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ARTICLE

HBx Downregulates miR-422a Expression via Activation of FOXG1/Q1/E1 in HepG2 Cells

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ABSTRACT

microRNA-422a (miR-422a) is downregulated in many hematopoietic tumors and solid tumors including hepatocellular carcinoma. We previously demonstrated that hepatitis B virus X protein (HBx) downregulated expression of miR-422a in HCC cell line HepG2 *in vitro*. However, we explore the mechanisms underlying this action. Forkhead box proteins (FOX) G1/Q1/E1 are known to negatively regulate miR-422a expression, and this prompted us to determine whether HBx suppresses miR-422a expression via activation of FOXG1/Q1/E1. The relationship between FOXG1/Q1/E1 and miR-422a in HepG2 cells stably expressing HBx was assessed with qRT-PCR. The correlation between HBx and FOXG1/Q1/E1 was determined with qRT-PCR and western blot *in vitro*. The cell cycle and CCK-8 assays were used to elucidate the consequence of miR-422a transfection in HepG2-hbx cells. FOXG1/Q1/E1 activated by HBx was found to be responsible, at least in part, for the downregulation of miR-422a in HepG2 cells. miR-422a transfection hampered the growth of HepG2-hbx cells by arresting cells in G1 phase. Both FOXG1/Q1/E1 and miR-422a may be suitable molecular targets for treatment of HBV-infected HCC.

KEYWORDS

HBx; hepatocellular carcinoma; FOXG1/Q1/E1; miR-422a

1 Introduction

miRNAs play important roles in the regulation of gene expression post-transcriptionally and are extensively involved in tumorigenesis [1]. Deregulation of miRNAs has been observed in multiple human diseases including hepatocarcinogenesis. miRNAs can be tumor suppressors or tumor promoters according to their expression and functions in specific tumors [2]. miR-422a downregulation can be observed in many hematopoietic tumors and solid tumors including hepatocellular carcinoma [3,4], leading to the activation of such oncogenes as *MAPK1*, *PIK3CA*, *CD73*, *IGF1/IGF1R*, and *FOXG1/Q1/E1* [4–9].

miRNA dysregulation in tumors can be attributed to genomic variations, epigenetic modification, and chaos in biogenesis process. In addition to known regulators, miRNAs are also regulated by protein-coding

genes, especially those encoding transcription factors (TFs). p53 promotes the maturation of miR-16-1, miR-143, and miR-145, which are tumor suppressors, in response to DNA damage [10]. c-Myc has been found to transcriptionally upregulate the onco-cluster of miR-17-92, while such tumor suppressors as let-7, miR-34a, and miR-16 were suppressed [11]. miR-422a silences the expression of FOXG1/Q1/E1, which is its own upstream regulator [4].

Because it is an onco-protein encoded by hepatitis B virus (HBV), hepatitis B virus X protein (HBx) is extensively involved in the initiation and progression of hepatocarcinogenesis [12]. HBx deregulates gene expression of hepatocytes by activating cell signaling pathways in cytoplasm and by binding TFs in nucleus, thereby contributing to malignant transition in hepatocytes [13]. Our team, for the first time, reported that HBx downregulated miR-16 family in HepG2 cells via activation of c-Myc, expanding its regulation of gene expression from protein-coding to non-coding genes [14]. Other teams have reported that the HBx transcript itself can directly mediate miR-15a/miR-16-1 repression in hepatocytes [15]. In addition to binding protein, HBx can also bind the DLEU2 lncRNA to activate gene expression in hepatocytes [16].

In a previous work, based on the pleiotropic functions of HBx, we explored their effects on the miRNA expression of hepatocytes. As in another report [17], miR-422a was downregulated by HBx in HepG2 cells in a microarray [14]. However, the mechanisms underlying the repression of miR-422a by HBx were not clearly established. In this research, we further confirmed that miR-422a was repressed by HBx transfection in HepG2 cells using qRT-PCR. As expected, FOXG1, Q1, and E1 were activated by HBx *in vitro*. Accordingly, si-FOXG1/Q1/E1 transfection hampered the HBx-induced miR-422a repression. Inversely, miR-422a transfection downregulated the expression of FOXG1/Q1/E1 in HepG2-hbx cells. Finally, miR-422a mimic transfection hampered the proliferation of HepG2-hbx cells by arresting cells in the G1 phase. In brief, we observed a preliminary HBx-FOXG1/Q1/E1-miR-422a pathway in HepG2-hbx cells. miR-422a may be an original molecular targeted agent suitable for treating HBV^+ HCC via silencing FOXG1/Q1/E1.

2 Materials and Methods

2.1 Cell Culture and Transfection

Untransfected HepG2 cells and HepG2 cells stably transfected with HBx expressing plasmid (HepG2-hbx) or empty vector (HepG2-vc) were constructed and cultured as described by our team [14]. The RNA nucleotides were transfected using Lipofectamine 3000 (Invitrogen). Except where otherwise specified, 50 nM of RNA nucleotides were transiently transfected in all experiments.

2.2 RNA Oligoribonucleotides

FOXG1/Q1/E1-specific siRNAs, negative control (NC), miR-422a mimic, and miRNA NC were purchased from Genepharma, Shanghai, China. The sequences of siRNAs and primers for PCR are provided in Tab. S1.

2.3 qRT-PCR and Western Blot

RNA was extracted using TRIzol reagent (Invitrogen). cDNA was synthesized with a PrimeScript™ RT Reagent Kit (Takara Biomedical Technology, Beijing, China). qRT-PCR for FOXG1/Q1/E1 and GAPDH were performed with TB Green® Premix Ex Taq™ II (Takara Biomedical Technology). The detection kits for miR-422a and U6 were provided by Genepharma; total cellular protein was extracted with RIPA buffer. The antibodies used in western blot were for FOXG1/Q1/E1 (ab18259, ab51340, ab236661; Abcam), HBx (sc-71239; Santa Cruz) and beta-actin (A1978; Sigma-Aldrich).

2.4 Cell Proliferation and Cell Cycle Assays

The growth of cells was detected with CCK-8 kit; the distribution of DNA content was determined using a commercial kit (KGA511, KeyGEN BioTECH, Nanjing, China).

2.5 Statistical Analysis

All data analysis was performed with SPSS 19.0, and $p < 0.05$ was set as significance level. The qRT-PCR results were analyzed using a Student's *t*-test.

3 Results

3.1 miR-422a was Suppressed by HBx in HepG2 Cells

To confirm previous results that had been found using a microarray method [14], we evaluated the significant miR-422a downregulation in HepG2 cells with stable and transient HBx transfection using qRT-PCR (Figs. 1A–1B). The western-blot results confirmed the HBx protein expression in HepG2 cells (Fig. 1C).

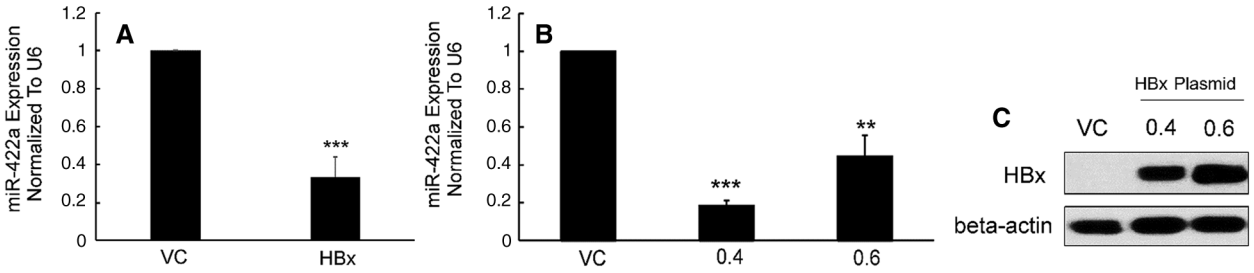

Figure 1: Stable and transient HBx transfection downregulated miR-422a expression in HepG2 cells. A) miR-422a expression was assessed in HepG2-hbx and HepG2-vc cells. B) HepG2 cells were transiently transfected with HBx plasmid (0.4 or 0.6 μg) or control. miR-422a expression was normalized to U6. C) Western-blot results confirmed the HBx protein expression in HepG2 cells after the transient transfection with HBx expressing plasmid

3.2 FOXG1/Q1/E1 was Upregulated in HepG2-hbx Cells

To clearly establish the mechanisms by which HBx downregulates miR-422a in HepG2 cells, we began by quoting a report that described a double-negative feedback loop between miR-422a and FOXG1/Q1/E1 in HCC [4]. We hypothesized that FOXG1, Q1, and E1 were activated by HBx and responsible for the miR-422a downregulation in HepG2 cells. As anticipated, qRT-PCR and western blot results confirmed the significant upregulation of FOXG1, Q1, and E1 in HepG2-hbx cells (Figs. 2A–2D). Moreover, the efficiency of si-FOXG1/Q1/E1 was also evaluated (Figs. 2A–2C). Collectively, our results, for the first time, showed that HBx could induce FOXG1/Q1/E1 activation in HepG2 cells *in vitro*.

3.3 miR-422a and FOXG1/Q1/E1 Showed Antagonistic Effects against Each Other in HepG2-hbx Cells

In a previous work, we showed the existence of an HBx-Lin28B-let-7 pathway in HepG2 cells [18]. We hypothesized that there was also an HBx-FOXG1/Q1/E1-miR-422a pathway in HepG2 cells. We confirmed that loss-of-FOXG1/Q1/E1 function using specific siRNAs significantly reactivated the expression of miR-422a in HepG2-hbx cells (Fig. 3D). Correspondingly, we verified that ectopically expressed miR-422a mimics could suppress the protein expression of FOXG1/Q1/E1 in HepG2-hbx cells (Figs. 3A–3C, three duplicates). In conclusion, we suggest that there may be not only a feed-forward HBx-FOXG1/Q1/E1-miR-422a pathway but also negative feedback between miR-422a and FOXG1/Q1/E1 (Fig. 4).

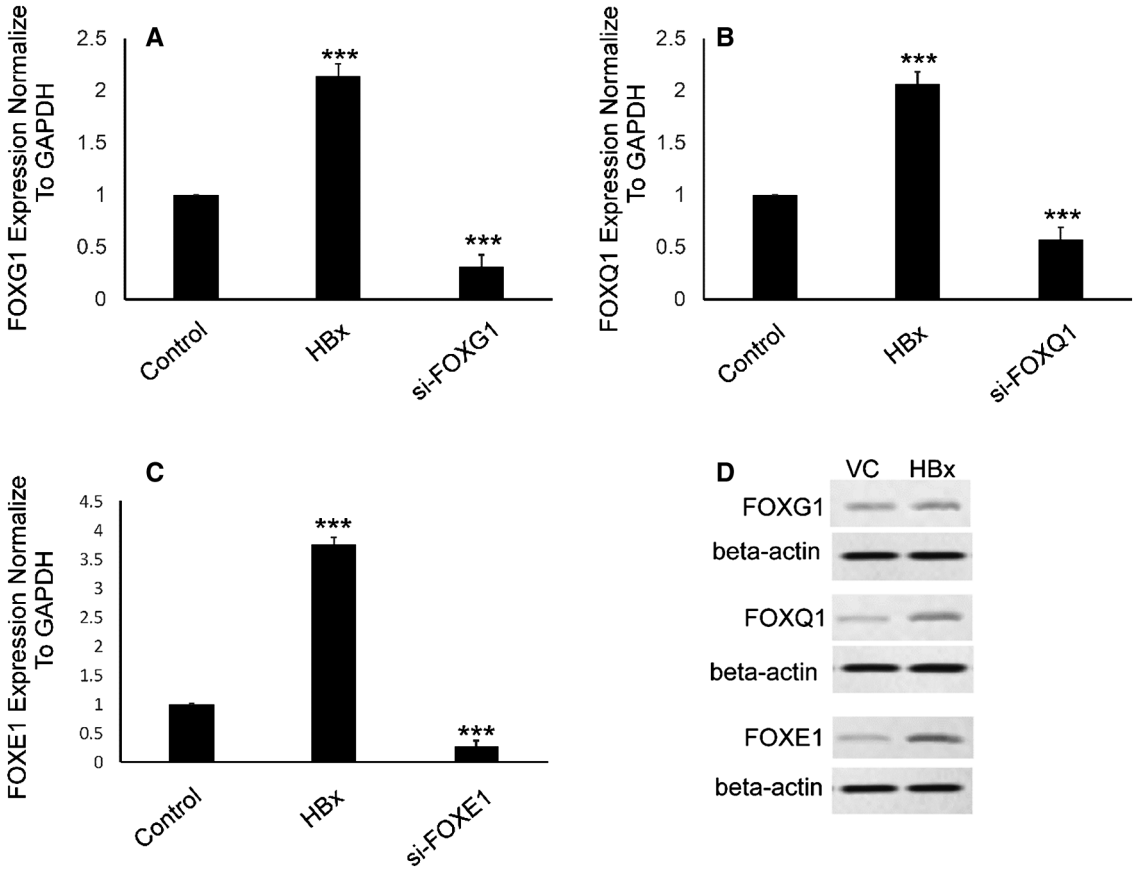

Figure 2: FOXG1, Q1, and E1 were upregulated in HepG2-hbx cells. A–C) qRT-PCR confirmed the elevation of FOXG1, Q1, and E1 mRNA in HepG2-hbx cells, while the FOXG1, Q1, and E1-specific siRNA efficiently silenced their expression. Expression of each was normalized to GAPDH. D) Western blot results validated the upregulation of FOXG1, Q1, and E1 protein in HepG2-hbx cells. Representative pictures are presented and beta-actin was used as reference gene

3.4 miR-422a Hampered the Proliferation of HepG2-hbx Cells by Arresting Cells in G1 Phase

To clearly establish the significance of the FOXG1/Q1/E1-mediated miR-422a downregulation in HCC, we sought to determine the consequence of restoring miR-422a in HepG2-hbx cells *in vitro*. CCK-8 analysis showed that, compared with NC-transfected group, ectopically expressed miR-422a significantly hampered HepG2-hbx cells growth at 48 h and 72 h after transfection (Fig. 5A). Cell cycle assay confirmed that miR-422a arrested HepG2-hbx cells in the G1 phase (Fig. 5B).

4 Discussion

HBx participates in the initiation and progression of HCC by deregulating coding genes of host hepatocytes. Increasing evidence confirmed that HBx can also regulate the non-coding genes including miRNA, lncRNA, and circRNA. We previously confirmed that HBx induced extensive miRNA downregulation in HepG2 cells including miR-422a with a microarray method [14], which contributed to carcinogenesis by re-activating multiple oncogenes including MAPK1, PIK3CA, CD73, IGF1/IGF1R, and FOXG1/Q1/E1 [4–9]. FOXG1/Q1/E1 can also transcriptionally suppress miR-422a expression in HCC [4]. miR-422a is also the target of long non-coding RNAs, such as OIP5-AS1, NT5E, DUXAP8, LINC00313, D63785 LINC01133, and LINC00858 [3,19–25]. However, the mechanisms underlying

miR-422a repression by HBx in HepG2 cells were not clearly established [14,17]. We confirmed the miR-422a downregulation in HepG2-hbx cells with qRT-PCR and found a tentative HBx-FOXG1/Q1/E1-miR-422a pathway in HepG2 cells *in vitro*.

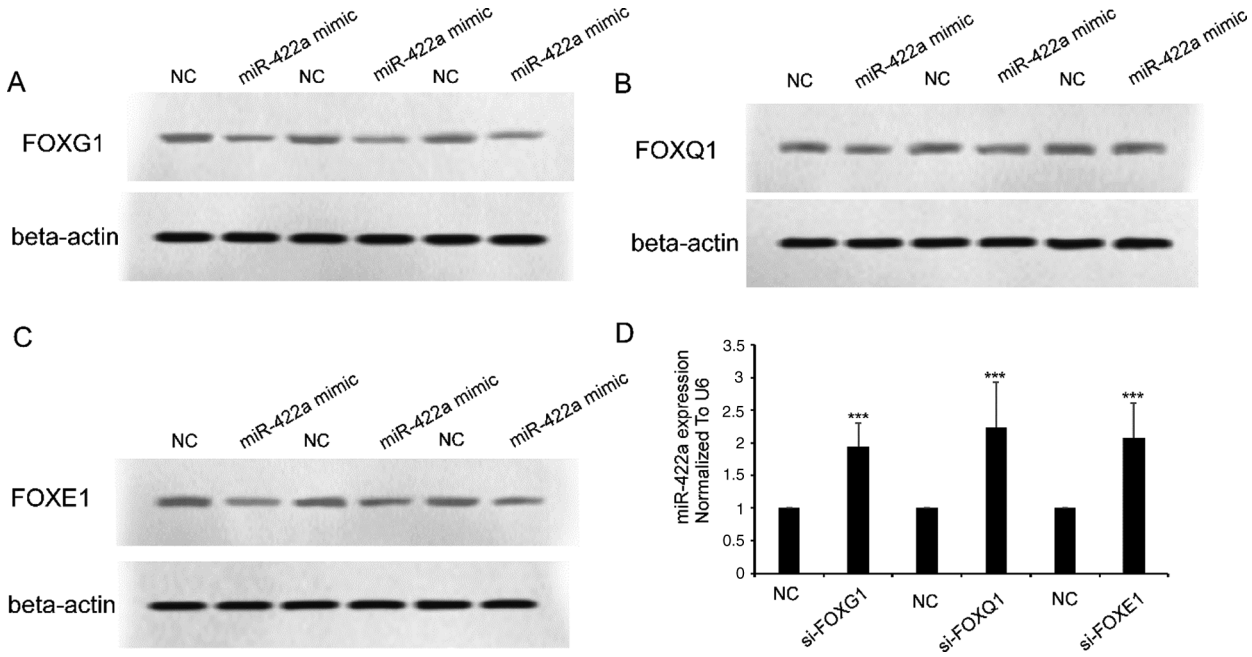

Figure 3: miR-422a and FOXG1/Q1/E1 antagonized against each other in HepG2-hbx cells. A–C) Western blot results showed that miR-422a transfection suppressed FOXG1/Q1/E1 expression in HepG2-hbx cells. D) miR-422a expression was rescued by FOXG1/Q1/E1-specific siRNA transfection in HepG2-hbx cells

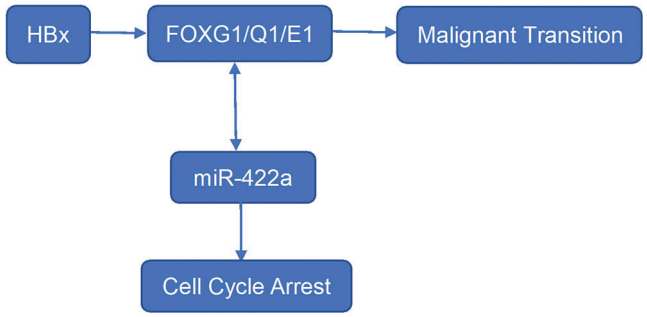

Figure 4: Preliminary HBx-FOXG1/Q1/E1-miR-422a pathway

FOX proteins are a superfamily of evolutionarily conserved transcriptional regulators, a loss or gain of FOX function can alter cell fate and promote tumorigenesis as well as cancer progression [26]. Several key members of the FOXA, FOXC, FOXM, FOXO, and FOXP subfamilies are heavily implicated in cancer, driving initiation, maintenance, progression, and drug resistance [27]. FOXM1, FOXC1, and FOXG1/Q1/E1 are involved in the initiation and progression of HCC [4,28–32]. For the first time, we found that HBx transfection activated FOXG1, Q1, and E1 expression in HepG2-hbx cells. FOXG1/Q1/E1 and miR-422a antagonize each other, striking a balance under normal conditions. Imbalance between FOXG1/Q1/E1/miR-422a can promote hepatocarcinogenesis [4]. Recently, we also identified a similar mechanism that

HBx downregulated let-7 via activation c-Myc-Lin28B pathway [18]. Our results again show the significance of the HBx-TFs-miRNA mechanism in transformation of HCC cells *in vitro*.

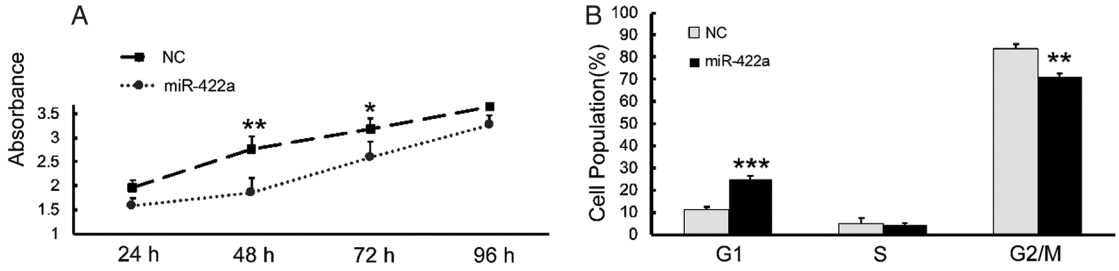

Figure 5: miR-422a hampered the proliferation of HepG2-hbx cells by arresting cells in G1 phase. A) The ectopic expression of miR-422a significantly hampered the proliferation of HepG2-hbx cells at 48 and 72 h. B) Cell cycle analysis indicated that the HepG2-hbx cells were arrested at G1 phase by miR-422a mimic

5 Conclusion

Our results elucidated the critical roles played by FOXG1/Q1/E1 in HBx-induced miR-422a downregulation. Targeting miR-422a/FOXG1/Q1/E1 loop might be a prospective treatment for HBV+ HCC patients.

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Data Availability: The data are available upon special request to the corresponding authors.

Author Contributions: Xiaofan Deng, Yamei Yang, and Xianfeng Gan performed the experiments and constructed the figures. Gang Wu designed the experiments and composed the manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Table S1: Sequences of RNA and DNA Oligonucleotides

Name	Sense Strand (5'-3')	Antisense Strand (5'-3')
SiRNA Duplxes		
si-FOXG1	GACCCUCUUUGCCAAGUUUTT	AAACUUGGCAAAGAGGGUCTT
si-FOXQ1	UCUGAGAACGAACAGGAAUTT	AUUCCUGUUCGUUCUCAGATT
si-FOXE1	UCCAGAGGAAGAUGAAUUUTT	AAAUUCAUCUUCCUCUGGATT
NC	UCACAACCUCCUAGAAAGAGUAGA	UACUCUUUCUAGGAGGUUGUUAUU
Name	Sense Primer (5'-3')	Antisense Primer (5'-3')
Primers for qRT-PCR		
FOXQ1	ctggcggagatcaacgagtacctcat	cgcagcaccttgacgaagcagt
FOXG1	ggctcacgctcaacggcatctacga	gcggcaccttcacgaagcacttgtt
FOXE1	ggaggtgctggctaccgtgaaggaa	gtgaggttgtggcggatgctgttctg
GAPDH	ccaaggtcatccatgacaac	tgtcataccagggtgagc