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Pattern of cell-to-cell transfer of microRNA by gap junction and its effect on the proliferation of glioma cells

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Abstract

MicroRNA is expected to be a novel therapeutic tool for tumors. Gap junctions facilitate the transfer of microRNA, which exerts biological effects on tumor cells. However, the length of microRNA that can pass through certain gap junctions composed of specific connexin remains unknown. To address this question, the present study investigated the permeability of gap junctions composed of various connexins, including connexin 43, connexin 32 or connexin 37, to microRNAs consisting of 18-27 nucleotides in glioma cells and cervical cancer cells. Results indicated that all of the microRNAs were able to be transferred from donor glioma cells to neighboring cells through the connexin 43 composed gap junction, but not the gap junctions composed of connexin 32 or connexin 37, in cervical cancer cells. Downregulation of the function of gap junctions comprising connexin 43 by pharmacological inhibition and shRNA significantly decreased the transfer of these microRNAs. In contrast, gap junction enhancers and overexpression of connexin 43 effectively increased these transfers. In glioma cells, cell proliferation was inhibited by microRNA-34a. Additionally, these effects of microRNA-34a were significantly enhanced by overexpression of connexin 43 in U251 cells, indicating that gap junctions play an important role in the antitumor effect of microRNA by transfer of microRNA to neighboring cells. Our data are the first to clarify the pattern of microRNA transmission through gap junctions and provide novel insights to show that antitumor microRNAs should be combined with connexin 43 or a connexin 43 enhancer, not connexin 32 or connexin 37, in order to improve the therapeutic effect.

KEYWORDS
connexin, gap junction, glioma, microRNA, proliferation

Abbreviations: 18α-GA, 18α-glycyrrhetinic acid; CBX, carbenoxolone; CDK6, cyclin-dependent kinase 6; Cx, connexin; miRNA, microRNA; RA, retinoic acid.

Yuexia Peng and Xiyan Wang contributed equally to this work.

1 | INTRODUCTION

MicroRNAs (miRNAs) are single-stranded small non-coding RNAs with a length of 18-27 nucleotides and regulate gene expression by targeting mRNAs for post-transcriptional silencing.[1-4] Studies have shown that miRNAs play an important role in tumorigenesis.[5,6] miRNAs can be divided into the following two types: oncogene miRNAs (onco-miRNAs) and tumor-suppressor miRNAs. Onco-miRNAs are usually highly expressed in tumor cells and promote the development and progression of the tumor. In contrast, tumor-suppressor miRNAs are often downregulated or absent in tumor cells, and they suppress tumor growth.[3,7] In recent years, miRNAs have been considered a novel therapeutic target for cancer.[8,9] These strategies include interfering with onco-miRNAs to suppress tumor growth and importing exogenous tumor-suppressor miRNAs into tumor cells to treat cancer.[10,11]

Gap junctions consist of two hemichannels, each provided by one of the adjacent cells. Six connexin (Cx) subunits form a hemichannel. Gap junctions form direct channels between neighboring cells.[12,13] Molecules with a weight <1.5 kDa (such as ions, secondary messenger and metabolic substances) can be delivered by gap junctions to the adjacent cell cytoplasm.[14] Studies show that gap junctions are crucial for maintaining the homeostasis of cells, coordinating the activity of cells and controlling the proliferation and differentiation of cells.[15] In the conventional view, gap junctions could only transfer substances that are between 1 and 1.5 kDa. The gap junction has a pore size of 1.0-1.5 nm,[16] which is the same size as the linear diameter of miRNA.[17] Therefore, miRNAs can theoretically be transferred through gap junctions. In recent years, studies have shown that astrocytes can transmit miR-4519 and miR-5096 to cocultured glioma U87 cells through gap junctions, thereby promoting the invasion of glioma cells.[18] Mesenchymal stem cells were shown to transfer miR-124 and miR-145 mimics to cocultured glioma U87 cells through gap junctions, decreasing the migration and self-renewal of glioma U87 cells.[19] In our previous study, we also confirmed that in glioma U87 cells, gap junctions delivered miR-124-3p and augmented its antitumor effect.[20] These findings suggest that gap junctions can transmit miRNA. However, there are still certain questions that remain, such as whether miRNAs consisting of 18-27 nucleotides can be passed through gap junctions composed of Cx43. It is also unclear whether gap junctions composed of other Cx differ in their capacity for miRNA delivery. In addition, it is unknown whether gap junctions composed of different Cx have different effects on the function of miRNAs. Solving these problems will provide a new strategy for the application of miRNA in tumor therapy.

The present study was conducted to explore whether miRNAs consisting of 18-27 nucleotides could be transferred through gap junctions between cancer cells. We further investigated the ability of gap junctions composed of three different types of Cx (Cx43, Cx32 or Cx37) to transmit these miRNAs and whether the different types of Cx affected the cell proliferation-inhibitory effect of miR-34a.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Glioma U87 and U251 cells and cervical cancer HeLa cells were obtained from ATCC. Cells were cultured in DMEM medium containing 10% FBS at 37°C in a humidified incubator with 5% CO_2. HeLa-Cx32 cells were stably transfected with the Cx32 plasmid with a bidirectional tetracycline-inducible promoter.[21]

2.2 | Transfection

MiR-34a mimics (RiboBio, Guangzhou, China) and Cy3-labeled miR-34a (Sigma Chemical Co., St Louis, MO, USA) were transfected into cells to upregulate their expression, and the control miRNA (miR-NC) (RiboBio, Guangzhou, China) had no homology for any human gene sequence. For stable knockdown of Cx43 expression in U87 cells, we transfected ShCx43 or GFP-labeled shCx43 plasmid (Cyagen Biosciences Inc., Santa Clara, CA, USA) into U87 cells. Sequences for the shRNAs targeting Cx43 were as follows:

shRNA 1: 5′-GAACCTACATCATCAGTAT-3′,
shRNA 2: 5′-CAGTCTGCCTTTCGTTGTA-3′.

For the stable overexpression of Cx43 in glioma U251 cells, we transfected the lentiviral plasmid Cx43 or GFP-Cx43 (Obio Technology, Shanghai, China) into U251 cells. To overexpress Cx37, the Cx37 plasmid and the control plasmid (Cyagen Biosciences Inc.) were respectively transfected into HeLa cells. All transfections were carried out using Lipofectamine 3000 according to the manufacturer's instructions.

2.3 | Coculture of cells

Donor cells were transfected with Cy3-labeled miR-34a for 24 hours and selected by BD influx flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) before the experiment. Receiving cells were stably labeled with GFP and not transfected with miRNA mimics. The donor cells and receiving cells were washed, trypsinized and mixed together in a ratio of 1:1. After 12 hours of coculture, cells were analyzed by confocal microscopy (LSM710, Zeiss, Jena, Germany) or BD influx flow cytometry (BD Biosciences).

2.4 | Quantitative real-time PCR

Total RNA was extracted using the Hipure miRNA Kit (Magen, Guangzhou, China) according to the manufacturer's protocol. qPCR was carried out according to the manufacturer's protocol (Transgen Biotech, Beijing, China).

Additionally, to examine the relative expression of Cx43, total RNA was isolated using the Hipure Total RNA Kit (Magen) according to the manufacturer's protocol. For qPCR, the primers for Cx43 were as follows: (forward) 5′-GGTCTGAGTGCCTGAACTTGCCT-3′ and (reverse) 5′-AGCCACACCTTCCCTCCAGCA-3′, and for GAPDH: (forward) 5′-GAAGGTGAAGGTCGGAGTC-3′ and (reverse) 5′-GAAGATGGTGATGGGATTTC-3′.

2.5 | Western blot analysis

Total proteins were harvested from the cells. Protein was separated using SDS-PAGE electrophoresis and transferred onto PVDF membrane (Millipore, Burlington, MA, USA). Monoclonal antibodies against Cx43 (1:8000; Sigma), Cx37 (1:2000; Sigma), Cx32 (1:2000; Santa Cruz Biotechnology, Dallas, TX, USA), GFP (1:2000; Cell Signaling Technology, Danvers, MA, USA), cyclin D1 (1:1000; Cell Signaling Technology), CDK6 (1:1000; Cell Signaling Technology), and β-tubulin (1:10 000; Sigma) were used. Immunoreactive bands were visualized using the Amersham ECL Plus Western Blotting Detection Kit (GE Healthcare, Chicago, IL, USA), and the bands were quantified by ImageJ software.

2.6 | Drug treatment of gap junctions

To inhibit the function of gap junctions in glioma U87 cells, we incubated the cells with 150 μmol/L CBX (Sigma) or 50 μmol/L 18α-GA (Sigma) for 6 hours.[22,23] In contrast, to enhance the function of gap junctions, we treated the cells with 10 μmol/L RA (Sigma) or 10 μmol/L galangin (China Pharmaceutical Biological Products Inspection Institute, Beijing, China) for 24 hours.[24] Galangin is a flavonoid compound that enhances gap junction function, increasing the cytotoxicity of the antitumor drugs cisplatin and oxaliplatin.[25,26]

2.7 | Parachute dye-coupling assay

The function of gap junctions was assessed using a parachute dye-coupling assay.[27] Cells were grown to 80%-90% confluency in 12-well plates. Donor cells from one well were double-labeled with 10 μg/mL calcein-AM (Invitrogen, Carlsbad, CA, USA) and 5 μg/mL CM-DiI (Invitrogen) in the dark for 30 minutes at 37°C. Unincorporated dye was removed by four consecutive washes with culture medium. Donor cells were then trypsinized and seeded onto the receiving cells at a 1:150 ratio and then incubated for 4 hours at 37°C. The parachute dye-coupling assay was implemented using a fluorescence microscope (Olympus IX71; Olympus, Tokyo, Japan). Average number of receiving cells (green fluorescence) around one donor cell (both green and red fluorescence) was considered as the measurement standard of the function of a gap junction.

2.8 | CCK-8 assay

Cell proliferation was assessed using CCK-8 (Dojindo Molecular Technologies, Rockville, MD, USA) according to the manufacturer's protocol. Five replicate samples were analyzed for each group. Cell survival rate was calculated by the following formula: survival rate = $OD_{miR\text{-}34a}/OD_{miR\text{-}NC}$.

2.9 | Cell cycle analysis

Cells were harvested, washed with PBS, and fixed with ice-cold 70% ethanol at 4°C overnight. The fixed cells were washed twice in cold PBS, resuspended in a volume of 100 μL of 10 mg/mL RNase (KeyGEN, Jiangsu, China) and 25 mg/mL propidium iodide (PI; KeyGEN), and incubated for 30 minutes at 37°C in the dark before being analyzed using flow cytometry (BD Biosciences).

2.10 | Statistical analysis

Statistical analysis was carried out using SPSS 13.0 and graphs were drawn by GraphPad Prism 6.0 software. Data were analyzed using one-way ANOVA or Student's *t* test. $^{*}P < 0.05$ or $^{**}P < .01$ were considered to be statistically significant.

3 | RESULTS

3.1 | Transfer of miRNAs consisting of 18-27 nucleotides between glioma U87 cells

Previous studies have reported that miRNAs composed of 22-23 nucleotides (such as miR-124, miR-145, miR-96 and miR-183) can be delivered from cell to cell through gap junctions.[19,20,28] In this context, we investigated whether exogenous synthetic miR-34a mimics that were composed of 22 nucleotides could be transferred between glioma U87 cells. We generated miR-34a mimics labeled with Cy3. U87 cells were transfected with the Cy3-labeled miR-34a, and these transfected cells served as donor cells. The donor cells were then cocultured with receiving cells, which were stably transfected with GFP in glioma U87 cells (U87-GFP). As presented in Figure 1A, the cells were observed by confocal microscopy after being cocultured for 12 hours, and the merged image shows that the cocultured U87-GFP (receiving cells) carried red Cy3-miR-34a mimics, which came from the donor cells. In addition, we obtained similar results through FACS analysis (Figure 1B). These results showed that miR-34a consisting of 22 nucleotides could be transferred between glioma U87 cells. To examine whether miRNAs composed of 18-27 nucleotides, including miR-1827, miR-144, miR-34a, miR-203a and miR-1183 (Table 1), could be transferred between glioma U87 cells, we carried out the coculture assay. U87 cells were transfected with exogenous miRNA mimics (Table 1) and served as donor cells. These donor cells were cocultured with U87-GFP receiving cells at a ratio of 1:1. After 12 hours of coculture, the U87-GFP receiving cells were selected by a BD influx flow cytometer based on the GFP label. Then, using qPCR analysis, expression of miRNAs in the U87-GFP receiving cells that were cocultured with the donor cells was shown to be significantly increased compared with the U87-GFP cells that were not cocultured with donor cells (Figure 1C). These results indicated that all miRNAs composed of 18-27 nucleotides could be transferred between glioma U87 cells.

3.2 | Gap junctions composed of Cx43 mediate the delivery of miRNAs consisting of 18-27 nucleotides between glioma U87 cells

To elucidate the role of gap junctions in the delivery of miRNA between glioma U87 cells, we manipulated the function of gap

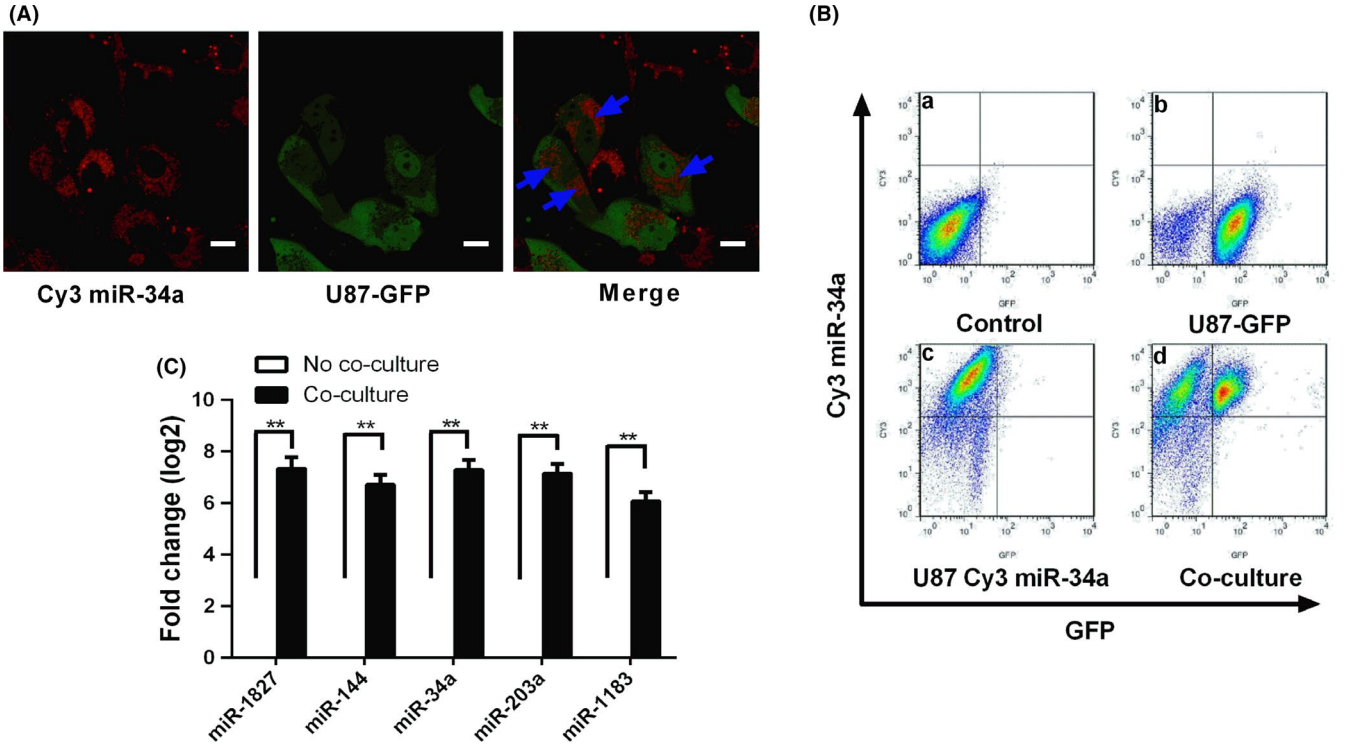

FIGURE 1 MicroRNA (miRNA) mimics composed of 18-27 nucleotides transferred between cocultured glioma U87 cells. A, U87 cells (donor cells) were transfected with Cy3-miR-34a mimics and cocultured with U87-GFP (receiving cells). Then, the cocultured cells were analyzed by confocal microscopy. Scale bar, 10 μm. B, Delivery of Cy3-miR-34a by U87 donor cells to U87-GFP receiving cells was analyzed using flow cytometry. a, Double negative cocultured cells; b, cocultured receiving cells, U87 cells stably transfected with GFP (U87-GFP); c, cocultured donor cells, U87 cells transfected with Cy3-miR-34a; d, cocultured cells with GFP and Cy3 positive. C, Expression of miRNAs in the receiving cells was detected by qPCR before and after the coculture. Columns represent the means of four experiments; bars represent SEM. $^{**}P < .01$

TABLE 1 MicroRNAs with a length of 18-27 nucleotides

miRNA	Sequence (mature miRNA)	Length (nucleotides)
miR-1827	UGAGGCAGUAGAUUGAAU	18
miR-144	UACAGUAUAGAUGAUGUACU	20
miR-34a	UGGCAGUGUCUUAGCUGGUUGU	22
miR-203a	AGUGGUUCUUAACAGUUCAACAGUU	25
miR-1183	CACUGUAGGUGAUGGUGAGAGUGGGCA	27

miRNA, microRNA.

junctions pharmacologically. Application of the gap junction inhibitors CBX and 18α-GA each significantly inhibited the transfer of dye between the cells, whereas the gap junction enhancers RA and galangin each increased the transfer of dye between glioma U87 cells (Figure 2A). Results from the coculture assay showed that both CBX and 18α-GA decreased the expression of miRNAs in the receiving cells by approximately 50% compared with the control group (Figure 2B), whereas RA and galangin increased the expression of miRNAs in the receiving cells by approximately 30% compared with the control group (Figure 2B). These results suggest that miRNAs consisting of 18-27 nucleotides could be delivered between glioma U87 cells through the gap junction composed of Cx43.

To confirm the role of gap junctions composed of Cx43 in the transfer of miRNAs, we carried out two experiments. The first experiment involved the knockdown of Cx43 expression using a shRNA plasmid in glioma U87 cells that endogenously expressed high levels of Cx43. The second experiment involved the upregulation of Cx43 expression by transfection of a Cx43 plasmid into glioma U251 cells in which the endogenous Cx43 expression was very low. Results of qPCR and western blot analyses showed that expression of Cx43 was reduced in U87 cells transfected with the Cx43 shRNA plasmid (Figure 3A-C). Parachute dye coupling assay indicated that gap junction function was reduced in U87 cells in which Cx43 expression was inhibited by knockdown of Cx43 (Figure 3D,E). Knockdown of Cx43 also markedly reduced the expression of miR-1827, miR-144,

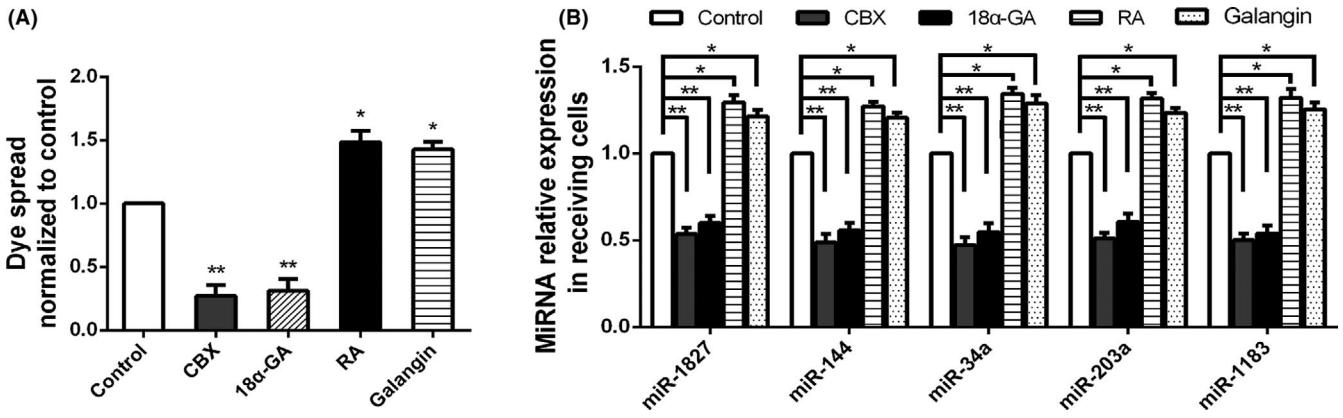

FIGURE 2 Gap junctions mediated the delivery of microRNAs (miRNAs) between glioma U87 cells. A, Parachute assay shows the degree of dye coupling in U87 cells. B, Expression of miRNAs in the receiving cells was assessed by qPCR after the coculture. Columns represent the means of four experiments; bars represent the SEM. **P* < .05, ***P* < .01. 18a-GA, 18α-glycyrrhetinic acid; CBX, carbenoxolone; RA, retinoic acid

miR-34a, miR-203a and miR-1183 in the receiving cells which were cocultured with the donor cells (Figure 3F). These results suggest that the knockdown of Cx43 expression significantly decreased the delivery of miRNAs consisting of 17-28 nucleotides between U87 cells.

We constructed U251 cells that stably overexpressed Cx43. Results from the western blot assay confirmed that Cx43 was overexpressed in these cells (Figure 4A). Function of gap junctions, determined by the parachute assay, was increased in U251 cells in which the expression of Cx43 was enhanced (Figure 4B). Expression of miR-1827, miR-144, miR-34a, miR-203a and miR-1183, as detected by qPCR, was not altered when the cells were cultured alone or cocultured with U251 cells (Figure 4C). Compared with the control, in the U251 cells that stably overexpressed Cx43, expression of miR-1827, miR-144, miR-34a, miR-203a and miR-1183 was markedly increased in the receiving cells that were cocultured with donor cells (Figure 4D). These results demonstrated that gap junctions composed of Cx43 mediated the transport of miRNAs consisting of 17-28 nucleotides between U251 cells.

3.3 | Permeability of gap junctions to miRNA is determined by the type of Cx

To investigate the effect of different types of Cx on the permeability of gap junctions to miRNAs, HeLa cells that did not express any Cx were transfected with Cx32 plasmid (named HeLa-Cx32), and the expression of Cx32 was induced with 1 μg/mL doxycycline (Dox) (Figure 5A). We observed that Cx32 expression in HeLa cells led to an increase in gap junction function (Figure 5B). Then, the GFP plasmid was transfected into and stably expressed in HeLa-Cx32 cells (named HeLa-Cx32-GFP, Figure S1). These HeLa-Cx32 cells were transiently transfected with miRNA mimics and used as donor cells, and the HeLa-Cx32-GFP cells were used as receiving cells. The donor cells were cocultured with receiving cells. Compared with the cells that were not cocultured, expression levels of miR-1827, miR-144, miR-34a, miR-203a and miR-1183 were not altered in the receiving cells in the coculture group (Figure 5C). These results suggest that the gap junctions composed of Cx32 could not transport miRNAs consisting of 18-27 nucleotides between HeLa cells.

To explore the permeability of gap junctions composed of Cx37 to miRNA, a Cx37 plasmid was transfected into and expressed stably in HeLa cells (named HeLa-Cx37). The Cx37 plasmid with a GFP label was transfected into and stably expressed in HeLa cells (named HeLa-Cx37-GFP) (Figure 5D). We showed that gap junction function was increased following the overexpression of Cx37 in the HeLa cells (Figure 5E). HeLa-Cx37 cells were transiently transfected with miRNA mimics and used as donor cells, and HeLa-Cx37-GFP cells were used as receiving cells. We carried out the coculture experiment as described above. Compared with the cells that were not cocultured, expression levels of miR-1827, miR-144, miR-34a, miR-203a and miR-1183 were not altered in the receiving cells in the coculture group (Figure 5F). These results suggest that gap junctions composed of Cx37 could not transit these miRNAs.

3.4 | Gap junctions composed of Cx43 enhanced the inhibitory effect of miR-34a on the proliferation of glioma cells

According to the abovementioned results, we clarified the pattern of cell-to-cell transfer of miRNA through gap junctions. Furthermore, we explored whether gap junctions composed of the different types of Cx had differing effects on the inhibitory role of miR-34a on the growth of tumor cells. MiR-34a is a well-recognized tumor suppressor gene that impedes tumorigenesis by inhibiting cell proliferation, inducing cell cycle arrest, promoting apoptosis and reducing metastasis.[19,20,28] The inhibitory effect of miR-34a on cell proliferation was first verified in U87 and U251 cell lines. Results of the CCK-8 assay indicated that miR-34a inhibited the proliferation of U87 and U251 cells in a concentration- and time-dependent method (Figure 6A,B). Compared with the miR-NC group, overexpression of miR-34a induced cell cycle arrest and decreased the expression of the cell cycle-regulated

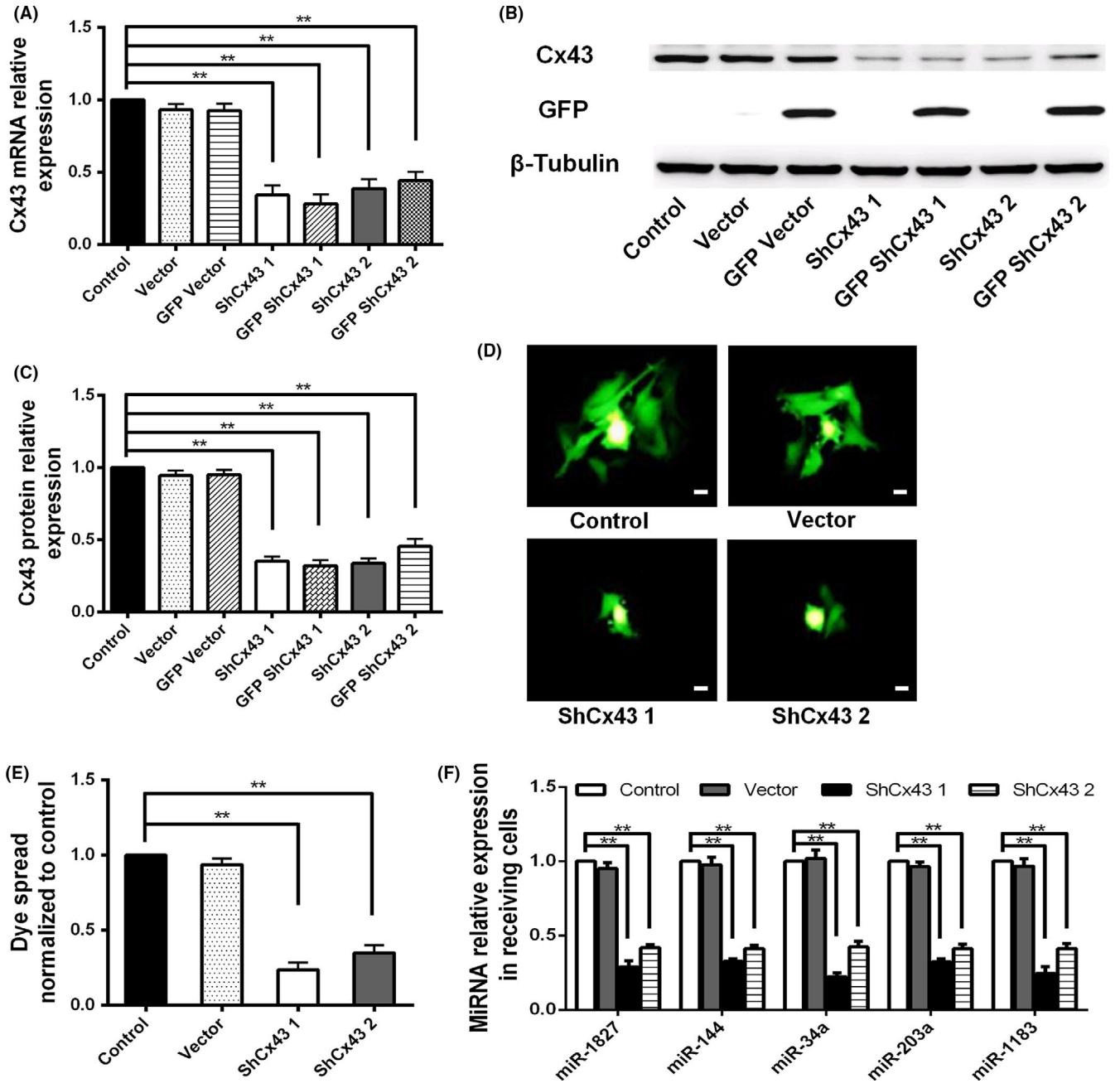

FIGURE 3 Effect of shRNA-mediated knockdown of connexin 43 (Cx43) expression on the transfer of microRNAs (miRNAs) between U87 cells. A, mRNA expression of Cx43 in U87 cells after the knockdown of Cx43 expression. B,C, Expression of Cx43 in U87 cells with shRNA stable transfection. GFP labeled U87 cells as the coculture receiving cells. D,E, Function of gap junctions in Cx43-deficient cells ($U87^{ShCx43}$) was analyzed by parachute assay. Scale bar, 10 μm. F, Compared with the control group, knockdown of Cx43 markedly decreased the expression of miRNAs in the receiving cells. Columns represent the means of four experiments; bars represent the SEM. $^{**}P < .01$

proteins cyclin D1 and CDK6 in U87 and U251 cells (Figure 6C,D). These results indicated that miR-34a inhibited the growth of glioma cell lines.

Next, we explored the effect of gap junctions composed of different types of Cx on the miR-34a-induced inhibition of glioma cell proliferation. Cx43 expression was knocked down using siRNA in glioma U87 cells (Figure 7A). Gap junction function was reduced in U87 cells in which Cx43 expression was inhibited (Figure 7B). We found that knockdown of Cx43 markedly attenuated the inhibition of cell proliferation, cell cycle arrest and the decreased expression of cyclin D1 and CDK6 induced by miR-34a overexpression in glioma U87 cells (Figure 7C-F). We also found that the percentage of Cy3-labeled miR-34a positive cells was approximately 70% compared with the control group and that knockdown of Cx43 reduced the percentage of Cy3 miR-34a positive cells (Figure 7G). These results indicated that the reduction in Cx43 expression significantly decreased the miR-34a-induced inhibition of cell proliferation. To confirm this result, upregulation of Cx43 expression was

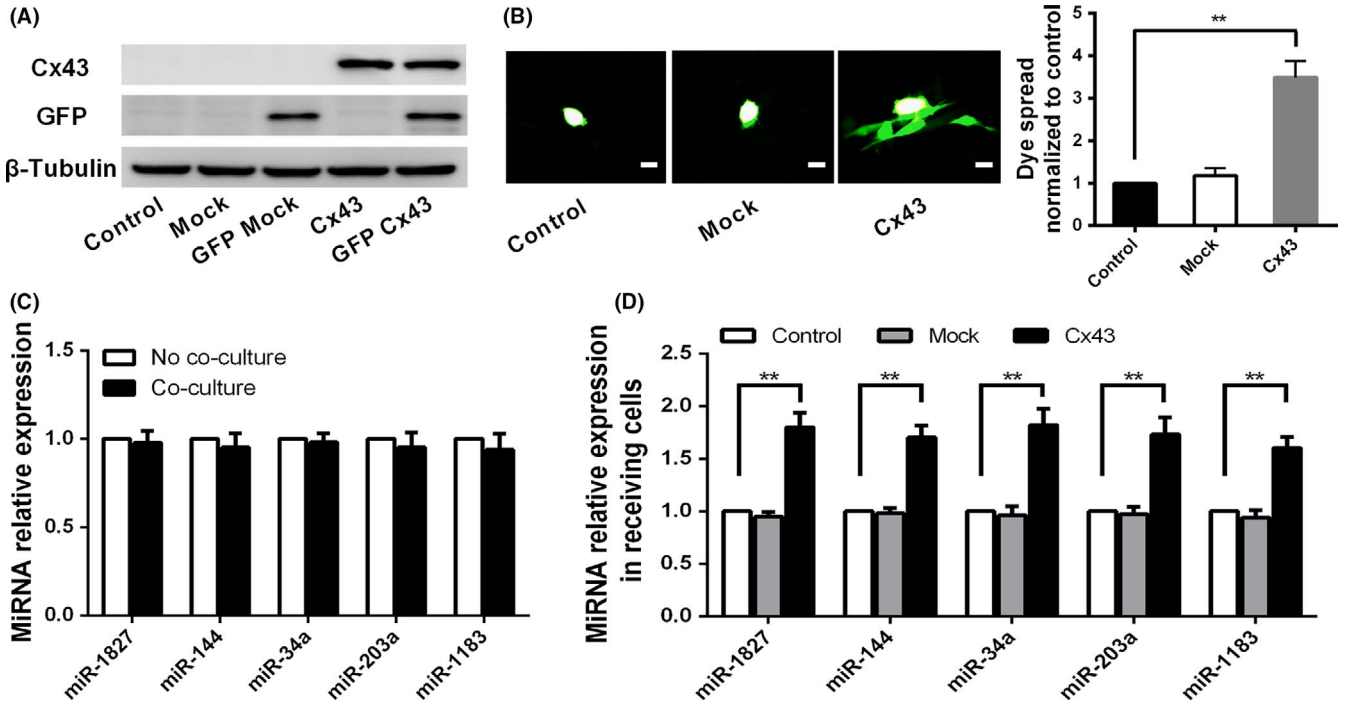

FIGURE 4 Effect of the overexpression of connexin 43 (Cx43) on the transfer of microRNAs (miRNAs) between U251 cells. A, Western blot analysis confirmed stable overexpression of Cx43 in U251 cells. GFP was used to label U251 cells as the coculture receiving cells. B, Function of gap junctions in U251 cells with overexpression of Cx43. Scale bar, 10 μm. C, Expression of miRNAs was not significantly different before and after coculture in the receiving cells. D, Compared with the control group, overexpression of Cx43 remarkably increased the expression of miRNAs in the receiving cells. Columns represent the means of five experiments; bars represent the SEM. $^{**}P < .01$

induced in glioma U251 cells to investigate whether Cx43 overexpression could enhance the inhibition of cell proliferation induced by miR-34a. As expected, the miR-34a-induced inhibition of cell proliferation was enhanced by Cx43 overexpression (Figure 8A). Cell cycle arrest and decrease of the expression of cyclin D1 and CDK6 were also increased by the overexpression of Cx43 in glioma U251 cells (Figure 8B-D). Percentage of Cy3 miR-34a positive cells was increased in glioma U251 cells after Cx43 overexpression (Figure 8E,F). Taken together, these results showed that gap junctions composed of Cx43 significantly enhanced the inhibitory effect of miR-34a on cell proliferation.

3.5 | Gap junctions composed of Cx32 or Cx37 did not alter the inhibitory effect of miR-34a on cell proliferation in cervical cancer cells

To investigate the influence of gap junctions composed of Cx32 or Cx37 on the inhibitory effect of miR-34a on cell proliferation in cervical cancer cells, Cx32 or Cx37 was overexpressed in HeLa cells using the abovementioned method. Similar to the U87 and U251 cells, miR-34a overexpression inhibited cell proliferation (Figure S2A,B), induced cell cycle arrest and decreased the expression of the cell cycle-regulated proteins cyclin D1 and CDK6 in HeLa cells (Figure S2C-F). However, the overexpression of either Cx32 or Cx37 did not affect this miR-34a-induced inhibition of cell proliferation (Figure S2A,B), cell cycle arrest or decrease in the expression of cyclin D1 and CDK6 in HeLa cells (Figure S2C-F). These results indicated that gap junctions composed of Cx32 or Cx37 did not affect the inhibitory effect of miR-34a on cell proliferation in HeLa cells.

4 | DISCUSSION

Accumulated evidence indicates that gap junctions mediate the delivery of miRNA from human macrophages to hepatocellular carcinoma cells,[29] from bone marrow stroma to breast cancer cells,[30] and between human microvascular endothelial cells and glioma U87 cells.[31] However, it remains unknown what length of miRNA can pass through a gap junction. To explore this problem, we synthesized a series of miRNAs consisting of 18-27 nucleotides and investigated the transfer of these miRNAs between glioma U87 cells. The present results showed that all exogenous miRNAs consisting of 18-27 nucleotides could be transferred between glioma U87 cells, and the cell-to-cell transfer of miRNAs was regulated by the manipulation of gap junction function. Inhibition of gap junction function with a gap junction inhibitor decreased miRNA delivery, whereas upregulation of gap junction function with a gap junction enhancer increased these transfers.

In the present study, two human glioma cell lines, U87 and U251 cells, were used. U87 cells endogenously expressed Cx43. The expression of Cx43 was shown to be repressed by transfection with a plasmid containing Cx43 shRNA. U251 cells in which the

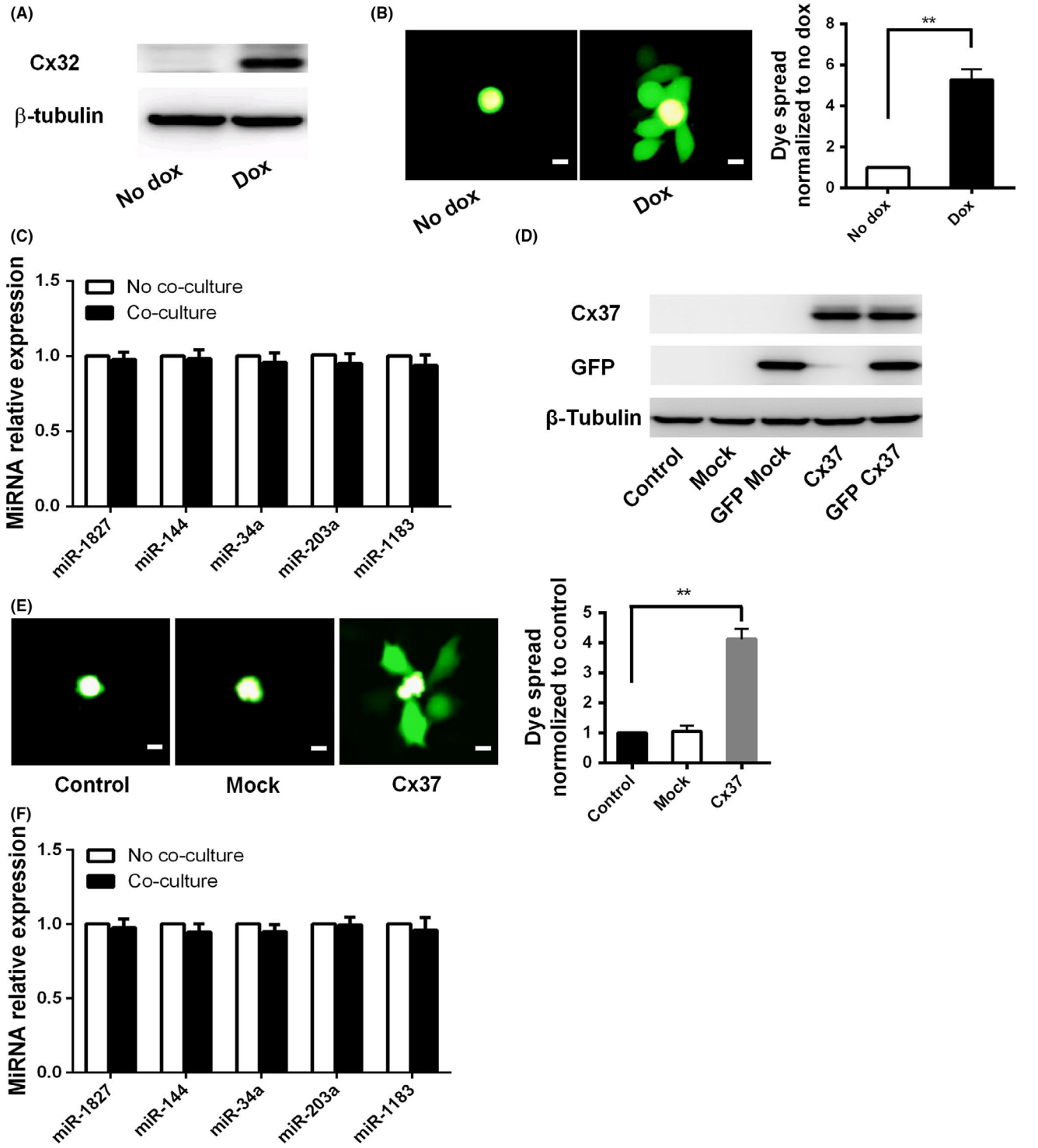

FIGURE 5 Ability of gap junctions composed of connexin 32 (Cx32) or connexin 37 (Cx37) to transfer microRNAs (miRNAs) between cervical cancer cells. A, Expression of Cx32 in HeLa-Cx32 cells after 48 h of doxycycline (Dox) treatment. B, Function of gap junctions in HeLa-Cx32 cells with induction of Cx32 expression. Scale bar, 10 μm. C, Expression of miRNAs in receiving cells was analyzed by qPCR. D, Expression of Cx37 in HeLa cells with Cx37 plasmid stable transfection. GFP was labeled in HeLa cells as the coculture receiving cells. E, Function of gap junctions in HeLa cells transfected with Cx37 plasmid. Scale bar, 10 μm. F, Expression of miRNAs was not significantly changed before and after the coculture. Columns represent the means of three experiments; bars represent the SEM. $^{**}P < .01$

endogenous expression level of Cx43 was low were transfected with the Cx43 plasmid, resulting in the overexpression of Cx43. Results from these studies indicated that gap junctions composed of Cx43 delivered miRNAs consisting of 18-27 nucleotides between glioma cells. The delivery of miRNAs was significantly decreased in U87 cells when the expression of Cx43 was downregulated,

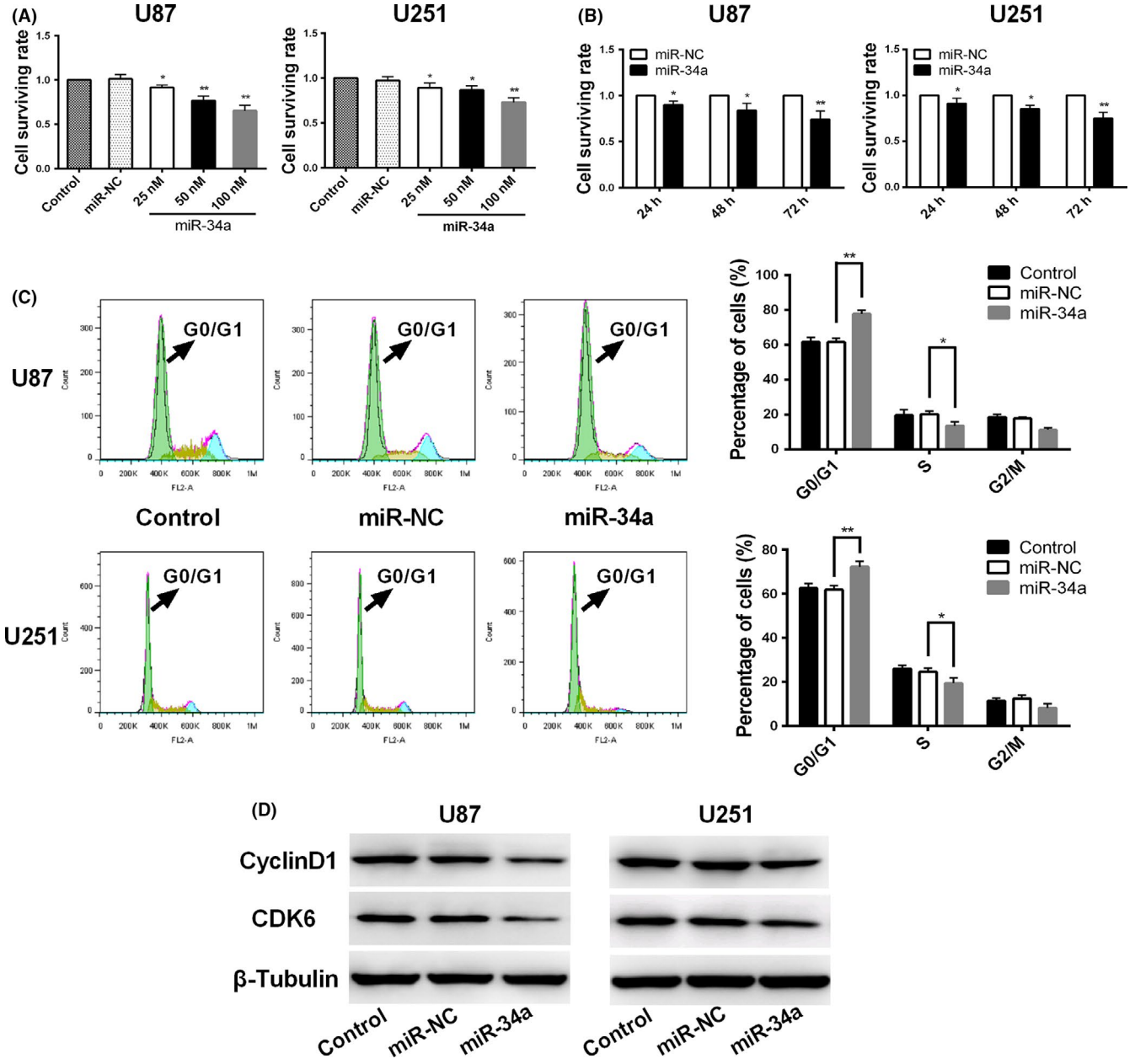

FIGURE 6 Overexpression of miR-34a inhibited glioma cell proliferation and induced cell cycle arrest. A,B, Dose- and time-dependent effect of miR-34a on cell proliferation in U87 and U251 cells by CCK-8 assay. C, Cell cycle analysis to evaluate the effects of miR-34a on cell cycle distribution. D, Expression of cell cycle-regulated proteins cyclin D1 and cyclin-dependent kinase 6 (CDK6) with miR-34a overexpression. Columns represent the means of three experiments; bars represent the SEM. *$P < .05$, **$P < .01$

whereas miRNAs transfers increased in U251 cells when Cx43 was overexpressed. Moreover, these results demonstrated that miRNAs consisting of 18-27 nucleotides were not delivered between U251 cells. This result was consistent with the report by Katakowski et al[32] in which miRNA could not be transferred between glioma U251 cells. In the present study, we found that overexpression of Cx43 restored the function of gap junctions, which enhanced miRNA delivery. Importantly, we demonstrated that the increase in the function of gap junctions through the overexpression of Cx43 enhanced the miR-34a-induced inhibition of cell proliferation, cell cycle arrest and the decrease in the expression of cyclin D1 and CDK6 in glioma U251 cells. This result suggests that patients suffering from glioma expressing Cx43 may benefit from treatment with antitumor miRNA mimics, as the antitumor miRNAs would be able to be transferred to a greater number of neighboring cells through gap junctions.

To date, 21 isoforms of Cx have been identified in humans, and the permeability of gap junctions composed of various Cx was shown to differ.[33,34] However, it remains unknown which type of Cx make up the gap junctions that transfer miRNA. In the present study, we showed that the capacity for gap junctions to transfer miRNAs consisting of 18-27 nucleotides was dependent upon the type of Cx. In

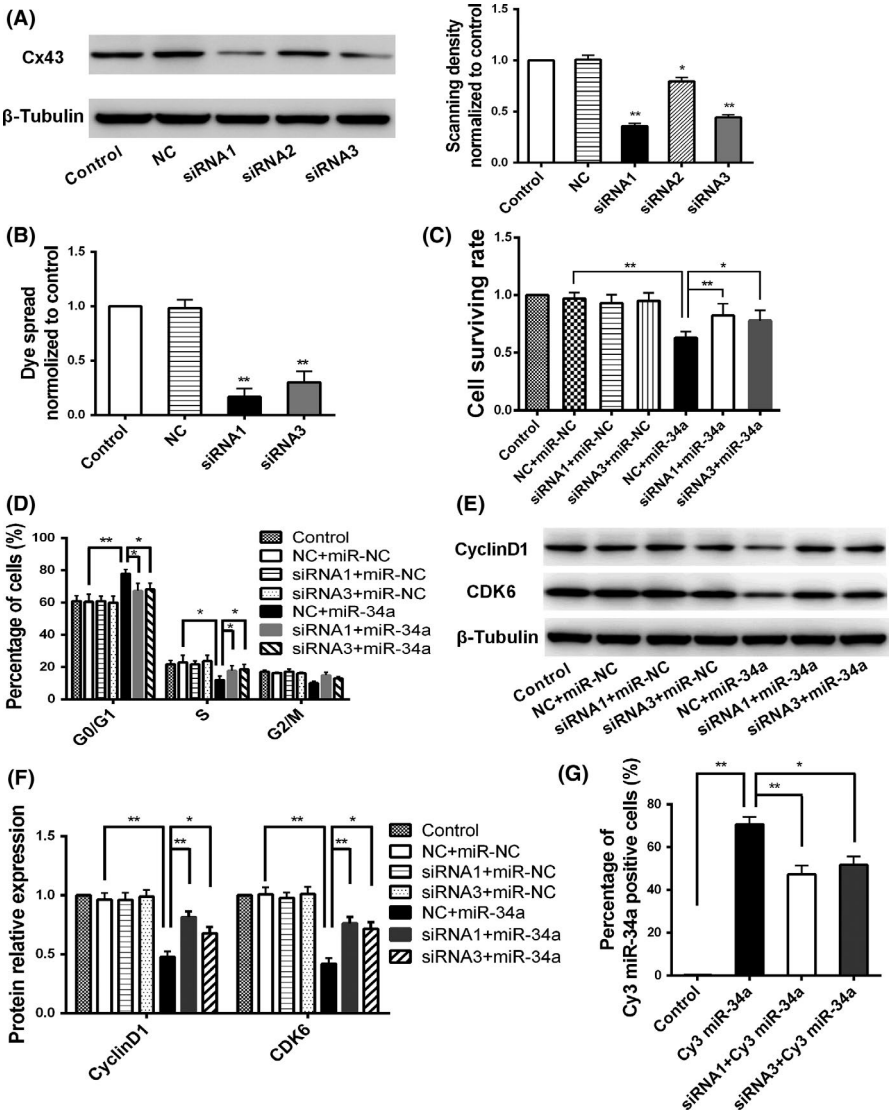

FIGURE 7 Effect of siRNA-mediated knockdown of connexin 43 (Cx43) expression on miR-34a-induced inhibition of proliferation in U87 cells. A, Western blot analysis of the siRNA-mediated knockdown of Cx43 expression in U87 cells. B, Histograms show the degree of dye coupling as measured by the parachute assay. C, CCK-8 assay was carried out to evaluate the effect of miR-34a on cell proliferation. D, The cell cycle was assessed by flow cytometry after miR-34a or siRNA transfection. E,F, Expression of cyclin D1 and cyclin-dependent kinase 6 (CDK6) in U87 cells transfected with miR-34a or siRNA. β-Tubulin was used as a loading control. G, Percentage of Cy3 miR-34a positive cells was measured in U87 cells after Cy3-labeled miR-34a or siRNA transfection. Columns represent the means of four experiments; bars represent the SEM. $^{*}P < .05$, $^{**}P < .01$

addition, we demonstrated that gap junctions composed of Cx43, but not Cx32 or Cx37, deliver these miRNAs. There are certain factors that influence the delivery of solutes through gap junctions, such as size, charge, hydrogen bonding and interactions with binding sites.[34-36] miRNAs are negatively charged molecules because they are anionic under physiological conditions. Currently, there is some evidence indicating that gap junctions composed of Cx30 do not transfer negatively charged molecules, such as miRNAs.[28] There may also be a similar weakly anion selective effect in gap junctions composed of Cx32.[37] Consistent with Cx30, we found that gap junctions composed of Cx32 or Cx37 did not deliver miRNAs consisting of 18-27 nucleotides. Gap junctions composed of various types of Cx show different permeability to ions as well as small substances, and they may synchronize and coordinate their specific roles in proliferation and differentiation.[34,38] Thus, the influence of gap junctions composed of other types of Cx on the delivery of miRNA warrants further exploration.

MicroRNA is a promising therapeutic agent against many diseases, including cancer.[4,9,39,40] However, several challenges limit miRNA-based clinical applications, including effective delivery of therapeutic miRNA, lack of specificity and induction of the immune response.[41-43] Gap junctions are selectively permeable, which allows for the exchange of secondary messenger molecules and metabolites between the cytoplasm and the extracellular environment.[16] Notably, transmission of miRNAs through gap junctions is efficient and specific

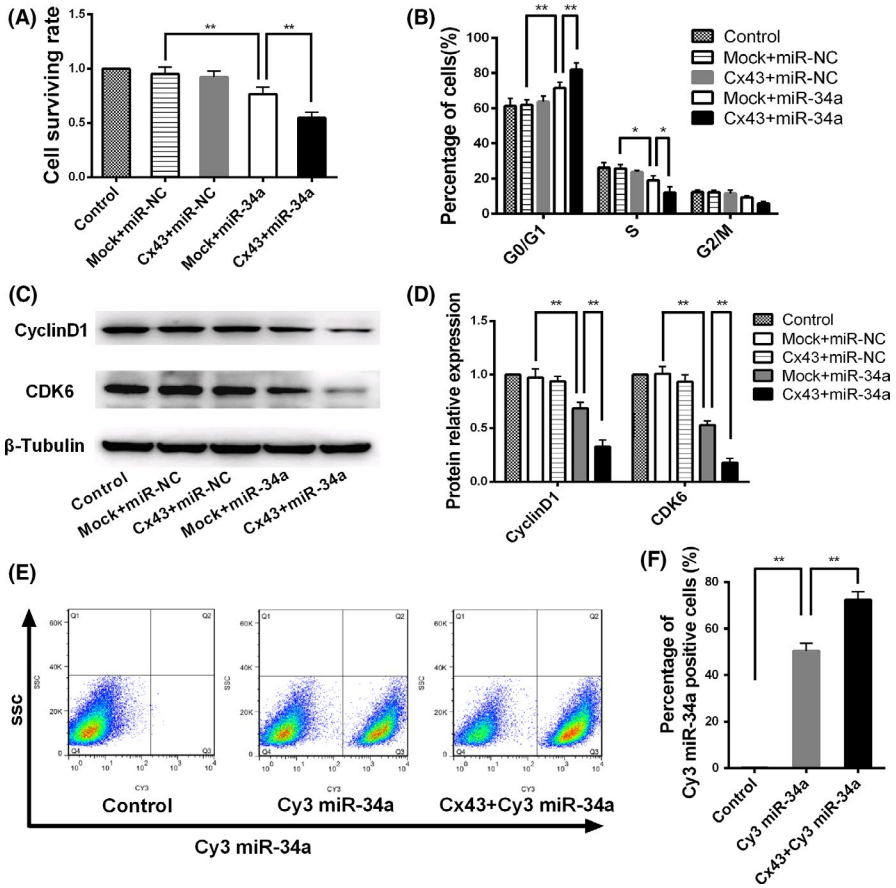

FIGURE 8 Overexpression of connexin 43 (Cx43) enhanced miR-34a-mediated inhibition of proliferation in U251 cells. A, Cell proliferation of different overexpressed groups was detected by CCK-8 assay. B, The cell cycle was analyzed in U251 cells with miR-34a or Cx43 plasmid transfection. C,D, Expression of cyclin D1 and cyclin-dependent kinase 6 (CDK6) in U251 cells transfected with miR-34a or Cx43 plasmid. β-Tubulin was used as a loading control. E,F, Percentage of Cy3 miR-34a positive cells was measured by flow cytometry after Cy3-labeled miR-34a or Cx43 plasmid transfection. Columns represent the means of four experiments; bars represent the SEM. $^{*}P < .05$, $^{**}P < .01$

because gap junctions transfer small molecules directly and they cannot go through the extracellular space.[44,45] Specificity is also reflected in the fact that the transfer of miRNAs through gap junctions depends on the type of Cx. Thus, manipulating the function of gap junctions by pharmacological means may provide a new approach for the development of miRNA-based clinical applications. For example, an increase in the gap junctions composed of Cx43 may significantly enhance the inhibitory effect of miR-34a on cell proliferation.

In conclusion, our study showed that miRNAs consisting of 18-27 nucleotides can be transferred between glioma U87 cells through gap junctions. Gap junctions composed of Cx43 delivered miRNAs consisting of 18-27 nucleotides between glioma cells, whereas gap junctions composed of Cx32 or Cx37 failed to transfer these miRNAs between cervical cancer cells. Moreover, gap junctions composed of Cx43 significantly enhanced the inhibitory effect of miR-34a on cell proliferation in glioma cells. It is well known that the effective delivery of therapeutic miRNA to cancer cells is a challenge that limits miRNA-based clinical applications. In the present study, we discovered a solution to this problem. In the future, antitumor miRNA can combine with Cx43 or a Cx43 enhancer, but not with Cx32 or Cx37, to improve the therapeutic effect and delivery of antitumor miRNA to a greater number of neighboring cancer cells. Taken together, these findings will promote the development of miRNA-based clinical applications and provide a new guiding strategy for miRNA-based cancer treatment.

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DISCLOSURE

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

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