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MicroRNA-185 regulates chemotherapeutic sensitivity in gastric cancer by targeting apoptosis repressor with caspase recruitment domain

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Gastric cancer remains the second leading cause of cancer deaths worldwide. Resistance to chemotherapy is a significant barrier for effective cancer treatment. Here, we identified miR-185 to be a contributor to chemosensitivity in gastric cancer. We observed low levels of miR-185 in gastric cancer cell lines and clinical tissues, compared with gastric epithelium cell line and noncancerous tissues. Furthermore, enforced expression of miR-185 increased the sensitivity of gastric cancer cells to low-dose chemotherapeutic agents, which alone cannot trigger significant apoptosis. Conversely, knockdown of endogenous miR-185 prevented high-dose chemotherapy-induced apoptosis. In elucidating the molecular mechanism by which miR-185 participated in the regulation of chemosensitivity in gastric cancer, we discovered that apoptosis repressor with caspase recruitment domain (ARC) is a direct target of miR-185. The role of miR-185 was confirmed in gastric tumor xenograft model. The growth of established tumors was suppressed by a combination therapy using enforced miR-185 expression and a low dose of anticancer drugs. Finally, we found that RUNX3 (Runt-related transcription factor) was involved in the activation of miR-185 at the transcriptional level. Taken together, our results reveal that RUNX3, miR-185 and ARC regulate the sensitivity of gastric cancer cells to chemotherapy.
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Gastric cancer remains the second leading cause of cancer deaths worldwide.[1] Chemotherapeutic agents are commonly used for various types of cancer therapy. However, the cytotoxicity and chemotherapy resistance became major barriers to its clinical application.[2,3] The underlying mechanisms of chemotherapy resistance in gastric cancer are not fully understood.

MicroRNAs (miRNAs) are a class of small noncoding RNAs with 21–25 nucleotides long and negative regulators of target gene by altering mRNA translation or stability.[4] MiRNAs functionally participated in a wide variety of physiological or pathological processes, including tumor.[5] Mounting evidence showed aberrant miRNA signatures in human cancers,[5] implying important roles of miRNAs during tumor progression. A great deal of miRNAs were identified as tumor suppressors;[6,7] some of these microRNAs have been proven to regulate chemosensitivity.[8,9] The involvement of miRNAs in chemoresistance need to be further investigated.

Apoptosis repressor with caspase recruitment domain (ARC) was initially discovered as an endogenous apoptosis inhibitor in the skeletal muscle and the heart.[10] It can antagonize both intrinsic and extrinsic apoptosis signaling pathway.[11] Recent studies showed ARC high expression[12,13] in many malignant tumors. Our previous work has proved that highly expressed ARC contributed to chemotherapy resistance in cancer cells by targeting the mitochondrial fission machinery. It was also demonstrated that ARC expression was downregulated in cancer cells following doxorubicin treatment,[14] but the mechanism is largely unknown.

It was reported that phosphorylation[15] and dephosphorylation[16] controlled ARC activity. Further studies showed that ARC could be modulated transcriptionally by p53 or Foxo3a.[17,18] In addition, ARC was downregulated through MDM2-dependent ubiquitination and degradation.[19] These investigations were mainly performed in cardiomyocytes. Preliminary studies in tumor cells demonstrated that phosphorylation of ARC[20] was required for its function. However, whether miRNA can regulate ARC in cancer cells is unknown.

The RUNX (Runt-related transcription factor) family consists of three members, RUNX1, RUNX2 and RUNX3. RUNX1 and RUNX2 were essential for hematopoiesis and osteogenesis, respectively.[21] RUNX3 was identified as a candidate tumor suppressor involved in gastric cancer.[22] In about 40–60% of human gastric cancers, loss of RUNX3 expression was observed.[22] However, the molecular mechanism by which RUNX3 suppressed the growth of cancer cells remains unclear.

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Abbreviations: ARC, apoptosis repressor with caspase recruitment domain; RUNX, Runt-related transcription factor; qRT-PCR, quantitative reverse transcription PCR; UTR, untranslated region; ChIP, chromatin immunoprecipitation
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Aberrant miR-185 expression was found in several types of cancers.[23] In this study, we focused on the function of miR-185 in gastric cancer. We found that miR-185 could sensitize gastric cancer cells to chemotherapy through negatively regulating ARC expression. Besides, miR-185 was upregulated by RUNX3 at the transcriptional level. These results were also confirmed in human gastric cancer samples and xenograft tumor models. Our results identify a novel regulatory pathway for apoptosis involving miR-185 and possibly provide a valuable insight into cancer therapy.

Results

MiR-185 enhances the sensitivity of gastric cancer cells to chemotherapy. Aberrant expression of miR-185 has been reported in several types of cancers.[23] To explore the potential role of miR-185 in human gastric cancer, we analyzed 25 pairs of human gastric cancer tissues and matched adjacent noncancerous tissues (Supplementary Table 1). Quantitive analysis revealed that miR-185 levels were significantly reduced in gastric cancer samples, compared with normal gastric tissues (Figure 1a). However, obvious decrease in miR-185 expression in cancer tissues was observed in 20 of 25 patients (Supplementary Figure 1a). In parallel, miR-185 expression was significantly reduced in all five collected gastric cancer cell lines, compared with that in human gastric epithelium cell line GES-1. The endogenous levels of miR-185 were relatively lower in SGC-7901, BGC-823 and MGC-803 (Figure 1b). When exposed to cisplatin (30 μM) or doxorubicin (2 μM), markedly elevated miR-185 levels were determined in SGC-7901 (Figure 1c) and MGC-803 cells (Figure 1d).

The elevation of miR-185 expression induced by cisplatin or doxorubicin treatment led us to consider whether miR-185 was involved in the regulation of anticancer drug-induced apoptosis. To this end, we produced a construct encoding miR-185. Enforced expression of miR-185 was confirmed by quantitative RT-PCR (qRT-PCR) (Supplementary Figure 1b). Overexpression of miR-185 alone had no significant effect on apoptosis (Figures 1e and f), which was consistent with the results of previous study.[24] To characterize the function of endogenous miR-185 in the apoptotic signaling pathway, which mediated chemotherapy, miR-185 antagomir was transfected into SGC-7901. MiR-185 levels were reduced by its specific antagomir. Cisplatin- or doxorubicin-induced apoptosis was attenuated by miR-185 antagomir (Figures 1g–i and Supplementary Figures 1c and d). A similar result was obtained in MGC-803 cells (Supplementary Figure 1e). Then, we attempted to investigate the influence of miR-185 on cell susceptibility to chemotherapy. Following low-dose cisplatin (3 μM) or doxorubicin (0.2 μM) treatment in gastric cancer cells, a limited amount of cells undergoing apoptosis was observed. Whereas when we enforced expression of miR-185, the apoptotic cells were significantly increased in response to the same dose of cisplatin or doxorubicin (Figures 1j and k and Supplementary Figure 1f). Taken together, these results suggest that miR-185 reduces chemotherapy resistance in gastric cancer.

ARC is a target of miR-185. To elucidate the potential mechanisms by which miR-185 regulates apoptosis, we performed a bioinformatic analysis by TargetScan and found that human ARC mRNA 3′-untranslated regions (UTRs) contained two binding sites for miR-185 (Figure 2a). We measured ARC protein levels in GES-1 and gastric cancer cells. Western blot results indicated that ARC was highly expressed in gastric cancer cells, whereas it was not detectable in GES-1. Endogenous levels of ARC were inversely associated with that of miR-185 in these cell lines (Figure 2b). Then, we attempted to evaluate if miR-185 modulated ARC expression. Enforced expression of miR-185 resulted in an obvious reduction of ARC protein levels, but not in ARC mRNA levels in SGC-7901 and MGC-803 cells (Figure 2c). In contrast, administration of miR-185 antagomir attenuated the decrease of ARC protein levels upon cisplatin (Figure 2d) or doxorubicin (Figure 2e) treatment. Therefore, it seems that miR-185 modulates ARC expression at the post-transcriptional level. To verify whether miR-185 directly targets ARC, we cloned ARC 3′-UTR containing two miR-185 binding sites downstream of the luciferase reporter gene (ARC 3′-UTR-Wt) to examine luciferase translation driven by the 3′-UTR of ARC. Synthesized miR-185 mimic or miRNA mimic control were transfected into HEK-293 cells to perform the luciferase assays. MiR-185 overexpression induced a decrease in the luciferase activity (Figure 2f). Besides, we generated mutated luciferase constructs ARC 3′-UTR-Mut1 and ARC 3′-UTR-Mut2, and mutations were introduced into the two miR-185 binding sites of ARC 3′-UTR, respectively. We found that both the binding sites were responsible for the role of miR-185 in regulating ARC expression (Figure 2f). Simultaneous mutations of two binding sites could nearly abolish the inhibitory effect of miR-185 on luciferase activity (Figure 2f). We further investigated the effect of miR-185 on ARC expression in gastric cancer cells, as shown by luciferase activity. The loss of luciferase expression driven by ARC 3′-UTR upon cisplatin treatment was attenuated by the site-directed mutations in ARC 3′-UTR (Figure 2g). This reduction of luciferase activity was also attenuated when transfecting miR-185 antagomir (Supplementary Figure 2). Thus, our data indicate that miR-185 is able to target ARC directly.

MiR-185 regulates chemotherapy resistance through ARC. Abundant studies have reported that ARC was expressed at high levels in cancer cells.[20] There is evidence implicating that ARC may be involved in carcinogenesis and chemotherapy resistance.[25] After cisplatin or doxorubicin treatment, we found a strong reduction of ARC protein levels, which is contrary to the alteration of miR-185 levels. However, ARC mRNA levels were not reduced as much as its protein levels (Figures 3a and b). We next explored the role of ARC in gastric cancer chemoresistance. High-dose cisplatin or doxorubicin triggers significant apoptosis. Enforced ARC expression diminished chemotherapy-induced apoptosis (Figures 3c and d). Whereas knockdown of endogenous ARC enhanced the sensitivity of gastric cancer cells to low-dose cisplatin (Figure 3e and Supplementary Figure 3a). We wondered whether miR-185 regulated chemotherapy sensitivity in gastric cancer cells by targeting ARC. Overexpression of miR-185 promoted low-dose cisplatin-induced cell death. ARC without 3′-UTR (ARC) showed a stronger inhibitory effect on cell death than ARC

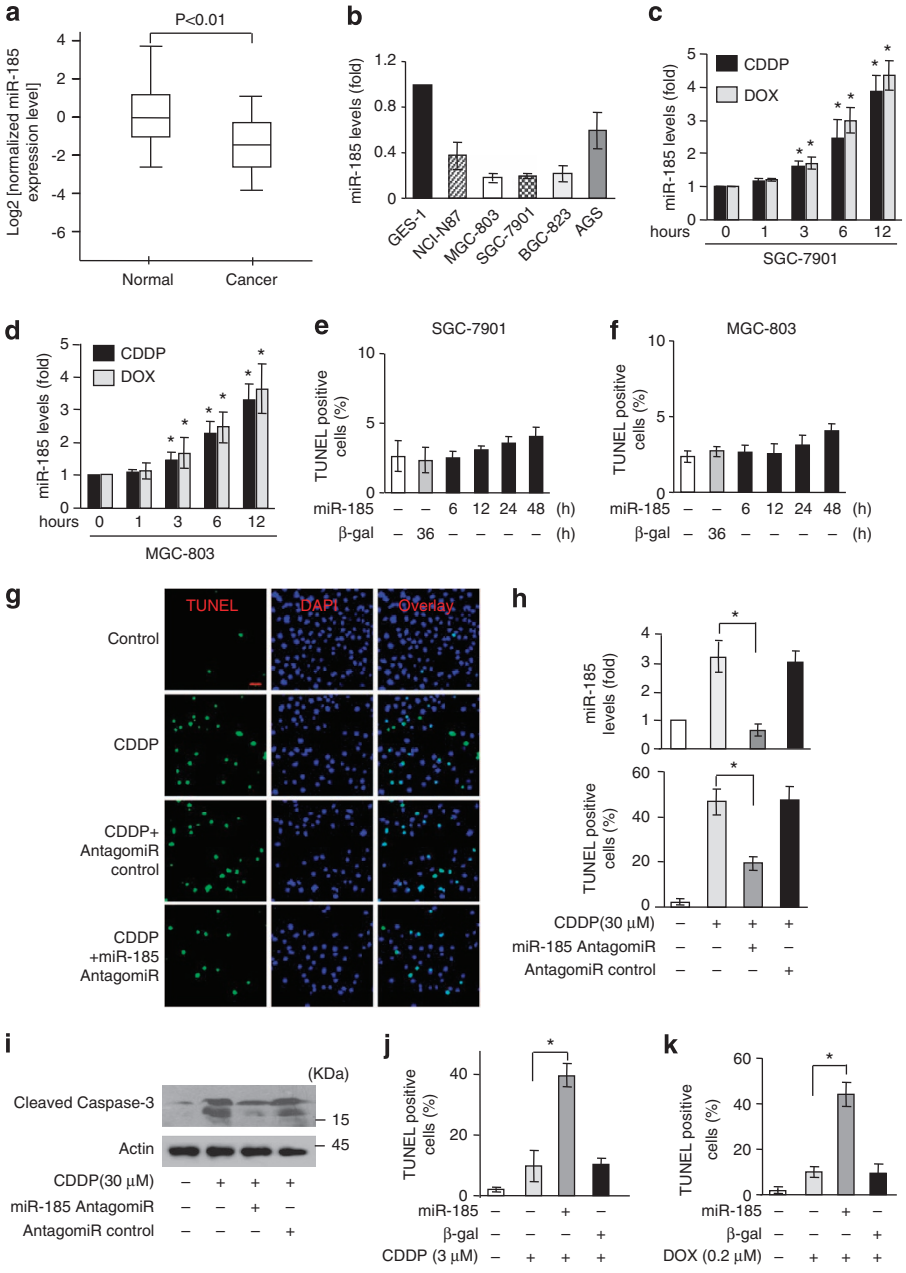

Figure 1 Enforced miR-185 enhances the chemosensitivity of gastric cancer cells. (**a**) MiR-185 expression levels in 25 pairs of human gastric cancer tissues and matched normal tissues. The boxes represent lower and upper quartiles separated by the median, and the excluding point was marked as a dot. (**b**) Real-time polymerase chain reaction (PCR) analysis of miR-185 levels in GES-1 and five gastric cancer cell lines. (**c** and **d**) MiR-185 levels in SGC-7901 (**c**) and MGC-803 cells (**d**) after 30 μM cisplatin (CDDP) or 2 μM doxorubicin (DOX) treatment as the indicated time. $^*P<0.05$ compared with 0 h. (**e** and **f**) Apoptosis detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay in SGC-7901 (**e**) and MGC-803 cells (**f**) overexpressing miR-185. (**g**–**i**) Inhibition of endogenous miR-185 using miR-185 AntagomiR (**h**, top) prevented apoptosis in SGC-7901 treated with 30 μM cisplatin for 24 h. Representative images show TUNEL-positive cells (**g**). Green, TUNEL-positive nuclei; blue, 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei, scale bar = 50 μm. Expression levels of miR-185 were detected (**h**, top).The apoptotic cells were counted (**h**, bottom). Cleaved caspase-3 was analyzed by western blot (**i**). (**j** and **k**) Ectopic expression of miR-185 enhanced apoptosis in SGC-7901 after 3 μM cisplatin (**j**) or 0.2 μM doxorubicin (**k**) treatment for 36 h. Error bars represent S.D. $^*P<0.05$

with 3′-UTR (ARC-3′-UTR) in the presence of miR-185 (Figure 3f and Supplementary Figure 3b). These results reveal that ARC contributes to chemotherapy resistance, which is regulated by miR-185.

MiR-185 is a transcriptional target of RUNX3. The expression of miRNAs can be regulated by transcriptional factors.[4] To elucidate the mechanism involved in the upregulation of miR-185 upon cisplatin or doxorubicin treatment, we analyzed the promoter sequence of miR-185. Three putative binding sites for RUNX3 were identified (Figure 4a). RUNX3 is a transcriptional factor that has a loss of function in gastric cancer.[22] Our findings that cisplatin or doxorubicin treatment increased RUNX3 expression

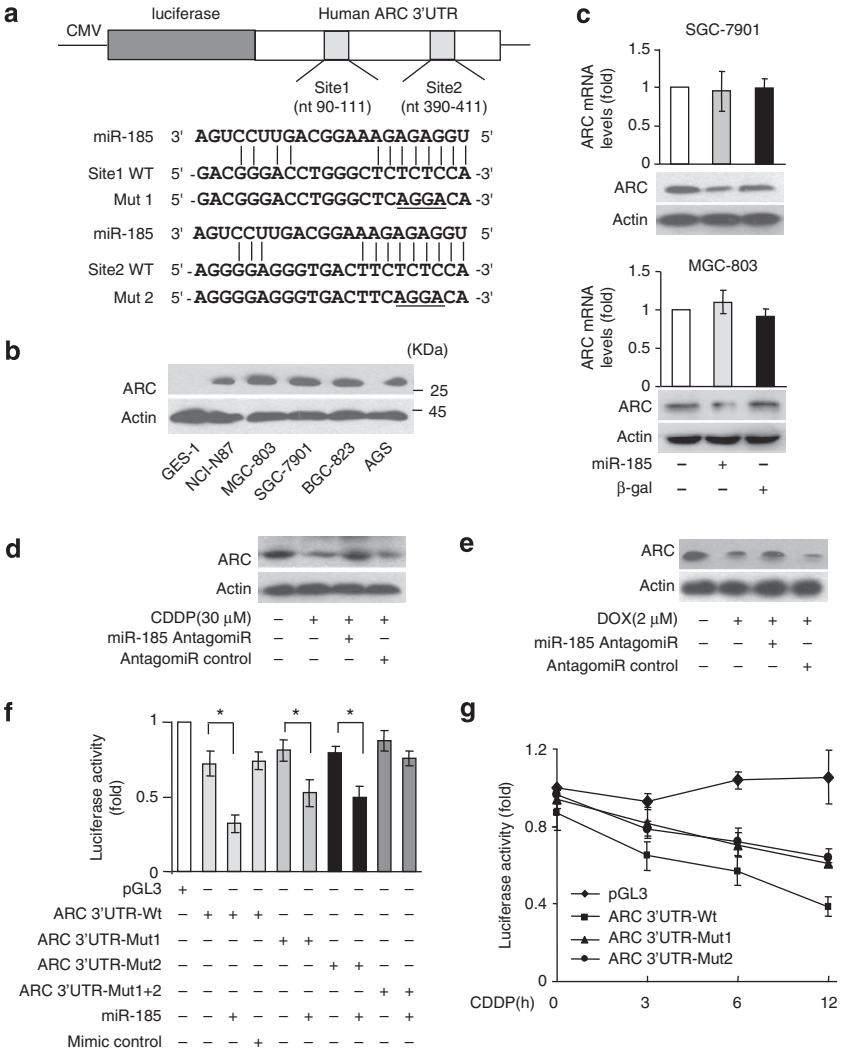

Figure 2 ARC is a direct target of miR-185. (**a**) Schematic diagram of the reporter constructs containing two putative miR-185 binding sites in human ARC 3′-UTR, mutations introduced into miR-185 binding sites were underlined. (**b**) Immunoblot of ARC protein in GES-1 and gastric cancer cells. (**c**) ARC mRNA levels and protein levels in SGC-7901 and MGC-803 cells overexpressing miR-185. (**d** and **e**) Knockdown of endogenous miR-185 attenuated decrease of ARC protein levels upon cisplatin (CDDP) (**d**) or doxorubicin (DOX) (**e**) treatment for 12 h in SGC-7901. (**f**) Luciferase activity detected in HEK-293 transfected with synthesized miR-185 mimic or mimic control, along with luciferase reporter constructs as indicated. (**g**) Luciferase activity of transfected reporter constructs in 30 μM cisplatin-treated SGC-7901. Error bars represent S.D. $^*P<0.05$. CMV, cytomegalovirus; β-gal, β-galactosidase; Mut, mutated; Wt, wild type

(Figures 4b and c) led us to consider whether RUNX3 participated in the upregulation of miR-185 expression. To test this hypothesis, the chromatin immunoprecipitation (ChIP) analysis was carried out to test the association of RUNX3 with the promoter of miR-185. It revealed that RUNX3 could directly bind to the binding site 3 (BS3) region but not the binding site 1 (BS1) and 2 (BS2) region in SGC-7901 cells treated with cisplatin or doxorubicin (Figure 4d). We next evaluated the transcriptional activity of miR-185 in response to RUNX3. MiR-185 promoter region was cloned upstream of luciferase reporter gene. Overexpression of RUNX3 induced a significant increase of miR-185 promoter activity, and this elevation was reversed by introducing mutations into the BS3 region of miR-185 promoter (Figure 4e). We determined that cisplatin or doxorubicin treatment increased miR-185 promoter activity in gastric cancer cells, whereas mutations in BS3 region arrested this elevation (Figure 4f). Finally, we found that enforced expression of RUNX3 led to a remarkable increment of miR-185 expression in SGC-7901 and MGC-803 cells (Figure 4g and Supplementary Figure 4). Collectively, our data indicate that miR-185 is directly upregulated by RUNX3 at the transcriptional level.

MiR-185 and ARC are necessary for RUNX3 tumor-suppressing activity. The involvement of miR-185 and ARC in regulating chemotherapy resistance led us to consider whether RUNX3 controls chemosensitivity and apoptosis in gastric cancer through this pathway. Reduced apoptosis of gastric epithelial cells was found in Runx3-deficient mice.[22] Besides, RUNX3 has been shown to

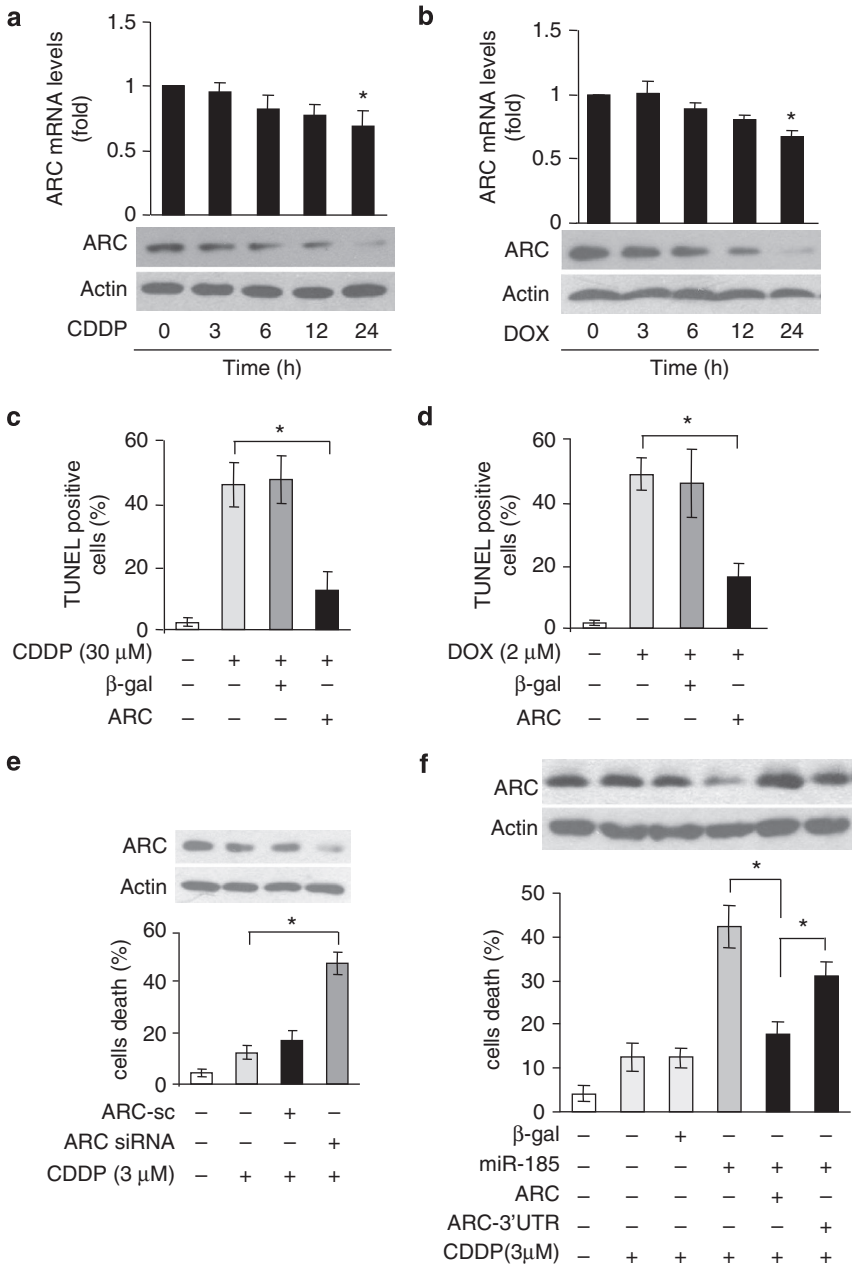

Figure 3 MiR-185 regulates chemotherapy resistance through ARC. (**a** and **b**) ARC mRNA levels (top) and protein levels (bottom) in SGC-7901 treated with 30 μM cisplatin (CDDP) (**a**) or 2 μM doxorubicin (DOX) (**b**). *$P<0.05$ compared with 0 h. (**c** and **d**) Ectopic expression of ARC prevented apoptosis in SGC-7901 treated with cisplatin (**c**) or doxorubicin (**d**) for 24 h. (**e**) Knockdown of endogenous ARC using its small interfering RNA (siRNA) increased cell death in SGC-7901 treated with cisplatin for 36 h, compared with ARC scrambled form (ARC-sc) group. Cell death was analyzed by trypan blue exclusion. (**f**) MiR-185 increased cell death in SGC-7901 in response to cisplatin treatment for 36 h, which was prevented by ARC. Error bars represent S.D. *$P<0.05$

mediate TGF-β-induced apoptosis.[26] Thus, we first tested whether RUNX3 influenced apoptosis in gastric cancer. An increment of apoptotic cells was observed in SGC-7901 cells overexpressing RUNX3 (Figure 5a). Concomitantly, ARC protein levels, but not its mRNA levels, were strongly reduced (Figure 5b). Silencing RUNX3 by using its siRNA attenuated the increase of miR-185 levels and reduction of ARC protein levels in response to cisplatin or doxorubicin (Figures 5c and d). Moreover, knockdown of endogenous RUNX3 diminished chemotherapy-induced cell death in SGC-7901 (Figures 5e and f).

Next, we were interested in determining the effect of RUNX3 on cell susceptibility to anticancer drugs. Strikingly, we found that RUNX3 overexpression led to an elevated amount of cells undergoing cell death, as well as an increase of miR-185 levels in response to low-dose cisplatin (Figure 5g). RUNX3 enhanced cisplatin sensitivity of gastric cancer cells.

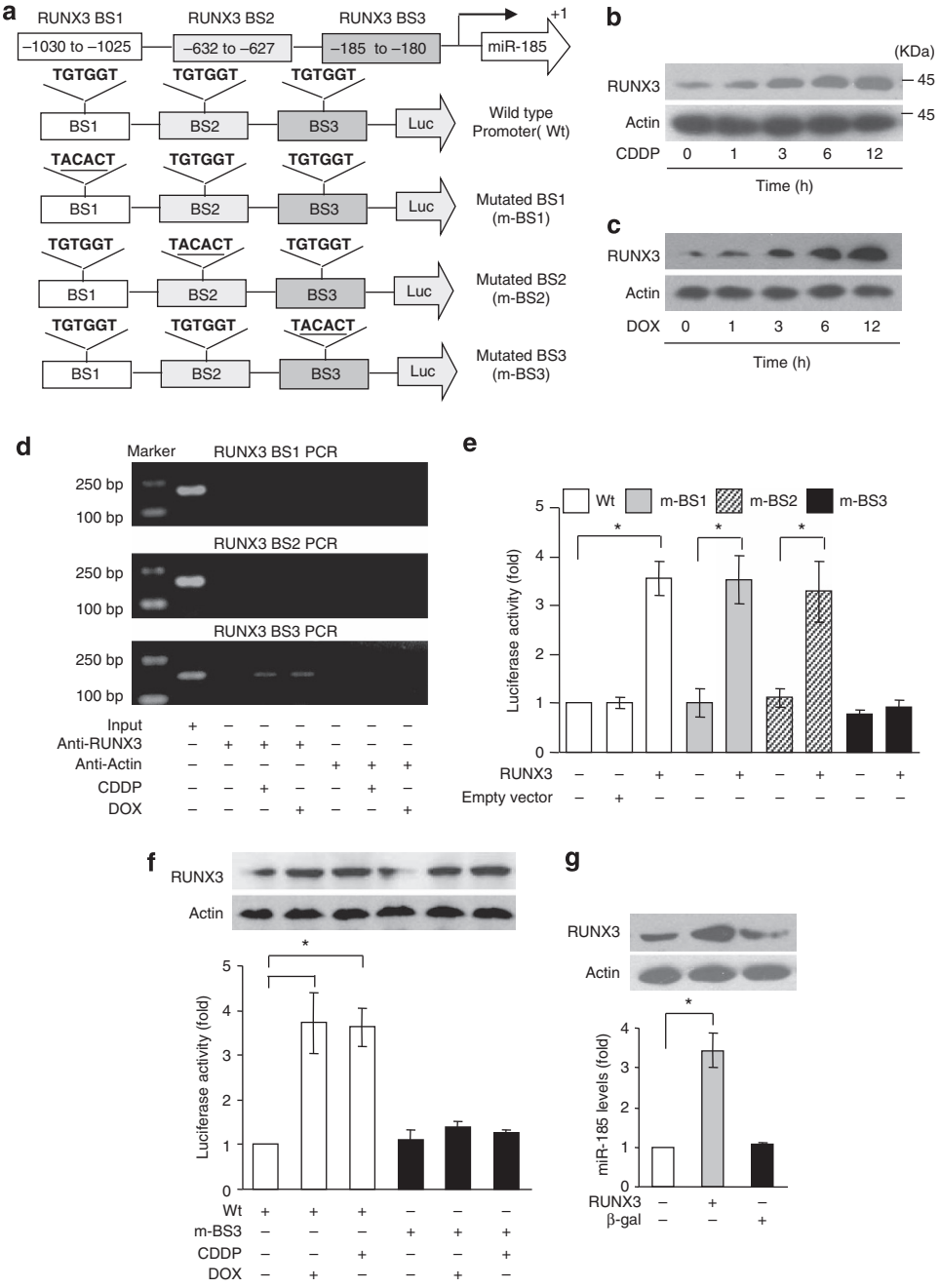

Figure 4 MiR-185 is a transcriptional target of RUNX3. (**a**) The promoter region of human miR-185 contains three optimal RUNX3 binding sites (BS1, BS2 and BS3). The mutations were introduced into the potential binding sites (m-BS1, m-BS2 and m-BS3). (**b** and **c**) Immunoblot of RUNX3 protein in SGC-7901 treated with 30 μM cisplatin (CDDP) (**b**) or 2 μM doxorubicin (DOX) (**c**). (**d**) ChIP analysis of RUNX3 binding to miR-185 promoter in SGC-7901 treated with 30 μM cisplatin or 2 μM doxorubicin for 12 h. (**e** and **f**) Luciferase activity of reporter constructs harboring wild-type (Wt) or mutated miR-185 promoter in HEK-293 cells transfected with plasmids encoding RUNX3 (**e**) or SGC-7901 cells treated with 30 μM cisplatin or 2 μM doxorubicin for 12 h (**f**, bottom). The expression levels of RUNX3 were detected by immunoblot (**f**, top). (**g**) Immunoblot of RUNX3 (top) and miR-185 levels (bottom) in SGC-7901 infected with adenoviral RUNX3. Error bars represent S.D. $^*P<0.05$

Subsequently, we considered whether miR-185 and ARC contributed to the function of RUNX3 in gastric cancer. Compared with antagomir control, administration of miR-185 antagomir reduced RUNX3-enhanced chemosensitivity (Figure 5h and Supplementary Figure 5a). This reduction was also observed in the presence of the construct encoding ARC. ARC without 3′-UTR exhibited a stronger inhibitory effect on cell death than ARC with 3′-UTR (ARC-3′-UTR) in the presence of RUNX3 (Figure 5i and Supplementary Figure 5b). These results indicate that RUNX3 mediates chemosensitivity and apoptosis in gastric cancer by modulating miR-185/ARC signaling pathway, although RUNX3 can also target other factors involved in apoptosis.[26,27]

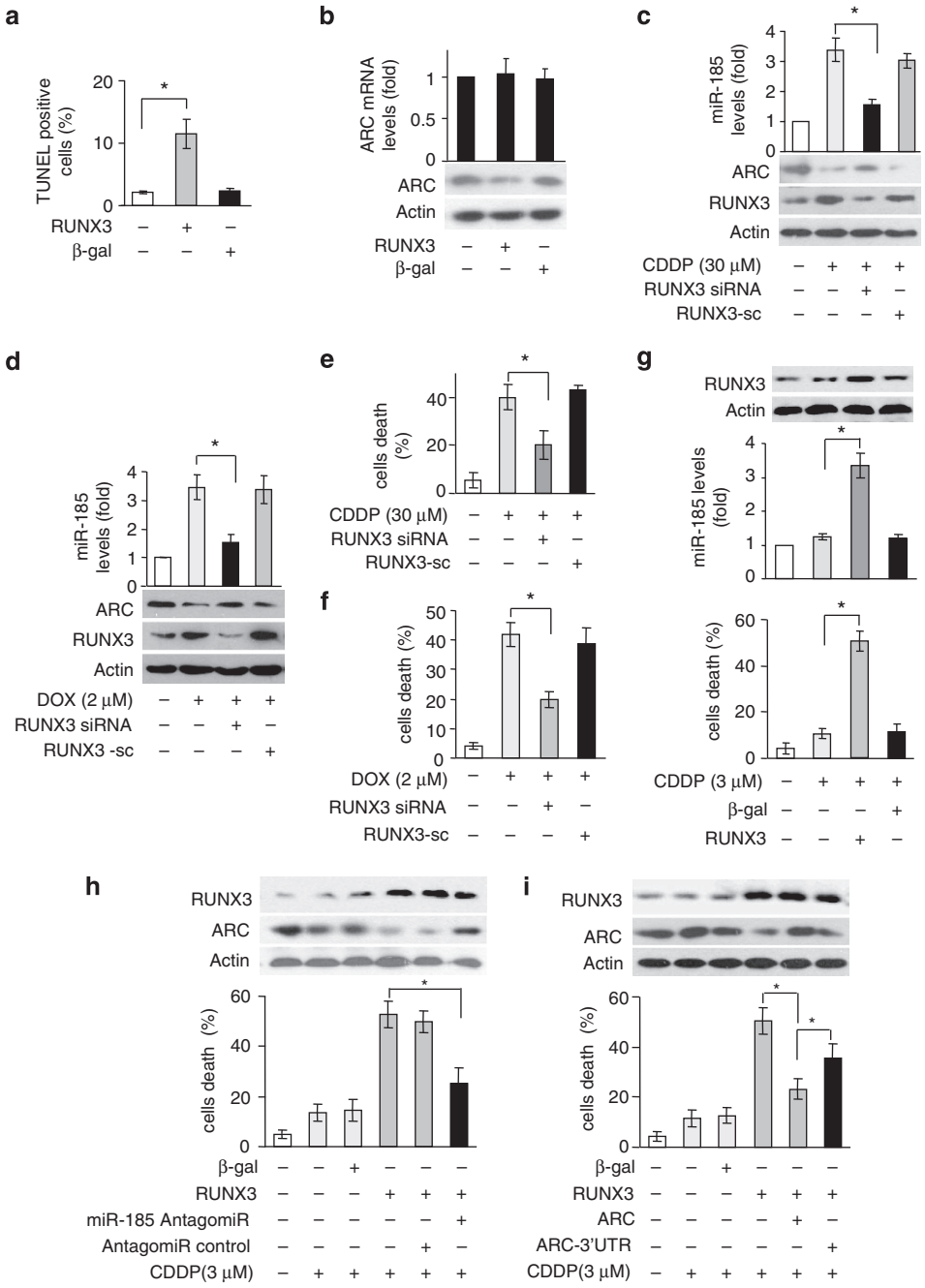

Figure 5 MiR-185 and ARC are necessary for RUNX3 tumor-suppressing activity. (**a** and **b**) Determination of apoptosis (**a**), analysis of ARC mRNA levels (**b**, top) and protein levels (**b**, bottom) in SGC-7901-overexpressing RUNX3. (**c** and **d**) Knockdown of endogenous RUNX3 using its small interfering RNA (siRNA) (bottom) attenuated upregulation of miR-185 expression (top) and downregulation of ARC (bottom) in SGC-7901 following cisplatin (CDDP) (**c**) or doxorubicin (DOX) (**d**) treatment for 12 h, compared with corresponding scrambled form (RUNX3-sc) group. (**e** and **f**) Knockdown of endogenous RUNX3 attenuated cell death in SGC-7901 treated with cisplatin (**e**) or doxorubicin (**f**) for 24 h. (**g**) Enforced expression of RUNX3 (top) increased miR-185 levels (middle) and cell death (bottom) in SGC-7901 following cisplatin treatment for 36 h. (**h** and **i**) RUNX3 increased sensitivity to cisplatin treatment for 36 h was abolished by miR-185 AntagomiR (**h**) or ARC (**i**) in SGC-7901. Error bars represent S.D. $^*P<0.05$. β-gal, β-galactosidase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling

MiR-185 enhances chemotherapeutic effect *in vivo*. It has been reported that miR-185 suppressed the proliferation of human colorectal cells.[28] Having demonstrated that miR-185 significantly increased the susceptibility of gastric cancer cells to chemotherapy *in vitro*, we further investigated the role of miR-185 in xenograft models. First, to test whether miR-185 alone could inhibit gastric cancer tumor growth, SGC-7901 cells stably overexpressing miR-185 or negative control were established. Overexpression of miR-185 was confirmed by qRT-PCR (Supplementary Figure 6a). The stable cells, SGC-7901-miR-185- or SGC-7901-negative control, were injected subcutaneously into nude mice. Xenograft tumor formation was monitored over a 34-day time course. Compared with controls, miR-185

overexpression resulted in a smaller tumor size (Figure 6a), suggesting that miR-185 suppressed gastric tumor growth *in vivo*. The mice were killed at day 34. As expected, we found significantly increased miR-185 levels in SGC-7901-miR-185-generated tumors, accompanied by the downregulation of ARC (Figure 6b).

Next, we attempted to assess miR-185 therapeutic activity against established tumors. SGC-7901 cells were subcutaneously inoculated into nude mice. When the tumor volumes reached 250–300 mm^3, intraperitoneal delivery of doxorubicin and/or intratumoral injection of miR-185 adenovirus were given every other day. Two different doses of doxorubicin, 2 and 4 mg/kg, were used in this study. During 2 weeks of therapy, we followed subcutaneous tumor growth and body weight of the mice. It was found that high-dose chemotherapy appeared as obvious inhibitors of tumor growth, and clear body weight loss indicated the severe toxicity of chemotherapy (Figure 6c and Supplementary Figure 6b). In comparison with high-dose chemotherapy, miR-185 alone or combined with low-dose doxorubicin did not exhibit obvious adverse effect, which was indicated by slight body weight loss (Supplementary Figure 6b). Combination therapy using low-dose doxorubicin and miR-185 successfully restrained the growth of established gastric tumor, as effective as high-dose chemotherapy (Figure 6c). Additionally, quantitative analysis of tumors from combination treatment group showed an elevation of miR-185 levels and remarkable reduction of ARC expression (Figure 6d), which may be contributed to enhanced apoptosis, as assessed by the TUNEL assay (Figure 6e and Supplementary Figure 6c) in xenograft models. These findings were consistent with our obtained results *in vitro*. To sum up, our data indicate that miR-185 increases gastric cancer chemosensitivity through suppressing ARC expression *in vivo*.

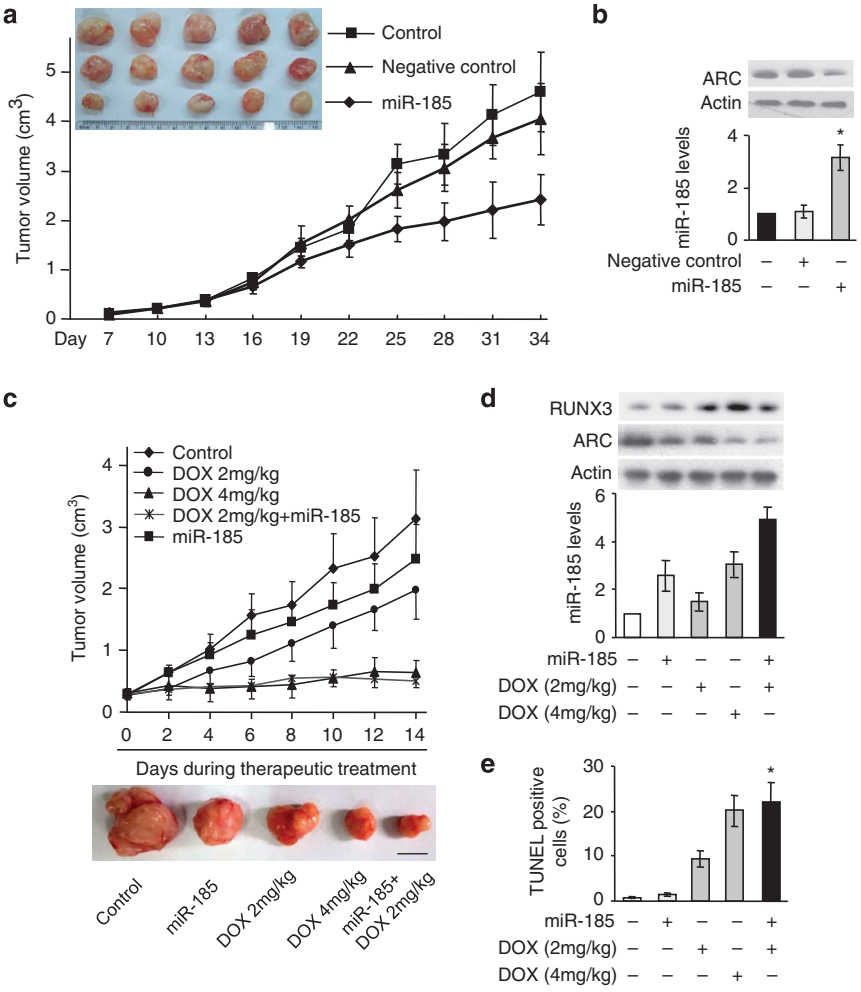

Figure 6 MiR-185 enhances the chemotherapeutic effect *in vivo*. (**a**) A total of 1×10^7 SGC-7901 cells stably overexpressing miR-185 or negative control were injected subcutaneously into BALB/c nude mice. Line graph shows tumor volumes measured every 3 days from day 7. Photographs show morphology of tumors at day 34, $n = 5$ each group. (**b**) Immunoblot of ARC protein (top) and miR-185 expression levels (bottom) in generated xenograft tumors shown in **a**, $^*P < 0.05$ compared with control. (**c**) A total of 1×10^7 SGC-7901 cells were injected subcutaneously into BALB/c nude mice. When tumors reached 250–300 mm^3, therapeutic treatment as indicated was given every other day. Tumor volumes were monitored during 2-week therapy ($n = 6$ each group) (top). At the end of this experiment, tumors were dissected and photographed. Representative images of tumors were shown (bottom), scale bar = 1 cm. (**d**) Western blot analysis as indicated and miR-185 expression levels in the xenograft tumors described in **c**. (**e**) Apoptosis detected in sections of xenograft tumors described in **c**, $n = 6$ each group. $^*P < 0.05$ compared with doxorubicin (DOX) (2 mg/kg) alone. Error bars represent S.D. TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling

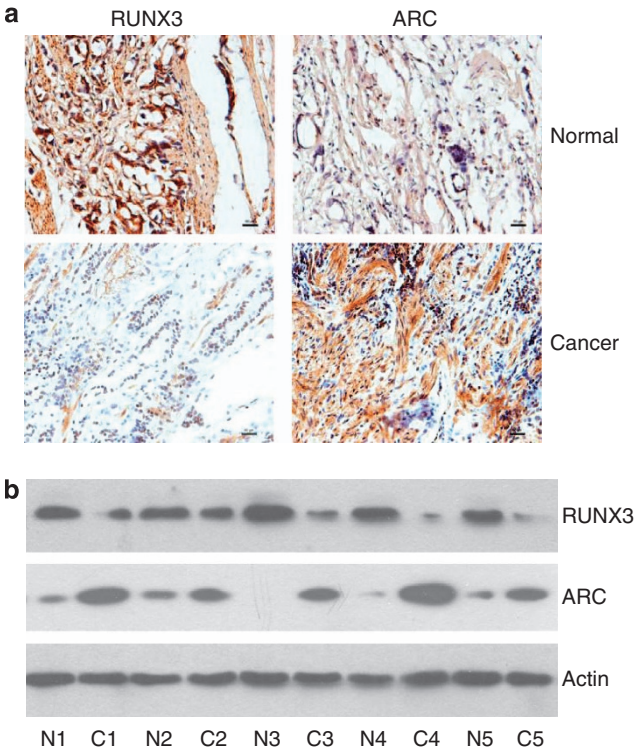

Figure 7 RUNX3 and ARC are dysregulated in human gastric carcinomas. (**a**) Immunohistochemistry staining of RUNX3 and ARC in paraffin sections of patient tissues, scale bar = 50 μm. (**b**) Immunoblot of RUNX3 and ARC proteins in five representative pairs of normal gastric tissues (N) and matched cancer tissues (C). The patient numbers were 1–5 as in Supplementary Table 1

RUNX3 and ARC are dysregulated in human gastric carcinomas. To further explore the potential clinical relevance of miR-185-mediated signaling pathway in human gastric cancer, we determined the expression levels of RUNX3 and ARC in human gastric tissues (Supplementary Table 1). Immunohistochemical staining showed loss of RUNX3 expression and high expression of ARC in tumor tissues (Figure 7a). This observation was further confirmed by immunoblotting of RUNX3 and ARC protein in these clinical samples. Five representative results were shown here (Figure 7b). According to our data, the inverse association between ARC expression and RUNX3 protein levels was indicated. These findings are consistent with the results in gastric cancer cell lines, and suggest that RUNX3–miR-185–ARC axis may be involved in the development of gastric cancer.

Discussion

Chemotherapy has an important role in the treatment of a variety of cancers, including gastric cancer. However, chemoresistance is a major challenge to effective treatment. It is quite urgent to explore potential mechanisms involved in chemotherapy resistance. Our current study identified miR-185 as a regulator in the sensitivity of gastric cancer cells to chemotherapy. We found that miR-185 was expressed at a lower level in several gastric cancer cell lines and human gastric cancer tissues. Enforced expression of miR-185 led to enhanced sensitivity of gastric cancer cells to low-dose chemotherapeutic agents. Furthermore, we discovered an miR-185-controlled apoptotic pathway involving ARC, which was regulated by RUNX3. Our conclusion was also supported by the results in nude mice model and clinical gastric cancer specimens.

Aberrant miR-185 expression has been identified in various cancers in previous studies. It has been shown that miR-185 was abundant in hepatocellular carcinoma.[29] However, there were opposite findings that decrease of miR-185 levels occurred in ovarian cancer,[30,31] glioma[32] and prostate cancer.[33] The function of miR-185 in cancer cells may be cell type-dependent.[34] In gastric cancer, lower expression levels of miR-185 were observed,[23,35] which was consistent with our findings. However, the exact function of miR-185 in gastric cancer is little known. Our results displayed miR-185 tumor-suppressing activity in gastric cancer xenograft model. Based on our experiments, induction of miR-185 could be a possible therapy strategy for gastric cancer.

MiR-185 suppressed the proliferation potential and tumor growth in colorectal cancer,[28] in non-small-cell lung cancer[24] and in ovarian cancer.[31] It inhibited invasion and migration in human prostate cancer xenograft model.[33] Moreover, miR-185 could sensitize high Six1-expressing cancer cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis.[31] MiR-152 and miR-185 cocontributed to chemosensitivity through epigenetic regulation in ovarian cancer.[34] These investigations implied that miR-185 was possibly related to apoptosis. However, the mechanism of miR-185 involvement in apoptosis signaling pathway is unclear. Our results showed that miR-185 increased anticancer drug-induced apoptosis by directly targeting ARC. MiR-185 has been shown to target other proteins in different types of cells. For instance, miR-185 regulated HIF-2α in human umbilical vein endothelial cells.[36] Six1,[31] androgen receptor,[33] Cdc42 and RhoA[28] were proven to be downregulated by miR-185 in cancer cells. Interestingly, miR-185 could disrupt DNA methylation by targeting DNA methyltransferase 1 in ovarian cancer[34] and gastric cancer.[35] In this context, we clearly demonstrated that ARC is a direct target of miR-185. To the best of our knowledge, this is the first report on the regulation of ARC expression mediated by an miRNA. ARC was highly expressed in various cancer cells. The function of miR-185 in these ARC-abundant cancer cells needs to be further explored.

Because of the limited number of human gastric cancer tissues we collected in this study, we did not analyze the relevance of miR-185 expression levels to clinicopathologic features and prognosis of these patients. The clinical significance of miR-185 during gastric cancer progression needs to be investigated in the further studies. Besides, miR-185 polymorphic nucleotide variations were discovered in breast cancer,[37] chronic lymphocytic leukemia and colorectal cancer.[38] It would be interesting to identify whether miR-185 SNPs exists and functions in gastric cancer.

Our present data revealed that RUNX3 enhanced miR-185 expression at the transcriptional level in gastric cancer. It has been reported that RUNX3 increased Bim expression[26] and decreased vascular endothelial growth factor levels[39] owing

to its transcriptional activity. Previous researches have reported that methylation modification was possibly related to RUNX3 expression level.[22] MDM2-mediated ubiquitination participated in the regulation of RUNX3 as well.[40] In this study, we observed upregulation of RUNX3 in human stomach cancer cells exposed to chemotherapeutic drugs. The mechanism by which RUNX3 was upregulated in response to anticancer agents remains to be determined.

The therapeutic effects of conventional anticancer agents were limited owing to chemoresistance and their toxicity. MiRNAs have been demonstrated to participate in cancer progression and resistance to chemotherapy, thereby acting as a potential candidate for therapeutic intervention. Anti-miR-21 in combination with S-TRAIL resulted in significantly increased apoptosis in murine glioma models.[41] Administration of anti-miR-221 or anti-miR-181b along with tamoxifen led to apparent reduction in breast tumor size.[42] Combination treatment of miR-200c and paclitaxel markedly decreased ovarian tumor burden.[43] Here, we report that restoration of miR-185 alone can inhibit gastric cancer tumor growth. Moreover, combination therapy using enforced miR-185 expression and lower dose chemotherapeutic drugs had an effective therapeutic activity against large established tumors, with decreased host toxicity. Our results are first to implicate that miR-185 can be considered as a target for gastric cancer treatment. It would be quite possible to develop miR-185-based combinatorial strategy for effective cancer therapy.

Taken together, we report here that miR-185 increases the chemosensitivity of gastric cancer cells *in vitro* and *in vivo*. It exerts tumor-suppressing function through negatively regulating ARC. Besides, miR-185 upregulation in response to cisplatin or doxorubicin treatment in gastric cancer cells is dependent on RUNX3 transcriptional activity. Our results suggest that miR-185 might be a novel therapeutic target for gastric cancer.

Materials and Methods

Reagents and cell culture. Cisplatin, doxorubicin and G418 were purchased from Sigma (St. Louis, MO, USA). Anti-ARC antibody and anti-RUNX3 antibody were obtained from Abcam (Cambridge, UK). Anticleaved caspase-3 antibody was obtained from Cell Signaling Technology (Danvers, MA, USA). Human gastric cancer cell line SGC-7901 was obtained as we described previously.[14] Human gastric epithelium cell, GES-1, and human gastric cancer cells, NCI-N87, MGC-803, BGC-823, AGS, were obtained from Beijing Institute for Cancer Research (Beijing, China). The cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C.

Clinical gastric cancer samples. Gastric cancer tissues and matched noncancerous gastric tissues from 25 patients (19 males and 6 females; mean age, 59.76 years, range, 44–81 years) with gastric cancer were collected from Beijing Military General Hospital (Beijing, China). These patients were randomly selected from the patient pool of the hospital's Gastrointestinal clinic; none of the patients received chemotherapy or radiotherapy. Human tissues were collected during gastroscopy and immediately frozen in liquid nitrogen. The study was approved by the ethics committee of the Beijing Military General Hospital. Informed consent was obtained from all study subjects.

Cell viability assay and TUNEL analysis. Cell death was determined by trypan blue exclusion. The trypan blue-positive and -negative cells were counted on a hemocytometer. TUNEL assay was performed using a kit from Roche Applied Science (Hamburg, Germany). The procedures were followed as per the instructions in the kit. The samples were imaged using a laser scanning confocal microscope (Zeiss LSM 510 META, Carl Zeiss, Jena, Germany).

Adenovirus. Adenovirus ARC and adenovirus β-galactosidase were as we described previously.[14] Constructions of adenoviral miR-185 and RUNX3 were described in Supplementary Information. All adenoviruses were amplified in HEK-293 cells. Adenoviral infection of cancer cells was performed as we described previously.[14]

Cell transfection with miRNA duplexes or miRNA inhibitors. The hsa-miR-185 duplexes were synthesized by GenePharma Co. Ltd (Shanghai, China). MiR-185 mimic sequence was, 5′-UGGAGAGAAAGGCAGUUCCUGA-3′. Mimic control sequence was, 5′-UUGUACUACACAAAAGUACUG-3′. Chemically modified antisense oligonucleotides (antagomirs) were used to inhibit endogenous miR-185 expression. The antagomir sequence was, 5′-UCAGGAACUGCCUUUC UCUCCA-3′. All the bases were 2′-O-methyl-modified (GenePharma Co. Ltd). The antagomir control sequence was, 5′-CAGUACUUUUGUGUAGUACAA-3′ (GenePharma Co. Ltd). Cells were transfected with miRNA duplexes (50 nM) or antagomirs (50 nM) using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions.

Reporter constructions and luciferase assay. The fragment of ARC 3′-UTR containing two miR-185 binding sites (sites 1 and 2) was amplified by PCR. The forward primer was 5′-ATGCTGCTGGAGCTGAATCGGAT-3′, and the reverse primer was 5′-ATAGAGCGCCTTTTGTGTAAGTC-3′. To generate reporter vector containing miR-185 binding sites, the PCR product was cloned downstream of the stop codon of the luciferase gene of pGL3 vector (Promega, Madison, WI, USA). To generate ARC 3′-UTR-Mut1 and ARC 3′-UTR-Mut2, the mutations (the wild-type ARC 3′-UTR site1: TCTCTCCA, ARC 3′-UTR-Mut1: TCAGGACA; the wild-type ARC 3′-UTR site 2: TCTCTCCA, ARC 3′-UTR-Mut2: TCAGGACA) were produced using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The two binding sites of miR-185 were mutated simultaneously to generate ARC 3′-UTR-Mut1 + 2.

For luciferase assay performed in HEK-293 cells, cells in 24-well plates were co-transfected with 200 ng per well luciferase reporter constructs, 400 ng per well miR-185 mimic or mimic control using Lipofectamine 2000 (Invitrogen). SV-Renilla luciferase plasmids of 5 ng per well served as the internal control. Cells were harvested at 24 h after transfection and the luciferase activity was detected using the Dual Luciferase Reporter Assay Kit (Promega) according to the manufacturer's instructions. A measure of 30 μl of protein samples was analyzed in a luminometer. Firefly luciferase activities were normalized to *Renilla* luciferase activity. The similar strategy was used to perform luciferase analysis in SGC-7901 cells.

The miR-185 promoter region (1297 bp) was amplified from human genomic DNA to generate wild-type promoter. The fragment containing three RUNX3 putative binding sites was amplified using the forward primer, 5′-GGGCAGAGCA GAGCTACAAATG-3′, and the reverse primer, 5′-AACCCAGCTCTAGCCAG CAGGT-3′. The PCR product was cloned into the reporter vector pGL4.17 (Promega). The introduction of mutations in three potential RUNX3 binding sites (wild-type: TGTGGT → mutated: TACACT) was carried out using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). Luciferase assay was performed as described.[44]

Immunoblotting. Immunoblotting was performed as we described.[14] Briefly, cells were lysed for 1 h at 4 °C in a lysis buffer (20 mmol/l Tris (pH 7.5), 2 mmol/l EDTA, 3 mmol/l EGTA, 2 mmol/l DTT, 250 mmol/l sucrose, 0.1 mmol/l phenylmethylsulfonyl fluoride, 1% Triton X-100) containing a protease inhibitor cocktail. Protein samples were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed using corresponding primary antibodies. Then, the horseradish peroxidase-conjugated secondary antibodies were used. Antigen–antibody complexes were tested by enhanced chemiluminescence.

qRT-PCR. Stem-loop qRT-PCR was carried out as described on an Applied Biosystems ABI Prism 7000 sequence detection system (Applied Biosystems, Carlsbad, CA, USA).[44] Total RNA was extracted using Trizol reagent. After DNase I (Takara, Japan) treatment, RNA was reverse transcribed with Reverse Transcriptase Kit (Toyobo, Osaka, Japan). Mature miR-185 levels were measured using SYBR Green Real-Time PCR Master Mix (Toyobo) according to the manufacturer's instructions. The sequences of miR-185 primers were: forward,

5′-CATGGAGAGAAAGGCAGT-3′; reverse, 5′-GTGCAGGGTCCGAGGT-3′. The levels of miR-185 analyzed by qRT-PCR were normalized to that of U6. The sequences of U6 primers were: forward, 5′-CTCGCTTCGGCAGCACA-3′; reverse, 5′-AACGCTTCACGAATTTGCGT-3′. Quantitative detection of ARC and RUNX3 was performed using the same strategy. The primers used for ARC were: forward, 5′-CGAGTCCGAAGATTCCTGA-3′; reverse, 5′-GACCCTCCGGAGTT TATTCA-3′. The primers used for RUNX3 were: forward, 5′-GACAGCCC CAACTTCCTCT-3′; reverse, 5′-CACAGTCACCACCGTACCAT-3′. The mRNAs levels were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequences of GAPDH primers were: forward, 5′-GTCGGAGTC AACGGATTTG-3′; reverse, 5′-TGGGTGGAATCATATTGGAA-3′.

ChIP Analysis. ChIP was carried out as we described previously.[45] Briefly, cells were washed two times with phosphate-buffered saline (PBS), and then were incubated for 10 min with 4% formaldehyde at room temperature. After washing two times with PBS, cells were lysed in a lysis buffer for 1 h at 4 °C. The cell lysates were sonicated into chromatin fragments (500–800 bp length). The samples were precleared with Protein A-agarose (Roche Applied Science) at 4 °C. The anti-RUNX3 antibody or anti-actin antibody was added and rocked overnight. Immunoprecipitates were captured with 10% (v/v) Protein A-agarose. To analyze RUNX3 binding to the promoter of miR-185, PCRs were carried out using primers that encompass RUNX3 BS1 or BS2 or BS3 of the miR-185 promoter. The primers were: BS1 (forward, 5′-GGACTCTTGAGATGAGATCAC-3′; reverse, 5′-CTCTCCATTCTGTCAACTGG-3′); BS2 (forward, 5′-TCCTGCAGATGTTCA GATGC-3′; reverse, 5′-CAACTCTCAGCATGTCCTTG-3′); BS3 (forward, 5′-C CAGATCAAGATATGGTGACC-3′; reverse, 5′-ACCTGTGACCTTGCCTTTG-3′).

Subcutaneous tumor xenograft model. For the experiments in Figure 6a, approximately 1×10^7 SGC-7901 cells, established stable SGC-7901-miR-185 or SGC-7901-negative control cells, were injected subcutaneously into the right flanks of female BALB/c nude mice (4–5 weeks old, 5 mice per group).The tumor size was measured with a caliper every 3 days. The tumor volume was calculated using the formula volume = length $\times$ width^2/2. The mice were killed at day 34, and the tumors were separated for further analysis. For the combination therapy experiments in Figure 6c, 1×10^7 SGC-7901 cells were injected subcutaneously into the right flanks of female BALB/c nude mice (4–5 weeks old). When tumors reached an average volume of 250–300 mm^3, the mice were randomly divided into five groups, six mice in each group. According to the experimental design, intraperitoneal delivery of doxorubicin and/or intratumoral injection of miR-185 adenovirus was administered every other day. Two different doses of doxorubicin (2 and 4 mg/kg) were used. During doxorubicin and/or miR-185 adenovirus treatment in established tumors, the tumor size and body weight of the mice were monitored every other day. At the end of the experiment, the mice were killed and the tumors were separated for further analysis. Animal experiments were reviewed and approved by the Animal Care Committee, Institute of Zoology, Chinese Academy of Sciences.

Statistical analysis. All statistical analyses were performed using the SPSS 13.0 statistical software package. The results are expressed as means ± S.D. of at least three independent experiments. The differences among experimental groups were evaluated by one-way analysis of variance. Paired data were determined by two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant.

Additional Materials and Methods are available in Supplementary Information.

Conflict of Interest

The authors declare no conflict of interest.

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