# NR5A2:

# A REGULATOR OF GLUCOSE METABOLISM

# INDEX

# NR5A2: A GLUCOSE METABOLISM REGULATOR

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Nuclear receptors are one of the largest groups of transcription factors in mammals, with 49 different members identified in the human genome. The metabolic nuclear receptors act as metabolic and toxicological sensors, enabling the organism to quickly adapt to environmental changes by inducing the appropriate metabolic genes and pathways. Ligands for these metabolic receptors are compounds from diet, intermediate metabolites, drugs, or environmental factors that, unlike other nuclear receptor ligands, are present in high levels. The interaction ligand-nuclear receptor induces a conformational change leading to the dissociation of corepressors and the recruitment of coactivators that ultimately will result in the activation of downstream target genes. Although most of metabolic receptors show a ligand-dependent activity, some members of this family do not have an established physiological ligand or display constitutive activity. These receptors are named orphan nuclear receptors (Fig. 2).

NR5A2 (Fig. 1) or liver receptor homolog-1 (LRH-1), is also known as pancreas hormone receptor (PHR-1), fetoprotein transcription factor (FTF), human B1-binding factor (hB1F) or CYP7A1 promoter binding factor (CPF), and belongs to the NR5A subfamily of nuclear receptors.

NR5A2, previously thought as an orphan nuclear receptor, exhibits constitutive activity [3, 4]. Crystallographic studies with recombinant mouse NR5A2 revealed that the constitutive activity of this nuclear receptor can be due to the stable conformation of the ligand binding pocket and its association with coactivator recruitment. Although several phospholipids, including phosphatidyl inositols (PI: PI(3,5)P2 and PI (3,4,5)P3, have been found to bind NR5A2 in vitro, its role as physiological ligand it is not yet established[5, 6]. In contrast, several corepressors are known to act by interacting with the ligand binding domain of NR5A2 [7], and several covalent modifications, including phosphorylation and sumoylation, are able to modulate NR5A2 activity (Fig. 3).

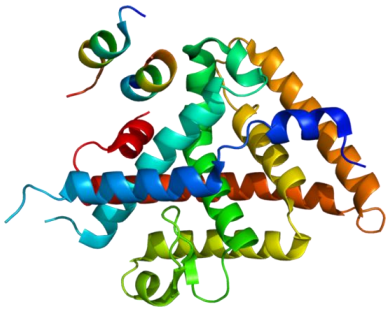

**Fig 1. Structure of NR5A2 protein**. The nuclear receptor has several domains: DNA binding domain with two zinc fingers, ligand binding domain, and a hinger region that links both, DBD and LBD.

## MECHANISM OF ACTION OF NUCLEAR RECEPTORS

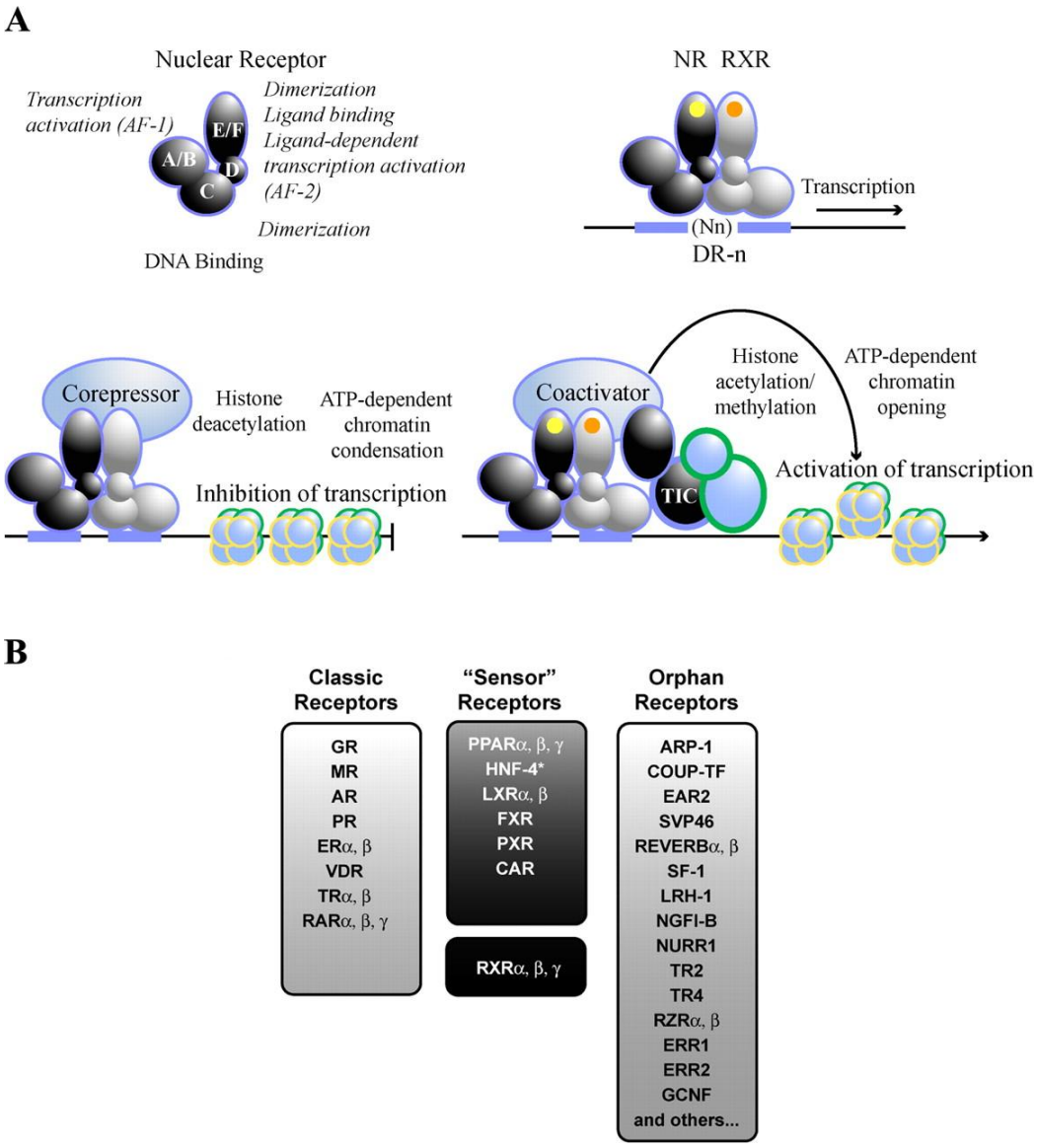


**Fig. 2 Nuclear receptor family**. A) Nuclear receptors can form an homodimer, or a heterodimer, often with the retinoic X receptor. In the presence of ligands, or due to an alternative pathway of activation such as phosphorylation, the corepressor is released and a coactivator is recruited, resulting in transcription enhancement. B) The three functional classes in nuclear receptor family: classic receptors bind hormones and are related to homeostasis, sensor receptors are metabolic receptors, and orphan receptors that have unknown ligand.

Phylogenetic tree analysis of the Ftz-F1 related subgroup of nuclear receptors in Drosophila allowed to indentify NR5A2. Homologs of the NR5A2 protein have now been found in several species. Except for FTF, all cDNAs corresponding to human NR5A2 are identical. NR5A2 is susceptible to alternative splicing to generate isoforms (Fig. 4) with different regulatory functions because at least two isoforms have been identified for human NR5A2 [8]. In contrast to most of the other metabolic receptors which do not dimerize with retinoid X receptor (RXR), the NR5A members bind as monomers to extended nuclear receptor hexomeric binding sites in the regulatory regions of their target genes. Specific recognition of the 3 base pair-long extension is ensured by the conserved Ftz-F1 box, a stretch of about 26 amino acid residues, located C-terminal from the DNA-binding domain.

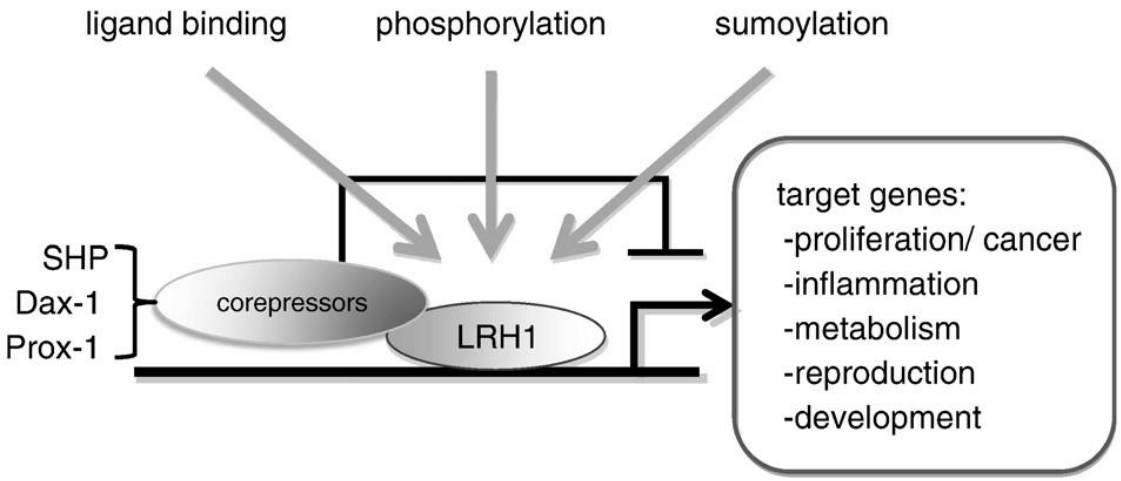


**Fig. 3. Regulation of LRH-1.** LRH-1 activity can be regulated by several mechanisms, which involve transcriptional coregulators, posttranslational modifications (mainly phosphorylation and sumoylation) and potentially phospholipid ligand binding (human LRH-1), although the latter mechanism is still not yet fully understood. Among the coregulators, the corepressors SHP, DAX-1 and PROX-1 are particularly relevant to modulate the constitutive activity of LRH-1 [9].

Like many nuclear receptors, NR5A2 shows pleiotropic functions, NR5A2 is known to participate in several processes related to metabolism, immune function, cell proliferation and reproduction.

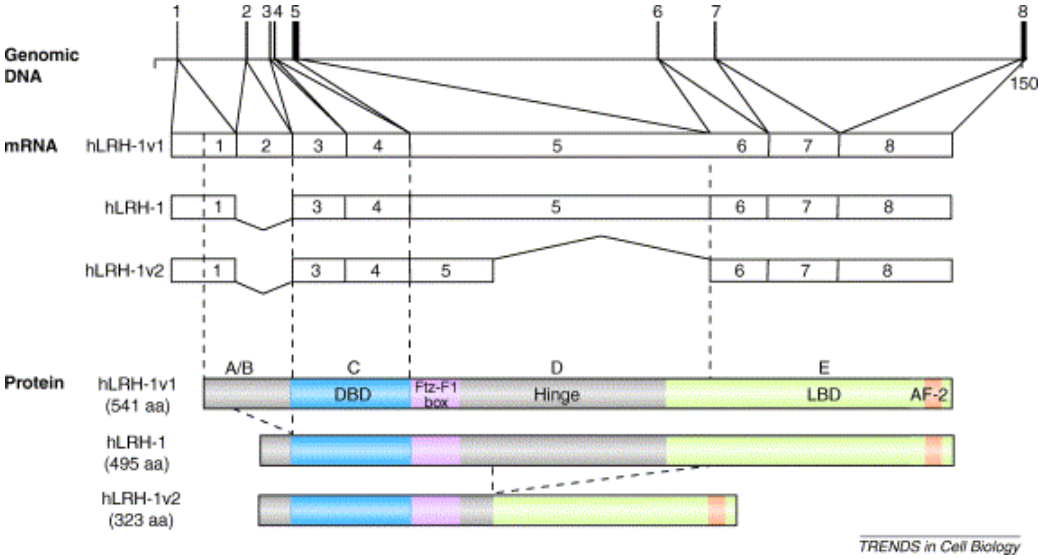


**Fig. 4. Structure of the gene, mRNA and protein isoforms of nuclear repector LHR-1.** Note the eight exons in DNA and the different splicing isoforms. hLRH-1v2 is the isoform present in HepG2 cell lines used in the experiments of our group [8]

## Molecular and physiological actions of NR5A2

### LIVER AND PANCREAS

The role of NR5A2 has been best studied in the liver. Target gene identification studies have showed NR5A2 as a key regulator of reverse cholesterol transport, a process by which cholesterol is transported from peripheral tissues by high density lipoproteins (HDL) to the liver[10, 11]. In addition, NR5A2 was also established as a critical regulator of bile acid homeostasis. In the liver, the NR5A2 role is closely related with the high-affinity interaction of NR5A2 with the nuclear receptor short heterodimer partner (SHP)[12]. When bile acids levels build up in the liver, SHP transcription is induced as a result of the activation of farnesoid X receptor (FXR), the nuclear bile acid receptor. It was demonstrated that an increase in SHP subsequently attenuates the transcriptional activity of NR5A2, leading to the repression of NR5A2 targets, including the rate-limiting enzyme of the bile acid biosynthesis pathway, cholesterol 7α-hydroxylase (CYP7A1) and the enzyme catalyzing the production of cholic acid, cholesterol 12α- hydroxylase (CYP8B1) [12-15]. Some studies showed that NR5A2 in mouse hepatocytes is not important for basal Cyp7a1 expression but in contrast essential for the expression of Cyp8b1 and consequently for bile acid composition. Others have confirmed these findings and have shown that in addition NR5A2 is not essential for the FXR mediated feedback regulation of bile acid synthesis.

GUT

In the gut, NR5A2 is involved to cell renewal. Thus, this nuclear receptor is most highly expressed in the proliferative cells residing in the base of the crypts, and its expression is progressively lost as cells differentiate and migrate towards the villi [9] .This pattern of expression mimics that of β-catenin, which is bound and activated by NR5A2 [16]. (see later).

ADIPOCYTES AND OVARY

NR5A2 is also expressed in adipocyte precursors and in steroidogenic tissues [17-19], including the adrenal and the reproductive organs. In the ovary, NR5A2 have higher levels in the granulosa cells, where it controls essential mechanisms for the maturation of ovarian follicles and for ovulation, including the appropriate regulation of the hormonal balance. In mice, a targeted mutation of the NR5A2 gene in the granulosa compartment produces in infertility as a result of impaired ovulation.

DEVELOPMENT AND CELL CYCLE

NR5A2 plays a master role in the initial biological processes ranging from the stem cell stage to the later phases of development (Fig. 6). Moreover, NR5A2 is involved in the embryonic expression of α-fetoprotein (AFP) and the early expression of other proteins essential for proper liver development.

NR5A2 is present from early morula stage and its deficiency is lethal already at day ~E6.5, indicating its important role during embryonic development. In gastrulation phase NR5A2 becomes to be restricted and is still present in the endoderm and in the primitive mesoderm. During mid-gestation the receptor is abundant in tissues derived from endoderm, such as liver, pancreas and intestine, and in bone, testis and several brain regions. In adult mice, LRH-1 is robustly expressed in the liver, exocrine pancreas, ovary and intestinal tract, all of them endodermic derived tissues. (Fig. 5)

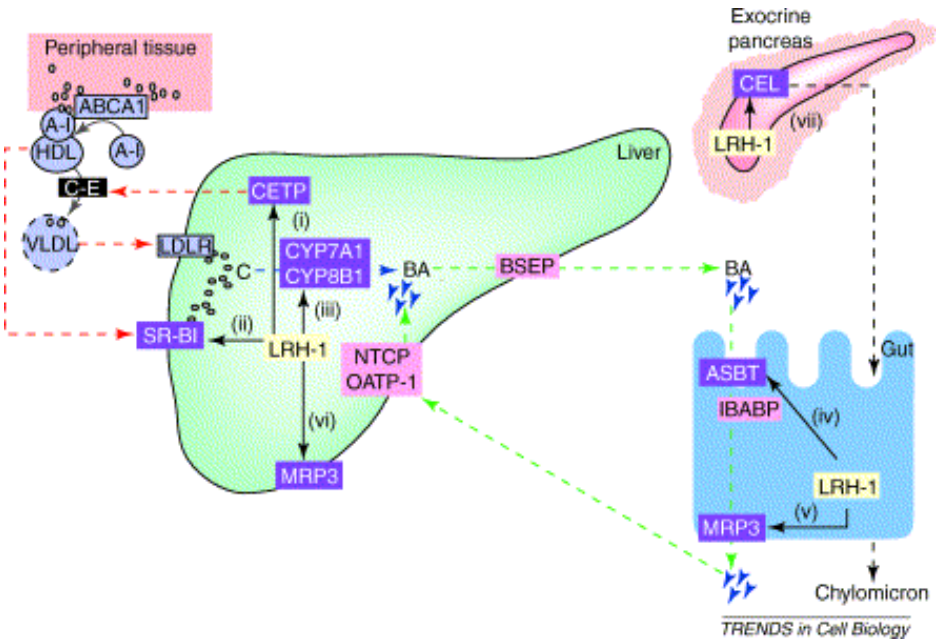


**Fig. 5. Endodermic derived tissues expressing LHR-1.** Liver, pancreas, and gut are some of the organs where NR5A2 role have been best studied. LRH-1 plays a key role in metabolism because several of its target genes are crucial to the regulation of different pathways of cholesterol and bile acid (BA) homeostasis, such as reverse cholesterol transport, BA synthesis, and enterohepatic circulation. [8]

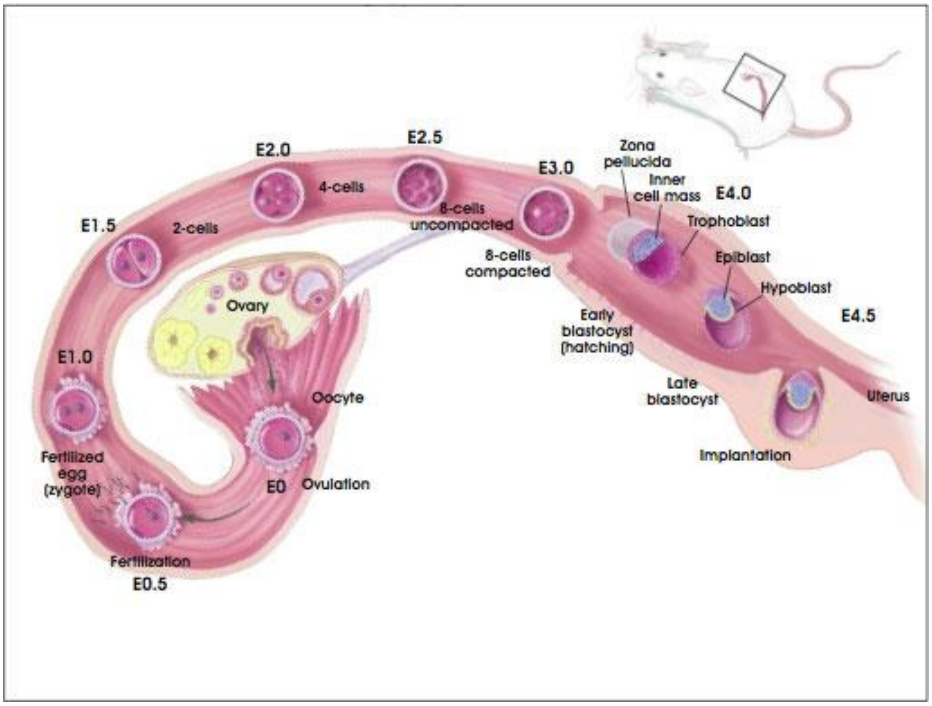


**Fig. 6. Phases of embryonic development in mice**. NR5A2 is present from the early phases of embryo development and highly expressed in embryonic stem cells (ES), in all cells in the morula stage (E2.5) and in the inner cell mass and trophectoderm in the blastocyst stage (E3.5). At E5.5 (egg cylinder), its expression becomes restricted to the visceral endoderm, while ectodermal cells become negative for NR5A2. (© Terese Wislow).

Moreover, an interesting function of NR5A2 in reprogramming has been demonstrated. NR5A2 can enhance the reprogramming of fibroblasts, using the four Yamanaka factors. Only four factors are required—Oct4, Sox2, Klf4, and Myc—to reprogram fibroblasts into pluripotent stem cells. Two nuclear receptors were able to enhance this activity: NR5A2 and the pregnane X receptor. (Fig. 8). NR5A2 can replace Oct4 but not Sox2 or Klf4, as one of the factors required for reprogramming. This is the first identification of a transcription factor that can replace Oct4 for reprogramming. [20, 21] This evidence suggests that there may be multiple ways of generating pluripotent stem cells.

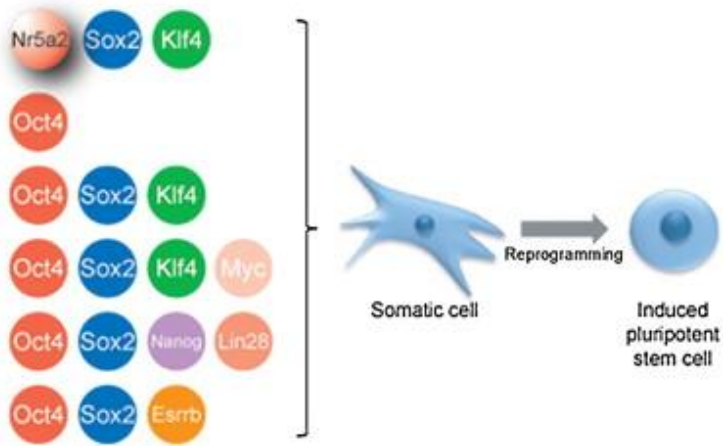


**Fig. 8. NR5A2 and iPSCs relationship**. Nr5a2 can replace Oct4 in the derivation of iPSCs from mouse somatic cells, and it can also enhance reprogramming efficiency. Sumoylation mutants of Nr5a2 with enhanced transcriptional activity can further increase reprogramming efficiency. Genome-wide location analysis reveals that Nr5a2 shares many common gene targets with Sox2 and Klf4, which suggests that the transcription factor trio works in concert to mediate reprogramming. Nr5a2 also works in part through activating Nanog.[20]

NR5A2 is related to cell cycle regulation throught two independent and complementary mechanisms, one consistent with its role as a transcription factor and another with its binding to β-catenin (Fig. 9). Both of them extend the role of NR5A2 from a simple DNA-binding transcription factor to a coactivator via its interaction with β-catenin [16].

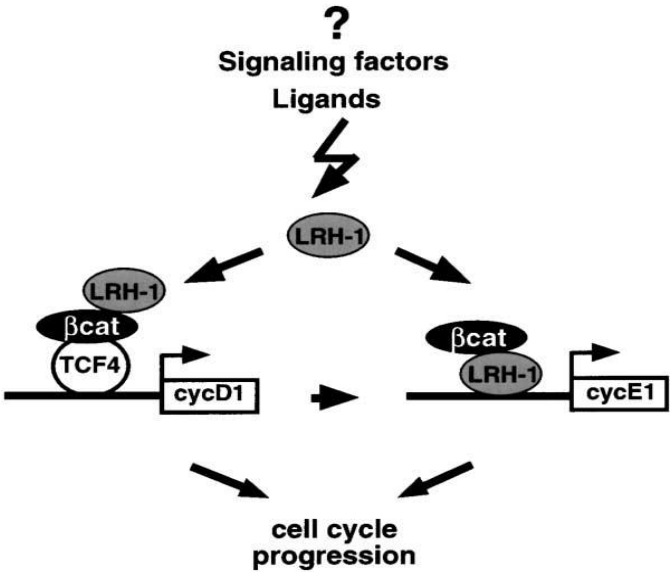


**Fig. 9. Dual Mechanism of LRH-1-Dependent Cell Proliferation.** LRH-1 promotes cell cycle progression by two mechanisms. On the left, LRH-1 acts as a coactivator for catenin, inducing the activation of cycline D1. On the right, LRH-1 acts directly on the cycline E promoter and catenin works as a LRH-1 coactivator.

CANCER

Related to its role in intestinal cell renewal, NR5A2 also modulates intestinal tumor formation in different cancer mouse models. LRH-1-haploinsufficiency significantly inhibits intestinal tumorigenesis both in the azoxymethane-induced intestinal tumor model and in APCmin/+ mice.

Apart from intestinal tumors, NR5A2 has also been associated to several other types of cancer. LRH-1 acts as protumorigenic factor by enhancing estradiol synthesis in preadipocytes, thus driving breast cancer growth. In addition, NR5A2 has been related to gastric, hepatic, pancreatic [14, 22] and endometrium and breast cancer [23, 24].

INFLAMATION

Nuclear receptors in general and LRH-1 in particular, have been repeatedly associated with different inflammatory pathways. NR5A2 has been linked in two ways with the inflammatory response. In one of them, the sumoylation of NR5A2 negatively regulates the expression of acute phase proteins (APPs) [13, 25]. The second pathway by which NR5A2 limits inflammation involves the regulation of extra-adrenal glucocorticoid production in gut [9]. NR5A2 seems to be essential for the local production of corticosterone via its regulation of the steroidogenic enzymes CYP11A1 and CYP11B1.

## PREVIOUS RESERCH

Genetical causes for obesity have always been of interest for our group. Knowing common genetical variances associated to obesity would allow treating efficiently the obese, helped by specific diet, exercise, medicine or surgery. A great effort was made to indentify common short nucleotide polymorphisms related to obesity. These SNPs might have a little but important role in the obese phenotype.[26]

In other hand, the relationship between hormonal balance in ovary and NR5A2 led our group to study the possible association between NR5A2 gene and different estrogen-related phenotypes. Studies in postmenopausical women revealed this association in three phenotypes: bond density, body mass index and diabetes. Table1. shows association between NR5A2 SNP and increased BMI.

| | | AGE | WEIGHT | HEIGHT | BMI |
|---|---|---|---|---|---|
| **CC** | **Mean** | 69,87 | 66,02 | 154,12 | 27,8122 |
| | **N** | 343 | 343 | 343 | 343 |
| | **Desvest** | 6,90 | 9,80 | 5,79 | 4,0431 |
| **CG** | **Mean** | 68,74 | 64,06 | 154,49 | 26,8076 |
| | **N** | 108 | 108 | 108 | 108 |
| | **Desvest** | 6,78 | 10,23 | 6,55 | 3,7342 |
| **GG** | **Mean** | 69,75 | 63,50 | 154,25 | 27,5460 |
| | **N** | 8 | 8 | 8 | 8 |
| | **Desvest** | 6,85 | 9,86 | 5,98 | 3,9658 |

**Table 1. Association studies between BMI and rs2816948**. A functional SNP located in NR5A2 promoter is related to BMI. The three possibilities CC, CG and GG showed statistical differences in BMI.

For a better understanding of NR5A2 implication in obesity our group examined the transcriptome variations due to the silencing of NR5A2. We decided to use hepatocarcinome cells (HepG2 cell line) because of its strong implication in metabolism. The analysis was done by RNA sequencing of RNA samples prepared from cultures treated with NR5A2- specific siRNA.

We found differences in approximately 4000 genes. The results confirmed these ones whose regulation by NR5A2 had been reported previously.

| Pathway | Diffex Genes | All Genes | % Diffex Genes | P value |
|---|---|---|---|---|
| Complement and coagulation cascades | 26 | 69 | 37,68% | 1,61E-10 |
| Glycolysis / Gluconeogenesis | 22 | 62 | 35,48% | 3,09E-08 |
| Glycine, serine and threonine metabolism | 12 | 31 | 38,71% | 8,18E-06 |
| Metabolism of carbohydrates | 26 | 101 | 25,74% | 4,30E-05 |
| Biological oxidations | 30 | 124 | 24,19% | 6,12E-05 |
| PPAR signaling pathway | 18 | 69 | 26,09% | 0,0005 |
| Drug metabolism - cytochrome P450 | 18 | 72 | 25,00% | 0,0011 |
| Hemostasis | 48 | 256 | 18,75% | 0,0019 |
| Retinol metabolism | 16 | 64 | 25,00% | 0,0021 |
| Metabolism of xenobiotics by cytochrome P450 | 17 | 70 | 24,29% | 0,0024 |
| Steroid hormone biosynthesis | 14 | 55 | 25,45% | 0,0031 |
| Tyrosine metabolism | 12 | 46 | 26,09% | 0,0046 |
| Metabolism of amino acids | 31 | 163 | 19,02% | 0,0097 |
| Starch and sucrose metabolism | 12 | 52 | 23,08% | 0,0187 |
| Type II diabetes mellitus | 11 | 47 | 23,40% | 0,0213 |
| Peroxisome | 16 | 78 | 20,51% | 0,0285 |
| Glutathione metabolism | 11 | 50 | 22,00% | 0,0381 |
| Total | 340 | 1409 | | |

**Table 2.** Metabolic pathways affected by NR5A2 silencing. Data obtained by RNA sequencing.

Most interestingly, they showed a significant association of NR5A2 with the expression of genes which encode proteins involved in carbohydrate metabolism (p= 4.3 x 10-5). The association is even stronger with those genes related to glycolysis and gluconeogenesis (p= 3.09 x 10-8) (Table 2). Among these genes it was the one coding for the catalytic subunit of glucose 6-phosphatase, an essential enzyme for the liberation of glucose from the hepatocyte and therefore a major enzyme for the control of plasma glucose levels. Also the catalytic subunit of the enzyme hexokinase 1 was altered.

The dependece of several carbohydrate genes was further confirmed by qPCR analysis of stable cells lines in which the expression of NR5A2 was supressed by the infection with lentiviral vectors coding for specfific NR5A2 shRNAs. In this cell lines the suppression of NR5A2 is more efficient (aproximately 5% of the control cells) and accordingly the differences on gene expression are more dramatic than those obtained with siRNA treatment.

A glucose consumption experiment was done to support our results. Finally, glucose measurement indicated the consumption is increased in those cell lines having a less proliferation.

## OBJECTIVES

Here we show the validation of the RNA sequencing results in cells overexpressing NR5A2, which support the previous dates in a NR5A2 KO cell line. Both of them seem to sustain that the NR5A2 association to diabetes could be understood by its role in the regulation of the expression of gene coding glycolysis and gluconeogenesis enzymes

Confirming the implication of NR5A2 in glucose metabolism
Obtaining statistical data for overexpressing NR5A2 and the affecting rate of tested genes.
Correlating silencing and overexpressing experiments.

## METHODOLOGY

### Cell culture

The hepatocarcinoma cell line HepG2 was cultured in Dulbecco's Modified Eagle Medium (DMEM), with L-glutamine and 4.5g/l of glucose (LONZA, Cat. N0 BE12-604F) plus 10% (vol/vol) fetal bovine serum (FBS) (LONZA Cat Nº DE14-801F) and 50u/ml of penicillin and 50μg/ml of streptomycin (Invitrogen, Cat. Nº15070). Cells were grown at 37Cº in 5% CO2.

### Construction of stable cells overexpressing NR5A2

A NR5A2 optimized sequence cDNA was synthesized *in vitro* (GENEART) and cloned into the Hind III and 3' ECORI cloning sites of the expression vector pCEFL (Fig 14) . Positive colonies were confimed by sequencing. HepG2 cells were transfected with the resulting plasmid in an amaxa nucleofector (LONZA), (10µg DNA/106 cells and T-028 program). Two stable clones clones were selected with G418 (750 mg/l) at 48h. No differences were found between the two clones. NR5A2 overexpression was tested both by inmunofluorescence and qPCR for both NR5A2 and the NR5A2 target ApoA1 [27].

### RNA extraction

RNA samples were extracted from the different samples at 24 or 48 hours after the medium renewal following transfection. RNAeasy plus columns (QIAGEN Cat. N0 74134) were used as recommended by the manufacturer. The samples were eluted in 50 µl of RNase free water.

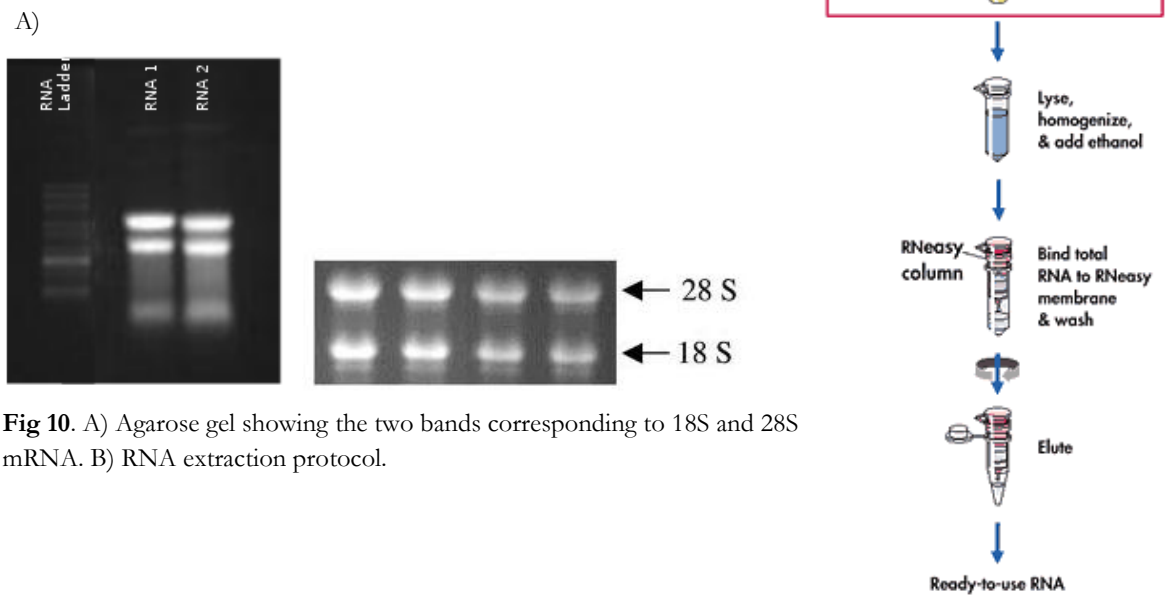


**Fig 10**. A) Agarose gel showing the two bands corresponding to 18S and 28S mRNA. B) RNA extraction protocol.

RNA quality was tested by electrophoresis in 1% formaldehyde/agarose gels (weight/vol) and run in 0,5% TAE. Two bands should be clearly observed attending to different molecular weight of the mRNA subunits 18S and 28S.(Fig. 10)

NanoDrop (Micro-Volume UV-Vis Spectrophotometer for Nucleic Acid and Protein Quantitation) was used to quantify the RNA concentration, using 10µl of sample

### cDNA synthesis

cDNA was synthesized from 1μg of total RNA from the different samples using the iScript rt cDNA synthesis kit (Bio-Rad (N0170-8890) and following the manufacturer's protocol.

| Components | Volume per Reaction |
|---|---|
| RNA | x (≈1μg) |
| 5x iScript Reaction Mix | 4 μl |
| iScript Reverse Transcriptase | 1 μl |
| Nuclease Free Water | x μl |
| Total Volume | 20 μl |

**Table 3. Reaction agents for cDNA synthesis.**

### Inmunofluorescence

HepG2 cells were grown in T-6 plate with 10mm diameter cover glasses until 50% confluence. Then, cover glasses were retired and washed with cold PBS 1x and then fixed with cold methanol during 5 minutes. The primary antibody incubation, anti-NR5A2 H-50 from Santa Cruz Biotechnology (sc-25389), was kept in wet camera during an hour and 1:100 concentration (vol/vol). The secondary antibody incubation, anti-rabbit IgG conjugated to a green fluorophore, was kept in wet camera during 45 minutes at 1:100 concentration (vol/vol). DAPI was used to dye nucleus. A ZEISS Imager M1 fluorescence microscopy was used for visualization.

### Glucose absorption assay

A cell density of 20,000 cells per T-24 well were seeded in DMEM with a glucose concentration of 1g/l. Samples of medium were collected at 24, 48 and 72 hours. The GOD-POD system was used to determine the glucose concentration by the absorbance at 505nm. The results were normalized by the cell number.

### Candidate target genes selection

Previous RNA sequencing in NR5A2 silencing experiments expression showed differences in several metabolism-related genes. From this battery of genes, the ones related to diabetes were chosen for PCR studies. S14 was used as a control gene and ApoA1 as a known target gene

## PCR

Termociclator C1000TM Thermal Cycler de Bio Rad was employed. 1µl of cDNA per sample in 12.5µl of total PCR mix was used. BIOMIXTM RED 2x de Bioline ( Cat. N0 BMR151-1011) provided the dNTPs and polimerase.

qPCR: Single Color Real Time PCR IQ5 de Bio Rad was used. 1µl of cDNA per sample in 12.5µl of total PCR mix was used. IQTM SYBR green supermix 2x (BIO RAD, Cat. N0 170-8882) Fig. 11)provided the polimerase, the dNTPs and SYBR green. The paired primers were used at 200nm total concentration and for the analysis the S14 was used to normalizing gene expression.

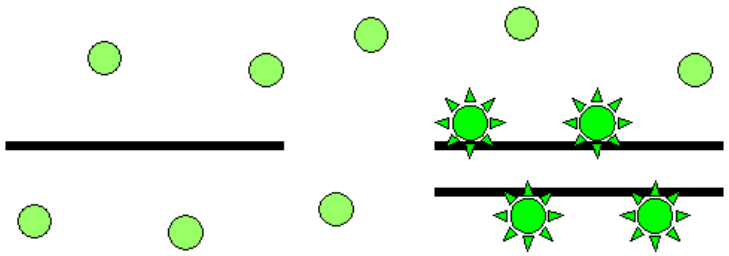

**Fig 11. SYBR green reaction**. The stain binds to double-stranded DNA and it is used as a dye for the quantification in real time PCR. The resulting DNA-dye-complex absorbs blue light ($\lambda_{max}$ = 488 nm) and emits green light ($\lambda_{max}$ = 522 nm).

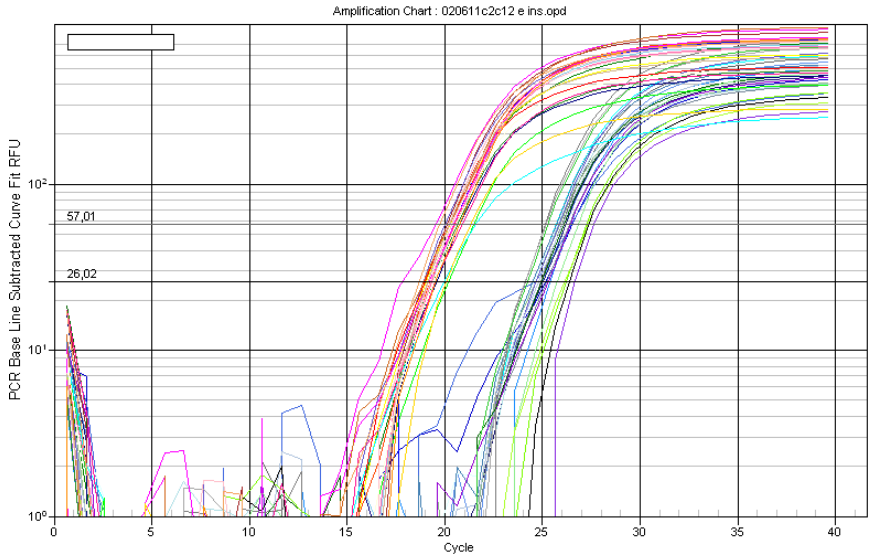


**Fig. 12 Ct determination**. The reference value to quantificate is the midpoint at the tangent, named ct. Thus, less ct means higher cDNA quantity.

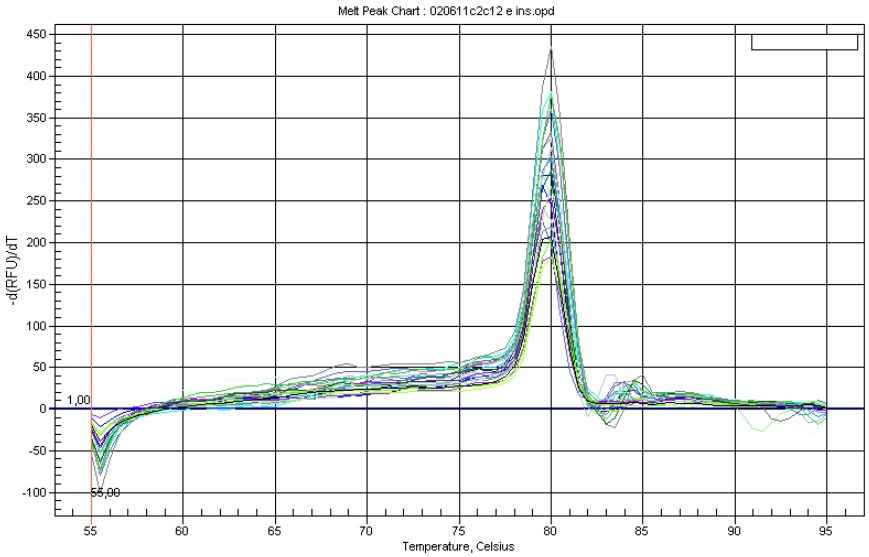


**Fig. 13. Amplification point**. It supplies the melting temperature of the cDNA fragment. Also, it shows the specific union between primers used and cDNA sample by the number of peaks.

Ct method.( Fig 12.) involves comparing the Ct values of the samples of interest with a control or calibrator such as a non-treated sample or RNA from normal tissue. The Ct values of both the calibrator and the samples of interest are normalized to an appropriate endogenous housekeeping gene (Fig. 13).

The comparative Ct method is also known as the 2–[delta][delta]Ct method, where [delta][delta]Ct=[delta]Ct,sample-[delta]Ct,reference
Here, [delta]CT,sample is the Ct value for any sample normalized to the endogenous housekeeping gene and [delta]Ct, reference is the Ct value for the calibrator also normalized to the endogenous housekeepinggene.

For the [delta][delta]Ct calculation to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal. This can be established by looking at how [delta]Ct varies with template dilution. If the plot of cDNA dilution versus delta Ct is close to zero, it implies that the efficiences of the target and housekeeping genes are very similar. If a housekeeping gene cannot be found whose amplification efficiency is similar to the target, then the standard curve method is preferred.

## Primers for PCR and qPCR

| Testing primers for HepG2 cell line | Testing primers for candidate target genes |
|---|---|
| NR5A2 Fw TACCGACAAGTGGTACATGGAA | G6PCFw TACGTCCTCTTCCCCATCTG |
| NR5A2 Re CGGTTGTGATGCTATTATGGA | G6PCRe CCTGGTCCAGTCTCACAGGT |
| APOA1 Fw AGCTTGCTAAGGTGGAGGT | FBP1Fw TCAACTGCTTCATGCTGGAC |
| APOA1 Re ATCGAGTGAAGGACCTGGC | FBP1Re CGTAGACCAGAGTGCGATGA |
| NR5A2 Overexp. Fw CAGAACTGCCAGATCGACAA | PFKFBP1Fw GTTTACCAGCTCGAGGCAAG |
| NR5A2 Overexp. Re CTGCCGCCTCTCATTCTATC | PFKFBP1Re AAAACCGCAACATGACCTTC |
| **Housekeeping** | FBP2Fw TCTTCCTGTACCCAGCCAAC |
| S14 Fw CCGGGATAGCTGTCCTGTTA | FBP2Re TAGCTGCCTGCCTGATTTTT |
| S14 Re TCCCTGCAAGCTCAATAGGT | HK1Fw AGACGCACCCACAGTATTCC |
| | HK1Re CGCATCCTCTTCTTCACCTC |
| | PPARAFw AGTCTCCCAGTGGAGCATTG |
| | PPARARe GTTGTGTGACATCCCGACAG |
| | PGC1AFw CCTGCATGAGTGTGTGCTCT |
| | PGC1ARe GCAAAGAGGCTGGTCTTCAC |
| | FABP1Fw GCAGAGCCAGGAAAACTTTG |
| | FABP1Re TCTCCCCTGTCATTGTCTCC |

## Statistics

Statistical analysis was performed with the two tailed Student t-test and error bar represents means s.e.m.± P value <0.05 was considered statistically significant. One, two or three asterisks indicate significance levels of 5, 2 or 1% respectively.

## Bionformatics

DNA and RNA sequences searching and oligos specificity validation http://www.ncbi.nlm.nih.gov/ and http://genome.ucsc.edu/ programs were used.

Oligos design: http://frodo.wi.mit.edu/primer3/were

qPCR analysis Bio-Rad IQ-5 software

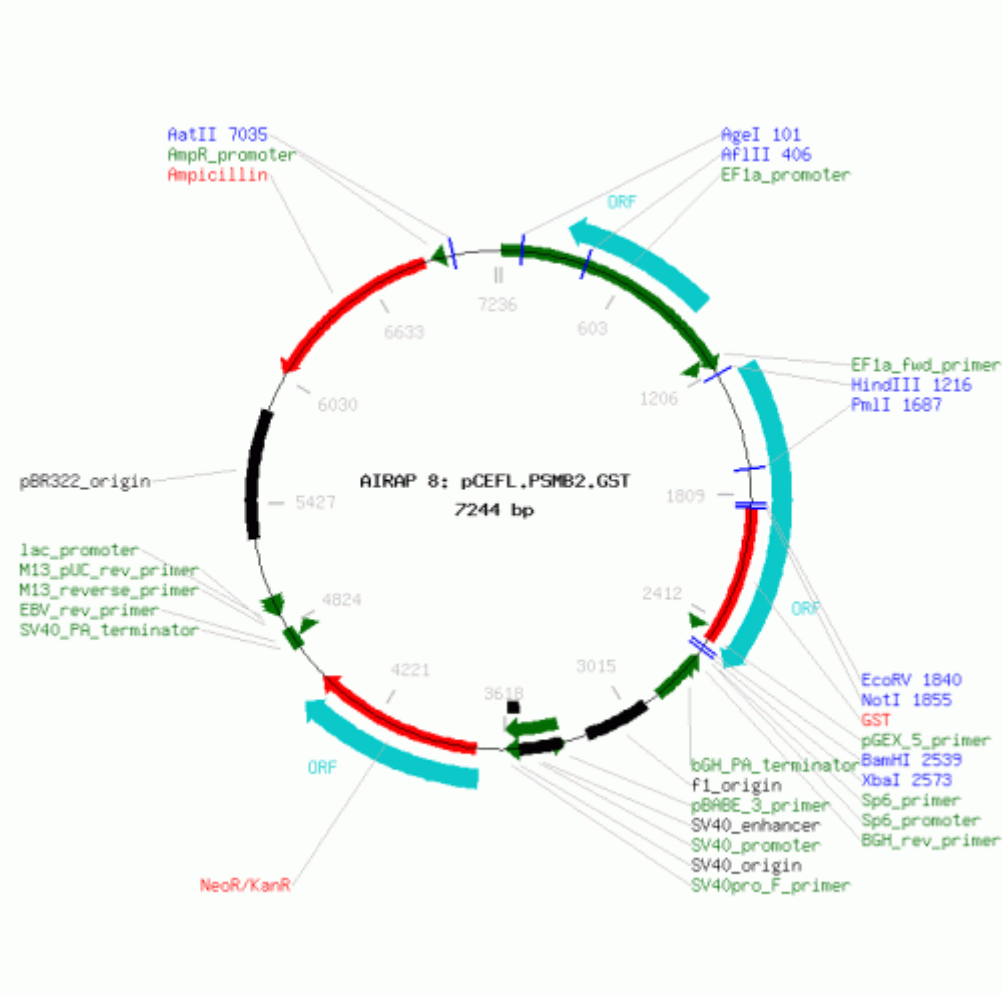


**Fig. 14. Eukaryotic plasmid used for the overexpressing NR5A2 construction**. Note the 5' Hind III and 3' ECORI sites and the gentemycin resistant region.

## MODEL VALIDATION

Two independent assays were made to confirm the over-expression of NR5A2 in cells: a qPCR for exogenous and endogenous NR5A2 and an inmunofluorescence for control HepG2 cells and pCEFL-NR5A2 cells. Agarose gel analysis of qPCR showed a band in transfected cells (marked with an arrow in the Fig.15). This band is not seen in non-transfected HepG2 cells. Also, the expression levels of ApoA1, a well-known NR5A2 target [27] were increased.(Fig.16). Inmunofluorescence showed nuclear localization of NR5A2 protein like the overlapping green and blue (DAPI) fluorophores. Note the higher intensity of green in NR5A2 overexpressing cells compared to control cells (Fig. 17).

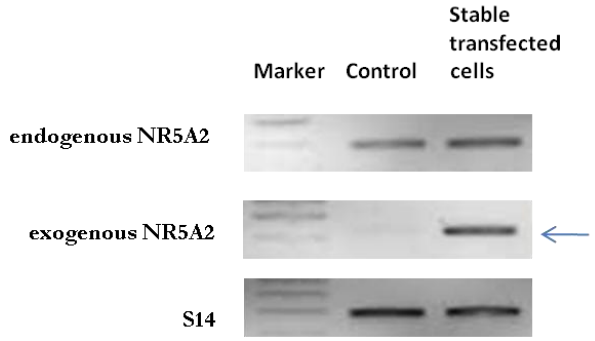


**Fig.15 Agarose gel analysis of qPCR;** Different specific primers were used for endogenous NR5A2 and exogenous NR5A2. S14 was used as a load control gene, NR5A2 transcript originated from exogenous NR5A2 gene was seen in the stable transfectant (blue arrow).

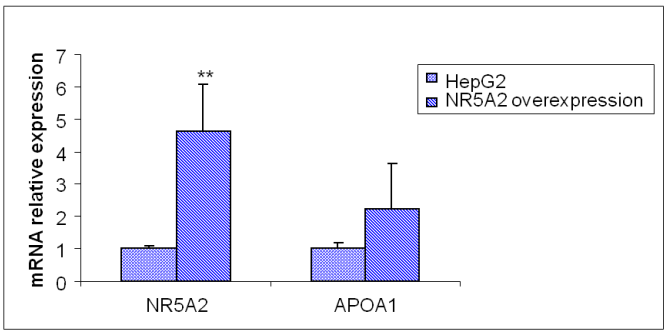


**Fig.16** Quantification **of qPCR results.** Columns indicated a 4-fold increase in the total NR5A2 mRNA levels ($p<0,01$) together with 2-fold increase in APOA1 mRNA levels.

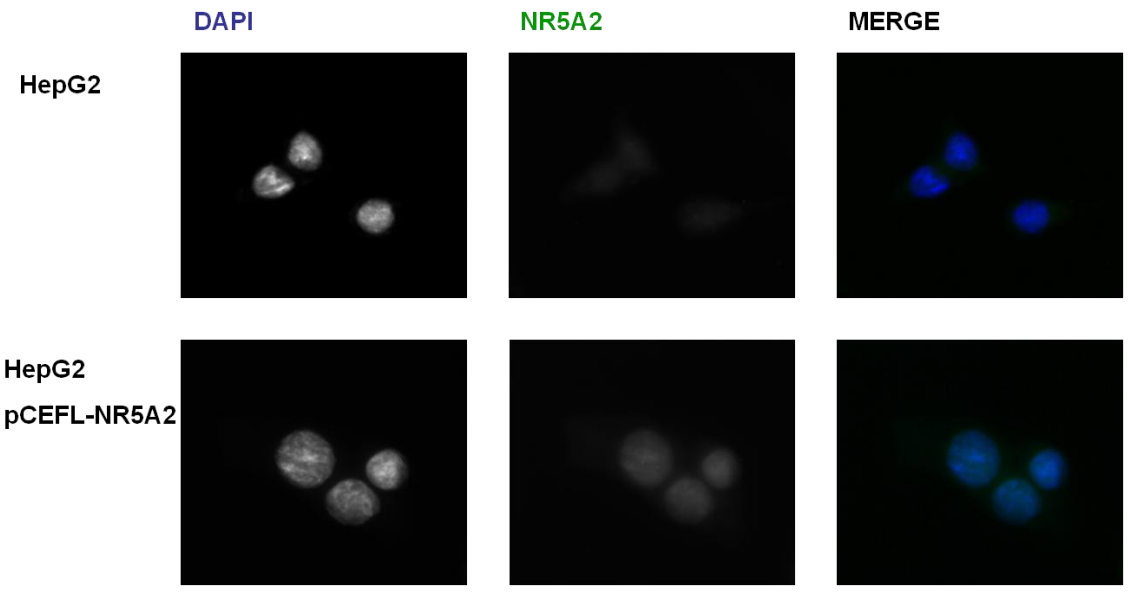


**Fig. 17 Inmunofluorescences in HepG2, against HepG2 pCEFL-NR5A2**. The specific nuclear localization is clearly observed in both cases. The higher fluorescence intensity in the pCEFL-NR5A2 cells, demonstrates the increase in NR5A2 protein levels.

## RESULTS

### Effects on gene expression

Several candidate genes, identified in previous work by Bolado (see previous research), were tested to demonstrate the relationship between glucose metabolism and the nuclear receptor NR5A2. A qPCR study was made for each one and the results were statistically treated and represented below (a short explanation of the metabolic function has been added for a better comprehension).

**Hexokinase I** is an enzyme which catalyzes the phosphorylation of glucose to yield glucose 6-phosphate, the reaction that initiates all physiologically relevant pathways of glucose utilization, including glycolysis and the pentose phosphate pathway. Figure 18 shows a decrease of approximately 3-fold in transfected cells comparing to control HepG2 cells (2,92±0,15004591) and p value= $3{,}2*10^{-5}$ . In previous silencing experiments hexokinase transcription levels showed an increase in cells treated with siRNA (data not published), demonstrating an inverse relationship with the overexpressing results.

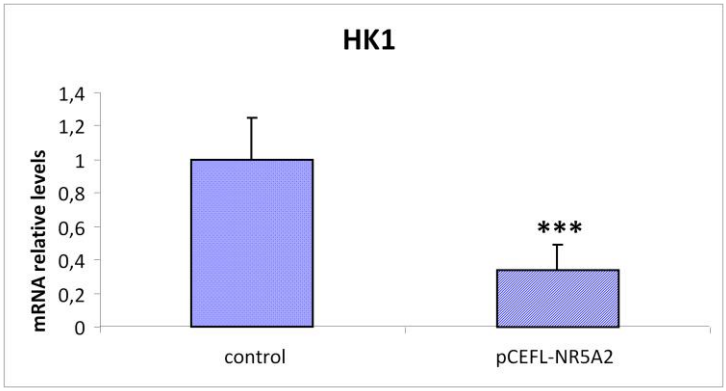


**Fig.18**. Hexokinase I expression decreases in cells overexpressing NR5A2 (p=3,23855E-05)

**Glucose 6-phosphatase** is an enzyme that hydrolyzes glucose-6-phosphate resulting in the creation of a phosphate group and free glucose. This catalysis completes the final step in gluconeogenesis and glycogenolysis and therefore plays a key role in the homeostatic regulation of blood glucose levels. Hexokinase I has the opposite function. Fig 19 shows a decrease of higher 3-fold in glucose 6-phosphatase transcription levels (3,21755556± 0,5019119) with p= 0,002937878 compared to control cells. These results are in accordance to the ones in silencing experiments, in which the RNAm levels were decreased (data not published).

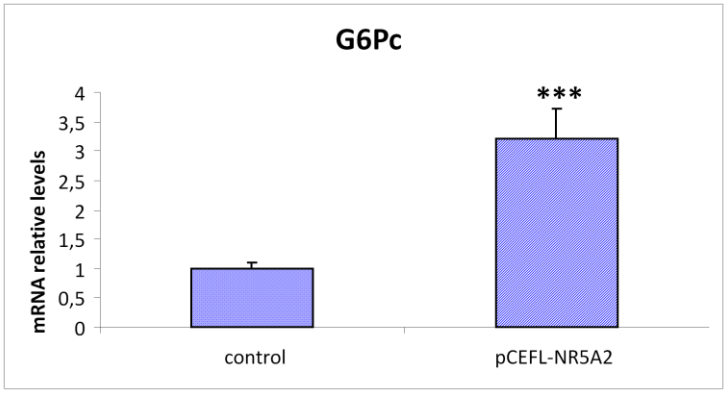


**Fig. 19** Glucose 6-phosphatase expression increases in cells overexpressing NR5A2 (p= 0,002937878)

**Phosphofructokinase/fructose 2,6 bifosfatase** is an allosteric and bifunctional enzyme. The kinase function works in glycolysis while phosphatase works in gluconeogenesis. Figure 20 shows an increase of approximately 2-fold in transcription levels in cells overexpressing NR5A2 (1,9482125± 0,98076693) compared to control cells, with a p value= 0,007159222. Again, these results are correlated to the silencing results, in which the transcription levels of this enzyme decreased.

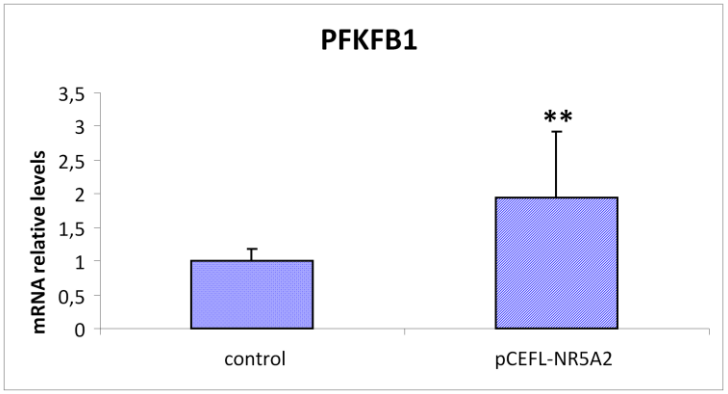


**Fig.20**. 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase expression increases in cells overexpressing NR5A2 (p=0,007159222)

**Fructose-1,6-bisphosphatase 1** is a gluconeogenesis regulatory enzyme, catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate in gluconeogenesis. Figure 21 shows a strong decrease in FBP1 levels in pCEFL-NR5A2 cells compared to control cells, higher 3-fold (0,26666667± 0,07527727) with a p value= $3{,}93402*10^{-5}$).These results, as expected, are the opposite to the silencing results.

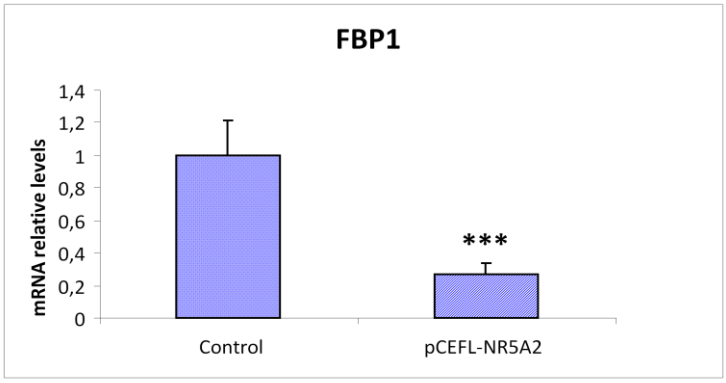


**Fig.21**. Fructose-1,6-bisphosphatase 1 expression decreases in cells overexpressing NR5A2 (p=3,93402E-05)

**Fatty acid binding proteins** bind long-chain fatty acids and other hydrophobic ligands and are also able to bind bile acids. It is thought that FABPs roles include fatty acid uptake, transport, and metabolism. Figure 22 shows a strong increase of FABP1 levels in cells overexpressing NR5A2, ~2,5-fold (2,656± 0,53794258) with a p value= $9{,}26557*10^{5}$. In silencing experiments, cells treated with shRNA showed decreased levels of this enzyme.

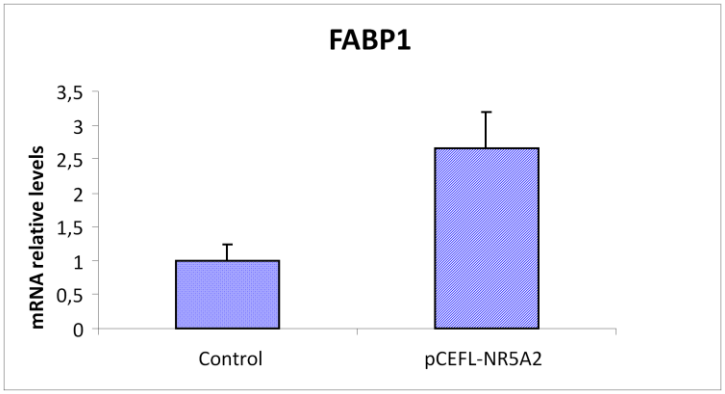


**Fig.22** Fatty acid binding protein expression increases in cells overexpressing NR5A2 (P=9,26557E-05)

**PPARA** is a transcription factor and a major regulator of lipid metabolism in the liver. Activation of PPAR-alpha promotes uptake, utilization, and catabolism of fatty acids by upregulation of genes involved in fatty acid transport and peroxisomal and mitochondrial fatty acid β-oxidation. Figure 23 shows an increase of approximately 2-fold in cells overexpressing NR5A2 (1,87±0,50765568) with a p value = 0,007546509. The RNAm levels were decreased in our previous silencing experiments.

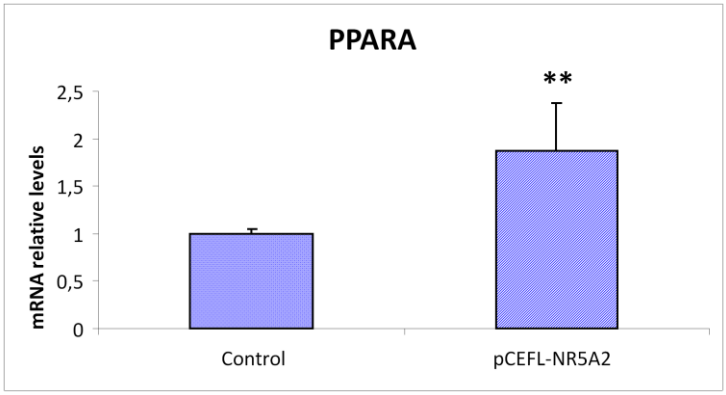


**Fig. 23** Peroxisome proliferator-activated receptor alpha expression increases in cells overexpressing NR5A2 (p=0,007546509)

**PGC-1α** is a transcriptional coactivator that regulates the genes involved in energy metabolism. This protein is thought to be a master integrator of external signals and may be also involved in controlling blood pressure, regulating cellular cholesterol homoeostasis, and the development of obesity. The cells treated with shRNA showed a decrease in PGC-1α transcription levels, just the opposite to the results obtained in overexpressing experiments, in which the pCEFL-NR5A2 cells show an increase of ~1,4-fold (1,361±0,38304192) with a p value = 0,163101928.

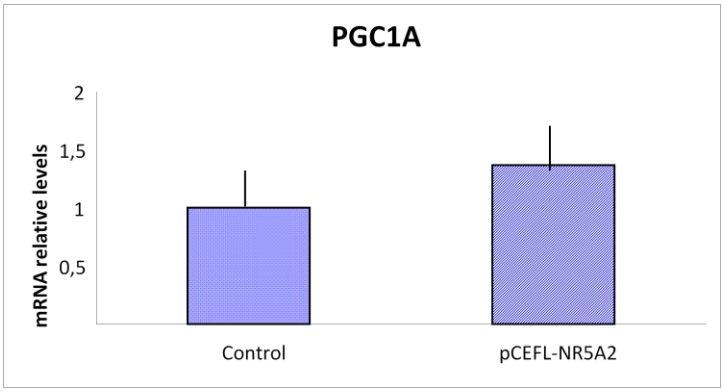


**Fig. 24** Peroxisome proliferator-activated receptor gamma coactivator 1-alpha expression increases in cells overexpressing NR5A2 (p=0,163101928).

**Effects on glucose metabolism**

Because of the involvement of NR5A2 in the regulation of genes related to glucose pathways, we performed an independent experiment where glucose concentration in the medium was measured. A specific medium was prepared with 1g/l of glucose. Twelve wells were inoculated with 20000 control cells and twelve wells with 20000 cells overexpressing NR5A2. The concentration of glucose was then measured at 72hours. Differences in the media were found. Our results showed the glucose concentration was smaller in medium of control cells than in medium of cells overexpressing NR5A2.

Figure 25 indicate that control cells removed a high concentration of glucose from the medium (1±0,08383234), whereas cells overexpressing NR5A2 removed less glucose from the medium (0,50499002±0,18962076) and the p value = 0,00318017.

Curiously, we observed the pCEFL cells have a faster proliferation than control cells. Because of that, the results of glucose consumption were measured normalizing with the number of cells per well.

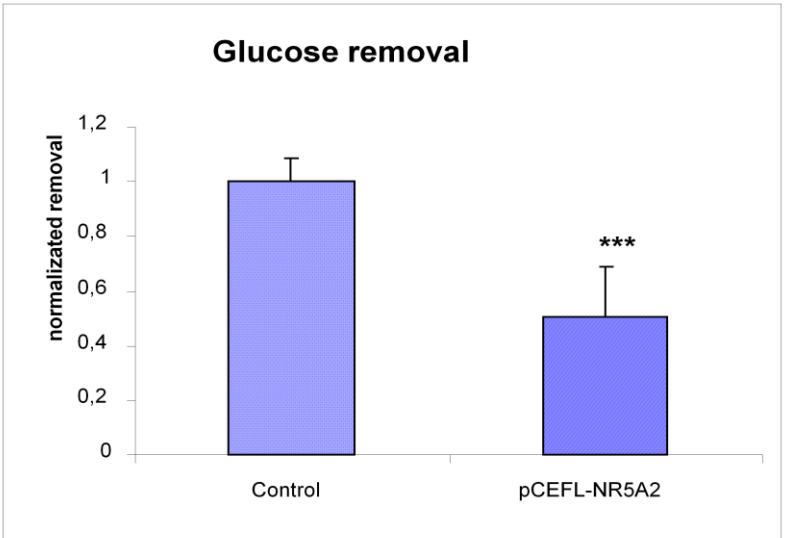


**Fig. 25** Removal of glucose from the medium in cells overexpressing NR5A2 (results normalized with the number of cells per well).

### Effects on proliferation

Changes in glucose concentration could be related to the proliferative rate. This suspect led us to test proliferation studies. Thereby, number of cells was counted by hemocitometer. From an initial concentration of 20.000 cells for both, control cells and stable transfectant cells, samples were collected and counted at 72h. Differences on proliferation were found between control HepG2 cells and cells overexpressing NR5A2.

The quantification showed that control cells have a slower growing rate than stable cloned cells. The pCEFL-NR5A2 has a higher proliferation rate ~1,9-fold (1,89±0,37388077) with a p value = 2,47134E-10.

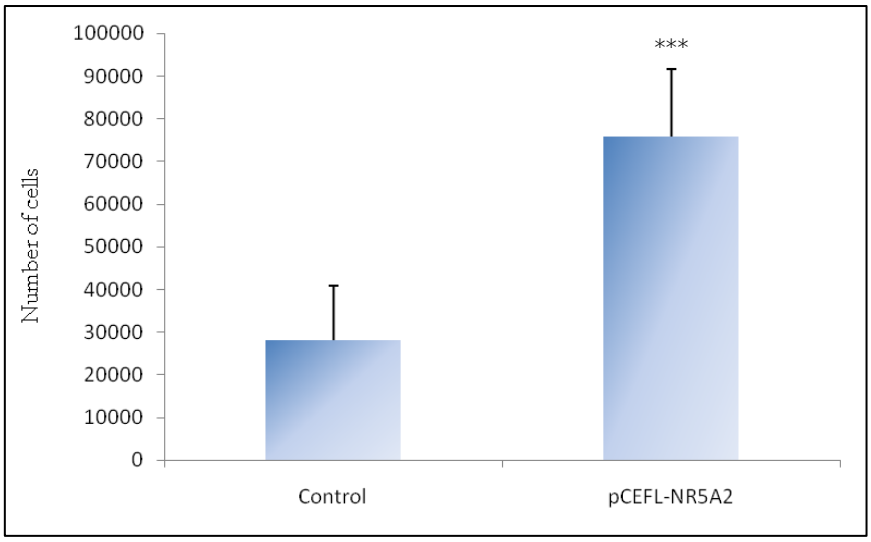


**Fig. 26.** Number of cells at 72h is higher in cells overexpressing NR5A2 comparing to control cells and is statistically significative with a p value = 2,47134E-10.

Curiously, changes in profiferation were not the consequence of glucose changes.

### Effects on cell and colonies morphology

Morphological differences were not observed between control cells and cells overexpressing NR5A2. However, their growing occurs in a distinct way. While normal HepG2 colonies grow horizontally along the entire plate surface, the cloned ones tend to grow upright producing roundy colonies (Fig 27 and 28).

## Control HepG2

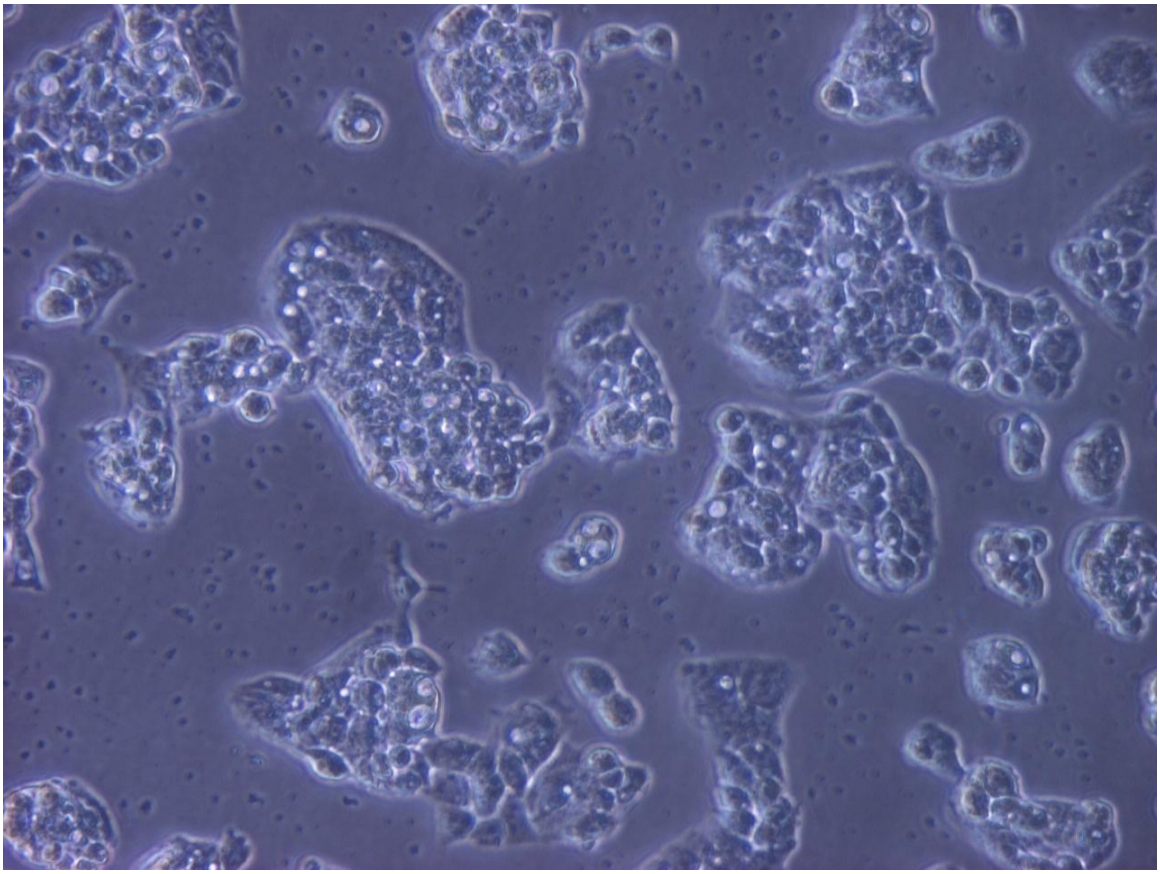

**Fig. 27. Cells with normal levels of NR5A2 expression grow along the plate.**

Control HepG2 cells form colonies that cover the plate horizontally. A plate containing DMEM is inoculated with approximately 50.000 cells. The whole plate becomes plenty in 7-10 days, and then trypsinization is needed to rise up cells. Those cells will be inoculate in a new plate and will begin a new cycle.

## Stable cloned cell line

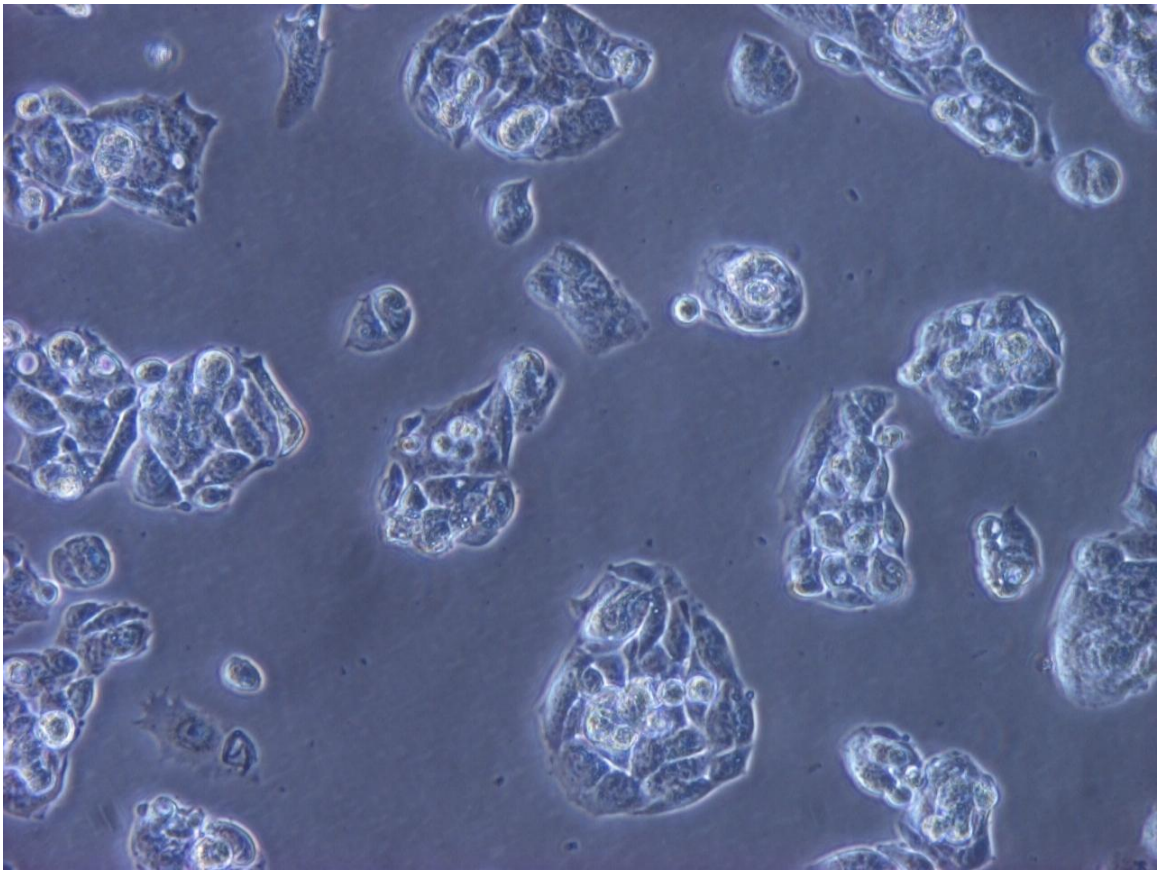

**Fig. 28 Colonies of cells overexpressing NR5A2 grow vertically.**

pCEFL HepG2 stable cell line overexpressing NR5A2 grows forming small circle colonies. These colonies grow preferentially one over another. Thus, seeing the whole plate covered is not a common situation. The growing rate of cells overexpressing NR5A2 is faster than control, getting the confluence between 1-3 days before in the same conditions.

## CONCLUSIONS

1. The previous NR5A2 silencing experiments treated with siRNA have been confirmed with the present results.

2. In HepG2 cells, overexpression of NR5A2 modifies the expression of several genes involved in lipids metabolism, glycolysis and gluconeogenesis:

- NR5A2 seems to regulate negatively the glycolysis pathway by acting on genes encoding enzymes as hexokinase-1, and positively in the gluconeogenesis pathway, by acting on the genes coding for enzymes as glucose 6-phosphatase and phosphofructokinase β1.

- Also NR5A2 works by increasing the transcription of the nuclear receptor PPARA, the coactivator PGA1A and the proteins FABP1, all of them related to fatty acids uptake, transport and metabolism.

3. In addition, our experiments showed that changes in NR5A2 expression are associated with changes in the concentration of glucose in the medium. Taken together, these results suggest that this nuclear receptor participates in the regulation of glucose consumption in cells and thus provide a possible mechanism to the association of NR5A2 genotypes to diabetes and obesity.

## DISCUSSION

Metabolic receptors are key regulators integrating the homeostatic control of (*1*) energy and glucose metabolism through peroxisome proliferator-activated receptor gamma (PPARγ); (2) fatty acid, triglyceride, and lipoprotein metabolism via PPARα; (*3*) reverse cholesterol transport and cholesterol absorption through the liver X receptors (LXRs) and liver receptor homolog-1 (LRH-1); (*4* ) bile acid metabolism through the farnesol X receptor (FXR), LXRs, LRH-1; and (5) the defense against xeno- and endobiotics by the pregnane X receptor/steroid and xenobiotic receptor (PXR/SXR). A strict coordination is needed to control the transcription of these metabolic systems, also depending on coregulators and others transcription factors.

An imbalance, either through chronic ligand excess or genetic factors, may cause an alteration in homeostasis of these circuits and lead to the pathogenesis of common metabolic diseases such as obesity, insulin resistance and type 2 diabetes, hyperlipidemia and atherosclerosis, and gallbladder disease. Further studies should explore the fact that many of these nuclear receptors are designed to respond to small molecules and turn them into therapeutic targets for the treatment of these disorders.

In our previous silencing experiments we found differences in a battery of genes associated with glucose homeostasis, suggesting that, apart from its role in cholesterol and bile acid metabolism, NR5A2 participates in the regulation of glucose metabolism. The confirming results in overexpressing experiments provide a possible evidence for the relationship between the NR5A2 variants and diabetes.

The best studied cause of insulin resistance is the excess of fat intake. This often leads to an hepatic glucose production or gluconeogenesis. In addition, the increased lipid levels in the liver and peripheral tissues promote the development of type 2 diabetes mellitus. We demonstrated that NR5A2, apart from its direct implication in glucose pathways by acting in enzymes transcription, can modulate the activity of other glucose or lipid regulators.

In overexpressing experiments, a NR5A2 increase has the opposite effect in hexokinase and glucose 6-phosphatase transcription, and the same occurs with phosphofructokinase-1 and fructose 1,6bi-phosphatase. While mRNA levels of glycolytic enzymes decrease, the ones of gluconeogenic enzymes increase. All of this leads to impair the glycolysis pathway and to stimulate the gluconeogenesis (Fig 31).

Fatty acid binding protein has a directly implication in lipid metabolism (Fig. 29), but also can acts through nuclear transcription mechanisms including enhanced fatty acid distribution to nuclei, interaction with PPARα in nuclei, and regulation of PPARα transcriptional activity of genes involved in long fatty acids uptake, oxidation, lipoprotein transport, and glucose metabolism. In cells overexpressing NR5A2, FABP1 transcription is altered and thus all the processes related [11].

Impaired mitochondrial functioning has also been involved in the aetiology of type 2 diabetes. Thus, it has been shown that mitochondrial oxidative phosphorylation is reduced in insulin-resistant subjects and that genes involved in mitochondrial oxidative metabolism are coordinately downregulated in type 2 diabetes Many of these oxidative genes are under the transcriptional control of peroxisome proliferator-activated receptor γ coactivator-1α which stimulates oxidative phosphorylation, mitochondrial biogenesis and the generation of oxidative type 1 muscle fibres.

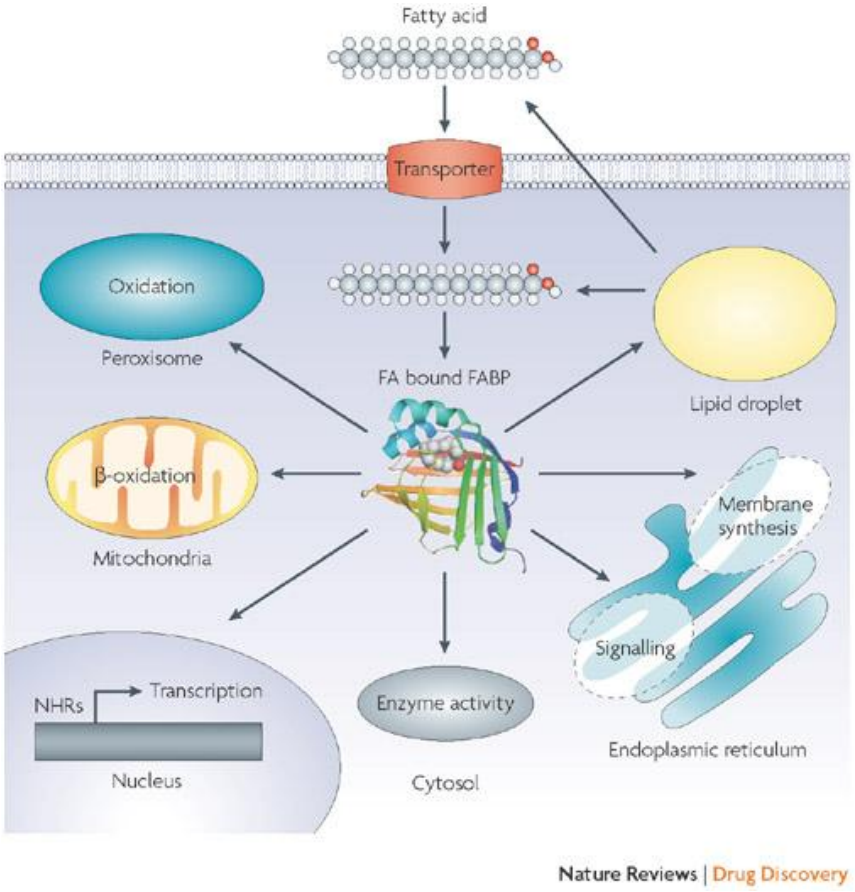


**Fig. 29** FABP1 protein interacts with fatty acid and can play different functions in the cell.

As said, PPARα plays a key role controlling fatty acid utilization pathways depending in physiologic conditions including fasting, nutritional alterations, and aging. The PPARα regulatory pathway has also been implicated in disease like cardiac hypertrophy, obesity, and diabetes mellitus. Moreover some nuclear receptor coactivators, such as PGC-1, serve to transducer physiologic input to changes in gene expression of the mitochondrial FAO enzyme genes.[2, 28]

PGC-1 is a coactivator molecule identified recently based on its ability to interact with PPARα. The observation that PGC-1 expression is induced by fasting and cold exposure, physiologic conditions known to increase cellular lipid utilization, suggested that PGC-1 may function as a regulator of mitochondrial β-oxidation (Fig 32).

In some studies [28], a severe hypoglycemia observed specifically in PPARα-deficient mice upon fasting, characterized by a 50% drop in blood glucose concentration after 24 hours of fasting, suggested a role for PPARα in glucose homeostasis. Several mechanisms may account for this fasting hypoglycemia, including normal glucose- 6-phosphate production in liver accompanied by the shift from glucose to glycogen production (Fig 30). Other authors attribute the fasting hypoglycemia to decreased production of lactate and hepatic glucose. Fasting induces the conversion of glycerol into glucose through the induction of several hepatic enzymes. The expression of these enzymes and of the glycerol transporters is PPARα-dependent. [29]

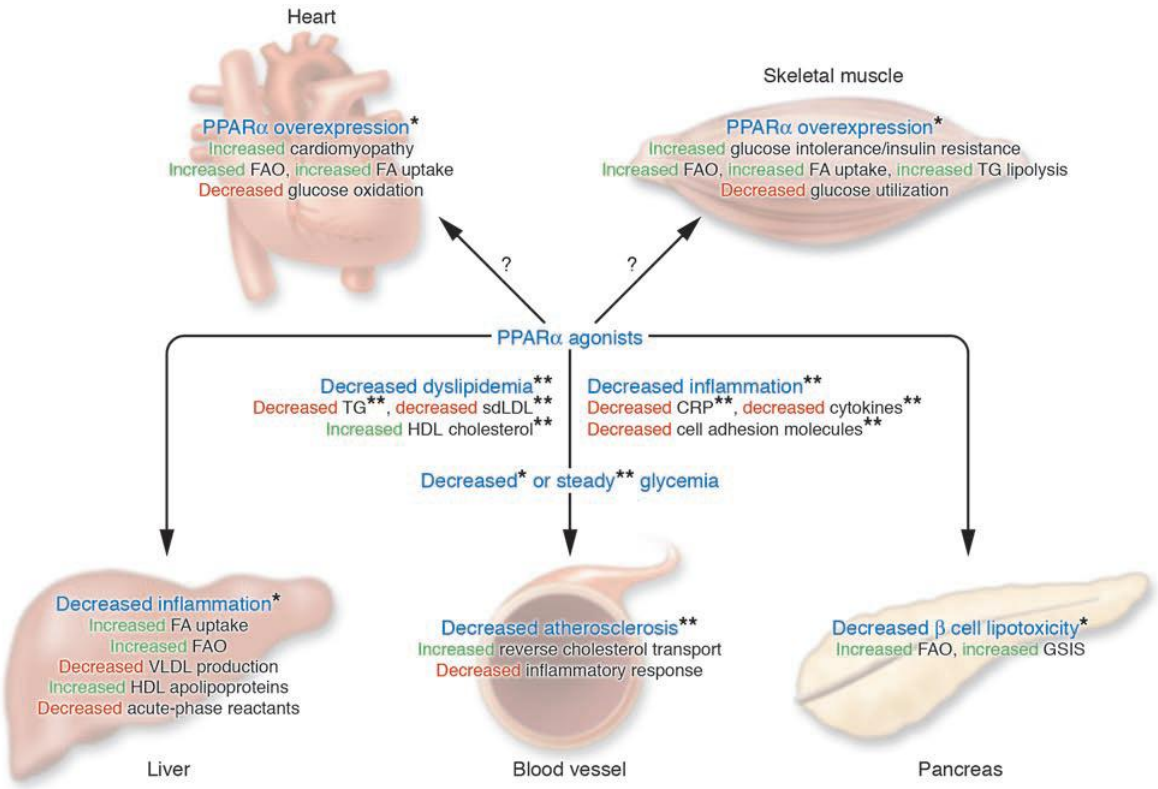


**Figure 30.** PPARα metabolic actions and potential pathophysiological consequences. The main effects of PPARα overexpression or of PPARα ligands in mice (denoted by a single asterisk) and in humans (denoted by a double asterisk) are shown. GSIS, glucose-stimulated insulin secretion Sorting out the roles of PPARα in energy metabolism and vascular homeostasis [28, 29].

## GLYCOLYSIS AND GLUCONEOGENESIS PATHWAYS

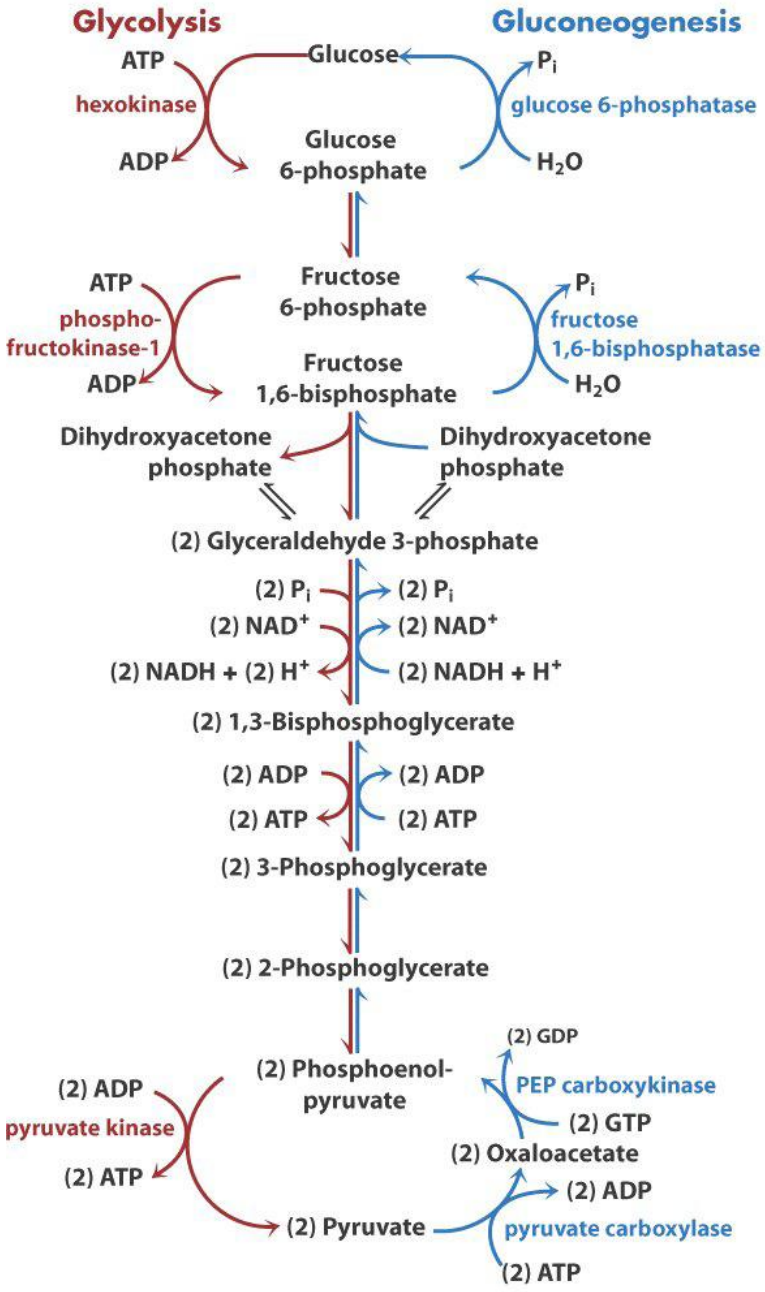


**Fig. 31. Glucose catalytic and anabolic pathways**. Red arrows follows the glycolisis pathway from glucose to pyruvate and blue arrows follows the gluconeogenesis , the opposite pathway. Key ezymes are mentioned. Picture courtesy of University of Valencia.

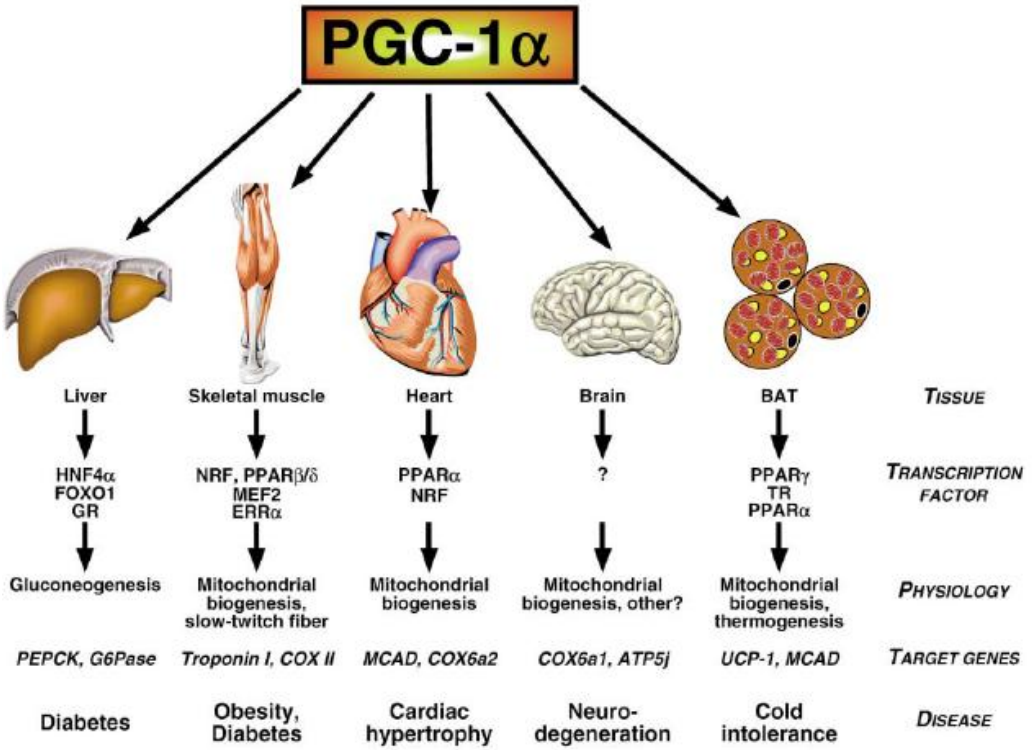


**Fig. 32.** Physiological processes where PGC-1 acts and the associated diseases. [1, 2]

Cells overexpressing NR5A2 also have a PPARα or PGC-1 altered expression, which ultimately results in changes in related glucose pathways.

In this way, our experiments show the importance of the NR5A2 as a regulator of the transcription of main genes implicated in glucose metabolism and supply a possible target for solving the diabetes pathology.

## FUTURE RESEARCH

Our results in glucose experiments indicate that cells overexpressing NR5A2 remove less glucose from the medium than control cells. The pathway that glucose follows is a new challenge. Glucose degradation as glycolisis, or alternative pathways as glucogenesis or fatty acid cycle could be the final step, although previous qPCR results seems to indicate the degradation pathway is more probable.

Recently, a synthetic phospholipid, the DPLC has been described as a NR5A2 ligand[30]. This model has been tested in mice with knock out NR5A2 in liver. Curiously the NR5A2-ligand interaction seems to have the opposite effect to our results, meaning NR5A2 as an antidiabetogenic agent. Thus, the nuclear receptor might work in a different way depending on the ligand or not union.

Nowadays we are testing the DPLC phospholipid model in HepG2 cell lines. For a better comprehension of the effects we will study on glucogen synthesis and fatty acids metabolism. Moreover the effects on insulin resistance will be tested in two specific models C2C12 in mice muscle, and 3T3-L1 in mice adipocytes. At the moment we have found differences in proliferation. The interaction PL-NR5A2 seems to promote a faster proliferation in silencing and control cells. The glucose consumption has been measured by the difference in the medium at 0, 24, 48 and 72h.

Morphological changes in HepG2 cells suggests a mesenchymatic transition in epithelium, so it could be of interest to explore NR5A2 role in differenciation.

Finally, using lentivirus to create knockout mice might be used to observe whole systemic effects.

**ACKNOWLEDGMENTS**

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