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# Early growth response-1 transcription factor promotes hepatic fibrosis and steatosis in long-term ethanol-fed Long-Evans rats

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## Abstract

**Background:** Previous studies demonstrated that the Long-Evans (LE) rats exhibited liver injury and lipid metabolic abnormalities after 8 weeks of ethanol feeding.

**Aims:** The goal of this study was to investigate if the LE rats develop more advanced hepatic abnormalities (e.g., fibrosis) after long-term feeding with an ethanol-containing Lieber–DeCarli diet. In addition, the contribution of early growth response-1 (EGR1) transcription factor to these pathological changes was assessed.

**Methods:** Long-Evans rats were fed an ethanol-containing or isocaloric control liquid diet for 18 months. Livers were processed for histological analyses, studies of fibrosis-related gene expression, cell fractionation and triglyceride measurement. Serum alanine aminotransferase (ALT) levels were assessed. DNA binding activities of p53 and the sterol regulatory element-binding protein-1c (SREBP1c) were analysed. The abundance of EGR1 and enzymes involved in fatty acid synthesis were determined. Chromatin immunoprecipitation was employed to study EGR1 binding to the SREBP1c promoter region.

**Results:** Ethanol feeding generated steatosis, chicken wire fibrosis and ALT elevations in the LE rats. Fibrosis was associated with the upregulation of EGR1 and its downstream target genes. EGR1 upregulation was associated with enhanced p53 activity and an increase in the cellular $p66^{shc}$ abundance. Steatosis was linked to the activation of SREBP1c. Importantly, EGR1 upregulation paralleled the expression and transcriptional activity of SREBP1c. Finally, EGR1 was shown to bind to the SREBP1c promoter region.

**Conclusions:** Long-term ethanol feeding promoted steatosis and fibrosis in LE rats via EGR1 activation. The highly abundant EGR1 bound to the SREBP1c promoter and contributed to the steatosis observed in the LE rat model.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Keywords**

alcoholic liver disease; Lieber; DeCarli diet; p53; SREBP1c

Alcoholic liver disease (ALD) is an alcohol-associated lifestyle disorder that is governed by gene–environment interactions. The complex interplay between the genetic and environmental factors is thought to be responsible for the fact that only 20–30% of unselected heavy drinkers develop steatohepatitis and less than 10% progress into cirrhosis (1). The molecular mechanisms behind the observed inter-individual differences in alcohol-related diseases and the pathophysiological details of ethanol-induced liver damage have not been fully elucidated.

Various animal models have been extensively employed to facilitate the understanding of the pathogenesis of ALD. Although these models have provided useful information about the various aspects of the disease, they all have certain limitations underlining the fact that a perfect animal model for human disease does not exist. Detailed comparison of the different animal models employed in ethanol-related hepatic research has been recently published (2). Lieber *et al*. provided convincing evidence that ethanol alone – without concomitant malnutrition – can induce hepatic steatosis in rats, similar to humans (3). The feeding regimen that became known as the Lieber–DeCarli diet (~36% ethanol-derived calories) has been extensively used to induce steatosis in various rat strains, although inflammation, fibrosis or cirrhosis have not been demonstrated. More recently, Tsukamoto *et al*. established a new rat model of ALD that exhibited steatosis, apoptosis, central necrosis, inflammation, portal and bridging fibrosis after feeding the animals with an ethanol-containing diet through an intragastrically placed catheter(4). Despite the more advanced pathological changes similar to the human form of ALD, the non-physiological nature of continuous ethanol infusion represents a substantial difference from oral consumption.

We previously assessed the susceptibility of three frequently used laboratory rat strains, Fischer, Sprague-Dawley and Long-Evans (LE) to ethanol-induced liver injury by feeding the animals with the Lieber–DeCarli diet for 8 weeks (5). Hepatic p53 activation correlated with the level of susceptibility towards ALD, as it was associated with apoptosis, oxidative stress and insulin resistance. In an attempt to produce more advanced ALD that exhibits fibrosis, we assessed the hepatic changes in the highly susceptible LE model after 18 months of chronic ethanol consumption using the Lieber–DeCarli diet.

In this study, we demonstrate that the LE rats exhibit fibrosis in addition to substantial steatosis after 18 months of *ad libitum* ethanol feeding. More importantly, two transcription factors – early growth response-1 (EGR1) and sterol regulatory element-binding protein-1c (SREBP1c) – have been identified, which orchestrate these pathological changes in the liver. Furthermore, EGR1 not only becomes a biologically relevant regulator of fibrogenesis in the livers of ethanol-fed LE rats but also contributes to the *de novo* fatty acid synthesis by binding to the promoter of SREBP1c and upregulating the expression of this transcription factor. These investigations establish that high-level expression of EGR1 is an important mechanism that promotes steatosis and fibrosis in the LE rat model of ALD.

## Materials and methods

### Animals and treatment

Male, 150–200 g outbred LE rats (Harlan Laboratories, Indianapolis, IN, USA) were fed *ad libitum* with ethanol-containing (35.5% ethanol-derived calories) or iso-caloric control liquid diet (Bioserv, Frenchtown, NJ, USA) for 18 months ($n$ = 11 and 10 respectively). In certain assays, we included samples from animals that were placed on these diets for only 8 weeks (5). Blood was collected by cardiac puncture. Livers were excised and snap-frozen in liquid nitrogen or processed for histological analysis and cell fractionation. Nuclear and cytosolic proteins were separated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Inc./Pierce, Waltham, MA, USA) from fresh liver tissue. The Lifespan Animal Welfare Committee of Rhode Island Hospital, Providence, RI approved all animal experiments.

### Histological studies

Routine haematoxylin-eosin, Oil Red O, Masson's tri-chrome and Sirius red collagen staining were carried out to assess hepatic steatosis and fibrosis.

### Biochemical assays

Serum alanine aminotransferase (ALT) levels were measured using a commercially available kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Serum adiponectin levels were assessed using an ELISA Kit (Invitrogen, Grand Island, NY, USA). Liver lysates were used to measure triglyceride and hydroxyproline with corresponding assay kits following the manufacturer's instructions (both kits from BioVision, Inc., Mountain View, CA, USA). Triglyceride levels were normalized to the protein content of the liver lysates. Protein concentration was determined using the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc./Pierce).

### p53 and sterol regulatory element-binding protein 1c electrophoretic mobility shift assays

The LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific, Inc./Pierce) was used to assess the *in vitro* binding activity of p53 and sterol regulatory element-binding protein 1c (SREBP1c), as previously described (5).

### Western-blot analysis

Tissue lysates and subcellular fractions were prepared using the PARIS™ Kit (Invitrogen), as previously described (5, 6). Primary antibodies against the following targets were employed: Shc, SREBP1c, SIRT1, stearoyl-CoA desaturase-1 (SCD1) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) fatty acid synthase (FAS) (BD Biosciences, San Jose, CA, USA), acetyl-CoA carboxylase (ACC), fibroblast growth factor (FGF)-basic (both from Abcam, Inc., Cambridge, MA, USA), EGR1, phospho-AMPKα (Thr 172) and total AMPKα (Cell Signalling Technology, Inc., Danvers, MA, USA). Loading controls included RNA polymerase II (Pol II) for nuclear extracts (Santa Cruz Biotechnology, Inc.) and p85 subunit of PI3K (Millipore, Billerica, MA, USA) for whole cell lysates.

### Real-time quantitative PCR and fibrosis gene profiling

The PARIS™ Kit (Invitrogen) was used to extract total RNA from the liver samples. Quantitative real-time PCR analysis was carried out, as previously described (6). The primers to assess the expression of SREBP1c and rat housekeeping genes (β-actin or TATA-box binding protein) were readily ordered (Realtimeprimers.com, Elkins Park, PA, USA), and the PCR programme was set up following the manufacturer's instructions. The rat adiponectin receptor-2 primers were previously reported (7). In the fibrosis gene profiling assay, 1 μg of RNA was reverse transcribed into cDNA using the $RT^2$ First Strand Kit, which then was used in the Rat Fibrosis $RT^2$ Profiler™ PCR Array (both from SABiosciences/Qiagen, Frederick, MD, USA) to assess the expression of 84 genes involved in tissue remodelling, following the manufacturer's instructions.

### Chromatin immunoprecipitation assay

A commercially available chromatin immunoprecipitation (ChIP) kit was used, following the manufacturer's instructions (Abcam, Inc.). The positive control antibody, anti-histone H3, was included in the kit. ChIP-grade antibodies against EGR1, p53 and uncoupling protein-2 (UCP2) were obtained from Santa Cruz Bio-technology, Inc.

### Transcription binding site analysis and polymerase chain reaction to amplify chromatin immunoprecipitation products

Online tools were employed to predict transcription factor binding sites in the promoter regions of rat SREBP1c (see Figure S3 for further details and PCR primers). The sequence of rat SREBP1c promoter has been previously published, and was used as a reference (8). The PCR reaction was set up using the QuantiTect SYBR Green PCR Kit (Qiagen, Inc, Valencia, CA, USA) following the manufacturer's instructions. The PCR reaction consisted of a denaturation step at 94°C for 15 s, annealing at 57°C for 30 s and extension at 72°C for 30 s. Melting curve analysis was included as assurance for quality control. The PCR products were separated on a 1% agarose gel. The fold enrichment compared with no antibody control was calculated.

### Statistical analysis

Data are presented as mean ± SEM and analysed using ® GRAPHPAD INSTAT software (GraphPad Software, Inc., La Jolla, CA, USA). Unpaired Student's *t* or one-way ANOVA with Tukey–Kramer post-test were employed. Significance was defined by $P < 0.05$.

## Results

### Lieber–DeCarli ethanol diet induces steatosis and fibrosis in the livers of Long-Evans rats

After 18 months on the ethanol-containing or corresponding control liquid diet, the rats were euthanized, and pathological changes in the liver were analysed. H&E staining revealed that the ethanol-fed LE rats developed severe micro-and macrovesicular steatosis in the central, periportal and random areas of the liver lobule. Steatosis was linked to disorganized liver architecture (Fig. 1A, panel b). In the control-fed animals (Fig. 1A, panel a), there were signs of minimal steatosis as well. In these rats, the fat distribution was mostly

random, and in a few animals, periportal steatosis was observed. There was a statistically significant difference in the percentage of hepatocytes exhibiting steatosis between control and ethanol-fed animals (10 ± 3.45% vs 33.182 ± 7.78%, respectively, $P = 0.0165$) with more macrovesicular steatosis in the ethanol-fed group. The presence of steatosis was further examined by Oil Red O staining that revealed more intense staining in the ethanol-fed rats (Fig. 1A, panel d) than in the controls (Fig. 1A, panel c). These findings were also confirmed by determining hepatic triglyceride content (Fig. 1B). The presence of steatosis in the control-fed animals was probably because of the fact that the liquid diet contained 35% kcal from fat as opposed to a typical rodent chow diet (10–17% kcal). The higher fat content of the liquid diet over the course of 18 months probably contributed to the steatosis observed in the control animals (personal communication with Dr. Jaime Lecker of Bioserv). As Figure 1A reveals, chronic ethanol feeding promoted abundant collagen accumulation and 'chicken wire' fibrosis as visualized by Sirius Red staining (Fig. 1A, panel f, arrowheads) and Masson's trichrome staining (Fig. 1A, panel h). In the control livers, no signs of collagen accumulation or fibrosis were visible (Fig. 1A, panel e&g). Collagen accumulation in the livers of ethanol-fed LE rats was also supported by the increased abundance of hydroxyproline (Fig. 1C), which is an important component of collagen. Ethanol-induced steatosis and fibrosis were also coupled with significant ALT elevations in the ethanol-fed LE rats (Fig. 1D).

## Chronic ethanol feeding promotes the expression of fibrosis genes

To further characterize the hepatic fibrosis seen in the ethanol-fed LE rats, we assessed the mRNA expression of 84 fibrosis-related genes. As shown in Figure 2A and Table S1, most of the studied genes were upregulated in the livers of ethanol-fed LE rats. Interestingly, the majority of the genes that were significantly upregulated by the chronic ethanol feeding are linked to the EGR1 transcription factor, which is a Smad-independent mediator of TGF-β signalling (9). EGR1 has been shown to increase the transcription of the profibrogenic cytokine TGF-β (10), connective tissue growth factor (CTGF) and FGF-basic among many others (9). Figure 2A and Table S1 demonstrate that the mRNA expression of CTGF and TGF-β2 were significantly increased (TGF-β1 was also upregulated, whereasTGF-β3 was downregulated, N.S.) in the livers of ethanol-fed LE rats. In addition, Western-blot analysis of the expression of FGF-basic protein revealed that it was up-regulated by ethanol feeding (Fig. 2B, densitometry data). EGR1 has also been linked to the upregulation of bcl-2 (10), an anti-apoptotic protein that was shown to promote hepatic stellate cell (HSC) proliferation and liver fibrosis (11). Importantly, the expression of bcl-2 was significantly increased in the livers of ethanol-fed LE rats, and it was coupled with increased expression of collagen type III, alpha 1 (Fig. 2A, Table S1), another indicator of increased HSC activation. Finally, chronic ethanol feeding markedly increased the expression of integrin beta 3. This molecular change may facilitate the effect of extracellular matrix on HSC prosurvival signalling and proliferation (12). Taken together, these data indicate that chronic ethanol feeding promoted the expression of genes that are involved in HSC proliferation, activation and fibrogenesis. In addition, the most pronounced upregulation was seen in the downstream targets of EGR1. Importantly, EGR1 is not only linked to fibrosis but also induces the transcription of p53 and may directly interact with this protein to increase its transcriptional activity (10). p53 was previously linked to liver injury in the ethanol-fed LE rats by promoting hepatocellular

apoptosis, oxidative stress and insulin resistance (5, 13). Thus, to further characterize the hepatocellular injury in the ethanol-fed LE rats, we assessed the expression and function of p53.

### p53 is transcriptionally active and promotes p66$^{shc}$ upregulation in the liver

p53 has been identified as a major determinant of ethanol-induced liver damage (5). It is also known that the lifespan determinant p66$^{shc}$ protein is indispensable for p53-mediated apoptosis and increases the generation of mitochondrial reactive oxygen species (ROS) (14). In addition, p66$^{shc}$ can also induce the phosphorylation and inactivation of the FoxO class transcription factors to subsequently diminish the antioxidant defence mechanisms in the liver (15). Abrogation of p66$^{shc}$ function significantly reduces ethanol-induced ROS production in hepatocytes and attenuates ethanol-induced liver damage in mice (16). In view of these recent findings, we assessed the abundance and *in vitro* DNA-binding activity of p53 in the livers of ethanol-fed rats. As demonstrated in Figure 3A,C, 18 months of ethanol feeding did not significantly alter the nuclear abundance of p53 compared with control, but it markedly increased the DNA-binding ability of p53 (Fig. 3A,B). This phenomenon translated into augmented transcriptional activity of p53 as revealed by the increased cellular abundance of p66$^{shc}$ (Fig. 3A,D).

### Hepatic fibrosis and steatosis are linked to the upregulation of early growth response-1 and sterol regulatory element-binding protein-1c

The two most prominent pathological changes that occurred in the livers of ethanol-fed LE rats were hepatic fibrosis and steatosis. Given the increased expression of EGR1-related genes, we were prompted to assess the nuclear abundance of EGR1. As shown in Figure 4A,B, chronic ethanol feeding markedly increased the nuclear expression of this transcription factor, providing an explanation for the upregulation of fibrosis-related genes (e.g., FGF-basic or CTGF) and the augmented p53 transcriptional activity in these rats. Importantly, fibrosis was also accompanied by substantial hepatic steatosis in the ethanol-fed LE rats. On the basis of previous observations, we suspected that the SREBP1c-mediated *de novo* fatty acid synthesis might have played a role in the generation of steatosis (5). SREBP1c activation is a critical step in the pathogenesis of ethanol-induced fatty liver disease and may occur a few weeks after the initiation of an ethanol-containing diet in mice (17). As SREBP1c activates a series of genes involved in fatty acid synthesis such as fatty acid synthase (FAS), acetyl-CoA-carboxylase (ACC), stearoyl-CoA desaturase-1 (SCD1), ATP citrate lyase and malic enzyme (18), we assessed the expression of SREBP1c messenger RNA, nuclear precursor (Figure S1A, B and C) and mature protein (Fig. 4A,C) as well as the cellular abundance of the downstream targets of the mature SREBP1c (Fig. 4A,D) in the ethanol-fed LE rats. Similar to the shorter ethanol-feeding regimen in murine models (19), the SREBP1c pathway was substantially upregulated, supporting the concept that *de novo* fatty acid synthesis contributed to the hepatic steatosis of this rat model.

Previous reports suggest that shorter (4 weeks) ethanol consumption suppresses the adiponectin/AMPK/SIRT1 signalling cascade at various levels: it decreases adiponectin secretion from the adipose tissue by promoting oxidative stress (20), curbs AMPK (19) and SIRT1 (21, 22) activation, which ultimately promote the activation of SREBP1c (17–19, 21).

Supporting these previous findings, in the chronically ethanol-fed LE rats, the circulating adiponectin levels were diminished, followed by a compensatory, non-significant increase in the hepatic adiponectin receptor-2 expression (Figure S2A and B). Subsequently, there was a downward trend in the expression of hepatic phospho-AMPK (Thr 172) and SIRT1 in the ethanol-fed LE rats; however, these changes were not statistically significant (Figure S1B,D and E). Therefore, we shifted the focus of our investigation to other mechanisms that may have contributed to the enhanced SREBP1c function in the chronic ethanol-fed LE rats.

Importantly, it has been recently reported that the genetic ablation of EGR1 in mice attenuated steatosis after ethanol feeding (23). In view of this finding, it was interesting to see that the nuclear expression of EGR1 paralleled the SREBP1c precursor expression (Fig. 4A), which led us to analyse the potential interaction between these two transcription factors. We hypothesized that EGR1 can bind to the promoter region of SREBP1c, and thus facilitates *de novo* fatty acid synthesis and steatosis.

## Early growth response-1 promotes sterol regulatory element-binding protein-1c expression and activation during chronic ethanol feeding

These experiments provide the initial description of fibrosis produced after 18 months of ethanol feeding in the LE rats that was not observed after only 8 weeks of ethanol consumption (5). It is also of interest in short-term ethanol-feeding experiments that the Lieber–DeC-arli ethanol diet failed to induce EGR1 expression in LE rats after 8 weeks (Fig. 5A,B), whereas EGR1 was markedly upregulated after 18 months on the same diet. In addition, EGR1 was also significantly higher in the 18 months isocaloric control-fed animals compared with the younger 8-week-fed controls suggesting that EGR1 may have other functions not directly linked to fibrogenesis. This hypothesis was further supported by the observation that the EGR1 expression correlated with the abundance of transcriptionally active SREBP1c, as assessed using EMSA (Fig. 5C,D). The hypothesis that EGR1 can bind to the rat SREBP1c promoter was tested using the ChIP assay (Fig. 5E,F) after identification of four potential EGR1 transcription factor binding sites in the SREBP1c promoter (Figure S3). The ChIP assay demonstrated that EGR1 could indeed bind to the SREBP1c promoter, whereas the other suspected and ethanol-induced transcription factor, p53 failed to do so (Fig. 5E). The employment of anti-EGR1 antibody resulted in a 35.7 ± 3.1 fold enrichment of the signal over no antibody (background) control (Fig. 5F). Importantly, we did not observe signal enrichment when anti-p53 or anti-UCP2 (mitochondrial protein) antibodies were used as controls. This finding links the enhanced EGR1 transcription factor expression to sustained SREBP1c activation. Thus, EGR1 may contribute to hepatic steatosis as well as fibrosis in ALD, produced by long-term ethanol consumption in the LE rat model. Our working model based on these findings is depicted in Figure 6, and it suggests that the cumulative effects of ageing-associated changes and chronic ethanol consumption may be responsible for the biologically relevant upregulation of EGR1, which then orchestrates many important aspects of ALD, including hepatocellular apoptosis, HSC activation and proliferation, fibrogenesis as well as steatosis.

## Discussion

In previous studies, 8 weeks of chronic ethanol feeding induced substantial steatosis, apoptosis, oxidative stress, hepatic and systemic insulin resistance in the LE rats (5, 13). In the present investigation, we tested if more advanced pathological changes would occur in this highly susceptible model after 18 months of chronic ethanol feeding using the same ethanol-containing Lieber–DeCarli diet. There is a need to develop a small animal model of liver fibrosis that involves *ad libitum* ethanol feeding conditions. Lieber and DeCarli were successful to induce perivenular fibrosis and cirrhosis in a baboon model using an adapted version of the their diet. However, only 30% of baboons exhibited cirrhosis after a time period of 5 years, and this finding was not reproducible by others (24). In our study, we were able to demonstrate that the LE model exhibited evidence of fibrosis after 18 months on the ethanol-containing Lieber–DeCarli diet in addition to substantial steatosis. Fibrosis was not present in the isocaloric control-fed animals; the steatosis that was seen in the controls is probably because of' the long-term consumption of the control liquid diet. Furthermore, 18 months of ethanol feeding induced the expression of EGR1, which subsequently increased the expression of profibrogenic genes (e.g., FGF-basic, CTGF) as well as enhanced the abundance of the transcriptionally active SREBP1c protein in the livers of LE rats. The ChIP assay confirmed that EGR1 bound to the promoter region of the rat SREBP1c in the 18 months ethanol-fed LE rats. In these animals, marked activation of EGR1 may represent a major mechanism of SREBP1c activation. Nonetheless, we considered the possibility that the ethanol-induced suppression of AMPK and SIRT1 would also contribute to the activation of SREBP1c in this long-term model of ALD, as both AMPK and SIRT1 have been reported to regulate SREBP1c in shorter *in vivo* models (18, 19, 21, 25). However, in the 18-month ethanol-fed LE rats, inhibition of the adiponectin/AMPK/SIRT1 signalling cascade was modest and concluded to be an unlikely source of the robust SREBP1c activation. Therefore, we hypothesize that ethanol may promote the abundance and function of SREBP1c through EGR1 activation in a context-dependent manner. During 8 weeks of ethanol feeding, SREBP1c was activated (Fig. 5C,D and (5) without a significant increase in the abundance of EGR1, indicating that previously described AMPK-dependent (19) and -independent (17) mechanisms may be the primary mediators of the SREBP1c regulation in the early phases of ALD. The increased expression of EGR1 may become relevant in the mechanisms of hepatic steatosis only after long-term ethanol feeding. Similar to the SREBP1c regulation, EGR1 may contribute to the increased transcriptional activity of p53 in a context-dependent manner.

One of the limitations of this study is that it is hard, if not impossible, to *in vitro* mimic the exact *in vivo* conditions that lead to sustained EGR1 upregulation followed by the activation of SREBP1c. To experimentally demonstrate the role of EGR1 in the regulation of SREBP1c, we plan to perform late-stage temporal *in vivo* EGR1 inhibition with anti-sense oligonucleotides (26) in the livers of old, ethanol-fed LE rats, followed by assessment of the SREBP1c pathway. The short half-life of SREBP1c suggests (25) that efficient inhibition of EGR1 should be rapidly followed by blunted SREBP1c signalling. A conditional EGR1 knock-out system established in LE rats would also be a possible, but technically challenging option. In addition, there are reports suggesting that peroxisome

proliferator-activated receptor-γ (PPAR-γ) agonists may inhibit the expression of EGR1 (27), and therefore, late-stage PPAR-γ agonist treatment may also be a feasible alternative to study the role of EGR1 upregulation in SREBP1c activation, although this approach is less specific, as it is also known to promote the adiponectin/AMPK/SIRT1 signalling cascade in ethanol-fed rodents (28). Finally, *ex vivo* investigations on primary hepatocytes isolated from long-term ethanol-fed LE rats could be used as experimental tools to assess the contribution of EGR1 to sustained SREBP1c activation by employing small-interfering RNA against EGR1.

The upstream mechanisms that lead to EGR1 activation may be linked to changes that occur during ageing and further facilitated by chronic ethanol consumption. EGR1 expression is induced by environmental stimuli that are associated with injury and stress (9). The long list of these stimuli includes oxidative stress (9) that may be a prime candidate for ageing-associated EGR1 upregulation. In addition, ethanol consumption can further exacerbate ROS generation that is linked to normal ageing.

The fact that EGR1 expression is enhanced in the LE rats fed an isocaloric control-diet for 18 months without obvious signs of fibrosis raises the question if the profibrogenic function of this transcription factor is secondary to other hepatic functions. Based on this investigation, the primary functions may include activation of *de novo* fatty acid synthesis by promoting SREBP1c expression in the liver.

In summary, we present the initial information that the LE rats develop fibrosis in addition to substantial micro-and macrovesicular steatosis after 18 months on the Lieber–DeCarli, ethanol-containing liquid diet. Although it takes almost 2 years to observe these changes, the strength of this model is that it involves physiological feeding conditions and would be available to many laboratories as a realistic animal model of ALD with many histological features similar to that found in the human disease. In this model, we observe that EGR1 contributes to *de novo* fatty acid synthesis by activating SREBP1c. The mechanisms of EGR1 upregulation remain unknown; nevertheless, this investigation provides evidence regarding the crosstalk between mediators of fibrosis and steatosis in a small animal model that exhibits both these pathological changes in the liver following long-term ethanol exposure.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **ACC** | acetyl-CoA carboxylase |
| **ALD** | alcoholic liver disease |
| **ALT** | alanine aminotransferase |
| **ChIP** | chromatin immunoprecipitation |
| **CTGF** | connective tissue growth factor |
| **EGR1** | early growth response-1 |
| **EMSA** | electrophoretic mobility shift assay |
| **FAS** | fatty acid synthase |
| **FGF** | fibroblast growth factor |
| **HSC** | hepatic stellate cell |
| **LE** | Long-Evans |
| **Pol ll** | RNA polymerase II |
| **ROS** | reactive oxygen species |
| **SCD1** | stearoyl-CoA desaturase-1 |
| **SREBP1c** | sterol regulatory element-binding protein-1c |
| **TGF-β** | transforming growth factor-β |
| **UCP2** | uncoupling protein-2 |

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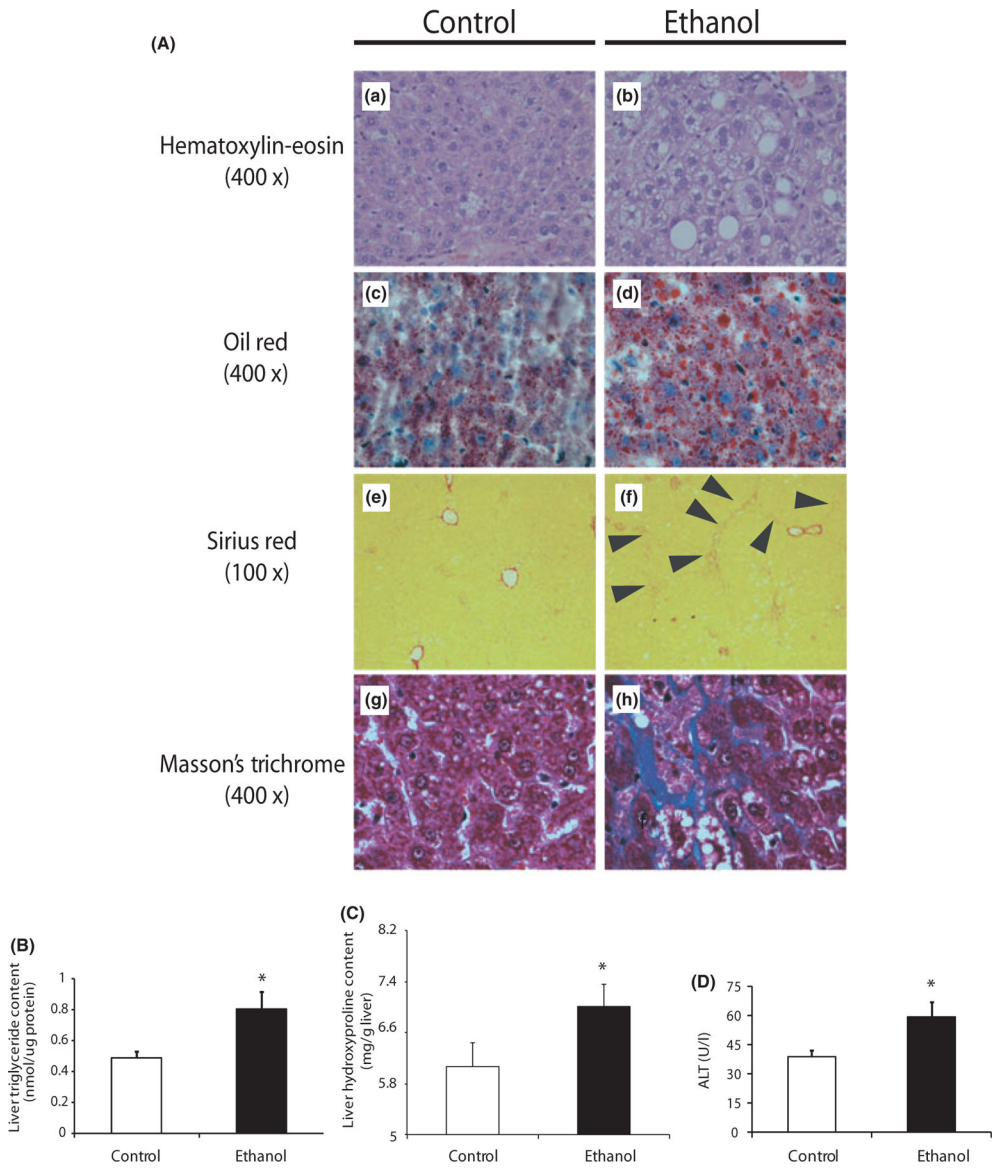


**Fig. 1.**

Long-term ethanol feeding promotes steatosis, fibrosis and ALT elevation in the LE model of ALD. H & E, Oil Red O, Sirius Red and Masson's trichrome staining (Fig. 1A) were performed on formalin-fixed or flash frozen liver sections. As Fig. 1A shows, ethanol-fed LE rats developed severe micro-and macrovesicular steatosis, disorganized liver architecture (panel b), whereas these changes were largely absent in the control-fed animals (panel a). The difference in steatosis was further confirmed using Oil Red O staining (panel d vs c). As Fig. 1A shows chronic ethanol feeding promoted abundant collagen fibre accumulation, 'chicken wire' fibrosis as visualized by Sirius Red staining (panel f, arrowheads) and Masson's trichrome staining (panel h), all these changes were absent in the control animals (panel e & g). Augmented collagen accumulation in the livers of ethanol-fed LE rats was also evidenced by the increased hepatic hydroxyproline content (Fig. 1C). Long-term

ethanol feeding significantly increased hepatic triglyceride content (Fig. 1B) and serum ALT levels (Fig. 1D). (*): control vs ethanol, $P < 0.05$.

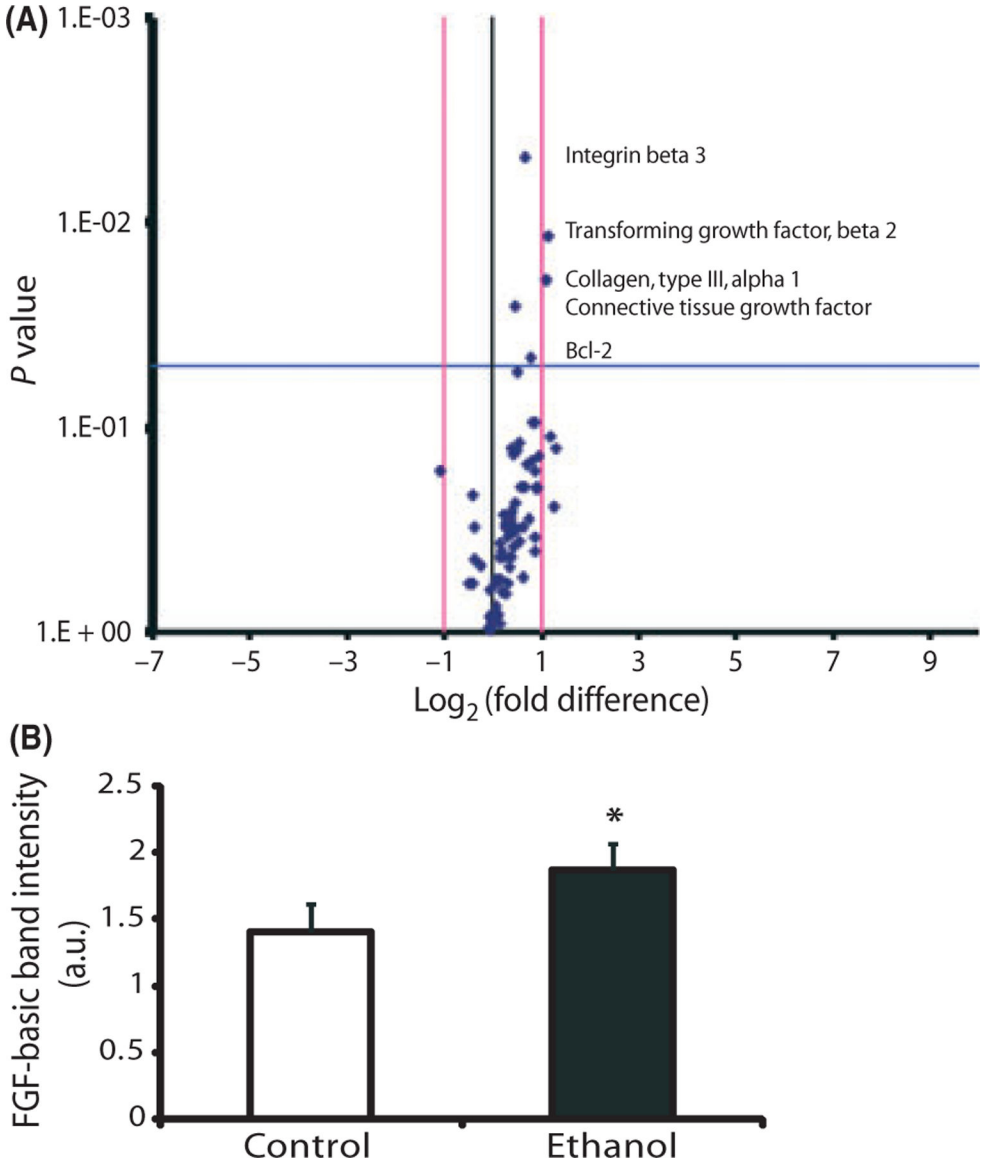


**Fig. 2.**

Chronic ethanol feeding promotes the expression of fibrosis-related genes. The RT$^2$ Profiler™ PCR Array Kit was employed to determine the expression of 84 fibrosis-related genes. (A) a volcano plot is shown. The black line indicates the gene expression level in the control-fed rats. The pink lines indicate the 'two fold-change in gene expression' threshold. The blue line indicates the desired threshold for the *P* value of the *t*-test, which is defined as 0.05. The names of the significantly upregulated genes are indicated. (B) it shows the result of a densitometry analysis, which was performed on the FGF-basic Western-blot ($n$ = 10/10). (*): control vs ethanol, $P < 0.05$.

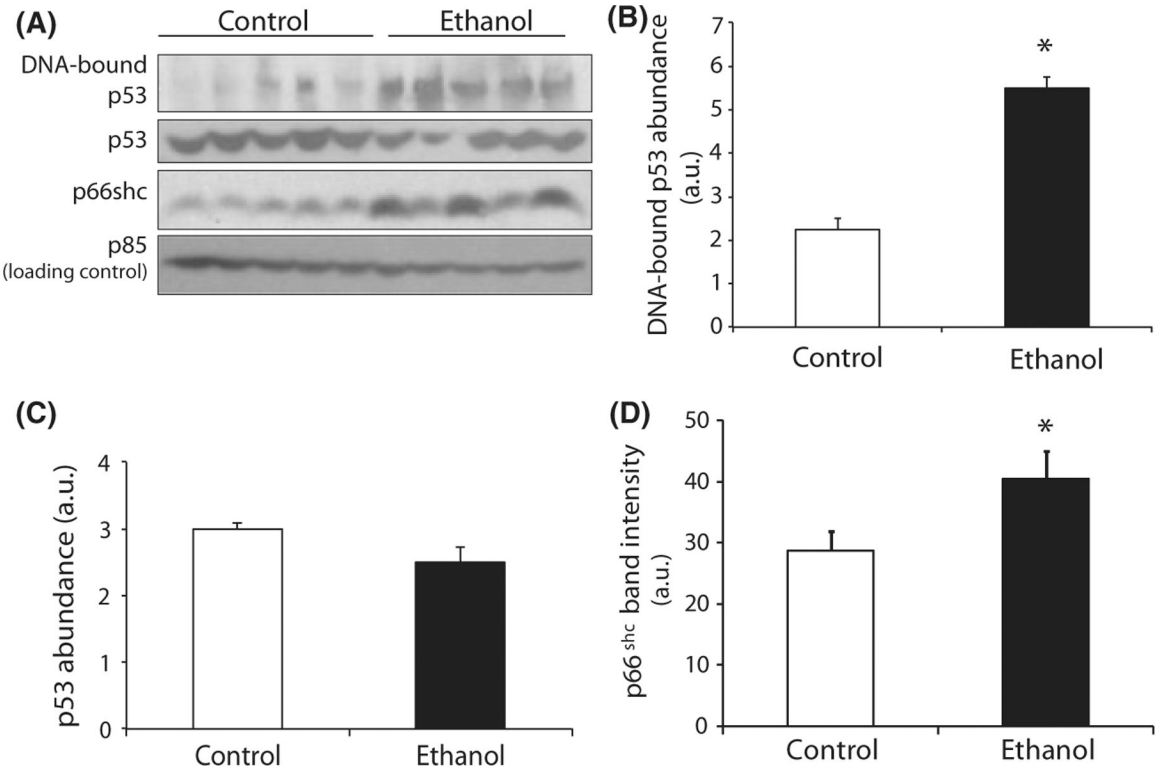


**Fig. 3.**

Chronic ethanol feeding enhances the transcriptional activity of p53 and promotes $p66^{shc}$ expression. Although 18 months of ethanol feeding did not significantly increase the nuclear abundance of p53 (Fig. 3A and C), it promoted the transcriptional activity of p53 as evidenced using the EMSA assay (Fig. 3A and B). Representative images are shown with corresponding densitometry analyses. Subsequently, chronic ethanol feeding increased the cellular abundance of $p66^{shc}$, a critical mediator of p53-induced apoptosis and oxidative stress in the context of ALD. The $p66^{shc}$ expression was assessed using Western blotting (Fig. 3A) and quantified using densitometry (Fig. 3D). p85 subunit of PI3K served as loading control (Fig. 3B). (*): control vs ethanol, $P < 0.05$.

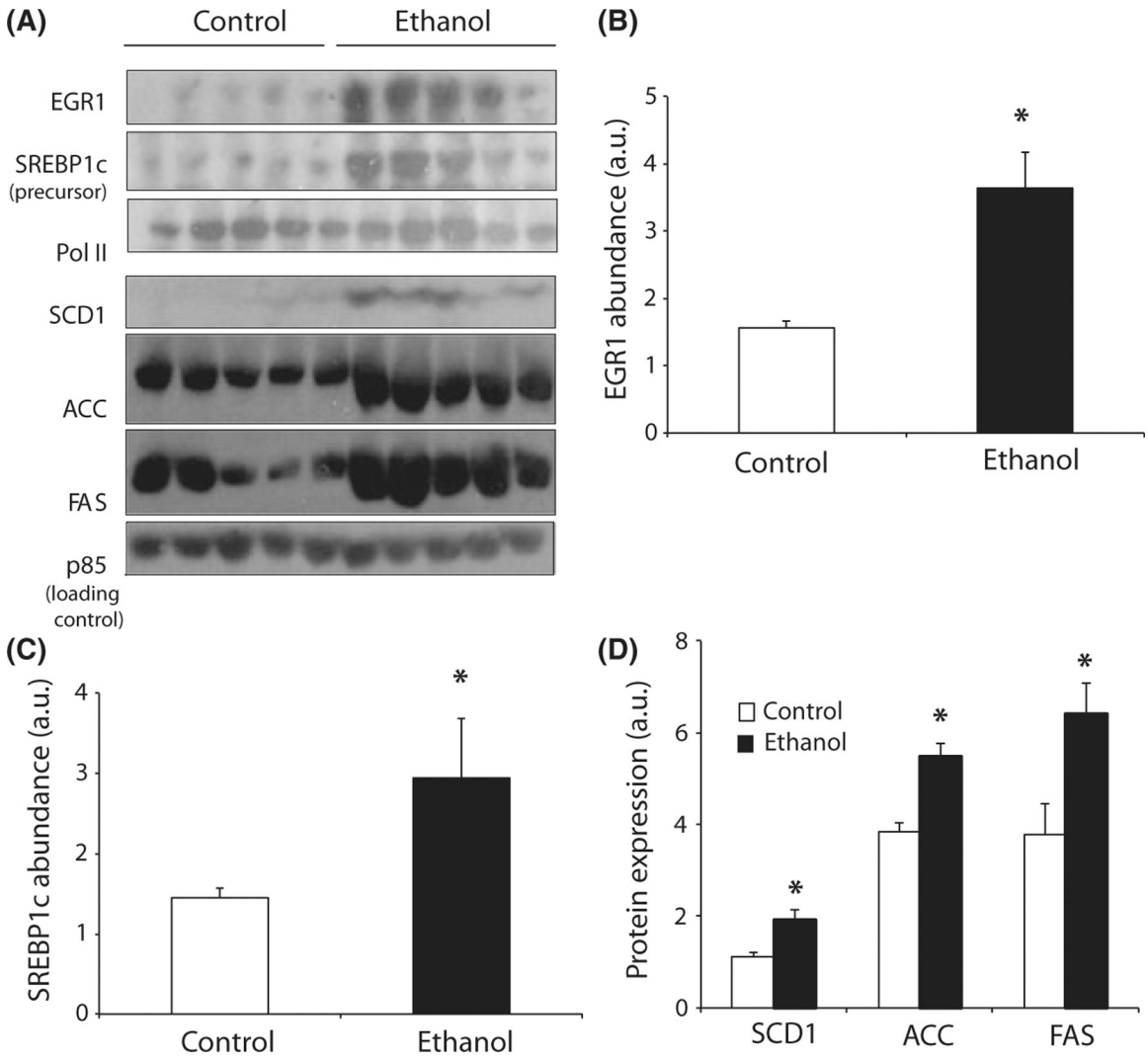


**Fig. 4.**

Chronic ethanol feeding increases the hepatic abundance of EGR1, SREBP1c and critical enzymes involved in *de novo* fatty acid synthesis. Nuclear abundance of EGR1 and SREBP1c precursor were assessed by Western blotting. Pol II served as a loading control (Fig. 4A, upper three panels). The results of densitometry are shown in Fig 4B. and Fig 4C. respectively. Ethanol-mediated induction of the SREBP1c pathway was evidenced by the upregulation of downstream targets, including SCD1, ACC and FAS that regulate *de novo* fatty acid synthesis. p85 subunit of PI3K served as a loading control (Fig. 4A, lower four panels). The results of densitometry for these proteins are shown in Fig. 4D. (*): control vs ethanol, $P < 0.05$.

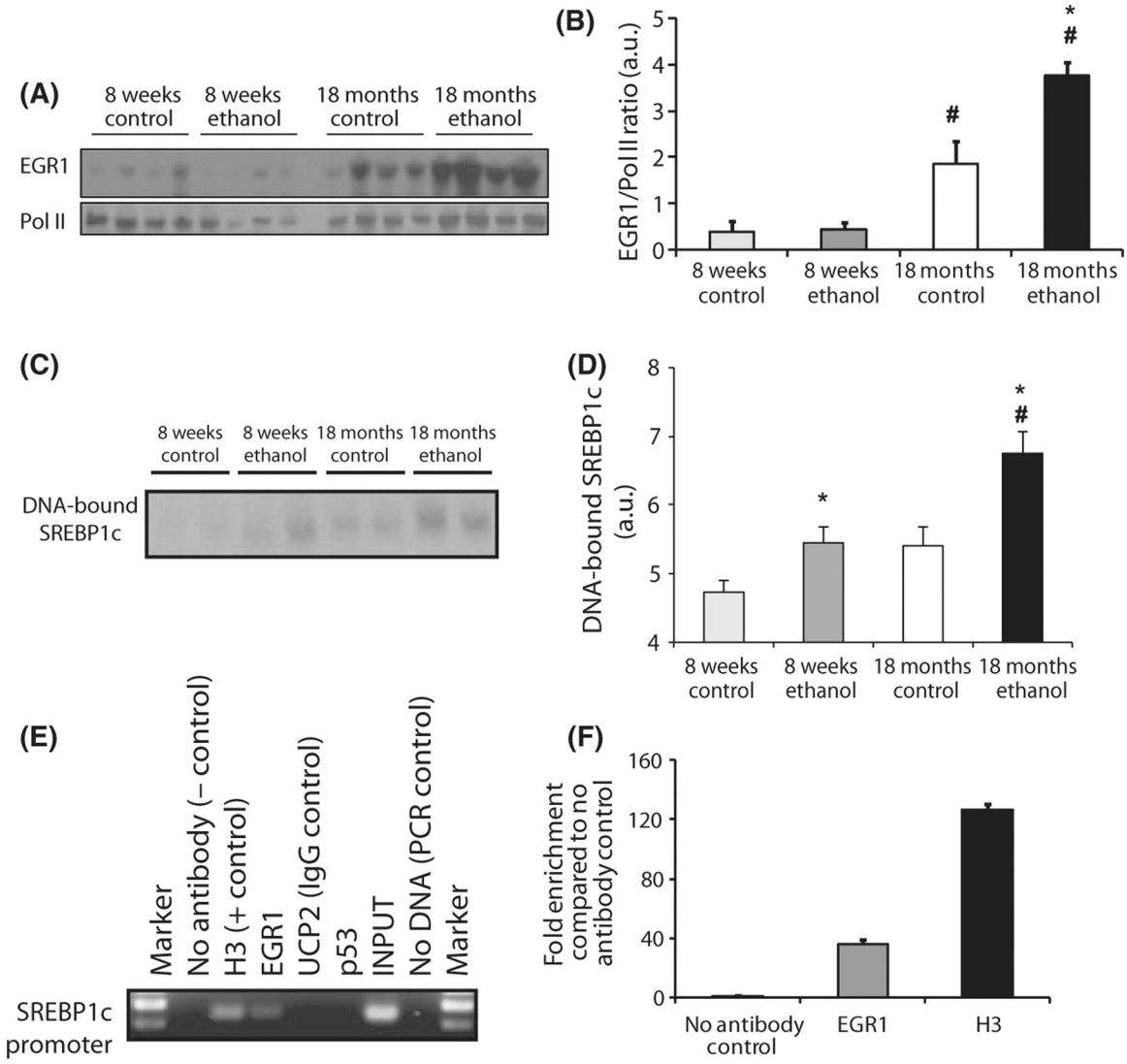


**Fig. 5.**

EGR1 becomes abundant in the livers of chronically ethanol-fed LE rats and promotes SREBP1c by binding to its promoter. The nuclear abundance of EGR1 was assessed by Western blotting in rats that were placed on ethanol-containing or isocaloric control liquid diets for 8 weeks or 18 months. (Fig. 5A, upper panel). Densitometry was performed (Fig. 5B). The EGR1 expression paralleled the *in vitro* DNA binding activity of SREBP1c, as shown in EMSA (Fig. 5C) quantified using densitometry (Fig. 5D). ChIP assay was carried out to assess EGR1 and p53 binding to SREBP1c promoter. Anti-UCP2 antibody was used as IgG negative control, anti-H3 antibody was used as positive control (Fig. 5E). Fold enrichment over no antibody control was calculated using Ct values (Fig. 5F). (*): control vs ethanol, (#): 18 months vs 8 weeks, $P < 0.05$.

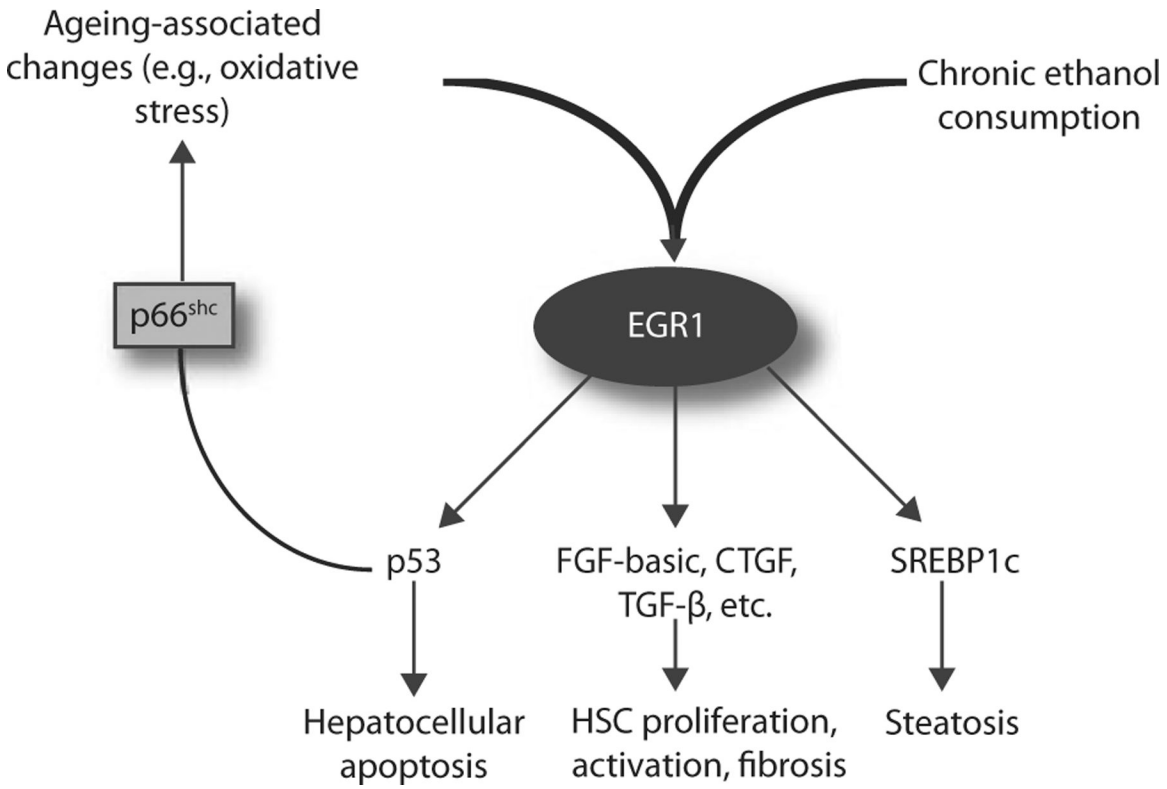


**Fig. 6.**

EGR1 contributes to hepatocellular apoptosis, fibrosis and steatosis in the long-term ethanol-fed LE rats. EGR1 becomes a biologically relevant transcription factor in the later phases of ALD. Ethanol potentiates ageing-associated mechanisms (e.g., oxidative stress) that may lead to the increased expression and function of EGR1. The highly abundant EGR1 increases the transcriptional activity of p53, promoting further oxidative stress and hepatocellular apoptosis. It also increases the transcription of numerous growth factors and profibrogenic proteins, favouring the activation of HSCs as well as the increased production of collagen. Finally, EGR1 enhances the expression and function of SREBP1c contributing to increased *de novo* fatty acid synthesis and steatosis.