Regulation of microRNA expression by hepatocyte growth factor in human head and neck squamous cell carcinoma

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Hepatocyte growth factor (HGF) is a multifunctional molecule that acts as mitogen, motogen, and/or morphogen in a variety of cells. MET, a specific receptor tyrosine kinase for HGF, is upregulated in various tumors including squamous cell carcinoma of the human head and neck (HNSCC), but how HGF affects the expression of downstream functional genes has not yet been elucidated in detail. In the present study, we examined the expression of microRNA (miRNA), non-coding small RNA that regulate cell proliferation and functions by interfering with the translation of target mRNA, with or without HGF stimulation in HNSCC cell line HSC3. Among several miRNAs, in which the expression was altered after HGF stimulation, we focused on miR-200c and miR-27b, both of which were drastically downregulated after HGF stimulation. Expression of ZEB1, a target mRNA for miR-200c, was upregulated 3 and 6 h after HGF stimulation, and that of E-cadherin, a downstream molecule of ZEB1, was downregulated 12 h after HGF stimulation. Expression of ST14/matriptase, an enzyme for extracellular matrix (ECM) degradation and HGF activation and a target mRNA for miR-27b, was drastically upregulated in the protein level after HGF stimulation, although it was not statistically altered in the mRNA level. These results suggest that miR-200c and miR-27b downregulated by HGF might play an important role in epithelial–mesenchymal transition mediated by ZEB1/E-cadherin and ECM degradation and HGF autoactivation mediated by ST14/matriptase, respectively. Altered expression of miRNA directly regulated by HGF might contribute enhanced progressive and invasive characteristics of HNSCC by regulating the translation of HGF-induced functional molecules. (Cancer Sci 2011; 102: 2164–2171)

Squamous cell carcinoma (SCC) is a common malignant tumor in the head and neck and constitutes 90% of malignancies in these regions.1,2 The incidence and mortality of head and neck SCC (HNSCC) is increasing despite intense efforts, and its 5-year survival rate is <50%.3,4 Several growth factors are considered to contribute to the carcinogenesis and progression of HNSCC.5 One of these factors, hepatocyte growth factor (HGF), is a multifunctional growth factor that acts as mitogen, motogen and/or morphogen in a variety of cells including squamous epithelial cells.4,5,6 MET, a specific receptor tyrosine kinase for HGF, is upregulated in various tumors including human HNSCC, and its signal transduction to the nucleus induces the expression of genes involving the progressive and invasive characteristics of HNSCC.5,7,8 However, how HGF affects downstream functional gene expression has not yet been elucidated in detail.

MicroRNA (miRNA) are non-coding small RNA (21–25 nucleotides) that regulate post-transcriptional gene expression by interfering with the translation of target mRNA.8,9 One miRNA might regulate the expression of several genes and over one-third of all protein-coding genes might be under translational control by miRNA.9 MiRNA are involved in a variety of cellular processes, including the regulation of cellular differentiation, proliferation and apoptosis.9,10 aberrant expression of miRNA is known to induce various human malignancies10–13 and they are known to be clearly classified by miRNA expression profiles.13,14

In the present study, we examined the expression of miRNA before and after HGF stimulation in HNSCC cell line HSC3, and showed the altered expression of several miRNA after HGF stimulation. We focused on miR-200c and miR-27b, both of which were drastically downregulated after HGF stimulation, because of their unique target genes involving downstream functional molecules directly regulated by HGF.

Materials and Methods

Reagents. Recombinant human HGF was obtained from the Research Center of Mitsubishi Tanabe Pharma Corporation (Yokohama, Japan). Antibodies used for immunohistochemistry and immunoblot analyses were as follows: anti-human E-cadherin (BD Transduction Laboratories, Flanklin Lakes, NJ, USA) and MET (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) mouse monoclonal antibodies, and anti-human ZEB1 (Abcam plc, Cambridge, UK), ST14/matriptase (Abcam plc) and β-actin (Cell Signaling Technology Inc., Danver, MA, USA) rabbit polyclonal antibodies. Anti-phospho-MET rabbit polyclonal antibody was prepared previously.15 MET kinase specific inhibitor SU11274 ([(3Z)-N-(3-chlorophenyl)-3-[(3,5-dimethyl-4-((4-methylpiperazin-1-yl)carbonyl)-1H-pyrrol-2-yl)methylene]-N-methyl-2-oxoindoline-5-sulfonamide] was purchased from Selleck Chemicals (Houston, TX, USA).

Human tissue samples and immunohistochemistry. Human tissue samples including HNSCC and adjacent normal squamous epithelial tissues were obtained from surgical specimens of patients with HNSCC in the University of Fukui Hospital, Fukui, Japan. Informed consent was obtained from patients before surgery and the protocol was approved by the ethical board of the Faculty of Medicine, University of Fukui. The tissue samples were routinely fixed with formalin, embedded with paraffin and stained with hematoxylin–eosin (HE). For immunohistochemistry, the sections were processed for antigen retrieval (autoclaving in 10 mM citrate buffer, pH 6.0 for 5 min), followed by treatment with 3% H2O2 in PBS for 10 min and washed in PBS twice. After blocking in Protein Block Serum-Free (Dako, Glostrup, Denmark) for 1 h at room temperature, the

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sections were incubated with primary antibodies for 16 h at 4°C. The sections were then washed in PBS and incubated with Envision-labeled polymer reagent (Dako) for 45 min at 37°C. The reaction was revealed with nickel, cobalt-3,3’-diaminobenzidine (Immunopure metal enhanced DAB substrate kit; ThermoFisher Scientific Inc. (Waltham, MA, USA) and the sections were counterstained with hematoxylin. In addition, frozen sections were prepared for mRNA extraction from some tissue samples. After staining with Cresyl Violet Stain (Applied Biosystems, Carlsbad, CA, USA), they were divided into SCC portions, adjacent normal squamous epithelia and non-epithelial stromal portions using a Laser-captured microdissection (LCM) system (Leica Microsystems, Wetzler, Germany) according to the manufacturer’s protocol.

**Cell lines and culture.** Two human HNSCC (tongue) cell lines, HSC3 and SAS, were obtained from RIKEN cell bank (Tsukuba, Japan). They were cultured in RPMI-1640 media (Sigma-Aldrich, St Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS) (Nichirei Biosciences, Tokyo, Japan) and 1% penicillin–streptomycin (Gibco Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere containing 5% CO2.

**Hepatocyte growth factor stimulation assay and extraction of total RNA.** For HGF stimulation assay, HSC3 and SAS cells were seeded at a density of 2.5 × 10^4/cm² in six-well plates. After culturing for 3 days, a designated concentration of HGF was added into the medium and the cells were cultured continuously for designated hours. Total RNA from human tissues and cultured cells was extracted using NucleoSpin RNA XS (TAKARA BIO Inc., Otsu, Japan) and TRIzol reagent (Gibco Invitrogen, respectively). They were quantified with NanoDrop spectrometer (NanoDrop Technology, Wilmington, DL, USA) and subjected to microarray, RT-PCR or quantitative RT-PCR (qRT-PCR) analysis. For the MET inhibition assay, the cells were treated with 10 mM SU11274 for 6 h before culturing with or without HGF stimulation (10 ng/mL). For the cell proliferation assay, HSC3 and SAS cells were seeded on 96-well plates and cultured with or without HGF. After the cells were cultured for 2 days and observed using phase-contrast micrographs, MTT reagent (Dojindo Molecular Technologies Inc., Kumamoto, Japan) was added in the medium (400 µg/mL) and the cells were incubated for 1 h further. The insoluble formazan product was extracted with an SDS-dimethyl formamide mixture. 200 µL of the solution was transferred to 96-well plates and absorbance was measured at a wavelength of 570 nm with a background reading at 660 nm on a spectrophotometric plate reader SPECTRA MAX 250 (Molecular Devices Inc., Sunnyvale, CA, USA).

**MicroRNA microarray analysis.** The quality of the microRNA was checked using Agilent 2100 bioanalyzer (Agilent technologies, Santa Clara, CA, USA) to make sure that the RNA lacked DNA contamination and that the RNA was not degraded. Sample labeling and miRNA microarray analysis were performed according to the miRXplore Universal Reference Service of Miltenyi Biotec Inc. (Bergisch Gladbach, Germany). Each microarray assay was performed three times using different sets of miRNA extracts and quantile normalization was performed.

**RT-PCR and qRT-PCR analyses.** Total RNA extracted from tissue samples were reverse-transcribed by pd(N)₆ random and oligo(dT)₁₂ mixed primers and SuperScript III reverse transcriptase (Gibco Invitrogen). They were subjected to PCR for 35 cycles (30 s at 94°C, 30 s at 60°C and 1 min at 72°C) using Takara Ex Taq Hot Start Version (TAKARA BIO Inc.). The primer sequences used were as follows: HGF (sense), 5’-GAAATGCGA- AACAGGTTTC-3’ and HGF (antisense), 5’-CAAAA- TCATCCAGGACGAC-3’; c-met (sense), 5’-TGTCGCCAGA- ATGGTCATAA-3’ and c-met (antisense), 5’-GGGAAGGAGG- TGGTACAACA-3’; β-actin (sense), 5’-CCAACCGCGGAGA- GATGA-3’ and β-actin (antisense), 5’-TCCATACGAGTCG- CAGTG-3’. Total RNA from cultured cells were subjected to Quantitative two-step RT-PCR according to the manufacturer’s manual (Applied Biosystems). The kits used were TaqMan MicroRNA Assay (for has-miR-27b, has-miR-200c and U6 small nuclear RNA as an inner control), TaqMan Gene Expression Assay (for ZEB1, E-cadherin, ST14/matriptase and GAPDH as an inner control), TaqMan MicroRNA Reverse Transcription kit and TaqMan Universal PCR Master Mix (Applied Biosystems). Real-time qRT-PCR was performed using StepOne Real-Time PCR System (Applied Biosystems). Reverse transcription was performed under the following conditions: 16°C for 30 min, 42°C for 30 min and 85°C for 5 min with a sample volume of 15 µL. Real-time PCR was performed as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min with a sample volume of 20 µL. The expression level was normalized to GAPDH or U6 expression used as an inner control and was determined using the following processes: the average C_i of the inner control (C_i inner) and the sample C_i were determined. This value was then entered into this formula: 2^(-ΔC_i) (C_i inner). Each qRT-PCR assay was performed three times using different sets of total RNA extracts and the value was expressed as an average of the three.

**Protein extraction and immunoblotting.** Cultured cells with or without HGF stimulation were lysed using CellLytic Cell Lysis Reagent (Sigma-Aldrich) and the cellular proteins were extracted. Samples were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using 4–12% gradient gel and transferred onto an Immobilon membrane (Millipore, Billerica, MA, USA). After blocking with 5% non-fat dry milk in 50 mM Tris-HCl (pH 7.5), 150 mM sodium chloride and 0.05% Tween 20 (TBS-T), the membrane was probed with each antibody in a designated concentration at 4°C overnight. After washing in TBS-T, the membrane was incubated with peroxidase-conjugated secondary antibody with 1% BSA/TBS-T for 30 min at room temperature. The labeled proteins were visualized with a chemiluminescence reagent ECL Plus (GE Healthcare, Buckinghamshire, UK) and the intensities were analyzed using a ImageQuantLAS4000 luminemaging analyzer (GE Healthcare). For the internal control of loading, samples were also probed with anti-β-actin. Each immunoblot was performed three times using different sets of protein extracts and the value was expressed as an average of the three.

**Transfection of miRNA into HSC3 cells.** Human non-processed miR-200c (Pre-miR has-mir-200c) and miR-27b (Pre-miR has-mir-27b) constructs and their control randomized construct (Pre-miR miRNA Precursor Negative Control) were purchased from Applied Biosystems. They were transfected using siPORT Amin Transfection Agent (Applied Biosystems) into HSC3 cells just after HGF stimulation. Cellular proteins were extracted similarly as described above and subjected to SDS-PAGE followed by immunoblotting.

**Invasion assay.** Invasion assay was achieved using 24-well BD Biocat Matrigel invasion chambers with 8-µm polycarbonate filters (Becton Dickinson, Bedford, MA, USA). Five thousand HSC3 cells transfected with miR-200c, miR-27b or control randomized miRNA were seeded on chamber plates and cultured with or without HGF (10 ng/mL) for 20 h. Invasive cells were stained with Giemsa solution and the number was counted under light microscope. The value (% invasion) was expressed as an average of three times.

**Statistical analysis.** Statistical parameters were assessed using the MEDCALC software program (MedCalc software, Mariakerke, Belgium) and significance was determined using either Mann–Whitney’s U-test or one-way analysis of variance. A P-value < 0.05 was considered to be significant. The values were expressed as the mean of three times with standard errors (SE).
Results

Expression of MET and HGF in HNSCC tissues and cell lines. In order to clarify the expression of MET and HGF, we performed RT-PCR and immunohistochemical analyses in five HNSCC tissue samples, and the expression was compared with adjacent normal squamous epithelia and non-epithelial stromal tissues. As shown in Figure 1(A), the expression of MET mRNA was found in all of these samples. Immunohistochemically, MET protein was stained in the cell surface of HNSCC cells, in the basal cells of normal squamous epithelium and in some stromal cells, probably endothelial cells (Fig. 1B). Hepatocyte growth factor was only detected in non-epithelial stromal cells but not in HNSCC and adjacent normal squamous epithelium both in RT-PCR and immunohistochemical analyses (Fig. 1). Next we examined the expression of MET and the response to HGF stimulation in two HNSCC cell lines, HSC3 and SAS. As shown in Figure 2(A), MET mRNA was abundantly expressed in both cell lines. In phase-contrast microscopy, both cells were obviously scattered 3 days after HGF stimulation (Fig. 2B). The cell growth ratio was also upregulated after HGF stimulation in SAS cells, but not in HSC3 cells (Fig. 2C). In order to simplify the analysis of miRNA regulation after HGF stimulation, we decided to use HSC3 cells in the following experiments because HGF signaling was thought to be involved in the invasive characteristics, such as scattering, but not in the proliferation of

Fig. 1. Expression of MET and hepatocyte growth factor (HGF) in human head and neck squamous cell carcinoma (HNSCC), adjacent normal squamous epithelial cells and non-epithelial stromal cells by RT-PCR analysis (A) and immunohistochemistry (B). Expression of MET mRNA was found in all HNSCC, normal squamous epithelial cells and stromal cells. Immunohistologically, MET protein was found in the cell surface of HNSCC cells, basal cells of normal squamous epithelium and some stromal cells (endothelial cells). HGF was detected only in non-epithelial stromal cells (insets in [B]), but not in HNSCC or adjacent normal squamous epithelium in both RT-PCR and immunohistochemical analyses. Bar, 100 μm.

Fig. 2. Expression of MET mRNA and the response to hepatocyte growth factor (HGF) stimulation in human head and neck squamous cell carcinoma (HNSCC) cell lines. Expression of MET mRNA was found in both SAS and HSC3 cell lines (A). In phase-contrast microscopy, both cell lines were obviously scattered 3 days after HGF stimulation (B). The cell growth ratio was also upregulated after HGF stimulation in SAS cells, but not in HSC3 cells (C). Expression of MET receptor tyrosine kinase protein and its phosphorylation after HGF stimulation in HSC3 cells were clarified by immunoblot analysis (D).
HSC3 cells. Expression of MET receptor tyrosine kinase protein and its phosphorylation after HGF stimulation in HSC3 cells were clarified by immunoblot analysis (Fig. 2D).

**Differential expression of miRNA in HSC3 cells before and after HGF stimulation by microarray analysis.** Next we examined the expression of miRNA before and after HGF stimulation using miRNA microarray analysis. Microarray was performed three times using different sets of miRNA extracts and the results were averaged and normalized with inner controls. Significantly upregulated (>3.0-fold increases) or downregulated (<0.5-fold increases) miRNA in HSC3 cells after HGF stimulation are listed in Table 1. Among these differentially expressed miRNA, we selected miR-200c and miR-27b for further study because of their unique target mRNA relative to epithelial–mesenchymal transition (EMT), and a cell surface proteinase for ECM degradation and HGF activation, respectively, but not to cellular proliferation.\(^{(16,17)}\) Net intensities of miR-200c and miR-27b after HGF stimulation were 0.50 and 0.80, and they were decreased 0.25-fold and 0.44-fold compared with those before HGF stimulation, respectively (Table 1).

**Downregulation of miR-200c and miR-27b after HGF stimulation validated by qRT-PCR analysis.** In order to validate the differential expression of miR-200c and miR-27b after HGF stimulation, we next performed real-time qRT-PCR to quantitate the expression levels of these miRNA before and 3, 6, 12 and 24 h after HGF stimulation in HSC3 cells. As shown in Figure 3(A), miR-200c expression was drastically downregulated 3 and 6 h after HGF stimulation \((P < 0.01)\) and then gradually recovered 12 and 24 h after stimulation. After the downregulation of miR-200c, miR-27b expression was downregulated maximally 6 h after HGF stimulation \((P < 0.01)\) and then gradually recovered 12 and 24 h after stimulation. Downregulation of miR-200c and miR-27b by HGF stimulation was almost completely inhibited by adding MET kinase-specific inhibitor SU11274 before HGF stimulation (Fig. 3B).

**Expression of target mRNA and the translated proteins for miR-200c and miR27b.** Next we examined the expression of known target mRNA for miR-200c and miR-27b by qRT-PCR analysis. As shown in Figure 4(A), the expression of ZEB1, a transcription factor and known target mRNA for miR-200c,\(^{(16)}\) was upregulated 6 h after HGF stimulation \((P < 0.05)\) and then gradually recovered up to 24 h after stimulation. The expression of E-cadherin, an adhesion molecule for epithelial cells and a downstream functional molecule for ZEB1,\(^{(16,18)}\) was downregulated 12 h after HGF stimulation \((P < 0.01)\). This result was also clarified as upregulation of ZEB1 \((2.80\text{-fold increase}, P < 0.05)\) and downregulation of E-cadherin \((0.73\text{-fold increase}, P < 0.05)\) in the protein level using immunoblot analysis (Fig. 4B,C). In contrast, mRNA expression of ST14/matriptase, an enzyme for ECM degradation and HGF activation and a known target mRNA for miR-27b,\(^{(17)}\) was not statistically upregulated after HGF stimulation (Fig. 4A). However, in the protein level, ST14/matriptase was drastically upregulated \((1.91\text{-fold increase}, P < 0.01)\) by western blot analysis (Fig. 4B,C).

**Inhibition of target molecule regulation and invasion by miR-200c and miR-27b transfection.** In order to clarify the target molecule regulation by miR-200c and miR-27b, we then performed the transfection of miR-200c and miR-27b into HSC3 cells. Statistically significant alteration of ZEB1, E-cadherin and ST14/matriptase expression after HGF stimulation \((P < 0.01)\) completely disappeared in HSC cells transfected with exogenous miR-200c or miR-27b (Fig. 5). Expression of ZEB1 (a target for miR-200c) was 146%, E-cadherin (a downstream molecule of ZEB1) was 130% and ST14/matriptase (a target for miR-27b) was 93% recovered compared with those of control HSC3 cells after HGF stimulation. Invasion assay showed that transfection of HSC3 cells with miR-200c or miR-27b partly suppresses the invasion induced by HGF stimulation (Fig. 6).

**Discussion**

MicroRNA are non-coding, small 21–25 nucleotide-length RNA that regulate cellular proliferation and function by interfering with the translation of target mRNA.\(^{(5)}\) Transcriptional control of gene expression is also reported to be controlled in part by miRNA.\(^{(13)}\) Recent studies have demonstrated that altered expression of several miRNA induces various human malignancies.\(^{(10–13)}\) Expression profiles have been established for many different cancers and seem to be unique to each cancer.\(^{(13,14)}\) In HNSCC, several profiles of miRNA expression using miRNA microarray analysis have been reported\(^{(20–23)}\) and we also showed miR-205 and miR-21 as specific markers for HNSCC in

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**Table 1. Differential expression of microRNA (miRNA) before and after hepatocyte growth factor (HGF) stimulation in HSC3 cells by statistical analysis of the miRNA microarray**

| miRNA   | Putative target gene | HGF(-) | HGF(+) | Fold increases | Reference |
|---------|----------------------|--------|--------|----------------|-----------|
|         | Downregulation (<0.5-fold) |
| let-7a  | RAS                  | 1.98   | 0.30   | 0.16           | Johnson et al.\(^{(37)}\) |
| miR-23a | MYC                  | 7.82   | 1.44   | 0.20           | Gao et al.\(^{(42)}\) |
| miR-205 | MED1                 | 39.34  | 9.68   | 0.25           | Mouillet et al.\(^{(43)}\) |
| miR-200c| ZEB1                 | 2.08   | 0.50   | 0.25           | Gregory et al.\(^{(16)}\) |
| miR-27a | ZBTB1                | 4.95   | 1.77   | 0.36           | Mertens-Talcott et al.\(^{(44)}\) |
| miR-27b | ST14/ matriptase     | 1.78   | 0.80   | 0.44           | Wang et al.\(^{(17)}\) |
| miR-16  | BCL2                 | 3.00   | 1.31   | 0.47           | Calin et al.\(^{(40)}\) |
|         | Upregulation (>3.0-fold) |
| miR-200a| ZEB2, CTNNB1         | 0.04   | 0.14   | 3.89           | Gregory et al.\(^{(16)}\) |
| miR-141 | ZEB2                | 0.49   | 5.12   | 11.90          | Bracken et al.\(^{(18)}\) |

Each value is shown in net intensity after quantile normalization.
However, transcriptional regulation of these miRNA has not yet been elucidated in detail. Hepatocyte growth factor (HGF) is a pleiotropic molecule that acts as mitogen, motogen and/or morphogen in a variety of cells including normal squamous epithelial cells and squamous carcinoma cells.\(^4\)–\(^6\) It is also known to be a scatter factor (SF)\(^4\) and involved in tumor–stromal interactions and EMT.\(^{25,26}\) MET, a specific receptor tyrosine kinase for HGF\(^\parallel\)SF, is upregulated in various tumors including human HNSCC and its signal transduction induces progression and invasiveness of HNSCC in a paracrine or autocrine manner.\(^5\)–\(^7\) Indeed, it has been reported that HGF/SF promotes cell migration and angiogenesis in human esophageal SCC,\(^12\) upregulates the expression of proangiogenic cytokines interleukin (IL)-8 and vascular endothelial growth factor (VEGF) through the activation of mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) and phosphatidylinositol 3\(^\parallel\)kinase (PI3K) signaling pathways in HNSCC,\(^28\) and induces the invasion of oral SCC by matrix metalloproteinase genes through the upregulation of the transcription factor E1AF.\(^29\) However, how HGF/SF and MET signaling cascade affects the expression of each specific downstream functional gene has not yet been elucidated in detail. In addition, there is a growing number of reports that describe the translational regulation of growth factors or their receptors, including HGF and MET, in cancer by miRNA,\(^30,31\) but less reports describe the regulation of miRNA expression by a growth factor itself to date.\(^32\) The present study clearly demonstrates that several miRNA affecting the translation of HGF-induced downstream functional molecules could also be regulated by HGF itself. Thus, the HGF signaling cascade might be involved in tumor progression and invasion through MET receptor tyrosine kinase in a paracrine or autocrine manner by directly regulating the transcription of downstream functional molecules as well as by interfering with the translation of these molecules through miRNA regulated by HGF.

In the present study, we found the altered expression of several miRNA after HGF stimulation. We focused on two miRNA, miR-200c and miR-27b, both of which were drastically downregulated after HGF simulation, because of their unique target mRNA affecting HGF-induced functional molecules. The target mRNA of miR-200c has been reported to be ZEB1, a transcriptional regulator of E-cadherin.\(^16,18\) ZEB1 mRNA was upregulated 6 h after HGF stimulation and its downstream functional
molecule E-cadherin mRNA was downregulated 12 h after HGF stimulation. These results suggest that HGF has an important role for EMT with cancer cell migration and scattering through the downregulation of miR-200c. Indeed, another HNSCC cell line, SAS cells, also shows marked downregulation of miR-200c after HGF stimulation (0.27-fold increase; data not shown).

**Fig. 5.** Inhibition of target molecule regulation in HSC3 cells transfected with miR-200c or miR-27b. Altered expression of target molecules was recovered after hepatocyte growth factor (HGF) stimulation when exogenous miR-200c or miR-27b was overexpressed in HSC3 cells (A). The mean extent of expression after HGF stimulation was 146% (ZEB1), 130% (E-cadherin) and 93% (ST14/matriptase) recovered compared with that in HSC3 cells transfected with control randomized miRNA (B). Statistical alteration of the expression after HGF stimulation completely disappeared when exogenous miR-200c or miR-27b was transfected into HSC3 cells (**P > 0.01).

**Fig. 6.** Suppression of the invasion in HSC3 cells transfected with miR-200c or miR-27b. HSC3 cells transfected with miR-200c or miR-27b after hepatocyte growth factor (HGF) stimulation (10 ng/mL) partly suppressed the invasion compared with those with control randomized miRNA.
In the present study, upregulated miRNA after HGF stimulation was limited. Among them, miR-200a and miR-141 were significantly upregulated (see Table 1), both of which are considered to regulate the translation of ZEB2 mRNA. (16,18) ZEB2 is a transcription factor that regulates the transcription of EMT-associated genes in cooperation with ZEB1. (16,18) Thus, upregulated miR-200a and miR-141 might be coordinated with downregulated miR-200c and miR-205 and then play an important role in the translational regulation of EMT-associated genes after HGF stimulation.

In summary, we examined the expression of miRNA before and after HGF stimulation in HNSCC cell line HSC3 and found the downregulation of miR-200c and miR-27b after HGF stimulation. The expression of ZEB1, a target mRNA for miR-200c, was upregulated, and that of E-cadherin, a downstream molecule of ZEB1, was downregulated after HGF stimulation. Expression of ST14/matriptase, an enzyme for ECM degradation and HGF activation and a target mRNA for miR-27b, was upregulated. The results of the present study suggest that miR-200c and miR-27b downregulated by HGF might play an important role for EMT and ECM degradation with HGF autoactivation followed by enhanced invasive characteristics of HNSCC (summarized in Fig. 7). This is the first report that HGF might directly regulate some miRNA that affect the expression of downstream functional molecules.

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Disclosure Statement

All authors of this paper have no conflict of interest.
