Chemotherapy-induced miRNA-29c/catenin-δ signaling suppresses metastasis in gastric cancer

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ABSTRACT

Chemotherapy has improved the survival of gastric cancer (GC) patients by unknown mechanisms. In this study, we showed that cisplatin and docetaxel used in GC treatment increase expression of miRNA-29 family members (miR-29s) and decrease expression of their oncogenic targets, mediating a significant part of the efficacious benefits of these chemotherapeutic agents. In particular, GC patients who experienced recurrences after chemotherapy tended to exhibit low levels of miR-29c expression in their tumors, suggesting that miR-29c activation may contribute to the chemotherapeutic efficacy. Enforced expression of miR-29s in GC cells inhibited cell invasion in vitro and in vivo by directly targeting catenin-δ (CTNND1). Drug treatment suppressed GC cell invasion by restoring miR-29c-mediated suppression of catenin-δ and RhoA signaling. In parallel, drug treatment also activated several tumor suppressive miRNAs, thereby decreasing expression of their oncogenic effector targets. Overall, our findings defined a global mechanism for understanding the efficacious effects of cytotoxic chemotherapy in GC.
INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer, and its prognosis remains poor despite adequate surgery with radical lymphadenectomy(1). Most GC patients demonstrate locally advanced disease at the time of diagnosis and may also require chemotherapy and/or radiotherapy(1-2). Recent studies have demonstrated an advantage for perioperative chemotherapy compared with surgery(3). However, the molecular mechanisms of chemotherapeutic drugs used for GC treatment are not well-established.

MicroRNAs (miRNAs) play critical roles in multiple biological processes by regulating mRNAs via cleavage or translational repression(4). The loss of homeostasis in miRNA/mRNA axes leads to relevant pathological events, including gastric carcinogenesis(5). Therefore, miRNAs could serve as potential biomarkers for GC clinical diagnosis and prevention. The microRNA-29 (miR-29) family consists of 3 members, miR-29a, miR-29b, and miR-29c, and has been shown to suppress cancer cell growth(6-9). Our previous study indicated that miR-29s suppressed GC cell proliferation or invasiveness via targeting CCND2 or MMP2, implying its tumor suppressive role(10). However, the impact of miR-29s on GC cell metastasis and the corresponding mechanisms are not well-established. Recently, Saito et al. reported that miR-29c could be activated by celecoxib, suggesting that selective COX-2 inhibitors may hold promise for GC treatment by restoring...
miR-29c(11). These findings suggested that miR-29s not only functioned as ts-miRs in GC, but also might serve as effective predictors for GC prevention.

Herein, we identify miR-29c as a potential biomarker for predicting the prognosis of GC patients who receiving chemotherapy. We further find that miR-29s act as metastasis suppressors by directly targeting catenin-δ (CTNND1) in GC. Moreover, we demonstrate that chemotherapeutic drugs represent their suppressive impact on GC cell invasion via remodifying the miR-29c-mediated catenin-δ/GTP Rho axis.

Materials and methods

Clinical specimens and cell lines

Tissues were collected as previously described(12). The patient characteristics are provided in Supplementary Table S1. The normal gastric epithelial cell line (GES-1) was a kind gift from Dr. Shi Juan. 293T and GC cell line, HGC27, MGC803, BGC823, and MKN45 cell lines were obtained from the American Type Culture Collection and grown in DMEM with 10% FBS (Hyclone) at 37°C in 5% CO2 cell culture incubator. Cell lines were tested 1 month before the experiment by methods of morphology check by microscopy, growth curve analysis, and mycoplasma detection according to the ATCC cell line verification test recommendations.

Quantification of RNA and protein

Total RNA was extracted from the cells and tissues with TRIzol reagent (Invitrogen). Quantitative real-time PCR (qRT-PCR) assay was conducted to
detect the ts-miRs expression and primers sequences are listed in Supplementary Table S3. Western blotting of proteins was performed as described previously(12). The antibodies included those against catenin-δ, PDK1, YWHAZ, GTP-Rho, Rho, Cofinin, Phosphorylated-Cofinfin, and GAPDH (catenin-δ, PDK1, and GAPDH from Abcam, others from CST).

Cell proliferation, migration, and invasion assays

The cellular proliferation rate was measured using CCK-8 (DOJINDO) as previously described(12). Scratch wound assay was conducted to detect cell migration and performed as described(13). Invasion assay was evaluated by the ability of cells passing through Matrigel-coated membrane matrix (BD Biosciences) and performed as described(12).

Affinity-precipitation of cellular GTP-Rho.

Active Rho Pull-Down was conducted in HGC-27 cells transfected with miR-29s through Active Rho Pull-Down and Detection kit (PIERCE) as described(13).

Immunohistochemistry and light microscopy

Immunohistochemistry was conducted to measure the catenin-δ, Ki-67, and Caspase 3 expression as described(14). The localization of catenin-δ in GC cells was detected using immunostaining as described(13).

Tumorigenicity and metastasis formation assay

All experimental procedures involving animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publications.
Nos. 80–23, revised 1996) and according to the institutional ethical guidelines of Peking Union Medical College for animal experiments. Tumorigenicity and metastasis formation assay were performed as described(14-15).

Detailed experimental procedures are provided in the Supplementary Materials and Methods.

Statistics

Each experiment was repeated at least three times. Student's t test (two-tailed) was performed and three-group data were analyzed using one-way analysis of variance. All statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). P-values <0.05 were considered statistically significant.

RESULTS

miR-29s expression is activated by cisplatin and docetaxel in GC cells.

To elucidate the role of miR-29s in GC chemotherapy, we determined their expression in GC cells treated with cisplatin or docetaxel. qRT-PCR analysis was performed to effectively discriminate miR-29a, miR-29b, and miR-29c expression (Fig. S1A-D) and then applied to evaluate their levels in GC cell lines, showing that miR-29s levels depicted significant reduction (Fig.1A). We next treated MGC803 and HGC27 cells, which have low miR-29s expression, with cisplatin and docetaxel (0-10³ mg/L), and the estimated IC50 values in
these cell lines were 0.9 and 0.09 mg/L for cisplatin in MGC803 and HGC27 cells, respectively, and 0.008 and 0.0009 mg/L for docetaxel in MGC803 and HGC27 cells, respectively (Fig. 1B, C). Further qRT-PCR analysis indicated that miR-29s expression was augmented following cisplatin and docetaxel treatment (Fig. 1D, E). Moreover, we observed that Mcl1 and Bcl2, which are validated miR-29s targets (6, 16), were decreased in drugs-treated GC cells (Fig. 1F, G). These findings suggested that chemotherapy may hold clinical promise for GC treatment through regulating miR-29s-mediated program.

**Deregulated miR-29c expression is associated with GC progression and survival.**

We next investigated the correlation between miR-29s and GC development. qRT-PCR was used to assess miR-29s levels in tissues from 166 cases of GC, and we found that miR-29a, miR-29b, and miR-29c were significantly down-regulated in majority of GC tissues examined when compared with matched normal gastric tissues (Fig. 2A). The association between miR-29s expression and histological grade was explored and no significant difference was found between well- and poorly-differentiated GC tissues (Fig. S2A-C). However, we observed that miR-29c down-regulation was correlated with a more extensive venous invasion ($P=0.02$) (Fig. 2B) and a more aggressive tumor phenotype ($P=0.05$) (Fig. 2C). No clear relationship was observed between decreased miR-29a or miR-29b expression and worse prognosis in GC patients (Fig. S2D-E). Kaplan-Meier survival analysis was then conducted.
based on cut-off values determined using receiver operating characteristic (ROC) curves, which demonstrated that higher miR-29c levels in patients were correlated with longer disease-free survival (DFS) (Fig. 2D-F; Supplementary Table S2).

We further investigated whether chemotherapy held better clinical promise for GC patients with higher miR-29s expression in tumors. Follow-up studies were performed with 66 cases of GC patients undergoing chemotherapy after resection, and these patients were divided into 2 groups based on recurrence 3 years after treatment. We observed that the tumors of GC patients who underwent recurrence had lower miR-29c levels (Fig. 2G; Fig. S2F).

Altogether, these results suggest that miR-29c is involved in GC development and may serve as an effective predictor for the prognosis of GC patients receiving chemotherapy.

**miR-29s suppress GC cell migration and invasion in vitro.**

Given the parallels between miR-29c and metastatic potential, we next investigated miR-29s’ role in GC cell movement. miR-29s were overexpressed in MGC803 and HGC27 cells (Fig. S3), and wound-healing assays were performed to assess GC cell migration. Scramble-treated MGC803 cells completely sealed linear scratch wounds between 12 and 36 h after injury, whereas miR-29s-treated MGC803 cells sealed only ~70% of the wound area after 36 h (Fig. 3A). Similar results were observed in HGC27 cells (Fig.
3B). *In vitro* invasion assays were also conducted to evaluate GC cell metastasis, and demonstrated that miR-29s significantly decreased invasive GC cells compared to scramble-treated cells (Fig. 3C, D).

**miR-29c inhibits GC cell metastasis *in vivo*.**

To confirm the *in vitro* findings, we conducted *in vivo* metastasis assays. In doing so, $5 \times 10^5$ viable HGC27 cells infected with Lenti-29c or Lenti-scr were resuspended in 0.1 ml phosphate-buffered saline and injected into the lateral tail veins of nude mice. Five weeks after injection, the animals were sacrificed, and the lungs and liver were dissected for microscopic histology. The number of liver metastases in mice injected with Lenti-29c-infected HGC27 cells was significantly lower than that in mice injected with Lenti-scr-infected cells (Fig. 3E, F). Hematoxylin and eosin (H&E) staining was also performed to assess the pathological properties of the liver tissues, which showed more metastatic nodules in Lenti-scr-treated compared to Lenti-29c treated mice (Fig. 3G). Similar results were observed with H&E staining in the lung tissues, although we did not observe significant changes in outward appearance (Fig. 3H).

**Catenin-δ is a direct target of miR-29s.**

To identify mRNA targets of miR-29s relevant to GC cell invasion, we interrogated the TargetScan(17) and miRanda(18) miRNA target prediction programs. Catenin-δ was identified as a possible target of miR-29s and encodes a member of the armadillo protein family, which functions in adhesion
between cells and signal transduction\(^\text{19}\). The 3´UTR of \textit{catenin-δ} houses a sequence that matches the seed sequence contained in miR-29s (Fig. 4A). Transfection of the \textit{catenin-δ}-3´UTR-luciferase reporter in combination with miR-29s mimics in 293T cells revealed that miR-29s repressed the luciferase activity of this reporter. Moreover, mutation of miR-29s sites abrogated this reduction in luciferase activity (Fig. 4B). Furthermore, elevating miR-29s in HGC27 or MGC803 cells reduced the catenin-δ protein level (Fig. 4C), whereas diminishing miR-29s activity using specific inhibitors (anti-29s) resulted in increased catenin-δ expression (Fig. 4D, Fig. S4). Because cisplatin and docetaxel treatment led to an increase of miR-29s levels in GC cells and catenin-δ is a miR-29s target, catenin-δ level may be decreased in GC cells treated by these drugs. Immunoblotting analysis confirmed this hypothesis (Fig. 4E, F). Moreover, loss of the predominant membrane localization of catenin-δ and gain of cytosolic or nuclear catenin-δ labeling closely correlated with cancer invasion\(^\text{20-21}\). Therefore, we further analyzed the levels of catenin-δ in the cytoplasm via immunostaining. Compared to scramble-treated cells, HGC27 cells transfected with miR-29s mimics demonstrated a significant decrease in catenin-δ expression (Fig. 4G).

We also validated the miR-29c-mediated catenin-δ axis in xenograft mouse tumors. miR-29c was overexpressed in HGC27 cells using a lentivirus and subsequent cell growth analysis indicated that miR-29c restoration suppressed HGC27 cell proliferation \textit{in vitro} and inhibited GC tumor formation \textit{in vivo} (Fig.
Immunohistochemistry analysis was performed in randomly selected xenograft mouse tumors, which showed that Lenti-29c-injected tumors expressed lower levels of catenin-δ than the controls. Ki-67 and caspase-3 expression analysis demonstrated that miR-29c retarded GC cell growth (Fig. 4H, I). These data indicated that catenin-δ was a functional target of miR-29s in GC.

**Catenin-δ is up-regulated in GC tissues with low levels of miR-29s.**

We next investigated whether catenin-δ was expressed at a higher level in GC tissues with down-regulated miR-29s. qRT-PCR and immunobLOTS were conducted for 20 pairs of GC tissues, demonstrating that the levels of miR-29s were decreased in these tissues (Fig. 4J; Fig. S6A). Additionally, catenin-δ expression was significantly up-regulated in the same specimens shown by immunobLOTS (Fig. 4K; Fig. S6B). Catenin-δ staining was also increased in GC tissues shown by immunohistochemistry analysis (Fig. 4L; Fig. S7). These findings indicated a significant inverse correlation between miR-29s and catenin-δ expression.

**Silencing catenin-δ decreases GC cell migration and invasion.**

Of note, loss of E-cadherin leads to catenin-δ mislocalization in the cytoplasm and disrupts its interaction with Rho GTPases and subsequent promotion of cell metastasis(22). Because miR-29s/ catenin-δ axis was established in GC cells, we reasoned that silencing catenin-δ should retard the ability of GC cells
to seal scratch wounds. As predicted, si_catenin-δ-treated HGC27 cells sealed wounds at a slower rate compared to si_Con treatment (Fig. 5A). Furthermore, \textit{in vitro} invasion assay was used to assess the effects of catenin-δ on HGC27 cell metastasis, demonstrating that si_catenin-δ treatment resulted in a significant decrease in invasive cells (Fig. 5B).

To further probe the correlation between cell phenotypic alterations and the miR-29s/catenin-δ axis, we performed a rescue assay that increased and then decreased the level of catenin-δ \textit{via} anti-miR-29s in combination with si_catenin-δ, as described(12). Immunoblotting was used to evaluate catenin-δ expression and demonstrated that the level of catenin-δ was altered under different co-transfection conditions in HGC27 cells (Fig. 5C). Furthermore, \textit{in vitro} invasion assay was conducted to better determine alterations in cell motility corresponding with catenin-δ protein level variations, and we observed that silencing catenin-δ to prevent induction by anti-29s-treatment led to an increase in invasive cells (Fig. 5D, E).

**Down-regulation of catenin-δ decreases F-actin \textit{via} Rho GTP activation.**

We next investigated the role of catenin-δ in the formation of filamentous actin (F-actin). HGC27 cells were transfected with either si_catenin-δ or si_Con for 48 h, and immunostaining was performed. As expected, silencing catenin-δ markedly suppressed actin organization, which was demonstrated by phalloidin staining compared to si_Con-treated cells (Fig. 5F, G).
The above findings prompted us to explore the possible mechanisms controlling this process. Because cytosolic catenin-δ dampens the activity of small Rho family GTPases and evokes cell migration, we examined the status of activated Rho in HGC27 cells treated with si_catenin-δ using a GST-fusion protein containing the Rho-binding domain of mouse Rhotekin. Immunoblotting revealed that si_catenin-δ resulted in a significant decrease in catenin-δ protein, and the level of activated RhoA was increased in si_catenin-δ-treated HGC27 cells compared to cells treated with si_Con (Fig. 5H). We further evaluated the expression of cofilin, a major downstream target of RhoA(23) and observed an increase in p-cofilin following 48 h treatment of HGC27 cells with si_catenin-δ, although no significant change was observed in total cofilin levels (Fig. 5H).

miR-29s increase F-actin and p-cofilin via activated Rho.

We next used an alternative approach to down-regulate catenin-δ, based on the fact that catenin-δ is a direct target of miR-29s in GC cells. Immunoblotting revealed miR-29s-treatment resulted in a decrease in catenin-δ protein levels as well as an increase in activated RhoA and p-cofilin compared to the scr-treatment (Fig. 5I). We also explored whether the up-regulation of miR-29s had a similar effect on actin organization as silencing catenin-δ in GC cells. Immunostaining analysis was conducted and showed that miR-29s restoration resulted in decreased actin organization in HGC27 cells (Fig. 5J, K).
These results demonstrate that miR-29s regulate GC cell movement by suppressing the catenin-δ pathway.

**Cytotoxic drugs treatment suppresses GC cell movement via regulating miR-29c-mediated catenin-δ axis.**

To investigate whether cytotoxic drugs represented their suppressive effect on GC cell movement, a series of rescue assays were conducted. In doing so, miR-29c inhibitor was transfected into GC cells after cisplatin and docetaxel treatment to re-modify miR-29c-mediated catenin-δ axis. qRT-PCR analysis revealed that cisplatin and docetaxel treatment led to an increase of miR-29c expression and this upregulation was dampened by anti-29c (Figure 6 A, B). We also observed that catenin-δ protein was decreased upon drugs treatment and increased with anti-29c transfection shown by immunoblots (Fig. 6C, D). The following wound healing assay and *in vitro* invasion assay indicated that miR-29c inhibition to prevent its induction by cisplatin or docetaxel treatment led to increased cell movement (Fig. 6E, F, G). These findings suggest that miR-29c-mediated catenin-δ axis is a target of cytotoxic drugs in GC cells.

**Clinical correlation among miR-29c, catenin-δ and tumor progression in GC patients.**

We further probed the clinical correlations among miR-29c, catenin-δ, and the TNM stage of GC patients. Expression analyses of miR-29c and catenin-δ were conducted with formalin-fixed paraffin-embedded (FFPE) tissues from
the 66 cases of GC mentioned above. qRT-PCR analysis indicated that miR-29c level was significantly decreased in majority of the examined GC samples (Fig. S8A-C). Catenin-δ protein in these FFPE tissues was evaluated by immunohistochemical staining, demonstrating a significant inverse correlation between miR-29c and catenin-δ (Fig. S8F; Supplementary Table S4) (miR-29c, $R^2=0.3382$, $P<0.0001$). We also observed that GC patients with venous metastasis tended to express high levels of catenin-δ (Fig. 7A, B). Moreover, GC tissues with low levels of miR-29c demonstrated increased catenin-δ protein levels (Fig. 7C, D). Furthermore, in-depth statistical analysis indicated an inverse association between miR-29c and catenin-δ and the TNM stage (Fig. 7E).

Together, these findings indicate that miR-29c is an independent prognostic factor of GC development.

**Drug-activating ts-miRs represent a crucial mechanism for GC chemotherapy.**

The above findings prompted us to further investigate the effect of chemotherapy on other ts-miRs validated in gastric carcinogenesis. HGC27 and MGC803 cells were treated with docetaxel and cisplatin, and qRT-PCR was performed to measure the expression of selected ts-miRs in these cells. The results indicated that most ts-miRs were up-regulated in GC cells upon docetaxel or cisplatin treatment (Fig. 7F). miR-375, which was validated as a
ts-miR in GC by negatively regulating PDK1 and YWHAZ(24), was the most significant of the increased miRNAs. We also demonstrated that cisplatin and docetaxel treatment led to a marked decrease in PDK1 and YWHAZ protein in GC cells (Fig. 7G, H). These data suggest that chemotherapeutic drugs may contribute to GC treatment via miR-375-mediated axes.

Altogether, our findings suggest that multiple gastric carcinogenesis-related programs, including ts-miR-mediated axis, are involved in the response to chemotherapy.

DISCUSSION

Our data revealed that miR-29s suppressed GC cell invasion, suggesting that miR-29s levels might be correlated with GC progression. To test this hypothesis, miR-29s expression analysis was conducted in 166 cases of GC tissues, demonstrating that GC tissues with poor differentiation tended to have low miR-29s levels, albeit statistically insignificant (miR-29a, \( P=0.84 \); miR-29b, \( P=0.27 \); miR-29c, \( P=0.90 \)) (Fig. S2A-C). These data are not in agreement with Saito’s report of 23 cases of GC tissues (14 differentiated and 9 undifferentiated)(11). However, we observed that miR-29c levels were dramatically decreased in GC patients with more aggressive tumor phenotypes, indicating that miR-29c may represent the most vital tumor suppressor in this family.

Our data also elucidated that catenin-δ is a novel target of miR-29s and
loss of miR-29c/catenin-δ homeostasis contributed to GC progression. Moreover, the miR-29c-mediated catenin-δ axis is chemotherapeutic target of cytotoxic drugs in GC cells. Actually, catenin-δ is a complicated protein and its function as tumor suppressor or oncogene depends on the subcellular localization(25). Accumulating reports demonstrate that catenin-δ is essential for adhesiveness in epithelial tissues, as well as increased protein stability and a reduction in E-cadherin turnover at the plasma membrane thereby function as tumor suppressor(26). Recent studies in vitro or in vivo confirm this idea(27-29). Because cisplatin and docetaxel treatment led to a decrease of catenin-δ proteins via modulating miR-29s expression, we next investigated whether these drugs held impact on E-cadherin levels. Our immunoblotting analysis indicated a significant increase of E-cadherin proteins in cell surface and cytoplasm but did not detect the expression of E-cadherin in nucleus of GC cells with drugs treatment (Fig. S9A, B). Meanwhile, miR-29c treatment resulted in a moderated increase of E-cadherin proteins in cell surface and cytoplasm (Fig. S9C, D). These data suggest that chemotherapeutic drugs lead to an increase of E-cadherin on cell surface probably via other unknown mechanism except via miR-29c-catenin-δ axis through comparing the effect of cytotoxic drugs to miR-29c treatment. As stated above, mislocalization of catenin-δ in the cytoplasm or nucleus resulted in the promotion of migration and invasion via regulating Rho GTPase activity and growth factor receptor signaling, implying its role as an oncogene(30-31). Interestingly, our
immunostaining analysis indicated a dramatic decrease of catenin-δ protein levels in the cytoplasm of GC cells treated with miR-29s mimics, suggesting that mislocalized catenin-δ functions as an oncogene.

The importance and mechanism of cancer chemotherapy have been extensively investigated. With the emergence of miRNA research, many groups have attempted to identify miRNA signatures associated with chemosensitivity or chemoresistance. In this study, miR-29s were validated as responsive ts-miRs in GC chemotherapy. These data promoted us to further investigate whether cytotoxic drugs held clinical promise through modulating a group of ts-miRs or oncomiRs in GC cells. To expand the scope of our findings, 22 known ts-miRs were selected and measured by qRT-PCR in cisplatin- and docetaxel-treated GC cells. As expected, most of these miRNAs were up-regulated, suggesting that chemotherapeutic drugs achieve clinical promise for GC prevention through activating ts-miRs, which may represent a global mechanism for GC chemotherapy. In addition, a previous study reported that the miR-29c levels were increased in GC cells following celecoxib treatment(11). In contrast to this study, another blocking agent for gastrointestinal tumors treatment, imatinib, was used to treat GC cells and led to a significant increase in miR-29c expression and a marked decrease in catenin-δ protein expression (Fig. S10), suggesting that selective inhibitors may exert similar effects on GC treatment through miR-29c-mediated axis. These findings demonstrate that miR-29c acts as not only a critical predictor of
GC chemotherapy but a powerful ts-miR for GC.

In conclusion, our findings indicate that loss of miR-29c/catenin-δ homeostasis contributes to GC progression. Re-modification of miR-29c-mediated axis may represent a realistic approach for GC prevention.

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Authors’ Contributions

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References


Figure Legends

Figure1. miR-29s expression is activated in GC cells following chemotherapeutic drugs treatment

A, qRT-PCR analysis was conducted to quantify the miR-29s level in GES-1, MGC803, HGC27, BGC823, and MKN45 cells. U6 was included as a control, and data were normalized to the level of GES-1 cells. B and C, IC50 values of cisplatin (B) and docetaxel
(C) were evaluated in GC cells. Cell viability was determined by using CCK-8 assay. D and E, qRT-PCR analysis of miR-29s expression in GC cells treated with Con (control, physiological saline), cisplatin (D) or docetaxel (E). F and G, Immunoblot analysis of the Mcl1 and Bcl2 protein level in GC cells treated with cisplatin (F) and docetaxel (G). GAPDH served as a loading control and data were normalized to Con treated cells. The numbers below the panels represent the normalized protein expression levels.

**Figure 2. miR-29c is an independent prognostic factor for GC development**

A, miR-29s expression was down-regulated in GC tissues examined by using qRT-PCR analyses. Tumor indicates GC tissues and Normal indicates the matched normal gastric tissues. B, miR-29c down-regulation was correlated with GC venous invasion. C, GCs with advanced TNM stages demonstrated decreased miR-29c expression. D, Kaplan-Meier survival curves for DFS in relation to miR-29C. E, Kaplan-Meier survival curves for DFS in relation to miR-29b. F, Kaplan-Meier survival curves for DFS in relation to miR-29b. Cut-off values for miR-29s (high/low expression) were determined by ROC analysis by using SPSS16.0 software. G, GC patients with low levels of miR-29c tended to recur after chemotherapy.

**Figure 3. miR-29s suppress GC cell migration and invasion**

A and B, Wound healing assay was conducted in MGC803 (A) and HGC27 cells miR-29s mimics or scramble transfection (Scr, a scrambled oligonucleotide used as negative control). Phase-contrast images were obtained immediately after wounding and at 12-h intervals up to 36 h. Wounds (in triplicate) were photographed, and the wound closure percentage from a representative experiment (n=3) was temporally measured using
AxioVison software. C and D, MGC803 (C) and HGC27 cells (D) were transfected with miR-29s mimics or scramble, and a subsequent in vitro invasion assay was performed. Representative images are shown (Magnification: 200×). The normalized ratio of invasive cells is shown in the right panels. E and F, Nude mice were injected with HGC27 cells infected with Lenti-29c (Lenti-miR-29c) or Lenti-scr (Lenti-scramble) through the lateral tail vein. Five weeks after injection, the mice were sacrificed, and the lungs and livers were dissected for microscopic histology (E). G and H, Histological analysis of sections from livers (G) and lungs (H) of mice injected with HGC27 cells treated with either Lenti-scr or Lenti-29c. The images shown in the top-right panel represent a magnified view of the boxed region indicated in the panel.

**Figure 4. Catenin-δ is a bona fide target of miR-29s in GC**

A, Sequence present in the 3′-UTR of catenin-δ targeted by miR-29s. The sequences shaded in gray represent mutants of the miR-29s-matched seed sequence. B, miR-29s expression decreased the luciferase activity when linked to the segment containing the target sequence within the 3′-UTR in catenin-δ mRNA. Mutation of the seed sequence abolished miR-29s-dependent repression. C, The protein level of catenin-δ in HGC27 and MGC803 cells upon miR-29s mimic or scr treatment, as measured by immunoblotting. GAPDH served as a loading control and data were normalized by Scr-treated GC cells. The numbers below the panels represent the normalized protein expression levels. D, miR-29s inhibitors (Anti-29a, Anti-29b, and Anti-29c) blocked the action of endogenous miR-29s in GC cells. The catenin-δ protein level was measured by immunoblotting. GAPDH served as a loading control and data were normalized by Anti-con (negative
control oligonucleotide) treated GC cells. **E and F,** Cisplatin and docetaxel treatment led to a decrease in catenin-δ protein levels in HGC27 (E) and MGC803 (F) cells, as shown by immunoblotting analysis. The numbers below the panels represent the normalized protein expression levels. GAPDH served as a loading control and data were normalized by control treated GC cells. **G,** Immunofluorescence microscopy of HGC27 cells stained with anti-catenin-δ and DAPI (nuclei), showing a decrease in catenin-δ staining after treatment with miR-29s mimics. Bars: 50 μm. **H,** Pathology analysis of tissue sections from recipient mice at 4 weeks post-injection. H & E staining and labeling with anti-catenin-δ, anti-Ki-67, and anti-caspase3 was performed. Bars: 50 μm. **I,** Normalization of catenin-δ, Ki-67, and caspase3 expression in the tissue sections. Data are mean ± SEM (n=3) and are representative of three independent experiments. **J,** qRT-PCR analysis of miR-29s expression in 8 pairs of GC samples. U6 snRNA served as a loading control. "△, C" indicates GC tissues; "▽, N" indicates the matched normal gastric tissues. The numbers indicates specimens of different GC patients. **K,** Immunoblot analysis of catenin-δ in 8 pairs of GC samples described as (H). GAPDH served as a loading control. The catenin-δ expression level is normalized to normal control and shown in the histogram. n=8. **L,** Representative images of catenin-δ immunohistochemistry analysis in the same samples described in (H). Magnification: 20×. Bars: 100 μm. The fold change was normalized to the normal control.

**Figure 5.** miR-29s suppress GC cell migration by negatively regulating the catenin-δ pathway

**A,** HGC27 cells were treated with si_catenin-δ (siRNA for catenin-δ) or si_Con (Control...
siRNA). Scratch wounds were made in confluent cells, and cells were allowed to migrate for 36 h in the presence of si_catenin-δ or si_Con. Bars: 250 μm. B, Representative images of in vitro invasion assay. Bars: 100 μm. In vitro invasion assay was conducted in HGC27 cells with indicated treatment. n=3. C, Immunoblots of catenin-δ in HGC27 cells 48 h after treatment with Anti-29s or Anti-con and subsequently treated for an additional 72 h with different combinations of si_catenin-δ or si_Con and Anti-miR-29s or Anti-con. The signal in each lane was quantified using Kodak Imaging software, and the expression ratio of catenin-δ to GAPDH was determined and shown. D, In vitro invasion assay was conducted as described in the Materials and Methods. Migration magnification: 200×. Bars: 100 μm. E, Normalized ratio in the in vitro invasion assay is shown in the bars. F, Immunofluorescence microscopy of HGC27 cells stained for phalloidin, showing a marked decrease in F-actin 48 h after treatment with si_catenin-δ. DAPI staining was used to show the nuclei. Bars: 50 μm. G, Percentage of cells with stress fibers is shown in the bars. H, Immunoblotting analysis in HGC27 cells showed a decrease in catenin-δ expression and an increase in GTP-Rho and p-cofilin expression 48 h after treatment with si_catenin-δ. GAPDH served as a loading control. I, Treatment with miR-29s mimics resulted in a significant decrease in catenin-δ protein levels and a dramatic increase in GTP-Rho and p-cofilin expression in HGC27 cells. GAPDH served as a loading control. J, Immunofluorescence microscopy of HGC27 cells stained for phalloidin, showing a marked decrease in F-actin 48 h after treatment with miR-29s mimics or scramble. DAPI staining was used to reveal the nuclei. K, Percentage of cells with stress fibers is shown in the bars.
Figure 6. Cisplatin and docetaxel treatment suppresses GC cell movement via regulating miR-29c-mediated catenin-δ axis

**A and B**, miR-29c expression was evaluated in GC cells treated by cisplatin (A) and docetaxel (B) with or without miR-29c inhibition. **C and D**, catenin-δ protein level was assessed in GC cells treated by cisplatin (C) and docetaxel (D) with or without miR-29c inhibition. **E and F**, wound healing assay was conducted in the rescue assay (MGC803, E; HGC27, F) and phase-contrast images were obtained immediately after wounding and at 12-h intervals up to 36 h. **G**, *In vitro* invasion assay was performed in the rescue assay. Representative images are shown (Magnification: 200×). The normalized ratio of invasive cells is shown in the bottom panels.

Figure 7. Clinical validation of the miR-29c-mediated catenin-δ axis in GC development

**A**, Relative levels of catenin-δ in GC patients with different TNM stages. n=66. **B**, Relative levels of catenin-δ in GC patients with or without metastasis. n=66. **C**, Relative levels of catenin-δ in GC patients with low and high miR-29c expression. **D**, Representative images showing positive catenin-δ staining in patients with low and high miR-29c expression. Bars: 100 μm. **E**, Correlation among miR-29c, catenin-δ, and TNM stage in GCs. **F**, qRT-PCR analysis quantifying the expression of candidate ts-miRs in HGC27 and MGC803 cells treated with cisplatin and docetaxel. **G**, qRT-PCR and immunoblotting analysis to assess the expression of miR-375 and its targets in GC cells treated with cisplatin. **H**, qRT-PCR and immunoblotting analysis to assess the expression of miR-375 and its targets in GC cells treated docetaxel. GAPDH served as a loading control and data
were normalized by Con-treated GC cells. The numbers below the panels represent the normalized protein expression levels.
Figure 1

(A) miR-29a, miR-29b, miR-29c

(B) Cisplatin

(C) Docetaxel

(D) Cisplatin

(E) Docetaxel

(F) Cisplatin

(G) Docetaxel
Figure 2

Panel A: Normalized fold expression of miR-29a, miR-29b, and miR-29c in tumor and normal tissue. 

Panel B: Venous invasion status for miR-29c, with miR-29c high (n=166, P=0.02).

Panel C: pTNM stage distribution for miR-29c, with miR-29c high (n=166, P=0.05).

Panel D: Survival rate analysis for miR-29c with Log Rank P=0.011.

Panel E: Survival rate for miR-29a, with miR-29a high (n=24) vs. low (n=53).

Panel F: Survival rate for miR-29b, with miR-29b high (n=14) vs. low (n=63).

Panel G: Survival rate for miR-29c, with P=0.03.
Figure 7

A

Relative Catenin-δ protein expression

TNM
Stage I  Stage II  Stage III  Stage IV
non-metastatic  metastatic

B

C

P=0.0085  P=0.002

D

Low miR-29c level  High miR-29c level

H&E
Catenin-δ

E

F

Cisplatin  Docetaxel

HGC-27  MGC-803  HGC-27  MGC-803

0.0  0.1  1.0  Con (μg/L)  0.0  0.01  0.001  0.1

miR-29c  pTNM stage  Catenin-δ

G

H

Cisplatin  Docetaxel

Normalized fold expression (miR-375)

PDK1  YWHAZ  GAPDH

Ratio: 1.0  0.4  0.3  1.0  0.5  0.6  1.0  0.3  0.4  1.0  0.6  0.6

Ratio: 1.0  0.3  0.3  1.0  0.3  0.4  1.0  0.2  0.7  1.0  2.7  2.7

Low  High

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Chemotherapy-induced miRNA-29c/catenin-δ signaling suppresses metastasis in gastric cancer

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