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TEAD1/4 exerts oncogenic role and is negatively regulated by miR-4269 in gastric tumorigenesis

Y Zhou[1,2,3,9], T Huang[1,2,3,4,9], J Zhang[1,2,3], CC Wong[2], B Zhang[5], Y Dong[2], F Wu[1], JHM Tong[1,3], WKK Wu[2,6], ASL Cheng[4,7], J Yu[2,4,8], W Kang[1,2,3,4] and KF To[1,2,3,4]

TEA domain (TEAD) transcription factors are key components of the Hippo–YAP1 signaling pathway, but their functional role and regulatory mechanisms remain unclear. This study aims to comprehensively explore the expression pattern and functional role of TEAD family in gastric carcinogenesis and investigate its regulation by microRNAs (miRNAs). The mRNA and protein expression of TEAD family were examined by quantitative reverse transcription–PCR (qRT–PCR) and western blot. Their functional roles were determined by in vitro and in vivo studies. The clinicopathological association of TEAD4 in gastric cancer (GC) was studied using immunohistochemistry on tissue microarray. The prediction of miRNAs, which potentially target TEAD1/4, was performed by TargetScan and miRDB. The regulation of TEAD1/4 by miRNAs was confirmed by qRT–PCR, western blot and luciferase assays. TEAD1/4 were overexpressed in GC cell lines and primary GC tissues. Knockdown of TEAD1/4 induced a significant anticancer effect in vitro and in vivo. TEAD1 was confirmed to be a direct target of miR-377-3p and miR-4269, while TEAD4 was negatively regulated by miR-1343-3p and miR-4269. Among them, miR-4269 was the most effective inhibitor of TEAD1/4. Ectopic expression of these miRNAs substantiated their tumor-suppressive effects. In primary GC tumors, downregulation of miR-4269 was associated with poor disease-specific survival and showed a negative correlation with TEAD4. TEAD1 and TEAD4 are oncogenic factors, whose aberrant activation are, in part, mediated by the silence of miR-377-3p, miR-1343-3p and miR-4269. For the first time, the nuclear accumulated TEAD4 and downregulated miR-4269 are proposed to serve as novel prognostic biomarkers in GC.

INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer and the third leading cause of cancer-related deaths worldwide.[1] In spite of its declining incidence and mortality within the recent decades, GC is still an emergent public health problem, particularly in Eastern Asia. Multiple risk factors have been reported to contribute to this cancer, such as Helicobacter pylori or Epstein–Barr virus infection, high-salt and low-vegetable diet, smoking, chronic gastritis with intestinal metaplasia.[2] Approximately 95% of GC are adenocarcinomas that are histologically classified as intestinal-, diffuse- or mixed type according to Lauren's characterization.[3] Even though patients who suffer from intestinal GC live longer than those with diffuse type, the overall survival remains poor, as most patients are diagnosed at an advanced stage[4] and there is a lack of effective therapies for them. Thus, there is an urgent need to investigate the molecular mechanisms underlying GC, to offer some clues for clinical instructions and to identify better biomarkers that predict prognosis.

Several dysregulated signaling pathways are involved in GC development, and the Hippo–YAP1 pathway has been shown to have a role in gastric carcinogenesis.[5,6] TEA domain (TEAD) transcription factors, also known as transcriptional enhancer factor, are crucial parts of Hippo–YAP1 signaling. In mammals, there are four members (TEAD1–4) with highly conserved domains. All of them contain a TEA domain for binding with DNA elements and a transactivation domain for interaction with transcription co-activators such as YAP1/TAZ. By binding with co-activators, TEADs function as key mediators in tumorigenesis, including liver cancer,[7–9] ovarian cancer,[10] breast cancer[11] and prostate cancer.[12] Three groups of co-activators have been identified and classified by Pobbati et al.,[13] including YAP1/TAZ, Vgll proteins and p160 family nuclear receptor co-activators.

[1]Department of Anatomical and Cellular Pathology, State Key Laboratory of Oncology in South China, Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong SAR, PR China; [2]Institute of Digestive Disease, Partner State Key Laboratory of Digestive Disease, The Chinese University of Hong Kong, Hong Kong SAR, PR China; [3]Li Ka Shing Institute of Health Science, Sir Y.K. Pao Cancer Center, The Chinese University of Hong Kong, Hong Kong SAR, PR China; [4]Shenzhen Research Institute, The Chinese University of Hong Kong, Shenzhen, PR China; [5]Department of Gastroenterology, The Affiliated Drum Tower Hospital of Nanjing University, Medical School, Nanjing, PR China; [6]Department of Anaesthesia and Intensive Care, The Chinese University of Hong Kong, Hong Kong SAR, PR China; [7]School of Biomedical Sciences, The Chinese University of Hong Kong, Hong Kong SAR, PR China and [8]Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Hong Kong SAR, PR China. Correspondence: Professor W Kang or Professor KF To, Department of Anatomical and Cellular Pathology, State Key Laboratory of Oncology in South China, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR, PR China.
E-mail: weikang@cuhk.edu.hk or kfto@cuhk.edu.hk
[9]These authors contributed equally to this work.
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However, the underlying mechanisms of TEAD transcription factors in gastric tumorigenesis stay poorly understood. Apart from their biological roles, regulation of TEADs by microRNA (miRNA) has not been investigated either. In this study, we aim to comprehensively reveal the expression pattern and functional role of TEAD family and perform an in-depth investigation to elucidate
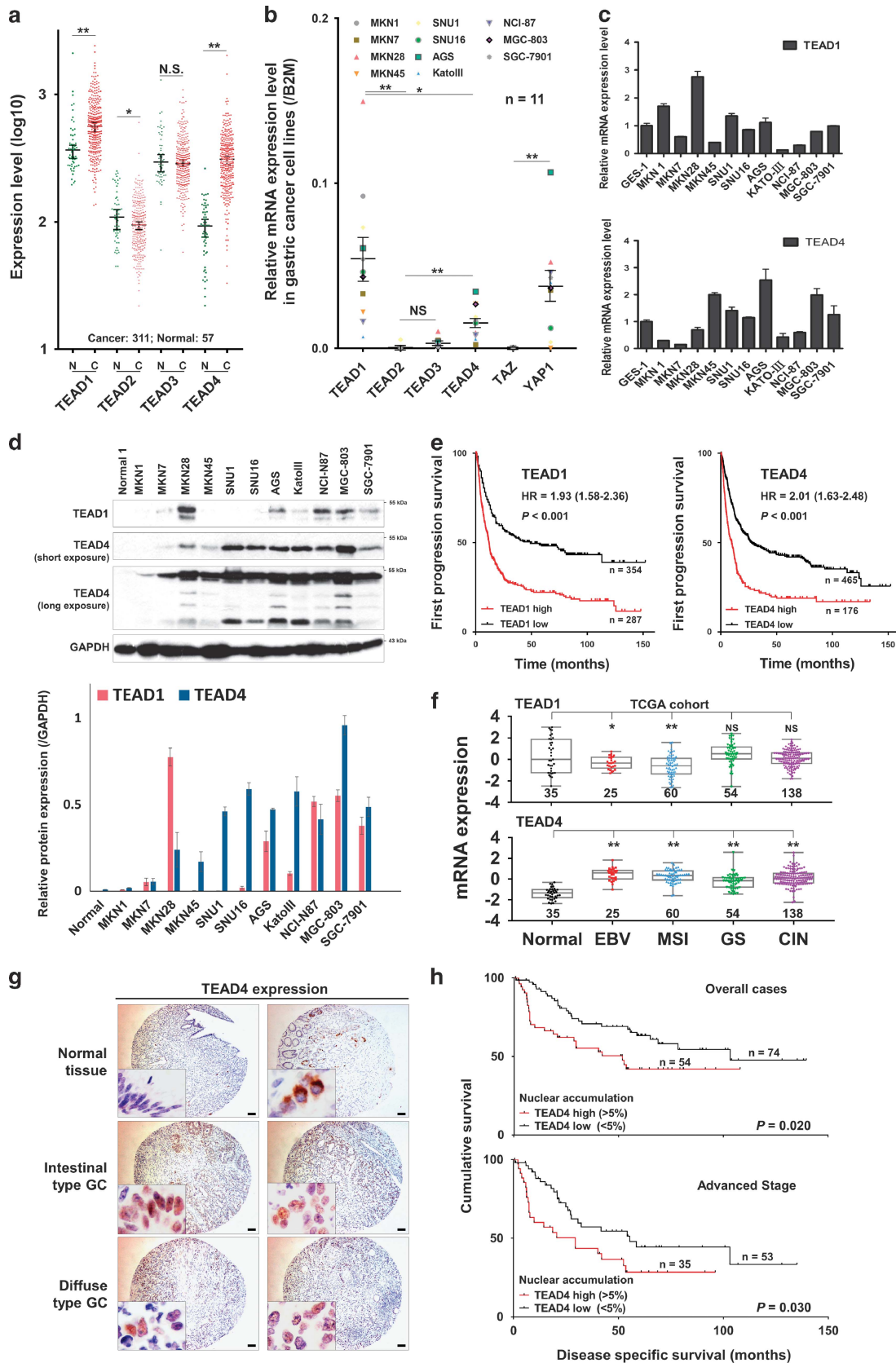

Figure 1. For caption see page 6520.

the miRNA deregulation mechanisms contributing to TEAD activation in GC.

RESULTS

TEAD1 and TEAD4 are overexpressed in GC

Through analyzing the expression microarray data from GENT data set, we found that both TEAD1 and TEAD4 were highly expressed in 311 GC tissues compared with the corresponding normal gastric tissues ($P < 0.001$; Figure 1a).[14] In GC cell lines, the expression levels of TEAD1 and TEAD4 were significantly higher than that of TEAD2 and TEAD3 (Figure 1b). In another published GC data set NCBI/GEO/GSE63089, TEAD1 and TEAD4 demonstrated elevated expression in GC samples compared with adjacent normal tissues ($n = 45$, $P < 0.001$; upper panel of Supplementary Figure S1a).[15] Nonetheless, only TEAD4 exhibited a concordant trend in The Cancer Genome Atlas (TCGA) cohort ($n = 32$, $P < 0.001$; lower panel of Supplementary Figure S1a). Opposite to the expression pattern of TEAD2/3, both mRNA and protein of TEAD1 and TEAD4 were highly expressed in most of the GC cell lines in contrast with immortalized gastric epithelium cell line GES-1 (Figures 1c and d; Supplementary Figure S1b). Overexpression of TEAD1 and TEAD4 was associated with poor progression-free survival in primary GCs according to GSE14210, GSE15459, GSE22377, GSE29272, GSE51105 and GSE62254 ($P < 0.001$; Figure 1e) by the analysis of Kaplan–Meier plotter (www.kmplot.com).[16] Similarly, the abundance of TEAD2/3 predicted unfavorable outcomes as well ($P < 0.001$; Supplementary Figure S1c). However, due to the upregulation in primary GC samples as well as in GC cell lines and concordant results between the expression patterns and survival situations, we only included TEAD1 and TEAD4 for further study. To elucidate the correlation of TEAD1/4 expression with the molecular classification of GC, TCGA cohort was analyzed.[17,18] TEAD1 upregulation was mostly found in genomically stable (GS) subtype, while high TEAD4 expression was strongly associated with Epstein–Barr virus-positive and microsatellite instability subtypes (Figure 1f).

We next performed immunohistochemistry to investigate TEAD4 protein expression in GC tissue microarray. In non-tumorous gastric epithelium, TEAD4 either displayed negative expression or was mainly localized in the cytoplasm of the epithelial cells. However, in both intestinal- and diffuse type of GC, TEAD4 was found to be localized in the nucleus of GC cells, marked by deep brown staining (Figure 1g). Accordingly, when using 5% positive GC cells as the cutoff for low- and high expression (Supplementary Figure S1d), TEAD4 nuclear accumulation was associated with poor disease-specific survival (overall cases, $P = 0.020$; advanced-stage cases, $P = 0.030$; Figure 1h). Supplementary Table S1 summarized the correlation of TEAD4 with other clinicopathologic parameters in 128 GC samples. It turned out that TEAD4 expression was not correlated with any of these parameters. By univariate Cox regression analysis, elder age, diffuse type, high grade, advanced T, N and M stage, lymph node metastasis and high expression of TEAD4 were related to poor outcome, respectively. More importantly, through multivariate analysis, upregulated TEAD4 was found to be still associated with poor prognosis ($P = 0.049$), together with elder age and advanced T, N and M stage. These data suggested that TEAD4 served as an independent prognostic biomarker for GC (Supplementary Table S2).

TEAD1/4 knockdown exerts anti-oncogenic effects *in vitro* and *in vivo*

As TEAD1/4 was upregulated in GC, small interfering RNA-mediated knockdown was used to investigate the functional role of TEAD1/4 in MGC-803 and SGC-7901 cells. TEAD1/4 were markedly decreased after knockdown by specific siRNAs in GC cell lines ($P < 0.001$; Figure 2a), whereas YAP1 expression was not affected by siTEAD1-1/2 or siTEAD4-1/2 treatment. TEAD1/4 knockdown suppressed cell proliferation in a 5-day MTT assay in

Figure 2. Downregulation of TEAD1 or TEAD4 exerts an anti-oncogenic role in GC. (**a**) Protein expression of TEAD1, TEAD4 and YAP1 after the siRNA-mediated knockdown in GC cell lines. (**b**) TEAD1/4 knockdown suppressed cell proliferation in a 5-day MTT assay in MGC-803 and SGC-7901 cells (**$P < 0.001$; unpaired *t*-test). (**c**) Knocking down TEAD1 or TEAD4 reduced monolayer colony formation (**$P < 0.001$; unpaired *t*-test). (**d**) Cell invasion ability was significantly inhibited by siTEAD1-1/2 or siTEAD4-1/2 (**$P < 0.001$; unpaired *t*-test). (**e**) Accumulation of G0/G1 cells was found in siTEAD1-1/2 or siTEAD4-1/2 transfectants compared with the mock control and scramble siRNA controls 24 h after transfection. (**f**) Both siTEAD1-1/2 and siTEAD4-1/2 induced senescence by the β-galactosidase staining in a 3-day transfection assay (**$P < 0.001$; unpaired *t*-test). (**g**) Western blot analysis of related cell cycle regulators and apoptotic markers. Cyclin D1, cyclin D3, CDK6 and p-Rb showed decreased expression, whereas p21 and p27 were uniformly upregulated in TEAD1- and TEAD4-depleted cells. (**h**) Both TEAD1 and TEAD4 knockdown in MGC-803 cells inhibited xenograft formation *in vivo* (**$P < 0.001$; unpaired *t*-test). (**i**) Enrichment plots of gene expression signatures for cell proliferation ($P = 0.002$) and survival ($P < 0.001$) according to TEAD4 mRNA expression levels. The barcode plot indicated the position of the genes in each gene set; red and blue colors represented the high and low expression of TEAD4, respectively. ES, enrichment score; NES, normalized enrichment score. (**j**) Correlation between TEAD1/4 mRNA expression and extents of methylation of promoter CpG sites in TCGA cohort (TEAD1: $n = 330$, $P = 0.003$; TEAD4: $n = 372$, $P < 0.001$; Pearson's correlation).

Figure 1. TEAD1 and TEAD4 are upregulated and predict poor prognosis in GC. (**a**) TEAD1 and TEAD4 displayed high expression in 311 GC samples compared with normal gastric tissues (http://medical-genome.kribb.re.kr/GENT/; *$P < 0.05$; **$P < 0.001$; NS, not significant; unpaired *t*-test). (**b**) TEAD1 and TEAD4 were upregulated in GC cell lines compared with TEAD2/3; meanwhile, expression of YAP1 is significantly higher than that of TAZ (**$P < 0.001$; unpaired *t*-test). (**c**) mRNA expression of TEAD1 and TEAD4 in 11 GC cell lines compared with immortalized gastric epithelium cell line GES-1. (**d**) High protein level of TEAD1 and TEAD4 was detected in most of the GC cell lines (upper panel). And the corresponding quantification by densitometry was shown at nether panel. (**e**) Overexpressed TEAD1 and TEAD4 were associated with poor progression-free survival in primary GCs ($P < 0.001$) from Kaplan–Meier plotter (NCBI/GEO/GSE14210, GSE15459, GSE22377, GSE29272, GSE51105 and GSE62254). (**f**) Distribution of TEAD1 and TEAD4 mRNA expression in four molecular subtypes of GC (*$P < 0.05$; **$P < 0.001$; TCGA cohort; unpaired *t*-test). (**g**) Immunohistochemistry images of TEAD4 in GC tissue microarray (Scale bars are 200 μm). TEAD4 exhibited negative or cytoplasmic expression in normal epithelium cells, but it was localized in the nucleus of the cancer cells. (**h**) High TEAD4 nuclear accumulation was associated with poor disease-specific survival (overall cases, $P = 0.020$; advanced-stage cases, $P = 0.030$; log-rank (Mantel–Cox) test).

MGC-803 and SGC-7901 cells ($P < 0.001$; Figure 2b). The cell growth-inhibitory effect was further confirmed by monolayer colony formation assay ($P < 0.001$; Figure 2c; Supplementary Figure S2a). Moreover, cell invasion ability was significantly inhibited by siTEAD1-1/2 or siTEAD4-1/2 ($P < 0.001$; Figure 2d; Supplementary Figure S2b).
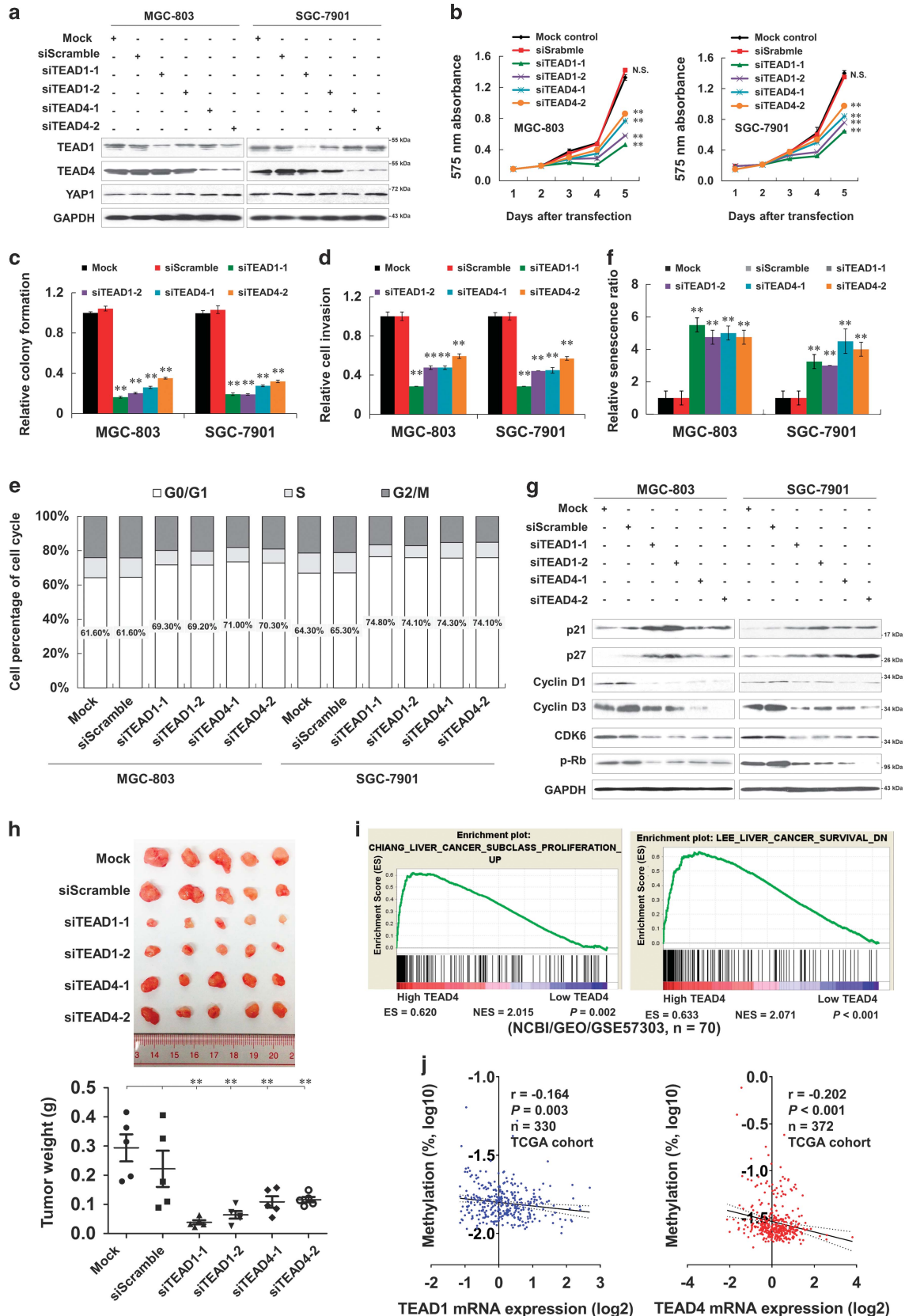
As growth-inhibitory effects were observed in siTEAD1-1/2- or siTEAD4-1/2-transfected cells, we analyzed cell cycle parameters by flow cytometry. Accumulation of G0/G1 cells and a decreased percentage of S phase cells were found in siTEAD1-1/2 or siTEAD4-1/2 transfectants compared with mock control or scramble siRNA controls (Figure 2e). In addition, cell senescence was determined by β-galactosidase staining in a 3-day transfection assay. Both siTEAD1-1/2 and siTEAD4-1/2 significantly induced cell senescence in three GC cell lines (Figure 2f), which was concordant with G0/G1-phase cell cycle arrest. The related cell cycle regulators and apoptosis markers were also examined by western blot (Figure 2g). Cyclin D1, cyclin D3, CDK6 and their downstream effector, p-Rb, demonstrated decreased expression, whereas p21 and p27 were uniformly upregulated in TEAD1- and TEAD4-silenced cells. The oncogenic role of TEAD1 and TEAD4 in gastric tumorigenesis was confirmed in vivo. Both TEAD1 and TEAD4 knockdown markedly inhibited the growth of tumor xenografts in nude mice ($P < 0.001$; Figure 2h). Consistent with the growth-promoting effect of TEAD4 in vitro and in vivo, gene set enrichment analysis[19–23] using a published GC data set NCBI/GEO/GSE57303 (Figure 2i) revealed that the whole set of cell proliferation-related genes were significantly enriched in TEAD4-upregulated cases ($P = 0.002$). In summary, TEAD1/4 is critical for GC development through inducing cell proliferation and preventing cell senescence. Moreover, TEAD4 abundance was negatively associated with longer cancer-related survival ($P < 0.001$).

To further illustrate the mechanism that underlies the overexpression of TEAD1/4 in GC, we analyzed copy number changes, somatic mutation and mRNA upregulation of TEAD1 and TEAD4 in the TCGA cohort. From the TCGA cohort analyzed by cBioPortal, 13% cases (34/258) have at least one alteration in TEAD1 or TEAD4 (Supplementary Figure S1e). Copy number changes of TEAD1 and TEAD4 were significantly correlated with mRNA expression ($P < 0.05$; Supplementary Figure S1f). Given that the proportion of this change only accounted for 13% of the total cases, genomic amplification was merely one of the multiple reasons for high TEAD1/4 mRNA expression in GC. Thereby, we checked the promoter methylation status of TEAD1/4 in GC. A range of 1500 bp in front of the first exon was included, where the promoter region was likely to be located in. In consequence, we found multiple methylated CpG islands (TEAD1: cg09113513 and cg22694703; TEAD4: cg13508391, cg14742305, cg25710178 and cg21637033). Withal the lower methylated level, the higher level of TEAD1 and TEAD4 mRNA was detected (Figures 2j; $P = 0.003$ and $P < 0.001$, respectively, TCGA cohort). Nevertheless, the correlation coefficients were rather low, which implied that apart from genomic and epigenetic regulation, post-transcriptional regulation might act as another mechanism on TEAD1/4 expression.

Regulation of TEAD1 and TEAD4 by miR-377-3p, miR-1343-3p and miR-4269

By TargetScan (www.targetscan.org), TEAD1 3′-untranslated region (UTR) was found to be potentially targeted by miR-873-5p (context score value: − 0.47), miR-377-3p (−0.17), miR-4269 (−0.38), miR-340 (−0.12) and miR-124 (−0.82). Meanwhile, miR-1343-3p (−0.57), miR-4269 (−0.63) and miR-375 (−0.11) might target TEAD4. All the putative binding sites were also predicted by miRDB (http://mirdb.org/miRDB/). In the first-round screening by quantitative reverse transcription–PCR, we found miR-377-3p, miR-1343-3p, miR-4269, miR-375 and miR-124 might regulate the expression of TEAD1 or TEAD4 (Supplementary Figure S3a). As miR-375 and miR-124 were well characterized in GC,[24–27] we chose three novel miRNAs, miR-377-3p, miR-1343-3p and miR-4269, for further investigation. The putative binding sites in 3′-UTR of TEAD1 or TEAD4 for these miRNAs were listed in Figure 3a. In both mRNA (Figure 3b) and protein (Figure 3c) levels, TEAD1 was decreased after overexpression of miR-377-3p or miR-4269 precursors, while ectopic expression of either miR-1343-3p or miR-4269 precursors inhibited the expression of TEAD4.

Next, we performed luciferase reporter assays to investigate the direct binding affinity between these miRNAs with the 3′-UTRs of TEAD1 and TEAD4. As shown in Figure 3d, both miR-377-3p and miR-4269 suppressed the relative luciferase activities of constructs encompassing the binding sites in TEAD1 3′-UTR. Similarly, luciferase activities were inhibited in the constructs containing wild-type binding sites in TEAD4 3′-UTR after ectopic expression of miR-1343-3p or miR-4269 ($P < 0.001$; Figure 3d). These results indicated that miR-377-3p and miR-4269 directly recognized binding sites in TEAD1 3′-UTR, and TEAD4 3′-UTR was directly targeted by miR-1343-3p and miR-4269 in GC cells.

miR-377-3p, miR-1343-3p and miR-4269 are tumor-suppressive miRNAs

To investigate the biological function of miR-377-3p, miR-1343-3p and miR-4269 in GC, their precursors were transfected into MGC-803 and SGC-7901 cells. Overexpression of these three miRNAs impaired cell proliferation in a 4-day MTT assay (Figure 4a). Consistently, miR-377-3p, miR-1343-39 and miR-4269 overexpression reduced colony size and number in monolayer colony formation assay, as compared with scramble miRNA ($P < 0.001$; Figure 4b; Supplementary Figure S3b). Ectopic expression of these three miRNAs also suppressed cell invasive ability of MGC-803 and SGC-7901 cells ($P < 0.001$; Figure 4c; Supplementary Figure S3c). Given that cell growth was inhibited by these miRNAs, cell cycle analysis was also performed. miR-1343-3p and miR-4269 overexpression increased the proportion of G0/G1-phase cells (Figure 4d; Supplementary Figure S3d). To investigate whether these three miRNAs exerts tumor-suppressive effects by inducing apoptosis, we performed fluorescence-activated cell sorting analysis of cells double-stained with Annexin V and 7-AAD, and found miR-377-3p and miR-4269 promoted late apoptosis (Figure 4e; Supplementary Figure S3e). Among these miRNAs, miR-4269 was the only one that was capable of inducing G0/G1 cell cycle arrest and late apoptosis, presenting its crucial role in gastric carcinogenesis. Furthermore, western blot of cell cycle regulators and apoptosis markers showed good concordance with the flow cytometry results (Figure 4f), further confirming these miRNAs' function as tumor suppressors.

To confirm whether the epigenetic modification was responsible for miR-377-3p, miR-1343-3p and miR-4269 downregulation in GC, we examined the expression of the three miRNAs after treating AGS, MKN1, NCI-N87 and MGC-803 cells with 5-Aza-2′-Deoxycytidine (5-Aza), Trichostatin A (TSA) or a combination of both. Expression of miR-377-3p was restored after 5-Aza or TSA treatment. Furthermore, the drug combination of 5-Aza and TSA was more effective for restoring miR-377-3p expression. For miR-1343-3p or miR-4269, the elevated expression was only observed in NCI-N87 or AGS, respectively, after treatment (Supplementary Figure S3f).

miR-4269 is involved in Hippo–YAP1 signaling by targeting TEAD1 and TEAD4

To evaluate whether all three miRNAs were able to regulate Hippo–YAP1 signaling by targeting TEAD1 or TEAD4, the expression of its downstream was examined after ectopic expression of miR-377-3p, miR-1343-3p or miR-4269 in GC cell lines. Interestingly, only miR-4269 effectively inhibited both mRNA and protein expression of connective tissue growth factor (CTGF), Cyr61 and c-Myc ($P < 0.001$; Figures 5a and b). The downstream effectors (CTGF, Cyr61 and c-Myc) of Hippo–YAP1 signaling were meanwhile downregulated by direct siRNA knockdown of TEAD1 or TEAD4, suggesting that miR-4269 regulated the downstream
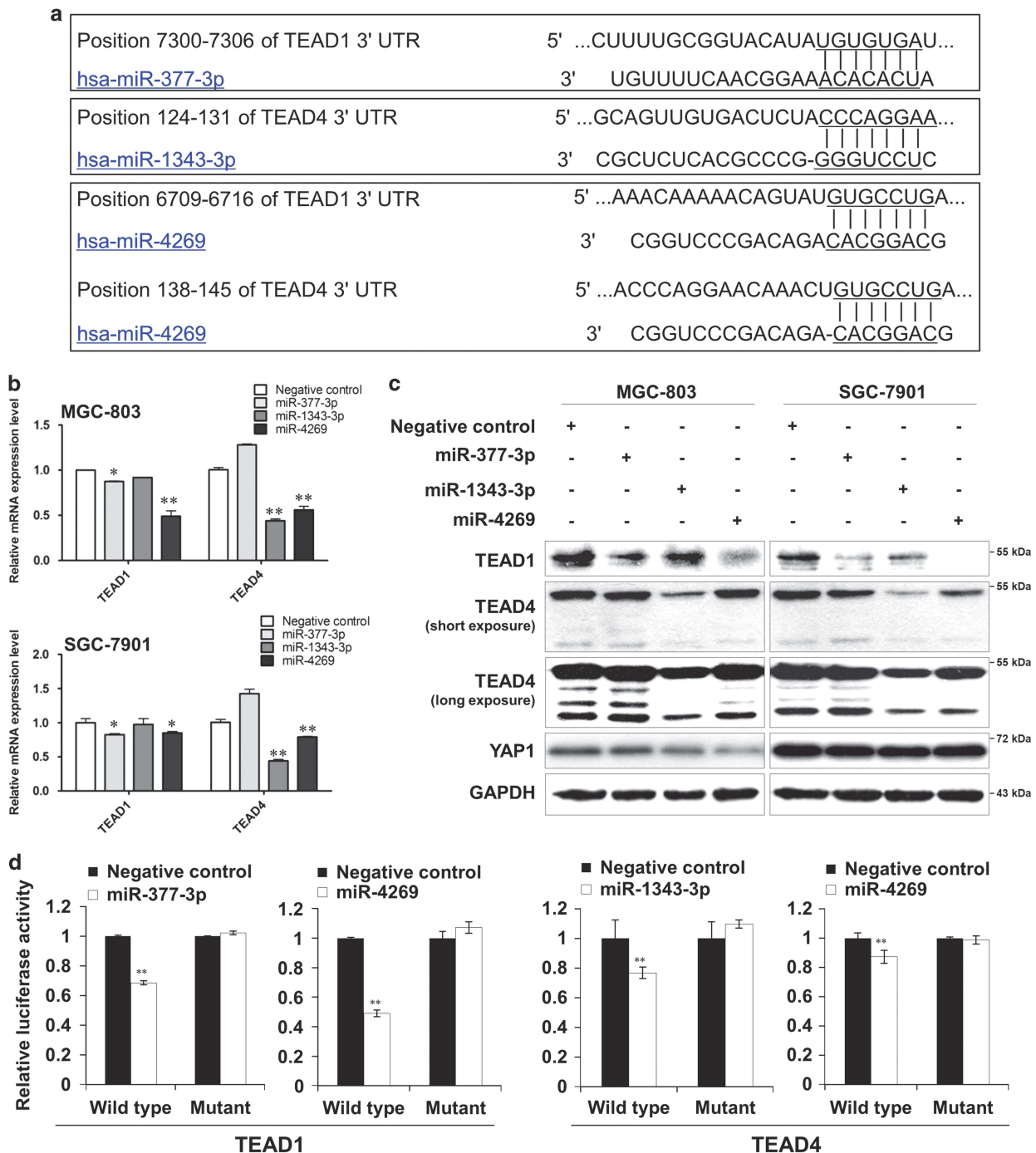
Figure 3. TEAD1 and TEAD4 are direct targets of miR-377-3p, miR-1343-3p and miR-4269. (**a**) Putative binding sites in 3′-UTR of TEAD1 or TEAD4 for the related miRNA binding. (**b**) mRNA expression of TEAD1 and TEAD4 in MGC-803 and SGC-7901 cells after ectopic expression of miR-377-3p, miR-1343-3p and miR-4269 (*P < 0.05; **P < 0.001; unpaired t-test). (**c**) Western blot analysis of TEAD1 and TEAD4 when overexpression of miR-377-3p, miR-1343-3p and miR-4269 in MGC-803 and SGC-7901 cells. (**d**) Both miR-377-3p and miR-4269 suppressed the relative luciferase activity of constructs encompassing the binding sites in TEAD1 3′-UTR. Meanwhile, after ectopic expression of miR-1343-3p or miR-4269, the luciferase activities were also inhibited in the constructs containing the wild-type binding sites in TEAD4 3′-UTR (**P < 0.001; unpaired t-test).

expression through targeting TEAD1 and TEAD4 (Supplementary Figure S4a). Similarly, after TEAD1/4 depletion, miR-4269 was incapable of regulating CTGF expression, which was revealed by luciferase activity assays (Supplementary Figure S4b). Decreased expression of miR-4269 was found in seven out of eleven GC cell lines compared with GES-1 cells (Figure 5c). To confirm whether

TEAD1 and TEAD4 are the functional targets of miR-4269, rescue experiments were conducted in MGC-803 and SGC-7901 cells. Western blot was applied to validate that TEAD1 and TEAD4 were successfully re-expressed in GC cells, respectively (Figure 5d). Re-expression of TEAD1 or TEAD4 partly diminished the tumor-suppressive effect of miR-4269 in MTT proliferation assays

($P < 0.05$; Figure 5e) and monolayer colony formation ($P < 0.001$; Figure 5f). Cell invasive ability ($P < 0.001$; Figure 5g) was also restored in part after re-overexpression of TEAD1 or TEAD4 in miR-4269-treated cells. On the other hand, when TEAD1 and TEAD4 were silenced by siRNAs, miR-4269 failed to exert its growth-inhibitory effect on cell proliferation (Supplementary Figure S4c), indicating that miR-4269 has a tumor-suppressive role mainly through targeting TEAD1 and TEAD4. Furthermore, both siTEAD1-1/siTEAD4-1 and miR-4269 promoted anticancer drug (5-fluorouracil and cisplatin) sensitivity in a dose-dependent manner of GC cells (*$P < 0.05$; **$P < 0.001$; Figure 5h). All these results suggested that TEAD1 and TEAD4 are main functional targets of miR-4269 in GC.

Downregulation of miR-4269 correlates with poor survival and shows negative correlation with TEAD4 in GC

The expression of miR-4269 was measured in 41 paired primary GC and adjacent normal samples. miR-4269 expression in GC displayed descending expression compared with adjacent non-tumorous tissues ($P = 0.006$; Figure 6a). Accordingly, our cohort ($n = 76$) was stratified into two groups (37 high- and 39 low-miR-4269 expression cases) based on the receiver-operating characteristic curve. The low-expression group showed a poor disease-specific survival compared with high-expression group ($P = 0.003$; Figure 6b). In addition, downregulation of miR-4269 was only marginally correlated with advanced stage by clinical correlation analysis ($P = 0.050$; Supplementary Table S3). By
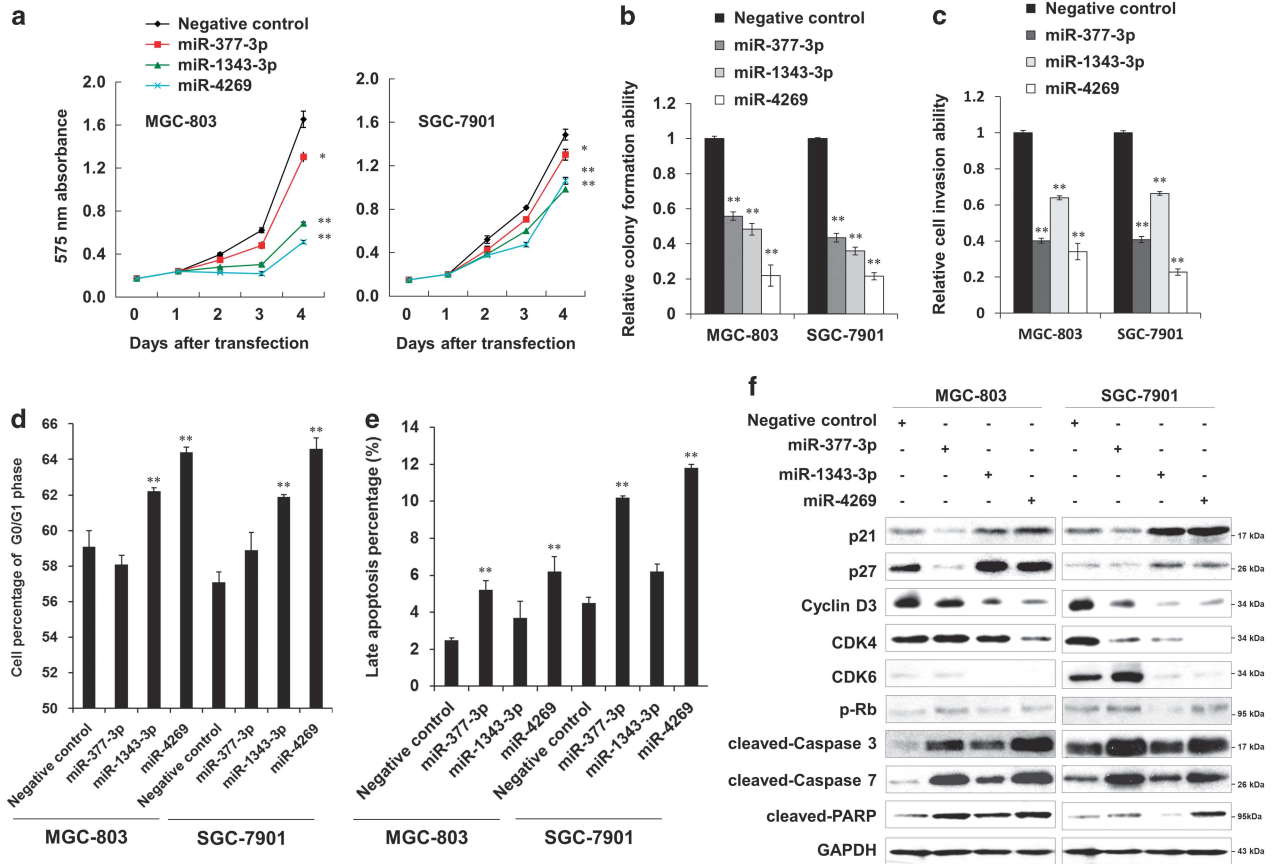

Figure 4. miR-377-3p, miR-1343-3p and miR-4269 are tumor-suppressive miRNAs. (**a**) Overexpression of miR-377-3p, miR-1343-3p and miR-4269 suppressed cell proliferation in a 4-day MTT assay (**$P < 0.05$; **$P < 0.001$; unpaired *t*-test). (**b**) Smaller colony size and reduced colony number were observed in miR-377-3p, miR-1343-3p and miR-4269 transfectants compared with negative control (**$P < 0.001$; unpaired *t*-test). (**c**) Cell invasion abilities were impaired by ectopic miR-377-3p, miR-1343-3p and miR-4269 expression in MGC-803 and SGC-7901 cells (**$P < 0.001$; unpaired *t*-test). (**d**) Cell cycle analysis by flow cytometry revealed that miR-1343-3p and miR-4269 overexpression increased the proportion of G0/G1-phase cells (**$P < 0.001$; unpaired *t*-test). (**e**) miR-377-3p and miR-4269 promoted late apoptosis in GC cells (**$P < 0.001$; unpaired *t*-test). (**f**) Western blot analysis of cell cycle regulators and apoptosis-related markers, validating the flow cytometry results.

Figure 5. Only miR-4269 regulates the downstream expression of Hippo–YAP1 signaling by targeting TEAD1 and TEAD4. (**a**) mRNA expression of CTGF and CYR61 after overexpression of miR-377-3p, miR-1343-3p or miR-4269 in MGC-803 and SGC-7901 cell lines (**$P < 0.001$; unpaired *t*-test). (**b**) Western blot analysis of CTGF, CYR61 and c-Myc upon ectopic expression of miR-4269. (**c**) miR-4269 presented decreased expression in seven out eleven GC cell lines compared with immortalized gastric epithelium cell GES-1. (**d**) Re-expression of both TEAD1 and TEAD4 was confirmed by western blot in the rescue experiments. (**e**) Re-overexpression of TEAD1 or TEAD4 partly diminished the tumor-suppressive effect of miR-4269 revealed by MTT proliferation assays (*$P < 0.05$; unpaired *t*-test). (**f**) Colony formation ability was partly restored in miR-4269-treated GC cells after re-expression of TEAD1 or TEAD4 (**$P < 0.001$; unpaired *t*-test). (**g**) TEAD1 or TEAD4 re-expression also revived cell invasive ability, which was previously impaired by miR-4269 (**$P < 0.001$; unpaired *t*-test). (**h**) Both siTEAD1-1/siTEAD4-1 and miR-4269 enhanced anticancer drug sensitivity of GC cells (*$P < 0.05$; **$P < 0.001$; unpaired *t*-test).

univariate analysis, female patients, diffuse type, advanced TNM stage, lymph node metastasis and low expression of miR-4269 were associated with shorter patient survival. However, by multivariate analysis, the only parameter that mattered in

disease-specific survival appeared to be advanced stage (Supplementary Table S4).

To validate the regulatory effect of miR-4269 on TEAD1 and TEAD4 in primary samples, the expression of TEAD1, TEAD4 and
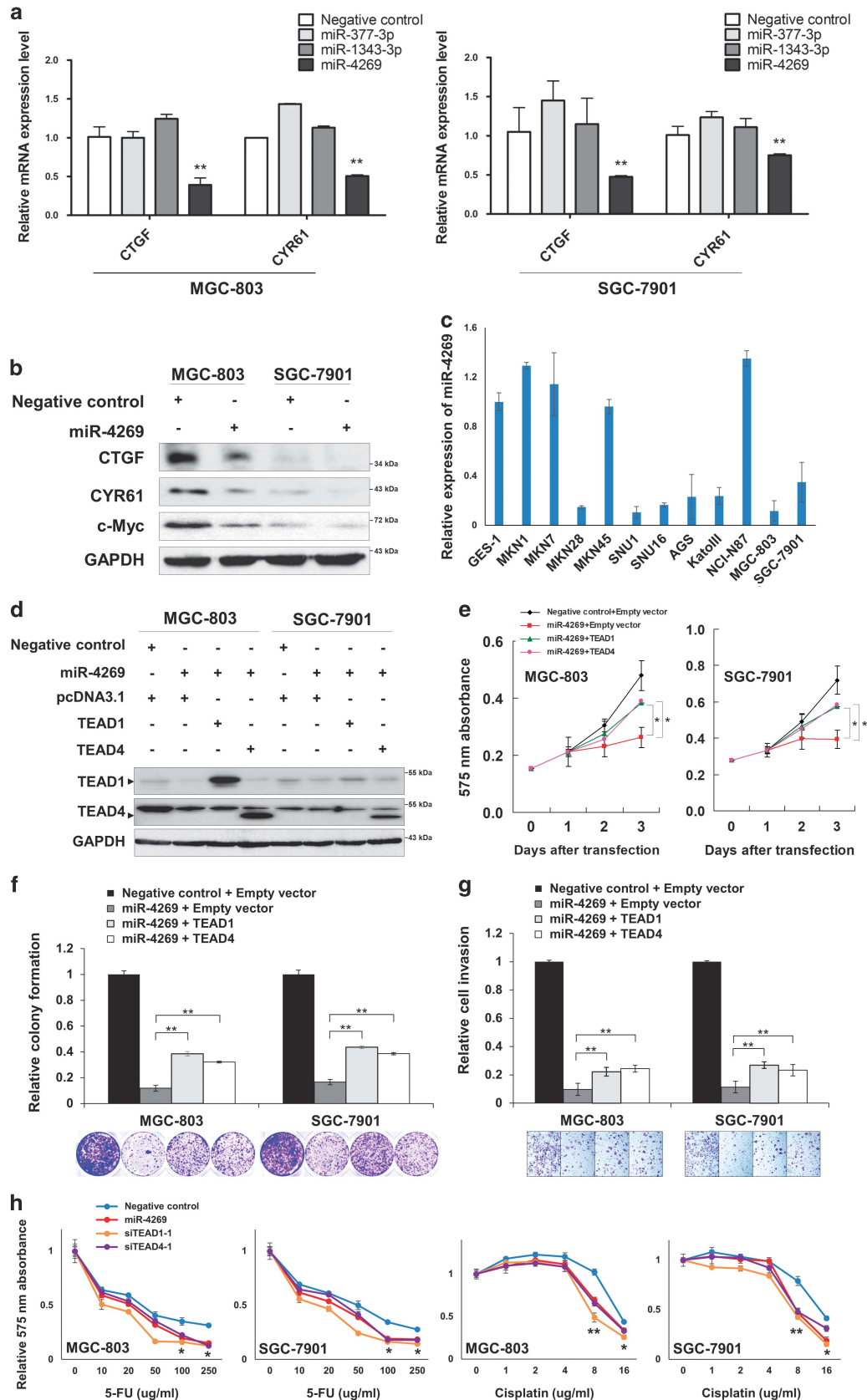
miR-4269 in 28 primary tumors were measured. TEAD4 mRNA ($P = 0.031$), instead of TEAD1 mRNA ($P = 0.535$), was negatively correlated with miR-4269 expression in primary GC (Figure 6c). In GC cell lines, we quantified the protein expression of TEAD1 and TEAD4 by ImageJ densitometry and detected a similar result: expression of miR-4269 showed a negative correlation with TEAD4 ($P = 0.017$), rather than TEAD1 ($P = 0.688$; Figure 6d). Meanwhile, we checked the expression of histone deacetylase 4 (HDAC4), the host gene of miR-4269, and found that HDAC4 exhibited decreased expression in cancer tissues in contrast with adjacent non-tumorous tissues ($n = 32$, $P = 0.045$; Figure 6e) in TCGA cohort. To simply explain this diminution, we again looked into the methylation status of the CpG islands, which were located within 1500 bp before the encoding region of HDAC4. As a result, two sites (cg00360072 and cg02457900) were uncovered to be severely methylated and their extents of hypermethylation were related to the decreased level of HDAC4 ($r = -0.190$, $P < 0.001$, TCGA cohort; Figure 6f). Thereby, as a product of this genomic locus, low-miR-4269 expression in some GC samples might be explained by hypermethylation as we described in Supplementary Figure S3f. Besides, expression of HDAC4 showed a marginally negative correlation with TEAD4 ($r = -0.100$, $n = 415$, $P = 0.050$; Figure 6g). All these findings suggested that miR-4269 downregulation was partly responsible for the aberrant TEAD4 activation in GC cells.

Schematic summary of miRNA regulation on TEAD1/4 in GC

In normal gastric epithelium, TEAD1 and TEAD4 were negatively modulated, which, in a way, was attributed to the normal expression of miR-377-3p, miR-1343-3p and miR-4269. However, the expression of these three miRNAs was downregulated or silenced in GC, thereby they lost the inhibitory effect on TEAD1/4. In addition, upregulated TEAD1/4, in turn, interacted with YAP1. As the central component of Hippo–YAP1 axis, YAP1 was translocated into the nucleus in cancer cells. As transcription factors, TEAD1 and TEAD4 exerted their oncogenic effect by activating the expression of downstream oncogenic factors CTGF, Cyr61 and c-Myc (Figure 7).

DISCUSSION

It has been well identified that TEAD transcription factor family is involved in the development of several types of cancer[28] and is overexpressed in breast cancer,[29,30] fallopian tube carcinoma,[31] germ cell tumor,[32] renal cell carcinoma,[33] medulloblastoma[34] and liver cancer.[35] In this study, TEAD1 and TEAD4, instead of TEAD2 and TEAD3, were found to be abundantly expressed, which means their function is predominant in this family and functional studies demonstrated their oncogenic role during gastric carcinogenesis. Moreover, we unraveled downregulation of multiple tumor-suppressive miRNAs as a novel mechanism by which, partially, TEAD1 and TEAD4 were overexpressed in GC, which in turn activated Hippo–YAP1 signaling pathway. Finally, TEAD4 and its repressor miR-4269 were identified as prognostic markers for GC patients, in keeping with previous reports.[36,37]

TEAD1 and TEAD4 are upregulated in GC according to our cohort and other publically available data sets. Functional investigations explicated that TEAD1 and TEAD4 were essential oncogenic factors in gastric carcinogenesis by promoting cell growth. It has been suggested that the aberrant activation of YAP1–TEAD complex contributes to the pathogenesis of various cancers. This complex drives cell growth and transformation through several target genes, including CTGF, Cyr61[38,39] and c-Myc.[40,41] In GC, Vgll4 was believed to be a suppressor for YAP1 activity through competing with TEAD, which was proved by both in vitro and in vivo experiments.[42] TEAD1 was reported to be involved in a feedback loop of miR-222/Vgll4/TEAD1, in which miR-222 repressed the activity of Vgll4.[36] Hence, TEAD proteins activate Hippo–YAP1 signaling pathway either through direct binding to YAP or indirectly by suppression of Vgll4. All these findings revealed a critical role of TEAD transcription factors and their co-activators in cancer development, and here we demonstrated their impact on GC development. Consistent with our data, a recent study uncovered a tripartite signaling complex involving RUNX3, which acted as a potent inhibitor of YAP1–TEAD-driven GC. They justified that RUNX3 physically interacted with the N-terminal region of TEAD through its Runt domain, which markedly reduced the DNA-binding ability of TEAD and attenuated signals conveyed by TEAD–YAP1 complex to the downstream.[43] Besides, recently a group of Korean scientists proposed TEAD4 as a therapeutic target for clinical intervention during GC treatment. More importantly, based on their 108 Korean cohorts, they attributed TEAD4 upregulation to hypomethylation.[37] The CpG spot proposed in their report, cg21637033, was also included in our study. Our results suggested that hypomethylation is one of the multiple mechanisms for the upregulation of TEAD1 and TEAD4 in gastric carcinogenesis.

Even though both genomic alteration and epigenetic change of TEAD1/4 were found significantly correlated in TCGA cohort, we still sought to improve the integrity of regulatory mechanisms of TEADs. Therefore, from a different angle, we investigated the role of miRNA dysregulation during the activation of TEAD1/4. Three novel tumor-suppressive miRNAs, miR-377-3p, miR-1343-3p and miR-4269, were confirmed to directly regulate the expression of TEAD1 or TEAD4. In particular, miR-4269 targeted both TEAD1 and TEAD4, and regulated the expression of downstream, including CTGF, CYR61 and c-Myc. By rescue experiments, we confirmed that TEAD1 and TEAD4 are functional targets for miR-4269 in GC. All these findings enriched our horizon of TEAD1/4 regulation by miRNAs, and ulteriorly revealed novel mechanisms underlying the aberrant activation Hippo–YAP1 pathway in GC tumorigenesis.

miR-377-3p, miR-1343-3p and miR-4269 are novel tumor-suppressive miRNAs whose functions are largely unknown. There were no reports about the functional role of miR-1343-3p and miR-4269 in tumorigenesis, while only several groups reported the tumor-suppressive role of miR-377-3p in various cancer types. In clear cell renal cell carcinoma, miR-377-3p exerted its tumor-suppressive function by targeting E26 transformation specific-1.[44] In hepatocellular carcinoma, miR-377-3p targeted T-lymphoma invasion and metastasis 1

Figure 6. miR-4269 downregulation correlates with poor survival and is negatively associated with TEAD4 expression in GC. (**a**) miR-4269 showed decreased expression in primary gastric tumors compared with paired adjacent non-tumorous tissues ($n = 41$, $P = 0.006$; paired t-test). (**b**) Downregulation of miR-4269 predicted a shorter disease-specific survival in primary GC samples ($P = 0.003$). (**c**) Expression correlation of TEAD1 ($P = 0.535$; Pearson's correlation) or TEAD4 ($P = 0.031$; Pearson's correlation) mRNA with miR-4269 in 28 primary tumors. (**d**) Expression correlation of miR-4269 with TEAD1 or TEAD4 protein expression in 11 GC cell lines. TEAD4 ($P = 0.017$; Pearson's correlation), instead of TEAD1 ($P = 0.688$; Pearson's correlation), showed negative correlation with miR-4269. (**e**) HDAC4 expression in paired primary GCs ($n = 32$, $P = 0.045$; paired t-test). (**f**) Correlation between HDAC4 mRNA expression and extents of methylation in related CpG sites ($n = 372$, $P < 0.001$; Pearson's correlation). (**g**) Expression correlation between HDAC4 and TEAD4 in TCGA cohort ($n = 415$, $P = 0.05$; Spearman's correlation).

and impaired T-lymphoma invasion and metastasis 1-promoted cell proliferation and invasion.[45] Our findings revealed that TEAD1 is a functional target for miR-377-3p and enriched the target pool for this tumor-suppressive miRNA. On the other hand, a salient anti-GC marker, miR-4269, was attested in our study for the first time. Owing to prominent P-values in both
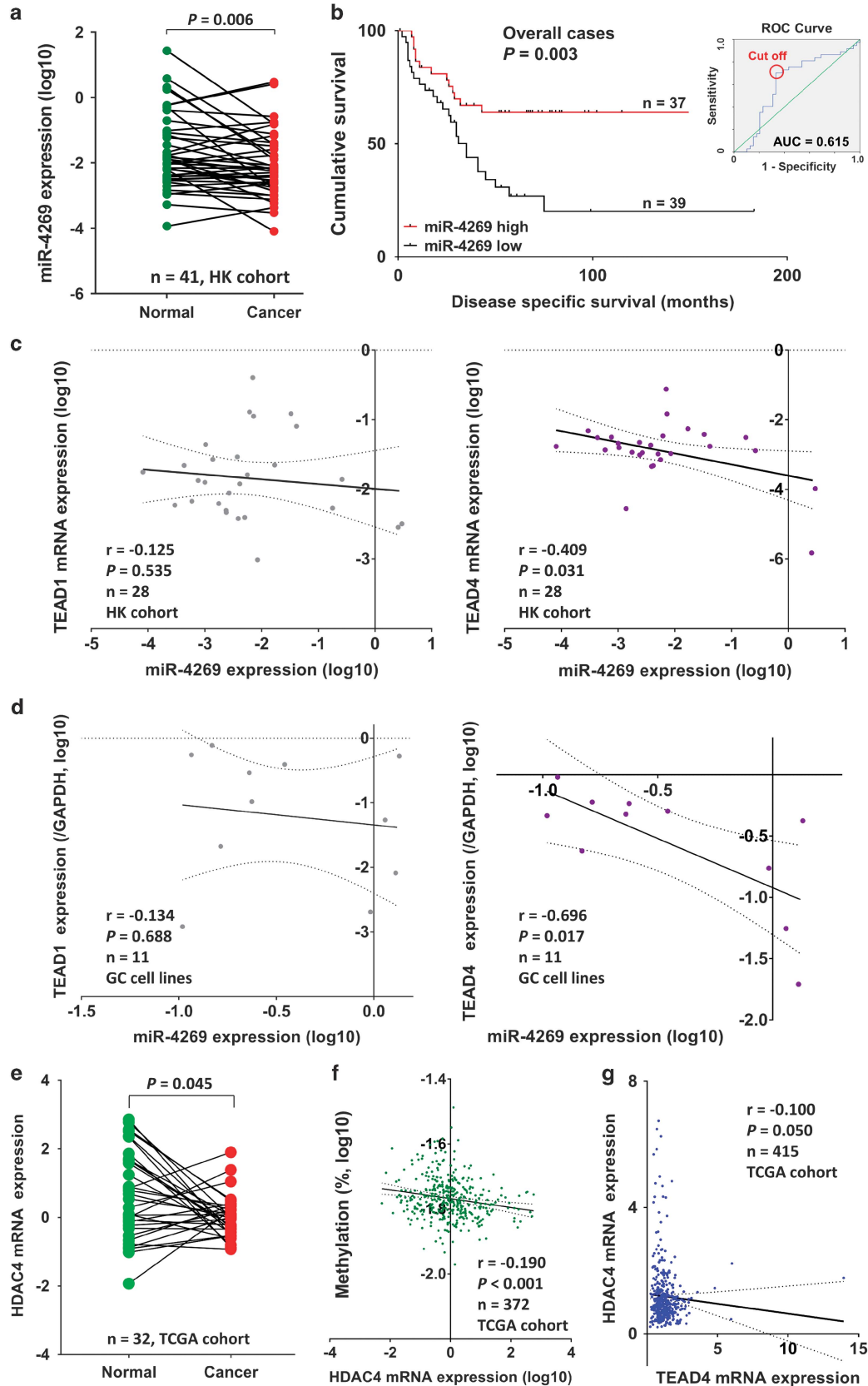
univariate and multivariate analysis, as well as high sensitivity and specificity, this miRNA served as a promising prognostic marker among GC patients.

In conclusion, the increasing knowledge about TEAD function and its regulation by dysregulated miRNAs will not only enhance our understanding of the underlying mechanisms of gastric tumorigenesis but also facilitate identification of novel prognostic biomarkers in the Hippo–YAP1 cascade and develop intervention therapies for GC.

MATERIALS AND METHODS

GC cell lines and clinical samples

Eleven human GC cell lines (AGS, KatoIII, MGC-803, MKN1, MKN7, MKN28, MKN45, NCI-N87, SGC-7901, SNU1 and SNU16) and GES-1, an immortalized gastric epithelial cell line, were cultured as reported.[46] A total of 129 patients diagnosed as GC between 1999 and 2006 at the Prince of Wales Hospital were recruited, whose formalin-fixed paraffin-embedded tissues were applied for this project. Another 76 paired frozen samples were also obtained from the same hospital (diagnosed between 1999 and 2010). The CUHK Clinical Research Ethics Committee approved the usage of human samples and Reference No. is CREC 2016.050.

Treatment of cell lines with 5-Aza and TSA

Cell lines, including AGS, NCI-N87, MGC-803 and MKN1, were treated with a demethylating agent (5-Aza) and histone deacetylases inhibitor.[47] For 5-Aza (Sigma, St Louis, MO, USA) treatment group, the cells were treated with 10 μM 5-Aza for 3 days. For TSA (Sigma) treatment group, 100 nM TSA was added to cells for 24 h. For combination, we treated the cells with 5-Aza for 4 days. In the following 24 h, TSA was added at 100 nM concentration. Control cultures were treated with an equal amount of vehicle dimethylsulfoxide (Sigma).

RNA extraction and quantitative reverse transcription–PCR

The procedures of RNA extraction was reported previously.[48] TRIzol reagent was purchased from Invitrogen, Carlsbad, CA, USA, and High-Capacity cDNA Reverse Transcription Kit was also from Applied Biosystems, Carlsbad, CA, USA. The primers of quantitative reverse transcription–PCR used were listed in Supplementary Table S5. Quantitative reverse transcription–PCR was performed as before.[49] Kits and reagents used were as follows: miR-377-3p (assay ID: #000566, Life Technologies, Carlsbad, CA, USA); miR-1343-3p (#463957); miR-4269 (#242701); and RNU6B (#001093). We repeated the experiment three times to get s.d.'s. Averages were defined as center values and s.d.'s were used for error bars.

Western blot analysis

Western blot analysis was performed in our previous study.[5] Some of the western blot results were quantified by densitometric scans using ImageJ (version 1.48p; W Rasband, National Institutes of Health; available at http://rsb.info.nih.gov/ij/) for the following Pearson's correlation analysis. The primary antibodies of TEAD1 (sc-376113), TEAD4 (sc-134071), CTGF (L-20) (sc-14939) and Cyr-61 (sc-374129) were commercially available from Santa Cruz (Dallas, TX, USA). YAP1 (ab52771) antibody was achieved from Abcam (Cambridge, MA, USA). TEAD2 (SAB2102402) and TEAD3 (AV38278) antibodies were obtained from Sigma-Aldrich (St Louis, MO, USA). Other primary antibodies were from Cell Signaling (Danvers, MA, USA), including p21 (#2946), p27 (#2552), p-Rb (Ser807/811) (#9308), cleaved-caspase 3 (Asp175) (#9661), cleaved-caspase 7 (Asp198) (#9491), cleaved-PARP (Asp214) (#9541), cyclin D1 (#2978), cyclin D3 (#2936), CDK4 (#12790), CDK6 (#3136), c-Myc (#9402) and GAPDH (#2118). Anti-Mouse IgG-HRP (Dako, Glostrup, Denmark, 00049039, 1:30 000) and anti-Rabbit IgG-HRP (Dako, 00028856, 1:10 000) were used for secondary antibodies.

Immunohistochemistry

Immunohistochemistry was also performed as in our earlier paper.[48] TEAD4 primary antibody (1:25, HPA056896) was from Protein Atlas Antibodies (Voltavägen, Bromma, Sweden). The nuclear accumulation of TEAD4 was assessed according to the ratio of GC cells with positive nuclear staining (low expression, ⩽5%; high expression, >5%).

miRNA and siRNA transfection for functional assays

miRNA precursors, including miR-377-3p (PM10524, Life Technologies), miR-1343-3p (PM20896), miR-4269 (PM16968), miR-873-5p (PM12405), miR-340 (PM12670), miR-124 (PM10691), miR-375 (PM10327) and scramble control (AM17110), were from Life Technologies. siRNAs, such as siTEAD1-1
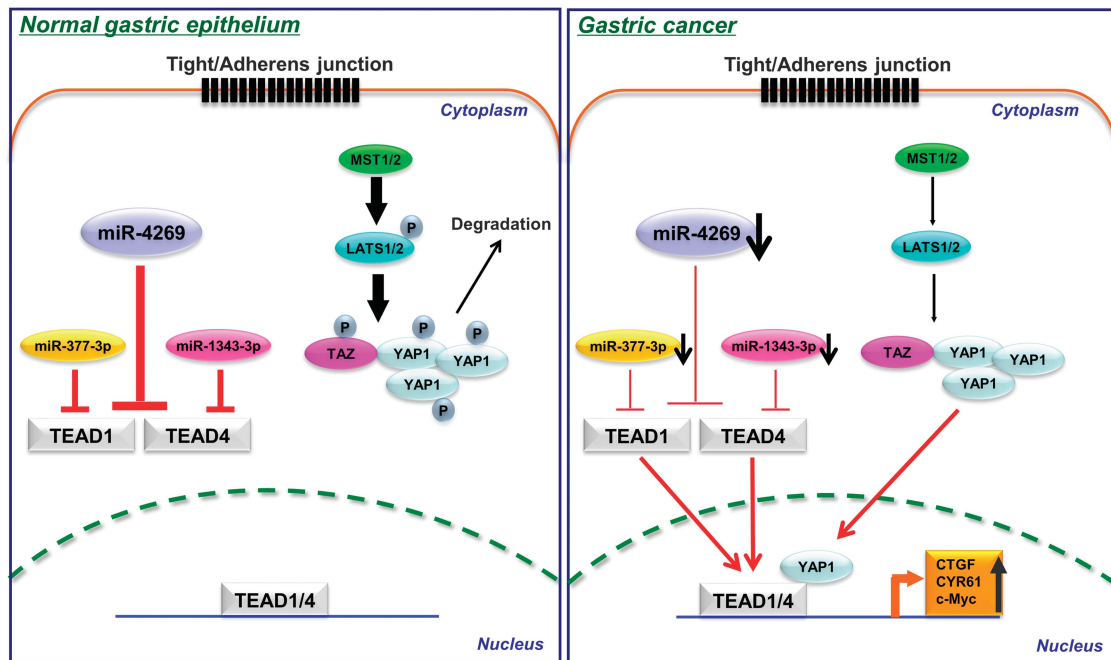

Figure 7. Schematic presentation of TEAD1/4 regulation by miR-377-3p, miR-1343-3p and miR-4269 in GC. TEAD1/4 showed low expression in normal gastric epithelium cells, which was partially inhibited by miR-377-3p, miR-1343-3p and miR-4269. However, the related miRNA showed decreased expression in GC, thus they loss the inhibition on TEAD1 and TEAD4. Via MST–YAP1 axis, YAP1–TEAD1/4 complex was translocated into the nucleus to promote gastric tumorigenesis by regulating the downstream expression.

(SI04181261), siTEAD1-2 (SI04237205), siTEAD4-1 (SI04131127) and siTEAD4-2 (SI04136069), were purchased from Qiagen (Valencia, CA, USA). We used Lipofectamine 2000 (Invitrogen) to perform all transfection assays. Cell functional tests were performed as before.[5] Flow cytometry was applied to perform cell cycle and apoptosis assays.[50]

In senescence experiments, MKN28, MGC-803 and SGC-7901 cells were transfected with siTEAD1, siTEAD4 or siScramble for 3 days. Cells were then stained with β-galactosidase (Kit, #9860, Cell Signaling) for 8 h and the senescence-positive cell populations were shown in pale green.[6] For rescue experiments, we transfected miR-4269 precursors or negative control in GC cells. Twenty-four hours after transfection, TEAD1- or TEAD4-expressing plasmid (#33109 and #24638, Addgene, Cambridge, MA, USA) or empty vector negative control (pcDNA3.1, Life Technologies) were then transfected with FuGENE HD (Roche, Nutley, NJ, USA). We collected cells after 24 h for western blot analysis and MTT assay.

Luciferase activity assays

The putative miR-377-3p- and miR-4269-binding sites in 3′-UTR of TEAD1, as well as the miR-1343-3p- and miR-4269-binding sites in TEAD4 3′-UTR were separately sub-cloned into pMIR-REPORT vector (Ambion, Austin, TX, USA). The sense and anti-sense of the oligonucleotides were listed in Supplementary Table S6. All the experimental procedures were reported previously.[48]

In vivo tumorigenicity model

The protocol of *in vivo* tumorigenicity model was described before.[48] Tumor weights were measured on day 25. All animal experimental procedures were approved by Department of Health, Hong Kong and CUHK Animal Ethics Committee. The Reference No. is 15-745 in DH/HA&P/ 8/2/1 Pt.53.

Statistical analysis

Some of the values are adjusted (log transformation) to be approximately normally distributed to meet the requirement of parametric tests. Corresponding statistical methods for each comparison and correlation was as previous.[48] We performed all the statistical analysis via SPSS software (version 22.0; SPSS Inc., Chicago, IL, USA; two-tailed, $P < 0.05$, statistically significant; two-tailed, $P < 0.001$, highly statistically significant).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

KFT and WK designed the experiments, offered direction and help on the whole project. YZ, TH, JZ, CCW, YD, FW and BZ conducted the experiments, analyzed the results and performed bioinformatics analysis. YZ, TH and WK drafted the manuscript. WKKW, ASLC and JY reviewed the manuscript and made significant revisions on the drafts. All authors read and approved the final manuscript.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)