Title

The effect of peri-conception hyperglycaemia and the involvement of the hexosamine biosynthesis pathway in mediating oocyte and embryo developmental competence

Running title

Hyperglycaemia, the HBP and oocyte competence

Keywords

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Authors and affiliations

Laura A. Frank[1], Melanie L. Sutton-McDowall[1], Robert B. Gilchrist[1] and Jeremy G. Thompson[1,2]

[1]The Robinson Institute, The Research Centre for Reproductive Health, School of Paediatrics and Reproductive Health, The University of Adelaide, Australia.

Correspondence

[2]Corresponding author: Jeremy Thompson, The Robinson Institute, Ground Floor, Norwich Centre, 55 King William Road, North Adelaide, SA 5006, Australia. Email jeremy.thompson@adelaide.edu.au

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Abbreviations used

ATP	adenosine triphosphate
BADGP	b*enzyl-2-acetamido*-2-deoxy-α-D-galactopyranoside
COC	cumulus-oocyte complex
DAPI	4’,6-diamidino-2-phenylindole

DHEA	dehydroepiandrosterone
DNA	deoxyribonucleic acid
dpc	days post coitum
ER	endoplasmic reticulum
G6PDH	glucose-6-phosphate dehydrogenase
GFPT	glutamine:fructose-6-phosphate amidotransferase
GlcNAc	N-acetylglucosamine
GVBD	germinal vesicle breakdown
HBP	hexosamine biosynthesis pathway
IVM	in vitro maturation
LH	luteinising hormone
mM	millimolar
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
O-GlcNAc	β-O-linked N-acetylglucosamine
O-GlcNAcase	β-N-acetylglucosaminidase
O-GlcNAcylation	β-O-linked glycosylation
OGT	O-linked β-N-acetylglucosaminyltransferase
PI 3-K	phosphoinositide 3-kinase
PPP	pentose phosphate pathway
RNA	ribonucleic acid
ROS	reactive oxygen species
SP1	specificity protein 1
TCA	tricarboxylic acid
UDP-GlcNAc	uridine diphosphate-N-acetylglucosamine
UV	ultraviolet

Abstract

The environment that the oocyte is exposed to during the peri-conception period can have a significant impact on oocyte developmental competence (the ability of the oocyte to support fertilisation and subsequent embryo development) and the long-term health of the resulting offspring. This is particularly true for maternal hyperglycaemia. While maternal hyperglycaemia during early pregnancy and beyond has been extensively studied, the effects on the oocyte itself, and the underlying mechanisms, remain largely unknown. However, there is increasing evidence for the role of the fuel-sensing hexosamine biosynthesis pathway in mediating the effects of hyperglycaemia in many different cell types. In this review, we will focus on the reproductive consequences of maternal hyperglycaemia during the peri-conceptual period and the role of the hexosamine pathway in mediating these processes.

1. Introduction

Maternal Type I or Type II diabetes, in which blood glucose levels are elevated, have long been associated with higher risks of a multitude of pregnancy complications, including spontaneous abortions, neonatal morbidity and mortality and congenital malformations (Becerra et al. 1990; Cornblath and Schwartz 1976; Farrell et al. 2002; Greene 1999; Norman and Reynolds 2011; Sadler et al. 1988). Many studies have examined the effect of hyperglycaemia on the early embryo. However, even when good glycaemic control is achieved during the first few weeks of pregnancy, there is still a significant risk of pregnancy complications, including fetal abnormalities, for women with diabetes (Bell et al. 2012; Dunne et al. 1999; Lapolla et al. 2008b; Ray et al. 2001).

Studies in animal models (including a significant body of work by Kelle Moley and her group) have demonstrated that hyperglycaemia may lead to increased fertility complications even prior to conception. Embryos derived from diabetic mice are growth retarded, have higher levels of apoptosis and a decrease in glucose uptake, and oocytes are smaller, slower to complete meiotic maturation and have altered mitochondrial distribution compared to those from non-diabetic mice (Chang et al. 2005; Colton et al. 2002; Wang and Moley 2010; Wang et al. 2009). These observations also extend to the surrounding cumulus and granulosa cells, with a

higher number of apoptotic events observed and a decrease in cumulus cell glucose uptake (Chang et al. 2005; Dunning et al. 2010).

One of the primary mechanisms by which somatic cells are negatively affected by hyperglycaemia is through increased glucose metabolism through the hexosamine biosynthetic pathway (HBP) (Brownlee 2001). The HBP is a fuel-sensing pathway, which converts glucose to UDP-N-acetylglucosamine (UDP-GlcNAc). This moiety can be attached to serine or threonine residues of proteins and act in an analogous manner to phosphorylation to regulate protein function; a post-translational modification called β-O-linked glycosylation (Whelan and Hart 2003). Altered β-O-linked glycosylation is increasingly recognised as being associated with a number of disease states including cancer, inflammatory conditions and neurodegenerative diseases (Hart et al. 2007; Yang and Suh 2013). 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).

Pre-ovulatory cumulus-oocyte complexes (COCs) and early embryos exposed to hyperglycaemic conditions display increased levels of β-O-linked glycosylation and a subsequent decrease in developmental competence in the mouse, cow and pig (Pantaleon et al. 2010; Sutton-McDowall et al. 2006). However, the mechanisms underpinning the adverse effect of excess HBP flux on the developing oocyte and embryo are unknown.

This review will briefly describe glucose metabolism in the developing oocyte, the clinical and biological consequences of hyperglycaemia and the role of the HBP and β-O-linked glycosylation in reproductive physiology.

2. The effects of hyperglycaemia on female reproduction

The concentration of glucose in ovarian follicular fluid parallels that of plasma at a slightly lower level (Gardner and Leese 1990; Leese and Lenton 1990; mouse, human), and is positively correlated with follicle size (Nandi et al. 2008; Sutton-McDowall et al. 2005; buffalo, sheep, cow). 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 (Unwin et al. 2002). Obesity and poor diet (modelled in mice fed a high fat diet (Jungheim et al. 2010)) are associated with pre-diabetes, impaired glucose tolerance and hyperglycaemia. Importantly, increasing body mass index is correlated with increasing glucose levels in follicular fluid in humans (Robker et al. 2009).

2.1. Clinical consequences of hyperglycaemia on female reproduction

In humans, maternal diabetes is associated with poor conception, difficulty maintaining pregnancies and delivery of babies with health problems (Becerra et al. 1990; Holing et al. 1998; Jungheim and Moley 2010; Lapolla et al. 2008b; Purcell and Moley 2011; Rich-Edwards et al. 1994). Women with diabetes have higher risks of spontaneous first-trimester abortion, preeclampsia, preterm birth and high birth weight babies (Greene 1999; Hedderson et al. 2003; Middleton et al. 2010; Mills et al. 1988). Similarly, obese women have increased risks of preeclampsia and spontaneous abortions (Dokras et al. 2006; Metwally et al. 2008; O'Brien et al. 2003). Consequently, large birth weight babies born to women with diabetes are at increased risk of developing metabolic syndrome (including insulin resistance) in childhood (Boney et al. 2005; Silverman et al. 1995). 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 6 - 10 % of pregnancies in women with diabetes; 3 - 5 fold higher than the general population (Lapolla et al. 2008a; Reece and Homko 2000). The degree of risk for diabetes-associated complications in pregnancy is dependent on the level of glycaemic control achieved, with the level of glycosylated haemoglobin positively correlated with the incidence of fetal malformations (Guerin et al. 2007; Lapolla et al. 2008b; Miller et al. 1981; Suhonen et al. 2000). The incidence of fetal anomalies doubles to 5 % with a maternal fasting blood glucose level of 6.6 mM; below the threshold of a diagnosis of diabetes (7 mM), and further increases to 30 % with a maternal fasting blood glucose > 14.3 mM (Schaefer et al. 1997). The occurrences of neural tube defects and skeletal and cardiovascular malformations in particular, are 18 times more common in babies born to mothers

with diabetes (Becerra et al. 1990; Farrell et al. 2002; Greene 1999), or obesity (Horal et al. 2004; Stothard et al. 2009).

A hyperglycaemic insult must occur within the first 6 weeks of gestation to produce an increased likelihood of congenital anomalies, as the increase is not seen in women with gestational diabetes only (Mills et al. 1979), which is usually diagnosed around the 24^{th}-28^{th} week of pregnancy (Seshiah et al. 2007). The earlier glycaemic control can be achieved, the less risk there is of malformations (Miller et al. 1981). 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, embryos derived from donor diabetic mothers developed into fetuses that 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.

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 remains a 3 - 5 times higher risk of spontaneous abortions and congenital anomalies for babies born to women with diabetes (Baccetti et al. 2002; Bell et al. 2012; Casson et al. 1997; El-Sayed and Lyell 2001). However, if a preconception treatment program is undertaken this risk is significantly reduced (Dunne et al. 1999; Pearson et al. 2007; Ray et al. 2001).

2.2. Biological consequences of hyperglycaemia: embryos

Abnormally high or low rates of maternal metabolism affect embryo development (Fleming et al. 2004; Leese et al. 2008). Under hyperglycaemic conditions, glucose uptake and expression of glucose transporters are reduced in pre-implantation embryos (Moley 1999; Moley et al. 1998a; Moley et al. 1998b). 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 had a higher incidence of fragmentation and contained fewer inner cell mass cells than blastocysts from normoglycaemic mothers (Lea et al. 1996), supporting the notion that a legacy of brief and early exposure to hyperglycaemia has long term consequences; 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 very high glucose (27 mM) or glucosamine (0.2 mM), producing fewer blastocysts, with reduced cell numbers compared to controls, and with increased apoptosis (Pantaleon et al. 2010). High levels of glucose inhibit the expression in mouse embryos of *Pax-3*, a gene required for neural tube closure (Chalepakis et al. 1994).

2.3. Biological consequences of hyperglycaemia: oocytes

Most diabetic rodent studies focus on pre-implantation embryo development or the period of foetal organogenesis, around days 9-11 (Wyman et al. 2008). However, consistent with clinical studies highlighting the benefits of pre-conception care, hyperglycaemia causes numerous perturbations in oocyte structure and function prior to fertilisation.

Hyperglycaemia induces apoptosis in 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); such mice have significantly higher proportions of apoptotic cells within ovarian follicles compared to control mice (Chang et al. 2005); a result also observed in a streptozotocin-induced diabetic mouse model. Increased apoptosis within the ovary and COC may partly be 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 even under 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 outcomes (Arnot et al. 1995; Bergh et al. 1998; Nataprawira et al. 1992; Wittmaack et al. 1994). Mitochondria are the most abundant organelle in the mammalian oocyte and early embryo, and play a critical role in oocyte maturation and early embryo development through the provision of ATP (Van Blerkom 2011). Impairment of mitochondrial function in general is strongly associated with diabetes (Rolo and Palmeira 2006); a family history of diabetes was associated with decreased ATP synthesis even before the onset of impaired glucose tolerance (pre-diabetes) in some individuals (Petersen et al. 2004). The mitochondria in oocytes from diabetic mice have altered structure and distribution, reduced energy production (as measured by tricarboxylic acid (TCA) cycle metabolites) and an increased mitochondrial DNA copy number (Wang and Moley 2010; Wang et al. 2009); interestingly, the latter is usually associated with increased oocyte competence (El Shourbagy et al. 2006; Santos et al. 2006; Tamassia et al. 2004). These authors attributed the increased copy number to a compensatory mechanism to ensure the adequate supply of ATP, possibly due to increased mitochondrial demand. Structurally, mitochondria in oocytes from streptozotocin-induced and Akita strain diabetic mice have a narrowed intermembrane space compared to those from non-diabetic mice. Other abnormalities included completely ruptured outer membranes and swelling of the mitochondria, indicative of impending mitochondria-dependent apoptosis (Wang et al. 2009).

Several mitochondria-related parameters (ATP, ROS, pyruvate dehydrogenase) have been associated with meiotic spindle assembly and chromosome alignment in oocytes (Johnson et al. 2007; Zhang et al. 2006). Many studies have shown delayed and decreased completion of meiotic maturation in oocytes from diabetic mice (Chang et al. 2005; Colton et al. 2002; Diamond et al. 1989; 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). In mouse oocytes, the spindle defects are diverse but include extra asters and mono-polar

spindles, with chromosome misalignments manifesting as one or more chromosomes displaced from the equator (Cheng et al. 2011; Wang et al. 2009). Subsequently, Cheng et al. (2011) performed islet transplantation (containing the insulin-producing beta cells) from non-diabetic to diabetic mice, and were able to reverse the effects of maternal diabetes on the observed spindle defects, chromosome misalignments and incidence of aneuploidy.

The endoplasmic reticulum (ER) is especially important in maturing oocytes for its role in Ca^{2+} signalling for the completion of meiosis, and preventing polyspermy (Runft et al. 2002). The distribution of the ER changes during the course of oocyte maturation and early embryo development. This is disrupted in oocytes and embryos from diabetic mice, possibly contributing to the reduced oocyte quality associated with hyperglycaemia (Zhang et al. 2013). Many of the adverse effects of hyperglycaemia, such as insulin resistance, are mediated through the PI3-K pathway. However, this does not appear to be the case with oocyte developmental competence, as down-regulation of the PI3-K pathway using inhibitors in bovine oocytes during maturation was unable to replicate the detrimental effects observed under hyperglycaemic conditions (Sutton-McDowall et al. 2006).

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 levels of meiotic resumption, (Downs and Hudson 2000; Funahashi et al. 2008; Hashimoto et al. 2000; mouse, cow, pig), cumulus expansion (Frank et al. 2012; Nandi et al. 2008; mouse, buffalo, sheep) and embryo development (Hendryx and Wordinger 1979; Wongsrikeao et al. 2006; pig, mouse). Glucose concentration throughout development must be maintained at an optimal level, and excess or absence results in reduced oocyte developmental competence (Thompson 2006). This concept is supported by recent work with mouse (Frank et al. 2012) and buffalo (Kumar et al. 2013) in vitro-matured oocytes, showing that optimal embryo development is achieved using a relatively low glucose concentration (dependent on species; mouse and buffalo 1 mM and 5.6 mM respectively) while higher concentrations (up to 30 mM, and 10 mM, respectively), or the absence of glucose, decreased development.

3. Glucose metabolism in the maturing cumulus-oocyte complex (COC)

The two cell types within the COC have different metabolic activities and glucose is consumed almost exclusively by the cumulus cells, due to the higher expression of key glucose transporters such as the insulin-sensitive facilitative glucose transporter SLC2A4, which has a high affinity for glucose (Nishimoto et al. 2006; Roberts et al. 2004; Williams et al. 2001; cow, mouse sheep). There are several documented pathways for glucose metabolism in the COC (Fig. 1), and with the exception of the polyol pathway all of these begin with the phosphorylation of glucose to glucose-6-phosphate by hexokinase (Sutton-McDowall et al. 2010).

Glycolysis accounts for the majority of the glucose taken up by the COC (Downs and Utecht 1999; Kumar et al. 2013; mouse, buffalo), and the rate of phosphofructokinase (PFK; one of the rate-limiting enzymes of glycolysis) activity remains constant throughout oocyte maturation (Cetica et al. 2002; Colton et al. 2003; Sutton-McDowall et al. 2010; cow, mouse). Cumulus cells have high PFK activity (Downs et al. 1996; mouse) whereas the oocyte itself has low levels of glucose consumption and limited PFK activity (Cetica et al. 2002; Harris et al. 2009; mouse, cow); instead relying on the cumulus cells to supply it with carboxylic acids (Biggers et al. 1967; Sutton-McDowall et al. 2010 mouse, cow). Indeed, in bovine COCs, cumulus cells metabolise 23-fold more glucose per ml tissue per hour than oocytes (Thompson 2006). Pyruvate is the preferred nutrient of the oocyte itself for energy production, and it is metabolised via the TCA cycle, fuelling oxidative phosphorylation (Biggers et al. 1967; Steeves and Gardner 1999).

A recent study measuring ATP in single mouse oocytes (Dalton et al. 2013) found variation in the amount of ATP from mitrochondria (measured in denuded oocytes) throughout in vitro maturation, including a notable drop in the level immediately following germinal vesicle breakdown (GVBD). When cumulus-enclosed oocytes were examined, the magnitude of the reduction in ATP following GVBD was reduced, and this effect was ablated with the inhibition of gap junction communication, highlighting the importance of the metabolic support provided by the cumulus cells to the oocyte.

The pentose phosphate pathway (PPP) accounts for relatively little of the glucose consumed by the COC (Sutton-McDowall et al. 2010), but plays a vital role in meiotic maturation of the oocyte (Downs et al. 1996; Sutton-McDowall et al. 2005); higher G6PDH (glucose-6-phosphate dehydrogenase; the enzyme which catalyses the first step of the PPP) activity in bovine oocytes compared to cumulus cells suggesting that the important site for PPP activity may be in the oocyte itself (Cetica et al. 2002). The PPP metabolises glucose to produce NADPH, an intracellular reductant that aids in the protection of cells from damage by reactive oxygen species by participating in the reduction of oxidised glutathione (Xu et al. 2005). Another product of the PPP is phosphoribosylpyrophosphate, the sugar component for de novo nucleic acid synthesis (Banfalvi 2006), hence participating in the regulation of oocyte meiotic maturation (Downs et al. 1998; Downs et al. 1996; mouse). In mice, inhibition of G6PDH results in a reduction in the number of oocytes ovulated, decreased blastocyst development and a subsequent reduction in litter size (Jimenez et al. 2013). Interestingly, in buffalo, the expression pattern of G6PDH during oocyte in vitro maturation and early embryo development is predictive of quality and developmental competence (Kumar et al. 2013).

The polyol pathway involves the conversion of glucose to sorbitol by aldose reductase followed by the production of fructose by sorbitol dehydrogenase. Aldose reductase has a low affinity for glucose and under normal conditions very little glucose is directed down this pathway. Little is known about the activity of this pathway within the COC (Sutton-McDowall et al. 2010).

The other significant pathway for glucose metabolism is the hexosamine biosynthesis pathway (Fig. 1), which will be the focus of the rest of this review.

4. The hexosamine biosynthesis pathway (HBP)

Under normoglycaemic conditions, approximately 1-3% of total glucose consumed by somatic cells 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 (Zachara and Hart 2004b). 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 the substrate for the majority of glycosylation in the cell, creating mucopolysaccharides; large glycosaminoglycan chains which are incorporated into proteoglycans in mucus, connective tissue, skin, tendons, cartilage and ligaments (Anderson et al. 2005) and importantly for reproduction, extra cellular matrix synthesis for cumulus cell mucification (Gutnisky et al. 2007) (Fig. 1).

Glutamine:fructose-6-phosphate amidotransferase (GFPT) 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). There are two isoforms of GFPT, which share ~75% homology in both humans and mice and both produce functional GFPT enzymes (Oki et al. 1999). The main difference between the two is localisation; in adult tissues, GFPT1 is more strongly expressed in the kidney and pancreas, while GFPT2 is preferentially expressed in the central nervous system. Both are expressed in the heart and placenta (DeHaven et al. 2001; Oki et al. 1999; Sayeski et al. 1994; Zhou et al. 1995). There is very limited data available on *Gfpt1* and *Gfpt2* expression in reproductive tissues, however both isoforms have been detected in the cumulus cells of bovine COCs matured in vitro (Caixeta et al. 2013).

4.1. β-O-linked glycosylation

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 or O-GlcNAcylation (for review see (Wells et al. 2003)). In contrast to the glycosaminoglycan-type glycosylation, the O-GlcNAc modification consists of a single O-linked N-acetylglucosamine residue, with no further additions such that a chain is not formed. First discovered in 1984 (Torres and Hart 1984), it is now estimated that β-O-linked glycosylation is as widespread as phosphorylation, and is found in all multicellular eukaryotes examined to date (Comer and Hart 2000; Roquemore et al. 1994; 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 β-O-linked

glycosylation have been characterized and purified, to date there is no known 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 β-O-linked glycosylation is recognised as one of the most common forms of post-translational modification of proteins, characterisation of this modification has only recently been pursued because of its comparatively recent discovery, as well as the lack of suitable tools for studying it (β-O-linked glycosylation was discovered nearly 30 years ago). In contrast, phosphorylation has been known for 70 years (Copeland et al. 2013; Whelan and Hart 2003).

4.1.1. Enzymes of β-O-linked glycosylation

Only one enzyme for the addition of O-GlcNAc has been discovered; O-linked β-N-acetylglucosaminyltransferase (OGT) (Kreppel et al. 1997b; Kreppel and Hart 1999; Lubas et al. 1997; Lubas and Hanover 2000). Its counterpart, O-GlcNAc specific β-N-acetylglucosaminidase (O-GlcNAcase) 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 β-O-linked glycosylation enzymes have been highly conserved throughout evolution (Gao et al. 2001; Kreppel et al. 1997b; Lubas et al. 1997). The crystal structure of human OGT in complex with its substrates has recently been resolved (Lazarus et al. 2011).

Consistent with observations of nuclear and cytoplasmic O-GlcNAc-modified proteins, OGT is not found in the golgi secretory pathway 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 β-O-linked glycosylation state, and is hyperglycosylated in response to elevated glucose (Akimoto et al. 2001), however the sites of β-O-linked glycosylation and their effect on the enzymatic activity of OGT remain unknown (Kreppel et al. 1997a).

4.1.2. β-O-linked glycosylation and phosphorylation

As well as the single residue nature of β-O-linked glycosylation, are other characteristics which make it more similar to phosphorylation than to other forms of glycosylation. Like phosphorylation, the addition/removal of 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/dephosphorylation (Slawson et al. 2006). Together with its enzymes, it is localised to cytosolic and nuclear proteins, in contrast to glycosaminoglycans which are secreted or membrane-bound (Van den Steen et al. 1998; Vosseller et al. 2001; Zachara and Hart 2004a). Unlike other forms of glycosylation that have highly complex, long-chain structures, β-O-linked glycosylation is reversible (Van den Steen et al. 1998) and hence more likely to be involved in dynamic cell signalling (Vosseller et al. 2001). Indeed, O-GlcNAc regulates protein function in a very similar manner to phosphorylation, with the two different modifications often targeting the same or adjacent sites on a protein in what has been described as a yin-yang relationship (Butkinaree et al. 2010; Comer and Hart 2000; Haltiwanger et al. 1997; Wang et al. 2008; Whelan and Hart 2003).

A reciprocal relationship between global phosphorylation and β-O-linked glycosylation levels has been demonstrated in several cell lines (Comer and Hart 2001; Lefebvre et al. 1999). In addition, there are site-specific observations of phosphorylation and β-O-linked glycosylation regulating each other at the same or neighbouring sites 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 modified by O-GlcNAc, and in glomerular mesangial cells inhibiting O-GlcNAcase results in a 4-fold increase in β-O-linked glycosylated 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 β-O-linked glycosylated SP1 and a 70-80% decrease in phosphorylated SP1 (Du et al. 2000).

4.2. Glucosamine as a hexosamine pathway substrate

Glucosamine is a hexose sugar which is transported into cells via facilitated glucose transporters (Uldry et al. 2002) but differs from glucose by the presence of an amino

group. Glucosamine is widely used as a hyperglycaemic mimetic (Marshall et al. 2004; Monauni et al. 2000), as it is metabolised via the HBP but is converted straight to glucosamine-6-phosphate, thereby bypassing the slowest (rate-limiting) step of the pathway when glucose is used as the starting substrate (Fig. 1) (Marshall et al. 2005; Nelson et al. 2000; Parker et al. 2004; Patti et al. 1999; Sutton-McDowall et al. 2006). Hence, glucosamine is 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). Consistent with these reports, mouse COCs matured in the presence of 2.5 mM glucosamine expand 4-fold more than those matured under control conditions (5.6 mM glucose), as a result of increased substrate in the glucosamine group for hyaluronic acid synthesis (Chen et al. 1993). In bovine COCs matured in vitro, the addition of glucosamine reduces total glucose consumption (Sutton-McDowall et al. 2004). One explanation is that while glucose-6-phosphate is a potent inhibitor of hexokinase, glucosamine-6-phosphate is a relatively weak one (Virkamaki and Yki-Jarvinen 1999), and more glucosamine-6-phosphate may be able to accumulate before the same level of negative feedback on hexokinase is reached (Pantaleon et al. 2010). Furthermore, during embryo development, treatment with 0.2 mM glucosamine had the same negative effect on mouse blastocyst development as 27 mM glucose (Pantaleon et al. 2010).

4.3. Currently known roles of the HBP

The HBP and β-O-linked glycosylation are known regulators of essential cellular processes such as the cell cycle (Haltiwanger and Philipsberg 1997; Slawson and Hart 2003; Slawson et al. 2002; Yang et al. 2012); protein transcription and translation (Comer and Hart 2000; Datta et al. 2001). Such regulation occurs during oocyte maturation (Eppig 1996; Grondahl 2008; Kang and Han 2011). Aberrant B-O-linked glycosylation is associated with a myriad of disease states (for reviews see (Hart et al. 2007; Yang and Suh 2013)) and in general, β-O-linked glycosylation is increased in unhealthy or pathological states compared to healthy cells. This is also the case when cells are exposed to many different types of stress in vitro. For example, O-GlcNAc levels increase rapidly in response to heat shock, ethanol, UV, hypoxia, reductive, oxidative or osmotic stress (Zachara et al. 2004). This may be a protective mechanism, as decreased OGT and β-O-linked glycosylation levels result

in cells that are less tolerant of stress (Hart et al. 2007; Zachara et al. 2004). Altered glycosylation status has long been associated with tumour growth (Fuster and Esko 2005), and many oncogene and tumor suppressor proteins are modified by O-GlcNAc (Chou and Hart 2001).

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 plays a role in both of 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 results in insulin resistance (Akimoto et al. 2007; Arias and Cartee 2005; Arias et al. 2004; McClain et al. 2002; Vosseller et al. 2002). 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). While there is no change in SLC2A4 expression in cells exposed to hyperglycaemia or glucosamine (Nelson et al. 2000), accelerated degradation of the protein occurs in these conditions (Thomson et al. 1997) as well as defective translocation of SLC2A4 to the plasma membrane in cultured insulin-resistant adipocytes (Nelson et al. 2000; Park et al. 2005).

4.4. The HBP and early development

Hexosamine pathway activity plays a crucial role in early development. Homozygous mutant mice for glucosamine-6-phosphate transferase (an enzyme acting downstream of GFPT in the HBP) die at 7.5 dpc after experiencing general proliferation defects (Nelkin et al. 1980). Moreover, β-O-linked glycosylation itself is vital for life even at the single cell level, with targeted deletion of the OGT locus in mice resulting in an ablation of embryonic stem cell viability (Shafi et al. 2000).

A recent study examining *Gfpt2* in developing mouse embryos found evidence of expression in the foregut endoderm at 8.5 dpc, and the myocardium underlying the cardiac cushions at 9.5 dpc (Woolford 2012). By 10.5 dpc expression in the heart

was largely lost and instead focused on the optic vesicle. No expression was seen in the placenta, while *Gfpt1* was observed here at 9.5 dpc. The author proposed that the function of *Gfpt2* at 8.5 dpc may be to facilitate O-GlcNAc modulation of fibroblast growth factor signalling important to heart development. At 9.5 dpc, glycosaminoglycan formation is part of cardiac cushion formation through swelling of the extracellular matrix. Interestingly, there was no effect on embryo or pup survival in mice generated with homozygous gene trap insertions (creating functionally null alleles of *Gfpt2*), suggesting that *Gfpt2* expression is not crucial to early mouse development and may be compensated for by *Gfpt1*.

Studies examining the role of the HBP in early embryo development have shown that excess flux during this time, whether as a result of hyperglycaemia or glucosamine treatment, induces negative consequences for the embryo. For example, Pantaleon et al. (2010) showed that 27 mM glucose (hyperglycaemia) or 0.2 mM glucosamine added to mouse embryo culture media from 18 – 90 h post-hCG, reduced embryo development, increased apoptosis and decreased cell number in the resulting blastocysts. BADGP (benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside, an inhibitor of OGT) at 2 mM rescued all these phenotypes in the hyperglycaemia treatment group, although only mild improvement was seen in the glucosamine group. This may reflect the relative potencies of each hexose in their capacity to stimulate the HBP and UDP-GlcNAc production (Marshall et al. 1991). Zygotes cultured in the complete absence of glucose failed entirely to form blastocysts. With a combination of control medium (0.2 mM glucose) and 5 mM glucosamine, blastocyst formation was completely ablated, while BADGP treatment in control medium alone reduced blastocyst development by 34% (Pantaleon et al. 2010). Taken together, these results imply that any significant perturbation of HBP flux during early cleavage is detrimental to embryo development. Additionally, the presence of 0.2 mM glucosamine in the absence of glucose stimulated embryo development by 50% compared to controls in which glucose was absent, indicating that while some HBP flux is beneficial, other pathways which do not metabolise glucosamine must also play roles during this period of development.

It also appears that the requirement for HBP flux during early cleavage can be satisfied with a short pulse of glucose or glucosamine at the cleavage stage (50 – 64

h post-hCG) (Pantaleon et al. 2008). Using mouse zygotes, the complete absence of glucose in culture to the blastocyst stage ablated embryo development, and as before, caused an increase in apoptosis and a decrease in blastocyst cell number. However, a 2 – 3 h pulse of glucose or glucosamine was enough to reverse development and apoptosis insults, although blastocyst cell number did not recover.

Pantaleon et al. (2008) also observed that a decrease in the mouse embryo in both mRNA and apical protein of the high-affinity glucose transporter SLC2A3 in the absence of glucose. This transporter is required for blastocyst formation, and hence it was hypothesised that the variations in HBP flux may affect SLC2A3 expression or function. Indeed, the glucose or glucosamine pulse was also able to restore both mRNA and protein expression of this transporter. Because of the ability of glucosamine to substitute for glucose in this role and the implication of the involvement of the HBP, the authors went on to examine the effect of inhibition of GFPT around the time of the pulse (30 min before, during, and 30 min after) using azaserine (which prevents entry of glucose into the HBP by inhibiting GFPT; Fig. 1). In the presence of azaserine, a glucose pulse was ineffective; however, a glucosamine pulse was able to rescue embryo development and SLC2A3 expression. These results strongly implicate the HBP as having a critical and temporally-sensitive role during early embryogenesis, at least in part through modulation of SLC2A3 levels. The pulse model used by Pantaleon et al. (2008) was based on studies by Chatot et al. (1994) who found a similar result. Mouse zygotes cultured in the absence of glucose failed to develop into blastocysts, however a pulse of glucose as short as 1 minute in duration was sufficient to support development to the blastocyst stage, if administered between 30 – 54 h culture (approximately 56 – 80 h post-hCG). This time frame was refined from an earlier study, in which the authors found that glucose addition at 24 h culture was too early, and 72 h culture too late, to support blastocyst development (Chatot et al. 1989).

Glucosamine 6-phosphate, the first intermediate in the HBP, is a competitive inhibitor of G6PDH (a key enzyme in the oxidative arm of the PPP). Since inhibition of G6PDH using dehydroepiandrosterone (DHEA) is known to block decidualization of endometrial stromal cells both in vitro and in vivo (Frolova et al. 2011; human, mouse). Tsai et al. (2013) tested this concept in vivo in mice, using a 60-day release

glucosamine pellet implanted in one uterine horn. Mice with the glucosamine pellet produced fewer live pups per litter than those with a control pellet, and once the active life of the pellet had expired the number returned to normal. The authors believe this may represent a potential non-hormonal, reversible contraceptive which acts by preventing the endometrium from becoming receptive to embryo implantation.

4.5. The HBP in oocyte maturation

4.5.1. Hexosamine biosynthesis in the COC - mucification and cumulus expansion

As the COC matures following the ovulatory LH surge, the cumulus cells around the oocyte undergo mucification and the matrix expands (Chen et al. 1993; Eppig 1981; Salustri et al. 1989); a phenomenon which assists in the transfer of the COC to the oviduct after ovulation and participates in fertilization (Tanghe et al. 2002). There is strong evidence for a role for cumulus expansion in follicle rupture and ovulation (Chen et al. 1993; Russell and Robker 2007), as mice with defective cumulus matrix formation are sub-fertile or infertile, primarily due to an impairment of ovulation (Robker et al. 2000). The precise mechanisms behind this phenomenon are unknown, although it has been suggested that expansion may promote the detachment of COCs from granulosa cells in preparation for ovulation, as well as providing a semi-solid mass to assist in propulsion of the COC from the follicle (Chen et al. 1993). It has also been demonstrated that cumulus cells from expanded, pre-ovulatory mouse COCs are more adherent to extracellular matrices such as collagen, found in the follicle wall, and may have proteolytic activity, thus actively participating via migratory mechanisms in their own release from the follicle (Alvino 2010).

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 tend to promote cumulus expansion (Assidi et al. 2013; Merriman et al. 1998; Mikkelsen et al. 2001). This concept is supported by recent evidence showing that bone

morphogenetic protein 15 or fibroblast growth factor 10, both of which increase developmental competence of bovine COCs in vitro (Carrette et al. 2001; Sturmey et al. 2009; Wilkinson and Gilbert 2004), increase mRNA of both *Gfpt1* and *Gfpt2* (Sutton-McDowall et al. 2012).

UDP-GlcNAc is an essential substrate for the cumulus cell production of hyaluronic acid by, the extracellular matrix glycosaminoglycan which supports the expansion of the cumulus matrix. In vitro, cumulus expansion is positively associated with the level of glucose present in maturation medium (Frank et al. 2012; mouse), and towards the end of maturation, there is an unusually high up-regulation of HBP activity, with approximately 25% of the total glucose metabolised via this pathway (Sutton-McDowall et al. 2004; cow). 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 bovine COCs (Gutnisky et al. 2007). Hence, to a degree, up-regulation of the HBP is essential for COC expansion.

4.5.2. The role of O-GlcNAc in oocyte maturation

As discussed, 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, glucosamine treatment significantly decreases glucose consumption and incorporation of radiolabelled glucose into the extracellular matrix by bovine COCs, indicative of the preferential use of glucosamine for cumulus expansion (Sutton-McDowall et al. 2004).

Using an *in vivo* model of peri-conception glucosamine treatment, younger mice (8 weeks) had a reduced litter size, while older mice (16 weeks) were not affected in this way but had litters with reduced fetal weight and increased congenital abnormalities (Schelbach et al. 2013). The detrimental effects of glucosamine treatment during oocyte *in vitro* maturation manifest post-compaction, with glucosamine treatment during IVM having no effect on meiotic maturation in cow, pig or mouse; however, in all species, morula and blastocyst development were severely inhibited in these conditions (Schelbach et al. 2010; Sutton-McDowall et al. 2006). A decrease in cleavage to the two-cell stage was also observed in the mouse study in the presence of glucosamine (Schelbach et al. 2010). BADGP supplementation in

IVM media rescued embryo development from COCs cultured in the presence of glucosamine in each study in a dose-dependent manner, highlighting the contribution of the HBP to the developmental competence of mammalian oocytes.

Recently we further dissected the impact of HBP flux on mouse COC maturation, and found that metabolism through this pathway of either glucose or glucosamine was crucial for subsequent developmental competence (Frank et al. 2012). Glucosamine alone was unable to support embryo development, and at higher levels (2.5 and 5 mM) was detrimental to measures of oocyte competence, supporting the notion that an intermediate level of β-O-linked glycosylation is optimal for cell viability (Yang et al. 2012). Glucosamine 6-phosphate is a competitive inhibitor of glucose-6-phosphate dehydrogenase (a key enzyme in the oxidative arm of the pentose phosphate pathway; PPP) (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 contribute to the decline in meiotic maturation completion observed in oocytes from diabetic mice (Chang et al. 2005; Colton et al. 2002; Diamond et al. 1989; Kim et al. 2007; Ratchford et al. 2007; Wang et al. 2010) or those exposed to high levels of glucosamine (Frank et al. 2012).

We also observed a temporal effect of HBP stimulation. The presence of glucose during the first hour of mouse *in vitro* maturation was critical to subsequent development, but glucosamine alone was able to substitute for glucose during this first hour. This result suggests that the HBP may be the only critical pathway during this period, although stimulation of the other pathways using glucose was necessary during the subsequent 17 hours for continued development (Frank et al. 2012). This is compatible with the dynamic nature of β-O-linked glycosylation, cycling on and off proteins at a rapid rate in response to environmental factors, especially glucose levels. Culture for one hour in the absence of glucose and subsequent perturbations in normal β-O-linked glycosylation patterns appears to be sufficient to cause long-term damage to the embryo.

To date there has been little research on the role of specifically β-O-linked glycosylation in oocyte maturation, as opposed to overall HBP activity, which also manifests in observations of cumulus expansion. It has been demonstrated that following exposure to glucosamine, β-O-linked glycosylation is elevated in bovine (Sutton-McDowall et al. 2006) as well as mouse (Fig. 2) COCs. We have also performed time-course experiments which revealed changes in β-O-linked glycosylation levels throughout in vitro maturation of mouse COCs even under control conditions (Frank et al., unpublished data). This is consistent with previously published reports showing that as *Xenopus* oocytes mature, the level of total cellular O-GlcNAc declines (Slawson et al. 2002).

Using mass spectrometry, we have identified 15 candidate proteins in mouse COCs as targets of this modification. One of these, Heat shock protein 90, was shown to have a detrimental effect on oocyte competence in its O-GlcNAcylated form, and interacts with OGT itself, possibly as a molecular chaperone (Frank et al. 2013). This work demonstrated for the first time the presence of OGT in the COC, and its potential role as a regulator of oocyte competence, in response to a hyperglycaemic environment.

5. Conclusions

The peri-conceptual environment is known to have a major impact on the developmental competence of oocytes and embryos and hence on subsequent fetal development. In particular, it is well known that hyperglycaemic conditions during this time are detrimental to subsequent embryo and fetal health, however the mechanisms for this effect are poorly understood. While 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, very little is known about the contribution of the HBP to oocyte and embryo early development. However, this lack of understanding is gradually being addressed, the mechanisms by which hyperglycaemia compromises fertility are becoming clearer and providing possible therapeutic targets.

Materials and Methods

Collection and in vitro maturation of mouse COCs was performed as described previously (Frank et al. 2012Frank et al. 2012). At 6 h maturation, COCs were fixed in 4% paraformaldehyde, then adhered to Cell-Tak (Becton Dickinson, NJ, USA) – coated slides and permeabilised in 0.25% Triton X-100 (USB Corporation, OH, USA). Blocking was performed for 2 h using 10% goat serum (Jackson ImmunoResearch, PA, USA) and 0.2% Tween 20 (Sigma Aldrich, MO, USA) and incubated overnight at 4°C with 1/250 primary antibody (CTD110.6 for anti-O-GlcNAc; Covance, NJ, USA) in blocking solution. On day 2 COCs were washed and incubated for 2 h with 1/250 secondary antibody (Alexa Fluor 488 goat anti-mouse IgM (Life Technologies, CA, USA) and 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining (Sigma Aldrich). COCs were mounted under a coverslip using fluorescence mounting medium (Dako, Glostrup, Denmark) and examined on an Olympus Fluoview FV10i laser scanning confocal microscope (Olympus, Tokyo, Japan). Both colours for images were collected simultaneously, and laser intensity settings remained uniform. Excitation/emission wavelengths in nm were 405/461 and 473/520 for DAPI and Alexa Fluor 488 respectively. A 60 x objective lens, type UPLSAP60xW, was used with 1.5 or 3.5 x digital zoom (for individual magnifications see Fig. 2).

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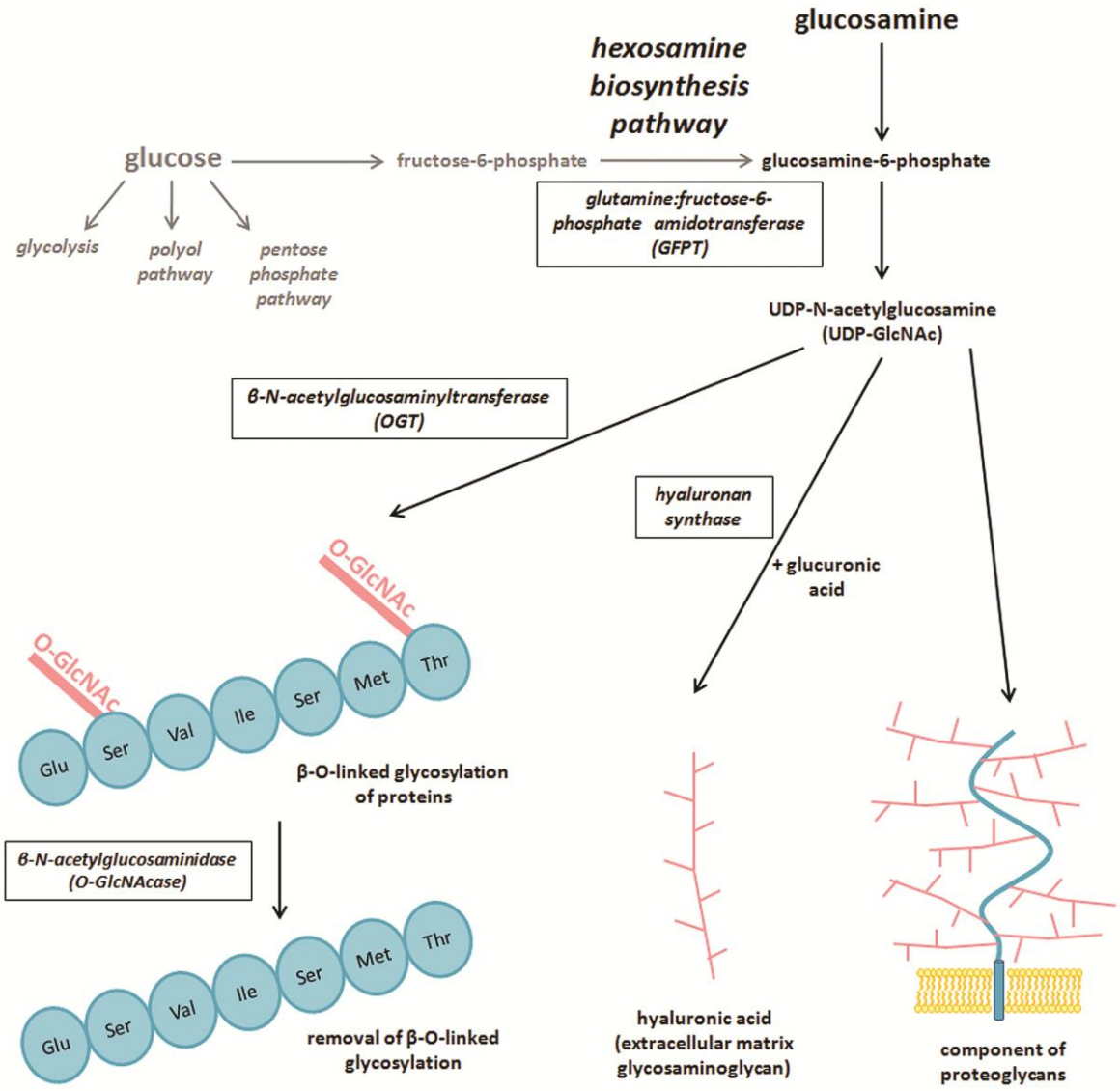

Figure 1. Glucose metabolism in cumulus-oocyte complexes (COCs)

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. UDP-N-acetylglucosamine, the end product of the hexosamine pathway, is an important component of proteoglycans and glycosaminoglycans, and the substrate for ß-O-linked glycosylation of a wide variety of proteins.

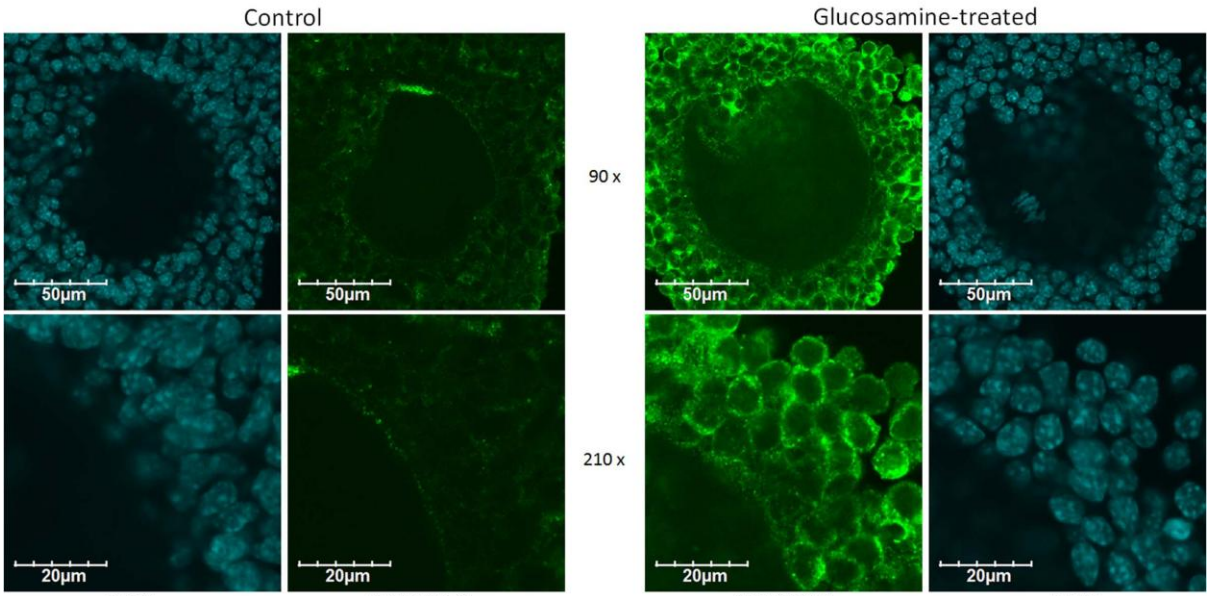

Figure 2. Immunohistochemical localisation of β-O-linked glycosylation in mouse COCs

Mouse COCs were collection after 6 h in vitro maturation in control medium (5.55 mM glucose) ± 2.5 mM glucosamine. CTD110.6 (green) shows O-GlcNAc and DAPI (blue) shows nuclear staining.