UNIVERZA V LJUBLJANI

FAKULTETA ZA FARMACIJO

ALJA KOZULIĆ – PIRHER

AKTIVACIJA SIGNALNE POTI mTOR IN EKSPRESIJA ŽILNEGA ENDOTELIJSKEGA RASTNEGA DEJAVNIKA V GASTROINTESTINALNIH NEVROENDOKRINIH CELICAH

mTOR PATHWAY ACTIVATION AND EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN GASTROINTESTINAL NEUROENDOCRINE CELLS

ENOVITI MAGISTRSKI ŠTUDIJ FARMACIJA

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CONTENT

ABSTRACT

Intestinal neuroendocrine tumours arise from the neuroendocrine cells diffused in the intestinal tract. They are highly heterogeneous and relatively rare. The cellular mechanisms underlying their development are not well understood, however a deregulation of the mammalian target of rapamycin (mTOR) pathway is described in 33% of tumours.

In order to further understand the mTOR pathway signalisation we created hypoglucidic stress model, which is based on the glucose depletion. Murine digestive neuroendocrine STC-1 cell line was used. Interestingly, cells survive hypoglucidic stress conditions and strongly activate mTOR pathway. By analysing the transcription of those cells, we saw that transcription of genes involved in lysosomal function and genes implicated in lipid metabolism was increased. According to the literature, these genes are associated with the activation of transcription factor EB-mTOR axis. The activation of this axis is connected with mTOR recruitment to the lysosomal surface. Surprisingly, we did not see such a colocalization. Therefore, the mechanism of mTOR pathway activation upon hypoglucidic stress remains unknown.

As vascular endothelial growth factor A (VEGF-A) expression was modified along development of those tumors, the expression of vascular endothelial growth factor B (VEGF-B) and vascular endothelial growth factor C (VEGF-C) has never been studied. According to our results, both genes were transcribed in STC-1 cells. Long term cultivation in 1mM glucose medium increased *Vegf b* mRNA expression, while *Vegf c* mRNA expression was not modified. Whether the increase was the consequence of mTOR activation, still remains to be confirmed. Furthermore, VEGF-B is synthesised. Its expression increases in hypoglucidic stressful conditions and is regulated by mTOR pathway. As VEGF-B is associated with the lipid uptake and lipid metabolism, its role in neuroendocrine cells during the glucose depletion will be further investigated. VEGF-C expression seems to be regulated by nutrient supply but also seems to be independent of mTOR activation. As VEGF-C is an important factor in lymphangiogenesis and cell survival, its role in tumor development and metastatic dissemination remains to be investigated.

Keywords: mammalian target of rapamycin (mTOR), lysosomes, vascular endothelial growth factor (VEGF), glucose deprivation, transcription factor EB (TFEB)

POVZETEK

Gastrointestinalni nevroendokrini tumorji nastanejo iz celic nevroendokrinega sistema. Uvrščamo jih med redke tumorje, vendar pa njihova pojavnost narašča. Glede na hormonsko aktivnost oziroma neaktivnost uvrščamo bolnike v dve skupini. V prvo spadajo bolniki z karcinoidnim sindromom, v drugo pa bolniki brez sindroma. Karcinoidni sindrom nastane zaradi obsežnega sproščanja bioaktivnih produktov tumorja in lahko ogrozi bolnikovo življenje. Povzroča nespecifične simptome kot so krči v trebuhu, pretirano potenje in driska, zato se velikokrat zamenjujejo s simptomi drugih, bolj pogostih bolezni prebavnega trakta. Bolniki iz druge skupine ne občutijo niti takšnih težav, zato je pri njih diagnoza še toliko bolj otežena. Bolezen največkrat odkrijejo v pozni fazi, zato je zdravljenje še toliko bolj težavno. Kljub napredku v razumevanju delovanja in razvoja teh tumorjev na molekularnem nivoju, je operativni poseg še vedno najuspešnejša metoda. Vendar pa le-ta ni vedno mogoč, še posebej takrat, ko je bolezen že v metastatski obliki. Za sistemsko zdravljenje nevroendokrinih tumorjev se največkrat uporabljajo analogi somatostatina, ki zavirajo izločanje številnih hormonov, kot so serotonin, inzulin, glukagon in gastrin ter zavirajo rast tumorja. Uporabljajo se tudi različne kombinacije kemoterapevtikov, vendar pa je njihova učinkovitost majhna, saj ima ta vrsta tumorjev nizko proliferativno aktivnost. Tretja možnost je tarčna terapija, in sicer z zaviralci angiogeneze in mTOR signalne poti. Nevroendokrini tumorji so načeloma dobro prekrvavljeni ter izločajo velike količine žilnega endotelijskega rastnega dejavnika A (VEGF-A). Zaviralci mTOR signalne poti, kot je na primer Everolimus, so se izkazali za učinkovito terapevtsko možnost, saj je v 33 % intestinalnih nevroendokrinih tumorjih opažena izrazita aktivacija te signalne poti. Čeprav je zadnje desetletje raziskav pomembno prispevalo k razumevanju nevroendokrinih tumorjev, ostaja veliko odprtih vprašanj.

Magistrska naloga je razdeljena na dva dela. V prvem delu smo poskušali razumeti posledice aktivacije mTOR signalne poti. Uporabili smo mišje intestinalne nevroendokrine celice (STC-1 celična linija). Ker je mTOR signalna pot občutljiva na koncentracijo hranilnih snovi v okolju, smo z modulacijo glukoze v celičnem mediju želeli vplivati na aktivacijsko stanje te poti. Celice smo gojili v standardnem DMEM gojišču z 25 mM koncentracijo glukoze ter ga po oseminštiridesetih urah zamenjali z gojiščem, ki je vseboval nižjo koncentracijo glukoze (5 mM, 2 mM ali 1 mM) in tako ustvarili šok zaradi znižanja koncentracije glukoze. V nasprotju z našimi pričakovanji ter objavljeno literaturo, je šok sprožil aktivacijo mTOR signalne poti. Ker je mTOR

signalna pot pomembna pri transkripciji genov, smo v nadaljevanju analizirali gene, ki so različno izraženi pri celicah, gojenih v 1 mM in 25 mM glukoznem okolju. Podatke smo pridobili iz analize transkriptoma, narejenega na STC-1 celicah, ki so bile gojene nekaj tednov v zgoraj naštetih gojiščih. Ugotovili smo, da je kar nekaj skupin genov, ki so udeležni v procesu znotrajceličnega transporta, bolj izraženih v razmerah, ko so celice podvržene pomankanju glukoze. Po temeljitem pregledu literature smo ugotovili, da je kar nekaj genov povezanih z aktivacijo mTOR signalne poti. Po podrobni analizi smo videli, da je v 1mM okolju povečana ekspresija genov kodirajočih za delovanje lizosomov. Te smo lahko razdelili v 2 skupini. V prvi skupini so bili geni, katerih transkripcija je povezana s prepisovalnim dejavnikom EB (TFEB). Prehod TFEB v jedro je povezan z aktivacijo mTOR signalne poti. V drugi skupini pa so bili geni, ki niso opisani kot direktne tarče TFEB-mTOR signalne poti. Če je ta signalna os resnično prisotna v STC-1 celicah, trenutno ne vemo oziroma ne moremo trditi.

STC-1 celice preživijo v okolju z znižano koncentracijo glukoze (1 mM) tudi do nekaj tednov, zato smo se osredotočili tudi na izražanje genov, ki so udeleženi v metabolizmu lipidov in katerih izražanje je povezano z aktivacijo TFEB-mTOR signalne poti. Ugotovili smo, da je bilo izražanje genov statistično značilno povečano. Ugotovili smo tudi, da je v 1mM glukoznem mediju povečano izražanje genov, ki so opisani kot regulatorji mTOR signalne poti, kot je na primer folikulin (FLCN). Ali folikulin resnično lahko aktivira mTOR signalno pot ob pomanjkanju glukoze v STC-1 celicah, pa ostaja neznanka.

V drugem delu magistrske naloge smo se osredotočili na ekspresijo žilnega endotelijskega rastnega dejavnika (VEGF). Ker je ekspresija žilnega endotelijskega rastnega dejavnika A (VEGF-A) že opisana pri intestinalnih nevroendokrinih celicah in tumorjih, smo se v raziskavi predvsem posvetili drugima dvema, to je žilnima endotelijskima rastnima dejavnikoma B in C (VEGF-B in VEGF-C). Ekspresija teh dveh dejavnikov še nikoli ni bila opisana pri intestinalnih nevroendokrinih tumorjih. Tako smo v mišji celični liniji STC-1 najprej validirali prepisovanje *Vegf-b* in *Vegf-c* genov v celicah, gojenih v 25 mM glukoznem gojišču s RT-qPCR metodo. S pomočjo prenosa western smo želeli preveriti njihovo prisotnost tudi na proteinskem nivoju. Nato smo uporabili model hipoglucidnega stresa, s katerim smo močno aktivirali mTOR signalno pot. Izmerili smo večje izražanje *Vegf-b* gena pri celicah, izpostavljenih pomanjkanju glukoze od 15-26 dni, medtem ko je bilo izražanje *Vegf-c* dokaj stabilno. Izražanje VEGF-B proteina je bilo povišano v 1mM vs 25 mM glukoznem gojišču in povišanje je bilo preprečeno, ko smo zavirali

mTOR signalno pot z rapamicinom (inhibitor mTOR signalne poti). To pomeni, da hipoglucidni stres preko aktivacije mTOR signalne poti pozitivno vpliva na izražanje VEGF-B. Izražanje VEGF-C se spreminja skladno s pomanjkanjem glukoze v mediju, vendar pa se zdi, da ni povezano z aktivacijo mTOR signalne poti. Nadaljevali smo z raziskovanjem VEGF-C izražanja v *in vivo* študiji. Uporabili smo mišji model, kjer so STC-1 celice vbrizgane v mišjo vranico ter se po 28 dneh pojavijo jetrni noduli. Lastnosti slednjih so zelo podobne tistim, ki jih opazimo pri slabo diferenciranem intestinalnem nevroendokrinem tumorju pri človeku. Ugotovili smo, da se VEGF-C izraža v mišjih jetrnih nodulih.

Magistrsko delo je odprlo veliko novih vprašanj, na katere trenutno še ne znamo odgovoriti. Zakaj pomanjkanje glukoze aktivira mTOR signalno pot še zdaleč ni razjasnjeno. Gre za nov pojav, ki nasprotuje trenutno sprejetim teorijam. Naši rezultati kažejo, da je njegova aktivacija povezana z ekspresijo VEGF-B. Kljub temu, da smo odkrili morebitne mehanizme regulacije izražanja VEGF-B v intestinalnih nevroendokrinih celicah, pa o vlogi tega dejavnika ne vemo nič. Tudi funkcija VEGF-C ostaja neraziskana.

Ključne besede: mTOR, žilni endotelijski rastni dejavnik, lizosom, pomanjkanje glukoze, TFEB.

LIST OF ABBREVIATIONS

4-EBP1- Eukaryotic translation initiation factor binding protein 1

AMPK - AMP-activated protein kinase

ANG1 - Angiopoietin 1

ATF - Activating transcription factor

ATG - Unc-51-like autophagy activating kinase

ATP - Adenosine triphosphate

BIP - Binding immunoglobulin protein

CHOP - CCAAT-enhancer-binding protein homologous protein

CLCN7 - Chloride channel 7

CLEAR - Coordinated Lysosomal Expression and Regulation

DNET - Digestive neuroendocrine tumors

DPP7 - Dipeptidyl-peptidase 7

eIF4 - Eukaryotic translation initiation factor

ER - Endoplasmic reticulum

FATP - Fatty acids transporter

FLCN - Folliculin

FKBP12 - FK506-binding protein of 12kDa

FNIP - Folliculin interacting protein

FRB - FKBP12-rapamycin binding domain

GβL - G protein beta protein subunit-like

GAP - GTPase – activating protein

GI-NETs - Gastrointestinal neuroendocrine tumors

GNS - N-acetylglucosamine-6-sulfatase

HIFα - Hypoxia inducible factor α;

HPS4 - Hermansky-Pudlak syndrome 4

HS - Hypoglucidic stress

IL1β - Interleukin-1 beta

IRES - Ribosome entry site

IRE1 - Inositol-requiring gene 1

IRS-1 - Insulin receptor substrate 1

KRAS - Kirsten rat sarcoma viral oncogene homolog

LAMP2 - Lysosome-associated membrane protein 2

LEDGF - Lens epithelium-derived growth factor

LKB1 - Liver kinase B1

MAP1LC3B - Microtubule – associated protein 1 light chain 3 beta

MCOLN1 - Mucolipin 1

MT1 - Metallothionein 1

mTOR - Mammalian target of rapamycin

mTORC1 - Mammalian target of rapamycin complex 1

mTORC2 - Mammalian target of rapamycin complex 2

NET - Neuroendocrine tumors

NEU1 - Neuroaminidase 1

NPC2 - Niemann Pick type C2

NRP - Neurophilin

P70S6K - P70 ribosomal protein S6 kinase

PDGF - Platelet-derived growth factor

PDK1 - Phosphoinositide-dependent kinase1

PERK - PKR-like endoplasmic reticulum kinase

PPARG - Peroxisome proliferator-activated receptor gamma

PPARGC1A - Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PPARGC1B - Peroxisome proliferator-activated receptor gamma coactivator 1-beta

PTEN - Phosphatase and tensin homolog

RHEB - Ras homolog enriched in brain

RTK - Receptor tyrosine kinase

RTEF-1 - Related transcriptional enhancer factor-1

SEER - American program surveillance, epidemiology and end results

SLC - Solute carrier

SREBP - Sterol regulatory element-binding protein

SQSTM1 - Sequestosome 1

TFEB - Transcriptional factor EB

TKI - Tyrosine kinase inhibitor

TMEM - Transmembrane protein

TNFβ - Tumor necrosis factor β

TPP - Tripeptidyl peptidase

TSC - Tuberous sclerosis

UPR - Unfolded protein response

UVRAG - UV radiation resistance associated

VEGF - Vascular endothelial growth factor

VEGFR - Vascular endothelial growth factor receptor

VPS - Vacuolar protein sorting

WHO - World health organisation

WIPI - WD repeat domain, phosphoinositide interacting 1

XBP1 - X-box binding protein 1

1. INTRODUCTION

1.1. Gastrointestinal tumors

Gastrointestinal neuroendocrine tumors (GI-NETs) are defined as rare neoplastic lesions which arise from endocrine cells of the digestive tract, most frequently in small intestine (41.8%), rectum (27.4%), and stomach (8.7%) (1). NETs are relatively rare tumors, but the incidence is increasing. According to American program surveillance, epidemiology and end results (SEER), the annual occurrence increased from 1.09 per 100,000 people in the 1970s to around 5 per 100,000 persons in 2004 (2). These tumors express both neuronal and endocrine markers and are able to retain the secretion potential. Indeed, some of NETs are able to over secrete these substances. Therefore, they are called functional neuroendocrine tumors and can cause so called carcinoid syndrome (3). The symptoms are nonspecific, including diarrhea, flushing, abdominal pain and often confused with others, more common gastrointestinal diseases. However, in more than 80% of cases, NETs do not massively release bioactive substances in bloodstream and are considered as non-functional tumors with low proliferation rate. These patients do not have any symptoms and therefore the diagnosis is even more difficult and delayed. Indeed 50% of NETs present regional or distant metastasis (most commonly in the liver) in the time of the diagnosis, which make the treatment even more complicated and unsuccessful (3,4).

Clinical classification divides NETs into three groups (Table I): well differentiated tumors (G1 and G2) and poorly differentiated tumors (G3). The classification is based on 3 major criteria: morphology, mitotic index and Ki67 expression (proliferation rate). Well differentiated NETs represent heterogeneous group of benign and low-grade tumors, with a slow progression and lower metastatic evolution. They develop mainly in small intestinal, appendix and pancreas. In contrast, poorly differentiated group is more aggressive, rapidly progressive and presents higher malignancy potential (5). They occur mainly in oesophageal, gastric, pancreatic and colorectal level (6).

Table I: WHO classification of neuroendocrine tumors (2010). *Neuroendocrine tumors are divided in 3 grades according to their morphology, mitotic index and Ki-67 count.*

	Morphology	Mitotic Index	Ki-67
Neuroendocrine neoplasm, grade 1 (G1)	Well differentiated	< 2 mitoses	≤ 2%
Neuroendocrine neoplasm, grade 2 (G2)	Well differentiated	2 - 20 mitoses	3-20%
Neuroendocrine carcinoma	Low differentiated	> 20 mitoses	> 20%

It has to be noticed, that studies of these tumors have been relatively challenging, mainly because of their two limiting properties: scarcity and heterogenecity. Consequently, our knowledge of their cellular biology is significantly lower in comparison with the others, more frequent cancers. More than 95% of DNETs are sporadic and the most known oncogenes (Kras, c-myc) or tumor suppressors deletions (p53, Pten) found in cancer are not altered in DNETs (6). However, a recent study of small intestinal neuroendocrine tumors revealed the alterations of the PI3K/Akt/mTOR pathways in 33% of the patients, suggesting the rationales for the use of mTOR pathway inhibitors, such as Everolimus (6). Clinical studies on the pancreatic neuroendocrine cancer found that the patients treated with Everolimus showed increased progression free survival. But the treatment did not cause tumor regression neither change in the patient's overall survival, pointing out the profound lack of understanding in pathway signalization and applications of target therapies (7). Indeed, the knowledge is built mainly on the clinical and histological investigations.

Nowadays, surgical restriction is still the only curative treatment of DNETs. Unfortunately, this treatment is not the option for patients that already had developed metastases at the time of the diagnosis. In the case of carcinoid syndrome when tumors release great amounts of hormones causing life-threatening complications, the restriction of the primary tumor can also be considered as option (8). However, patients with the progressive disease could be treated by the chemotherapeutic drugs, such as etoposide and cisplatin as the

first line therapy in poorly differentiated DNETs (9). Besides the traditional chemotherapeutic approaches, DNETs might be treated by the three main axis of pharmacological therapy:

1. Analogues of somatostatins, which have antitumor activity in particular for the slowly progressive DNETs with limited hepatic invasion. They are also used for the management of the carcinoid syndrome. Somatostatin is an inhibitor of various hormone secretions, including serotonin, insulin, gastrin and glucagon. As the NETs cells are rich in the somatostatin receptors, different analogues, such as Octreotide and Lanreotide are used in order to prevent excessive hormone secretion (10).
2. Drugs inhibiting the angiogenesis, in the case of well-differentiated tumors, as hypervascularization is one of the most important characteristic of these tumors. Commonly used drugs are tyrosine kinase inhibitors (TKI) which directly target the vascular endothelial growth factor receptors (VEGFRs) and prevent its downstream signalisation. Besides TKI, anti-angiogenic agent Bevacizumab that targets vascular endothelial growth factor A (VEGF-A) is currently in clinical trial. It prevents binding of VEGF-A to it receptors (11).
3. Targeting intracellular mTOR pathway which is implicated in the DNETs cancerogenesis. Indeed, targeted therapies can be given in a combination with the chemotherapeutic agents in order to increase the efficiency of the treatment. Different associations are possible, depending on the patient state, tumor grade and responsiveness (11) (Figure 1).

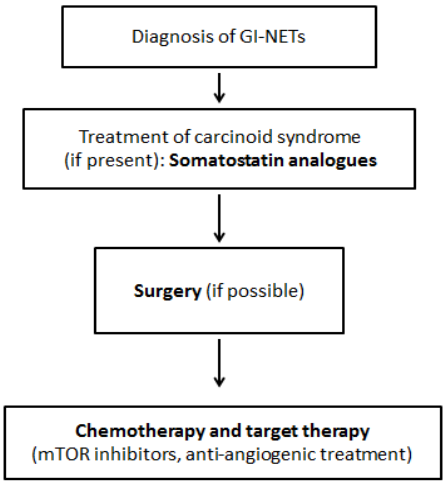


Figure 1: Simplified scheme of gastrointestinal NETs management. *After the diagnosis, patients are treated with somatostatin analogues when carcinoid syndrome is present. Surgery is the first choice if possible. When the patients exhibit disease progression, important hepatic invasion or bone metastasis, chemotherapy and target therapies are the only option* (10).

1.2. mTOR pathway activation, modulation and its effects

The mammalian target of rapamycin (mTOR) signalling network integrates intracellular as well as extracellular signals and serves as central regulator of the cell growth, proliferation, survival and metabolism. Generally, it has been shown that mTOR pathway is activated in various cellular processes, such as survival and angiogenesis (12).

The mTOR protein is a 289-kDa serine-threonine kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and its conserved throughout the evolution. It forms two distant multiprotein complexes, mTORC1 and mTORC2 (Figure 2). The great breakout in understanding its function came from the use of bacterial macrolide called rapamycin. Once intracellular, rapamycin binds to FK506-binding protein of 12kDa (FKBP12) and interacts with the FKBP12-rapamycin binding domain (FRB) of mTOR leading to the inhibition of mTORC1. It was shown that in it can also inhibit mTORC2 activity by blocking its assembly (12). Indeed, there is a complex crosstalk between both complexes as well as the establishment of feedback axis when using rapamycin or its analogues. Rapamycin can also inhibit the negative feedback loops, which regulate prosurvival and proliferative effectors. This loops are relevant in many cancer cell lines as well as clinical samples and could be involved in partial response of tumors (13,14). mTORC1 signalisation was often found to be significantly deregulated in human cancers. For instance, in the case of breast, colorectal, prostate cancer, lymphoma and many others (15–19).

The mTOR pathway can be modulated by insulin, growth factors, hormones, as well as intracellular energetic status which depends on the cellular metabolism and availability of nutrients. Cancer cells are generally associated with the reprogramming of energy metabolism, one of the emerging hallmarks of cancer (20). These anomalous characteristic was first described by Oto Warburg, who noted that even in the presence of oxygen the cancer cells favour glycolysis despite of lower efficiency of ATP production. However, glucose depletion which can occur during tumor development, often leads to decrease in intracellular AMP/ATP

ratio. This event further activates AMP-activated protein kinase (AMPK) which phosphorylates tuberous sclerosis 2 (TSC2) and therefore increases its GAP activity towards Ras homolog enriched in brain (Rheb). Consequently, mTORC1 activation is reduced (21). AMPK has also been described as negative regulator of mTORC1 activity by directly phosphorylating Raptor (22).

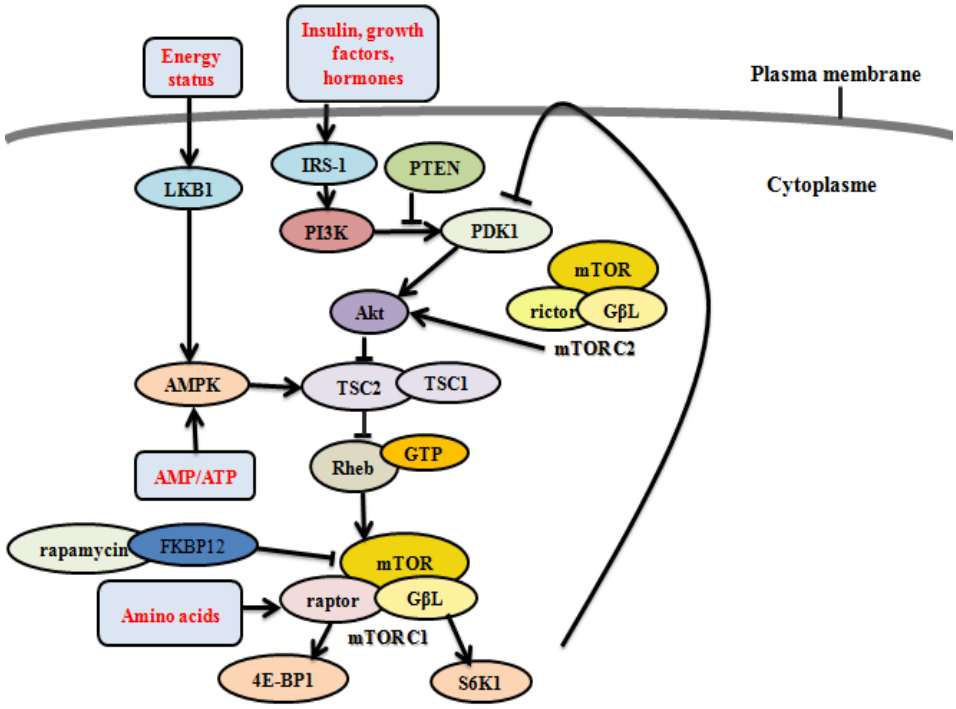


Figure 2: Simplified scheme of mTOR pathway. *The PI3K/AKT/mTOR pathway is activated by growth factors, mitogens and hormones. Nutrients (amino acids, glucose) also activate mTOR. Activation of PI3K initiates a cascade of events: PDK1 activates AKT which phosphorylates TSC2, thereby rendering TSC1/TSC2 complex unstable and inactive. Rheb, the small G protein, is no longer inhibited by the GAP (GTPase-activating protein) activity of TSC2. The AMP-activated protein kinase (AMPK) also phosphorylates and enhances the activity of TSC2 under energy starvation. mTOR is present in two complexes, mTORC1 and mTORC2. mTORC1 is a large multi-protein complex and contains two known partners, Raptor and GβL, that promote the activation of P70S6K1 and the phosphorylation of 4-EBP1. Rapamycin allosterically inhibits mTORC1. mTORC2 contains GβL and rictor. It phosphorylates AKT and triggers activation of mTORC1* (23).

Glucose starvation can perturb many cellular processes and normal function of cellular organelles. Endoplasmic reticulum (ER) can be an effective glucose-sensing apparatus that establishes functional links between altered glucose supply and metabolism adaptation allowing cells to survive harsh environment which occur in the process of tumor development and growth. In fact, it was already published that glucose depletion leads to ER stress (24). ER stress prevents correct protein folding within ER, mainly because of the alterations in N-linked protein glycosylation and the decrease in ATP abundance. Aberrant Ca^{2+} regulation in the ER can also cause protein unfolding (25). Therefore, cells respond to ER stress by activating signal transduction cascade called unfolded protein response (UPR) (Figure 3). UPR restores the ER protein folding capacity thanks two 3 sensors present in the ER membrane: inositol-requiring gene 1 (IRE1), PKR-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6). Activation of all branches leads to the translocations of X-box binding protein 1 (XBP1), ATF6p50 and ATF4 to the nucleus, where they act as the transcription factors for survival as well as apoptotic genes, such as CCAAT-enhancer-binding protein homologous protein (CHOP) and binding immunoglobulin protein (BIP). The PERK branch triggers phosphorylation of eukaryotic Initiation Factor 2 (eIF2) and decreases the protein synthesis (26). Finally, the homeostasis can be established to eliminate the defective cells. Therefore, ER stress could play a controversial role: role in the cellular survival by increasing the capacity of cellular adaptation to environmental changes or by activating the programmed cell death when the stresses are two strong (27). ER stress has been also associated with the mTOR activation where final goal is to sustain the cellular survival. Moreover, it can function downstream as well as upstream the mTORC1 signalization (28). In the case of prolonged ER stress, the cell can turn on adaptive mechanisms that decrease their sensivity to other stresses. In addition, this leads to a survival advantages and development of more aggressive profile (24). Therefore, the duration of ER stress determine the cellular faith. Taking into account the data from our laboratory, digestive neuroendocrine cell lines (STC-1, GluTag) submitted to hypoglucidic stress (HS) trigger UPR and activate mTOR pathway (unpublished data). This pathway helps cells to adapt and survive the unfavorable conditions by changing gene transcription profile and protein expression. Taken together, UPR signaling is elicited by a variety of physiological inputs that include both, favorable and unfavorable

growth conditions, where bilateral crosstalk between mTORC1 and UPR occurs (28). (Figure 4)

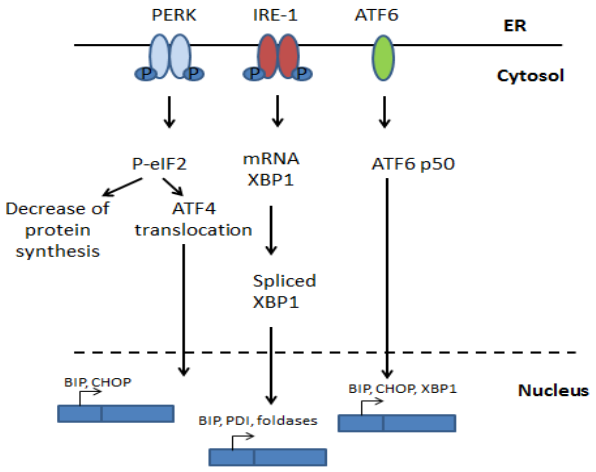


Figure 3: The three branches of unfolded protein response (UPR). *First function of UPR is to reduce the protein load within ER by lowering protein synthesis mediated by PERK axis. Second function is to increase the folding capacity of ER by upregulating UPR target genes transcription for chaperons and enzymes. This can be achieved by all three branches of the UPR. Finally, if homeostasis can not be established, the programmed cell death is triggered* (28).

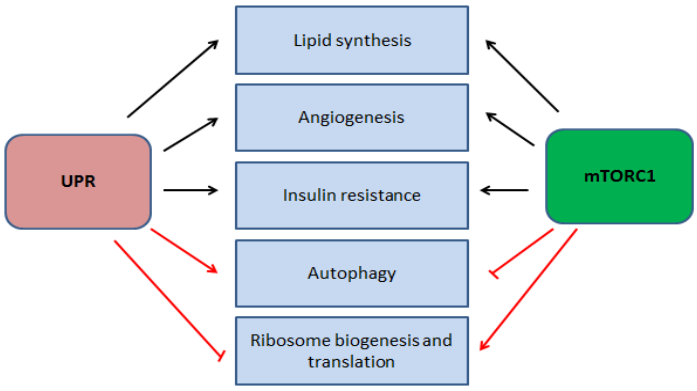


Figure 4: Crosstalk between mTORC1 and UPR signalization. *UPR and mTORC1 influence each other's activities. The effects of their activations can be positive and synergistic (lipid synthesis, angiogenesis, insulin resistance; black arrows). Contrary, certain processes could be regulated inversely, like autophagy and ribosome biogenesis (red arrows)* (28).

Growth factors and nutrients are the best-characterized cellular activators of mTORC1. But there are also others factors that regulate its activation states, such us inflammation and Wnt signalization (29). Furthermore, changes in the mTORC1 activation deeply affect the gene transcription. The mechanisms by which mTORC1 operates are mainly indirect: by the phosphorylation of others proteins which can shuttle between nucleus and cytoplasm and are implicated in the process of transcription. Indeed, only one article suggests that mTOR protein could translocate to the nucleus (30). Most of transcription factors that are regulated by this complex are involved in the metabolic pathways. The mTOR pathway promotes lipid biogenesis and inhibits the autophagy process (23). For instance, Peterson et al. found the mechanism by which mTORC1 modulates sterol regulatory element-binding protein (SREBP), the master regulator of lipo- and sterolgenic gene transcription. Indeed, mTORC1 influences nuclear entry of LIPIN1, the negative regulator of lipid gene expression. When activated, mTORC1 phosphorylates LIPIN1, which is retained in the cytoplasm. Therefore, the gene promoter is not occupied and SREBP can be bound. Consequently, the promoter activity is increased and the gene transcription is enhanced (31). In addition, SREBP must be released from the ER proteolytically by the control of nutrient state impulses. This shows the importance of the proper functioning of all cellular machineries, allowing cell to proliferate and survive (32). However, it has to be acknowledged that mTORC1 can regulate gene expression through other processes, such as epigenetic mechanisms by directly affecting RNA stability and degradation. mTORC1 is also positive regulator of HIFα (hypoxia induced transcription factor α) (33). HIFα promotes tumorigenesis by ensuring adaptive, proliferative and survival advantages of cancer cells mainly by modulating energy metabolism and oxygenation. To increase oxygenation, the VEGF-A transcription and translation is increased. HIFα activity is frequently elevated in cancer (23,34).

Furthermore, mTORC1 activation is also associated with transcription of genes implicated in intracellular trafficking and cellular organelles formation. Indeed, it is recognized as a key regulator of the transcription factor EB (TFEB) (35,36). TFEB is basic Helix-Loop-Helix (bHLH) leucine zipper transcription factor that regulates lysosomal biogenesis and their function. Indeed, formation of lysosomes is transcriptionally regulated. Lysosomal genes contain one or more repetitions of a 10-base pair motif (GTCACGTGAC) which is usually localized within 200 base pairs of the transcription initiation side. This

motive is called Coordinated Lysosomal Expression and Regulation (CLEAR) elements (37). Nevertheless, TFEB overexpression resulted in the increased number of the lysosomes in many cell types. Accordingly, TFEB is considered as a master regulation of the lysosome biogenesis and their formation (38). Lysosomal biogenesis genes are divided into three groups:

1. Vacuolar-adenosine triphosphatase (v-ATPases), which can sense intracellular amino acid availability as well as are used for intra-lysosomal acidification.
2. Lysosomal membrane proteins is heterogeneous group of structural proteins, ion channels, nutrient sensing machinery, trafficking and fusion proteins, catabolic enzymes and different transporters. They are essential for building up lysosomes and are involved in transport of metabolites resulting from hydrolytic degradation, as well as for interaction and fusion with other cellular membrane systems. In addition, trafficking pathways of proteins are closely linked to the biogenesis of this compartment (39).
3. Lysosomal hydrolases include protein families such as the sulphatases, glycosidases, peptidases, phosphatases, lipases and nucleases. They allow lysosomes to hydrolyse different biological substrates (40). Furthermore, lysosomes are very important in the process of autophagy. Autophagy is a crucial cellular clearance process that allows the degradation of cellular components. Therefore, is very important in the recycling of molecules, especially in unfavorable cellular circumstances, such as nutrient deprivation.

Once more, TFEB was found to be the main regulator of autophagic genes transcription (Figure 5). By binding to CLEAR element on the autophagy gene promoters, TFEB triggers the transcription of UVRAG, WIPI, MAP1LC3B, SQSTM1, VPS11, VPS18 and ATG9B. The overexpression of this genes triggers autophagosome formation and activation of the autophagy (38,41). Furthermore, it is well established that mTORC1 can also regulate autophagy independently of TFEB translocation. When nutrients are in excess, mTORC1 is activated, phosphorylates ATG proteins and inhibits the autophagic process. The ability of mTORC1 to modulate TFEB activation as well as ATG phosphorylation suggests that mTORC1 might exhibit brother regulatory role in cellular clearance. It monitors two different mechanisms which have the same outcome. Ferguson et al. showed that mTORC1 phosphorylates TFEB at the lysosomal surface, which promotes binding to 14-3-3 proteins and inhibits its transport to the nucleus. Contrary, impaired mTORC1 signalization reduces TFEB

phosphorylation which can be rapidly shifted to the nucleus where it accumulates and orchestrates the expansion of lysosomal and autuphagic compartements (36) (Figure 5). Further on, there have also been other proteins described, that are able to modulate mTORC1 signalization. Folliculin (FLCN) was identified as one of them. In different cell lines, we are talking about the FLCN-mTORC1-TFEB axis (42).

Settembre et al. reported that TFEB also regulate lipid catabolism which makes its function even more complicated. TFEB overexpression in mouse liver led to increased expression of different group of genes important in lipid breakdown. In this study TFEB induced peroxisome proliferator activated receptorγ- (Pparg) and PPARγ co-activator 1α (Pgc1α, also known as Ppargc1a) transcription. This group of genes is a major regulator of lipid metabolism in the liver. Furthermore, *PGC1α* promoter has identified CLEAR sites. The results suggest that TFEB directly regulates *PGC1α* gene expression during starvation response, by binding to CLEAR elements (43) (Figure 5).

Lysosomes are membrane-bound organelles with acidic lumen and a single lipid-bilayer membrane. They contain several types of hydrolases involved in a degradation of specific substances. Indeed there is around 60 different soluble hydroxylases that are active in acidic environment and play role in the execution of degradation processes. For instance, they are able to break down glycosaminoglycanes, sphingolipides, glycogen and proteins. Lysosomes can also secrete their content in the process of exocytosis. In this process, lysosomes fuse their membrane with plasma membrane through Ca^{2+} mediated mechanism that leads to release of lysosomal content. Lysosomes play important part in the signaling pathways that are involved in cell metabolism and growth, especially in connection with mTORC1 activation machinery. The importance of the correct lysosomal functioning was evaluated by the consequences that appear when the lysosomal biogenesis is decreased. This leads to the increase of intracellular debris, protein aggregates and the decrease in organelles clearance. However, not much is known how lysosomal function varies in different cell types, life stages and individuals, as well as impact of different psychological and pathological conditions (40). Lysosomal dysfunction play important role in human disease and the processes of aging with a decline in the lysosmal function and accumulation of intracellular material (40,44). The lysosomes are gaining on importance also in the cancer cells. They are more numerous, larger and more active than in normal cells (45).

A second form of regulatory input on mTORC1 is its spatial organization. Activation of mTORC1 by Rheb–GTP occurs on the cytosolic surface of lysosomes. According to Sabatini's group, which focuses its research mainly on amino acids deprivation, the mTORC1 complex is translocated to the lysosome in accordance with the amino acids availability (46). Lysosomes are known to be an important pool of amino acids as they serve as major site of protein degradation and are used as an intracellular storage. Thus, intra-lysosomal quantity of amino acids was found to play important role in mTORC1 recruitment to lysosomal surface when there is increase in amino acid availiability. Once recruited, mTORC1 is tethered and activated by the Rag GTPases as well as Rheb protein. Rheb was found to be sensitive on oxygene, energy and growth factor inputs and could also modulate the mTORC1 activation state. However, to have fully activated mTORC1 pathway, both Rag and Rheb activation is necessaire (46).

In conclusion, activation of mTORC1 signaling is a multi-step process that depends on the inputs of cellular signaling cascades and the availability of nutrients, energy, and oxygen. In order to be activated, mTORC1 has to be recruited to the lysosomal surface (28). Therefore, lysosomes are not just vast bags but have significant role in the mTOR pathway signalization which is very important in NETs.

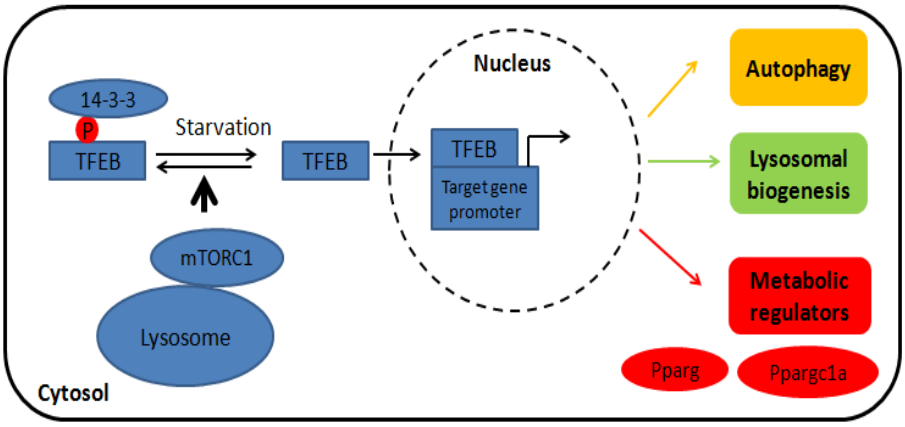


Figure 5: Scheme of TFEB-mTORC1 signalization network. *TFEB is involved in cellular adaptation to the nutrient deprivation. Nutrient starvation causes TFEB translocation in nucleus, where it binds to lysosomal, autophagic and metabolic genes promoters. It enhances the gene transcription leading to proper use of energy stores and ensuring cell survival. When sufficient nutrient amount is in the cellular environment, mTORC1 is recruited to lysosome,*

activated and able to phosphorylate TFEB. TFEB is bound to 14-3-3 protein and retained in the cytoplasm (38).

Neuroendocrine tumors retain the secretory proprieties of neuroendocrine cells. The cytoplasm of NETs cells contain secretory granules of variable size as well as shapes, where the secretory products are stored. These tumors have extensive rough endoplasmic reticulum (ER) that is responsible for the biosynthesis of the peptide pro-hormones. They are further transported to the Golgi apparatus and packed in the granules, which are afterwards shifted towards cell membrane. Lysosomes are important for degradation, signalisation as well as secretion and are necessary for having efficient intracellular trafficking (Figure 6). Since secretory cells synthetize high amount of the proteins, all 3 branches (ER-Golgi-Lysosomes) has to work properly. Moreover, in the case of the nutrient deprivation, when synthesis and correct protein folding is at risk, the secretory apparatus has to be reorganised. Therefore, the cells have to first sense the decrease of glucose in intracellular environment and secondly use different mechanisms in order to adapt to the changes. One the mechanism in secretory cells might be UPR and mTOR pathway activation (47).

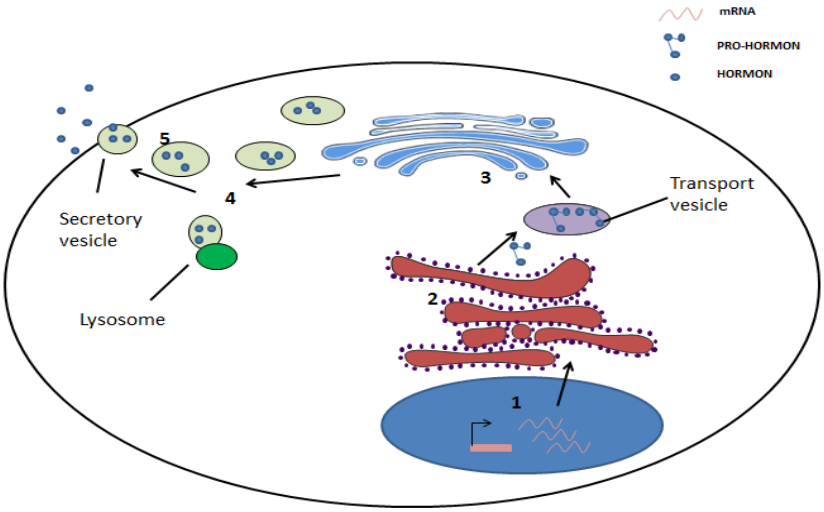


Figure 6: Secretory apparatus of neuroendocrine cells: ***(1)*** *gene transcription in nucleus which generates mRNAs that are translated at the ribosomes of endoplasmic reticulum* ***(2)*** *where pro-hormones are synthetized. Pro-hormones are further processed at the Golgi apparatus* ***(3)*** *and packed in the vesicles* ***(4)****. The vesicles are then transported towards cellular membrane, where they fuse and secrete theirs content in the extracellular space* (47).

1.3. Vascularisation of neuroendocrine tumors and the VEGF family

The other important feature of well-differentiated tumors is their hypervascular character, with a high intratumoral density (48). The cells are able to synthetise and secrete great amount of angiogenic factors, which allow them to build efficient blood vessel network. The synthesis is correlated with mTOR activation and the amount of the secreted factors could be reduced by the rapamycin treatment (49). In contrast, poorly differentiated tumors display lower density with the signs of hypoxia. Altogether, intratumoral vascularization was found to be higher in the benign tumors than malignant and was associated with good prognosis, prolonged survival, low risk of local invasion and metastatic dissemination (50,51). The results point out the importance of angiogenesis for GI-NETS development, mainly caused by the vascular endothelial growth factor A (VEGF-A) secretion (Figure 7).

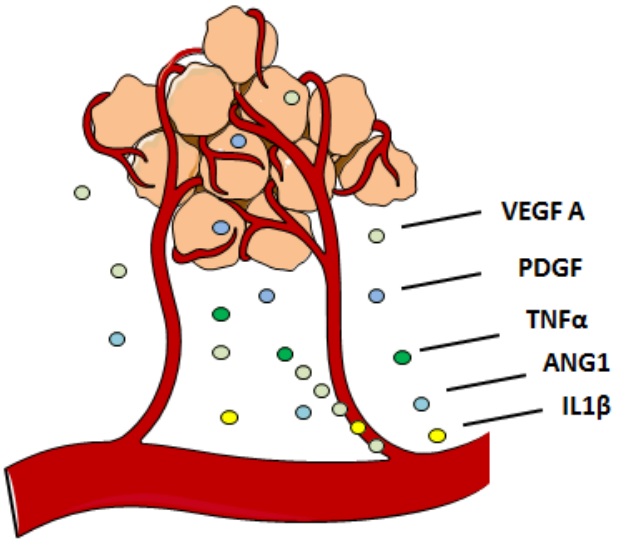


Figure 7: The process of angiogenesis. *Tumor cells secrete growth factors, of which VEGF-A is the most important. VEGF-A binds to VEGFR1 and VEGFR2 on endothelial cells (EC). There are also other important factors involved in the process, such as PDGF, TNFα, ANG1 and IL1β. Angiogenesis is referred as new blood vessels formation from pre-existing one.*

The VEGF (vascular endothelial growth factor) family consists of 7 members, but only 5 are present in mammals: VEGF-A, VEGF-B, VEGF-C, VEGF-D and P1GF (placental growth factor). VEGF A was identified and isolated as an endothelial cell-specific mitogen

that has the capacity to induce physiological and pathological angiogenesis (52). VEGF-A is often referred as VEGF, although this nomenclature is obsolete and misleading. Nowadays, it is clear that VEGF-A is not the only member of the family as later on the others were discovered. However, in old publications VEGF-A is noted as VEGF.

VEGF family members differ in expression pattern, receptor specificity and biological functions. Moreover, it has become apparent that the function of VEGFs is not limited only to angiogenesis and permeability. Nowadays their function is also associated with cell survival, fatty acids uptake and in immune reponse. It has been discovered that in tumor cells, the family members signal in autocrine and paracrine manner. Their signalization was found to be implicated in tumorogenesis, when binding to the VEGF receptor tyrosine kinases (RTKs) and neurophilins (NRPs). VEGF A binds to the VEGF receptor (VEGFR) 1, also known as FLT1, with the higher affinity than to VEGFR2, also known as FLK1 and KDR. However, it is unable to bind to VEGFR3, the main receptor for VEGF-C family member (53,54) (Figure 8). VEGF-C signalization is mainly associated with the lymphangiogenesis as well as cellular survival (55). VEGF-C undergoes series of proteolytic cleavages, which increase its affinity to VEGFR3. Nascent VEGF-C is consisted of a signal sequence, an N-terminal extension, the VEGF-homology domain and C-terminal which has cysteine-rich sequences (56). After the signal sequence is removed, two VEGF-C precursors are held together by intermolecular disulphide bonds forming an antiparallel homodimer. Afterwards, this dimer undergoes proteolytic process which increases protein binding affinity to VEGFR2 and VEGFR3. Final, mature form of VEGF-C is in form of noncovalently linked homodimer (57). VERGFRs expression is very important in certain types of cancers and can contribute to the tumor progression by modulation of proliferation, survival and migration (58,59). Many studies have correlated the VEGF-C expression with the clinical relevance. For instance, VEGF-C expression has been associated with lymphatic invasion, lymph node metastasis and poor survival in some of gastrointestinal malignancies (60,61). However, there are less data about the regulation of VEGF-C transcription.

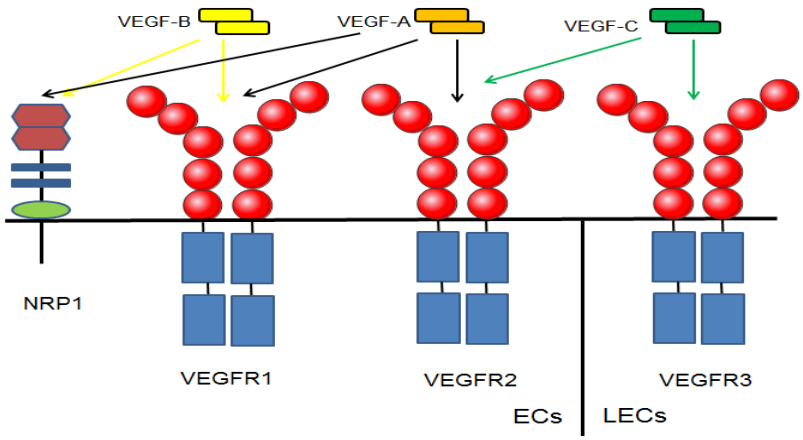


Figure 8: VEGFs, theirs receptors and coreceptors. *VEGF-A binds to the VEGFR1 and R2 as well as to coreceptor NRP1 (black arrows). VEGF-B binds to VEGFR1 and NRP1 (yellow arrows). However it is unable to bind to the other two receptors. VEGF-C binds to VEGFR2 and VEGFR3 (green arrows)* (62).

Contrary to the VEGF-A, VEGF-B gene deletion do not cause embryonic death. Besides, the studies of gene deletion or overexpression in mice do not cause any obvious phenotypes (63,64). Therefore, VEGF-B was long time considered as remarkably challenging to understand. It was initially believed to be an angiogenic factor, like the other members of VEGF family. Indeed, it shares 47% homology to VEGF 165 in amino acides sequences (62). But in fact, VEGF-B was found to be barely angiogenic. In comparison with the others members of the VEGF family, VEGF-B still remains at least studied and understood. Various studies tried to define VEGF-B role under different pathological conditions. In 2010 Hagberg et al. published that VEGF-B regulates endothelial fatty acid uptake (65). The authors demonstrated that secreted VEGF-B signals in paracrine manner to the endothelial cells which express NRP1 and VEGFR1. The binding causes the upregulation of the vascular fatty acids transporters (FATPs) and subsequent transport of long chain fatty acids through the endothelial layer of the vessels *in vivo*. Furthermore, VEGF-B overexpression in mouse heart caused downregulation of gene cluster that regulates fatty acid oxidation and increase in genes regulating oxidation pathway. This points out that VEGF-B role is organ specific. Actually, there is no data about regulation of the VEGF-B expression during different pathological events, nor tumour development. Indeed, there is no data about the signalling pathways that could modulate its expression neither about the circumstances that favour its expression.

2. AIM OF THE STUDY

mTOR pathway is involved in cancerogenesis and is deregulated in NETs. To study the modulation of mTOR activity and its role in NETs, particularly in the synthesis of VEGF, *in vitro* studies using STC-1 cell line as well as *in vivo* xenograft model will be done. Our study will be organised in 4 independent parts. First two parts will focus on the mTOR pathway activation, while second two will focus on the expression of VEGFs:

1. According to literature, mTOR pathway is sensible to nutrient availability. Therefore an hypoglucidic stress (HS) model will be used. This model, in which concentration of glucose is decreased, will modulate the state of mTOR signalisation. Western blot will be used to determine the activation state of this pathway by examining the phosphorylation of two main downstream targets: P70S6K1 and 4-EBP1. According to literature, we hypothesize that glucose depletion causes a decrease in the mTOR pathway's activation, as will be studied by the phosphorylation of its target proteins.
2. The effect of mTOR modulation on the gene transcription in HS environment will be studied by the analysis of microarray data. The analysis will focus on deregulated genes that might be influenced by HS and consequently mTOR pathway. Furthermore, their promoters and function in the cell will be explored. We will also try to find deregulated transcription factors associated with the mTOR pathway. This part of work will include review of published articles and comparison of the data. We hypothesized that genes transcribed in mTOR-dependent manner would be significantly downregulated in HS conditions.
3. VEGF-A is very important in the NETs and its biosynthesis should be enhanced by mTOR pathway. Its expression and modulation has been described in previous work of our laboratory. In opposite to VEGF-A, the VEGF-B and VEGF-C gene expression has never been described in NETs. Therefore, RT-qPCR will be used to evaluate expression on mRNA level. In the case of positive results, we will explore the presence of VEGF-B and VEGF-C at the protein level using Western blot. Their expression will also be evaluated *in vivo* using xenograft model and investigation of hepatic nodules that occur after dissemination from the spleen. If confirmed, we will use the HS model in order to further

investigate whether glucose deprivation could modulate their mRNA and protein expression.

4. Finally, the relation between mTOR signalling and VEGF-B and -C expression will be investigated. This part will be done only if expression of these two factors will be confirmed in standard cell incubation conditions in pervious part. We will use: 1./ rapamycin, the mTORC1 allosteric inhibitor, 2./ siRNA against RAPTOR, an essential protein of mTORC1. The protocol of transfection will be developed. If the changes of VEGFs expression will be detected, HS model and ER stress inductors will be used to modulate mTOR pathway activity. This will allow to further understand the potentially new mechanisms of VEGFs regulation.

3. MATERIALS AND METHODS

3.1. Cell line, glucose deprivation model

The STC-1 cell line, a gift of G. Rindi (Department of Pathology and Laboratory Medicine, Roma, Italy), is derived from neuroendocrine intestinal tumors (RIP1 Tag2/RIP2PyST1 tumors) from double transgenic mice carrying transgenes RIP1 Tag2 or RIP2PyST1 (for large T Ag SV40 (Tag) or polyoma small T antigen (PyST)). STC-1 cells retain the capacity to synthesize and secrete neuroendocrine peptides such as secretin, cholecystokinin, peptide YY, gastric inhibitory polypeptide or proglucagon (66). Cells were routinely cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 2 mM glutamine and antibiotics (100 UI/ml penicillin, 100 µg/ml streptomycin), in 5% CO_2 at 37°C. The cells were seeded and maintained 48 h in standard medium containing 25 mM of glucose. In some experiments, the medium was replaced with fresh media containing 5 mM, 2 mM or 1 mM of glucose for the indicated times (Figure 9). To inhibit mTOR pathway, cells were treated at 11 nM rapamycin (*S*elleckchem, USA). To induce ER stress, cells were treated with thapsigargin (Applichem, UK) at 300 nM, bortezomib (Selleckchem, USA) at 30 nM or brefeldin A (TOKU-E, USA) at 3 µM. (Table II)

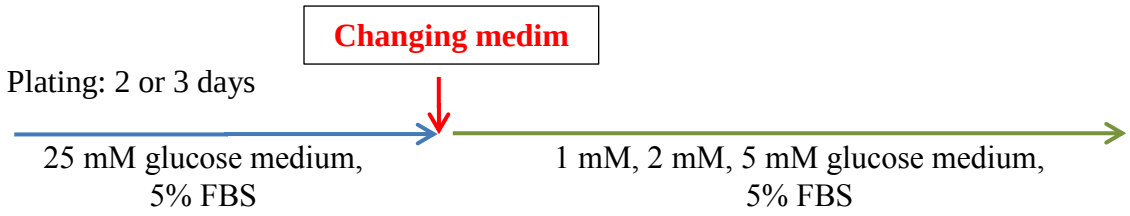


Figure 9: *In vitro* hypoglucidic stress (HS) model of STC-1 cells culture. *The cells are plated for two or three day in standard cultivation conditions (25 mM glucose concentration). Then the medium is changed with the depleted one (1 mM, 2 mM or 5 mM).*

Table II: List of drugs used in experiments.

Drug	Concentration	Classification	Reference
Rapamycin	11 nM	mTOC1 inhibitor	*S*elleckchem, USA
Thapsigargin	300 nM	ER stress inductor	Selleckchem, USA
Bortezomib	30 nM	ER stress inductor	Applichem, UK
Brefeldin A	3 µM	ER stress inductor	TOKU-E, USA

3.2. Cell Transfection

For the knockdown assay, siRNA against Raptor and control siRNA were used (DharmaFECT Mouse SmartPOOL, Dharmacon). The cells were transfected with JetPEI Polyplus reagent (Genycell Biotech, Spain) by using double reverse transfection protocol. Transfection agent was prepared by adding siRNA to the Jet Prime Kit and distributed in wells, giving final concentration 100nM siRNA per well. Then cells were added at a final density of 100.000 cells per well. The cells were incubated for 24h. Afterwards, the transfection procedure was repeated. The cells were incubated for another 24h.(Figure 10)

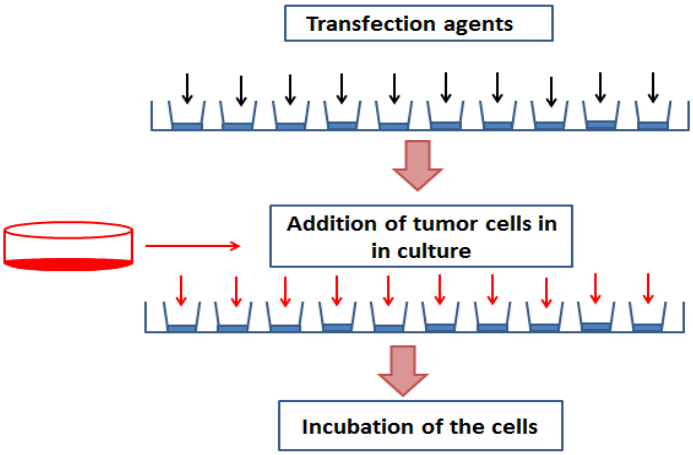


Figure 10: Reverse transfection procedure. *Firstly, the transfection agents are divided in the wells. Afterwards, the cells are added in each well (100.000 cells/well) and incubated in standard conditions (5% CO_2 at 37°C condition).*

3.3. Protein and Western Blot Analysis

Cells were seeded at a density of $0{,}15.10^6$ cells per well in 6-well plates and maintained 48 hours under normal culture condition. Cells were then washed with cold PBS and lysed in cold solubilization buffer (pH 8) containing 150 mM NaCl, 50 mM Tris-base, 2 mM EDTA, 0.5% deoxycholate, 0.1% SDS, 1% NP40, 2 mM Orthovanadate and 20 mM NaF. Cell lysates were centrifuged at 13,000 rpm for 15 min at 4°C and the supernatant was stored at -80°C. Protein yield was quantified using the Bradford protein assay kit.

Total protein lysates (20 µg) were separated by electrophoresis on a 7.5% SDS-PAGE gel and transferred to a PVDF membrane (Millipore, Billerica, Mass., USA). Saturation was performed in a 0.1% Tween 20 Tris-buffered saline solution containing 5% non-fat dry milk

for 1 h. The membranes were hybridized with primary antibodies overnight at 4°C, washed and incubated with the corresponding immunoperoxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories; Beckman Coulter France, Roissy, France) for 1 h at room temperature. Immunodetection was performed using electrochemiluminescence (ECL Western Blotting Detection System; Covalab, Villeurbanne, France) and the ChemiDoc XR5 machine (Bio-Rad, Marnes la Coquette, France). Antibodies phospho-P70S6K1 Thr389 (No. 9234), P70S6K1 (No. 9202), CHOP (SC-575), VEGF-C (No. 374628) were purchased from Santa-Cruz Biotechnology (Santa Cruz, Calif., USA); antibody VEGF-B (No.185696) from Abcam (Cambridge, UK); antibody phospho-PERK Thr980 (No. 3179), PERK (No. 3192), phospho-eIF2α (No. 3597), eIF2α (No. 2103) from Cell signaling Technology (Beverly, Ma.,USA); phospho-4-EBP1 The40 (No. 2411-1) and 4-EBP1 (No. 1557-1) from Epitomics (Burlingame, Calif., USA) and α-TUBULIN from Sigma-Aldrich (St. Louis, Mo., USA) (Table III).

Table III: The list of antibodies used for Western blot analysis.

Primary Antibody	Dilution	Secondary Antibody	Reference
VEGF-B	1/1000	Goat anti-rabbit	Abcam (Cambridge, UK)
VEGF-C	1/1000	Goat anti-mouse	SantaCruz(California, USA)
phospho-4-EBP1	1/5000	Goat anti-rabbit	Epitomics (Burlingame, Calif., USA)
4-EBP1	1/1000	Goat anti-rabbit	Epitomics (Burlingame, Calif., USA)
phospho-p70S6K1^{thr389}	1/1000	Goat anti-rabbit	SantaCruz(California, USA)
p70S6K1	1/1000	Goat anti-rabbit	SantaCruz(California, USA)
Phospho-PERK	1/1000	Goat anti-rabbit	Cell Signaling Technology (Beverly, Mass., USA)
PERK	1/1000	Goat anti-rabbit	Cell Signaling Technology (Beverly, Mass., USA)
Phospho-eIF2α	1/1000	Goat anti-rabbit	Cell Signaling Technology (Beverly, Mass., USA)
eIF2α	1/1000	Goat anti-rabbit	Cell Signaling Technology (Beverly, Mass., USA)
CHOP	1/1000	Goat anti-rabbit	SantaCruz(California, USA)
TUBULIN	1/2000	Goat anti-rabbit	Sigma-Aldrich (St. Louis, Mo., USA)

Primary and secondary antibodies and the dilutions used in experiment.

3.4. Quantitative Real Time Polymerase Chain Reaction (Q RT-PCR)

The total cellular RNA was extracted from the cells using PureLink RNA Mini Kit (Life Technologies) by following the manufacturer's protocol. RNA was quantified by UV spectrophotometry and quality was evaluated by gel electrophoresis. RNA (1 μg) was reverse transcribed by using Quanti Tech Reverse Transription (RT) Kit (Qiagen). RT reaction was performed at 42 ^{0}C for 15 minutes (min), followed by heating at 95 ^{0}C for 4 min. The cDNA was stored at -20°C until qPCR was performed. Before the assay cDNA were diluted with nuclease free water (Thermo Scientific) to obtain the final dilution of 1/100. Oligonucleotides used in the assay are described in Table IV. The RT-q PCR was carried out in 96 well plates by using previously diluted samples (10μl/well) and the manually prepared mix of Maxima SYBER Green/ROX qPCR Master Mix 2X from Thermo Scientific (9vL/well) and the primer solution of reverse and forward primers (1μL/well). For each sample, duplicate or triplicate determinations were made. The PCR standard curve was calculated with the serially diluted solutions (1/5; 1/25; 1/125; 1/625; 1/3125) of cDNA, gathered from the STC-1 cell line, which was routinely cultivated in 25 mM glucose DMEM medium. The optimal temperature was chosen for each experiment using the following criteria: efficiency (E) 90-110%, slope = 3,1-3,6 and coefficient of determination=1. The size of transcripts was evaluated by electrophorese on 2% agarose gels containing Syber Green with DNA molecular marker. The products gave a single band expected size (VEGF-B: 66kb and VEGF-C: 72kb), as well as the melting curve showed only one pick in each experiment.

The cDNA templates were subjected to a 5 min initial denaturation at 95^0 C prior to 40 cycles of PCR (95 ^{0}C for 10 s and 1 min at 55,7 ^{0}C for VEGF-C and 57^{0}C for VEGF-B, per cycle). Three housekeeping genes were used as internal controls: hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) gene, 5'-CTGGTGAAAAGGACCTCTCGAA-3' (forward) and 5-CTGAAGTACTCATTATAGTCAAGGGCAT-3' (reverse); TATA-Binding Protein (*Tbp*) gene, 5-ATTCTCAAACTCTGACCACTGCACCGTTG-3' (forward) and 5'-TTAGGTCAAGTTTACAGCCAAGATTCACGGTAG-3' (reverse) and ribosomal protein L4 (*Rpl4*) gene, 5'-TGAAGAACCTGAGAATCATGT-3' (forward) and 5'-CCTGGCGAAGAATGGTAT-3' (reverse). The others primers used in experiments: vascular

endothelial growth factor b (*Vegf b) 5'*- TGCCATGGATAGACGTTTATGC-3'(forward) and 5'-TGCTCAGAGGCACCACCAC-3'(reverse); vascular endothelial growth factor c (*Vegf c)* 5'- AAGACCGTGTGCGAATCGA -3' (forward) and 5'-ACACAGCGGCATACTTCTTCAC-3' (reverse) (Table IV). The number of mRNA quantity in each well was extrapolated from the corresponding standard curve using the Bio-Rad CFX Manager software. The relative expression of VEGF-C and VEGF-B was calculated as follows: ΔCt (target gene) = Ct (target gene) – Ct (housekeeping genes). ΔΔCt=ΔCt (target gene) - ΔCt (standard) mean of target gene. The relative copies of the target gene were determined as $2^{-\Delta\Delta Ct}$ (67).

Table IV: Primers used in the experiment.

Target	Forward Primer (5'-3')	Reverse Primer (5'-3')	T(^{0}C)	mRNA sequence source
VEGF B	TGCCATGGATAGACGTTTATGC	TGCTCAGAGGCACCACCAC	57	NM_011697.3
VEGFC	AAGACCGTGTGCGAATCGA	ACACAGCGGCATACTTCTTCAC	55,7	NM_009506.2

The sequence of forward (5'-3') and reverse (5'-3') and primers, optimal temperature and source of the mRNA sequence are presented in table below.

3.5. Transcriptomic analysis

mRNA expression array analysis was carried out thanks to ProfileXpert-LCMT platform, using Illumina Iscan. The chip used was BeadChip MouseWG-6 v2 (Illumina), containing gene-specific probes used to detect labeled cRNAs. The chip used can detect 19,100 genes and 25,600 transcripts. Samples analyzed came from standard (25 mM glucose) or long time glucose depletion conditions (5 mM and 1 mM, days 15, 19, 26). Gene set enrichment analysis was performed by using Genome studio software (v.2011, 1). For the differential gene expression analysis, which compares deregulated genes in 1 mM versus 25 mM glucose conditions (as well as 5 mM vs 25 mM and 1 mM vs 5 mM), a fold change ≥1.5 or p-value ≤0.05 were chosen as criteria (Student test). Gene annotation enrichment analysis was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery) tool with a p-value enrichment ≤0.5. The deregulated genes were organized in four groups: BP (Biological Process), CC (Cellular component), MF (Molecular Function) and

KEGG (KEGG pathway).The number of deregulated genes was calculated for each group and subgroup of genes as well as the percentage of deregulated genes in each pathway.

3.6. Xenograft model

Four-week-old female Swiss nu/nu mice were obtained from Charles River Laboratories (L'Arbresle, France). For xenografting procedure, 50 µl of a solution containing STC-1 cells adjusted to a final concentration of $5 \cdot 10^7$ cells/ml were injected into the spleen, from where they disseminated into the liver, through the portal vein, to form intrahepatic tumor nodules. Animals were sacrificed at day 28, livers were excised and prepared for histological analysis, tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Further on, 4-µm-thick sections were prepared according to conventional procedures to further perform immunofluorescence analysis (Figure 11).

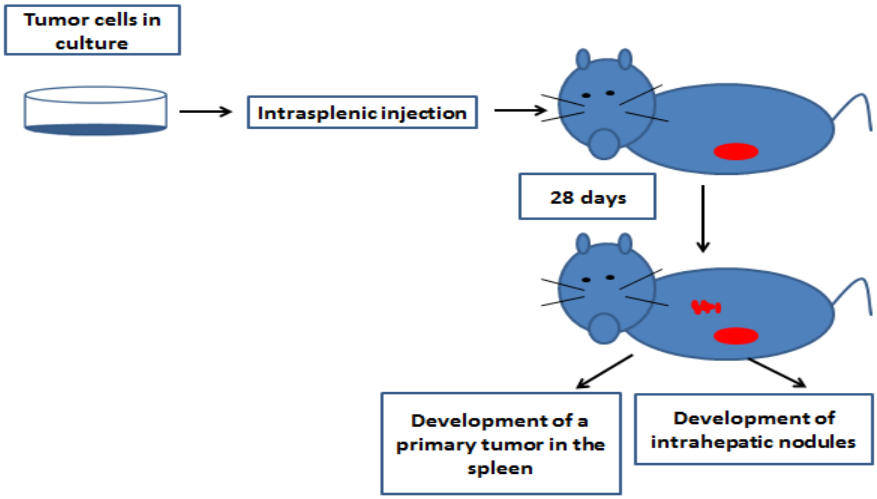


Figure 11: Mouse xenograft model. *STC-1 cells were injected in to mice spleen. After 28 days the mice were scarified. Livers were collected and sliced in thick sections.*

3.7. Imunofluorescence analysis

Immunofluorescence was performed using a VEGF-C antibody from Santa-Cruz Biotechnology, (Santa Cruz, Calif., USA). Antigen retrieval was performed by incubating sections for 35 min in a citrate buffer (pH 7,3) preheated at 98°C. Sections were then incubated with primary antibodies for 30 minutes, washed with PBS and incubated with

secondary fluorescent Alexa Fluor-488 (Life Technologies, Carlsbad, CA, USA). Afterwards, they were once again washed with the PBS and incubated with Hoescht (1/1000eme - Life Technologies) for 15minutes. Negative controls were achieved by omission of the primary antibody. Microscopic pictures were taken with Nikon Eclipse 80I.

For the mTOR-LAMP2 colocalization essay, STC-1 cells were grown on glass coverslips inserted into 12-well plates and maintained 72 hours under normal culture conditions. Cells were then fixed for 10 minutes at room temperature in 3.7% cold formaldehyde, washed with PBS, and submitted to immunofluorescence staining. Cells were incubated in PBS 0.3% Triton and 4% Bovine Serum Albumin and stained overnight with primary antibodies: LAMP2 (ab 13524) from Abcam (Cambridge, UK) and mTOR (7C10) from Cell Signaling Technology (Beverly, Mass., USA). They were incubated with secondary fluorescent Alexa Fluor 488 or -568-conjugated antibodies (Life Technologies, Carlsbad, CA, USA) for 1 hour and counterstained with DAPI (D3571, Life Technologies). Finally, pictures were taken using a Leica SP5X confocal laser scanning microscope or a Zeiss inverted microscope Axio Observer.D1.

3.8. Statistical analyses

For the RT-qPCR experiments, the data is presented as mean +/- standard error of the mean (+/-SEM). For further analysis, one-way ANOVA with post-hoc Turkey test was performed. $p < 0,05$ was considered as statistically significant (*).

4. RESULTS

4.1. mTORC1 activation upon HS

As mTOR signalization plays important role in neuroendocrine tumors, we have further investigated the effect of glucose depletion on its activation. The mTOR pathway activation was studied by the evaluation of phosphorylation state of two main downstream targets: P70S6K1 and 4-EBP1. As shown in Figure 12, 4h incubation in 5 mM, 2 mM or 1 mM glucose media caused the increase of their phosphorylation. As STC-1 cells survive long term HS stress (data from laboratory), the activation of mTOR pathway was also studied after longer period of glucose depletion. mTOR pathway was found to be activated even after 19 days incubation in depleted media (Figure 12).

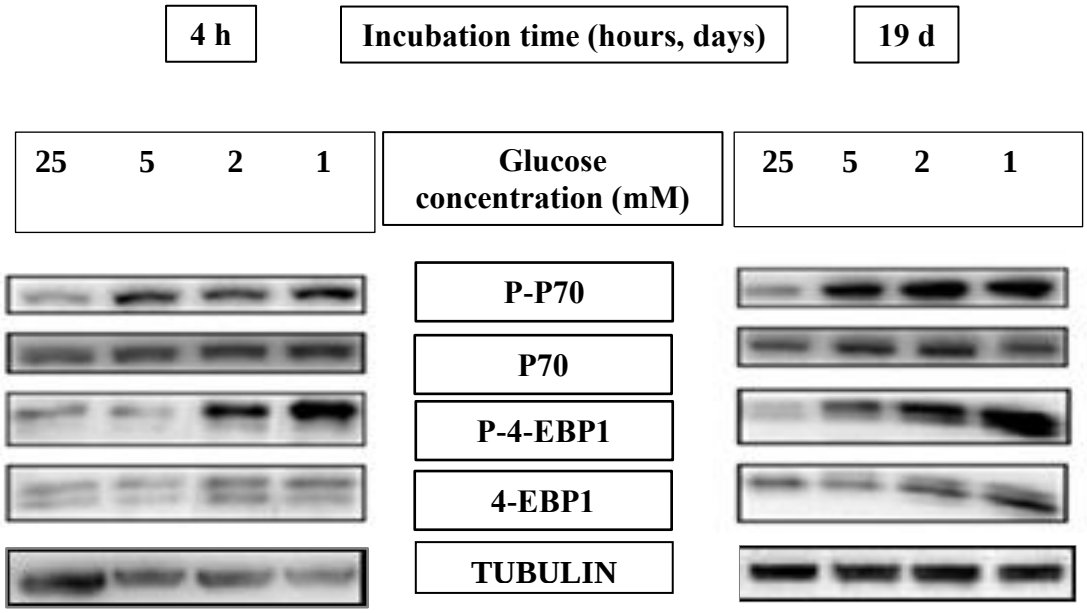


Figure 12: mTORC1 activation upon HS in STC-1 cells. *The cells were incubated 4h and 19 days in 25 mM, 5 mM, 2 mM and 1 mM glucose media. The activation state of mTOR pathway was indicated by the phosphorylation of P70S6K1 and 4-EBP1.Western blot is representative of 3 independent experiments*.

4.2. Long term HS modulates the transcription of genes implicated in intracellular trafficking and organelle formation

In order to understand the effect of mTOR activation on the process of gene transcription, STC-1 cells were submitted to long term HS (more than 15 days). By using microarray technique, mRNA expression was measured and the data were analyzed. According to the results, some biological pathways were significantly enriched (Table VI) (Fold change>1,5; p≤0,05). DAVID analysis showed significant deregulation in transcription of genes coding for different cellular components involved in the cellular trafficking (endocytic vesicles, vacuoles, cytoplasmic vesicles), protein synthesis (endoplasmic reticulum), proteolytic processing, protein maturation (Golgi apparatus) as well as in the process of cellular degradation (lysosomes, lytic vacuoles, phagocytic vesicles, endosomes). All gene clusters were significantly deregulated (p≤ 0.05). (Table VI)

Table VI: Long term HS modulates transcription of genes implicated in intracellular trafficking and organelle formation (DAVID).

GO.ID	GO. Term	P Value	Fold Enrich -met	All Genes	Deregulatd Genes	Deregulated Rate
0031902	**late endosome membrane**	0,008	8,830	8	4	50,00%
0030670	**phagocytic vesicle membrane**	0,041	8,831	6	3	50,00%
0005765	**lysosomal membrane**	0,003	5,887	18	6	33,33%
0030666	**endocytic vesicle membrane**	0,088	5,887	9	3	33,33%
0045335	**phagocytic vesicle**	0,088	5,887	9	3	33,33%
0044437	**vacuolar part**	$1x10^{-4}$	5,046	35	10	28,57%

0055037	**recycling endosome**	0,041	5,046	14	4	28,57%
0010008	**endosome membrane**	$6x10^{-4}$	4,541	35	9	25,71%
0044440	**endosomal part**	$6x10^{-4}$	4,541	35	9	25,71%
0005774	**vacuolar membrane**	0,004	4,415	28	7	25,00%
0031228	**intrinsic to Golgi membrane**	0,008	3,86	32	7	21,88%
0005770	**late endosome**	0,003	3,613	44	9	20,45%
0030176	**integral to endoplasmic reticulum membrane**	0,036	3,211	33	6	18,18%
0005764	**lysosome**	$1x10^{-4}$	2,381	178	24	13,48%
0031227	**intrinsic to endoplasmic reticulum membrane**	0,072	2,377	52	7	13,46%
0000323	**lytic vacuole**	$1,9x10^{-4}$	2,368	179	24	13,41%
0005773	**vacuole**	$8,474x10^{-5}$	2,338	204	27	13,24%
0005789	**endoplasmic reticulum membrane**	0,001	2,324	152	20	13,16%
0042175	**nuclear envelope-endoplasmic reticulum network**	$7x10^{-4}$	2,318	160	21	13,13%
0044432	**endoplasmic reticulum part**	$4,45705x 10^{-5}$	2,294	231	30	12,99%
0005768	**endosome**	$8,934x10^{-5}$	2,173	252	31	12,30%
0044433	**cytoplasmic vesicle part**	0,088	1,859	95	10	10,53%

0000139	**Golgi membrane**	0,046	1,805	137	14	10,22%
0044431	**Golgi apparatus part**	0,092	1,479	227	19	8,37%

The mRNA expression array analysis was performed on cells exposed to replete (25 mM glucose) or deplete (1 mM glucose) media, on 4 samples corresponding to 2 independent experiments and 3 different times of depletion (15, 19, 26 days). BeadChip MouseWG-6 v2 was used and Genome studio software (v.2011, 1) analysis was performed (Fold Change ≥ 1,5; p-value ≤0,05). The table contains number of all genes in the gene cluster groups, number of deregulated genes and calculated percent of deregulated genes reported as deregulated rate.

Among the deregulated groups of genes (DAVID analysis), we further examine the genes of cellular trafficking which were significantly upregulated or downregulated (p≤0,05) and might be related to the mTOR pathway activation. According to our results, the most upregulated genes were involved in vacuole (n=24), endosome (n=26) and lysosome (n=21) formation and functioning (Figure 13).

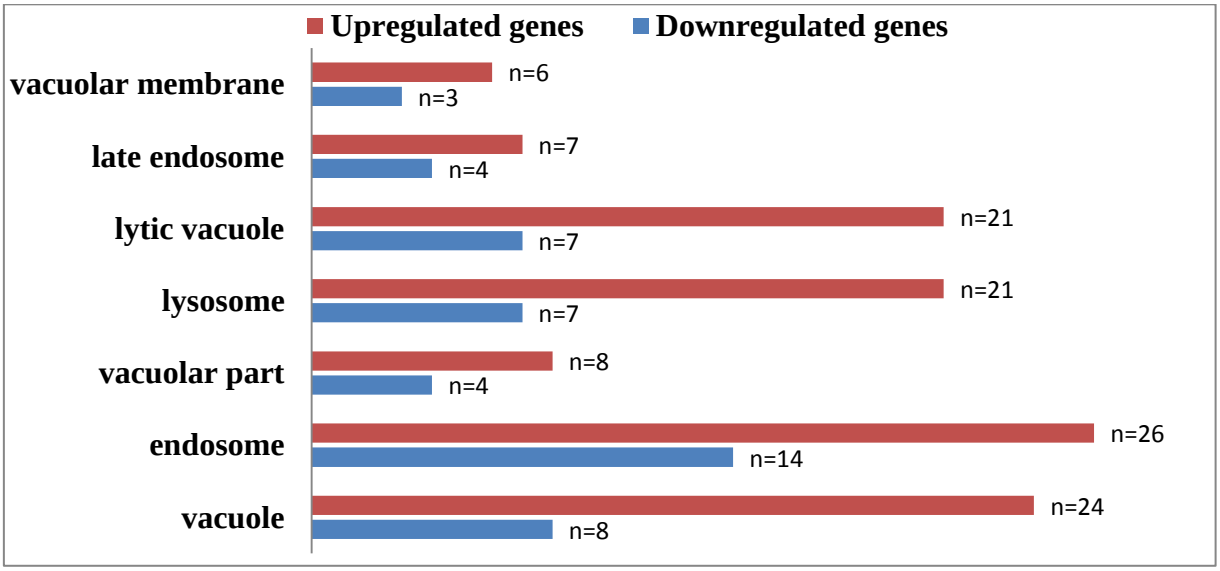


Figure 13: Long term HS significantly up- or downregulates the mRNA expression of vacuolar, endosomal and lysosomal genes. *The microarray analysis was performed on cells exposed to replete (25 mM glucose) or deplete (1 mM glucose) media, on 4 samples corresponding to 2 independent experiments and 3 different times of depletion (15, 19, 26*

days). The number of deregulated genes in each group is indicated for each Biological Process (n).

4.3. Long term HS increases mRNA expression of lysosomal genes and genes linked to mTORC1-TFEB axis

As mTOR pathway is closely correlated with the lysosomal biogenesis and autophagy, their expression pattern was explored. HS caused the increase in expression of lysosomal genes (Figure 14B, Figure 14). *Mcoln1, Clcn7, Gns, Neu1, Tmem55b, Tpp1* and *Cd63* have identified CLEAR elements and are direct targets of TFEB. Regarding our results, *Neu1* is the most expressed gene in standard culture conditions (25 mM). The second is *Gns*. The other genes are notably less expressed. *Cd63* seems to be the least expressed in 25 mM glucose medium. However, looking at the fold changes in mRNA expression (1 mM vs 25 mM), *Cd63* expression is the most increased in 1mM condition (FC=7,477). Others genes are more or less equally increased for around 1,5 - 2 times (Figure 14B).

Regarding others conditions, the statistically difference in mRNA expression was found for *Neu1* (FC=1,556; p=0,007), *Tpp1* (FC=1,550; p=0,019) and *Cd63* (FC=5,358; p=0,044) in 5 mM vs 25 mM glucose environment. There was no significant difference in the mRNA expression in 1 mM vs 5 mM glucose conditions.

The mRNA expression of autophagic genes (*Atg family, Uvrag, Wipi, Map1lc3b, Sqstm1, Vps11, Vps18*), targets of TFEB or mTOR pathway, was not significantly increased or decreased, when comparing all three culture conditions (1 mM vs 25 mM, 5 mM vs 25 mM and 1 mM vs 5 mM).

According to the established division of TFEB targeted lysosomal genes in 3 groups (membrane proteins, hydrolases and v-ATPases), we found that only group of genes coding for lysosomal membrane proteins was significantly upregulated in 1mM vs 25 mM glucose medium. The mRNA expression of genes coding for the subunits of v-ATPase and hydroxylase, were not found to be statistically different when comparing all conditions of glucose depletion.

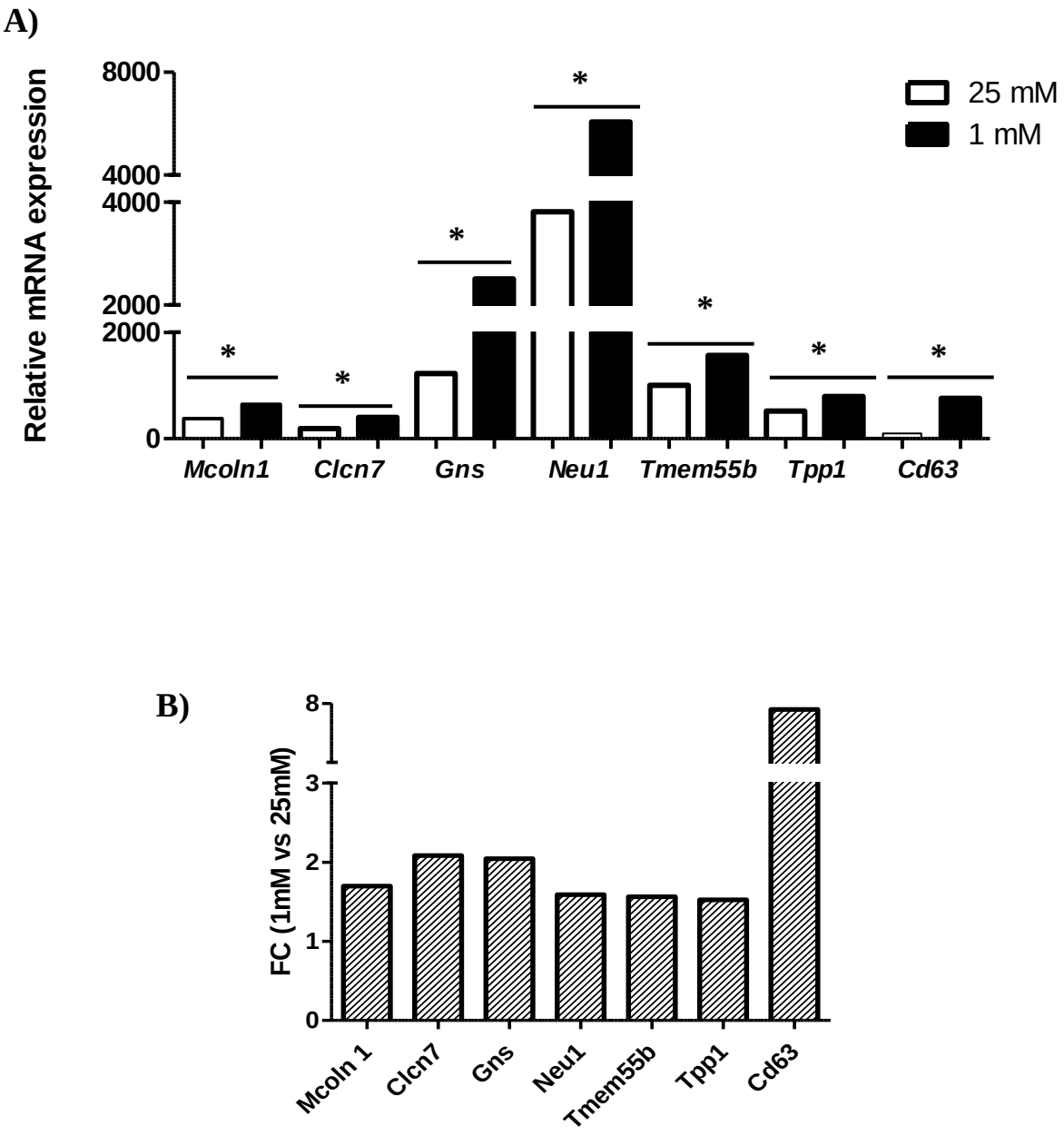
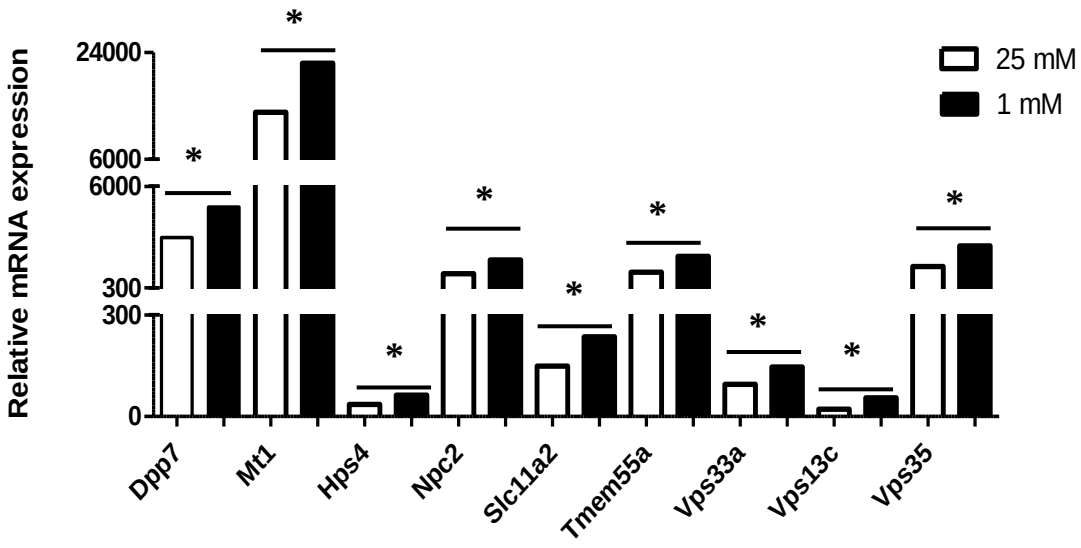


Figure 14: Long term HS increases lysosomal TFEB target gene expression. *A.) Relative mRNA expression of TFEB target genes in 1mM vs 25 mM glucose environment. mRNA level was significantly increased for all genes: Mcoln1 (p=0,042), Clcn7(p=0,027), Gns (p=0,024), Neu1(p=0,007), Tmem55b (p=0,002), Tpp1(p=0,034) and Cd63 (p=0,035). B.) The mRNA FC (1 mM vs 25 mM) gene expression pattern. Mcoln1 (FC=1,698), Clcn7 (FC=2,085), Gns (FC=2,044), Neu1 (FC=1,589), Tmem55b (FC=1,563), Tpp1(FC=1,523) and Cd63 (FC=7,477).*

The analysis also showed the significant mRNA increase of lysosomal genes in 1mM vs 25 mM glucose medium (Figure 15) that are not described as direct targets of mTOR-TFEB signalization. According to results, *Mt1* is the highest expressed gene in standard culture conditions (25 mM). Following are *Dpp7*, *Npc2*, *Tmem55a* and *Vps35*. Less expressed are *Slc11a2* and *Vps33a*. *Hps4* and *Vps13c* are the least expressed genes (Figure 15A). Incubation in 1 mM vs 25 mM glucose medium caused significant increase in the mRNA fold change expression (Figure 15B). Almost all genes were around 1,5 times more expressed, in exception of *Vps13c* whose expression increased for 2,42 times (Figure 15). *Vps13c* mRNA was also found to be significantly increased in 1 mM versus 5 mM conditions (FC=1,757; p=0,001) as well as *Slc11a2* (FC=1,681; P=0,029). There were no changes in mRNA expression comparing 1 mM vs 5 mM conditions.

A)



B)

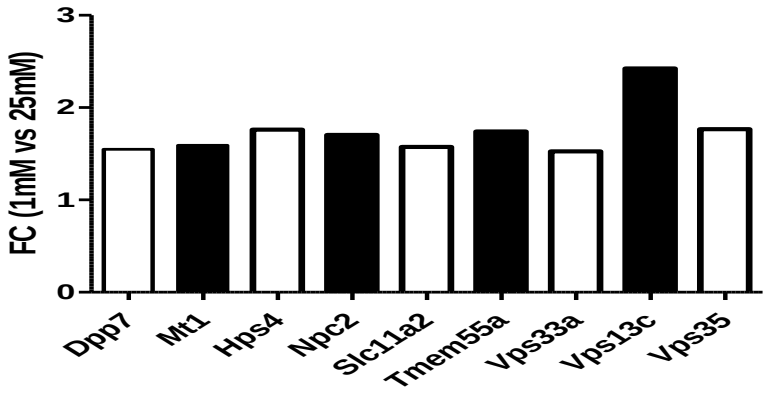


Figure 15: Long term HS increases lysosomal gene expression. *A.) Relative mRNA expression lysosomal genes that are not targets of TFEB (1 mM vs 25 mM glucose environment). mRNA level was significantly increased for all genes: Dpp7 (p=0,013), Mt1(p=0,003), Hps4 (p=0,002), Npc2 (p=0,05), Slc11a2 (p=,0412), Tmem55a (p=0,001), Vps33a (p=0,0423), Vps13c (p=0,010), and Vps35 (p=0,008). B.) The mRNA FC (1 mM vs 25 mM) expression pattern. Dpp7 (FC=1,549), Mt1 (FC=1,591), Hps4 (FC=1,760), Npc2 (FC=1,703), Slc11a2 (FC=1,573), Tmem55a (FC=1,741), Vps33a (FC=1,527), Vps13c (FC=2,423) and Vps35 (FC=1,767).*

4.4. Increased mRNA expression of lipid metabolism genes linked to mTORC1-TFEB axis signalization upon long term HS

As, STC-1 cells survive long term HS (data from laboratory), they have to adapt to the new environmental disadvantages. Therefore, an important question considers the determination of alternative energy source on which cells could rely on when glucose is depleted. When focusing on the metabolic pathways, the transcriptomic analysis showed general mRNA increase of genes involved in lipid metabolism. Some of them are described as targets of mTOR-TFEB signalization. These are genes for peroxisome proliferator activated receptors and its coactivators (*Pparg, Ppargc1a, Ppargc1b*). As in shown in Figure 16A, all three genes have similar mRNA expression rate in standard cultivation condition. However, the glucose depletion (1 mM) caused significant increase of *Ppargc1a* (p=0,0003), *Pparg* (p=0,0067) and P*pargc1b* (p=0,0223) mRNA (Figure 16A). The mRNA expression FC (1mM

vs 25 mM) analysis showed that *Ppargc1a* is the most expressed gene (FC=4,087), *Pparg* is the following (FC=3,273). The least expressed gene from this group is P*pargc1b* (FC=2,067) (Figure 16 B). Comparing mRNA expression in 1 mM vs 5 mM glucose medium, the increase of *Ppargc1a* (FC=1,667, p=0,007) and *Pparg* (FC= 1,929, p=0,025) was observed (Figure 17). *Ppargc1a* (FC= 2,447, p=0,0107) mRNA expression was also increased in 5 mM glucose medium in comparison with standard condition (25 mM).

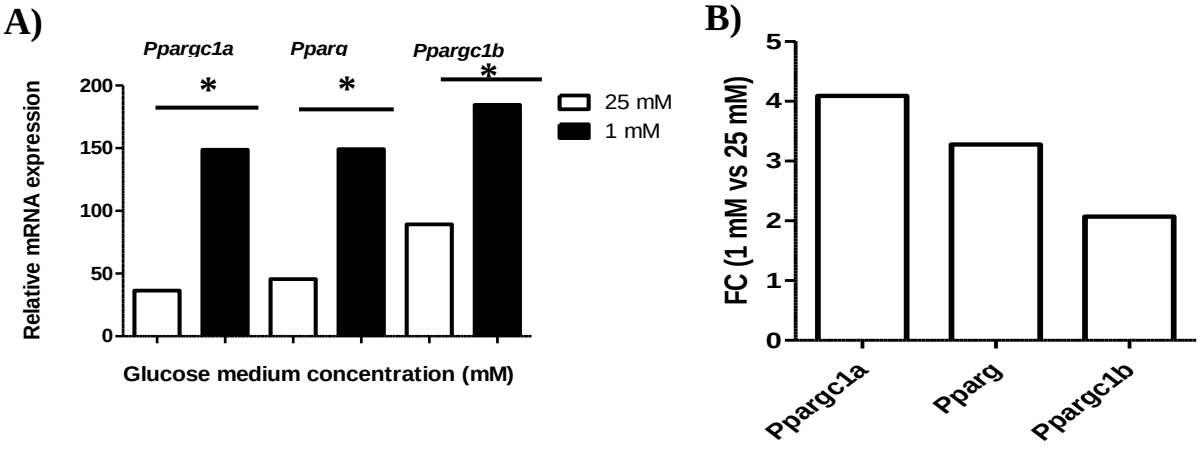


Figure 16: Long term HS (1 mM vs 25 mM) causes the increase in mRNA expression of lipid metabolism genes linked to mTORC1-TFEB axis signalization. *A.) Relative mRNA expression of TFEB lipid metabolism target genes. mRNA level was significantly increased in 1mM environment versus 25 mM glucose concentration. Ppargc1a (p=0,001), Pparg (p=0,007) and Ppargc1b (p=0,022). B.) The mRNA FC (1 mM vs 25 mM) expression pattern. Ppargc1a (FC=4,087), Pparg (FC=3,273) and Ppargc1b (FC=2,067).*

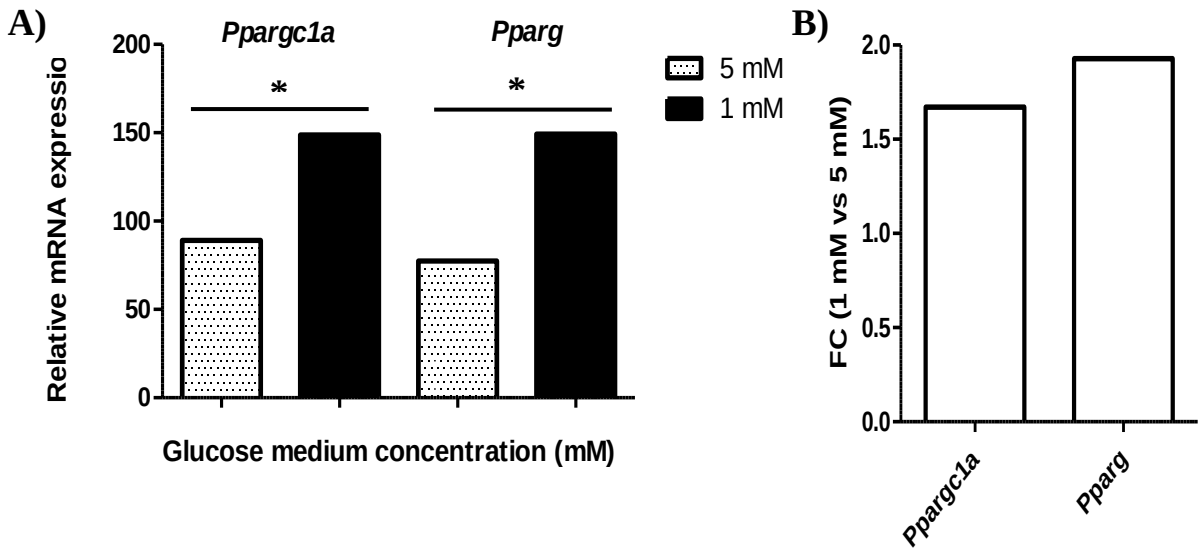


Figure 17: Long term HS (1 mM vs 5 mM) causes the increase in mRNA expression of lipid metabolism genes linked to mTORC1-TFEB axis signalization. *A.) Relative mRNA expression of TFEB lipid metabolism target genes. mRNA level was significantly increased in 1mM environment versus 5 mM glucose concentration. Ppargc1a (p=0,007) and Pparg (p=0,025). B.) The mRNA FC (1 mM vs 5 mM) expression pattern. Ppargc1a* (FC=1,667) and *Pparg* (FC= 1,929).

4.5. Long term HS increases *Flcn* and *Fnip1* mRNA expression.

Microarray analysis showed the increase of *Flcn* and *Fnip1* mRNA expression (1mM vs 25 mM glucose concentration), two genes coding for proteins that modulates the mTOR activation in the nutrient availability dependent manner. Regarding the results, 1mM HS caused the increase of *Flcn* (p=0,007) and *Fnip1* (p=0,002) mRNA expression (Figure 18A). mRNA expression of *Flcn* was increased for 2,463 times and for *Fnip1* 3,245 times (25 mM vs 1 mM) (Figure 18B). Comparing 5 mM vs 25 mM glucose conditions, *Fnip1* mRNA expression was significantly upregulated (p=0,0384, FC=1,65). The increase was not significant when comparing 1 mM vs 5 mM conditions. Whether *Flcn* and *Fnip1* are able to interact with the mTOC1 and modulate the activation of pathway still remains to be studied.

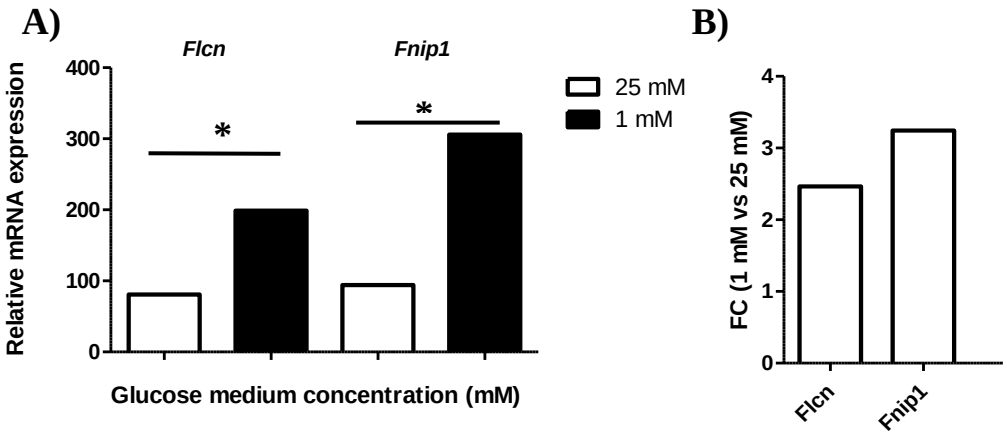
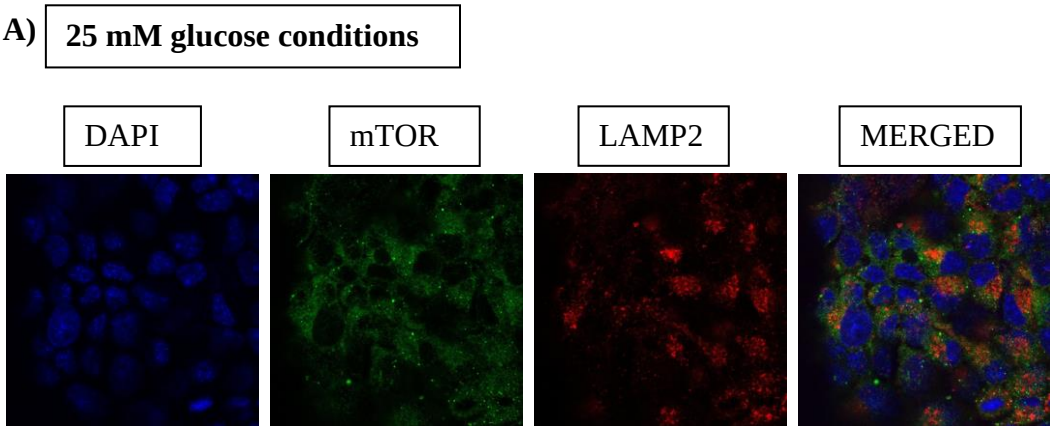


Figure 18: Long term HS increases *Flcn* and *Fnip1* mRNA expression *A.) Relative mRNA expression of Flcn and Fnip1 in 25 mM and 1 mM glucose environment. mRNA expression was significantly increased in 1mM environment versus 25 mM glucose concentration. Flcn (p=0,006) and Fnip1 (p=0,017) B.) The mRNA FC (1 mM vs 25 mM) expression pattern. Flcn (FC=2,463) and Fnip1 (FC=3,245).*

4.6. mTOR and lysosome do not colocalize upon long term HS

According to literature, mTORC1 has to be recruited to the lysosomal surface in order to be activated. This recruitment favors its activation and depends on the nutrient availability. As HS triggers the activation of mTORC1, we hypothesized that we would find the colocalization of mTOR proteins and lysosomes. By running immunofluorescence analysis mTOR and lysosome (LAMP 2 marker) colocalization was studied. However, colocalization in 25 mM as well as in 1mM glucose culture conditions was not observed (Figure 19).



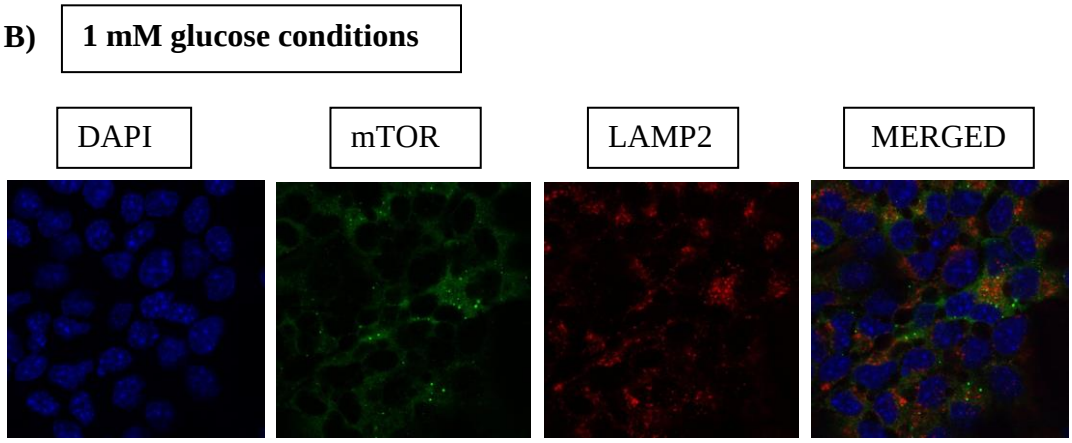


Figure 19: Lysosome and mTOR do not colocalize. *A.) mTOR and LAMP2 localization in 25 mM glucose medium (A) and in 1 mM glucose medium (19 days incubation) (B). mTOR (green), LAMP2 (red), DAPI (blue). The results are representative of three independent experiments.*

4.7. Long term HS may increase *Vegf b* mRNA expression but not *Vegf c* mRNA

The VEGF-A expression was previously found to be very important in the neuroendocrine tumors as well as in STC-1 line and related with mTOR activation. But the expression of other members of VEGF family is unknown. The expression of *Vegf b mRNA* and *Vegf c mRNA* in STC-1 cell line was studied in the cells submitted to a long lasting HS for 15, 19 and 26 days. Microarray data analysis showed *Vegf-b* gene is expressed in STC-1 cell line in standard cultivation conditions. Additionally, it showed significant increase of *Vegf b* mRNA (FC=1,571; p=0,040) expression in cells submitted to long term hypoglucidic stress (Figure 20). In contrary, significant difference of *Vegf c* mRNA expression as well as *Vegf a* was not found when comparing 1 mM and 5 mM, 5 mM and 25 mM and 1 mM vs 25 mM glucose medium.

In order to evaluate the microarray data, RT-qPCR performed. Our first results showed that mRNA expression varied strongly among the different experiments, with no statistical differences between the different glucose concentrations. Nevertheless, *Vegf b* mRNA expression may increase in 1mM in comparison with the control group in each conditions; this variation need to be confirmed with others experiments. Contrary to *Vegf b, Vegf c* mRNA

expression might be more stable (Figure 21). However, further confirmation of these results is needed.

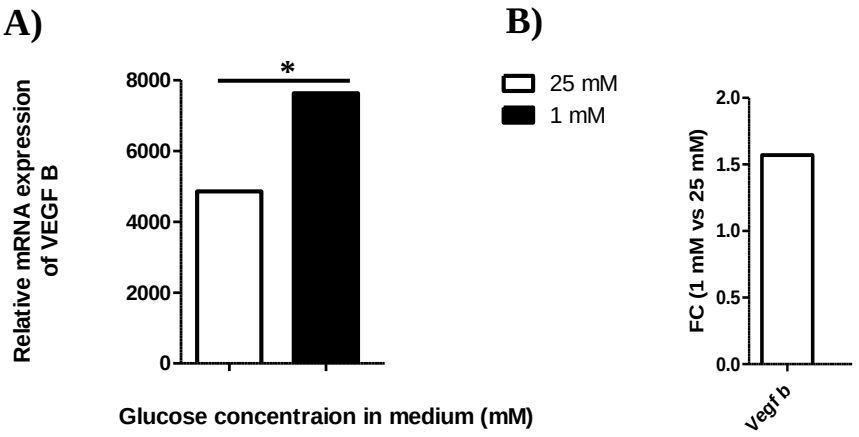


Figure 20: Long term HS increase *Vegf b* mRNA expression (microarray analysis). *Relative mRNA expression of Vegf b during long term HS. Vegf b mRNA expression (p=0,040) was significantly increased in 1 mM vs 25 mM glucose medium (incubation for 15, 19 and 26 days). B.) The Vegf b mRNA expression FC (1 mM vs 25 mM). Vegf b (FC=1,571).*

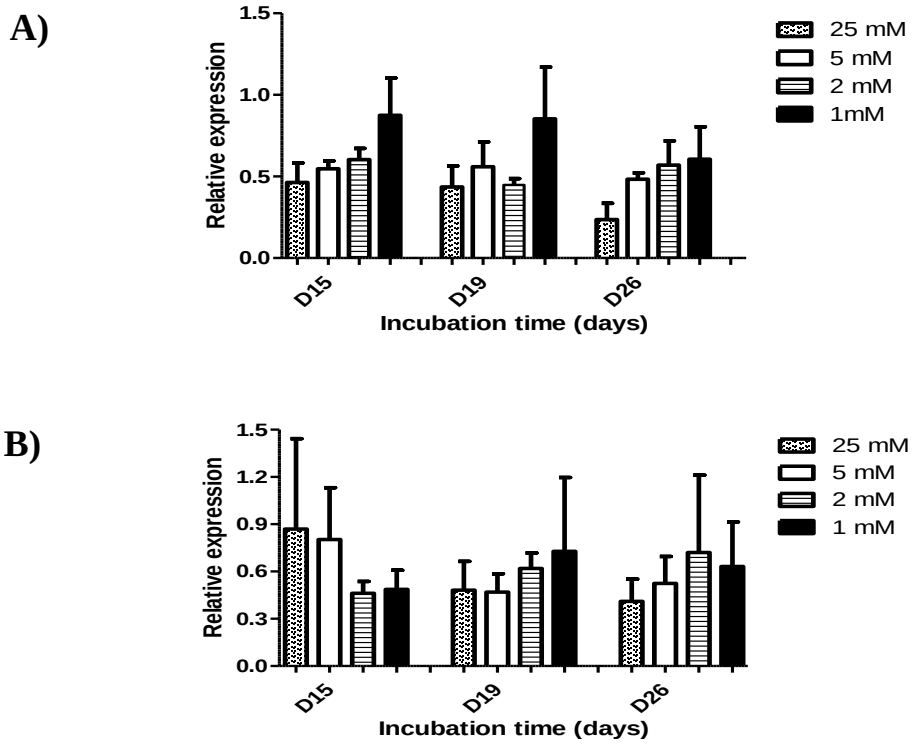


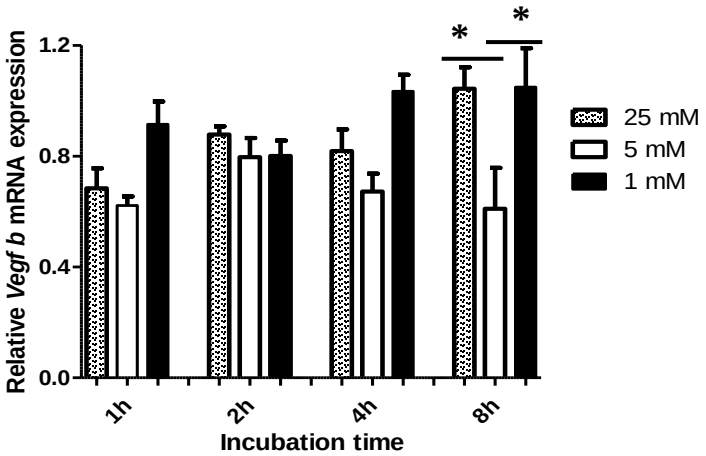
Figure 21: Long term HS may increase *Vegf b* mRNA expression but not *Vegf c* (RT-qPCR). *A)Vegf b B)Vegf c mRNA expression in STC-1 cells incubated in 25 mM, 5 mM, 2 mM and 1 mM glucose medium for 15, 19 and 26 days. Expression was measured by the RT-qPCR (n=3, mean+/- SEM).*

4.8. *Vegf b* and *Vegf c* mRNA expression during first hours of HS

In order to know whether *Vegf c* and *Vegf b* mRNA expression can be regulated earlier on, the expression of *Vegf b* and *–c* mRNA was studied in the first 8h of HS (Figure 22). According to the result, there was no major difference in *Vegf c* expression in cells incubated in 5 or 1mM glucose medium in comparison with the 25 mM standard condition (Figure 22B). In contrast, *Vegf b* mRNA expression varied (Figure 22A). Incubation in 1 mM glucose medium displayed tendency to increase mRNA expression in the first hour and later on at 4h and 8h of incubation. Moreover, there was a significant difference between mRNA expression in cells incubated in 25 mM and 5 mM as well as 5 mM and 1 mM after 8h (Figure 22A).

As STC-1 cells strongly activated mTOR pathway in the first hours of HS (Figure 12A), this activation could modulate *Vegf b* and *Vegf c* expression. Cells were treated with a mTOR inhibitor, rapamycin, before the induction of HS and the effects on their expression were studied. The treatment with rapamycin modified the expression of *Vegf b*, depending on the incubation time (Figure 23A). A significant difference of *Vegf c* mRNA fold change between 25 mM and 5 mM was observed (Figure 23B).

A)



B)

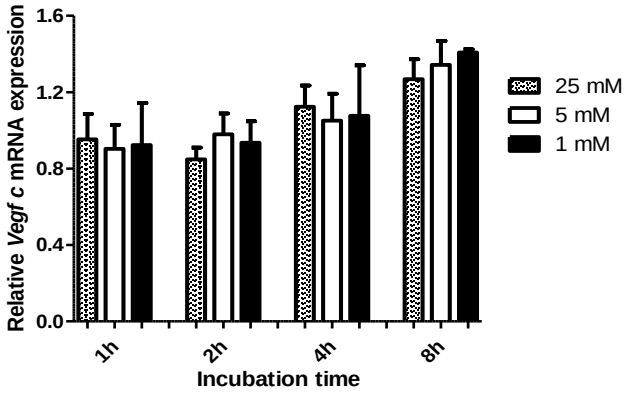
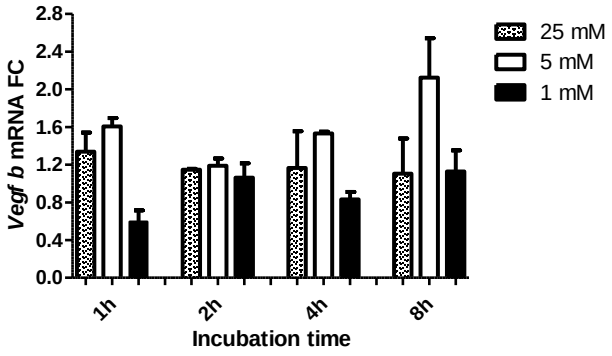


Figure 22: *Vegf b* and *Vegf c* mRNA expression during first hours of HS. *A) Vegf b B) Vegf c mRNA expression in STC-1 cells incubated in 25 mM, 5 mM, 2 mM and 1 mM glucose medium for the indicated time. Expression was measured by the RT-qPCR (n=3).*

A)



B)

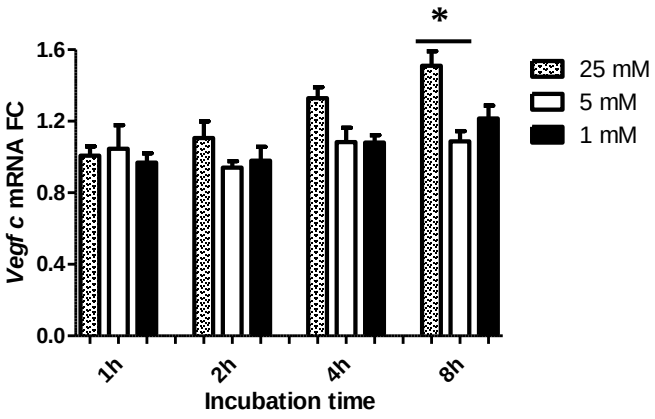


Figure 23: FC of *Vegf b* and *Vegf c* mRNA expression during first hours of HS after rapamycin treatment. *A)Vegf b B)Vegf c mRNA expression between cells treated with rapamycin and non-treated ones. The values are presented as mean+/-SEM (n=3).* $p < 0.05$. Expression was measured by the RT-qPCR (n=3).*

4.9. VEGF-B expression is increased by HS

If *Vegf b* and *Vegf c* mRNA are expressed in STC-1 cell line, are they translated into the corresponding proteins? By performing Western blot, we observed that both proteins were present in 25 mM standard conditions (Figure 24). Regarding the effect of HS, the cells incubated for up to 48h in 5 mM or 1 mM glucose medium had an increased expression of VEGF-B (Figure 24A). The same pattern was observed after long lasting HS (22 days, Figure 24B). VEGF-C expression was only studied after a long lasting HS. It is strongly expressed in 25 mM glucose conditions and is also increased by HS. In those experiments phosphorylation status of P70S61 kinase was used as a control of the activation of mTOR pathway by HS.

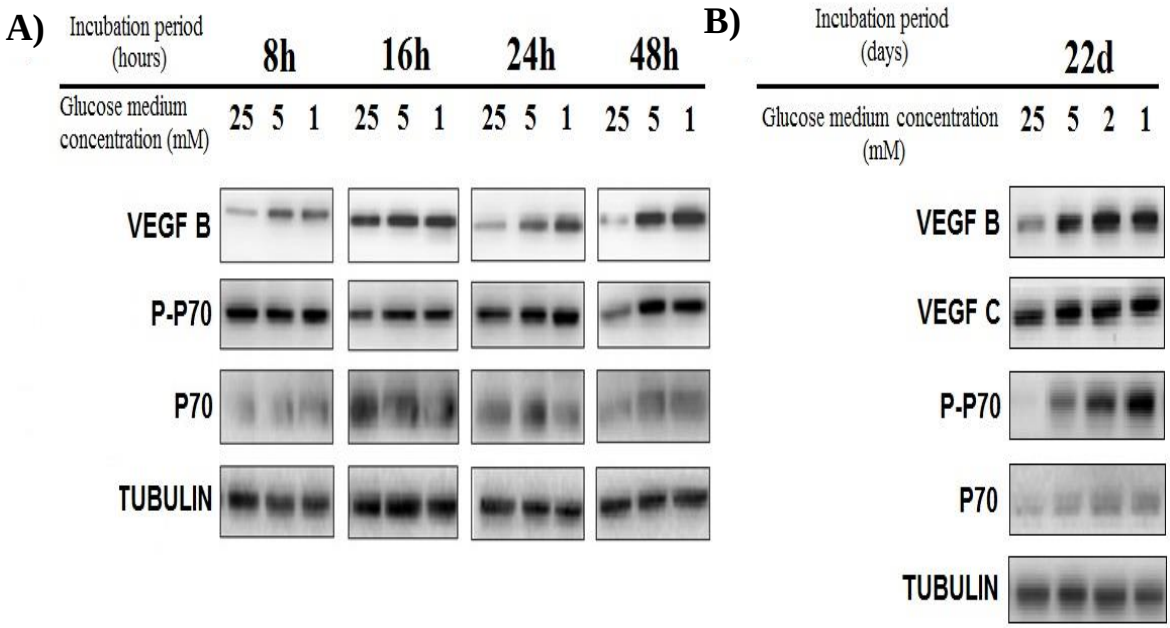
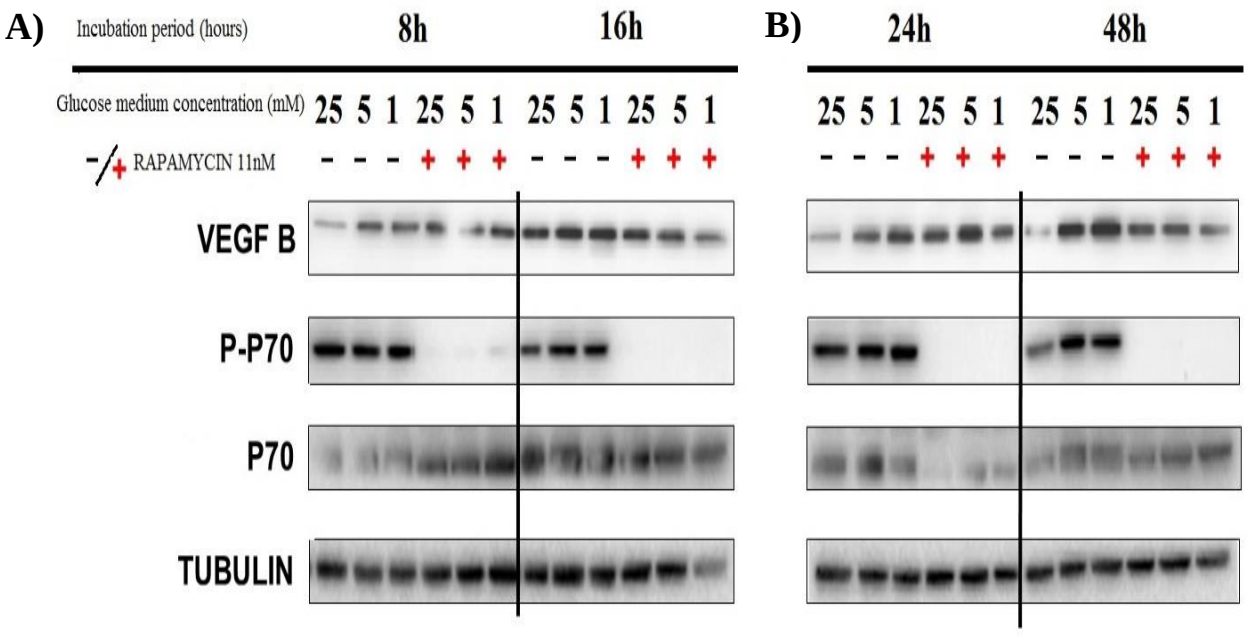


Figure 24: VEGF-B and -C expression in standard condition or after a short or a long lasting HS. *A) VEGF-B expression in the first 48 h of HS. B) VEGF-B and -C expression after 22 days of glucose depletion. Long term HS leads to an increase of VEGF-B expression. VEGF-C expression is slightly increased, with the strong expression in basal condition. Western blots representative of 3 experiments.*

4.10. Role of mTOR in VEGF-B expression upon HS

As HS modulates mTOR pathway activity, we further investigated whether this pathway could be related to the HS-induced expression of VEGF-B. Cells were treated with rapamycin and submitted to glucose depletion for different time periods. The inhibition of mTOR pathway was checked by the phosphorylation status of P70S6 kinase, which was completely abolished. After 24h rapamycin treatment VEGF-B expression decreased (Figure 25B) only in 1 mM glucose condition, while the expression was increased in 5 mM. Forty-eight hours rapamycin treatment decreased the VEGF-B expression in both HS conditions, 5 mM and 1 mM (Figure 25B). Controversy, 8h, 24h and 48h treatment in 25 mM condition increased VEGF-B. In long lasting HS, here 13 days, rapamycin treatment is also associated to an increased expression of VEGF-B in the standard condition, and to a decreased expression of VEGF-B in 1 mM (Figure 25C). Concerning VEGF-C, its expression did not decrease after the mTOR pathway inhibition.



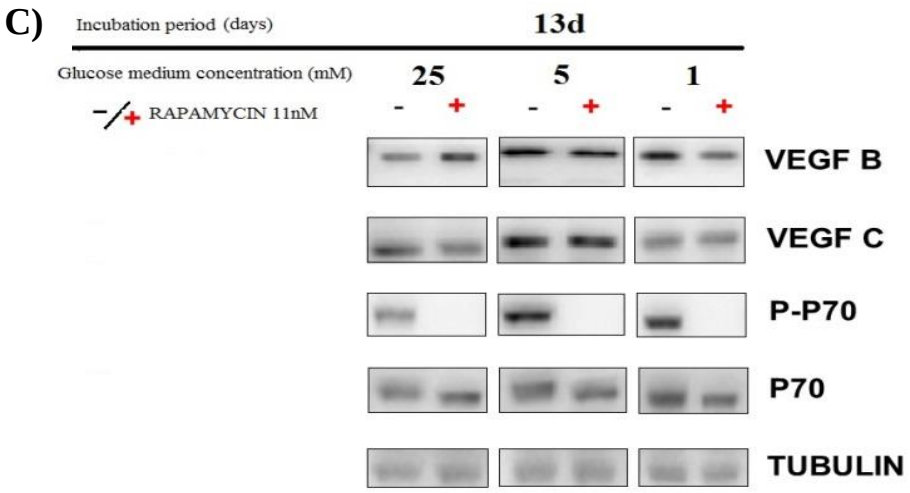


Figure 25: VEGF-B protein expression seems to depend on mTOR activation only after 48h of HS. *Comparison of VEGF-B expression after A) 8h and 16h B) 24h and 48h of HS and rapamycin treatment C) 13 days of HS and 24h of rapamycin treatment. Western blots representative of 3 experiments.*

4.11. Role of mTORC1 in VEGF-C expression

Parallelly to the experiments studying the effect of rapamycin on VEGF-B and –C expression, we developed a double transfection method to downregulate Raptor, a protein essential for the mTORC1 activation, using siRNA against Raptor, as a non-pharmacological tool to inhibit mTOR pathway. As shown in Figure 26, the knockdown of Raptor was only partial. Moreover, the phosphorylation of P70S61 kinase did not decrease, but increased in comparison with control. Accordingly, we were unable to further investigate the effects of mTOR activation on VEGF-C expression. As this unexpected result was from one experiment, further experiments must be done with more efficient silencing of Rapor to confirme this unexpected effect on phosphorylation of P70S61 kinase.

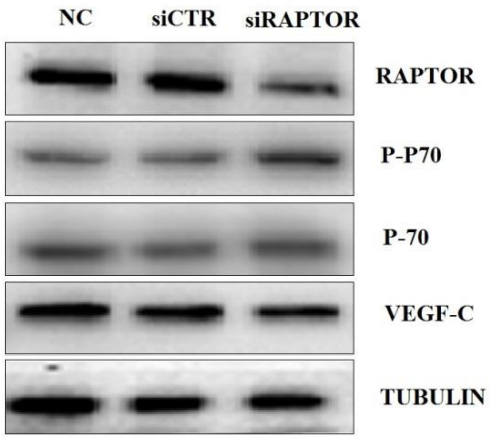


Figure 26: Downregulation of Raptor. *Knockdown of Raptor was made by the use of siRNA against Raptor. The degree of knockdown, as well as the efficacy of mTOR pathway inhibition was determined by probing for Raptor and phospho-P70S6K1 by Western blot. (NC=negative control, siCTR= siRNA control); n=1*

4.12. *In vivo* VEGF-C expression in hepatic nodules

As VEGF-C was expressed *in vitro,* we wondered whether VEGF-C would be detected *in vivo* in a murine model of STC-1 intrasplenic xenograft, characterized by an hepatic dissemination (68). Particularly the variation of expression of VEGF-C during the development of the hepatic nodules was of interest. Results showed that VEGF-C is expressed in the hepatic nodules 28 days after xenograft challenge (Figure 27).

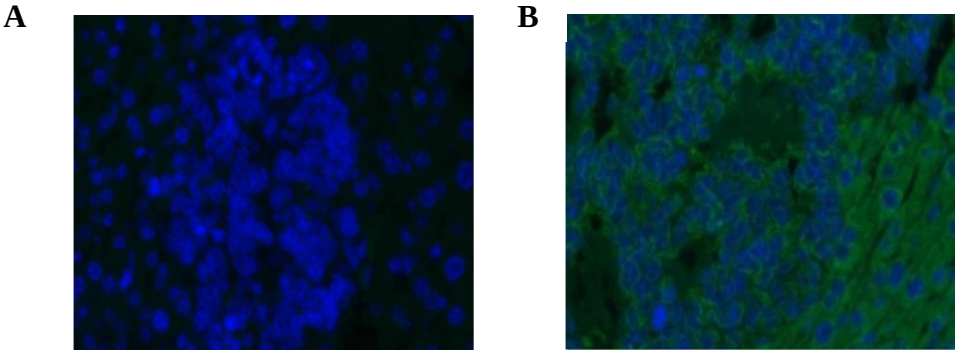


Figure 27: *In vivo* VEGF-C expression in hepatic nodules derived from intrasplenic xenograft. *Intrahepatic nodules resulted from the dissemination of STC-1 intrasplenic*

xenograft (28 days). A) Negative control with secondary antibody and Hoechst (blue). B) VEGF-C (green). The experiment representative of 2 mouse xenografts.

4.13. The role of ER stress in regulation of VEGF-B and VEGF-C expression

As HS modulates VEGF-B expression, we further investigated the potential conditions that could modify the VEGFs expression. Data from laboratory suggested that HS causes the ER stress which in turn activates mTOR pathway. Therefore, we used three different inductors of ER stress (thapsigargin, bortezomib and brefeldin A). The aim was to further examine which mechanisms of ER stress are important for VEGF-B and VEGF-C expression. The activation of mTOR pathway was evaluated by the phosphorylation of P-70S6K1. Additionally, ER stress induction was examined by the phosphorylation of PERK and its downstream effector eIF2α, as well as by induced expression of CHOP. According to results, ER inducers provoked different activation of downstream targets.

Thapsigargin (Tg) is inhibitor of sarco/endoplasmic reticulum Ca^{2+} ATPase. According to results, Tg did not induce the increase in VEGF-B expression after 8h and 17h incubation period. However, long term incubation with Tg increased VEGF-B expression in comparison with 8h incubation period. VEGF-C expression followed pattern of VEGF-B after induction of ER stress by using Tg. Actually, 17h treatment increased VEGF C expression in comparison with the 8h incubation, although the expression was still decreased in comparison with the control (Figure 28).

Brefeldin A inhibits protein transport form ER to Golgi apparatus and induces ER stress. The treatment caused the increase of VEGF-B expression after 8h and 17h of culture in comparison with control. The VEGF-C expression was induced after 8h of incubation, while 17h incubation did not lead to an increase (Figure 28).

Bortezomib, the proteasome inhibitor, caused an increase of VEGF-B expression after 17h of incubation, but did not increase VEGF-C expression (Figure 28).

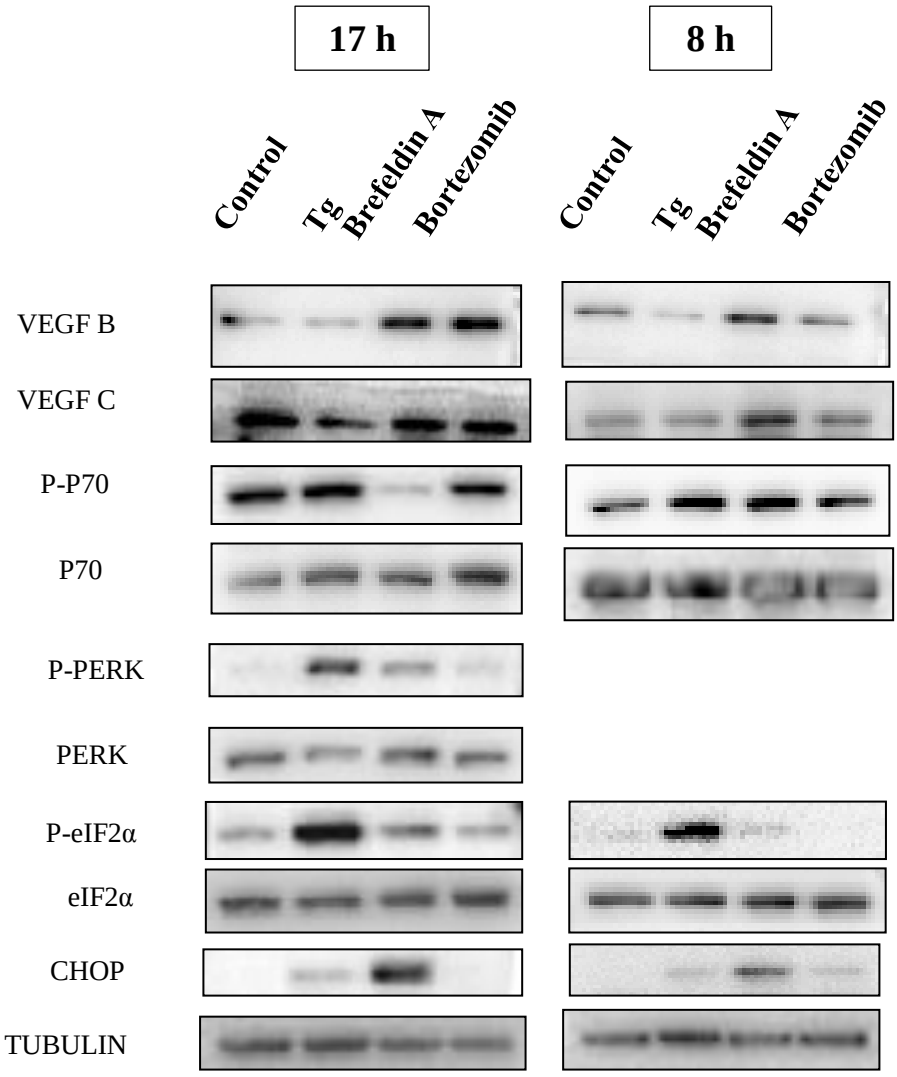


Figure 28: VEGF B and VEGF C expression after ER stress induction. *The cells were incubated in 25 mM glucose medium. ER stess was induced by the use of thapsigargin (Tg, 300nM), brefeldin A (3μM) and bortezomib (30nM) after 8h or 17 h incubation. The induction was verified by the phosphorylation ER effectors. The activation of mTOR pathway was followed by the phosphorylation of P-70. (n=1)*

5. DISCUSSION

To study the correlation between mTOR and hypoglucidic environment, the hypoglucidic stress (HS) model was developed. STC-1 cell line was used in experiments. STC-1 cells are representative of poorly differentiated human digestive neuroendocrine tumors in *in vivo* observation (68). Surprisingly, the HS activated mTOR pathway and forced STC-1 cell to adapt and survive long term depletion of glucose (data from laboratory, Figure 12). These findings are not in agreement with the literature which associates the activation of this pathway with excessive energetic status (23). The glucose starvation should force cells to save the energy since the main fuel of cancer cells is decreased. The activation of mTOR pathway is known to induce the phosphorylation of 4-EBP1 and P70S6K1, two downstream targets, implicated in the cap-dependent protein translation (23). Indeed, phosphorylation of the 4-EBP1 releases the eukaryotic translation initiation factor (eIF4) which takes part in the eIF4F complex initiating cap-dependent protein translation, a major driver of the cellular proliferation. Phosphorylation of P70S6K1 upregulates ribosomal biosynthesis and enhances the translational capacity of the cell. The phosphorylation was completely abolished when cells were treated with the rapamycin (23) (Figure 25).

Rapamycin is allosteric inhibitor of mTORC1, which efficiency depends on the cell line. For instance, in STC-1 rapamycin induces a total abolishment of P70S6K1 phosphorylation (Figure 25) as well as strong decrease in 4-EBP1 phosphorylation. But not all types of cells exhibit the same profile. Sabatini et al. showed that cells are differently sensitive to mTORC1 inhibition by rapamycin (23). The determinate factors are still unknown. It could be that mTORC1 activation depends on the different intracellular signalization and energetic status of the cell (23). Rapamycin, the mTORC1 inhibitor, induced different feedback loops that lead to the cell resistance (69). It is unknown if those loops are present in STC-1 cells.

The mechanism by which glucose deprivation leads to strong mTOR pathway activation has not been identified. Our findings opened a lot of new questions about the functioning of the mTOR pathway. However, some explanations can be offered:

1. In the term of complex formation, nutrient deprivation can lead to stabilization of protein interactions within the complex. Indeed, two states of raptor-mTOR interactions can be distinguished depending on nutrient conditions. For instance, cells growing in a nutrient-

rich environment, have the raptor-mTOR interaction in a low stability state which is associated with high mTOR kinase activity. Contrary, in nutrient poor environment the raptor-mTOR interaction is in a high stability state linked with the decrease mTOR kinase activity (70). Sabatini et al. also reported that redox-sensitive mechanisms contribute to the regulation of interaction between raptor and mTOR (71).

2. The quantity of mTORC1 could be more important in HS conditions. To explore this option we are currently trying to coimmunopercipitate (COIP) raptor-mTOR complex, by using antibody against RAPTOR.
3. STC-1 cells could form complex with another protein when glucose is depleted. For instance, in 2014 a new partner of mTORC1 was identified. Astrin is a negative modulator of mTORC1 upon metabolic and redox stimuli (72). In order to identify other possible partners, mass spectrometry will be done after COIP of mTOR in STC-1 cells.
4. Well identified proteins regulating mTORC1 activation could be differently bound in HS. With COIP we could investigate if the quantity of inhibitors/activators of this pathway changes upon HS.

mTOR pathway is not only important in protein translation, but is also considered as an important regulator of transcription. In order to identify its possible gene transcriptional targets, a microarray experiment was performed. The mTOR pathway was activated by the use of HS model. Interestingly, there we found a significant increase in the transcription of the genes involved in intracellular trafficking and organelle formation (Table VI). Indeed, mRNA expression of many lysosomal genes was significantly upregulated in 1 mM glucose medium in comparison with standard culture conditions (25 mM glucose concentration) (Figure 13). Furthermore, many of these genes were recognized as targets of TFEB. According to the literature, mTORC1 activation leads to cytoplasmic TFEB retention. Additionally, Brugarolas et al. showed that this is not always the case. TSC2 null cells, in which mTOR pathway is overactivated, can translocate TFEB into the nucleus (73). Indeed, only strong mTORC1 activation leads to an increased phosphorylation of TFEB (on three different serine residues). In the nucleus, TFEB triggers transcription of lysosomal and autophagy genes. But our results showed an increase only in the lysosomal TFEB target genes (Figure 14). Genes associated with autophagy were not found to be significantly expressed. This was expected since the mTOR pathway is activated in glucose depleted conditions. Detailed analysis showed that

only one of the three lysososmal biogenesis subgroup genes, coding for lysosomal membrane proteins, was increased. It is possible that during HS, certain epigenetic modifications occur, such as chromatin remodeling or gene methylation. This would explain why TFEB might not increase the mRNA expression of the other two subgroups, lysosomal hydroxylases and vATPases. Alternatively others transcriptional factors could be involved. Besides TFEB, there are three identified transcription factors that belong in the same family: MITF, TFEC and TFE3 (42). All of them have slightly different profile of gene expression which depends on the cell type and the developmental stage. Thus, they share some common elements of regulation, such as the response to variations in the amount of nutrients in the environment. All of them were found to translocate in the nucleus in case of starvation. However, only TFEB and TFEC can bind to CLEAR elements (37,42). In January 2014, Puertollano et al. showed that TFE3 can also be associated with the mTOR activation (42). Studying our microarray analysis data and literature findings, the TFEB transcriptional profile was the most similar with ours, in comparison to the transcriptional profiles of other family members (TFE3, TFEC and MITF). It is known that TFEB regulates the expression of PPARG group of genes implicated in lipid metabolism (43). Moreover, microarray analysis showed that HS caused the increase of its expression, just as Settembre et al. suggested (Figure 16). However, according to our results, the cultivation of STC-1 in 5 mM glucose medium already increased mRNA expression in comparison with the expression in 25 mM glucose environment (Figure 17). However, in these conditions the expression of other lysosomal groups of genes was not increased. That might suggest that other regulatory factors could be involved. For instance, mTOR pathway activation or glucose deprivation itself could trigger the translocation of transcriptional enhancers. This would mean that this network is even more complicated as previously thought, as enhancers could serve to fine-tune the expression (Figure 29).

According to the major findings from Sabatini's laboratory, mTORC1 has to be translocated to the lysosomal surface in order to be activated (46). Therefore the eventual mTOR-lysosome coolocalisation in HS environment was studied by an immunofluorescence essay. Regarding our results, the colocalization was not observed (Figure 19), although mTOR pathway is strongly activated in HS (Figure 12). Our experiences did not allow us to conclude why this unexpected behavior was observed. However, the activation of mTOR pathway and non-lysosomal localization could have a broader meaning. It might be the characteristic of the

neuroendocrine cells in general, since similar behavior was also found in others types of neuroendocrine cells (data from laboratory).

These neuroendocrine secretory cells produce high amount of the proteins, meaning that they must have an excessive gene transcription, protein folding and proceeding, packing, vesicles transportation as well as proper lysosomal function. Therefore transcription-ER-Golgi-vesicules-lysosomal axis has to be highly active and organized. If the mTOR spatial organization in the secretory cells is also important for its activation, more immunofluorescence studies are needed. For instance, the eventual mTOR - ER, mTOR - Golgi colocalization could be performed, since mTOR has the sequence for ER and Golgi targeting (74).

Finally, strong HS stress increased FLCN and FNIP1 mRNA expression (Figure 18). This opens possibility that nutrient sensing could be modulated by these two proteins as they appear to be positive regulators of mTORC1. Reduced amount of FLCN inhibits mTORC1 activity in cultured cells and in mice (75,76). FLCN helps cell to organize rapid and robust reactivation of mTORC1 when switching from nutrient depleted state to nutrient related one (42). However, colocatization with mTOR needs to be studied.

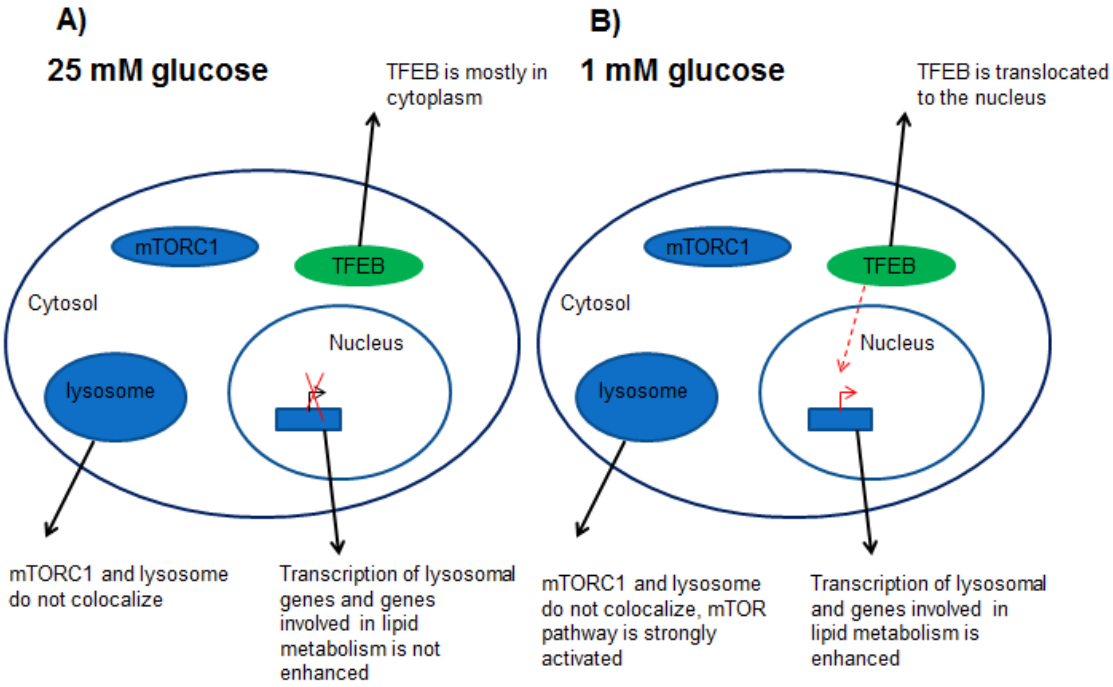


Figure 29: Possible signalization of STC-1 cells in HS environment. *A.) In standard culture condition (25 mM glucose), mTOR pathway is not strongly activated. Lysosomes and mTORC1 do not colocalize. TFEB might not be shifted in the nucleus in greater amount. Therefore, we don't observe the strong increase in transcription of TFEB target genes. B.) Although we do not observe mTORC1 and lysosome collocalization, we have intense mTOR pathway activation in 1mM HS conditions. The activation might strongly increase TFEB phosphorylation and its translocation into the nucleus. Once there, TFEB orchestrates transcription of its targets. However, others enhancers and transcriptional activators could be involved.*

As mTOR pathway is important modulator of VEGF-A expression in NETs, we dedicated the next part of research work on exploring the expression of other vascular endothelial growth factors and their possible connection with the mTOR pathway. Contrary to VEGF-A, that is expressed in neuroendocrine cells and human neuroendocrine tumors, the expression of VEGF-B and VEGF-C has not been described yet (68). To answer this question, expression of *Vegf b* and *Vegf c* mRNA has been studied. Both *Vegf* mRNAs were found in STC-1 cell line and theirs expression was confirmed on the protein level (Figure 21).

To evaluate the effect of hypoglucidic conditions on *Vegf b* and *Vegf c* mRNA expression, HS model was used. As *Vegf c* expression is increased in certain stressful conditions, such as oxidative stress, hypoxia or thermal stress (77), we hypothesized that HS could modify the mRNA expression. Unexpectedly, *Vegf c* mRNA expression was not increased in the first 8 hours of HS in comparison with normal cultivation conditions (Figure 22B). By analyzing microarray data we found that *Vegf c* mRNA does not significantly vary upon HS.

The *Vegf b* transcriptional regulation is less investigated. Only one article demonstrated that *Vegf b* transcription is regulated by related transcriptional enhancer factor-1 (RTEF-1) in endothelial cells (78). Moreover, *Vegf b* half-life was found to be stable (half-life more than 8h), even in different stressful conditions, such as hypoxia and serum depletion, yet there was no data about the effect of the glucose depletion (79). According to our results, only long term cultivation in 1mM condition increased the *Vegf b* expression (Figure 20, 21A). Yet, the significant difference of *Vegf b* mRNA expression observed with microarray experiments

was not confirmed by RT-qPCR (Figure 20, 21A). At least in the first hours of hypoglucidic stress the increase seems not to be regulated by the mTOR pathway (Figure 23A).

Whether, the *Vegf c* transcription is regulated by mTOR pathway in STC-1 cell line remains to be examined. Only few reports documented a correlation between VEGF-C and hypoxia inducible factor α (HIFα), the factor associated with the hypoxic conditions as well as with mTOR pathway activation (80,81). This year, the *Vegf c* translation was demonstrated to be increased in hypoxic conditions, switching from cap-dependent to internal ribosome entry site (IRES) dependent translational mechanism independently of *Vegf c* transcription as well as HIFα expression (82). Furthermore, in certain conditions, such as oxidative or thermal stress, lens epithelium-derived growth factor (LEDGF) binds to the stress response element (STRE) on VEGF-C promoter and controls its transcription in rat glioma and human non-small cell lung carcinoma (77). However, it is unknown if LEDGF is expressed in STC-1. To study VEGF-C regulation by mTOR, we tried to downregulate Raptor, an essential component of mTORC1 (23) (Figure 26). Although, we succeeded to decrease the protein expression, the phosphorylation of P70S6K1 did not diminish. On the contrary, phosphorylation seemed to increase. Thereby, downregulation by silencing Raptor and the role of mTOR pathway on VEGF-C expression has to be further investigated.

The activation of mTOR pathway is known to induce cap-dependent protein translation, a major driver of the cellular proliferation. Phosphorylation of P70S6K1 enhances the translational capacity of the cell. However, IRES dependent translational mechanism is usually set up in stressful conditions or when the cap-dependent mechanism is encountered (83). Our preliminary results showed that rapamycin treatment did not modified the *Vegf c* mRNA expression in standard cultivation condition (Figure 23B). Furthermore, the VEGF-C protein expression was not decreased by rapamycin treatment either (Figure 25C). Therefore, a switch between cap-dependent to IRES dependent VEGF-C translation could occur in STC-1 cells.

These results opened new questions not only about VEGF-C expression regulation, but also about its role in digestive neuroendocrine tumors. Firstly, it would be compelling to see whether VEGF-C is expressed in human samples of neuroendocrine tumors correlated with the clinical issues. Secondly, VEGF-C could be indirectly implicated in the response to the mTOR target therapy since a relapse is observed. Inhibitors of mTOR pathway, such as Everolimus,

are used as a part of the therapy (7). The treatment increases the progression free survival, mainly by decreasing the proliferation rate and tumor growth. Couderc et al. showed that the mTOR inhibitors decrease the expression and consequently the secretion of VEGF-A (84). VEGF A shears the same receptor as VEGF C, but the opposite of VEGF C, it has a high affinity. If the VEGF-C is expressed in human intestinal-NETs and its synthesis and secretion is not decreased by the mTOR inhibition, the VEGF-C signalization could gain in importance. Another drug used could trigger similar behavior: Bevacizumab, anti-VEGF A humanized monoclonal antibody. As this mAb neutralizes VEGF-A and prevents its binding to the receptors, signaling induced by VEGF C would be in favor.

VEGF-C can act on multiple targets. NRP2 and VEGFR2 expression have already been proven in our laboratory on STC-1 cell line as well as on human digestive neuroendocrine tumors. Therefore, VEGF-C could play important role in autocrine signaling. VEGF-C could also have a paracrine effects, inducing endothelial survival as well as cellular migration (85). By targeting lymphatic cells, the limphangiogenesis as well as the acceleration of lymphatic dissemination could also be triggered. Whether VEGF-C is synthetized and favors the metastatic development in neuroendocrine tumors was not investigated. Our results showed that VEGF-C is detectable in hepatic nodules, which occur after the dissemination from the spleen (Figure 27). Whether VEGF-C is secreted from the STC-1 cells has to be investigated as well as a colocalization of VEGF-C with the neuroendocrine marker needs to be done.

Poorly differentiated intestinal NETs express VEGF-A and are prone to neoangiogenesis (68). Neoangiogenesis is described as chaotic procedures leading to disrupted and leaking vasculature. As a result the so called blood lakes appear (86). Because of lack of flow, fresh nutrients and oxygen are not delivered. This causes the hypoxic and also low nutrients hypoglucidic stress. According to our results, hypoglucidic conditions cause an increase of the VEGF-B that is regulated by the mTOR pathway (Figure 30). This opens two main predictions: 1./ Long term use of antiangiogenic therapy, can in fact worsen the situation making cells more aggressive (87). The cells would be better adapted to such an environment. Indeed, the drugs such as Bevacizumab, would be able to decrease the intratumoral blood vessel density, but in a longer period it would enable the selection process towards more aggressive phenotypes (20). 2./ Rapamycin treatment which was able to decrease the increased

expression of VEGF-B in HS, might not be delivered to the hypoglucidic areas. The areas with decreased glucose concentrations would also experience lower rapamycin concentration values, meaning that the mTOR inhibitors could not be fully efficient due to the supply deficit.

The role of VEGF-B in STC-1 cell line is still unknown. It is known that STC-1 as well as human GI-NETs express NRP-1. If the VEGF-B was secreted, the autocrine loop could be established, helping cells in glucose depleted areas, to favor lipid uptake and β-oxidation (88).

As the STC-1 cells survive in long term hypoglucidic conditions, they are prone to make metabolic adaptation. The transcriptomic analysis of the cells submitted to the long term HS suggested the metabolic changes towards lipid metabolism. The mechanism underlying this metabolic adaptation is under study. First results showed that HS activates the endoplasmic-reticulum (ER) stress which regulates lipid metabolism (data from laboratory) (89). Therefore, the cells were treated with 3 different ER stress inducers (thapsigargin, brefeldin A, bortezomib). The difference in their mechanism of action allowed us to further investigate which processes could be important in VEGF-B and VEGF-C regulation. Interestingly, not all of them induced an increase of their expression, even though the mTOR pathway was activated. For instance, thapsigargin, the inhibitor of SARKO/ER Ca^{2+} ATPase did not cause the increase in their expressions. In the contrary, it seems that the expression was decreased (Figure 28). Moreover, it could be that HS does not trigger ER stress by creating intracellular Ca^{2+} imbalance. However, further conformations are needed.

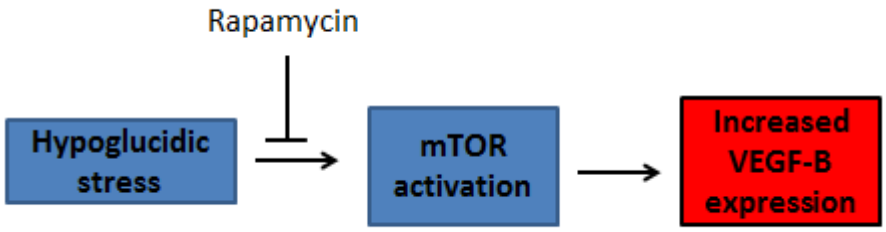


Figure 30: Possible regulation of VEGF B expression by mTOR pathway activation in HS. *HS (1 mM) leads to the strong activation of mTOR pathway which increases VEGF B expression.*

6. CONCLUSIONS

HS strongly activates mTOR pathway and leads to changes in the transcription profile. Indeed, microarray analysis showed increased mRNA expression of genes implicated in lysosomal biogenesis and lipid metabolism that are target of TFEB-mTORC1 axis. However, the direct connection between mTOR activation and the transcription modulation still remains to be further investigated. Despite the mTOR pathway activation lysosomes and mTOR proteins do not colocalize. What is the underlying mechanism behind its activation remains unknown.

As VEGF –A plays important role in NETs we have further investigated the other VEGFs whose expression in DNETs has never been described yet. We found that *Vegf b* and *Vegf c* are expressed in STC-1 cell line. Furthermore, VEGF-B expression was increased in hypoglucidic conditions and induced by mTOR pathway activation. However, the role of VEGF-B remains to be investigated. Nevertheless, as the activation of mTOR in HS conditions and VEGF-B regulation has never been discussed, the confirmation of results on others cell lines would be interesting. VEGF-C expression might depend on the glucose supply and could be independent of mTOR pathway. As the VEGF-C was defined as factor associated with the development of metastasis it would be interesting to study its role in of NETs development.

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