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Tumor Necrosis Factor-α-Induced Protein-8 Like-2 (TIPE2) Upregulates p27 to Decrease Gastic Cancer Cell Proliferation

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ABSTRACT

The pathogenesis of gastric cancer is not completely understood. Tumor necrosis factor-α-induced protein-8 like-2 (TIPE2) has recently been identified as a novel negative regulator gene of the immune system, and studies in mice and humans have suggested its inhibitory action in both inflammation and cancer. In this study, we examined the expression levels of TIPE2 in human gastric cancer tissues and also samples of paraneoplastic control tissue, and found that TIPE2 expression was reduced in gastric cancer. To investigate the role of TIPE2 in gastric cell carcinogenesis, a TIPE2 plasmid was introduced into gastric cell lines and TIPE2 function was examined. Colony-forming assays showed that restoration of TIPE2 expression in gastric cells significantly suppressed cell proliferation. Analysis by flow cytometry showed that the number of cells in the S phase of the cell cycle was reduced concomitant with TIPE2 expression, and cell apoptosis was maintained at a low level. Microarray and western blot analyses revealed that TIPE2 selectively up-regulated N-ras and p27 expression. The role of p27 in mediating TIPE2-associated cell growth inhibition was verified by a p27 siRNA interference assay. In this study, we proved that TIPE2 is an inhibitor of gastric cancer cell growth, and suggest that TIPE2 might promote a p27-associated signaling cascade that leads to restored control of the cell cycle and cell division. Our results provide a new molecular mechanism by which TIPE2 may regulate proliferation of gastric cells. J. Cell. Biochem. 116: 1121–1129, 2015.

KEY WORDS: TIPE2; GASTRIC CANCER; CELL CYCLE; p27

Gastric cancer is one of the most common malignant tumors, and accounts for about 10% of all invasive cancers worldwide. It also is probably the second leading cause of cancer deaths [Roder, 2002]. The incidence of gastric cancer varies greatly across populations, with almost two-thirds of gastric cancer cases and deaths occurring in less developed regions [Yang, 2006]. Despite the

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decline in its incidence in the West, gastric cancer remains the most frequent type of cancer in Asia. In China, the total number of cases and deaths from gastric cancer have increased concomitant with the huge demographic changes which have been ongoing [Yang et al., 2004, 2005]; resulting in an urgent need to find more effective treatments and new targets for therapy. While the pathogenesis of cancer is incompletely understood, it is believed that dysfunctions of immune regulation, such as persistent inflammation, significantly contribute to the pathogenesis of tumors [Nathan and Ding, 2010]. Therefore, anti-inflammatory molecules represent potential drug candidates for cancer therapy. TNFAIP8 like-2 (TIPE2), a member of the tumor necrosis factor-α-induced protein-8 (TNFAIP8) family, was recently identified as a novel negative regulator gene of the immune system which can independently maintain immune homeostasis [Sun et al., 2008]. In vitro experiments have demonstrated that TIPE2 inhibits activator protein 1 (AP-1) and NF-κB stimulation. TIPE2 knockout cells showed hyper-responsiveness to Toll-like receptor (TLR) and T cell receptor (TCR) activation [Sun et al., 2008]. These results suggest that TIPE2 expression in certain cells is closely associated with immune system disorders, and may even trigger some developmental diseases. Similarly, TIPE2 knockout mice suffered autoimmune diseases which produced severe multiple organ infiltration of mononuclear cells at 3 months after birth. Additionally, serological tests have demonstrated that levels of several inflammatory cytokines including IL-1, IL-6, IL-12, and anti-inflammatory cytokine IL-10 in TIPE2 knockout mice are significantly elevated when compared to levels in normal mice.

TIPE2 is expressed not only in human lymphoid tissues, but also in some non-lymphoid tissues, such as those found in the nervous, digestive, urinary, respiratory, and reproductive systems [Li et al., 2009; Zhang et al., 2010; Xi et al., 2011; Zhang et al., 2011; Kong et al., 2013], suggesting that TIPE2 may have roles beyond immune regulation. Professor Chen's group first reported the functions of TIPE2 in regulating cell death and migration [Gus-Brautbar et al., 2012; Zhang et al., 2013]. In detail, TIPE2 competitively binds to the Ras-interacting domain of RalGDS family proteins, preventing Ras from forming an active complex, and thereby inhibits activation of Ras downstream signaling molecules, Ral and AKT. As a result, TIPE2 expression induces cell death and inhibits Ras-induced tumor formation, providing a molecular bridge from inflammation to cancer.

In the present study, we initially examined the expression level of TIPE2 in atrophic gastritis and gastric cancer tissues. The results showed that TIPE2 expression was reduced in gastric cancer tissues. We then introduced the TIPE2 plasmid into the gastric cancer cell lines AGS and BGC-823 and investigated the function of the gene. Colony-forming cell assays demonstrated that restoration of TIPE2 in those gastric cancer cells significantly suppressed cell proliferation. Next, the mechanism for the inhibitory effect on proliferation was explored by testing the cells for apoptosis and cell cycle stage using flow cytometry. The results showed that cell apoptosis was maintained at a low level while expression of TIPE2 was being restored; however, cell cycle analysis showed that more gastric cancer cells had entered their S phase as TIPE2 expression increased. An analysis of signal transduction data revealed that TIPE2 had upregulated N-ras and p27 expression, thereby inhibiting cells from moving into S phase and mitosis, leading to suppressed cell proliferation. This report describes a new molecular mechanism by which TIPE2 regulates cell proliferation in gastric cells.

MATERIALS AND METHODS

PATIENT SAMPLES

Newly endoscopic biopsy specimens from 15 surgically resected gastric tumors and its adjacent paraneoplastic tissues were obtained from The Second Hospital of Shandong University (Jinan, Shandong, China). All human tissue samples (11 males and 4 female) with gastric cancer in September, 2013. All persons gave their informed consent prior to their inclusion in the study. The tissues were kept at −80°C and used for subsequent analysis. All studies were reviewed and approved by the ethic committee of Shandong University (Jinan, Shandong Province, China).

IMMUNOHISTOCHEMISTRY FOR TIPE2 PROTEIN EXPRESSION

The immunohistochemical analysis was performed according to the previous report [Zhang et al., 2011]. Briefly, sections were thawed and fixed in acetone for 10 min at 20°C. The tissue was rehydrated in 0.1 M PBS for 5 min. Next, the sections were incubated with the blocking goat serum for 10 min, rabbit anti-TIPE2 polyclonal antibody ((Boster Co. Ltd, Wuhan, China; dilution 1:50) for 60 min, and then with peroxidase-labeled goat anti-rabbit IgG antibody (Maixin Bio, Fuzhou, China) for 15 min at room temperature. DAB peroxidase substrate kit (Maixin Bio, Fuzhou, China) was used to produce the enzymatic reaction. The sections were finally counterstained with hematoxylin. All slides were scored by evaluating the percentage and intensity of stained cells as previously described (H-score, Budwit-Novotny et al., 1986). Specifically, staining intensity, 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining. The percentage of positive cells is as follows: 0, <1%; (1) 1%–10%; (2) 10–25%; (3) 25%–50%; (4) >50%. The staining intensity multiply the percentage of positive cells for each slide to produce a final grade of TIPE2 expression: 0, total score = 0; 1+, total score = 1–3; 2+, total score = 4–6; 3+, total score = 7–9; 4+, total score = 10–12.

CELL CULTURE AND TRANSFECTIONS

Gastric adenocarcinoma cell line AGS and gastric cancer cell line BGC-823 cells were maintained in our laboratory. AGS cells were cultured in Ham's F-12 medium (HyClone, Utah) containing 10% FCS and 1% penicillin-streptomycin. BGC-823 cells were cultured in RPMI-1640 (Life Technologies, CA) supplemented with 10% FCS (Tianhang Co. Ltd, Hangzhou, China) and 1% penicillin-streptomycin. The full-length human TIPE2 cDNA expression plasmid pRK5-tipe2 and control plasmid pRK5-mock were kindly offered by Professor Youhai Chen (University of Pennsylvania) and has been described previously [Zhang et al., 2011]. FuGENEs HD Transfection Reagent (Roche Applied Science, Basel, Switzerland) was used for transfection. All transfection procedures were performed according to the manufacturer's instructions.

RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR

Total cellular RNA was extracted with Trizol (Life Technologies, CA) according to the protocol provided by the manufacturer. First-strand cDNA was synthesized from 1 μg total cellular or tissue RNA using the RevertAid TM First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, MA) with random primers. Then cDNA was amplified for quantitative real-time PCR, the specific primers used were as follows: for TIPE2, forward primer 5′-CTGAGTAAGATGGCGGGTCG-3′ and reverse primer 5′-TCTGGCGAAAGCGGGTAG-3′; for β-actin, forward primer 5′-AGTTGCGTTACACCCTTTCTTG-3′ and reverse primer 5′-CACCTTCACCGTTCCAGTTTT-3′; for p16, forward primer 5′-TTCCTGGACACGCTGGTGGT-3′ and reverse primer 5′-CTATGCGGG-CATGGTTACTGC-3′; for p18, forward primer 5′-TTTGAAAGACC-GAACTGG-3′ and reverse primer 5′- AACAACCTCATTCCTCCC-3′; for p21, forward primer 5′-CTGTGATGCGCTAATGGCG-3′ and reverse primer 5′-AAGTCGAAGTTCCATCGCTCA-3′; for p27, forward primer 5′-GGTTAGCGGAGCAATGCG-3′ and reverse primer 5′-TCCACA-GAACCGGCATTTG-3′; for p57, forward primer 5′-GTCCCTCCGCAG-CACATCCA-3′ and reverse primer 5′-GGTTCTGGTCCTCGGCGT-TCA-3′; for N-ras, forward primer 5′-GAAACCTCAGCCAAGACC-3′ and reverse primer 5′-GCAATCCCATACAACCCT-3′. The real-time PCR reactions were performed at: 95°C, 10 s (denaturation); 55°C, 30 s (annealing); 72°C, 30 s (extension) for 35 cycles. The real-time PCR reactions were performed on the ABI7000 Fast Real-Time PCR System with SYBR Premix Ex Taq TM according to the procedures.

WESTERN BLOT ANALYSIS

Western Blot Analysis was performed as described previously [Wang et al., 2011]. Briefly, cell lysates (20 μg/lane) were separated on 10% SDS polyacrylamide gel and then were transferred to a poly (vinylidene fluoride) membrane. TIPE2, p27, and H-ras protein was detected by a rabbit polyclonal IgG (Boster Co. Ltd, Wuhan, China) and visualized by the enhanced chemiluminescence system (Amersham, Arlington Heights, IL). The density of the bands was quantitated using the NIH image software package (Version 1.61). The intensity of TIPE2, p27 and H-ras expression was judged by the ratio of their expression in experiment groups to their corresponding expression in control groups, and a ratio of more than 1.0 was considered to be an indication of over-expression.

COLONY FORMATION ASSAY

Gastric cell line AGS and BGC-823 cultured in a 6-well plate (2×10^5/well) was transfected with pRK5-tipe2 and its control plasmid pRK5-mock using the FuGENEs HD Transfection Reagent (Roche Applied Science, Basel, Switzerland). After a certain time of growth, the cells were digested with trypsin and counted, 300 cells were transferred to a new well of 6-well plate and medium containing 10% FBS serum was added to make up the volume of 3 mL. After a week's growth at 37°C (BGC-823 for 2 weeks), the formation of cell clones could be visually seen. After washed 3 times with PBS buffer, the cells were fixed for 10 min with 1 mL of methanol in each well at room temperature. Then 1 mL diluted Giemsa dye was added to each well and incubated at room temperature for about 20–25 min. Finally, the wells were washed with PBS until no residual background Giemsa dye was observed and the 6-well plate was scanned for colony counting and analysis.

WOUND HEALING ASSAY

Confluent AGS and BGC-823 cell monolayers on six-well tissue culture plastic dishes were transfected with pRK5-tipe2 and its control plasmid pRK5-MOCK for 72 h. Wounds (500 μmol/L) were generated in each plate using a thin disposable tip. Cultures were rinsed with PBS and replaced with fresh quiescent medium containing 10% fetal bovine serum, wounds were visualized using a phase-contrast microscope [Mauro et al., 1999].

CELL APOPTOSIS AND CELL CYCLE ANALYSIS

5×10^5 AGS cells in a well of 6-well plates were transfected with varied concentrations of pRK5-tipe2 and its control plasmid pRK5-mock, incubating for varied time periods before the cells were digested and harvested by centrifugation. Then the cells were fixed gently (drop-by-drop) in 75% ethanol overnight at −20°C and then re-suspended in PBS containing 50 mg/L PI, 1g/L RNase, and 0.1% Triton X-100. After 30 min at 37°C in the dark, the cells were analyzed with flow cytometry equipped with an argon laser at 488 nm. Then cell cycle was determined and analyzed [Deitch et al., 1982]. Apoptosis was quantified by annexin V/propidium iodide staining (BD Biosciences) [Vermes et al., 1995; Van Engeland et al., 1998].

MICROARRAY ANALYSIS

The microarray chip consisted of 27,326 different human cDNAs (Angilent, Wilmington), in which house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as internal control. The cDNAs from pRK5-tipe2 transfected AGS cells were labeled with Cy3, and the cDNAs from the control pRK5-mock transfected AGS cells were labeled with Cy5. The labeled cDNAs were hybridized with microarray chip under standard conditions according to manufacturer's instructions. The data was analyzed by Molecule annotation system 3.0.

siRNA INTERFERENCE

Chemical modified Stealth siRNA targeting p27/Kip1 and control siRNA were from RiboBio Co., Ltd. (Guangzhou, Guangdong, China). The sequence for p27 siRNA was 5′-GGCUGCUAAGAAGAUGAGU-3′. Cells were transfected with siRNA by the Lipofectamine 2000 method (Life Technologies, CA).

STATISTICAL ANALYSIS DATA ANALYSIS

Data were expressed as mean ± standard deviation (SD). Differences between two groups were compared using the Student's *t*-tests. All experiments were repeated at least three times and $P < 0.05$ was considered statistically significant.

RESULTS

EXPRESSION OF TIPE2 WAS REDUCED IN GASTRIC CANCER CELLS

We first used immunohistochemistry to examine TIPE2 expression in gastric cancer and paraneoplastic control tissues collected from gastric cancer patients; the presence of gastric cancer was confirmed

by H&E staining (Fig. 1A). As shown in Figure 1A, the intensity and proportion of TIPE2-positive stained cells were significantly reduced in cancer samples compared to the high levels of TIPE2 expression in samples of adjacent paraneoplastic tissues (Figs. 1A and B).

Next, the expression of TIPE2 in these gastric tissues was verified at the mRNA level. RNA was extracted from both gastric cancer and adjacent paraneoplastic control tissue, reverse-transcribed, and amplified by PCR using the TIPE2 primer pair. The PCR products were separated by agarose gel electrophoresis and visualized to estimate the initial levels of TIPE2 mRNA. The results demonstrated that after 35 cycles of PCR amplification, the levels of TIPE2 mRNA in gastric cancer tissues were much lower (Supplemental Fig. 2A, undetectable) compared to levels in adjacent paraneoplastic control tissues. This result was also verified by quantitative real-time PCR results (Supplemental Fig. 2B, the TIPE2 cDNA ratio of adjacent paraneoplastic control tissues to tumor was 34.86, $P = 0.0047$). To test the accuracy of the PCR amplification, the gel electrophoresis DNA bands for the PCR products were collected, extracted, and sequenced. The sequencing results were then aligned with data in the Basic Local Alignment Search Tool (BLAST) to identify regions of similarity between the amplified sequences and sequences contained in the NCBI database. The results revealed that the amplified products were a 99% match for the Homo sapiens tumor necrosis factor alpha-induced protein 8-like 2 (TNFAIP8L2) mRNA and genome sequence (Supplemental Fig. 3). The combined results of our studies proved that TIPE2 expression was truly reduced in gastric cancer tissue, implying that TIPE2 may participate in preventing the occurrence and developmental of gastric cancer.

EFFECTS OF TIPE2 RESTORATION ON CELL PROLIFERATION AND MIGRATION

To simulate TIPE2 expression in gastric cell lines and investigate the function of TIPE2 in carcinogenesis, we transfected gastric epithelial cells lines AGS and BGC-823 with TIPE2 expression plasmid pRK5-tipe2. Our previous work had shown that the basal expression of TIPE2 in these two gastric cancer cell lines was low, and that TIPE2 expression could be restored 72 h after transfection with pRK5-tipe2 (Supplemental Fig. 4).

To assess the effects of TIPE2 expression on cell proliferation and migration in vitro, AGS and BGC-823 cells were examined for changes in growth and migration following transfection with pRK5-tipe2. As shown in Figure 2A and Supplemental Figure 5, cells expressing TIPE2 showed significantly decreased colony-forming capability when compared to control cells transfected with a mock plasmid. The colony forming ability of AGS cells transfected with pRK5-tipe2 plasmid was reduced by ~60% compared to control cells. The largest difference in growth rates between pRK5-tipe2 and mock transfected cells was found at day 3, whereas colonies with restored TIPE2 expression showed growth rates similar to those of cells derived from the parental cell lines. Furthermore, colony-forming assays conducted using AGS cells transfected with different amounts of pRK5-tipe2 showed that TIPE2 inhibited gastric epithelial cell growth in a dose-dependent manner (Fig. 2B), which is in line with the pattern of TIPE2 expression in this cell line.

We next examined the directional migration of AGS cells with the wound-healing assay. The "wound" was created in confluent cell cultures (time zero), and migration of cells into the gap was monitored after 72 h. Figures 2C and D show AGS cells growing into the wound. However, after 72 h, the wound was still visible, and the speed at which the TIPE2 over-expressing cells migrated into the wound was almost same as that of the control cells. These data showed that restoration of TIPE2 expression significantly suppressed proliferation of gastric cells, but had minimal effect on cell migration.

TIPE2 EXPRESSION SUPPRESSED GASTRIC EPITHELIAL CELLS ENTERING S PHASE OF THE CELL CYCLE

We next studied AGS cells by flow cytometry to test whether cell apoptosis and the cell cycle were modulated concomitant with TIPE2

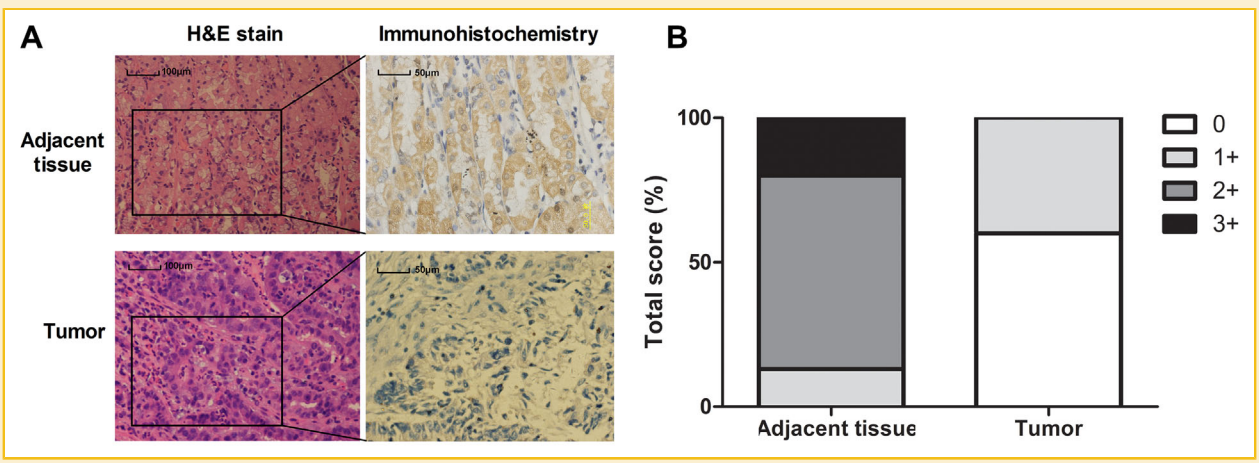

Fig. 1. Expression levels of TIPE2 in gastric cancer cells. (A) H&E staining and immunohistochemistry of the gastric cancers and adjacent tissues; (B) All slides were scored based on both staining intensity and the percentage of positive cells. The immunohistochemical staining of TIPE2-positive cells is shown in brown. Original magnification: H&E stain, ×200; Immunohistochemistry, ×400. Rabbit anti-TIPE2 polyclonal antibody ((Boster Co. Ltd, Wuhan, China; dilution 1:50) was used for immunohistochemistry. TIPE2 signal was scored as described in the Materials and Methods.

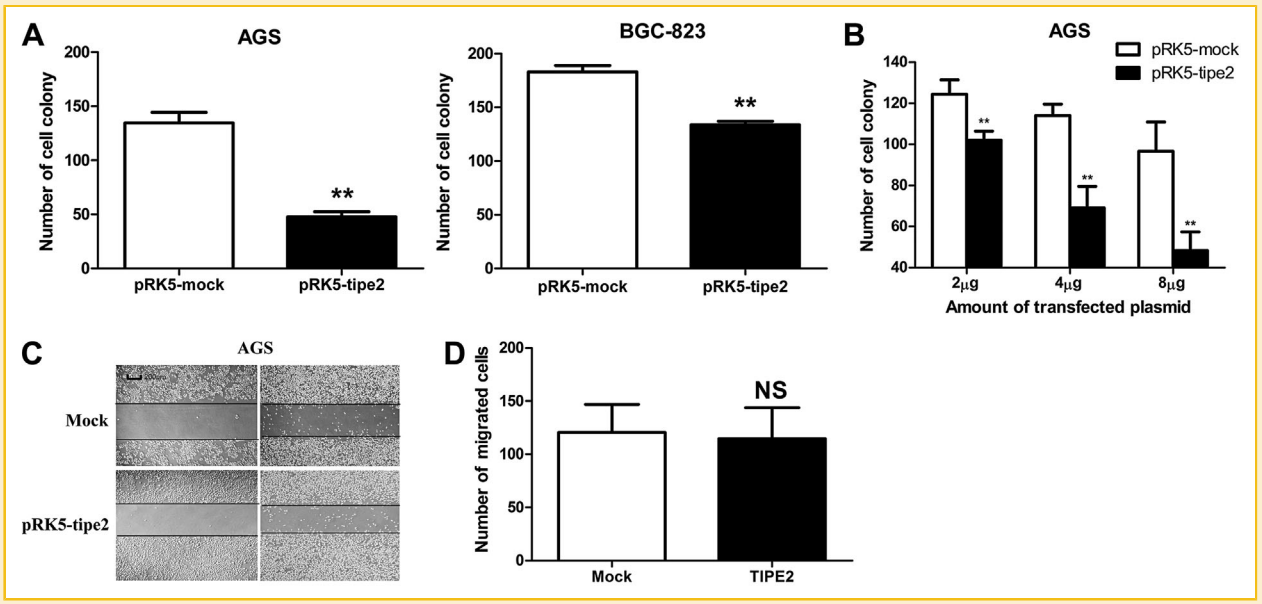

Fig. 2. TIPE2 expression significantly suppressed proliferation of AGS and BGC-823 cells, but had minimal effect on cell migration. (A) Cells expressing TIPE2 demonstrated significantly decreased colony-forming capability in comparison to controls, $P < 0.01$; (B) TIPE2 inhibited gastric epithelial cell growth in a dose-dependent manner, $P < 0.01$; (C) TIPE2 expression in AGS cells had a minimal effect on cell migration; (D) Quantification of wound healing assay results. Colony formation and wound healing assays were performed as described in Materials and Methods.

administration. Following transfection with the pRK5-tipe2 plasmid, AGS cells were collected and analyzed at time points of 24, 48, and 72 h. Results showed low percentages of apoptotic cells at all three time points (Q2 + Q4 <4%) both before and after transfection (data at 72 h is shown in Fig. 3A and Supplemental Fig. 6), suggesting that cell apoptosis is not the key factor in TIPE2-induced cell growth suppression. However, a cell cycle study showed that the percentage of S phase cells significantly decreased as TIPE2 expression was restored (Fig. 3B and Supplemental Fig. 7). Additionally, the data also showed that TIPE2 triggered S phase decay in a dose- and time-dependent manner (Fig. 3B). At a TIPE2 dose of 4 μg, the number of cells in S phase was reduced by ~30% compared to mock control cells. With regard to time dependence, the difference in the numbers of S phase cells became apparent at 48 and 72 h after transfection, and the difference reached a peak at the 72 h time point. In summary, these combined results indicate that TIPE2 had a negative effect on the cell cycle process, and thereby inhibited DNA synthesis and proliferation of gastric epithelial cells, which is in line with results of the colony forming assays.

N-RAS AND p27 ARE INVOLVED IN TIPE2-ASSOCIATED CELL CYCLE ARREST

To determine which molecular agents are involved in TIPE2-induced cell cycle arrest, cDNA microarray assays were conducted to analyze changes in gene expression during the process of TIPE2 restoration. Among the up-regulated genes, N-ras expression was increased by

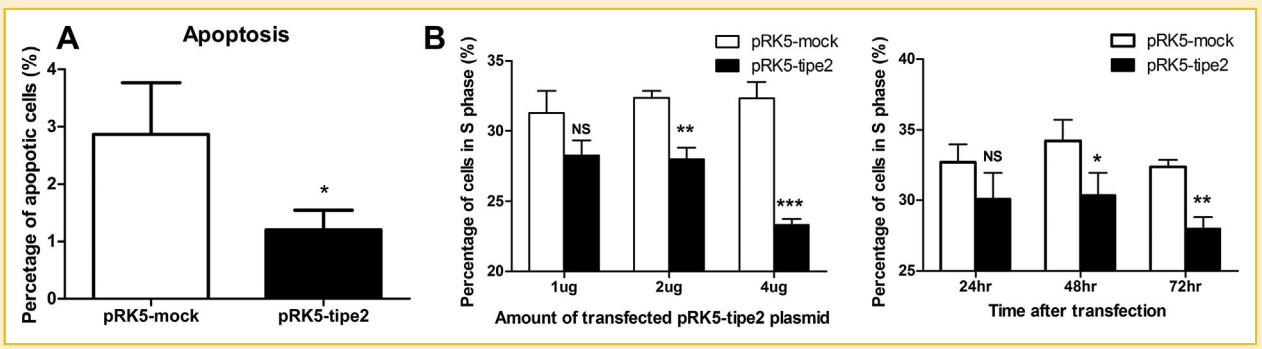

Fig. 3. TIPE2 expression suppressed gastric epithelial cells at the S phase of the cell cycle. (A) Both pRK5-tipe2 transfected and non-transfected cells showed low levels of apoptosis; (B) percentage of cells in S phase decreased significantly as TIPE2 expression was restored, and decay at cellular S phase occurred in a dose- and time-dependent (2 μg pRK5-tipe2 applied) manner. Cell apoptosis and cycle analysis were performed as described in Materials and Methods.

>2-fold, and this increased expression was verified by RT-qPCR (Figs. 4A and B) and Quantification of activated Ras (Supplemental Fig. 8). Nonetheless, the N-Ras mRNA level increased in tumor tissues comparing to adjacent control tissues, implying the existance of other activation pathways in tumor microenvironment with impaired homeostasis (Supplemental Fig. 9 A). Additionally, the expression levels of all cyclin-dependent kinase inhibitor genes were tested using microarray assays and RT-qPCR (Fig. 4A). The microarray results showed that the most up-regulated cyclin-dependent kinase inhibitor gene was p27 (approximately 1.5 fold), others tested showed no significant changes in expression during the process of TIPE2 restoration. RT-qPCR and western blot results showed that TIPE2 restoration up-regulated p27 protein expression in AGS cells (Figs. 5A, B, C, and Supplemental Fig. 10), this variation was verified in tumor tissues (Supplemental Fig. 9B), in line with the association of ras with p27 that had been proven. Moreover, siRNA interference studies showed that the colony forming capacity of AGS cells was restored as the p27 protein was knocked down (Fig. 5D). These results suggest that TIPE2 induced cell cycle arrest through regulation of p27 protein levels.

DISCUSSION

Although the causes of cancer are complex, and only partially understood, clinical studies have found altered expression of several specific biological molecules during development of cancer. Such changes in expression may play crucial roles in a number of processes likely to be important for tumor progression, such as cell proliferation, cell motility, cell adhesion, invasion, cell survival, and angiogenesis [Hartwell and Kastan, 1994; Woodburn, 1999; Coussens et al., 2002; Ferrara, 2002; Ghobrial et al., 2005].

TIPE2 induces cell death and inhibits Ras-induced tumor formation, and thus provides a molecular bridge leading from inflammation to cancer. Several papers support the notion that TIPE2 is an essential negative regulator of inflammation and carcinogenesis [Lou and Liu, 2011]. Li Zhang et al. first reported TIPE2 expression in several types of non-immune cells such as lung, stomach, and liver [Zhang et al., 2011]. Separate studies in patients with systemic lupus erythematosus, chronic hepatitis B, and chronic hepatitis C found significantly reduced levels of TIPE2 expression in peripheral blood mononuclear cells, as compared to expression in healthy individuals [Li et al., 2009; Xi et al., 2011; Kong et al., 2013]. Finally, a group led by Professor Youhai Chen reported that TIPE2 down-regulation was associated with human hepatocellular carcinogenesis [Gus-Brautbar et al., 2011]. However, there have been no previous investigations examining TIPE2 expression and regulatory mechanisms in gastric cancer. In our study, TIPE2 was found to be highly expressed in control samples consisting of paraneoplastic stomach tissues, but expression was down-regulated to almost undetectable levels in gastric cancer samples. We thus speculated that TIPE2 might also play a role in restricting the development of gastric cancer, and could possibly be useful for prevention of gastric cancer.

The cell cycle process is essential for life-sustaining activities, including DNA synthesis and cell division [Vermeulen et al., 2003]. Cell division is divided into stages of mitosis and interphase, which include G1, S, and G2 phases [Norbury and Nurse, 1992]. DNA is replicated in the S phase, which is preceded by a gap (G1), during which the cell prepares for DNA synthesis. This is followed by a gap (G2) during which the cell prepares for mitosis [Vermeulen et al., 2003]. Throughout their long evolution, cells have developed a robust regulatory mechanism to ensure the orderly progression of the cell cycle. In many cases, a failure to arrest proliferation releases cells with highly unstable genomes that may evolve into cancer cells. Therefore, a negative regulatory mechanism may play an important role in preventing tumorigenesis [Hartwell and Kastan et al., 1994].

Cyclin-CDK complexes are regulated by cyclin-dependent kinase inhibitors (CDKIs), which generally inhibit cell cycle progression [Lloyd et al., 1999]. Two families of cdk inhibitors negatively

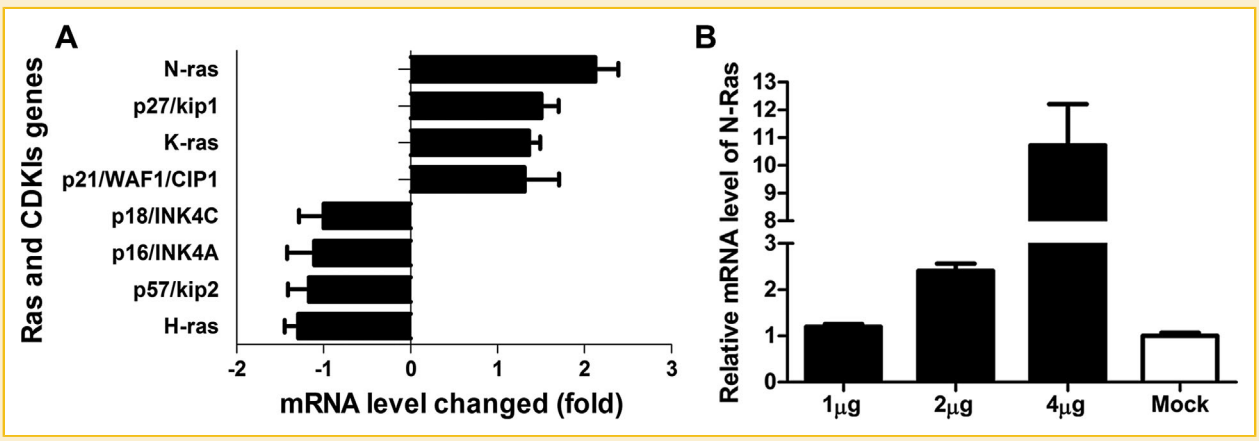

Fig. 4. TIPE2 restoration up-regulated N-Ras expression. (A) Microarray analysis showed that N-ras expression increased >2-fold concurrent with TIPE2 restoration; whearas H-ras gene down-regulated 1.29 folds and K-ras up-regulated only 1.39 folds. Cyclin-dependent kinase inhibitor genes expressed as following: p16/INK4A, −1.11 folds; p18/INK4C, 1.002 folds; p21/WAF1/CIP1, 1.31 folds; p27/kip1, 1.47 folds; p57/kip2, −1.17 folds. (B) This increased expression was verified by RT-qPCR at different concentrations of pRK5-tipe2 transfection. All ras genes and their downstreaming p21, p27 as well as other cyclin-dependent kinase inhibitor genes were tested. Microarray analysis and RT-qPCR were performed as described in the Materials and Methods.

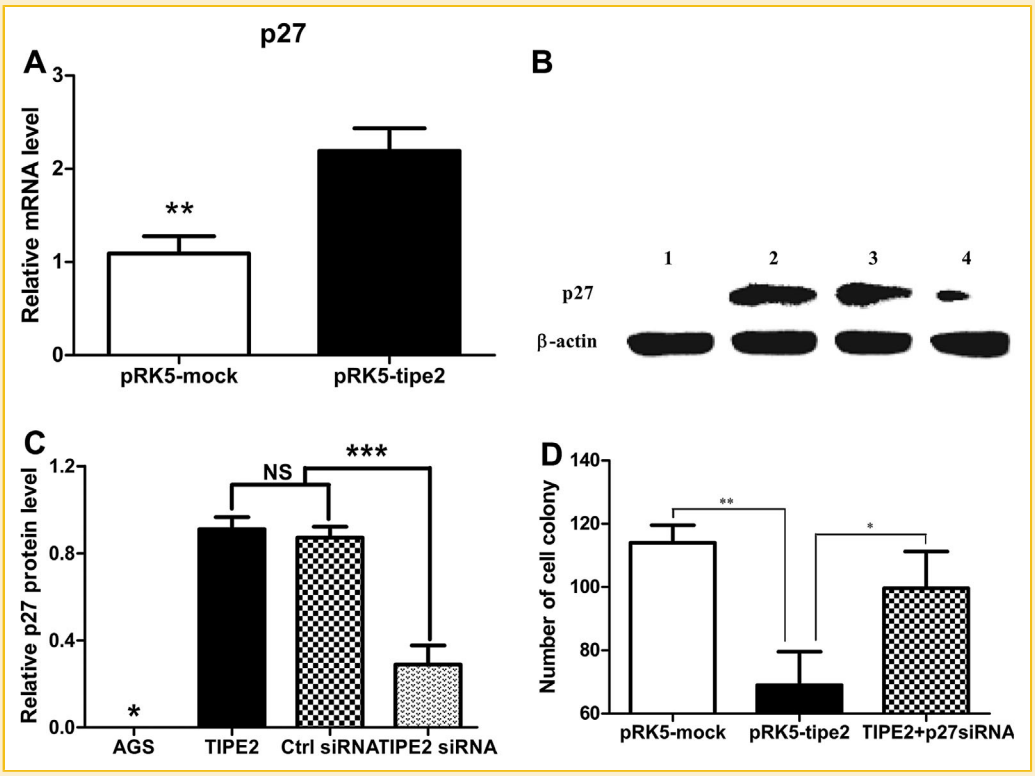

Fig. 5. p27 was involved in TIPE2-associated cell cycle arrest. (A) TIPE2 restoration up-regulated p27 protein expression in AGS cells; (B) siRNA significantly knocked down p27 expression: 1, AGS; 2, AGS with pRK5-tipe2 transfection; 3, AGS with TIPE2 expression interfered by Control siRNA; 4, AGS with TIPE2 expression interfered by p27 siRNA. (C) Quantification p27 knocked down western blot results; (D) Colony forming capacity of AGS cells was restored as p27 protein was knocked down; western blot, colony formation assay, and RT-qPCR were performed as described in Materials and Methods.

regulate the cell cycle. The inhibitor of cdk4 (INK4) family members, such as p16, specifically bind cdk4 and cdk6 to inhibit cyclin D association. Members of the kinase inhibitor protein (KIP) family, including p21 and p27, bind and inhibit cyclin bound cdks. Proteins p21 and p27 are inhibitors of cdk2, which is essential for DNA replication and may also function in the assembly and activation of cdk4 and cdk6 complexes [Hartwell and Kastan, 1994; Zhang et al., 1994; Labaer et al., 1997; Cheng et al., 1999], and over-expression of the kip proteins leads to cell cycle arrest [Xiong et al., 1993].

Binding of p27 to CDK2 triggers large conformational changes in and around the catalytic cleft of CDK2 which inhibit the enzymatic activity of CDK-cyclin complexes, resulting in cellular arrest at the G1 phase [Polyak et al., 1994; Russo et al., 1996]. Inhibition of p27 expression with antisense oligonucleotides prevents cell cycle arrest, indicating that p27 is an essential component in the pathway that controls the cell cycle [Coats et al., 1996]. Accumulation of p27 within cells blocks their entry into S phase and subsequent proliferation. In this study, our results in gastric epithelial cell lines demonstrated that TIPE2 significantly inhibited cell proliferation by inducing a p27-associated signaling cascade that led to restored control of the cell cycle and a reduction in the proportion of cells in S-phase. This cascade of events provided a molecular mechanism by which TIPE2 could regulate proliferation of gastric cells. However, we did not observe a similar effect of TIPE2 on cellular migration. This may have been because the gastric cancer cell line was different from the previously studied WT and TIPE2$^{-/-}$ macrophage cells, regarding the presence of filamentous actins and microtubules.

Previous studies demonstrated that Ras is an upstream molecule of p27; therefore, elucidation of pathways and molecules involved in regulating p27 may provide mechanistic links between oncogene activation and cell cycle deregulation in cancers [Hengst and Reed, 1996; Takuwa et al., 1997]. The association of ras with p27 has also been proven in other studies. For instance, fibroblasts transfected with a dominant negative ras allele showed accumulation of p27 and G1 arrest [Vlach et al., 1996; Aktas et al., 1997; Kawada et al., 1997; Takuwa and Takuwa, 1997; Montagnoli et al., 1999], and co-expression of ras and myc caused a loss of p27 [Ui et al., 1995; Garlich et al., 2008]. These study results imply that ras becomes inhibited as p27 expression is up-regulated, and ras is required for p27 degradation during the transition from G1 to S phase. Professor Youhai Chen found that TIPE2 can act as a suppressor of Ras oncogenes, and thus prevent downstream activation of Ral and AKT [Gus-Brautbar et al., 2012]. Over-expression of TIPE2 can reduce Ras-induced tumor formation, and p27 might be a mediator in this process. In our study, we found that expression of N-ras was significantly increased during TIPE2 restoration, and this is consistent with previous studies which proved Ras was a critical factor in TIPE2-induced carcinogenesis inhibition. Our results also

showed that p27 had a role in this process, by acting to decrease cell proliferation. However, in contrast to previous reports that expression of ras causes a loss of p27, our results demonstrated that TIPE2 triggers N-Ras expression and accumulation of p27. The different result in our study could be explained by Professor Youhai Chen′s model in which TIPE2 suppresses binding of N-ras to downstream molecules, leading to increased N-ras expression via a feedback mechanism. In conclusion, our study showed that the TIPE2 molecule is an inhibitor of gastric cancer cell growth, and suggests that TIPE2 triggers a p27 associated signaling cascade that leads to restored control of the cell cycle and cell division. These findings describe a new molecular mechanism by which TIPE2 regulates gastric cell proliferation.

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