Biochemical and Biophysical Research Communications 375 (2008) 315–320

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

miR-122 targets an anti-apoptotic gene, *Bcl-w*, in human hepatocellular carcinoma cell lines

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ARTICLE INFO

Article history:
Received 21 July 2008
Available online 8 August 2008

Keywords:
miR-122
Bcl-w
Hepatocellular carcinoma

ABSTRACT

miR-122, a hepato-specific microRNA (miRNA), is frequently down-regulated in human hepatocellular carcinoma (HCC). In an effort to identify novel *miR-122* targets, we performed an *in silico* analysis and detected a putative binding site in the 3′-untranslated region (3′-UTR) of *Bcl-w*, an anti-apoptotic Bcl-2 family member. In the HCC-derived cell lines, Hep3B and HepG2, we confirmed that *miR-122* modulates *Bcl-w* expression by directly targeting binding site within the 3′-UTR. The cellular mRNA and protein levels of *Bcl-w* were repressed by elevated levels of *miR-122*, which subsequently led to reduction of cell viability and activation of caspase-3. Thus, *Bcl-w* is a direct target of *miR-122* that functions as an endogenous apoptosis regulator in these HCC-derived cell lines.

Hepatocellular carcinoma (HCC) is among the top 10 most prevalent cancers worldwide [1] and accounts for 80–90% of liver cancers [2]. Like other cancers, aberrant gene regulation features significantly in HCC. Using microarray analysis, several reports that profile the gene expression of HCC patients have identified numerous pathways (e.g., proliferation, cell cycle regulation, apoptosis, angiogenesis) that may be dysregulated during hepatocarcinogenesis [3]. In particular, the deregulated expression of proteins involved in cell cycle regulation, DNA repair [4], and apoptosis regulation [5] has been extensively described as a crucial event in the carcinogenetic process that leads to HCC development. Recently, a new class of small noncoding RNAs (miRNA) has been discovered [6] and implicated as playing a key role in development as well as in carcinogenesis [7]. By binding to the complementary sequences of their target mRNAs (mostly in the 3′-UTR), miRNAs are able to induce mRNA degradation or translational repression [8]. Dysregulation by miRNAs may affect previously known oncogenes or tumor-suppressor genes, thereby having implications on carcinogenesis.

miR-122 is a liver-specific miRNA that is expressed in the developing liver and at high levels in the adult liver, where it makes up 70% of all miRNAs [9,10]. The most well-known function of *miR-122* in the mammalian liver is to regulate lipid and cholesterol metabolism [11]. *miR-122* down-regulation has been reported in rodent and human HCCs [12,13], suggesting that its function is associated with hepatocarcinogenesis. In HCC-derived cell lines, miR-122 directly targets cyclin G1 (*CCNG1*) by binding its 3′-UTR. An inverse correlation between *miR-122* and *CCNG1* exists in primary liver carcinoma, further emphasizing the importance of *miR-122* in HCC pathogenesis [13]. In vertebrates, each miRNA has been predicted to target ~200 transcripts [14]. A search for other *miR-122* regulatory targets that may be involved in the progression of HCC using online prediction algorithms has identified *Bcl-w*, which harbors a putative *miR-122* binding site in its 3′-UTR. Bcl-w is an anti-apoptotic Bcl-2 family member [15]. Although mutations in anti-apoptotic Bcl-2 family genes (*Bcl-xL*, *Mcl-1*, *Bcl-w*, and *a1*) have not been identified as a cause of tumors, high expression levels of these proteins can contribute to carcinogenesis in cooperation with other proto-oncogenes [16]. The expression of Bcl-w has been detected at relatively high levels in certain epithelium-derived tumor cell lines, such as colonic, cervical, and breast cancer cells [17]. In gastric adenocarcinomas, Bcl-w suppresses cancer cell death by blocking SAPK/JNK activation [18] and by promoting cell invasion by inducing metalloproteinase-2 (MMP-2) expression [19]. *Bcl-w* expression was modulated by Met/HGF receptor (*c-met*) in human colorectal cancers, and it inhibits apoptosis [20]. Moreover, *Bcl-w* was up-regulated in autoimmune hepatitis (AIH)-associated cirrhosis [21] and may play a role in hepatocarinogenesis.

In this study, we demonstrate that the expression level of cellular *miR-122* can be elevated or inhibited by RNA polymerase II-based miRNA-like siRNA expression vectors or a synthetic miRNA

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doi:10.1016/j.bbrc.2008.07.154

duplex, and subsequent experiments using this system provide evidence that *miR-122* can directly repress the Bcl-w protein level by targeting binding sites in the 3′-UTR. Down-regulation of Bcl-w by *miR-122* results in a decrease in the Bcl-w/Bax ratio, ultimately leading to apoptosis in HCC-derived cell lines.

Materials and methods

Construction of miR-122 expression plasmids. Paired oligonucleotides including cohesive ends and a specific sequence for the sense and anti-sense *miR-122* strands were annealed and cloned into the corresponding ends created by BsmBI digestion in the pSM-155 vector (pSM-vector), a kind gift from Dr. Guangwei Du (Stony Brook University, NY, USA) [22]. This cloning step generates vectors that express sense (pSM-122_S) and anti-sense (pSM-122_AS) *miR-122*. The oligonucleotides are listed in Supplementary Table S1.

Cell culture and transfection. Hep3B, HepG2 and HeLa cells (ATCC Number: HB-8064, HB-8065, and CCL-2, respectively) were cultured in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum and penicillin/streptomycin (Invitrogen). *miR-122* duplex RNA (miR-122_D) and negative control RNA (miR-NC) were purchased from Integrated DNA Technologies (IDT). siRNAs that target *Bcl-w* (Bcl-w_siR; GenBank Accession No. NM_004050, region 3301–3321, 5′-CTCGGTCCTGCGATTATTAAT), and Bcl-xL (Bcl-xL_siR; GenBank Accession No. NM_001191, region 642–662, 5′-GGCAGGCGACGAGTTTGAACT) were synthesized by Sigma according to a published report [23]. The day before transfection, cells were seeded in antibiotic-free medium. Plasmids and RNA duplexes transfection were carried out using Lipofectamine 2000 in accordance with the manufacturer's guidelines (Invitrogen). EGFP expression from pSM-122_S and pSM-122_AS was monitored 24 h post-transfection using an Olympus IX70 fluorescent microscope equipped with a BP450-480 pass excitation filter and a BA515 barrier emission filter. Photographs were taken with a CCD camera (Diagnostics Instruments) mounted to the microscope and processed using Spot software (Diagnostics Instruments).

miR-122 target prediction. Computer-based programs were used to predict potential *miR-122* targets. Using "has-miR-122" as a search term, we queried *PicTar* [14] (http://pictar.bio.nyu.edu/) and *TargetScan 4.2* [24] (http://www.targetscan.org/). An *miR-122*/*Bcl-w* 3′-UTR duplex was predicted by RNAhybrid [25] (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/). Prediction algorithms and known miRNAs change over time, and the analysis included here is from May 2008.

Real-time quantitative RT-PCR. The expression level of *miR-122* was measured in cells transfected with pSM-122_S, pSM-122_AS, pSM-Vector, miR-122_D, or miR-NC using the NCode™ miRNA First-Strand cDNA Synthesis Kit and NCode™ SYBR® Green miRNA qRT-PCR Kit (Invitrogen) as described previously [26]. The level of *U6* RNA was measured and used to normalize the relative abundance of *miR-122*.

The expression levels of *CCNG1*, *Bcl-w*, and *Bcl-xL* were measured in cells 24 h post-transfection using High Capacity cDNA Reverse Transcription kit and Power SYBR Green PCR master Mix (Applied Biosystems) as described previously [27]. The primers used are listed in Supplementary Table S1.

Construction of 3′-UTR reporter plasmids and luciferase assays. The 3′-UTRs of *CCNG1* and *Bcl-w* were cloned downstream of the *Renilla* luciferase gene (*Xho*I/*Not*I sites) in the psiCheck-2 plasmid (Promega) and designated as psi-CCNG1 and psi-Bcl-w, respectively. (Primers are listed in Supplementary Table S1.) Hep3B cells were co-transfected with either *miR-122* (pSM-122_S or miR-122_D), anti-sense *miR-122* (pSM-122_AS) or negative controls (pSM-Vector or miR-NC) and target reporter plasmid using Lipofectamine 2000 (Invitrogen). The transfections and luciferase activity measurements were performed according to the manufacturer's instructions in the Invitrogen Lipofectamine 2000/Promega Dual-luciferase kit. Relative protein levels were expressed as *Renilla*/firefly luciferase ratios.

Western blots. Hep3B and HepG2 cells were transfected in six-well plates with the indicated concentration of miR-NC, miR-122_D, Bcl-w_siR, or Bcl-xL_siR. After transfection, cells were cultured for 96 h. Intermediate samples at 48 and 72 h were collected and analyzed by western blot to assess Bcl-w, Bax, and Bcl-xL expression as described [23]. Band signals were acquired in the linear range of the scanner using densitometric software (Quantity One, Bio-Rad). The ratio between the Bcl-w and the corresponding β-actin bands was used to quantitate Bcl-w modulation by *miR-122*.

Cell viability and apoptosis assays. For cell viability assays, the cells were transfected with different quantities of miR-122_D as indicated. Seventy-two hours post-transfection, cell viability was measured by a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases (cell proliferation reagent WST-1; Roche). To detect apoptosis, caspase-3 activity was assayed using the EnzChek Caspase-3 Assay Kit #2 (Molecular Probes) according to the manufacturer's instructions. Measured fluorescence levels were normalized to the fluorescence levels of non-treated cell lysates.

Statistical analysis. Data are represented as means ± SD of three independent experiments, each performed in triplicate. Statistical significance between treatment and control groups was analyzed using the Student's *t* test ($P < 0.05$ was regarded as significant and indicated with *).

Results

Expression of miR-122 in cells transfected with miR-122-expressing vectors and synthetic miR-122 duplex

In order to study the influence of *miR-122* on the expression of putative targets in HCC-derived cell lines, we changed the functional level of *miR-122* in Hep3B and HepG2 cells as well as an unrelated HeLa cell line. To induce *miR-122* expression in these cells, we first constructed sense and anti-sense *miR-122* sequences into an miRNA-like siRNA vector [22]. This cloning step generated pSM-122_S and pSM-122_AS, vectors that express mature and anti-sense sequences of *miR-122*, respectively. After transfecting pSM-122_S and pSM-122_AS, the expression of a co-expressed fluorescent marker was monitored (Fig. 1A; only Hep3B was shown). Total RNA was reverse transcribed, and the relative amount of *miR-122* RNA was measured by real-time qRT-PCR analysis. As expected, the expression level of *miR-122* was elevated by transfection of pSM-122_S in these cells (Fig. 1B). However, the decreased of miR-122 expression in response to transfection with pSM-122_AS was only observed in Hep3B cells, which may due to the relatively low level of endogenous miR-122 in HepG2 and HeLa cells [13,28,29]. In parallel, we evaluated the expression level of *miR-122* in these cells transfected with a synthesized *miR-122* RNA duplex that has been functionally validated *in vivo* [30]. The intracellular level of *miR-122* was also increased by transfecting synthetic miRNA (Fig. 1B). These methods allowed us to control the cellular levels of *miR-122*.

miR-122 acts directly at the Bcl-w 3′-UTR

Identifying miRNA-regulated gene targets is a necessary step to understand miRNA functions. *miR-122* is the most abundantly expressed miRNA in human liver, and is known to regulate lipid and

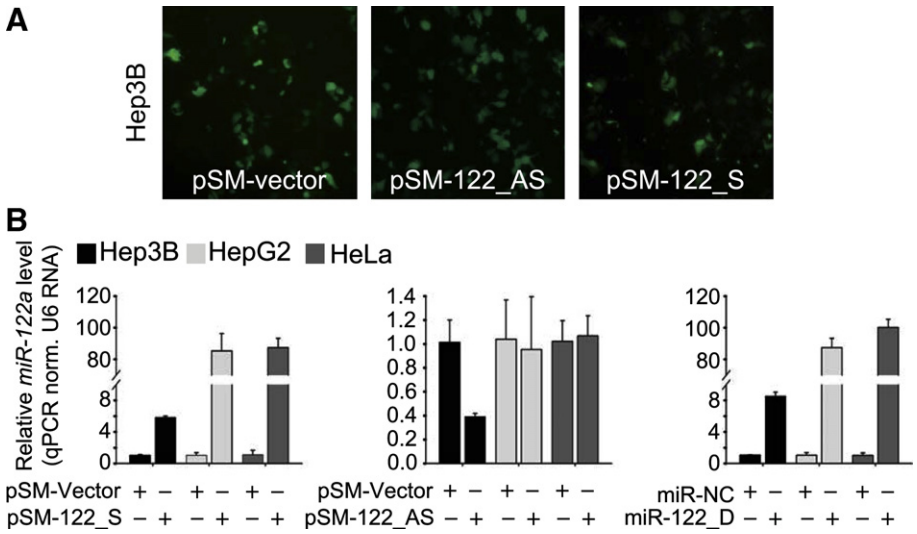

Fig. 1. *miR-122* expression by miR-like siRNA vector. (A) pSM-122_S, pSM-122_AS, and pSM-vector were transfected into Hep3B cells. EGFP reporter co-expression in Hep3B was monitored. (B) Cells were harvested after EGFP expression was confirmed 24 h post-transfection, and total RNA was extracted, followed by qRT-PCR. *miR-122* expression was normalized by U6 RNA in Hep3B, HepG2 and Hela cells. *miR-122* expression in these cells transfected with *miR-122* duplex (miR-122_D) or negative control (miR-NC) was also measured. $^{*}P < 0.05$.

cholesterol metabolism [11]. However, the targets of *miR-122* in hepatocarcinogenesis remain unclear. The only validated *miR-122* target involved in HCC is *CCNG1* [13]. To identify putative *miR-122* targets that may contribute to tumorigenesis, an *in silico* strategy was employed. By querying PicTar [14] and TargetScan [24], an anti-apoptotic BCL2 family member, *Bcl-w*, was identified in both programs as a putative *miR-122* target gene. The prediction results and score are listed in Fig. 2A; as a validated *miR-122* target, *CCNG1* is shown as a reference. Moreover, the PicTar prediction showed conserved binding sites on the 3′-UTR of *Bcl-w* across vertebrate species, including human, chimpanzee, dog, mouse, and rat (Fig. 2B). This observation suggests that *miR-122* targeting to *Bcl-w* may be evolutionarily conserved. The free energy of *miR-122* binding to the *Bcl-w* targeting site was calculated using RNAhybrid (mfe:-17.5 kcal/mol), and the predicted structure is shown in Fig. 2C.

To substantiate that *miR-122* is a direct regulator of *Bcl-w*, a 317-base pair fragment of the 3′-UTR that contains the putative

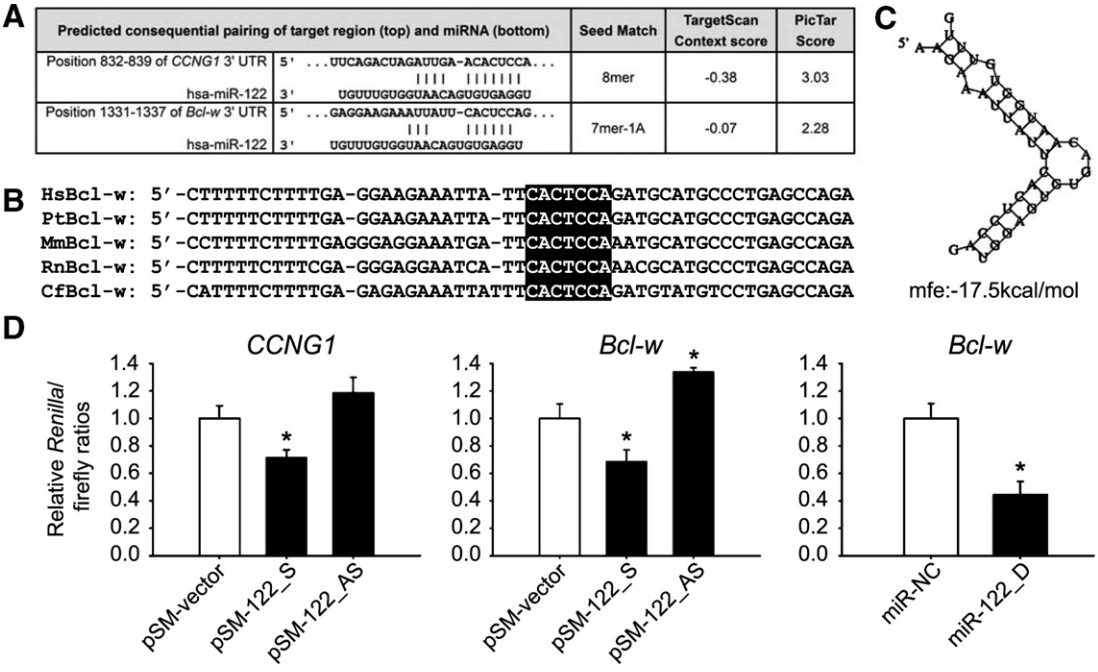

Fig. 2. Effect of the putative *miR-122* binding site derived from the *Bcl-w* 3′-UTR on luciferase expression. (A) Putative binding sites of *miR-122* in the *CCNG1* and *Bcl-w* 3′-UTR regions as detected by TargetScan and Pictar. (B) *Bcl-w* 3′-UTR sequences that contain the putative *miR-122* targeting site are highly conserved among vertebrate species. The *miR-122* seed sequence is boxed. Sequences listed are HsBcl-w: human, NM_004050; PtBcl-w: chimpanzee, Ensembl Transcript ID: ENSPTRT00000011308; MmBcl-w: mouse, NM_007537; RnBcl-w: rat, NM_021850; and CfBcl-w: dog, Ensembl Gene ID: ENSCAFG00000011482. (C) Predicted RNA duplex structure of *miR-122*/*Bcl-w* targeting site generated by RNAhybrid. (D) Luciferase activity in Hep3B cells transiently co-transfected with the 3′-UTR of *CCNG1* or *Bcl-w* reporter vector, and the expression vector for the sense strand of *miR-122* (pSM-122_S and miR-122_D), the anti-sense strand of *miR-122* (pSM-122_AS), or a negative control (pSM-vector and miR-NC). Data represent means ± SD of three independent experiments, each performed in triplicate. $^{*}P < 0.05$.

miR-122 binding site was cloned into the psiCHECK-2 reporter vector, downstream of the luciferase gene. The 3′-UTR of *CCNG1* was cloned into the same vector as a positive control [13]. Upon co-transfection in Hep3B cells, the *miR-122*-expressing vector (pSM-122_S) significantly repressed the expression of reporter construct, and anti-sense *miR-122* (pSM-122_AS) increased the expression of the reporter construct relative to a vector-only control (pSM-vector) (Fig. 2D). Luciferase activity was again reduced with the co-transfection of the *miR-122* duplex (miR-122_D). The repression of luciferase activity was more effective with miR-122_D than the *miR-122*-expression vector (Fig. 2D). This difference may occur because *miR-122* duplexes were immediate substrates for RNA-induced silencing complex (RISC), whereas the vector-derived *miR-122* precursor required transcription and dicing to be processed into mature miRNAs.

Bcl-w expression is down-regulated by miR-122

To determine whether changing the functional level of cellular *miR-122* influenced the endogenous expression of *Bcl-w*, cells were transfected with pSM-122_S and pSM-122_AS. Real-time qRT-PCR was performed at 24 h post-transfection. The mRNA expression levels of *Bcl-w* were reduced to 77%, 81%, and 42% in Hep3B, HepG2, and HeLa cells, respectively, when compared to the vector-only control (pSM-vector) (Fig. 3A). As a positive control, the mRNA expression levels of *CCNG1* were reduced to 76% in Hep3B cells (Fig. 3A). The mRNA expression level of *CCNG1*, however, was increased 1.3-fold upon transfection of pSM-122_AS, while no significant changes were observed in the level of *Bcl-w* in these cells, suggesting that additional mechanisms control *Bcl-w* expression. *Bcl-w* mRNA down-regulation was also reproduced when transfected with *miR-122* duplex (miR-122_D) and the siRNA that targets *Bcl-w* (Bcl-w_siR) (Fig. 3A). We next examined the *Bcl-w* protein level in response to the elevated *miR-122* level. Cells were transfected with miR-122_D, miR-NC, and Bcl-w_siR. The Bcl-w protein level was significantly reduced in cells transfected with *miR-122* duplex (47% and 53%) and *Bcl-w* siRNA (76% and 78% in Hep3B and HepG2, respectively) relative to miR-NC (Fig. 3B). Bcl-w has been demonstrated to form a complex with pro-apoptotic Bax protein, and a reduced Bcl-w/Bax ratio has been shown to induce spermatogonial and spermatocyte apoptosis in the testis [31]. We therefore evaluated the Bax protein level in cells transfected with *miR-122*. The results showed no significant changes in the Bax protein level (Fig. 3B), and a reduced Bcl-w/Bax ratio (49.8% and 52.7% in Hep3B and HepG2, respectively) was observed as expected. While the reduction of the *Bcl-w* mRNA level caused by miR-122_D and Bcl-w_siR was comparable (Fig. 3A), the reduction of Bcl-w protein was more significant. This suggests that *miR-122* modulates Bcl-w expression at the post-transcriptional level. To further confirm that *miR-122* modulates the Bcl-2 system by specifically repressing Bcl-w expression, we analyzed the expression of another anti-apoptotic Bcl-2 family member, Bcl-xL, in the cells with elevated *miR-122* expression. There is no *miR-122* targeting site detected in the 3′-UTR of *Bcl-xL*. The results of this experiment show no significant change of Bcl-xL mRNA and protein levels (Fig. 3A, B). This suggests that *miR-122* specifically targets to *Bcl-w* in these cells.

Bcl-w repression by miR-122 reduces cell viability and activates caspase-3 in the HCC-derived cell lines

The Bcl-w/Bax ratio was reduced in cells with an increased *miR-122* level. To investigate the biological importance of *Bcl-w* as a target of *miR-122*, we measured the cell viability and caspase-3 activity following transfection with different doses of miR-122_D. As shown in Fig. 4A and B, respectively, cell viability was decreased, and caspase-3 activity was increased, when elevating the functional level of cellular *miR-122*. To further confirm that

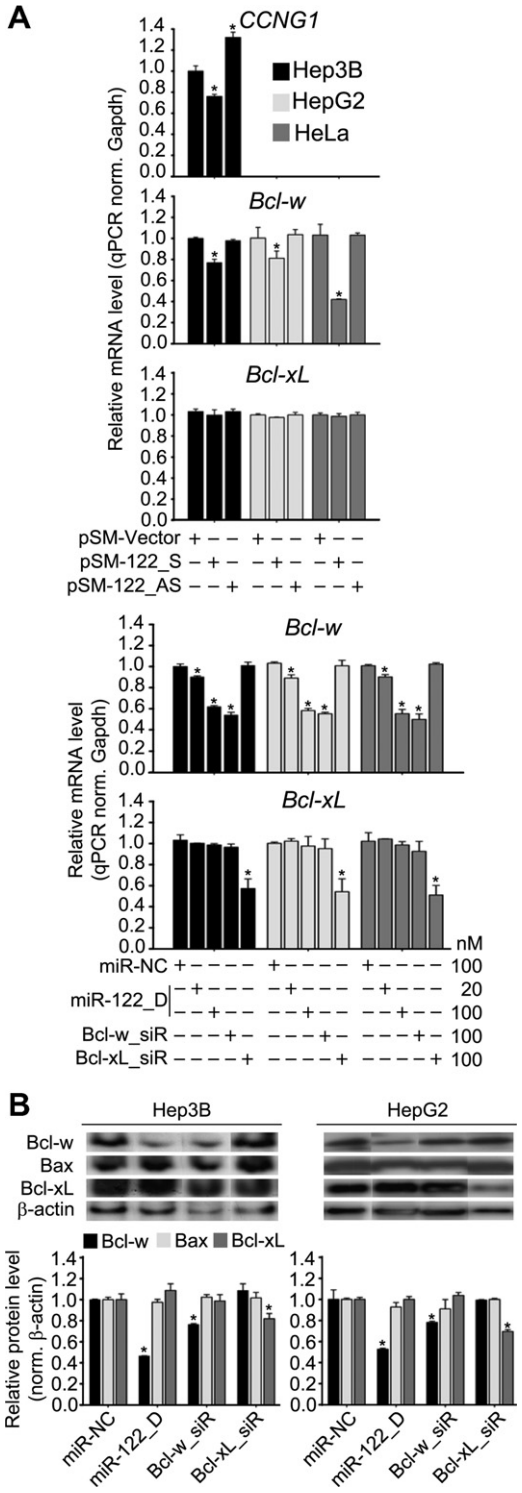

Fig. 3. *Bcl-w* expression is repressed by *miR-122*. (A) The relative mRNA levels of *Bcl-w* and *Bcl-xL* were measured in cells transfected with pSM-122_S, pSM-122_AS, miR-122_D, Bcl-w_siR, and Bcl-xL_siR and compared to negative controls (pSM-vector and miR-NC) by qRT-PCR 24 h post-transfection. As a validated *miR-122* target, the mRNA level of *CCNG1* was also measured in Hep3B cells as a positive control. (B) Western blot analysis of Bcl-w, Bax, and Bcl-xL protein level after miR-122_D, Bcl-w_siR, or Bcl-xL_siR transfection. Cells were collected 72 h after transfection. All treatments had a final conc. of 100 nM. The relative Bcl-w, Bax, and Bcl-xL protein levels in each treatment were quantified as shown in the lower panel. $^{*}P < 0.05$.

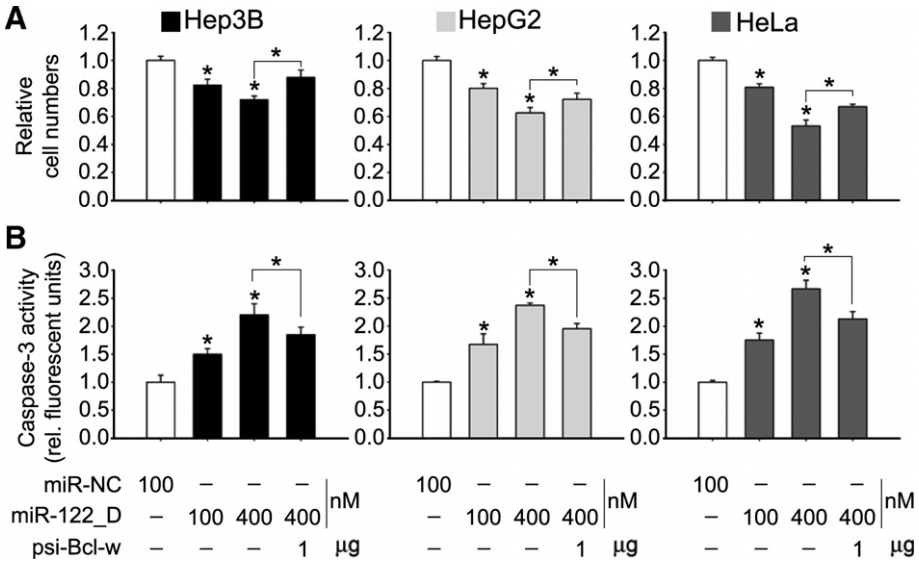

Fig. 4. Repression of Bcl-w by *miR-122* reduces cell viability and induces apoptosis. Cells were transfected with the indicated doses of miR-122_D. At 72 h post-transfection, cell viability (A) and caspase-3 activity (B) were measured. psi-Bcl-w was co-transfected in the rescue experiments. $^{*}P < 0.05$.

miR-122 is specifically responsible for the reduction of cell viability and the activation of caspase-3 by targeting *Bcl-w*, we co-transfected the *Bcl-w* 3′-UTR reporter construct, which harbors the *miR-122* targeting site, to compete with endogenous *Bcl-w* transcripts. The results showed a partial rescue of cell numbers and decreased caspase-3 activity (Fig. 4A and B, respectively). These data confirm that Bcl-w down-regulation by *miR-122* triggers apoptosis and that the level of *miR-122* expression is important for this mechanism.

Discussion

Although several miRNAs have been reported to be involved in controlling apoptosis and cancer formation [7,32], the only validated target gene of *miR-122*, a liver-specific miRNA that is down-regulated in HCC, is *CCNG1*. Here, we report the modulating effects of *miR-122* on *Bcl-w* expression by directly targeting the 3′-UTR region of *Bcl-w* mRNA. *miR-122* appears to inhibit Bcl-w at the post-transcriptional level in HCC-derived cell lines (Hep3B and HepG2) and a cervical carcinoma cell line (HeLa). The results of *Bcl-w* targeting by *miR-122* may have implications in the pathogenesis of HCC. Our data suggest that decreased *miR-122* expression helps cells evade cell death, a cardinal feature of cancer cells. Therefore, the reduced levels of *miR-122* in HCC not only results in chromosomal instability through deregulation of *CCNG1* but also enhances anti-apoptotic activity through an increase in the Bcl-w/Bax ratio. However, controversial *miR-122* expression profile observations have been reported in HCC with different pathological causes. For example, replication of the hepatitis C virus (HCV) was reported to depend on the status of *miR-122* expression [29], and the expression level of *miR-122* was not significantly decreased in HCV-associated HCC [28]. Further systematic research is required to clarify the role of deregulated *miR-122* in HCC.

Acknowledgments

We thank Dr. Guangwei Du for kindly providing the pSM plasmids. This work was supported by Grant # 96-2628-B-001-008 to Jen-Leih Wu from the National Science Council of Taiwan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.07.154.

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