrogen (Hong *et al.*, 2010).

Dysregulations of DNA repair, cell cycle checkpoint control and the production of the oestradiol associated with BRCA1 disruption can have consequences on the proliferative role of GCs, and therefore affect the quality of the ovarian follicle and the oocyte. This has been suggested to be the reason why a low number of mature oocytes was found in women with the BRCA1 mutation in response to ovarian stimulation (Derks-Smeets *et al.*, 2017).

1.3 Early embryonic development

The preimplantation period around one week in human and four days in rodents shows a high sensitivity to environmental signals. During this period, the embryo despite the small number of cells displays a high level of plasticity and self-organisation, and it is also vulnerable by its regulated supply and environmental status. Therefore, any disturbance around this period could have consequences in the foetal outcome (Fleming *et al.*, 2018).

Embryogenesis is the process where the fertilised oocyte goes through several divisions (cleavage) arising new cells (blastomeres) and to form a blastocyst which then implants in the uterus wall and to subsequently form the foetus (Figure 1.3).

Fertilisation triggers the completion of meiosis in the oocyte, followed by the formation of the 1-cell embryo containing the haploid paternal and maternal pronuclei. Each pronucleus undergoes DNA replication before starting mitosis to produce the 2-cell embryo containing two diploid zygote nuclei. Following two additional cleavages, the 8-cell embryo undergoes compaction (Hamatani *et al.*, 2004). During this process, the embryo (now called morula) increases cell-cell contact and polarises along the radial axis of the embryo to end with a compacted embryo with polarised outer (apical) and non-polar inner (basolateral) cells (Gasperowicz and Natale, 2011; Frum and Ralston, 2015).

The first cell fate decision starts around 16-cell stage embryo where blastomeres differentiate to generate two distinct cell lineages: trophectoderm (TE) and inner cell mass (ICM) (Chazaud and Yamanaka, 2016) (Figure 1.3). The TE cells have the characteristics of transporting epithelium, apical-basal polarity, intercellular junctions, and transport of ions across the TE. These features make TE essential for the blastocyst formation, and processes such as cell polarisation, compaction, and blastocoel formation at E3.0-3.25 in mouse embryos. On the other hand, the ICM cells are pluripotent and undifferentiated due to the expression of pluripotency transcription factors (such as OCT4 and NANOG).

Subsequent cleavage divisions and cavitation (blastocoel formation) lead to the formation of the blastocyst: a > 32-cell embryo with a developing fluid-filled internal blastocoel (Figure 1.3) followed by the second cell fate decision. Lineage studies indicate that PE cells form the yolk sac and EPI the foetus and extraembryonic mesoderm (Strumpf *et al.*, 2005). Both cell fate decision periods require the specific interaction of transcription factors that facilitate the differentiation and maintenance of cell identity; these are called lineage markers and define the character of each type of cells.

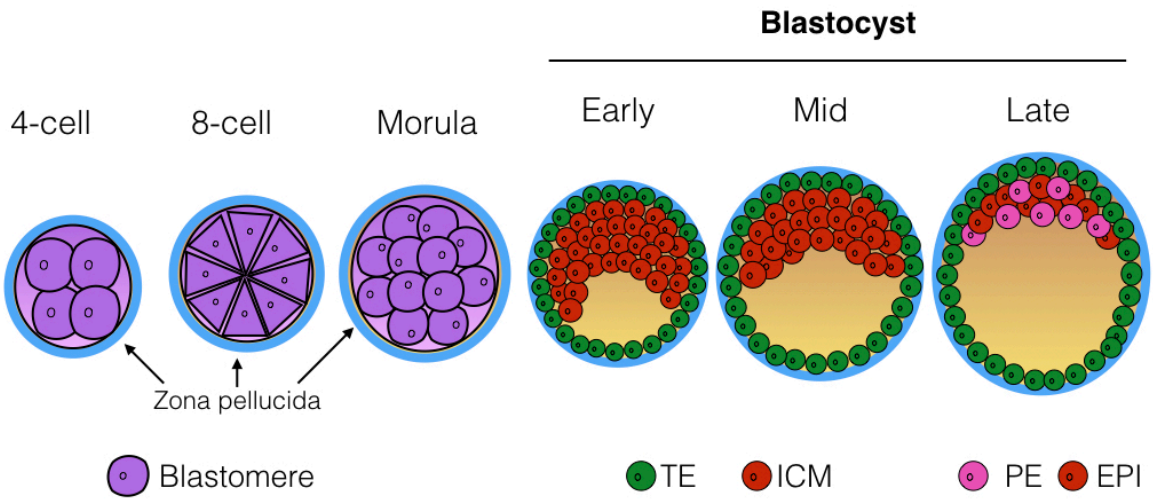

Figure 1.3 Embryogenesis before implantation

Development of the mouse embryo since 4-cell stage (E2.0) until the late blastocyst stage (E3.5).

1.3.1 Lineage markers during preimplantation period in the mouse embryo

The process of differentiation in the blastocyst is characterised by diverse patterns of gene expression between the different lineages. At 8-cell stage embryo, the polarity is regulated by the HIPPO signalling pathway, which also determines the patterns of expression of specific transcription factors in each cell lineage (Gasperowicz and Natale, 2011; Frum and Ralston, 2015). In the outer-polarised cells (TE cells) HIPPO is repressed allowing YAP/TEAD4 complex to be active and promote the expression of TE determination genes such as CDX2 and repress ICM genes such as SOX2 (Gasperowicz and Natale, 2011; Frum and Ralston, 2015).

In mouse embryos, in both cell fate decisions, cells acquire the expression of specific transcription factors in each cell lineage. OCT4, SOX2 and NANOG are enhanced in ICM and have a role in the promotion of the pluripotency avoiding differentiation, whereas CDX2 and EOMES are upregulated in TE cells and GATA4 and GATA6 are present in the EPI cells (Chazaud and Yamanaka, 2016). Each transcription factor has a role in the maintenance of specific cell characteristics and the survival of each cell lineage (See Figure 1.4).

1.3.1.1 Pluripotency markers of ICM

The POU domain transcription factor OCT4 (POU5F1) is expressed throughout the early embryo (4-cell stage) until the blastocyst stage where it is restricted to ICM cells. *Oct4* is essential for the pluripotency of embryonic stem (ES) cells, its reduction leads to cell differentiation and induction of TE genes such *Cdx2* with the formation of cells with TE-phenotype (Niwa, Miyazaki and Smith, 2000; Hough *et al.*, 2006). Maternal-zygotic deletion of *Oct4* leads to an increased level of *Nanog* in the embryo, suggesting a modulating role for *Nanog* expression in maintaining ICM cell differentiation. Moreover, deletion of *Oct4* during the morula to blastocyst stage also affects PE formation, showing decreased levels of *Sox17* (Le Bin *et al.*, 2014).

SOX2 (SRY-related HMG-box gene 2) is a transcription factor detected at the early 2-cell stage nuclei but is later be mainly restricted to ICM and subsequently in EPI cells of the blastocyst. Sox2 knock-out embryos form abnormal ICMs and die after implantation (Avilion *et al.*, 2003). Despite described as a pluripotent factor restricted to ICM cells, SOX2 has also been described in cytoplasm and nuclei of TE cells (Keramari *et al.*, 2010). SOX2 binds with OCT4 to activate the transcription of pluripotent genes in ES cells regulating *Nanog* expression. *Sox2* transcription is regulated by an enhancer containing a composite Sox-Oct cis-regulatory element that SOX2 and OCT4 bind in a combinatorial interaction (Chew *et al.*, 2005). By depleting SOX2 (maternal and zygotic source) embryos in culture arrest at the morula stage and fail to form the cavity and do

not progress beyond 30 cells (Keramari *et al.*, 2010). The embryos also showed downregulation of TE markers such as *Tead4, Cdx2, Yap* and *Eome*s and extracellular signal factors such as *Fgfr2* and *Fgf4* proteins, but no change in *Oct4*, *Nanog* or *Gata6*, suggesting that SOX2 plays a role in TE differentiation control.

NANOG is a homeodomain transcription factor, which is expressed from the early morula stage, becomes restricted to the ICM in the blastocyst, to later be exclusively found in EPI cells (Komatsu and Fujimori, 2015). NANOG is a pluripotency and ES cell self-renewal factor and is regulated by the binding of the same composite Sox2-Oct cis-regulatory element that regulates SOX2 and OCT4, in its proximal promoter (Rodda *et al.*, 2005). Reduction of *Nanog* in ES cells is correlated with cell differentiation and the induction of extraembryonic endoderm genes such as *Gata4* and *Gata6* (Hough *et al.*, 2006). Meanwhile, in embryos, disruption of *Nanog* inhibits differentiation of ICM into EPI and possibly PE due to a decrease of *Gata4* (PE marker) expression at E3.5 (Mitsui *et al.*, 2003; Messerschmidt and Kemler, 2010).

1.3.1.2 Transcription factors markers for TE cells

CDX2, caudal-like transcription factor, was primarily described to be ubiquitously present in all blastomeres at 8-cell stage and required for TE differentiation. However, mutant embryos for CDX2 showed normal polarisation (Strumpf *et al.*, 2005; Ralston and Rossant, 2008), which suggested the presence of a maternal pool of CDX2 which leads in the process of compaction and cavity formation. Indeed, depletion of maternal and zygotic CDX2 leads to an arrested development before blastocyst cavitation, altered cell polarity markers and even a failure in compaction (Jedrusik *et al.*, 2010).

Among the roles of CDX2 is the promotion of TE determination genes such as Eomes (Eomesodermin), a T-box transcription factor, and the repression the expression of ICM genes (such as OCT4 and NANOG) in TE cells after blastocyst formation (Figure 1.4). A loss-of-function of CDX2 alters the expression of *Oct4* and *Nanog,* with these pluripotency factors now being expressed in TE cells instead of being restricted to the ICM (Rossant and Tam, 2009). OCT4, NANOG and CDX2 are regulated reciprocally; the three can bind to Cdx2 and Nanog loci in undifferentiated mouse ES cells. Nanog knockout embryos showed low *Cdx2* in OCT4-positive ICM cells of $Nanog^{-/-}$ embryos, but the complete absence of Cdx2 in ICM of $Nanog^{+/+}$ or $Nanog^{+/-}$ (Chen *et al.*, 2009). Induction of *Cdx2* in mES cells decreased *Oct4* and *Nanog* expression through modification in the histones and loss of chromatin accessibility at both *Oct4* and *Nanog* regulatory elements (Carey *et al.*, 2014)

TEAD4 is an early transcription factor expressed in all blastomeres (IMC and TE cells), which plays a key role in the regulation of TE lineage development. Deletion of TEAD4 in embryos affects the transcription factor patterns of ICM/TE cells, decreasing CDX2 and increasing OCT4. Moreover, the cells fail to form blastocoels and the embryos die before implantation (Nishioka *et al.*, 2008). TEAD4 regulates CDX2 expression through YAP1 (Yes-associated protein 1) and HIPPO signalling (Chen *et al.*, 2010). In inner cells of the embryo YAP1 is phosphorylated and excluded from the nucleus, TEAD4 remains inactive, and these cells adopt an ICM fate. In the outer cells, the non-phosphorylated YAP1 remains in the nucleus allowing TEAD4-mediated CDX2 expression and promoting TE fate (Nishioka *et al.*, 2009) (See Figure 1.4).

1.3.1.3 Transcription factors for EPI and PE fate

The second cell fate decision occurs in mouse embryos at E3.5-3.75 where ICM differentiates into EPI and PE cells, each with respective markers. NANOG and SOX2, previously key markers of ICM remain restricted to EPI cells. Meanwhile, PE cells require the expression of GATA6/4 and SOX17 among other transcription factors. This segregation is related to NANOG and GATA6 expression which are expressed in a random ("salt and pepper") pattern in E3.5 mouse embryos. This pattern is regulated by FGF signalling, where EPI cells express TGF4 ligand and PE the FGFR2 receptor, essential in the mutual repression of NANOG and GATA6. In embryos null for *Fgf4* or *Grb2* (FGF4 downstream target) all ICM cells adopt an EPI identity expressing NANOG (Chazaud *et al.*, 2006) (See Figure 1.4). Null-SOX2 or NANOG mutant embryos show downregulation of GATA6/4 and the SOX17 expression suggesting that SOX2 and NANOG are necessary for the initial recruitment of PE genes at E3.5 (Frankenberg *et al.*, 2011; Wicklow *et al.*, 2014).

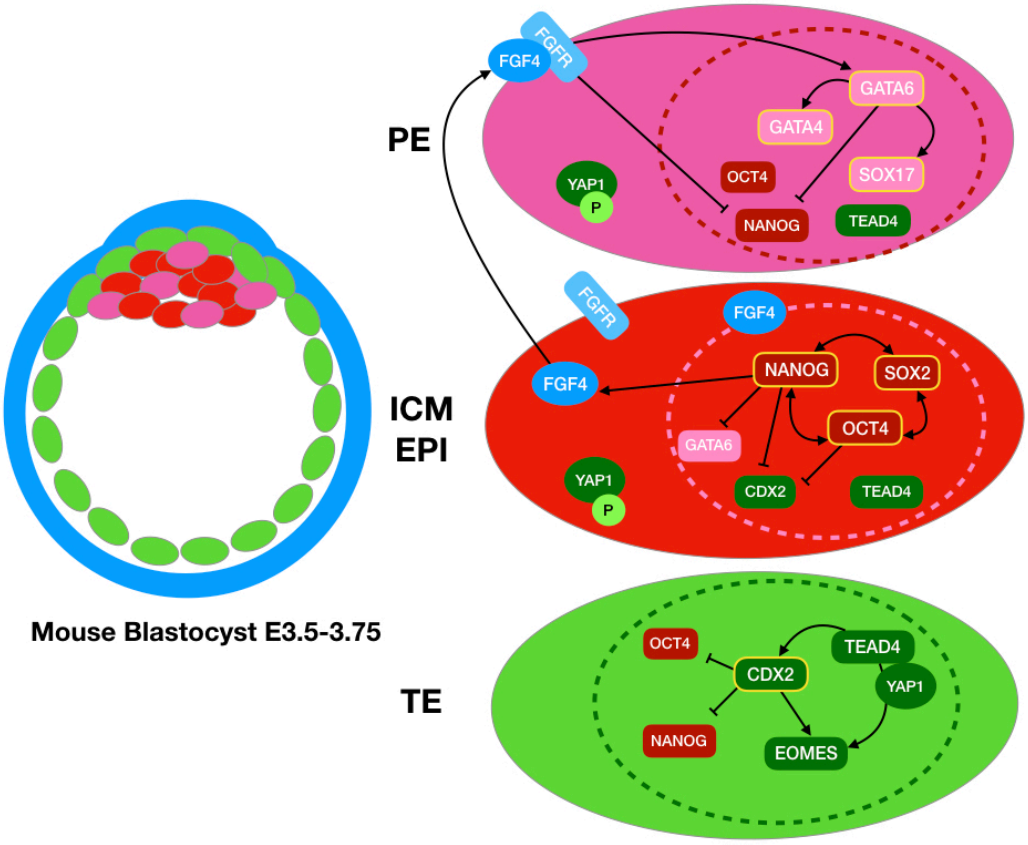

Figure 1.4 Regulation of TE, ICM (EPI) and PE markers

In TE cells TEAD4 with YAP1 upregulates CDX2 and EOMES, meanwhile CDX2 represses the expression of pluripotency factors. ICM cells are characterised by the presence of OCT4, SOX2 and NANOG, where CDX2 is downregulated and YAP1 is phosphorylated and present in the cytoplasm. ICM segregates into EPI (NANOG positive) and PE (GATA6 positive) cells, which is regulated by FGF signalling.

1.3.2 Alterations during preimplantation

Adverse developmental processes experienced before conception and during preimplantation can affect embryo and foetal development with further consequences for the long-term health of the offspring (Fleming *et al.*, 2004; Watkins and Fleming, 2009). Studies of *In-vitro* fertilisation (IVF), have shown that the composition of the media that embryos are cultured in may have an impact on the quality of embryos and influence negative foetal outcomes (Dumoulin *et al.*, 2010). Moreover, the manipulation of the embryo by IVF has been shown to have consequences in later life, for example at 8 and 18 years old, children conceived by IVF showed higher blood pressure and glucose levels compared to controls (Ceelen *et al.*, 2008).

Maternal nutrition around conception is also an essential factor that can modulate embryo development. Low protein diet (LPD) studies in mice have shown that exposure during the pregnancy or exclusively during preimplantation, not only increases birth and placenta weight, but also increases the blood pressure of mice in adulthood (Kwong *et al.*, 2000; Watkins, Ursell, *et al.*, 2008; Watkins, Wilkins, *et al.*, 2008). Additionality, maternal undernutrition during gestation or preimplantation in sheep has been shown to delay myogenesis in the foetal quadriceps (Lie *et al.*, 2014, 2015). These studies suggest that the periconceptional period is highly sensitive to the environment, and alterations in this period lead to long-term consequences on future health. However, the effects of the maternal diet around conception can be detected earlier, for examples at the blastocyst stage. Rat embryos (E4.25) exposed to LPD during the preimplantation period showed a significant reduction in cell numbers (ICM and TE lineage), suggesting a slower rate of cellular proliferation associated with a lower mitotic index (Kwong *et al.*, 2000). Moreover, the effects were sex-specific, showing that male blastocysts, and not female, from LPD group showed a decrease of the expression of imprinted genes from the blastocyst stage onwards (Kwong *et al.*, 2006). The effects observed in the blastocyst associated with changes in the cell lineage after maternal diet modification were accompanied by changes in the expression of genes related to proliferation and differentiation processes. The mechanism by which early life environment may modulate such changes has been suggested to involve the altered epigenetic regulation of genes.

1.4 Epigenetic mechanisms

The mechanisms by which early life nutrition may affect future life are not fully established, however it has been suggested to involve the altered epigenetic regulation of genes. Epigenetics is the study of heritable changes in gene expression that occur without changes in the original DNA sequence (Bishop and Ferguson, 2015). Epigenetic processes control gene activity in a tissue-specific manner, and the central mechanisms are DNA methylation, histone modifications and non-coding RNAs. DNA methylation at cytosine-guanine (CpG) dinucleotides is a major epigenetic modification of the genome; this plays an important role in regulating gene expression and is essential for normal mammalian development. DNA methylation works in concert with histone modification to regulate gene expression.

One good model which demonstrates the importance of epigenetics and its effect on phenotype is the Agouti mouse. The Agouti viable yellow allele (A^{vy}) determines the colour of the mouse coat. Hypomethylation of this metastable allele drives ectopic agouti expression, causing a yellow coat colour instead of the black wild type. The administration of maternal methyl-supplemented diet increased the methylation A^{vy}, and changes the phenotype of offspring leading to a darker mouse coat (Wolff *et al.*, 1998). DNA methylation at A^{vy} is established in the early embryo development and then maintained throughout life. However, offspring of a dam carrying A^{vy} have variegated of coat colour, showing a somatic reversion of the phenotype. This is explained by parental imprinting effect. The yellow coat and the methylated A^{vy} were given by a maternal inheritance suggesting a complete erasure of the epigenetic modification in the male germline but incomplete in the female germline (Morgan *et al.*, 1999). Waterland et al. tested the transgenerational inheritance of maternal methyl-supplemented diet at A^{vy}. In a three-generation cumulative exposure study indicates that diet-induced hypermethylation at A^{vy} is not inherited transgenerationally suggesting that environment might support the maintenance of epigenetic information in the developing germline, but the allele is resistant to the environmentally induced acquisition of information (Waterland, Travisano and Tahiliani, 2007).

Genomic imprinting refers to epigenetic variations that involve heritable changes in the expression of genes dependent upon allelic inheritance. In this process, maternal and paternal genomes become distinguishable from each other as a result of gamete-specific differential methylation (Biliya and Bulla, 2010). All imprinted genes are essential for normal development, foetal growth, nutrient metabolism and adult behaviour. Imprinted genes are usually clustered and contain CpG-rich regions that are DNA-methylated only on one of the two parental chromosomes (differentially methylated regions, DMRs) (Li and Sasaki, 2011).

1.4.1 DNA methylation

DNA methylation is a modification of the chromatin that consists of the addition of the methyl group to the cytosine residues in CpG dinucleotides, which results -usually in gene silencing. This modification is heritable through mitosis, allowing a directed differentiation process and the propagation of lineage-specific markers over many cell divisions (Messerschmidt, Knowles and Solter, 2014). In mammals, there are two episodes where the methylation is resetting: during germ cell development, DNA methylation resets parent-of-origin based genomic imprints and restores totipotency to gametes; and between fertilisation and preimplantation, when the methylation marks are removed from the paternal and maternal DNAs (Santos and Dean, 2004).

1.4.1.1 DNA methylation during embryogenesis

The epigenetic imprints established in the germline are maintained throughout the development and adult life, therefore the methylation imprints at DMRs escape from this epigenetic reprogramming. To maintain or establish DNA methylation, DNA methyltransferases (DNMTs) are indispensable. DNMT1o (oocyte-specific isoform) maintains the imprints at the single cell stage in preimplantation development. DNMT1 knockout mice showed that a lack of zygotic DNMT1 induces demethylation of imprinted *loci* in the blastocyst, indicating that DNMT1 is a prerequisite for the maintenance of methylation imprints in the preimplantation embryo (Hirasawa *et al.*, 2008). Moreover, the foetus of homozygous null DNMT1 females die in the late part of gestation (Howell *et al.*, 2001).

During the preimplantation period, the embryo after the fifth cell cycle undergoes the first cell differentiation process accompanied by *de novo* DNA methylation. This process mediated by DNMT3A and DNMT3b gives rise to stable silencing of genes related to the maintenance of pluripotency and the establishment of the first two cell lineages, ICM and TE. Both methyltransferases participate in the methylation of *Oct4* and *Nanog* during embryonic cell differentiation, and a disruption of either gene leads to a drastic reduction in the methylation level and increased expression of OCT4 and NANOG (J.-Y. Li *et al.*, 2007). Therefore, the level of methylation in the ICM differ from TE, showing a higher level in ICM cells (Santos *et al.*, 2002; Shi and Wu, 2009).

1.4.1.2 DNA methylation in the ovary

Methyl donor diets (including high amounts of methionine, choline or folate) provides S-adenosylmethionine (SAM), the primary methyl donor for most methylation reactions, and thereby modify DNA methylation. Modifications of folate intake in humans and mice have demonstrated the alteration of the DNMTs in a variety of tissues including cervix and endometrial cells (Piyathilake *et al.*, 2008; Ding *et al.*, 2012). In the ovary, DNMTs have been detected in the nuclei of mouse oocytes, and their expression decreases with age, the same as the DNA methylation status (Yue *et al.*, 2012; Petrussa, Van de Velde and De Rycke, 2014). In the ovary of PCO gilts with high levels of homocysteine in the follicular fluid increased expression of Dnmt1 was found in the oocyte together with changes in the methylation status (Jia *et al.*, 2016).

1.4.2 Histone modifications

DNA in the cell is wrapped around a set of histone proteins. Histones are important both to reduce the effective size of the genome but also for gene regulation. The tail domains of the histone which are rich in positively charged amino acids interact with the negatively charged DNA (Andrews and Luger, 2011). These tails are subject to a range of modifications which include acetylation, methylation, phosphorylation, ubiquitylation and sumoylation (Kouzarides, 2007). Histone modification either affects the chromatin structure or provides a binding site for effector proteins which in turn regulate gene expression. Among these modifications are the trimethylated residues Lys9 and Lys27 of histone H3 (H3K9me3 and H3K27me3, respectively) which are the most well-studied histone marks of a repressive chromatin state. Acetylated histones H3K9ac or trimethylated H3K4me3 are the most well-known markers of an active chromatin state (Teperek-Tkacz *et al.*, 2011).

1.4.2.1 Polycomb repressive complex

Polycomb group (PcG) protein are constituted by a large and conserved group of proteins which function as epigenetic modifiers and regulate transcription (See Figure 1.5). PcGs play a central role in cell differentiation and the maintenance of the cell identity (Di Croce and Helin, 2013). The two major complexes are: polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2/3 (PRC2/3), both associated with chromatin condensation. PRC1 catalyses the monoubiquitylation of histone H2A (H2AK119), and it is constituted by RING1B (also known as RING2/RNF2), which has the E3 ubiquitin ligase activity and one component of ring finger protein (PCGF1-6). Canonical PRC1 is recognised by the presence of one chromobox protein (CBX), which

binds the H3K27me3, and one Polyhomeotic (PH) homolog protein (HPHC) (See Figure 1.5) (Entrevan, Schuettengruber and Cavalli, 2016). Meanwhile, PRC2 which participates in cell proliferation, stem cell plasticity, carcinogenesis and X inactivation, contain an embryonic ectoderm development (EED), suppressor of Zeste 12 homolog (SUZ12), in combination with adipocyte enhancer-binding protein 2 (AEBP2) and retinoblastoma-binding protein p48 (RbAp48) and either the histone methyltransferases (HMTases) EZH1 or EZH2 (See Figure 1.5) (Chittock *et al.*, 2017).

PRC1 and PRC2 are typically co-localised at target sites throughout the genome. However, both are intimately interconnected, as the enzymatic activity of each complex influences the other occupancy on chromatin. The recruitment of PRC2 to catalyse H3K27me3 is subsequently recognised by the CBX present in PRC1, leading to H2AK119ub1 placement and polycomb chromatin domain formation (See Figure 1.5) (Blackledge, Rose and Klose, 2015).

The PRC2 core subunit EZH2, EED and SUZ12 participate in the ovary and embryo development. In the case of PRC1, the BMI1 (PCGF4) is also expressed in the ovary. Both groups of subunits and their relation to the ovary and the embryo are described below.

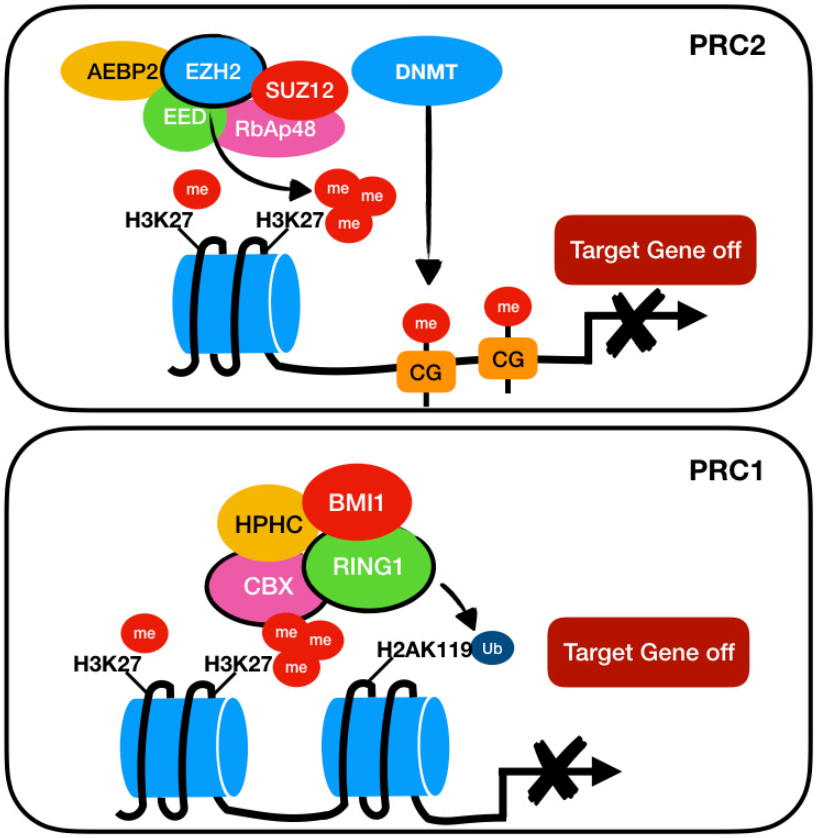

Figure 1.5 Polycomb repressive complex (PRC) 1 and 2 and their activity

EZH2 part of PRC2 catalyses the trimethylation of H3K27 which also recruits DNMTs and methylation of the CpG to silence the gene expression. In PRC1, CBX binds to H3K27me3 and BMI1 (PCGF4) binds to the RING1A/B ubiquitinates H2AK119 also repressing the transcription of the target gene.

1.4.2.2 PRC1: BMI1

B lymphoma Mo-MLV insertion region 1 homolog (BMI1) is the best-known oncogene (PCGF4) that pairs with the RING1A/B subunit and mediates the E3 ligase activity in PRC1 (See Figure 1.5). The ability of BMI1 and PRC1 to bind to chromatin is dependent on PRC2 function, and it has been proposed that this is primarily achieved via binding to the H3K27me3 mark (Bracken *et al.*, 2007). BMI1 participates in gene silencing by regulating chromatin structure on genes related to stem cell fate decisions, as well survival genes, stem cell-associated genes and, anti-proliferative genes like Ink4a/Arf locus (Park, Morrison and Clarke, 2004). The Ink4a locus, which encodes $p16^{INK4A}$ and $p14^{ARF}$ (p19 in mouse) genes is associated with senescence and apoptosis respectively, BMI1 deficiency is associated with an increase of $p16^{INK4A}$ in neural stem cells, reducing the rate of proliferation (Molofsky *et al.*, 2003; Park *et al.*, 2003). Additionality, BMI1 knock-out mice showed

impaired mitochondrial function, an increase in reactive oxygen species and engagement of the DNA damage response pathway (Liu *et al.*, 2009). Certainly, monoubiquitination of BMI1 is induced at sites of DNA breaks damage, associating the role of BMI1 in DNA repair response (Ginjala *et al.*, 2011). Accumulation of BMI1 at DNA lesion immediately after UVA-micro-irradiation together with BRCA1 and H2AX (both DNA damage markers) was observed in HeLa cells (Suchánková *et al.*, 2015).

In the ovary, BMI1 is expressed in a high percentage of ovarian epithelial cells (OEC), and it is associated with advanced cancer stage and higher grades of carcinomas (Abd El Hafez and El-Hadaad, 2014). However, recently BMI1 has been studied due to its interaction with the P4 receptor (PR) regulating uterine P4 responsiveness in mice (Xin *et al.*, 2018). Moreover, this role has been conserved from mouse to human, since there was a close association with loss of BMI1 and PR sensitivity in the endometrium of miscarrying women in the same study.

1.4.2.3 PRC2: EZH2 and EED

Enhancer of zeste homolog 2 (EZH2) exhibits a SET domain, which functions as HMTase on H3K27me, an important mark in regulating cell differentiation. PRC2 activity results to be necessary for cell differentiation repressing developmental control genes (Margueron and Reinberg, 2011). Additionally, EZH2 can operate as a recruitment platform for DNA methyltransferases and is essential for DNA methylation by recruiting DNMTs to specific DNA regions (See Figure 1.5) (Viré *et al.*, 2006). EZH2 is negatively regulated by phosphorylation via PI3K/AKT signalling pathway (Cha, 2005). A number of hormones such as E2, and androgen receptors (ER and AR respectively), and LH hormone (See Figure 1.6) can influence EZH2 activity through this mechanism (Cha, 2005; Bredfeldt *et al.*, 2010; Ma *et al.*, 2017). Moreover, among the targets of PRC2 and EZH2 are the differentiation-specific genes such as members of the GATA, SOX, FOX, POU family and components of Wnt, TGF-β, NOTCH, FGF and retinoic acid signalling (Simon and Lange, 2008).

Overexpression of EZH2 has been observed in some cancers such as breast, prostate, and ovarian. Immunohistochemistry analysis of human ovarian carcinoma tissue samples showed an upregulation of EZH2 as compared with normal ovaries, and its overexpression has been associated with advanced stages of ovarian cancer and poor prognosis (Lu *et al.*, 2010; Rao *et al.*, 2010; Guo *et al.*, 2011; Fu *et al.*, 2015). Moreover, an inverse correlation was detected between EZH2 and the CDK inhibitor p57 in ovarian tissue, silencing EZH2 expression by shRNA in ovarian cancer cell increased of p57 and inhibited cell proliferation and decreased the cells ability to migrate (Guo *et al.*, 2011). In cancer cell lines, EZH2 has been suggested to promote cell invasion

and/or metastasis. Rao *et al*. suggested that EZH2 might regulate cell migration/invasion via the regulation of E-cadherin and/or TGF-β1 expression (Rao *et al.*, 2010). In other studies, depletion of EZH2 in EOC cells increased BRCA1 protein in the nucleus, whereas cytoplasmic protein was decreased, suggesting that depletion of EZH2 resulted in nuclear translocation of BRCA1 protein (Li *et al.*, 2014). Wang et al., have shown that BRCA1 interacts with EZH2 in mES cells and human breast cancer cells. A decrease of BRCA1 increases EZH2 and H3K27me3 blocking differentiation and enhancing breast cancer cell migration and invasion (See Figure 1.6) (Wang *et al.*, 2013).

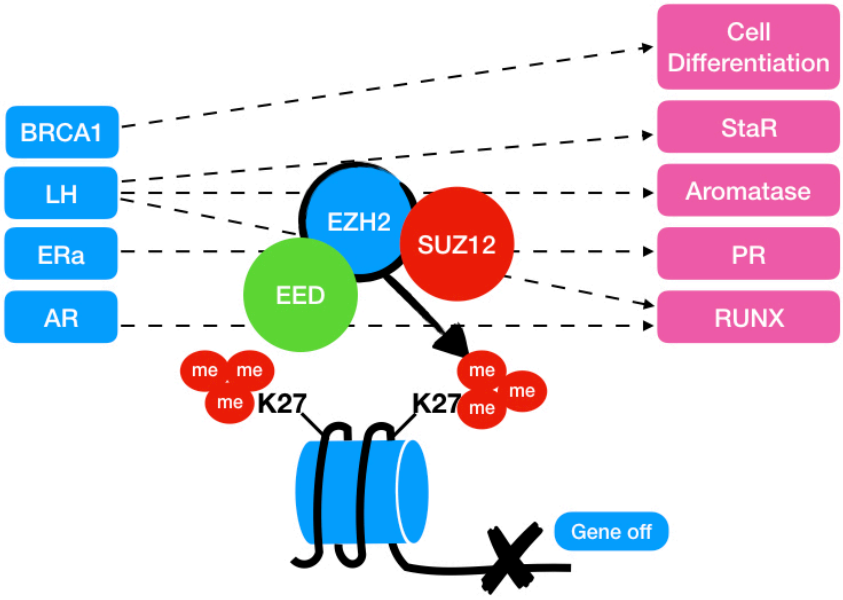

Figure 1.6 Regulation of EZH2

EZH2 is regulated by diverse factor (left, blue boxes) with modulation of the expression of different target genes (right, pink boxes), which modulate differentiation and cell proliferation.

During mouse embryogenesis, the PRC2 also has a pivotal role. H3K4 and H3K27 tri-methylation appear from the 2-cell stage onwards, and at the blastocyst stage H3K27me3 can be detected in ICM and TE but is predominant in the ICM cells (Dahl *et al.*, 2010; Paranjpe and Veenstra, 2015). Embryos from mice with knockout of PRC2 components die after implantation stage and display severe defects during gastrulation and a decrease of the epigenetic plasticity of pluripotent epiblast (Erhardt *et al.*, 2003; Aloia, Di Stefano and Di Croce, 2013). In embryos, *Ezh2* expression peaks during the zygote stage, then gradually decreases from the 2-cell stage, exhibiting an inverse pattern when compared with *Oct4* and *Sox2*. Moreover, further studies showed that *Ezh2* expression was markedly suppressed by OCT4 and SOX2 alone in a dose-dependent manner (Wu *et al.*, 2014).

EED, also part of PRC2, is required for EZH2 and SUZ12 stability. EED binds to H3K27me3 producing a positive feedback loop enhancing the enzymatic activity of EZH2 (Margueron *et al.*, 2009). EED-deficient ES cells display a disruption of PRC2 and H3K27 methylation in PRC2 target genes, increasing GATA3, GATA4 and GATA6 expression in mutant EED cells (Boyer *et al.*, 2006). During embryogenesis, EED participates in the maintenance of the paternal X chromosome inactivation by the histone hypoacetylation and the repression of the chromatin structure (Wang *et al.*, 2001). EED and EZH2 are both co-localised on the X chromosome in TE cells, however, they are not lineage exclusive (Ferguson-Smith and Reik, 2003).

In mouse ES cells, EED was detected as a downstream target of STAT3 and OCT4. Both transcription factors bind to the EED promoter decreasing EED activity and inducing loss of the H3K27me3 mark on differentiation factors genes (Ura *et al.*, 2008). EED and PRC2 are necessary for differentiation processes in ES cells, OCT4-bound genes such as NANOG and SOX2 become deregulated in EED-deficient ES cells (Obier *et al.*, 2015).

The evidence presented above suggests that both DNA and histone methylation play a fundamental role in cell specification and differentiation. As one-carbon metabolism, which provides the methyl groups for virtually all these methylation reactions is mostly dependent upon dietary methyl donors and cofactors (Glier, Green and Devlin, 2014; Friso *et al.*, 2017). It has been suggested that variation in the dietary intake of methyl donors and cofactors (choline, betaine, folate and methionine) may influence DNA and histone methylation with long-term consequences for health (Pauwels, Ghosh, Duca, Bekaert, Freson, Huybrechts, Langie, *et al.*, 2017). In this project, the synthetic form of folate, folic acid was examined concerning its role in the modulation of the proliferation and differentiation processes and is discussed below.

1.5 One-Carbon Metabolism

One-carbon metabolism is integrated for pathways centred on folate metabolism (folate cycle), the remethylation cycle of methionine (methionine cycle) and the transsulfuration pathway of homocysteine. These pathways provide methyl groups for biological methylation reactions of proteins, phospholipids and nucleic acid. Environmental, nutritional and endocrine alterations can affect one-carbon metabolism which may result in changes in cell function, metabolism, growth and proliferation (Kalhan, 2016; Friso *et al.*, 2017).

Diets with altered levels one-carbon metabolites have shown consequences in female reproduction. A study in women showed that maternal biomarkers of one-carbon metabolism could have a role in the embryonic development at the first trimester of the pregnancy (Parisi *et al.*, 2017). Moreover, dietary interventions around conception have shown changes in DNA methylation patterns of offspring and program the long-term consequence in their health in adulthood (Ikeda *et al.*, 2012).

1.6 Folate and folic acid

Folate is a water-soluble vitamin and present naturally in food such as fruits and leafy green vegetables. Folate participates in one-carbon metabolism involved in the biosynthesis of purines, thymidylate and methyl groups with the participation of other cofactors such as choline and betaine (See Figure 1.7) (Ducker and Rabinowitz, 2017). Mammals cannot synthesise folate; therefore diet or supplementation is the only source of this vitamin. The role of folate in DNA methylation through the methionine/homocysteine cycle and the participation in cell cycle and proliferation through its role in DNA synthesis has been most studied (Guéant *et al.*, 2013).

The bioavailability of folate as polyglutamate is lower compared to the monoglutamate form, the latter approaching 100% under fasting conditions (Eichholzer, Tonz and Zimmermann, 2006; Winkels *et al.*, 2007). Folic acid (FA), the oxidised form also known as pteroylmonoglutamic acid, is more stable to temperature and pH conditions than folate, which has led to its use in supplementation and food fortification (Scaglione and Panzavolta, 2014).

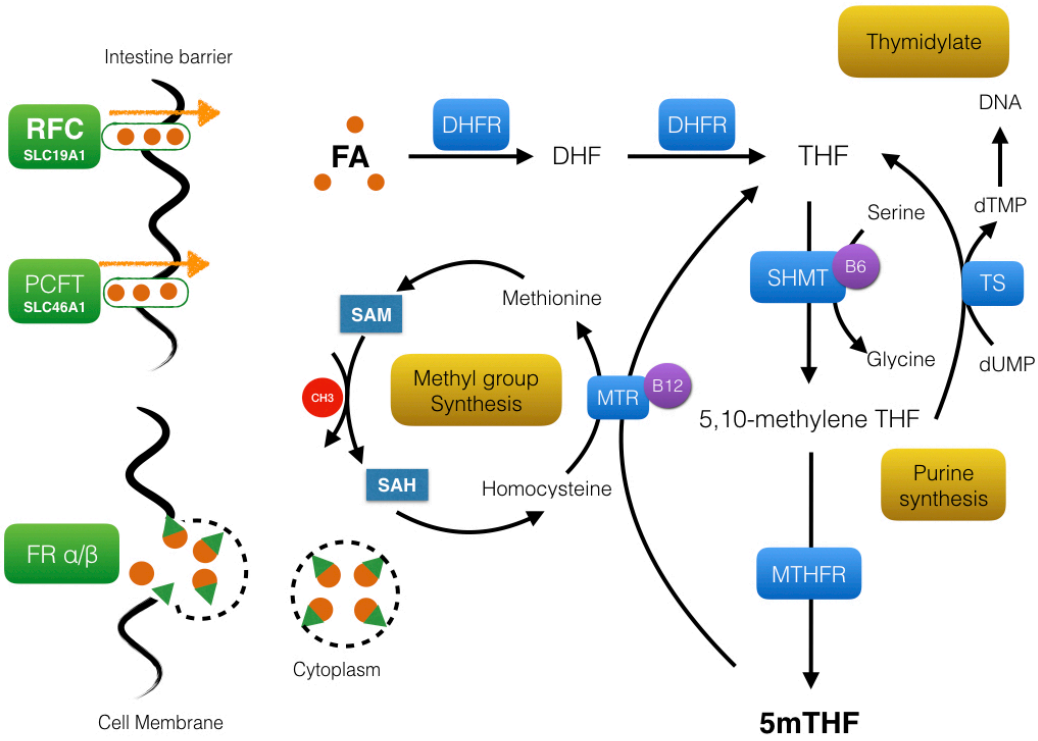

Figure 1.7 Folate and FA absorption and main functions

Simplified model of absorption of folate and FA (folic acid) in the intestinal barrier and cell membrane and the main routes of the one-carbon metabolism.

1.6.1 Folate absorption and metabolism

Folate is absorbed and incorporated into cells through the members of the superfamily of solute carriers RFC and PCFT, and the folate receptors (FRs). The reduced folate carrier (RFC or SLC19A1) is the major transporter of folates to the systemic tissues at their ambient neutral pH with low affinity for FA. This transporter mediates cellular uptake of reduced folates (5mTHF) and is widely expressed in gut, liver, pancreas, kidney and colon.

The proton-coupled folate transporter (PCFT or SLC46A1) is a low-pH-dependent folate transporter with a similar affinity for reduced (5mTHF) and FA. Besides the intestine, PCFT has been identified in liver, kidney, colon, spleen and placenta. At the cell surface, folate receptors (FRs) are expressed in the cell membrane-anchored by a glycosylphosphoinositol domain. FRα is expressed on the membrane of epithelial tissues, specifically in the apical brush-border of renal tubular cells, retinal pigment epithelium, and the choroid plexus. In the case of FRβ, the receptor is present in the placenta and hematopoietic tissues. Both receptors, transport folate through receptor-mediated endocytosis (Zhao *et al.*, 2011).

The absorption of folate and FA is a saturable process. Therefore high FA intake induces a significant decrease in intestinal and renal folate uptake due to downregulation of the expression of all transporters (Ashokkumar *et al.*, 2010; Dev, Ahmad Wani and Kaur, 2011). On the contrary, low intake of folate leads to an increase of intestinal folate uptake by increasing the number of transporters RFC and PCFT (Wani, Thakur and Kaur, 2012). Folate is absorbed in the proximal small intestine by PCFT in an acidic microenvironment (pH 5.8-6.0). Dietary folate, mainly 5-methyltetrahydrofolate (5mTHF), is ingested as polyglutamates and has to be hydrolysed to monoglutamate by a γ-glutamyl hydrolase in the intestine.

FA has to be reduced to dihydrofolate (DHF) and then to tetrahydrofolate (THF) before being released to circulation (Choi *et al.*, 2014). This step is mediated by dihydrofolate reductase (DHFR), which is essential for the metabolism of both folate and FA. DHFR has been detected in the enterocyte and mainly in the liver participating in the rate-limited conversion of the FA and folate to 5mTHF (Bailey and Ayling, 2009). THF is then metabolised by the enzyme serine hydroxymethyltransferase (SHMT) to produce glycine and 5,10-methylene-THF to later generate 5mTHF by the action of the riboflavin (B2)-dependent enzyme methylenetetrahydrofolate reductase (MTHFR) (Figure 1.8) (Scaglione and Panzavolta, 2014).

Folate participates in two main metabolic cycles: the synthesis of thymidylate and purines and the synthesis of methionine from homocysteine. 5mTHF gives the methyl group for homocysteine remethylation in a B12 vitamin-dependent reaction by the enzyme 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR). The 10-formyl-THF serves as a donor of one-carbon groups for purine biosynthesis; meanwhile, 5,10-methylene-THF participates as a cofactor for the conversion of dUMP to dTMP for nucleotide biosynthesis (See Figure 1.8).

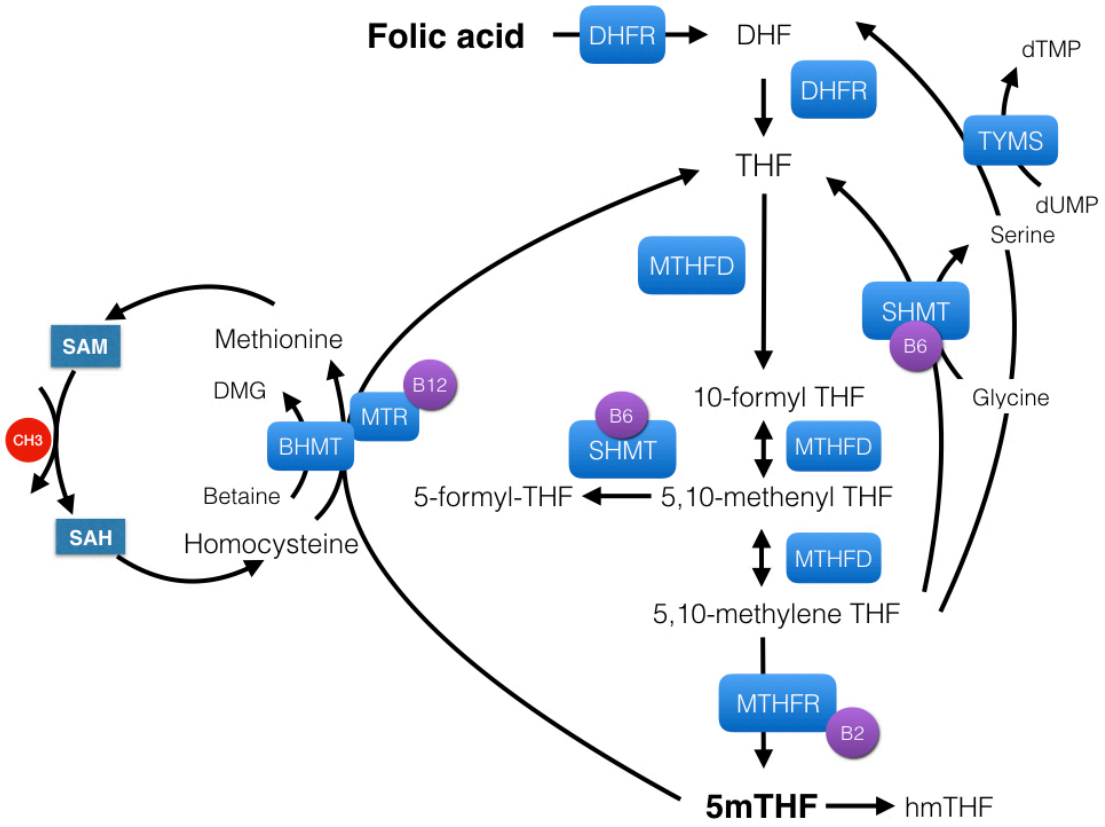

Figure 1.8 Folic acid and its metabolites

Folate metabolism and its metabolites: DHF: Dihydrofolate, THF: Tetrahydrofolate, dTMP: deoxythymidine monophosphate, dUMP: deoxyuridine monophosphate. Enzymes (in blue) DHFR: Dihydrofolate reductase. TYMS: Thymidylate Synthase, MTHFD: Methylenetetrahydrofolate dehydrogenase SHMT: Serine hydroxymethyltransferase, MTR: 5-methyltetrahydrofolate-homocysteine methyltransferase, TS: Thymidylate Synthase, BHMT: Betaine-homocysteine methyltransferase, MTHFR: Methylenetetrahydrofolate reductase.

1.6.2 Role of folate during pregnancy

During pregnancy, the requirements for folate increases due to rapid cell replication and the growth of foetal, placental and maternal tissue (Institute of Medicine, 1998). Low levels of folate in blood are associated with risk of neural tube defects (NTD) during pregnancy (Osterhues et al. 2013). NTD are common complex congenital malformations of the central nervous system (such as anencephaly and spina bifida) resulting from the failure of neural tube closure during the third and fourth weeks of gestation (Au, Ashley-Koch and Northrup, 2010). In the UK, the prevalence of NTD pregnancy was 1.28 per 1000 total births between 1991 and 2012, similar to what is reported in other countries without fortification strategies (Au, Ashley-Koch and Northrup, 2010; Morris *et al.*, 2016).

In the early '90s, studies have shown that FA supplementation reduces the risk of NTD pregnancy by an estimated 72% (MRC Vitamin Study Research Group, 1991). Consequently, US Public Health recommended all women in reproductive age consume 400µg of FA daily, and in the case of women with a previous pregnancy with NTD, the recommendation was to consume 4mg of FA per day (Centers for Disease Control and Prevention, 2002). However, as most of the pregnancies are not planned, in 1996 US Food and Drug Administration required the fortification of flour and grains in the United States with 150µg/100g by January 1998. Since then, more than seventy countries around the world have followed this strategy of food fortification, which has been successful in the reduction of the incidence of NTD (Crider, Bailey and Berry, 2011; Imbard, Benoist and Blom, 2013). However, there is an increasing concern about the possible side effect of the high consumption of FA for all the population (Smith, Kim and Refsum, 2008). Several studies in human and animal models have shown possible detrimental consequences of the FA supplementation, some of them are discussed below. However, there is not a conclusive decision about if this strategy of fortification should be moderated and/or reconsidered.

1.6.3 Consequences of folate and FA consumption

1.6.3.1 Unmetabolised FA

After more than a decade of fortification with FA, much discussion has taken place about the safety of persistent exposure to elevated levels of this vitamin in all groups of ages. High levels of unmetabolized folic acid (UMFA) have been identified due to the limited ability to reduce FA to 5-MTHF by the enzyme DHFR in the gut and the liver (Patanwala *et al.*, 2014). High levels of unmetabolized folic acid (UMFA) have been reported in US children, adolescents and adults, but also in children exposed to voluntary FA fortification in Ireland (Pfeiffer *et al.*, 2015; Vaish *et al.*, 2016). Meanwhile, in countries with non-mandatory fortification, 400µg of FA supplement use is sufficient to detect UMFA in the serum of elderly people in Germany (Obeid *et al.*, 2016). Evidence has shown that 200µg of FA is the threshold dose at which UMFA appears in serum in human (Sweeney, McPartlin and Scott, 2007). To investigate how the intestine metabolises high doses of FA, Wistar rats were fed with ten times FA recommendation (20 mg/kg) for 10 or 60 days. The results showed significant reduction of intestinal folate uptake in the shorter intervention (acute supplementation), decreasing the number of transporters RFC and PCFT at the protein level (Dev, Ahmad Wani and Kaur, 2011).

1.6.3.2 Physiological consequences associated with folate cycle disruption

There is plenty of evidence about the detrimental effect of low folate on embryo development, which involves intrauterine growth restriction, placental and cardiovascular abnormalities as well as NTD (Taparia *et al.*, 2007). Low folate and reduced co-enzymes necessary for its metabolism are associated with an embryonic growth delay and even death at 10dpc (Piedrahita *et al.*, 1999; Pickell *et al.*, 2009). However, folate is crucial even earlier, ovarian follicles express folate receptors and its co-enzymes, and it has been showed to be essential for successful preimplantation development (Meredith *et al.*, 2016; Strandgaard *et al.*, 2017). IVF studies have shown that folate status can modulate the ovarian response and then the reproductive outcomes (Twigt *et al.*, 2011; Gaskins *et al.*, 2015). The use of FA supplementation reduced the levels of homocysteine which are associated with a better chance of clinical pregnancy (Berker *et al.*, 2009; Ocal, Ersoylu and Cepni, 2012).

After all the evidence of low folate and its consequences, it is certainly clear that appropriate levels of folate in blood are necessary for an optimal embryo and foetal development. However, there is not the same consensus about the effect of continued exposure to high doses of FA, specifically before and during pregnancy. Studies in human and animals have shown alterations in the embryo development and foetal outcomes (birthweight, placenta size, and methylation

altered patterns). A systematic review showed that FA supplementation altered foetal outcomes increasing the birth weight in the woman using FA supplementation compared to the placebo group (Fekete *et al.*, 2012). Using an animal model, Keating et al. showed that rats fed with a high level of FA (40 mg, 20-fold higher than the recommended intake) diet during pregnancy and lactation period had an increase in birthweight and weight gain in female offspring. This effect persisted at 3 and 13 months of age. The female offspring continued to have a higher weight compared to the control group, besides higher levels of insulin and decreased levels of adiponectin compared to controls rats (Keating *et al.*, 2015).

At the embryonic stage, high maternal periconceptional folate status in women was associated with a reduced embryo size between the 6^{th} and 12^{th} weeks of gestation (Van Uitert *et al.*, 2014). Using a rodent model, Pickell et al., showed that embryos at 10.5 and 14.5dpc had a significant developmental delay and growth retardation denoted by a lower embryonic length and weight, compared to embryos with an adequate maternal diet (Pickell *et al.*, 2011). Disruption of the folate cycle (by methotrexate, which inhibits DHFR) before embryo implantation showed a decreased number of ICM and TE cells, arrested development and decreased cell division, all associated with retarded growth (O'Neill, 1998; B. Zhang *et al.*, 2015). These results in rodents suggest that periconceptional high FA diet affects embryo development.

The use of FA supplementation during pregnancy has been associated with changes in the levels of DNA methylation in cord blood in a gender-specific manner of genes related to the brain development, leptin and insulin sensitivity and also imprinted genes such as H19 involved in the control of growth and differentiation (Qian *et al.*, 2016; Pauwels, Ghosh, Duca, Bekaert, Freson, Huybrechts, A. S. Langie, *et al.*, 2017; Caffrey *et al.*, 2018). Rats fed with 2 and 5mg of FA during the first, second, third week or the whole pregnancy showed an increase in global DNA methylation in the brain of pups from dams supplemented with FA during the first week of gestation. However, just supplementation in late gestation or throughout pregnancy led to lower expression in *Erα*, *Gr* and *Ppar-α* genes in the liver, without changes in the brain. The plasma folate levels in pups were higher in all the supplemented groups compared with the control group, but the analysis of folate in tissue showed that brain and liver had higher levels of folate at different time points. These results demonstrate that even one week of supplementation is enough to change methylation patterns, and maternal FA supplementation affects folate concentrations, DNA methylation and gene expression in offspring in a gestation-period-dependent and organ-specific manner (Ly *et al.*, 2016).

The use of FA supplementation is higher in women of reproductive age that plan to be pregnant compared to other population subgroups (Murto *et al.*, 2015). Some of them are doubly exposed

(supplementation and fortification) showing constant high levels of FA in the blood (See 1.6.3.1). As folate can affect both DNA synthesis and methylation, high levels of FA intake may affect follicle development, oocyte growth and embryo development. To date, there have been very few studies investigating the effect of FA supplementation on the ovary or embryo development. Indeed, oocyte quality is a prerequisite for good embryo quality and foetal viability. That is why expanding the knowledge of the effect of high FA diet in the ovary and embryo development can help us to determine the potential effect in the future life.

1.7 Research hypothesis

The aim of this project was to identify the effect of FA supplementation on ovarian tissue and embryonic development.

The hypotheses of this project are:

- FA induces persistent effects on the expression of genes involved in proliferation and cell differentiation in the ovary of adult mice leading to persistent effects on ovarian function.
- Periconceptional FA supplementation alters embryonic development at the blastocyst stage.

1.7.1 Specific Aims

- Determine the FA effect on ovarian expression of genes related to cell differentiation and follicle development of adult mice.
- Determine changes in the ovarian follicle number at diestrus stage after four weeks of high FA supplementation in adult mice.
- Determine if changes observed in the ovaries immediately after high FA diet in ovarian tissue can persist in time.
- Determine the effect of periconceptional supplementation with FA on the embryo and cell lineage number and on mRNA and protein expression of cell lineage markers at the blastocyst stage.

Chapter 2 Methods

2.1 Animals procedures

2.1.1 Materials

Table 2.1 Reagents for animals procedures

Reagent/Chemical	Supplier
AIN93-M diet	TestDiet, USA
Ethanol	Fisher, UK
CO2 Gas	BOC, UK
Bovine Serum albumin (BSA) A3311-10G	Sigma-Aldrich, UK
Phosphate buffered saline (PBS)	Sigma-Aldrich, UK
Polyvinil-pyrrolidone (PVP)	Sigma-Aldrich, UK

2.1.2 Animal housing conditions

All animal procedures were performed in accordance with the Animals (Scientific Procedures) Act, 1986 under UK Home Office Project Licence. The project consisted of three different animal models using FA supplementation.

All the animals used were virgin C57BL/6 female mice housed on a 12 h light: dark cycle. Mice were housed in groups of 2 or 4 in plastic, grated metal-topped MB3 cages. Cages contained wood chip bedding, shredded paper for nesting and for environmental enrichment a crimson Perspex nesting houses and compressed cotton wool pellets. The room temperature was of 20.5 +/- 2°C and humidity 50-55%. Individual mouse weights and food intake were monitored per week.

2.1.3 Animal diet

After a week of acclimation, mice were randomly assigned to receive control diet (Table 2.2) or high FA diet (Table 2.3). Basal daily recommendation (BDR) of folate for rodents is between 1-2mg/kg of food, therefore our control diet contained 1mg of FA/kg of food because it was similar to the chow diet that the animals received before the experiment. The FA supplemented diet containing 5mg of FA (5x BDR) was selected to represent the likely increase in FA seen in the general population after supplementation and intake of fortified food (>2mg/d of folic acid) (Crider, Bailey and Berry, 2011).

Table 2.2 Control diet composition

Ingredients	%	Ingredients	%
Corn Starch	46.5192	AIN 93M Mineral Mix	3.5000
Dextrin	15.5000	AIN-93 Vit/100PPM Folate	1.0000
Casein – Vitamin Testes	14.0000	Choline Chloride	0.2500
Sucrose	10.0000	L-Cystine	0.1800
Powdered Cellulose	5.0000	Red Dye	0.0500
Soybean Oil	4.0000	t-Butylhydroquinone	0.0008
Energy			%
Protein			13.6
Fat (ether extract)			9.7
Carbohydrates			76.7

TestDiet Ain-93M with 1-PPM Folate. Modification of TestDiet® AIN-93M Semi-purified diet with 1 PPM total folate. For the complete information of the diet see Appendix A.1.1.

Table 2.3 High folic acid diet composition

Ingredients	%	Ingredients	%
Corn Starch	46.5192	AIN 93M Mineral Mix	3.5000
Dextrin	15.5000	AIN-93 Vit/500PPM Folate	1.0000
Casein – Vitamin Testes	14.0000	Choline Chloride	0.2500
Sucrose	10.0000	L-Cystine	0.1800
Powdered Cellulose	5.0000	Yellow Dye	0.0500
Soybean Oil	4.0000	t-Butylhydroquinone	0.0008
Energy			%
Protein			13.7
Fat (ether extract)			9.8
Carbohydrates			76.5

TestDiet Ain-93M with 5-PPM Folate. Modification of TestDiet® AIN-93M Semi-purified diet with 5 PPM total folate. For the complete information of the diet see Appendix A.1.2.

2.1.4 Oestrus cycle stage identification

The Oestrus cycle in mice consists of four phases referred to, respectively, as proestrus, oestrus, metestrus, and diestrus. Proestrus corresponds to the human follicular phase of the menstrual cycle and is characterised by elevated levels of oestradiol, although remaining levels of progesterone are still in the circulation at the beginning of this phase. Oestrus corresponds to ovulation. Metestrus is equivalent to the human luteal phase characterised by elevated levels of both progesterone and oestradiol. The next phase, diestrus, is defined as a late luteal phase when progesterone is still at high levels during this phase (Wood *et al.*, 2007; Hong *et al.*, 2010).

Once the animals completed the diet treatment, the oestrus cycle was checked by vaginal secretion analysis following Byers et al. and Caligioni C methods (Caligioni, 2009; Byers *et al.*, 2012). Once a day, saline solution was gently flushed into the vagina three or five times. All fluid was collected in an Eppendorf tube and a small proportion of the vaginal fluid was observed in a glass slide under the stereo microscope Olympus SZX10. Three types of cells can be observed, leukocytes, nucleated epithelial cells and cornified epithelial cells. The four stages of the cycle are defined according to the types and proportion of these cells (Figure 2.2). Proestrus, presents predominantly nucleated epithelial cells, oestrus characterised by cornified epithelial cells, metestrus were a mix of the three cells with a predominance of leukocytes, finally, diestrus presents a high number of leukocytes (Figure 2.2).

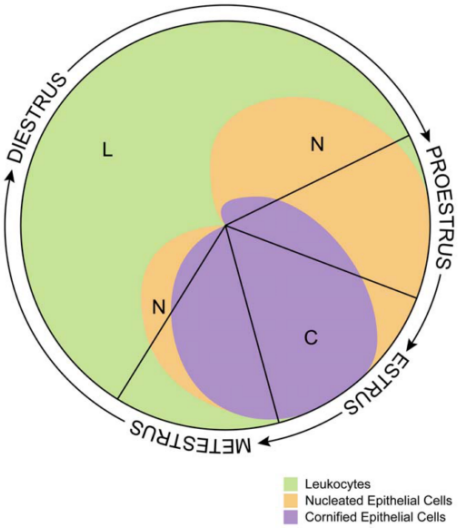

Figure 2.2 Oestrus cycle identification tool

The classification of the oestrus cycle is based in the proportion of leukocytes (green), nucleated (orange) and cornified cells (purple) (Byers *et al.*, 2012).

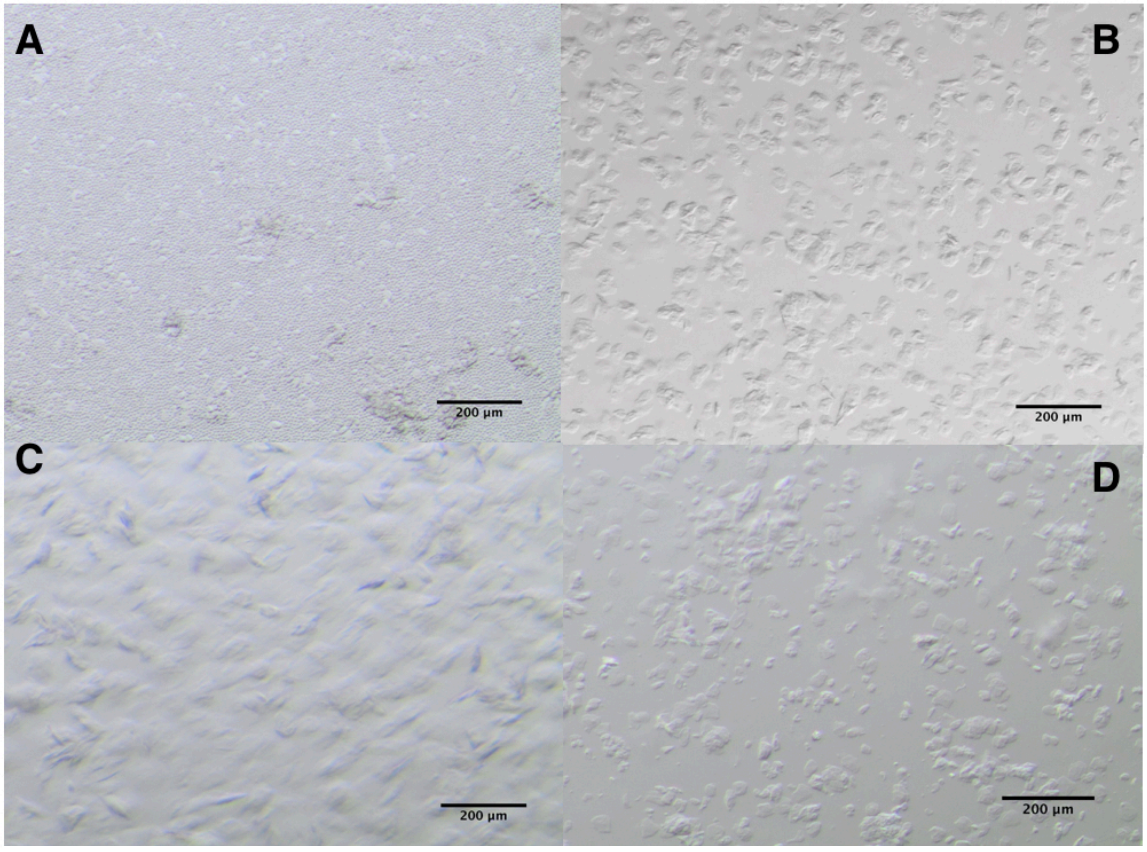

Figure 2.1 Vaginal smear during oestrus cycle

Vaginal secretion from mice at different stage of oestrus cycle. A) Diestrus, B) Proestrus, C) Oestrus, and D) Metestrus. Bar scale 200 µm.

2.1.5 Animal dissection

Animals were culled by CO2 exposure followed by cervical dislocation (Animal Scientific Procedures Act 1986, Schedule 1).

2.1.6 Blood collection

After cervical dislocation, a blood sample was collected by cardiac puncture using a 25mm needle and a 1.2ml EDTA tube. Blood samples were centrifuged at 3000 *x* g for 10 minutes at 4°C, plasma was collected and stored at -80°C for further analysis. In addition, the ovaries, mammary glands, liver, muscle, abdominal adipose tissue and brain were collected and immediately snap-frozen on liquid nitrogen prior to storage at -80°C.

2.1.7 Tissue collection

After blood collection, animals were placed on a corkboard and the limbs were pinned in supine position. Absolute ethanol was applied to the skin and fur across the thorax. A vertical incision in the thorax was made to start the dissection, to later peel back the sink to expose the mammary glands.

For non-pregnant animals, the tissue collection started with mammary glands, followed by ovaries, uterus, liver, fat and muscle. In the case of the pregnant animals, after the first incision, the ovaries and uterus were placed immediately in a petri dish with saline solution to flush the uterus. An additional student continued with the dissection meanwhile the embryos were collected.

2.1.8 Mating and check plug

After the four weeks of diet with control or high FA, animals used for the embryo project were mated with C57BL/6 male (age between 2-6 month old). The mating period usually takes variable time (between 1-4 days), depending the stage of the female cycle (unknown). The first day of mating one female was placed in male cage at 4pm and left until next morning (9 am) to check the vaginal plug. A successful mating was confirmed by the visual presence of a semen plug in the female vagina (plug-positive) and defined as 0.5dpc. In case of no presence of a vaginal plug (plug-negative), the female was placed back to her cage and mated with the same male at 4pm of the same day. At 0.5dpc the females started the preimplantation period and were assigned to receive either a control or high FA diet.

At 3.5pdc females were culled by cervical dislocation, ovary, oviduct and uterus were extracted for the collection of the embryos. If after flushing the oviduct and the uterus no embryos were found, the mouse was classified as false-positive plug (positive presence of the semen plug, but no embryos available).

2.1.9 Embryo collection at 3.5pc

Using a light microscope (Wild Heerbrugg, Germany), the uterus was flushed with pre-warmed (at 37ºC) H6BSA medium (H6 medium supplemented with either 4mg/ml BSA, H6 information Appendix A.2) using a 30G needle and 1ml syringe. Immediately after that, using a glass Pasteur pipette and a mouth pipette, the embryos were collected and grouped by stage (Figure 2.3). Depending on inner cell mass (ICM) and blastocoel cavity, blastocysts were classified in three types. Morula in contrast does not present any sign of a cavity (Figure 2.4).

- Early blastocyst: blastocyst in which the blastocoel cavity is less than half of the volume of the embryo
- Middle blastocyst: blastocyst in which the blastocoel cavity is half or more than half of the volume of the embryo
- Late blastocyst: blastocyst in which the blastocoel cavity completely fills the embryo and the zona pellucida is thinned

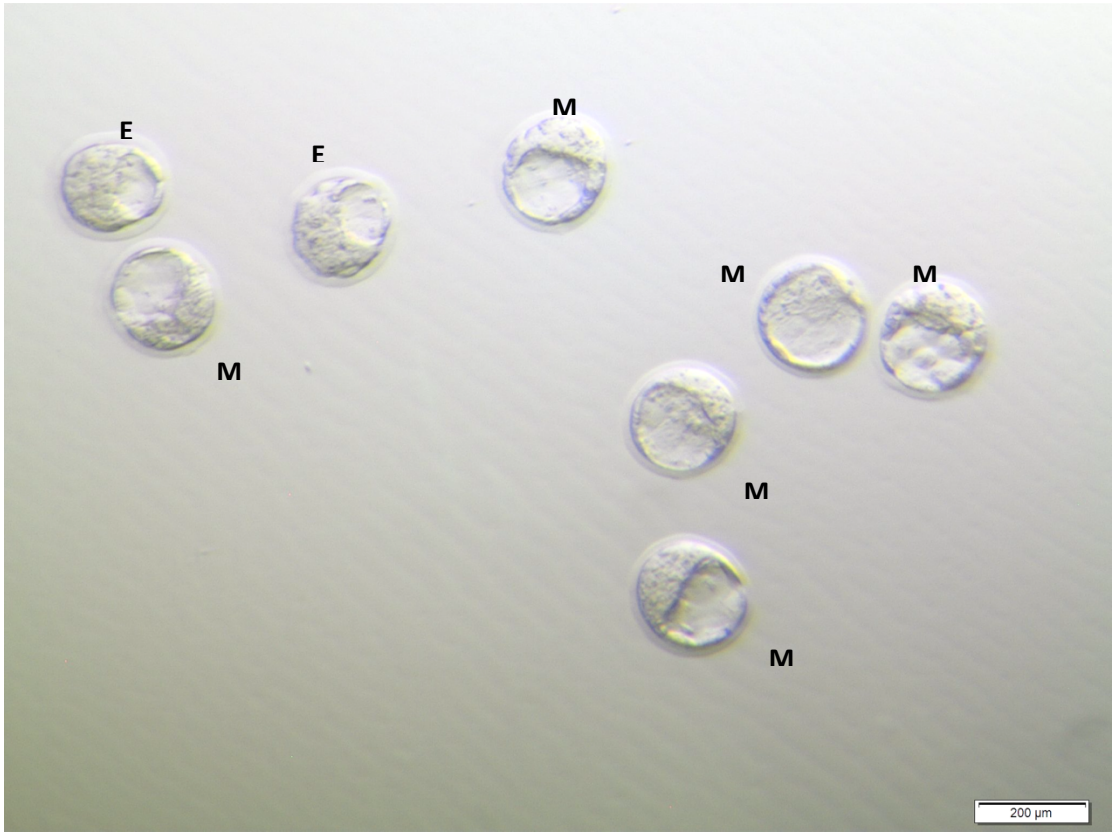

Figure 2.3 Embryos form C57BL6 mouse at 3.5dpc

Late, middle (M) and early (E) blastocyst were classified by the size of the cavity and proportion of ICM cells. Bar scale 200 µm.

Among the embryos found at 3.5dpc, there were some that showed to be degenerated. Two classification were made from embryos that did not look like blastocyst or morula stage: degenerated oocyte, which looked like an unfertilised oocyte that had disintegrated (Figure 2.5), and degenerated blastocyst, an embryo with a similar size to a blastocyst, but without a cavity and presenting an irregular shape (Figure 2.6). Degenerated embryos were counted in the analysis of developmental stages of all the embryos per mother, however were not used for any assay in this project. The female mice which just had degenerated oocytes were removed from analysis of this project.

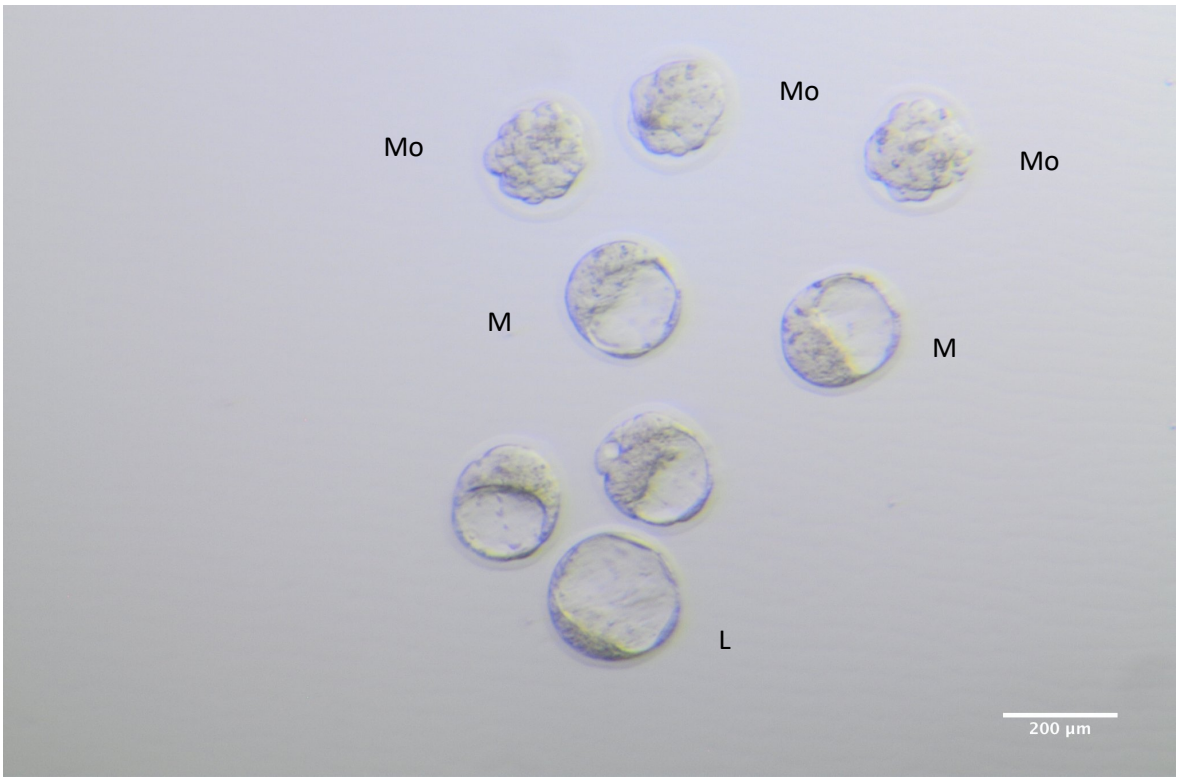

Figure 2.4 Embryos classification

Blastocysts were classified like early, middle (M) or late (L) by the size of the cavity and proportion of ICM cells. Morula has not sign of cavity (Mo). Bar scale 200 µm.

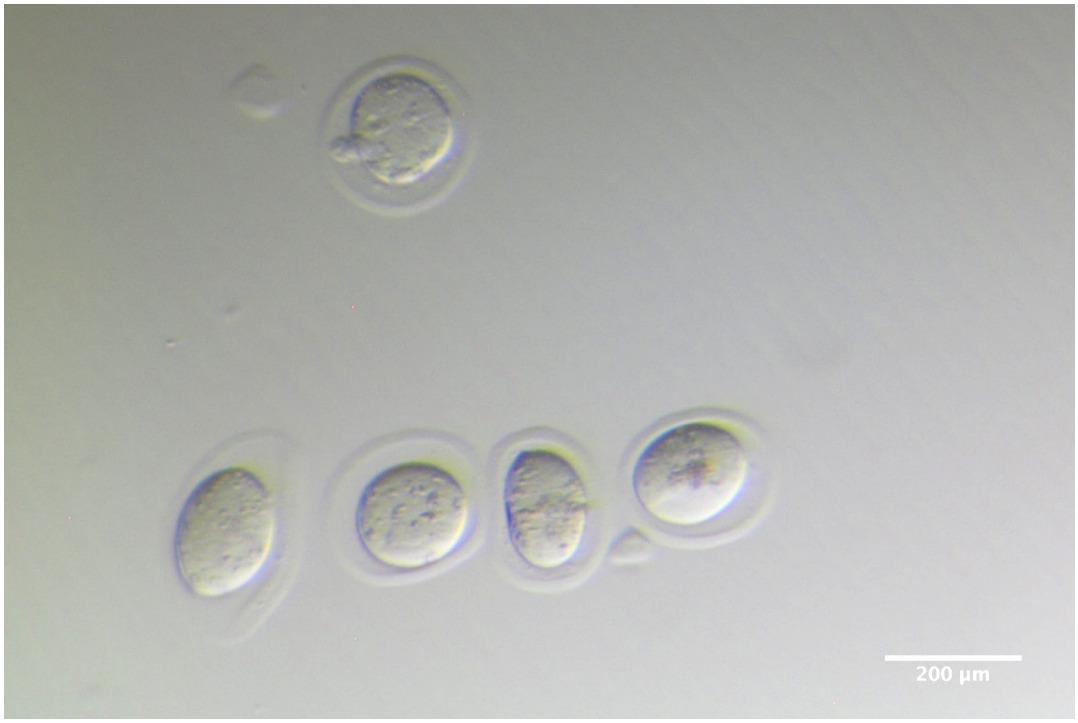

Figure 2.5 Degenerated oocytes collected at 3.5dpc

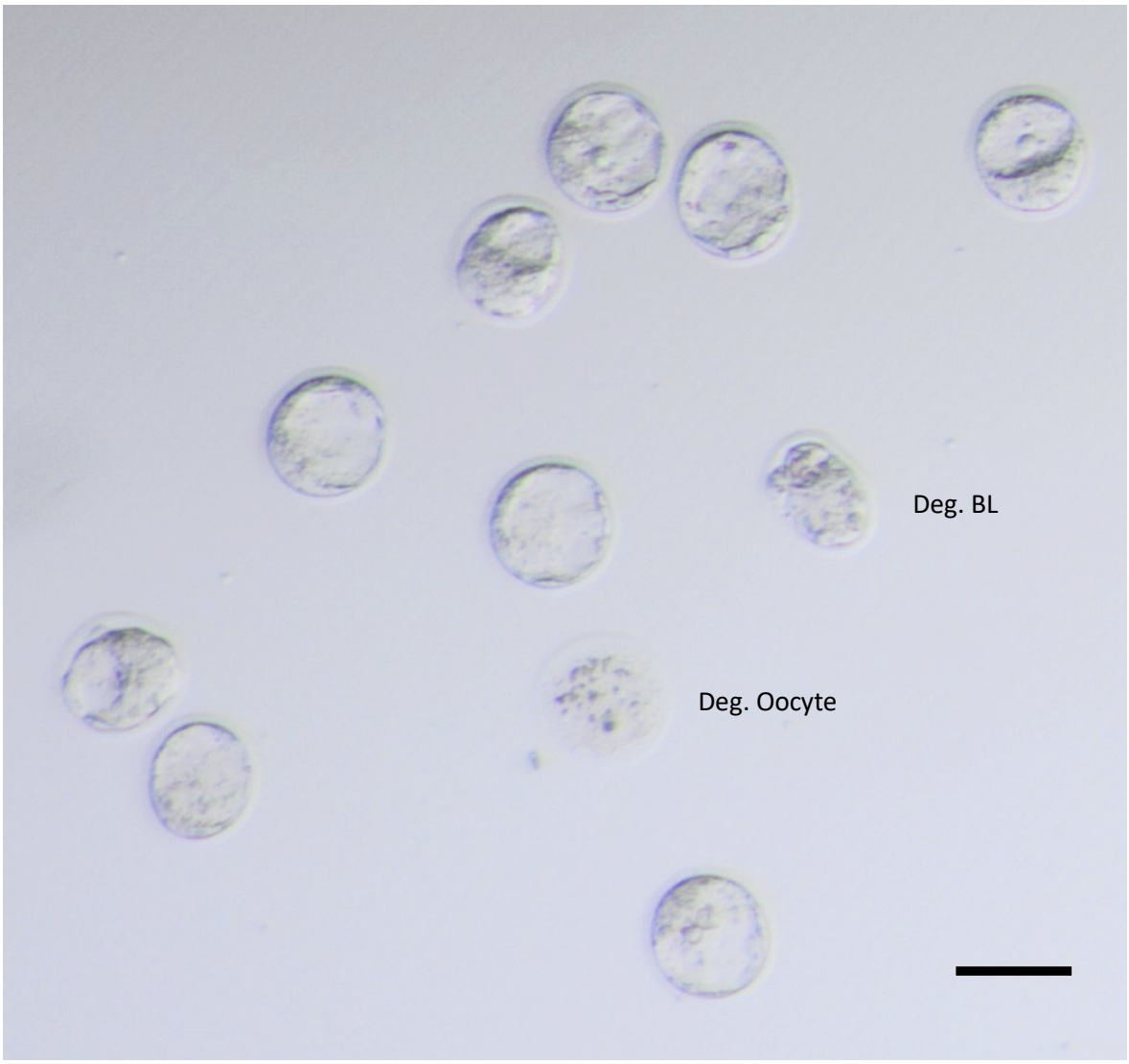

Figure 2.6 Degenerated oocyte and blastocyst

Blastocysts were classified by the size of the cavity and proportion of ICM cells. Degenerate oocyte and blastocyst (BL) were identified and count them. Bar scale 200 µm.

All embryos collected at 3.5dpc were washed through three 50 µL PBS drops supplemented with 1 % of Polyvinyl-pyrrolidone (PVP). For qPCR assay, embryos were transferred individually into nuclease-free thin walled non-stick tubes in a minimal volume (< 1 µL) and immediately snap-frozen on dry ice prior to storage at -80°C. Alternatively, embryos were used for staining assays as described in Differential cell labelling of blastocysts and Immuno-labelling.

2.2 Folate assay in plasma

Plasma extracted immediately after blood extraction of C57BL/6 mice (See 2.1.6). Plasma samples were sent to Bevital laboratories in Bergen, Norway for the assessment of folate. The method used was base in a microbiological assay, using a chloramphenicol resistant strain of *Lactobacillus casei* (*L. casei*), which measure the biologically active folate species in plasma. Folate (a polyglutamate) supports the growth of *L.casei* once it has deconjugate to monoglutamate by glutamyl hydrolase to then form mono and di-glutamates.

2.3 FA assay in plasma

Plasma extracted immediately after blood extraction of C57BL/6 mice (See Blood collection 2.1.6). Plasma samples were sent to Bevital laboratories in Bergen, Norway for the assessment of FA and its metabolites using liquid chromatography mass spectrometry (LC-MS). This assay allowed the detection of more metabolites than the folate assay. 5mTHF, 5fTHF, FA, hmTHF and the folate catabolites pABG and apABG were measured (see Figure 1.8 for FA metabolites).

2.4 RNA extraction

2.4.1 Materials

Table 2.4 Reagents for RNA extraction

Reagent/Chemical	Supplier
mirVana miRNA isolation Kit (AM1561)	Invitrogen, UK
Acid-Phenol: Chloroform	Ambion, UK
DNAse/RNAse free H2O	Fisher, UK
Agarose	Sigma-Aldrich, UK
Gel red	Sigma-Aldrich, UK
50 X Tris-acetate-EDTA (TAE) buffer	Fisher, UK
1 kb ladder	Fermentas, UK
100bp DNA ladder	Promega, USA
Loading buffer	Invitrogen, UK

2.4.2 RNA extraction from ovarian tissue

Ovarian tissue was crushed under liquid nitrogen, using a pestle and mortar. Tissue was not allowed to thaw during crushing. RNA was extracted from pulverised tissue using the mirVana miRNA isolation Kit following the manufacturer's protocol. To lyse the tissue, 1ml of lysis buffer was added per 0.1g of tissue and mixed by hand until all visible clumps are dispersed. A total of 1/10 volume of miRNA homogenate additive was added to the lysis buffer and incubated on ice for 10 min. After adding the acid-Phenol: Chloroform at an equal volume to the sample lysate volume and the miRNA homogenate additive, the sample was vortexed for 30-60 seconds (sec) and centrifuged for 5 minutes at 10,000 x g at room temperature to separate the aqueous and organic phases. After centrifugation, the aqueous phase was carefully removed and transferred to a fresh tube. 100% ethanol at 1.25 times the volume was added, mixed thoroughly and placed onto the filter cartridge and centrifuged for 15 sec (10,000 x g) to pass the mixture through the filter. After discarding the flow-through, the filter was washed with miRNA Wash solution 1 and twice with miRNA Wash solution 2/3. After centrifugation to remove residual fluid, the filter was transferred to a fresh collection tube. 100µL of pre-heated nuclease free water was then added to the filter and centrifuged at 17,000 x g for 20-30 sec.

RNA was quantified using Nanodrop ND-1000 spectrophotometer Abs (A260/A280) (Thermo Scientific, UK). 1µL of RNA elution was analysed measuring the absorbance at 260nm. A 260/280 ratio has been used as a measure of purity of the sample. A 260/280 ratio of ~1.8-2.0 was considered acceptable.

RNA consists primarily of ribosomal RNA (rRNA), 28S and 18S rRNA species. The radio 28S/18S was used as an indicator that the purified RNA was intact and had not been degraded. mRNA quality was assessed by electrophoresis of total RNA. Samples (250ng) mixed with loading buffer were run in a 0.8% agarose gel containing Red gel intercalating nuclei acid stain gel for 10 minutes at 120V. Under the UV transluminator, the 28S and 18S band were visualized (Figure 2.7). The RNA samples were stored at -80°C.

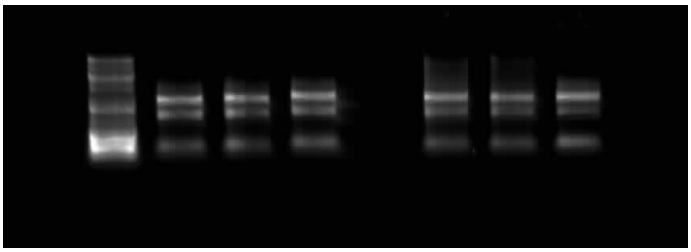

Figure 2.7 Agarose gel of ovarian RNA

Agarose gel with 250 ng of ovarian RNA. Visual assessment of the 28S and 18S rRNA on agarose gel.

2.4.3 RNA extraction from blastocysts

Poly A+ RNA was extracted from single blastocyst using the Dynabeads mRNA direct kit. The poly-A tail is a long chain of adenine nucleotides that is added to the mRNA to increase its stability during RNA processing.

Individual embryos were lysed in 150 µL lysis buffer for 10 minutes; meanwhile Dynabeads were washed twice in lysis buffer. 10 µL of Dynabeads were added to each sample and roller-incubated for 10 minutes at room temperature. After incubation, the bead-mRNA complex was concentrated by a magnet, its supernatant was removed and placed on dry ice immediately and saved at -70 °C freezer (used later for DNA extraction). Beads were washed twice with Buffer A and three times with Buffer B before suspending them in 10 µL of nuclease free water. For the elution, tubes were incubated in a heating block at 65 °C for 2 min, and placed immediately on ice in the magnet to remove the supernatant before use in the reverse transcription reaction.

Due to the small amount and volume of RNA of the final elution, RNA was not quantified or assessed by electrophoresis gel. However, previous quantification using Nanodrop with a single blastocyst showed between 0.5 and 1 ng of RNA per µL.

2.5 DNase treatment

2.5.1 Materials

Table 2.5 Reagents for DNase treatment

Reagent/Chemical	Supplier
DNase I, Amplification grade (D5307)	Sigma-Aldrich, UK
10x reaction buffer (R6273)	Sigma-Aldrich, UK
Stop Solution (S4809)	Sigma-Aldrich, UK

2.5.2 DNase treatment of ovarian tissue

After RNA extraction and prior to cDNA synthesis, any contaminating DNA was removed by DNase I treatment. 1µg of RNA was treated with DNase I in 10x reaction buffer provided in the kit, mixed and incubated at room temperature for 15 minutes. 1µL of Stop Solution (50mM EDTA) was added to the samples to inactivate the DNase I. Samples were then heated at 70°C for 10 minutes to denature both DNase I and the RNA using a Veriti 96 well-plate Thermal Cycler (Applied Biosystems, UK). The RNA was then stored at -80°C.

2.5.3 DNase treatment of blastocysts

After RNA extraction DNase treatment was not performed on the blastocyst samples due to the small concentration of RNA per sample.

2.6 Reverse Transcription

2.6.1 Materials

Table 2.6 Reagents for reverse transcription

Reagent/Chemical	Supplier
Deoxynucleotide Trisphosphates (dNTPs) (U1240)	Sigma-Aldrich, UK
Random Nonamers (R7647-100UL)	Sigma-Aldrich, UK
Reverse Transcriptase 5X reaction buffer (M531A)	Promega, UK
Dnase and Rnase free water	Fisher, UK
M-MLV Reverse Transcriptase (M1705)	Promega, UK

2.6.2 Reverse transcription from ovarian tissue

cDNA was made using 1 µg of RNA treated with DNase I. To 1 µg of RNA, 1 µL of 10mM dNTP's mix and 1µL of Random Nonamers (50 µM) were added, mixed and incubated at 70°C for 10 minutes. After incubation, 4µL of Buffer Reverse Transcriptase, 5µL of nuclease free water and 1µL of M-MLV Reverse transcriptase enzyme (200 units) were added to the sample. The samples were then incubated at 21°C for 10 minutes, followed by 50 minutes at 37°C and 90°C for 10 minutes. cDNA samples were diluted to 100 µL (10ng/µL) and were stored at -20°C.

2.6.3 Reverse transcription from blastocysts

For cDNA conversion, immediately after RNA extraction, 8 µL of RNA elution were used for sample, meanwhile the remaining 2µL for a negative control of reverse transcription (RT-). RT- was performed for every RNA sample containing the same mix of RT except the M-MLV reverse transcriptase enzyme. This negative control allows to the detection of genomic DNA during qPCR.

The reverse transcription reaction was performed exactly as described for tissue samples, described above. Prepared blastocyst cDNA was diluted to 60 µL with nuclease free water to use in qPCR.

2.7 Real-time PCR

For real-time PCR, all assays were run in the Light Cycler 480 II Roche Real-Time PCR system using the Software 1.5.1 version. All reactions were run in duplicate included reference and target genes, with their respective standard curve points and NTC (no template control). The gene expression of target genes was normalised with reference genes selected according to GeNorm analysis (Biogazelle, Belgium) (Vandesompele *et al.*, 2002).

2.7.1 Materials

Table 2.7 Reagents for qPCR

Reagent/Chemical	Supplier
TaqMan Gene expression Assays (Table 2.8)	Invitrogen, UK
TaqMan Gene expression Master Mix (4369016)	Invitrogen, UK
QuantiFast SYBR Green PCR Kit (204054)	Qigen, UK
QuantiTect Primer Assay (Table 2.8 and Table 2.9)	Qigen, UK
Dnase and Rnase free water	Fisher, UK

2.7.2 Real-time PCR from ovarian tissue

Depending on the gene of interest, different approaches were used to measure the level of mRNA. For genes highly expressed in the ovary, incorporation of intercalating dyes (SYBR Green) was used, whereas for lowly expressed genes (e.g *Pouf5f1* and *Brca1*), assays containing hydrolysis probes (TaqMan) were used to allow precise measurement of the amount of PCR product per cycle. Each assay has its own advantages and disadvantages; SYBR Green is a cheaper method that does not involve a specific probe and enables the amplification of any double-stranded DNA sequence (dsDNA) to be measured. Meanwhile, TaqMan qPCR detection is based on the additional use of probes providing a specific detection of the PCR product using the fluorogenic probe, reducing false positives. Among the disadvantages of these methods are, the nonspecific binding of SYBR to any dsDNA and the higher cost of TaqMan associated with the use of a unique probe for each target.

In order to assess the specific amplification of the target by SYBR Green, the melting curve was monitored after every run. The presence of a single peak was interpreted as the amplification of one single product (Figure 2.8). Moreover, the plain expression of NTC (blue line in Figure 2.8) showed the no presence of contamination of the reaction.

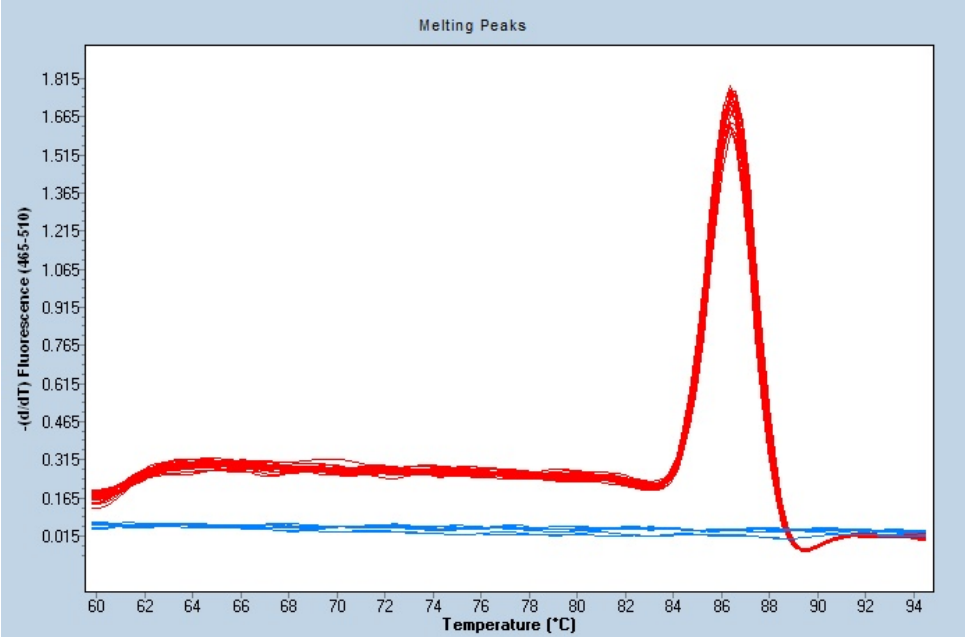

Figure 2.8 Melting curve example

Red lines represent the cDNA samples, and the blue the NTC samples.

For SYBR green measurements, 1.5 µL of cDNA (15 ng/µL) was used with 2x Quantifast SYBR Green, 10X QuantiTect primer assay and RNase-free water to complete a volume reaction of 10 µL. The cycling conditions were: an initial heat activation of 5 minutes at 95°C, and 40 cycles of denaturation for 10 sec at 95°C and annealing/extension for 30 sec at 60°C. The melting curve conditions were 15 sec at 95°C and 1 minute at 60°C. In the case of TaqMan assays, a higher concentration of cDNA was used as these were more lowly expressed transcripts. Then 4 µL cDNA (40 ng/µL) was used with 2x TaqMan gene Master Mix, 20x TaqMan gene expression assay and RNase-free water to complete a volume reaction of 20µL total. The cycling conditions were: an initial heat activation of 10 minutes at 95°C, followed by 40 cycles of denaturation for 15 sec at 95°C and annealing/extension for 1 min at 60°C. The list of the primers assay used are listed in the Table 2.8.

The results of the qPCR are expressed in Ct values which represent the cycle number at which the fluorescence signal, associated with the amplicon accumulation, crosses the threshold. The threshold was set automatically by the software within the exponential phase of the amplification curve.

Table 2.8 List of primer for qPCR used for ovarian tissue

Gene	Assay ID	Company/ Cat.no.
Pou5f1	Mm03053917_g1	TaqMan 4331182
Brca1	Mm01249840_m1	TaqMan 4331182
Ezh2	Mm_Ezh2_1_SG	QuantiTect QT01063671
Bmi1	Mm_Bmi1_1_SG	QuantiTect QT00165298
Dnmt1	Mm_Dnmt1.1_SG	QuantiTect QT00157990
Gdf9	Mm_Gdf0_1_SG	QuantiTect QT00249410
Fshr	Mm_Fsfr_1_SG	QuantiTect QT00122472
Lhcgr	Mm_Lhcgr_1_SG	QuantiTect QT00101990
Pgr	Mm_Pgr_1_SG	QuantiTect QT00114534
Cyp19a1	Mm_Cyp19a1_1_SG	QuantiTect QT00099792
Slc46a1	Mm_Slc46a1_1_SG	QuantiTect QT00159600
Slc19a1	Mm_Scl19a1_1_SG	QuantiTect QT00493479
FRa	Mm_Fra_1_SG	QuantiTect QT00096159
Sdha	Mm_Sdha_2_SG	QuantiTect QT01037666
18S	Mm_Rn18s_3_SG	QuantiTect QT02448075
Actb	Mm_Actb_1_SG	QuantitTect QT00095242
Gapdh	Mm_Gapdh_3_SG	QuantiTect QT01658692
Ppia	Mm_Ppia_1_SG	QuantiTect QT00247709
Ubc	Mm_Ubc_1_SG	QuantiTect QT00245189

2.7.3 qPCR from blastocyst

All the assays were performed using SYBR Green in a volume of 10 µL, using 5 µL of 2x Quantifast SYBR Green, 1 µL of primer, 3 µL of blastocyst cDNA and 1 µL of RNA-free water.

As DNAse treatment was not performed in these samples and to ensure no DNA amplification, all the primers were designed to span an exon-intron boundary. In addition, agarose gels after qPCR were used to check the presence of the correctly sized amplicon, and melting curve verification of a single product for each run. The list and the sequences of the primers used for blastocyst gene expression are in Table 2.9

Table 2.9 List of primers for blastocyst gene expression

Gene	Assay ID	Amplified Exon	Cat.No.
H2afz	Mm_H2afz_1_SG	2/3	QT00137319
Sptbn1	Mm_Sptbn1_1_SG	29/30	QT00129108
Ezh2	Mm_Ezh2_1_SG	14/15/16	QT01063671
Eed	Mm_Eed_1_SG	8/9	QT00172424
Gata6	Mm_Gata6_1_SG	1/2	QT00171297
Sox17	Mm_Sox17_1_SG	1/2	QT00160720
Slc46a1	Mm_Slc46a1_1_SG	4/5	QT00159600
Mthfr	Mm_Mthfr_1_SG	9/10/11	QT00198114

Gene		Sequence
Ppib[+]	For	TTCTTCATAACCACAGTCAAGACC
	Rev	ACCTTCCGTACCACATCCAT
Tuba[*]	For	CTGGAACCCACGGTCATC
	Rev	GTGGCCACGAGCATAGTTATT
Oct4A[§]	For	CACGAGTGGAAAGCAACTCA
	Rev	TTGGTTCCACCTTCTCCAAC
Nanog[æ]	For	TGCTTACAAGGGTCTGCTACT
	Rev	GAGGCAGGTCTTCAGAGGAA
Cdx2[æ]	For	CTGCCACACTTGGGCTCT
	Rev	CTGCTGCTGCTTCTTCTTGA
Dnmt1[+]	For	GCTACCAGTGCACCTTTGGT
	Rev	ATGATGGCCCTCCTTCGT
Dnmt3l[+]	For	AACCGACGGAGCATTGAA
	Rev	CCGAGTGTACACCTGGAGAGT

List of primers for qPCR used for blastocyst samples. [+](Watkins *et al.*, 2017), [*](Lucas *et al.*, 2011), [§](Kashani *et al.*, 2014), and [æ]from J. Eckert, University of Southampton.

2.7.4 Quantification of gene expression by standard curve method

To quantify the gene expression in ovarian and embryo samples, the standard curve method was used. RNA was extracted from an ovary/embryo of a non-supplemented mouse and converted to cDNA, pooled and diluted 2-fold 5 times generating six serial dilutions. The dilutions were run in duplicate next to every assay performed (example of cyclophilin A, in Table 2.10). With the average Ct values and the known concentration of each point a graph with trendline and equation and R-squared (R^2) was displayed (Figure 2.9)

Table 2.10 Standard curve values example for cyclophilin A (CYC) gene

Standard Sample	Dilution	Concentration (ng)	Average Ct Value	Standard Deviation (SD)
A	1X	43	17.94	0.04
B	2X	21.5	18.92	0.06
C	2X	10.8	20.06	0.14
D	2X	5.4	20.80	0.02
E	2X	2.7	21.90	0.03
F	2X	1.3	22.92	0.07

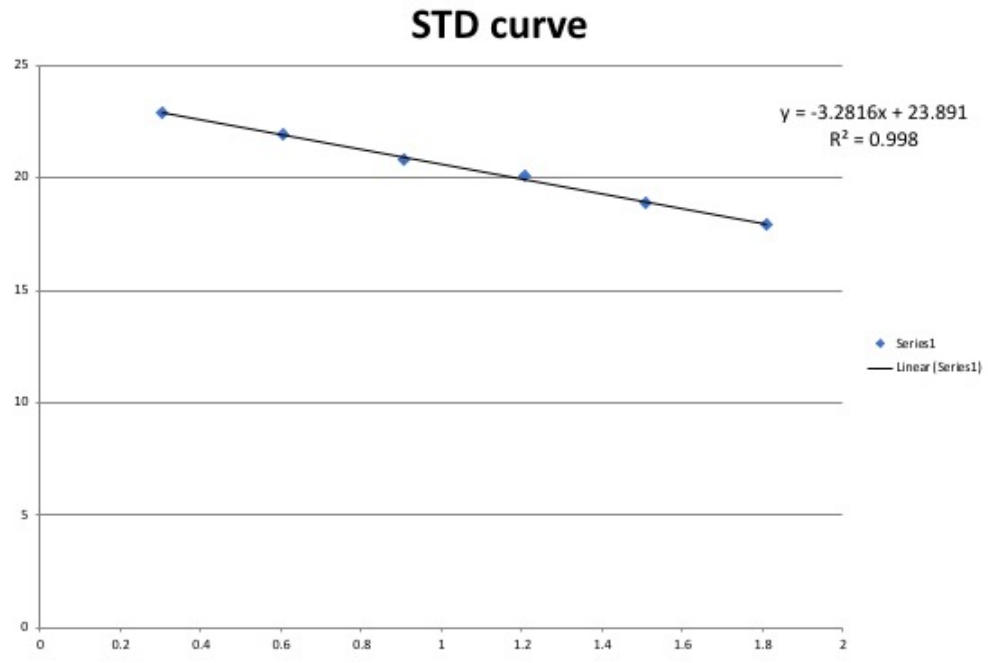

Figure 2.9 Graph example of standard curve of CYC

Example of a standard curve graph of CYC using the logarithm of the known concentration of six points of serial dilution of cDNA against the Ct values of each point. From the slope an equation and R^2 are displayed.

The interpretation of the equation and the correlation coefficient of plot allow the definition of the efficiency of the used primers. Precisely, the slope of the line suggests the efficiency of the assay, where a slope between -3.1 and -3.6 are considerable acceptable (90 and 110% efficient, respectively).

Therefore, using the equation from the plot, new values per sample can be calculated considering the efficiency of the primers using the equation: Quantity = $10^{(ct-b)/m}$, where b is the intercept (23.891) and m is the slope (-3.2816) of the linear regression of the Figure 2.9.

2.8 GeNorm

To identify the best reference genes, a set of six potential reference genes were run in a qPCR with six samples per group. For each project a geNorm assay was run: ovaries from mice culled at PND102/130/186 from Chapter Result I (Figure 2.10), ovaries from mice culled at PND102 and diestrus stage (Figure 2.11), ovaries from mice culled at PND130 (Figure 2.12) and blastocyst collected at 3.5dpc (Figure 2.13). Among the genes used for mouse ovarian tissue were: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ubiquitin C (UBC), beta-2-microglobulin (B2M), RNA18S5 (18S), cyclophilin A (CYC), actin beta (ACTB) and succinate dehydrogenase complex flavoprotein subunit A (SDHA). In the case of blastocyst samples genes well-expressed at the embryo were used: TATA-Box-Binding protein (TBP), Spectrin Beta, Non-Erythrocytic 1 (SPTBN1), Phosphoglycerate Kinase 1 (PGK1), H2A Histone Family Member Z (H2AFZ), Peptidylprolyl Isomerase B (PPIB) and Tubulin Alpha (TUBA).

Results were analysed by Biogazelle qbase plus 3.0 (Biogazelle, Belgium), and target genes with the greatest stability (M value) were selected as reference genes (average geNorm M $\leq$ 0.5). The M value indicates the average expression stability value of remaining reference genes at each step during stepwise exclusion of the least stable reference gene. The optimal number of reference genes was calculated accorfrding to the geNorm V (variation) value (geNorm V < 0.15). This value represents the variation between two sequential normalization factors containing an increasing number of genes (Vandesompele *et al.*, 2002).

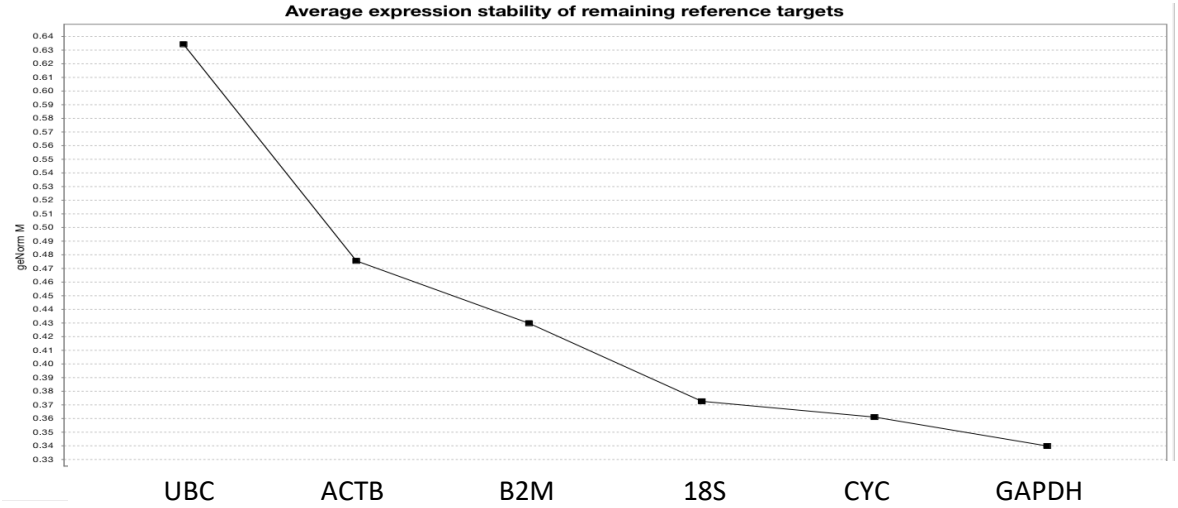

Figure 2.10 GeNorm result ovaries at PND102

UBC, ACTB, B2M, 18S, CYC and GAPDH were tested in six samples per group. The optimal number of reference targets were two: CYC and GAPDH (lower values geNorm M, right side graph).

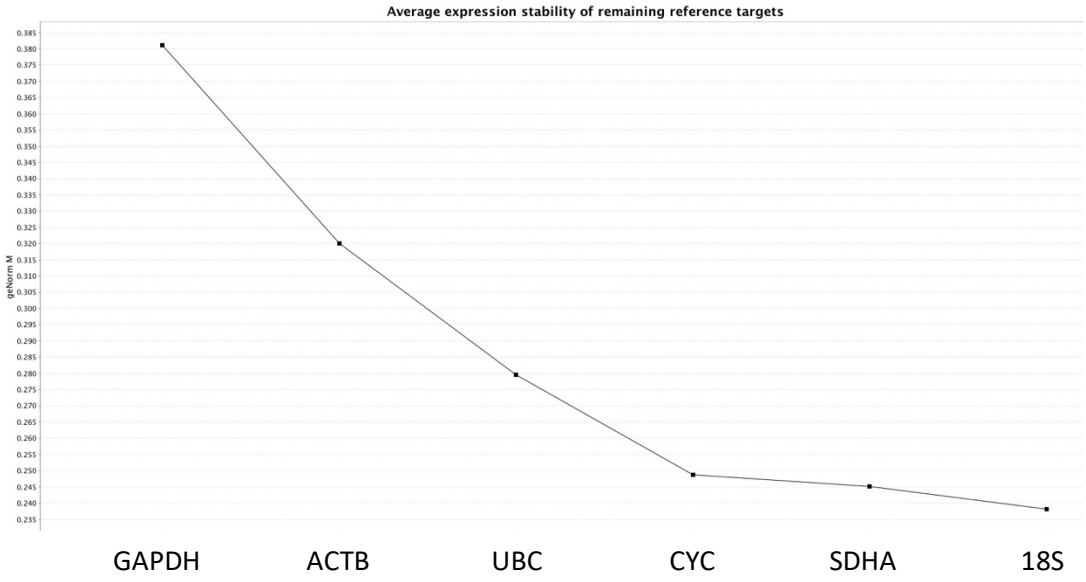

Figure 2.11 GeNorm result ovaries PND102 at diestrus stage

GAPDH, ACTB, UBC, CYC, SDHA and 18S were tested in six samples per group. The optimal number of reference targets were two: SDHA and 18S (lower values geNorm M, right side graph).

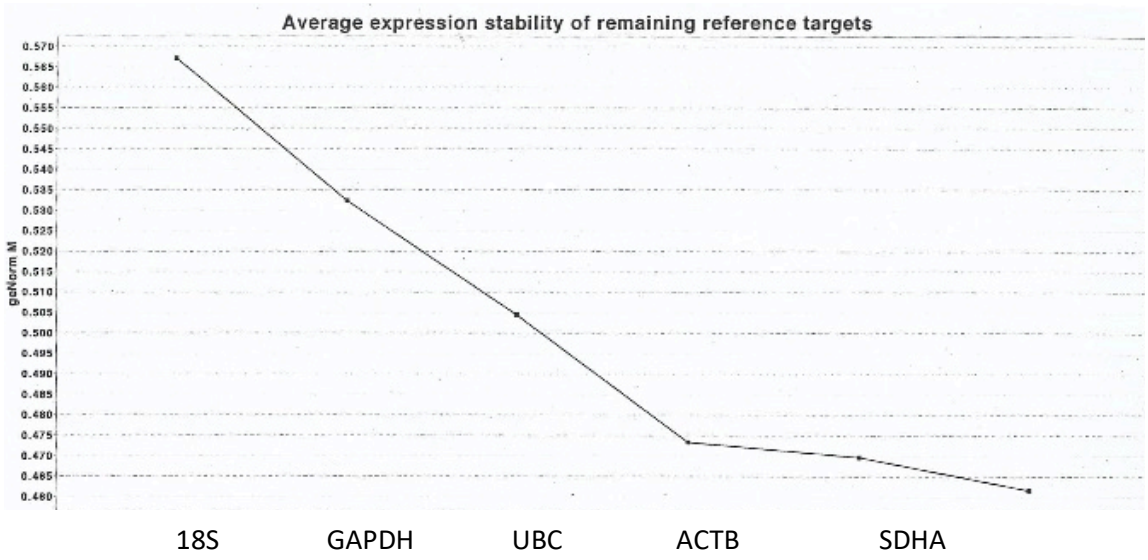

Figure 2.12 GeNorm result of ovaries PND130

18S, GAPDH, UBC, ACTB, SDHA and CYC were tested in six samples per group. The optimal number of reference targets were two: SDHA and CYC (lower values geNorm M, right side graph).

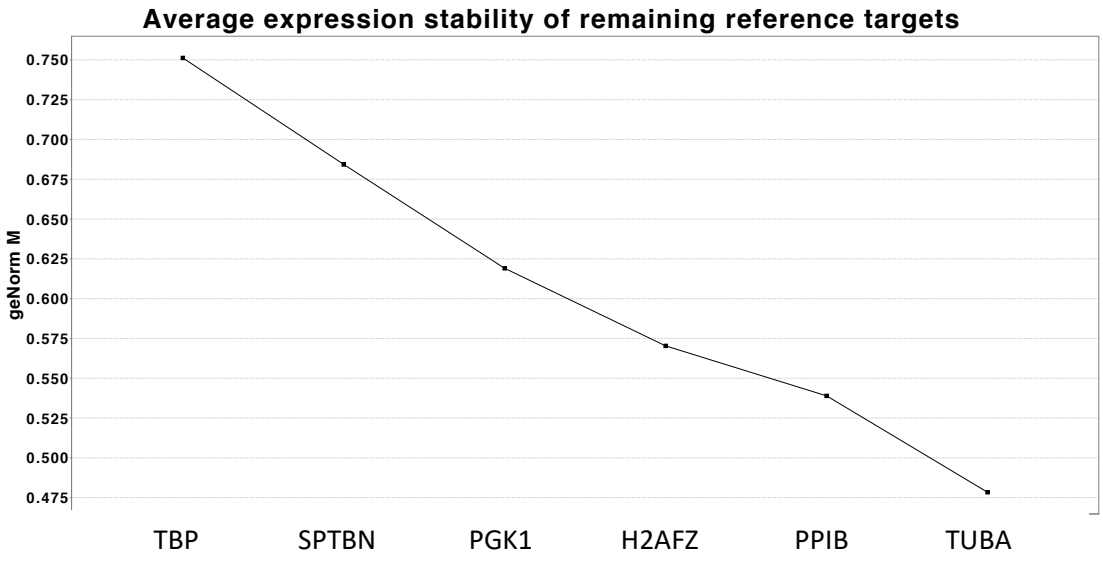

Figure 2.13 GeNorm result of male middle blastocyst

TBP, SPTBN, PGK1, H2AFZ, PPIB and TUBA were tested in five samples per group. The optimal number of reference targets were three: H2AFZ, PPIB and TUBA (lower values geNorm M, right side graph).

2.9 DNA extraction

2.9.1 Materials

Table 2.11 Reagents DNA extraction

Reagent/Chemical	Supplier
Glycogen	Fisher, UK
Sodium Acetate 3M	Sigma-Aldrich, UK
Ethanol	Fisher, UK
KAPA Express extract (KK7150)	KapaBiosystems, UK
10X Buffer with 15mM MgCl2 (203203)	Qigen, UK
Deoxynucleotide Trisphosphates (dNTPs)	Sigma-Aldrich, UK
HotStarTaq DNA Polymerase (203203)	Qigen, UK
Dnase and Rnase free water	Fisher, UK

2.9.2 DNA extraction from embryos and genotyping

To determine the sex of each embryo DNA was extracted from RNA supernatant. 1µl of Glycogen (20 µg/µl) and 0.1 volume of 3M Sodium Acetate were added to the supernatant from RNA extraction. Subsequently, 2.5 volume of absolute ethanol was added and vortexed and kept at -80°C for 30 minutes. After that, samples were centrifuged at 4°C for 15 minutes at 10,000 x g and supernatant was discarded. Every pellet was washed in 70% ethanol, vortexed and spun for 15 minutes at 10,000 x g at 4°C and supernatant was discarded. The final pellet was dried removing the excess of liquid, and resuspended in 5µl of nuclease free water.

To generate male and female controls, DNA was extracted from mice tails using KAPA Biosystem extract kit. 2mm of a mouse-tail was mixed with 10X Kapa express Extract buffer, Kapa express extract Enzyme and nuclease free water, according to the manufacture's protocol. For lysis, the sample was heated to 75°C for 10 minutes, followed by 5 minutes at 95°C for enzyme inactivation. Samples were centrifuged to pellet cellular debris and the DNA extracted was diluted 1:10 and stored at -20°C.

DNA was used for sex determination through Nested PCR. 5 µl of DNA were added to a tube containing master mix with 10X Buffer containing 15 mM $MgCl_2$, 2.5 mM dNTP mix, 2.5 µM each outer primer (Table 2.12 from Kunieda et al. 1992), 25 mM MgCl2, 5U/µl HotStarTaq polymerase and nuclease free water until a complete volume of 50 µl. The PCR conditions were: 95°C for 15

min for activation, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 1 min to finish at 72°C for 10 min. 5µl of template from the previous PCR were used for the second PCR with the same reagents except $MgCl_2$ and using inner primers (Table 2.13) at the same PCR conditions.

Table 2.12 Outer primers for genotyping (Kunieda *et al.*, 1992)

Gene		Outer primers
Polymorphic X Chromosome microsatellite locus (DXNd-3)	For Rev	GAGTGCCTCATCTATACTTACA TCTAGTTCATTGTTGATTAGTT
Sex-determining region Y (SRY)	For Rev	TCTTAAACTCTGAAGAAGAGAC GTCTTGCCTGTATGTGATGG
Zinc finger Y-chromosome (ZFY)	For Rev	AAGATAAGCTTACATAATCACATGGA CCTATGAAATCCTTTGCTGCACATGT

Table 2.13 Inner primers for genotyping (Kunieda *et al.*, 1992)

Gene		Inner primers
Polymorphic X Chromosome microsatellite locus (DXNd-3)	For Rev	ATGCTTGGCCAGTGTACATAG TCCGGAAAGCAGCCATTGGAGA
Sex-determining region Y (SRY)	For Rev	GTGAGAGGCACAAGTTGGC CTCTGTGTAGGATCTTCAATC
Zinc finger Y-chromosome (ZFY)	For Rev	GTAGGAAGAATCTTTCTCATGCTGG TTTTTGAGTGCTGATGGGTGACGG

The PCR product was run in 1.5% agarose gel at 120V for at least 60 minutes to identify the bands associated with female or male sex. Three bands representing ZFY, SRY and DXNd were defined as male and a single band (DXNd) was defined as a female embryo. Some samples could not be identified due to unclear visualisation on the gel Figure 2.14.

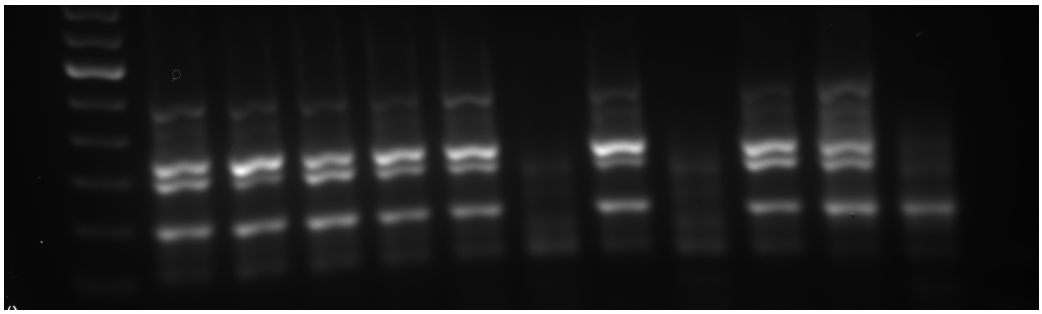

Figure 2.14 Agarose gel for genotyping

Agarose gel with PCR products from blastocyst genotyping. 3 bands correspond to a male blastocyst, and only one band is positive for female. Top band ZFY (217bp), middle band SRY (147bp) and bottom band DXNd (111bp). In the right side of the figure male (M) and female (F) controls. Ladder arrows: red (100bp), yellow (200bp) and blue (300bp).

2.10 Histological analysis of the ovarian tissue

2.10.1 Materials

Table 2.14 Reagents for histological analysis of the ovary

Reagent/Chemical	Supplier
Formaldehyde	Sigma-Aldrich, UK
Clearene	Leica, UK
Ethanol	Sigma-Aldrich, UK
Mayer's Haematoxylin	See Appendix A.3
Eosin	See Appendix A.3
Pertex mounting media	Leica, UK

2.10.2 Haematoxylin and eosin (H&E) staining

Tissue samples were fixed in 10% neutral buffered formaldehyde and processed for histological analysis by embedding in paraffin. Tissues fixed sections were cut at 4µm thickness and mounted on glass slides. Every ten sections, one section was stained with haematoxylin and eosin (H&E) for morphological analysis of structure and follicle distribution.

Before the tissue section is stained, the paraffin must be removed by a process called clearing. Therefore, sections were dewaxed in the solvent Clearene for 10 minutes twice. After that, all sections were hydrated through graded alcohols to water for five minutes per solution. The slides were stained in Mayers Haematoxylin for five minutes, followed by a five minutes wash in running tap water. The second stain was with Eosin for five minutes followed by a brief rinse in water. All the samples were dehydrated through alcohols twice for one minute each and cleared in Clearene three times for two minutes before mounting the slide in pertex. At the end of the process, cell nuclei will be stained blue (by Haematoxylin) and the cytoplasm pink (by Eosin).

2.10.2.1 Analyses of follicle number

The number of follicles was counted every ten sections. To prevent multiple counts of the same follicle, only those follicles with a visible oocyte nucleus were included. Follicles showing evidence of atresia such as pyknotic granulosa cells or signs of degeneration such as deformed shape, loss of nuclear membrane and/or fragmentation were excluded and counted be separated (Figure 2.16).

Follicle classification was based on the following characteristics (Figure 2.15):

- Primordial follicle: One partial or complete layer of flattened granulosa cells surrounding the oocyte
- Primary follicle: One complete layer or more than 50% of cuboidal granulosa cells surrounding the oocyte
- Secondary follicle: Two or more layers of granulosa cells surrounding the oocyte
- Pre-antral follicle: A secondary follicle with small follicular antrum
- Antral follicle: Oocyte surrounded by several layers of granulosa cells and a defined antrum
- Corpus luteum: Residual follicular wall of granulosa and theca cells (Figure 2.17).

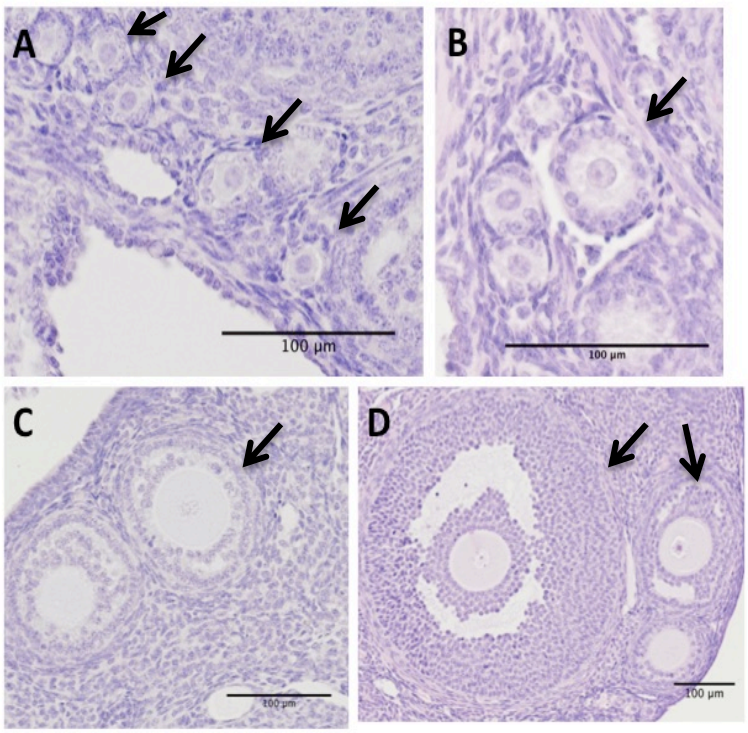

Figure 2.15 Photomicrographs of cross-sections of ovaries stained with H&E

Primordial (black arrows, A), primary (black arrow, B), secondary (black arrow, C), pre-antral (right black arrow, D) and antral (left black arrow, D) follicles were defined according to proportions and type of cells surrounding the oocyte. Scale 100 µm.

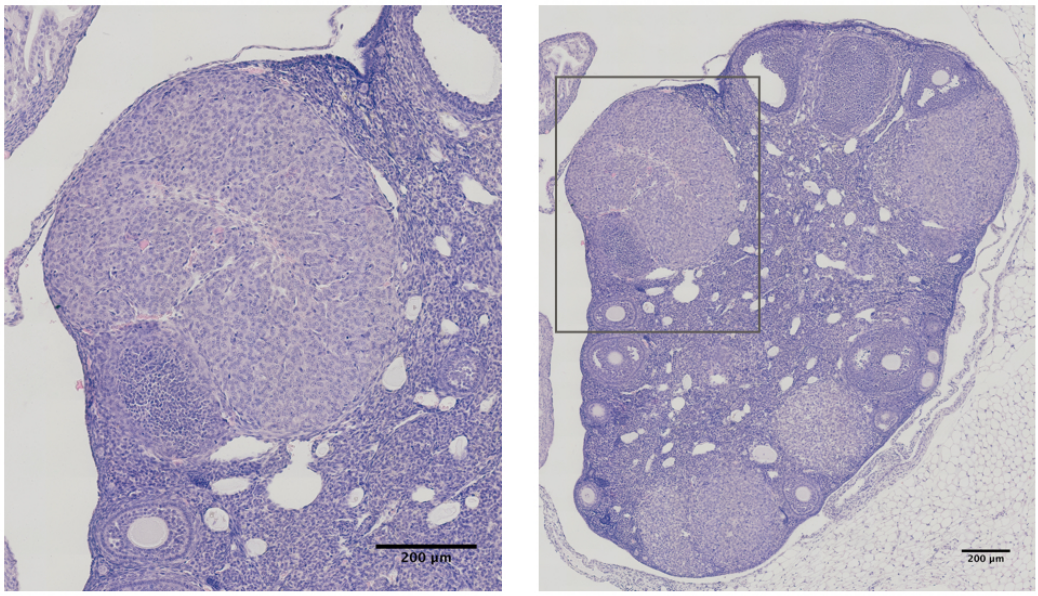

Figure 2.17 Corpus luteum of an ovary stained with H&E

Corpus luteum (CL) of an ovary stained with H&E. Scale 200 µm.

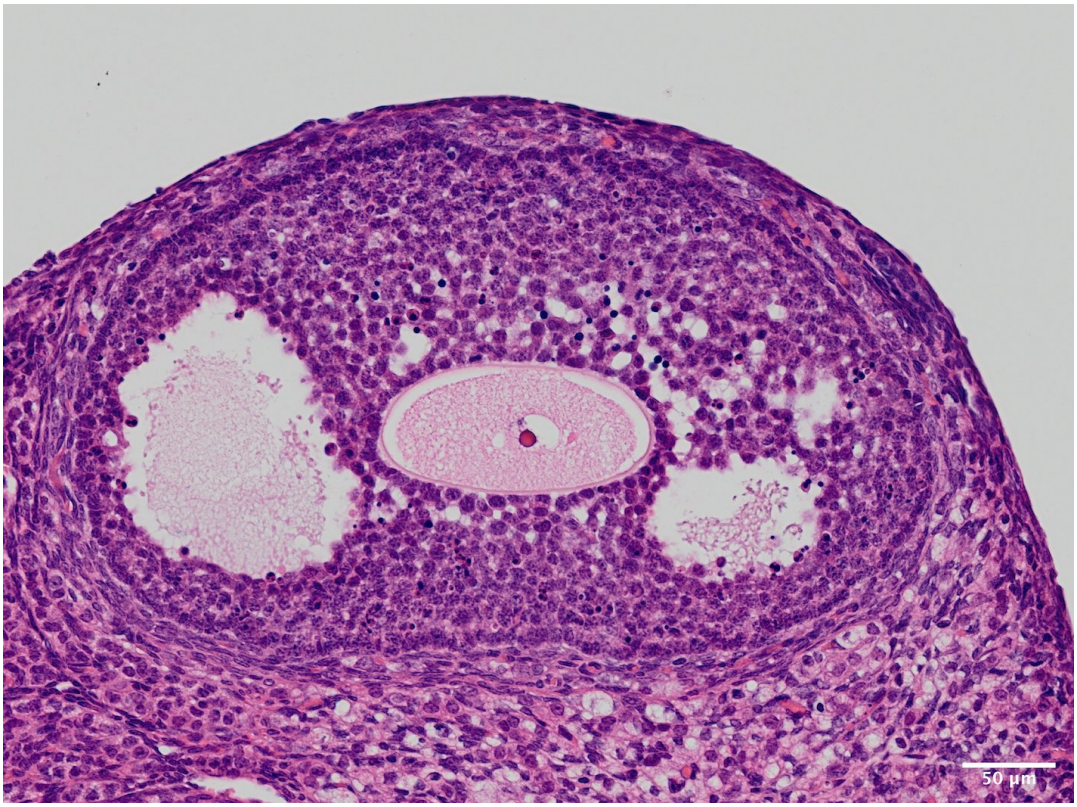

Figure 2.16 Atretic follicle example

Atretic follicle with signs of pyknotic granulosa cells and deformed oocyte shape. Scale 50 µm.

2.10.2.2 Volume

The area of each ovary and CL was measured every ten sections using the software ImageJ version 2.0 to determine the volume. The volume (*V*) of the ovary and CL was calculated using ratio (*R*) of the ovary/CL, previously calculated from the area (*A*) ($A = \pi\ x\ R^2$), and the known thickness (*h*) of the section (4 µm) ($V = \pi\ x\ h\ x\ R^2$).

2.11 Immunohistochemistry (IHC)

2.11.1 Materials

Table 2.15 Reagents for IHC

Reagent/Chemical	Supplier
Clearene	Leica, USA
Ethanol	Sigma-Aldrich, UK
Hydrogen Peroxide	Fisher, UK
Methanol	Fisher, UK
Citric Acid Monohydrate	Fisher, UK
Sodium Hydroxide 1M	Sigma-Aldrich, UK
Avidin-Biotin Complex Kit Sp-2001	Vector, Peterborough. UK
Bovine Serum albumin (BSA) A3311-10G	Sigma-Aldrich, UK
Vectastain. Avidin Biotin Elite Complex (ABC kits)	Vector, Peterborough. UK
DAB Stable buffer	Biogenex
Sodium azide	Sigma-Aldrich, UK
DAB Chromogen solution	Biogenex
Mayer's Haematoxylin	See Appendix
Pertex mounting media	Leica, USA

Tris Buffered Saline (TBS)	Supplier
Sodium Chloride	Sigma-Aldrich, UK
Tris	Sigma-Aldrich, UK

Blocking solution	Supplier
Dubecco's Medium	Sigma-Aldrich, UK
Foetal Calf Serum PAA	Fisher, UK
BSA	Sigma-Aldrich, UK
Goat Serum	Vector, Peterborough. UK

2.11.2 IHC ovary

Ovary sections were deparaffinised in clearene (10 min twice) and rehydrated through graded alcohols (5 min x 3 times) to 70%. 0.5% hydrogen peroxide in methanol was then used to inhibit endogenous peroxidase (10 min) followed by three washes with TBS for two minutes.

For the antibodies used, as suggested by the manufacturers, microwave citrate treatment was performed as antigen retrieval for the antibodies used. 0.01M citrate buffer pH6.0 was placed in a container with the slides with the ovarian sections. To generate a balance in the heat exposure, two other boxes containing buffer and extra blank slides were placed in the microwave. The slides were heated for 25 minutes at 50% power (800W, Sharp Microwave). After this time, slides were washed in running water for two minutes and then washed with TBS (5 min twice).

After the antigen retrieval, the slides were removed from the buffer and exposed to avidin and biotin solution for 20 min each, with three washes of TBS between each incubation. Blocking solution (Table 2.15) containing 5% serum was used to block for 20 minutes at room temperature. After blocking, slides were incubated with primary antibody (Table 2.16) at appropriate dilutions in TBS overnight at 4ºC. The next morning slides were washed with TBS three times for five minutes each, and incubated with biotinylated anti-rabbit goat antibody for 30 minutes and washed with TBS again. Thirty minutes of Avidin biotin-peroxidase complexes incubation was used for immunodetection and visualisation of biotinylated antibody. After three washes with TBS, slides were incubated with 3,3'Diaminobenzidine (DAB) for 5 minutes, rinse in TBS and washed in running water for 5 minutes. Mayer's haematoxylin was used to counterstain sections. Before mounting, sections were dehydrated through graded alcohols, cleared in clearene before finally mounted in pertex.

Table 2.16 Antibodies for IHC of ovary sections

Protein	Source	Code / Company	Dilution
Anti-gamma H2AX	Rabbit polyclonal	Abcam / Ab2893	1:1000
Anti-Brca1	Rabbit polyclonal	Abcam / Ab191042	1:500
Anti-Oct4	Rabbit polyclonal	Abcam/Ab18976	1:200
Secondary Biotinylated	Rabbit	Vector	1:1000

2.12 Differential cell labelling of blastocysts

2.12.1 Materials

Table 2.17 Reagents for differential labelling

Reagent/Chemical	Supplier
Acid Tyrodes	Sigma-Aldrich, UK
H6 Medium	See Appendix A.2
Bovine Serum albumin (BSA) A3311-10G	Sigma-Aldrich, UK
Trinitrobenzene sulphonic acid (TNBS) (P2297)	Sigma-Aldrich, UK
Polyvinil-pyrrolidone (PVP)	Sigma-Aldrich, UK
Anti-Dinitrophenol (Anti-DNP) antibody produced in goat (D9781)	Sigma-Aldrich, UK
Propidium iodine (PI)	Sigma-Aldrich, UK
Guinea pig complement serum (S1639)	Sigma-Aldrich, UK
Phosphate buffered saline (PBS)	Sigma-Aldrich, UK
Ethanol	Sigma-Aldrich, UK
Bisbenzimide Hoechst 33258	Sigma-Aldrich, UK
Glycerol	Sigma-Aldrich, UK

2.12.2 Differential cell labelling of blastocysts

This method was performed according Hardy et al. 1989 with modifications from Eckert et al. 2012. The assays consisted in the identification of the nuclei of the outer TE cells undergoing antibody-mediated complement lysis during immunosurgery and labelled with the fluorochrome propidium iodine.

After the collection of the embryos, blastocysts were washed three times in H6BSA medium. The zona pellucida was removed using Acid Tyrodes (pH 2,3 at 37°C) in a glass cavity block. Afterwards, embryos were let to rest for 10-20 minutes in a large drop of H6BSA in a new cavity block at 37°C. Outer cells of the blastocysts were labelled using Trinitrobenzene sulfonic acid (TNBS, 1ml TNBS plus 9 ml H6-PVP, pH 7.4). Blastocysts were incubated for 10 minutes at room temperature with TNBS and then washed three times in H6-PVP or until the yellow/orange colour from the TNBS was removed. After that, blastocysts were incubated with anti-dinitrophenol (anti-DNP 1 mg/ml of H6PVP) and washed three times in H6PVP.

Blastocysts were placed and incubated in propidium iodine (PI) and Guinea pig complement and put in the incubator (37°C) for 10 minutes. After the end of the incubation, blastocysts were washed three times in H6BSA. For the fixation of the blastocysts, absolute ethanol was used together with Bisbenzimide (Hoechst 33258, 2.5 mg/ml in distilled water) at 4°C for at least 30 minutes or until the image was taken. Hoechst was used as a nuclear marker. For mounting, blastocysts were place in drops of glycerol, covered with a coverslip and images taken with the fluoresce microscope Nikon Elipse Ti to take Z-stack images using the software NIS elements (version 4.30).

Outer cells of the blastocyst exposed to the anti-DNP were lysed by the complement treatment, allowing the PI to enter the lysed trophectoderm (TE) cells and stain the nuclei red. Inner cell mass cells (ICM) were protected against the PI, since the reagent does not go through the single cell layer formed by the TE cells. Instead, ICM were stained by the second fluorochrome during the final alcohol fixation (Hoechst 33342) (Figure 2.18)

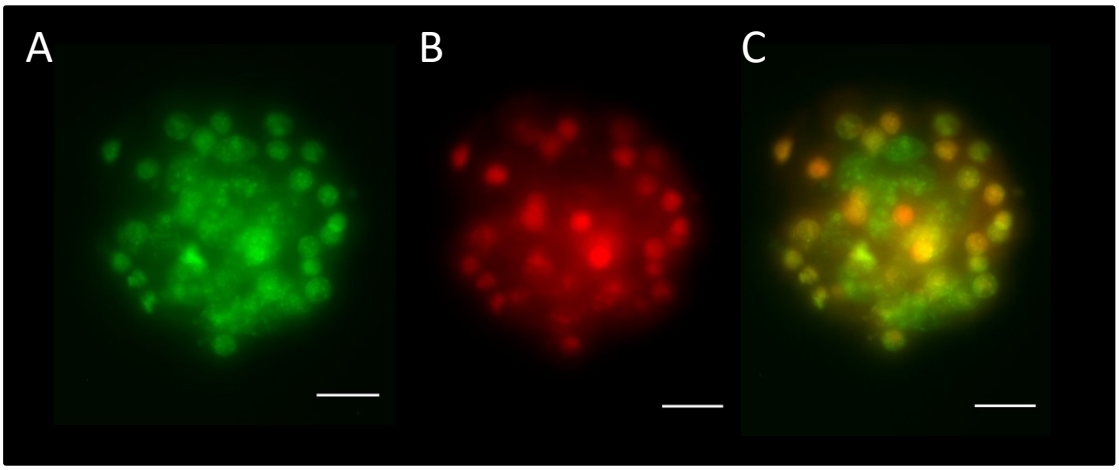

Figure 2.18 Differential labelling in a blastocyst example

Hoechst dyed all the nuclei/Hoechst (green) (A) and propidium iodide (red) just the outer cells (trophectoderm cells) (B). Merge image show yellow (green + red) TE cells and green ICM (B). Scale 20 µm.

2.13 Immuno-labelling

2.13.1 Materials

Table 2.18 Reagents for immuno-labelling

Reagent/Chemical	Supplier
Hydrochloric acid solution HCL 2M	Sigma-Aldrich, UK
Poly-L-lysine hydrobromide (PLL) (P8954)	Sigma-Aldrich, UK
Acid Tyrodes	Sigma-Aldrich, UK
H6	See Appendix A.2
Paraformaldehyde	Sigma-Aldrich, UK
Bovine Serum albumin (BSA) A3311-10G	Sigma-Aldrich, UK
Phosphate buffered saline (PBS)	Sigma-Aldrich, UK
Ethanol	Sigma-Aldrich, UK
Ammonium chloride NH_4Cl	Sigma-Aldrich, UK
Triton X-100	Sigma-Aldrich, UK
Tween-20	Sigma-Aldrich, UK
DAPI 4',6-Diamidine-2'-phenylindole dihydrochloride (D9542)	Sigma-Aldrich, UK
Citifluor PBS solution AF3 (AGR1322A)	Agar Scientific, UK

2.13.2 Chamber preparation

Metal penny repair washes (M6 x 40 size) were used to build metal chambers. After washing carefully, a 22mm coverslip previously treated with HCL 1M was attached to the penny washer with super-glue. After two days, the chambers were ready to use. The same day of the collection of the embryos, chambers were washed twice with PBS and treated with PLL 1.5mg/ml in PBS for 20 min on a hot plate at 60°C

2.13.3 Immuno-labelling blastocyst

All blastocysts collected from the uterus of the female mouse were treated with warmed acid tyrodes to remove the zona pellucida (60-90 secs), they were then left to recover in H6BSA for 20 min at 37°C. After that, blastocysts were fixed with 4% PFA at room temperature for 10 minutes, before washing in PBS-PVP drops for at least 3 times. Embryos were placed carefully in metal

chambers treated with PLL and filled with PBS, and gently liquid was blow over them until they sink down and stick to the bottom of the chamber.

Permeabilization of the embryos was performed with 0.25% Triton X-100in PBS for 15 minutes and washes were done with PBS containing 0.1%Tween 20 (PBST). Neutralisation of the fixation was done by incubation of the embryos in ammonium chloride solution for 10 minutes. After three washes with PBST, embryos were blocked with blocking solution containing PBST and 3% BSA for 30 minutes. Overnight incubation with the primary antibody (Table 2.19) was done with the same blocking solution at 4°C in the dark.

The next morning three washes of 10 minutes each were performed before incubation with the secondary antibody (Table 2.20) in PBST + 3% BSA for 1 hour at room temperature in the dark. After that, embryos were washed three times before incubation with DAPI for 20 minutes at room temperature in the dark. DAPI was removed by washing with PBST. 10µL of Citifluor antifading and mounting solution was added to the chamber containing PBST and immediately covered with a coverslip avoiding air-bubbles. The coverslip was sealed with nail varnish and chambers were kept at 4°C in the dark until confocal analysis.

Imaging was performed with a Leica SP8 Confocal microscope using a 63x glycerol objective and LAS-X software. Images were acquired by accumulation of z-series every 2µm of the whole embryos. The fluorescence signal was given by Alexa 488, 568 and 647 laser (Figure 2.19). For the analysis of the stained embryos, a negative control embryo (without primary antibody) was used per every batch of embryos stained. Negative controls did not show background staining (Figure 2.20).

Table 2.19 Primary antibodies used for immunodetection in blastocysts

Protein	Source	Code / Company	Dilution
Cdx2	Mouse monoclonal	Ab157524 / Abcam	1:100
Nanog	Rabbit monoclonal	Ab80892 /Abcam	1:100
Gata4	Rabbit polyclonal	Ab84593 /Abcam	1:100-1:200
Gata4	Goat polyclonal	AF2606 /R&D System	1:100-1:200
Sox17	Goat polyclonal	AF1924 /R&D System	1:100-1:200
Gata6	Goat polyclonal	AF1700 / R&D System	1:50-1:500

Table 2.20 Secondary antibodies used for immunodetection on blastocyst

Source	Code / Company	Dilution
Donkey Anti-mouse Alexa Fluor 488	Ab150105 /Abcam	1:500
Donkey Anti-goat Alexa Fluor 647	Ab150131 /Abcam	1:500
Donkey Anti-rabbit Alexa Fluor 568	Ab175470 /Abcam	1:500

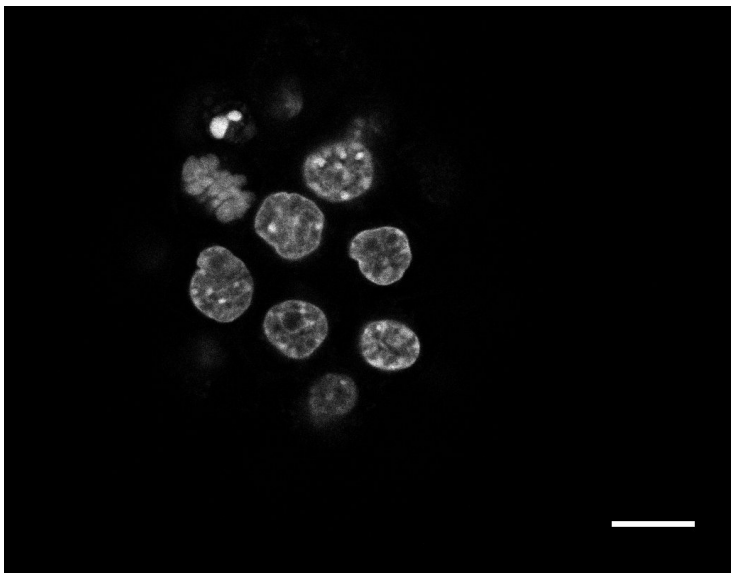

Figure 2.20 Negative control for Immunodetection

Blastocyst incubated with DAPI and secondary antibodies used as negative control. Scale 20 µm

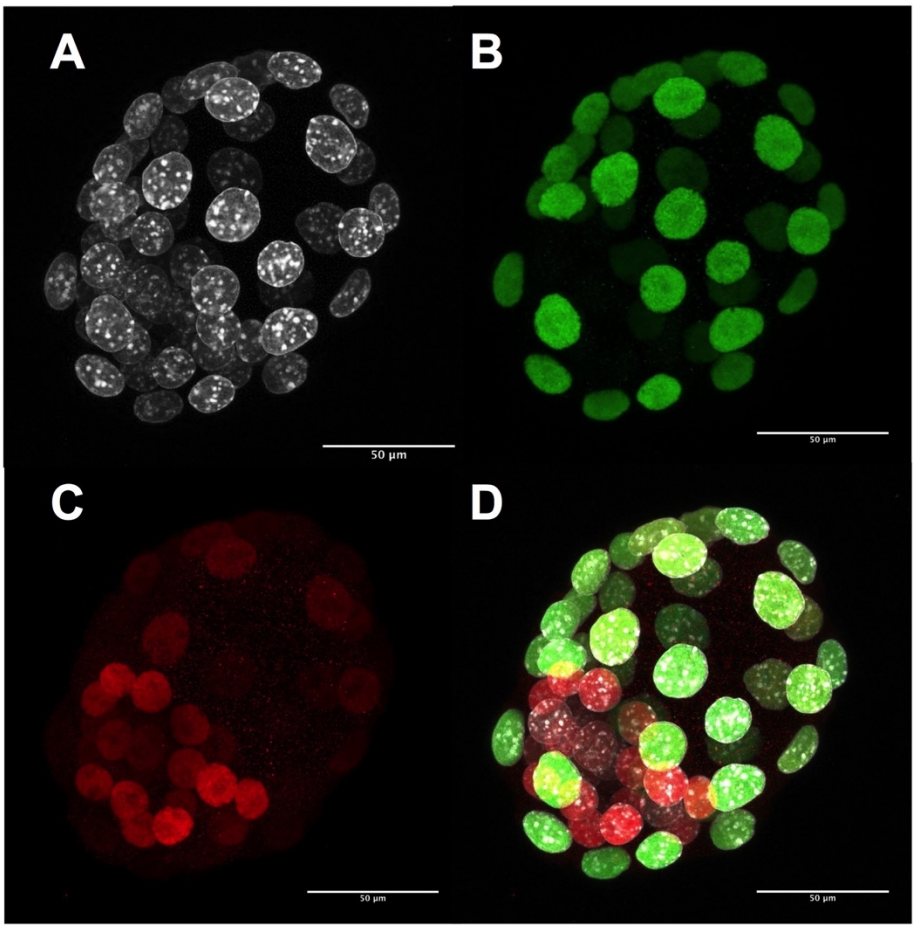

Figure 2.19 Immunodetection CDX2 and NANOG

Immunodetection CDX2 (green) and NANOG (red) cells in blastocyst. Images correspond to DAPI (A), CDX2 (B), NANOG (C) and merge (D). Scale 50 µm.

2.14 Statistical analysis

All the statistical analysis was conducted using STATA statistical software version 12 for Mac or SPSS v24 for Mac. To assess the normality of variables, the Shapiro-Wilk test was used. Student unpaired *t*-test was used for ovarian gene expression analysis after FA supplementation. However, the number of samples in the histological analysis led to no certainly of normal distribution hence, no parametric Mann-Whitney test was used for the analysis of the percentage of follicles per mother. For the analysis of food intake (AUC) and body weight of three groups (embryo project) one-way Anova was used.

For all the analysis related to embryos (total embryo number, embryo cells, gene expression and immunodetection) a multilevel random effect regression model (Watkins, Ursell, *et al.*, 2008) was used to take account of a potential maternal-embryo hierarchical association.

Chapter 3 Results I

Effect of high folic acid supplementation during adulthood on genes related to the follicle development in the ovary

3.1 Introduction

The genome responds to environmental conditions through functional adaptations modulated by epigenetic mechanisms. Nutrition can influence epigenetic modifications during early life, and these changes are often maintained resulting in persistent changes in gene activity and expression (Jiménez-Chillarón *et al.*, 2012). A growing number of studies have shown that folic acid (FA) can induce several alterations in the epigenome (Friso *et al.*, 2017). However, these may be dependent upon both the dose and timing of exposure. Recent studies have suggested that high levels of FA supplementation can increase cancer risk (Kotsopoulos, Kim and Narod, 2012). For example Ly et al., have shown that FA supplementation during pregnancy or the peri-pubertal period increases the number of mammary tumours (Ly *et al.*, 2011). However, further research is needed to identify the mechanisms by which FA supplementation may influence cancer risk. To date, most of the studies have focussed on the effect of FA supplementation on breast cancer susceptibility. Ovarian cancer shares many common features with breast cancer, including the deletion and inactivation of the tumour suppressor gene BRCA1; however, there are no current studies examining the effect of FA on the ovary.

Breast cancer susceptibility gene 1 (BRCA1) is a tumour suppressor gene, which plays a central role in DNA repair. While it is mutated in inherited forms of both breast and ovarian cancer, it has also been reported to be hypermethylated in sporadic forms of breast and ovarian cancer (Puppe *et al.*, 2009). Studies by M Burton (in preparation) have shown that BRCA1 expression is altered in the mammary gland of mice fed with a FA supplemented diet. FA supplementation also induced changes in OCT4, a pluripotency factor, and polycomb genes, which have been shown to play a pivotal role in the initiation and progression of many types of cancer. These genes have also been shown to be important for ovarian cancer development, OCT4 is expressed in ovarian surface epithelial cells (OSE) and has been shown to be over-expressed in ovarian carcinoma (Zhang *et al.*, 2013). Over-expression of EZH2, the catalytic subunit of PRC2, and BMI1, an essential component of PRC1, have been also detected in human ovarian carcinomas (Honig *et al.*, 2010; Rao *et al.*, 2010).

Chapter 3

The aim of this chapter was to identify the effect of the FA supplementation on the expression of genes involved in genome stability and tissue differentiation in the ovary of adult mice. The genes to be examined were BRCA1, OCT4, EZH2 and BMI1.

3.2 Experimental Design

35 Virgin C57BL/6 adult (PND74) mice were randomly assigned to receive control (1mg FA/kg food) or high FA diet (5mg FA/kg food) for four weeks. The high FA diet (5X BDR) exemplifies a human population exposed to food fortification and supplementation with FA (>1000mg FA/day). After four weeks, on PND102, five animals per group were culled. Meanwhile, the rest of the animals were fed a control diet for an additional four (PND 130, n=10 per group) or twelve weeks (PND186, n=10 per group) (Figure 3.1).

Food and weight were monitored weekly. Animal dissection, blood and organ collection were performed by M Burton. Both ovaries from each animal were kept at –80C until further analysis.

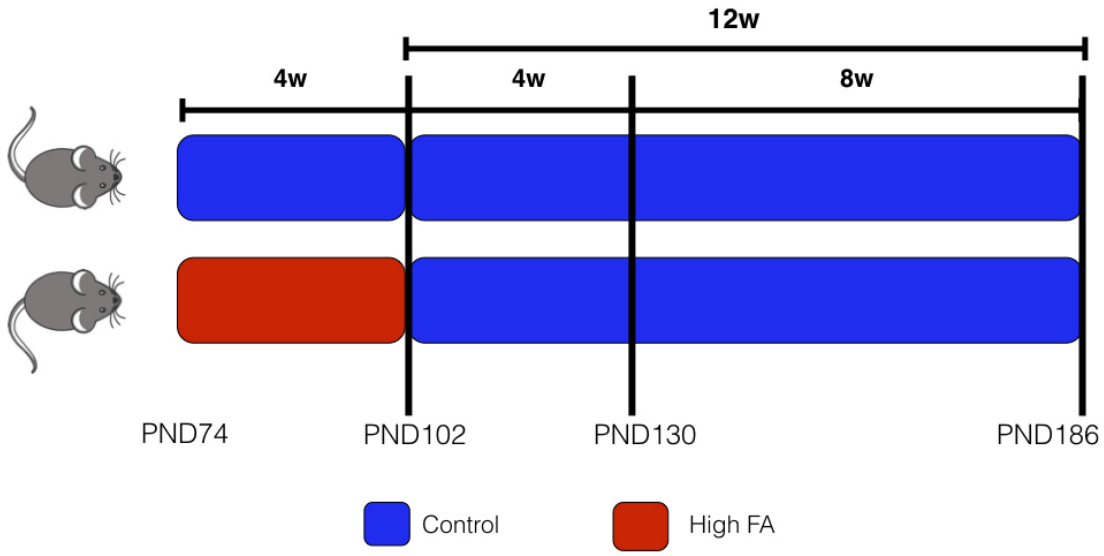

Figure 3.1 Animal model from PND74 to PND186

Virgin adult female C57BL76 mice were fed either a control diet or a high FA diet for four weeks (from PND74 to PND102). Some animals were also called at PND102, meanwhile others continued with control diet and culled at PND130 or PND186.

3.3 Results

3.3.1 Gene expression in the ovary

Previous studies had shown that the expression of BRCA1, OCT4 and the polycomb genes EZH2 and BMI1, were altered in the mammary gland after FA supplementation. As these genes also play a pivotal role in ovarian development and cancer progression, the effect of FA supplementation on the expression of these genes in the ovary was investigated. Gene expression was normalised to *Gapdh* and *Cyc* as references genes selected after geNorm analysis (See Figure 2.10). The analyses are shown at three-time points: PND102; immediately after supplementation, PND130; four weeks after the end of supplementation and PND186; twelve weeks after the end of supplementation.

3.3.1.1 Gene expression of Brca1

BRCA1 is a member of a family of genes involved in DNA repair. BRCA1 is often mutated in inherited forms of breast and ovarian cancer, or methylated and silenced in sporadic forms. However, in healthy ovarian tissue, BRCA1 is expressed in the nuclei of GCs and oocytes (D. Zhang *et al.*, 2015). FA supplementation decreased *Brca1* expression in the ovaries ($P = 0.03$) at PND102, but there was an increase in expression at PND130 ($P = 0.0001$) four weeks after the end of supplementation. However, there was no difference between the groups at PND186 ($P = 0.55$).

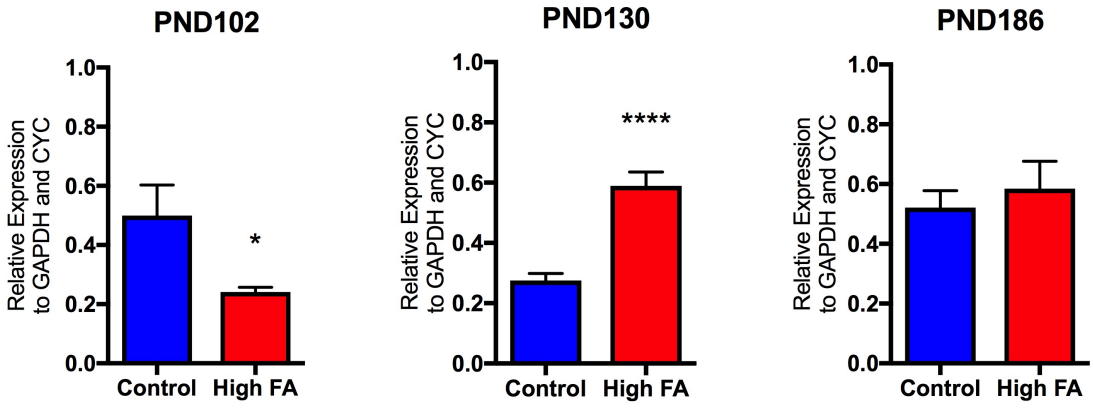

Figure 3.2 Gene expression of Brca1 in ovaries at PND102, 130 and 186

Gene expression level of *Brca1* in ovarian tissues removed from adult C57BL/6 mice at PND102, 130 and 186. The gene expression was normalised to reference genes *Gapdh* and *Cyc*. The results are shown as mean ± SEM (n = 5-10 mice per group). T-test analysis. * $P < 0.05$, **** $P < 0.0001$

3.3.1.2 Gene expression of cell differentiation control genes

The development of the ovarian follicles comprises an orchestra of genes regulated by hormones and growth factors. The hormone signalling pathways includes not only transcription factors such as OCT4, but also the recruitment of epigenetic machinery including the polycomb complex (LaVoie, 2005).

3.3.1.2.1 Gene expression of Oct4

Octamer-binding transcription factor 4 (OCT4), is expressed in the oocyte and it is essential for the recruitment of primordial follicle in the cycle of follicle development (Bahmanpour *et al.*, 2013). FA supplementation did not alter *Oct4* expression at PND102 (*P* = 0.11). However, there was an increase in Oct4 expression four weeks after the end of supplementation on PND130 (P = 0.0007), but this increase in expression did not persist at twelve weeks after the end of supplementation (PND186, *P* = 0.50).

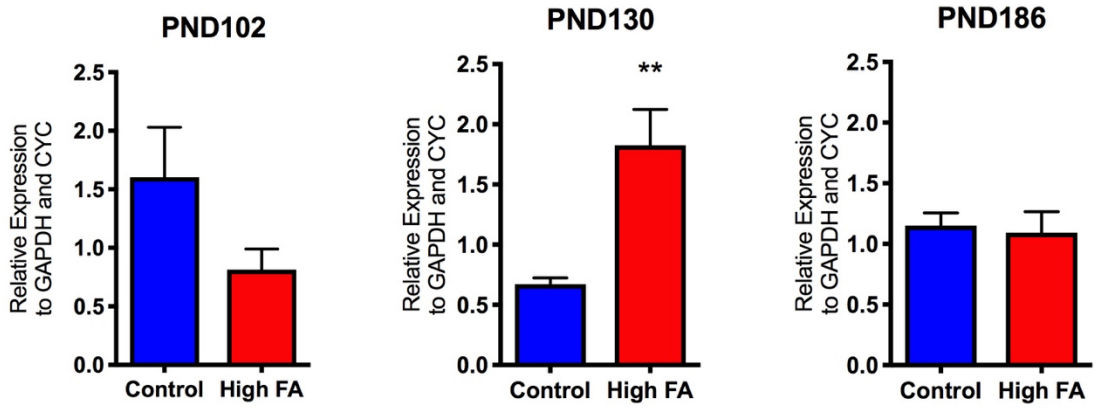

Figure 3.3 Gene expression of Oct4 in ovaries at PND102, 130 and 186

Gene expression level of *Oct4* in ovarian tissues removed from adult C57BL/6 mice at PND102, 130 and 186. The gene expression was normalised to reference genes *Gapdh* and *Cyc*. The results are shown as mean ± SEM (n = 5-10 mice per group). T-test analysis. * *P* < 0.05

3.3.1.2.2 Gene expression of chromatin modifiers

Polycomb group (PcG) proteins are epigenetic chromatin modifiers which play a critical role in stem cell renewal and the maintenance of lineage-specific gene expression programs (Jacobs and van Lohuizen, 2002). They form two complexes, Polycomb repressive complex 1 and Polycomb repressive complex 2 (PRC1 and PRC2), which work together to mediate stable transcriptional repression through trimethylation of histone H3 at lysine 27 (H3K27me3), which is catalysed by the PcG protein EZH2. Polycomb proteins regulate BMI, which is a member of the PRC1, and mediates gene silencing by regulating chromatin structure on genes related to DNA damage response, senescence control and stem cell fate decisions (Bhattacharya *et al.*, 2015).

3.3.1.2.2.1 EZH2

EZH2 is expressed in the ovarian epithelial cells and GCs of preovulatory follicles, which also express high levels of H3K27me3 (Li *et al.*, 2010; Maekawa *et al.*, 2016). *Ezh2* was downregulated immediately after FA supplementation (PND102, P = 0.01) but upregulated by PND130 (P = 0.0006). However, there was no change at PND186 on the *Ezh2* gene expression in the ovaries (P = 0.18).

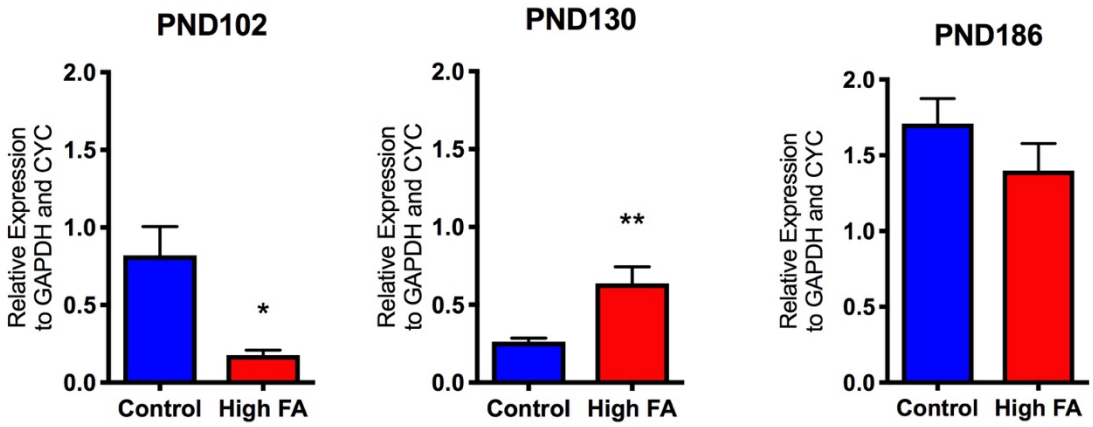

Figure 3.4 Gene expression of Ezh2 in ovaries at PND102, 130 and 186

Gene expression level of *Ezh2* in ovarian tissues removed from adult C57BL/6 mice at PND102, 130 and 186. The gene expression was normalised to reference genes *Gapdh* and *Cyc*. The results are shown as mean ± SEM (n = 5-10 mice per group). T-test analysis. * $P < 0.05$, ** $P < 0.01$

3.3.1.2.2.2 BMI1

BMI1 is highly expressed in ovarian cancer cells and mediates gene silencing together with EZH2 (Abd El Hafez and El-Hadaad, 2014). In this project, FA supplementation decreased *Bmi1* expression in the ovary at PND102 (P = 0.02). However, four weeks after the end of supplementation, ovaries showed an increase in *Bmi1* expression (P = 0.09), but a decrease at PND186 (P = 0.03).

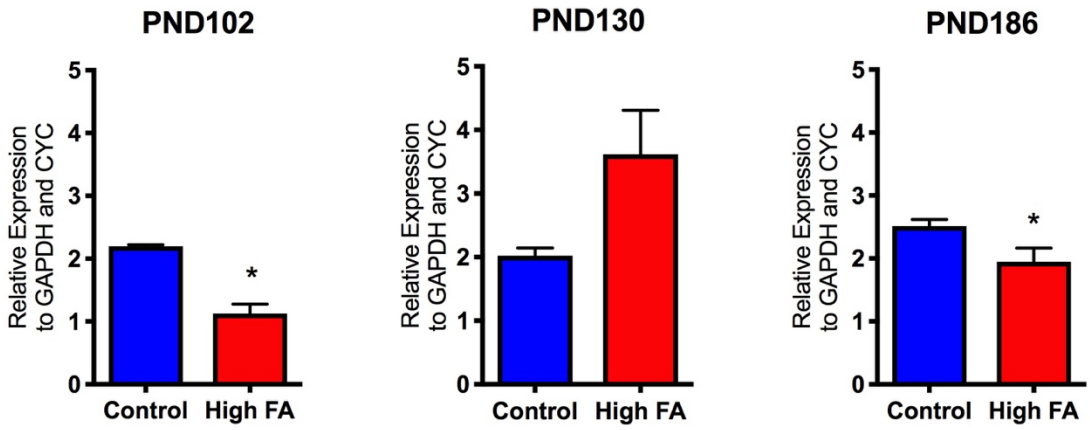

Figure 3.5 Gene expression of Bmi1 in ovaries at PND102, 130 and 186

Gene expression level of *Bmi1* in ovarian tissues removed from adult C57BL/6 mice at PND102, 130 and 186. The gene expression was normalised to reference genes *Gapdh* and *Cyc*. The results are shown as mean ± SEM (n = 5-10 mice per group). T-test analysis. * $P < 0.05$

3.4 Discussion

The link between FA supplementation and cancer risk has been highly controversial. Some, but not all, studies have shown that folate intake is inversely related to cancer risk. However, more recent studies have suggested that there is a U shaped relationship between folate/FA intake and cancer risk suggesting high supplementation may lead to increased tumour formation (Cole *et al.*, 2007; Figueiredo *et al.*, 2009; Fife *et al.*, 2011; Chen *et al.*, 2014; Mortensen *et al.*, 2015). In a prospective study, women with BRCA1/2 mutations and high plasma folate had 3.2-fold increased risk of developing breast cancer compared to women with lower folate levels (S. Kim *et al.*, 2016). In rodents, supplementation of FA has shown an increased risk of mammary adenocarcinomas and tumour progression after carcinogen DMBA exposure when a mammary tumour is induced (Ly *et al.*, 2011; Deghan Manshadi *et al.*, 2014).

To examine the effect of FA supplementation on the expression of genes associated with ovarian cancer risk, mice were fed with control or high FA diet for four weeks and ovaries analysed at three different time-points after supplementation. The results showed that immediately after supplementation (PND102) there was a decrease in the expression of *Brca1* and chromatin modifiers *Ezh2* and *Bmi1*. However, these outcomes were not always maintained twelve weeks after supplementation. On the contrary, *Brca1* and *Ezh2* expression was higher in the FA supplemented group compared to controls, four weeks after the end of supplementation, with no changes at PND186. In the case of *Oct4*, there was an increase in its expression at PND130, but this was not maintained to PND186.

In this project, genes related to DNA stability and differentiation were analysed at the mRNA level, and so whether such changes were mirrored at the protein level are not known. Western blotting would allow protein expression to be assessed and histological analysis would provide information about the localisation of those changes.

OCT4 is a pluripotency marker essential in the development of the follicle and mainly present in the oocyte (Monti and Redi, 2009). In stem cells, OCT4 participates in the regulation of differentiation and proliferation, and it is regulated by EZH2 (Pursani *et al.*, 2017). In this project, FA supplementation led to an increase in the expression of *Oct4* and *Ezh2,* which suggests a shared regulation. Increased *Oct4* expression in the ovaries by FA may impact on the regulation of the follicle cycle and/or the quality of the oocyte. However, further analysis is necessary to determine the genuine effect in the ovarian follicle.

EZH2 plays an important role in the aetiology of several tumours. Expression of EZH2 at both protein and mRNA levels have been correlated with the histological grade of ovarian cancer (Lu *et*

al., 2007; Guo *et al.*, 2011). Over-expression was associated with an increase in proliferation rate, while knockdown of EZH2 expression in ovarian carcinoma cell lines leads to cycle arrest, reduced growth and inhibition of cell migration (Visser *et al.*, 2001; Rao *et al.*, 2010). *Ezh2* was downregulated after four weeks of FA supplementation (PND102), however was then upregulated four weeks after supplementation had ended (PND130). This may mark a compensation mechanism, but suggests that changes in *Ezh2* expression may alter the level of histone methylation and the expression of key pluripotency and developmental control genes. Upregulation of the methyltransferase has been associated with the silencing of target-genes related to growth control such as CDKN1C ($p57^{KIP2}$), which is inactivated in certain cancers and is associated with regulation of cell growth and differentiation (Kavanagh and Joseph, 2011). Given the pivotal role that EZH2 plays, it will be essential to determine whether levels of H3K27 methylation change after FA supplementation and the downstream effects.

BMI1, which catalyses the mono-ubiquitination of histone H2A and stabilises PRC2, has a vital role in the maintenance of self-renewal, differentiation capacities and prevention of senescence. Additionality, BMI1 has been implicated in different malignancies including ovarian cancer. Its expression is associated with advanced stage and higher grades of ovarian cancer, including shorter patient survival (Zhang, Sui and Xin, 2008; Abd El Hafez and El-Hadaad, 2014). BMI1 acts by regulation of genes related to stem cell fate decisions, activation of telomerase activity, and repression of INK4A/ARF locus which encodes $p16^{INK4A}$ and $p14^{ARF}$, two tumour suppressor and cell cycle regulator genes (Park *et al.*, 2003; Park, Morrison and Clarke, 2004). Moreover, BMI1 has a role in mitochondrial homoeostasis and DNA repair that is independent of the INK4A/ARF locus pathway. KO mice for BMI1 showed significant mitochondrial dysfunction and increased ROS (reactive oxygen species) levels which could be sufficient to induce DNA damage (Liu *et al.*, 2009). Ginjala et al., showed recruitment of BMI1 at sites of DNA breaks and its role in ubiquitination of H2A at lysine 119 to contributes to efficient DNA repair (Ginjala *et al.*, 2011). Ovaries from adult mice fed with high levels of FA showed a decrease of *Bmi1*, which could be associated with increased mitochondria dysfunction, increased ROS production and DNA damage. This study also showed that FA supplementation affected *Brca1* expression, which also plays an essential role in DNA damage detection and repair, as well as cell cycle arrest. In the ovary, BRCA1 controls cell proliferation; an increase in BRCA1 expression has been observed in GCs of developing follicles, while BRCA1 is downregulated in large and antral follicles. Moreover, BRCA1 has been described as a risk factor for breast and ovarian cancers in humans, inactivation of BRCA1 in GCs leads to the development of cyst tumours in the ovaries and uterine horns (Antoniou *et al.*, 2003; Chodankar *et al.*, 2005). FA supplementation of adult mice showed a decreased in *Brca1* expression immediately after the end of supplementation. These results

together with a decrease in *Bmi1*, could mean an alteration in the balance of DNA repair/damage checkpoint responses regulating the cell cycle or promoting apoptosis. Increased *Brca1* expression at the later timepoint may reflect a response to the increased DNA damage. Interestingly, *Ezh2* and *Brca1* showed the same temporal changes in gene expression at PND102 and PND130 in adults. Studies have suggested that *Ezh2* and *Brca1* interact and therefore alteration in one gene may therefore influence the other. For example, Gonzalez et al., reported that *Ezh2* downregulation in ER-negative breast cancer cells depends on BRCA1 upregulation to decrease proliferation and progression of G_2/M through the cell cycle (Gonzalez *et al.*, 2009). In contrast, BRCA1 knockdown increased EZH2 occupancy and H3K27me3 level in the promoter of EZH2 target genes such as HOXA9.

This study suggests that FA supplementation, even at relatively moderate levels can induce long term effects on the expression of key genes involved in ovarian cancer initiation and progression. It will be important to determine whether these changes at the RNA level are also mirrored by downstream functional effects and indeed whether similar changes are seen in humans. Although, there are a few limitations concerning the experiment: the oestrus cycle was not checked at the moment of culling, and differences in the hormone levels could have an impact in the gene expression analysis. The ovaries are in a constant cycle that includes the follicular and luteal phase with endocrine fluctuations of oestrogen, FSH and LH. Oestradiol level has an essential role in the ovary; it allows the development of the follicle and the release of the healthy oocyte. *Brca1* expression is known to be closely related to oestradiol levels in the ovary. Mutant mice that do not express BRCA1 in their GCs showed an increase of oestrogen, and an increase in the proestrus stage of the cycle, which is characterised by high levels of the hormone (Hu *et al.*, 2005; Hong *et al.*, 2010). Additionally, these results are focused just on the mRNA levels of gene expression analysis, and it will be important to determine whether the protein levels are also changed. Furthermore, it is essential to mention that the number of animals available immediately after the supplementation was lower (five animals per group) compared with the 4 and 12 weeks after the supplementation (ten animals per group).

3.5 Conclusion

There is extensive evidence of the detrimental effect of low folate diet can have for NTD and the risk of cancer (Hursthouse *et al.*, 2011; S. Kim *et al.*, 2016). However, the effect of high levels of FA supplementation has not been fully examined. Despite the limitations of this project, the results presented here show; firstly, that a high FA diet altered genes associated with DNA stability such as BRCA1, and differentiation such as OCT4, EZH2 and BMI1 in the ovary. Secondly, the effects of FA supplementation were often observed after the end of supplementation, although in many cases the direction of the effect was reversed. These changes in expression suggest there may be other alterations in ovary on follicle morphology or developmental markers upon FA supplementation. This will be examined in the following chapter.

Chapter 4 Results II

Effect of high FA supplementation on the morphology and follicle development in the ovary of adult mice at diestrus stage

4.1 Introduction

The development of ovarian follicles involves endocrine and paracrine factors, which facilitate the coordination of follicle growth for successful ovulation. Hormone-regulated follicular development involves the integration of gonadotropin-releasing hormone (GnRH), the anterior pituitary release of gonadotropins (LH and FSH) and ovarian sex steroids (oestradiol and progesterone).

Primordial follicles remain quiescent until puberty when they develop into primary and later follicle stages. After primordial follicle activation, members of the TGFβ family such as activin and GDF9 regulate the development of secondary follicles in a gonadotropin-independent process. LH and FSH regulate the growth of the early antral to the preovulatory follicle, where FSH is the major stimulator of the follicle growth. Oestradiol (E2) production is regulated by FSH stimulating aromatase P450 expression in mural GCs of preovulatory follicles, inhibiting cell apoptosis and promoting cell development (Matsuda *et al.*, 2012). LH controls the process of ovulation through the stimulation of progesterone (P4) and inhibition of E2 synthesis by GCs, which leads to the follicle rupture and formation of the corpus luteum (CL).

The follicular developmental process involves differentiation and proliferation factors. OCT4, a pluripotent marker related to the silencing of developmental control genes, is modulated by gonadotropins during the differentiation of GCs and oocytes (Bagheripour *et al.*, 2017). BRCA1, a tumour suppressor gene is also present in GCs. It has been reported to be a key regulator of basal aromatase expression, as indicated by an inverse correlation between *Brca1* and aromatase mRNA and protein levels observed in a GC line (Hu *et al.*, 2005). The development of the follicle also involves epigenetic mechanisms. Methylation, acetylation and ubiquitination are some of the modifications of the chromatin associated with the regulatory effects of the endocrine and paracrine factors.

Any disturbance of the microenvironment around the oocyte may have detrimental effects on the female gamete. Before ovulation, GCs and the oocyte depend on the diffusion of blood plasma

nutrients which cross the blood follicular barrier and secretory activity of both granulosa and theca cells (Forges *et al.*, 2007; Revelli *et al.*, 2009). There is growing evidence that one-carbon metabolites, which includes folate, cobalamin and homocysteine, influence follicle development and fertility (Forges *et al.*, 2007). These nutrients are co-factors in the biosynthesis of amino acids, nucleotides and DNA methylation; processes that are critical for the correct regulation of the ovulatory cycle. However, not all the evidence is conclusive. Folate and homocysteine concentrations in the follicular fluid have been inversely correlated with the follicular diameter in women undergoing *in vitro* fertilisation (IVF) suggesting a possible detrimental effect on the follicle development (Boxmeer, Brouns, *et al.*, 2008; Boxmeer *et al.*, 2009).

Previously, high FA diet altered genes related to follicle development and cell differentiation, however, there was no oestrus evaluation of the mice before culling them. For that reason, the aim of this project was to determine the effect of a FA diet on ovarian function, at a specific oestrus stage, on the expression of genes related to proliferation and differentiation, as identified in the previous chapter (See Chapter Results I), and also determine the effect of FA supplementation on ovarian morphology.

4.2 Experimental Design

24 Virgin C57BL/6 adult (PND74) mice were randomly assigned to receive control (1mg FA/kg food) or high FA diet (5mg FA/kg food). Food and weight were monitored weekly. After four weeks of treatment, all mice were culled at the diestrus stage of the oestrus cycle to avoid alterations on the hormones levels associated with the oestrus cycle (PND102) (Figure 4.1). Animal dissection, blood and organ collection is described in Methods sections 2.1.5 and 2.1.7.

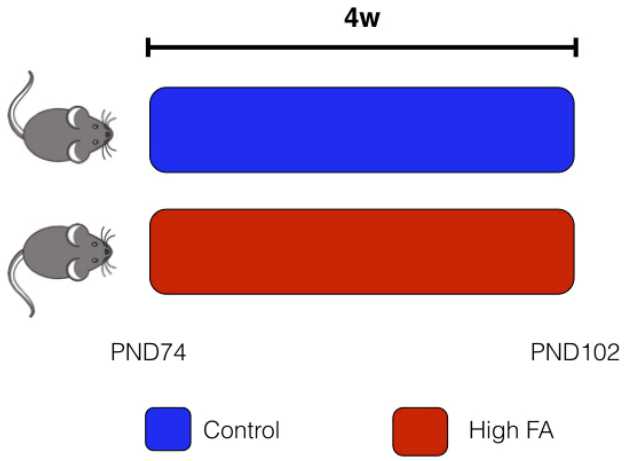

Figure 4.1 Animal model PND102 at diestrus stage

Adult female C57BL/6 mice (PND74) were fed with control and high FA diet during four weeks and culled at diestrus stage (PND102).

4.3 Results

4.3.1 Weight and energy intake measurements

At PND74 animals weight from control and high FA group did not differ between groups (*P* = 0.58). Food per cage and individual body weight were monitored at the same hour weekly during the experimental period. The animals showed no changes in body weight (Figure 4.2) or energy intake (Figure 4.3) with the high FA diet during the four-week period.

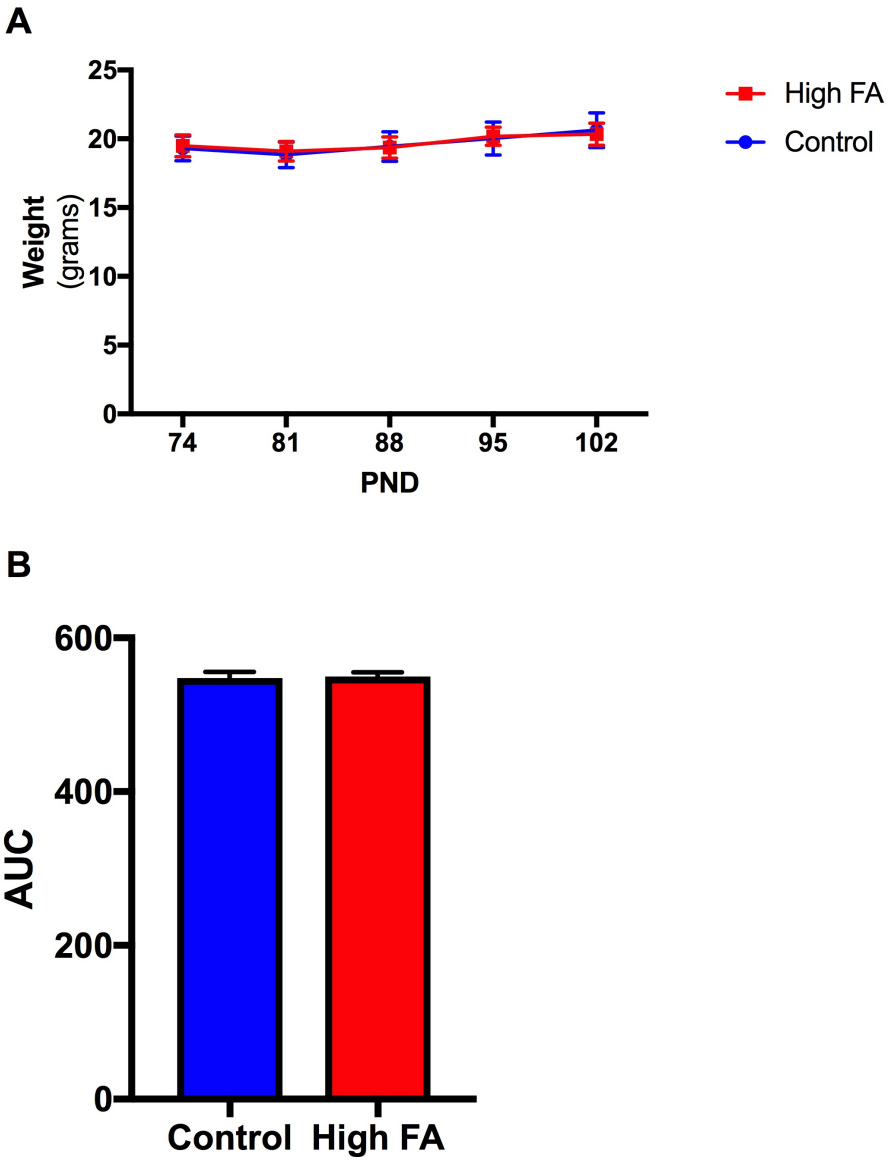

Figure 4.2 Body weight during four weeks of diet between PND74 to PND102

Body weight of C57BL/6 female mice during four weeks of high FA diet (A). Mean ± SD. Area under the curve (AUC) analysis of the body weight (B). Mean ± SEM. n = 12 animals per group. T-Test

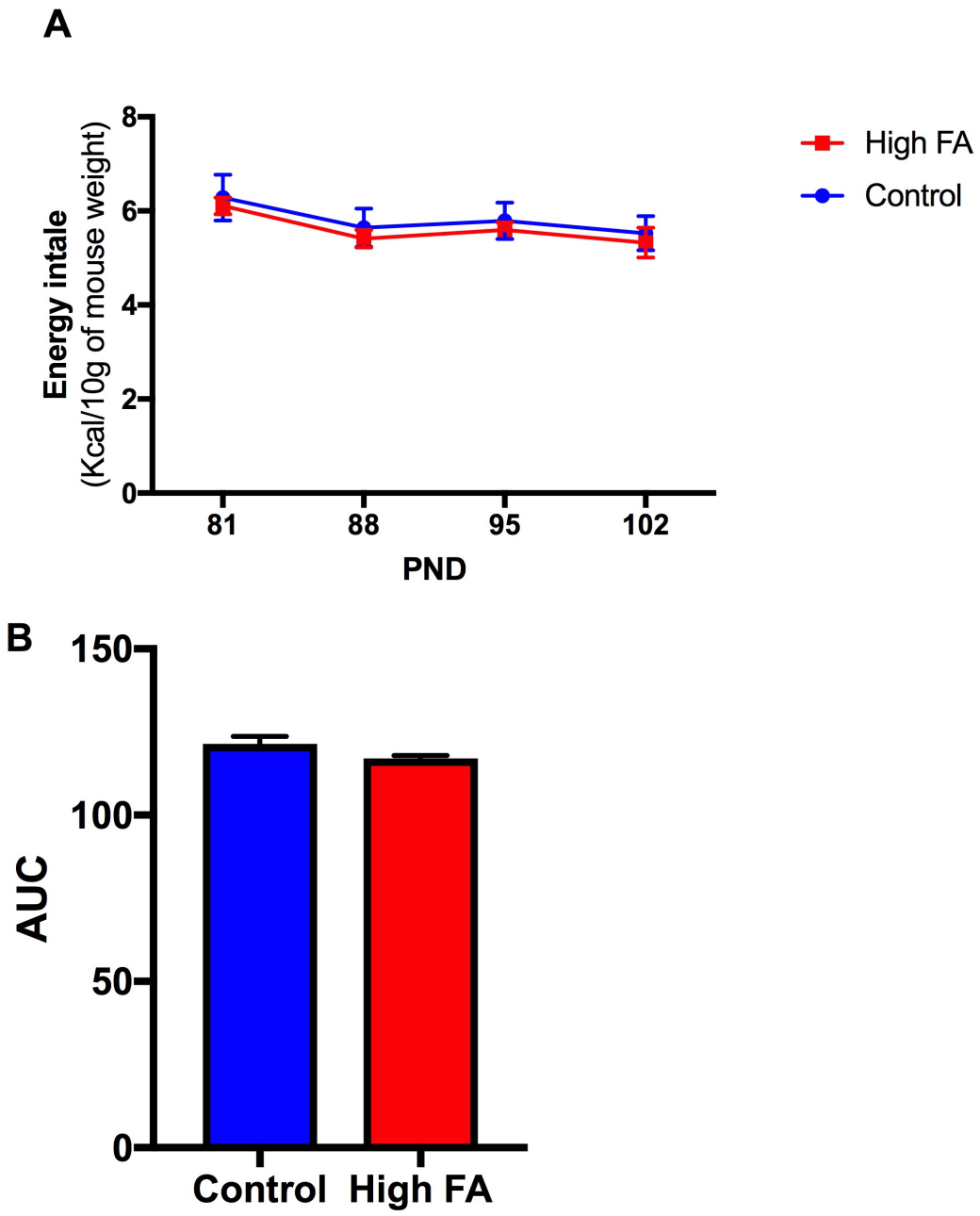

Figure 4.3 Energy intake during four weeks of diet between PND74 to PND102

Energy intake of C57BL/6 female mice after four weeks of high FA diet (A). Mean ± SD. Area under the curve (AUC) analysis of the body weight and food consumption per cage (B). Mean ± SEM. n = 12 animals per group. T-Test

4.3.2 Folic acid and metabolites in plasma

FA is metabolised throughout several steps including diverse transporters and enzymes (See Figure 4.5). To confirm that the increased FA supplied in the diet in the high FA group did affect FA and folate levels in the mice, plasma samples were analysed by LC-MS/MS to assess folate and folate catabolites. Methyltetrahydrofolate (mTHF), the most abundant bioactive form of folate was significantly higher in the high FA group ($P < 0.0001$). Similarly, levels of unmetabolised FA were higher in the high FA group ($P = 0.01$), with levels of unmetabolised FA being almost negligible in the control samples. pABG (para-aminobenzoylglutamic acid), a catabolite of folate usually generated after oxidation of folate, was higher in the supplemented group ($P = 0.04$).

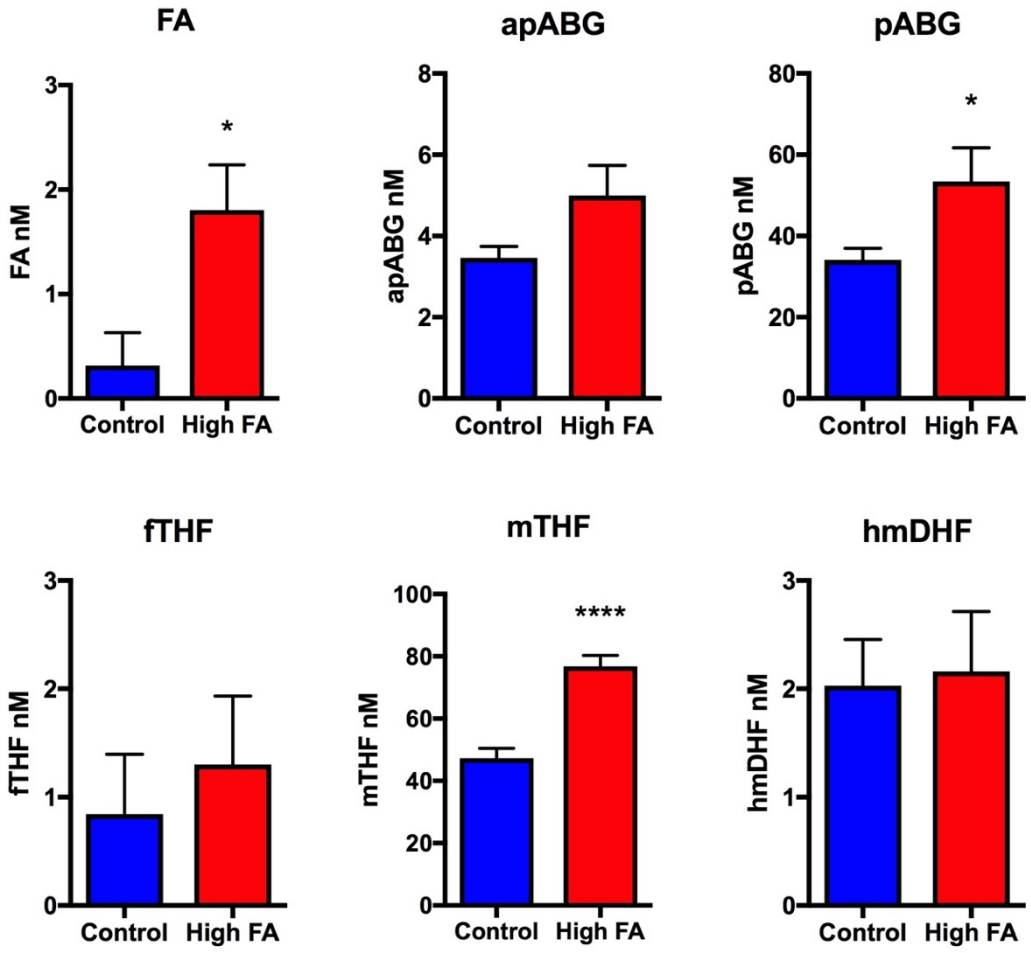

Figure 4.4 FA and FA metabolites in plasma of female mice PND102

Plasma folate and its metabolites mTHF (5-methyl-tetrahydrofolate), FA (folic acid), fTHF (5-formyl-tetrahydrofolate), hmDHF (4-alfa-hydroxy-5-methyl-tetrahydrofolate), apABG (acetyl-para-aminobenzoylglutamic acid) and pABG (para-aminobenzoylglutamic acid). The results are shown as mean ± SEM (n = 8 mice per group). T-test analysis. **** $P < 0.0001$, * $P < 0.05$

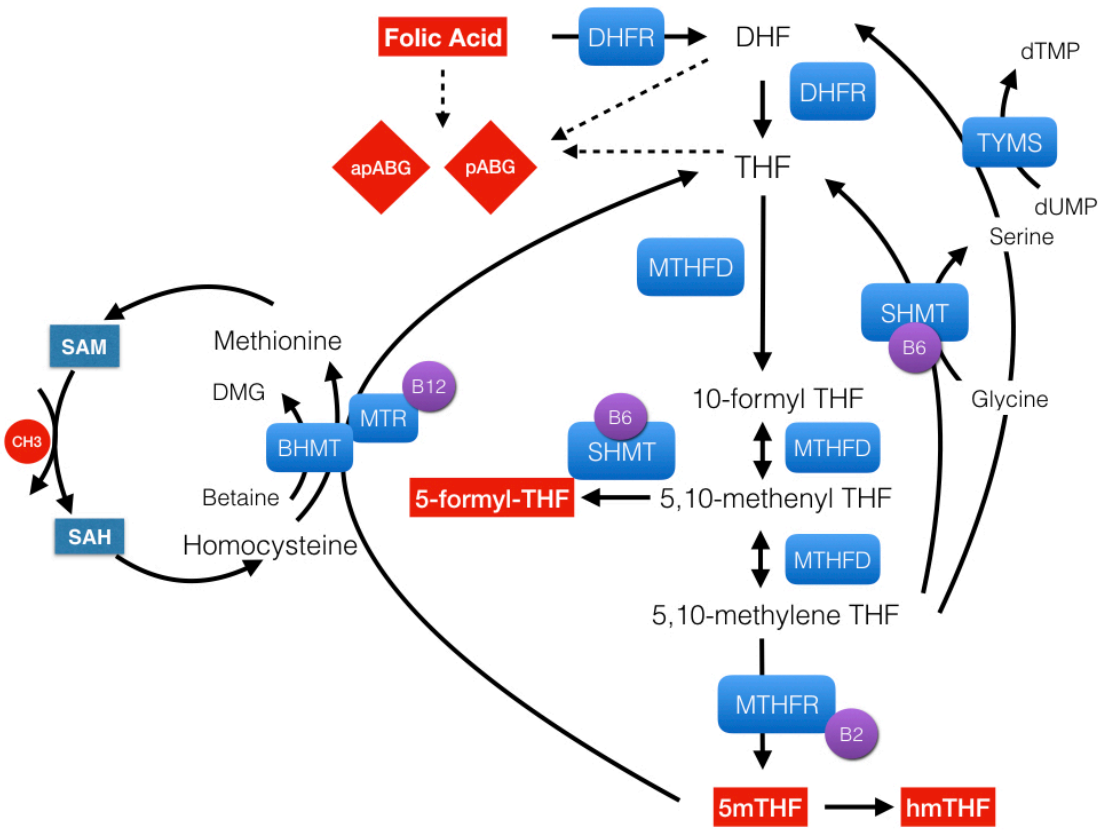

Figure 4.5 Folic acid cycle

Folic acid metabolism and its metabolites in red: apABG (acetyl-para-aminobenzoylglutamic acid), pABG (para-aminobenzoylglutamic acid), 5-formyl-THF (5-formyl-tetrahydrofolate), 5mTHF (5-methyl-tetrahydrofolate) and hmDHF (4-alfa-hydroxy-5-methyl-tetrahydrofolate).

4.3.3 Gene expression in the ovary

To determine the effect of FA on gene expression in the ovarian tissue of adult mice at diestrus stage, one ovary per female was isolated, and qPCR was performed using specific primers for follicle development control and cell differentiation (See primers list Table 2.8). The gene expression was normalised to *Sdha* and *18s* as references genes, previously selected by geNorm analysis (See Figure 2.11).

4.3.3.1 Gene expression of folate transporters

The folate machinery has been reported to be expressed in the ovary, specifically folate receptor (FR) and the reduced-folate carrier (RFC) have been detected in the oocyte and GCs (Meredith *et al.*, 2016). In this project, the expression of *Folr1* was not detected, however, the folate transporters *Slc46a1* (which encodes RFC) and *Slc19a1* (which encodes PCFT) were detected and analysed. The ovaries showed significant downregulation of *Pcft* (P = 0.02) but not of *Rfc*1 transporter (P = 0.2) after high FA exposure.

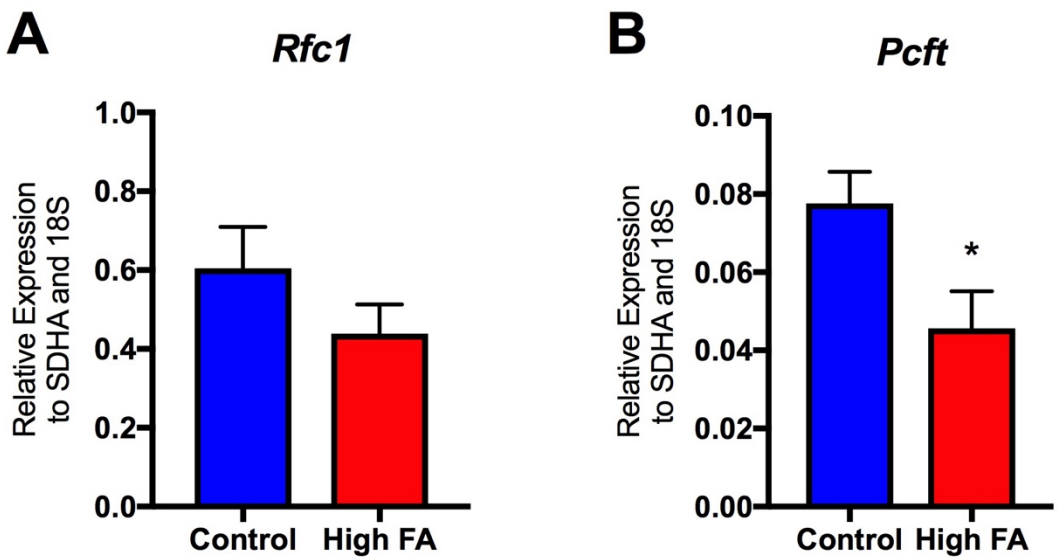

Figure 4.6 Gene expression of folate transporters in ovaries at PND102

Folate transporters *Rfc1* (SLC19A1) and *Pcf1* (SLC46A1) gene expression level in ovarian tissue removed from adult C57BL/6 mice at diestrus stage. The gene expression was normalised to *Sdha* and *18s* used as reference genes. The results are shown as mean ± SEM (n = 12 mice per group), T-test analysis. * $P < 0.05$.

4.3.3.2 Gene expression of follicle development regulation factors

The development of the follicle is regulated by several factors that include paracrine and endocrine factors. The expression of these factors depends on the type of cell (somatic or oocyte), the follicle developmental stage and the oestrus cycle. In this project, the effects of high FA intake on differentiation and proliferation-regulatory genes were analysed.

4.3.3.2.1 Gene expression of Gdf9

Growth differentiation factor-9 (GDF9) belongs to the TGFβ family and is expressed throughout all stages of follicle development, but is essential for the development of early stage follicles (Cook-Andersen *et al.*, 2016). There was no effect of a high FA intake on the expression of *Gdf9* in the ovaries.

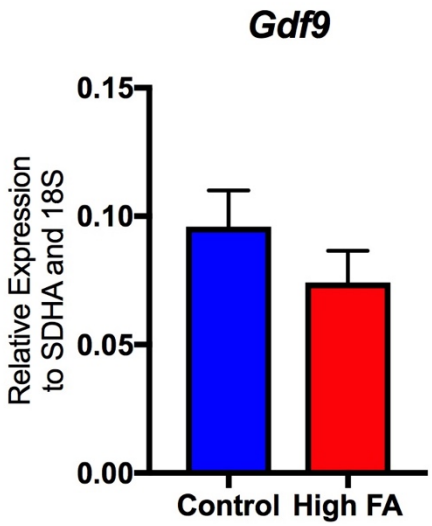

Figure 4.7 Gene expression of Gdf9 in ovaries at PND102

Gdf9 gene expression level in ovarian tissues removed from adult C57BL/6 mice at diestrus stage. The gene expression was normalised to *Sdha* and *18s* used as reference genes. The results are shown as mean ± SEM. (n = 12 mice per group). T-test analysis.

4.3.3.2.2 Gene expression of gonadotropin hormone receptors

The pituitary gonadotropin hormones FSH and LH are essential for the ovarian follicle development during cyclic recruitment, which involves the growth of small antral follicles to pre-ovulatory stages (Cheng *et al.*, 2002). FSHR that is expressed in GCs and normal ovarian surface epithelium regulates the ovarian steroid production and GC proliferation. Meanwhile, LHR is expressed in mural GCs of antral follicles and corpus luteum (CL). The binding of LH to LHR stimulates the steroidogenic activity (Hsueh *et al.*, 2015). Here, there was a reduction of *Fshr* (P = 0.04), but no change in *Lhcgr* (P = 0.15) ovarian expression (Figure 4.8) in the mice fed the high FA diet for 4 weeks compared to control ovaries.

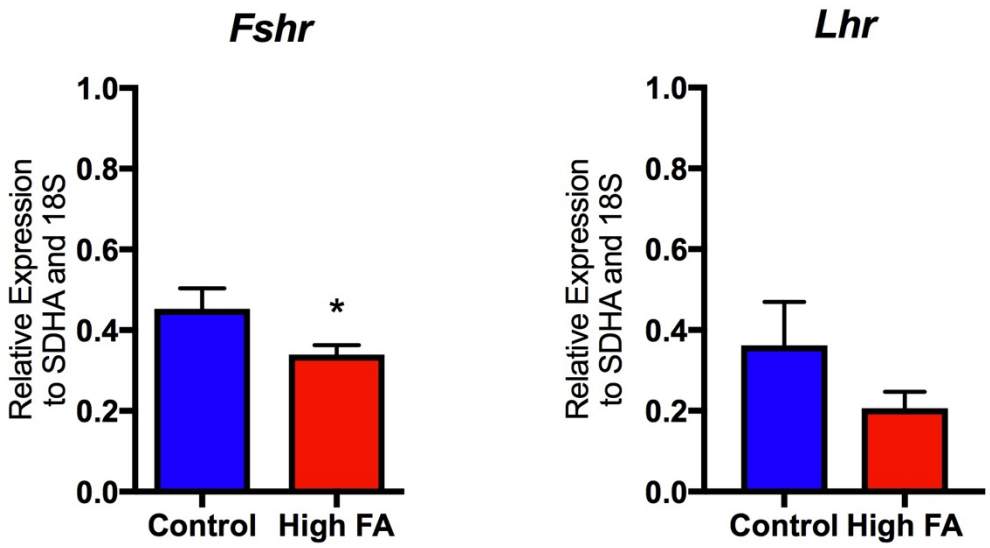

Figure 4.8 Gene expression of gonadotropin hormone receptors in ovaries at PND102

Fshr and *Lhcgr* receptors gene expression levels in ovarian tissues removed from adult C57BL/6 mice at diestrus stage. The gene expression was normalised to *Sdha* and *18s* used as reference genes. The results are shown as mean ± SEM (n = 12 mice per group). T-test analysis. * $P < 0.05$

4.3.3.3 Gene expression of Brca1

BRCA1, breast cancer susceptibility gene 1, is a member of a family of genes involved in DNA repair. In the previous chapter, *Brca1* was found to be downregulated immediately after FA supplementation. Here, a downregulation of *Brca1* expression was also observed in the FA supplemented group compared to the control group ($P = 0.03$) (Figure 4.9).

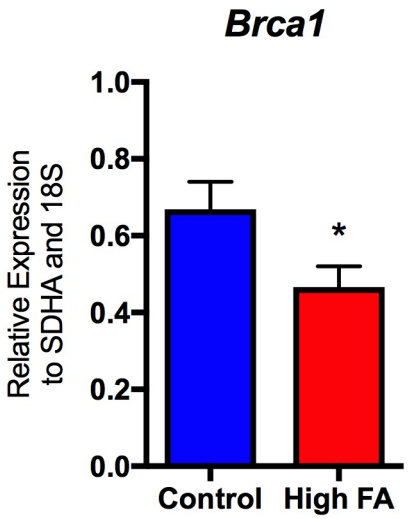

Figure 4.9 Gene expression of *Brca1* in ovaries at PND102

Gene expression level of *Brca1* in ovarian tissues removed from adult C57BL/6 mice at diestrus stage. The gene expression was normalised to *Sdha* and *18s* used as reference genes. The results are shown as mean ± SEM (n = 12 mice per group). T-test analysis. * $P < 0.05$

4.3.3.4 Gene expression of cell differentiation control genes

The development of the follicle compresses an orchestra of genes regulated by hormones and growth factors to guarantee successful ovulation and CL formation. The hormone signalling pathways include not only transcription factors but also recruitment of epigenetic machinery (LaVoie, 2005).

4.3.3.4.1 Gene expression of Oct4

In the previous chapter, OCT4, a pluripotency factor that is expressed in the oocyte, was not affected by FA intake. However, at diestrus stage FA supplementation in female adult mice induced a decrease in ovarian *Oct4* ($P = 0.03$) expression (Figure 4.10).

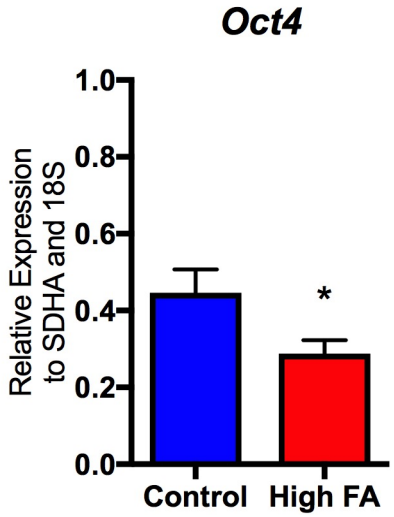

Figure 4.10 Gene expression of differential factor *Oct4* in ovaries at PND102

Gene expression level of *Oct4* in ovarian tissues removed from adult C57BL/6 mice at diestrus stage. The gene expression was normalised to *Sdha* and *18s* used as reference genes. The results are shown as mean ± SEM (n = 12 mice per group). T-test analysis. * $P < 0.05$

4.3.3.4.2 Gene expression of chromatin modifiers

EZH2 (PRC2), which mediates H3K27me3, and BMI1 (PRC1), were downregulated in the ovaries immediately after FA supplementation. In this project, at diestrus stage, the high FA group also showed a decrease on the expression of *Ezh2* and *Bmi1* compared with the control group (P = 0.02 and *P* = 0.03, respectively) (Figure 4.11).

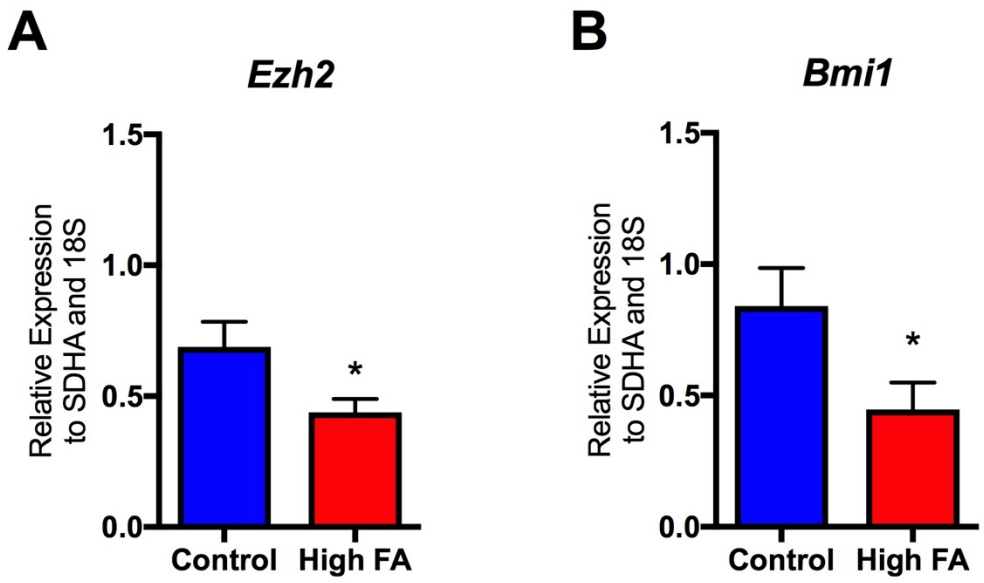

Figure 4.11 Gene expression of chromatin modifiers in ovaries at PND102

Ezh2 and *Bmi1* gene expression level in ovarian tissues removed from adult C57BL/6 mice at diestrus stage. The gene expression was normalised to *Sdha* and *18s* used as reference genes. The results are shown as mean ± SEM (n = 12 mice per group). T-test analysis. * $P < 0.05$

4.3.3.4.3 Gene expression of DNA methyltransferase 1

The folate status is associated with DNA methylation levels and alteration of DNA methyltransferases (DNMTs). DNMT1 is the maintenance methyltransferase, which has a preference for hemimethylated DNA and is essential to maintain double strand methylation after genomic replication. DNMT1 has been reported to be expressed in the cytoplasm and nuclei of oocytes and GCs (Petrussa, Van de Velde and De Rycke, 2014). After four weeks of high FA diet, ovaries showed a lower expression of *Dnmt1* ($P = 0.03$) (Figure 4.12).

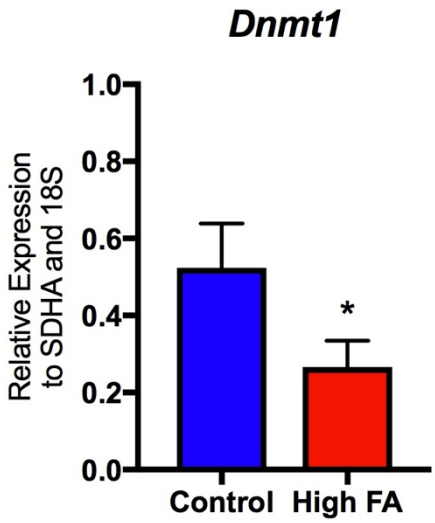

Figure 4.12 Gene expression of DNA methyltransferase in ovaries at PND102

Dnmt1 gene expression level in ovarian tissues removed from adult C57BL/6 mice at diestrus stage. The gene expression was normalised to *Sdha* and *18s* used as reference genes. The results are shown as mean ± SEM (n = 12 mice per group). T-test analysis. * $P < 0.05$

4.3.4 Ovarian morphology

Alteration in the ovarian morphology, including changes in the number of follicles per ovary, can affect the oocyte quality and quantity available for ovulation. To determine the effect of FA on the number of ovarian follicles, one ovary per female was fixed, embedded in paraffin and sectioned before staining with Haematoxylin and Eosin (H&E) (See Methods 2.10.2). One every ten ovarian sections of the ovary were analysed and the total number of follicles and the different stages of development were assessed. Follicles were classified as primordial, primary, secondary, and antral (See Methods 2.10.2.1). Corpus luteum (CL) and atretic follicles were also counted and analysed as a percentage related to the total number of follicles.

4.3.4.1 Ovarian follicle number

All the ovarian follicles with a visible oocyte nucleus and without evidence of atresia were counted. On average 107.7 ± 31.7 follicles were counted per ovary. A lower number of follicles were observed in the high FA group (control: 116.3 ± 29.9; high FA: 100.3 ± 33.7), however, this difference was not statistically significant (P =0.38) (Figure 4.13).

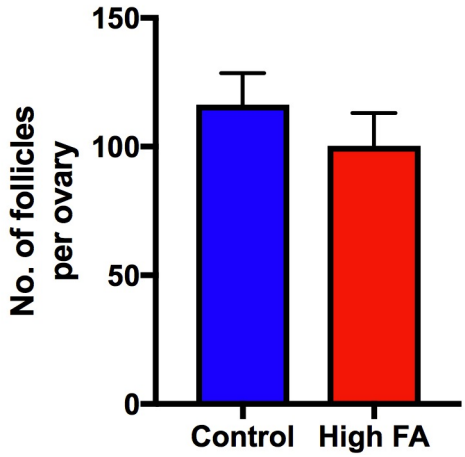

Figure 4.13 Total number of follicles per ovary at PND102

Total number of follicles per ovary. All values are reported as means ± SEM. n = 6-7 animals per group. Mann Whitney test.

4.3.4.1.1 Ovarian follicle stage proportion

The effect of FA supplementation on the developmental stage of the ovarian follicle development was also assessed (Figure 4.14). Again, no significant difference was found between control and high FA groups. Follicles were grouped into early (primordial, primary and secondary) and later (pre-antral and antral) stages of development (Figure 4.15). There was no change in the proportion of follicles per ovary in the different developmental stages after high FA diet (P = 0.15).

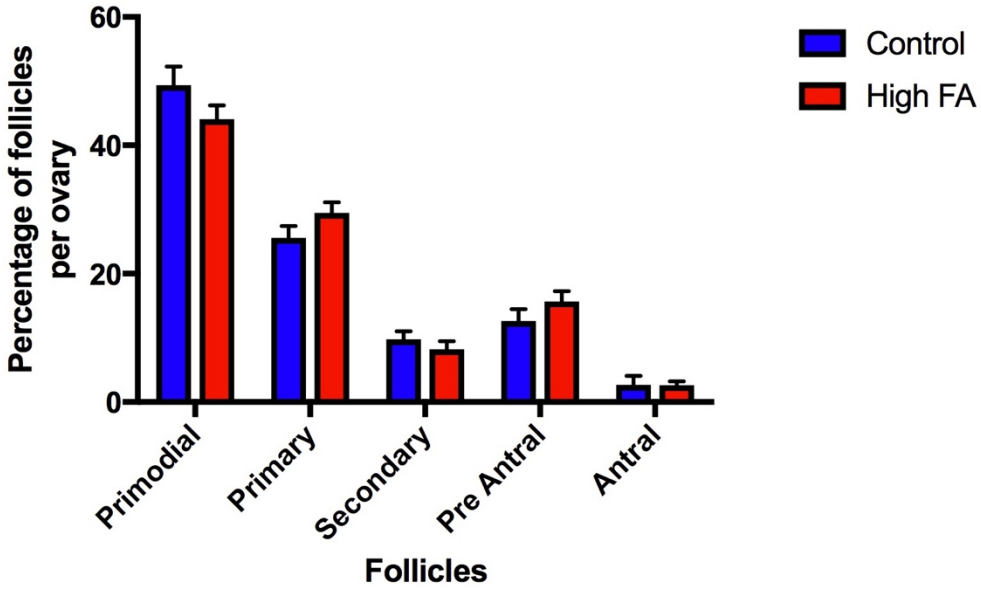

Figure 4.14 Ovarian follicle proportion after high FA diet at PND102

Percentage of follicle number of adult C57BL/6 ovary stained with Haematoxylin and eosin (H&E). Primordial, primary, secondary, pre-antral and antral follicles were counted every ten sections. All values are reported as means ± SEM. n = 6-7 animals per group. Mann Whitney test.

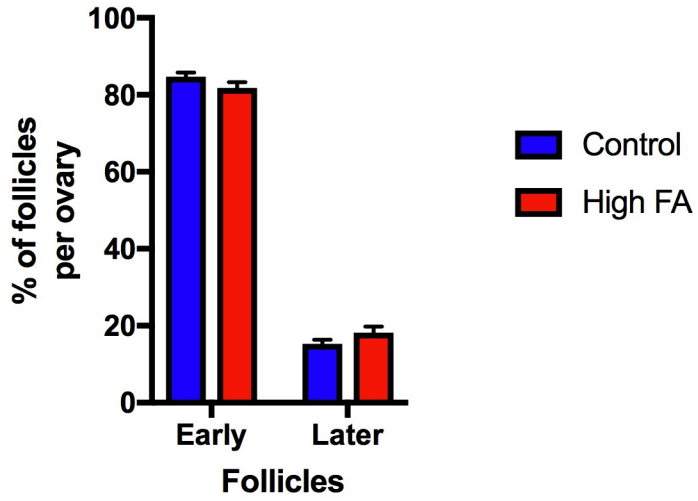

Figure 4.15 Percentage of early and later follicles per ovary after high FA diet

Ovarian follicles grouped by early (primordial, primary and secondary) and later (pre-antral and antral) stage of the development. All values are reported as means ± SEM. n = 6-7 animals per group. Mann Whitney test.

4.3.4.1.2 Atretic follicle number

Atretic follicles (described in Methods 2.10.2.1) were observed in all the ovaries, but only when the nucleus of the oocyte was visible was considered. FA supplementation increased the number of the atretic follicles per ovary (P = 0.005) compared to the control group (Figure 4.17A). Moreover, the percentage of atretic follicles about the total number of follicles showed an increase compared to the control group (P = 0.01) (Figure 4.17B).

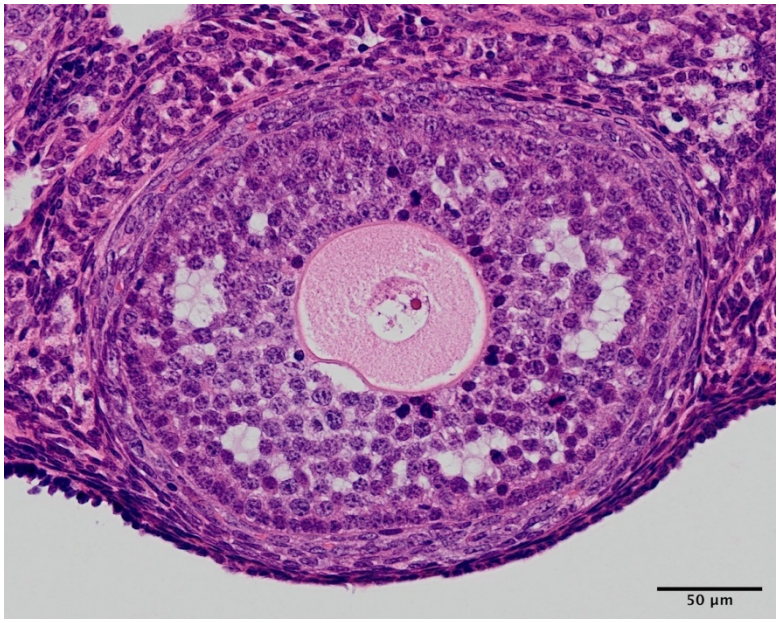

Figure 4.17 Atretic follicle

Atretic pre-antral follicle from high FA group with pyknotic granulosa cells and deformed oocyte. Scale 50 µm.

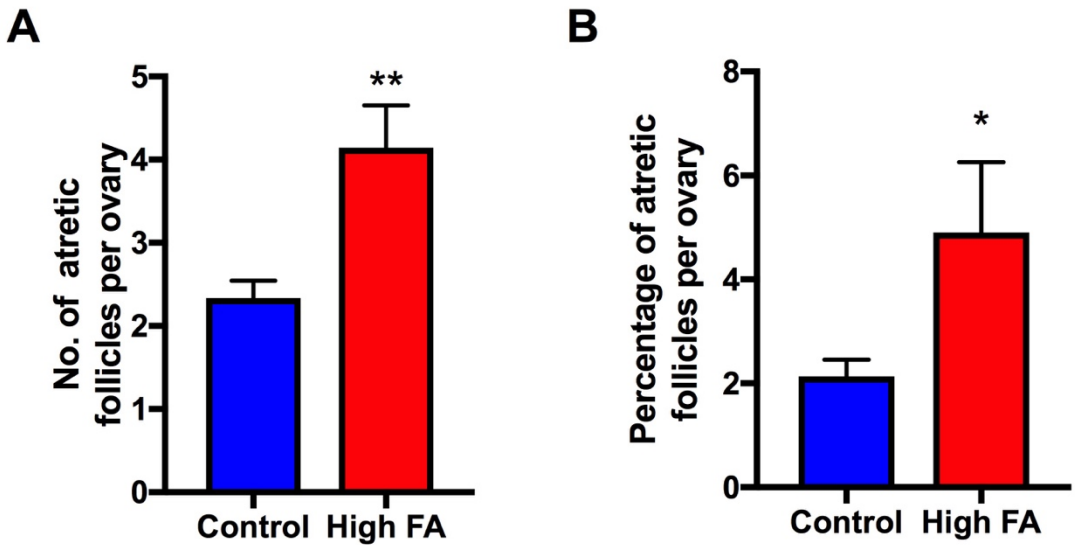

Figure 4.16 Atretic follicles

Number of atretic follicles per ovary (A). Percentage of atretic follicles per total of follicle per ovary (B). All values are reported as means ± SEM. n = 6-7 animals per group. Mann Whitney test, ** $P < 0.01$, * $P < 0.05$.

4.3.4.1.3 Corpus luteum number

The corpus luteum (CL) is a transient endocrine gland formed after ovulation (luteal phase) from the secretory cells of the ovarian follicles. After four weeks of control or high FA diet, the animals were culled at diestrus stage, which is part of the luteal phase and is characterised by the presence of CL. On average 4.7±1.3 CL were found per ovary. There was no significant difference between the total number of CL per ovary between the groups (*P* = 0.19) (Figure 4.18A). However, there was a trend towards an increase in the percentage of CL about the total number of follicles per ovary (*P* = 0.07) (Figure 4.18B).

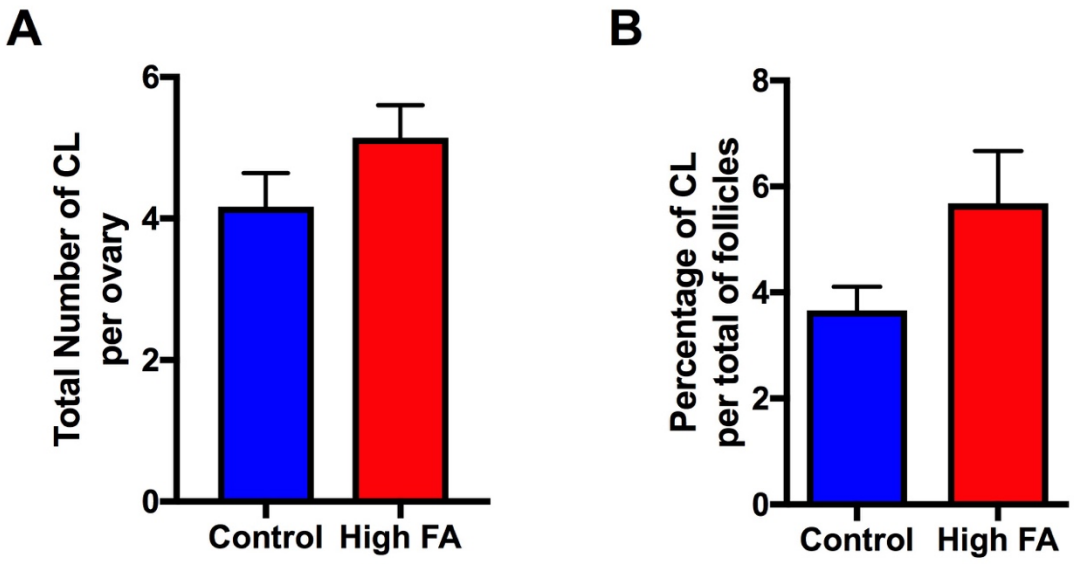

Figure 4.18 Corpus luteum of ovaries PND102 after high FA diet

Total number of CL per ovary (A). Percentage of CL per total number of follicles per ovary (B). All values are reported as means ± SEM. n = 6-7 animals per group. Mann Whitney test.

4.3.4.2 Ovarian volume

The ovarian volume was measured using the area and ratio of each ovary section (See Methods 2.10.2.2). FA supplementation did not alter the volume of the ovary compared with the control group (Figure 4.19) .

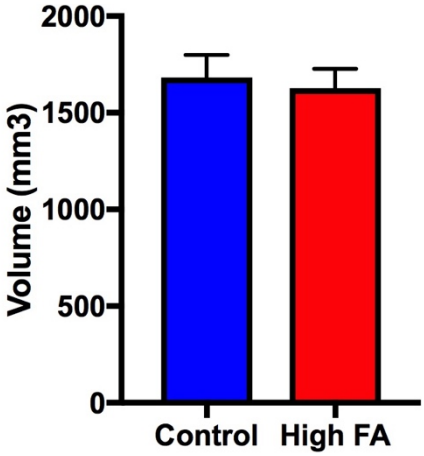

Figure 4.19 Volume per ovary per group at PND102 after high FA diet

Volume per ovary per group (mm^3). All values are reported as means ± SEM. n = 6-7 animals per group. Mann Whitney test.

4.3.4.2.1 CL volume

The volume of each CL was determined using the area of each CL (See Methods 2.10.2.2) and expressed as the percentage of total volume of all CL per ovary. FA supplementation did not alter the volume of CL per ovary (Figure 4.20).

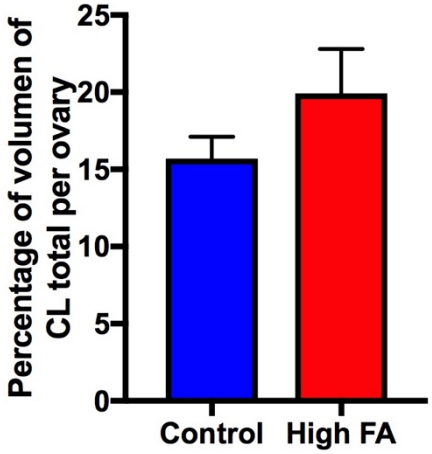

Figure 4.20 Percentage of the volume of CL per ovary

All values are reported as means ± SEM. n = 6-7 animals per group. Mann Whitney test.

4.3.5 Ovary immuno-localisation of Oct4 and H2AX

Changes in the gene expression observed previously in the ovary could be due to changes in the number of the different cell types within the ovary rather than changes in the level of expression of the transcripts within the cells. In order to determine whether a high FA diet changed the number of cells expressing the different transcripts and to confirm that an RNA change was accompanied by a change in protein expression, immunohistochemistry was also carried out.

Immunohistochemistry (IHC) was performed using antibodies against OCT4 and H2AX. OCT4 IHC showed a cytoplasmic localisation in the oocyte. However, quantification of OCT4 was not carried out due to time constraints. In the case of anti-gamma H2AX, the IHC showed differences in staining across the same follicle between sections making the quantification of protein expression difficult. Therefore, H2AX IHC was used as a technique to determine the localisation of the proteins rather than quantification of the protein. As observed in Figure 4.26, continuous sections of the same ovary showed different staining, causing a subjective selection of the appropriate sample to analyse. More time for the analysis of the picture could allow an average quantification of the immuno-staining.

4.3.5.1 Oct4 immuno-localisation

The analysis of *Oct4* mRNA expression showed downregulation in the high FA group. Therefore, an immunodetection assay was performed to identify the localisation of this protein to analyse then if the changes in the mRNA are correlated with the protein level observed by IHC. Previous studies have reported that OCT4 is expressed in the cytoplasm of GCs of preovulatory follicle, luteal cells and ovarian stromal cell in all the oestrus cycle stages (Esmaeilian *et al.*, 2012; Bagheripour *et al.*, 2017)

Here, the IHC of OCT4 showed expression mainly in the cytoplasm of the oocyte of the primary to antral follicles (Figure 4.21 and Figure 4.22). However, the staining produced a high background, which was evident in the cytoplasm of CL and interstitial cells with a 1:100 dilution. Higher dilutions of the OCT4 antibody were also used to reduce this background. However, OCT4 staining then became very faint (Figure 4.23 and Figure 4.24).

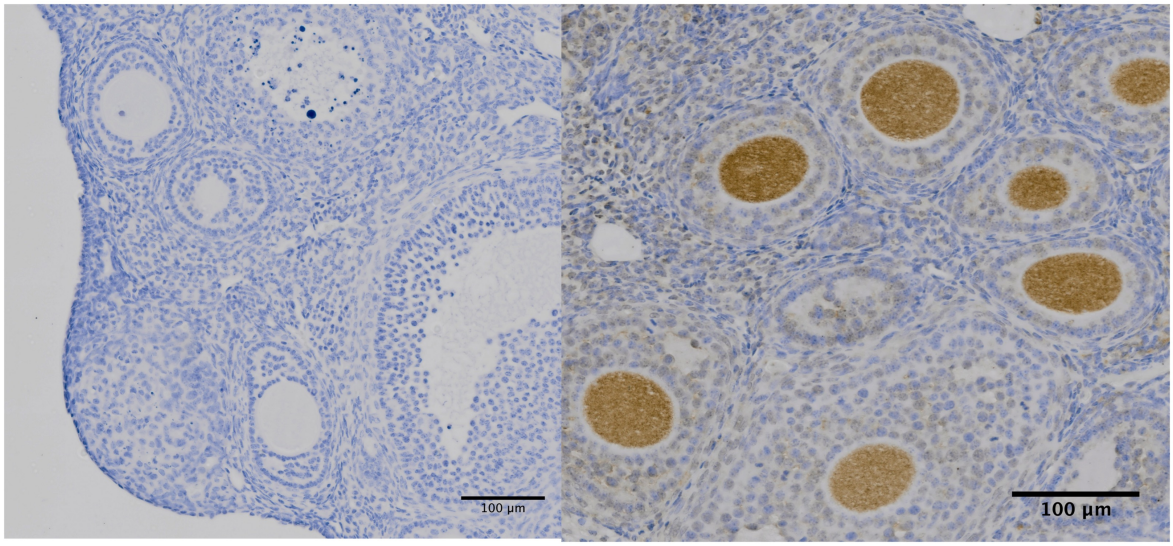

Figure 4.21 Ovarian IHC of OCT4

Ovarian IHC of OCT4 at 1:100 dilutions. Negative control (without primary antibody) left and OCT4 1:100 dilution, right. Scale 100 µm.

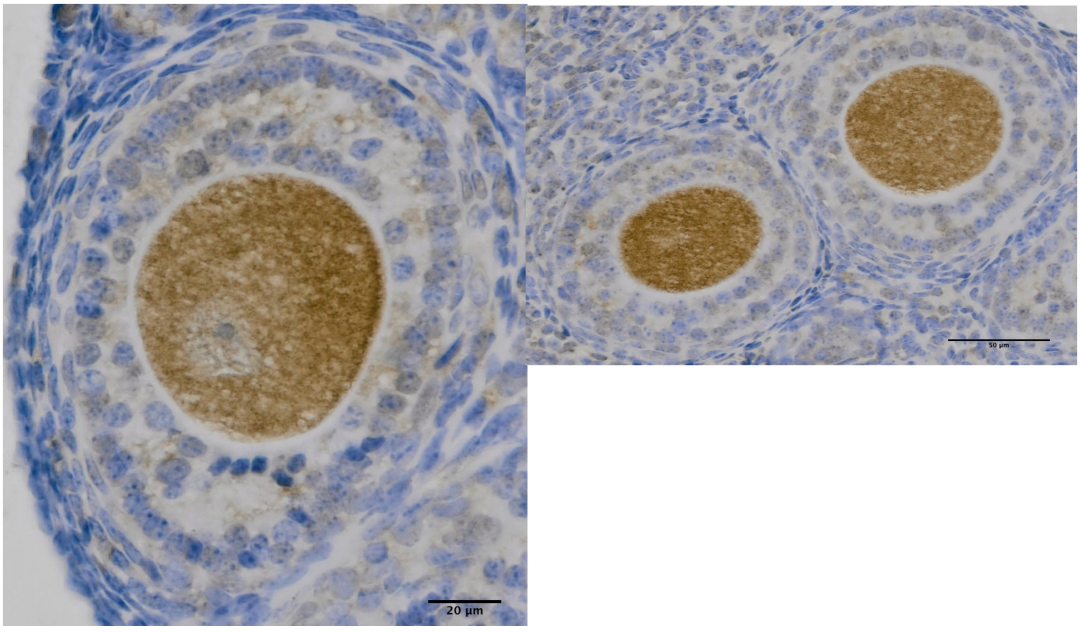

Figure 4.22 Follicles stained with OCT4 IHC

Secondary follicles stained with OCT4 IHC (test sample). Left, secondary follicle with a visible nucleus, OCT4 is mainly present in the cytoplasm of the oocyte and partially in the nucleus. Scale 20 um. Right, secondary follicles with no visible nuclei stained with OCT4. Scale 50 µm.

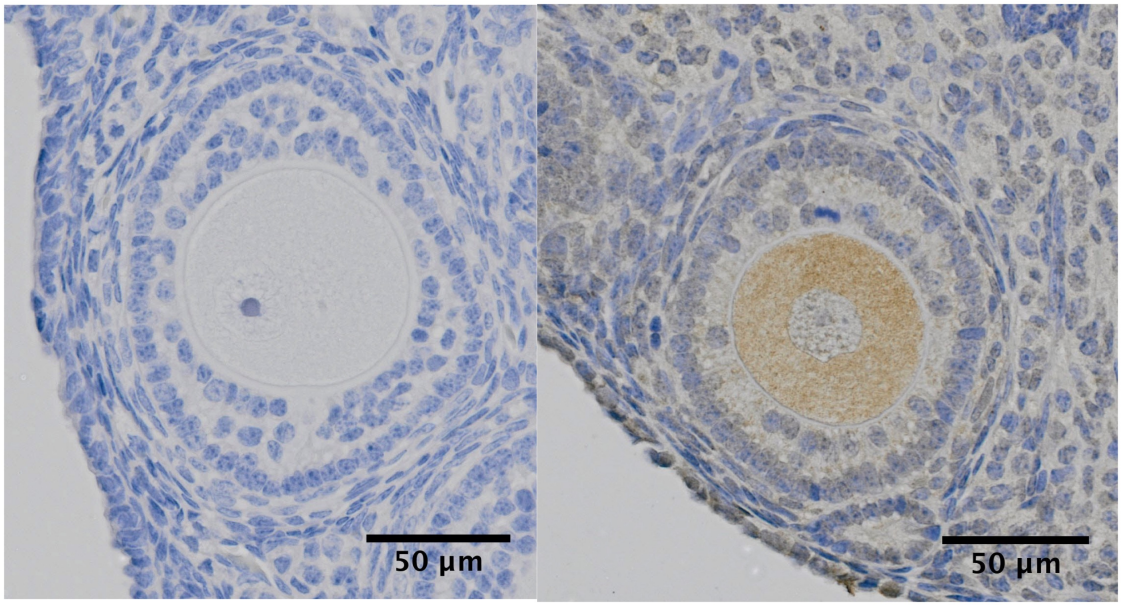

Figure 4.23 OCT4 1:200 IHC

Negative control without OCT4 antibody (left). 1:200 OCT4 dilutions (right). Scale 50 µm.

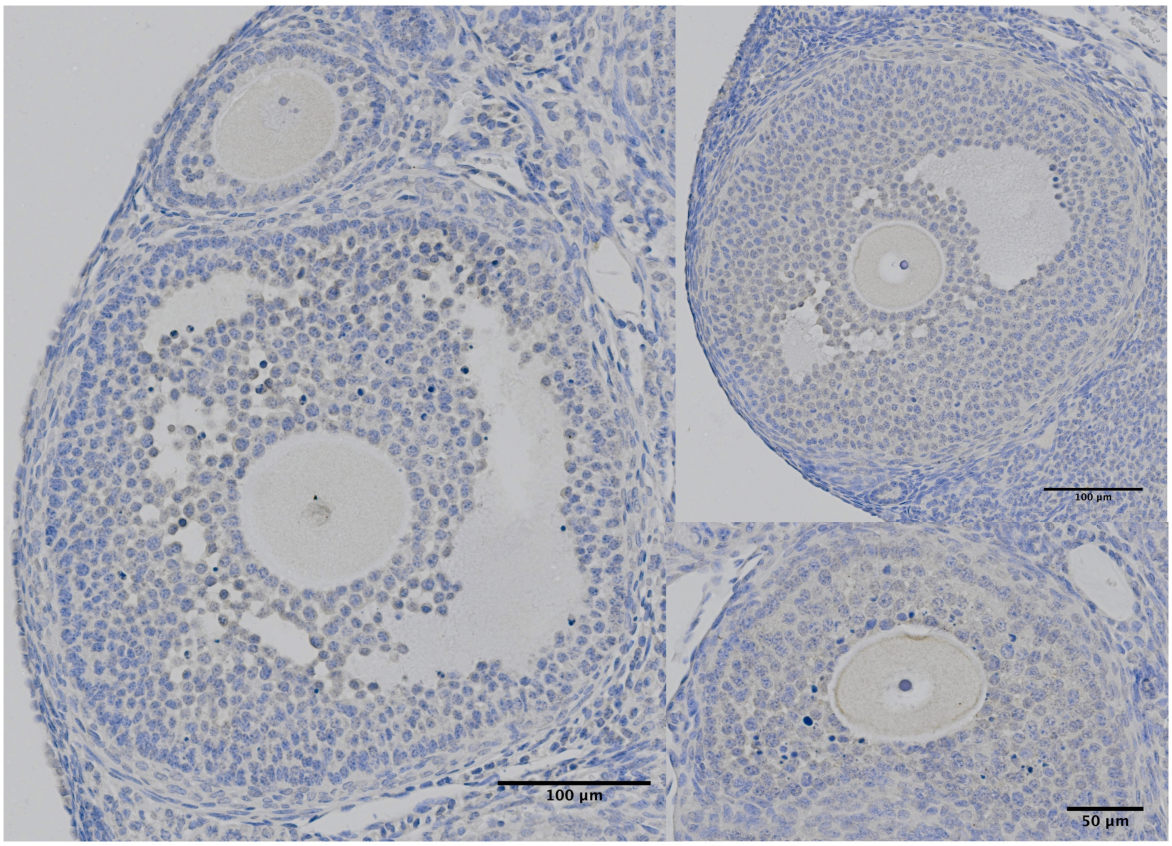

Figure 4.24 OCT4 at 1:200 in large follicles IHC

4.3.5.2 H2AX immuno-localisation

As *Brca1* expression in the high FA group was downregulated, antibodies against BRCA1 were also used to stain the ovarian sections. However, this was not successful (See Appendix B.1). Therefore, anti-gamma H2AX antibody, a marker for DNA damage was used. Anti-gamma H2AX (H2AX139ph) is phosphorylated on Serine-140 in response to DNA double strand breaks (DSBs) and has been shown to be recruited to areas of DNA damage.

H2AX was observed in the nucleus of the oocyte of primary to antral follicles and also granulosa and theca cells. The quantification of the H2AX expression was not possible due to the time required to analyse. The Figure 4.25 shows early stage follicles (primary and secondary) of control and high FA group showing high variability within each group in the oocyte nucleus, which made complicated the election of the adequate sample to analyse. The same follicle (in continuous sections) showed different levels of stain, making it difficult to identify an objective method to analyse the sections (Figure 4.26).

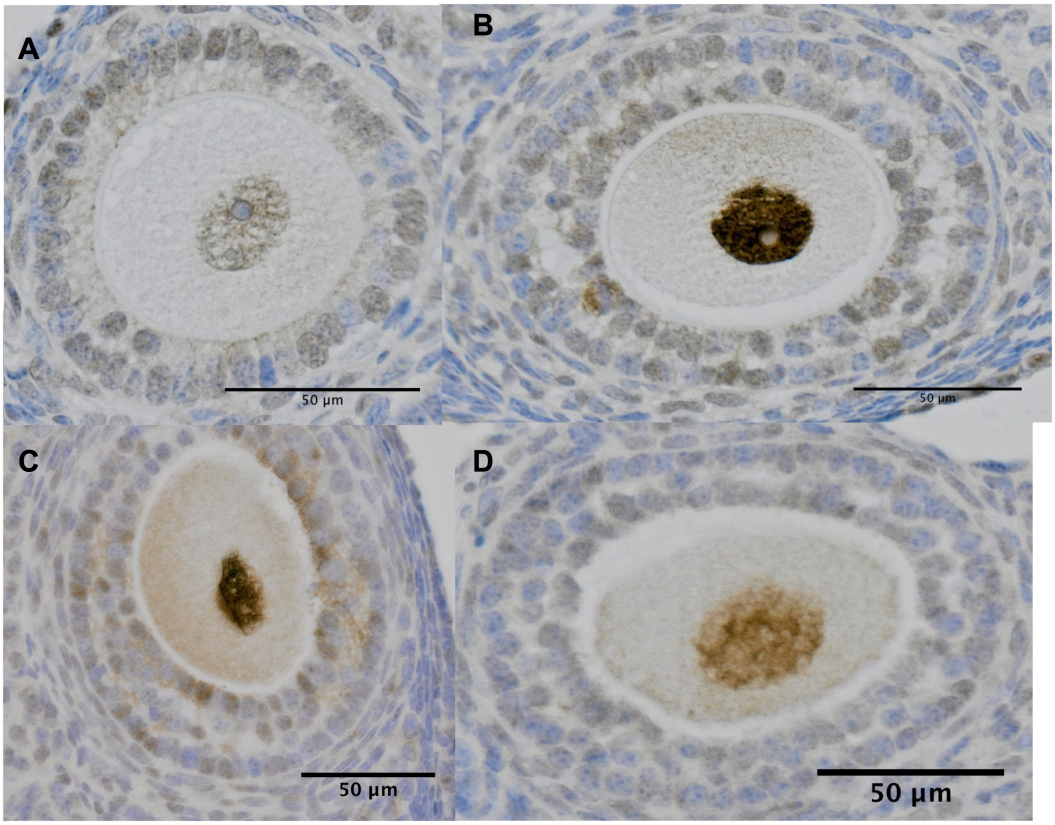

Figure 4.25 Anti-gamma H2AX IHC in early follicles

Early follicles stained with H2AX. Primary (A) and secondary (B) follicle from the control group. Secondary follicles (C and D) from ovaries of high FA group. Scale 50 µm.

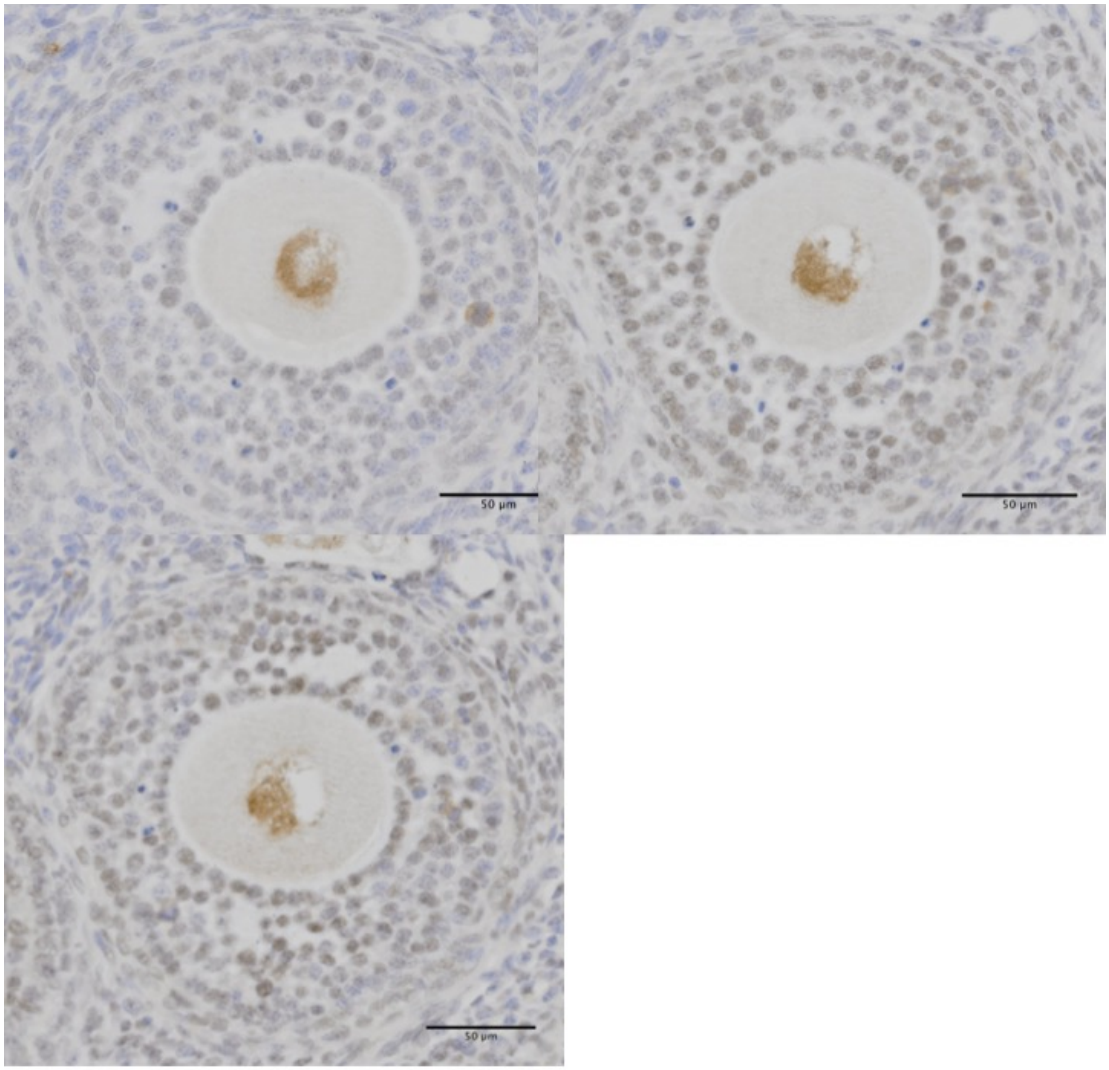

Figure 4.26 Anti-gamma H2AX ovarian IHC

Pre-antral follicle from the same ovary of high FA group. Three continuous ovarian sections stained with H2AX showed different levels of the tone of the staining. Scale 50 µm.

4.4 Discussion

The ovary comprises complex machinery where by many factors play an essential role for the maturation and growth of the oocyte during the development of the follicle (See Introduction 1.2.21.2.2). During the highly proliferative process of developing ovarian follicles can be susceptible to changes in the follicular fluid content. Nutrition can modulate the ovarian follicular fluid and follicle environment, thereby influencing ovarian stimulation and fertility (Twigt *et al.*, 2011; Steegers-Theunissen *et al.*, 2013). Irregular levels of one-carbon metabolites in the follicular fluid have been reported to induce alterations in follicle development, embryo quality and pregnancy rate in Artificial Reproductive Technology (ART) studies (Boxmeer, Brouns, *et al.*, 2008; Berker *et al.*, 2009; Boxmeer *et al.*, 2009; Ocal, Ersoylu and Cepni, 2012). However, little is known about the effect of high levels of FA intake on normal ovarian and follicle development (Gaskins, Afeiche, *et al.*, 2014).

In this project, feeding adult female mice a high FA diet for four weeks did not induce changes in the proportion of ovarian follicles at the diestrus stage. However, an increase in atretic follicles was observed in the high FA group. This result could suggest increased apoptosis during follicle development, which in turn may lead to a reduction numbers of healthy follicles. Moreover, decreased expression of genes related to the follicle development such as *Fshr*, *Oct4* and *Brca1* were also observed. These changes were accompanied by a downregulation of epigenetic writers-the polycomb genes (*Ezh2* and *Bmi1*) and *Dnmt1* (See Table 4.1). As these genes play a critical role in follicle growth, such changes are likely to affect both the quality of the dominant follicle and then the oocyte.

Table 4.1 Summary gene expression results in the ovary at PND102

Roles	Genes	High FA diet effect
Folate transport	*Pcft*	↓
Oocyte development	*Gdf9*	-
	Oct4	↓
Differentiation	*Oct4*	↓
	Ezh2 – Bmi1 – Dnmt1	↓
Gonadotropin hormone	*Fshr*	↓
DNA damage	*Brca1*	↓

↓= Decrease, - = no change

The CL is a transient reproductive gland with the important function of producing progesterone (P4). Studies in animals have shown that CL size is positively correlated with P4 plasma levels (Rocha *et al.*, 2019). In this project, CL analyses showed a trend toward an increase in the proportion of CL in relation to the total number of follicles per ovary in the high FA group. However, although total volume of those CL was not significantly higher, alterations in the P4 levels secreted by CL cannot be discarded.

According to the literature, the presence of OCT4 can fluctuate depending on the developmental stage of the follicle and the oestrus cycle. During proestrus, where the FSH peak occurs, higher levels of OCT4 are reported compared to any other stages of the cycle. In this project, the IHC showed that OCT4 is mainly present in the cytoplasm of the oocyte of both small and large follicles and weakly expressed in the cytoplasm of GCs (Patel *et al.*, 2013; Bagheripour *et al.*, 2017). OCT4 plays a key role in oocyte development, together with GDF9 it is necessary for the primordial follicle recruitment, and they are regulated by the NOBOX gene (Rajkovic *et al.*, 2004; Choi and Rajkovic, 2006). In this project, *Gdf9* decreased after FA supplementation; however, this was not statistically significant. Additionally, gonadotropins also regulate OCT4 in the oocyte. Exogenous FSH and LH hormone stimulation in mice showed upregulation of *Oct4* in oocytes, suggesting a potential role for *Oct4* in the recruitment of oocyte for their maturation (Monti *et al.*, 2006; Monti and Redi, 2009). In this project, the decrease of *Oct4* accompanied by the downregulation of *Fshr,* could suggest the control of OCT4 by the gonadotropin hormones. Decreases in OCT4 in the oocyte compromise not only the follicle development but also the future zygote pluripotency. Although the quantification of OCT4 protein was not possible during this project due to the time involved and the number of sections needed. It will be needed to see if the mRNA changes do affect protein expression.

FSHR, a marker for GCs cells, is essential for the differentiation and proliferation of GCs. Knockdown of *Fshr* in mice led to the arrested development of follicles, with a fewer number mature follicles and anovulatory infertility (Siegel *et al.*, 2013). Inhibition of the FSH-FHSR interaction has also been reported to lead to an increase in apoptosis in rats GCs and G1 arrest (Navalakhe *et al.*, 2013). In this project, FA supplementation induced a decrease in *Fshr* without changes in the total number of follicles. Moreover, there was a trend to an increase in the number of CL. These results suggest that FA might increase apoptosis in GCs, reduce FSHr due to the lower number of GCs and reduce the proportion of growing follicles increasing the volume of CL.

One function of FSH is to induce differentiation of GCs which produce ovarian steroid hormone by inducing the expression of the aromatase (CYP19A1) enzyme. Folate and homocysteine have been shown to modify steroidogenic and gonadotropin hormone levels depending on the stage of the

cycle. In the case of folate, high levels in plasma were associated with higher levels of P4 during the luteal phase and lower levels of FSH during ovulation. In contrast, homocysteine levels are inversely correlated to higher levels of FSH, lower P4 during the luteal phase and lower E2 across the cycle (Michels *et al.*, 2017). Diestrus is part of the luteal phase during the oestrus cycle, characterised by high levels of P4 from the large CL formed after ovulation. Change of *Fshr* after high FA diet may suggest alterations in the gonadotropin-steroidogenic-hormone regulation.

In the ovary, the BRCA1 expression has been reported in the GC of developing follicles, and in a small proportion of mural cells of large antral and preovulatory follicles, becoming restricted to cumulus GCs. *Brca1* ovarian expression showed a significant decrease after the FA supplementation . However, IHC for BRCA1 was not successful, and so the expression of H2AX was examined instead. This DNA damage marker was detected in the nuclei of oocyte and GCs. However, uneven staining of H2AX in the follicles , meant analysis was not possible.

A decrease in BRCA1 is associated with loss of DNA repair in ageing animal models. Knockout of BRCA1 in GCs showed more follicles with high levels of degenerating cells (pyknosis) as well as fewer CL (Upton *et al.*, 2015). A decrease of phosphorylated H2AX and BRCA1 in primordial follicles suggested an impaired DNA repair machinery (Govindaraj *et al.*, 2017). In this project, there was no analysis of apoptosis (e.g. TUNEL analysis). However, a greater number of atretic follicles were observed in the high FA group, which could be associated with the reduction in *Brca1* levels. Although BRCA1-cell localisation is necessary to clarify if the lower expression of *Brca1* is associated with atretic follicles. Another role of BRCA1 is in the modulation of oestrogen biosynthesis in GCs. BRCA1 represses the expression of aromatase in cumulus and mural GCs in mice. A decrease in BRCA1 may, therefore, lead to an increase in aromatase and higher oestrogen levels (Hu *et al.*, 2005). Hong et al. showed that adult mice that do not express BRCA1 in their GCs, have an increased length of the follicular phase, specially the proestrus phase (Hong *et al.*, 2010).

During development, the ovarian follicle goes through activation and inactivation of several genes at the different stages of the cycle coordinated by transcription factors regulated by hormone pathways. This regulation is mediated by the recruitment of enzymes, which alter chromatin structure and hence modulating transcription. EZH2 and BMI1 are subunits of PRC2 and PRC1, which are often co-recruited in the regulation of differentiation genes. In this work, *Ezh2* and *Bmi1* expression decreased in the high FA diet group immediately after supplementation . EZH2 has been shown to participate in the regulation of steroidal hormone synthesis. In GCs of preovulatory follicles of rats, EZH2 binds to aromatase gene (CYP19A1) which shows high levels of H3K27me3, inhibiting oestradiol synthesis (Lee *et al.*, 2013; Maekawa *et al.*, 2016). Consequently, after the LH surge when the levels of CYP11A1 mRNA increase, the binding activity of EZH2 to the

promoter region decreases, changing chromatin structure and allowing transcription (Okada *et al.*, 2016). A higher intake of FA may be expected to increase methyl group availability for methylation. However, in this study downregulation of the methyltransferase EZH2 was observed, suggesting a feedback mechanism and repression of methyl transferases .

PRC1 comprise several proteins, which include BMI1 and E3 ubiquitin ligase activity that mediates mono-ubiquitination of H2AK119 and the silencing of the gene expression. BMI1 is overexpressed in diverse types of cancer and is associated with a poor prognosis due to its function in cell self-renewal, proliferation and differentiation processes (Honig *et al.*, 2010; Yang *et al.*, 2010; B. W. Kim *et al.*, 2016). Recently the interaction of BMI1 with the progesterone receptor (PR) was described. Together with the E3 ligase and in a PRC1-independent manner BMI1 modulates the response capacity of the PR in the uterus allowing embryonic implantation in human and mouse models (Xin *et al.*, 2018). In this project, *Bmi1* and *Ezh2* downregulation suggest that modification of chromatin structure may coordinate the effect of the FA in the ovary. Further experiments are required to determine the full impact of the downregulation of these epigenetic modifiers.

DNA methylation is also part of the epigenetic orchestra necessary in the ovary. Oocytes lacking DNMT1 can be fertilised and complete the process of implantation, however after E14 almost all foetuses die (Howell *et al.*, 2001). Even when methylation patterns were normal in those oocytes, embryos showed a loss of allele-specific expression and methylation at specific imprinted loci (Howell *et al.*, 2001). The expression of DNMTs in the oocyte is also related to the ageing effect. Oocytes from older mice show lower methylation status, *Dnmt* expression and pregnancy rate compared with oocytes from younger mice (Yue *et al.*, 2012).

Together with changes in follicle development-related genes, after a high FA diet, the expression of folate transporters was also altered. Ovaries exhibited lower expression of the carrier-mediated transporters, which is likely to be associated with the saturation of absorption after high levels of 5m-THF in the plasma. In humans, physiological doses of FA are absorbed by PCFT and RFC; however, larger doses taken via supplementation of FA- tend to saturate these transporters and promote the absorption by passive diffusion (by FOLR1/2) (Tam, O'Connor and Koren, 2012). Elevated consumption of FA displays high and constant levels of FA and unmetabolised FA on plasma for people exposed to FA supplementation and/or fortification (Tam, O'Connor and Koren, 2012; Pfeiffer *et al.*, 2015). Here, exposure to a high FA diet showed elevated levels of unmetabolised FA and folate metabolites after four weeks. Prolonged exposure to these high levels in plasma can have further consequences not only in the ovary but also in other organs as has been reported in the brain, liver and mammary gland (Burdge *et al.*, 2009; Deghan Manshadi *et al.*, 2014; Ly *et al.*, 2016).

4.5 Conclusion

After four weeks of a high FA diet, ovaries showed downregulation of genes related to folate transport, oocyte and follicle growth and differentiation that may have consequences for the appropriate development of the follicle. However, morphological analyses showed no changes in the proportion of follicles but an increase of atretic follicles in the high FA group. IHC showed that OCT4 is present in the oocyte, confirming its role in the oocyte development and possible participation in the primordial follicle recruitment.

The proportion of ovarian follicles after FA supplementation did change significantly. These results could be related to the short time of treatment, which is not sufficient to induce alterations in the number of follicles. Therefore, further analyses at later time points after the end of supplementation will be required to assess the long term impact of FA supplementation on follicle development. However, an increase in the atretic follicles was observed, which could compromise ovarian function and oocyte quality and have future repercussions for ovulation and embryo development.

Chapter 5 Results III

Sustained effect of high FA diet on ovarian morphology and follicle development.

5.1 Introduction

Diets with modified levels of FA have been associated with functional alterations in organs like liver, mammary gland, and ovary (Burdge *et al.*, 2009; Deghan Manshadi *et al.*, 2014; Ly *et al.*, 2016; Michels *et al.*, 2017). Specifically, in the ovary, changes in the follicular fluid associated with the maternal FA consumption have suggested possible consequences in the follicular development and later on, implications for oocyte and embryo growth (Boxmeer, Brouns, *et al.*, 2008; Boxmeer, Steegers-Theunissen, *et al.*, 2008). However, some effects associated with altered FA intake could not be observed immediately, or during the period the animals received the diet, but rather after time of the end of supplementation or throughout the life course. Kotsopoulos et al., showed that a folate-deficient diet fed throughout weaning and until adulthood in rats did not change DNA methylation, in contrast, when the folate-deficient diet was restricted between weaning and puberty , there was a significant increase in DNA methylation in the liver of the adult offspring (Kotsopoulos, Sohn and Kim, 2008).

During adulthood, the follicle development in the ovary continues with active GC proliferation to recruit oocytes for ovulation. During this process, several growth and hormone factors are involved (See Introduction 1.2.2). Having seen changes four weeks after high FA diet in the Chapter 3 Results I, the intention of this project was looking for a persistence effect after FA supplementation at the same stage of the oestrus cycle. Specifically, to determine if the FA effect found at PND102 (Chapter 4 Results II) persist at PND130. Therefore, the gene factors examined here were in the majority the same analysed before to assess the ovarian follicle growth.

5.2 Experimental Design

10 Virgin C57BL/6 adult (PND74) mice were randomly assigned to receive control (1 mg FA) or high FA diet (5mg FA) (See Methods 2.1.3). After four weeks (PND102), both control and high FA groups were fed with the control diet (1mg FA) for four further weeks (PND130) (Figure 5.1). Food and weight were monitored weekly during the eight weeks of the project. Mice were culled at diestrus stage of the oestrus cycle at PND130. Animal dissection and organ collection is described in Methods sections 2.1.5 and 2.1.7.

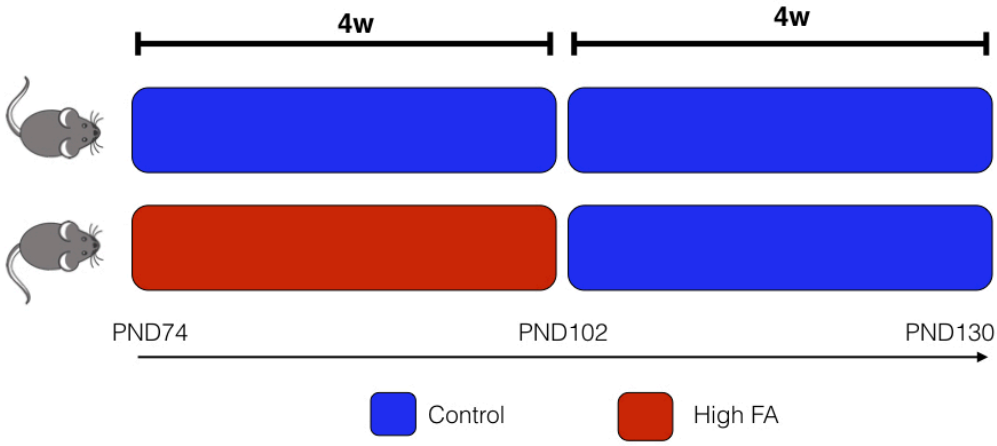

Figure 5.1 Animal model PND130

Virgin adult female C57BL/6 mice (PND74) were fed with control and high FA diet during four weeks (PND102) to then change to control diet for four weeks more (PND130).

5.3 Results

5.3.1 Weight and energy intake measurements

At PND74 animals weight from control and high FA group did not differ between groups. Food per cage and individual body weight were monitored weekly between PND74 and PND130. In the Figure 5.2A and Figure 5.3A, the dotted line at PND102 represents the change of diet for high FA group, where both groups were fed with control diet. There was no change in the body weight (Figure 5.2) or energy intake (Figure 5.3) of the control or high FA groups .

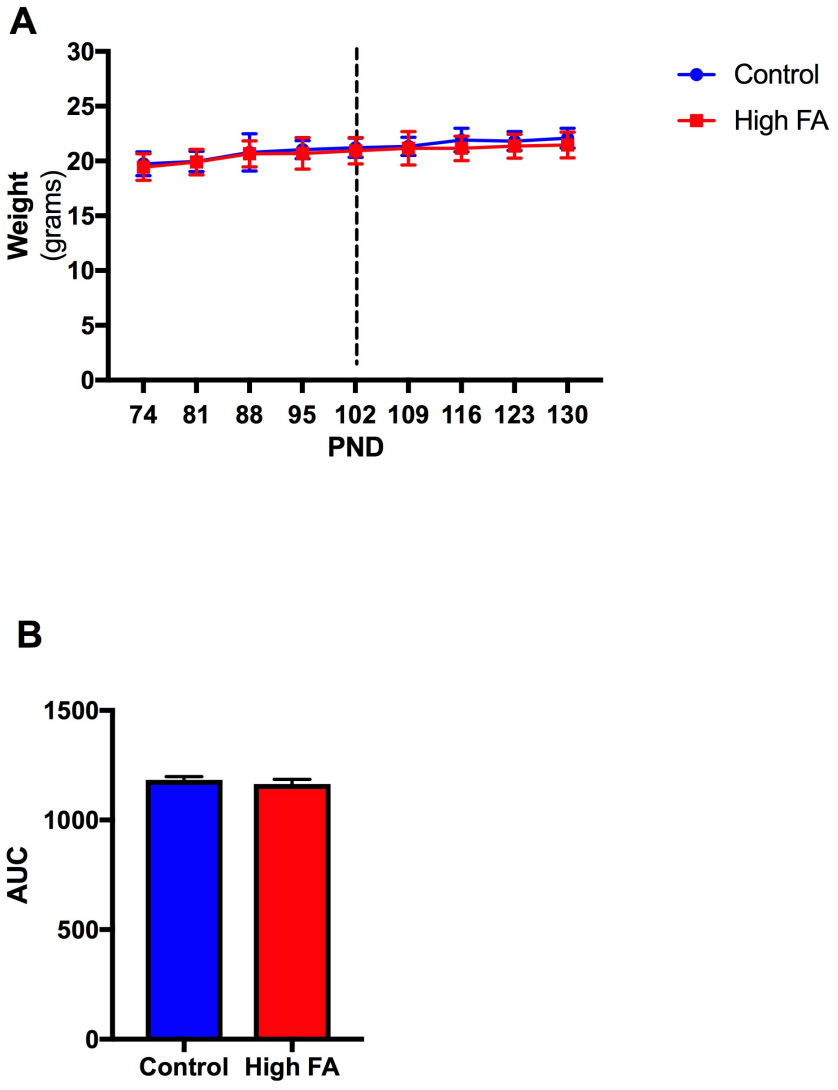

Figure 5.2 Body weight during eight weeks of diet between PND74 and PND130

Body weight of C57BL/6 female mice during eight weeks of control or high FA diet (A). Mean ± SD. Area under the curve (AUC) analysis of the body weight (B). Mean ± SEM. n = 10 animals per group. T-Test analysis

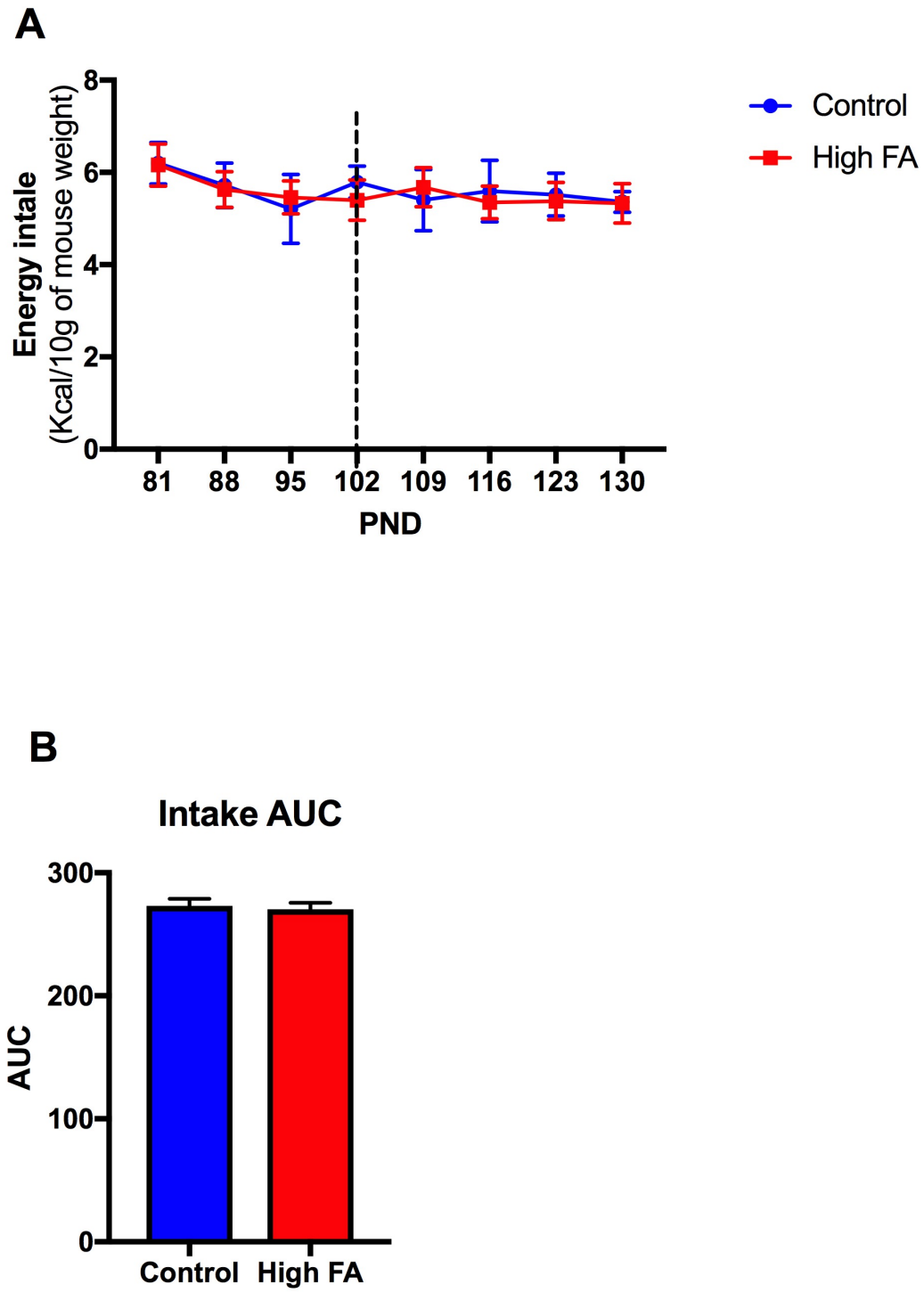

Figure 5.3 Energy intake during eight weeks of diet between PND74 and PND130

Energy intake of C57BL/6 female mice after eight weeks of control or high FA diet (A). Mean ± SD. Area under the curve (AUC) analysis of the body weight (B). Mean ± SEM. n = 10 animals per group. T-Test analysis.

5.3.2 Gene expression in ovarian tissue

Changes in the differentiation and developmental control genes and growth factor genes may have consequences for follicle development and affect the normal functioning of the ovary. To determine if high FA diet can have a persistent effect on gene expression in the ovary of adult mice, one ovary per female mouse was isolated and analysed by qPCR with specific primers for developmental control and cell differentiation (See primers list Table 2.8). The gene expression was normalised to *Sdha* and *Cyc* as references genes selected after geNorm analysis (See Figure 2.12).

1.1.1.1 Gene expression of follicle development regulation factors

The development of the follicle is regulated by several components which include paracrine and endocrine factors. The expression of these factors depends on the type of cell (somatic or oocyte) and follicle and oestrous stage. In the previous chapter, follicle development-control factors such as *Gdf9, Fshr, Lhchr, Brca1* and *Oct4* and chromatin modifiers such as *Ezh2, Bmi*1 and *Dnmt1* were analysed immediately after high FA diet at PND102 (See Table 4.1 for summary). In this project, the gene analysed were the same and a few additional genes to give a better understanding about the FA effect, and to identify if the changes persist until four weeks after finished the diet at PND130.

1.1.1.1.1 Gene expression of *Gdf9*

Growth differentiation factor-9 (GDF9) marker for early follicle development is mainly expressed in the oocyte and promotes proliferation and differentiation of GCs. Previously *Gdf9* did not change at PND102. However, in this project *Gdf9* showed a trend toward an increase in expression at PND130 four weeks after the end of high FA diet ($P = 0.07$) (Figure 5.4).

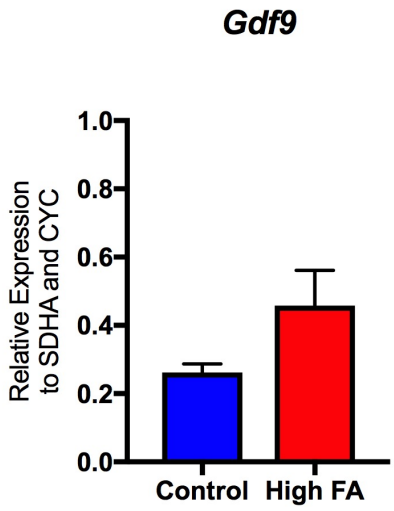

Figure 5.4 Gene expression of *Gdf9* in ovaries at PND130

Gdf9 gene expression level in ovarian tissues removed from adult C57BL/6 mice at diestrus stage. The gene expression was normalised to *Sdha* and *Cyc* used as reference genes. The results are shown as mean ± SEM. n = 10 mice per group. T-test analysis.

1.1.1.1.2 Gene expression of gonadotropin hormone receptor

FSH represents the main stimulator of follicle development after pre-antral stage. The gonadotropin hormone receptor (*Fshr*) was downregulated by high FA diet immediately after the end of supplementation at PND102, however four weeks after the end of supplementation, *Fshr* expression showed a trend toward an increase in ovaries of the high FA group at PND130 (*P* = 0.06) (Figure 5.5).

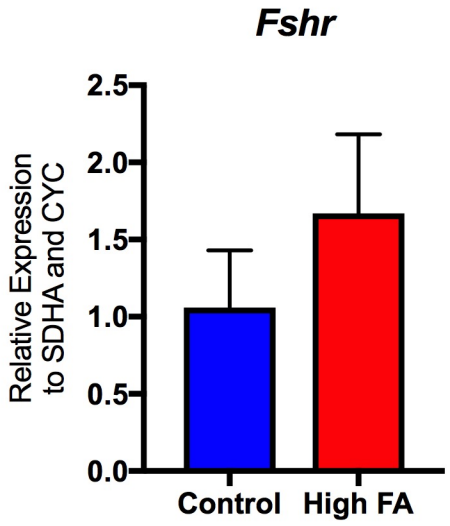

Figure 5.5 Gene expression of *Fshr* in ovaries at PND130

Fshr gene expression level in ovarian tissues removed from adult C57BL/6 mice at diestrus stage. The gene expression was normalised to *Sdha* and *Cyc* used as reference genes. The results are shown as mean ± SEM. n = 10 mice per group. T-test analysis

1.1.1.1.3 Gene expression of steroidogenic hormone-related genes

Progesterone receptor (PR) is expressed in GCs of preovulatory follicles in response to the ovulatory surge mediated by gonadotropin hormones. PR mediates the transcriptional effect of progesterone in cell proliferation and differentiation during ovulation and luteinisation (Gava *et al.*, 2004). The enzyme cytochrome P450 aromatase (and its encoding gene CYP19A1) is expressed in GCs of large antral ovarian follicles where it is essential for the oestrogen synthesis. The expression of both, *Pgr* and *Cyp19a1* was not altered by FA supplementation at PND 130 did not show changes after four weeks of termination of the high FA diet (Figure 5.6).

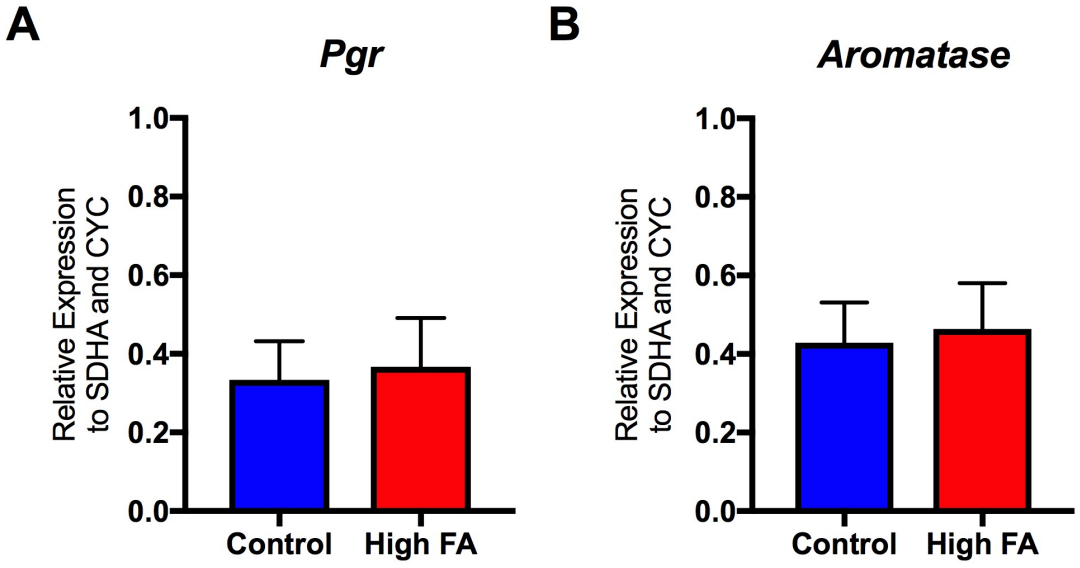

Figure 5.6 Gene expression of steroidogenic hormone in ovaries at PND130

Pgr (A) and *Cyp19a1* (which encodes *Aromatase*) (B) gene expression level in ovarian tissues removed from adult C57BL/6 mice at diestrus stage. The gene expression was normalised to *Sdha* and *Cyc* used as reference genes. The results are shown as mean ± SEM. n = 10 mice per group. T-test analysis.

1.1.1.2 Genes expression of *Brca1*

Mutations in the BRCA1 gene have been associated with inherited forms of breast and ovarian cancer (Venkitaraman, 2002; Mersch *et al.*, 2015). In the ovary, Brca1 has been reported to be expressed in the GCs and oocyte nuclei. BRCA1 plays a key role in DNA repair and the regulation of aromatase expression in GCs which is associated with its role as a tumour suppressor gene (Hu *et al.*, 2005; D. Zhang *et al.*, 2015).

Previously, at PND102 Brca1 showed a downregulation in the ovary after feeding a high FA diet. However, after four weeks after the end of supplementation, *Brca1* expression in the high FA group is similar to control group, without significant difference (Figure 5.7).

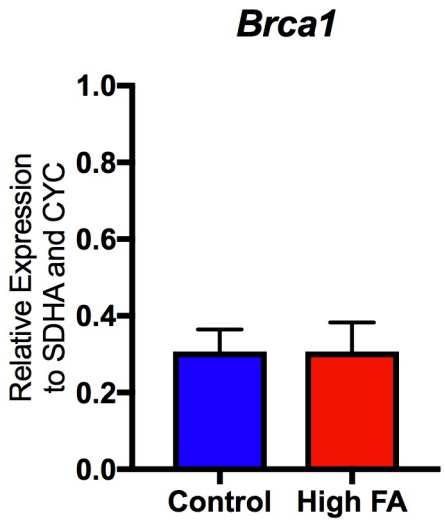

Figure 5.7 Gene expression of *Brca1* in ovaries at PND130

Brca1 gene expression level in ovarian tissues removed from adult C57BL/6 mice at diestrus stage. The gene expression was normalised to *Sdha* and *Cyc* used as reference genes. The results are shown as mean ± SEM. n = 10 mice per group. T-test analysis. * $P < 0.05$

1.1.1.3 Gene expression of cell differentiation control genes

The follicle development comprises an orchestra of transcription factors to guarantee a successful ovulation. The hormone signalling pathways which trigger the network of transcription factors required to growth includes the participation of epigenetic machinery such as chromatin modifiers part of PRC1/2 and DNA methyltransferases (DNMTs).

1.1.1.3.1 Gene expression of *Oct4*

OCT4 has been reported to be expressed oocytes of small and large follicles. *Oct4* was downregulated upon high FA group in the ovary at PND102. However, at PND130, four weeks after the end of supplementation *Oct4* expression was upregulated in the high FA group ($P = 0.01$) (Figure 5.8).

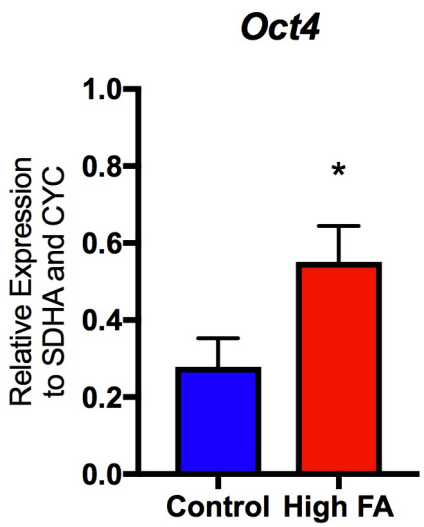

Figure 5.8 Gene expression of *Oct4* in ovaries at PND130

Oct4 gene expression level in ovarian tissues removed from adult C57BL/6 mice at diestrus stage. The gene expression was normalised to *Sdh1* and *Cyc* used as reference genes. The results are shown as mean ± SEM. n = 10 mice per group. T-test analysis. * $P < 0.05$

1.1.1.3.2 Gene expression of chromatin modifiers

Ezh2 and *Bmi1* subunit of PRC2 and PRC1 respectively, showed a downregulation in expression in the ovaries immediately after feeding with high FA diet at PND102. However, at PND130, *Ezh2* and *Bmi1* expression was increased in the high FA group 4 weeks after the end of supplementation ($P = 0.03$ and $P = 0.01$ respectively) (Figure 5.9).

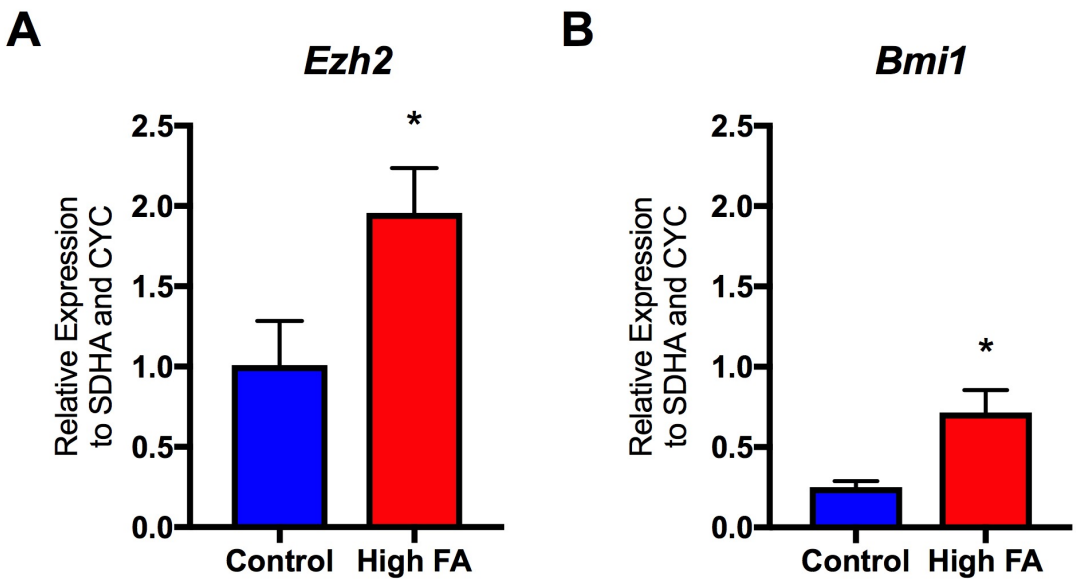

Figure 5.9 Gene expression of chromatin modifiers in ovaries at PND130

Ezh2 (A) and *Bmi1* (B) gene expression level in ovarian tissues removed from adult C57BL/6 mice at diestrus stage. The gene expression was normalised to *Sdha* and *Cyc* used as reference genes. The results are shown as mean ± SEM. n = 10 mice per group. T-test analysis. * $P < 0.05$

1.1.1.3.3 Gene expression of DNA methyltransferase 1

FA supplementation has been associated with alteration in DNA methylation in different organs (Ly *et al.*, 2016). DNA methylation is mediated by DNA methyltransferases, where DNMT1 is related to the maintenance methylation. *Dnmt1* gene expression was downregulated in the high FA group at PND102. However, 4 weeks after the end of supplementation *Dnmt1* showed no significant difference compared to control ovaries (P = 0.23) (Figure 5.10).

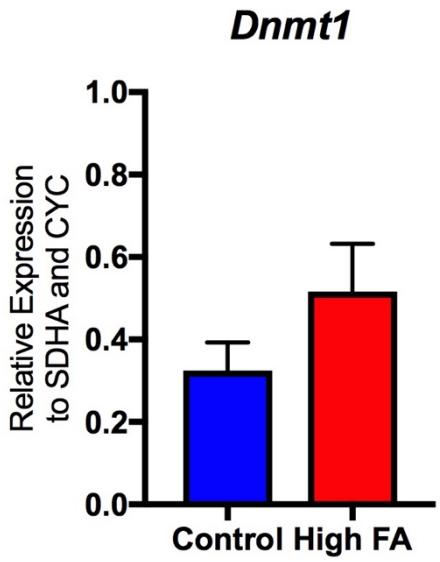

Figure 5.10 Gene expression of *Dnmt1* in ovaries at PND130

Dnmt1 gene expression level in ovarian tissues removed from adult C57BL/6 mice at diestrus stage. The gene expression was normalised to *Sdha* and *Cyc* used as reference genes. The results are shown as mean ± SEM. n = 10 mice per group. T-test analysis.

5.4 Discussion

In the previous chapter, feeding with a high FA diet for four weeks led to a decrease in the expression of genes such as *Fshr*, *Oct4*, *Brca1* and epigenetic-related genes *Ezh2*, *Bmi1* and *Dnmt*1 in the ovary at PND102. In this chapter, the expression of these genes was examined four weeks after the end of supplementation to determine if high FA diet induced a persistent change in the expression of these genes. Here, FA supplementation induced a persistent change in the expression of the expression of *Oct4*, *Ezh2* and *Bmi1* but the change in expression observed at PND130 was in the opposite direction at PND102 (See Table 5.1). Morphological analysis was not possible in this model due to the time required for sectioning samples and count follicles. Such analyses would allow to examining the persistent effect of FA on the ovary and the different follicle stages.

Table 5.1 Summary gene expression results in the ovary at PND102 and PND130

Roles	Genes	At PND102	At PND130
Oocyte development	*Gdf9*	-	↑*
	Oct4	↓	↑
Differentiation	*Oct4*	↓	↑
	Ezh2 – Bmi1	↓	↑
	Dnmt1	↓	-
Gonadotropin hormone	*Fshr*	↓	↑*
DNA damage	*Brca1*	↓	-

↑= increase, ↓= decrease, - = no change, *trend (P <0.1).

GDF9 is an oocyte-specific growth factor essential for the early development but highly expressed during all stages of the follicle development. GDF9 participates in the oocyte-control of the differentiation of the surrounding GCs at the pre-antral stage and the inhibition of luteinisation of GC preventing progesterone (P4) secretion (Spicer *et al.*, 2006). Diet deficient in methyl groups has shown an increase in homocysteine together with an increase of GDF9 in the oocyte of sheep, suggesting the modulation of this oocyte-specific gene by the levels of one-carbon metabolites (Kanakkaparambil *et al.*, 2009). In this project, *Gdf9* did not show a significant change, however, did show a trend toward an increase in the ovary. Higher levels of FA in the plasma, usually are associated with lower levels of homocysteine, in that case, the release of high FA diet could induce an increase of homocysteine level that might impact on *Gdf9* levels. The *Gdf9* levels observed at PND102 were generally lower compared to the expression of *Gdf9* observed in this chapter. This difference suggests the susceptibility and then variability of the assay. Despite being

an oocyte-specific factor, changes in *Gdf9* expression cannot be associated with a proportion of oocytes without an additional analysis at protein level.

OCT4 is a pluripotency marker which maintains cells undifferentiated state, and its absence facilitates the differentiation of cells (Monti *et al.*, 2006). OCT4 is expressed in the nucleus and cytoplasm of the oocyte in early and antral follicles, but also in some GCs. In the oocyte, OCT4 has a role in the early follicular growth and the selection of the oocyte for ovulation. In GC, OCT4 is upregulated under ovarian stimulation with gonadotropin hormones and by NOBOX transcription factor (Monti *et al.*, 2006).

Oct4 expression was affected by FA supplementation at both PND102 and PND130. However, its expression at PND130 was upregulated four weeks after finishing the diet. This increase of *Oct4* is simultaneal to *Fshr* (trend) and *Ezh2/Bmi1* gene expression which suggests a possible communal effect and a possible decrease in GCs proliferation. As it was described before (Chapter 4 Results II 4.4), FSH regulates OCT4 in oocytes, and ovarian cancer cell lines (Monti *et al.*, 2006; Zhang *et al.*, 2013) and EZH2 binds to OCT4 promoter decreasing its expression in ES cells (Pursani *et al.*, 2017). Therefore, these results suggest that FA regulate *Fshr* which through polycomb protein alters *Oct4* expression.

The expression of *Fshr* was also upregulated four weeks after the end of high FA diet. FSH plays a role in later stages of the follicle development, promoting LHR and the steroidogenic production of oestrogen. However, no changes were observed in *Aromatase* which metabolises androstenedione to estrone, or in progesterone receptor (PR). These results may suggest there are no alterations associated with the steroidogenic synthesis at least at the mRNA level. FSH receptor has been reported to be affected by the level of one-carbon metabolites in other studies. GCs exposed to homocysteine *in-vitro* showed an increase in cell proliferation and increased *Fshr* mRNA levels (Kanakkaparambil *et al.*, 2009). Therefore, changes observed in *Fshr* in the ovary can be linked to the altered folate/homocysteine ratio. An upregulation of the *Gdf9*-oocyte marker and the *Fshr*-GC marker suggest an increase in the follicle number, however, morphological analysis is necessary to corroborate this hypothesis.

FA and its metabolites were not measured in this project as high levels of folate was not expected at PND130. Previous studies in human have reported a *T*-max of 80-120 minutes for meals containing 1mg of FA (Kelly *et al.*, 1997; Sweeney *et al.*, 2006). Therefore, after four weeks of control diet in both groups, the levels of folate and FA in plasma should be similar.

The upregulation of the genes observed here compared with the previous chapter could reflect a compensatory mechanism once the high FA diet ended. During high FA diet, the ovary showed

downregulation of the genes related to the follicle growth, however, after four weeks of the normal diet, the ovaries showed an increase in the expression. This occurs except for *Brca1* and *Dnmt1* expression, upon returns to the control diet, both returned to a similar expression to the control group. The compensatory effect observed here it has been previously documented in folate-deficient diet and DNA methylation pattern, where the changes were not observed until the animals change the diet to normal folate concentration (Kotsopoulos, Sohn and Kim, 2008). These results suggest the importance of the timing of FA supplementation and subsequent supply of FA in the diet.

5.5 Conclusion

The consequences of a high FA diet can continue even four weeks after finishing the supplemented diet. Changes in the chromatin modifiers *Ezh2* and *Bmi*1 at PND130 were in the opposite direction than PND102 even when animals were at the same stage of the oestrus cycle. The same was observed with *Fshr* and *Oct4*. These results suggest: first, the changes in *Fshr-Oct-Ezh2/Bmi1* could be connected so then change simultaneously, second, the subsequent upregulation at mRNA level (after downregulation during high FA diet) could be a compensatory mechanism after return to a normal level of FA, and third, the high FA diet has later consequences that can differ from the immediate outcome. More analyses are necessary to prove and analyse the implications of high FA in the ovary.

Chapter 6 Results IV

Effect of high FA diet on embryo development

6.1 Introduction

Adverse early life environment during pregnancy leads to embryonic metabolic adaptations that can result in an increased risk of chronic disease, such as hypertension, type 2 diabetes and cardiovascular diseases (Barker *et al.*, 1993; Barker, 1995). DOHaD, the developmental origin of health and disease hypothesis proposes that the adaptation of the foetus to an adverse uterine environment can affect its growth and metabolism, leading to physiological changes which persist throughout the life course (Fleming, Velazquez and Eckert, 2015).

During the periconceptional period, nutritional perturbations can modify development throughout gestation, altering the physiology and metabolic health of the adult offspring (Ashworth, Toma and Hunter, 2009; Fleming *et al.*, 2012). During the development of the follicle in the ovary, factors such as the preconceptional diet can have an impact on the amount of nutrients in the follicular fluid, follicle development and oocyte maturation resulting in changes of the quantity/quality of the oocyte expulsed during ovulation (Ashworth, Toma and Hunter, 2009). After fertilisation, there is a second sensitive stage called preimplantation, which is a critical stage for the establishment of the developing organism (Wrenzycki *et al.*, 2005). The blastocyst is a vulnerable structure because it consists of only a small number of cells and has a limited nutritional supply. Studies have shown that environmental factors during this stage of development can induce long-term effects on the health of the offspring both *in vivo* (maternal diet) and *in vitro* (embryo culture) (Fleming *et al.*, 2004; Watkins and Fleming, 2009). During this period two main differentiation events occur. Firstly, segregation between the inner cell mass (ICM) and trophectoderm (TE) cells during blastocyst formation, and secondly, the differentiation of ICM into primitive endoderm (PE) or epiblast (EPI) which is completed by E4.5. Each lineage is characterised by the presence of different markers that define the stage of the development. POU5F1, NANOG and SOX2 are pluripotent factors present in ICM, and CDX2, EOMES and TEAD4 are part of TE. In the second differentiation event, GATA6 and SOX17 are expressed which are essential for PE formation, whereas NANOG remains present in EPI cells.

Unbalanced nutrition throughout pregnancy can modify the normal response to environmental conditions in the future life (Vanhees *et al.*, 2013). Maternal low protein diet (LPD) during the

preimplantation period in a mouse model reduced birthweight in female offspring and increased postnatal growth rate, whereas male offspring showed elevated blood pressure in adulthood. Additionally, rat embryos (E4.25) showed a significant reduction in cell numbers (ICM and TE lineage), suggesting a slower rate of cellular proliferation (Kwong *et al.*, 2000). A rat model has shown that maternal LPD during preimplantation alters the expression of imprinted genes in the male blastocyst at E4.0 only, showing a gender-specific effect (Kwong *et al.*, 2006). These results propose that periconceptional nutrition has an impact on embryo development. Beside LPD, one-carbon metabolism also shows to be essential for the embryonic development.

Low levels of maternal RBC folate has been associated with a slower embryo development (Parisi *et al.*, 2017), meanwhile high levels of homocysteine are associated with a poor embryo quality (Boxmeer *et al.*, 2009). A diet high in methionine has been reported to induce transcriptional changes (mostly down-regulation) in genes related to embryo development (Peñagaricano *et al.*, 2013). However, the effect of preconceptional supplementation with high folic acid (FA) in the blastocyst is not well understood.

The objectives of this study were to determine the effect of periconceptional high FA diet on embryo development at the blastocyst stage, and the effect of high FA diet exclusively during the preimplantation period.

6.2 Experimental Design

Virgin C57BL/6 adult (PND74) female mice were randomly assigned to three different experimental groups; the Control group where mice were fed with a diet consisting of 1mg FA/kg food (recommended daily requirement) throughout the experimental period; the preimplantation (P.I) group where mice were fed with 1mg of FA for four weeks before pregnancy, and changed to high FA diet (5mg FA/kg of food) for the first three days of the pregnancy, and periconceptional (P.C) group fed with a high FA diet (5mgFA/kg of food) for four weeks prior to conception and for the first three days of pregnancy (Figure 6.1).

Female mice were naturally mated with a male C57BL/6 overnight, and checked the next morning for the presence of a vaginal plug. The presence of a vaginal plug was defined as successful mating and was designated as 0.5-day post-coitum (dpc). All female mice were culled at 3.5dpc and embryos were collected by flushing the mouse uterus. Animal dissection, blood and organ collection is described in the sections 2.1.5, 2.1.6 and 2.1.7. 98 females were used in this model, however only 75 became pregnant and used for analysis. This number represents 76.5% of the total animals planned for the model, which can be explained by a false positive plug or the non-presence of a vaginal plug after five days mating (See Methods 2.1.8 section). There was no difference in the number of false positive pregnant mice between the groups.

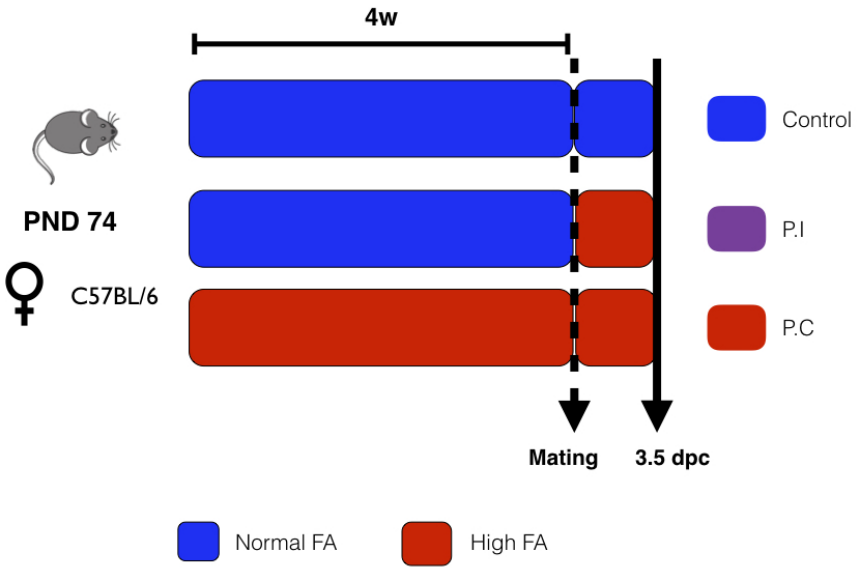

Figure 6.1 Animal model embryo analysis

Virgin adult female C57BL/6 mice were fed with normal and high doses of FA during 4 weeks and mated with male C57BL/6 mice. Female mice were culled at 3.5 dpc and embryos were collected.

6.3 Results

6.3.1 Weight and energy intake measurements

At PND74, the body weight of the animals did not differ among the three groups. To ensure mice consumed the diet and maintained normal growth and feeding patterns, body weight and food intake per cage were monitored weekly and analysed by the area under the curve (AUC). There was a significant increase in body weight between the P.C group compared to the control and P.I groups (P = 0.003 and P = 0.02 respectively) (Figure 6.2). However, no changes in energy intake (Kcal per 10 grams of body weight) before and after conception were observed between the different dietary groups (Figure 6.3 and Figure 6.4)

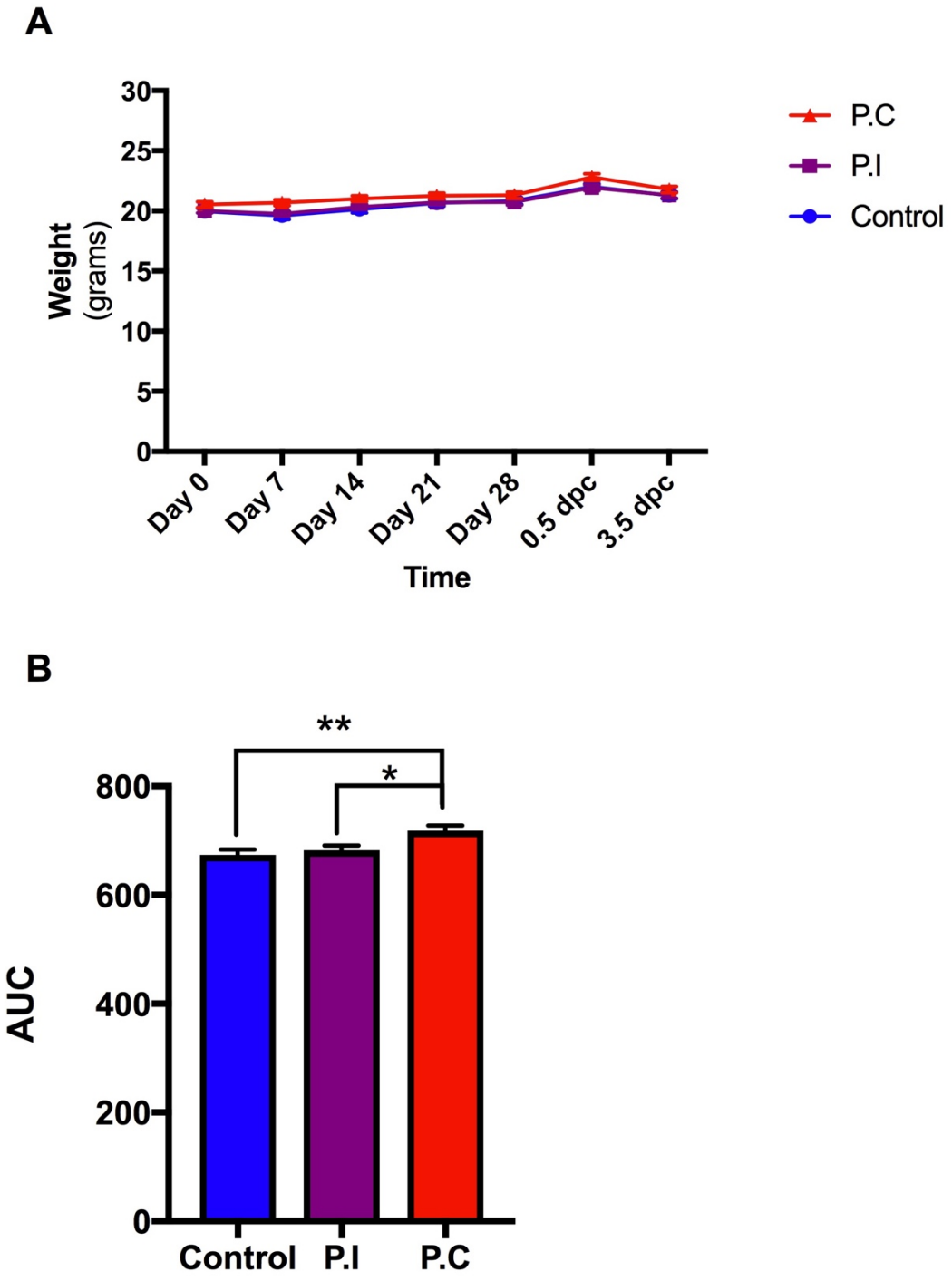

Figure 6.2 Body weight during periconceptional supplementation with FA

Body weight of C57BL/6 female mice four weeks before the pregnancy and until 3.5dpc (A). Area under the curve (AUC) analysis of the body weight (B). Mean ± SEM. n = 25/24/26 animals per group. Anova test, Bonferroni Post-hoc. *P< 0.05, **P<0.01

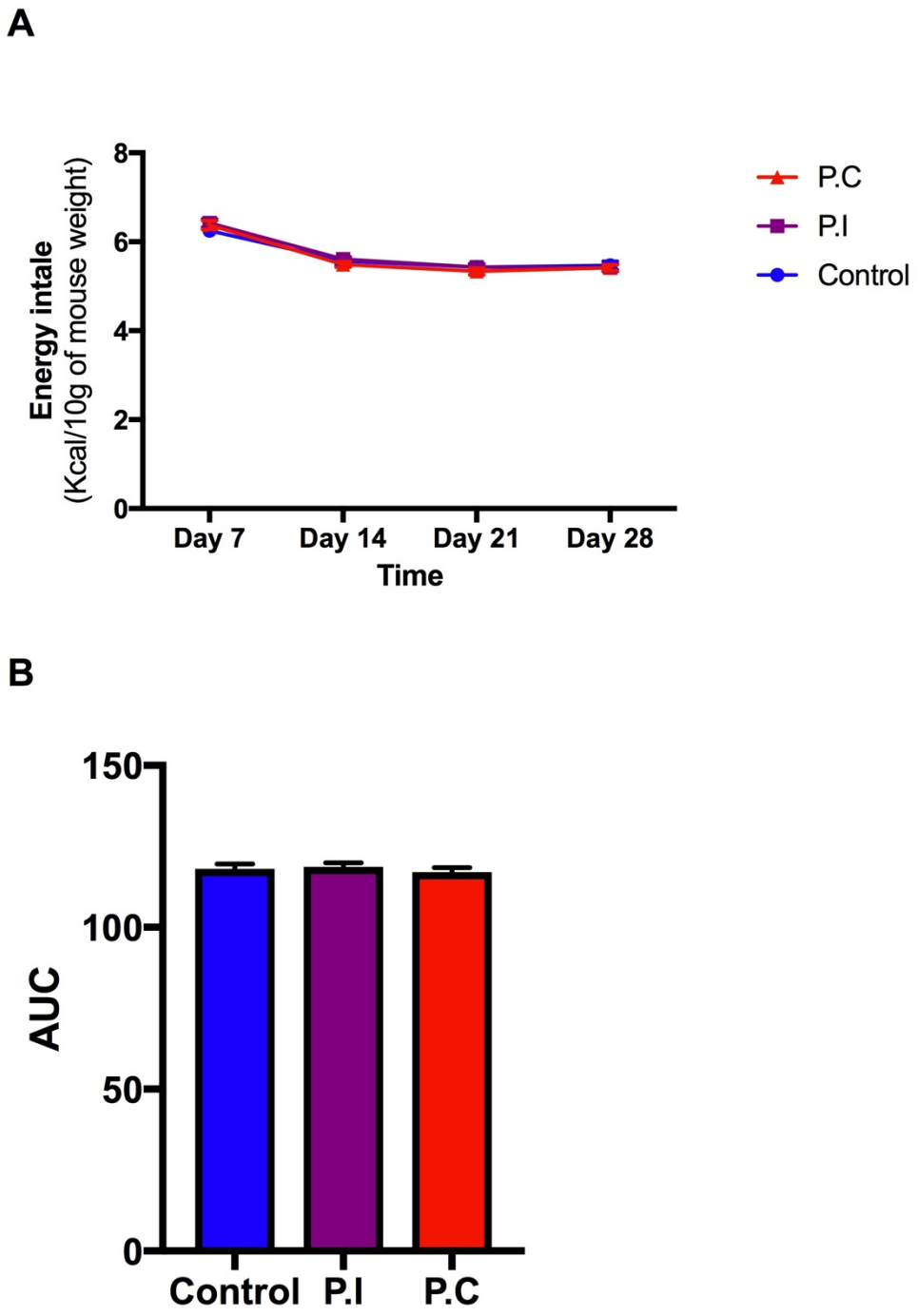

Figure 6.3 Energy intake during preconception

Energy intake of C57BL/6 female mice four weeks before the pregnancy (A). Area under the curve (AUC) analysis of the energy intake per group (B). Mean ± SEM. n = 25/24/26 animals per group. Anova test, Bonferroni Post-hoc

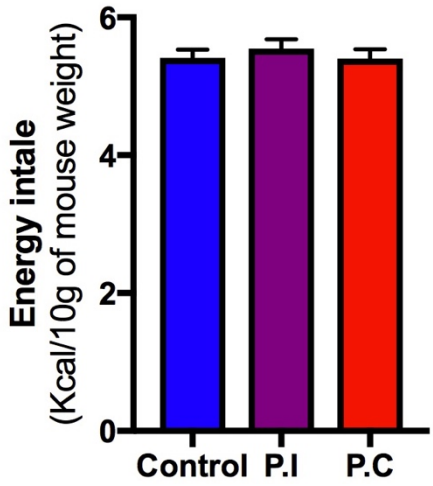

Figure 6.4 Energy intake during the preimplantation period.

Energy intake of C57BL/6 female mice during the preimplantation period (0.5-3.5dpc). Mean ± SEM. n = 25/24/26 animals per group. Anova test, Bonferroni Post-hoc.

After 28 days of the diet treatment, the females were placed with a male mouse at 4pm for mating. The next morning at 9am the presence of a vaginal plug was assessed, if the result was negative, the female was placed again with the same male at 4pm on the same day, until finding a positive plug (See Methods 2.1.8 section). The total number of days taken before successful mating occurred was analysed (Figure 6.5). For the P.C group, the mice took more time to mate compared to control group (*P* = 0.02).

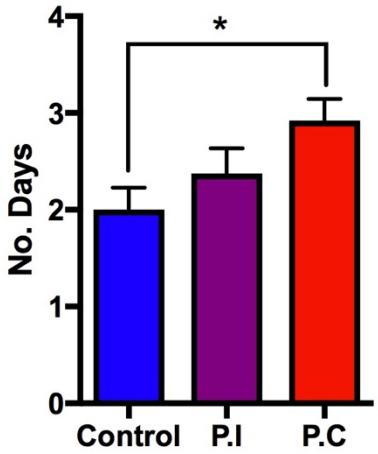

Figure 6.5 Days of mating

Number of days taken until a successful mating of female with the male C57BL/6 mice. Mean ± SEM, n = 25/24/26 animals per group. *P< 0.05, Anova test, Bonferroni Post-hoc.

6.3.2 Plasma folate analysis

At 3.5dpc the plasma folate was measured on six random pregnant mice per group. After the diet treatment, there were not any significant differences among the groups (Figure 6.6). However, an increase was observed in the supplemented groups, where the P.C group showed the highest level of folate on plasma compared with the control group.

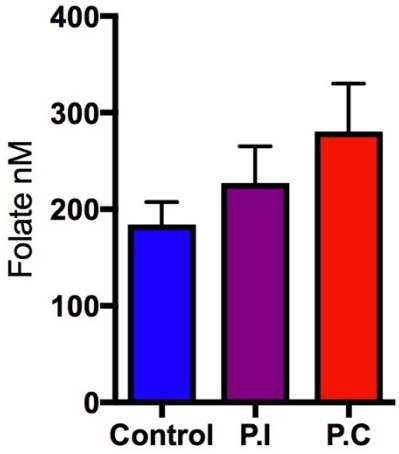

Figure 6.6 Folate plasma level of pregnant mice

Folate plasma level of pregnant female mice exposed to normal or high FA supplementation. Mean ± SEM, n = 6 mice per group. Anova test, Bonferroni Post-hoc.

6.3.3 Number of embryos available at 3.5pdc

The number of embryos and the stage of development at the collection time point can provide information about early growth during the preimplantation period. The figure below shows the total number of embryos (excluding degenerated oocytes and blastocysts per group (See Methods 2.1.9 section for definition of degenerated oocyte and blastocyst) collected at 3.5dpc in the three dietary groups (Figure 6.7). To assess the stage of the development, all embryos collected after flushing the mouse uterus were divided in different categories late, middle and early blastocyst, morula and degenerated oocyte or embryo (Figure 6.8). The supplementation with FA exclusively during preimplantation reduced the number of embryos and blastocyst observed at 3.5pdc (*P* = 0.03) compared with a periconceptional supplementation. However, there are no differences among the stages of those blastocysts.

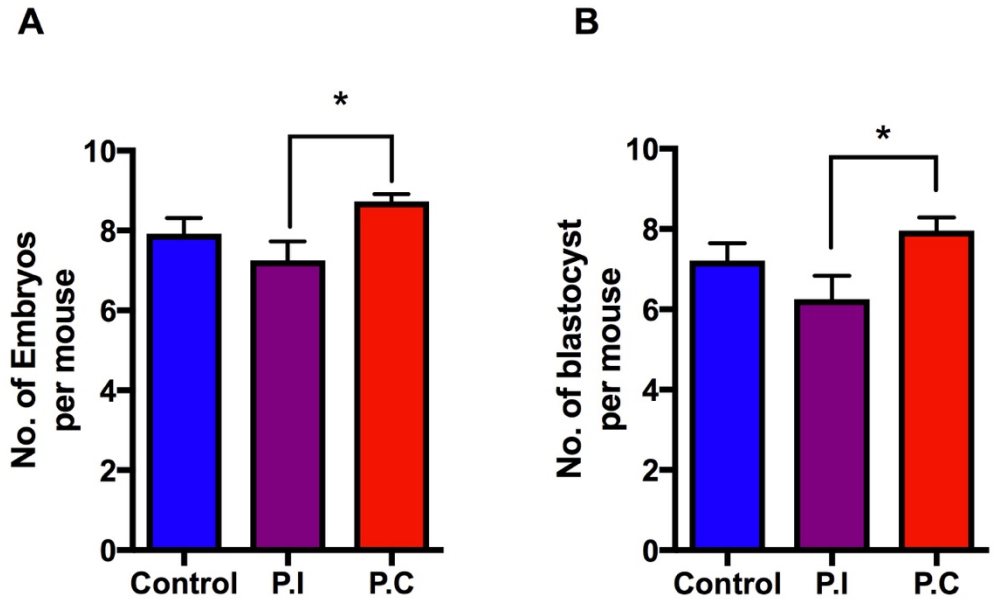

Figure 6.7 Number of embryos available at 3.5pdc.

Number of embryos (blastocysts and morula) (A) and blastocyst (B) available at 3.5pdc. Analysis by a multilevel random effect regression model taking account of potential maternal-embryo hierarchical association. Mean ± SEM, n = 25/24/26 animals per group.

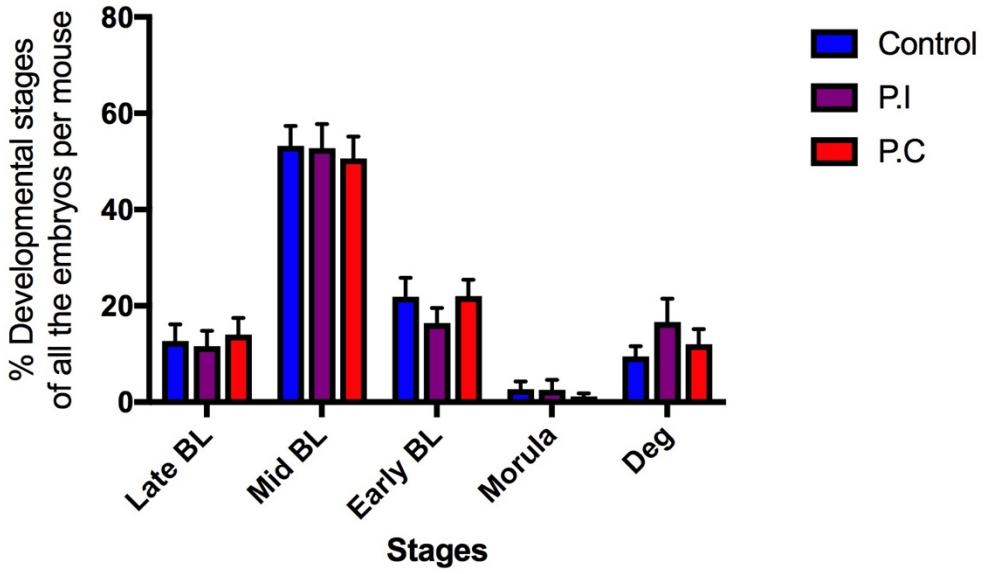

Figure 6.8 Percentage of developmental stages of embryos at 3.5dpc

Percentage of different developmental stages of all embryos per mouse at 3.5pdc. Blastocysts (BL) were grouped according the proportion of the embryo cavity in early, middle or late blastocyst. Degenerative embryo (Deg) was defined as deteriorated embryo or oocyte. Analysis by a multilevel random effect regression model taking account of potential maternal-embryo hierarchical association. Mean ± SEM, n = 25/24/26 animals per group.

6.3.4 Differential cell labelling of blastocyst at 3.5dpc

The first lineage decision during mouse development is the establishment of TE and ICM lineages, morphologically distinguishable at the blastocyst stage. Before blastocyst formation, compaction occurs, where blastomeres acquire apicobasal polarity. In subsequent cell division, outer cells remain polarised and generate the TE cells, meanwhile ICM stay in the centre of the blastocyst.

A differential cell labelling staining was performed in all the blastocysts to identify the number of TE and ICM cells. This assay allows the identification of the outer cell layer (TE) and can determine changes in the development of the blastocyst associated with the FA diet.

Periconceptional supplementation with FA (P.C) showed a decrease in the number of TE cells compared with the control group (P = 0.03) (Figure 6.9). Moreover, the same group showed a lower number of total cells in the blastocysts compared to the P.I group (P = 0.015). The proportion of ICM and TE cells is an indicator for blastocyst development. The radio of ICM/TE was increased in the P.C group compared with the control group (P = 0.03) (Figure 6.10). These analyses were done taking into account the maternal-embryo association and were not affected by the number of embryos per mother.

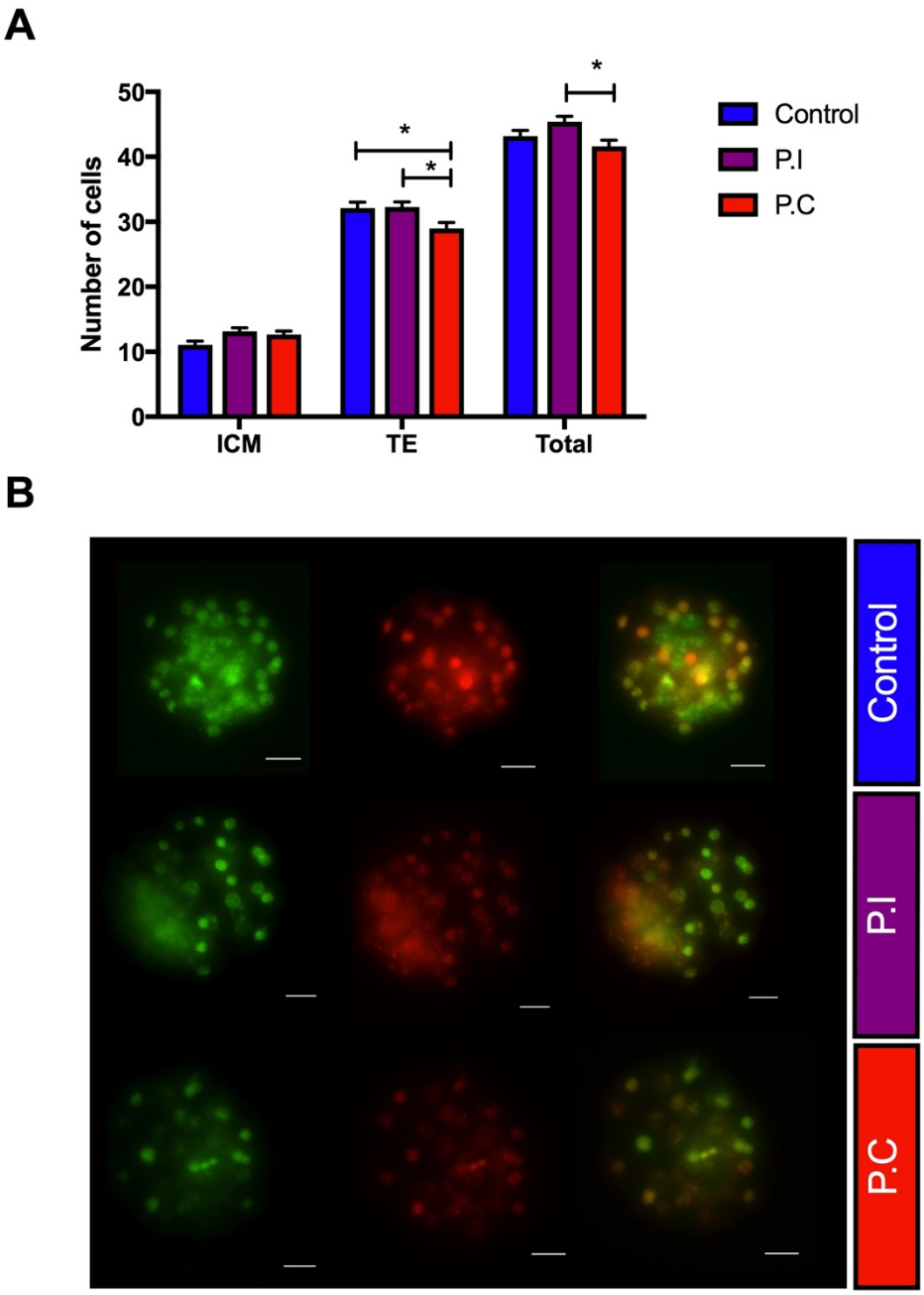

Figure 6.9 . Differential labelling of inner and outer cells in blastocyst

Number of cells identified as ICM or TE cells by differential cell staining (A). Bars indicate mean ± SEM, n = 8 animals per group. Analysis by a multilevel random effect regression model taking account of potential maternal-embryo hierarchical association. *$P < 0.05$. Representative pictures of blastocyst stained with Hoechst 33258 (green) and propidium iodide (red) (B). Hoechst coloured all the nuclei of the blastocyst, whereas propidium iodide coloured TE cells. Scale bar 20 µm.

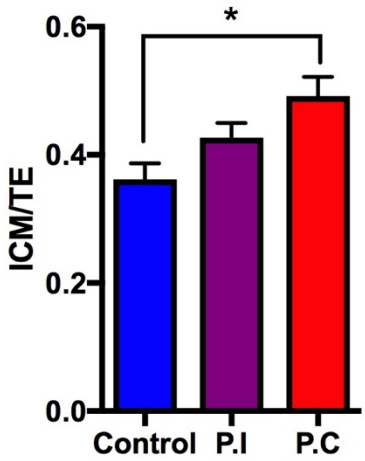

Figure 6.10 ICM/TE cell ratio of blastocyst

Bars indicate mean ± SEM, n = 8 animals per group. Analysis was conducted using a multilevel random effect regression model taking account of potential maternal-embryo hierarchical association. *$P < 0.05$ compared to control group (no differences between the P.I and P.C groups)

6.3.5 Gene expression in male blastocyst at 3.5pdc

During embryonic development cells differentiate and each cell lineage (e.g. ICM, TE, PE and EPI) acquires a different gene expression profile (See Introduction 1.3.1). In this process, epigenetic mechanisms have an active role (See Introduction 1.4.1.11.4.2). Given the differences observed in TE number and ICM/TE ratio, the expression of key regulators of TE differentiation and ICM maintenance was assessed by qPCR.

Figure 6.8 shows the middle blastocyst was the most frequent stage of the blastocyst found at 3.5dpc (>50%). To avoid further variables that could impact on the embryo development, embryo culture to control the stage of development was discarded, and only embryos collected directly from the mother uterus were used. Eighty middle blastocysts were used for RNA extraction, which were genotyped to determine the sex (61% were male, 9% were female and 30% were inconclusive) (See Methods 2.9.2 section). Because the males were the most abundant sex, gene expression assays were performed in one male middle blastocyst per mother (n = 10 mothers per group). Due to the small number of female blastocysts, the same assay could not be repeated. Some studies refer to the hypothesis of a sex-dependent growth rate differences in IVF embryos, which suggests that male embryos develop faster than females during preimplantation (Kochhar, Peippo and King, 2001; Dumoulin *et al.*, 2005). This theory could explain the greater number of male embryos at the middle blastocyst stage, however, a complete analysis of all the embryos available at 3.5dpc could help to determine changes in the growth speed.

The gene expression was normalised using *H2afz*, *Ppib* and *Tuba* as reference genes previously selected by geNorm analysis (See Figure 2.13). qPCR was performed using primers assays previously optimised and verified beforehand using gel electrophoresis and melting curve analysis (See Methods 2.7.4 section, Figure 2.8 and Appendix C.1 for agarose gels)

6.3.5.1 Gene expression of lineage markers

CDX2, a caudal-type homeodomain transcription factor has been detected from the 8-cell stage, but is specifically expressed in TE at the blastocyst stage (Ralston and Rossant, 2008). A decrease of *Cdx2* expression was observed in blastocysts from mothers exposed to high levels of FA. The preimplantation and periconceptional high FA diet induced a decrease in *Cdx2* expression in the P.I (P = 0.01) and the P.C group (P = 0.0008) compared to the control group (Figure 6.11).

OCT4, a octamer-binding and NANOG, a homeobox transcription factor are pluripotency markers both required for the maintenance of ICM fate and pluripotency of ES cells (Loh *et al.*, 2006). OCT4A is the isoform of OCT4 which is the main variant responsible for the stemness properties in pluripotent cells (Farashahi Yazd *et al.*, 2011). NANOG, a homeobox gene, is also expressed in ICM cells at the blastocyst stage and becomes EPI-specific in the late blastocyst. A decrease in the expression of both markers was observed in periconceptional high FA diet in both the P.I and P.C groups. *OCT4* downregulation was identified in the blastocyst from the P.I and P.C groups (P = 0.005 and P = 0.01, respectively) (Figure 6.11). *Nanog* was downregulated in the blastocyst form the P.I and P.C groups, although it was only significant in the P.C (P = 0.01 C v P.C, P = 0.06 C v P.I) (Figure 6.11).

GATA6, GATA family 6 transcription factor is expressed in PE cells at the blastocyst stage. Together with NANOG, it can be co-expressed in ICM cells at the early blastocyst stage, however following embryo development to middle blastocyst, ICM cells will differentiate into PE or EPI cells, after which they express either GATA6 or NANOG (Schrode *et al.*, 2014). *Gata6* was downregulated significantly in the P.I group (P = 0.03), whereas the P.C group showed a trend toward a decrease (P = 0.07) compared to the control group (Figure 6.11).

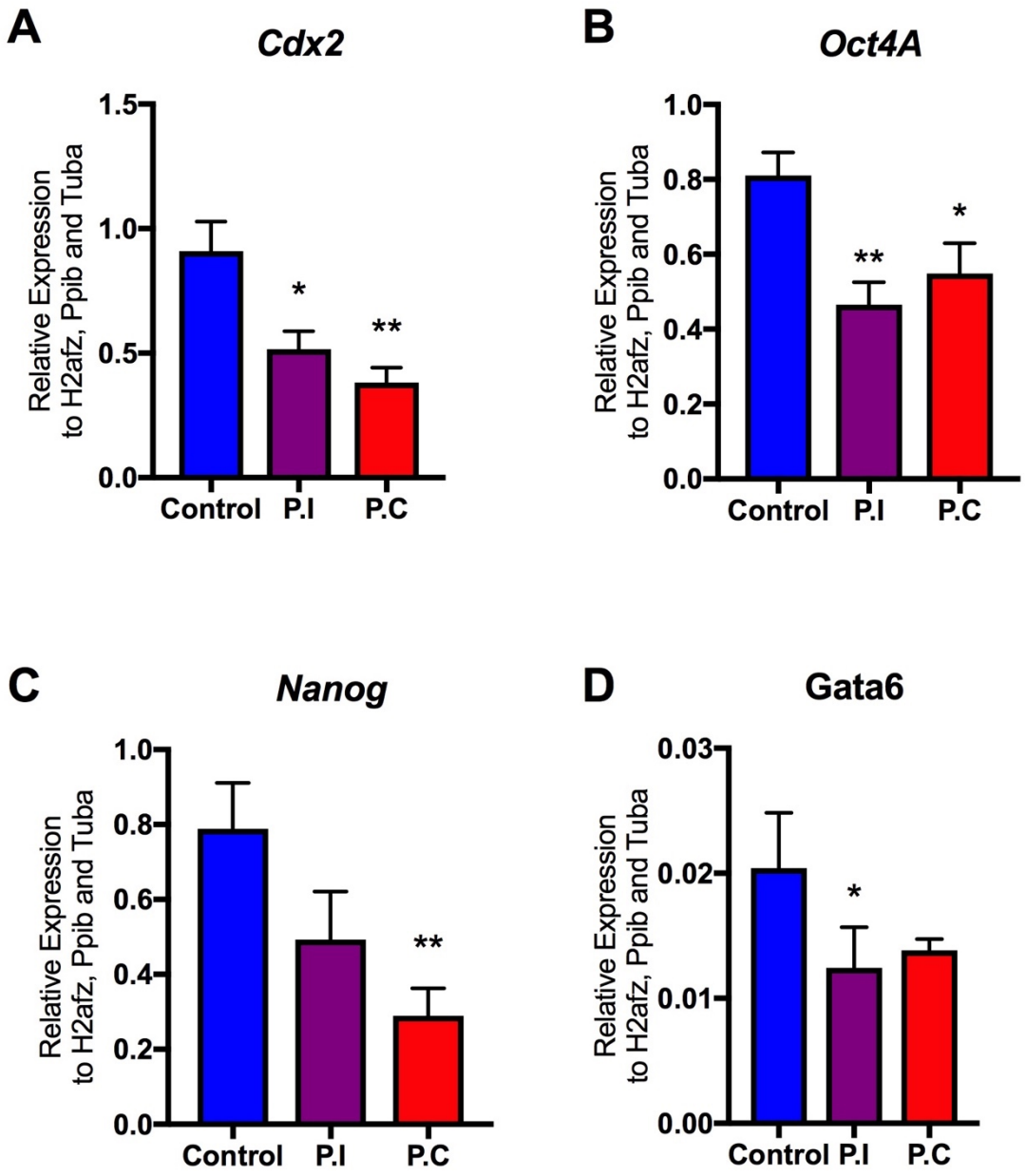

Figure 6.11 Gene expression of lineage markers

Gene expression of lineage markers *Cdx2* (A), *Oct4A* (B), *Nanog* (C) and *Gata6* (D) at middle blastocyst stage. Bars indicate mean ± SEM. Analysis was conducted using a multilevel random effect regression model taking account of potential maternal-embryo hierarchical association. **P* < 0.05, ***P* < 0.01 compared to control group.

6.3.5.2 Gene expression of epigenetic markers

During embryogenesis, epigenetic mechanisms play a crucial role in regulating the balance between pluripotency and differentiation. In particular the polycomb repressive complex (PRC1/PRC2), which mediates H3K27 trimethylation and DNMTs, which catalyse DNA methylation, regulate the expression of the pluripotency genes and differentiation specific genes. (See Introduction section 1.4).

6.3.5.2.1 Gene expression of chromatin modifiers

Enhancer of zeste homolog 2 (EZH2), is a catalytic subunit of PRC2, whereas the embryonic ectoderm development (EED) is a non-catalytic subunit that stabilises EZH2. Both subunits are expressed in the blastocyst and participate in the regulation of the differentiation and proliferation.

Ezh2 and *Eed* are part of PRC2 polycomb that is involved in mouse embryonic development. Male middle blastocyst showed significant decrease in *Ezh2* gene expression only in the P.I group (P = 0.02) (Figure 6.12A). However, *Eed* was down regulated in the P.I and P.C group (P = 0.01 and P <0.0001, respectively) (Figure 6.12B).

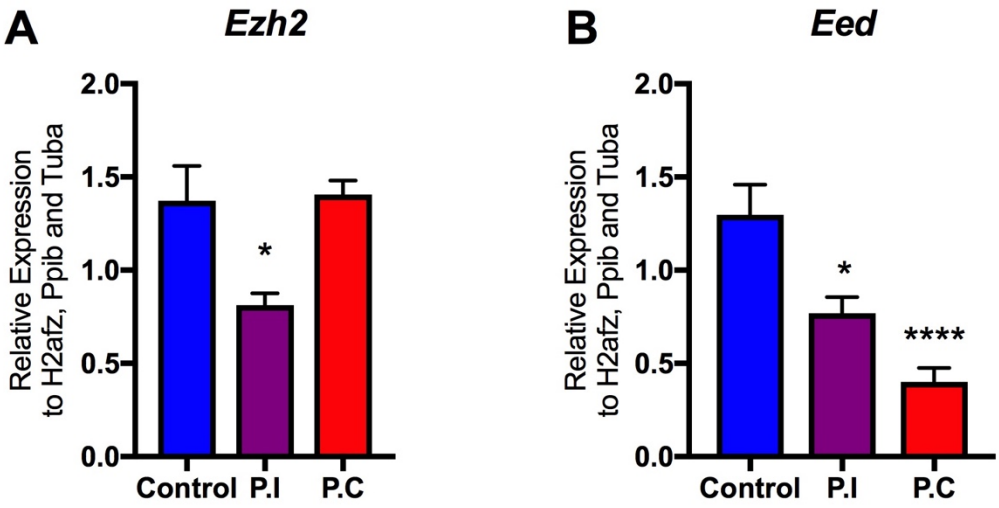

Figure 6.12 Gene expression of chromatin modifiers

Gene expression of *Ezh2* and *Eed* at middle blastocyst stage. Bars indicate mean ± SEM. Analysis was conducted using a multilevel random effect regression model taking account of potential maternal-embryo hierarchical association. *P < 0.05, ****P < 0.0001 compared to control group.

6.3.5.2.2 Gene expression of DNA methyltransferases

DNA methylation and DNA methyltransferases (DNMT's) participate in the embryonic development, genome stability, X chromosome inactivation and genomic imprinting. DNMT1 has a role as a maintenance methyltransferase, which methylates hemimethylated DNA. Meanwhile, DNMT3A and DNMT3B participate in *de novo* DNA methylation of non-methylated DNA. DNMT3L –without enzymatic activity- is essential for maternal imprint establishment and binds directly to DNMT3A or 3B to potentiate their activity. All these DNMT's have been reported in the mouse embryo during the preimplantation period (Petrussa, Van de Velde and De Rycke, 2014). Due to limited cDNA, only *Dnmt1* and *Dnmt3l* were analysed and both showed good amplification (See Appendix C.1). High FA diet did not change the expression of *Dnmt1* and *Dnmt3l* at middle blastocyst stage (Figure 6.13).

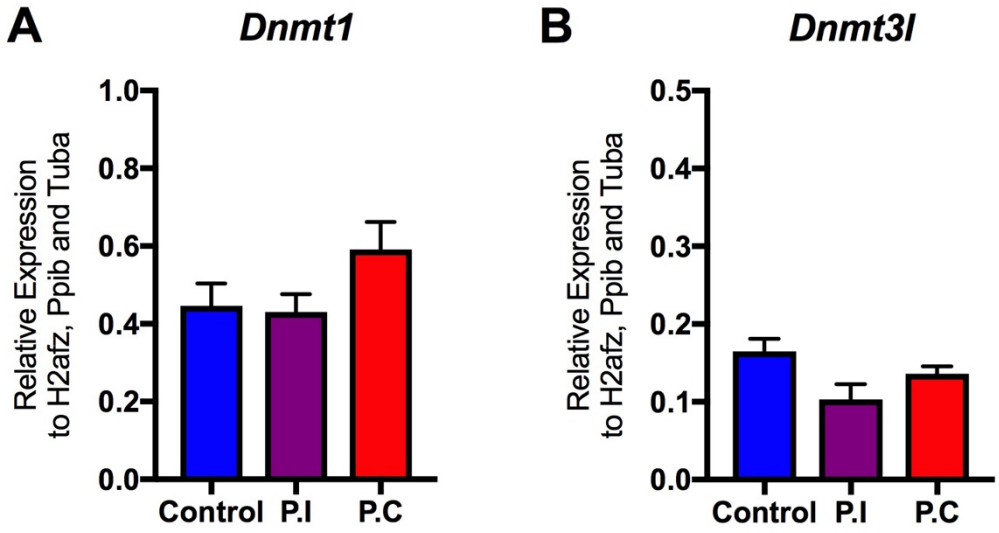

Figure 6.13 Gene expression of DNMTs

Gene expression of *Dnmt1* and *Dnmt3l* at middle blastocyst stage. Bars indicate mean ± SEM. *Dnmt3l* values are expressed as mRNA x10. Analysis was conducted using a multilevel random effect regression model taking account of potential maternal-embryo hierarchical association.

6.3.5.3 Gene expression of folate transporter

Folate receptor and transporters have been described to be expressed in the oocyte and blastocyst during preimplantation (Kwong *et al.*, 2010). However, *Slc46a1* (encoded for *Pcft1*) was the only folate transporter detected by qPCR the single middle blastocyst. Mutation in folate transporters and receptors are characterised by a delayed embryonic development, morphological abnormalities including NTD or embryo death (Taparia *et al.*, 2007). Nevertheless, after high FA diet were no changes in *Slc46a1* gene expression between the groups (Figure 6.14).

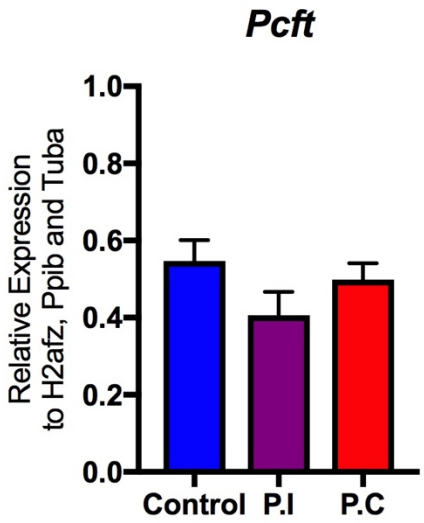

Figure 6.14 Gene expression of folate transporter

Gene expression of *Slc46a1* (which encodes *Pcft1)* at middle blastocyst stage. Bars indicate mean ± SEM. Analysis was conducted using a multilevel random effect regression model taking account of potential maternal-embryo hierarchical association.

6.3.6 Immunofluorescence detection of ICM and TE markers

Given the changes observed in the number of TE cells and lineage markers at mRNA, an immunofluorescence (IF) staining was performed to determine the localization and cell-positive number of CDX2 and NANOG at the protein level. Primitive endoderm markers such as GATA4, GATA6 and SOX17 were analysed to assess the differentiation of the cells. However, none of these antibodies worked effectively in the blastocysts (See Methods 2.13.3 and Appendix C.2). Cell nuclei were counterstained with DAPI (4',6'-diamidino-2-phenylindole dihydrochloride) solution.

For these assays, 28 females were mated , but only 64% of them became pregnant due to a false-positive plug or the presence of no plug after five days of mating. All plug positive females were used for this assay (See Methods 2.1.8 section for false-positive plug definition). An unexpected higher proportion of false-positive pregnancies were detected, resulting in 18 animals and 86 embryos being analysed.

The IF showed CDX2-positive nuclei as green and NANOG-positive nuclei as red. Outer cells of the blastocysts were stained with CDX2, meanwhile NANOG was predominantly present within the inner cells. However, some outer cells showed double staining (yellow colour) (Figure 6.15).

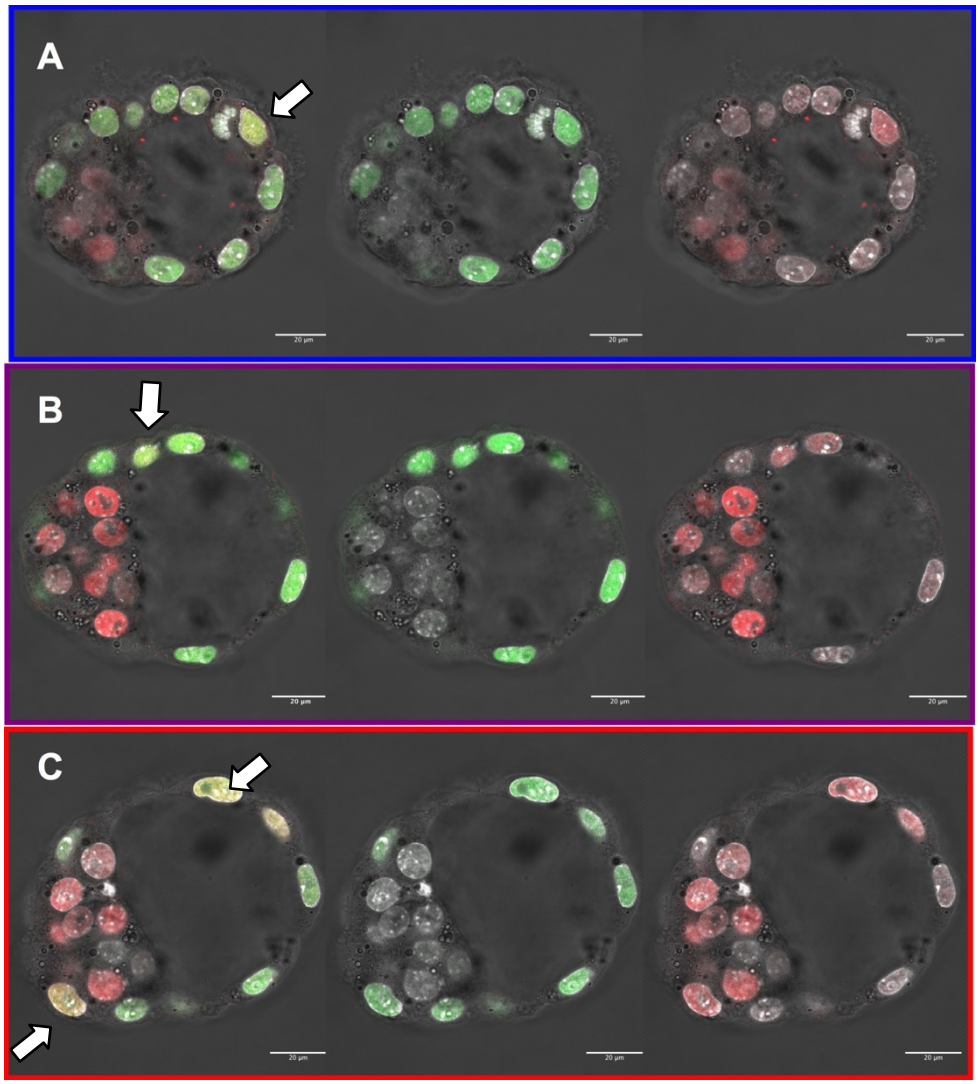

Figure 6.15 . Immunofluorescence detection of CDX2 and NANOG in 3.5dpc blastocyst

Immunofluorescence detection of CDX2 (green) and NANOG (red) performed in E3.5 blastocysts from Control (A, blue box), P.I (B, purple box) and P.C group (C, red box). CDX2 and NANOG were co-detected in some outer cells (white arrows). Nuclei were counterstained with DAPI (gray). Scale bar 20µm. Negative control was used in every staining (See Methods Figure 2.20)

6.3.6.1 Total cell number in blastocyst at E3.5

The number of cells was assessed by counting the number of DAPI-positive nuclei observed per blastocyst. The total number of cells was lower in the P.I group compared to the control group (*P* = 0.016) (Figure 6.16A). In some cells, chromosomes were clearly observed, instead of a well-rounded nucleus, demonstrating that these cells were undergoing cell division (See Figure 6.17). A separate count was made of the cells undergoing division. This analysis showed that the P.I group had an increased number of cells undergoing cell division (*P* = 0.02) and even in relation to the total number of cells (*P* = 0.008) (Figure 6.18). Moreover, when cells undergoing division were included to the total number of cells, the difference between control and the P.I group was still significant (*P* = 0.03) (Figure 6.16B).

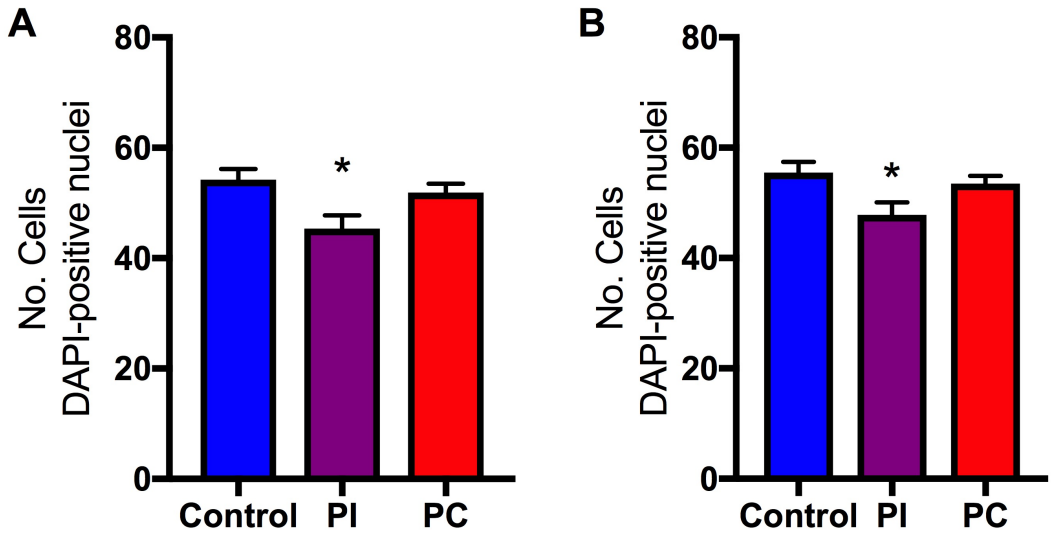

Figure 6.16 Quantification of DAPI-positive nuclei in blastocyst

Quantification for DAPI-positive nuclei in blastocysts from mothers exposed to normal or high FA excluding (A) and including (B) cells undergoing division. Analysis was conducted using a multilevel random effect regression model taking account of potential maternal-embryo hierarchical association. * P < 0.05 compared to control group. Bars indicate mean ± SEM, n = 86 blastocysts examined, (2-9 per mother).

Double and non-stained nuclei were also observed. To analyse this number, nuclei stained with NANOG and CDX2 were defined as double-stained, and nuclei with neither CDX2 nor NANOG were define as non-stained. No differences were found in the number of cells double stained for NANOG and CDX2 or non-stained (Figure 6.19).

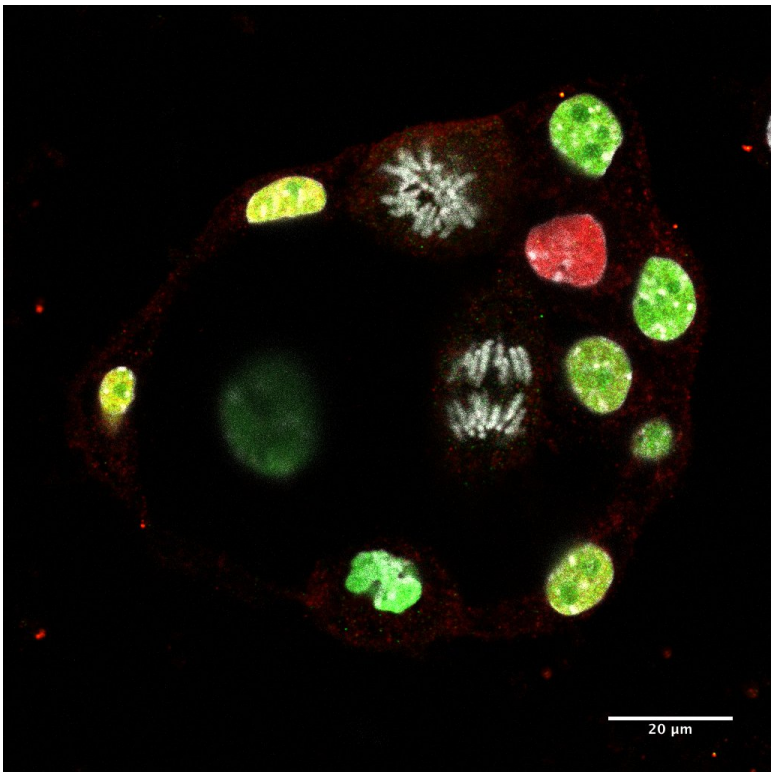

Figure 6.17 Visualisation of cells undergoing division

Immunofluorescence detection of CDX2 (green) and NANOG (red) performed in E3.5 blastocysts. Nuclei and chromosomes undergoing mitosis were counterstained with DAPI (gray). Scale bar 20µm.

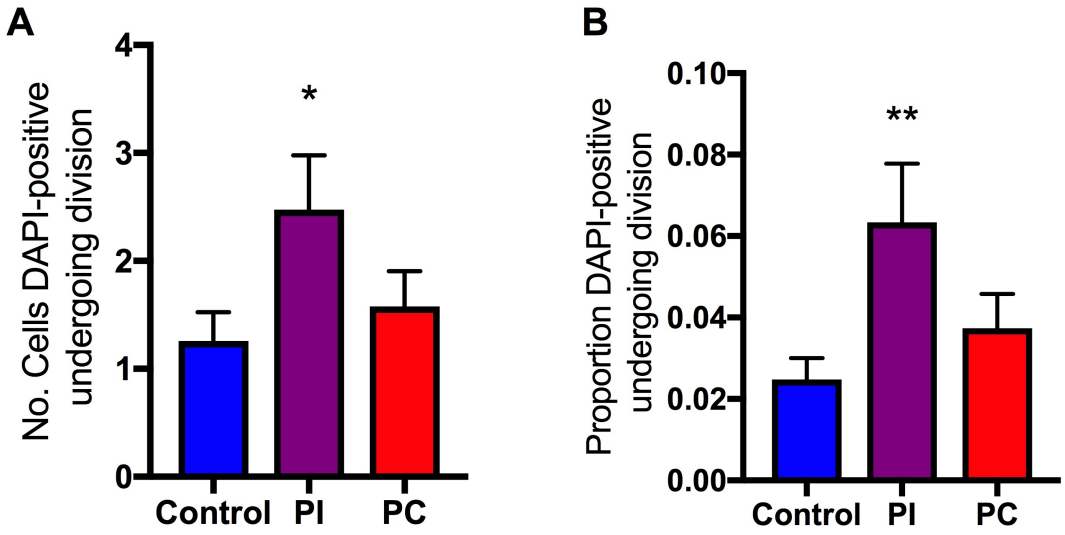

Figure 6.18 Quantification for DAPI-positive nuclei cell undergoing division

Number of cells undergoing division (A). Proportion of cells undergoing division by total cell number (B). Analysis was conducted using a multilevel random effect regression model taking account of potential maternal-embryo hierarchical association. * $P < 0.05$, ** $P < 0.01$ compared to control group. Bars indicate mean ± SEM, n = 86 blastocysts examined, (2-9 per mother)

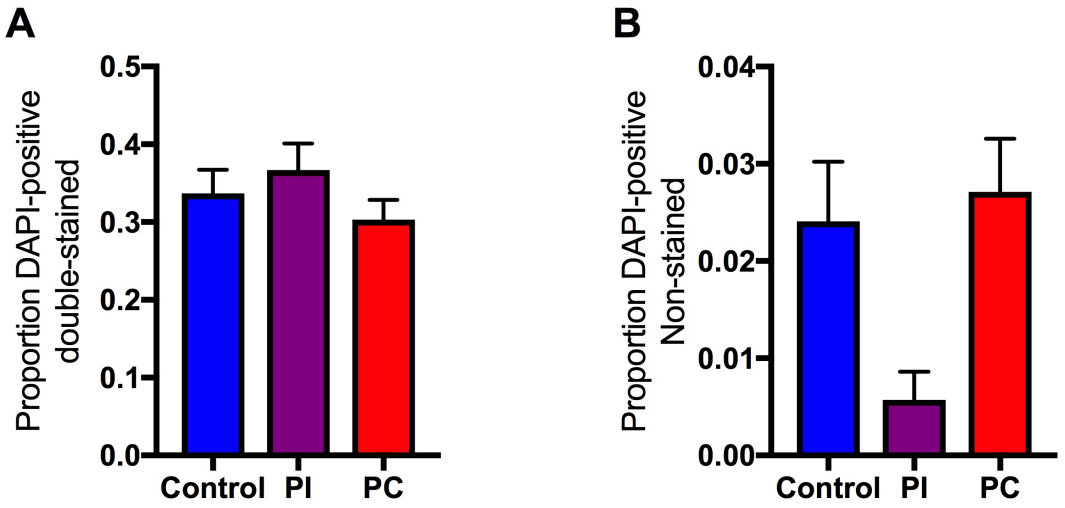

Figure 6.19 Quantification of double stained and non-stained cells

Proportion of double stained cells for Cdx2 and Nanog antibody (A). Proportion of non-stained cells (excluding cells in division) (B). Analysis was conducted using a multilevel random effect regression model taking account of potential maternal-embryo hierarchical association. Bars indicate mean ± SEM, n = 86 blastocysts examined, (2-9 per mother)

6.3.6.2 Quantification of CDX2-positive cells in blastocyst at E3.5

Similar to the previous results from the differential labelling and gene expression experiments, CDX2-positive cells were lower in the FA supplemented groups compared to the control group (*P* = 0.005 for the P.I group, and P = 0.04 for the P.C group) (Figure 6.20A). To identify the number of nuclei expressing exclusively CDX2, the number of double stained cells was subtracted from the total number of CDX2-positive cells (Figure 6.20B). CDX2 expression was lower in the P.I (*P* = 0.002) and P.C group (*P* = 0.04).

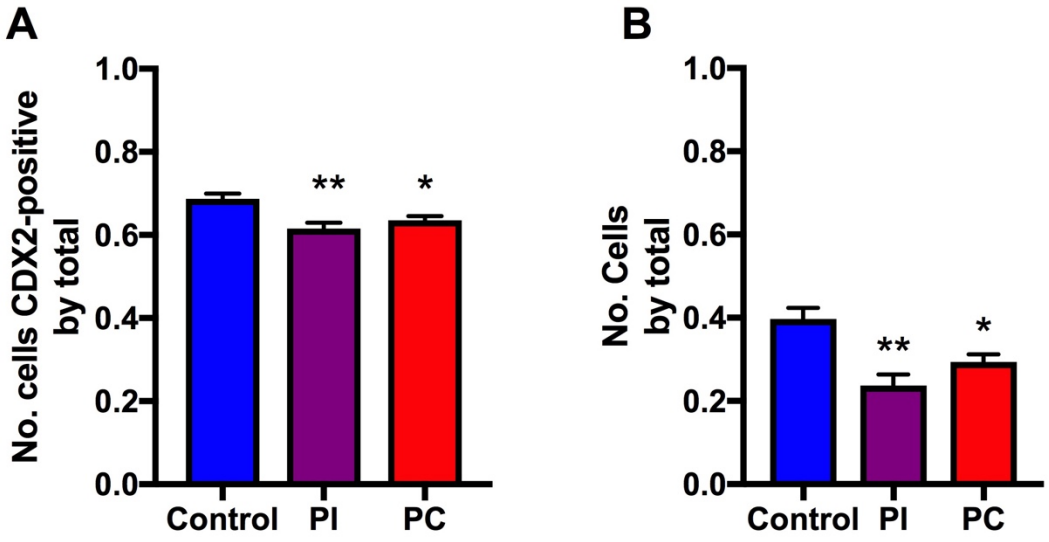

Figure 6.20 Quantification of CDX2-positive cells in blastocyst at 3.5dpc

The Proportion of CDX2-positive cells by total number of cells (A). Proportion of CDX2-positive cells without the cells stained also for NANOG (B). Number expressed by total number of cells. Analysis was conducted using a multilevel random effect regression model taking account of potential maternal-embryo hierarchical association. * $P < 0.05$, ** $P < 0.001$ compared to control group. Bars indicate mean ± SEM, n = 86 blastocysts examined, (2-9 per mother)

6.3.6.3 Quantification of NANOG-positive cells in blastocyst at E3.5

NANOG, a transcription and pluripotency factor described as a marker for ICM, was used for the IF to detect changes in the ICM that could be correlated with the gene expression assay. At the protein level, no changes were observed among the groups when the NANOG-positive cells were analysed in relation to the total number of cells (Figure 6.21A). However, when the double-stained cells were removed from the proportion of NANOG-positive cells, an increase of NANOG was observed in the P.I group (*P* = 0.001) (Figure 6.21B).

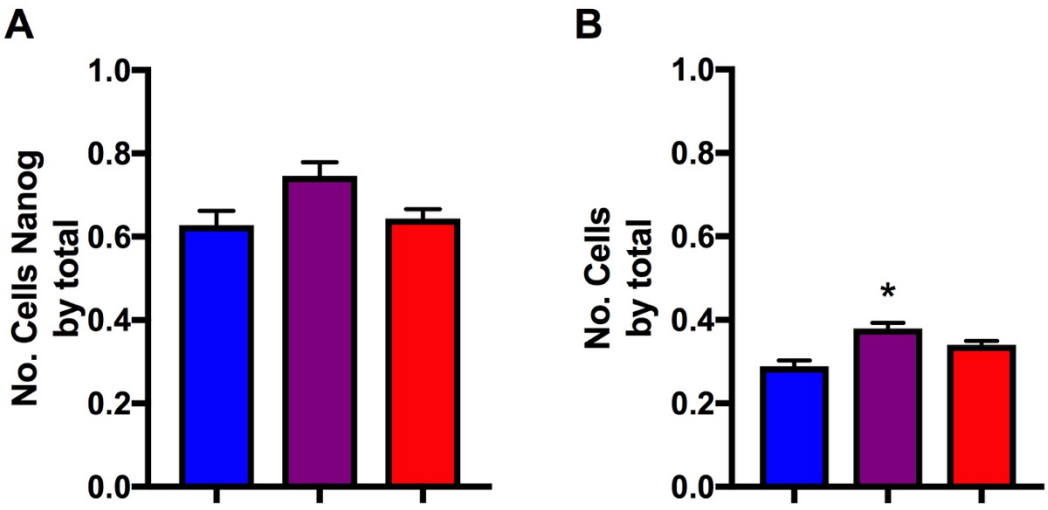

Figure 6.21 Quantification of NANOG-positive cells in blastocyst at 3.5dpc

Proportion of NANOG-positive cells by total number of cells (A). Proportion of NANOG-positive cells without cell stained for CDX2 (B). Number expressed by total number of cells. Analysis was conducted using a multilevel random effect regression model taking into account a potential maternal-embryo hierarchical association. *P < 0.05 compared to control group. Bars indicate mean ± SEM, n = 86 blastocysts examined, (2-9 per mother).

6.4 Discussion

Nutritional perturbations before and during pregnancy can modify development throughout gestation and determine the physiological and metabolic health of the adult offspring (Ashworth, Toma and Hunter, 2009; Fleming *et al.*, 2012). Folate is an essential nutrient for the synthesis of amino acids, purines and thymidylate and the production of the universal methyl group donor S-Adenosylmethionine (SAM). During embryogenesis, one-carbon metabolism is fundamental for the optimal development of the blastocysts (Ikeda *et al.*, 2012). Whilst the consequences of inhibiting this pathway by deficient methyl donor diet around conception have been well described (Deng *et al.*, 2008; Kim *et al.*, 2009; B. Zhang *et al.*, 2015), the effect of larger doses of folic acid (FA) has not been defined. To assess the effect of a high FA diet on embryo development, mice were fed with a normal or high FA diet for four weeks before pregnancy or just during the preimplantation period. FA in both time periods –preimplantation and periconceptional- altered not only the development of the blastocyst at the mRNA and protein level but also the proportion of ICM/TE cells and the number of embryos on 3.5dpc. These results support the idea that oocyte maturation, fertilisation and preimplantation development are particularly sensitive to high FA diet (See Table 6.1 for a summary of the results).

Preimplantation is a sensitive period where environmental perturbations can have significant effects on embryo growth (Kwong *et al.*, 2000, 2006; Watkins, Ursell, *et al.*, 2008). In this chapter, the maternal consumption of high FA diet exclusively during preimplantation (P.I group), showed alteration an increase of NANOG (only at protein level) and a decrease of CDX2 at both mRNA and protein level. Moreover, a decreased number of total cells was observed, potentially compensated by an increased number of cells undergoing division registered. These results are consistent with changes observed with LPD during the same period of time, suggesting that nutritional changes during preimplantation can have an impact on the development of the embryo.

In the case of the P.C group, the decrease in CDX2, *Oct4* and *Nanog* were not observed with changes in total embryo number, however, the blastocysts showed a lower number of TE cells. This group was exposed to the effect of FA not only *in-utero* but also during follicular development and several ovulatory cycles, therefore targeting oocyte and oocyte maturation, whereas the P.I group targets were only fertilised embryos. Additionally, female exposed to four weeks of preconceptional high FA diet conveyed in a delay in the mating process, spending in average one more day with the male than mice exposed to normal FA (n= 75 mice).

The first chapter, showed no effect of the FA on the ovarian follicle number, however, decreased ovarian gene expression of *Oct4* and epigenetic markers such as *Ezh2* and *Bmi1* were observed,

suggesting that the preconceptional high FA affects in the ovulatory cycle. The impact of the diet in the one ovulatory cycle has been described using LPD for 3.5 days before mating where adult offspring displayed higher blood pressure and reduced exploratory behaviour (Watkins, Wilkins, *et al.*, 2008).

Table 6.1 Summary results of high FA diet effect in the embryo at 3.5dpc

High FA diet	Preimplantation (P.I)	Periconceptional (P.C)
Body weight and intake	-	Higher body weight
Mating time	-	Longer mating
Embryo number	Lower Embryo Number*	-
Differential labelling	-	Lower number of TE Higher ICM/TE ratio
Gene expression	Lower *Cdx2*, *Oct4*, *Gata6*, *Ezh2* and *Eed*	Lower *Cdx2*, *Oct4*, *Nanog* and *Eed*
Immunodetection	Lower number of cells Higher mitotic index Lower CDX2 High NANOG	Lower CDX2

* Compared to P.C group.

The effect of a high FA diet on the lineage markers and the number of cells at the blastocyst stage has not been previously reported. However, inhibition of dihydrofolate reductase through the use of methotrexate has shown a negative effect in the embryo development, including reduced ICM and TE cells (B. Zhang *et al.*, 2015) and a reduced cell division (O'Neill, 1998). The decrease in transcription factors such as *Cdx2*, *Oct4*, *Nanog* and *Gata6* in the blastocyst observed here, suggest a delay in the development of the blastocyst. The same delay in growth has been reported at E10.5 in mice using high doses of FA before and during pregnancy (Pickell *et al.*, 2011). Moreover, a study performed in human pregnancy showed that low and high maternal RBC folate levels were associated with a smaller embryo size at the sixth and the 12th week of gestation (Van Uitert *et al.*, 2014). However, changes observed in those embryos were not translated to an altered birthweight, suggesting a later compensation in embryo development.

One of the most significant effects of the FA on the blastocyst is the impact in the TE cells (reduced number of cells and CDX2). CDX2 is a transcription factor expressed specifically in TE cells at the blastocyst stage and is maintained in Exe, whereas CDX2 protein is expressed since earlier stages of development (Strumpf *et al.*, 2005; Ralston and Rossant, 2008). CDX2 is involved in many cellular processes including polarisation during TE formation and the maintenance of this lineage. CDX2 maternal and zygotic null embryos show reduced development and increased cell death from the morula stage onwards (Jedrusik *et al.*, 2015). The decrease in CDX2 could be

induced before the middle blastocyst is formed, which could have an impact on the number of TE cells available at 3.5pdc, resulting in a decrease in TEAD4 (an upstream gene of CDX2), a regulator of TE lineage. TEAD4 activates CDX2 in the outer cells, which will repress OCT4/NANOG expression, promoting the specification and development of the cell lineage (Nishioka *et al.*, 2008). More analysis before the blastocyst formation could help to determine if the effect to high FA diet is present in morula and earlier stages, and also identify changes in other transcription factors as TEAD4.

The lower number of TE cells and lower CDX2 expression observed in the P.C group can have consequences in the further differentiation and the nutrition supply function, as the TE cells give rise to trophoblast cells and the placenta. Therefore, these results could have a high impact on implantation and foetal development. Alteration on the foetal and placenta weight after maternal high FA-supplemented diet have been reported in the foetus at E17.5 and birth (Penailillo *et al.*, 2015; Bahous *et al.*, 2017). Further work is needed to determine if the decrease of TE cells is associated with a change in placenta or birthweight.

The effect of the altered proportion of TE and ICM cells has have been described in LPD models (Watkins *et al.*, 2007; Mitchell *et al.*, 2009), and in some cases, can be associated with different outcomes in later life. Kwong et al. showed that rats exposed to maternal LPD during preimplantation altered birthweight, postnatal growth and increased blood pressure up to 12w of age. At the blastocyst stage, Kwong observed that the blastocyst had a lower number of cells (ICM and TE) compared with a normal protein diet. This alteration was linked to a slower rate of cell proliferation due to a significantly reduced mitotic index in response to an LPD (Kwong *et al.*, 2000). Folate alters cell proliferation, and specific inhibition of folate cycle has been shown to reduce the cell proliferation of the embryo (O'Neill, 1998). Meanwhile, human placenta cell line cultures exposed to high FA reduced cell viability and increased proliferation rate (Ahmed *et al.*, 2016). Concordant with that, in this project, high FA diet increased the mitotic index (cell undergoing division) in the P.I group. Inhibition of the folate cycle (by methotrexate, MTX) between 8-cell and the blastocyst stage has been shown to decrease ICM and TE cell number, the fraction of transferred blastocyst that resulted in foetuses at E10.5, and increase evident resorption sites (B. Zhang *et al.*, 2015). This suggests that the folate pathway is important for implantation, and the development of the placenta TE cells.

OCT4, NANOG and CDX2 play an essential role in the first cell fate choice in the embryo, the differentiation of ICM and TE. These transcription factors regulate each other showing high levels of CDX2 and lower OCT4 in TE, and low CDX2 and high OCT4 in the ICM. CDX2 activates the TE transcriptional program and represses the pluripotent network (OCT4, NANOG and SOX2) during

the blastocyst formation (Huang *et al.*, 2017). Embryos CDX2-deficient fail to repress OCT4 and NANOG expression in the TE lineage, and a subsequently fail to hatch and implant (Strumpf *et al.*, 2005; Wu *et al.*, 2010). In this project, P.I blastocyst showed low levels of CDX2 and high NANOG in the IF assay in proportion to the total number of cells, suggesting a retarded growth at 3.5dpc, whereas the cell division was increased to potentially compensate for the lower cell number. On the other hand, P.C blastocysts showed a decrease in CDX2, and only a decrease in the TE cells but no in the total number of cells, suggesting that the continue supplementation with FA had a lower impact compared to the change from normal to high FA diet during preimplantation.

In this project, *Oct4* was downregulated in both supplemented group, and previously FA also altered *Oct4* in the ovary which is mainly present in the oocyte. Mohanty et al. showed that the folate receptor (FRα) up-regulates *Oct4* and *Sox2* in cranial neural crest cells maintaining the pluripotent state of the cells (Mohanty *et al.*, 2016). Here we observed the opposite, FA down-regulates the pluripotent factors and the polycomb genes but increased cell division, this could be explained due to the differences between *in-vitro* and *in-vivo* models. However, more analysis would help to understand the mechanism how FA modifies differentiation/proliferation in the blastocyst.

Altered transcription factors expression analysed in this project may reflect changes in the epigenetic machinery associated with cell differentiation and proliferation. This epigenetic machinery is essential for the lineage cell fate and any alteration of the chromatin modifiers can have a lethal outcome on the embryo growth. After a high FA diet, *Ezh2* was decreased in the P.I group, whereas *Eed*, its interacting partner, was downregulated in both supplemented FA groups. EZH2-EED complex plays an essential role during embryogenesis, EZH2 is upregulated upon fertilisation but remains present in all stages of the preimplantation period. The absence of EZH2 is associated with an arrested development where *Ezh2*-null embryos at E7.5 had a smaller size and showed a retarded growth *in-vivo*. Meanwhile, *Ezh2*-deficient blastocysts kept in culture showed an impaired outgrowth of ICM/TE cells dying after a short time in culture (O'Carroll *et al.*, 2001). Loss of *Ezh2* in mouse embryos, besides inducing a delayed growth at the blastocyst stage, showed an increased apoptotic index in ICM cells, decrease of *Oct4*, *Nanog* and *Sox2* and decreased level of H3K27me3 that conferred the importance of EZH2 in the ICM fate lineage (Huang *et al.*, 2014). Studies have described how transcription factors such as *Oct4*, *Nanog* and *Sox2* are direct targets of EZH2 and EED in ICM and ES cells and with the aim of silencing differentiation factors (Ura *et al.*, 2008; Wu *et al.*, 2014). In contrast to the polycomb repressor genes, no alterations in *Dnmts* were observed at 3.5dpc in the middle blastocyst stage. At this stage, the process of remethylation of the DNA starts to generate the differentiation of the cell

lineages. In that context, it could be that changes in the *Dnmts* associated with the FA levels have not happened yet, as changes in *Ezh2* and *Eed* were observed.

6.5 Conclusion

In conclusion, high FA diet before and/or during the preimplantation affects embryo development inducing slower embryo growth and a reduced number of embryos available for implantation. However, the specific effect of FA supplementation is dependent upon the time of supplementation. High FA diet exclusively during P.I period induced a greater impact on the blastocyst than continued supplementation through the preconceptional and PI period. The blastocyst from the first group displayed a retarded development with changes in ICM, TE and PE markers, in contrast, the blastocyst from P.C group had the main impact in TE cells.

Folate is essential for the embryo development; however, an excess of this nutrient is known to be detrimental at the blastocyst stage. Further analysis post-implantation could provide a greater understanding of the effect of a high FA diet on the trophoblast cells and foetal development. Further analysis after middle blastocyst stage would determine if the P.I embryos with an increased cell division can compensate for the decreased number of cells.

The effects of folate deficiency are associated with inconclusive neural tube development and embryo delay (Van Uitert *et al.*, 2014; Parisi *et al.*, 2017). The results shown here suggest that the effect of high FA supplemented diet during the oocyte maturation, fertilisation and the preimplantation period may have similar negative effects usually associated with low folate intake.

Chapter 7 Results V

Effects of long-term FA supplementation in C57BL/6 female mice and their offspring

7.1 Introduction

Women are recommended to take FA before and during pregnancy to reduce the risk of neural tube defects (NTD). Oliver et al. showed that 80% of European women take FA supplementation during pregnancy (Oliver *et al.*, 2014). Furthermore, in countries with FA fortification like the US, 10% of women using supplementation exceeded the tolerable upper limit (1mg/day) (Hoyo *et al.*, 2011). Additionality, the preconceptional use of FA supplementation has been associated with success pregnancy rate and better oocyte quality in IVF studies (Gaskins, Afeiche, *et al.*, 2014). However, new evidence has shown that high FA intake in women also can affect foetal outcomes such as birthweight and induce higher postnatal weight gain (Pastor-Valero *et al.*, 2011; Grieger and Clifton, 2014). Further studies are required to determine the effects of FA supplementation before and during pregnancy for the mother and the possible consequences that could have for the new-born.

In the previous result chapter, we observed that FA decreased the number of trophectoderm (TE) cells at the blastocyst stage. Moreover, FA supplementation exclusively during preimplantation showed a delay in the embryo development with a reduction in cell number and a lower number of embryos available at 3.5dpc. These results suggested that FA intake could modify the development of the embryo with potential downstream consequences for the offspring. Therefore, the aim of this chapter was to identify the effect of the long-term FA supplementation in the mother and female offspring after lactation. In our previous studies, we examined the effect of FA supplementation after four weeks of supplementation on ovarian and embryo development. Differently, in this project, the female mice were exposed during preconception, pregnancy and lactation, which may induce a significantly higher impact on the ovary and offspring than observed before.

7.2 D.1 Methods

30 C57BL/6 female mice at PND74 were fed with control or high FA diet for four weeks before mating, during pregnancy and lactation. At weaning five litters including the mother were culled by CO2. Animal dissection, blood and organ collection is described in the sections 2.1.5, 2.1.6 and 2.1.7. To determine the following effects of FA supplementation, from eight litters per group only one female offspring and the mother were kept alive and continue for five weeks more receiving control diet. Dam and female offspring were culled after at diestrus stage (PND179 dam, PND56 offspring) (Figure 7.1).

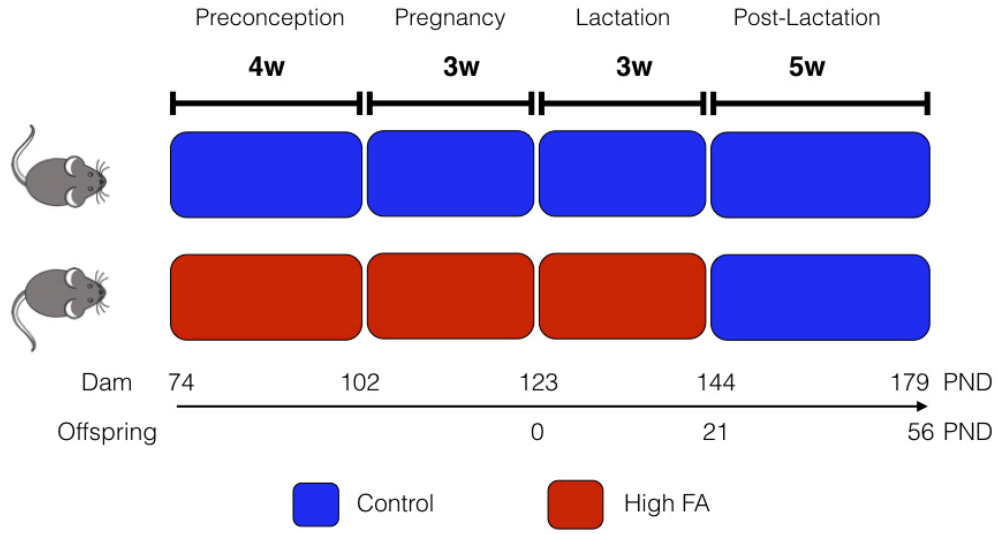

Figure 7.1 Animal Model long-term FA supplementation

Virgin adult female C57BL/6 mice (PND74) were fed with control and high FA diet during four weeks before mating, pregnancy and lactation. At weaning dams and one female offspring per litter were fed with control diet for five weeks more.

7.3 Results

7.3.1 The gestational length and litter outcomes

Feeding the mice with high FA diet before conception and during pregnancy did not alter the length of the gestation or the litter size or gender of the offspring.

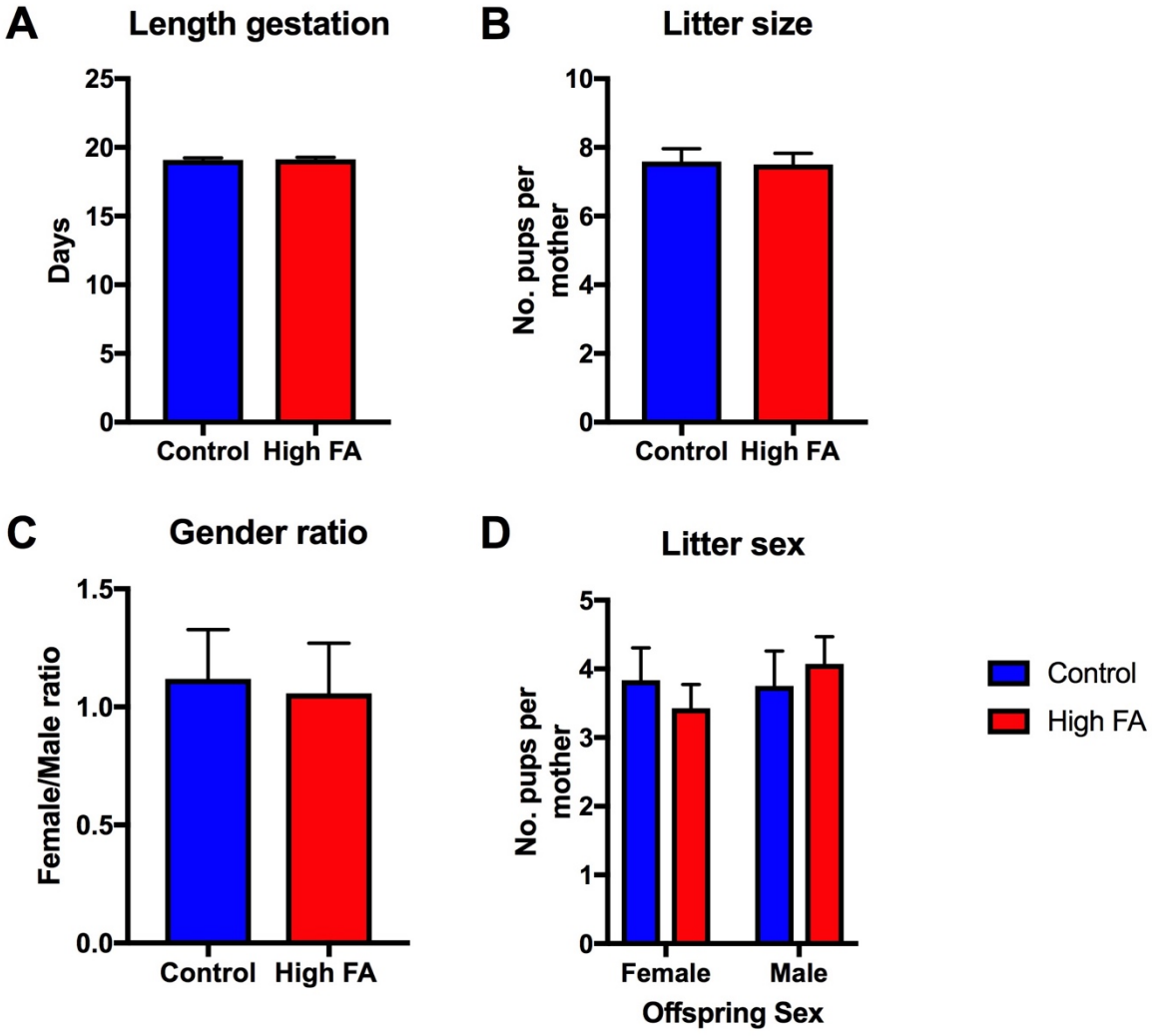

Figure 7.2 Gestational length and litter outcomes

Gestational length, litter and gender analyses of C57BL76 mice after periconceptional FA supplementation. Mean +/- SEM. n = 30 litters. T-Test analysis

7.3.2 Body weight measurements

At weaning (three weeks old), all offspring per litter were weighed. After analyses were made by the dietary group and by group and sex, no difference in weight was observed between the control and high FA dietary groups.

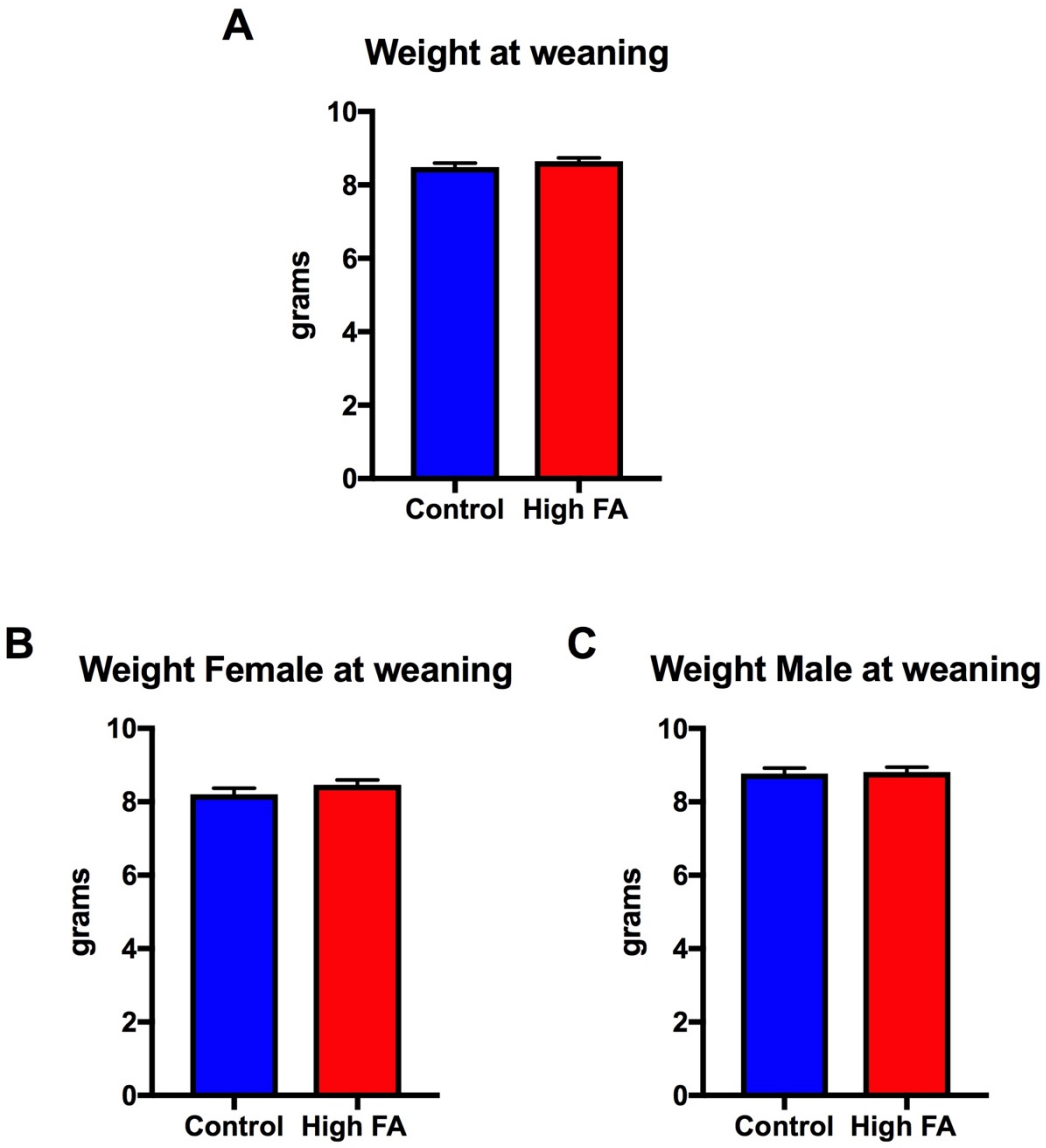

Figure 7.3 Body weight at weaning after FA supplementation

Body weight of C57BL76 mice after periconceptional FA supplementation. Mean +/- SEM. n = 30 litters. T-Test analysis

7.4 Discussion

In this project the effect of FA supplementation has been studied in different animals models that involved non-pregnant and pregnant mice in the preimplantation period. Hence, the aim of this chapter was to identify if the changes in embryo development observed before are detected at the end of weaning. Preliminary data showed that FA supplementation did not alter the gestational length and foetal outcome.

FA supplementation has shown alter not only DNA methylation patterns but also foetal outcomes such as embryo development, birthweight and placenta size in human and animal models (Van Uitert *et al.*, 2014; Keating *et al.*, 2015; McKay *et al.*, 2016; Bahous *et al.*, 2017). In this project, FA supplementation did not affect the litter size, sex ratio or length of the gestation. Results from the previous chapter Results IV showed a reduction in TE cell number, and delayed expression of TE differentiation markers. These results suggest changes in placental formation and function, impacting on embryo development and potentially the birthweight and future growth of the offspring. However, unfortunately, we could not measure birthweight in this experiment as we did not want to disturb the litters immediately after birth. However, the weight of the offspring at weaning was not different between the groups, suggesting that any effects on TE development and placental formation had been ameliorated over time. It would be interesting, however, to have measured the placental size and embryo number and size during gestation.

In the previous chapter embryo genotyping showed a majority of male embryos at the middle blastocyst stage, which may suggest differences in growth rate or an effect on fertilisation. However, the results presented here showed that the sex ratio of the litter after periconceptional high FA diet did not change. Further experiments with a larger number of animals will be needed to confirm whether there is a sex effect or not of FA supplementation.

In this animal model, mice were continuously exposed to FA through preconception, pregnancy and lactation, while previously the mice were given FA either preconception and preimplantation or just through the preimplantation period, before being fed a control diet. Indeed, we reported that the most marked changes in the blastocyst were seen in the mice given the high FA diet just during the preimplantation period. This may suggest that the switch back to the control diet is a crucial factor.

One limitation of this experiment was the lack of analysis of gene expression in the ovary or ovarian morphology in either the mother or offspring. It would have been interesting to compare such mice with earlier results and to see whether the changes in the ovarian morphology of the mothers were also observed in the offspring. Analyses of gene expression and morphology of the

ovaries of mothers (G0) would allow the identification of any effect of the FA on the ovarian function. Moreover, analyses in the ovaries of offspring would let to identify if the effect of the supplementation in the second generation (G1) can be observed.

7.5 Conclusion

FA supplementation before pregnancy and until lactation did not alter the length of gestation, size and sex of the litter. Moreover, continue supplementation until weaning did not alter the body weight of the offspring at 3w old. Further analyses are necessary to determine the effect of FA on the ovaries of the mother and offspring after periconceptional supplementation.

Chapter 8 Discussion

According to the DOHaD hypothesis, any maternal diet disruption before and during pregnancy can have consequences for further development. There is extensive evidence of the detrimental effects of a low folate diet during pregnancy on the development of the embryo, which leads to intrauterine growth restriction, placental and cardiovascular abnormalities in later life (Taparia *et al.*, 2007; Au, Ashley-Koch and Northrup, 2010). There is also evidence that a maternal diet high in folic acid (FA), results in increased levels of FA in the blood and delayed embryonic development (Van Uitert *et al.*, 2014), altered birthweight (Pastor-Valero *et al.*, 2011; Fekete *et al.*, 2012) and is associated with changes in the methylation of specific genes in the cord blood (Steegers-Theunissen *et al.*, 2009; Joubert *et al.*, 2016; Qian *et al.*, 2016; Pauwels, Ghosh, Duca, Bekaert, Freson, Huybrechts, A. S. Langie, *et al.*, 2017). However, the developmental effects of a high FA diet in the mother have not been fully examined. In this project, for the first time, we examined the effect of high FA diet on ovarian function and subsequently on the embryo development.

In the first results chapter, ovaries from mice exposed to high FA diet for four weeks showed alteration in the expression of genes related to cell differentiation. However, these animals were not culled at the same stage of the oestrus cycle which could interfere in the interpretation of the results. In the two presented animal models in Chapter Results II and III, oestrus cycle was assessed at the end of the experiment and the results showed the same trend in the gene expression of *Oct4*, *Ezh2* and *Bmi1* as the first result chapter, suggesting that the alteration of these factors were independent of the oestrus cycle, and might be maintained during the whole cycle.

FA supplementation during adulthood decreased the expression of genes related to the follicle growth, such changes may impact on the quality of the follicle, although no difference was observed on the follicle stage or number. The effect of FA supplementation was initially examined immediately after supplementation, but we also examined whether the effects could persist as FA intake can potentially impact one-carbon metabolism and the epigenetic regulation of genes. Here, we found that a number of the changes seen immediately after FA supplementation persisted four weeks after the end of supplementation suggesting that FA intake may induce long-term changes in gene expression potentially through the altered epigenetic regulation of genes. Unfortunately, morphological analysis was not possible due to the time required for the analysis, but changes in the expression of genes which play such a central role in regulating cell differentiation and function, suggest that further studies are worthwhile to determine whether these changes are observed at the protein level and what are the downstream consequences. Finally, to determine if the changes observed in the oocyte and GCs markers, affect the follicle

and oocyte quality, female mice fed an FA supplemented diet were mated and culled at 3.5dpc. The periconceptional supplementation with FA effectively induced a delay in the mating process suggesting an alteration of the oestrus cycle which might be associated with altered level of hormones. There was also a decrease in the expression of ICM and TE lineage markers in the embryo and a reduced number of TE cells in the blastocyst. These results suggest that the preimplantation period is sensitive to changes in the FA supply and that high FA diet could have possible consequences in the further development of the placenta and the foetus.

In order to explain the effects observed in this project after FA supplementation, is necessary first analyse the metabolism of FA and the possible explanations of its impact in the ovary and blastocyst.

8.1 Effect of high FA diet on FA metabolism

Over the past two decades, the intake of FA has risen dramatically due to the increased consumption of vitamin supplements, fortification of staple food in many countries and the advice for pregnant women to take periconceptional FA supplements. Studies in humans have shown that after an intake over 200µg of FA per day unmetabolised FA can be detected in the blood (Sweeney, McPartlin and Scott, 2007), suggesting that the transporters and coenzymes necessary for the absorption and metabolism of FA can reach a point of saturation. The consequences of high FA intake and levels of unmetabolised FA in the bloodstream over many years which is now what populations are increasingly being exposed to is unknown.

Studies have shown that FA supplementation induces the downregulation of folate transporters and coenzymes necessary for its metabolism even leading to a pseudo-MTHFR deficiency and altered methyl metabolism (Bailey and Ayling, 2009; Christensen *et al.*, 2015; Bahous *et al.*, 2017). This dysregulation induced by a high FA intake has been suggested to have similar adverse effects to those seen in individuals with low folate intake (See Figure 8.1) (Henry *et al.*, 2017). Human and animal studies have shown that low and high FA diets alter embryo growth and foetal outcomes such as birthweight and placenta development. Both low and high FA diets are associated with similar long-term effects (See Figure 8.1). In this project, high levels of FA and 5mTHF were observed in the plasma of high FA diet compared to controls animals, and *Pcft* was downregulated in the ovary of the same mice (at PND102). The same inverse association has been reported between folate intake and the expression of RFC and PCFT *in-vitro* previously (Ashokkumar *et al.*, 2010). These changes suggest that the transcription of the folate transporters is dependent upon folate concentrations. To detect if the high FA diet used in this project induced similar effects to those observed after feeding a low FA diet, it would be necessary to analyse the

activity of the coenzymes involved in the one-carbon metabolism (e.g. DHF, MTHFR or MTR), and also determine the levels of vitamin B12 and B6 which also could be altered.

In this project, we analysed folate levels in the plasma of the mice using the *L. casei* assay. In this analysis, performed in pregnant mice at 3.5dpc, only the metabolised folate (monoglutamate form) is determined. Despite feeding the mice a diet with a level of FA 5 times the recommended daily allowance, this assay did not show any significant differences among the groups, although the FA supplemented group did have a higher concentration of folate, this increase was not significant. The use of this technique in humans has shown that the level of folate in plasma is lower than in red blood cells; therefore the use of whole blood instead plasma is recommended (Shane, 2011). These results suggested that the folate assay was not the optimum to reflect the impact of feeding an FA supplemented diet. Therefore, a FA assay was performed with the plasma of non-pregnant female mice at PND102. Using LC-MS, the levels of FA and its metabolites were determined. This analysis showed a significant increase in FA and 5mTHF in the FA supplemented group.

The FA dose used in the high-FA group in this project was 5-fold the basal recommendation, this amount represents an intake of >2mg of FA in women, which could imitate an use of high supplementation dose or simultaneous exposure to supplementation and fortification with FA. The consequences of continuous exposure to high FA intake are not defined yet; however, the exposure to FA fortification should be considered to the moment to recommend FA supplements.

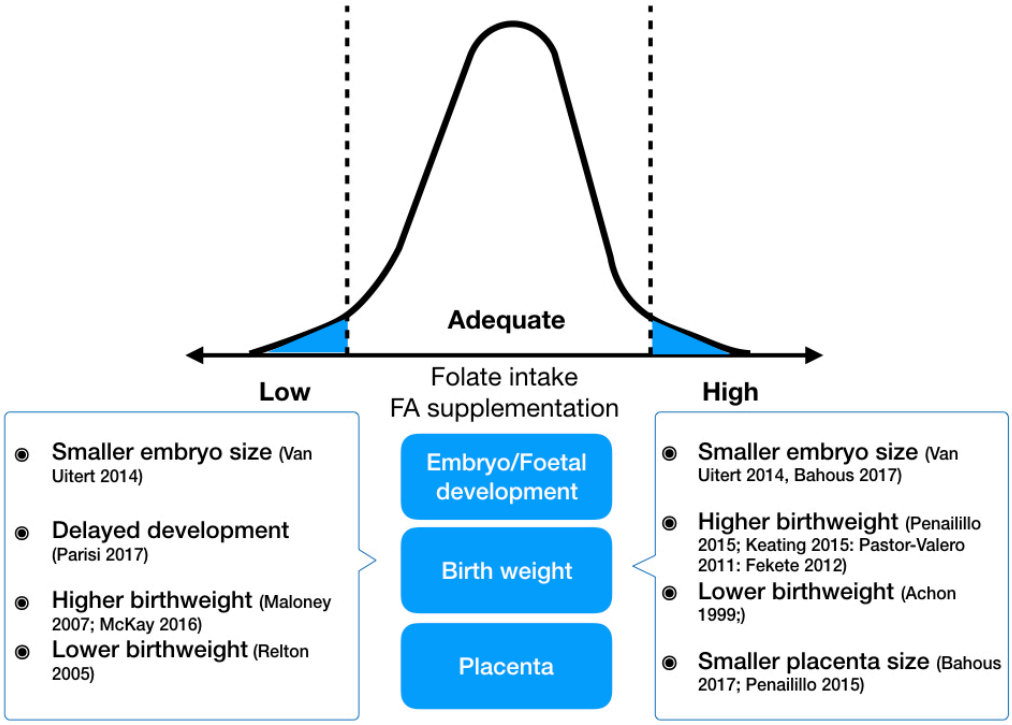

Figure 8.1 FA and folate intake curve and its consequences in early development

Low and high levels of folate and FA in blood have consequences in human and animal models in the early development, birthweight and placenta.

8.2 Effect of high FA diet in the adult ovary

The first aim of this project was to determine if high FA diet induces persistent effects on the expression of genes involved in proliferation and cell differentiation in the ovary of adult mice after four weeks of supplementation. FA supplementation induced a downregulation in genes related to the oocyte (*Oct4*) and GCs (*Fshr*) development. Additionally, genes related to the control of differentiation and proliferation (*Ezh2, Bmi1* and *Dnmt1*) were also reduced in the ovary after FA supplementation. Variations in the quality and proportion of oocyte and GCs were not determined by the morphological analyses made in this project. The histological analysis performed here allowed us to count the number and proportion of follicles available at PND102, which provided the number of oocytes per ovary (oocyte = follicle) but not the number of GCs. Moreover, and more importantly, the quality of the follicles could not be analysed by the same technique. To assess oocyte quality, an *in-vitro* culture of oocytes and GCs from these ovaries would be required to identify changes in the proliferation rates and pathways downstream of these changes. During the morphological analyses, an increase in the number of atretic follicles was observed in the high FA ovaries. This result suggests that FA might increase the number of apoptotic GCs in the follicle which could be associated with lower levels of *Fshr*, however, further analysis is necessary to determine if those changes are connected.

Alteration of one-carbon metabolism cycle has been shown to induce dysregulation of gonadotropin (Kanakkaparambil *et al.*, 2009) and steroid hormones levels (Gaskins *et al.*, 2012; Li *et al.*, 2015; Michels *et al.*, 2017). Moreover, the use of FA supplementation was associated with an increased length of the oestrus cycles (Cueto *et al.*, 2015). This suggests that hormonal disruption could be involved in the gene expression changes observed in this project. Interestingly the mice fed the FA supplemented diet showed a delayed time to mating compared to the control, which might suggest an alteration of the length of the oestrus cycle and hormonal dysregulation. Additionally, the proportion of CL per total number of follicles trended to increase in the high FA group, which could be associated with increased levels of progesterone. If FA supplementation is affecting the oestrus cycle, which may explain the mating difference, an extended analysis of the stages of the oestrus cycle could help to identify any cycling variation.

We also examined whether the effects of FA supplementation persisted after the end of supplementation. The changes observed at PND102 immediately after the end of the diet did not all persist. To the contrary, a number of the genes whose expression was altered immediately after supplementation at PND102 exhibited the opposite direction of the effect of FA supplementation at PND130. There is not enough information to determine the reason for the difference between these two-time points. However, it may reflect a compensatory mechanism

after the cessation of the high FA diet. High levels of FA are associated with lower levels of homocysteine, the end of the FA supplemented diet may, therefore, lead to an increase the levels of homocysteine inducing the opposite effects observed during high FA diet (See Figure 8.2).

The key pluripotency factor, OCT4, was most persistently dysregulated by FA in the ovary. This alteration in the expression of OCT4 could affect the oocyte growth and the further pluripotent capacity of the zygote. OCT4 is a pluripotency factor which maintains the undifferentiated status of the stem cells. During follicle development, OCT4 is regulated by NOBOX and gonadotropin hormones (FSH and LH). However, OCT4 has also been reported to be altered by the inhibition of the folate cycle reducing OCT4-positive cells in hES cells (Hara *et al.*, 2010). Additionally, folate receptor alpha (FRα) regulates OCT4 in neural stem cells by binding to the enhancer and promoter region (Mohanty *et al.*, 2016). *Oct4* expression was affected in both ovaries (PND102 and PND130 model) and blastocysts from FA supplemented mice (P.I and P.C), suggesting OCT4 sensitivity to FA levels. More analyses are necessary to determine how FA is modulating the *Oct4* expression, directly or via intermediates such as hormones.

The receptor of FSH was also altered by high FA diet, the dysregulation of FSHR has been associated with alterations of the proliferative properties of GCs and the number of large follicles (Burns *et al.*, 2001). Several studies have shown that the response of the ovary to hormone stimulation with gonadotropin can be modulated by the one-carbon metabolites status (Forges *et al.*, 2007; Kanakkaparambil *et al.*, 2009; Twigt *et al.*, 2011). In this project, the females after high FA diet showed an altered mating period, which could be related to an oestrus cycle dysregulation and then alteration of circulating steroid and gonadotropin hormones.

Epigenetic mechanisms could be involved in mediating the effects seen here. One of the consequences of disrupted one-carbon metabolites status are the changes in DNA methylation. Mammary gland, placenta and cord blood have shown changes in the DNA methylation associated with the folate levels (Ly *et al.*, 2011; Mikael *et al.*, 2013; Pauwels, Ghosh, Duca, Bekaert, Freson, Huybrechts, A. S. Langie, *et al.*, 2017). In this project, the polycomb repressive complex subunits like EZH2 and BMI1 were affected consistently by the intake of a high FA diet. These changes suggest that FA intake may affect key components of the epigenetic machinery leading to downstream effects on wider gene expression profiles. The methylation status of DNA in the ovary was not assessed in this project but would have been interesting to study along with changes in histone modification particularly H3K27, the repressive modification induced by EZH2 and the polycomb complex. FA has been widely reported as a nutritional factor that alters the methylation pattern in different tissues and even could persist and be detected during adulthood, (Anderson, Sant and Dolinoy, 2012; Richmond *et al.*, 2018). Those changes have generally been

associated with increased levels of methylation rate and an increase in the expression of methyltransferases. However, this is not always the case, and there are examples where high FA leads to hypomethylation and a decrease in DNMTs (Liu *et al.*, 2016). In this project, *Ezh2* and *Bmi1* were downregulated in the high FA diet group, together with downregulation of *Dnmt1*. These changes were seen at the same time as changes in *Fshr* and *Oct4*, which suggests they may be connected. Moreover, since EZH2 expression and activity is regulated by hormone levels (E2, androgens and LH), any alteration in hormone levels could affect EZH2-target genes associated with proliferation in the ovary (Bredfeldt *et al.*, 2010; Ma *et al.*, 2017). More detailed analyses are necessary to determine the methylation status and the binding sites of these epigenetic regulators of the gene expression.

The effects of FA supplementation in the ovary are not entirely elucidated. Moreover, a more extended supplementation period (longer than four weeks) could give a better understanding of the possible outcomes of the diet and the long-term effects.

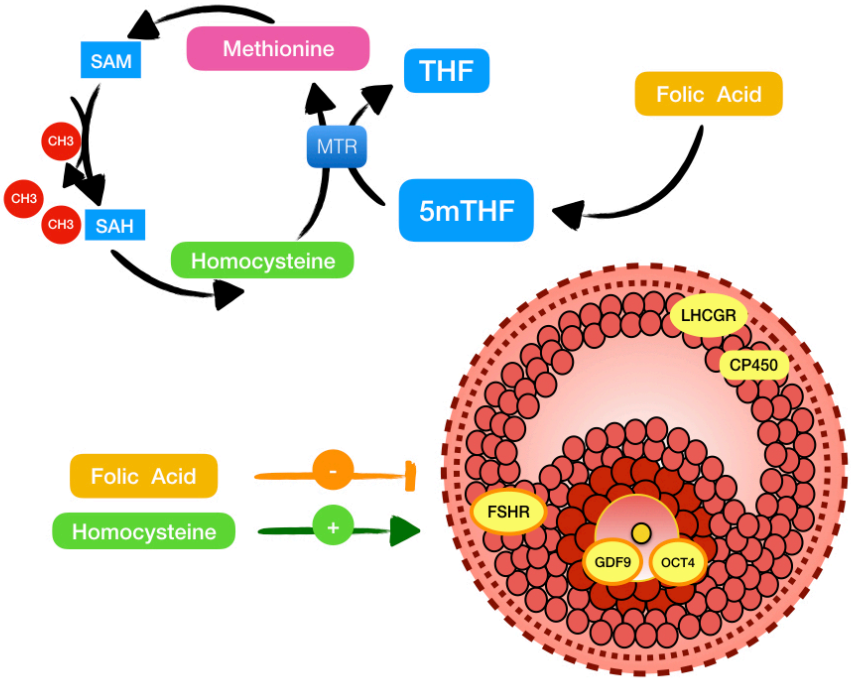

Figure 8.2 Hypothesis proposed about FA and homocysteine targets in the follicle

FA decreased the expression of *Fshr*, *Gdf9* and *Oct4* (all in the yellow border). After four weeks of the release of the high FA diet, the same genes showed an upregulation, which suggested could be linked with an increase of homocysteine level.

8.3 Effect of high FA diet in the blastocyst

The changes observed at PND102 within the ovary poised the question if periconceptional supplementation with FA can affect embryogenesis and whether changes could be detected at the blastocyst stage. We found that FA decreased the expression of *Oct4*, *Nanog* and *Eed* in the whole blastocyst, likewise, CDX2 was reduced at the mRNA and protein level. These changes in gene expression were accompanied by a decrease in the number of TE cells within the embryo. These results suggest that FA can affect ICM and TE lineage markers and also the number of TE cells. Depletion of CDX2 is linked to dysregulation of OCT4 and NANOG expression and an arrested development before blastocyst cavitation (Jedrusik *et al.*, 2010). The decrease in CDX2 observed in this project might not affect cavity formation, but could affect further development. The possible consequences of these outcomes could be seen in the further differentiation of these cells to form the placenta. In the literature, there is evidence that the birthweight and the placental size are sensitive to the FA supplementation during pregnancy (Keating *et al.*, 2015; Penailillo *et al.*, 2015; Bahous *et al.*, 2017). However, the effects are not always in the same direction, the use of diets containing 8 and 40 mg of FA during pregnancy have shown increase the body weight of female offspring in rats and mice (Keating *et al.*, 2015; Penailillo *et al.*, 2015). In contrast, the use of 20 mg in mice showed a reduced foetal weight at E17.5 day (Bahous *et al.*, 2017).

FA supplementation just during the preimplantation period also affected embryo development and showed not only a decrease in the expression *Oct4* and *Gata6* but also an increase in NANOG. There was also a decrease in the number of total cells which suggest a delayed development. In comparison with the periconceptional FA supplementation, the effect of the high FA diet during the first three days of pregnancy led to a lower number of embryos available. A decrease in the ICM and TE cells has been previously associated with increased resorption sites after inhibition of the folate cycle (B. Zhang *et al.*, 2015). However, despite the detection of degenerated blastocysts and oocytes, there were no significant differences among the different dietary groups. However, we did not analyse earlier time points by flushing the oviduct nor examined the uterine wall for resorption sites, hence embryo loss earlier or due to accelerated implantation cannot be excluded.

Additionally, the blastocysts found showed an altered expression pattern of lineage markers of ICM, TE and PE and also less total number of cells yet higher mitotic index. Despite these results, there was no significant difference in the morphological stage of development of the blastocysts collected. These results imply that even when embryos had a lower number of cells, they looked

similar to control blastocysts. A later post-implantation analysis could help to detect if the increasing mitotic rate observed compensated for the lower number of cells in the blastocyst. These results demonstrate the high sensitivity of the preimplantation period to the folate status affecting the oocyte maturation and embryo growth.

Dynamic epigenetic regulation could be at play here. In both models (periconceptional and preimplantation FA supplementation) there is a downregulation of *Ezh2* and *Eed* in the middle blastocyst. However, the synchronised downregulation of *Ezh2* and *Eed* is exclusive to the P.I group, with an increased level of NANOG. EZH2-EED complex has been shown to be regulated by the pluripotency factor OCT4 (Ura *et al.*, 2008; Wu *et al.*, 2014) and depletion of PRC2 core subunit was associated with a delayed embryonic development and a decrease of H3K27me3 (O'Carroll *et al.*, 2001; Hinkins *et al.*, 2005; Huang *et al.*, 2014). Therefore, these results suggest that downregulation of *Ezh2* and *Eed* might induce an upregulation of NANOG through a potential decrease of the repressive mark H3K27me3, allowing the maintenance of the pluripotency state of ICM cells.

In order to understand the possible consequences of the FA effects observed at 3.5dpc, a fourth animal model was prepared. Females mice were fed with control or high FA diet for four weeks before mating, pregnancy and lactation. In this model, FA supplementation before and during pregnancy did not affect the litter size, sex ratio or length of the gestation suggesting that FA not increase the resorption sites or the embryo loss previously suggested by the results of the P.I group. However, there was not a group of mice with exclusive supplementation during preimplantation (simulating the P.I embryo group), which showed the most marked changes at the blastocyst stage. Birthweight could not be analysed to avoid any disturbance immediately after birth. However, the weight of the offspring at weaning did not differ between the groups.

8.4 Limitations

Among the limitations of the project is the detection of each follicle cell marker only at the mRNA level. Even when specific reference genes are used to normalise gene expression, and all the animals were in the same stage of the oestrus cycle, variation in the proportion of cells (mural, cumulus GCs or theca cells) could affect the real significance of the changes observed in the ovary. Therefore, the localisation of the proteins by IHC analysis would be essential to determine whether these changes at the mRNA level are reflected at the protein level as well as determine the site of localisation. OCT4 and H2AX (a marker of DNA damage) staining were performed in ovaries at PND102; however, both were not quantified due to time limitations. To understand the implications of FA on the ovary, it is essential to see if changes observed at mRNA level are translated to protein variations. Moreover, in the second model (at PND130) no morphological analyses were made. At PND102 there was no effect of the FA on the number of follicles; however, later analysis could help to understand the consequences in ovarian morphology.

In the case of the analyses made in the blastocysts, the analysis of a PE endoderm marker (such as GATA6 and SOX17) was not possible by immunodetection. As shown in Appendix C.2, none of the PE markers used displayed good quality staining despite extensive optimisation efforts. Identification by IF of other genes that changed at mRNA level would also be necessary, for example, the detection of EZH2, EED or even H3K27me3 to detect in which lineage the changes seen by gene expression occur.

Finally, the ovaries of the mother and the offspring after periconceptional FA supplementation were not analysed due to the time required. The post-supplementation analysis in the ovary of the dam exposed for around 3 months of high FA diet (preconception, pregnancy and lactation) would replicate the release factor appreciated at PND130 and then might show a persistent effect. For the same reasons, it would be necessary to analyse the ovaries of the offspring once they acquire the sexual maturity.

8.5 Conclusion

In conclusion, a high FA intake for four weeks before pregnancy could modify essential genes involved in follicle growth. Moreover, these changes may also have an effect on embryo growth. This study has also shown supplementation during the preimplantation period alone can have significant repercussions for the development of the blastocyst, affecting lineage markers of ICM, TE and PE cells. The further consequences of the changes in the blastocyst are not elucidated in this project, but it is crucial now to understand the potential effect on the placenta and the foetus.

Women are encouraged to increase FA intake to decrease the incidence of NTD. The results presented here suggest that periconceptional consumption of high levels of FA (approximately > 2mg of FA) could have consequences for the ovary and the foetal development. Determining the safe upper limit for FA is critical to find a healthy balance to achieve an optimal intake, avoid NTD and decrease the side effect of high FA intake.

8.6 Future directions

8.6.1 Hormonal analysis of mice at PND102

Alterations in the plasma levels of one-carbon metabolites have shown an effect on the levels of steroid hormones and altered follicle development (Kanakkaparambil *et al.*, 2009; Michels *et al.*, 2017). Due to the high levels of FA in the plasma, it is expected that homocysteine levels decrease. There is evidence that shows homocysteine alters FSHR and the proliferation of GCs (Kanakkaparambil *et al.*, 2009). In parallel, the steroid hormones (E2 and P4) have shown to be modulated by FSHR and BRCA1 (Burns *et al.*, 2001; Chand, Simpson and Clyne, 2009), and both factors were altered by FA supplementation. Therefore, in order to understand if the changes observed at the mRNA level in *Fshr*, *Oct4* and *Brca1* have downstream effects on follicle growth, the measurement of homocysteine, oestradiol (E2) and progesterone (P4) will be required.

Another alternative, to assess if FA alters the oestrus cycle, is to evaluate the length of each oestrus phase after FA supplementation. In this project, oestrus cycle was detected just at the end of the supplementation; however, longer examination of the cycles can provide information whether there is any alteration that could explain the difference in the mating process. Folate intake in women have shown an increase in the cycle length, and folate serum was associated with an increase in progesterone during the luteal phase, suggesting that FA can have some effect in the ovarian cycle (Cueto *et al.*, 2015; Michels *et al.*, 2017).

8.6.2 Pathway analysis of the gene expression analysis

The best alternative to analyse the global effect of the FA supplementation in the ovary and determine the pathways affected would be an RNA-Seq analysis. The sequencing of the ovarian RNA would allow the detection of all the genes that significantly change after supplementation and with that permit a full pathway analysis. This type of analysis need to be done using total RNA, without segregation which could result in an imprecise assay. However, using markers for the type of cells (e.g. FSH/AMH for GCs, GDF9/NOBOX for oocyte and CYP17A1 for theca cells) could help to localise the changes in the expression.

8.6.3 Protein analysis

After determining the effect of FA supplementation at the mRNA level in the ovary, protein localisation and quantification would be necessary to determine the impact of FA supplementation. For that IHC and immunofluorescence (IF) detection would allow identifying if the changes in mRNA are translated to protein level. However, due to the difficulties observed with IHC, *in-situ* hybridisation (ISH) could be also an alternative at least to localise expression.

In the case of the blastocyst, IF detection of PRC2 subunits (EZH2 and EED) or H3K27me3 could allow the localisation and quantification at the protein level of the changes of these proteins.

8.6.4 Epigenetic regulation of the gene expression

After the analysis of the gene expression, the next step could be the detection of regulating epigenetic marks. As changes in *Ezh2*, *Bmi1* and *Eed* were associated with the high FA diet, and it will be essential to identify the binding sites of these molecules by chromatin immunoprecipitation (ChIP).

Appendix A Methods

A.1 Diet

A.1.1 Control diet AIN-93M with 1 mg FA

TestDiet AIN-93M w/ 1 PPM Folate-Red **5AFJ**

DESCRIPTION

Modification of TestDiet® AIN-93M Semi-Purified Diet with 1 PPM total Folate. Dyed red.

Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location. Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.

*Product Forms Available**	*Catalog #*
1/2" Pellet	1813521-209

**Other Forms Available On Request*

INGREDIENTS (%)	
Corn Starch	46.5192
Maltodextrin	15.5000
Casein - Vitamin Tested	14.0000
Sucrose	10.0000
Powdered Cellulose	5.0000
Soybean Oil	4.0000
AIN 93M Mineral Mix	3.5000
AIN-93 Vit/100PPM Folate	1.0000
Choline Chloride	0.2500
L-Cystine	0.1800
FD&C Red 40 Lake	0.0500
t-Butylhydroquinone	0.0008

NUTRITIONAL PROFILE [1]

Protein, %	***13.0***
Arginine, %	0.49
Histidine, %	0.36
Isoleucine, %	0.67
Leucine, %	1.21
Lysine, %	1.02
Methionine, %	0.36
Cystine, %	0.23
Phenylalanine, %	0.67
Tyrosine, %	0.71
Threonine, %	0.54
Tryptophan, %	0.15
Valine, %	0.80
Alanine, %	0.39
Aspartic Acid, %	0.90
Glutamic Acid, %	2.86
Glycine, %	0.27
Proline, %	1.65
Serine, %	0.77
Taurine, %	0.00
Fat, %	***4.1***
Cholesterol, ppm	0
Linoleic Acid, %	2.04
Linolenic Acid, %	0.31
Arachidonic Acid, %	0.00
Omega-3 Fatty Acids, %	0.31
Total Saturated Fatty A	0.60
Total Monounsaturated Fatty Acids, %	0.88
Polyunsaturated Fatty Acids, %	2.16
Fiber (max), %	***5.0***
Carbohydrates, %	***72.1***
Energy (kcal/g) [2]	***3.77***

From:	kcal	%
Protein	0.518	13.7
Fat (ether extract)	0.368	9.8
Carbohydrates	2.886	76.5

Minerals	
Calcium, %	0.51
Phosphorus, %	0.31
Potassium, %	0.36
Magnesium, %	0.05
Sodium, %	0.14
Chloride, %	0.27
Fluorine, ppm	1.0
Iron, ppm	39
Zinc, ppm	35
Manganese, ppm	11
Copper, ppm	6.0
Cobalt, ppm	0.0
Iodine, ppm	0.21
Chromium, ppm	1.0
Molybdenum, ppm	0.14
Selenium, ppm	0.22
Vitamins	
Vitamin A, IU/g	4.0
Vitamin D-3 (added), IU/g	1.0
Vitamin E, IU/kg	78.8
Vitamin K, ppm	0.75
Thiamin Hydrochloride, ppm	6.0
Riboflavin, ppm	6.5
Niacin, ppm	30
Pantothenic Acid, ppm	16
Folic Acid, ppm	1.1
Pyridoxine, ppm	5.8
Biotin, ppm	0.2
Vitamin B-12, mcg/kg	27
Choline Chloride, ppm	1,750
Ascorbic Acid, ppm	0.0

1. Formulation based on calculated values from the latest ingredient analysis information. Since nutrient composition of natural ingredients varies and some nutrient loss will occur due to manufacturing processes, analysis will differ accordingly. Nutrients expressed as percent of ration on an As-Fed basis except where otherwise indicated.
2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.

Figure 8.3 AIN-93M Control diet

A.1.2 High FA diet AIN-93M with 5 mg FA

TestDiet AIN-93M w/ 5PPM Folate- Yellow **5844**

DESCRIPTION

Modification of TestDiet® AIN-93M Semi-Purified Diet with 5 PPM total Folate. Dyed yellow.

Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location. Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.

*Product Forms Available**	*Catalog #*
1/2" Pellet	1816048-209

**Other Forms Available On Request*

INGREDIENTS (%)	
Corn Starch	46.5192
Maltodextrin	15.5000
Casein - Vitamin Tested	14.0000
Sucrose	10.0000
Powdered Cellulose	5.0000
Soybean Oil	4.0000
AIN 93M Mineral Mix	3.5000
AIN-93 Vit/ 500PPM Folate	1.0000
Choline Chloride	0.2500
L-Cystine	0.1800
FD&C Yellow 5 Lake	0.0500
t-Butylhydroquinone	0.0008

NUTRITIONAL PROFILE [1]

Protein, %	***13.0***
Arginine, %	0.49
Histidine, %	0.36
Isoleucine, %	0.67
Leucine, %	1.21
Lysine, %	1.02
Methionine, %	0.36
Cystine, %	0.23
Phenylalanine, %	0.67
Tyrosine, %	0.71
Threonine, %	0.54
Tryptophan, %	0.15
Valine, %	0.80
Alanine, %	0.39
Aspartic Acid, %	0.90
Glutamic Acid, %	2.86
Glycine, %	0.27
Proline, %	1.65
Serine, %	0.77
Taurine, %	0.00
Fat, %	***4.1***
Cholesterol, ppm	0
Linoleic Acid, %	2.04
Linolenic Acid, %	0.31
Arachidonic Acid, %	0.00
Omega-3 Fatty Acids, %	0.31
Total Saturated Fatty A	0.60
Total Monounsaturated Fatty Acids, %	0.88
Polyunsaturated Fatty Acids, %	2.16
Fiber (max), %	***5.0***
Carbohydrates, %	***72.1***
Energy (kcal/g) [2]	***3.77***

From:	kcal	%
Protein	0.518	13.7
Fat (ether extract)	0.368	9.8
Carbohydrates	2.886	76.5

Minerals	
Calcium, %	0.51
Phosphorus, %	0.31
Potassium, %	0.36
Magnesium, %	0.05
Sodium, %	0.14
Chloride, %	0.27
Fluorine, ppm	1.0
Iron, ppm	39
Zinc, ppm	35
Manganese, ppm	11
Copper, ppm	6.0
Cobalt, ppm	0.0
Iodine, ppm	0.21
Chromium, ppm	1.0
Molybdenum, ppm	0.14
Selenium, ppm	0.22
Vitamins	
Vitamin A, IU/g	4.0
Vitamin D-3 (added), IU/g	1.0
Vitamin E, IU/kg	78.8
Vitamin K, ppm	0.75
Thiamin Hydrochloride, ppm	6.0
Riboflavin, ppm	6.5
Niacin, ppm	30
Pantothenic Acid, ppm	16
Folic Acid, ppm	5.1
Pyridoxine, ppm	5.8
Biotin, ppm	0.2
Vitamin B-12, mcg/kg	27
Choline Chloride, ppm	1,750
Ascorbic Acid, ppm	0.0

1. Formulation based on calculated values from the latest ingredient analysis information. Since nutrient composition of natural ingredients varies and some nutrient loss will occur due to manufacturing processes, analysis will differ accordingly. Nutrients expressed as percent of ration on an As-Fed basis except where otherwise indicated.
2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.

Figure 8.4 AIN-93M high FA diet

A.2 H6 medium

Table 8.1 H6 medium reagents

Reagent/Chemical for H6 medium	Concentration
Stock B	
Deionised water, sterile	10 ml
Sodium hydrogen Carbonate ($NaHCO_3$)	0.2106 g
Stock E	
Deionised water, sterile	50 ml
Hepes	2.9785 g
Stock F	
Sodium Chloride (NaCl)	4.720 g
Potassium Chloride (KCl)	0.110 g
Sodium Dihydrogen Orthophosphate($NaH_2PO_4.2H_2O$)	0.060 g
Magnesium Chloride hexahydrate ($MgCl_2.6H_2O$)	0.100 g
D-Glucose	1.000 g
DL-Lactic Acid	3.4 ml
Deionised water, sterile	Made up to 100 ml
Stock G	
Deionised water, sterile	10 ml
Pyruvic Acid	0.030 g
Penicillin G Potassium	0.060 g
Streptomycin Sulphate	0.050 g
Stock H	
Deionised water, sterile	10 ml
Calcium Chloride Dihydrate	0.260 g
20% Sodium Cloride	
Deionised water, sterile	10 ml
Sodium Chloride (NaCl)	2.000 g

H6 medium was used for flushing the uterus and wash embryo before assays. Information by T Fleming group.

Appendix A

Table 8.2 Preparation H6BSA

To prepare 100 ml of H6BSA	Concentration
Stock B	
Deionised water, sterile	78 ml
Stock B	1.6 ml
Stock E	8.4 ml
Stock F	10 ml
Stock G	1.0 ml
Stock H	1.0 ml
20% NaCl	0.6 ml
Bovine Serum albumin (BSA) A3311-10G	0.4 g

pH adjusted to 7.4; osmolarity adjusted to 207-280 mOsm; sterile filtered (0.22 uM filter); aliquots stored at 4°C

A.3 Haematoxylin and Eosin (H&E) preparation

H&E preparation is suitable for paraffin and frozen sections. To prepare Mayers Haematoxylin has to be dissolved with alum and sodium iodate in distilled water by standing overnight at room temperature. The chloral hydrate and citric acid are added, mixed and boiled for five minutes. After cooling, solution was filtered and stored. In the case of Eosin, Eosin and calcium chloride were mixed in water thoroughly and filtered.

Table 8.3 H&E reagents

Reagent/Chemical for Mayers Haematoxylin	**Concentration**
Hematoxylin	1 g
Potassium or ammonium alum	50 g
Sodium iodate	0.2 g
Citric Acid	1 g
Chloral hydrate	50 g
Distilled water	1,000 ml
Reagent/Chemical for Eosin	
Eosin Yellowish	5 g
Calcium chloride	5 g
Tap water	500 ml

Source Histology Research Unit (HRU), University of Southampton.

Appendix B IHC

B.1 BRCA1 immuno-localisation

After *Brca1* downregulation in the gene expression analysis, localisation of BRCA1 can suggest the impact on the ovary function and follicle development. BRCA1 has been detected in GCs of developing follicles (primary to antral), but decrease in large antral or preovulatory follicles, where its expression is restricted to cumulus GCs (Hu *et al.*, 2005). However, BRCA1 IHC showed to be faintly present in the cytoplasm of the oocyte (Figure 8.5). No nuclear stained was observed using different dilutions of the antibody .

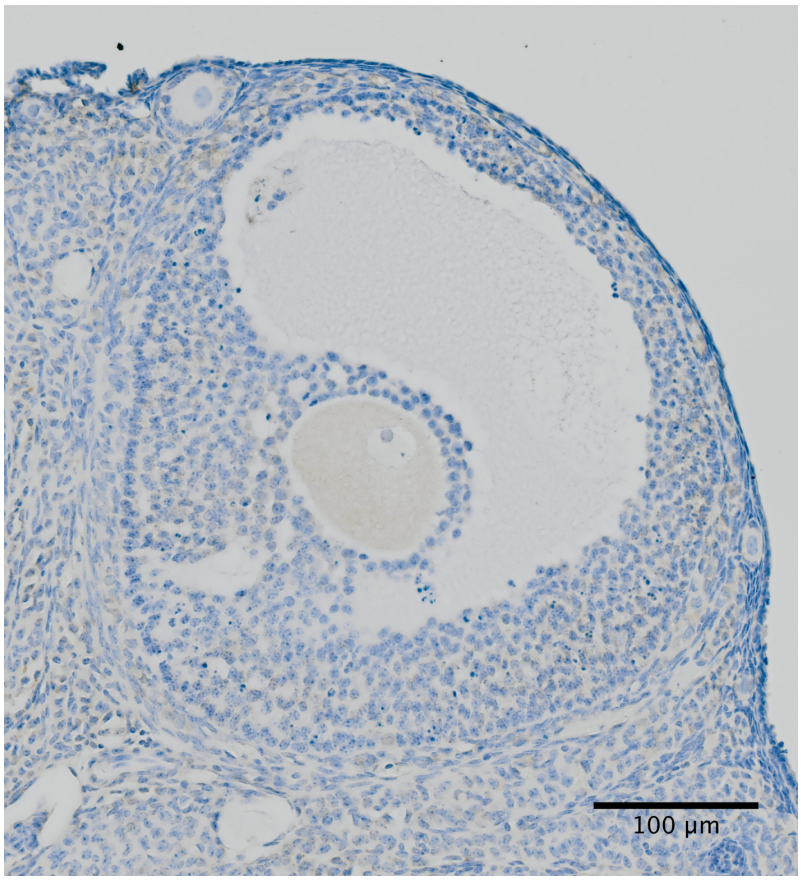

Figure 8.5 BRCA1 IHC in ovary PND102

BRCA1 1:500 dilution IHC test in antral follicle. Scale 100 µm.

Appendix C Blastocyst

C.1 Optimisation qPCR middle blastocyst

Every primer assay performed in the qPCR of the blastocyst was previously checked by the presence of a single amplicon in the agarose gel and a single pick in the melting curve (See Methods Section 2.7.3). Images below show the agarose gel of qPCR product of single blastocyst during the optimisation of the process. Among the DNMTs, *Dnmt3a* and *Dnmt3b* were not used in this project due to both showed a weak expression (Figure 8.6). The same weak expression was observed with *Sox17* gene, therefore *Gata6* was used as PE marker (Figure 8.7).

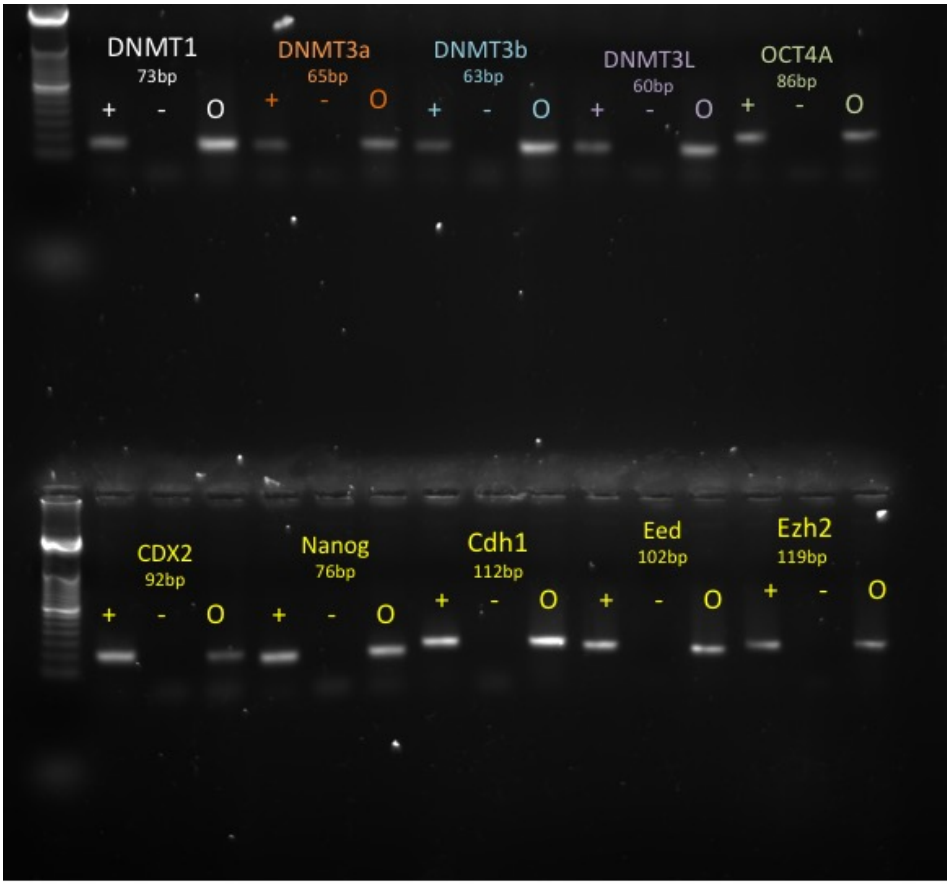

Figure 8.6 Agarose gel optimisation qPCR of single blastocyst

Agarose gel using 5ul of qPCR product of a single middle blastocyst test. (+) represent the blastocyst sample, (-) is the control (RT -) without transcriptase reverse enzyme and (O) is an ovary sample.

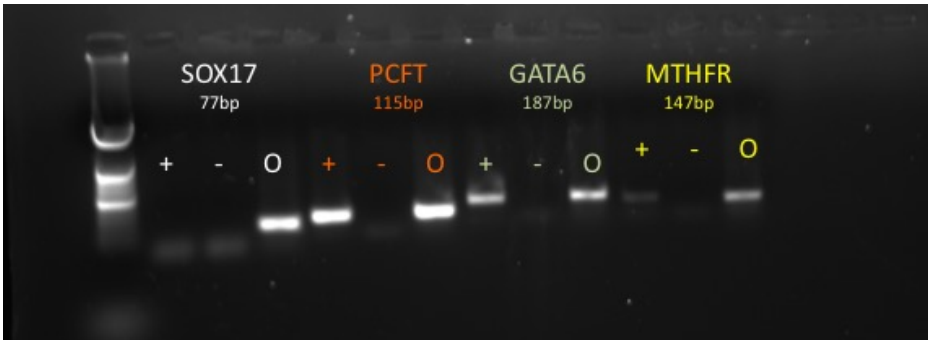

Figure 8.7 Agarose gel optimisation qPCR of single blastocyst (2)

Agarose gel of qPCR of 5ul product of a single middle blastocyst test. (+) represent the blastocyst sample, (-) is the control RT without transcriptase reverse enzyme and (O) is an ovary sample.

C.2 Primitive endoderm markers for IF on embryos

During the optimisation of the IF in the blastocysts, different markers were used to identify primitive endoderm (PE) cells (See Methods 2.13.3 Table 2.19). However, none of the antibodies were successful in determine PE nuclei. hGATA6, hGATA4, rabbit GATA4 and hSOX17, despite some of these are human antibodies, those have been used for other groups in published articles (Bolton *et al.*, 2016; Panamarova *et al.*, 2016; Goolam and Zernicka-Goetz, 2017). The samples use for the antibody tests were blastocysts collected at 3.5dpc, and also blastocysts kept in culture for 24h in KSOM media at 37C to reach a later stage (reaching more than 60 cells per blastocyst) (Figure 8.9, Figure 8.8, Figure 8.11 and Figure 8.12). Variation on fixation (using 4%PFA and 1% paraformaldehyde), permeabilization (using between 0.25-1% PBS-T), washes and blocking (with different concentrations of BSA 0-3%) were used, as well increasing antibody dilutions (Figure 8.10).

One of the main problems associated with PE markers was that the antibody has difficulties to penetrate the embryo. All embryos were without zona pellucida, and even when two type of fixation and different concentrations of PBS-Triton (permeabilization solution) were used, no nuclear staining was observed.

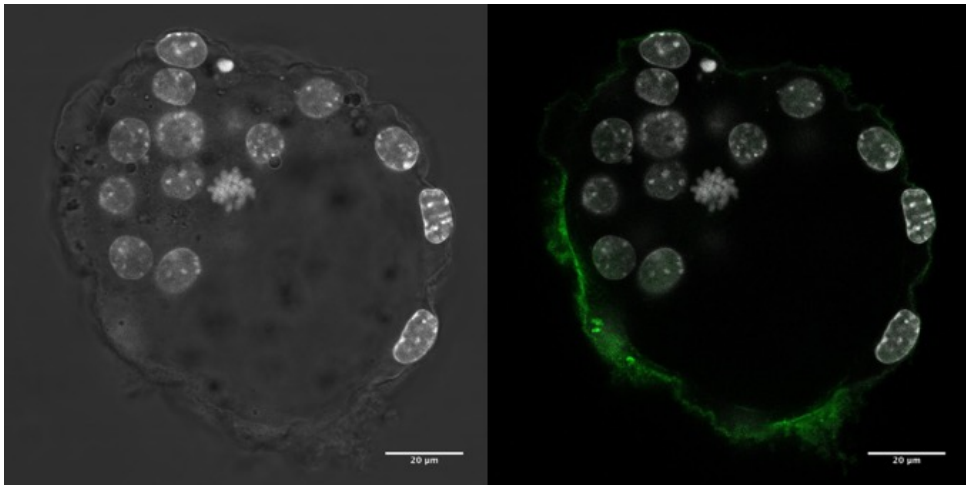

Figure 8.9 IF GATA6 in 3.5dpc blastocyst

Blastocyst fixed with 4%PFA and blocking PBS-T-BSA. Goat hGATA6 (green) 1:400 and DAPI (gray) 1:500 dilutions. Scale 20 µm.

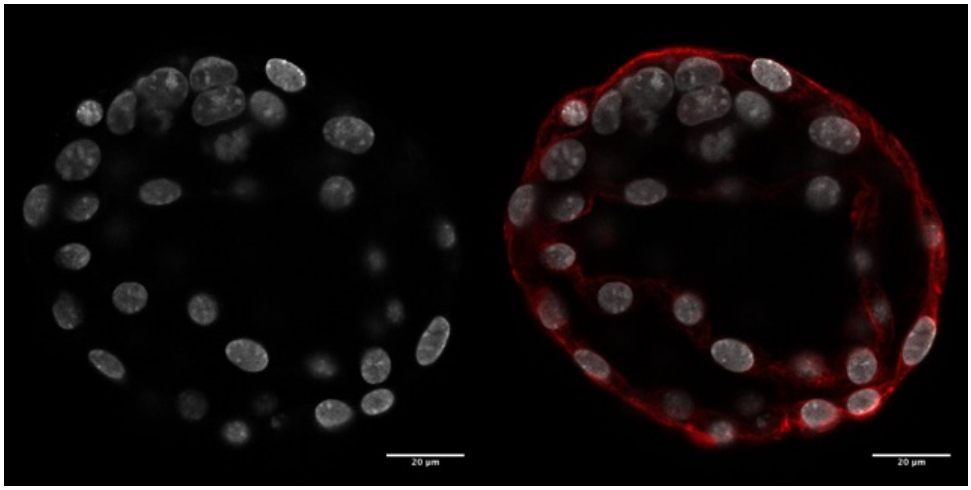

Figure 8.8 IF GATA6 in 3.5dpc blastocyst kept in culture for 24h

Blastocyst fixed with 4%PFA and blocking PBS-T-BSA. Goat hGATA6 (red) 1:200 and DAPI (gray) 1:500 dilutions. Scale 20 µm.

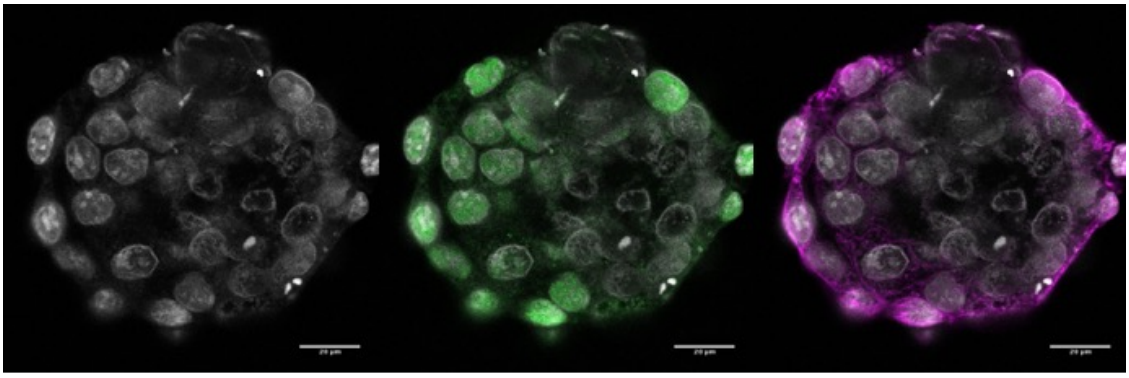

Figure 8.10 IF GATA6 and CDX2 in 3.5dpc blastocyst

Blastocyst fixed 10 minutes with 1% paraformaldehyde. Goat hGATA6 (pink) 1:100, mCDX2 (green) 1:100 and DAPI (gray) 1:500 dilutions. Scale 20 µm.

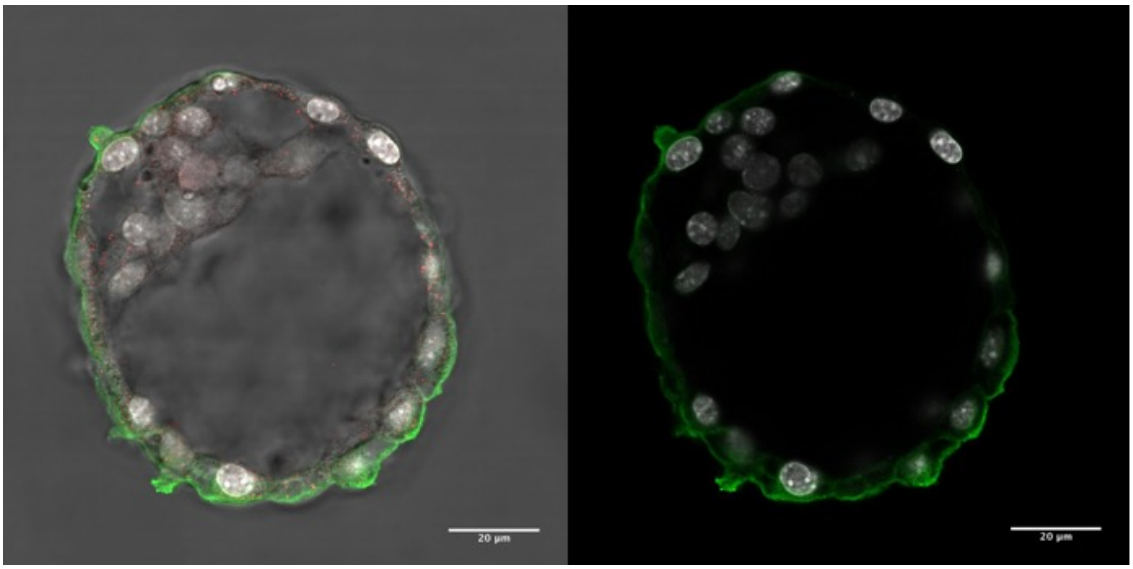

Figure 8.11 IF NANOG and GATA4 in 3.5dpc blastocyst kept in culture for 24h

Fixation 4%PFA. Rabbit NANOG (red) 1:100, goat hGATA4 (green) 1:200 and DAPI (gray) 1:500 dilutions. Scale 20 µm.

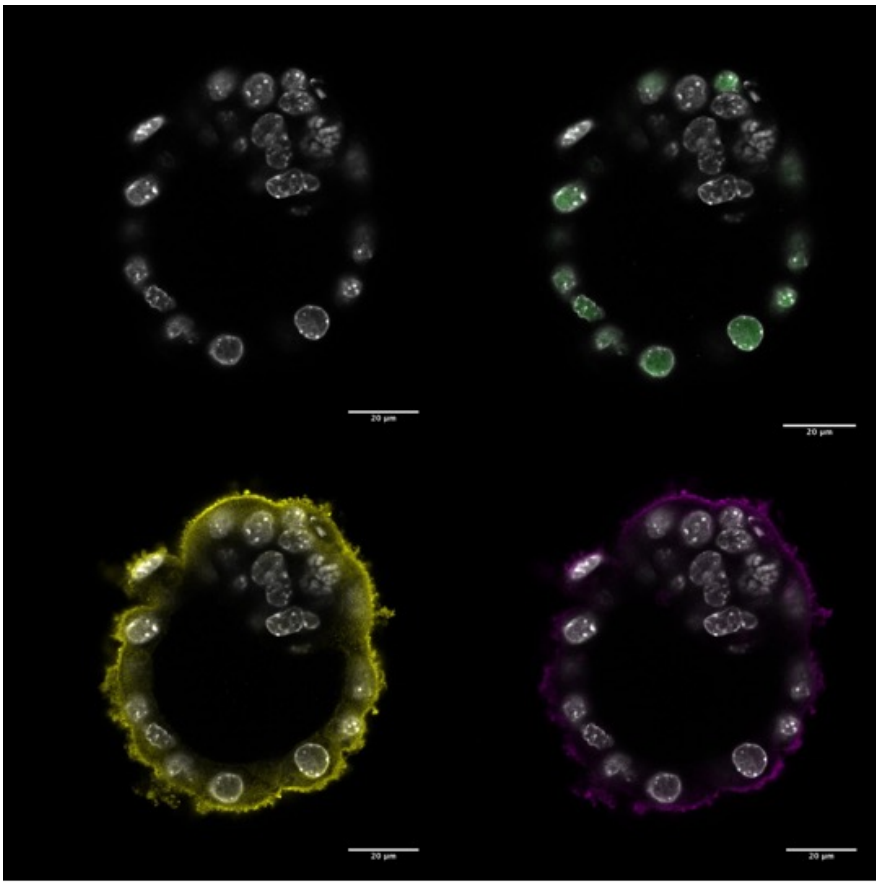

Figure 8.12 IF CDX2, SOX17 and GATA4 in 3.5dpc blastocyst kept in culture for 24h

Fixation 4%PFA, Blocking PBS-T-BSA, mouse CDX2 (green) 1:100, goat hSOX17 (yellow) 1:100, rabbit GATA4 (purple) 1:100 and DAPI (gray) 1:500 dilution. Scale 20 µm

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