Long non-coding RNA GAS5 aggravate renal epithelial cell apoptosis in cisplatin-induced AKI by regulating miR-205-5p

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Research

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Abstract

Introduction: The present study focuses on the interaction between long non-coding RNA GAS5 and microRNA-205-5p and their roles in cisplatin-induced acute kidney injury.

Methods: Human kidney tubular cells (HK-2) were used to simulate acute renal injury induced by cisplatin with the consequent fluctuating expression levels of GAS5 and MIR-205-5p being determined respectively. Furthermore, the modulating effects of miR-205-5p and GAS5 in cisplatin-induced apoptosis of renal tubular epithelial cells and the possible binding sites between them were evaluated.

Results: The results depicted that the expression of GAS5 was significantly up-regulated after AKI induced by cisplatin, while inhibiting the increase of expression would alleviate the apoptotic-promoting effect of cisplatin on renal tubular epithelial cells. MIR-205-5p is negatively regulated by GAS5, thus down-regulation of GAS5 will consequently elevate the expression of miR-205-5p and further alleviate the damage of HK-2 cells induced by cisplatin.

Conclusions: In conclusion, in cisplatin-induced AKI, the expression of GAS5 was increased and consequently inhibited that of miR-205-5p by direct binding, which eventually aggravate the renal tubular epithelial injury, indicating their potential of being important diagnostic markers and therapeutic targets in the treatment of cisplatin-induced AKI.

Background

Acute kidney injury (AKI),being a common clinical disease with an up to 20% incidence in hospitalized patients, has a significant relevance with the deterioration of renal diseases and mortality(1). Several statistical clinical studies with fairly large sample size had delineated that the severity of AKI is evidently correlated with mortality, demonstrated by the four-week survival rate of severe AKI patients being only 50%(2). In recent years, the increasing incidence and mortality of AKI has been extensively confirmed, which has consequently aroused the vigilance and widespread concern of this none-negligible, pernicious and thorny disease (2).

Cisplatin is a metal coordination compound of platinum which has a molecular structure of planar quadrilateral (3). Although firstly synthesized in 1844, it was not until the discovery of its inhibiting cell division characteristic in 1960 that cisplatin became an indispensable constituent of antineoplastic therapies and was extensively exerted in the treatment of diverse cancers of head and neck, lung, ovary, testicle and bladder until today (3). Although cisplatin has exceptional anti-tumor properties, various parlous side effects, including nephrotoxicity, electrolyte imbalance, bone marrow toxicity and ototoxicity, were gradually confirmed by clinical practice, and has consequently become a critical constraint restricting the further development of cisplatin-based chemotherapy in the field of cancer treatment (4). In fact, adequate studies have depicted that cisplatin-induced AKI is a elusive pathophysiological process with complex mechanisms and interaction of multiple factors(5). Being one of the most common and

fatal complications among hospitalized patients treated with cisplatin, the discovery of the potent drug to alleviate this disease with elucidation of underlying mechanism have become a top priority.

In recent years, various members of non-coding RNAs were identified and depicted to correlate with diverse metabolic reactions through elaborated interaction with each other, the most common of which are long-non coding RNAs (lncRNAs) and micro RNAs (miRs) (6, 7). lncRNAs has been delineated to be key link of multiple path physiological procedures, covering various fields such as apoptosis, cell proliferation and migration (8). It was revealed that growth arrest-specific 5 (GAS5), as a member of lncRNAs, plays a role of apoptotic gene and thus have the potential of being promising non-invasive biomarker for various diseases including AKI (9). Being a member of non-coding endogenous short RNA, miRs can interact with the untranslated 3'-terminal of messenger RNA (mRNAs),hence, inhibit the expression of relevant genes(10, 11). Despite the potential regulatory mechanisms of lncRNAs and miRs on apoptosis may be intrinsically associated with cisplatin-induced AKI, the related studies are scarce. MiR-205-5p was abnormally expressed in various cancers, by directly affected cell proliferation, invasion, migration and cisplatin-resistant(12–14). Bioinformatics analysis showed a targeting relationship between miR-205-5p and GAS5. In view of the present situation, this paper, focusing on lncRNA GAS5 and miR-205-5p, through various experimental methods, intends to investigate their possible therapeutic effects in cisplatin-induced AKI, and briefly elucidate their underlying mechanisms.

Methods

having received bone marrow or organ transplantation, or having acquired immunodeficiency syndrome were the exclusive criteria for this study. All the research protocol of the present study was approved by the Human Ethics Committee with all the patients who participated in this study having signed the informed consent before the beginning of the experiment.

Evaluation of GAS5 in cisplatin-induced AKI

Serum samples were obtained by centrifuging the blood samples from the subjects at 5000g, 4℃ for 3 min. After centrifuging, the serum were administrated with the miRNeasy Mini kit (QIAGEN, Germany) to get the total RNA of individual patients respectively with the correlated cDNA further synthetized exerting RT2 PreAMP cDNA Synthesis Kit(QIAGEN, Germany). Furthermore, RT2 SYBR® Green qPCR Master Mix (QIAGEN, Germany), StepOnePlus thermo-cycler (Applied Biosystems) and $2^{-\Delta\Delta Ct}$in method were exerted to perform the qRT-PCR aiming to evaluation the fluctuation of GAS5's expression levels. The association of GAS5 and Creatinine clearance rate (CCr) were also revealed by Pearson's correlation coefficient by SPSS 19.0 (SPSS, Chicago, IL), aiming to investigate the potential pernicious effects in this pathological disease.

Lentiviral vector construction and infection

The LV10-CMV-RFP-Puro vector (GenePharma, Shanghai, China) was administrated with si-GAS, a short-hairpin RNA directed against GAS5, while the negative sequence was exerted for negative controlling.

After receiving the lentiviral expression vectors, the 293T cells were further subjected to virus particles incubated by Polybrene (Sigma, St. Louis, Missouri, USA) and an exclusion procedure of puromycin. Besides, the GAS5 cDNA was multiplied with PfuUltra II Fusion H DNA Polymerase (Stratagene, Agilent Technologies, Santa Clara, CA, USA), followed by the subcloning to EcoRI and HindIII of pcDNA3.1 vector (Invitrogen, USA) to construct pcDNA-GAS5. The mimics and inhibitors of miR-205-5p and NC mentioned were provided by GenePharma (Shanghai, China), while Lipofectamine 2000 (Life Technologies Corporation, Carlsbad, CA) were exerted to transfect the cells. The sequences for GAS5 siRNA were 5'-GCGAGCGCAATGTAAGCAA-3' Non targeting sequence (5'-ACGUGACACGUUCGGAGAATT-3') was used as a negative control.

miR-205-5p mimic, 5'-UCCUUCAUUCCACCGGAGUCUG-3'; miR-NC mimic, 5'-UCGCUUGGUGCAGGUCGGGAA-3'; miR-205-5p inhibitor, 5'-CAGACUCCGGUGGAAUGAAGGA-3'; miR-NC inhibitor, 5'-CAGUACUUUUGUGUAGUACAA-3'

qRT-PCR

After the total RNA was isolated from the cells, the cDNA was obtained exerting High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) with that of miRNAs being fabricated by TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). TaqMan Universal Master Mix II, TaqMan miRNA assays for miR-205-5p and U6, and TaqMan gene expression assays for GAS5 and GAPDH were further exerted for qRT-PCR. Expressions were calculated utilizing $2^{-\triangle\triangle Ct}$ method. The primers utilized are:

GAS5 F:5′-GGCAAATGAGCACTAAAG -3 R:5′- CACCCACTCCTCTATCTACA -3′

GAPDH F:TCAACGACCCCTTCATTGACC R: CTTCCCGTTGATGACAAGCTTC

miR-205-5p forward, 5'-TCCTTCATTCCACCGGAGTCTG-3' and reverse, 5'-GCGAGCACAGAATTAATACGAC-3'; U6 forward, 5'-ATTGGAACGATACAGAGAAGATT-3' and reverse, 5'-GGAACGCTTCACGAATTTG-3'

Western blot

Before experiment, the HK-2 cells was plated in a 6-well plate at a rate of 2 × 106 cells/well and incubated for 24 h. Cells were lysed and 50 μg protein was isolated utilizing SDS-PAGE. The polyvinylidene fluoride membrane were exerted to receive the protein sample underwent electrophoresis and then administrated with the blockage of non-fat milk. The antibodies of GAPDH (ab9485, 1:5000, Abcam) and cleaved Caspase-3 (ab49822, 1:1500, Abcam) were further exerted to administrate the foregoing membranes, followed by incubation of Goat Anti-Rabbit IgG H&L (HRP, ab205718, 1:3000, Abcam) or Goat Anti-Mouse IgG H&L (HRP, ab6789, 1:3000, Abcam), with the relevant protein levels eventually revealed by enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA).

Luciferase reporter analysis

The GAS5-wt (embodying the specific sequence able to bind with miR-205-5p) and GAS5-mut (embodying the mutation of foregoing sequence) were obtained from Genescript (Nanjing, China). The luciferase analysis was performed by the dual-luciferase reporter analysis system (Promega, USA) after the HEK 293T cells' being co-transfected with miR-205-5p or miR-NC and GAS5-wt or GAS5-mut. According to the manufacturer's protocol, a dual-luciferase reporter assay system (Promega, USA) was used to detect the luciferase activity after co-transfection in HEK 293T cells for 48 h.

Pull-down analysis

The HK2 cells underwent a transfection of biotinylated probe (Sangon, Shanghai, China) targeting GAS5 and miR-205-5p, and 48h later, the HK2 cells were lysed, the products of which received the incubation of Dynabeads M-280 Streptavidin (Invitrogen, Carlsbad, CA, USA). The qRT-PCR was exerted for analyzing the bound RNAs.

RNA Immunoprecipitation (RIP) analysis

The Magna RIP RNA-Binding Protein IP Kit (Millipore, Bedford, MA, USA) andAgo2 antibody (2897, Cell Signaling, Danvers, MA, USA) were exerted for RIP analysis, by which the expression levels of GAS5 and miR-205-5p were consequently revealed.

HK-2 model culture

Antibiotics (100 U/ml penicillin G + 100 μg/ml streptomycin + 0.25 μg/ml amphotericin B, Invitrogen) and Dulbecco's Modified Eagle Media/F12 with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C in a 100% humidified atmosphere of 5% CO_2-95% air were exerted for culturing the HK-2 cells (American Type Culture Collection, Manassas, VA). The foregoing cells' conservation of original phenotypic and metabolic characteristics of the proximal tubule during the culturing procedure were confirmed.

Apoptosis analysis

According to the manufacturer's, the cells were transfected for 48h and further stained for 15min in the dark at room temperature with Annexin V and PI (Annexin V-FITC Apoptosis Detection Kit, eBioscience), the HK-2 cells' apoptosis status were evaluated by BD FACSCalibur flow cytometer (BD Biosciences, California, USA) and Cell Quest software (BD Biosciences). After all the cells being classified as alive, early-apoptotic, late-apoptotic and dead, the apoptosis status was consequently revealed by the early-apoptotic ratio. All the above analysis was repeated triplicately.

Statistical Analysis

The data were administrated with one-way ANOVA and Dunnett's multiple comparison tests or paired *t* test (version 5.00; Graph Pad Prism Software Inc., San Diego, CA). $P<0.05$ was selected for representing statistical significance.

Results

The expression of GAS5 and miR-205-5p in the cisplatin-induced AKI patients and in HK-2 cells

Comparing to their corresponding, GAS5 was upregulated while miR-205-5p was reduced in AKI group (Fig. 1A, 1B, $P < 0.01$), indicating that GAS5 and miR-205-5p may be of importance in cisplatin-induced AKI. Furthermore, the expression levels increase of GAS5 was to revealed to correlating with the dose of Cisplatin. And the expression of miR-205-5p was decreased as well as in dose dependent manner. (Fig. 1C and 1D)

The apoptosis-promoting effects were significantly inhibited by suppressing GAS5 in Cisplatin-stimulated HK-2 cells.

In order to explore the role of GAS5 in cisplatin-induced AKI, we induced in the cell model using Cisplatin. TheHK-2 cells with consistently suppressed expression levels of GAS5 were obtained by being transfected with si-GAS5 or pc-GAS5 (Fig. 2A), after which the inhibitory effects the two mentioned above and the apoptosis-promoting effects of GAS5 were further evaluated. As shown in Fig. 2, the rate of cleaved-casepase5/Gapdh(Fig. 2B), the activity of caspase-3(Fig. 2C), and the apoptosis rate (Fig. 2D and 2E) show that cisplatin induced apoptosis (control group). However, the regulation of GAS5 reduced this apoptosis induced by Cisplatin, on the contrary, the downregulation of GAS5 aggravates this effect. It was revealed that the Cisplatin-stimulated apoptosis was significantly suppressed after the decrease of GAS5 ($P < 0.05$).

The apoptosis-promoting effects were significantly amplified by suppressing miR-205-5p in Cisplatin-stimulated HK-2 cells.

The HK-2 with diverse expression levels of miR-205-5p were obtained by being transfected with miR-205-5p mimic and miR-205-5p inhibitor, after which the inhibitory effects the two mentioned above and the apoptosis-inhibiting effects of miR-205-5p were further evaluated (Fig. 3A**)**.. In Cisplatin-stimulated HK-2 cells model group, It shows that the up-regulation of miR-205-5p inhibits cell apoptosis, while the down-regulation of miR-205-5p promotes cell apoptosis in western blot(Fig. 3A and Fig. 3B) and flow cytometry(Fig. 3D and 3E).

Reciprocal inhibition existed between miR-205-5p and GAS5 in HK-2 cells.

Upregulation of GAS5 reduced miR-205-5p expression and downregulation of GAS5 induced miR-205-5p expression in both control group(-) and cisplatin-stimulated group(+) (Fig. 4A). Targetscan, Starbase, and microRNA.org were exerted to reveal that GAS5 can directly bind with miR-205-5p and consequently serves as the predictive target of it (Fig. 4B). Besides, the luciferase activity was suppressed by miR-205-5p with the foregoing trend being significantly reversed by Mut(Fig. 4C). Furthermore, it was confirmed that miR-205-5p can significantly pull down while the mut of that can not (Fig. 4D). Similar trends was

also revealed by *GAS5* probe(Fig. 4E), collectively suggesting the direct interacting relationship between miR-205-5p and GAS5.

Up-Regulation of miR-205-5p mostly reversed GAS5-induced apoptosis in Cisplatin-stimulated HK-2 cells

Cisplatin-stimulated HK-2 cells with consistent high expression levels of GAS5 were transfected by miR-205-5p mimic, revealing that the apoptosis-promoting effects of GAS5 can be significantly inhibited by miR-205-5p (Fig. 5A, 5B).

Discussion

Being members of none-coding RNAs, GAS5 and miR-205-5p were repeatedly delineated to be evidently involved in the regulation of apoptotic mechanisms in various pathophysiological procedures (15–18). Functional experiments have further revealed that GAS5 is able to perform two-pronged functions of inhibiting the proliferation and promoting apoptosis of various cell types (19). These cellular mechanisms of GAS5 are not only significantly involved in drug resistance mechanism of specific cell type like tumor, but also play an critical role in normal cells' damage tolerance under many pathophysiological procedures. miR-205-5p was proved to be one of the downstream factors of GAS5's cell proliferation modulation (20). It was revealed to be of importance in kidney development, homeostasis and pathology, and participate in various histopathological changes such as the occurrence and deterioration of tubulointerstitial sclerosis and terminal stage glomerulopathy in diverse types of kidney disease (21). Interestingly, recent studies have depicted the crossroad-like role of miR-205-5p as an apoptosis-suppressing gene or as an anti-survival gene under different physiological conditions, leaving the potential correlation and underlying mechanism between GAS and cisplatin-induced AKI rather complicated and thus need to be delineated urgently (22). So far, however, to the best of our knowledge, the related research is still rare. In the present study, it is confirmed that GAS5 was significantly increased in AKI group compared with their corresponding, with miR-205-5p being evidently reduced in the mean time. Besides, these changes in expression were further proved to be dose-dependent with cisplatin, jointly making the involvement of GAS5 and miR-205-5p in this cisplatin-induced AKI preliminarily confirmed.

The interaction between lncRNAs and microRNAs was delineated to elaborately modulate the elusive balance of survival and programmed death (23). In the present study, through the evaluation of cell apoptosis in the Cisplatin-stimulated HK-2 cells, it was revealed that the down-regulation of GAS5 significantly correlate with the alleviation of cell apoptosis, while that of miR-205-5p conversely delivered a apoptosis-promoting effect, which conforms to the revealed role of miR-205-5p to elevate the resistance of oxidative and endoplasmic reticulum stresses and consequently be in favor of cell survival recent years (24, 25). Furthermore, the administration with miR-205-5p mimic also suppressed the severe apoptotic procedures in the present study. The above-mentioned conforms to the general regulation pattern of GAS5 and miR-205-5p on apoptosis in the pathophysiological procedures of various cell types, and also indicates the intrinsic causal relationship between GAS5, miR-205-5p and cisplatin-induced AKI

(26). A antagonistic relationship between GAS5 and miR-205-5p in this pathological procedure was also implied.

To further elucidate the contradictory modulating effects between GAS5 and miR-205-5p in cisplatin-induced AKI, the fluctuation of their expression levels after interaction were evaluated. As presented in Fig. 4A, a reciprocal inhibitory relationship of miR-205-5p and GAS5 in HK-2 cells was suggested by the result that upregulation of GAS5 can significantly down-regulate the expression levels of miR-205-5p, and vice versa, further confirming the antagonistic relationship between them.

It was suggested that miR-205-5p can directly interact to *GAS5*via specific bingding site (27). In the present study, this potential interacting site structure of GAS5 and miR-205-5p was further evaluated by bioinformatics analysis. As shown in Fig. 4B, it was revealed to be a functional binding site in transcription productsGAS5, elucidating the interaction between them at the molecular structure level, which conforms to the reported characteristics of these two members of none-coding RNAs to directly bind with each other (28, 29). The relative luciferase activity was decreased by up-regulated miR-205-5p expression, with this inhibition further reversed with the administration of GAS5-mut,conforming to the demonstration of binding site and antagonistic expression results mentioned above. Moreover, in this study, bi-directional pull-down assay was further exerted to confirm their putative binding structure. It was revealed that GAS5 was significantly pulled down by by biotinylated miR-205-5p, while the foregoing trend was further reversed by miR-205-5pmut, collectively suggesting their reciprocal inhibitory interaction synthetically. Finally, a significant reversed of GAS5's cell apoptosis-promoting effects by administration of miR-205-5p directly confirmed that miR-205-5p participates in GAS5's modulation mechanism with an antagonistic role. In conclusion, the present study revealed that lncRNA GAS5 can aggravate renal epithelial cell apoptosis in cisplatin-induced AKI by antagonistically interacting with miR-205-5p, and thus have the potential of being promising therapeutic target of hindering the development of this parlous disease.

The present study have some limitations: Initially, it was proved that CMTM4, ZEB2 and PTEN can perform as downstream regulators of miR-205-5p, and thus have the potential to be of importance in the GAS5's apoptosis modulation mechanism of cisplatin-induced AKI (10, 21, 26, 30–33). Suggesting that they can be exerted as complementary indicators in follow-up studies to render the mechanism revealed more comprehensive. Secondly, the presented study was mainly performed in vitro, leaving the complex interaction between specific factors in vivo environment being not properly simulated, making the comparative experiment with in vivo evaluation of the mechanism underlying cisplatin-induced AKI further warranted. Finally, as a member of microRNAs like miR-205-5p, miR-34 was also revealed to interact with GAS5 in regulation of many pathological procedures, thus may potentially participate in the elusive mechanism underlying this disease (6). Its role remains to be explored in our subsequent research to illuminate the holistic mechanism of the apoptosis modulating effects of miR-205-5p and GAS5.

Conclusion

The expression of GAS5 in cisplatin-induced AKI was increased and consequently inhibited that of miR-205-5p by direct binding, which eventually aggravate the renal tubular epithelial injury. We indicated the potential of GAS5 being important diagnostic markers and therapeutic targets in the treatment of cisplatin-induced AKI.

Abbreviations

qRT-PCR: quantitative real-time PCR, cDNA: complementary DNA,

Declarations

Acknowledgement

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Ethical approval and Consent to participate

All procedures performed in studies involving human participants were in accordance with the Ethical Standards of the Institutional Research Committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written consent was obtained from study participants. All the research protocol of the present study was approved by the Human Ethics Committee of The First Affiliated Hospital of Xiamen University with all the patients who participated in this study having signed the informed consent before the beginning of the experiment.

Consent for publication

Not applicable

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors contribution

Yinan L designed the study, analyzed the data, performed the experiments, prepared the manuscript

Min Zhang, Hui Bi, Shaoyan Wang, Xuejuan Sun, analyzed the data, performed the experiments, prepared the manuscript

All authors have read and approved the manuscript.

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Figures

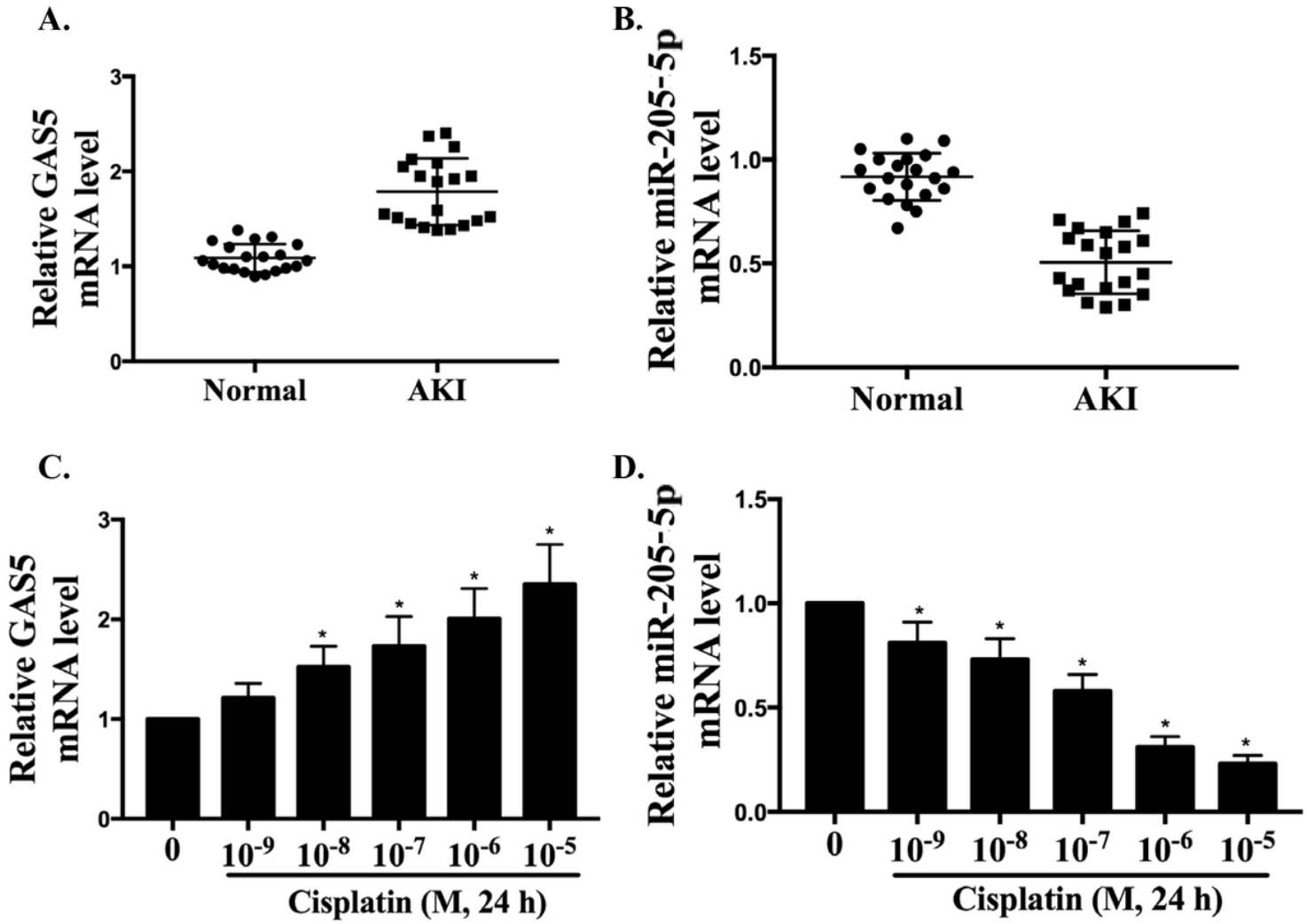

Figure 1

The expression of GAS5 and miR-205-5p in the cisplatin-induced AKI patients and in HK-2 cells. A. B. The relative expression level of GAS5 and miR-205-5p in cisplatin-induced AKI patients and healthy volunteers. N=20. C and D. The expression level of GAS5 and miR-205-5p in HK-2 cells treated with different concentration of Cisplatin for 24 hours. N=5. *p<0.05 vs ctrl group..

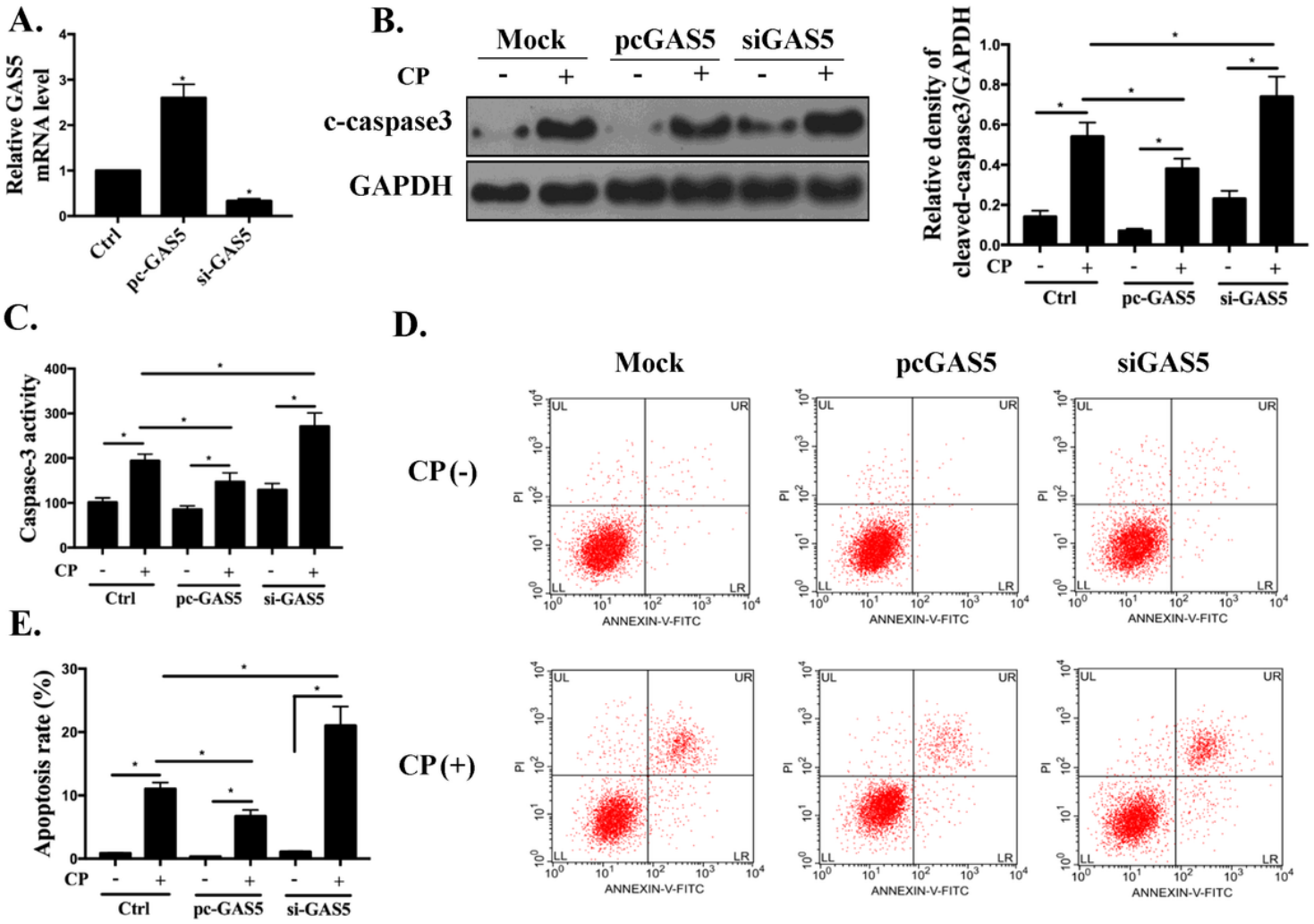

Figure 2

Association between GAS5 inhibition and apoptosis of in Cisplatin-stimulated HK-2 cells. A. mRNA level of LncRNA-GAS5 in different groups. B. Western blot assay of cleaved-caspase3 in HK-2 cells administrated with 10-6M Cisplatin. C. caspase3 activity. D and E. flow cytometry examined the cell apoptosis and apoptosis rate. N=5. *p<0.05.+: HK-2 cells administrated with 10-6M Cisplatin, -: control HK-2 cells

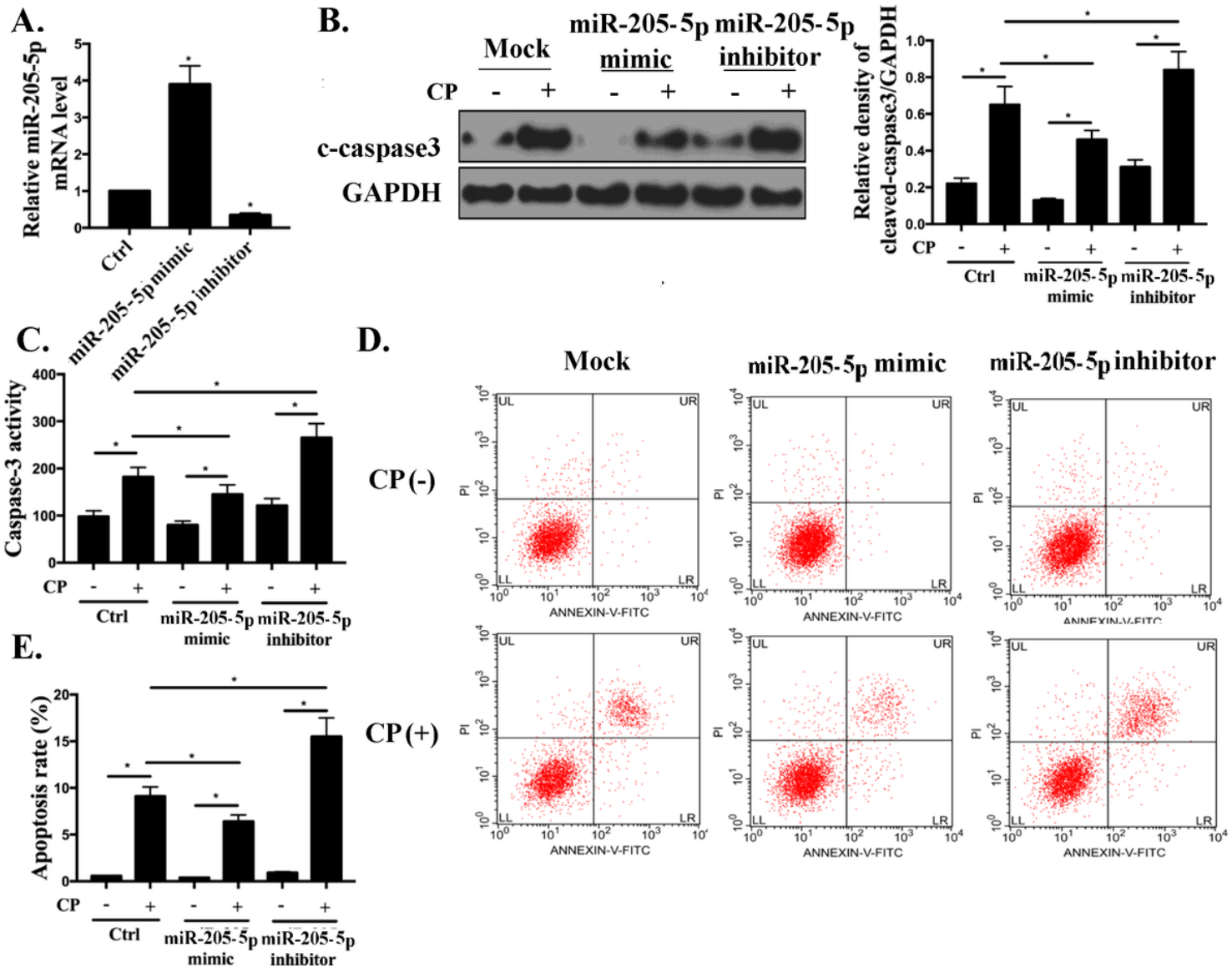

Figure 3

Effects of miR-205-5p on the cell apoptosis in cisplatin-stimulated HK-2 cells. A. mRNA level of miR-205-5p in different groups. B. Western blot assay of cleaved-caspase3 in cells administration with 300uM CoCl2. C. caspase-3 activity. D and E. flow cytometry examined the cell apoptosis and apoptosis rate. N=5, *p<0.05.+: HK-2 cells administrated with 10-6M Cisplatin, -: control HK-2 cells

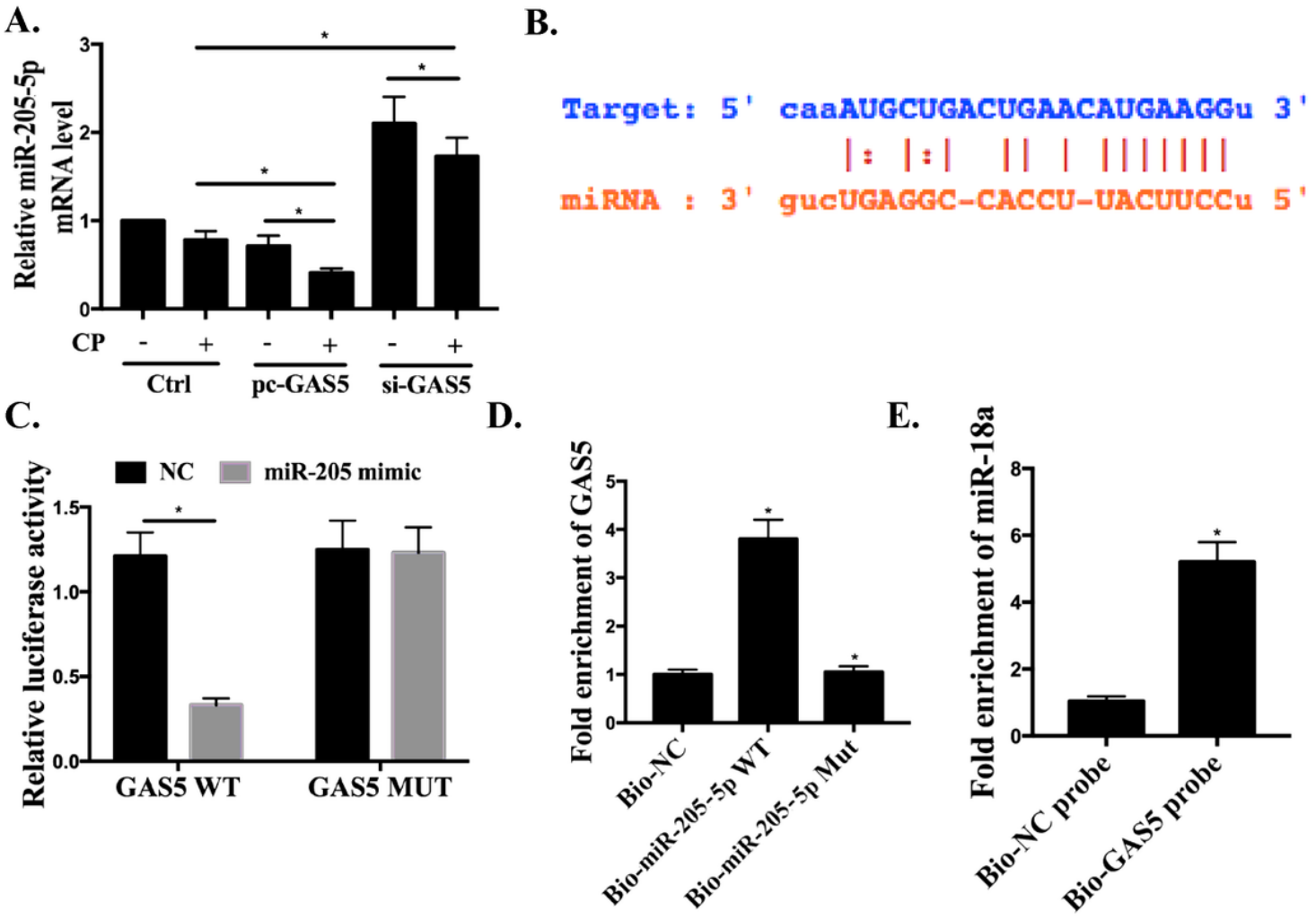

Figure 4

Reciprocal inhibition between miR-205-5p and GAS5 in Cisplatin-stimulated HK-2 cells. A.miR-205-5p expression in cells transfected with pc-GAS5 or si-GAS5. B. Putative binding sites of miR-205-5p within GAS5 mRNA. C. The luciferase activities were suppressedby transfection of WT vector and miR-205-5p. D. Evaluation of GAS5 expression levels after being pulled down by miR-205-5p. E. Evaluation of miR-205-5p expression levels after being pulled down by GAS5. N=5,*p<0.05, +: HK-2 cells administrated with 10-6M Cisplatin, -: control HK-2 cells

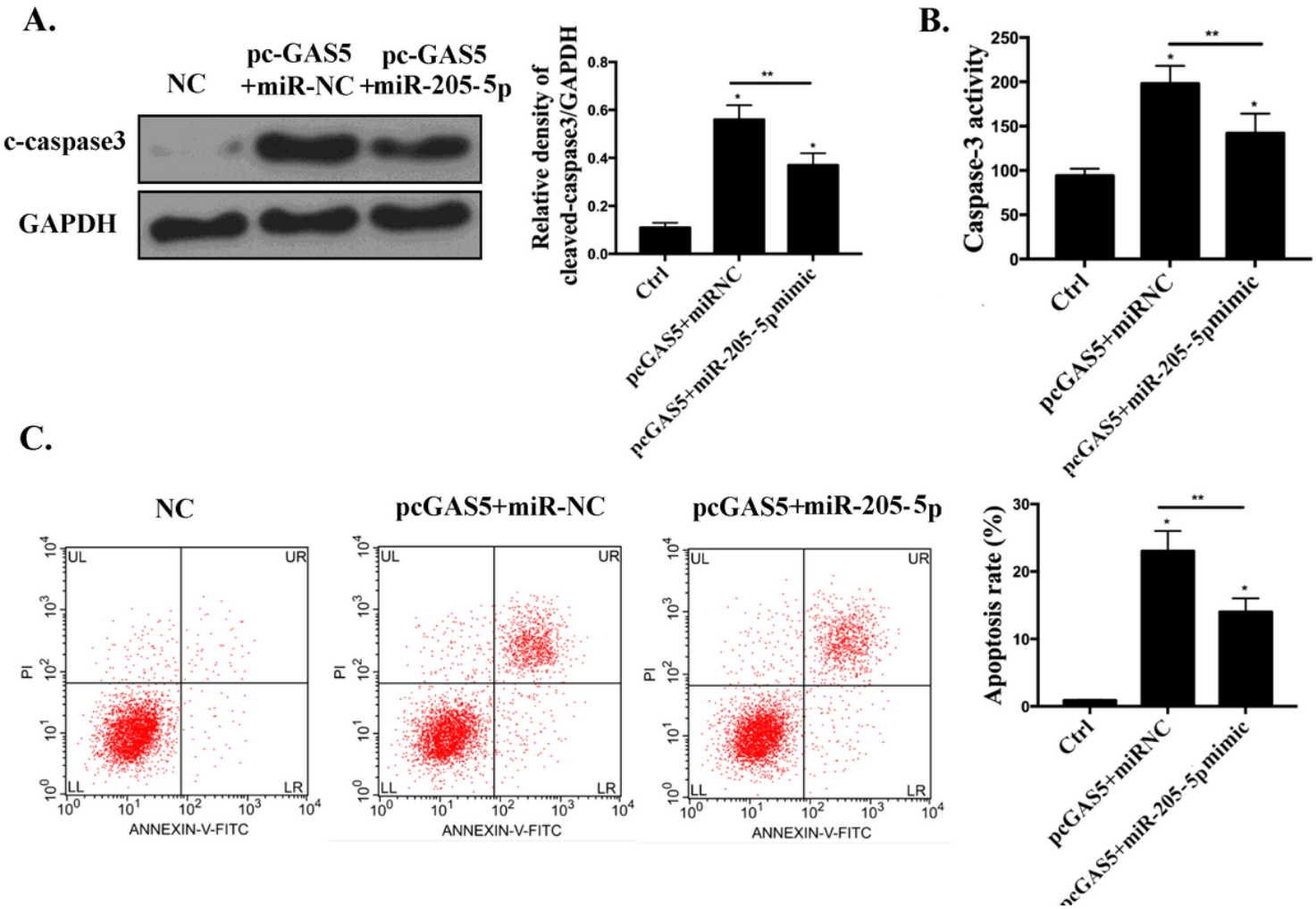

Figure 5

Up-Regulation of miR-205-5p mostly reversed GAS5-induced apoptosis in Cisplatin-stimulated HK-2 cells. A. Western blot assay of cleaved-caspase3. B. caspase-3 activity. C. flow cytometry examined the cell apoptosis. N=5, *p<0.05 vs ctrl group, **p<0.05 pcGAS5+miRNC vs pcGAS5+miR205-5p mimic.