Hepatocyte Notch signaling deregulation related to lipid metabolism in women with obesity and non-alcoholic fatty liver

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- ***What is already known about this subject?***

The deregulation of the Notch pathway seems to play pathogenic roles in liver fibrosis. Moreover, it has been suggested that the inhibition of this pathway in the liver may be therapeutically beneficial in patients with non-alcoholic fatty liver disease (NAFLD).

- ***What does your study add?***

In this study, we aimed to explore the relationship between the Notch signaling pathway and the degree of NAFLD. Additionally, we wanted to investigate whether this pathway is related to hepatic lipid metabolism. The main result was, in a well-characterized cohort of women with morbid obesity (MO) with NAFLD, the downregulation of hepatic HES5 expression, which positively correlate with the hepatic expression of HES1, HEY1 and Notch3. Moreover, the novelty of this work lies in the fact that expression of Notch proteins and ligands positively correlates to hepatic lipid metabolism-related gene expression and Toll-like receptors (TLR).

Abstract

Objective: In this cohort study, we aimed to explore the relationship between the Notch signaling pathway and the degree of NAFLD. Moreover, we wanted to investigate whether this pathway is related to hepatic lipid metabolism and TLR.

Methods: We used real-time polymerase chain reaction (RT-PCR) analysis to evaluate the hepatic expression level of all genes studied (Notch1, Notch2, Notch3, Notch4, HES1, HES5, HEY1 and HEY2) in hepatic tissue from two cohorts: women with morbid obesity (MO) (n=57) with normal liver histology (NL, n=20) or with NAFLD (n=37).

Results: In women with MO with NAFLD, we found downregulation of hepatic HES5 expression. This expression correlated positively with the hepatic expression of HES1, HEY1 and Notch3. We also found a positive correlation between HES5 expression and SREBP1c and between Notch3 and several genes related to hepatic lipid metabolism (LXRα, FXR, SREBP1c, ACC1, FAS, PPARα, CPT1, CROT, ABCA1, and ABCG1). Finally, we found a positive correlation between Notch 2 and TLR2, TLR4 and TLR9 and a positive relationship between Notch 1 and TLR9.

Conclusion: Taken together, these findings suggest that hepatic expression of Notch proteins and ligands in relation to lipid metabolism pathways in the liver could have a role in NAFLD pathogenesis.

Abbreviations

ABCA1, ATP binding cassette subfamily A member 1

ABCG1, ATP binding cassette transporters G1

ACC1, acetyl-CoA carboxylase 1

ALT, alanine aminotransferase

AST, aspartate aminotransferase

BMI, body mass index

CPT1a, carnitine palmitoyltransferase 1a

CROT, carnitine O-octanoyltransferase

FAS, fatty acid synthase

FXR, farnesoid X receptor

HES, hairy enhancer of split

HES1, Transcription factor HES-1

HES5, Transcription factor HES-5

HEY, Hes-related family

HEY1, Transcription factor HEY-1

HEY2, Transcription factor HEY-2

LXRα, liver X receptors α

MO, morbid obesity

NAFLD, non-alcoholic fatty liver disease

NASH, non-alcoholic steatohepatitis

PPARα, peroxisome-proliferator-activated receptor α

SREBP1c, sterol regulatory element-binding protein 1c

T2DM, type 2 diabetes mellitus

TLR, Toll-like receptor

Introduction

Non-alcoholic fatty liver disease (NAFLD) affects approximately 25% of the global population and approximately 37% of subjects with obesity, paralleling worldwide increases in obesity and metabolic syndrome. Non-alcoholic steatohepatitis (NASH) is the severe form of NAFLD and, if left untreated, can evolve into end-stage liver diseases such as liver cirrhosis and hepatocellular carcinoma (1). The risk of liver-related mortality increases exponentially with an increase in fibrosis stage (2). The underlying precise mechanisms of disease pathogenesis and NAFLD progression have just begun to be understood. The classic "multiple hit" theory of NAFLD pathogenesis states that lipid accumulation initiates hepatic steatosis and subsequently triggers multiple insults (hormones/adipokines secreted from the adipose tissue, inflammation, dysregulated fat metabolism, lipotoxicity, oxidative stress, mitochondrial dysfunction, genetic and epigenetic factors, intestinal dysbiosis and angiogenesis) (3), ultimately inducing NASH and cirrhosis (4). In this sense, a more detailed understanding of the molecular pathogenesis of NAFLD, particularly NASH, is needed to develop more rational strategies for the prevention and treatment of this common liver disease. Currently, there is no approved drug treatment that specifically targets NASH.

Notch proteins are a family of receptors involved, first, in cell differentiation during embryogenesis, participating in different processes such as gene expression in somitogenesis, vasculogenesis, regulating endocardial and hematopoietic stem cells, among others. Second, these proteins are involved in the homeostasis of different tissues, such as the lung, skin, liver or intestine. In addition, this signaling pathway is emerging as an important regulator of both innate and adaptive immune system development and function (5,6). Notch signaling has crucial implications in metabolic syndrome (7) via M1 versus M2 macrophage specification regulation, among other mechanisms (8,9) as shown Figure 1.

There are only a handful of classical Notch target genes, among which the best characterized belong to the hairy enhancer of split (HES) and Hes-related (HEY) family, which execute most biological processes and partially underlie the target specificity of

different Notch receptor paralogs. However, countless other genes can also be regulated in parallel with these direct Notch target genes (10). Finally, different diseases have been described with deregulation in this signaling pathway, both neoplasic and nonneoplasic ones. Additionally, fibrosis occurs in different organs, such as the lung, heart and liver (11). Regarding the liver, Notch signaling is important for the proper development of the biliary tree and is also involved in liver regeneration and repair, liver metabolism, liver fibrosis and cancer of the liver (12-15). Moreover, the possibility of using mitigating therapies of Notch against different types of fibrosis has been investigated, for example, against cholestatic liver fibrosis (16). It is important to note that a recent study investigated the inhibition of Notch signaling as a therapeutic strategy against fibrosis caused by NAFLD (17). As Romeo suggested recently (18), the regulation of Notch signaling represents a therapeutic objective against fibrosis in patients with NASH. However, as the authors stated, there may be additional Notch-regulated factors involved in the pathogenesis of NASH/fibrosis yet to be identified, such as liver-enriched long noncoding RNAs (19) or lipid metabolism (6).

Therefore, in the present project, we first explored the role of the Notch pathway in the pathogenesis of NAFLD by studying the hepatic expression of Notch1, Notch2, Notch3, Notch4 and the Notch target genes HES1, HES5, HEY1 and HEY2 in a cohort of patients with MO and NAFLD. In addition, as lipid metabolism seems to be involved in the pathogenesis of NAFLD and may be related to Notch pathway regulation, our second aim was to investigate the association between the hepatic expression of lipid metabolism-related genes (LXRα, FXR, SREBP1c, ACC1, FAS, PPARα, CPT1, CROT, ABCA1 and ABCG1) and the expression of Notch1-4 and the Notch target genes. Finally, given that the Notch and Toll like-receptors (TLRs) pathways have been described to cooperate to activate the Notch target genes (20), another objective of the study was to analyze the relationship between the liver expressions of both pathways' genes.

Methods

Subjects

The study was approved by the institutional review board (Institut Investigació Sanitària Pere Virgili (IISPV) CEIm; 23c/2015), and all participants gave written informed consent. The study population consisted of 57 Caucasian women with morbid obesity (BMI > 40 kg/m^2). Liver biopsies were obtained during planned laparoscopic bariatric surgery. All liver biopsies were indicated for clinical diagnosis. The exclusion criteria were as follows: (1) subjects who had alcohol consumption higher than 10 g/day; (2) patients who had acute or chronic hepatic, inflammatory, infectious or neoplasic diseases; (3) women who were menopausal or undergoing contraceptive treatment; (4) women with diabetes receiving pioglitazone or insulin; and (5) patients treated with antibiotics in the previous 4 weeks.

Sample size

Accepting an α risk of 0.05 and a β risk of less than 0.2 in a bilateral contrast, 24 subjects per group are needed to detect a difference ≥ 0.2 units. It is assumed that the common standard deviation is 0.3.

Liver pathology

Liver samples were scored by experienced hepatopathologists using methods described elsewhere (21). According to their liver pathology, women with MO were subclassified into: normal liver (NL) histology (n=20) and NAFLD (n=37) [SS (micro/macrovesicular steatosis without inflammation or fibrosis, n=22) and NASH (Brunt Grades 1-2, n=15)]. None of the patients with NASH in our cohort presented fibrosis.

Biochemical analyses

All of the subjects included underwent physical, anthropometric and biochemical assessments. Blood samples were obtained from subjects with obesity and control subjects. Biochemical parameters were analyzed using a conventional automated analyzer after 12

hours of fasting. Insulin resistance (IR) was estimated using the homeostasis model assessment of IR (HOMA1).

Gene expression in the liver

Liver samples collected after bariatric surgery were conserved in RNAlater (Qiagen, Hilden, Germany) at 4ºC and then processed and stored at -80ºC. Total RNA was extracted from liver by using the RNeasy mini kit (Qiagen, Barcelona, Spain). Reverse transcription to cDNA was performed with the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Madrid, Spain). Real-time quantitative PCR was performed with the TaqMan Assay predesigned by Applied Biosystems for the detection of LXRα (Hs00173195_m1), FXR (HS00231968_M1), SREBP1c (Hs01088691_m1), ACC1 (Hs00167385_m1), FAS (Hs00188012_m1), PPARα (Hs00947538_m1), CPT1a (Hs00912671_m1), CROT (Hs00221733_m1), ABCA1 (Hs01059118_m1), ABCG1 (Hs00245154_m1), HES1 (Hs00172878_m1), HES5 (Hs01387463_g1), HEY1 (Hs00232618_m1), HEY2 (Hs00232622_m1), NOTCH1 (Hs01062014_m1), NOTCH2 (Hs01050702_m1), NOTCH3 (Hs00166432_m1), NOTCH4 (Hs00965889_m1), TLR2 (Hs02621280_s1), TLR4 (Hs00152939_m1), TLR9 (Hs00370913_s1) and 18S ribosomal RNA (Fn04646250_s1) that was used as a housekeeping gene. All reactions were carried out in duplicate in 96-well plates using the 7900HT Fast Real-Time PCR systems (Applied Biosystems, Foster City, CA, USA)

Statistical analysis

The data were analyzed using the SPSS/PC+ for Windows statistical package (version 23.0; SPSS, Chicago, IL, USA). The Kolmogorov-Smirnov test was used to assess the distribution of variables. Continuous variables were reported as the mean±SD**;** non-continuous variables were reported as the median and 25-75th percentile and categorical variables were shown as counts (percent). The different comparative analyses were performed using a nonparametric Mann-Whitney U test or Kruskal-Wallis test, according to the presence of two or more groups. The strength of the association between variables was calculated using Pearson's

method (parametric variables) and Spearman's ρ correlation test (nonparametric variables). P-values < 0.05 were statistically significant.

Results

Baseline characteristics of subjects

The main characteristics of the study cohort, including anthropometric and biochemical parameters, are shown in Table 1. We classified the subjects according to hepatic histology as MO with NL histology (n=20) and MO with NAFLD (n=37) (Table 1), which were comparable in terms of age, weight, BMI, insulin, HOMA1, glycosylated hemoglobin (HbA1C), cholesterol, triglycerides, gamma-glutamyltransferase (GGT) and alkaline phosphatase (ALP) and blood pressure. However, biochemical analyses indicated that the levels of glucose, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly higher in women with MO with NAFLD than in women with MO and NL.

Evaluation of the liver Notch receptors and ligands expression according to liver histology

One of the objectives of the present study was to evaluate the hepatic expression of Notch receptors and their ligands in a cohort of women with MO. We observed that in women with MO with NAFLD, the hepatic gene expression of HES5 was significantly lower than in women with MO with normal hepatic histology (Figure 2). Then, we categorized the subjects with MO and NAFLD in terms of their histological severity into simple hepatic steatosis (SS) and NASH. In this case, we did not observe any significant differences in the hepatic gene expression of the Notch receptors and ligands studied (data not shown).

Correlations between the HES5 hepatic expression with the liver expression of other Notch receptors and ligands

When we analyzed the association of HES5 hepatic expression with the other Notch receptors and ligands studied, we observed that HES5 correlated positively with HES1, HEY1 and Notch3 (Table 2).

Correlations of liver Notch receptor and ligand expression with liver biology parameters

We studied the relationship of liver Notch receptors and ligand expression with liver biology parameters, and we demonstrated a positive correlation between HES1 liver expression and GPT levels (r=0.292, p=0.029) and between HEY1 and GGT (r=0.284, p=0.034) and Notch4 and GGT (r=0.297, p=0.026).

Correlations of the liver Notch receptors and ligands expression with glucose metabolic parameters

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Correlations of liver Notch receptor and ligand expression with genes involved in lipid metabolism and biochemical lipid parameters

To add to the current knowledge about the role of Notch signaling in the pathogenesis of NAFLD in relation to lipid metabolism-related genes, we analyzed the correlation between the expression of the liver Notch receptors and ligands and the liver expression of LXRα, FXR, SREBP1c, ACC1, FAS, PPARα, CPT1, CROT, ABCA1 and ABCG1. The results shown in Figure 3 indicate that, in our cohort of women with MO, the hepatic gene expression of HES5 was positively associated with the hepatic expression of some key genes involved in lipid metabolism (ABCG1, CROT, and SREBP1c).

Moreover, the hepatic gene expression of HEY2 was positively associated with the expression of SREBP1c (r=0.2948, p=0.0443), and the hepatic gene expression of Notch1 was also positively associated with the expression of SREBP1c (r=0.2937, p=0.0451).

Regarding biochemical lipid parameters, we also observed that hepatic HEY2 expression correlated negatively with LDL cholesterol (r=-0.352, p=0.028). However, we were unable to

relate the hepatic expression of Notch proteins and ligands with other lipid metabolic circulating parameters.

Correlations of liver expression of TLRs (TLR2, TLR4 and TLR9) with liver Notch receptor and ligand expression

When we studied the correlation of liver expression of TLRs (TLR2, TLR4 and TLR9) with liver Notch receptor and ligand expression, we found a positive correlation between Notch 2 and TLR2, TLR4 and TLR9 ($r=0.303$, $p=0.039$; $r=0.355$, $p=0.014$; $r=0.327$, $p=0.025$, respectively). Also, we have found a positive relationship between Notch 1 and TLR9 ($r=0.406$, $p=0.005$).

Discussion

In this study, we found, in a well-characterized cohort of women with MO with NAFLD, downregulation of hepatic HES5 expression, which positively correlates with the hepatic expression of HES1, HEY1 and Notch3. The novelty of this work lies in the fact that the expression of Notch proteins and ligands positively correlates with hepatic lipid metabolism-related gene expression.

Although several rodent studies have been related to Notch signaling activation and NAFLD (22,23), it was not until 2013 that Valenti et al. demonstrated that Notch proteins and ligands were expressed in the liver of lean and subjects with obesity and that increased activation of this pathway positively correlated with gluconeogenic gene expression and hyperglycemia in a cohort of patients with morbid obesity undergoing bariatric surgery. In a validation cohort across a range of BMIs, they confirmed the positive association between HES/HEY family genes and insulin resistance and demonstrated an independent positive association with hepatic fat content. Finally, hepatic Notch signal activation correlated with measures of liver inflammation, suggesting that it may represent a marker of the transition from SS to NASH (24). In our study, although we found downregulation of hepatic HES5 in women with MO with NAFLD, we could not demonstrate upregulation of the Notch pathway or an association with the presence of NASH. Moreover, we were unable to relate hepatic expression of Notch proteins and ligands with glucose metabolic parameters.

Zhu et al., in a very interesting study, described that the number of HES1+ hepatocytes, but not of nonparenchymal cells (NPCs), was increased in patients with NASH. Moreover, in a longitudinal analysis using paired baseline and end-of-treatment biopsy specimens from the Pioglitazone versus Vitamin E versus Placebo for the Treatment of Nondiabetic Patients with Non-alcoholic Steatohepatitis (PIVENS) trial, they found that Notch activity, specifically in hepatocytes, tracks with NASH severity. In this study, in patients with NASH and in a mouse model of diet-induced NASH, the authors concluded that hepatocyte Notch activity facilitates liver fibrosis (17). In our work, we could not study the relationship with fibrosis because none

of the patients with NASH in our cohort presented fibrosis. This fact could be explained in part by the fact that our cohort is made up of middle-aged women with no other cause of liver disease than obesity and insulin resistance.

On the other hand, it has been described that increased hepatic lipid content can be caused by nutrient-induced activation of mammalian target of rapamycin (mTOR). This activation increases basal Akt activity, leading to a self-perpetuating lipogenic cycle. Pajvani et al. showed that inhibition of Notch signaling prevented hepatosteatosis by blocking mTOR complex 1 (mTORC1) activity. They also demonstrated that Notch signaling increased mTORC1 complex stability, augmenting mTORC1 function and SREBP1c-mediated lipogenesis and that inhibition of hepatic Notch signaling protects from fatty liver by reducing de novo lipogenesis (23). In this sense, we studied the relationship between Notch proteins and ligand hepatic expression with lipid metabolic parameters and hepatic lipid metabolism-related gene expression. We found a negative relationship between hepatic HEY2 expression and LDL cholesterol. Moreover, we described for the first time that hepatic HES5 expression was positively related to the hepatic expression of some key lipid metabolism-related genes in our cohort of women with MO. Therefore, the maladaptive hepatocyte Notch response in NAFLD is related to the expression of hepatic lipid metabolism-related genes.

In the present study, we have described a positive correlation between Notch and TLRs pathways, accordingly to some *in vitro* studies, in which the Notch and TLRs pathways cooperate to activate the Notch target genes, including the transcriptional repressors HES1 and HEY1 (25,20,26).Further human studies are needed to corroborate this relationship. It is important to note here that although our cohort made it possible to establish clear relationships between women with morbid obesity with NAFLD and deregulation of hepatic Notch protein and ligand expression, without the interference of confounding factors such as sex or age, these results cannot be extrapolated to men, women of other ages or over- and normal-weight subjects.

Conclusions

The hepatic expression of Notch proteins and their ligands seems to play a role in regulating lipid metabolism pathways in the liver, which could have implications in NAFLD pathogenesis.

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Table 1. Anthropometric and biochemical variables of the cohort with obesity classified according to the histopathological characteristics.

Variables	NL (N = 20) Mean ± SD	NAFLD (N = 37) Mean ± SD	p - value
Age (years)	45.33±10.15	47.81±9.03	0.321
Weight (kg)	119.70±16.52	112.86±13.24	0.086
BMI (kg/m²)	43.88±6.43	43.78±4.69	0.942
Glucose (mg/dL)	82.96±20.98	101.88±46.23*	**0.026**
Insulin (mUI/L)	11.43±8.64	11.87±11.84	0.887
HOMA1	10.51±15.34	15.32±21.93	0.462
HbA1c (%)	5.49±0.30	5.92±1.14	0.054
Cholesterol (mg/dl)	180.83±32.38	177.14±44.16	0.733
HDL-C (mg/dL)	46.88±11.16	42.51±9.55	0.180
LDL-C (mg/dL)	112.69±25.26	106.55±34.06	0.478
Triglycerides (mg/dL)	105.94±26.43	128.52±83.24	0.154
AST (UI/L)	21.88±10.90	32.48±22.75*	**0.014**
ALT (UI/L)	22.36±8.87	36.04±23.98*	**0.002**
GGT (UI/L)	27.80±33.18	31.04±32.08	0.659
ALP (UI/L)	65.23±15.64	68.45±13.70	0.457
SBP	118.94±17.02	117.71±12.465	0.781
DBP	65.684±10.38	65.92±9.37	0.934

NL, normal liver; NAFLD, non-alcoholic fatty liver disease; BMI, body mass index; HOMA1, homeostatic model assessment method-insulin resistance; HbA1c, glycosylated hemoglobin; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; ALP, alkaline phosphatase; SBP, systolic blood pressure, DBP, diastolic blood pressure. Insulin resistance was estimated using homeostasis model assessment of IR (HOMA1). Data are expressed as the mean ± SD. *Significant differences between NL group and NAFLD group ($P < 0.05$).

Table 2. Correlations between the HES5 hepatic expression with the liver expression of other Notch receptors and ligands.

	HES5	
Genes	**r**	**p-value**
HES1	0.380	**0.004***
HEY1	0.371	**0.005***
HEY2	0.172	0.200
NOTCH1	0.180	0.181
NOTCH2	0.025	0.852
NOTCH3	0.334	**0.011***
NOTCH4	0.195	0.146

HES, hairy enhancer of Split; HES1, Transcription factor HES-1; HES5, Transcription factor HES-5; HEY, Hes-related family; HEY1, Transcription factor HEY-1; HEY2, Transcription factor HEY-2. *Positive correlation between the HES5 hepatic expression with the liver expression of HES1, HEY1 and NOTCH3.

Figure 1. Notch regulates gluconeogenesis and lipogenesis of hepatocytes and activates inflammatory cells. Notch signaling, which starts with the interaction between delta-like protein receptor or Jag1 receptor and Notch1-4 receptors, serves to promote PI3K-mediated survival and differentiation through HES repression of PTEN (27). Thus far, the insulin-PI3K-AKT pathway activates mTOR, which in turn activates Srebp1c, a key factor that turns on transcription of Fasn. FASN is a rate-limiting enzyme of lipogenesis that promotes the synthesis of TAG, which generates the accumulation of lipid droplets in the cytoplasm (6). This accumulation initiates some inflammatory responses in other cells, which results in the activation of stellate cells, generating an inflammatory microenvironment (18). In addition, Notch signaling regulates hepatic glucose production through synergy with FoxO1. Transcriptionally active FoxO1 is phosphorylated by AKT and excluded from the nucleus, avoiding the transcription of G6pc. This enzyme promotes the transport of glucose out of the cell (6). Jag1, Jagged1; PI3K, phosphatidylinositol 3-kinase; HES, hairy enhancer of split; PTEN, phosphatase and tensin homolog; Akt, serine/threonine kinase; mTOR, mammalian target of rapamycin; Srebp1c, sterol regulatory element-binding protein 1c; Fasn, fatty acid synthase; TAG, triglyceride; FoxO1, forkhead box protein O1; G6pc, glucose-6-phosphatase catalytic subunit.

Figure 2. Differential hepatic expression of HES5 between women with MO with NL histology and women with MO with NAFLD. A.U; arbitrary units; MO, morbidly obesity; NAFLD, non-alcoholic fatty liver disease. NL; normal liver. $p < 0.05$ was considered statistically significant.

Figure 3. Correlation of the liver expression of Notch ligand HES5 with the liver expression of genes involved in lipid metabolism. ABCG1, ATP binding cassette transporters G1; CROT, carnitine O-octanoyltransferase; SREBP1c, sterol regulatory element-binding protein 1c; HES, hairy enhancer of split; HES5, Transcription factor HES-5. The strength of association between variables was calculated using Spearman's r correlation test. $p<0.05$ is considered statistically significant.

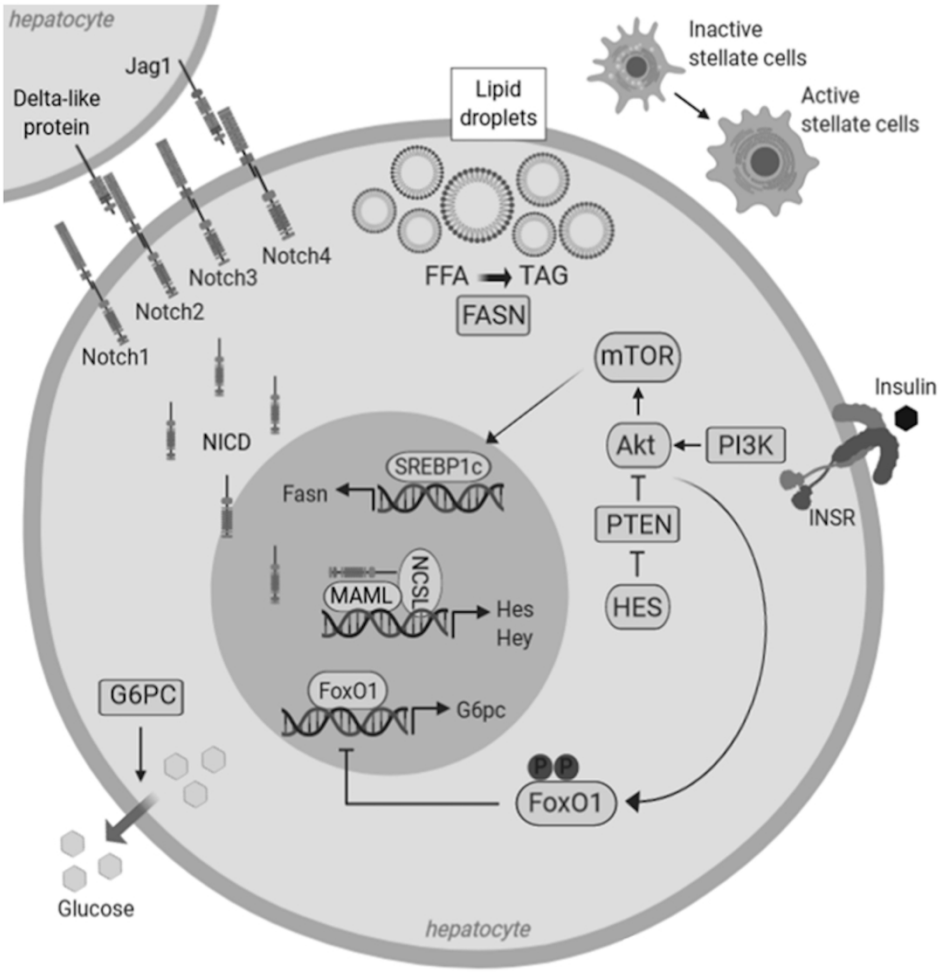

Figure 1. Notch regulates gluconeogenesis and lipogenesis of hepatocytes and activates inflammatory cells. Notch signaling, which starts with the interaction between delta-like protein receptor or Jag1 receptor and Notch1-4 receptors, serves to promote PI3K-mediated survival and differentiation through HES repression of PTEN(21). Thus far, the insulin-PI3K-AKT pathway activates mTOR, which in turn activates Srebp1c, a key factor that turns on transcription of Fasn. FASN is a rate-limiting enzyme of lipogenesis that promotes the synthesis of TAG, which generates the accumulation of lipid droplets in the cytoplasm(6). This accumulation initiates some inflammatory responses in other cells, which results in the activation of stellate cells, generating an inflammatory microenvironment(18). In addition, Notch signaling regulates hepatic glucose production through synergy with FoxO1. Transcriptionally active FoxO1 is phosphorylated by AKT and excluded from the nucleus, avoiding the transcription of G6pc. This enzyme promotes the transport of glucose out of the cell(6). Jag1, Jagged1; PI3K, phosphatidylinositol 3-kinase; HES, hairy enhancer of split; PTEN, phosphatase and tensin homolog; Akt, serine/threonine kinase; mTOR, mammalian target of rapamycin; Srebp1c, sterol regulatory element-binding protein 1c; Fasn, fatty acid synthase; TAG, triglyceride; FoxO1, forkhead box protein O1; G6pc, glucose-6-phosphatase catalytic subunit.

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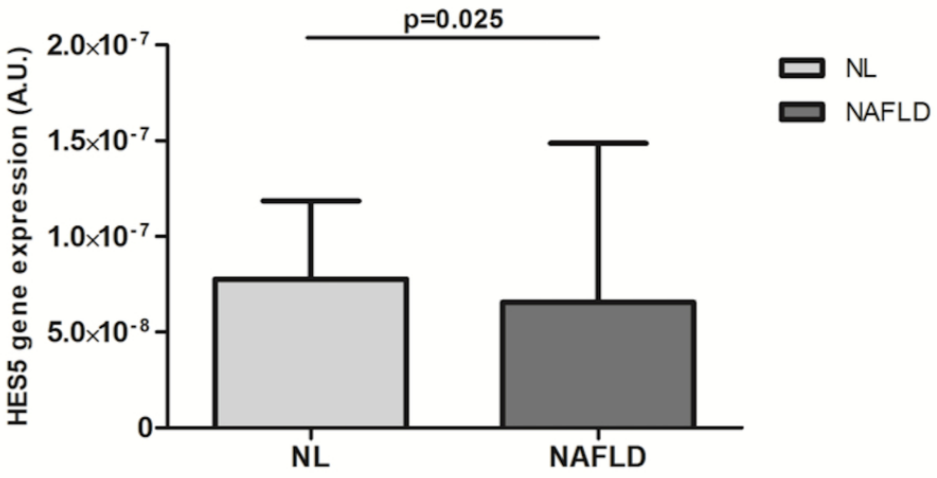

Figure 2

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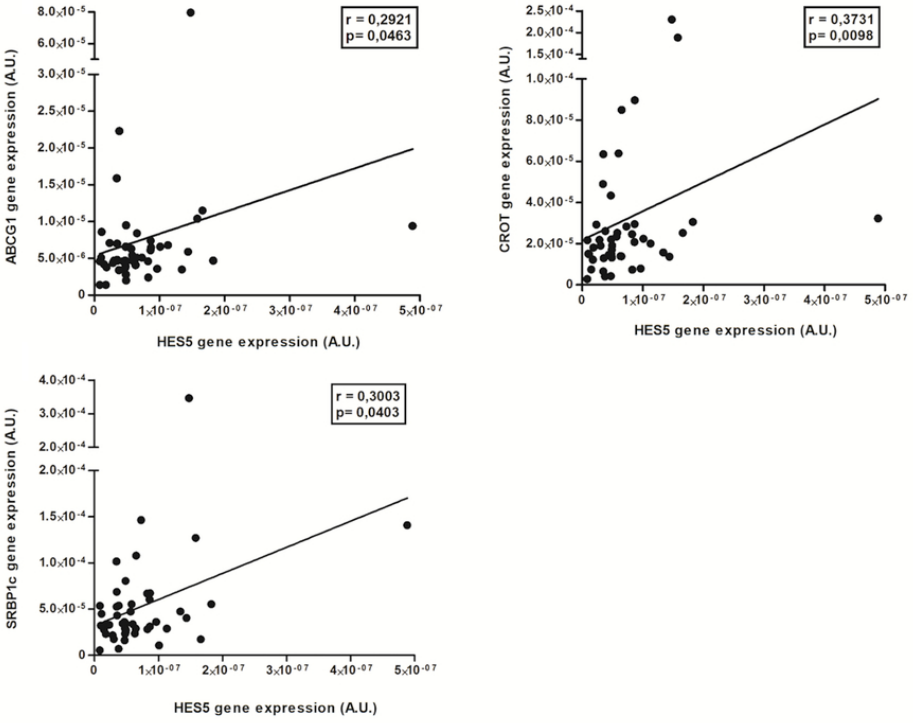

Figure 3

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