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Functional screening for miRNAs targeting Smad4 identified miR-199a as a negative regulator of TGF-β signalling pathway

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ABSTRACT

The transforming growth factor-β (TGF-β) signalling pathway participates in various biological processes. Dysregulation of Smad4, a central cellular transducer of TGF-β signalling, is implicated in a wide range of human diseases and developmental disorders. However, the mechanisms underlying Smad4 dysregulation are not fully understood. Using a functional screening approach based on luciferase reporter assays, we identified 39 microRNAs (miRNAs) as potential regulators of *Smad4* from an expression library of 388 human miRNAs. The screening was supported by bioinformatic analysis, as 24 of 39 identified miRNAs were also predicted to target *Smad4*. MiR-199a, one of the identified miRNAs, was inversely correlated with Smad4 expression in various human cancer cell lines and gastric cancer tissues, and repressed Smad4 expression and blocked canonical TGF-β transcriptional responses in cell lines. These effects were dependent on the presence of a conserved, but not perfect seed paired, miR-199a-binding site in the *Smad4* 3′-untranslated region (UTR). Overexpression of miR-199a significantly inhibited the ability of TGF-β to induce gastric cancer cell growth arrest and apoptosis *in vitro*, and promoted anchorage-independent growth in soft agar, suggesting that miR-199a plays an oncogenic role in human gastric tumourigenesis. In conclusion, our functional screening uncovers multiple miRNAs that regulate the cellular responsiveness to TGF-β signalling and reveals important roles of miR-199a in gastric cancer by directly targeting Smad4.

INTRODUCTION

The transforming growth factor-β (TGF-β) signalling pathway plays pivotal roles in a variety of biological processes. The ligand-activated TGF-β receptors phosphorylate Smads, allowing these cytoplasmic transducers to form an active nuclear transcriptional complex upon association with Smad4 (1). Smad4 is a central transducer of the TGF-β pathway, and it plays complex and contradictory roles during tumourigenesis (2). The tumour-suppressive effects of TGF-β signalling are largely due to its ability to inhibit cell proliferation and trigger apoptosis. Accumulating data suggest that dysregulation of Smad4 is closely associated with a variety of human cancers. *Smad4* mutations have been found in juvenile polyposis (3), colorectal cancer with distant metastasis (4), pancreatic cancer and other types of cancer (5–7). In addition, aberrant Smad4 expression is also observed in various tumour types (8–10). Targeted inactivation of Smad4 in mouse models has demonstrated the physiological functions of Smad4 in the suppression of tumourigenesis and metastasis (11–20).

As a common cellular mediator, the abundance and activity of Smad4 must be strictly controlled to ensure the proper cellular response to TGF-β signals. Smad4 activity and stability are regulated by post-translational modifications such as sumoylation (21,22), ubiquitination (23,24) and deubiquitination (25); however, the exact

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mechanisms of *Smad4* post-transcriptional control remain elusive.

MicroRNAs (miRNAs) are endogenous ~22 nt single-stranded RNAs, which play important gene-regulatory roles by pairing and post-transcriptionally regulating the expression of their target mRNAs (26). Increasing evidence suggests that miRNAs are implicated in the cellular response to TGF-β signalling in a variety of different contexts. MiRNAs have been found to target the TGF-β superfamily receptors (27–29), Smads (30–35) and multiple components of the TGF-β signalling pathway (36–39). Conversely, we and others have found that miRNAs regulated by TGF-β signals also affect TGF-β-regulated physiological or pathological processes (40–44). However, no systemic identification of the miRNAs that target the TGF-β signalling pathway or modulate TGF-β responses has been reported.

In this study, we performed a functional screening for miRNAs that regulate *Smad4*, a central transducer of both the TGF-β and bone morphogenetic protein (BMP) signalling pathways. Using *Smad4* 3′-UTR and *Smad4*-dependent CAGA-Lux luciferase reporter assays, we identified 39 miRNAs that potentially regulate *Smad4* expression from an expression library containing 388 human miRNAs. Among the identified miRNAs, miR-199a expression was inversely correlated with Smad4 levels in human cancer cell lines and gastric cancer tissues. We therefore investigated the effects of miR-199a on the modulation of TGF-β signalling and its contribution to human gastric cancer.

MATERIALS AND METHODS

Vector construction

The CAGA-Lux and BMP response elements (BRE-Lux) reporter plasmids were the kind gifts of Ye-Guang Chen (Tsinghua University, Beijing, China). For the expression of miRNAs, 300–600-bp genomic fragments of human miRNA precursors were amplified by PCR and subcloned into pIRES2-EGFP (Clontech, Palo Alto, CA, USA) or pCDNA3.1 (Invitrogen, Carlsbad, CA, USA). To stably inhibit miR-199a function, the synthetic adaptors 5′-GAT CTGAACAGGTAG TCTGAACACTGGGGTACCTG CAGAACAGGTAGTCTGAACACTGGG-3′ and 5′-TC GACCCAGTGTTCAGACTACCTGTTCTGCAGGTA CCCCAGTGTTCAGACTACCTGTTCA-3′, which contain two perfect complementary sequences to mature miR-199a and a 9-bp interval sequence, were inserted into pSuperior.retro.puro, and then the H1 promoter and a tandem anti-miR-199a repeat sequence was subcloned into pIRES2-EGFP. The fragments containing the Cyto MegaloVirus (CMV) promoter and miR-199a precursor or anti-199a were then subcloned into the pAD-Track-CMV adenoviral vector to generate infectious adenovirus.

The *Smad4* 3′-UTR was cloned into the pGL3-CM as previously described (43) between the *Bgl* II and *Mlu* I sites. Overlapping PCR was performed to mutate the miR-199a target site in the *Smad4* 3′-UTR, using two additional primers, and the products were subcloned into pGL3-CM.

Luciferase reporter assay

The reporter plasmids were co-transfected using Lipofectamine 2000 reagent (Invitrogen) with the miRNA or anti-miRNA expression plasmids and the vector phRG-TK (Promega, Madison, WI, USA), which expresses synthetic Renilla luciferase to normalize the transfection efficiency. Luciferase activities were measured using the Dual-Luciferase Reporter Assay reagent (Promega) on a LB 960 Centro XS3 luminometer (Berthold Technologies, GmbH & Co. KG, Bad Wildbad, Germany). For the TGF-β response assay, the cells were stimulated with 5 ng/ml TGF-β1 or 25 ng/ml BMP4 for 12 h before the luciferase assay. Each experiment was performed in triplicate, and the data represent the mean ± SD of three independent experiments.

Bioinformatic analysis

Smad4-targeting miRNAs were predicted and downloaded from the respective databases, and then compared with the list of miRNAs predicted by the functional screening.

Pathway enrichment analysis of the individual miRNA-target genes was performed in DIANA (http://diana.cslab.ece.ntua.gr/pathways/) using the TargetScan 5 method at a significance level of $P < 0.05$. Disease pathways were dismissed from further analysis to focus on signalling and metabolism pathways. MiRNA-pathway relationships were analysed, and the degrees of each node were calculated using Cytoscape software (45).

Cell culture and transfection

SNU-16 (gastric carcinoma, American Type Culture Collection (ATCC) CRL-5974) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum; NIH-3T3 (fibroblast, ATCC CRL-1658) cells were cultured in DMEM supplemented with 10% fetal calf serum; HaCat (keratinocyte, CLS 300493) cells were cultured in Minimum Essential Medium (MEM) supplemented with non-essential amino acids and 10% fetal bovine serum; and HepG2 (hepatocellular carcinoma, HB-8065), MDA-MB-453 (breast cancer, ATCC HTB-131), MDA-MB-231 (breast cancer, ATCC HTB-26), MDA-MB-468 (breast cancer, ATCC HTB-132), MCF-7 (breast cancer, ATCC HTB-22), AGS (gastric carcinoma, ATCC CRL-1739), BGC-823 (gastric carcinoma, ILCS HTL98007), SGC-7901 (gastric carcinoma) (46) and MGC-803 (gastric carcinoma) (46) were cultured in Roswell Park Memorial Institute formulation 1640 (RMPI-1640) supplemented with 10% fetal bovine serum. For transient transfections, the cells were transfected with the aforementioned constructs in 6-cm flasks, and protein lysates and total RNAs were collected at the indicated time points. For stable transfection, AGS and SNU-16 cells were transfected with the miRNA expression construct or corresponding control construct in 6-well plates and selected using G418.

Northern blot

Total RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer's instructions, and RNA

quality was assessed by 1% agarose gel electrophoresis and ethidium bromide staining. Northern blot analysis was performed as described (43) using 20 μg total RNA samples. The miR-199a (MIMAT0000231) probe was synthesized by Invitrogen Biotechnology Co., Ltd (Beijing, China) as follows: 5′-GAACAGGTAGTCTGAACACT GGG-3′ and labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (New England Biolabs, Beijing, China).

Real-time RT-PCR miRNA precursor and mRNA analysis

Reverse transcription was performed using the mRNA-selective PCR kit (DRR025A, TaKaRa, Dalian, China). Briefly, 2 μg of total RNA was reverse transcribed using Avian Myeloblastosis Virus (AMV) reverse transcriptase XL with oligo dT primers or random N6 primers. The real-time PCR primers used are available on request. Real-time PCR was performed using the LightCycler system (Roche Ltd, Basel, Switzerland) with FastStart DNA Master SYBR Green (Fermentas, Beijing, China), and expression was quantified relative to a housekeeping gene β-actin. All experiments were repeated at least three times.

Western blot

Western blot was carried out as previously described (43) using antibodies against Smad4 (ab40759, Abcam, Cambridge, MA, USA), p21 (#2946; Cell Signalling Technology, Danvers, MA, USA), Bim (B-7929; Sigma, St Louis, MO, USA), Plasminogen Activator Inhibitor-1 (PAI-1) (sc-8979; Santa Cruz Biotechnology, CA, USA), Smurf1 (sc-25510; Santa Cruz), JunB (sc-8051; Santa Cruz) and GlycerAldehyde-3-Phosphate DeHydrogenase (GAPDH) (TA-08; Zhong Shan, Beijing, China).

Flow cytometry

For the cell-cycle analysis, $2–3 \times 10^6$ cells were fixed in 70% cold ethanol, treated with RNase A and stained with propidium iodide (Sigma). For the quantification of apoptosis, the cells were washed in cold PBS, incubated with Annexin V-PE and 7-Amino-Actinomycin D (7-AAD) (Southern Biotech, Birmingham, AL, USA) for 15 min in the dark and analysed within 1 h. Flow cytometry was performed using a Fluorescence-Activated Cell Sorting (FACS) Calibur Flow Cytometer with CellQuest 3.0 software (BD Biosciences, San Jose, CA, USA) or winMDI software (V2.9; Purdue University, West Lafayette, USA). All analyses were performed in triplicate, and 20 000 gated events/sample were counted.

Statistical analysis

Student *t* tests were performed to assess the significance of treatments vs. controls. The relationship between the expression of miR-199a and Smad4 in gastric cells and tissues was determined using the Spearman rank correlation. $P < 0.05$ were considered statistically significant.

RESULTS

Identification of 39 miRNAs that target *Smad4* by systematic functional screening

To identify miRNAs that target human *Smad4* systemically, we created the Luc-Smad4 reporter construct (Figure 1a), in which human *Smad4* 3′-UTR was inserted downstream of firefly luciferase gene (43) to screen for miRNAs that downregulate the activity of the luciferase reporter gene. We constructed an expression library containing 388 human miRNAs (Supplementary Table S1), and high level expression of miR-145 and miR-146b was confirmed in transfected NIH-3T3 fibroblast cells by Northern blot (Figure 1b). Each of the miRNA expression vectors was individually co-transfected into NIH-3T3 cells with the Luc-Smad4 reporter construct, and the luciferase activities were assayed 48 h later. Initial screening identified that 83 of the 388 miRNAs were capable of suppressing luciferase expression (Figure 1a and Supplementary Table S1).

The 83 potential *Smad4*-targeting miRNA candidates were then individually co-transfected with the CAGA-Lux reporter, in which luciferase expression is induced by TGF-β1 in a *Smad4*-dependent manner. From this screening, we identified 39 miRNAs that repressed TGF-β1-induced luciferase expression (Figure 1a and c). We examined Smad4 protein expression after transfection of 10 of the randomly selected potential candidate miRNAs using Western blot. Each of the miRNAs repressed Smad4 expression to a different extent (Figure 1d). As readouts of TGF-β1 signalling, we examined the expression of JunB and p21 using Western blot analysis and observed that all of the four miRNAs tested significantly inhibited TGF-β1 signalling in HepG2 cells (Figure 1e). MiR-146b, miR-199a and miR-633 were among the most potent regulators of *Smad4* expression and TGF-β signalling (Figure 1d and e). Taken together, these data suggest that the identified miRNAs potentially target Smad4 and regulate Smad4-mediated signal transduction.

We compared the results of the screening with four prevailing miRNA-target prediction programs, including TargetScan (47), miRanda (48), microcosm (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5) and PITA (26). Of the 39 miRNAs that were identified as direct regulators of *Smad4* in the functional screening, 24 miRNAs were also predicted to target *Smad4* by at least one of the four miRNA-target prediction programs and 12 were predicted by at least two programs, which favours the liability of our screening (Supplementary Figure S1). Consistent with this, pathway enrichment analysis based on predicted miRNA target genes (49) revealed that 19 miRNAs were predicted with a low false discovery rate (FDR) ($P < 0.05$), and another 14 miRNAs with a moderate FDR ($P < 0.1$) to regulate TGF-β signalling pathway (Figure 1f, g and Supplementary Table S1). There are 15 miRNAs that were not predicted to target *Smad4* by any of the four programs, which we interpreted as an advantage of functional screening over bioinformatic predictions due to the unconventional nucleotide paring.

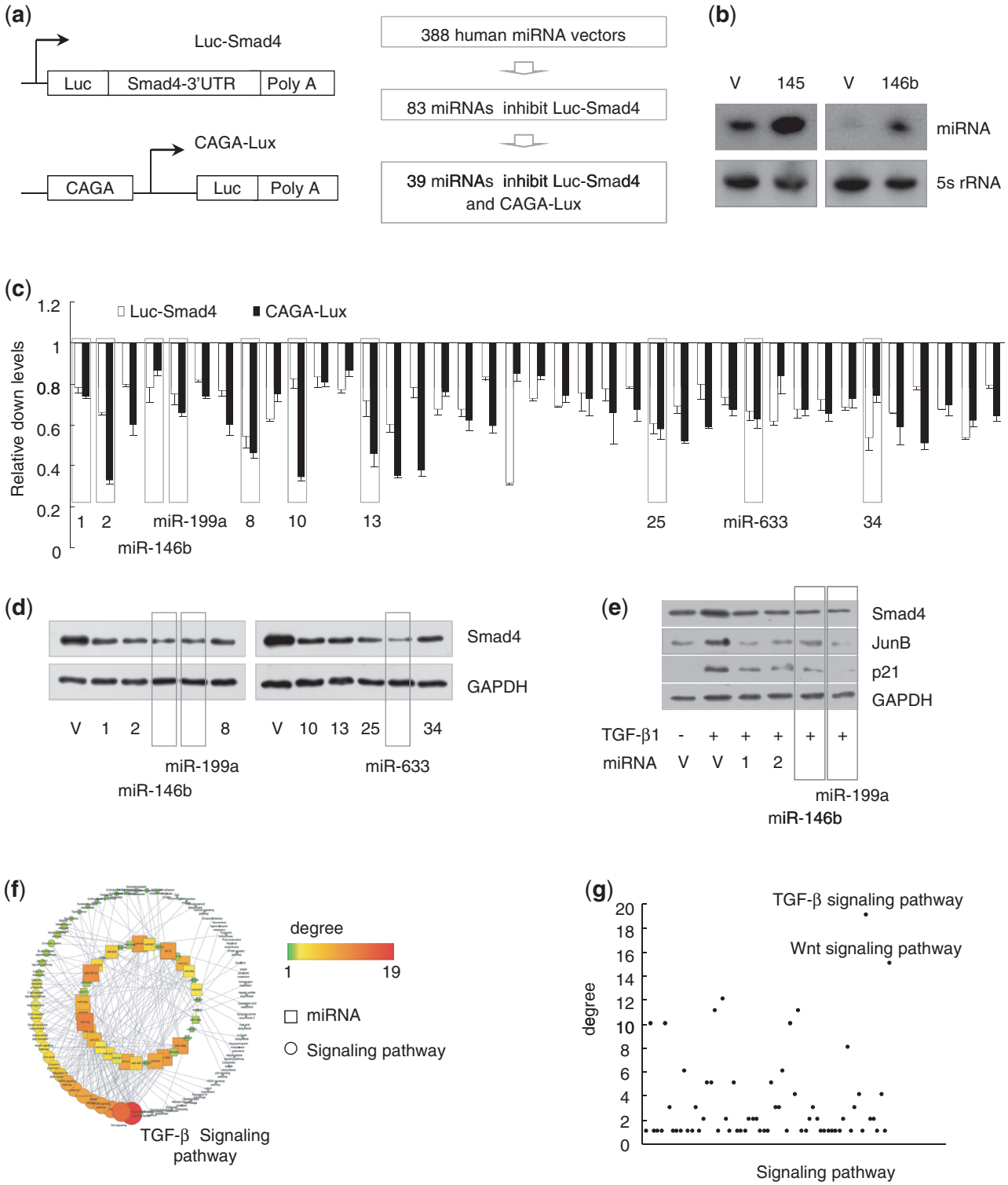

Figure 1. Identification of miRNAs targeting *Smad4*. (**a**) Panel illustrating the functional luciferase miRNA screening constructs and the procedure. (**b**) Northern blot of RNA extracts from NIH-3T3 cells transfected with miR-145, miR-146b or control vectors; 5s rRNA was used as a loading control. (**c**) Inhibition of Luc-Smad4 and CAGA-Lux reporter activities by 39 miRNAs identified. The bars indicate the relative luciferase activities in miRNA-transfected cells normalized by values from empty vector-transfected cells. (**d** and **e**) Ectopic expression of candidate miRNAs suppressed Smad4 expression (d), as well as the expression of its downstream target genes (JunB and p21) (e) in HepG2 cells. (**f**) Interacting network between miRNA candidates and signalling pathways inferred from mirPath database. (**g**) Degree analysis of the network from (f), indicating that 19 of the identified *Smad4*-targeting miRNAs regulate the TGF-β pathway ($P < 0.05$), which constitutes the top signalling pathway targeted by the identified 39 miRNAs.

An inverse correlation between miR-199a and Smad4 expression in human gastric cancer

We next checked whether the expression of these miRNAs was correlated with Smad4 expression in pathological setting. We first checked expression of seven miRNA candidates and Smad4 in seven breast and gastric cancer cell lines. Among examined miRNAs, miR-199a expression showed a best inverse correlation with Smad4 in the cell lines tested (Figure 2a, b and Supplementary Figure S2). Downregulation of Smad4 in gastric cancer tissues has been well documented (50,51), whereas miR-199a has been reported to be upregulated in gastric cancer tissues (52). Therefore, we further tested whether miR-199a inversely correlates with Smad4 in gastric cancer. As expected, in 15 pair-matched human gastric cancer specimens, reduced Smad4 expression was detected in 60% (9 of 15) of the gastric cancer tissues using Western blot, whereas Northern blot indicated that miR-199a was upregulated in 67% (10 of 15) of the gastric cancer tissues (Figure 2c). Spearman rank correlation analysis confirmed the negative correlation between miR-199a and Smad4 expression in gastric cancer tissues ($\rho = -0.769$, $P = 0.026$) (Figure 2d). Although miR-146b and miR-633 showed a similar effect with miR-199a on suppressing Smad4 expression (Figure 1c and d), miR-146b was not upregulated in gastric cancer (Supplementary Figure S3a and b), whereas miR-633 was hardly detectable in both human normal and gastric cancer tissues (Supplementary Figure S3b), indicating that the expression of miR-146b or miR-633 is not responsible for downregulation of Smad4 in gastric cancer tissues. All these data suggest that miR-199a might negatively regulate expression of Smad4 and plays roles in human gastric cancer.

MiR-199a impairs Smad4-dependent TGF-β responses

We next validated whether miR-199a could regulate the cellular responses to TGF-β signalling. First, we confirmed that overexpression of miR-199a in NIH-3T3 cells (Figure 3a) significantly repressed the luciferase activity of the CAGA-Lux reporter gene in response to TGF-β1 treatment, whereas 2′-OMe-modified antisense oligonucleotide anti-miR-199a increased the luciferase activity, and restoration of Smad4 rescued inhibition of CAGA-Lux activity by miR-199a (Figure 3b). Overexpression of miR-199a also greatly reduced the mRNA expression levels of the endogenous targets of TGF-β signalling, *PAI-1* and

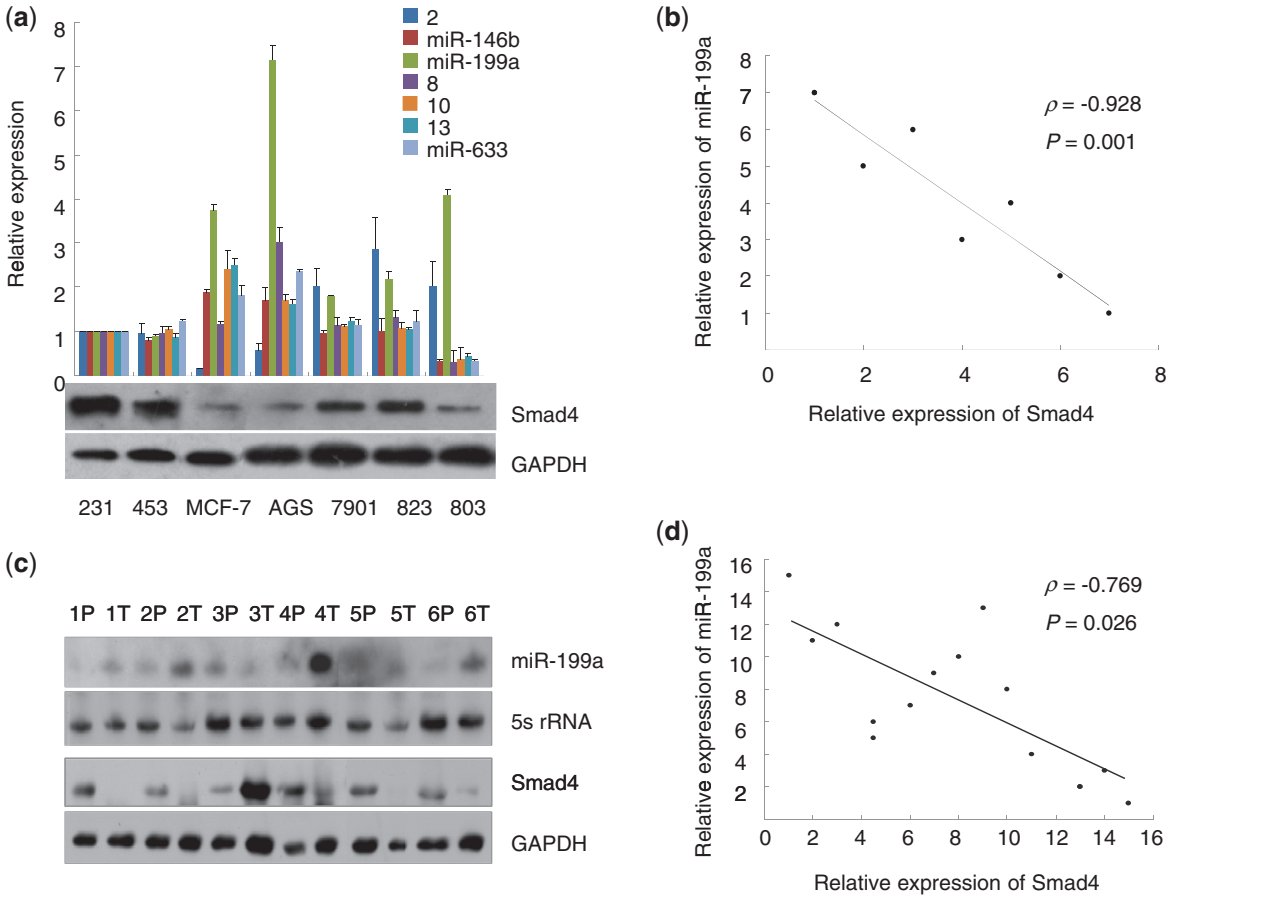

Figure 2. Inverse correlation between miR-199a and Smad4 expression. (**a**) The levels of miR-199a and six other miRNAs by real time PCR, as well as Smad4 protein levels by Western blot, were assessed in MDA-MB-231 (231), MDA-MB-453 (453), MCF-7, AGS, SGC-7901(7901), BGC-823(823) and MGC-803 (803) cells. (**b**) Spearman rank correlation analysis demonstrated an inverse correlation between miR-199a and Smad4 expression in the cells tested in (a), $n = 7$. (**c**) miR-199a levels, as assessed by Northern blot, were inversely correlated with Smad4 protein levels in human gastric cancer specimens. (**d**) Spearman rank correlation analysis of (c), $n = 15$. T refers to tumour tissues; P refers to peritumoural tissue.

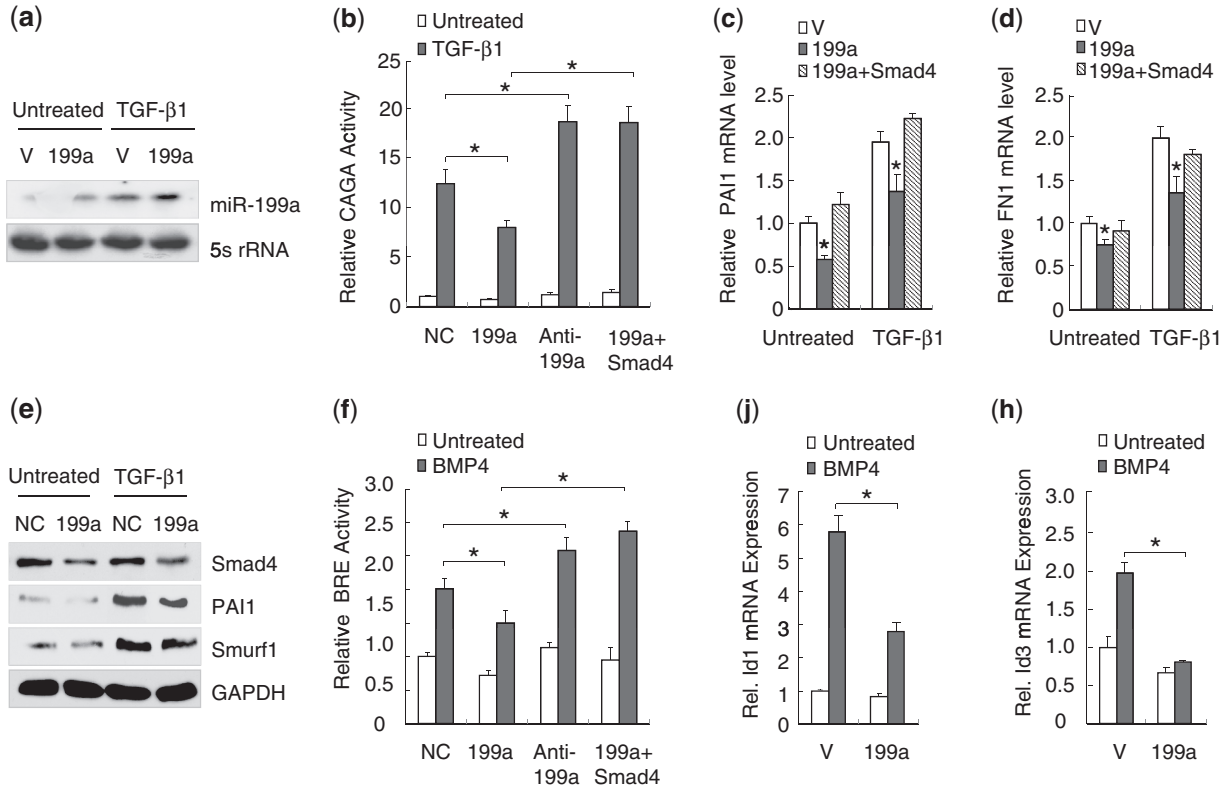

Figure 3. miR-199a partially blocks TGF-β signalling in NIH-3T3 cells. (**a**) Ectopic expression of miR-199a in NIH-3T3 cells treated with or without TGF-β1, detected by Northern blot using 5s rRNA as a loading control. (**b**) Ectopic expression of miR-199a inhibited TGF-β/Smad4-responsive CAGA-Lux activity, which was rescued by restoration of Smad4, whereas antisense inhibitor anti-199a increases CAGA-Lux activity. (**c** and **d**) Ectopic expression of miR-199a suppressed TGF-β1-induced expression of target genes, PAI-1 and FN1, assessed by real time PCR analysis. (**e**) Inhibition of expression of Smad4 and its downstream target genes, PAI-1 and Smurf1, by miR-199a mimics, as detected by Western blot. (**f**) Suppression of BMP4/Smad4-responsive BRE-Lux activity by miR-199a was rescued by restoration of Smad4. (**g** and **h**) Ectopic expression of miR-199a suppressed BMP4/Smad4 target genes ID1 and ID3, as assessed by real time PCR analysis. V refers to empty vector; 199a refers to miR-199a expression construct, anti-199a refers to modified 2'-OMe antisense inhibitors of miR-199a. $^*P < 0.05$.

fibronectin 1 (*FN1*), and this effect was reversed by co-expression of *Smad4* (Figure 3c and d). Transfection of miR-199a mimics resembled the effects of miR-199a overexpression, as evidenced by downregulation of Smad4 and its downstream target genes *PAI-1* and *Smurf1* (Figure 3e). These data suggest that miR-199a might regulate TGF-β signalling via targeting *Smad4*.

As Smad4 is a shared mediator of both TGF-β and BMP signalling, we examined whether miR-199a could also negatively modulate BMP signalling. As expected, overexpression of miR-199a also repressed BMP4-induced BRE-Lux luciferase reporter gene activity (Figure 3f) and expression of the BMP4 target genes *Id1* (Figure 3g) and *Id3* (Figure 3h). The regulation of Smad4 expression and TGF-β signalling by miR-199a was further validated in different cell lines (Supplementary Figure S4).

Smad4 is a target of miR-199a

Existing bioinformatic tools based on stringent seed-pairing rules failed to identify miR-199a-binding sites in the *Smad4* mRNA 3′-UTR. However, predictions based on overall base pair alignment, irrespective of the seed sequence, successfully identified a potential miR-199a-binding site (paired with miR-199a nucleotides 4–22), which is conserved through evolution (Figure 4a). To test whether *Smad4* is a bona fide target of miR-199a, the predicted miR-199a-binding site was mutated in the different paired region of *Smad4* 3′-UTR (Smad4 (ΔT1, ΔT2 and ΔT3) in Figure 4a). Mutations destroying the pairing with nucleotides 6–10 (ΔT3) or nucleotides 11–16 (ΔT2) significantly abolished the repression of miR-199a on reporter gene activity, whereas mutations destroying the pairing with nucleotides 17–22 (ΔT1) slightly alleviated the repression of miR-199a (Figure 4b). To further confirm this regulation, we ectopically co-expressed miR-199a with mutant *Smad4* cDNA, in which the target site paired with nucleotides 6–10 of miR-199a was mutated, in *Smad4*-null MDA-MB-468 cells. Consistent with the results of the luciferase assays, co-expression of miR-199a significantly repressed ectopic wild-type *Smad4*, but not mutant *Smad4* expression (Figure 4c). These data demonstrate that the predicted miR-199a-paired region is required for the regulation of *Smad4* by miR-199a.

MiR-199a impairs TGF-β-induced cell-cycle arrest and apoptosis in gastric cancer cells

To explore the roles of miR-199a in human gastric cancer, we carried out experiments in SNU-16 cells because they have a good response to canonical TGF-β signalling. SNU-16 cells were stably transfected with a

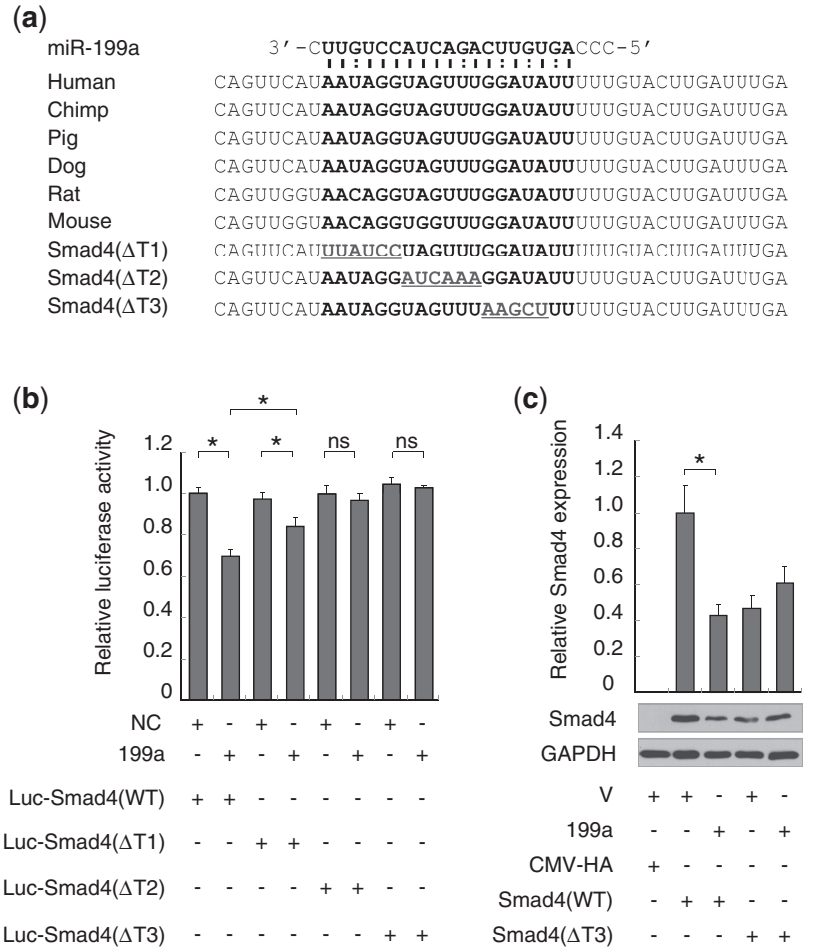

Figure 4. miR-199a directly targets *Smad4*. (**a**) Sequence alignment of putative miR-199a-binding site in the *Smad4* 3′-UTR, showing a high level of complementarity and sequence conservation. Lines show Watson–Crick pairing, whereas dashed lines show G:U wobble base pairs. Unpaired region in mutated constructs were underlined. (**b**) Overexpression of miR-199a reduced the luciferase activity of the wild-type Luc-Smad4 reporter, whereas this regulation was alleviated when the miR-199a target-binding site in *Smad4* 3′-UTR was mutated as indicated. (**c**) Overexpression of miR-199a in *Smad4*-null MDA-MB-468 cells downregulated ectopic expression of wild-type Smad4, but not expression of Smad4 with a mutated miR-199a-binding site in the 3′-UTR (Smad4[ΔT3]), as determined by Western blot. V refers to vehicle vector for miR-199a, 199a refers to miR-199a expression construct, CMV-HA is a vehicle vector for Smad4. $*P < 0.05$.

miR-199a-expressing vector, and overexpression of miR-199a was confirmed by Northern blot (Figure 5a). The cells were treated with TGF-β1, and as expected, expression of Smad4 and its downstream target JunB and p21, as well as transcriptional reporter CAGA-Lux, were repressed (Figure 5a, c and d). We observed similar results in cells transfected with Smad4 siRNA (Figure 5b). Moreover, repression of TGF-β1-induced p21 expression by miR-199a can be completely rescued by Smad4 restoration (Figure 5d).

Additionally, treatment with TGF-β1 for 12 h induced robust cell-cycle arrest in control cells, whereas these effects were effectively inhibited in miR-199a-overexpressing or Smad4 siRNA-transfected cells (Figure 6a and b). Sustained treatment with TGF-β1 for 24 h induced severe apoptosis, which was again impaired in miR-199a-overexpressing or Smad4-depleted cells (Figure 6c and Supplementary Figure S5). Consistently, anti-miR-199a enhanced TGF-β1-induced apoptosis of SNU-16 cells (Figure 6d).

We further examined the effects of miR-199a on the anchorage-independent growth of SNU-16 cells in soft agar. As shown in Figure 7, both colony size and number were reduced on TGF-β1 treatment, whereas overexpression of miR-199a partially abolished the TGF-β1 suppression of colony growth. These data suggest that miR-199a exerts a growth and survival-promoting effect at least partially by antagonizing TGF-β signalling in gastric cancer cells.

DISCUSSION

In this study, we established a functional screening method, based on comprehensive quantitative luciferase assays, to identify novel miRNAs that regulate expression of a specific gene, *Smad4*, irrespective of seed or sequence pairing and evolutionary conservation. The sensitivity and specificity of the screening was enhanced by using two independent luciferase reporter constructs (Luc-Smad4 and CAGA-Lux), which minimized false-positivity and

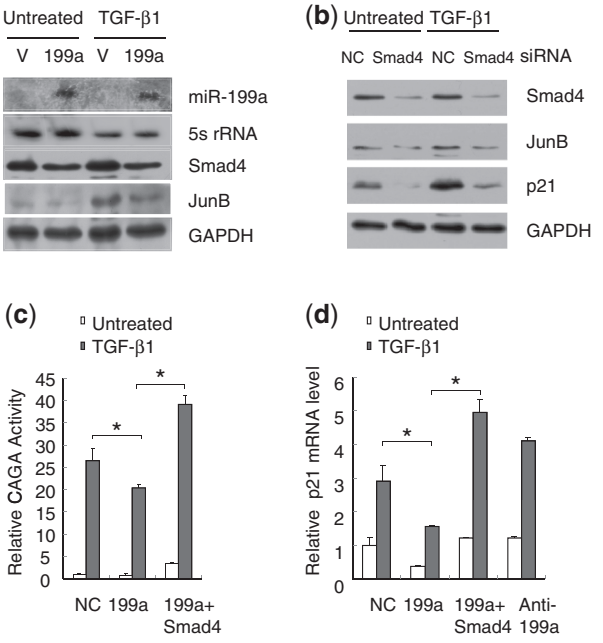

Figure 5. miR-199a inhibits TGF-β transcriptional response in SNU-16 gastric cancer cells. (**a**) Top 2 panels: overexpression of miR-199a was confirmed by Northern Blot using 5s rRNA as a loading control; bottom 3 panels: Smad4 expression and induction of JunB by TGF-β1 were inhibited upon miR-199a overexpression. (**b**) Downregulation of JunB and p21 expression upon Smad4 knockdown by siRNA. (**c**) miR-199a inhibited TGF-β1-responsive CAGA-Lux activity, which can be rescued by Smad4 restoration. (**d**) Suppression of TGF-β1-induced p21 expression by miR-199a can be rescued by Smad4 restoration. $*P < 0.05$.

false-negativity associated with the limited weightings used by existing bioinformatic algorithms. Our screening strategy was supported by currently available bioinformatic algorithms, as more than 60% of the miRNAs identified in the functional screening were also predicted to target *Smad4* by the bioinformatic algorithms (Supplementary Figure S1). In addition, DIANA mirPath analysis suggested that the identified miRNAs regulate the TGF-β signalling pathway (Figure 1f and g). The results of the screening are supported by a previous study which showed that miR-146a and miR-146b, two of the identified miRNAs, target *Smad4* in an acute promyelocytic leukemia cell line (53) and thyroid cancer (35), respectively. Recently, a similar strategy has been proved to be an effective method for the identification of miRNAs targeting *p21* (54). Importantly, using the functional screening, we were able to identify *Smad4*-targeting miRNAs that were not predicted by current bioinformatic algorithms. We demonstrated that *Smad4* is a bona fide target of miR-199a, although it was not predicted by any algorithm based on stringent seed pairing. These results proved the sensitivity and specificity of our screening methods, and we propose that luciferase-based screening strategies are suitable for the identification of miRNAs targeting other protein-coding genes.

We identified a class of miRNAs that post-transcriptionally regulate *Smad4*, suggesting miRNAs play an important role in the cellular responses to TGF-β signalling. TGF-β signalling is known to play a complicated role in tumourigenesis, as inactivation of TGF-β tumour-suppressive function and/or activation of TGF-β tumour-promoting function are of vital importance in TGF-β-associated tumourigenesis. Previous work from our and other laboratories has provided solid evidence to support the hypothesis that Smad4-mediated TGF-β signalling is a critical barrier against tumourigenesis and metastasis in both mice and humans (13,15,55). Tight regulation of Smad4 expression and activity is critical for cells to properly respond to TGF-β signalling, and previous studies have shown that Smad4 expression and degradation can be regulated by post-translational modifications (56,57). In this work, we identified a group of miRNAs that could modulate TGF-β signalling via targeting *Smad4,* indicating that Smad4 expression can be precisely regulated by miRNAs at the post-transcriptional level, to ensure a proper cellular response to TGF-β signalling. Our results also suggest that the predicted *Smad4*-targeting miRNAs may have important roles in tumourigenesis and metastasis. In support of this idea, let-7b, miR-142-5p, miR-146a, miR-199a, miR-199b and miR-372 have been shown to be involved in acute myeloid leukemia (58,59), whereas miR-146a, miR-146b, miR-199a, miR-199b, miR-373 and miR-520c have been demonstrated to play a role in breast cancer metastasis (60–64). It would be interesting to further investigate whether these miRNAs act synergistically, and examine how they regulate the tumour-suppressive effects of TGF-β.

We observed that miR-199a promoted the growth and survival of human gastric cancer cells by regulating TGF-β signalling. MiR-199a overexpression significantly downregulated Smad4, leading to diminished TGF-β-induced growth arrest and apoptosis. Previous work has proposed a controversial function for miR-199a in carcinogenesis and tumour progression, as miRNA profiling of clinical samples has shown that miR-199a is upregulated in hepatoblastoma (65), Acute Myelogenous Leukemia (AML) (66) and cervical carcinoma (67), and downregulated in hepatocellular carcinoma (68–70) and ovarian cancer (71). Functional analyses indicate that miR-199a may suppress cell growth, cancer migration, invasion and metastasis in testicular cancer and endometrial stromal cells (64,72), and restrict the response to NF-KB signalling in type II epithelial ovarian cancer cells (73). MiR-199a is upregulated and associated with gastric cancer progression (52), and miR-199a may promote gastric cancer growth through inhibition of MAP3K11 (74). In this study, we showed that overexpression of miR-199a conferred a growth advantage to gastric cancer cells, at least partially through promoting cell proliferation and protecting cells from TGF-β-induced apoptosis, suggesting that miR-199a may play an oncogenic role in gastric tumourigenesis via regulation of *Smad4*. As TGF-β signalling plays a complicated role in tumour development and progression, we propose that miR-199a may also exert complex regulatory effects on tumour cells. As miR-199a can be upregulated by TGF-β1 in human primary pulmonary

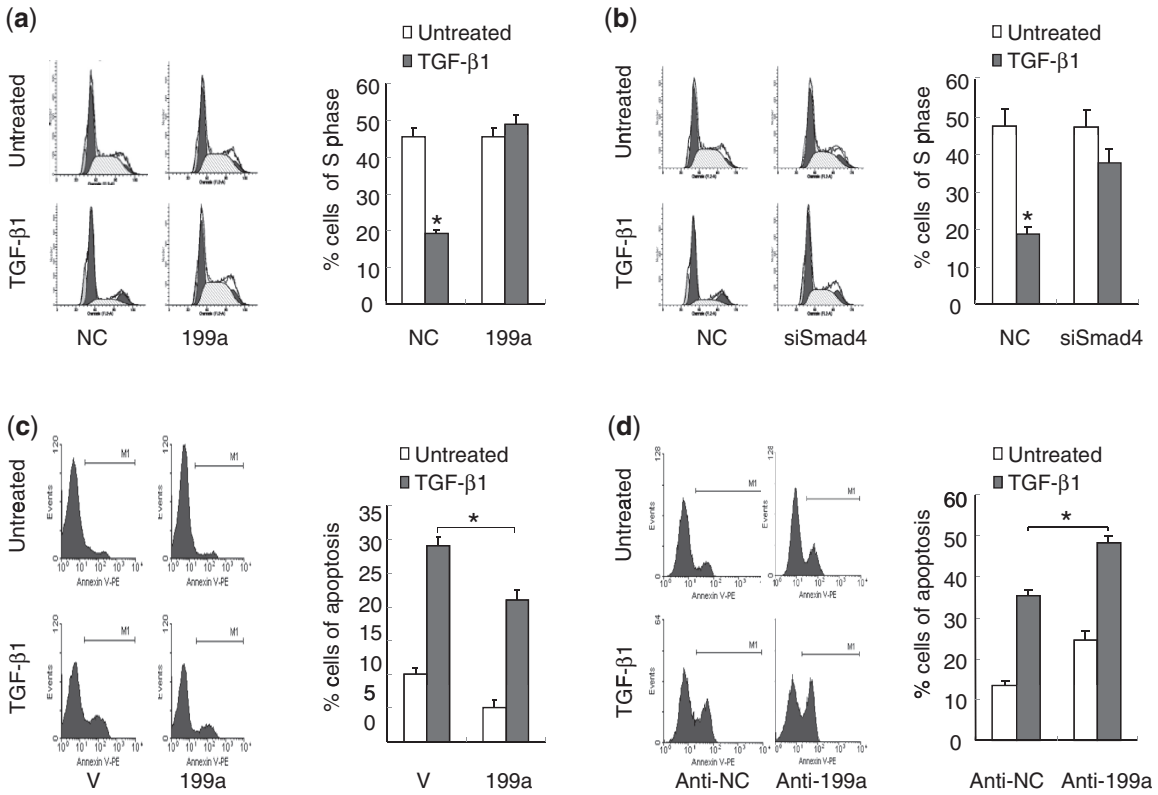

Figure 6. miR-199a attenuates the effects of TGF-β1 on proliferation and apoptosis. (**a**) miR-199a mimics reverted S phase reduction induced by TGF-β1 (1 ng/ml for 12 h), as assessed by FACS analysis of DNA content. (**b**) Smad4 knockdown by siRNA blocked S phase reduction induced by TGF-β1 (1 ng/ml for 12 h). (**c**) Stable overexpression of miR-199a attenuated apoptosis induced by TGF-β1 (1 ng/ml for 24 h), as assessed by FACS analysis of Annexin V-positive cells. (**d**) Inhibition of miR-199a (Anti-199a) enhanced TGF-β1-induced apoptosis compared to the negative control (Anti-NC). $*P < 0.05$.

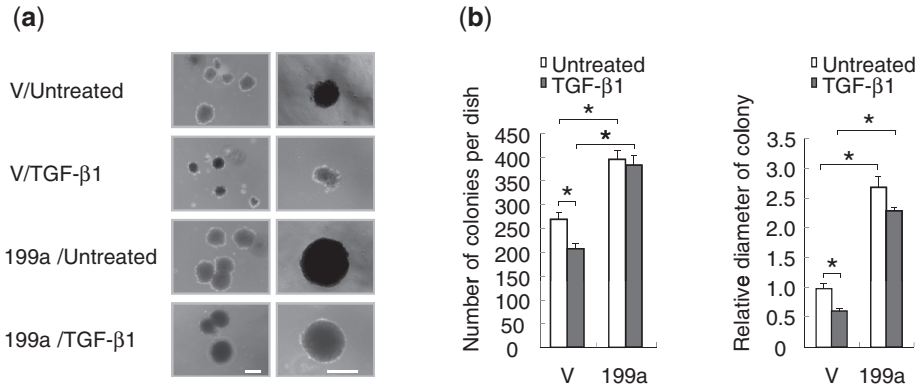

Figure 7. miR-199a promotes SNU-16 anchorage-independent growth, which is reduced by TGF-β1. (**a**) Cells transfected with indicated constructs were grown in soft agar with or without TGF-β1 (1 ng/ml). Representative images were shown. Scare bar = 100 μm. (**b**) Statistical analysis indicated that both colony size and number were reduced by TGF-β1 treatment, whereas miR-199a promoted colony growth and partially abolished TGF-β1 effects. Colonies with more than 50 cells were counted. $*P < 0.05$.

artery smooth muscle cells (75) and NIH-3T3 fibroblast cells (Figure 3a), it is possible that TGF-β and miR-199a may form a negative feedback loop to ensure proper and moderate TGF-β responses. However, we failed to observe induction of TGF-β1 on miR-199a in SNU-16 and HepG2 cells (Figure 5a and Supplementary Figure S6). These data indicate that the regulatory mechanisms of miR-199a in response to TGF-β signalling vary in different cell types.

In conclusion, this study identified a group of miRNAs predicted to target *Smad4*, of which miR-199a was demonstrated to directly target *Smad4* and modulate the response to TGF-β signalling in cell lines and gastric cancer. This study expands our understanding of the

mechanisms underlying the post-transcriptional regulation of *Smad4* and its ability to mediate TGF-β signalling. The information obtained in this study will be useful for further study of the function and mechanism of miRNAs regulating TGF-β-mediated biological processes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table 1 and Supplementary Figures 1–6.

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