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Final Degree Project

Characterization of MANF expression and secretion under serum and glucose deprivation in vitro

Marta Medina Carbonero

Institute of Biotechnology, University of Helsinki

Supervised by Dr. Päivi Pulkkila and M.Sc. Emilia Galli

Universitat de Vic – Universitat Central de Catalunya

Supervised by Dr. Josep Bau Macià

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Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.

Marie Curie

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SELECTED ACRONYMS AND ABBREVIATIONS

ARMET	Arginine-Rich, Mutated in Early stage Tumors
ARP	Arginine Rich Protein
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
BSA	bovine serum albumin
CDNF	Cerebral Dopamine Neurotrophic Factor
CNS	Central Nervous System
DMEM	Dulbecco's Modified Eagle Medium
E	primer efficiency
eIf2α	eukaryotic initiation factor 2 alpha
ELISA	Enzyme – linked immunosorbent assay
ER	endoplasmic reticulum
FBS	Fetal bovine serum
Grp78	Glucose regulated protein 78kDa
HRP	horseradish peroxidase
Hyou1	Hypoxia up-regulated protein 1
IRE1	Inositol-requiring enzyme 1
MANF	Mecencephalic Astrocyte-derived Neurotrophic Factor
NTFs	Neurotrophic factors
PAA	polyacrylamide
PBS	phosphate buffered saline
PD	Parkinson's Disease
PERK	Pancreatic ER kinase
RT	room temperature
SAPLIPs	saposin-like proteins
SDS	sodium dodecyl sulfate
UPR	Unfolded Protein Response
XBP1	X-box binding protein 1

SUMMARY OF FINAL DEGREE PROJECT – Degree in Biotechnology

Title: Characterization of MANF expression and secretion under serum and glucose deprivation *in vitro*

Key words: MANF, PD, NTFs, ER, ELISA, mRNA, qPCR, MIN6, EA.hy926 and Grp78

Author: Marta Medina Carbonero

Supervisors: Dr. Josep Bau Macià (UVIC-UCC), Dr. Päivi Pulkkila (UH, BI) and M.Sc. Emilia Galli (UH, BI)

Mesencephalic Astrocyte-derived Neurotrophic Factor (MANF) was discovered to promote neuronal survival in rodent models of Parkinson's disease (PD) and cerebral ischemia. MANF belongs to a novel evolutionary conserved family of neurotrophic factors (NTFs) and it is an endoplasmic reticulum (ER) stress response protein. MANF was more recently shown to promote beta cell proliferation and survival and its levels in serum are increased at the onset of type 1 diabetes in children.

MANF protein and mRNA expression is widespread in most human and mouse organs. However, MANF expression and secretion levels from cells depending on the availability of energy sources have not been previously studied and, study it was the main objective of the project. Characterization of MANF expression and secretion was done *in vitro* from mouse insulinoma 6 (MIN6) and human endothelial (EA.hy926) cell lines cultured under serum or glucose deprivation. Enzyme-linked immunosorbent assay (ELISA) specific for human and mouse MANF were used to characterize intracellular and secreted MANF protein levels. Moreover, MANF protein and ER stress markers were analyzed by using western blotting. The mRNA expression of MANF and ER stress markers was also analyzed using quantitative PCR method.

The results indicated that intracellular MANF levels are up-regulated under glucose deprivation in MIN6 and EA.hy926 cell lines. However, secreted MANF levels increase when glucose concentration in the medium decreases only in beta pancreatic insulin producers cells (MIN6), in contrast to EA.hy926 cells, indicating that responses to glucose deprivation are different in the two cell lines. Furthermore, intracellular and secreted MANF levels increase when serum concentration is decreased in both MIN6 and EA.hy926 cell lines. The studied human endothelial cell line present increased levels of Grp78, an ER stress marker, after serum and glucose deprivation.

1. INTRODUCTION

1.1. MANF was discovered to present neurotrophic activity

Neurotrophic factors (NTFs) are an important group of secreted proteins which regulate life and death of neurons during development and promote the regeneration of neurons from injury (Lindholm and Saarma, 2010).

Mesencephalic Astrocyte-derived Neurotrophic Factor (MANF), together with homologous Cerebral Dopamine Neurotrophic Factor (CDNF), were discovered to promote neuronal survival in rodent models of cerebral ischemia and Parkinson's Disease (PD) (Lindholm and Saarma, 2010), a neurodegenerative brain disease where progressive loss of midbrain dopamine neurons affects motor performance (Dauer and Przedborski, 2003).

Since MANF and CDNF proteins are structurally different from other known NTFs, they form a novel family of NTFs (Lindholm and Saarma, 2010).

It has been shown that MANF and CDNF are the most potent factors protecting and repairing the dopaminergic neurons in the rat 6-hydroxydopamine model of PD (Lindholm et al., 2007; Voutilainen et al., 2009). In addition, MANF mRNA is up-regulated *in vivo* in animal's model of brain ischemia and epileptic insults (Lindholm et al., 2008) and it can also rescue efficiently cortical neurons in rat stroke models (Airavaara et al., 2009).

Although CDNF is not found in invertebrates such *Drosophila*, they express a protein similar to mammalian MANF (Palgi et al., 2009). Recently, it was shown that MANF regulates dopaminergic neuron development in larval zebrafish (Chen YC et al., 2012) and the analysis of *Drosophila* MANF mutants show problems in the membrane traffic, and metabolic changes (Palgi et al., 2012).

1.2. Structure of MANF

The first announcement of human Arginine Rich Protein (ARP) gene characterized it as an oncogene mutated in renal cell carcinomas (Shrindhar et al., 1996). ARP (or ARMET; Arginine-Rich, Mutated in Early stage Tumors) was later described as a gene

which encodes protein with neurotrophic activities and the protein was re-named as Mesencephalic Astrocyte-derived Neurotrophic Factor: MANF (Petrova et al., 2003). MANF was originally identified from the culture medium of rat type-1 astrocyte ventral mesencephalic cell line 1 (VMCL1) based on its trophic effects on cultured embryonic dopaminergic neurons (Petrova et al., 2003).

Human MANF (hMANF) gene (4351 bp) is located in the short arm of chromosome 3 (3p21.2) and encodes for 18kDa protein, which consists of 179 amino acids together with 21 amino acids of pre-sequence. This protein contains 8 cysteines with highly conserved spacing among multicellular organisms (Petrova et al., 2003; Lindholm et al., 2007). The secondary structure is dominated by alpha-helices (47%) and random coils (37%). In terms of three-dimensional structure, MANF consists of two-domain protein (Figure 1), in which the N-terminal domain is similar to the saposin-like proteins (SAPLIPs), that may interact with lipids and membranes (Parkash et al., 2009). The C-terminal domain, homologous to SAP-domains (Hellman et al., 2011), contains a CXXC motif, with possible reductase activity (Parkash et al., 2009), and an Endoplasmic Reticulum (ER)-retrieval sequence RTDL, which has been suggested to bind to KDEL-receptors (Henderson et al., 2013). KDEL receptors in the Golgi recognize KDEL and KDEL-like sequences and mediate protein retrieval from the Golgi back to the ER (Raykhel et al., 2007).

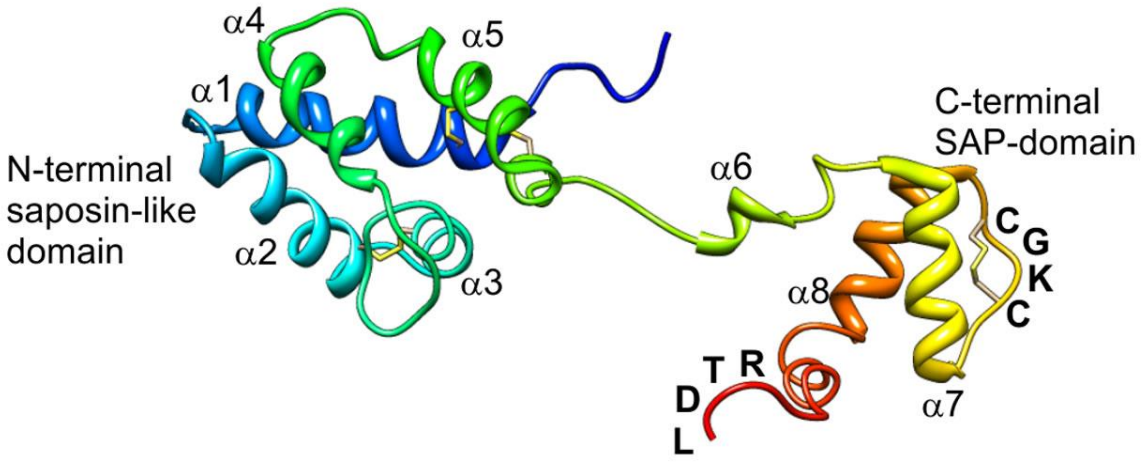

Figure 1. Schematic three-dimensional structure of human MANF (Lindström et al., 2013)

1.3. Expression of MANF

MANF protein and mRNA expression is widespread in most human and mouse organs (Lindholm et al., 2008).

Studies of brain MANF expression show that it is chiefly neuronal (Lindholm et al., 2008; Wang et al., 2014). In central nervous system (CNS), MANF was detected in several brain regions, such as cerebral cortex, hippocampus, olfactory bulb, cerebellum and spinal cord, among others (Lindholm et al., 2008). Furthermore, in the nigrostriatal dopamine system, it is shown that MANF is expressed in the striatum and substantia nigra (Lindholm et al., 2008).

In addition to the nervous system, MANF is widely expressed in non-neuronal tissues, especially in secretory tissues, such as pancreas, salivary gland and in testicular spermatocytes (Lindholm et al., 2008). Moreover, a recent study show MANF protein is also present in human blood serum (Galli et al., 2016).

1.4. MANF outside the CNS

A recent report shows that MANF is up-regulated in autoimmune diseases and inflammatory diseases, such as rheumatoid arthritis, suggesting that MANF may be a negative regulator of inflammation (Chen et al., 2015). In addition, MANF has been identified with an immune modulatory function, biases immune cells toward an anti-inflammatory phenotype, thereby promoting tissue repair in both vertebrates and invertebrates (Neves et al., 2016).

Plus, it is also demonstrated that MANF has a protective activity in cardiomyocytes from cardiovascular ischemia (Glemblotski et al., 2012).

Additionally, complete and pancreas-specific conditional knockout of MANF in mice caused a rigorous diabetes characterized by postnatal reduction of beta-cell mass, since their increased apoptosis and decreased proliferation (Lindahl et al., 2014). Moreover, MANF specifically promotes beta cell proliferation and survival, indicating

that MANF is a therapeutic candidate for the treatment of human type 1 of diabetes (Lindahl et al., 2014).

Furthermore, serum MANF levels are increased at the onset of type 1 diabetes in children (Galli et al., 2016). Serum MANF levels are also increased after therapeutic fasting in humans (Galli, Drinda, Lindholm, unpublished). However, the origin of serum MANF and how the levels of circulating MANF are regulated is currently unknown.

1.5. MANF is an ER stress response protein

Endoplasmic Reticulum (ER) is the cellular organelle responsible for synthesis, folding and modification of proteins destined to the secretory pathway and endosomal compartments (Ron and Walter, 2007). ER is also an important site of Ca^{2+} storage and synthesis of sterols and lipids (Ron and Walter, 2007).

High quantity of unfolded proteins in the ER leads to ER stress and the induction of unfolded protein response (UPR), which is a signal-transduction pathway with the aim to restore the normal function of ER (Szegezdi et al., 2006). Via UPR, the protein folding capacity and degradation of misfolded proteins is increased in the cell and protein translocation across ER membrane is reduced.

Intracellularly, MANF localizes to the luminal ER, where it interacts with the chaperone Grp78 (Glembotski et al., 2012). MANF is an ER stress response protein (Apostolou et al., 2008) and it is secreted in response to experimental ER stress *in vitro* (Apostolou et al., 2008; Lindholm and Saarma, 2010; Glembotski et al., 2012). Microarray studies have identified MANF as an UPR up-regulated gene (Holtz and O'Malley, 2003; Girardot et al., 2004). Furthermore, *in vitro* studies have shown that if there is an induction of UPR, MANF is up-regulated in several mammalian cell lines (Mizobuchi et al., 2007; Apostolou et al., 2008; Tadimalla et al., 2008).

There are three principal UPR pathways mediated by the ER transmembrane receptors: inositol-requiring enzyme 1 (IRE1), pancreatic ER kinase (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (Szegezdi et al., 2006). In addition, the transcription factor X-box binding protein 1 (XBP1) regulates UPR target gene as well (Szegezdi et al., 2006).

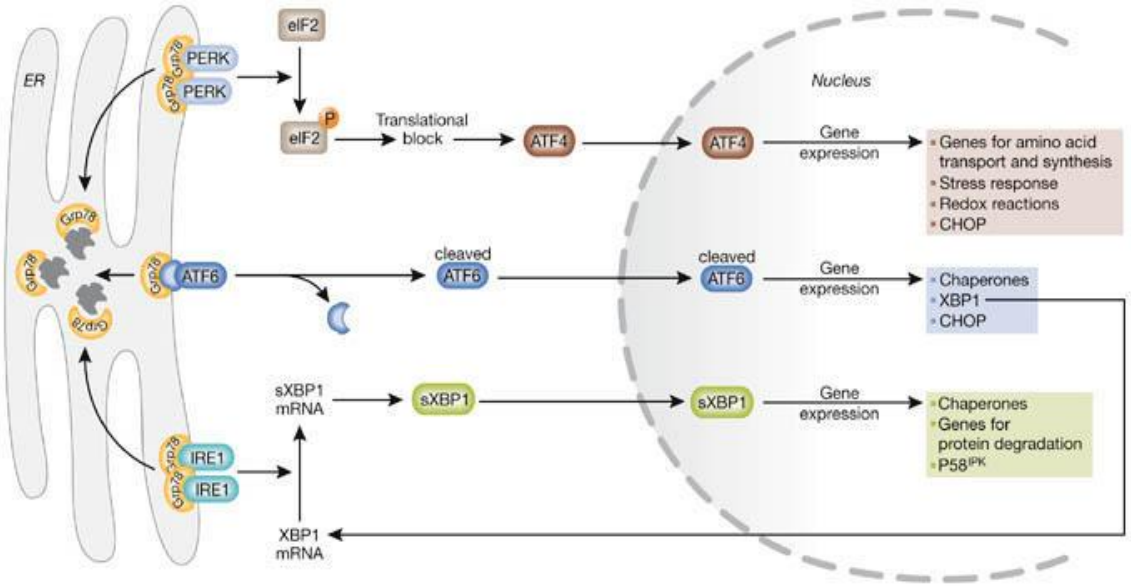

Figure 2. UPR activation. There are three pathways mediated by ER transmembrane receptors: IRE, ATF6 and PERK. (Szegezdi et al., 2006).

In normal stress-free ER, transmembrane receptors PERK, ATF6 and IRE1 are associated with chaperone Grp78 (glucose regulated protein 78 kDa; also it is called BiP) (Szegezdi et al. 2006). Accumulation of unfolded proteins dissociates Grp78 and induces the start of UPR. PERK gets activated and phosphorylates eukaryotic initiation factor 2 alpha (eIf2α), which prevents protein translation at the same time that enables translation of transcription factor ATF4. ATF4 induces transcription of genes required for return to ER stability, such as CHOP. ATF6 is activated by a cleaver process and regulates the expression of ER chaperones and XBP1. RNA of XBP1 is spliced by IRE1. Spliced XBP1 protein (sXBP1) regulates transcription of chaperones and genes involved in protein degradation. (Szegezdi et al., 2006). (Figure 2).

Another ER stress marker is the gene called Hypoxia up-regulated protein 1 (Hyou1), which encodes for glucose regulated protein 170 (Grp170) that it is an ER chaperone, also up-regulated during UPR condition.

Although there is evidence that MANF is an UPR-up-regulated protein, its role during ER stress remains unclear.

2. AIMS OF THE STUDY

Conditions regulating MANF secretion from cells are not well known. Preliminary data from Prof. Saarma's Laboratory suggest that availability of energy sources regulates MANF expression and secretion. The main objective of the project was to study how serum and glucose starvation affect MANF expression and secretion in cancer cell lines by using cell culture and molecular biology techniques. We also wanted to know whether the changes in MANF expression and secretions are related to ER stress induced by starvation.

The specific aims were:

I. To characterize MANF expression and secretion *in vitro* under serum deprivation.
II. To characterize MANF expression and secretion *in vitro* under glucose deprivation.
III. To study if the changes in MANF expression/secretion are related to expression of ER stress markers.

3. MATERIALS AND METHODS

3.1. Cell culture methods

Two cell lines were used: Human endothelial cell line EA.hy926 and mouse insulinoma 6 (MIN6). The cell lines were chosen because 1) they express MANF abundantly, and 2) endothelial cells in blood vessels and pancreatic insulin producing beta cells might be potential sources of circulating MANF *in vivo*.

EA.hy926 cell line was cultured in 10cm plate using Dulbecco's Modified Eagle Medium (DMEM) containing 25mM of glucose, plus 10% of serum (FBS; fetal bovine serum) and 1% of Penicillin-Streptomycin (PeSt) as a growth medium.

MIN6 is a pancreatic beta cell line from mice. MIN6 cells were cultured in 10cm plate using DMEM containing 25mM of glucose and L-glutamine (sigma D5796), plus 70μM of 2-mercaptoethanol, 10% of FBS and 1% of PeSt as a growth medium.

Both cell lines had been cultured at 37 °C in 5% CO_2 incubator.

3.1.1. Serum or glucose deprivation

FBS treatments used were 0%, 1% and 10% of FBS. The basal medium for EA.hy926 cells FBS treatments was composed of DMEM, 1% of PeSt and 0.5% of bovine serum albumin (BSA). However, the basal medium for MIN6 cells FBS treatments was composed of DMEM containing glucose and L-glutamine (sigma D5796), 1% of PeSt, 70μM of 2-mercaptoethanol and 0.5% of BSA. (Table 1)

Table 1. Components of FBS treatments medium. The volumes of each component are expressed per 1ml of final volume.

Treatment	FBS (μl)	PBS (μl)	Basal media (μl)
0% FBS	0	100	900
1%FBS	10	90	900
10% FBS	100	0	900

Glucose treatments used were 0mM, 1mM, 5mM, 10mM and 25mM of glucose. The basal medium for EA.hy926 cells glucose treatments was composed of no-glucose DMEM (Thermo Fisher Scientific, cat. no. 11966-025), 1% of PeSt and 10% of dialyzed FBS (dFBS). The basal medium for MIN6 cells glucose treatments was composed of no-glucose DMEM (Thermo Fisher Scientific, cat. no. 11966-025), 70μM of 2-mercaptoethanol, 1% of PeSt and 10% of dFBS. (Table 2)

Table 2. Components of glucose treatments medium. The volumes of each component are expressed per 1ml of final volume.

Treatment	20mM glucose (μl)	PBS (μl)	Basal media (μl)
0mM glucose	0	22.5	977.5
1mM glucose	0.9	21.6	977.5
5mM glucose	4.5	18	977.5
10mM glucose	9	13.5	977.5
25mM glucose	22.5	0	977.5

3.2. Enzyme-linked immunosorbent assay (ELISA)

3.2.1. Sample preparation

For ELISA samples, EA.hy926 and MIN6 cells were seeded on 12-well plates at cell density of $20x10^4$ and $130x10^4$, respectively, and incubated overnight. The next day, wells were washed with phosphate buffered saline (PBS) and added with 0.5ml of treatment medium. After 24h of incubation under different treatments, medium and cell lysate samples were collected.

For this procedure, cells were placed on ice and medium was removed and placed into tubes. These medium samples were centrifuged at 2000rpm during 5min at 4°C, to remove detached cells and cell debris. Supernatant was changed into new tubes.

Immediately after medium was removed, cells were washed once with cold PBS and 200μl of lysis buffer (Appendix 8.1) were added to each well. Cells with lysis buffer were incubated on ice during 30min. Samples were collected in tubes, which were

centrifuged at 12000rpm during 20min at 4°C, to pellet the nuclei. Supernatant containing soluble proteins was placed into new tubes.

Samples were frozen at -20 °C.

3.2.2. Total protein concentration measurement

Total protein concentration from cell lysates was quantified by using improved Lowry method (DC Protein Assay, Bio-Rad Laboratories, Hercules, CA).

The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. There are two steps that lead to color development. Absorbance was read at 750nm, using a plate reader (VICTOR, Perkin Elmer).

3.2.3. ELISA method and analysis

Samples from EA.hy926 and MIN6 cell lines were analyzed by sandwich ELISA specific for human MANF (hMANF), in case of EA.hy926, and mouse MANF (mMANF), in case of MIN6. Both ELISA procedures were developed and optimized in Saarma's Laboratory.

<u>hMANF ELISA</u>

MaxiSorp (Nunc, Fisher Scientific) 96-well microtiter plates were coated overnight at 4°C with goat anti-hMANF polyclonal antibody (R&D Systems) at 1µg/ml concentration in 0.05M carbonate coating buffer (Appendix 8.2).

Carbonate coating buffer with the antibody was removed, and the plate was incubated for 2h with blocking buffer (PBS added with 0.05% of Tween20 (PBST) and 1% of casein) at room temperature (RT). After washing with PBST, standard samples of recombinant hMANF (P-101-100, Icosagen) ranging from 31.25 to 2000pg/ml, diluted medium and cell lysate samples were added on the plate in duplicate measurements and it was incubated overnight at 4°C in 100rpm agitation.

The used detection antibody was horseradish peroxidase (HRP) – linked mouse anti-hMANF (Icosagen, clone 4E12) diluted in casein blocking buffer. After washing four times, the detection antibody was incubated on the plate at 1µg/ml concentration for 5h in agitation at RT, and protected from the light. Before adding the detection reagent, the plate was washed four times again. For detection, 3,3',5,5'-tetramethylbenzidine was used according to the kit protocol (DuoSet ELISA Development System, R&D Systems). The absorbance was read using plate reader (VICTOR, Perkin Elmer) at 450nm and 540nm (for wavelength correction).

mMANF ELISA

As in hMANF ELISA, 96-well plates were coated overnight with goat anti-hMANF polyclonal antibody (R&D Systems) at 1µg/ml concentration in carbonate coating buffer.

After washing once the plate with PBST, the plate was blocked for 2h at RT with the blocking buffer (1% casein in PBST). After incubation, wells were washed once again and the standard samples of recombinant hMANF (P-101-100, Icosagen) ranging from 62.5 to 1000pg/ml, diluted medium and cell lysate samples were applied in duplicate measurements and the plate was incubating overnight at 4°C in agitation (100rpm).

Detection antibody, rabbit anti-MANF, C-terminal (LSBio, LS-B2688, lot 54167) at 0.5µg/ml concentration in casein blocking buffer, was incubated on the plate for 3h in agitation (100rpm) at 37°C. Then, the secondary antibody, HRP-linked donkey anti-rabbit (GE Healthcare), was incubated for 2h more in agitation at RT and protected from the light. Washing with PBST was repeated four times before adding the detection antibody, before the application of secondary antibody and also before the addition of detection reagent. As hMANF ELISA, the detection was done using 3,3',5,5'-tetramethylbenzidine. The absorbance was read using plate reader (VICTOR, Perkin Elmer) at 450nm and 540nm (for wavelength correction).

For both ELISAs, antibodies and samples were added in 100µl volume to each well. Furthermore, cell lysate samples were diluted 1:100 and medium samples were diluted 1:2 in casein blocking buffer.

The ELISA results were normalized with the total protein concentration measured in each sample (MANF ng/mg total protein).

3.3. Western blot

3.3.1. Sample preparation

Samples from cell lysates were analyzed with western blot assay (The same samples that were analyzed with ELISA, see 3.2.1. section for more details about cell lysate sample preparation).

Once the total protein concentration from cell lysate was measured (see 3.2.2. section for more details), 40µg from each sample were taken and mixed together with 20µl of 5 times Laemmli buffer (LB). LB contains β-mercaptoethanol, which reduces the intra and inter-molecular disulfide bonds; sodium dodecyl sulfate (SDS) detergent, that denatures the proteins and subunits and gives each an overall negative charge so that each will separate based on size; bromophenol blue, that serves as a dye front that runs ahead of the proteins and also serves to make it easier to see the sample during loading, and glycerol, that increases the density of the sample so that it will layer in the sample well. (Appendix 8.4)

Each mix was brought up to 100µl using purified water. To ensure the protein denaturation, samples were boiled at 95°C during 5min.

3.3.2. Gel preparation, running and protein transfer

Proteins were separated by 12% of polyacrylamide SDS-PAGE electrophoresis.

Gels were prepared manually between two glass plates in a gel caster. Firstly, the polymerization of the separation gel was done, with a 12% of polyacrylamide (PAA).

Then, the polymerization of the stacking gel was done, with a comb inserted at the top to create the sample wells. (Appendix 8.3)

Samples were loaded with a volume of 25µl per lane. Therefore, 10µg of total protein were loaded per lane, since the samples were prepared at 0.40µg/µl concentration of total protein.

Gels were running during around 75min at 20mA per gel in running buffer. Then, the proteins were transferred into nitrocellulose membrane in western blotting buffer at 100V during 1h. The transfer was done on ice to avoid possible overheating. The membranes were blocked in western blot blocking solution (5% of non-fat milk) in agitation for 1h before adding the primary antibodies. (Appendix 8.4)

3.3.3. Antibodies

The membranes were stained with antibodies against MANF (18kDa), Grp78/Bip (78kDa), Actin (42kDa), p-eIf2α (38kDa) and t-eIf2α (38kDa) as primary antibodies diluted with western blot blocking solution (Table 3). Membranes were incubated overnight with primary antibodies at 4°C in agitation.

Table 3. Used primary antibodies for western blot detection for each cell line.

Cell line	Primary antibody	Manufacturer	Used dilution
EA.hy956	Rabbit anti-Bip (clone C50B12)	cell signaling technology	1:1000
	Mouse anti-Actin (clone AC-40)	sigma A4700	1:1000
	Mouse anti-MANF (clone 4E12)	Icosagen	1:1000
	Rabbit anti p-eIf2α	cell signaling technology	1:1000
	Rabbit anti t-eIf2α	cell signaling technology	1:1000
MIN6	Rabbit anti-Bip (clone C50B12)	cell signaling technology	1:1000
	Mouse anti-Actin (clone AC-40)	sigma A4700	1:1000
	Goat anti-MANF	R&D Systems	1:500
	Rabbit anti p-eIf2α	cell signaling technology	1:1000
	Rabbit anti t-eIf2α	cell signaling technology	1:1000

The proteins MANF, Grp78 and actin were detected from the same gel run by cutting the membranes into three pieces according to the molecular weight marker; whereas p-eIf2α and t-eIf2α proteins were detected from another gel run.

After the incubation in primary antibodies solution, the membranes were washed with western blot washing buffer three times (15min each time). HRP-labeled secondary antibodies were: rabbit anti-goat (Dako) diluted 1:1500, goat anti-mouse (Dako) diluted 1:3000 and donkey anti-rabbit (GE Healthcare) diluted 1:3000, depending on primary antibodies (dilutions were done with western blot blocking solution). Secondary antibodies were incubating during 1h in agitation at RT. Then, membranes were washed 4 times (15min each time) with western blot washing buffer before adding substrate reagent.

For actin bands, Chemiluminescense ECL Blotting Substrate, 1:1 detection reagent (Pierce, Thermo Scientific) was used. For Bip, MANF, p-eIf2α and t-eIf2α Super Signal ELISA Femto maximum sensitivity substrate 1:1 detection reagent (Pierce, Thermo Scientific) was used.

The chemiluminescence was captured and developed on X-Ray films. First, blots were placed in between two layers of plastic wrap in a film cassette. Then, in darkness, X-Ray films were placed on the top of the blots and were exposed. The time of exposition depends on the samples; the goal is get the signal clearly while the background remains low. In this study, the exposition time varied between 1 and 10 minutes. The exposed films were developed using X-Ray film processor (OptiMax, PROTEC).

Membranes where p-eIf2α was detected were stripped from the attached antibodies and re-stained for the detection of t-eIf2α. Membranes under stripping buffer (Appendix 8.4) were taken to water bath at 55°C during 15min. Then, the container with the membranes and the stripping buffer was transferred onto a shaker and was incubated for another 15min at RT (altogether 30min, not more). Membranes were rinsed four times with PBS and then they were washed 3 times with western blot washing buffer (15min each time). After that, membranes are ready for blocking again and for the addition of new primary antibody.

3.3.4. Image processing and quantitation

The developed films were scanned and the scanned images were processed on Adobe Photoshop CC 2015 program. Specifically, the images were straightened, each line of bands was cut independently and the bands of the molecular weight marker were marked. Furthermore, text was added for indicate at which sample corresponds each band and the images were transformed to greyscale images. No processing was performed on the individual bands.

For quantitation the western blot bands, ImageJ program was used (Image processing and analysis in Java). This program is based in the measurement of the color of each pixel, which has a color value: For 8-bit images, Black=0 and White=255, and for 16-bit images, Black=0 and White=65535. Images were analyzed with black background. The used value is the Integrated Density (IntDen), that the program calculates with the following formula: IntDen = (Mean pixel value) x (number of pixels).

MANF and Grp78/Bip IntDen value bands were normalized with actin IntDen values detected from the same sample and separated on the same membrane. However, p-eIf2α IntDen value bands were normalized with t-eIf2α IntDen value bands detected from the same sample and from the same membrane.

3.4. Quantitative PCR

3.4.1. RNA isolation and determination of RNA concentration

For mRNA samples, EA.hy926 and MIN6 cells were seeded on 6-well plates at cell density of $40x10^4$ and $217x10^4$, respectively. The next day, wells were washed with PBS and added with 2ml of treatment medium. After 24h of incubation with the treatment medium, RNA was extracted from each sample by using TRIZOL reagent (Invitrogen) following manufacturer's instructions. (Appendix 8.5)

RNA concentration from each sample was analyzed by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Furthermore, the purity of the RNA was evaluated with the measurements of 260/280 (absorbance at 260 nm and 280nm) and 260/230 (absorbance at 260nm and 230nm) ratios. For RNA samples, the value of

260/280 should be around 2; if it is lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at 280nm. The value 260/230 should be between 2.0-2.2 in RNA samples and, if it is lower, it may indicate the presence of contaminants which absorb at 230nm, such as EDTA, carbohydrates and phenol (e. g. TRIZOL reagent, a phenolic solution). Only the samples that gave accepted values in these ratios were taken in account.

3.4.2. cDNA synthesis

Before cDNA synthesis, 1µg of RNA from each sample was DNAse treated to degrade possible DNA contaminations (Appendix 8.6). Then, the DNAse treated RNA was used for the reverse transcription reaction using Reverd Aid Premium reverse transcriptase (Thermo Scientific) and oligo(dT) as primers (Oligomer). (Appendix 8.7)

3.4.3. Quantitative PCR method

Quantitative PCR (qPCR) was performed for analyze changes in gene expression of MANF in the cells after 24h of treatment. Furthermore, markers of ER stress were quantified as well; Grp78, CHOP and spliced XBP1 in the study of EA.hy926 cell line, and Hyou1, Grp78, total and spliced XBP1 in the study of MIN6 cell line. Actin was used for normalize the signal from the proteins of interest.

The qPCR for the amplification of each gene was performed in a reaction mix containing SYBR green I master (LightCycler 480, Roche), sense and antisense primers (Table 4), equal amount of cDNA samples and sterile water.

The human primer efficiencies were determined by using dilutions samples of cDNA coming from untreated cells. For EA.hy926 qPCR results analysis, these obtained primer efficiencies were used. However, for MIN6 samples, the used mouse primer efficiencies were calculated before.

Table 4. Primers used for amplify MANF and ER stress markers, depending on cell line.

cell line	protein	strand	primer sequences (5'- 3')
EA.hy926	MANF	sense	GGC AAA GAG AAT CGG TTG TG
		antisense	CAG ATC TTC TCC ACA GGG ATG
	CDNF	sense	AGG GGC CGA CTG TGA AGT
		antisense	TTT TCT TAT AGT GTC CAG CGA AAA
	Grp78	sense	AGC TGT AGC GTA TGG TGC TG
		antisense	AAG GGG ACA TAC ATC AAG CAG T
	CHOP	sense	CAG AGC TGG AAC CTG AGG AG
		antisense	TGG ATC AGT CTG GAA AAG CA
	spliced XBP1	sense	TGC TGA GTC CGC AGC AGG TG
		antisense	GCT GGC AGG CTC TGG GGA AG
	Actin	sense	TCC CTG GAG AAG AGC TAC
		antisense	GTA GTT TCG TGG ATG CCA CA
MIN6	MANF	sense	GAC AGC CAG ATC TGT GAA CTA AAA
		antisense	TTT CAC CCG GAG GTT CTT C
	Hyou1	sense	TAC TCC CGT TCC TTG GCT GAA G
		antisense	GGC TGT GGG AGT GTT GTC ATT G
	Grp78	sense	ATA AAC CCC GAT GAG GCT GT
		antisense	CAT CAA GCA GTA CCA GAT CAC C
	total XBP1	sense	CAC CTT CTT GCC TGC TGG AC
		antisense	GGG AGC CCT CAT ATC CAC AGT
	spliced XBP1	sense	GAG TCC GCA GCA GGT G
		antisense	GTG TCA GAG TCC ATG GGA
	Actin	sense	CCA GTT CGC CAT GGA TGA C
		antisense	GAG CCG TTG TCG ACG ACC

3.5. Statistical analysis

For the analysis of differences between the treatment groups, one-way ANOVA followed by Tukey *post hoc* test was used. Results are expressed as mean ± SEM and considered significant at $p<0.05$ (*$p<0.05$, **$p < 0.01$, ***$p < 0.001$).

4. RESULTS

4.1. ELISA analysis of MANF protein levels after serum or glucose deprivation

4.1.1. ELISA results reveal increase in MANF protein levels under serum deprivation

In respect to MIN6, the amount of MANF protein was statistically significantly higher from those cells treated with 0% of serum, in the medium (120.2%, p=0.002) and in the cell lysate (110.3%, p=0.013), than the cells treated with 10% of serum.

However, there were no differences between the MANF levels from the cells treated with 1% and 10% of serum, neither in the medium (p=0.267) or the cell lysate (p=0.215) (Fig. 3A).

Regarding EA.hy926 samples, the MANF levels were statistically higher from those cells treated with 0% of serum, both intracellular (113.8%, p=0.041) and secreted MANF (124.7%, p=0.018), compared to the cells treated with 10% of serum.

Nevertheless, the quantity of MANF from the medium and cell lysate between the cells treated with 1% and 10% of serum was not significantly different (p=0.261, p=0.111, respectively) (Fig. 3B).

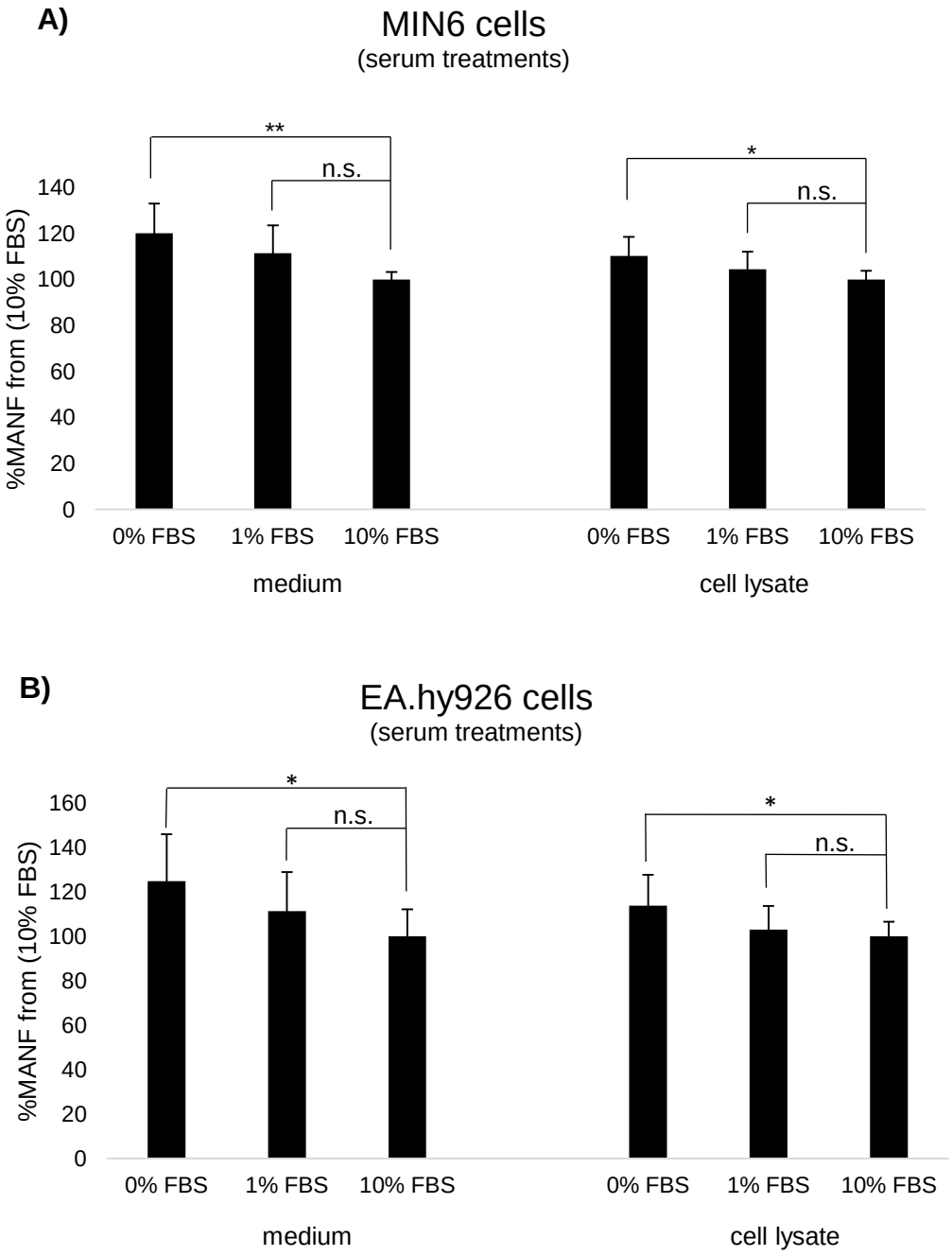

Figure 3. MANF levels from cell lysate and medium of MIN6 (A) and EA.hy926 (B) cells under serum (FBS) starvation were quantified with MANF ELISA. Those samples were collected after 24h since the treatments were applied. MANF is expressed in percentage compared from the amount of MANF in 10% FBS treatment. Results are considered significant at $p<0.05$ (*$p<0.05$, **$p < 0.01$, ***$p < 0.001$) and the error bars correspond to the standard deviation.

4.1.2. ELISA results reveal increase in MANF protein levels under glucose deprivation

In MIN6 cells, as in the medium as in the cell lysate, there were significant differences between the amounts of MANF in different glucose treatments. In medium, the abundance of MANF was significantly higher in all the treatments compared with the concentration of MANF in 25mM of glucose treatment. In detail, the levels of secreted MANF were 124.2%, comparing the cells treated with 10mM to the cells treated 25mM of glucose (p=0.011), 126.1% comparing the cells treated with 5mM to the cells treated with 25mM of glucose (p=0.006), 147.3% comparing the cells treated with 1mM to the cells treated with 25mM of glucose (p=0.000) and 167.3% comparing the cells treated with 0mM to the cells treated with 25mM of glucose (p=0.000). In other words, the MANF secreted quantity increases when a lower amount of glucose is available. Similarly to medium, the amount of intracellular MANF was significantly higher in the cells treated with 0mM (148.7%), 1mM (149.1%) and 5mM (129.3%) of glucose than the intracellular MANF levels in MIN6 cells treated with 25mM of glucose (p=0.000 in all three cases). However, there were no differences in MANF amounts from cell lysate between MIN6 cells treated with 25mM and 10mM of glucose (p=0.054) (Fig. 4A).

On the other hand, in EA.hy926 cell line only significant differences were found in intracellular MANF levels when those cells were treated with glucose starvation. The amount of MANF in the cell lysate was significantly higher in the cells treated with 0mM of glucose (171.5%) than the cells treated with 25mM of glucose (p=0.000) and also significantly higher in the cells treated with 1mM of glucose (143.6%) compared to the cells treated with 25mM of glucose (p=0.001). No significantly differences were found in the cell lysate in the cells treated with 5mM and 10mM of glucose compared to the cells treated with 25mM of glucose (p=0.454 and p=0.985, respectively). But, although there was a trend, any difference was found in the medium from the cells treated with different concentration of glucose (p=0.350) (Fig. 4B).

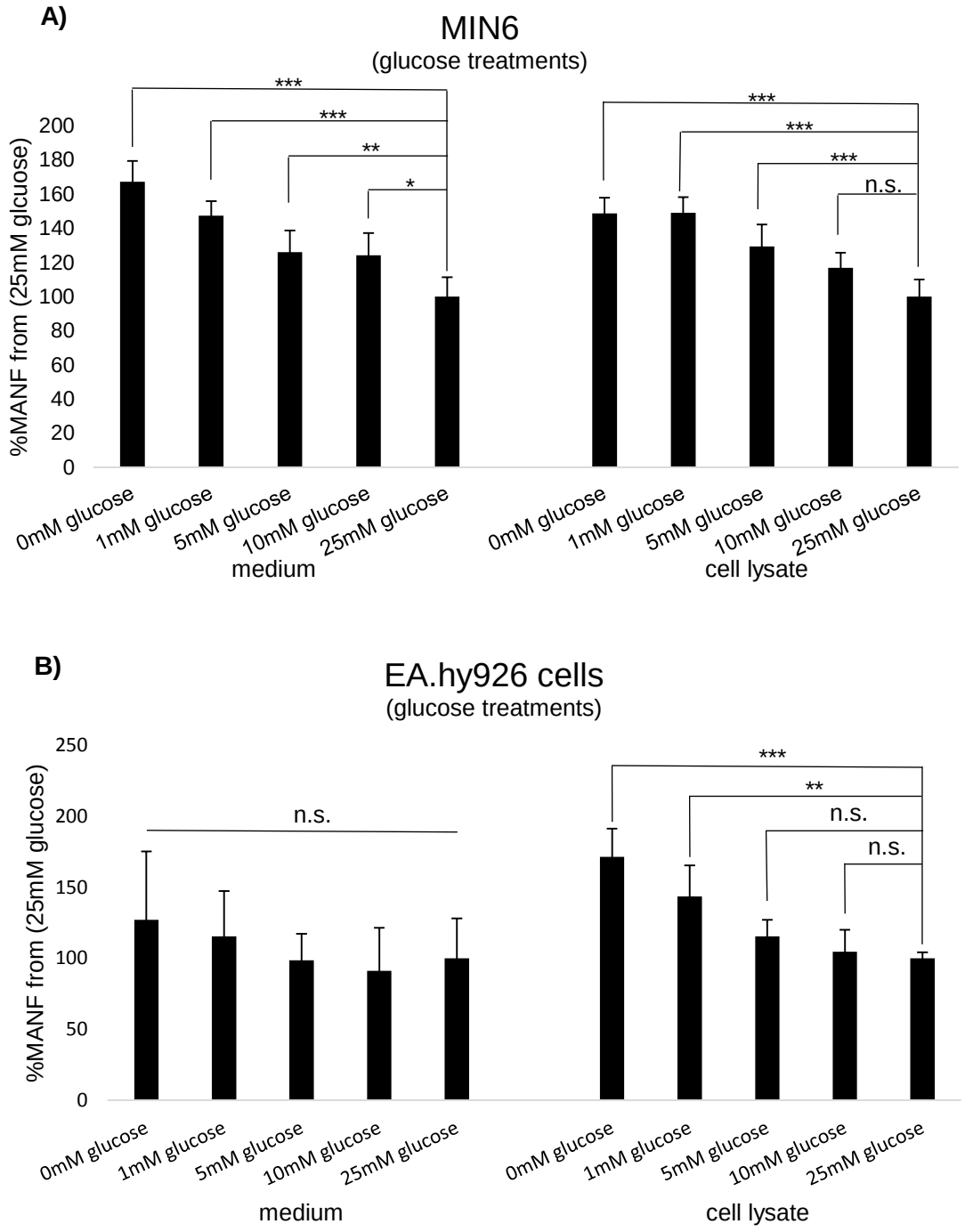

Figure 4. MANF ELISA was done for quantify MANF levels from cell lysate and medium of MIN6 (A) and EA.hy926 (B) cells that were treated with glucose starvation during 24h. The amount of MANF is expressed in percentages and it is compared from the MANF levels of the samples treated with 25mM of glucose. Results are considered significant at $p<0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$) and the error bars represent the standard deviation.

4.2. Western blot analysis of ER stress markers after serum or glucose deprivation

Western blot analyses of ER stress markers were done from the cell lysates of MIN6 and EA.hy926 cells incubated 24h with different glucose and serum concentrations. With this assay, MANF and the ER stress markers Grp78 and phosphorylated eIF2α were detected. To allow comparison between the results, MANF and Grp78 have been standardized with actin and p-eIF2α has been standardized with total eIF2α. The quantifications are relative values of the intensity of each band, compared with the values of the cells treated with 10% of serum or 25mM of glucose, depending on the experiment.

4.2.1. Analysis of ER stress markers in serum starvation

Referring to serum treatments in EA.hy926 cells, MANF intensity was higher in the cells treated with 0% serum (118.8%) and 1% of serum (109.7%), than the cells treated with 10% of serum (p=0.002 and p=0.025, respectively). Grp78 was significantly higher in the cells treated with 0% of serum (111.8%) than the cells treated with 10% of serum (p=0.000), although there were not differences in the intensity of Grp78 signal between the cells treated with 1% of serum (100.45%) and 10% of serum (p=0.965). However, any differences in phosphorylated eIF2α detection were not found between all the treatments (p=0.275). (Fig. 5A)

In that case, the bands detected were pretty clear. But, although is easy to differentiate between the bands, MANF and actin bands were not perfectly delimitated. MANF bands show a clear trend, increasing MANF signal with low concentration of serum in the treatments. Also Grp78 bands follow a subtle trend, which increases with less concentration of serum. (Fig. 5B)

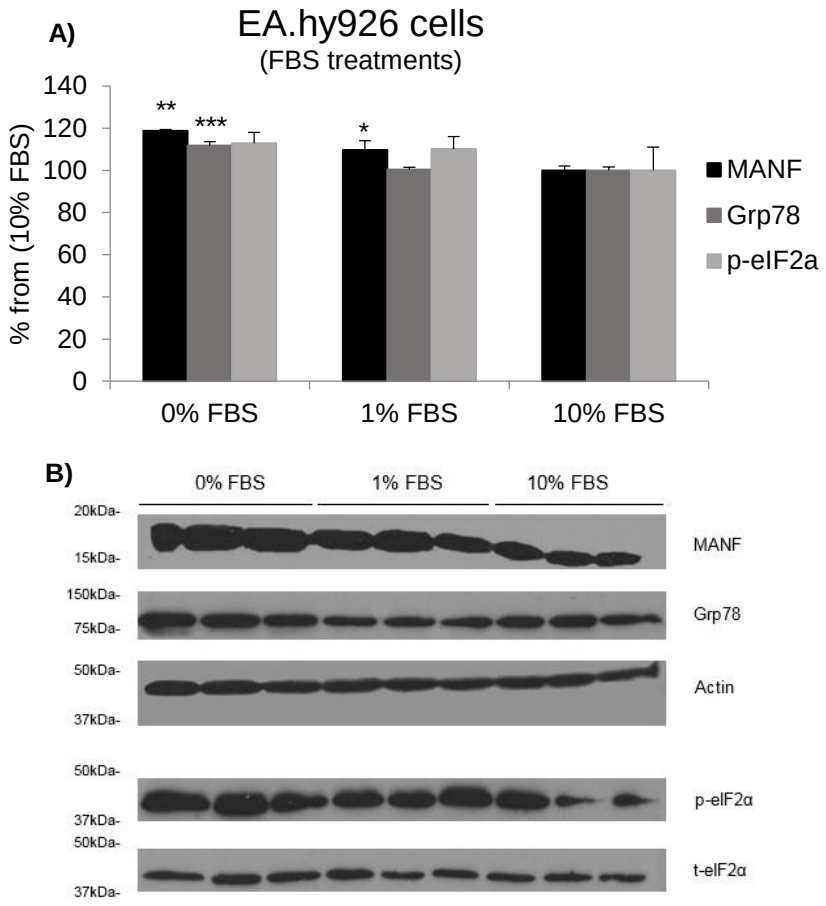

Figure 5. MANF, Grp78 and p-eIF2α proteins were detected, by using western blot assay, of the cell lysate from the EA.hy926 cells treated with serum starvation. The quantitation of the bands was done using ImageJ program and the relative band intensities are expressed as percentage of the band intensities of samples treated with 10% of serum. The results are considered significant at $p<0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$) and the error bars represent the standard deviation (A). The bands were developed on X-Ray films (B).

In case of MIN6 cell line, the results were not clear enough. Referring to the serum treatments, MANF was significantly lower in the cells treated with 0% of serum (89.8%) compared with the cells treated with 10% of serum (p=0.025). However, there was no difference in MANF levels between the cells treated with 1% of serum (101.4%) and 10% of serum (p=0.862). Furthermore, neither of the treatments presented differences between the intensities of Grp78 bands (p=0.083). The intensity of phosphorylated eIF2α was significantly higher in the cells treated with 0% of serum (112.9%) than the cells treated with 10% of serum (p=0.020), but no differences were present between the amount of phosphorylated eIF2α of the cells treated with 1% of serum (106.8%) and the cells treated with 10% of serum (p=0.189). (Fig. 6A)

However, not all the detected bands were pretty clear. Although actin bands seem to be the same size and well defined, MANF bands were not well delimitated. Moreover, three bands were detected with the anti-Grp78 antibody. Besides, t-eIF2α bands were neither well defined and not all the phosphorylated eIF2α bands look like adequately delimitated. (Fig. 6B)

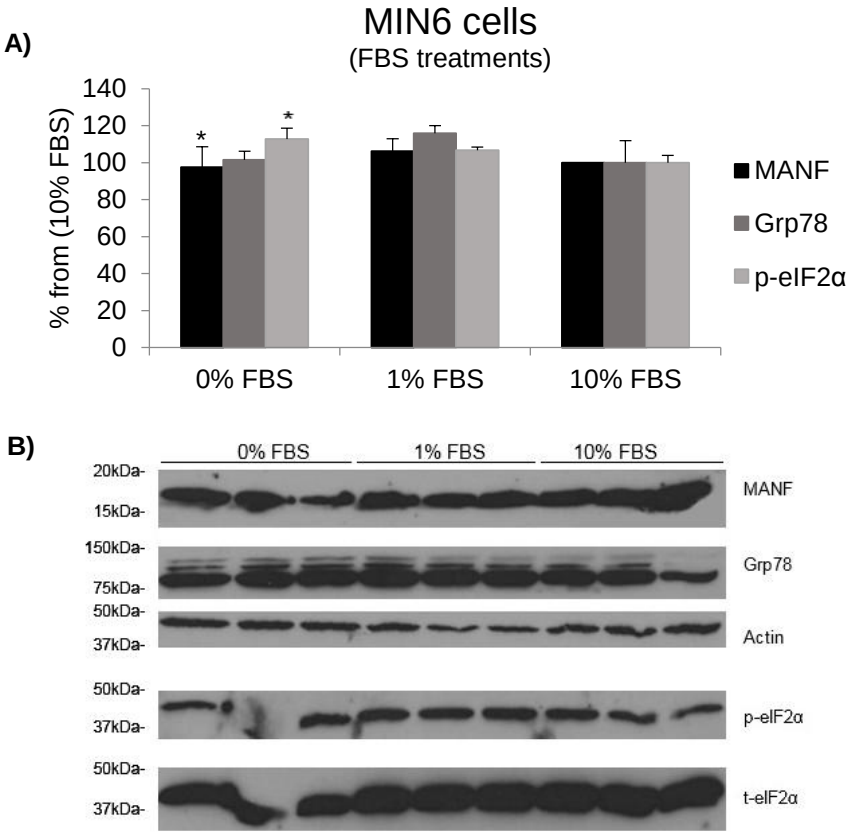

Figure 6. Western blot analysis of the cell lysate samples from MIN6 cells under serum deprivation during 24h. The quantification of the bands was done using the program ImageJ. The band intensities are expressed as percentage of the intensity relative to the intensity obtained from samples treated with 10% of serum. The results are considered significant at $p<0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$) and the error bars represent the standard deviation (A). The bands were developed on X-Ray films (B).

4.2.2. Analysis of ER stress markers in glucose deprivation

Regarding glucose treatments to EA.hy926 cell line, MANF intensity detected was higher in the cells treated with 0mM of glucose (116.8%) and 1mM of glucose (115.5%) than the cells treated with 25mM of glucose (p=0.016 and p=0.022, respectively). But, there was no difference in MANF signal between 5mM of glucose (110.6%) and 25mM

of glucose treatments (p=0.075). Grp78 intensity was higher in the cells treated with 1mM of glucose (119.6%) than the cells treated with 25mM of glucose (p=0.026). However, there were no differences in Grp78 signal comparing 25mM of glucose treatment with 0mM (115.8%) and 5mM (110.3%) of glucose treatments (p=0.052 and p=0.179, respectively). The intensity from phosphorylated eIF2α bands was higher in cells treated with 0mM (116%), 1mM (123.6%) and 5mM (115.1%) of glucose than in cells treated with 25mM of glucose (p=0.027, p=0.007 and p=0.033, respectively). (Fig. 7A)

MANF bands show a clear trend; MANF increases with decreasing glucose concentration. Furthermore, similar trends are present in Grp78 and p-eIF2α bands; they increase with decreasing concentration of glucose. (Fig. 7B)

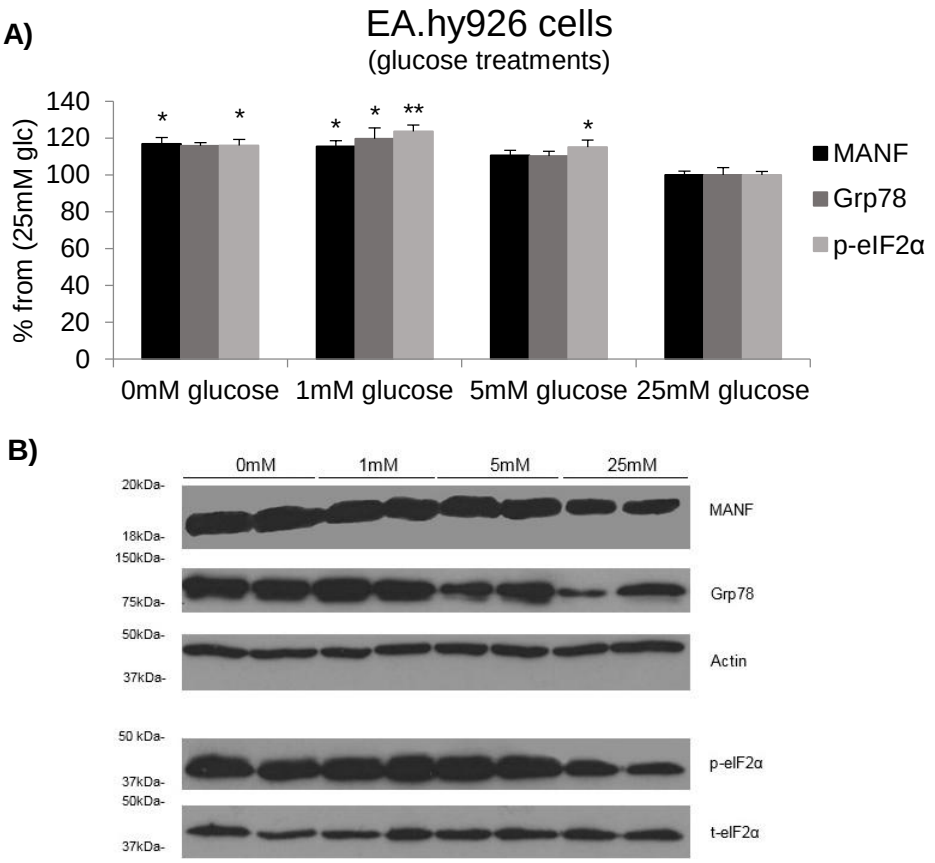

Figure 7. Western blot of cell lysates from the EA.hy926 cells treated 24h with glucose deprivation. With the program ImageJ, the quantification was done and the band intensities are expressed as percentage of the intensity relative to the intensity obtained from samples treated with 25mM of glucose. The results are considered significant at p<0.05 (*p<0.05, **p<0.01, ***p<0.001) and the error bars represent the standard deviation (A). The bands were developed on X-Ray films. (B).

Regarding glucose treatments to MIN6 cells, only the intensity of MANF was significantly higher in the cells treated with 5mM of glucose (114.5%) than the cells treated with 25mM of glucose (p=0.048). The intensity of MANF do not present differences between the cells treated with 0mM (106.4%) and 25mM of glucose (p=0.387), neither between the cells treated with 1mM (114.2%) and 25mM of glucose (p=0.051). Any phosphorylated eIF2α (p=0.165) or Grp78 (p=0.065) intensity differences were not found between all the treatments. (Fig. 8A)

Otherwise, MANF detected bands are well detected, but, again the detection of Grp78 correspond in two bands and not well defined and even some bands don not appear. Furthermore, not all the actin bands were well delimitated. Only a trend with MANF bands was appreciable, which increases when glucose decreases. (Fig. 8B)

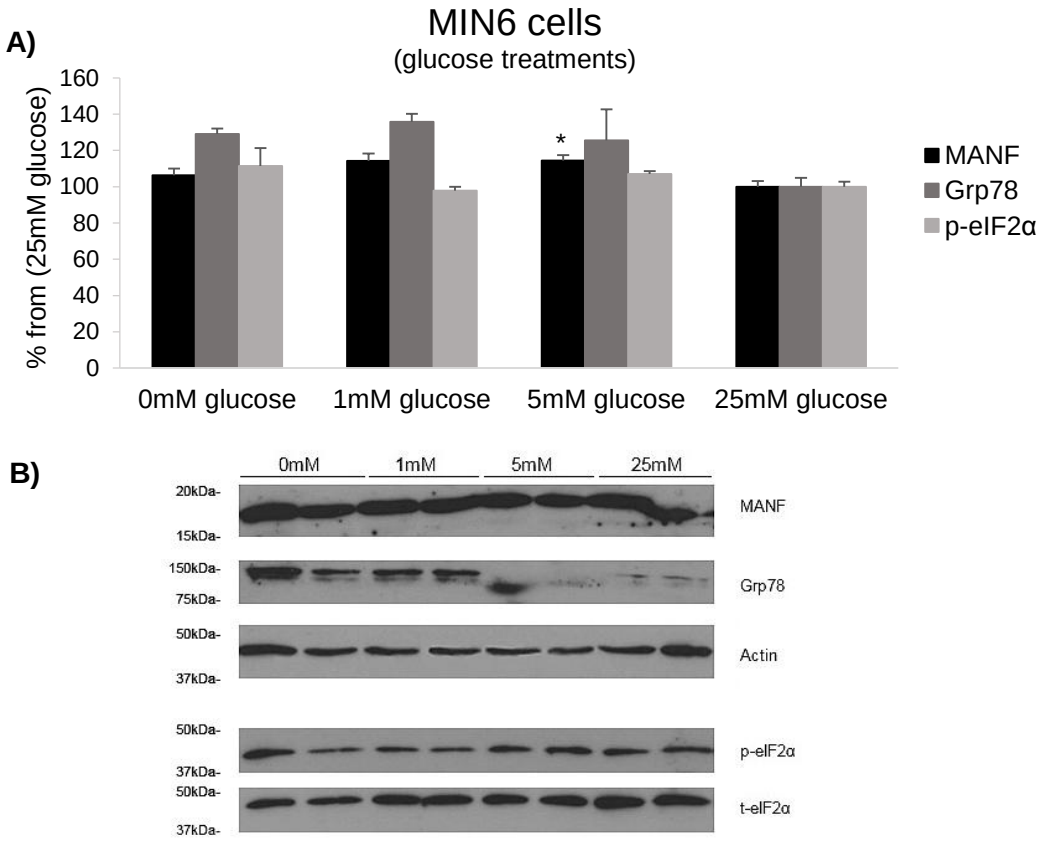

Figure 8. Western blot analysis was done from cell lysate of MIN6 cells treated 24h with glucose treatments. The bands have been quantified with ImageJ program and the band intensities are expressed as percentage of the intensity relative to the intensity obtained from samples treated with 25mM of glucose. The results are considered significant at p<0.05 (*p<0.05, **p<0.01, ***p<0.001) and the error bars represent the standard deviation (A). The bands were developed on X-Ray films (B).

4.3. Quantitative PCR analysis of ER stress gene expression after serum or glucose deprivation

4.3.1. Determination of primer efficiencies

For data analysis of the qPCR results, mouse primer efficiency values used were obtained from Saarma's Laboratory data. However, we determined human primer efficiency values using the cDNA reverse transcribed from the mRNA of untreated EA.hy926 cells.

The cDNA was serially diluted 1:10 for test each primer. Standard curve was constructed by plotting the log of the dilution factor against the crossing point (Cp) value obtained during amplification of the sample with each primer (Fig. 9).

Primer efficiency, E, is calculated from the slope of the standard curve using the following formula:

$$E = 10^{(-1/\text{slope})}$$

Ideally, the amount of PCR product will perfectly double during each cycle of exponential amplification; that is, there will be a 2-fold increase in the number of copies with each cycle, and then the slope would be -3.3. This translates to a reaction efficiency of 2.

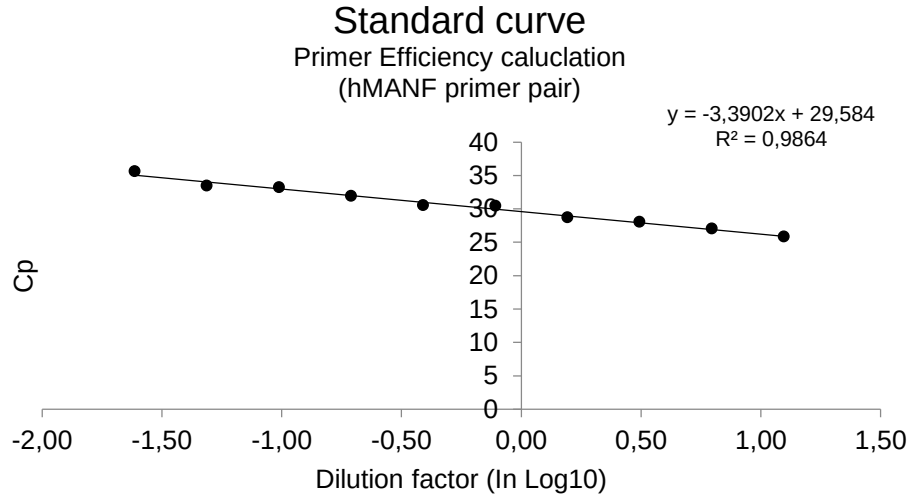

Figure 9. A typical standard curve used for the determination of primer efficiency. In that case, for determine the efficiency of human MANF primer pair.

The calculated primer efficiencies of each human primer pair used are E=1.97 for MANF, E=1.92 for CDNF, E=2.10 for Grp78, E=2.07 for CHOP, E=2.21 for spliced XBP1 and E=1.87 for Actin. (Table 5)

Table 5. Primer efficiency values (E) for each human primer pair used.

protein	E
MANF	1.97
CDNF	1.92
Grp78	2.10
CHOP	2.07
spliced XBP1	2.21
Actin	1.87

4.3.2. Quantitative PCR results from the study samples were inconclusive

The expression of MANF and ER stress markers was analyzed by qPCR from those cells treated with serum or glucose deprivation. The standardization of the values actin was used. The results were not clear enough and it seems that there was not any appreciable trend.

In EA.hy926 cells, besides from MANF gene, its homologous CDNF gene was analyzed as well. Furthermore, also the ER stress markers Grp78, CHOP and spliced XBP1 were analyzed. But, the signals of CDNF and spliced XBP1 were not enough, so the data from these genes is not shown.

In serum treatments, the cells treated with 0% of serum, MANF, Grp78 and CHOP gene expression was higher than the cells treated with 10% of serum, but in all of the three genes the expression was higher in the cells treated with 1% of serum, compared with both 10% and 0% treatments. (Table 6)

In glucose treatments, MANF expression was higher in cells treated with 0mM of glucose than the cells treated with 25mM of glucose. However, the expression of MANF was lower in the cells treated with 5mM of glucose than the cells treated with 25mM of glucose. Expression of Grp78 and CHOP genes was higher in the cells treated with 0mM and 5mM of glucose, than the cells treated with 25mM of glucose. (Table 6)

Table 6. Quantitative PCR results from EA.hy926 cells under treatments of serum and glucose deprivation. The values are the averages of the change fold from each repeat. (n = number of repeats taken into account, SD = standard deviation).

gene of interest	**MANF** (average ± SD)	n	**Grp78** (average ± SD)	n	**CHOP** (average ± SD)	n
Serum concentration						
0 %	1.67 ± 0.26	2	1.26 ± 0.04	2	1.38 ± 1.33	2
1 %	3.93 ± 0.00	1	2.74 ± 0.00	1	2.24 ± 0.00	1
10 %	1.00 ± 0.00	1	1.00 ± 0.54	2	1.00 ± 0.15	2
Glucose concentration						
0mM	5.12 ± 0.00	1	12.21 ± 0.00	1	20.31 ± 0.00	1
5mM	0.77 ± 0.25	2	5.44 ± 5.55	2	9.55 ± 12.29	2
25mM	1.00 ± 0.41	2	1 .00 ± 0.16	2	1.00 ± 0.37	2

Referring to MIN6 cells, the expression of MANF and the ER stress markers Grp78, spliced XBP1 and Hyou1 was analyzed. In serum treatments, the expression of MANF, Grp78, spliced XBP1 and Hyou1 genes was slightly higher in the cells treated with 1% of serum compared to the expression of the cells treated with 10% of serum. However, the expression of MANF, Grp78 and spliced XBP1 genes was higher in the cells treated with 10% of serum than the cells treated with 0% of serum. Expression of Hyou1 gene was higher in the cells treated with 0% of serum than the cells treated with 1% and 10% of serum. (Table 7)

In glucose treatments, MANF and spliced XBP1 expression was lower in the cells treated with 0mM and 5mM of glucose compared to the expression in the cells treated

with 25mM of glucose. However, the expression of Grp78 and Hyou1 genes was higher in the cells treated with 0mM of glucose than the cells treated with 25mM of glucose, although the expression of these two genes was lower in the cells treated with 5mM of glucose than the cells treated with 25mM of glucose. (Table 7)

Table 7. Quantitative PCR results from MIN6 cells under serum and glucose starvation treatments. The values are the averages of the change fold from each repeat. (n = number of repeats taken into account, SD = standard deviation).

Gene of interest	**MANF** (average ± SD)	n	**Grp78** (average ± SD)	n	**spliced XBP1** (average ± SD)	n	**Hyou1** (average ± SD)	n
Serum concentration								
0 %	0.90 ± 0.22	3	0.83 ± 0.16	3	0.68 ± 0.19	3	1.30 ± 0.25	3
1 %	1.18 ± 0.32	3	1.04 ± 0.08	3	1.03 ± 0.21	3	1.14 ± 0.21	3
10 %	1.00 ± 0.37	3	1.00 ± 0.22	3	1.00 ± 0.56	3	1.00 ± 0.16	3
Glucose concentration								
0mM	0.49 ± 0.09	3	1.14 ± 0.08	3	0.13 ± 0.06	3	1.32 ± 0.35	3
5mM	0.39 ± 0.10	3	0.48 ± 0.05	3	0.19 ± 0.06	3	0.64 ± 0.12	3
25mM	1.00 ± 0.12	3	1.00 ± 0.17	3	1.00 ± 0.07	3	1.00 ± 0.32	3

5. DISCUSSION

MANF is a secreted protein (Apostolou et al., 2008; Lindholm and Saarma, 2010; Glembotski et. al. 2012), which intracellularly localizes to the luminal ER (Apostolou et al., 2008). It has been demonstrated that MANF interacts with the chaperone Grp78 (Glembotski et. al. 2012) in the ER. Furthermore, it has been reported that MANF is an ER stress response protein (Apostolou et al., 2008) and its gene is up-regulated when UPR is activated. Moreover, it is known that also MANF secretion is increased in ER stress (Mizobuchi et al., 2007; Apostolou et al., 2008; Glembotski et al., 2012; Oh-Hashi et al., 2012)

ELISA results show that intracellular and secreted MANF is increasing under serum starvation, in both human endothelial EA.hy926 and mouse beta pancreatic MIN6 cell lines. The serum provides to the cells wide variety of nutrients and trophic factors. Therefore, the obtained results reveal the evidence that the secretion and the production of MANF is linked to the availability of nutrients for the cells, producing then, more MANF when there is a lack of nutrients.

ELISA results on MIN6 cells under glucose starvation are especially interesting, since these pancreatic cells are insulin producers. It is clear that the MANF protein levels increase when glucose decreases. Furthermore, both intracellular and secreted MANF levels produced in MIN6 cells under glucose starvation were higher than the MANF produced levels in MIN6 cells under serum starvation. Moreover, it is known that serum starvation may affect cell viability and if prolonged induce cell death. In that case, if there was a lower concentration of cells after they were treated under serum deprivation, it would be normal if the protein levels were less. However, the viability of the cells was not affected by visual inspection. Therefore, MIN6 cells responses are more intense when there is a lack of glucose than when there is a lack of serum. Our study suggests, then, that MANF has an important role in the glucose metabolism in the beta cells, as an intracellular protein and as a secreted protein.

However, MANF ELISA results show that the EA.hy926 cells respond differently under glucose deprivation treatments compared to MIN6 cells. In both MIN6 and EA.hy926 cells intracellular MANF levels are increased by glucose deprivation, even more in case

of EA.hy926 cells. Differently from MIN6 cells, glucose deprivation does not increase MANF secretion from EA.hy926 cells. These results suggest that in this endothelial cell line, MANF secretion is not regulated by glucose levels in the culture media.

It is known that MANF secretion is induced by chemically-induced ER stress in cell culture. Serum and glucose deprivation are also known to induce ER stress. Thus we wanted to study whether increased MANF secretion and expression of ER stress markers are related in glucose or serum starvation. Unfortunately the analysis of ER stress markers by western blotting and qPCR did not give conclusive results. Referring to the western blot results, only the EA.hy926 cells under serum and glucose starvation show clear results. Although no differences were found in phosphorylated eIF2a levels, it is clear that intracellular MANF levels increase when the availability of glucose or serum decreases, in accordance with the MANF ELISA results. Moreover, there are evidences that also Grp78 levels increases when glucose or serum decreases. Therefore, these results suggest that when there is a lack of nutrients Grp78 gets activated. Moreover, it can be said that Grp78 is up-regulated in glucose starvation and intracellular MANF is regulated by glucose as in the case of Grp78, and, probably, the cells are under ER stress when are under glucose and serum deprivation.

The western blot results from MIN6 cells are not clear enough for discuss about them. In the detected bands is clear that the used antibodies do not work perfectly: The bands are not well delimitated and even in the Grp78 detection more than one band appeared. Furthermore, the problem might be technical as well; since the western blot bands were diffused and with a high background, could be because too much protein was loaded, due to an error during the total protein concentration measurement or during the western blot sample preparation. Therefore, it is hard to conclude something from these results.

The analyses of the expression of MANF and ER stress markers genes by quantitative PCR were absolutely inconclusive, since any trend is appreciable. The PCR is a multi-step method (isolation of RNA, reverse transcription reaction and the quantitative PCR run) and it is extremely sensitive. Any mistake during any of these steps can interfere in the results. Furthermore, the amounts used during this technique are really small, so any little mistake can be reflected with a big error in the results. The problem might be the mRNA degradation before the qPCR analysis; RNA is very sensitive to degradation

and it could affect differently in each gene. Hence, if RNA degradation occurs, the results are not reliable. Additionally, it is a method that requires a lot of repetitions for get good, stable and conclusive results.

Therefore, both western blot and qPCR experiments need more optimization and more replications for reliable results.

Future prospects for this project could be to analyze the parameters that reflects energy metabolism directly, such ATP levels. Hence, to determine if intracellular and secreted MANF levels are linked to the cellular energy metabolism.

6. CONCLUSIONS

I. Intracellular MANF levels are up-regulated under glucose deprivation in beta pancreatic MIN6 and human endothelial EA.hy926 cell lines.

II. Secreted MANF levels increase when glucose decrease only in the beta insulin producers cells (MIN6) in contrast to EA cells, suggesting that responses for glucose deprivation are different in the two cell lines.

III. Intracellular MANF is up-regulated by glucose as well as Grp78 indicating that similarly to Grp78, MANF is a glucose regulated protein.

IV. EA.hy926 cells present increased levels of Grp78 under glucose and serum deprivation, indicating ER stress in the cell.

V. Both intracellular and secreted MANF levels increase when serum available decreases, in both MIN6 and EA.hy926 cell lines.

7. REFERENCES

Airavaara, M., Shen, H., Kuo, C. C., Peränen, J., Saarma, M., Hoffer, B., & Wang, Y. (2009). Mesencephalic astrocyte-derived neurotrophic factor reduces ischemic brain injury and promotes behavioral recovery in rats. *Journal of Comparative Neurology*, *515*(1), 116-124. doi: 10.1002/cne.22039.

Airavaara, M., Harvey, B. K., Voutilainen, M. H., Shen, H., Chou, J., Lindholm, P., Lindahl, M., Tuominen, R. K., Saarma, M., Hoffer, B., & Wang, Y. (2012). CDNF protects the nigrostriatal dopamine system and promotes recovery after MPTP treatment in mice. *Cell transplantation*, *21*(6), 1213-1223. doi: 10.3727/096368911X600948

Apostolou, A., Shen, Y., Liang, Y., Luo, J., & Fang, S. (2008). Armet, a UPR-up-regulated protein, inhibits cell proliferation and ER stress-induced cell death. *Experimental cell research*, *314*(13), 2454-2467. doi: 10.1016/j.yexcr.2008.05.001

Bäck, S., Peränen, J., Galli, E., Pulkkila, P., Lonka-Nevalaita, L., Tamminen, T., Voutilainen, M. H., Raasmaja, A., Saarma, M., Männistö, P. T., & Tuominen, R. K. (2013). Gene therapy with AAV2-CDNF provides functional benefits in a rat model of Parkinson's disease. *Brain and behavior*, *3*(2), 75-88. doi: 10.1002/brb3.117

Chen, Y. C., Sundvik, M., Rozov, S., Priyadarshini, M., & Panula, P. (2012). MANF regulates dopaminergic neuron development in larval zebrafish. *Developmental biology*, 370(2), 237-249. doi: 10.1016/j.ydbio.2012.07.030

Chen, L., Feng, L., Wang, X., Du, J., Chen, Y., Yang, W., Zhou, C., Cheng, L., Shen, Y., Fang, S., Li, J., & Shen, Y. (2015). Mesencephalic astrocyte-derived neurotrophic factor is involved in inflammation by negatively regulating the NF-κB pathway. *Scientific reports*, 5. doi: 10.1038/srep08133

Dauer, W., & Przedborski, S. (2003). Parkinson's disease: mechanisms and models. *Neuron*, *39*(6), 889-909.

Galli, E., Härkönen, T., Sainio, M. T., Ustav, M., Toots, U., Urtti, A., Yliperttula, M., Lindahl, M., Knip, M., Saarma, M., & Lindholm, P. (2016). Increased circulating concentrations of mesencephalic astrocyte-derived neurotrophic factor in children with type 1 diabetes. *Scientific Reports*, *6*. doi: 10.1038/srep29058.

Girardot, F., Monnier, V., & Tricoire, H. (2004). Genome wide analysis of common and specific stress responses in adult Drosophila melanogaster. *BMC genomics*, *5*(1), 74.

Glembotski, C. C., Thuerauf, D. J., Huang, C., Vekich, J. A., Gottlieb, R. A., & Doroudgar, S. (2012). Mesencephalic astrocyte-derived neurotrophic factor protects the heart from ischemic damage and is selectively secreted upon sarco/endoplasmic reticulum calcium depletion. *Journal of Biological Chemistry*, *287*(31), 25893-25904. doi: 10.1074/jbc.M112.356345

Hellman, M., Arumäe, U., Yu, L. Y., Lindholm, P., Peränen, J., Saarma, M., & Permi, P. (2011). Mesencephalic astrocyte-derived neurotrophic factor (MANF) has a unique mechanism to rescue apoptotic neurons. *Journal of Biological Chemistry*, *286*(4), 2675-2680. doi: 10.1074/jbc.M110.146738

Henderson, M. J., Richie, C. T., Airavaara, M., Wang, Y., & Harvey, B. K. (2013). Mesencephalic astrocyte-derived neurotrophic factor (MANF) secretion and cell surface binding are modulated by KDEL receptors. *Journal of Biological Chemistry*, *288*(6), 4209-4225. doi: 10.1074/jbc.M112.400648

Holtz, W. A., & O'Malley, K. L. (2003). Parkinsonian mimetics induce aspects of unfolded protein response in death of dopaminergic neurons. *Journal of Biological Chemistry*, *278*(21), 19367-19377.

Lindahl, M., Danilova, T., Palm, E., Lindholm, P., Võikar, V., Hakonen, E., Ustinov, J., Andressoo, J. O., Harvey, B. K., Otonkoski, T., Rossi, J., & Saarma, M. (2014). MANF

is indispensable for the proliferation and survival of pancreatic β cells. *Cell reports*, *7*(2), 366-375. doi: 10.1016/j.celrep.2014.03.023

Lindahl, M., Saarma, M., & Lindholm, P. (2016). Unconventional neurotrophic factors CDNF and MANF: Structure, physiological functions and therapeutic potential. *Neurobiology of Disease*. Advance online publication. doi: 10.1016/j.nbd.2016.07.009

Lindholm, P., Voutilainen, M. H., Laurén, J., Peränen, J., Leppänen, V. M., Andressoo, J. O., Lindahl, M., Janhunen, S., Kalkkinen, N., Timmusk, T., Tuominen, R. K., & Saarma, M. (2007). Novel neurotrophic factor CDNF protects and rescues midbrain dopamine neurons in vivo. *Nature*, *448*(7149), 73-77.

Lindholm, P., Peränen, J., Andressoo, J. O., Kalkkinen, N., Kokaia, Z., Lindvall, O., Timmusk, T., & Saarma, M. (2008). MANF is widely expressed in mammalian tissues and differently regulated after ischemic and epileptic insults in rodent brain. *Molecular and Cellular Neuroscience*, 39(3), 356-371. doi: 10.1016/j.mcn.2008.07.016

Lindholm, P., & Saarma, M. (2010). Novel CDNF/MANF family of neurotrophic factors. *Developmental neurobiology*, *70*(5), 360-371. doi: 10.1002/dneu.20760

Lindström, R., Lindholm, P., Kallijärvi, J., Yu, L. Y., Piepponen, T. P., Arumäe, U., Saarma, M., & Heino, T. I. (2013). Characterization of the structural and functional determinants of MANF/CDNF in Drosophila in vivo model. *PloS one*, *8*(9), e73928. doi: 10.1371/journal.pone.0073928

Mizobuchi, N., Hoseki, J., Kubota, H., Toyokuni, S., Nozaki, J. I., Naitoh, M., Koizumi, A., & Nagata, K. (2007). ARMET is a soluble ER protein induced by the unfolded protein response via ERSE-II element. *Cell structure and function*, *32*(1), 41-50.

Neves, J., Zhu, J., Sousa-Victor, P., Konjikusic, M., Riley, R., Chew, S., Qi, Y., Jasper, H., & Lamba, D. A. (2016). Immune modulation by MANF promotes tissue repair and regenerative success in the retina. *Science*, *353*(6294), aaf3646. doi: 10.1126/science.aaf3646

Oh-hashi, K., Tanaka, K., Koga, H., Hirata, Y., & Kiuchi, K. (2012). Intracellular trafficking and secretion of mouse mesencephalic astrocyte-derived neurotrophic factor. *Molecular and cellular biochemistry*, *363*(1-2), 35-41. doi: 10.1007/s11010-011-1155-0

Palgi, M., Lindström, R., Peränen, J., Piepponen, T. P., Saarma, M., & Heino, T. I. (2009). Evidence that DmMANF is an invertebrate neurotrophic factor supporting dopaminergic neurons. *Proceedings of the National Academy of Sciences*, *106*(7), 2429-2434. doi: 10.1073/pnas.0810996106

Palgi, M., Greco, D., Lindström, R., Auvinen, P., & Heino, T. I. (2012). Gene expression analysis of Drosophilaa Manf mutants reveals perturbations in membrane traffic and major metabolic changes. *BMC genomics*, 13(1), 1. doi: 10.1186/1471-2164-13-134

Parkash, V., Lindholm, P., Peränen, J., Kalkkinen, N., Oksanen, E., Saarma, M., Leppänen, V. M., & Goldman, A. (2009). The structure of the conserved neurotrophic factors MANF and CDNF explains why they are bifunctional. *Protein Engineering Design and Selection*, *22*(4), 233-241. doi: 10.1093/protein/gzn080

Petrova, P. S., Raibekas, A., Pevsner, J., Vigo, N., Anafi, M., Moore, M. K., Peaire, A.E., Shridhar, V., Smith, D. I., Kelly, J., Durocher, Y., & Commissiong J.W. (2003). MANF: a new mesencephalic, astrocyte-derived neurotrophic factor with selectivity for dopaminergic neurons. *Journal of Molecular Neuroscience*, *20*(2), 173-187.

Raykhel, I., Alanen, H., Salo, K., Jurvansuu, J., Nguyen, V. D., Latva-Ranta, M., & Ruddock, L. (2007). A molecular specificity code for the three mammalian KDEL receptors. *The Journal of cell biology*, *179*(6), 1193-1204.

Ron, D., & Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. *Nature reviews Molecular cell biology*, *8*(7), 519-529.

Shridhar, V., Rivard, S., Shridhar, R., Mullins, C., Bostick, L., Sakr, W., Grignon, D., Miller, O. J., & Smith, D. I. (1996). A gene from human chromosomal band 3p21. 1 encodes a highly conserved arginine-rich protein and is mutated in renal cell carcinomas. *Oncogene*, *12*(9), 1931-1939.

Szegezdi, E., Logue, S. E., Gorman, A. M., & Samali, A. (2006). Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO reports*, *7*(9), 880-885.

Tadimalla, A., Belmont, P. J., Thuerauf, D. J., Glassy, M. S., Martindale, J. J., Gude, N., Sussman, M. A., & Glembotski, C. C. (2008). Mesencephalic astrocyte-derived neurotrophic factor is an ischemia-inducible secreted endoplasmic reticulum stress response protein in the heart. *Circulation research*, *103*(11), 1249-1258. doi: 10.1161/CIRCRESAHA.108.180679

Voutilainen, M. H., Bäck, S., Peränen, J., Lindholm, P., Raasmaja, A., Männistö, P. T., Saarma, M., & Tuominen, R. K. (2011). Chronic infusion of CDNF prevents 6-OHDA-induced deficits in a rat model of Parkinson's disease. *Experimental neurology*, *228*(1), 99-108. doi: 10.1016/j.expneurol.2010.12.013

Voutilainen, M. H., Bäck, S., Pörsti, E., Toppinen, L., Lindgren, L., Lindholm, P., ... & Tuominen, R. K. (2009). Mesencephalic astrocyte-derived neurotrophic factor is neurorestorative in rat model of Parkinson's disease. *The Journal of Neuroscience*, 29(30), 965. doi: 10.1523/JNEUROSCI.0833-09.2009

8. APPENDIX: Supplementary materials and methods information

8.1. Lysis buffer

Stock solution

- 173mM NaCl
- 20mM Tris (pH 8.0)
- 2.5mM EDTA
- 1% NP40 (=IGEPAL CA-630)
- 10% glycerol
- MiliQ water

Added Upon use

- 0.5mM sodium orthovanadate
- Protease inhibitor cocktail tablet (1/10ml)

8.2. Carbonate coating buffer

0.05M carbonate coating buffer

Add of 0.035M of sodium bicarbonate and 0.015M of sodium carbonate in purified water. Adjust the pH to 9.6 using 1N HCl / 1N NaOH.

8.3. SDS-PAGE

The used components for the separating gel were (12% PAA):

	For a 8ml of gel
• 30% PAA	3.2ml
• purified water	2.8ml
• 10% APS	50ul
• TEMED	5ul
• separating buffer gel*	2ml

The used components for the stacking gel were:

	For a 6ml of gel
• 30% PAA	0.6ml
• purified water	3.9ml
• 10% APS	50ul
• TEMED	5ul
• stacking buffer gel*	1.5ml

*Components of separating buffer gel and stacking buffer gel:

	For 200ml of buffer
Separating buffer gel:	
• 3M Tris-HCl (pH 8.8)	100ml
• 20% SDS	4ml
• purified water	96ml
Stacking buffer gel:	
• 1M Tris-HCl (pH 6.8)	100ml
• 20% SDS	4ml
• purified water	96ml

8.4. Western blot buffers

- Laemmli Buffer (LB): 60mM Tris-Cl at pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue.

- Running buffer: 25mM Tris, 190mM glycine; 0.1% SDS at pH 8.3 in purified water.

- Western blotting buffer: 25mM Tris, 190mM Glycine and 20% Methanol in purified water.

- Western blot washing buffer (TBST): Addition of 10x tris-buffered saline (TBS) and 0.1% of tween 20 in purified water.

- Western blot blocking solution: 5% milk in TBST.

- Stripping buffer: To prepare 250 ml, mix 1.74ml of β-mercaptoethanol, 25ml of 20% SDS and 15.63 ml of 3M tris-HCl (pH 6.7). Bring to 250ml with purified water.

8.5. Protocol of RNA isolation by TriReagent (Invitrogen)

1. Take culture plate under the hood (TriReagent contains phenol)
2. Collect the medium into a container, subsequently add 1ml of TriReagent per well
    a. Pipet several times up and down
    b. Collect into 2ml tubes (can be sealed properly)
    c. Incubate at RT for 5min
3. Add 0.2ml of chloroform per tube
    a. Shake vigorously by hand for 15 sec.
    b. Incubate at RT for 2-3min
4. Centrifuge samples at 10.5 krpm for 15min at 4°C
5. Transfer the upper, colorless phase into new tubes
    a. Be careful not to pipet the middle white phase containing DNA
6. Add 0.5ml of isopropyl alcohol (isopropanol, 2-propanol)
    a. Mix by pipetting
    b. Incubate at RT for 10min
7. Centrifuge samples at 10.5 krpm for 10min at 4°C
    a. Carefully remove the supernatant, discard
8. Wash the RNA with 1ml of 75% ethanol
    a. Mix by vortexing (so that the pellet is detached from the tube wall)
9. Centrifuge samples at 7 krpm for 5min at 4°C
    a. Carefully remove ethanol, discard
    b. Repeat the washing step
10. Freeze the samples under ethanol

8.6. DNase treatment

RNA	1 ug
RQ1 10x reaction buffer (Promega)	1µl
RQ DNase (Promega)	1µl/ 1µg RNA
Sterile water	up to 10µl

1. Incubate at 37°C for 30min
2. Add 1µl of RQ1 DNase stop solution (Promega)
3. Incubate at 65°C for 10min
4. Proceed with reverse transcription reaction

8.7. Reverse Transcription reaction (cDNA synthesis)

Oligo dT 18-mer 0.5 ug/ul (Oligomer)	1 µg
1µg DNAse treated RNA	11µl
dNTP mix - 10mM each (Thermo Scientific)	1µl
Sterile water	1.5µl

1. Incubate at 65°C for 5min. Spin to collect the contents. Put the tubes on ice.
2. Add:

5x RT buffer (Thermo Scientific)	4 µg
RNase inhibitor (Thermo Scientific)	0.5µl
Revert aid premium RT (Thermo Scientific)	1µl

3. Mix gently and spin down
4. Incubate at 55°C for 40min. Heath inactivate at 85°C for 5min
5. Spin down and store the samples at -20°C