THE ROLE OF THE HEXOSAMINE BIOSYNTHESIS PATHWAY AND β-O-LINKED GLYCOSYLATION IN DETERMINING OOCYTE DEVELOPMENTAL COMPETENCE

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Abstract

Maternal diabetes and conditions such as obesity in which blood glucose levels are elevated are associated with reduced fertility and poor pregnancy outcomes. Many studies have examined the effects of hyperglycaemia on the early embryo and fetus; however, it is becoming increasingly evident that the peri-conceptual environment surrounding the oocyte has a significant impact on developmental competence and the long-term health of offspring.

In this thesis, I aimed to investigate the role of the hexosamine biosynthesis pathway (HBP) in oocyte developmental competence. The HBP is a glucose-metabolising pathway which can also be up-regulated by glucosamine, a potent hyperglycaemic mimetic which enters the HBP downstream of the rate-limiting enzyme. The HBP produces uridine diphosphate-N-acetylglucosamine, which can be used for the β-O-linked glycosylation (O-GlcNAcylation) of proteins, regulating their function in a similar manner to phosphorylation.

Firstly I established the effect of hyper- and hypo-glycaemic conditions during in vitro maturation (IVM) of mouse cumulus-oocyte complexes (COCs) on a range of measures associated with oocyte developmental competence, including cumulus expansion, meiotic maturation, cleavage and blastocyst development rates. A low (1 mM) glucose concentration achieved optimal oocyte competence, and glucose supplementation during only the first hour of IVM was necessary and sufficient to support oocyte maturation and embryo development to the blastocyst stage. Glucosamine was able to substitute for glucose during this first hour.

In the absence of glucose throughout IVM, glucosamine was not able to increase developmental competence, and at higher concentrations (2.5 and 5 mM) had a detrimental effect on these outcomes. These experiments underscored the importance of the other glucose metabolic pathways, during COC maturation, and supported the concept that excess flux through the HBP has detrimental consequences.

Using Western blots and immunohistochemistry, it was shown that both glucosamine and high glucose levels induced an increase in total O-GlcNAcylation in COCs, which was reduced in the presence of an inhibitor of the β-O-linked glycosyltransferase enzyme. Several specific proteins were identified using mass spectrometry as potential targets of O-GlcNAcylation in COCs, including heat-shock protein 90 (HSP90, both α and β isoforms). While glucosamine treatment of COCs significantly decreased blastocyst development rate, inhibiting HSP90 with 17-allylamino-17-demethoxygeldanamycin during IVM in the presence of glucosamine recovered blastocyst rates to control levels. This effect was not due to an increase in overall HSP90 levels, since inhibiting HSP90 in control COCs did not affect blastocyst rate. These results suggest O-GlcNacylated HSP90 has an aberrant function in the COC.

This study is the first to examine in detail O-GlcNAcylation levels in the COC, and their correlation to oocyte developmental competence. HSP90 was identified as a potential target of O-GlcNAcylation in the COC, and subsequently shown to mediate oocyte developmental competence. This research is significant because of the increasing numbers of women wishing to become pregnant who have high blood glucose levels due to diabetes, obesity or poor diet. I have generated critically needed knowledge towards understanding how these lifestyle factors affect fertility and identifying possible avenues for new therapies.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Laura Alice Frank

2012

Dedication

I dedicate this thesis to my grandfather, Professor William Herdman Elliott.

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Publications arising from this thesis

1. **FRANK L. A.,** SUTTON-MCDOWALL M. L., RUSSELL D. L., WANG, X., FEIL, D., GILCHRIST R. B. & THOMPSON J. G. (2012) *Effect of varying glucose and glucosamine concentration* in vitro *on mouse oocyte maturation and developmental competence.* Reproduction, Fertility and Development (accepted, in press).

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1. **FRANK L. A.,** SUTTON-MCDOWALL M. L., RUSSELL D. L., GILCHRIST R. B. & THOMPSON J. G. (2012) *Proteomic analysis of β-O-linked glycosylated proteins extracted from glucosamine-treated mouse cumulus-oocyte complexes reveals HSP90 glycosylation as a potential regulator of oocyte competence.* Society for Reproduction and Fertility Conference 2012, Edinburgh, UK.

2. **FRANK L. A.,** SUTTON-MCDOWALL M. L., RUSSELL D. L., GILCHRIST R. B. & THOMPSON J. G. (2011) *Proteomic analysis of β-O-linked glycosylated proteins extracted from glucosamine-treated mouse cumulus-oocyte complexes reveals HSP90 glycosylation as a potential regulator of oocyte competence.* Society for Reproductive Biology Annual Scientific Meeting, Cairns, Australia.

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4. **FRANK L. A.,** SUTTON-MCDOWALL M. L., RUSSELL D. L., GILCHRIST R. B. & THOMPSON J. G. (2010) *Mouse oocyte require hexosamine biosynthesis pathway substrates during the first hour of in vitro maturation for subsequent embryo development.* Australian Society for Medical Research South Australian Annual Scientific Meeting, Adelaide, Australia.

5. **FRANK L. A.,** SUTTON-MCDOWALL M. L., RUSSELL D. L., LANE, M., GILCHRIST R. B. & THOMPSON J. G. (2009) *The effect of altering glucose levels during collection and maturation of mouse oocytes on subsequent developmental competence.* Faculty of Health Sciences Postgraduate Research Expo, the University of Adelaide, Adelaide, Australia.

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Abbreviations

17AAG	17-allylamino-17-demethoxygeldanamycin
ADP	adenosine diphosphate
ANOVA	analysis of variance
APS	ammonium persulphate
AR	aldose reductase
ATP	adenosine triphosphate
BADGP	benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside
BMI	body mass index
BMP15	bone morphogenetic protein 15
BSA	bovine serum albumin
cAMP	cyclic AMP (adenosine monophosphate)
CEI	cumulus expansion index
COC	cumulus-oocyte complex
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOHaD	Developmental Origins of Health and Disease
DON	6-diazo-5-oxo-L-norleucine
eCG	equine chorionic gonadotrophin
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid

ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
FOX01a	forkhead box protein 01a
FSH	follicle-stimulating hormone
G6PDH	glucose-6-phosphate dehydrogenase
GDF9	growth differentiation factor 9
GFPT	glutamine:fructose-6-phosphate amidotransferase
GlcN	glucosamine
GlcNAc	N-acetylglucosamine
GnRH	gonadotrophin-releasing hormone
GRP	glucose-regulated protein
GV	germinal vesicle
GVBD	germinal vesicle breakdown
H_2O	water
HbA1c	glycosylated haemoglobin
HBP	hexosamine biosynthesis pathway
HSP90	heat-shock protein 90
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
IRS	insulin receptor substrate
IVF	in vitro fertilisation
IVM	in vitro maturation

JNK	c-Jun N-terminal kinase
kg	kilogram
KILLER	tumor necrosis factor–related apoptosis-inducing ligand receptor
L	litre
LB	Laemmli buffer
LH	luteinising hormone
LSD	least-significant difference
mg	milligram
MII	metaphase II
mL	millilitre
mM	millimolar
mRNA	messenger RNA (ribonucleic acid)
NAD+/NADH	nicotinamide adenine dinucleotide
NADP+/NADPH	nicotinamide adenine dinucleotide phosphate
O-GlcNAc	β-O-linked N-acetylglucosamine
O-GlcNAcase	β-N-acetylglucosaminidase
O-GlcNAcylated	β-O-linked glycosylated
O-GlcNAcylation	β-O-linked glycosylation
OGT	O-linked β-N-acetylglucosaminyltransferase
PBS	phosphate buffered saline
PFK	phosphofructokinase
PI	propidium iodide
PI 3-K	phosphoinositide 3-kinase

PPP	pentose phosphate pathway
PUGNAc	O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate
PVDF	polyvinylidene
REDOX	reduction-oxidation
RIPA	radioimmunoprecipitation
RNA	ribonucleic acid
RT	room temperature
SDH	sorbitol dehydrogenase
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SLC2Ax	solute carrier family 2 (facilitated glucose transporter), member x
SP1	specificity protein 1
SV40	simian vacuolating virus 40
TCA	tricarboxylic acid
Thr	threonine
TEMED	*N,N,N',N'*-tetramethylethylenediamine
TRAIL	tumor necrosis factor–related apoptosis-inducing ligand
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
UDP-GlcNAc	uridine diphosphate-N-acetylglucosamine
UV	ultraviolet

CHAPTER 1

THE EFFECTS OF PRE-CONCEPTION HYPERGLYCAEMIA AND THE INVOLVEMENT OF THE HEXOSAMINE BIOSYNTHESIS PATHWAY IN MEDIATING OOCYTE DEVELOPMENTAL COMPETENCE

1.1 INTRODUCTION

Maternal diabetes and other conditions such as obesity in which blood glucose levels are elevated have long been associated with a higher risk of a multitude of pregnancy complications, including spontaneous abortions, neonatal morbidity and mortality and congenital malformations (Cornblath and Schwartz 1976; Sadler et al. 1988; Becerra et al. 1990; Greene 1999; Farrell et al. 2002). Numerous studies have examined the effect of hyperglycaemic conditions on early embryogenesis. However, even when optimal glycaemic control is achieved in the first few weeks of pregnancy, there is still a significant risk of pregnancy complications and neonatal abnormalities among diabetic women compared to normoglycaemic women (Dunne et al. 1999; Ray et al. 2001; Lapolla et al. 2008). Even before conception, diabetic oocytes are smaller, slower to complete meiotic maturation and have altered mitochondrial distribution compared to non-diabetic oocytes (Colton et al. 2002; Chang et al. 2005; Wang et al. 2009). It is becoming increasingly evident that the environment that the oocyte is exposed to during the pre-conception period has a significant impact on its developmental competence (the ability of the oocyte to support fertilisation and subsequent embryo development) and the long-term health of the resulting offspring (Young et al. 1998; Tarin et al. 1999).

The detrimental effects of hyperglycaemia on the developing oocyte are at least in part mediated by the hexosamine biosynthesis pathway (HBP) (Sutton-McDowall et al. 2006; Schelbach et al. 2010), a fuel-sensing pathway which utilises glucose to produce uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc). These UDP-GlcNAc moieties can be attached to serine or threonine residues of proteins and act in an analogous manner to phosphorylation to regulate protein function, a modification called β-O-linked glycosylation (O-GlcNAcylation). Altered O-GlcNAcylation is being associated with an increasing number of disease states including cancer, inflammatory conditions and neurodegenerative diseases (Hart et al. 2007). Notably, it is also implicated as a primary mechanism behind the development of insulin resistance and pancreatic β-cell destruction in type 2 diabetes (Marshall et al. 1991; Yang et al. 2008).

Embryos as early as the one-cell zygote stage, or pre-ovulatory oocytes exposed to hyperglycaemic conditions display increased levels of O-GlcNAcylation and a subsequent decrease in developmental competence (Sutton-McDowall et al. 2006; Pantaleon et al. 2010). However, the mechanism behind the influence of the HBP on the developing oocyte and embryo is unknown.

This review will describe glucose metabolism in the developing oocyte, the clinical and biological consequences of hyperglycaemia on the oocyte and subsequent embryo and the current knowledge of the role of the HBP and O-GlcNAcylation in reproductive physiology and elsewhere.

1.2 FOLLICULOGENESIS AND OOCYTE MATURATION IN MAMMALS

Folliculogenesis is the process of follicle and oocyte growth from the primordial stage, an oocyte surrounded by a single later of flattened somatic granulosa cells, through to a pre-ovulatory follicle (Fig. 1.1). Females are born with a finite number of primordial follicles which varies between species, approximately 4,270 in the mouse (Gosden and Telfer 1987). The first transition of the primordial follicle to the primary stage is defined by the conversion of the granulosa cells to a cuboidal shape (McGee and Hsueh 2000). As the granulosa cells proliferate and form multiple layers, the follicles are regarded as pre-antral or secondary follicles. At this stage another somatic cell layer, the theca, is formed and separated from the granulosa cells by the basement membrane. Thecal cells express receptors for luteinising hormone (LH) and granulosa cells begin to express receptors for follicle-stimulating hormone (FSH). However, up until this stage, pre-antral follicle growth is gonadotrophin-independent (Edson et al. 2009) and instead depends on autocrine and paracrine factors such as the oocyte secreted factors, bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) (Gilchrist et al. 2008), and kit-ligand (granulosa cell-secreted) (Joyce et al. 1999). While the oocyte secretes glycoproteins to form the zona pellucida layer separating it from the surrounding granulosa cells (Johnson and Everitt 2004), the oocyte and granulosa cells maintain contact through trans-zonal processes and gap junctions (see section 1.3).

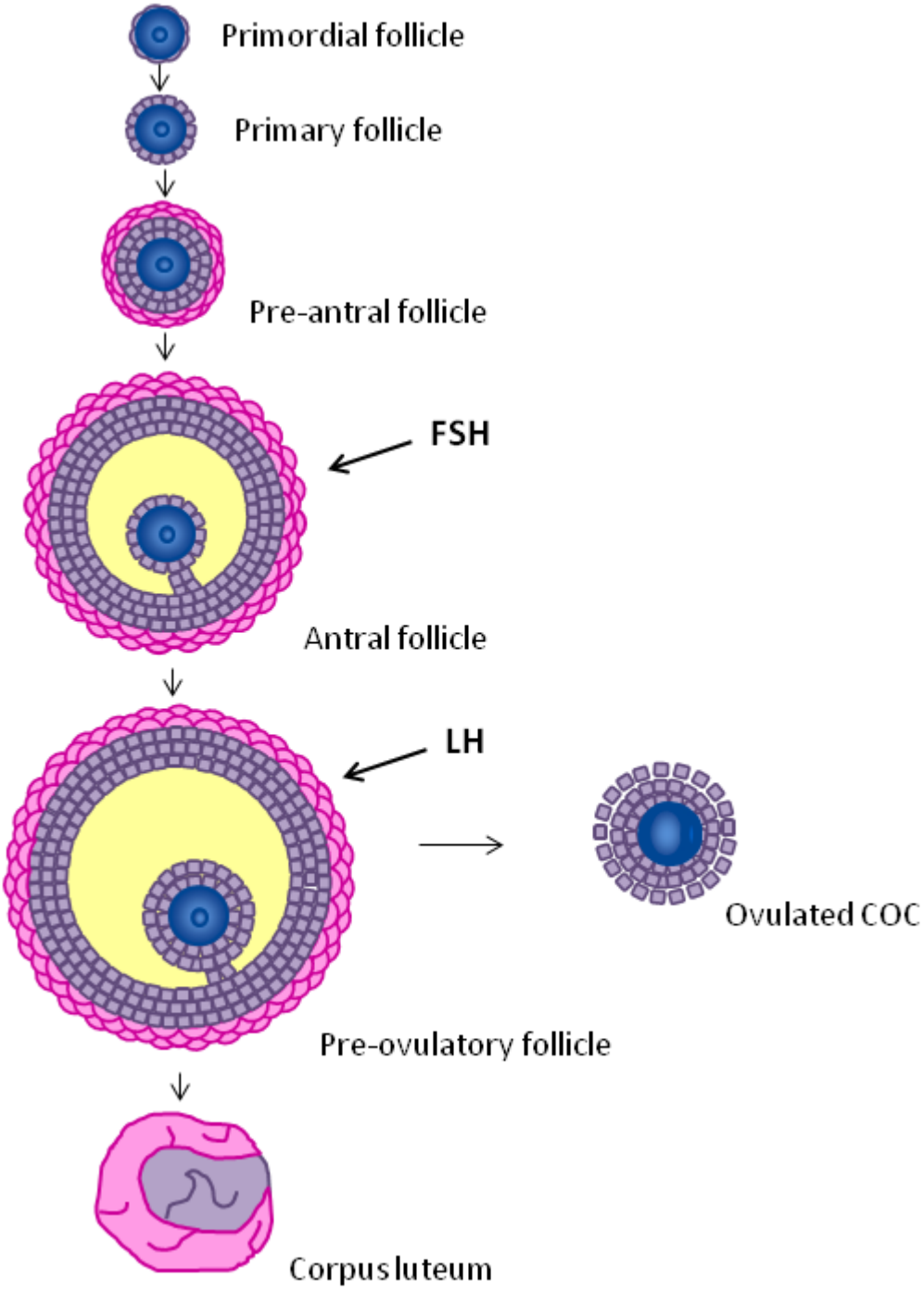

Figure 1.1 Folliculogenesis

Primordial follicles consist of an immature oocyte (blue) surrounded by a layer of flattened somatic granulosa cells (purple). The transition to a primary follicle is marked by the transformation of these cells into a cuboidal shape. Pre-antral follicles develop layers of a second type of somatic cells, theca cells (pink), and antral follicles contain a single fluid-filled antrum (yellow), which increases in size under the influence of follicle-stimulating hormone (FSH). The oocyte directs the differentiation of a sub-population of granulosa cells into cumulus cells, forming the cumulus-oocyte complex (COC). Pre-ovulatory follicles which do not undergo atresia respond to the luteinising hormone (LH) surge to induce the final maturation of the oocyte, ovulation and corpus luteum formation.

Further proliferation of the granulosa cells is accompanied by a buildup of follicular fluid between them, consisting of secretions from the granulosa cells as well as serum components which are filtered through the follicle from the surrounding vasculature (Johnson and Everitt 2004). This eventually creates a single fluid-filled antrum, which defines the antral follicle. Under the control of the hypothalamus, gonadotrophin-releasing hormone stimulates FSH and LH production by the pituitary gland. Antral follicles are now gonadotrophin-dependent and rely on FSH to promote follicle growth and further granulosa cell proliferation. As the antrum increases in size, the oocyte directs differentiation of a sub-population of surrounding granulosa cells into cumulus cells and forms a unit with these cells which becomes suspended in antral fluid and is connected to the granulosa cells only via a 'stalk' (Johnson and Everitt 2004). This unit is called the cumulus-oocyte complex (COC). Out of all follicles reaching the antral stage, only 0.1 % will continue on under the influence of FSH to further proliferate and become mature pre-ovulatory follicles (Barnett et al. 2006), with the remainder undergoing atresia, the apoptosis of all follicular cells including the oocyte.

Until the antral stage, the oocyte is arrested in the follicle at the prophase I stage of meiosis. In response to the LH surge, a complex network of other hormones and signalling cascades is activated, ultimately leading to the production of Maturation Promoting Factor (MPF) (Masui and Markert 1971). MPF is a ubiquitous cytoplasmic factor which induces entry into the cell cycle (both for mitosis and meiosis (Sunkara et al. 1979; Nelkin et al. 1980)), including in the arrested oocyte where meiosis resumes and proceeds to metaphase II. COC maturation encompasses not only meiotic (nuclear) maturation but also cytoplasmic maturation, a process which is not clearly defined but is described as the process in which the oocyte acquires the capability to undergo subsequent fertilisation and embryo development, and may involve mRNA and protein accumulation (Krisher 2004). Another aspect of COC maturation is the expansion of the cumulus matrix, facilitated by the production of extracellular matrix protein hyaluronan by the cumulus cells (see section 1.3.4). In vitro, the resumption of meiosis can be triggered by mechanical release of the COC from the follicle (Pincus and Enzmann 1935).

The LH surge is responsible for inducing ovulation of mature COCs and subsequently the formation of the corpus luteum from the remaining granulosa and theca cells. In this review, COC maturation refers to these final stages of development before ovulation.

1.3 GLUCOSE METABOLISM IN THE MATURING CUMULUS-OOCYTE COMPLEX

Within mammalian antral follicles, oocytes are surrounded by a support network of cumulus cells (specialised somatic cells), the whole unit collectively known as the cumulus-oocyte complex (COC) (see section 1.2). The oocyte and cumulus cells are tightly connected via trans-zonal processes, and gap junctions at the end of these allow the transfer of low molecular weight molecules (~ < 1000 daltons) (Eppig 1991; Bruzzone et al. 1996). Bi-directional communication between the oocyte and cumulus cells is enabled through these gap junctions and paracrine signalling (Gilchrist et al. 2004). The cumulus cells provide metabolic support for the oocyte (Colonna and Mangia 1983) as well as small molecules such as cyclic AMP (cAMP) which regulate oocyte meiotic maturation (Salustri and Siracusa 1983; Eppig and Downs 1984; Buccione et al. 1990). Oocyte-secreted factors such as BMP15 and GDF9 regulate the differentiation of granulosa cells to cumulus cells and the growth and metabolism of the cumulus cells, including cumulus expansion in the final stages of oocyte maturation (Gilchrist et al. 2008). The importance of the bi-directional communication within the COC is highlighted by the failure of both folliculogenesis and oogenesis in mice which are deficient in connexin-37 or connexin-43 (the building blocks of gap junctions between the oocyte and cumulus cells) (Simon et al. 1997; Ackert et al. 2001). For these reasons, glucose metabolism and its effects on oocyte developmental competence will be discussed in relation to the COC as a whole.

Glucose is an essential catabolic and anabolic substrate during both spontaneous and ligand-induced COC maturation in vitro (Downs and Hudson 2000; Sutton et al. 2003; Schelbach et al. 2010). In vivo, the components of follicular fluid are mainly derived from blood by filtration of plasma by follicles (Gosden et al. 1988) and the glucose concentration parallels that of plasma at a slightly lower level (see section 1.6). There are several documented pathways for glucose metabolism in the COC (Fig. 1.2), and with the exception of the polyol pathway all of these begin with the phosphorylation of glucose to glucose-6-phosphate by hexokinase (EC 2.7.1.1).

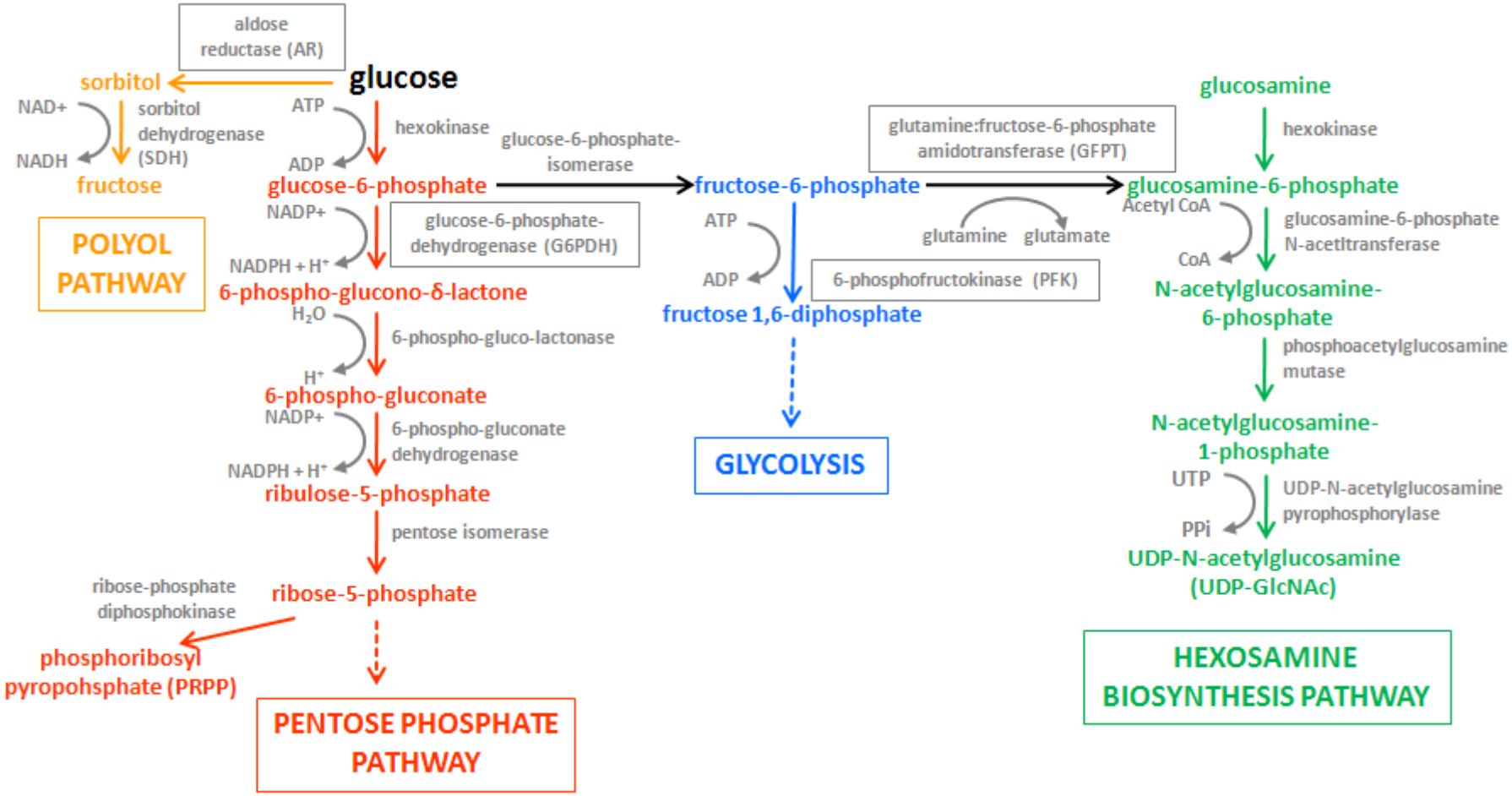

Figure 1.2 Glucose metabolism in cumulus-oocyte complexes (COCs)

Glucose is known to be metabolised through four pathways in the COC: the polyol pathway, the pentose phosphate pathway, glycolysis and the hexosamine biosynthesis pathway. Enzymes shown in boxes are rate-limiting enzymes for each pathway.

1.3.1 Glycolysis and Energy Production in the COC

Glycolysis accounts for the majority of the glucose taken up by the COC (Downs and Utecht 1999), and the rate of phosphofructokinase (PFK; one of the rate-limiting enzymes of glycolysis, EC 2.7.1.11) activity and glycolysis appears to remain constant throughout maturation (Cetica et al. 2002; Colton et al. 2003; Sutton-McDowall et al. 2010). Glucose consumption by the COC is almost exclusively by the cumulus cells, which have functional differences from the oocyte facilitating this metabolism. As well as transporters SLC2A1, SLC2A3 and SLC2A8, which are expressed in both oocytes and cumulus cells (human, rhesus monkey, sheep and cow, (Dan-Goor et al. 1997; Augustin et al. 2001; Zheng et al. 2007; Pisani et al. 2008)), cumulus cells express the insulin-sensitive facilitative glucose transporter SLC2A4. SLC2A4 is not present in the oocyte and has a high affinity for glucose (Williams et al. 2001; Nishimoto et al. 2006), and cumulus cells have high PFK activity (Downs et al. 1996). The oocyte itself has low glucose consumption (Biggers et al. 1967; Leese and Barton 1984) as well as low glycolytic and PFK activity (Cetica et al. 2002; Harris et al. 2009), and relies on the cumulus cells to supply it with pyruvate (Biggers et al. 1967; Sutton-McDowall et al. 2010). Pyruvate is the preferred nutrient of the oocyte and is metabolised via the tricarboxylic acid (TCA) cycle and oxidative phosphorylation; therefore ATP production within the oocyte is dependent on mitochondrial oxidative phosphorylation (Steeves and Gardner 1999). Pyruvate remains the preferred substrate for energy production from the COC stage through early embryo development until the 8-cell stage in mice; the failure of early embryos to survive on glucose may be due to high ATP levels in the early embryo, as ATP is a powerful allosteric inhibitor of PFK (Biggers and Borland 1976). Developmental competence of oocytes is correlated with glycolytic rate despite the known low level of activity in the oocyte. Oocytes from sheep and cattle with higher developmental capacity, such as in vivo matured oocytes or those derived from adult donors, have increased metabolism via glycolysis than those with reduced developmental capacity such as in vitro matured oocytes or those derived from pre-pubertal donors (O'Brien et al. 1996; Steeves and Gardner 1999).

Another important aspect of energy production in the COC is the production of ATP from endogenous lipid supplies. This occurs in the mitochondria via β-oxidation, and while the mouse oocyte has a relatively low lipid density compared to other species (Sturmey et al. 2009), β-oxidation has been shown to be important during oocyte maturation (Dunning et al. 2010). An essential co-factor involved in β-oxidation, L-carnitine, has been shown to increase early bovine embryo development when present in culture media in the absence of any other energy substrates (Sutton-McDowall et al. 2012). Interestingly however there was no interactive effect between carbohydrate (glucose, lactate and pyruvate) and L-carnitine supplementation on ATP production.

1.3.2 The Pentose Phosphate Pathway

The pentose phosphate pathway (PPP) accounts for relatively little of the glucose that is consumed by the COC (Sutton-McDowall et al. 2010), but plays a vital role in meiotic maturation of the oocyte. The PPP uses glucose to produce NADPH, the principle intracellular reductant for all cells and used to protect them from damage by reactive oxygen species, as well as in other reductant-requiring processes (Xu et al. 2005). Another product of the PPP is phosphoribosyl pyrophosphate, the sugar component for de novo nucleic acid synthesis (Banfalvi 2006).

In contrast to PFK activity, the activity of glucose-6-phosphate dehydrogenase (G6PDH, the rate-limiting enzyme of the oxidative phase of the PPP, EC 1.1.1.49) in cow oocytes is higher than in cumulus cells (Cetica et al. 2002), and the PPP is a key pathway influencing oocyte meiotic maturation (Downs et al. 1996; Downs et al. 1998). It has previously been shown that blocking glycolytic activity does not influence the meiotic maturation of mouse oocytes (Downs et al. 1996), and in contrast the use of PPP stimulators leads to a dose-dependent increase in germinal vesicle breakdown (the first stage of meiotic maturation) (Downs et al. 1998; Downs and Utecht 1999). Furthermore, inhibiting the PPP in pig oocytes reduces the rate of meiotic completion (Herrick et al. 2006). These results are supported by the observations that the addition of glucose to in vitro maturation (IVM) medium increases rates of meiotic maturation in cow and pig oocytes (Sutton-McDowall et al. 2005; Funahashi et al. 2008), and that medium containing pyruvate as the sole metabolite results in fewer mouse oocytes maturing (Downs and Hudson 2000).

1.3.3 The Polyol Pathway

The polyol pathway involves the conversion of glucose to sorbitol by aldose reductase (AR, EC 1.1.1.21) followed by the production of fructose by sorbitol dehydrogenase (SDH, EC 1.1.1.14). AR has a low affinity for glucose and under normal conditions very little glucose is directed down this pathway (Sutton-McDowall et al. 2010). However, under hyperglycaemic conditions hexokinase can become saturated and as much as 33 % of glucose taken up by the cell is metabolised via this pathway (Brownlee 2001). Excess flux of glucose down the polyol pathway is thought to play a role in the complications of diabetes, as intracytoplasmic accumulation of sorbitol and fructose increases osmotic stress, and also oxidises NADPH to NADP+ and reduces NAD+ to NADH, limiting the availability of these cofactors available for necessary REDOX reactions in the cell (Brownlee 2001). Both AR and SDH are expressed in reproductive tissues, with the highest level of both protein and enzyme activity found in the ovary (Kaneko et al. 2003). AR is also expressed in rat granulosa cells and oocytes, and SDH in the oocyte (Iwata et al. 1990; Kaneko et al. 2003). It has been suggested that the granulosa cells may convert glucose to sorbitol and fructose to provide the oocyte with alternative energy substrates to glucose, although this is not supported by experiments showing a decline in meiotic

maturation rates when fructose is the sole energy substrate provided (Wongsrikeao et al. 2006). This may be due to an absence of the fructose-specific transporter SLC2A5 (Burant et al. 1992), demonstrated in studies of the rat ovary (Kol et al. 1997) and sheep granulosa cells (Campbell et al. 2010).

1.3.4 The Hexosamine Biosynthesis Pathway

In somatic cells under normoglycaemic conditions, approximately 1 – 3 % of total glucose consumed by the cell is directed down the HBP (Marshall et al. 1991; Sayeski and Kudlow 1996), which produces UDP-GlcNAc (Marshall et al. 2004). The HBP is a highly conserved pathway which functions in all cell types as a link between nutrient levels and cell signalling using UDP-GlcNAc for β-O-linked glycosylation (O-GlcNAcylation; see section 1.4) (Zachara and Hart 2004). This link has been studied in most detail in relation to the glucose-mediated development of insulin resistance (Marshall et al. 1991; Yang et al. 2008). UDP-GlcNAc is also an important component of many mucopolysaccharides, large glycosaminoglycan chains which are incorporated into proteoglycans in mucus, connective tissue, skin, tendons, cartilage and ligaments (Anderson et al. 2005). In bacteria UDP-GlcNAc is used for teichoic acid synthesis, and in fungi, insects and crustaceans for the production of chitin (Milewski 2002).

Glutamine:fructose-6-phosphate amidotransferase (GFPT, EC 2.6.1.16) catalyses the first step of the HBP, converting fructose-6-phosphate to glucosamine-6-phosphate using glutamine as a co-factor (Badet et al. 1987). GFPT is the rate-limiting enzyme of the HBP and is strongly allosterically inhibited by the end-product UDP-GlcNAc (Milewski 2002). Although it is an essential enzyme, short-term inhibition of GFPT is not necessarily lethal (Bates et al. 1966). There are two isoforms of GFPT in humans and mice: GFPT1, which is ubiquitous, and GFPT2 which is preferentially expressed in the central nervous system (Sayeski et al. 1994; Zhou et al. 1995; Oki et al. 1999; DeHaven et al. 2001).

HBP regulation in the maturing COC is a unique situation. As the COC matures following the ovulatory LH surge, the cumulus cells around the oocyte undergo mucification and the matrix expands (Eppig 1981; Salustri et al. 1989; Chen et al. 1993), a phenomenon which assists in the transfer of the COC to the oviduct after ovulation in preparation for subsequent fertilization (Tanghe et al. 2002). UDP-GlcNAc is an essential substrate used for production of hyaluronic acid, the extracellular matrix glycosaminoglycan which supports the expansion of the cumulus matrix (Fig. 1.3). Towards the end of in vitro COC maturation, there is an unusually high upregulation of HBP activity, with approximately 25 % of the total glucose metabolised via this pathway (Sutton-McDowall et al. 2004). Preventing the entry of glucose into the HBP using an inhibitor of GFPT (6-diazo-5-oxo-L-norleucine) decreases cumulus expansion and reduces glucose uptake by COCs (Gutnisky et al. 2007).

1.3.4.1 Glucosamine

Glucosamine is another hexose sugar which can be transported into cells via facilitated glucose transporters (Uldry et al. 2002), differing from glucose by the presence of an amino group. Glucosamine is widely used as a hyperglycaemic mimetic especially in in vitro systems (Monauni et al. 2000; Marshall et al. 2004), as it is metabolised via the HBP but bypasses GFPT, the rate-limiting step (Fig. 1.2) (Patti et al. 1999; Nelson et al. 2000; Marshall et al. 2005). For this reason, it is also a more potent stimulator of HBP activity than glucose; in adipocytes glucosamine is estimated to be at least 40 times more effective than glucose at mediating desensitization of the insulin-responsive transport system (Marshall et al. 1991). Furthermore, during embryo development experiments 0.2 mM glucosamine had the same negative effect on blastocyst development as 27 mM glucose (Pantaleon et al. 2010). Consistent with these reports, mouse COCs matured under control conditions expand to only 25 % of the volume of COCs matured in the presence of glucosamine, as a result of increased substrate for hyaluronic acid synthesis (Chen et al. 1993). This may be due to the fact that while glucose-6-phosphate is a potent inhibitor of hexokinase, glucosamine-6-phosphate is a relatively weak one (Virkamaki and Yki-Jarvinen 1999). More glucosamine-6-phosphate may accumulate before the same level of negative feedback on hexokinase is reached (Pantaleon et al. 2010).

1.4 β-O-LINKED GLYCOSYLATION (O-GLCNACYLATION)

An alternative fate of UDP-GlcNAc is its attachment to the hydroxyl groups or serine or threonine residues of proteins, a post-translational modification called β-O-linked glycosylation (O-GlcNAcylation, Fig. 1.3) (for review see (Wells et al. 2003)). In contrast with the glycosaminoglycan-type glycosylation, the O-GlcNAc modification consists of a single O-linked N-acetylglucosamine residue, with no further additions to form a chain. First discovered in 1984 (Torres and Hart 1984), it is now estimated that O-GlcNAcylation is as widespread as phosphorylation, and has been found in all multicellular eukaryotes examined to date (Roquemore et al. 1994; Comer and Hart 2000; Wells et al. 2001). Thousands of key cytosolic and nuclear proteins are known to be modified by O-GlcNAc: in one study, out of approximately 5,000 human sequences examined from SWISS-PROT, over 4,600 had at least one predicted O-GlcNAc site (Gupta and Brunak 2002). While the enzymes of O-linked glycosylation have been characterized and purified, to date there is no definitive recognition sequence for O-GlcNAc attachment (Julenius et al. 2005). A 'fuzzy' motif is marked by the close proximity of proline and valine residues, a downstream tract of serines and no leucine or glutamine residues in the near vicinity (Gupta and Brunak 2002). Although O-GlcNAcylation is now recognised as one of the most common forms of post-translational modification, it is only now starting to be well characterized because of its comparatively recent discovery (in contrast, phosphorylation has been known for 70 years) as well as the technical difficulties associated with studying it (Whelan and Hart 2003).

Levels of UDP-GlcNAc increase directly in response to increasing concentrations of glucose or glucosamine (Comer et al. 2001; Parker et al. 2004; Sutton-McDowall et al. 2006). It has also been reported that O-GlcNAc levels increase under glucose deprivation conditions (Cheung and Hart 2008; Taylor et al. 2008; Kang et al. 2009). However, it has since been shown that the majority of O-GlcNAcylation induced by glucose deprivation is actually an N-linked GlcNAc modification, which is not regulated by the specific β-O-linked glycosylation regulating enzymes (see section 1.4.1) and can be inhibited by tunicamycin, an N-glycosylation inhibitor (Isono 2011). This is supported by results from experiments showing that inhibition of the enzyme which attaches O-GlcNAc moieties was unable to reduce an increase in O-GlcNAcylation levels in embryos cultured in glucose deprivation conditions (Pantaleon et al. 2010).

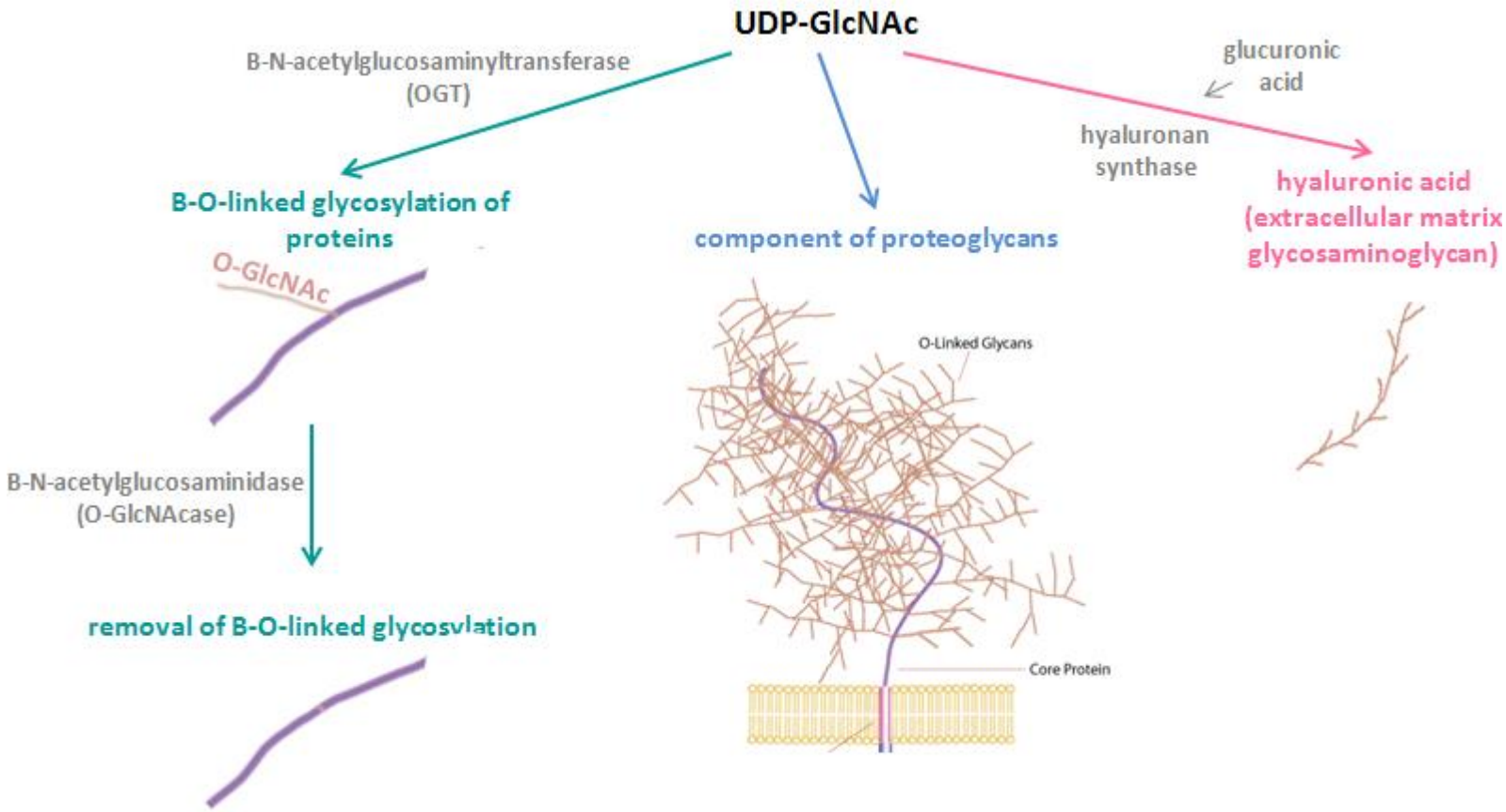

Figure 1.3 Fates of uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc)

UDP-GlcNAc can be used for β-O-linked glycosylation or as a component of mucopolysaccharide chains for proteoglycans. Protein cores are represented in purple and attached glycosylation moieties in pink. Hyaluronic acid is a glycosaminoglycan synthesized in the maturing COC as an extracellular matrix protein to support cumulus expansion, and is the only glycosyaminoglycan which is not synthesized on a core protein but instead secreted directly into the extracellular space (Montreuil et al. 1997). Proteoglycan diagram modified from (Sigma-Aldrich 2011).

1.4.1 Enzymes of β-O-linked Glycosylation

Only one enzyme for the addition of O-GlcNAc has been discovered, O-linked β-N-acetylglucosaminyltransferase (OGT, EC 2.4.1.255) (Kreppel et al. 1997; Lubas et al. 1997; Kreppel and Hart 1999; Lubas and Hanover 2000). Its counterpart, O-GlcNAc specific β-N-acetylglucosaminidase (O-GlcNAcase, EC 3.2.1.169) is the only known enzyme which removes O-GlcNAc from molecules (Dong and Hart 1994; Gao et al. 2001; Wells et al. 2002) (in comparison, there are hundreds of kinases and phosphatases (Webb 1992)). Both of these enzymes have been highly conserved throughout evolution (Kreppel et al. 1997; Lubas et al. 1997; Gao et al. 2001) from *Caenorhabditis elegans* to *Homo sapiens*, although no evidence of either of them (or O-GlcNAcylation) or of any homologue has been found in *E. coli* or yeast species to date (Iyer and Hart 2003). OGT has been shown to be essential for life even at the single cell level, and targeted deletion of the OGT locus resulted in embryonic lethality (Shafi et al. 2000).

Consistent with observations of nuclear and cytoplasmic O-GlcNAc-modified proteins, OGT is not found in the golgi secretory pathway along with enzymes for other types of glycosylation, but instead is concentrated in the nucleocytoplasmic compartment (Holt and Hart 1986). The OGT gene is X-linked, mapping to region q13 which is often associated with neurodegenerative disorders (Shafi et al. 2000). Interestingly, OGT itself is regulated by its own intrinsic O-GlcNAcylation state, and is hyper-glycosylated in response to elevated glucose (Akimoto et al. 2001), however the sites of O-GlcNAcylation and their effect on the enzymatic activity of OGT are unknown (Kreppel et al. 1997).

1.4.2 β-O-linked Glycosylation and Phosphorylation

As well as the single residue nature of O-GlcNAcylation, it has other characteristics which make it more similar to phosphorylation than to other forms of glycosylation. Like phosphorylation, O-GlcNAc turns over more rapidly than the polypeptide backbone (Chou et al. 1992; Roquemore et al. 1996), and has been shown to cycle on and off proteins on a time scale similar to that for phosphorylation/de-phosphorylation (Slawson et al. 2006). Together with its enzymes it is localised to cytosolic and nuclear proteins, in contrast with glycosaminoglycans which are secreted or membrane-bound (Van den Steen et al. 1998; Vosseller et al. 2001; Zachara and Hart 2004). Also, unlike other forms of glycosylation, O-GlcNAcylation is reversible (Van den Steen et al. 1998). These features suggest that while complex long-chain glycosylation is not likely to be involved in dynamic cell signalling, O-GlcNAc is in a position to do so (Vosseller et al. 2001). Indeed, O-GlcNAc regulates protein function in a very similar manner to phosphorylation, with the two modifications often targeting the same or adjacent sites on a protein in what has been described as a yin-yang relationship (Haltiwanger et al. 1997; Comer and Hart 2000; Whelan and Hart 2003; Wang et al. 2008; Butkinaree et al. 2010).

A reciprocal relationship between global phosphorylation and O-GlcNAcylation levels has been demonstrated in several cell lines (Lefebvre et al. 1999; Comer and Hart 2001). Site-specific observations of phosphorylation and O-GlcNAcylation regulating each other at the same or neighbouring sites have been made on many proteins, including the oestrogen receptor β (Cheng et al. 2000), the SV40 T antigen (Medina et al. 1998), c-Myc (Chou et al. 1995) and RNA polymerase II (Kelly et al. 1993). The transcription factor, specificity protein 1 (SP1), is known to be modified by O-GlcNAc, and in glomerular mesangial cells inhibition of O-GlcNAcase results in a 4-fold increase in O-GlcNAcylated SP1 and a 30 % decrease in serine/threonine phosphorylated SP1 (Haltiwanger et al. 1998). A similar result was shown in aortic endothelial cells, where hyperglycaemia induces a 1.7-fold increase in O-GlcNAcylated SP1 and a 70 – 80 % decrease in phosphorylated SP1 (Du et al. 2000).

There is an interesting relationship between tyrosine phosphorylation and O-GlcNAcylation. While no O-GlcNAcylation of tyrosine residues has been observed (Wells et al. 2001), tyrosine phosphorylation occurs at a significantly higher rate on O-GlcNAc-modified proteins than the overall rate (68 % vs. 2 %) (Mishra et al. 2011). Mishra et al. have hypothesised that phosphorylation may be a pre-requisite for O-GlcNAcylation (almost all O-GlcNAc-modified proteins discovered so far are also phosphoproteins), and that tyrosine phosphorylation as well as that of serine and threonine interacts with O-GlcNAcylation in some way (Mishra et al. 2011). They also suggest that the wide range of simultaneous changes in O-GlcNAcylation observed in many experimental studies under different conditions cannot be accounted for by changes in the levels of OGT and O-GlcNAcase alone, and that the phosphorylation status of target proteins may play a significant role in determining O-GlcNAcylation status. In the brain, OGT forms a complex with protein phosphatase-1 (Wells et al. 2004), suggesting a direct mechanism by which O-GlcNAcylation may be coupled to de-phosphorylation of specific proteins (Rexach et al. 2008).

1.4.3 Molecular Mechanism of O-GlcNAc Modulation of Proteins

O-GlcNAc levels respond to many stimuli including mitogens, growth factors, cellular stages of development and nutrient levels (Zachara and Hart 2004; Love and Hanover 2005). O-GlcNAc modifications can alter the behaviour of proteins in several ways: they can change enzyme activity (Du et al. 2001), regulate protein-protein interactions (Roos et al. 1997), influence DNA binding or sub-cellular localisation (Duverger et al. 1996; Juang et al. 2002; Gao et al. 2003) and affect the half-life and proteolytic processing of the protein (Cheng et al. 2000). The transcription factor SP1, involved in transcription in early development, is an example of an extensively O-GlcNAc modified protein (Jackson and Tjian 1988) which falls under several of these categories. Hyper-O-GlcNAcylation of SP1 in response to hyperglycaemia or glucosamine treatment prevents its degradation via the proteasome (Han and Kudlow 1997), inhibits interactions with TATA-binding protein-associated factor and holo-SP1

(Roos et al. 1997) and reduces its ability to activate proteins involved in diabetes, such as endothelial nitric oxide synthase (Du et al. 2001).

1.4.4 Currently Known Roles of β-O-linked Glycosylation

The HBP and O-GlcNAcylation are known to be involved in controlling essential cellular processes such as cell cycle regulation (Haltiwanger and Philipsberg 1997; Slawson et al. 2002; Slawson and Hart 2003), protein transcription and translation (Comer and Hart 2000; Datta et al. 2001). The role of O-GlcNAc in cell cycle regulation has been investigated extensively in *Xenopus* oocytes by Slawson et al. (Slawson et al. 2002; Slawson et al. 2005; Slawson et al. 2006). It was found that in M-phase, OGT is concentrated at the mitotic spindle and midbody, and overexpression of OGT results in a polyploid phenotype with faulty cytokinesis (Slawson et al. 2005). In another study, alloxan (an inhibitor of OGT) blocked the G2/M transition in *Xenopus* oocytes in a concentration-dependent manner, and injection of O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate (PUGNAc), an O-GlcNAcase inhibitor, into the oocyte accelerated the maturation process (Dehennaut et al. 2007).

Aberrant O-GlcNAcylation is associated with myriad disease states (for review see (Hart et al. 2007)), and in general, O-GlcNAcylation is increased in the unhealthy or pathological state compared to healthy cells. This is also the case when cells are exposed to many different types of stress in vitro: O-GlcNAc levels increase rapidly in response to heat shock, ethanol, ultra-violet (UV) light, hypoxia, reductive, oxidative or osmotic stress (Zachara et al. 2004). This may be a protective mechanism, as decreased OGT and O-GlcNAcylation levels result in cells that are less tolerant of stress (Zachara et al. 2004; Hart et al. 2007).

1.4.4.1 Diabetes and Insulin Signalling

The most widely studied example of the HBP as a fuel-sensing pathway coupled to cell signalling is in diabetes mellitus. First implicated in the development of type 2 diabetes in 1991 (Marshall et al. 1991), the HBP has now been shown to play a role in both the major pathologies of diabetes: insulin resistance and the decline in pancreatic β-cell function. Insulin resistance is defined as the reduced ability of insulin to lower blood glucose, and it is well known that increasing flux through the HBP, raising O-GlcNAc levels using PUGNAc (an inhibitor of O-GlcNAcase), or overexpression of OGT result in insulin resistance (McClain et al. 2002; Vosseller et al. 2002; Arias et al. 2004; Arias and Cartee 2005; Akimoto et al. 2007). This is in part due to impaired glucose transport into cells expressing the insulin-responsive transporter SLC2A4 (primarily skeletal and heart muscle and adipocytes) (Buse 2006). There have been various mechanisms described to explain this effect. While there is no change in SLC2A4 expression in cells exposed to hyperglycaemia or glucosamine (Nelson et al. 2000), studies have shown accelerated degradation of the protein in these conditions (Thomson et al. 1997) and defective translocation of SLC2A4 to the plasma membrane in cultured insulin-resistant adipocytes (Nelson et al. 2000; Park et

al. 2005). Supporting the role of the HBP in this phenomenon is data from a transgenic mouse model which overexpresses GFPT in muscle and fat cells. These mice became insulin-resistant, which was associated with impaired translocation of SLC2A4 to the plasma membrane (Cooksey et al. 1999). It is believed that a significant part of the block in SLC2A4 function may be the impaired docking or fusion of the SLC2A4-containing vesicle when it reaches the plasma membrane (Nelson et al. 2002). Munc18c regulates the docking/fusion step, and is known to be modified by O-GlcNAc (Chen et al. 2003). Hyperglycaemia or glucosamine disrupts insulin-mediated trafficking of Munc18c, possibly due to altered O-GlcNAcylation of this protein (Nelson et al. 2002). As well as this, many proteins in SLC2A4 transport vehicles as well as SLC2A4 itself are modified by O-GlcNAc (Buse et al. 2002; Park et al. 2005).

Decline in pancreatic β-cell function can be caused by both a decrease in insulin synthesis and destruction of the β-cells, and glucosamine treatment is commonly used to induce both these effects in vitro (Marshall et al. 2004; Park et al. 2007). Hyper-O-GlcNAcylation in pancreatic β-cells leads to reduced insulin secretion, which can be recovered with benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP, an inhibitor of OGT) treatment (Park et al. 2007). Andreozzi et al. (2004) demonstrated that upregulation of the HBP using either hyperglycaemia or glucosamine led to impairment in insulin-stimulated phosphorylation of two tyrosine residues in insulin receptor substrate-1 (IRS-1), as well as an increase in phosphorylation on two serine residues. Phosphorylation of the serine residues is known to be stimulated by enhanced activation of c-Jun N-terminal kinase (JNK) and extracellular single-regulated kinase (ERK)1/2 by high glucose or glucosamine. Serine phosphorylation of IRS-1 interferes with tyrosine phosphorylation of the protein and subsequently its ability to interact with downstream phosphoinositide 3-kinase (PI 3-K) sub-units. By increasing serine phosphorylation of IRS-1 through JNK and ERK1/2, hyperglycaemia and glucosamine altered the tyrosine phosphorylation and hence the downstream PI 3-K/protein synthesis pathway, leading to impaired insulin synthesis. Under hyperglycaemic conditions, inhibition of GFPT with azaserine reversed the serine phosphorylation of IRS-1, highlighting the involvement of the HBP in the process (Andreozzi et al. 2004). Hyperglycaemia and glucosamine are also known to mediate destruction of pancreatic β-cells (Efanova et al. 1998; Federici et al. 2001; Andreozzi et al. 2004). Similar to impaired insulin synthesis, this process has also been linked to the HBP through O-GlcNAcylation of IRS-1 and IRS-2 and subsequent impairment of the PI 3-K signalling pathway in pancreatic β-cells. Through several intermediate steps, impaired PI 3-K signalling leads to increased forkhead box protein 01a (FOX01a) activation, causing abnormally sustained expression of the pro-apoptotic protein Bim. This effect can be reversed with azaserine (a GFPT inhibitor), further confirming the role of the HBP in the destruction of the β-cells (D'Alessandris et al. 2004).

Recently, these results have been underscored with the elucidation of a molecular mechanism by which the activity of OGT can be linked to the development of insulin resistance (Yang et al. 2008). It was demonstrated that after insulin-regulated phosphatidylinositol 3,4,5-triphosphate is activated and recruited to the plasma membrane, it binds and recruits OGT through a novel phosphoinositide-binding domain. OGT subsequently modifies O-GlcNAcylation of insulin signalling proteins.

1.4.4.2 Cancer

Altered glycosylation status has long been associated with tumour growth (Fuster and Esko 2005), and many oncogene and tumour suppressor proteins are modified by O-GlcNAc (Chou and Hart 2001). The activity of GFPT is higher in actively proliferating cells such as fetal tissues and hepatocytes (Kikuchi et al. 1971; Richards and Greengard 1973), and this includes cancer cells: increased GFPT activity has been reported in Yoshida sarcoma, ascites hepatoma-130 and Ehrlich ascites sarcoma cells when compared to normal mouse liver cells (Kikuchi et al. 1971). Threonine (Thr)-58 of c-Myc was the first identified O-GlcNAc site on an oncoprotein (Chou et al. 1995). The c-myc gene encodes a helix-loop-helix-leucine zipper protein which is involved in the regulation of transcription during cell proliferation, differentiation and programmed cell death. The Thr-58 site of c-Myc is also a known phosphorylation site in vivo (Lutterbach and Hann 1994), and the phosphorylation status of Thr-58 regulates the transactivation activity of c-Myc (Chou and Hart 2001). O-GlcNAcylation has also recently been reported to play a role in cancer metastasis, with increased O-GlcNAcylation correlated with enhanced migratory and invasive capabilities of breast cancer cells. Gu et al. (2010) found increased O-GlcNAcylation in breast tumour cells compared with adjacent tissues, and specifically identified p120 and β-catenin as being O-GlcNAcylated. The enhanced migratory ability of the cells appeared to be mediated by a reduction in E-cadherin at the cell surface, a key molecule involved in epithelial cell-cell adhesion. E-cadherin itself is not modified by O-GlcNAc, however the cytoplasmic 'tail' of E-cadherin is reliant on binding to p120 and β-catenin for transportation to the membrane and stabilization at the cell surface, which the authors suggest is disrupted by O-GlcNAcylation in breast cancer cells (Gu et al. 2010).

1.4.4.3 Alzheimer's Disease

Not all disease states are associated with hyper-O-GlcNAcylation. O-GlcNAc has been implicated in the aetiology of Alzheimer's disease (Iyer and Hart 2003); both the beta amyloid pre-cursor protein (Griffith et al. 1995) and the microtubule-associated protein tau (Arnold et al. 1996) have been shown to be extensively modified by O-GlcNAc. In brain samples from Alzheimer's patients, a lower level of O-GlcNAcylation and an increase in phosphorylation was detected on tau protein compared to non-Alzheimer's samples, and the regulation of tau phosphorylation by O-GlcNAcylation was confirmed in pheochromocytoma-12 cells which overexpress tau (Liu et al. 2004). Interestingly, the O-GlcNAcase gene maps to the late-onset Alzheimer's disease locus (Blatch and Lassle 1999; Nobile and Pitzalis

1999; Bertram et al. 2000; Myers et al. 2000), and OGT maps to the X-linked Parkinson's Dystonia locus (Shafi et al. 2000).

1.4.4.4 Endoplasmic Reticulum Stress

The endoplasmic reticulum (ER) is an organelle which is vital for the folding, export and processing of newly synthesized proteins. ER stress occurs when the influx of unfolded proteins exceeds the folding or processing capabilities of the ER and is induced by a variety of conditions, including hyperglycaemia (Lindenmeyer et al. 2008) and glucosamine treatment (Werstuck et al. 2006; Matthews et al. 2007). The ER stress response to hyperglycaemic conditions has mostly been studied in the context of diabetes. Pancreatic β-cells have a highly developed ER, and ER stress is necessary for aspects of β-cell failure and insulin resistance (Nakatani et al. 2005; Laybutt et al. 2007). In vitro glucosamine treatment of human aortic smooth muscle cells, monocytes and hepatocytes induces ER stress and subsequent disregulation of lipid metabolism (Werstuck et al. 2006). This leads to the accumulation of cholesterol in treated cells, a contributing factor to vascular disease which patients with diabetes are at increased risk of developing. Glucosamine treatment also causes an increase in the ER stress marker 78 kDa glucose-regulated protein (GRP78) in human astroglial cells (Matthews et al. 2007), and expression of both GRP78 and GRP94 (another ER stress marker) were found to be increased in the livers of diabetic mice (Nakatani et al. 2005).

1.4.4.5 Glucosamine as a Dietary Supplement

Glucosamine is one of the most commonly taken non-vitamin dietary supplements, with sales in Australia increasing by 60 % between 2003 and 2008, to $2.13 billion (Lavelle 2010). It is used to relieve the symptoms of osteoarthritis (Anderson et al. 2005), and this effect is achieved through the incorporation of GlcNAc into mucopolysaccharide chains (see section 1.3.4) which are an important component of cartilage, although recent evidence suggests that there is minimal, if any, effect of glucosamine compared to placebo (Wandel et al. 2010). No adverse effects have been found in a study of humans taking the recommended dose of glucosamine (23.1 mg/kg body weight) for 66 weeks (Anderson et al. 2005). Healthy subjects were found to have a serum glucosamine concentration of 0.04 mM (Monauni et al. 2000), and it is estimated that intake of the recommended oral dose of glucosamine would result in a serum level of 0.06 mM (Anderson et al. 2005). No effect of the recommended oral dose of glucosamine have been found to date on serum glucose levels, insulin levels or on the results of an oral glucose tolerance test (Yu et al. 2003; Tannis et al. 2004).

1.4.4.6 Oocyte Developmental Competence

The role of the HBP in COC maturation was first studied in the context of the synthesis of hyaluronic acid during cumulus expansion (Chen et al. 1993; Sutton-McDowall et al. 2004). Using in vitro maturation (IVM), it has been shown that in the presence of glucosamine there is a significant decrease

in glucose consumption by cow COCs, and less incorporation of radiolabelled glucose into the extracellular matrix (Sutton-McDowall et al. 2004). Recently, O-GlcNAcylation has been examined and found to be elevated in cow COCs (Sutton-McDowall et al. 2006) and mouse embryos (Pantaleon et al. 2010) exposed to glucosamine. Glucosamine treatment during IVM does not affect meiotic maturation in cow, pig or mouse; however in all species blastocyst development was severely inhibited in these conditions (Sutton-McDowall et al. 2006; Schelbach et al. 2010). A decrease in cleavage rate was also observed in the mouse study in the presence of glucosamine (Schelbach et al. 2010). BADGP (the in vitro inhibitor of OGT) in IVM media was able to rescue embryo development from COCs cultured in the presence of glucosamine in a dose-dependent manner, highlighting the contribution of the HBP to the developmental competence of these oocytes. Many of the adverse effects of hyperglycaemic conditions on insulin resistance are mediated through the PI 3-K pathway (see section 1.3.4.4 a)). However, this does not appear to be the case with oocyte developmental competence, as downregulation of the PI 3-K pathway using inhibitors in cow oocytes during maturation was unable to replicate the results observed using glucosamine treatment (Sutton-McDowall et al. 2006).

1.5 DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE

The first hypothesis to state that conditions during in utero growth can affect the long-term health of the foetus was the Barker hypothesis. This was based on several epidemiological studies carried out by David Barker, which found a high correlation between low birth weight and adult cardiac and metabolic disorders (stroke, type 2 diabetes, dyslipidaemia), and death from ischemic heart disease (Barker and Osmond 1988; Barker et al. 1989; Barker 1995). These findings generated interest in the study of maternal under-nutrition during gestation, and subsequent studies investigating the impact of nutritional and environmental conditions at various time throughout the peri-conceptual period and gestation. Together, the results led to the formulation of the Developmental Origins of Health and Disease (DOHaD) hypothesis, that a stimulus or insult during a sensitive period of development can have long-term effects (Wadhwa et al. 2009).

Most studies which investigate the effects of DOHaD are focused on how different insults or stimuli manifest themselves in the phenotype of the offspring, with few investigating the mechanisms behind them (Sinclair and Singh 2007). However, there have been three main mechanisms suggested, specifically in relation to the peri-conceptual period of development: reduced proliferation, metabolic stress and abnormal gene expression and regulation (Thompson et al. 2002; Fleming et al. 2004).

A slowing of growth rate or reduction in cell number in the developing pre-implantation embryo is commonly observed in response to sub-optimal conditions, both in vitro and in vivo (Lane and Gardner

1997; Lighten et al. 1998; Kwong et al. 2000; Spanos et al. 2000). It is hypothesised that reduced embryonic cell numbers result in a limited supply of cells for future fetal and placental development (Fleming et al. 2004), and indeed it has been shown that reduced inner cell mass (ICM; the cells which will go on to form the embryo) numbers may be a cause of fetal growth retardation in a diabetic rat model (Lea et al. 1996). Embryos exposed to sub-optimal conditions during development also have increased glucose uptake as a stress response and exhibit upregulated glucose metabolism (Lane and Gardner 1998; Khurana and Niemann 2000; Fleming et al. 2004), the consequences of which are discussed in section 1.6.2.1.

During early embryo development, there is genome-wide de-methylation and re-methylation of embryonic DNA (Morgan et al. 2005). In the mouse zygote there is active de-methylation (removal of the methyl group from existing 5-methylcytosines) of the paternal DNA, followed by passive de-methylation (lack of addition of the methyl group due to absence of inhibition of DNA methyltransferase 1 during successive rounds of DNA replication, (Wu and Zhang 2010)) during pre-implantation development and de novo re-methylation after implantation (Santos et al. 2002). Environmental conditions are well known to influence epigenetic status, and methylation patterns in the offspring of mice and rats has been demonstrated to be influenced by the level of methyl donors or protein in the maternal diet (Wolff et al. 1998; Reik et al. 2001). Importantly, many genes which regulate fetal growth and development are imprinted genes, which are expressed selectively from either the maternal or paternal allele during development and are mainly exempt from the global passive de-methylation described above (Reik and Walter 2001; Santos et al. 2002). Conditions during pre-implantation development are known to alter the DNA methylation of several imprinted genes in mouse and sheep models (Doherty et al. 2000; Young and Fairburn 2000).

1.6 THE EFFECTS OF HYPERGLYCAEMIA ON FEMALE REPRODUCTION

The concentration of glucose in follicular fluid parallels that of plasma at a slightly lower level (Table 1.1) (Gardner and Leese 1990), and is positively correlated with follicle size (Sutton-McDowall et al. 2005; Nandi et al. 2008). As well as being the defining pathology of diabetes, elevated blood glucose levels are present in cases of pre-diabetes or impaired glucose tolerance, where glucose levels are higher than normal but not high enough for a diagnosis of diabetes (Diabetes Australia 2011). Obesity and poor diet are also associated with hyperglycaemia, which can be induced in mice fed a high fat diet (Jungheim et al. 2010). In humans, increasing body mass index (BMI) is correlated with increasing glucose levels in follicular fluid (Robker et al. 2009).

Species	Condition	Plasma glucose (mM)	Follicle glucose (mM)	Oviductal glucose (mM)	Uterine glucose (mM)	Notes	Reference
Human	normal	<5.55					1
	pre-diabetic	5.55 - 6.99					1
	diabetic	≥7.00					1
			3.44 ± 0.17			Patients undergoing IVF, unexplained infertility	2
			3.39 ± 0.91			Patients undergoing IVF, tubal occlusion	3
			3.44 ± 0.18			Patients undergoing IVF, unexplained, tubal occlusion or male factor infertility, non-stimulated	4
			3.26 ± 0.09			Patients undergoing IVF, unexplained, tubal occlusion or male factor infertility, stimulated	4
			3.72 ± 0.40			Patients undergoing IVF, measured after one freeze-thaw cycle at -70°C	5
				3.11 ± 0.64		Follicular phase of menstrual cycle	6
				0.50 ± 0.21		Mid-cycle	6
				2.32 ± 0.54		Luteal phase of menstrual cycle	6
					3.15 ± 0.31	Constant throughout menstrual cycle	6
Mouse		11.71 ± 0.76				During natural oestrous	7
	Akita model*	16.65					8
			0.46 ± 0.08			Ovaries stimulated	7
				3.40 ± 0.14		Ovaries stimulated, cumulus present	9
				5.19 ± 0.20		Ovaries stimulated, cumulus absent	9
				1.09 ± 0.13		COCs present	7
				1.65 ± 0.28		COCs not present	7
					0.61 ± 0.06		7
Rat		5.8 ± 0.2					10
		5.00 ± 0.21					11
	diabetic	23.3 ± 0.5				Streptozotocin-induced diabetes	10
	diabetic	29.73 ± 1.26				Streptozotocin-induced diabetes	11
	diabetic	22.2				Streptozotocin-induced diabetes	12
Sheep			1.40 ± 0.22			Adult cycling non-pregnant sheep	13
			1.17 ± 0.14			Small follicles	14
			1.46 ± 0.11			Medium follicles	14
			1.65 ± 0.13			Large follicles	14
Buffalo			2.42 ± 0.31			Adult cycling non-pregnant buffalo	13

Species	Condition	Plasma glucose (mM)	Follicle glucose (mM)	Oviductal glucose (mM)	Uterine glucose (mM)	Notes	
Goat			1.40 ± 0.34			Antral follicles	15
Rabbit				1.46 ± 0.24		Oestrus rabbits	16
Pig		5.19 ± 0.45				Pre-ovulatory	17
		4.62 ± 0.26				Post-mating	17
				0.97 ± 0.20		Pre-ovulatory	17
				0.25 ± 0.05		Post-mating	17
Cow		5.57 ± 0.14					18
			4.91 ± 0.19			Dominant follicles, mean throughout oestrus cycle	18
			4.58 ± 0.24			Non-dominant follicles, mean throughout oestrous cycle	18
			2.30 ± 0.25			5mM stimulated follicles	19
			3.92 ± 0.12			10mM stimulated follicles	19
			1.4 ± 0.2			Small antral follicles	20
			2.2 ± 0.3			Medium antral follicles	20
			2.3 ± 0.2			Large antral follicles	20

Table 1.1 Plasma, follicle, oviductal and uterine glucose concentrations in various species

References: 1: (American Diabetes Association 2011). 2: (Davoodi et al. 2005). 3: (Gull et al. 1999). 4: (Leese and Lenton 1990). 5: (Huyser et al. 1993). 6: (Gardner et al. 1996). 7: (Harris et al. 2005). 8: (Chang et al. 2005). 9: (Gardner and Leese 1990). 10: (Pampfer et al. 1997). 11: (Xu et al. 2005). 12: (Lacy et al. 1991). 13: (Nandi et al. 2008). 14: (Nandi et al. 2007). 15: (Herrick et al. 2006). 16: (Leese and Barton 1985). 17: (Nichol et al. 1992). 18: (Orsi et al. 2005). 19: (Johnson et al. 2001). 20: (Sutton-McDowall et al. 2005). *Akita mice spontaneously develop diabetes.

1.6.1 Clinical Consequences of Hyperglycaemia on Female Reproduction

It is well established that maternal diabetes is associated with poor conception rates and difficulties with maintenance of a pregnancy and delivery of a healthy baby (Becerra et al. 1990; Rich-Edwards et al. 1994; Holing et al. 1998; Lapolla et al. 2008; Jungheim and Moley 2010; Purcell and Moley 2011). Women with diabetes are more at risk of having a spontaneous first-trimester abortion, pre-eclampsia, pre-term birth or a high birth weight baby (Mills et al. 1988; Greene 1999; Hedderson et al. 2003; Middleton et al. 2010), and obese women have also been shown to be at increased risk of pre-eclampsia and spontaneous abortions (O'Brien et al. 2003; Dokras et al. 2006; Metwally et al. 2008). Babies who are large for gestational age born to diabetic women are at increased risk of developing metabolic syndrome (including insulin resistance) in childhood (Silverman et al. 1995; Boney et al. 2005), and in rodent models, streptozotocin-induced diabetes (streptozotocin mediates destruction of the pancreatic β-cells) during pregnancy results in high birth weight pups with increased pancreatic insulin content (Oh et al. 1988).

Congenital anomalies occur in around 6 – 10 % of diabetic pregnancies (3 - 5 fold higher than the general population) (Reece and Homko 2000; Lapolla et al. 2008). The degree of risk for diabetic complications in pregnancy is dependent on the level of glycaemic control achieved, with the level of glycosylated haemoglobin (HbA1c) positively correlated with the rate of fetal malformations (Miller et al. 1981; Suhonen et al. 2000; Guerin et al. 2007; Lapolla et al. 2008). Schaefer et al. examined the rates of major congenital anomalies associated with increasing levels of fasting serum glucose. These authors reported that the rate of anomalies doubled (to 5.2 %) with a maternal fasting blood glucose level of 6.6 mM, below the threshold of a diagnosis of diabetes (7 mM), and further increased to 30.4 % with a maternal fasting blood glucose > 14.3 mM (Schaefer et al. 1997). The rate of neural tube defects and skeletal and cardiovascular malformations in particular are approximately 18 times more common in babies of diabetic women (Becerra et al. 1990; Greene 1999; Farrell et al. 2002), and are also increased in babies of obese women (Stothard et al. 2009). It has been demonstrated that high levels of glucose, or glucosamine, inhibit the expression of Pax-3 (a gene required for neural tube closure) in embryos (Chalepakis et al. 1994), and increase the levels of neural tube defects in vivo (Horal et al. 2004).

The hyperglycaemic insult must occur within the first six weeks of gestation to produce increased rates of congenital anomalies (Mills et al. 1979); the increase is not seen in women with gestational diabetes only (Mills et al. 1979), which usually presents around the 24th-28th week of pregnancy (Diabetes Australia 2011). The earlier glycaemic control can be achieved, the less risk there is of malformations. Even so, there is mounting evidence to suggest that even very brief exposure to hyperglycaemia is enough to cause malformations in the offspring. Wyman et al. (2008) performed zygote transfers from diabetic to non-diabetic mice, and observed that despite transferring the embryos to a normoglycaemic

environment within 24 hours of fertilisation, foetuses derived from donor diabetic mothers were significantly smaller than controls and had increased incidences of deficient neural tube closure, hydrocephaly and limb defects. Exposure to hyperglycaemia for 96 hours (to the blastocyst stage) further increased the risk of resorption or miscarriage, however this study did not address the possible contributions of sperm damage induced by conception in the hyperglycaemic environment (Wyman et al. 2008).

The effect of the timing of exposure to hyperglycaemia extends to the pre-conception period. In human studies, even if optimal glycaemic control is achieved within the first few weeks of pregnancy, there is still a 3 – 5 times higher risk of spontaneous abortions and congenital anomalies in diabetic women (Casson et al. 1997; El-Sayed and Lyell 2001; Baccetti et al. 2002); however if a pre-conception treatment program is undertaken this risk is significantly reduced (Dunne et al. 1999; Ray et al. 2001; Pearson et al. 2007).

1.6.2 Biological Consequences of Hyperglycaemia on Female Reproduction

1.6.2.1 Embryos

Abnormally high or low rates of maternal metabolism are known to affect embryo development (Fleming et al. 2004; Leese et al. 2008). Under hyperglycaemic conditions it has been shown that both glucose uptake and expression of glucose transporters are reduced in pre-implantation embryos (Moley et al. 1998; Moley et al. 1998; Moley 1999). In a non-obese diabetic mouse model, significantly fewer embryos reached the blastocyst stage of development in diabetic mice compared to control mice, and this discrepancy was reversed by treating the mothers with insulin (Moley et al. 1991). Blastocysts recovered from diabetic rats were found to be more fragmented and to have fewer ICM cells than blastocysts from normoglycaemic mothers (Lea et al. 1996). Supporting the observation that the effects of hyperglycaemia continue after the initial insult is removed, zygotes removed from diabetic mice and transferred to non-diabetic surrogates display retarded rates of development to the two-cell stage in vivo, and embryos recovered after cleavage and cultured in vitro similarly show a significant delay in their progression to the blastocyst stage (Diamond et al. 1989). Early embryogenesis alone is susceptible to hyperglycaemic damage, with mouse embryos treated from the zygote stage in vitro with 27 mM glucose or 0.2mM glucosamine producing fewer blastocysts, with reduced cell numbers compared to controls, and an increase in apoptosis (Pantaleon et al. 2010). BADGP, an inhibitor of OGT, was able to reduce all of these effects.

1.6.2.2 Oocytes

Most diabetic rodent studies focus on pre-implantation embryo development or the period of fetal organogenesis, around days 9 – 11 (Wyman et al. 2008). However, consistent with clinical studies

highlighting the benefits of pre-conception care, hyperglycaemia is known to cause numerous perturbations in oocyte structure and function prior to fertilisation.

Hyperglycaemia induces apoptosis in both follicles and COCs. The Akita mouse model carries an autosomal dominant mutation which results in the spontaneous development of diabetes (hypoinsulinaemia and hyperglycaemia) (The Jackson Laboratory 2000). In one study using these mice, 64 % of ovarian follicles were terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) positive (indicating DNA damage and cell death) vs. 37 % in control mice. TUNEL positive staining was also significantly increased in COCs from Akita mice, and these results were also seen in a streptozotocin-induced diabetic mouse model (Chang et al. 2005). Increased apoptosis within the ovary and COC may be partly due to the up-regulation of expression of several cell death signalling proteins (TRAIL and KILLER) found in cumulus cells from diabetic mice (Chang et al. 2005). Oocyte size is also affected, with oocytes from diabetic and obese mice smaller than control oocytes (Chang et al. 2005; Jungheim et al. 2010). Smaller oocyte size is also observed in mice with even mild hyperglycaemia that is below the diabetic threshold (Jungheim et al. 2010). The link between maternal hyperglycaemia and small oocytes also translates to humans, with smaller oocytes collected from obese women undergoing in vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) than from non-obese women (Marquard et al. 2011). Data from human studies of patients undergoing IVF has shown that both small oocyte size and cumulus cell apoptosis correlate with poor pregnancy rates (Nataprawira et al. 1992; Wittmaack et al. 1994; Arnot et al. 1995; Bergh et al. 1998), possibly due to growth reduction (see section 1.5).

The mitochondria in oocytes from diabetic mice have altered structure and distribution, reduced function (as measured by TCA cycle metabolites) and an increased mitochondrial DNA copy number (Wang et al. 2009; Wang and Moley 2010), the latter usually associated with increased oocyte competence. The authors attribute the increased copy number to a compensatory mechanism to ensure the adequate supply of ATP, possibly due to increased demand. These findings may partly explain the depletion in ATP observed with upregulation of the HBP in several cell lines (Hresko et al. 1998; Marshall et al. 2004).

Many studies have shown delayed and decreased completion of meiotic maturation in oocytes from diabetic mice (Diamond et al. 1989; Colton et al. 2002; Chang et al. 2005; Kim et al. 2007; Ratchford et al. 2007; Wang et al. 2010), accompanied by spindle defects and chromosome misalignments during maturation (Chang et al. 2005). Glucosamine 6-phosphate is a competitive inhibitor of G6PDH (Kanji et al. 1976), and significantly less glucose is metabolised through the PPP in COCs from diabetic mice compared to controls (Colton et al. 2003). This downregulation of the PPP in response to upregulation of the HBP may be a contributing factor to the decline in meiotic maturation rates observed.

While excess glucose is clearly detrimental, the supply of some glucose is a fundamental requirement for oocyte and embryo health (Sutton et al. 2003), and its absence reduces rates of meiotic resumption, (Downs and Hudson 2000; Hashimoto et al. 2000), cumulus expansion (Nandi et al. 2008) and embryo development (Hendryx and Wordinger 1979; Wongsrikeao et al. 2006). Glucose concentration throughout development must be maintained at an optimal level, and excess or absence results in reduced developmental competence (Thompson 2006).

1.7 CONCLUSIONS

The pre-conception environment is known to influence the developmental competence of the oocyte. In particular, it is well known that hyperglycaemic conditions during this time are detrimental to subsequent embryo and fetal health; but the mechanisms for this effect are poorly understood. The importance of the HBP as a fuel sensing and regulatory signalling pathway and O-GlcNAc as the effector is becoming increasingly evident in a wide variety of fields, including recently oocyte developmental competence. However, there is still very little known about the contribution of the HBP or the mechanisms by which it acts to affect developmental competence, particularly under hyperglycaemic conditions. Understanding these will generate critically needed knowledge towards how hyperglycaemia affects fertility, and identify possible therapeutic targets.

1.8 HYPOTHESES AND AIMS

1.8.1 Hypothesis 1

HBP activity regulated by glucose or glucosamine alters the developmental competence of the oocyte in a concentration-dependent manner

Experimental aims for Hypothesis 1

1. Assess the effects of physiological and non-physiological concentrations of glucose during IVM of mouse COCs on meiotic maturation, cumulus expansion, cleavage and blastocyst development rates.

2. Determine the contribution of the HBP to oocyte developmental competence of mouse COCs and analyse the effects on meiotic maturation, cumulus expansion, cleavage and blastocyst development rates.

1.8.2 Hypothesis 2

The effects on oocyte and embryo health observed after manipulation of HBP activity are due to altered O-GlcNAcylation of one or more proteins in the COC which are critical to oocyte developmental competence.

Experimental aims for Hypothesis 2

1. Examine overall protein O-GlcNAcylation levels in COCs matured in various glucose and glucosamine concentrations.

2. Identify proteins found to be differentially O-GlcNAcylated under varying glucose and glucosamine concentrations.

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS AND SOLUTIONS

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Media, buffers and solutions were prepared using Milli Q water (Millipore, MA, USA). Recipes for media and solutions can be found in Appendix 1.

2.2 ANIMAL PROCEDURES

2.2.1 Mice

CBA x C57BL6 F1 hybrid mice were obtained from the University of Adelaide's Waite campus and kept in the Animal House at the Medical School, University of Adelaide, North Terrace campus, under a 14 : 10 hour light : dark cycle with access to food and water *ad libitum*. Female mice were sourced as pre-pubertal (21 - 22 days old) and male mice were obtained at 6 – 8 weeks old. All experimental procedures were approved by the University of Adelaide Animal Ethics Committee (Medical).

2.2.2 Ovarian Stimulation

Stimulation of ovarian follicles was achieved by an intraperitoneal injection of 5 IU equine chorionic gonadotrophin (eCG; Folligon, Intervet, Boxmeer, The Netherlands) in 0.1 mL 0.9 % saline (Baxter Healthcare Pty. Ltd., NSW, Australia).

2.3 CUMULUS-OOCYTE COMPLEX (COC) AND EMBRYO CULTURE

All procedures after ovary and epididymides/vasa deferentia collection were performed on warming stages calibrated to maintain medium in dishes at 37 °C. COC and embryo culture and fertilisation was performed in incubators at 37 °C under paraffin oil (Merck, Darmstadt, Germany), in humidified air comprising 6 % CO_2/5 % O_2/89 % N_2. Maturation, fertilisation and embryo culture media were pre-equilibrated for at least 4 h prior to use at 37 °C in a humidified 6% CO_2 atmosphere, and collection medium pre-warmed to 37 °C. See Appendix 1, 8.1 for collection and maturation media composition.

2.3.1 COC Collection and Maturation

Forty-six hours post-eCG injection, female mice were humanely sacrificed by cervical dislocation. Ovaries were dissected out and placed into warm (37 °C) collection medium, and follicles punctured with a 30 gauge needle to release the COCs. COCs with a morphologically normal and unexpanded cumulus cell vestment were placed into a fresh dish and remained in collection medium for a total time of one hour. COCs were washed in maturation medium, transferred into maturation dishes (50 µL medium/COC in groups of 30) and matured for up to 18 hours. This volume was used as it overcomes

the problem of maintaining glucose concentration despite the high glucose consumption of maturing COCs (Sutton-McDowall et al. 2004), and previous studies in our laboratory have indicated that it is necessary to observe the effect of glucosamine when using physiologically relevant glucose levels in the mouse (Schelbach et al. 2010).

2.3.2 Fertilisation

Wash, fertilisation and culture media were Research Vitro Wash, Research Vitro Fertilization and Research Vitro Cleave respectively from Cook Medical (William A. Cook Australia Pty. Ltd., QLD, Australia). Male mice, which had previously been assessed for mating ability (not less than 3 days prior), were used as sperm donors for in vitro fertilisation. Mice were sacrificed by cervical dislocation and the epididymides and vasa deferentia were collected into warm (37 °C) wash medium, cleaned of excess fat and tissue and transferred into 1mL of fertilisation medium. Sperm were extracted into the medium and allowed to capacitate for one hour prior to addition to fertilisation drops (10 µL capacitated sperm added to 90 µL fertilisation drop). After 17 hours of maturation COCs were washed once in fertilisation medium and transferred to fertilisation drops (including sperm). COCs and sperm were incubated together for four hours, before COCs were transferred to wash medium and cumulus cells removed mechanically using a Gilson pipette (Gilson, Inc., WI, USA). Presumptive zygotes were washed in culture medium and placed in culture drops (4 – 7 per 10 µL drop).

2.3.3 Embryo Culture and Assessment

Approximately 25 hours post-insemination (Day 2), embryo development was assessed and any embryos which had not developed to the two-cell stage were removed from culture drops. On Day 5 (at approximately 102 hours post-insemination), embryo development stage was assessed and scored, using the scoring system reported by (Gardner and Lane 2004) (Fig. 2.1). Degenerate oocytes were identified by a characteristic dark and granulated cytoplasm with a flat appearance (live oocytes appear convex).

2.4 ASSESSMENT OF CUMULUS EXPANSION INDEX (CEI)

After 17 hours of maturation the cumulus expansion index (CEI) was scored using the system of scoring reported by Vanderhyden (Vanderhyden et al. 1990) (Fig. 2.2). An operator-blinded system was used, as well as measurements of COC diameter taken to confirm correct scoring.

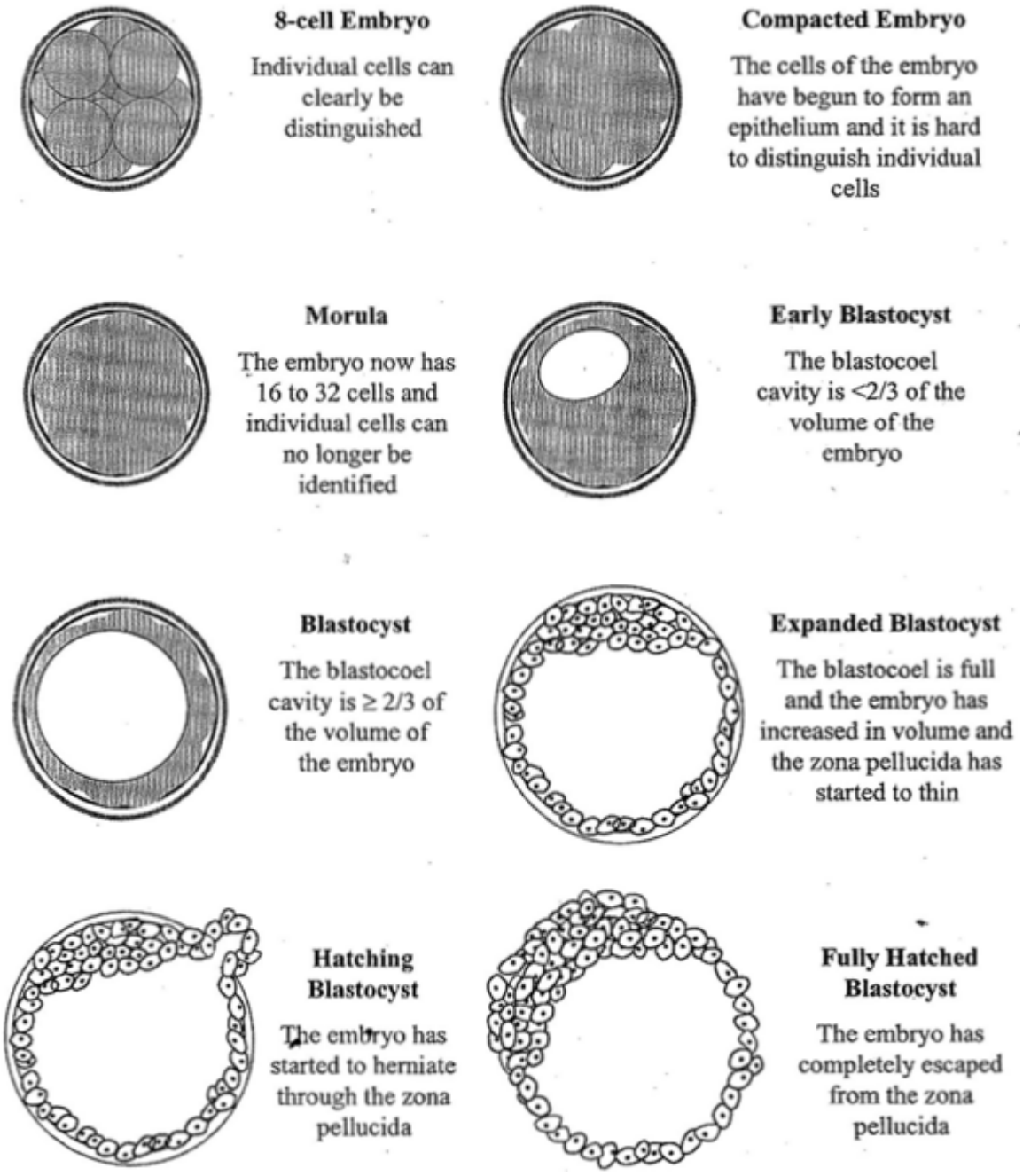

Figure 2.1 Categories for embryo development scoring on Day 5.
Any embryos which had not reached the 8-cell stage were classified as either degenerate, 2-cell or ≥3 cell. Diagram from (Gardner and Lane 2004).

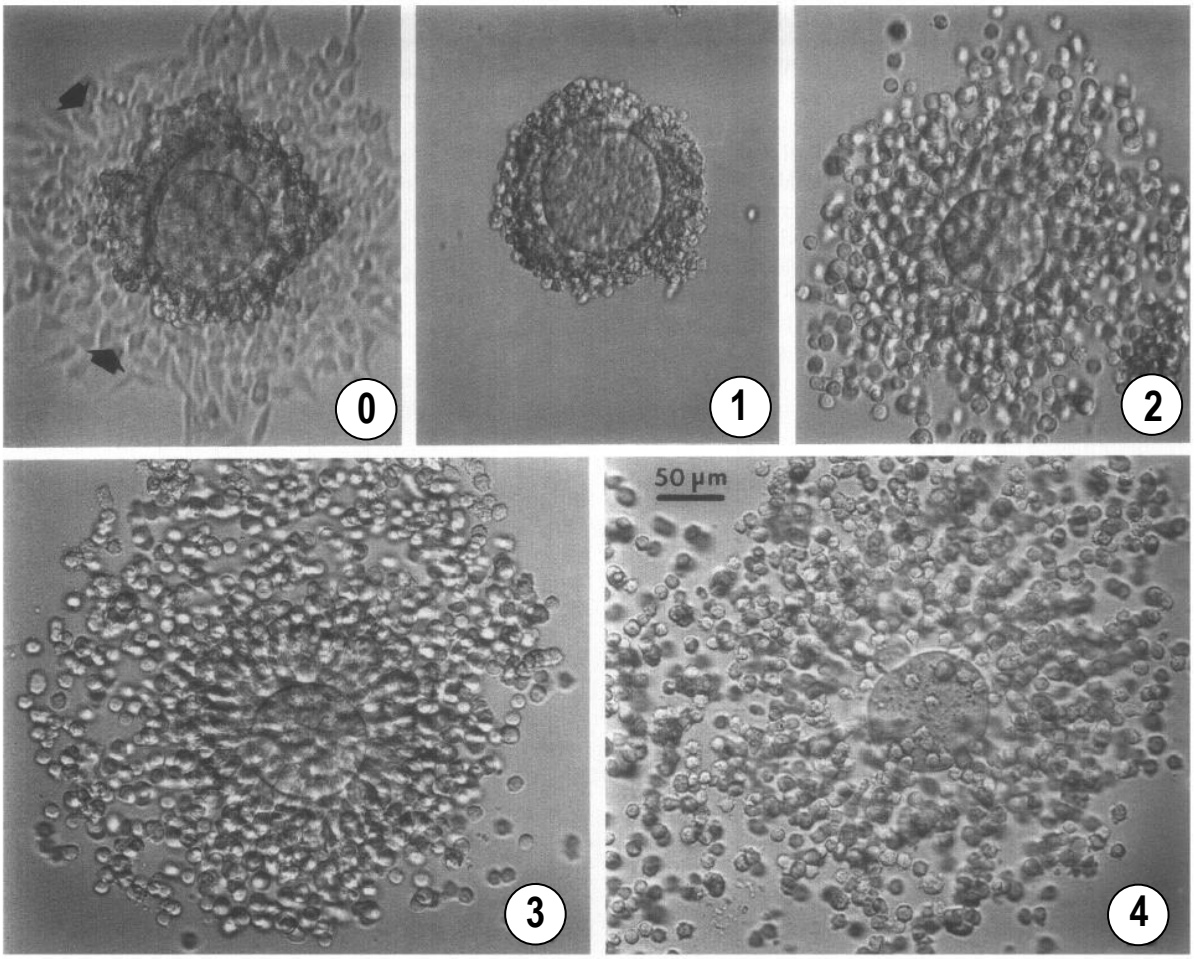

Figure 2.2 Cumulus expansion scoring system according to Vanderhyden, Caron et al.
A score of 0 indicates no detectable response, and the cumulus cells have begun to detach and plate down onto the dish. A score of 1 denotes the minimum observable response, with no detachment of cumulus cells and a 'glistening' appearance. Complexes with only the outer layer of cumulus cells expanded are given a score of 2, and for 3 all layers except the corona radiata have expanded. A score of 4 indicates the maximum expansion, including corona radiata ((Vanderhyden et al. 1990) (including diagram)).

2.5 ASSESSMENT OF MEIOTIC MATURATION

Meiotic maturation was assessed using 4',6-diamidino-2-phenylindole (DAPI), a fluorescent stain for DNA. After 17 hours of maturation, COCs were denuded mechanically using a Gilson pipette and oocytes fixed for 30 minutes in 4 % paraformaldehyde in phosphate buffered saline (PBS). Oocytes were washed in 0.01 % bovine serum albumin (BSA; ICPbio, Glenfield, New Zealand) in PBS and transferred to 3 µM DAPI (in H_2O) for 15 minutes. Oocytes were washed again in 0.01 % BSA, collected in 4 µL wash medium and placed on a slide next to an 8 µL drop of anti-fade reagent (ProLong Gold, Invitrogen, CA, USA) so that the drops combined when a coverslip was placed on top. Using a Nikon Eclipse TE2000-E microscope with UV laser (330 – 380 nm, DAPI excitation at 358 nm, emission at 461 nm) oocytes were then classified into germinal vesicle (GV), germinal vesicle breakdown (GVBD) or metaphase II (MII) stages (Fig. 2.3).

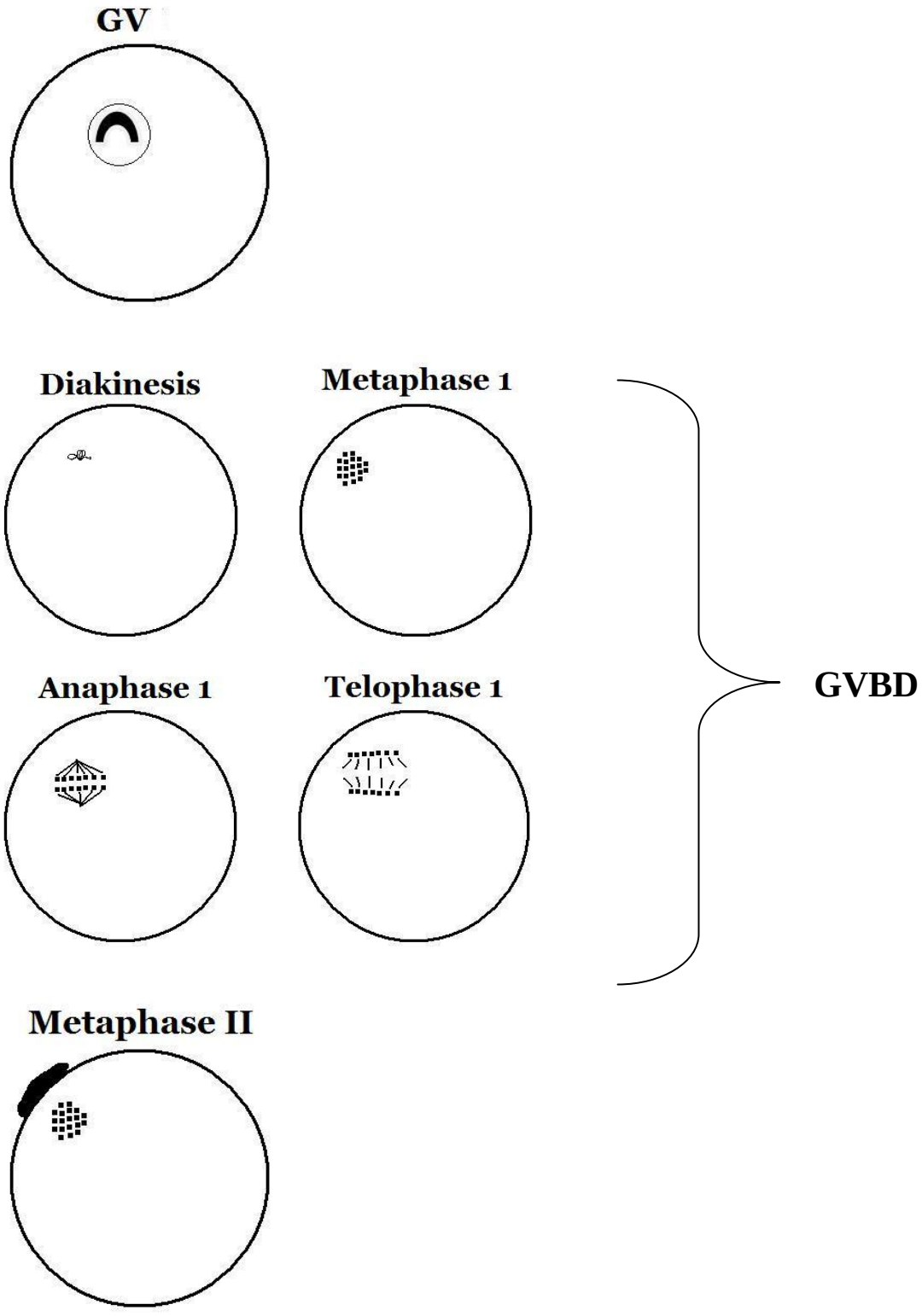

Figure 2.3 Meiotic maturation stages

In germinal vesicle (GV) stage, the nuclear membrane is visible and chromatin condensed in a ring or horseshoe shape around the nucleolus. Germinal vesicle breakdown (GVBD) incorporates several stages (diakinesis, metaphase I, anaphase I and telophase I) and is indicated by a lack of visible nuclear membrane and chromatin in a conformation of one of these stages. Oocytes in metaphase II (MII) display chromosomes on the metaphase plate as well as a small polar body.

2.6 IMMUNOFLUORESCENCE

COCs for immunofluorescence were fixed at the time of collection in 4 % paraformaldehyde in PBS for at least one hour. A standard glass slide (no coating) was pre-cleaned with 70 % ethanol, and a Dako Pen (Dako, Glostrup, Denmark) used to draw a circle on the slide. The inside of the circle was then coated in 5 µL Cell-Tak (BD Biosciences, NJ, USA) and allowed to dry. COCs were carefully placed onto the Cell Tak and allowed to adhere for 2 – 3 minutes, and immunofluorescent staining carried out as described in Appendix 1, 8.2.2.

The primary antibody used for detection of O-GlcNAcylation was CTD110.6 (anti-O-GlcNAc antibody; Covance, NJ, USA), diluted 1 in 250 in blocking solution (see Appendix 1, 8.2.1), and secondary antibody was Alexa Fluor 488 goat anti-mouse IgM (µ chain) (Invitrogen, CA, USA), diluted 1 in 250 in blocking solution. Propidium iodide (PI) was also included as a nuclear stain.

Images were visualized on an Olympus Fluoview FV10i laser scanning confocal microscope (Olympus, Tokyo, Japan). All colours for images were collected simultaneously, and laser intensity settings remained uniform for each run. The laser used for CTD fluorescence (green) had an excitation wavelength of 473 nm and an emission wavelength of 520 nm, and for PI fluorescence (red) an excitation wavelength of 559 nm and an emission wavelength of 619 nm. The lens used was a 60 x objective with no digital zoom, type UPLSAP60xW.

2.7 IMMUNOPRECIPITATION

COC samples for immunoprecipitation were collected in a minimal volume of medium (number of COCs varied according to experiment, see individual chapters), snap frozen in liquid nitrogen at the time of collection and stored at -80 °C. For immunoprecipitation, COC samples were thawed, 50 µL 1 : 9 protease inhibitor cocktail : radioimmunoprecipitation (RIPA) buffer mixture added to the tube and repeatedly pipetted to lyse COCs. Antibody was then added (detailed protocols for each antibody are described in Chapters 4 and 5) and incubated with rotation overnight at 4 °C. The following day, 10 µL Pierce Protein L beads (Thermo Fisher Scientific, MA, USA) was added and samples rotated at 4 °C for a further 4 – 5 hours. Samples were then centrifuged at 20,238 rcf for two minutes (eppendorf Centrifuge 5424, Hamburg, Germany), the supernatant removed and the pellet of beads resuspended in RIPA buffer. This process was repeated three times, and after the final centrifugation, the pellet was resuspended in 5 x Laemmli buffer (LB) and boiled for seven minutes in an egg cooker (Sunbeam Poach and Boil, NSW, Australia). The sample was centrifuged for a further two minutes and supernatant removed and used for Western blotting.

2.8 WESTERN BLOTTING

COC samples were either immunoprecipitated prior to Western blotting (see section 2.7) or thawed and lysed in 20 µL 1 : 9 protease inhibitor cocktail : RIPA buffer mixture and 5 µL 5 x LB and boiled for 5 minutes in an egg boiler. Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 4 – 7.5 % gels, and transferred onto a polyvinylidene fluoride (PVDF; Millipore, MA, USA) membrane overnight. Molecular weight markers ranging from 10 – 250 kDa (Precision Plus Protein Dual Color Standards, Bio-Rad, CA, USA) were used on every gel. Gel and buffer recipes are listed in Appendix 1, 8.4. Wet transfer of proteins to membranes was performed in Bio-Rad Mini Trans-Blot system overnight at 4°C, 20 mA. Washing and antibody incubation protocols varied according to the antibody used: see individual chapters for descriptions. Colorimetric blots were developed using Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega, WI, USA). Chemiluminescent blots were developed using the Amersham enhanced chemiluminescence (ECL) kit and hyperfilm (GE Healthcare, Buckinghamshire, UK).

Quantification of protein bands on Western blots was performed using ImageJ version 1.44p (Rasband 1997 - 2011). ImageJ output (a measure of the pixel density of the selected area of the membrane) was analysed for each time point. The raw data was tested for normality and the appropriate tests applied (see section 2.9).

2.9 STATISTICAL ANALYSES

Data were tested for normality and significnace using SPSS version 18.0.2 (Predictive Analytics SoftWare (PASW), IBM, NSW, Australia). Data were tested for normality using the Shapiro-Wilk test. All proportional data were arc-sine transformed and if normally distributed, analysed using a one-way analysis of variance (ANOVA) and comparisons made by least-significant difference (LSD) post-hoc test. Data which were not normally distributed (including CEI scores) were analysed using a Kruskal-Wallis test followed by Mann-Whitney U tests if significance was found. A p value of < 0.05 was accepted as significant. For embryo development experiments, cleavage rates were expressed as a proportion of total oocytes matured, and blastocyst rates as a proportion of cleaved. Tests were weighted by the number of oocytes per treatment group for each replicate.

CHAPTER 3

EFFECTS OF HEXOSAMINE BIOSYNTHESIS PATHWAY SUBSTRATES DURING COLLECTION AND IN VITRO MATURATION OF MOUSE COCS ON OOCYTE DEVELOPMENTAL COMPETENCE

3.1 INTRODUCTION

The metabolic environment that the cumulus oocyte complex (COC) is exposed to during maturation affects the developmental competence of the oocyte (Sutton-McDowall et al. 2010). The composition of the culture medium during in vitro COC maturation (IVM) is well documented to influence subsequent embryo development (van de Sandt et al. 1990; Rose and Bavister 1992; Rose-Hellekant et al. 1998) and the rate of live births following embryo transfer (van de Sandt et al. 1990). Carbohydrates are important components of the medium which can affect the developmental competence of the oocyte (Herrick et al. 2006), and glucose, lactate and pyruvate are all essential for the growth of oocytes (Nandi et al. 2008). Glucose in particular has been shown to affect oocyte developmental competence in many in vitro systems and several species (Hendryx and Wordinger 1979; Downs and Mastropolo 1994; Hashimoto et al. 2000; Khurana and Niemann 2000) as well as in vivo (Moley et al. 1991; Lea et al. 1996). During IVM, the metabolism of glucose is certainly influenced by the concentration in the medium (Downs and Utecht 1999), and insufficient glucose limits the substrate available for nucleic acid synthesis and energy production (Downs et al. 1998) and impairs nuclear maturation (Sutton-McDowall et al. 2005) and embryo development (Rose-Hellekant et al. 1998; Eppig et al. 2000; Ali and Sirard 2002).

Three commonly used measures to assess oocyte developmental competence are the meiotic maturation stage, cumulus expansion index and embryo (cleavage and blastocyst stage) development rates. Meiotic maturation of mammalian oocytes involves progression from prophase I, where oocytes are arrested following completion of oogenesis, the breakdown of the germinal vesicle (GVBD) to metaphase II (MII) of meiosis when the first polar body is extruded (Eppig et al. 1994; Sutton-McDowall et al. 2010)). This occurs in response to the preovulatory gonadotrophin-releasing hormone (GnRH) and luteinising hormone (LH) surges in vivo (Purcell and Moley 2011), see section 1.2), or to mechanical release from the follicle in vitro (Edwards 1965). The pentose phosphate pathway (PPP) is considered to be important in determining meiotic maturation (Downs et al. 1996; Downs et al. 1998), and glucose is known to increase rates of meiotic maturation in vitro (Downs et al. 1996; Downs et al. 1998; Sutton-McDowall et al. 2005; Sato et al. 2007; Funahashi et al. 2008). While meiotic maturation is not necessarily an indicator of the overall level of oocyte developmental competence (Moor and Trounson 1977), it is necessary to allow fertilisation of the oocyte and further development (Iwamatsu and Chang 1972; Niwa and Chang 1975). Alterations in expression of genes involved in meiotic regulation have been found in oocytes from human patients with low fertilisation rates (Wood et al. 2007).

Cumulus expansion during COC maturation is facilitated by the increased production of hyaluronic acid, the extracellular glycosyaminoglycan which supports the matrix as it expands (Eppig 1981; Salustri et al. 1989; Chen et al. 1990). The precursor of hyaluronic acid is uridine diphosphate-N-acetylglucosamine

(UDP-GlcNAc), the end product of the hexosamine biosynthesis pathway (HBP) (see section 1.3.4). In vivo, there is strong evidence for a role for cumulus expansion in follicle rupture and ovulation (Chen et al. 1993; Russell and Robker 2007), with mice with defective cumulus matrix formation sub-fertile or infertile, primarily due to impairment of ovulation (Robker et al. 2000). In vitro, cumulus cell expansion itself is not a direct predictor of oocyte developmental competence (Ali and Sirard 2002; Luciano et al. 2004), however IVM conditions which promote developmental competence (the inclusion of follicle-stimulating hormone, epidermal growth factor and serum in the medium) also generally promote cumulus expansion (Merriman et al. 1998; Mikkelsen et al. 2001). As well as examining meiotic maturation and cumulus expansion, it is important to assess cleavage and blastocyst development rates following fertilisation, as not all oocytes exhibiting complete meiotic maturation and expanded cumulus matrices are competent to support cleavage (Kito and Bavister 1997), or may undergo the first cleavage division but are unable to progress to the blastocyst stage (van de Sandt et al. 1990; Rose and Bavister 1992; Rose-Hellekant et al. 1998; Sutton-McDowall et al. 2006; Schelbach et al. 2010).

The contribution of the HBP during COC maturation has only recently begun to be investigated, but has a significant impact on oocyte developmental competence. Glucosamine supplementation during the IVM phase, used to selectively upregulate the HBP, has no effect on nuclear maturation or cleavage rates in bovine or porcine oocytes (Sutton-McDowall et al. 2006). However, this supplementation severely impairs blastocyst development in cow, pig and mouse as well as decreasing cleavage rates in the mouse (Sutton-McDowall et al. 2006; Kimura et al. 2008; Schelbach et al. 2010). This was accompanied by an increase in detectable β-O-linked N-acetylglucosamine (O-GlcNAc) levels in cow COCs using immunofluorescence (Sutton-McDowall et al. 2006).

The aim of my experiments was firstly to establish a model of glucose dose-response in our spontaneous IVM system for mouse COCs, using meiotic maturation, cumulus expansion and embryo development as markers of oocyte developmental competence. Secondly, the contribution of the HBP to these measures was examined using glucosamine alone. This is the first study to use glucosamine in the absence of glucose during IVM of oocytes.

3.2 METHODS

All protocols are described in Chapter 2. Data are presented as mean value ± standard error of the mean (SEM).

3.2.1 Experimental Design

Experiment 1: dose-response effect of glucose concentration throughout IVM

The effect of increasing doses of glucose on embryo development, cumulus expansion and meiotic maturation was examined using glucose concentrations of 0, 1, 3, 10 or 30 mM glucose or 30 mM sucrose as an osmolarity control in IVM medium. These chosen concentrations represented a range from below physiological levels (~0.46 mM in the mouse follicle (Harris et al. 2005)), to true hyperglycaemic conditions even when accounting for substrate depletion in the media. (30 mM). For cumulus expansion experiments, the average number of COCs scored in total per group was 215 (n = 6 replicates). These data were not normally distributed, and therefore presented as median ± standard error of the median and analysed using Kruskal-Wallis and Mann-Whitney U tests. Three replicates each were performed for meiotic maturation and embryo development, with an average of 25 and 24 COCs matured per treatment per replicate respectively. All of these data were normally distributed and analysed using a one-way ANOVA.

Experiment 2: dose-response effect of glucosamine concentration throughout IVM

Glucosamine concentrations of 0, 0.5, 1, 2.5 or 5 mM in the absence of glucose were used during IVM to examine the contribution of the HBP to various measures of oocyte developmental competence. These concentrations were based on the effective dose of 2.5 mM shown in previous studies (Sutton-McDowall et al. 2006; Schelbach et al. 2010), and extending the range to 0 or 5 mM. A control group containing 0.5 mM glucose in collection and 5.55 mM glucose during maturation was also included, based on commonly used glucose concentrations in defined IVM media Three replicates were performed of each experiment, with an average of 19 and 28 COCs matured per treatment per replicate for meiotic maturation and embryo development respectively. All data were normally distributed and therefore analysed with a one-way ANOVA, except for the degenerate data from each experiment which was analysed using Kruskal-Wallis and Mann-Whitney U tests.

Experiment 3: effect of presence or absence of glucose during the first hour of IVM

During preliminary experiments, it became evident that the glucose concentration in the collection media appeared to be exerting an effect of its own. The total time that COCs were in collection media was calculated and determined to be consistently one hour. From this point, COC collection was timed to ensure a one hour exposure in each experiment, and experiments to measure cumulus expansion, meiotic maturation and embryo development were designed for collection and maturation medium containing either 0 mM or 10 mM glucose. A control group using standard glucose concentrations of 0.5

mM in collection and 5.55 mM in maturation was included, as well as an osmolarity control group using maturation medium with 10 mM sucrose alone. Three replicates were performed of each experiment, with an average of 108 COCs scored per treatment for cumulus expansion data and 28 and 30 COCs matured per treatment per replicate for meiotic maturation and embryo development experiments respectively. Cumulus expansion data were not normally distributed and therefore presented as median values and analysed using Kruskal-Wallis and Mann-Whitney U tests. All meiotic maturation and embryo development data were normally distributed and analysed using one-way ANOVAs.

Experiment 4: effect of glucosamine supplementation during the first hour of IVM

Embryo development was examined after collection in medium containing 0 or 1 mM glucose ± 2.5 mM glucosamine, and maturation in medium containing 0 or 5.55 mM glucose, based on commonly used glucose concentrations in defined IVM media. Four replicates were performed with an average of 24 COCs matured per treatment per replicate. All data were normally distributed and analysed using one-way ANOVAs.

3.3 RESULTS

A model of glucose dose-response in IVM medium was established using concentrations ranging from 0 mM to 30 mM (hyperglycaemic in vivo). Cumulus expansion index (CEI) was dependent on glucose concentration. In the absence of glucose there was almost no expansion (Fig. 3.1), increasing to a CEI of approximately 2.5 with low (1 mM or 3 mM) glucose concentrations and approximately CEI = 3 with higher (10 mM or 30 mM) concentrations. COCs cultured in the presence of 30mM sucrose displayed no expansion and cumulus cells did not plate down (CEI = 0.9 ± 0.02).

The ability of COCs to mature in vitro in the presence of glucose was established (Fig. 3.2). Although there were no significant differences between the levels, the lowest MII rate observed was in the absence of any hexose sugar, suggesting that glucose supports maturation in vitro. Such an effect is well established in the literature (see section 3.4).

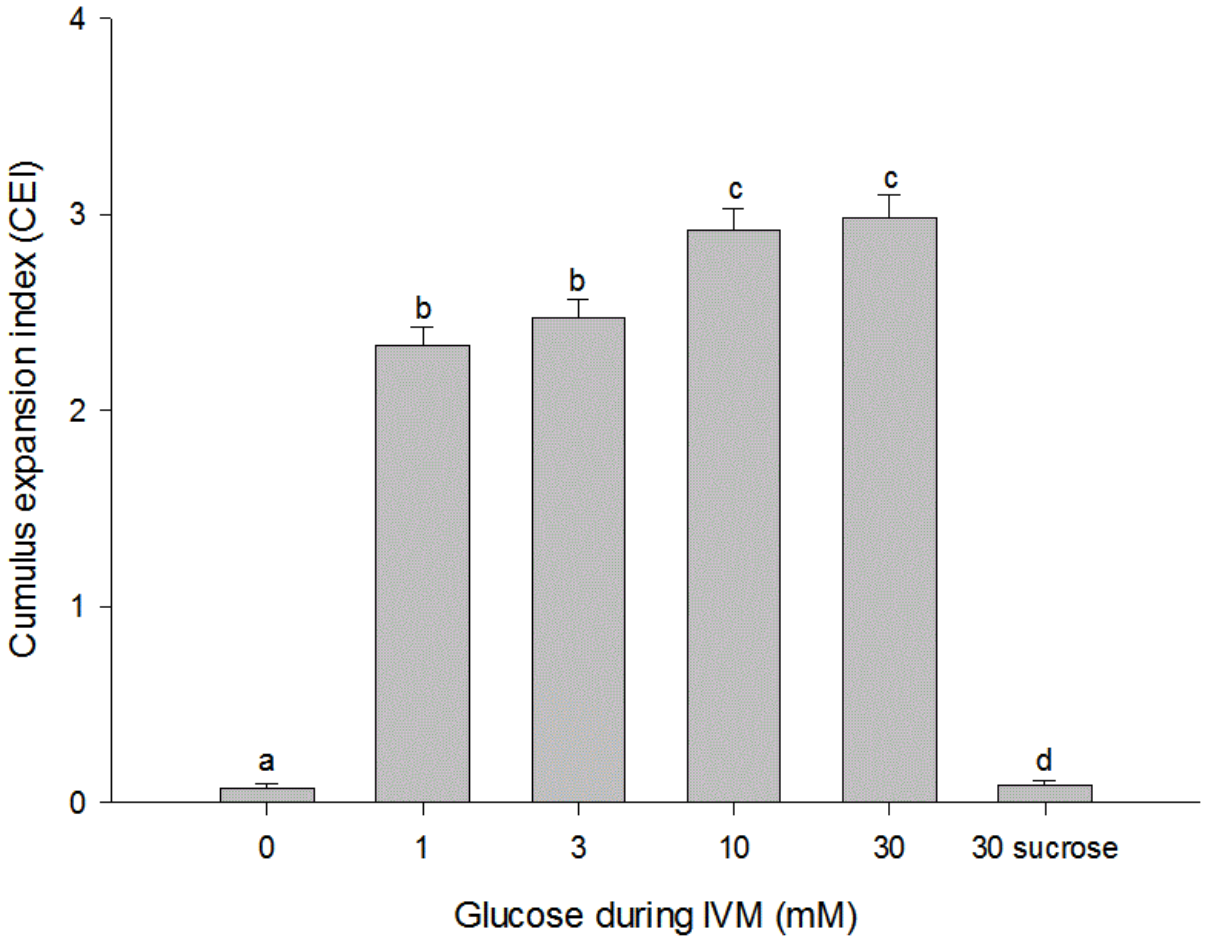

Figure 3.1 Cumulus expansion indices following glucose dose-response in IVM

Cumulus expansion was measured using the Vanderhyden scoring system for cumulus-oocyte complexes (COCs) (Vanderhyden et al. 1990) following in vitro maturation (IVM) in media containing various glucose concentrations. Average number of COCs scored per group was 215, n = 6 replicates. Groups with different superscripts differ significantly ($p < 0.05$). Data are presented as median ± standard error of the median.

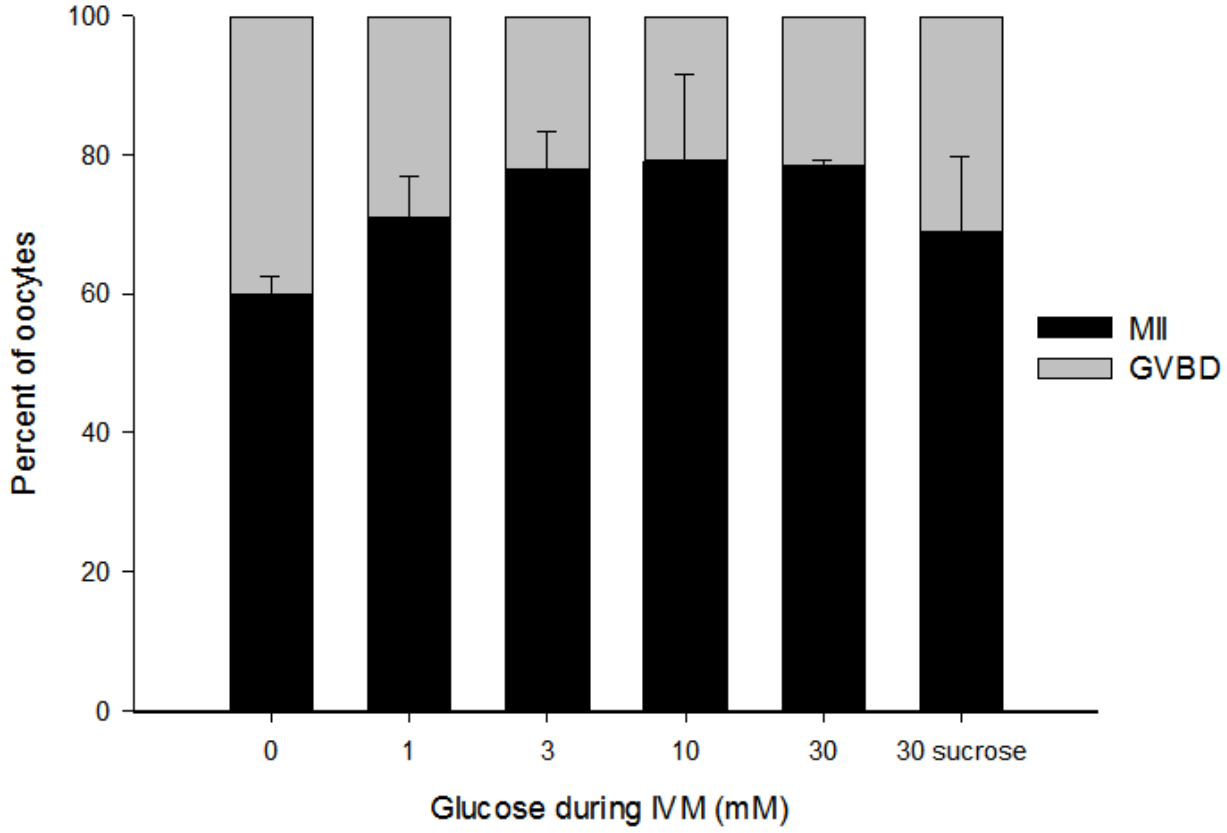

Figure 3.2 Meiotic maturation stage following glucose dose-response in IVM

Meiotic maturation was assessed using 4',6-diamidino-2-phenylindole (DAPI) staining following in vitro maturation (IVM) in media containing various glucose concentrations. No germinal vesicle (GV) stage oocytes were observed. GVBD denotes germinal vesicle breakdown, MII denotes metaphase II. Average number of cumulus-oocyte complexes (COCs) scored per group was 25, n = 3 replicates. Data are presented as mean ± SEM.

The effect of glucose concentration on cleavage rate was minimal, although the 10 mM glucose group achieved the highest cleavage rate of 93.5 ± 3.3 % (significantly higher than the 0 mM, 3 mM and 30 mM groups, $p < 0.05$, Fig. 3.3). However, there was a clear effect of glucose concentration during IVM on blastocyst development. The absence of glucose resulted in low blastocyst development (16.3 ± 7.0 %). However, at low glucose (1 mM) embryo development rate peaked at 67.3 ± 8.6 % (significantly higher than 0 mM, $p < 0.05$) and this decreased linearly as glucose concentration increased. 30 mM glucose produced significantly fewer blastocysts than the 1 mM group (25.4 ± 9.4 %, $p < 0.05$), and interestingly the osmolarity control group using 30 mM sucrose produced an intermediate blastocyst rate, despite confirming that the sucrose used had no contaminating glucose..

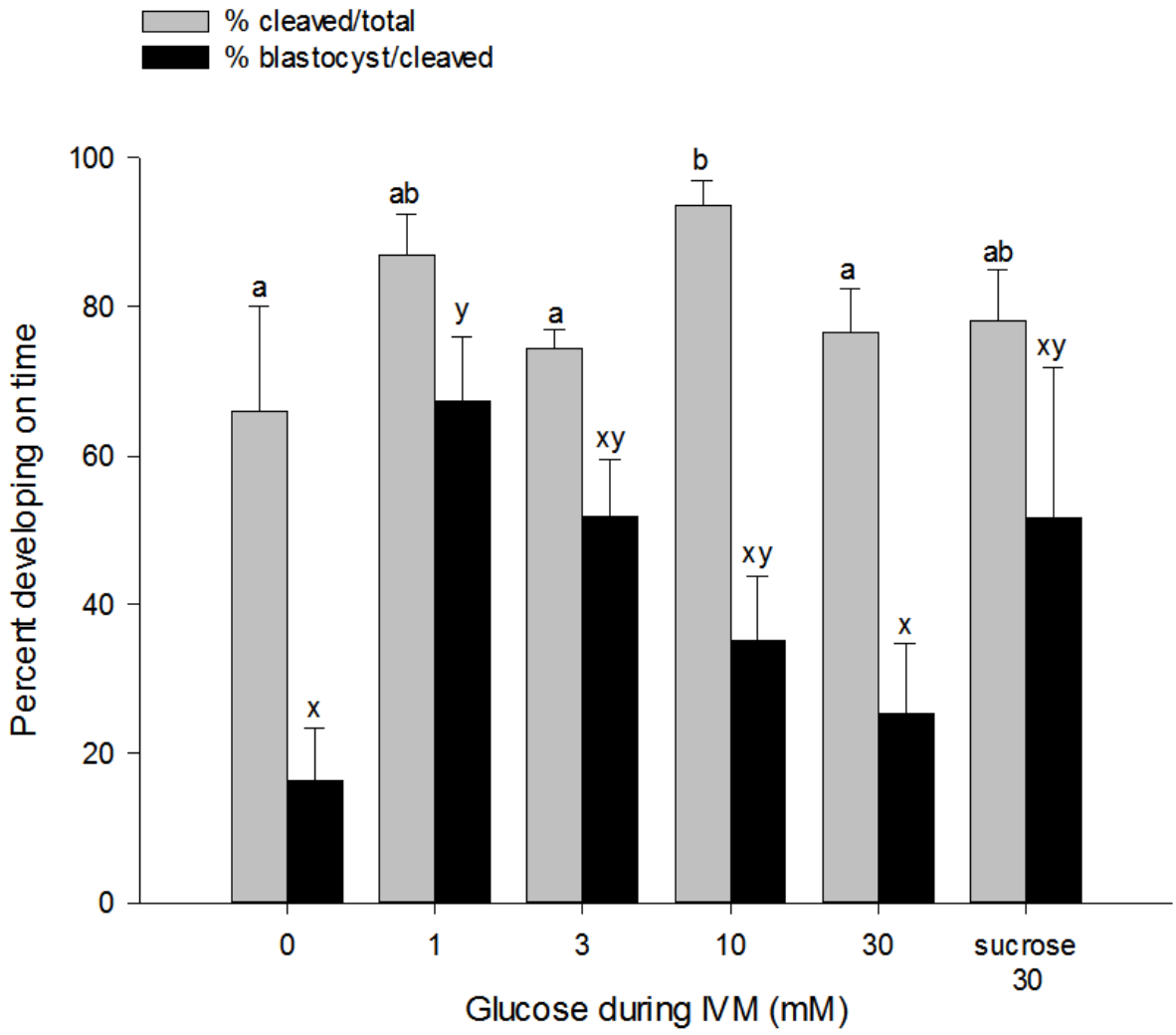

Figure 3.3 Cleavage and blastocyst development following glucose dose-response in IVM
Cleavage rate was assessed on Day 2 and blastocyst rate on Day 5, following in vitro maturation (IVM) in media containing various glucose concentrations. Average number of cumulus-oocyte complexes (COCs) matured per group was 24, n = 3 replicates. Groups with different superscripts differ significantly ($p < 0.05$). Data are presented as mean ± SEM.

Glucosamine dose-response experiments were also performed to examine the contribution of the HBP alone during IVM. Cumulus expansion indices were unable to be scored using the Vanderhyden system (Vanderhyden et al. 1990), because glucosamine supplementation causes dramatically increased expansion of the matrix as well as 'stickiness' of the cumulus cells which are readily dissociated from the complex. This has also been reported in a previous study where mouse COC IVM medium was supplemented with glucosamine (Schelbach et al. 2010).

The effect of the glucosamine dose-response model on meiotic maturation was measured using 4',6-diamidino-2-phenylindole (DAPI) staining (Fig. 3.4). The absence of glucose in IVM media resulted in a significant decrease in MII rate compared to the control group (90.0 ± 10.0 % vs. 61.7 ± 6.4 % respectively, $p < 0.05$). None of the glucosamine concentrations tested were able to increase this rate, and with higher concentrations (2.5 and 5 mM) there was a further significant decrease in the proportion of oocytes reaching MII (18.7 ± 4.5 % and 8.8 ± 8.1 % respectively, $p< 0.05$). The presence of 2.5 or 5 mM glucosamine in IVM media also resulted in a significantly higher number of oocytes which had degenerated by the end of the 17h maturation period (28.9 ± 2.0 % and 30.0 ± 9.0 % respectively, $p < 0.05$ all other groups).

Glucosamine concentration during IVM did not significantly affect cleavage rates (Fig. 3.5), except in the 5 mM group which had a significantly lower rate than the 1 mM group (60.1 ± 20.2 % vs. 93.7 ± 12.6 % respectively, $p < 0.05$). As seen in the meiotic maturation experiments, 5 mM glucosamine resulted in a significant increase in the proportion of oocytes degenerating (49.8 ± 22.1 % vs 0.9 ± 0.9 % in control, $p < 0.05$). Blastocyst development rates showed a similar pattern to MII rates, with a drop between the control group and zero glucose group (although not significant). Glucosamine supplementation did not increase the blastocyst rate and 5 mM glucosamine significantly decreased the rate compared to the control group (8.6 ± 8.6 % 58.2 ± 11.6 % respectively, $p < 0.05$).

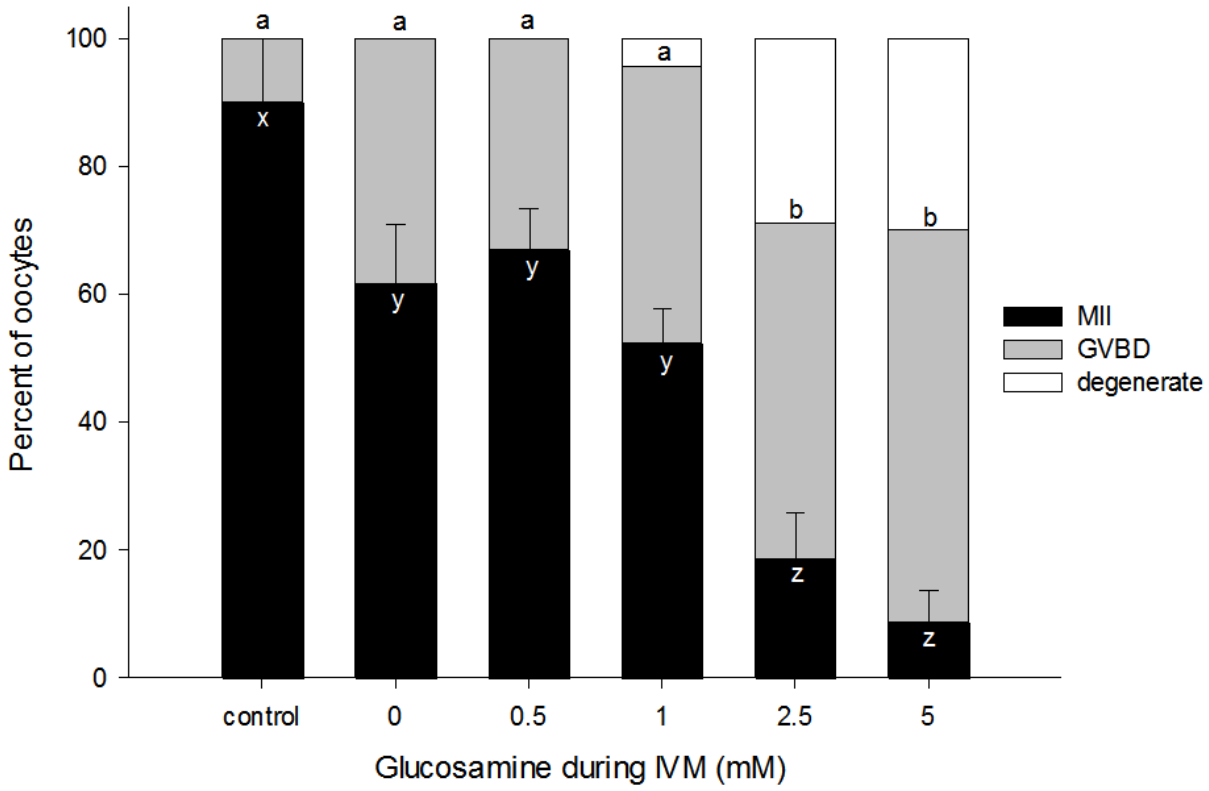

Figure 3.4 Meiotic maturation stage following IVM in various glucosamine concentrations
Meiotic maturation was assessed using 4',6-diamidino-2-phenylindole (DAPI) staining following in vitro maturation (IVM) in media containing various glucosamine concentrations. No germinal vesicle (GV) stage oocytes were observed. GVBD denotes germinal vesicle breakdown, MII denotes metaphase II. Average number of oocytes scored per group per replicate was 19, n = 3 replicates. Groups with different superscripts differ significantly ($p < 0.05$). Data are presented as mean ± SEM. Control = 0.5 and 5.55 mM glucose in collection and maturation respectively.

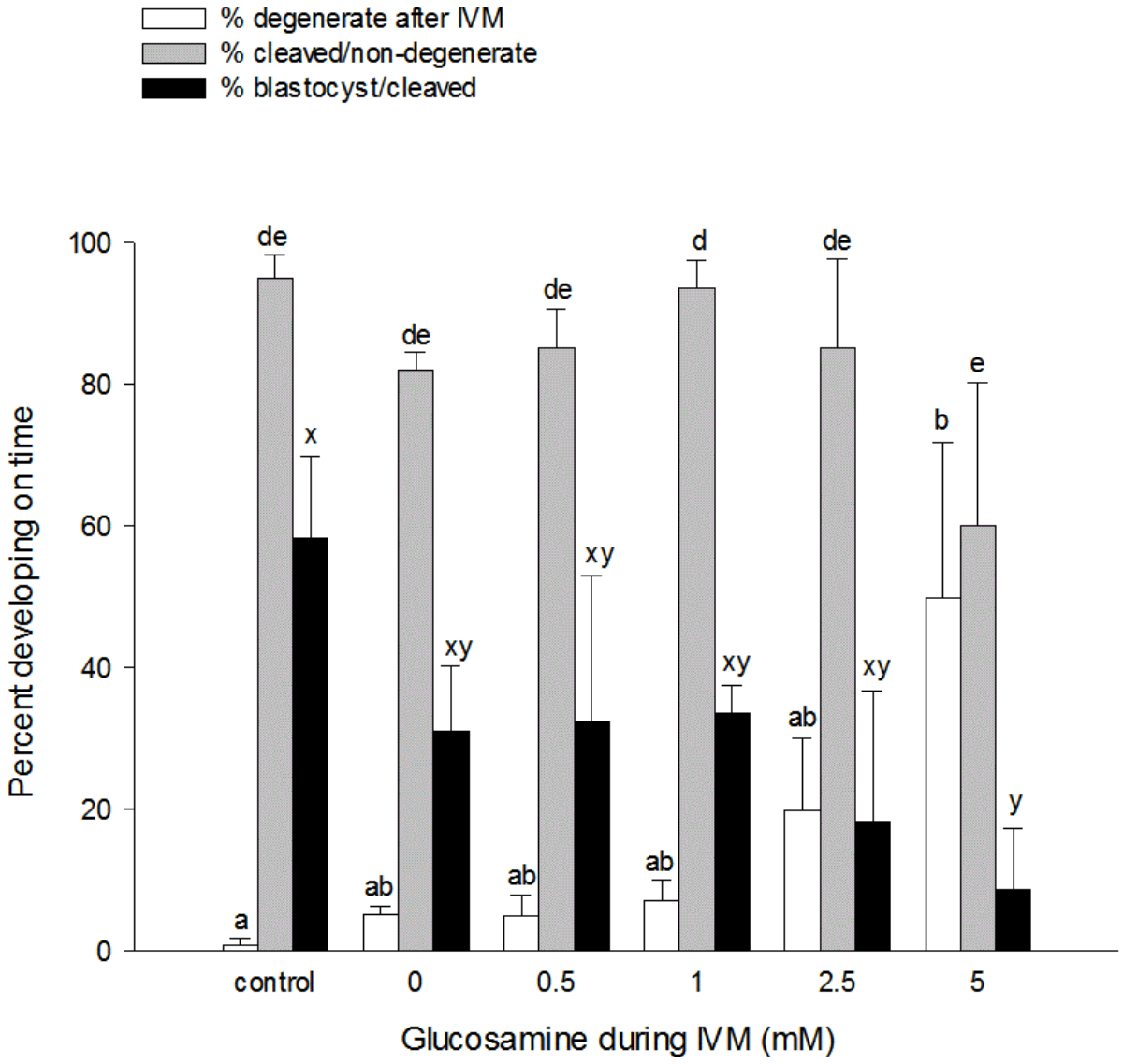

Figure 3.5 Cleavage and blastocyst development following glucosamine dose-response in IVM

Cleavage rate was assessed on Day 2 and blastocyst rate on Day 5, following in vitro maturation (IVM) in media containing various glucosamine concentrations. Oocytes degenerate after fertilization (fert.) were measured as a proportion of cumulus-oocyte complexes (COCs) matured; cleavage rate was measured as cleaved from those not degenerate at the end of fertilisation. Average number of COCs matured per group was 31, n = 3 replicates. Groups with different superscripts differ significantly ($p < 0.05$). Data are presented as mean ± SEM. Control = 0.5 and 5.55 mM glucose in collection and maturation respectively.

During preliminary experiments for the glucose dose-response model, it became evident that using a standard collection medium containing 0.5 mM glucose before separating COCs into treatment groups for maturation negated any effect of varying glucose concentrations during maturation on CEI. Experiments were set up to investigate this effect. Collection medium was used to collect ovaries and aspirate and sort COCs before washing and transfer into maturation medium; time in collection medium was 1 h. The presence of fetal calf serum (FCS) in maturation medium also overrode the effect of different glucose concentrations between collection and maturation media on both CEIs and MII rates (see Appendix 2, 9.1), therefore bovine serum albumin (BSA) was used as the protein source in all experiments described, with fetuin added to all media for embryo development experiments to overcome the zona hardening which occurs in mouse oocytes under serum-free conditions (Schroeder et al. 1990).

The concentration of glucose in both collection and maturation contributed to CEI (Fig. 3.6). If no glucose was present during maturation, CEI was close to zero regardless of glucose in collection medium. Glucose (10 mM) during maturation only (0 mM in collection) increased the CEI significantly (0 mM = 0.14 ± 0.05 vs. 10 mM 2.75 ± 0.13, $p < 0.05$) and groups for which glucose was present in both collection and maturation regardless of concentration had an increased score again (3.66 ± 0.13 and 3.35 ± 0.14, collection/maturation 10mM/10mM and control group respectively, $p < 0.05$ vs. all other groups). 10 mM sucrose during maturation did not influence the CEI.

Meiotic maturation rates were less influenced by changing glucose concentrations between collection and maturation (Fig. 3.7). COCs collected and matured in the absence of glucose displayed MII rates 20 % lower than the control group (59.2 ± 2.0 % vs. 81.0 ± 2.5 %, $p < 0.05$). In all other groups approximately 80 % of oocytes had reached MII by the end of maturation, indicating that glucose in either collection or maturation medium is sufficient for nuclear maturation.

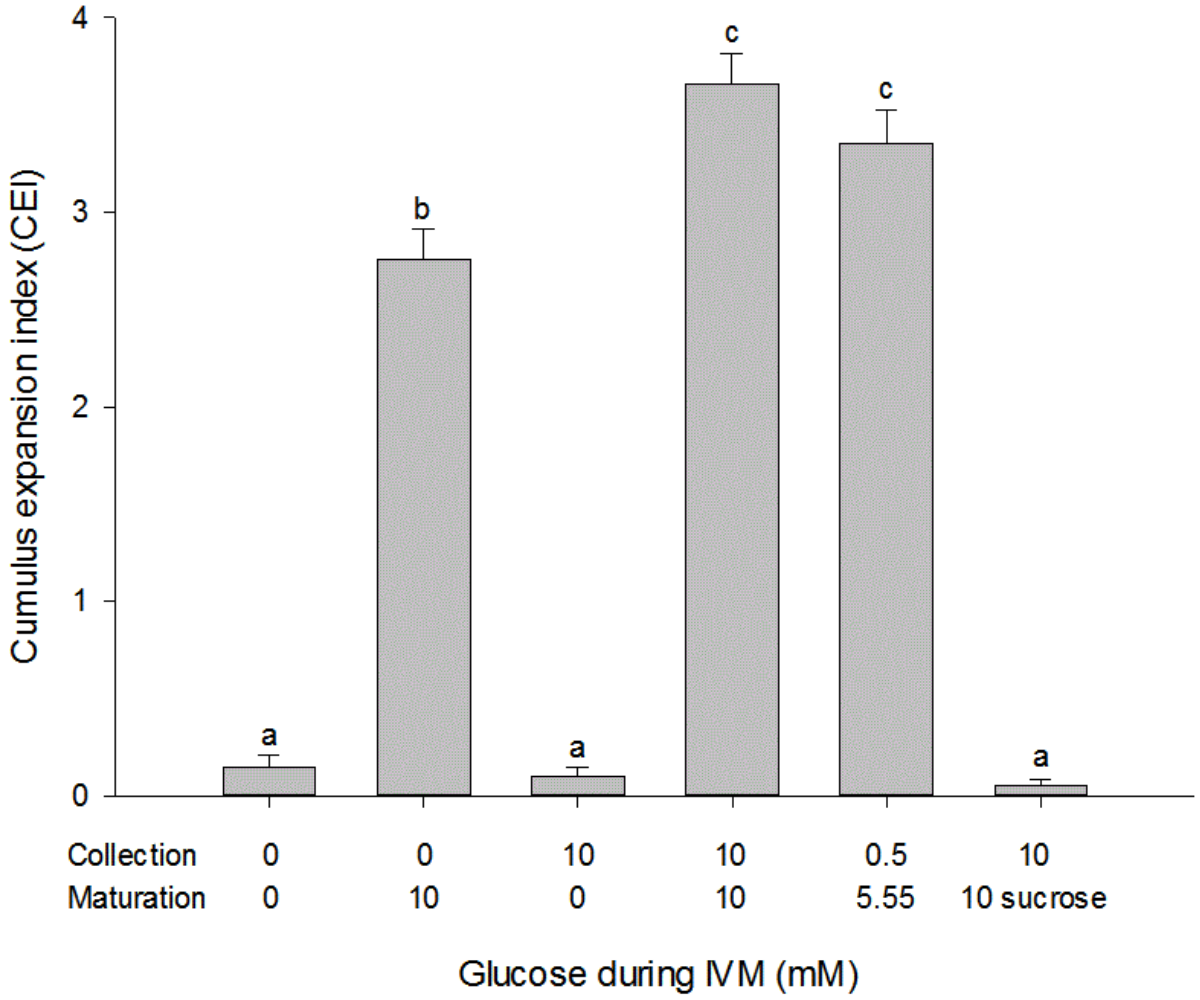

Figure 3.6 Cumulus expansion indices following collection and in vitro maturation in various glucose concentrations

Cumulus expansion was measured using the Vanderhyden scoring system for cumulus-oocyte complexes (COCs) (Vanderhyden et al. 1990) following collection and maturation in media containing various glucose concentrations. Average number of COCs scored per group was 108, n = 3 replicates. Groups with different superscripts differ significantly ($p < 0.05$). Data are presented as median ± standard error of the median.

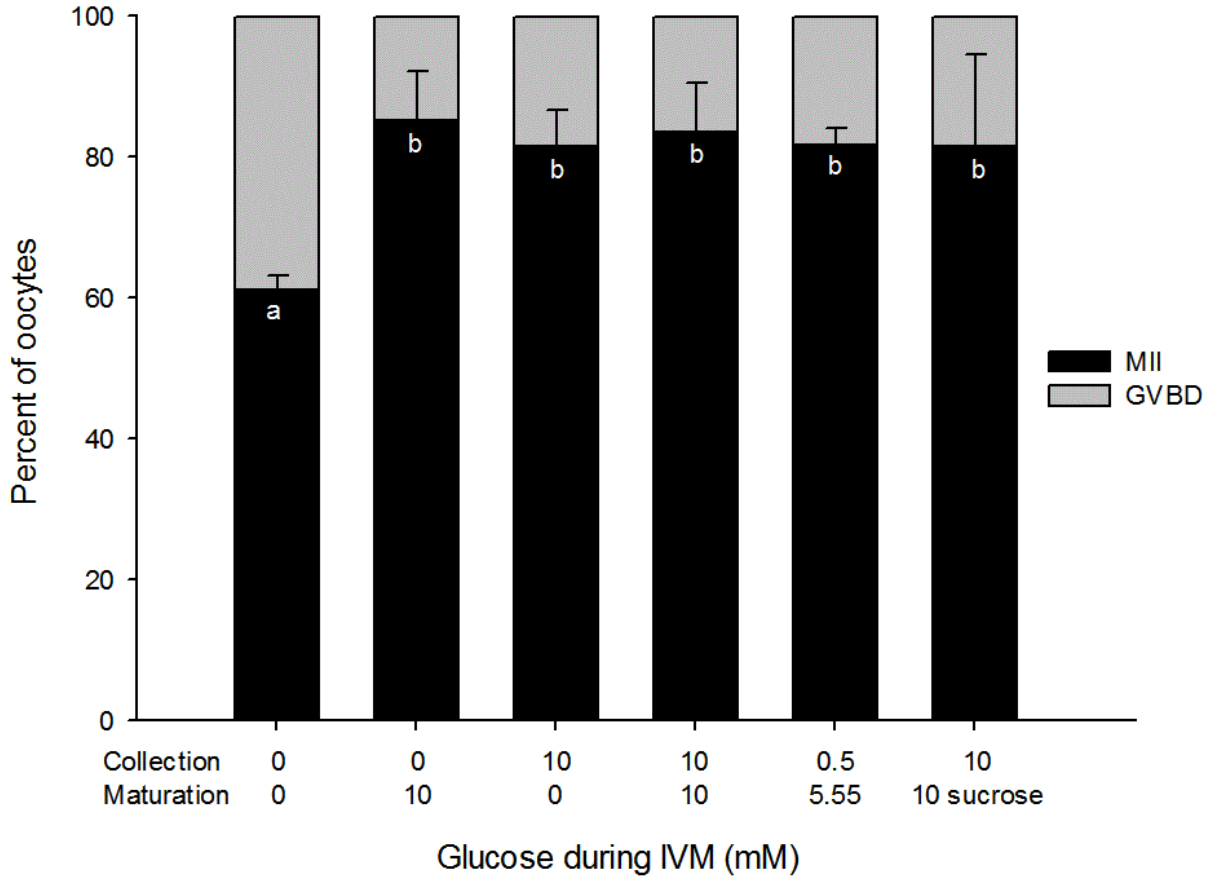

Figure 3.7 Meiotic maturation stage following collection and in vitro maturation in various glucose concentrations

Meiotic maturation was assessed using 4',6-diamidino-2-phenylindole (DAPI) staining following collection and in vitro maturation in media containing various glucose concentrations. No germinal vesicle (GV) stage oocytes were observed. GVBD denotes germinal vesicle breakdown, MII denotes metaphase II. Average number of cumulus-oocyte complexes (COCs) scored per group per replicate was 28, n = 3 replicates. Groups with different superscripts differ significantly ($p < 0.05$). Data are presented as mean ± SEM.

Cleavage rates followed the same pattern as nuclear maturation rates (Fig. 3.8), with 0 mM glucose in collection and maturation media resulting in significantly lower cleavage rates (60.1 ± 3.6 % vs. 81.5 ± 6.3 % in control, $p < 0.05$). No other treatment resulted in cleavage rates different to control. Glucose was necessary during the collection phase to support subsequent embryo development, since the absence of glucose during this first hour decreased blastocyst rates compared to control (17.8 ± 3.7 % and 18.5 ± 1.1 % collection/maturation 0 mM/0 mM and 0 mM/10 mM respectively vs. control 56.9 ± 2.5 %, $p < 0.05$). If 10 mM of glucose was present during collection, this was sufficient to overcome the absence of glucose during maturation, with no difference in blastocyst rates compared to control (46.5 ± 3.3 %), although 10mM glucose in both collection and maturation media significantly lowered blastocyst development (25.8 ± 4.0 %, $p < 0.05$).

Given the significant effects of glucose in the collection phase of IVM, the role of the HBP during this time was investigated by testing the effects of glucosamine supplementation. Glucosamine (2.5 mM) was provided in the absence or presence of glucose at various concentrations. Consistent with the previous experiments, when no glucose was present during collection the blastocyst rate was very low (18.4 ± 6.9 %, Fig. 3.9). However, the addition of 1 mM glucose or 2.5 mM glucosamine was able to significantly increase this rate (45.4 ± 10.8 % and 55.1 ± 16.5 % respectively, $p < 0.05$) when standard (5.55 mM) glucose was subsequently used in maturation medium. The addition of both 1 mM glucose and 2.5 mM glucosamine did not have an additive effect on blastocyst rate, nor did increasing the glucose concentration in collection to 10 mM. An additional experiment was conducted and demonstrated that the total time spent in collection medium also influences on time embryo development in these conditions (see Appendix 2, 9.2).

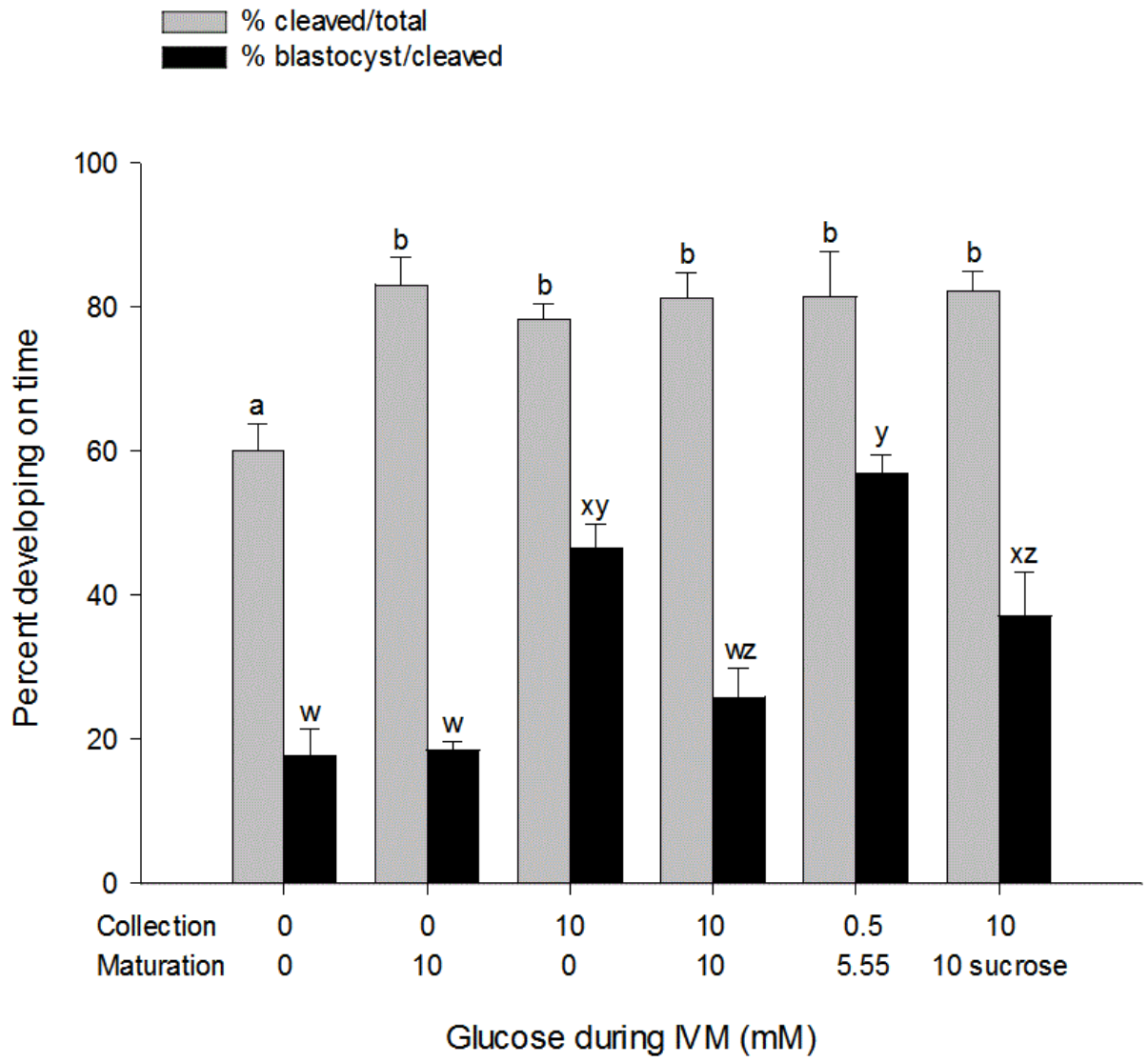

Figure 3.8 Cleavage and blastocyst development following collection and in vitro maturation in various glucose concentrations

Cleavage rate was assessed on Day 2 and blastocyst rate on Day 5, following collection and maturation in media containing various glucose concentrations. Average number of cumulus-oocyte complexes (COCs) scored per group per replicate was 30, n = 3 replicates. Groups with different superscripts differ significantly ($p < 0.05$). Data are presented as mean ± SEM.

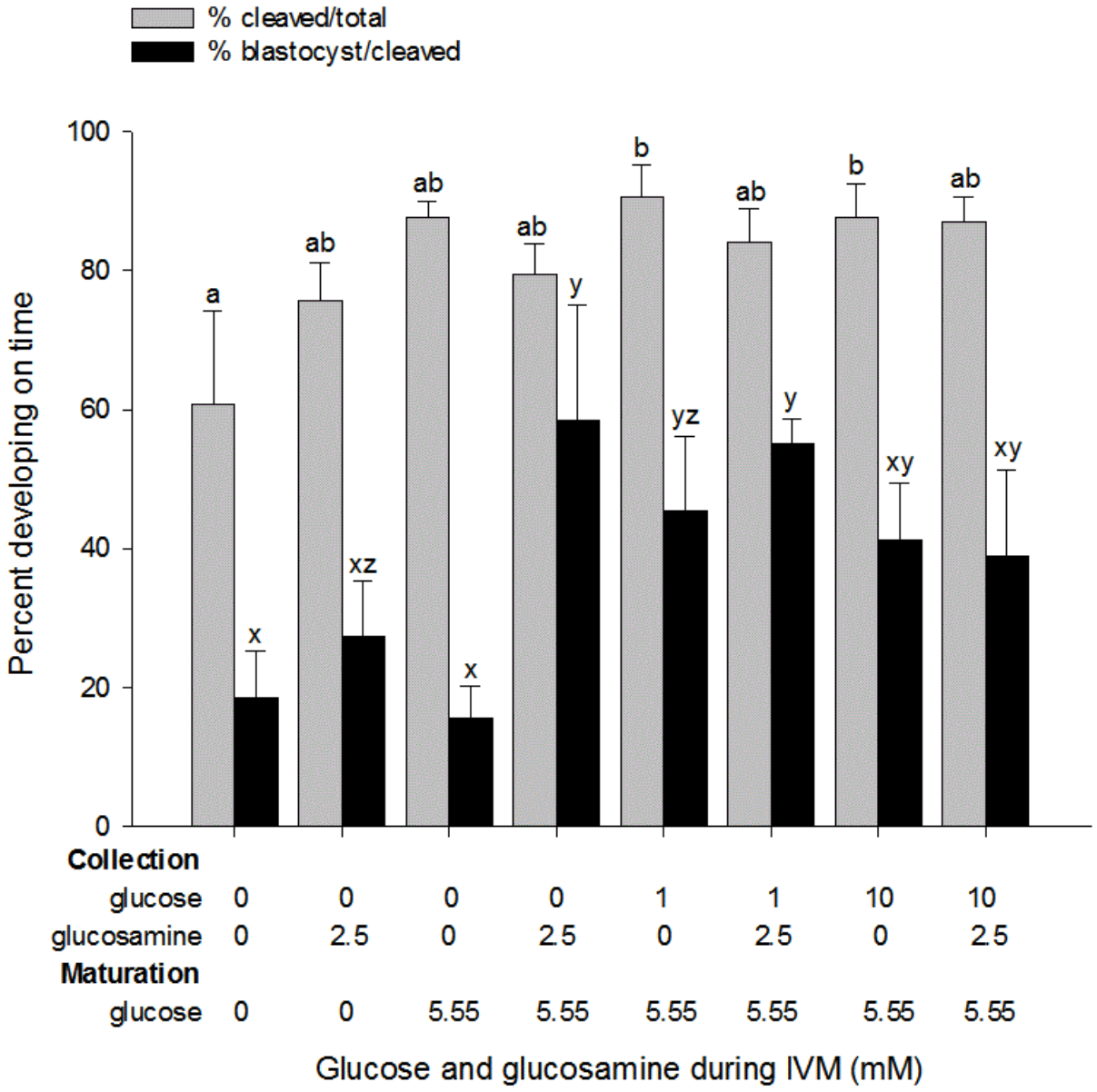

Figure 3.9 Cleavage and blastocyst development following collection and in vitro maturation in various glucose concentrations ± glucosamine supplementation

Cleavage rate was assessed on Day 2 and blastocyst rate on Day 5, following collection and in vitro maturation in media containing various glucose concentrations ± glucosamine supplementation (2.5 mM). Average number of cumulus-oocyte complexes (COCs) scored per group per replicate was 24, n = 4 replicates. Groups with different superscripts differ significantly ($p < 0.05$). Data are presented as mean ± SEM.

3.4 DISCUSSION

Glucose affects every aspect of COC maturation, including meiotic and cytoplasmic maturation and cumulus cell expansion (Sutton-McDowall et al. 2010). One glucose metabolic pathway in particular, the HBP, has recently been investigated in oocytes and embryos and appears to play a critical role in their development (Sutton-McDowall et al. 2006; Kimura et al. 2008; Pantaleon et al. 2010; Schelbach et al. 2010). The HBP is selectively upregulated by glucosamine (Marshall et al. 2005), and hence the aim of this chapter was to establish glucose and glucosamine dose-response models in a mouse IVM system which could be used for subsequent experiments to investigate the role of the HBP in oocyte developmental competence. Using several indicators associated with oocyte developmental competence we have shown that glucose is necessary to support embryo development to the blastocyst stage and improves meiotic maturation rates and cumulus expansion indices. However, good cumulus expansion is not a pre-requisite for subsequent development. Furthermore, we have investigated the role of glucose and glucosamine supplementation during the first hour of spontaneous IVM specifically, during which COCs are collected, and found that the presence of glucose is necessary to support embryonic development. Glucose during this collection period alone was able to support development to the blastocyst stage, and furthermore, glucosamine is able to substitute for glucose during this time provided glucose is present during maturation.

The glucose dose-response results observed in these experiments are consistent with several other publications showing that glucose is necessary during IVM for optimal embryo development, and that concentrations too high or low are detrimental to oocyte developmental competence (Eppig et al. 2000; Hashimoto et al. 2000; Clark et al. 2011). The rate of meiotic maturation to the MII stage in the absence of glucose was significantly lower than in all other groups, a finding which has also been observed in other studies in the mouse (Fagbohun and Downs 1992; Eppig et al. 2000) and cow (Hashimoto et al. 2000). Bovine COCs cultured in medium containing glucose at physiological concentrations or lower display decreased meiotic maturation rates (Sutton-McDowall et al. 2005), and supra-physiological concentrations of glucose leads to optimal oocyte maturation and growth of cumulus and granulosa cells in buffalo and sheep (Nandi et al. 2008). While we did not test a glucose concentration lower than mouse physiological follicular fluid levels (approximately 0.46 mM (Harris et al. 2005)), glucose supplementation at 1 mM or higher (supra-physiological) appeared able to support meiotic maturation at higher rates than occurred in the absence of glucose. This is consistent with previous work showing that concentrations of 1 mM glucose or above during IVM of mouse COCs were all able to induce GVBD at the same rate (Eppig et al. 2000).

Cumulus expansion requires the production of hyaluronic acid from UDP-GlcNAc, the end product of the HBP and this pathway is greatly upregulated in vitro in the maturing COC to support the expansion (see

section 1.3.4). Consequently, in the absence of glucose little cumulus expansion was observed. CEI increased with increasing amounts of glucose.

While glucose concentration during IVM had little overall effect on cleavage rates, there was a clear dose-response effect on blastocyst development. With 0 mM glucose there was very low blastocyst development, with the highest rate observed at the lowest concentration (1 mM glucose), then a steady decrease as the glucose concentration increased towards 30 mM. This pattern supports a similar study performed by Eppig et al. (2000), where 1 mM glucose during IVM was also able to sustain optimal levels of blastocyst development. However, in that study, no significant decrease in blastocyst rates was observed with higher glucose concentrations (5.5 or 27.8 mM). This may be due to a higher COC: volume ratio than was used in the current study, dampening the effects of a high glucose concentration (volume used was not described by Eppig et al. 2000). A significantly lower cleavage rate was also observed in the Eppig study with 0 mM glucose and observed in experiments in this chapter as well. The low CEIs observed in the 0 mM glucose group may be contributing to this, as low CEIs are known to be associated with poor fertilisation rates (Yeo 2010), and it has been demonstrated that incubation with hyaluronic acid induces sperm capacitation and the acrosome reaction in a dose-dependent manner (Gutnisky et al. 2007). Incubation with 6-diazo-5-oxo-L-norleucine (DON; an inhibitor of the HBP and therefore, hyaluronic acid synthesis) negatively impacts fertilisation success (Chen et al. 1993). The decrease in meiotic maturation rates in the absence of glucose, as well as possible defects in cytoplasmic maturation, may be causes of the decrease in fertilisation in this group.

The experiments performed with glucosamine supplementation alone revealed that in the absence of glucose throughout IVM, glucosamine is unable to facilitate meiotic maturation or embryo development. In previous studies in both the cow and mouse, glucosamine supplementation at 2.5 mM in IVM media in the presence of glucose resulted in a significant decrease in blastocyst development rates (Sutton-McDowall et al. 2006; Schelbach et al. 2010). This effect was also seen in the experiments presented in this chapter, although it was not significant until 5 mM glucosamine was used. This may be explained by a decreased flux through the HBP in the 2.5 mM glucosamine group in this study compared to published work (all performed in the presence of glucose), which was increased using 5 mM glucosamine alone to produce the same detrimental effect on blastocyst development. However, contrary to results previously published, the two higher glucosamine concentrations tested (2.5 and 5 mM) also caused a significant decrease in MII rates. A possible explanation for this may be the inhibition of the PPP, a key pathway in meiotic maturation, by the HBP. Glucosamine 6-phosphate, an intermediate in the HBP, is a competitive inhibitor of glucose 6-phosphate dehydrogenase (G6PDH), the first rate-limiting enzyme of the PPP (Kanji et al. 1976). It may be that in the absence of glucose this competitive inhibition is

enhanced compared to glucose-supplemented conditions, downregulating the PPP and subsequently decreasing the rate of meiotic maturation.

An unexpected finding from these experiments was that the presence of glucose during the first hour of IVM (the collection phase) was necessary and sufficient to support embryo development to the blastocyst stage. This effect did not appear to be dependent on meiotic maturation or cleavage rate, as both of these outcomes were scored at the same level to controls when glucose was present at some stage during IVM (not necessarily in collection). Similarly, cumulus expansion did not appear to confer developmental competence as 10 mM glucose during IVM resulted in the highest CEI but a significantly lower blastocyst development rate than the control group. Glucose was necessary in the maturation phase to elicit cumulus expansion, although the presence of glucose during collection also increased the score further. Together these results support the idea that cumulus expansion itself during IVM does not necessarily indicate good developmental competence (Lonergan et al. 1994).

Previously, some correlation between the degree of cumulus expansion and embryo development has been demonstrated: Rose-Hellekant et al. (1998) found that low CEIs (< 1.0) for bovine COCs were associated with very poor developmental competence (2 – 3 % blastocyst development from cleaved). A score of ≥ 1.8 was associated with significantly increased blastocyst rates, however there was no further improvement with higher CEIs. Our results support these findings to a degree, except for the treatment groups with 10 mM glucose during collection followed by 0 mM glucose during maturation. This group exhibited negligible cumulus expansion, but blastocyst rates were not different to the control group. The discrepancy between our results and those previously described may be partly due to the fact that in the bovine study, differing CEIs were produced by varying the substrates provided during IVM, and not just the level of glucose. The groups that displayed low CEIs in the Rose-Hellekant et al. (1998) study were supplemented with pyruvate + glucose or pyruvate + glutamine only. Both glucose and glutamine are required for the first step of the HBP, which would explain why both groups had very low CEIs. Following this, during maturation the absence of glutamine, an essential substrate for the first step of the HBP, in the first group would have severely limited the pool of UDP-GlcNAc available for both hyaluronic acid synthesis and β-O-linked glycosylation (O-GlcNAcylation) during IVM. The absence of glucose in the second group would have affected metabolism through the PPP (the key pathway involved in meiotic competence (Downs et al. 1996)) and glycolysis, and both these insults could have produced the low blastocyst rates accompanying the low CEIs (although endogenous triglyceride stores would have compensated at least in part for the lack of glucose for glycolysis). The authors do not provide a description of how long the COCs were in “wash” medium for before being transferred to maturation drops, but the wash medium used did not contain glucose, so for at least a short period prior

to maturation the COCs were glucose deprived, which I have shown in this study to be detrimental to blastocyst development.

In my study, the low CEIs of the groups described above are due to the absence of glucose during the maturation phase of IVM. However, both groups were provided with glucose during the collection phase. It appears that while this is not enough to support cumulus expansion, the provision of glucose during this short time is sufficient to stimulate meiotic completion and good cleavage and blastocyst development rates.

Meiotic maturation completion rates were the same regardless of which phase of IVM glucose was present in, which suggests that the COCs' PPP may not be sensitive to the timing of glucose provision in the current experimental system. It is possible that the absence of glucose during the first hour is delaying the resumption and completion of meiotic maturation. However, a time course was not undertaken in this study and since the first polar body is extruded around 9 – 12 hours of maturation in mice (Edwards and Gates 1959; Polanski 1997) a delay would most likely not have been noticeable at the 17 h time point. The other pathway which may be affected by the absence of glucose during the first hour of IVM is glycolysis. An absence of glucose would mean an absence of substrate for pyruvate and lactate production by the cumulus cells which can then be supplied to the oocyte, although these were present in the collection medium so the extent of influence of this is unclear. It has been demonstrated that there are three distinct 'bursts' of ATP increase in the mouse oocyte as it matures. These bursts accompanied the redistribution of mitochondria in the oocyte, and oocytes with altered mitochondrial distribution are known to have poor developmental competence (Yu et al. 2010). Further studies to measure ATP production in COCs from the treatment groups described here would begin to address this effect in the current system.

To further investigate the possible mechanism behind this first-hour effect, glucosamine was added as a substitute for glucose during the collection phase. While glucose during collection is able to overcome the absence of glucose during maturation, glucosamine supplementation was sufficient during collection only if glucose is present during maturation. Together, the results from these experiments suggest activation of the HBP is essential during the first hour of IVM, while the timing of activation of the other glucose-requiring pathways during IVM is not as specific. These results are the first to demonstrate the critical importance of the collection phase of IVM, an aspect which many may not be aware of but which may have a significant impact on results.

The results presented in this chapter answer the aims for Hypothesis 1 (see section 1.8.1), that HBP activity regulated by glucose or glucosamine alters the developmental competence of the oocyte in a concentration-dependent manner. The aims for Hypothesis 1 were as follows:

1. Assess the effects of physiological and non-physiological concentrations of glucose during IVM of mouse COCs on meiotic maturation, cumulus expansion, cleavage and blastocyst development rates.

2. Determine the contribution of the HBP to oocyte developmental competence of mouse COCs and analyse the effects on meiotic maturation, cumulus expansion, cleavage and blastocyst development rates.

I have assessed the effects of physiological and non-physiological concentrations of glucose during IVM on meiotic maturation, cumulus expansion, cleavage and blastocyst development rates, as well as examining the contribution of the HBP to oocyte developmental competence using glucosamine. The HBP was shown to contribute significantly to oocyte developmental competence especially during the first hour (collection period) of IVM. The models established here are used in subsequent chapters to further investigate the role of the HBP and mechanisms behind the observed effects of hexosamine substrates on oocyte developmental competence.

CHAPTER 4

β-O-LINKED GLYCOSYLATION IN MOUSE CUMULUS-OOCYTE COMPLEXES DURING IN VITRO MATURATION

4.1 INTRODUCTION

Reversible β-O-linked glycosylation (O-GlcNAcylation) of proteins is gaining recognition as an important regulatory mechanism of cytosolic and nuclear proteins (Van den Steen et al. 1998; Wells et al. 2003; Slawson et al. 2006). Some O-GlcNAc modifications are necessary for cell viability in mammals (Vocadlo et al. 2003; O'Donnell et al. 2004). However, excessive O-GlcNAcylation, resulting from excess flux through the hexosamine biosynthesis pathway (HBP) or inhibition of β-N-acetylglucosaminidase (O-GlcNAcase), is often detrimental to cell survival and function (Marshall et al. 1991; McClain et al. 2002; Arias et al. 2004; Yang et al. 2008). Conversely, too little O-GlcNAcylation can also disrupt normal cellular function, for example a lack of O-GlcNAc modification on the tau protein in the human brain which allows hyper-phosphorylation is implicated in the aetiology of Alzheimer's disease (Liu et al. 2004).

There are two primary antibodies which are most commonly used to detect O-GlcNAcylation: RL2, raised against a nuclear pore complex-lamina fraction isolated from rat liver envelopes, which have multiple O-GlcNAcylation sites (Snow et al. 1987), and CTD110.6, raised against an O-GlcNAcylated RNA polymerase II carboxyterminal peptide (Comer et al. 2001). Although all O-GlcNAc antibodies are raised against a specific epitope and only recognise a subset of O-GlcNAcylated proteins, RL2 is particularly dependent on the structure of the protein to which the O-GlcNAc modification is attached. In contrast, CTD110.6 is not as dependent on epitope structure and recognises the widest range of O-GlcNAcylated proteins (Zachara et al. 2002; Whelan and Hart 2006; Ahrend et al. 2008) and is used throughout the experiments described within this thesis.

The aim of the experiments in this chapter was to examine the overall level of O-GlcNAcylation in mouse cumulus-oocyte complexes (COCs) at various time points throughout in vitro maturation (IVM). One of the main challenges with the study of O-GlcNAcylated proteins is that the stoichiometry of the O-GlcNAc modification is often very low (Ahrend et al. 2008), and confounding this is that both of the most commonly used antibodies to detect O-GlcNAc have relatively low binding affinities (Teo et al. 2010). To increase the opportunities to detect O-GlcNAcylated proteins, immunoprecipitation was performed prior to Western blotting for some experiments (see section 2.7).

4.2 MATERIALS AND METHODS

All protocols are described in Chapter 2. Data are presented as mean value ± standard error of the mean (SEM). Benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP) used in IVM medium was dissolved and stored in dimethyl sulfoxide (DMSO) at -80 °C.

4.2.1 Immunoprecipitation

Immunoprecipitation was performed as described in Chapter 2, using 0.6 µL CTD110.6 (Covance, NJ, USA) per 50 COCs.

4.2.2 Western Blotting

For all Western blots shown in this chapter, the primary antibody used was CTD110.6 to detect O-GlcNAcylation, diluted 1/1000. Secondary antibody was alkaline phosphatase-conjugated anti-mouse IgM (Rockland, PA, USA) diluted 1/2500. Washing and antibody staining protocol was modified from that published by Zachara et al. (Zachara et al. 2002), and is described in Appendix 3 (section 10.1). Blots were also probed for β-actin as a housekeeper, using anti-β-actin−peroxidase diluted 1/2,000,000 and included in the same primary antibody step as anti-HSP90. The specificity of CTD110.6 for O-GlcNAc in COC samples was confirmed using competitive inhibition (see section 10.1.3). Chemiluminescence and colorimetric developing of Westerns were both trialled during preliminary workup for this antibody and it was found that colorimetric blots were clearer for COC samples. Therefore all blots described were developed colorimetrically using Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega, WI, USA). Blots included 5 ng or 0 – 10 ng bovine serum albumin conjugated-N-acetylgluocsamine (BSA-GlcNAc) in a standard loading curve as a positive control and 100 ng bovine serum albumin (BSA, not glycosylated) and 125 ng ovalbumin (N-linked but not β-O-linked glycosylated) as negative controls. ImageJ output (a measure of the pixel density of the selected area of the membrane) was analysed for each time point. The raw data was tested for normality and the appropriate tests applied (see section 2.9).

4.2.3 Experimental Design

Experiment 1: effect of glucosamine and BADGP on O-GlcNAcylation levels in the COC during IVM

Following a 1 h collection period, groups of 50 COCs were collected after 0, 6 h, 12 h or 18 h of IVM and snap frozen. Standard collection and maturation media were used (0.5 mM and 5.55 mM glucose respectively, based on commonly used glucose concentrations in defined IVM media), and glucosamine (2.5 mM) and BADGP (2.5 mM) treatment used to examine the effect of upregulation and inhibition of O-GlcNAcylation. Treatment groups were 1) control, 2) + glucosamine (GlcN) and 3) + GlcN + BADGP. BADGP is an inhibitor of O-linked β-N-acetylglucosaminyltransferase (OGT, the enzyme which attaches O-GlcNAc to proteins). The concentrations of glucosamine and BADGP used were those which are

associated with known embryo development outcomes, demonstrated in previous studies in our laboratory (Schelbach et al. 2010) as well as in Chapter 3. O-GlcNAcylation levels were assessed following Western blotting ± immunoprecipitation, using ImageJ version 1.44p (Rasband 1997 - 2011). Two replicates were performed with immunoprecipitation and three without, using 50 COCs per treatment per replicate. Densitometry data obtained from the non-IP Westerns was normally distributed, and therefore analysed using a one-way ANOVA.

Experiment 2: dose-response effect of glucose concentration throughout IVM

The effect of increasing doses of glucose on O-GlcNAcylation in COCs was examined using glucose concentrations of 0, 1, 3, 10 or 30 mM glucose, and one group with 30 mM glucose and 2.5 mM BADGP. O-GlcNAcylation levels were assessed following Western blotting, using ImageJ version 1.44p (Rasband 1997 - 2011).

Experiment 3: immunohistochemical analysis of O-GlcNAcylation levels at 6h IVM

Treatment groups for Experiment 3 were as described for Experiment 1. COCs were collected at 6h maturation and fixed in 4% paraformaldehyde overnight. Whole COCs were adhered on Cell-Tak (BD Biosciences) –coated slides and immunohistochemically stained using CTD110.6 for O-GlcNAc and propidium iodide (PI) for nuclear staining (see section Appendix 1, section 8.2). Briefly, COCs were permeabilised in 0.25 % Triton X-100, blocked using goat serum (Jackson Immuno) and incubated overnight with 1/250 CTD110.6. On day 2 COCs were washed and incubated for 2 hours at room temperature with 1/250 Alexa Fluor 488 goat anti-mouse IgM (Invitrogen) and 30 minutes with PI, then mounted under a coverslip and examined using confocal microscopy. For competitive inhibition the primary antibody step was performed in the presence of 15.4 mM free GlcNAc. The representative images shown are from one of three replicate, where 7, 11, 9, 3, 5, and 4 images were taken of COCs from the control, glucosamine, BADGP, competitive inhibition, no primary antibody and no secondary antibody groups respectively, over three replicates. All images shown here are from one replicate.

4.3 RESULTS

For the first experiment, samples were immunoprecipitated with CTD110.6 prior to Western blotting to enrich for O-GlcNAcylated proteins. Densitometry analysis was carried out on proteins detected above 75 kDa, to ensure exclusion of any IgM antibody bands and because these proteins were the clearest on developing the Westerns (areas measured are shown in boxes (Fig. 4.1 A). Densitometry was also calculated using protein bands in sections between antibody bands (also shown in boxes, Fig. 4.1 A) and this produced the same pattern of intensity however due to higher variability the significance was lost (see Appendix 3, 10.1.4). Throughout IVM, glucosamine treatment increased O-glycosylation within the COC (Fig. 4.1 B). COCs exposed to both glucosamine and BADGP had similar levels of O-glycosylation to the control group at all time points. The prominent bands observed at 25, 50 and 75 kDa are the fragments of the antibody used to immunoprecipitate the O-GlcNAcylated proteins (confirmed during preliminary workup) and were not included in the intensity analysis.

Proteins from COCs collected at the 6 h time point using the same three treatment groups described above were also separated on an SDS-PAGE gel without being immunoprecipitated to confirm the pattern of O-GlcNAcylation (Fig. 4.2 A). The levels of O-glycosylation in each group mirrored the pattern found in the immunoprecipitated samples, and glucosamine supplementation significantly increased O-GlcNAcylation compared to the control group, while the addition of BADGP significantly decreased this level (Fig. 4.2 B).

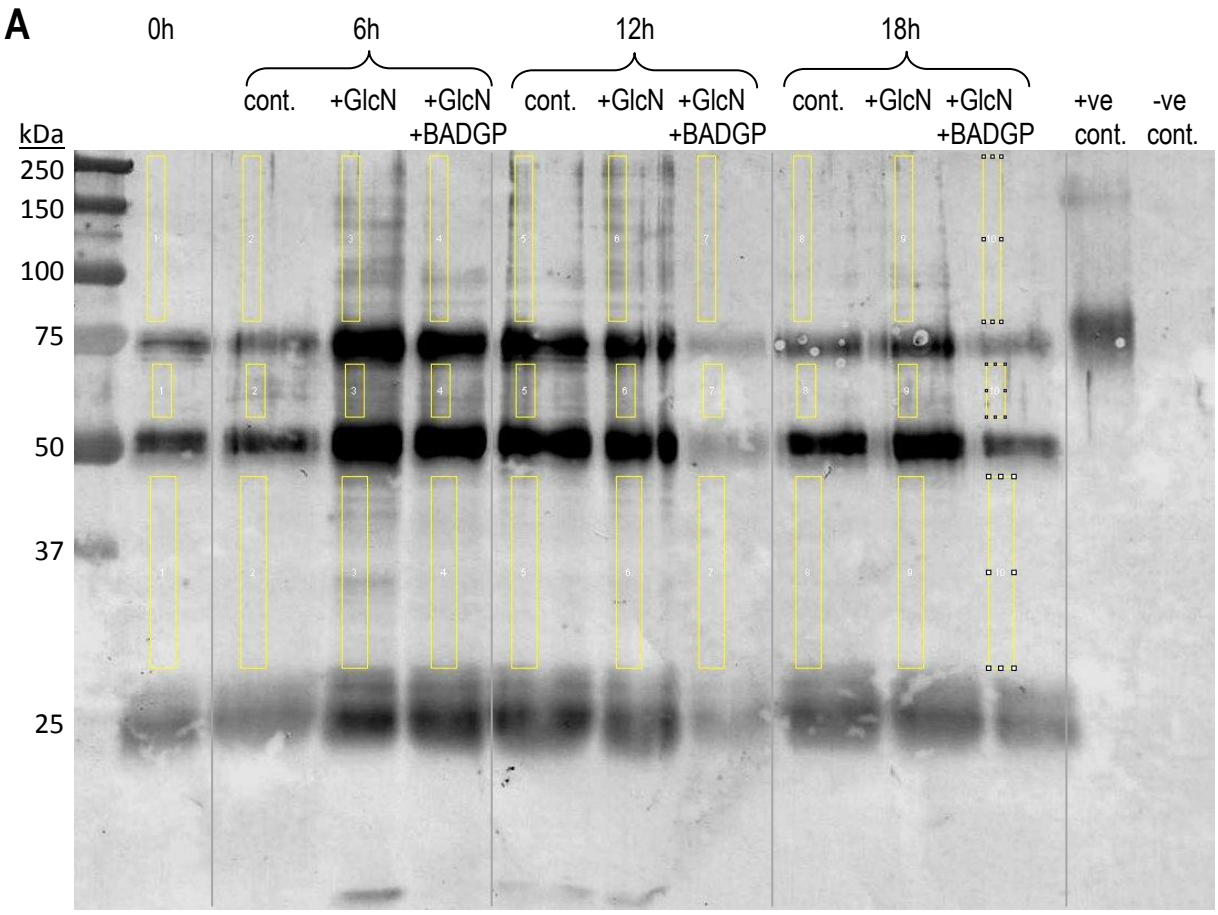

Figure 4.1 A Representative Western blot of O-GlcNAcylation in COCs throughout IVM ± glucosamine and BADGP

Cumulus-oocyte complexes (COCs) (50 per lane) were collected at 0 h, 6 h, 12 h or 18 h in vitro maturation (IVM) in control medium ± 2.5 mM glucosamine (GlcN) and 2.5 mM benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP). Proteins were immunoprecipitated using CTD110.6 for O-GlcNAc, separated using SDS-PAGE, Western blotted and stained using CTD110.6. Positive control (+ve) was BSA-GlcNAc, negative controls (-ve) were BSA and ovalbumin. IgM bands are clearly visible at 25, 50 and 75 kDa, areas used for densitometry analysis are shown in yellow boxes.

B

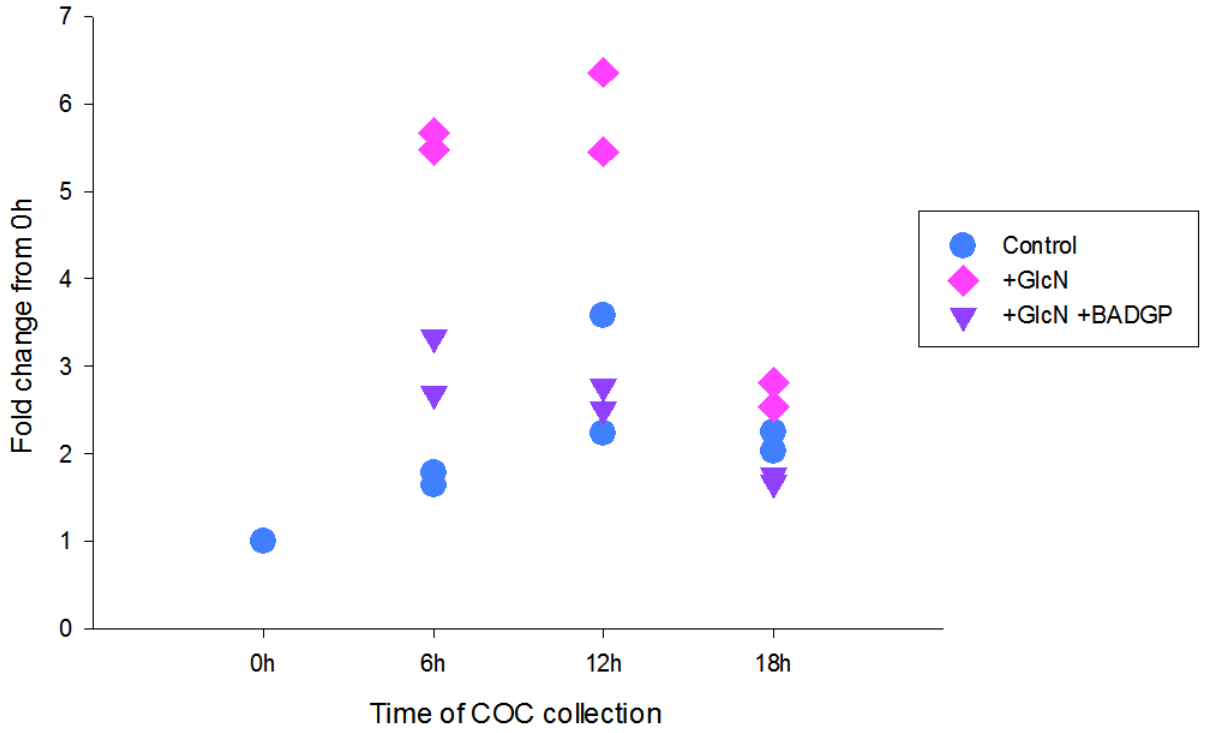

Figure 4.1 B Densitometry analysis of Western blots of O-GlcNAcylation in COCs throughout IVM ± glucosamine and BADGP

Cumulus-oocyte complexes (COCs) were collected at 0 h, 6 h, 12 h or 18 h in vitro maturation (IVM) in control medium ± 2.5 mM glucosamine (GlcN) and 2.5 mM benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP). Proteins were immunoprecipitated using CTD110.6 for O-GlcNAc, separated using SDS-PAGE, Western blotted and stained using CTD110.6. (Fig. 4.1 A). Densitometry analysis of bands from the Western blots was carried out using ImageJ version 1.44p (Rasband 1997 - 2011), and represents protein bands detected above 75 kDa. n = 2 replicates with 50 COCs per treatment group.

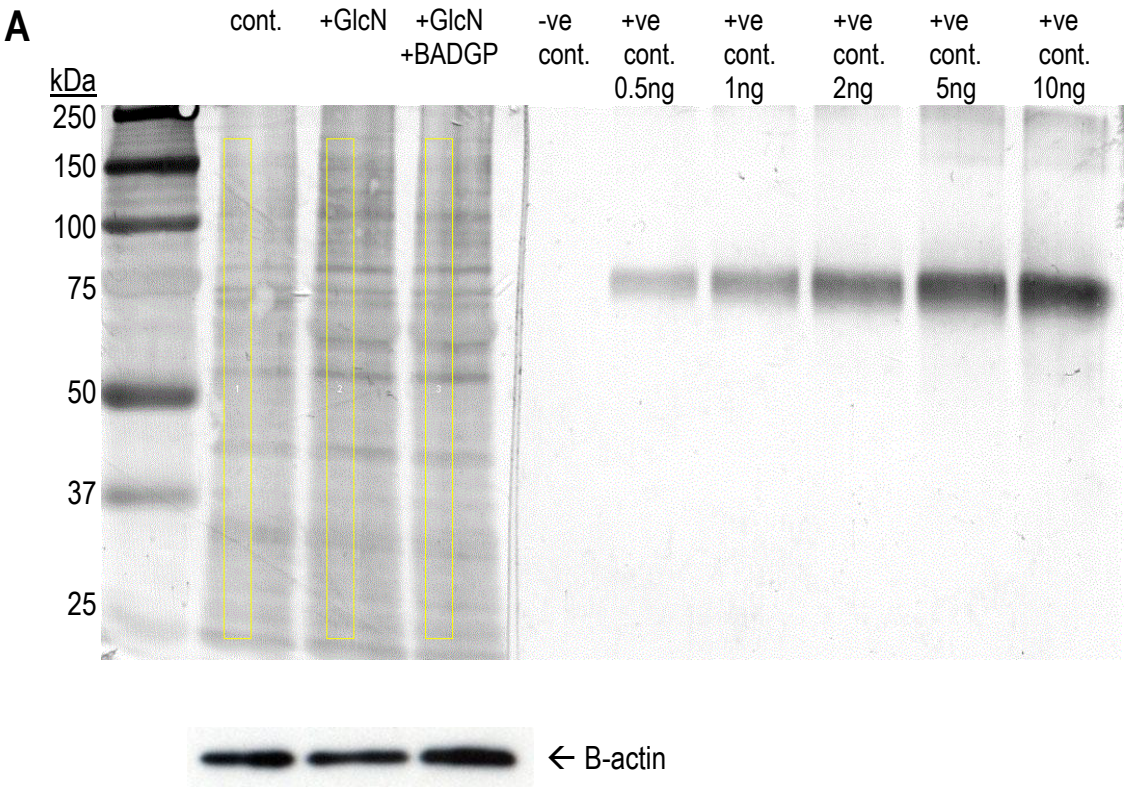

Figure 4.2 A Representative Western blot of O-GlcNAcylation in COCs after 6 h IVM ± glucosamine and BADGP

Cumulus-oocyte complexes (COCs) (50 per lane) were collected at 6 h in vitro maturation (IVM) in control medium ± 2.5 mM glucosamine (GlcN) and 2.5 mM benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP). Proteins were separated using SDS-PAGE, Western blotted and stained using CTD110.6 for O-GlcNAc and β-actin as a loading control. Areas used for densitometry analysis are shown in yellow boxes. The positive control (+ve) was BSA-GlcNAc, used in a standard curve at the stated concentrations, and negative controls (-ve) were BSA and ovalbumin.

B

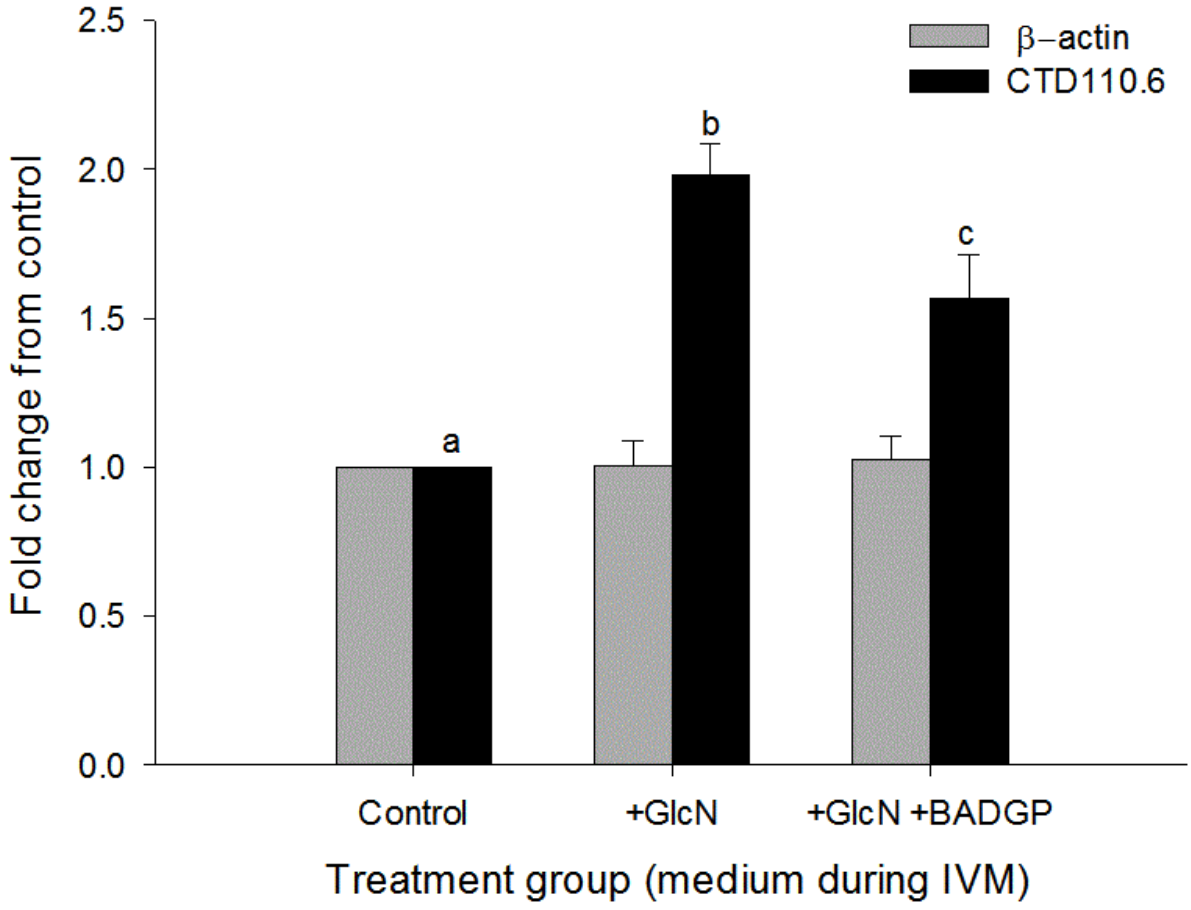

Figure 4.2 B Densitometry analysis of Western blots of O-GlcNAcylation in COCs after 6 h IVM ± glucosamine and BADGP

Cumulus-oocyte complexes (COCs) (50 per lane) were collected at 6 h in vitro maturation (IVM) in control medium ± 2.5 mM glucosamine (GlcN) and 2.5 mM benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP). Proteins were separated using SDS-PAGE, Western blotted and stained using CTD110.6 for O-GlcNAc and β-actin (Fig. 4.2 A). Densitometry analysis of bands from the Western blots was carried out using ImageJ version 1.44p (Rasband 1997 - 2011). Groups with different superscripts differ significantly ($p < 0.05$). n = 3 replicates with 50 COCs per treatment group. Data are presented as mean ± SEM.

Since glucosamine was used as a hyperglycaemic mimetic in the current study, Western blots were also performed on COCs cultured in different glucose concentrations for 6 h, including one group with 30 mM glucose and 2.5 mM BADGP (Fig. 4.3 A). A linear regression for the dose-response effect of glucose (for the 1 mM, 5.55 mM and 30 mM glucose groups) revealed an R-squared value of 0.93 with a significance of < 0.06, and there is a trend of decreased O-GlcNAcylation levels in the 30 mM glucose group with BADGP supplementation compared to no BADGP (Fig. 4.3 B, $P < 0.07$).

For immunohistochemistry, treatment groups were as described for the Western blots (Fig. 4.1 A and 4.2 A), 1) control, 2) + glucosamine (GlcN) and 3) + GlcN + BADGP. For competitive inhibition, the primary antibody step was performed in the presence of 15.4 mM free GlcNAc. Immunohistochemical localisation of O-GlcNAc with CTD110.6 revealed extensive positive staining in the cumulus cells, with intensity increasing in COCs treated with glucosamine and was decreased to levels similar to the control group control when BADGP was present (Fig. 4.4 A). To confirm antibody specificity, COCs from the glucosamine treatment group were stained using CTD110.6 in the presence of 15.4mM free GlcNAc to compete away the antibody, which appears to decrease the staining intensity to control levels.

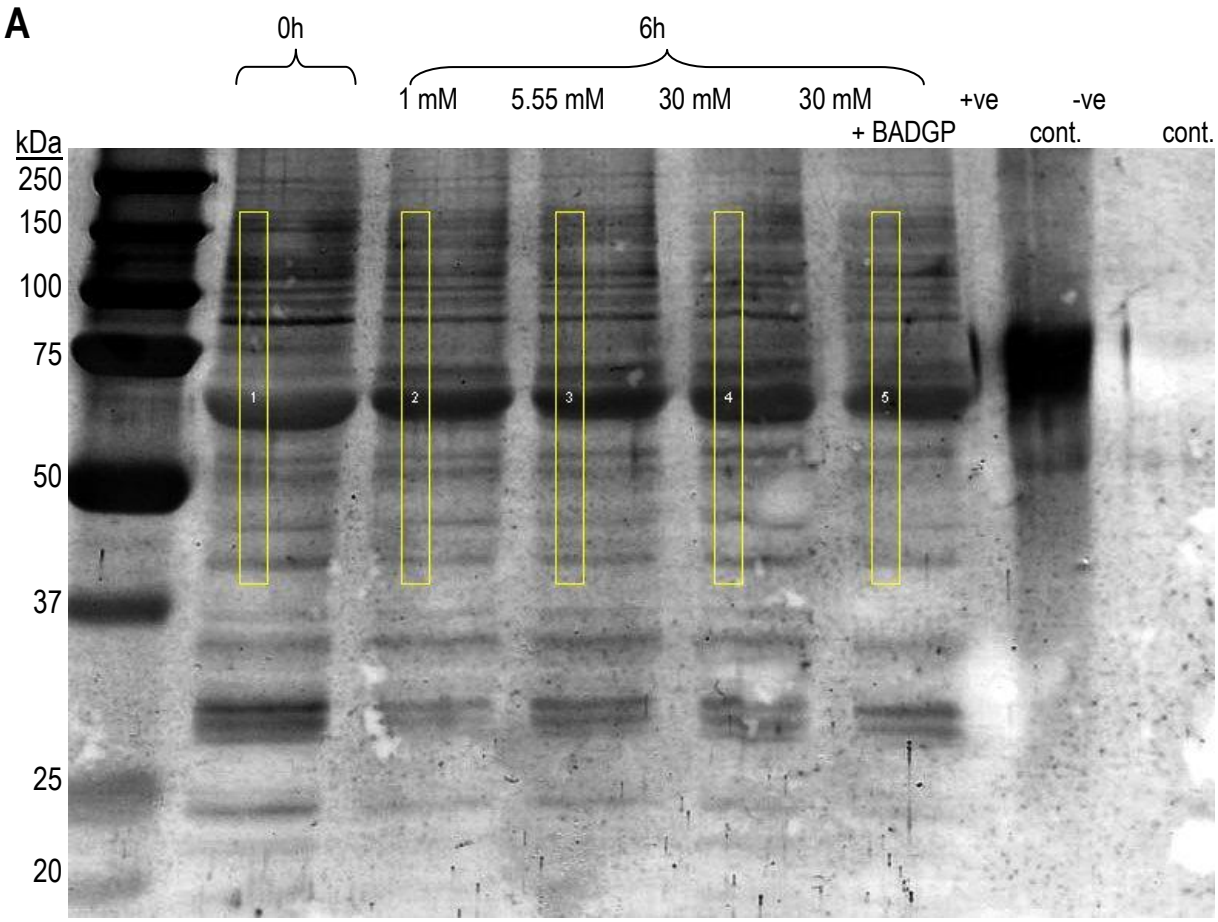

Figure 4.3 A Representative Western blot of O-GlcNAcylation in COCs after 6 h IVM in various glucose concentrations

Cumulus-oocyte complexes (COCs) (50 per lane) were collected at 0 h or 6 h in vitro maturation (IVM) in medium containing various glucose concentrations (listed above in mM). BADGP denotes 2.5 mM benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP) in maturation medium. Proteins were separated using SDS-PAGE, Western blotted and stained using CTD110.6 for O-GlcNAc. Positive control (+ve) was BSA-GlcNAc, negative controls (-ve) were BSA and ovalbumin. Areas used for densitometry analysis are shown in yellow boxes.

B

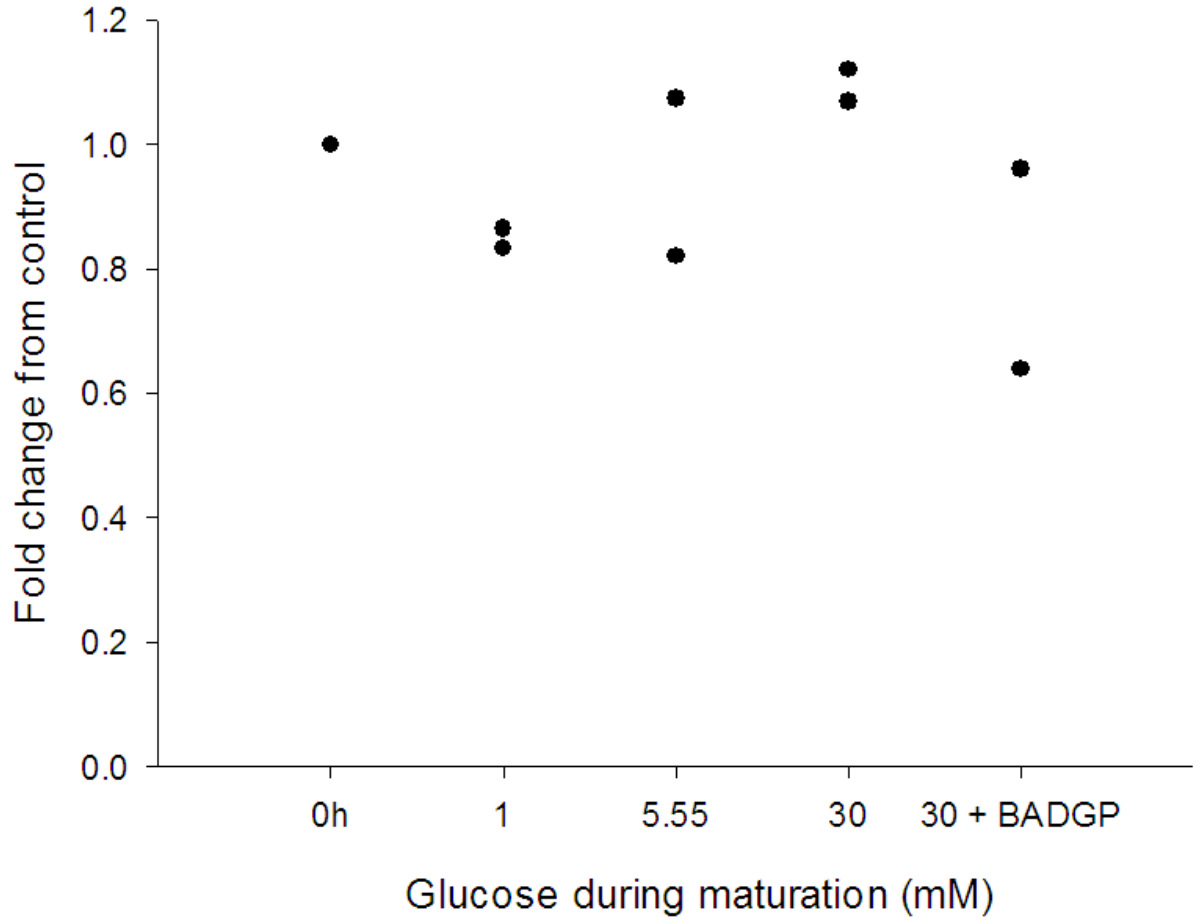

Figure 4.3 B Densitometry analysis of Western blots for O-GlcNAcylation in COCs after 6 h IVM in varying glucose concentrations

Cumulus-oocyte complexes (COCs) were collected at 0 h or 6 h in vitro maturation (IVM) in media containing various glucose concentrations. BADGP denotes 2.5 mM benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP) in maturation medium. Proteins were separated using SDS-PAGE, Western blotted and stained using CTD110.6 for O-GlcNAc. Densitometry analysis of bands from the Western blots was carried out using ImageJ version 1.44p (Rasband 1997 - 2011). n = 2 replicates with 50 COCs per treatment group.

` PI CTD110.6 combined

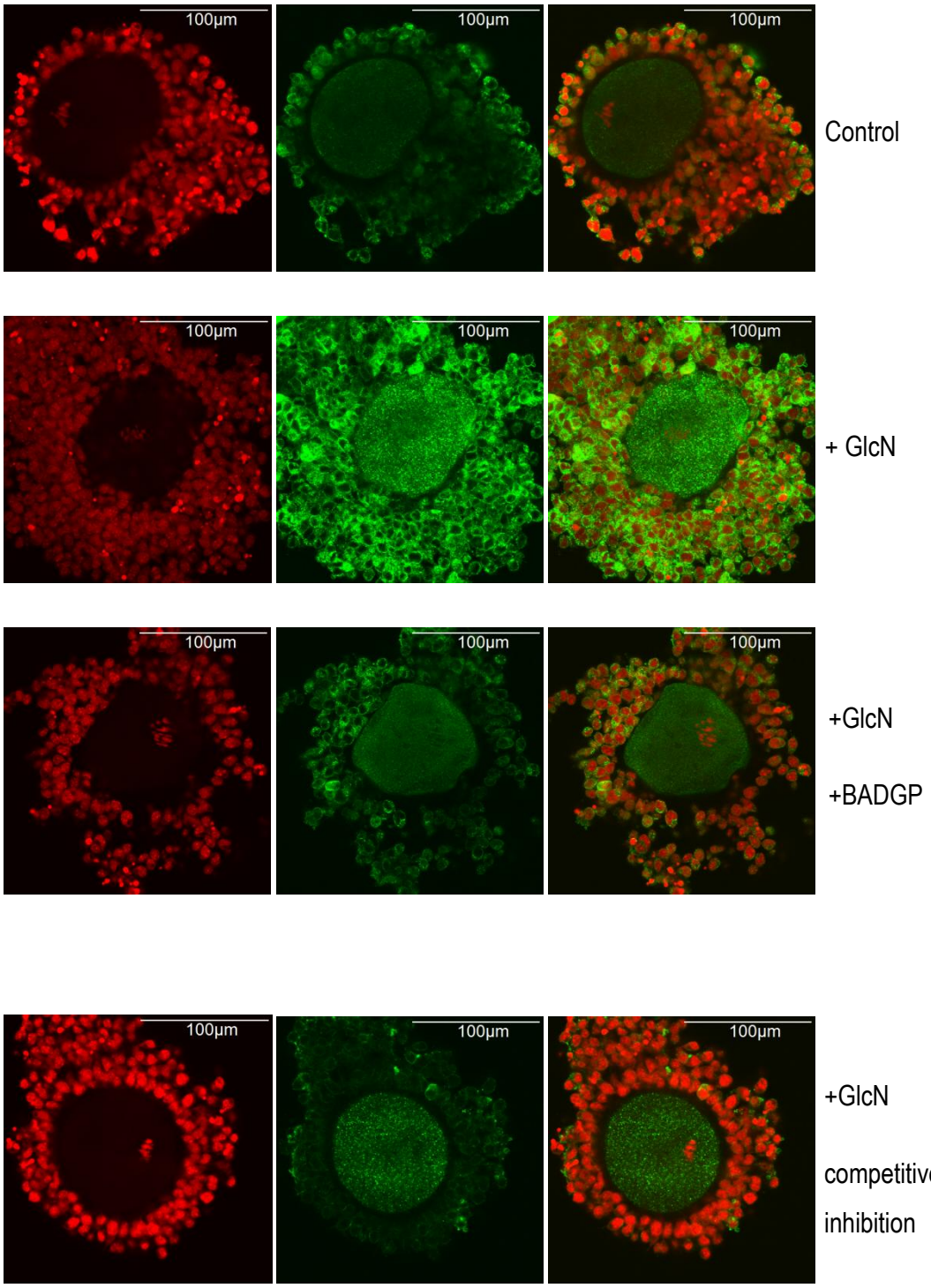

Figure 4.4 A Immunohistochemistry of O-GlcNAcylation in COCs after 6 h IVM ± glucosamine and BADGP

Cumulus-oocyte complexes (COCs) were collected after 6 h in vitro maturation (IVM) in control medium ± glucosamine (GlcN) and benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP). Propidium iodide (PI, red) shows nuclear staining, CTD110.6 (green) shows O-GlcNAc. Competitive inhibition was achieved by adding free GlcNAc during the primary antibody step.

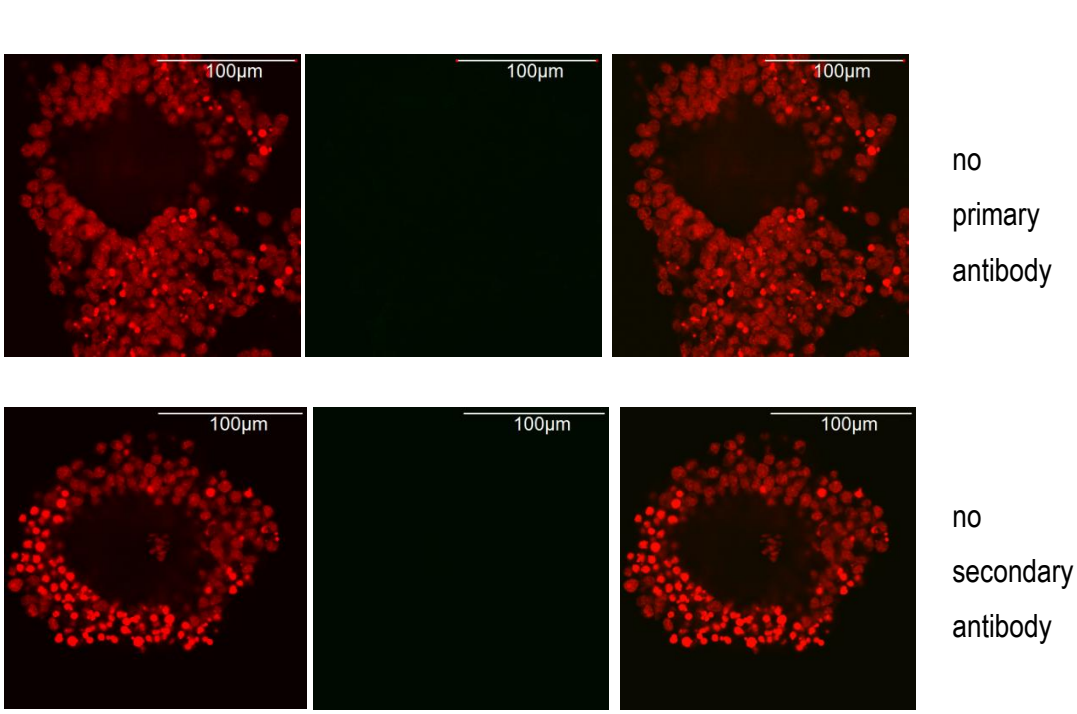

Figure 4.4 B Controls for immunohistochemistry of O-GlcNAcylation in COCs after 6 h IVM ± glucosamine and BADGP

Cumulus-oocyte complexes (COCs) were collected after 6 h in vitro maturation (IVM) in control medium ± glucosamine (GlcN) and benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP). Propidium iodide (PI, red) shows nuclear staining, CTD110.6 (green) shows O-GlcNAc. No antibody (primary or secondary) controls are shown.

4.4 DISCUSSION

It is well documented that glucosamine treatment or O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate (PUGNAc, an inhibitor of O-GlcNAcase) increases O-GlcNAc levels detectable by Western blot in other cell types including the Jurkat (human T lymphocyte) line (Comer et al. 2001) rat skeletal muscle, liver (Arias and Cartee 2005) and neonatal cardiomyocytes (Champattanachai et al. 2008). Increased O-GlcNAc levels have also been observed by Western blot in the pancreas of Goto-Kakizaki rats (a type 2 diabetic model) compared to control rats (Akimoto et al. 2007). The results of the present study support these observations, as both glucosamine and hyperglycaemic conditions increased detectable O-GlcNAcylation levels, shown by Western blot and immunohistochemistry.

It is not surprising that glucosamine was able to increase O-GlcNAcylation levels significantly while glucose only induced a modest increase, as it is well known that glucosamine is much more potent at stimulating UDP-GlcNAc production (approximately 40 times, as measured in adipocytes (Marshall et al. 1991)). It is possible that using more sensitive detection methods for the Western blots, such as the enhanced chemiluminescence (ECL)-advance kit (Amersham, GE Healthcare, Buckinghamshire, UK) would reveal proteins which are not as highly O-GlcNAcylated and provide a more complete picture of overall O-GlcNAcylation levels. However, the aim of this chapter was not to quantify the absolute levels of O-GlcNAcylation but rather to examine the pattern of O-GlcNAcylation between treatment groups. It is also possible to add PUGNAc to inhibit the removal of O-GlcNAc from proteins, hence increasing the detectable levels (Whelan et al. 2010). However, the treatment groups used for this chapter were chosen because they have previously been used in studies examining embryo development, and we aimed to correlate O-GlcNAcylation levels with known developmental outcomes.

Glucosamine supplementation, which led to the highest level of O-GlcNAcylation in these experiments, significantly reduces blastocyst formation following IVM in mouse, pig and cow (Sutton-McDowall et al. 2006; Schelbach et al. 2010). Similarly, co-culture with BADGP and glucosamine during IVM reduced O-GlcNAcylation levels in COCs and rescued blastocyst development. Together these results suggest that increased levels of O-GlcNAcylation in COCs are associated with reduced developmental competence. A similar phenomenon has been seen in pancreatic β-cells. When O-GlcNAcylation was increased in β-cells following glucosamine or streptozotocin treatment, cell viability was decreased. The combination of glucosamine and streptozotocin treatment further reduced the percent of viable cells (Park et al. 2007).

In COCs matured in control medium (5.55 mM glucose), or 1 mM glucose, which was shown in Chapter 3 to support optimal embryo development, there are still detectable levels of O-GlcNAcylation. This result supports previous findings that some O-GlcNAcylation is necessary for normal cellular function (Slawson et al. 2005), and when cow COCs were stained for O-GlcNAcylation in a previous study there

was a positive signal from the control group COCs as well (Sutton-McDowall et al. 2006). The lower levels of detectable O-GlcNAcylation observed at the 18 h time point of the time course experiment in this chapter are consistent with previously published reports showing that as *Xenopus* oocytes mature the levels of total cellular O-GlcNAc decline (Slawson et al. 2002).

In the immunohistochemistry experiment, in all groups proteins within the oocyte itself appear to be positively stained for O-glycosylation. While this staining appears to increase with glucosamine treatment, neither BADGP nor competitive inhibition of CTD110.6 was able to decrease it. This result is in contrast to that previously shown by Sutton-McDowall et al. (Sutton-McDowall et al. 2006), who found staining for O-GlcNAcylation in the cumulus cells but not in the oocyte in cow COCs. Supporting this, a previous study demonstrated that effect of glucosamine on blastocyst development was mediated by the cumulus cells, with denuded mouse oocytes not responding to glucosamine supplementation compared to control medium (Schelbach et al. 2010). A possible explanation for the difference observed in immunohistochemical staining could be the different antibodies used, as the bovine staining was performed using RL2, or it may be a species difference between mouse and bovine COCs (either the amount of O-GlcNAcylation in the oocyte or the efficiency of staining).

When immunoprecipitation was performed on the COC samples prior to Western blotting, fragments of the antibody were also part of the supernatant loaded onto the SDS-PAGE gel. Accordingly, the antibody fragments were detected on the Western blot along with the proteins of interest (at approximately 25 kDa for the light chain, 50 kDa for the heavy chain and 75 kDa for undissociated antibody, confirmed by control experiments during workup and also consultation with Adelaide Proteomics facility staff). Since the same amount of antibody was used for each immunoprecipitation sample, it was expected that the antibody bands on the Western blot would appear at the same staining intensity, however this was not the case. Interestingly, the intensity of the antibody bands appeared to match up with the pattern of intensity of the protein bands. A possible explanation for this may be that antigen (O-GlcNAcylated protein) binding to CTD110.6 induced conformational change of the antibody which then affected the ability of the antibody to be bound by the Protein L beads, and subsequently retained and carried through to the final supernatant mixture. Allosteric conformational change induced by antigen binding has previously been reported, and the change subsequently affected the binding of the antibody to Protein G or A beads (Oda et al. 2003) (Protein L beads were not tested in the study). Furthermore, the uneven antibody bands seem to be an antibody-specific effect, as RL2 was also tested and produced a Western blot with consistent strength bands (Appendix 3, section 10.1.4.2). However, overall, RL2 did not give as clear or consistent results as CTD110.6 and hence CTD110.6 was used for experiments in this chapter. An attempt to overcome the antibody band detection was made using double immunoprecipitation, where one antibody was used during the immunoprecipitation step and the

other during Western blotting. However, this had limited value as the two antibodies pick up different subsets of proteins, so there was always a reduction in the number of proteins appearing on the final Western blot as some were lost at the immunoprecipitation stage and some during Western staining. As well as this problem, there was some cross-reactivity of the secondary antibodies, where the anti-IgG secondary still picked up the IgM bands.

The experiments described in this chapter are the first to examine levels of O-GlcNAcylation in the mouse COC throughout maturation. We have also investigated the effects of glucosamine supplementation, hyperglycaemic glucose concentrations and inhibition of OGT on O-GlcNAcylation, and were able to relate these to previously observed developmental outcomes. The results obtained support many studies in which increased O-GlcNAcylation is detrimental, but also those which show that some O-GlcNAcylation is necessary for normal cellular function.

CHAPTER 5

IDENTIFICATION OF β-O-LINKED GLYCOSYLATED PROTEINS IN MOUSE COCS

5.1 INTRODUCTION

Altered β-O-linked glycosylation (O-GlcNAcylation) of specific proteins is implicated as a possible mechanism in several disease states, such as insulin receptor substrate-1 in diabetes (D'Alessandris et al. 2004), tau in Alzheimer's disease (Arnold et al. 1996) and c-Myc in cancer (Chou et al. 1995). O-GlcNAcylation can change the behaviour and function of the protein in several ways, by altering protein-protein interactions, subcellular localisation, or half-life (Duverger et al. 1996; Roos et al. 1997; Cheng et al. 2000; Juang et al. 2002; Gao et al. 2003). It can also alter the phosphorylation state of proteins which may be a critical regulator of function, as in the case of tau (Liu et al. 2004).

The results in Chapter 4, together with previous studies, show that total O-GlcNAcylation is increased in cumulus-oocyte complexes (COCs) which have poor developmental competence after in vitro maturation (IVM) in medium containing glucosamine (Sutton-McDowall et al. 2006; Kimura et al. 2008; Schelbach et al. 2010). The aim of the experiments in this chapter was to identify specific β-O-linked glycosylated (O-GlcNAcylated) proteins within the mouse COC. In previous studies, glucosamine treatment (Park et al. 2007) or high glucose levels (25 mM) (Walgren et al. 2003) were shown to either upregulate or cause novel O-GlcNAcylation on 96 % and 100 % of proteins identified, respectively. Therefore in this study, I attempted to identify proteins in COCs with either newly appeared or significantly increased O-GlcNAcylation when exposed to the potent hyperglycaemic mimetic glucosamine during IVM.

Where candidate proteins were identified using mass spectrometry, the second aim of this chapter was to examine the possible role of one or more of these proteins in the COC during maturation. Because of the technical difficulties associated with the study of O-GlcNAcylation (Ahrend et al. 2008), there are many O-GlcNAcylated proteins still to be discovered as the post-translation modifications vary between different cell types and under different conditions. Hence, many published studies are focused on identifying as many proteins as possible using 2-dimensional gel electrophoresis and mass spectrometry, and less on further examining the roles that specific proteins may play in cell biology. In this chapter I aimed for a balance between identifying some proteins by mass spectrometry following 1-dimensional gel electrophoresis but also choosing one or more of the most promising candidates for further investigation, to relate the mass spectrometry results back to oocyte developmental competence under hyperglycaemic conditions.

5.2 METHODS

All protocols are described in Chapter 2. Data are presented as mean value ± standard error of the mean (SEM). Benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP) and 17-(Allylamino)-17-demethoxygeldanamycin (17AAG) used in IVM medium were dissolved in dimethyl sulfoxide (DMSO) and aliquots were stored at -80 °C.

5.2.1 Immunoprecipitation

Immunoprecipitation was performed as described in Chapter 2. For Experiment 1, 50 COCs were immunoprecipitated with 0.6 µL CTD110.6 (anti-O-GlcNAcylation antibody; Covance, NJ, USA), and for Experiment 2, 30 COCs per group were immunoprecipitated with 4 µL anti-HSP90 (BD Biosciences, NJ, USA).

5.2.2 Western Blotting

For all Western blots shown in this chapter, the primary antibody used was a pan anti-HSP90 (BD Biosciences, NJ, USA), diluted 1/2500. The secondary antibody was goat polyclonal antibody to mouse IgG (HRP-conjugated, abcam, Cambridge, UK), diluted 1/5000. Washing and antibody staining protocol is described in Appendix 3 (section 10.2). Blots were developed using an Amersham enhanced chemiluminescence (ECL) kit and hyperfilm (GE Healthcare, Buckinghamshire, UK), and included 2 µg HeLa cell lysate (Enzo Life Sciences Inc., NY, USA) as a positive control. Some blots were also probed for β-actin as a housekeeper, using anti-β-actin−peroxidase diluted 1/20,000 and included in the same primary antibody step as anti-HSP90. Quantification of protein bands was performed using ImageJ version 1.44p (Rasband 1997 - 2011). ImageJ output (a measure of the pixel density of the selected area of the membrane) was analysed for each time point. The raw data was tested for normality and the appropriate tests applied (see section 2.9).

5.2.3 Silver Staining and Mass Spectrometry

Silver staining was performed on proteins separated by SDS-PAGE (protocol described in Chapter 2) using the SilverQuest Silver Staining Kit (Invitrogen, CA, USA) which is compatible with mass spectrometry. Gels were provided to the Adelaide Proteomics facility where specific bands were excised, destained and analysed using liquid chromatography-electrospray ionisation ion-trap mass spectrometry, to identify potential O-glycosylation targets (for full report see Appendix 3 section 10.3). Using this method, each time a peptide is matched to a protein, it is given a score based on the degree of homology with the identified protein. The Combined Ion Score is the sum of all these individual matches for each protein; the higher the score, the more likely that the identified protein was present in the sample analysed.

5.2.4 Experimental Design

Experiment 1: identification of specific proteins which are O-GlcNAcylated under hyperglycaemic conditions

Following a 1 h collection period, COCs were cultured for 6 h of IVM in standard medium (0.5 mM glucose in collection and 5.55 mM in maturation, based on commonly used glucose concentrations in defined IVM media) supplemented with 2.5 mM glucosamine, then groups of 50 COCs were collected and immunoprecipitated as described in Chapter 2. The immunoprecipitated proteins were separated on a 4 % - 10 % SDS-PAGE gel (Appendix 1, section 8.4), silver stained, specific bands were excised and analysed using mass spectrometry to identify potential O-GlcNAcylation targets. One replicate was performed of the mass spectrometry identification.

Experiment 2: levels of overall and O-GlcNAcylated HSP90 at 6 h in vitro maturation

Following a 1 h collection period, groups of 15 or 30 COCs (for overall and O-GlcNAcylated HSP90 Western blots respectively) were collected after 6 h of IVM and snap frozen. Standard collection and maturation media (see Appendix 1, 8.1) were used (0.5 mM and 5.55 mM glucose respectively, based on commonly used glucose concentrations in defined IVM media), ± 2.5 mM glucosamine ± 2.5 mM BADGP in maturation to examine the effect of upregulation and inhibition of O-GlcNAcylation. Treatment groups were 1) control, 2) + glucosamine (GlcN) and 3) + GlcN + BADGP. BADGP is an inhibitor of O-linked β-N-acetylglucosaminyltransferase (OGT, the enzyme which attaches O-GlcNAc to proteins). Overall HSP90 levels were assessed following Western blotting, and O-GlcNAcylated HSP90 levels assessed by immunoprecipitating samples with CTD110.6 to capture O-GlcNAcylated proteins prior to Western blotting. Densitometry analysis of bands for all Westerns was performed using ImageJ version 1.44p (Rasband 1997 - 2011). Two replicates were performed after immunoprecipitation and three without, using 30 and 15 COCs per treatment per replicate respectively. For non-IP Westerns, data was normally distributed and therefore analysed using a one-way ANOVA (see section 2.9).

Experiment 3: effect of inhibition of HSP90 during in vitro maturation of COCs

Embryo development was examined after collection and maturation in control media ± glucosamine ± 17AAG (a specific inhibitor of HSP90 activity). Control and glucosamine treatment groups were as described for Experiment 2. 17AAG was added to maturation medium at a concentration of 0.1 μM. Five replicates were performed with an average of 28 COCs matured per treatment per replicate. Data were normally distributed and therefore analysed using a one-way ANOVA.

5.3 RESULTS

Mass spectrometry analysis of proteins from COCs collected at 6 h IVM + glucosamine revealed 15 potential targets of O-GlcNAcylation (Table 5.1). Eleven of these 15 proteins have been previously described in the literature as targets of O-GlcNAcylation, including heat-shock protein 90 (HSP90), the α form of which had the highest Combined Ion Score of any protein identified in this study (for full report see Appendix 3, section 10.3). As 11/15 proteins had been already described as O-GlcNAcylated, this partially verified the selectivity of the antibody immunoprecipitation techniques. One class of proteins which was well represented was structural proteins, with fibronectin, actin and tubulin. Several others identified are involved in protein folding, such as protein disulfide isomerase and protein disulfide isomerase A3, and both isoforms of HSP90 (α and β).

HSP90 is a molecular chaperone which assists in protein folding by stabilizing unfolded proteins and preventing their aggregation, and is upregulated several fold in cells undergoing stress (Zhao et al. 2001; Picard 2002). It is one of the most abundant cytosolic proteins, accounting for approximately 1 % of total protein in unstressed cells, and has been described as one of the 'stickiest' proteins in the cytosol, acting as a molecular glue in the cell and overseeing the folding of a wide range of proteins (Lai et al. 1984; Csermely et al. 1998). HSP90 is vital for early development; fetal mice lacking HSP90β die mid-gestation because of defective placental development (Voss et al. 2000). Because of its important role as a protein chaperone and the wide range of proteins regulated by HSP90 activity, and the high Combined IonScore of both HSP90 isoforms determined by the mass spectrometry, I decided to focus on this protein for further investigation. The anti-HSP90 antibody used for Western blots in this chapter detects both isoforms of HSP90, which share 86 % homology in mice (Moore et al. 1989) and are likely the result of a gene duplication as they are structurally and functionally very similar (Picard 2002).

Name	Combined Ion Score	Previously identified as O-GlcNAcylated (Reference)
Heat shock protein HSP 90-α	633	Yes (1, 4, 7)
Protein disulfide-isomerase A3	631	No
Heat shock protein HSP 90-β	450	Yes (1, 4)
Fibronectin	343	No
Actin, cytoplasmic 1 Actin, cytoplasmic 2	332	Yes (4, 5, 6, 7)
Elongation factor 1-α 1 Elongation factor 1-α 2	115	Yes (1, 4, 6, 7)
Glucose-6-phosphate 1-dehydrogenase X	114	Yes (6)
Zona pellucida sperm-binding protein 3	110	No
Zona pellucida sperm-binding protein 2	101	No
Tubulin α-1A chain Tubulin α-1B chain Tubulin α-1C chain	89	Yes (2, 4, 6, 7)
Protein disulfide-isomerase	83	Yes (3), precursor protein (5)

Table 5.1 β-O-linked glycosylated proteins identified in glucosamine-treated COCs

Cumulus-oocyte complexes (COCs) were collected following 6 h in vitro maturation with glucosamine supplementation, proteins immunoprecipitated using CTD110.6 for O-GlcNAc and identified by mass spectrometry. References: 1: (Wells et al. 2002). 2: (Walgren et al. 2003). 3: (Sprung et al. 2005). 4: (Nandi et al. 2006). 5: (Park et al. 2007). 6: (Gurcel et al. 2008). 7: (Teo et al. 2010). Combined Ion Score is the sum of all individual peptide scores matched to the protein.

Firstly, the level of O-GlcNAcylation of HSP90 was tested across 3 groups: control, glucosamine (GlcN) and GlcN + BADGP. Following 6 h of IVM, COCs were collected in groups of 30, snap frozen and used for immunoprecipitation with CTD110.6 to capture O-GlcNAcylated proteins. Immunoprecipitated samples were run on an SDS-PAGE gel and Western blotted using the anti-HSP90 antibody for detection. Densitometry analysis of bands on the Western blot revealed an approximate 40-fold increase in O-GlcNAcylated HSP90 in the glucosamine treated COCs compared to the control group (Fig. 5.1) or GlcN + BADGP treatment.

Next, total HSP90 levels were determined by using COC samples collected at 6 h IVM from the same treatment groups (control, glucosamine (GlcN) and GlcN + BADGP) and separating the proteins on an SDS-PAGE gel with no immunoprecipitation. This was followed by detection with the pan anti-HSP90, using anti-β-actin as a loading control (Fig. 5.2 A). Overall levels of HSP90 were not different between treatment groups (Fig. 5.2 B).

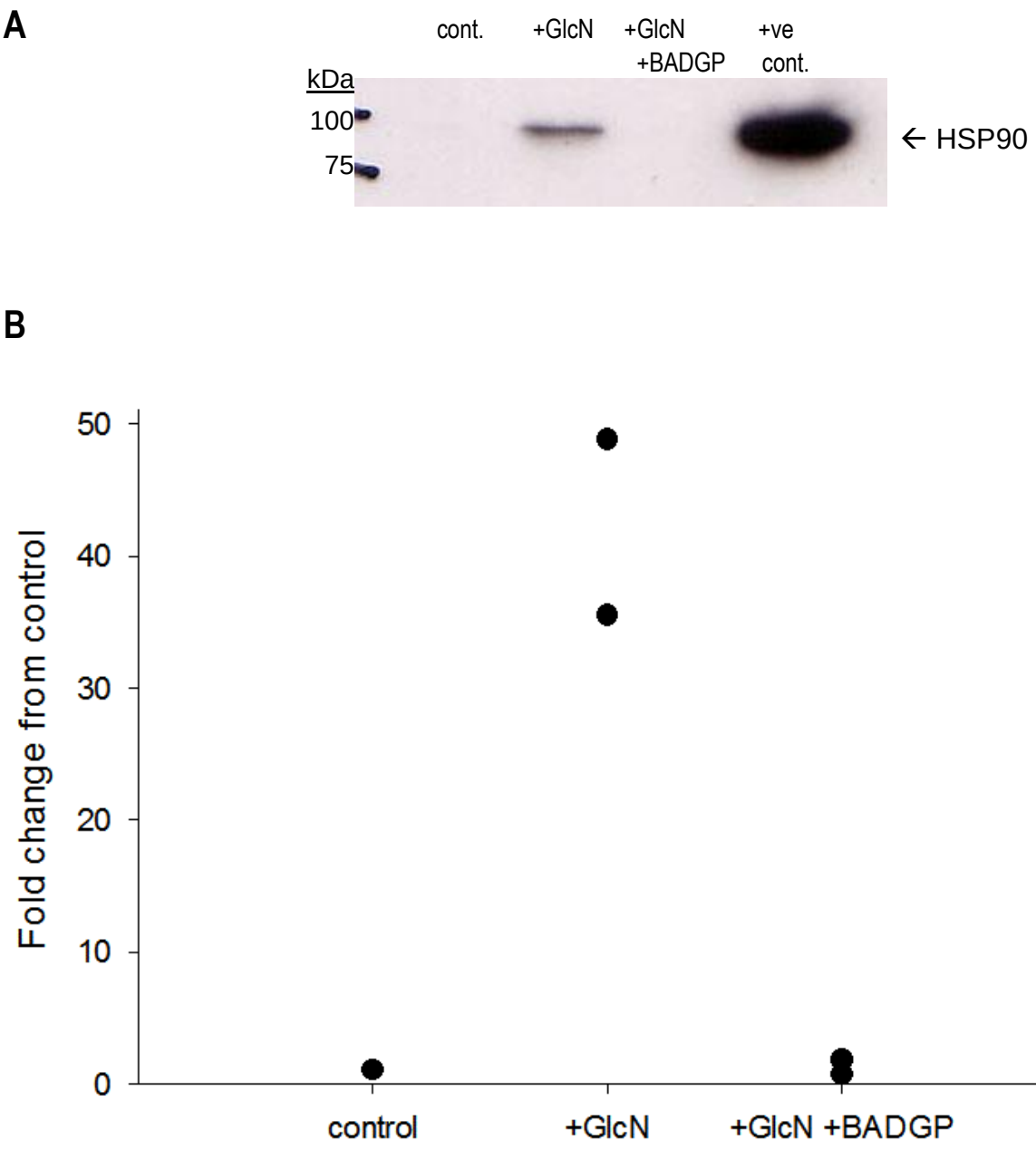

Figure 5.1 Levels of β-O-linked glycosylated HSP90 in COCs at 6 h IVM ± glucosamine and BADGP

Cumulus-oocyte complexes (COCs) (30 per lane) were collected at 6 h in vitro maturation (IVM) in control medium ± 2.5 mM glucosamine (GlcN) and 2.5 mM benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP). **A)** Proteins were immunoprecipitated using CTD110.6 for O-GlcNAc, separated using SDS-PAGE, Western blotted and stained using anti-HSP90. Positive control (+ve) was HeLa cell lysate. **B)** Bands were quantified using ImageJ version 1.44p (Rasband 1997 - 2011). Groups with different superscripts differ significantly ($p < 0.05$), n = 2 replicates.

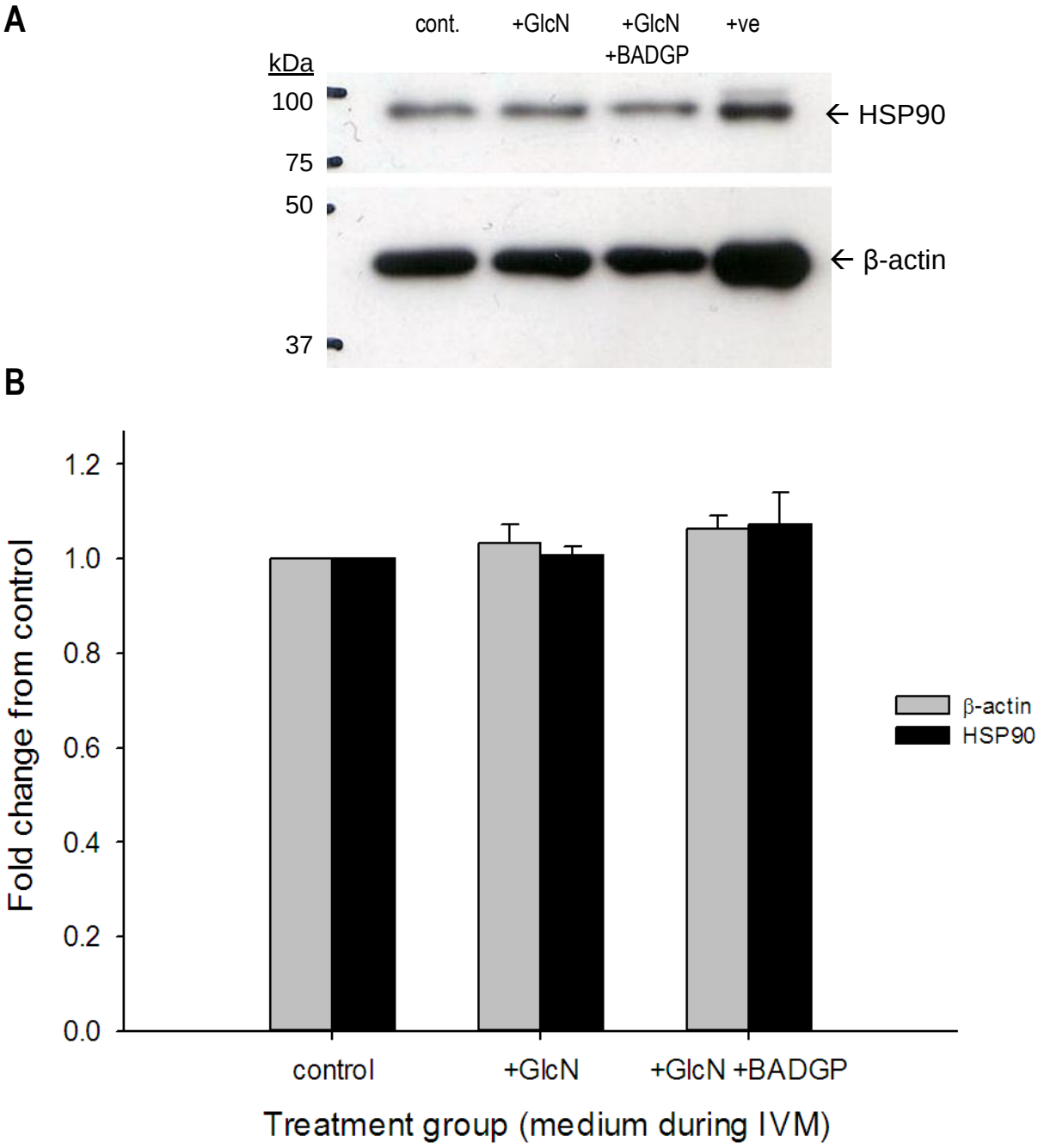

Figure 5.2 Levels of total HSP90 in COCs at 6 h IVM ± glucosamine and BADGP

Cumulus-oocyte complexes (COCs) (15 per lane) were collected at 6 h maturation in control medium ± 2.5 mM glucosamine (GlcN) and 2.5 mM benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP). **A)** Proteins were separated using SDS-PAGE, Western blotted and stained using anti-HSP90 and β-actin as a loading control. Positive control (+ve) was HeLa cell lysate. **B)** Bands were quantified using ImageJ version 1.44p (Rasband 1997 - 2011), n = 3 replicates. Data are presented as mean ± SEM.

To investigate the role of HSP90 in mouse COCs during maturation, 17-(Allylamino)-17-demethoxygeldanamycin (17AAG), a specific inhibitor of HSP90 activity, was added to the maturation medium during IVM ± glucosamine, and on time embryo development assessed. A dose-response of 17AAG was carried out to determine an appropriate dose, as well as toxicity testing which confirmed that 17AAG supplementation during COC in vitro maturation had no effect on subsequent embryo development under control conditions (see Appendix 3, 10.4.2). Neither glucosamine nor 17AAG had an effect on cleavage rates. The presence of glucosamine significantly reduced blastocyst development compared to the control group (Fig. 5.3) (control = 37.5 ± 3.5 % vs. glucosamine = 21.0 ± 2.8 %; $p < 0.05$). However, supplementation of IVM cultures with 17AAG recovered blastocyst development to rates comparable to the control group (+GlcN +17AAG = 37.7 ± 4.5 %).

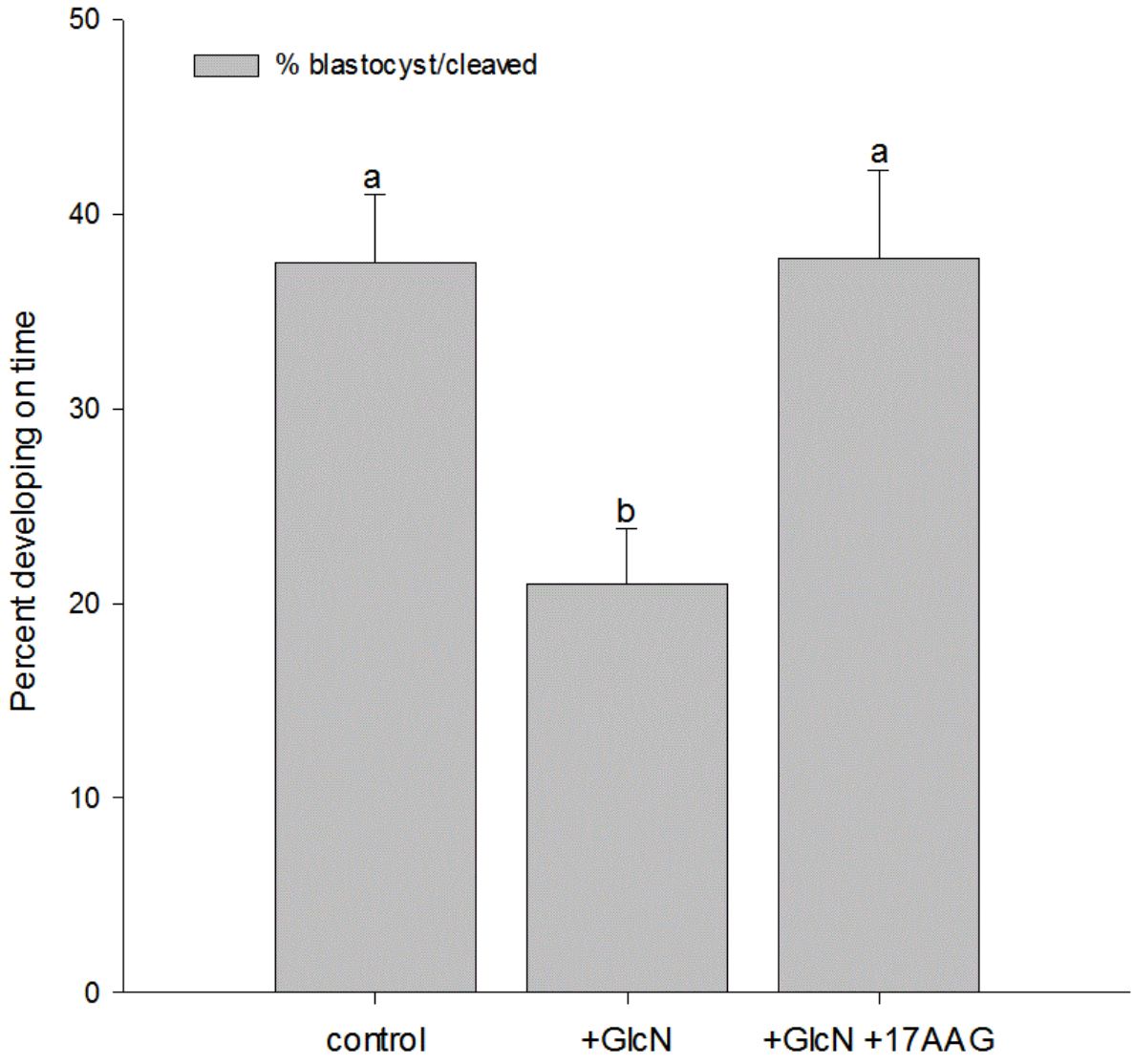

Figure 5.3 Blastocyst development following inhibition of HSP90 during in vitro maturation ± glucosamine

Blastocyst rate was assessed on Day 5, following collection and maturation in control medium ± glucosamine (GlcN) supplementation (2.5 mM) and 17-(Allylamino)-17-demethoxygeldanamycin (17AAG, 0.1 µM). Groups with different superscripts differ significantly ($p < 0.05$). Average number of cumulus-oocyte complexes (COCs) matured per group per replicate was 28, n = 5 replicates. Data are presented as mean ± SEM.

5.4 DISCUSSION

After confirming in Chapter 4 that glucosamine supplementation during IVM of mouse COCs increased total O-GlcNAcylation levels, the aim of this chapter was to identify some of the specific proteins which were O-GlcNAcylated under these conditions. Immunoprecipitation of proteins using CTD110.6 to capture O-GlcNAcylated proteins followed by mass spectrometry revealed 15 candidate proteins, four of which have not been previously reported as O-GlcNAcylated in the literature. While the number of proteins identified is not as high as in other studies, this can be accounted for by the 1-dimensional electrophoresis used instead of 2-dimensional, in which proteins are separated to a greater extent and easier to distinguish. This method was not used in this study as the aim was not to identify as many proteins as possible, and those that were identified would presumably be those which are most heavily O-GlcNAcylated (high stoichiometry) and are relatively highly abundant. They would also have been proteins on which the O-GlcNAc moiety is accessible to the antibody: the O-GlcNAc on some proteins can be buried in protein folding, and not able to be recognized by the antibody unless the protein is fully denatured (Wang et al. 2007).

Several of the identified proteins, such as fibronectin, actin and tubulin-α, are structural proteins. Fibronectin levels correlate with follicular size and oocyte maturity in human follicular fluid and when the cell-binding capacity of fibronectin is inhibited, this prevents both spontaneous and gonadotrophin-releasing hormone (GnRH)-induced resumption of meiosis as well as cleavage in mouse oocytes (Hung et al. 1989). Fibronectin also promotes bovine embryo development in serum-free medium (Larson et al. 1992). Actin is an important component of the cytoskeleton and interestingly is known to influence the remodelling of connexin-43 gap junctions (Theiss and Meller 2002), vital for folliculogenesis in the mouse (Ackert et al. 2001). Gap junctional communication is impaired in cultured astrocytes injected with anti-actin antibodies (Theiss and Meller 2002), which suggests a possible role for O-GlcNAcylation of actin in the COC: the O-GlcNAcylation may impair actin's function in some way, and in turn disrupt the gap junctional communication between the cumulus cells and the oocyte. This communication is vital for COC maturation (see Chapter 1, section 1.3). Tubulin-α forms microtubules in the cell, which assist in maintaining cell shape, guiding chromosome segregation and transport and motility of proteins (Hadfield et al. 2003). In drosophila specialised forms of tubulin-α are required for oocyte meiosis and cleavage (Matthews et al. 1993).

Two proteins identified, zona pellucida sperm-binding proteins 2 and 3, are expressed specifically in the oocyte (Lunsford et al. 1990). While they have not previously been identified as O-GlcNAcylated, they are known glycoproteins (long-chain type) (Roller and Wassarman 1983), and the glycosylation plays a role in the acrosome reaction of sperm at fertilisation (Chakravarty et al. 2008).

Glucose-6-phosphate dehydrogenase X is the rate-limiting enzyme of the oxidative phase of the pentose phosphate pathway (see Chapter 1, 1.3.2), and has previously been identified as O-GlcNAcylated, although it is unknown how this may affect the protein's function (Gurcel et al. 2008).

Elongation factor 1-α and protein disulfide-isomerase (PDI) are two proteins involved in the synthesis and processing of new proteins. Elongation factor 1-α catalyses the binding of aminoacyl-transfer RNA to the ribosome, regulating the rate of protein elongation during translation (Condeelis 1995). After translation, PDI catalyses disulfide bond formation (chemical cross-linking of specific cysteines) during protein folding (Wilkinson and Gilbert 2004). The roles of O-GlcNAcylated elongation factor 1-α and PDI in the COC have not been investigated, although it may be speculated that any change in their function would affect the significant amount of protein synthesis occurring during oocyte maturation. It is interesting to note the presence of both PDI and heat shock protein 90 (HSP90) in the candidate list, as both are known to have chaperone roles (Picard 2002; Wilkinson and Gilbert 2004). While PDI is located in the endoplasmic reticulum and HSP90 is a cytoplasmic protein, the combination of the effect of excess O-GlcNAc on both of these proteins may mean that inhibition of correct protein assembly and folding downstream plays a significant role in the effect of hyperglycosylation on COCs and other cell types.

The protein I chose to focus on for further investigation was HSP90, as it had the highest Combined IonScore (α isoform) and third highest (β isoform) out of all the proteins identified by the mass spectrometry. HSP90 levels have been examined during oogenesis and found to be increased during this time in the mouse, turtle and drosophila (Zimmerman et al. 1983; Morange et al. 1984; Barnier et al. 1987; Harry et al. 1990; Curci et al. 1991). Increases in HSP90 mRNA expression also accompany many major changes in cellular structure and organisation during embryonic development (Csermely et al. 1998). Originally discovered as one of the proteins whose abundance increases under conditions of heat stress (Picard 2002), an increase in HSP90 levels can be induced in cells by almost any form of stress, including ethanol and cocaine treatment, acidic pH, nutrient deprivation and fluctuations in oxygen supply (Gabai and Kabakov 1994; Miles et al. 1994; Salminen et al. 1997). HSP90 is one of the most 'passive' molecular chaperones: there is no evidence for a role for HSP90 in recognizing and catalysing the folding of newly synthesized proteins (Mayer and Bukau 1999) but rather it stabilises proteins that have unfolded and have no tertiary structure, but a defined secondary structure (Jakob et al. 1995). HSP90 itself does not increase the rate of protein re-folding (it is not a true catalyst) but holds the unfolded protein to prevent its aggregation with others, allowing the protein another chance at folding correctly (Hartl 1996; Zhao et al. 2001).

Western blots of COC proteins immunoprecipitated with CTD110.6 confirmed the presence of O-GlcNAcylated HSP90 in glucosamine-treated COCs and the absence of this form of HSP90 in the control and BADGP treatment groups. This suggests that under normal conditions HSP90 exists in an un-O-GlcNAcylated form. It was also important to perform Western blots for the total amount of HSP90 present in each treatment group, because HSP90 is a stress protein and glucosamine is known to induce stress (Werstuck et al. 2006) (see Chapter 1, 1.4.4.4)). However I found that there were no differences were seen in total HSP90 levels in any group.

The inhibition of HSP90 during IVM was achieved through the use of 17AAG, an inhibitor of HSP90 which has been clinically trialled to the phase II stage as a tumour suppressor in various types of cancer (Modi et al. 2011; Oh et al. 2011). HSP90 does not function as a stand-alone protein but associates with HSP70, HSP40, other chaperone and linking proteins (Hernandez et al. 2002). It contains a nucleotide-binding pocket and ADP is bound to this pocket while the super-chaperone complex is being formed and the client protein loaded on. The replacement of ADP with ATP stimulates conformational change of HSP90, releasing the first co-chaperones and recruiting another set, which facilitate folding and stabilising of the client protein. HSP90 then holds the client protein in a state receptive to its stimulus or ligand. However, if the client protein stimulus fails to appear, the chaperone machinery reverses, ATP is hydrolysed to ADP and once HSP90 is returned to a complex with HSP70, the client protein can be targeted for degradation by the proteasome. It is unknown how exactly the protein is targeted but appears to be dependent on the re-association of HSP90 with HSP70 (summary from (Isaacs et al. 2003)). 17AAG binds to the nucleotide binding pocket of HSP90 with much higher affinity than either ADP or ATP (Isaacs et al. 2003), but induces a conformation similar to the ADP-bound form of HSP90, effectively causing constitutive activity of the complex which degrades client proteins (Schneider et al. 1996).

During preliminary toxicity testing, I found that 17AAG had no effect on cleavage or blastocyst development rates when added to control media. However, in the presence of glucosamine (which significantly reduced blastocyst development) 17AAG was able to recover blastocyst development to rates comparable to control levels. Since levels of total HSP90 were constant between treatment groups, it is unlikely that 17AAG simply inhibited an excess of HSP90 in the glucosamine-treated COCs. Together with the Western blot result showing elevated O-GlcNAcylated HSP90 in glucosamine-treated, but not control or BADGP treated COCs, it suggests that there is something about the function of O-GlcNAcylated HSP90 which is detrimental to oocyte developmental competence.

Interestingly, Zhang et al. (2010) have recently shown that inhibition of HSP90 in bovine aortic endothelial cells exposed to high glucose or glucosamine dramatically reduces the expression and half-life of OGT, the enzyme which attaches O-GlcNAc to proteins, and subsequent O-GlcNAcylation. This suggests that OGT may be a client protein of HSP90. If this is the case, it brings up the question of how O-GlcNAcylation of HSP90 itself would affect its regulation of OGT folding.

Phosphorylation of HPS90 is known to be linked to its chaperoning function (Zhao et al. 2001). If it were a case of the reciprocal O-GlcNAcylation/phosphorylation relationship, it would be expected that O-GlcNAcylation of HSP90 might inhibit or down-regulate its function; however this is contrary to the evidence from the experiment in this chapter, that inhibition of HSP90 in fact has the opposite effect on embryo development from O-GlcNAcylation (O-GlcNAcylation in hyperglycaemic conditions is counteracted by HSP90 inhibition). It may be that the O-GlcNAcylation of HSP90 is not on the same site or sites close enough to affect the critical site for phosphorylation. It is unknown what the critical site(s) are which are important for HSP90 function, although one site which can be phosphorylated in vitro is threonine 5 (Lees-Miller and Anderson 1989). A search on the YinOYang server predicts that this site on HSP90α is likely to be both phosphorylated and O-GlcNAcylated (Gupta and Brunak 2002).

If O-GlcNAcylation is not inhibiting the function of HSP90, another hypothesis is that O-GlcNAcylation somehow enhances HSP90 activity. However, after communication with the head of a leading HSP90 laboratory (Didier Picard, University of Geneva) it was confirmed that there is only one known study which has reported HSP90 forms with increased activity, and these were as a result of randomly-induced point mutations (Zurawska et al. 2010; Picard 2011).

There is another possible mechanism for the observed effect of 17AAG in glucosamine-treated, but not control COCs: there may be different forms of HSP90 complex present in these COCs. This hypothesis is based on the activity of HPS90 in cancer, where it chaperones the correct folding of many oncogenic proteins important for tumour progression (Isaacs et al. 2003). The appeal of 17AAG as an anti-cancer drug derives partly from the fact that HSP90 acts concomitantly on many proteins which the tumour depends on for survival, as well as 17AAG's preferential targeting of tumour cells (Kamal et al. 2003; Sreedhar et al. 2004). This preferential action can be partly explained by the finding that HSP90 in tumour cells is present entirely in the complexed form with the co-chaperones mentioned above, compared to normal cells in which HSP90 is present in its un-complexed latent form (Kamal et al. 2003). The complexed form of HSP90, but not the latent form, binds ADP (or 17AAG), which accounts for the higher affinity of 17AAG for the tumour HSP90 over the normal cell form. The authors hypothesise that this altered conformation of HSP90 could be caused by the gradual accumulation of mutant and over-expressed cell signalling proteins, engaging HSP90 more actively in chaperoning the folding of these proteins (Kamal et al. 2003).

While the possibility has not been explored in this study, the mechanism of selective activity of 17AAG on tumour cells may be applicable to the glucosamine-treated COCs in these experiments. It is possible that in COCs exposed to hyperglycaemic conditions, the O-GlcNAcylation of HSP90 causes disruption in the balance of complexed to un-complexed HSP90, and greater affinity of 17AAG for the protein in glucosamine-treated than in control COCs.

The experiments conducted in this chapter identify potential targets of O-GlcNAcylation in COCs matured under hyperglycaemic conditions. O-GlcNAcylation of HSP90, one of the most promising candidate proteins due to a high ion score and effect on a wide range of client proteins, was confirmed using immunoprecipitation and Western blotting. Inhibition of the O-GlcNAcylated form of HSP90 in glucosamine-treated COCs resulted in an increase of blastocyst rates to control levels, and this was not due to an increase in the total amount of HSP90. These results are the first to identify candidate proteins which may be targeted by O-GlcNAcylation in COCs cultured under hyperglycaemic conditions.

CHAPTER 6

DISCUSSION

It is now well-established that environmental and metabolic insults occurring early in development can have long-term health implications (Barker 1995; Fernandez-Gonzalez et al. 2007). In particular, maternal hyperglycaemia during the peri-conceptual period negatively affects oocyte developmental competence (defined as the ability of the oocyte to undergo successful fertilisation and embryo development) (Dunne et al. 1999; Hashimoto et al. 2000; Sutton-McDowall et al. 2006; Lapolla et al. 2008; Schelbach et al. 2010). The need to further study this effect is driven by the increasing prevalence of metabolic conditions such as diabetes and obesity, as well as lifestyle choices like poor diet, all of which are associated with hyperglycaemia (Robker et al. 2009; Jungheim et al. 2010; Diabetes Australia 2011).

The effects of poorly controlled diabetes on the health of the mother and baby both during and after pregnancy have been reported for several decades. Mothers who have elevated blood glucose levels are at increased risk of preeclampsia and spontaneous abortions, and their babies are more likely to have high birth weight and congenital anomalies (Cornblath and Schwartz 1976; Mills et al. 1979; Sadler et al. 1988; Becerra et al. 1990; Greene 1999; Farrell et al. 2002; Hedderson et al. 2003; Middleton et al. 2010). Many trials have demonstrated that early glycaemic control during pregnancy (measured by a low glycosylated haemoglobin level) reduces the risk of these complications (Miller et al. 1981; Schaefer et al. 1997; Dunne et al. 1999). The best preventive measure for diabetic women wishing to become pregnant is pre-conception care to control blood glucose levels (Lapolla et al. 2008). This implicates the un-fertilised, maturing oocyte as a target for hyperglycaemia-induced damage, a hypothesis which has been supported by studies in animal models (Diamond et al. 1989; Chang et al. 2005; Kim et al. 2007; Wang et al. 2009; Jungheim et al. 2010; Wang and Moley 2010). However, the mechanisms by which maternal hyperglycaemia prior to conception causes long-term detrimental effects on offspring remained unknown.

The aim of my thesis was to investigate the biochemical mechanism by which hyperglycaemia reduces the developmental competence of the oocyte. Specifically, I hypothesized that up-regulation of the hexosamine biosynthesis pathway, with a concomitant increase in β-O-linked glycosylation of COC proteins, was the cause of the perturbed development under periconception hyperglycemia.

In Chapter 3, I assessed the effects of glucose concentrations (hypo- to hyper-glycaemic levels) during mouse cumulus-oocyte complex (COC) in vitro maturation (IVM) on a range of outcomes associated with developmental competence. Glucose concentration during IVM had no clear effect on cleavage rate, however optimal blastocyst development rates were achieved with the lowest concentration of glucose tested, 1 mM. With increasing glucose concentrations, blastocyst development rates decreased in a linear fashion up to 30 mM glucose, with 30 mM glucose resulting in blastocyst development no different to the poor rates observed in the complete absence of glucose.

Meiotic maturation rates were not affected by glucose concentration except in the complete absence of glucose, where fewer oocytes completed nuclear maturation to the metaphase II (MII) stage. Cumulus expansion was negligible in the absence of glucose and increased gradually with increasing glucose concentrations.

These observations support those previously published, including 1) the absence of glucose during IVM is detrimental to subsequent embryo development, cumulus expansion and meiotic maturation (Hendryx and Wordinger 1979; Downs and Hudson 2000; Hashimoto et al. 2000; Wongsrikeao et al. 2006; Nandi et al. 2008) and 2) high glucose levels inhibit embryo development and meiotic maturation (Diamond et al. 1989; Hashimoto et al. 2000; Colton et al. 2002; Ratchford et al. 2007). The first of these findings can be explained by a lack of substrate for the glucose metabolic pathways, all of which are important during COC maturation and contribute to oocyte developmental competence (reviewed by (Sutton-McDowall et al. 2010)). The pentose phosphate pathway plays a major role in meiotic maturation (Downs et al. 1996; Downs et al. 1998) and metabolises glucose to produce the sugar component of new nucleic acids; glycolysis produces ATP and substrates for the TCA cycle and cumulus expansion is dependent on the production of uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) through the hexosamine biosynthesis pathway (HBP). UDP-GlcNAc production is also necessary for post-translational modifications of proteins by β-O-linked glycosylation (O-GlcNAcylation), a phenomenon investigated further in subsequent experiments. The second finding, that glucose levels comparable with those observed in diabetic animals in vivo cause a decrease in oocyte competence, supports studies which have found various metabolic, structural and functional defects in diabetic oocytes (Diamond et al. 1989; Colton et al. 2002; Kim et al. 2007; Wang et al. 2010), as well as reduced embryo development in hyperglycaemic conditions. The dose-response effect of glucose shown in Chapter 3 confirmed the effect of hyperglycaemia on the oocyte before fertilisation, and provided a framework for subsequent experimental designs in Chapters 4 and 5.

The finding that glucose was essential during the first hour of IVM (the collection phase) was unexpected but explored further by substituting glucosamine for glucose, showing that the COC requires at least one of these HBP substrates during this time for blastocyst development. Glucose during the first hour was able to suffice even if none was provided during the subsequent 17 h maturation phase, while glucosamine was only able to achieve this if glucose was present during maturation. Cumulus expansion was negligible in COCs matured in the absence of glucose for the 17 h maturation period, however if glucose was present during the first hour, blastocyst development was comparable to the control group. This observation is consistent with others suggesting that in vitro, cumulus expansion is not necessary for optimal embryo development (Ali and Sirard 2002; Luciano et al. 2005; Gutnisky et al. 2007). Meiotic maturation was the same in all groups provided with glucose in at least one phase of

IVM. Since even very poor cumulus expansion could result in good embryo development rates and the timing of glucose provision was not critical to meiotic maturation, these results pointed to the importance of the activation of the HBP during the first hour of IVM. This hypothesis was strengthened when glucosamine was found to be able to substitute for glucose during this time, as glucosamine is only metabolised via the HBP.

COC maturation is a complex process during which the oocyte must synthesise and accumulate proteins and RNA necessary to support its development through the fertilisation stage and until the first cleavage (in mouse) (Flach et al. 1982), and involves the co-operation of many different proteins. The necessary HBP activation during the first hour of IVM does not appear to be for the purpose of cumulus expansion, since expansion was able to proceed when glucose was present during the maturation phase only. Rather, I suspect the crucial role of the HBP during the first hour lies with β-O-linked glycosylation, although O-linked β-N-acetylglucosaminyltransferase (OGT, the enzyme which attaches the O-GlcNAc moiety to proteins) inhibition experiments were not performed in this study to confirm this and should be key future experiments. O-GlcNAc is a dynamic modification which can be cycled on and off of proteins on a similar timescale to phosphorylation (Slawson et al. 2006), and regulates protein function in a number of different ways. The wide range of protein classes affected likely includes at least some involved in COC maturation, and combined with the rapid cycling of this modification it is perhaps not surprising that the interruption of the supply of HBP substrates even for an hour affects oocyte developmental competence.

Increasing glucose concentrations, or glucosamine treatment, increase the level of detectable O-GlcNAcylation in a wide range of cell types (Comer et al. 2001; Park et al. 2007; Champattanachai et al. 2008). In pancreatic β-cells, increased O-GlcNAcylation was associated with reduced cell viability (Park et al. 2007). In the experiments presented in this thesis, glucosamine supplementation during embryo culture increased positive O-GlcNAc antibody binding and reduced developmental potential. While reduced antibody binding for O-GlcNAc in bovine oocytes in the presence of BADGP (benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside, an inhibitor of OGT) has been reported (Sutton-McDowall et al. 2006), to date no studies have examined the pattern of O-GlcNAcylation in the mouse oocyte. To address this knowledge gap, in Chapter 4 I used Western blotting and immunohistochemistry to quantify and localise O-GlcNAcylation in the mouse oocyte under various conditions. Similar to the results obtained by Park et al. (2007), using Western blotting I found O-GlcNAcylation levels were lowest in the most viable/developmental competent group of COCs (those matured in 1 mM glucose). There was a positive correlation between glucose concentration and detectable O-GlcNAcylation and a trend towards reduced O-GlcNAc antibody binding when BADGP was added. In a time course study, I found higher O-GlcNAcylation in glucosamine-treated COCs compared to control COCs (although not significant at 18

h), which was reduced with BADGP treatment. There also appeared to be variability of O-GlcNAcylation within the control group over the time points tested, which fits in with the concept of O-GlcNAcylation as a highly dynamic modification (Vosseller et al. 2001).

Previous studies in mice have determined that the cumulus cells are necessary for the effect of glucosamine on the oocyte (Schelbach et al. 2010) and extensive positive staining for O-GlcNAc in the cumulus cells of control and glucosamine-treated cow COCs, with very little in the oocyte (Sutton-McDowall et al. 2006) supports this. Consistent with these reports, immunohistochemistry of mouse COCs in Chapter 4 revealed the same pattern of O-GlcNAcylation as with Western blotting, detectable mainly in the cumulus cells. It has been suggested that the relatively low level of O-GlcNAcylation in glucosamine-treated oocytes is due to the inability of the oocyte to take up glucosamine (Schelbach et al. 2010), because the glucose transporter which performs this task is poorly functional in oocytes. Further experiments could include examining O-GlcNAcylation staining of oocytes exposed to hyperglycaemic levels of glucose, because while the oocyte does not express the insulin-sensitive transporter SLC2A4, it does express SLC2A1, SLC2A3 and SLC2A8 (Dan-Goor et al. 1997; Augustin et al. 2001; Zheng et al. 2007; Pisani et al. 2008).

The Western blotting and immunohistochemistry results from Chapter 4 can be correlated with developmental outcomes for the mouse from Chapter 3 and previously published studies (Schelbach et al. 2010). COCs matured in the lowest concentrations of glucose (or hyperglycaemic conditions + BADGP) have the highest rate of blastocyst development (Sutton-McDowall et al. 2006; Schelbach et al. 2010). Increased O-GlcNAcylation is seen in the glucosamine-treated and hyperglycaemic glucose groups, which produce compromised blastocyst development rates. This supports published observations that hyperglycaemic conditions cause excessive O-GlcNAcylation of proteins (Comer and Hart 2001; Parker et al. 2004).

The aim of Chapter 5 was to identify some of these proteins and investigate their role in the maturing COC.

Mass spectrometry analysis is becoming a useful tool for identifying proteins modified by O-GlcNAc (Ahrend et al. 2008). Lists of O-GlcNAcylated proteins have been published for many different cell types and under different conditions and the modification affects many protein classes including vesicle proteins, kinases, cytoskeletal proteins, transcription factors, proteasomal proteins, polymerases, metabolic enzymes and RNA-processing proteins (Hart et al. 2007). The methods used in Chapter 5 to identify potential targets of O-GlcNAc in the mouse COC were immunoprecipitation using an anti-O-GlcNAc antibody followed by one-dimensional SDS-PAGE, silver staining and mass spectrometry. This approach only captures proteins in which the O-GlcNAc modification was visible to the antibody (not

hidden by the conformation of the protein). Subsequently on the silver gel, proteins with low O-GlcNAc stoichiometry would have shown up as bands which were too small or faint to analyse. To identify more protein targets in the future, two-dimensional SDS-PAGE should be used, with staining of proteins using the anti-O-GlcNAc antibody and observing which spots shifted under different glycaemic conditions.

However, while one aim of this chapter was to identify proteins potentially modified by O-GlcNAc, I also wanted to spend some time focusing on a specific candidate protein(s) of particular interest, to relate these identified protein(s) back to oocyte developmental competence and their role in the COC. Using immunoprecipitation to capture O-GlcNAcylated proteins from glucosamine-treated COCs, followed by mass spectrometry, 15 potential targets of O-GlcNAcylation were identified. The identified proteins ranged from structural to protein folding to metabolic, and heat-shock protein 90 (HSP90), a molecular chaperone, was chosen to investigate further as it had the highest Combined IonScore and is known to regulate the folding of a wide range of proteins and is regarded as a "stress-response protein", which was therefore an attractive choice.

The presence of O-GlcNAcylated HSP90 in glucosamine-treated COCs was confirmed using immunoprecipitation for O-GlcNAcylated proteins and detection using anti-HSP90 antibody. These Western blots revealed approximately 40 times more O-GlcNAcylated HSP90 in glucosamine-treated COCs than in control or glucosamine + BADGP-treated COCs. This suggests that in conditions which are conducive to good oocyte developmental competence, HSP90 is present within the COC, but not O-GlcNAcylated, and that the O-GlcNAcylation of this protein may alter its function in the COC. To test if this was the case, I used the specific HSP90 inhibitor, 17-(Allylamino)-17-demethoxygeldanamycin (17AAG), during IVM and scored embryo development. Somewhat surprisingly, 17AAG supplementation under control conditions (inhibition of non-O-GlcNAcylated HSP90) had no effect on either cleavage or blastocyst rates. However, in the presence of glucosamine (increased O-GlcNAcylated HSP90), 17AAG was able to recover blastocyst development.

In the majority of cases of O-GlcNAcylation of specific proteins, excessive O-GlcNAcylation has an inhibitory or negative effect on the function of the target protein (Hart et al. 2007). However there are examples of the opposite occurring, such as in Alzheimer's disease, where O-GlcNAcylation is actually protective against hyper-phosphorylation of the protein tau, which is associated with progression of the disease (Liu et al. 2004). In the current study, it appears that O-GlcNAcylation does not have an inhibitory effect on HSP90, since inhibition of HSP90 using 17AAG has the opposite effect from glucosamine treatment. This brings up the question of how O-GlcNAcylation is affecting HSP90 function, if not inhibiting it. It is possible that O-GlcNAcylation of residues on HSP90 causes an increase in activity or change of function, via conformational change, altering its affinity for co-factors, affecting the rate of degradation or its sub-cellular location. One example of upregulation of HSP90 activity is in

many types of tumour, and 17AAG is currently in clinical trials as a tumour suppressor (Modi et al. 2011; Oh et al. 2011). It is not entirely understood how cancerous cells achieve this upregulation of HSP90 activity but in cancerous cells, HSP90 exists only in a form complexed to a set of co-chaperones which facilitate protein folding and stabilisation and never in its latent, un-complexed form as in normal cells (Kamal et al. 2003). This in turn suggests that HSP90 is constitutively ready to assist many proteins necessary for tumour survival. 17AAG effectively shuts down this chaperone complex by changing the conformation of HSP90 so that it attracts a different set of co-factors, which induce degradation of client proteins instead of folding and stabilisation (Isaacs et al. 2003). Recently, OGT itself has been identified as a potential candidate protein for HSP90 folding, since when HSP90 is inhibited (in bovine aortic endothelial cells), the half-life of OGT, and subsequently O-GlcNAcylation, are significantly reduced (Zhang et al. 2010).

I hypothesise that this ability of glycosylated HSP90 to maintain OGT activity in a positive feedback loop is a possible mechanism for the observed effects of both glucosamine and 17AAG on mouse COCs during IVM (Fig. 6.1). The O-GlcNAcylation of HSP90 increases its activity, possibly by altering its affinity for different sets of co-factors, as seen in tumour cells. This subsequently up-regulates the correct folding and activity of OGT, which continues the O-GlcNAcylation of HSP90 and creates a positive feedback loop. OGT attaches O-GlcNAc to a myriad of other proteins, causing altered activity to those important during COC maturation. 17AAG is able to prevent this positive feedback loop by inhibiting HSP90 activity, reducing OGT half-life and effectiveness, and the downstream proteins are not as heavily O-GlcNAcylated.

In the preliminary dose-response experiments of 17AAG during IVM, 10 μM 17AAG reduced the blastocyst rate significantly. This is consistent with my hypothesis, as complete inhibition of HSP90 in this scenario would prevent it from carrying out chaperoning function on OGT at all, presumably reducing the occurrence of O-GlcNAcylation to nearly zero, and it is known that some O-GlcNAcylation is necessary for normal cellular function. Complete inhibition of HSP90 would also prevent it chaperoning its wide range of other protein clients, many of which may also be important in COC maturation.

My hypothesis may not be restricted to just the COC. This mechanism of glycosylated HSP protecting OGT activity under hyperglycaemic conditions could be tested in other cell types known to express HSP90 and sensitive to hyperglycaemic perturbation.

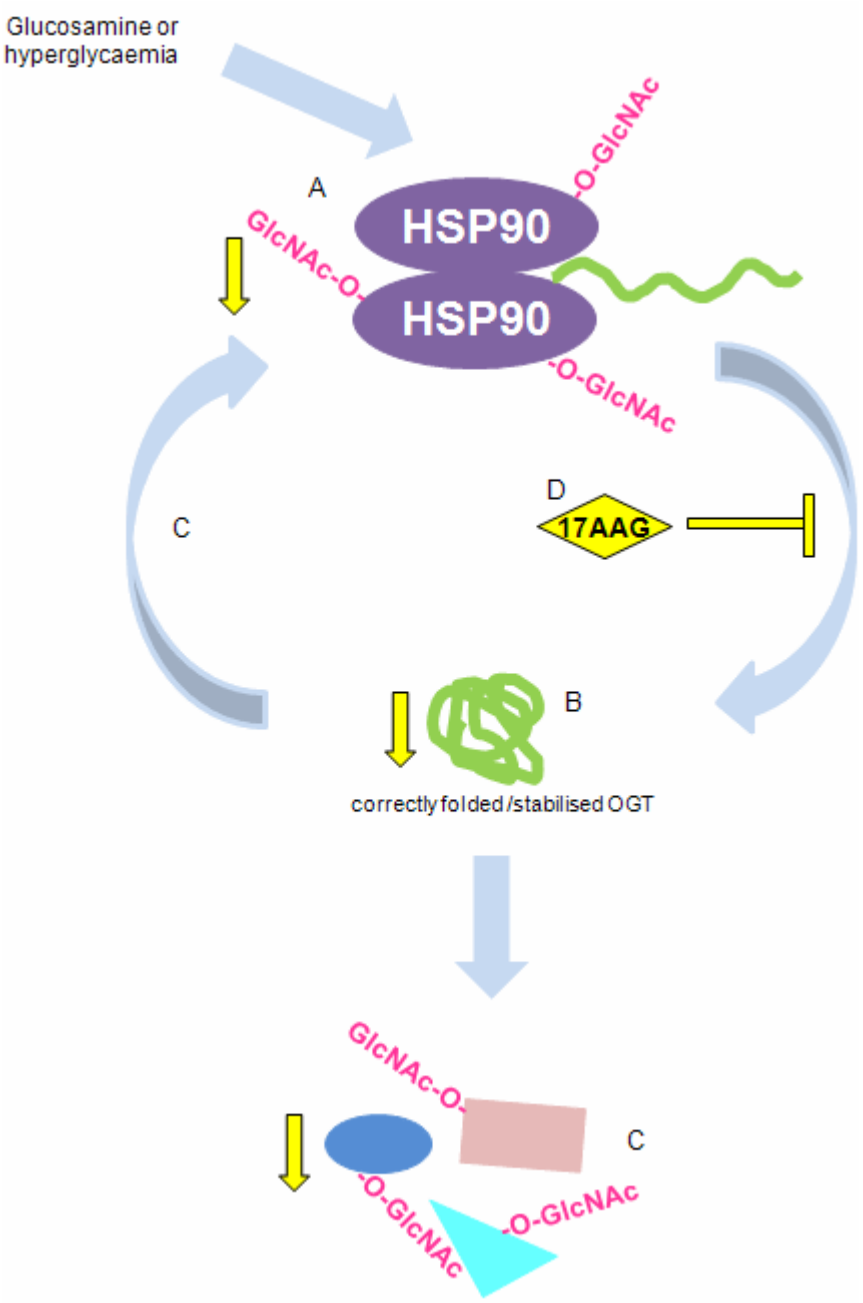

Figure 6.1 Hypothesis for the mechanism of glucosamine and 17AAG action in mouse COCs

A) Hyperglycaemic conditions, including glucosamine supplementation, cause excess β-O-linked glycosylation (O-GlcNAcylation) of heat-shock protein 90 (HSP90). B) O-GlcNAcylation induces increased HSP90 activity, subsequently increasing the folding or stabilisation rate of O-linked β-N-acetylglucosaminlytransferase (OGT), a client protein. C) OGT further glycosylates HSP90 in a positive feedback loop, as well as causing excessive O-GlcNAcylation on other target proteins important in cumulus-oocyte complex (COC) maturation. D) 17-(Allylamino)-17-demethoxygeldanamycin (17AAG), an inhibitor of HSP90, interrupts this positive feedback loop. Effects of 17AAG are shown by the yellow arrows.

While the experiments presented in this thesis have focused on excessive O-GlcNAcylation as a cause of reduced oocyte developmental competence, they are complementary to the vast body of literature demonstrating that structural and functional defects in diabetic oocytes, differential methylation and abnormal gene expression during early development are all detrimental to reproductive outcomes.

The clinical effects of hyperglycaemia during pregnancy have been known since the early 1900s, when diabetic pregnancies first became more than a rare phenomenon, due to the increasing availability of insulin therapy (Skipper 1933). Research has gradually highlighted the importance of the peri-conceptual period to the long-term health of babies, and studies into the effect of hyperglycaemia, specifically on early development, have shown that the oocyte itself is susceptible to damage by hyperglycaemia prior to conception. The results generated in this thesis are the first to examine O-GlcNAcylation in the mouse COC and to identify specific proteins which are potential targets of aberrant O-GlcNAcylation under hyperglycaemic conditions. They provide new leads for future investigations into possible therapies and interventions which may improve outcomes for mothers with hyperglycaemia.

CHAPTER 7

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Wilkinson, B. and H. F. Gilbert (2004). "Protein disulfide isomerase." Biochim Biophys Acta 1699(1-2): 35-44.

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APPENDIX 1
MEDIA, SOLUTIONS AND PROTOCOLS

Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

8.1 COC COLLECTION AND MATURATION MEDIA

Media for collection and maturation media were as described in Table 8.1, with various glucose and glucosamine concentrations (see individual chapters). Media were supplemented with bovine serum albumin (BSA, ICPbio, Glenfield, New Zealand), 4 mg/mL and 3 mg/mL in collection and maturation respectively. Maturation medium was supplemented with 50 mIU/mL recombinant human follicle-stimulating hormone (FSH) (Organon, Oss, The Netherlands), and 1 mg/mL fetuin was used in both media for all embryo development experiments as it prevents the zona hardening observed in mouse oocytes under serum-free culture conditions (Schroeder et al. 1990).

Component	Concentration (mM)	
	Collection	Maturation
Sodium chloride	95.00	115.00
Potassium chloride	5.50	6.00
Magnesium sulphate heptahydrate	1.00	0.80
Sodium dihydrogen phosphate	0.30	-
Potassium dihydrogen phosphate	-	2.00
Sodium bicarbonate	5.00	27.50
Sodium pyruvate	0.32	0.40
Sodium L-lactate	9.97	-
Calcium chloride dihydrate	1.80	1.80
Taurine	0.10	-
3-(N-Morpholino)propanesulfonic acid (MOPS)	20.00	-
Phenol red	0.01	-
Gentamicin	75 mg/L	75 mg/L
Glutamax 1*	1.0 mL/L	1.0 mL/L
Non-essential amino acids (NEAA)*	1.0 mL/L	1.0 mL/L
Essential amino acids (EAA)*	-	2.0 mL/L

Table 8.1 Simple collection and maturation media used for all experiments

*Glutamax 1, NEAA (100 x stock) and EAA (50 x stock) all from GIBCO, Invitrogen, CA, USA.

8.2 IMMUNOFLUORESCENCE

8.2.1 Immunofluorescence Solutions

Permeabilising solution

0.25 % Triton X-100 (United States Biochemical Corp., OH, USA)

Made up in phosphate buffered saline (PBS)

Blocking solution

10 % goat serum (Jackson ImmunoResearch, PA, USA)

0.2 % Tween 20

Made up in PBS

Primary antibody solution

CTD110.6 (anti-O-GlcNAc antibody; Covance, NJ, USA), diluted 1 in 250 in blocking solution

Secondary antibody solution

Alexa Fluor 488 goat anti-mouse IgM (μ chain) (Invitrogen, CA, USA), diluted 1 in 250 in blocking solution

Loading medium

40 % Confocal loading medium (simple collection medium, Table 8.1, minus phenol red)

40 % PBS

20 % Glycerol

8.2.2 Immunofluorescence Protocol

All steps performed on COCs adhered to slides using Cell-Tak (BD Biosciences, NJ, USA) (see section 2.6).

1. Remove excess paraformaldehyde from the slide, 2 x wash with PBS

2. Incubate with permeabilising solution for half an hour at room temperature (RT)

3. Remove permeabilising solution, 2 x wash with PBS

4. Incubate with blocking solution for 2 hours at RT

5. Remove blocking solution, add primary antibody solution and leave on overnight at 4 °C.

6. Remove primary antibody solution, 3 x wash with PBS

7. Incubate with secondary antibody solution for 2 hours at RT

8. Remove secondary antibody solution, 2 x wash with PBS

9. Incubate with propidium iodide (PI) 1.5 μM in PBS for half an hour at RT

10. Remove PI, 1 x wash with PBS, add loading media and examine under confocal microscope

8.3 IMMUNOPRECIPITATION SOLUTIONS

Radioimmunoprecipitation (RIPA) buffer

10 mM Tris(hydroxymethyl)aminomethane (Tris) pH 7.4

150 mM Sodium chloride

1 mM Ethylenediaminetetraacetic acid (EDTA)

1 % Triton X-100 (United States Biochemical Corp., OH, USA)

Made up in Milli Q H_2O

5 x Laemmli buffer (LB)

50 % Glycerol

10 % Sodium dodecyl sulphate (SDS)

0.5 % Bromophenol blue

250 mM Tris pH 6.8

10 µL β-Mercaptoethanol added to 100 µL 5 x LB immediately before use

8.4 WESTERN BLOTTING

8.4.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Gels

10 % separating gel

4.9 mL Milli Q H_2O

2.5 mL 40% Acrylamide/Bis (Bio-Rad, CA, USA)

2.5 mL 1.5 M Tris pH 8.8 (in Milli Q H_2O)

100 µL 10 % SDS (in Milli Q H_2O)

100 µL 10% ammonium persulfate (APS, in Milli Q H_2O)

10 µL *N*,*N*,*N*',*N*'-Tetramethylethylenediamine (TEMED)

Allow to set for 45-60 minutes before adding stacking gel

4 % stacking gel

3.4 mL Milli Q H_2O

500 µL Acrylamide/Bis

1.25 mL 0.5M Tris pH 6.8

500 µL 10 % SDS (in Milli Q H_2O)

25 µL 10% APS

5 µL TEMED

Allow to set for 60 minutes before use

8.4.2 Western Blot Buffers

10 x Running buffer

30.3 g Tris

144 g Glycine

10 g SDS

Made up to 1 L in Milli Q H_2O

Transfer buffer

30 g Glycine

15 g Tris

Made up to 2 L in Milli Q H_2O

APPENDIX 2

EFFECT OF FETAL CALF SERUM, GLUCOSAMINE SUPPLEMENTATION AND TIME IN COLLECTION ON MARKERS OF OOCYTE DEVELOPMENTAL COMPETENCE

9.1 FETAL CALF SERUM SUPPLEMENTATION IN MATURATION MEDIUM

Preliminary experiments for the glucose dose-response model of in vitro maturation (IVM) described in Chapter 3 showed that the presence of 2 % fetal calf serum (FCS) in maturation medium annulled the effect of varying glucose levels between collection and maturation on both CEI (Fig. 9.1) and meiotic maturation rates (Fig. 9.2). This was demonstrated by providing glucose (varying levels) in collection or maturation medium only, with 0 mM glucose in the opposite phase, maturing COCs for 17 h and examining cumulus expansion and meiotic maturation. The effect of FCS on both these measures is most likely due to the presence of glucose in FCS (3.06 – 6.94 mM) (Branzoi et al. 2010; Branzoi et al. 2010; Innovative Research 2011) coupled with various growth factors and inter-α-trypsin inhibitor. A 2 % FCS concentration would result in 0.06 – 0.14 mM additional glucose in the maturation medium, which may have been sufficient to facilitate cumulus expansion and meiotic maturation. Serum also contains an inter-α-trypsin inhibitor (Chen et al. 1992), which stabilises the cumulus matrix as it expands, and without serum, the cumulus cells tend to dissociate from the oocyte more readily. A stable cumulus matrix may be able to retain sufficient glucose to stimulate some expansion and meiotic maturation.

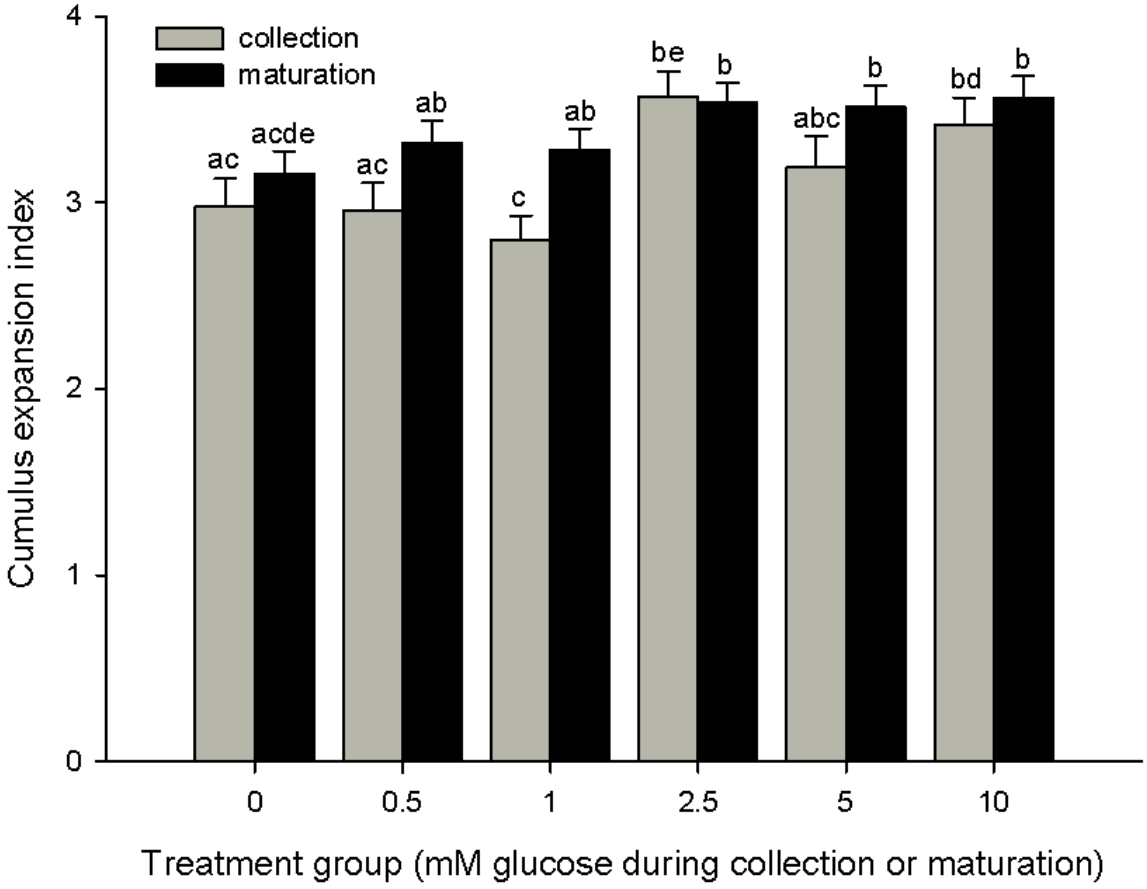

Figure 9.1 Cumulus expansion indices following glucose supplementation during collection or in vitro maturation only

Cumulus expansion was measured using the Vanderhyden scoring system for cumulus-oocyte complexes (COCs) (Vanderhyden et al. 1990) following glucose supplementation during the collection or in vitro maturation phase only, with serum present in both phases. Average number of COCs scored per group was 122. Groups with different superscripts differ significantly ($p < 0.05$).

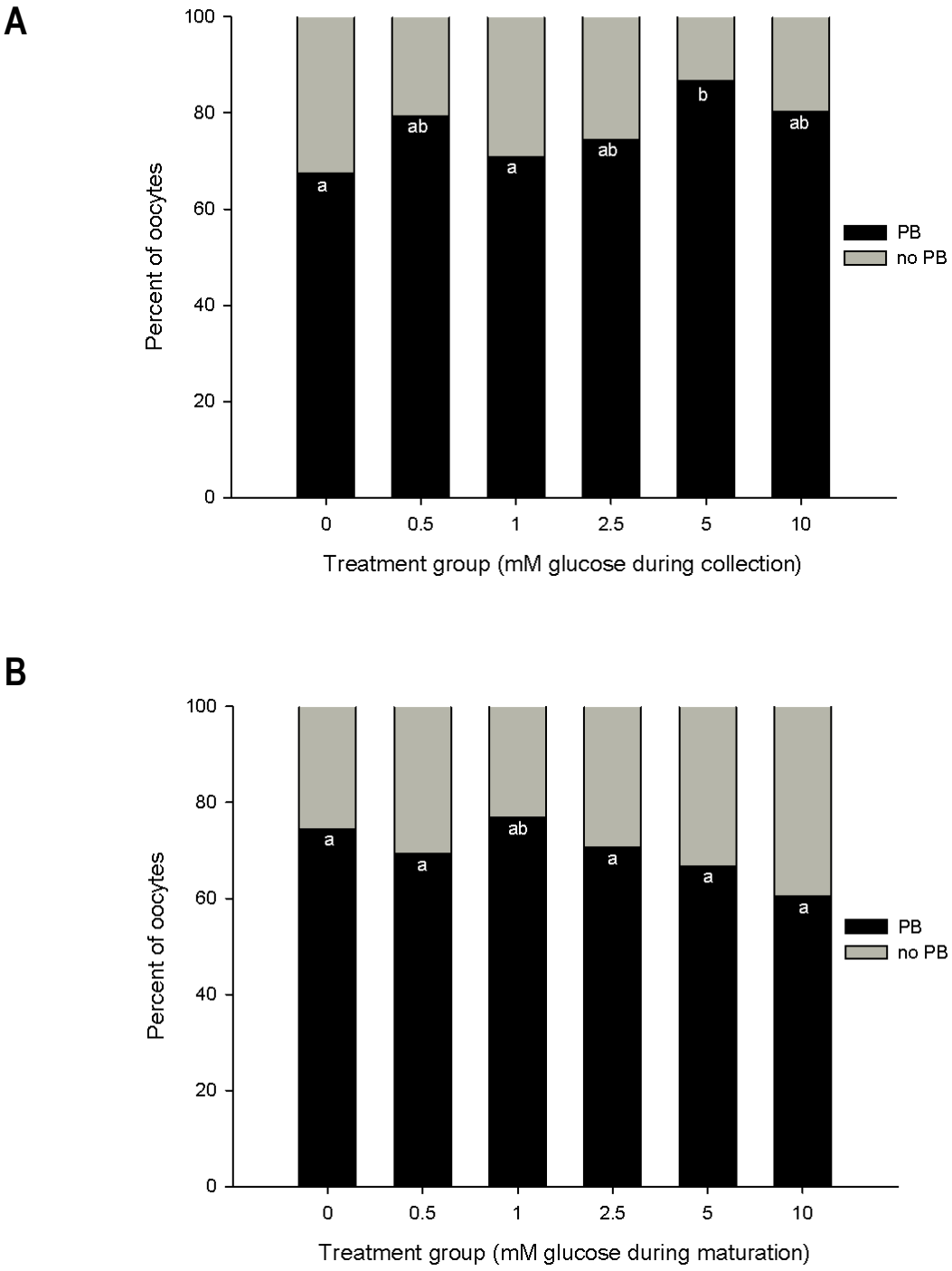

Figure 9.2 Meiotic maturation stage following glucose supplementation during collection or in vitro maturation only

Meiotic maturation was assessed by the presence of a polar body (PB) or no polar body, following glucose supplementation during the collection or in vitro maturation phase only, with serum present in both phases. Groups with different superscripts differ significantly ($p < 0.05$). **A)** Glucose present in collection phase only. Average number of cumulus-oocyte complexes (COCs) per group was 24, n = 3 replicates. **B)** Glucose present in maturation only. Average number of COCs per group was 32, n = 4 replicates.

9.2 EFFECT OF TIME IN COLLECTION ON EMBRYO DEVELOPMENT

Following the discovery that hexosamine biosynthesis pathway (HBP) substrates are necessary during the first hour of IVM (the collection phase), an experiment was designed to investigate how specific this timing was. COCs were held in collection medium for either 0.5 or 1.5 hours, supplemented with no further additives, 1 mM glucose or 2.5 mM glucosamine (the last two shown to increase blastocyst development rates compared to no HBP substrate supplementation in Chapter 3). Maturation medium for all groups contained 5.55 mM glucose. There was no significant difference between COCs cultured in the absence of glucose and glucosamine during collection for 0.5 or 1.5 hours, however the 1.5 hour group trended toward a lower blastocyst rate (38.4 ± 11.6 % vs. 22.9 ± 2.9 % respectively). When COCs were held for 1.5 hours, glucosamine supplementation significantly increased the blastocyst rate compared to no supplementation (62.2 ± 5.7 % vs. 22.9 ± 2.9 %, $p < 0.05$), as did glucose supplementation although not significantly (46.4 ± 7.9%). These results suggest that the time spent in collection medium does have an impact on the developmental competence of the COC and that even 0.5 hours can make a difference. This is further dependent on whether or not HBP substrates are provided during this time.

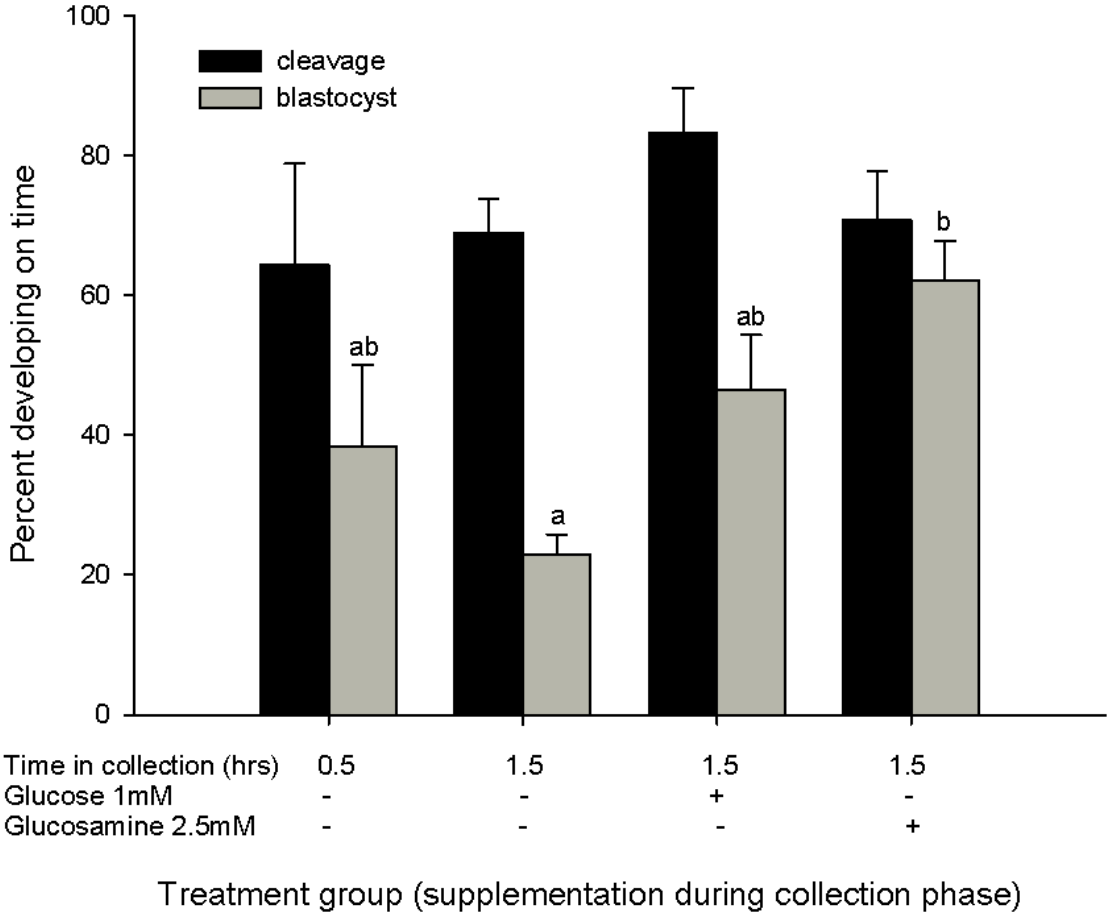

Figure 9.3 Effect of time in collection and hexose substrates on embryo development
Cleavage rate was assessed on Day 2 and blastocyst rate on Day 5, following collection in media containing either 0 or 1 mM glucose and 0 or 2.5 mM glucosamine. Maturation medium was standard containing 5.55 mM glucose. Average number of cumulus-oocyte complexes (COCs) matured per group was 41, n = 2 replicates. Groups with different superscripts differ significantly ($p < 0.05$).

APPENDIX 3

ADDITIONAL WESTERN BLOTS, MASS SPECTROMETRY REPORT AND 17AAG PRELIMINARY WORK

10.1 CTD110.6 WESTERN BLOTS

10.1.1 Buffers

Tris-buffered saline, high Tween 20 (TBS-HT)

150 mM NaCl

10 mM Tris-HCl pH 7.5

0.3 % v/v Tween 20

Made up in Milli Q H_2O

Tris-buffered saline with deoxycholic acid (TBS-HD)

150 mM NaCl

10 mM Tris-HCl pH 7.5

1.0 % v/v Triton X-100

0.1 % w/v SDS

0.25 % w/v Deoxycholic acid

Made up in Milli Q H_2O

10.1.2 Protocol

The protocol for CTD110.6 Western blots was modified from (Zachara et al. 2002). Blocking was achieved using a high concentration (3 %) of Tween 20, which has been shown to be a substitute for milk or bovine serum albumin (BSA) (Batteiger et al. 1982), since some milk proteins are β-O-linked glycosylation (O-GlcNAcylated) and BSA is used as a control in these Western blots.

1. Remove blots from transfer tank, rinse 1 x 5 min with TBS-HT and block for 1 h with TBS-HT at room temperature (RT)

2. Incubate blots overnight at 4 °C on rocking platform with primary antibody (CTD110.6, Covance, NJ, USA) diluted 1/1000 in TBS-HT

3. Wash blots 2 x 10 min with TBS-HD at RT

4. Wash blots 3 x 10 min with TBS-HT at RT

5. Incubate blots for 50 min at RT with secondary antibody (alkaline phosphatase-conjugated anti-mouse IgM (Rockland, PA, USA) diluted 1/2500 in TBS-HT

6. Wash blots 2 x 10 min with TBS-HD at RT

7. Wash blots 3 x 10 min with TBS-HT at RT

8. Develop using Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega, WI, USA)

10.1.3 Competitive Inhibition of CTD110.6 for Western Blot

To ensure that CTD110.6 was specific for O-GlcNAc in cumulus-oocyte complex (COC) samples, a competitive inhibition Western blot was performed. The primary antibody incubation step was performed ± 15.4 mM free GlcNAc. The free GlcNAc was able to eliminate any signal from the COC sample and greatly reduce the signal from the positive control, confirming the specificity of CTD110.6 for O-GlcNAc (Fig. 9.1). This experiment was performed during preliminary workup according to the competitive inhibition protocol previously published by the authors of the original CTD110.6 paper (Comer et al. 2001), and accordingly the secondary antibody used for this Western was goat anti-mouse IgM peroxidase for chemiluminescence.

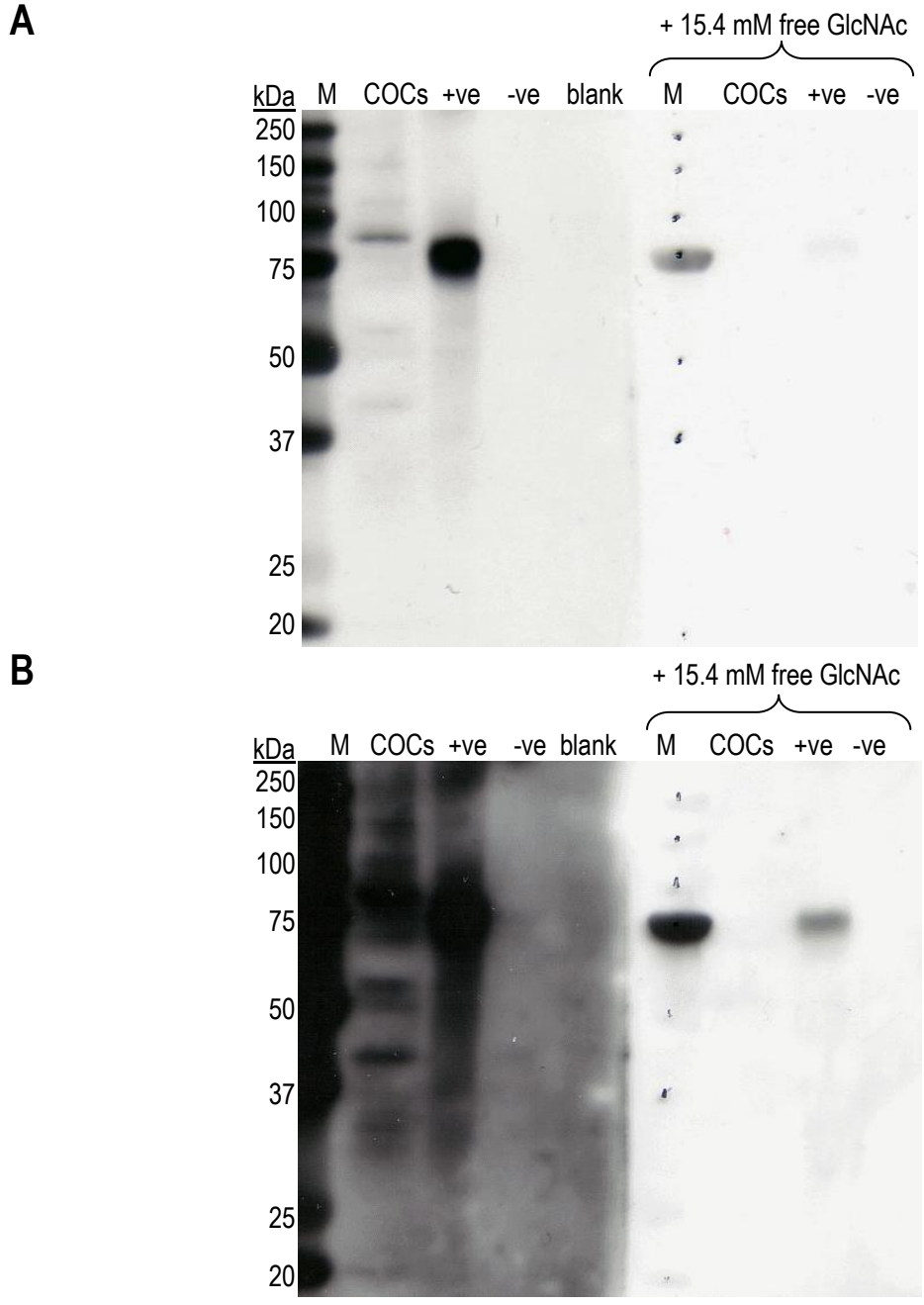

Figure 10.1 Competitive inhibition of CTD110.6 staining on COC samples

Cumulus-oocyte complexes (COCs) (50 per lane) were collected at 0 h and snap frozen. Proteins were separated using SDS-PAGE, Western blotted and stained using CTD110.6. Positive control (+ve) was BSA-GlcNAc, negative controls (-ve) were BSA and ovalbumin. M denotes molecular weight markers. Competitive inhibition (right half of each membrane) was achieved by performing the primary antibody incubation in the presence of 15.4 mM free GlcNAc. **A)** Two minute exposure of film to membrane. **B)** 20 minute exposure of film to membrane.

10.1.4 Densitometry Analysis of β-O-linked Glycosylation in COCs Throughout Maturation ± Glucosamine and BADGP

10.1.4.1Analysis of Western Blot in Fig. 4.1 A Including All Protein Sections

For results in Chapter 4 (Fig. 4.1 B) densitometry analysis was carried out on proteins detected above 75 kDa, to ensure exclusion of any IgM antibody bands and because these proteins were the clearest on developing the Westerns, but densitometry was also calculated using protein bands in sections between antibody bands (Fig. 10.2) and this produced the same pattern of intensity. However, due to higher variability the significance was lost.

10.1.4.2 Immunoprecipitation Using RL2

When immunoprecipitation was performed prior to Western blotting in experiments in Chapter 4, the antibody fragments themselves were visible on the Western blot. This is a well documented phenomenon, however since the same amount of antibody was used for each immunoprecipitation sample the antibody bands were expected to be consistent and this was not the case when using CTD110.6 as the immunoprecipitation antibody. To investigate this further, the same experiment (Fig. 4.1 A) was performed using RL2 as the antibody. Groups of 50 COCs were collected after 0 h, 6 h, 12 h or 18 h of in vitro maturation and snap frozen. Standard collection and maturation media were used (0.5 mM and 5.55 mM glucose respectively) and groups 2 and 3 were supplemented with 2.5 mM glucosamine in maturation. Group 3 also included 2.5mM benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP), an inhibitor of O-linked β-N-acetylglucosaminyltransferase (OGT, the enzyme which attaches O-GlcNAc to proteins). When RL2 was used, the antibody bands appear consistent in strength on the Western blot (Fig. 9.3), supporting the idea that the uneven bands observed with CTD110.6 are antibody-specific, possibly due to conformational change of the antibody upon antigen (O-GlcNAcylated protein) binding (see section 4.4).

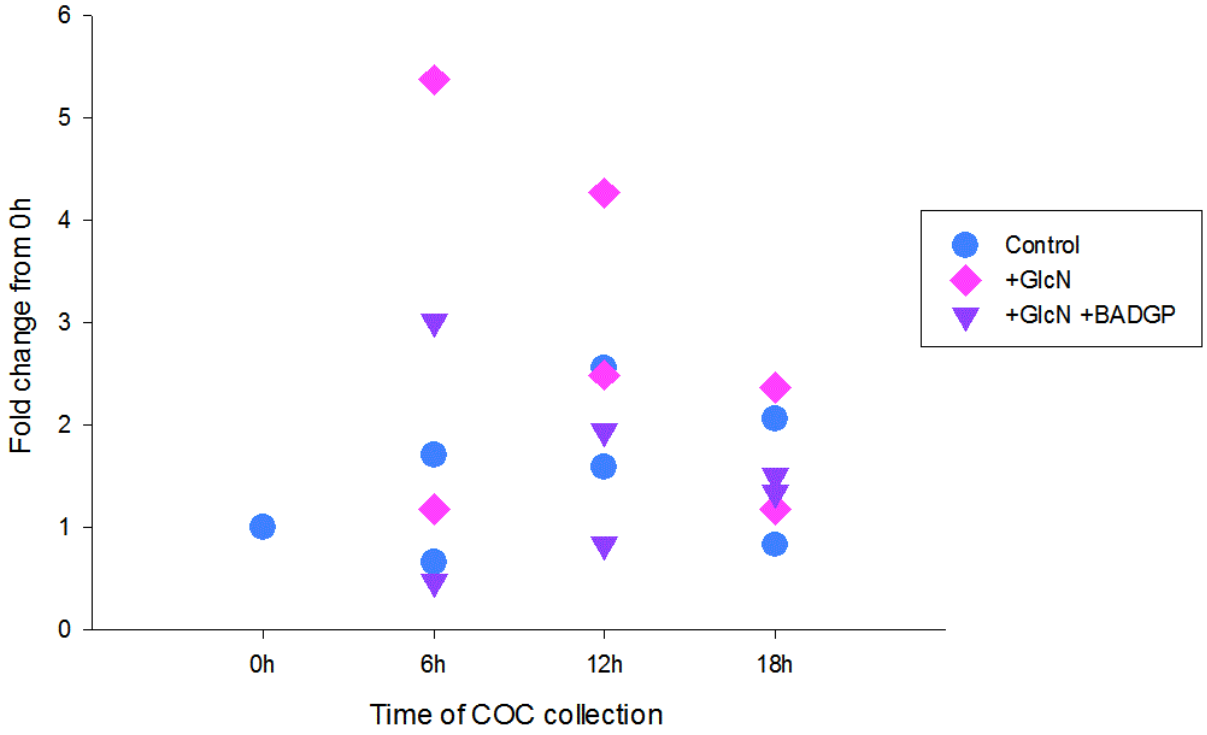

Figure 10.2 Densitometry analysis of Western blots of O-GlcNAcylation in COCs throughout IVM ± glucosamine and BADGP

Cumulus-oocyte complexes (COCs) were collected at 0 h, 6 h, 12 h or 18 h in vitro maturation (IVM) in control medium ± 2.5 mM glucosamine (GlcN) and 2.5 mM benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP). Proteins were immunoprecipitated using CTD110.6 for O-GlcNAc, separated using SDS-PAGE, Western blotted and stained using CTD110.6. (Fig. 4.1 A). Densitometry analysis of bands from the Western blots was carried out using ImageJ version 1.44p (Rasband 1997 - 2011), and represents all protein bands shown in yellow boxes. n = 2 replicates with 50 COCs per treatment group.

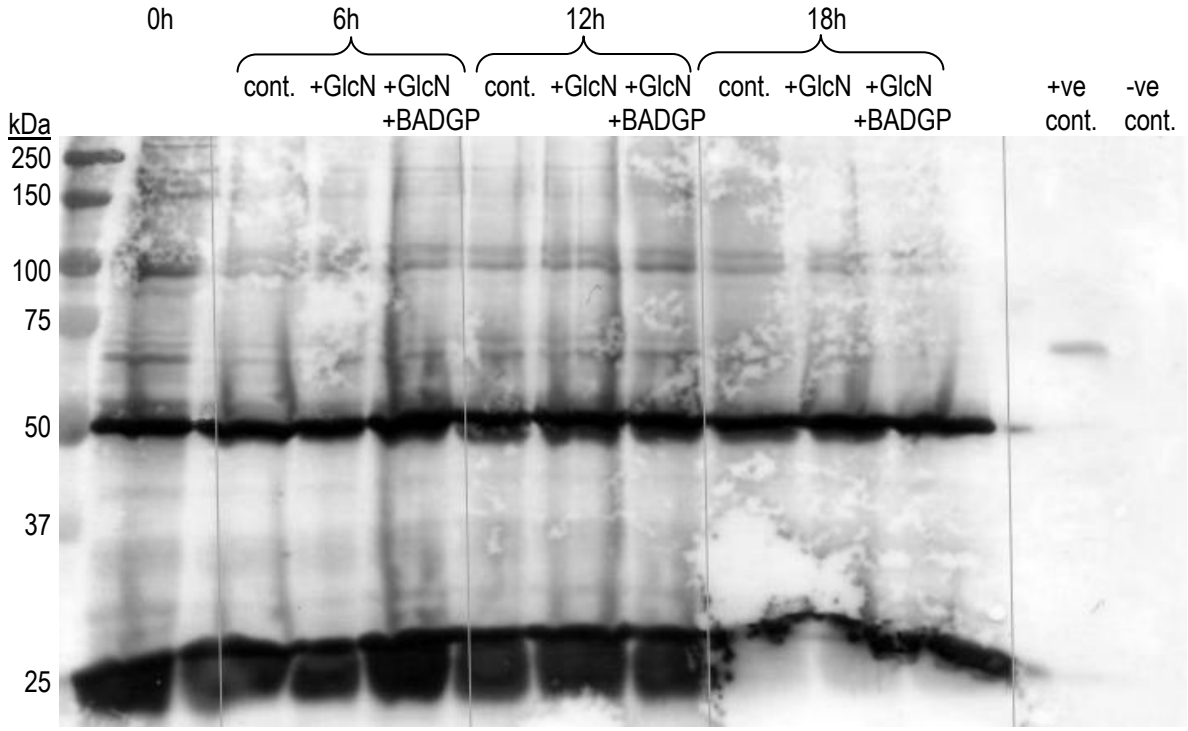

Figure 10.3 Western blot of O-GlcNAcylation in COCs throughout IVM ± glucosamine and BADGP

Cumulus-oocyte complexes (COCs) (50 per lane) were collected at 0 h, 6 h, 12 h or 18 h in vitro maturation (IVM) in control medium ± 2.5 mM glucosamine (GlcN) and 2.5 mM benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP). Proteins were immunoprecipitated using RL2 for O-GlcNAc, separated using SDS-PAGE, Western blotted and stained using RL2. Positive control (+ve) was BSA-GlcNAc, negative controls (-ve) were BSA and ovalbumin. IgG bands are clearly visible at 25 and 50 kDa.

10.2 HEAT-SHOCK PROTEIN 90 (HSP90) WESTERN BLOTS

10.2.1 Buffers

Tris-buffered saline with Tween 20 (TBST)

150 mM NaCl

50 mM Tris-HCl pH 7.5

0.05 % v/v Tween 20

Made up in Milli Q H_2O

Blocking solution

5 % skim milk powder (Woolworths Limited, NSW, Australia)

Made up in TBST

10.2.2 Staining Protocol for HSP90

1. Remove blots from transfer tank, rinse 1 x 5 min with TBST and block for 1 h with blocking solution at room temperature (RT)

2. Incubate blots overnight at 4 °C on rocking platform with primary antibody (anti-HSP90 BD Biosciences, NJ, USA) diluted 1/1000 in TBS-HT

3. Wash blots 3 x 5 min with TBST at RT

4. Incubate blots for 1 h at RT with secondary antibody (goat polyclonal antibody to mouse IgG (HRP-conjugated, abcam, Cambridge, UK)), diluted 1/5000 in blocking solution

6. Wash blots 3 x 5 min with TBST at RT

7. Develop using Amersham enhanced chemiluminescence (ECL) kit and hyperfilm (GE Healthcare, Buckinghamshire, UK)

10.3 MASS SPECTROMETRY REPORT FROM ADELAIDE PROTEOMICS

Protein Identification Report

16 November 2010

ADELAIDE PROTEOMICS CENTRE

For: Laura Frank
Job: 10-066

Sample preparation

Bands were excised from the gel manually (Figure 1), washed in 500 μL of 50 mM ammonium bicarbonate (NH_4HCO_3) and processed as follows:

- Destained with 30 mM potassium ferricyanide and 100 mM sodium thiosulfate
- Reduced with 0.5 μmol dithiothretiol (DTT) in 100 mM ammonium bicarbonate
- Alkylated with 2.75 μmol iodoacetamide (IAA) in 100 mM ammonium bicarbonate
- Digested with 100 ng of sequencing grade modified trypsin (Promega) in 5 mM ammonium bicarbonate + 10% acetonitrile (ACN)
- Extracted with 1% formic acid (FA) in water, 1%FA in 50% ACN and 100% ACN

The volumes of the resulting peptide extracts were reduced by vacuum centrifugation to approximately 1 μL.

Liquid chromatography-electrospray ionisation ion-trap (LC-eSI-IT) mass spectrometry

Data acquisition

Vacuum concentrated samples were resuspended with 0.1%FA in 2% ACN to total volume of ~8 μL. LC-eSI-IT MS/MS was performed using an online 1100 series HPLC system (Agilent Technologies) and HCT Ultra 3D-Ion-Trap mass spectrometer (Bruker Daltonics). The LC system was interfaced to the MS using an Agilent Technologies Chip Cube operating with a ProtID-Chip-150 (II), which integrates the enrichment column (Zorbax 300SB-C18, 4 mm, 40 nL), analytical column (Zorbax 300 SB-C18, 150 mm x 75 μm), and nanospray emitter. Five microlitres of sample was loaded on the enrichment column at a flow rate of 4 μL/min in Mobile Phase A (0.1% FA in 2% v/v ACN) and resolved with 1-30% gradient of Mobile Phase B (0.1% FA in 98% w/v CAN) over 32 minutes at 300 nL/min. Ionizable species ($300 < m/z < 2{,}200$) were trapped and the two most intense ions eluting at the time were fragmented by collision-induced dissociation. Active exclusion was used to exclude a precursor ion for 15 seconds following the acquisition of two spectra.

Data analysis

MS and MS/MS spectra were subjected to peak detection and de-convolution using DataAnalysis (Version 3.4, Bruker Daltonics). Compound lists were exported into BioTools (Version 3.1, Bruker Daltonics) then submitted to Mascot (Version 2.2). The specifications were as shown in Table 1.

Peter Hoffmann 8303 5507 Peter.Hoffmann@adelaide.edu.au
Megan Penno 8303 4903 Megan.Penno@adelaide.edu.au
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School of Molecular & Biomedical Science
Molecular Life Science building 1.50
University of Adelaide, SA, 5005

Results

The protein identities of the spots obtained by LC-eSI-IT MS are summarised in Table 1. This Table includes the following information:

- The name of the identified protein and the organism from which the protein was identified. Proteins are ranked by their combined ion scores (i.e. the sum of individual peptide scores for the protein). It is not always possible to uniquely identify a single protein from a set of detected peptides. Where a set of peptides identifies multiple proteins, all matching protein identifiers are listed.
- The accession number of the protein in the database
- The combined ion score for all queries (i.e., MS/MS spectra) matched to a single protein.
- The number of queries that were matched to a single protein and the total number of queries resulting from that LC-eSI-IT MS run.
- The sequences of the identified peptides. A peptide is bold weighted if it is it's first appearance in the protein summary for the sample. Peptides shown in grey have already been reported in higher placing protein identifications.
- The independent ion scores for each of the matched peptides and the cut off score, whereby peptides with ion scores above this threshold indicate identity or extensive homology.
- The exponentially modified protein abundance index (emPAI value) as an approximate measure of relative quantitation. This is calculated from the number of observed peptides relative to that predicted from the matched sequence. In general, higher scores indicate greater relative abundance in that sample (i.e., the gel spot or band). Developed by Ishihama and colleagues, the key publication is Ishihama *et al*, 2005, Molecular & Cellular Proteomics, 4, 1265-1272.
- The predicted molecular weight (MW) and isoelectric point (pI) of the matched protein based on the values provided in the MASCOT Summary Report

Protein identifications were made on the basis of having at least two matching unique peptides with individual ion scores above the specified threshold. These unique peptides were required to have different sequences or different variations of the same sequence, for example, containing a modified residue or missed cleavage site. Multiple charge states were not considered as unique. Identities assigned based on only a single peptide hit should be considered as tentative and further experimental evidence is required to confirm their presence in the sample.

Report prepared by

Note:
All samples (gels, digests, etc.) not completely used for the analysis are stored at the APC for 12 months and then discarded.

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Figure 1

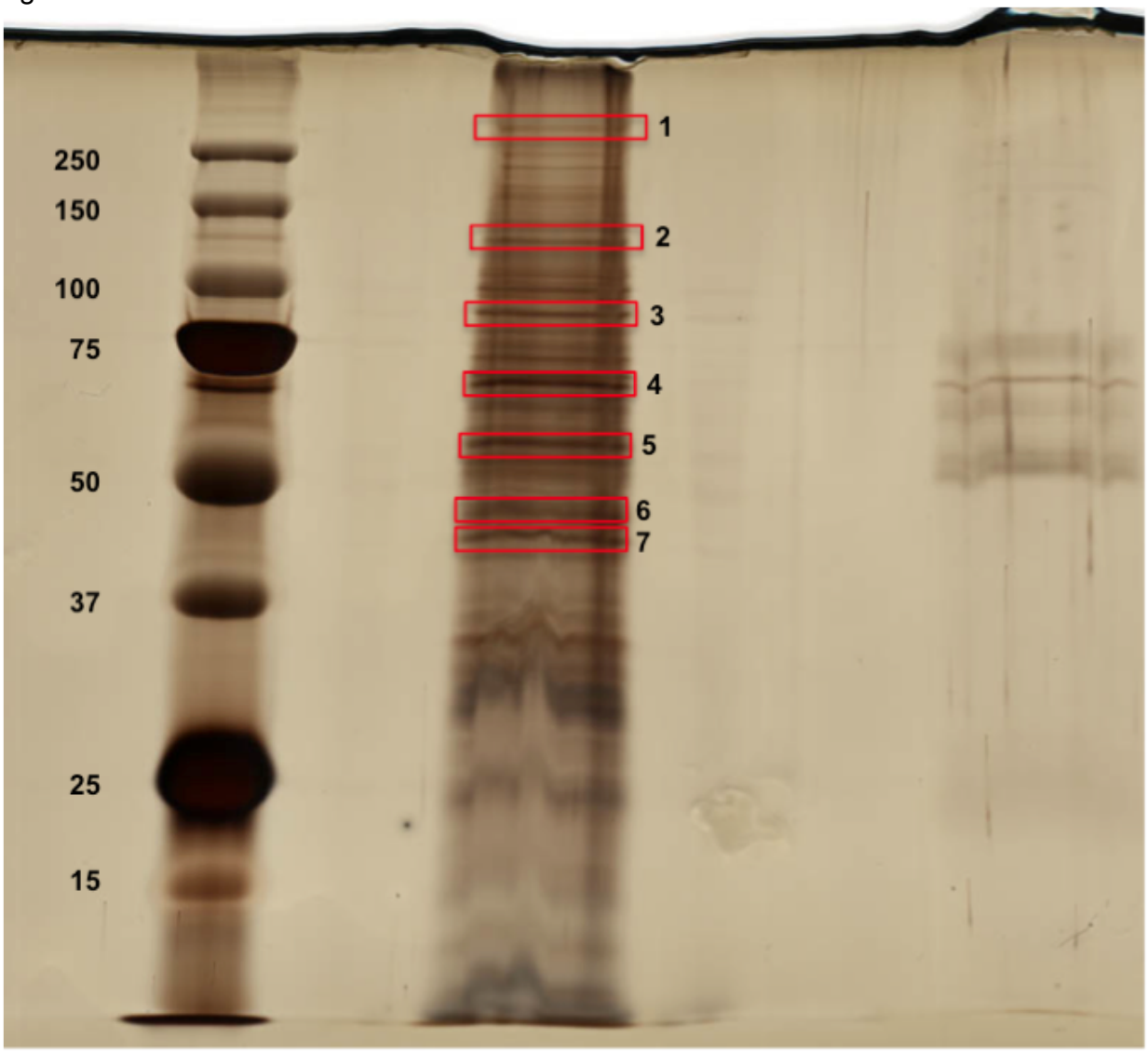

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Table 1

Combined LC-eSI-IT MS MASCOT search results summary.

Sample reference: 1

Mascot parameters
Database: SwissProt
Taxonomy: Mus musculus (house mouse)
Enzyme: semiTrypsin (2 missed cleavages)
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Mass tolerances: MS 0.3Da, MS/MS 0.4Da (Monoisotopic)

Protein	Accession	Predicted MW(kDa) / pI	ID / Total queries	Combined IonScore	Sequence coverage (%)	emPAI
Fibronectin [Mus musculus]	FINC_MOUSE	276.0 / 5.4	20 / 530	343	8	0.1

Peptide sequences	IonScore (Identity / homology threshold)
R.GEWACIPYSQLRDQCIVDDI.T	1(40/16)
R.HALQSASAGSGSFTDVR.T	26(41/26)
R.FTNIGPDTMR.V + Oxidation (M)	12(42/19)
R.EESPPLIGQQATVSDIPR.D	25(41/19)
V.SIIAVNGR.E	7(38/17)
R.PAQGVITTLENVSPPR.R	16(42/15)
R.GDSPASSKPVSINYK.T	7(41/20)
K.ATGVFTTLQPLR.S	46(42/36)
R.TVLVTWTPPR.A	29(42/36)
R.SSPVIIDASTAIDAPSNLR.F	69(39/38)
R.WLPSTSPVTGYR.V	14(42/19)
K.TEIDKPSQMQVTDVQDNSISVR.W + Oxidation (M)	27(41/22)
R.ITYGETGGNSPVQEFTVPGSK.S	36(41/23)
R.APITGYIIR.H	58(40/35)

Sample reference: 6

Mascot parameters
Database: SwissProt
Taxonomy: Mus musculus (house mouse)
Enzyme: semiTrypsin (2 missed cleavages)
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Mass tolerances: MS 0.3Da, MS/MS 0.4Da (Monoisotopic)

Protein	Accession	Predicted MW(kDa) / pI	ID / Total queries	Combined IonScore	Sequence coverage (%)	emPAI
Elongation factor 1-alpha 1 [Mus musculus]	EF1A1_MOUSE	50.4 / 9.1	5 / 530	115	7	0.2
Elongation factor 1-alpha 2 [Mus musculus]	EF1A2_MOUSE	50.8 / 9.1			7	

Peptide sequences	IonScore (Identity / homology threshold)
K.IGGIGTVPVGR.V	59(41/36)
R.LPLQDVYK.I	36(42/41)
R.EHALLAYTLGVK.Q	24(40/22)

Sample reference: 2

Mascot parameters
Database: SwissProt
Taxonomy: Mus musculus (house mouse)
Enzyme: semiTrypsin (2 missed cleavages)
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Mass tolerances: MS 0.3Da, MS/MS 0.4Da (Monoisotopic)

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Protein	Accession	Predicted MW(kDa) / pI	ID / Total queries	Combined IonScore	Sequence coverage (%)	emPAI
Zona pellucida sperm-binding protein 2 [Mus musculus]	ZP2_MOUSE	81.4 / 6.1	6 / 589	101	4	0.0
Peptide sequences					**IonScore (Identity / homology threshold)**	
K.VVGGYQVNIR.V					56(42/34)	
K.FPYETCTIK.V					19(42/22)	
K.TFAFVSEAR.G					29(42/31)	

Sample reference: 7

Mascot parameters
Database: SwissProt
Taxonomy: Mus musculus (house mouse)
Enzyme: semiTrypsin (2 missed cleavages)
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Mass tolerances: MS 0.3Da, MS/MS 0.4Da (Monoisotopic)

Protein	Accession	Predicted MW(kDa) / pI	ID / Total queries	Combined IonScore	Sequence coverage (%)	emPAI
Actin, cytoplasmic 1 [Mus musculus]	ACTB_MOUSE	42.1 / 5.3	29 / 632	332	28	0.8
Actin, cytoplasmic 2 [Mus musculus]	ACTG_MOUSE	42.1 / 5.3			28	
Peptide sequences					**IonScore (Identity / homology threshold)**	
K.EITALAPSTMK.I					42(42/34)	
K.QEYDESGPSIVHR.K					40(42/30)	
R.AVFPSIVGRPR.H					29(40/26)	
R.VAPEEHPVLLTEAPLNPK.A					37(41/28)	
R.KDLYANTVLSGGT.T					6(41/21)	
R.DLTDYLMK.I + Oxidation (M)					13(42/26)	
K.SYELPDGQVITIGNER.F					59(41/35)	
V.FPSIVGRPR.H					48(41/-)	
K.EITALAPSTMK.I + Oxidation (M)					41(42/37)	
R.GYSFTTTAER.E					39(42/27)	
R.LDLAGRDLTDYLMK.I + Oxidation (M)					29(41/29)	

Sample reference: 3

Mascot parameters
Database: SwissProt
Taxonomy: Mus musculus (house mouse)
Enzyme: semiTrypsin (2 missed cleavages)
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Mass tolerances: MS 0.3Da, MS/MS 0.4Da (Monoisotopic)

Protein	Accession	Predicted MW(kDa) / pI	ID / Total queries	Combined IonScore	Sequence coverage (%)	emPAI
Heat shock protein HSP 90-alpha [Mus musculus]	HS90A_MOUSE	85.1 / 4.9	30 / 588	633	20	0.4
Peptide sequences					**IonScore (Identity / homology threshold)**	
R.NPDDITNEEYGEFYK.S					97(41/34)	
K.ADLINNLGTIAK.S					60(41/36)	
R.YYTSASGDEMVSLK.D + Oxidation (M)					26(42/23)	
R.TLTIVDTGIGMTK.A + Oxidation (M)					93(41/36)	
R.ELISNSSDALDKIR.Y					29(42/31)	
R.ALLFVPR.R					26(42/29)	
K.DQVANSAFVER.L					63(42/34)	
R.GVVDSEDLPLNISR.E					78(41/-)	
K.ELHINLIPSK.Q					10(42/23)	
R.ELISNSSDALDK.I					44(42/30)	
K.HIYFITGETK.D					34(42/38)	

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K.FYEQFSK.N	28(42/35)
K.EGLELPEDEEEK.K	20(42/25)
R.APFDLFENR.K	52(42/37)

Protein	Accession	Predicted MW(kDa) / pI	ID / Total queries	Combined IonScore	Sequence coverage (%)	emPAI
Heat shock protein HSP 90-beta [Mus musculus]	HS90B_MOUSE	83.6 / 5.0	24 / 588	450	17	0.3
Peptide sequences				**IonScore (Identity / homology threshold)**		
R.APFDLFENKK.K				7(42/18)		
K.ADLINNLGTIAK.S				60(41/36)		
R.TLTLVDTGIGMTK.A + Oxidation (M)				93(41/36)		
R.GVVDSEDLPLNISR.E				78(41/-)		
K.IDILPNPQER.T				23(41/33)		
S.GDEMTSLSEYVSR.M				2(40/18)		
R.ELISNASDALDKIR.Y				1(41/19)		
R.APFDLFENK.K				9(42/17)		
R.RAPFDLFENK.K				50(41/-)		
R.ELISNASDALDK.I				62(42/32)		
K.EGLELPEDEEEK.K				20(42/25)		
K.SIYYITGESK.E				36(42/37)		
R.NPDDITQEEYGEFYK.S				29(41/23)		

Protein	Accession	Predicted MW(kDa) / pI	ID / Total queries	Combined IonScore	Sequence coverage (%)	emPAI
Zona pellucida sperm-binding protein 3 [Mus musculus]	ZP3_MOUSE	47.2 / 6.1	4 / 588	110	8	0.1
Peptide sequences				**IonScore (Identity / homology threshold)**		
R.VSVDTDVVR.F				35(42/38)		
K.LVQPGDLTLGSEGCQPR.V				48(40/19)		
R.NTLYITCHLK.V				27(42/29)		

Sample reference: 4

Mascot parameters
Database: SwissProt
Taxonomy: Mus musculus (house mouse)
Enzyme: semiTrypsin (2 missed cleavages)
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Mass tolerances: MS 0.3Da, MS/MS 0.4Da (Monoisotopic)

No identification

Sample reference: 5

Mascot parameters
Database: SwissProt
Taxonomy: Mus musculus (house mouse)
Enzyme: semiTrypsin (2 missed cleavages)
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Mass tolerances: MS 0.3Da, MS/MS 0.4Da (Monoisotopic)

Protein	Accession	Predicted MW(kDa) / pI	ID / Total queries	Combined IonScore	Sequence coverage (%)	emPAI
Protein disulfide-isomerase A3 [Mus musculus]	PDIA3_MOUSE	57.1 / 5.9	27 / 616	631	46	1.0
Peptide sequences				**IonScore (Identity / homology threshold)**		
R.LAPEYEAAATR.L				49(42/32)		
R.FAHTNIESLVK.E				7(42/20)		
K.LNFAVASR.K				43(42/-)		
K.YGVSGYPTLK.I				56(42/36)		
K.QAGPASVPLRTEEEFKK.F				33(41/16)		
A.SDVLELTDENFESR.V				85(41/40)		

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K.QAGPASVPLR.T	31(41/27)
R.DLFSDGHSEFLK.A	45(42/-)
R.YLKSEPIPESNEGPVK.V	2(41/14)
K.IFRDGEEAGAYDGPR.T	11(42/22)
K.VVVAENFDDIVNEEDK.D	49(41/21)
K.MDATANDVPSPYEVK.G + Oxidation (M)	47(42/29)
R.ELNDFISYLQR.E	54(41/32)
-.MRFSCLALLPGVAL.L + Oxidation (M)	7(39/21)
K.GFPTIYFSPANK.K	31(42/31)
K.RLAPEYEAAATR.L	22(42/21)
K.DASVVGFFR.D	27(41/36)
R.LKGIVPLAKVDC.T	5(39/22)
R.EATNPPIIQEEKPK.K	12(42/24)
K.FISDKDASVVGFFR.D	4(41/17)
K.FVMQEEFSR.D + Oxidation (M)	38(42/39)

Protein	Accession	Predicted MW(kDa) / pI	ID / Total queries	Combined IonScore	Sequence coverage (%)	emPAI
Glucose-6-phosphate 1-dehydrogenase X [Mus musculus]	G6PD1_MOUSE	59.7 / 6.1	7 / 616	114	14	0.1
Peptide sequences				**IonScore (Identity / homology threshold)**		
K.LEEFFAR.N				11(41/24)		
R.NSYVAGQYDDAASYK.H				60(41/23)		
R.GGYFDEFGIIR.D				11(42/20)		
K.EMVQNLMVLR.F + 2 Oxidation (M)				10(42/21)		
R.DGLLPEDTFIVGYAR.S				26(41/27)		
Y.VYGSRGPTEADELMK.R				3(42/24)		

Protein	Accession	Predicted MW(kDa) / pI	ID / Total queries	Combined IonScore	Sequence coverage (%)	emPAI
Tubulin alpha-1A chain [Mus musculus]	TBA1A_MOUSE	50.8 / 4.9	5 / 616	89	10	0.1
Tubulin alpha-1B chain [Mus musculus]	TBA1B_MOUSE	50.8 / 4.9			10	
Tubulin alpha-1C chain [Mus musculus]	TBA1C_MOUSE	50.6 / 5.0			10	
Peptide sequences				**IonScore (Identity / homology threshold)**		
R.AVFVDLEPTVIDEVR.T				44(41/25)		
K.DVNAAIATIK.T				20(42/20)		
K.VGINYQPPTVVPGGDLAK.V				29(40/23)		

Protein	Accession	Predicted MW(kDa) / pI	ID / Total queries	Combined IonScore	Sequence coverage (%)	emPAI
Protein disulfide-isomerase [Mus musculus]	PDIA1_MOUSE	57.5 / 4.8	5 / 616	83	8	0.1
Peptide sequences				**IonScore (Identity / homology threshold)**		
K.THILLFLPK.S				16(39/27)		
R.EADDIVNWLK.K				14(42/23)		
K.VDATEESDLAQQYGVR.G				54(41/23)		
E.ALAAHK.Y				3(40/21)		

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10.4 PRELIMINARY WORK FOR 17AAG EXPERIMENTS

10.4.1 Dose-response of 17AAG

To determine an appropriate dose of 17-(Allylamino)-17-demethoxygeldanamycin (17AAG) supplementation, a dose-response experiment was carried out by testing doses of 0.01, 0.1, 1.0 or 10.0 µM 17AAG during the maturation phase of IVM in the presence of glucosamine (Fig. 9.4). Standard collection and maturation media were used (0.5 mM and 5.55 mM glucose respectively), and glucosamine (2.5 mM) added to all groups except control. Following this experiment, 0.1 µM 17AAG was chosen as the dose to use for future experiments, as in the presence of glucosamine it increased the blastocyst rate significantly compared to no treatment (40.8 ± 5.0% vs. 26.0 ± 3.8% respectively, $p < 0.05$). Doses of 17AAG higher than 0.1 µM caused a significant decrease in cleavage rate ($p < 0.05$ vs. all other groups) and 10 µM decreased the blastocyst rate significantly compared to all other groups (1.7 ± 1.7%, $p < 0.05$).

10.4.2 Toxicity Testing of 17AAG

After an appropriate dose of 17AAG had been found (see section 9.4.1), I tested the effect of 17AAG treatment under control conditions. Standard collection and maturation media were used (0.5 mM and 5.55 mM glucose respectively), and 17AAG added to the second group at a concentration of 0.1 µM. This experiment revealed no effect of 17AAG on embryo development under control conditions (Fig. 9.5).

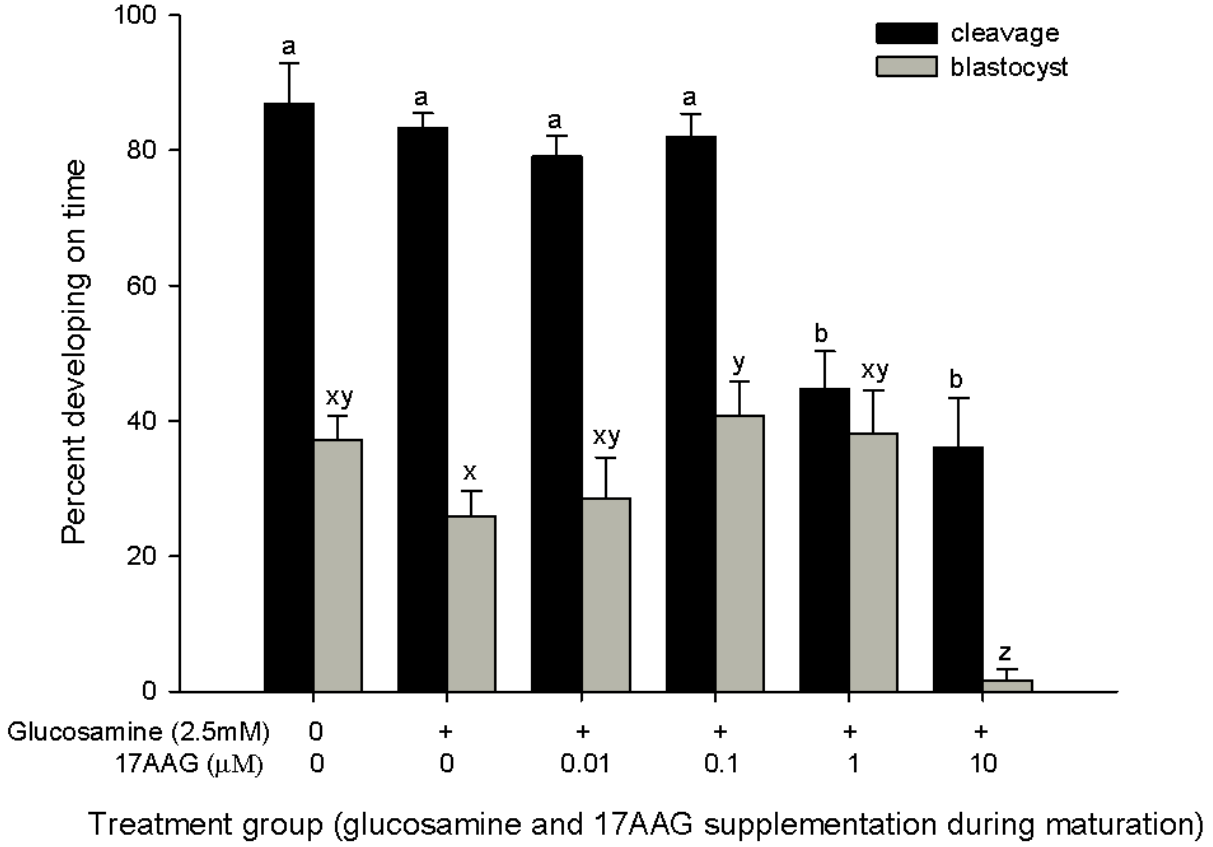

Figure 10.4 Cleavage and blastocyst development following inhibition of HSP90 during IVM ± glucosamine

Cleavage rate was assessed on Day 2 and blastocyst rate on Day 5, following collection and in vitro maturation (IVM) in control medium ± glucosamine (2.5 mM) and 17-(Allylamino)-17-demethoxygeldanamycin, an inhibitor of heat-shock protein 90 (HSP90) (17AAG, doses as shown). Groups with different superscripts differ significantly ($p < 0.05$). Average number of cumulus-oocyte complexes (COCs) matured per group per replicate was 29, n = 5 replicates.

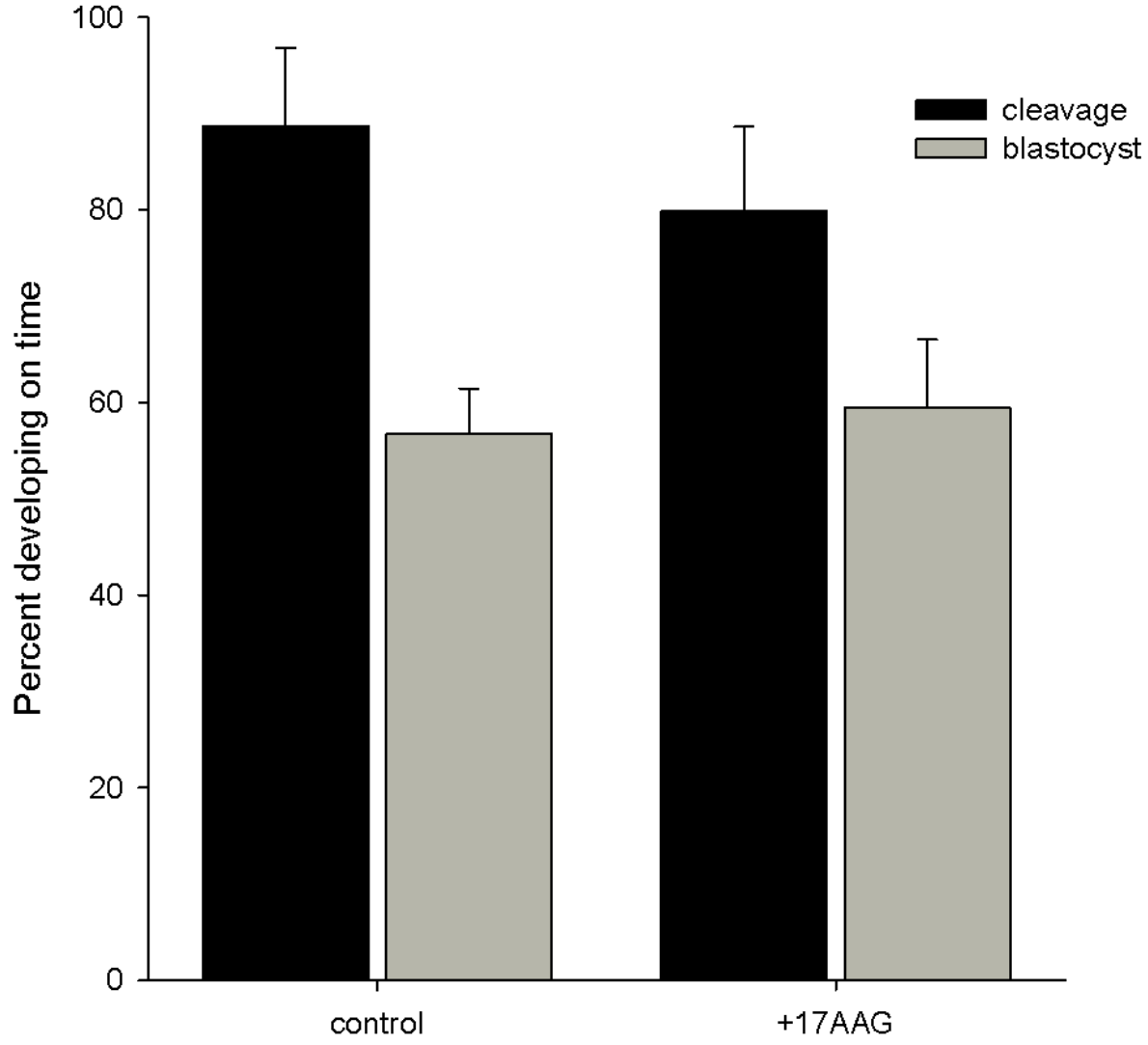

Figure 10.5 Cleavage and blastocyst development following inhibition of HSP90 during IVM
Cleavage rate was assessed on Day 2 and blastocyst rate on Day 5, following collection and in vitro maturation (IVM) in control medium ± 17-(Allylamino)-17-demethoxygeldanamycin, an inhibitor of heat-shock protein 90 (HSP90) (17AAG, 0.1 μM). Average number of cumulus-oocyte complexes (COCs) matured per group per replicate was 27, n = 3 replicates.