Title

Hyperglycaemic conditions perturb mouse oocyte *in vitro* developmental competence via beta-O-linked glycosylation of Heat shock protein 90

Running title

Glycosylation of HSP90 and oocyte maturation

Authors

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Abstract

Study question: What is the effect of beta-O-linked glycosylation (O-GlcNAcylation) on specific proteins in the cumulus-oocyte complex (COC) under hyperglycaemic conditions?

Summary answer: Heat shock protein 90 (HSP90) was identified and confirmed as being O-GlcNAcylated in mouse COCs under hyperglycaemic conditions (modelled using glucosamine), causing detrimental outcomes for embryo development.

What is known already: O-GlcNAcylation of proteins occurs as a result of increased activity of the hexosamine biosynthesis pathway, which provides substrates for cumulus matrix production during COC maturation, and also for O-GlcNAcylation. COCs matured under hyperglycaemic conditions have decreased developmental competence, mediated at least in part through the mechanism of increased O-GlcNAcylation.

Study design, size, duration: This study was designed to examine the effect of hyperglycaemic conditions (using the hyperglycaemic mimetic, glucosamine) on O-GlcNAc levels in the mouse COC, and furthermore to identify potential candidate proteins which are targets of this modification, and their roles in oocyte maturation.

Participants/materials, setting, methods:

COCs from 21-day old superovulated CBA x C57BL6 F1 hybrid female mice were matured *in vitro* (IVM). Levels of O-GlcNAcylated proteins, HSP90 and O-GlcNAc transferase (OGT, the enzyme responsible for O-GlcNAcylation) in COCs were measured using Western blot, and localization observed using immunocytochemistry. For glycosylated HSP90 levels, and to test OGT-HSP90 interaction, immunoprecipitation was performed prior to Western blotting. Embryo development was assessed using *in vitro* fertilization and embryo culture post-maturation.

Main results and the role of chance: Addition of the hyperglycaemic mimetic glucosamine to IVM medium for mouse COCs increased detectable O-GlcNAcylated protein levels (by Western blot and immunocytochemistry), and this effect was reversed using an OGT inhibitor ($P < 0.05$). HSP90 was identified as a target of O-GlcNAcylation in the COC, and inhibition of HSP90 during IVM reversed glucosamine-induced decreases in oocyte developmental competence ($P < 0.05$). We also demonstrated the novel finding of an association between HSP90 and OGT in COCs, suggesting a possible client-chaperone relationship.

Limitations, reasons for caution: *In vitro* maturation of COCs was used so that treatment time could be limited to the 17 h of maturation prior to ovulation. Additionally, glucosamine, a hyperglycaemic mimetic, was used because it specifically activates the hexosamine pathway which provides the O-GlcNAc moieties. The results in this study should be confirmed using *in vivo* models of hyperglycaemia and different HSP90 inhibitors.

Wider implications of the findings: This study leads to a new understanding of how diabetes influences oocyte competence and provides insight into possible therapeutic interventions based on inhibiting HSP90 to improve oocyte quality.

Study funding/competing interest(s):

This work was supported by a Program Grant from the National Health and Medical Research Council, Australia, ID 453556. J. G. T. is a recipient of funding from and a consultant to Cook Medical Pty Ltd. The other authors have no conflicts of interest to declare.

Keywords

Hyperglycaemia, oocyte developmental competence, hexosamine pathway, HSP90, O-GlcNAc

Introduction

Reversible beta-O-linked glycosylation of proteins (the addition of N-acetylglucosamine; O-GlcNAcylation) is gaining recognition as an important regulatory mechanism of cytosolic and nuclear proteins (Van den Steen et al. 1998; Slawson, Housley, and Hart 2006; Wells, Whelan, and Hart 2003). O-GlcNAcylation regulates protein function in a manner similar 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 (Comer and Hart 2000; Butkinaree, Park, and Hart 2010; Wang, Gucek, and Hart 2008; Haltiwanger et al. 1997; Whelan and Hart 2003). O-GlcNAcylation is involved controlling essential cellular processes such as cell cycle regulation (Slawson and Hart 2003; Slawson et al. 2002; Haltiwanger and Philipsberg 1997; Drougat et al. 2012), protein transcription and translation (Comer and Hart 2000; Datta et al. 2001) and DNA damage/repair pathways (Zachara et al. 2011). It is implicated in a myriad of disease states including cancer, inflammatory conditions and neurodegenerative diseases (Hart, Housley, and Slawson 2007; Slawson and Hart 2011). Some O-GlcNAc modifications are necessary for cell viability in mammals (Vocadlo et al. 2003; O'Donnell et al. 2004). However, excessive O-GlcNAcylation or inhibition of the removing enzyme beta-N-acetylglucosaminidase (O-GlcNAcase) is often detrimental to cell survival and function (Marshall, Bacote, and Traxinger 1991; McClain et al. 2002; Arias, Kim, and Cartee 2004; Yang et al. 2008). Conversely, too little O-GlcNAcylation can also disrupt normal cellular function (Liu et al. 2004; Yuzwa et al. 2011).

In contrast with glycosaminoglycan-type glycosylation, the O-GlcNAc modification consists of a single beta-O-linked N-acetylglucosamine residue, and no chain is formed by addition of further residues. 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 (Wells, Vosseller, and Hart 2001; Comer and Hart 2000; Roquemore, Chou, and Hart 1994). However, the role of O-GlcNAc in various systems, including the reproductive system, is only now starting to be characterized because of its comparatively recent discovery as well as the technical difficulties associated with studying it (Whelan and Hart 2003). In somatic cells, under normoglycaemic conditions approximately 1 – 3% of total glucose consumed by the cell is directed down the hexosamine biosynthesis pathway (HBP) (Sayeski and Kudlow 1996; Marshall, Bacote, and Traxinger 1991), which produces UDP-GlcNAc, the substrate for O-GlcNAcylation (Marshall, Nadeau, and Yamasaki 2004). The HBP was first implicated in the development of type 2 diabetes in 1991 (Marshall, Bacote, and Traxinger 1991), and it has now been shown to play a significant role in both the major pathologies of diabetes: insulin resistance and the decline in pancreatic beta-cell function (Yang et al. 2008; Marshall, Bacote, and Traxinger 1991). Increasing flux through the pathway, raising O-GlcNAc levels using PUGNAc (O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate), an inhibitor of O-GlcNAcase), or overexpression of the transfer enzyme beta-O-linked N-acetylglucosamine transferase (OGT), all result in insulin resistance (Arias and Cartee 2005; Arias, Kim, and Cartee 2004; McClain et al. 2002; Vosseller et al. 2002; Akimoto et al. 2007).

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; Holing et al. 1998; Lapolla, Dalfra, and Fedele 2008; Purcell and Moley 2011; Jungheim and Moley 2010; Rich-Edwards et al. 1994).In studies of diabetic women, even if optimal glycaemic control is achieved within the first few weeks of pregnancy (embryonic and early fetal

development), there is still a 3 – 5 times higher risk of spontaneous abortions and congenital anomalies in these women (El-Sayed and Lyell 2001; Casson et al. 1997; Baccetti et al. 2002). However, if a pre-conception treatment program is undertaken this risk is significantly reduced (Dunne et al. 1999; Pearson et al. 2007; Ray, O'Brien, and Chan 2001), implicating the period of oocyte maturation as a critical window of oocyte susceptibility to damage.

Many studies examining the role of the HBP use glucosamine as a hyperglycaemic mimetic, as it is specifically metabolised by the HBP, enters the pathway downstream of the rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (Patti et al. 1999; Marshall, Nadeau, and Yamasaki 2005; Nelson, Robinson, and Buse 2000) and hence is a potent stimulator. Using this treatment, mouse embryos exposed from the zygote stage *in vitro* to 27 mM glucose (approximately the blood glucose level of diabetic mice, vs. normoglycaemic ~8 mM (Chu et al. 2006; Fox et al. 2011; Ozcan et al. 2006)) or 0.2 mM glucosamine have been shown to produce fewer blastocysts, with reduced cell numbers compared to controls, and an increase in apoptosis (Pantaleon et al. 2010). Benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP), an inhibitor of OGT, was able to reduce all of these effects.

Consistent with these findings, peri-conception glucosamine treatment in mice *in vivo* causes a range of defects (reduced litter size, increased congenital abnormalities and reduced fetal weight) depending on the age of the mother (Schelbach et al. 2013). *In vitro,* O-GlcNAcylation is elevated in bovine cumulus-oocyte-complexes (COCs) exposed to glucosamine (Sutton-McDowall et al. 2006) and while glucosamine treatment during IVM does not affect meiotic maturation of cow, pig or mouse oocytes, blastocyst development was severely inhibited (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 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. Recently, in our laboratory we have shown that flux through the HBP during the first hour of IVM appears to be critical for developmental competence, whereas high levels of glucosamine supplementation alone (in the absence of glucose) throughout IVM were detrimental to competence measures (Frank et al. 2013). These results support the concept that an intermediate level of HBP flux is optimal.

Given the important role of HBP in oocyte developmental competence, the aim of the current study was to examine O-GlcNAc levels in mouse COCs and to identify potential targets of this modification.

Methods

Animals

CBA x C57BL6 F1 hybrid mice (females 21 days old, males 6 – 8 weeks old) were maintained in the Animal House at the Medical School, The University of Adelaide, under a 14:10 hour light:dark cycle with *ad libitum* access to food and water.

Ethical approval

All experimental procedures were carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and approved by The University of Adelaide Animal Ethics Committee (Medical).

Chemicals

Unless otherwise specified all reagents and antibodies were purchased from Sigma-Aldrich (MO, USA). BAGDP and 17-(Allylamino)-17-demethoxygeldanamycin (17AAG; A.G. Scientific, CA, USA) were dissolved and stored in DMSO at -80°C. The final concentration of DMSO in culture media for each was 0.27% and 0.01% respectively.

Media

All media used were as previously described (Frank et al. 2013). Briefly, simple mouse IVM media supplemented with fatty acid-free bovine serum albumin (BSA; ICPbio, Glenfield, New Zealand) were used for collection and IVM of COCs, with 50 mIU/mL recombinant human follicle-stimulating hormone (Organon, Oss, The Netherlands) added to maturation media only. Media used for embryo development experiments were also supplemented with 1 mg/ml fetuin to prevent zona hardening. For embryo production following IVM, Research Vitro Wash, Fertilization and Cleave media were used (Cook Medical, QLD, Australia). Maturation 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. Where media was supplemented with glucosamine, a dose of 2.5 mM was used. This has been shown in previous studies to be an effective inhibitory dose for mouse *in vitro* COC culture at the volumes and levels of glucose (5.6 mM) used and it induces a significant decrease in subsequent blastocyst rate and meiotic completion but does not totally ablate development (Schelbach et al. 2010; Frank et al. 2013).

COC collection and IVM

Female mice were administered 5 IU equine chorionic gonadotrophin (eCG; Folligon, Intervet, Boxmeer, The Netherlands) as an intraperitoneal injection. 46 h post-eCG injection, ovaries were collected, COCs were aspirated and were held in collection medium for one hour. COCs were washed once in collection medium, once in maturation medium and matured for up to 18 h in a volume of 50 µL medium/COC at 37 °C under paraffin oil, in humidified air comprising 6% CO_2/5% O_2/89% N_2.

In vitro fertilisation and embryo culture

In vitro fertilisation (IVF) and embryo culture were carried out as described previously (Frank et al. 2013). Briefly, after 17 h of maturation COCs were placed in fertilisation medium for 4 h with sperm which had been allowed to capacitate for 1 hour prior to co-incubation. Presumptive zygotes were transferred to culture drops, cleavage rate assessed on Day 2 of culture and embryo developmental stage assessed on Day 5.

Immunoprecipitation

COC samples for immunoprecipitation were processed by adding a 1:9 Protease Inhibitor Cocktail (PIC): radioimmunoprecipitation assay (RIPA) buffer solution and antibody and incubating with rotation overnight at 4 °C (RIPA buffer: 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 (USB Corporation, OH, USA)). For immunoprecipitation, 0.6 µL CTD110.6 (anti-O-GlcNAc antibody; Covance, NJ, USA) was used per 50 COCs or 2 µL anti-OGT per 50 COCs. On day 2 of the protocol, 10 µL Protein L beads (Thermo Fisher Scientific, MA, USA) were added to each sample and rotated at 4 °C for a further 4 - 5 h. Samples were then washed and centrifuged three times at 20,000 g for two minutes (Eppendorf Centrifuge 5424, Hamburg, Germany) with RIPA buffer to remove all supernatant. Beads were boiled in 1 x

Laemmli buffer (LB; 50% glycerol, 10% SDS, 0.5% bromophenol blue, 250 mM Tris, 10% β-mercaptoethanol added immediately before use) for 7 minutes and centrifuged, and the supernatant used for Western blotting.

Western blots

For all Western blots, samples were lysed in the PIC:RIPA mixture, LB added and samples boiled for 7 minutes. Proteins were then separated on a 7.5% SDS-PAGE gel and wet-transferred to a polyvinylidene fluoride membrane (GE Healthcare, Little Chalfont, UK) overnight. Molecular weight markers were Precision Plus Protein Dual Color Standards (Bio-Rad, CA, USA).

O-GlcNAc blots

Blocking and washing solutions and protocol were as described previously (Zachara et al. 2002), using 1/1000 CTD110.6 (anti-O-GlcNAc) and 1/2500 alkaline phosphatase-conjugated anti-mouse IgM (Rockland, PA, USA). Blots included BSA-conjugated-N-acetylglucosamine (BSA-GlcNAc) as a positive control (5 ng unless otherwise specified) and 100 ng of each of BSA (not glycosylated) and ovalbumin (N-linked but not β-O-linked glycosylated) as negative controls. Control experiments were performed using competitive inhibition with 15.4mM free GlcNAc to further confirm specificity of CTD110.6 (not shown). Blots were developed using Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega, WI, USA). Blots performed without immunoprecipitation were co-stained with horseradish peroxidase (HRP)-conjugated anti-beta-actin as a loading control (1/1,000,000) and developed using the ECL system (GE Healthcare, Little Chalfont, UK) after colorimetric development.

HSP90 blots

Membranes were blocked for one hour in 5% skim milk in Tris-buffered saline with Tween 20 (TBST; 150 mM NaCl, 50 mM Tris-HCl, 0.05 % Tween 20, adjusted to pH 7.6), washed 3 x 5 minutes with TBST, and incubated with 1/2500 pan anti-HSP90 (Becton Dickinson, NJ, USA; this antibody targets both alpha and beta isoforms of the protein) in 5% milk overnight at 4 °C. On day 2, membranes were washed as described above, incubated with 1/5000 HRP-conjugated goat polyclonal anti-mouse IgG in 5% milk at room temperature for 1 hour, washed again and developed using the ECL system (GE Healthcare, Little Chalfont, UK). Blots included 1 µg HeLa cell lysate (Enzo Life Sciences, NY, USA) as a positive control and were also stained with HRP-conjugated anti-beta-actin as a loading control (1/1,000,000).

Immunocytochemistry

COCs were collected at 6 h of maturation and fixed in 4% paraformaldehyde overnight. Whole COCs were adhered on Cell-Tak (Becton Dickinson, NJ, USA) –coated slides, permeabilised in 0.25% Triton X-100 (USB Corporation, OH, USA), blocked for 2 h using 10% goat serum (Jackson ImmunoResearch, PA, USA) and 0.2% Tween 20 and incubated overnight at 4°C with 1/250 primary antibody (CTD110.6 for anti-O-GlcNAc, anti-HSP90 or anti-OGT) 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) or goat anti-rabbit IgG (Abcam, Cambridge, UK), Alexa Fluor 594 goat anti-mouse IgG or donkey anti-goat IgG (Abcam), and counter-stained for 30 minutes with propidium iodide (PI) or 4’,6-diamidino-2-phenylindole (DAPI), mounted under a coverslip and examined 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. Excitation/emission wavelengths in nm were 559/619, 405/461, 473/520 and 559/618 for PI, DAPI, Alexa Fluor 488

and Alexa Fluor 594 respectively. A 60 x objective lens, type UPLSAP60xW, was used with 0 – 8.5 digital zoom (for individual magnifications see figure legends). For quantitative assessment of fluorescence, the mean gray scale intensity of an area of defined size was measure per COC. To ensure consistency this area was centred over and included the entire ooctye for each picture. For competitive inhibition in Experiment 2, the primary antibody step was performed in the presence of 15.4 mM free GlcNAc.

Silver staining and mass spectrometry

Samples were separated on a 7.5% SDS-PAGE gel as described for Western blotting, and stained using the SilverQuest Silver Staining Kit (Invitrogen, CA, USA) which is compatible with mass spectrometry. Gels were provided to the Adelaide Proteomics facility (The University of Adelaide, SA, Australia) where specific bands were excised, destained and analysed using liquid chromatography-electrospray ionisation ion-trap mass spectrometry, to identify potential O-GlcNAcylation targets. 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.

Statistical analyses

Data were analysed using SPSS version 18.0.2 (Predictive Analytics Software, IBM, NSW, Australia). Quantification of protein bands on Western blots was performed using ImageJ version 1.44p (Rasband 1997 - 2012). Data were tested for normality using the Shapiro-Wilk test and, if normally distributed, analysed using a one-way analysis of variance (ANOVA) and comparisons made by least-significant difference post-hoc test. Data which were not normally

distributed 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. Embryo development data were assessed using Chi Square analysis.

Results

Experiment 1: Western blot analysis of O-GlcNAcylation levels in the COC during IVM

Groups of 50 COCs were collected at 6 h of culture and snap frozen. Control group COCs were matured in standard media. Groups 2 and 3 were treated with 2.5 mM glucosamine in maturation medium, and group 3 also included 2.5 mM of the OGT inhibitor BADGP. Data from preliminary experiments (Supp. Fig. 1) showed that throughout IVM (6, 12 and 18 h), glucosamine treatment increased O-GlcNAcylation within the COC. The increase relative to the control was greatest at 6 h of maturation, therefore this time point was used for the experiments discussed here. Proteins from COCs collected at the 6 h time point (Fig. 1) showed a pattern of increased O-GlcNAcylation with glucosamine treatment ($P < 0.05$ compared to control, $n = 4$ replicates) which was reduced with the addition of BADGP ($P < 0.05$).

Experiment 2: Immunocytochemical analysis of O-GlcNAcylation levels in the COC at 6 h IVM

Treatment groups for Experiment 2 were as described in Experiment 1. Immunocytochemical localisation of O-GlcNAc with the CTD110.6 antibody in whole COCs collected after 6 h of culture revealed extensive positive staining in the cumulus cells (Fig. 2; representative images from $n = 3$). Compared to the control group, staining intensity increased in COCs treated with glucosamine (2.3-fold higher, $P < 0.05$) and this increase was eliminated to control levels by co-treatment with BADGP ($P < 0.05$). For representative immunocytochemistry controls please see Supplementary Figure 2.

Experiment 3: Identification of specific proteins which are O-GlcNAcylated in glucosamine-treated COCs

Immunoprecipitation of 100 glucosamine-treated COCs collected at 6 h of maturation was performed as described, and the immunoprecipitated product was separated on a 7.5% SDS-PAGE gel which was then silver stained. Specific bands were excised, destained and analysed using liquid chromatography-electrospray ionisation ion-trap mass spectrometry to identify potential O-glycosylation targets. Mass spectrometry analysis revealed 15 potential targets of O-glycosylation (Table 1). Eleven of these 15 proteins have been previously described in the literature as being O-GlcNAcylated, including HSP90, the alpha form of which had the highest Combined Ion Score of any protein identified in this experiment. One class of proteins which was well represented was structural proteins, with fibronectin, actin and tubulin identified, as well as several involved in protein folding including protein disulfide isomerase and protein disulfide isomerase A3, and both isoforms of HSP90 (alpha and beta).

Experiment 4: Total and O-GlcNAcylated HSP90 in COCs after 6 h IVM

HSP90 is a molecular chaperone which responds to cellular stress by stabilizing unfolded proteins and preventing their aggregation. Glucosamine is known to induce stress in various cell types, therefore the total levels of HSP90 in each treatment group were examined using Western blot analysis on COCs using treatment groups as described in Experiment 1. There was no difference in the total amount of HSP90 protein in COCs between treatment groups at 6h (Fig. 3A, n = 3). However, when proteins were immunoprecipitated using CTD110.6 to pull down O-glycosylated proteins and then detected with anti-HSP90, there was a dramatically higher level of O-glycosylated HSP90 found in COCs from the glucosamine treatment group, than either the

control or glucosamine + BADGP groups (Fig. 3B, n = 2). Co-localisation of HSP90 and O-GlcNAcylation was observed in both the oocyte and cumulus cells (Fig. 3C; representative images from n = 3). For representative immunocytochemistry controls please see Supplementary Figure 2.

Experiment 5: Effect of inhibition of HSP90 during IVM of COCs

Previous work in our laboratory has shown that the presence of glucosamine during IVM significantly reduces oocyte developmental competence of several species including mice (Sutton-McDowall et al. 2006; Schelbach et al. 2010). The amount of O-GlcNAcylated HSP90 present in these COCs appears to be higher (Fig. 3B) than in those which are known to have good developmental competence (control and BADGP groups). Therefore it was hypothesised that inhibiting O-GlcNAcylated HSP90 during COC maturation would be beneficial to oocyte developmental competence. Control and glucosamine groups were as described for Experiment 1; HSP90 inhibitor 17AAG was added to the maturation medium of groups 2 and 4 at a concentration of 0.1 µM. Supplementing IVM media with 17AAG did not affect blastocyst development under control conditions (Fig. 4). However, when added to the group developmentally compromised by the hyperglycaemic mimetic glucosamine, 0.1 µM 17AAG was able to restore embryo development (57.3% vs. 76.7% respectively, $P < 0.05$, n = 3 with average 27 COCs/group/replicate). There was no difference in the cleavage rate between treatment groups.

Experiment 6: Interaction of HSP90 with OGT

To test for a direct interaction between HSP90 and OGT, proteins from COCs matured for 6h were immunoprecipitated using anti-OGT, then separated via SDS-PAGE and detected using

anti-HSP90. While a very small amount of HSP90 was detected in the no-antibody control immunoprecipitation, this was dramatically enriched in the immunoprecipitation using anti-OGT (Fig. 5A). Co-localisation of HSP90 and OGT was also observed in both the oocyte and cumulus cells using immunocytochemical staining (Fig. 5B; representative images from n = 2). For representative immunocytochemistry controls please see Supplementary Figure 2.

Discussion

In this study, we examined levels of O-GlcNAcylation on proteins within mouse COCs, identified HSP90 as a target protein, and investigated its potential role in oocyte maturation under hyperglyacemic conditions. Treatment with the hyperglycaemic mimetic glucosamine during IVM increased O-GlcNAcylated protein levels in COCs (by Western blot and immunohistochemistry), especially by 6h of maturation. Inhibition of O-GlcNAcylated HSP90 (confirmed using immunoprecipitation and Western blot) during IVM was able to reverse glucosamine-induced decreases in oocyte developmental competence, suggesting an aberrant function of O-GlcNAcylated HSP90. Using immunoprecipitation, we demonstrated the novel finding of an association between HSP90 and OGT in COCs, suggesting that OGT may be a client protein of HSP90. When combined, these results reveal that glucosamine treatment caused O-GlcNAcylation of HSP90, with negative consequences for subsequent embryo development.

We chose a 6h time-point to examine differences in O-linked glycosylation across treatments, as this time-point appeared to be a particularly active period for this post-translation protein modification in the presence of glucosamine. We acknowledge that the results reported here may differ if we had chosen other time points.

It is well documented that glucosamine treatment or PUGNAc increases O-GlcNAc levels detectable by Western blot in other cell types including the Jurkat (human T lymphocyte) cell line (Comer et al. 2001), rat skeletal muscle, liver (Arias and Cartee 2005) and neonatal cardiomyocytes (Champattanachai, Marchase, and Chatham 2008). Increased O-GlcNAc levels have also been observed 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 glucosamine increased detectable O-GlcNAcylation levels, shown by Western blot and immunocytochemistry. Glucosamine supplementation, which led to the highest level of O-GlcNAcylation in these experiments, significantly reduces blastocyst formation following oocyte 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 embryo development. Together these results suggest that increased levels of O-GlcNAcylation in COCs are associated with reduced oocyte developmental competence. A similar phenomenon has been seen in pancreatic β-cells. When O-GlcNAcylation was increased in β-cells following glucosamine or streptozotocin (diabetes-inducing) treatment, cell viability was decreased. The combination of glucosamine and streptozotocin treatment further reduced the percent of viable cells (Park et al. 2007). Our results are consistent with all of these studies, providing further evidence for the detrimental effect of excess O-GlcNAcylation on cellular function.

In mouse COCs matured in control medium (5.55 mM glucose) there were 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), including the detection in bovine COCs of O-GlcNAc in the control group (Sutton-McDowall et al. 2006).

Using immunocytochemistry, in all groups proteins within the oocyte itself appear to be positively stained for O-GlcNAcylation. 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 the oocyte in bovine 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 treatment compared to control medium (Schelbach et al. 2010). A possible explanation for the difference observed in immunocytochemical staining could be the different antibodies used (bovine, 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 detection).

Immunoprecipitation of proteins using the CTD110.6 antibody to capture O-GlcNAcylated proteins in glucosamine-treated COCs, followed by mass spectrometry, revealed 15 candidate proteins. While validation of the O-GlcNAcylation status of all the proteins was beyond the scope of this study, four of these have not been previously reported as O-GlcNAcylated in the literature and are potential novel targets.

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, Tsuiki, and Yemini 1989). Fibronectin also promotes bovine embryo development in serum-free medium (Larson, Ignotz, and Currie 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, Rees, and Kaufman 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.

We focused on HSP90, the two isoforms of which share 86% homology in mice (Moore et al. 1989) and are likely the result of gene duplication as they are structurally and functionally very similar (Picard 2002). 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) as well as during oogenesis in the mouse, turtle and Drosophila (Zimmerman, Petri, and Meselson 1983; Curci et al. 1991; Barnier et al. 1987; Morange et al. 1984; Harry, Williams, and Briscoe 1990). Western blots of COC proteins immunoprecipitated with the CTD110.6 antibody 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 maturation 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). However, there were no differences seen in total HSP90 levels in any treatment group.

Inhibition of HSP90 during oocyte maturation was achieved through the use of 17AAG, an inhibitor of HSP90 which has been clinically trialed to the phase II stage as a tumour suppressor in various types of cancer (Modi et al. 2011; Oh et al. 2011). We 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 reduces 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 the function of HSP90 in a O-GlcNAcylated state is detrimental to oocyte developmental competence.

The experiments conducted in this study are the first to examine levels of O-GlcNAcylation in the mouse COC during IVM. We also identified potential targets of O-GlcNAcylation in COCs matured under hyperglycaemic conditions, using the hyperglycaemic mimetic glucosamine. Inhibition of the O-GlcNAcylated form of HSP90 (one of the most promising candidate proteins) 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 oocytes under hyperglycaemic

conditions. However, while they are an important step in suggesting a possible mechanism for the decrease in developmental competence observed under hyperglycaemic conditions, this work was performed *in vitro* and needs to be extended through the use of *in vivo* models. Glucosamine is commonly used as a hyperglyacemic mimetic, and specifically up-regulates the HBP and subsequent O-GlcNAcylation, which we have previously shown to be associated with poor developmental competence of COCs. However, it is not metabolized through other glucose metabolic pathways (glycolysis and the pentose phosphate pathway), and further work is currently exploring alternative in vivo models of hyperglycaemia.

Authors' roles

All authors contributed to experiment design, interpretation of data and manuscript revision. L.A.F. was also responsible for data analysis and manuscript drafting, and L.A.F. and H.M.B. for data acquisition.

Acknowledgements

We would like to gratefully acknowledge Professor Didier Picard at the University of Geneva, for helpful discussions, and the assistance with animal work and mass spectrometry from the Laboratory Animal Services and Adelaide Proteomics Centre staff respectively, at the University of Adelaide

Funding

This project was funded by a National Health and Medical Research Council Program Grant, ID 453556.

Conflicts of interest

J. G. T. receives funding from and is a consultant to Cook Medical Pty Ltd. The other authors have no conflicts of interest to declare.

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691

Name	UniProt ID	Combined Ion Score	Previously identified as O-GlcNAcylated (reference)
Heat shock protein HSP 90-alpha	HS90A_MOUSE	633	Yes [1-3]
Protein disulfide-isomerase A3	PDIA3_MOUSE	631	No
Heat shock protein HSP 90-beta	HS90B_MOUSE	450	Yes [1, 2]
Fibronectin	FINC_MOUSE	343	No
Actin, cytoplasmic 1 Actin, cytoplasmic 2	ACTB_MOUSE ACTG_MOUSE	332	Yes [2-5]
Elongation factor 1-alpha 1 Elongation factor 1-alpha 2	EF1A1_MOUSE EF1A2_MOUSE	115	Yes [1-3, 5]
Glucose-6-phosphate 1-dehydrogenase X	G6PD1_MOUSE	114	Yes [5]
Zona pellucida sperm-binding protein 3	ZP3_MOUSE	110	No
Zona pellucida sperm-binding protein 2	ZP2_MOUSE	101	No
Tubulin alpha-1A chain Tubulin alpha-1B chain Tubulin alpha-1C chain	TBA1A_MOUSE TBA1B_MOUSE TBA1C_MOUSE	89	Yes [2, 3, 5, 6]
Protein disulfide-isomerase	PDIA1_MOUSE	83	Yes [7], precursor protein [4]

Table 1

Table 1. Beta-O-linked glycosylated (O-GlcNAcylated) proteins identified in glucosamine-treated mouse cumulus-oocyte-complexes (COCs)

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

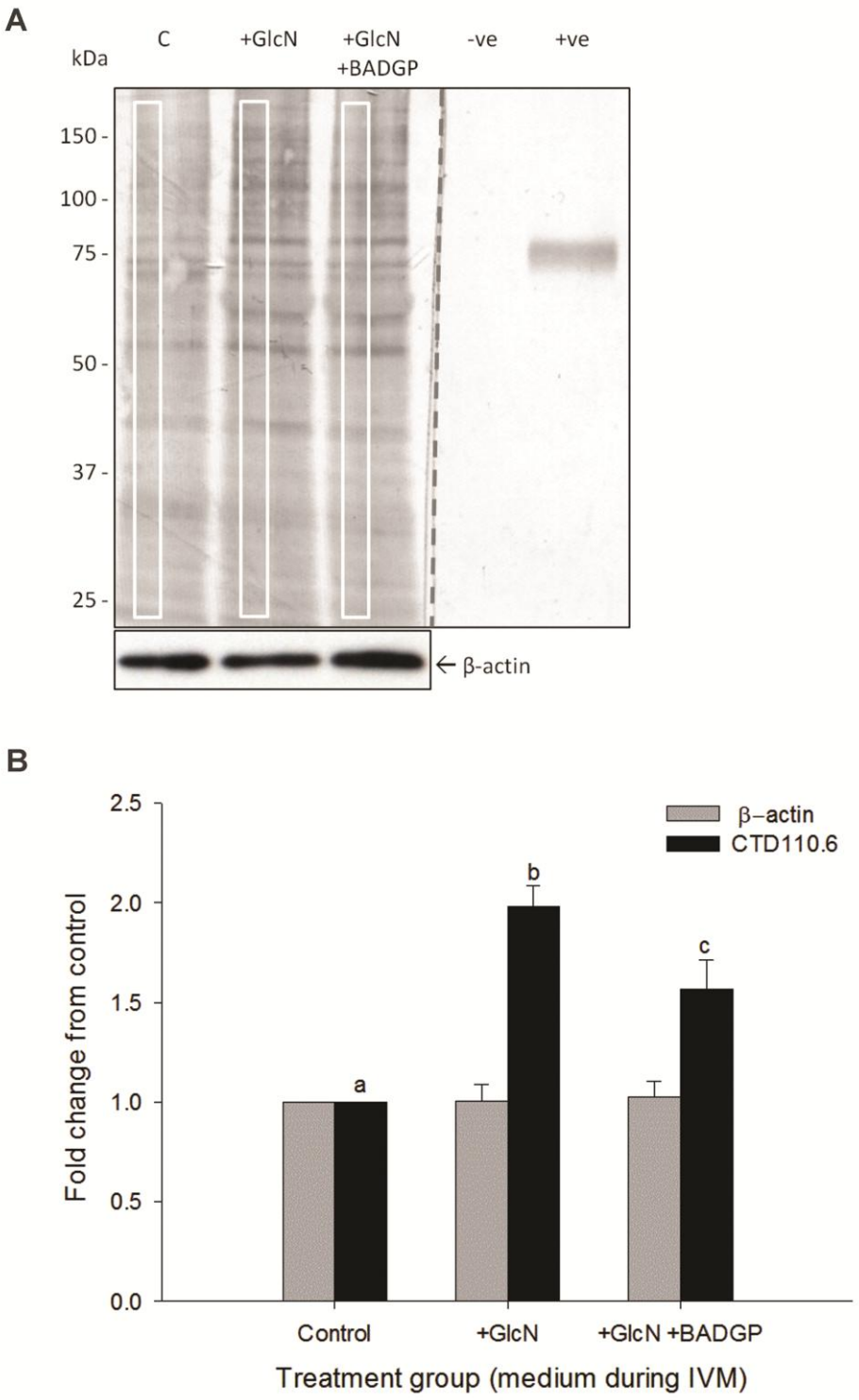

A
kDa
C
+GlcN
+GlcN
+BADGP
-ve
+ve
150 -
100 -
75 -
50 -
37 -
25 -
← β-actin
B
β–actin
CTD110.6
Fold change from control
2.5
2.0
1.5
1.0
0.5
0.0
a
b
c
Control
+GlcN
+GlcN +BADGP
Treatment group (medium during IVM)

Figure 1

Figure 1 Beta-O-linked glycosylation (O-GlcNAcylation) of proteins in cumulus-oocyte-complexes (COCs)

COCs (50 per lane) were cultured in control medium (C), ± 2.5 mM glucosamine (GlcN), ± 2.5 mM benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP). Proteins were separated using SDS-PAGE, Western blotted and probed using the O-GlcNAc antibody CTD110.6 or anti-β-actin as a loading control. Positive control (+ve) was 0.5 ng BSA-GlcNAc, negative controls were 100 ng each BSA and ovalbumin. (A) Representative Western blot. The membrane was cut before staining on dotted line shown; areas used for densitometry analysis are shown in white boxes. (B) Densitometry analysis. Data are presented as mean ± SEM, n=4.

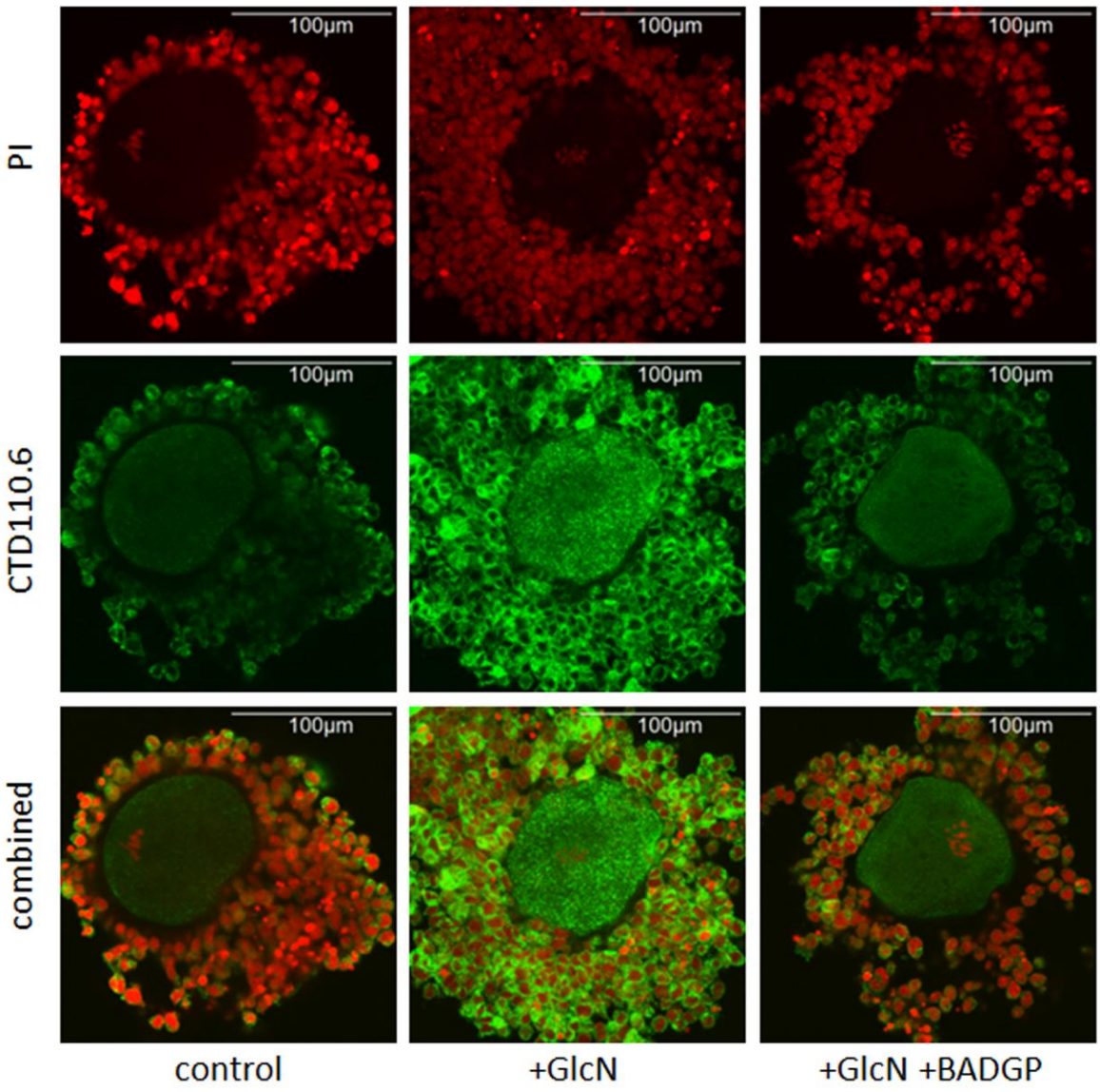


Figure 2

Figure 2 Immunocytochemistry of beta-O-linked glycosylation (O-GlcNAcylation) in cumulus-oocyte-complexes (COCs)

COCs were collected after 6 h IVM in control medium ± 2.5 mM glucosamine (GlcN) ± 2.5 mM benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP). Propidium iodide (PI, red) shows nuclear staining, CTD110.6 (green) shows O-GlcNAc at 60x magnification.

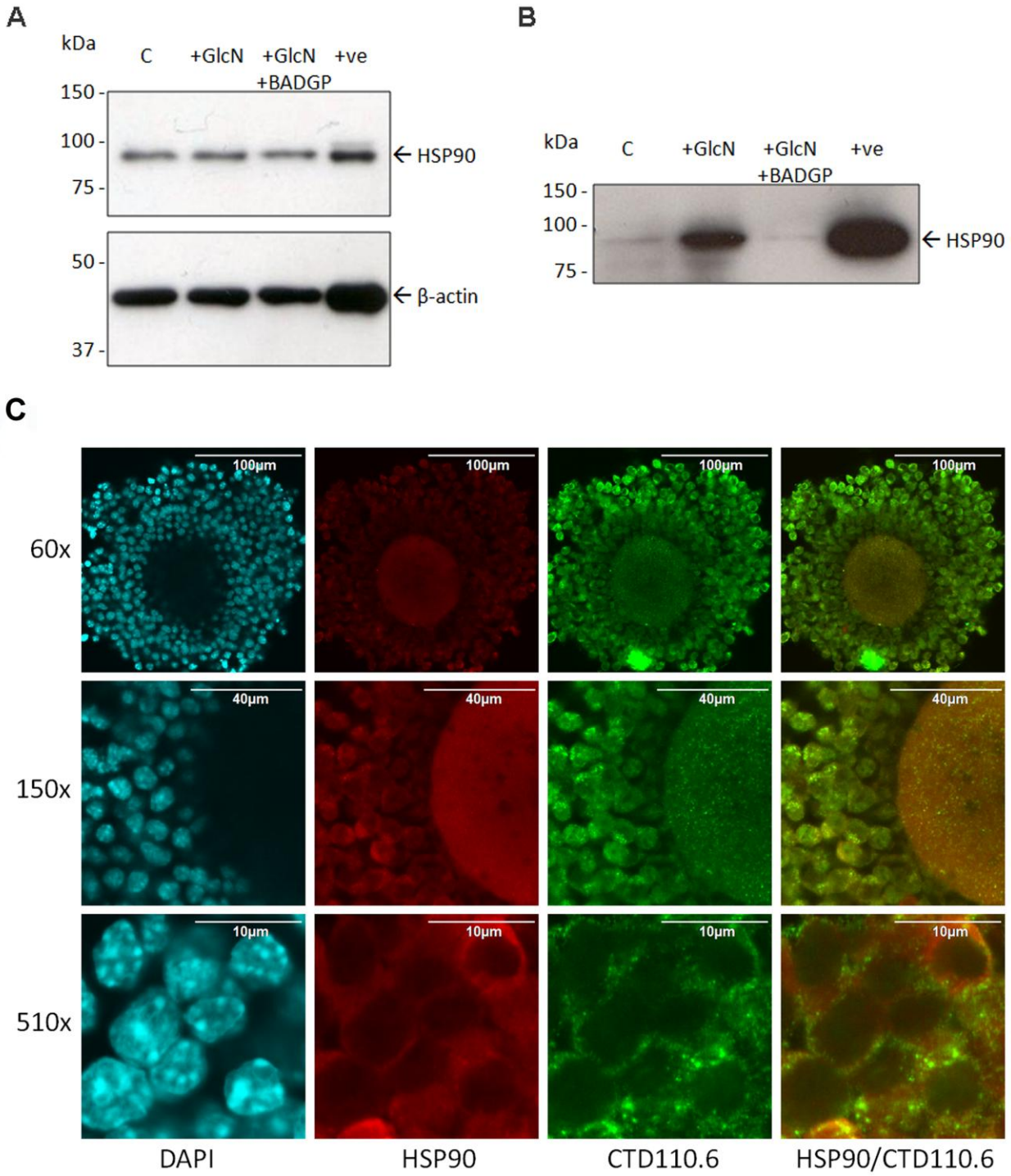

A
kDa
C
+GlcN
+GlcN +BADGP
+ve
150
100
75
50
37
HSP90
β-actin
B
kDa
C
+GlcN
+GlcN +BADGP
+ve
150
100
75
HSP90
C
60x
150x
510x
100μm
40μm
10μm
DAPI
HSP90
CTD110.6
HSP90/CTD110.6

Figure 3

Figure 3 Total and beta-O-linked glycosylated (O-GlcNAcylated) Heat shock protein 90 (HSP90) in cumulus-oocyte-complexes (COCs)

COCs (15 per lane) were collected after 6 h IVM in control medium ± 2.5 mM glucosamine (GlcN) ± 2.5 mM benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP). Proteins were separated using SDS-PAGE, Western blotted and probed using an anti-HSP90 antibody. Positive control (+ve) was 4 μg HeLa cell lysate. (A) Total levels of HSP90. β-actin was used as a loading control. (B) Proteins were immunoprecipitated using CTD110.6 for O-GlcNAc prior to Western blotting. (C) Immunocytochemical localisation of HSP90 (red) and O-GlcNAcylation (green) in glucosamine-treated COCs at various magnifications. 4',6-Diamidino-2-phenylindole (DAPI) was used for nuclear staining.

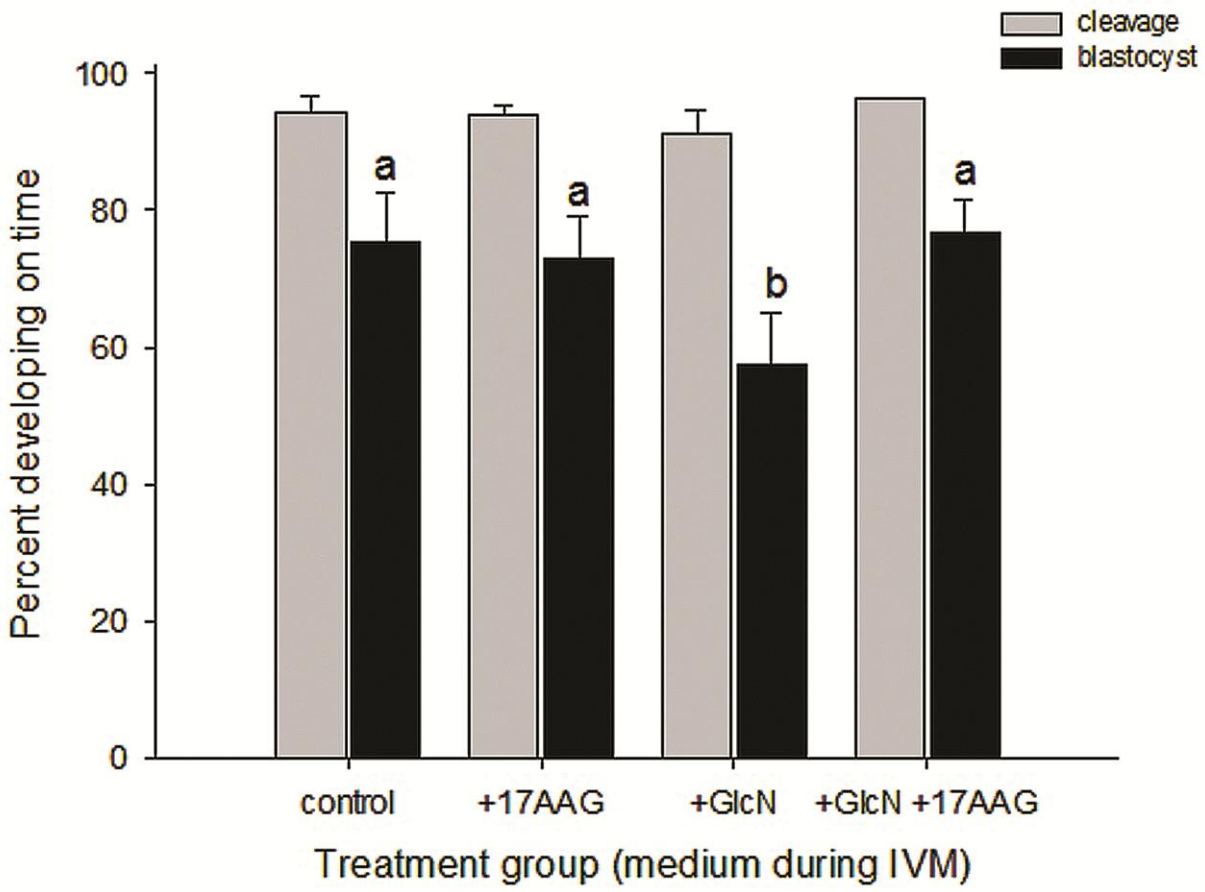

cleavage
blastocyst
Percent developing on time
100
80
60
40
20
0
a
a
b
a
control
+17AAG
+GlcN
+GlcN +17AAG
Treatment group (medium during IVM)

Figure 4

Figure 4 Embryo development following inhibition of Heat shock protein 90 (HSP90) during *in vitro* maturation (IVM)

Cleavage and blastocyst (day 5) rates were assessed, following collection and oocyte maturation in control medium ± glucosamine (GlcN; 2.5 mM) and the HSP90 inhibitor 17-(allylamino)-17-demethoxygeldanamycin (17AAG, 0.1 μM). Groups with different superscripts differ significantly. Data are presented as mean ± SEM.

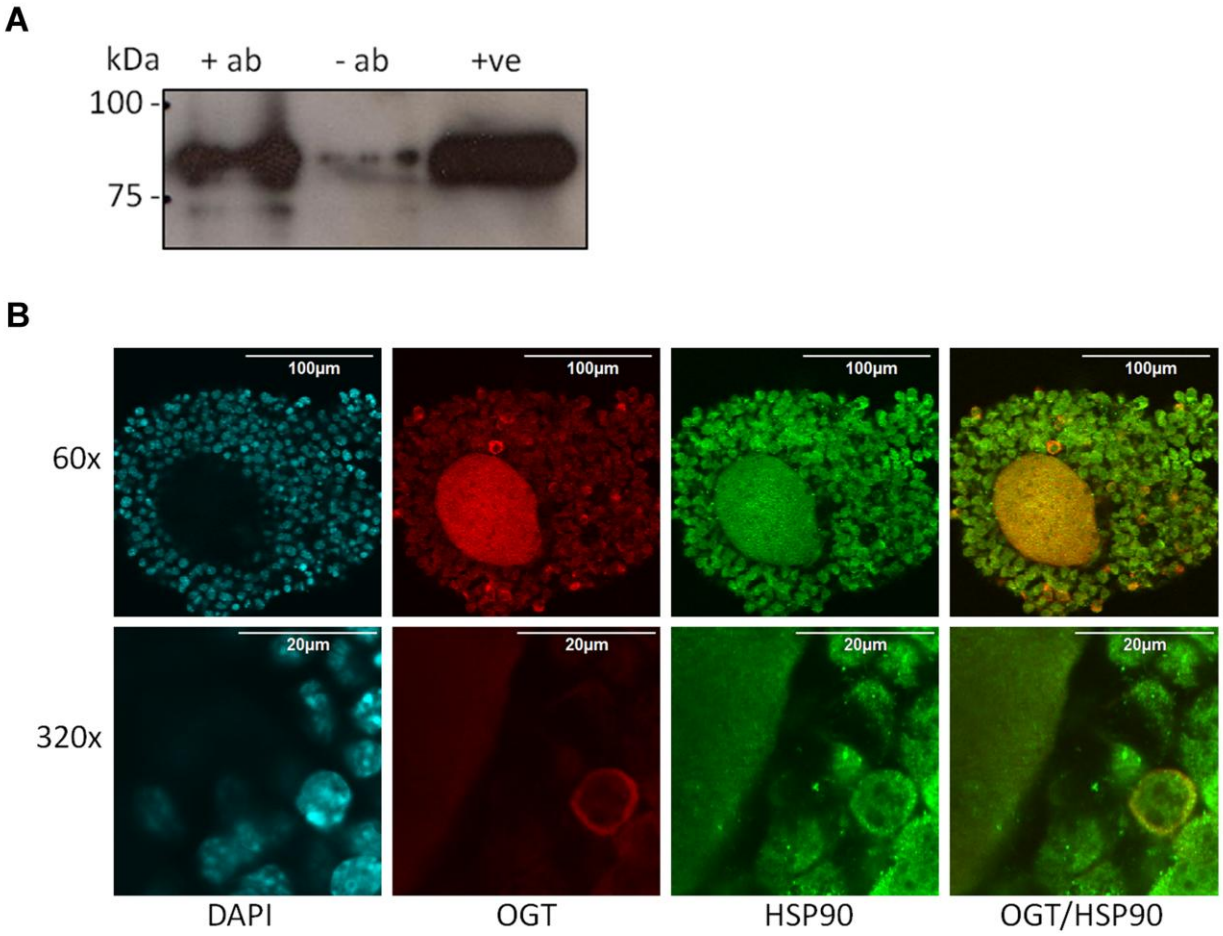
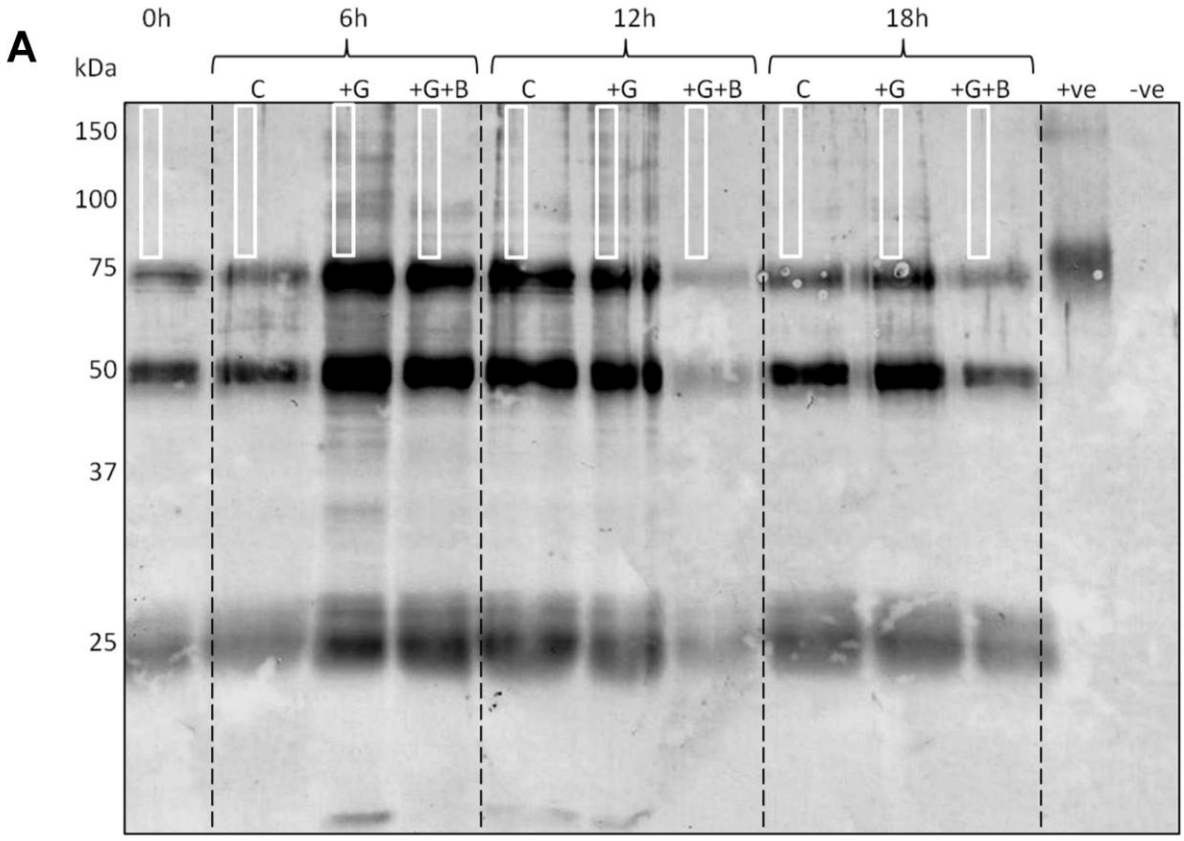
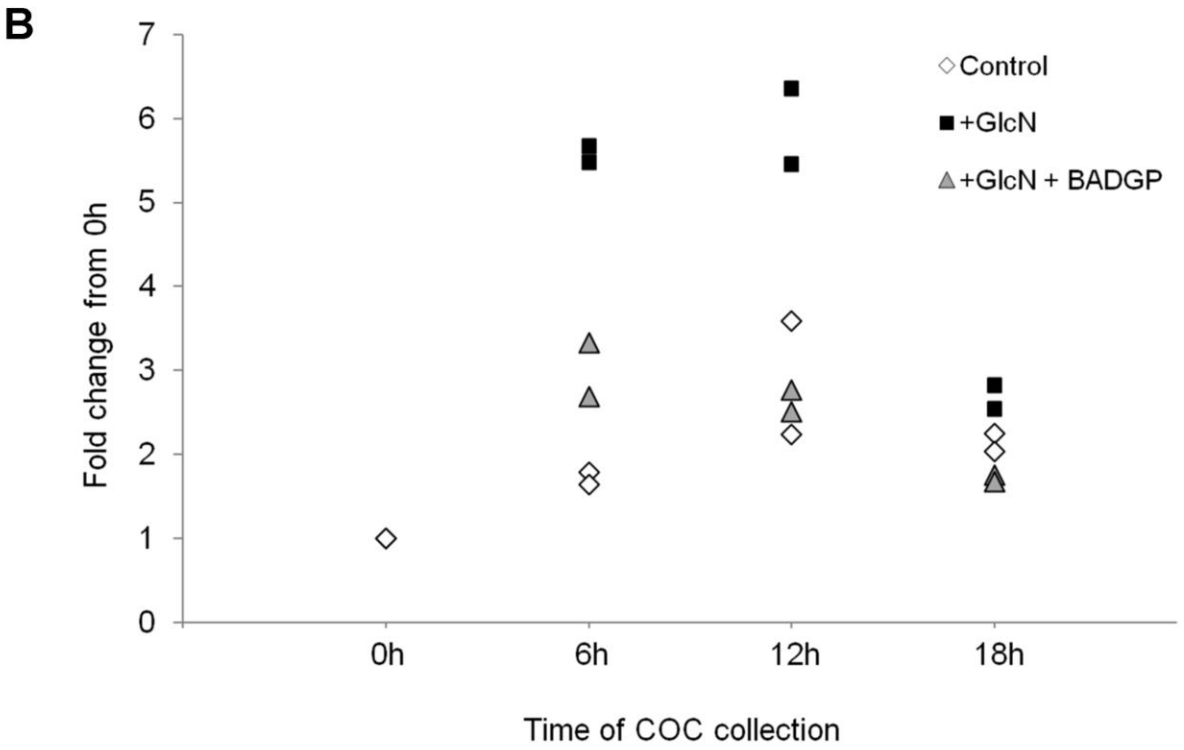

A
kDa
+ ab
- ab
+ve
100 -
75 -
B
60x
320x
100µm
20µm
DAPI
OGT
HSP90
OGT/HSP90

Figure 5

Figure 5 Heat shock protein 90 (HSP90) is associated with beta-O-linked N-acetylglucosamine transferase (OGT) in cumulus-oocyte-complexes (COCs)

(A) COCs (50 per lane) were immunoprecipitated using anti-OGT, separated using SDS-PAGE, Western blotted and probed using an anti-HSP90 antibody. Positive control (+ve) was 4 µg HeLa cell lysate. "+ ab" and "- ab" refer to whether or not primary antibody was used for immunoprecipitation. (B) Immunocytochemical localisation of HSP90 (green) and OGT (red) in COCs at various magnifications. 4',6-Diamidino-2-phenylindole (DAPI) was used for nuclear staining.

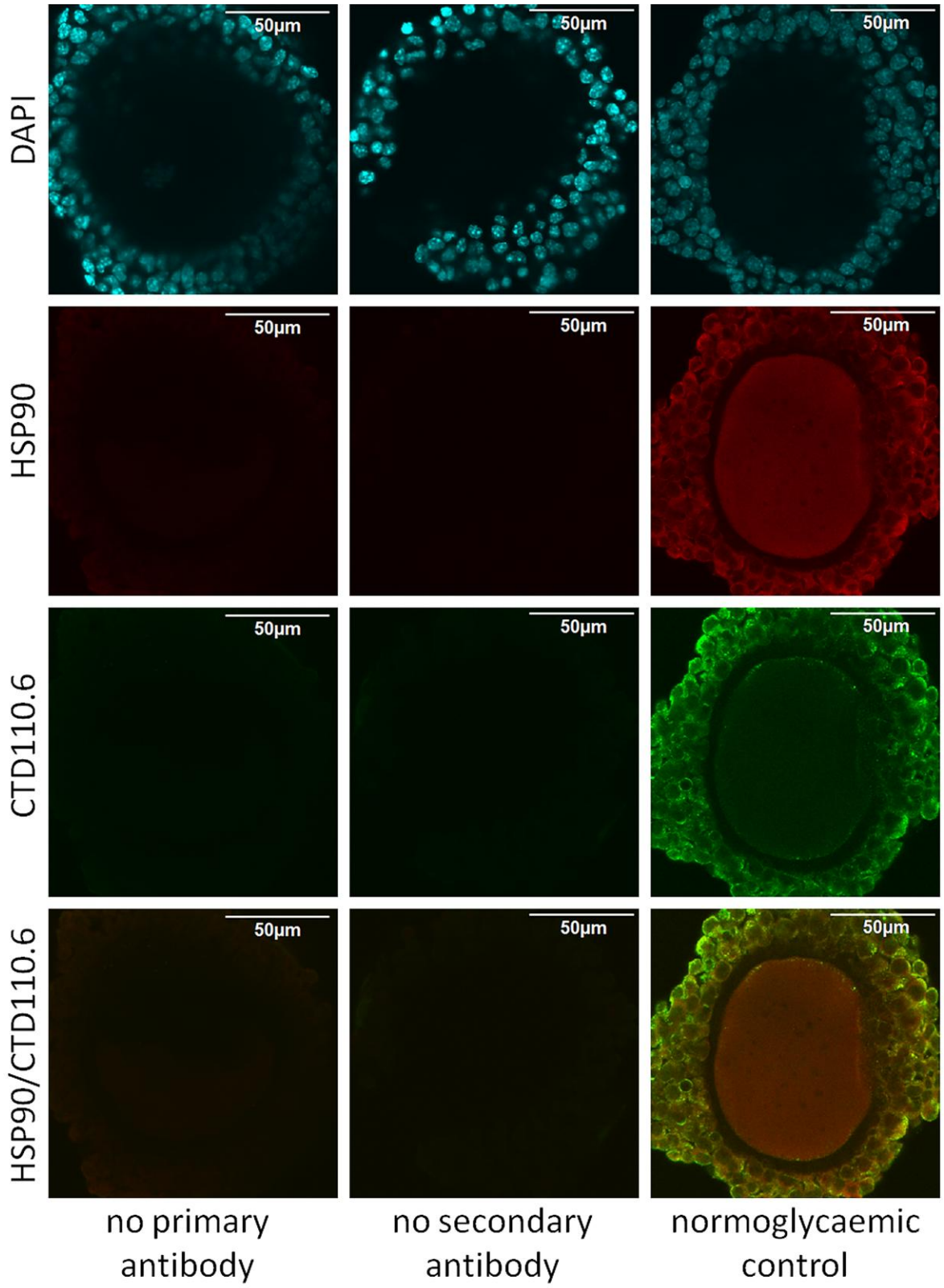




Supplementary Figure 1

SUPPLEMENTARY Figure 1 Preliminary data: beta-O-linked glycosylation (O-GlcNAcylation) in cumulus-oocyte complexes (COCs) throughout *in vitro* maturation (IVM)

COCs (50 per lane) were cultured in control medium (C), ± 2.5 mM glucosamine (GlcN), ± 2.5 mM benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP). Proteins were immunoprecipitated using the O-GlcNAc antibody CTD110.6, separated using SDS-PAGE, Western blotted and probed using CTD110.6. Positive control (+ve) was 0.5 ng BSA-GlcNAc, negative controls were 100 ng each BSA and ovalbumin. (A) Representative Western blot. Areas used for densitometry analysis are shown in white boxes and were restricted to > 75 kDa to avoid any influence of the clearly visible IgM bands at 25, 50 and 75 kDa. (B) Densitometry analysis.



Supplementary Figure 2

SUPPLEMENTARY Figure 2 Representative controls for immunocytochemistry in cumulus-oocyte complexes (COCs)

COCs were collected after 6 h *in vitro* maturation in control medium ± 2.5 mM glucosamine (GlcN) (no antibody controls performed on glucosamine-treated COCs). Immunocytochemical localisation of Heat shock protein 90 (red) and O-GlcNAcylation (green) COCs at 90x magnification. 4',6-Diamidino-2-phenylindole (DAPI) was used for nuclear staining. Normoglycaemic and no antibody controls shown.