miR-655 Is an EMT-Suppressive MicroRNA Targeting ZEB1 and TGFBR2

Yosuke Harazono1,5, Tomoki Muramatsu1,4, Hironori Endo1,5, Narikazu Uzawa5, Tatsuyuki Kawano6, Kiyoshi Harada5, Johji Inazawa1,3,4,* Ken-ichi Kozaki1,2,3,*

1 Department of Molecular Cytogenetics, Tokyo Medical and Dental University, Tokyo, Japan, 2 Department of Therapeutic Genomics, Medical Research Institute and School of Biomedical Science, Tokyo Medical and Dental University, Tokyo, Japan, 3 Department of Genome Medicine, Hard Tissue Genome Research Center, Tokyo Medical and Dental University, Tokyo, Japan, 4 Global Center of Excellence (GCOE) Program for International Research Center for Molecular Science in Tooth and Bone Diseases, Tokyo Medical and Dental University, Tokyo, Japan, 5 Department of Maxillofacial Surgery, Tokyo Medical and Dental University, Tokyo, Japan, 6 Department of Esophagogastric Surgery, Tokyo Medical and Dental University, Tokyo, Japan

Introduction

The epithelial-to-mesenchymal transition (EMT) is an essential biological process with remarkable morphological changes between the epithelial and mesenchymal states [1], and plays key roles in embryonic development, cancer and other diseases [2–5].

During the acquisition of EMT characteristics, cancer cells lose the expression of genes that promote cell-cell contact, such as E-cadherin and the miR-200 family, and gain the expression of mesenchymal markers, such as vimentin, fibronectin, and N-cadherin, leading to enhanced cancer cell migration and invasion [6–8] and to confer drug resistance characteristics on cancer cells [9]. Therefore, the development of EMT inhibitors may provide novel strategies for the prevention, diagnosis and treatment of cancers.

MicroRNAs (miRNAs) are endogenous small non-protein-coding RNAs of 19–22 nucleotides. These single-stranded RNAs are considered to play crucial roles in many normal cellular processes [10,11,12], and the multistep processes of carcinogenesis and cancer progression [13–15], depending on their specific gene targets. Furthermore, the many achievements in the field of the discovery of tumor-suppressive miRNAs (TS-miRNAs) and in vitro/in vivo delivery technology may offer the possibility of new therapeutic approaches for cancer. Since one miRNA can target an unpredictable number of messenger RNAs (mRNAs) of protein-coding genes on a genome-wide scale, the clinical applications of miRNAs for cancer therapies are considered better than those of short interfering RNAs (siRNAs). In addition, among miRNA-based approaches by in vitro delivery including the use of DNA plasmids or viral vectors, miRNA replacement therapy using double-stranded RNAs (dsRNAs) mimicking TS-miRNAs may be one of the most promising, offering hope for new cancer therapies [15,16].

Recently, the miR-200 family (miR-141, -200a, -200b, -200c, and -429) and miR-203 have been demonstrated as EMT-suppressive miRNAs directly targeting ZEB1 and ZEB2 [17]. The miR-200-ZEB1-E-cadherin axis has been clarified to be a crucial pathway downstream of TGF-b in EMT while reciprocal repression between ZEB1 and the miR-200 family has recently been reported to promote EMT and invasion in cancer cells [18–22]. Actually, EMT-induced cancer cells were also reported to be more efficient at forming cancer stem cells with invasive and tumorigenic phenotypes [23]. Therefore, EMT-suppressive miRNAs in cancers
have been considered to be important diagnostic markers and new therapeutic agents for human malignancies.

Herein, we show the identification of a novel EMT-suppressive miRNA by function-based screening using 470 synthetic miRNAs and the detailed characterization of the miRNA and its direct targets. The function-based screening makes it possible to analyze the biological effects of a large number of dsRNAs on cancer cells directly. In addition, this approach has already proved successful in the exploration of dsRNAs having oncogenic or tumor-suppressive effects on cancer cells [24–27]. In the present study, to detect the promoter activity of CDH1/E-cadherin by measuring the fluorescence intensity of ZsGreen1 protein in our function-based screening, we established a unique cell-based reporter system using a pancreatic cancer cell line, Panc1, having phenotypic plasticity at EMT/mesenchymal-to-epithelial transition (MET). The present study is the first to show clearly that miR-655 targets ZEB1 and TGFBR2 inducing inactivation of the TGF-β signaling pathway, involving the miR-200-ZEB1-E-cadherin axis, strongly suggesting a potential role for miR-655 as a prognostic marker and therapeutic agent in human cancers.

Materials and Methods

Cell Lines and Primary Tumor Samples

The culture conditions for the pancreatic cancer [28], esophageal squamous-cell carcinoma (ESCC) [29] and oral squamous cell carcinoma (OSCC) [30,31] cell lines were reported previously. These cell lines were authenticated in previous studies with array-based comparative genomic hybridization (aCGH) analyses [28,29]. A breast cancer cell line, MDA-MB-231, was purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in the medium recommended by the manufacturers. Primary ESCCs and OSCCs were obtained with the written consent of each patient after approval by a local ethics committee of Medical Research Institute and Faculty of Medicine, Tokyo Medical and Dental University (Approval ID: 2010-5-2).

Transfection with Synthetic miRNAs and Small Interfering RNAs (siRNAs)

10 nM of dsRNA mimicking human mature miRNA or control non-specific miRNA (Ambion, Austin, TX; Thermo Scientific Dharmacon, Lafayette, CO) was transfected individually into cells using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA). The function-based screening was performed using Pre-miRTM miRNA Precursor Library-Human V3 (Ambion) in duplicate [26,27]. The numbers of viable cells were assessed by the colorimetric water-soluble tetrazolium salt (WST-8) assay (Cell counting kit-8; Dojindo Laboratories, Kumamoto, Japan). The reporter construct was generated using the pZsGreen1-Luciferase Vector (Clontech Laboratories, Palo Alto, CA). The fluorescence intensity of the ZsGreen1 protein was measured by ARVO x (Perkin Elmer, Waltham, MA).

Transwell Migration and Invasion Assay

Transwell migration and invasion assays were carried out in 24-well modified chambers precoated with (invasion) or without (migration) Matrigel (BD BioCoat, BD Biosciences, Franklin Lakes, NJ) as described elsewhere [32]. Cells in serum-free medium were transferred into the upper chambers. After incubation, the cells that migrated into the lower chambers with 10% FBS as the chemoattractant were fixed and stained with the Diff-Quick stain (Sysmex, Kobe, Japan), and counted in 5 random fields. Each assay was performed in triplicate.

Real-time Reverse Transcription-PCR and miRNA Target Predictions

Real-time reverse transcription-PCR (RT-PCR) was performed as described elsewhere [33]. Predicted targets for miRNAs and their target sites were analyzed using microRNA.org. All samples were analyzed in a duplicated manner.

Western Blot Analysis and Luciferase Activity Assay

An anti-CDH1 (#610181) antibody (BD Biosciences), an anti-TGFBR2 (SC-220), an anti-TGFBR1 (SC-398) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), an anti-ZEB1 (#33968), an anti-Snaill (#3879S), an anti-Phospho-Smad2/3 (Ser465/467 and Ser423/424, respectively) (#3101 and #9520, respectively) antibodies (Cell Signaling Technology, Beverly, MA), an anti-Smad2/3 (ab40853 and ab28379, respectively) antibodies (Abcam, Cambridge, MA) were used in Western blotting. Immunohistochemistry was performed as described elsewhere [32]. Luciferase constructs were made by ligating oligonucleotides containing the wild type or mutated sequence of 3' UTR target sites downstream of the luciferase gene in the pMIR-REPORT luciferase vector (Ambion). Luciferase activity was measured as described elsewhere [33].

Statistical Analysis

The association between clinicopathological characteristics and status of miR-655 expression in ESCC patients was evaluated with χ² or Fisher’s exact test (Table S2). A p-value less than 0.05 was defined as being statistically significant. In Kaplan-Meier curves, differences between subgroups were tested with the log-rank test. Differences between subgroups were tested with the Mann-Whitney U test.

Results

Establishment of a Cell-based Reporter System for Investigating CDH1/E-cadherin-promoter Activity in Panc1 Cells

To perform function-based screening of EMT-suppressive miRNAs, we established a cell-based reporter system. The promoter region of the CDH1/E-cadherin gene located in the 5' untranslated region (5' UTR) and Exon1 (from nt −1001 to +57 relative to the transcription start site, TSS, at the 5' end of the gene) was prepared by genomic PCR using specific primers (Table S1), and was inserted into the pZsGreen1-1 vector at the multiple cloning site upstream of the promoterless ZsGreen1 gene (Fig. 1A) [34]. We first transfected the above-mentioned construct into Panc1 cells, the phenotypic plasticity of which had already been confirmed in previous studies [35,36], and then carried out cloning of stable transfectants by limiting dilution. Furthermore, among many clones, the most-reactive single-cell clone, PECadZsG-Panc1, was selected by measuring the fluorescence intensity of the ZsGreen1 protein induced by transient transfection of miR-200b or -200a, already known as EMT-regulating miRNAs. In this clone 96 hours after transfection with 10 nM of dsRNA mimicking miR-200a or -200b, the fluorescence intensity of ZsGreen1 was remarkably increased as compared with that in the control counterparts (Fig. 1B). RT-PCR and Western blot analyses also showed a consistent correlation between the fluorescence intensity of ZsGreen1 and expression levels of CDH1/E-cadherin mRNA and protein in these transfectants. Consequently, we judged that ZsGreen1 expression was tightly regulated under the CDH1/E-cadherin-promoter in our cell-based reporter system for function-based screening of EMT-suppressive miRNAs.
Function-based Screening of EMT-suppressive miRNAs with our cell-based Reporter System

To identify EMT-suppressive miRNAs, we performed function-based screening, in which the fluorescence intensity of ZsGreen1 was made an index, using our cell-based reporter system and 470 dsRNAs at 10 nM. Figure 1C and Table S2 demonstrate results of this screening in a Panc1 stable clone, PEcadZsG-Panc1, 96 hours after transient transfection with each dsRNA. In Table 1, 17 miRNAs, the relative fluorescence intensity of which remarkably increased in our screening (>2.2-fold change of mean fluorescence intensity compared with the control counterpart), were enrolled as candidate EMT-suppressive miRNAs. Among these candidates, we excluded well-known EMT-suppressive miRNAs, such as miR-200a and -200c [8,21,37,38], and selected 10 miRNAs (miR-96-5p, -132-3p, -183-5p, -139-5p, -217, -520d-3p, -526b-3p, -629-3p, -655, and -200b-3p) showing a consistent positive correlation between two sets of fluorescence data taken from the fluorescence microplate reader (Table S2). In a Western blot analysis in the parental Panc1 cells 96 hours after transient transfection with these miRNAs, miR-520d-3p and miR-655, as well as miR-200b-3p, were confirmed to upregulate expression of the E-cadherin protein markedly, whereas only slight effects of other miRNAs were observed (Fig. 1D). Moreover, the relative fluorescence intensity of miR-655 was clearly higher than that of miR-520d-3p (Table 1), suggesting miR-655 to be a prime candidate for EMT-suppressive miRNA.
Table 1. Summary of 17 miRNA genes selected as candidates for EMT-suppressive miRNAs in functional-based screening using a stable Panc1 clone transfected with a reporter construct containing a promoter sequence of CDH1/E-cadherin in the 5' upstream region of the ZsGreen1 reporter gene and Pre-miRTM miRNA Precursor Library - Human V3 (Ambion).

| Pre-miRTM miRNA Precursor | Mature Sequence | Ratio of fluorescence intensity of ZsGreen1 (RFI)* | Ratio of growth level (RG)** | Relative fluorescence intensity (RFI/RG) |
|--------------------------|-----------------|--------------------------------------------------|------------------------------|----------------------------------------|
|                          |                 | Mean | SD   | Mean | SD   | Mean | SD   |
| hsa-miR-200c-3p          | UAAUACUGCCGGGUAAUGAUGG | 5.22 | 0.05 | 0.86 | 0.08 | 5.99 | 0.15 |
| hsa-miR-200b-3p          | UAAUACUGCCGGUAUGAUGGAC | 4.91 | 0.09 | 1.06 | 0.14 | 4.74 | 0.47 |
| hsa-miR-655              | AAUAUAUCAUGGUUAACCUUUU | 4.09 | 0.00 | 1.06 | 0.06 | 3.87 | 0.07 |
| hsa-miR-200a-3p          | UUAGACUGUCUGUAACGAUGU | 4.22 | 0.03 | 1.28 | 0.04 | 3.30 | 0.16 |
| hsa-miR-132-3p           | UAAGACUGUCAGCCAGGUGCU | 2.53 | 0.02 | 0.83 | 0.08 | 2.93 | 0.01 |
| hsa-miR-526b-3p          | AAAGUGCUUCCUUUUAGAGGC | 3.27 | 0.03 | 1.28 | 0.02 | 2.58 | 0.06 |
| hsa-miR-302c-3p          | UAAGUCAGCAUCUGUUGUGGUC | 2.37 | 0.15 | 1.36 | 0.04 | 2.49 | 0.19 |
| hsa-miR-373-3p           | GAAGUGCUUCAGAUUUUGGGGUU | 2.93 | 0.11 | 1.20 | 0.06 | 2.43 | 0.21 |
| hsa-miR-217              | UACUGCAUCAGGAUCAGUUGAU | 2.66 | 0.06 | 1.11 | 0.04 | 2.42 | 0.20 |
| hsa-miR-629-3p           | GUUUCUCCAAUGCUGACCAUGC | 3.45 | 0.12 | 1.56 | 0.12 | 2.40 | 0.96 |
| hsa-miR-302d-3p          | UAAGUCUCACAGAUUGAGGUGU | 3.04 | 0.04 | 1.29 | 0.16 | 2.33 | 0.11 |
| hsa-miR-361-5p           | UUAUCAGACUUCUCAGGGGUAC | 2.35 | 0.15 | 1.00 | 0.16 | 2.36 | 0.13 |
| hsa-miR-96-5p            | UUGGCGACUUGCCUUGUUGUUGGC | 1.63 | 0.18 | 1.43 | 0.12 | 2.33 | 0.05 |
| hsa-miR-139-5p           | UCUCACUGCAUCAGUUCUGCU | 2.30 | 0.06 | 1.06 | 0.03 | 2.22 | 0.16 |
| hsa-miR-183-3p           | UCUCAGUCUGUGACUACUCUG | 2.76 | 0.01 | 1.24 | 0.00 | 2.12 | 0.01 |
| hsa-miR-520d-3p          | AAAGUCUCUCUCUCUGGUGGGGUU | 1.57 | 0.69 | 1.18 | 0.09 | 2.02 | 0.30 |
| hsa-miR-181d             | ACAUUCAGUUGUGUGGCUUGGGU | 2.27 | 0.01 | 1.29 | 0.06 | 2.20 | 0.00 |

*The ratio of fluorescence intensity of ZsGreen1 (RFI) in cells 4 days after transfection with each dsRNA was normalized to that in control transfectants (Pre-miRTM Negative Control #1, Ambion).

**The ratio of growth level (RG) of viable cells assessed by WST8 assay 4 days after transfection with dsRNAs. WST-8 assay was employed to normalize the number of viable cells relative to the control transfectants. doi:10.1371/journal.pone.0062757.t001
EMT-suppressive Effects of miR-655 on Mesenchymal-like Cancer Cells having Phenotypic Plasticity at EMT/MET

The expression profile of miR-655 was compared with that of each of seven typical EMT-related genes (CDH1/E-cadherin, miR-141, -200a, -200b, -200c, -205, and TGFBR2) in a panel of 23 pancreatic cancer cell lines and a breast cancer cell line, MDA-MB-231 (Fig. 2A and Fig. S2). We noticed a consistent positive correlation among expression profiles of miR-200 family members and a slight correlation between CDH1/E-cadherin and miR-200 family members. Although no correlation between the expression pattern of miR-655 and that of any of these marker genes was found in this panel, these cell lines all showed lower expression of miR-655 than the miR-200 family and miR-205, as compared with a normal pancreas. Marked down regulation of miR-655 expression was also observed in 97.7% (42/43) of ESCC and 94.7% (18/19) of OSCC cell lines (<0.5-fold expression) (Fig. S3A and B). Moreover, the expression of endogenous miR-655 was higher in MCF7 and MCF10A (human breast epithelial cells) than MDA-MB-231, suggesting that downregulation of miR-655 might contribute to phenotypic stabilization of mesenchymal feature in MDA-MB-231 cell line (Fig. S3C).

To confirm the EMT-suppressive effects of miR-655 on mesenchymal-like pancreatic or breast cancer cells having phenotypic plasticity at EMT/MET, we ectopically introduced 10 nM of synthetic dsRNA mimicking mature miR-655 into Panc1, KP1N, KP4-4 and MDA-MB-231 cells. The Panc1 and KP1N cell lines are miR-655-high expressers, while the KP4-4 and MDA-MB-231 cell lines are miR-655-low expressers (Fig. 2A). However, in all four cell lines 96 hours after transfection with miR-655, a morphological shift toward the epithelial phenotype was induced (Fig. 2B) consistent with an upregulation of CDH1/E-cadherin expression at the mRNA and protein levels (Fig. 2C). In addition, we confirmed that ectopic expression of miR-655 increased CDH1/E-cadherin expression at the mRNA and protein levels in an ESCC cell line, TE8, and an OSCC cell line, HSC2 (Fig. S4), although a morphological shift toward the epithelial phenotype in these cell lines was not observed (data not shown). These EMT-suppressive effects of miR-655 were observed in a miR-200 family-independent manner (data not shown). To take into consideration off-target effects of dsRNAs, these EMT-suppressive effects of miR-655 were also confirmed using two kinds of dsRNAs purchased from independent companies (Fig. S5). Notably, effects of overexpression of exogenous miR-655 on cell growth were not constant in these cell lines (Fig. 2D), whereas the number of cells that migrated through the uncoated or Matrigel-coated membranes in cell migration or invasion assays, respectively, was significantly decreased in all miR-655-transfectants compared with their control counterparts (Fig. 2E and 2F). These results suggest that miR-655 may suppress EMT in mesenchymal-like cancer cells.

Expression Analysis of miR-655 in Primary ESCC and OSCC Cases

We investigated the normal human tissue distribution and tumor expression of endogenous miR-655 by TaqMan RT-PCR analysis. Among 22 normal tissues, upregulation of miR-655 expression was observed in brain, cervix, esophagus and placenta (>2-fold increase compared with a normal pancreas) (Fig. 3A). We next examined the expression level of the miR-655 transcript in primary tumors of ESCC and OSCC, respectively. Expression levels of miR-655 in tumors as compared with paired non-tumorous mucosae were markedly reduced in 44.8% (13/29) and 60.9% (14/23) of primary ESCC and OSCC cases, respectively (<0.5-fold expression) (Fig. 3B). Furthermore, to evaluate the clinical significance of miR-655 expression in ESCC, we categorized the patients into two groups based on the mean value: a low miR-655 group (n = 18) and a high miR-655 group (n = 11). In Kaplan-Meier survival curves for 29 patients with ESCC expressing higher and lower levels of miR-655, univariate analyses of overall and non-recurrent survival with log-rank tests demonstrated a significant association between higher levels of miR-655 expression and a better survival rate (Fig. 3C, P = 0.0399, log-rank test), whereas the miR-655 expression in each tumor was not associated with clinicopathological features (Table S3). These findings suggest that miR-655 expression may significantly correlate with prognosis in ESCC. We could not analyze the prognostic significance of miR-655 expression in OSCC because complete survival data was not included in our clinical data. Finally, we analyzed miRNA-target associations at the mRNA level in ESCC and OSCC primary samples (Fig. S6A), however significant correlations were not found between miR-655 expression and expression of ZEB1 or TGFBR2 transcripts as well as a large number of miRNAs and their targets, indicating that not only miR-655 but also other unknown molecules including transcription factors might regulate ZEB1 and TGFBR2 expression. Similar to result in primary samples, there were no correlations between miR-655 expression and mRNA or protein expression of these targets in ESCC cell lines because of remarkable reduction of miR-655 expression in almost ESCC cell lines (Fig. S6B).

Characterization of ZEB1 and TGFBR2 as Novel Direct Targets of miR-655

We searched the websites microRNA.org (http://www.microRNA.org/) and Target Scan Human 6.2 (http://www.targetscan.org/) [39,40] for direct targets of miR-655, and focused on ZEB1 and TGFBR2 as potential candidates, respectively. Although expression levels of ZEB1 and TGFBR2 tended to be lower in pancreatic cancer cell lines and a breast cancer cell line relative to a normal pancreas (Fig. 4A), transcript and protein levels of these candidate genes were markedly reduced in mesenchymal-like pancreatic or breast cancer cells 96 hours after transfection with dsRNAs mimicking miR-655 (Fig. 4B). We obtained the same results using other sets of dsRNAs purchased from independent sources (Fig. S7). Moreover, in the luciferase reporter assay with vectors containing the wild type or a mutated 3’-UTR target site of ZEB1 (region 4) and TGFBR2 (region 1) downstream of the luciferase reporter gene, we detected statistically significant reductions in luciferase activity in wild type constructs, but not in mutant constructs (Fig. 4C and Fig. S8A and B), indicating that ZEB1 and TGFBR2 were novel direct targets of miR-655. We confirmed that the treatment with TGF-b could induce EMT accompanied by the upregulation of TGFBR2, TGFBR1, SNAI1/Snail, ZEB1, and PAI-1 and the downregulation of CDH1/E-cadherin at mRNA and protein levels in KP1N cells. Furthermore, overexpression of exogenous miR-655 significantly inhibited the upregulation and downregulation of these EMT-regulatory genes in KP1N cells treated with and without TGF-b, respectively (Fig. 4D and Fig. S9), whereas miR-655 could not change levels of phosphorylated Smad2/3 in these cells. Our findings strongly suggest that the TGF-b-induced EMT can be suppressed by miR-655, independently of miR-200 family members, through translational inhibition of ZEB1 and TGFBR2 in cancer cells (Fig. 4E).
Discussion

EMT plays a crucial role in many stages not only in embryonic development but also in cancer progression [3,6]. Cancer cells undergoing EMT are endowed with more aggressive phenotypes, such as mesenchymal and stem cell-like features, resulting in the acquisition of malignant properties, such as invasion, metastasis, recurrence, and drug resistance [4,5,41]. The evidence for EMT, including our own [32,35], led us to consider that the development of EMT inhibitors might provide opportunities for both prevention and treatment of cancer. Therefore, to identify EMT-suppressive miRNAs, we performed here the function-based

Figure 2. EMT-suppressive effects of miR-655 on mesenchymal-like cancer cells having phenotypic plasticity at EMT/MET. A. TaqMan real-time RT-PCR analysis of CDH1/E-cadherin and miR-655 in a panel of 23 pancreatic cancer cell lines and a breast cancer cell line, MDA-MB-231. Relative expression levels of transcripts of CDH1/E-cadherin and miR-655 were quantified in comparison to GAPDH and RNU6B, respectively, to normalize the initial input of total RNA. Bar graphs show the ratio of the expression level in these cell lines to that in normal pancreas (Ambion). B. Representative results of phase contrast images (Upper) and CDH1/E-cadherin protein expression level detected by immunofluorescence staining (Lower) in Panc1, KP1N, KP4-4 and MDA-MB-231 cells 96 hours after transfection with 10 nM of ds-NC or dsRNA mimicking miR-655 (ds-miR-655) (Ambion). C. TaqMan real-time RT-PCR analysis (Upper) and Western blot (Lower) analysis of mRNA and protein levels of CDH1/E-cadherin, respectively, in Panc1, KP1N, KP4-4 and MDA-MB-231 cells 96 hours after transfection of 10 nM of ds-NC or ds-miR-655. Asterisks (*), statistical analysis with the Mann-Whitney U test. D. Growth curves in Panc1, KP1N, KP4-4 and MDA-MB-231 cells after transfection of 10 nM of ds-NC or ds-miR-655. Each data point represents the mean of duplicate determinations (bars, SD) in these experiments. Asterisks (*), statistical analysis with the Mann-Whitney U test. E. Representative phase micrographs of Panc1, KP1N, KP4-4 and MDA-MB-231 cells transiently transfected with 10 nM of ds-NC or ds-miR-655 in cell migration and invasion assays in vitro using uncoated and Matrigel-coated transwell-chamber culture systems (Becton Dickinson), respectively. At 48 hours after transfection of dsRNA, cells were transferred into the upper chamber of the transwell (4×10⁴ cells per well). The migrating or invading cells on the lower surface of filters were fixed and stained with the Diff-Quik stain 48 hours after cell transfer. F. Quantification of the cell migration (Left) and invasion (Right) shown in Figure 3B. Bar graphs show the percentage (%) of miR-655-transfectants migrating (Left) or invading (Right) through uncoated or Matrigel-coated filters, respectively, relative to control-transfectants. Asterisks (*), statistical analysis with the Mann-Whitney U test.

doi:10.1371/journal.pone.0062757.g002
screening of 470 dsRNAs mimicking mature human miRNAs using mesenchymal-like cancer cells, Panc1.

Over the last few years, we have focused on miRNAs as key post-transcriptional regulators of gene expression and previously identified four tumor-suppressive miRNAs (TS-miRNAs) directly targeting oncogenic genes in oral squamous cell carcinoma (OSCC) and hepatocellular carcinoma (HCC) using expression- and DNA methylation-based screening, respectively [15,33,42]. In recent studies, we successfully performed function-based screening with a cell proliferation assay for 327 synthetic miRNAs and identified two TS-miRNAs directly targeting Rictor in OSCC and endometrial cancer (EC) [15,26,27]. In the present study, a unique cell-based reporter system for investigating CDH1/E-cadherin-promoter activity in the Panc1 cell line was established.

Figure 3. Expression analysis of miR-655 in primary ESCC and OSCC cases. A, TaqMan real-time RT-PCR analysis of endogenous miR-655 in 22 normal human tissues (Ambion and Clontech). Marked upregulation of miR-655 expression (>2-fold increase compared with pancreas) was observed in brain, cervix, esophagus and placenta. B, Expression profiles of miR-655 in a panel of paired tumorous and non-tumorous tissues from primary ESCC and OSCC cases. Bar graphs show the ratio of the expression level in tumors (T) to those in their paired normal mucosae (N). C Kaplan-Meier survival curves for high and low miR-655 groups based on TaqMan real-time RT-PCR. In univariate analyses of overall and non-recurrent survival with log-rank tests, a high level of miR-655 expression was significantly associated with a much better survival rate among patients with ESCC (P = 0.0359, log-rank test).

doi:10.1371/journal.pone.0062757.g003

Figure 4. Characterization of ZEB1 and TGFBR2 as novel direct targets of miR-655. A, Expression analysis for ZEB1 (top) and TGFBR2 (bottom) in a panel of 23 pancreatic cancer cell lines and a breast cancer cell line, MDA-MB-231, using TaqMan real-time RT-PCR. Relative expression levels of transcripts of ZEB1 and TGFBR2 were quantified in comparison to GAPDH to normalize the initial input of total RNA. Bar graphs show the ratio of the expression level in each cell line to that in a normal pancreas (Ambion). B, TaqMan real-time RT-PCR analysis and Western blot analysis of ZEB1 (left) and TGFBR2 (right) in Panc1, KP1N and MDA-MB-231 cells 96 hours after transfection with 10 nM of dsRNA mimicking miR-655 (ds-miR-655) or control non-specific miRNA (ds-NC) (Ambion). C, Confirmation of ZEB1 and TGFBR2 as direct targets of miR-655. Left, Schema of putative binding sites of miR-655 in the 3'-UTR region of ZEB1 and TGFBR2. Right, Results of luciferase reporter assays in Panc1 cells 48 hours after cotransfection of pMIR-REPORT luciferase vectors containing wild-type (Wt) or mutated (Mut) 3'-UTR target sites of ZEB1 or TGFBR2 for miR-655, ds-miR-655 or ds-NC, and pRL-CMV internal control vector. These sites were analyzed using microRNA.org and Target Scan Human 6.2. D, Suppressive effects of ds-miR-655 on TGF-b-induced EMT in KP1N cells. The results of Western blotting of TGFBR2, Smad1, ZEB1, E-cadherin, TGFBR1, Smad2/3 and phosphorylated Smad2/3 in KP1N cells 72 hours after treatment with or without TGF-b (5 ng/ml) and transfection with ds-miR-655 or ds-NC, simultaneously. TGF-b-treated cells were compared with untreated cells. E, Schema of regulation of the ZEB1-E-cadherin axis and TGF-b signaling pathway by miR-655 through downregulation of ZEB1 or TGFBR2 in cancer cells.

doi:10.1371/journal.pone.0062757.g004
and used in the function-based screening of EMT-suppressive miRNAs. In this system, the CDH1/E-cadherin-promoter was employed to monitor MET, because the transcription of CDH1/E-cadherin is known to be repressed during EMT and activated during MET [26]. Panc1 has already been used as an in vitro experimental model for assessing induction of EMT [35,36]. Since Panc1 was also confirmed to be a mesenchymal-like cell line with phenotypic plasticity at EMT/MET [35], we used it in the establishment of our cell-based reporter system. Actually, miR-200a, -200b and -200c were discovered as EMT-suppressive miRNAs in previous studies, emerged at the top of a list of results of screening, suggesting the present cell-based reporter assay to be a powerful tool for high-throughput function-based screening of miRNAs, siRNAs and chemical compounds having EMT-suppressive effects.

Here, we successfully identified miR-655 for the first time as a novel EMT-suppressive miRNA through function-based screening. This miRNA had mostly remained uncharacterized in the field of cancer research. The miR-655 gene is located within a non-coding region at 14q32.31, which harbors 50 intergenic miRNA genes within a limited region of 198 kb. Although notable copy number aberrations were not detected at 14q32.31 by our aCGH analyses using a panel of pancreatic cancer [29], ESCC [29] and OSCC [30,31] cell lines, the expression of miR-134 and miR-370 located at this locus was described to be significantly lower in gastrointestinal stromal tumors (GISTs) with 14q loss and also in GISTs with tumour progression [43]. In the present study, the expression of miR-655 was largely downregulated in a panel of pancreatic cancer, ESCC and OSCC cell lines, and a breast cancer cell line, MDA-MB-231. Moreover, we found a significant correlation between higher levels of miR-655 expression and a better survival rate in patients with ESCC, suggesting miR-655 expression to be a promising prognostic marker for ESCC.

In the present study, ZEB1 and TGFB2 were identified as direct targets of miR-655. These targets have been revealed to be major components of TGF-b signaling pathways and to induce EMT through repression of CDH1/E-cadherin [17,19,44]. ZEB1 was identified first as a strong predictor of poor survival and distant metastasis in colorectal adenocarcinoma, breast cancer [45,46], and lung adenocarcinoma [47]. High TGFB2 expression was also correlated with a shorter overall survival in estrogen receptor-negative breast cancer [48], miR-520c, miR-373, and miR-211 were described as miRNAs targeting TGFB2 and contributing to the induction of MET [25,49] although these three miRNAs were not identified as prime candidates of EMT-suppressive miRNA in our screening. The miR-200 family members are typical EMT-suppressive miRNAs targeting several components of TGF-b signaling pathways, including the miR-200-ZEB1-E-cadherin axis, which is crucial in EMT and was described to be deregulated in mesenchymal-like cancer cells [17,18,20]. Although the miR-200 family and miR-205, like miR-655, target ZEB1, their biological functions were found to differ from those of miR-653. First, besides ZEB1, TGFB2 was characterized as a direct target of miR-655 in our study, but not the miR-200 family and miR-205. Second, our studies past and present, have showed the mesenchymal-specific downregulation of miR-200 expression in a panel of OSCC [35] and pancreatic cancer cell lines, respectively, but not miR-205 and miR-655 expression. These differences between miR-655 and miR-200 family members indicate the biological function of each EMT-suppressive miRNA in physiological and pathophysiological processes, including EMT/MET. In addition, several components of TGF-b signaling pathways, TGFB2, Snail and ZEB1, were reduced directly or indirectly by overexpression of miR-655 in cancer cells treated with or without TGF-b. Recent studies have demonstrated that a miRNA significantly decreased signal output over time, by reducing the concentration of several components in a signaling cascade [50,51]. These results strongly support our findings that a single EMT-suppressive miRNA may target several EMT-inducible components of a specific signaling pathway and coordinate their expression. On the other hand, overexpression of miR-655, as well as the miR-200 family, induced significant morphologic changes and inhibited cell migration and invasion in 3 pancreatic cancer cell lines and a breast cancer cell line, MDA-MB-231. These observations suggest the EMT-suppressive effects of miR-655 to be essential for cancer progression.

In conclusion, we established a unique cell-based reporter system for monitoring the promoter activity of CDH1/E-cadherin. By using the system for the first time we identified miR-655 as a novel EMT-suppressive miRNA, the biological meaning of which was different from that of the miR-200 family. Overexpression of miR-655 remarkably increased E-cadherin expression and suppressed cell motility in several cancer cell lines, clearly indicating that this miRNA is a strong suppressor of EMT. In ESCC, miR-655 expression demonstrated a significant association with a better prognosis. Furthermore, ZEB1 and TGFB2, which are cardinal components of the TGF-b signaling pathway, were characterized as direct targets of miR-655. Our results suggest the potential of the EMT-suppressor miR-655 targeting ZEB1 and TGFB2 as a prognostic marker and therapeutic agent for cancer.

Supporting Information

Figure S1 Fluorescence micrographs of a stable Panc1 clone 96 hours after transient transfection with dsRNA in functional-based screening using Pre-miRTM miRNA Precursor Library - Human V3 (Ambion). The Panc1 clone was established by transfection with a reporter construct containing a promoter sequence of CDH1/E-cadherin in the 5’ upstream region of the Zeb1 reporter gene and cloning using limiting dilution (see Fig. 1A, and 1B). Each dsRNA was transfected individually into the clone. These 17 miRNA genes were selected as candidates for EMT-suppressive miRNAs in functional-based screening (see Table 1, Fig. 1C, and Table S2).

Figure S2 Expression profiles of known EMT-related genes, miR-141, -200a, -200b, -200c, -205 and VIM in a panel of 23 pancreatic cancer cell lines and a breast cancer cell line, MDA-MB-231 (see Fig. 2A and 4A). Bar graphs show the ratio of the expression level in these cell lines to that in a normal pancreas tissue (Ambion) by TaqMan real-time RT-PCR analysis.

Figure S3 Expression profiles of miR-655 in a panel of 43 ESCC cell lines. (A) and 18 OSCC cell lines (B). Bar graphs show the ratio of the expression level in ESCC and OSCC cell lines to that in normal esophageal tissue (Ambion). C. Expression profiles of miR-655 in normal esophagus and mammary gland, MCF7, MCF10A (human breast epithelial cells) and MDA-MB-231.

Figure S4 TaqMan real-time RT-PCR analysis (Upper) and Western blot (Lower) analysis of mRNA and protein levels of CDH1/E-cadherin, respectively, in TE8 and HSC2 cells 96 hours after transfection of 10 nM of ds-NC or ds-miR-655.

Figure S5 TaqMan real-time RT-PCR analysis (Upper) and Western blot (Lower) analysis for CDH1/E-cadherin in Panc1,
KP1N and MDA-MB-231 cells 96 hours after transfection of 10 nM of ds-NC or ds-miR-655 (Thermo Scientific Dharmacon). (PPT)

**Figure S6 A.** The correlations between miR-655 and ZEB1/ TGFBR2 on mRNA levels in ESCC/OSCC primary samples. **B.** The correlations between miR-655 and ZEB1/TGFBR2 on mRNA and protein levels. The quantification of each protein band in the result of Western blotting was done using LAS-3000 with MultiGauge software (GE Healthcare, Tokyo, Japan). Pearson’s test was performed to determine the degree of correlation between two variables. (PPT)

**Figure S7** TaqMan real-time RT-PCR analysis (Upper) and Western blot (Lower) analysis for ZEB1 (left) and TGFBR2 (right) in Panc1, KP1N and MDA-MB-231 cells 96 hours after transfection of 10 nM of ds-NC or ds-miR-655 (Thermo Scientific Dharmacon). (PPT)

**Figure S8 A.** Complementary miR-655 seed sequence and PCR region in the 3’ UTR of ZEB1 (Upper) and TGFBR2 (Lower). These sites were analyzed using microRNA.org and Target Scan Human 6.2. **B.** Results of luciferase reporter assays in Panc1 cells 48 hours after cotransfection of pMIR-REPORT luciferase vectors containing wild-type of ZEB1 or TGFBR2 for miR-655, ds-miR-655 or ds-NC, and the pRL-CMV internal control vector. Asterisks (*), statistical analysis with the Mann-Whitney U test. (PPT)

**Figure S9** TaqMan real-time RT-PCR analysis for CDH1/E-cadherin (left) and PAL-1 (right) in KP1N cells 96 hours after transfection of 10 nM of ds-NC or ds-miR-655 (Ambion). Cells were analyzed 72 hours after treatment with or without TGF-b (5 ng/ml) and transfection with ds-miR-655 or ds-NC, simultaneously. (PPT)

**Table S1** Primers used in this study. (XLS)

**Table S2** Summary of functional-based screening using 470 dsRNAs mimicking mature miRNAs (Pre-miR™ miRNA Precursor Library - Human V3, Ambion) and a stable Panc1 clone transfected with a reporter construct containing a promoter sequence of CDH1/E-cadherin in the 5’ upstream region of the Zeb1 promoter gene. (XLS)

**Table S3** Correlation between clinicopathological characteristics and status of miR-655 expression in primary ESCC cases. (XLS)

**Acknowledgments**
We thank Ayako Takahashi and Rumi Mori for their skillful technical assistance. We are also grateful to Erina Tomouchi (an undergraduate student in Faculty of Dentistry, Tokyo Medical and Dental University) for her invaluable technical support in the establishment of our cell-based reporter system.

**Author Contributions**
Conceived and designed the experiments: JI KK. Performed the experiments: YH. Contributed reagents/materials/analysis tools: NU TK KH. Provided technical support: TM HE.

**References**
1. Greenburg G, Hay ED (1982) Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. J Cell Biol 95: 333–339.
2. Thiery JP (2002) Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2: 442–454.
3. Thiery JP, Acloque H, Huang HY, Nieto MA (2009) Epithelial-mesenchymal transitions in development and disease. Cell 139: 871–890.
4. Itozaki M, Minena S, Yokobori T, Iida H, Bepari A, et al. (2010) Epithelial-mesenchymal transition in cancer development and its clinical significance. Cancer Sci 101: 293–299.
5. Micluzi DS, Farabaugh SM, Ford HL (2010) Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. J Mammary Gland Biol Neoplasia 15: 117–134.
6. Kalluri R, Weinberg RA (2009) The basics of epithelial-mesenchymal transition. J Clin Invest 119: 1420–1428.
7. Zhang J, Ma L (2012) MicroRNA control of epithelial-mesenchymal transition and metastasis. Cancer Metastasis Rev.
8. Li Y, VandenBoom TG, Kong D, Wang Z, Ali S, et al. (2009) Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in cancer cells. Cancer Res 69: 6704–6712.
9. Sahabia M, Emmadi S, Reddiugh G, Julian S, Prevost G, et al. (2008) Molecular signature and therapeutic perspective of the epithelial-to-mesenchymal transition in epithelial cancers. Drug Resist Update 11: 123–131.
10. Ambros V (2004) The functions of animal microRNAs. Nature 431: 350–355.
11. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281–297.
12. He L, Hanlon GJ (2004) MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 5: 522–531.
13. Esquela-Kerscher A, Slack FJ (2006) Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer 6: 259–269.
14. Onoda H, Takahashi T (2007) MicroRNAs in biological processes and carcinogenesis. Carcinogenesis 28: 2–12.
15. Kozaki K, Inazawa J (2012) The Zeb1/miR-200 feedback loop controls Notch signalling in cancer cells. EMBO J 30: 770–782.
16. Suzuki E, Imoto I, Pimkhaokham A, Nakagawa T, Kamata N, et al. (2007) miR-655 inhibits EMT in cancer cells. Mol Cell 40: 841–849.
17. Bracken CP, Gregory PA, Kolesnikoff N, Bert AG, Wang J, et al. (2008) A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. Cancer Res 68: 7846–7854.
18. Gregory PA, Bracken CP, Smith E, Bert AG, Wright JA, et al. (2011) An autocrine TGF-beta/ZEB/miR-200 signaling network regulates establishment and maintenance of epithelial-mesenchymal transition. Mol Biol Cell 22: 1684–1689.
19. Okano H, Miyazaki T, Kinoshita K, Yamada Y, Yoshida T (2010) Functional screening identifies a microRNA, miR-491 that induces apoptosis by targeting Bel-4XL in colorectal cancer cells. Int J Cancer 127: 1072–1080.
20. Levy C, Khade M, Ilopoulos D, Janas MM, Schulten S, et al. (2010) Intronic miR-211 assumes the tumor suppressive function of its host gene in melanoma. Mol Cell 40: 841–849.
21. Burk U, Schubert J, Wellner U, Schmalhofer O, Vincan E, et al. (2008) A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep 9: 582–589.
22. Brabletz S, Rajlich K, Meidolf S, Burk U, Niedermann G, et al. (2011) The ZEB1/miR-200 feedback loop controls Notch signalling in cancer cells. EMBO J 30: 770–782.
23. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanad E, et al. (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 133: 704–715.
24. Nakano H, Miyazawa T, Kinoshita K, Yamada Y, Yoshida T (2010) Functional screening identifies a microRNA, miR-491 that induces apoptosis by targeting Bel-4XL in colorectal cancer cells. Int J Cancer 127: 1072–1080.
25. Levy C, Khade M, Ilopoulos D, Janas MM, Schulten S, et al. (2010) Intronic miR-211 assumes the tumor suppressive function of its host gene in melanoma. Mol Cell 40: 841–849.
26. Tsuruta T, Kozaki K, Uesugi A, Furuta M, Hirasawa A, et al. (2011) The tumor suppressor miR-218 targets the mTOR component Rictor and inhibits AKT phosphorylation in oral cancer. Cancer Res 71: 5650–5662.
27. Uesugi A, Kozaki K, Tsuruta T, Furuta M, Hirasawa A, et al. (2011) The tumor suppressor microRNA miR-218 targets the mTOR component Rictor and inhibits AKT phosphorylation in oral cancer. Cancer Res 71: 5650–5662.
28. Suzuki A, Shibata T, Shimada Y, Murakami Y, Horii A, et al. (2008) TGF-beta/ZEB/miR-200 signaling network regulates epithelial-mesenchymal transition in cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes Dev 22: 894–907.
29. Burk U, Schabert J, Wellner U, Schmalhofer O, Vincan E, et al. (2008) A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep 9: 582–589.
30. Suzuki A, Shibata T, Shimada Y, Murakami Y, Horii A, et al. (2008) Bcl-X(L) in colorectal cancer cells. Int J Cancer 127: 1072–1080.
31. Haruki S, Imoto I, Kozaki K, Matsui T, Kawachi H, et al. (2010) Frequent silencing of protocadherin 17, a candidate tumour suppressor for esophageal squamous cell carcinoma. Carcinogenesis 31: 1027–1036.
squamous-cell carcinomas by aberrant promoter hypermethylation. Oncogene 26: 7921–7932.

31. Nakamura E, Kozaki K, Tsuda H, Suzuki E, Pimkhaokham A, et al. (2008) Frequent silencing of a putative tumor suppressor gene melatonin receptor 1A (MTNR1A) in oral squamous-cell carcinoma. Cancer Sci 99: 1390–1400.

32. Ono H, Imoto I, Kozaki K, Tsuda H, Matsui T, et al. (2012) SIX1 promotes epithelial-mesenchymal transition in colorectal cancer through ZEB1 activation. Oncogene.

33. Kozaki K, Imoto I, Mogi S, Omura K, Inazawa J (2008) Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in oral cancer. Cancer Res 68: 2094–2105.

34. Berx G, van Roy F (2009) Involvement of members of the cadherin superfamily in cancer. Cold Spring Harb Perspect Biol 1: a003129.

35. Kurasawa Y, Kozaki K, Pimkhaokham A, Muramatsu T, Ono H, et al. (2012) Stabilization of phenotypic plasticity through mesenchymal-specific DNA hypermethylation in cancer cells. Oncogene 31: 1963–1974.

36. Yu J, Ohuchida K, Mizumoto K, Sato N, Kayashima T, et al. (2010) MicroRNA, hsa-miR-200c, is an independent prognostic factor in pancreatic cancer and its upregulation inhibits pancreatic cancer invasion but increases cell proliferation. Mol Cancer 9: 169.

37. Wellner U, Schubert J, Burk UC, Schmalhofer O, Zhu F, et al. (2008) The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. Nat Cell Biol 11: 1487–1495.

38. Korpalo M, Lee ES, Hu G, Kang Y (2008) The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. J Biol Chem 283: 14910–14914.

39. Betel D, Wilson M, Gabow A, Marks DS, Sander C (2008) The microRNA.org resource: targets and expression. Nucleic Acids Res 36: D149–153.

40. Betel D, Koppal A, Agius P, Sander C, Leslie C (2010) Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. Genome Biol 11: R90.

41. Singh A, Settleman J (2010) EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. Oncogene 29: 4741–4751.

42. Furuta M, Kozaki KI, Tanaka S, Arii S, Imoto I, et al. (2010) miR-124 and miR-203 are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma. Carcinogenesis 31: 766–776.

43. Haller F, von Heydebreck A, Zhang JD, Gunawan B, Langer C, et al. (2010) Localization- and mutation-dependent microRNA (miRNA) expression signatures in gastrointestinal stromal tumours (GISTs), with a cluster of co-expressed miRNAs located at 14q32.31. J Pathol 220: 71–86.

44. Li I, Shi JY, Zhu GQ, Shi B (2012) MiR-17–92 cluster regulates cell proliferation and collagen synthesis by targeting TGFβ pathway in mouse palatal mesenchymal cells. J Cell Biochem 113: 1235–1244.

45. Spaderna S, Schmalhofer O, Hlubek F, Berx G, Eger A, et al. (2006) A transient, EMT-linked loss of basement membranes indicates metastasis and poor survival in colorectal cancer. Gastroenterology 131: 830–840.

46. Spaderna S, Schmalhofer O, Hlubek F, Dammert A, Bauer K, et al. (2008) The transcriptional repressor ZEB1 promotes metastasis and loss of cell polarity in cancer. Cancer Res 68: 537–544.

47. Liu Y, Yan X, Liu N, Zhou J, Liu J, et al. (2012) Lentivirus-delivered ZEB-1 small interfering RNA inhibits lung adenocarcinoma cell growth in vitro and in vivo. J Cancer Res Clin Oncol.

48. Buck MB, Fritz P, Dippon J, Zugmaier G, Knabbe C (2004) Prognostic significance of transforming growth factor beta receptor II in estrogen receptor-negative breast cancer patients. Clin Cancer Res 10: 491–498.

49. Keklikoglou I, Koerner C, Schmidt C, Zhang JD, Heckmann D, et al. (2011) MicroRNA-520/373 family functions as a tumor suppressor in estrogen receptor-negative breast cancer by targeting NF-κB and TGF-β signaling pathways. Oncogene.

50. Linsley PS, Schelter J, Burchard J, Kubokawa M, Martin MM, et al. (2007) Transcripts targeted by the microRNA-16 family cooperatively regulate cell cycle progression. Mol Cell Biol 27: 2240–2252.

51. Ebert MS, Sharp PA (2012) Roles for microRNAs in conferring robustness to biological processes. Cell 149: 515–524.