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miR-301a Promotes Pancreatic Cancer Cell Proliferation by Directly Inhibiting Bim Expression

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ABSTRACT

It is well known that microRNAs (miRNAs) play an important role in many diseases, including tumorigenesis. However, the mechanisms by which miRNAs regulate pancreatic cancer (PC) development remain poorly understood. In the present study, we assayed expression level of miR-301a in PC tissues by real-time PCR, and defined the target gene and biological function by luciferase reporter assay and Western blot analysis. We first verified that the expression level of miR-301a was significantly increased in PC tissues. Moreover, miR-301a overexpression promoted PC cell proliferation, whereas its depletion decreased cell proliferation. We further demonstrated that miR-301a directly targeted 3′-UTR of Bim gene, and inhibited its protein expression in vitro and in vivo. Importantly, Bim re-expression reduced PC cell proliferation induced by miR-301a. These data suggest an important role of miR-301a in the molecular etiology of PC and implicate the potential application of miR-301a in PC therapy. J. Cell. Biochem. 113: 3229–3235, 2012.

KEY WORDS: miR-301a; Bim; PANCREATIC CANCER; PROLIFERATION; APOPTOSIS

Pancreatic cancer (PC) is one of the most aggressive malignancies with worst prognosis, which is the fourth leading cause of cancer-related deaths in the world [Lebedeva et al., 2006]. Despite efforts made in multiple fields, there has been little success in improving the disease-free survival rate of patients [Chen et al., 2009]. The advances in suitable therapy for the purpose of increasing survival rate have been limited because the pathophysiological mechanisms causing this are not known. Therefore, revealing the molecular mechanism for the PC development is indispensable for developing effective therapy.

Bcl-2 family proteins appear to control a distal step in what might represent a final common pathway for apoptotic cell death [Yan et al., 2003]. The Bcl-2 family consists of both anti-apoptotic and pro-apoptotic proteins with the balance of these proteins regulating cell apoptosis [Drury et al., 2010]. Pro-apoptotic Bcl-2 family members are further characterized as multi-domain or BH3-only proteins. BH3-only proteins such as Bim sense apoptotic stimuli and initiate apoptosis through activation of multi-domain pro-apoptotic proteins such as Bak and Bax [Willis et al., 2005]. Depletion of Bim enhances anchorage-independent survival in both transformed and non-transformed cells [Marani et al., 2004; Woods et al., 2007]. Overexpression of Bim can trigger cell apoptosis by inducing cytochrome *c* release from mitochondria [Liu et al., 2007]. However, the regulation of Bim expression remains unclear.

There is increasing evidence that post-transcriptional regulation of gene expression, mediated by microRNAs (miRNAs), plays an important role in the control of cell proliferation, apoptosis, and tumorigenesis [Hwang and Mendell, 2006; Saito et al., 2009; Bao et al., 2011]. miRNAs are non-coding RNAs that have been highly conserved during evolution and have emerged recently as potent regulators of gene expression [Zeng et al., 2002]. Overexpression of oncogenic miRNAs or underexpression of tumor suppressor miRNAs plays pivotal roles in tumorigenesis [Bo et al., 2011]. Dong et al. [2011] demonstrated that Bcl-2 upregulation induced by miR-21 via a direct interaction was associated with apoptosis and chemoresistance in MIA PaCa-2 PC cells. Aberrant expression of miRNAs, such as miR-15a and miR-214 resulted in different cellular effects in PC [Zhang et al., 2010]. Downregulation of miR-15a contributed to

Abbreviations used: miRNA, microRNA; miR-301a, microRNA-301a; PC, pancreatic cancer; Bim, Bcl-2 interacting mediator of cell death.

The authors declared that they have no conflicts of interest.

Additional supporting information may be found in the online version of this article.

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proliferation of PC cells, whereas upregulation of miR-214 in PC specimens might be related to the poor response of PC cells to chemotherapy. Zhang et al. [2010] further demonstrated that miR-15a directly targeted multiple genes relevant in PC, suggesting that it may serve as a novel therapeutic target for treatment of the disease. A recent study showed that miR-301a is involved in PC progression. Lee et al. [2007] investigated aberrant miRNA expression in PC, and showed increased levels of miR-301a in PC tissues. Lu et al. [2011] certified that miR-301a downregulated NF-κB-repressing factor (Nkrf) and elevated NF-κB activation. However, little is known whether miR-301a directly regulates cell proliferation in PC development.

In the study, we showed that upregulated miR-301a promoted PC cell proliferation, whereas its depletion decreased cell proliferation. We further demonstrated that miR-301a directly targeted 3′-UTR of Bim gene. Importantly, Bim re-expression reduced PC cell proliferation induced by miR-301a.

MATERIALS AND METHODS

CELL CULTURES AND TISSUE SAMPLES

Thirteen specimens of PC tissues and their adjacent benign tissues were collected at Cancer Hospital, Fudan University, China from 2008 to 2010 (Table I). Written informed consent for the biological studies was obtained from the patients involved in the study or from their parents/guardians. The study was approved by the Ethics Committee of the Fudan University.

BxPC-3 and Hs766T human PC cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 medium containing 10% FBS (GIBCO, Carlsbad, CA). HEK293 cells were maintained in DMEM containing 10% FBS and cultured at 37°C with 5% CO_2.

REAL-TIME POLYMERASE CHAIN REACTION (PCR)

Total RNA was extracted by using Trizol reagent (Invitrogen, Carlsbad, CA), and the reverse-transcription reactions were performed using an M-MLV Reverse Transcriptase kit (Invitrogen). The gene-specific stem–loop RT primers for miRNAs, which were released by the Sanger Institute, were designed according to Chen et al. [2005]. Real-time PCR was performed using a standard SYBR Green PCR kit (Toyobo, Osaka, Japan) and a Rotor-Gene RG-3000A (Corbett Life Science, Sydney, New South Wales, Australia) according to the instructions from the respective manufacturer. U6 and β-actin were used as references for miRNAs and RNAs, respectively. Each sample was analyzed in triplicate. The $2^{-\Delta\Delta Ct}$ method was used to quantify the relative levels of gene expression.

TABLE I. Clinic-Pathological Characteristics of the Patients With Pancreatic Cancer

Sample	Age	Gender	Tumor stage	Collection
1	43	M	IV	Surgical resection
2	70	M	III	Surgical resection
3	60	F	III	Surgical resection
4	55	M	II	Surgical resection
5	57	F	IV	Surgical resection
6	62	M	III	Surgical resection
7	41	M	I	Surgical resection
8	39	M	IV	Surgical resection
9	59	M	I	Surgical resection
10	64	M	IV	Surgical resection
11	73	F	III	Surgical resection
12	70	F	II	Surgical resection
13	64	M	I	Surgical resection

LUCIFERASE REPORTER ASSAY

HEK293 cells ($1–2 \times 10^5$ cells/well) were plated in a 24-well plate and then cotransfected with 20 nM of either miR-301a mimics or miRNA control, 40 ng of either pGL3-Bim-3′-UTR-WT or pGL3-Bim-3′-UTR-MUT, and 4 ng of pRL-TK (Promega, Madison, WI) using Lipofectamine™ 2000. HEK293 cells were collected 48 h after transfection and analyzed using the Dual-Luciferase Reporter Assay System (Promega). The pRL-TK vector was cotransfected as an internal control to correct for differences in both transfection and harvest efficiencies. Transfections were performed in triplicate and repeated at least thrice in separate experiments.

WESTERN BLOT ANALYSIS

Western blot analysis was carried out on 10% SDS–PAGE. Briefly, proteins were electrotransferred onto nitrocellulose filter. After blocking for 1 h in PBS with 0.1% Tween 20 (PBST) and 5% BSA, the membranes were incubated over night with specified primary antibody in PBST containing 5% BSA. Detection was carried out by the use of HRP conjugated IgG and DAB assay kit (Sigma, St. Louis, MO). The antibodies used included Bim (Cell Signaling Technology, Beverly, MA) and β-actin (Sigma). Western blot was scanned and band intensity was quantified by using Quantity One Imaging Software from Bio-Rad.

TREATMENT OF BxPC-3 WITH miR-301a MIMICS OR ITS INHIBITOR

Human PC cells BxPC-3 and Hs766T were plated in 24- or 6-well plates and miR-301a mimics (GCUCUGACUUUAUUGCACUACU) or its inhibitor (AGTAGTGCAATAAAGTCAGAGC; Ambion, Austin, TX) were transfected at a final concentration of 20 nM by using Lipofectamine 2000 (Invitrogen). Cells were harvested for Western blot analysis or cell death assay at the indicated time points.

CELL PROLIFERATION ASSAY

Cell proliferation was determined by using CyQUANT Cell Proliferation Assay (Invitrogen) according to the manufacturer's protocol. The fluorescence intensity was measured by using a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA).

pcDNA-Bim was constructed to re-express Bim by introducing a *Bam*HI–*Eco*RI fragment containing the Bim-mRNA into the same sites in pcDNA3.1. The following primer sequences were selected: sense primer, 5′-CGGGATCCCGATGGCAAAGCAACCTTCTG-3′, antisense primer, 5′-GGAATTCCCTATTTCTCTAACCTCCTTGC-3′. Bim was re-expressed in BxPC-3 cells treated with miR-301a, and the numbers of cells per well were measured by the absorbance (450 nm) of reduced WST-8 at 12, 24, 48, 60, and 72 h, respectively.

STATISTICAL ANALYSIS

All data were expressed as the mean ± standard deviation (SD) from at least three separate experiments. The differences between groups

were analyzed using the Student's *t*-test. The difference was deemed statistically significant at $P < 0.05$.

RESULTS

miR-301a LEVEL IS SIGNIFICANTLY INCREASED IN PANCREATIC CANCER

In order to assess the role of miR-301a in PC development, we firstly evaluated the different expression of miR-301a in PC tissues and their adjacent benign tissues by using quantitative real-time PCR. Figure 1 showed that miR-301a was expressed at low levels in benign tissues, whereas miR-301a level was significantly increased in PC tissues. Compared with the adjacent non-cancerous tissues, miR-301a level was significantly upregulated in 11 pairs of PC tissues (Fig. 1). These data indicate that upregulation of miR-301a may be related to PC development.

miR-301a PROMOTES CELL PROLIFERATION AND SUPPRESSES CELL APOPTOSIS IN VITRO

To study the biologic role of miR-301a in cell proliferation, the PC cell lines BxPC-3 or Hs766T treated with miR-301a were analyzed. The miR-301a levels were significantly increased in BxPC-3 cells transfected with miR-301a mimics (Fig. 2A), and upregulated miR-301a promoted BxPC-3 cell proliferation (Fig. 2B). Furthermore, downregulated miR-301a by its inhibitor inhibited BxPC-3 cell proliferation (Fig. 2C,D). Upregulated miR-301a also promoted Hs766T cell proliferation (Fig. 2E,F), together with decreased caspase-3 processing (Fig. 2G). These results suggest that upregulated miR-301a positively regulates PC cell growth.

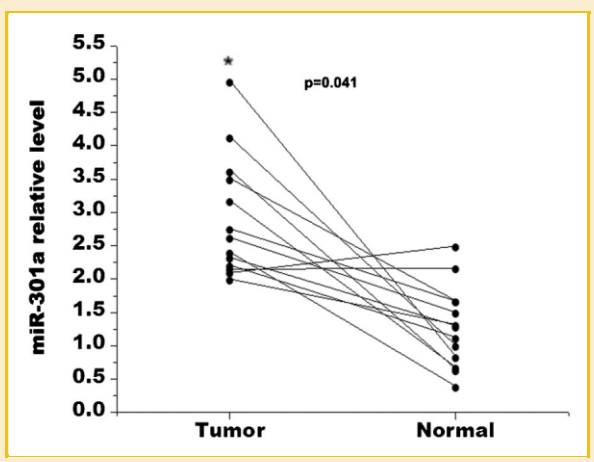

Fig. 1. miR-301a level was significantly increased in pancreatic cancer tissues. The analysis of the miR-301a expression level was performed in pancreatic tumor tissues (n = 13) and their adjacent benign tissues. Total RNA was subjected to real-time RT-PCR to analyze the expression level of miR-301a in each sample. U6 was used as a reference for miRNAs. Relative miR-301a level of every sample was calculated with respect to a normal control. Each sample was analyzed in triplicate. The $2^{-\Delta\Delta Ct}$ method was used to quantify the relative gene expression levels.

miR-301a DIRECTLY TARGETS BIM 3′-UTR

In order to identify the target gene of miR-301a in regulating PC cell proliferation, we searched for candidate genes using TargetScan5.2 (http://www.targetscan.org/) and miRBase (http://www.mirbase.org/) miRNA databases. The Bim gene is one of the predicted target genes of miR-301a (Fig. 3A), and the binding site sequence of miR-301a (TTGCACTG) appears in isoforms 3, 4, and 10. We constructed a firefly luciferase reporter contained 3′-UTR of Bim, and co-transfected HEK293 cells with the pGL3-promoter-Bim-3′-UTR and miR-301a. The reporter assay showed that miR-301a was able to significantly repress luciferase expression of pGL3-promoter-Bim-3′-UTR (Fig. 3B), and mutation of 4 nt in the miR-301a target sequence led to complete abrogation of the suppressive effect (Fig. 3B). We further observed that miR-301a overexpression decreased the Bim protein content in BxPC-3 cell (Fig. 3C). We next assayed the Bim mRNA and protein level in the PC tissues, and a significant negative correlation was observed between Bim expression levels and the miR-301a expression levels in vivo (Fig. 3D). These observations confirm that miR-301a inhibits endogenous Bim in PC cells.

miR-301a PROMOTES PANCREATIC CANCER PROGRESSION BY REPRESSING BIM

Bim is an important determinant of cells proliferation or apoptosis in cancer development, and downexpression of Bim results in abnormal cell growth. Therefore, we speculated that the role of miR-301a in PC cell growth was mediated by regulating endogenous Bim expression. The inhibition of Bim by miR-301a was partially recovered after co-transfection with a plasmid-expressing Bim (Supplementary Fig. 1). Figure 4 showed that BxPC-3 or Hs766T cell proliferation was inhibited when Bim was re-expressed in BxPC-3 cells treated with miR-301a. These data confirm that miR-301a promotes PC progression, at least in part by repressing endogenous Bim.

DISCUSSION

Recent studies showed that miRNAs are involved in several important biological events, such as cell growth and tumorigenesis [Basu et al., 2011; Zhang et al., 2011]. miRNAs are known to act as regulators in PC cell growth and to regulate PC development [Wang and Sen, 2011]. Deregulation of some miRNAs, including miR-148, miR-132, miR-15a, and miR-214, have been observed in PC [Zhang et al., 2010, 2011; Liffers et al., 2011]. Liffers et al. demonstrated that miR-148a was downregulated in human pancreatic ductal adenocarcinomas and regulated cell survival by targeting CDC25B. Downregulation of miR-132 by promoter methylation contributed to PC development. Downregulation of miR-15a contributed to proliferation of PC cells, whereas upregulation of miR-214 in PC specimens might be related to the poor response of PC cells to chemotherapy. Patel et al. [2011] demonstrated the involvement of miR-301a in immediate induction of plasminogen activator inhibitor-1 by placental growth factor in human pulmonary endothelial cells. Recently, the relationship between miR-301a

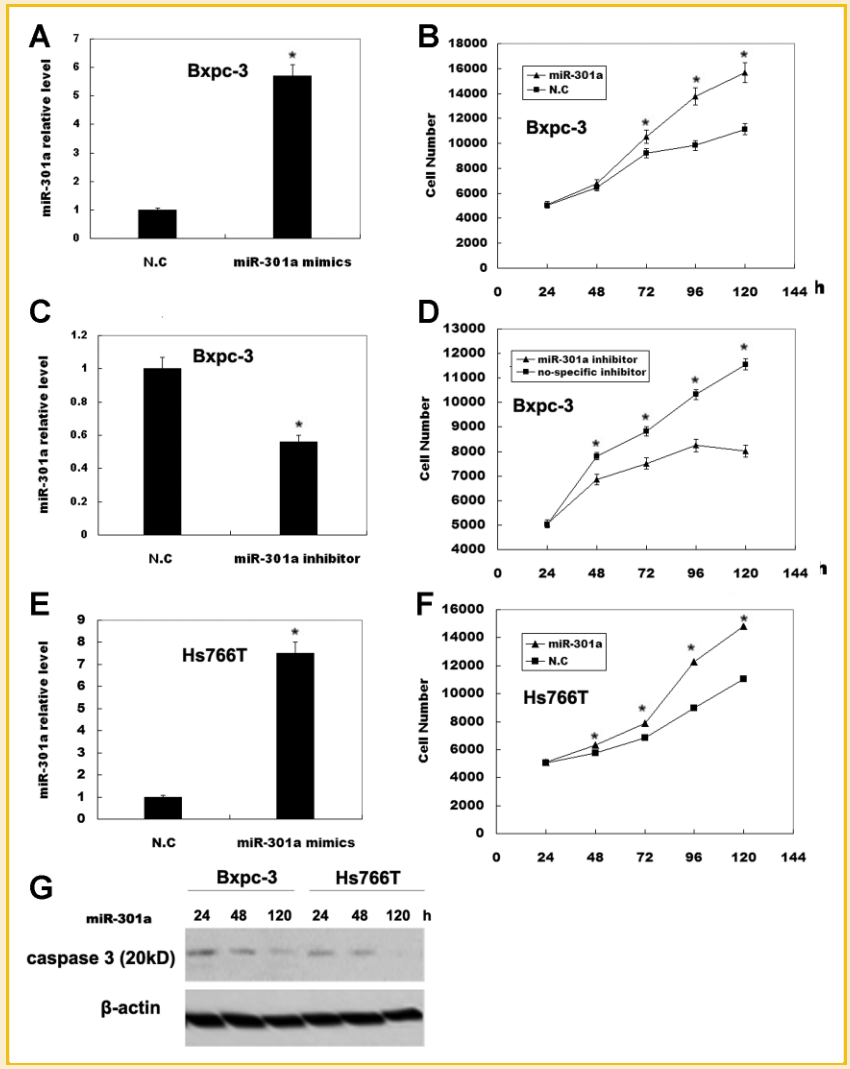

Fig. 2. miR-301a promoted pancreatic tumor cells proliferation. A: BxPC-3 cells were treated with mature miR-301a and miR-301a expression level was assayed by real-time PCR. The negative control (N.C.) is miR control (UUCUCCGAACGUGUCACGUTT). B: BxPC-3 cells were transiently transfected with miR-301a mimics, and at the indicated time points, the numbers of cells per well were measured by using CyQUANT Cell Proliferation Assay kit. C,D: BxPC-3 cells were transiently treated with miR-301a inhibitor, and the numbers of cells per well were measured by using CyQUANT Cell Proliferation Assay kit. The results show data from at least three independent experiments, expressed as the mean ± SD. $^{*}P < 0.05$ versus miR control or inhibitor control. E,F: Hs766T cells were transiently treated with miR-301a, and the numbers of cells per well were measured by using CyQUANT Cell Proliferation Assay kit. $^{*}P < 0.05$. G: Western blot analysis for active caspase-3 (20 kDa) after BxPC-3 and Hs766T cells were treated with miR-301a for 24, 48, and 120 h.

and PC was reported [Lu et al., 2011]. Lu et al. [2011] demonstrated that miR-301a downregulated Nkrf and elevated NF-κB activation. However, little is known whether miR-301a directly regulates cell proliferation in PC development.

In the study, we firstly verified that the expression level of miR-301a was significantly increased in PC tissues. miR-301a overexpression promoted PC cell proliferation, whereas its depletion decreased cell proliferation. We further demonstrated that miR-301a directly targeted 3′-UTR of Bim gene, and inhibited its expression. Importantly, Bim re-expression reduced PC cell proliferation induced by miR-301a. These data confirm that miR-301a promoted PC progression, at least in part by repressing endogenous Bim. Additionally, we found that miR-301a targets ZEB1 and ZEB2 mRNAs. However, miR-301a could not regulate PC cell metastasis by targeting ZEB1 and ZEB2. There are already several miRNAs implicated in PC development, such as miR-148 and miR-214. We also found that miR-214 overexpression contributed to PC cell growth, whereas miR-148 did not regulate PC cell proliferation. Currently, we did not found the target gene of miR-214 in regulating PC cell growth although miR-214 has predicted targets sites in the Bim 3′UTR.

The Bcl-2 family consists of both anti-apoptotic and pro-apoptotic proteins with the balance of these proteins regulating cell apoptosis. The life-death switch is governed by the BH3-only

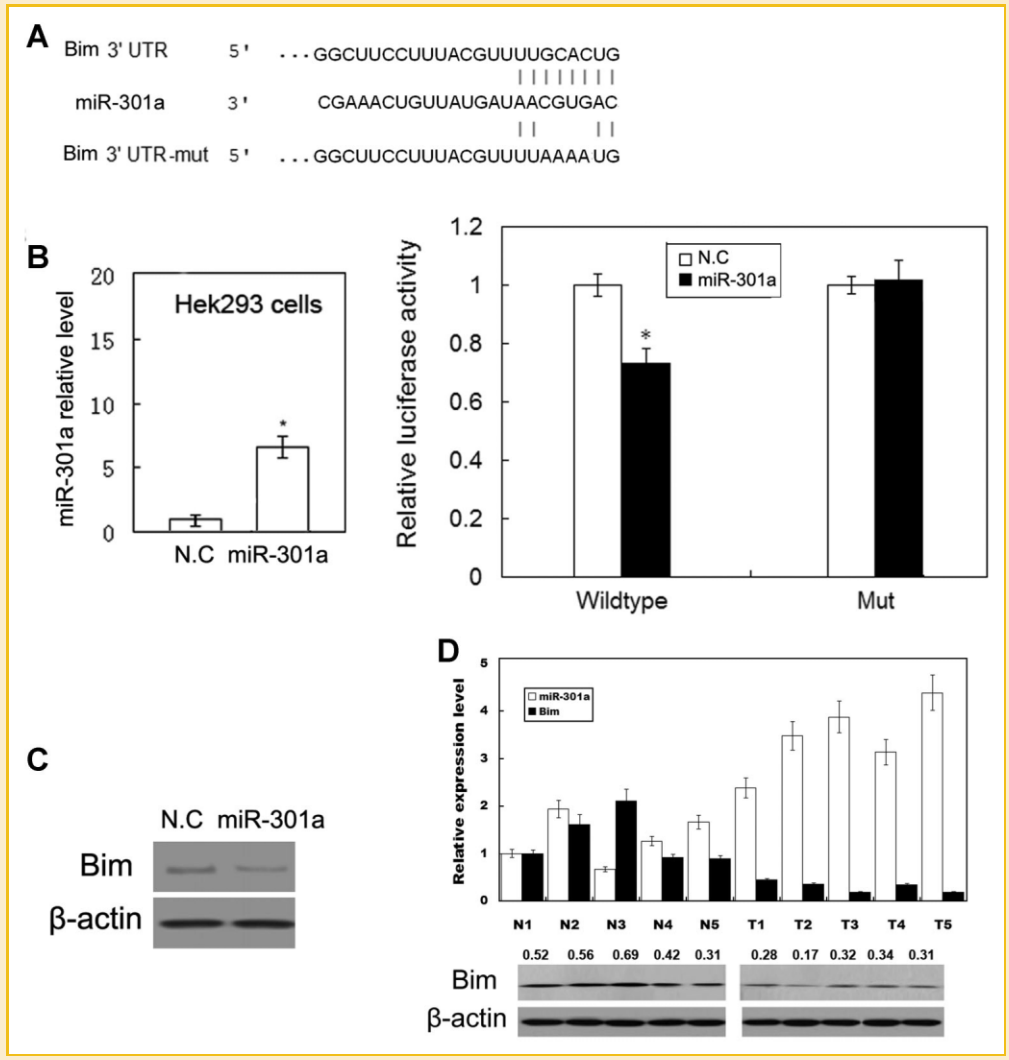

Fig. 3. miR-301a directly targeted Bim 3′-UTR. A: Schematic representation of the miR-301a site in Bim 3′-UTR. B: The 3′-UTR reporter assay was carried out in HEK293 cells that overexpressed miR-301a. PGL3-promoter-Bim-3′-UTR-WT or PGL3-promoter-Bim-3′-UTR-MUT was co-transfected with pRL-TK using Lipofectamine 2000. Luciferase assays were performed 48 h after transfection using the Dual-Luciferase Reporter Assay System. Firefly luciferase activity was standardized to Renilla luciferase control. An asterisk indicates significantly different from the N.C. ($P < 0.05$). C: Western blot analysis for endogenous Bim protein level using antibodies against Bim in BxPC-3 cells. N.C. is miR control (UUCUCCGAACGUGUCACGUTT). D: Real-time PCR and Western blot analysis for Bim level in the randomly selected pancreatic cancer tissues, and a significant negative correlation was observed between Bim expression levels and the miR-301a expression levels in vivo. Relative miR-301a or Bim mRNA level was calculated with respect to N1. Western blot was scanned and band intensity was quantified by using Quantity one software (Bio-Rad).

members of the Bcl-2 family, which reside upstream of Bax and Bak, and promote apoptosis by directly activating Bax or Bak, or by antagonizing the antiapoptotic activity of prosurvival Bcl-2 family members [Cheng et al., 2001; Greenhough et al., 2010]. Bim is one of the most powerful BH3-only proteins, able to engage all prosurvival proteins [Certo et al., 2006]. Therefore, Bim is a tumor suppressor. Chen et al. [2011] showed that one of the mechanism of Myc inhibiting multiple myeloma cell apoptosis is through Myc activates miR-17-92 cluster and subsequently down-modulates proapoptotic protein Bim. Chen et al. [2004] demonstrated that overexpression of Bim alpha3 results in cell apoptosis. Bim expression and activity is controlled by both transcriptional and post-translational mechanisms. For example, Bim is transcriptionally upregulated by FoxO3a in response to cytokine deprivation or PI3K-Akt pathway inhibition and by CHOP-C/EBPα under conditions of ER stress [Dijkers et al., 2000; Greenhough et al., 2010]. Qian et al. [2011] showed that miR-24 inhibited apoptosis by repressing Bim expression by post-translational mechanisms in mouse cardiomyocytes.

In conclusion, our data demonstrate that upregulated miR-301a promoted PC cell proliferation by inhibiting Bim expression in vitro and in vivo. Importantly, Bim re-expression reduced PC cell proliferation induced by miR-301a. These data suggest an important role of miR-301a in the molecular etiology of PC and implicate the potential application of miR-301a in PC therapy.

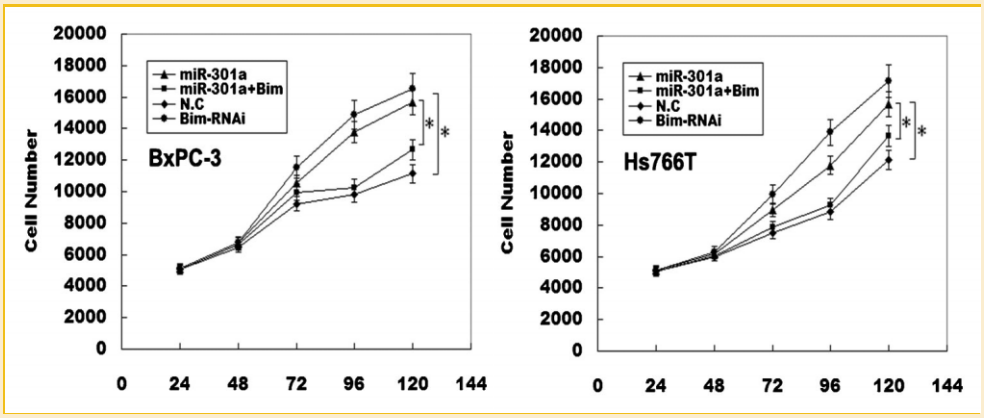

Fig. 4. miR-301a promoted pancreatic cancer progression by inhibiting Bim. Bim was re-expressed in pancreatic cells by co-transfecting pcDNA-Bim with with miR-301a, and the numbers of cells per well were measured by the absorbance (450 nm) of reduced WST-8 at 12, 24, 48, 60, 72 h, respectively. The results show data from at least three independent experiments, expressed as the mean ± SD. $^{*}P < 0.05$. N.C. is miR control (UUCUCCGAACGUGUCACGUTT).

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