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A novel RNA oligonucleotide improves liver function and inhibits liver carcinogenesis in vivo

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Abstract

Hepatocellular carcinoma (HCC) occurs predominantly in patients with liver cirrhosis. Here, we show an innovative RNA-based targeted approach to enhance endogenous albumin production whilst reducing liver tumour burden. We designed short-activating RNAs (saRNA) to enhance expression of C/EBP α (CCAAT/enhancer-binding protein- α), a transcriptional regulator and activator of albumin gene expression. Increased levels of both C/EBP α and albumin mRNA in addition to a 3-fold increase in albumin secretion and 50% decrease in cell proliferation was observed in C/EBP α -saRNA transfected HepG2 cells.

Intravenous injection of C/EBP α -saRNA in a cirrhotic rat model with multifocal liver tumours increased circulating serum albumin by over 30% showing evidence of improved liver function. Tumour burden decreased by 80% ($p = 0.003$) with a 40% reduction in a marker of pre-neoplastic transformation.

Since C/EBP α has known anti-proliferative activities via retinoblastoma, p21 and cyclins; we used mRNA expression liver cancer specific microarray in C/EBP α -saRNA transfected HepG2 cells to confirm down-regulation of genes strongly enriched for negative regulation of apoptosis, angiogenesis and metastasis. Up-regulated genes were enriched for tumour suppressors and positive regulators of cell differentiation. A quantitative PCR and Western-blot analysis of C/EBP α -saRNA transfected cells suggested that in addition to the known anti-proliferative targets of C/EBP α , we also observed suppression of IL6R, c-Myc and reduced STAT3 phosphorylation.

Conclusion—We demonstrate for the first time that a novel injectable saRNA-oligonucleotide that enhances C/EBP α expression successfully reduces tumour burden and simultaneously improves liver function in a clinically relevant liver cirrhosis/HCC model.

Keywords

Liver cirrhosis; Hepatocellular Carcinoma; Short-activating RNA; PAMPAM dendrimer nanoparticles; C/EBP α transcription factor

INTRODUCTION

Human hepatocellular carcinoma (HCC) is currently the third most common cause of cancer related mortality worldwide.¹ The majority of patients with HCC develop malignant tumours from a background of liver cirrhosis. Currently most patients are diagnosed at an advanced disease stage and therefore the 5 year survival for the majority of HCC patients remain dismal.² Surgical resection, loco-regional ablation and liver transplantation are currently the only therapeutic options which have the potential to cure HCC. However, based on the evaluation of individual liver function and tumour burden only about 5–15% of patients are eligible for surgical intervention.³

Most eukaryotic cells use RNA-complementarity as a mechanism for regulating gene expression. One example is the classic RNA interference (RNAi) pathway which uses double stranded short interfering RNAs to knockdown gene expression via the RNA-induced silencing complex (RISC).⁴ It is now established that short duplex RNA oligonucleotides also have the ability to target the promoter regions of genes and mediate

transcriptional activation of these genes and they have been referred to as RNA activation (RNAa), antigene RNAs (agRNAs), short-activating RNA (saRNA).^{5–8} SaRNA induced activation of genes appears to be conserved in other mammalian species including mouse, rat, and non-human primates and is fast becoming a popular method for studying the effects of endogenous up-regulation of genes.⁵ SaRNAs have recently been designed to activate expression of genes such as p21 as potential therapy for the treatment of HCC or prostate cancer thus demonstrating a promising novel approach for adjuvant therapy.^{9,10}

With the same iterative approach that we previously used to design saRNAs specific for Kruppel-like factor 4 (Klf4), c-Myc and MafA^{7,11}, we generated saRNA molecules to up-regulate transcript levels of the CCAAT/enhancer-binding protein alpha (C/EBPα) gene.

C/EBPα is a leucine zipper protein that is conserved across humans and rats. This transcription factor is enriched in hepatocytes, myelomonocytes, adipocytes, as well as mammary epithelial cells including other cell types.¹² In the adult liver, C/EBPα is defined as functioning in terminally differentiated hepatocytes whilst rapidly proliferating hepatoma cells express only a fraction of C/EBPα.¹³ C/EBPα is known to up-regulate p21, a strong inhibitor of cell proliferation through the up-regulation of retinoblastoma and inhibition of Cdk2 and Cdk4^{14,15}. Since the binding sites for the family of C/EBP transcription factors are present in the promoter regions of numerous genes that are involved in the maintenance of normal hepatocyte function and response to injury (including albumin, interleukin 6 response, energy homeostasis, ornithine cycle regulation and serum amyloid A expression)^{16–20}; we determined the therapeutic benefit of up-regulating expression of C/EBPα in cirrhotic rats with compromised liver function and HCC by using saRNA as a safe and potentially clinically translatable method of targeted gene up-regulation.

For targeted *in vivo* delivery, we complexed C/EBPα-saRNA into the structurally flexible triethanolamine (TEA)-core poly (amidoamine) (PAMAM) dendrimer.²¹ The *in vivo* efficacy of these nanoparticles have previously been evaluated where biodistribution studies show that the dendrimers preferentially accumulate in peripheral blood mononuclear cells and liver with no discernible toxicity.²¹ Here we demonstrate the therapeutic effect of intravenously injecting C/EBPα-saRNA-dendrimers in a clinically relevant rat liver tumour model.⁴⁴

After three doses through tail vein injection at 48hour intervals, the treated cirrhotic rats showed significantly increased serum albumin levels within one week. More important was the unexpected observation that liver tumour burden significantly decreased in the C/EBPα-saRNA-dendrimer treated groups. This study demonstrates, for the first time, that gene targeting by small RNA molecules can be used by systemic intravenous administration to simultaneously ameliorate liver function and reduce tumour burden in cirrhotic rats with HCC.

EXPERIMENTAL PROCEDURES

Full methods for designing short-activating RNA, animal experiments, nuclease activity, assessment of tumour burden, immuno-staining, qRT-PCR, gene microarray profiling, ChIP-

seq analysis, gene ontology enrichment analysis and gene methylation analysis are available in the Supporting Information.

Design of short activating RNA oligonucleotides

The gene sequence of albumin and C/EBP α was selected for designing short activating RNA molecules for its specific activation using the parameters previously described.⁷

Transfection of saRNA oligonucleotides into HepG2 and rat-liver epithelial cell lines

HepG2 is a liver cell line derived from a human hepatoblastoma that is free of known hepatotropic viral agents and expresses genes involved in a wide variety of liver-specific metabolic functions.²² HepG2 cells were cultured in Roswell Park Memorial Institute medium (RPMI) supplemented with 100 units/ml penicillin, 0.1mg/ml streptomycin, 2mmol/L glutamine (Sigma) and 10% fetal bovine serum (Labtech International). For C/EBP α -saRNA transfection, cells were grown to 60% confluency in 24 well plates prior to transfection of 5, 10 and 20 nmoles of saRNA using Nanofectamine (PAA, UK) following the manufacturer's protocol. This process was repeated three times at 16 hours intervals before cells were harvested for isolation of total RNA for mRNA analysis.

Albumin ELISA

Rat liver epithelial cells and HepG2 cells were cultured in phenol-red free RPMI media in the presence of charcoal stripped FCS. Following three sets of saRNA transfections at 8 hours, 16 hours and 24 hours, the culture media was collected for total murine albumin ELISA (Assay Max, Albumin ELISA, Assay Pro USA) following the manufacturer's instructions.

WST-1 assay

Cell proliferation was quantified at 16, 24 and 96 hours following C/EBP α -saRNA transfection by mitochondrial dehydrogenase expression analysis, using WST-1 reagent following the manufacturer's guideline (Roche, UK). Briefly, the WST-1 reagent was used at 1:100 dilution to plates and incubated for one hour. The enzymatic reaction was measured at 450 nm using Bio-Tek ELISA reader.

Isolation of total RNA from cell lines

Total RNA extraction from cell lines was performed using the RNAqueous-Micro kit (Ambion, UK) following the manufacturer's instructions. Briefly, the cells were gently centrifuged followed by 3 pulses of sonication at Output 3 in Lysis buffer (Ambion, UK). The cell lysates were then processed through an RNA binding column, followed by multiple washes and elution. The total RNA isolated was quantified by a Nanodrop 2000 spectrophotometer. 500ng of total extracted RNA was processed for elimination of genomic DNA followed by reverse transcription using the QuantiTect[®] Reverse Transcription kit from Qiagen.

Animal experiments

We used a clinically relevant rat liver tumour model previously described.²³ For *in vivo* therapy C/EBP α -saRNA was reconstituted with 100 μ l of RNase/Dnase free H₂O; 50 μ l of 20nM saRNA oligonucleotide and 50 μ l of (TEA) core PAMAM dendrimer, previously described.^{24,25} 10 cirrhotic animals were treated with 3 $\times$ doses via tail vein injections in the 1st week. Control animals (n=10) were injected with equal volume of PBS or scramble-saRNA. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

RESULTS

Expression level of C/EBP α and Albumin in HepG2 Cells transfected with C/EBP α -saRNA

We assessed the effect of transfecting C/EBP α -saRNA on C/EBP α and albumin transcript levels. Both C/EBP α (Fig. 1A) and albumin transcripts (Fig. 1B) increased over two fold. Increasing the amounts of C/EBP α -saRNA (5, 10, and 20 nM (nanomoles) dose dependently enhanced C/EBP α transcript levels (Fig. 1C). The maximum expression of albumin was achieved with 5 nM of C/EBP α -saRNA, with no further dose dependent increase at higher saRNA levels (Fig. 1D). Analysis of the promoter regions of C/EBP α (Fig. 1E), the binding box of albumin (DBP) (Fig. 1F) and albumin (Fig. 1G) showed the presence of the core C/EBP α binding motifs (GCAAT) thus supporting targeting of both transcripts by C/EBP α -saRNA induced up-regulation of C/EBP α . An EpiTect Methyl PCR assay also demonstrated reduced methylation at the CpG-island of both C/EBP α and DBP promoters following transfection of C/EBP α -saRNA (Figs. 2A and 2B).

To determine the biological relevance of increased albumin mRNA transcripts in C/EBP α -saRNA transfected HepG2 cells, a human albumin specific enzyme-linked-immunosorbent assay (ELISA) was performed. Secreted albumin peptide was detected in the culture media of the transfected cells (Fig. 2C).

To establish if enhanced albumin secretion in HepG2 cells by C/EBP α -saRNA also affected other hepatocytes specific functions and maintenance of hepatocyte differentiation, we measured expression levels of the ornithine cycle enzyme ornithine transcarbamylase (OTC) and alpha-fetoprotein (AFP). C/EBP α -saRNA caused an increase in OTC levels (Fig. 2D) suggesting an improved ability of urea production. The expression level of AFP decreased (Fig. 2E) indicative of the negative regulation typically observed with normal hepatocytes.²⁶ In addition to the observed gene changes described, we also observed that C/EBP α -saRNA caused a marked down-regulation of HepG2 cell proliferation (Fig. 2F). This observation confirms the known anti-proliferative effects of C/EBP α .^{14,27}

Intravenous injection of C/EBP α -saRNA in male Wistar rats bearing liver cirrhosis/HCC promoted increased circulating levels of albumin, amelioration of liver function and a reduced tumour burden

The stability of C/EBP α -saRNA was initially tested in circulating serum by performing a nuclease activity assay using blood samples from C/EBP α -saRNA treated rats. We observed

a significant reduction in the stability of C/EBP α -saRNA duplex by 48 hours (Figs. 3A and 3B). We thus injected cirrhotic rats over a period of one week with repeat doses of C/EBP α -saRNA-dendrimer. Measurement of circulating albumin showed a significant increase of over 30% after three doses of C/EBP α -saRNA-dendrimer injection when compared to PBS control or scramble-saRNA-dendrimer control groups (Fig. 3C). Further blood analysis demonstrated that the worsening of bilirubin levels was significantly less in the C/EBP α -saRNA-dendrimer treated group by at least 17% when compared to both control groups (Fig 3D). There was also a significant drop in levels of the liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) by at least 10% and 30% respectively in the C/EBP α -saRNA-dendrimer treated group when compared to both control groups (Fig 3E and 3F). Histological examination of the liver showed a significant reduction in tumour nodules from C/EBP α -saRNA-dendrimer injected rats when compared to both control groups (Fig 4A and 4B). These results were consistent with immunohistology studies of tissue sections from C/EBP α -saRNA treated rat liver stained for placenta-form of glutathione S-transferase (GST-*p*). Independent conclusions by two pathologists suggested that there was evidence of reduced carcinogenesis by treatment of C/EBP α -saRNA-dendrimer when compared to the PBS control or scramble-saRNA-dendrimer control groups. Furthermore there were no differences in liver fibrosis between the PBS control; scramble-saRNA-dendrimer or C/EBP α -saRNA-dendrimer treated groups (Fig. 4C). The average density of positive staining for GST-*p* from control groups was 70 ($\pm$ 5.0 %), and that from C/EBP α -saRNA-dendrimer injected rats was 32 ($\pm$ 6.5%). Since overexpression of GST-*p* is observed during rat liver pre-neoplastic state and neoplastic transformation^{28,29}, this data suggests that C/EBP α -saRNA-dendrimer treatment may reduce this process.

Total RNA extracted from liver biopsies of 7 animals from each group were screened for transcript levels of albumin (Fig. 5A), C/EBP α (Fig. 5B), hepatocyte nuclear factor 4- α (HNF4 α) (Fig. 5C) and hepatocyte nuclear factor 1- α (HNF1 α) (Fig. 5D). A significant increase in mRNA level was observed for all the factors, consistent with the role of HNF4 α in hepatocyte differentiation together with C/EBP α and HNF1 α in promoting expression of albumin. Taken together, lower mRNA levels of hepatocyte growth factor (HGF) (Fig. 5E) and increased levels of 4-hydroxyphenylpyruvic acid dioxygenase (HPD1) (Fig. 5F) and plasminogen (Fig. 5G) are suggestive of improved liver function in these cirrhotic rats treated with C/EBP α -saRNA-dendrimer.³⁰

Pathway gene microarray analysis suggests that C/EBP α -saRNA contributes to up-regulation of tumour suppressor genes and down-regulation of genes involved in liver cancer

To investigate other liver specific factors that might be affected in response to C/EBP α -saRNA; we analysed the gene expression profile of a panel of 84 liver cancer specific genes (Qiagen/SABiosciences Human Liver Cancer RT² profilerTM) in C/EBP α -saRNA transfected HepG2 cells (Fig 6). Of particular interest was the observed up-regulation of 20 genes (Table 1), 18 of which are known tumour suppressor genes in HCC (Table 3) including RB. The most significantly up-regulated (over 3 fold) included the death agonist gene BH3-interacting domain (BID), and tumour protein 53 gene (TP53), encoding p53.

BID interacts with BCL2-associated X protein (BAX) which in turn is up-regulated by wild type p53 to regulate cell cycle arrest and apoptosis in response to DNA damage.^{31,32}

Growth arrest and DNA-damage-inducible, 45 beta (GADD45B), also up-regulated, is a member of the growth arrest DNA damage inducible gene family associated with cell growth control where together with p53 induces hepatoprotection in HepG2 cells.³³ Deleted in Liver Cancer 1 (DLC1) gene is a reported tumour suppressor for human liver cancer inhibiting cell growth and proliferation, as well as inducing apoptosis.³⁴ Our data suggests that DLC1 is up-regulated in C/EBP α -saRNA transfected HepG2 cells (Table 3).

Runt-related transcription factor-3 (RUNX3) is a member of the runt domain family of transcription factor and has been frequently been observed in HCC where its expression is significantly lower than in surrounding normal tissue.³⁵ Since ectopic expression of RUNX3 reverses epithelial-mesenchymal transition (EMT) in HCC cells³⁶, we also observed, in the C/EBP α -saRNA transfected HepG2 cells, an up-regulation of RUNX3 (Table 3) and down-regulation of 4 genes involved in EMT. These included CTNB1 (encoding β -catenin), Hepatocyte growth factor (HGF), Small body size mothers against decapentaplegic homolog 7 (SMAD7), and Transforming factor beta 1 (TGFB1) (Table 4).

Suppression of cytokine signalling 3 (SOCS3) was also detected. SOCS3 is a member of the STAT-induced STAT inhibitor (SSI) which functions as negative regulators of cytokine signalling. Decreased expression of SOCS3 is correlated with increased phosphorylation of STAT3 in HCC.³⁷ SOCS3 furthermore has been implicated in negatively regulating cyclin D1 (CCND1), and anti-apoptotic genes including XIAP, survivin (BIRC5), and myeloid leukaemia cell differentiation protein (MCL1).³⁸ Here, we observed a significant increase in expression of SOCS3 (Table 3) and a significant decrease in STAT3, CCND1, XIAP, BIRC5 and MCL1 expression (Table 4). Similar to the *in vivo* observations of reduction in GST-*p* (Fig. 2D), the array data also confirmed down-regulation in expression of GSTP1 (Table 4).

Overall, the down-regulated genes were strongly enriched for functions related to negative regulation of apoptosis and cell death (gene ontology (GO) terms GO:0043066 and GO:0060548; p-values 2×10^{-9} and 2×10^{-9} , respectively), whereas the up-regulated genes were enriched for functions related to positive regulation of cell differentiation (GO:0045597; p = 5×10^{-3}).

Transfection of C/EBP α -saRNA in HepG2 suppresses STAT3, IL6R and cMyc in HepG2 cells

Previously published reports demonstrate that IL6R promote hepatic oncogenesis by directly activating STAT3 and in turn up-regulating expression of c-Myc.³⁹ Since a ChIP-Seq analysis of these 3 genes show the presence of C/EBP α binding sites within their promoter regions (Figs. 7A, 7B, and 7C), we assessed whether transfection of C/EBP α -saRNA in HepG2 cells would affect expression levels of these three factors. We observed a significant reduction in mRNA levels of STAT3 (Fig. 7D), cMyc (Fig. 7E) and IL6R (Fig. 7F) when compared to untransfected cells. This trend in gene reduction was also observed for MYC and STAT in our previously described gene expression array (Table 2, in bold). When the

methylation status of the CpG islands at the promoter regions of STAT3 (Fig. 8A), MYC (Fig. 8B) and IL6R (Fig. 8C) were assessed using EpiTect Methyl II PCR assay (Qiagen), an increase in methylation state at the promoters of all three genes was detected. A Western blot also confirmed a reduction in the phosphorylation status of STAT3 and in the protein level of IL6R (Fig. 8D). Collectively, we show that *in vivo* delivery of C/EBP α might have a positive effect in assisting liver function and decreasing aberrant cell proliferation in a cirrhotic/HCC setting.

DISCUSSION

Hepatocellular carcinoma (HCC) develops in most patients from a background of liver cirrhosis and accounts for 90% of all liver cancers. Although much progress has been made in targeting therapy to HCC, few of these treatments have had much impact in patient outcome.

The initial aim of this investigation was to study the therapeutic potential of using saRNAs to help ameliorate liver function in a clinically relevant rat model of liver cirrhosis with hepatocellular carcinoma. By enhancing expression of the gene encoding C/EBP α , a liver enriched transcription factor that enhances albumin and confers anti-mitotic activity; we primarily sought to increase circulating albumin in these rats. Using our previously published concept of designing short activating RNA oligonucleotide to increase the expression of a target gene^{7,11}, C/EBP α -saRNA was generated. This was initially tested in the hepatocellular carcinoma line (HepG2) where introduction of the saRNA oligonucleotide led to increased transcript levels of C/EBP α and albumin. Both genes furthermore contained the recognition motif of C/EBP α , CGAAT within their promoter regions. It was therefore unsurprising to detect a loss in methylation status at their CpG islands following transfection of C/EBP α -saRNA.

The biological significance of increasing albumin transcript levels in C/EBP α -saRNA transfected cells corresponded well with the increased secretion of albumin. Interestingly, we found that the maximum albumin gene expression was achieved at 5 nM of C/EBP α -saRNA with no further increase at higher saRNA levels. In addition to the albumin gene, we also found increased gene expression in other important biological markers such as ornithine cycle enzyme ornithine transcarbamylase (OTC) and alpha-feto-protein (AFP).⁴⁰

To test the potential therapeutic value of the C/EBP α -saRNA, we subsequently performed an *in vivo* study using an HCC rat model. For targeted delivery of C/EBP α -saRNA we linked the duplex RNA molecule to cationic PAMAM dendrimers. These nanoparticle have previously been evaluated where biodistribution studies demonstrate that they preferentially accumulate in peripheral blood mononuclear cells and the liver with no discernible toxicity.²⁵ Intravenous injection of C/EBP α -saRNA-dendrimers over a course of one week showed a significant improvement by 30% in the circulating levels of albumin where compared to PBS control or scramble-saRNA-dendrimer control groups. Changes in bilirubin levels showed a 10% improvement in the C/EBP α -saRNA group when compared to the control groups. Additionally a 10% improvement in AST levels and 30% improvement in ALT levels were observed in the C/EBP α -saRNA treated group when

compared to the control groups. More significant was the reduction in tumour burden and the inhibition of pre-neoplastic lesions as detected by a 40% reduction in GST-*p* staining in the liver sections from the C/EBP α -saRNA treated group. From a clinical perspective, this represents a very attractive therapeutic avenue since the expression level of C/EBP α in matched tumour tissues and non-tumour tissues of HCC patients is down-regulated in the majority of tumour specimens. Moreover, patients with tumour samples showing higher levels of C/EBP α have a longer survival rate than those patients with tumour samples in which the expression of the C/EBP α is lower.⁴¹ Our data supports this evidence suggesting that up-regulation of C/EBP α provides a strong anti-proliferative role in hepatocytes.^{14,42}

To better understand the global molecular effect of C/EBP α -saRNA more specific to liver cancer, we performed a liver cancer pathway gene expression profile analysis. Such analysis of whole tumours is frequently confounded by the presence of cell types other than those with a transformed phenotype.⁴³ Therefore we profiled the gene expression changes brought about by C/EBP α -saRNA in HepG2 cells.

The expression pattern of the liver cancer genes varied greatly between untransfected and C/EBP α -saRNA transfected HepG2 cells. After normalisation and cluster analysis, several important genes were significantly altered in expression. From the list of 20 genes that were up-regulated, 18 were known tumour suppressor genes. Of note was the up-regulation of RB, TP53, BID and BAX to regulate cell cycle and apoptosis. The down-regulation of key genes were also noted, in particular ADAM17, a metalloproteinase reported as being a pathological feature of HCC.⁴⁴ ADAM17 is known to cause the shedding of receptor ligands such as epidermal growth factor (EGF) and tumour necrosis factor alpha (TNF α)^{45,46} thus preventing regulation of key signaling events for normal cell signaling.

Upon further analysis of the tumour suppressor genes, we noticed a pathway-defined trend where key effector genes of the tumour suppressors were down-regulated. Examples of this included repression of RHOA following up-regulation of the tumour suppressor DLC1; or up-regulation of RUNX3 to reverse expression of the oncogenes involved in epithelial-mesenchymal transition (EMT). Here we observed down-regulation of CTNNB1 (β -catenin), HGF, SMAD7 and TGFB1. We also observed increased expression of the tumour suppressor SOC3, a known regulator of apoptosis and cell adhesion. Concomitantly, we also observed down-regulation of the associated SOC3 oncogenes including STAT3, cyclin-D1 (CCND1), XIAP, BIRC5 and MCL1. STAT3 activation together with IL6R is known to enhance hepatic oncogenesis as part of a feedback loop⁴⁷, and moreover perturbation in any of the components from this network is sufficient to suppress HCC.³⁹ Here, we demonstrated by gene expression analysis and detection of hypermethylation within the gene promoters, that both STAT3 and IL6R were down-regulated following C/EBP α -saRNA transfection. In addition to the well characterised anti-mitotic activity of C/EBP α involving retinoblastoma, p21 and the cyclin dependent proteins, our data here suggests that C/EBP α may regulate other liver specific oncogenic pathways including c-Myc (MYC).⁴⁸ Our observed reduction in the EMT factors; the positive regulation of apoptosis and down-regulation of IL6R, STAT3 and MYC, and the presence of numerous C/EBP α binding motifs within the promoter regions of these three genes provide a novel landscape to further

study the role of C/EBP α in improving the function of hepatocytes in a cirrhotic/HCC setting.

In summary, we initially designed saRNAs targeting the liver enriched transcription factor C/EBP α with the aim of addressing hypoalbuminemia. This was successfully done *in vitro* and *in vivo*. In the course of this work we also confirmed the well known anti-proliferative effects of C/EBP α in a clinically relevant cirrhotic/HCC model. In addition to regulating known targets of C/EBP α that controls cell proliferation we demonstrated using a liver cancer specific gene array analysis that C/EBP α potentially targets numerous other oncogenes and tumour suppressor genes which must be further investigated. C/EBP α -saRNAs therefore may have a profound effect at the transcriptional level for liver cancer. Currently, most therapeutic disciplines such as surgery, chemotherapy, radiotherapy and biologics are associated with variable decrease of liver dysfunction.^{49,50} The data presented here offers a new approach to targeting liver cancer cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

HCC	Hepatocellular carcinoma
C/EBPα	CCAAT/enhancer binding protein alpha
saRNA	short activating RNA
OTC	Ornithine transcarbamylase
AFP	Alphafetoprotein
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase

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Fig. 1. Transfection of C/EBPα-saRNA in HepG2 cells regulates expression of C/EBPα and albumin

(A) HepG2 cells were transfected with 20 nM C/EBPα-saRNA and harvested for total RNA extraction and reverse transcription for quantitative analysis of C/EBPα and (B) albumin gene expression. (C) A dose escalation of C/EBPα-saRNA demonstrates its effect over 96 hours (hr) on C/EBPα gene expression and (D) albumin gene expression. Data represents mean $\pm$ SD. The panels show the genomic region containing (E) C/EBPα, (F) DBP and (G) Albumin (ALB) 2000 nucleotides upstream and downstream of each gene where all have one or more C/EBPα binding sites. The figure panels show the chromosomal coordinates ("Scale" and chromosome identifier), C/EBPα binding sites ("C/EBPα"; black boxes), occurrence of the C/EBPα binding motif ("GCAAT motif"; black vertical lines), and RefSeq genes (blue boxes and lines) within the genomic regions.

Fig. 2. Transfection of C/EBP α -saRNA in HepG2 cells regulates hepatocyte function and affects cell proliferation

(A) Methylation assay of the CpG islands at the promoter regions of C/EBPA and (B) DBP demonstrated reduction in methylation when compared to control. Data represents mean $\pm$ SD. (C) An enzyme linked immunosorbent assay (ELISA) specific for human albumin detected a significant increase of albumin secretion following transfection of 20 nM CEPBA-saRNA. Data represents mean $\pm$ SD. (D) Expression of the gene encoding ornithine cycle enzyme Ornithine transcarbamylase (OTC) increased in C/EBP α -saRNA transfected cells suggesting an improved ability of urea production. (E) Decreased expression of the gene encoding alphafetoprotein (AFP) suggested improved regulation of cell differentiation. Data represents mean $\pm$ SD. (F) A WST-1 cell proliferation assay on HepG2 cells over 96 hr transfected with increasing amounts of C/EBP α -saRNA. Results show a dose dependent reduction in cell proliferation. Data represents mean $\pm$ SD.

Fig. 3. Intravenous injection of C/EBPα-saRNA-dendrimer in male Wistar rats with liver cirrhosis and HCC shows improved liver function

(A) C/EBPα-saRNA-dendrimer was tested for nuclease sensitivity in rat serum for the indicated times. RNA stability was visualised on a 2% denaturing agarose gel and (B) quantified by densitometry analysis. (Data represents mean $\pm$ SD, $n=3$). (C) C/EBPα-saRNA injected rats showed a significant change in circulating levels of albumin when compared to PBS control (Control) or scramble-saRNA-dendrimer control groups. (D) Changes in bilirubin levels suggested that C/EBPα-saRNA-dendrimer injected rats had at least a 10% improvement when compared to both control groups. Changes in (E) aspartate aminotransferase (AST) and alanine aminotransferase (ALT) demonstrated at least a 10% and 30% improvement in values when compared to the control groups. Data represents mean $\pm$ SD.

Fig. 4. Intravenous injection of C/EBPα-saRNA-dendrimer in male Wistar rats with liver cirrhosis and HCC shows reduced tumour burden

(A) Liver tumour nodules were visibly reduced in C/EBPα-saRNA injected rats when compared to both PBS control and Scramble-saRNA control groups. (B) Tumour burden was assessed by the volume of all tumour nodules with a diameter in excess of 3 mm. C/EBPα-saRNA injected rats had significantly reduced tumour burden after two weeks of treatment when compared to both control groups. Data represents mean $\pm$ SD. (C) 2 μ m liver sections from PBS control, scramble-saRNA-dendrimer control and C/EBPα-saRNA-dendrimer injected rats were immunostained for expression of placenta-form glutathione-S-transferase (GST-p). PBS control rats showed 70% ($\pm$ 5.0 %) of positive staining for the pre-neoplastic marker, scramble-saRNA-dendrimer injected rats showed 64% ($\pm$ 10.0 %) of positive staining whilst C/EBPα-saRNA-dendrimer injected rats only showed 32% ($\pm$ 6.5 %) of positive staining.

Fig. 5. Intravenous injection of C/EBP α -saRNA-dendrimer in male Wistar rats with liver cirrhosis and HCC positively regulates expression of factors for liver function

(A) Total RNA extracts from 7 control rats vs 7 C/EBP α -saRNA injected rats were analysed for albumin gene expression, (B) C/EBP α gene expression, (C) HNF4 α gene expression and (D) HNF1 α gene expression showed an increase in these factors. (E) Decreased mRNA levels encoding HGF and (F) increased levels of hydroxyphenylpyruvic acid dioxygenase (HPD1) and (G) plasminogen indicated positive regulation of cell proliferation and improved liver function. Data represents mean $\pm$ SD.

Fig. 6. Microarray analysis of 84 liver cancer pathway genes
Negative regulation of genes (in green) or positive regulation of genes (in red) following transfection of 20 nM C/EBPα-saRNA in HepG2 cells. Control (CONTROL-1 to 4) and C/EBPA-saRNA transfected (C/EBPA-1 to 4) are shown as 4 repeats.

Fig. 7. Transfection of C/EBPα-saRNA in HepG2 cells results in negative regulation of cell proliferating factors

STAT3, c-Myc (MYC) and IL6R have one or more C/EBPα binding sites. The panels show the genomic region 2000 nucleotides upstream and downstream of (A) STAT3 (B) MYC and (C) IL6R. Figure panels shown are as described in (Figure 1 E–G). C/EBPα-saRNA transfected HepG2 cells show negative regulation in mRNA expression levels of genes encoding (D) STAT3, (E) c-Myc and (F) Interleukin 6 receptor (IL6R). Data represents mean $\pm$ SD.

Fig. 8. Transfection of C/EBPα-saRNA in HepG2 cells targets STAT3, c-Myc and IL6R signalling

A methylation assay of the CpG islands at the promoter regions of (A) STAT3, (B) MYC and (C) IL6R demonstrated hypermethylation when compared to control. Data represents mean $\pm$ SD. (D) A Western blot analysis showed decreased phosphorylation of STAT3 at residues 705 and 727 and down-regulation of IL6R in cells transfected with C/EBPα-saRNA.

Table 1
Gene expression (up-regulated by C/EBP α -saRNA)

Clustered analysis of genes positively regulated following transfection of C/EBP α -saRNA in HepG2 cells.

Gene Symbol	Fold Up-Regulation	T-TEST
	C/EBP α saRNA/Control Group	p value
BAX	1.12	0.0027
BID	13.58	0.0001
CASP8	6.69	0.0000
DAB2IP	2.59	0.0042
DLC1	4.84	0.0001
FAS	1.64	0.0004
FHIT	2.84	0.0021
GADD45B	3.35	0.0001
HHIP	1.59	0.0054
IGF2	9.75	0.0001
LEF1	17.86	0.0001
PTEN	1.28	0.0013
PTK2	2.87	0.0001
RB1	1.96	0.0001
RUNX3	6.01	0.0002
SMAD4	1.72	0.0019
SOCS3	6.52	0.0003
TGFBR2	3.71	0.0025
TNFSF10	3.53	0.0003
P53	3.91	0.0018

Table 2
Gene expression (down-regulated by C/EBP α -saRNA)

Clustered analysis of genes negatively regulated following transfection of C/EBP α -saRNA in HepG2 cells.

Gene Symbol	Fold Down-Regulation	T-TEST
	C/EBP α saRNA/Control Group	p value
ADAM17	-2.19	0.0007
AKT1	-1.78	0.0220
ANGPT2	-1.61	0.0097
BCL2	-2.77	0.0177
BCL2L1	-1.77	0.0014
BIRC2	-2.83	0.0054
BIRC5	-18.53	0.0014
CCL5	-35.1	0.0001
CCND1	-6.45	0.0001
CCND2	-2.40	0.0001
CDH1	-3.04	0.0002
CDH13	-5.52	0.0001
CDKN1A	-2.53	0.0016
CDKN1B	-1.74	0.0145
CDKN2A	-6.75	0.0001
CFLAR	-7.12	0.0003
CTNNB1	-1.59	0.0081
CXCR4	-2.73	0.0004
E2F1	-1.40	0.0174
EGF	-8.63	0.0074
EGFR	-2.58	0.0064
EP300	-3.28	0.0001
FADD	-1.15	0.0001
FLT1	-36.26	0.0001
FZD7	-2.00	0.0004
GSTP1	-1.97	0.0019
HGF	-11.07	0.0001
HRAS	-3.69	0.0001
IGFBP1	-7.36	0.0001
IGFBP3	-17.56	0.0001
IRS1	-1.61	0.0010
ITGB1	-2.48	0.0001
KDR	-13.5	0.0001
MCL1	-2.47	0.0031
MET	-1.30	0.0247
MSH2	-1.03	0.0057

Gene Symbol	Fold Down-Regulation	T-TEST
	C/EBP α RNA/Control Group	p value
MSH3	-3.63	0.0002
MTDH	-1.73	0.0014
MYC	-1.76	0.0132
NFKB1	-1.48	0.0365
NRAS	-29.47	0.0001
OPCML	-1.51	0.0074
PDGFRA	-1.54	0.0019
PIN1	-1.15	0.0012
PTGS2	-2.76	0.0001
PYCARD	-1.49	0.0178
RAC1	-1.46	0.0069
RASSF1	-4.59	0.0001
RELN	-2.09	0.0005
RHOA	-1.46	0.0019
SFRP2	-1.73	0.0092
SMAD7	-3.46	0.0002
SOCS1	-7.04	0.0001
STAT3	-13.06	0.0006
TCF4	-9.96	0.0008
TERT	-1.65	0.0005
TGFA	-9.27	0.0001
TGFB1	-1.80	0.0002
TLR4	-4.81	0.0007
TNFRSF10B	-1.07	0.0063
VEGFA	-1.30	0.0429
WT1	-6.674	0.0010
XIAP	-2.93	0.0228
YAP1	-1.58	0.0058

Table 3
Up-regulation of tumour suppressor genes by C/EBP α -saRNA

Analysis of tumour suppressor genes up-regulated following transfection of C/EBP α -saRNA in HepG2 cells.

Gene Symbol	Gene Function	Fold Up-Regulation	T-TEST
		C/EBP α saRNA/Control Group	p value
BAX	Apoptosis	1.12	0.0027
BID		13.58	0.0001
CASP8	Apoptosis, angiogenesis	6.69	0.0000
DLC1	Apoptosis, Ras/Raf/MEK/ERK, small GTPase-mediated signalling	4.84	0.0001
FAS		1.64	0.0004
FHIT		2.84	0.0021
GADD45B	Apoptosis, cell cycle	3.35	0.0001
RUNX3		6.01	0.0002
SOCS3	Apoptosis, adhesion & proteolysis	6.52	0.0003
TNFSF10		3.53	0.0003
PTEN	Cell cycle, PI3K/AKT, adhesion & proteolysis, angiogenesis	1.28	0.0013
RB1	Cell cycle, classical WNT, Ras/Raf/MEK/ERK, small GTPase-mediated signalling	1.96	0.0001
IGF2	Cell cycle, IGF/IGFR signalling	9.75	0.0001
TP53	DNA damage, Ras/Raf/MEK/ERK, small GTPase-mediated signalling	3.91	0.0018
DAB2IP	Small GTPase-mediated signalling	2.59	0.0042
HHIP	Hedgehog signalling	1.59	0.0054
SMAD4	TGF β signalling, epithelial to mesenchymal transition	1.72	0.0019
TGFBR2	TGF β signalling, angiogenesis	3.71	0.0025

Table 4
Analysis of genes down-regulated by C/EBP α -saRNA

Analysis of genes down-regulated following transfection of C/EBP α -saRNA in HepG2 cells.

Gene Symbol	Gene Function	Fold Down-Regulation	T-TEST
		C/EBP α saRNA/Control Group	p value
CCND1	Classical Wnt, cell cycle, DNA damage	-6.45	0.0001
CDKN2A	Classical Wnt, Ras/Raf/MEK/ERK & Small GTPase-mediated signalling, cell cycle	-6.75	0.0001
CTNNB1	Classical Wnt, epithelial to mesenchymal transition (EMT), angiogenesis	-1.59	0.0081
FZD7	Classical Wnt	-2.00	0.0004
MTDH		-1.73	0.0014
PIN1		-1.15	0.0012
TCF4		-9.96	0.0008
SMAD7	TGF β signalling, EMT, adhesion & proteolysis	-3.46	0.0002
TGFB1	TGF β signalling, EGFR signalling, EMT, immune & inflammatory response	-1.80	0.0002
AKT1	PI3K/AKT signalling, adhesion & proteolysis	-1.78	0.0220
IRS1	PI3K/AKT signalling	-1.61	0.0010
IGFBP1	IGF/IGFR signalling	-7.36	0.0001
IGFBP3		-17.56	0.0001
IRS1		-1.61	0.0010
YAP1	Hippo signalling	-1.58	0.0058
CDKN1A	Ras/Raf/MEK/ERK & small GTPase-mediated signalling, cell cycle, CDKN1A	-2.53	0.0016
HRAS	Ras/Raf/MEK/ERK & small GTPase-mediated signalling	-3.69	0.0001
NRAS		-29.47	0.0001
RAC1	Ras/Raf/MEK/ERK & small GTPase-mediated signalling, immune & inflammatory response, adhesion & proteolysis	-1.46	0.0069
RHOA		-1.46	0.0019
RASSF1	Ras/Raf/MEK/ERK & small GTPase-mediated signalling, cell cycle	-4.59	0.0001
ADAM17	EGFR signalling, adhesion & proteolysis	-2.19	0.0007
CDH13	EGFR & small GTPase-mediated signalling, adhesion & proteolysis, angiogenesis	-5.52	0.0001
EGF	EGFR signalling, angiogenesis	-8.63	0.0074
EGFR	EGFR signalling, adhesion & proteolysis	-2.58	0.0064
TGFA	EGFR signalling	-9.27	0.0001
HGF	MET/HGF signalling, EMT	-11.07	0.0001
MET	MET/HGF signalling	-1.30	0.0247
RELN	Small GTPase-mediated signalling, adhesion & proteolysis	-2.09	0.0005
CDKN1B	Cell cycle	-1.74	0.0145
MYC		-1.76	0.0132
E2F1	Cell cycle, apoptosis	-1.40	0.0174
EP300	Cell cycle, apoptosis, adhesion & proteolysis	-3.28	0.0001
BCL2	Apoptosis	-2.77	0.0177

Gene Symbol	Gene Function	Fold Down-Regulation	T-TEST
		C/EBPAsaRNA/Control Group	p value
BCL2L1		-1.77	0.0014
BIRC2		-2.83	0.0054
BIRC5		-18.53	0.0014
FADD		-1.15	0.0001
GSTP1		-1.97	0.0019
MCL1		-2.47	0.0031
TERT		-1.65	0.0005
TNFRSF10B		-1.07	0.0063
WT1		-6.674	0.0010
CFLAR	Apoptosis, adhesion & proteolysis	-7.12	0.0003
MSH2	Apoptosis, DNA damage	-1.03	0.0057
PYCARD	Apoptosis, adhesion & proteolysis	-1.49	0.0178
CCL5	Immune & inflammatory response	-35.1	0.0001
CXCR4		-2.73	0.0004
NFKB1		-1.48	0.0365
PTGS2		-2.76	0.0001
TLR4		-4.81	0.0007
CDH1	Adhesion & proteolysis	-3.04	0.0002
EGFR		-2.58	0.0064
EP300		-3.28	0.0001
HGF		-11.07	0.0001
ITGB1		-2.48	0.0001
OPCML		-1.51	0.0074
SOCS1		-7.04	0.0001
TERT		-1.65	0.0005
ANGPT2	Angiogenesis	-1.61	0.0097
FLT1		-36.26	0.0001
KDR		-13.5	0.0001
PDGFRA		-1.54	0.0019
VEGFA		-1.30	0.0429