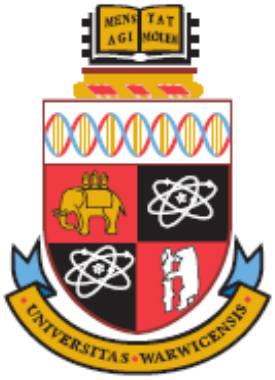

Role of Mammalian Target of Rapamycin (mTOR) Signalling in BeWo Trophoblast Differentiation and Fusion

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Medicine

University of Warwick, Warwick Medical School

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To my beloved country, Libya

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My special and profound thanks go to my family for their unceasing support and encouragement to have this work accomplished.

Declaration

I declare that all the work presented in this thesis is my own unless stated otherwise and was performed under the supervision of Professor Dimitris Grammatopolous at the University of Warwick and University Hospitals Coventry and Warwickshire and has not been submitted for a degree to any other university or institution. I have acknowledged all the sources of information by providing references and web links.

Isma A Alkawafi,

January 2015

Abstract

Foetal growth and development is closely related to the placental transport function, since any defect in this function is associated with pathological foetal growth. Trophoblast differentiation into a multinucleate syncytium is a key biological mechanism. Transcriptional regulation of fusogenic genes to promote fusion and capacity for placental hormonogenesis is achieved by a coordinated action of signals such as cAMP and MAPKs. The mammalian target of rapamycin (mTOR) acts as a placental growth signalling sensor, and this has been implicated in the pathophysiology of diseases such as intrauterine growth restriction (IUGR). This is also implicated in the pathological mechanism of diabetes mellitus, a disease which is associated with excess nutrient availability and insulin resistance. Many researchers have reported a relationship between changes in placental amino acid transporter activity and altered foetal growth. The signalling pathway of the protein kinase mTOR has been proposed to regulate cellular growth in response to growth factors, nutrient and amino acids by inducing transcription and translation. The role of mTOR signalling pathway in early development is confirmed by post implantation lethality in mouse following a complete deletion of mTOR gene. mTOR is an evolutionarily conserved member of the phosphatidylinositol-3-OH (PI-3)-kinase-related kinase (PIKKs), and its upstream activators PI3K and Akt appear to be involved in the control of trophoblast fusion. The precise role of mTOR in the trophoblast differentiation mechanisms is not well understood.

Previous studies have shown that, fusion processes in BeWo cells is stimulated by inhibition of intracellular calcium and down regulation of phosphatidylinositol 3 kinase (PI3K)/Akt pathway. Since PI3K/Akt pathway is the upstream signalling of mTOR pathway, we conducted this study to investigate the role of mTOR signalling pathway in BeWo cells differentiation. Our in-vitro studies showed that inhibition of mTOR by rapamycin, reduced forskolin-induced hCG release, whereas syncytin-1 and -2 mRNA expression was substantially augmented. These findings led us to investigate the effect of forskolin-driven adenylyl cyclase activation on mTOR expression and activity. Forskolin treatment for 24h significantly reduced mTOR protein expression. Moreover, within 10min of forskolin treatment, there was a substantial reduction in basal mTOR phosphorylation at Ser2481 and Ser2448, which is required for mTOR activity. This was associated with increased phosphorylation of p70S6K, at Ser371.Immunohistochemistry showed increased mTOR expression and phospho-mTOR (Ser2448/2481) staining in GDM placenta compared to controls.

Our results identify distinct actions of mTOR on the biochemical and morphological differentiation of BeWo trophoblasts. In addition, it showed increased expression and activity of mTOR in gestational diabetes mellitus (GDM).

List of Abbreviations

AC	Adenyl cyclase
AICAR	5-aminoamidazole 4-carboxyamide
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
ATG13	Autophagy-related gene 13
8-Br-cAMP	8- Bromoadenosine- 3', 5'- cyclic monophosphate
11β-HSD2	11-Beta-Hydroxysteroid Dehydrogenase Type 2
cAMP	3, 5-cyclicmonophosphate
CREB	cAMP response element binding
DEPTOR	DEP domain containing mTOR interacting protein
4EBP1	Eukaryotic initiation 4E binding protein
ERK	Extracellular signalling-regulated kinase
FIP200	Focal adhesion kinase family-interacting protein of 200 Kd
FKBP12	12 KDa FK506 binding protein
FKBPs	FK506 binding proteins
FoxO1	Forkhead box protein O1
FSH	Follicle stimulating hormone
GCM-1	Glial cell missing-1
GPCR	G-protein coupled receptors
HBP	Hexosamine biosynthetic pathway
hCG	Human chorionic gonadotropin
HEELP	Haemolysis (elevated liver) enzymes(low platelet) count
hPL	Human placental lactogen
IGF	Insulin like growth factor
IRS-1	Insulin receptor substrate 1
IUGR	Intrauterine growth restriction

LH	leutinizing hormone
MAPK	Mitogen activated protein kinase
MFSD2	Major Facilitator Superfamily Domain Containing 2
mLST8	Mammalian lethal with sec-13 protein 8
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex-1
mTORC2	Mammalian target of rapamycin complex-2
O-Glc-NAc	O-N-acetylglucosamine
OGT	*O*-GlcNAc transferase
OGTT	Oral glucose tolerance test
PDK1	Phosphoinositide-dependent kinase-1
PI3-K	Phosphoinositide 3-kinase
PIKKs	PI3K related protein kinase
PKA	Protein kinase A
PPARg	Peroxisome proliferator-activated receptor g
RAPTOR	Regulatory associated protein of mTOR
RDR	D mammalian retrovirus
RICTOR	Rapamycin insensitive compound of mTOR.
Rheb	Ras homolog enriched in brain
SREBP	Sterol Regulatory Element Binding Protein
TSH	Thyroid stimulating hormone
TSC	Tuberous sclerosis complex
ULK1.	un-51-like kinase 1

Conference output

1. **Alkawafi, I**. Vatish, M. Grammatopoulos, D. Role of mTOR signalling in BeWo trophoblast differentiation and fusion. Oral presentation at The Endocrine Society's 95th Annual Meeting and Expo, San Francisco June 15–18, 2013.
2. **Alkawafi, I**. Bari, M F. Vatish, M. Grammatopoulos, D. Gestational Diabetes Is Associated with Increased Activity of the Placental mTOR Pathway: Characterisation of Mechanisms Controlling Trophoblast Differentiation and Endocrine Function. Poster presentation at 61st Annual Meeting of The Society for Gynaecologic Investigation (SGI) Florence-Italy March 26-29, 2014.

Chapter One

Introduction

1.1. The placenta

The placenta is the main organ that maintains fetal development and lead to a successful pregnancy. Its main function is to provide an immunological barrier that separates foetal from maternal blood, to produce important hormones and cytokines and to transport oxygen and important nutrient between mother and fetus (Roos et al., 2009).

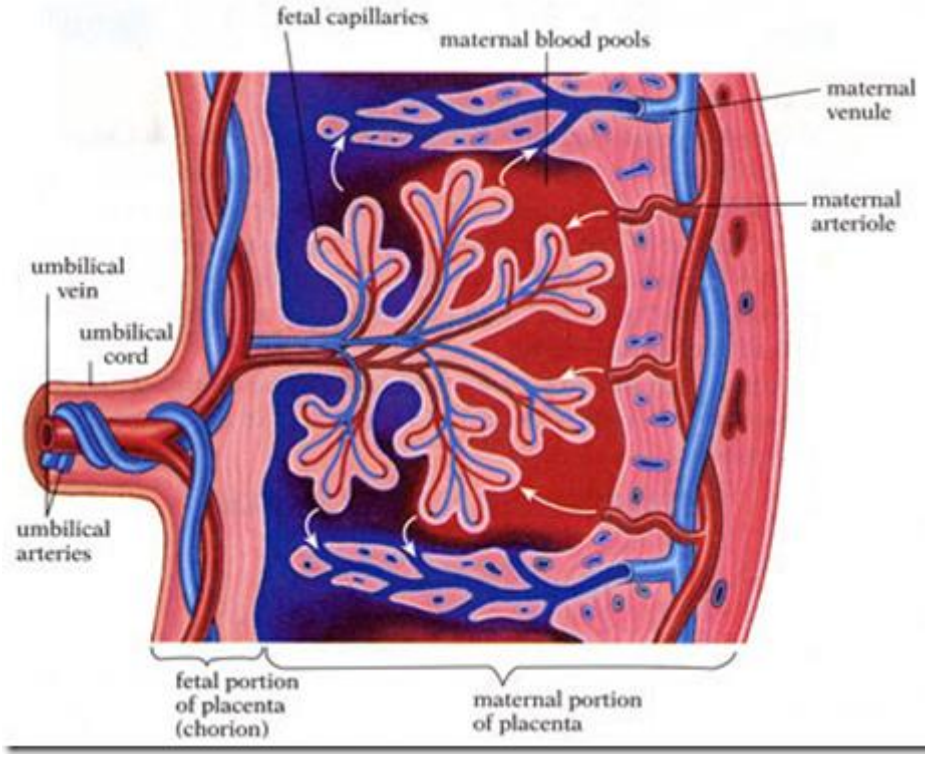

Figure 1.1: Structure of chorionic villus, Adapted from http://imueos.wordpress.com/2010/05/25/placenta-function/.

The precursor cells for the inner cell mass are formed following the first cell division of the fertilized ovum and develop into the fetus. The outer cell layer proliferates and forms the trophoblastic cell mass which differentiates into villous and extravillous cytotrophoblast (Chen and Olson, 2005). Cytotrophoblast is a mononucleated cell which plays a major role in the implantation and placental development (Malassine

and Cronier, 2002). Cytotrophoblasts situated between the syncytiotrophoblast and the basement membrane and are considered as the precursor cells for the syncytiotrophoblast (Handwerger, 2010). Throughout pregnancy, cytotrophoblast cells are separated from the developing fetus through a layer of syncytiotrophoblast (Knöfler et al., 2001). In extra villous space, cytotrophoblasts are characterised by invasive behaviour and allow penetration into maternal deciduae and myometrium (Pijnenborg et al., 1980). Groups of cytotrophoblasts grow rapidly to form the primary villi which are then converted to secondary villi following the invasion by foetal mesenchyme. Inside the mesenchyme, capillaries are formed which convert the secondary villi to tertiary (Castellucci et al., 1990b).

In the villous space, cytotrophoblasts fuse to form a syncytiotrophoblast, which forms continuous, uninterrupted, multinucleated cells situated in direct contact with maternal blood, with the function of ensuring fetal growth. In addition, it plays a major role in controlling feto-maternal exchange of nutrient and gases and also producing hormones such as oestrogen, human placental lactogen (hPL) and human chorionic gonadotropin (hCG) (Knöfler et al., 2001). Many studies have shown that, isolated cultured cytotrophoblasts in medium enriched with foetal calf serum have the ability to fuse forming a multinucleated syncytium which secrete hPL, hCG and steroids similar to hormones secreted by STB *in-vivo* (Kliman et al., 1986).

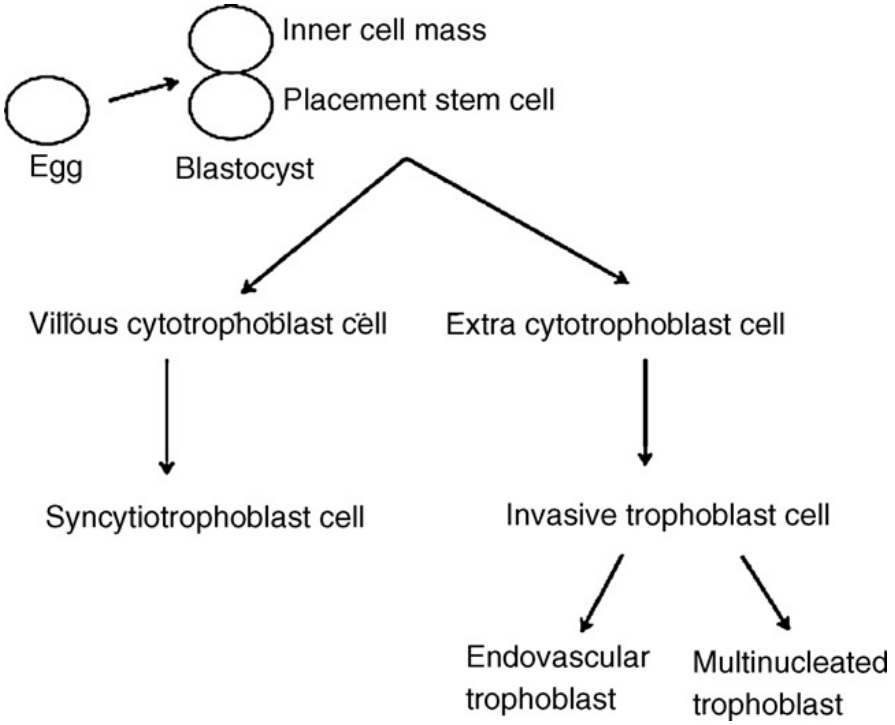

Figure 1.2: The cytotrophoblast two differentiation pathways.

The precursor cells for the inner cell mass are formed following the first cell division of the fertilized ovum which develops into foetus. The other cell forms placental stem cells which differentiate into villous and an extravillous cytotrophoblast, villous cytotrophoblasts differentiate into syncytiotrophoblast, whereas the extra villous cytotrophoblast differentiates into endovascular trophoblast. Adapted from (Handwerger, 2010).

1.1.1. Trophoblast fusion

Cell-cell fusion is a common and highly controlled process that occurs in almost all organisms including yeast, myoblast, placental trophoblast and macrophage fusion (Chen and Olson, 2005).

Syncytial fusion is a process in which the separating plasma membrane of two adjacent cells dissolute, resulting in assembly of a multinucleated structure termed as syncytium. This structure is derived and preserved by continued fusion of mono-

nucleated cells (Chen and Olson, 2004). This process does not occur spontaneously, but rather a number of different and distinct intracellular factors are required to activate and control this processes (Huppertz et al., 2006).

Like most highly differentiated cells, the syncytiotrophoblast does not possess any proliferative ability. Moreover, its nuclei demonstrate average rate of RNA synthesis and continuous fusion of cytotrophoblast with syncytiotrophoblast is essential to maintain this layer throughout pregnancy (Huppertz et al., 1998). Recently, a group of studies have demonstrated an association between disorganisation of syncytial trophoblast fusion and complication of pregnancy such as intrauterine growth restriction (IUGR) and pre-eclampsia (Huppertz and Kaufmann, 2002). In normal pregnancy, the endovascular trophoblast invades the uteroplacental arteries and modifies it into dilated and elastic vessels which is independent of maternal vasomotor control. In preeclampsia, both the interstitial and the endovascular invasion of trophoblast are reduced resulting in increased uteroplacental vascular resistance and hypertension (Kaufmann et al., 2003).

1.1.1.1. Fusogenic genes

Fusion of intracellular membranes is certainly associated with hormone secretion and vesicles uptakes (Huppertz et al., 2006). In human placenta, syncytiotrophoblast depends on syncytial fusion of cytotrophoblast cells for the contribution of new material. Syncytiotrophoblast necrosis and death result within days in the absence of constant fusion with cytotrophoblast (Castellucci et al., 1990a).

Furthermore, various studies have shown that syncytiotrophoblast contains fewer uridine than cytotrophoblasts, indicating that transcription is low in syncytiotrophoblast, and the syncytiotrophoblast depends largely on cytotrophoblast

for new protein synthesis (Huppertz et al., 1999). Around 8 percent of the human genome has a retroviral origin (Lander et al., 2001). However, research on non-defective endogenous retrovirus genes resulted in detection of 16 genes, two of them are capable of inducing cell to cell fusion and are highly expressed in human placenta (de Parseval et al., 2003, Mi et al., 2000). The discovery of the envelope (env) protein of human endogenous retrovirus (HERV-W) was the important discovery in the mechanism of syncytiotrophoblast fusion (Mi et al., 2000). Northern blot analysis demonstrated the expression of syncytin mRNA in placenta and testes (Mi et al., 2000). In addition, researchers have identified the presence of two homologus genes to human syncytin 1 and 2 in mice called syncytin A and B (Dupressoir et al., 2005).

Syncytin 1 and syncytin 2 are encoded by HERV-W and HERV-FRD genes respectively and play an important role in trophoblast fusion (Lee et al., 2001, Mi et al., 2000). It has been shown that fusion of BeWo cells and primary trophoblasts is inhibited following transfection with anti-syncytin 1 antiserum. Furthermore, syncytin 1 leads to fusion of non-fusogenic COS cells, which means that this protein is able to induce fusion in non-fusogenic cells. In addition, by using a specific monoclonal antibody, various studies have demonstrated that syncytin 2 is expressed in the cytoplasm and membrane of villous trophoblast. And in the second trimester, the expression of this protein was observed in the cytotrophoblast cells at the site of contact with syncytiotophoblast. (Malassine et al., 2007, Oren-Suissa and Podbilewicz, 2007, Vargas et al., 2009). Expression of syncytin-1 is observed in villous and extra villous cytotrophoblast (Frendo et al., 2003, Malassine et al., 2005). Malassine, et al have shown for the first time the expression of syncytin 2 in villous cytotrophoblast (Malassine et al., 2007).

1.1.1.2. Regulation of syncytin induced trophoblast fusion

The processes of trophoblast fusion is tightly controlled; uncontrolled fusion can lead to the exhaustion of a regenerative pool of villous cytotrophoblast, whereas a lower rate of fusion results in a function defect in syncytiotrophoblast (Pötgens et al., 2004). In addition to syncytin, different proteins have been suggested to be involved in the syncytia fusion process such as Glial Cell Missing (GCM1), a transcription factor that regulates syncytin expression (Black et al., 2004).

The D mammalian retrovirus (RDR, also known as ASCT2 or ATB^0 or SLC1A5) was identified as the syncytin 1 receptor (Blond et al., 2000). The addition of forskolin to BeWo choriocarcinoma cells and the reduction of oxygen concentration have no effect on the mRNA expression of RDR, even though both conditions affect syncytin-1 mRNA expression (Huppertz et al., 2006).

The major facilitator superfamily domain containing 2A (MFSD2A) is the receptor for syncytin 2 and it mediates its fusogenic activities, and both of them are expressed in the placenta (Esnault et al., 2008).

Recently, the relationship between the cell fusion process and PI3K/AKT signalling pathway (the upstream signalling of mTORC1) has been demonstrated by Vatish et al who have revealed that, in addition to an increased cAMP level, the fusion process is associated with an inhibition of PI3K/Akt pathway and a decrease in intracellular calcium level (Vatish et al., 2012).

One of the regulators of syncytin 1 expression is glial cell missing-1 (GCM1), which is a placental transcription factor that controls mRNA expression through two GCM1 binding sites (Yu et al., 2002). GCM was first isolated from Drosophila melanogaster

and two isoforms (GCM1 and GCM2) have been identified in humans and rodents (Kim et al., 1998).

The expression of GCM1 gene in mammals is tissue specific and is limited to thymus, placenta and kidney (Hashemolhosseini et al., 2002). The importance of this protein in placental development was confirmed by KO studies in mice that showed embryonic lethality due to a defect in syncytiotrophoblast development following deletion of GCM1 protein (Schreiber et al., 2000). Researchers have also demonstrated that in both BeWo cells and primary trophoblast cells, cAMP or cAMP-driven protein kinase A (PKA) pathway induces syncytin 1/2 and GCM1 expression (Knerr et al., 2005).

1.1.2. Human chorionic gonadotropin (hCG)

Human chorionic gonadotropin is a placental glycoprotein hormone which is necessary for human reproduction and fetal viability. It is a heterodimeric hormone which consists of β-subunit with 145 amino acids and α-subunit with 92 amino acids connected with disulphide bounds at cys110 and cys26 of β and α-subunits respectively (Lapthorn et al., 1994). The family of glycoprotein hormones also include follicle stimulating hormone (FSH), leutinizing hormone (LH) and thyroid stimulating hormone (TSH). The α-subunit is common to all glycoprotein hormones, while β-subunit is unique and gives each hormone its specific biological and immunological function (Pierce and Parsons, 1981).

The α-subunit of hCG hormone is encoded by a gene located on chromosome 6 (Fiddes et al., 1979) whereas β subunit is encoded by six genes located on chromosome 19 (Policastro et al., 1986). Measurement of hCG is used as a marker to monitor the

progress of successful pregnancy, ectopic pregnancy, Down syndrome and some hCG secreting tumour (Xing et al., 2001).

The β-subunit of hCG is used as a marker of pregnancy since it is the first hormone released by trophoblast after implantation. Its proposed functions include the stimulation of continuous secretion of steroid hormones by corpus luteum, modulation of implantation and regulation of immunological adaptation during pregnancy (Rull and Laan, 2005).

Shi et al have shown that hCG plays a role in trophoblast differentiation from cytotrophoblast to multinucleated syncytiotrophoblast which produces a large amount of hCG compared with cytotrophoblast (Shi et al., 1993). In theca-interstitial cells, LH/hCG stimulates mTORC1 signalling pathway through phosphatidylinositol-3-kinase/Akt pathway for the regulation of androgen biosynthesis (Palaniappan and Menon, 2010).

1.1.3. Signalling molecules in the placenta

1.1.3.1. Cyclic adenosine monophosphate (cAMP)

Cyclic AMP is involved, as a second messenger, in intracellular signalling in response to various hormones, neurotransmitters and growth factors. It plays a key role in many cellular processes such as cell fusion and gene transcription (Tasken and Aandahl, 2004). cAMP level is regulated by the enzyme adenylyl cyclase that controls cAMP synthesis and the enzyme cAMP phosphodiesterases4 (PDE4) which controls cAMP degradation (Houslay, 2010). cAMP activation includes binding of an extracellular protein to a G protein-coupled receptors (GPCR) which in turn activates the enzyme adenylyl cyclase resulting in the generation of cAMP. (Keryer et al., 1998). Once it is generated, it interacts with various cell proteins including cAMP dependent protein

kinas A (PKA), which regulates most of cAMP effects and consists of two classes known as type I and type II PKA (Tasken and Aandahl, 2004). PKA consists of two catalytic subunits (C) and one regulatory subunit (R), on each R subunit cAMP binds to two sites called A and B regulatory subunits. Three catalytic subunits (Cα, Cβ, and Cγ) and four regulatory subunits (RIα, RIβ, RIIα, and RIIβ) have been identified. RIα and RIIα are expressed in all cell types, whereas RIβ and RIIβ are tissue specific. Furthermore, RI isoform is localized mainly in the cytosol, whereas RII isoform is targeted to structures such as nuclear membranes, cytoskeleton and cytoplasmic organelles such as Golgi apparatus. Extracts from human placenta showed the expression of both PKA type I and type II (Keryer et al., 1998).

cAMP plays a key role in BeWo choriocarcinoma cells and primary trophoblast differentiation. Studies have shown that cAMP/PKA signaling pathway stimulates the expression of both syncytin and GCM1 in BeWo cells and primary trophoblast (Knerr et al., 2005). In addition, it has been shown that in BeWo cells ERK 1/2 and p38MAPK are the downstream effectors of adenylyl cyclase, and they play a key role in hCG secretion and fusogenic gene expression (Delidaki et al., 2011).

1.1.3.2. Mammalian target of rapamycin (mTOR)-The placental nutrient sensor

During the last decade mTOR, (also called FRAP, RAFT, RAPT, or SEP) (Wullschleger et al., 2006) has appeared as the main regulator of cell growth and metabolism. Abnormalities in mTOR signalling pathway have been linked to aging and to human diseases such as type 2 diabetes, cancer and obesity, which may indicate that targeting mTOR function could help treating many human diseases (Laplante and Sabatini, 2012).

mTOR is one of the phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family (PIKKs), together with ATM, ATR, DNA-PK, and hSMG1. They all have C-terminal protein kinase domains which are similar to the lipid kinase PI3K, hence giving the family its name. It is a huge protein (289kD) (Ballou and Lin, 2008) and is considered as an atypical serine/threonine protein kinase which incorporates various upstream signals to control growth-related processes involving mRNA translation, ribosomal biogenesis, autophagy and cellular metabolism (Laplante and Sabatini, 2012). While P1KKs family respond to geno-toxic stress, mTOR responds to stress related to nutrient and energy.

Furthermore, unlike PIKK family, in addition to its kinase domain, mTOR has many heat repeat in its N-terminal end as well as FAT domain of vague function (Sengupta et al., 2010). mTOR presents in two different complexes (figure 1.3), mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), mTORC1 consists mainly of mTOR, mammalian lethal with sec-13 protein 8 (mLST8) also known as GBL, DEP domain contains mTOR interacting protein (DEPTOR), raptor (regulatory associated protein of mTOR) and is considered to be rapamycin sensitive (Hara et al., 2002).

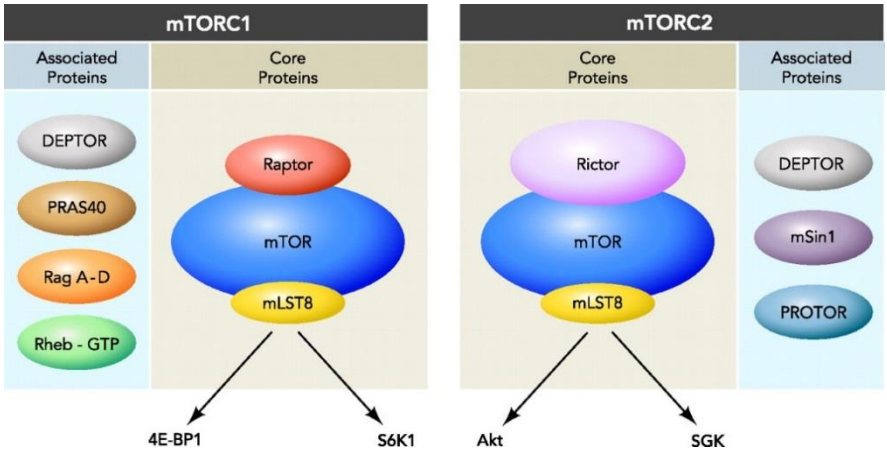

Figure 1.3: Protein component of mTORC1 and mTORC2

The core protein of mTORC1 are mTOR, raptor, and mLST8. In addition, it also binds to non-conserved proteins, which include PRAS40, DEPTOR, and Rheb-GTP. mTORC2 consists of core proteins mTOR, rictor, and mLST8, in addition to mSin1, DEPTOR, and PROTOR/PPR5. The mTORC2 regulates cell structure and survival by signaling to SGK and Akt. Adapted from (Frost and Lang, 2011).

Two members of this protein complex GBL and raptor, the two mTOR interacting proteins, are essential for the phosphorylation of S6K1 and 4E-BP1 (the downstream effectors of mTORC1) both *in vivo* and i*n vitro* (Kim et al., 2002).

Expression of the phosphorylated forms of P70S6K and the inhibition of the 4EBP1 are used as a measure of the activity of mTORC1 (Roos et al., 2007).

mTORC2, in addition to mTOR and GBL, contains the rapamycin insensitive component of mTOR (rictor) and mammalian stress-activated map kinase-interacting protein 1 (mSin1) (Laplante and Sabatini, 2012).

Compared to mTORC1, mLST8 is important for mTORC2 activation both *in-vivo* and *in-vitro* (Guertin et al., 2006). Similar to mTORC1, mTORC2 is inhibited when Deptor binds to the FAT domain of mTOR (Peterson et al., 2009).

mTORC2, by phosphorylating and activating the Akt (Ser473)/PKB, plays a role in controlling cell proliferation and survival (Sarbassov et al., 2005). Even though mTORC2 is said to be rapamycin insensitive, it has been mentioned that prolonged exposure to rapamycin leads to sequestration of mTORC1 which reduces the accessibility of the mTOR for the mTORC2 assembly (Sarbassov et al., 2006).

1.1.3.2.1 Domain structure of mTOR

The 2459 amino acid protein of mTOR consists of highly preserved domains (Kim et al., 2002), specifically the heat repeat sequence located in the NH2-terminal part of mTOR (Perry and Kleckner, 2003), the kinase domain located in the COOH-terminal, and it is similar to the catalytic domain of the PI3K (figure 1.4). Between the NH2-terminal and the kinase domain situated the FRB domain, which is the binding site for rapamycin-FKBP12 complex (Sekulic et al., 2000).

PRAS40 has been categorized as a negative regulator of mTORC1(Wang et al., 2007). The role of the other mTOR binding protein is still uncertain (Yip et al., 2010). Earlier studies showed that raptor may play a role in controlling mTORC1 assembly and mediating mTORC1 activity and subcellular localization (Hara et al., 2002).

It has been shown that the FATC domain is a very important for mTOR activity and the removal of even one amino acid from this domain inhibits mTOR activity (Peterson et al., 2000). Furthermore, some studies have suggested that both FATC and FAT domains act together to expose the catalytic domain of mTORC1 (Sekulic et al., 2000). Raptor is also important for mTORC1 activity since Raptor-free mTORC1 fails to phosphorylate 4E-BP1, but it is still able to phosphorylate S6K1 (Yip et al., 2010). However, prolonged loss of mLST8 has no impact on mTORC1 activity although it affects the assembly and action of mTORC2 (Guertin et al., 2006).

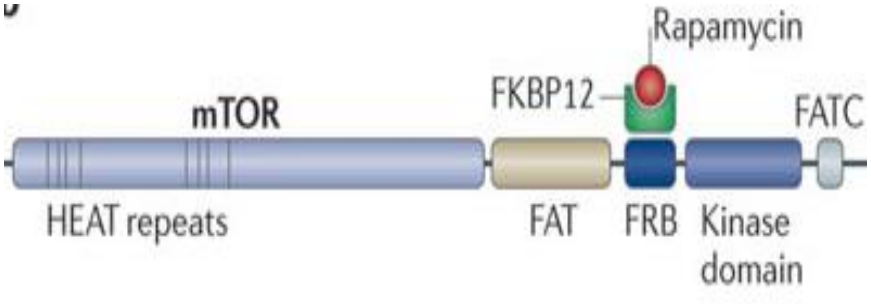

Figure 1.4: Domain structure of mTOR Adapted from (Bové et al., 2011).

1.1.3.2.2 Downstream effectors of mTORC1

mTORC1 plays a major role in controlling growth in response to upstream inputs (Sengupta et al., 2010). The activation of mTOR is initiated when Akt is phosphorylated through PI3K dependent or independent pathway leading to downstream activation of various cellular pathways. Akt inhibits the tuberous sclerosis complex 1 and 2 (Tsc1 & Tsc2) which are known inhibitors to mTOR. The activated Akt also leads to phosphorylation of mTOR at Ser2448, and subsequent phosphorylation (activation) of P70S6K at Thr389 and phosphorylation (inhibition) of eukaryotic initiation 4E binding protein-1 (4EBP-1). The later phosphorylation is required for completely activation of this pathway, since when it is bound to elf, it inhibits further protein synthesis (Hay and Sonenberg, 2004).

One of the main substrates of mTOR is the 40s ribosomal protein s6 kinas. S6K1, the main isoform of the S6k, regulates cell growth proliferation and differentiation by playing a major role in ribosome biogenesis, cell cycle progression and protein synthesis (Duvel et al., 2010).

P70s6k has been identified as the kinase that controls the processes of phosphorylation of 40S ribosomal protein S6. Inhibition of p70s6k activation *in-vivo* by the

immunosuppressant rapamycin will interfere with the ability of the cell progression through G1 phase of the cell cycle (Ferrari et al., 1993). Since it plays a role in cell growth and insulin sensitivity, abnormal activity of S6K1 has a major role in the progression of tumours, diabetes mellitus, obesity and aging (Dann et al., 2007). mTORC1 phosphorylate S6K1 at Thr389 (Fingar and Blenis, 2004). For P70s6k Ser 371, it has been mentioned that, Ser371 is crucial for Thr389 phosphorylation and in turn S6K1 activity. However, it is still vague how Ser371 is phosphorylated (Dann et al., 2007).

Protein synthesis

Cell growth requires a high energy level in order to keep protein synthesis at a high rate. Since this process is started when adequate levels of nutrient (amino acid and energy) and growth factor are available, mTOR is believed to control protein translation by integrating environmental signals from amino acids and growth factors (Sengupta et al., 2010).

mTORC1 regulates the entire translational processes and also the translation of messenger RNA (mRNAs) to stimulate cell growth and proliferation. *In vitro* studies have shown that mTOR controls protein synthesis partly by phosphorylating the downstream signalling p70s6k and eukaryotic initiation factor 4E (elF-4E) binding protein 1 (4E-BP1). These two compounds subsequently facilitate translation and protein synthesis (Ma and Blenis, 2009). When dephosphorylated, the 4E-BPs inhibits mRNA translation (Pause et al., 1994), this inhibitory effect is blocked when mTORC1 induces 4E-BP1 phosphorylation (Dowling et al., 2010).

Regulation of autophagy

Autophagy is a physiological process in which cells degrade cytoplasmic proteins and organelles inside lysosomes (Klionsky, 2007). Production of energy and protein synthesis can be maintained through autophagy since the degradation of protein complexes and cellular organelles provide the anabolic processes with biological material (Laplante and Sabatini, 2009).

mTORC1 is the key regulator of autophagy; when growth factors and nutrients are available, mTORC1 inhibits autophagy initiation (Noda and Ohsumi, 1998). Autophagy promoting states include starvation, growth factor deficiency, infection and oxidative stress. mTORC1 is also reactivated when amino acids are released from the degraded proteins, and the activated mTORC1 will eventually restore the intracellular lysosome population (Yu et al., 2010).

This process is required for cellular adaptation during starvation and for the recycling of dead organelles. Inhibition of mTORC1 leads to the formation of autophagosomes which results in the degradation of the cellular components (Laplante and Sabatini, 2012)**.** Autophagy is strongly stimulated by the inhibition of mTORC1 (Martina et al., 2012).

mTORC1 regulates autophagy via a group of protein complexes which consists of un-51-like kinase 1 (ULK1) that is the key protein in autophagy initiation. Focal adhesion kinase family-interacting protein of 200kD (FIP200), and autophagy-related gene 13 (ATG13) (Laplante and Sabatini, 2009). ULK1, ATG13 and FIP200 form a complex which activates ULK1 (Ganley et al., 2009). In rich nutrient conditions, mTORC1 binds to the Ulk1-Atg13-FIP200 complex, by direct binding of Raptor to Ulk1/2, mTOR then phosphorylates ATG13 leads to down regulation of ULK1 kinase, thereby

inhibiting autophagy. In addition, AMPK indirectly leads to the induction of autophagy by inhibiting mTORC1 through phosphorylation of Raptor or TSC2 (Ravikumar et al., 2010).

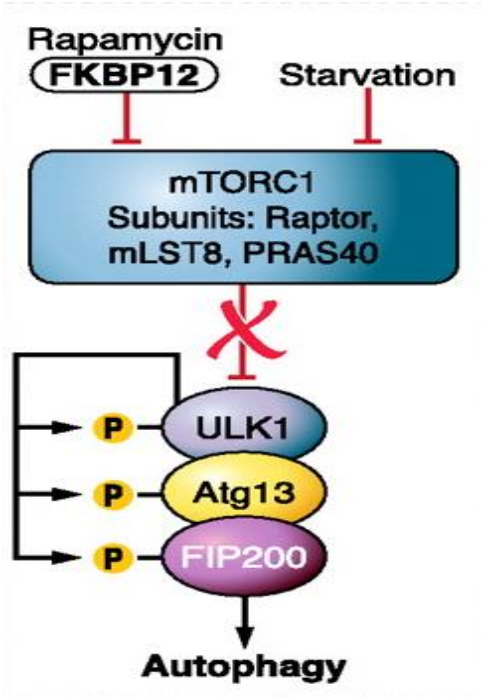

Figure 1.5: m TORC1 and autophagy regulation

Ulk1 form complex with Atg13, and FIP200. Under rich growth conditions, mTORC1 phosphorylates Ulk1 and Atg13, thus inhibiting the Ulk1/2 kinase activity. In starvation, Ulk1/2 are rapidly dephosphorylated, and Ulk1/2 phosphorylates Atg13 and FIP200 converting the complex to the pre-autophagosomal membrane and autophagy initiation. Adapted from (Ravikumar et al., 2010).

Nutrient transport

Some researchers have suggested that amino acids transporters are among the down streams of mTORC1 (Edinger, 2007). Some supporting studies reported the inhibition of mRNA expression of some amino acid transporters when BJAB (EBV-negative Burkitt's lymphoma) cell line was treated with rapamycin (Peng et al., 2002). Furthermore, it was reported that in Drosophila, the surface expression of cationic amino acid transporters is stimulated by mTOR (Hennig et al., 2006).

In L6 myotubes, rapamycin inhibits the up-regulation of system A amino acid transporter activity by leucin (Peyrollier et al., 2000). Amino acids are transported across the plasma membranes by transporter proteins, the activity of three of them has been shown to be altered when foetal growth is changed (Roos et al., 2009).

The activity of System A transporter (transports small neutral amino acids such as alanine, glutamine and serine) has been found to be increased in microvillous plasma membrane (MVM) isolated from pregnancy complicated with fetal overgrowth. However, the activity of this system was shown to be down regulated in IUGR. The other amino acids transport system is system L which transports big amino acids such as leucine was found to be down regulated in MVM and basement plasma membrane of pregnancy with IUGR (Jansson et al., 2002, Mahendran et al., 1993).

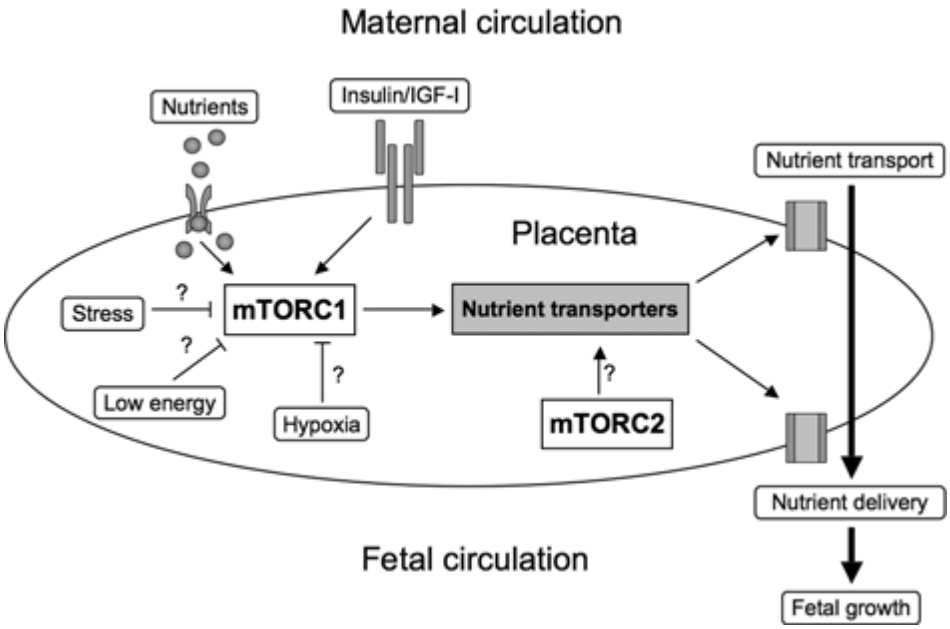

Figure 1.6: A model of mTOR signalling pathway regulating nutrient transport across placenta adapted from (Roos et al., 2009).

Lipogenesis

Many studies suggested that mTORC1 plays a role in adipogenesis since the inhibition of mTORC1 with rapamycin leads to impairment of adipogenesis. In contrast, up regulation of mTORC1 signalling, by silencing TSC1/2 expression through siRNA (small interfering RNA), induces adipogenesis. The downstream signal transducer of mTORC1, 4E-BP1 is thought to mediate this effect by regulating the translation of peroxisome proliferator-activated receptor (PPARg) (Lamming and Sabatini, 2013). In rat hepatocyte, mTORC1 has been shown to be involved in regulating the transcription factor Sterol Regulatory Element Binding Protein (SREBP) which is involved in activation of lipogenic enzymes (Owen et al., 2012).

Another evidence for the role of mTORC1 in lipogenesis is the failure of mice with S6K1gene knockdown to gain weight because of defective adipocytes generation (Carnevalli et al., 2010).

Negative feedback regulator of IRS1 by S6K1

The presence of negative feedback loop from mTORC1-S6K1 signalling pathway to the IRS-PI3K-PDK1-Akt pathway has been shown by various studies (Harrington et al., 2004, Um et al., 2004). S6K1 directly phosphorylate and inactivate IRS1 function leading to uncoupling of IRS-1 from PI3K resulting in PI3K inhibition (Um et al., 2004). It has been postulated that the inhibition of insulin-PI3K signalling is related to metabolic disorder such as obesity and diabetes. Aberrant activation of mTOR signalling pathway because of TSC deficiency or increased nutrient might lead to the inhibition of PI3K pathway and contribution in development of insulin resistance and type 2 diabetes (Wullschleger et al., 2006).

1.1.3.2.3 The upstream effectors of mTOR

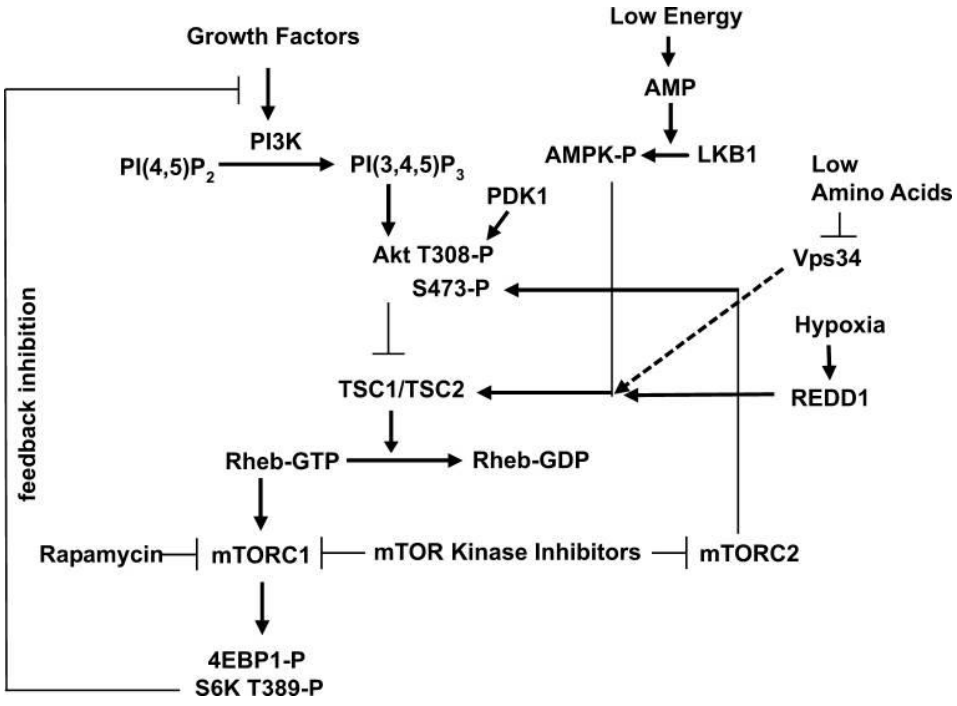

Figure 1.7: mTOR signalling pathway

Activation of PI3K by growth factor resulted in increased production of PI3, 4, 5-triphosphate. Akt then binds to PI (3, 4, 5). P3 leads to phosphorylation and activation of Thr308 by PDK1and Ser 473 by mTORC2. The complex, TSC1/TSC2, inhibits m TORC1 by converting the GTPase activity of Rheb to inactive GDP-bound form.TSC1 /TSC2 complex can be phosphorylated and inhibited by Akt which maintains the Rheb in the active GTP-bound form. AMPK activates TSC1/TSC2 complex which in turn inhibits mTORC1, as in low energy conditions. adapted from (Ballou and Lin, 2008).

Growth factor

Growth factors control mTOR signalling pathway through PI3/Akt pathway. Insulin or insulin like growth factor (IGF) binding to their corresponding receptors leads to phosphorylation of insulin receptor substrate (IRS-1) and PI3K recruitment. The association of PI3K to IRS-1 phosphorylate phosphatidylinositol-4,5-phosphate (PIP2) to phosphatidylinositol-3,4,5-phosphate (PIP3) (Wullschleger et al., 2006)).

Akt controls mTORC1 via tuberous sclerosis complex (TSC). TSC1 & 2 form a complex that can convert Rheb to its inactive GDP-bound and consequently suppresses mTORC1 activity. However, IGF-1 activates Akt which in turn phosphorylates TSC2 at Ser939 which will maintain Rheb in its GTP-bound forms to stimulates mTORC1 down signalling to S6K and 4EBP1 (Ballou and Lin, 2008).

A main advance in the understanding of regulation of mTOR signalling by growth factor and Akt was through the discovery of TSC1 and TSC2 proteins (also known as hamartin and tuberin respectively) as the upstream regulators of mTORC1. TSC1 and TSC2 are encoded by tuberous sclerosis complex 1 (TSC1) and tuberous sclerosis complex 2 (TSC2) genes (Cheadle et al., 2000). In Drosophila, mutation in TSC1 or TSC2 leads to increased cell and organ size (Hay and Sonenberg, 2004).

Nutrient

Amino acid has been suggested to stimulate mTORC1 activity by inhibiting TSC1/2, or through activation of Rheb, (Gao et al., 2002). Amino acids limited availability, especially the unavailability of leucine, leads to inactivation of S6K1 and 4E-BP1. In contrast, in a condition where leucine availability is restored phosphorylation and activation of S6K1 and 4E-BP1 are retained in an mTORC1 dependent way (Hay and Sonenberg, 2004). Previous studies have shown that silencing TSC1/TSC2 expression leads to amino acids deprivation resistance, which indicates that TSC1/TSC2 might play a role in mTOR regulation by amino acids (Gao et al., 2002).

Energy and Oxygen level

mTORC1 is affected by the energy condition of a cell through AMP-activated protein kinase (AMPK). Low energy, high adenosine monophosphate/adenosine triphosphate

(AMP/ATP) ratio and low oxygen level lead to activation of AMPK, which is considered to be one of the major mTOR controlling substances and an intracellular energy-sensor which is stimulated by even minimal change in ATP level (Kahn et al., 2005). The upstream kinas for AMPK is suggested to be the tumour suppressor LKB1 which in conjunction with AMP responds to energy deprivation by activating AMPK, which consequently activates TSC2 leading to down regulation of mTORC1 signalling (Wullschleger et al., 2006).

An increase in AMP/ATP ratio activates TSC1 and converts Rheb to its GDP form resulting in inhibition of mTORC1. When activated, AMPK inhibits energy requiring processes such as protein synthesis and up regulates ATP-producing processes like fatty acid oxidation (Inoki et al., 2003).

The effect of AMPK mediated pathway on mTOR activity can be demonstrated by measuring S6K1 phosphorylation (Kimura et al., 2003). In agreement, cell exposure to 5-aminoamidazole 4-carboxyamide (AICAR, an AMP analog), inhibits S6K1 and 4E-BP1 phosphorylation, leading to the inhibition of mTORC1 signalling (Inoki et al., 2003). A study by Kinura et al has shown that the mTOR and AMPK signalling pathways are linked, the evidence for this is that, AICAR inhibits p70s6k phosphorylation in a time dependent manner, second the reagents that activate AMPK inhibit p70s6k in various cell lines including HEK293 cells line, third rapamycin-resistant p70s6k is also resistant to AICAR (Kimura et al., 2003).

In addition, researchers have shown that mTORC1 might act as a sensor to the hypoxic status of the cell via two homologues proteins; the expression of transcriptional regulation of DNA damage protein response 1 and 2 (REDD1 and REDD2) (Brugarolas et al., 2004). REED is the downstream effector of AKT and the upstream

of TSC1/2. Hypoxia up regulates REDD1 that stimulates TSC2 and subsequently inhibits mTORC1. REDD also inhibits mTORC1 through LKB1/AMPK signalling pathway. Furthermore, prolonged hypoxia resulted in increased AMP/ATP ratio and activation of AMPK signalling (Wullschleger et al., 2006).

1.1.3.2.4 mTORC2

mTORC2 was believed to be rapamycin insensitive since acute treatment with rapamycin does not affect mTORC2. Rapamycin-FKBP12 complex (this complex binds to and inhibits mTORC1) does not bind to mTORC2, since mTORC2 lack Raptor (Sarbassov et al., 2004). mTORC2, unlike mTORC1, does not respond to nutrient but it does respond to insulin through PI3K dependent mechanism (Laplante and Sabatini, 2012). In addition to its activation by PI3K-P1P3, AKT is also activated by mTORC2 by phosphorylation at the hydrophobic site (Ser473), while the kinase that phosphorylates Akt at Thr308 is PDK1 (Sarbassov et al., 2005). mTORC2 plays major roles in proliferation, metabolism and cell survival. Those processes are highly dependent on Akt activation, mTORC2 phosphorylates (and consequently activates) Akt at Ser473 which stimulates these processes through various effectors such as the transcription factors forkhead box protein O1 (FoxO1) and FoxO3a, the later control gene expression implicated in cellular metabolism, stress resistance, apoptosis and cell cycle arrest. Cytoskeleton organization is also controlled by mTORC2. Some studies have shown that mTORC2 knock down negatively affects actin polymerization and they proposed that mTORC2 regulates actin cytoskeleton by phosphorylating and activating protein kinase Cα; however, the mechanism by which mTORC2 controls this has not been identified (Laplante and Sabatini, 2009).

1.1.3.2.5 Rapamycin

Rapamycin is a macrocyclic antibiotic generated by the bacterium streptomyces hygroscopicus, a streptomycete that was found in a soil sample from Rapa Nui Island in the early 1970s (Sehgal, 1998). Initially, it was used for its antifungal activity, but it was also discovered to have a potent immunosuppressive effect (which was considered to be undesirable side effect) since it inhibits the proliferation of B and T cells. It also inhibits mammalian cell proliferation and has anti-tumour activity (Sehgal, 2003). The PI3K/Akt/mTOR pathway controls cell growth and metabolism, a function that is affected by rapamycin and other mTOR kinas inhibitors (Xue et al., 2009).

Mechanism of action of rapamycin

The discovery of mTOR came as a result of studies investigating the intracellular target of the rapamycin-FKBP complex (Brown et al., 1994). Early studies have reported that binding rapamycin-FKBP complex to mTORC1 results in structural changes that weaken mTOR-raptor assembly (Kim et al., 2002). Rapamycin belongs to a group of macro-cyclic immunosuppressive agents that bind to a specific cytosolic binding proteins in order to produce its cellular action. Rapamycin and FK506 bind to the same immunophyllins known as FK506 binding proteins (FKBPs) (Sehgal, 1998).

Even though there is no doubt that rapamycin-FKBP1 complex inhibits the kinase activity of mTOR to block downstream signals *in-vivo* (Brown et al., 1995), the mechanisms of inhibition of mTOR activity by rapamycin remain unclear (Fingar and Blenis, 2004).

Various FKBP family have been identified, genetic studies have reported that the 12kDa FK506 binding protein (FKBP12) is the most important binding protein with

which rapamycin forms a complex that interacts with and inhibits mTOR when it is an element of mTORC1 (since mTORC2 lack raptor), to produce its immunosuppressive effect (Brown et al., 1994).

The amino acid terminal of mTOR has FKBP12-rapamycin binding domain (FRB domain) that is located between the kinase and FAT domains of mTORC1 (Figure 1.4). FKBP12-rapamycin complex binds to FRB domain and exerts its inhibitory effect.

- This mechanism allows the inhibition of phosphorylation/activation of p70S6K and translation of special mRNA encoding protein (Dumont and Su, 1996).
- mTORC1 auto phosphorylation is also inhibited by rapamycin/FKBP12 complex, this complex also weakens mTOR activity through destabilizing mTOR-Raptor complex (Kim et al., 2002).
- mTOR also stimulates protein translation by phosphorylating eIF-4E binding proteins (4E-BP1) (Brunn et al., 1997), when phosphorylated, 4E-BP1 affinity for eIF-4E is decreased. This leads to translation of mRNAs and consequently cell growth and proliferation, these activities are inhibited by rapamycin (Sehgal, 2003).

However, some studies argued that rapamycin appears less effective in the treatment of some cancers possibly due to the loss of negative feedback effect of mTORC1/S6K on Akt which leads to phosphorylation of Akt through mTORC2 at Ser473 (Laplante and Sabatini, 2012).

Furthermore, upon activation by mTORC1, S6K phosphorylates insulin receptor substrate-1(IRS-1) which reduces the capability of GFs to activate receptor tyrosine

kinase and when P70S6K is inhibited by rapamycin, this negative feedback effect of P70S6K is lost (Harrington et al., 2004).

Recently a model for the inhibition of mTORC1 by rapamycin has been suggested where the binding of FKBP12-rapamycin complex to mTORC1 leads to conformational alteration in mTOR which affects the interaction between mTOR and raptor. Furthermore, the rapamycin-FKBP12 complex blocks the binding site for S6K1. The binding of another complex to mTORC1 or augmented effect of the first complex causes a complete fragmentation of mTORC1 and elimination of 4E-BP1 phosphorylation (Yip et al., 2010).

1.1.4. Models for studying human placental function

BeWo cell is a human trophoblast choriocarcinom-derived cell line, characterised by cytotrophoblastic properties and reserve numerous placental differentiation markers. They also have the ability to produce human chorionic gonadotrophin (hCG), alkaline phosphatase and placental lactogen (hPL) (Nickel et al., 1993).

BeWo cells have a slow fusion rate which can be initiated by treating BeWo cells with forskolin (Wice et al., 1990). The incubation of these cells with forskolin resulted in the activation of adenylyl cyclase (AC), and an increased production of Cyclic AMP (cAMP). cAMP in turn induces the differentiation of cytotrophoblast to syncytiotrophoblast and syncytia formation, the fused cytotrophoblast then produces various hormones such as human chorionic gonadotropin (hCG) similar to first trimester trophoblasts (Orendi et al., 2010). 8-Bromoadenosine-3',5'-cyclic monophosphate (8-Br-cAMP) an exogenous cAMP analogue can also be used to induce cell differentiation (Chen et al., 2011).

Morphological appearance of undifferentiated BeWo cells is similar to primary trophoblast with nearby cells position with apical projection, (Bode et al., 2006).

Unlike BeWo cells, JEG-3 cells do not fuse in response to cAMP analogue, but they have the ability to secrete hormones like hPL, estradiol, estrone, hCG and progesterone (CHOU, 1982, Kohler and Bridson, 1971).

Coutifaris *et al* have examined E-cadherin, the Ca^{+2} dependent cell adhesion molecule, during the differentiation of BeWo cells, JEG-3 cells and cytotrophoblast cells from human chorionic villi. They noticed that only JEG-3 cells were unable to form multinucleated syncytium (Coutifaris et al., 1991).

In this work I have used the BeWo choriocarcinoma cell line to investigate the effect of mTOR on their fusigenic capacity and hCG production and this is achieved by using BeWo cells in their undifferentiated (forskolin untreated) and differentiated (forskolin treated) forms.

1.1.5. Gestational diabetes mellitus as a disease of placenta

Gestational diabetes mellitus (GDM) is defined as glucose intolerance of any degree with onset or first detection during pregnancy (Metzger, 1991), and is characterised by decreased glucose clearance monitored during oral glucose tolerance test (Bari et al., 2014).

According to the NICE Guideline 2015 (NICE, 2015), GDM is diagnosed if the woman has either

- A fasting plasma glucose level of 5.6 mmol/l or above or
- A 2-hour plasma glucose level of 7.8 mmol/l or above.

It has been estimated that 2-3% of all pregnancies are complicated by diabetes, and the gestational diabetes represents 90% of all cases of diabetes diagnosed during pregnancy (Bentley-Lewis et al., 2008). In spite of development in clinical management of diabetes, there is still increased perinatal morbidity in pregnancy complicated by GDM (Jansson et al., 2002). GDM is characterised by decreased insulin secretion associated with insulin resistance (Vejrazkova et al., 2014). The nature and extent of structural and functional changes in placenta of GDM depend on glycaemic control and modality of treatment (Desoye and Hauguel-de Mouzon, 2007).

1.1.5.1. Pathophysiology

The role of insulin is to regulate the storage and release of glucose, amino acid and fat. Insulin stimulates the enzyme lipoprotein lipase resulted in lipolysis of triglycerides in blood and release of free fatty acids (FFA). Insulin stimulates the transport of FFAs to adipocytes which are then stored as lipids. In addition, by binding with insulin receptors, it stimulates glucose entrance to the cells and enhances the storage of some glucose as glycogen (Sonksen and Sonksen, 2000). However, pregnancy itself is potentially diabetogenic. Prolactin, cortisol and progesterone and hPL; these hormones inhibit IRS-1 phosphorylation leading to profound insulin resistance (Kuhl, 1998).

GDM is connected to placental function and a number of placental hormones and molecules have been involved in insulin resistance in GDM (Desoye and Hauguel-de Mouzon, 2007). Human pregnancy is associated with a series of metabolic changes that result in adipose tissue growth in early pregnancy and insulin resistance with increased lipolysis in late gestation. Early pregnancy is also associated with increased insulin secretion from pancreatic β-cell, however, insulin resistance is either decreased, unchanged or even increased. Late pregnancy is characterized by insulin

resistance and increased lipolysis with increased free fatty acids formation. To maintain euglycemia in the mother, normal pregnancy is associated with 200% increase in insulin secretion and 50% decrease in glucose disposal by insulin (Barbour et al., 2007).

The changes in late pregnancy involve decreased insulin receptors phosphorylation, reduced expression of IRS-1 and inhibited phosphoinositide 3-kinase (PI3-kinase) response to insulin, the purpose of these changes is to spare glucose, amino acids and fatty acids for the fetus (Vejrazkova et al., 2014).

GDM characterized by decreased insulin secretion coupled with increased insulin resistance induced by pregnancy. At cellular level, women with GDM show signs of subclinical inflammation with increased TNF-α, which is shown to impair insulin activity by inhibiting adiponectin, (insulin-sensitizing hormone) and subsequently reducing insulin receptor tyrosine kinase activity. Insulin receptors are highly expressed by placenta relative to other tissues and their location develops and changes as pregnancy progresses. Early in gestation, they are located in syncytiotrophoblast microvillous membrane, whereas at term, they are mainly found in the endometrium (Desoye et al., 1994).

Tumour necrosis factor α (TNFα) has also been shown to play a role in GDM. All cytokines including TNFα, leptin and resistin are expressed by placenta and adipocytes. Some of these cytokines play a major role in controlling insulin action which might indicate a possible interaction between placenta and adipose tissue in the pathophysiology of GDM and insulin resistance (Desoye and Hauguel-de Mouzon, 2007).

In adipose tissue, the peroxisome proliferator-activated receptor γ (PPARγ) decreases with increased lipolysis and acceleration of IR of the adipose tissue. This will lead to the elevation of post prandial FFAs and an increased glucose production by the liver. Thus, decreased adiponectin production, effect of placental hormones, in addition to increased lipolysis, all these factors result in severe insulin resistance (IR) in muscle, adipose tissue and liver in women with GDM (Barbour et al., 2007).

As mentioned above, many factors contribute to the development of insulin resistance in GDM which include changes in growth hormones and cortisol secretion which act as insulin antagonists, secretion of human placental lactogen which alters fatty acid and glucose metabolism that results in increased lipolysis and decrease glucose uptake, in addition to oestrogen and progesterone which affect insulin glucose balance (Ural and Repke, 2008).

1.1.5.2. The Risk factors for GDM

Various risk factors are associated with the development of GDM. According to the Nice guideline (NICE, 2015) the following are considered as risk factors for developing GDM:

- history of macrosomia (birth weight > 4.5kg),
- family origin of an ethnic group with a higher rate of type II diabetes (South Asian, black Caribbean, Middle Eastern),
- high body mass index (above 30kg/m^2),
- and family history of diabetes (first-degree relative with diabetes).

Other risk factors include history of unexplained stillbirth and essential hypertension and hypertension related to pregnancy (Xiong et al., 2001).

It has been shown in a meta-analysis of literature which estimated that the risk of developing GDM in obese and severely obese women is four and eight times respectively compared to normal weight pregnant women (Chu et al., 2007). Furthermore, it has been found that obese pregnant women are found to have elevated fasting insulin level with increased insulin resistance compared with lean pregnant women (Catalano et al., 2003).

Maternal age older than 25years, in addition to previous history of GDM and persistent glucosuria, are also known risk factors for GDM. However, in 50% of GDM no risk factors were identified (Xiong et al., 2001, Ural and Repke, 2008).

1.1.5.3. Consequences of GDM

Normal pregnancy requires adequate proliferation and invasion of trophoblast throughout implantation and placentation. However, the changes in maternal environment in diabetes can affect this processes (Weiss et al., 2001). GDM is among the complications that interfere with normal placental and foetal development which may lead to increased incidence of congenital malformation and growth retardation in the foetus. Exposure of placenta to diabetic environment may result in functional and structural abnormalities including villous immaturity, increase in the surface area of exchange and thickening of the basement membrane, inadequate transfer of nutrient from mother to foetus may result in abnormal growth of the developing foetus (Desoye and Hauguel-de Mouzon, 2007). It has been reported by various studies that hyperglycaemia prevents proliferation of cell-lines of first trimester model such as BeWo cells, which indicates that hyperglycaemia resulted in impairment of placental growth in the first trimester of uncontrolled diabetic pregnancy (Weiss et al., 2001).

Since glucose is transferred easily across the placenta, states of maternal hyperglycaemia would be associated with foetal hyperglycaemia. Among those complications is foetal macrosomia which is characterised by an excessive amount of fat deposition and large shoulders. Macrosomic babies are prone to many complications during labour, such as shoulder dystocia. Infants of diabetic mothers are prone to hypoglycaemia due to excessive insulin and the cessation of transplacental glucose delivery from maternal blood. Furthermore, premature infants are predisposed to respiratory distress syndrome, in addition to the risk of developing obesity and type 2 diabetes later in life (Coustan, 2013). Macrosomia in GDM may lead to cephalo-pelvic disproportion which indicates caesarean section (Crowther et al., 2005). Epidemiological studies of GDM have shown that GDM associated with maternal obesity and women with GDM are at an increased risk of obstetric complication and future occurrence of type 2 diabetes mellitus (Feig et al., 2008).

1.1.5.4. Screening for gestational diabetes

In women with risk factors the test used for gestational diabetes is the 2-hour 75 g oral glucose tolerance test (OGTT) (NICE, 2015). Women with previous history of GDM should be offered early self-monitoring of blood glucose or a 2-hour 75 g OGTT after booking (whether in the first or second trimester), and a further 75 g 2-hour OGTT at 24–28 weeks if the results of the first OGTT are normal. The 75g OGTT should be offered at 24-28 weeks for women with other risk factors for gestational diabetes (NICE, 2015).

As mentioned above, gestational diabetes is diagnosed if a woman has either (NICE, 2015)

- a fasting plasma glucose level of 5.6 mmol/litre or above or
- a 2-hour plasma glucose level of 7.8 mmol/litre or above.

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1.1.6. Association between mTOR signalling pathway and placental pathology

In a study conducted by Wen et al in immortalised cell line (HTR-8/SVneo), which originates from human trophoblast, they have shown that growth factor angiopoietin-2 (Ang-2) and glucose stimulate trophoblast differentiation through the mTOR signalling pathway. The same study has demonstrated that, activation of mTOR pathway by Ang-2 was PI-3 kinase dependent, whereas glucose induced mTOR up regulation was PI-3 kinase independent. This identified mTOR as an important player of regulating placental trophoblast proliferation (Wen et al., 2005). In addition it has been suggested that invasive trophoblast differentiation is regulated by mTOR signalling pathway (Pollheimer and Knöfler, 2005).

Furthermore, a study conducted by Jansson et al reported that low protein diet in pregnant rats is associated with decrease placental activity of insulin, leptin and mTOR signalling pathway as a result of maternal protein restriction, which indicates that maternal hormones and placental mTOR signalling might play a role in regulating placental amino acid transport mechanism (Jansson et al., 2006).

mTOR is shown to be a key placental signalling pathway that regulates placental system A activity, and inhibition of mTOR in placenta reduces the activity of system L amino acid transporter (Roos et al., 2007). mTOR functions as an integrator of signalling molecule (such as hormones, growth factors, and nutrient) of maternal, foetal, and placental origin. In pregnancy complicated by IUGR, there is decreased activity of mTOR signalling, while in pregnancy complicated by obesity, mTOR

activity of placenta increased and might contribute in the high risk of delivering large babies (Lager and Powell, 2012).

1.2. Thesis Hypothesis and Aims

Vatish et al have shown that in addition to the increased cAMP, the process of BeWo cells fusion is associated with reduced intracellular calcium and inhibition of Type 1 tphosphatidyinositol 3 kinase (PI3K)/Akt signalling (Vatish et al., 2012). Since PI3K/Akt is the up-stream signalling of mTOR and mTOR signalling pathway is associated with many placental pathology and intrauterine growth restriction, we hypothesised that mTOR regulates the function of differentiated (forskolin treated) BeWo cells. We aimed to characterise the role of mTOR and its downstream signalling in placental biology.

To accomplish this aim, we have used the BeWo cells which have the ability to differentiate and are a well-established *in-vitro* model to study placental biological function (Manley et al., 2005).

In the BeWo cell line we investigated the role of mTOR in the following:

(a) Expression of fusogenic genes such as syncytin 1 and 2.

(b) Endocrine capacity of cells during differentiation and syncytialization by monitoring secretion of human chorionic gonadotropin (hCG) which is also a hormonal marker of differentiation and by measuring hCG we also monitor biochemical differentiation.

(c) Expression of molecules important for placental endocrine function such as 11-βHSD2.

(d) The effect of BeWo cells differentiation on expression of mTOR and its related components.

In placenta, we measured the expression of mTOR and its related components in placentae obtained from normal pregnancy compared with placenta from pregnancy complicated with GDM.

Chapter Two

Materials and Methods

2.1Materials

The listed chemicals obtained from different companies are as follows:

2.1.1. Chemicals and Solvents

Product	Producer or Supplier
Absolute Alcohol	Hayman Ltd, Witham, UK
Acetic acid	Sigma-Aldrich
Acrylamide	Roche, Lewes, UK
Agarose	Gibco BRL, Paisley, UK
Ammonium persulphate	Sigma-Aldrich
Bovine albumin serum (BSA)	Sigma-Aldrich
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich
Ethedium Bromide	Sigma-Aldrich
Ethanol	Hayman Ltd, Witham, UK
Isopropanol	Sigma-Aldrich
Methanol	Hayman Ltd, Witham, UK
TRITON X-X100	Sigma-Aldrich

2.1.2. Enzymes

The product	Producer or supplier
dNTP(deoxynucleotide triphosphates)	Bioline
DNase 1 (RNase free)	Qiagen
Random hexamers	Promega
Reverse transcriptase	Fermentas/Promega
RNase Inhibitor	Fermentas/Promeg
Taq DN Polymerase	Fermentas/Promega
Trypsin (0.25%)	Sigma-Aldrich
5x buffer	Fermentas/Promega

2.1.3. General buffers and solution

- **DNA loading buffer**

  1m Tris-HCL pH 7.5 40% glycerol (v/v), 0.1% bromphenol blue (w/v).

- **Dulbecco's Phosphate buffered saline (PBS)**

  0.132g$CaCl_2$ x $2H_2O$, 0.2gKCl, KH_2PO_4, 0.1gMCl_2 x $6H_2O$, 8.0gNaCl, 1.15g Na_2PO4, up to 1000ml in distilled water.

- **Pen/Strep**

  100u/ml penicillin and 100 µg/ml streptomycin in PBS.

- **Protease Inhibitor Cocktail**

  1.6mg/ml benzamidine HCL, 1mg/ml phenanthroline, 1mg/ml aprotinin, 1mg/ml pepststin A, 1mg/ml leupeptin in absolute ethanol.

- **RIPA buffer (Santa Cruz # sc-24948)**

  50mM Tris-HCL (pH 7.4), 150nM NaCl, 1mM PMSF, 1mM EDTA, 5µg/ml aprotinin, 5µg/ml leucopeptin, 1% Triton X-100, 1% sodium deoxycholate, 0.1%SDS.

- **Trypsin EDTA in HBSS without Ca^2 and mg^2 (Trypsin-EDTA)**

  0.4gKCl, 0.06g H_2PO_4, 8g NaCl, 0.35g NaHCO3, 0.048gNa_2HPO_4, 1g D-glucose, 10ml phenol red 0.1%, 2.5 g Trypsin (1:250).

  2x Sample Buffer for Laemmli SDS-PAGE (Sigma#s34010).

- **Blocking buffer**

  1x TBS, 0.1% Tween-20 with 5%w/v BSA.

- **Resolving gel (10%)**

  4ml acrylamide (30% 37.5:1), 3ml 1.5M Tris-HCL pH 8.8, 120µl 10% SDS, 4.8ml distilled water, 40µl 10% ammonium persulphate, 10µl TEMED.

- **Resolving gel (7%)**

  5.84ml distilled water, 3ml 1.5M Tris-HCL pH 8.8, 3ml acrylamide (30% 37.5:1), 120µl 10% SDS, 40µl 10% ammonium persulphate, 10µl TEMED.

- **Stacking gel (10%)**

  1.5ml acrylamide (30% 37.5:1), 2.5ml 0.5M Tris-HCl pH 6.8, 100µl 10% SDS, 5.8 ml distilled water, 100µl 10% ammonium sulphate, 10µl TEMED.

- **2x SDS loading buffer (stock) (2x SDS sample buffer)**

  6% SDS, 20% glycerol in 0.12 M Tris-HCL pH 6.8.

- **SDS loading buffer (working) (SDS sample buffer)**

  900µl 2x SDS-PAGE loading buffer, 100µl of mercaptoethanol, 50µl of 0.08%bromophenol blue.

- **Stripping Buffer (Odyssey Infrared Imaging System)**

  25mM glycine pH 2, 2% SDS.

- **Stripping buffer (ECL detection)**

  30ml of water, 6ml of 10% SDS, 1875ml of 1M Tris-Hall pH6.8 and 233µl of β-mercaptoethanol.

- **Transfer buffer**

  7.575 g Tris-HCL, 36g glycine, 500ml methanol, distilled water to 2.500ml.

- **Trypsin-EDTA in HBSS without Ca^{2+} and Mg^{2+} (trypsin-EDTA)**

  0.4g KCl, 0.06g H2PO4, 8g NaCl, 0.35g NaHCO3, 0.048g Na_2HPO_4, 1g D-glucose, 10ml phenol red 0.1%, 2.5G TRYPSIN (1:250), 0.38G EDTA up to 1000 ml in distilled water.

- **Western Transfer buffer**

  7.5g Tris-HCl, 36g glycine, 500ml methanol, distilled water to 1000ml.

- **Western Wash Buffer TBS/T(500ml)**

  50ml 10x TBS in 450 ml distilled water, add 0.5ml Tween-20.

- **10%Ammonium persulphate (APS)**

  0.1g ammonium persulphate in 1ml distilled water.

- **10x TBS**

  7.575g Tris-base, 80g NaCl, up to 1000ml distilled water, pH 7.6.

- **Wash buffer TBS/T (500ml)**

  50ml 10x TBS in 450ml distilled water, 0.5ml Tween-20.

2.1.4. Miscellaneous

The product	Producer or supplier
BCA Protein assay kit	Pierce Cramlington, UK
Biotinylated Protein Ladder	Fermenta
Chemiluminescent agent (ECL kit)	Amersham, Italy
GeneElute Mammalian Total RNAKit	Qiagen and Sigma-Aldrich
Laemmeli Loading Buffer	Biorad
Mercaptoethanol	Sigma-Aldrich

Odyssey blocking buffer	LI-COR Biosciences
TaqMan Gene Expression Assay	Applied Biosystem

2.2 Chemicals

Forskolin was purchased from Calbiochem/Merck Biosciences (Nottingham. UK).

Rapamycin Ready Made Solution, 2.5mg/mL in DMSO (2.74mM), was purchased from Sigma-Aldrich.

InSolution™ AMPK Inhibitor, Compound C (1mg/250µl) solution in DMSO from Calbiochem/Merck Biosciences (Nottingham. UK).

AICAR from Sigma-Aldrich.

The following antibodies were all from cell signalling technology

Total mTOR (7C10) Rabbit mAb (#2983), phospho-mTOR (ser2481) (#2974), phospho-mTOR (ser2448) (D9C2) XP® (#5536).

Total Akt # 2938, phospho Akt Thr308 #9275 and P.Akt ser473 #9271.

AMPKα (#23A3), Phospho-AMPKα (Thr172) (#40H9).

p70S6 kinase (#9202), Phosph-p70S6 kinase Ser371 (#9208) and Thr389 (#9206).

OGT Antibody (#5368**),** O-GLcNac (CTD110.6) was purchased from Cell New England Biolabs (Hitchin-UK).

Beta-actin antibody was purchased from Abcam (Cambridge, UK).

11βHSD2 antibody was purchased from Santa Cruz Biotechnology (California).

IRDye 800CW Goat anti-Rabbit IgG (H + L) was from Li-Cor (UK limited).

The secondary horse-radish peroxidase (HRP)-conjugated immunoglobulin `was from DAKO (Ely, Cambridgeenswere, UK).

IRDye 800-conjugated goat anti-rabbit and IRDye 700-conjugated goat anti-mouse antibody were purchased from Rockland immunochemical.

SYBER green Master Mix and PCR reaction mixture for quantitative RT-PCR was purchased from applied Biosystems (Warrington, UK).

2.3 Cell Culture

All techniques involving cell culture used in this research project were carried out aseptically in a sterile cell culture hood. Before and after each experiment, the flow chambers (cabinets) were sprayed with 70% ethanol and all cell culture ware were supplied in a sterile condition from BD Falcon. All pre-treatments and stimulations were carried out at 37°C in a humidified incubator at 5% CO2**.** Gloves were worn at all-times throughout cell culture procedures. Only BeWo cell line was used during this research project.

2.3.1Maintenance and sub-culture of BeWo cells

BeWo cells were cultured in F-12K Nutrient mixture Kaighn's Modification (1X) liquid with L-glutamine from Gibco (Paisley, UK), with 10% fetal bovine serum (FBS) heat in-activated (Biosera UK, East Sussex, UK) and 1% penicillin/streptomycin from Sigma Aldrich which was formulated to contain 10.000 U/ml penicillin;10 mg/ml. Aliquots were incubated at -20ºC until required. Cells were grown at 37°C within a humidified atmosphere at 5% CO2. The media was changed every other day till the cells are confluent. When cells became 70-80% confluent, they were then washed with pre-warmed PBS at 37ºC after aspiration of the medium. The washing with PBS is to remove debris and traces of FBS. Next, 2ml of trypsin

containing 0.02% of EDTA in HBSS without calcium was added in 75 cm² flask. The cells then incubated in the incubator at 37ºC for 2-3 min. When the cells detached from the base of the flask, which can be confirmed by observing the cells under microscope, 7ml of pre-warmed media were added in order to neutralise the effect of trypsin EDTA. Cells were then transferred to 25-75cm² flask or 6-12 well plates. Differentiation of BeWo cells was achieved by incubating confluent undifferentiated BeWo cells with 100µM forskolin for 24hr, cell differentiation was monitored by examining the cells under light microscope for multinucleated cells.

BeWo cells were generous gift from Dr. Jing Chen.

2.3.2Treatment of BeWo cells

BeWo cells were maintained in culture media as described above. Cells then seeded in 6-well plates for treatment. Once confluence (70-80%) was achieved, the media were then removed and the cells were rinsed with warm PBS. Next cells were pre-treated with inhibitors for one hour before the cells being treated with final treatments (see table 2.1). Cells were treated for either 10 minutes when protein phosphorylation was measured; alternatively, cells were treated for 24 hours when expression of total proteins was measured.

At the beginning of this work the author have treated BeWo cells with forskolin only at different time point (5minutes, 10minutes, 24hours and 48hours). However at 5minutes, neither T.mTOR nor phospho-mTOR were affected by forskolin. At 10 minutes forskolin exerts an inhibitory effect on mTOR phosphorylation at Ser2448 and Ser2481 but it has no effect on T.mTOR. At 24 and 48 hours both Total and phospho-mTOR were inhibited by forskolin. I have then decided to choose the time point 10min (the first time point where phospho-mTOR inhibited by forskolin) to test

for the phospho-protein and 24hrs to test for T.protein. I have also used T.mTOR as a loading control for phospho-mTOR analysis, since at this time point T.mTOR is not inhibited by forskolin.

Table 2.1: Treatment design of BeWo cells

Treatment of the first group of BeWo cells	Treatment of the second group of BeWo cells	Treatment of the third group of BeWo cells
Basal (untreated cells)	Basal	Basal
Forskolin	Forskolin	Forskolin
Forskolin+rapamycin	Forskolin+Compound C	Forskolin+AICAR
Rapamycin	Compound C	AICAR

The selection of treatment concentration used in this thesis was based on previously published studies and manufacturer's data.

Forskolin 100µM was used to induce the syncytialisation process in BeWo cells Forskolin-diluted in DMSO at a concentration of 0.1M to final concentration of 100µM, this is the concentration which has been shown to produce maximum concentration of cAMP (which is required for BeWo cells syncytialisation) within few minutes, and this higher concentration of cAMP can be maintained for at least 48hours in the presence of 100µM forskolin (Wice et al., 1990). Furthermore the use of this concentration was based on previously published studies (Delidaki et al., 2011, Egawa et al., 2008, Al-Nasiry et al., 2006).

Forskolin is a chemical compound isolated from the root of Plectranthus barbatus (Coleus Forskohlii). Forskolin was prepared at a concentration of 100µM by using DMSO since other solvents like ethanol inhibits the activation of adenylyl cyclase by forskolin (Huang et al., 1981). Furthermore, the culture media contain heat inactivated foetal calf serum, as the unheated one has a phosphodiesterase, the enzyme which degrades cAMP (Wice et al., 1990).

The concentration for the inhibitors used in these experiments is shown in (table2.2).

Table 2.2: Inhibitors used and their concentration and pre-incubation time

inhibitor	Form	Final concentration (M).	Pre-incubation time (minutes)
Rapamycin	In Solution (DMSO)	100nM	60
Compound C	In Solution (DMSO)	10µM	60
AICAR	Powder	0.5mM	60

Compound C is AMP-activated protein kinase inhibitor (AMPK) which functions as a key energy sensor in the cell. When it binds to AMP, it leads to its phosphorylation and activation by the upstream kinase, whereas its binding to ATP leads to inactivation of AMPK (Kimball, 2006). The mammalian target of rapamycin (mTOR) has been identified as one of the AMPK downstream, and it has been mentioned that there is a negative cross talk between mTORC1 and AMPK, since the activation of AMPK leads to inhibition of mTORC1 through TSC1/2 dependent or independent mechanism (Inoki et al., 2003).

5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), AICAR is frequently used as an activator of AMP-activated protein kinase (AMPK) which controls the response of the cells to energy changes and stress; its activity is also regulated by starvation, hypoxia, ischemia and exercise (Hardie, 2003). Inside the cell, AICAR is converted into the monophosphorylated nucleotide 5-aminoimidazole-4-carboxamide-1-dribofuranosyl-5_-monophosphate (ZMP) by the enzyme adenosine kinase and it is similar to AMPK effect although ZMP is less potent than AMPK. Furthermore, ZMP does not affect the intracellular level of adenosine monophosphate (AMP), adenosine diphosphate (ADP) or adenosine triphosphate (ATP) (Corton et al., 1995).

2.3.3Cells cryopreservation and revive

To store the cells for a long period, a stock of the cells was conserved in a liquid nitrogen, cells were trypsinized and centrifuged at 1000 rpm for 5 minutes. Media were removed and the cells then re-suspended with 5ml of freezing media which contain 90% FBS and 10% DMSO. 1 ml of this suspension was then transferred to 1ml cryovials (Nalgene). Vials were kept in cryofreeezing container (Fisher Scientific, Loughborough UK) containing isopropanol and stored overnight at -80ºC before transferring the vials to liquid nitrogen. To revive cells from liquid nitrogen, cells were warmed slightly in a water bath for 1-2 minutes, the content then mixed with pre-warmed culture medium and then centrifuged at 1000 rpm for 4 min and re-suspended with 5ml of culturing media and then transferred to 25 cm² flask. The media were then changed after 24hrs.

2.3.4 Counting cells using haemocytometer

Cells count allows an accurate platting of the cells, and to determine the cells density a haemocytometer was used. Chemicals used: tryptan blue (Sigma cat no T181154) diluted in 1:1 PBS. The tryptan blue is used to stain any dead cells which should not be included in the cell counting. 10ml of fresh media is put in T75 flask, following the removal of trypsin. Then 500µl of the cell suspension is mixed with 500µl of diluted tryptan blue, and then 20µl of the suspension is loaded into a haemocytometer chamber. Under a microscope the number of unstained cells was counted.

Number of the cells per ml= number of cells over a large square X dilution factor X 10000.

2.4 Measurement of hCG secretion

BeWo cells were cultured in 6-well plates; when they reached 70-80% confluent, cells were treated with the appropriate treatment at the specific concentration (Table 2.1 and 2.2) for 24hrs. Subsequently, the supernatant was collected and centrifuged at 500 xg for 5 min at 4°C in order to remove cell debris. All samples were then stored at -80°C. The quantity of secreted hCG was determined by the Department of Biochemistry (University Hospital Coventry and Warwickshire NHS Trust) using the Elecsys® Intact hCG+b electrochemiluminescence immunoassay (ECLIA) and the fully automated modular analytics E170 testing system from Roche Diagnostics (Mannheim, Germany).

2.5 Signalling assay

2.5.1 Preparation of soluble proteins from cultured cells for SDS-PAGE Immunoblot Analysis

Following treatments, cells were washed with ice-cold PBS and lysed in RIPA (200µLfor each well of 6-well plate), containing protease and phosphatase inhibitors.

Culture plates were then incubated on ice for 3-5 minutes. Cells lysate were then transferred to 1.5 ml microcentrifuge tubes. The BCA protein assay kit (Peirce, Rockford, IL) was used for protein quantification. Cell lysates were then mixed with the calculated amount of Laemmli buffer.

After sonication for 10 sec, cell lysates were boiled for 10 min at 97^0C and then cooled on ice. Lysate then centrifuged at 10,000x g for 5 min at 4^0C to remove cell debris and the supernatant was stored at -20^0C until required.

2.5.2 Preparation of soluble proteins from frozen placental fragment for SDS-PAGE

Previously frozen placental pieces obtained from woman with gestational diabetes (GDM) and BMI matched controls (ethically approved) sliced and defrosted in ice-cold lysis buffer (20mM Tris–Cl, pH7.5, 150mM NaCl, 1% Triton-X-100, 10mM NaF, and 1mM EDTA) supplied with 1mM Na3VO4 (Sodium orthovanadate, 1mM)**,** PMSF and protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA). Next, the samples were homogenized and then exposed to constant agitation at 4^0C for 3hrs. Tissue lysates were then centrifuged at 4^0C for 5 minutes. The BCA protein assay was used to determine the protein concentration of the supernatant. The sample

was then mixed with Laemmli buffer and incubated at 98^0C for 5 minutes and then left on ice for 2 minutes. Sample was then stored at -20 until use.

2.5.3 Determination of protein concentration

The protein concentration from the cell lysate was measured using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL USA), with the bovine serum albumin (BSA), as reference (table 2.3).

Table 2.3: Bovine serum albumin (BSA) standard serial dilution.

Vial	BSA Concentration (mg/ml)
A	2
B	1.5
C	1
D	0.75
E	0.5
F	0.25
G	0.125
H	0.025
I	0

The principle of this method is based on the reduction of Cu^{+2} to Cu^{+1} by bicinchoninic acid with the highly selective and sensitive colometric detection of the cuprous cation (Cu^{+1}). The reaction involves two main stages, first is the chelation of copper with

protein in an alkaline environment leading to the formation of a blue coloured complex (Biuret reaction). In the following step, the cuprous cation (Cu^{+1}) reacts with BCA reagent forming a purple coloured reaction product. The BCA/copper complex is water soluble and showing a strong linear absorbance at 562nm with increasing protein concentration. The assay was performed according to the manufacturer's instruction. The standards were prepared in lysis buffer (RIPA). Standard curve was generated using linear regression fit with Graph Pad Prism R 4.0 software. The protein concentration of each sample was measured using standard curve.

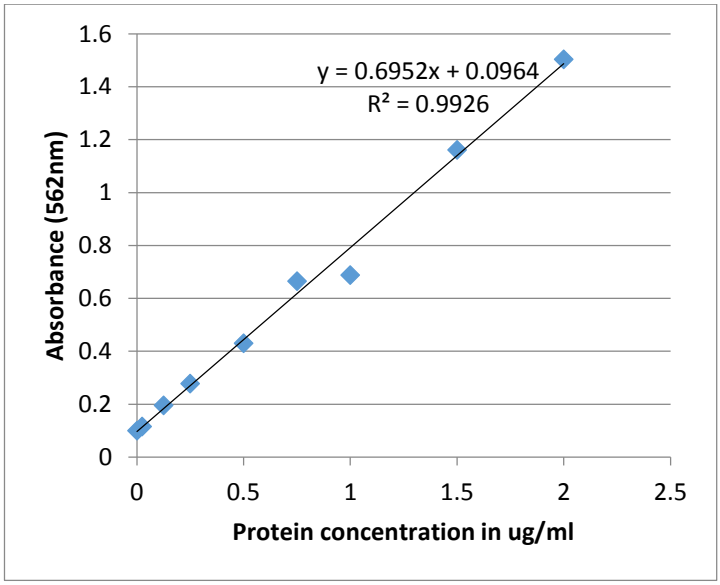


Figure 2.1: Standard curve obtained by using Bovine Serum Albumin (BSA) dilution

2.6 Western Blotting

Protein level in cell lysates from both treated and untreated BeWo cells was measured using Western blotting. Denaturation of the proteins in cell lysates made by heating at 98ºC for 10 minutes. Lysates was then loaded into the stacking gel wells against the Fermentas protein marker. Equal quantities of proteins were added in each well (as measured by BCA assay). Electrophoresis was run at 15-20 amp, until the dye had

reached the bottom of the gel. The resolving gel was washed with transfer buffer before transfer to nitrocellulose membrane (Amersham Bioscience, Milan, Italy) .The samples were transferred for 60 min at 100V.

Solution components	Resolving Gel (ml)		Stacking gel (ml)
	7.5%	10%	4.5%
Distilled H2O	5.84	4.84	5.8
30% acrylamide mix	3	4	1.5
1.5M Tris (pH 8.8)	3	3	-
0.5 M Tris (pH 6.8)	-		2.5
10%SDS	0.120	0.120	0.100
10% ammonium persulphate	0.04	0.04	0.100
TEMED	0.01	0.01	0.01

2.6.1 Visualisation of proteins using the Odyssey Infrared Imaging System

Following transfer, the nitrocellulose membrane was blocked with 10ml Odyssey blocking buffer (Licor-Bioscience, Cambridge, UK) for 1hr at room temperature with gentle agitation in order to reduce the non-specific binding of the antibody to the membrane. After this, the membrane is incubated with primary antibody (1:1000 dilution) in 5% BSA overnight at 4^0C under gentle agitation. Membrane was washed with 15mls of Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) three times 10 minutes each, before incubation in dark with secondary antibody conjugated

to a fluorescent dye (1:5000) in LI-COR blocking buffer. The membrane was washed again three times with 15 ml of TBS-T, and once with TBS, 10 min each. Finally, membranes were processed, proteins were identified using Odyssey Infrared Imaging System (Li-COR Bioscience, Cambridge, UK). Results were normalised for total protein or β-actin. β.actin is commonly used as a loading control in BeWo cells experiment and appears to give a satisfactory results (Delidaki et al., 2011, Neelima and Rao, 2008).

All primary antibodies used in this project were diluted in 5% BSA in 1:1000 dilution. This concentration was shown to give clear bands in the membranes after development, with minimal non-specific signals; although higher antibodies concentration (1:500) was also evaluated but excessive background signals were observed.

2.6.2 Stripping of membranes following Odyssey infrared imaging detection

In order to re-probe membranes with various antibodies and to remove unrequired background signals, antibodies were stripped off by washing membranes with stripping buffer. The membrane was washed with 20ml of stripping buffer (25mM glycine buffer, pH 2, and 2% SDS) at room temperature on shaker (2X 15min). Membrane was blocked in Li-COR blocking buffer following two washes, 15 min each with TBS and then re-probed with appropriate antibody.

2.6.3 Visualization of proteins using Enhanced Chemiluminescense (ECL)

Following transfer, nitrocellulose membrane was initially blocked with TBS-T and 5 % (w/v) BSA (Sigma-Aldrich, Willingham, UK) for 1hr at room temperature, on a

rolling platform. The membrane was then incubated with the primary antibody diluted in 10 ml of TBS-T containing 5% BSA, overnight at 4^0C. Then, the membrane was washed with 15ml TBS-T (3x15min), prior to incubation with the appropriate horseradish peroxidase-conjugate secondary antibodies (1:2000 final dilution) for 1hr at room temperature. Following another washing step with TBS-T (3x15min) and two washes in TBS (2x15 min), proteins were visualized by exposing the membrane to X-ray films using the ECL-plus detection system (GE Healthcare, Amersham, UK). The Scion Image software (Scion Corporation, Maryland, USA) was used to quantify band densities. Stripping of the membrane and re-probing with an anti-βactin antibody (1:10000) were used to confirm equal protein loading in each well (as mentioned in page 53).

2.6.4 Stripping of membranes following ECL detection

The membrane was incubated in stripping buffer (62.5mM Tris-HCL pH 6.8, 100m M β-mercapto-ethanol, 2% SDS) for 30 min at 55^0C with agitation. This was followed by washing the membrane with TBS-T (3x15min). Then the membrane was blocked with a blocking buffer containing TBS-T in 5% BSA. Next, the membrane was washed 3 times for 30 minutes with TBS-Tween (containing 0.05% tween-20) before incubation with blocking buffer and addition of primary and secondary antibodies, as mentioned above.

2.7 Molecular biology techniques

2.7.1 mRNA extraction

70-80% confluent cells were plated in 6 well plates and treated with different treatment at the indicated concentration for 24 hr. RNase Plus mini kit (Qiagen, Crawley West Sussex) was used for total RNA extraction, according to manufacturer's instruction.

The concentration of RNA was determined by using UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, USA).

2.7.2 RT-PCR (reverse transcription polymerase chain reaction)

Total RNA extracted from BeWo cells was reverse transcripted into cDNA using RevertAid First Strand cDNA Synthesis Kit. In a total volume of 20µl, 500 ng of RNA was used for first strand cDNA synthesis as following:

Firstly, 500ng RNA, 1 µl random oligo (dT) (# S0131) and RNase free H_2O up to 12.5µl, all were mixed in sterile Eppendorf tube and heated at 65^0C for 5min, the mixture was then cooled down in ice, before adding the master mix which consists of 5X reaction buffer (4µl), RNase Inhibitor (#E00381) 0.5µl, dNTP Mix 10mM (#R0191) 2µl (1mM final concentration), and 1µl of RevertAid H Minus Reverse Transcriptase, the mixture was then incubated at 25^0C for 5 min,42^0C for 60 min and 70^0C for 10 min.

2.7.3 Gel electrophoresis of DNA

To further analyse the DNA, 10-20 µl of the sample with $1/5^{th}$ volume of DNA loading buffer was loaded. Electrophoresis was carried in 1 XTBE at 80-120 V for 1-2h the nucleic acid then visualised under UV light.

2.8 Polymerase chain reaction (PCR)

In this study, SYBER Green was used for mRNA detection experiments. In this technique, fluorescence was detected and monitored over time during the polymerase chain reaction (PCR).

2.8.1 Quantitative real time polymerase chain reaction (RT-PCR)

Applied Biosystem fast 7500 machine was used to perform PCR, by using SYBER green master mix in 20µl. Master mix was made by mixing the 1µl of forward and reveres primers (pre-optimized concentration), 10µl of SYBER green master mix and 6µl of water (total volume 18µl), then added to the suitable wells depending on the map designed for the 96 wells plate (MicroAmp™ Fast Optical 96-Wells Reaction Plate, Applied Biosystems, Cat.No. 4314320) The reaction was performed in a triplicate with a 2µl of suitable cDNA added to each well. Plates were closed and centrifuged at 1000g for 1min. The machine was then adjusted to fast SYBER green. All samples were labelled according to wells, melting curve then added, and the plate was positioned inside the machine and the program was initiated. PCR conditions consisted of enzyme activation at 95°C for 20sec, followed by 40 cycles of denaturation at 95°C for 3s, 60°C for 30s, followed by melting curve analysis. Quantitative amounts of syncytin were calculated with reference to GAPDH using the comparative ΔΔCT method for relative quantification.

However, at the beginning of this work the author have also tried ACTB as a reference gene along with GAPDH but the results were more satisfactory with GAPDH. Furthermore, in BeWo cells, GAPDH is frequently used as a reference gene in RT-PCR experiments and seems to give a satisfactory results (Delidaki et al., 2011, Neelima and Rao, 2008, Pascolo et al., 2003), so the author decided to use GAPDH as a reference gene.

The sequence of those genes is listed below in table 2.4

Table 2.4: Primer sequence used for RT-PCR analysis

Gene of Interest/ Size	Forward Primer (5'-3')	Reverse Primer ('5-3')
Syncytin-1 bp187	TCATATCTAAGCCCCGCAAC	TGATCTTGCAAGGTGACCAG-
Syncytin-2 bp 231	AGCAGCCGTAGTCCTTCAAA-	AGGGGAAGAACCCAAGAGAA-

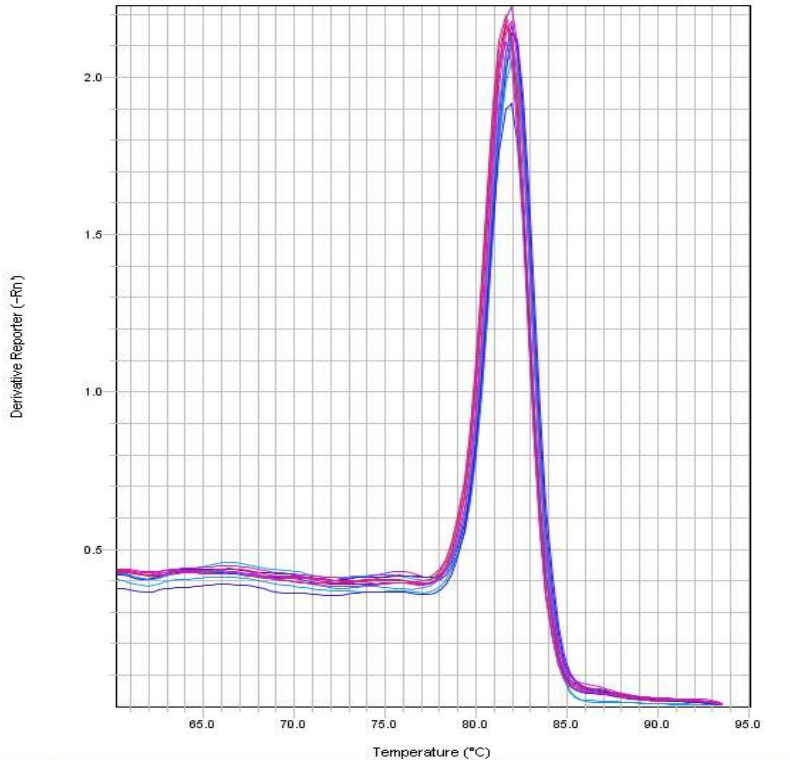


Figure 2.2: Melt Curves analysis from RT-PCR. A single melting peak seen for all samples.

2.9 Immunohistochemistry staining using NovoLink Max Polymer Detection System (500 tests) RE7150-K

According to the manufacturer's instruction, the following steps were followed for immunohistochemistry staining of previously frozen placental slides.

The previously frozen slides was first immersed in pre-cooled acetone for 10 min to fix the tissue sections, and then left to dry. The slides was then washed with PBS (three times, 5 min each) then incubated in 0.3% peroxidase block (#RE7101) solution in PBS for 30min at room temperature in order to block any endogenous peroxidase activity.

The slides was then washed with PBS three times, 5min each and incubated with protein blocking solution (#RE7102) for 1hr at room temperature. The blocking buffer was then poured off from the slides and the slides were washed three times 5min each before they were incubated with primary antibody diluted in 5% bovine serum albumin (BSA) overnight in cold room. Next day, the slides were washed twice in PBS 5min each, and then incubated with Post Primary Block for 30min (RE7111), followed by washing with PBS twice 5min each. The slides were then incubated with NovoLink polymer and secondary antibody for 30 min and next washed with PBS twice 5min each. To reveal the colour of the antibody staining, the slides was incubated with freshly made DAB solution for 5min. The slides were then rinsed with water 2 changes, 5min each.

The slides were then counterstained by immersing in Haematoxylin for 1-2 min and then washed in water for 5 min. The tissue slides were then dehydrated in alcohol 4 changes (95%, 95%, 100% and 100%) and cleared in three changes of xylene and

mounted. The colour of the antibody staining in the tissue section was observed under microscopy.

2.10 Data analysis and statistical methods

In experiments of protein phosphorylation, total and phosphorylated protein levels was quantified using the Odyssey Infrared Imaging system or Scion image software (when ECL detection system was used). Background was corrected by using default settings and measurements of band intensities were used to obtain raw data. The resulting protein expression was calculated by the following formula *(phosphor/total)* and expressed as a fold change from basal, using this formula *(treated/basal).*

In experiments of gene expression level, mRNA was normalized to a calibrator with the ΔΔCt method. Results were expressed as n-fold difference in gene expression relative to GAPDH The calibrator was further determined as follows: 2-(ΔCt sample –ΔCt calibrator) where Ct (threshold cycle) is the fractional cycle number at which the PCR fluorescent signal passes a specific threshold.

The statistical analysis was carried out using Student's unpaired t-test (which was used to compare two samples) or one-way ANOVA followed by the Tukey multiple comparison test using Prism software (GraphPad Prism, version 5.0; GraphPad Inc., SanDiego, CA USA). Values were considered statistically significant at $P < 0.05$. Each experiment was repeated at least three times. Blots are representative of one experiment, and graphs represent the mean ± SEM of three independent experiments.

Student unpaired t-test and ANOVA is commonly used to analyse data from BeWo cells (Madigan et al., 2010, Vatish et al., 2012). ANOVA is also commonly used to analysis data from RT-PCR (Brunner et al., 2004). Furthermore, the author have

chosen ANOVA to avoid type 1 error that result from performing multiple two sample t-test (statistics, 2013).

Chapter Three

Results

Introduction

It has been demonstrated that syncytin 1 and 2 and glial cell missing (GCM1) mRNA expression in trophoblast is associated with increased hCG secretion in the cell medium (Knerr et al., 2005). The formation of syncytium and its maintenance is one of the key mechanisms at the feto-maternal interface. Since understanding this process plays an important role in the management of pregnancy complication such as preeclampsia, where disturbed trophoblast fusion has been thought to be one of the causes (REDMAN and SARGENT, 2000). cAMP/PKA pathway plays a key role in up-regulation of fusogenic gene expression and the associated hCG production (Keryer et al., 1998, Knerr et al., 2005). The role of syncytin in inducing trophoblast fusion and hCG secretion has also been demonstrated by other studies which showed that syncytin mRNA expression was linear with cytotrophoblast differentiation and hCG secretion, which indicates syncytiotrophoblast formation. Furthermore, they found that *in-vitro* activation of cytotrophoblasts by cAMP is accompanied by increased hCG and fusogenic gene expression (Frendo et al., 2003).

Various studies have shown that mTOR is involved in placental signaling and it acts as growth signaling sensor in placenta (Wen et al., 2005) and its expression is down regulated in IUGR (Roos et al., 2007). In addition, mTOR gene disruption in mice resulted in impaired trophoblast formation (Gangloff et al., 2004). Recently Vatish et al have shown that the process of BeWo cells differentiation and cAMP secretion is associated with a reduction in intracellular calcium and inhibition of PI3K/Akt signaling pathway (the upstream effector of mTOR signaling pathway) (Vatish et al., 2012).

Based on the findings of previous studies, our study is aimed at investigating the role of mTOR in BeWo cells differentiation and also the effect of syncytialised BeWo cells on expression of mTOR and its components.

3.1.Effect of mTOR on Forskolin-Induced BeWo Cells Fusion

In this experiment, in order to evaluate the role of mTOR on forskolin induced hCG secretion in BeWo cells, the level of hCG was measured in culture media. In addition, the role of mTOR on forskolin induced mRNA expression of fusogenic gene as syncytin 1 and 2 was measured by using quantitative RT-PCR. Specific inhibitors were used to determine the signalling pathways involved in these effects. The morphological changes were assessed under a light microscope.

In this experiment, BeWo cells were incubated with F12K media supplemented with 10% foetal calf serum in 6-well plates; when the cells reached 70-80% confluent, BeWo cells then pre-treated with the inhibitors for 60 min (table 2.2), before they being treated with forskolin in the presence and absence of inhibitors (as shown in table 2.1) for 24 hr.

The level of hCG was assessed in the media collected from treated and untreated BeWo cells. The quantity of secreted hCG was determined by the Department of Biochemistry (University Hospital Coventry and Warwickshire NHS Trust) using the Elecsys® Intact hCG+b electrochemiluminescence immunoassay (ECLIA) and the fully automated modular analytics E170 testing system from Roche Diagnostics (Mannheim, Germany).

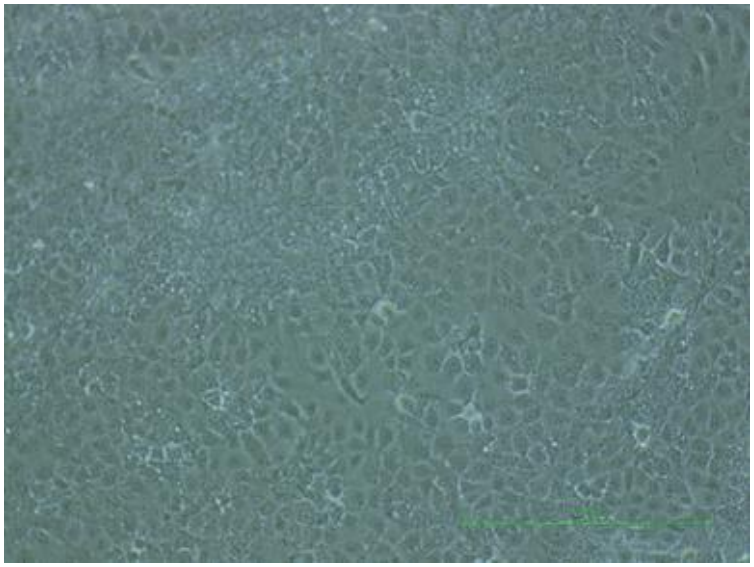
The results showed that, in forskolin-treated BeWo cells, forskolin increased hCG secretion by approximately 8 folds compared to basal (Figure 3.2).whereas treatment of BeWo cells with forskolin in the presence of either rapamycin or AICAR, resulted

in decreased hCG secretion by approximately 6 folds (figure 3.2 and figure3.4). Whereas compound C exerts no effect on forskolin-induced hCG secretion (figure 3.3).

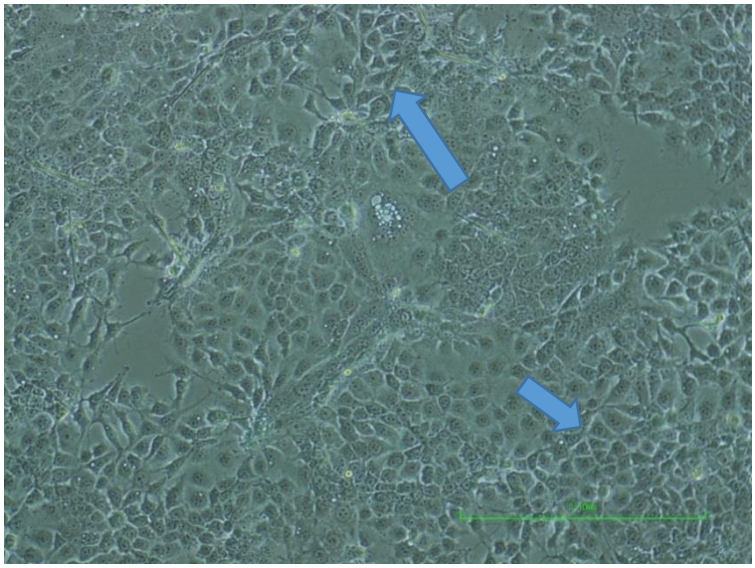
Quantitative RT-PCR showed that incubation of BeWo cells with forskolin for 24h resulted in up-regulation of syncytin 1 and syncytin 2 mRNA expression by approximately 4 and 8 folds respectively (Figure.3.5). Interestingly, this effect was further enhanced (8 and 10 folds respectively) when rapamycin was added to forskolin-treated BeWo cells (figure 3.5). However, compound C (AMPK inhibitor) and AICAR showed no effect on forskolin induced syncytin mRNA expression (figure3.6 and figure 3.7.respectively).

This might indicate that the effect of mTOR on forskolin-induced fusogenic gene expression is AMPK independent. In agreement with this, one study has shown that AMPK phosphorylation at Thr172 is essential for forskolin-stimulated tight junction formation in BeWo cells. They have also shown that AICAR and compound C have no effect on forskolin-induced cell fusion, which might indicate that AMPK is not implicated in this fusion process (Egawa et al., 2008, Zheng and Cantley, 2007).

This suggests diverse and distinct actions of mTOR on the biochemical and morphological differentiation of BeWo cells: a positive action on the endocrine phenotype coupled with a negative effect on fusogenic capacity.



A



B

Figure 3.1: Fusion of forskolin treated BeWo cells.

BeWo cells were studied under light microscope using a 20X objective and photographed digital microscope camera (Olympus, Tokyo, Japan), shows untreated BeWo cells (A-top) and BeWo cells after treatment with forskolin 100µM for 24h (B-bottom). The blue arrow indicates fused cytotrophoblast cells. Scale Bar = 100µm.

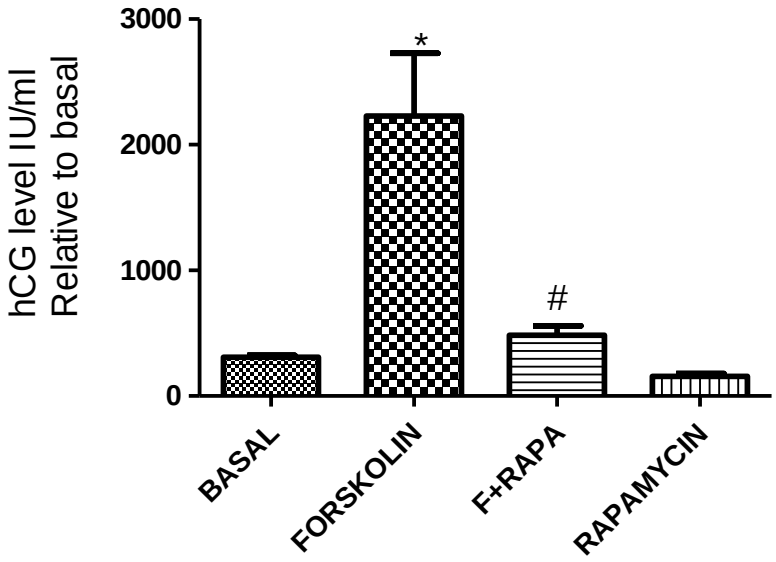


Figure 3.2: hCG release from BeWo cells treated with forskolin with/without rapamycin

BeWo cells were treated with either 100µM forskolin, forskolin (100µM) + rapamycin (100nM) or rapamycin (100nM) only for 24h. The medium was collected after 24 hours of incubation and the level of hCG (IU/ml) released in the medium was determined (as described in materials and methods chapter). Data represent the mean ± SEM of three independent experiments each analysed in triplicate *$*P<0.05$* of forskolin-treated cells compared to basal, *$\#P<0.05$* of forskolin+rapamycin-treated cells compared to forskolin-treated cells.

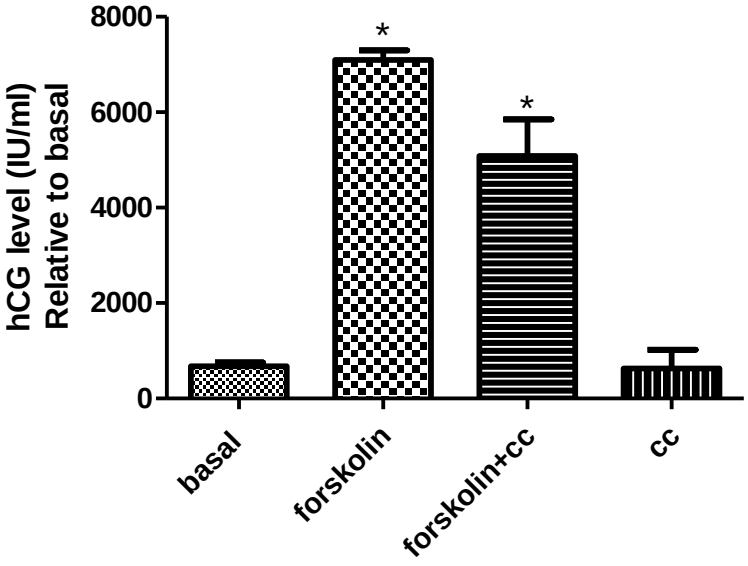


Figure 3.3: hCG release from BeWo cells treated with forskolin with or without compound C for 24hr

BeWo cells were treated with 100µM forskolin, forskolin 100µM+ compound C (10 µM) or compound C (10µM) only. The medium was collected after 24hr of incubation and the level of hCG (IU/ml) released in the medium was determined. Data represent the mean ± SEM of three independent experiments each in triplicate. *$P<0.05$ of forskolin-treated or forskolin+CC-treated BeWo cells compared with basal cells (no treatment).

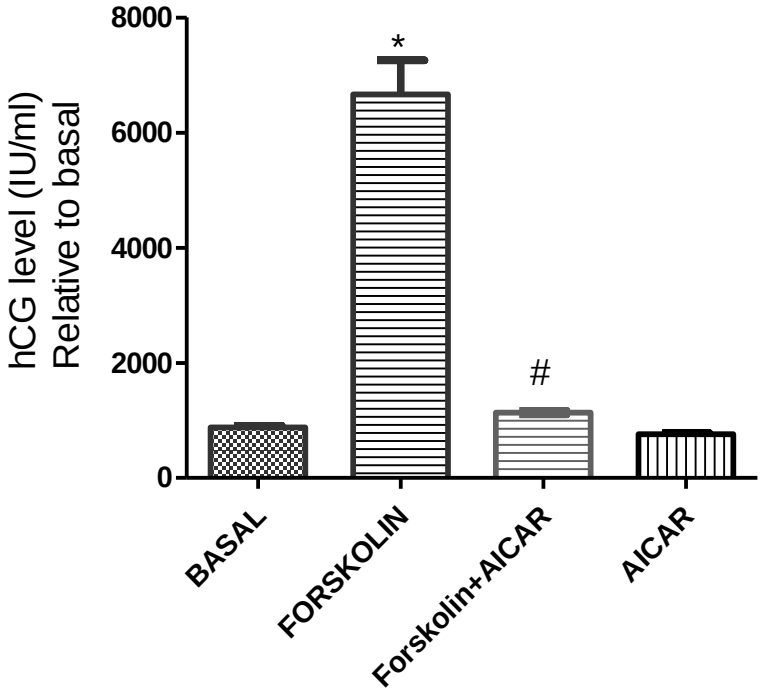


Figure 3.4: hCG release from BeWo cells treated with forskolin with/without AICAR for 24h

Level of hCG (IU/ml) released from BeWo cells treated with 100µM forskolin, forskolin (100µM) +AICAR (0.5mM) or AICAR (0.5mM) only. Data represent the mean ± SEM of three independent experiments each in triplicate *$P<0.05$* of forskolin compared with basal, #P<0.05 of forskoloin+AICAR treated cells compared to forskolin-treated BeWo cells.

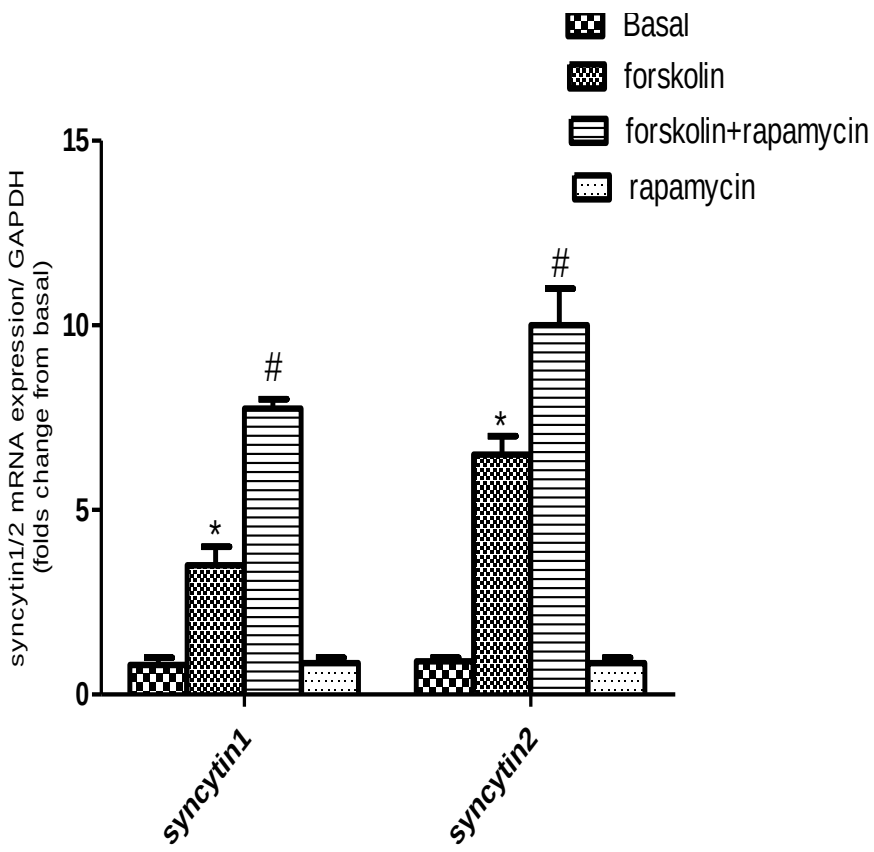


Figure 3.5: Expression of syncytin 1 and 2 mRNA in BeWo cells treated with forskolin with or without rapamycin for 24 h

BeWo cells were treated with forskolin 100µM in the presence or absence of rapamycin 100nM or rapamycin only for 24h and the expression level of targets mRNA was determined by RT-PCR as described in material and method chapter. Relative mRNA for syncytin 1 and syncytin 2 expression was normalized against the house keeping gene GAPDH. Data represent mean values ± SEM from three independent experiments each in triplicate. *$*P < 0.05$* of forskolin-treated compared to basal cells. *$\#P<0.05$* (forskolin+rapamycin-treated compared to forskolin-treated cells).

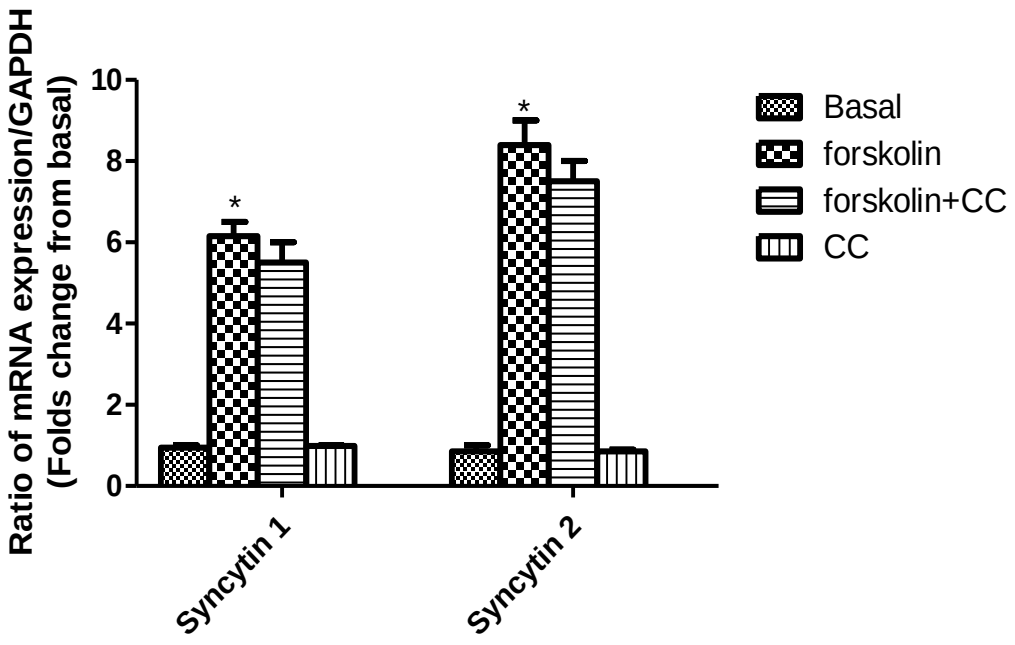


Figure 3.6: Expression of syncytin 1 and syncytin2 mRNA on BeWo cells treated with forskolin with or without compound C.

BeWo cells were treated with either forskolin (100µM), forskolin 100µM+compound C (10µM) or compound C (10µM) for 24 hr and the expression level of targets mRNA was determined by RT-PCR as described in material and method, relative mRNA expression was normalized against the house keeping gene (GAPDH) Data represent mean values ± SEM from three independent experiments. *$P < 0.05$ of forskolin treated cells compared with basal (untreated cells).

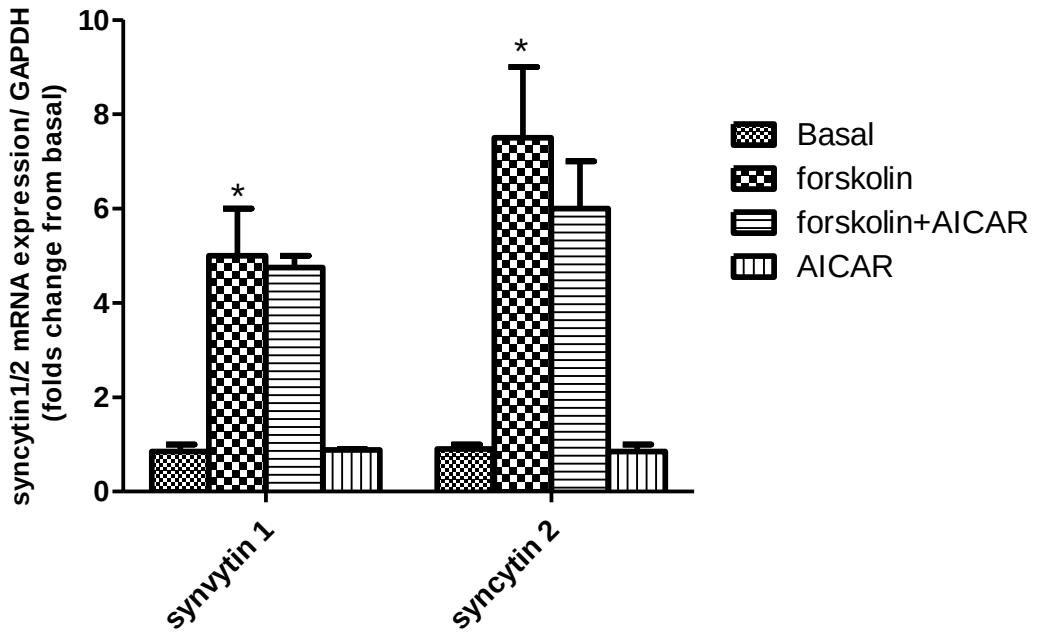


Figure 3.7: Expression of syncytin 1 and syncytin2 mRNA in BeWo cells treated with forskolin with or without AICAR.

BeWo cells were treated with either forskolin (100µM) or forskolin (100µM) +AICAR (0.5mM) or with AICAR only for 24hr and the expression level of targeted mRNA was determined by RT-PCR as described in material and method, relative mRNA expression was normalized against the house keeping gene (GAPDH) Data represent mean values ± SEM from three independent experiments each in triplicate. *$*P < 0.05$* of forskolin treated compared with basal (untreated cells).

3.2.Effect of mTOR on forskolin induced 11βHSD2 expression

11-βHSD2 is NAD dependent enzyme and is present mainly in kidney, placenta and pancreas (Krozowski et al., 1995). Most of foetal glucocorticoids originate from the mother and the placental enzyme 11βHSD2 converts active cortisol to inactive cortisone. This action is very important in placenta which allows 11-βHSD2 to act as a protector that prevent the passage of maternal glucocorticoid to the foetus (Edwards et al., 1988). In placenta 11βHSD2 is expressed mainly in syncytiotrophoblst (Krozowski et al., 1995).

Even though glucocorticoids are essential for intrauterine foetal growth and development, excessive intrauterine exposure to glucocorticoids can cause intrauterine growth restriction and could lead to hypertension and diabetes mellitus in adult life (Seckl, 2001).

The effect of mTOR on 11βHSD2 enzyme production by BeWo cells was also investigated by applying Western immunoblotting in cell lystaes from treated (for 24h) and untreated BeWo cells (see table 2.1) 11βHSD2 specific antibody was used.

Results showed that the treatment of BeWo cells with forskolin resulted in approximately 7 folds increase in 11βHSD2 secretion (figure 3.8).

However, neither rapamycin nor compound C affects forskolin-stimulated 11βHSD2 secretion (figures 3.8 and 3.9). Whereas AICAR has a negative effect on forskolin-induced 11βHSD2 secretion by BeWo cells (Figure 3.10). The results might indicate that mTOR pathway has no effect on 11βHSD2 secretion by BeWo cells, and the inhibitory effect of AICAR on 11βHSD2 secretion could be through mTOR independent pathway.

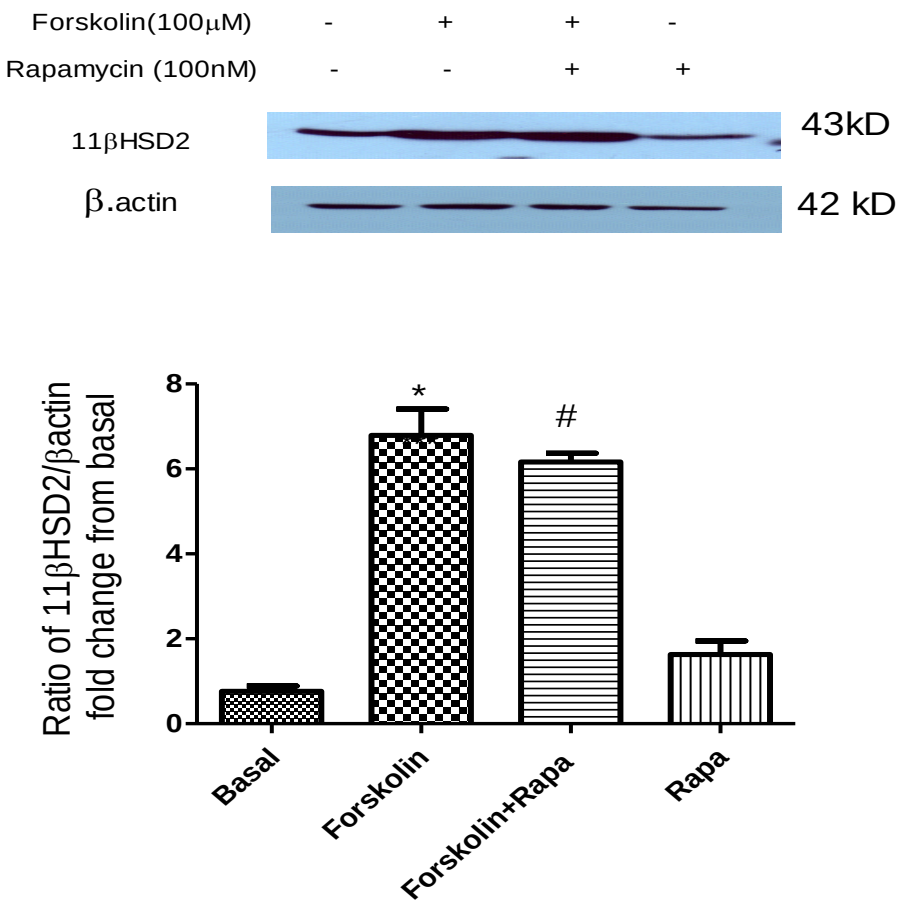


Figure 3.8: Expression of 11βHSD2 in BeWo cells treated with forskolin with or without rapamycin.

BeWo cells were treated with the indicated treatment. The expression level of 11βHSD2 was measured in BeWo cell lysate by Western immunoblotting using the ECL Western blotting detecting reagent (VMR, UK) for protein visualisation. Result were normalised for β.actin. Blots are representative. Data represent the mean ± SEM of three independent experiments. *$P < 0.05$ forskolin-treated compared to basal. # $P<0.05$ of forskolin+rapamycin treated cells compared to rapamycin treated cells.

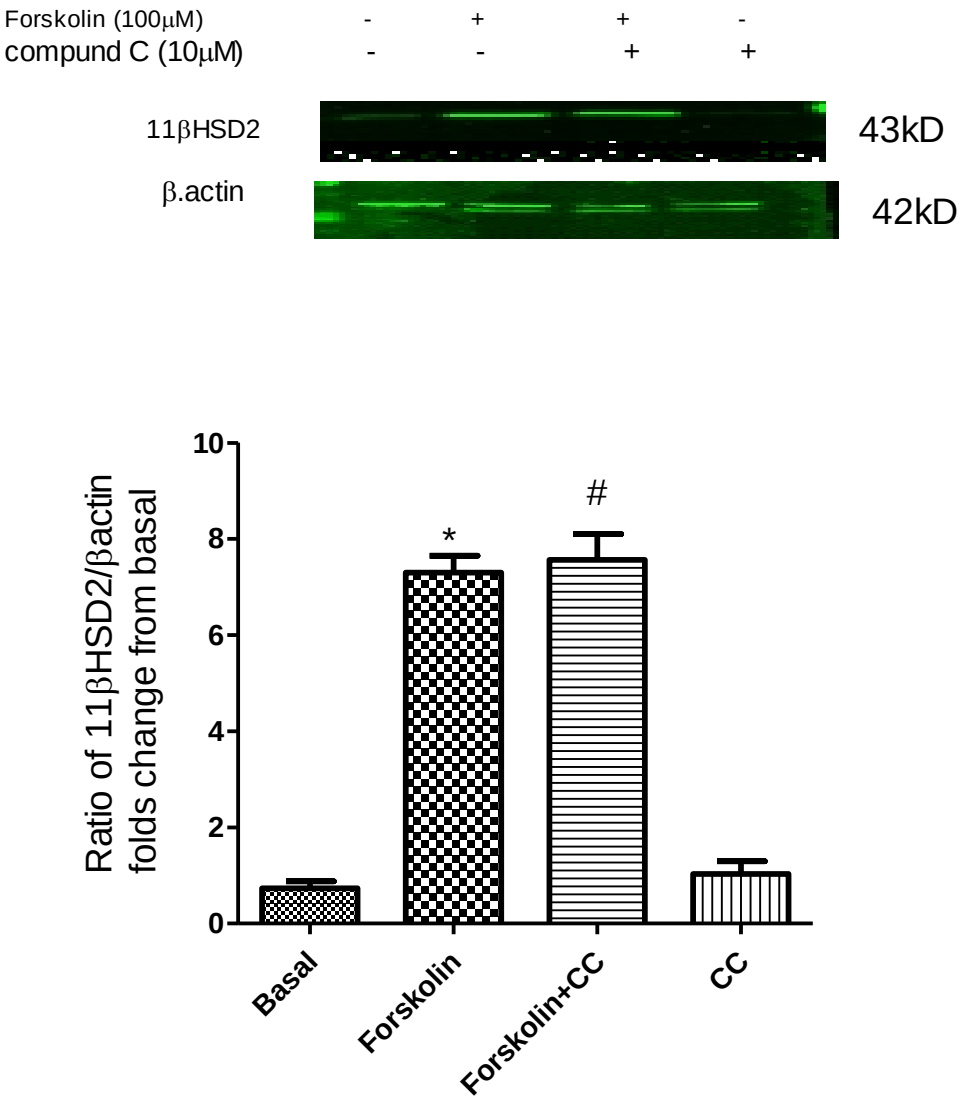


Figure 3.9: Expression of 11βHSD2 in BeWo cells treated with forskolin with or without compound C.

BeWo cells were treated with forskolin with or without compound C as indicated for 24h. The expression level of 11βHSD2 was measured in BeWo cell lysate by Western immunoblotting using the Odyssey detection system. Results were normalised for β.actin. Data represent the mean ± SEM of three independent experiments. Blots are representative *P<0.05 (forskolin-treated compared to basal cells) #P<0.05 forskolin+compound C-treated compared to compound C-treated cells.

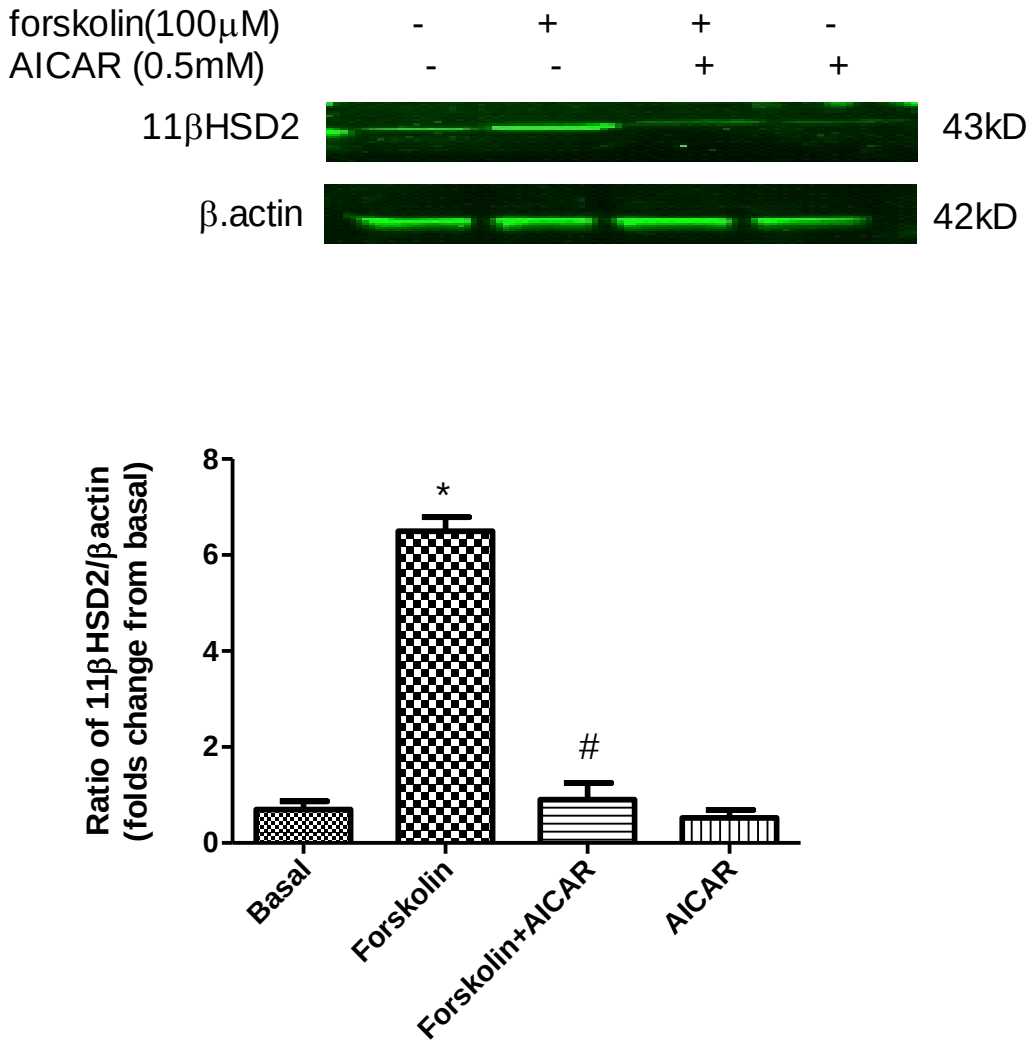


Figure 3.10: Expression of 11βHSD2 in BeWo cells treated with forskolin with or without AICAR.

BeWo cells were treated with forskolin with or without AICAR as indicated for 24h. The expression level of 11βHSD2 was measured in BeWo cell lysate by Western immunoblotting using the Odyssey detection system. Results were normalised for β.actin. Data represent the mean ± SEM of three independent experiments. *$P < 0.05$, (forskolin-treated compared to basal), #$P<0.05$ (forskolin+AICAR-treated compared to forskolin-treated cells).

3.3. Effect of BeWo cells differentiation on expression of mTOR and its related components

Our previous finding led us to investigate the effect of forskolin-driven adenylyl cyclase activation and intracellular cAMP production on mTOR expression and activity.

To measure the expression of P.mTOR (Ser-2448/2481), total mTOR, P.Akt (Ser473) and P.p70s6k (Ser371 and Thr389), I applied a western blot analysis on treated and untreated BeWo cells. BeWo cells were treated for 10 min when protein phosphorylation was measured, since the process of phosphorylation usually takes few minutes to start, however, when total protein was measured, BeWo cells were incubated with treatment for 24 hr as was previously explained in chapter 2 (2.3.2 Treatment of BeWo cells).

I found that forskolin almost eliminates phosphorylation of mTOR at Ser-2448 and 2481, and these effects were maintained when either rapamycin or AICAR was added to forskolin-treated BeWo cells (figure3.11 and 3.14 respectively). However, compound C showed no effect on mTOR phosphorylation at ser2448 at 10min. (figure3.12). However, when I tested the effect of compound C on P-mTOR (Ser2448) at 24h it showed up-regulation of phosphorylation at Ser2448, whereas the inhibitory effect of forskolin on mTOR phosphorylation was maintained even at 24hr (figure3.13).

Forskolin also inhibited the expression of T.mTOR (Figure 3.15), and this inhibitory effect was maintained when either rapamycin or AICAR were used with forskolin in treating BeWo cells (Figure 3.15. figure 3.17). However, the addition of compound C to forskolin-treated BeWo cells resulted in up-regulation of T.mTOR expression

(figure 3.16). Collectively, these results showed that forskolin almost eliminates mTOR expression and phosphorylation.

To further investigate the effect of forskolin-induced BeWo cells differentiation on mTOR signalling pathway, I have tested the effect of forskolin on Akt (Ser473) phosphorylation. The results showed that forskolin inhibits Akt phosphorylation at Ser473 within 10 minutes of treatment (Figure 3.18). Akt (Ser473) is mTORC2 substrate and when activated, it resulted in up-regulation of mTORC1 signalling pathway, this indicates that forskolin inhibits the upstream stimulator of mTORC1 as well as its downstream effectors (P.p70s6k Thr389).

The addition of either rapamycin, or AICAR (figure3.18, 3.20) to syncytialised (forskolin-treated) BeWo cells resulted in no change in the inhibitory effect of forskolin, on Akt phosphorylation. However, treatment of BeWo cells with rapamycin alone resulted in up-regulation of Akt phosphorylation (figure 3.18), and this activation could be indirectly through mTORC2, since rapamycin also inhibits P.p70s6k which exerts a negative feedback effect on mTORC2. It has also been observed that rapamycin can lead to an early transitory increase in Akt the effect that was explained due to the up-regulation of IRS1 (insulin receptor substrate1) which stimulates PI3K pathway and consequently phosphorylate Akt at Ser473 (O'Reilly et al., 2006).

Forskolin exerts inhibitory effect on p70s6k phosphorylation at Thr389 (the direct downstream effector of mTORC1) (figure 3.21), and this inhibitory effect might be due to inhibition of mTOR phosphorylation by forskolin. Rapamycin also inhibited p70s6k phosphorylation at Thr389 (figure 3.21), whereas treating BeWo cells with compound C alone resulted in up-regulation of P.p70s6k (Thr389) (figure 3.22).

The experiment has also shown that forskolin stimulates P.p70s6k phosphorylation at Ser371 (figure 3.23); this effect is inhibited when either rapamycin (figure3.23), compound C (figure 3.24) or AICAR (figure3.25) were used with forskolin in treating BeWo cells.

I have also measured the expression of P.AMPK (Thr172) in order to know if the effect of forskolin on mTOR expression and phosphorylation is AMPK dependent. The results showed that, forskolin has no effect on AMPK phosphorylation (at 10min) even when either rapamycin (figure 3.26) or AICAR (figure 3.28) was used with forskolin in treating BeWo cells. However, the addition of compound C to forskolin-treated BeWo cells resulted in inhibition of AMPK phosphorylation (10min) (figure 3.27).

AICAR is AMPK stimulator and since AICAR produced no effect on AMPK phosphorylation at 10 min (figure 3.28), I treated BeWo cells again with either forskolin, forskolin+AICAR or AICAR only for 24h. Interestingly, at 24hr, AICAR stimulated AMPK phosphorylation at (Thr172) (figure 3.29).

At 24h forskolin showed no effect on AMPK phosphorylation, and this is contradicting the results showed by (Egawa et al., 2008) who have found that long term treatment (>12hr) of BeWo cells with forskolin stimulates AMPK phosphorylation at Thr172. However, they also noticed that forskolin has no effect on AMPK phosphorylation on short term treatment (<12h). This might indicate that forskolin-induced inhibition of T.mTOR at 24hr is AMPK independent. The results showed that inhibitory effect of compound C on AMPK phosphorylation can be demonstrated at 10 min (figure 3.27) whereas the stimulatory effect of AICAR on AMPK phosphorylation can be demonstrated at 24h (figure 3.29).

3.3.1. Expression of P.mTOR (Ser2448/2481) expression

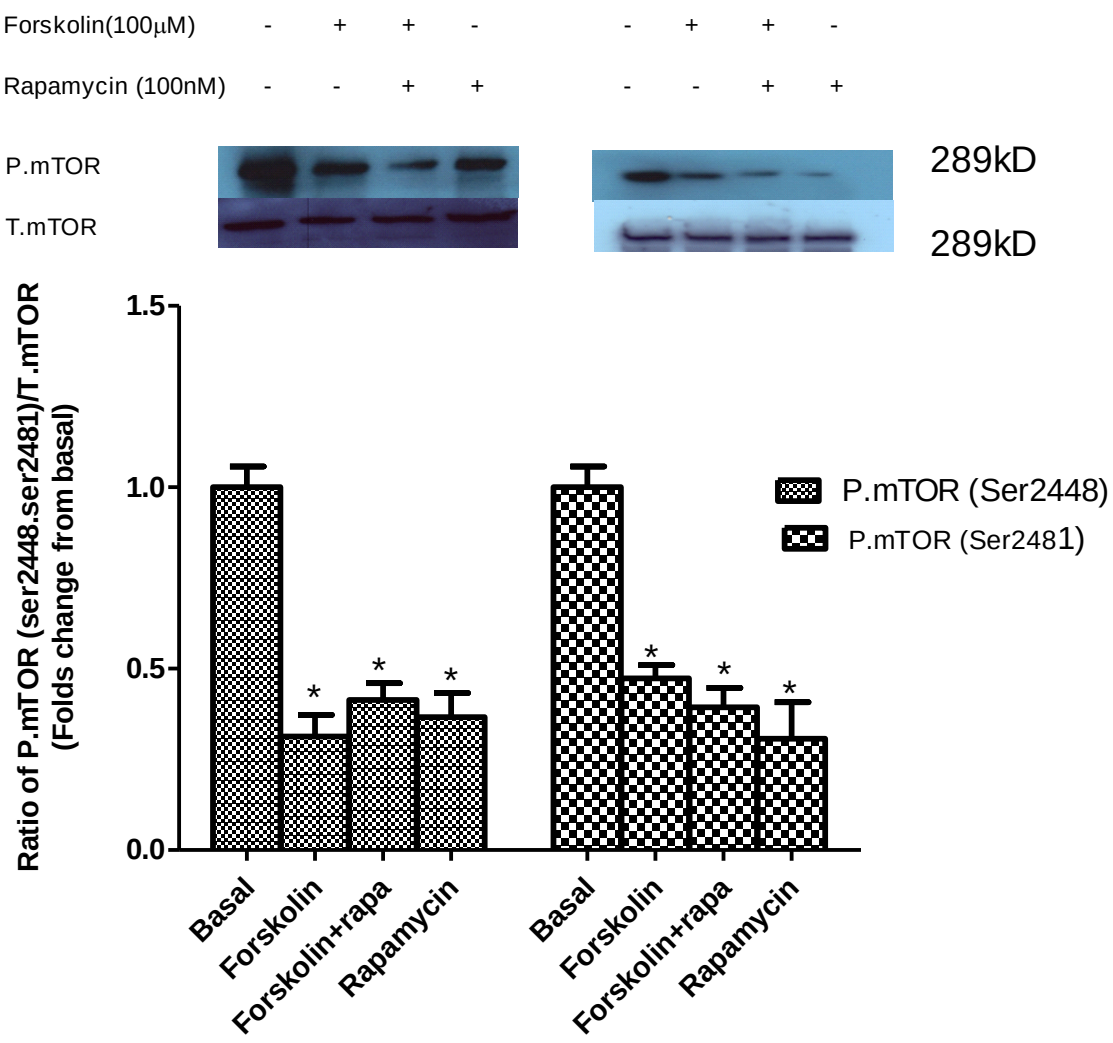


Figure 3.11: Expression of phospho-mTOR (Ser2448 and Ser2481) in BeWo cells treated with forskolin with or without rapamycin (10 minutes).

Phosphorylation levels of target proteins were detected in cell lysates using specific primary phospho-antibody. Antibody for total mTOR was used as loading control. ECL Western blotting detecting reagent (VMR, UK) was used for protein visualization. Blots are representative and data represent the mean ± SEM of three independent experiments, quantified against total protein (P/T).*$P < 0.05$ of treated BeWo cells compared to basal (untreated).

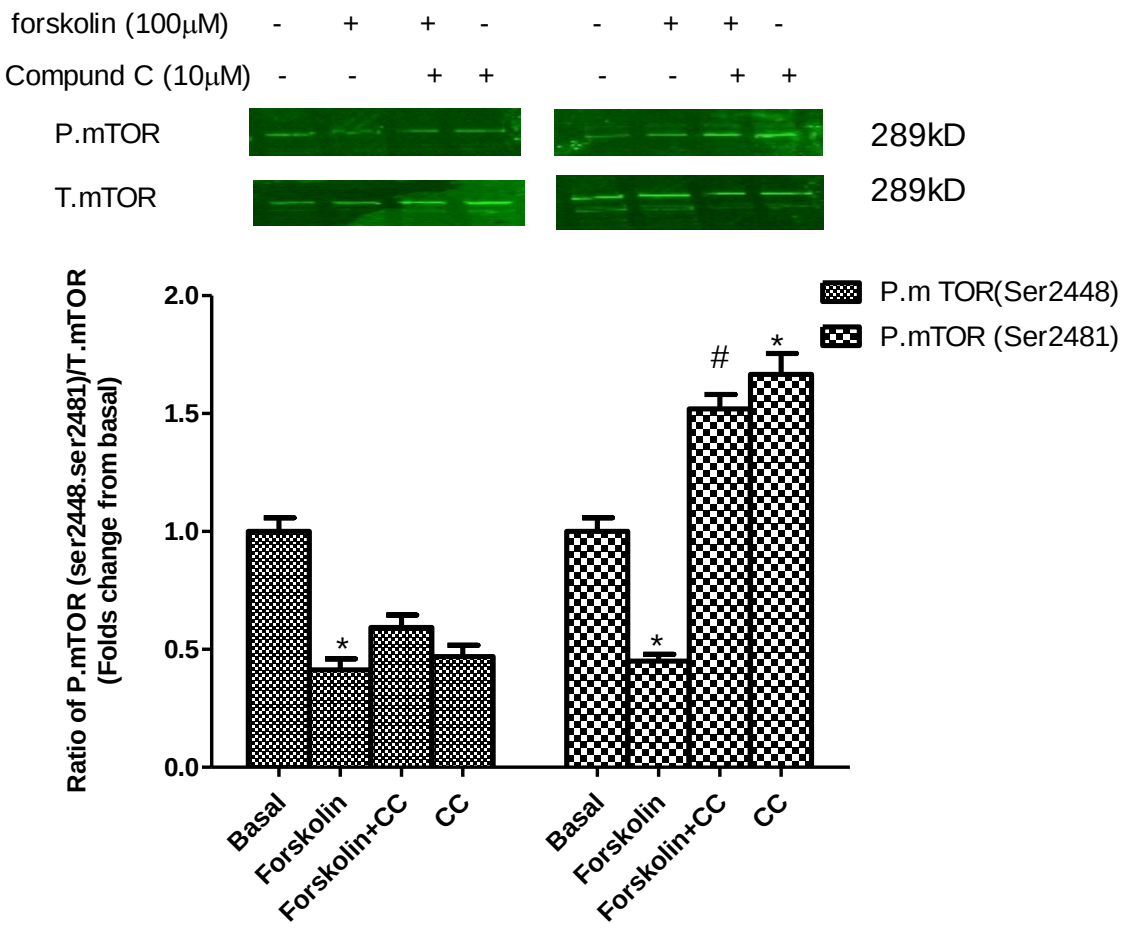


Figure 3.12: Expression of phospho-mTOR (Ser2448 and Ser2481) in BeWo cells treated with forskolin with or without compound C (CC) for 10 min.

Cells were treated with forskolin (100μM), forskolin (100nM) + compound C (10μM), or compound C only (10μM) for 10min then phosphorylation of target proteins was detected in cell lysates using immunoblotting. Antibody for total mTOR was used as loading control, Odyssey system was used for protein visualization. Blots are representative and data represent the mean ± SEM of at least three independent experiments. *$P < 0.05$ of treated cells compared to basal, # P<0.05 (forskolin+CC compared to forskolin treated cells).

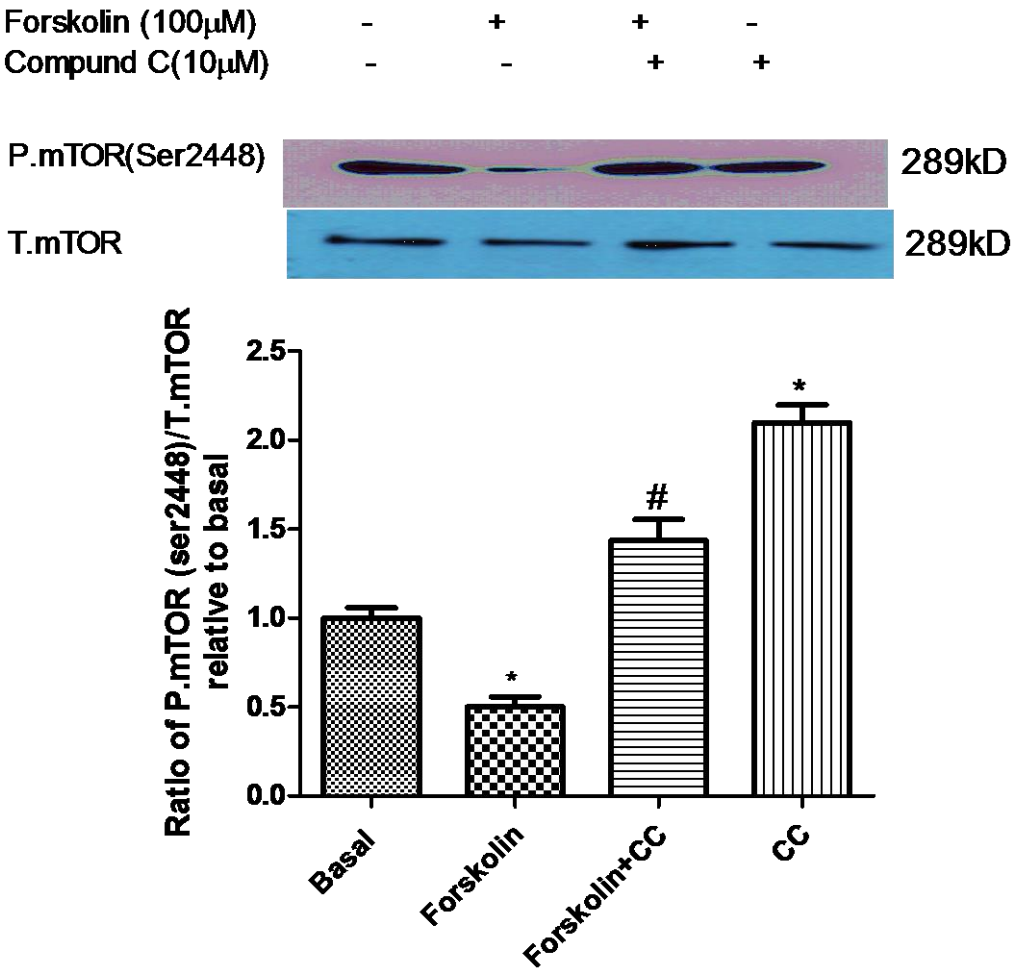


Figure 3.13: Expression of phospho-mTOR (Ser2448) in BeWo cells treated with forskolin with or without compound C (CC).

BeWo cells were treated with the indicated treatment for 24h and phosphorylation levels of target proteins were detected in cell lysates using specific primary phospho-mTOR (Ser2448) antibody. Antibody for total mTOR was used as loading control. Blots are representative and data represent the mean ± SEM of three independent experiments quantified against total protein (P/T).*$P < 0.05$(forskolin and compound C treated cells compared to untreated cells*)*, *#P<0.05(*forskolin+compoundC treated cells compared to basal cells).

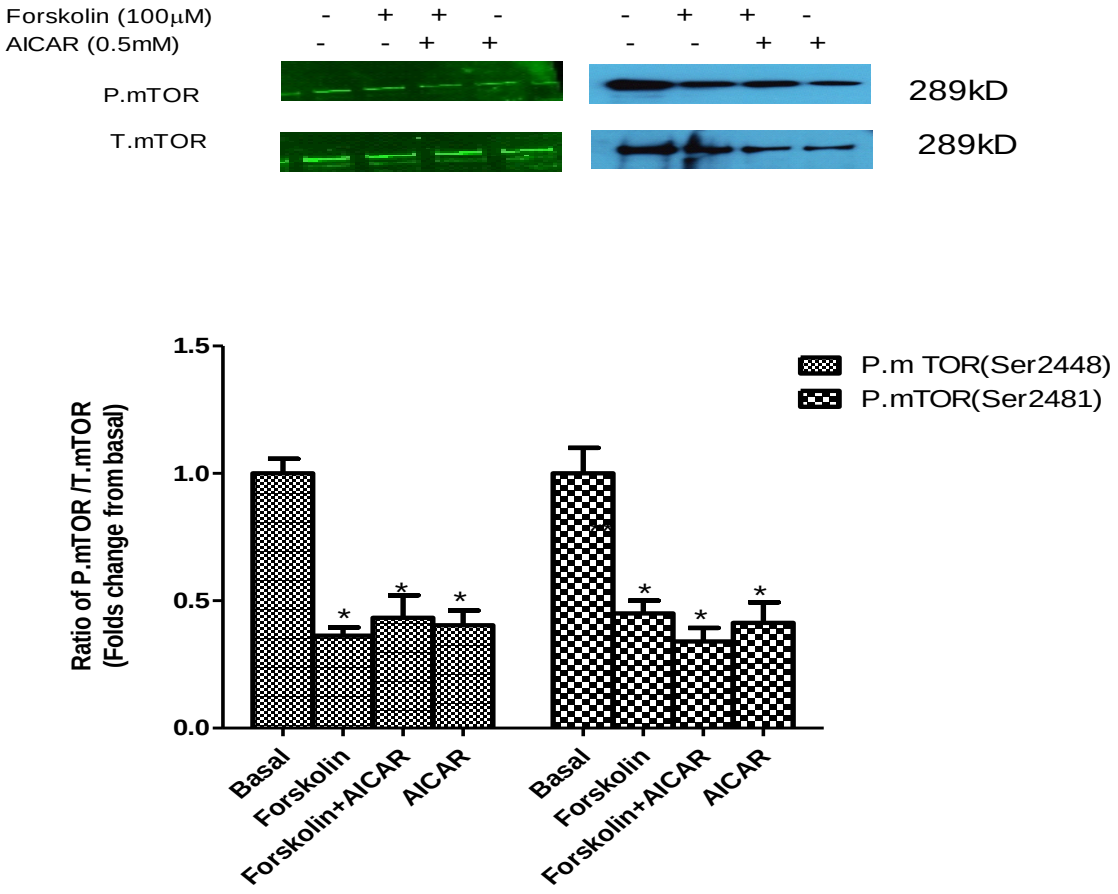


Figure 3.14: Expression of phospho-mTOR (Ser2448 and Ser2481) in BeWo cells treated with forskolin with or without AICAR for 10 min.

Cells were treated with either forskolin 100nM, forskolin (100µM) +AICAR (0.5mM) or AICAR (0.5mM) for 10min then phosphorylation levels of target proteins was measured. Odyssey infrared system and ECL Western blotting detecting reagent (VMR, UK) were used for protein visualization, data represent mean ± SEM of three independent experiments. Bar graphs represent fold increase relative to basal conditions. *$P<0.05$* of treated BeWo cells compared to basal.

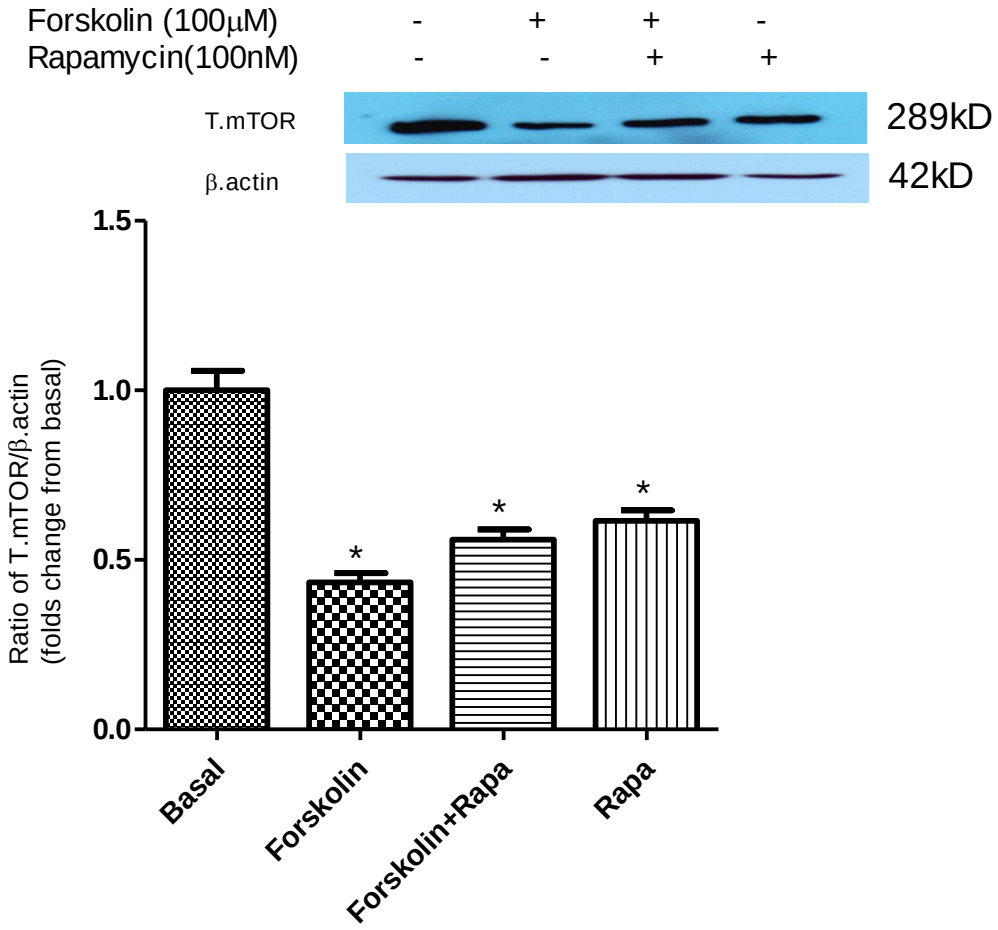


Figure 3.15: Expression of total mTOR in BeWo cells treated with forskolin with or without rapamycin for 24 h.

BeWo cells were treated with the indicated treatment for 24hr next, the expression levels of total proteins was detected in cell lysates using immunoblotting. Antibody for β-actin was used as a loading control, data represent mean ± SEM of at least three independent experiments. Bar graphs represent fold change compared to basal, *$P<0.05$ (treated BeWo cells compared to basal).

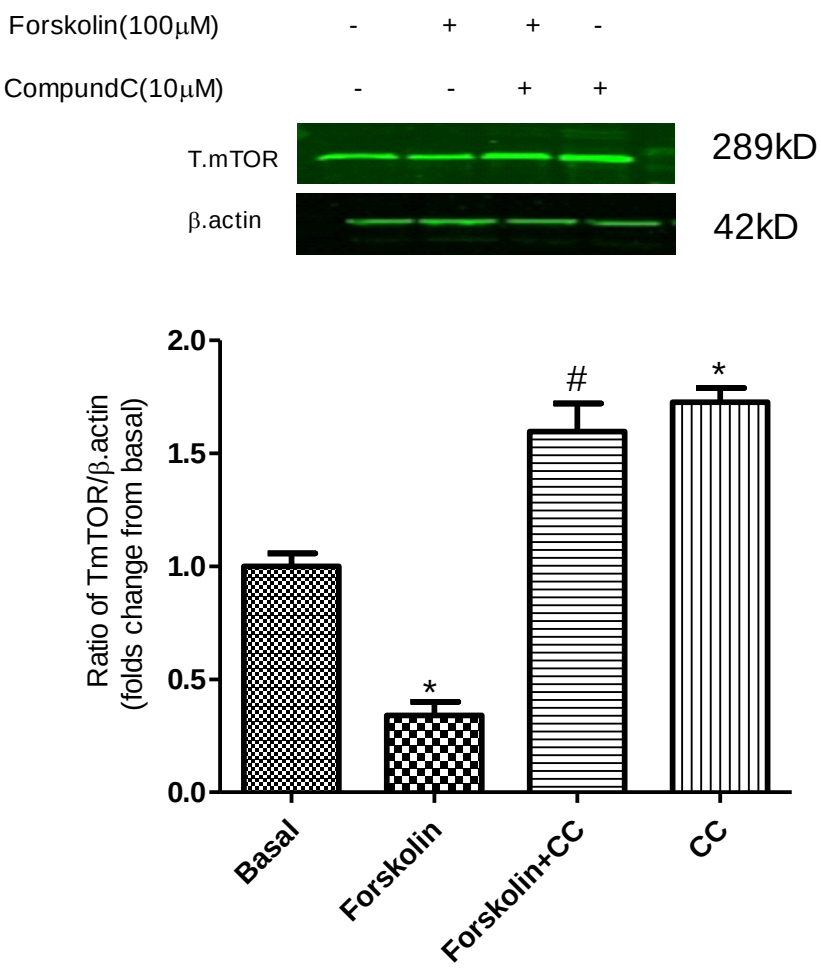


Figure 3.16: Expression of total mTOR in BeWo cells treated with forskolin with or without Compound C.

Cells were treated with the indicated treatment for 24hr, then the expression levels of total proteins were detected in cell lysates using immunoblotting. Data represent mean ± SEM of three independent experiments *(*P <0.05*forskolin and compound C treated BeWo cells compared to basal), #P<0.05 (forskolin+CC treated cells compared to forskolin treated cells).

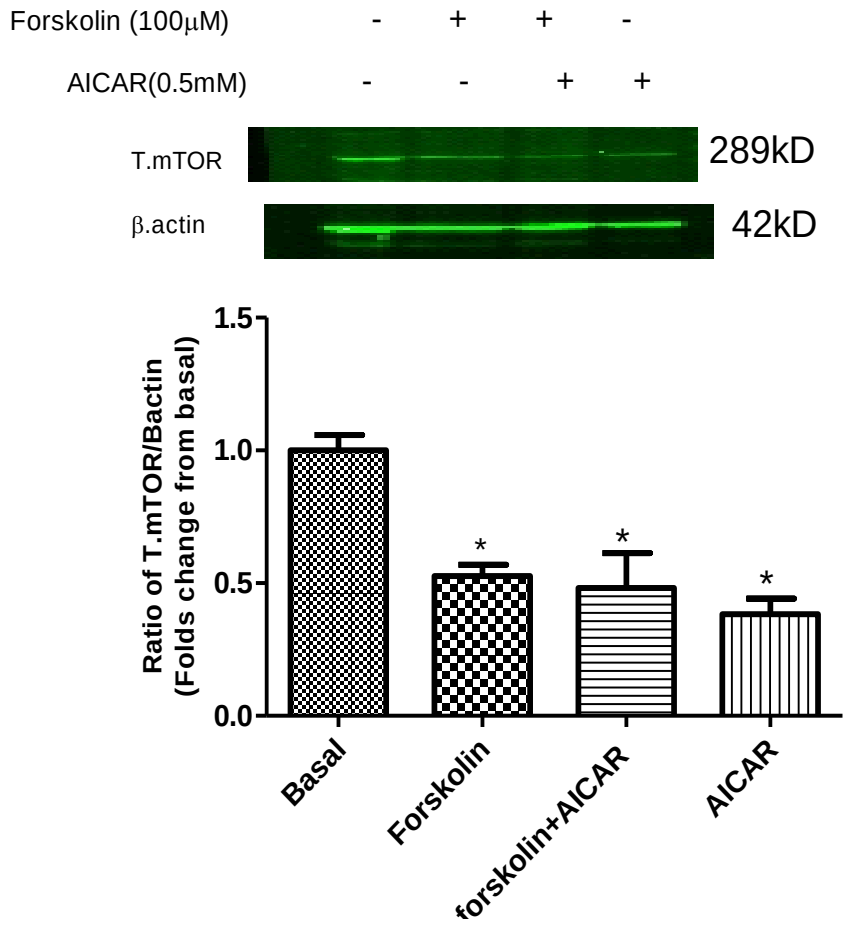


Figure 3.17: Expression of total mTOR in BeWo cells treated with forskolin with or without AICAR for 24h.

Cells were treated with the indicated treatment for 24hr., then the expression levels of T.mTOR proteins were detected in cell lysates using immunoblotting, data represent the mean ± SEM of three independent experiments, Bar graphs represent folds change from basal (*$P<0.05$ of treated BeWo cells compared to basal).

3.3.2. Expression of PAkt (Ser473) in BeWo cells

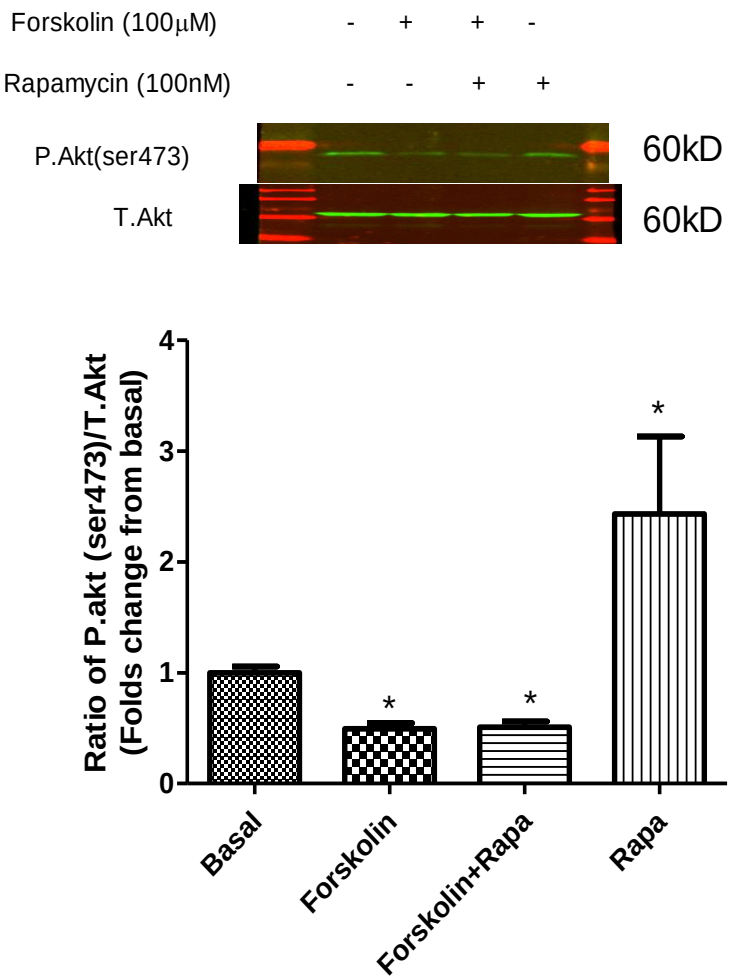


Figure 3.18: Expression of P.Akt in BeWo cells treated with forskolin with or without rapamycin.for 10 min.

BeWo cells were treated with forskolin (100mM) with or without rapamycin (100nM) for 10min, the expression of P.Akt (Ser473) was determined by immunoblotting using the Odyssey detection system for protein visualization. Total Akt was used as a loading control. Data represent the mean ± SEM from three independent experiments.* *P<0.05*(treated BeWo cells compared to basal).

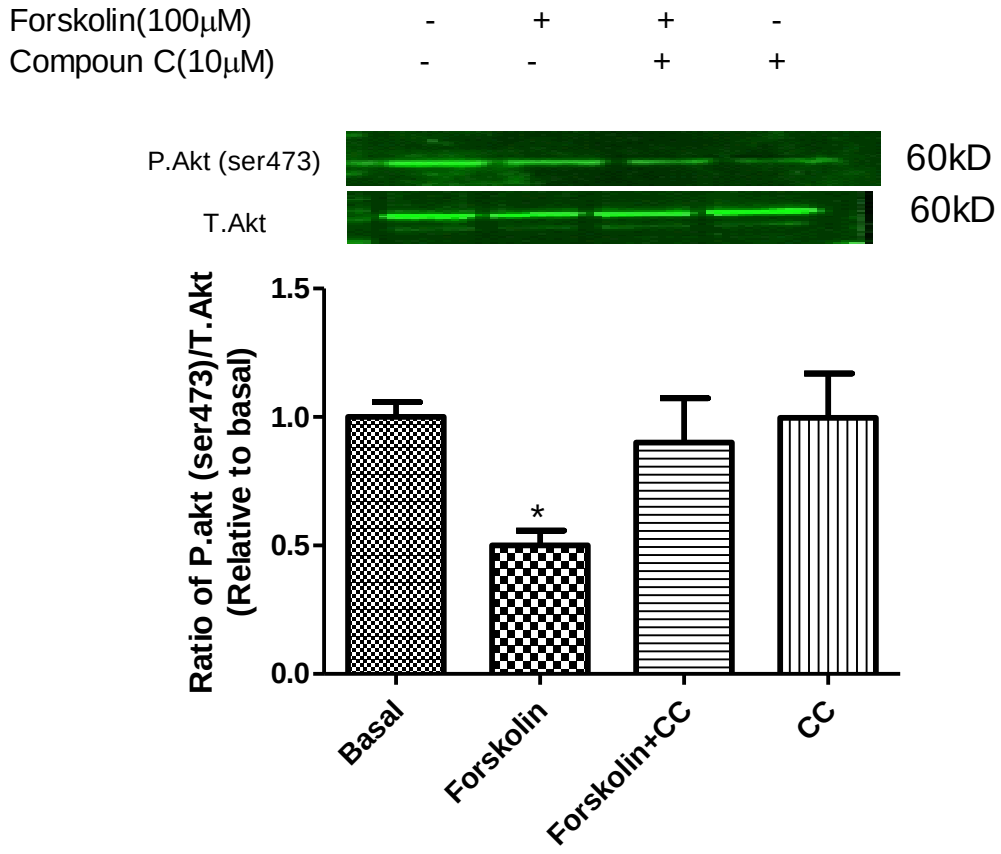


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Figure 3.19: Expression of P.Akt in BeWo cells treated with forskolin with or without compound C

BeWo cells were treated with the indicated treatment for 10 min. The expression of PAkt (ser473) was determined in treated and untreated BeWo cells using western immunoblotting. Data represent the mean ± SEM from three independent experiments and bar graph represent the folds of changes from basal. Blots are representative. * *P<0.05* (forskolin treated cells compared to basal).

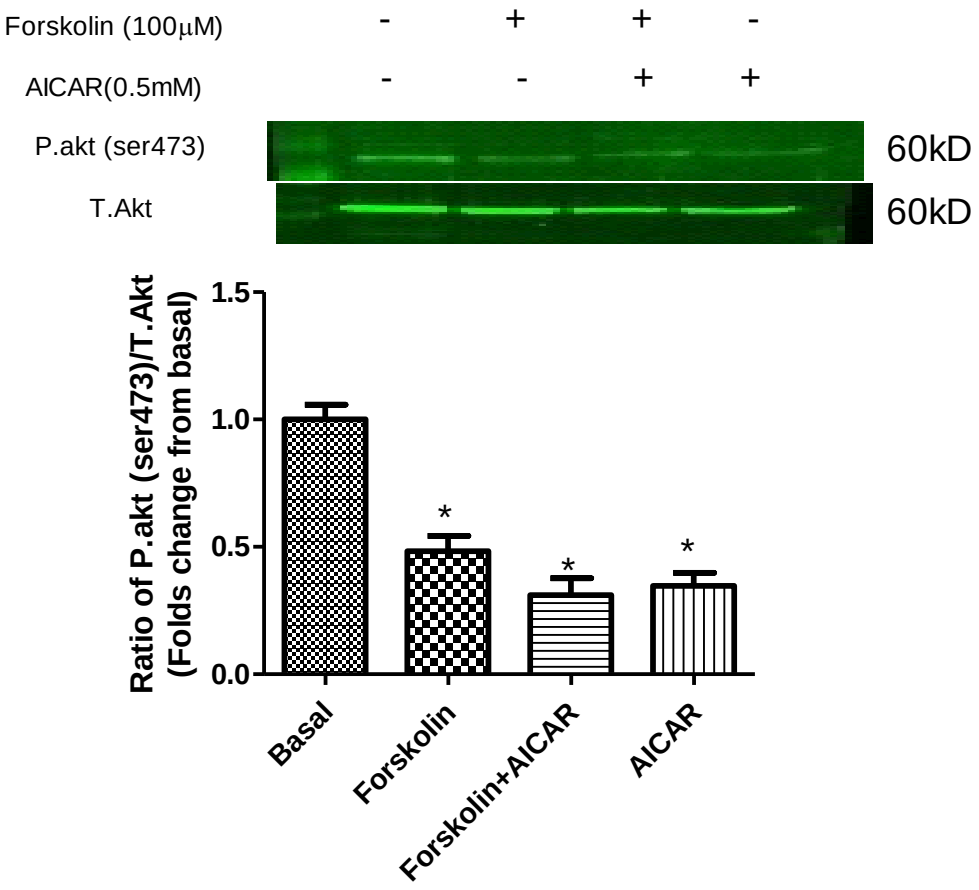

Figure 3.20: Expression of P.Akt in BeWo cells treated with forskolin with or without AICAR.

Cells were treated with forskolin (100mM) with or without AICAR (0.5mM) for 10 min, P.Akt expression was determined by immunoblotting using the Odyssey detection system for protein visualisation. Data represent the mean ± SEM from three independent experiments. * $P<0.05$ treated cells compared to basal.

3.3.3. Expression of P.p70s6k (Ser371 and Thr389)

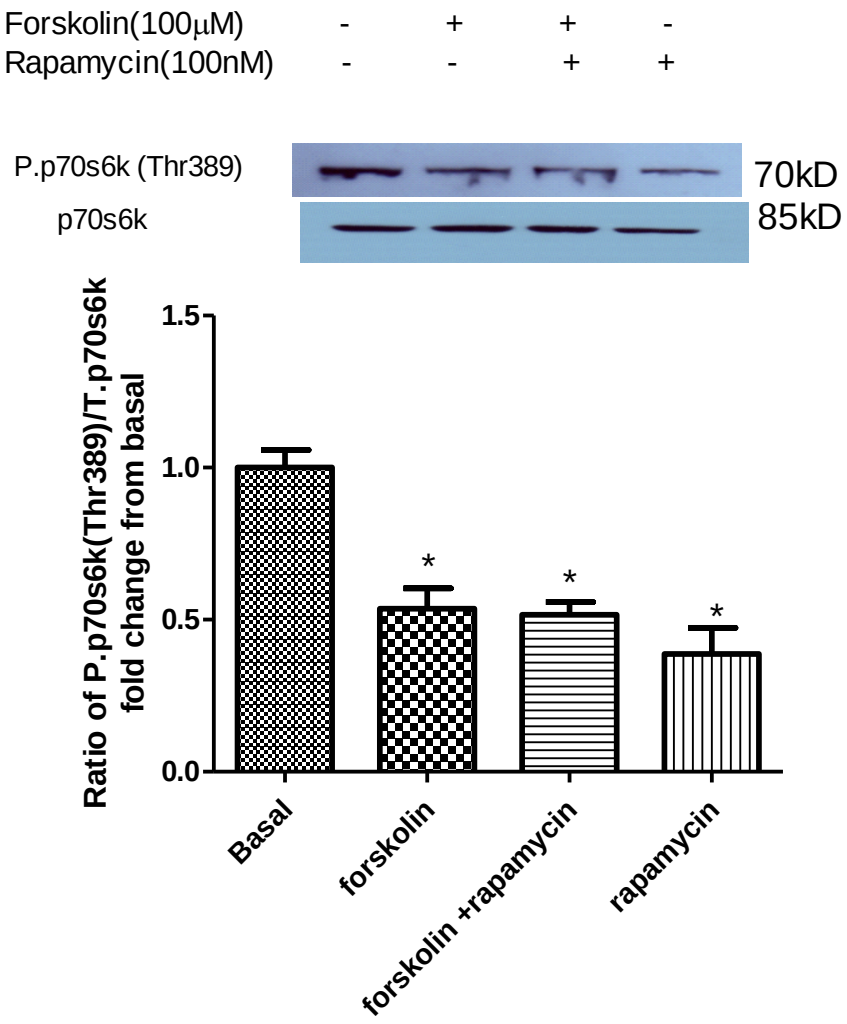

Figure 3.21: Expression of p70s6k (Thr389) in BeWo cells treated with forskolin with or without rapamycin (10min).

Western immunoblot detecting the expression of P.p70s6k (Thr389) in treated and untreated BeWo cells. Blots are representative and data are presented in mean ± SEM of three independent experiments. **P<0.05* of treated cells compared with basal.

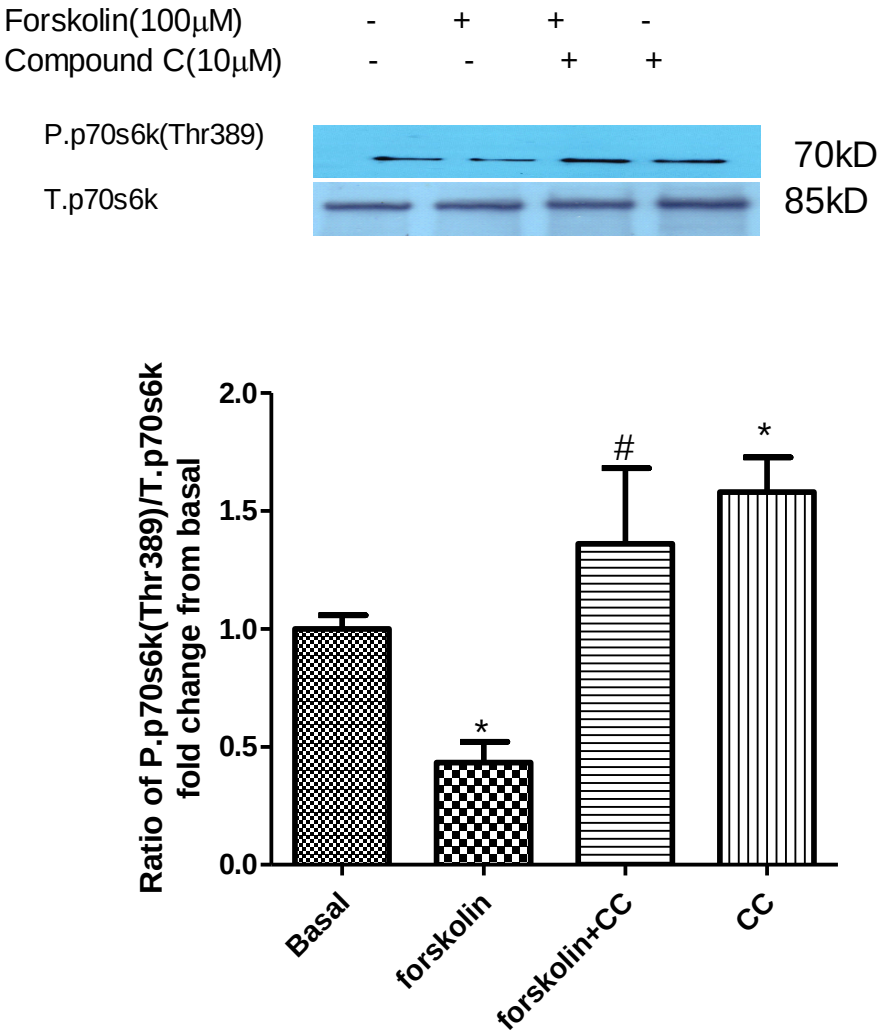

Figure 3.22: Phosphorylation of p70s6k (Thr389) in BeWo cells treated with forskolin with or without compound C (10μM) for 10 min.

Cell lysates were prepared from treated and untreated BeWo cells and target proteins were assayed on western blot. Blots are representative and data are presented as mean ± SEM of at least three independent experiments. *$P<0.05$ of forskolin treated and compound C-treated cells compared to basal. #P<0.05 forskolin+compound C-treated cells compared to forskolin-treated cells.

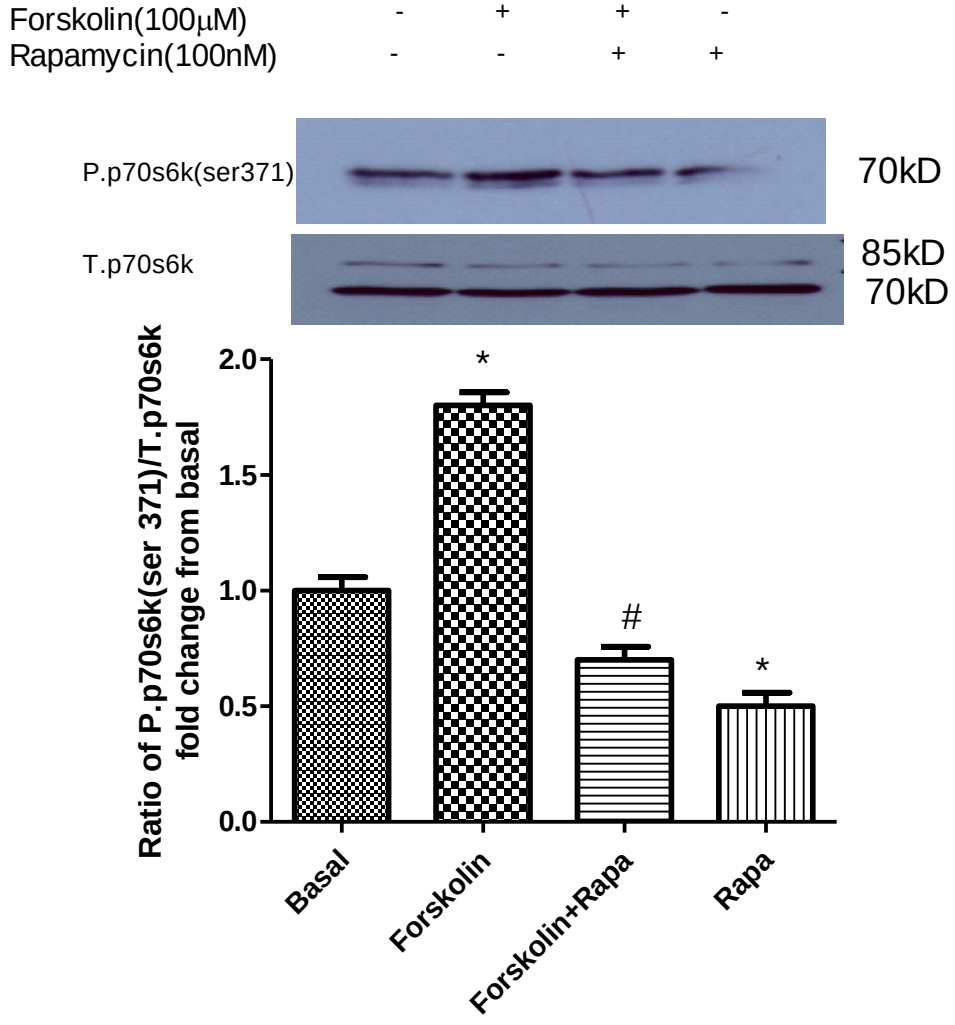

Figure 3.23: Expression of P.p70s6k (Ser371) in BeWo cells treated with forskolin with or without rapamycin (100nM) for 10 min.

Cell lysates were prepared from treated and untreated BeWo cells and target proteins were assayed on western blot. Total P70S6K was used as a loading control. Data are presented as mean ± SEM of three independent experiments. **P<0.05* (rapamycin treated and forskolin treated BeWo cells compared to basal). #P<0.05 (forskolin+rapamycin treated cells compared to forskolin treated cells).

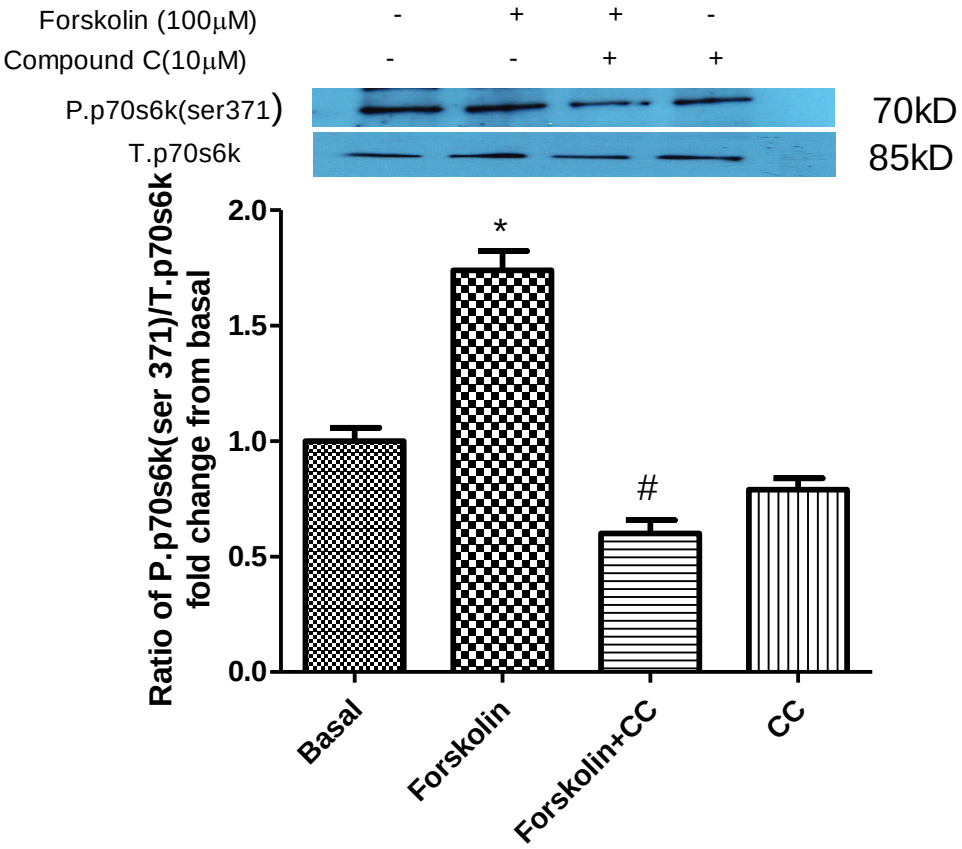

Figure 3.24 : Phosphorylation of p70s6k (Ser371) in BeWo cells treated with forskolin with or without compound C (10min.).

Cells were treated with the indicated treatment for (10mins). Western blotting was used for measurement of p70s6k phosphorylation (Ser371). Blots are representative and data represent the mean ± SEM of three independent experiments.*$P<0.05$ (forskolin treated compared to untreated cells) #$P<0.05$ (forskolin+CC treated cells compared to forskolin treated cells).

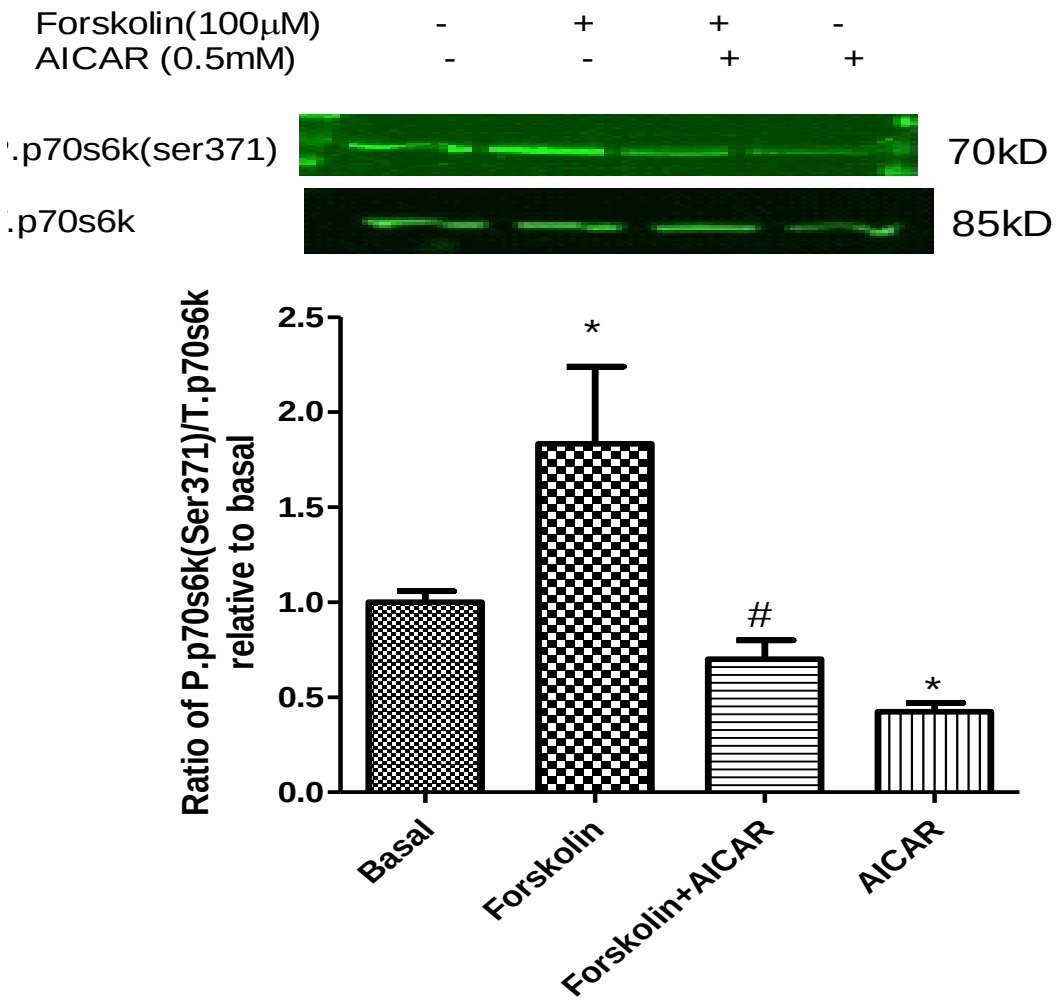

Figure 3.25: Phosphorylation of p70s6k (Ser371) in BeWo cells treated with forskolin with or without AICAR (10 min).

BeWo cells were treated with the indicated treatment for 10 min. Cell lysates were prepared and the expression of P.p70s6k (Ser371) was assayed on western blot. Total P70S6K was used as a loading control. Blots are representatives and data are presented in mean± SEM of three independent experiments. **P<0.05 (*treated compared to basal), #P<0.05(Forskolin + AICAR-treated compared to Forskolin-treated BeWo cells).

3.3.4. Expression of P.AMPK (Thr172)

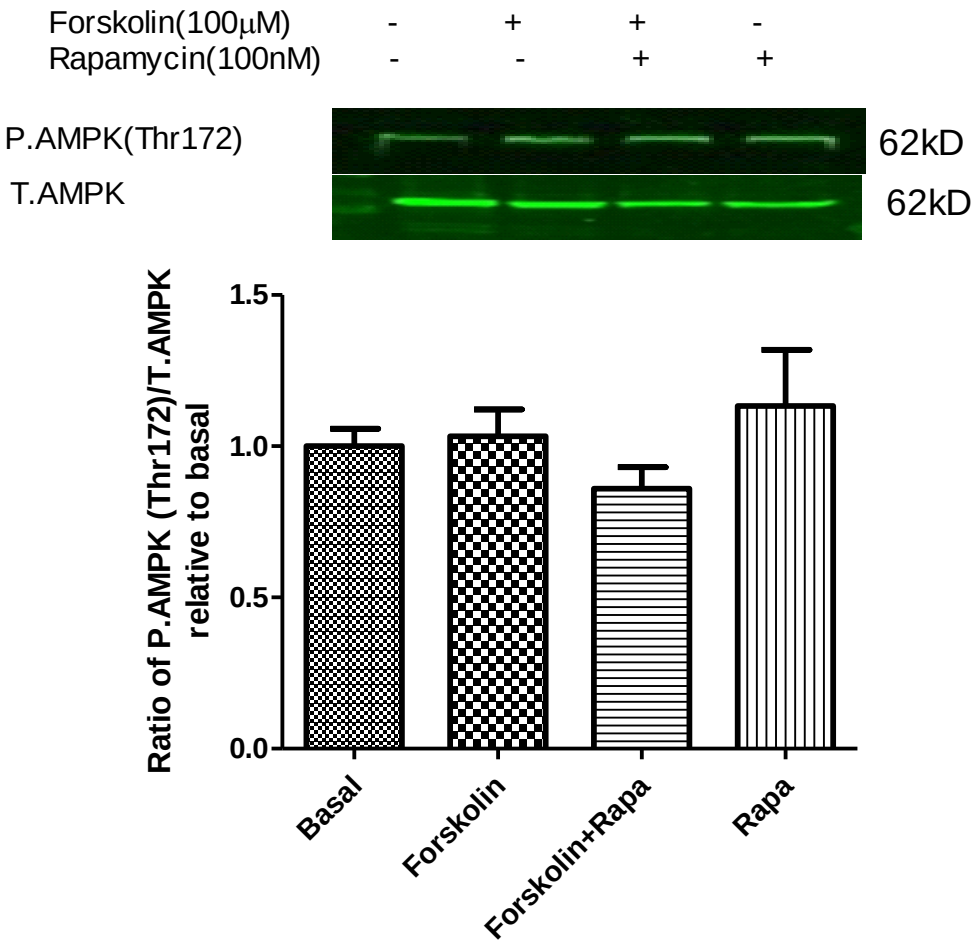

Figure 3.26: Expression of P.AMPK (Thr172) in BeWo cells treated with forskolin with or without rapamycin (10 min).

Expression of P.AMPK (Thr172) was measured on western blot. Total AMPK was used as a loading control and odyssey infrared system was used for protein visualization. Blots are representative. Data are presented in mean ± SEM of three independent experiments.

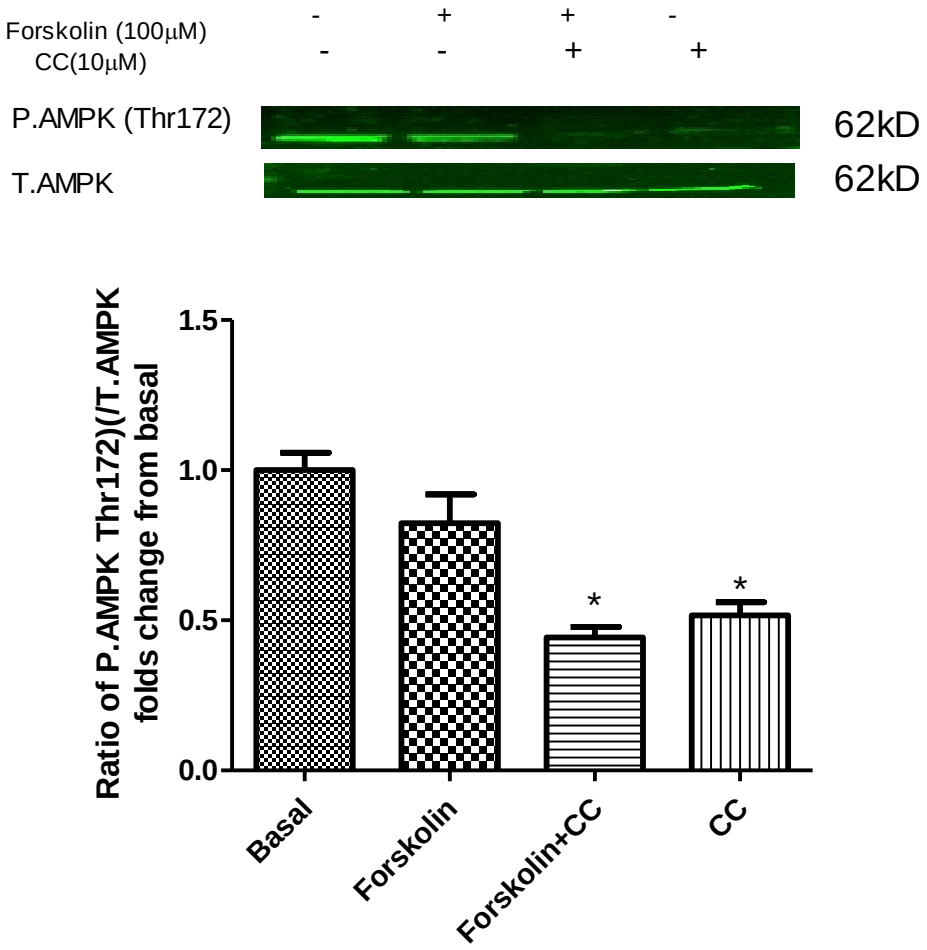

Figure 3.27: AMPK phosphorylation in BeWo cells treated with forskolin with or without compound C (CC) for 10min.

Measurement of AMPK phosphorylation in treated and untreated BeWo cells using Western immunoblotting. Blots are representative and data are presented in mean ± SEM of three independent experiment. *$P<0.05$ of treated cells compared with basal.

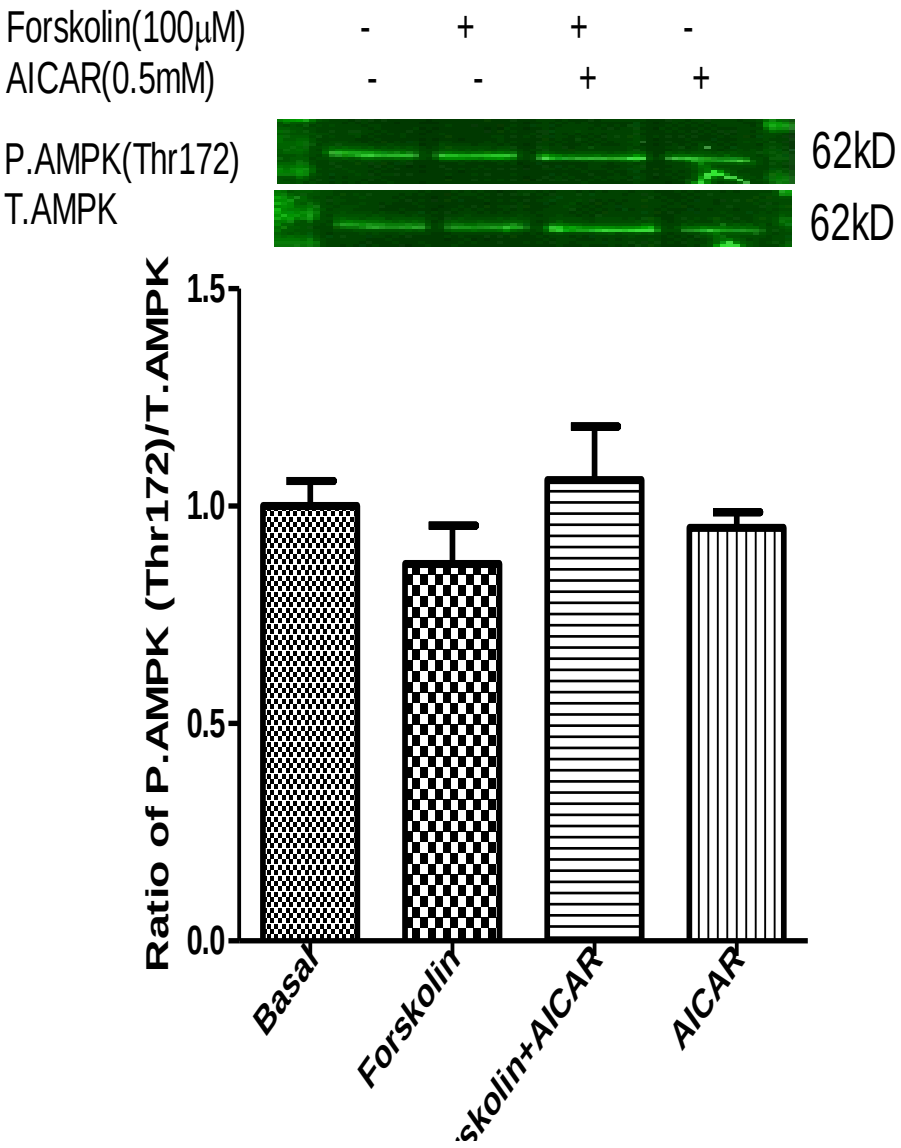

Figure 3.28: AMPK phosphorylation in BeWo cells treated with forskolin with or without AICAR (10 min).

Cell lysates were prepared and the expression of P.AMPK (Thr172) was measured using western blot. Total AMPK was used as a loading control. Blots are representative and data are presented in mean ± SEM of three independent experiments.

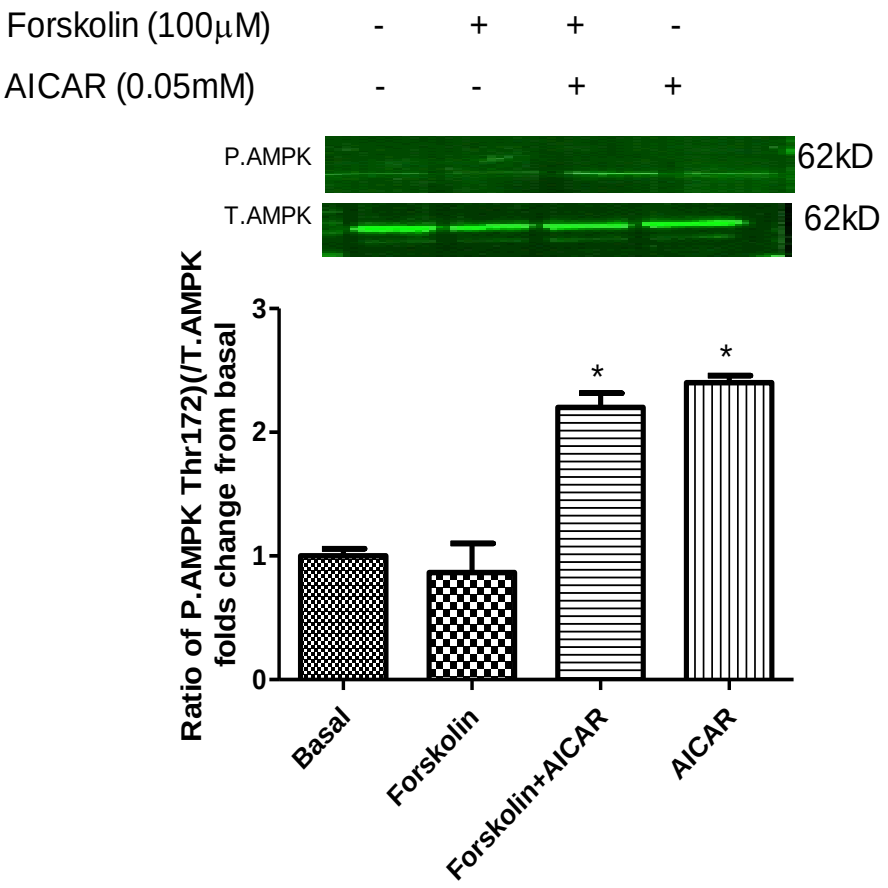

Figure 3.29: Expression of P.AMPK in BeWo cells treated with forskolin with or without AICAR for 24h.

Cells were treated with the indicated treatment for (24h). Cell lysates were prepared and AMPK phosphorylation was assayed by western immunoblotting. Total AMPK was used as a loading control Data are presented in a mean ± SEM of three independent experiments and blots are representative.*$P<0.05$ of treated cells compared to basal.

3.4.Expression of mTOR and it is related component in normal and GDM placenta

Our previous findings (which suggested the involvement of mTOR in the processes of BeWo cells differentiation) and the results of a previous study conducted by Jansson et al that have shown increased expression of mTOR signalling pathway in placenta from obese pregnant women, characterised by increased insulin resistance (Jansson et al., 2013). And because insulin is the upstream activator of Phosphatidylinositol 3-kinase (PI3K) which controls most of the metabolic actions of insulin, and it also activates mTORC1 through Akt (Taniguchi et al., 2006), all these prompt me to investigate the hypothesis that mTOR signalling is activated in the placenta from the mothers with GDM compared to normal pregnant women.

I measured the expression and activity of 11βHSD2, mTOR and its related compounds in GDM placentas and compared normal placentas. Tissue lysates were prepared from previously frozen placentas obtained from GDM patients and BMI matched controls (as described in materials and methods 2.5.2). Five GDM (BMI >30) and 6 matched controls were used. The demographics of the women from both group are presented in table 3.1.

Table 3.1: Biometric characteristics between normal and GDM pregnant women

characteristic	Normal (n=6) Mean	GDM (n=5) Mean
Age (years)	28.8(22-34)	31.5(27-36)
BMI (kg/m²)	24.4 (23.5-26.8)	34.5 (32.9-36.3)
Gestational age (weeks)	39.4 (38-41)	38.(37-39)

I applied Western immunoblotting on placental lysates to measure the expression of mTOR and its related components. I also compared the expression of 11β-HSD2 in GDM compared to normal placenta.

The results showed increased expression of 11βHSD2 protein in placenta from GDM by 2 folds (figure 3.30). The expressions of T.mTOR and P.mTOR (Ser2448/2481) were also increased in GDM compared to control (figure 3.31). In addition, the expressions of P.p70s6k (Ser371 and Thr389) were both increased in GDM placenta compared to control (Figure3.32).

Furthermore, the expression of phospho-Akt (Ser473), which is the indicator of mTORC2 activity, is increased in GDM placenta compared to control (Figure 3.33). However, there was no significant difference in the expression of P.Akt (Thr308) in GDM compared to normal placenta (Figure 3.33). P.Akt (Thr308 and Ser473) represent the upstream activator of mTOR signalling pathway The expression of P.AMPK (Ser172) in GDM placenta was lower than in normal placenta (figure 3.34).

Immunohistochemistry showed increased staining of mTOR and P.mTOR (Ser2448/2481) in GDM placenta, compared to control (figures 3.35, figure3.36 and figure 3.37).

3.4.1. Expression of 11βHSD2 in normal and GDM placentas

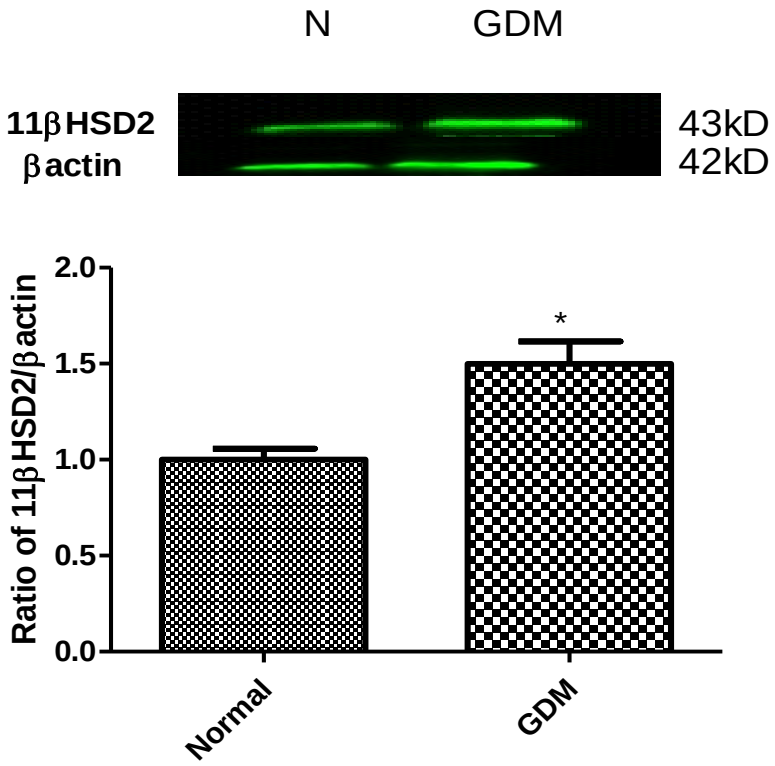

Figure 3.30: Expression of 11β HSD2 in normal (N) and GDM (D).

Placental lysates were prepared from previously frozen normal and GDM placentas as mentioned in the material and method, and the target proteins were assayed on western blot using the Odyssey detection system for protein visualization. β.actin was used as a loading control. Data represent the mean ± SEM of three independent experiments.*P < 0.05 GDM compared with normal.

3.4.2. Expression of total and phospho-mTOR (Ser2448/2481) in normal and GDM placentas

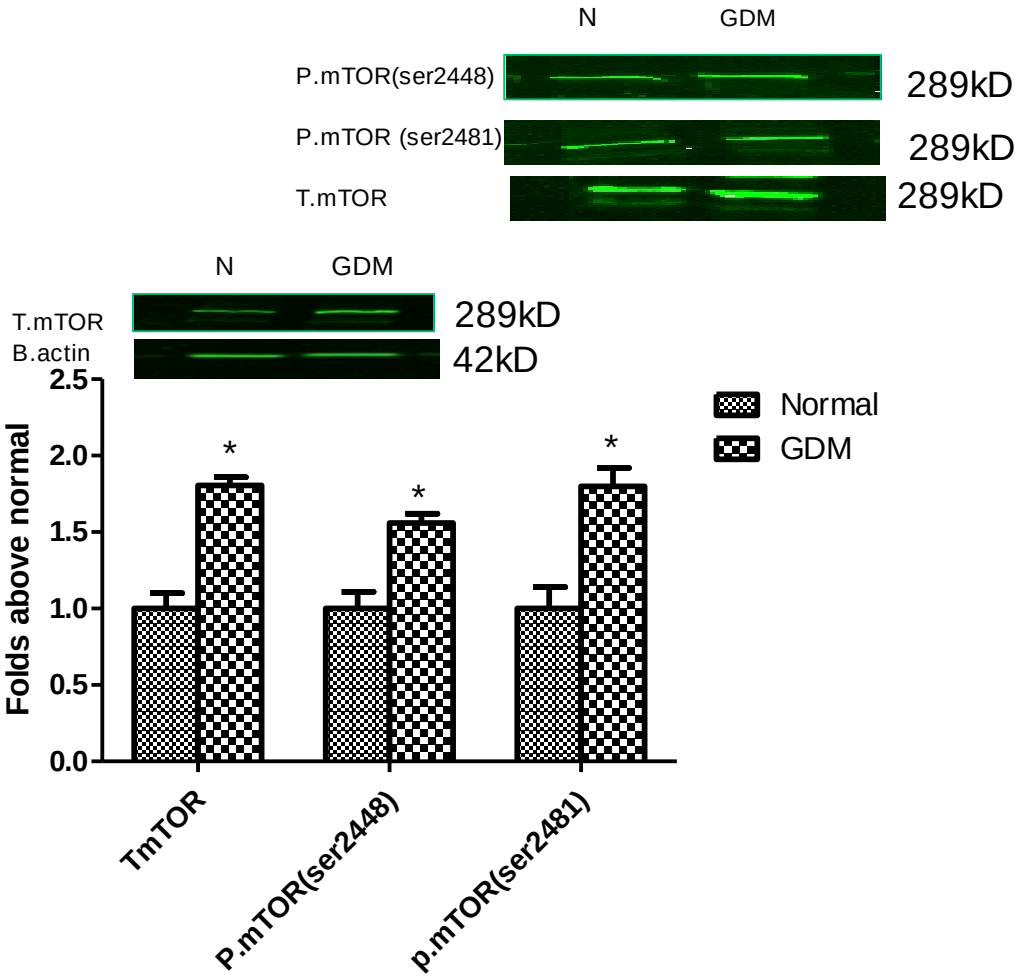

Figure 3.31: Expression of total and P.mTOR (Ser2448 and Ser2481) in normal (N) and GDM placenta

Placental lysates were prepared from previously frozen normal and GDM placenta, and target proteins were assayed on western blot using the Odyssey detection system. T.mTOR was used as a loading control for P.mTOR (Ser2448 and Ser2481) and β actin was used as a loading control for T.mTOR. Blots are representative and data represent the mean ± SEM of three independent experiments. *$P<0.05$ GDM compared with normal.

3.4.3. - Expression of P.p70sk (ser371 and Thr389) in normal and GDM placenta

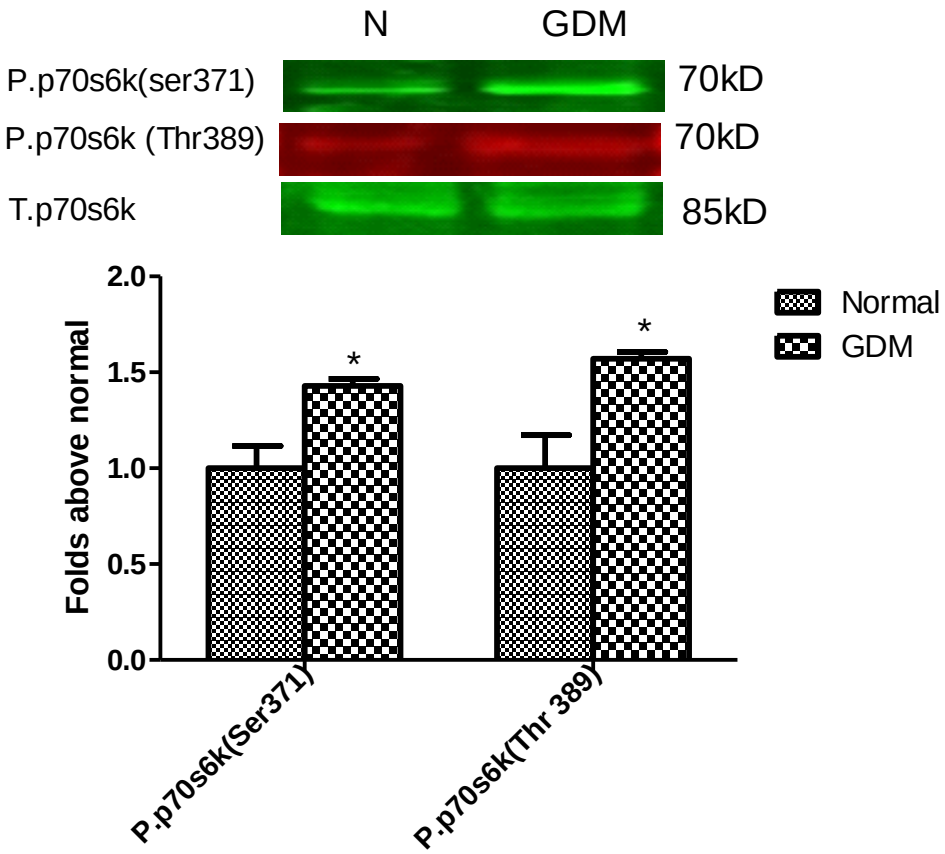

Figure 3.32: Expression of P.p70s6k (Ser371 and Thr389) in normal (N) and GDM placenta.

Placental lysate were prepared from previously frozen normal and diabetic placenta target proteins were assayed on western blot using the Odyssey detection system. Total p70s6k was used as a loading control. Data represent the mean ± SEM of three independent experiments. *$P < 0.05$ GDM compared with normal.

3.4.4. Expression of P.Akt (Ser473 and Thr308) in normal and GDM placentas:

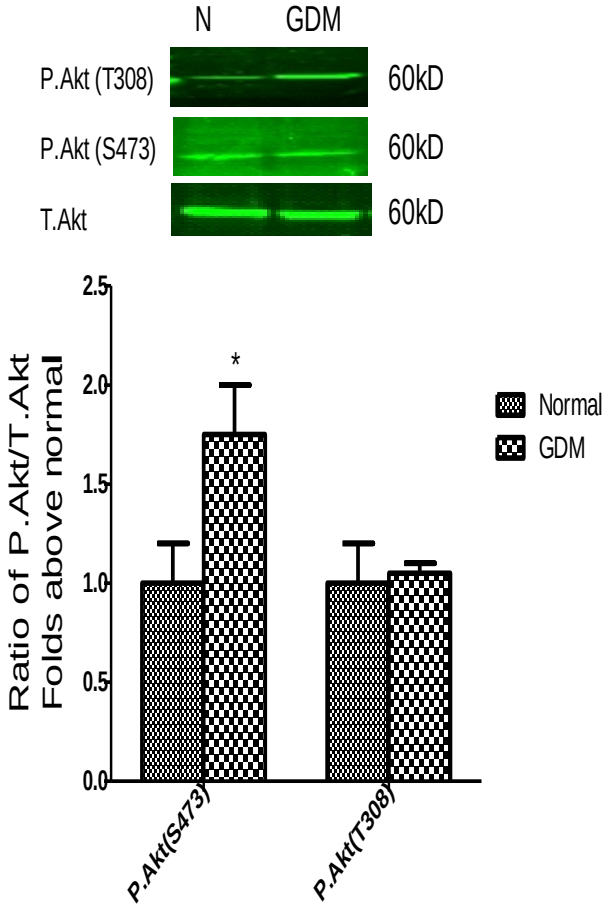

Figure 3.33: Expression of PAkt (Ser473 and Thr308) in normal (N) and GDM placenta.

Placental lysates were prepared from previously frozen normal and GDM placenta, and target proteins were then assayed on western blot using the Odyssey detection system. T.Akt was used as a loading control. Blots are representative and data represent the mean ± SEM of three independent experiments. *$P < 0.05$ GDM compared with control.

3.4.5. Expression of P.AMPK (Thr172) in normal and GDM placentas

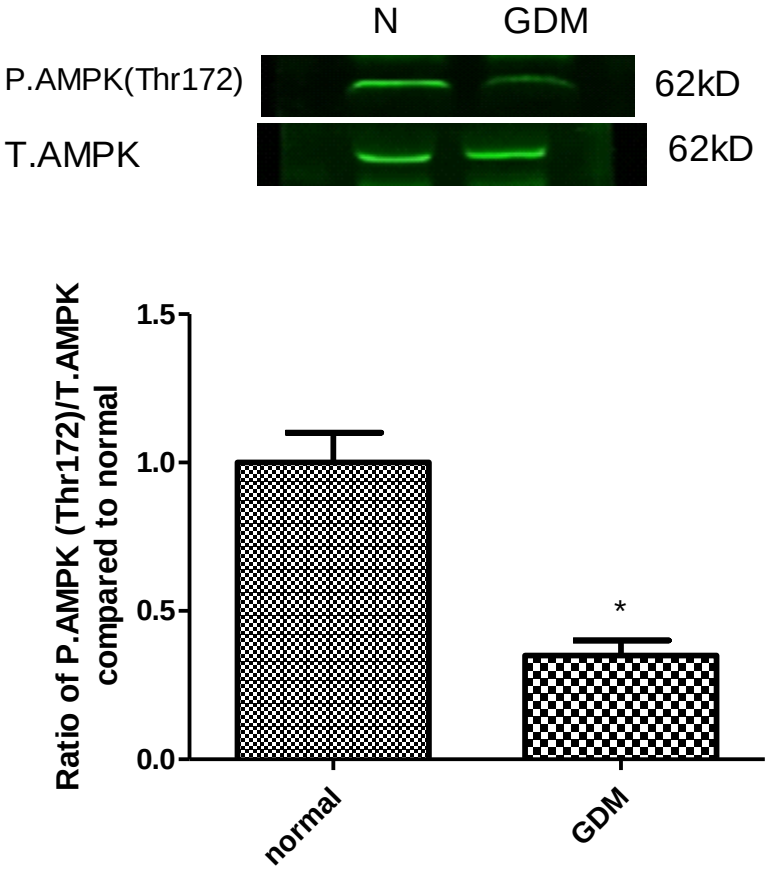

Figure 3.34: Expression of P.AMPK in normal (N) and GDM placenta.

The target proteins were assayed on western blot using the Odyssey detection system for protein visualization. T.AMPK was used as loading control. Data represent the mean ± SEM of three independent experiments.*$P < 0.05$ GDM compared with normal.

3.4.6. Immunohistochemistry: phospho-mTOR (Ser2448) staining in normal and GDM placentas

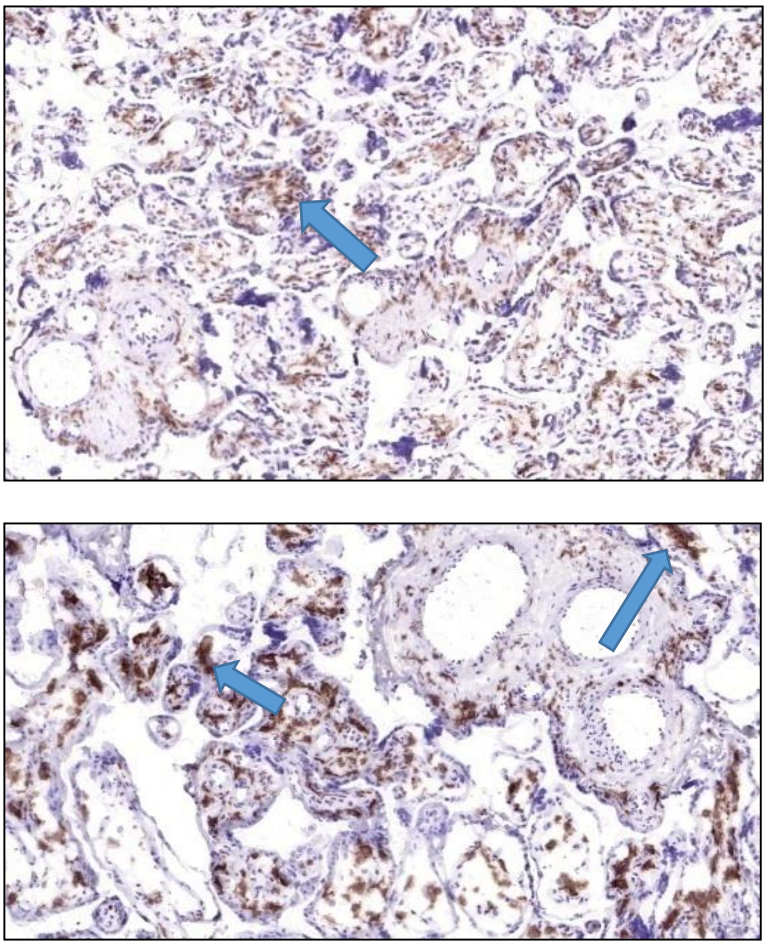

Figure 3.35: Immunohistochemistry of P.mTOR (Ser2448) staining in human normal (top) and GDM (bottom) placentas.

Placental staining of phospho-mTOR (Ser2448) (D9C2) XP Rabbit mAb in 1:200 performed on serial sections of placenta as described previously in material and methods. P.mTOR staining was higher in GDM compared to control. Most of the staining located in cytotrophoblasts (arrows).

3.4.7. Immunohistochemistry: phospho-mTOR (Ser2481) staining in normal and GDM placentas

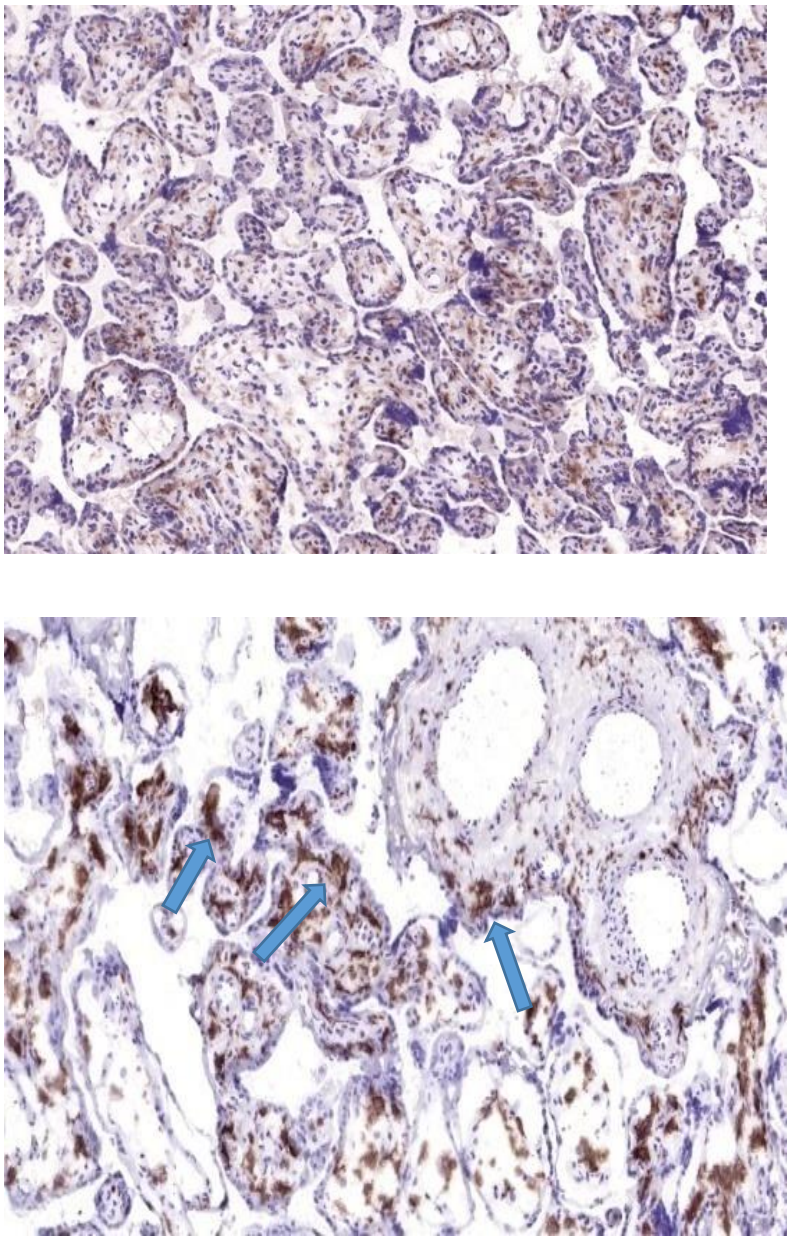

Figure 3.36: Immunohistochemistry of P.mTOR (Ser2481) staining in human normal (top) and GDM (bottom) placentas.

Placenta; staining of Phospho-mTOR (Ser2481) antibody (#2974) in 1:200 dilution, performed on serial sections as described previously in material and methods. .P.mTOR staining was higher in GDM compared to control. Most of the staining located in cytotrophoblast (arrows).

3.4.8. Immunohistochemistry: T.mTOR staining in normal and GDM placentas

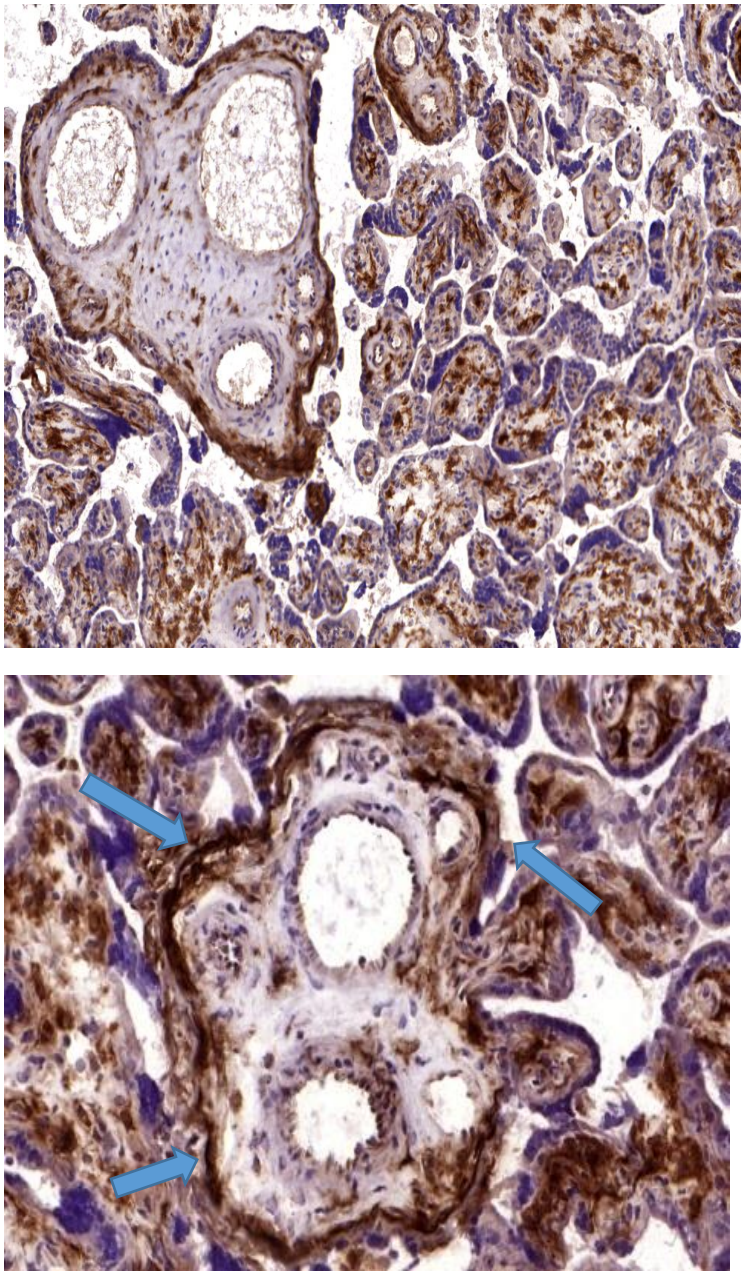

Figure 3.37: Immunohistochemistry of T. mTOR staining in human normal (top) and GDM (bottom) placentas

This is performed on serial sections of placenta as described previously in material and methods, Anti-mTOR antibody (ab2732) was used as primary antibodies with 1:200 dilution. T.mTOR staining was higher in GDM compared to control. Most of the staining located in cytotrophoblast (arrow).

3.5. Expression of OGT and O-GLcNAc protein in BeWo cells and placenta

Introduction

The deleterious effect of hyperglycaemia involves different mechanisms, among those is the O-GlcNac Glycosylation (O-GlcNAclation) (Alejandro et al., 2014) which consists of adding of a single *N*-acetylglucosamine (GlcNAc) to serine or threonine residues of proteins in the nucleus and cytoplasm. It is a posttranslational modification and unlike other glycosylation events which occur in Golgi bodies or in endoplasmic reticulum. It is a reversible process and is regulated by the enzymes *O*-GlcNAc transferase (OGT) which catalyse the addition of O-GlcNAC, and *N*-acetylglucosaminidase (*O*-GlcNAcase) which catalyse the removal of O-GlcNAC (Walgren et al., 2003). The highest concentration of O-GLcNAc occur in nuclear protein (Hanover et al., 1987). O-linked N-acetylglucoseamine (O-GlcNAc) was first discovered in early 1980s (Torres and Hart, 1984). Nearly 500 proteins of important cellular processes were identified as an O-linked N-acetylglucoseamine target. O-Glc-Nac is linked to various human diseases, an increased O-Glc-Nac level has been linked to the aetiology of insulin resistance, hyperglycemia and metabolic abnormalities (Park et al., 2010). Through hexosamine biosynthetic pathway (HBP) 2-3% of glucose in β-cells is used for the synthesis of uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc), which acts as an OGT substrate. (Alejandro et al., 2014).

It is well known that GDM induces hyperglycaemic environment in the placenta (Rudge et al., 2011) that resulted in increased activity of hexamine biosynthesis pathway and in turn increased O-GlcNAcylation (Alejandro et al., 2014). Based on this and the role of the O-Glc-Nacylation in pathology of insulin resistance, and the

increased expression of mTOR in GDM. I hypothesized that mTOR is involved in O-GlcNAcylation and my aims in this experiments are:

- To compare the expression of O-GlcNAc and OGT proteins in GDM placenta with normal placenta.
- To compare the expression of O-GlcNAc and OGT proteins in normal placenta with BeWo cells.
- To compare the expression of O-GlcNAc and OGT proteins between untreated and rapamycin treated BeWo cells cultured in normal (7mM) or in high (20mM) glucose media.

3.5.1. Expression of O-GlcNac and OGT protein in normal and GDM placentas

In this experiment, western immunoblotting was applied using cell lysates prepared from normal placenta and GDM placentas (as explained in materials and methods), specific O-GlcNAc and OGT antibody were used.

The result showed increased expression of O-GlcNAC and OGT protein in GDM placentas compared to control (Figure 3.38).

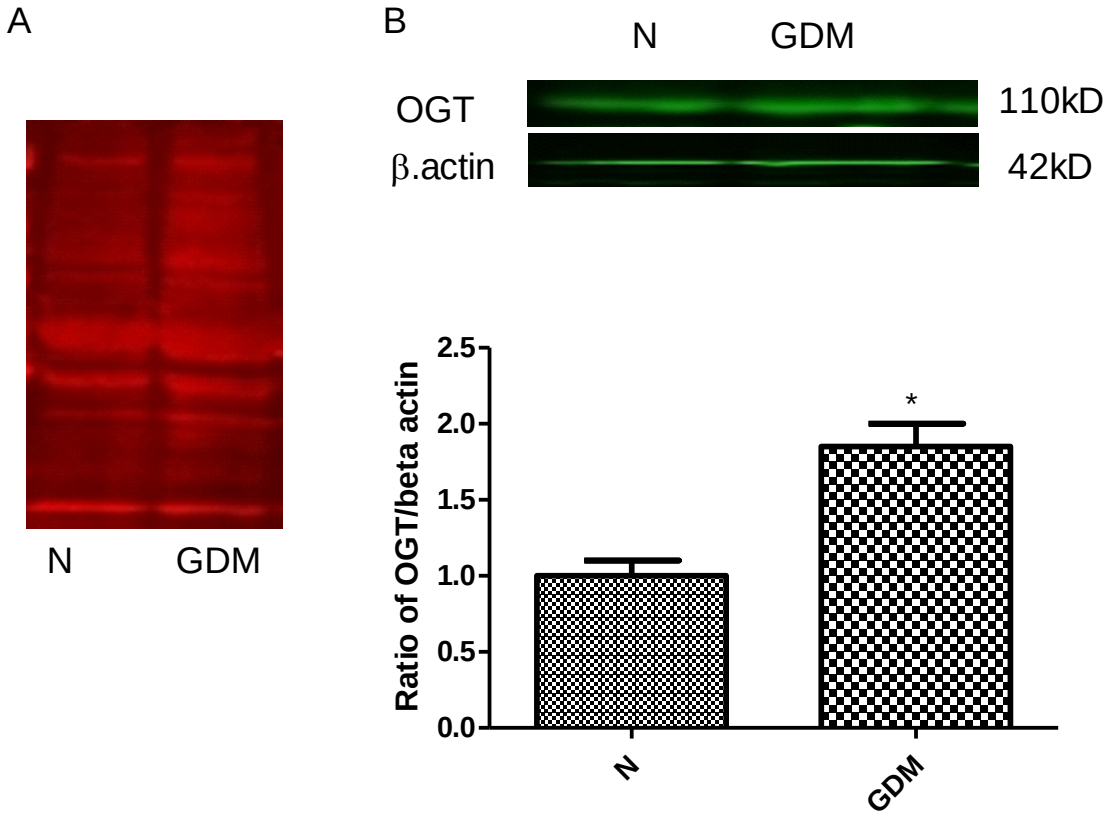

Figure 3.38: Expression of OGlcNAc and OGT proteins in normal (N) and GDM placentas.

Placental lysates were prepared from previously frozen normal and GDM placenta. OGlcNAc (A) and OGT (B) proteins were assayed on western blot using OGlcNAc and OGT specific antibody. The Odyssey detection system was used for protein visualization. Blots are representative. The data represent Mean ± SEM of three independent experiments. *$P < 0.05$ *GDM* compared to normal.

3.5.2. Expression of O-GlcNac protein in BeWo cells and normal placenta

BeWo cell lysates and placental lysates were prepared and subjected to Western immunoblotting for the measurement of OGT and O-GlcNac protein expression. The result showed decreased expression of OGT and O-GlcNac proteins in normal placenta compared to BeWo cells (figure 3.39).

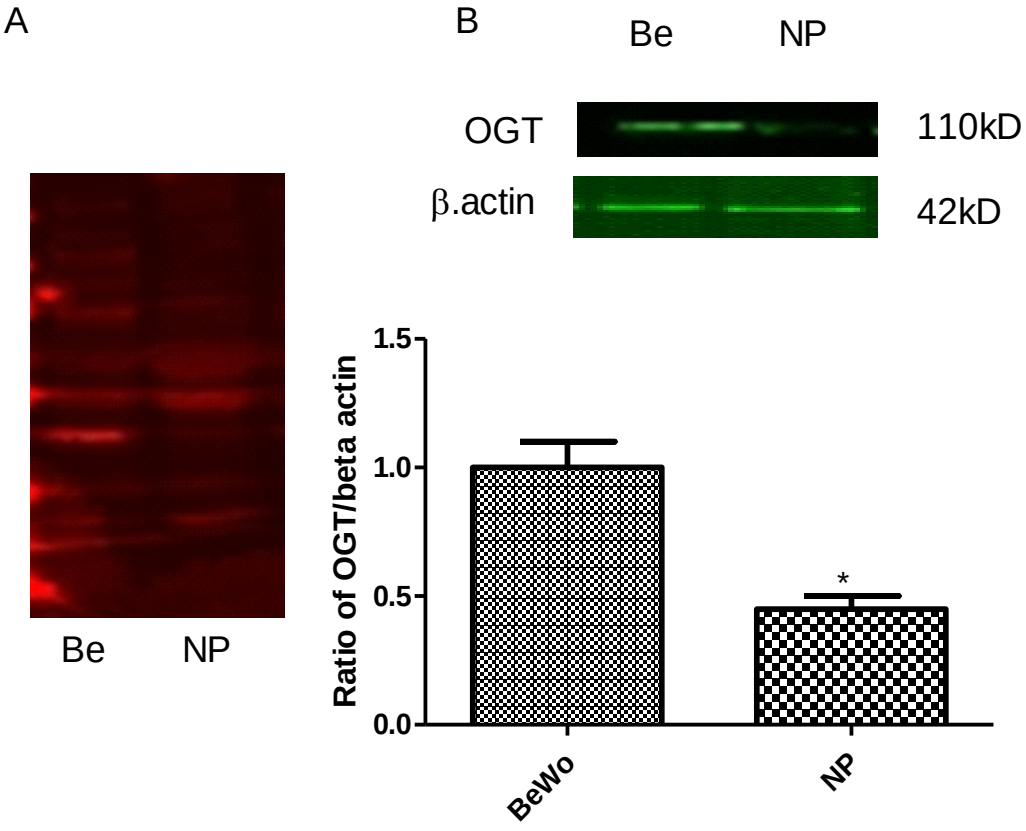

Figure 3.39: Expression of OGlcNAc and OGT proteins in BeWo cells and normal (NP) placentas. Lysates were prepared from BeWo cells and normal placenta (as mentioned in materials and methods chapter),O-GlcNac(A) and OGT (B), proteins were assayed on western blot using O-GlcNAc and OGT specific antibody the Odyssey detection system was used for protein visualization. The data represents Mean ± SEM of three independent experiments. *$P<0.05$ NP compared to BeWo cells.

3.5.3. Expression of O-GlcNac and OGT protein in BeWo cells treated or untreated with rapamycin (100nM) and cultured in F12K media with normal (7mM) or high (20mM) glucose concentration for 48hr

In this experiment, BeWo cells were cultured in F12K medium contains 7mM glucose (this is the normal glucose concentration contained in F12K media which is used for BeWo cells culture); when the cells were 70% confluent, media were changed and BeWo cells were incubated in media with either normal glucose concentration (7mM) with or without rapamycin (100nM), or with high glucose concentration (20mM) with or without rapamycin 100nM) for 48hr. Following incubation, cell lysates were prepared from treated and untreated BeWo cells and processed for SDS-PAGE electrophoresis.

For the high glucose concentration; two different concentration of glucose was tried by the author (20mM and 25mM), since those concentrations was considered to be the hyperglycemic condition in term placenta trophoblast and BeWo, JAR and JEG-3 cells (Weiss et al., 2001, Hahn et al., 1998). At the 25mM concentration, the growth of the cells was not satisfactory. Consequently and after preliminary testing the decision to fix the glucose concentration at 20mM was made.

The result showed that, the overall expressions of O-GlcNac and OGT proteins were higher in untreated BeWo cells compared to rapamycin treated BeWo cells. The expression was also higher in BeWo cells, which were cultured in high glucose media compared with the cells cultured in normal media. (Figures 3.40 and 3.41).

3.5.3.1. Expression of O-GlcNac and OGT protein in BeWo cells treated or untreated with rapamycin and cultured in normal media (with 7mM) or high glucose media (20mM) for 48 h.

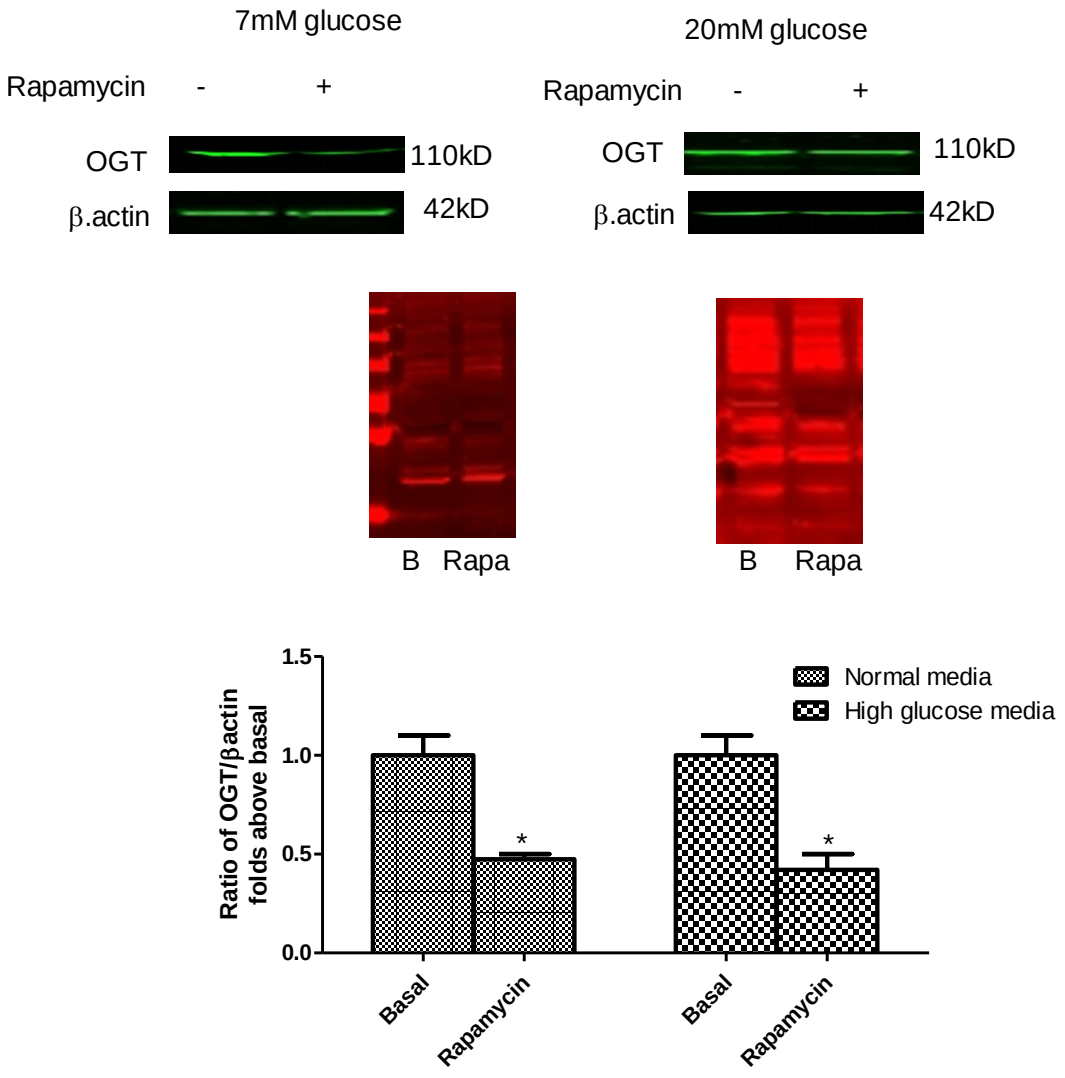

Figure 3.40: Expression of OGlcNAc and OGT protein in BeWo cells treated or untreated with rapamycin and cultured in normal media or high glucose media.

Lysates were prepared from BeWo cells treated or untreated with rapamycin and cultured in normal glucose media or high glucose media for 48h and O-GlcNAc and OGT protein expression from both group were assayed using western immunoblotting

The graph represent Mean ± SEM of three independent experiments.*P<0.05 of rapamycin treated cells compared to basal.

Chapter Four

General Discussion and Conclusion

Discussion

It is well recognised that cAMP and mTOR signalling pathways control important cellular processes such as proliferation, metabolism, and protein synthesis and cell survival. Thus, understanding the pathway that joins the two signalling is very crucial (Xie et al., 2011). Despite its role in cancer, there is no enough data regarding the effect of mTOR signalling pathway on gestational trophoblastic diseases (GTDs) (Mparmpakas et al., 2010). GTDs is a group of diseases affecting placenta and consists of complete hydatidiform mole, invasive mole, placental site trophoblastic tumour and choriocarcinoma (Fulop et al., 2004). The role of mTOR signalling pathway in trophoblast cell differentiation and proliferation is still not fully known (Wen et al., 2005). Researchers have shown that mTOR is important for early embryo growth and proliferation. They reported that mTOR lacking embryos die after implantation due to impaired cell proliferation (Vargas et al., 2009, Gangloff et al., 2004).

In BeWo cells, many pathways have shown to be involved in hCG secretion and fusogenic gene expression. For example, cAMP/PKA pathway plays a major role in trophoblast differentiation (Keryer et al., 1998). Furthermore, ERK1/2 and p38 MAPK pathways, the activated down stream of adenylyl cyclase, have been demonstrated to play distinct roles in fusogenic gene expression and hCG secretion ((Delidaki et al., 2011)). A decrease in syncytin 1 expression may be associated with the development of complication during pregnancy as it has been reported by previous study (Wilson et al., 2003), where they found an association between the development of preeclampsia and HEELP syndrome and a decrease in syncytin 1 expression.

Fusion of cytotrophoblast is not just associated with morphological changes, but it is also characterised by changes in trophoblast transport and endocrine activities

(Pötgens et al., 2002). The relationship between mTOR protein and hCG hormone has been demonstrated in various studies. One study has reported that hCG up regulates mTOR kinase signalling pathway in theca interstitial cells, (Palaniappan and Menon, 2010), while in granulosa cells in ovulating follicle in mice, inhibition of hCG resulted in down regulation of mTOR kinase activity (Siddappa et al., 2014).

Researchers have found that a combination of a calcium channel inhibitor and PI3K/Akt inhibition augmented forskolin-induced BeWo cell fusion. However, inhibition of P13K/Akt pathway alone was not enough to stimulate BeWo cell fusion. This indicates that many signalling pathways are involved in processes of BeWo cells syncytjalization (Vatish et al., 2012). Therefore, the aims of this work were to investigate the role of mTOR in fusogenic gene expression and hCG secretion by syncytialised BeWo cells and the effect of BeWo cells differentiation on expression of mTOR and its related components. In addition, I investigated the expression and activity of mTOR in placentas obtained from gestational diabetes (GDM) patients and BMI matched controls.

For the purpose of this study, BeWo cell lines were used as substitutes for placental trophoblasts. As I have mentioned earlier, BeWo cell line originates from human choricarcinoma cells and when incubated with forskolin, it showed an increased level of intracellular cAMP in a time and dose dependent manner, which resulted in cells fusion and syncytia formation (multinucleated cell) which has the ability to secrete hCG hormone (Wice et al., 1990).

In this study, several aspects of the role of mTOR on BeWo cells differentiation were investigated. First, I investigated the role of mTOR on forskolin-induced fusogenic gene expression. The results showed that, forskolin-treated BeWo cells showed

increased expression of fusogenic genes. In agreement with previous experiment which showed that stimulation of BeWo cells with forskolin resulted in either increased expression of fusogenic genes (Delidaki et al., 2011) or decreased expression of other genes such as mTOR and 4EBP genes. This suggested that forskolin changed the expression of various genes when it is used for BeWo cells differentiation (Mparmpakas et al., 2010).

This experiment showed that mTOR might inhibit forskolin-induced fusogenic gene expression since the addition of rapmycin to forskolin-treated BeWo cells resulted in significant increase in expression of syncytin 1 and 2.

The next part of this study was to investigate the effect of mTOR on forskolin-induced hCG secretion. hCG secretion by BeWo cells represents the biochemical marker of BeWo cells differentiation. The result showed that, rapamycin and AICAR have negative effect on forskolin-induced hCG secretion, which might indicate that mTOR is important for forskolin to induce hCG secretion by BeWo cells. However, compound C has no effect on forskolin-induced hCG secretion.

In this study, by using the BeWo choriocarcinoma cell line and discerning pathway inhibitors, I have shown a dual action of mTOR on BeWo cell differentiation, a stimulatory effect on hCG secretion associated with inhibitory effect on fusogenic gene expression.

Since compounds C and AICAR have been used in this experiment, it can be mentioned that I have also tested the effect of AMPK (mTOR signalling pathway inhibitor) on hCG secretion and fusogenic gene expression.

Furthermore, forskloin, through the activation of cAMP pathway, has been proven to increase level of 11-βHSD2 mRNA and activity in JEG-3 cells (Pasquarette et al., 1996). In addition, *in-vitro* studies have proven that in cultured syncytiotrophoblast of term human placenta, the oxidase activity of 11βHSD is referred mainly to 11βHSD2 (Sun et al., 1998).

The results showed that mTOR exerts no effect on 11βHSD2 secretion by forskolin-treated BeWo cells since neither compound C, nor does rapamycin affect 11βHSD2 release by forskolin-stimulated BeWo cells. However, AICAR may have a direct inhibitory effect on forskolin-induced 11βHSD2 secretion.

mTOR activity is controlled by many environmental signals, however, the exact mechanism of how different signals regulate mTOR remains vague. Among those signals is cAMP which was identified as one of the mTORC1 regulators. Researchers have noticed that the effect of forskolin-increased intracellular cAMP on Akt/mTOR/S6k1 pathway differs from one cell type to another. In isolated rat islets, forskolin increased p70s6k phosphorylation in a dose dependent manner, and this effect was independent of insulin and was inhibited by rapamycin (Kwon et al., 2004).

Another study demonstrated that in skeletal muscles, forskolin inhibited the stimulatory effect of insulin on Akt/mTOR pathway (Richmond et al., 2009). In addition, various studies have revealed that cAMP has an inhibitory effect on Akt phosphorylation at Ser473, which is essential for Akt protein kinase activity. cAMP also prevents the translocation of PDK1 to the plasma membrane which further inhibit Akt activity (Fang et al., 2000, Kim et al., 2001, Li et al., 2000). One study have shown a different mechanism through which cAMP inhibits mTORC1 activity where

elevated cAMP resulted in disassembly of both mTORC1 and mTORC2 (Xie et al., 2011).

I have shown that treatment of BeWo cells with forskolin almost eliminates mTOR phosphorylation at Ser2448 and Ser2481. Furthermore, the expression of T.mTOR was also inhibited and this might suggest that inhibition of mTOR signalling pathway is crucial for BeWo cells differentiation. Researchers have reported that the syncytialisation of BeWo cells results in down regulation of mTOR mRNA expression and its downstream signalling (p70s6k) when compared to untreated cells. They have referred this to the cessation of BeWo cells proliferation and the start of differentiation event (Mparmpakas et al., 2010). Coop et al have investigated the effect of prolonged treatment with rapamycin on the phosphorylation of mTOR Ser2448 and mTOR Ser2481 in HEK293 and U205 cells. They found that phosphorylation of mTOR 2448 in rapamycin treated cells was markedly reduced with either acute (1hr) or prolonged treatment (24hrs). In contrast, phosphorylation of 2481 was not changed after the first one hour of treatment while it was completely absent after prolonged treatment (24 hrs.) (Copp et al., 2009). AICAR also inhibits mTOR phosphorylation at Ser2448 in acute lymphocytic leukaemia (ALL) cells (Leclerc et al., 2010).This might indicate that P.mTOR (Ser2448) is one of mTORC1 components and P.mTOR (Ser2481) is probably part of mTORC2 (Copp et al., 2009).

In BeWo cells, the results revealed that Akt phosphorylation at Ser473 was also inhibited by forskolin and up-regulated in rapamycin treated cells. The crosstalk between Akt and mTOR complex can be explained by positioning mTOR both in the upstream (mTORC2) and downstream (mTORC1) of Akt, since mTORC2 stimulates Akt phosphorylation at Ser473, while phosphorylation of Akt at Thr308 activates mTORC1 (Vincent et al., 2011). This was further confirmed by the fact that Akt

phosphorylation at Ser473 was selectively absent in mTORC2 lacking cells (Bhaskar and Hay, 2007).

Up-regulation of Akt phosphorylation at Ser473 by rapamycin could be due to the inhibition of p70s6k activity (the downstream target of mTORC1) which exerts a negative feedback effect on mTORC2-stimulated Akt phosphorylation. The activation of Akt phosphorylation at Ser473 by rapamycin has weakened its antineoplastic effect. This was observed following the treatment of tumour cells with rapamycin (Sun et al., 2005). This is a consequence of inhibition of negative feedback loop of IRS-1 on PI3K/AKT which is mTOR dependent, so by inhibiting mTOR, rapamycin also inhibits this feedback loop leading to activation of AKT (O'Reilly et al., 2006). This effect has also been demonstrated in rhabdomyosarcoma cell line where treatment of this tumour by rapamycin resulted in up-regulation of Akt phosphorylation at Ser473 (Wan et al., 2006).

One of important lower stream targets of mTOR is P.p70s6k (Thr389), an mTORC1 substrate which is important for S6K1 activity. However, little is known regarding the regulation of p70s6K (Ser371) (Dann et al., 2007, Shin et al., 2011).

In BeWo cells, forskolin inhibited P.p70s6k (Thr389) phosphorylation whereas it stimulated the phosphorylation at Ser371. P.p70s6k (Thr389) is the marker for mTORC1 activity, and this indicates that forskolin down regulates the upstream (Akt) and downstream (P.p70s6k Thr389) effectors of mTORC1. Some studies have shown that the regulation of Ser371 and Thr389 is different. For example, rapamycin completely inhibits Thr389 phosphorylation, while it only slightly inhibits Ser371 phosphorylation (Varma and Khandelwal, 2007). Another difference is that Ser371 is not affected by serum starvation and insulin while Thr389 is considerably affected

(Shin et al., 2011). In addition, Tortin, an mTOR catalytic inhibitor and LY294002, a PI3-K/Akt pathway inhibitor have no effect on Ser371 phosphorylation, whereas both resulted in complete inhibition of Thr389 phosphorylation (Shin et al., 2011). Here we have shown for the first time that forskolin stimulates P.p70s6k phosphorylation at Ser371.

I have also compared the expression of mTOR and its components in GDM placenta to normal placenta. In vitro studies have shown that hyperglycaemia results in inhibition of proliferation of first trimester model (BeWo, JEG3), hence, impaired function of placenta in first trimester due to hyperglycaemia can be expected (Weiss et al., 2001).

A large group of evidence demonstrated that in pregnancy complicated by diabetes, there is an association between increased foetal growth and increased expression and activity of placental glucose and amino acid transporters (Jansson et al., 2002). Nutrient transport across placenta is controlled by foetal, maternal and placental factors; among the placental factors is the mTOR which is considered to be a positive controller of amino acid transport (Roos et al., 2007). Furthermore, it has been reported that in a gestational diabetic mother, placental leucin transport is increased compared to large for gestational age. This was associated with an increase in the activity of system A amino acid transport in microvillous plasma membrane (MVM) of placenta from diabetic mother (Jansson et al., 2002).

In-vitro researches have shown that the upstream regulators of mTOR such as leptin, insulin and IGF-I stimulate placental amino acid transport (Jansson et al., 2003) and mTOR positively regulates amino acid transport across placenta. This might indicate

that mTOR plays an important role in gestational diabetes, and abnormality in mTOR activity might be one of the causative factors for GDM pathology.

Western mmunoblotting showed increased expression of total and phospho-mTOR and its downstream effectors (P.p70s6k Thr389) in placentas from pregnancy complicated with GDM compared with normal placentas. Furthermore, immunohistochemistry revealed increased total and phospho-mTOR staining in placentas from GDM compared to normal placentas. This indicates that, mTOR might play a role in pathophysiology of GDM.

Surprisingly, there was no difference in the expression of P.Akt (Thr308) between GDM placentas and the control. This might indicate that the mTOR pathway in GDM due to P.Akt (Ser473) activation. mTORC2 expression is also increased in GDM placentas as demonstrated by increased expression of P.mTOR 2481 and increased phosphorylation of Akt (Ser473).

GDM placenta also showed increased expression of 11βHSD2 compared to normal. Numerous studies have tested the effect of various protein on the expression of 11βHSD2 in placenta and BeWo cells. One study has reported that epinephrine and nor-epinephrine down regulate placental 11βHSD2 gene expression in a manner faster than forskoin induced 11βHSD2 expression (Sarkar et al., 2001). Placental 11βHSD2 expression is also inhibited by steroid (estrogen, progesterone) (Sun et al., 1998). On the other hand, retinoic acid (Tremblay et al., 1999), forskolin (Pasquarette et al., 1996) and cAMP (Sun et al., 1998) up-regulate 11β HSD2 protein expression.

In pregnancy complicated by IUGR, it has been reported that the gene expression of 11-βHSD2 is significantly reduced compared with normal pregnancy which might

indicate that 11-βHSD2 is a major regulator of foetal growth during human pregnancy (McTernan et al., 2001).

Our finding is in agreement with another study conducted by (Ma et al., 2012) in which they reported that the expression of 11βHSD2 was up-regulated at both mRNA and protein level in placenta of patients with GDM compared to normal placenta.

Various studies have shown the role of protein *O*-GlcNAcylation in glucose induced insulin resistance (McClain et al., 2002, Vosseller et al., 2002). However, the relationship between O-GlcNac and PI3K/Akt/mTOR pathway is contradicting. Some studies have shown that increased activity and expression of OGT protein and O-GlcNac acylation in muscle, liver cells and fat cells resulted in inhibition of insulin signalling, whereas other studies showed that O-GlcNac acylation does not induce insulin resistance (Krzeslak et al., 2014).

Here, I have shown increased expression of OGT and O-GlcNac protein in GDM placenta compared to normal placenta and also in BeWo cells compared to normal placenta. The results have also shown decreased expression of both proteins in rapamycin treated BeWo cells compared to untreated cells. This might indicate the association between mTOR signalling pathway and O-GlcNAcylation which needs to be further investigated.

Conclusion

In conclusion, I have shown that inhibition of mTOR by rapamycin reduced forskolin-induced hCG release, whereas syncytin-1 and -2 mRNA expression was substantially augmented. I have also demonstrated that within 10min of forskolin treatment, there was a substantial reduction in basal mTOR phosphorylation at Ser2481 and Ser2448, which is required for mTOR activity. Additionally, increased expression of total and phospho-mTOR (Ser2448/2481) staining in GDM placenta. All these results suggest novel, distinct roles for mTOR in the control of trophoblast differentiation and may provide an explanation for the mechanisms of increased placental and fetal size in GDM.

Further experiments investigating the effect of mTOR in a time and dose dependent manner are required to support my results and to further investigate the role of mTOR on BeWo cells differentiation. Future work should also include studying the effect of mTOR on hCG and steroid hormone secretion from placental explant of normal and GDM placentas to further strengthen my experimental finding on BeWo cells. Future experiments should also include a mass spectrometry to identify if mTOR is among the proteins which are involved in O.Glc-Nac modification.

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