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Survivin-targeting miR-542-3p overcomes HER3 signaling-induced chemoresistance and enhances the antitumor activity of paclitaxel against HER2-overexpressing breast cancer

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Abstract

Elevated expression of HER3, which interacts with HER2 in breast cancer cells, confers chemoresistance via phosphoinositide 3-kinase (PI-3K)/Akt-dependent upregulation of Survivin. However, the underlying mechanism is not clear. Ectopic expression or specific knockdown of HER3 in HER2-overexpressing breast cancer cells did not alter *Survivin* mRNA levels and Survivin protein stability, supporting the notion that HER3 signaling may regulate specific miRNAs that target *Survivin* to alter its protein translation. Here we showed that overexpression and specific knockdown of HER3 reduced and enhanced expression of two *Survivin*-targeting miRNAs, miR-203 and miR-542-3p, in breast cancer cells, respectively. While the specific inhibitor of either miR-203 or miR-542-3p attenuated an anti-HER3 antibody-induced downregulation of Survivin, inhibition of miR-542-3p exhibited a better efficacy than miR-203 inhibition did. Consistently, miR-542-3p mimic was much more effective than miR-203 mimic not only in inhibition of Survivin, but also in enhancement of paclitaxel-induced apoptosis in HER2-overexpressing breast cancer cells. Moreover, the combination of miR-542-3p mimic and paclitaxel, as compared with either agent alone, significantly inhibited *in vivo* tumor growth of HER2-overexpressing breast cancer cells. Collectively, our data indicated that the HER3/PI-3K/Akt signaling upregulates Survivin via suppression of miR-203 and miR-542-3p. Because miR-542-3p has three binding sites on the 3′-UTR of *Survivin* mRNA, its mimic was able to

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Conflicts of interest

The authors declare no conflict of interest.

effectively downregulate Survivin *in vitro* and *in vivo*. Thus, miR-542-3p-replacement therapy is an excellent approach to overcome HER3-mediated paclitaxel resistance and significantly enhances the antitumor activity of paclitaxel against HER2-overexpressing breast cancer.

Keywords

HER3; Survivin; miRNA; Paclitaxel resistance; Breast cancer

1. Introduction

The HER3 receptor is a unique member of the erbB family of receptor tyrosine kinases (RTKs), which are often aberrantly activated in a wide variety of human cancers [1,2]. Unlike other family members, HER3 has no or little intrinsic kinase activity [3,4]. It frequently co-expresses and interacts with other RTKs in cancer cells to activate oncogenic signaling, such as the PI-3K/Akt pathway, MEK/MAPK pathway, Src kinase, etc. [3,5,6]. Although recent studies have identified oncogenic *erbB3* gene mutations in colon and gastric cancers [7], overexpression of HER3 receptor is still the major mechanism for its enhanced signaling, which is associated with poor clinical outcomes in patients with solid tumors [8]. HER3 signaling has been shown to play a pivotal role in the development of *erbB2*-altered (gene amplification and/or overexpression) breast cancer [9,10], castration-resistant prostate cancer (CRPC) [11], and ovarian cancer [12,13]. Mechanistic studies have indicated that one of the major functions of HER3 signaling is to cause cancer treatment failure [14,15]. We have shown that elevated expression of HER3 renders HER2-overexpressing breast cancer cells resistant to tamoxifen [16], trastuzumab (Herceptin), lapatinib [17,18], and the chemotherapeutic drug paclitaxel [19]. Thus, effective inhibition of HER3 is thought to be required to overcome drug resistance and to effectively treat HER2-overexpressing breast cancer. In studying the molecular basis of HER3-induced paclitaxel resistance, we found that overexpression of HER3 led to a PI-3K/Akt-dependent upregulation of Survivin [19]. Specific knockdown of Survivin significantly enhanced paclitaxel-induced apoptosis in the otherwise resistant, HER3-expressing breast cancer cells [19], suggesting that Survivin is a valuable target for chemo-sensitization to abrogate HER3-mediated paclitaxel resistance. Nonetheless, the precise mechanism by which HER3 signaling upregulates Survivin in HER2-overexpressing breast cancer cells remains unclear.

Survivin is a dual-function protein acting as a critical inhibitor of apoptosis (IAP) and a key regulator of cell cycle progression [20]. It is produced in embryonic tissues and undetectable in most adult tissues. Overexpression of Survivin is observed in almost all human malignancies and increased Survivin correlates with poor clinic outcomes, tumor recurrence, and drug resistance in cancer patients [21,22]. Because of its selective expression in tumor tissues, Survivin has been recognized as an attractive therapeutic target [20,23]. Several approaches, including use of transcriptional inhibitor YM155, antisense oligonucleotide, gene therapy, and immunotherapy, have been designed to target Survivin [21,22,24,25]. YM155, a leading inhibitor, has become the first choice in Survivin-related studies and is being used in clinical trials of cancer treatment (https://clinicaltrials.gov/ct2/results?term=YM155&Search=Search). However, concerns have been raised regarding the

mechanism of action and specificity of YM155 [26]. A recent study revealed that YM155 failed to improve response rates to paclitaxel and carboplatin in patients with advanced non-small cell lung cancer (NSCLC) [27]. This failure is probably due to the lack of specificity of YM155 and its insufficient inhibition of Survivin in patients [24]. It is believed that novel strategy/agents that can effectively downregulate Survivin *in vivo* are required to increase chemotherapeutic efficacy, thereby reducing the risk of relapse and improving the survival of cancer patients.

In the current study, we focused on elucidating the molecular basis of HER3 signaling-induced upregulation of Survivin in HER2-overexpressing breast cancer cells. Using both an *in vitro* cell culture system and an *in vivo* tumor xenograft model, we also investigated whether the newly identified mechanism-based strategy, miRNA-replacement therapy, can effectively inhibit Sur-vivin, thereby overcoming HER3-mediated paclitaxel resistance and significantly enhancing the antitumor activity of paclitaxel against HER2-overexpressing breast cancer.

2. Materials and methods

2.1. Reagents and antibodies

The miRIDIAN has-miR-203 and has-miR-542-3p specific inhibitors, mimics, and their negative controls were purchased from Thermo Scientific Dharmacon (Lafayette, CO). In vivo-jetPEI® *in vivo* DNA and siRNA delivery reagent was purchased from Polyplus-transfection® SA (New York, NY). The Akt inhibitor VIII was purchased from EMD Chemicals, Inc. (Gibbstown, NJ). Paclitaxel (Ben Venue Labs, Inc., Bedford, OH) was obtained from University of Colorado Hospital pharmacy. The fully human anti-HER3 antibody MM-121 was kindly provided by Merrimack Pharmaceuticals Inc. (Cambridge, MA).

The primary antibodies used for western blot analyses were obtained as follows: Survivin (6E4) (Abcam, Cambridge, MA); Mcl-1 (Santa Cruz Biotechnology, Inc., Dallas, TX); Bcl-xl, caspase-8 (1C12), caspase-3 (8G10), and PARP (Cell Signaling Technology, Inc., Beverly, MA); β-actin (AC-75, Sigma-Aldrich, St. Louis, MO). All other reagents were purchased from Sigma-Aldrich unless otherwise specified.

2.2. Cells and cell culture

Human breast cancer cell lines SKBR3, BT474, MDA-MB-453, and HCC1954 were obtained from the American Type Culture Collection (Manassas, VA). The trastuzumab-resistant subline BT474-HR20 was described previously [17]. The identity of all cell lines was confirmed with DNA profiling by the University of Colorado Cancer Center's DNA Sequencing Core facility. Cell lines were free of mycoplasma contamination, as determined by the MycoAlert™ Mycoplasma Detection Kit (Lonza Group Ltd., Basel, Switzerland) once every three months. All cell lines were maintained in DMEM/F-12 (1:1) medium containing 10% FBS, cultured in a 37 °C humidified atmosphere containing 95% air and 5% CO_2, and split twice a week.

2.3. Transfection of cells with miRNA mimic or inhibitor

Cell transfection with miRNA mimic, inhibitor, or controls was carried out using HiPerFect Transfection Reagent (QIAGEN Inc., Valencia, CA) as described previously [28].

2.4. Quantification of apoptosis

An apoptotic enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics Corp., Indianapolis, IN) was used to quantitate cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) as previously reported [17,19,28].

2.5. Western blot analysis

Protein expression was determined by western blot assays as described previously [18,19,28]. Equal amounts of total cell lysates were boiled in Laemmli SDS sample buffer, resolved by SDS-PAGE, transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA), and probed with the primary antibodies described in the figure legends.

2.6. Reverse transcription (RT)-PCR and quantitative real-time (qRT)-PCR

Total RNA was extracted using a modified chloroform/phenol procedure (TRIZOL®, Invitrogen, Carlsbad, CA). First-strand cDNA was generated using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Human *Survivin* mRNA expression was examined by conventional RT-PCR as we described previously [28–30]. To quantify the human *Survivin* and *erbB3* mRNA levels, qRT-PCR was performed using the Absolute* Blue qPCR Master Mixes (Thermo Fisher Scientific Inc., Waltham, MA) according to the manufacturer's protocol. The expression of *β-actin* was used as an internal control for both conventional RT-PCR and qRT-PCR. All qRT-PCR reactions were carried out on a 7500 Fast Real-Time PCR system (Applied Biosystems). Sequences of the specific primers were reported previously [28,31].

2.7. Analysis of miRNA expression

The expression levels of miRNAs were determined as described previously [28,31]. In brief, total RNA, including small RNA, was extracted and purified using the miRNeasy® Mini Kit (QIAGEN Inc.). TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) was used to generate cDNA with the primers specific to the mature miRNA. The expression levels of miR-203, miR-542-3p, Let-7c, and miR-29b were then measured by qRT-PCR using TaqMan Assays (Assay ID: 000507, 001284, 000379, 000413, respectively; Applied Biosystems). RNU6B (Assay ID: 001093; Applied Biosystems) was used as an internal control. The relative miRNA levels were calculated by the comparative Ct method (ΔΔCt).

2.8. Immunohistochemistry (IHC)

IHC was performed as described previously [31–33]. The specificity of all antibodies - Ki67 (ThermoFisher Scientific; rabbit monoclonal SP6; 1:500 dilution in TBST + 1% BSA), cleaved caspase-3 (Cell Signaling Technology; rabbit polyclonal; 1:1000), and Survivin (Epitomics, Burlingame, CA; rabbit monoclonal EP2880Y; 1:100) - were confirmed by both positive and negative controls. For Survivin, endometrial cancer tissues originating from

ovary were used as positive controls. For Ki67 and cleaved caspase-3, human tonsil tissues were used as positive controls. For the negative controls, the same cells/tissues were used without addition of the primary antibodies. Antibody complexes were visualized with IP Flex DAB (Biocare Medical, Concord, CA). All sections were counterstained with Mayer's hematoxylin and nuclei were blued with 1% ammonium hydroxide (v/v). The sections were then cleared with xylene and mounted with synthetic resin and a cover glass.

2.9. Tumor xenograft model

Athymic nu/nu mice (Charles River Laboratories, Inc., Wilmington, MA) were maintained in accordance with the Institutional Animal Care and Use Committee (IACUC) procedures and guidelines approved by the University of Colorado Anschutz Medical Campus Animal Care and Use Committee. Five × 10^5 HCC1954 cells in 100 μL of PBS mixed with 50% Matrigel (BD Biosciences, San Jose, CA) were injected subcutaneously into the 6-week-old female mice as described previously [31–33]. Therapy began when the tumor volume reached ~50 mm^3. The tumor-bearing mice were randomly assigned into four groups (n = 5): 1) control mice receiving intra-peritoneal (i.p.) injection of 100 μL PBS and intratumoral injection of 5 μg miRNA negative mimic; 2) mice receiving i.p. injection of 100 μL PBS and intratumoral injection of 5 μg has-miR-542-3p mimic; 3) mice receiving i.p. injection of paclitaxel (6 mg/kg) in 100 μL PBS and intratumoral injection of 5 μg miRNA negative mimic; 4) mice receiving i.p. injection of paclitaxel (6 mg/kg) and intratumoral injection of 5 μg has-miR-542-3p mimic. For *in vivo* administration of miRNA mimic, the negative control or miR-542-3p mimic was complexed with *in vivo*-JetPEI at an N/P ratio of 6 in 5% glucose solution (a total of 20 μL) for intratumoral injection. The mice were treated twice a week for three weeks. The animals' health status was monitored daily for weight loss or signs of altered motor ability while in their cages. At the end of study, mice were euthanized according to the approved IACUC protocol. Tumors from all animals were excised and embedded in paraffin for histology and IHC analyses.

2.10. Statistical analysis

Statistical analyses of the experimental data were performed using the two-sided student's *t*-test. A *P*-value < 0.05 was deemed statistically significant. All statistical analyses were conducted with the software StatView v5.1 from SAS Institute Inc. (Cary, NC).

3. Results

3.1. The HER3 receptor regulated Survivin expression independent of gene transcription and protein stability in HER2-overexpressing breast cancer cells

In studying the contribution of HER3 receptor to paclitaxel resistance in HER2-overexpressing breast cancer cells, we generated stable *erbB3*-transfectants (SKBR3.B3.pool, SKBR3.B3.1, SKBR3.B3.2) and empty vector control clone (SKBR3.Neo.1) from SKBR3 cells [19]. The increased HER3 interacted with HER2, leading to paclitaxel resistance via a PI-3K/Akt-dependent upregulation of Survivin but not the functionally related molecules Mcl-1 and Bcl-xL [19]. To further elucidate the molecular mechanism by which HER3 signaling enhanced expression of Survivin, we first evaluated if HER3 expression might affect *Survivin* mRNA levels. Total RNA samples obtained from

SKBR3 cells and their *erbB3*-transfectants as well as the vector control clone were subjected to the conventional RT-PCR analyses of *Survivin* and *β-actin*. We found that elevated expression of HER3 in SKBR3 cells did not increase the mRNA levels of *Survivin* (Fig. 1A, left). Similar results were also observed in another HER2-overexpressing breast cancer HCC1954 cell line stably or transiently transfected with an expression vector containing human *erbB3* cDNA (Supplementary Fig. S1). Conversely, specific knockdown of HER3 expression with a shRNA sequence, which was proven to be specific and effective in previous studies [17,19], in SKBR3 and MDA-MB-453 cells (both are HER2-overexpressing breast cancer cell lines) did not decrease *Survivin* mRNA levels (Fig. 1A, right). To confirm these observations, qRT-PCR assays were then performed. Our studies revealed that changes of HER3 expression in HER2-overexpressing breast cancer cells had no significant effect on *Survivin* mRNA levels (Fig. 1B). We next examined whether HER3 expression might alter Survivin protein stability. The series SKBR3 cell lines were treated with cycloheximide (CHX), a protein synthesis inhibitor, for 4, 8, or 16 h. The cells were then collected and subjected to western blot analyses of Survivin. Quantitative analysis showed that the degradation rates of Survivin protein remained similar in HER3-low (SKBR3.neo.1 and SKBR3) and HER3-high (SKBR3.B3.pool and SKBR3.B3.1) expressing cells (Fig. 1C). Collectively, our data demonstrated that overexpression of HER3 upregulates Survivin via a mechanism independent of gene transcription and protein stability, and it may enhance Survivin protein translation in HER2-overexpressing breast cancer cells.

3.2. Modulation of HER3 signaling specifically influenced expression of two Survivin-targeting miRNAs, miR-203 and miR-542-3p

It is well-known that miRNAs, a class of small noncoding RNAs, negatively regulate gene expression at the post-transcriptional level. A miRNA generally binds to the 3′ UTR of its target mRNA to regulate gene expression by promoting mRNA degradation or repressing protein translation [34,35]. Numerous studies have demonstrated that miRNAs play highly diverse roles in the development of human cancers [36–39]. As no significant change of *Survivin* mRNA levels was found upon alterations of HER3 in HER2-overexpressing breast cancer cells (Fig. 1A and B), we hypothesized that HER3/PI-3K/Akt signaling inhibited certain miRNA(s) to relieve the translational suppression of Survivin. We thus tested if manipulation of HER3 expression altered expression of the miRNAs that target *Survivin* mRNA. qRT-PCR revealed that ectopic expression of HER3 in SKBR3 cells (SKBR3.B3.1 and SKBR3.B3.2 vs SKBR3.neo.1) significantly reduced the expression levels of miR-203 and miR-542-3p (Fig. 2A), both of which have been reported to target *Survivin* mRNA [40,41]. The increased HER3 had no effect on the other two *Survivin*-targeting miRNAs, miR-494 and miR-320a [42] (data not shown). Consistent with our findings on Mcl-1 and Bcl-xL [19], elevated expression of HER3 had little effect on miR-29b and Let-7c (Fig. 2A), that target *Mcl-1* and *Bcl-xL*, respectively [43,44]. Furthermore, overexpression of HER3 in SKBR3 cells via transient infection with the lentivirus containing human *erbB3* cDNA significantly reduced expression levels of miR-203 and miR-542-3p, but not miR-29b and Let-7c (Fig. 2B). Similarly, using HCC1954-*erbB3* stable clones and HCC1954 cells transiently transfected with *erbB3* cDNA, we also found a significant reduction in levels of both miR-203 and miR-542-3p upon increased HER3 expression (Supplementary Fig. S2). In contrast, specific knockdown of HER3 (Fig. 2C) or an Akt inhibitor (Fig. 2D)

significantly increased the expression levels of miR-203 and miR-542-3p, but not miR-29b and Let-7c, in both SKBR3.B3.1 and SKBR3.B3.2 cells. Taken together, these data strongly supported that activation of HER3/PI-3K/Akt signaling specifically suppresses expression of miR-203 and miR-542-3p, thereby upregulating Survivin in HER2-overexpressing breast cancer cells.

3.3. HER3 blockade downregulated Survivin in HER2-overexpressing breast cancer cells via induction of miR-203 and miR-542-3p

We have shown that Survivin plays a critical role in HER3-mediated paclitaxel resistance in HER2-overexpressing breast cancer cells [19], and HER3 blockade with the anti-HER3 monoclonal antibody MM-121 overcomes this resistance via specific downregulation of Survivin [33]. Our current data suggested that reduction of miR-203 and miR-542-3p is the major mechanism resulting in upregulation of Survivin. Thus, we speculated that MM-121 might induce expression of both miR-203 and miR-542-3p to inhibit Survivin in breast cancer cells co-expressing HER2 and HER3. Indeed, treatment of SKBR3.B3.1, SKBR3.B3.2, and MDA-MB-453 cells with MM-121 significantly enhanced expression of both miR-203 and miR-542-3p in a time-dependent manner (Fig. 3A). Since the parental SKBR3 cells also express a large amount of HER3 [19], MM-121 treatment was able to markedly induce expression of both miR-203 and miR-542-3p in SKBR3 cells (Supplementary Fig. S3). In contrast, MM-121 had little effect on expression of the two miRNAs in MDA-MB-231 cells, which express significantly lower levels of HER3 as compared with those expressed by SKBR3 cells [19]. These data suggested that the anti-HER3 antibody MM-121 can only increase expression levels of miR-203 and miR-542-3p in breast cancer cells highly expressing HER3.

To determine whether the induction of miR-203 and miR-542-3p played a causal role in MM-121 inhibition of Survivin, specific miRNA inhibitors were used. While the inhibitor of either miR-203 or miR-542-3p was able to reverse the inhibitory effect of MM-121 on Survivin, inhibition of miR-542-3p appeared more effective than miR-203 inhibition in attenuation of MM-121 activity. Combinations of the miRNA inhibitors most potently abrogated MM-121-induced downregulation of Survivin (Fig. 3B and C), suggesting that both miR-203 and miR-542-3p contributed to MM-121-mediated reduction of Survivin. These results were not only in agreement with our previous findings [19,33] and the data shown above (Figs. 1 and 2), but also emphasized that induction of miR-203 and miR-542-3p may serve as a useful biomarker upon HER3 blockade to specifically reduce Survivin in HER2-overexpressing breast cancer cells.

3.4. MiR-542-3p mimic overcame HER3-mediated paclitaxel resistance and significantly enhanced the antitumor activity of paclitaxel against HER2-overexpressing breast cancer

Recent studies have supported miRNAs as promising therapies for human cancers, and several miRNA-targeted therapeutics are now being tested in clinical trials of cancer treatments [39,45]. We wondered if miR-203 or miR-542-3p could be developed as a novel therapeutic to downregulate Survivin, thereby overcoming HER3-mediated chemoresistance in HER2-overexpressing breast cancer. We found that inhibition of miR-542-3p seemed to be more effective than miR-203 inhibition in attenuation of the suppressive activity of

MM-121 toward Survivin (Fig. 3B and C). Further bioinformatics analysis showed that miR-542-3p has three binding sites on the 3′-UTR of *Survivin* mRNA, whereas miR-203 has one [46]. These exciting findings inspired us to hypothesize that miR-542-3p might be more effective than miR-203 in inhibition of Survivin in HER2-overexpressing breast cancer cells. To test this hypothesis, SKBR3.B3.1 or SKBR3.B3.2 cells transfected with either control miRNA mimic, miR-203 mimic, or miR-542-3p mimic were subjected to the following experiments. First, western blot assays revealed that a smaller amount of miR-542-3p mimic (10 nmol/L) was more efficient than a greater amount of miR-203 (40 nmol/L) to specifically downregulate Survivin, whereas the miRNA mimics had no significant effect on the expression of Mcl-1 and Bcl-xL (Fig. 4A). Similar results were also obtained from HCC1954-pLEX, HCC1954-*erbB3*, and BT474-HR20 (BT474-derived trastuzumab-resistant subline [17]) cells (Supplementary Fig. S4A). Second, we examined whether the miRNA mimics could overcome HER3-mediated paclitaxel resistance. MiRNA mimic-transfected SKBR3.B3.1 or SKBR3.B3.2 cells were then treated with paclitaxel for an additional 24 h. Our data showed that miR-542-3p mimic (10 nmol/L), as compared with miR-203 mimic (40 nmol/L), more profoundly enhanced paclitaxel-induced apoptosis, indicated by increased PARP cleavage (a hallmark of apoptosis), caspase-3 activation (Fig. 4B), and histone-associated DNA fragments detected by an apoptotic ELISA (Fig. 4C). We also observed that miR-542-3p mimic (2.5 nmol/L), as compared with control mimic or miR-203 mimic (10 nmol/L), in combination with paclitaxel potently induced PARP cleavage and caspase-3 activation in HCC1954-pLEX, HCC1954-*erbB3*, and BT474-HR20 cells (Supplementary Fig. S4B). Thus, the mimic of miR-542-3p was able to effectively inhibit Survivin and dramatically overcome HER3-mediated paclitaxel resistance in HER2-overexpressing breast cancer cells.

To develop the *Survivin*-targeting miR-542-3p as a novel therapeutic against HER2-overexpressing breast cancer, we took advantage of a tumor xenograft model established from HCC1954 cells to test the chemo-sensitization activity of miR-542-3p *in vivo*. When the tumor volume reached ~50 mm^3, the tumor-bearing mice were treated with either negative control miRNA mimic (miR-NC, 5 μg/100 μL PBS), miR-542-3p mimic (5 μg/100 μL PBS), paclitaxel (6 mg/kg) and miR-NC mimic (5 μg/100 μL PBS), or paclitaxel (6 mg/kg) and miR-542-3p mimic (5 μg/100 μL PBS). All treatments were carried out via intraperitoneal injection of PBS/paclitaxel and/or intratumoral injection of miR-NC/miR0542-3p twice a week for three weeks. Our data showed that treatment with either paclitaxel or miR-542-3p alone slightly reduced tumor growth, and the inhibitory effects were not statistically significant (Fig. 5A). However, the combination of miR-542-3p and paclitaxel significantly inhibited tumor growth as compared with either agent alone (Fig. 5A). These data suggested that miR-542-3p can enhance paclitaxel-mediated antitumor activity against HER2-overexpressing breast cancer in *in vivo* tumor xenograft models. After six treatments, the remaining tumors obtained from each group were subjected to histology and IHC analyses. Treatment with either miR-542-3p or paclitaxel alone appeared to have little effect on tumor mass architecture, whereas less tumor mass and larger empty spaces between tumor cells were observed upon the combined treatment (Fig. 6A, H & E). As our *in vitro* studies showed that miR-542-3p mimic profoundly facilitated paclitaxel-induced apoptosis in HER2-overexpressing breast cancer cells (Figs. 4 & S4), we investigated

whether the combination of miR-542-3p mimic and paclitaxel would exert similar effects on cell proliferation and apoptosis *in vivo*. Tumor tissues obtained from the animal experiments were then examined by IHC analyses of the cell pro-liferative marker Ki67 and the apoptosis marker cleaved caspase-3 as well as the expression of Survivin. Treatment with miR-542-3p mimic alone significantly downregulated Survivin, slightly reduced Ki67-positive tumor cells, and increased the tumor cells with cleaved caspase-3 (Fig. 6A and B). Paclitaxel alone significantly decreased the tumor cells expressing Ki67 and Survivin and slightly induced caspase-3 cleavage in the tumor cells. Importantly, the combination of miR-542-3p and paclitaxel than either agent alone exhibited more profound activity to reduce Ki67-positive cells, downregulate Survivin, and increase the tumor cells with cleaved caspase-3 (Fig. 6B). Taken together, our data indicated that the mimic of miR-542-3p effectively reduced Survivin and potently enhanced paclitaxel-mediated antitumor activity against HER2-overexpressing breast cancer via inhibition of proliferation and induction of apoptosis *in vivo*.

4. Discussion

Recent advances in our understanding of the unique features of the HER3 receptor in cancer biology indicate that activation of HER3 signaling is one of the major mechanisms resulting in human cancer treatment failure [14,15]. It is believed that effective targeting of HER3 is required to enhance cancer treatment in the clinic. Because the HER3 receptor possesses weak or no kinase activity [3,4], therapeutic strategy against HER3 is limited to a blocking antibody. Several anti-HER3 antibodies are currently being tested in preclinical studies [47] and clinical evaluations (http://www.clinicaltrials.gov). To date, no HER3-targeted therapy has been approved for cancer treatment. We have shown that overexpression of HER3 confers paclitaxel resistance via PI-3K/Akt- dependent upregulation of Survivin in HER2-overexpressing breast cancer cells [19], and the anti-HER3 antibody MM-121 is able to downregulate Survivin and abrogate the resistance [33]. Thus, identification of the crucial downstream mediator(s) that relay(s) HER3 signaling-induced Survivin expression and paclitaxel resistance shall facilitate the development of novel approaches to inhibit HER3 signaling and thereby enhance the therapeutic efficacy of paclitaxel against HER2-overexpressing breast cancer. This study not only identified miR-203 and miR-542-3p as the crucial mediators downstream of HER3/PI-3K/Akt signaling, but also supported the rational design of a miRNA-based therapeutic strategy inhibiting Survivin to overcome HER3-mediated paclitaxel resistance.

Although targeting of Survivin has long been researched in use of cancer treatment [20,48], the currently available strategies lack specificity and effectiveness [21]. The research data presented here provided us an exciting opportunity to examine a novel miRNA-based approach to inhibit Survivin. To determine whether miR-203 and miR-542-3p may be useful *Survivin*-targeted therapeutics, we first performed bioinformatics analyses and discovered that miR-542-3p has three binding sites on the 3′-UTR of *Survivin* mRNA, whereas miR-203 has one [46]. This interesting observation inspired us to test the hypothesis that the miRNAs with multiple binding sites on the 3′-UTR of *Survivin* mRNA shall be more effective than those with a single binding site in downregulation of Survivin. Indeed, both our *in vitro* cell culture studies and *in vivo* animal experiments showed that introduction of

miR-542-3p mimic, even a lesser amount than miR-203 mimic, not only exhibited more potent activity to specifically downregulate Survivin, but also markedly increased paclitaxel-mediated antitumor effects via significant inhibition of proliferation and induction of apoptosis. Our data strongly suggested that miR-542-3p-replacement therapy holds potential to be developed as a novel strategy inhibiting Survivin, thereby enhancing the therapeutic efficacy of paclitaxel against HER2-overexpressing breast cancer. Our study also supported the hypothesis that functional cooperation exists among the multiple binding sites of one miRNA, which agrees with our recent report showing that miR-125a, miR-125b, and miR-205 acted in concert to inhibit HER3 expression in HER2-overexpressing breast cancer cells [28]. It is likely that the multiple binding sites of one miRNA and the "sister" miRNAs, which have common targets [49], act synergistically to repress the target. Thus, the miRNAs with multiple binding sites may be more promising in miRNA-replacement therapy. Further investigations are warranted to validate miR-542-3p as a novel, effective *Survivin*-targeted therapeutic for cancer treatment. Since miR-542-3p functions as a tumor suppressor in human cancers [50,51], its antitumor activity has been evaluated in bladder cancer, colorectal cancer, hepatocellular carcinoma, and melanoma. Some of the studies also found that miR-542-3p targeted *Survivin* to exert antitumor effects [52,53]. Interestingly, combinations of the Survivin inhibitor (YM155) and miR-542-3p more potently promoted apoptosis of colon cancer cells [52]. In addition, miR-542-3p inhibited tumor growth, cancer cell migration and invasion, and/or metastasis by targeting cortactin (*CTTN*) or OTUB1 in colorectal cancer cells [54,55], the serine/threonine protein kinase, PIM1 in melanoma [56], and FZD7/Wnt signaling in hepatocellular cancer cells [57]. It is clear that miR-542-3p exerts anticancer activity via inhibition of multiple targets and signaling pathways.

The molecular mechanism by which HER3 signaling downregulates miR-203 and miR-542-3p in HER2-overexpressing breast cancer cells remains elusive. Interestingly, epigenetic silencing of the two miRNAs via promoter methylation has been frequently observed in a wide variety of human cancers [50,58–60], including breast cancer [51,61]. In addition, histone modification, including acetylation and methylation of specific lysine residues [62], is another important epigenetic mechanism governing gene expression [63]. It is unknown if HER3 signaling regulates expression of miR-203 and/or miR-542-3p via histone modifications. Our recent profiling studies of histone modifications revealed that elevated expression of HER3 significantly altered a number of histone H3 marks, including H3K9 acetylation (H3K9ac), H3K4 dimethylation (H3K4me2), and H3K9me2. RNA-seq analyses also revealed that increased HER3 expression affected the mRNA levels of several enzymes controling histone modifications (data not shown). We are currently testing the hypothesis that histone modifications contribute to HER3 signaling-induced downregulation of miR-203 and/or miR-542-3p in HER2-overexpressing breast cancer cells.

In summary, we demonstrated that HER3/PI-3K/Akt signaling specifically inhibits miR-203 and miR-542-3p to upregulate Survivin in HER2-overexpressing breast cancer cells. The mimic of miR-542-3p exhibited a remarkable inhibitory effect on Survivin, and thereby potently facilitated paclitaxel-induced apoptosis in HER2-overexpressing breast cancer cells *in vitro* and *in vivo*. Our data suggested that miR-542-3p mimic is an effective therapeutic agent inhibiting Survivin, and miR-542-3p-replacement therapy should significantly enhance the antitumor activity of paclitaxel against HER2-overexpressing breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.canlet.2018.01.065.

Abbreviations

RTKs	Receptor tyrosine kinases
PI-3K	Phosphoinositide 3-kinase
CHX	Cycloheximide
ELISA	Enzyme-linked immunosorbent assay
IHC	Immunohistochemistry
i.p	Intraperitoneal

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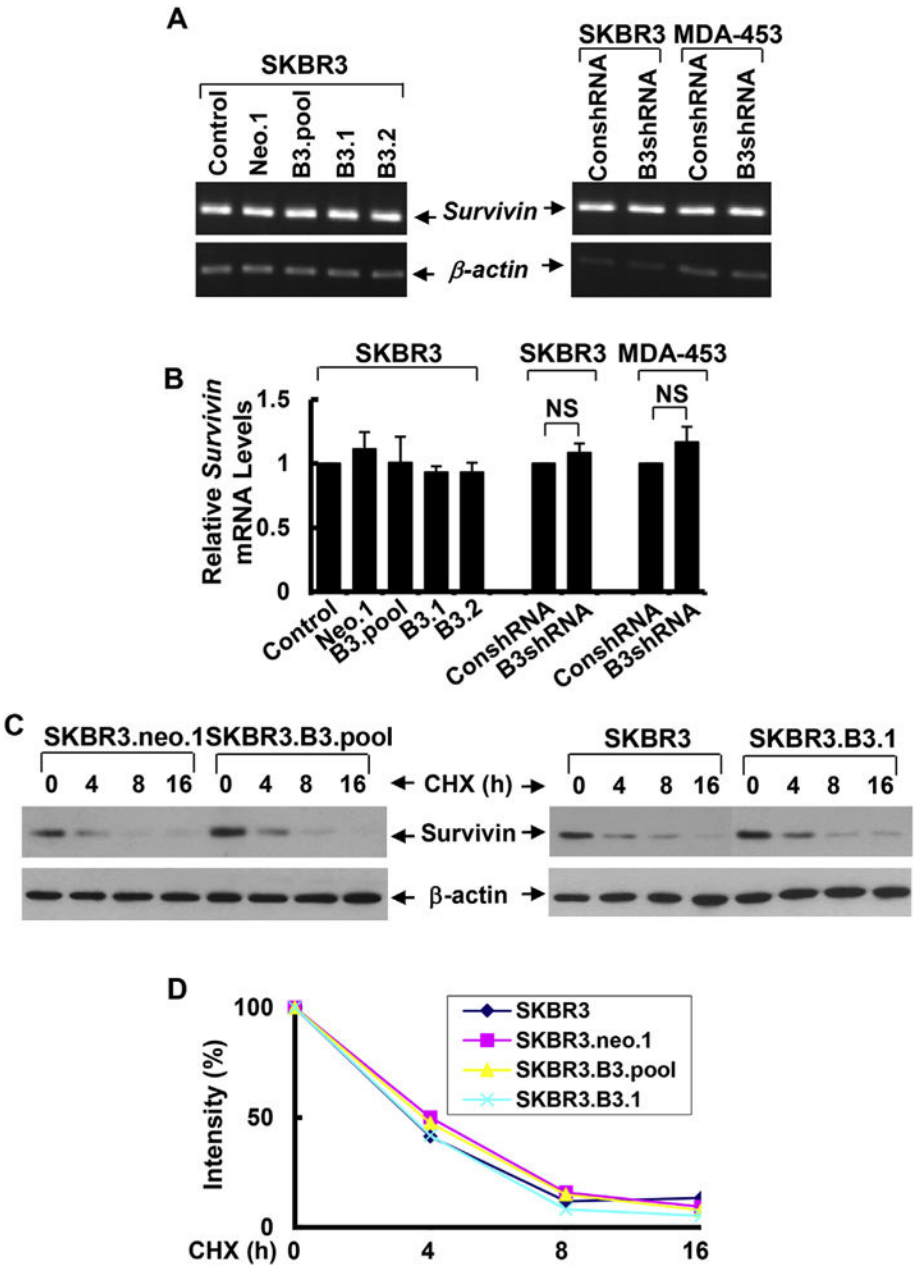

Fig. 1. Ectopic expression of HER3 or specific knockdown of HER3 does not affect Survivin mRNA levels and its protein stability in HER2-positive breast cancer cells

SKBR3 cells and the expression vector transfectants (Neo.1, B3.pool, B3.1, and B3.2) or SKBR3 and MDA-MB-453 (MDA-453) cells transfected with a specific shRNA against human *HER3* were subjected to total RNA extraction. **A**, First-strand cDNA was synthesized using a reverse transcription kit. The partial coding sequence of *Survivin* or *β-actin* was amplified with specific primers. The PCR products were separated on a 1.8% agarose gel containing ethidium bromide and visualized under UV light. **B**, The mRNA levels of *Survivin* were measured by qRT-PCR. *Bars*, S.D. Data represent the results of three independent experiments. **C**, SKBR3 cells or the *HER3*-transfectants were treated with cycloheximide (CHX) for 0, 4, 8, or 16 h. Total cell lysates of each cell line collected at

indicated time points were examined by western blot analyses of Survivin. **D**, The intensities of Survivin signals were determined by densitometry analyses and normalized to control (0 h), which was set as 100%.

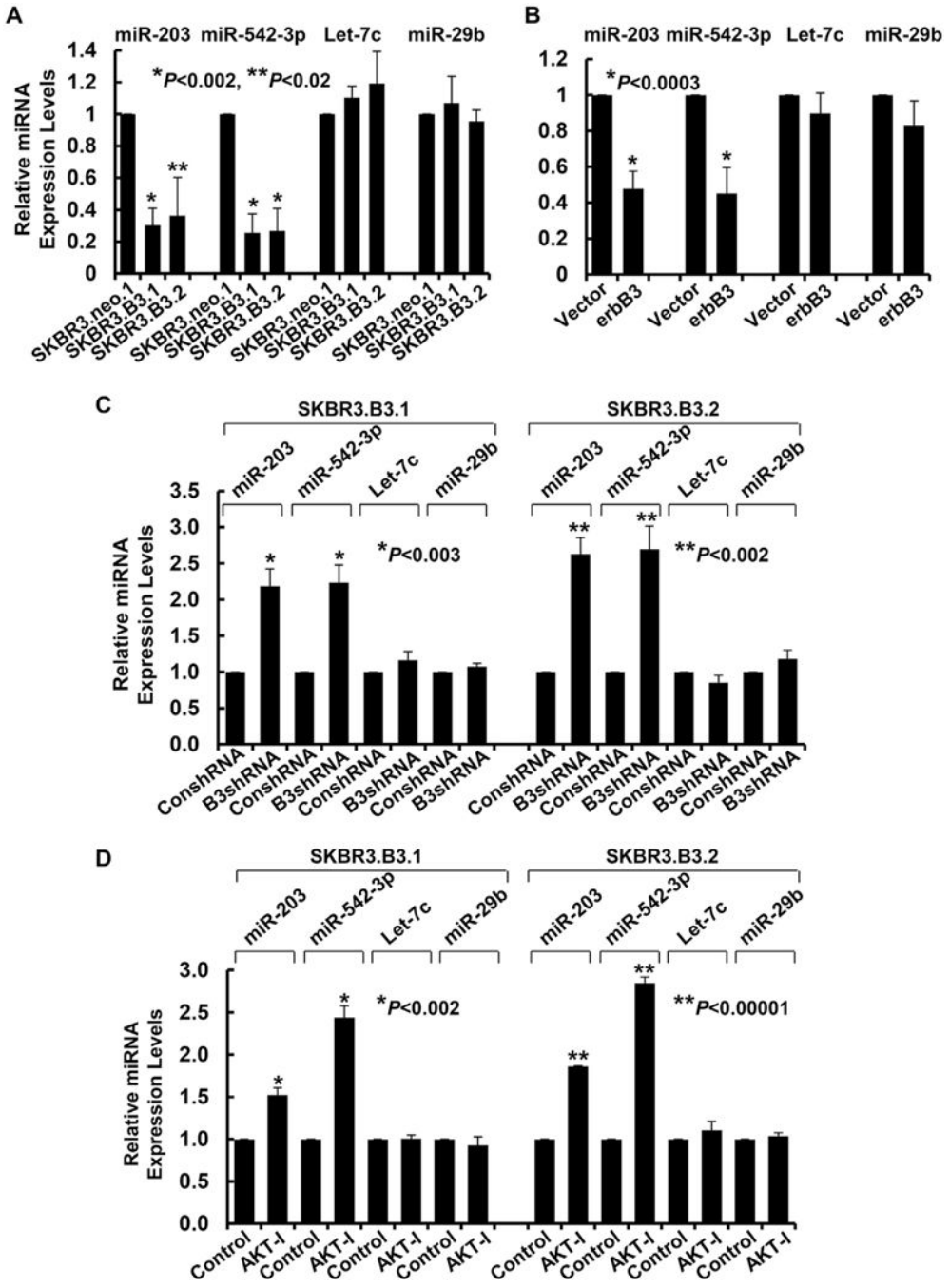

Fig. 2. Modulation of the HER3/Akt signaling specifically influences expression of two *Survivin*-targeting miRNAs, miR-203 and miR-542-3p

Cells were collected and subjected to total RNA extraction, inclusive of the small RNA fraction. The expression levels of miR-203, miR-542-3p, Let-7c and miR-29b were measured by qRT-PCR using TaqMan miRNA assays. All results were normalized according to the internal control RNU6B. *Bars*, S.D. Data represent the results of three independent experiments. A & B, Ectopic expression of HER3 (A) or transient transfection with an expression vector containing human *HER3* cDNA (B) in SKBR3 cells significantly reduced the expression levels of miR-203 and miR-542-3p, but not Let-7c and miR-29b. C, Specific

knockdown of HER3 expression in SKBR3.B3.1 and SKBR3.B3.2 sublines significantly enhanced expression of miR-203 and miR-542-3p, but not Let-7c and miR-29b. D, A specific Akt inhibitor significantly enhanced expression of miR-203 and miR-542-3p, but not Let-7c and miR-29b, in SKBR3.B3.1 and SKBR3.B3.2 cells.

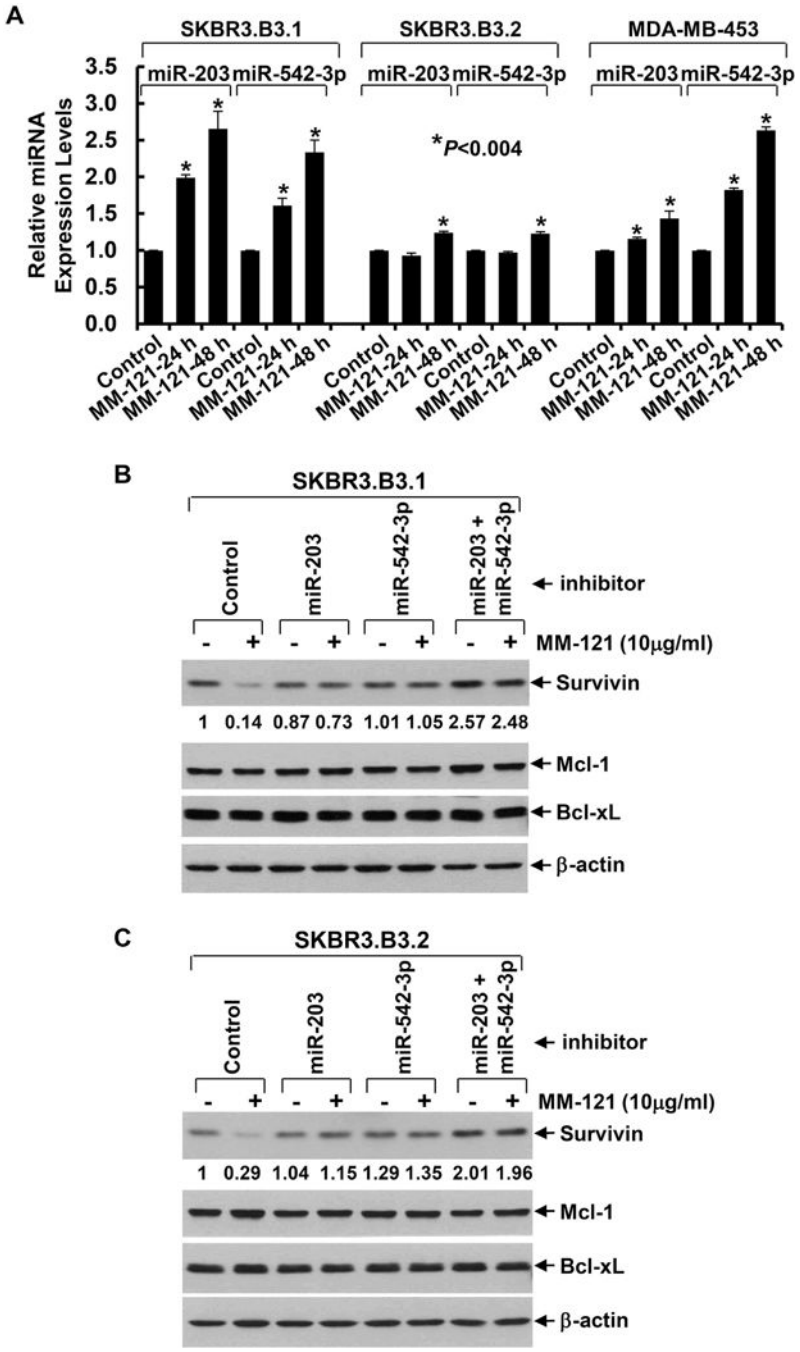

Fig. 3. Blockade of HER3 with monoclonal antibody MM-121 downregulates Survivin via induction of miR-203 and miR-542-3p in HER2-positive breast cancer cells

A, SKBR3.B3.1, SKBR3.B3.2, and MDA-MB-453 cells treated with MM-121 (10 μg/mL) for 24 or 48 h were collected and subjected to total RNA extraction. The expression levels of miR-203 and miR-542-3p were measured by qRT-PCR using TaqMan miRNA assays. RNU6B was used as an internal control. *Bars*, S.D. Data show the representative of three independent experiments. **B** & **C**, SKBR3.B3.1 or SKBR3.B3.2 cells were transfected with either negative control miRNA inhibitor (Control) or the specific inhibitor(s) of miR-203

(miR-203) and/or miR-542-3p (miR-542-3p). After 24 h, the cells were then untreated or treated with MM-121 (10 μg/mL) for another 24 h. Cells were collected and subjected to western blot analyses with specific antibody against Survivin, Mcl-1, Bcl-xL, or β-actin. The densitometry analyses of Survivin signals are shown underneath, and the arbitrary numbers indicate the intensities of each cell line relative to controls, defined as 1.

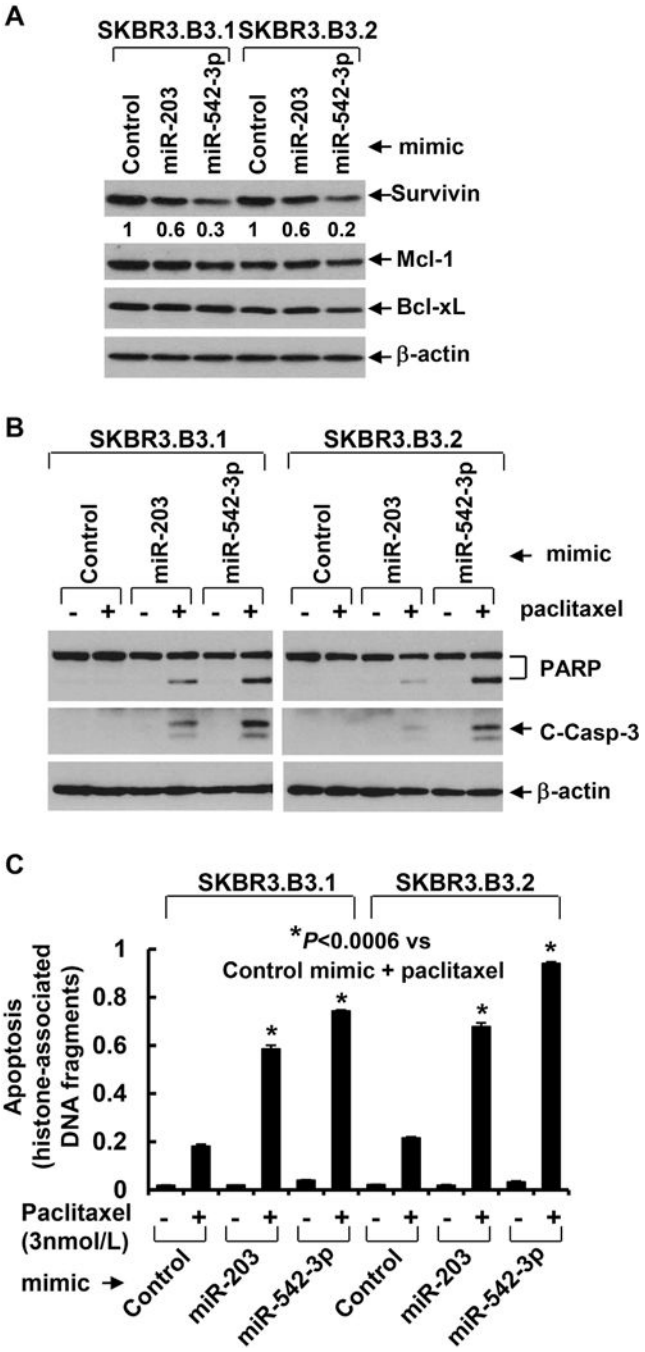

Fig. 4. The mimic of miR-542-3p effectively downregulates Survivin and significantly enhances paclitaxel-induced apoptosis in HER2-positive breast cancer cells

SKBR3.B3.1 or SKBR3.B3.2 cells were transfected with the negative control miRNA mimic (Control, 40 nmol/L) or the mimic of miR-203 (40 nmol/L) or miR-542-3p (10 nmol/L) for 24 h. **A**, Cells were collected for western blot analyses of Survivin, Mcl-1, Bcl-xL, or β-actin. **B** & **C**, The cells were then untreated or treated with paclitaxel (3 nmol/L) for another 24 h and subjected to western blot analyses of PARP, cleaved caspase-3 (C-Casp-3), or β-

actin (**B**) or a specific apoptosis ELISA (**C**). *Bars*, S.D. Data represent the results of three independent experiments.

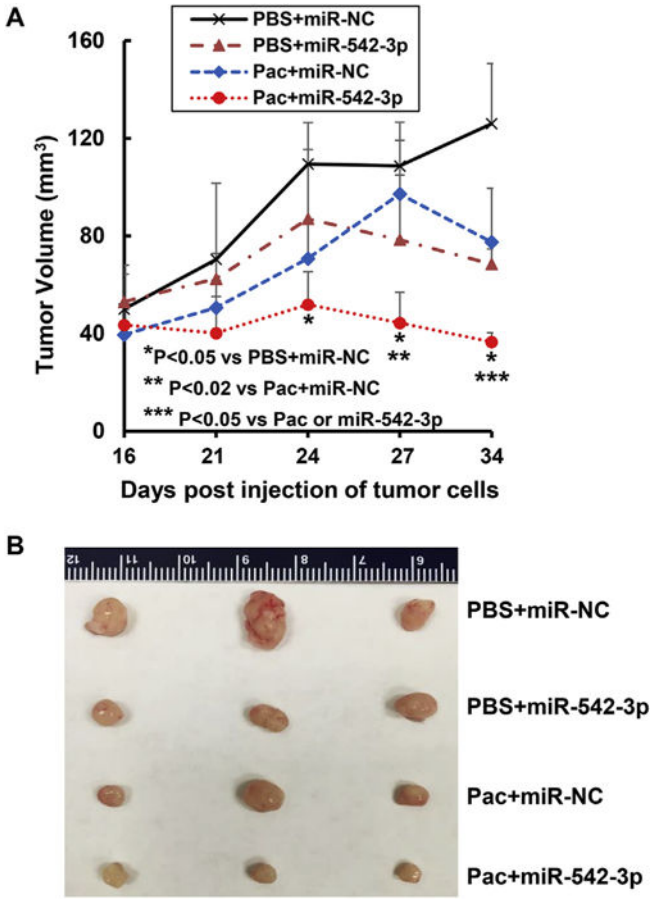

Fig. 5. The miR-542-3p mimic in combination with paclitaxel significantly inhibits *in vivo* growth of tumor xenografts. HCC1954 cells were injected subcutaneously in nude mice to establish tumor xenografts

The tumor-bearing mice (n = 5) received treatments of PBS + negative control miRNA mimic (miR-NC), PBS + miR-542-3p mimic (miR-542-3p), paclitaxel (Pac) + miR-NC, or Pac + miR-542-3p as described in the Materials and methods. After six treatments, the mice were euthanized at day 34 post injection of tumor cells. All tumors were excised for histology and IHC analysis. **A**, The graphs show the tumor growth curves. *Bars*, SD. **B**, Data show the representative tumors from each treatment group.

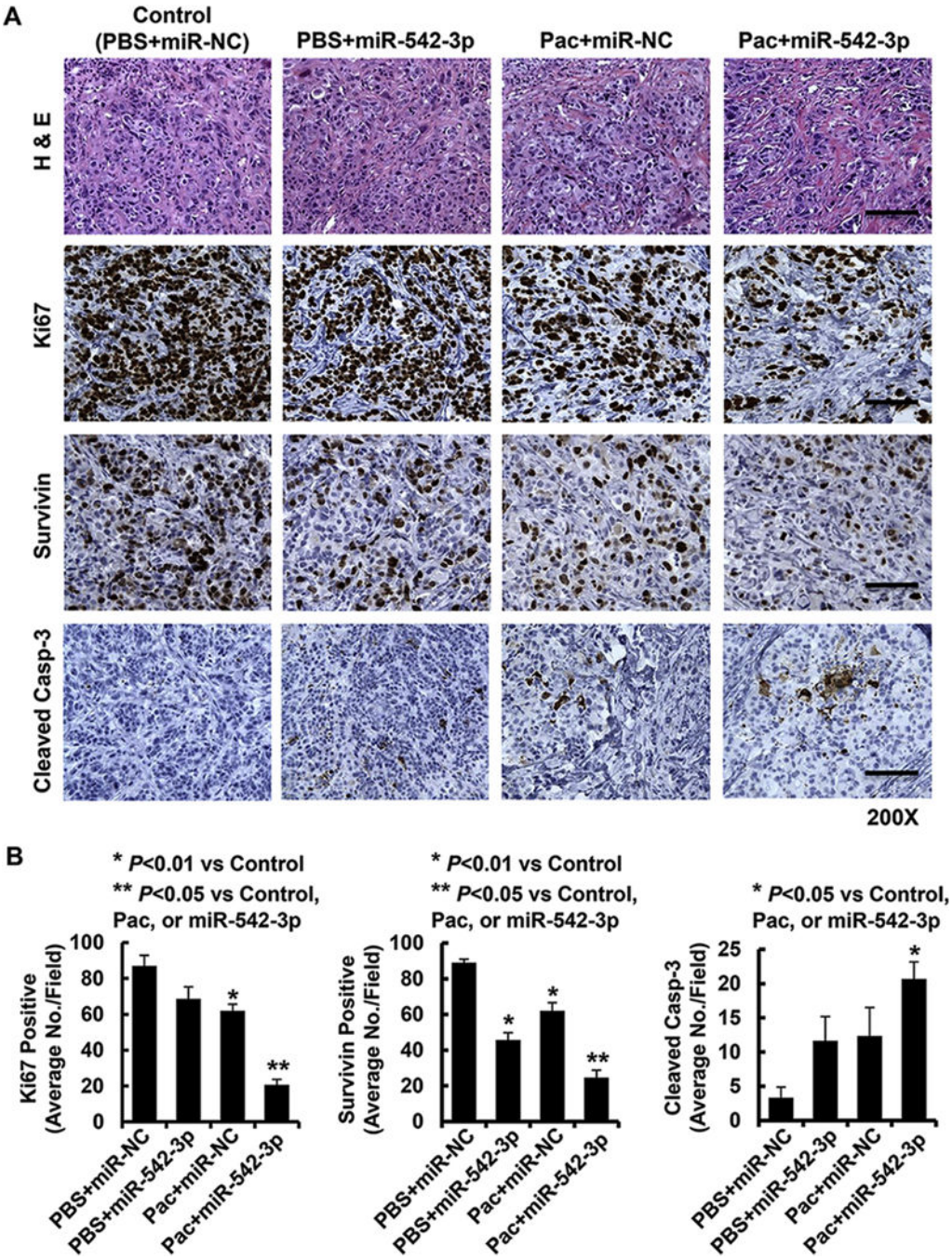

Fig. 6. The combination of miR-542-3p and paclitaxel significantly downregulates Survivin, inhibits proliferation, and induces apoptosis of HCC1954 breast cancer cells *in vivo*

The tumors obtained from the animal experiments were evaluated by IHC analysis of Ki67, Survivin and cleaved caspase-3. **A**, Data show the representative images of representative tumors with hematoxylin and eosin (H & E) staining and immunostaining of Ki67, Survivin and cleaved caspase-3 (Cleaved Casp-3). **B**, The IHC slides were observed by two independent personnel. The tumor cells with positive staining of Ki67, Survivin, or cleaved caspase-3 were counted from three randomly selected areas in each slide. The three areas

were first identified by scanning the entire slide at ×10 magnification, and then the positively stained cells were counted at ×20 magnification using an Olympus B×40 microscope (Tokyo, Japan). A minimum of 500 tumor cells were evaluated. If differences occurred between spot intensities, the most positive spot was considered. The evaluations were recorded as percentages of positively stained target cells in each field. The bar graphs show the mean percentages of positively stained cells in each field. *Bars*, SD.