Table II. Multivariate analysis with a Cox proportional hazards regression model

Characteristic	Univariate analysis	Multivariate analysis	Hazard ratio (95% CI)
Age (≥60 years)	0.22	0.15	
Gender (male)	0.92	0.53	
HCV status (positive)	0.28	0.82	
Cirrhosis (positive)	0.15	0.066	
Tumor size (≥50 mm)	<0.01*	0.011*	2.21 (1.18–4.12)
No. of tumor nodule (multiple)	<0.01*	<0.01*	2.67 (1.38–5.62)
Tumor differentiation (poor)	<0.01*	0.031*	2.34 (1.33–4.11)
Capsular formation (absence)	0.18	0.36	
Vascular invasion (presence)	0.062	0.10	
TNM stage (III + IV versus I + II)	<0.01*	0.020*	2.35 (1.14–4.82)
AFP (≥20 ng/ml)	0.18	0.36	
HSF1 expression (high)	0.018*	0.040*	2.07 (1.22–3.50)
BAG3 expression (high)	0.043*	0.056	

AFP, alpha-fetoprotein; CI, confidence interval; HCV, hepatitis C virus; TNM, tumor node metastasis.
*Significant *P* value.

HCC cells; accordingly, HSF1 deficiency inhibited the development of HCC. Furthermore, clinicopathological analysis demonstrated a significant correlation between HSF1 or BAG3 protein levels and prognosis. Our results demonstrate the importance of HSF1 in human HCCs and suggest inhibition of HSF1 as a novel strategy to target that subset of HCC patients in whom this protein is overexpressed.

Supplementary material

Supplementary Materials and methods, Table I and Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

Funding

Ministry of Education, Culture, Sports, Science and Technology, Japan (to N.S.); Japan Society for the Promotion of Science (24390185, 24659359); Ministry of Health, Labour and Welfare Japan; Japan Health Sciences Foundation; grants-in-aid for scientific research (22300317) and Uehara Memorial Foundation (to S.M.).

Conflict of Interest Statement: None declared.

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Received December 4, 2012; revised August 22, 2013; accepted August 28, 2013

Hepatology Research

Hepatology Research 2013 doi: 10.1111/hepr.12188

Original Article

Involvement of miRNA-29a in epigenetic regulation of transforming growth factor-β-induced epithelial–mesenchymal transition in hepatocellular carcinoma

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Aim: Epithelial–mesenchymal transition (EMT) is a crucial process during cancer invasion and metastasis, which is accompanied by the suppressed expression of E-cadherin initiated by stimuli such as transforming growth factor (TGF)-β. Recent studies have shown that the epigenetic regulation of E-cadherin could be an alternate mechanism of EMT induction in hepatocellular carcinoma (HCC). miRNA-29a (miR-29a) is involved in the epigenetic regulation of genes by targeting DNA methyltransferases (DNMT), which methylate CpG islands to suppress the transcription of genes. We studied the involvement of miR-29a in TGF-β-induced EMT in HCC cells.

Methods: We treated human HCC cell lines with TGF-β to induce EMT. To investigate DNA methylation in EMT, cells were treated with a methylation inhibitor, 5-Aza-2′-deoxycytidine (5-Aza) and methylation status of CpG islands in the E-cadherin promoter was examined using methylation-specific PCR. Precursor miR-29a (pre-miR-29a) was electroporated to force the expression of miR-29a in HCC cells in order to study the role of miR-29a in EMT.

Results: TGF-β transformed HCC cells into a spindle-shaped morphology accompanied by a decrease of E-cadherin with the induction of methylation of its promoter. Pretreatment of the cells with 5-Aza blocked this suppression of E-cadherin, indicating the involvement of DNA methylation. TGF-β increased DNMT3B and DNMT1 and decreased miR-29a expression. The forced expression of miR-29a abrogated the suppression of E-cadherin induced by TGF-β.

Conclusion: miR-29a could regulate TGF-β-induced EMT by affecting DNA methylation via the suppression of DNMT. These observations reveal the epigenetic regulation of genes by miRNA as a unique mechanism of EMT in HCC.

Key words: E-cadherin, epithelial–mesenchymal transition, hepatocellular carcinoma, miR-29a

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is the third most common cause of cancer death worldwide.[1] Treatments for HCC at the early stage include hepatic resection and liver transplantation. Percutaneous radiofrequency ablation and transarterial chemoembolization are good options for patients without the indication for resection or transplantation.[2,3] However, patients with advanced disease such as HCC with portal venous invasion or extrahepatic metastases do not have effective treatment options, and their prognosis is extremely poor.

Epithelial–mesenchymal transition (EMT) is an essential process associated with the propensity for invasion and metastases of many types of neoplasms including HCC.[4–6] The process of EMT is characterized by a reduction of intercellular adhesion accompanied by the loss of epithelial markers such as E-cadherin and an increase in motility with the acquisition of mesenchymal markers such as fibronectin and vimentin.[4] The process of EMT is initiated by the suppression of E-cadherin, a hallmark of EMT, promoted by EMT regulating transcriptional factors such as Snail, Slug and Twist.[7] However, recent studies have shown that the epigenetic regulation of E-cadherin could be the mechanism that induces EMT.[8,9] Alterations of the DNA methylation status could cause chromosomal instability and inacti-

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Received 15 January 2013; revision 17 June 2013; accepted 18 June 2013.

vation of tumor-suppressor genes, which are commonly observed in human neoplasms.[9] E-cadherin has CpG islands in its promoter region and the CpG hypermethylation could be involved in the inactivation of E-cadherin, which leads to EMT.[8] Furthermore, CpG hypermethylation has been reported in 49% of human HCC tissues.[10] DNA methyltransferases (DNMT) are key regulators of the methylation of CpG islands.[11] It has been reported that increased expression of DNMT1 was associated with the stages, portal venous invasion and prognosis of HCC.[12] Increased expression of DNMT3B was also reported in HCC tissues.[13]

miRNA are a type of small non-coding RNA with lengths of 18–23 nucleotides that play an important role in the suppression of the transcription of mRNA. miR-29a is reported as one of the onco-miR, which are miRNA involved in carcinogenesis and cancer progression by acting as an oncogene or an oncosuppressor. miR-29a directly targets DNMT3B[14] and DNMT1,[15] which play a crucial role in the DNA methylation of CpG islands. miR-29a has been reported to be suppressed in HCC tissues.[16,17] We have reported that miR-29a could modulate the expression of MEG3, a long non-coding RNA involved in the proliferation of HCC, by affecting the methylation status of CpG islands in its promoter region through the suppression of DNMT.[18]

We hypothesized that miR-29a, which directly targets DNMT, plays a role in the process of EMT induction. Here we show a new mechanism in which miR-29a regulates TGF-β-induced EMT via the epigenetic regulation of E-cadherin.

METHODS

Chemicals and drugs

RECOMBINANT HUMAN TGF-β was purchased from PeproTech (Rocky Hill, NJ, USA). The methylation inhibitor 5-Aza-2′-deoxycytidine (5-Aza) was purchased from Sigma-Aldrich (St Louis, MO, USA). Antibodies for E-cadherin, fibronectin, DNMT3B and DNMT1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for vimentin and Snail were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody for β-actin was purchased from Sigma-Aldrich. Specific antibodies for histone 3 with tri-methylation at lysine 4 (H3K4me3), histone 3 with di-/tri-methylation at lysine 9 (H3K9me2/3) and histone 3 with tri-methylation at lysine 27 (H3K27me3) were purchased from Cell Signaling Technology.

Cell lines and culture

Human HCC cell lines PLC/PRF/5 and HepG2 were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). The cells were maintained in growth medium consisting of Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (Invitrogen) at 37 °C with 5% CO_2. PLC/PRF/5 cells were used at their passage 8–12 for all experiments. HepG2 cells were used at passage 4–6 for all experiments.

Cytotoxic assay

Cells (2000–5000 cells/well) were seeded on a 96-well collagen-coated plate and incubated at 37 °C overnight to allow the cells to attach. Various concentrations of TGF-β or diluent control were added to the cells. After 24–72 h, cell viability was assessed using the CellTiter 96 AQueous assay kit (Promega, Madison, WI, USA).

Immunoblotting

Hepatocellular carcinoma cells were washed with phosphate-buffered saline, lysed with CelLytic M buffer (Sigma) and incubated for 15 min at room temperature. The protein concentration of the lysates was determined using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of proteins from each sample were mixed with NuPAGE LDS Sample Buffer (Invitrogen) and separated by sodium dodecylsulfate polyacrylamide gel electrophoresis using NuPAGE Novex 4–12% Bis-Tris Gels (Invitrogen). The proteins on the gels were then transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with a blocking buffer (LI-COR Bioscience, Lincoln, NE, USA) and incubated with the primary antibodies. The protein of interest was visualized and quantified using IRDye700- and IRDye800-labeled secondary antibodies (Rockland, Gilbertsville, PA, USA) and a LI-COR Odyssey Imaging System (LI-COR).

Real-time quantitative reverse transcription polymerase chain reaction (qRT–PCR) for specific genes

RNA was extracted from HCC cells with Trizol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was generated from a total of 600 ng of DNase I-treated RNA using a High Capacity cDNA RT Kit (Applied Biosystems, Carlsbad, CA, USA). The transcribed cDNA was diluted 50 times with DNase-free water and real-time qRT–PCR was performed using

a StepOnePlus Real-Time PCR System (Applied Biosystems) to detect E-cadherin mRNA with SYBR green I (PowerSYBR Green PCR Master Mix; Applied Biosystems). The determined threshold cycle (CT) was normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control, and the relative amount of E-cadherin expression was determined using the comparative CT method. The following PCR primers were used: E-cadherin primers, forward 5′-TCGACACCCGATTCAAAGTGG-3′, reverse 5′-GTGG GTTATGAAACCGTAGAGG-3′; and GAPDH primers, forward 5′-CGACAGTCAGCCGCATCTT-3′, and reverse 5′-CCCCATGGTGTCTGAGCG-3′.

Transfection of siRNA against Snail

Hepatocellular carcinoma cells were electroporated with siRNA against Snail (Mission siRNA, Hs_SNAI1_9785; Sigma) or negative control (Sigma) using a 4D-Nucleofector (Lonza, Basel, Switzerland) with an SF Cell Line 4D-Nucleofector X Kit (Lonza) by program EH-100. After 48 h, the cells were collected and used for further experiments.

Methylation-specific real-time PCR analysis

DNA was extracted by using the NucleoSpin Tissue kit (Macherey-Nagel, Duren, Germany). DNA (100 μg) was treated with bisulfite using the Methyl Easy Xceed Rapid DNA Bisulphite Modification Kit (Human Genetic Signatures, Sydney, New South Wales, Australia) according to the manufacturer's instructions. An equal amount of bisulfite-treated DNA was used for real-time quantitative PCR using EpiScope MSP kit (TaKaRa Bio, Tokyo, Japan) in order to determine the DNA methylation status of the CpG islands in the promoter region of E-cadherin. The sequences of methylation-specific PCR primers, which have been previously reported by Lim *et al.*,[19] were as follows: methylated, forward 5′-TTTTAGGTTAGAGGGTTATC GC-3′, reverse 5′-ACCAATCAACAACGCGAA-3′; and unmethylated, forward 5′-TAGTAATTTTAGGTTAGAGG GTTATTGT-3′, reverse 5′-CAACCAATCAACAACACAAA-3′. Amplification was as follows: 95°C for 30 s followed by 45 cycles at 98°C for 5 s, 55°C for 30 s and 72°C for 60 s. The comparative CT method was used to calculate differences in methylation between samples using the following formula: percentage of methylation (%) = $100 \times 1 / (1 + 2^{[CT\ methylated - CT\ unmethylated]})$. When the fluorescence of SYBR green I was not detected, 40 was used to calculate the percentage. EpiScope unmethylated HCT116 DKO gDNA (Takara Bio) and EpiScope Methylated HCT116 gDNA (Takara Bio) were used as methylated DNA control and unmethylated DNA controls.

Chromatin immunoprecipitation (ChIP) quantitative PCR

Chromatin immunoprecipitation assay was performed using the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology) to examine the level of histone methylation according to the manufacturer's instructions. Briefly, cells were fixed with formaldehyde for 10 min at room temperature for cross-linking. Chromatin was digested with micrococcal nuclease into 0.2–1-kb DNA/protein fragments. Immunoprecipitations of cross-linked chromatin were performed with antibodies specific to H3K4me3, H3K9me2/3 and H3K27me3. Real-time PCR was performed to quantify the amount of E-cadherin promoter region in the immunoprecipitates. The sequences of the primers used were as follows: forward 5′-CAGGTGAACCCTCAGCCAATC-3′, and reverse 5′-ACTGACTTCCGCAAGCTCACA-3′. Input fraction was diluted 1:49 and was used as a control to normalize the CT values. The immunoprecipitated chromatin interact with E-cadherin promoter (IP) was expressed as a percentage of the total input chromatin (input) using the following formula: percent input (%) = $2 \times 2^{(CT\ input - CT\ IP)}$.

Real-time qRT–PCR for miR-29a

RNA was extracted from HCC cells with Trizol reagent (Invitrogen) according to manufacturer's instructions. cDNA was generated using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems). The transcribed cDNA was diluted 50 times with DNase-free water, and real-time qRT–PCR was performed with TaqMan miRNA assays (Applied Biosystems) with a StepOnePlus Real-Time PCR System (Applied Biosystems). The determined CT was normalized with snRNA U6 (U6) as the endogenous control, and the relative amount of miR-29a expression was described using the comparative CT method.

Transfection of miRNA precursor and inhibitor

Hepatocellular carcinoma cells were electroporated with the miRNA precursor of miR-29a (pre-miR-29a; Applied Biosystems), miR-29a inhibitor (anti-miR-29a; Applied Biosystems) or negative control (Applied Biosystems) at 100 μM using a 4D-Nucleofector (Lonza) with an SF Cell Line 4D-Nucleofector X Kit (Lonza) by program EH-100. After 48 h, the cells were collected and used for further experiments.

Statistical analysis

Data were analyzed by ANOVA followed by Fisher's protected least significant difference test. Results were considered to be statistically significant at $P < 0.05$.

RESULTS

Cytotoxicity of TGF-β on HCC cells

TRANSFORMING GROWTH FACTOR-β is a multifunctional growth factor that controls cell growth,

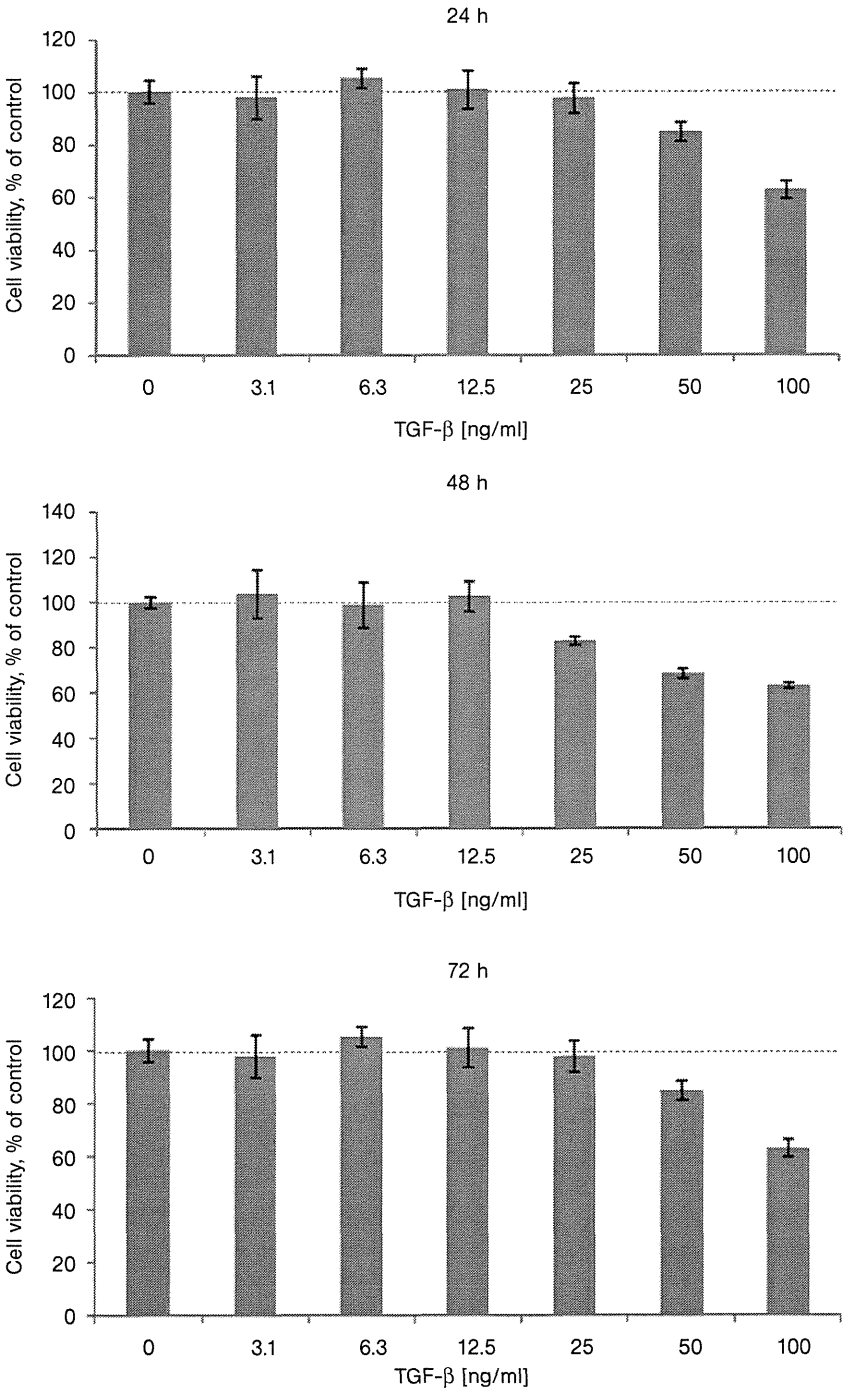

Figure 1 Effect of transforming growth factor-β (TGF-β) on the cell viability of hepatocellular carcinoma (HCC) cells. PLC/PRF/5 cells were seeded on 96-well plates at 2000–5000 cells/well and treated with ~100 ng/mL of TGF-β for 24–72 h. The viability of the cells was determined using a metabolic assay (MTS assay; Promega). Bars represent the mean ± standard error of the mean of six separate determinations.

and it can induce cell death in a variety of cell types. We first examined the cytotoxic effect of TGF-β on HCC cells in order to determine the concentration of TGF-β that would not induce cell death. As shown in Figure 1, TGF-β treatment for 24–72 h did not affect the viability of the PLC/PRF/5 cells at doses of 0–12.5 ng/mL. Thus, we chose doses of 1–10 ng/mL for TGF-β for the following experiments to induce EMT on HCC cells.

Induction of EMT in HCC cells by TGF-β

We treated the PLC/PRF/5 cells with TGF-β to induce EMT. In a stable condition, PLC/PRF/5 cells grow in a monolayer with polygonal morphology, forming clusters on a collagen-coated dish, as shown in Figure 2(a, left panel). After treating the PLC/PRF/5 cells with TGF-β at 10 ng/mL for 48 h, the cells became spindle shaped and grew more separately from each other without cell-to-cell contact (Fig. 2a, right panel). During this transformation, a reduction in the protein expression of E-cadherin and an increase in the protein expression of fibronectin was observed by immunoblotting (Fig. 2b).

Suppression of E-cadherin induced by TGF-β

We validated the suppression of E-cadherin expression induced by TGF-β at both mRNA level and protein level in two human HCC cell lines, PLC/PRF/5 and HepG2. As we expected, when we treated HCC cells with TGF-β (10 ng/mL) for 72 h the expression of E-cadherin mRNA was reduced by 48.2% in HepG2 and by 37.6% in PLC/PRF/5 (Fig. 3a). Similarly, a reduction in the protein expression of E-cadherin was observed in both cell lines (Fig. 3b).

Involvement of DNA methylation in EMT induced by TGF-β

The expression of E-cadherin has been reported to be regulated by the DNA methylation of CpG islands in its promoter region of the E-cadherin gene.[8,11] We next sought to clarify the involvement of DNA methylation in TGF-β-induced EMT by using a DNA methylation inhibitor, 5-Aza. We pretreated PLC/PRF/5 cells with 10 μM of 5-Aza or diluent control for 120 h before inducing EMT with TGF-β (10 ng/mL, 72 h). As shown in Figure 4(a), TGF-β without pretreatment with 5-Aza reduced the expression of E-cadherin mRNA by 53.3%. Pretreatment of the cells with 5-Aza (10 μM, 120 h) abrogated this reduction of E-cadherin mRNA (Fig. 4a). Similar results were obtained at the E-cadherin protein expression level. Treatment of PLC/PRF/5 cells with TGF-β reduced the protein expression of E-cadherin by 48% (Fig. 4b). Vimentin is described as one of the mesenchymal markers most commonly associated with EMT. As shown in Figure 4(b), induction of vimentin was observed as the protein expression of E-cadherin decreased after TGF-β treatment. The reduction of E-cadherin protein induced by TGF-β was abrogated by pretreating the cells with 5-Aza (Fig. 4b). Next, we examined the methylation status of CpG islands in the E-cadherin promoter. Methylation-specific real-time PCR was performed to determine the level of methylation of CpG islands which contains E-boxes.[19] After the

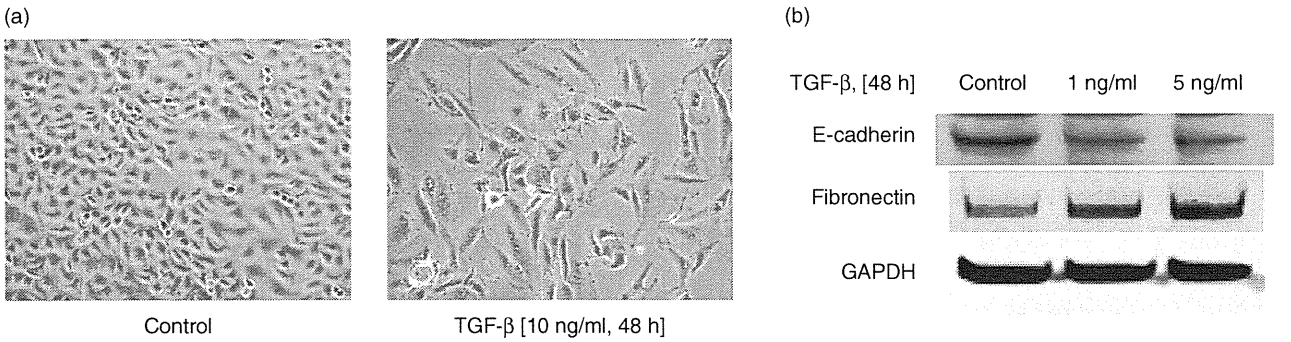

Figure 2 Induction of epithelial–mesenchymal transition (EMT) by transforming growth factor-β (TGF-β) in hepatocellular carcinoma (HCC) cells. (a) PLC/PRF/5 cells were seeded on a collagen-coated 10 cm dish at 1 000 000 cells/dish and were treated with 10 ng/mL of TGF-β for 48 h. The morphology of the cells was observed and images were captured using microscopy. (b) PLC/PRF/5 cells were seeded on 3.5-cm dishes and were treated with ~5 ng/mL of TGF-β for 48 h. Protein was extracted, separated using sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Expression of E-cadherin and fibronectin was detected using specific antibodies.

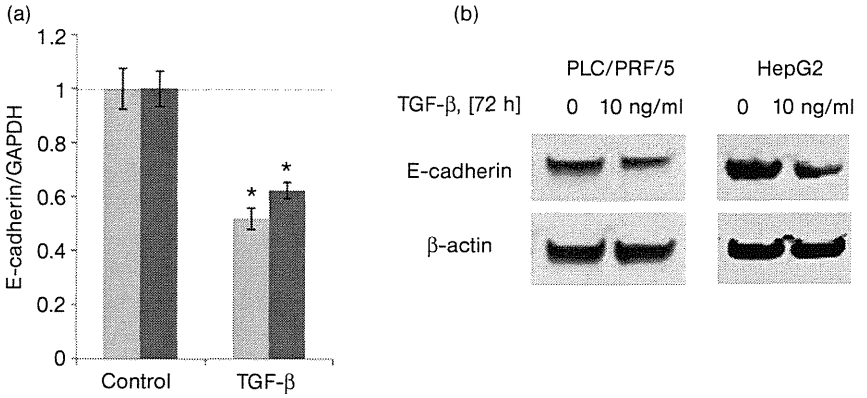

Figure 3 Suppression of E-cadherin expression induced by transforming growth factor-β (TGF-β). Hepatocellular carcinoma (HCC) cells were seeded on a collagen-coated 10-cm dish at 1 000 000 cells/dish and were treated with 10 ng/mL of TGF-β for 72 h. (a) The expression of E-cadherin mRNA was measured using real-time quantitative reverse transcription polymerase chain reaction. The expression was calculated as a relative value to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Bars express mean value of relative expression ± standard error of the mean of three determinants. $^*P < 0.05$. (b) Protein was extracted, separated using sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The expression was detected using specific antibodies. ▒, HepG2; ■, PLC/PRF/5.

treatment of PLC/PRF/5 cells with TGF-β (10 ng/mL) for 96 h, the methylation of E-cadherin promoter was induced (35.5 ± 1.96%), which was blocked by pretreatment with 5-Aza (Fig. 5). These observations indicate the involvement of DNA methylation during the induction of EMT by TGF-β.

The methylation of histone, one of histone modification, is reported to be another mechanism of epigenetic regulation of gene expression. Methylation of histone 3 on lysines has been reported to modulate the transcription activity of genes. Tri-methylation of H3K4me3 promotes activation of transcription of some genes whereas

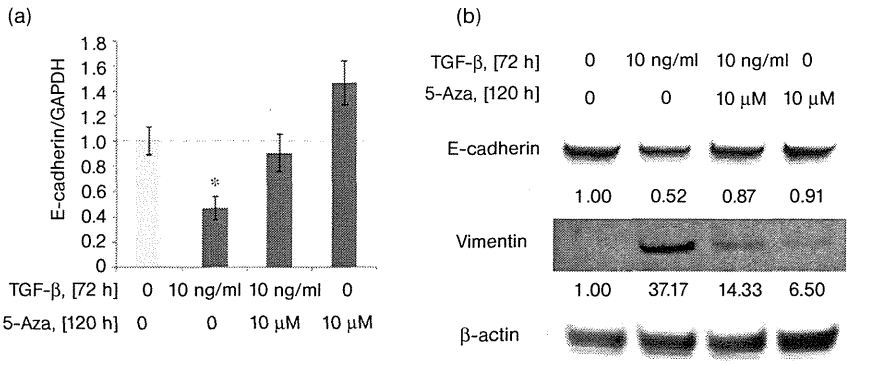

Figure 4 Effect of methylation inhibitor 5-Aza-2′-deoxycytidine (5-Aza) on transforming growth factor-β (TGF-β)-induced suppression of E-cadherin. PLC/PRF/5 cells were pretreated with 5-Aza (10 μM) for 120 h followed by the treatment with TGF-β (10 ng/mL) for 72 h. (a) The expression of E-cadherin mRNA was measured using real-time quantitative reverse transcription polymerase chain reaction. The expression was calculated as a relative value to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Bars express mean value of relative expression ± standard error of the mean of three determinants. $^*P < 0.05$. (b) The expression of E-cadherin protein was examined using immunoblotting. Protein was extracted, separated using sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Protein expression of E-cadherin was detected using a specific antibody. The intensity of the bands were quantified using densitometry.

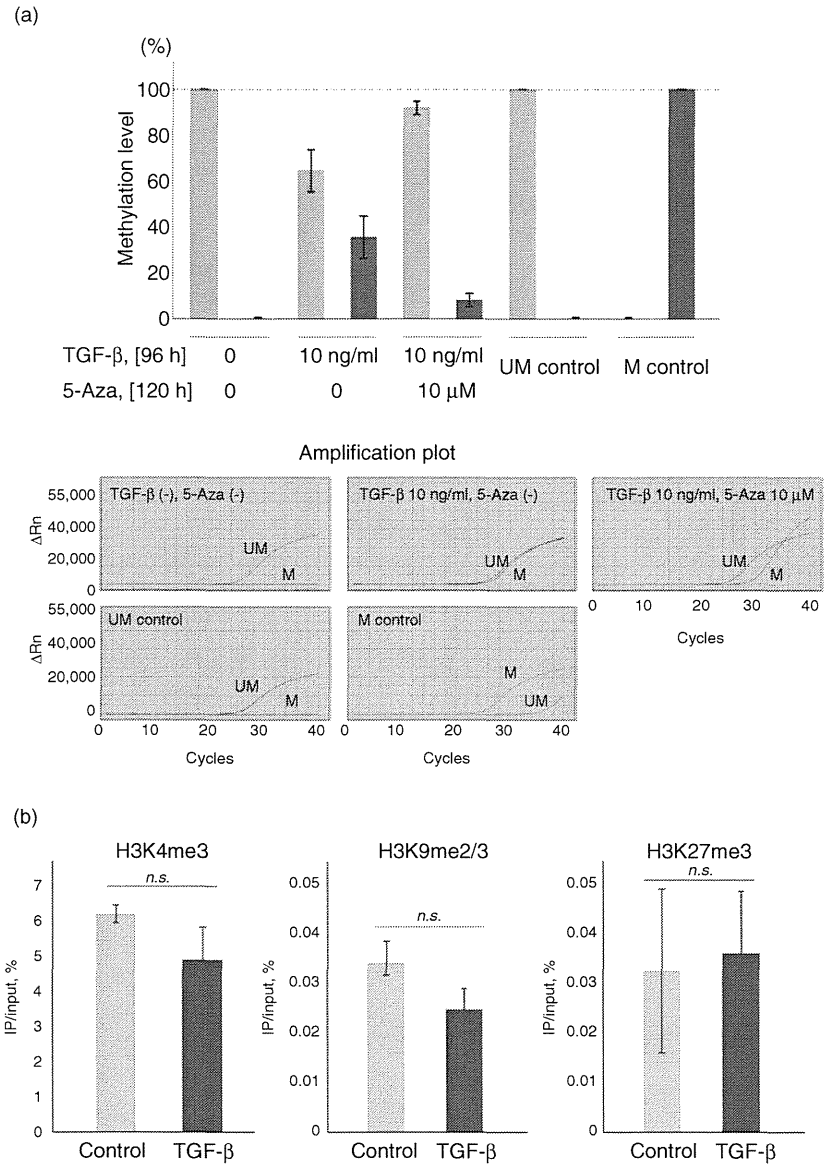

Figure 5 Effect of transforming growth factor-β (TGF-β) on methylation status of E-cadherin promoter. (a) PLC/PRF/5 cells were pretreated with 5-Aza-2′-deoxycytidine (5-Aza) (10 μM) for 120 h followed by the treatment with TGF-β (10 ng/mL) or diluent control for 96 h. DNA was extracted from cells and was treated with bisulfite using the Methyl Easy Xceed Rapid DNA Bisulphite Modification Kit (Human Genetic Signatures). An equal amount of DNA was used for methylation-specific real-time polymerase chain reaction (PCR) with primers that detect unmethylated or methylated promoter of E-cadherin. The percentage of methylated or unmethylated promoter was calculated as described in Methods. The bars represent mean percentage of three independent experiments ± standard error of the mean (SEM) (upper panel). Representative graphs of the amplification plots for the PCR reaction are shown (lower panel). UM, unmethylated; M, methylated. Effect of TGF-β on methylation histone 3. (b) PLC/PRF/5 cells were pretreated with 5-Aza (10 μM) for 120 h followed by the treatment with TGF-β (10 ng/mL) or diluent control for 96 h. Chromatin immunoprecipitation (ChIP) was performed using the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology) and specific antibodies for histone 3 with tri-methylation at lysine 4 (H3K4me3), histone 3 with di-/tri-methylation at lysine 9 (H3K9me2/3) and histone 3 with tri-methylation at lysine 27 (H3K27me3). Real-time PCR was performed to quantify the amount of E-cadherin promoter region in the immunoprecipitates (IP) and the chromatin interact with E-cadherin promoter was expressed as a percentage of the total input chromatin as described in Materials and Method section. The bars represent mean percentage of three independent experiments ± SEM. *n.s*, not significant. ▒, unmethylated; ■, methylated.

tri-methylation of H3K9me3 and H3K27me3 promotes suppression of transcription.[20–22] We performed ChIP quantitative PCR to investigate whether the methylation of histone was involved in TGF-β-induced EMT. PLC/PRF/5 cells were treated with TGF-β (10 ng/mL) or diluent control for 96 h and immunoprecipitation was carried out using specific antibodies for H3K4me3, H3K9me2/3 and H3K27. The level of modification was examined by detecting E-cadherin promoter using real-time PCR. As shown in Figure 5(c), the level of histone methylation in three sites was not altered by TGF-β treatment.

Involvement of Snail in TGF-β-induced EMT

The regulation of E-cadherin expression is crucial in EMT induction and the underlying mechanism of this regulation is reported to be a zinc finger homolog Snail, which suppress the mRNA expression of E-cadherin by binding to E-cadherin promoter. We knocked down the expression of Snail protein using siRNA. The protein expression of snail was induced by TGF-β treatment, as the expression of E-cadherin decreased and vimentin increased (Fig. 6). The knockdown of Snail induced the re-expression of E-cadherin and a decrease of vimentin,

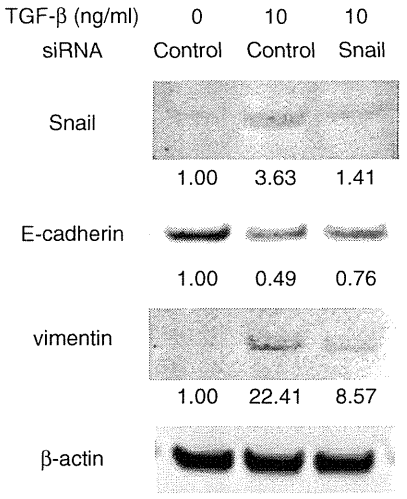

Figure 6 Involvement of Snail in transforming growth factor-β (TGF-β)-induced epithelial–mesenchymal transition (EMT). PLC/PRF/5 cells were electroporated with siRNA against Snail (MISSION siRNA, Sigma) or negative control (Sigma) using the 4-D Nucleofector kit (Lonza) followed by treatment with TGF-β (10 ng/mL) or diluent control for 96 h. Protein was extracted, separated with sodium dodecylsulfate polyacrylamide gel electrophoresis, and detected with specific antibodies for Snail, E-cadherin and vimentin.

but it was not to the same level before TGF treatment, which indicates that the regulation of E-cadherin expression could depend on not only Snail but also other mechanisms.

Suppression of the expression of miR-29a by TGF-β

DNA methyltransferases have been described as key regulators of the DNA methylation of CpG islands, and the increased expression of DNMT has been reported in human HCC tissues.[11] DNMT1 and DNMT3B have been reported to be involved in aberrant DNA methylation in cancer development and proliferation, and are direct targets of miR-29a. Having shown the involvement of DNA methylation in the induction of EMT by TGF-β, we hypothesized that miR-29a could be involved in TGF-β-induced EMT. We examined whether TGF-β modulates the endogenous expression of miR-29a in HCC cells. HCC cells were treated with TGF-β or diluent control for approximately 72 h and the expression of miR-29a was measured using real-time qRT–PCR at various time points. Treatment of HCC cells with TGF-β (10 ng/mL) significantly induced the suppression of the expression of miR-29a by 27.8% in PLC/PRF/5 cells and by 26.7% in HepG2 cells at 3 h (Fig. 7a,b). A question arose whether the suppression of miR-29a itself could induce EMT in HCC cells. We transfected anti-miR-29a or negative control in PLC/PRF/5 cells. The morphology of cells was not altered by the suppression of miR-29a (data not shown) and there was no obvious difference in the protein expression of E-cadherin, vimentin, fibronectin and Snail (Fig. 7c), which indicate that miR-29a may not be a master regulator of EMT, although it probably has a role in regulation of EMT.

Modulation of DNMT by miR-29a and TGF-β

Because we have shown that the expression of miR-29a was modulated by TGF-β in HCC cells, we next sought to clarify whether TGF-β modulates the protein expression of DNMT. First, we examined whether miR-29a could modulate the protein expression of DNMT in HCC cells, as reported in other type of cells. We electroporated pre-miR-29a or negative control in HCC cells using 4D-Nucleofector (Lonza) and, after 48 h, the protein expression of DNMT3B and DNMT1 was examined using immunoblotting. As we expected, the protein expression of DNMT3B and DNMT1, which are the direct targets of miR-29a, decreased after the forced expression of miR-29a in the PLC/PRF/5 cells (Fig. 8a). Next, we examined whether TGF-β could modulate the expression of DNMT. HCC cells were treated with

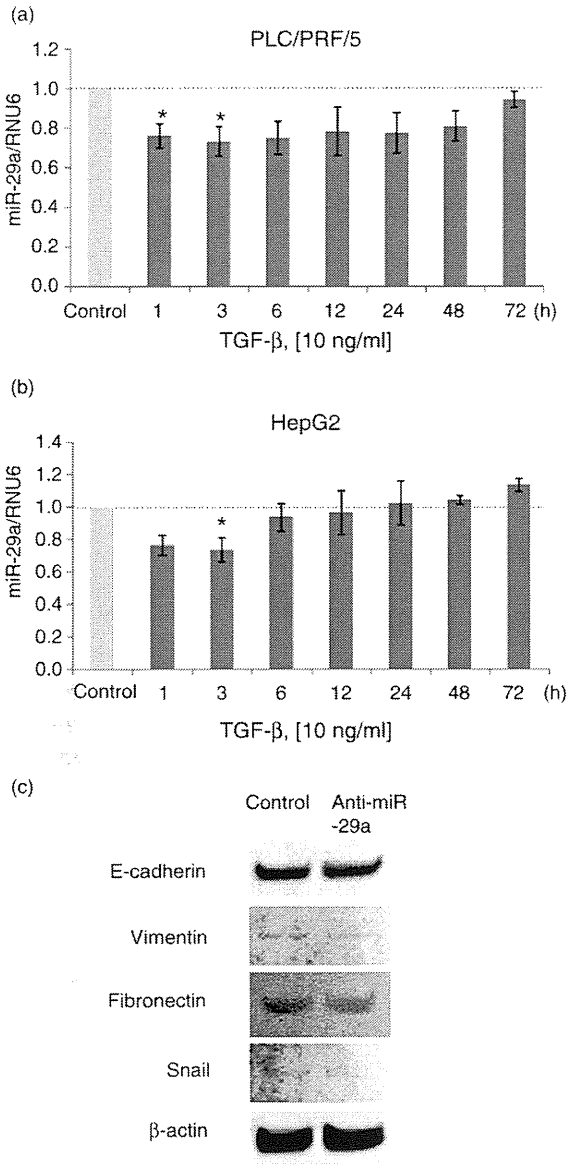

Figure 7 The expression of miR-29a under transforming growth factor-β (TGF-β) treatment. PLC/PRF/5 cells (a) and HepG2 cells (b) were treated with TGF-β or diluent control for ~72 h. The expression of miR-29a was measured using real-time quantitative reverse transcription polymerase chain reaction at the indicated time points. The expression was calculated as a relative value to the expression of RNU6. The bars express mean value of relative expression ± standard error of the mean of three determinants. $^*P < 0.05$. Effect of miR-29a knock-down on hepatocellular carcinoma (HCC) cells. (c) PLC/PRF/5 cells were electroporated with miR-29a inhibitor (anti-miR-29a; Applied Biosystems) or negative control. After 72 h, protein was extracted and immunoblotting was performed to detect the expression of E-cadherin, vimentin, fibronectin and Snail using specific antibodies.

10 ng/mL of TGF-β for 72 h and the protein expression of DNMT3B and DNMT1 was examined using immunoblotting. After treating the cells with TGF-β, the expression of DNMT3 and DNMT1 was increased 42% and 34% in PLC/PRF/5, and 21% and 18% in HepG2, respectively (Fig. 8b).

Regulation of EMT by miR-29a

Finally, we sought to examine the involvement of miR-29a in TGF-β-induced EMT. We forced the expression of miR-29a in HCC cells and examined whether the suppression E-cadherin expression induced by TGF-β was altered or not. The cells were electroporated with pre-miR-29a or negative control, and after 48 h, the cells were treated with TGF-β (10 ng/mL) or diluent control for 72 h. In cells transfected with the negative control, the expression of E-cadherin mRNA was reduced by 71.0% in HepG2 and 57.2% in PLC/PRF/5 after TGF-β treatment (Fig. 9a). In the cells transfected with pre-miR-29a, this effect was significantly abrogated (Fig. 9a). Similar results were observed at the protein level of E-cadherin expression. In cells transfected with the negative control, the expression of E-cadherin protein was reduced by 52% and vimentin was increased 9.97-fold after TGF-β treatment. This effect was partially abrogated with the forced expression of miR-29a in PLC/PRF/5 cells (Fig. 9b). We examined the methylation status of CpG islands in the E-cadherin promoter using methylation-specific real-time PCR. As shown in Figure 10, the methylation of the E-cadherin promoter was induced (27.3 ± 9.15%) after the treatment of PLC/PRF/5 cells with TGF-β (10 ng/mL) for 96 h. Transfection with pre-miR-29a on PLC/PRF/5 cells partially abrogated this induction of promoter methylation (Fig. 10).

DISCUSSION

IN PREVIOUS REPORTS, EMT was described as being regulated by transcription factors such as Snail and Twist, which cause the suppression of E-cadherin expression, a hallmark of EMT.[4–7] In this study, we show a possible mechanism of the regulation of EMT mediated by an miRNA via the epigenetic regulation of E-cadherin (Fig. 11). We show that miR-29a could modulate E-cadherin expression through the regulation of DNMT (Fig. 11). This is the first report showing the role of a miRNA in regulating EMT through the epigenetic regulation of gene expression.

Transforming growth factor-β is a multifunctional growth factor that controls cell growth, while its roles in carcinogenesis and the development of HCC remain

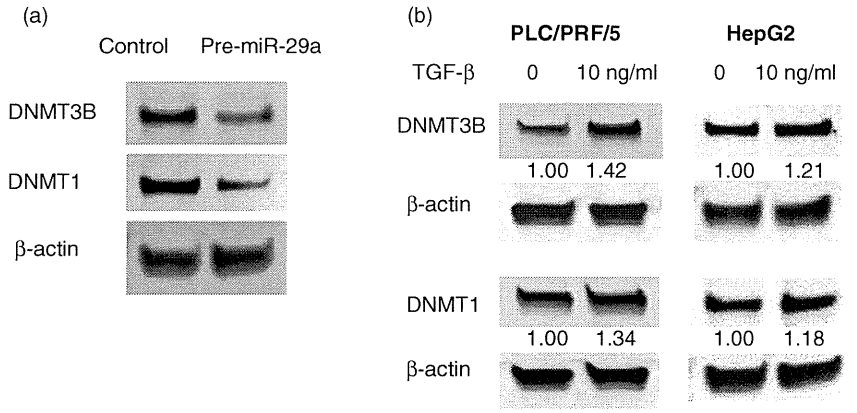

Figure 8 Effect of transforming growth factor-β (TGF-β) or miR-29a forced expression on DNA methyltransferases (DNMT). (a) PLC/PRF/5 cells were transfected with precursor of miR-29a or negative control with scrambled sequences and after 48 h the protein expression of DNMT3B and DNMT1 was detected using immunoblotting. Protein was extracted, separated using sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Protein expression of DNMT was detected using specific antibodies. (b) Hepatocellular carcinoma (HCC) cells were treated with 10 ng/mL of TGF-β for 72 h and the protein expression of DNMT3B and DNMT1 was detected using immunoblotting. Protein was extracted, separated using sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. Protein expression of DNMT was detected using specific antibodies.

unclear. TGF-β shows growth inhibitory effects on HCC cell growth,[23,24] although it stimulates the invasion and metastasis of HCC by promoting migration and invasiveness.[25,26] However, TGF-β signaling could induce EMT on various types of tumor cells including HCC,[7,27,28] and increased serum concentrations have been reported in HCC patients,[29–31] suggesting that the promotion of tumor development could be one of the main features of TGF-β in HCC. The regulation of E-cadherin expression is crucial in EMT induction[32,33] and the underlying mechanism of this regulation is reported to be a zinc finger homolog Snail and Slug,

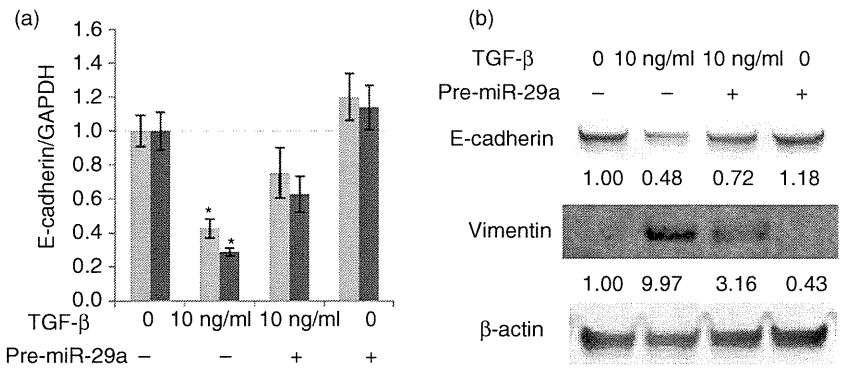

Figure 9 Effect of miR29a forced expression on suppression of E-cadherin induced by transforming growth factor-β (TGF-β). Hepatocellular carcinoma (HCC) cells were transfected with precursor of miR-29a (pre-miR-29a) or negative control and after 48 h the cells were treated with 10 ng/mL of TGF-β for 72 h. (a) The expression of E-cadherin mRNA was measured using quantitative reverse transcription polymerase chain reaction. The expression was calculated as a relative value to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Bars express mean value of relative expression ± standard error of the mean of three determinants. $^*P < 0.05$. (b) The expression of E-cadherin protein was detected using immunoblotting. Protein was extracted, separated using sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The expression of E-cadherin protein was detected using a specific antibody. Density of the bands was quantified and the expression level was calculated as a relative value to the expression of β-actin. ▒, HepG2; ■, PLC/PRF/5.

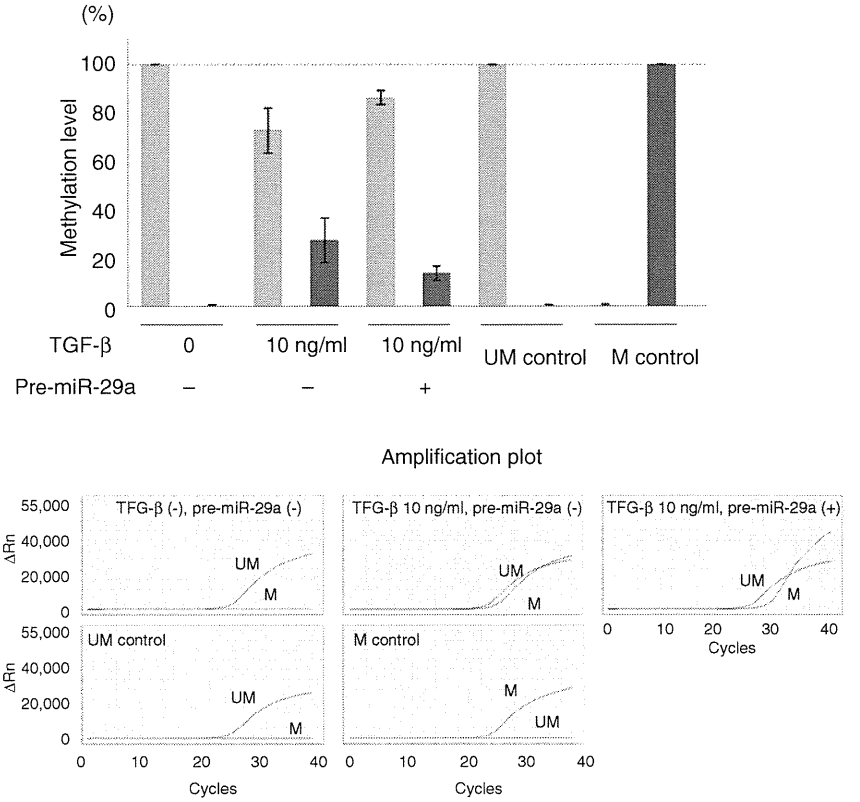

Figure 10 Effect of miR-29a forced expression of transforming growth factor-β (TGF-β)-induced methylation of E-cadherin promoter. PLC/PRF/5 cells were transfected with pre-miR-29a or negative control and after 48 h cells were treated with TGF-β (10 ng/mL) or diluent control for 96 h. DNA was extracted from cells and was treated with bisulfite using the Methyl Easy Xceed Rapid DNA Bisulphite Modification Kit (Human Genetic Signatures). An equal amount of DNA was used for methylation-specific real-time polymerase chain reaction (PCR) with primers that detect unmethylated or methylated promoter of E-cadherin. The percentage of methylated or unmethylated promoter was calculated as described in Methods. The bars represent mean percentage of three independent experiments ± standard error of the mean (upper panel). Representative graphs of the amplification plots for the PCR reaction are shown (lower panel). UM, unmethylated; M, methylated. ▒, unmethylated; ■, methylated.

which can suppress the expression of E-cadherin mRNA by binding to the E-box element of the E-cadherin promoter.[34–36] In this report, we found that E-cadherin expression could be regulated by methylation of its promoter region, indicating that epigenetic regulation via miR-29a could be a novel mechanism in the regulation of EMT. Moreover, our results suggest a possible mechanism in the regulation DNMT by TGF-β. DNMT are key regulators of DNA methylation, and are involved in the aberrant methylation of CpG islands in the promoter region of cancer-associated genes.[37,38] DNMT1 and DNMT3B have been reported to show increased expression in HCC tissues and are associated with a poor prognosis in HCC patients.[11–13] In other types of cells, it has been reported that TGF-β increases the expression of DNMT,[39,40] but the underlying mechanism of this process is unclear. DNMT3B and DNMT1 are targets of miR-29a.[14,18,41] In this study, we found that TGF-β could

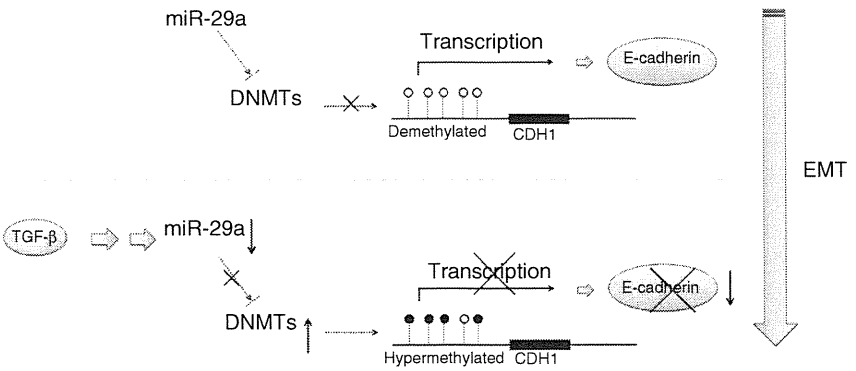

Figure 11 Proposed model of the involvement of miR-29a in transforming growth factor-β (TGF-β)-induced epithelial–mesenchymal transition (EMT). Upper panel, stable condition. Lower panel, EMT. CDH1, protein coding region of E-cadherin; DNMT, DNA methyltransferases.

reduce the expression of endogenous miR-29a and that the forced expression of miR-29a suppressed the protein expression of DNMT. miR-29a could be a mechanism linking the TGF-β signaling to the increased expression of DNMT.

In this study, we found that TGF-β could reduce the endogenous expression of miR-29a. It has been reported that TGF-β could suppress miR-29a expression in hepatic stellate cells[42] and renal cells,[43] but the mechanism of the regulation of miR-29a by TGF-β is completely unclear. miR-29a has been reported to show decreased expression in many types of neoplasms including HCC,[44–46] and it is considered to be a potential tumor suppressor.[47,48] Moreover, it has been reported that the degree of miR-29 downregulation was associated with poor survival in HCC patients.[44] In our results, the suppression of miR-29a level induced by TGF-β seemed slight, which was approximately 20% by 48 h (Fig. 5a,b), however, re-expression of miR-29a could partially abrogate the effect of TGF-β (Fig. 9). Some miRNA, such as miR-200, have been reported to act in switch-like manners.[49] miR-29a may behave as one of the switches during EMT and play a role in promoting a metastatic and invasive phenotype induced by the enhanced aberrant signaling of TGF-β.

In conclusion, our study demonstrates a role of miR-29a in the regulation of EMT in HCC. These results identify a new role of miRNA in contributing to the malignant features of HCC represented by EMT, suggesting that miRNA involved with EMT could be a new therapeutic target in the treatment of advanced HCC.

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