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ARTICLE

Mechanisms involved in the activation of C/EBPα by small activating RNA in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is generally accompanied by high mortality and low cure rate. CCAAT enhancer-binding proteins (CEBPs) are transcriptional regulators that play a key role in maintaining liver function. Altered expression of C/EBPα and C/EBPβ occurs in many tumours including HCC. saRNAs are small double-stranded RNAs that enhance target gene expression at the transcriptional level. In this report, we activate CEPBA with saRNAs and suppress CEBPB with siRNAs in cells that represent three different degrees of HCC. We performed functional assays to investigate the effects of enhancing C/EBPα and its downstream targets, p21 and albumin across these lines. We also used Mass-spectrometry (MS) subsequent to a ChIP pull-down assay to characterise the components of the protein complex involved in regulating saRNA function. Putative saRNA interacting protein candidates that were identified by MS were knocked-down with siRNAs to investigate its impact on saRNA activity. We confirmed CEBPA-saRNA decreased proliferation and migration in the differentiated lines (HepG3/Hep3B). The undifferentiated line (PLCPRF5) showed saRNA-induced increase in CEBPA but with no loss in proliferation. This effect was reversed when CEBPB was suppressed with CEBPB-siRNA. When interrogating saRNA mode of action; three saRNA interacting proteins, CTR9, HnRNPA2/B1 and DDX5 were identified by MS. Targeted knock-down of these two proteins (by siRNA) abrogated saRNA activity. This study provides insight into how different HCC lines are affected by CEBPA-saRNAs and that endogenous abundance of CEBPB and saRNA accessory proteins may dictate efficacy of CEBPA-saRNA when used in a therapeutic context.

Introduction

Hepatocellular carcinoma (HCC) is the second highest cause of cancer-related death and the fifth most common malignancy worldwide [1, 2]. Decades of investigations have provided insight into the molecular pathogenesis of HCC, where the majority of studies have been focused on transcription factors and their influence in aberrant cell

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growth. One promising target for cytostatic therapy is the liver-enriched CCAAT/enhancer-binding protein family (C/EBP) of transcription factors, which play a pivotal role in liver metabolism, regeneration, and differentiation. The family members C/EBPα and C/EBPβ regulate hepatocyte proliferation and function, and help to maintain homoeostasis of cell metabolism. The endogenous levels of *CEBPA* in the liver are typically lower in people with liver diseases; for example, *CEBPA* is downregulated in most HCC tumours compared to the corresponding tumour-free liver regions [3, 4]. *CEBPA* produces two translational isoforms, a full-length 42-kDa protein and a truncated 30-kDa protein. Accumulating evidence suggests that the 42-kDa isoform of *CEBPA* functions as a tumour suppressor, despite both isoforms being transcriptionally active [5, 6]. *CEBPA* activation has previously been shown to inhibit the migration of HCC cells through the suppression of epithelial to mesenchymal transition (EMT) induced by epidermal growth factor receptor (EGFR)/β-catenin signalling [7].

C/EBPβ promotes tumorigenesis by modulating the expression of genes encoding cytokines and chemokines, and by regulating cell cycle progression and apoptosis [8, 9]. *CEBPB* knockdown has previously been shown to activate CEBPA expression by stimulating the expression of the transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) and dislodging histone deacetylase 1 (HDAC1) from the *CEBPA* promoter [10, 11]. There is a dynamic interaction between C/EBPα and C/EBPβ during liver regeneration. A high ratio of C/EBPα to C/EBPβ suppresses cell proliferation by repressing cell cycle and acute phase response genes and activating metabolic genes, whereas a low ratio of C/EBPα to C/EBPβ has an opposite effect [12, 13].

Transcriptional activation of *CEBPA* (42-kDa) using a 21-nucleotide double-stranded small activating RNA (saRNA) significantly improves liver function in animal models of liver cirrhosis and HCC and decreases ascites in an acute liver failure animal model [14, 15]. This saRNA has now been developed for clinical use as MTLCEBPA [16] in a Phase 1 HCC trial (clinicaltrials.org: NCT02716012).

The purpose of this study was to provide a better understanding of how oligonucleotide induced changes in C/EBPA and C/EBPB expression affects HCC cells. Additionally, we further explored the mode of saRNA activity across different types of HCC cell lines. We identified a pool of novel saRNA-interacting proteins. These included heterogeneous nuclear riboprotein A2/B1 (hnRNPA2/B1) and DEAD-box helicase 5 (also known as ATP-dependent RNA helicase 5; DDX5). Targeted knockdown of these protein candidates by siRNA demonstrated their key involvement with saRNA activity.

Results

SaRNA-mediated activation of *CEBPA* regulates C/EBP downstream targets in HCC cells

C/EBPα has been reported to regulate the proteolytic cleavage of C/EBPβ into its 32 kDa (LAP) and 20 kDa (LIP) isoforms whilst C/EBPβ heterodimerises with C/EBPα to regulate cell proliferation and differentiation [17, 18]. C/EBPα also regulates protein levels of p21 which suppresses kinase activity by binding to CDKs, thus leading to the inhibition of cell cycle progression [19, 20]. To determine how CEBPA-saRNA affects the expression of CEBPA and its main downstream targets (p21 and albumin) in hepatocytes; three different HCC cell lines were used. HepG2 and Hep3B represents differentiated HCC whilst PLC/PRF/5 represents undifferentiated HCC. *CEBPA* activation by *CEBPA*-saRNA significantly increased the transcript and protein levels of CEBPB, CDKN1A (p21) and albumin in HepG2 (Fig. 1a, b) and Hep3B (Fig. 1c, d) cells, but not in PLC/PRF/5 cells (Fig. 1e, f). This suggests that saRNA-induced activation of *CEBPA* regulates C/EBPA downstream targets- C/EBPβ and p21 in differentiated HCC (represented by HepG2 and Hep3B) whilst access of the saRNA to the regulatory regions of CEBPA may be more challenging in undifferentiated HCC lines (as represented by PLCPRF5). CEBPA induced upregulation of albumin (when compared to HepG2 cells) was diminished in Hep3B and PLCPRF5 cells. Since Hep3B and PLC/PRF/5 cells express Hepatitis B virus S surface antigen this may have a dominant negative impact on CEBPA accessing the promoter region of albumin in these cells [21, 22].

CEBPA/CEBPB/p21 signalling axis shows cell-specific differences between HepG2, Hep3B and PLCPRF5 HCC lines

To investigate the biological effect of suppressing *CEBPB* expression in HCC cell lines, small interfering RNAs (siRNAs) were used to silence the expression of *CEBPB*. siRNA induced silencing of *CEBPB* in HepG2 cells led to increased protein expression of *CEBPA* by 1.7-fold and of p21 by 3-fold (Fig. 1b). In Hep3B cells, silencing of CEBPB only led to an increase in expression of p21 (Fig. 1d). In PLCPRF5 cells, silencing of CEBPB led to a 2-fold increase in CEBPA protein expression, with no changes seen in expression of p21 or albumin (Fig. 1f). The different responses of these three HCC lines by CEBPB silencing demonstrates a very dynamic regulatory role of CEBPB and CEBPA in the disease model these cells represents.

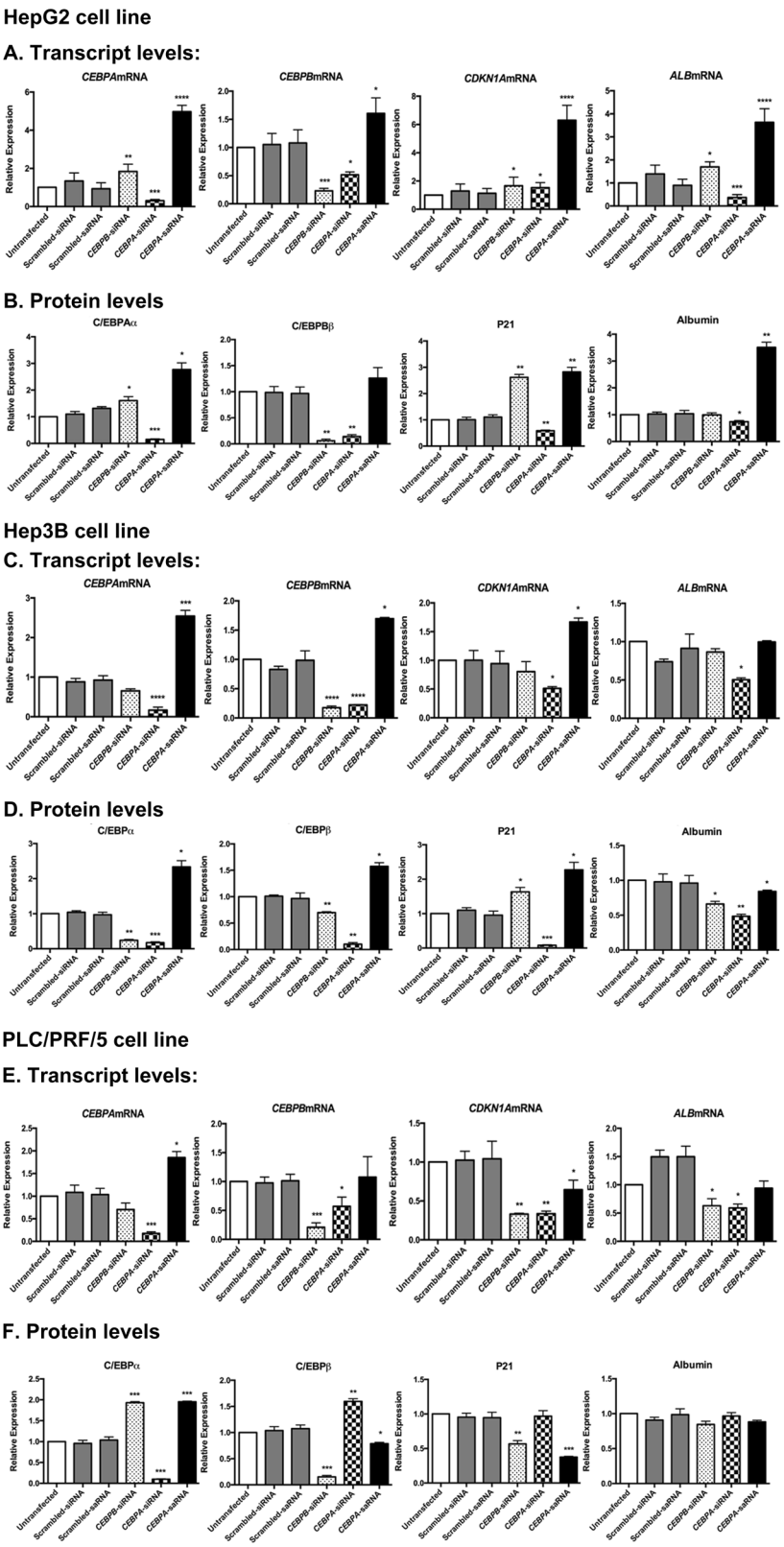
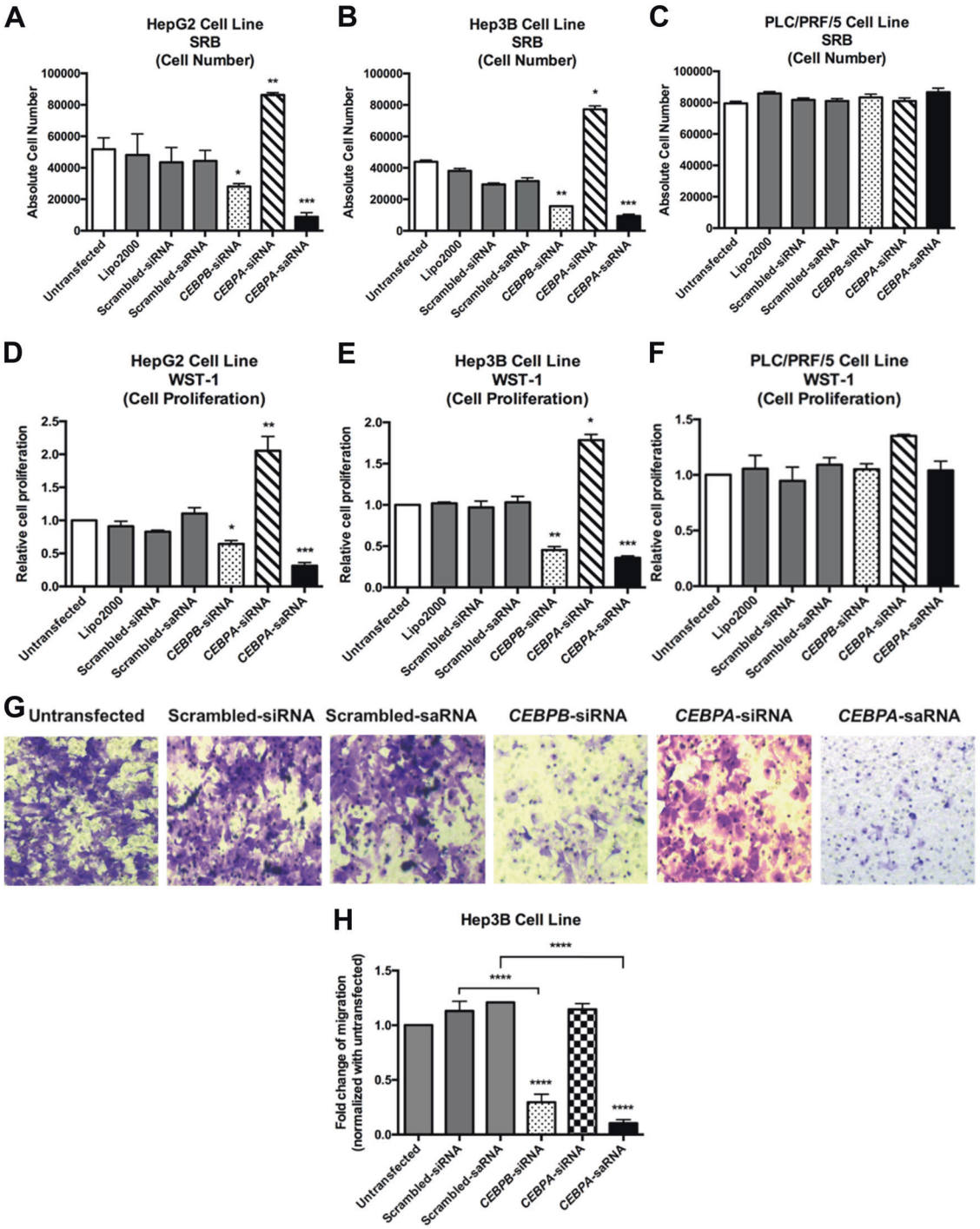


Fig. 1 Regulation in expression of C/EBPα downstream targets, p21 and albumin in hepatocellular carcinoma cell lines. Cells were transfected with 20 nM of scrambled saRNA or *CEBPA*-saRNA, or 10 nM of scrambled siRNA, *CEBPA*-siRNA or *CEBPB*-siRNA. Cells were harvested at 72 h after seeding for total protein extraction, total RNA extraction and real-time PCR analysis of mRNA expression. The bar graphs show the relative expression levels of *CEBPA*, *CEBPB*, p21 (*CDKN1)* and albumin (mean ± SD ($n = 3$)) in **a** HepG2, **c** Hep3B and **e** PLC/PRF/5 cells. Relative expression was calculated using the Livak method of $2^{-\Delta\Delta CT}$, normalised to *GAPDH*. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Relative protein band intensity of C/EBPα, C/EBPβ, P21 and albumin in **b** HepG2, **d** Hep3B and **f** PLC/PRF/5 cells was quantified from Western blots. β-actin was used as a loading control for the SDS-PAGE



CEBPA activation or *CEBPB* suppression inhibits proliferation of differentiated HCC cells

In HepG2 and Hep3B cells, siRNA silencing of CEBPB was sufficient in enhancing the anti-proliferative effects of endogenously expressed CEBPA. As a consequence, siRNA induced silencing of CEBPB showed similar inhibition in cell proliferation (measured by WST-1 proliferation assay and total cell numbers (SRB assay)) as seen in cells were CEBPA levels were enhanced using CEBPA-saRNA (Fig. 2a–e). PLCPRF5 cells, which represents a more aggressive undifferentiated HCC line, showed no response to its rate of proliferation when treated with CEBPB-siRNA or CEBPB-saRNA (Fig. 2c–f).

◀ **Fig. 2** Effects of C/EBPα and C/EBPβ on cytotoxicity, proliferation and migration in hepatocellular carcinoma cells. HepG2, Hep3B and PLF/PRC/5 cells were cultured and transfected with 20 nM of scrambled saRNA or CEBPA-saRNA, or 10 nM of scrambled siRNA; *CEBPA*-siRNA or *CEBPB*-siRNA in standard 96-well plates. Cytotoxicity was measured using sulphorhodamine B (SRB) assay. Representative bar charts showing the calculated cell numbers for **a** HepG2 cells, **b** Hep3B and **c** PLF/PRC/5 cells after each treatment using a titration curve based on the optical density (OD) value measured using spectrophotometry. Data represents the calculated cell numbers (mean ± SD ($n = 3$). Cell proliferation was assessed using WST-1 assay and OD values were measured at 10 min intervals. Representative bar charts showing the relative cell proliferation for each treatment condition in **d**, HepG2, **e** Hep3B and **f** PLF/PRC/5 cells normalised to the negative control (untransfected group). Data represent relative cell proliferation (mean ± SD ($n = 3$)). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Hep3B cells were grown and transfected with 20 nM of scrambled saRNA or CEBPA-saRNA, or 10 nM of scrambled siRNA *CEBPA*-siRNA or *CEBPB*-siRNA in standard 96-well plates. A transwell assay was carried out to measure migration of the treated cells. **g** Crystal violet stained images showing migrated cells after each treatment. (**h**) Representative bar charts showing the relative cell migration (mean ± SD ($n = 3$)) counted from nine randomly selected fields under a ×10 magnification microscope. Values are shown relative to the untransfected group. **** $p < 0.0001$

CEBPA activation or *CEBPB* suppression inhibits Hep3B cell migration

Cell migration plays a critical role in cellular processes, including the invasion and metastasis of tumour cells. Since the migratory ability of Hep3B cells is more pronounced than HepG2 or PLCPRF5 cells [23], we investigated whether activation of CEBPA expression (by CEBPA-saRNA) or suppression of CEBPB (by CEBPB-siRNA) would affect Hep3B cell migration. A trans-well migration assay was performed (Fig. 2g, h) in Hep3B cells transfected with CEBPA-saRNA or CEBPB-siRNA. We observed a significant reduction by 60–70% in cell proliferation when CEBPB expression was suppressed by CEBPB-siRNA (Fig. 2h). This effect was similar when CEBPA expression was increased by CEBPA-saRNA in Hep3B cells. Interestingly no changes were observed when CEBPA alone was suppressed by CEBPA-siRNA. In this setting it appears that CEBPB is the dominant regulator of cell migration in Hep3B and this effect is supported but not reliant on CEBPA. This observation has also been previously reported where the endogenous expression ratio of CEBPA to CEBPB expression can greatly impact on the treatment response of HCC cells [13]. This led us to further investigate what the impact of combining the two conditions (CEBPA-saRNA and CEBPB-siRNA) would be on these three HCC lines.

CEBPB knockdown improves HCC cell line response to CEBPA-saRNA

To investigate the combined activity of enhancing CEBPA expression whilst suppressing CEBPB expression; co-transfection studies were performed with *CEBPA*-saRNA and CEBPB-siRNA in HepG2, Hep3B and PLCPRF5 cells. 10 nM of CEBPA-saRNA combined with 10 nM of CEBPB-siRNA caused a 50% reduction in HepG2 cells proliferation. Increasing the amount of CEBPA-saRNA to 20 nM combined with 10 nM of CEBPB-siRNA caused a more pronounced 80% reduction in HepG2 cell proliferation (Fig. 3a–d). Hep3B cell also behaved similarly with a 68% reduction in proliferation when transfected with 10 nM CEBPA-saRNA/10 nM CEBPB-siRNA. This effect increased to 82% reduction in proliferation with 20 nM CEBPA-saRNA/10 nM CEBPB-siRNA (Fig. 3b–e). PLCPRF5 cells showed no change in proliferation when transfected with CEBPA-saRNA or CEBPB-siRNA alone (Fig. 2c–f); however, when transfected with 10 nM CEBPA-saRNA together with 10 nM CEBPB-siRNA, PLCPRF5 cells showed a 48% reduction in proliferation (Fig. 3c–f). This effect increased to 68% reduction in proliferation with 20 nM CEBPA-saRNA/10 nM CEBPB-siRNA demonstrating that the suppression of CEBPB was sufficient to sensitise PLCPRF5 cells to the anti-proliferative effects caused by CEBPA-saRNA.

Identification of proteins that interact with *CEBPA*-saRNA in HCC cells

To better understand the mechanism by which *CEBPA*-saRNA mediates its effects, we carried out pull-down protein assays with a biotinylated *CEBPA*-saRNA to characterise the protein components it interacts with in HepG2, Hep3B and PLCPRF5 cells. After confirming activity of the biotinylated CEBPA-saRNA (Supplementary Figure 1A), an affinity pulldown assay was performed using streptavidin sepharose beads. The eluted protein/biotinylated-CEBPA-saRNA complex was then separated onto a 4–12% gradient SDS-polyacrylamide gel for electrophoresis. The separated protein bands were visualised with Coomassie stain (Supplementary Figure 1B) for manual extraction and mass-spectroscopy sequencing of each visible band (Supplementary Figure 1B, lanes 5 (Bio-SCR) and 6 (Bio-C/EBPA)). Only cells transfected with biotinylated duplex saRNA (Bio-SCR and Bio-C/CEBPA) showed resolution of affinity purified protein bands. Pulldown from the biotinylated *CEBPA*-saRNA group showed more protein bands than the untreated and biotinylated scrambled saRNA groups (Supplementary Figure 1C). Collectively, 35 CEBPA-saRNA interacting protein bands from HepG2 and 49 CEBPA-saRNA interacting protein bands from Hep3B cell lines were isolated for analysis. Only 12 bands were resolved from PLCPRF5 cells (Supplementary Table 1).

To better understand the roles of the identified CEBPA-saRNA interacting proteins, we clustered the sequenced protein identities into several groups on the basis of their

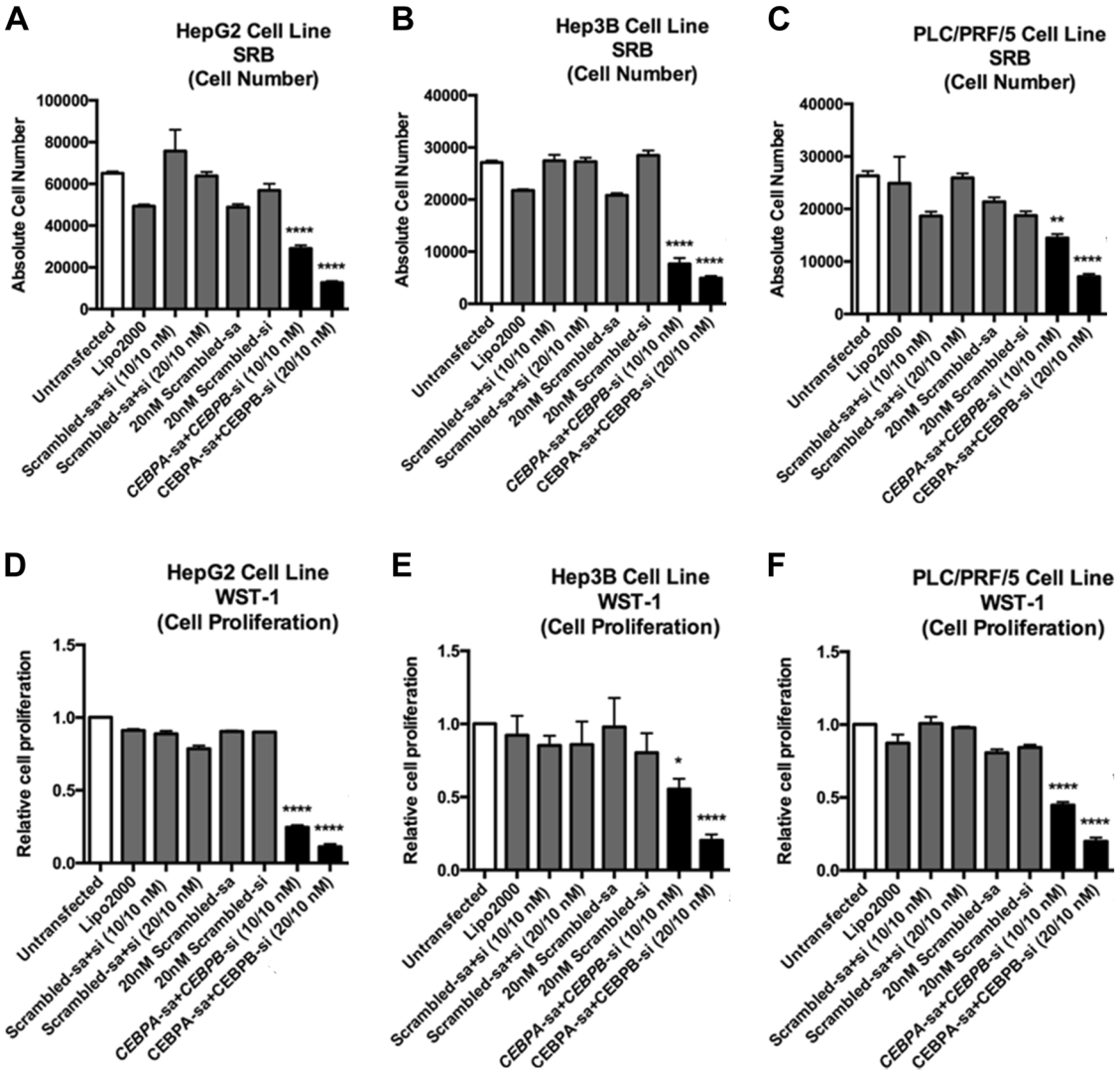


Fig. 3 Effects of combination of *CEBPA*-saRNA and *CEBPB*-siRNA on cytotoxicity and proliferation of hepatocellular carcinoma cells. HepG2, Hep3B and PLC/PRF5 cells were grown in standard 96-well plates and transfected with *CEBPA*-saRNA and *CEBPB*-siRNA. Cells were co-transfected with CEBPA-saRNA and CEBPB-siRNA to examine potential additive effect. Cytotoxicity was measured using sulphorhodamine B (SRB) assay. Representative bar charts showing the calculated cell numbers for **a** HepG2 cells, **b** Hep3B and **c** PLF/PRC/5 cells after each treatment. Data represent cell numbers (mean ± SD ($n = 3$)). Cell proliferation was assessed using WST-1 assay. Representative bar charts showing the relative cell proliferation assessed for each treatment condition in **d** HepG2, **e** Hep3B and **f** PLF/PRC/5 cells normalised to the negative control (untransfected group). Data represents relative cell proliferation (mean ± SD ($n = 3$))

function. This included RNA shuttling, stabilisation, folding, transcription and metabolism (Supplementary Table 2). In addition to this we also divided the list based on their reported subcellular localisation: cytoplasmic, nuclear or present in both cellular compartments.

CTR9, DDX5 and *HNRNPA2B1* carries important regulatory function for *CEBPA*-saRNA activity

To better understand the identity of the proteins isolated in complex with CEBPA-saRNA, we compared all of the proteins sequenced in our analysis with CDKN1A-saRNA interacting proteins recently reported by Portnoy et al. in PC3 cells [24]. 11 protein hits from HepG2 and Hep3B cells matched those identified by Portnoy et al. (Supplementary Table 1). Only three saRNA interacting protein identities from PLC/PRF5 cells matched with the Portnoy et al., report (Supplementary Table 1). We therefore further characterised how these three proteins (CTR9, DDX5 and hnRNPA2/B1) regulated CEBPA-saRNA activity.

CTR9 has previously been described to be involved in transcriptional elongation and also validated as an saRNA

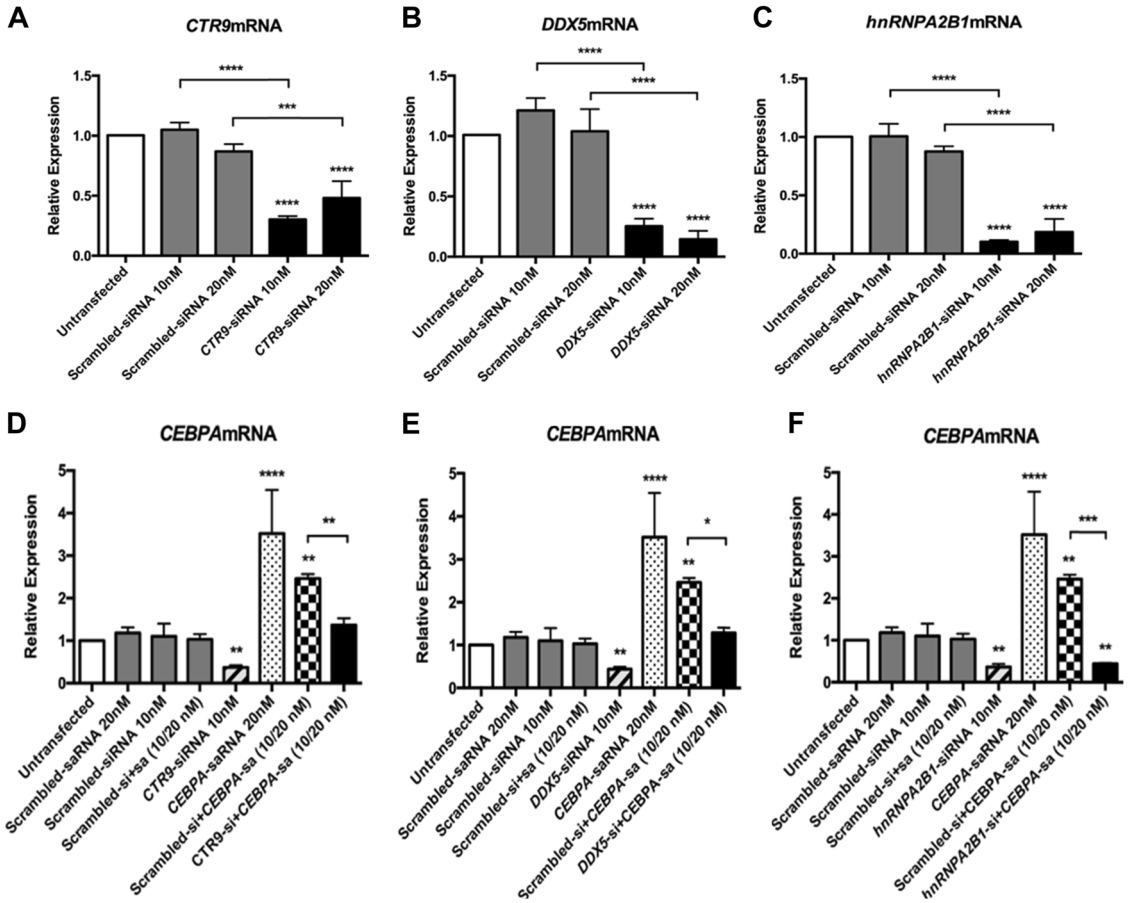


Fig. 4 siRNA knockdown of *CEBPA*-saRNA interacting protein complex-binding factors. Cells were transfected with 10 or 20 nM scrambled or targeted siRNA to knockdown **a** *CTR9*, **b** *DDX5* and **c** *HNRNPA2B1*. Cells were co-transfected with 20 nM *CEBPA*-saRNA or scrambled saRNA and 10 nM scrambled or targeted siRNA against **d** *CTR9*, **e** *DDX5* or **f** *HNRNPAB1*. The bar graphs show the relative expression levels of *CEBPA* (mean ± SD ($n = 3$)). Relative expression was calculated using the Livak method of $2^{-\Delta\Delta CT}$ normalised to *GAPDH* as a housekeeping gene. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

interacting protein by Portnoy et al. [18] [24]. HnRNPA2/B1 affects pre-mRNA processing, mRNA metabolism and transport. DDX5 is a member of the DEAD box protein family, which are associated with many cellular processes that involve alteration of RNA secondary structures, including translation initiation, nuclear and mitochondrial splicing, and ribosome and spliceosome assembly.

To identify whether one or more of these proteins were involved in saRNA-mediated activation of *CEBPA* in HCC cells, we first transfected HepG2 cells with siRNAs targeting these putative saRNA-interacting proteins to confirm efficient knockdown of these factors. Compared to untransfected HepG2 cells, we observed a significant decrease in the transcript levels of *CTR9*, *DDX5* and *HNRNPA2B1* with 0.7- (Figs. 4a), 0.8- (Fig. 4b) and 0.9-fold (Fig. 4c), respectively. We then investigated the relationship between *CTR9* (Fig. 4d), *DDX5* (Fig. 4e) and *hnRNPA2/B1* (Fig. 4f) with *CEBPA*-saRNA by knocking down each of these factors using siRNA in the presence of *CEBPA*-saRNA in HepG2 cells. When *CTR9* (Fig. 4d), *DDX5* (Fig. 4e) or *HNRNPA2B1* (Fig. 4f) was suppressed, a significant decrease in the stimulation of *CEBPA* transcription by *CEBPA*-saRNA (over 1.5-fold) was observed compared to those of the positive controls which were transfected with *CEBPA*-saRNA and a scrambled-siRNA alone. These results indicate that the endogenous expression levels of *CTR9*, *DDX5* or *hnRNPA2/B1* can potentially regulate the activity of *CEBPA*-saRNA. The enrichment of isolated saRNA interacting proteins on either the guide strand (AS) or passenger strand (SS) is a new and intriguing discovery (Supplementary Tables). We are uncertain if the differences in protein identities pulled down on either the AS or the SS is dependent on the cell line. As yet we cannot exclude that although accessory proteins may predominantly bind to the AS strand, since this is the characterised guide strand [16]; they may still remain attached to either strands post Ago2 processing. We are currently studying this mechanism and anticipate this will be in a future publication.

Table 1 The relevance of CEBPA-saRNA and CEBPB-siRNA co-treatment in HCC

Differentiated HCC	Undifferentiated HCC
Low expression of CEBPA	Low expression of CEBPA
Mid expression of CEBPB	High expression of CEBPB
CEBPA-saRNA sufficiently increases CEBPA to release from CEBPB inhibition	CEBPA-saRNA not increased sufficiently to release from CEBPB inhibition
Increased CEBPA reduces cell proliferation (via p21)	No reduction in cell proliferation
CEBPB-siRNA further enhances biological activity of CEBPA. Differentiated cells show more pronounced reduction in proliferation	CEBPB-siRNA releases its inhibition on CEBPA activity. Undifferentiated cells can start to show reduced cell proliferation
DDX5, hnRNPA2B1, CTR9 amongst other saRNA interacting proteins are important for saRNA activity	DDX5, hnRNPA2B1, CTR9 amongst other saRNA interacting proteins are important for saRNA activity

As yet it is not clear whether undifferentiated cells also have reduced expression of saRNA interacting proteins

Discussion

The aim of the present study was to investigate the biological consequence of increasing CEBPA expression using CEBPA-saRNA in different HCC types and to characterise saRNA interacting proteins for further understanding its mode of action.

We observed that CEBPA regulates proliferation of HepG2 and Hep3B and that this activity is further enhanced when CEBPB is suppressed. It is reported that the endogenous ratio of CEBPA to CEBPB influences how these transcription factors regulate cell proliferation [13]. This feature was most prominently observed in PLCPRF5 cells (representing a more aggressive HCC cell line) where increased CEBPA expression by CEBPA-saRNA was able to exert its anti-proliferative effect only when CEBPB expression was suppressed by siRNA. The endogenous expression levels of CEBPB therefore may likely have an impact on treatment outcome when CEBPA is upregulated by CEBPA-saRNA.

We used a proteomics approach to identify CEBPA-saRNA interacting proteins in the different HCC cell lines, and identified novel candidate proteins. We classified these proteins into six main functional groups. Those involved in mRNA shuttling, transcription, translation, and transcription stabilisation, metabolism and apoptosis.

We identified 11 proteins from differentiated HCC cells (HepG2 and Hep3B) and three proteins from undifferentiated HCC cell (PLCPRF5) that bound to *CEBPA*-saRNA. These three proteins (CTR9, DDX5 and hnRNAP2B1) have also been reported to bind to *CDKN1A*-saRNA, suggesting that they are important regulators of saRNA activity [24].

CEBPA-saRNA showed interaction with a larger population of protein in the differentiated HepG2 and Hep3B cells that were responsive to CEBPA activation than in undifferentiated non-responsive PLC/PRF/5 cells. These findings suggest that undifferentiated HCC cells may show reduced responsiveness to CEBPA-saRNA when compared to differentiated HCC cells as they may lack or have reduced endogenous expression of the factors that bind and mediate the transcriptional activity of saRNA (Table 1). This observation opens the opportunity to explore if non-responsive HCC might be transformed into becoming more sensitive to saRNA induced activity by upregulating key RITA components such as CTR9, hnRPNPA2/B1 or DDX5.

Since CEBPA-saRNA is currently in a phase I clinical trial (as MTLCEBPA) to treat severe liver cancer in patients (clinical trials.org: NCT02716012); its expansion to treat non-alcoholic fatty liver disease (NAFLD); non-alcoholic steatohepatitis (NASH) or other diseases where CEBPA plays a key role, will become more relevant once its interaction with CEBPB and the effects of saRNA regulatory proteins are better defined for each disease context (Summary Table 1). The endogenous levels of CEBPB may have an impact on how patients may respond to activation of CEBPA. We are the first to report that co-treatment of CEBPA-saRNA and CEBPB-siRNA may give an advantage to the anti-tumour activity driven by CEBPA. Since the endogenous levels of CTR9, hnRNPA2/B1 and DDX5 are crucial to enhancing saRNA activity; regulating their expression may be an additional step towards optimising saRNA therapy. Characterisation of the saRNA transcriptional activation machinery in the context of disease cell types will undoubtedly allow a more efficient approach to developing future clinical applications for saRNA.

Methods

Cell line selection and cell culture

All cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HepG2 (HB-8065; ATCC, Manassas, VA, USA) cells were

cultured in Roswell Park Memorial Institute medium (RPMI, Gibco, Thermo Fisher Scientific, Waltham, MA, USA), PLC/PRF/5 (CRL-8024; ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and Hep3B (HB-8064; ATCC, Manassas, VA, USA) cells were cultured in modified Eagle's medium (MEM, Gibco, Thermo Fisher Scientific, Waltham, MA, USA). These cell media were supplemented with 10% foetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), 100 units/ml penicillin, 0.1 mg/ml streptomycin and 2 mmol/l glutamine (Labtech International, Sussex, UK). All cells were cultured at 37 ℃ in a 5% humidified CO_2 incubator.

Design of saRNAs

The design of the saRNA used in this study has been previously described [14]. Briefly, a bioinformatics method was used to design saRNAs to specifically activate *CEBPA*. First, information including the genomic orientation, location, and transcriptional configuration of the target (*CEBPA*) was downloaded from the UCSC Ref Seq database (University of California, Santa Cruz). Second, the read direction of the target gene was obtained from the UCSC Spliced EST track (University of California, Santa Cruz), and transcripts antisense and adjacent to the target gene were indexed. According to this method, antisense transcripts were identified that (i) overlapped the promoter and mRNA at the 5′ end of the target; (ii) overlapped with the mRNA of the target; and (iii) were primarily 20–100 kb downstream of the poly-adenylation site of the target. Third, the antisense genomic sequence was downloaded from a region of a fixed size that covered both upstream and downstream of the transcription start site (TSS). The typical region covered 500 nt upstream and downstream of the TSS, but larger or smaller region sizes could also be used. Finally, the siRNA was designed on the basis of the antisense target sequence with particular and effective downregulation by using the detailed method described below. (a) A siRNA design algorithm (GPboost) was used to clarify efficacious siRNAs; (b) all efficacious siRNAs with aaaa, cccc, gggg, or uuuu and GC substance were removed (<20% or >55%); (c) all efficacious siRNA with a Hamming distance (<2 to all potential off-target transcripts) were alternatively removed; and (d) a given figure of non-overlapping siRNA remainders, classified according to the predicted siRNA knockdown efficacy, was returned. The two highest scoring saRNAs were returned for a provided antisense target sequence. Synthesised double-stranded *CEBPA*-saRNA (Sigma-Aldrich, St. Louis, MO, USA) was labelled with a biotin moiety at both ends of the strand (sense and antisense).

Transfection of RNA oligonucleotides

For transfection with saRNAs, the cells were grown to 60% confluency in 24-well plates and transfected with different concentrations of saRNA by using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. These transfected cells were subsequently harvested at 72 h after transfection and RNA was extracted for mRNA expression analysis, as described below.

The siRNAs (Supplementary Table 4) were all purchased from Thermo Scientific. For transfection with siRNAs, the cells were grown to 70% confluency in 24-well plates and transfected with different concentrations of RNA by using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. These transfected cells were subsequently harvested at 48 h after transfection and RNA was extracted for mRNA expression analysis, as described below.

Total RNA extraction and real-time quantitative PCR

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The cell pellets in each sample tube were lysed with Buffer RLT (Qiagen, Valencia, CA, USA) at 4 °C for 10 min. The lysates were subsequently transferred to QIAshredder columns and centrifuged. Subsequently, each sample was mixed with 70% ethanol and transferred to an RNeasy Mini spin column in a collection tube. After centrifugation, the samples were washed and eluted with Buffer RW1 and Buffer RPE, respectively. Subsequently, RNase-free water was directly pipetted to each spin column membrane, and the columns were centrifuged. The total RNA in the collection tubes was subsequently quantified with a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Genomic DNA was removed, and reverse transcription was conducted using a QuantiTech Reverse Transcription kit (Qiagen, Valencia, CA, USA). Finally, cDNA was synthesised for real time-polymerase chain reaction (RT-PCR). Primers were purchased from Qiagen (Valencia, CA, USA) (Supplementary Table 5).

Quantitative analysis of the expression of target genes and a control housekeeping gene (gluceraldehyde-3-phosphate-dehydrogenase (*GAPDH)*) was performed using a QuantiFast SYBR Green PCR Kit (Qiagen, Valencia, CA, USA). Briefly, gene-specific primers and the cDNA template were added to SYBR Green PCR Master Mix (SYBR® Green I dye, AmpliTaq Gold® DNA polymerase, dNTPs with dUTP and optimised buffer; Qiagen, Valencia, CA, USA). The Applied Biosystems 7900HT Fast-Real-Time System was used to amplify cDNA at one cycle of 95 °C for 10 min, and 40 cycles (95 °C for 15 s and 60 °C for 40 s) for

fluorescence data collection, followed by dissociation curve analysis. The Applied Biosystems RQ Manager was used to analyse the amplification of the relative expression levels of cDNA samples in triplicate. The relative expression of target genes was calculated using the comparative Ct method (Livak method) and described as fold differences normalised to the housekeeping gene. The Livak method was used to calculate the relative expression between the non-transfected groups and *CEBPA*-saRNA-transfected groups according to the following steps: the Ct of *CEBPA* was normalised to that of the housekeeping gene for both the non-transfected group and *CEBPA*-saRNA-transfected group: $\Delta Ct = Ct$ target gene$-Ct$ housekeeping gene. The ΔCt of the *CEBPA*-saRNA group was normalised to the ΔCt of the non-transfected group: $\Delta\Delta Ct = \Delta Ct$ transfection $-\Delta Ct$ control. The relative expression was calculated: relative expression $= 2-\Delta\Delta Ct$.

SDS-polyacrylamide gel electrophoresis and western blot analysis

5 mg of each protein sample was loaded into the wells of precast polyacrylamide gels (NuPAGE® Novex® 4–12% Bis-Tris gels from Invitrogen, Waltham, MA, USA) and an electrophoresis system (XCell SureLock™ Mini Cell, Invitrogen, Waltham, MA, USA) was used to separate the protein samples for ~2 h at 125 volts DC according to the manufacturer's instructions. The proteins were subsequently prepared for transfer onto nitrocellulose membranes using Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA, USA). Transfer buffer soaked electrode papers, SDS-polyacrylamide gel and nitrocellulose membrane were then laid onto the semi-dry system in order. Protein transfer was performed at 400 mA for 2 h. Protein transfer was confirmed by using Ponceau S Staining Solution (P7170, Sigma-Aldrich, St. Louis, MO, USA) to detect the protein bands transferred onto the nitrocellulose membrane.

The western blot procedure was performed using the SNAP i.d.® 2.0 Protein Detection System (Millipore, Billerica, MA, USA). Primary antibodies against β-actin (ab8226; Abcam, Cambridge, UK), C/EBPα (ab40761; Abcam, Cambridge, UK), CEBP/β (ab18336; Abcam, Cambridge, UK), p21 (ab18209; Abcam, Cambridge, UK) and albumin (ab106582; Abcam, Cambridge, UK), and goat anti-rabbit (ab97051; Abcam, Cambridge, UK) and goat anti-mouse (ab97023; Abcam, Cambridge, UK) secondary antibodies were purchased from Abcam (Cambridge, UK). The primary (1:1000) and secondary antibodies (1:2000) were respectively diluted in 10× phosphate-buffered saline containing Tween 20 (PBST) and 0.5% dried skim milk. Subsequently, the blots were incubated with 5 ml of the primary antibody solution for 10 min at room temperature, and washing three times. The same procedure was conducted for incubation of the blots with secondary antibodies. Finally, Luminata™ HRP Chemiluminescence Detection Reagent (Luminata Forte Western HRP substrate, Millipore, Billerica, MA, USA) was used to detect the secondary antibody according to the manufacturer's instructions. The blots were visualised with a C-DiGit® Blot Scanner ((LI-COR Biotechnology, Cambridge, UK) by using Image Studio™ software ((LI-COR Biotechnology, Cambridge, UK). To reprobe with a new antibody, the previous antibody on the blot was removed by stripping the membrane with 1× stripping buffer (Sigma-Aldrich, St. Louis, MO, USA) for 15 min and washing three times with PBST at room temperature.

Sulphorhodamine B colorimetric assay

Cytotoxicity was assayed by measuring the number of cells labelled using sulphorhodamine B (SRB). Briefly, a standard plate containing different absolute cell numbers (8000, 10,000, 20,000, 40,000, 60,000 and 80,000) was set aside for a no-growth control after 2–3 h incubation, and the remaining assay plates were set up containing different absolute cell numbers per well (HepG2: 12,000; Hep3B: 10,000; PLC/PRF/5: 8000), and incubated for 24, 48, 72 and 96 h. The cells were fixed with 10% trichloroacetic acid at 4 ℃ for 1 h, washed four times with slow-running tap water and air-dried at room temperature. The cells were subsequently stained with 0.057% SRB solution, and this was followed by solubilisation of protein-bound SRB with 10 mM Tris-base solution. The optical density (OD) was measured at 510 nm by using a BIO-Tek plate reader [25]. A SRB standard curve was established by using the OD values from the standard plate (no-growth control), thus allowing the absolute cell numbers in each treatment group to be calculated.

WST-1 cell proliferation assay

Cell proliferation assays were performed using WST-1 reagent (Roche diagnostics, UK) to analyse the activity of cellular mitochondrial dehydrogenases according to the manufacturer's instructions. Briefly, the cells were seeded onto 96-well plates and transfected with 20 nmol of RNA by using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA). Cells were analysed at 24, 48, 72 and 96 h after transfection. The initial medium was removed, and a 1:100 dilution of WST-1 reagent was added to each well. Plates were incubated at 37 °C for one hour and readings at 420 nm were taken at intervals of 10 min with a reference reading at 620 nm by using the BIO-Tek plate reader.

Transwell cell migration assay

Cell migration assays were performed using 8.0 μm pore size Transwell™ permeable supports (24-well format, Corning Costar, USA). The cells were trypsinised, pelleted and re-suspended in serum free medium, and 100 μl of cell suspension was placed on top of the filter membrane in the upper Transwell chamber and incubated for 10 min (37 °C, 5% CO_2) to allow the cells to settle. The lower compartment was loaded with 600 μl of medium containing 10% foetal bovine serum by using a pipette to form a chemotactic gradient. After 16 h of incubation, the remaining cells on the filter side of the upper chamber were removed with a cotton swab, and the cells that migrated through the top membrane were fixed with 1 ml of 70% ethanol for 10 min, stained with 1% crystal violet in 2% ethanol, and rinsed in water. The migrated cells on the undersides of filters were viewed with a 10 X magnification microscope (Olympus, Tokyo, Japan) and counted in 10 high-power fields to calculate the average number of migrated cells per insert. The crystal violet-stained cells were dissolved in 33% acetic acid, and the absorbance was measured at 600 nM by using BIO-Tek ELISA reader.

saRNA complex immunoprecipitation

Cells were transfected with 20 nM of biotin-labelled scrambled or *CEBPA*-saRNA, formaldehyde cross-linked, and harvested for 72 h. The cells were lysed with SDS-chromatin immunoprecipitation buffer, and the supernatant was collected after centrifuge for precipitation. The biotin-labelled saRNA complex was subsequently immunoprecipitated from the supernatant with streptavidin beads at 4 °C overnight, separated via SDS-PAGE and stained with Coomassie blue. Bands on the gel were excised and digested with an In-Gel Tryptic Digestion Kit (Thermo Scientific, Wilmington, DE, USA) according to the manufacturer's instructions. Peptide samples were purified and concentrated with Pierce C18 Spin Columns (Thermo Scientific, Wilmington, DE, USA) according to the manufacturer's instructions. The samples were dried with a SpeedVac and suspended in 1–2 μl of Matrix solution before Liquid Chromatography-Mass Spectrometry (LC-MS) analysis.

Statistical analysis

Data represent the mean ± standard deviation (SD). Student's *t*-test, non-parametric statistical tests and ANOVA (one-way and two-way) were performed for the statistical analysis.

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Author contributions VR, NH and KW-H designed the experiments. XYZ performed the experiments. VR and NH supervised the project. VR and XYZ analysed the data, XYZ wrote the paper. VR, NH and KW-H revised the manuscript. JF, HCJ, PS, JR and NH had comments and discussion on the results.

Compliance with ethical standards

Conflict of interest VR, PS, JJR and NH own stock in MiNA (Holdings) Limited. All the remaining authors declare that they have no conflict of interest.

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