

# Survivin-targeting Artificial MicroRNAs Mediated by Adenovirus Suppress Tumor Activity in Cancer Cells and Xenograft Models

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Survivin is highly expressed in most human tumors and fetal tissue, and absent in terminally differentiated cells. It promotes tumor cell proliferation by negatively regulating cell apoptosis and facilitating cell division. Survivin's selective expression pattern suggests that it might be a suitable target for cancer therapy, which would promote death of transformed but not normal cells. This was tested using artificial microRNAs (amiRNAs) targeting survivin. After screening, two effective amiRNAs, which knocked down survivin expression, were identified and cloned into a replication-defective adenoviral vector. Tumor cells infected with the recombinant vector downregulated expression of survivin and underwent apoptotic cell death. Further studies showed that apoptosis was associated with increases in caspase 3 and cleaved Poly (ADP-ribose) polymerase, and activation of the p53 signaling pathway. Furthermore, amiRNA treatment caused blockade of mitosis and cell cycle arrest at the G2/M phase. *In vivo*, survivin-targeting amiRNAs expressed by adenoviral vectors effectively delayed growth of hepatocellular and cervical carcinomas in mouse xenograft models. These results indicate that silencing of survivin by amiRNA has potential for treatment of cancer.

*Molecular Therapy—Nucleic Acids* (2014) 3, e208; doi:10.1038/mtna.2014.59; published online 4 November 2014

**Subject Category:** siRNAs, shRNAs, and miRNAs Therapeutic proof-of-concept

## Introduction

Cancer cells are characterized by defective regulation of proliferation and apoptosis resulting in uncontrolled cell growth, which eventually leads to the demise of the organism.<sup>1</sup> Several mechanisms reduce apoptosis of cancer cells thereby aberrantly prolonging their life span.<sup>2,3</sup>

Survivin, also called baculoviral inhibitor of apoptosis repeat-containing 5 or BIRC5, is the smallest member of the inhibitor of apoptosis family. It is evolutionarily highly conserved and its expression is restricted to tumor cells and fetal tissues but missing in terminally differentiated cells. Expression of survivin is controlled by the cell cycle and it is only detectable in cells during G2/M transition. Survivin's main role is to block apoptosis by inhibiting Fas and Bax pathways and the activity of caspases 3 and 7. In addition, survivin localizes to the mitotic spindle during cell division and may thereby promote proper separation of chromosomes.<sup>2,4–14</sup> As survivin is selectively expressed in tumors, it provides a suitable target for therapy as has been shown *in vitro* and *in vivo*.

RNA interference (RNAi) has been widely applied to biological studies and it provides a promising therapeutic strategy to human disease. MicroRNAs (MiRNAs), small single-stranded sequences of ~22 nucleotides, are considered to play important roles in cell proliferation, apoptosis, and cancer progression. They are characterized by their expression patterns, which are developmentally regulated, tissue specific, or steadily expressed in the whole organism.<sup>15–17</sup> Exogenous RNAi has been expressed in mammalian cells and animals in response to artificial microRNA (amiRNA) transcripts among a background of cellular miRNAs.<sup>18</sup> AmiRNAs are regarded as regulatory molecules by sequence-specific base pair that

binds to the targeted mRNA, contributing to direct mRNA degradation or translational inhibition.<sup>19,20</sup> Owing to the specificity and efficiency in gene silencing, it has been increasingly investigated whether amiRNA targeted to oncogenes can correct aberrant transcript levels in cancer cells.<sup>21–24</sup> Therefore, expression of survivin can be manipulated by amiRNAs that target the survivin transcripts.

To test if this affects tumor cell survival and growth, we developed E1-deleted adenoviral vectors expressing survivin-specific amiRNAs. *In vitro* and *in vivo* experiments showed that repressing survivin in hepatocellular carcinoma and cervical cancer cell lines resulted in their diminished proliferation and increased apoptosis in culture and in reduced tumor progression in xenograft mouse models. Overall, these data show that survivin-specific amiRNA gene transfer may provide treatment options for a wide range of human malignancies.

## Results

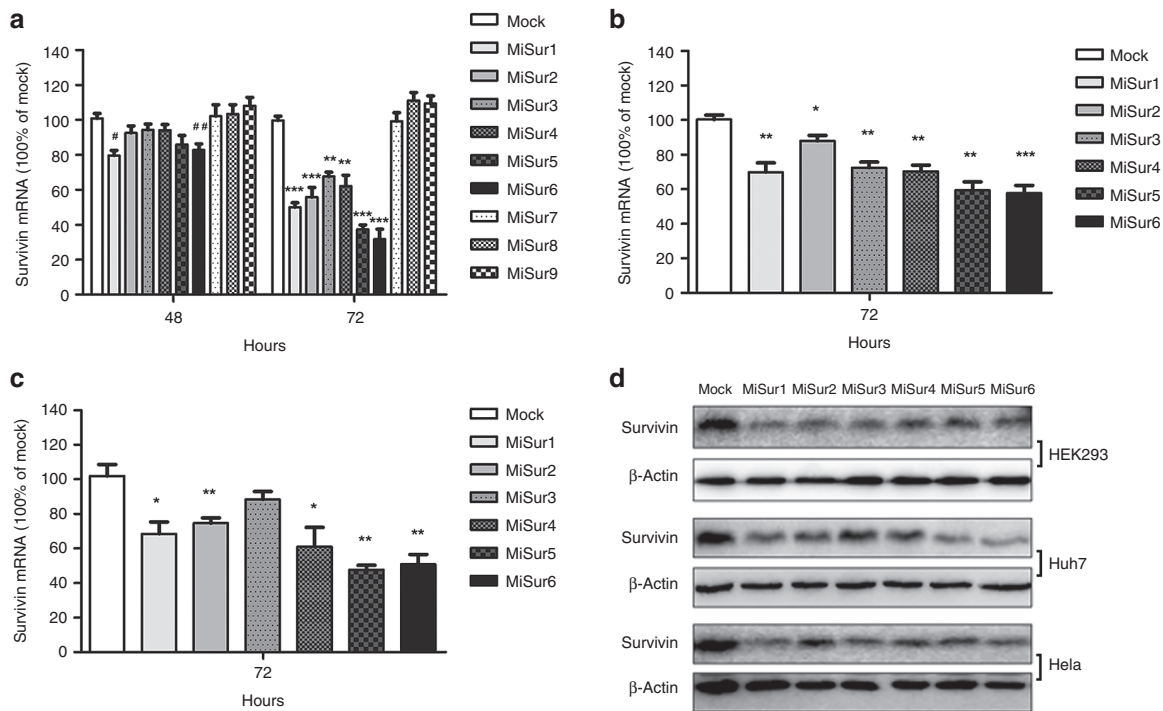
### Survivin-targeting amiRNAs inhibit cancer cell proliferation

To identify amiRNAs that could potentially reduce expression of survivin, we established a screen to test nine candidate amiRNAs. AmiRNA clones were expressed in the backbone of the BLOCK-iT miR RNAi expression vector, which can be transfected transiently into cultured cells. A dramatic change was observed by 72 hours in transfected HEK 293 cells, in which survivin mRNA was reduced by six of the nine amiRNAs (**Figure 1a**). The same amiRNAs also reduced the expression of survivin in the hepatocellular carcinoma cell

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**Keywords:** artificial microRNA; cancer treatment; survivin

Received 24 July 2014; accepted 24 September 2014; published online 4 November 2014. doi:10.1038/mtna.2014.59



**Figure 1** Survivin-targeting amiRNAs reduce survivin mRNA and protein expression in HEK 293 and cancer cells. (a) Survivin mRNA was quantitated by real-time polymerase chain reaction at 48 and 72 hours, respectively after transient transfection of HEK 293 cells with amiRNA vectors. Relative mRNA levels of survivin are shown in reference to actin expression. (b,c) Survivin mRNA was quantitated at 72 hours after transient transfection of Huh7 (b) and HeLa (c) cells. Relative mRNA levels of survivin are shown in reference to actin expression. (d) Expression of survivin protein in HEK 293 and cancer (Huh7, HeLa) cells at 72 hours after transient transfection. Protein was detected by western blot with  $\beta$ -actin as a loading control. Columns, mean; bars,  $\pm$  SEM; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  versus controls. Each data point represents the average of five repeated samples, and each experiment was performed three times.

line Huh7 and the cervical carcinoma cell line HeLa (Figure 1b,c). Reduced levels of survivin transcripts corresponded to reduced levels of survivin protein in all of the three cell lines (Figure 1d). These results show that survivin expression can readily be reduced by specific amiRNAs.

As transduction of cancer cells with plasmid expressing the survivin-targeting amiRNAs yielded promising results, we next tested a more efficient adenovirus (Ad) vector gene delivery system. To this end, we constructed and purified two recombinant Ad vectors expressing amiRNAs MiSur5 and MiSur6 (Ad-MiSur5, Ad-MiSur6). Next, we tested the effect of Ad-MiSur5 and Ad-MiSur6 vectors on levels of survivin mRNA in Huh7 cells in a time-dependent as well as dose-dependent manner. As shown in Figure 2a, infection of cells with Ad-MiSur5 or Ad-MiSur6 led to a three- to fivefold decrease in survivin mRNA compared to that in cells infected with a control vector. Reduction in survivin transcripts was already observed after 24 hours but increased over time. The effect was dose-dependent and the most pronounced reductions in survivin transcripts were obtained with the  $10^{10}$  vp dose of Ad vectors. Suppression of survivin expression led to cells death (Figure 2b,c) and reduction in the cells ability to form colonies (Figure 2d, e) as has been described previously.<sup>6,25</sup>

The effect of Ad vector-delivered surviving-targeting amiRNA on proliferation of Huh7 and HeLa cells was further assessed with a MTT assay. Again results confirmed that MiSur5 or MiSur6 inhibited cell proliferation in a dose- and

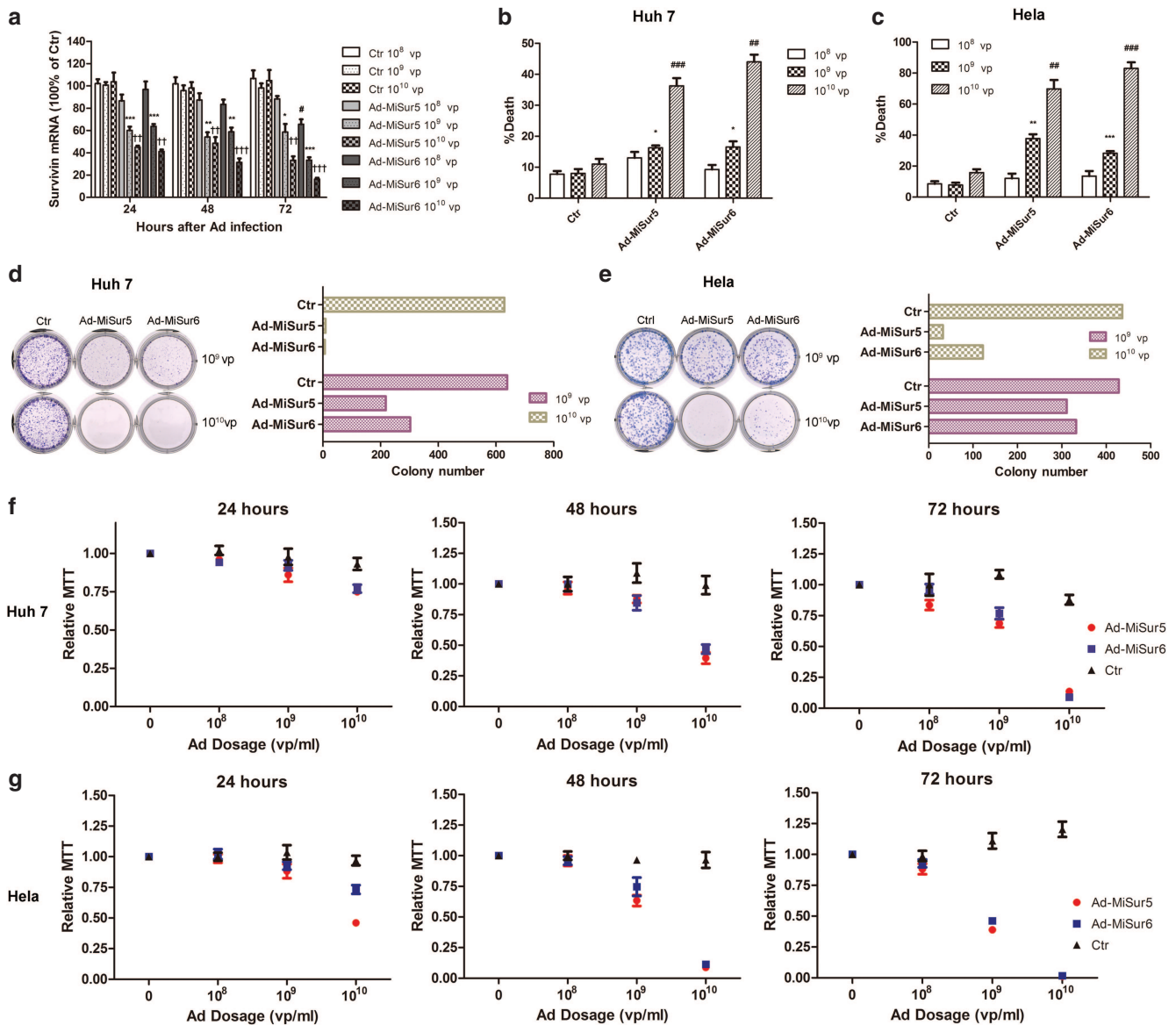
time-dependent manner. Complete inhibition of proliferation of either of the cell lines was achieved by 72 hours after infection of cells with  $10^{10}$  vp of Ad vectors (Figure 2f,g).

In summary, two of the Ad-expressed amiRNAs, MiSur5 or MiSur6, show substantial inhibition of tumor cell growth and survival *in vitro*.

### Survivin-targeting amiRNAs induce cancer cell apoptosis by the activation of cleaved caspase 3 and the elevation of proapoptotic Bim protein

It is known that survivin prevents apoptosis of human cancer cells.<sup>5,26</sup> To test if knock-down of survivin suppresses tumor cell growth by inducing cell death, we analyzed apoptosis of Huh7 and HeLa cells that were infected with Ad-MiSur5, Ad-MiSur6, or a control vector. As shown in Figure 3a,b, both amiRNA expressing vectors induced apoptosis of the cancer cells as evidenced by positive staining for annexin alone (early apoptosis) or annexin with propidium iodide (PI) (late apoptosis).

We examined if treatment with survivin-targeting amiRNAs caused over time accumulation of cleaved caspase 3. Ad-MiSur5- or Ad-MiSur6-treated Huh7 (Figure 3c) and HeLa (Figure 3d) cells showed significantly augmented levels of cleaved caspase 3, which increased over time. Increases in activated caspase 3 were more pronounced in cells infected with high doses of vector. Other apoptosis markers such as Bim and cleaved Poly (ADP-ribose) polymerase also became augmented (Figure 3e,f).

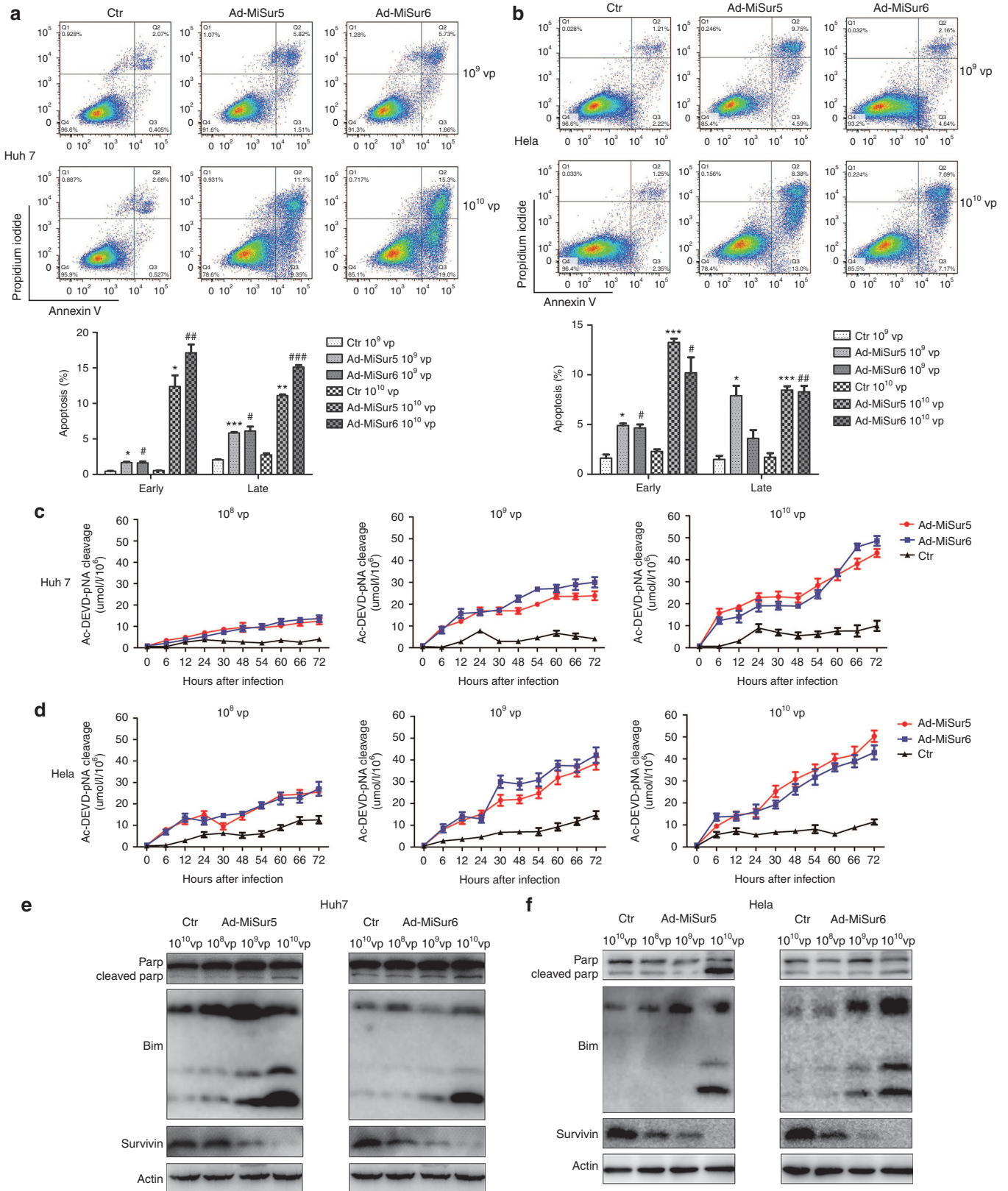


**Figure 2** Recombinant Ad vector-expressed amiRNAs significantly suppress cancer cell growth. (a) Survivin mRNA was quantitated at the indicated time after infection of cells with Ad vectors expressing amiRNAs (Ad-MiSur5, Ad-MiSur6) with increasing doses ( $10^9$  vp,  $10^9$  vp,  $10^{10}$  vp). (b,c) Recombinant Ad vectors expressing amiRNAs promote cell death in cancer cells. Huh7 and HeLa cells were infected with the indicated amounts of Ad-MiSur5, Ad-MiSur6, or control vector. After 72 hours, cell death was measured by trypan blue staining. Data are presented as the mean  $\pm$  SEM of quadruplicate samples and are representative of three independent experiments. (d,e) Colony formation assay with Huh7 and HeLa cells. Huh7 and HeLa cells were infected with the indicated doses of Ad-MiSur5, Ad-MiSur6, or a control vector in six-well cell plates and cultured in media for 3 weeks. Cell colonies were stained by Giemsa solution. Left, colony formation by Huh7 (d) and HeLa (e) cells. Right, quantification of colony numbers. Graphs are representative of three independent experiments. (f,g) Effect of survivin-targeting amiRNAs on cancer cell viability. Huh7 (f) and HeLa (g) cells were treated with Ad vectors at the indicated doses for 24–72 hours, and cell viability was quantified by MTT assay. Data are presented as the mean  $\pm$  SEM of octuplicate samples and are representative of three independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  experimental samples versus controls.

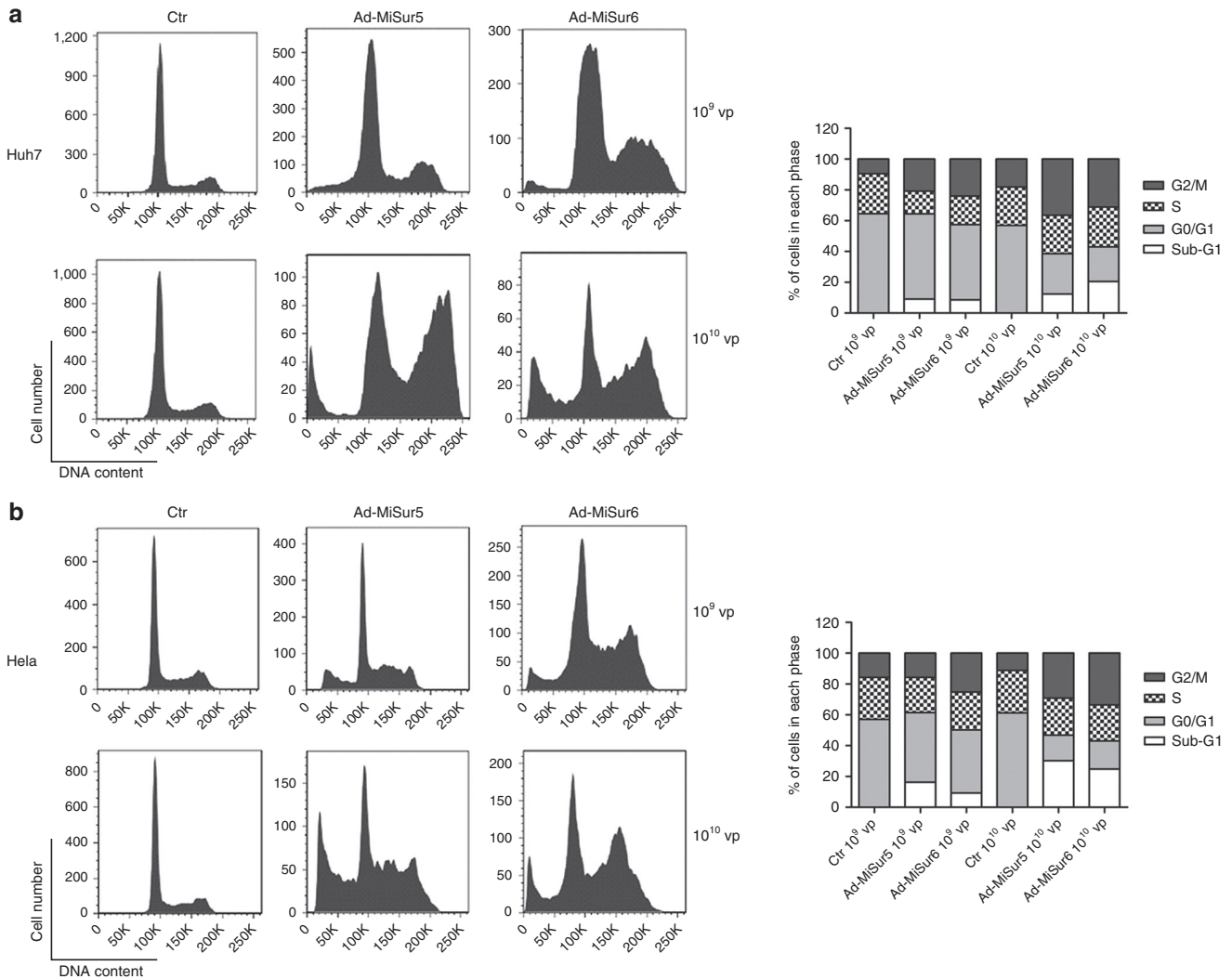
### Survivin-targeting amiRNAs arrest cell cycle in G2/M

Survivin associates with microtubules of the mitotic spindle at the start of cell division and is needed to ensure proper separation of chromosomes into the two daughter cells.<sup>13,27</sup> In the absence of surviving, cells are unable to pass through mitosis. We confirmed that high dose of Ad vector encoding survivin-targeting amiRNAs caused accumulation of cells at the G2/M phase (Figure 4a,b) and sub-G1 phase (apoptosis).

Expression of survivin is inhibited by p53 and it is feasible that in turn survivin represses p53 expression. Indeed Ad-MiSur5 or Ad-MiSur6 infected Huh7 and HeLa cells had increased levels of p53, phosphorylated p53, and p21 a downstream target of p53 (Figure 5a,c), thus suggesting that cell cycle arrest depended on the p53 pathway. Furthermore, levels of the retinoblastoma protein (Rb) increased while phosphorylated Rb and cyclin E and D declined (Figure 5b,c).



**Figure 3** Ad vector-expressed amiRNAs induce cancer cell apoptosis. (a,b) Left images, flow cytometry analysis of apoptosis was evaluated by propidium iodide (PI)/AnnexinV staining of Huh7 (a) and HeLa (b) cells 72 hours after infection with 10<sup>9</sup> vp and 10<sup>10</sup> vp of Ad vectors. Right, analysis of cancer cell apoptosis in bar graph. Early apoptosis: AV<sup>+</sup>/PI<sup>-</sup>(%); later stage apoptosis/secondary necrosis: AV<sup>+</sup>/PI<sup>+</sup>(%). Data are presented as the mean  $\pm$  SEM of triplicate samples and are representative of three independent experiments. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001 experimental samples versus controls. (c,d) Kinetics of caspase 3 activation were assayed in Huh7 (c) and HeLa (d) cells treated with



**Figure 4** Ad vectors expressing amiRNAs causes cell cycle arrest in G2/M. (a,b) Forty-eight hours after infection, fixed Huh7 (a) and HeLa (b) cancer cells were labeled with propidium iodide staining and DNA content was measured by flow cytometry. Percentage of cells in the sub-G1, G0/G1, S, and G2/M phases are shown in the column charts. Data represent one out of three experiments and each experiment was performed in triplicate.

### Survivin-targeting amiRNAs inhibit tumor growth in nude xenografts model

To determine if Ad-MiSur5 and Ad-MiSur6 vectors could affect tumor progression *in vivo*, we established a subcutaneous Huh7 cell xenograft mouse model. Nude mice were challenged with 10<sup>6</sup> Huh7 cells in matrigel given subcutaneously and 2 weeks later tumors were injected with the 10<sup>10</sup> vp of the Ad vectors. As shown in **Figure 6a** and **Supplementary Figure S1**, injection of the Ad vectors delayed tumor growth. Staining of tumor sections showed increased expression of cleaved caspase 3 upon Ad-MiSur5 and Ad-MiSur6 treatment (**Figure 6b**). Percentages of dead cells within tumors increased in treated mice (**Figure 6c**), while percentages of cells positive for the proliferation marker Ki67 declined (**Figure 6d**).

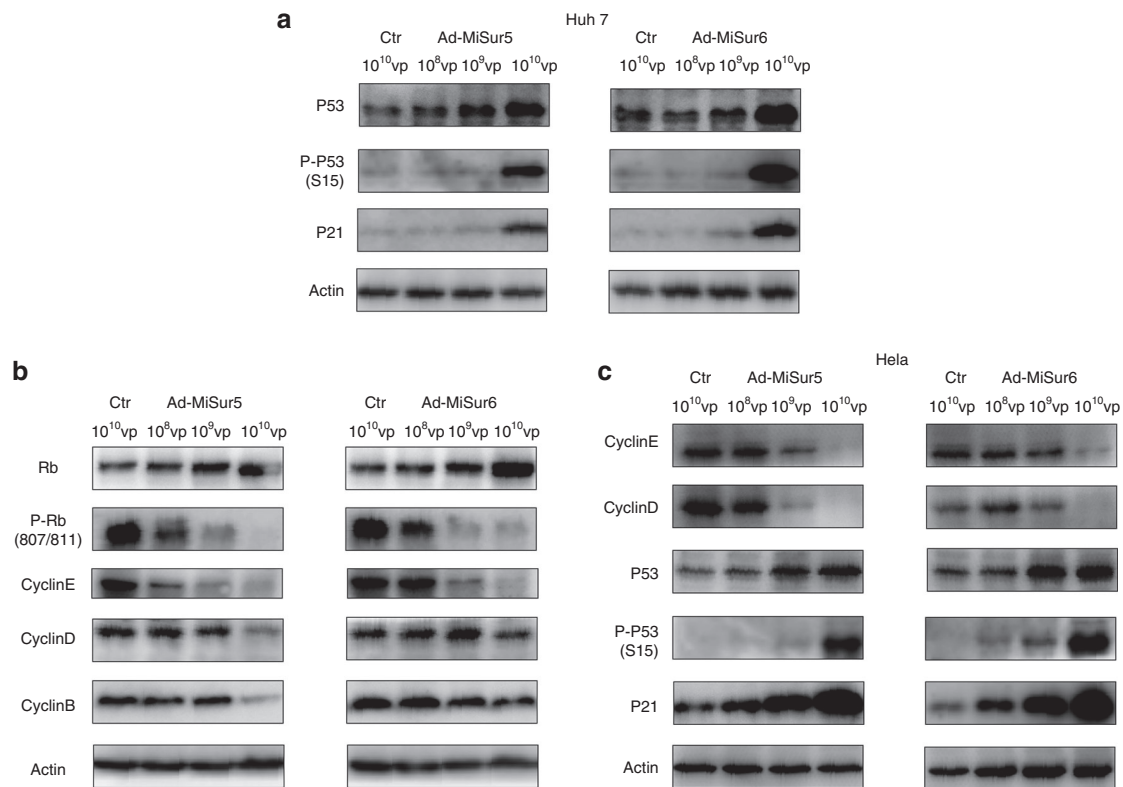
We repeated the experiment with HeLa xenografts and again observed upon Ad vector treatment a delay in tumor progression (**Figure 7a**) associated with an increase in tumor cell apoptosis (**Figure 7b**).

Overall, these results show that survivin targeting amiRNAs delivered by Ad vectors directly into the tumor significantly extend survival of tumor bearing mice by causing massive tumor cell apoptosis.

### Discussion

Survivin is considered to be a potential target for anticancer therapy.<sup>8,28-30</sup> Attenuation of survivin expression in function

10<sup>9</sup> vp, 10<sup>9</sup> vp, and 10<sup>10</sup> vp of Ad vector over a time course. Activities were determined by incubation of 10<sup>6</sup> cells with substrates, Ac-DEVD-pNA. In the graph, each bar represented the mean ± SEM (*n* = 5). (e,f) Western blot analysis for the protein levels of Poly (ADP-ribose) polymerase (PARP), cleaved PARP, and bim were used to measure apoptosis of Huh7 (e) and HeLa (f) cells infected with Ad vectors expressing control sequences (10<sup>10</sup> vp), MiSur5 (10<sup>8</sup> vp-10<sup>10</sup> vp) or MiSur6 (10<sup>8</sup> vp-10<sup>10</sup> vp). β-actin was used as a loading control.



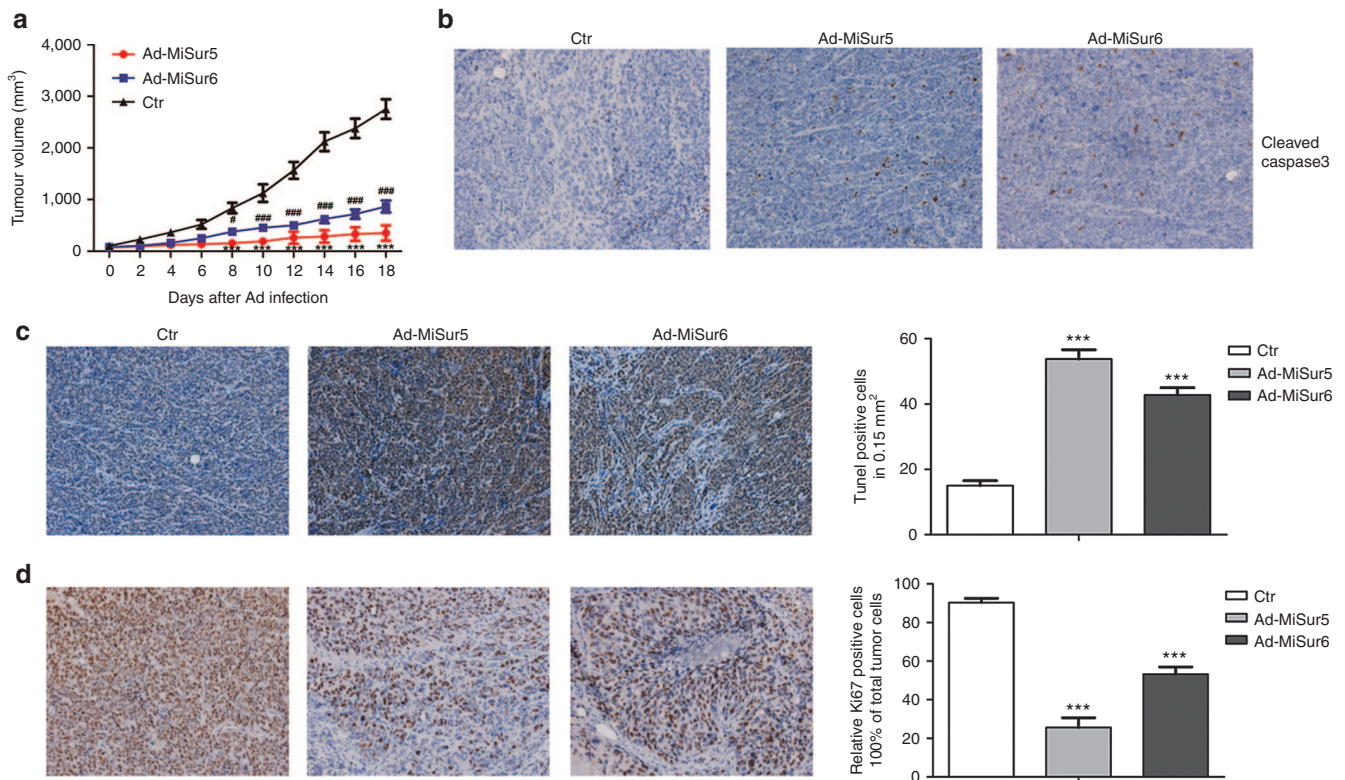
**Figure 5 Ad vectors expressing amiRNAs activate p53-p21 signaling.** (a) Western-blots to detect p53, phosphorylated p53 and p21 were conducted 72 hours after infection of Huh7 cells with Ad vectors expressing amiRNA or control sequences. (b) Western blots for Rb, phosphorylated Rb, and cyclins in Huh7 cells infected with Ad vectors expressing amiRNA or control sequences. (c) Western-blot analysis of HeLa cells as in (a) and (b).

has been shown to induce apoptosis and inhibit tumor growth in multitypes of cancer by using antisense oligonucleotides, small interfering RNAs (siRNAs), or dominant-negative mutants *in vivo*.<sup>31–34</sup> Previous studies have shown that elimination of survivin expressing tumors cells by cytolytic CD<sup>8+</sup> T cells, which spontaneously develop in some cancer patients or can be induced by specific vaccines provides clinical benefits.<sup>30,35,36</sup> Silencing of survivin by siRNA has been demonstrated to inhibit tumor growth by triggering apoptosis or overcoming drug resistance.<sup>32,33</sup> It is commonly used for *in vitro* investigations to transfect siRNAs for direct targeting survivin mRNA. However, this approach has many limitations including disadvantages pharmacokinetic profile, ineffective delivery into cells, poor stability *in vivo*, and difficulty to perform clinical trials. The vector-based systems of high efficiency that facilitate engineered shRNA expression are driven by polymerase III promoter.<sup>37,38</sup> Unfortunately, there are some limits on their tissue-specific and cytotoxicity.

Previous studies have shown that key cellular pathways and checkpoint for virus replication are controlled by miRNAs. In eukaryotic organisms, siRNAs can be formed from double-stranded RNAs by an enzyme Dicer, RNase III family member, and then incorporate into an enzyme complex, RISC, to target mRNA complementary to the siRNA for specific cleavage and degradation. Since translational arrest of target mRNAs is processed only by partial complementarity to the miRNA, which is often sufficient for effective regulation, allowing a single miRNA to regulate large numbers of

targets. The amiRNAs are siRNA sequence embedded in native pri-miRNAs scaffold involving a hairpin structure and driven by polymerase II promoter. The stem-loop structure pri-miRNA is cleaved by nuclear RNase III Drosha to generate a small hairpin precursor, 70 nucleotides pre-miRNAs. With the export from nuclear, the pre-miRNAs are processed by Dicer into mature miRNA molecule and incorporated into miRNA-containing RNA-induced silencing complex. Several studies illustrated a higher efficiency and safety in the use of amiRNAs comparing with shRNAs.<sup>19</sup> Recently, amiRNAs have been applied to mammalian cell targets as a new tool for repressing gene expression, not only a single gene, but multigenes.<sup>18,39</sup> This in turn has led to the development of amiRNAs to silence oncoproteins that are essential for tumor cell proliferation. Here we focus on survivin, which is a highly suited target for cancer therapy as it is selectively expressed in a wide variety of cancer cells but virtually absent in terminally differentiated normal tissues.

MiRNAs are undergoing clinical testing. Two general approaches are being tested, miRNA agonists to knock-down the functions of oncoproteins to which tumor cells have become addicted or miRNA antagonists to restore loss of function of tumor suppressors.<sup>40,41</sup> MiRNAs are also tested as treatments for chronic infections or degenerative diseases.<sup>42–44</sup> MiRNAs are attractive therapeutics; delivery methods are well established through already gained experience with silencing RNA. Also, they are expected to cause minimal side effects as they are already expressed in

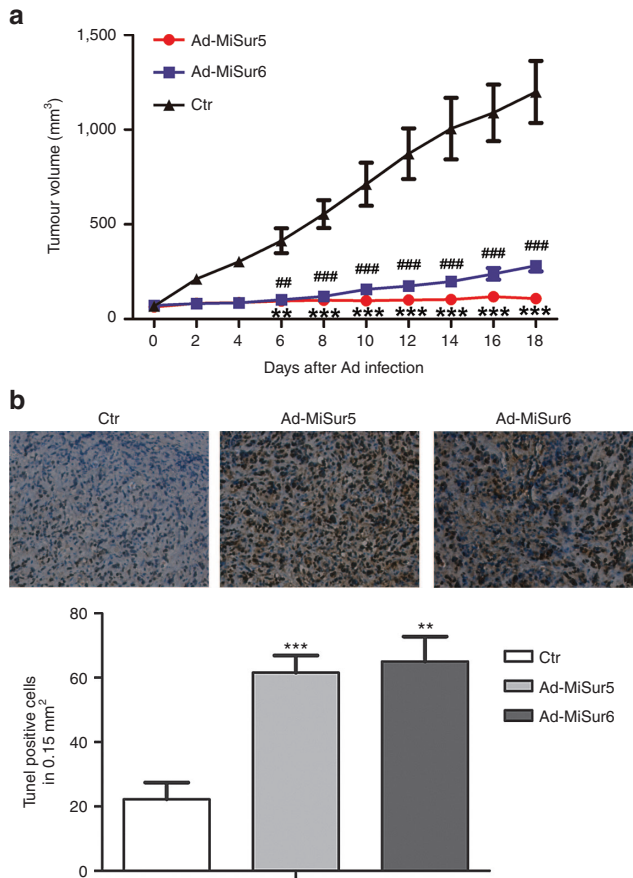


**Figure 6 Ad vectors expressing amiRNAs inhibit tumor growth in nude mouse Huh7 cell xenografts.** (a) Tumor growth was measured every second day with calipers of female nude mice inoculated subcutaneously with Huh7 cells after the infection of  $10^{10}$  vp Ad-MiSur5, Ad-MiSur6, control vectors. Each data point represents the average from five mice and each bar represents the mean  $\pm$  SEM. (b) Apoptotic cells were identified by staining for cleaved caspase 3 (brown) in Huh7 tumors at 3 weeks after injected of Ad vectors expressing amiRNA or control sequences. Tumor tissues were analyzed microscopically ( $n = 4$  mice for each group). (c) Left images, TUNEL staining (brown) shows apoptosis of tumor cells after amiRNAs or control treatment. Right, quantification of TUNEL positive cells ( $n = 4$  mice for each group) after treatment; each bar represents the mean  $\pm$  SEM. (d) Left, Ki67 staining of nude mice xenograft sections. Right, quantification of Ki67-positive cells ( $n = 4$  mice for each group) after treatment; each bar represents the mean  $\pm$  SEM. Data are representative of three independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  experimental samples versus controls.

normal cells. The most advanced clinical trial is using a miR-122 antagonist targeted to the liver of patients with chronic hepatitis C virus infection. Clinical results are very promising by showing not only a significant reduction in viral loads but also no dose-limiting side effects and no development of viral escape mutants. Still in preclinical testing for treatments of cancer are inhibitors for miR-21 for hepatocellular carcinoma and miR-10b for treatment of glioblastoma. Agonists that being tested include let-7 for treatment of lung cancer, mi-R16, miR-34a, and miR-146a for treatment of solid cancers.<sup>41,45</sup> Delivery methods to reduce off target effects need to be improved and chemical modification to increase the amiRNA's stability, target specificity, and ability to cross membranes are being developed. Overall, targeting miRNAs is evolving into a very promising approach for cancer treatment, and as shown here, knockdown of survivin through amiRNA can at least in preclinical models reduce progression of different solid tumors.

In this study, we developed a strategy for cancer therapy based on Ad vector delivery of survivin-targeting amiRNAs. In the past, viral vectors have predominantly used in anticancer therapy.<sup>46,47</sup> Virus-based vaccine is employed for the delivery of exogenous DNA sequences into specific tumor. Ad vector

has been attracted much attentions on cancer gene therapy due to its large cloning capacity, efficient transduction, and producing high-titer stocks.<sup>48,49</sup> Although Hela expresses wild-type p53/p21, Huh7 expresses mutant p53 and defective p21, our results show that survivin-targeting amiRNAs reduced proliferation of two very different human carcinoma cells. We performed cell cycle studies to illustrate common apoptosis mechanisms at increasing p53/p21 protein level. Reduced cell growth was linked to an arrest of cells in the G2/M phase, which could have been caused by increased activation of p53 pathways suggesting that the G2/M arrest is dependent on p53 activation. The important tumor suppressor, p53 activates a series of cellular genes in the checkpoint of G2/M. We also demonstrated that amiRNAs could induce p53 phosphorylation at Ser 15, a hint of apoptosis by inhibiting survivin. It will be important to detect whether survivin-targeting amiRNAs-induced p53 upregulation and cleaved Poly (ADP-ribose) polymerase activation is confined to cancer cells. In addition, as it is crucial for cancer remission, knock-down of survivin causes increased tumor cell apoptosis. Most importantly, these observations from *in vitro* cultures could be extended to *in vivo* human cancer models of immunocompromised mice.



**Figure 7** Ad vectors expressing amiRNAs inhibit tumor growth in nude mouse HeLa cell xenografts. (a) Tumor growth curve (b) terminal deoxynucleotidyl transferase dUTP nick end labeling staining. Data are presented as in Figure 6.

## Materials and Methods

**Artificial miRNA design and generation of plasmids microRNA.** Artificial miRNAs were designed by BLOCK-iT Pol II miR RNAi Designer (Life Technologies, Grand Island, NY), which identifies an optimal target site within a gene to induce gene knockdown. Nine appropriate sequences of single-stranded oligonucleotides were generated and annealed, and respectively cloned into the miRNA expression vector pcDNA 6.2-GW/miR (Invitrogen, Carlsbad, CA) following the protocol of BLOCK-iT Pol II miR RNAi Expression Vector Kit (Invitrogen).

**Cell lines, transfection, and bacterial strains.** Human embryonic kidney 293 (HEK 293) cells, human hepatocellular carcinoma cells (Huh7), and human cervical cancer cells (Hela) were grown in Dulbecco's modified Eagle's medium medium with 10% fetal bovine serum and 100 units/ml of penicillin. All cells were maintained in a humidified 37 °C incubator with 5% CO<sub>2</sub>. Transfections were carried out using Lipofectamine 2000 (Invitrogen) for HEK 293 cells or Lipofectamine LTX (Invitrogen) for Huh7 cells and Hela cells, according to the manufacturer's instructions. pcDNA 6.2-GW/miR plasmids and pShuttle plasmids (Clontech, Mountain View, CA) were

transformed into the bacterial strain TOP10 (Invitrogen) at 37 °C in S.O.C medium.

**Construction and production of recombinant adenoviruses.** The expression cassettes of artificial miRNAs from pcDNA 6.2-GW/miR plasmids were amplified by polymerase chain reaction (PCR) and restriction enzyme sites *Mfe* I on the 5' UTR and *Pst* I on the 3' UTR were added, respectively, then subcloned into the pShuttle vector (sense, 5'-GCT-GCA-ATT-GGA-TGT-ACG-GGC-CAG-ATA-TAC-G-3'; antisense, 5'-CCA-GCT-GCA-GGC-TAT-GAC-CAT-GTA-ATA-CG-3'). After digestion with *I-Ceu*I and *PI-Sce*I, the amiRNA expressing cassettes were cloned from pShuttle into the E1 domain of the molecular clones of adenovirus of human serotype 5 (Ad). Recombinant Ad vectors were rescued by transfection of plasmid DNA into HEK 293 cells. The Ad vectors were purified by cesium chloride density-gradient centrifugation and virus particle content was determined by spectrophotometry (260 nm). The plasmid construct and recombinant adenovirus genome of each vector were confirmed by restriction enzyme digestions.<sup>50</sup>

**Real-time PCR.** Total cellular RNA was extracted from each sample (~1 × 10<sup>6</sup> cells) using Trizol Reagent (Invitrogen). RNA (1 µg) from each sample was reverse transcribed using a high-capacity RNA-to-cDNA synthesis kit (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. A total of 15 µl of specific real-time PCR mixture (7.5 µl SYBR Green PCR Master Mix, 0.5 µl of 10 µmol/l forward and reverse primers each, 1.5 µl of H<sub>2</sub>O, and 5 µl of cDNA), surviving transcripts, and actin were amplified from the cDNA in triplicate experiments by FastStart Universal SYBR Green Master (Roche Diagnostics, Indianapolis, IN). The survivin primers were: sense, 5'-TTC-AAG-GAG-CTG-GAA-GGC-T-3', antisense, 5'-CAC-TTT-CTC-CGC-AGT-TTC-CTC-AA-3'. The actin primers were: sense, 5'-ACC-GAG-CGC-GGC-TAC-AG-3', antisense, 5'-CTT-AAT-GTC-ACG-CAC-GAT-TTC-C-3'. The ABI 7900HT Fast Real-time PCR system (Applied Biosystems) was used for PCR amplification and detection. The relative quantitative PCR program consisted of the initial process of 10 minutes at 95 °C and 40 cycles of 1 minute at 95 °C, 30 seconds at 55 °C, and 30 seconds at 72 °C. Target transcript levels were calculated from the comparative C<sub>t</sub> method and were normalized by actin products in each sample. Melting curves for each PCR mixture were generated to ensure the purity of amplification products.

**Western blot analysis.** Levels of survivin protein were measured by western blot analyses. Plasmid transfected or virus-infected cells were lysed in a radioimmunoprecipitation assay lysis buffer containing 50 mmol/l Tris (pH 7.4), 150 mmol/l NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mmol/l ethylenediaminetetraacetic acid (pH 8.0), 2 mmol/l phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 1 mmol/l Na<sub>3</sub>VO<sub>4</sub>, protease inhibitor cocktail (Roche), and the phosphatase inhibitor cocktail (Roche). Cell debris was removed by centrifugation after freeze-thawing of cells and protein concentrations were measured using the bicinchoninic acid protein assay (Pierce,



Rockford, IL). Equal amounts of total extracted proteins (30 µg) were applied to each lane on 10, 12, or 14% SDS-PAGE. Following transfer to PVDF membranes (Millipore, Bedford, MA), protein expression levels were detected after incubation with primary antibodies for survivin (Santa Cruz Biotechnology, Santa Cruz, CA), p53, p-p53, p21, Rb, p-Rb, cyclin B, cyclin D, cyclin E, and Bim (Cell Signaling Technology, Danvers, MA), respectively, and subsequently with secondary antibodies conjugated with horseradish peroxidase (Sigma-Aldrich, St Louis, MO). The expression of β-actin (Sigma-Aldrich) was measured as a normalization control for protein loading. The signal was detected using a chemiluminescence detection system (GE Health Life Sciences, Pittsburgh, PA).

**Cell death assays.** Both Huh7 cells and Hela cells ( $1 \times 10^6$ /well) were seeded in six-well 35-mm diameter culture plates and were infected with different doses of recombinant adenovirus-expressed survivin-targeting amiRNAs and scramble controls, respectively. Seventy-two hours later, cells were trypsinized, washed, and pelleted. Resuspended cells were stained with trypan blue.<sup>51</sup> A hemocytometer (Thermo Fisher Scientific, Madison, WI) was used to count dead and live cells under light microscopy. An average of four fields was analyzed for each sample.

**Cell growth-inhibitory assays.** Cells ( $8 \times 10^3$ /well) were seeded in 96-well plates and cell survival was determined by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay, as previously described.<sup>52</sup> Treated and control cells were incubated at 37 °C for 4 hours, after adding 5 µg/ml MTT reagent and cleavage was determined by using a multiwell scanning spectrophotometer (Thermo Fisher Scientific) at a wavelength of 490 nm.<sup>53</sup> The survival of amiRNA-treated cells was normalized to control cells. The growth-inhibitory effect was measured for 3 consecutive days by MTT assay. The experiments were performed in eight replicates for each sample, and repeated in triplicate.

**Colony formation assays.** Cells ( $1 \times 10^3$ /well) were seeded in six-well plates and infected with different concentrations ( $10^9$  and  $10^{10}$  vp) of recombinant Ad vectors expressing survivin-targeting amiRNAs and scramble controls. Each group of infected cells was cultured in medium with 5% FBS in triplicate wells. Three weeks after infection, colonies were fixed with cold methanol and stained with a Giemsa solution, and then scored using an Elispot reader.<sup>54,55</sup> The total number of surviving cells, extrapolated from amiRNA-treated cells, when compared with cells treated with scramble amiRNAs, was plotted on a dose-response column chart.

**Caspase 3 activity assay.** Caspase 3 activity was assayed based upon caspase 3 hydrolysis of peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA), to release p-nitroaniline (pNA) according to the caspase 3 colorimetric assay (Sigma-Aldrich).<sup>56,57</sup> AmiRNA-treated and control cells were centrifuged and supernatants removed. Cell pellets were washed twice with ice-cold PBS, resuspended in lysis solution, and incubated on ice for 15 minutes. The lysates were centrifuged, the appropriate substrates were added, and the reaction solution was incubated at 37 °C for

2 hours. Caspase 3 activity was measured at a wavelength of 405 nm.

**Flow cytometry analysis for apoptosis and cell cycle.** Trypsinized cancer cells were washed twice with ice-cold PBS and incubated with annexin-V-FITC/PI (Sigma-Aldrich) in binding buffer<sup>58</sup>; cell fluorescence was immediately determined by a FACScan machine (BD, Franklin Lakes, NJ).

For cell cycle analysis, trypsinized cancer cells were washed twice with ice-cold PBS and fixed with 70% ethanol at 4 °C overnight. Cells were washed with PBS twice and resuspended in 500 µl of 50 µg/ml PI (Sigma-Aldrich) and 100 µg/ml RNase A (Life Technologies, Carlsbad, CA). Cell cycles were determined by measuring DNA content of each cell population by flow cytometry.

**Xenograft liver cancer model.** Human Huh7 cells ( $5 \times 10^6$ ) were suspended in 100 µl matrigel (BD) and injected subcutaneously into the flanks of nude mice at 7–8 weeks of age. When tumors were palpable ~10 days after cell implantations, mice were randomly distributed into treated ( $n = 10$ ) and control ( $n = 10$ ) groups receiving an injection of  $10^{10}$  vp recombinant Ad vector expressing survivin-targeting amiRNAs or scramble amiRNAs, respectively. Tumor size was recorded with the formula “ $a \times b^2 \times 0.5$ ” ( $a$  is the longest and  $b$  is the shortest radius of the tumor in millimeters as measured by a caliper in 2-day intervals).<sup>59</sup> Half of the mice in each group were sacrificed 18 days after initial treatment; the others were maintained until excessive tumor burden required their euthanasia (**Supplementary Figure S2**). All animal experiments were approved by the Institutional Animal Care and Use Committee at the Institut Pasteur of Shanghai and the animals were cared for in accordance with the institutional guidelines.

**Histology and immunohistochemistry.** Tumors were fixed with 1 ml 4% PFA overnight and dehydrated in ethanol, paraffin-embedded, and cut into 5 µm sections after hematoxylin and eosin (H&E) staining. Immunohistochemistry was performed as previously described.<sup>60</sup> Briefly, sections were microwaved for 25 minutes in 0.01 mol/l citrate buffer (pH = 6.0), cooled, treated with normal goat serum (1:5) for 20 minutes, and incubated with the primary antibody (cleaved-caspase 3, Cell Signaling Technology, Beverly, MA; Ki67 and NCL-ki67p, Leica Biosystems, Newcastle Upon Tyne) at 4 °C overnight. Biotin-conjugated second rabbit anti-mouse antibody was applied for 15 minutes at 37 °C, followed by incubation of sections with enzyme-conjugated peroxidase-streptavidin for 15 minutes at 37 °C. Sections were washed thoroughly with TBST after each incubation. Immunoreactivity was visualized for 3 minutes with a diaminobenzidine substrate kit that stained positive cells brown. Sections were dehydrated in 100% (v/v) alcohol, cleaned in toluene, and mounted with permount.

**Statistical analysis.** All graphs were created with Graphpad Prism 5 software (GraphPad Software, La Jolla, CA) and statistical analyses were performed with SPSS software version 16.0 (SPSS, Chicago, IL). ANOVA was applied to compare relative mRNA levels between groups. A  $P$  value of less than 0.05 was considered statistically significant.

## Supplementary material

**Figure S1.** Female nude mice inoculated subcutaneously with Huh7 cells and then infected with  $10^{10}$ vp Ad-MiSur5, Ad-MiSur6, control vectors are shown.

**Figure S2.** Depletion of survivin prolongs lifetime of xenograft nude mice.

**Acknowledgments.** This work was supported by grants from Natural Science Foundation of China (31170871 and 31370929), “Knowledge Innovation Program” and “100 Talent Program” from Chinese Academy of Sciences, and the Shanghai Pasteur Foundation. We gratefully acknowledge Ying Yu, Institute for Nutritional Sciences, Chinese Academy of Sciences, for his generous assistance and reagents.

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