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Characterization of O-GlcNAc signalling in human placenta

by

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Declaration

I declare that this thesis has been entirely composed by myself as the result of my own original research work. The contribution by others has been clearly indicated and acknowledged. This thesis has not been submitted for the award of any other degree in any University.

Abstract

The aim of this thesis is to investigate the role of the recently discovered post-translational modification, O-GlcNAcylation, in the regulation of human placenta physiology. The rationale for this research work arises from the increasing evidence showing that O-GlcNAc signalling is a key mediator of cellular functions involved in a wide range of chronic diseases. However, there is a paucity of studies investigating its role in human placenta. Abnormal placenta physiology is considered as an underlying cause of poor pregnancy outcomes including miscarriage, preeclampsia and intrauterine growth alterations, the latter associated with increased risk for the development of chronic disorders later in life. Despite its importance, some of the molecular mechanisms controlling placental formation and function are still poorly understood. In this thesis, the BeWo cell line, a validated model to study *in vitro* mechanisms occurring in human trophoblast, and term placental biopsies, were employed. A transient OGT-depleted BeWo cell line was generated using a small interfering RNA (siRNA) methodology, and was used to identify the role of O-GlcNAc transferase (OGT) and its biochemical mark O-GlcNAcylation during the formation of syncytiotrophoblast, a crucial structure controlling the majority of placental functions. In addition, an antibody-based enrichment method, followed by proteomic analysis, was used to identify O-GlcNAc-modified proteins in BeWo extracts. Term placental biopsies were used to investigate correlations between OGT and feto-maternal parameters. Overall, the results obtained in this research work support a critical role for OGT in the control of syncytiotrophoblast formation via mechanisms involving PKA-MAPKs signalling. Importantly, the proteomic analysis showed that O-GlcNAc-modified proteins belong to a wide range of functional classes, in particular, transcription and chromatin remodelling factors. Finally, maternal body mass index (BMI) and depression target OGT signalling in term placentas with consequences on the activity of regulators of fetal growth and mechanisms of placental response to stress. Taken together, this thesis uncovered novel roles for placental OGT in controlling a wide range of placental mechanisms that are crucial to human reproduction success and identified O-GlcNAc signalling as the molecular link between maternal environment and *in utero* fetal programming of human diseases.

List of Abbreviations

µl: microliters

µM: micromolar

11β-HSD2: 11β-hydroxysteroid dehydrogenase 2

4E-BP1: eukaryotic initiation factor 4E-binding protein 1

AC: adenylyl cyclase

ADHD: attention-deficit hyperactivity disorder

AMP: adenosine monophosphate

AMPK: AMP-activated protein kinase

ANOVA: analysis of variance

ATP: adenosine triphosphate

BM: basal membrane

BMI: body mass index

BSA: bovine serum albumin

BW: birth weight

BWC: birth weight centile

cAMP: cyclic AMP

CBP: CREB-binding protein

CGRP: calcitonin-gene related peptide

co-IP: co-immunoprecipitation

CREB: cAMP response element-binding protein

CRH: corticotropin-releasing hormone

DAVID: database for annotation, visualization and integrated discovery

EPDS: Edinburgh Postnatal Depression Scale

ERK1/2: extracellular signal-regulated kinase 1/2

ESC: embryonic stem cell

ETD: electron-transfer dissociation

FBS: fetal bovine serum

FDR: false discovery rate

FFAs: free fatty acids

FHR: fetal heart rate

GBD: global burden of disease

GCM1: glial cells missing transcription factor 1

GDM: gestational diabetes mellitus

GFAT: glutamine:fructose-6-phosphateamidotransferase

GlcNAc: N-acetylglucosamine

GLUT: glucose transporter

GO: gene ontology

GPCR: G protein-coupled receptor

GSK3: glycogen synthase kinase 3

h: hour/hours

HbA1c: hemoglobin a1c protein

HBP: hexosamine biosynthetic pathway

HCD: higher energy collisional dissociation

hCG: human chorionic gonadotropin

HexNac: N-acetylhexosamine

HPA: hypothalamic–pituitary–adrenal

hPGH: human placenta growth hormone

hPL: human placenta lactogen

IGF-1: insulin-like growth factor 1

IP: immunoprecipitation

IRS-1: insulin receptor substrate 1

IUGR: intrauterine growth restriction

kDa: kilodalton

LAT: large-neutral amino acid transporter

LGA: large for gestational age

LH/CG-R: luteinizing hormone/choriogonadotropin receptor

mA: milliampere

MAPK: mitogen-activated protein kinase

mg: milligrams

Min: minutes

mM: millimolar

MS: mass spectrometry

mTOR: mammalian target of rapamycin

mTORC1: mammalian target of rapamycin complex 1

MVM: microvillous membrane

NF-kB: kappa-light-chain-enhancer of activated B cells

NLS: nuclear localization sequence

nmol: nanomolar

NPC: nuclear pore complex

ns: non significative

OGA: O-glycoprotein 2-acetamino-2-deoxy-β-glucopyranosidase or O-GlcNAcase

O-GlcNAc: O-Linked β-N-acetylglucosamine

OGT: O-linked β-N-acetylglucosamine transferase or O-GlcNAc transferase

OGTT: oral glucose tolerance test

PBS: phosphate-buffered saline

PIP3: phosphatidylinositol 3,4,5 trisphosphate

PKA: protein kinase A

PrE: primitive endoderm

PVN: paraventricular nucleus

S6K1: ribosomal protein S6 kinase beta-1

SDS: sodium dodecyl sulfate

Ser: serine

SGA: small for gestational age

siRNA: short (or small) interfering RNA

SNAT: sodium-coupled neutral amino acid transporter

SP1: specificity protein 1

TET: ten-eleven translocation

TG: triglyceride

Thr: threonine

UDP-GlcNAc: uridine diphosphate N-acetylglucosamine

Chapter 1

Introduction

1.1 Human placenta

1.1.1 Formation of human placenta

Approximately 4–5 days after fertilization of human oocyte, the blastocyst is segregated into two lineages: the inner cell mass , which will give origin to the embryo, and the trophectoderm, precursor of all placental cells (Hamilton and Boyd, 1960). At day 6–7 post-conception, the trophectoderm makes contact and fuses with the endometrium, the epithelium of the uterine mucosa (Figure 1.1, A). At this stage, the stem cells of the trophectoderm generate the early mononuclear cytotrophoblast and the multinuclear primary syncytium, which penetrates in the maternal decidua, a specialized tissue formed from the endometrium under the influence of progesterone (Figure 1.1, B) (Okada et al., 2018; Schlafke and Enders, 1975). At 14 days after fertilization, the blastocyst is completely embedded in the decidua. At this stage, cytotrophoblast cells rapidly proliferate and project toward the primary syncytium to form finger-like extensions into the maternal decidua thus forming the primary villi (Figure 1.1, C) (Hertig et al., 1956; Turco and Moffett, 2019). During the following days, the villous tree develops by continuous proliferation and cell fusion of cytotrophoblast which gives origin to the multinuclear syncytiotrophoblast layer, the mother/fetus interface (Castellucci et al., 2000). By day 18 post-fertilization, fetal capillaries appear within the core of the villi to form the tertiary villi (Hamilton and Boyd, 1960). The recruitment of pericytes, specialized cells that wrap around the endothelial cells of fetal capillaries, stabilizes placental vascular network which further expands to finally connect to the vasculature of the fetus (Burton et al., 2009; Knöfler et al., 2019). At 4 weeks post-implantation, the formation of the basic placental structure is completed (Turco and Moffett, 2019).

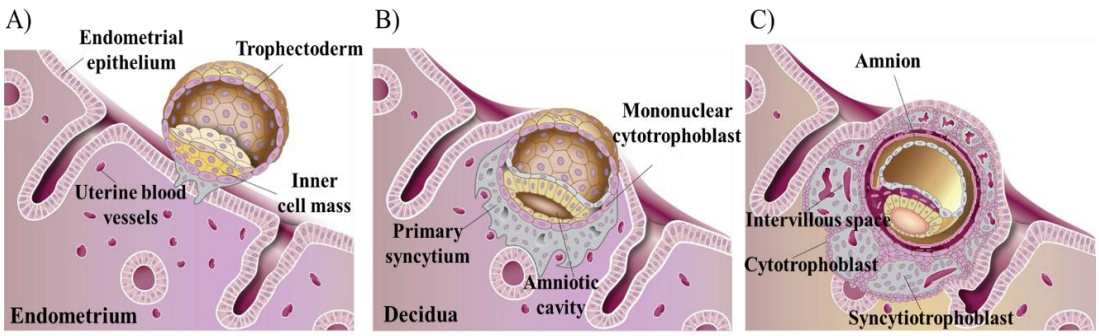


Figure 1.1 Early stages of human placentation and syncytiotrophoblast formation. A) At 6-7 days post-fertilisation, the trophectoderm of the blastocyst makes contact and fuses with the endometrial epithelium of the uterus. B) The stem cells of the trophectoderm give origin to mononuclear trophoblast cells from which the primary syncytium develops by cell fusion. C) 14 days after fertilization, primary villi are formed by sustained proliferation of cytotrophoblast which takes contact with the decidua and fuses to form syncytiotrophoblast, the feto-maternal interface (Adapted from Turco and Moffett, 2019).

1.1.2 Signalling pathways regulating syncytiotrophoblast formation

The syncytiotrophoblast originates from asymmetrical cell division and fusion of villous cytotrophoblast cells (Kliman et al., 1986). The proliferation of the cytotrophoblast layer increases significantly within two weeks post-conception when the villous tree is forming and relies on physical contact with syncytiotrophoblast (Forbes et al., 2008). Human chorionic gonadotropin (hCG), a key hormone secreted by syncytiotrophoblast, is the best characterized factor which promotes syncytialization via autocrine mechanisms (Pidoux et al., 2007; Shi, 1993; Yang et al., 2003). It has been suggested that hCG may constitute the earliest signal which triggers the formation of the primary syncytium. In fact, although hCG is almost exclusively secreted by the mature syncytiotrophoblast, it is also produced by the human embryo as early as 7 days post-fertilization (Bonduelle et al., 1988).

The best characterized molecular signalling pathway which promotes syncytiotrophoblast formation is the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) pathway activated by several factors including hCG and calcitonin-gene related peptide (CGRP) (Green et al., 2006; Shi et al., 1993). In human trophoblast, the hCG binding to the luteinizing hormone/choriogonadotropin receptor (LH/CG-R), a rhodopsin-like G protein-coupled receptor (GPCR) of 674 amino acids, induces conformational changes in the receptor and mechanical activation and release of protein G which activates, in turn, the adenylyl cyclase (AC). AC catalyses the conversion of ATP to cyclic AMP (cAMP) increasing the intracellular levels of this second messenger (Keryer et al., 1998; Pidoux et al., 2007) (Figure 1.2). Mostly, the biological effects of cAMP are mediated via PKA. The latter exists as a tetramer consisting of two regulatory and two catalytic subunits. When cAMP levels are low, the tetramer remains intact and the enzyme inactive. When the intracellular cAMP rises, two cAMP molecules bind to each regulatory subunit inducing a conformational change which causes the release of the catalytic subunits (Kim et al., 2007). The liberated catalytic subunits promote the transfer of ATP terminal phosphates to protein substrates at serine (Ser) and threonine (Thr) residues on the Arg-Arg-X-Ser/Thr consensus motif (Shabb, 2001). In trophoblast, activation of PKA results in the downstream activation of cAMP response element-binding protein (CREB) via phosphorylation at Ser133 and the recruitment of the transcriptional co-activators CREB-binding protein (CBP) and P300 which initiate the transcription of specific

genes involved in trophoblast differentiation including hCG, glial cells missing transcription factor 1 (GCM1) and the fusogenic proteins syncytin-1 and syncytin-2 (Chen and Cheong, 2011; Gerbaud and Pidoux, 2015; Gupta et al., 2016) (Figure 1.2). Syncytin-1 and syncytin-2 are two transmembrane proteins encoded by the envelope genes of human endogenous retrovirus (HERVs) (Blaise et al., 2003; Sha et al., 2000). Their expression increases during trophoblast differentiation and they are both essentials for syncytium formation (Frendo et al., 2003; Vargas et al., 2009). In addition, induction of cAMP/PKA signalling regulates the expression of cadherin and connexin, two key players in syncytiotrophoblast formation, which mediate cell–cell adhesion and assembly of the fusogenic machinery (Gerbaud and Pidoux, 2015). In particular, E-cadherin is important for the formation of adherens junctions, intercellular adhesive structures, during the aggregation of mononuclear cytotrophoblasts while its protein expression diminishes during cell fusion (Coutifaris et al., 1991).

A second key signalling pathway controlling syncytiotrophoblast formation is the mitogen-activated protein kinase (MAPK) cascade. During pregnancy, a sophisticated cross-talk between cAMP/PKA pathway and ERK1/2 and p38 MAPKs regulates different processes including placental leptin expression (Ge et al., 2011; Maymó et al., 2010), protection from maternal stress via 11β-HSD2 enzyme (Shu et al., 2014), decidualization (Yoshino et al., 2003), extravillous trophoblast migration (McKinnon et al., 2001) and syncytiotrophoblast formation (Vaillancourt et al., 2012; Daoud et al., 2005; Delidaki et al., 2011; Nadeau and Charron, 2014). The latter has been widely explored *in vitro*. In BeWo cells, ERK1/2 and p38 are both activated downstream of cAMP/PKA and contribute to hCG production and syncytin-2 expression via different mechanisms. In addition, p38 regulates gene expression of syncytin-1 by controlling GCM1 expression (Delidaki et al., 2011).

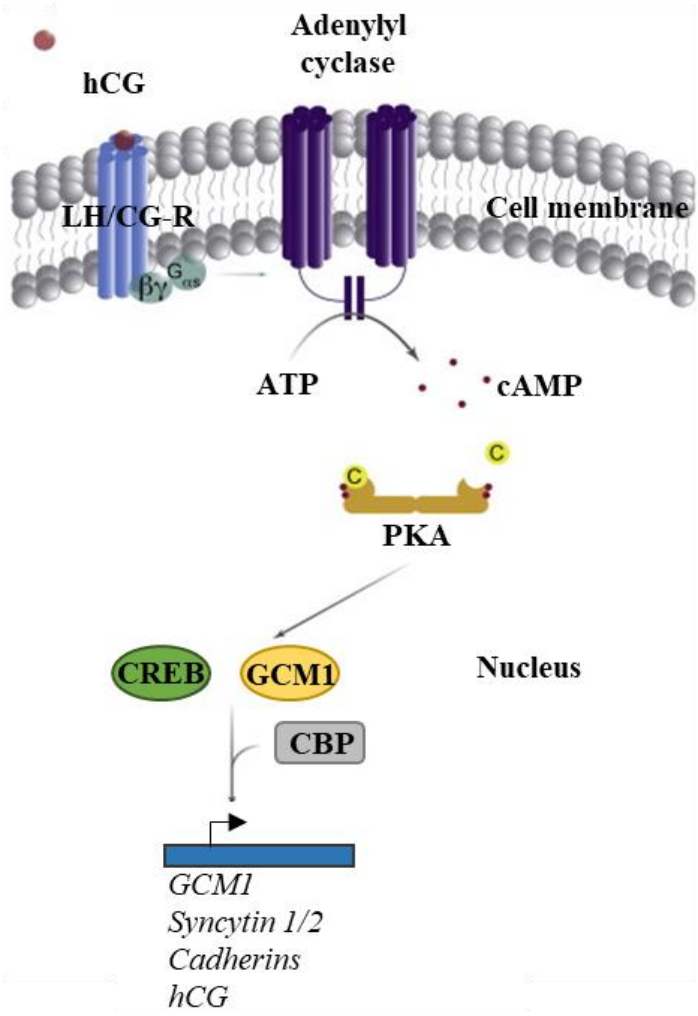


Figure 1.2 Cyclic AMP signalling pathway regulates syncytiotrophoblast formation. hCG binds to the LH-CG receptor, a G protein-coupled receptor (GPCR), which activates adenylyl cyclase to generates cAMP. Four molecules of cAMP bind to PKA regulatory subunits, which releases and activates PKA catalytic subunits which, in turn, enter the nucleus and activate CREB and GCM1 to promote transcription of specific markers of trophoblast differentiation (Adapted from Gerbaud and Pidoux, 2015).

1.1.2.1 Experimental models to study human trophoblast differentiation

Primary cultures and cell lines. Considering the complex dynamic of the placenta physiology, it is not surprising that the study of its diverse functions, including syncytiotrophoblast formation, at different stages of development remains limited. Human primary cytotrophoblast constitutes the most valid model to study cell fusion and syncytialization in human trophoblast (Cotte et al., 1980). The availability of placenta specimens, however, is limited for ethical issues making primary trophoblast cells difficult to be obtained. In addition, primary cytotrophoblast cells cannot be replicated in culture and spontaneously differentiate within about 12 h of plating (Li and Schust, 2015) limiting their application for intensive biochemical and functional studies. Moreover, cytotrophoblast isolation procedures are prone to contamination with low levels of fibroblasts that multiply more rapidly than the cytotrophoblast cells themselves in culture (Frank et al., 2001; James et al., 2016, 2007).

For that reason, alternative self-renewing cellular model systems are widely employed to enable functional studies of placentation. While such systems have their disadvantages compared to primary cells, including genetic abnormalities due to their cancerogenic origin and genotypic and phenotypic variations caused by serial culture passages, they are advantageous in terms of reproducibility, stability and proliferation, enabling researchers to evaluate the effect of therapeutic agents on protein expression as well as physiological processes including syncytial fusion (Orendi et al., 2011). The choriocarcinoma cell lines BeWo, JAR and JEG3, remain the most widely employed *in vitro* model for placental research as they are easy to handle and propagate (Hannan et al., 2010). Although these cells represent a cancer cell model, and their behaviour may diverge significantly in many aspects from a physiological condition, they display various key characteristics and functions of human placental trophoblast cells. In particular, BeWo cells display most of the characteristics of villous trophoblast, including syncytial fusion and the ability to form a barrier with low permeability as well as secretion of hormones including hCG, hPL, progesterone and estradiol (Liu et al., 1997; Orendi et al., 2010; Wolfe, 2006). Recently, a comparative study was conducted to evaluate the endocrine and transport properties of different human trophoblast-derived cell lines including BeWo, JAR and JEG3. The functional evaluation focused on characteristics displayed by the placental barrier including tight junction formation, sodium fluorescein retardation, trans epithelial resistance, glucose

transport and hormone secretion (Rothbauer et al., 2017). Interestingly, BeWo and JAR displayed high glucose transport, which was similar to data derived from term placenta explants. In addition, among the tested cell lines, the choriocarcinoma cell line BeWo resulted the best model for studies on syncytial fusion (Rothbauer et al., 2017).

Murine models. Mouse experimental models, and particularly loss-of-function mutants with disrupted placental development, have proven to be very useful in providing novel insights in the understanding of molecular mechanisms underlying human placental function and disorders. Indeed, human and mouse placentas show many structural and functional similarities. For instance, the labyrinth layer in mice is analogous, in terms of function, to the chorionic villi of the human placenta constituting the site of the feto-maternal exchanges. Moreover, both labyrinth and human placental villi, are covered by the syncytiotrophoblast which takes direct contact with maternal blood (Rossant and Cross, 2001). Interestingly, a recent study compared the molecular similarities between the murine labyrinth and the human villous tree by assessing the proteome and gene expression profile discovering that almost 80% of ortholog genes, evolved in parallel with the diversification of species, are co-expressed in human and mouse (Cox et al., 2009). Studies on loss-of-function mutants have shown that many of these genes are important for a correct trophoblast formation in mice and are also crucial in the analogous cell type in humans. In particular, in mice lacking *Gcm1* gene the labyrinthine trophoblasts did not differentiate and placentas fail to form the labyrinthine layer, necessary for feto-maternal nutrient and gas exchange, resulting in placenta insufficiency whereas all other layers of the murine placenta formed normally indicating a specific role of *Gmc1* in trophoblast differentiation (Schreiber et al., 2000). Similar studies showed a critical role for p38 MAPK in controlling murine labyrinthine formation as targeted inactivation of p38 or its upstream activator Mekk3, resulted in insufficient oxygen and nutrient transfer across the placenta along with severe growth retardation which were no longer present when p38 was reintroduced (Adams et al., 2000; Yang et al., 2000). Interestingly, the existence of two murine endogenous retroviral genes, syncytin-A and syncytin-B, homologous to the human syncytin genes, was also reported (Dupressoir et al., 2005). More recently, it has been demonstrated that, homozygous syncytin-A null mouse embryos die in utero at mid-gestation due to specific disruption of the architecture of the syncytiotrophoblast, with the trophoblast

cells failing to fuse into a syncytial layer (Dupressoir et al., 2009) suggesting the importance of this fusogenic protein for the syncytiotrophoblast formation in both humans and mice.

Although many similarities, the murine and human placentas are very different from an endocrine point of view limiting the employment of this model when studying endocrine functions of human placenta. One of the unique characteristics of human placenta, in fact, is the secretion of steroidogenic hormones throughout pregnancy which occurs in much larger amounts in humans than in any other mammal (Malassine et al., 2003). For instance, no β-hCG genes have been found in mice (Maston and Ruvolo, 2002) suggesting that this major and fundamental hormone for human pregnancy is not produced during murine gestation. A major difference is also detectable in the regulation and function of a crucial placental enzyme, the 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2), which will be extensively explored later in this thesis. In humans, 11β-HSD2 protects the fetus from the exposure to an excess of maternal stress hormones throughout pregnancy and its downregulation is associated with intrauterine growth retardation highlighting its fundamental role in the regulation of fetal growth (Burton and Waddell, 1999). However, in mice, this enzyme is switched off at mid-gestation (Brown, 1996) suggesting a different regulation in mouse and human placentas.

1.1.3 Functions of human placenta: role of placental barrier

During pregnancy, the placenta fulfils a variety of vital functions ranging from immunological acceptance, nourishment and protection of the embryo to endocrine control of pregnancy, maternal metabolism and fetal growth (Gude et al., 2004; Murphy et al., 2006; PrabhuDas et al., 2015). The mature placenta appears as a discoidal organ constituted by a basal plate and a chorionic plate enclosing the intervillous space filled with maternal blood (Figure 1.3). The basal plate is the maternal site of the placenta in contact with the uterine wall and is where the uterine arteries and veins are directed to and from the intervillous space. The chorionic plate is the fetal side of the placenta and gives origin to umbilical cord and branched chorionic villi floating in the intervillous space rich in maternal nutrients. The umbilical vein transports nutrient and oxygen from the mother to the fetus while the arteries transport waste products from the fetus. In a longitudinal section, a chorionic villous appears as a multilayer structure called “placental barrier” formed by 1) trophoblast cells (syncytiotrophoblasts and cytotrophoblasts) that cover the surface of the villous tree; 2) connective tissue forming the villous core which contains mesenchymal cells, macrophages and fibroblasts; 3) endothelium of fetal capillaries (Figure 1.3) (Benirschke et al., 2012; Chatuphonprasert et al., 2018).

The predominant functions of the placenta are operated by the syncytiotrophoblast. The syncytiotrophoblast, from the Greek *syn* - "together", *cytio* - "of cells", *tropho* - "nutrition" and *blast* - "bud", is a multinucleated layer taking origin from continuous proliferation and fusion of the inner cytotrophoblast cells. It is characterized by an apical membrane (or maternal-facing microvillous membrane MVM), in direct contact with the maternal circulation, and a basal membrane (BM) facing the fetal capillaries. The syntytiotrophoblast represents the primary site of nutrient and gas exchange between the mother and the fetus thus preventing the direct contact of maternal and fetal blood (Lager and Powell, 2012). In addition to its role in sustaining fetal metabolism, syncytiotrophoblast produces a multitude of factors and hormones crucial for both placental and fetal development and gives protection to the fetus against a variety of insults (Berga et al., 2016; Cottrell, 2009; Zeldovich et al., 2013) (Figure 1.3).

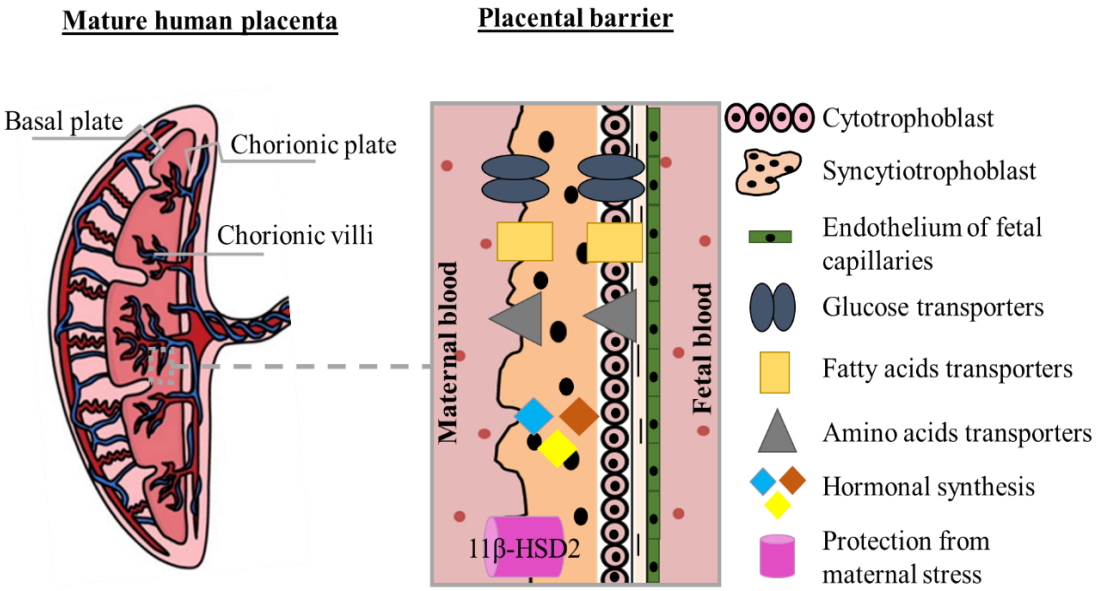


Figure 1.3 Cartoon depicting structure and functions of placental barrier. The mature placenta appears as a discoidal organ with the chorionic villi floating in the intervillous space filled with maternal blood. In longitudinal sections, the villi appear as three-layered structures. The apical membrane of syncytiotrophoblast takes contact with the maternal blood whereas the basal membrane faces fetal circulation. Syncytiotrophoblast fulfils the majority of placenta functions including transport of macronutrients, production of placental hormones and constitutes both a physical and enzymatic barrier against a variety of insults coming from the maternal environment. (Adapted from Lager and Powell, 2012)

1.1.3.1 Transport of macronutrients glucose, amino acids and lipids

Flux across the syncytiotrophoblast represents the rate-limiting steps in maternal-fetal transfer of most important nutrients as only smaller solutes are highly permeable through the syncytial membranes (Brett et al., 2014; Lager and Powell, 2012; Smith et al., 1992). Most sites of known mechanisms of cellular transport are localized to the maternal- and fetal-facing plasma membrane surfaces of the syncytiotrophoblast (Sideri et al., 1984; Smith et al., 1992). Indeed, glucose, amino acids and lipids enter fetal circulation through nutrient-specific transport proteins located within the MVM and BM (Figure 1.3). Hence, it is commonly referred to as facilitated diffusion (Schneider, 1991; Brett et al., 2014; Lager and Powell, 2012).

Glucose. Glucose is the main source of energy for both fetus and placenta (Hauguel et al., 1983). The vast majority of fetal glucose derives from mother (Kalhan et al., 2000; Staat et al., 2012). *In vitro* experiments demonstrate that almost 75% of glucose that enters the placenta reaches the fetal side unchanged (Hauguel-de Mouzon et al., 2001). Glucose transfer across the placenta occurs via specialized glucose transporters (GLUTs). The density of glucose transporter proteins is considerably greater within the MVM than within the BM of syncytiotrophoblast. This suggests that a consistent part of the glucose picked up from maternal circulation is used to fulfil placental metabolic needs (Illsley, 2000; Jansson et al., 1993). GLUT1 is the principal isoform involved in glucose transport across the placenta. Its expression is higher within MVM compared to BM (Baumann et al., 2002; Jansson et al., 1993). However, the density of GLUT1 in the BM increases significantly in the first half of pregnancy reaching a plateau from late second trimester onward suggesting increased fetal glucose demand toward term (Jansson et al., 1993). Interestingly, during the first trimester of pregnancy, placental glucose transport responds to insulin, while during the third trimester glucose uptake is not regulated by insulin (Castillo-Castrejon and Powell, 2017; Anette Ericsson et al., 2005). This can be due to the fact that GLUT1 is the predominant placental glucose transporter expressed throughout pregnancy which increases toward term, while, on the contrary, the insulin-sensitive isoform GLUT4, is expressed during the first trimester but is downregulated in term placentas (Stanirowski et al., 2017). Insulin-like growth factor I (IGF-1), instead, increases the expression of GLUT1 in the BM enhancing transepithelial glucose transport (Baumann et al., 2014). Unlike GLUT1, which is ubiquitously expressed in all

placental cells, GLUT3 shows a more restricted expression pattern, which suggests specialized functions for this glucose transporter (Xu et al., 2015). The GLUT3 isoform is expressed in cytotrophoblasts during early gestation but is localized primarily to the fetal vascular endothelium during late gestation (Brown et al., 2011). It seems to be mainly involved in positive regulation of glucose flux across the placenta by decreasing the umbilical arterial glucose concentration (Illsley, 2000). As for GLUT9, the placenta is one of the few tissues that express both splice variants, GLUT9a and GLUT9b, which are localized within the BM and MVM respectively (Augustin et al., 2004; Bibee et al., 2011).

Amino acids. Amino acids provide the essentials for protein synthesis. Therefore, the concentration of amino acids results higher in fetal circulation compared to maternal circulation highlighting the great demand of the fetus for these macronutrients to grow (Jansson, 2001). Amino acids transport across syncytiotrophoblast is regulated by 15 distinct families of transporters differently distributed between MVM and BM. The rate-limiting step in placental amino acids transport occurs across MVM (Glazier et al., 1997). The most well-characterized placental amino acid transport systems are System A and System L (Jansson, 2001). System A is more highly expressed in the MVM of the syncytiotrophoblast and regulates the transport of small neutral amino acids (SNAT) such as alanine, serine and glycine. Its activity is promoted by different hormones including insulin, leptin and IGF-1 (Jansson et al., 2003; Roos et al., 2009b). On the other hand, System L controls the placenta transport of large neutral amino acids (LAT) such as leucine and its activity is predominantly stimulated by glucose and insulin (Roos et al., 2009b).

Fatty acids. In the maternal circulation, the fatty acids source is in the form of triglycerides (TGs). At the placenta barrier, specific placental TG lipases, such as endothelial lipase and lipoprotein lipase expressed at the MVM, break down TGs into free fatty acids (FFAs) to make them available for placental uptake (Duttaroy, 2009; Gauster et al., 2007). Three membrane systems have been implicated in placental FFAs transport and include a family of fatty acid transport proteins (FATPs) and fatty acid translocase (FAT/CD36), located on both MVM and BM, and a fatty acid binding protein (FABPpm) exclusively found within MVM (Campbell et al., 1998). Similarly to amino acid transporters, the expression and activity of placental fatty acid transporters is influenced by insulin, IGF-1 and leptin (Magnusson-Olsson et al., 2006; Mousiolis et al., 2012).

1.1.3.2 Protective function

The syncytiotrophoblast constitutes a physical barrier against the invasion of a wide range of pathogens (Koi et al., 2002; Robbins et al., 2010; Zeldovich et al., 2013). Rupture or damage of syncytial barrier may constitute a portal for infectious agents to enter fetal compartment (Robbins et al., 2012, 2010). Along with physical barrier activity, the syncytiotrophoblast offers enzymatic defence against a vast variety of xenobiotics coming from maternal compartment thus preventing their cellular accumulation and transfer across the placenta (Myllynen et al., 2005). A crucial enzyme in the syncytiotrophoblast is 11β-HSD2 that offers protection to the fetus from an excess of maternal stress hormones, glucocorticoids, by catalysing the conversion of the human glucocorticoid, cortisol, to its inactive metabolite, cortisone (Yang, 1997). Glucocorticoids are steroid hormones produced predominantly by the adrenal gland which mediate the response of the body to acute stress or daily life challenges (McEwen, 2007). During pregnancy, physiological levels of glucocorticoids guarantee the correct development for most fetal organs in preparation for extrauterine life including lung, heart, kidney, and gut (Seckl, 1998). However, elevation of maternal circulating glucocorticoids as a consequence of maternal stressors, including mood disorders (Conradt et al., 2013; O'Donnell et al., 2012; Ponder et al., 2011) and malnutrition (Bellisario et al., 2015; Chivers and Wyrwoll, 2017; Cottrell et al., 2012; Panchenko et al., 2016), leads to a failure in 11β-HSD2 barrier, including decreased enzyme levels or inhibition of the enzymatic ability to deactivate cortisol, resulting in increased exposure of the fetus to maternal glucocorticoids. Increased glucocorticoids exposure during *in utero* life induces fetal growth derangements with long-term consequences on the offspring's health in both human and rodent models (Cottrell, 2009; Maccari et al., 2003; Matthews, 2000; Seckl and Holmes, 2007).

1.1.3.3 Endocrine function

The syncytiotrophoblast is the primary source of placental hormones during much of pregnancy. The placental endocrine activity is involved in regulating many important aspects of gestation and post-parturition including regulation of maternal metabolism such as mobilization of nutrients, pregnancy maintenance, delivery and lactation (Costa, 2016; Napso et al., 2018) (Figure 1.4). Some placental hormones are secreted

into the fetal circulation to regulate also fetal development and growth (Murphy et al., 2006).

hCG: The first and best characterized protein hormone produced by the syncytiotrophoblast is the human chorionic gonadotropin (hCG). It belongs to the glycoprotein family and is constituted by a α-subunit, shared by all the family members, and a β-subunit characteristic of each hormone. Beta-hCG measurement in maternal blood and urine is commonly employed as a pregnancy test (Cole, 2012). Levels of β-hCG increase exponentially during early pregnancy followed by a plateau during the late first trimester and decline slowly until the end of pregnancy (Cole, 1997). Throughout pregnancy, hCG has been shown to have numerous functions in the placenta, uterus and possible in the fetus (Cole, 2010). From the time of implantation until 3 to 4 weeks post-fertilization, hCG, produced by trophoblast cells, controls the production of progesterone from ovarian corpus luteal cells. As pregnancy progresses, the syncytiotrophoblast is mature enough to take over the production of progesterone (Tuckey, 2005). Progesterone, a steroid hormone synthesised from maternal cholesterol, in turn, is important in inhibition of myometrium contractility, promotion of uterine quiescence and decidualization of endometrium (Graham and Clarke, 1997). Besides controlling progesterone production, hCG promotes placentation by acting in an autocrine manner to stimulate syncytium formation thus participating in the generation and integrity of placental barrier (Shi et al., 1993; Yang et al., 2003). Moreover, hCG promotes the expression of 11β-HSD2 in the syncytiotrophoblast thus participating in maintaining the defence against maternal glucocorticoids excess (Ni et al., 2009). In the uterus, hCG promotes angiogenesis and vasculogenesis during pregnancy ensuring maximal blood supply to the developing placenta and nutrients to the growing fetus (Zygmunt et al., 2003). Furthermore, hCG takes part in regulating the immune response by reducing the macrophage activity at the placenta-uterine interface thus promoting maternal immune tolerance toward the forming fetal tissues (Schumacher, 2017). Finally, hCG contributes to myometrial relaxation during the course of pregnancy while its decrease prior the onset of labour (Edelstam et al., 2007), along with reduction in the number of myometrial hCG receptors (Zuo et al., 1994), suggest a role for hCG signalling in the onset of parturition.

Estrogens: In addition to progesterone and hCG production, syncytiotrophoblast constitutes the major source of estrogens, synthetized from maternal cholesterol,

which include: estrone (E1), estradiol (E2), estriol (E3) and estetrol (E4). During the 1st week of gestation, the estrogens are produced by ovarian corpus luteum under the stimulation of hCG. As pregnancy progress, the syncytiotrophoblast takes over the synthesis of these steroids producing around 100-120 mg/day at term (Chatuphonprasert et al., 2018). Estradiol is the most abundant steroid produced during pregnancy (Loriaux et al., 1972). Estrogens mainly promote blastocyst implantation by stimulating endometrial growth and differentiation (Groothuis et al., 2007). In addition, estradiol stimulates the uteroplacental blood flow by promoting vasodilatation of uterine and placental arteries (Corcoran et al., 2014) and takes part in labour initiation by stimulating the contraction of human myometrial cells (Di et al., 2001).

Hormones regulating maternal metabolism: A number of hormones produced by the syncytiotrophoblast, including human placenta lactogen (hPL) and human placenta growth hormone (hPGH), which share high homology and function, takes part in the regulation of maternal metabolism to ensure the mobilization of energy resources needed during pregnancy. Glucose is the main source of energy for both placenta and fetus and, for that reason, pregnancy has a major impact on glucose homoeostasis via insulin-mediated signalling (Butte, 2000; Leturque et al., 1987). Insulin secretion increases significantly during the first part of gestation to promote maternal lipid accumulation whereas insulin insensitivity arises during mid-gestation to promote hepatic gluconeogenesis and limit glucose uptake in maternal tissues thus providing higher levels of circulating glucose for the growing fetus (Bell and Bauman, 1997; Butte, 2000). Maternal serum levels of hPL rises in relation to the fetus and placenta size with maximum levels reached near term, suggesting a critical role of this hormone in placental and fetal growth (Bhagavan, 2002). Indeed, hPL reduces sensitivity to insulin in maternal peripheral tissues increasing glucose concentration in maternal circulation and promotes lipolysis of maternal fat storage to produce free fatty acids which, in part, become available fuel for the mother, utilized in substitution of glucose, and in part are converted in ketones which freely cross the placenta contributing to the formation of fetal brain (Bronisz et al., 2018; Handwerger and Freemark, 2000).

Neuroendocrine hormones: During pregnancy, placenta becomes the main source of neuroendocrine hormones by replacing much of the mother's pituitary and hypothalamic functions (Krieger, 1982; Voltolini and Petraglia, 2014). An example is the corticotropin-releasing hormone (CRH) secreted mainly by the paraventricular

nucleus (PVN) of the hypothalamus. CRH is synthesized in the syncytiotrophoblast from first to third trimester and maternal serum levels rise exponentially throughout pregnancy to peak at labour (Grammatopoulos, 2008; Petraglia et al., 1988; Riley et al., 1991). It targets multiple fetal–maternal tissues and is involved in the regulation of several functions throughout pregnancy including hormone secretion, immune response, glucose transport, vascular tonus and myometrial contractility (Gao et al., 2012; Voltolini and Petraglia, 2014). Interestingly, in contrast to the hypothalamus, placental CRH release is positively regulated by glucocorticoids (Robinson et al., 1988) suggesting increased demand for CRH biological functions near term (Petraglia et al., 2010). Indeed, at term, CRH regulates timing of parturition via estrogens and progesterone-mediated mechanisms which control the contractile properties of the myometrium (Majzoub and Karalis, 1999; Gangestad et al., 2012; Thomson, 2013). In addition, CRH stimulates cortisol and dehydroepiandrosterone sulphate (DHEAS) production from fetal adrenal gland at the end of pregnancy, suggesting a role in fetal lung maturation and adaptive mechanisms in response to the stress of parturition (Pepe and Albrecht, 1995; Voltolini and Petraglia, 2014).

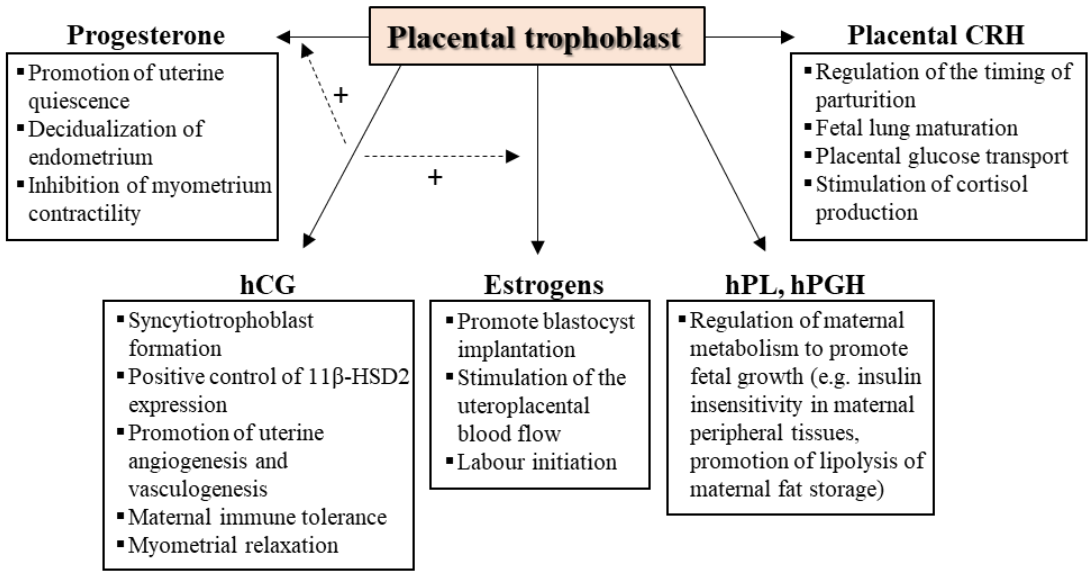


Figure 1.4 Endocrine activity of the human placental trophoblast. The syncytiotrophoblast is the primary source of placental hormones during much of pregnancy. Placental hormones regulate many important aspects of gestation including mobilization of maternal nutrients for fetal growth, placentation, pregnancy maintenance and parturition.

1.1.4 Placental mechanisms of *in utero* programming: contribution of maternal overnutrition and stress

According to the paradigm of the Developmental Origins of Health and Disease (DoHAD) postulated by Barker 27 years ago (Barker et al., 1993), perinatal exposure to suboptimal environmental conditions, including maternal metabolic alterations and stress, deranges fetal growth and "program" the offspring to develop chronic diseases, including cardiovascular disorders, obesity, and diabetes, later in life (Curhan et al., 1996; Leon et al., 1998). It is now widely accepted that changes in fetal growth are the result of maladaptive placental responses to maternal environment which include aberrant placental development and function with major impact on placental size and morphology, alterations in the expression of nutrient transporters in the placental barrier and changes in the placental response to stress and epigenetic mechanisms (Fowden et al., 2006; Longtine and Nelson, 2011; Wu et al., 2004). Although it is now widely accepted that alterations in maternal physiology can be passed to the fetus via maladaptive changes in placenta development and function, the exact mechanisms through which the placenta acquires disease properties during maternal metabolic complications, especially overweight and obesity, and during distress states such as mood disorders, need further investigations.

1.1.4.1 Epidemiologic evidence of *in utero* programming

Maternal obesity: Obesity is the leading preventable cause of mortality in the developed countries classified as a disease by the American Medical Association in 2013 (Barness et al., 2007; Mokdad et al., 2004). In 2015, 600 million adults (12%) and 100 million children were classified as obese among the world's population (GBD 2015 Obesity Collaborators, 2017). Likewise, the prevalence of obesity in women at the reproductive age, as well as the number of women entering pregnancy obese, have risen rapidly in the last two decades (Flegal et al., 2012; Heslehurst et al., 2010).

Obesity is the most common problem in obstetrics that affects enormously both mother and child health in the short- and long-term (Catalano and Shankar, 2017). In particular, it is associated to spontaneous abortion, stillbirth and reduced likelihood of natural labour at term along with post-term pregnancy and occurrence of complications during delivery (Chu et al., 2007; Denison et al., 2008). In addition, maternal obesity significantly increases the risk for developing hypertensive disorders,

pre-eclampsia and gestational diabetes mellitus (GDM) during pregnancy as well as postpartum diabetes and hyperglycemia (Catalano and Ehrenberg, 2006; O'Brien et al., 2003; Weiss et al., 2004; Yogev and Langer, 2008).

The consequences for the offspring born from obese mothers are also severe. Epidemiological studies showed that maternal obesity significantly deranges offspring's growth increasing the risk for macrosomia (birthweight ≥4500 g), fat mass and fetal overgrowth (Ehrenberg et al., 2004; Metzger, 2010; Sewell et al., 2006). In addition, offspring born from pregnancy complicated by obesity and GDM are more likely to develop childhood and adult obesity and type 2 diabetes mellitus (Drake and Reynolds, 2010; Hochner et al., 2012; Reynolds et al., 2010). Macrosomia itself is a strong predictor of BMI at 1 year of age as well as obesity and cardiovascular risk at adulthood (Lindberg et al., 2012). Moreover, a strong association between maternal obesity and offspring neurodevelopmental and psychiatric morbidity has been reported (Edlow, 2017). In particular, children born from mothers with a metabolic condition, including obesity, are at risk for autism and developmental delay (Girchenko et al., 2018; Krakowiak et al., 2012; Li et al., 2016). In addition, maternal obesity has been associated with symptoms of attention-deficit hyperactivity disorder (ADHD) in the child and difficulty in the control of emotionality (Rodriguez, 2010; Rodriguez et al., 2008). Likewise, the risk for developing schizophrenia is increased up to 7.78-fold in children born from obstetric complications including diabetes and emergency cesarean section often associated to maternal obesity (Cannon et al., 2002; Chu et al., 2007). Finally, infants born larger than normal have a higher likelihood of developing anxiety and depression during adolescence and as adults (Colman et al., 2012; Van Lieshout et al., 2020).

Maternal stress and mood disorders: Epidemiological evidence shows that up to 13% of women experience depressive symptoms at some point during gestation or within one year from parturition (Gavin et al., 2005). Similarly, the prevalence for anxiety symptoms has been found to increase with the trimester of pregnancy reaching 24.6% in the third trimester as revealed recently by a meta-analysis study (Dennis et al., 2017). Clinical studies show that symptoms of antenatal depression as well as moderate to severe life stress and maternal anxiety in women are associated with an increased risk for premature delivery, low birthweight and increased neonatal crying (Dunkel Schetter and Tanner, 2012). Moreover, it has been demonstrated that a distressed psychological state in the mother influences many aspects of fetal

physiology. For instance, perceived stress, depression and anxiety disorders during pregnancy, can change fetal heart rate (FHR) activity which, in turn, is a predictor of neurobehavioral disturbance in childhood (Kinsella and Monk, 2009). In addition to FHR, the fetal activity and sleep pattern have been shown to be influenced by maternal psychological states, suggesting that maternal mood exerts a major impact on the activity of the fetal nervous system (Kinsella and Monk, 2009). Indeed, clinical studies showed that maternal distress particularly affects fetal brain development and influences children's long–term mental health. For instance, exposure to traumatic events and chronic stress during pregnancy have been associated with emotional and cognitive problems in the child, including an increased risk of attentional deficit/hyperactivity, anxiety, and language delay (Talge et al., 2007). Moreover, maternal mental disorders such as anxiety and depression are associated with increased risk for child emotional problems, depression in adolescence and low levels of general cognitive development in childhood (Stein et al., 2014). Two principal pathways have been proposed for the *in utero* transmission of maternal stress to the fetus and include the dysregulation of the maternal-fetal hypothalamic–pituitary–adrenal (HPA) axis, which will be discussed later in this thesis Chapter, and disruption of placenta physiology and development due to variations in uterine artery flow (Kinsella and Monk, 2009).

1.1.4.2 Role of placental nutrient transport in fetal programming

Observation in humans showed that, a low caloric intake is associated to a lower placental weight and thickness (Roseboom et al., 2011) whereas higher maternal body weight correlated with larger placenta (Strøm-Roum et al., 2016; Thame et al., 2004). Early stages of gestation constitute a sensitive window during which placental structures are formed and the placenta is more susceptible to developmental alterations. For instance, the glucose transport system is particularly affected by changes in maternal metabolism as glucose uptake responds to insulin in primary villous during early pregnancy but not at term (Ericsson et al., 2005a; Ericsson et al., 2005b). In addition, GLUT1 levels increase in early pregnancy representing a critical event since metabolic perturbations may further stimulate its expression (Jansson et al., 1999). Accordingly, placental glucose transporters along with amino acid transporters, respond to adverse maternal metabolic conditions including, obesity, diabetes and malnutrition (Das et al. 1998; Illsley 2000). In particular, pre-existing

diabetes and GDM are associated with increased expression of GLUT transporters in the BM of the syncytiotrophoblast and significantly higher uptake of glucose compared to normal pregnancies (Bibee et al., 2011; Gaither et al., 1999; Jansson et al., 1999). According to the Pedersen hypothesis, maternal hyperglycemia increases transplacental passage of glucose inducing fetal hyperglycemia and insulin secretion leading to increased fetal adipose mass accumulation and macrosomia at birth (Pedersen, 1971; Vambergue and Fajardy, 2011). Besides diabetes, recent observations showed that placental expression of GLUT1 is positively associated with maternal sugar intake in normal pregnancies (Brett et al., 2015). Furthermore, in obese women giving birth to large infants, placental GLUT1 expression and system A activity are up-regulated in BM and MVM respectively (Acosta et al., 2015; Jansson et al., 2013). Similarly, observations in animal models consuming high-fat diet periconceptionally showed fetal overgrowth along with up-regulation of transplacental glucose, increased expression of GLUT1 and SNAT2 amino acid transporter (Jones et al., 2009).

1.1.4.3 Placental nutrient-sensing system in fetal programming

According to the placental nutrient-sensing model, the placenta mediates the allocation of nutrients between mother and fetus by coordinating changes in maternal metabolism and physiology with fetal growth and requirements. This is possible due to the presence of sensors, expressed by the syncytiotrophoblast, which are able to detect maternal nutritional stimuli, such us glucose, fatty acids, amino acids and ATP, and adjust cell metabolism and placental transport in response to nutrients availability and fetal demand (Figure 1.5). Extreme conditions can activate or disactivate these nutrient-sensing mechanisms in the attempt to restore homeostasis with consequences on fetal development. The best characterized nutrient sensing pathways in the placenta are the mammalian target of rapamycin complex 1 (mTORC1) and the AMP-activated protein kinase (AMPK), but a novel candidate, the hexosamine biosynthetic pathway (HBP), has lately emerged (Díaz et al., 2014; Dimasuay et al., 2016; Hart et al., 2019; Jansson and Powell, 2013).

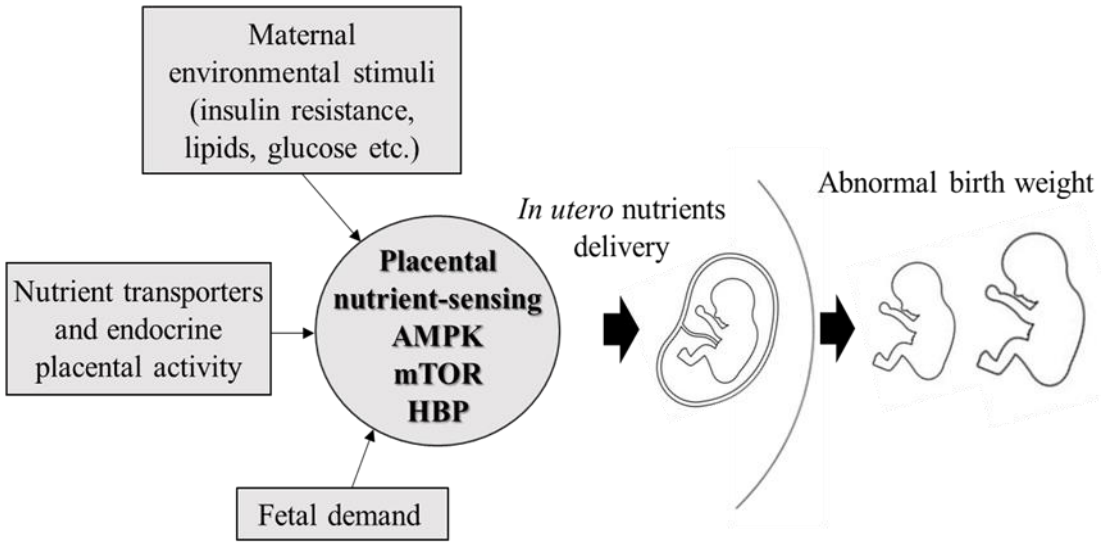


Figure 1.5 Representation of the placental nutrient-sensing model. The placenta integrates both maternal and fetal signals with information from nutrient-sensing signalling pathways to match fetal demand with maternal supply thus regulating pregnancy physiology, placental growth, and nutrient transport to the fetus.

AMPK: AMPK activity as a cellular energy sensor is well known in different cell types (Hardie et al., 2012). In response to depletion of ATP and rise in AMP levels, AMPK maintains energy balance within the cell by switching off anabolic pathways, such as protein synthesis, and turning on processes generating ATP by increasing, for example, glucose uptake and fatty acid oxidation (Garcia and Shaw, 2017). According to its role as a placental nutrient sensor, AMPK is activated during maternal calorie restriction (Ma et al., 2011), whereas it is decreased in the placenta of over-nourished obese ewes (Zhu et al., 2009), in pregnant rats feeding high-fat diet (Gaccioli et al., 2013) and in obese women (Jansson et al., 2013) all conditions associated with derangement in fetal growth and alterations of birth weight.

mTORC1: mTOR is a ubiquitously expressed serine/threonine kinase constituted by two complexes, mTORC1 and mTORC2. In particular, mTORC1 controls cell growth, proliferation, and metabolism in response to nutrient availability and growth factor signalling by regulating protein synthesis via activation of the downstream targets, ribosomal S6 kinases (S6K1 and S6K2) and eukaryotic initiation factor 4E-binding proteins (4E-BP1 and 4E-BP2) (Ma and Blenis, 2009; Wang and Proud, 2006). In addition, it controls the transcription of metabolic gene network including genes

involved in amino acids, lipids and nucleotide metabolism (Düvel et al., 2010). In the placenta, mTORC1 is highly expressed in the syncytiotrophoblast (Roos et al., 2007) and its activation is regulated by glucose and amino acid concentrations (Roos et al., 2009b) as well as insulin and IGF-1 (Shang and Wen, 2018; Taniguchi et al., 2006). In turn, mTORC1 stimulates both system A and L amino acid transporters thus regulating amino acids delivery to the fetus (Roos et al., 2009a). In agreement with its role as a placental nutrient sensor, mTORC1 is activated during maternal obesity associated with fetal overgrowth in both human (Jansson et al., 2013) and animal models of high-fat diet-induced obesity (Gaccioli et al., 2013; Rosario et al., 2016), whereas it is inhibited in human intrauterine growth restriction (IUGR) (Roos et al., 2007; Yung et al., 2008) and in IUGR induced by a poor diet in animal models (Kavitha et al., 2014; Rosario et al., 2011). Importantly, AMPK exerts an inhibitory effect on mTORC1 and the two placental sensors have been found to be inversely activated in placentas from GDM pregnancies and in obese mothers giving birth to large babies (Jansson et al., 2013; Martino et al., 2016), suggesting the presence of a placental AMPK-mTORC1 interplay which regulates fetal growth.

HBP: The nutrient-sensing HBP is activated in a nutrient-dependent fashion, especially by glucose, to produce the end product uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), the substrate for the O-GlcNAc transferase (OGT) which modifies post-translationally thousands of nucleocytoplasmic proteins thus controlling a myriad of cellular processes within multiple tissues (Love and Hanover, 2005; Vaidyanathan and Wells, 2014). However, the role of the HBP and O-GlcNAc modification in nutrient-sensing within the placenta is less known. Preliminary studies showed that, HBP is active in first trimester human placenta and influences villous hormone production and IGF-stimulated trophoblast turnover (Acharya et al., 2012). In murine models, manipulation of HBP and O-GlcNAc signalling causes disturbance in the vasculature and shape of the placenta along with abnormal expression of GLUT1 transporters and fetal growth restriction (Yang et al., 2015). Similarly, increased flux through the HBP, induced by stimulation with glucose and glucosamine, leads to aberrant placental physiology and fetal growth in rodent models suggesting a potential role of the HBP pathway and O-GlcNAc modification of proteins in placental growth and function (Dela Justina et al., 2017; Palin et al., 2013).

1.1.4.4 Maternal hypothalamic–pituitary–adrenal (HPA) axis and 11β-HSD2 activity

In humans, the HPA axis controls the synthesis and release of the stress hormone cortisol. Physical or emotional stress, but also metabolic stimuli such as hypoglycemia, induce the release of CRH from the hypothalamus, which stimulates the secretion of the adrenocorticotropic hormone (ACTH) from the anterior pituitary gland which, in turn, controls the synthesis and secretion of cortisol from the adrenal cortex (Figure 1.6) (Smith and Vale, 2006). In a physiological state, cortisol restores homeostasis after stress response. This occurs via activation of glucocorticoid receptors (GRs), which are present in almost every cell in the body. When the ligand-receptor complex is formed, it translocates to the nucleus where it regulates the transcription of genes mainly associated to the anti-inflammatory response and regulation of metabolism (Sapolsky et al., 2000). The HPA axis activity changes significantly during pregnancy and lactation as an adaptive response to the modifications occurring in maternal physiology (Brunton et al., 2008). Moreover, in the fetus, cortisol plays a crucial role in the maturation of many organs contributing to the formation of respiratory, renal, and cardiovascular systems (Seckl, 1998). Normal pregnancy is a state of transient hypercortisolemia in part due to increased levels of placental CRH in the second half of pregnancy which stimulates the release of the ACTH from the anterior pituitary gland and consequently the release of cortisol from the adrenal (Mastorakos and Ilias, 2006) (Figure 1.6). Increases in plasma cortisol can be detected as early as the 11^{th} week of gestation reaching almost 5-fold between the first trimester and delivery (Lindsay and Nieman, 2005). The fetus is protected from maternal hypercortisolism by the activity of placental 11β-HSD2 which inactivates cortisol by converting it into cortisone, while 11β-HSD1 drives the opposite reaction by converting cortisone into cortisol (Figure 1.6). During most of the pregnancy, the conversion of cortisol into cortisone by 11β-HSD2 predominates whereas, in late gestation, placental 11β-HSD2 activity markedly declines and maternal glucocorticoid concentrations rise to favour fetal organ maturation (Agnew et al., 2018; Fowden et al., 1998). However, the protection offered by the 11β-HSD2 barrier in early gestation can be weakened by maternal disease and mood disorders such as anxiety or depression (Ponder et al., 2011), metabolic disorders (McTernan et al., 2001), infection and inflammation (Chapman et al., 2013; Chisaka et al., 2005). These conditions lead to an increase in maternal circulating cortisol along with decreased

expression of placental 11β-HSD2, promoting increased transfer of maternal glucocorticoids to the fetus. Because maternal cortisol levels are much higher than fetal levels, even moderate variations in placental 11β-HSD2 expression or activity can significantly alter fetal cortisol exposure (Duthie and Reynolds, 2013). As the forming fetal organs, particularly brain, liver and kidney, highly express GRs, increased exposure to cortisol impinges on the basic structure of immature organs affecting, subsequently, their functions and predisposing the offspring to an increased risk for neurodevelopmental and cardiometabolic problems in adulthood (Duthie and Reynolds, 2013; Lindsay and Nieman, 2005).

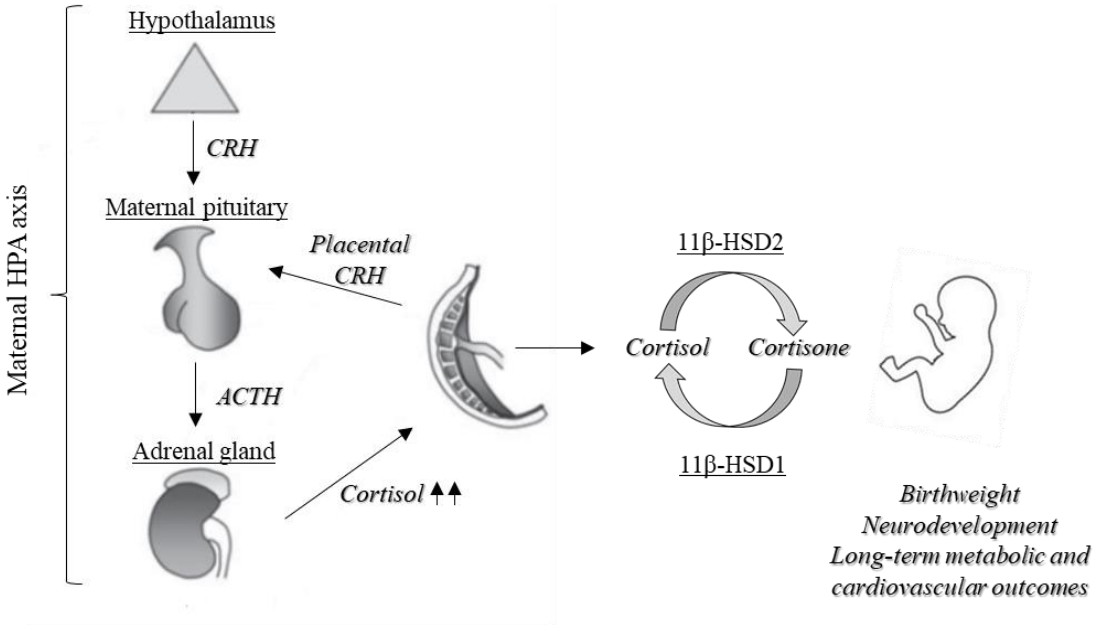


Figure 1.6 Placental barrier to maternal stress hormones. The secretion of maternal cortisol is controlled by the HPA axis and the majority of this hormone is inactivated in the placenta by the 11β-HSD2 enzyme which converts cortisol into cortisone. In normal pregnancies, only a little portion of cortisol reaches the fetus to ensure organ maturation. In this respect, glucocorticoids program *in utero* many aspects of the offspring's physiology at adulthood including metabolism and brain functions (Adapted from Duthie and Reynolds, 2013).

1.1.4.5 Fetal programming and placental epigenetics

Epigenetic mechanisms may also contribute to transmit maternal alterations to the growing fetus. The term “epigenetics” refers to inheriting mechanisms of variation in gene expression which are not associated to changes in the DNA nucleotide sequence such as DNA methylation and histone acetylation. One proposed mechanism via which maternal nutritional status may alter placental epigenetic modifications is by altering the availability of methyl donors, such as folate (Waterland and Jirtle, 2003). Observations in humans showed that, maternal glucose metabolism dysregulation preferentially affects DNA methylation of placental genes involved in energy and glucose metabolism along with genes regulating fetal and placental growth (Bouchard et al., 2010; Lesseur et al., 2014; Ruchat et al., 2013). Recent studies conducted in both humans and murine models showed that the placental epigenetic machinery is also sensitive to maternal obesity associated with adverse pregnancy outcomes (Mitsuya et al., 2017; Panchenko et al., 2016). In humans, the methylation status of specific subsets of genes, including genes controlling energy homeostasis and genes associated with cardiometabolic risk, is particularly affected in the placenta of babies born with abnormal birth weight (Chen et al., 2018; Díaz et al., 2017). These data suggest that changes in DNA methylation may provide a molecular mechanism to explain the impact of maternal nutrition and metabolism on fetal programming of diseases.

Maternal stress and mood disorders can also induce changes in placental gene expression via epigenetic mechanisms particularly affecting the expression of genes involved in the regulation of placental glucocorticoid pathway with potential implications for fetal neurodevelopment. For instance, maternal anxiety and perceived stress have been linked to increased placental methylation of the gene encoding the 11β-HSD2 enzyme (Monk et al., 2016; Conradt et al., 2013) while maternal depression has been associated with increased placental methylation of the gene encoding glucocorticoid receptors, NR3C1 (Conradt et al., 2013). Recent studies have also found that levels of war trauma and chronic stress were associated with placental DNA methylation levels of four key genes regulating the HPA axis activity including CRH, CRH binding protein (CRHBP), which binds CRH to regulate its actions, NR3C1, and FKBP5, a co-chaperone protein which modulates glucocorticoid receptor activity (Kertes et al., 2016).

1.2 O-GlcNAc signalling

1.2.1 The hexosamine biosynthetic pathway (HBP) and O-GlcNAc modification

An increasing body of evidences point to a key regulatory role for glucose in mediating cellular signalling. Although most cellular glucose is metabolized by glycolysis to release energy in the form of ATP, almost 1–5% enters the HBP where it is converted to the final product UDP-GlcNAc, a nucleotide sugar extensively involved in cell signalling (Marshall et al., 1991). The UDP-GlcNAc formed in the HBP is the substrate for the downstream UDP-N-acetylglucosamine-peptide N-acetylglucosaminyltransferase, also known as O-linked β-N-acetylglucosamine transferase or O-GlcNAc transferase (OGT) which catalyses the addition of a single N-acetylglucosamine (GlcNAc) to the hydroxyl groups of Ser and Thr residues on thousands of nucleocytoplasmic proteins through an O-glycosidic linkage (O-GlcNAc), while the O-glycoprotein 2-acetamino-2-deoxy-β-glucopyranosidase or O-GlcNAcase (OGA) catalyses the removal of O-GlcNAc from the target proteins (Ma and Hart, 2014). In the HBP, the glutamine:fructose-6-phosphateamidotransferase (GFAT), which converts fructose-6-phosphate to glucosamine-6-phosphate (GlcN-6-P), is sensitive to UDP-GlcNAc levels and represents the rate-limiting step to HBP flow and O-GlcNAcylation levels within the cell (McKnight et al., 1992) (Figure 1.7).

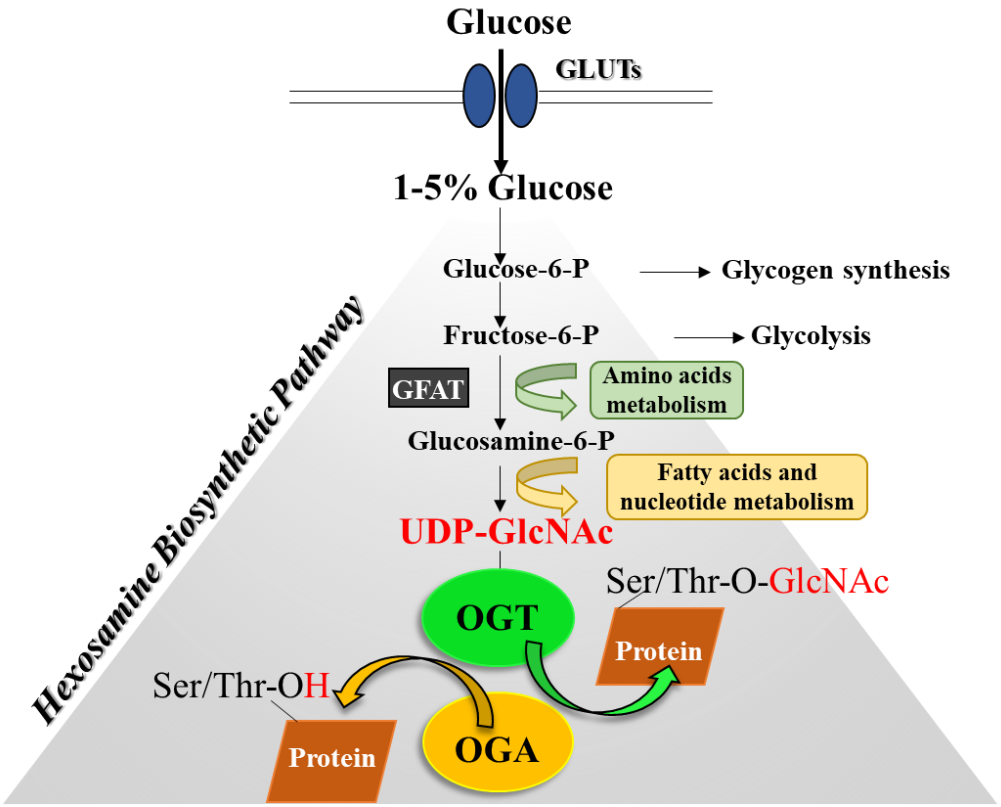


Figure 1.7 Representation of the hexosamine biosynthetic pathway (HBP) and protein O-GlcNAcylation. In addition to glycolysis and glycogen synthesis, 1–5% of glucose which enters cells is converted in the nucleotide sugar UDP-GlcNAc in the HBP. GFAT, mediates the conversion of fructose-6-P to glucosamine-6-P and is considered the rate-limiting step of the HBP activity. UDP-GlcNAc is the substrate of OGT which catalyses the transfer of O-GlcNAc on Ser and Thr residues of nucleocytoplasmic proteins, while OGA catalyses the removal of the sugar. Amino acids, fatty acids and nucleotides participate also in the biosynthesis of UDP-GlcNAc.

1.2.2 OGT and OGA enzymes

OGT and OGA alone control the O-GlcNAc cycling of thousands of nucleocytoplasmic and mitochondrial proteins. This is possible due to a finely controlled regulation of these two enzymes which includes alternative splicing, cellular compartmentalization, high structural adaptability and post-translational modifications.

OGT was found in all mammalian tissues but it is particularly abundant in insulin-sensitive and high-energy tissues including pancreas, brain, liver, heart and skeletal muscle (Gao et al., 2001; Lubas et al., 1997; Nolte and Müller, 2002). This enzyme is encoded by the *Ogt* gene (~43 kb) and the transcript is alternatively spliced to generate three different isoforms: 1) a nucleocytoplasmic isoform (ncOGT or OGT, 110 kDa), which constitutes the most studied OGT isoform containing a putative nuclear localization sequence (NLS) and mainly expressed within the nucleus but also abundant in the cytoplasm; 2) a mitochondrial isoform (mOGT, 103 kDa) and 3) a short isoform (sOGT, 75 kDa) both linked to apoptosis (Hanover et al., 2003) (Figure 1.8). The three isoforms contain a variable number of tetratricopeptide (34-amino acid) repeats (TPRs) on the N-terminal domains and a common C-terminal domain with two catalytic regions (Wrabl and Grishin, 2001). The TPR motifs are packed into anti-parallel α-helices and contain conserved "asparagine ladder" and "hinge-like" domains enabling the enzyme to adapt and promiscuously bind to a large variety of different substrate proteins (Jínek et al., 2004; Lazarus et al., 2011). Deletion studies demonstrated that the TPRs is important in the O-GlcNAcylation of many substrates including nucleoporins (Lubas and Hanover, 2000) and RNA Polymerase II C-terminal domain (Comer and Hart, 2001) as well as in the homo-dimerization and auto-glycosylation of OGT associated with an attenuated O-GlcNAcylation of specific targets (Jínek et al., 2004; Whisenhunt et al., 2006).

Less is known about OGA. The enzyme is a monomeric hexosaminidase (~130 kDa) which is encoded by the human Mgea5 gene (~34 kb). There are two isoforms, the full-length (OGA-L) and the short isoform (OGA-S). OGA-L is localized to the cytosol and nucleus whereas OGA-S was found primarily in lipid droplets (Figure 1.8) (Keembiyehetty et al., 2011; Wells et al., 2002). OGA cleaves the β-linked GlcNac at neutral pH. Interestingly, the catalytic efficiency for OGA is similar across different protein substrates, suggesting that O-GlcNAc itself, rather than the structure

of the target protein, modulates OGA's specificity (Shen et al., 2012). OGA contains an N-terminal N-acetyl-β-D-glucosamindase domain and, within its C-terminal domain, a region which shares sequence homology with histone acetyltransferase (HAT) enzymes which seems to play a role in OGA nuclear localization (Schultz and Pils, 2002).

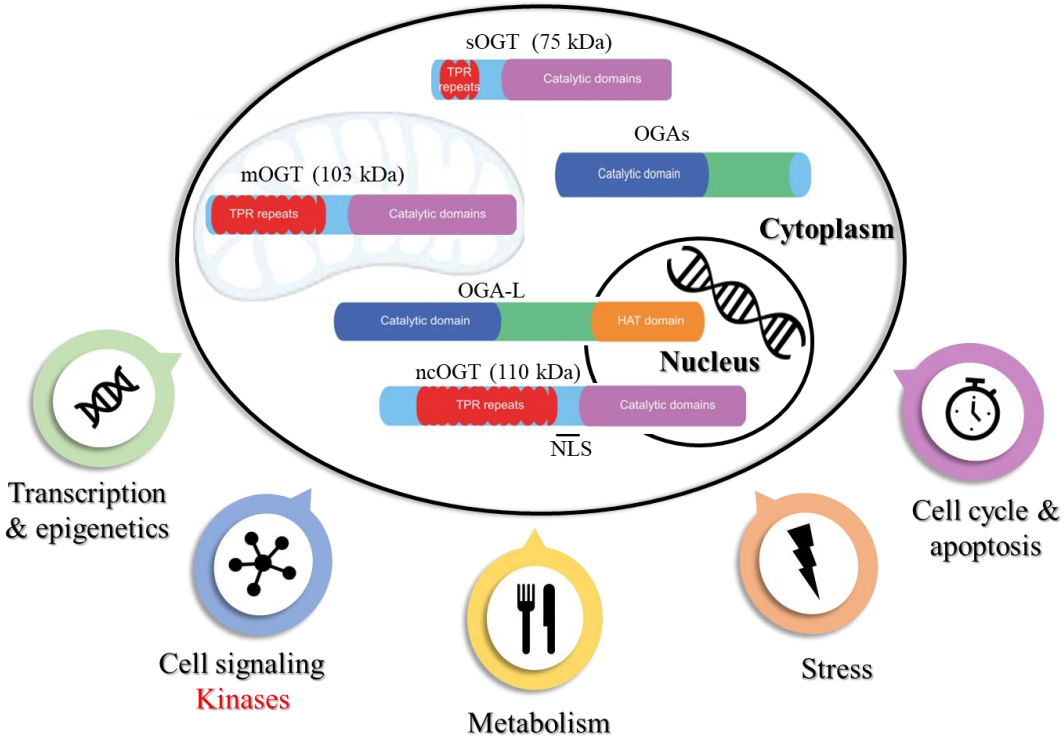


Figure 1.8 Representation of the OGT and OGA isoforms and cellular functions controlled by the OGT/OGA interplay. There are three isoforms for the OGT enzyme with different molecular weights. The ncOGT (110 kDa) is the most studied and well-known isoform and is characterized by the presence of a nuclear localization sequence (NLS). The OGA-L isoform can also localize to the nucleus via its HAT domain. The ncOGT and OGA-L isoforms can control alone the O-GlcNAc cycling on thousands of nucleocytoplasmic proteins thus regulating almost all the cellular functions.

1.2.3 Diverse roles of O-GlcNAcylation in biological processes

Protein O-GlcNAcylation is one of the most abundant post-translational modifications within the cell and is conserved among all metazoans. It is ubiquitously found in all cellular compartments and occurs on proteins belonging to almost all the functional classes, with the highest density on nucleoporins, transcription factors and chromatin remodelling proteins. The discovery of the O-GlcNAc modification is relatively recent and occurred by chance in 1980 when Torres and Hart noticed that most of the ^{3}H-Gal used to label proteins on the cell surface of lymphocytes, was unexpectedly incorporated on intracellular proteins (Torres and Hart, 1984). Indeed, O-GlcNAcylation differs from the canonical protein glycosylation for many aspects. First, it occurs on nuclear and cytoplasmic proteins and is independent from the endoplasmic reticulum (ER) and the Golgi apparatus where, instead, secreted and cell surface proteins are glycosylated. Secondly, unlike the very complex pattern of glycans found on extracellular proteins, the O-GlcNAc modification is constituted by only one sugar unit which is not further elongated or modified. Finally, the O-GlcNAc modification is reversible and its cycling occurs very quickly in response to a variety of stimuli due to the dynamic interplay occurring between OGT and OGA (Hart et al., 2007). Consistent with the above mentioned characteristics, the role of O-GlcNAcylation is to regulate nearly all the aspects of cellular processes and physiology. In particular, the O-GlcNAc modification is enriched in the nucleus where regulates transcription and epigenetics and is abundantly present on nucleoporins at the nuclear pore complex (NPC) where mediates bidirectional trafficking between cytoplasm and nucleus. Besides transcription, O-GlcNAcylation regulates translation and ribosome biogenesis although much more work is needed in this area. Interestingly, the O-GlcNAc modification widely regulates cell signalling dynamics and protein degradation often in interplay with other post-translational modifications and, as a cellular stress sensor, protects cells from acute stress including heat, high salt, ultraviolet light and hypoxia. Importantly, the O-GlcNAc modification is at the nexus of macronutrients metabolism and works as a nutrient sensor by coupling fluctuations in the availability of nutrients including glucose, amino acids and lipids, with adaptation of downstream signalling pathways (Copeland et al., 2008; Groves et al., 2013; Hart, 2015; Lewis and Hanover, 2014; Ruan et al., 2013; Zhu et al., 2016).

The vast majority of the cellular functions mentioned above, particularly the control of transcription and signalling, are regulated by a finely tuned interplay between O-GlcNAcylation and phosphorylation which occurs with three principal mechanisms: 1) many proteins, including the RNA polymerase II, are reciprocally modified at the same Ser and Thr sites under different conditions, suggesting that phosphorylation and O-GlcNAcylation are mutually exclusive modifications (Du et al., 2001; Kelly et al., 1993); 2) in other cases, they can occur at proximal sites. For example, in order to get activated, the calcium/calmodulin-dependent protein kinase type IV (CAMKIV) is first de-O-GlcNAcylated and then phosphorylated on a residue close to the de-O-GlcNAcylated site. Mutation of the major site of O-GlcNAcylation on CAMKIV results in a constitutively active enzyme (Dias et al., 2009); 3) O-GlcNAcylation and phosphorylation may exist simultaneously at different sites such as on the insulin receptor substrate (IRS) proteins (Ball et al., 2006) (Figure 1.9).

Consistently with its extensive regulatory role of key cellular functions, dysregulation in O-GlcNAcylation levels is directly associated to the aetiology of the major chronic diseases including metabolic disorders, cancer, cardiovascular diseases and neurodegenerative disorders (Dias and Hart, 2007; Ma and Hart, 2013; Slawson et al., 2010).

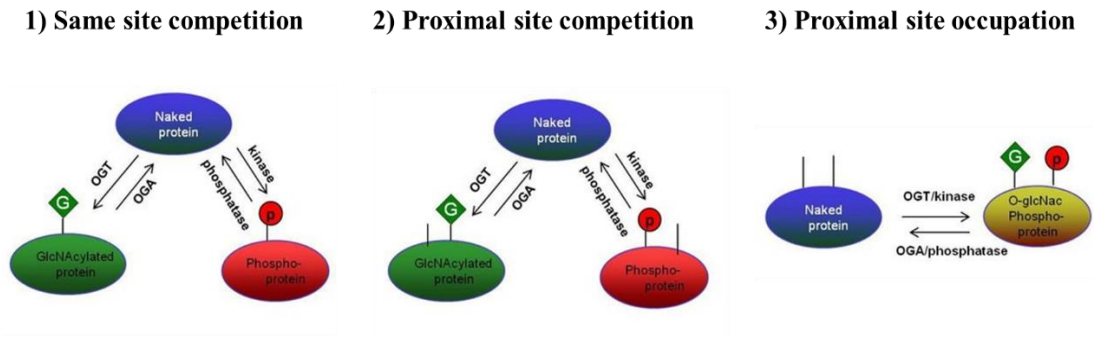


Figure 1.9 Cross-talk between O-GlcNAcylation and phosphorylation. The three main mechanisms occurring during modification of the nucleocytoplasmic proteins are represented (Adapted from Kanwal et al., 2013).

1.2.3.1 O-GlcNAcylation and signalling

O-GlcNAcylation is particularly important for kinases regulation and *vice versa*. Studies employing protein arrays showed that, O-GlcNAc modification occurs on almost all families of kinases with over one-half of all kinases being modified by the sugar (Dias et al., 2012). On the other hand, OGT and OGA are regulated by phosphorylation (Bullen et al., 2014; Song et al., 2008) and both enzymes have been found in protein complex containing both kinases and phosphatases indicating that phosphorylation and glycosylation occur dynamically on protein targets (Zeidan and Hart, 2010). The reciprocal regulation between O-GlcNAcylation and phosphorylation is particularly evident for metabolic signalling pathways. In cell responsive to insulin, upon insulin stimulation, the OGT associates with phosphoinositides at the plasma membrane where it is directly phosphorylated and activated by insulin receptor (IR). Once activated, OGT modifies other components of the insulin signalling cascade to turn off insulin signalling (Whelan et al., 2008; Yang et al., 2008). OGT can be also phosphorylated by AMPK, the major energy sensing kinase within cells as previously discussed. Phosphorylation by AMPK can influence OGT substrate binding. On the other hand, the activity of AMPK is positively regulated by O-GlcNAcylation (Bullen et al., 2014; Xu et al., 2014) (Figure 1.10). A dynamic interplay exists also between OGT and the insulin-regulated mitotic protein glycogen synthase kinase (GSK3). GSK3 mediates the phosphorylation of OGT at Ser3 or Ser4 enhancing its activity. Treatment with a GSK3 inhibitor alters global protein O-GlcNAcylation in mammalian cells (Kaasik et al., 2013; Wang et al., 2007). On the contrary, GSK3 is a substrate for OGT and its activity is perturbed by altering the O-GlcNAc cycling (Kazemi et al., 2010). These findings strongly suggest an extensive cross-talk between essential nutrient-sensitive enzymes which dynamically regulate each other to control cellular metabolism (Figure 1.10).

Importantly, the interplay between O-GlcNAcylation and phosphorylation can influence the nuclear localization of OGT itself along with the activity of many transcription factors involved in cell growth. Indeed, for many proteins including c-Myc, Sp1 and mTOR, the nuclear form is more O-GlcNAcylated than the cytoplasmic form and their re-localization to the cytoplasm can be induced by silencing OGT gene (Dauphinee et al., 2005; Zachara and Hart, 2006).

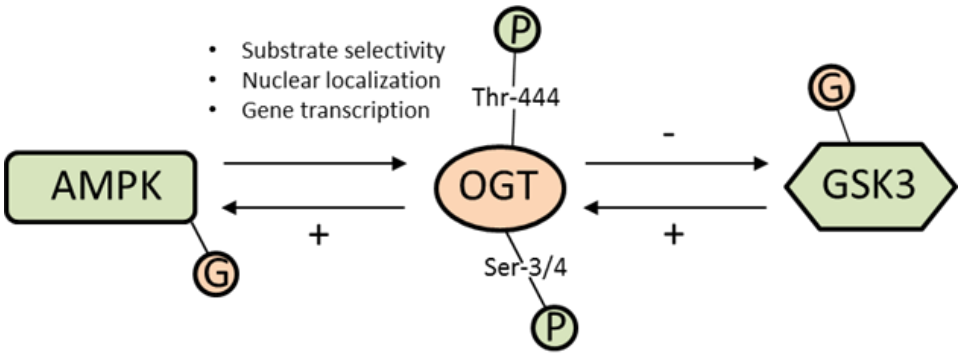


Figure 1.10 Reciprocal regulation of OGT and kinases. OGT modifies and activates AMPK whereas inhibits GSK3. On the other hand, OGT can be phosphorylated and activated by GSK3 whereas AMPK phosphorylation occurring on Thr-444 can regulate OGT activity by altering its substrate selectivity, nuclear localization and gene transcription.

1.2.3.2 O-GlcNAcylation in transcription and epigenetics

Recently, O-GlcNAcylation has emerged as fundamentally important for nutrient regulation of gene expression.

Nearly all factors associated with RNA polymerase II transcription are modified by O-GlcNAc in mammals (Vaidyanathan et al., 2014). More importantly, O-GlcNAcylation is highly present on the carboxy-terminal domain (CTD) of RNA polymerase II and regulates the pre-initiation complex assembly and initiation of transcription in interplay with phosphorylation thus controlling the expression of a multitude of genes in higher eukaryotes (Bond and Hanover, 2013; Lu et al., 2016). Accordingly, OGT and OGA are highly localized at the transcription initiation sites of thousands of genes. *In vitro* studies showed that inhibition of OGT and OGA blocked transcription prior to initiation, but not after elongation (Lewis and Hanover, 2014; Ranuncolo et al., 2012).

In addition, it has been demonstrated that OGT and OGA are localized to the chromatin-remodeling complexes where they contribute to regulate both activation and repression of transcription by modifying histones and chromatin-remodeling enzymes (Hanover et al., 2012; Sakabe et al., 2010). Indeed, O-GlcNAc is part of the

histone code and all the four subunits of the histone core are O-GlcNAcylated in their tail regions (Sakabe et al., 2010). Furthermore, O-GlcNAcylation controls DNA methylation and thus gene repression. As an example, the ten-eleven translocation (TET) family of enzymes responsible for DNA demethylation, target OGT to chromatin and are regulated by O-GlcNAcylation themselves (Chen et al., 2012; Deplus et al., 2013; Vella et al., 2013). Moreover, OGA and OGT co-localize at repressed promoters with mSin3A, a core component of a large multiprotein corepressor complex with histone deacetylase activity, suggesting another mechanism via which O-GlcNAc cycling can dynamically intervene in epigenetic regulation (Whisenhunt et al., 2006; Yang et al., 2002).

Finally, OGT is a polycomb gene. Members of this gene family encode for a group of proteins which repress the expression of large sets of genes, including HOX genes, and are responsible for regulating tissue patterning during development further supporting a central role played by protein O-GlcNAcylation in controlling transcriptional mechanisms and development (Gambetta et al., 2009; Sinclair et al., 2009).

1.2.4 O-GlcNAcylation in the pathogenesis of chronic disorders

1.2.4.1 O-GlcNAcylation in diabetes

Pathologies including diabetes, diabetes-related complications and obesity are all associated, directly or indirectly, with alterations in the nutritional status of the organism.

Due to its ability to regulate several cellular processes in relation to the flux of nutrients entering the HBP, especially glucose, alterations in O-GlcNAc modification are involved in the pathophysiology of diabetes. Accordingly, in both humans and animal models, glucose toxicity and hyperglycemia-induced insulin resistance, two major hallmarks of type 2 diabetes, have been directly linked to abnormal O-GlcNAcylation (Buse, 2005; Copeland et al., 2008; Dias and Hart, 2007; Lehman et al., 2005; Slawson et al., 2010). O-GlcNAc modification is abundant on proteins belonging to the insulin signalling pathway and increased O-GlcNAcylation, induced by hyperglycemia, affects particularly insulin-sensitive organs including pancreatic islets, liver, adipose and muscle tissue. Accordingly, overexpression of even moderate levels of OGT (20%) in mice, specifically targeted to adipose and muscle tissue, was sufficient to cause insulin resistance (McClain et al., 2002). Similarly, overexpression of OGT in liver resulted in impairment of insulin-responsive genes and the establishment of insulin resistance (Copeland et al., 2008; Yang et al., 2008).

The molecular mechanisms underlying the onset of insulin resistance during hyperglycemic conditions, have been widely investigated. Studies suggest that increase of O-GlcNAcylation at the expense of phosphorylation on key transcription factors and components of insulin signalling pathway perturbs the response to insulin resulting in diminished glucose uptake in the muscle and fat tissues and increased gluconeogenesis in the liver (Copeland et al., 2008; Ma and Hart, 2013).

In adipocytes, increased O-GlcNAc reduces the phosphorylation of the phosphoinositide 3-kinase (PI3K) p85 binding motif of insulin receptor substrate 1 (IRS1) resulting in a concomitant reduction in tyrosine phosphorylation of IRS-1 and insulin signalling (Whelan et al., 2010).

In the liver, O-GlcNAcylation regulates insulin signalling in physiologic conditions. The binding of insulin to its tyrosine kinase receptor activates the intracellular substrates IRS1/2 thus initiating PI3K-Akt pathways to increase glucose uptake and metabolism in cells. PI3K activation gives rise to phosphatidylinositol 3,4,5

trisphosphate (PIP3) which recruits OGT to the plasma membrane where, O-GlcNAcylation of specific components, leads to the attenuation of insulin signalling. However, upon chronic hyperglycemia, abnormally high O-GlcNAcylation leads to abnormal diminishing of insulin sensitivity (Figure 1.11). In parallel, hyper-O-GlcNAcylation increases the activity of many transcription factors and co-activators involved in promoting gluconeogenic/lipogenic gene transcription. As an example, FoxO1 responds to hyperglycemia through elevated O-GlcNAcylation in the liver. Diabetes-induced O-GlcNAcylation of hepatic FoxO1, in turn, increases the expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), two rate-limiting enzymes in gluconeogenesis (Ma and Hart, 2013; Housley et al., 2008) (Figure 1.11). Similarly, the carbohydrate-responsive element-binding protein (ChREBP), a central regulator of lipid synthesis in the liver, is hyper-O-GlcNAcylated during hyperglycemia and translocates to the nucleus to initiate the transcription of lipogenic genes (Ma and Hart, 2013) (Figure 1.11).

Furthermore, O-GlcNAcylation of the ubiquitously expressed transcription factor Sp1 contributes to the development of diabetes-related complications. Studies showed that hyper-O-GlcNAcylation occurs at multiple sites on Sp1 during acute hyperglycemia and diabetes leading to its nuclear accumulation, increased DNA binding, enhanced transcriptional activity and protein stability (Kudlow, 2006; Solomon et al., 2008; Copeland et al., 2008). Increased transcriptional activity of hyper-O-GlcNAcylated Sp1 has been associated with the development of diabetic atherosclerosis via enhancement in the transcription of the transforming growth factors TGF-α, TGF-β, and plasminogen activator inhibitor (PAI)-1, all involved in the atherosclerotic process (Özcan et al., 2010). Moreover, O-GlcNAc modification of Sp1 mediates hyperglycemia-induced upregulation of the vascular endothelial growth factor A (VEGF-A), a proangiogenic protein whose expression contributes significantly to retinal lesions and neovascularization in diabetic retinopathy in both human and rat retinal cells (Donovan et al., 2014).

All together these studies suggest that a chronically sustained flux through the HBP and increased O-GlcNAcylation of key proteins regulating metabolic pathways may represent one mechanism via which hyperglycemia promotes insulin resistance and the tissue damage observed in diabetes.

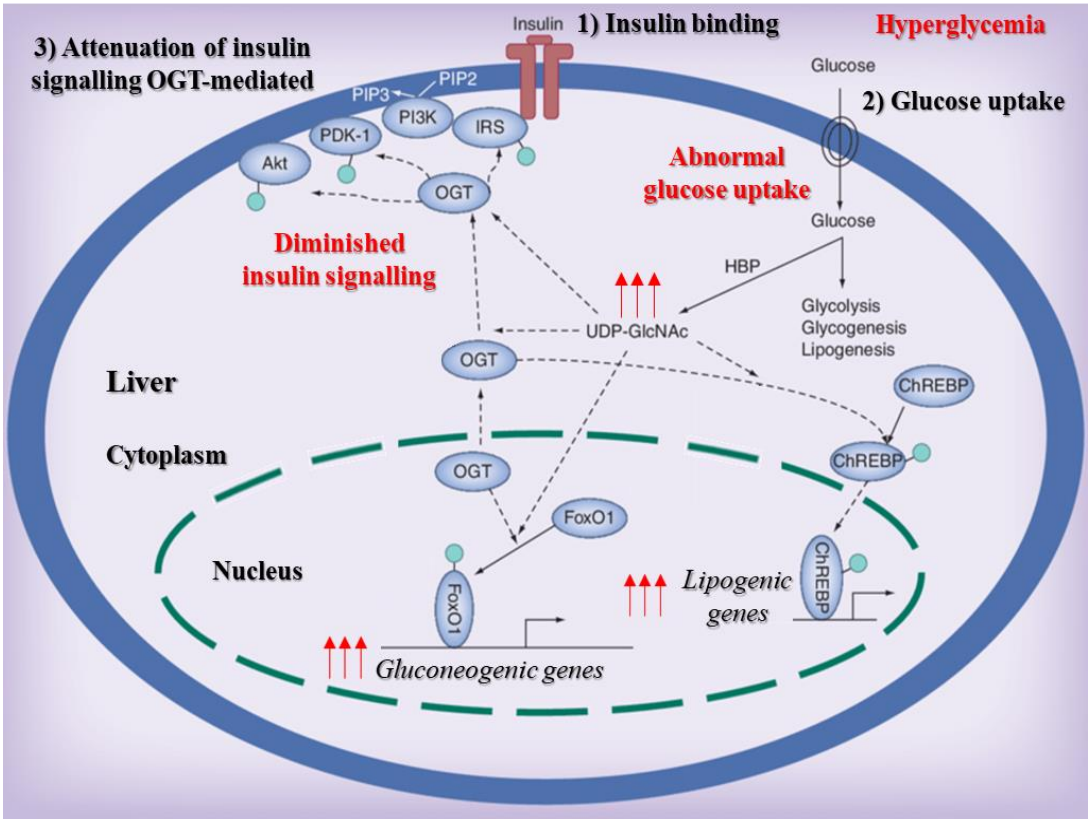


Figure 1.11 O-GlcNAc modification regulates insulin signalling in the liver. In physiological conditions (black), insulin binding to its tyrosine kinase receptor activates the intracellular substrates IRS1/2, initiating PI3K-Akt signalling which leads to increased glucose uptake. OGT is recruited to the plasma membrane, where the O-GlcNAcylation of several components of the insulin signalling pathway (green circles) leads to an attenuation of insulin response. Upon chronic hyperglycemia (red), the UDP-GlcNAc pool increases leading to high O-GlcNAcylation of proteins, including elements of the insulin signalling pathway and some key transcription factors, diminishing insulin sensitivity and stimulating gluconeogenic/lipogenic gene transcription (Adapted from Ma and Hart, 2013).

1.2.4.2 O-GlcNAcylation in cancer metabolism

Many cancer types exhibit enhanced O-GlcNAcylation levels and abnormal expression of OGT and OGA. In many cases, the histological grade of the tumor and cancer progression correlate with higher levels of OGT and O-GlcNAc and reduced levels of OGA (Ferrer et al., 2016; Yehezkel et al., 2012), whereas downregulation of OGT via RNA interference (RNAi) or pharmacological inhibition blocked cell growth in *in vitro* studies using different cancer cells (Ferrer et al., 2014).

The repromming of cellular metabolism and stress response by O-GlcNAc-mediated mechanisms is thought to enable cancer cells to resist stressful microenvironments and promote tumor growth. For instance, O-GlcNAcylation regulates cancer metabolism and sustains cellular survival via regulation of the hypoxia-inducible factor (HIF-1) pathway (Ferrer et al., 2014). HIF-1 promotes adaptive responses of the tumor cells to their environment by activating the transcription of downstream genes important to maintain tumor survival and progression such as GLUT1 (Masoud and Li, 2015). Human breast cancer cells display high levels of HIF-1 and OGT, and low OGA levels which correlate with poor patient outcome. Downregulation of O-GlcNAcylation in these cells promotes HIF-1 proteasomal degradation and leads to endoplasmic reticulum-mediated apoptosis whereas overexpression of a stable form of HIF-1 or GLUT1 prevents these effects (Ferrer et al., 2014). Cross-talk between O-GlcNAcylation and the AMPK/mTOR pathway is also emerging as a key mechanism regulating cellular metabolism and growth in normal physiology as well as in cancer. The mTOR pathway plays a major role in coordinating cell growth and for that reason is critical to cancer biology. Indeed, it is hyperactivated in human cancers due to activation and inactivation of its positive and negative regulators respectively. For instance, LKB1 negatively regulates mTOR signalling via its substrate AMPK. Loss of LKB1 mediates mTOR activation promoting cell growth in human cancers (Zhou et al., 2013). It has been shown that downregulation of O-GlcNAcylation in cancer cells enhanches LKB1 and AMPK activity decreasing activation of mTOR effectors. On the contrary, overexpression of the glucose transporter GLUT1 reversed LKB1 and AMPK activation (Ferrer et al., 2014, 2016). Taken together these findings suggest that OGT can regulate the major pathways controlling cellular metabolism and stress response in cancer cells including HIF-1, GLUT1 and AMPK/mTOR pathway.

1.2.5 O-GlcNAc modification and pregnancy

1.2.5.1 O-GlcNAc modification during pre-implantation, implantation, and embryo development

Both OGT and OGA are extremely conserved from worm to humans and are essential for embryonic implantation, development and survival (Gao et al., 2001; Kreppel et al., 1997). During pre-implantation, OGT mediates blastocyst formation and cell proliferation which are both impaired by hyperglycemic conditions in early mouse embryos (Pantaleon et al., 2010). Moreover, OGT regulates the timing of differentiation in mouse embryonic stem cells by maintaining the undifferentiated state (Miura et al., 2018; Shi et al., 2013). In addition, O-GlcNAcylation may intervene in fetal immune tolerance by modulating the activity of natural killer (NK) cells at the maternal-fetal interface (Lima et al., 2018; Yao et al., 2004).

As for embryo development, knockdown of OGT results in morphological defects, impaired embryonic growth and decreased cell survival in zebrafish embryos (Webster et al., 2009). Similarly, in murine models carrying OGT deletion, the embryogenesis is blocked at day five (O'Donnell et al., 2004; Shafi et al., 2000), while knock-out of OGA led to impaired cellular division, embryonic growth and early post-natal death (Yang et al., 2012). Interestingly, another study showed that, the negative effects displayed by mouse embryos carrying a mutation for GlcNAc-6-phosphate acetyltransferase (EMeg32), a crucial enzyme of the HBP involved in *de novo* UDP-GlcNAc synthesis, were prevented by nutritional restoration of UDP-GlcNAc levels suggesting that aberrations in the embryonic development can be mediated by decreased efficiency of the HBP via reduced O-GlcNAcylation (Boehmelt et al., 2000). On the other end, increased O-GlcNAcylation induced by maternal hyperglycemia may impact negatively on fetal neurodevelopment. *In vivo* inhibition of OGT and reduction of O-GlcNAcylation in diabetic pregnant mice decreased the rate of neural tube defects (NTDs) in the embryos, suggesting that OGT and O-GlcNAcylation can be suitable candidate targets to prevent birth defects in hyperglycemic pregnancies (Kim et al., 2017).

1.2.5.2 O-GlcNAc modification and placental programming

Both OGT and OGA are extensively expressed in placentas (Lubas et al., 1997; Gao et al., 2001). The main role of the O-GlcNAc modification in the regulation of placenta function has emerged in relation to its role as a cellular sensor of stress. In several cell types, O-GlcNAc modification acts as a sensor of stress which rapidly increases in response to different stressors and cellular injuries to promote cell survival (Groves et al., 2013; Martinez et al., 2017). Recently, studies conducted in rodents identified OGT as a placental marker of prenatal stress and demonstrated that, the placental expression of OGT changes according to the type and intensity of stress experienced by the mother, affecting the offspring's development in a sexually dimorphic manner (Howerton et al., 2013; Lima et al., 2018; Olivier-Van Stichelen et al., 2014; Pantaleon et al., 2017). Indeed, OGT is a X-linked gene which escapes X-inactivation as demonstrated in mouse trophoblast cells. This means that OGT remains more expressed in placentas from female fetuses compared to males. It has been suggested that the differences observed in the placental OGT dosage may account for sex-specific sensitivity to the intrauterine environment thus contributing to gender-dependent susceptibility to *in utero* programming of chronic disease (Olivier-Van Stichelen et al., 2014).

The role of placental OGT as a nutrient sensor is poorly explored in human pregnancies. Nevertheless, data on rodents point toward a role for O-GlcNAcylation as a good candidate to link maternal diet and offspring development. Accordingly, maternal diet, either poor or high in fat content, increases the gene expression of OGT preferentially in the placenta of female mice (Mao et al., 2010). In addition, severe and mild maternal hyperglycemia in rats, resulted in increased O-GlcNAcylation of kappa-light-chain-enhancer of activated B cells (NF-kB) which was associated with augmented production of proinflammatory cytokines, placental morphometric alterations and reduced placental index (Dela Justina et al., 2018, 2017). Furthermore, in the human choriocarcinoma BeWo cell line and in first trimester explants, stimulation of HBP flux with glucosamine was associated to increased protein O-GlcNAcylation, enhanced distribution of O-GlcNAc modified proteins in the nuclear speckles as well as reduced trophoblast proliferation and placental secretion of leptin, a key regulator of placental amino acid uptake. In agreement with these *in vitro* data,

placentas from mice treated with glucosamine throughout pregnancy were significantly smaller than those from control mice (Palin et al., 2013).

Although evidence emerging from studies in rodent models strongly suggest a link between O-GlcNAc signalling and key pathways regulating placental adaptation to maternal environment and fetal growth, including mechanisms of placental stress response and nutrient transport, few efforts have been made to investigate the role of OGT and its biochemical signatures in human placenta in relation to maternal health status and the potential implications for fetal development.

1.3 Thesis aims

Little is known about the placental molecular mechanisms governing the transmission of maternal metabolic stimuli to the growing fetus. Less is known about the role of protein O-GlcNAcylation in the human placenta. However, an increasing number of studies have linked O-GlcNAc signalling to the alterations of cellular metabolism underlying the pathophysiology of chronic disorders including diabetes, cancer and neurodegenerative diseases, highlighting the urgent need to elucidate the role of this recently identified post-translational modification also in the human placenta. It is widely accepted that a healthy placenta is crucial to guarantee the well-being of the mother and in programming the health of the future generation. Thus, the overall aim of this thesis is to investigate the role played by OGT and O-GlcNAcylation in some aspects of placenta physiology. In particular, the main objectives of this research work are:

- developing a method to characterize global O-GlcNAc-modified proteins in the human trophoblast cell line BeWo. This part of the study will allow to acquire a broader overview of potential OGT targets with a pivotal role in regulating trophoblast physiology.

- investigating the roles played by OGT in regulating the molecular mechanisms underlying the formation of the syncytiotrophoblast using BeWo cells. The pathways governing trophoblast differentiation are well-characterized and include the activation of a phosphorylation cascade involving several protein kinases. The evaluation of a potential interplay between phosphorylation and O-GlcNAcylation in the regulation of key kinases involved in mediating the differentiation process will allow to obtain a clearer understanding of the mechanisms underlying the formation of syncytiotrophoblast.

- evaluating *ex-vivo*, in human term placental biopsies, the potential association between O-GlcNAc signalling and maternal alterations, including obesity and depression, and exploring the effects on placental nutrient-sensing and stress response mechanisms and the potential implications for fetal outcomes.

Chapter 2

Materials and Methods

2.1 Subjects

Pregnant women were recruited at the Chelsea and Westminster Hospital in London where informed written consent and approval of the ethics committee were obtained (REC No: 14/LO/0592). All the procedures were conducted in accordance with the Helsinki Declaration. Study subjects were 34 healthy women aged 21 to 45 years with BMI ranging from 19 to 45.4 (kg/m^2) related to the first trimester of pregnancy (11-14 weeks) and giving birth to infants with a birth weight (BW) ranging from 2630 to 4440 g. The inclusion criteria for this study were good general health, normal gestation length (>37 weeks), mother age above 20 years old, while women with diabetes, chronic diseases, or pregnancy complications such as preeclampsia were excluded. Samples were randomly selected. Three women giving birth to small for gestational age babies (SGA, birth weight centile (BWC)<10) and 5 mothers giving birth to large for gestational age (LGA, BWC>90) were not excluded from the cohort study. Maternal depressive symptoms were assessed in early pregnancy (11-14 weeks) at the time of recruitment, using the Edinburgh Postnatal Depression Scale (EPDS) which was validated for assessing depression also during pregnancy by Cox and colleagues (Cox et al., 1996). A value ≥13, on a scale ranging from 0 (low depression) to 30 (high depression), is used in the test to identify maternal depression (Cox et al., 1996).

2.2 Oral glucose tolerance test (OGTT) and HbA1c measurement

At 28-30 weeks of gestation all women underwent full oral glucose tolerance test (OGTT) and haemoglobin A1c (HbA1c) test as part of standard clinical care at the Chelsea and Westminster Hospital where samples collection and analysis were performed. For the OGTT, used as a diagnostic tool for gestational diabetes, blood glucose levels were measured at fasting and 2 h after 75 g of oral glucose load. The OGTT measures the concentration of blood glucose after fasting for 8 h and the body's ability to metabolise glucose at 2 h after consuming a glucose drink. Individuals without diabetes display glucose levels below 5.6 mmol/L at fasting and below 7.8 mmol/L after 2 h from glucose administration. The HbA1c test is employed to measure the average blood glucose carried by the red blood cells over the last 2–3 months and, in this study, was performed on the same day as the OGTT.

2.3 Placenta collection and sample preparation for immunoblotting

Placentas were collected and processed at the Chelsea and Westminster Hospital. Soon after delivery, placentas were collected and immediately placed on ice for dissection. Small pieces of villous tissue were then snap frozen in liquid nitrogen and stored for further analysis. For immunoblotting sample preparation, 5 mg of frozen tissue was homogenized still frozen in 200 µl of ice-cold Pierce™ RIPA buffer with the following composition: 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS (Thermo Scientific Pierce) supplemented with 1% of Halt™ Protease and Phosphatase Inhibitor Single-Use Cocktail (Thermo Fisher Scientific). Samples were then clarified by centrifugation at 14000 g for 10 min at 4 °C and the supernatant was collected in sterilized 0.5 ml Eppendorf tubes and kept at -80 °C until immunoblotting analysis.

2.4 Cell culture and treatments

2.4.1 Storage, reviving and propagation of BeWo cells

BeWo cells (American Type Culture Collection) were stored in liquid nitrogen in 2 ml cryogenic vials with a mixture 1:9 of dimethyl sulfoxide (DMSO, Sigma-Aldrich) and fetal bovine serum (FBS, Sigma-Aldrich). For each experiment, cells were revived by thawing at 37 °C in a water bath, resuspended by gentle pipetting and transferred to a 15 ml centrifuge tube with 5 ml of culture medium and centrifuged to eliminate DMSO. Cells were resuspended in growing medium Ham's F-12K medium supplemented with 10% FBS and 1% of 1:1 penicillin-streptomycin 10000 U/mL sterilized solution (VWR International), transferred to a T75 sterilized flask and grown at 37 °C under a humidified atmosphere of 5% CO_2. Medium was changed every two days. At confluence cells were trypsinized to dissociate using a solution of 0.5% trypsin-EDTA (Gibco, Thermo Fisher Scientific), resuspended in fresh medium and centrifuged at 200 rpm at 37 °C for 5 min to eliminate trypsin. For each experiment, the number of cells seeded was determined using a hemocytometer (Hirschmann Instruments).

2.4.2 Protein extraction from BeWo cells

At the end of each experiment, cells were washed in ice-cold PBS and total protein content was extracted by lysing cells in 100 µL/well of ice-cold RIPA buffer (25 mM

Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with 1% of Halt™ Protease and Phosphatase Inhibitor Single-Use Cocktail for 5 min on ice. Samples were then clarified by centrifugation at 14000 g for 10 min at 4 °C and the supernatant was collected in sterilized 0.5 ml Eppendorf tubes and analysed by immunoblotting.

2.4.3 Treatments

2.4.3.1 Trophoblast differentiation forskolin-induced

Forskolin (Cayman Chemical) was dissolved in DMSO and used at a concentration of 100 µM to induce differentiation of BeWo cells as previously described (Delidaki et al., 2011). Briefly, Bewo cells were seeded at a density of 0.3 x 10^6 in a 6-well plate and grown as a monolayer of mononucleated cells (cytotrophoblast) at 37 °C under a humidified atmosphere of 5% CO_2 in Ham's F-12K medium supplemented with 10% FBS and 1% pen/strep until they reached 70% of confluence. Afterwards, BeWo cells were treated with forskolin to activate the cAMP/PKA pathway and induce syncytialization or with the same volume of DMSO used as a control for 48 h.

2.4.3.2 Experimental hyperglycemia

Glucose treatment was used to create hyperglycemic conditions to study the response of global protein O-GlcNAcylation and the role of AMPK as a cellular energy sensor. Bewo cells were grown in a 6-well plate and the growing medium, formulated with a concentration of 5 mM, was supplemented with glucose powder suitable for cell culture (Sigma Aldrich) to a final concentration of 15 mM which was applied to the cells to mimic hyperglycaemic exposure to glucose for 48 h.

2.4.3.3 Enhancement of protein O-GlcNAcylation with PUGNAc

To increase global protein O-GlcNAcylation in BeWo cells, PUGNAc (O-(2-acetamido-2deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate) (Sigma-Aldrich), an inhibitor of OGA, the enzyme which removes O-GlcNAc moieties from Ser and Thr residues, was used. PUGNAc was dissolved in sterilized water (vehicle) and used at a concentration of 100 µM as suggested in the literature (Park et al., 2005). The corresponding controls were treated with the same volume of vehicle only.

2.4.3.4 Inhibition of PKA with H89

H89 (Tocris Biotechne) was dissolved in sterilized water and used at a concentration of 5 µM to specifically inhibit PKA. The dose was chosen on the basis of a cell titration experiment consisting of 1 h pretreatment of BeWo cells with 0, 1, 5 or 10 µM of H89 followed by stimulation with 100 µM of forskolin for 10 min, the time needed to have the maximum forskolin-induced activation of PKA in BeWo cells (Delidaki et al., 2011). The activation of PKA was estimated by determining the activation of the well-known PKA downstream targets, CREB and ERK, and was measured as the phosphorylation of Ser133 and Thr202/Tyr204 by immunoblotting respectively (Figure 2.1).

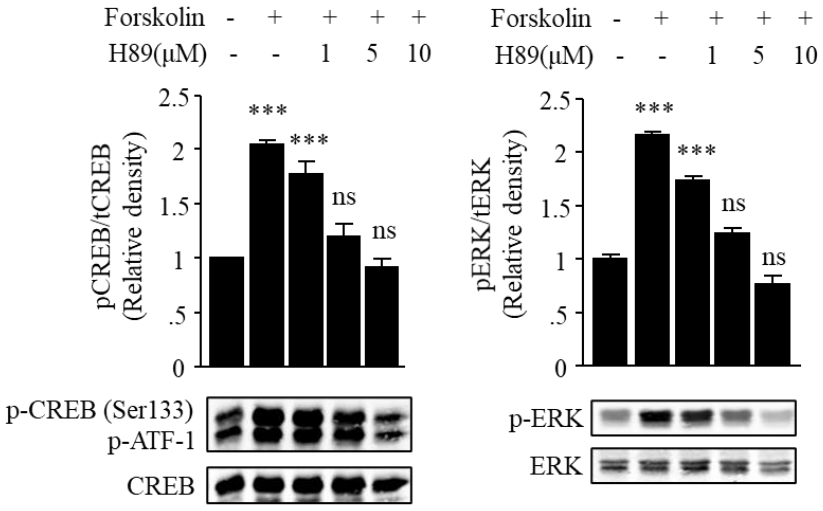


Figure 2.1 Dose-dependent inhibition of PKA by H89 in BeWo cells. BeWo cells were pretreated for 1 h with different doses of H89 before inducing activation of PKA with 100 µM of forskolin for 10 min. Activation of PKA downstream targets CREB and ERK was measured by immunoblotting. N=3. Data are expressed as mean ± SEM; p values were determined using t test. ***p<0.001 vs control.

2.4.3.5 Transient gene silencing using short interfering RNA

BeWo cells were transiently transfected with Silencer® Select small interfering (si)RNAs for OGT (s16093), GLUT1 (s12925) and negative control (4390844). siRNAs used in this study were designed, validated and purchased from Thermo Fisher Scientific. BeWo cells were seeded at a density of 0.3 x 10^6 in a 6-well plate and treated when they reached 60% of confluence with 10 µM of siRNA against OGT or GLUT1 and siRNA control prepared as follow. Five nmol of lyophilized siRNA were resuspended using 50 µL of nuclease-free water for a final concentration of 100 µM and further diluted to make a 10 µM working solution. For each well, 3 µL of siRNA 10 µM solution were diluted with 150 µL of Opti-MEM® medium (Thermo Fisher Scientific) and mixed 1:1 with a solution made with 9 µL of Lipofectamine® RNAiMAX (Thermo Fisher Scientific) diluted with 150 µL of Opti-MEM® medium. A volume of 250 mL of the mixture were added to each well and cells were incubated at 37 ºC for the duration of the experimental procedure.

2.5 Fluorescence microscopy

Microscope glass coverslips 0.17 mm thick (Thermo Scientific), were sterilized in 100% ethanol and let to dry under a sterile hood before to be transferred to a 12-well plate. Cells were then seeded to a density of 0.1 x 10^6 cells/well and incubated at 37 °C under a humidified atmosphere of 5% CO_2 until they reached a confluency of 60%. Cells were then treated according to the experimental design. At the end of treatment, the culture medium was removed and the coverslips transferred to a new sterile well plate to be washed 3 times with PBS. Cells were fixed with 100% ice-cold methanol for 20 min at -20 ºC. Each coverslip was then washed 3 times with PBS and blocked for 1 h in 5% bovine serum albumin (BSA, VWR Chemicals) and 0.3% Triton X-100 (Sigma Aldrich) prepared in PBS. After blocking, the coverslips were incubated overnight with the primary rabbit polyclonal antibody against the membrane protein E-cadherin (sc-7870, Santa Cruz) diluted 1:200 in an antibody buffer solution 1% BSA and 0.3% Triton X-100 prepared in PBS. The day after, coverslips were rinsed 3 times with PBS and incubated with the goat anti-rabbit IgG (H+L) conjugated to DyLight™ 800 4X PEG (#5151, Cell Signaling) diluted 1:800 in the antibody buffer solution in the dark for 1 h at room temperature. The coverslips were then rinsed 3 times with PBS and incubated with the blue-fluorescent DNA stain DAPI (sc-3598, Santa Cruz)

for 10 min at room temperature, washed 3 times with PBS, mounted on a microscope slide in DPX Mounting medium (Sigma-Aldrich) and dried overnight at +4 ^{o}C. Images were collected at a magnification of 20X on an Olympus IX71 fluorescence microscope (Olympus Optical).

2.6 RNA extraction

Total RNA was extracted using the kit GenElute RTN70-1kt (Sigma Aldrich) according to the manufacturer's instructions. RNase- and DNase-free barrier tips were used in the procedure to avoid contaminations. All the reagents used in this process were provided with the kit except for 70% ethanol solution. Cells grown in 6-well plate to 90-100% confluence, were washed with PBS at room temperature and lysed using 250 µL of the Lysis Solution provided and prepared with 1% 2-mercaptoethanol. The lysate was then transferred to a GenElute filtration column and spinned down by centrifugation at 14000 g for 2 min to remove cellular debris and shears DNA. An equal volume of 70% ethanol solution was then added to the filtered lysate and the mixture was transferred to a GenElute binding column to isolate the RNA from the lysate/ethanol mixture by centrifugation. Afterwards, the binding column was washed with 500 µL of Wash Solutions 1 and 2 and transferred to a clean collection tube. The RNA was released from the column using 50 µL of Elution Buffer and 1 min centrifugation at 14000 g.

2.7 RNA quantification and quality control

The concentration and quality of the total RNA isolated was determined by spectrophotometric analysis using a NanoDrop ND-1000 (Thermo Fisher Scientific). Only samples with a ratio of absorbance A260/A280 between 1.8 and 2.1 were used as a ratio appreciable lower than ~2.0 may indicate contamination with proteins or ethanol used for the extraction which absorb at or near 280 nm.

2.8 Protein quantification

Total proteins content was quantified in BeWo cells and term placenta extracts using the Thermo Scientific™ Pierce™ BCA Protein Assay kit (Thermo Fischer Scientific) based on the bicinchoninic acid (BCA) assay. The kit provides: 1) BCA Reagent A containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide; 2) BCA Reagent B containing 4% cupric sulfate;

3) BSA at a concentration of 2 mg/mL in 0.9% saline and 0.05% sodium azide. Briefly, a standard curve was prepared by diluting the BSA standard with RIPA buffer as the diluent according to Figure 2.2, A. The BCA Working Reagent (WR) was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). Ten μL of each standard or unknown sample were then pipetted in a 96-well polystyrene microplates in replicates and 200 μL of BCA WR were added to each well. The plate was thoroughly mixed and incubated at 37°C for 30 min. Proteins reduce the cupric ion present in Reagent B from Cu+2 to Cu+1 whereas the BCA in Reagent A reacts with Cu+1 resulting in a purple colour whose intensity is proportional to protein concentration. The absorbance was read at 562 nm on a Tecan Sunrise microplate Reader using a Magellan® Software. Figure 2.2 B shows a typical standard curve obtained with this assay. The derived equation was used to calculate the unknown protein amount for each sample.

A)

Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

B)

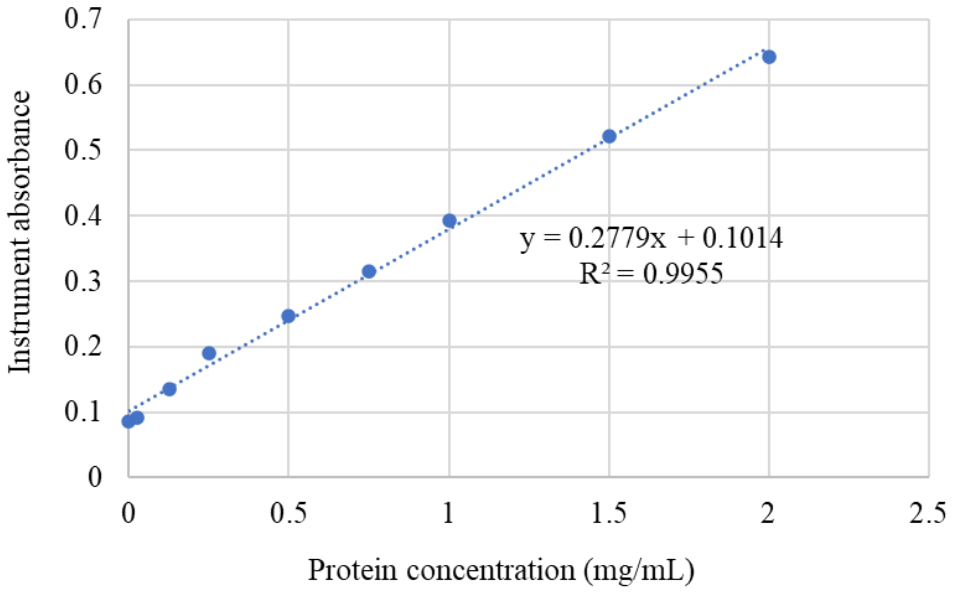


Figure 2.2 Albumin standard for protein determination. A) Preparation of diluted BSA standards. B) Typical standard curve obtained after reading absorbance at 562 nm. The x axis represents known protein concentration in the BSA standard. The linear equation of the calibration curve is used to calculate the unknown concentration of protein in the samples.

2.9 Sample preparation for immunoblotting

After protein concentration assay, samples were boiled for 5 minutes at 95°C in Laemmli SDS sample buffer (Thermo Fisher Scientific) to be denatured. The buffer is composed by 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl. The 2-mercaptoethanol reduces the molecular disulfide bonds between the thiol groups of cysteines destabilizing the protein. The SDS detergent coats and denatures proteins giving an overall negative charge which allows proteins to move through an electrical field towards the anode during electrophoresis. The bromophenol blue facilitates sample loading in the electrophoresis gel.

2.10 Immunoblotting

An amount of 10-20 µg protein along with 5-10 µl of PageRuler™ Prestained Protein Ladder (Thermo Fischer Scientific), were loaded in 1D SDS-PAGE gel prepared according to a Bio-RAD® recipe (Table 1) and were separated by electrophoresis according to their size using a TRIS-glycine-SDS running buffer pH 8.3 (Alfa Aesar) with a constant current setting of 50 mA. The separated proteins were transferred from the gel to a nitrocellulose membrane in cold Western Blot transfer buffer (Thermo Scientific) supplemented with 20% of methanol in a vertical mini-gel electrophoresis tank (Bio-Rad) under a constant voltage of 100 V applied for 1 h. Membranes were then blocked with Odyssey Blocking Buffers (LI-COR Biosciences) at room temperature for 1 h and incubated overnight at 4°C with primary antibodies diluted in 5% BSA at different concentrations depending on the antibody efficiency (Table 2). After washing 3 times in Tris-buffered saline (TBS) supplemented with 0.1% Tween™ 20 (Fisher BioReagents™) (TBST), membranes were incubated at room temperature for 1 h with 1:15000 secondary anti-mouse IgG (HL) (DyLight 680 Conjugate) or anti-rabbit IgG (HL) (DyLight 800 4X PEG Conjugate) antibody (Cell Signaling) followed by other 3 washes in TBST. Fluorescence emission was detected and quantified for each protein band using an Odyssey infrared imaging system (LICOR Biosciences).

Table 1. Recipes for stacking and resolving gels

Components	Stacking gel	Resolving gel		
		8%	12%	X%
30% Acrylamide/Bis	1.98 ml	4 ml	6 ml	0,5 x X=A ml
0.5 M Tris-HCl, pH 6.8	3.78 ml	—	—	—
1.5 M Tris-HCl, pH 8.8	—	3.75 ml	3.75 ml	3.75 ml
10% SDS	150 µl	150 µl	150 µl	150 µl
Distilled deionized water	9 ml	7 ml	5 ml	11 - A ml
TEMED	15 µl	7.5 µl	7.5 µl	7.5 µl
10% APS	75 µl	75 µl	75 µl	75 µl

The letter A designates the volume of 30% Acrylamide/Bis solution required to produce the specified percent gel (X%). Adapted from http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6201.pdf

Table 2. List of primary antibodies used for immunoblotting experiments

Protein	Antibody ID	Tipology	Dilution
GLUT1	sc-377228; Santa Cruz	Mouse monoclonal	1:500
OGT	#24083; Cell Signaling	Rabbit monoclonal	1:1000
O-GlcNAc	MA1072; Applied Biosystem	Mouse monoclonal	1:1000
OGA	sc-376429; Santa Cruz	Mouse monoclonal	1:1000
ERK1/2	#9102; Cell Signaling	Rabbit polyclonal	1:1000
p-ERK1/2	#9101; Cell Signaling	Rabbit polyclonal	1:1000
p38	#9212; Cell Signaling	Rabbit polyclonal	1:1000
p-p38 (Thr180/Tyr182)	#9211; Cell Signaling	Rabbit polyclonal	1:1000
E-cadherin	sc-7870; Santa Cruz	Rabbit polyclonal	1:2000
AMPKα	#2532; Cell Signaling	Rabbit polyclonal	1:1000
p-AMPKα (Thr172)	#2531; Cell Signaling	Rabbit monoclonal	1:500
ACC	#3662; Cell Signaling	Rabbit polyclonal	1:1000
p-ACC (Ser79)	#3661; Cell Signaling	Rabbit polyclonal	1:500
p-4E-BP1 (Ser65)	sc-293124; Santa Cruz	Mouse monoclonal	1:500
11β-HSD2	sc-20176; Santa Cruz	Rabbit polyclonal	1:1000
β-actin	sc-47778; Santa Cruz	Mouse monoclonal	1:2000

2.11 Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was performed in two separate reactions: first, total RNA was reverse transcribed into cDNA, then the cDNA was amplified by PCR and the product quantified using fluorescent dyes. In this thesis work, RT-qPCR was performed to quantify gene expression after transfection with siRNAs as well as the expression of specific markers of differentiation in BeWo cells.

2.11.1 Genomic DNA removal

Before performing cDNA synthesis, the extracted RNA samples underwent DNA removal using the Invitrogen™ DNase I, Amplification Grade kit (Thermo Fisher Scientific) to digest any residual genomic DNA. One µg of RNA was diluted in DEPC-treated water up to 8 µL and mixed with 1 µL of 10X DNase I Reaction Buffer and 1 µL of DNase I Amp Grade provided with the kit. Samples were then incubated for 15 min at room temperature and 1 µL of EDTA was added to initiate the DNA removal. The reaction was conducted at 65 °C for 10 min.

2.11.2 cDNA synthesis

cDNA was synthesized by reverse transcription using the High-Capacity RNA-to-cDNA™ kit (Applied Biosystems™) according to the manufacturer's instructions. The kit provides: 1) 50 µL of 20X Enzyme mix containing the reverse transcriptase MuLV and RNase inhibitor protein; 2) 500 µL of 2X RT Buffer Mix which includes dNTPs, random octamers, and oligo dT-16. Reagents were mixed and diluted as described in Table 3 for a final volume of 20 µL. To start the reverse transcription reaction, samples were incubated at 37°C for 60 min followed by 95°C for 5 min and finally hold at 4°C.

Table 3. cDNA synthesis using High-Capacity RNA-to-cDNA™ Kit

Component	Volumes
2X RT Buffer Mix	10.0 µL
20X RT Enzyme Mix	1.0 µL
RNA sample	up to 9 µL
Nuclease-free H2O	to 20 µL
Total per reaction	**20.0 µL**

2.11.3 SYBR® Green-based quantitative RT-qPCR

Following reverse transcription, RT-qPCR was performed in duplicates using the SensiFAST SYBR Lo-ROX kit (Bioline) in a 96-well PCR plate. One µL of cDNA solution, 1 µL of reverse and forward primer (Sigma-Aldrich), 7 µL of nuclease-free water (Invitrogen) and 10 µL of SYBR® Green were mixed and added to each well. Primers were purchased commercially (Sigma-Aldrich), and the amplicon length was 22 base pairs. The primer sequences used are listed in Table 4. RT-qPCR was performed using the Applied Biosystems™ 7500 Real-Time PCR machine and the setting conditions used are shown in Table 5. The mRNA expression for each target gene was normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reference gene. Fold changes in the mRNA expression levels were calculated using the ΔΔCt quantitation method.

Table 4. Sequences of forward and reverse primers used for RT-qPCR

Gene ID	Protein	Forward and reverse sequence primer
SLC2A1	GLUT1	5′-TTGCAGGCTTCTCCAACTGGAC-3′; 5′-CAGAACCAGGAGCACAGTGAAG-3′
OGT	OGT	5′-CAGGAAGGCTATTGCTGAGAGG-3′; 5′-CGGAACTCACATATCCTACACGC-3′
ERVW-1	Syncytin-1	5′-CGCAACTGCTATCACTCTGCCA-3′; 5′-CAGACAGTGACTCCAAGTCCTC-3′
ERVFRD-1	Syncytin-2	5′-ACCAGCTACCTGGGCATATCAG-3′; 5′-GAGGCATTGGTGAATCGACTGG-3′
GCM1	GCM1	5′-AGTGAACACAGCACCTTCCTCC-3′; 5′- TTGGACGCCTTCCTGGAAAGAC-3′
DUSP23	DUSP23	5′-AGATCGACCGCTTCGTGCAGAT-3′; 5′-CCGCTCCTTCACCAGGTAACAG-3′
GAPDH	GAPDH	5′-GTCTCCTCTGACTTCAACAGCG-3′; 5′-ACCACCCTGTTGCTGTAGCCAA-3′

Table 5. Setting conditions for RT-qPCR

Stage	Temperature	Time	Notes
Pre-PCR	60 °C	1 min	
Holding	95 °C	2 min	Polymerase activation
Cycling (40X)	95 °C 60 °C	5 s 30 s	Denaturation Annealing/extension
Melt curve	95 °C 60 °C	15 s 1 min	Melting curve

2.11.3.1 Melting curve analysis

The SYBR® Green I dye is a double-stranded DNA binding dye which detects PCR products as they accumulate during PCR cycles. The method enables the melting curve analysis which allows to check for nonspecific product formation. The melting curve is generated when double-stranded DNA, with the incorporated dye, is heated and melts to generate a single-stranded DNA. In the process, a sudden decrease in fluorescence is detected when the melting temperature is reached as a consequence of the dissociation of the DNA strands and subsequent release of the fluorescent dye giving origin to the melting curve. As the melting temperature of nucleic acids is affected by factors such as their length or the presence of base mismatches, the presence of PCR artifacts can be detected by analysing the shape of the melting curve thus reducing the need for time-consuming gel electrophoresis. A single-peaked melt curve chart is interpreted as representing a pure, single amplicon whereas additional peaks in the melting curves indicate the presence of nonspecific products such as primer dimerization. The melting curves generated from RT-qPCR experiments in this study were single-peaked curves, verifying the presence of a single PCR product.

2.12 Immunoprecipitation and co-immunoprecipitation assay

The immunoprecipitation was performed using the anti-O-GlcNAc antibody RL2 (Thermo Fisher Scientific) to isolate O-GlcNAc-modified proteins from a complex mixture of proteins extracted from BeWo cells. The co-immunoprecipitation assay was used to study the physical interaction between OGT protein and PKA or 11β-HSD2 in BeWo extracts (Figure 2.3). Cell lysates were diluted using PBS to reduce the concentration of the detergents present in the protein extraction buffer RIPA and 300 µg of total protein, corresponding to approximately 300-350 µL of cell lysate, were incubated overnight under gentle rotation at 4 °C with primary antibodies anti-OGT or anti-O-GlcNAc added at a concentration of 1:50. The day after, 20 µL of A/G protein agarose beads (Santa Cruz) were added in each tube and incubated under gentle rotation for 4 h at 4 °C followed by centrifugation at 2000 g for 5 min at 4 °C to spin down the beads. The supernatant was then collected to evaluate the immunoprecipitation efficiency whereas beads were washed 3 times with 200 µl of ice-cold PBS followed by centrifugation after each washing. Beads were finally resuspended in 20 µL of Laemmli buffer, boiled for 5 min at 95 °C in order to dissociate the immunocomplex from the beads and centrifuged to collect the supernatant containing the proteins of interest. Samples were further analyzed by immunoblotting to detect the protein of interest using specific antibodies or SDS-PAGE for mass spectrometry analysis.

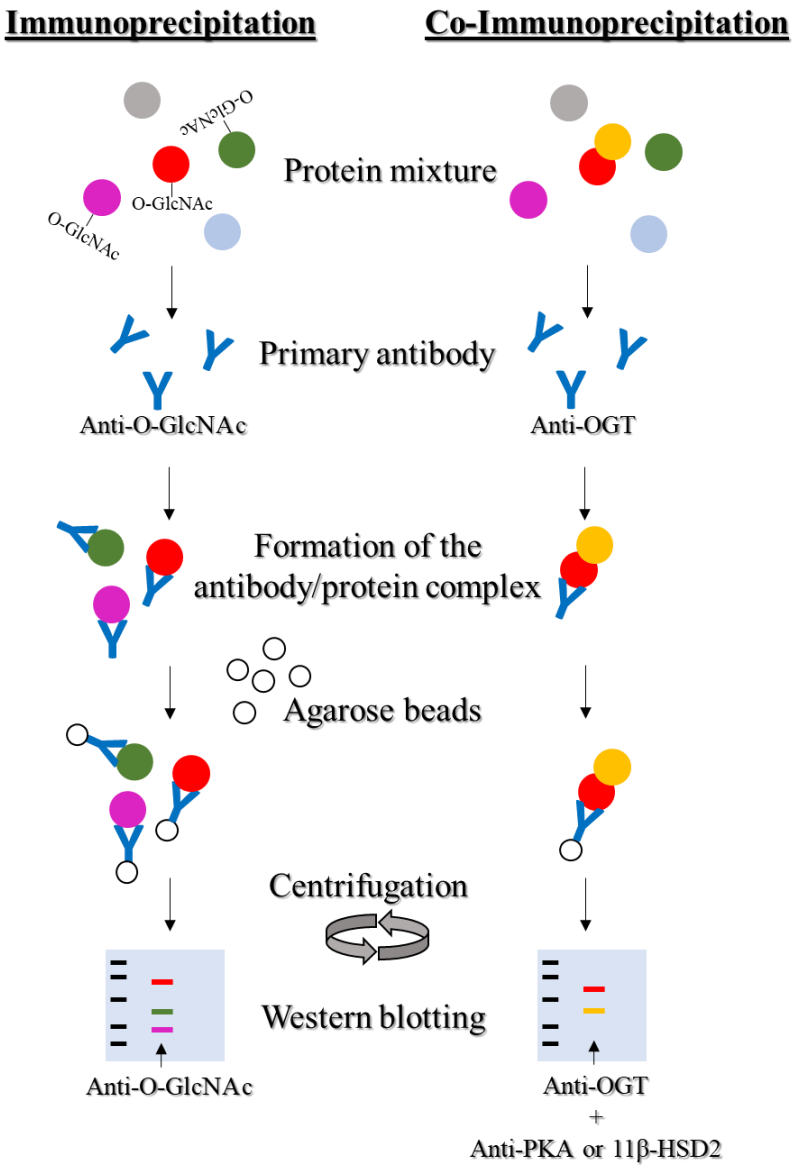


Figure 2.3 Schematic procedure of the agarose beads-based immunoprecipitation and co-immunoprecipitation assay. The O-GlcNAc-modified proteins were immunoprecipitated using a specific antibody against O-GlcNAc (RL2) while anti-OGT antibody was used to pull down proteins physically associated with OGT in the co-immunoprecipitation assay. Agarose beads were used to bind specifically to the antibody. Once coupled to agarose beads, centrifugation was used to pull down and purify the antibody/protein complex. O-GlcNAc-modified proteins isolated by immunoprecipitation were visualized by immunoblotting using anti-O-GlcNAc antibody while anti-PKA/11β-HSD2 antibodies were used to evaluate protein-protein interaction with OGT after co-immunoprecipitation.

2.13 Coomassie blue staining

After isolation of O-GlcNAcylated proteins by immunoprecipitation and separation by SDS-PAGE electrophoresis, proteins on the gel were visualized using the Bio–Safe™ Coomassie Stain (Bio-Rad) to confirm that the immunoprecipitation was successful before processing samples for proteomic analysis. The gel was first washed in double-distilled water (ddH_2O) to remove running buffer residues before being incubated in the Coomassie staining solution for 1 h at room temperature under gentle shaking. The gel was then washed for 30 min in ddH_2O and gel lanes were cut and stored in ddH_2O at 4 °C until processing for proteomic analysis.

2.14 In-gel protein digestion for proteomics

Each step was conducted at room temperature under shaking at 650 rpm unless otherwise specified. The whole gel lane was cut in small pieces and destained in 50% ethanol prepared in 50 mM ammonium bicarbonate (ABC) buffer for 20 min until the Coomassie was completely removed from the gel. After dehydrating the gel using 100% ethanol for 5 min, a reduction/alkylation step was performed by adding 10 mM of tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) and 40 mM of 2-chloroacetamide (CAA) for 5 min at 70 °C with gentle vortex. The gel pieces were then washed 3 times using 50% ethanol in 50 mM ABC for 20 min and underwent a second dehydration in 100% ethanol for 5 min. The tryptic digestion was then performed using 2.5 ng/µL trypsin solution and incubating samples overnight at 37 °C. The day after, trypsin was collected in a clean tube and peptides were further extracted from the gel by sonication in bath in a solution 25% acetonitrile 5% formic acid for 10 min. Peptides in solution were concentrated using a vacuum evaporator to bring the volume down to 20 µL and were resuspended in 2.0% acetonitrile + 0.1% trifluoroacetic acid solution to a final volume of 50 µL. Samples were then transferred to a mass spec vial and stored at -20 °C until mass spectrometry (MS) analysis.

2.15 MTT assay

The MTT assay kit (Sigma-Aldrich) was used to measure cellular metabolic activity as an indicator of cell viability after performing OGT gene silencing. The MTT assay is based on the conversion of the water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) compound to insoluble purple formazan crystals by metabolically active cells. The formazan is then solubilized and the concentration is

determined by measuring the optical density at 570 nm (Figure 2.4). BeWo cells were seeded at 1×10^3 cells/well in a 96-well culture plate, allowed to attach and stabilize for 24 h and treated with siRNA or control siRNA for 24 h and 48 h. At the end of the siRNA treatment, the medium was replaced with 100 µL of fresh culture medium and 10 µL of MTT reagent was added to each well at a concentration of 0.5 mg/mL. The plate was incubated for 3 h at 37°C before adding 100 µL of the MTT solubilization solution to each well followed by a second incubation at 37°C for 4 h in a humidified chamber. Finally, absorbance was measured at 570 nm with a microplate Tecan Sunrise ELISA Reader using a Magellan® Software.

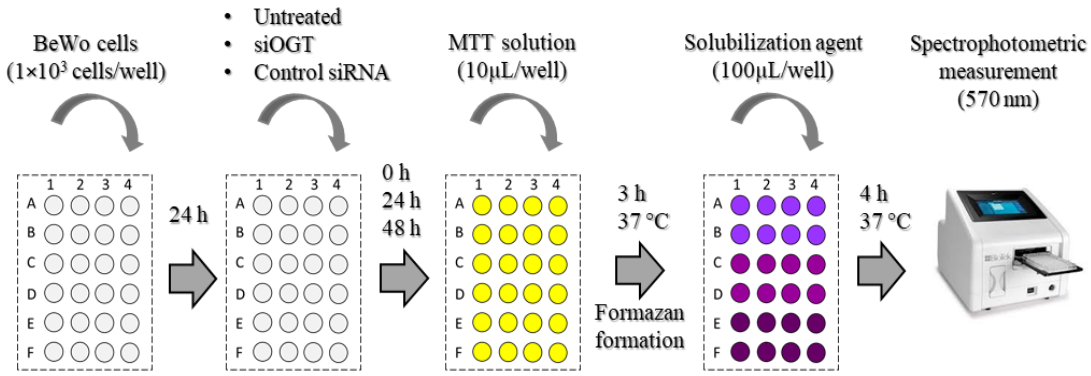


Figure 2.4 Schematic representation of the MTT assay procedure. The MTT solution was added to BeWo cells at 0, 24 and 48 h after treatment with siOGT or control siRNA. A group of untreated cells was also included in the experiment. After incubating MTT-treated cells for 3 h at 37 °C to allow the formation of the formazan crystals, a solubilizing solution was added followed by incubation for 4 h at 37 °C. Absorbance was measured at 570 nm. The amount of absorbance is proportional to the amount of living cells in each well.

2.16 Assessment of β-hCG and cortisol in the culture media

The culture media of BeWo cells was collected at the end of each experiment and stored at -20 °C for the measurement of β-hCG, a marker of human trophoblast differentiation, and cortisol, a steroid hormone belonging to the glucocorticoid class which regulates placental response to stress and fetal growth. Briefly, 200 µL of culture media were collected in a polypropylene test tubes and analyzed using electrochemiluminescence immunoassay (Roche Diagnostics) by the Department of Biochemistry (University Hospitals Coventry and Warwickshire NHS Trust).

2.17 *In silico* analysis for proteomics study

Three biological replicates of peptides from O-GlcNAc-enriched and non-enriched BeWo extracts, were subjected to fractionation using high performance liquid chromatography (HPLC) and analyzed by an Orbitrap Fusion with UltiMate 3000 RSLCnano System (Thermo Scientific) using a combination of two "softer" fragmentation methods, Higher energy Collisional Dissociation (HCD) and Electron-Transfer Dissociation (ETD). HCD has the advantage to generate a distinct HexNAc oxonium ion (m/z 204) and a series of HexNAc fragments (m/z 186, m/z 168, m/z 144, m/z 138 and m/z 126) which serve as diagnostic tools for O-GlcNAc modification whereas ETD produces sufficient fragmentation ions for confident peptide identification and preserves, at the same time, the O-GlcNAc-modified residues for accurate site assignment (Zhao et al., 2011). Data file analysis for N-acetylhexosamine (HexNAc) peptides and peptide fragment assignation used search against the Uniprot_Human database and was performed using the MaxQuant software (version 1.6.10.43). Protein abundance analysis was performed by a two-sided t-test, and significance was determined on the basis of a false discovery rate (FDR) of <0.05 and hyperbolic curve threshold of S0=0.1 using the Perseus software (version 1.6.2.2). S0 is defined as the "artificial within groups variance" and controls the relative importance of the t-test p value and difference between means (Tusher et al., 2001). The significantly O-GlcNAc-enriched proteins were further analysed using the publicly-available annotation platform Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 to perform a Gene Ontology (GO) analysis to identify biological and functional categories. A predictive model was applied to the identified O-GlcNAc sites using the web-based tool OGTsite

(http://csb.cse.yzu.edu.tw/OGTSite/) and an interacting network analysis was conducted using the Search Tool for the Retrieval of Interacting Genes (STRING) database (https://string-db.org/). The proteomics analysis presented in this thesis has been mainly performed by the staff members at the Proteomics Research Technology Platform at the University of Warwick with the exception of the analysis conducted using DAVID, OGTsite and STRING.

2.18 Statistical analysis

Data were analysed using a Student t-test to compare two groups or 1-way and 2-way analysis of variance (ANOVA) to compare more than two groups. Multiple comparison tests were performed in case of ANOVA analysis using a Tukey's post-hoc test. A linear regression model was applied to evaluate linear relationships between two variables. The StatView software (version 5.0.1.0, Abacus Concepts, CA, USA) was used to carry out statistical analysis. A level of probability set at $p<0.05$ was used as statistically significant. Data are presented graphically as means ± SEM.

Chapter 3

Identification and site mapping of O-GlcNAc-modified proteins in BeWo cells

3.1 Introduction

The main goal of the work presented in this Chapter was to identify O-GlcNAc-modified proteins in human placental trophoblast BeWo cells using MS. The approach used to achieve the Chapter's aim is summarised in the workflow diagram in Figure 3.1. O-GlcNAc signalling is emerging as a major regulator of cellular functions in response to a variety of stressful and metabolic stimuli. Thus, identifying O-GlcNAc-modified proteins in the human trophoblast can be crucial to better understand the molecular basis underlying the pathophysiological adaptations occurring in the placenta during pregnancy complications. One major challenge when studying O-GlcNAcylation is associated to low abundance and substoichiometric occupancy of O-GlcNAc sites which requires the application of enriching methods upstream MS (Ma and Hart, 2014). In addition, the O-GlcNAc site mapping is limited by the susceptibility of the β-O-glycosidic bond which anchors the GlcNAc moiety to the amino acid chain and is easily lost during the conventional fragmentation methods employed in proteomics such as the Collision-Induced Dissociation (CID) (Chalkley and Burlingame, 2001). By taking advantage of the recently identified O-GlcNAc monoclonal antibodies and the combination of two "softer" fragmentation methods, Higher energy Collisional Dissociation (HCD) and Electron-Transfer Dissociation (ETD), O-GlcNAcylated proteins were first enriched from BeWo protein extracts, then characterized and O-GlcNAc sites were mapped. HCD has the advantage to generate a distinct HexNAc oxonium ion (m/z 204) and a series of HexNAc fragments (m/z 186, m/z 168, m/z 144, m/z 138 and m/z 126) which are used as diagnostic tools for identifying O-GlcNAc-modified sites whereas ETD produces sufficient fragmentation ions for confident peptide identification and preserves, at the same time, the O-GlcNAc-modified residues for accurate site assignment (Zhao et al., 2011). Following enrichment and MS, a Gene Ontology (GO) analysis was performed in order to assign biological and functional roles to the proteins characterized as O-GlcNAc-modified, in the attempt to identify single proteins or clusters with a pivotal role in trophoblast physiology. This is the first study which combines O-GlcNAc-specific antibody enrichment followed by HCD/ETD analysis for profiling O-GlcNAc-modified proteins in human trophoblast.

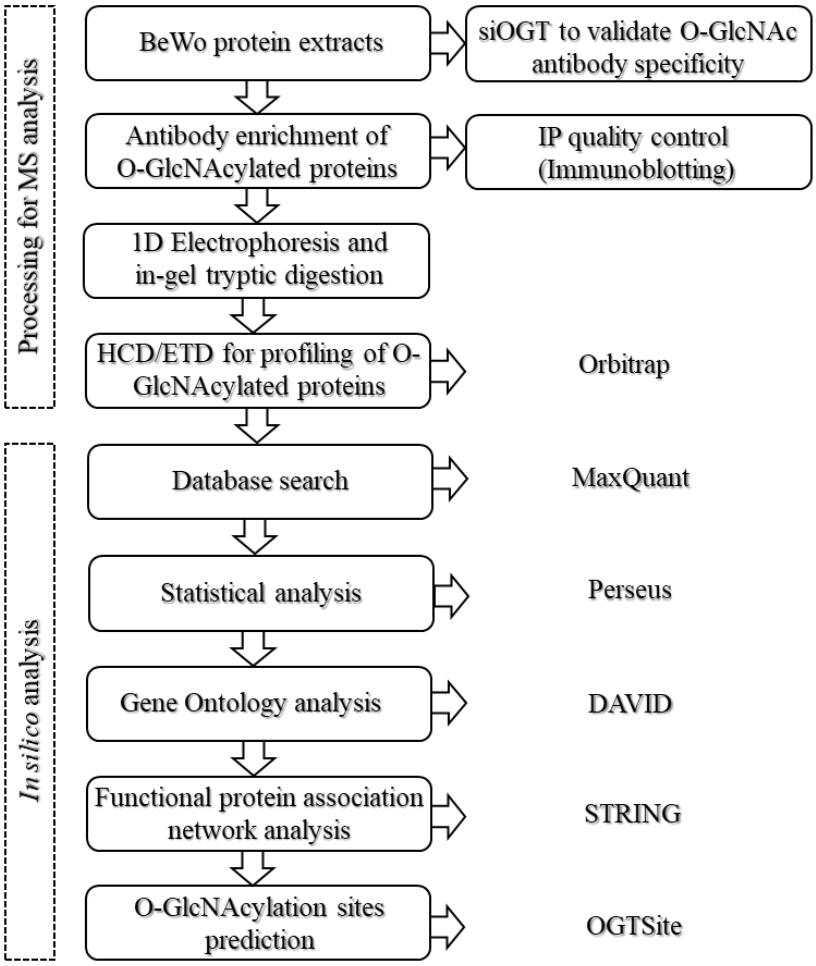


Figure 3.1 Workflow diagram summarizing the approach used to characterize O-GlcNAcylated proteins in BeWo cell extracts. Proteins were extracted and O-GlcNAc-enriched by IP with a specific antibody. The IP efficiency was verified by immunoblotting and both enriched and not enriched samples underwent MS analysis using a combined HCD/ETD fragmentation method. MS data were searched against the Uniprot_Human database using the MaxQuant software (version 1.6.10.43) and statistically analysed using Perseus (version 1.6.2.2). A Gene Ontology (GO) and a protein-protein association network analysis were performed on O-GlcNAc-enriched proteins in order to assign biological roles and functional interconnection. Finally, a predictive model was applied to the identified O-GlcNAc sites using the web server, OGTsite.

3.2 Sample processing for HCD/ETD MS analysis

3.2.1 Antibody specificity validation before O-GlcNAc enrichment of BeWo protein extracts

The pharmacological inhibition of OGT is still a major challenge due to the lack of specificity and poor cell permeability showed by many inhibiting agents (Trapannone et al., 2016). As OGT is the unique enzyme responsible for protein O-GlcNAcylation, a siRNA was used here to perform specific silencing of endogenous OGT. Afterwards, OGT protein expression and the corresponding amount of O-GlcNAcylation was measured by immunoblotting at 4, 9, 24 and 48 h after transfection with siOGT, control siRNA or no treatment (NT) (Figure 3.2, A). Results showed that after 9 h onward, siOGT significantly reduced both OGT protein expression and global protein O-GlcNAcylation compared to both NT and control siRNA (Figure 3.2. B and C, siOGT vs control siRNA or NT, OGT protein expression: 9 h: $p<0.05$, 24 h: $p<0.01$ and 48 h: $p<0.001$; O-GlcNAcylation: 9 h: $p<0.05$; 24 and 48 h: $p<0.001$). By performing an MTT assay after 24 and 48 h post-transfection, cell viability was significantly reduced in siOGT or control siRNA compared to NT ($p<0.001$) possibly due to the toxic effects of the reagent used for transfection. However, there was no difference in cell survival between siOGT and control siRNA treatment suggesting that the knockdown of OGT had no effect *per se* on cell viability (Figure 3.2, D).

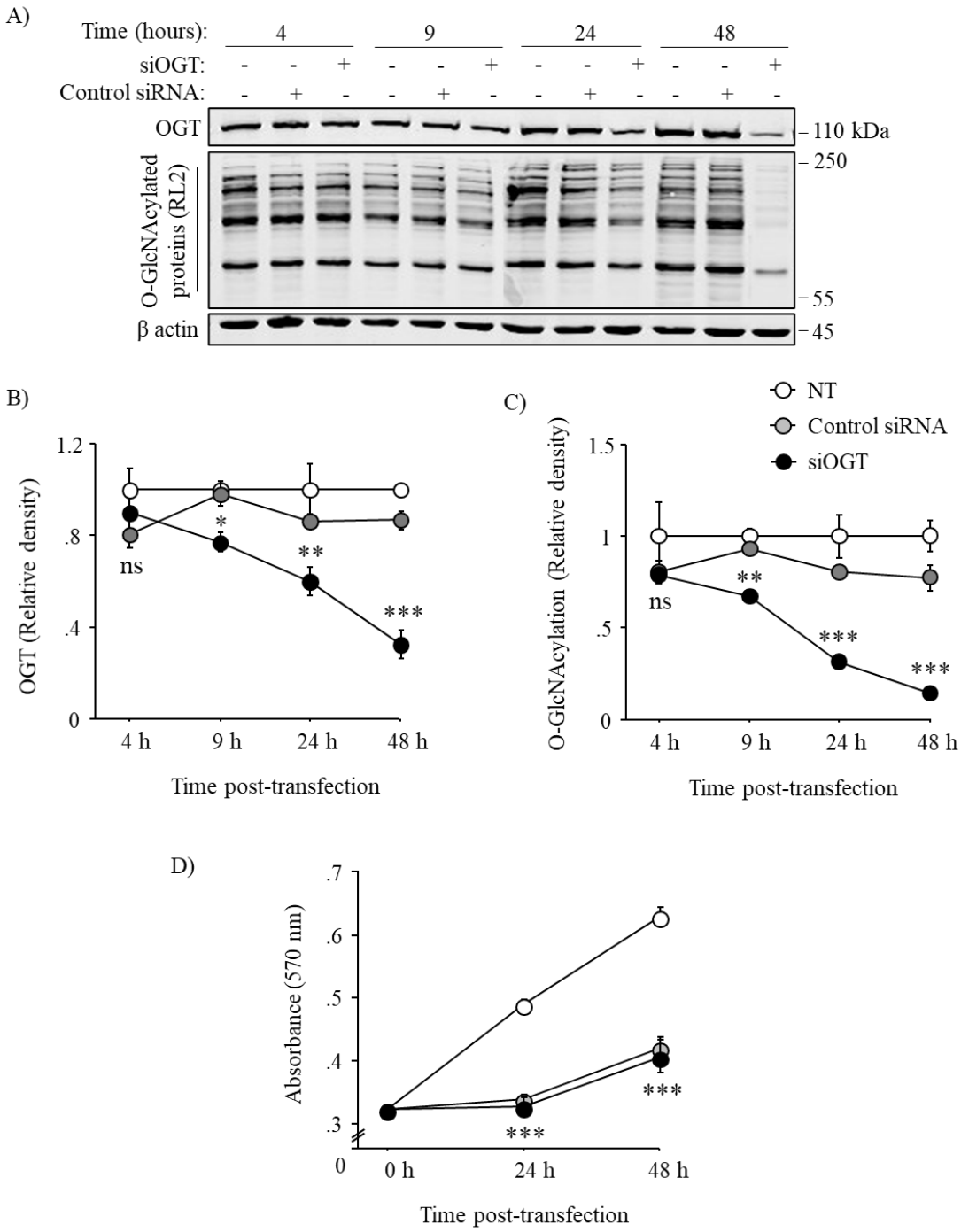


Figure 3.2 Validation of the anti-O-GlcNAc antibody (RL2) using siRNA against OGT in BeWo cells. A) Representative immunoblotting showing OGT and O-GlcNAcylation protein levels in NT, control siRNA and siOGT-treated cells at 4, 9, 24 and 48 h post-transfection. B) Relative density for OGT and C) protein O-GlcNAcylation. Normalization to β actin. N=3. D) MTT assay showing cell viability at 24 and 48 h post-transfection. Data are expressed as mean ± SEM; ns=non-significant, *p<0.05, **p<0.01, ***p<0.001.

3.2.2 Antibody-based enrichment of O-GlcNAcylated proteins in BeWo extracts

The enrichment of O-GlcNAcylated protein is a fundamental step before applying MS detection due to the substoichiometric site occupancy of O-GlcNAc. After testing its specificity against O-GlcNAc proteins, the antibody RL2 was used to purify O-GlcNAcylated proteins from BeWo whole extracts using IP before applying MS analysis (Figure 3.3). The immunoprecipitates were analysed using fluorescent immunoblotting to verify the efficiency and specificity of IP. In Figure 3.3 O-GlcNAc-modified proteins in whole extracts (lane 1), whole extracts after IP (lane 2), IgG control (lane 3) and purified O-GlcNAcylated proteins (lane 4) are shown. O-GlcNAcylated proteins are almost 3 times more represented after purification with the anti-O-GlcNAc antibody (lane 4) compared to the not-enriched whole extracts in lane 1 indicating a robust enrichment after IP; on the contrary, O-GlcNAc proteins are almost 3 times lower after IP in the supernatant (lane 2). The intensities for all the lanes are reported in the table in Figure 3.3. After IP validation, proteins from both whole extracts and O-GlcNAc-enriched samples derived from three biological replicates were separated by SDS-PAGE, stained with Coomassie blue for protein visualization and gel lanes were cut out. After destaining and in-gel trypsin digestion of proteins, samples underwent MS analysis for protein identification and quantification (Figure 3.3).

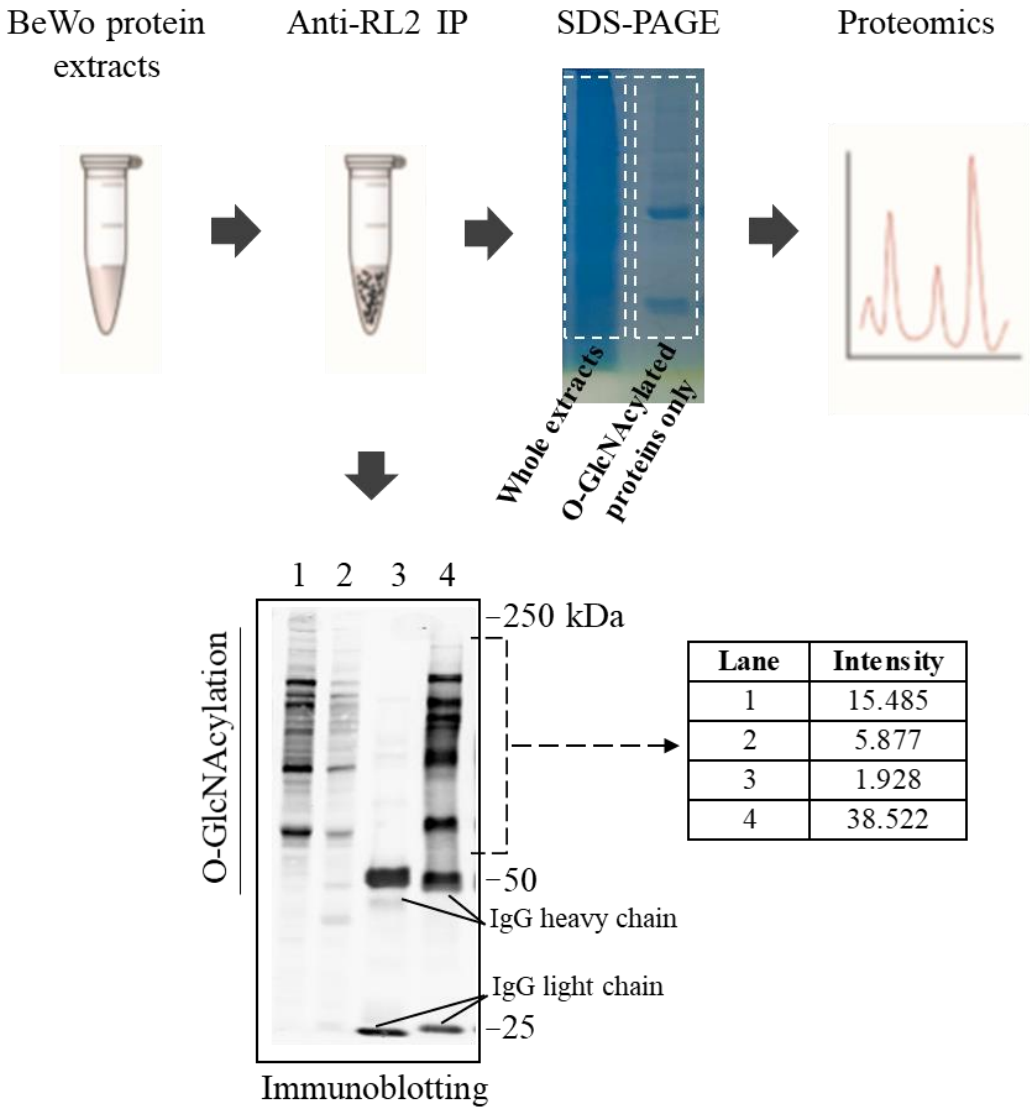


Figure 3.3 Representation of the antibody-based enrichment method used to purify O-GlcNAcylated proteins upstream MS analysis in BeWo extracts. O-GlcNAcylated proteins were purified from BeWo extracts using a specific antibody (RL2) against O-GlcNAc-modified proteins. The IP efficiency was checked by visualizing O-GlcNAcylated proteins using immunoblotting in whole extract (lane 1), whole extracts after IP (lane 2), IgG control (lane 3) and O-GlcNAc-enriched proteins in the immunoprecipitate (lane 4). The density of the signal was measured for each lane using the Odyssey software and is reported in the table. Whole extracts and O-GlcNAc-purified proteins only were run on agarose gel, stained with Coomassie blue to be visualized and gel lanes were excised, proteins digested with trypsin and identified by MS.

3.3 *In silico* analysis

3.3.1 Characterization and cellular localization of O-GlcNAc-modified proteins enriched from BeWo extracts

A two-stage HCD/ETD fragmentation method was applied to analyse non-enriched extracts and O-GlcNAc-enriched proteins. The acquired data were searched against the Uniprot_Human database using MaxQuant software. A total of 2334 proteins was identified by MS, 210 of which were significantly more represented in the O-GlcNAc-enriched samples compared to the non-enriched ones as graphed in the volcano plot in Figure 3.4, A. Data were analysed by two-sided t-test using Perseus software. The hyperbolic lines in the volcano plot represent a statistical cut-off based on FDR ($p<0.05$) and S0=0.1. The S0 controls the relative importance of the t-test p value taking into account the difference between means (Tusher et al., 2001) so the hyperbolic lines in the graph are based on both the p value and "within groups difference of means". The significantly enriched proteins represent with high confidence O-GlcNAc-modified proteins and are listed in Table 6 according to their p values for the significance of enrichment. However, there is the possibility that some of the 210 O-GlcNAc-enriched proteins here identified by MS may have been co-purified during the co-immunoprecipitation assay because tightly associated to O-GlcNAcylated proteins, for example as a part of multiproteic complexes and may not be O-GlcNAc-modified themselves. For that reason, a manual database (neXtProt, PhosphoSitePlus®) and literature search (Zhao et al., 2011; Lund et al., 2016; Kang et al., 2013; Ahmad et al., 2011; Ha and Lim, 2015; Zeidan et al., 2010; Hiromura et al., 2003; Duan et al., 2018; Liu et al., 2016; Ruan et al., 2013; Gurel et al., 2014) were performed to verify which of the O-GlcNAc-enriched proteins, identified in this study, were already characterized as O-GlcNAc-modified. Results showed that 99/210 (47%) proteins were already identified as O-GlcNAcylated according to the literature and database search (shown in blue in Table 6), while 106/210 (50%) were found to interact with OGT in interactome studies (shown in yellow in Table 6) for a total of 60 proteins (shown in green in Table 6) which both interact with OGT and are O-GlcNAcylated representing with high probability O-GlcNAc-modified proteins. Finally, 61/210 proteins (shown in grey in Table 6) were found to be neither O-GlcNAc-modified nor interacting with OGT and, for that reason, they might represent novel O-GlcNAcylated or co-purified proteins (Figure 3.4, B). Finally, of the 210 O-

GlcNAc-enriched proteins identified in this study, 36% is located exclusively in the nucleus, 21% is cytoplasmic and 33% is shared between nucleus and cytoplasm as predicted by GO analysis. Interestingly, a significant amount of these proteins (10%) belong to the nuclear envelope (Figure 3.5, A). These data are further confirmed by immunocytochemistry analysis using anti-O-GlcNAc antibody RL2 which showed higher fluorescence inside and surrounding the nucleus (Figure 3.5, B and C).

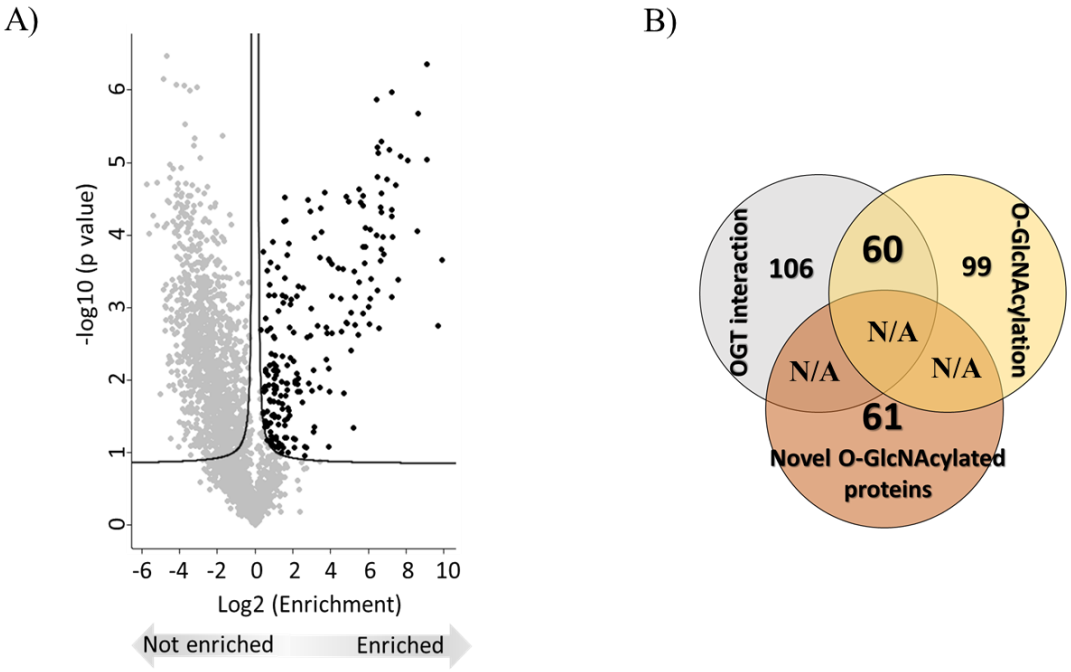


Figure 3.4 Characterization of putative O-GlcNAcylated proteins enriched from BeWo extracts and identified by HCD/ETD analysis. A) Volcano plot showing, in black, the 210 proteins which were significantly enriched by immunoprecipitation with anti-RL2 and representing putative O-GlcNAc-modified proteins. Data are plotted as significance (-log10 of the p value) versus fold change of the enrichment (Log2) on the y and x axes, respectively. Statistical analysis was performed by two-sided t-test using Perseus. The black hyperbolic curved lines represent the threshold for statistical significance with FDR of 0.05 and S0 (minimal fold change) of 0.1. B) Schematic representation of results originated from manual literature and database search for proteins already known to interact with OGT or be O-GlcNAc-modified. N/A: not applicable.

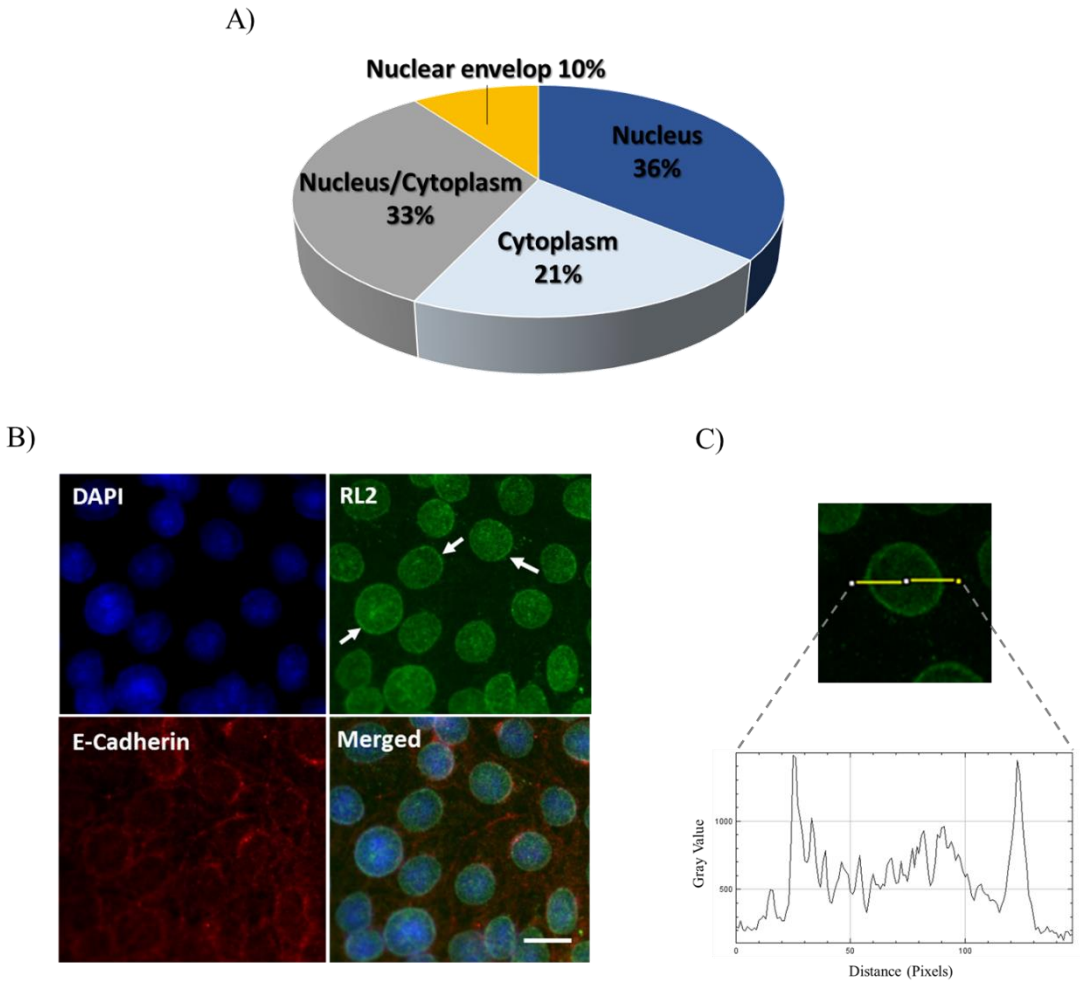


Figure 3.5 Cellular compartmentalization of O-GlcNAcylated proteins in BeWo. A) Pie chart showing the subcellular localization of the 210 O-GlcNAc-enriched and identified proteins in BeWo extracts as predicted by GO analysis. B) Immunocytochemistry showing cellular localization of O-GlcNAcylated proteins recognized by the anti-O-GlcNAc antibody, RL2 (green) in BeWo cells. C) Two-dimensional graph displaying changes in pixel intensity along a line drawn across a cell stained with anti-O-GlcNAc antibody. The analysis was performed with ImageJ 1.49v software on different cells and a representative example is shown here. The X-axis of the graph represents distance along the drawn line and the Y-axis is the corresponding pixel intensity.

Table 6. Putative O-GlcNAcylated proteins purified from BeWo and identified by MS

Gene ID	Protein Name	-Log10(p value)
NUP62	Nuclear pore glycoprotein p62	5,87
NUP54	Nucleoporin p54	5,68
NUP98	Nuclear pore complex protein Nup98-Nup96	4,80
TFG	Protein TFG	4,53
HIST1H1B	Histone H1.5	4,37
SEC24B	Protein transport protein Sec24B	3,80
RPL3	60S ribosomal protein L3	3,72
SETD1A	Histone-lysine N-methyltransferase SETD1A	3,65
RPSA	40S ribosomal protein SA	3,62
HSPA1B	Heat shock 70 kDa protein 1B	3,55
RPS14	40S ribosomal protein S14	3,32
PRPF40A	Pre-mRNA-processing factor 40 homolog A	3,29
RBM25	RNA-binding protein 25	3,27
HSPA9	Stress-70 protein, mitochondrial	3,14
HSPA5	Endoplasmic reticulum chaperone BiP	2,95
FUS	RNA-binding protein FUS	2,71
NONO	Non-POU domain-containing octamer-binding protein	2,59
PRPF19	Pre-mRNA-processing factor 19	2,58
SFPQ	Splicing factor, proline- and glutamine-rich	2,22
RPL27	60S ribosomal protein L27	2,13
HNRNPA2B1	Heterogeneous nuclear ribonucleoproteins A2/B1	2,11
SMARCA2	Probable global transcription activator SNF2L2	1,94
WDR5	WD repeat-containing protein 5	1,89
HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1	1,86
RPL11	60S ribosomal protein L11	1,83
HSPA4	Heat shock 70 kDa protein 4	1,82
YBX1	Nuclease-sensitive element-binding protein 1	1,55
HUWE1	E3 ubiquitin-protein ligase HUWE1	1,52
RANBP2	E3 SUMO-protein ligase RanBP2	1,50

YY1	Transcriptional repressor protein YY1	1,50
NUP153	Nuclear pore complex protein Nup153	5,97
TRIM21	E3 ubiquitin-protein ligase TRIM21	5,04
NUP214	Nuclear pore complex protein Nup214	5,02
QSER1	Glutamine and serine-rich protein 1	4,77
CSNK2A1	Casein kinase II subunit alpha	4,59
BAP1	Ubiquitin carboxyl-terminal hydrolase BAP1	4,54
OGT	UDP-N-acetylglucosamine--peptide N-acetylglucosaminyltransferase 110 kDa subunit	4,45
TET3	Methylcytosine dioxygenase TET3	4,08
HNRNPU	Heterogeneous nuclear ribonucleoprotein U	3,90
KANSL3	KAT8 regulatory NSL complex subunit 3	3,84
HIVEP1	Zinc finger protein 40	3,64
DMAP1	DNA methyltransferase 1-associated protein 1	3,53
HCFC1	Host cell factor 1	3,39
RAE1	mRNA export factor	3,34
DIDO1	Death-inducer obliterator 1	3,24
HCFC1	Host cell factor 1	3,12
TET2	Methylcytosine dioxygenase TET2	3,12
AHNAK	Neuroblast differentiation-associated protein AHNAK	3,11
POM121	Nuclear envelope pore membrane protein POM 121	2,92
SPEN	Msx2-interacting protein	2,64
NUP160	Nuclear pore complex protein Nup160	2,30
CARM1	Histone-arginine methyltransferase CARM1	2,13
CAD	CAD protein	2,07
NUP205	Nuclear pore complex protein Nup205	1,87
RTN4	Reticulon	1,72
NUP50	Nuclear pore complex protein Nup50	1,70
NUP188	Nucleoporin NUP188 homolog	1,69
ASXL1	Putative Polycomb group protein ASXL1	1,18
ASXL2	Putative Polycomb group protein ASXL2	1,07
PROSER1	Proline and serine-rich protein 1	4,25
RPS3	40S ribosomal protein S3	4,20

FLG2	Filaggrin-2	4,04
SLC25A5	ADP/ATP translocase 2	3,88
PHF12	PHD finger protein 12	3,83
ALB	Serum albumin	3,67
SLC16A1	Monocarboxylate transporter 1	3,17
RAB14	Ras-related protein Rab-14	3,16
PSPC1	Paraspeckle component 1	3,16
DSG1	Desmoglein-1	3,15
ZC3H18	Zinc finger CCCH domain-containing protein 18	2,99
DCD	Dermcidin	2,79
NXF1	Nuclear RNA export factor 1	2,78
FAM208B	Protein FAM208B	2,75
DDX54	ATP-dependent RNA helicase DDX54	2,72
HRNR	Hornerin	2,72
TUBB4B	Tubulin beta-4B chain	2,68
RPS16	40S ribosomal protein S16	2,68
CSNK2B	Casein kinase II subunit beta	2,61
DHX9	ATP-dependent RNA helicase A	2,36
PSMD3	26S proteasome non-ATPase regulatory subunit 3	2,34
RPLP0	60S acidic ribosomal protein P0	2,15
RPN1	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	2,06
GTF3C3	General transcription factor 3C polypeptide 3	1,98
COPA	Coatomer subunit alpha	1,97
LMNB1	Lamin-B1	1,93
ARCN1	Archain 1, isoform CRA_a|Coatomer subunit delta	1,90
SLC2A3	Solute carrier family 2, facilitated glucose transporter member 3	1,90
TUBA1C	Tubulin alpha-1C chain	1,88
ZNF326	DBIRD complex subunit ZNF326	1,84
MRPS26	28S ribosomal protein S26, mitochondrial	1,70
TMEM33	Transmembrane protein 33	1,68
RAB35	Ras-related protein Rab-35	1,61
XPO1	Exportin-1	1,57

RPS10	40S ribosomal protein S10	1,49
KDM1B	Lysine-specific histone demethylase 1B	1,46
CNP	2',3'-cyclic-nucleotide 3'-phosphodiesterase	1,38
CHD1	Chromodomain-helicase-DNA-binding protein 1	1,37
IQGAP1	Ras GTPase-activating-like protein IQGAP1	1,36
RAB11A	Ras-related protein Rab-11A	1,34
HACD3	Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase 3	1,19
COPB2	Coatomer subunit beta'	1,18
SCAF11	Protein SCAF11	1,14
ATP2A2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	1,12
AHCTF1	Protein ELYS	1,08
EIF2B3	Translation initiation factor eIF-2B subunit gamma	1,07
RBM27	RNA-binding protein 27	3,54
POU2F1	POU domain, class 2, transcription factor 1	5,29
UBAP2	Ubiquitin-associated protein 2	5,17
TET1	Methylcytosine dioxygenase TET1	5,08
POM121C	Nuclear envelope pore membrane protein POM 121C	4,58
RUVBL1	RuvB-like 1	4,48
SP3	Transcription factor Sp3	4,34
PRRC2C	Protein PRRC2C	4,10
SRCAP	Helicase SRCAP	3,61
UBAP2	Ubiquitin-associated protein 2	3,60
RAN	GTP-binding nuclear protein Ran	3,51
HSPA8	Heat shock cognate 71 kDa protein	3,40
EWSR1	RNA-binding protein EWS	3,17
NUP88	Nuclear pore complex protein Nup88	2,75
UBAP2	Ubiquitin-associated protein 2	2,73
JMJD1C	Probable JmjC domain-containing histone demethylation protein 2C	2,62
NFRKB	Nuclear factor related to kappa-B-binding protein	2,41
SEC23IP	SEC23-interacting protein	2,20
RACK1	Receptor of activated protein C kinase 1	1,42

WNK1	Serine/threonine-protein kinase WNK1	6,36
CIC	Protein capicua homolog	4,69
ANKHD1	Ankyrin repeat and KH domain-containing protein 1	4,63
ANKRD17	Ankyrin repeat domain-containing protein 17	4,46
BASP1	Brain acid soluble protein 1	4,19
NUP58	Nucleoporin p58/p45	4,05
EMSY	BRCA2-interacting transcriptional repressor EMSY	3,98
TCERG1	Transcription elongation regulator 1	3,96
ATP5F1A	ATP synthase subunit alpha, mitochondrial	3,76
SP1	Transcription factor Sp1	3,65
NUP93	Nuclear pore complex protein Nup93	3,06
SAP30BP	SAP30-binding protein	2,66
LUZP1	Leucine zipper protein 1	2,32
MEF2D	Myocyte-specific enhancer factor 2D	2,28
MARK2	Serine/threonine-protein kinase MARK2	1,95
UBAP2L	Ubiquitin-associated protein 2-like	1,83
CLASP2	CLIP-associating protein 2	1,20
PRRC2B	Protein PRRC2B	1,13
WNK2	Serine/threonine-protein kinase WNK2	1,07
CDK12	Cyclin-dependent kinase 12	1,07
MARK3	MAP/microtubule affinity-regulating kinase 3	0,99
SAP130	Histone deacetylase complex subunit SAP130	0,95
KRT1	Keratin, type II cytoskeletal 1	5,21
SIX4	Homeobox protein SIX4	5,13
SEC23B	Protein transport protein Sec23B	4,52
NFYA	Nuclear transcription factor Y subunit alpha	4,41
KRT14	Keratin, type I cytoskeletal 14	4,38
RUVBL2	RuvB-like 2	4,32
LYZ	Lysozyme	3,99
SS18	Protein SSXT	3,97
KRT9	Keratin, type I cytoskeletal 9	3,73
YEATS4	YEATS domain-containing protein 4	3,69

ACTR6	Actin-related protein 6	3,49
KRT2	Keratin, type II cytoskeletal 2 epidermal	3,38
TBC1D4	TBC1 domain family member 4	3,14
KRT10	Keratin, type I cytoskeletal 10	3,14
GDPD3	Lysophospholipase D GDPD3	3,04
KRT6A	Keratin, type II cytoskeletal 6A	3,01
FBXO22	F-box only protein 22	2,85
KRT5	Keratin, type II cytoskeletal 5	2,76
DHCR7	7-dehydrocholesterol reductase	2,74
MYOF	Myoferlin	2,65
GRN	Progranulin	2,64
H1F0	Histone H1.0	2,59
CDK11B	Cyclin-dependent kinase 11B	2,26
DARS	Aspartate--tRNA ligase, cytoplasmic	2,20
PFKL	ATP-dependent 6-phosphofructokinase, liver type	2,19
VPS72	Vacuolar protein sorting-associated protein 72 homolog	2,15
ACTL6A	Actin-like protein 6A	2,12
PTHLH	Parathyroid hormone-related protein	2,08
SLC7A5	Large neutral amino acids transporter small subunit 1	2,02
CAV1	Caveolin-1	2,02
NSRP1	Nuclear speckle splicing regulatory protein 1	1,95
PRKD2	Serine/threonine-protein kinase D2	1,95
PHKB	Phosphorylase b kinase regulatory subunit beta	1,95
HEXB	Beta-hexosaminidase subunit beta	1,93
ATP2B4	Plasma membrane calcium-transporting ATPase 4	1,87
FERMT2	Fermitin family homolog 2	1,86
KPRP	Keratinocyte proline-rich protein	1,84
KRT16	Keratin, type I cytoskeletal 16	1,82
SNTB2	Beta-2-syntrophin	1,77
STX18	Syntaxin-18	1,77
COBLL1	Cordon-bleu protein-like 1	1,66
ARHGEF16	Rho guanine nucleotide exchange factor 16	1,59

RAB8A	Ras-related protein Rab-8A	1,58
BCAP31	B-cell receptor-associated protein 31	1,53
DYNC1H1	Cytoplasmic dynein 1 heavy chain 1	1,51
GSTZ1	Glutathione transferase zeta 1	1,48
STT3B	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3B	1,44
SERPINH1	Serpin H1	1,43
ATP6V1H	V-type proton ATPase subunit H	1,40
PRKCH	Protein kinase C eta type	1,35
CPVL	Probable serine carboxypeptidase CPVL	1,33
AAMP	Angio-associated migratory cell protein	1,28
KRT77	Keratin, type II cytoskeletal 1b	1,27
FHL2	Four and a half LIM domains protein 2	1,23
DNMT3B	DNA (cytosine-5)-methyltransferase 3B	1,21
CEMIP2	Cell surface hyaluronidase	1,20
SEC23A	Protein transport protein SEC23	1,17
LEO1	RNA polymerase-associated protein LEO1	1,17
RABGAP1	Rab GTPase-activating protein 1	1,17
ZNF384	Zinc finger protein 384	1,10
COL4A1	Collagen alpha-1(IV) chain	1,08
SURF4	Surfeit locus protein 4	1,06
REXO4	RNA exonuclease 4	1,00

Search for OGT interactome studies was performed on https://www.nextprot.org/ and on Zhao et al., 2011. Search for protein O-GlcNAcylation was performed on https://www.phosphosite.org/ and on literature (Zhao et al., 2011; Lund et al., 2016; Kang et al., 2013; Ahmad et al., 2011; Ha and Lim, 2015; Zeidan et al, 2010; Hiromura et al., 2003; Duan et al., 2018; Liu et al., 2016; Ruan et al., 2013; Gurel et al., 2014).

According to the literature and database search, proteins that both interact with OGT and are known to be O-GlcNAcylated are shown in green in the table; proteins that only interact with OGT are shown in yellow; proteins that are known to be O-GlcNAc-modified are shown in blue; novel putative O-GlcNAcylated proteins are shown in grey at the bottom of the table.

3.3.2 Mapping of O-GlcNAcylation sites

For the identification of the O-GlcNAc sites, each raw spectral file originated from the HCD/ETD fragmentation of BeWo proteins, was searched against the Uniprot_Human database using the MaxQuant software allowing for HexNAc modification on serine/threonine (+203.079 Da). Indeed, once fragmented, peptides containing O-GlcNAcylation sites show a distinctive fragmentation profile characterized by the HexNAc oxonium ion with a specific mass-to-charge ratio (m/z) of 204 (m/z 204). A series of HexNAc fragments are also produced at high intensity (m/z 186, m/z 168, m/z 144, m/z 138 and m/z 126) in the HCD/ETD fragmentation spectra (Figure 3.6) (Zhao et al., 2011). A total of 114 O-GlcNAc sites on 22 proteins, 4 of which were novel O-GlcNAc-modified proteins, were identified in BeWo extracts using the HCD/ETD fragmentation method and engine search (Table 7). Not surprisingly, O-GlcNAc sites were found mainly on the 210 proteins significantly enriched by the O-GlcNAc antibody suggesting that primarily O-GlcNAcylated proteins were purified with the antibody-based enrichment method used in this study. By comparison with the literature and database search, 69 sites, were not previously reported and might constitute novel O-GlcNAcylation sites (marked in bold in Table 7). A predictive model analysis, based on the computational evaluation of the amino acids motif surrounding the O-GlcNAcylation sites as substrate for the OGT enzyme, was also applied to the novel identified O-GlcNAc sites using the web-based tool OGTsite (Wu et al., 2014). Studies have demonstrated that OGTsite predicts O-GlcNAcylation by providing a ~84% accuracy (Kao et al., 2015). Results showed that 10/69 O-GlcNAc-modified sites identified in this study are predicted as O-GlcNAc-modified by the OGTsite tool and are shown in red in Table 7. Interestingly, this analysis revealed that the most pronounced feature surrounding the O-GlcNAc modification site is the abundance of hydrophobic amino acids Proline (P), Valine (V), and Alanine (A), located around position -2 and -3 relative to the Ser/Thr (S/T) at position 0 (Table 7).

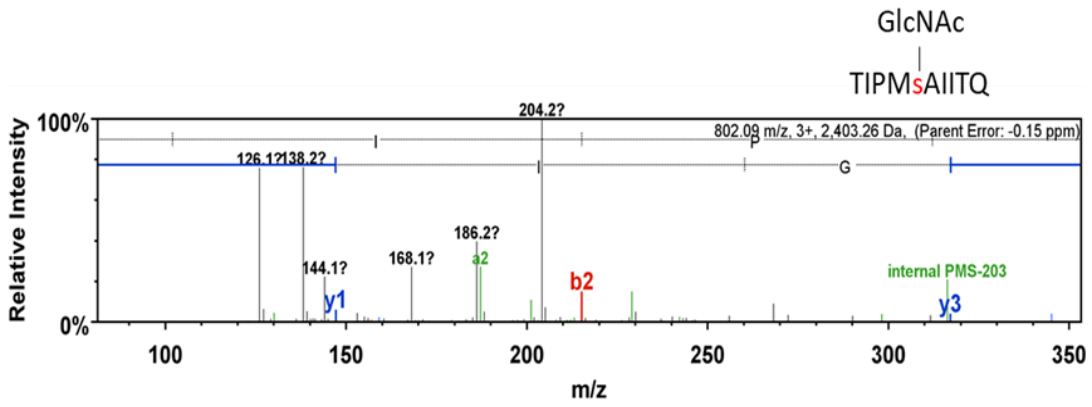
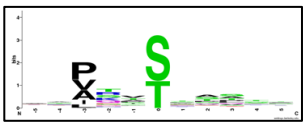
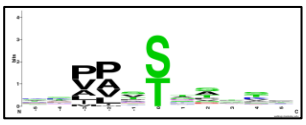
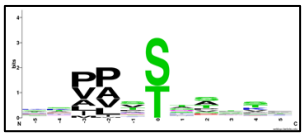
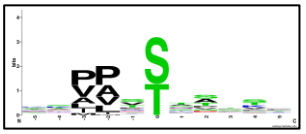
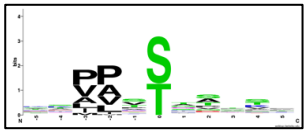
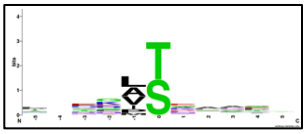
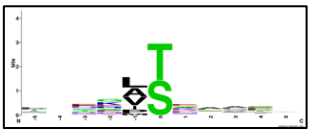
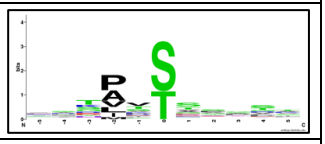
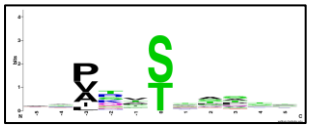


Figure 3.6 Typical ion spectra generated by the fragmentation of an O-GlcNAcylated peptide. The spectrum shows the fragmentation of HCF-1 on the peptide (K)TIPmsAIITQAGATGVTSSPGIK(S). The HexNAc oxonium ion (m/z 204) and its fragments (m/z 186, m/z 168, m/z 144, m/z 138, and m/z 126) are produced at high intensity and are distinctive for O-GlcNAc identification sites.

Table 7. O-GlcNAc sites identified in BeWo extracts

Gene ID	Protein name	Modification sites	Substrate motif
ERP44	Endoplasmic reticulum resident protein 44	**T367, T369**	
TLDC1	TLD domain-containing protein 1	**S425**	
POLDIP3	Polymerase delta-interacting protein 3	**T138**	
UBQLN2	Ubiquilin-2	**S116**	
UBAP2L	Ubiquitin-associated protein 2-like	S445, T446	
UBAP2	Ubiquitin-associated protein 2	**T336**, **T338**, **S368**, **T389**, **T391**, **S421**, S485, T487, **T1010**, **T1009**	
RBM27	RNA-binding protein 27	T539	
ANKRD17	Ankyrin repeat domain-containing protein 17	**S1705**, **S1821**	
POM121	Nuclear envelope pore membrane protein POM 121	**T686**, **T688**	
SRCAP	Helicase SRCAP	**T2229**, **T2235**, **S2239**, **T2406**, **T2412**, **S2416**	
PRRC2C	Protein PRRC2C	**T2244**, **T2242**	
HCFC1	Host cell factor 1	T495, **T498, S518**, T566, S569, T579, T583, T586, T619, S620, T625, T627, T642, T651, T652, **T689**, T698, **T771**, **S775**, T779, T797, T800, T801, T805, S806, T808, T861, **S863**, S1234, T1243, **T1488**, **S1497**	   
KRT1	Keratin, type II cytoskeletal 1	**S502**	
NUP98	Nuclear pore complex protein Nup98-Nup96	**T223**	
SEC24B	Protein transport protein Sec24B	**S315**, S342	
POM121C	Nuclear envelope pore membrane protein POM 121C	**S663**, **T665**	

EMSY	BRCA2-interacting transcriptional repressor EMSY	T283, **S284**	
QSER1	Glutamine and serine-rich protein 1	**S51**, **S52**, **T233**, **S715**	
NUP153	Nuclear pore complex protein Nup153	**T515**, S543, S544, **T548, S564, T630, S764,** T890, S891, S895, S911, S912, S937, S938, S1023, **T1112, S1113, T1114, S1115, S1154,** T1156	
PROSER1	Proline and serine-rich protein 1	**S595**, **S596**, **T597**, **T601**	
NUP214	Nuclear pore complex protein Nup214	**T598**, **S1044**, **S1045**, **T1051**, **T1134**, **S1136**, **T1137**, T1201, S1207, **S1211**, **S1324**, **S1549**, **S1887**	 
WNK1	Serine/threonine-protein kinase WNK1	**T1600**, T1848	

All the O-GlcNAcylation sites showed in this table have been identified in this study using HCD/ETD fragmentation and search engine for the HexNAc ion in the Uniprot_Human database. ***In bold*** *are indicated the potentially novel O-GlcNAc sites which are not reported in literature, while in red are shown the modified sites predicted in this study using the OGTsite tool with the corresponding OGT substrate motifs. The manual search for O-GlcNAcylation sites was performed on https://www.phosphosite.org/ and on literature (Zhao et al., 2011; Lund et al., 2016; Kang et al., 2013; Ahmad et al., 2011; Ha and Lim, 2015; Zeidan et al, 2010; Hiromura et al., 2003; Duan et al., 2018; Liu et al., 2016; Ruan et al., 2013; Gurel et al., 2014).*

3.3.3 Gene Ontology (GO) analysis of O-GlcNAc-enriched proteins identified by MS

In order to assign a putative molecular and biological function to the 210 O-GlcNAc-enriched proteins identified in this study, a GO analysis was performed using the web-accessible program DAVID. By agglomerating information on tens of millions GO annotation terms from a variety of public bioinformatics databases, DAVID provides significantly enriched biological and functional annotation terms and condenses large input lists of genes which share the same annotations into single functional groups (Dennis et al., 2003). To quantify the degree of enrichment for each functional group, DAVID compares the annotations associated to the user's list of genes with the distribution of the annotations among the whole human genome background assigning a p value of annotation enrichment to each group of genes sharing the same annotation term. A p value ≤ 0.05 indicates that a certain group of genes is specifically associated (enriched) with a specific biological or functional category more than a random chance as compared to human background. As graphed in Figure 3.7 A, the GO molecular functions analysis identified "protein binding", "RNA binding", "DNA binding" and "chromatin binding" as the most significantly enriched annotations, suggesting that the O-GlcNAcylated proteins identified in this study might be involved in epigenetic regulation at level of both DNA and RNA. Indeed, the top-ranked categories in terms of percentage of genes for the GO biological functions analysis (Figure 3.7, B), are associated with transcription and regulation of transcription, in agreement with the GO molecular function analysis, followed by terms associated to transport across the nucleus.

A gene functional classification analysis was also performed in DAVID for the 210 O-GlcNAc-enriched proteins. This type of analysis classifies the user's list of genes into functionally related gene groups based on their GO term annotations and has the advantage of ranking the importance of different gene groups by assigning them an enrichment score. A higher score for a certain group indicates that the gene members in that group are involved in more important (enriched) terms in a given study, therefore more attention should go to them (Huang et al., 2009). An enrichment score of 1.3 is equivalent to non-log scale 0.05. Groups with scores ≥ 1.3 are statistically significantly enriched and are shown in Table 8 for this study. Results indicated that 56/210 of the O-GlcNAc-enriched proteins identified in this study were allocated in 5 functional clusters with a significant enrichment score ranging from 15.8 to 3.5.

Interestingly, the top ranking group of genes, in terms of statistical significance, belong to components of nuclear envelope involved in the transport across the nucleus, followed by proteins involved in regulation of transcription, translation and a group of protein kinases (Table 8) suggesting that these groups of genes may play major biological roles in the context of O-GlcNAc signalling in BeWo cells.

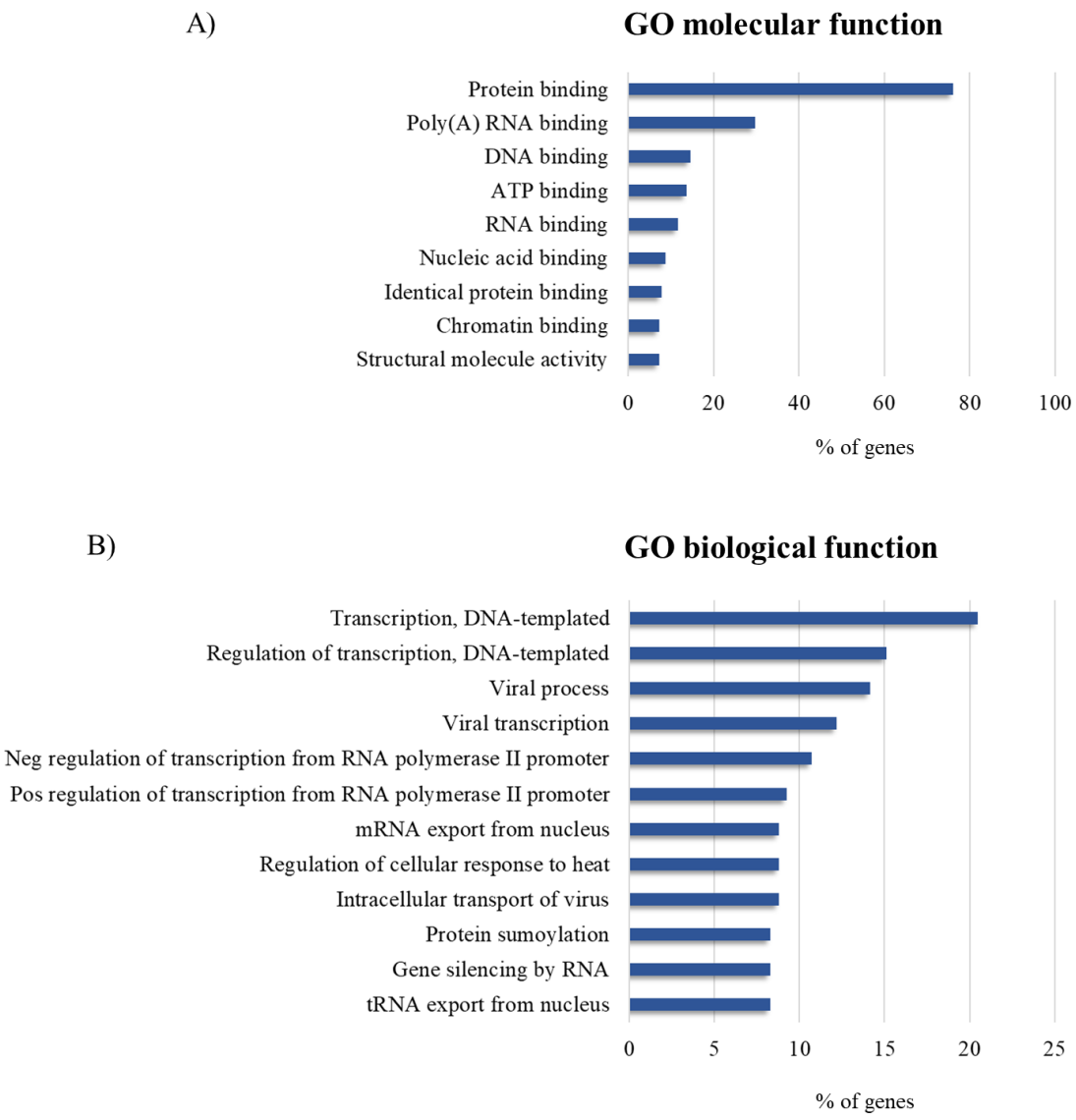


Figure 3.7 Gene Ontology (GO) analysis for the O-GlcNAc-enriched proteins identified by MS in BeWo extracts. Statistically significantly enriched annotation terms are displayed for A) GO molecular functions and B) GO biological functions analysis with the corresponding percentage of genes showed on the x axis.

Table 8. Gene functional classification analysis

Group 1	Enrichment Score: 15.8	Enriched GO terms
NUP50	nucleoporin 50	GO:0016925 protein SUMOylation GO:0031047 gene silencing by RNA GO: 0006406 mRNA export from nucleus GO: 0007077 mitotic nuclear envelope disassembly
NUP58	nucleoporin 58	
NUP214	nucleoporin 214	
POM121	POM121 transmembrane nucleoporin	
RAE1	ribonucleic acid export 1	
RANBP2	RAN binding protein 2	
NUP62	nucleoporin 62	
NUP153	nucleoporin 153	
NUP54	nucleoporin 54	
NUP98	nucleoporin 98	
NUP88	nucleoporin 88	
NUP93	nucleoporin 93	
NUP188	nucleoporin 188	
NUP205	nucleoporin 205	
NUP160	nucleoporin 160	
POM121C	POM121 transmembrane nucleoporin C	
Group 2	**Enrichment Score: 10.9**	
SAP130	Sin3A associated protein 130	GO:0006351 transcription, DNA-templated
EMSY	BRCA2 interacting transcriptional repressor	
SAP30BP	SAP30 binding protein	
CIC	capicua transcriptional repressor	
VPS72	vacuolar protein sorting 72 homolog	
NFRKB	nuclear factor related to kappaB binding protein	
Group 3	**Enrichment Score: 9.9**	
RPL11	ribosomal protein L11	GO:0006364 rRNA processing GO:0003735 structural constituent of ribosome GO:0006412 translation
RPL27	ribosomal protein L27	
RPL3	ribosomal protein L3	
RPSA	ribosomal protein SA	
RPLP0	ribosomal protein lateral stalk subunit P0	
RPS14	ribosomal protein S14	
RPS16	ribosomal protein S16	
RPS10	ribosomal protein S10	
Group 4	**Enrichment Score: 9.4**	
HNRNPA1	heterogeneous nuclear ribonucleoprotein A1	GO:003676 nucleic acid binding
SFPQ	splicing factor proline and glutamine rich	
RBM25	RNA binding motif protein 25	
PSPC1	paraspeckle component 1	
HNRNPA2B1	heterogeneous nuclear ribonucleoprotein A2/B1	
SPEN	spen family transcriptional repressor	
NONO	non-POU domain containing, octamer-binding	

Group 5	Enrichment Score: 3.5	
CDK11B	cyclin dependent kinase 11B	
WNK2	WNK lysine deficient protein kinase 2	
PRKCH	protein kinase C eta	
PRKD2	protein kinase D2	GO:0004674 protein serine/threonine kinase activity
WNK1	WNK lysine deficient protein kinase 1	
MARK2	microtubule affinity regulating kinase 2	
CSNK2A1	casein kinase 2 alpha 1	
CDK12	cyclin dependent kinase 12	
MARK3	microtubule affinity regulating kinase 3	

Enrichment score of 1.3 is equivalent to non-log scale 0.05. Groups with scores ≥1.3 are statistically significantly enriched and here represented. Only clusters having at least 6 members are shown.

3.3.4 Protein-protein interaction network analysis

To further investigate the relationship between the O-GlcNAc-enriched proteins identified in BeWo, a protein-protein interaction network analysis was constructed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING), a freely available database based on known and predicted protein-protein interactions. The results confirmed what observed in the GO analysis, showing that the majority of the O-GlcNAc-enriched proteins identified by MS in this study, are physically and functionally associated and are mainly involved in regulation of transcription (23%) (yellow), translation (10%) (red) and nucleocytoplasmic transport (13%) (green) (Figure 3.8). This analysis has also predicted that OGT is in a direct protein-protein interaction with 21 of the 210 O-GlcNAc-enriched proteins identified here (Figure 3.9) many of which are involved in chromatin modification (blue area, Figure 3.8) and regulation of transcription. Interestingly, 18 of the 21 proteins identified in this analysis were already found to interact with OGT following a manual search as described in paragraph 3.3.1. However, by manual search a total of 106 proteins were identified suggesting that, of the two methods used to predict which proteins are in a physical association with OGT, the manual search seems to represent a more effective method.

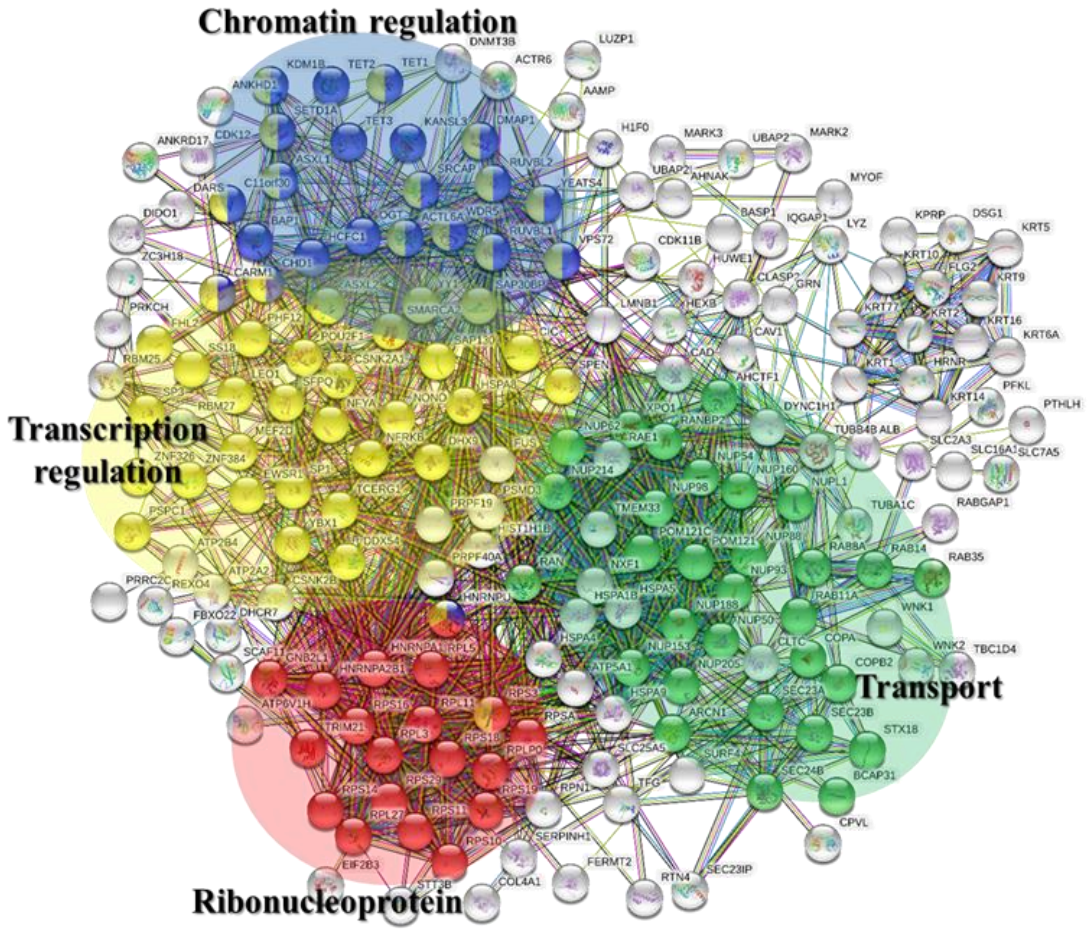
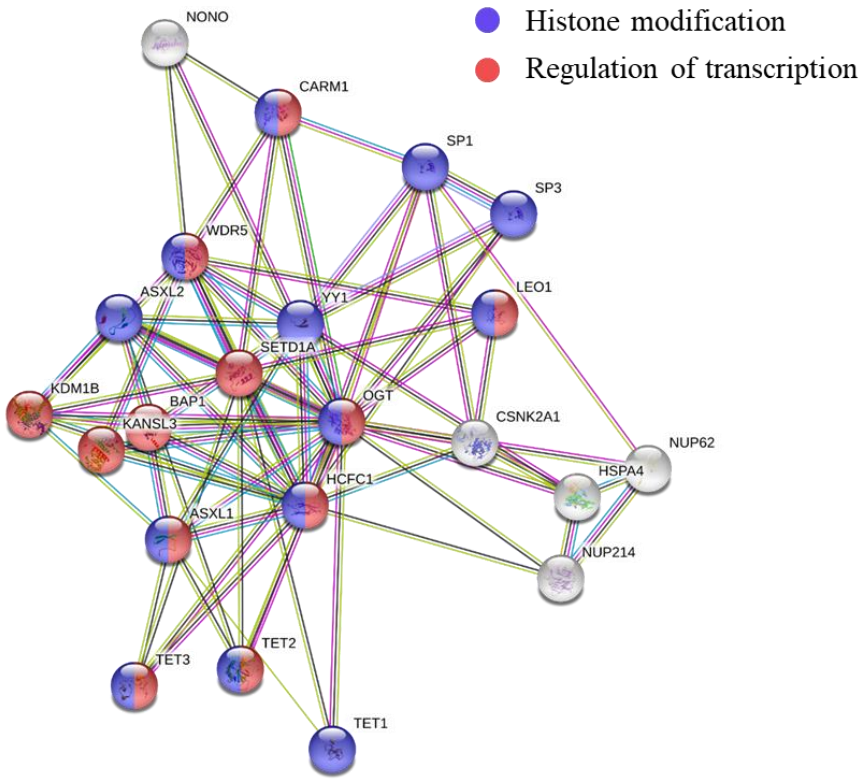


Figure 3.8 Protein-protein association network analysis of the O-GlcNAc-enriched proteins in BeWo using STRING database. The four functional classes identified in this analysis are highlighted with different colours. The lines connecting the spheres represent known or predicted protein-protein interactions according to the STRING protein interaction database.



Gene ID	Full name
OGT	O-linked N-acetylglucosamine (O-GlcNAc) transferase
TET1	Ten-eleven translocation methylcytosine dioxygenase 1
TET3	Ten-eleven translocation methylcytosine dioxygenase 2
TET2	Ten-eleven translocation methylcytosine dioxygenase 3
ASXL1	Putative Polycomb group protein ASXL1
SETD1A	Histone-Lysine N-Methyltransferase SETD1A
KDM1B	Lysine-specific histone demethylase 1B
KANSL3	KAT8 Regulatory NSL Complex Subunit 3
BAP1	BRCA1 Associated Protein 1
LEO1	LEO1 Homolog, Paf1/RNA Polymerase II Complex Component
HCFC1	Host Cell Factor C1
CARM1	Coactivator Associated Arginine Methyltransferase 1
WDR5	WD Repeat Domain 5
SP1	Sp1 Transcription Factor
SP3	Sp3 Transcription Factor
ASXL2	Putative Polycomb group protein ASXL2
YY1	YY1 Transcription Factor
CSNK2A1	Casein Kinase 2 Alpha 1
NONO	Non-POU Domain Containing Octamer Binding
NUP62	Nucleoporin 62
NUP214	Nucleoporin 214
HSPA4	Heat Shock Protein Family A (Hsp70) Member 4

Figure 3.9 Protein-protein interaction network analysis for the OGT enzyme. OGT is among the O-GlcNAc-enriched proteins in BeWo extracts and interacts with 21/210 of the identified proteins as predicted by the STRING model. The majority of these proteins are factors which regulate chromatin structure and transcription.

3.4 Discussion

As described in Chapter 1-Introduction, protein O-GlcNAcylation controls most cellular processes. As a "cellular sensor" it is highly dynamic and changes rapidly in response to many stimuli including stress and metabolic variations (Zachara and Hart, 2004). Abnormal levels of O-GlcNAcylation on nucleocytoplasmic proteins are associated with chronic diseases including cancer, diabetes and neurodegenerative disorders (Yang and Suh, 2014). Thus, identifying new OGT targets and O-GlcNAc modification sites is crucial to evaluate the impact that changes in O-GlcNAc cycling may produce on cellular processes especially in the human placenta where functional adaptations can program the health of future generations (Sferruzzi-Perri and Camm, 2016).

By using an antibody-based enrichment method followed by the HCD/ETD peptide fragmentation, 210 putative O-GlcNAc-modified proteins were significantly enriched by the anti-O-GlcNAc antibody and identified in BeWo cell line, 61 of which are potentially novel. In addition, 114 O-GlcNAc sites on 22 proteins were identified, 69 of which may constitute putative novel sites. A GO and protein-protein association network analysis predicted that the identified proteins are functionally interconnected and regulate common biological processes within the cell including regulation of transcription, mainly via control of chromatin structure, regulation of translation, protein degradation and transport across the nucleus. In the context of trophoblast physiology, these proteins are involved at different levels in both placentation and fetal development (Figure 3.10).

One of the most significantly enriched cluster of O-GlcNAcylated proteins found in this study includes many nucleoporins, components of the nuclear pore complex (NPC), a fundamental barrier between nucleus and cytoplasm which controls virus replication, nuclear envelope disassembly during mitosis, gene expression and SUMOylation (Texari and Stutz, 2015; Flatt and Greber, 2015). Nucleoporins are among the most O-GlcNAcylated proteins and loss of O-GlcNAc modification leads to their increased ubiquitination, proteasomal degradation and downstream malfunction of the nuclear pore which explains why O-GlcNAcylation of the NPC is highly conserved among metazoans (Y. Zhu et al., 2016). The high content in O-GlcNAc moieties on nucleoporins justifies why they are highly enriched by the anti-O-GlcNAc antibody in this study and explains the abundance of modification sites, 26

of which novel, identified by MS on Nup214, Nup153, Nup98, POM121 and POM121C. In human reproduction, NCP marks the implantation window in human endometrium (Guffanti et al., 2008), regulates trophoblast differentiation from cytotrophoblast into syncytiotrophoblast (Mayhew et al., 1999) and is involved in fetal development since several nucleoporins are mutated in some inherited diseases (Schreiber and Kennedy, 2013). Moreover, alterations in protein SUMOylation, a post-translational modification which occurs copiously at the NPC, are associated with preeclampsia, growth restriction and inflammation (Baczyk et al., 2013; Baczyk et al., 2018).

Many proteins, known to regulate gene transcription and chromatin structure, were also significantly O-GlcNAc-enriched and identified in this study. The majority of them are known to be in a physical stable interaction with the OGT enzyme. An example is the transcriptional coregulator host cell factor-1 (HCF-1), encoded by the gene HCFC1 and one of the most heavily O-GlcNAcylated protein. Up to 32 sites were identified here for HCF-1, 7 of which are potentially novel. OGT is required for HCF-1 maturation whereas the latter is required for stabilizing OGT in the nucleus (Daou et al., 2011). In this way, they regulate the activity of protein complexes involved in epigenetic control via chromatin modifications. In the SET1/COMPASS complex, for example, OGT is assisted by the two methylcytosine dioxygenases TET2 and TET3 in sustaining, via O-GlcNAcylation of HCF-1, the tri-methylation of histone H3 Lys4 (H3K4me3), an epigenetic modification involved in early mammalian development (Deplus et al., 2013). Moreover, OGT and HCF-1 are part of the polycomb repressive complex PR-DUB and, together with the lysine-specific histone demethylase 1B (KDM1B), the transcriptional regulators ASXL1 and ASXL2 and the deubiquitinating enzymes BAP1, all significantly enriched by the anti-O-GlcNAc antibody in this study, regulate the ubiquitination status and activity of H2A, one of the five main histones involved in the structure of chromatin in eukaryotic cells (Dey et al., 2012). A mutation that disrupts the catalytic activity of the PR-DUB complex, abolishes H2A deubiquitination *in vitro* and HOX genes repression *in vivo*. The latter are master regulators of embryonic development (Scheuermann et al., 2010; Deschamps and van Nes, 2005), also involved in placentation and trophoblast syncytialization (Murthi et al., 2012; Novakovic et al., 2017). Furthermore, 8 of the 10 components of the SRCAP complex (SRCAP, DMAP1, VPS72, RUVBL1, RUVBL2, ACTL6A, ACTR6, ACTIN, YEATS4 and ZNHIT1) (Feng et al., 2018),

were found among the O-GlcNAc-enriched proteins identified in this study and 6 novel O-GlcNAc sites were identified for SRCAP. This chromatin remodelling complex controls the deposition of the histone variant H2A.Z at promoters of transcription factors, including SP1, during replication (Wong et al., 2007). Both SRCAP and H2A.Z are essential for normal gene expression via recruitment of RNA polymerase II and TATA-binding protein (Adam et al., 2001). In mammal reproduction, H2A.Z is essential for both embryonic development and trophoblast differentiation (Kafer et al., 2015) while SRCAP serves as a coactivator for CREB and the glucocorticoid receptors, both crucial for a fully functional placenta and fetal development (Gupta et al., 2016; Turkay et al., 2012). Taken together these findings suggest a central role played by OGT in regulating the histone code and controlling different multiprotein complexes within the nucleus (Sakabe et al., 2010).

Besides transcription and epigenetic mechanisms, gene regulation may occur also at level of ribosomes as suggested by differences existing between expression of transcriptome and proteome (Schwanhüusser et al., 2011). It has been demonstrated that O-GlcNAcylation of ribosomal proteins influences the role of these cellular components with implications on ribosome performance (Dierschke et al., 2019). However, much more research is required in this field. In the present study many core ribosomal proteins were found to be O-GlcNAc-modified in agreement with literature (Zeidan et al., 2010), suggesting a second mechanism via which protein O-GlcNAcylation might control gene expression in human trophoblast.

Protein O-GlcNAcylation is often in interplay with phosphorylation and ubiquitination (Ruan et al., 2013; Guinez et al., 2008). Here, many proteins involved in ubiquitination were found to be O-GlcNAcylated including UBAP2, UBAP2L, UBQLN2, TRIM21, BAP1, HUWE1, PRPF19 and 9 novel O-GlcNAcylation sites were identified for UBAP2 and UBAP2L. Ubiquitination influences protein localization and activity and marks misfolded and damaged proteins for proteasomal degradation thus maintaining cellular homeostasis. For this reason, suboptimal ubiquitination of proteins is involved in the pathophysiology of a broad range of human diseases including cancer, viral infection, neurodegenerative disorders, diabetes and inflammation (Petroski, 2008). In the placenta of both rodents and humans, alterations in the ubiquitination system are associated with intrauterine growth retardation (Harbers et al., 1996; Chen et al., 2015). Another important cluster of proteins identified here as O-GlcNAc-enriched proteins belong to a group of kinases involved in trophoblast physiology, identifying

the potential of O-GlcNAcylation to modify kinase networks. In particular, the casein kinase 2 (CK2), highly expressed in the syncytiotrophoblast, is known to be regulated on its catalytic subunit by O-GlcNAc on Ser347 (Chen et al., 2015). Inhibition of CK2 decreases proliferation, invasion and syncytialization of trophoblast cells and is associated to preeclampsia in human placenta (Abi Nahed et al., 2020). In addition, WNK1 kinase, highly expressed in the placenta (O'Reilly et al., 2003), is the most represented protein enriched by the anti-O-GlcNAc antibody in this study. Although its role has not yet been well explored, WNK kinases are known for being positive regulators of canonical Wnt/β-catenin signalling (Serysheva et al., 2013) which regulates many aspects of mammalian reproduction including placentation (Tepekoy et al., 2015; Knöfler and Pollheimer, 2013).

In conclusion, the findings presented in this Chapter demonstrated that, the antibody-based enrichment method, followed by HCD/ETD analysis, is a valid approach for the detection and site assignment of O-GlcNAc modification and, in combination with GO analysis, it offers a global perspective for studing the function of a broad range of O-GlcNAcylated proteins involved in the regulation of placenta physiology and diseases. Furthermore, what has strongly emerged from this study is that the OGT enzyme is highly versatile and able to modify different classes of proteins involved in diverse biological processes. More importantly, these data position OGT and O-GlcNAcylation at the "front-stage" in the epigenetic control of human trophoblast and support further research to dissect placental OGT roles as the cellular sensor linking maternal environmental cues to *in utero* fetal programming of human diseases.

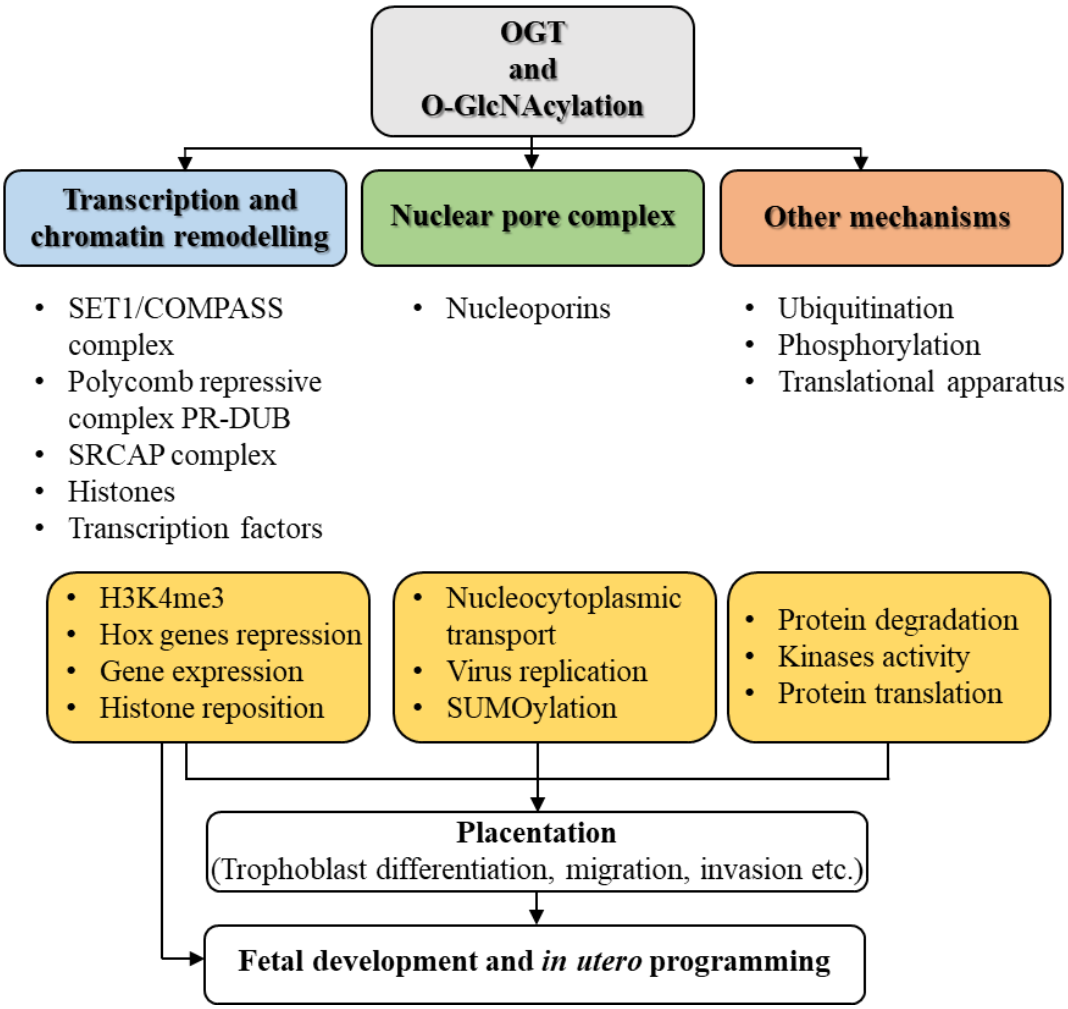


Figure 3.10 Schematic representation of O-GlcNAc-modified proteins in BeWo trophoblast cells and their biological function. The scheme summerizes the main findings emerged in this thesis Chapter and shows the potential mechanisms through which protein O-GlcNAcylation might influence placentation and fetal development.

Chapter 4

Role of OGT and O-GlcNAcylation in the regulation of human trophoblast differentiation

4.1 Introduction

The formation of syncytiotrophoblast is a critical step in placentation, fetal development and pregnancy success. The syncytial layer establishes a feto-maternal interface around the highly vascular placental villi and facilitates the passive exchange of nutrients and gases between the mother and the embryo (Knöfler et al., 2019). Moreover, the syncytiotrophoblast produces important placental hormones throughout pregnancy including hCG which is crucial for placental maturation, pregnancy establishment and maintenance as well as fetal growth (Cole, 2010; Murphy et al., 2006). The *in vivo* fusion process, which gives origin to the syncytiotrophoblast in the human placenta, can be reproduced *in vitro* using the human choriocarcinoma cell line, BeWo. This cell line grows in culture as a monolayer of mononucleated cytotrophoblast cells which can be induced to aggregate and fuse to form a non-proliferative and endocrine active multinucleated syncytium by activating the AC/cAMP cascade. The molecular mechanisms underlying trophoblast differentiation into syncytiotrophoblast are well-established. The intracellular increase of cAMP activates the PKA-CREB axis along with ERK1/2 and p38 MAPKs signalling in a PKA-dependent manner leading to increased expression of markers of differentiation, such as the fusogenic proteins syncytin-1 and syncytin-2, and enhanced secretion of a panel of placental hormones including hCG (Daoud et al., 2005; Delidaki et al., 2011; Wice et al., 1990).

The aim of this thesis Chapter was to investigate to which extent OGT and its biochemical mark O-GlcNAcylation are important in regulating trophoblast differentiation in BeWo cells. To achieve this, the OGT-depleted BeWo cell line was generated using siRNA against OGT and was induced to differentiate using forskolin, an AC activator. Expression levels of the fusogenic proteins syncytin-1 and syncytin-2 were investigated by RT-qPCR whereas immunohistochemistry was used to detect morphological changes and areas of cell fusion. The secretion of β-hCG was detected by chemiluminescence while differences in signalling pathways downstream PKA were detected by immunoblotting using specific antibodies.

4.2 Forskolin-induced differentiation in BeWo cells as a model to study syncytiotrophoblast formation

4.2.1 Morphological and biochemical differentiation in forskolin-treated BeWo cells

In order to set up a suitable *in vitro* model of trophoblast differentiation using BeWo cells, a previously validated protocol was applied in this study (Delidaki et al., 2011). Undifferentiated choriocarcinoma BeWo cells were grown as a monolayer of mononucleated cells to 80% of their confluence before being treated with 100 μM of forskolin or DMSO for 48 h. Forskolin activates AC and enhances the intracellular cAMP levels thus activating PKA and its downstream targets involved in the trophoblast differentiation process. In Figure 4.1 A, the effect of forskolin or DMSO on cellular morphology is captured with immunofluorescence microscope. Nuclei are stained with blue-fluorescent DNA stain DAPI and cellular membranes with anti-E-cadherin antibody. In absence of forskolin, the cytotrophoblast maintains a contiguous monolayer with prominent E-cadherin staining at the borders of each cell (Figure 4.1 A, upper panel). In contrast, after 48 h of forskolin treatment, clustered nuclei appeared along with loss of E-cadherin integrity (Figure 4.1 A, lower panel, yellow arrows). Reduced E-cadherin protein expression in forskolin-treated cells compared to DMSO treatment was further confirmed by immunoblotting analysis of cell lysates ($p<0.01$, Figure 4.1 B). Gene expression of the fusogenic proteins syncytin-1 and syncytin-2 was also significantly increased in forskolin-treated cells compared to DMSO ($p<0.001$, Figure 4.1 C) along with levels of secreted β-hCG as measured in the culture media by chemiluminescence ($p<0.001$, Figure 4.1 D). Phosphorylation at Thr202/Tyr204 and Thr180/Tyr182, the activation sites of ERK1/2 ($p<0.01$) and p38 ($p<0.05$) respectively, was significantly higher after 48 h of forskolin treatment compared to DMSO, whereas phosphorylation of CREB at Ser-133 was downregulated in forskolin-treated cells compared to controls ($p<0.05$) (Figure 4.1, E and F).

It has been shown that in BeWo cells, the maximum activation of CREB, p38 and ERK1/2 is reached 10 min after stimulation with 100 μM forskolin (Delidaki et al., 2011) as also shown in Figure 4.2 where the activation of CREB ($p<0.01$, A), ERK1/2 ($p<0.001$, B) and p38 ($p<0.01$, C) is represented as significantly increased in forskolin-treated cells compared to DMSO. In contrast, 1 h pre-treatment with H89, an inhibitor of PKA, significantly prevented the activation of CREB, ERK1/2 or p38, compared to

vehicle treatment ($p<0.01$ for p38 and CREB; $p<0.001$ for ERK1/2). As expected, prolonged H89 treatment (48 h) reduced by 80% β-hCG secretion forskolin-induced compared to DMSO treatment ($p<0.001$, Figure 4.2 D), confirming that trophoblast differentiation in BeWo is PKA-dependent.

Taken together these results demonstrated that forskolin treatment induces morphological and endocrine differentiation in BeWo cells via PKA-dependent mechanisms and therefore constitutes a suitable *in vitro* model to further study trophoblast differentiation.

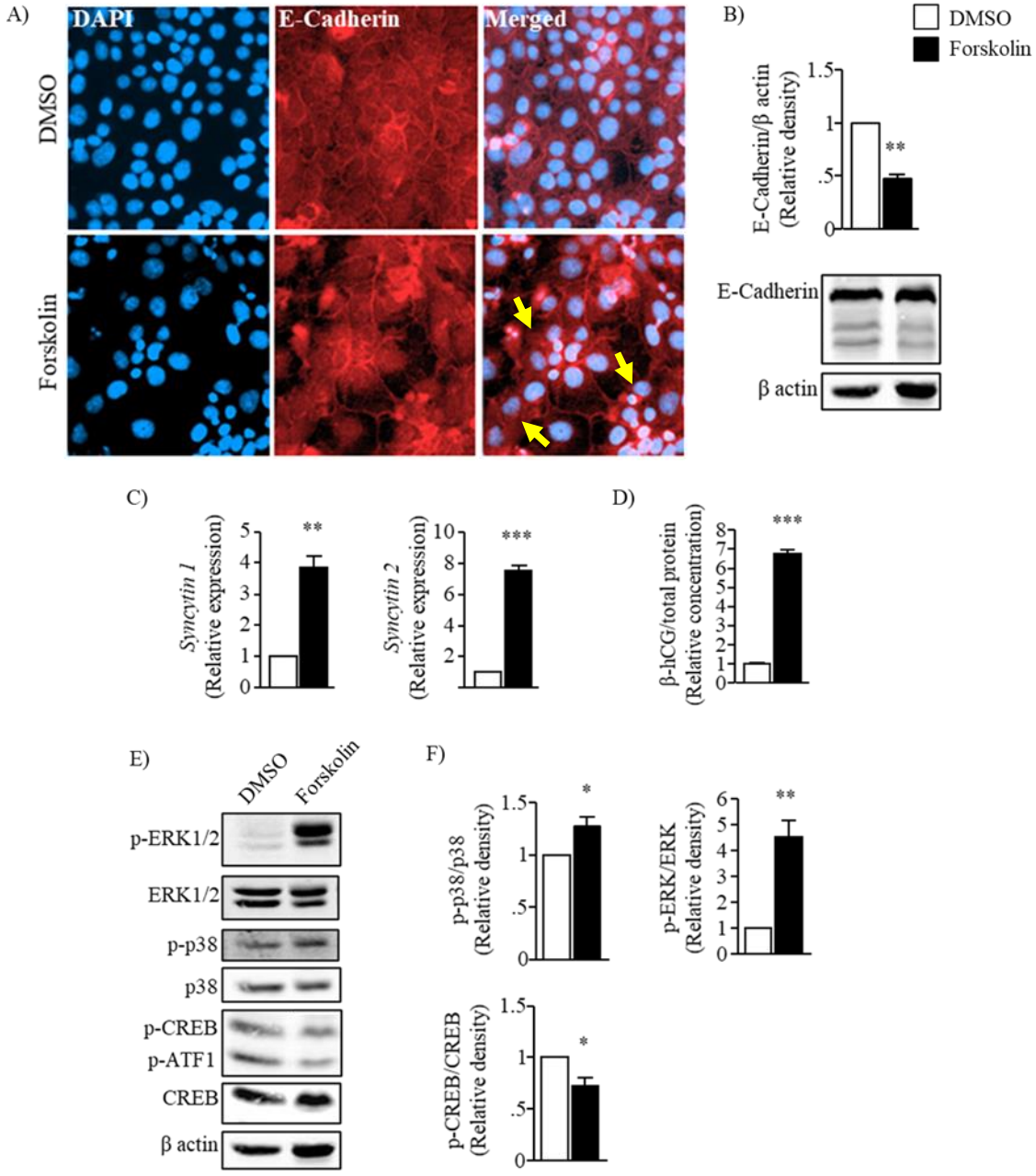


Figure 4.1 Morphological and biochemical profiling of forskolin-induced differentiation in BeWo cells. BeWo cells were grown as a mononucleated monolayer (cytotrophoblast) until approximately 80% of confluence before being treated with 100 µM forskolin for 48 h to differentiate into syncytiotrophoblast or with DMSO as a control. A) Double-label immunofluorescence microscopy of BeWo cytotrophoblast (upper) and syncytiotrophoblast (lower). Cells were fixed and stained with DAPI (blue) or an antibody against the membrane protein E-cadherin (red). Representative immunoblot and densitometry analysis of B) E-cadherin normalized against β actin and E-F) phosphorylated activation sites of p38, ERK1/2 and CREB

normalized against their total protein. C) RT-qPCR analysis of relative amounts of syncytin-1 and syncytin-2 gene expression normalized to GAPDH mRNA levels. D) Relative concentration of secreted β-hCG measured in the media by chemiluminescence and normalized to total protein content. N=3. Data are expressed as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 vs DMSO.

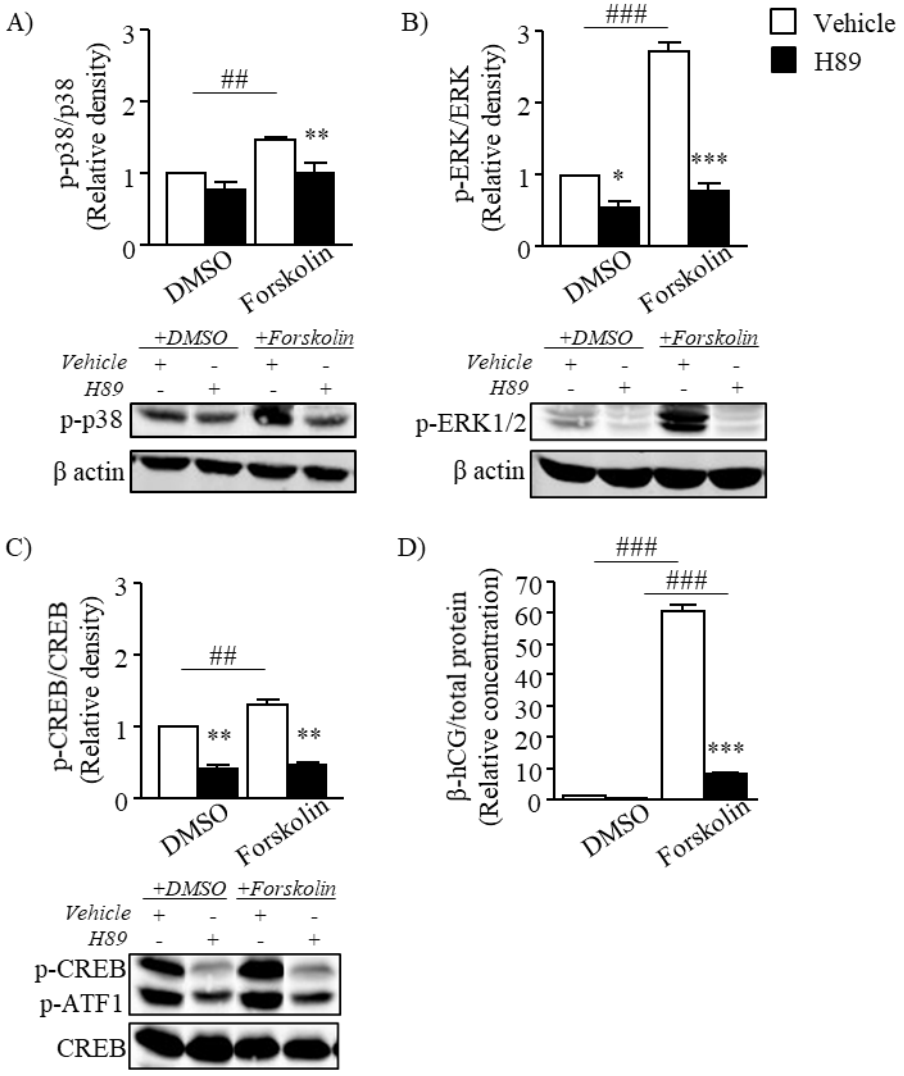


Figure 4.2 Effect of PKA inhibition on forskolin-induced downstream signalling initiation and differentiation in BeWo cells. Representative immunoblots and densitometry analysis of phosphorylation and activation of p38 (A), ERK1/2 (B) and CREB (C) measured by immunoblotting and normalized against total protein forms in BeWo cells pre-treated with 5 μM of H89 or vehicle for 1 h followed by 100 μM of forskolin or DMSO for 10 min. D) Relative concentration of secreted β-hCG normalized to the total protein content and measured by chemiluminescence in the media of BeWo cells pre-treated with 5 μM of H89 or vehicle for 1 h followed by forskolin or DMSO for 48 h. N=3. Data are expressed as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 vs vehicle. ##p<0.01, ###p<0.001 vs DMSO.

4.2.2 The O-GlcNAc system is downregulated during forskolin-induced differentiation in BeWo cells

It has been reported that, global O-GlcNAc modification and OGT levels are downregulated when pluripotent embryonic stem cells (ESCs) are induced to differentiate (Jang et al., 2012; Kim et al., 2009; Miura et al., 2018). Indeed, O-GlcNAcylation of key signalling components works as a biochemical checkpoint that prevents the onset of differentiation and maintains ESCs in their undifferentiation state (Miura et al., 2018). To see whether a similar regulatory mechanism controls BeWo differentiation, levels of OGT, OGA and global O-GlcNAcylation were measured by immunoblotting after 48 h of forskolin or DMSO treatment (Figure 4.3 A). Results showed that, in forskolin-treated cells, the levels of global O-GlcNAcylation ($p<0.01$) and protein expression of OGT ($p<0.01$) were significantly downregulated compared to DMSO treatment (Figure 4.3 B). Surprisingly, also the protein levels of OGA, the enzyme responsible for the removal of O-GlcNAc, were downregulated in forskolin-differentiated cells compared to DMSO ($p<0.001$), suggesting that the reduction in global O-GlcNAcylation during BeWo differentiation is associated to OGT downregulation only rather than to enhanced OGA activity.

These data are in agreement with what observed during ESCs differentiation and suggest that downregulation of OGT and its biochemical mark O-GlcNAcylation might constitute an important step during trophoblast differentiation.

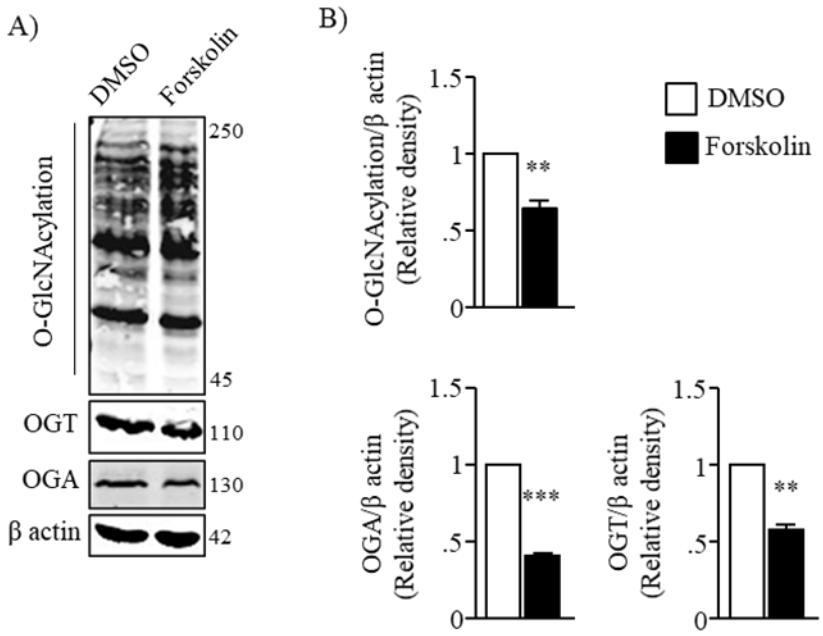


Figure 4.3 Downregulation of the O-GlcNAc system in forskolin-induced differentiation of BeWo cells. Representative immunoblot (A) and densitometry analysis (B) of global O-GlcNAcylation, OGA and OGT protein expression in differentiated BeWo cells treated for 48 h with forskolin and non-differentiated cells treated with DMSO. N=3. Data are expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 vs DMSO.

4.2.3 Knockdown of OGT recapitulates the effects of forskolin inducing spontaneous differentiation in undifferentiated BeWo cells

As demonstrated in paragraph 4.2.2 of this Chapter, the OGT system is downregulated during forskolin-induced differentiation from cytotrophoblast into syncytiotrophoblast in BeWo cells suggesting that reduced O-GlcNAc protein modification might constitute a biochemical mechanism important for the differentiation process. To further investigate this aspect, OGT was knocked down using siRNA in undifferentiated BeWo cells and markers of differentiation were measured afterwards. After 48 h from transfection, global O-GlcNAc levels ($p<0.001$) and OGT protein expression ($p<0.01$) were significantly lower in OGT-depleted cells compared to control siRNA treatment. Interestingly, OGA protein levels were also significantly downregulated in siOGT cells compared to control treatment ($p<0.01$) (Figure 4.4 B) suggesting that the OGA activity is regulated by cellular O-GlcNAcylation levels as already demonstrated in several human cell lines (Zhang et al., 2014) and in paragraph 4.2.2 of this Chapter. In presence of 48 h siOGT treatment, clustered nuclei and derangement of the cell membrane appeared (Figure 4.4 A, lower panel, yellow arrows), in contrast with the well-organized monolayer of mononucleated cytotrophoblast observed in control siRNA treatment (Figure 4.4 A, upper panel). Accordingly, E-cadherins were downregulated in siOGT lysates compared to controls as shown by immunoblotting ($p<0.01$) (Figure 4.4 C). Moreover, gene expression of the fusogenic proteins syncytin-1 and syncytin-2 ($p<0.01$, Figure 4.4 D) and β-hCG secretion ($p<0.01$, Figure 4.4 F) were significantly up-regulated in OGT-depleted cells compared to siRNA controls. However, there was no difference in the activation of signalling pathways downstream of PKA between the two treatments (Figure 4.4 E) suggesting that the expression of markers of differentiation observed in the OGT-depleted cytotrophoblast cells could be PKA-independent.

Taken together these observations demonstrated that OGT depletion in undifferentiated BeWo cells induced spontaneous differentiation suggesting that O-GlcNAcylation might act as a suppressor of differentiation in human trophoblast.

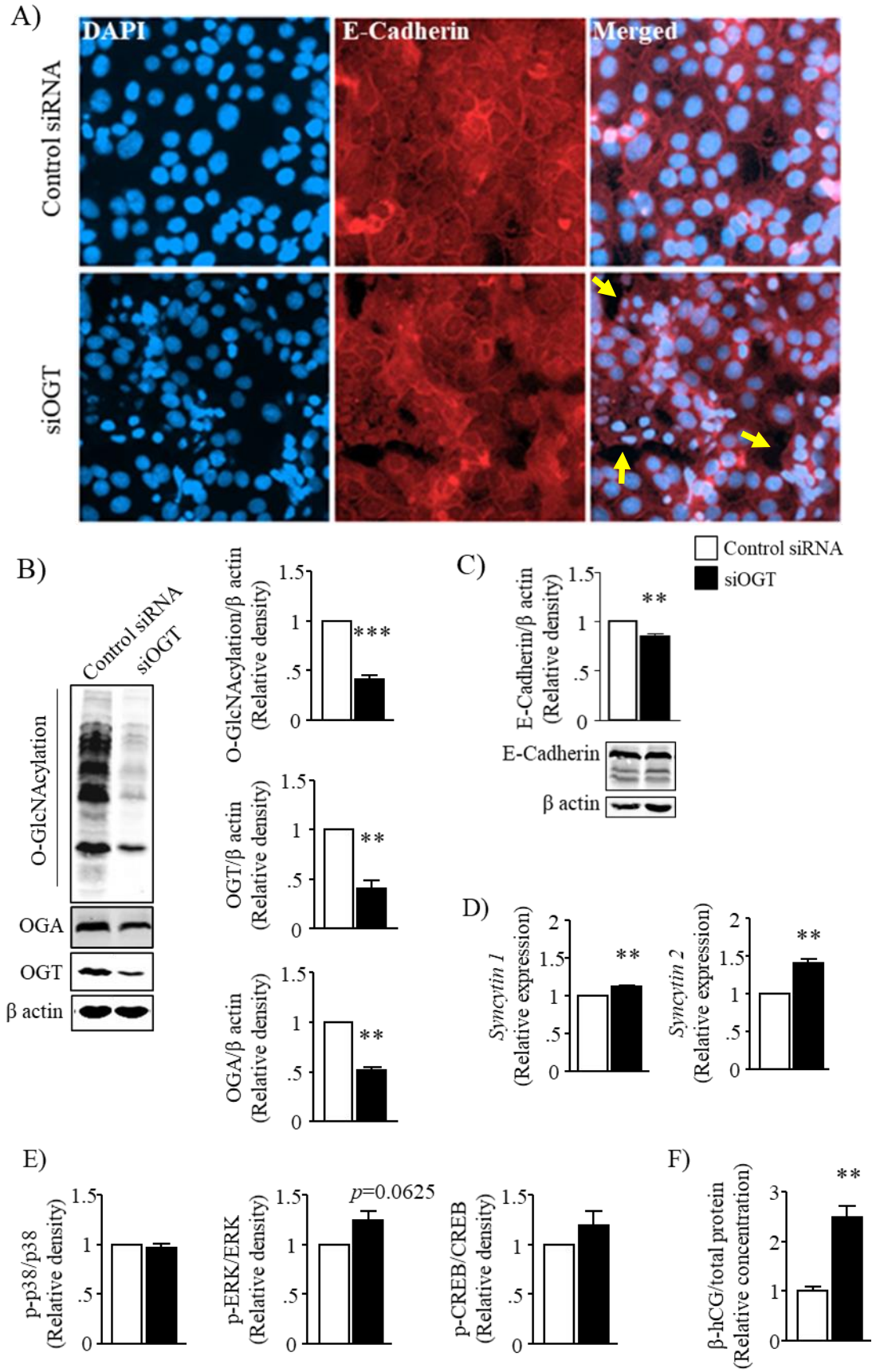

A)
DAPI
E-Cadherin
Merged
Control siRNA
siOGT
B)
Control siRNA
siOGT
O-GlcNAcylation
OGA
OGT
β actin
O-GlcNAcylation/β actin (Relative density)
OGT/β actin (Relative density)
OGA/β actin (Relative density)
***
**
**
C)
Control siRNA
siOGT
E-Cadherin/β actin (Relative density)
E-Cadherin
β actin
**
D)
Syncytin 1 (Relative expression)
Syncytin 2 (Relative expression)
**
**
E)
p-p38/p38 (Relative density)
p-ERK/ERK (Relative density)
p=0.0625
p-CREB/CREB (Relative density)
F)
β-hCG/total protein (Relative concentration)
**

Figure 4.4 Markers of differentiation after 48 h knockdown of OGT in undifferentiated BeWo cells. A) Double-label immunofluorescence microscopy of BeWo cytotrophoblast treated with control siRNA (upper panel) and siOGT (lower panel) for 48 h, fixed and stained with DAPI (blue) or anti-E-cadherin antibody (red). Representative immunoblot and densitometry analysis of B) O-GlcNAcylation, OGT and OGA protein and C) E-cadherin levels in BeWo extracts from control siRNA or siOGT treatment. Protein levels are normalized against β actin. D) RT-qPCR analysis of relative amounts of syncytin-1 and syncytin-2 gene expression normalized to GAPDH mRNA levels. E) Densitometry analysis of phosphorylated forms of p38, ERK1/2 and CREB normalized against their total protein and F) relative concentration of secreted β-hCG measured in the culture media and normalized to the total protein content. N=3. Data are expressed as mean ± SEM; **p<0.01, ***p<0.001 vs control siRNA.

4.3 Role of OGT and O-GlcNAcylation in forskolin-induced differentiation in BeWo cells

4.3.1 O-GlcNAc up-regulation reduces differentiation in forskolin-treated BeWo cells

As shown in paragraph 4.2.2, OGT and protein O-GlcNAcylation were downregulated during forskolin-induced differentiation in BeWo cells. In order to examine whether the attenuation of O-GlcNAc signalling is important in syncytiotrophoblast formation, BeWo cells were differentiated in presence of 100 μM PUGNAc, an OGA inhibitor, or vehicle. PUGNAc treatment significantly increased the global O-GlcNAc modification levels compared to vehicle ($p<0.05$) without modifying OGT protein levels confirming that inhibition of OGA promotes the accumulation of O-GlcNAc modification on nucleocytoplasmic proteins. On the contrary, both OGA gene and protein expression ($p<0.01$) were significantly increased by PUGNAc treatment compared to vehicle (Figure 4.5 A and B) probably in response to the inhibitory effects of PUGNAc on OGA activity as already observed in studies on pancreatic β cells (Filhoulaud et al., 2019). In parallel, PUGNAc significantly reduced ERK1/2 activation in forskolin-treated cells ($p<0.01$) while there was only a tendency toward decrease for the activation of p38 ($p=0.056$) (Figure 4.5 C). Instead, β-hCG secretion levels were strongly suppressed by PUGNAc by almost 50% compared to vehicle ($p<0.01$) (Figure 4.5 D). Similarly, the gene expression of both syncytins along with GCM1 and DUSP23, two key factors in promoting placental cell fusion via PKA/cAMP-mediated mechanisms (Lin et al., 2011), was significantly reduced by PUGNAc treatment compared to vehicle ($p<0.01$) (Figure 4.5 E).

All together, these results demonstrated that the attenuation of O-GlcNAc signalling is critical for forskolin-induced differentiation in BeWo cells and that inhibition of OGA using PUGNAc shifts the balance of the OGT/OGA interplay towards increased OGT activity and protein O-GlcNAcylation impairing downstream trophoblast differentiation PKA-mediated.

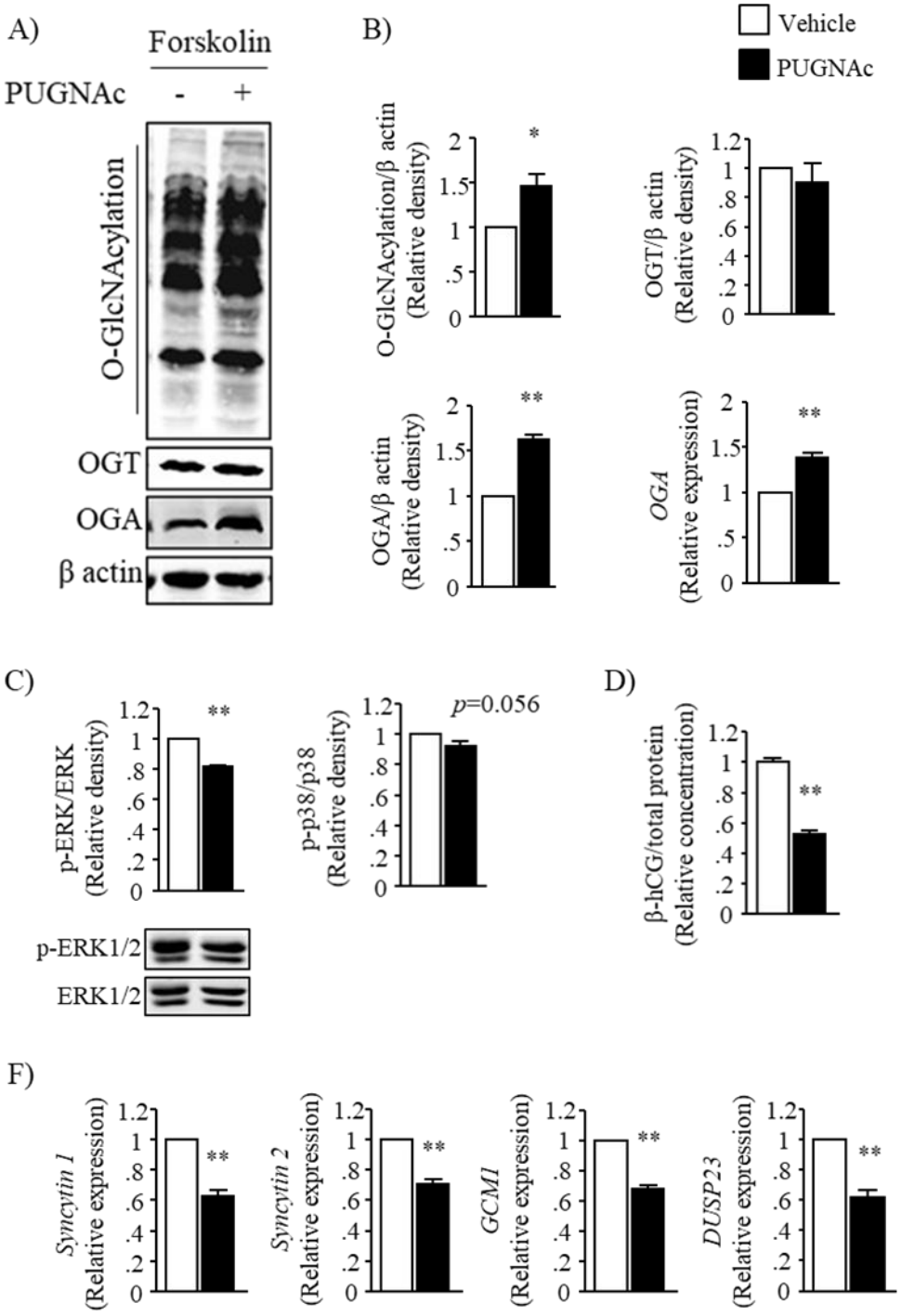


Figure 4.5 Markers of differentiation in forskolin-treated BeWo cells underwent pharmacological up-regulation of O-GlcNAcylation. Cells were induced to differentiate with 100 μM of forskolin for 48 h in presence of 100 μM of the OGA inhibitor PUGNAc or vehicle. A) Representative immunoblot and B) densitometry analysis of global levels of O-GlcNAcylation and OGT and OGA protein levels as well as gene expression of OGA in PUGNAc- or vehicle-treated cells. C)

Densitometry analysis of phosphorylated forms of ERK1/2 and p38 normalized against their total protein as measured by immunoblotting in protein lysates and D) relative concentration of secreted β-hCG normalized to total protein content and measured in the culture media. E) RT-qPCR analysis of relative amounts of syncytin-1, syncytin-2, GCM1 and DUSP23 gene expression normalized to GAPDH mRNA levels. N=3. Data are expressed as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 vs vehicle.

4.3.2 Knockdown of OGT enhances forskolin-induced differentiation in BeWo cells

As demonstrated previously in this Chapter, O-GlcNAc signalling is attenuated during forskolin-induced differentiation in trophoblast BeWo cells. Therefore, the differentiation response to forskolin was investigated in OGT-depleted BeWo cells and results are reported in this paragraph. BeWo cells were treated with control siRNA or siOGT and induced to differentiate 48 h later using 100 μM forskolin. After 48 h of stimulation with forskolin, extensive areas of syncytialization were observed in OGT-depleted cells compared to control siRNA (Figure 4.6 A, bottom panel, yellow arrows). Interestingly, these areas are characterized by small nuclei. A regression in the nuclear size has already been described in the syncytialized areas of human term placentas and represents the morphological feature of syncytiotrophoblast turnover characterized by shrinkage of the nuclei, nucleolar regression and chromatin condensation typical of cells in late apoptosis (Mayhew et al., 1999, 2014). In parallel to morphological changes, secreted levels of β-hCG were significantly increased in OGT-depleted cells compared to control siRNA in response to forskolin ($p<0.001$, Figure 4.6 C). As for the signalling pathways, the forskolin-induced activation of both p38 ($p<0.001$) and ERK1/2 ($p<0.01$) was enhanced in siOGT treatment compared to control siRNA (Figure 4.6 B).

Together, these data demonstrate that the effect of forskolin in inducing differentiation in BeWo cells is potentiated in presence of reduced levels of OGT suggesting that O-GlcNAc signalling may exert a regulatory role on the PKA-mediated molecular mechanisms underlying trophoblast differentiation.

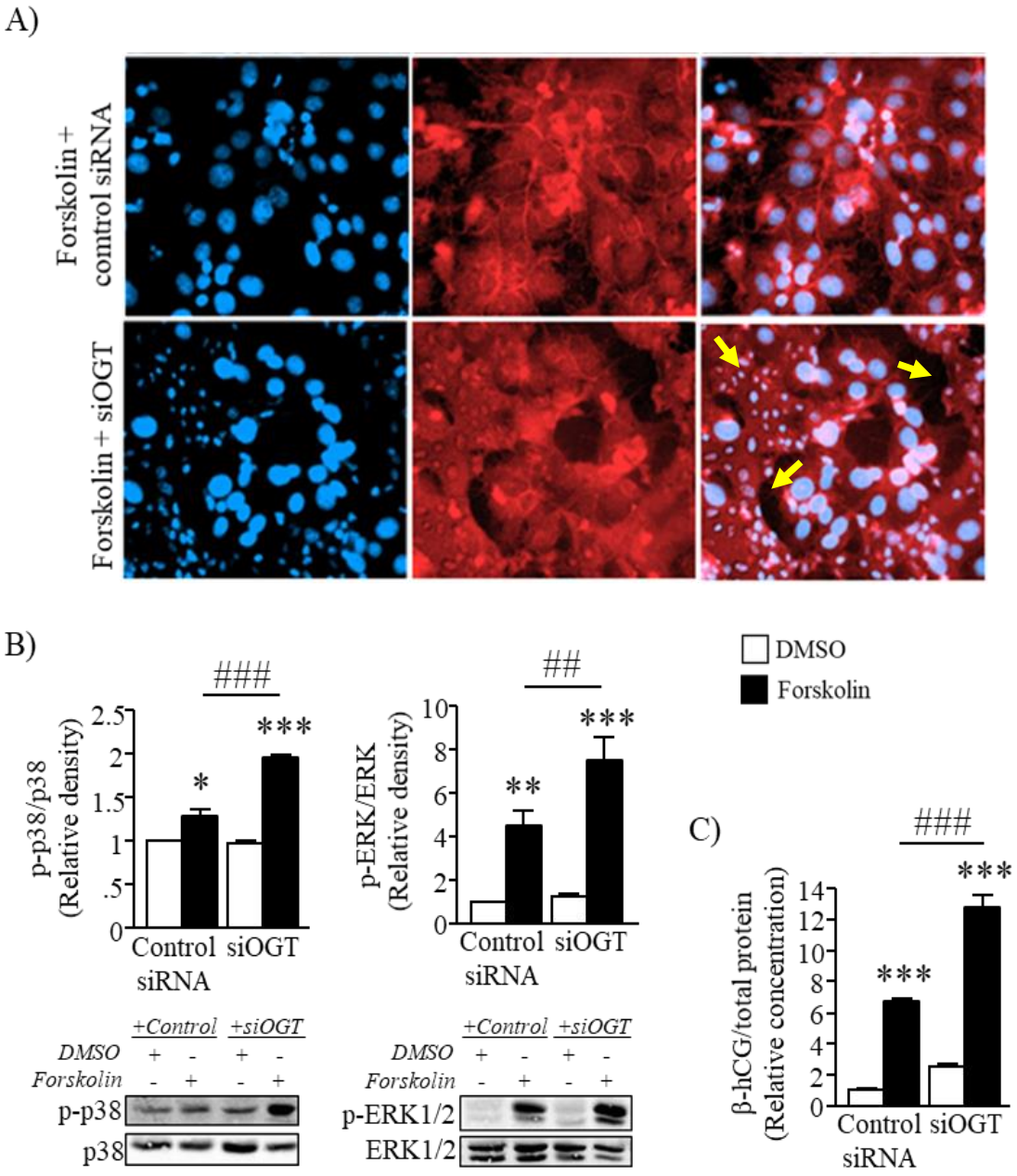


Figure 4.6 Morphological and endocrine forskolin-induced differentiation in OGT-depleted BeWo cells. BeWo cells were treated with control siRNA or siOGT for 48 h and induced to differentiate with 100 μM of forskolin for 48 h or treated with DMSO. A) Double-label immunofluorescence microscopy showing DAPI (blue) or anti-E-cadherin (red) immunostaining of differentiated BeWo cells treated with siOGT or control siRNA. B) Representative immunoblot and densitometry analysis of phosphorylated sites of activation for ERK1/2 and p38 normalized against the total protein form in protein lysates and C) relative concentration of secreted β-hCG

measured in the culture media of BeWo cells pre-treatment with control siRNA or siOGT for 48 h and induced to differentiate with forskolin for 48 h or treated with DMSO. β-hCG levels are normalized to the total protein content. N=3. Data are expressed as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 vs DMSO. ## p<0.01, ###p<0.001 vs control siRNA.

4.3.3 Restoration of O-GlcNAcylation prevents up-regulation of forskolin-induced differentiation observed in OGT-depleted BeWo cells

To test whether restoration of O-GlcNAcylation prevents the enhancement of forskolin-induced differentiation observed when OGT is silenced, BeWo cells were treated with siOGT or control siRNA in presence of PUGNAc, to inhibit OGA activity and enhance O-GlcNAcylation, or vehicle as control for 24 h before being induced to differentiate with forskolin for 48 h. PUGNAc treatment partially restored the pool of O-GlcNAcylated proteins in siOGT cells compared to vehicle ($p<0.05$) with no impact on OGT protein levels (Figure 4.7 B) suggesting that a residual O-GlcNAc activity was still present in the cells after OGT silencing. The large areas of syncytialization observed in OGT-depleted cells (Figure 4.7 A, middle panel) are no longer detected in cells treated with PUGNAc (bottom panel) which morphologically resemble more to control cells (upper panel). Accordingly, the up-regulation of β-hCG levels and gene expression of both syncytins observed in siOGT cells treated with vehicle were significantly downregulated in PUGNAc treatment ($p<0.001$) (Figure 4.7 C and D). Moreover, the activation status of ERK1/2 ($p<0.01$) and p38 ($p<0.05$) observed in OGT-depleted cells treated with vehicle was returned to baseline levels by PUGNAc treatment, whereas no significant effects were exerted by PUGNAc on CREB activation (Figure 4.7 E).

Taken together, these results confirm that downregulation of O-GlcNAc signalling facilitates forskolin-induced differentiation in BeWo cells and further corroborate the possibility of a repressive role played by O-GlcNAc signalling on mechanisms regulating human trophoblast differentiation.

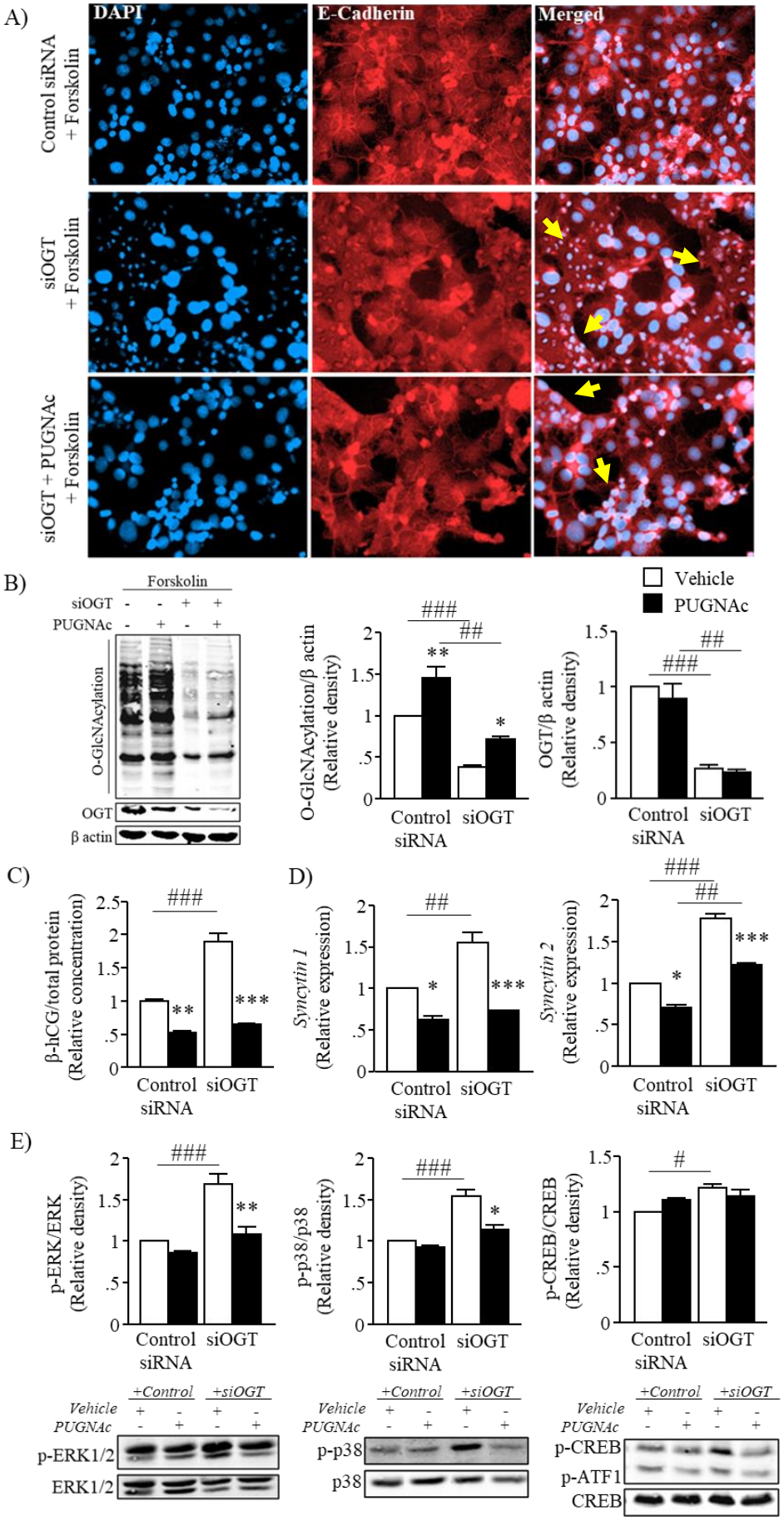

A)
DAPI
E-Cadherin
Merged
Control siRNA + Forskolin
siOGT + Forskolin
siOGT + PUGNAc + Forskolin
B)
Forskolin
siOGT
PUGNAc
O-GlcNAcylation
OGT
β actin
Vehicle
PUGNAc
O-GlcNAcylation/β actin (Relative density)
OGT/β actin (Relative density)
Control siRNA
siOGT
C)
β-hCG/total protein (Relative concentration)
D)
Syncytin 1 (Relative expression)
Syncytin 2 (Relative expression)
E)
p-ERK/ERK (Relative density)
p-p38/p38 (Relative density)
p-CREB/CREB (Relative density)
+Control
+siOGT
Vehicle
PUGNAc
p-ERK1/2
ERK1/2
p-p38
p38
p-CREB
p-ATF1
CREB

Figure 4.7 Effects of O-GlcNAc restoration on morphological and biochemical forskolin-induced differentiation in OGT-depleted BeWo cells. Cells were treated with siOGT or control siRNA for 48 h in presence of PUGNAc or vehicle and induced to differentiate for 48 h with 100 μM forskolin. A) Double-label immunofluorescence microscopy. Cells were fixed and stained with DAPI (blue) or anti-E-cadherin antibody (red) after treatments. Representative immunoblot and densitometry analysis of B) O-GlcNAcylation and OGT protein normalized against β-actin and E) phosphorylation of the activation sites for ERK1/2, p38 and CREB normalized against the corresponding total protein measured in the cell lysates. C) Relative concentration of secreted β-hCG measured in the culture media and normalized to the total protein content. D) RT-qPCR analysis of relative amount of syncytin-1 and syncytin-2 gene expression normalized to GAPDH mRNA levels. N=3. Data are expressed as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 vs vehicle. #p<0.05, ##p<0.01, ###p<0.001 vs control siRNA.

4.4 Cross-talk between PKA and O-GlcNAc signalling during BeWo differentiation

4.4.1 PKA partially mediates the up-regulation of forskolin-induced differentiation observed in OGT-depleted BeWo cells

In paragraph 4.3.2 of this Chapter, it was demonstrated that the effect of forskolin on BeWo differentiation was significantly potentiated when O-GlcNAc was depleted by siRNA against OGT. On the contrary, up-regulation of global O-GlcNAcylation by PUGNAc reduced forskolin-induced differentiation suggesting that O-GlcNAcylation might exert an inhibitory effect on signalling pathways controlling trophoblast differentiation. It is well-established that cAMP/PKA signalling is the major route to trigger trophoblast differentiation. Moreover, it has been reported that, in neurons, OGT and PKA are functionally associated (Griffith and Schmitz, 1999). However, it is not known whether a similar interaction occurs also in trophoblast cells. Using co-immunoprecipitation, here it is shown that PKA and OGT physically interact in BeWo cells (Figure 4.8 A). Furthermore, pre-treating siOGT BeWo cells with the PKA inhibitor H89 before inducing differentiation with forskolin partially prevented the up-regulation of gene expression of both syncytins ($p<0.01$) and GCM1 ($p<0.05$) observed in OGT-depleted cells treated with vehicle only (Figure 4.8 B) suggesting that the increased expression of genes promoting differentiation observed in OGT-depleted cells occurs via PKA potentiation rather than an unrelated pathway. Similarly, an almost complete suppression in the forskolin-induced secretion of β-hCG was observed in OGT-depleted cells treated with H89 compared to vehicle ($p<0.001$) (Figure 4.8 C) suggesting that the up-regulation of β-hCG production observed in OGT-depleted cells is also controlled by PKA-mediated mechanisms.

Taken together, these findings suggest that PKA and OGT are physically associated and that PKA mediates in part the abnormal differentiation induced by forskolin when OGT is silenced in BeWo cells.

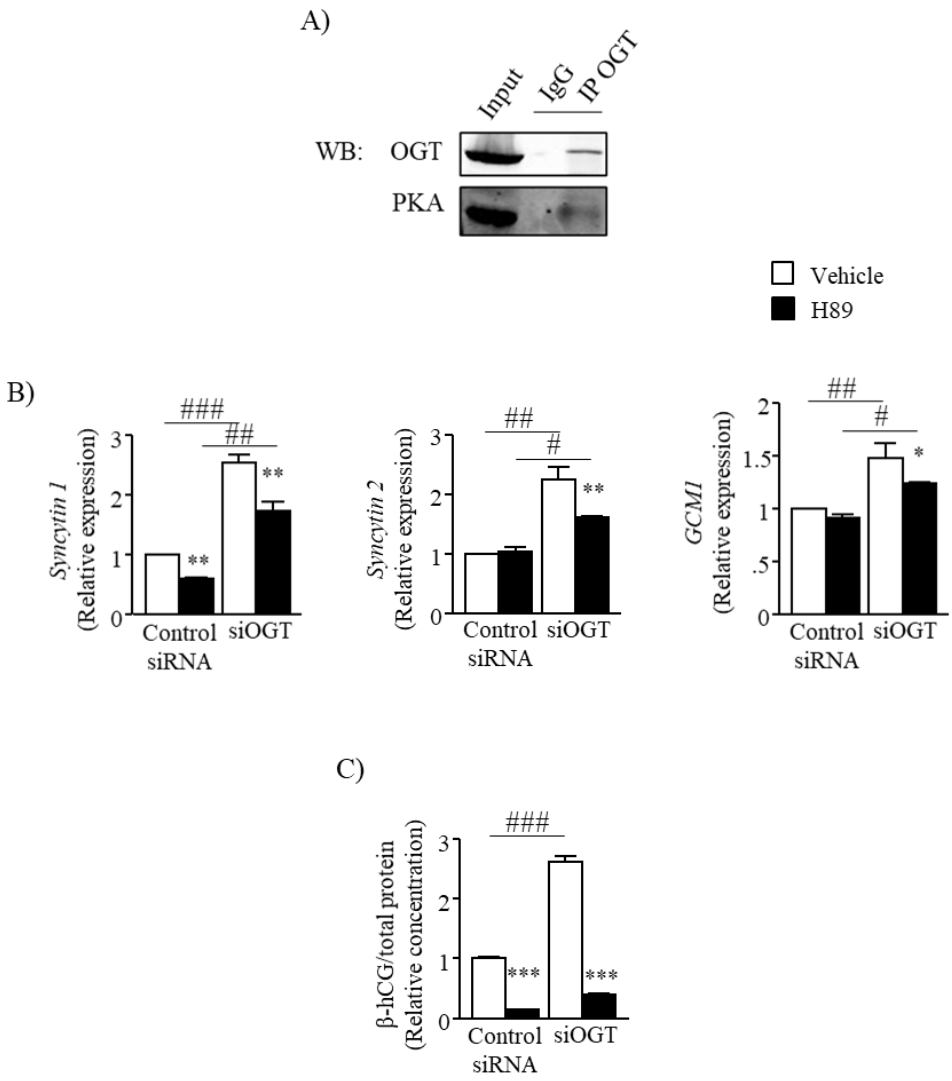


Figure 4.8 Inhibition of PKA in differentiated OGT-depleted BeWo cells. A) Association of OGT and PKA shown by co-immunoprecipitation assay. BeWo lysates were immunoprecipitated with IgG or anti-OGT antibody and blots were probed with antibodies against OGT and PKA. B) RT-qPCR analysis of relative amounts of syncytin-1, syncytin-2 and GCM1 gene expression normalized to GAPDH mRNA levels and C) relative concentration of secreted β-hCG normalized to total protein content. For the data shown in B) and C) BeWo cells were treated with siOGT or control siRNA for 48 h and induced to differentiate with 100 μM forskolin for 48 h in presence of 1 h pre-treatment with H89 or vehicle. N=3. Data are expressed as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 vs vehicle. #p<0.05, ##p<0.01, ###p<0.001 vs control siRNA.

4.4.2 Depletion of OGT alters the activation profile of targets downstream of PKA in BeWo cells

As shown in paragraph 4.2.1 of this Chapter, ERK1/2, p38 and CREB are activated downstream of PKA within 10 min of forskolin treatment and selective inhibition of PKA with H89 prevented their activation and downstream endocrine differentiation of BeWo cells. To test whether the knockdown of OGT changes the activation dynamics of signalling downstream of PKA, siOGT and control siRNA BeWo cells were stimulated for 30 min with 100 μM of forskolin and phosphorylation of ERK1/2, p38 and CREB on their activation sites was measured at 0, 10, 20 and 30 min after forskolin treatment by immunoblotting (Figure 4.9 B-D-F). The 30-min stimulation was selected in order to obtain a more complete activation profile of targets downstream of PKA after reaching their maximal activation within 10 min of forskolin treatment. In control siRNA cells, forskolin significantly increased CREB activation 10 min after treatment remaining sustained at 20 and 30 min as compared to baseline ($p<0.01$). In contrast, in OGT-depleted cells, the basal CREB activation was increased and forskolin had no additional effect compared to control siRNA curve (Two-way ANOVA: interaction Time x siRNA treatment: $p<0.01$, Figure 4.9 A-B). As for ERK1/2, the enzyme activation was increased of approximately 6-fold 10 min after forskolin in both siOGT and control cells and decreased steadily over the next 20 min remaining still higher 30 min after stimulation compared to baseline in both siRNA treatments. However, at baseline ($p<0.01$) and 30 min after forskolin treatment ($p<0.05$), the activation of ERK1/2 was significantly higher in siOGT cells compared to control siRNA (Figure 4.9 C-D). Finally, in control siRNA, forskolin significantly upregulated p38 activation 10 min after treatment which remained sustained at 20 and 30 min compared to baseline ($p<0.01$). In siOGT cells, however, p38 activation was significantly upregulated compared to control siRNA at baseline ($p<0.001$) as well as 10 and 20 min after forskolin treatment ($p<0.01$) (Figure 4.9 E-F).

These data suggest that OGT may alter the dynamics of PKA mechanisms affecting the initiation of signalling involved in trophoblast differentiation.

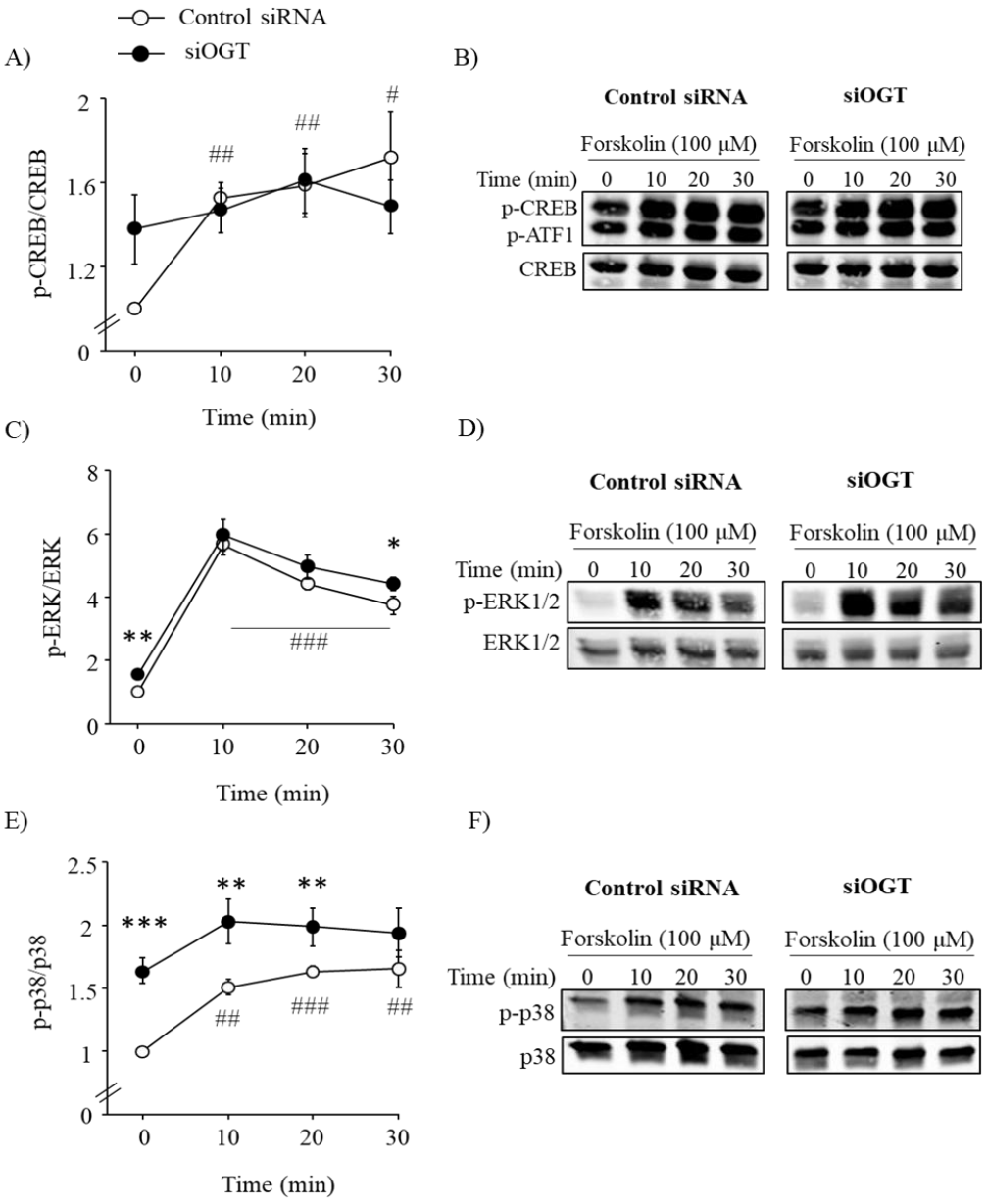


Figure 4.9 Activation kinetics of targets downstream of PKA in OGT-silenced or control siRNA-treated BeWo cells. Undifferentiated BeWo cells were treated for 48 h with siOGT or control siRNA before being stimulated with 100 µM forskolin for 30 min. Activation of CREB (A-B), ERK1/2 (C-D) and p38 (E-F) was measured by immunoblotting at 0, 10, 20 and 30 min after forskolin treatment. N=3. Data are expressed as mean ± SEM. A two-way ANOVA with Time and siRNA treatment as independent variables was used followed by Tukey's post-hoc test. *p<0.05, **p<0.01, ***p<0.001 vs control siRNA. #p<0.05, ##p<0.01, ###p<0.001 vs time 0 (baseline).

4.6 Discussion

In human placenta, the syncytiotrophoblast, which takes origin from the fusion of cytotrophoblast cells forming the inner layer of trophoblast, supplies the fetus with nutrients, gases and protection and constitutes the primary source of pregnancy hormones. Therefore, the integrity and functionality of the syncytiotrophoblast layer is vital for pregnancy success. Defective syncytialization of cytotrophoblast cells during placentation may result in the occurrence of clinical pregnancy complications such as preeclampsia, *in utero* fetal growth restriction and Down's syndrome (Massin et al., 2001; Roland et al., 2016; Ruebner et al., 2010). For these reasons, a deeper understanding of signalling pathways regulating trophoblast differentiation in human placenta is crucial to target molecular mechanisms that might trigger placental dysfunctions.

The formation of syncytiotrophoblast is under a precise control and coordinated activity of multiple factors (Gauster and Huppertz, 2008). Some of these mechanisms have been well-characterized in both cell lines and primary cells. In BeWo as well as in primary trophoblast cells, the interplay between cAMP-PKA and downstream MAPK pathways regulates hCG secretion and expression of fusogenic genes promoting cytotrophoblast differentiation into syncytiotrophoblast (Delidaki et al., 2011; Daoud et al., 2005). In this Chapter, the role of the interplay of OGT and OGA in regulating syncytiotrophoblast formation was investigated in trophoblast BeWo cells by enhancing or depleting protein O-GlcNAcylation using the OGA inhibitor PUGNAc or a specific siRNA against OGT respectively. Results showed that OGT is a negative regulator of differentiation in BeWo cells. Accordingly, O-GlcNAc signalling was downregulated during differentiation while pharmacological increase of O-GlcNAcylation by OGA inhibition reduced BeWo differentiation as evidenced by suppression of syncytiotrophoblast markers such as β-hCG, GCM1 and syncytins. On the contrary, downregulation of OGT by gene silencing reduced protein O-GlcNAcylation and OGA expression promoting spontaneous differentiation in cytotrophoblast BeWo cells and potentiating the effects of forskolin (Figure 4.10).

As demonstrated here, OGT is physically associated to the catalytic subunit of PKA and depletion of OGT enhanced PKA response to forskolin leading to increased β-hCG secretion, syncytin-1 and syncytin-2 gene expression and increased areas of syncytialization. These effects were prevented by specific inhibition of PKA

suggesting that OGT may control trophoblast differentiation via PKA-mediated mechanisms. Although this study has generated novel evidence that depletion of OGT alters PKA-induced signalling dynamics, it did not clarify the exact mechanism via which OGT controls PKA activity. It is well-established that O-GlcNAc modification competes with phosphorylation for the occupancy of Ser/Thr residues. It is also known that, phosphorylation of Thr197 on the catalytic subunits of PKA by pyruvate dehydrogenase kinase 1 (PDK1) is essential for its activation (Cheng et al., 1998). Although more evidence is needed, it is attractive to postulate that O-GlcNAcylation of Thr197 on PKA might constitute a possible mechanism via which OGT regulates PKA activation during differentiation of BeWo cells.

The forskolin-mediated activation of PKA-CREB-syncytin-1 axis is one of the most well-characterized pathways involved in the fusion of trophoblast BeWo cells (Malhotra et al., 2015). In neurons, O-GlcNAcylation of PKA promotes its translocation to the nucleus and enhances CREB activation (Xie et al., 2016). In the present study, however, O-GlcNAcylation may exert a negative effect on the PKA-CREB axis as in OGT-depleted BeWo cells CREB activation is sustained even in absence of the PKA activator, forskolin. Up-regulation of PKA-CREB signalling may then lead to increased expression of downstream targets including β-hCG and syncytins thus enhancing differentiation as observed in siOGT cells compared to controls. The discrepancy in this study and the one mentioned above is likely to be due to functional differences existing between trophoblast and neuronal cells.

ERK1/2 and p38 kinases are also activated downstream of cAMP/PKA and both play a critical role in regulating trophoblast differentiation as demonstrated in BeWo cells and cytotrophoblast explants (Daoud et al., 2005; Delidaki et al., 2011; Shu et al., 2014). In BeWo cells, both kinases are involved in hCG release and syncytin-2 expression via independent mechanisms. In addition, p38 controls gene expression of syncytin-1 via GCM1 (Delidaki et al., 2011). As shown here, the activation of both p38 and ERK1/2 is maximal after 10 min from forskolin treatment and it is significantly upregulated also after 48 h from the treatment suggesting a mechanism of long-term activation induced by forskolin. Interestingly, the activation kinetics of both ERK1/2 and p38 changes in OGT-depleted cells following forskolin stimulation, with p38 showing more pronounced forskolin-induced activation in siOGT BeWo cells compared to control siRNA. In agreement with that, enhancing global O-GlcNAcylation using PUGNAc significantly affected the gene expression of the two

targets downstream of p38, syncytin-1 and GCM1, whereas their expression was significantly upregulated in OGT-depleted cells in a PKA-dependent manner. However, OGT might interact directly with p38 to regulate BeWo differentiation. Studies showed that OGT interacts and regulates p38 activation in a manner dependent on cell type and experimental conditions (Goldberg et al., 2011; Moriwaki and Asahi, 2017). Future studies will be dedicated to further investigate the functional importance of p38/OGT association in the context of trophoblast differentiation.

Overall, the findings presented in this Chapter provide novel evidence supporting a central role for protein O-GlcNAcylation as a negative regulator of trophoblast differentiation via PKA-dependent mechanisms and demonstrate that unbalance in OGT/OGA interplay can alter trophoblast syncytialization with pronounced effects on syncytiotrophoblast morphology and hCG secretion in BeWo cells. Interestingly, cells respond to OGT depletion by adjusting OGA levels accordingly (Figure 4.10). This observation has been already reported in studies conducted on several human cell lines suggesting an adapting mechanism probably aimed to restore O-GlcNAc homeostasis (Zhang et al., 2014).

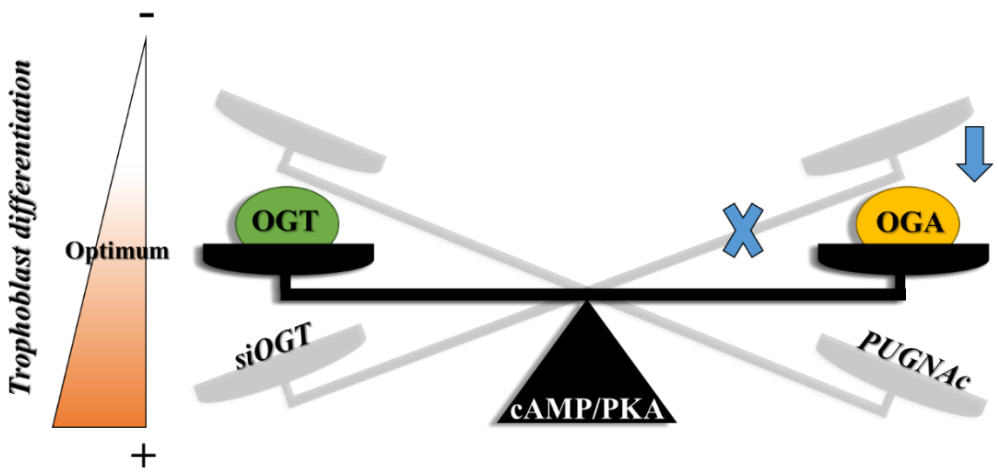


Figure 4.10 OGT/OGA interplay in the regulation of trophoblast differentiation. In BeWo trophoblast cells, blocking OGA activity with PUGNAc prevents the removal of O-GlcNAc modification from the nucleocytoplasmic proteins enhancing global protein O-GlcNAcylation which, in turn, inhibits the expression of markers of differentiations including fusogenic proteins and hCG. On the contrary, depletion of OGT using a specific siRNA induces overexpression of markers of differentiation and increased areas of syncytialization in a PKA-dependent manner. Cells respond to OGT depletion by adjusting OGA protein expression accordingly.

Chapter 5

Role of O-GlcNAc signalling in placental adaptation to maternal BMI

5.1 Introduction

It is well established that high pre-pregnancy BMI is strongly associated with fetal overgrowth (Gaudet et al., 2014) and that changes in the growth trajectories during *in utero* development increase the risk for chronic diseases later in life (Barker et al., 1993).

As discussed in Chapter 1 - Introduction, a suboptimal maternal milieu, including metabolic alterations, can interfere with placental physiology by altering placental gene expression, signalling and morphology with dramatic consequences on nutrients transport and fetal growth (Connor et al., 2020; Lewis et al., 2013; M. Li et al., 2011). The majority of the studies have been focusing on maternal diabetes. However, it is emerging that glucose transport across the placenta plays a critical role in regulating fetal growth even in absence of maternal diabetes. Indeed, maternal glucose levels below those diagnostic of diabetes are strongly associated with increased birth weight (HAPO Study Cooperative Research Group, 2002). Moreover, GLUT1, which in humans controls placental glucose transport capacity, is associated with maternal BMI. It has been shown that heavier babies born from obese mothers are exposed to increased glucose levels during *in utero* life via changes in GLUT1 transporters, regardless maternal glucose levels (Acosta et al., 2015). Nevertheless, studies investigating the molecular mechanisms underpinning placental dysfunctions and fetal overgrowth in relation to maternal BMI remain still very limited.

Protein O-GlcNAcylation is a good candidate signalling pathway to mediate maternal metabolic disfunctions to the fetus via regulation of placenta physiology. First, OGT is extensively expressed in the human placenta (Gao et al., 2001). Second, O-GlcNAc cycling regulates almost all the biological processes within cells and the O-GlcNAc modification is present on a wide range of proteins belonging to a variety of functional classes in trophoblast cells, as demonstrated in Chapter 3. Finally, OGT activity depends on the concentration of the donor substrate, the UDP-GlcNAc, which is produced from nutrients flowing through the HBP, especially glucose (Hart and Akimoto, 2009). Accordingly, O-GlcNAc cycling is perturbed in many tissues and organs during chronic diseases characterized by alterations in glucose metabolism including obesity, diabetes and cancer (Ma and Hart, 2013; Slawson et al., 2010).

Therefore, it is plausible to hypothesize that changes in nutrient flux through the placenta, particularly glucose, can affect placental OGT activity and protein O-

GlcNAcylation thus altering placental physiology. To date, however, there are only two studies conducted in hyperglycemic rats which investigates the consequences of perturbations in protein O-GlcNAcylation on placenta (Dela Justina et al., 2017, 2018). The present study sought to examine the impact of maternal BMI on placental OGT and protein O-GlcNAcylation levels and characterize, in the trophoblast cell line BeWo, interactions between OGT and AMPK, one of the placental nutrient sensor extensively involved in the regulation of fetal growth.

5.2 Maternal BMI is positively associated with circulating blood glucose levels and birth weight

Women were divided into two groups, lean (BMI<30, n=8) or obese (BMI>30, n=18), based on their BMI ($p<0.0001$), calculated by taking into account maternal body weight and height (kg/m^2) at 11-14 weeks of gestation. Maternal age, gestational age and plasma levels of protein-A (PAPP-A), a pregnancy marker of adverse perinatal outcomes, were not different between the two groups (Table 9). Although BW showed no statistical differences between maternal groups ($p=0.28$), BWC was significantly higher for babies born to obese mothers compared to those born to normal weight mothers ($p=0.038$) (Table 9). Indeed, both BW ($p=0.0349$, $R^2=0.172$) and BWC ($p=0.013$, $R^2=0.231$) were positively correlated to maternal BMI (Table 10). As for the OGTT, glucose blood levels at fasting and 2 h after the oral glucose load were below the diagnostic threshold for diabetes which is defined as a fasting value below 5.6 mmol/L and below 7.8 mmol/L at 2 h (Table 9). However, both maternal fasting glucose ($p=0.049$) and HbA1c ($p=0.0085$) levels were significantly higher in obese women compared to normal weight subjects (Table 9) and positively correlated each other ($p=0.0058$, $R^2=0.287$; Table 10). Furthermore, a positive correlation was found between maternal BMI and both maternal fasting glucose ($p=0.0177$, $R^2=0.221$) and HbA1c ($p=0.0156$, $R^2=0.229$) (Table 10).

Taken together these data suggest that, although non-diabetic, obese mothers showed higher levels of glucose in their blood at 28-30 weeks of gestation and gave birth to heavier babies.

Table 9. Clinical characteristics of the population study

Parameters	BMI <30	BMI >30	*P* value
Subjects number	8	18	
Maternal age, years	33.7 ±0.5	30.9 ±1.6	0.19
Ethnicity			
White	7	12	
Other	1	6	
Booking body mass index (BMI), kg/m^2 #	**24 ±1.4**	**37.2 ±1.2**	**<0.0001 *****
HbA1c, mmol/mol ##	**27 ±0.6**	**32 ±1.2**	**0.0085 ****
Maternal fasting glucose mmol/L ##	**4.3 ±0.1**	**4.6 ±0.09**	**0.049***
2 h glucose mmol/L##	5.9±0.2	5.9±0.3	0.92
Gestational age at delivery, weeks	40.2 ±0.3	39.5 ±0.2	0.07
PAPP-A	1.2 ±0.3	1.2 ±0.2	0.93
Birth weight, g	3358.7 ±198.5	3598.8 ±115	0.28
Birth weight percentile (BWC)	**40.1 ±12.1**	**69.6 ±7.2**	**0.038 ***
Mode of delivery			
Vaginal delivery	4	7	
Elective caesarean	2	2	
Emergency caesarean	2	9	
Parity (n)			
Nulliparous	6	13	
Parous	2	5	
Males	2	13	
Females	6	5	

 # The BMI (kg/m^2) refers to the first trimester of pregnancy (11-14 weeks).

HbA1c, maternal fasting glucose and 2 h glucose levels refer to the third trimester of pregnancy (28-30 weeks).

*Data are expressed as mean ± SEM. Differences between groups were analyzed using a t-test. * p<0.05, ** p<0.01, ***p<0.001 vs BMI <30.*

Table 10. Linear relationships between relevant clinical data

Independent variables	Dependent variables	Slope coefficient (β)	R^2	*p* value
BMI	Fasting glucose	+0.022	0.221	0.0177
	HbA1c	+0.284	0.229	0.0156
	BWC	+2.068	0.231	0.0130
	BW	+27.508	0.172	0.0349
HbA1c	Fasting glucose	+0.043	0.287	0.0058

5.3 Positive association between GLUT1 and O-GlcNAcylation in placental biopsies

In order to investigate whether protein O-GlcNAcylation, highly sensitive to glucose fluctuations, responds to the maternal obesogenic environment via glucose-dependent mechanisms, clinical data were correlated with biochemistry data obtained from placental biopsies. Protein levels of O-GlcNAcylation, OGT and GLUT1, the most abundant glucose transporter constitutively present in the placenta, along with total and phosphorylated AMPK, were measured in placenta homogenates by immunoblotting using specific antibodies (Figure 5.1, A and B). Interestingly, maternal BMI was positively associated with placental OGT levels ($p=0.0294$, $R^2=0.183$) which, in turn, were positively correlated with protein O-GlcNAcylation levels ($p=0.0023$, $R^2=0.326$) (Figure 5.1, C and D respectively). Moreover, protein O-GlcNAcylation was strongly correlated with placental GLUT1 ($p<0.0001$, $R^2=0.507$) (Figure 5.1, E). Interestingly, the latter was positively associated with both BWC ($p=0.0056$, $R^2=0.279$) (Figure 5.1, F) and BW ($p=0.0105$, $R^2=0.243$), but not with any of the maternal parameters (data not shown). Previous reports suggested a functional interaction between OGT and AMPK in metabolic tissues (Bullen et al., 2014; Gélinas et al., 2018). Here, a similar interaction was explored in placental biopsies by correlating phosphorylation of AMPK on its activation site (Thr172) with OGT and protein O-GlcNAcylation levels. Interestingly, a significant negative correlation was found between AMPK activation and OGT protein expression ($p=0.0247$, $R^2=0.193$) (Figure 5.1, G).

Taken together, these data suggest that placenta responds to maternal BMI by upregulating the protein expression of OGT and downstream O-GlcNAcylation levels. This is associated with reduced activity of the nutrient sensor AMPK. Placental GLUT1 protein expression, which showed a positive association with neonatal weight, might be involved in enhancing placental OGT and protein O-GlcNAcylation levels by increasing intracellular glucose availability in mothers with higher BMI.

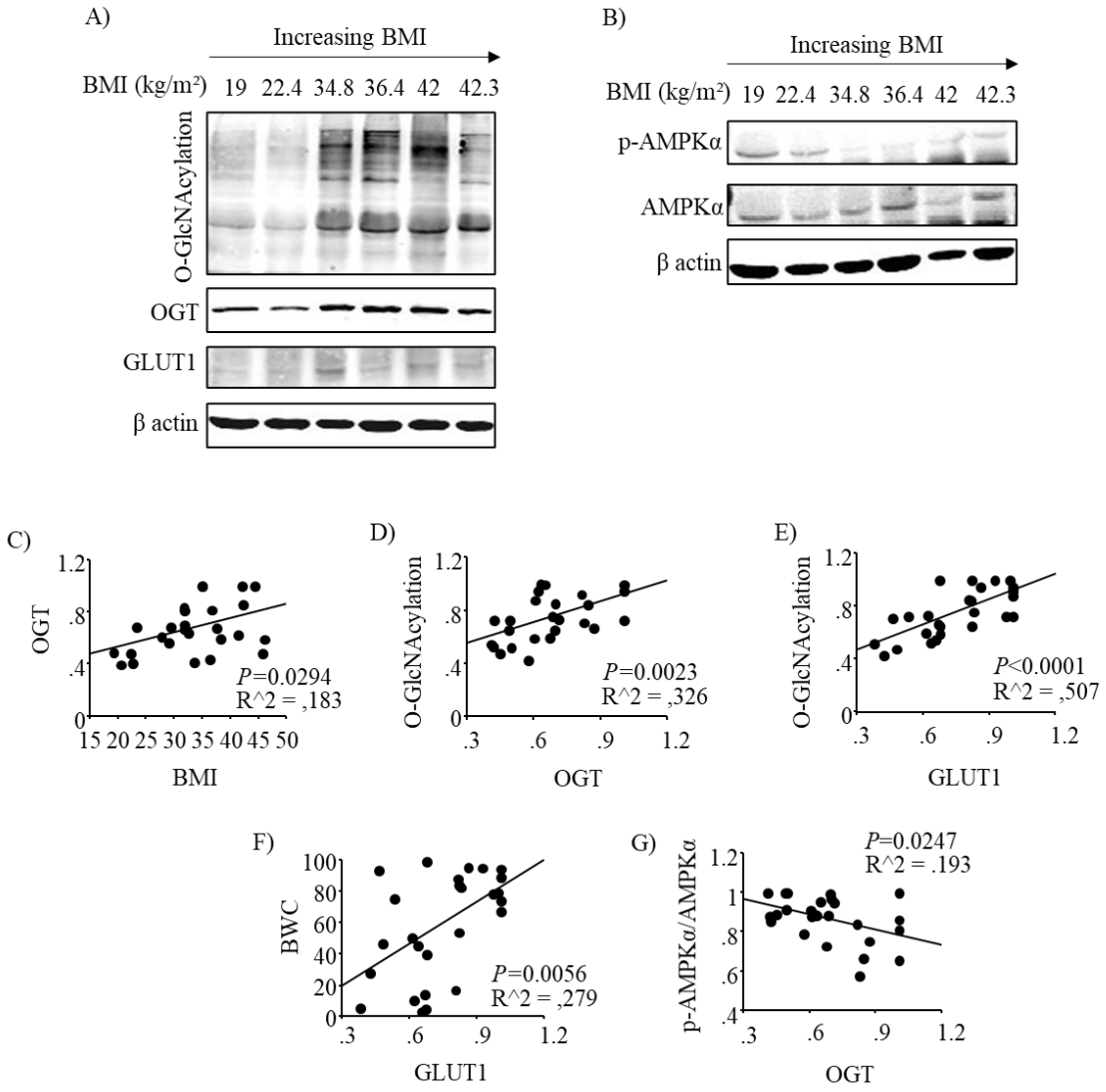


Figure 5.1 Association between maternal BMI and biochemical data in placental biopsies. Representative immunoblots showing A) global O-GlcNAcylation signal as well as OGT, GLUT1 and β actin protein levels and B) phosphorylated and total levels of AMPK in placental homogenates from women with increasing BMI. C-G) Linear association between protein levels normalized to β actin or total protein amount and feto-maternal characteristics. Regression lines and p values are shown. N=26. R^2=coefficient of determination.

5.4 Role of OGT and GLUT1 in regulating protein O-GlcNAcylation in BeWo cells

The effects of OGT on trophoblast biological responses were further investigated *in vitro* by using BeWo cells. Interestingly, by comparing immunoblots derived from placenta or BeWo cell extracts and stained with the anti-O-GlcNAc antibody, a strong correlation in the O-GlcNAc signal profile emerged between placenta and BeWo samples (Figure 5.2). The O-GlcNAc signal was detected on proteins with a broad range of molecular weights (10-250 kDa). Four areas of high signal intensity (58-62, 80-95, 110-120 and 140-235 kDa) were evident on immunoblots from both placenta and BeWo samples. Well-known OGT targets, such as HCF-1, chromatin remodeling factors and components of the nuclear pore complex, are included within the four areas of high signal intensity and were identified in BeWo cells as O-GlcNAcylated proteins by MS as discussed in Chapter 3. The fact that the areas of O-GlcNAc signal intensity overlap between BeWo and placenta extracts suggests that similar clusters of proteins are O-GlcNAcylated in human term placenta and BeWo cells further validating the use of BeWo cell line for this research study.

To investigate any direct effects of glucose and a possible role of GLUT1 on OGT activity in the trophoblast, BeWo cells depleted of either OGT or GLUT1 using specific siRNAs for 48 h, were incubated with normal (5 mM) or high (15 mM) glucose concentrations for further 48 h. Significant downregulation of OGT and GLUT1 mRNA expression was confirmed by RT-qPCR after 48 h from siRNA treatment (Figure 5.3) ($p<0.001$ for both siOGT and siGLUT1). Exposure of control siRNA cells to 15 mM glucose led to a significant increase in global protein O-GlcNAcylation compared to 5 mM ($p<0.01$). OGT depletion significantly reduced the amount of O-GlcNAcylated proteins in both 5 and 15 mM glucose treatments compared to control siRNA ($p<0.001$) (Figure 5.4 A and B). In these cells, O-GlcNAcylation levels were not affected by increased glucose concentrations. Similarly, in GLUT1-depleted cells, the levels of O-GlcNAcylation were significantly downregulated in both 5 and 15 mM glucose treatments ($p<0.001$), and also in these cells, the ability to respond to higher glucose concentrations, by rising protein O-GlcNAcylation, was abolished (Figure 5.4 A and B).

These findings suggest that, in the trophoblast cell line BeWo, an excess of glucose induces a significant increase in global protein O-GlcNAcylation by mechanisms which involve both OGT enzymatic activity and GLUT1 expression.

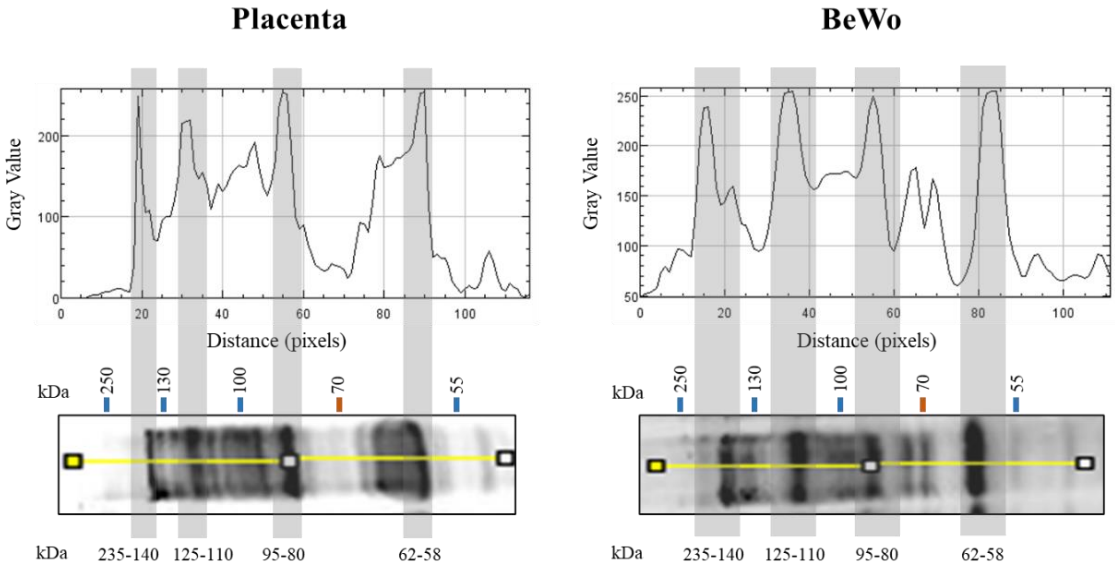


Figure 5.2 Densitometry profiles comparing global O-GlcNAcylation signal in protein extracts from placental biopsies and BeWo cells. Modified proteins were detected by immunoblotting using the anti-O-GlcNAc antibody RL2 and quantified using ImageJ 1.49v. Four areas with greater intensity were identified and protein molecular weights are indicated in kDa. The two-dimensional graphs display changes in pixel intensity along a line drawn across the immunoblotting lanes. The X-axis of the graph represents distance along the drawn line and the Y-axis is the corresponding pixel intensity.

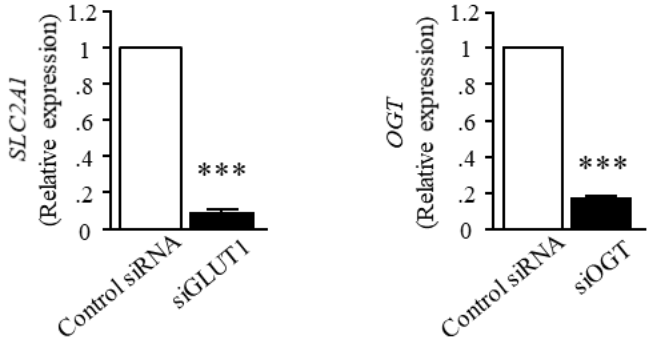


Figure 5.3 Gene expression of GLUT1 and OGT after 48 h transfection with specific siRNAs. N=3. Data are expressed as mean ± SEM; ***p<0.001 vs control siRNA.

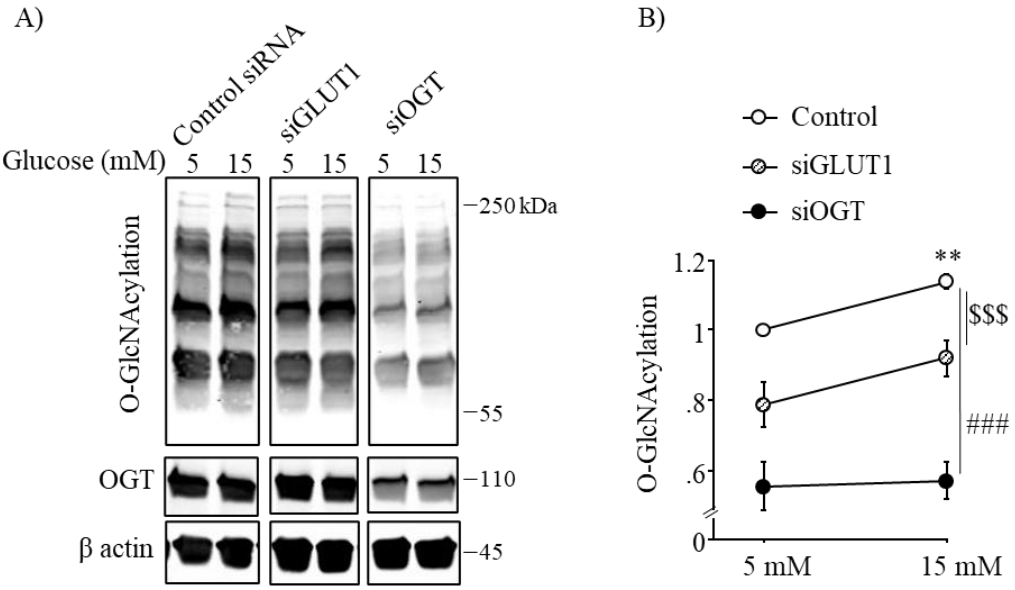


Figure 5.4 Effect of GLUT1 and OGT downregulation on glucose-induced protein O-GlcNAcylation. BeWo cells were treated for 48 h with control siRNA (lanes 1-2), siRNA against GLUT1 (lanes 3-4), and against OGT (lanes 5-6) and with 5 or 15 mM glucose for further 48 h. Representative immunoblots (A) or densitometric analysis of total O-GlcNAc signal normalized to β actin levels (B) are shown. N=3; $$$, ### $p<0.001$ vs control siRNA; ** $p<0.01$ vs 5 mM glucose. Data are means ± SEM.

5.5 Interactions between AMPK, PKA and OGT in trophoblast BeWo cells.

A functional interaction between the nutrient sensors OGT and AMPK, that regulates nutrient-sensitive intracellular processes, has previously been reported in C2C12 myotubes (Bullen et al., 2014). Here, a similar interaction was investigated in BeWo cells. The two metabolic sensors showed opposite responses to an extracellular glucose load. Compared to normal glucose (5 mM) treatment, exposure of BeWo cells to hyperglycemia (15 mM) significantly decreased phosphorylation of both AMPK at Thr172 ($p<0.001$) and its downstream target acetyl-CoA carboxylase (ACC) at Ser79 ($p<0.01$). The anti-ACC antibody used in this study recognizes both ACC-1 and ACC-2 isoforms. In contrast, phosphorylation of 4E-BP1 at Ser65 was increased ($p<0.01$), suggesting enhanced mTOR activity. In parallel, a significant increase in global protein O-GlcNAcylation signal ($p<0.01$) was observed although no changes in OGT protein expression were found ($p=0.08$) (Figure 5.5).

Previous studies in metabolic tissues identified AMPK regulation by the cAMP/PKA pathway (Gélinas et al., 2018). This was also evident in BeWo cells, since activation of adenylyl cyclase and PKA by forskolin, led to a significant increase in the activation of AMPK ($p<0.01$) and ACC ($p<0.01$) (Figure 5.6). Conversely, 4E-BP1 activation was reduced ($p<0.01$). Forskolin treatment also reduced OGT protein expression ($p<0.001$) and activity ($p<0.01$) compared to DMSO-treated cells (Figure 5.6), confirming an antagonistic functional response of AMPK and OGT to PKA activation in BeWo cells.

As shown in Chapter 4, OGT and PKA are physically and functionally associated and their interplay contributes to regulate target proteins downstream of PKA including MAPKs. Here, the role of OGT on PKA-AMPK interactions was explored. Depletion of OGT by siRNA in BeWo cells resulted in enhanced phosphorylation of AMPK ($p<0.01$) and ACC ($p<0.05$) in response to forskolin without affecting AMPK expression levels (Figure 5.7). This interaction between OGT, PKA and AMPK was further investigated, by inhibiting PKA activity using H89. As shown in Materials and Methods (Chapter 2, Figure 2.1), preliminary titration experiments identified 5 μM H89 as an effective dose to significantly inhibit forskolin-induced phosphorylation of two downstream targets of PKA, CREB and ERK1/2. In OGT-depleted cells, inhibition of PKA reduced significantly the overall stimulatory effect of forskolin on AMPK phosphorylation ($p<0.001$) (Figure 5.8). Interestingly, in control siRNA cells,

H89 treatment significantly increased expression of OGT ($p<0.01$) suggesting that PKA might also exert an inhibitory effect on OGT protein expression. This agrees with the previously observed downregulation of OGT protein expression and activity in forskolin-stimulated cells (Figure 5.7).

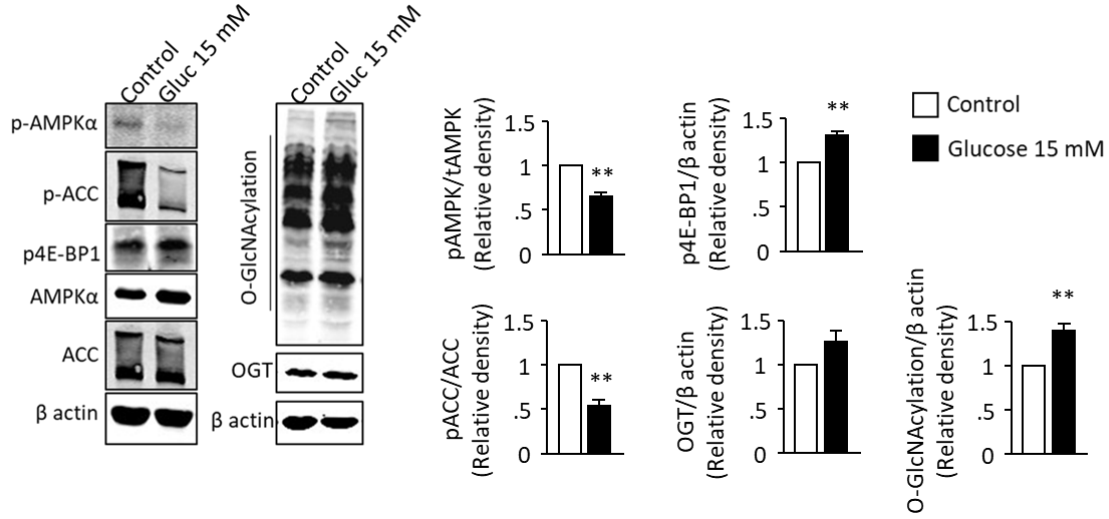


Figure 5.5 Opposite response of the nutrient sensors OGT and AMPK to a glucose load in BeWo cells. Representative immunoblots and densitometry analysis showing the effect of glucose (15 mM) treatment on AMPK activation and protein O-GlcNAcylation. BeWo cells were treated for 48 h with 5 or 15 mM glucose and activation of AMPK and downstream targets ACC and 4E-BP1, as well as OGT protein levels and global O-GlcNAcylation, were determined using specific antibodies. N=3; ** $p<0.01$ vs Control (5 mM). Data are means ± SEM.

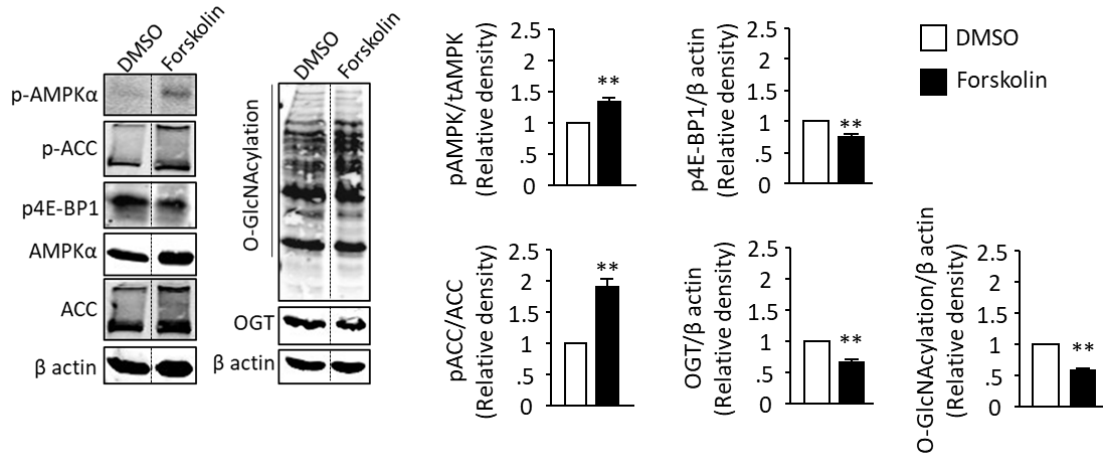


Figure 5.6 Antagonistic response of AMPK and OGT to PKA activation in BeWo cells. Representative immunoblots and densitometry analysis showing the effect of forskolin (100 µM) treatment on AMPK activation and protein O-GlcNAcylation. BeWo cells were treated for 48 h with forskolin to activate PKA and p-AMPK as well as activation of downstream targets ACC and 4E-BP1, and O-GlcNAc signalling were determined by immunoblotting using specific antibodies. N=3; ** p<0.01 vs DMSO. Data are means ± SEM.

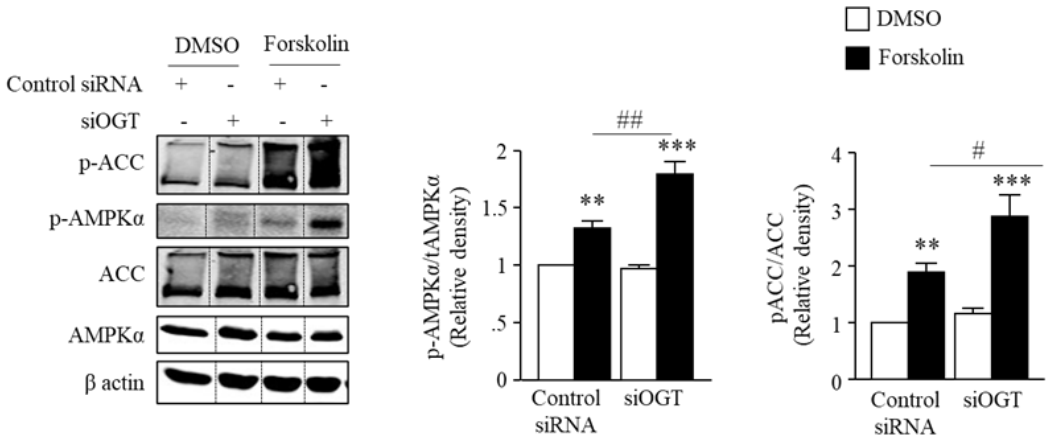


Figure 5.7 Role of OGT on PKA-AMPK interactions forskolin-induced in BeWo cells. Following treatment with siOGT or control siRNA for 48 h and stimulation with 100 µM forskolin or DMSO for further 48 h, phosphorylation or total protein expression of AMPK and its downstream target ACC was determined by immunoblotting. Representative immunoblots and densitometry analysis are shown. N=3; ***p<0.001, ** p<0.01 vs DMSO; ## p<0.01, # p<0.05 vs Control siRNA. Data are means ± SEM.

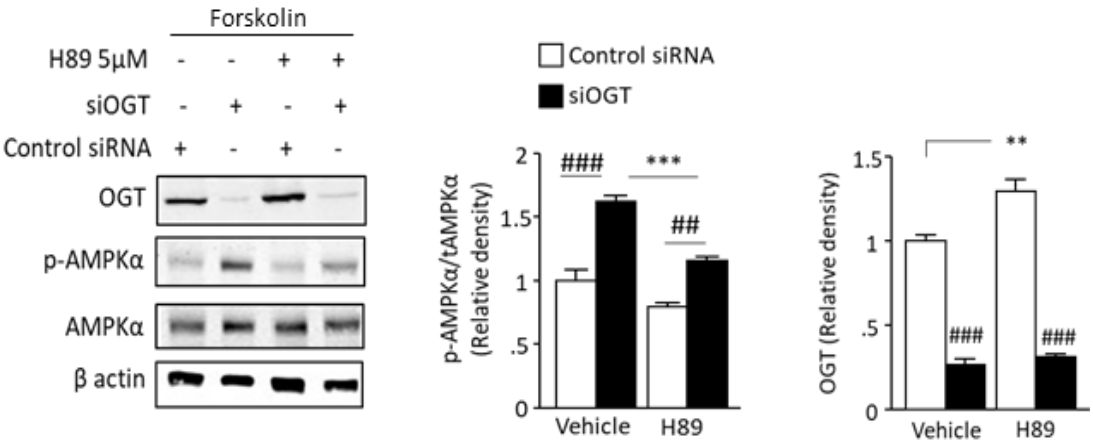


Figure 5.8 Effect of PKA inhibition by H89 on OGT expression and AMPK signalling in forskolin-treated BeWo cells. Representative immunoblots and densitometry analysis showing the effects of PKA inhibition using 5 μM H89 on the activation of AMPK and OGT protein expression in forskolin-stimulated BeWo cells pre-treated for 48 h with siOGT or control siRNA. N=3; *** $p<0.001$, ** $p<0.001$ vs vehicle; ### $p<0.001$, ## $p<0.01$ vs Control siRNA. Data are means ± SEM.

5.6 Discussion

It is well established that maternal BMI is an important determinant of neonate birth weight. At the same time, abnormal weight at birth is associated with increased risk to develop chronic diseases later in life. The presence of nutrient-sensing systems, including AMPK, mTOR and HBP, allows the placenta to detect changes occurring in maternal metabolism and adapts its physiology and functions according to the stimuli coming from the maternal environment. However, the placental response to changes in maternal physiology may become maladaptive during suboptimal maternal conditions leading to negative consequences on the growing fetus. In this context, many studies have been focusing on evaluating the impact of maternal diabetes on placental physiology and fetus health. On the contrary, the effect of maternal BMI on placental function in women without diabetes, but with impaired metabolic homeostasis, remains largely unexplored although it represents a crucial area of research to identify important clues during the 'pre-diabetes' stage of disease. This is the first study that investigates the impact of maternal BMI on O-GlcNAcylation and OGT enzyme in term placentas. Results showed that, maternal BMI exhibited positive association with neonatal birth weight which, in turn, was associated with increased expression of the placental glucose transporter GLUT1. This is the main GLUT isoform expressed by the syncytiotrophoblast plasma membranes, particularly abundant on the maternal-facing MVM (Illsley, 2000). Expression of GLUT1 on the fetal-facing BM of the syncytiotrophoblast, however, has been shown to constitute the rate-limiting step in transplacental glucose transfer (Illsley et al., 1987) and, increased abundance of GLUT1 on the BM, may promote increased glucose delivery to the fetus and enhanced fetal growth (Acosta et al., 2015; Jansson et al., 1999), in agreement with what found in this study.

It is likely that, in response to enhanced trophoblast glucose uptake and transplacental glucose transport in women with higher BMI, adaptive changes lead to increased placental expression of OGT and protein O-GlcNAcylation levels. The direct link between hyperglycemia and increased protein O-GlcNAcylation has been already demonstrated in a variety of biological systems investigating diabetes and diabetes-associated complications (Ma and Hart, 2013). Although no direct association between maternal circulating glucose and placental protein O-GlcNAcylation was detected in this study cohort, it is possible that increased glucose uptake through GLUT1, along

with enhanced levels of circulating glucose and increased OGT protein expression in response to maternal obesity, act synergistically to raise the cellular UDP-GlcNAc pool in the HBP and downstream protein O-GlcNAcylation. Certainly, as demonstrated with the *in vitro* data, both OGT and GLUT1, as well as glucose availability, directly control the amount of protein O-GlcNAcylation in BeWo cells.

The interplay between OGT and the placental sensor AMPK was also evaluated in this part of the thesis (Figure 5.9). Studies in obese pregnant ewes identified downregulation of AMPK and its downstream target ACC (Zhu et al., 2009). Moreover, in obese non-diabetic women giving birth to large babies, placental AMPK activity was inversely correlated to maternal BMI and birth weight (Jansson et al., 2013), suggesting that the activity of AMPK is impaired during maternal obesity with negative outcomes on the fetus. The results presented in this thesis Chapter support and extend these observations since, for the first time in the human placenta, a negative correlation between AMPK activation and OGT expression was found. Studies conducted in metabolic tissues have already identified a functional cross-talk between OGT and AMPK showing that these two nutrient sensors cooperatively regulate nutrient-sensitive intracellular processes that mediate cellular metabolism, growth, proliferation, and tissue function (Bullen et al., 2014; Gélinas et al., 2018). As a major cellular energy sensor, AMPK is activated in response to increased AMP/ATP ratio in conditions associated with energy deprivation (Lin and Hardie, 2018). In various cellular settings, AMPK alters OGT substrate selectivity by promoting interaction with specific proteins and competes with OGT for the modification of the same or proximal site on target proteins (Gélinas et al., 2018). In the placenta, AMPK-OGT interactions might mediate the effects of maternal obesity and hyperglycemia on the fetus, since activation of placental AMPK in humans is thought to be involved in global downregulation of metabolism and mitotoxicity (Landau et al., 2019). The *in vitro* experiments conducted in this part of the thesis, confirmed the presence of a direct glucose-activated OGT-driven pathway that diminishes AMPK effects on ACC and increases 4E-BP1 activity. This might alter the balance between placental lipid synthesis and oxidation pathways, through inhibition of ACC activity, and cell growth and protein synthesis, through enhancement of 4E-BP1 activity. Accordingly, previous studies in large for gestational age (LGA) infants born to obese women identified 4E-BP1 as one of the mTOR intracellular targets that exhibit increased levels in response to impaired AMPK (Jansson et al., 2013).

An interesting facet of the OGT-AMPK cross-talk is the antagonistic effects exerted by PKA on OGT to promote AMPK activation. This study suggests multiple strands of interactions: on one hand the cAMP/PKA pathway can attenuate OGT expression and activity, whereas OGT promotes impairment of PKA signalling involved in AMPK activation and ACC phosphorylation (Figure 5.9). Given that PKA-AMPK interaction might be also important for tight junction formation in trophoblast cells during forskolin-induced syncytialization (Egawa et al., 2008), OGT might be important in regulating AMPK-mediated mechanisms underlying the formation of placental barrier as already observed for the PKA-MAPK pathway in Chapter 4.

Put together, these results expand the understanding around the complex interplay of signalling between OGT, AMPK/mTOR and PKA in placental trophoblast cells. This mechanism appears to be activated in response to maternal BMI and excessive exposure of trophoblast to nutrients in the maternal environment, such as glucose, might derange metabolic outcomes and have important consequences for placental adaptation and accelerated fetal growth.

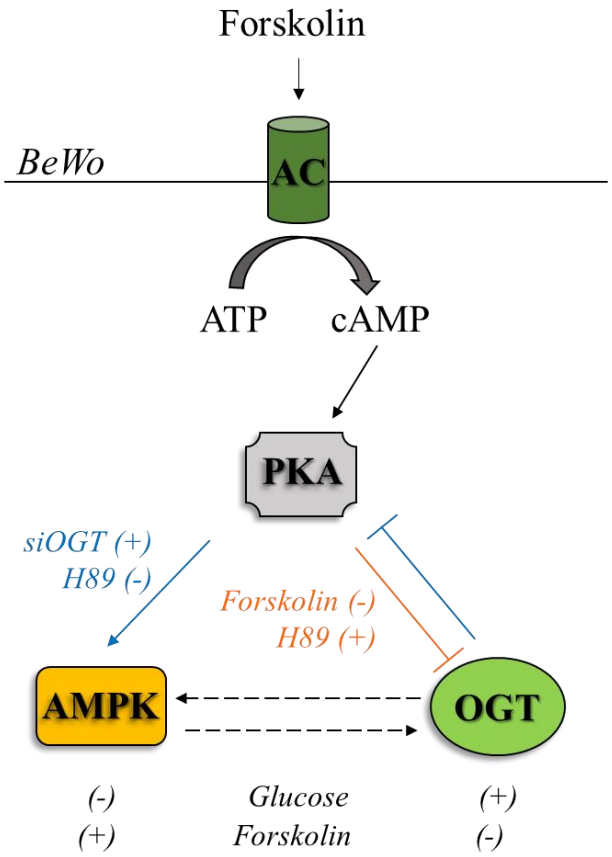


Figure 5.9 Proposed OGT/PKA interplay controlling AMPK activity in trophoblast BeWo cells. Glucose upregulates OGT and downregulates AMPK activity, whereas forskolin upregulates AMPK and reduces O-GlcNAcylation suggesting that the two nutrient sensors OGT and AMPK are regulated in an opposite way by different stimuli in BeWo cells. The activation of AMPK, in forskolin-differentiated BeWo cells, is increased in siOGT-treated cells while inhibition of PKA by H89 prevented activation of AMPK siOGT-induced suggesting that OGT exerts a negative control on AMPK activity via PKA-mediated mechanisms (blue). In addition, PKA controls OGT protein expression as activation of PKA by forskolin reduces while inhibition of PKA by H89 upregulates OGT protein expression (orange). Besides PKA-mediated mechanisms, a direct interplay between OGT and AMPK might also exist.

Chapter 6

The OGT/11β-HSD2 interplay and possible roles in placental adaptations to maternal depression

6.1 Introduction

As discussed in Chapter 1 - Introduction, a major function of the placenta is to prevent excessive exposure of the fetus to the maternal stress hormone cortisol. This hormone is synthesised by the HPA axis and released in the circulation. One of its functions is to restore homeostasis after stress response via activation of nuclear glucocorticoid receptors (GRs), which are present in almost every cell in the body (Sapolsky et al., 2000). The activity of the HPA axis changes considerably during pregnancy and lactation as an adaptive response to the modifications occurring in maternal physiology (Brunton et al., 2008). Moreover, in the fetus, cortisol controls the maturation of many organs contributing to the formation of respiratory, renal, and cardiovascular systems (Seckl, 1998). Particularly during early pregnancy, the fetus is protected from an excess of maternal cortisol by the activity of placental 11β-HSD2 which inactivates the hormone by converting it into cortisone. However, the protection offered by the 11β-HSD2 barrier can be weakened by maternal diseases and mood disorders such as anxiety or depression (Ponder et al., 2011). Because maternal cortisol levels are much higher than fetal levels, even moderate variations in placental 11β-HSD2 expression or activity can significantly alter fetal cortisol exposure (Duthie and Reynolds, 2013) impinging on the basic structure of immature organs, particularly fetal brain which expresses high levels of GRs, thus affecting organ function and predisposing the offspring to an increased risk for neurodevelopmental and cardiometabolic disorders in adulthood (Duthie and Reynolds, 2013; Lindsay and Nieman, 2005).

Recently, the OGT enzyme has emerged as a placental biomarker of maternal stress as in rodent models of prenatal chronic stress or exposure to glucocorticoids, placental O-GlcNAc signalling is perturbed with long-term consequences on the central nervous system of the offspring (Howerton et al., 2013; Pantaleon et al., 2017). In this part of the thesis the role of maternal depression on placental O-GlcNAc signalling and 11β-HSD2 expression was evaluated in term placental tissues. Moreover, the interplay between OGT and 11β-HSD2 activity was studied in OGT-depleted BeWo cells. The trophoblast BeWo cell line has been shown to mimic the biological function of placental villous trophoblast including placental 11β-HSD2 barrier properties and constitutes a widely employed model to study 11β-HSD2 activity (Mark and Waddell, 2006; Zhu et al., 2016).

6.2 O-GlcNAc signalling is increased in the placenta of depressed mothers

Maternal depression has been associated with reduced expression of the placental barrier 11β-HSD2 and increased exposure of the fetus to maternal cortisol (Seth et al., 2015). To evaluate whether the placental O-GlcNAc pathway is targeted by maternal depression, term placentas from 8 non-depressed and 8 depressed mothers were used in this study to measure the 11β-HSD2 and OGT protein levels as well as the global O-GlcNAc-modified proteins using immunoblotting. Depression was diagnosed at the time of recruitment using the Edinburgh Postnatal Depression Scale (EPDS). The neonates body weight was also used as a measure of pregnancy outcomes as it is well-established that women presenting depressive symptoms are at increased risk of having babies with low birth weight (Chang et al., 2014).

There was a statistical tendency for placental 11β-HSD2 protein levels to be downregulated in depressed compared to non-depressed women ($p=0.06$) (Figure 6.1 A). However, there was no statistical significance in the birth weight between babies born from non-depressed and depressed mothers (Figure 6.1 B). Interestingly, both OGT protein levels ($p<0.01$) and the global O-GlcNAcylation ($p<0.05$) were significantly increased in the placenta from depressed mothers compared to non-depressed group (Figure 6.1 C). Moreover, OGT protein levels were negatively correlated to 11β-HSD2 protein expression ($p=0.05$, $R^2=0.242$) (Figure 6.1 D).

To further investigate the possibility of an interplay between OGT and 11β-HSD2, a co-IP assay was performed using protein extracts from placental tissues to evaluate the physical association between these two enzymes. Results are shown in Figure 6.2 and confirmed that, in term placental tissues, there is a protein-protein association between OGT and 11β-HSD2.

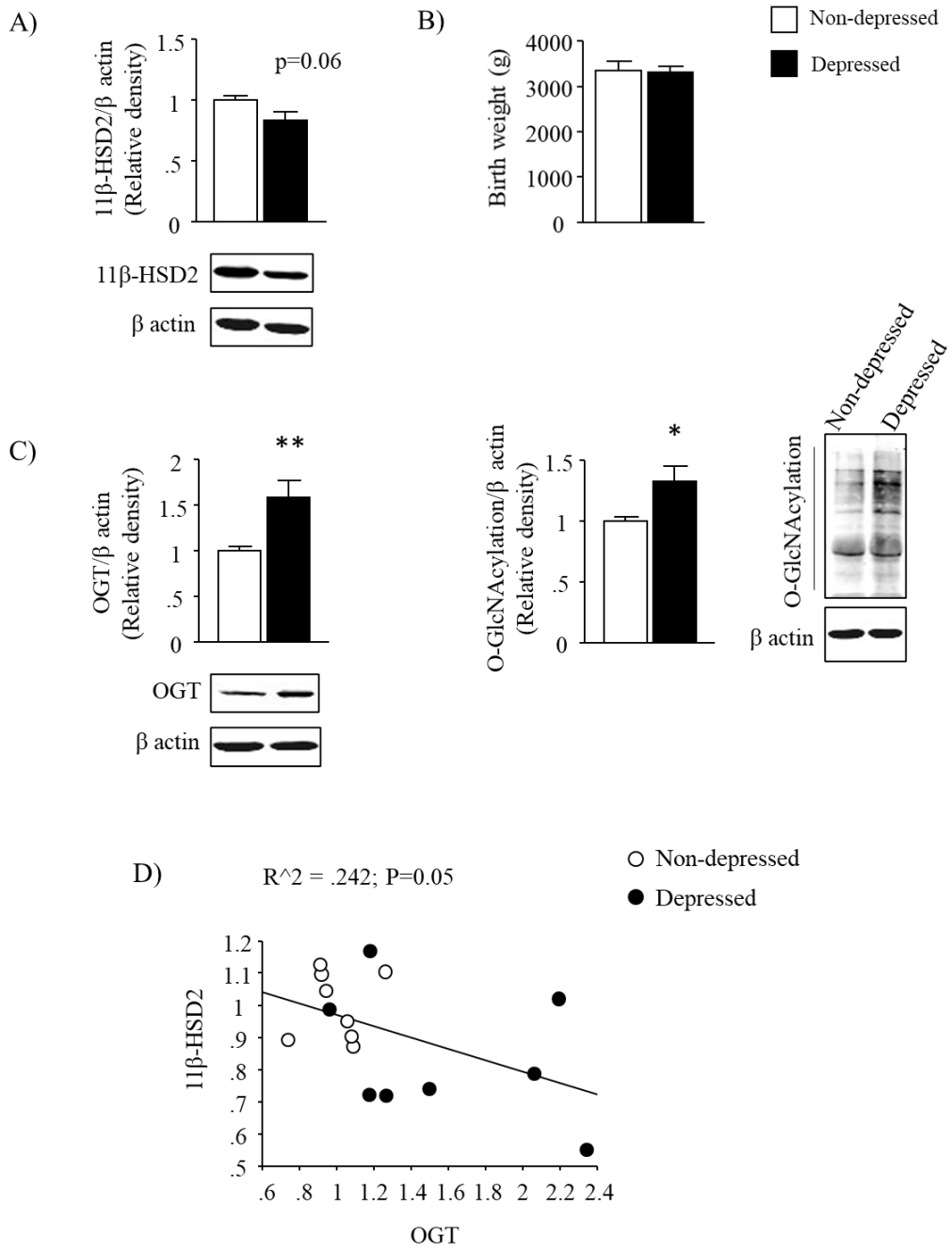


Figure 6.1 O-GlcNAc signalling and 11β-HSD2 protein expression in term placentas from non-depressed and depressed women. Representative immunoblots and densitometry analysis of A) 11β-HSD2 protein and C) OGT protein and O-GlcNAcylation levels in homogenates of term placentas and B) birth weight of babies born to non-depressed and depressed mothers. D) Linear association between 11β-HSD2 and OGT protein expression in placentas from non-depressed and depressed mothers. Data are expressed as mean ± SEM; *p<0.05, **p<0.01 vs non-depressed.

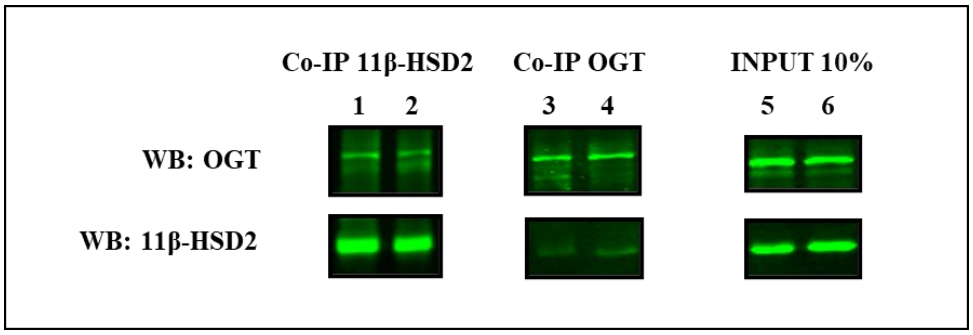


Figure 6.2 Co-IP assay showing the 11β-HSD2/OGT association in term placentas. 11β-HSD2 (lanes 1-2) or OGT (lanes 3-4) protein was immunoprecipitated from protein extracts of term placentas from non-depressed mothers using specific antibodies and immunoprecipitates as well as 10% input, the protein extract before immunoprecipitation (lanes 5-6), were immunoblotted with both anti-11β-HSD2 and anti-OGT primary antibodies to be visualized. Two biological replicates are shown on each immunoblot.

6.3 Activity of 11β-HSD2 in OGT-depleted BeWo cells

To further investigate the functional meaning of the 11β-HSD2/OGT association, the BeWo cell line was used in this study to evaluate whether downregulation of OGT expression might affect the enzymatic activity of 11β-HSD2, one of the major components of the placental barrier. As previously discussed, the role of 11β-HSD2 is to prevent that an excess of maternal cortisol reaches the fetus by converting it into the inactive metabolite cortisone. In this experiment, OGT expression was transiently silenced in BeWo cells using siRNA for 48 h. The culture medium was then changed to fresh medium and both control siRNA and siOGT cells were treated with 1 μM of cortisol for 3 h. The culture medium was then collected and the levels of remaining cortisol were measured using an electrochemiluminescence immunoassay, as a measure of the enzymatic activity of 11β-HSD2 (Zhang et al., 2015) (Figure 6.3 A). Results indicated that, although the protein levels of 11β-HSD2 showed no statistical difference between control siRNA and siOGT-treated cells ($p=0.892$), the amount of cortisol detected in the culture medium of the siOGT-treated cells after 3 h from cortisol treatment was significantly lower compared to the levels of cortisol detected in the control siRNA-treated cells ($p<0.05$) (Figure 6.3 B).

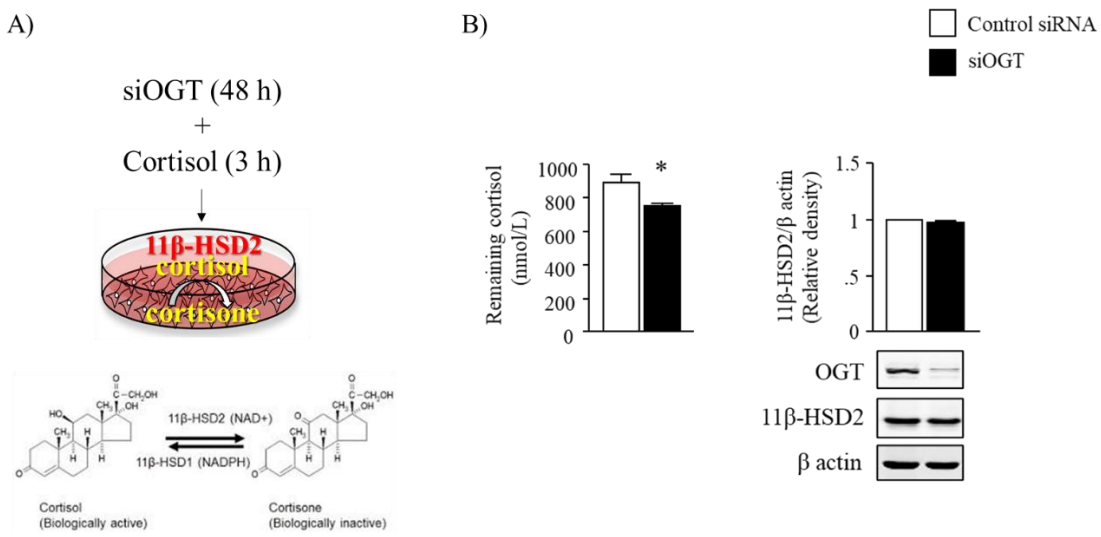


Figure 6.3 Effect of siOGT on exogenous cortisol levels in BeWo cells. A) BeWo cells were treated with siOGT or control siRNA for 48 h followed by treatment with 1 µM cortisol for 3 h. B) The residual cortisol remaining in the medium was measured by electrochemiluminescence while the 11β-HSD2 protein levels were measured by immunoblotting. Cortisol concentration is normalized to protein concentration. 11β-HSD2 levels are normalized to β actin. N=6. Data are expressed as mean ± SEM; *p<0.05 vs control siRNA.

6.5 Discussion

Previous studies using rodent models demonstrated that placental OGT and its biochemical activity can be targeted by maternal stress (Howerton et al., 2013; Pantaleon et al., 2017). However, the effect on placental O-GlcNAc signalling seems to depend on the nature of the stressor. In particular, Howerton and colleagues found that placental O-GlcNAc signalling is downregulated when pregnant mice underwent a chronic stress procedure and this was associated with neurodevelopmental alterations in the offspring (Howerton et al., 2013). On the other hand, Pantaleon and colleagues found that administration of corticosterone, a synthetic glucocorticoid, in pregnant mice, significantly increased O-GlcNAc signalling in the placenta impacting negatively on key mediators of cell survival such as the AKT pathway (Pantaleon et al., 2017). Although with contrasting results, these two studies suggest that the placental O-GlcNAc signalling pathway can detect increased maternal stress initiating downstream responses and constitutes, for this reason, a placental sensor of stress. Mood disorders, including maternal depression, are states of distress associated with hyperactivation of the HPA axis, increased maternal cortisol levels and impairment of the placental barrier system regulated by 11β-HSD2. All together, these adaptive changes to maternal environment may significantly affect fetal development and birth weight programming the offspring's physiology (O'Donnell et al., 2012; O'Donnell and Meaney, 2017; Stein et al., 2014).

In this part of the study, the hypothesis that maternal depression might target the placental O-GlcNAc signalling system was tested. The protein levels of OGT and global protein O-GlcNAcylation were measured in term placentas from non-depressed and depressed mothers and the functional interaction between OGT and 11β-HSD2 was evaluated in BeWo cells. Results indicated that O-GlcNAc signalling is upregulated in the placenta of depressed mothers compared to control group. Although the levels of maternal glucocorticoids are not available for this study, the upregulation of OGT and O-GlcNAcylation in placentas from depressed mothers is in agreement with what observed in the rodent model of corticosterone administration reported by Pantaleon and colleagues (Pantaleon et al., 2017). Many studies found that upregulation of protein O-GlcNAcylation occurs in different cellular models following the application of a diverse array of acute stress stimuli and constitutes a protective cellular mechanism in response to stress (Groves et al., 2013; Martinez et

al., 2017; Zachara and Hart, 2004). According to these studies, stress-induced changes in O-GlcNAcylation reprogram cellular pathways by promoting survival, for example via upregulation of heat shock protein expression, and by inhibiting proteins that promote cell death, as for example the pro-apoptotic transcription factors NF-kB and CHOP, collectively increasing cellular protection (Martinez et al., 2017). However, on the long-term, upregulation of protein O-GlcNAcylation is detrimental to cell physiology and has been associated with the development of many clinical conditions including hypertension, heart failure, glucose toxicity and type II diabetes (Copeland et al., 2008; Ma and Hart, 2013; Marsh et al., 2014), as already extensively discussed in Chapter 1 - Introduction. In the context of placenta, a chronic upregulation of protein O-GlcNAcylation, as a result of a persistent emotional distress, may have devastating consequences on the placental mechanisms which regulate fetal programming. For instance, OGT and O-GlcNAcylation are key regulators of transcription. Many transcription factors and chromatine-remodelling proteins interact directly with OGT and are O-GlcNAc-modified. In Chapter 3, it has been demonstrated that O-GlcNAcylation of key proteins involved in transcription regulation and epigenetic control occurs also in trophoblast cells. It is well-established that changes in the placental epigenetic code is one of the principal mechanisms via which maternal stressors mediate a rearrangment in the placental gene expression (Bale, 2015; Monk et al., 2016). The consequences of a reorganization at level of gene transcription, occurring during *in utero* life, are amplified by the fact that tissues and fetal organs are rapidly developing and are more vulnearble to changes in reprogramming trajectories. In this regard, OGT constitutes a crucial link between maternal stress and the implications of placental epigenetics in long-term programming of diseases (Nugent and Bale, 2015).

Besides regulation at the transcriptional and epigenetic level, the OGT enzyme may act more upstream by regulating directly the activity of the placental barrier 11β-HSD2. In this study, a physical interaction between OGT and 11β-HSD2 has emerged in term placentas. Moreover, a functional study conducted in BeWo cells has demonstrated that, when OGT is depleted from the cell, exogenous cortisol is metabolized more efficiently than in control siRNA-treated cells. Cortisol is a steroid hormone and passively diffuses across the cell membrane due to its lipophilic nature (Oren et al., 2004). BeWo cells are not able to synthesize cortisol *de novo* and studies demonstrated that 11β-HSD2 is the only enzyme able to metabolize cortisol in the

placenta (Benediktsson et al., 1997; Stirrat et al., 2018). Thus, although the levels of the metabolite cortisone were not measured in this study, it is possible to speculate that the cortisol levels remaining in the culture medium after 3 h from cortisol administration are primarily due to the 11β-HSD2 activity which metabolises cortisol inside the cell. OGT depletion might, somehow, upregulate 11β-HSD2 activity in BeWo cells resulting in a decrease of cortisol in the culture medium of siOGT cells compared to control siRNA treatment. It could be possible that, the presence of OGT or O-GlcNAc modification at critical amino acid residues might modify the conformation of the enzyme limiting the access to cortisol. The steric impediment might be removed when OGT is downregulated allowing enhanced cortisol metabolism. The interplay OGT/OGA may also play a critical role in regulating the interaction between OGT and 11β-HSD2. For instance, it has been demonstrated that in different experimental conditions, OGA works as an adaptor protein recruiting OGT to specific substrates (Yang and Qian, 2017). However, further studies are needed to better identify the exact mechanism via which O-GlcNAc signalling might regulate 11β-HSD2 activity in the placenta. Future studies should include measurement of 11β-HSD2 activity after overexpressing OGT in the cells and co-IP assay involving OGA to study the formation of the OGT-OGA-11β-HSD2 complex.

In summary, this part of the thesis demonstrates that, during maternal depression, the protein expression of OGT is promoted leading to increased placental levels of protein O-GlcNAcylation. This might have implications at level of the placental gene expression profile as well as on the enzymatic activity of the placental glucocorticoid barrier, 11β-HSD2, with long-term consequences on the offspring's future health (Figure 6.4).

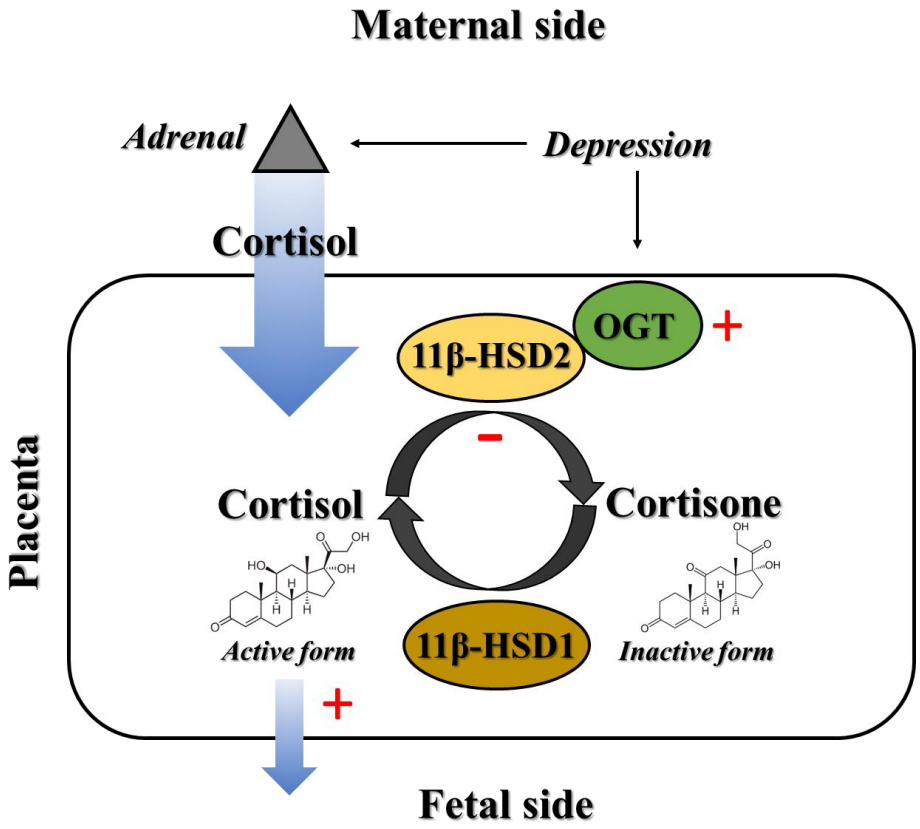


Figure 6.4 Potential role of OGT in regulating 11β-HSD2 activity during maternal depression. The 11β-HSD2 enzyme constitutes the principal placental barrier to maternal cortisol by converting the majority of maternal cortisol into the inactive metabolite cortisone. OGT might constitute a negative regulator of 11β-HSD2 activity as depletion of OGT in BeWo cells reduced the amount of cortisol in the medium. Upregulation of OGT protein expression and activity, in term placentas, can be induced by maternal depression. This might impair the activity of the 11β-HSD2 barrier increasing the transport of cortisol to the fetus.

Chapter 7

Final discussion

7.1 Discussion

Over the last decades, O-GlcNAc modification of nucleocytoplasmic proteins has emerged as a major regulator of multiple cellular functions and a critical signalling pathway linking metabolism with many pathophysiological conditions (Yang and Suh, 2014). However, its role in human placenta has not been investigated yet.

The purpose of this thesis was to explore to which extend OGT, and its biochemical mark O-GlcNAcylation, are involved in the regulation of different aspects of trophoblast physiology with a particular focus on the formation of syncytiotrophoblast. In addition, the potential implication of O-GlcNAc modification, as a placental sensor of maternal metabolism and stress, was evaluated both *in vitro* and in term placental biopsies. Overall, the results obtained strongly suggest a major role played by the O-GlcNAc modification in regulating the formation of syncytiotrophoblast and its endocrine function and identified, at the same time, the OGT enzyme as a key mediator of the signalling cascades linking maternal metabolism to fetal growth. Moreover, this study has identified that, during maternal depression, key mechanisms that regulate the placental response to maternal stress can be targeted by OGT. Finally, this research work demonstrated, for the first time, that in trophoblast BeWo cells, a wide range of proteins, involved in controlling epigenetic mechanisms and transcription, are putatively O-GlcNAc-modified, suggesting that this still poorly understood post-translational modification has the potential to act as the molecular bridge between maternal environmental cues and epigenetic mechanisms of *in utero* programming. Figure 7.1 summarizes the main findings emerged in this research project and illustrates the different mechanisms via which placental OGT and O-GlcNAcylation can control placenta formation and functions and, indirectly, fetal developmental trajectories.

In this thesis, the role of O-GlcNAcylation in syncytiotrophoblast formation and function was investigated in human trophoblast BeWo cells. Throughout pregnancy, syncytiotrophoblast fulfils several important placental functions including transport of nutrients to the fetus, protection against a variety of insults and production of placental hormones (Gude et al., 2004). Given its crucial role in many aspects of pregnancy, alterations in syncytiotrophoblast morphology or function are associated with negative pregnancy outcomes including preeclampsia and changes in fetal growth trajectories with potential short- and long-term impact on the health of both mother and fetus

(Backes et al., 2011; Redman and Staff, 2015; Roland et al., 2016; Sferruzzi-Perri and Camm, 2016). As demonstrated in this thesis work, depletion of the OGT enzyme induced spontaneous differentiation of BeWo cells and enhanced the formation of areas of syncytialization and production of the hCG hormone during forskolin-induced differentiation, suggesting that certain levels of O-GlcNAcylation are necessary to control syncytiotrophoblast formation via mechanisms involving the PKA-ERK1/2-p38 signalling pathway. Similar results were observed in ESCs by Miura and colleagues who showed that the O-GlcNAc modification on the activation site of PKCζ was required for the maintenance of the undifferentiated state of ESCs, while upregulation of O-GlcNAc levels prevented activation of the PKCζ-MEK-ERK1/2 pathway and the ability of naïve ESCs to differentiate into primitive endoderm (PrE) cells, one of the first cell lineages generated by the embryo (Miura et al., 2018). Taken together these findings suggest that, by regulating similar signalling cascades, protein O-GlcNAcylation may play a major role in controlling the timing of cell lineage differentiation in the embryo and the formation of syncytiotrophoblast from cytotrophoblast cells, two crucial steps in embryogenesis and placentation.

Thus, changes in placental OGT levels may have a significant impact on placenta formation constituting a real risk for the developing fetus. In the pioneering study conducted by Howerton and colleagues (Howerton et al., 2013; Howerton and Bale, 2014), which brought to light the first evidence on the importance of placental OGT and O-GlcNAc signalling during *in utero* development, downregulation of placental OGT expression, following prenatal maternal stress in mice, was associated with adverse consequences on the offspring's neurodevelopment. Importantly, in this thesis work, it was demonstrated that changes in OGT and O-GlcNAc signalling affect both morphology and endocrine activity of the syncytiotrophoblast via interplay with PKA-mediated mechanisms. For instance, the production of the hCG hormone appeared to be particularly influenced by changes in the O-GlcNAc signalling pathway in BeWo cells showing significant upregulation in OGT-depleted cells and downregulation when O-GlcNAcylation was enhanced by PUGNAc. It is known that hCG can contribute, both directly and indirectly, to fetal development. Indeed, high hCG concentrations during the first trimester of pregnancy are associated with increased fetal growth in a sex-specific manner (Barjaktarovic et al., 2017), whereas elevation of hCG during the second trimester appears to be associated with fetal growth restriction and preterm delivery (Gonen et al., 1992; Lieppman et al., 1993; Onderoğlu

and Kabukçu, 1997). Moreover, studies on both humans and rodents showed that, hCG can cross the blood–brain barrier and that the hCG receptors are expressed in multiple fetal organs, including fetal and neonatal rat brains, strongly suggesting that hCG can act directly on regulating fetal neurodevelopment (al-Hader et al., 1997; Lei et al., 1993). Interestingly, hCG also controls the expression of 11β-HSD2 (Shu et al., 2014), a crucial placental enzyme that regulates the transfer of maternal glucocorticoids through the placenta protecting, on one hand, the fetus from an excess of maternal stress hormones and regulating, on the other hand, fetal development and growth (Seckl and Holmes, 2007). Importantly, the novel findings emerged in this research work link O-GlcNAc signalling to syncytiotrophoblast formation and hCG production, raising the question on whether a functional interconnection of OGT, hCG and 11β-HSD2 may exist in human placenta.

Certainly, this thesis has identified an association between OGT and 11β-HSD2 in term placental biopsies and BeWo cells. It is known that the 11β-HSD2 enzyme is targeted by maternal stressors, such as mood disorders, and that suboptimal expression and activity of placental 11β-HSD2 are associated with abnormal fetal development and poor health condition in adulthood (Cottrell and Seckl, 2009; Lesage et al., 2006). As discussed previously, the abnormalities observed in fetal growth as a result of 11β-HSD2 defects are due to an increased transplacental passage of glucocorticoids that target the growing fetal organs altering their structure and development as well as their functionality in adult life. In this thesis, it was shown that placental O-GlcNAc signalling is upregulated during maternal depression and that OGT and 11β-HSD2 are physically associated in term placentas. More importantly, it has emerged that depletion of OGT in BeWo cells appeared to alter the activity of 11β-HSD2 enhancing its ability to metabolize cortisol suggesting a novel functional interplay between OGT and 11β-HSD2 that opens new exciting opportunities in the study of placental glucocorticoids signalling and fetal programming of human diseases.

As discussed above, certain levels of OGT activity are important for a proper syncytiotrophoblast formation. However, similarly to OGT depletion, enhancement of placental O-GlcNAc signalling can result in negative outcomes. It has been shown that, increased hyperglycemia-induced O-GlcNAcylation prevents embryo implantation and development in mice (Pantaleon et al., 2010) and alters placenta morphology and placental inflammatory response in rats (Dela Justina et al., 2017, 2018). However, studies investigating the link between maternal metabolic diseases

and O-GlcNAc signalling have not been performed yet in human placenta. According to its role as a nutrient sensor, the O-GlcNAc modification is very sensitive to changes in the flux of nutrients entering the HBP, particularly glucose. Elevated O-GlcNAcylation has been associated with glucose toxicity and insulin resistance in insulin-sensitive tissues during diabetes and diabetic complications (Ma and Hart, 2013). However, in this thesis more attention was given to investigate the potential link between the placental O-GlcNAc modification and maternal BMI rather than maternal diabetes. This mainly because, high maternal body weight is associated with a higher risk of GDM (Chu et al., 2007) and constitutes an important area of research in terms of pre-diabetes interventions. Moreover, pregnancy outcomes associated with maternal obesity and overweight are as poor as those observed in diabetic pregnancies (Catalano, 2010), making necessary a better understanding of the molecular mechanisms linking suboptimal maternal BMI to fetal development. As demonstrated in Chapter 5 and supported by multiple other studies, mothers with higher BMI give birth to heavier babies. In addition to that, here it was shown that increased maternal BMI was associated with higher circulating glucose levels. Moreover, the placenta of heavier babies expressed more GLUT1 transporters, the primary isoform involved in the transplacental passage of glucose. These observations are in agreement with the literature and suggest that maternal BMI determines neonatal weight, in part, via mechanisms involving placental glucose uptake even in absence of diabetes (Acosta et al., 2015; Lowe et al., 2019). According to the Pedersen hypothesis, fetal overgrowth is the result of increased transplacental transfer of glucose which stimulates fetal secretion of insulin and increases adiposity (Pedersen, 1971). In this context, one aim of this thesis was to investigate the role played by the placental OGT system in mediating maternal metabolic perturbations to the fetus. Interestingly, a positive association between maternal BMI and placental OGT protein levels was identified. In addition, both OGT and GLUT1 protein levels were positively correlated with the levels of global protein O-GlcNAcylation in placental biopsies and silencing of OGT or GLUT1 significantly reduced the global O-GlcNAc modification in BeWo cells. Taken together, these findings show, for the first time, that maternal BMI targets the OGT expression in human placentas determining the increase of protein O-GlcNAcylation, in part, via mechanisms involving placental glucose transport.

The consequences of the upregulation of O-GlcNAc signalling can negatively impact the placenta function at different levels interfering with a variety of mechanisms

involved in controlling fetal growth as discussed here and summarized in Figure 7.1. A crucial candidate target of OGT, critically involved in controlling fetal growth, is AMPK. As shown in Chapter 5, higher OGT protein levels correlated with lower AMPK activation in term placentas, raising the possibility that the two nutrient sensors might act in an antagonistic manner in response to environmental stimuli. AMPK is the master regulator of cellular energy status switching on and off cell metabolism according to ATP availability. For instance, AMPK works as a negative regulator of mTOR signalling to limit protein synthesis and cell growth when cell energy is low and *vice versa*. As demonstrated in Chapter 5, in conditions of excessive glucose in the culture medium of BeWo cells, AMPK was turned off whereas 4E-BP1, which controls protein synthesis downstream of mTOR, was activated along with the O-GlcNAc signalling. Alterations of the placental AMPK-mTOR axis have been associated with suboptimal fetal growth and weight at birth as a consequences of caloric restriction or overnutrition in both humans and animal models (Jansson et al., 2013). As emerged in Chapter 5, in BeWo cells, OGT controls the activation of AMPK in a PKA-dependent manner. Indeed, when OGT is silenced and PKA is activated by forskolin, AMPK activation is significantly enhanced but not in presence of a PKA inhibitor. Taken together, these observations suggest that AMPK and OGT are functionally associated via PKA-dependent mechanisms and that by targeting the OGT system, maternal metabolic alterations can affect metabolic pathways controlling fetal growth such as the AMPK-mTOR signalling pathway. AMPK has been already recognised as a therapeutic target and AMPK activators, such as metformin, are employed to restore a variety of pregnancy complications including GDM, preeclampsia and fetal growth alterations (Kumagai et al., 2018). Thus, this study offers new insights regarding a potential OGT-AMPK-mTOR interplay which may regulate fetal growth during maternal obesity and provides new promising directions for future research investigation.

One of the most intriguing goals concerning this thesis project was to understand to which extent the O-GlcNAc modification might be involved in controlling trophoblast physiology and fetal development by acting directly on mechanisms regulating placental gene expression. Epigenetic mechanisms have been identified as critical mediators of *in utero* programming due to their ability to dynamically integrate environmental signals into gene expression. It is well-established that the maternal metabolic status can impact on the placenta “transcriptome” and “epigenome” with

consequences on the developing fetus. Importantly, it has been shown that DNA methylation can be particularly influenced by changes in maternal nutrition and metabolism during early life. For instance, placental DNA methylation is able to dynamically adapt to changes in maternal glycemic levels throughout pregnancy (Cardenas et al., 2018). More importantly, alterations in the placental methylome profile have been associated with maternal obesity, pre-pregnancy BMI and gestational weight gain and involve, primarily, genes that control placental and fetal growth, including genes that regulate glucose metabolism, such as leptin and adiponectin, as well as genes involved in mediating glucocorticoid signalling, such as GRs and 11β-HSD2 (Drake et al., 2012; Mitsuya et al., 2017; Nogues et al., 2019; Shrestha et al., 2020).

As discussed previously, OGT activity is strictly related to the amount of nutrients entering cells. It is also well-known that OGT is a major regulator of the transcriptional and chromatin remodelling machinery which makes O-GlcNAc signalling a good candidate to link environmental exposure to nutrients with placental gene transcription. In Chapter 3, the "O-GlcNAcome" was explored in the trophoblast BeWo cell line. This was the first study that used a proteomic approach to identify O-GlcNAc-modified proteins in a human trophoblast cell line. Overall, it has emerged that O-GlcNAcylation is particularly abundant on factors regulating transcription and chromatin remodelling. Many of the identified proteins belong to protein complexes involved in methylation of histones. For instance, many components of the Set1/COMPASS complex, which controls the tri-methylation of histone H3 Lys4 (H3K4me3), commonly associated with the activation of transcription of nearby genes (Martin and Zhang, 2005), were enriched by the anti-O-GlcNAc antibody in BeWo extracts. As suggested by the literature, OGT appears to have a central role in regulating the activity of the Set1/COMPASS complex. Briefly, the formation of TET2/3–OGT interaction induces O-GlcNAcylation of HCF-1 transcription coregulator, allowing the assembling of the complex and the binding of SETD1A methyltransferase to chromatin, an event necessary for histone H3K4me3 and subsequent transcriptional activation. Reduction of either TET2/3 or OGT activity results in a direct decrease in H3K4me3 and concomitant decrease of transcription (Deplus et al., 2013). In mammalian cells, H3K4me3 is particularly enriched at transcriptionally active promoters where it recruits mostly positive transcription regulators (Martin and Zhang, 2005). Surprisingly, the H3K4me3 modification has

been found in syncytiotrophoblast cells marking the presence of active transcription (Ellery et al., 2009), and it has been shown to 'prime' the gene activation of the human growth hormone (hPGH), a placental hormone greatly involved in fetal growth, placental development and maternal adaptation to pregnancy, in fusing primary cytotrophoblast cells isolated from human term placentas (Kimura et al., 2007). Misregulation of histone H3K4me3 modification is associated with aberrations in neurodevelopment. Interestingly, in mouse placenta, H3K4me3 seems to control OGT gene expression itself. In particular, prenatal stress reduced the association of H3K4me3 at the OGT promoter, suggesting a transcriptional mechanism via which maternal stress can regulate the expression of OGT in the placenta (Howerton et al., 2013). Besides acting on regulating the epigenetic machinery, O-GlcNAcylation is also present on many transcription factors, including Sp1 and Sp3, as emerged in Chapter 3. Importantly, Sp1 regulates the transcription of many crucial genes in the syncytiotrophoblast including GLUT1 (Okamoto et al., 2001), 11β-HSD2 (Li et al., 2011) and syncytin-1 (Cheng and Handwerger, 2005). O-GlcNAcylation of Sp1 has been shown to increase its stability, nuclear localization and transcriptional activity and has been implicated in diabetes and diabetic complications in many tissues (Du et al., 2000; Geraldes et al., 2009).

Taken together, these last observations suggest that protein O-GlcNAcylation might control important trophoblast functions, including glucose transport across the placental barrier and glucocorticoids metabolism, via direct regulation of placental gene expression and that maternal disorders, known to affect placental physiology and fetal development, such as obesity or mood disorders, can mediate their deleterious effects via changes in placental transcriptome and epigenome involving the O-GlcNAc signalling pathway (Figure 7.1).

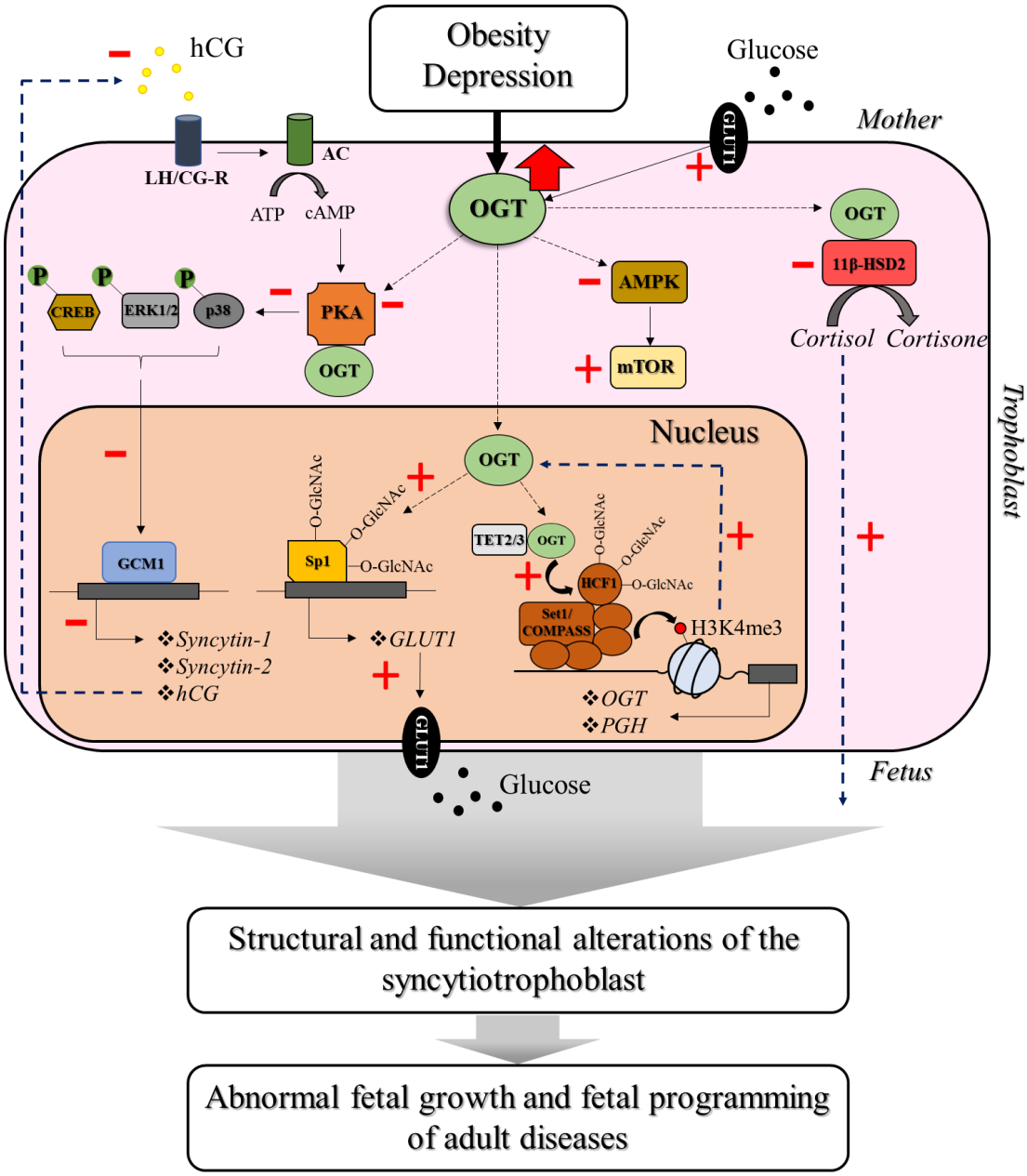


Figure 7.1 Summary of the potential mechanisms by which placental O-GlcNAc signalling can participate in fetal programming. Maternal obesity or depression enhances the cellular levels of OGT and protein O-GlcNAcylation in the trophoblast thus perturbing the activity of signalling pathways involved in the regulation of trophoblast formation and function with potential consequences on fetal growth and increased risk for developing chronic diseases in adulthood. By interacting with PKA, enhanced OGT activity can interfere with the phosphorylation and activation of targets downstream of PKA, including CREB, ERK1/2 and p38, reducing the expression of specific markers of trophoblast differentiation such as fusogenic proteins, the hCG

hormone and the transcription factor GCM1. The hCG hormone itself plays a crucial role in regulating syncytiotrophoblast formation via a positive feedback mechanism that can be impaired during upregulation of O-GlcNAc signalling. Alterations of the cAMP/PKA signalling pathway are associated with aberrant trophoblast differentiation which, in turn, can affect fetal growth. Increased OGT activity can also interfere with the activation of the nutrient sensor AMPK. Reduced activation of AMPK is associated with increased activity of mTOR signalling which promotes placental nutrient transport and fetal growth (Jansson et al., 2012). Furthermore, by interacting with 11β-HSD2, OGT can also impair cortisol metabolism exposing the fetus to increased transplacental levels of maternal glucocorticoids which target the developing fetal organs. Finally, inside the nucleus, increased OGT activity can stabilize and promote the transcriptional activity of the transcription factor Sp1 potentially increasing gene expression of GLUT1 which, in turn, can promote increased delivery of glucose to the fetus and further potentiate protein O-GlcNAcylation. Upregulation of O-GlcNAc signalling can also lead to increased O-GlcNAcylation of the transcription factor HCF-1 promoting the activity of the Set1/COMPASS complex which, by increasing the tri-methylation of histone H3 Lys4 (H3K4me3), can induce further gene transcription of OGT itself and enhance the expression of the human placental growth hormone (hPGH), involved in fetal growth and placental development (Kimura et al., 2007).

Chapter 8

Conclusions

8.1 Conclusions, limitations and future research

This thesis work has contributed significantly to identify a critical role for OGT and protein O-GlcNAcylation in the regulation of human placental physiology bringing novel knowledge to a poorly explored field of research. Overall, the findings that emerged from this research work revealed that O-GlcNAcylation is involved at many levels in regulating trophoblast physiology and function. Only some aspects have been investigated more in-depth due to a limitation of resources and time. Particular attention was dedicated to understanding the role of OGT in syncytiotrophoblast formation since this is the placental structure that carries out several important placental functions. The experiments performed using the human trophoblast cell line BeWo identified a clear role for OGT as a negative regulator of syncytiotrophoblast differentiation. Although the BeWo cell line is a widely employed model to study trophoblast differentiation, showing remarkable similarities with crucial functional aspects of human trophoblast, including endocrine activity and transport characteristics, there are some limitations associated with the use of cell lines. In particular, the gene expression profile of BeWo cells is cancerous and studies showed that there is a poor correlation with that of trophoblast cells in primary culture (Novakovic et al., 2011). For that reason, primary trophoblast cells, although more difficult to obtain and manipulate, are certainly physiologically more similar to an *in vivo* scenario and should be employed in future experiments to overcome the limitations associated with the use of cell lines.

Interesting findings have also emerged from the analysis of term placentas in women with BMI ranging from lean to obese, where a positive association between maternal BMI and placental OGT signalling was identified along with the reduced activity of AMPK, a crucial nutrient sensor involved in the regulation of fetal growth. This is the first study conducted on human term placentas which links maternal BMI to changes in O-GlcNAcylation levels of placental proteins, suggesting that OGT might constitute a key molecular bridge between maternal metabolism and placental regulation of fetal growth. However, a major limitation associated with this part of the research can be identified in the low number of placenta samples available. Future studies need to be carried out on a larger sample size which will allow for multifactorial regression analysis of variables. In addition, a more homogeneous enrichment of placentas from female and male fetuses is needed in order to address sex differences associated with

OGT expression in the placenta. As an X-linked gene, OGT has been shown to escape X-inactivation remaining more expressed in the placenta of females compared to males. The role of OGT in mediating placental functions according to the sex of the placenta is a crucial area for future research since it may explain the sex-specific increased risk for developing a number of diseases that are programmed during *in utero* life, including neurodevelopmental and metabolic disorders.

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