Universitat Politècnica de València - ETSIAMN

Biotechnology degree

Insulin Receptor Substrate 2 (IRS2): a role in Hepatic Stellate Cells

Final degree work

Patricia Badía Folgado

Academic Year 2017/2018

July 2018, Valencia

Tutor: Dr. Luke Noon

Tutor UPV: Dr. Máximo Ibo Galindo Orozco

Título: Sustrato 2 del Receptor de Insulina (IRS2): función en las Células Hepáticas Estrelladas

Lugar de realización: Centro de Investigación Príncipe Felipe

Fecha: Julio de 2018

Localidad: Valencia

Titulación: Grado en Biotecnología

Tutor: Dr. Luke Noon

Tutor UPV: Dr. Máximo Ibo Galindo Orozco

Resumen:

La resistencia a la insulina (IR) es una característica de la diabetes mellitus tipo 2 y, en muchos casos, la IR en el hígado se asocia con una expresión reducida de las proteínas del sustrato receptor de insulina (IRS), concretamente IRS-1 e IRS-2. Estudios recientes han revelado una estrecha relación entre la IR y la progresión de las enfermedades hepáticas crónicas, como la disfunción hepática, la fibrosis y la cirrosis. Las células estrelladas hepáticas (HSC) son fundamentales para la patogénesis de la cirrosis hepática y se cree que contribuyen a la reparación del hígado y a la fibrogénesis. Estas células son las principales productoras de tejido conectivo en un hígado fibrótico, pero también son responsables de dirigir la reparación epitelial mediante la expresión de factores paracrinos, incluido el factor 7 de crecimiento de fibroblastos. Las HSCs quiescentes se activan por lesión; migran al sitio donde se ha producido el daño, se diferencian en miofibroblastos productores de matriz y participan en la resolución de la fibrosis sufriendo apoptosis o volviendo a un fenotipo quiescente. Sin embargo, los eventos moleculares que regulan el ciclo de lesión de las HSCs no se comprenden completamente y, por esta razón, el objetivo de este proyecto fue evaluar cómo los cambios en la sensibilidad a la insulina les afectaba. Esto se estudió sobreexpresando *IRS2* en células humanas derivadas de HSC-LX2. Irs2 funciona como un andamio intracelular que acopla los receptores de insulina/IGF-1 a las moléculas efectoras de señalización. El análisis de señalización celular se realizó por Western blot, mientras que las técnicas de inmunofluorescencia, MTT y RT-PCR se utilizaron para caracterizar líneas celulares estables con el objetivo de cuantificar la supervivencia, la proliferación y los cambios fenotípicos que podrían ayudar a comprender cómo la señalización de insulina afecta a las HSCs. Los resultados mostraron que la sobreexpresión de Irs2 aumentó la sensibilidad a la insulina de las células LX2, y esto condujo a una reducción de su supervivencia. Finalmente, las conclusiones sugieren que la reversión fibrogénica podría promoverse mejorando la sensibilidad a la insulina.

Palabras clave: IRS2, sobreexpresión, células hepáticas estrelladas, resistencia a la insulina, cirrosis.

Title: Insulin Receptor Substrate 2 (IRS2): a role in Hepatic Stellate Cells

Abstract:

Insulin resistance (IR) is a feature of type 2 diabetes mellitus and, in many cases, IR in liver is associated with reduced expression of insulin receptor substrate (IRS) proteins, specifically IRS-1 and IRS-2. Recent studies have revealed a close relationship between IR and the progression of chronic liver diseases, such as liver dysfunction, fibrosis and cirrhosis. Hepatic stellate cells (HSCs) are central to the pathogenesis of liver cirrhosis and are thought to contribute to liver repair and fibrogenesis. These cells are the principal producers of the connective tissue in a fibrotic liver, but they are also responsible for directing epithelial repair by expression of paracrine factors, including fibroblast growth factor 7. Quiescent HSCs are activated by injury; they migrate to the location of damage, transdifferentiate into myofibroblasts and participate in the resolution of fibrosis by undergoing apoptosis or reverting back to a quiescent phenotype. However, the molecular events that regulate the HSC injury cycle are not completely understood and, for this reason, the aim of this project was to evaluate how changes in insulin sensitivity affected them. This was studied by overexpressing *IRS2* in human HSC-derived-LX2 cells. Irs2 functions as an intracellular scaffold that couples the insulin/IGF-1 receptors to signalling effector molecules. Cell signalling analysis was performed by Western blot, whilst immunofluorescence, MTT assays and RT-PCR analysis were used to characterize stable cell lines with the aim to quantify survival, proliferation and phenotypic changes that could help to understand how insulin signalling affects HSCs. Results showed that mouse Irs2 overexpression increased insulin sensitivity of LX2 cells, and this lead into a reduced survival of cells. Therefore, conclusions suggested that fibrogenic reversion could be promoted by improving insulin sensitivity.

Key words: IRS2, overexpression, hepatic stellate cells, insulin resistance, cirrhosis.

ACKNOWLEDGEMENTS

En primer lugar, me gustaría agradecerle a Luke haberme acogido en esta pequeña familia del I-23, por haber confiado en mi y por haber estado pendiente de mi trabajo hasta el último día, incluso desde Inglaterra.

También agradecerles a “MJ” y Fátima haber sido mis profesoras durante estos seis meses y por haberme dedicado todo el tiempo que he necesitado. Por animarme y por hacerme reír siempre que ha hecho falta. Porque aparte de supervisoras, han sido amigas, y eso es lo mejor que me llevo de estos seis meses.

Gracias a Arantxa y Amparo, por haberme ayudado siempre que he necesitado algo, y gracias a todo el laboratorio en general. Siempre admiraré la gran capacidad de organización que tenéis para coincidir media horita y poder comer todos juntos. Hacíais los días de Western más llevaderos. Ojalá el resto de laboratorios en los que esté se parezcan, aunque sea un poquito, al vuestro.

Y finalmente, gracias a mis padres por todo el esfuerzo que llevan haciendo durante años para que así pueda estar haciendo lo que me gusta, y para que pueda seguir haciéndolo.

TABLE OF CONTENTS

TABLE OF FIGURES

ABBREVIATIONS

Col: Collagen

DM: Diabetes Mellitus

ECM: Extracelular Matrix

FGF7: Fibroblast Growth Factor 7

FOXO: Factors of the Forkhead box O

GFAP: Glial Fibrillary Acidic Protein

HNF4α: Hepatocyte Nuclear Factor 4α

HSC: Hepatic Stellate Cell

IF: Intermediate Filament

IGF-1: Insulin-like Growth Factor

IGF1R: Insulin-like Growth Factor receptor

IL: Interleukin

IR: Insulin Resistance

IRS2: Insulin receptor substrate 2

KRLB: Kinase Regulatory Loop Binding

OPN: Osteopontin

PDGF: Platelet-derived Growth Factor

PH: Pleckstrin Homology

PIP_3: Phosphatidylinositol (3,4,5)-triphosphate

PTB: Phosphotyrosine-binding

qHSC: quiescent Hepatic Stellate Cell

SEC: Sinusoidal Endothelial Cell

TGF: Transforming Growth Factor

TNF: Tumour Necrosis Factor

α-SMA: Actin alpha-smooth muscle

1. INTRODUCTION

1.1. Insulin resistance

Type 2 diabetes mellitus (DM) is reaching epidemic proportions worldwide, as it is one of the most common metabolic disorders in the world (Wilcox, 2005). It develops because of multifactorial origin, such as ethnic origin, genetic factors and previous gestational diabetes; which combine with advanced age, overweight and obesity, unhealthy diet, physical inactivity and smoking. Nonetheless, the main causes of most of the cases of diabetes worldwide (90%) are overweight and obesity, along with physical inactivity (World Health Organization, 2016). As a consequence, Type 2 DM prevalence has increased considerably during the recent years, and it has become one of the main health problems in the world, affecting approximately 378 million people worldwide, and no cure has been found yet (International Diabetes Federation, 2013). The prevalence of type 2 diabetes is higher in patients who are affected by liver diseases and a link between the severity of liver injury and type 2 DM has been observed (Hickman & Macdonald, 2007). Studies have identified type 2 DM as a marker of fibrosis and of its faster progression (Hui et al., 2003) as its development in patients with cirrhosis has been identified, but new studies are demonstrating that the initiation and progression of liver injury are caused by the prevalence of diabetes (Hickman & Macdonald, 2007). It is known that insulin resistance (IR) has a main role in its development (Shulman, 2000). IR is defined as a clinical state in which high or normal insulin concentration generates a reduced biologic response, so hepatic glucose output is increased due to the reduced inhibition by insulin (Cefalu, 2001). Therefore, as a result of reduced glucose uptake by peripheral tissues and an increased hepatic glucose output, hyperglycemia is produced. (Michael et al., 2000).

Insulin and Insulin-like Growth Factor 1 (IGF-1) control several biological processes by acting on two similar tyrosine kinase receptors. Their activation initiates a cascade of phosphorylation states that ends up with the activation of enzymes that control many responses implicated in metabolism and growth. Insulin/IGF-1 signalling involves many different regulation points, which are controlled both positively and negatively to establish an appropriate intensity and signal duration. However, if these signalling pathways are perturbated, IR can be produced (Boucher et al., 2014). Different factors including hepatic parenchymal cell damage, alcohol, portal systemic shunting and hepatitis C virus are responsible for the development of this resistance (Kawaguchi et al., 2011) and it is associated with different chronic liver diseases, such as liver dysfunction, fatty non-alcoholic liver disease, chronic viral hepatitis, hemochromatosis, alcoholic liver disease, fibrosis, and cirrhosis (Hickman & Macdonald, 2007; Kawaguchi et al., 2011; Mohamed et al., 2009).

Liver cirrhosis is the final stage of different liver diseases, and fibrosis is its precursor. It is characterised for the degeneration and necrosis of hepatocytes, replacement of liver parenchyma by fibrotic tissues and loss of liver function (Ferrell, 2000). The main cells involved in this disease are the activated hepatic stellate cells (HSCs), and their repeated cycles of apoptosis and regeneration contribute significantly to its development (Zhou et al., 2014). At present, no effective strategies to treat liver cirrhosis have been found, as the molecular mechanisms of this disease are not quite understood (Zhou et al., 2014). However, spontaneous resolution of liver fibrosis has been observed in different animal models (Iredale, 2008) and several studies of fibrosis recovery have reported that elimination of activated HSCs by apoptosis or inactivation led to the regression of fibrosis, suggesting that elimination of activated HSCs is a crucial step in the event of fibrosis regression (Kisseleva et al., 2012).

1.2. Hepatic stellate cells

Hepatic stellate cells (HSCs), known as well as fat-storing cells, Ito cells, lipocytes, perisinusoidal cells, or vitamin A-rich cells, which store vitamin A among other retinoids, are central to the pathogenesis of liver cirrhosis and are thought to contribute to liver repair and fibrogenesis, as it was mentioned previously (Götze et al., 2015; Zhou et al., 2014). They reside within the perisinusoidal space of Disse in the liver, which is lined between parenchymal cells and fenestrated sinusoidal endothelial cells (SECs) (Sawitza et al., 2009). Their activation plays a main role in fibrosis, as it was explained in the previous section. After having a deregulation during the normal healing process in chronic liver injury and being exposed to inflammatory cytokines such as platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, tumour necrosis factor (TNF)-α and interleukin (IL)-1, extracellular matrix (ECM) is accumulated, including type I collagen (ColI) (Kisseleva et al., 2012; Zhou et al., 2014). Myofibroblasts are stromal cells which show myoid characteristics, are involved in the production and remodelling of the ECM scar in fibrosis and strongly contribute to liver fibrosis (Kisseleva et al., 2012; Lepreux & Desmoulière, 2015). HSCs are a major source of these myofibroblasts and of connective tissue in a fibrotic liver, but they are also responsible for directing epithelial repair by expression of paracrine factors, including fibroblast growth factor 7 (FGF7) (Schumacher & Guo, 2016).

During liver injury, quiescent HSCs (qHSCs) migrate to the location of damage, get activated, transdifferentiate into myofibroblasts and participate in the resolution of fibrosis by producing extracellular matrix and by releasing growth factors to stimulate the liver regeneration and to produce fibrous scars. After fibrosis resolution, HSCs undergo apoptosis or revert back to an inactivated state which it is thought that is similar to, but different from, the previous quiescent phenotype (Figure 1) (Kisseleva et al., 2012; Schumacher & Guo, 2016). However, it is not well understood which the fate of these cells is, and different studies have been performed to try to understand it.

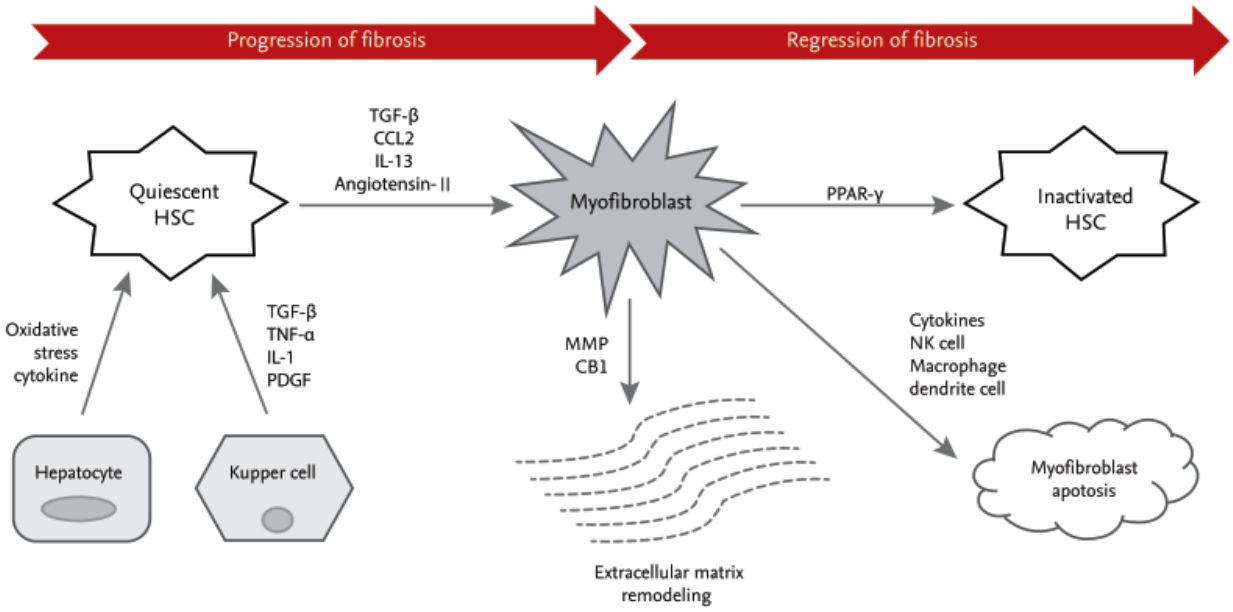


Figure 1. Hepatic stellate cell (HSC) differentiation process during progression and regression of fibrosis. During the HSC injury cycle, HSCs undergo differentiation from quiescent cells to myofibroblasts. Exposition to inflammatory cytokines, transforming growth factors and small molecules enhances this process (Jung & Yim, 2017).

1.2.1. Fibroblast growth factor 7 (FGF7)

FGF7 is an important mitogen for different types of epithelial cells (Rubin et al., 1989) and it regulates their migration and differentiation, being a potent survival factor under stress conditions (Werner, 1998). It is produced by mesenchymal cells and it binds to its high-affinity receptor FGFR2-IIIb (Steiling et al., 2004). Different studies have demonstrated that expression of FGF7 is highly regulated after liver injury, and in transgenic mice, the expression of FGFR2-IIIb^{-} mutant in hepatocytes has remarkably inhibited their proliferation after injury. For this reason, it is thought that FGF7 plays a key role in repair after liver injury, participating in fibrosis resolution (Steiling et al., 2003).

1.2.2. Cell markers

Kisselva et al. (2012) analysed the different types of cells by considering their expression markers. Myofibroblasts (activated HSCs), which are supposed to be present only during the production of fibrous scar and disappear with fibrosis resolution, are GFAP^{+}, Desmin^{+}, α-SMA^{+} and Coll^{+}(Bataller & Brenner, 2005); whilst qHSCs (or inactivated HSCs), which are present in normal liver, are GFAP^{+}, Desmin^{+}, α-SMA^{-} and Col^{-}.

Glial Fibrillary Acidic Protein (GFAP) is a member of intermediate filaments (IF) which maintains mechanical strength and structure of cells (Bai et al., 2013), and it has been documented that it is a useful marker of early activation of HSCs (Perkins, 2007). It has low expression levels in the state of qHSC, whilst this expression increases considerably when HSCs get activated (Geerts et al., 2001). With regards to actin alpha-smooth muscle (α-SMA), it is an actin isoform and a specific marker for smooth muscle cell differentiation (Skalli et al., 1986). Therefore, its expression will be used to identify activated HSCs, which show a myofibroblastic phenotype (Carpino et al., 2005). Similarly, Collagen Type I and III (Col I and III) will be used to observe how ECM appears and is eliminated, as collagen is one of the main proteins that are produced during ECM accumulation, leading to scar deposition and liver fibrosis (Geerts et al., 2001; Tacke & Trautwein, 2015). Vimentin will be used as an HSCs marker, as it is an IF protein in normal adult tissues, and many cells express it in a wider spectrum during development, tissue injury, or when in culture (Geerts et al., 2001). With regard to differentiation, hepatocyte nuclear factor 4α (HNF4α) is an important transcription factor in liver differentiation, as well as APOA2, which is a differentiation marker (Liu et al., 2015). Therefore, these cell markers will be used as well to analyse this feature. Osteopontin (OPN), encoded by the gene SPP1, Seth et al., (2014) demonstrated that it has a key role in functions of HSCs such as signalling, cell migration and activation of fibrinolysis, ECM and fibrogenic pathways; and some studies have indicated that it might be activated specifically for fibrogenic liver repair (Wen et al., 2016). Finally, an interesting cell marker for detecting cellular senescence that will be useful for understanding the HSC injury cycle will be the tumor suppressor p53, as it plays a key role in this event (Collado et al., 2007; Nishizawa et al., 2016). Cellular senescence consists in an antiproliferative program that leads to permanent growth arrest in the cell (Kuilman et al., 2010). Recent studies have shown that p53 can regulate it and that this protein can lead to cell cycle arrest, DNA repair and apoptosis when it becomes active. Its deletion reduces HSC senescence, leading to extensive liver fibrosis (Jin et al., 2016).

1.3. Insulin receptor substrate 2 (IRS2)

Several studies have demonstrated that in liver, IR is associated with reduced expression of insulin receptor substrate (IRS) proteins, specifically IRS1 and IRS2 (Taniguchi et al., 2005), which act as scaffolds to organize and mediate signalling complexes (Boucher et al., 2014; Taniguchi et al., 2005). These proteins are involved in the mediation of many responses in insulin sensitive tissues, specifically somatic growth and carbohydrate metabolism. This has been shown in some studies where Irs-2$^{-/-}$ knockout mice have developed diabetes as a consequence of severe insulin resistance paired with the loss of β-cell function (Valverde et al., 2003; Withers et al., 1998). IRS2 is a large protein (>1,200 amino acid residues) that contains an amino-terminal pleckstrin homology (PH) domain and phosphotyrosine-binding (PTB) domains (~100 a.a. each) followed by long, unstructured carboxyl-terminal tail regions (Copps & White, 2012) and a kinase regulatory loop binding (KRLB) domain, that works directly with the catalytic site of the insulin receptor, but not the Insulin-like Growth Factor receptor (IGF1 R) (Wu et al., 2008). It has been studied that IRS2 plays a central role in lipid metabolism and ERK activation in the Ras/ERK pathway (Huang et al., 2005). However, the pivotal one that links IRS proteins to the metabolic actions of insulin is the PI3-kinase (PI3K) and Akt pathway (Boucher et al., 2014). In the current study, the gene of this protein *(IRS2)* will be overexpressed in LX2 cells, and later analysis will be performed to get a better understanding of insulin sensitivity and to know how this gene overexpression could alter HSCs survival and transdifferentiation.

1.3.1. PI3K/Akt pathway

As shown in Figure 2, Insulin and IGF-1 receptors get activated by their ligands, which preferentially bind to their own receptors. However, they can also bind to the alternate receptor with reduced affinity (Belfiore et al., 2009). After binding, a cascade of phosphorylation events is initiated. Once the ligands bind to their receptors, a conformational change is produced and then, receptors auto phosphorylate, leading to the recruitment and phosphorylation of IRSs proteins. IRS proteins activate the PI3K/Akt pathway by the recruitment and activation of PI3K, generating then the second messenger phosphatidylinositol (3,4,5)-triphosphate (PIP_3). Next, PIP_3 recruits PDK-1 to the membrane and activates it, which phosphorylates and activates Akt, and induces downstream signalling, that leads in the regulation of glucose transport, lipid synthesis, gluconeogenesis, glycogen synthesis and control of the cell cycle and survival (Boucher et al., 2014). Stimulation of both glycogen and protein synthesis is produced by the phosphorylation of GSK-3 by Akt, which when is not phosphorylated, inhibits it. This leads to the dephosphorylation of GSK-3 substrates such as glycogen synthase and eukaryotic initiation factor 2B (Ef2b), producing the synthesis of these products (Inglehart et al., 2007). Furthermore, Akt regulates glucose by phosphorylating transcription factors of the Forkhead box O (Foxo) at several sites, providing docking sites for different binding proteins. These transcription factors promote gluconeogenesis and negatively regulate the expression of genes that promote glucose utilization, including those involved in glycolysis, the pentose-phosphate shunt pathway and lipogenesis (Zhang et al., 2006), and their phosphorylation by Akt inhibits them and prevents them to translocate into the nucleus, where they can suppress gene expression (Brunet et al., 1999).

1.3.2. Ras/ERK pathway

Once Insulin and IGF-1 bind to their receptors, another pathway is activated independently of PI3K/Akt, in which IRS and Shc proteins are recruited - this is the Ras/ERK pathway (Figure 2). After the recruitment of IRS and Shc proteins, Grb2 binds to them and proteins like son-of-sevenless (SOS) bind to Grb2. SOS is a guanine nucleotide exchange factor (GEF) for Ras, which catalyses the switch of membrane-bound Ras from an inactive, GDP-bound form (Ras-GDP) to an active, GTP-bound form (Ras-GTP). Ras-GTP then stimulates and interacts with Raf, which stimulates its target MEK, that simultaneously phosphorylates and activates the MAP kinases ERK, which are important as they have a main role in cell proliferation and differentiation, regulation of gene expression and in extranuclear events such as cytoskeletal reorganization (Boucher et al., 2014).

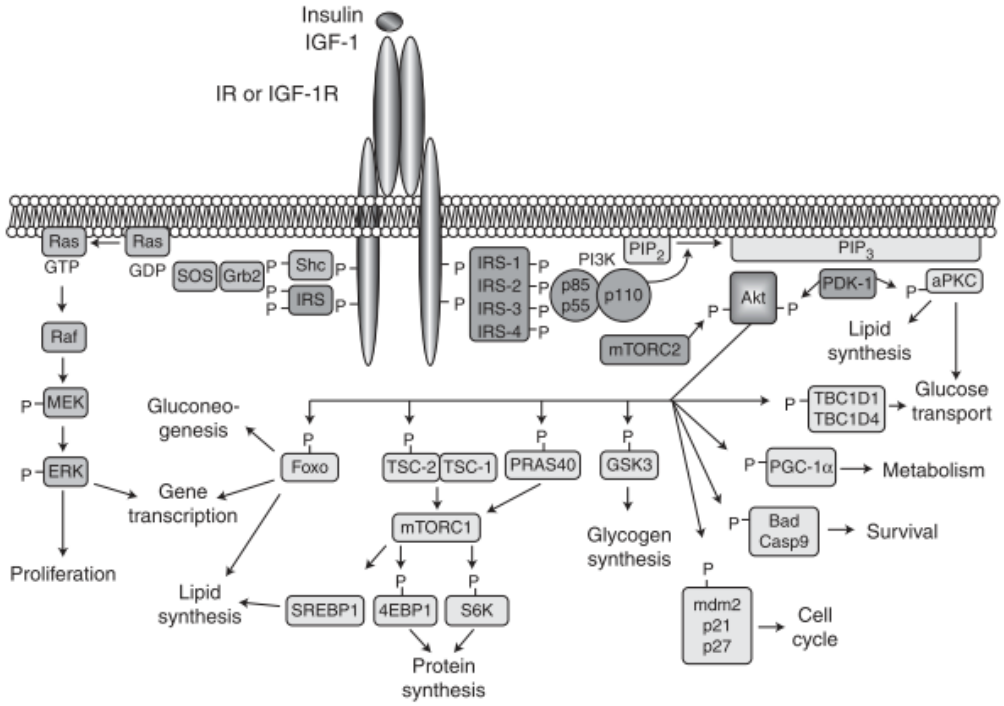


Figure 2. Insulin and IGF-1 pathways. Activation of insulin and IGF-1 receptors by their ligands initiates a cascade of phosphorylation events that lead in the production of different processes such as regulation of glucose transport, lipid synthesis, gluconeogenesis, glycogen synthesis and control of the cell cycle and survival (Boucher et al., 2014)

1.4. In vitro model: human HSC-derived-LX2 cells

In the present study, LX-2 Human Hepatic Stellate Cell Line will be used (#SCC064, Millipore). LX-2 was generated by immortalization of primary human hepatic stellate cells with the SV40 large T antigen followed by selective culture of early passaged cells in low serum media conditions (Millipore, 2016).

Control cells will simulate activated HSCs, whilst Mitomycin C treated cells will be the inactivated HSCs. Within these two conditions, two other conditions will be established. Control LX2 cells will have GFP overexpression, whilst target cells will have *Irs2* overexpression. Both genes were overexpressed using the lentiviral system, and this was performed previously by the laboratory.

1.5. Statistical analysis

Data were statistically analysed with GraphPad Prism 6 software using 2way Anova analysis or Student's paired t-test, depending on the number of conditions/variables compared. Results were expressed as the means ± SEM and values of $p < 0.05$ were considered statistically significant.

2. HYPOTHESIS

Previous results have shown that *Irs2-/-* mouse has enhanced fibrosis and impaired liver regeneration. This lead us to think that IRS2 might be implicated in fibrogenic reversion in liver. As it was mentioned previously, HSCs regulate fibrosis in liver. Therefore, our aim was to test whether an overexpression of IRS2 might contribute to an increase of insulin sensitivity in human HSC LX2. Therefore, our hypothesis states that by artificially increasing this insulin sensitivity in HSCs via exogenous expression of Irs2, fibrogenic reversion could be promoted.

3. OBJECTIVES

3.1. Detection of changes of sensitivity in insulin/IGF-1 pathways

Insulin/IGF-1 pathways play a key role in the development of insulin resistance and type 2 DM. For this reason, one of the objectives of the present study was to detect if *Irs2* overexpression produced any changes in insulin sensitivity. For studying this, mouse *Irs2* was overexpressed in human HSC-derived-LX2 cells and then were stimulated either with insulin or IGF-1, and different phospho-proteins from the signalling pathways were quantified by Western blot.

3.2. Understanding HSC injury cycle

The molecular events that regulate HSC injury cycle are not completely understood. For this reason, the next aim of this project was to evaluate how changes in expression of *Irs2* altered cells in their survival, proliferation and phenotype. This was studied by overexpressing of mouse *Irs2* in human HSC-derived-LX2 cells and was performed in two different conditions: control, which were the activated HSCs; and Mitomycin C cells, which simulated inactivated HSCs, as this treatment inhibited cell cycle. All this was analysed by MTT assays, Western blot and by coculture analysis by RT-PCR and immunofluorescence.

Finally, with all these experiments it was possible to characterize this stable cell line of human HSC-derived-LX2 cells.

4. MATERIALS AND METHODS

4.1. Cell culture

Isolated human HSC-derived-LX2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) High Glucose, pyruvate (Gibco), containing 2% heated inactivated fetal bovine serum (FBS) (Sigma), 1% streptomycin/penicillin (Gibco) and 1% L-glutamine (Gibco), and were grown until they reached confluence. Once the cultures reached it, they were trypsinised (0.25% trypsin EDTA) (Gibco) for 2 minutes. Then, trypsin was inactivated with DMEM medium and cells were centrifuged at 3000 rpm for 3 minutes. Finally, they were resuspended and passaged at a ratio of 1:5 in a 10 cm dish. Subsequent passages were performed to prepare the different experiments.

4.2. Inactivation

When cell cultures reached 80% of confluence, half of them were inactivated by adding 100 µL of Mitomycin C (Sigma-Aldrich) to the medium, followed by an incubation of 3 hours and a half. Five washes with sterile PBS were subsequently carried out. Finally, cultures were trypsinised as described previously and cells were counted and seeded in different dishes depending on their following experiment.

4.3. Growth assays

To assess a combination of cell proliferation, metabolic activity and cell survival, LX2 cells (GFP and IRS2) were seeded at a density of $2{\cdot}10^3$ cells/well in a 96-well plate and cultured for 48 hours, whereas to assess cell growth in inactivated cells (treated with mitomycin C), cells were cultured in a pre-treated gelatin (Sigma-Aldrich) 96-well plate at a density of $15{\cdot}10^3$ cells/well for 5 days. In both cases, cells were seeded using 100 µl DMEM 2% FBS media and after 24 hours, media was changed to HepaRG and incubated at 37 °C. After cell incubation, 20 µl of MTT solution (0.5 mg/mL, Sigma-Adrich) was added for 4 hours to each well in dark conditions. After the MTT treatment, media was aspirated. Resulting formazan crystals were dissolved using 100 µl/well of dymethylsulfoxide (Sigma-Aldrich) and optical density was measured at 570 nm using an automated microplate reader (Victor PerkinElmer). Four independent experiments were done using four replicates, as shown if Figure 3.

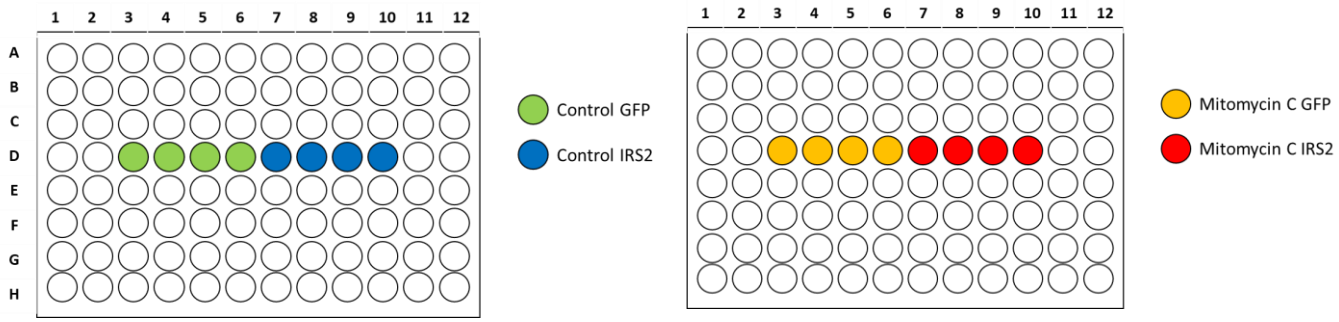


Figure 3. Growth assays design. Control cells were seeded at a density of $2{\cdot}10^3$ cells/well and cultured for 3 days. Mitomycin C treated cells were seeded at a density of $15{\cdot}10^3$ cells/well and cultured for 5 days.

4.4. Cell signalling experiment

Control cells (GFP and IRS2) were cultured in a 6-well cell culture plate (170.000 cells/well) in DMEM 2% FBS as showed in Figure 4. After 24 hours, the medium was changed to Williams Medium E (Gibco) (HepaRG medium), containing 11% Fetal Clone III (Thermo Scientific Fisher), 1% streptomycin/penicillin (Gibco), 1% L-glutamine (Gibco), 0.1% hydrocortisone (Sigma-Adrich) and 0.05% insulin (Sigma-Aldrich). One day later, 3 washes every 15 minutes with Serum Free Media (SFM) were performed. Subsequently, the cell culture was incubated during 4 hours with SFM and after this 4-h starvation period, cells were treated with 1nM insulin and 1nM IGF1. After hormone incubation for 10 minutes at 37 °C, the dish was placed on ice to stop the cellular metabolism and media were removed. Cells were washed with cold phosphate-buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1.44 g Na_2HPO_4, 1L ddH_20, pH=7.4) and then were collected with ice-cold lysis solution containing 5% Glycerol P (Sigma-Aldrich), 4% Complete (Roche), 0.2% Sodium Orthovanadate (Sigma-Aldrich), 2% PMSF (Roche) and 88.8% Lysis buffer 3% BSA in TBST (2.423 g Tris, 8.766 g NaCl, 10 mL Tween 20, 990 mL ddH2O, pH=7.5).

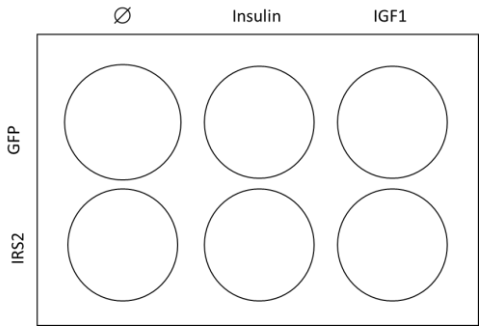


Figure 4. Signalling experiment design. Cells were seeded at a density of 170.000 cells/well and were cultured for 48h. After 4h of starvation, cells were stimulated with [1nM] of insulin and IGF-1.

4.5. Protein study

To evaluate and understand the molecular events that regulate HSC injury cycle, both control and mitomycin C cells, were seeded in a 10 cm pre-treated gelatin dish at a density of $1.2 \cdot 10^6$ cells/dish and cultured for 3 days. In both cases, cells were seeded using 10 mL DMEM 2% FBS media and after 24 hours, media was changed to HepaRG and incubated at 37 °C. After 48 hours, dishes were scraped with ice-cold lysis solution and cells were stored at -80 °C.

4.6. Western blot

To analyse proteins from both cell signalling experiment and protein study, Western blot was performed.

4.6.1. Proteins extraction

Samples were placed on ice and were lysed by sonication at a frequency of 30kHz with an intermittent pulse of 10 seconds on and 15 seconds off, and it was performed three times per sample. Lysed samples were then incubated for 30 minutes on ice. Next, samples were centrifuged at 13000 rpm for 15 minutes at 4 °C. Supernatant was then collected and stored at -80 °C.

4.6.2. Protein preparation and separation

Proteins were quantified and prepared with 6x loading and lysis buffer. Depending on the molecular weight of the target proteins, two different percentages of gels of polyacrylamide were prepared, of 7% and 15%. Samples were running at 70 V during the first 30 minutes. Then, current was changed to 120 V.

4.6.3. Transfer

Before starting with the transfer, the membrane of nitrocellulose was activated during 5 minutes with methanol. Then, transfer was started. For the largest proteins, transfer buffer with 20% of methanol was used and a transfer of 100V was carried out for 2 hours; whereas for the smallest proteins, transfer buffer without methanol was used and the process was performed overnight at 30 V. After transfer, the nitrocellulose membrane was blocked with TBST 3% BSA for 1 hour. Primary antibody was then added and incubated overnight at 4°C. The next day, membrane was washed, and secondary antibody was added and incubated for 2 hours at room temperature. Finally, membrane was developed with the developer kit (Thermo Scientific).

4.6.4. Antibodies

Antibodies against the following proteins were used: P-GSK 3β (1/500, rabbit, Santa Cruz), P-ERK (1/500, mouse, Santa Cruz), P-FOXO1 and 4 (1/1000, rabbit, Cell Signaling), P-AKT (1/1000, Cell Signaling) and actin (1/50000, rabbit, Santa Cruz) for the cell signalling Western blot and IRS2 (rabbit, Santa Cruz), GFP (chicken, Santa Cruz), GFAP (mouse, Santa Cruz), p53 (mouse, Santa Cruz), α-sma (rabbit, Abcam), FGF7 (mouse, Santa Cruz) for the protein study.

4.7. Co-culture

To evaluate survival, proliferation and phenotypic changes of LX2 cells over time, both control and mitomycin C cells (GFP and IRS2), were seeded at a density of $2.5 \cdot 10^4$ cells/well in a 24-well plate with pre-treated gelatin coverslips and cultured for different timepoints (5, 9 and 15 days); whereas to analyse their RNA, the same density of cells was seeded in a pre-treated gelatin 24-well plate without coverslips for 15 days. In both cases, cells were seeded using 0.5 mL of DMEM 2% FBS media. After 24 hours, Hepa RG (Sigma-Aldrich) cells were thawed by placing cryovials in 37 °C water bath and resuspended in HepaRG media. Subsequently, DMEM media of the previous day was aspirated and 0.5 mL of Hepa RG cells were added to each well. One experiment was done using two replicates, as shown in Figure 5.

Figure 5. Co-cultures design. Cells were seeded at a density of $2.5 \cdot 10^4$ cells/well. (A) Co-culture with coverslips with different timepoints. (B) Co-culture of 15 days for RNA extraction.

4.8. Immunofluorescence

At the time points of 5, 9 and 15 days, the media of the co-culture (Figure 5A) was aspirated, and cells were fixed on the coverslips with 4% paraformaldehyde (PFA) (Sigma-Aldrich) for 10 minutes at room temperature. Subsequently, cells were washed 3 times with PBS and stored at 4 °C. Once all cells were fixed, they were permeabilized with PBS [0.5% Triton x100] for 15 minutes at room temperature. Afterwards, cells were washed twice with PBS and blocked with PBS [1% BSA; 5% Horse Serum; 0.2% Triton x100] for 1 hour at room temperature. Primary antibodies were then added in blocking buffer. Each coverslip required 60 µl of antibody mix and these were mixed together for a triple staining. Cells were incubated overnight at 4 °C. After 24 hours, cells were washed 6 times with PBS and secondary antibodies were added in PBS [1% BSA; 0.2% Triton x100] and incubated for 2 hours at room temperature. After incubation, cells were washed six times with PBS and 2 times with H_2O. Subsequently, coverslips were mounted onto slides with Fluorescent Mounting Medium (Dako). Finally, slides were placed in a cardboard slide folder and stored at 4 °C.

4.8.1. Primary antibodies

The primary antibodies used for the immunofluorescence were: Vimentin (1/300, mouse, Santa Cruz) and HNF4-α (1/100, rabbit, Santa Cruz).

4.8.2. Secondary antibodies

The secondary antibodies used were Hoechst (1/2000, Sigma-Aldrich), α-rabbit (647, Santa Cruz) and α-mouse (488, Santa Cruz).

4.8.3. Microscopy and image acquisition

Conventional fluorescence microscopy with immersion oil was used with the objective lens of 40x and images of the samples that were prepared by immunofluorescence were taken, all of them at the same exposure.

4.9. RT-PCR

4.9.1. RNA extraction

For a later RNA extraction, LX2 cells were collected from the co-culture at day 15 by using RLT buffer with β-mercaptoethanol. First, DMEM media was aspirated and cells were washed with PBS. After washing, 350 µl/well of RLT buffer with 10 µl/mL β-mercaptoethanol were added. Then cells were collected by scrapping and stored at -80 °C. Total RNA was extracted with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions.

4.9.2. Retro-transcription

Total RNA was reverse transcribed to complementary DNA (cDNA) using the RNA to cDNA EcoDry Premix (Takara). The reaction was performed for 60 minutes at 42˚C (annealing), (cDNA synthesis), and 10 minutes at 70˚C (enzyme denaturation).

4.9.3. Quantitative PCR

Comparative quantitative real time polymerase chain reaction (RT-PCR) was performed with Lightcycler 480 (Roche). cDNA (X µl) was used in each PCR reaction. The housekeeping gene RPL19 was used as a reference gene for normalisation and H_2O was used as a negative control. The primer pairs (forward/reverse) for APOA1 were (TCGCAGCAACTGTGCTACTC) and (TCTGGGCTCTTGACCTTCTC); for FGFRII-IIIβ: (TGCTGGCTCTGTTCAATGTG) and (GGCGATTAAGAAGACCCCTA); for SPP1: (GCCGAGGTGATAGTGTGGTT) and (CCATGTGTGAGGTGATGTCC); for α-sma: (CTGAGCGTGGCTATTCCTTC) and (CAGTGGCCATCTCATTTTCA); for COLA1: (TGGTGACAAGGGTGAGACAG) and (CTCCAGAGGGACCTTGTTCA) and for COLA3 (GGTGAGCCTGGTAAGAATGG) and (CTTGCCATCTTCGCCTTTAG). The PCR reaction was catalysed by TB Green™ Premix Ex Taq ™ II (Takara), which generated the fluorescence signals as well during each of the 47 cycles, in proportion to the quantities of double stranded DNA (denaturation 5 minutes at 95˚C; amplification 10 seconds at 95 ˚C, 20 seconds at 54 ˚C, 30 seconds at 72 ˚C; melting 5 seconds at 95˚C, 1 minute at 65 ˚C and continuous detection at 97 ˚C; cooling 30 seconds at 40˚C).

5. RESULTS AND DISCUSSION

5.1. Overexpression of *Irs2* in early passages.

As the main objective of this study was to analyse which changes mouse *Irs2* overexpression produced in LX2 cells behaviour and insulin sensitivity, the first step was to prove that this overexpression was effective. Thus, control GFP and IRS2 LX2 cells were seeded under the same conditions. Three days later, cells were collected, and proteins were extracted for finally analysing them by Western blot.

Western blot was performed to know if *Irs2* had been over expressed correctly. As shown in Figure 6, the first passage (passage 10) had *Irs2* over expression (cells were infected at passage 3 and 5). However, two passages later (n=2) this over expression was no longer observed. From this, it was concluded that all the experiments would have to be done in early passages, and later passages should be discarded.

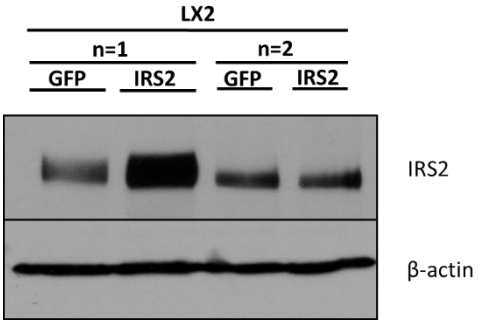


Figure 6. Western blot of IRS2. Control GFP and IRS2 LX2 cells were analysed. IRS2 was overexpressed in passage 10 (n=1), but this overexpression disappeared two passages later (p12, n=2).

5.2. *Irs2* overexpression and insulin sensitivity

It is known that insulin and IGF-1 pathways play a key role in the development of IR and Type 2 DM. To study how overexpression of *Irs2* changed phosphorylation of some of the proteins of these pathways, cells were stimulated with insulin and IGF-1.

Generally, it was observed that *Irs2* overexpression increased phosphorylation of most of the signalling pathways proteins, more significantly when cells were stimulated with IGF-1 (Figure 7 and 8). Except in the case of p-ERK (Figure 8C), where no important differences were observed except for the insulin stimulation, where phosphorylation level decreased with the overexpression. It is important to notice that ERK phosphorylation is involved in cell proliferation and differentiation, and this decrease after insulin stimulation could have an effect on cells behaviour. In the case of p-FOXO 1 and 4, only significant differences could be found when cells were stimulated with IGF-1.

In conclusion, these results suggested that *Irs2* overexpression altered downstream signalling pathways consistent with an increase in insulin/IGF-1 sensitivity.

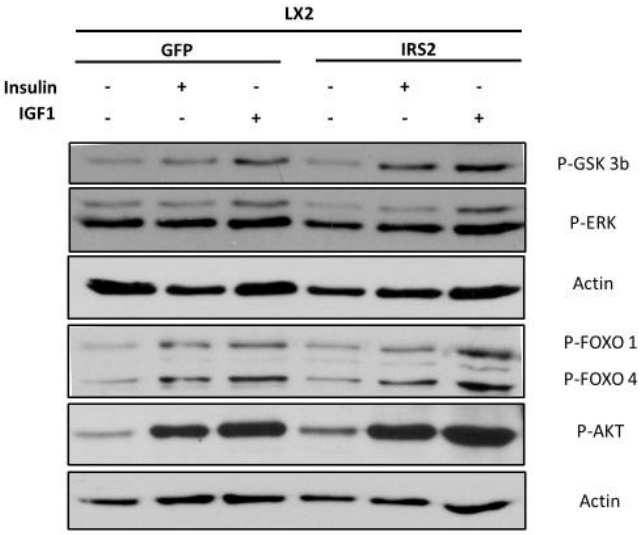


Figure 7. Western blot of insulin/IGF-1 pathways phosphorylated proteins in GFP and IRS2 cells. A general increase in phosphorylation expression was observed in all the cases, except in P-ERK protein.

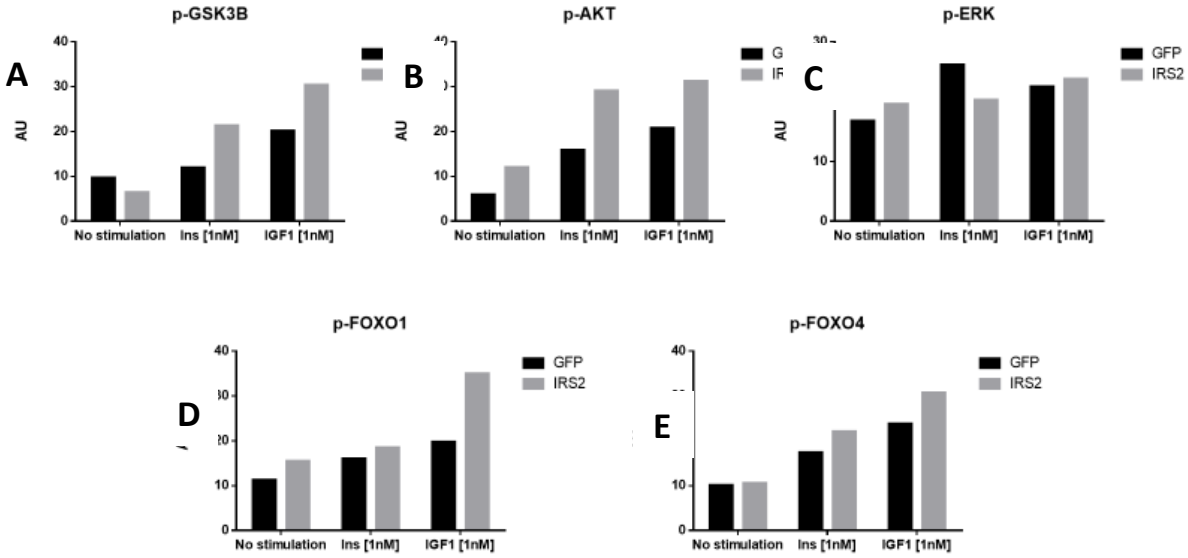


Figure 8. Results of signalling experiment. n=1 experiment. Cells were stimulated with [1nM] of insulin and IGF1. Generally, IRS2 overexpression enhanced proteins phosphorylation. (A and B) Phosphorylation of p-GDK3B and p-AKT increased with Irs2 overexpression. (C) Phosphorylation of p-ERK was not increased significantly with Irs2 overexpression, but it decreased when cells were stimulated with insulin. (D and E) Phosphorylation of p-FOXO 1 and 4 was only increased with IGF-1 stimulation.

5.3. Growth assays of control and mitomycin C treated GFP and IRS2 cells

After checking that LX2 cells had over expressed *Irs2*, and observing that this overexpression changed their insulin sensitivity, the next step was to know which effects *Irs2* overexpression had on their ability to grow.

As explained previously, it is important to completely understand the HSC injury cycle and insulin signalling pathways. IR is involved in these events, and for this reason, it is needed to know which changes are produced in the cell line of interest when *Irs2* is overexpressed. Therefore, one of the key points of this study was to know if LX2 cells survived for longer when they were

inactivated with Mitomycin C. It has been seen that Irs2 contributes to increase cell viability and reduces apoptosis in JAK2-mutated cells (de Melo Campos et al., 2016). Therefore, the aim of this experiment was to see how over expression of *Irs2* contributed to the survival in both control and mitomycin C treated LX2 cells.

Results showed that over expression of *Irs2* in control cells did not have a significant change in their growth characteristics as judged by MTT assay ($p > 0.005$, Fig. 9A). Whilst in mitomycin C treated cells, a significant difference ($p = 0.0162$; $p < 0.05$, Fig. 9B) between GFP and IRS2 cells was observed, where this over expression reduced survival. As a conclusion, it could be suggested that overexpression of *Irs2* in LX2 cells contributed to their death following Mitomycin C cell cycle arrest. Some studies have demonstrated that a modification in PI3K/Akt and Ras/ERK pathways can produce unexpected responses. For example, Irs2 could promote an increase in proliferation of HSCs. However, this event could display elevated reactive oxygen species (ROS) levels, which could be responsible of the depletion of these cells and a reduction of their lifespan (Martelli et al., 2010). Furthermore, the failure to sustain expression of the transgene (section 5.1.) could be explained as well by this selective disadvantage. Therefore, this could be an explanation of these results.

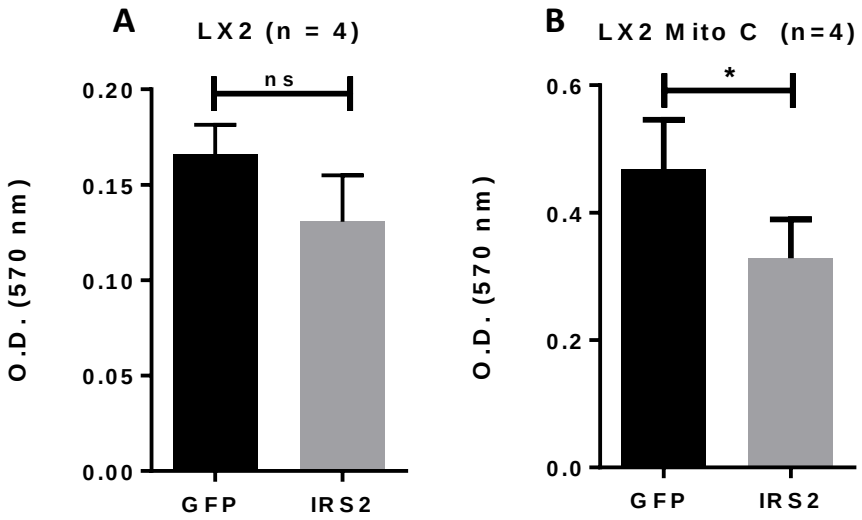


Figure 9. Results of growth assays. (A) To assess cell growth in control cells, LX2 cells were seeded at a density of $2 \cdot 10^3$ cells/well in a 96-well plate and cultured for 48 hours. (B) Whilst mitomycin C treated LX2 cells (GFP and IRS2) were cultured in a pre-treated gelatin 96-well plate at a density of $15 \cdot 10^3$ cells/well for 5 days. Four independent experiments with four replicates per condition were performed. Ns $p < 0.05$ and * $p > 0.05$.

Moreover, these conclusions were related with Figure 10, where LX2-Irs2 looked bigger and fewer in number in both conditions (Mitomycin C and IRS2). A possible explanation could be that Irs2 promotes growth whilst inhibiting proliferation. This could mask differences in the MTT assays because the cells proliferated less but "grew" bigger. Following Mitomycin C, they cannot proliferate so combination of cell numbers changing by death and static cell growth in the absence of proliferation could be seen.

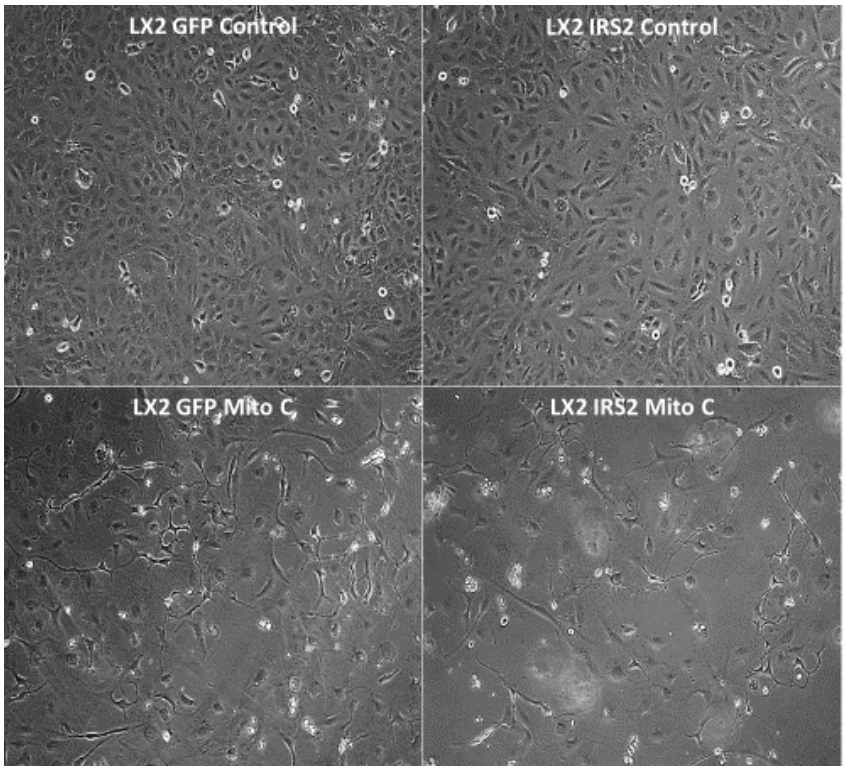


Figure 10. Microscopy 20x images. Images of cell cultures in 10 cm dishes were taken by day 3 in p10 at 20x. Mitomycin C treated and IRS2 cells survived less than contro and GFP.

5.4. P53 expression in LX2 cells

Since it looked like Irs2 might accelerate cell death following Mitomycin C, the next step was to know if there was more p53 expression when cells were treated with Mitomycin C. For studying this, p53 was analysed by Western blot, in an n = 2 experiment.

The four different conditions were compared (Figure 11) and only a significant difference was found between GFP control and Mitomycin C cells, in which Mitomycin C cells increased p53 expression. No significant differences between the other groups were evident ($p > 0.05$, Fig. 11). However, generally it was observed that p53 expression increased with IRS2 cells and with mitomycin C treatment, consistent with the hypothesis that Irs2 is inhibitory to cell growth/survival in HSCs.

These results can be related with the previous section (5.3.), where a decrease of survival was found in both conditions: Mitomycin C and IRS2 cells. As it is known, p53 plays a key role in cellular senescence, where its expression enhances this event by cell cycle arrest, DNA repair or apoptosis. Therefore, an increase in its expression could explain a lower survival of cells (Jin et al., 2016). Nonetheless, this was an n = 2 experiment, and more analysis would have to be performed to get a significant conclusion.

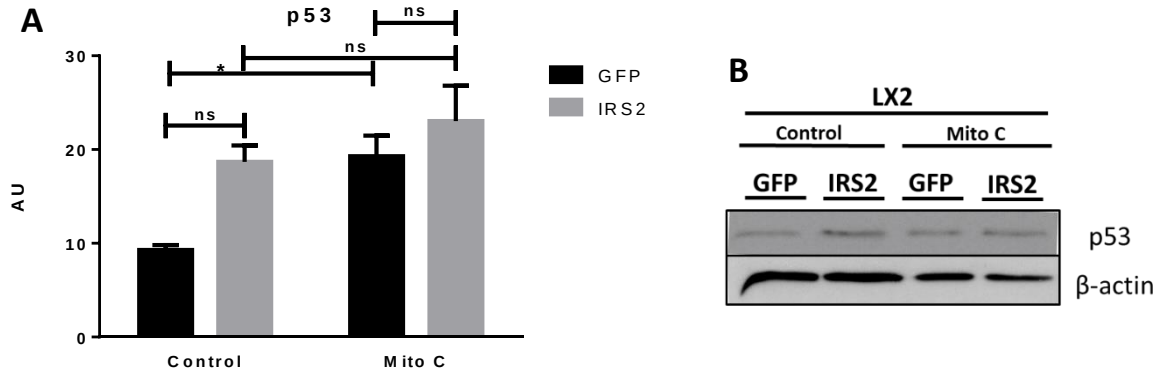


Figure 11. Results of p53 Western blot analysis. (A) Quantified p53 expression of control and Mitomycin C treated GFP and IRS2 cells was compared (n=2 for each group). Only a significant difference was found between control and Mitomycin C GFP cells. Data were statistically analysed using a paired t test. Results were expressed as the means ± SEM. * $p > 0.05$, n = 2. (B) Western blot from where p53 was quantified. Control and Mitomycin C GFP and IRS2 cells (n=2) were harvested.

5.5. FGF7 expression in LX2 cells

The previous results demonstrated that Irs2 might contribute to a loss of cell survival. For this reason, it was thought that studying FGF7 expression would be interesting. As it is known, FGF7 is an important mitogen and plays a key role in repair after liver injury, participating in fibrosis resolution and enhancing proliferation (Steiling et al., 2003). Therefore, FGF7 expression was analysed by Western blot to know how *Irs2* over expression had modified its secretion in control cells.

A significant difference was found between control GFP and IRS2 cells, which suggested that *Irs2* overexpression might contribute to a decrease in FGF7 expression. This result could be related with the less survival of over expressed IRS2 cells observed previously. If IRS2 cells do not survive as long as GFP, less FGF7 could be secreted. Another possibility could be that FGF7 was secreted into the medium. Therefore, not all the FGF7 produced could be detected by Western blot and other techniques would have to be considered.

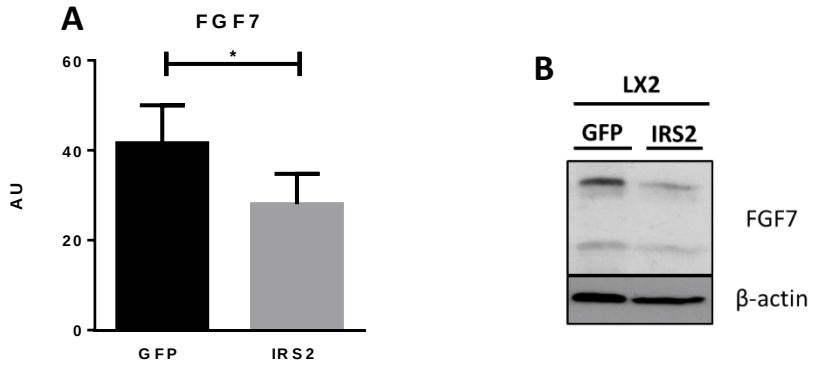


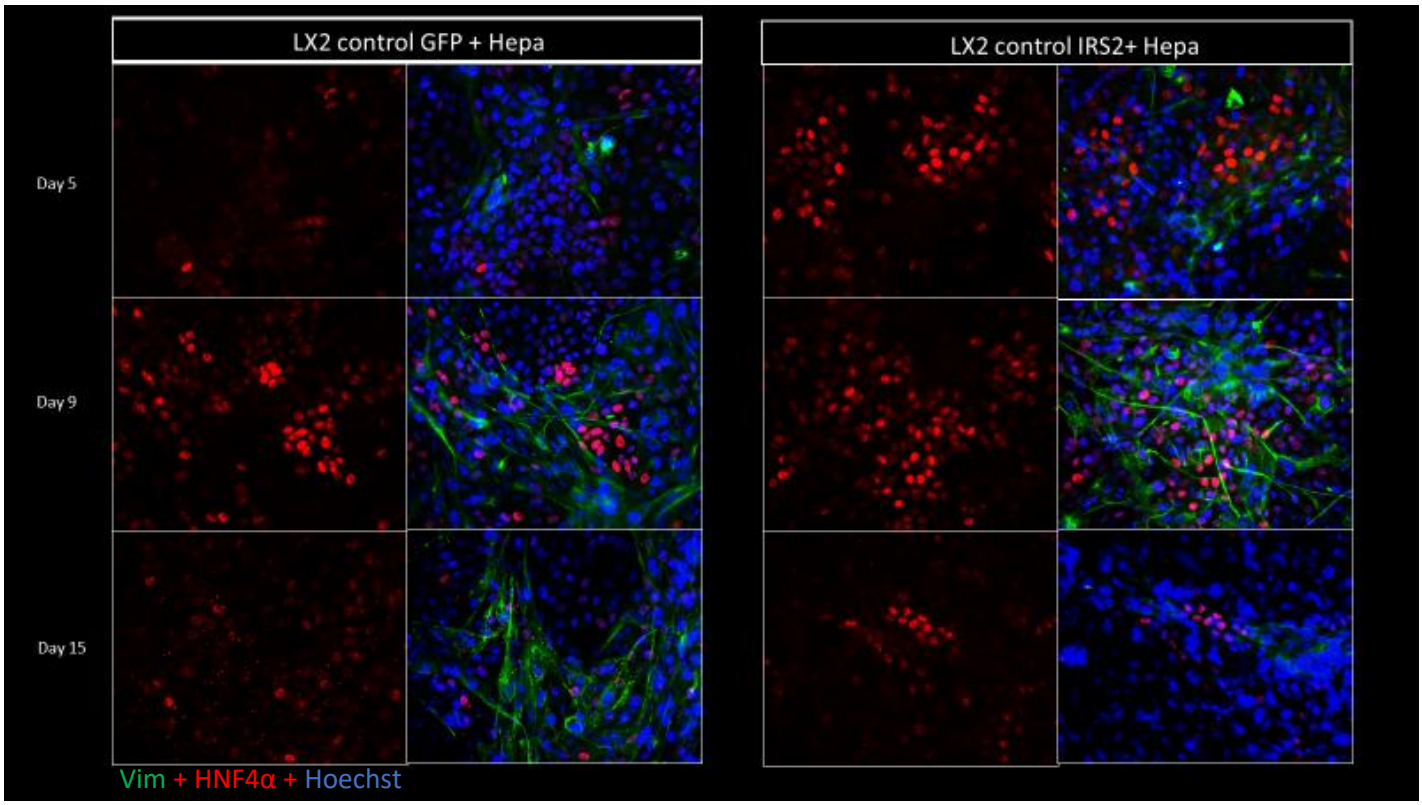
Figure 12. Results of FGF7 Western blot analysis. (A) Quantified FGF7 expression was compared between control GFP and IRS2 cells (n=4). A significant difference was found between GFP and IRS2 cells (p=0.0421). Data were statistically analysed using a paired t test. Results were expressed as the means ± SEM. * $p > 0.05$. (B) Western blot from where FGF7 was quantified. Control GFP and IRS2 cells (n=4) were harvested.

5.6. Survival of LX2 with HepaRG cells in co-culture.

Monocultures indicated that *Irs2* overexpression might contribute to reduce cell survival. Therefore, co-culture of LX2 with HepaRG cells was performed to have more realistic results as, in this way, it would be possible to study natural interactions between the two cell lines, improve their culturing and establishing synthetic interactions between them (Goers et al., 2014). Co-cultures with the four different conditions were cultured once for different timepoints and were analysed by immunofluorescence. Images then were taken and analysed qualitatively.

Generally, images (Figure 13) suggested the same that was proposed previously, which was that LX2 cells had a lower survival when were treated with Mitomycin C and when *Irs2* was overexpressed. In control cells (Figure 13A), by day 15 Irs2-LX2 cells were not present anymore, as vimentin expression was no longer observed. Whilst in Mitomycin C conditions (Figure 13B), by day 5 it was clear that GFP had more vimentin expression than IRS2, showing a lower survival of IRS2 cells.

A



B

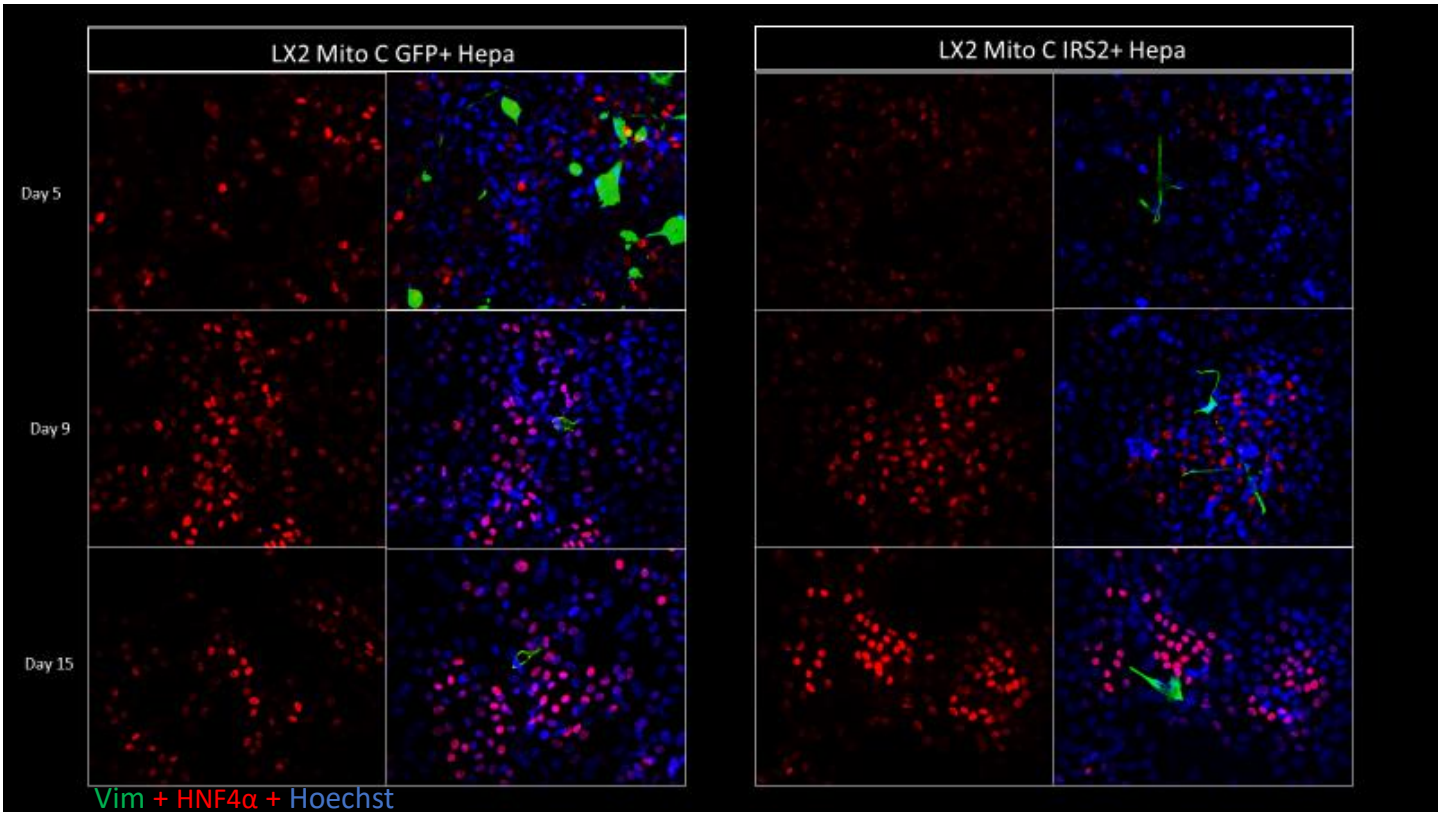


Figure 13. Vimentin, HNF4α AND Hoechst staining images. Images were merged with ImageJ software. (A) Control GFP and IRS2 LX2 with HepaRG co-cultures at different timepoints. By day 15 no survival of IRS2 LX2 cells (vimentin) was observed. (B) Mitomycin C GFP and IRS2 LX2 with HepaRG cells co-cultures at different timepoints. Big differences by day 5 are observed in LX2 survival, where GFP survive more.

5.7. FGFR2-IIIb

To completely analyse FGF7 expression, mRNA of its high-affinity receptor FGFR2-IIIb was analysed by RT-PCR from a co-culture of LX2 with HepaRG cells in the four different conditions. A significant difference was found between mitomycin C GFP and IRS2 cells, where IRS2 expressed less FGFR2-IIIb. Furthermore, FGFR2-IIIb expression increased significantly when cells were treated with Mitomycin C (Figure 14). This last result coincides with literature, which indicates that when HSCs get inactivated after liver injury repair (in this case treated with Mitomycin C), expression of FGF7 and FGFR2-IIIb increase significantly, participating in fibrosis resolution (Schumacher & Guo, 2016).

Whilst in the case of IRS2 cells, FGFR2-IIIb was less expressed, which matches with the conclusion obtained previously (section 5.5.), where FGF7 expression decreased with *Irs2* over expression. This could discard the possibility of not detecting FGF7 because of its secretion into the medium and it could be related with the shorter survival of *Irs2* overexpressed cells.

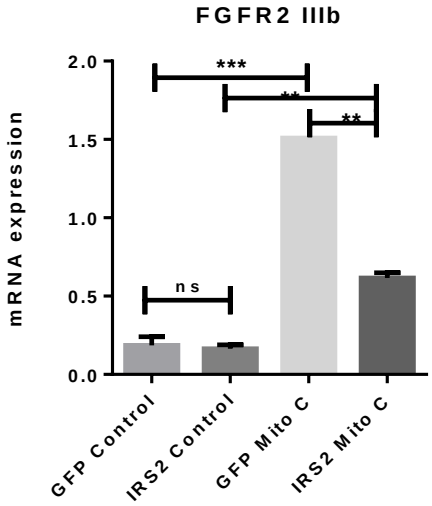


Figure 14. Results of FGFR2-IIIb mRNA expression quantification. mRNA quantification of FGFR2-IIIb was compared between control and Mitomycin C GFP and IRS2 cells (n=1, with two duplicates). Significant differences were found between GFP control and mitomycin C ($p < 0.001$), IRS2 control and mitomycin C ($p < 0.01$) and mitomycin C GFP and IRS2 cells ($p < 0.01$). Data were statistically analysed using a 2way ANOVA. Results were expressed as the means ± SEM.

5.7. Fibrosis and differentiation

Liver fibrosis consists in the excessive accumulation of ECM proteins, including collagen, and this occurs in most of chronic liver diseases (Bataller & Brenner, 2005). For studying how mouse *Irs2* over expression alters fibrosis and differentiation of LX2 cells, different experiments were performed. GFAP and α-sma expression were analysed by Western blot; whilst α-sma, APOA2, collagen Type I and III and SPP1 by RT-PCR. However, it is important to notice that the qRT-PCR was performed just once (n = 1) and no significant results were obtained.

5.7.1. Alpha-sma and GFAP expression

Proteins of control and Mitomycin C treated GFP and IRS2 cells were extracted and GFAP and α-sma were analysed by Western blot. No significant differences of GFAP and α-sma expression were observed neither between IRS2 and GFP cells or between control and mitomycin C

conditions (Figure 15). However, a slight decrease of both cell markers expression was observed once cells were treated with Mitomycin C.

This last result of GFAP expression coincides with the study of Geerts et al. (2001), which states that GFAP has high expression levels when HSCs are activated (control cells, Figure 8A), whilst its expression decreases when HSCs are in a quiescent state, which it is thought to be similar to but different from their inactivated state (in this case with Mitomycin C, Figure 8A). However, differences were no significant.

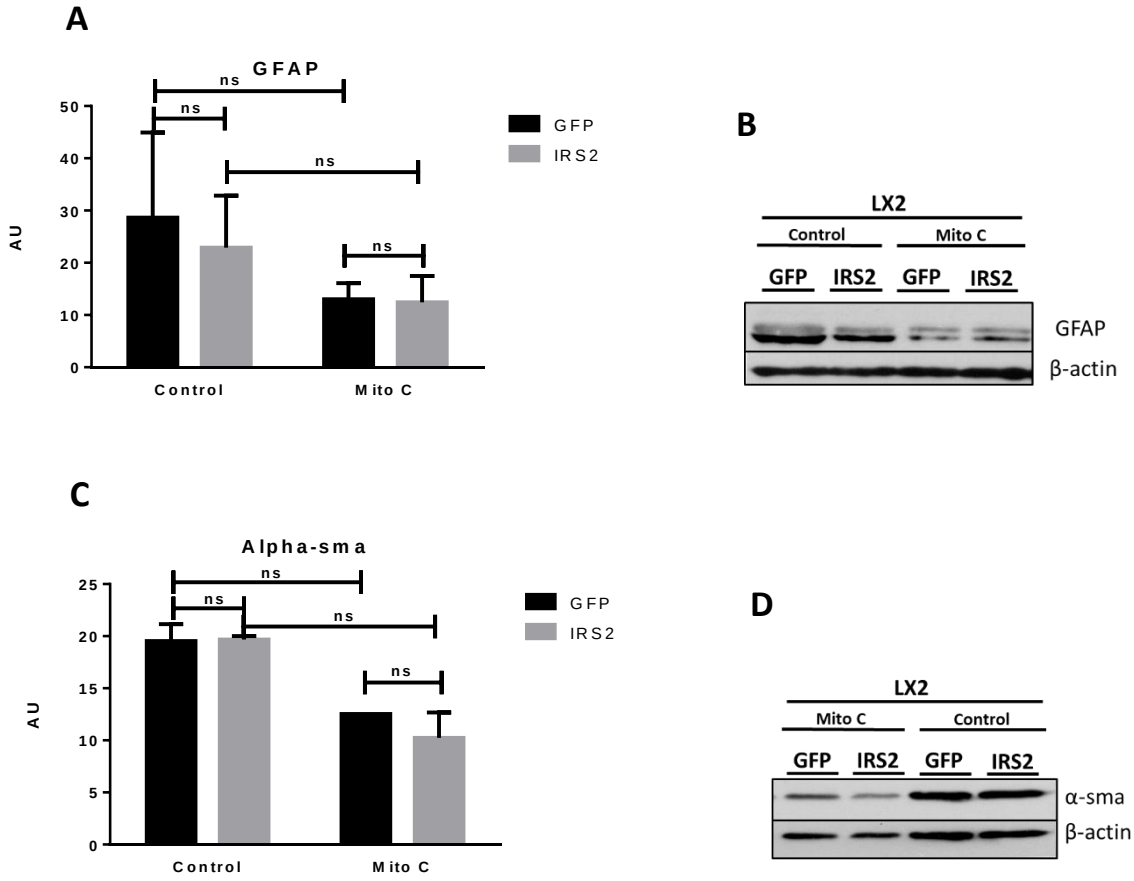


Figure 15. Results of GFAP and Alpha-sma Western blot analysis. Data were statistically analysed using a 2way Anova. Results were expressed as the means ± SEM. (n = 4). (A) Quantified GFAP expression of control and Mitomycin C treated GFP and IRS2 cells was compared (n=4 for each group). No significant differences were found between the four conditions ($p > 0.05$). (B) Western blot from where GFAP was quantified. Control and Mitomycin C GFP and IRS2 cells were harvested. (C) Quantified Alpha-sma expression of control and Mitomycin C treated GFP and IRS2 cells was compared (n=4 for each group). No significant differences were found between the four conditions ($p > 0.05$) (D) Western blot from where Alpha-sma was quantified. Control and Mitomycin C GFP and IRS2 cells were harvested.

Furthermore, mRNA of α-sma was analysed by RT-PCR. Similar results to the Western blot analysis were obtained (Figure 16). However, in this case significant differences were found between control and Mitomycin C treated cells, where the α-sma mRNA expression of the last ones decreased significantly after inactivating them with Mitomycin C. However, no differences were found between GFP and IRS2 cells.

Results confirmed what other studies have presented, which is that activated HSCs (Control in Figure 16) are supposed to show a myofibroblastic phenotype, expressing high levels of α-sma, whilst when HSCs get inactivated or get back to a quiescent state, this myofibroblastic

phenotype disappears and α-sma expression levels decrease (Carpino et al., 2005). Nonetheless, with regard to GFP and IRS2, results suggest that *Irs2* overexpression does not affect significantly their inactivated state or reversion to a quiescent phenotype.

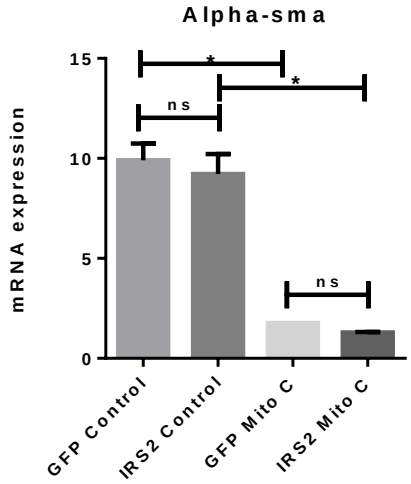


Figure 16. Results of Alpha-sma mRNA expression. Quantified mRNA of Alpha-sma expression of control and Mitomycin C treated GFP and IRS2 cells was compared (n=1, with 2 duplicates). Significant differences between control and Mitomycin C treated cells were found, where mRNA expression of Mitomycin decreased significantly ($p < 0.05$). Data were statistically analysed using a 2way Anova. Results were expressed as the means ± SEM.

5.7.2. Differentiation: APOA2 expression

APOA2 consists in a differentiation marker, and an analysis of its mRNA from the co-cultures was performed by RT-PCR to know how differentiation patterns changed between the four conditions.

Results showed significant differences between control and Mitomycin C cells, where inactivated cells increased APOA2 mRNA expression; whilst between Mitomycin C GFP and IRS2 cells, IRS2 decreased its expression (Figure 17). These results suggested that inactivation of cells might enhance HSCs differentiation. However, they suggested as well that *Irs2* overexpression might reduce it when cells got inactivated.

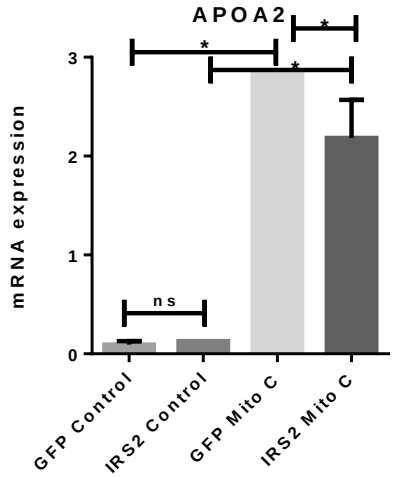


Figure 17. Results of APOA2 mRNA expression. Quantified mRNA of APOA2 expression of control and Mitomycin C treated GFP and IRS2 cells was compared (n=1, with 2 duplicates). Significant differences between control and Mitomycin C treated cells were found, where mRNA expression of Mitomycin increased significantly ($p < 0.05$), as well as between Mitomycin C GFP and IRS2 cells, where IRS2 decreased APOA2 expression ($p < 0.05$). Data were statistically analysed using a 2way Anova. Results were expressed as the means ± SEM.

5.7.3. Fibrosis: Collagen Type I and III and SPP1 expression

As it was mentioned previously, liver fibrosis involves accumulation of ECM proteins, in which collagen is included, and in which osteopontin plays a key role in its resolution (Wen et al., 2016). For this reason, their mRNA was analysed by RT-PCR.

Results of collagen type I and III showed significant differences between control and Mitomycin C cells, in which Mitomycin C treated cells reduced significantly Col I and III expression ($p < 0.005$). This result could be related with literature, which indicates that during fibrosis, where HSCs get activated (control), ECM proteins accumulate, including collagen. However, when HSCs stop being activated and change to an inactivated or quiescent state (Mitomycin C), ECM proteins disappear, which coincides with the results obtained (Schumacher & Guo, 2016). Whilst results of SPP1 expression showed the opposite. Significant differences were found between control and mitomycin C cells, in which in this case inactivated cells increased the expression of the gene ($p < 0.05$). This result coincides as well with literature, which indicates that OPN participates in fibrosis resolution by up-regulating its expression, and it is confirmed to enhance viability and wound healing (Wen et al., 2016). In conclusion, huge differences in fibrosis between activated and Mitomycin C treated cells were observed, as fibrosis depends on proliferation of these cells in the cocultures. Therefore, this assay was capable of detecting these differences, and reflected reduced survival of the LX2-Irs2 compared to control. Nonetheless, no significant differences were found between GFP and IRS2 cells in any of the three cases at the level of the mRNA. But it is important to notice that this assay was performed only once and at one timepoint (day 15). Therefore, the model was not the right one, as it was not long enough (n = 1), and no significant conclusions could be suggested.

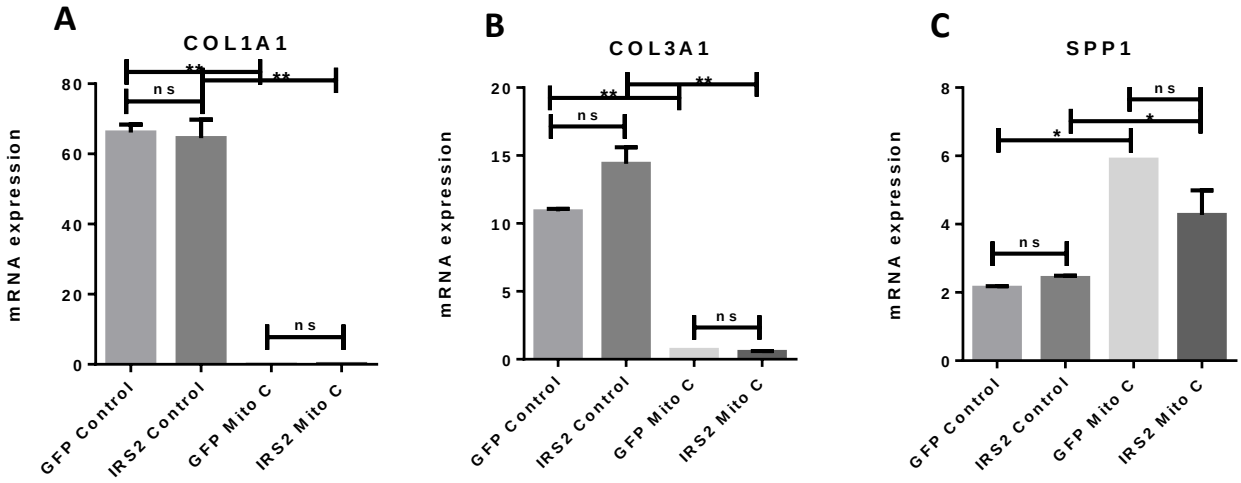


Figure 18. Results of Col1A1, Col3A1 and SPP1 mRNA expression. Quantified mRNA of COL1A1, COL3A1 and SPP1 expression of control and Mitomycin C treated GFP and IRS2 cells was compared (n=1, with 2 duplicates). Data were statistically analysed using a 2way Anova. Results were expressed as the means ± SEM. (A, B) Significant differences between control and Mitomycin C treated cells were found, where mRNA expression of Mitomycin decreased significantly ($p < 0.005$). No significant differences between GFP and IRS2 groups were found. (C) Significant differences between control and Mitomycin C treated cells were found, where mRNA expression of Mitomycin increased significantly ($p < 0.05$). No significant differences between GFP and IRS2 groups were found.

6. CONCLUSIONS

The purpose of the current study was to know if *Irs2* overexpression contributed to increase insulin sensitivity of LX2 cells and, if this happened, how this would change HSCs behaviour. The hypothesis presented suggested that *IRS2* might increase insulin sensitivity, and this would lead in a reduction of activated HSCs survival, which could lead in a decrease of fibrosis progression.

Generally, this study was rigorous enough as most of the experiments were n = 4, except for the signalling experiment and cocultures (n = 1). After analysing and discussing the results obtained, the study suggests that mouse Irs2 overexpression alters insulin/IGF-1 pathways, increasing insulin and IGF-1 sensitivity, as in general phosphorylation of the involved proteins increased notably. Furthermore, results have suggested as well that this Irs2 overexpression does not contribute to a longer survival of LX2 cells, as IRS2 cells have shown in all the experiments a decrease in their survival in both control and Mitomycin C conditions. Therefore, it could be concluded that *Irs2* overexpression reduces the lifespan of both activated and inactivated HSCs, or that these cells do not survive because of a selective disadvantage. These findings will be of interest to fibrosis therapies, as a reduction of activated HSCs survival or its faster disappearance might reduce the accumulation of fibrous scar and enhance fibrosis resolution. Therefore, *Irs2* overexpression could be considered for the development of future fibrosis and cirrhosis therapies.

Nonetheless, further research needs to be done to deepen the importance of *Irs2* overexpression in HSCs. More immunofluorescence assays should be done for later analysing samples by In-cell technology, which would allow proliferation and differentiation to be quantified. Furthermore, it would be interesting to perform ELISA assays to know if FGF7 is secreted into the medium or if its secretion is significantly reduced when *Irs2* is overexpressed. Finally, it would be interesting to study cells senescence with new markers such as p21, caspase-3 and Ki67, to see if results coincide with the p53 expression ones.

However, the current study has suggested an important conclusion, which is that increased insulin/Irs2 leads to reduced proliferation and survival of HSCs in monoculture and coculture - suggesting that fibrogenic reversion can be promoted by improving insulin sensitivity.

7. REFERENCES

BAI, X., SAAB, A. S., HUANG, W., HOBERG, I. K., KIRCHHOFF, F., & SCHELLER, A. (2013). Genetic Background Affects Human Glial Fibrillary Acidic Protein Promoter Activity. *PLoS ONE*, *8*(6). https://doi.org/10.1371/journal.pone.0066873

BATALLER, R., & BRENNER, D. (2005). Liver fibrosis. *The Journal of Clinical Investigation*, *115*(2), 209–218. https://doi.org/10.1172/JCI200524282.The

BELFIORE, A., FRASCA, F., PANDINI, G., SCIACCA, L., & VIGNERI, R. (2009). Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. *Endocrine Reviews*, *30*(6), 586–623. https://doi.org/10.1210/er.2008-0047

BOUCHER, J., KLEINRIDDERS, A., & KAHN, C. R. (2014). Insulin Receptor Signaling in Normal. *Cold Spring Harb Perspect Biol 2014*, *6*, a009191. https://doi.org/10.1101/cshperspect.a009191

BRUNET, A., BONNI, A., ZIGMOND, M. J., LIN, M. Z., JUO, P., HU, L. S., … GREENBERG, M. E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. *Cell*, *96*(6), 857–868. https://doi.org/10.1016/S0092-8674(00)80595-4

CARPINO, G., MORINI, S., GINANNI CORRADINI, S., FRANCHITTO, A., MERLI, M., SICILIANO, M., … GAUDIO, E. (2005). Alpha-SMA expression in hepatic stellate cells and quantitative analysis of hepatic fibrosis in cirrhosis and in recurrent chronic hepatitis after liver transplantation. *Digestive and Liver Disease*, *37*(5), 349–356. https://doi.org/10.1016/j.dld.2004.11.009

CEFALU, W. T. (2001). Insulin resistance: cellular and clinical concepts. *Experimental Biology and Medicine (Maywood, N.J.)*, *226*(1), 13–26. https://doi.org/10.1177/153537020122600103

COLLADO, M., BLASCO, M. A., & SERRANO, M. (2007). Cellular Senescence in Cancer and Aging. *Cell*. https://doi.org/10.1016/j.cell.2007.07.003

COPPS, K. D., & WHITE, M. F. (2012). Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. *Diabetologia*. https://doi.org/10.1007/s00125-012-2644-8

DE MELO CAMPOS, P., MACHADO-NETO, J. A., EIDE, C. A., SAVAGE, S. L., SCOPIM-RIBEIRO, R., DA SILVA SOUZA DUARTE, A., … TRAINA, F. (2016). IRS2 silencing increases apoptosis and potentiates the effects of ruxolitinib in JAK2V617F-positive myeloproliferative neoplasms. *Oncotarget*, *7*(6), 6948–6959. https://doi.org/10.18632/oncotarget.6851

FERRELL, L. (2000). Liver pathology: Cirrhosis, hepatitis, and primary liver tumors. Update and diagnostic problems. *Modern Pathology*, *13*(6), 679–704. https://doi.org/10.1038/modpathol.3880119

GEERTS, A., ELIASSON, C., NIKI, T., WIELANT, A., VAEYENS, F., & PEKNY, M. (2001). Formation of normal desmin intermediate filaments in mouse hepatic stellate cells requires vimentin. *Hepatology*, *33*(1), 177–188. https://doi.org/10.1053/jhep.2001.21045

GOERS, L., FREEMONT, P., & POLIZZI, K. M. (2014). Co-culture systems and technologies: Taking synthetic biology to the next level. *Journal of the Royal Society Interface*. https://doi.org/10.1098/rsif.2014.0065

GÖTZE, S., SCHUMACHER, E. C., KORDES, C., & HÄUSSINGER, D. (2015). Epigenetic changes during hepatic stellate cell activation. *PLoS ONE*, *10*(6). https://doi.org/10.1371/journal.pone.0128745

HICKMAN, I. J., & MACDONALD, G. A. (2007). Impact of Diabetes on the Severity of Liver Disease. *American Journal of Medicine*, *120*(10), 829–834. https://doi.org/10.1016/j.amjmed.2007.03.025

HUANG, C., THIRONE, A. C. P., HUANG, X., & KLIP, A. (2005). Differential contribution of insulin receptor substrates 1 versus 2 to insulin signaling and glucose uptake in L6 myotubes. *Journal of Biological Chemistry*, *280*(19), 19426–19435. https://doi.org/10.1074/jbc.M412317200

HUI, J. M., SUD, A., FARRELL, G. C., BANDARA, P., BYTH, K., KENCH, J. G., … GEORGE, J. (2003). Insulin Resistance Is Associated with Chronic Hepatitis C and Virus Infection Fibrosis Progression. *Gastroenterology*, *125*(6), 1695–1704. https://doi.org/10.1053/j.gastro.2003.08.032

INGLEHART, R., AMERICAN, T., SCIENCE, P., & DEC, N. (2007). the Renaissance of, *82*(4), 1203–1230.

International Diabetes Federation. (2013). *Atlas de la diabetes de la FID*. *International Diabetes Federation*. https://doi.org/2-930229-80-2

IREDALE, J. (2008). Defining therapeutic targets for liver fibrosis: Exploiting the biology of inflammation and repair. *Pharmacological Research*. https://doi.org/10.1016/j.phrs.2008.06.011

JIN, H., LIAN, N., ZHANG, F., CHEN, L., CHEN, Q., LU, C., … ZHENG, S. (2016). Activation of PPARγ/P53 signaling is required for curcumin to induce hepatic stellate cell senescence. *Cell Death & Disease*, *7*, e2189. https://doi.org/10.1038/cddis.2016.92

KAWAGUCHI, T., TANIGUCHI, E., ITOU, M., SAKATA, M., SUMIE, S., & SATA, M. (2011). Insulin resistance and chronic liver disease. *World Journal of Hepatology*, *3*(5), 99–107. https://doi.org/10.4254/wjh.v3.i5.99

KISSELEVA, T., CONG, M., PAIK, Y., SCHOLTEN, D., JIANG, C., BENNER, C., … BRENNER, D. A. (2012). Myofibroblasts revert to an inactive phenotype during regression of liver fibrosis. *Proceedings of the National Academy of Sciences*, *109*(24), 9448–9453. https://doi.org/10.1073/pnas.1201840109

KUILMAN, T., MICHALOGLOU, C., MOOI, W. J., & PEEPER, D. S. (2010). The essence of senescence. *Genes and Development*. https://doi.org/10.1101/gad.1971610

LEPREUX, S., & DESMOULIÈRE, A. (2015). Human liver myofibroblasts during development and diseases with a focus on portal (myo)fibroblasts. *Frontiers in Physiology*. https://doi.org/10.3389/fphys.2015.00173

LIU, K., GUO, M.-G., LOU, X.-L., LI, X.-Y., XU, Y., JI, W.-D., … DUAN, J.-C. (2015). Hepatocyte nuclear factor 4alpha induces a tendency of differentiation and activation of rat hepatic stellate cells. *World Journal of Gastroenterology*, *21*(19), 5856–5866. https://doi.org/10.3748/wjg.v21.i19.5856

MARTELLI, A. M., EVANGELISTI, C., CHIARINI, F., GRIMALDI, C., CAPPELLINI, A., OGNIBENE, A., & MCCUBREY, J. A. (2010). The emerging role of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin signaling network in normal myelopoiesis and leukemogenesis. *Biochimica et Biophysica Acta - Molecular Cell Research*. https://doi.org/10.1016/j.bbamcr.2010.04.005

MICHAEL MD, KULKARNI RN, POSTIC C, PREVIS SF, SHULMAN GI, MAGNUSON MA, K. C. (2000). Loss of insulin signaling in hepatocytes leads to s... *Molecular Cell*, *6*, 87–97. Retrieved from

https://www.cell.com/molecular-cell/pdf/S1097-2765(05)00015-8.pdf

Millipore. (2016). SCC064 | LX-2 Human Hepatic Stellate Cell Line, *114*. Retrieved from https://www.emdmillipore.com/US/en/product/LX-2-Human-Hepatic-Stellate-Cell-Line-,MM_NF-SCC064

MOHAMED, H. R., YAQOOT, M., -AZZIZ, A., KKALED, ;, ZALATA, R., & ABDEL-RAZIK, A. M. M. (2009). Relation of Insulin Resistance and Liver Fibrosis Progression in Patients with Chronic Hepatitis C Virus Infection. *International Journal of Health Sciences, Qassim University*, *3*(2). Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3068815/pdf/ijhs-3-2-0177.pdf

NISHIZAWA, H., IGUCHI, G., FUKUOKA, H., TAKAHASHI, M., SUDA, K., BANDO, H., … TAKAHASHI, Y. (2016). IGF-I induces senescence of hepatic stellate cells and limits fibrosis in a p53-dependent manner. *Scientific Reports*, *6*. https://doi.org/10.1038/srep34605

PERKINS, J. D. (2007). Glial Fibrillary Acidic Protein as an Early Marker of Hepatic Stellate Cell Activation in Chronic and Posttransplant Recurrent Hepatitis C. *Liver Transplantation*, *13*(3), 465–466. https://doi.org/10.1002/lt.

RUBIN, J. S., OSADA, H., FINCH, P. W., TAYLOR, W. G., RUDIKOFF, S., & AARONSON, S. A. (1989). Purification and characterization of a newly identified growth factor specific for epithelial cells. *Proceedings of the National Academy of Sciences of the United States of America*, *86*(3), 802–806. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2915979

SAWITZA, I., KORDES, C., REISTER, S., & HÄUSSINGER, D. (2009). The niche of stellate cells within rat liver. *Hepatology*, *50*(5), 1617–1624. https://doi.org/10.1002/hep.23184

SCHUMACHER, J. D., & GUO, G. L. (2016). Regulation of Hepatic Stellate Cells and Fibrogenesis by Fibroblast Growth Factors. *BioMed Research International*, *2016*, 1–8. https://doi.org/10.1155/2016/8323747

SHULMAN, G. I. (2000). Cellular mechanisms of insulin resistance. *The Journal of Clinical Investigation*, *106*(2), 171–176. https://doi.org/10.1172/JCI10583.On

SKALLI, O., ROPRAZ, P., TRZECIAK, A., BENZONANA, G., GILLESSEN, D., & GABBIANI, G. (1986). A monoclonal antibody against alpha-smooth muscle actin: a new probe for smooth muscle differentiation. *The Journal of Cell Biology*, *103*(6 Pt 2), 2787–2796. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3539945

STEILING, H., MÜ, M., WERNER, S., & HELLERBRAND, C. (2004). Activated Hepatic Stellate Cells Express Keratinocyte Growth Factor in Chronic Liver Disease. *American Journal of Pathology*, *165*(4), 1233–1241. https://doi.org/10.1016/S0002-9440(10)63383-4

STEILING, H., WÜSTEFELD, T., BUGNON, P., BRAUCHLE, M., FÄSSLER, R., TEUPSER, D., … WERNER, S. (2003). Fibroblast growth factor receptor signalling is crucial for liver homeostasis and regeneration. *Oncogene*, *22*(28), 4380–4388. https://doi.org/10.1038/sj.onc.1206499

TACKE, F., & TRAUTWEIN, C. (2015). Mechanisms of liver fibrosis resolution. *Journal of Hepatology*, *63*(4), 1038–1039. https://doi.org/10.1016/j.jhep.2015.03.039

TANIGUCHI, C. M., UEKI, K., & KAHN, C. R. (2005). Complementary roles of IRS-1 and IRS-2 in the hepatic regulation of metabolism. *Journal of Clinical Investigation*, *115*(3), 718–727. https://doi.org/10.1172/JCI23187

VALVERDE, A. M., BURKS, D. J., FABREGAT, I., FISHER, T. L., CARRETERO, J., WHITE, M. F., & BENITO, M. (2003). Molecular mechanisms of insulin resistance in IRS-2-deficient

hepatocytes. *Diabetes*, *52*(9), 2239–2248. https://doi.org/10.2337/diabetes.52.9.2239

WEN, Y., JEONG, S., XIA, Q., & KONG, X. (2016). Role of osteopontin in liver diseases. *International Journal of Biological Sciences*. https://doi.org/10.7150/ijbs.16445

WERNER, S. (1998). Keratinocyte growth factor: a unique player in epithelial repair processes. *Cytokine & Growth Factor Reviews*, *9*(2), 153–165. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9754709

WILCOX, G. (2005). Insulin and insulin resistance. *The Clinical Biochemist. Reviews / Australian Association of Clinical Biochemists*, *26*(2), 19–39. https://doi.org/10.1016/S0025-7125(03)00128-7

WITHERS, D. J., GUTIERREZ, J. S., TOWERY, H., BURKS, D. J., REN, J. M., PREVIS, S., … WHITE, M. F. (1998). Disruption of IRS-2 causes type 2 diabetes in mice. *Nature*, *391*(6670), 900–904. https://doi.org/10.1038/36116

World Health Organization. (2016). Global Report on Diabetes. *Isbn*, *978*, 88. https://doi.org/ISBN 978 92 4 156525 7

WU, J., TSENG, Y. D., XU, C. F., NEUBERT, T. A., WHITE, M. F., & HUBBARD, S. R. (2008). Structural and biochemical characterization of the KRLB region in insulin receptor substrate-2. *Nature Structural and Molecular Biology*, *15*(3), 251–258. https://doi.org/10.1038/nsmb.1388

ZHANG, W., PATIL, S., CHAUHAN, B., GUO, S., POWELL, D. R., LE, J., … UNTERMAN, T. G. (2006). FoxO1 regulates multiple metabolic pathways in the liver effects on gluconeogenic, glycolytic, and lipogenic gene expression. *Journal of Biological Chemistry*, *281*(15), 10105–10117. https://doi.org/10.1074/jbc.M600272200

ZHOU, W. C., ZHANG, Q. B., & QIAO, L. (2014). Pathogenesis of liver cirrhosis. *World Journal of Gastroenterology*, *20*(23), 7312–7324. https://doi.org/10.3748/wjg.v20.i23.7312

APPENDICES

Appendix 1: *Irs2* silencing and insulin sensitivity

In this case, a different pattern from the analysed in the current study with Irs2 overexpression was observed. Generally, it was observed that Irs2 silencing decreased phosphorylation of most of the signalling proteins when these were stimulated with IGF-1. Whilst, in general, no significant differences were observed between control and shIRS2 cells with insulin stimulation.

In conclusion, these results suggested that *Irs2* silencing altered downstream signalling pathways in a different way than with Irs2 overexpression.

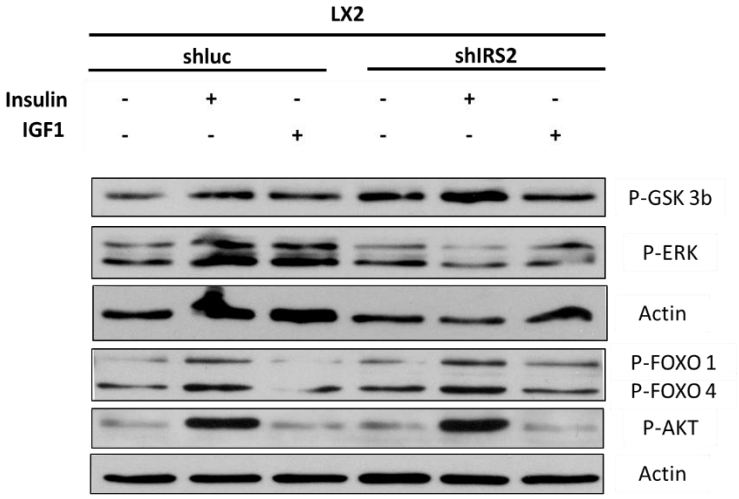


Figure 19. Western blot of insulin/IGF-1 pathways phosphorylated proteins in GFP and shIRS2 cells. A different pattern of phosphorylation was observed from the Irs2 overexpression cells.

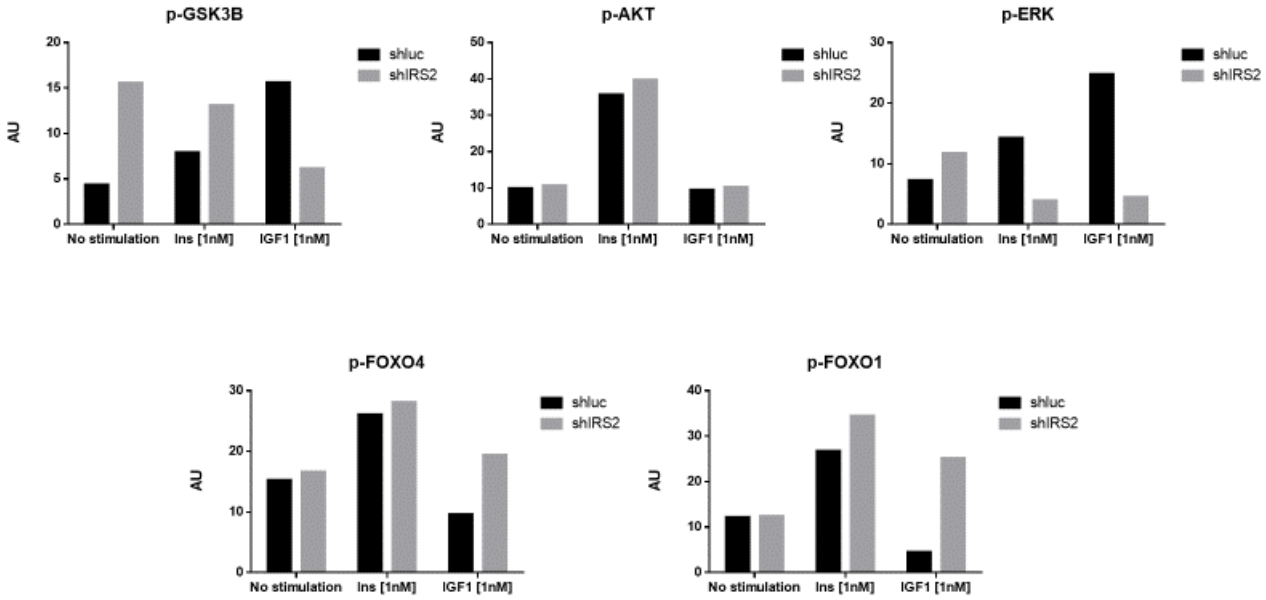


Figure 20. Results of signalling experiment with shIRS2 cells. n=1 experiment. Cells were stimulated with [1nM] of insulin and IGF1.