Isabel Alexandra Pinto Carrilho do Rosário
Licenciatura em Bioquímica

New mechanisms that regulate the expression of genes implicated in the process of ketogenesis

Dissertação para obtenção do Grau de Mestre em Biotecnologia

Orientador: Prof. Dr. Pedro F. Marrero González,
Prof. Titular, Facultat de Farmàcia, Universitat de Barcelona
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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Setembro, 2012

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Ninguém sabe que coisa quer.
Ninguém conhece que alma tem,
Nem o que é mal nem o que o bem.
(Que ânsia distante perto chora?)
Tudo é incerto e derradeiro.
Tudo é disperso, nada é inteiro.
Ó Portugal, hoje és nevoeiro…

A Mensagem
III. Os Tempos
Quinto
Nevoeiro

Fernando Pessoa

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Abstract

Fatty acid oxidation and ketogenesis play an important role in metabolic adaptation of the liver. Ketogenesis is a crucial process, especially in fasting. It is the last step of lipid metabolism and provides energy in the form of ketone bodies for the extra hepatic tissues, saving glucose for vital tissues, as the brain. In this project there were studied mechanisms by which genes involved in ketogenesis are regulated.

In the first part, the hypothesis was to test whether the FoxO family members, FoxO3a and FoxO1, were responsible for HMGCS2 induction by p53 activation.

HMGCS2 is a key regulator of the ketone body production, and it was published by the laboratory where this project was developed that it is a FoxO3a/FoxO1 target. P53 was described to stimulate FAO in conditions of food restriction, and that this could be a part of its effect as a suppressor gene, related to the *Warburg effect*.

During the development of this project, it was seen that FoxO3a and FoxO1 were not related to HMGCS2 induction, when there is p53 activation, induced by DNA damage with doxorubicin treatment.

In the second part, Fsp27, from the CIDE family, was studied. Fsp27 is a lipid droplet associated protein. Its expression is regulated by HMGCS2 activity and this gene is expressed during early fasting. Fsp27 is able to down-regulate FAO when it's overexpressed. The objective was to seek a regulatory role of Fsp27 in mouse liver.

During the development of this project, it was confirmed that this protein had a nuclear localization and that its expression would affect PPAR signaling pathway. In AML12 cells, Fsp27 inhibited the activation of the HMGCS2 promoter by PPARα. The hypothesis was that Fsp27 would be sequestering PPARα's endogenous ligands, since this inhibition would disappear when the cells were treated with the pharmacological agonist WY14643.

KEYWORDS: Ketogenesis; Metabolic adaptation; Warburg effect; DNA damage

Resumo

A oxidação dos ácidos gordos e a cetogénese têm um papel importante na adaptação metabólica do fígado. A cetogénese é um processo crucial, especialmente em jejum. É o último passo do metabolismo dos lípidos, fornecendo energia na forma de ácidos gordos para os tecidos extra hepáticos, guardando a glucose para os tecidos vitais, como o cérebro. Neste projecto foram estudados mecanismos pelos quais genes envolvidos na cetogénese são regulados.

Na primeira parte, a hipótese passa por testar se os membros da família FoxO, FoxO3a e FoxO1, são responsáveis pela indução de HMGCS2, por activação de p53.

O gene HMGCS2 regula a produção de corpos cetónicos, sendo publicado pelo laboratório onde este projecto foi desenvolvido que é alvo de FoxO3a/FoxO1. Foi descrito que o p53 estimula a FAO em condições de restrição de comida e que isto poderia fazer parte do seu efeito como gene supressor, relacionado com o *efeito de Warburg.*

Durante o desenvolvimento deste projecto, verificou-se que FoxO3a e FoxO1 não estão relacionados com a indução de HMGCS2, quando há activação de p53, induzida por danos no DNA, induzidos por doxorubicina.

Na segunda parte, a proteína Fsp27, da família CIDE, foi estudada. Fsp27 é uma proteína associada a gotas lipídicas. A sua expressão é regulada pela actividade de HMGCS2 e este gene é expresso durante o jejum. Sobre expresso, Fsp27 regula negativamente a FAO. O objectivo seria procurar um papel regulador de Fsp27 no fígado de rato.

Durante este projecto, foi confirmada a localização nuclear da proteína e descoberto que a sua expressão afecta a sinalização por PPAR. Em células AML12, Fsp27 inibe a activação do promotor de HMGCS2 por PPARα. A hipótese seria que Fsp27 estaria a sequestrar ligandos endógenos de PPARα, visto que esta inibição desaparece quando as células são tratadas com o agonista farmacológico WY14643.

Palavras-chave: Cetogénese; adaptação metabólica; Efeito de Warburg ; lesão de DNA.

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List of Abbreviations

ABCB1 –ATP-Binding Cassette sub-family B member 1

ACC- Acetyl CoA Carboxylase

ADP – Adenosine Diphosphate

AGC family- Protein Kinase A, Protein Kinase G and Protein Kinase C

AMP- Adenosine Monophosphate

AMPK- AMP-activated Protein Kinase

Atg7-Autophagy Related Protein 7

ATM- Ataxia- Telangiectasia Mutated

ATP- Adenosine Triphosphate

Bad- Bcl-2-Associated Death Promoter

BAT- Brown Adipose Tissue

BSA- Bovine Serum Albumin

C/EBPα- CCAAT- Enhancer Binding Proteins

cAMP – cyclic Adenosine Monophosphate

CBP- Creb Binding Protein

CIDE- Cell Death Inducing DFF45-like Effector

CIDEA- Cell Death Inducing DFF45-like Effector A

CIDEB- Cell Death Inducing DFF45-like Effector B

CIDEC- Cell Death Inducing DFF45-like Effector C

CK2- Caseine Kinase II

CPTI- Carnitine Acyltransferase I/ Carnitine palmitoyl Transferase I

CPTII- Carnitine Acyltransferase II/ Carnitine Palmitoyl Transferase II

DNA – Deoxyribonucleic Acid

dNTPs- Deoxynucleotide Triphosphate

Doxo- Doxorubicin

DRAM- Damage-Regulated Autophagy Modulator

DYRK1- Dual specificity tyrosine-phosphorylation-regulated kinase 1

ERK-1/2 –Extracellular signal-Regulated Kinases 1/2

$FADH_2$ – Flavine Adenine Dinucleotide

FAO- Fatty Acid Oxidation

FAS – Fatty Acid Synthase

FFA- Free Fatty Acids

FoxO – Forkhead box 'Other' proteins

FoxO1- Forkhead box 'Other' 1

FoxO3a - Forkhead box 'Other' 3a

Fsp27- Fat Specific Protein 27

GADD45a – Growth Arrest and DNA Damage- inducible protein A

GLUT1-Glucose Transporter 1

GLUT4- Glucose Transporter 4

HAT- Histone Acetyl Transferase

HIPK2- Homeodomain-Interacting Protein Kinase 2

HMGCL- 3- Hydroxy-3-Methylglutaryl- CoA Lyase

HMG-CoA reductase – 3-Hydroxy-3-Methyl-Glutaryl CoA Reductase

HMGCS2- 3- Hydroxy-3-MethylGlutaryl- CoA synthase

HSL- Hormone-Sensitive Lipase

IGF-1 – Insulin-Like Growth Factor I

IGF-1R- Insulin-Like Growth Factor Receptor

IKK- IκB Kinase

IRS– Insulin Response Sequences

JNK- c-Jun N-terminal Kinase

LCAS- Long Chain Acyl CoA Synthase

LKB1- Liver Kinase B1

LXR- Liver X Receptor

MAPK- Mitogen-Activated Protein Kinase

MDM2– Mouse Double Minute

MDR- Multidrug Resistance

MDR1- Multidrug Resistance Gene

MST1- Macrophage Stimulating 1 (hepatocyte growth factor like)

mTOR- mammamial Target of Rapamycin

NAD+ - Nicotinamide Adenine Dinucleotide, oxidizing agent

NADH- Nicotinamide Adenine Dinucleotide, reducing agent

NADPH- Nicotinamide Adenine Dinucleotide Phosphate, reduced form

NFkB- Nuclear Factor kappa-light-chain-enhancer of activated B cells

NQO1-Reduced Nicotinamide Adenine Dinucleotidequinone Oxidoreductase 1

NQO2- Reduced Nicotinamide Adenine Dinucleotidequinone Oxidoreductase 2

OCTN2- Organic Cation/ Carnitine Transporter

PBS- Phosphate Buffered-Saline

PCAF- P300/CBP- Associated Factor

PCR – Polymerase Chain Reaction

PEPCK- Phosphoenolpyruvate Carboxykinase

PI-3K– Phosphoinositide-3 Kinase

PKB / Akt – Protein Kinase B

PMSF- Phenylmethylsulfonyl

PPAR- Peroxisome Proliferator Activated Receptor

PPARα- Peroxisome Proliferator Activated Receptor α

PPARβ- Peroxisome Proliferator Activated Receptor β

PPARγ- Peroxisome Proliferator Activated Receptor γ

PPRE-Peroxisome Proliferator Response Elements

PTEN- Phosphatase and Tensin Homolog

PVDF-Polyvinylidene Fluoride

RNA – Ribonucleic Acid

ROS – Reactive Oxygen Species

RT- Room Temperature

RXR- Retinoid X Receptor

Sesn1- Sestrin 1

Sesn2- Sestrin 2

SGK- Serum and Glucocorticoid inducible kinase

SiCtl- Small interference RNA control

siRNA- Small interference RNA

SirT1- Sirtuin 1

SKP2- S-phase Kinase-Associated Protein 2

Sp1 – Specificity protein 1

SREBP-1 – Sterol Regulatory Element-Binding Protein 1

SREBP-2 – Sterol Regulatory Element-Binding Protein 2

SSRP1- Structure Specific Recognition Protein 1

TAG- Triacylglycerol

TGFα- Transforming Growth Factor alpha

TIGAR- TP53-Induced Glycolysis and Apoptosis Regulator

TP53- Tumor Protein 53

TSG101- Tumor Suppressor Gene 101

VLDL- Very Low Density Lipoprotein

WAT – White Adipose Tissue

XOR- Xanthine Oxidoreductase

1. Introduction

1.1 Fatty Acid Oxidation and Ketogenesis

Fatty acids are the main energy reserve in the body, and as so they are considered fuel molecules. They are stored in triglycerides, which are highly concentrated stores of metabolic energy. They are reduced and anhydrous.

In mammals, the accumulation of triglycerides is typically in the cytoplasm of adipocytes. The synthesis, storage and mobilization of the triglycerides occurs in these adipocytes. There is also storage of triglycerides in the muscle, for its own use.

Before the peripheral tissues can use the lipid energy, it is necessary that they pass through three stages of processing. The first step is the degradation of the triglycerides into fatty acids and glycerol by lipases.[1]

Lipolysis, a process that is active during fasting, is activated by β-adrenergic cathecolamines (like epinephrine) and it can be easily suppressed by insulin signaling. The release of fatty acids from the adipocytes is mediated by HSL (Hormone-Sensitive Lipase). The transcription of this protein is activated during the final steps of adipocyte differentiation, increases during fasting and it is repressed by both insulin and glucose. The protein Perilipin is also involved in lipolysis and coats the lipid droplet that contains the triglycerides reserves of the cells.[2]

They are then released from the adipose tissue, and transported by the blood stream to the energy requiring tissues. After that, the fatty acids need activation to CoA-esters by thiokinases and transportation to the mitochondria. The short- and medium- chain fatty acids can pass directly into the mitochondria, because of the permeability of the inner mithocondrial membrane. To transport the activated long chain fatty acids it is necessary a special transport system. The fatty acids are conjugated with carnitine, catalyzed by carnitine acyltransferase I /carnitine palmitoyl transferase I (CPTI).

Acyl CoA compounds are converted to acylcarnitines by carnitine-acylcarnitine translocase and then, on the matrix membrane, are transesterified to acyl-CoA by CPTII (carnitine palmitoyltransferase type II). A carnitine transporter (OCTN2) will transport carnitine into the cells, except in the hepatocytes.

Inside the mitochondria, the fatty acids go under β-oxidation (FAO) and acyl-CoA is shortened by two carbon atoms, to generate one acetyl CoA molecule and reducing equivalents (NADH and $FADH_2$) in each subsequent cycle, that are linked to the citric acid cycle and the mitochondrial respiratory chain. ATP will be consequently generated by oxidative phosphorylation in aerobic tissues.[1][3][4]

In cases of fasting or diabetes, the energy requirements are essentially satisfied by FAO, with the ketone bodies derived from acetyl-CoA.

Mitochondrial FAO is an essential process, especially in newborns, that have low glycogen reserves and rely mainly on fatty acids for energy.[3]

In these cases, the amounts of insulin are low, and the glucagon is high, which will lead to an augmented release of fatty acids from the adipose tissue. The energy necessary to gluconeogenesis is provided and also high amounts of acetyl-CoA are synthetized. In the liver, the acetyl-CoA is transformed into ketone bodies, by the enzymes HMGCS2 and HMGCL.[3][5]

In normal cells, except lipogenic tissues, the mammalian fatty acid synthase – a complex multifunctional enzyme that contains seven catalytic domains and a phosphopantetheine prosthetic group on a single 260 KDa polypeptide- activity is minor, because fatty acids are provided by the dietary fat. Mammalian FAS is down-regulated by dietary fat in lipogenic tissues (liver, adipose tissue), but its activity is described to be higher in many cancers.[6][7]

The predominant product of FAS is Palmitate, a saturated 16-carbon fatty acid. It is synthesized *de novo* from the substrates acetyl-CoA, malonyl-CoA and NADPH (Figure 1.1). It has been shown that inhibition of FAS will lead to elevated levels of malonyl-CoA, inhibition of CPTI and inhibition of fatty acid oxidation.[6]

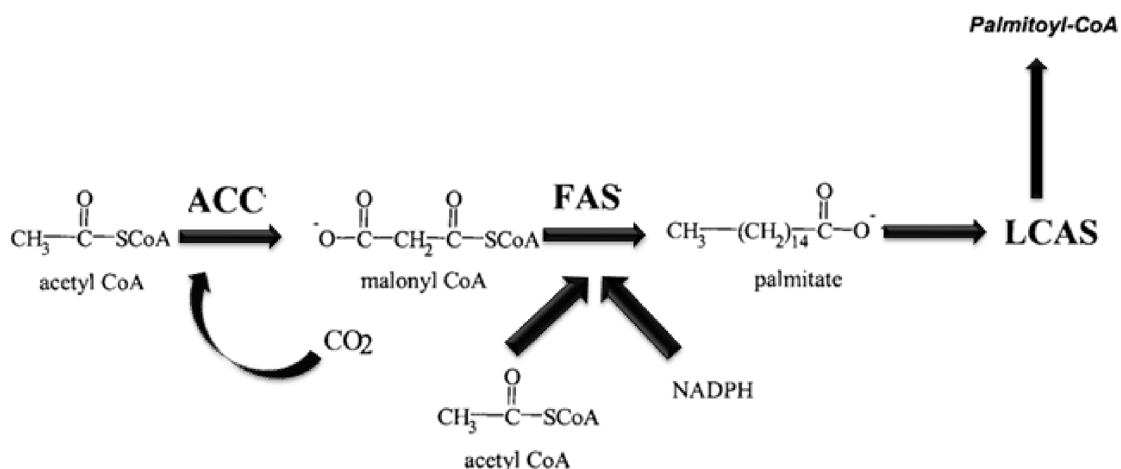

Figure 1.1 -Scheme of Fatty acid synthesis pathway. ACC (Acetyl-CoA carboxylase), a regulatory enzyme of fatty acid synthesis, carboxylates acetyl-CoA, producing malonyl-CoA. Free palmitate is produced from NADPH, acetyl-CoA and malonyl-CoA. LCAS (Long chain acyl-CoA synthase) esterifies CoA to palmitate, producing palmitoyl-CoA. Adapted from [6]**.**

Ketogenesis is the last step of the lipid metabolism and it is an important process, especially in the fasted state, because it provides the energy needed for the extra hepatic tissues, and as so, glucose is reserved for vital tissues, like the brain.

The ketone body production has three control points, the adipocyte lipolysis, the mitochondrial fatty acids entry, controlled by the inhibition of CPTI by malonyl-CoA and mitochondrial 3-hydroxy-3-methylglutaril CoA synthase, that catalyzes the irreversible first step of the ketone body synthesis.[4]

1.2 3-Hidroxy-3-MethylGlutaryl-CoA Synthase

Mitochondrial 3-hidroxy-3-methylglutaryl-CoA synthase (HMGCS2) is a gene that regulates the ketone body production, in liver and extrahepatic tissues. It catalyzes the condensation of acetyl-CoA with acetoacetyl-CoA, to generate acetoacetate, β-hydroxibutyrate and NAD+. cAMP, insulin and dexamethasone can vary its mRNA levels. [5][8]

HMGCS2 is controlled by nutritional and hormonal effects, and its activity is modulated by posttranscriptional mechanisms of succinylation and desuccinylation.[9]

In rats, the hepatic ketogenic capacity increases during the post-natal development or fasting, and HMGCS2 expression increases in liver. This effect is also observed in high fat diets, but is suppressed by insulin.[10]

1.2.1 HMGCS2 Regulation

The rat mitochondrial HMGCS2 promoter has three main regulatory sequences. 1) an insulin-responsive sequence, at the position -211 respecting to the transcriptional start site, that binds proteins from the forkhead family of transcription factors; 2) a peroxisome proliferator-activated receptor element, at the position -104, that binds retinoid X receptor-peroxisome proliferator-activated receptor heterodimers and 3) a Sp1-binding site, at the position -56, that binds proteins from the Sp1 family of transcription factors. The Sp1 binding site and the peroxisome proliferator-activated receptor element are present in similar positions in the human promoter.[8][10]

1.2.2 HMGCS2 and PPAR

The expression of HMGCS2 is stimulated by peroxisome proliferator-activated receptor α (PPARα). PPARα is a fatty-acid activated nuclear receptor that regulates metabolic changes in the liver, associated with starvation.

It was described that mHMGCS2 protein interacts physically with PPARα *in vitro*, leading to a transcriptional activation of HMGCS2 *in vivo*, and it also contains a nuclear-receptor interacting consensus motif (LXXLL), common to many nuclear hormone receptor-interacting cofactors, and that is required for efficient binding to PPARα. This facts, with the knowledge that mHMGCS2 accumulates in the nucleus in the presence of co-expressed PPARα, suggests that mHMGCS2 may be a co-regulatory factor for PPARα.[11]

Sirtuin-1 (SirT1) is a NAD+-dependent protein deacetylase that may also contribute to the metabolic adaptation to fasting. PPARα signaling has alterations when this protein is deleted from hepatic cells.

FAO and ketogenesis are induced by human HMGCS2, and its expression is necessary for PPARα-mediated induction of FAO.[5]

HMGCS2 mRNA levels increase in response to starvation, fat-feeding and diabetes and during the early neonatal period.

The high levels of plasma glucagon and low insulin levels may be the cause of the increased expression of the HMGCS2 gene in the liver of starved, newborn, suckling and fed rats.

The oxaloacetate is consumed in high amounts by gluconeogenesis in cases of type one diabetes, but the breakdown of fat generates large amounts of acetyl-CoA. The acetyl-CoA would normally be directed to the tricarboxylic acid cycle, condensed with oxaloacetate, but since the concentration of oxaloacetate is lowered, it is used to produce excess of acetoacetate and D-3-hydroxybutyrate – *ketone bodies.*[1][8]

1.2.3 HMGCS2 and FoxO family of transcription factors

The HMGCS2 promoter transcriptional activation is mediated by a network of recruited transcription factors, and this can explain why there is an induction of this gene expression in the liver, under pathologic and physiologic conditions. In tissues that oxidize fatty acids, this gene was proposed to prevent the acetyl-CoA accumulation, which will impair the rates of the fatty acid oxidation, so a lack in the induction of HMGCS2 can impair β-oxidation.[10]

The members of the FoxO family of transcription factors, such as FoxO3a, are involved in the regulation of HMGCS2 gene by insulin, through phosphorylation by phosphoinositide-3 kinase (PI-3K)/PKB transduction pathway that regulates FoxO expression. The inhibition of the PI-3K blocks the insulin repression, and on the other hand, when an active form of PKB/Akt is overexpressed (downstream PI-3K target in the insulin signaling pathway) represses the FoxO3a/FKHRL1 induced expression of HMGCS2.[8]

It has been shown that HMGCS2 is down-regulated in poorly differentiated carcinomas and although the importance of its expression in tumor progression is not known, there is the hypothesis that some specific dietary manipulations can affect cancer incidence if this gene expression or ketone body production plays a role in tumor development/progression.[10]

1.3 FoxO family of transcription factors

The Forkhead box ‘Other’ proteins, are a subgroup of the Forkhead transcription factor family and they are described as having an important role in mediating the effects of insulin and also of growth factors on various physiological functions- cell proliferation, apoptosis and metabolism. They are the mammalian orthologs of *Caenorhabditis elegans* DAF16. [12][13]

1.3.1 The Forkhead transcription factor family

The Forkhead transcription factor family is characterized by a 100-amino acid conserved monomeric DNA-binding domain, ‘forkhead box’, and it is present in all eukaryotes. The DNA binding domain folds into a variant of the helix-turn-helix motif and is made up of three α helices and two characteristic large loops, or wings. It is called the helix-DNA binding domain. The FoxO family was initially discovered in humans at chromosomal translocations, suggesting that they can play an important role in tumor development.[14][15]

There are four mammalian FoxO members, FoxO1/FKHR/FoxO1a, FoxO3/FKHRL1/FoxO3a, FoxO4/AFX and FoxO6, and all of these share a high protein homology.[12][13]

All the FoxO members are expressed in all tissues in mammals, in different degrees. FoxO1 mRNA is mainly expressed in adipose tissue, FoxO3a mRNA is highly expressed in the brain, FoxO4 mRNA is mostly expressed in the heart and finally, FoxO6 mRNA is expressed mainly in the developing brain.[14]

These proteins can modulate the expression of several genes, which are involved in processes like autophagy, apoptosis, DNA damage repair, oxidative stress, cell differentiation, cell cycle progression and glucose metabolism (Table 1.1). An irregular pattern of FOXO activation or regulation will lead to disorders of apoptotic pathways, proliferation and cell cycle regulation.[16]

Table 1.1 -FoxO target genes involved in metabolism.[12]

Genes	FoxO-effect	Organ or cell type	Metabolic effects
G6Pase	Upregulation	Liver, kidney	Increased gluconeogenesis
G6Pase transporter	Upregulation	Liver	Increased gluconeogenesis
PEPCK	Upregulation	Liver, kidney	Increased gluconeogenesis
IGFBP-1	Upregulation	Liver	Inhibition of IGF-1
PGC-1α	Upregulation	Liver	Increased gluconeogenesis
PDK4	Upregulation	Muscle, liver	Glucose saving
LPL	Upregulation	Muscle	Triglyceride clearance; fatty acid metabolism
HMG-CoA synthase	Upregulation	Liver	Ketone body production
PDX-1	downregulation	Pancreatic β-cell	Inhibition of β-cell differentiation
p21	Upregulation	Adipocyte	Inhibition of fat cell differentiation
AdipoR1/2	Upregulation	Liver, muscle	Fatty acid oxidation, glucose uptake

Abbreviation: HMG, hydroxymethyl glutaryl.

Their working mechanism is either to activate or repress multiple target gene expression. In the process of tumor suppression, they regulate genes that induce apoptosis such as Bim, Trail and Fas L, in cell cycle regulation they interact with p21, p27 and cyclin D1.Concerning DNA damage repair, they regulate GADD45a and in autophagy Atg7.[16]

1.3.2 FoxO proteins as insulin and growth factors targets

The FoxO proteins have been described as targets of insulin action, and also targets of growth factors. They interact with Insulin Response Sequences (IRS), similar to the ones existing in the PEPCK gene (phosphoenolpyruvate carboxykinase) through phosphatidylinositol 3-kinase (PI3K) and protein kinase B signaling.[12]

PI3K is activated through binding of insulin, IGF-1 (Insulin like growth factor I) or other growth factors to tyrosine kinase receptors, and as consequence, several serine/threonine kinases are also activated, including the Akt family protein kinases and the related serum and glucocorticoid inducible kinase (SGK). All the mammalian FoxO members share the same regulatory sites for AKT/PKB, with the exception of FoxO6. They have three consensus phosphorylation sites (RXRXXS/T) of Akt – Thr32, Ser253 andSer315 in FoxO3 sequence, Thr24, Ser256 and Ser319 in human FoxO1- but other AGC family kinases can phosphorylate in the same sites, although Akt and SGK phosphorylate a different combination of sites.[13] [14][15]

1.3.3 Posttranslational modifications of FoxO factors

Phosphorylation of FOXO factors by Akt (Figure 1.2) will provoke a relocation of FOXO proteins from the nucleus to the cytoplasm and as so, its inactivation. Ser256 (human FoxO1) functions as a *gatekeeper* and its phosphorylation is required for the subsequent phosphorylation ofThr24 and Ser 319. The phosphorylation of Ser319 will lead to phosphorylation of Ser322 and Ser325 by casein kinase 1. All these phosphorylation events together with phosphorylation of Ser329 by DYRK1, function as a nuclear export signal, forming a complex with the nuclear export proteins Ran and Crm-1. The inactivation of FoxO by phosphorylation leads to down regulation of proapoptotic genes induced by FoxO family.[12][15][17]

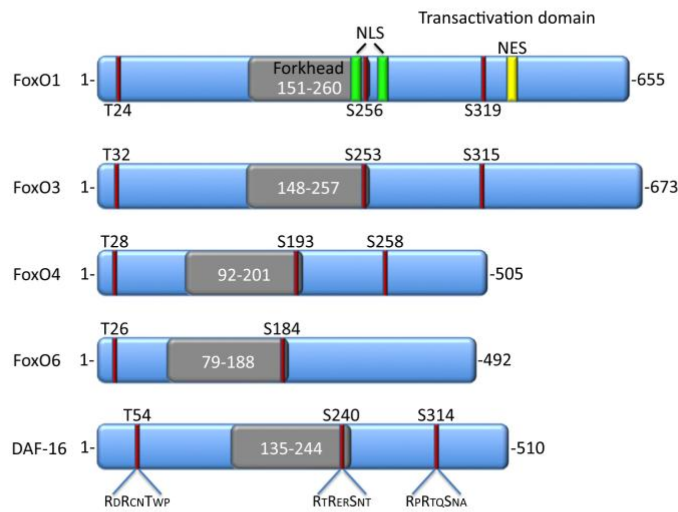

Figure 1.2 - Conserved AKT phosphorylation sites in FoxO proteins. Representation of mammalian and *C.Elegans* FoxO isoforms and the corresponding AKT phosphorylation sites. There are also represented the locations ofthe forkhead domain and the nuclear export (NES) and nuclear localization sequence (NLS).[13]

These phosphorylation events mediate the binding of 14-3-3 proteins, and this is described to contribute to FoxO3 accumulation in the cytoplasm (Figure 1.4).14-3-3 proteins belong to a family of evolutionary conserved modulator proteins and they regulate multiple signaling pathways in the cell. Through binding to specific Ser/Thr- phosphorylated motifs on target proteins, these proteins can modulate enzymatic activity, protein stability, cellular localization or association with other proteins. In consequence of FoxO phosphorylation by Akt and subsequent binding of 14-3-3 protein, transcriptional activity is reduced.

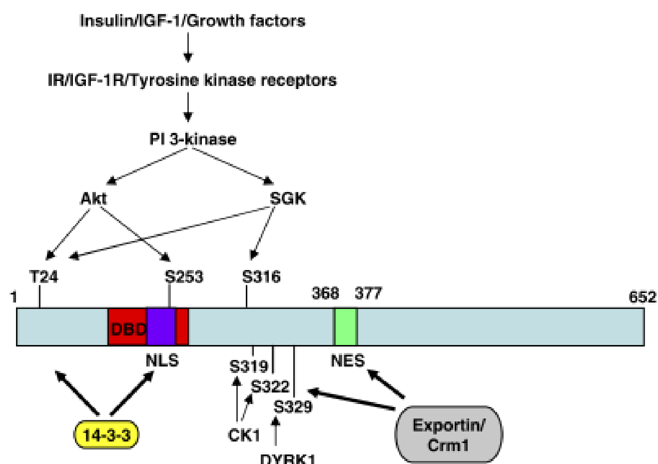

Figure 1.3 -Phosphorylation and regulation of nuclear export of FoxO. [15]

Some other modifications in FoxO1 were described, as acetylation and phosphorylation by MST1 that affect DNA binding. [13]

FoxO transcription factors up-regulate genes from the glucose metabolism, like glucose-6-phosphatase, that converts glucose-6-phosphate to glucose, and PEPCK, that converts oxaloacetate to phosphoenolpyruvate. The glucose metabolism is connected with the protein stability, and this is probably mediated by AMP-activated kinase.

Another regulation pathway is given by the c-Jun N-terminal kinase (JNK) that phosphorylates Thr447 and Thr451, positively regulating FoxOs.[12] [14][15]

FoxO activity can be modulated by acetylation directly on lysine residues mediated by CBP (Creb binding protein), P300 and PCAF, but there are some controversial results. In some cases there is an increase in the activity and in others there is a decrease. The recruitment of CBP/P300 will cause histone acetylation, and this leads to a positive control for transcription. On the other hand, the FoxO acetylation also diminishes DNA-binding, and this leads to a negative control. Since this is a reversible regulation, FoxOs can be deacetylated by class-I histone deacetylases and the class-III, NAD-dependent histone deacetylases-sirtuins Sirt1-. The effect of this deacetylation vary according to the target genes, but it mainly enhances FoxO's transcriptional activity. [13][15]

FoxO protein levels are regulated by its degradation, and it has been described that insulin decreases FoxO1 protein levels, by ubiquitination and further degradation in a PI3K-dependent manner, in HepG2 cells.The signaling for ubiquitination and degradation can be given by phosphorylation. FoxOs can be phosphorylated by Akt,ERK-1/2 and IKK. SKP2 binds AKT-phosphorylated FoxO1 at Ser256 and MDM2 binds ERK-phosphorylated FoxOs, they are both E3 ligases. MDM2 can signal FoxO for two different things, it can lead to mono-ubiquitination, that translocates FoxO to the nucleus and activates transcription, and poly-ubiquitination that targets for degradation. Also, 14-3-3 binding and Akt phosphorylation are involved in the degradation pathway.

Arginine methylation is another posttranslational modification that will lead to FoxO exclusion from the nucleus.

In human cancers, there are many tumor suppressors that can also be degraded by the ubiquitin pathway, like p53, $p27^{KIP1}$,p130 and $p57^{KIP2}$.[13][15]

1.3.4 FoxO transcription factors and Drug Resistance

It has been described that FoxO transcription factors also have a role in promoting drug resistance. MDR (Multi-Drug Resistance) is characterized by enhanced cell survival, increased DNA damage repairs and modified cellular drug uptake, efflux or metabolism. The signaling PI3K/Akt pathway is increased in cancerous cells. FoxO members, especially FoxO3, are active in response to anticancer

drugs, as doxorubicin, that induce apoptosis by generating ROS (reactive oxygen species). Oxidative stress induces Foxo3 activation, by its nuclear translocation in mammalian cells, and it also induces the stress activated kinase p38-MAPK. It was also shown that, despite their ability to execute cell cycle arrest and death programs, FoxO can also promote cell survival by amplifying PI3-K/Akt signaling. The multidrug resistant gene (MDR1 or ABCB1- cellular membrane transporter that mediates ATP-dependent efflux of a wide variety of hydrophobic anti-cancer drugs like taxanes, anthracyclines and other chemotherapeutic drugs, that enter freely in the cells by passive diffuson) is a target of FoxO3 in leukaemic cells. The drug-resistance is probably acquired by re-programming of post-translational modifications.[18]

In paclitaxel treatments, JNK can activate FoxO3a indirectly by inhibiting PI3K-AKT or directly through FoxO phosphorylation. ERK and Akt inactivation by JNK also induces FoxO3a nuclear localization and regulation of target genes (p27kip1 and Bim). Treatment with doxorubicin induces cell cycle arrest and cell death in breast cancer cells, and subsequent p38 induction, with FoxO3a nuclear relocalization. The doxorubicin induced nuclear localization of FoxO3a was shown to be due to phosphorylation of Ser-7, by the stress activated protein kinase p38. This event is likely to exist in every cell lines. P38 can also phosphorylate and activate tumor suppressors p53 and p73, in response to UV radiation and chemotherapeutic drugs.[19]

FoxO3 is also a p53 target gene, and together they can be part of a regulatory transcriptional network, important in aging and cancer. This topic will be further discussed below.[20]

1.4 Tumor suppressor p53

The tumor suppressor p53 is a 53 KDa protein. It has anti proliferative effects as growth arrest, apoptosis and cell senescence. It was discovered to be essential in cell cycle arrest, in response to irradiation and DNA-damaging agents, and also to regulate gene expression as a sequence specific transcription factor, forming a homotetramer on the target gene response elements. Induction of stress signals as oncogene assaults, chemo- or radiation-induced DNA damages, hypoxia or nucleotide depletion leads to an accumulation of p53 protein in normal cells, triggering the transcription of various p53 target genes. P53 is considered as a *cellular gatekeeper*. In 50% of all human cancers, p53 exhibits mutations, leading to accumulation of a dysfunctional protein. In the other 50%, there are abnormalities with the p53 pathway. In normal cells, p53 has been shown to also have a role, in homeostatic regulation of energy-producing processes, coordination of overall rate of biosynthesis and mobilization of defenses against reactive oxygen species.[21][22][23][24]

1.4.1 p53 – Stabilization and Activation

There are two necessary processes by which p53 is induced in response to DNA-damage, stabilization and activation (from the 'latent' to active form).[23]

The pathways by which p53 is degraded include Mdm2 (mouse double minute 2) and JNK proteins, that are responsible for p53 constitutive instability. Mdm2 is a product of an oncogene amplified in several tumors and it's a p53 transcription factor inhibitor. In response to genotoxic stress, Mdm2 binds to p53 at residues 17-22 and signals p53 for degradation. Some enzymes can be activated that will modify p53 and its interaction with its negative regulator, and also some signals can prevent the degradation of p53 by inducing $p14^{ARF}$ which blocks Mdm2.[21][23]

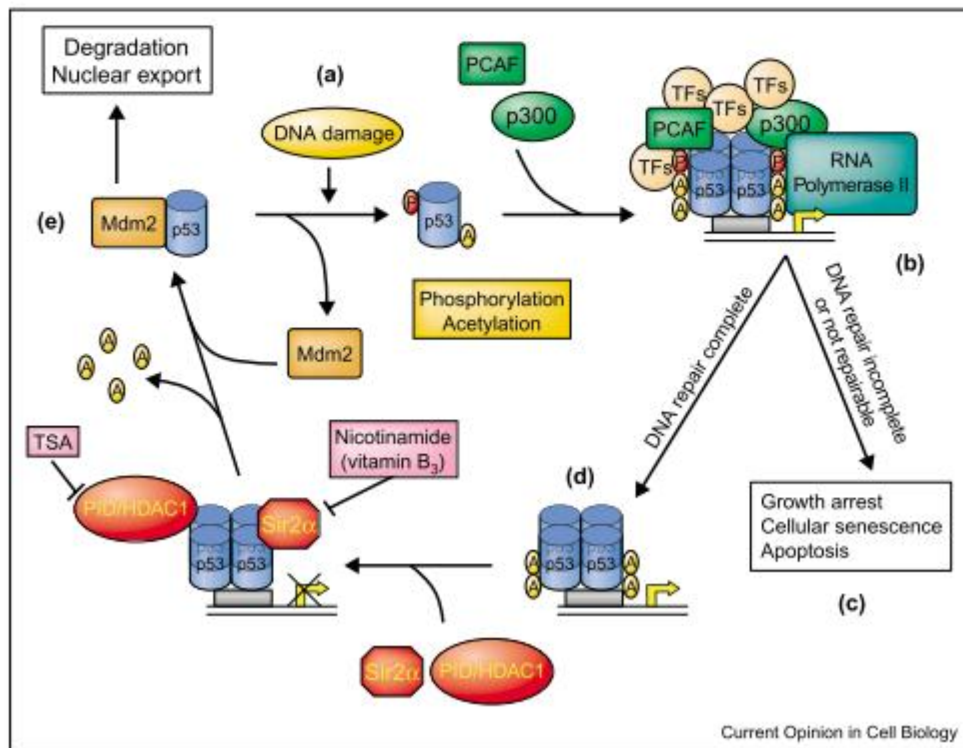

Figure 1.4 - Mechanisms for p53 transcriptional activation. a) DNA damage induces phosphorylation and acetylation events, p53 tetramerization and transcriptional activation. b) Through unknown mechanisms, the cell may choose a particular fate on the basis of its DNA repair status. C) When DNA repair is not complete or not repairable, the cell may choose growth arrest, apoptosis, or cellular senescence. D) On the other hand, if the repair of DNA is complete, deacetylases (such as Sir2α and PID/HDACI) may provide crucial p53 deacetylation activity to shut off p53-dependent transcription. E) Mdm2 regulates p53 levels until the next DNA damage signal is received. Nicotinamide and TSA are Sir2α and PID/HDAC1 inhibitors, respectively. A- acetylation; P- Phosphorylation; PCAF- p300/CBP associated factor; TFs- transcription factors; TSA – trichostatin A.[22]

JNK, another E3 ubiquitin-ligase, can play two different roles when it comes to p53 stabilization. If it's inactive, it binds to 97-116 residues and targets p53 to degradation by the proteasome. If it's activated, it phosphorylates threonine 81 and activates p53, repressing its degradation. The signals that activate JNK are the same that lead to p53 accumulation, like UV, X-radiation and oxidative stress by hydrogen peroxide. The p53-JNK interaction is in this cases prevented by a peptide.[23]

Other post-translational modifications can be responsible for p53 stabilization. For example, acetylation can promote stabilization and also transcriptional activity, but Mdm2 can inhibit this acetylation, mediated by CBP and p300. Ubiquitination and acetylation occur ate the same sites at the carboxyl terminus.[22]

Activation of p53, that will complement its stabilization, can occur by phosphorylation and acetylation. The most important residue that undergoes phosphorylation in UV-induced apoptosis is Ser46, by p53AIP1, p53DINP1 or HIPK2. These events may be dependent of post-translational modifications on p53. Ser392 is also phosphorylated in response to UV radiation, by a protein complex containing CK2, hSpt16 and SSRP1.[22]

Acetylation is another important modification for p53 activation. It is an important modification of histones that leads to an increased transcriptional activity. The acetylation processes have three potential roles: in the first place, they contribute to p53 stabilization; they can also induce conformational rearrangements in the C-terminus that increase DNA binding capacity, and finally, these processes can regulate the compartmentalization of p53 between nucleus and cytoplasm.[23]

CBP/p300 is a co-activator of p53, and possesses a histone acetyl transferase (HAT) activity. The acetylation consequences vary from increased DNA binding, enhancement of stability and changes in protein-protein interactions, and it's enhanced in stress situations. The residues that suffer acetylation are mainly Lys372, Lys 381, Lys 382 and also Lys320.

Acetylation can be reversed by Sirt1, and it's crucial for p53 ubiquitination and degradation.[22]

1.4.2 p53- Ubiquitination and Degradation

In normal cells, p53 is ubiquitinated in the nucleus and the shuttled to the cytoplasm, where it's recognized for degradation by the proteasome. When DNA damage occurs, p53 induction is triggered due to the need of increasing its levels. The ubiquitination inhibition and further degradation, is made by the tumor suppressor gene 101 (TSG101) and c-Abl.[22]

P53 can be stabilized by Mdm2-independent mechanisms, like stabilization by reduced nicotinamide adenine dinucleotide (NADH) quinone oxidoreductase 1 (NQO1) and NQO2, specific for oxidative stress and that prevents degradation of p53.[21][22]

1.4.3 AMP-Activated Protein Kinase regulation

When there are low stress signals or even none, p53 regulates the availability of nutrients, growth factors and hormones, and the overall rate of biosynthesis with energy status of the cell.[21]

For cells to undergo division it is necessary that the energy supplies are enough to produce ATP and to macromolecular synthesis. When the energy supplies are low, and so is the ATP levels, adenylate cyclase converts molecules of ADP to AMP and ATP, to compensate.

There are evidences that G1/S transition in mammalian cells depends on glucose availability by AMPK (AMP-activated protein kinase). AMPK can phosphorylate its substrates and turn off ATP consuming pathways; also it can activate energy production, by stimulating glucose uptake, fatty acid oxidation and mitochondrial repair.[21][25]

If there is a state of glucose restriction a reversible cell cycle arrest is necessary, but the presence of amino acids and growth factors is required, to support the TOR-dependent growth.[25]

The AMPK has one catalytic unit (α) and two regulatory units (β and γ). When there is a signal of DNA-damage, p53 activates transcription of the genes for β-subunits of AMPK. These subunits are involved in the localization and activity of this kinase.

AMPK controls p53, by inducing modifications and activation of p53 promoter. An upstream kinase, LKB1, also control p53 activity, by forming a complex and by direct or indirect phosphorylation of the residues Ser15 and Ser392.[21]

It seems that AMPK activates a nutrient-sensitive signaling pathway that leads to a p53-dependent cell cycle arrest, when there is energy deficiency.

Although the activation of p53 by DNA-damage leads to a low rate of survival, its metabolic activation is related to enhance survival ability by the cells to glucose depletion. [25]

The cell cycle arrest proposed at G1/S transition, in the presence of low glucose levels is a metabolic checkpoint that depends on AMPK and also p53, but occurs even in conditions when mTOR pathway is not inhibited. The mTOR pathway is an important switch between catabolic and anabolic processes. Its stimulation on the presence of nutrients, growth factors and hormones will lead to a major protein synthesis, increasing cell growth and proliferation.[21][26]

As it was said, p53 regulates β-subunits of AMPK, activating TSC2, inhibition of Raptor and downregulation of mTOR kinase. It can also be downregulated by activation of AMPK by p53 modulated Sesn1 and Sesn2 (sestrins). Inhibition of the mTOR pathway by p53 can also lead to an increase in autophagy. P53 can also induce autophagy by inducing the p53-activated gene DRAM.[21]

The stabilization and transcriptional activation of p53 in this metabolic checkpoint occurs by direct phosphorylation of Ser15 by AMPK, leading to cell survival. On the contrary, it is also been described that under the same conditions, glucose depletion, AMPK activation phosphorylates Ser46, up-regulating p53 and leading to cellular apoptosis. The AMPK- and p53- dependent responses to low glucose levels can vary according to the cellular type.[21][26]

1.4.4 Doxorubicin induces genotoxic stress, activating p53

Doxorubicin, an anthracycline antibiotic, is widely used in cancer treatment, produces genotoxic stress both in carcinoma and non-carcinoma cells. Its side effects in non-carcinoma cells limit its utilization, especially in cardiomyocites. ROS have been described has having an important role in doxorubicin-induced cardiomyopathy, and some antioxidants have been shown to decrease this negative effects.

A recent study showed that in doxorubicin treated MEF cells, there was an activation of AMPK, leading to an increase in apoptosis. In the same study, it was seen that in treatments under 4 hours, there would be an inhibition of apoptosis, probably due to an increase in ROS.

When there is signaling for DNA-damage, the ataxia- telangiectasia mutated (ATM) protein kinase is activated, inducing a two-phase dynamics of p53, that can be responsible for anti- or pro-apoptotic functions. When cells lack AMPK, ATM is phosphorylated, and that could be the reason for the increased sensitivity of these cells to doxorubicin-induced DNA damage. So, AMPK may play an important protective role against doxorubicin induced cellular apoptosis.

The accumulation of p53 is necessary for doxorubicin induced cellular death, and could be induced by an ATM dependent pathway. In MEF cells treated with doxorubicin, AMPK phosphorylates Ser15 of p53.

AMPK activation will interfere with p53 stability through p53 acetylation, meaning that an inhibition of AMPK increases doxorubicin induced p53 acetylation. This acetylation can be reverted by Sirt1 (class III NAD+-dependent deacetylase) as it was said here before. During doxorubicin-induced genotoxic stress in MEFs, AMPK also regulates p53 stability and function, by regulation of Sirt1 mediated deacetylation.[26]

1.4.5 P53 can negatively regulate IGF-1-AKT pathway

As it was already mentioned in the previous chapter, Akt or protein kinase B, stimulates cell proliferation by blocking $p27^{kip1}$ and activating c-myc and cyclin D1. It inhibits Bad, Mdm2 and FoxO proteins through activation of NFkB, decreasing apoptosis.

IGF-1, insulin-like growth factor, and insulin regulate Akt pathway, by signaling the availability of nutrients in the organism. Its binding to tyrosine kinase receptor IGF-1R recruits PI3K.

The protein p53 can regulate in a negative manner the IGF-1-Akt pathway. The activation of p53 leads to a decrease of the activity of IGF-1/Akt pathway and several changes in metabolism. IGF-BP3 encodes an IGF1 encoding protein, and its activation by p53 inhibits IGF1. P53 also stimulates PTEN, reducing Akt activity, glycolysis, fatty acid synthesis and increased β-oxidation of lipids.[21]

1.4.6 p53 and metabolism

Some of the effects of p53 inhibition are deficient mitochondrial biogenesis, a decrease in oxygen consumption and stimulation of glycolysis, seen by increasing levels of lactate. There are evidences that mitochondrial ATP synthesis is dependent on p53, like for example an increase in ATP synthesis by mitochondrial respiration, comparing to glycolysis. When p53 is inhibited, the values are reversed, and the there is more ATP produced by glycolysis.

P53 can also affect the glucose metabolism. It can retard glucose uptake by repressing GLUT1 and GLUT4, that encode glucose transporters, and it can stimulate glucose uptake by increasing the transcription of Hexokinase II gene, that converts glucose to glucose-6-phosphate. An up-regulation of Hexokinase II is seen in cancer cells, but a mild activation can be a part of the pro-survival functions of p53, helping the recovery from the metabolic checkpoint.[21]

1.4.7 The Warburg effect

Cancerous cells exhibit a specific metabolic pattern, presenting a shift from respiration to fermentation. This is called the Warburg effect, and it's characterized by a bigger production of lactate from glucose than in normal cells, performing lactic fermentation even in aerobic conditions. As for the normal cells, they present the Pasteur effect, that is characterized by inhibition of lactate production in the presence of oxygen .[27][28]

Metabolic regulation can be affected by every major oncogene and tumor suppressor, with varying mechanisms.

As it was already referred, glycolysis is suppressed by p53, by elevating expression of TP53-induced glycolysis and apoptosis regulator TIGAR- a fructose-2,6-biphosphatase. It also promotes oxidative phosphorylation, by enhancing the expression of cytochrome c oxidase-2, necessary for the assembly of the cytochrome c oxidase complex of the electron transporter chain. An inhibition/loss of p53 in tumor cells facilitates the Warburg effect, increasing glycolysis and decreasing oxidative phosphorylation.[28]

1.4.8 FoxO and p53

In situations of stress stimuli or nutrient deprivation, the FoxO3a transcription factor interacts with p53 in the nucleus, at least when both proteins are overexpressed. They also share similar target genes, as p21, GADD45, WIP1, PA26. Both p53 and FoxOs are phosphorylated and acetylated in response to stress stimuli and UV radiations, and both bind to SIRT1 deacetylase. [14]

P53 induces the activation of SGK that inhibits FoxO3a by its phosphorylation and relocalization to the cytoplasm. On the other hand, FoxO3a prevents p53 from repressing SIRT1 gene expression. SIRT1, with increased expression when insulin is low, deacetylates p53 and FoxO transcription factors.[14]

P53 regulates the expression of FoxO3a by binding to a site in the second intron of the gene.

Recent studies suggest that there could be a connection between genes that extend lifespan and suppressing tumorigenesis. FoxO3 and p53 may be part of a common regulatory complex. It was found that in MEFs and lymphocytes, p53 is a direct upstream transcriptional activator of the FoxO3

gene in response to DNA-damage by doxorubicin. FoxO3 is not necessary for p53 dependent cell cycle arrest, but it may have a role in p53-dependent apoptosis.[20]

FoxO3 transcription factor functions may increase p53 pro-apoptotic activity. P53 and its transactivating isoform TA-p73 bind to p53 response element (p53RE) of the endogenous FoxO3 gene in the adult mouse liver, recruits the acetyltransferase p300 activating the chromatin structure and expression of FoxO3.[29]

1.5 Peroxisome Proliferator-Activated Receptors

The peroxisome proliferator-activated receptors are ligand activated transcription factors that mediate the effect of unsaturated fatty acids and certain drugs on pathways. They belong to the nuclear receptor superfamily and have three different subtypes, PPARα (NR1C1), PPARβ/δ(NR1C2) and PPARγ (NR1C3).[30][31]

Although they share a high level of sequence and structural homology, each one of them has distinct physiological functions and different tissue expression patterns. PPARα is found mainly in brown adipose tissue, liver, kidney, heart and skeletal muscle, tissues with high rates of fatty acid catabolism. The genes controlled by this PPAR are the ones involved in reverse cholesterol transport and degradation of free fatty acids through peroxisomal and β-oxidation pathways. PPARγ is found especially in adipose tissue, and at lower levels in heart, colon, kidney, spleen, intestine, skeletal muscle, liver and macrophages. Its main role is in adipocyte gene expression and differentiation, but also as a regulator of target genes involved in glucose and lipid metabolism. PPARβ/δ is the least studied PPAR, and it's believed to be expressed ubiquitously. It may have a role in the regulation of fatty acid catabolism, energy metabolism and reverse cholesterol transport, additionally it was also shown that it could improve insulin resistance and reduce plasma glucose in animal models of type 2 diabetes.[31]

1.5.1 Trancriptional regulation by PPAR

Transcriptional regulation by PPARs requires heterodimerization with RXR (Retinoid X Receptor), also a member of the nuclear receptor superfamily. The activation of the PPAR/RXR heterodimer requires a ligand, and then it binds to DNA response elements (PPRE), to induce transcription. These DNA response elements are present in and around the promotor of the target genes. PPARs can also repress transcription by interfering with other transcription factors- transrepression.[30]

It is described that PPARs modulate the expression of genes involved in regulating glucose, lipid and cholesterol metabolism. Interferences in these pathways can lead to obesity, diabetes and cardiovascular disease, making PPARs attractive therapeutic targets.[31]

In the present study, it was mainly PPARα that was studied, and also PPARγ.

1.5.2 PPARα

As it was already referred, PPARα is highly expressed in organs with a significant catabolism of fatty acids. It was seen that this member of the PPAR family is activated by natural fatty acids and the identification of target genes was centered on cellular lipid metabolism, mainly in hepatocytes. They also can attenuate inflammatory responses. The first target gene identified for PPARα was acyl CoA, involved in peroxisome fatty acid β-oxidation. After that another target genes were found, that were present on transport and cellular uptake of fatty acids, intracellular fatty acid binding and activation, microsomal ω-oxidation, peroxisomal β-oxidation and ketogenesis, synthesis of lipoproteins and glycerol metabolism.[30]

PPARα doesn't only regulate oxidation of fatty acids, but it also regulates the metabolism of amino-acids. It was found to interfere in the expression of genes involved in trans- and deamination of amino-acids and urea synthesis.[30]

The increase in the expression of fatty acid oxidation, ketogenic genes and also cytosolic and mitochondrial glycerol 3- phosphate was shown to be PPARα dependent, in fasting. These evidences showed that PPARα is a direct stimulator of hepatic glycerol metabolism and influences hepatic glucose production during fasting .[30]

Studies made with activation of PPARα by its pharmacological ligand, WI14643, showed that there was an increased expression of genes involved in lipid and glucose metabolism and genes associated with peroxisome biogenesis, cell surface recognition function, transcription, cell cycle and apoptosis.

Besides the liver, PPARα is also highly expressed in the small intestine due to exposure to its agonist via diet, where it was shown to induce transcription factors and enzymes connected to sterol and bile acid metabolism, like SREBP-1. On the other hand, genes involved in cell cycle and differentiation, apoptosis, and host defense repressor. In the white adipose tissue, although it's expressed in much lower levels than PPARγ, it may influence adipose tissue remodeling. In the skeletal muscle, it was shown to be involved a metabolic reprogramming in muscle fibers, characterized by a switch from glucose utilization to fatty acid oxidation pathways, which leads to muscle glucose intolerance and insulin resistance.[30]

1.5.3 PPARβ

The PPARβ isoform is the least studied of this family and it is thought to have merely a housekeeping role. Lack of PPARβ may lead to multiple developmental and homeostatic abnormalities, like decreased adipose mass, myelination defects, altered skin inflammatory responses, a shift in muscle fiber composition towards type I muscle fibers and impaired wound healing.[30]

1.5.4 PPARγ

The main role of the third member of this family, the PPARγ, is in adipogenesis and its effects have been studied in 3T3-L1 adipocyte model. It is the key transcription factor in the adipogenesis program and it is essential for adipocyte survival. PPARγ also regulates lipid metabolism, insulin sensitivity, cardiovascular disease, inflammation, organ development and tumor formation.[30][32]

1.5.5 PPARα and Metabolism

PPARα importance in energy stores was shown by experiences made with PPARα-deficient mice that developed hypoglycemia, hypoketonemia and fatty liver, in fasted states. Although fatty acids are thought to be natural ligands for PPARα, this is not fully understood .[33]

Carbohydrate and lipid metabolism is also coordinated in hepatocytes by SREBPs proteins (sterol regulatory element binding proteins). SREBP-1c stimulates fatty acid synthesis, is involved in *de novo* lipogenesis when carbohydrates are abundant and is affected by levels of glucagon and insulin, critical hormones in glucose homeostasis and the oxygen receptor LXR. SREBP-2 stimulates cholesterol synthesis and its expression is affected by cholesterol availability. SREBPs are both regulated by fasting/feeding and can be affected by fatty acids.

PPARα and SREBPs activation by hepatic fatty acids can have different sources. Fatty acids can come from “new” fat, if they’re synthesized from carbohydrates. The “new” fat is retained in the liver, for storage, energy production or synthesis of structural and signaling lipids, or exported in VLDL to peripheral tissues for metabolism or storage. The stored/ “old” fat is released from adipose tissue into the circulation. When fatty acids are not utilized for energy, they return to the liver. It is thought that the fate of the fatty acids may depend on their site of origin.[33]

It was shown that mice in the fasted state lacking the capacity to synthesize fatty acids – FAS inhibition- had the same response that PPARα-deficient mice, developing fatty liver, hypoglycemia and decreased expression of PPARα target genes. These consequences could be reverted with the administration of a potent PPARα agonist. On the contrary, the cholesterol phenotype in the FAS

knock-out mice was not dependent on diet. The serum and hepatic cholesterol, the increasing SREBP-2, decreased HMG-CoA reductase gene expression regardless the dietary fat availability can also be normalized by pharmacologically activating PPARα, so the metabolic abnormalities in FAS inhibited mice may be caused by the PPARα unavailability.[33]

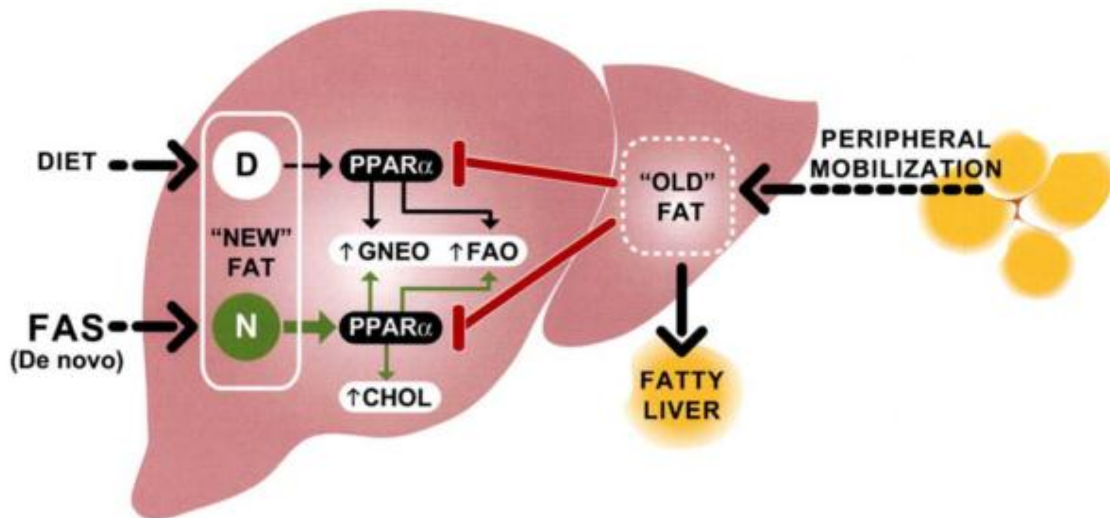

Figure 1.5 - Model for differential effects of hepatic lipid." New" fat is constituted by fat absorbed from the diet or synthezised via FAS in the liver, capable of activating PPARα to ensure normal glucose and lipid homeostasis. The "old" fat constitutes a different hepatic compartment, derived from peripheral mobilization of adipose stores, and it doesn't seem to activate PPARα as effectively as "new" fat, leading to fatty liver. Dietary fat, contrary to de novo synthesized fat, is inadequate for the maintenance of cholesterol homeostasis, suggesting different PPARα pools.[33]

The model described in literature is that the new fatty acids are PPARα endogenous activators. FAS knock-out mice are protected by the hypoglycemia/steatohepatitis when new fat is absorbed by diet activating PPARα, promoting gluconeogenesis and fatty acid oxidation. New fat produced by FAS accesses a separate pool of PPARα that stimulates cholesterol biosynthesis, gluconeogenesis and fatty acid oxidation. It was concluded that specific fatty acid pools synthesized by hepatic FAS or derived from the diet activate PPARα to impact glucose and lipid metabolism. The same doesn't occur with the old fat, the one stored in the liver after transfer from the periphery (Figure 1.5).[33]

Since FAS is a cytosolic enzyme, and PPARα is a nuclear factor, it is necessary a mechanism to deliver the cytosolic product to the nucleus and that could make the cell distinguish between old fat and new fat. One possibility is that lipid binding proteins specific for new fat shuttles fatty acids to the nucleus. Another possibility is that PPARα can shuttle from the nucleus to the cytoplasm with the aid of chaperones, promoting an interaction with the new fat pool.[33]

1.6 Cell Death-Inducing DNA fragmentation factor 45-like effector (CIDE) C/Fsp27

The Cell Death-Inducing DNA fragmentation factor 45-like effector (CIDE) protein family members were identified initially as mediators of apoptotic cell death. There are three members in this family, Cidea, Cideb and Cidec (Fsp27 in mouse). Cidea is highly expressed in BAT, Cideb is expressed mainly in liver, and at lower levels in kidney, small intestine and colon. Cideb can form homo- or heterodimers with other CIDE family members, in heterologous cells like 293T and COS-7 cells, to induce caspase-independent cell death. Cidea regulate apoptosis by being induced by TGF-β, and it was found that it also regulates energy homeostasis in BAT by regulating lipolysis and thermogenesis. Cideb was also found to have an important role in metabolism, as it regulates diet-induced obesity, liver steatosis and insulin sensitivity, controlling lipogenesis and FAO in liver.[34]

1.6.1 Fsp27 and metabolism

The third member of the CIDE family is Cidec/Fsp27. It is a 27 KDa protein expressed mainly in WAT. Fsp27 was found to be a novel lipid droplet that is involved in large unilocular lipid droplets in adipocytes and is required for the accumulation of triacylglycerols, also decreasing fatty acid oxidation in these cells. The lipid droplets are composed by a core of TAG, surrounded by a phospholipid monolayer with various proteins. It is also described that the overexpression of this protein in non-adipose tissues, results in spontaneous lipid accumulation .[35][36]

When the nutritional role of FSP27 was studied, it was found that between the dietary manipulations (intermittent fasting, caloric restriction, fasting –refeeding, and fasting), the only that up-regulates Fsp27 gene expression is intermittent fasting, promoting lipid deposition on adipose tissue. It was also described that PPARγ, C/EBPα, and insulin are involved in the nutritional regulation of Fsp27.[35]

It is described that another dietary manipulation, caloric restriction, down-regulates human Cidec and that is probably involved in fatty acid metabolism in cultured adipocytes.[37]

TAG are synthesized from FFAs and glucose in WAT in fed state. These TAG are stored in large lipid droplets. In fasted state, WAT generates the hydrolysis of stored TAG, generating FFAs and glycerol, releasing them into the circulation, so they can be used as energy by other tissues. In contrast with WAT, where the cytoplasm of adipocytes is almost entirely occupied with a large (10-200 μm in diameter), unilocular, TAG-rich lipid droplet, cells of the liver, muscle, heart, kidney, intestine, BAT and mammary gland accumulate small lipid droplets.

Depletion of Fsp27 induces multilocularization of large lipid droplets, and this may be related with an increase in lipolysis. The multilocularization will increase the total surface of lipid droplets and probably increases the access of lipases to stored lipids. On the other hand, the unilocular lipid droplets may

prevents lipolysis by minimizing the accessibility of stored lipid to lipases, and are crucial for efficient TAG storage in WAT.[36]

In Fsp27-KO mice, it was seen a lean phenotype, an increased metabolic rate and resistance to diet-induced obesity. This is probably caused by the decreased TAG storage, the increased mitochondrial biogenesis and the increased energy expenditure in these mice's WAT. The increase of FFAs and their metabolites in FSP27-KO mice may stimulate member of the PPAR family in WAT and induce mitochondrial biogenesis. FFAs have been described to activate PPARα and PPARδ. An increase in mitochondrial biogenesis and FFA oxidation in adipocytes may be caused by an increase in intracellular lipolysis.[36]

Perilipin, a lipid-droplet associated protein, regulates the storage of triglycerides and it's highly expressed in differentiated adipocytes. Its transcription is controlled by PPARγ2, one of the PPARγ isoforms. It is described that Cidec colocalizes with perilipin in the surface of lipid droplets and that cells lacking Cidec mRNA have increased lipolysis. It was shown that PPARγ2 regulates the transcriptional activity of Cidec in differentiated adipocytes. [32]

Cidea was found to be highly expressed in the mammary glands of pregnant and lactating mice, its deficiency led to a reduced milk lipid secretion and premature pup death. This was found to be due to the role of Cidea in inducing XOR (xanthine oxidoreductase) expression, an essential factor for milk lipid secretion. C/EBPβ mediates the interaction between XOR and Cidea. This member of the CIDE family is located in the nucleus in lactating mammary glands, BAT and cultured MECs. The residues responsible for Cidea interaction with C/EBPβ were found to be crucial for its nuclear location and XOR induction. Cidea is considered a C/EBPβ transcriptional coactivator. Fsp27 is also located in the nucleus, and in lipid droplets, and interacts with C/EBPβ in white adipocytes, so there is the possibility that it also functions as a coactivator of C/EBPβ.

2. Goal of this thesis

This thesis has as main purpose to study new mechanisms that regulate genes involved in ketogenesis.

It was already known from previous studies that doxorubicin, a DNA damaging agent, induced p53 activation, and this will lead to an induction of HMGCS2. Here, the objective was to see if the FoxO family members, FoxO3a and FoxO1, were responsible for HMGCS2 induction by p53 activation.
The second one implied finding a regulatory role for Fsp27 in mouse liver and its role in PPAR signaling.

After checking this hypothesis, it was decided to proceed to a different objective,
With the growing interest for the role of Fsp27, here the objective was to seek for a regulatory role for Fsp27 in mouse liver, involving PPAR signaling.

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3. Materials and Methods

3.1 DNA obtainment

To obtain the DNA of the constructs used in this work, first a PCR was made, with Taq polymerase, dNTPS, specific primers and the DNA (genomic or cDNA) to amplify. The amplified product was run in an agarose gel to check the product size. The band of interest was cut from the gel and purified with the *Wizard SV Gel and PCR Clean System kit* (Promega), according to the manufacturer's instructions.

After quantification of the purified product on *Nanodrop 1000 Spectrophotometer*, the product was inserted in a pGEM-T vector, according to the protocol *pGEM-T and pGEM-T easy vector* (Promega).

The DNA was transformed into competent cells DH5α (E.coli), previously prepared by salt washes ($CaCl_2$) to permeabilize them, and frozen at -80^{0}C, and then plated on agar plates, with ampicillin, X-gal and IPTG, for selection.

The plates were left at 37ºC o/n, and then the positive white colonies were chosen, that can't metabolize X-gal, having the polilynker disrupted because of the insert. The negative blue colonies have a functional LacZ.

The positive colonies were left to grow o/n in 2mL LB inoculums, with antibiotic (ampicillin).

The DNA from the inoculums was obtained with the *Kit Pureyeld Plasmid Miniprep System* (Promega). After quantification, the insert was released from pGEM-T, with restriction enzymes specific for the sequence amplified (Annex – 6.1 Constructs), ran in an agarose gel, the bands were purified and finally, the insert was cloned into the vector of interest (pGL3b, pcDNA3, pDS-red-N1 express).

A new transformation was made, with plates with ampicillin for pGL3b and pcDNA3 vectors, and with kanamycin for pDsRed-Express-N1 vector, and in the next day a PCR screening was made, to check which colonies were positive. The positive colonies shown in the agarose gel were sent to sequence and when the results came, the DNA was obtained with the *PureLink HiPure Plasmid Filter Maxiprep Kit* (Invitrogen).

3.2 Cell lines and Maintenance

To develop this project there were used two different cell lines. First, it was used the **AML12** (alpha mouse liver) cell line (ATCC CRL-2254) , a cell line established from hepatocytes from a mouse (CD1 strain line MT42) transgenic for human TGFα.

Cells were cultured in DMEM/F12 (1:1) – Dulbecco's Modified Eagle's Medium/Nutrient F12-Ham (**Gibco ref.31330-038**), supplemented with 10% (v/v) Fetal Bovine Serum, antibiotics penicillin (100 units/mL) and streptomycin (100 µg/mL), 0.1 µM dexamethasone (**Sigma-Aldrich D4902**) and 5 µg/ml insulin, 5 µg/mL transferrin and 0.005 µg/mL selenium (**Roche- 11 074 547 001**). For the doxorubicin treatments, the medium used was DMEM No glucose 1x (**Gibco ref. 11966025**), with L-Glutamine.

For the SiRNA transfections, the medium used was the DMEM/F12, supplemented the same way as before, but without antibiotics.

The other cell line utilized was the **HeLa** (Human cervical adenocarcinoma) cell line (ATTCC NO.CCL-2), cultured in DMEM – Dulbecco's Modified Eagle's Medium, prepared in the laboratory, supplemented with 10% Fetal bovine serum, antibiotics penicillin (100 units/mL) and strepromycin (100ug/mL)- **Gibco 15140-122**, L-glutamine 200 mM – **Gibco 25030-024**, and sodium bicarbonate solution 7.5%- **Sigma Aldrich S8761.**

The cells were incubated at 37ºC in a humidified atmosphere with 5%CO2. The culture medium was changed every 2 or 3 days, and to pass the cells, they were first rinsed twice with PBS, and then a 0,05% trypsin-EDTA (**Gibco 15400-054)** solution would be added. After about 4 minutes for AML12 cells, and 2 minutes for HeLa cells at 37ºC, the trypsin-EDTA was deactivated by adding complete growth medium. The cells were then plated for maintenance with dilutions according to their confluence, or counted to proceed to experiments.

For transfections, a serum-free medium was used –OptiMem I (**Gibco 31985-047**).

3.3 Reagents used for cell line treatments

Doxorubicin hydrochloride (**Sigma-Aldrich D1515**)- Anthracycline antibiotic used as a chemotherapeutic drug. Dissolved in Ethanol for 1 mM;

WY14643 (**Sigma-Aldrich,C7081**)- Selective PPARα agonist. Dissolved in DMSO to 10 nM.

3.4 Reagents used for transfection experiments

Lipofectamine LTX reagent (**Invitrogen, 15338-100**);

Dharmafect 4 transfection Reagent (**Thermo Scientific, T-2004-01**).

3.5 Transient Transfection and Luciferase assays

AML12 cells were plated at $1x10^5$cell/well, in a 24 MW 24 hours before transfection. To tranfect the DNA, it was used the Lipofectamine LTX reagent, at a proportion of 2.5 µL LTX to 1 µg of DNA. 48 hours after transfection, cellular extracts were prepared by washing with PBS and harvested in 100 uL 1x Passive lysis buffer (**Promega**) for analysis of Luciferase activity. Aliquots of 10 µL of lysates were used, with the Dual-Luciferase Reporter Assay System (**Promega- E1960**). The relative Luciferase activity was given as the ratio between relative luciferase unit/relative Renilla unit.

3.6 siRNA transfection

FoxO1, FoxO3a and SiControl nontargeting (**D-001210-01**) were purchased from Dharmacon (**Thermo Fisher**). AML12 cells were plated in 6-well plates, at $2.5x10^5$ cell/well. The transfections were performed according to Dharmacon instructions, with 6,4 µL of Dharmafect-4 and 25 nM of siRNA final concentration per well. After 72 hours of transfection, the cells were rinsed with PBS and then total protein and RNA extracts were isolated, for Western Blot assays and real time PCR analysis, respectively.

3.7 Protein extraction

The cells plated in a 6-well plate, were washed twice with cold PBS, and then harvested with a rubber policeman. Then, the cells were centrifuged for 10 minutes, 1000 x g at 4ºC. After discarding the supernatant, the pellet was lysed with 40-100 µL of NP40 lysis buffer, with agitation for 15-20 minutes at 4ºC, and after that centrifuged for 5-10 minutes ate 12000 x g at 4ºC. The supernatant recovered corresponded to the total protein extracts, and was stored at -80ºC.

NP40 lysis buffer – Tris-HCl 50 mM pH8; NaCl 150 mM, NP40 1%, supplemented with protease inhibitors (**Sigma Aldrich**) and 0.1 mM phenylmethylsulfonyl (PMSF).

The protein concentrations were estimated by the Bradford method, using the Bio-Rad protein assay with bovine serum albumin (BSA) as a standard.

3.8 Western Blot Analysis

The extracted proteins were resolved in an 8% polyacrylamide gel, and then transferred for about 2 hours at 200 mA, or o/n at 80 mA, to a PVDF membrane (**Milipore**). After this, the membranes were blocked for about an hour, with a blocking solution of PBS 1% Tween, with 5% low fat milk, at RT. After the blocking of the membrane, the primary antibody is incubated, o/n at 4ºC or at RT for at least 2 hours. Three washes were performed, with PBS 1% Tween, before incubation with the secondary antibody, one hour at RT. For tubulines, the blocking solution was TBS 0.1% Tween, with 5% low fat milk, and as so, the washes were made with TBS 0.1% Tween. Finally, another three washes were required and then the blots were incubated for 1 minute with substrate for peroxidase and chemiluminescence's enhancer (**EZ-ECL Chemiluminescence Detection Kit for HRP- Biological Industries- 20 500 120**) and immediately exposed to X-Ray film.

Table 3.1 -List of antibodies used in this project.

Protein	Primary Antibody	Secondary antibody
P53	Rabbit anti-p53 (FL393): sc6243 *(Santa Cruz Biotechnology)*	Donkey anti-rabbit IgG, whole Ab ECL antibody, horseradish peroxidase (HRP) conjugated (Amersham biosciences, NA934V)
Actines	Rabbit anti-actines A2066 *(Sigma-Aldrich)*	Donkey anti-rabbit IgG, whole Ab ECL antibody, horseradish peroxidase (HRP) conjugated (Amersham biosciences, NA934V)
Tubulines	Mouse anti-tubulines- CP06 *(Calbiochem)*	Goat anti-mouse IgG, whole Ab ECL antibody, horseradish peroxidase (HRP) conjugated (Amersham biosciences, NA931V)

3.9 RNA extraction and analysis

The cultured AML12 cells were washed twice with cold PBS and then recovered with a rubber policeman, on TRI reagent solution (**Ambion AM9738**). The RNA was extracted according to manufacturer's instructions. The RNA was ressuspended in DEPC-treated water (**Sigma-Aldrich**) and its concentration and purity (A_{260}/A_{230}; A_{260}/A_{280})were checked in *NanoDrop 1000* spectrophotometer (**Nanodrop technologies, Inc. Thermo Scientific**). To avoid contaminations with genomic DNA, a DNAse I (**Ambion- AM1906**) treatment was made before proceeding.

After the extraction, cDNA was synthesized from 1 µg of total RNA by M-MLV reverse transcriptase (**Invitrogen-28025-013**) with random primers (**Roche Diagnostics**- **1103473001**) and dNTPs (**Attend Bio-GC013-001**), according to the manufacturer's instructions.

The mRNA levels were studied by cDNA analysis with a quantitative real-time polymerase chain reaction (qPCR), with the ABI Prism 7700 Sequence detection system (**Applied Biosystems**). The mRNA levels were normalized with 18S ribosomal RNA determined by Eukaryotic 18S rRNA endogenous control (**Applied Biosystems**). The mRNA samples were analyzed always in duplicate, in 96-wells plates.

qPCR analyses: initial activation step -50ºC/2 min;95ºC/10 min; 50 cycles of denaturation- 90ºC/15s; annealing/extension-60º/1 min. The results were obtained by the comparative Ct method and then expressed as folds of the control experiment, to further analysis.

Probes (Applied Biosystems):

mHMGCS2- Mm00550050_m1

mFoxO1- Mm00490672_m1

mFoxO3a- Mm01185722_m1

3.10 Fluorescence assays

For the fluorescence assays, HeLa cells were plated in 24-well plates and 24 hours after plating, the cells were transfected. After 48 hours of transfection, the cells were seen in a Fluorescence microscope LEICA DMI 4000B.

The images were recorded in the software Leica Application Suite and analyzed in the software MacBiophotonics Image J Software.

4. Results & discussion

4.1 Induction of gene expression by Doxorubicin

The first objective was to confirm the induction of the HMGCS2 gene, as well as the induction of the members of the FoxO family, FoxO1 and FoxO3a.

To do that, it was used the *AML12* cell line (alpha mouse liver), grown in a glucose rich medium. The cells were then treated with doxorubicin, a DNA damaging agent, in previously optimized conditions, for six hours in the presence of a medium with low glucose, to promote starving and stress responses, and as so amplify p53 induction.

With a Western Blot analysis (Figure 4.1), it can clearly be seen the induction of p53 protein levels. This induction will lead to the transcription of p53 target genes.

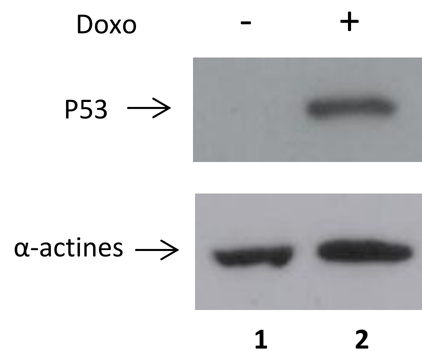

Figure 4.1 - Doxorubicin promotes p53 protein expression in AML12 cells. Western Blot of total protein extracts of AML12 cells incubated with 3 uM of doxorubicin, for 6 hours, using antibodies to p53 and actines, as control. Lane 1) Untreated cells; lane 2) Doxorubicin treated cells.

To analyze the FoxO and HMGCS2 mRNA levels, after a 6 hour doxorubicin treatment the AML12 cells were recovered and the RNA was extracted. Then a Real Time PCR assay was performed.

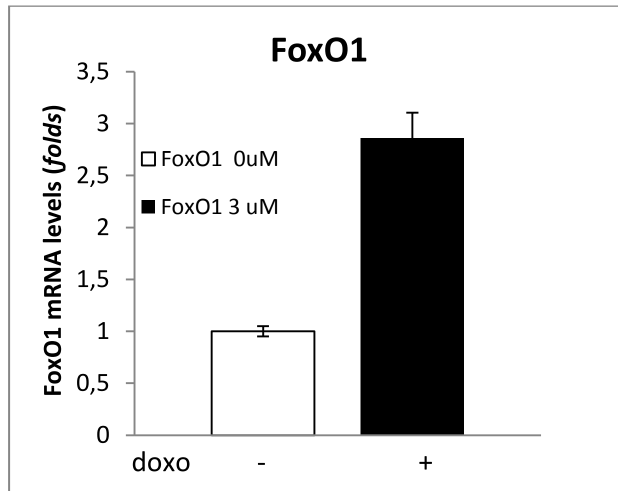

Figure 4.2 -Gene induction in response to doxorubicin. AML12 cells were incubated with 3 uM of doxorubicin, for 6 hours. The RNA was then extracted and a Real Time PCR was performed, to determine the mRNA levels of FoxO1.

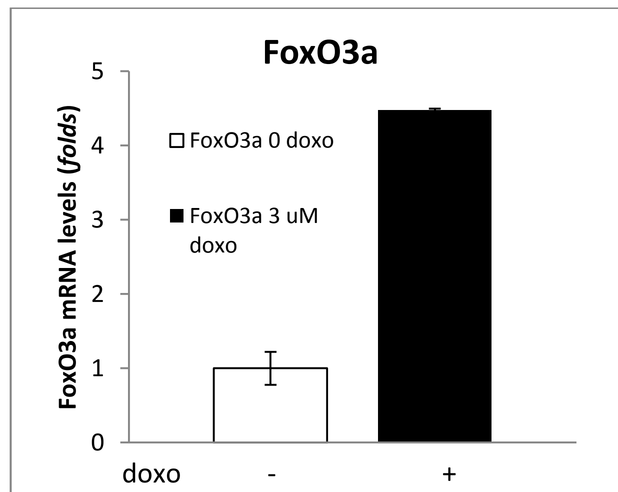

Figure 4.3 -Gene induction in response to doxorubicin. AML12 cells were incubated with 3 uM of doxorubicin, for 6 hours. The RNA was then extracted and a Real Time PCR was performed, to determine the mRNA levels of FoxO3a.

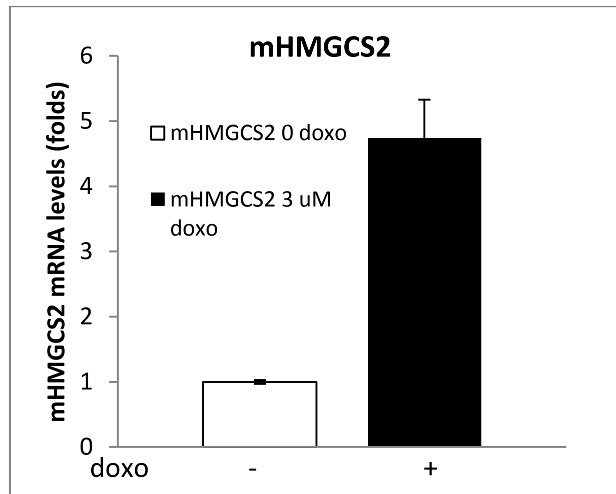

Figure 4.4 - Gene induction in response to doxorubicin. AML12 cells were incubated with 3 µM of doxorubicin, for 6 hours. The RNA was then extracted and a Real Time PCR was performed, to determine the mRNA levels of HMGCS2.

As it can be seen, there is an increase of 2.5 folds for FoxO1 mRNA levels (Figure 4.2), 4.5 for FoxO3a (Figure 4.3) and about 5 folds for HMGCS2 (Figure 4.4). So, as it was expected, the induction of p53 by DNA damaging, and amplified by starving and stress responses, promotes the induction of the FoxO3, a described target of p53 [20] , and also, it can be seen an induction of the FoxO1 mRNA levels. As for the HMGCS2, described as up-regulated by the FoxO transcription factors, in the ketone body production, in particular, by the FoxO3a member, that has a binding site in the promoter of the HMGCS2 [8][12], also shows the expected behavior.

4.2 Regulation of HMGCS2 expression by the Fork Head Family of transcription factors

4.2.1 Regulation by FoxO1

The first member to be inhibited was FoxO1. To do that it was necessary to transfect a small interference RNA, with a concentration of 25 nM in the AML12 cells.

The doxorubicin treatment was made 6 hours before recovering the cells, 72 hours after transfection, to extract protein and RNA. To normalize, the wells that weren't transfected with the FoxO1 siRNA, were transfected with a control small interference RNA.

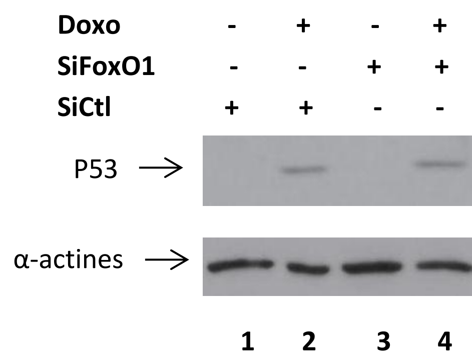

Figure 4.5 -Doxorubicin promotes p53 protein expression in AML12 cells, even in the absence of FoxO1. Western Blot of total protein extracts of AML12 cells incubated with 3 μM of doxorubicin, for 6 hours, using antibodies to p53 and actines, as control. Lane 1) transfection of 25 nM of SiCtl; lane 2) transfection of 25 nM o SiCtl and treatment with doxorubicin; lane 3) transfection of 25 nM of SiFoxO1; lane 4) transfection of 25 nM of SiFoxO1 and treatment with doxorubicin.

As it had been seen previously, the doxorubicin treatment induces the p53 activation and this activation doesn't suffer any kind of alteration in the absence of FoxO1 (Figure 4.5).

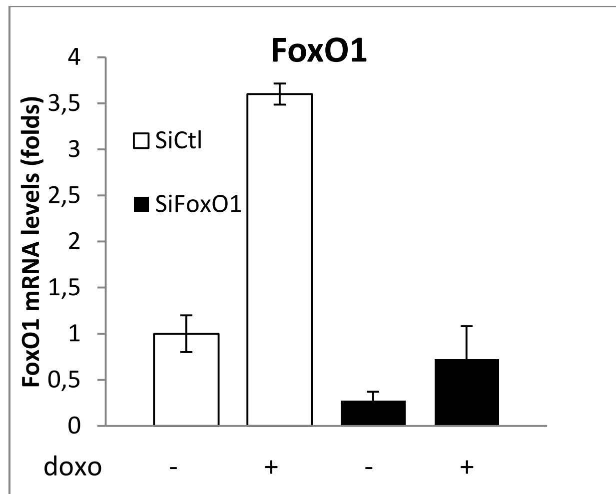

Figure 4.6 -Gene induction in response to doxorubicin. AML12 cells were transfected with 25 nM of SiFoxO1 and 25 nM of SiCtl for 72 hours, and treated with 3 μM of doxorubicin 6 hours before the cells were recovered. The RNA was then extracted and a Real Time PCR was performed, to determine the mRNA levels of FoxO1.

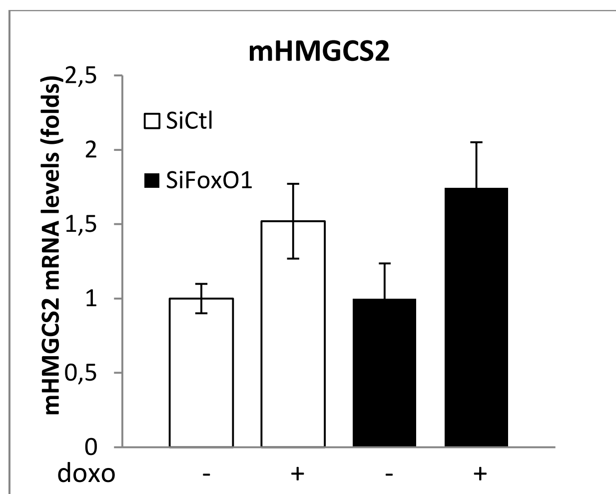

Figure 4.7 - Gene induction in response to doxorubicin. AML12 cells were transfected with 25 nM of SiFoxO1 and 25 nM of SiCtl for 72 hours, and treated with 3 uM of doxorubicin 6 hours before the cells were recovered. The RNA was then extracted and a Real Time PCR was performed, to determine the mRNA levels of HMGCS2.

The 3,5 fold induction of the expression of FoxO1, was lost in the wells transfected with the small interference RNA of FoxO1 (Figure 4.6), which means that the small interference RNA of the FoxO1 works properly, and it’s inhibiting its activity.

The activity of HMGCS2 doesn’t suffer any alteration, showing a doxorubicin induction, in both SiCtl transfected cells and SiFoxo1 transfected cells (Figure 4.7).

With these results it is possible to say that FoxO1 transcription factor is not the responsible for the induction of HMGCS2. Another possibility is that the lack of FoxO1 could be masked by the activity of other members of the FoxO family.

4.2.2 Regulation by FoxO3a

After establishing that FoxO1 is not responsible for the HMGCS2 induction, mediated by p53 activation, the role of the member FoxO3a was studied. In the same way that it was done for FoxO1, 25 nM of a small interference RNA for FoxO3a was transfected in AML12 cells, and they were treated with 3 uM of doxorubicin 6 hours before recovering the cells, 72 hours after transfection.

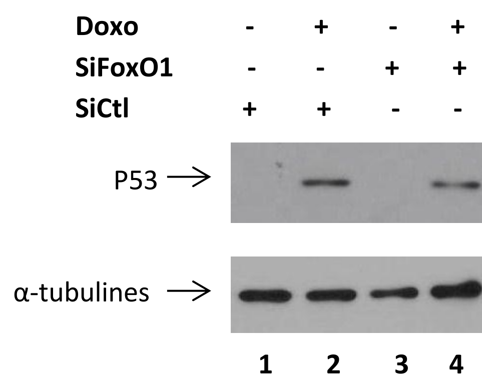

Figure 4.8 -Doxorubicin promotes p53 protein expression in AML12 cells, even in the absence of FoxO3a. Western Blot of total protein extracts of AML12 cells incubated with 3 µM of doxorubicin, for 6 hours, using antibodies to p53 and tubulin, as control. Lane 1) transfection of 25 nM of SiCtl; lane 2) transfection of 25 nM o SiCtl and treatment with doxorubicin; lane 3) transfection of 25 nM of SiFoxO3a; lane 4) transfection of 25 nM of SiFoxO3a and treatment with doxorubicin.

In this case, it can be seen the same behavior as before, the p53 activity is induced by the treatment with doxorubicin (Figure 4.8, lane 2 and 4), and there are no alterations when the FoxO3a activity is inhibited by a small interference RNA (Figure 4.8, lane 3 and 4).

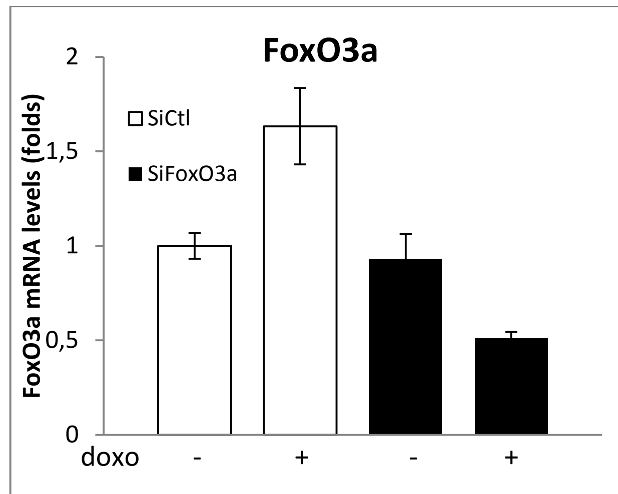

Figure 4.9 - Gene induction in response to doxorubicin. AML12 cells were transfected with 25 nM of SiFoxO3a and 25 nM of SiCtl for 72 hours, and treated with 3 µM of doxorubicin 6 hours before the cells were recovered. The RNA was then extracted and a Real Time PCR was performed, to determine the mRNA levels of FoxO3a.

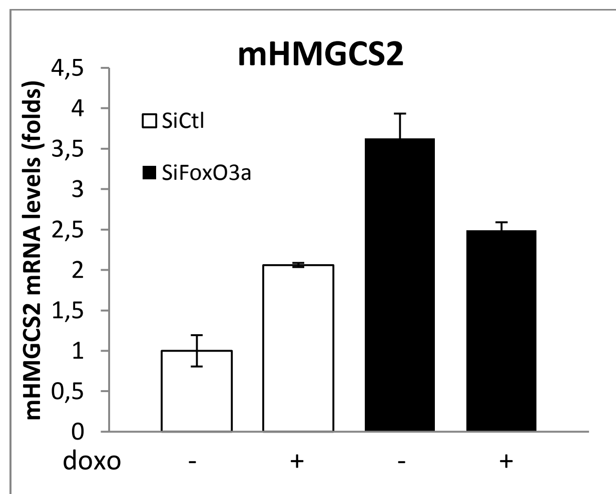

Figure 4.10 -Gene induction in response to doxorubicin. AML12 cells were transfected with 25 nM of SiFoxO3a and 25 nM of SiCtl for 72 hours, and treated with 3 µM of doxorubicin 6 hours before the cells were recovered. The RNA was then extracted and a Real Time PCR was performed, to determine the mRNA levels of HMGCS2.

The success of the siFoxO3a transfection can be determined by the lack of induction seen in the mRNA levels of the cells transfected with the small interference RNA for FoxO3a and treated with doxorubicin, opposing to the cells treated with doxorubicin, but only transfected with SiCtl (Figure 4.9), showing that the SiFoxO3a is in fact inhibiting the activity of FoxO3a.

Despite the positive response of the SiFoxO3a, when the HMGCS2 gene is analyzed an unexpected result appears. It seems that the HMGCS2 mRNA levels show an induction with the doxorubicin

treatment, but when the Sifoxo3a transfected cells are observed, a bigger induction appears, even in the untreated cells (Figure 4.10).

This observation leads to the conclusion that the small interference RNA for the FoxO3a may have some *off-target effects*, meaning that the introduction of the siRNA in the cells may originate some non-specific results.

4.3 Fsp27 cellular location

It was described recently that Fsp27 had a nuclear location. [38] To confirm that, a different cell line was used, since the AML12 cell line did not show any fluorescence transfection results. HeLa (Human Cervical Adenocarcinoma) cells, a cell line easier to transfect, were co- transfected with both Fsp27-pDsRed-Express-N1 and H2B-GFP-N1, this last construct will give a green color to the nucleus. After 48 hours of transfection, the cells were seen on a Fluorescence microscope and pictures were made with the green fluorescence (*nucleus*), the red fluorescence (*Fsp27*) and then with the help of a specific software, a merge of the two figures was made.

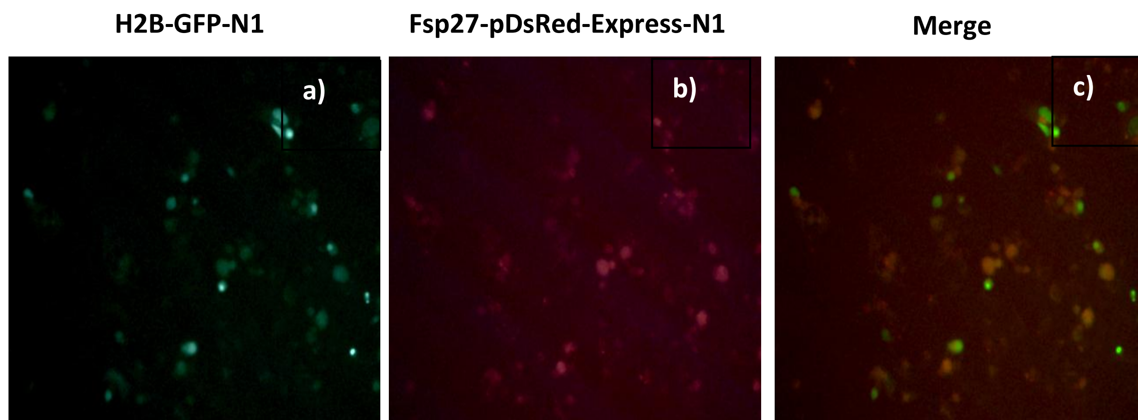

Figure 4.11 - Fsp27 cellular localization. HeLa cells were co-transfected with Fsp27-DsRed-Express-N1 (500 ng) and H2B-GFP (500 ng). After 48 hours of transfection, it was seen in the fluorescence microscope a) in green the H2B-GFP-N1 in the nucleus, b) in red color the Fsp27-pDsRed-Express-N1 and then a c) merge of the two was made with the help of the MacBiophotonics Image J Software.

Observing Figure 4.11, we can see that in 4.11.c), the merge between the green fluorescence (the nucleus) and the red fluorescence (Fsp27) shows that there is an overlap (orange), meaning that there is a co-localization between H2B-GFP-N1 and Fsp27-pDsRed-Express-N1, in the nucleus.

This confirms the cellular location of Fsp27.

4.4 Role of Fsp27 in PPAR signaling

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors, belonging to the nuclear receptor superfamily, that act as fatty acid sensors in metabolic active organs. They mediate the effect of certain drugs and unsaturated fatty acids on gene expression. There are three subtypes know, the PPARα, PPARβ/δ and the PPARγ. [30]

In this part of the project the aim is to study if the expression of Fsp27 affects PPAR signaling.

4.4.1 PPARα

Here, it was used the HMGCS2 promoter, a key part of the ketogenesis, to study the effect of Fsp27 in PPARα signaling.

The AML12 cells were transfected and the luciferase activity was read 48 hours after.

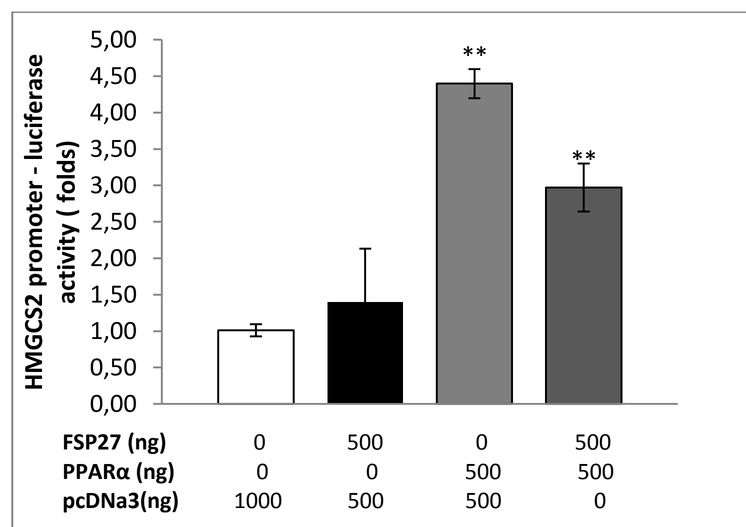

Figure 4.12 - HMGCS2 promoter activity, in the presence of Fsp27 and PPARα. The AML12 cells were co-transfected with pcDNA3, PPARα-pcDNA3 and Fsp27-pcDNA3, and incubated for 48hours. The results of normalized luciferase activities (means+-SD), from three independent experiments, are expressed in folds. (P<0.005)**

In Figure 4.12, it can be seen that there is a high activity of the promoter when only the PPARα expression vector is transfected, which makes sense with its role in metabolism, and its high expression in liver.

When both Fsp27 and PPARα expression vectors are transfected, it is observed a significant decrease in the activation shown by PPARα. It seems that Fsp27 is repressing the activation by PPARα and this will be further studied.

4.4.2 PPARγ

PPARγ, another member of the peroxisome proliferator-activated receptors family, plays an important role in the lipid and glucose metabolism and its expression it's predominant in adipose tissue, since it is essential for adipocytes survival.[30]

The same assays that were performed with PPARα were performed with PPARγ, to conclude if Fsp27 also interferes with the HMGCS2 activation by the transcription factor.

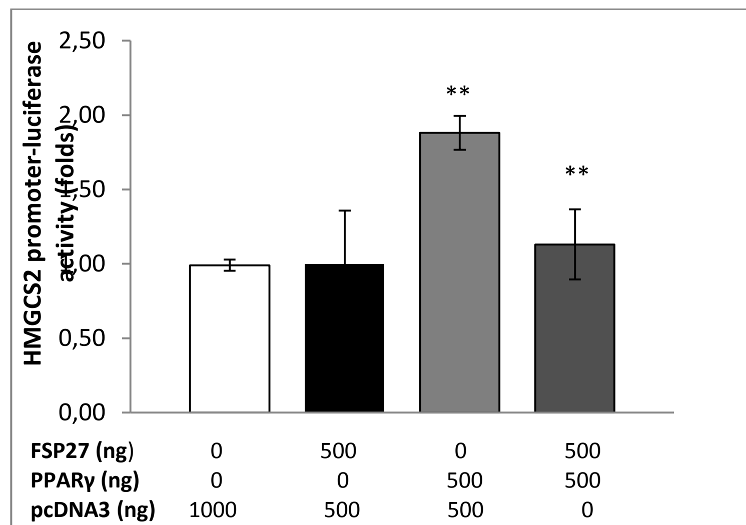

Figure 4.13 -HMGCS2 promoter activity, in the presence of Fsp27 and PPARγ. The AML12 cells were co-transfected with pcDNA3, PPARγ-pcDNA3 and Fsp27-pcDNA3, and incubated for 48hours. The results of normalized luciferase activities (means+-SD), from three independent experiments, are expressed in folds. (P<0.005).**

Although the same behavior is observed, a decrease in the promoter activation when both Fsp27 and PPARγ are transfected in the AML12 cells is seen (Figure 4.13), the activation shown when only the PPARγ is transfected is lower than the one shown by PPARα (Figure 4.12).

This is understandable, since PPARγ is highly expressed in adipocytes, opposing to PPARα that is highly expressed in hepatocytes, and for this experiences the cells used are from mouse liver.

4.4.3 Interaction of PPARα agonist WY 14643 with Fsp27

Since PPARα had a better response with the HMGCS2 promoter in AML12 cells, the interaction between this transcription factor and Fsp27 will be further explored.

One of the hypotheses for the decrease in the activation of the HMGCS2, when both Fsp27 and PPARα were present, was that Fsp27 could be quenching an endogenous ligand of PPARα.

To explore the hypothesis, a pharmacological agonist of PPARα, WY 14643, was used in the presence of the transcription factor.

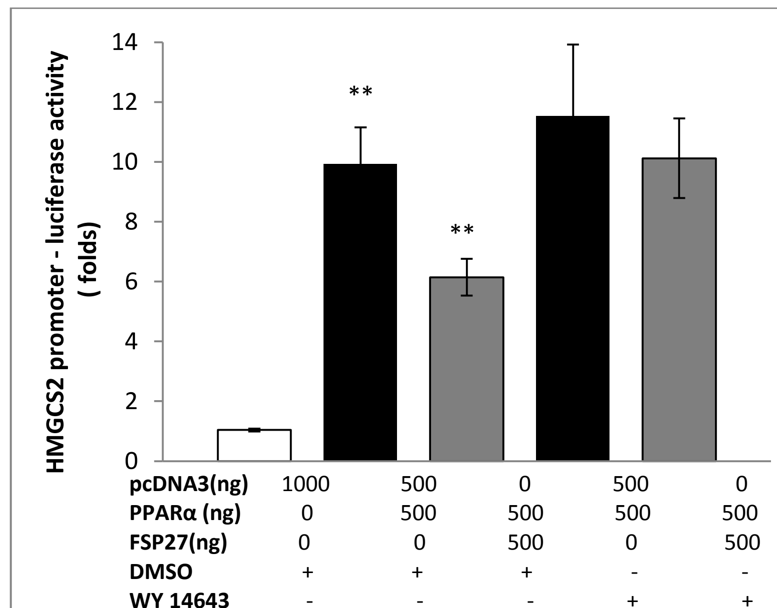

Figure 4.14 -HMGCS2 promoter activity, in the presence of Fsp27 and PPARα. The AML12 cells were co-transfected with pcDNA3, PPARγ-pcDNA3 and Fsp27-pcDNA3, and incubated with 10μM of the pharmacological agonist WY14643 for 24 hours. The results of normalized luciferase activities (means+-SD), from three independent experiments, are expressed in folds. (P<0.005).**

The treatment with the PPARα pharmacological agonist WY 14643 didn't induce the activation of the promoter when only PPARα expression vector is transfected (Figure 4.14 -black bars). This could be because of the existence of endogenous ligands of PPARα, namely fatty acids.

Despite this result, when the cells transfected with both Fsp27 and PPARα were treated with the pharmacological agonist, it seems that the repression observed decreases, and the activation rises to the values of the cells without Fsp27 (Figure 4.14 – grey bars).

One of the hypotheses is that Fsp27 was sequestering the PPARα endogenous ligands, and that is why the activity decreases, and when these cells are treated with the agonist, it has an available site to bind to PPARα and as so, induces its activation. That would explain why the pharmacological agonist would only have an effect when the Fsp27 is present.

4.4.4 Future perspectives

To explore the hypothesis that Fsp27 has an effect in PPAR signaling, by sequestering its endogenous ligands and as so, repressing its activity, one of the options would be to inhibit the fatty acid synthesis pharmacologically (C75/cerulenin) or genetically (shRNA), inhibiting FAS, the key enzyme that regulates the anabolic process.

5. Conclusions

In the first part of this work, the induction of HMGCS2 gene, FoxO3a and FoxO1 was confirmed, using a DNA damaging agent, doxorubicin, inducing stress responses. The tumor suppressor p53 was also activated in response to the DNA damage, as expected.

Despite the observed behavior, it was seen that HMGCS2 induction, by p53 activation, wasn't dependent of FoxO1.

Concerning the Foxo3a results, it can't be concluded if this member of the FoxO family is involved in the HMGCS2 induction, because of the *off target* effects of its siRNA.

P53 activation didn't show any modifications in the siRNA experiments, having a constant expression pattern.

In the second part of the work, it was confirmed that the protein Fsp27 had a nuclear location, in HeLa cells.

Studying the interaction of Fsp27 and PPARα signaling pathway it was found that PPARα endogenous ligands, probably fatty acids, could be sequestered by Fsp27, repressing its activation. This was confirmed with the use of the PPARα's pharmacological agonist WY 14643.

To further corroborate this, fatty acid synthesis should be inhibited, by inhibiting FAS. The expected behavior should be that the pharmacological agonist shouldn't need the presence of Fsp27 to activate PPARα.

This hypothesis follows a model described in literature that indicates that "new" fatty acids are PPARα endogenous activators. New fat produced by FAS accesses a separate pool of PPARα, stimulating cholesterol biosynthesis, gluconeogenesis and FAO. It is said that specific fatty acid pools synthesized by hepatic FAS or derived from the diet activate PPARα to impact glucose and lipid metabolism. On the other hand, old fat stored in the liver doesn't have those effects.

6. Annex

6.1 Constructs

6.1.1 mHMGCS2 promoter construct

The promoter of the mouse mitochondrial HMGCS2 gene was cloned in the pGL3-basic vector, a reporter vector that contains a modified backbone coding region for firefly (*Photinus pyralis*) luciferase, optimized for monitoring the transcriptional activity in transfected eukaryotic cells.[39]

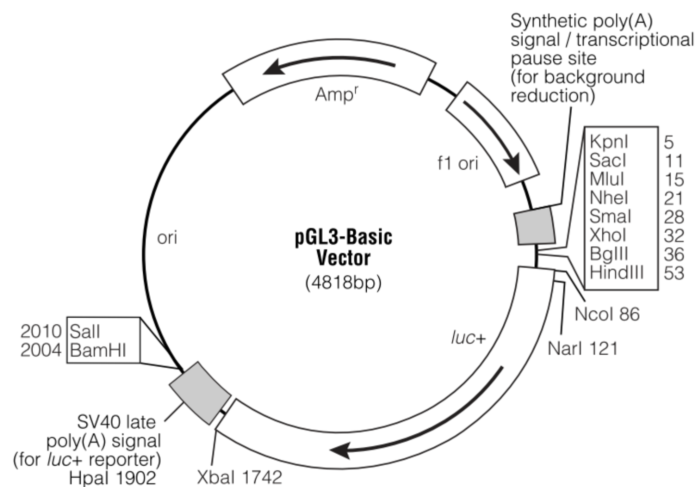

Figure 6.1 - pGL3- Basic Vector circle map.

The genomic DNA was cloned with the enzymes XhoI and MluI and with the following nucleotides: forward: 5' ACGCGTAATCAGGAGGCTGCAGAGAC 3', primer reverse: 5'CTCGAGACAGAAAGCCAGCAGTCCAC 3'.

6.1.2 Fsp27 protein constructs

To clone the native protein in the pcDNA3 vector, it was used cDNA, cloned from the translation initiation site ATG, to the stop codon TGA, with the enzymes HindIII and EcoRI. The following oligonucleotides were used: primer forward: 5' AAGCTTTGACAAGGATGG ACTACGCC 3', primer reverse: 5' GAATTCACTCGGGTCTTCATTGCAGC 3'.

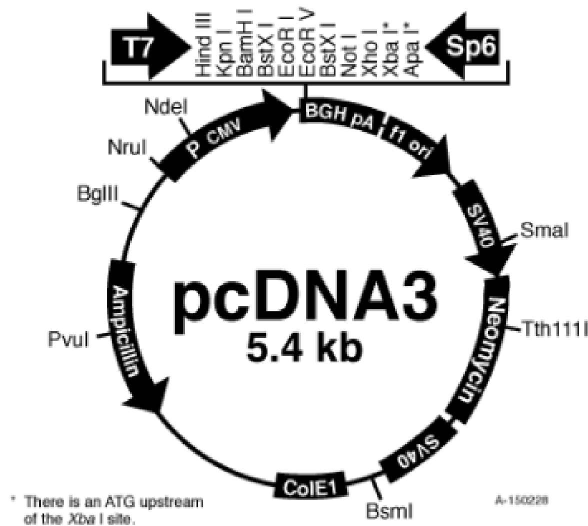

Figure 6.2 - pcDNA3 vector circle map.

The Fsp27 protein was also cloned in a GFP tagged vector. The same enzymes and the same primer forward were used, although the oligonucleotide corresponding to the primer reverse had to be different, to exclude the region containing the stop codon of the Fsp27 protein. The primer reverse used was: 5' GAATTCTTGCAGCSTCTTCAGACAGG 3'.

The vector used was the pDsRed-Express-N1, a mammalian expression vector that encodes a variant of *Discosoma sp.* Red fluorescent protein, the DsRed-Express.

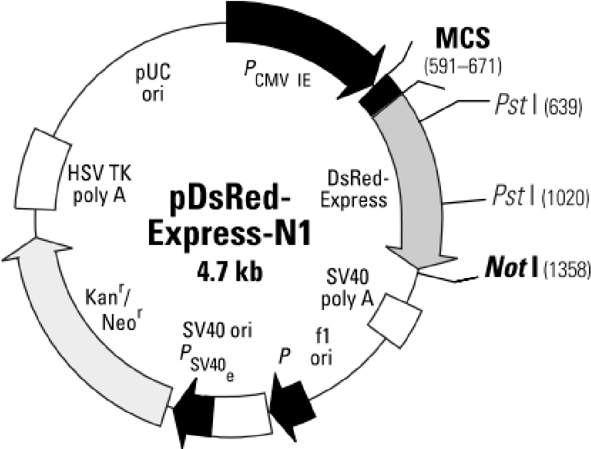

Figure 6.3 -pDsRed-Express-N1 vector circle map.

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