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MicroRNA-345 induces apoptosis in pancreatic cancer cells through potentiation of caspase-dependent and -independent pathways

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Background: Previously, miR-345 was identified as one of the most significantly downregulated microRNAs in pancreatic cancer (PC); however, its functional significance remained unexplored.

Methods: miR-345 was overexpressed in PC cells by stable transfection, and its effect on growth, apoptosis and mitochondrial-membrane potential was examined by WST-1, Hoechst-33342/Annexin-V, and JC-1 staining, respectively. Gene expression was examined by quantitative reverse-transcription-PCR and/or immunoblotting, and subcellular fractions prepared and caspase-3/7 activity determined by commercially available kits. miR-345 target validation was performed by mutational analysis and luciferase-reporter assay.

Results: miR-345 is significantly downregulated in PC tissues and cell lines relative to normal pancreatic cells, and its expression decreases gradually in PC progression model cell lines. Forced expression of miR-345 results in reduced growth of PC cells because of the induction of apoptosis, accompanied by a loss in mitochondrial membrane potential, cytochrome-*c* release, caspases-3/7 activation, and PARP-1 cleavage, as well as mitochondrial-to-nuclear translocation of apoptosis-inducing factor. These effects could be reversed by the treatment of miR-345-overexpressing PC cells with anti-miR-345 oligonucleotides. *BCL2* was characterised as a novel target of miR-345 and its forced-expression abrogated the effects of miR-345 in PC cells.

Conclusions: miR-345 downregulation confers apoptosis resistance to PC cells, and its restoration could be exploited for therapeutic benefit.

Pancreatic cancer (PC) is one of the deadliest malignancies. With an estimated 48 960 diagnoses and nearly 40 560 deaths in 2015, it is considered the fourth leading cause of cancer-related death in the United States (Siegel *et al*, 2015). Owing to the lack of specific symptoms, early detection is not possible and most PCs are diagnosed in advanced and metastatic stage, leaving limited options for therapy (Feldmann and Maitra, 2008). Most current therapies provide no or marginal survival benefits and therefore 5-year overall survival rate of PC patients has stayed at 3–6% for past few decades (Arora *et al*, 2013). Clearly, there is an urgent need to identify novel diagnostic/therapeutic targets, understand the molecular mechanisms underlying the aggressive nature of this

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malignancy, and develop alternative strategies and novel therapeutics for effective treatment of this devastating disease.

MicroRNAs (miRNAs) are small (~22-nucleotides) noncoding RNAs that act as important regulators of gene expression (Bhardwaj *et al*, 2010; Srivastava *et al*, 2013). miRNAs act by specifically binding to the 3′ untranslated region (UTR) of their cognate target mRNAs and subsequently cause either their degradation or inhibit translation (Bhardwaj *et al*, 2010). To date, ~2469 miRNAs have been identified in humans (Friedlander *et al*, 2014; Zearo *et al*, 2014), and emerging evidence suggests that miRNAs potentially regulate ~30–80% of all protein-coding genes (Lu and Clark, 2012). These miRNAs control various key cellular processes, such as proliferation, apoptosis, differentiation, and development, and are implicated in several human diseases, including cancer (Bhardwaj *et al*, 2010; Ryan *et al*, 2010). Earlier, it was demonstrated that ~52.5% of miRNA genes are located in cancer-associated genomic regions, or in fragile sites, thus strongly suggested their role in cancer (Calin *et al*, 2004). Depending on the mRNA targets, miRNAs may function either as oncogenes (by inhibiting tumour suppressor genes) or as tumour suppressors (by inhibiting oncogenes) and thus have an important role in the progression and pathogenesis of cancer (Bhardwaj *et al*, 2010). According to recently published studies, a number of miRNAs have been shown to be differentially expressed in PC (Srivastava *et al*, 2014). These aberrantly expressed miRNAs have diverse pathological functions, such as regulating cell cycle progression, apoptosis, chemoresistance, angiogenesis, tumorigenicity, and metastatic potential (Srivastava *et al*, 2014). miR-345 was reported to be one of the top aberrantly expressed miRNAs in PC exhibiting significant downregulation (Bloomston *et al*, 2007; Lee *et al*, 2007; Szafranska *et al*, 2007); however, its role in PC pathogenesis has remained unexplored thus far.

In the current study, we demonstrate that miR-345 is significantly downregulated in PC, and its restoration in PC cells diminishes growth and induces apoptosis through activation of caspase-dependent and -independent pathways. Moreover, we show that miR-345 directly targets the 3′UTR of BCL2 and suppresses its expression at the protein level. Furthermore, we present evidence that the unopposed expression of BCL2 leads to abrogation of pro-apoptotic effects of miR-345. Together, these findings highlight the pro-apoptotic function of miR-345 in PC and support its utility as a candidate for diagnostic and therapeutic target.

MATERIALS AND METHODS

Cell culture and pancreatic tissue specimens. Human PC cell lines (MiaPaCa, Panc1, Colo-357, HPAF, ASPC-1, Panc10.05, Panc02.03, Panc03.27, BXPC3, CFPAC, CAPAN1, and SW1990) were maintained as monolayer cultures in DMEM or RPMI 1640 media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA) and 100 μM of penicillin and 100 $\mu g\,ml^{-1}$ of streptomycin (Invitrogen). The hTERT-HPNE cells were maintained in Medium D containing 1 volume of M3 base (InCell Corp., San Antonio, TX, USA), 3 volumes of glucose-free DMEM, 5.5 mM glucose, $10\,ng\,ml^{-1}$ epidermal growth factor, $50\,\mu g\,ml^{-1}$ gentamicin (all from Sigma-Aldrich, St Louis, MO, USA), and 5% fetal bovine serum. All the PC cell lines were cultured in humidified atmosphere at 37 °C with 5% CO_2 and media were replaced as needed. Cell lines were tested intermittently for mycoplasma contamination. Frozen pancreatic tissue (normal and malignant) specimens were obtained through the cooperative human tissue network at the University of Alabama at Birmingham under an institutional review board-approved protocol.

Plasmids and transfections. For the generation of stably miR-345-overexpressing cell lines, MiaPaCa and Panc1 cells were transfected with miR-345 expression (pCMV-miR-345) or control (pCMV-Neo) plasmid (Origene, Rockville, MD, USA) using X-tremeGENE HP DNA transfection reagent (Roche, Indianapolis, IN, USA) as per the manufacturer's instructions. Stable populations of transfected cells were selected in media containing $200\,\mu g\,ml^{-1}$ of G418 (Gibco, Grand Island, NY, USA), expanded and examined for stable miR-345 overexpression. For inhibition of miR-345 function, miR-345-overexpressing cells (Panc1-miR-345 and MiaPaCa-miR-345) were transiently transfected with anti-miR-345 or miRNA inhibitor negative controls (Ambion, Austin, TX, USA) at 30 nM concentration using X-tremeGENE HP DNA transfection reagent. For BCL2 overexpression, cells were transiently transfected with pcDNA3-BCL2 construct (lacking 3′UTR region) generated by Stanley Korsmeyer Laboratory (procured through Addgene, Cambridge, MA, USA, plasmid # 8768) or pcDNA3 (Invitrogen) as a control vector using X-tremeGENE HP DNA transfection reagent.

RNA isolation and real-time quantitative reverse-transcription PCR (qRT-PCR). Total RNA was extracted using TRIzol reagent as per the manufacturer's instruction (Invitrogen). Complementary DNA (cDNA) was synthesised using the high-capacity cDNA reverse transcription kit following manufacturer's instructions (Applied Biosystems, Carlsbad, CA, USA). Expression level of mature miR-345 was examined by following the strategy as described earlier (Srivastava *et al*, 2011). To examine the expression level of BCL2, real-time qRT-PCR was performed in 96-well plates using cDNA and SYBRGreen Master Mix (Roche) on an iCycler system (Bio-Rad, Hercules, CA, USA) with specific primers. Threshold cycle (Ct) values for miR-345 and BCL2 were normalised against Ct values for U6 small nuclear RNA and GAPDH, respectively, and a relative fold change in expression was calculated. Details of the primers used in the study are provided in Supplementary Table 1. The PCR conditions used were: cycle 1: 95 °C for 10 min, cycle 2 (x40): 95 °C for 10 s and 58 °C for 45 s.

Cell growth assays. PC cells (5×10^3 cells per well) were seeded in to 96-well plates, and cultured up to 5 days. Media were replaced every day with fresh complete media. Cell growth was monitored each day using WST-1 assay kit (Roche Diagnostics, Mannheim, Germany) as described earlier (Bhardwaj *et al*, 2014; Deshmukh *et al*, 2015). Separately, miR-345-overexpressing cells were transiently transfected with BCL2 for 24 h, cultured up to 5 days and growth monitored as described above.

Hoechst 33342 staining. Cells (2.5×10^5) were grown in glass-bottom fluorodishes, washed with PBS, and incubated with $10\,mg\,ml^{-1}$ Hoechst 33342 (Life Technologies, Grand Island, NY, USA) in the dark at room temperature for 10 min. Cells were then observed and photographed using a Nikon Eclipse TE2000-U fluorescent microscope (Nikon Instruments Inc, Melville, NY, USA).

Apoptosis assay. Cells (1×10^6) were seeded in to six-well plates. After 24 h, culture medium was replaced with fresh complete medium and cells were allowed to grow for next 48 h. Subsequently, the extent of apoptosis was measured as previously described (Srivastava *et al*, 2012; Arora *et al*, 2015). In brief, cells were harvested and stained with 7-amino-actinomycin (7-AAD) and PE Annexin V using commercially available kit (BD Pharmingen, San Diego, CA, USA) followed by flow cytometry on a BD-FACS Canto II (Becton-Dickinson, San Jose, CA, USA). Percentage of the apoptotic cells was calculated using DIVA software version 6.1.3 (Becton-Dickinson).

Mitochondrial membrane potential ($\Delta\psi$m) determination. Cells (1×10^6) grown in six-well plates were treated with $20\,\mu g\,ml^{-1}$ of JC-1 dye (Life Technologies) for 20 min and incubated at 37 °C in the dark. Following incubation, cells were harvested by

trypsinising, washed, resuspended in 500 μl of PBS, and analysed by flow-cytometry on a BD-FACS Canto II. Percentage of loss in $\Delta\psi$m was calculated using DIVA software version 6.1.3.

Subcellular fractionation. The cytoplasmic, mitochondrial, and nuclear fractions from low- and high-miR-345-expressing PC cells were prepared using commercially available kit as per the manufacture's protocol (Mitosciences, Oregon, OR, USA).

Western blot analysis. Protein isolation and western blotting were performed as described earlier (Srivastava *et al*, 2011; Tyagi *et al*, 2014). Immunodetection was carried out using specific antibodies (1 : 1000) against BCL2, cytochrome *c*, cleaved caspase-3, cleaved caspase-7, PARP-1, apoptosis-inducing factor (AIF; rabbit monoclonal), HDAC (mouse monoclonal; Cell Signalling, Danvers, MA, USA), α-tubulin (rabbit monoclonal), and VDAC (goat monoclonal; Santa Cruz Biotechnology, Dallas, TX, USA). β-Actin (mouse monoclonal; 1 : 20 000; Sigma-Aldrich, St Louis, MO, USA), α-tubulin, VDAC, and HDAC served as an control for total, cytoplasmic, mitochondrial, and nuclear fractions, respectively. All secondary antibodies (Santa Cruz Biotechnology) were used at 1 : 2500 dilution. Proteins were visualised with the SuperSignal West Femto Maximum sensitivity substrate kit (Thermo Scientific, Logan, UT, USA) and the signal detected using an LAS-3000 image analyzer (Fuji Photo Film Co., Tokyo, Japan).

Caspase-3/7 activity assay. Caspase-3/7 activity was determined using a commercially available Caspase-Glo 3/7 assay kit (Promega, Madison, WI, USA) as per the manufacturer's protocol. Briefly, cells were cultured in 96-well plate for 48 h and 100 μl of Caspase-Glo 3/7 reagent was added. Subsequently, cells were incubated at room temperature for 1 h and luminescence measured using VICTOR-X3 Multilabel Plate Reader (Perkin Elmer, Santa Clara, CA, USA).

Dual luciferase 3′UTR-reporter assay. For the validation of *BCL2* as a direct target of miR-345, cells were transiently co-transfected for 24 h with 200 ng of pLuc3U-BCL2 target-reporter plasmid containing BCL2 3′UTR region (Signosis, Santa Clara, CA, USA) along with 0.25 μg of control reporter plasmid (pRL-TK; Promega; plasmid containing a *Renilla reniformis* luciferase gene downstream of the thymidine kinase (TK) promoter. Moreover, as a control, we also generated a mutant BCL2 3′UTR (MUT-BCL2 3′UTR) reporter construct by site-directed mutagenesis in the putative target region of miR-345 using Quickchange XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) and transiently transfected as described above. After 48 h of transfection, cells were harvested in reporter lysis buffer (Promega). Firefly and Renilla luciferase activities were measured using a dual-luciferase assay kit (Promega) according to the manufacturer's instructions. The data are represented as the ratio of firefly to Renilla luciferase activity.

Statistical analysis. All the experiments were performed at least three times and numerical data expressed as mean ± s.d. The expression profiles of miR-345 in malignant pancreatic versus normal tissues were analysed using unpaired one-tailed Student's *t*-test. A pairwise comparison was performed to check if miR-345 had lower expression level in PCs than in NPs. A value of $P<0.05$ was considered to be significant throughout the study.

RESULTS

miR-345 is downregulated in PC. We examined the expression of miR-345 in a set of normal ($n=7$) and cancerous ($n=21$) pancreatic tissues, in an *in vitro* progression model cell lines (hTERT-HPNE and derived lines; Campbell *et al*, 2008) and a panel of established PC cell lines. Our data reveal that miR-345 is downregulated in the majority of PC cases as compared with the normal pancreas. Relative mean expression of miR-345 in the normal pancreatic tissues is significantly higher (41.93, *P*-value$\leqslant$0.001) than that of the malignant pancreatic tissues (11.85; Figure 1A). Furthermore, we observe that the expression of miR-345 is lost gradually in PC progression model cell lines (Figure 1B); whereas a differential expression pattern is seen among various established PC cell lines (Figure 1C). Notably, expression of miR-345 is relatively downregulated in poorly differentiated cell lines as compared with well-differentiated cell lines (Figure 1C). Altogether, our data demonstrate the downregulated expression of miR-345 in PC.

miR-345 promotes apoptosis in PC cells. To investigate the biological relevance of miR-345 downregulation in PC, we stably overexpressed it in two poorly differentiated PC cell lines, Panc1 and MiaPaCa (exhibiting low endogenous expression of miR-345). miR-345 overexpression was confirmed by real-time RT-PCR analysis. The data show that Panc1-miR-345 and MiaPaCa-miR-345 cells have ~63- and ~51-fold overexpression of miR-345, respectively, as compared with the control cell lines (Supplementary Figure 1). Growth analyses of miR-345-transfected cells revealed ~57.8% and ~52.6% reduction in overall growth by 5th day in Panc1-miR-345 and MiaPaCa-miR-345, respectively, relative to control vector (Panc1-Neo and MiaPaCa-Neo)-transfected cell lines (Figure 2A). Morphological examination demonstrated that miR-345-overexpressing PC cells became round and shrunken, and many of them detached from the bottom of the culture plate (data not shown). Therefore, we next examined the effect of miR-345 overexpression on chromatin condensation and nuclear fragmentation by Hoechst 33342 staining to confirm if the reduced growth and morphological alterations resulted from cellular apoptosis. Data demonstrate the presence of cells with fragmented nuclei and condensed chromatin in miR-345-overexpressing PC cells (Figure 2B), suggesting apoptosis induction upon restoration of miR-345 in PC cells. We then confirmed apoptosis induction by performing Annexin V and 7-AAD staining followed by flow cytometry. The data reveal a higher apoptotic index (Annexin V-positive/7-AAD-negative cells) in Panc1-miR-345 (48.5%) and MiaPaCa-miR-345 (36.5%) cells, as compared with their respective control cells, that is, Panc1-Neo (21.2%) and MiaPaCa-Neo (17%; Figure 2C). Importantly, the effect of miR-345 restoration on apoptosis was abolished following treatment of miR-345-overexpressing PC cells with anti-miR-345 (Figure 2C). Taken together, these results indicate that miR-345 promotes apoptosis in PC cells.

miR-345 activates mitochondrial pathways of apoptosis in PC. Mitochondria are known to have a central role in apoptosis (Elkholi *et al*, 2014; Bhat *et al*, 2015). Therefore, we examined the effect of miR-345 on the mitochondrial membrane potential ($\Delta\psi$m), and localisation/activation of the key proteins involved in apoptosis. Our flow cytometry analysis revealed a high proportion (35.6% and 29.2%, respectively) of damaged mitochondria/loss of $\Delta\psi$m in miR-345-overexpressing Panc1 and MiaPaCa cells as compared with their respective controls (Figure 3A). Moreover, we observed that the loss of $\Delta\psi$m was associated with increased levels of cytochrome *c* in the cytosol with a concomitant decrease in the mitochondria of miR-345-overexpressing cells (Figure 3B). Similarly, we also observed increased levels and activity of effector caspases (cleaved caspases-3 and -7) (Figure 3C and Supplementary Figure 2) along with PARP-1 cleavage in miR-345-overexpressing PC cells (Figure 3C). Interestingly, the effects of miR-345 overexpression on $\Delta\psi$m, cytochrome *c* translocation, and activation of caspases were attenuated by treatment with miR-345 inhibitor (Figure 3A–C). To explore the possibility of caspase-independent apoptosis, we examined the levels of AIF, known to induce apoptosis in a caspase-independent manner (Cande *et al*,

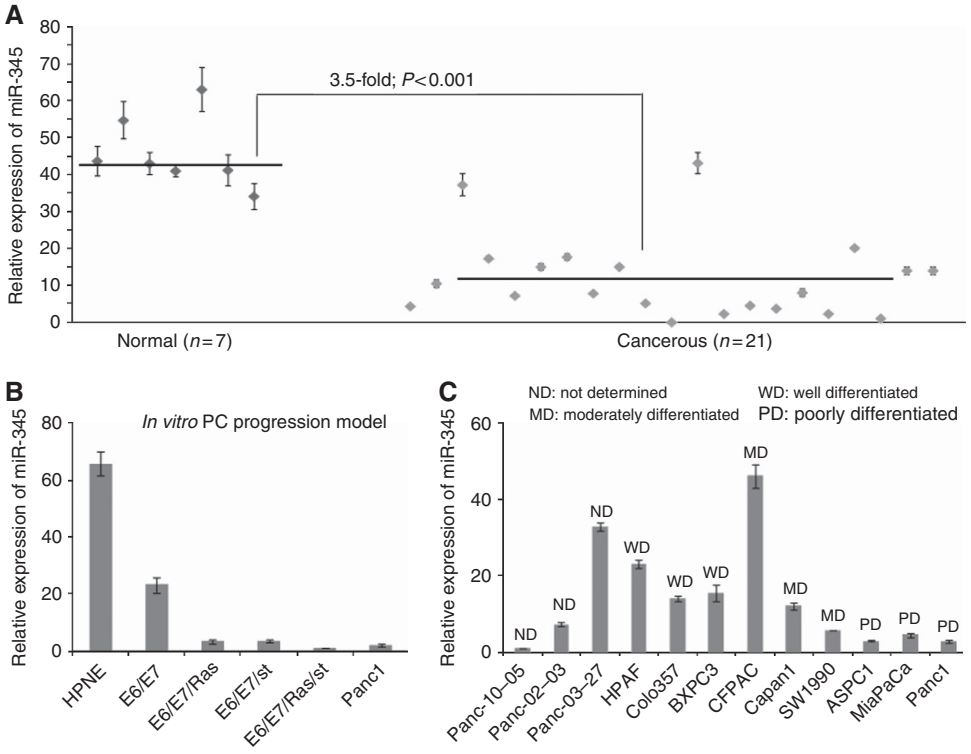

Figure 1. miR-345 is downregulated in pancreatic cancer. (**A**) miR-345 expression profiling was performed in normal ($n=7$) and cancerous ($n=21$) pancreatic tissue specimens. A decreased expression of miR-345 is observed in majority of cancer tissues as compared with the normal pancreas. Dark lines indicate mean expression level of miR-345 of the group and the fold reflects the ratio between the mean values of miR-345 expression in normal *vs* cancer cases. (**B**) Expression analysis of miR-345 in hTERT-HPNE PC *in vitro* progression model demonstrates a gradual decrease in the expression of miR-345. (**C**) A differential expression pattern of miR-345 is observed in PC cell lines. All the poorly differentiated cell lines exhibit relatively lower expression as compared with the well-differentiated cell lines. U6 small nuclear RNA served as an internal control. A relative quantity of miR-345 was determined using the $2^{-\Delta\Delta CT}$ method. Bars represent the mean ± s.d. ($n=3$).

2004). The data reveal that the level of nuclear AIF is increased with a concomitant decrease in its level in the mitochondrial fraction in miR-345-overexpressing Panc1 and MiaPaCa cells (Figure 3D). Furthermore, we observe that the level of mitochondrial AIF is regained in miR-345-overexpressing cells when they are treated with anti-miR-345 (Figure 3D). Together, our findings suggest that miR-345 induces apoptosis in PC cells by promoting caspase-dependent as well as -independent pathways of apoptosis.

miR-345 negatively regulates *BCL2* through direct binding to its 3′UTR. To identify the target of miR-345, we performed *in silico* analysis using the algorithms Target Scan (http://www.targetscan.org) and miRanda (http://www.microrna.org), and identified *BCL2*, an important molecule of the anti-apoptotic gene family, as putative target of miR-345. Further investigation revealed the presence of a 8-mer-binding site for miR-345 in the 3′UTR of the *BCL2* transcript (Figure 4A). To validate the potential targeting of BCL2 by miR-345, we examined its expression in a miR-345-overexpressing Panc1 and MiaPaCa cells. Our investigation revealed no change in the expression of *BCL2* at the transcript level (Figure 4B; upper panel); however, its expression decreased at the protein level in both Panc1-miR-345 and MiaPaCa-miR-345 cells as compared with their respective control cells (Figure 4B; upper panel), thus suggesting its translational repression by miR-345. To test whether *BCL2* is a direct target of miR-345, control and miR-345-overexpressing PC cells were transiently transfected with a luciferase reporter plasmid containing a region of *BCL2* 3′UTR having a wild-type or mutated miR-345 target site (Figure 4C). As shown in Figure 4D, our data demonstrate that miR-345 significantly suppressed the luciferase activity of the reporter plasmid with wild-type-*BCL2* 3′UTR in Panc1-miR-345 and MiaPaCa-miR-345 (~69% and ~83%, respectively) as compared with that in control cells. Furthermore, cells transfected with mutated-3′UTR did not show any response to the suppressor activity of miR-345 (Figure 4D). Altogether, our data suggest that *BCL2* is a direct target of miR-345.

BCL2 is involved in the miR-345-mediated activation of apoptotic pathways in PC cells. Following identification of *BCL2* as a direct target of miR-345, we further examined its significance in miR-345-mediated induction of apoptosis of PC cells. For this, expression vector of BCL2, which encodes the entire coding sequence of BCL2, but lacks the 3′UTR, was transiently transfected into the miR-345-overexpressing PC cells (Panc1-miR-345 and MiaPaCa-miR-345), and the effects on proteins associated with apoptosis pathways were analysed. Our immunoblot analysis shows that forced expression of BCL2 efficiently blocked the miR-345-induced activation of caspases, cleavage of PARP-1, and prevents the nuclear translocation of AIF (Figure 5A). Furthermore, we also examined the effect of BCL2 overexpression on the miR-345 decreased growth of PC cells. Our data demonstrate that forced expression of BCL2 abrogated the growth inhibitory effect of miR-345 in both Panc1 and MiaPaCa cells (Figure 5B). Together, these data suggest that miR-345 promotes apoptosis by suppressing the expression of BCL2, which triggers the mitochondrial pathway of apoptosis (Figure 6).

DISCUSSION

The recent discovery of miRNAs has revealed a novel mechanism of gene regulation and provided new avenues for cancer research (Srivastava *et al*, 2014). These miRNAs are highly deregulated in various malignancies, including PC, and their aberrant expression

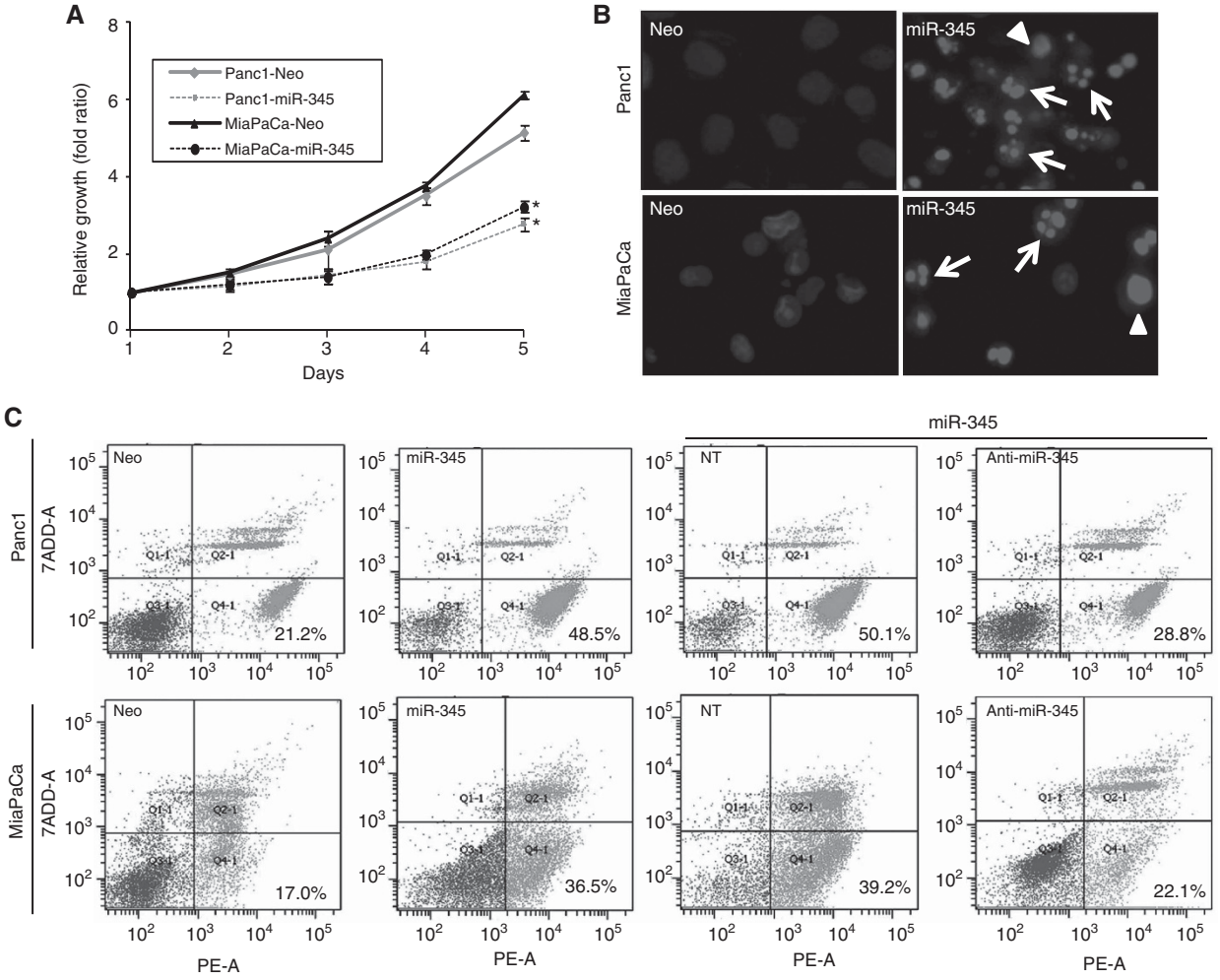

Figure 2. miR-345 restoration induces apoptosis in PC cells. (**A**) Cell growth was monitored by WST-1 assay daily up to 5 days. Data are presented as fold change in comparison to the cell growth on day 1. Data (mean ± s.d., $n = 3$; *$P < 0.05$) show decreased growth of miR-345-overexpressing PC cells relative to their respective controls. (**B**) PC cells were cultured up to 48 h in glass bottom fluorodishes, stained with Hoechst 33342, and examined and photographed using a fluorescent microscope. miR-345-overexpressing PC cells show apoptotic characteristics as evident by condensed (arrowhead) and fragmented (arrow) nuclei. Micrographs represent random fields of view (magnification × 400). (**C**) miR-345-overexpressing PC cells along with their control cells were seeded in six-well plate and media replaced after 24 h. After next 48 h incubation, cells were washed, stained with PE Annexin V, and analysed for apoptosis by flow cytometry. In parallel, miR-345-overexpressing PC cells were transiently transfected with anti-miR-345 or non-target (NT) sequence control, cultured up to 48 h and processed for apoptosis analysis. Histograms are representative of three independent experiments (Panc1, upper panel; MiaPaCa, lower panel). Data suggest a pro-apoptotic role of miR-345 in PC cells.

has been linked with initiation, progression, and dissemination of cancer cells. miR-345, which was identified to be one of the most significantly downregulated miRNA in PC (Bloomston *et al*, 2007; Lee *et al*, 2007; Szafranska *et al*, 2007), was studied for its functional role in PC. In corroboration with published reports, we also observed significant downregulation of miR-345 in majority of pancreatic tumour tissues and established PC cell lines. Moreover, we also observed a progressive loss of miR-345 in an *in vitro* progression model of PC. Although the mechanism(s) underlying miR-345 downregulation in PC is yet to be determined, it is likely that both genetic and non-genetic mechanisms are involved. An earlier study by Loukopoulos and co-workers demonstrated that chromosomal region 14q32-33, which harbours the gene encoding miR-345, is frequently lost in PC (Loukopoulos *et al*, 2007). Low expression of miR-345 has been observed in other malignancies as well and associated with clinicopathological features. miR-345 downregulation correlated with lymph node metastasis in clinical specimens of colorectal cancer, and its restoration suppressed colon cancer cell proliferation and invasiveness (Tang *et al*, 2011). Moreover, Pogribny *et al* (2010) identified a role of miR-345 downregulation in the development of cisplatin resistance in breast cancer cells. These studies strongly suggest that downregulation of miR-345 might be an important event in PC initiation and/or progression at least in a significant number of cases.

Apoptosis is a tightly regulated cell death process that has a critical role in growth and tissue homeostasis. In fact, the inherent ability to resist apoptosis is a hallmark of tumorigenesis. Moreover, preclinical studies in PC have shown that inhibition of apoptosis yields favorable response to gemcitabine cytotoxicity (Bauer *et al*, 2015; Guo *et al*, 2015). Therefore, enormous research has been done to understand the mechanisms of apoptosis resistance, associated regulatory pathways, and molecular targets for the development of novel anti-cancer therapies. In this regard, our study identified miR-345 downregulation as an important mechanism that confers apoptosis resistance to PC cells through mitochondrial pathways. Mitochondria have been very well recognised for their central role in regulating apoptosis. It is considered that loss of the mitochondrial membrane potential

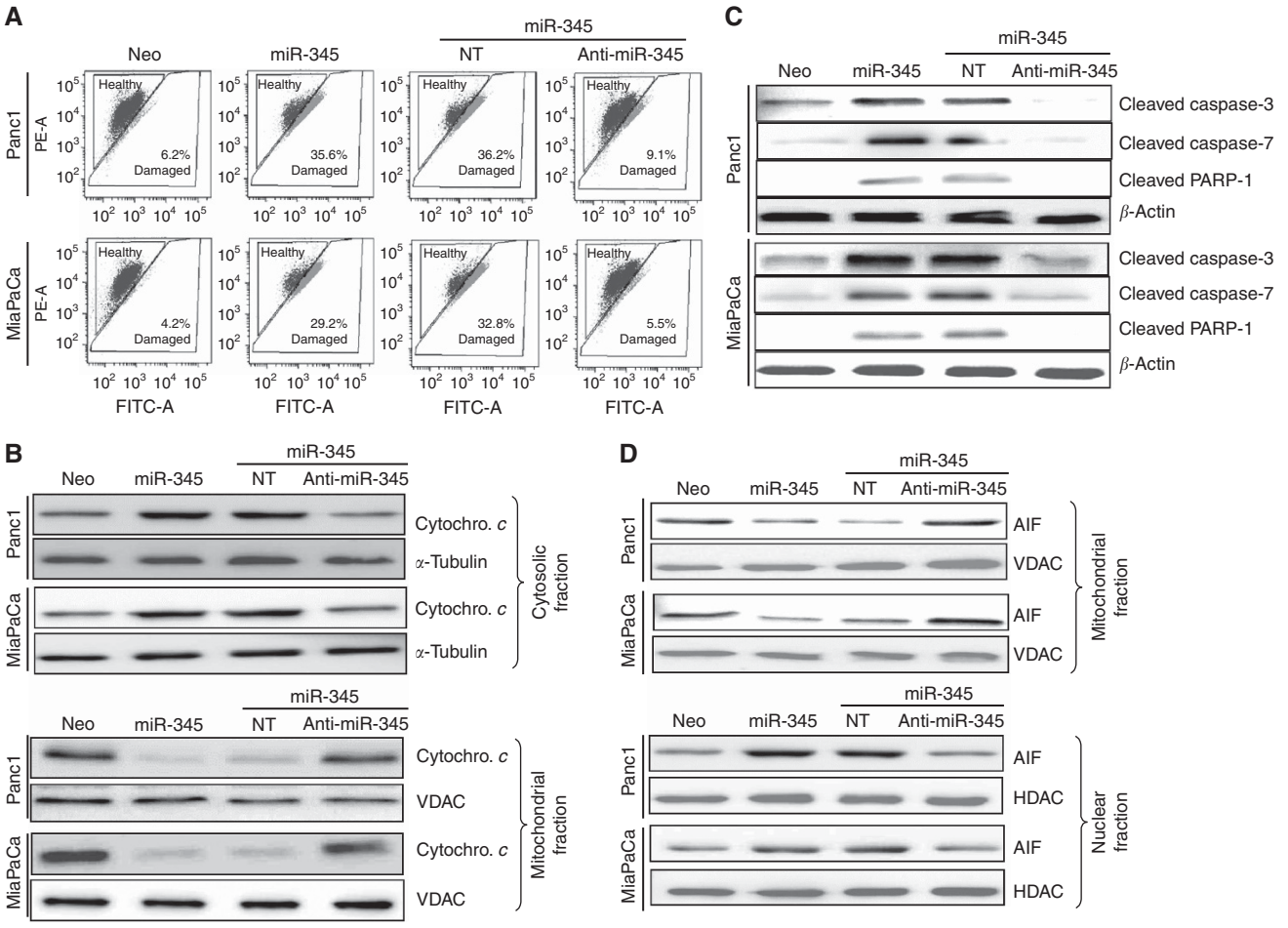

Figure 3. miR-345 promotes apoptosis through mitochondrial pathways. (**A**) miR-345-overexpressing PC (Panc1-miR-345 and MiaPaCa-miR-345) cells were transiently transfected with anti-miR-345 or non-target (NT) sequence control. After 48 h of transfection, cells were stained with JC-1 as described in Materials and Methods and subjected to flow cytometry. Dot plot shows cell population with intact mitochondria (having high $\Delta\psi m$; in blue); and population with damaged mitochondria (having low $\Delta\psi m$; in orange). Results demonstrate a loss of $\Delta\psi m$ in miR-345-overexpressing PC cells, an effect that was abrogated upon treatment with anti-miR-345. (**B**–**D**) Total, cytoplasmic, nuclear, or mitochondrial protein fractions were collected from low- and high-miR-345-expressing PC cells; and high-miR-345-expressing cells transiently transfected with anti-miR-345 or NT sequence control. Thereafter, expression of (**B**) cytochrome *c* (in cytosolic and mitochondrial fractions), (**C**) cleaved caspase-3, caspase-7, and PARP-1 (in total fraction) and (**D**) AIF (in nuclear and mitochondrial fractions) was examined by immunoblot assay. *β*-Actin, *α*-tubulin, VDAC, and HDAC were used as loading control for the total, cytosolic, mitochondrial, and nuclear fractions, respectively.

($\Delta\Psi m$) is the important event for the mitochondria-mediated apoptosis pathways (Gupta *et al*, 2009). Following the collapse of $\Delta\Psi m$, cytochrome *c* is released into the cytosol, where it forms complex with apoptotic protease-activating factor-1 and ATP. This complex then binds to pro-caspase-9, and causes its cleavage, which further initiates the activation of effector caspase-3 and -7. These effector caspases then cleave PARP-1, a known cellular substrates of caspases, whose cleavage by caspases is considered to be a characteristic of caspase-dependent apoptosis (Elmore, 2007). Along these lines, we demonstrated a disruption of $\Delta\Psi m$ in PC cells following miR-345 overexpression, release of cytochrome *c* from mitochondria to cytosol, and further activation of effectors caspase-3, caspase-7, and subsequent PARP-1 cleavage. Although caspase activation is considered a hallmark of apoptotic cell death, mitochondrially mediated apoptosis induction also occurs in a caspase-independent manner (Borner and Monney, 1999; Sperandio *et al*, 2000). AIF is localised to the mitochondrial intermembrane space, and upon apoptotic stimuli, AIF translocates to the nucleus where it causes chromatin condensation and nuclear fragmentation, resulting in cell death (Cande *et al*, 2004). Interestingly, in our study, we found that miR-345 overexpression induced the translocation of AIF to the nucleus. Thus, miR-345-induced apoptosis involved both cytochrome *c* (caspase-dependent) and AIF (caspase-independent) in the mitochondrial apoptotic pathway.

Findings from our study are quite interesting as we identified anti-apoptotic BCL2 to be a direct target of miR-345. Our study suggested that miR-345 downregulation-mediated upregulation of BCL2 could be a key event in triggering apoptosis resistance in PC cells. Several lines of evidence show that BCL-2 is overexpressed in variety of malignancies, including PC (Bold *et al*, 2001; Sun *et al*, 2002; Scherr *et al*, 2014), and its elevated levels have been shown to be associated with apoptosis-resistance and enhanced metastatic potential in PC (Bold *et al*, 1999, 2001). BCL2 keeps a check on the release of cytochrome *c* and AIF from mitochondria by preventing the disruption of mitochondrial membrane potential (Susin *et al*, 1996; Harris and Thompson, 2000; Tait and Green, 2010). We demonstrated that exogenous expression of BCL2 abrogated the miR-345-induced activation of caspases and prevented the translocation of AIF to the nucleus, favouring cell survival. Yang *et al* (1997) in their study investigated that overexpression of BCL2 prevents the initiation of the cellular apoptotic programme by

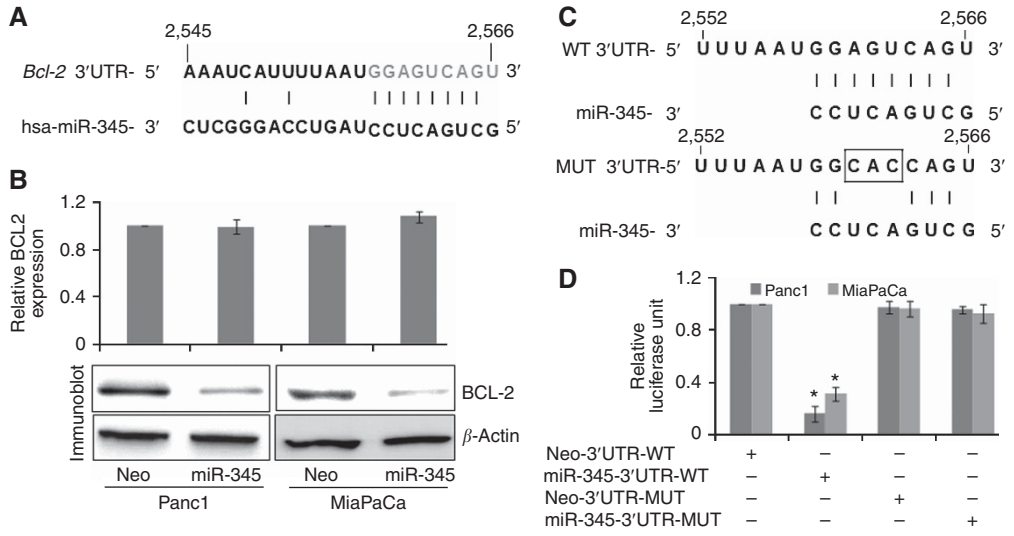

Figure 4. miR-345 suppresses BCL2 expression in PC cells through directly targeting its 3′UTR. (**A**) *In silico* analysis (using algorithms of Target Scan and miRanda) showing miR-345-binding sites in *BCL2* 3′UTR. (**B**) Total RNA and protein from control and miR-345-overexpressing PC cells were isolated and expression of *BCL2* was examined by qRT-PCR and immunoblot analysis. GAPDH and *β*-actin served as internal controls for RT-PCR and immunoblot analysis, respectively. Data show that expression of *BCL2* remained unaltered at the transcript level, but was repressed at the protein level in both Panc1-miR-345 and MiaPaCa-miR-345 cells. (**C**) Schematic representation of BCL2 3′UTR with either wild-type (WT) or mutant (MUT) miR-345 target site. *MUT*-3′UTR construct carries three nucleotides variation in the seed matching region of the target site to disrupt binding of miR-345. (**D**) PC cells were grown in six-well plate and transiently co-transfected with the luciferase promoter-reporter plasmids containing WT or MUT BCL2-3′UTR along with pRL-TK construct (transfection efficiency control) for 24 h. Thereafter, total protein was collected in passive lysis buffer and subsequently firefly/Renilla luciferase activities were measured using a dual-luciferase assay system. Data (mean ± s.d.; $n=3$) are presented as fold change in normalised luciferase activity. $^*P<0.05$. Results show that miR-345 suppresses the luciferase activity of the reporter plasmid containing WT 3′UTR of BCL2.

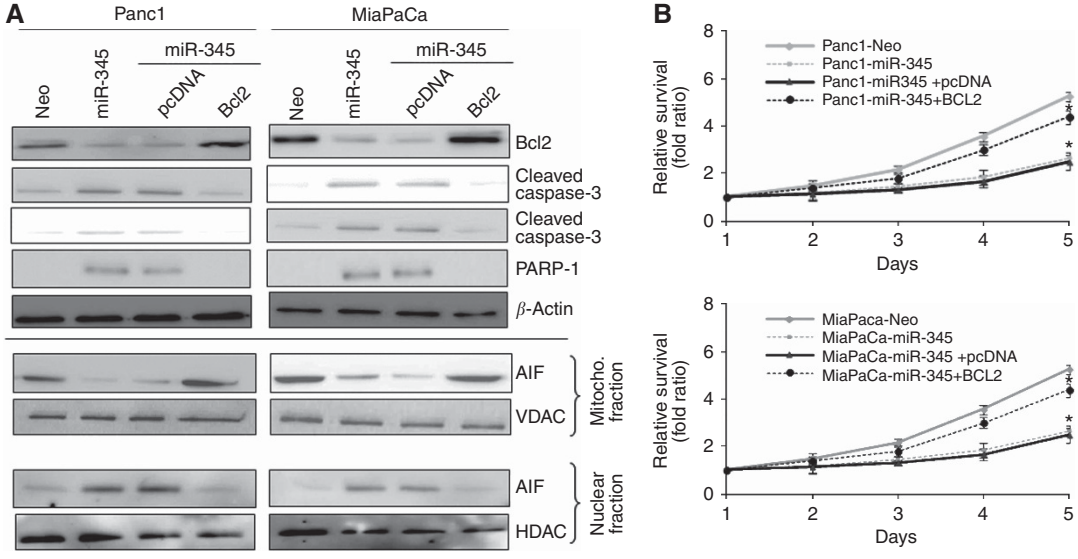

Figure 5. miR-345-mediated downregulation of BCL2 activates apoptosis. (**A**) miR-345-overexpressing PC cells were transiently transfected with BCL2-overexpressing (pcDNA3-BCL2) or control (pCDNA3) plasmids. After 48 h of transfection, total, nuclear, and mitochondrial fractions were collected and effect on the expression of BCL2, cleaved caspases-3 and -7, and cleaved PARP-1 (in total) and AIF (in nuclear and mitochondrial) was examined by immunoblot analysis using specific antibodies. *β*-Actin, VDAC, and HDAC were used as loading control for the total, mitochondrial, and nuclear fractions, respectively. Data demonstrate that forced expression of BCL2 blocked the miR-345-induced activation of caspases, PARP-1, and prevents the nuclear translocation of AIF. (**B**) Panc1-miR-345 and MiaPaCa-miR-345 cells were transiently transfected with BCL2-overexpressing or control plasmids. After 24 h of transfection, cells were trypsinised, counted, and seeded (5×10^3 per well) in 96-well plate. Thereafter, cell growth was monitored by WST-1 assay daily upto 5 days. Data (mean ± s.d.; $n=3$) presented as fold change as compared with the cell growth on day 1. $^*P<0.05$. Results show that forced expression of BCL2 abrogated the growth inhibitory effect of miR-345 in PC cells.

blocking the release of cytochrome *c* from mitochondria. Moreover, a separate study by Susin *et al* (1998) demonstrated that BCL2 interferes with permeability transition pores and prevents the release of AIF. In this regard, the ability of miR-345 to downregulate the anti-apoptotic gene BCL2 is highly significant.

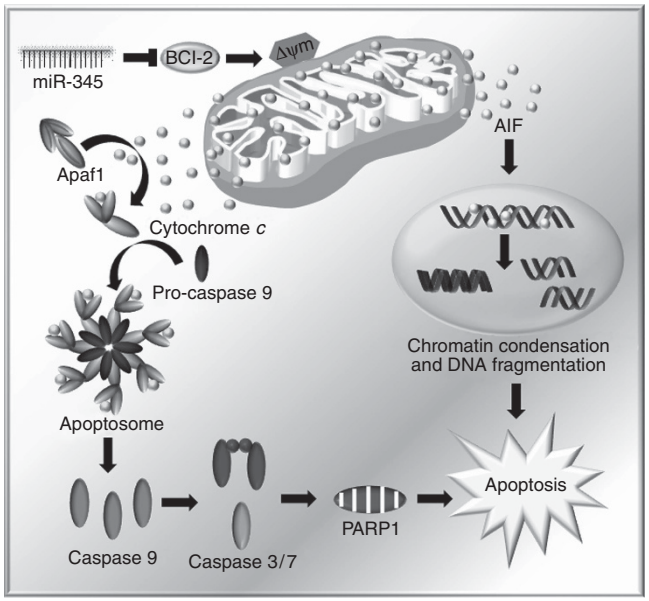

Figure 6. The proposed mechanistic model of miR-345-induced apoptosis in PC cells. The anti-apoptotic BCL2 protein guards the outer mitochondrial membrane and controls apoptosis by maintaining the mitochondrial permeability. miR-345 inhibits BCL2 through its direct binding, and its inhibition result in the loss of ΔΨm, which allows cytochrome *c* to be released from the mitochondria into the cytosol. Released cytochrome *c* then binds with apoptotic protease-activating factor-1 and procaspase-9, forms apoptosome, activates caspases-9 and -3/7 and PARP1 cleavage, and causing apoptosis. At the same time of cytochrome *c* release, AIF is also liberated from the mitochondria, translocates to the cell nucleus, and causes DNA fragmentation and condensation resulting in cell death.

In summary, we showed that downregulation of miR-345 was a frequent event in PC, and this downregulation significantly correlated with PC progression. Furthermore, ectopic expression of miR-345 in PC cells dramatically reduced cell growth and induced apoptosis. We further identified BCL2 as functional target of miR-345, and proved the involvement of the mitochondrial pathway in miR-345-promoted apoptosis. Our findings thus suggest an essential role of miR-345 in regulating apoptosis, and implicate the potential application of miR-345 in PC therapy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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