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# OncomiR-181a promotes carcinogenesis by repressing the extracellular matrix proteoglycan decorin in hepatocellular carcinoma

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## Abstract

**Background** Proteoglycans are important tumor microenvironment extracellular matrix components. The regulation of key proteoglycans, such as decorin (DCN), by miRNAs has drawn attention since they have surfaced as novel therapeutic targets in cancer. Accordingly, this study aimed at identifying the impact of miR-181a in liver cancer and its regulatory role on the extracellular matrix proteoglycan, DCN, and hence on downstream oncogenes and tumor suppressor genes.

**Results** DCN was under-expressed in 22 cirrhotic and HCC liver tissues compared to that in 11 healthy tissues of liver transplantation donors. Conversely, miR-181a was over-expressed in HCC liver tissues compared to that in healthy liver tissues. In silico analysis predicted that DCN 3'UTR harbors two high-score oncomiR-181a binding regions. This was validated by pmirGLO luciferase reporter assay. Ectopic miR-181a expression into HuH-7 cells repressed the transcript and protein levels of DCN as assessed fluorometrically and by western blotting. DCN siRNAs showed similar results to miR-181a, where they both enhanced the cellular viability, proliferation, and clonogenicity. They also increased Myc and E2F and decreased p53 and Rb signaling as assessed using reporter vectors harboring p53, Rb, Myc, and E2F response elements. Our findings demonstrated that miR-181a directly downregulated the expression of its direct downstream target DCN, which in turn affected downstream targets related to cellular proliferation and apoptosis.

**Conclusion** To our knowledge, this is the first study to unveil the direct targeting of DCN by oncomiR-181a. We also highlighted that miR-181a affects targets related to cellular proliferation in HCC which may be partly mediated through inhibition of DCN transcription. Thus, miR-181a could be a promising biomarker for the early detection and monitoring of liver cancer progression. This would pave the way for the future targeting of the oncomiR-181a as a therapeutic approach in liver cancer, where miR-181a-based therapy approach could be potentially combined with chemotherapy and immunotherapy for the management of liver cancer.

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**Keywords** Decorin (DCN), microRNA-181a (miR-181a), Hepatocellular carcinoma (HCC), Liver cancer

## Introduction

Hepatocellular carcinoma (HCC) ranks as the third most common cause of cancer-related death worldwide, posing a constant threat in both developed and developing countries [1]. In Egypt, liver cancer has the highest incidence, mortality, and prevalence, according to the Global Cancer Statistics 2020 report [1]. Alarming, the burden of HCC in Egypt has been on the rise with a doubling in the incidence rate among patients with chronic liver disease over the period of a decade [2]. Several environmental and biological factors contribute to the etiology or progression of HCC, particularly hepatitis C virus (HCV) infection where, until the recent advent of direct acting antiviral agents against HCV, Egypt has had one of the highest prevalence rates of this viral infection in the world [3, 4]. Moreover, liver cancer is highly resistant and refractory to various therapeutic interventions; a recent prospective study of HCC management concluded the poor survival of the patients with HCV-related liver cancer with the most common therapeutic modalities. HCV-infected HCC patients face several significant treatment challenges. Antiviral resistance in HCV-related HCC, which is often diagnosed at an advanced stage, limits the treatment options. While recent advancements in antiviral therapy have improved the treatment outcomes, the risk of disease recurrence and limited access to care in certain regions remain significant challenges. Although this study was conducted in Egypt, the authors reported findings similar to those reported from high-income countries [5, 6]. Another reason behind this therapeutic challenge is the tumor microenvironment, which can foster recurrent *de novo* HCC tumors, thus rendering HCC treatment as challenging [7].

The extracellular matrix is an important component of the tumor microenvironment and is primarily composed of proteoglycans, which have a core protein and glycosaminoglycan chains. Proteoglycans maintain tissue homeostasis on the physiological level and show dramatic changes in their expression levels in carcinogenic tissues. Decorin (DCN) is a small multifunctional cellular or pericellular matrix proteoglycan and structural constituent of the hepatic microenvironment. Its name stems from its ability to “decorate” the collagen fibrils, thus regulating matrix assembly and homeostasis by fibrillogenesis [8]. However, there was a paradigm shift in our understanding of the emerging role of DCN in impeding the growth of tumor cells [9, 10], where it was manifested that DCN affects various biological processes, including cell growth, differentiation, proliferation, adhesion, spread and migration, and regulates inflammation and fibrillogenesis [11]. Accordingly, two chief footprints

have emerged for DCN on the functional level, namely, preserving the cellular structure and controlling different signal transduction pathways with an eventual culmination of tumor suppressive effects.

Several studies have reported higher levels of DCN in normal liver tissue compared to those in tumorous liver tissues [12, 13]. Interestingly, the sequential evolution of liver cirrhosis into HCC is marked by the progressive reduction of DCN levels, suggesting a critical role for this proteoglycan in cancer progression [14]. Of note, HCC patients with higher expression of DCN have more favorable survival rates [13, 15]. Mechanistically, DCN potently suppresses cell proliferation, survival, migration, and angiogenesis and could thus generate a powerful antitumorigenic signal [16–19]. Moreover, introduction of recombinant human DCN increases apoptosis, inhibits cell proliferation, and induces G0/G1 phase arrest through reactivating the expression of cyclin-dependent kinase inhibitor 1 C (p57Kip2) [20]. In a carcinogen-induced HCC mouse model, DCN knockout mice showed enhanced tumor predominance compared with wild-type mice [21]. Therefore, regulating DCN by non-coding RNAs, specifically miRNAs, might represent a cornerstone in understanding the behavior of DCN in liver cancer.

Several miRNAs are aberrantly expressed in HCC tissues; thus, these small endogenous molecules emerged as potential biomarkers and novel molecules or targets for tumor therapy. The first small-interfering RNA was granted FDA approval as a therapeutic agent in 2018, and several candidate miRNA therapeutics are in clinical development or clinical trials [22]. In this age of rapidly developing RNA technology, the use of miRNA mimics or repressors is continuously gaining traction as antitumor therapeutic molecules. The mechanistic epigenetic role of miRNAs as potential upstream microregulators of DCN in HCC and their subsequent impact on downstream targets and cancer progression pose a significant research gap. Therefore, this study aimed at identifying the mechanistic role of miR-181a as a possible upstream microregulator of DCN in HCC and hence its impact on the regulation of downstream transcription factors and cancer progression.

## Patients and methods

### Patients

Liver biopsies were obtained from 22 HCC patients and 11 healthy donors during liver transplantation at the Kasr El Ainy and Dar El-Fouad hospitals in Egypt. The clinical data of patients and healthy volunteers is shown in Table 1. The experiments were undertaken with the

**Table 1** Clinical parameters and characteristic features of non-metastatic HCC patients and healthy volunteers

	Parameters	Average ± SD <sup>a</sup>
HCC and Cirrhotic Patients	Age	49 ± 13.5
	Sex (male% / female %)	95.5% / 4.5%
	Ethanol abuse	None
	Aspartate aminotransferase (AST) (U/L)	100.5 ± 65.8
	Alanine aminotransferase (ALT) (U/L)	85.6 ± 95.6
	Alkaline phosphatase (U/L)	110.2 ± 60.7
	Serum albumin (g/dL)	4.6 ± 1.5
	Serum AFP (ng/mL)	155.7 ± 22.3
	HCV Ab	100% (22 HCC patients)
	HBV Ab	18.2% (4/22 HCC patients)
Healthy Controls (Liver Donors)	Age	31 ± 10.5
	Sex (male% / female %)	70% / 30%
	Ethanol abuse	None
	Diabetic	None
	Hypertensive	None
	HCV status <sup>b</sup>	None
	HBV status <sup>c</sup>	None

<sup>a</sup> Data is presented as average ± SD

<sup>b</sup> HCV status was determined using anti-HCV antibody and/or HCV viral RNA quantification

<sup>c</sup> HBV status was determined using anti-HBc and anti-HBs antibodies and by detection of HBsAg

understanding and written informed consent of each patient. The inclusion criteria of the study patients with HCC included patients with : (1) histologically confirmed diagnosis of HCC; (2) age range of 20–65 years; (3) confirmed HCV infection; and (4) no history of other primary cancers. The exclusion criteria included: (1) the presence of other active malignancies; (2) severe comorbidities that could interfere with study participation or outcomes; and (3) lymph node metastasis. The inclusion criteria for the healthy donors included: (1) age- and sex-matching with the patients with HCC; (2) no history of liver disease or other chronic conditions; and (3) no current or past history of cancer.

The study methodologies conformed to the ethical standards set by the declaration of Helsinki. The ethical committees of Cairo University and the German University in Cairo approved the study methodologies. Liver disease assessment for each patient is shown in Table 2.

#### Extraction and quantification of microRNA and mRNA from liver tissues and HuH-7 cell line

MirVana microRNA Isolation Kit (Ambion, USA) and BIOZOL RNA Extraction Reagent (Bioer Technology, China) were used, as per the manufacturer's protocol, for total mRNA extraction from liver biopsies and HuH-7 cells, respectively. Extracted RNA was then reversed

**Table 2** Liver disease assessment for each patient: Number/size of focal lesions, scoring, and PT-INR of each HCC patient according to the Milan criteria

Patients	Number of focal lesions	Size of focal lesions	MELD <sup>a</sup> Score	Child-Pugh Score	PT-INR <sup>b</sup>
Patient 1	3 focal lesions	1.5 cm, 1 cm, and 1 cm	11	C	1.4
Patient 2	Unifocal	2.5 cm	15	C <sub>10</sub>	1.6
Patient 3	3 focal lesions	2 cm, 2.5 cm, and 3 cm	10	C <sub>11</sub>	2.4
Patient 4	3 focal lesions	2 cm, 2 cm, and 3.5 cm	10	C	2
Patient 5	Unifocal	1.5 × 2 cm	16	C	1.4
Patient 6	3 focal lesions	3 × 4 cm, 1 cm, and 1 cm	12	B	1.5
Patient 7	Unifocal	4 cm	11	B <sub>7</sub>	1.5
Patient 8	3 focal lesions	4 cm, 1 cm, and 1 cm	8	A <sub>5</sub>	1.3
Patient 9	3 focal lesions	1 cm, 1 cm, and 1.5 cm	17	C	1.5
Patient 10	Unifocal	2.5 cm	10	B <sub>8</sub>	1.4
Patient 11	2 focal lesions	1 cm and 1.7 cm	15	B <sub>8</sub>	1.4
Patient 12	3 focal lesions	1 cm each	17	C <sub>11</sub>	1.8
Patient 13	Unifocal	3 cm	13	B <sub>7</sub>	1.6
Patient 14	3 focal lesions	3 cm, 1.5 cm, and 2 cm	15	B	1.6
Patient 15	3 focal lesions	1 cm, 1 cm, and 4 cm	12	B <sub>7</sub>	1.14
Patient 16	2 focal lesions	3 cm and 1.5 cm	19	C <sub>12</sub>	1.9
Patient 17	2 focal lesions	1.5 cm and 3 cm	16	C <sub>10</sub>	1.55
Patient 18	3 focal lesions	2.5 cm, 2.5 cm, 1.5 cm	9	B <sub>7</sub>	1.18
Patient 19	3 focal lesions	1.5 cm, 1 cm, and 1 cm	11	B <sub>8</sub>	1.19
Patient 20	Unifocal	2 cm	18	C	1.7
Patient 21	Unifocal	1.5 cm	17	C	1.55
Patient 22	3 focal lesions	3 cm, 2.5 cm, and 1 cm	12	B	1.6

<sup>a</sup> MELD: Model for End-stage Liver Disease score

<sup>b</sup> PT-INR: international normalized ratio for prothrombin time

transcribed into complementary DNA (cDNA) (Applied Biosystems, USA). cDNA was amplified and quantified using TaqMan quantitative real-time polymerase chain reaction (RTqPCR) (Applied Biosystems). The housekeeping genes, RNU6B and beta-2 microglobulin (B2M) were used to normalize the relative expression of miR-181a and DCN in each sample, respectively. The  $2^{-\Delta\Delta C_t}$  method was used to calculate the relative gene expression.

#### Cell culture and transfection

The HuH-7 cells that were used in all cell culture experiments were kindly provided by Prof. Kai Breuhahn (Institute of Pathology, University Heidelberg, Heidelberg, Germany). The HuH-7 cells were cultured in DMEM supplemented with 10% FBS, 1% mycozap/penicillin/streptomycin (Lonza, Switzerland). HiPerFect Transfection Reagent (Qiagen, Germany) was used, as per the manufacturer's protocol, to transfect cells with siRNAs against DCN, miR-181a mimics, or negative control oligonucleotides (Qiagen, Germany).

#### Bioinformatics

To predict microRNAs that putatively target the 3'untranslated region (3'UTR) of the DCN transcript, the online bioinformatics software microrna.org (<http://www.microrna.org>) and Target Scan (<http://www.targetscan.org/>) were used.

#### MTT cell viability assay

4,5-Dimethyl thiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) was added to HuH-7 cells 48 h post-transfection. This was followed by incubation for 4 h and adding ethanol/DMSO solution for cell lysis. Optical density was measured at 572 nm, and relative viability was calculated.

#### BrdU cell proliferation assay

BrdU Cell Proliferation ELISA kit (Roche Applied Biosystems, Germany) was used, as per manufacturer's protocol, to assess proliferation 48 h post-transfection of HuH-7 cells. Luminescence was measured and expressed as relative light units per second (RLUs/s).

#### Colony-forming assay

HuH-7 cells were transfected with miR-181a or DCN siRNA. Then, 48 h post-transfection, the cells were detached and imbedded in a layer of 0.36% soft agarose superimposed on another agarose layer of 0.76%. The plates were then incubated for two weeks to colonize. Colonies were stained with 0.05% crystal violet and counted.

#### Microplate fluorimetry for protein quantification

Transfected HuH-7 cells were fixed using 4% paraformaldehyde. Blocking solution was added followed by overnight incubation with primary mouse monoclonal antibody against DCN (Santa Cruz, USA). After 24 h, Texas Red-conjugated goat anti-mouse secondary antibody (Santa Cruz, USA) solution was added and incubated in the dark for 1 h. Fixed cells were mounted by adding DAPI Fluoroshield Mounting Medium (Abcam, UK) to counterstain the nuclei and prevent rapid photobleaching of TR fluorescence. Fluorescent images were captured using Zeiss Immunofluorescent microscope. Perkin Elmer Wallac 1420 VICTOR2™ fluorometer was used to quantify fluorescence of immunolabeled proteins in each well. Excitation and emission wavelengths of TR fluorochrome (596 and 615 nm, respectively) normalized to the fluorescence of nuclei after DAPI staining using its excitation and emission wavelengths (360 and 460 nm, respectively) were used.

#### Protein extraction and Western blot

HuH-7 cells were lysed using RIPA buffer (Serva) containing 1X phosphatase inhibitors and 1X protease inhibitors (ThermoFisher Scientific). After centrifugation, protein concentrations in the supernatants were measured using Modified Lowry protein assay kit (ThermoFisher Scientific). Denaturation at 95 °C for 5 min was performed, then 40 µg of protein lysates were run on a 10% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane (Serva), using Bio-Rad western blot system (Bio-Rad). The membrane was incubated overnight with primary antibody against DCN (R&D Systems) at 4 °C on a shaker with a concentration of 3 µg in 5% BSA as well as GAPDH (Cell Signaling Technologies) overnight at 4 °C with a dilution of 1:1000 in 5% BSA. After three washes with TBS-T, the membrane was incubated for 1 h with the respective secondary antibodies as follows: secondary anti-mouse antibody (dilution, 1:2,500) in 5% milk (Santa Cruz Biotechnology) was used for DCN, and secondary anti-rabbit antibody (dilution, 1:10,000) in 5% milk (Cell Signaling Technology) was used for GAPDH. Protein bands were visualized using Pierce ECL Western blotting substrate (ThermoFisher Scientific), and relative band intensity was quantified using ImageQuant software.

#### miR-181a binding confirmation using reporter constructs

Wild-type constructs (WT) were made by designing oligonucleotide inserts containing the miR-181a-binding regions in the 3'UTR of DCN, and ligating these inserts downstream to a luciferase reporter gene in the pmir-GLO Dual-Luciferase microRNA Target Expression Vector (Promega, USA). To ensure site-specific binding, mutant constructs (MUT) were also prepared, where the ligated oligonucleotide inserts did not contain the

binding region nucleotides. Table 3 shows the oligonucleotide insert sequences that were designed and ligated to form the wild-type and mutant constructs for each of the two miR-181a/DCN 3'UTR binding regions. HuH-7 cells were transfected with 2 µg of either the WT or MUT construct using SuperFect transfection reagent (Qiagen). After 24 h, cells were co-transfected in a 96-well plate with miR-181a mimics (2 µL; ~22.4 ng) using HiPerFect transfection reagent (0.75 µL; Qiagen) and 25 µL serum-free DMEM. Relative luciferase activity was measured after 48 h using Steady-Glo® Luciferase Reporter Assay Kit (Promega, USA).

#### Analysis of downstream oncogenes and tumor suppressor transcription factors

Using SuperFect transfection reagent, HuH-7 cells were transfected with four different vectors containing cis-acting enhancer response elements specific for the cell cycle regulatory proteins, p53, Rb, Myc, and E2F, upstream of a sensitive luciferase reporter gene (pp53-TA-Luc, pRb-TA-Luc, pMyc-TA-Luc, and pE2F-TA-Luc, respectively; Clontech, Germany). This setup allows for the measurement of transcriptional activity driven by these specific regulatory elements. As a control, pLuc vector containing an unspecific binding site (pTAL-Luc; Clontech, Germany) was used. After 24 h, cells were co-transfected with siRNAs against DCN or with miR-181a mimics. Upon binding of the respective protein to its response element, a downstream luciferase gene is expressed and assayed 48 h later using Steady-Glo® Luciferase Assay Kit (Promega, Germany), as per the manufacturer's instructions. Luminescence was used to calculate the transcriptional activity of each regulatory protein and plotted as percentage transcriptional activity relative to cells transfected with the vector alone. Unspecific luminescence detected by the reagents and empty pTAL-Luc vector (baseline luminescence) was subtracted from all the values before plotting.

#### Statistical analyses

Gene expression is expressed in relative quantitation ( $RQ=2^{-\Delta\Delta Ct}$ ). All data are expressed as mean ± standard

error of the mean (SEM), unless otherwise stated. Unpaired Student's *t* test (parametric and two-tailed) was used for all the experiments to compare between two groups. All experiments were performed in quadruplicates ( $n=4$ ) and repeated at least three times, unless otherwise stated. A *p*-value less than 0.05 was considered as statistically significant, where \*\*\* =  $p<0.001$ , \*\* =  $p<0.01$ , \* =  $p<0.05$  and ns=statistically not significant. All the data were statistically analyzed using GraphPad Prism 5.00 software.

## Results

### Expression profiling of DCN and miR-181a in liver tissues and HuH-7 cells

The small leucine-rich pericellular proteoglycan, DCN, an important component of the hepatic microenvironment, acts as a tumor suppressor in a variety of cancers, including HCC [9, 10]. To establish its expression levels in a cohort of Egyptian HCV-infected HCC patients, DCN mRNA was screened in both tumorous and adjacent cirrhotic liver tissues from HCC patients undergoing liver transplantation and compared to normal liver tissues from healthy liver donors. DCN was significantly under-expressed in both cirrhotic ( $p=0.0016$ ) and HCC tissues ( $p=0.0059$ ) when compared to those taken from healthy livers. Similarly, HCC cell line HuH-7 showed significantly lower expression levels of DCN compared to healthy liver tissues ( $p=0.0016$ , Fig. 1a). Next, it was crucial to screen for the expression levels of miR-181a in the HCC and healthy liver tissues. miR-181a levels were significantly overexpressed in the HCC tissues when compared to those in the healthy liver tissues ( $p=0.0244$ , Supplementary Figure S1).

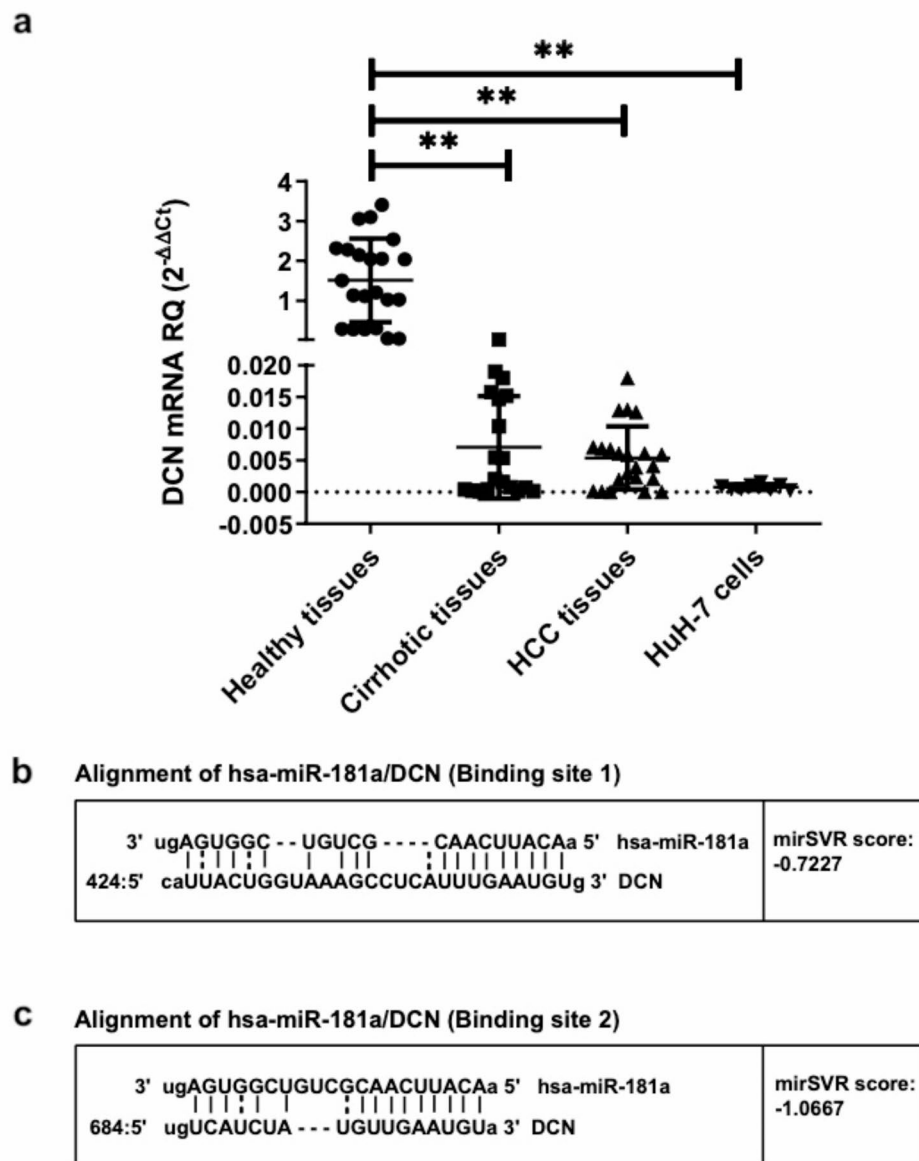
### Bioinformatics to predict an upstream microregulator of DCN

Since DCN was found to be severely suppressed in cancerous liver tissues and given that miRNAs are predicted to regulate approximately 30% of all protein-coding genes, bioinformatics was performed to identify a miRNA with high potential to be targeting and downregulating this proteoglycan. Using two different computational

**Table 3** Oligonucleotide design for hsa-miR-181a/DCN 3'UTR inserts (wild-type and mutant)

Insert name	Oligonucleotide sequence*
Binding site 1 (wild-type)	Sense: 5'- CCATTACTGGTAAAGCCTCAT <b><u>TTGAATG</u></b> TGTGAATTT-3' Antisense: 5'-CTAGAAATTCACAC <b><u>CATTCAA</u></b> ATGAGGCTTTACCAGTAATGGAGCT-3'
Binding site 1 (mutant)	Sense: 5'- CCATTACTGGTAAAGCCTCATTGTGAATTT-3' Antisense: 5'-CTAGAAATTCACAATGAGGCTTTACCAGTAATGGAGCT-3'
Binding site 2 (wild-type)	Sense: 5'- CGGTAACCTTATGTCATCT <b><u>ATGTTGA</u></b> ATGTAAGCTTAT-3' Antisense: 5'-CTAGATAAGCTTACAT <b><u>TCAACAT</u></b> AGATGACATAAGTTACCGAGCT-3'
Binding site 2 (mutant)	Sense: 5'-CGGTAACCTTATGTCATCTATGTAAGCTTAT - 3' Antisense: 5'-CTAGATAAGCTTACATAGATGACATAAGTTACCGAGCT-3'

\*the miR-181a binding regions are indicated by bold and underlined font

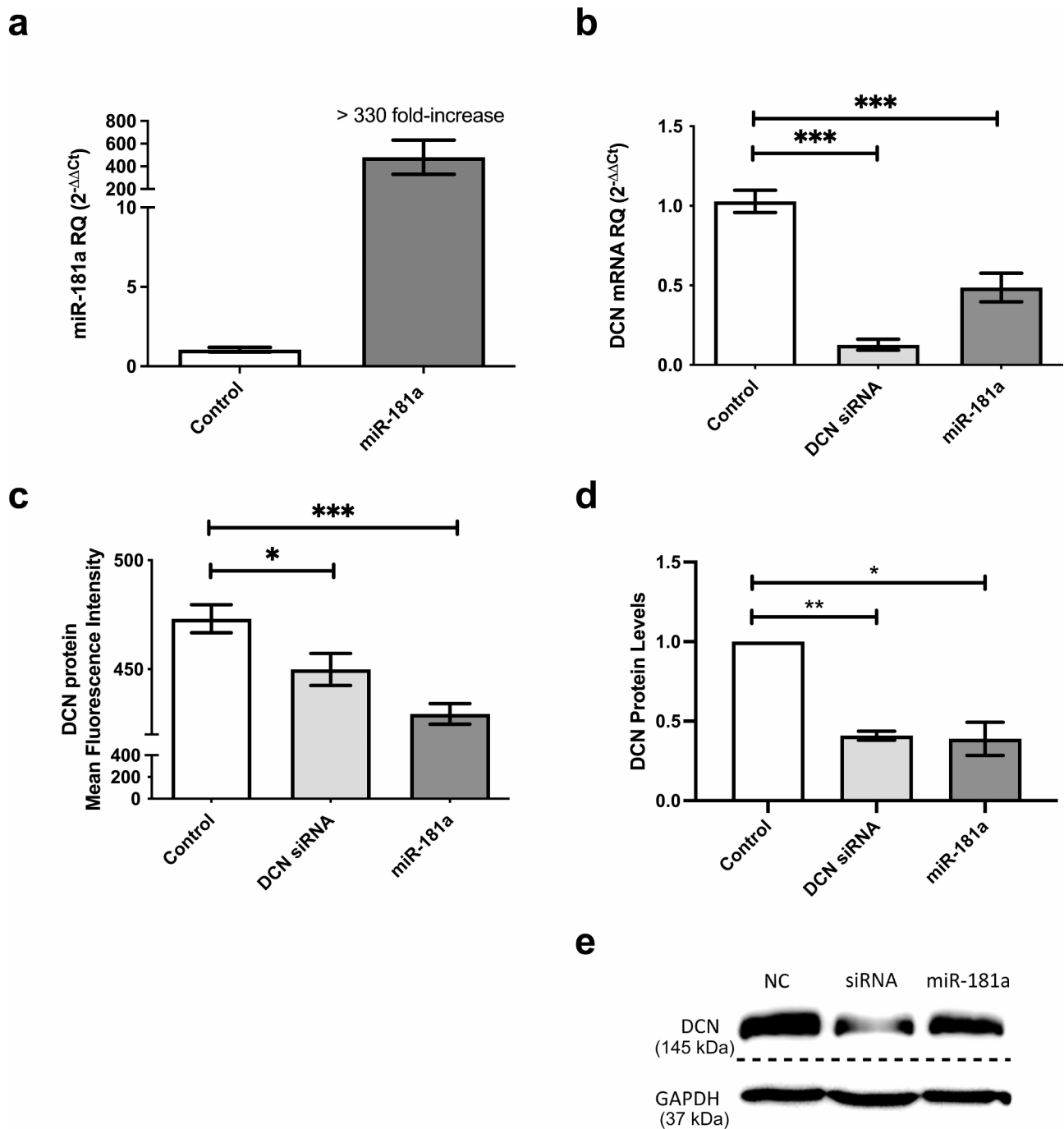


**Fig. 1** Expression profiling of DCN in liver tissues and predicted targeting of DCN 3'UTR by miR-181a. **(a)** The expression of DCN was investigated in healthy, cirrhotic, and HCC liver biopsies in addition to HuH-7 cells. Each tissue sample was carried out in duplicates and repeated three times. **(b, c)** Alignment of the two high-score binding sites on DCN transcript with miR-181a seed sequence, as predicted by microRNA.org. Vertical lines indicate complementarity between the binding region of the mRNA and the seed sequence of the miRNA, while the dots indicate mismatches or GU wobbles. Also shown are the mirSVR scores, which factor in multiple features of the predicted miRNA: mRNA duplex, including (1) duplex features which includes base pairing at the seed region and 3'end of the miRNA; (2) sequence features which include A/U composition near the target sites and secondary structure accessibility; and (3) global features such as length of the UTR, relative position of the target site in the UTR and conservation score. The MirSVR downregulation scores correlate linearly with the extent of downregulation. Asterisks indicates statistically significant differences, where  $**p < 0.01$

online algorithms, namely microRNA.org (<http://www.microRNA.org>) and Target Scan (<http://www.targetscan.org/>), hsa-miR-181a was predicted to target the 3'UTR of DCN at two different binding regions with perfect complementarity between the 8-nucleotide seed sequence of miR-181a and its target sites (Fig. 1b, c). Binding scores given by the software represent the hybridization energy exerted for miR-181–DCN mRNA binding, where the lower the energy, the higher the predicted binding [23].

#### Impact of miR-181a on DCN mRNA and protein expression in HuH-7 cells

To explore the effect of miR-181a on its predicted downstream target, DCN, synthetic oligonucleotide miR-181a mimics were transfected into HuH-7 cells. Ectopic delivery of mimics led to a more than 330-fold increase in intracellular levels of miR-181a in transfected HuH-7 cells ( $p=0.0096$ ), confirming transfection efficiency (Fig. 2a). Overexpression of miR-181a resulted in a



**Fig. 2** Impact of miR-181a on DCN mRNA and protein in HuH-7 cells. **(a)** HuH-7 cells were transfected with miR-181a mimics and the efficiency of miR-181a delivery was confirmed on the mRNA level using RTqPCR. **(b)** Relative expression of DCN was determined using RTqPCR in HuH-7 cells transfected with miR-181a mimics or specific siRNAs against DCN as a positive control. **(c)** Relative fluorescence intensity of DCN protein was quantified using a multilabel counter fluorometer after fluorescent labeling of transfected HuH-7 cells, and readings were plotted as % mean fluorescence intensity (MFI). The experiment was carried out in quadruplets and repeated three times. **(d)** Relative band intensity was quantified using ImageQuant software after western blotting to determine the DCN protein levels in transfected HuH-7 cells. The experiment was carried out in duplicates and repeated three times. **(e)** Representative image of the western blot results. The loading controls were run on the same gel. Then, the blot was cropped during the experiment as a different set of secondary antibodies was used for DCN and GAPDH. Thus, the figure represents two grouped blots from different parts of the same blot (the three upper bands represent the blot that was incubated with DCN antibody, while three lower bands represent the blot that was incubated with GAPDH antibody). The dashed line delineates the cropping site. The original uncropped blots of DCN and GAPDH are presented in Additional file 1 (Supplementary Figure S2). Asterisks indicate statistically significant differences, where \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

significant downregulation of DCN mRNA ( $p < 0.0001$ ), similar to the decrease caused by knockdown of DCN using specific siRNAs ( $p < 0.0001$ , Fig. 2b). Moreover, both DCN siRNAs and miR-181a mimics produced a marked reduction of DCN protein levels, measured both fluorimetrically ( $p = 0.0240$  for DCN siRNAs and  $p < 0.0001$  for miR-181a, Fig. 2c) and by western immunoblotting ( $p = 0.0023$  for DCN siRNAs and  $p = 0.0278$  for miR-181a, Fig. 2d, e). The original uncropped blots of DCN and GAPDH are presented in Additional file 1 (Supplementary Figure S2).

#### Binding confirmation of miR-181a to the 3'UTR of DCN

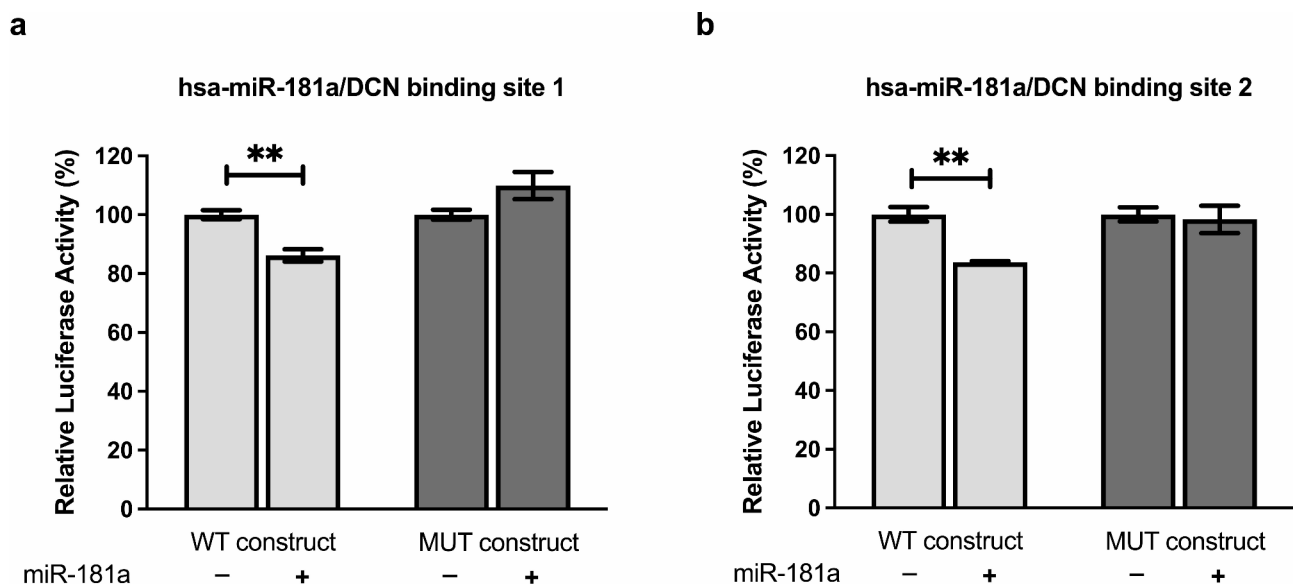
To determine whether the observed effects of miR-181a on DCN are mediated through direct targeting of the DCN 3'UTR, binding confirmation using luciferase reporter constructs was performed for each of the two predicted binding regions. For both WT binding site constructs, luciferase activity was inhibited upon co-transfection with miR-181a mimics compared to cells transfected with the WT constructs alone ( $p = 0.0060$  and  $p = 0.0029$  for binding site 1 and 2, Fig. 3a and b, respectively). Luciferase activity, however, was unaffected in cells co-transfected with miR-181a and MUT constructs compared to cells transfected with MUT construct alone, confirming the specificity of these miR-target gene interactions. These observations validate the independent and site-specific direct binding of miR-181a to two binding regions within the DCN 3'UTR (Fig. 3a, b).

#### Impact of miR-181a on viability, proliferation, and clonogenicity in HuH-7 cells

Having validated the direct targeting of DCN by miR-181a, it was important to assess how the regulation of this tumor suppressor proteoglycan would affect various characteristic properties of tumor cells. MTT, BrdU, and colony-formation assays were performed after transfection of miR-181a mimics. Ectopic expression of miR-181a into HuH-7 cells significantly increased cellular viability ( $p < 0.0001$ ), proliferation ( $p < 0.0001$ ), and clonogenicity ( $p = 0.0037$ , Fig. 4a–c). These findings were similar to those incurred by knockdown of DCN ( $p = 0.0391$ ,  $p = 0.0064$ , and  $p = 0.0395$ , for viability, proliferation, and clonogenicity, respectively) signifying the oncogenic role of miR-181a in liver cancer, which is in part mediated through targeting of DCN. Representative images for the colony forming assay are shown in Supplementary Figure S3.

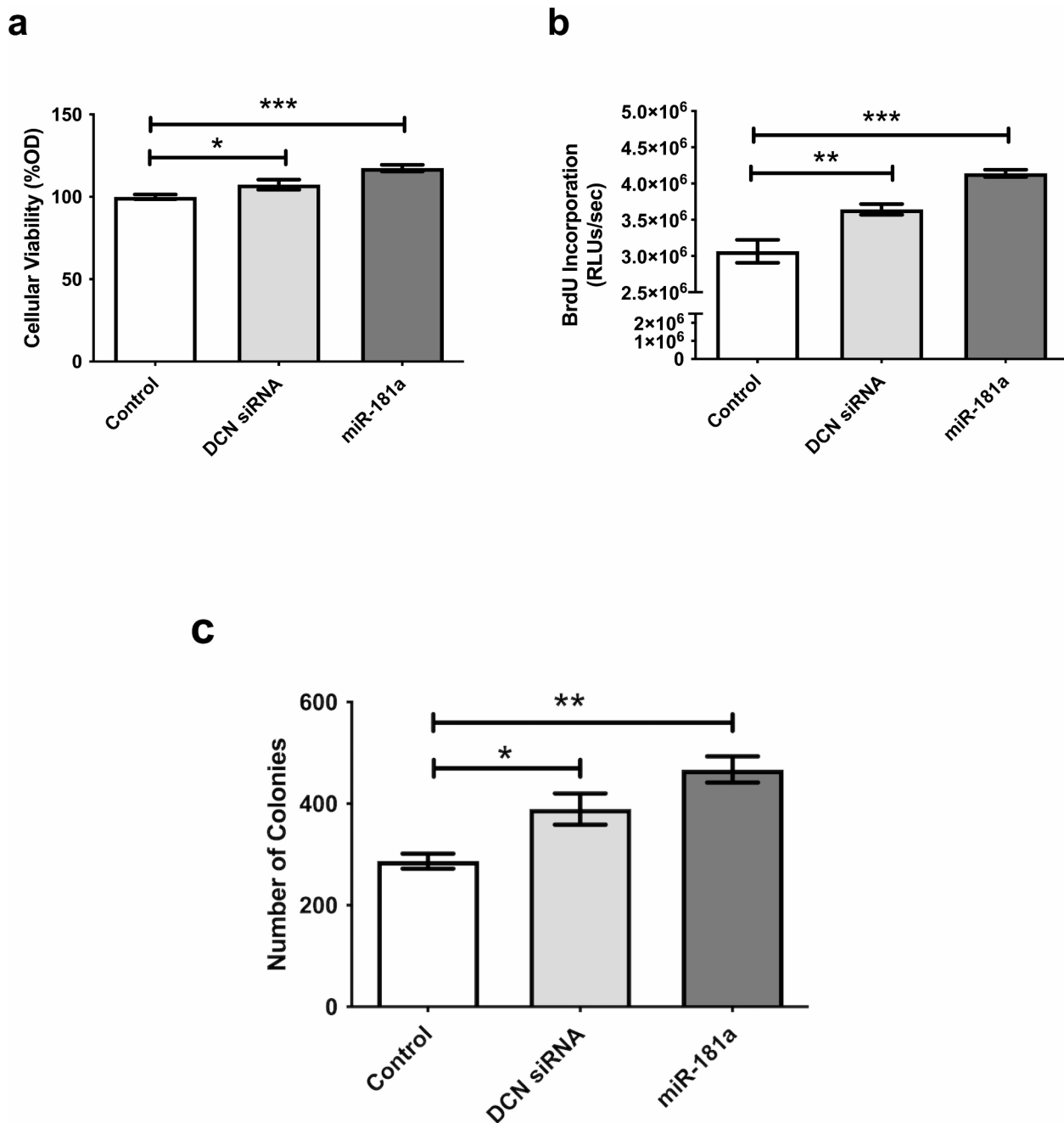
#### Effect of miR-181a on oncogenes and tumor suppressor transcription factors in HuH-7 cells

Given the role of DCN in regulating the cell cycle, it was interesting to verify the effect of its upstream microregulator, miR-181a, on the induction of key signaling pathways controlling apoptosis and proliferation. Various reporter vectors containing *cis*-acting enhancer elements specific to p53, Rb, Myc, or E2F, upstream of a sensitive luciferase reporter gene were used. These vectors allow for the measurement of transcriptional activity driven by these specific regulatory elements. After treatment of cells with miR-181a mimics, binding of these transcription factors to their enhancer elements



**Fig. 3** miR-181a targeting of DCN. Huh-7 cells were co-transfected with miR-181a mimics and either the wildtype (WT) or mutant (MUT) construct for each of the two predicted binding regions on DCN 3'UTR. Luciferase reporter assay was used to validate the independent and site-specific binding of miR-181a to (a) the first and (b) the second predicted binding regions on DCN 3'UTR. Asterisks indicates statistically significant differences, where \*\*  $p < 0.01$

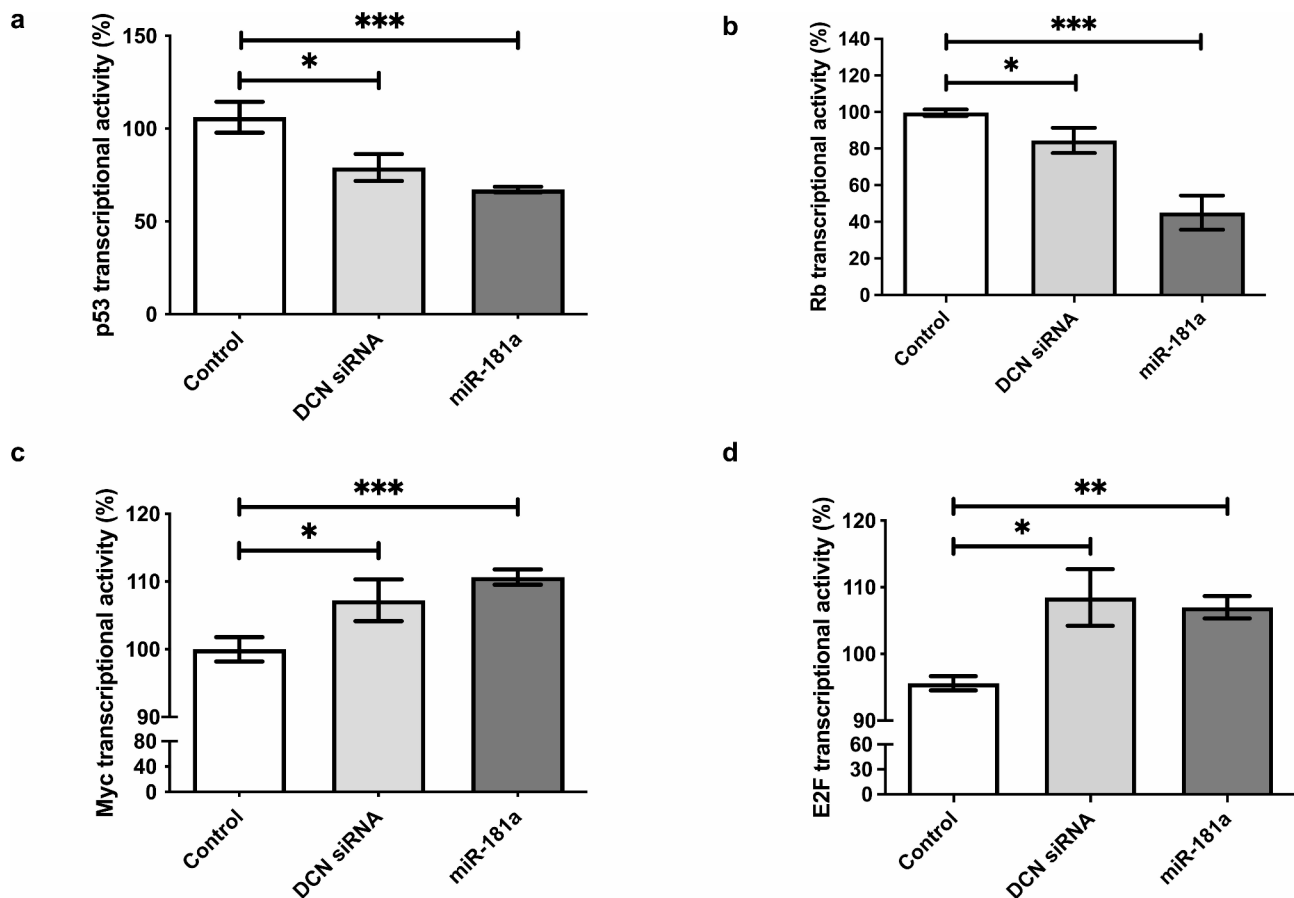




**Fig. 4** Impact of miR-181a on viability, proliferation, and clonogenicity of HuH-7 cells. HuH-7 cells were transfected with miR-181a mimics or specific siRNAs against DCN as a positive control, and **(a)** MTT assay for cellular viability, **(b)** BrdU incorporation assay for cellular proliferation, and **(c)** colony-forming assay for clonogenicity were performed. Representative images for the colony forming assay are shown in Supplementary Figure S3. Asterisks indicates statistically significant differences, where \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$

was monitored via luciferase activity to quantify the activation or repression of each signaling pathway. Transfection of miR-181a markedly repressed signaling through the tumor suppressors p53 ( $p = 0.0007$ ) and Rb ( $p < 0.0001$ , Fig. 5a and b, respectively). Moreover, miR-181a mimicking significantly activated the signaling pathways of proto-oncogenes Myc ( $p < 0.0001$ ) and E2F ( $p = 0.0018$ , Fig. 5c and d, respectively). The effects on all

four key transcription factors were paralleled in cells in which DCN was knocked down ( $p = 0.0401$ ,  $p = 0.0234$ ,  $p = 0.0476$ , and  $p = 0.0264$ , for p53, Rb, Myc, and E2F, Fig. 5a–d, respectively).



**Fig. 5** Impact of miR-181a on various oncogenes and tumor suppressor transcription factors. HuH-7 cells were transfected with reporter vectors containing *cis*-acting enhancer elements specific to cell cycle regulating transcription factors (a) p53, (b) Rb, (c) Myc, or (d) E2F, upstream of a sensitive luciferase reporter gene, and luciferase activity was used to quantify the activation or repression of each signaling pathway after knockdown of DCN or miR-181a mimicking. Asterisks indicates statistically significant differences, where \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$

## Discussion

DCN represents a potent inhibitor of tumor growth and migration by modulating the cell-signaling pathways and deposition of tumor stroma. Moreover, the lack of DCN allows for the development of tumors [24]. Being an essential macromolecule of the tumor microenvironment, DCN is downregulated in numerous cancers, including endometrial carcinoma, colorectal cancer, breast cancer, and giant cell tumor of bone [25–28]. Furthermore, DCN was reported to be the most downregulated gene during HCC progression, where its transcript expression levels showed a stepwise decline from cirrhosis to adjacent non-tumor and HCC tumor tissues [14]. Consistent with this, we also found that DCN was significantly under-expressed in liver cancer. This is supported by several other studies reporting the downregulation of DCN in HCC using various methodologies and confirmed by studies analyzing publicly available gene expression datasets for cirrhotic, HCC, and non-tumorous liver samples [13–16, 29–31]. While our data did not reveal any further reduction in DCN levels between the

adjacent cirrhotic liver tissues and tumor samples, both cirrhotic and HCC tissues had significantly lower DCN expression when compared to healthy liver tissues. It is possible, however, that expanding the sample size would unveil a similar pattern of gradual decline in DCN levels throughout the process of cirrhosis progression to HCC.

In the present study, using bioinformatics algorithms, we identified miR-181a as a promising miRNA which may be regulating DCN. Most studies indicate that miRNAs associate with specific sequences at the 3' UTR of their target mRNAs to cause either translational repression or target mRNA cleavage, where fully complimentary targets are cleaved while targets with central mismatches to the miRNA are translationally silenced [32]. Similar to the findings reported by Brockhausen et al., Chang et al., and Yang et al. [33–35], our study revealed that miR-181a is overexpressed in HCC liver tissues compared to that in healthy liver tissues. On the other hand, the study by Korhan et al. revealed that miR-181a is downregulated in HCC and suppresses motility, invasion, and branching-morphogenesis. However, that study used human liver

tissue microarray samples containing 12 normal and 19 cirrhotic liver tissues, and 48 HCC tissues to detect the relative miR-181a-5p expression levels in the respective liver tissues by *in situ* hybridization [36]. Conversely, we used primary tissues from Egyptian patient cohorts. Thus, the different experimental models used might have influenced the observed discrepancy in the miR-181a expression pattern, potentially leading to different functional outcomes.

For further confirmation, we retrieved TCGA data regarding the differential expression pattern of miR-181a and DCN in HCC and normal liver tissues. The findings revealed the overexpression of miR-181a in 370 HCC tissues compared to that in 50 normal liver tissues. They also revealed the down expression of DCN in 374 HCC tissues compared to that in 50 normal liver tissues (Supplementary Figure S4 A and B).

Next, we revealed that miR-181a decreases DCN not only at the protein level, but also on the mRNA level, indicating that the miR-181a–DCN interaction most likely results in degradation of the DCN transcript. Indeed, bioinformatics software predicted two perfectly complementary binding regions within the 3'UTR of DCN with high binding potential to miR-181a. The site-specific binding of this miRNA to both regions was independently confirmed with luciferase reporter assays. Thus, the tumor suppressor proteoglycan DCN is shown for the first time to be directly targeted by miR-181a in HCC. Despite the scarce literature about the epigenetic regulation of DCN, our findings conform with hypertrophic scar studies which showed that miR-181b, a miRNA closely related to miR-181a, directly targets DCN thereby decreasing its levels in hypertrophic scar fibroblasts [37, 38]. Interestingly, multiple previous studies have reported miR-181a to be up-regulated in cirrhotic and HCC liver tissues compared to that in normal liver tissues [35, 39–41]; this pattern of miR-181a expression is the inverse of what we have observed for its target gene, DCN, which further endorses the repressive action of miR-181a on the DCN transcript.

Next, we assessed the impact of miR-181a-mediated DCN repression on cancer progression and whether the effects would simulate those caused by silencing of DCN via siRNAs. Functionally, repressing DCN by miR-181a or siRNAs resulted in increased cell viability, proliferation, and colony-forming ability of the human HCC cell line HuH-7. These data are in agreement with previous studies that have characterized the antitumorigenic role of DCN where cellular proliferation was inhibited, while apoptosis was enhanced in a time- and dose-dependent manner *in vitro* by culturing HuH-7 cells with DCN [42]. Moreover, DCN downregulated integrin  $\beta$ 1 protein expression thereby inhibiting HCC cell invasion and migration [12]. These findings were also reflected *in vivo*

where several studies revealed that DCN gene delivery diminished tumor occurrence, while genetic ablation of DCN led to increased tumor formation [10, 29, 43]. The reported function of miR-181a in HCC has been fairly controversial. miR-181a directly inhibited Egr1, reduced Gab2 protein levels, and repressed c-Met activation, thereby hindering HCC cell proliferation and migration, and diminishing branching-morphogenesis and invasion [36, 44, 45]. Thus, miRNA may be a tumor suppressor miRNA. However, the findings reported by multiple previous studies that used *in vitro* and *in vivo* HCC models go in line with our findings, attributing tumor-promoting properties to miR-181a; liver-specific knockout of the miR-181 family in primary liver cancers in mice decreased liver tumor size and inhibited tumor progression [46]. Moreover, miR-181a mimics induced an *in vitro* EMT-like change in human hepatocyte cell lines which could be reversed by a miR-181a inhibitor [39]. Moreover, miR-181a decreased apoptosis and increased cell proliferation of HCC cells *in vitro* and enhanced tumor growth *in vivo*, through targeting various genes including Atg5, Fas, RASSF1, PTEN, CDKN1 $\beta$ , and E2F7 [35, 47–50]. Hence, from our study, the tumor suppressor proteoglycan DCN can be added to the repertoire of target genes directly regulated by miR-181a, offering another mechanism by which this miRNA mediates its oncogenic effects in HCC.

DCN is a natural pan-inhibitor of cell surface receptor tyrosine kinases (RTKs), thus affecting downstream signaling pathways which regulate the cell cycle. Remarkably, in DCN-deficient mice, tumorigenesis was reported to be associated with the downregulation of the cyclin-dependent kinase inhibitors p21WAF1 and p27Kip1 and a concurrent upregulation of beta-catenin [51]. Other studies have highlighted the impact of DCN on cell cycle proteins where, in experimental animal models of hepatocarcinogenesis with DCN deficiency, multiple RTKs were constitutively phosphorylated and activated with a simultaneous increase in phosphorylation and inactivation of the tumor suppressor protein Rb, as well as higher protein levels of the proto-oncogene c-Myc [10, 43]. On the other hand, when DCN was overexpressed in mouse models of hepatocarcinogenesis, the activity of several RTKs was suppressed while the tumor suppressor p53 function was enhanced [29, 52]. Mechanistically, in this study we provide evidence for how the oncogenic miR-181a-mediated repression of DCN may affect the regulation of key cell cycle proteins and hence cancer progression. Similar to the effect of siRNAs, repression of DCN through the ectopic delivery of miR-181a into HuH-7 cells harboring reporter vectors with response elements for various cell cycle regulating proteins resulted in distinctly repressed signaling of the tumor suppressors p53 and Rb, and markedly activated

signaling of the oncogenes Myc and E2F. Taken together, these findings suggest that DCN deficiency caused by miR-181a can be permissive for tumorigenesis.

While our expression profiling of DCN and MiR-181a was performed on human tissue samples which have precious clinical value in research, as a limitation to our study all other work was performed *in vitro*; therefore, it would be recommended to investigate the effect of the conditional knockout or knock-in of miR-181a in animal models to confirm the effects of this miRNA on its multiple downstream direct target genes including DCN, as well as oncogenes and tumor suppressor genes downstream of DCN.

## Conclusions

The findings of this study revealed that oncomiR-181a directly targets the tumor suppressor proteoglycan DCN, leading to enhancing the oncogenic properties of HCC cells. Hence, it would be of clinical significance to explore its diagnostic and therapeutic potential in liver cancer. miR-181a could be a promising biomarker for the early detection and monitoring of liver cancer progression. In addition, measuring the miR-181a levels in liver biopsies could be incorporated into existing diagnostic and surveillance strategies to assess HCC progression. Finally, potential therapeutic strategies based on targeting miR-181a could include developing miRNA inhibitors or mimics to modulate miR-181a expression and its downstream effects on tumorigenesis. This miR-181a-based therapy approach could be potentially combined with chemotherapy and immunotherapy for the management of liver cancer.

## Abbreviations

cdNA	Complementary DNA
DCN	Decorin
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
RLUs	Relative light units

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12876-024-03413-6>.

Supplementary Material 1

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None.

## Author contributions

RAA contributed to the study concept and design, execution of experiments, acquisition of data, statistical analysis, interpretation of data, and writing of the manuscript. RBEAE optimized, performed, and analyzed the western blot experiments. RAY assisted in the execution of functional analysis experiments and acquisition of their data. MMA assisted in the execution of functional analysis experiments. HZ assisted in optimization of the western blot experiments. The surgeons, KAH and GE, provided tissue samples and clinical data. KB contributed to the study concept and provided critical revision of the

manuscript for important intellectual content. NE and IOF contributed to the western blot experiments and writing of the manuscript. AIA contributed to the study concept, design, supervision, and provided critical revision of the manuscript for important intellectual content. All authors have contributed to and agreed on the content of the final version of the manuscript.

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## Data availability

The datasets generated and/or analyzed during the current study are not publicly available to protect the patients' privacy, but are available from the corresponding author on reasonable request.

## Declarations

### Ethics approval and consent to participate

The study methodologies conformed to the ethical standards set forth by the declaration of Helsinki. The ethical committees of Cairo University and the German University in Cairo approved the study methodologies. The experiments were undertaken with the understanding and written informed consent of each patient.

### Consent for publication

Not applicable.

### Competing interests

The authors declare no competing interests.

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