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Original Article

microRNA-196b promotes esophageal squamous cell carcinogenesis and chemoradioresistance by inhibiting EPHA7, thereby restoring EPHA2 activity

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Abstract: Esophageal cancer (EC) is extremely aggressive and has a very poor survival rate. Esophageal squamous cell carcinoma (ESCC) accounts for 80% of all ECs worldwide, with the majority of the remaining 20% being esophageal adenocarcinoma (EAC). Due to its occult and insidious presentation, ESCC is typically diagnosed and treated in its advanced stages, thereby limiting the success of present therapeutic modalities. microRNAs (miRNAs) can function as tumor suppressors or oncogenes, playing critical roles in cancer initiation and progression by regulating target genes in oncogenic pathways. In the current study, we demonstrated that microRNA-196b (miR-196b) is one of the most upregulated miRNAs in both ESCC and EAC. miR-196b was overexpressed in ESCC and EAC cell lines, cellular exosomal RNAs, and ESCC tissue samples. Functional studies revealed that miR-196b acted as an oncomiR by directly targeting a tumor suppressor, ephrin type-A receptor 7 (EPHA7). EPHA7 abrogates the activity of ephrin type-A receptor 2 (EPHA2), a key molecule involved in the epithelial-to-mesenchymal transition (EMT) and MAPK/ERK pathways, mediating resistance to UV and chemoradiotherapy in both ESCC and EAC. Taken together, these findings suggest that miR-196b is a strong candidate molecular target for EC treatment.

Keywords: Esophageal cancer, esophageal squamous cell carcinoma, miR-196b, chemoradioresistance, EPHA7, EPHA2

Introduction

Esophageal cancer (EC), which comprises two major subtypes, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), is one of the deadliest of all human cancers, with an extremely aggressive natural history and a very poor survival rate [1]. ESCC accounts for 80% of all ECs worldwide [2]. ESCC is typically diagnosed and treated at advanced stages, thereby limiting the success of present therapeutic modalities. Despite many advances in diagnosis and treatment, the 5-year survival rate for all patients diagnosed with EC ranges from 15% to 20% [3]. Radiation therapy and combined chemoradiotherapy have been used for patients with most EC patients. Despite reduction of tumor burden, these treatments do not usually induce complete remission. Molecular alterations related to chemotherapy responsiveness can also constitute therapeutic targets to overcome potential chemotherapy resistance. MicroRNAs (miRNAs) are small, single-stranded, non-coding RNAs that usually downregulate target messenger RNA (mRNA) expression [4]. miRNAs can function as tumor suppressors or oncogenes, playing critical roles in cancer initiation and progression by inhibiting target genes in oncogenic pathways [5]. Unlike messenger RNAs (mRNAs), miRNAs are stable not only in body fluids [6], but also in formalin-fixed, paraffin-embedded

(FFPE) tissues [7]. miRNAs can be packaged into extracellular vesicles (EVs) [8-12] or bound to proteins [13, 14], thereby being transported from primary to metastatic sites [10], which makes miRNAs attractive therapeutic targets. By cross-referencing our RNA data with others' from NCBI, we identified miR-196b as one of the most significantly overexpressed miRNAs in ESCC. We investigated in detail the function of miR-196b and its target gene, erythropoietin-producing hepatocellular carcinoma cell receptor A7 (EPHA7), in EC pathogenesis and potential therapy.

Materials and methods

Cell culture

All EC cell lines used in this study were kindly provided by Dr. Stephen J. Meltzer's lab (Johns Hopkins University). The ESCC cell lines KYSE-70 and KYSE-180 were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin and streptomycin. EAC cell lines, FLO-1 and JHU-Ad1 were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS, 1% penicillin and streptomycin. The non-cancerous epithelial esophageal cell line HET-1A was purchased from ATCC and cultured in the base medium for this cell line (BEBM) along with BEGM kits (Catalog No. CC-3170, Lonza) except GA-1000 (gentamycin-amphotericin B mix). All cell lines were maintained at 37°C with 5% CO_2 in a humidified incubator.

Tissue specimens and microdissection

EC tissue blocks (n=40) were retrieved from archives at Chengdu Military General Hospital. Esophageal lesions were confirmed by histopathologic diagnosis following endocytoscopy and/or surgical excision. Formalin-fixed, paraffin-embedded (FFPE) tissues were subjected to microdissection into adjacent matched normal esophageal epithelium, dysplasia, and carcinoma as described previously [15].

Preparation of exosome and isolation of RNA and protein from exosome

Exosome isolation was performed using the Total Exosome Isolation kit (Cat# 4478359, Thermo Fisher) according to manufacturer's instructions. Briefly, the FBS-free culture medium was centrifuged to remove cell debris. The conditioned medium was collected and concentrated using a Pierce Protein Concentrator PES (NMWL) 10 kD (Thermo Fisher). The concentrated medium was mixed with the Total Exosome Isolation reagent. The samples were incubated at 4°C overnight and then subjected to centrifugation at 10,000 g at 4°C for 1 h. The exosome pellets were resuspended and stored in -20°C or immediately processed with the Total Exosome RNA and Protein isolation kit (Cat# 4478545, Thermo Fisher) to isolate the exosomal RNA and protein.

RNA extraction and quantitative real-time reverse transcription-PCR (qRT-PCR)

Total RNA from cultured cells and FFPE samples was isolated and quantitated as described previously [16]. miR-196b (Acc#: MIMAT0001080) expression was assayed using the Taqman MiRNA Reverse Transcript Kit (Cat# 4366596, Thermo Fisher) with primer (5'-UAGGUAGUUUCCUGUUGUUGGG-3'), and target genes EPHA7 and EPHA2 were analyzed using the Real-Time™ SYBR Green (Bio-Rad Laboratories, Berkeley, CA) as described previously [17].

miRNA and plasmid transfection

miRNA transfection was performed as described [16, 17]. Briefly, miRNA precursors (miR-196b-5p mimic, inhibitor and mock control) were transiently transfected into the EC cell lines by the Lipofectamine RNAiMAX (Life Technologies) using the Opti-MEM I Reduced Serum Medium (Life Technologies). Cells were subjected to further analysis after 24 h, 48 h and 72 h post-transfection. For rescue experiments, MSCV-EPHA7 plasmid containing human full length EPHA7 cDNA was kindly provided by Dr. Michael A. Teitell at UCLA and the pCLXSN-EPHA2 plasmid containing full-length human EPHA2 cDNA was purchased from Addgene (Plasmid #102755) or their corresponding empty vectors MSCV and pCLXSN were transfected into miR-196b mimic or inhibitor transiently transfected EC cells respectively using the FuGENE reagent (Promega), 48 h after miRNA transfection as described previously [16].

Protein extraction and Western blotting

Cell lysates were prepared using the RIPA Buffer (ThermoFisher) according to the manu-

facturer's protocol, and Western blot analysis with chemiluminescent detection was performed as described [18]. The following antibodies and dilution factors were used: anti-rabbit EPHA7 (PA130296, Invitrogen; 1:800), anti-rabbit EPHA2 (PA514574, Invitrogen; 1:1000), anti-Rabbit Phospho-Src (Tyr416) (2101, Cell Signaling; 1:1000), anti-rabbit Phospho-p44/42 MAPK (Thr202/Tyr204) (9101, Cell Signaling; 1:1000), anti-rabbit beta actin (PA1-183, Invitrogen; 1:2000).

Matrigel invasion assays

Matrigel invasion assays were performed using the BD BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences, 354480) as previously described [19]. Briefly, 500 µl of warm serum-free DMEM medium was added to the upper and lower chambers to rehydrate for 2 h in a 37°C incubator, then 8×10^4 cells transfected by either miR-196b mimic or inhibitor with the mock controls for 24 h were seeded onto the top chamber of pre-wetted inserts. Then the Matrigel chamber with cells was incubated in a 37°C humidified incubator with 5% CO_2 for 24 h. The cells were then fixed, stained with the Diff-Quick staining solution and counted (five microscope fields under the 10× lens). The experiments were performed in duplicates for each cell line twice. Cells were counted on five non-overlapping random fields for each chamber and four chambers were counted for each experimental point, with the percentage of invasive cells being normalized to corresponding controls.

Dual luciferase reporter assay

Cells were plated (7×10^5 cells/well) in 24 well plates and co-transfected with 100 ng of pEZX-EPHA7-3'UTR plasmid DNA (wild type and mutant) expression clones inserted downstream of a secreted Gaussia luciferase (GLuc) reporter, and 100 ng pEZX-miR-196b or the pEZX-MT scrambled control plasmid DNA (mock), using the FuGENE Transfection Reagent (Promega). Luciferase activities were determined using the Secrete-PairTM Dual Luminescence Assay Kit (*Genecopoeia*). GLuc luciferase activities were then normalized by SEAP luciferase expression for each sample.

UVC/Chemosensitivity and MTT assays

For MTT cell proliferation assay, the cells transfected with miR-196b mimic and inhibitor or their corresponding mock controls were washed with PBS after 48 h transfection. Then 100 µl of MTT working solution (0.5 mg/ml MTT in optiMEM) was added to each well, and incubated at 37°C for 3 h. Then the MTT solution was removed and 100 µl of DMSO was added to each well for additional 30 min incubation in a 37°C incubator. Color development was measured at 490 nm using a spectrophotometer plate reader (BIO-TEK Instruments), and quantified as per the manufacturer's protocol (Promega, USA). For MTT chemosensitivity assay, the cells transfected with miR-196b mimic and inhibitor or their corresponding mock controls were treated with UVC (5, 10 and 20 J/m^2) and various concentrations of paclitaxel (1, 10 and 100 µM), cisplatin (5, 25 and 125 µM), 5-fluorouracil (5-FU, 2.5, 5 and 10 µM), or epirubicin (50, 100 and 200 nM), respectively. After 48 h, MTT was added and absorbance was measured as described above.

Statistical analysis

miR-196b expression in clinical samples was analyzed by the exact two-sided binomial test. Data were expressed as mean ± standard error (S.E.). Permutation tests were performed for MTT assays between control and miR-196b mimic or inhibitor transfected groups. The student's t-test (two tailed) was applied to Matrigel assay between control and miR-196b or inhibitor transfected groups. *P*-values less than 0.05 were considered statistically significant.

Results

Identifying miRNAs significantly dysregulated in EC by systematic literature review

To explore the differentially expressed miRNAs in EC, we conducted a systematic review of studies to evaluate the miRNA expression in EC. We searched PubMed and ISI Web of Science online databases for the relevant human studies on diagnostic miRNAs involving EC, published in English language journals up to January 2020. The following terms were used for the literature search: microRNA (s), miRNA (s), esophageal cancer (s), esophageal neoplasm (s), stomach neoplasm (s), stomach

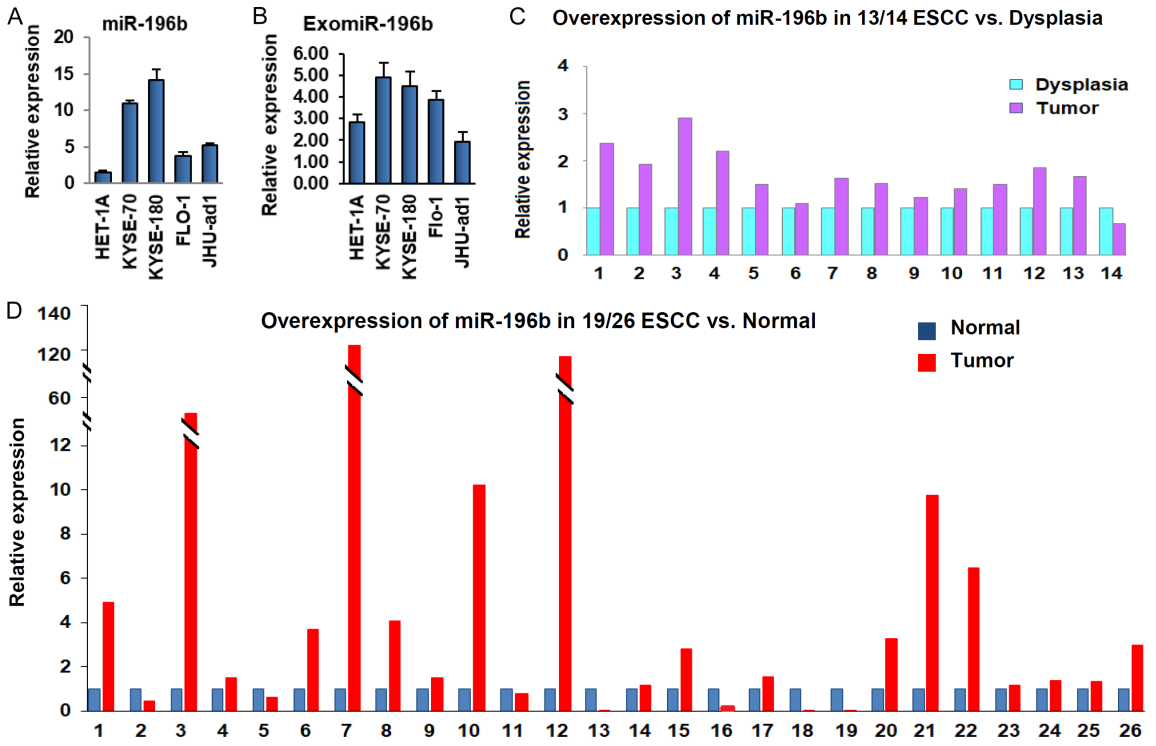

Figure 1. Expression of miR-196b in secreted exosomes (exomiR-196b) of EC cell lines, and EC cohort by qRT-PCR. A. Expression of miR-196b in EC cell lines by qRT-PCR. B. exomiR-196b overexpression in cell culture medium of EC cell line compared to that in non-cancerous epithelial esophageal cell line HET-1A, except JHU-ad1. C. miR-196b expression in pure populations of tumor cells and dysplasia microdissected from FFPE tissues. The level of miR-196b expression was significantly increased in 13 of 14 ESCC compared to their adjacent dysplasia. D. The level of miR-196b expression was significantly increased in 19 of the 26 in ESCC compared with the adjacent normal tissues.

cancer (s) and gastric cancer (s). A manual search of the references listed by studies retrieved from the online databases was also performed to identify additional studies. The systematic review was drafted in accordance with the PRISMA guidelines [20]. Eligibility criteria included case-control studies evaluating blood or tissue-based miRNA expression profiles. Among 206 eligible studies, there are 97 deregulated miRNAs using either blood or tissue samples. miR-196b is one of most commonly upregulated miRNA in ESCC. A list of representative miRNAs involving EC was compiled in Supplementary Materials (Supplementary Table 1).

Overexpression of miR-196b in EC cell lines, exosomes and EC tissues

To verify the expression of miR-196b in EC, we performed qRT-PCR in EC cell lines first. As expected, overexpression of miR-196b was observed in both ESCC (KYSE-70 and KYSE-180) and EAC (FLO-1 and JHU-ad1) cell lines, compared to non-cancerous epithelial esophageal cell line HET-1A (**Figure 1A**). We then tested if miR-196b can be detected in exosomes secreted from EC cells in cultured medium. Consistent with the cellular RNA data, miR-196b expression in exosomes was also increased in cell culture medium of three EC cell lines, compared to that of HET-1A, but not in EAC cell line, JHU-ad1 (**Figure 1B**).

To analyze miR-196b expression in EC patients, we used the archival FFPE samples, which were microdissected into normal, dysplastic, and tumor cells, followed by total RNA isolation and qRT-PCR. We found that miR-196b expression was significantly increased in 13 of 14 (93%) ESCC tumor cells when compared to their adjacent dysplasia (**Figure 1C**) while 19 of 26 (73%) when compared to their adjacent normal cells (**Figure 1D**). These results confirmed that overexpression of miR-196b is a frequent event during the progression of ESCC, suggesting miR-

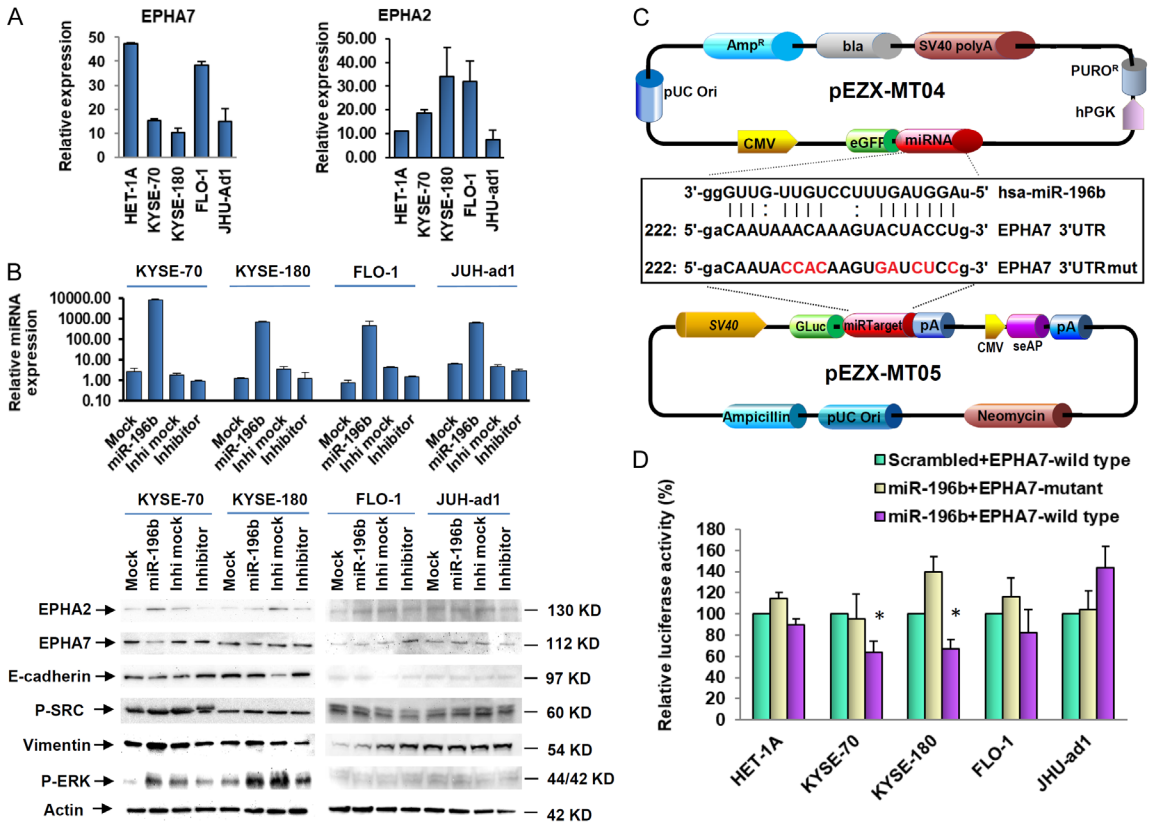

Figure 2. Expression of miR-196b and its target genes in EC. (A) Expression of EPHA7 and EPHA2 in EC cell lines by qRT-PCR. An inverse correlation between miR-196b (A) and EPHA7 expression pattern and a positive correlation between miR-196b and EPHA2 expression pattern were observed in EC cell lines. (B) Force expression of miR-196b (top) results in decreased EPHA7 and MET marker E-cadherin and increased EPHA2, EMT marker vimentin, ERK and SRC phosphorylation. (C) Map of the plasmids, pEZX-MT04 containing miR-196b and pEZX-MT05 containing 3'-UTR of EPHA7 to illustrate the location of miR-196b binding site at the 3'-UTR of EPHA7, and its mutant control sequence. (D) Dual luciferase reporter assay. Cotransfection with pEZX-miR-196b and pEZX-EPHA7 3'UTR wild type significantly decreased the luciferase activities compared to the cotransfection with pEZX-miR-196b and EPHA7 3'UTR mutant/miR-196b scrambled control and EPHA7 3'UTR wild type sequence in ESCC cell lines. The data were reported as mean ± S.D. from three independent experiments (*P<0.05).

196b might serve an oncogenic biomarker for human ESCC diagnosis.

miR-196b directly targets EPHA7 in EC

Using the bioinformatics platforms, TargetScan and microrna.org [21, 22], we identified a list of potential target genes of miR-196b, including GATA6, HOXC8, HOXB7, ARHGAP28, EPS15 and EPHA7. We narrowed down the functional pathways by enrichment analysis using different algorithms, and identified the prominent EPH pathway. EPHA7, a member of the Eph family of receptor tyrosine kinases, has attracted growing attention as a tumor suppressor as it is downregulated in a variety of cancers especially in ESCC [23-25]. However, its biological roles and the underlying molecular mechanisms are still unclear. Therefore, we focused on the regulatory role of miR-196b over EPHA7. We observed an inverse correlation between miR-196b and EPHA7 expression in EC cell lines at both mRNA and protein levels (**Figure 2A**). Consistent with this, EPHA7 was significantly downregulated in miR-196b transfected EC cells compared to the mock transfected ones, while EPHA7 was upregulated in miR-196b inhibitor transfected cells compared to the inhibitor mock transfected ones (**Figure 2B**). To confirm the specificity of miR-196b targeting EPHA7, we performed luciferase reporter assays by co-transfection of pEZX-MT05 vector (GeneCopoeia) containing the miR-196b binding site (either wild type or mutant sequences) in the EPHA7 3'UTR region and pEZX-MT04 vector containing miR196b (**Figure 2C**) or scram-

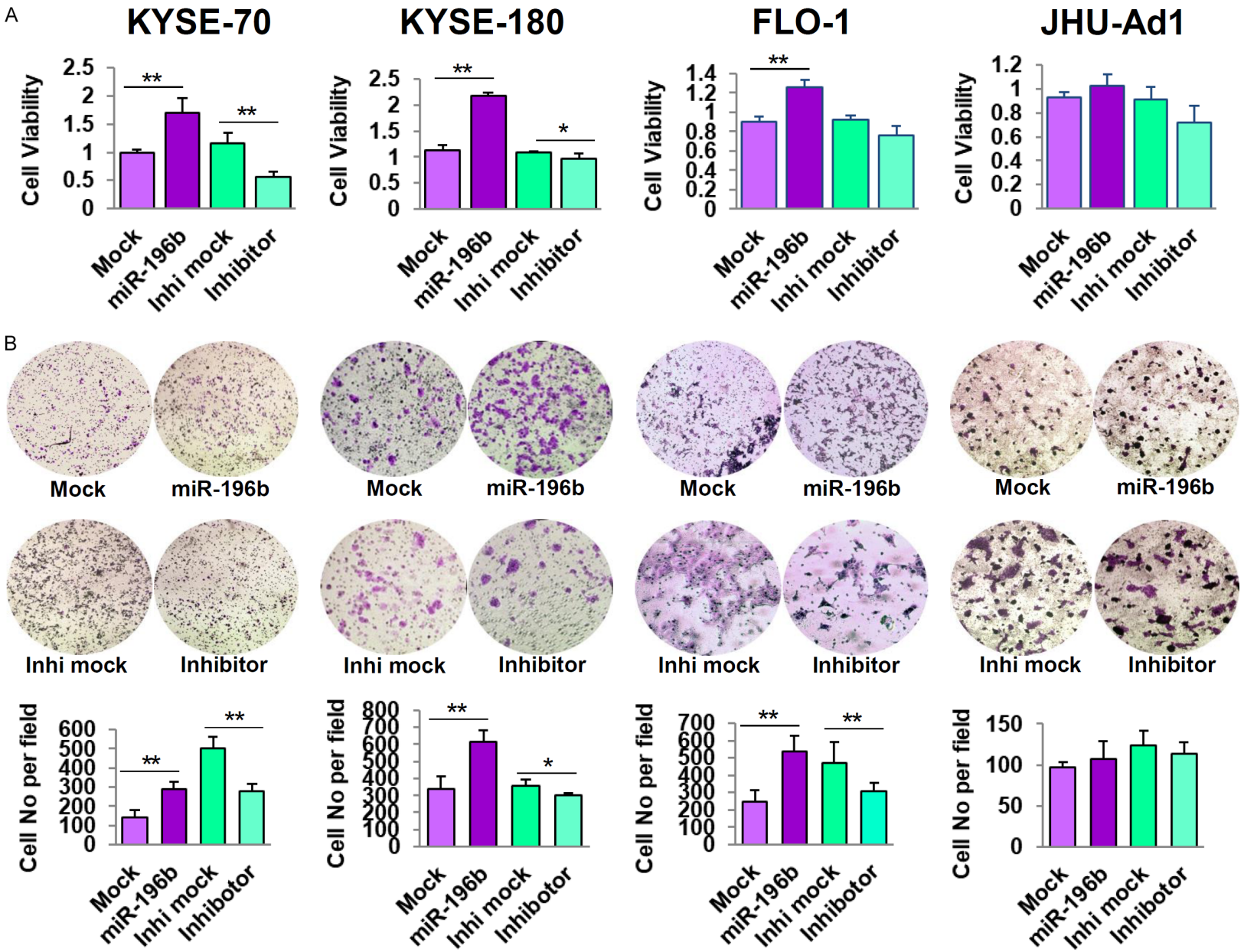
A
KYSE-70
KYSE-180
FLO-1
JHU-Ad1
Cell Viability
Mock
miR-196b
Inhi mock
Inhibitor
B
Mock
miR-196b
Inhi mock
Inhibitor
Cell No per field
Inhibotor

Figure 3. miR-196b promotes proliferation and increases invasive ability of EC cell lines. A. Effects of miR-196b on cell proliferation were determined by MTT assay. The proliferation was increased after transfection of miR-196b mimic in EC cell lines compared to the mock control in ESCC cell lines KYSE-70 and KYSE-180 and in EAC cell line FLO-1 but not to a significant degree in JHU-ad1 cells. Values represent the mean ± S.D. for three independent experiments. (**P<0.01, *P<0.05). B. Transwell assays with matrigel were performed for the invasive activity of EC cells transfected with either miR-196b mimic or the mock control. Overexpression of miR-196b significantly induces cell invasion in ESCC cell lines KYSE-70 and KYSE-180 and in EAC cell line FLO-1 but not significant in JHU-ad1 cells. Invasive ability of the cells was displayed as a percentage of the absolute cell numbers (bottom). Results are displayed as mean data ± SE. (**P<0.01, *P<0.05). Five fields of unit area on each membrane or whole membrane were counted for cell numbers, and the experiments were repeated three times with triplicates.

bled control. After successful co-transfection of the vectors containing miR-196b and EPHA7 3'UTR wild type sequences into cell lines, we observed significantly decreased luciferase activities in ESCC cells KYSE-70 and KYSE-180, compared to that co-transfected with either miR-196b/EPHA7 3'UTR mutant sequences or scrambled control/EPHA7 3'UTR wild type sequences. Although not statistically significant, we observed decreased luciferase activity in miR-196b/EPHA7 3'UTR wild type cotransfected in EAC cell line FLO-1 (**Figure 2D**) as well, but not in JHU-ad1. These data suggest that miR-196b directly targets EPHA7 by binding to its 3'UTR in EC.

miR-196b abrogates the blocking of EPHA7 to EPHA2 and its downstream oncogenic signal

EPH receptors and their ligands are the largest family of receptor tyrosine kinases which regulate physiological and pathological processes in tumor development and progression [26]. EPHA7 was shown to significantly inhibit tumor growth via blocking EPHA2 phosphorylation and its oncogenic signals [27]. EPHA2 overexpression is associated with poor survival [28] and poor prognosis in ESCC [29]. We sought to determine whether miR-196b overexpression can rescue the EPHA2 pathway by inhibiting EPHA7. We observed an inverse correlation between EPHA2 and EPHA7 expression in EC cell lines (**Figure 2A**), consistent with the observation of the expression of the two genes in lymphoma [30]. We then examined the expression of EPHA2 in miR-196b transfected cells. Forced expression of miR-196b results in significant downregulation of EPHA7 and upregulation of EPHA2. Conversely, inhibition of endogenous miR-196b blocked EPHA7 and restored EPHA2 expression (**Figure 2B**). These data suggest that miR-196b negatively regulates EPHA7 expression, which in turn activates EPHA2 activity.

EPHA2 has been demonstrated to activate the MAPK/ERK pathway [31], which was inhibited by EPHA7 [40, 41]. We next examined the possibility that miR-196b might restore EPHA2 downstream signals by blocking EPHA7. Transfection of miR-196b significantly induced SRC family kinases phosphorylation compared to the mock transfected ESCC cell lines, although the change was not significant in EAC cell lines (**Figure 2B**). Conversely, transfection of miR-196b inhibitor reduced SRC family kinases phosphorylation (**Figure 2B**). Hence, miR-196b acts, at least in part, to promote EPHA2 downstream oncogenic signaling in ESCC.

miR-196b facilitates cell proliferation and invasion in EC by targeting EPHA7 and restoring EPHA2

We next sought to demonstrate the functional role of miR-196b in EC proliferation and invasion. We first transfected miR-196b mimic and controls to EC cell lines, respectively, and examined cell proliferation rate using MTT assays. Interestingly, overexpression of miR-196b significantly accelerated cell proliferation (**Figure 3A**) compared to the mock control in ESCC cell lines KYSE-70 and KYSE-180 and EAC cell line FLO-1 (P<0.01) and also slightly increased in EAC cell line JHU-ad1 although the change was not statistically significant. Conversely, transfection of miR-196b inhibitor significantly inhibited cell proliferation compared to the inhibitor mock control in ESCC cell lines KYSE-70 and KYSE-180 (P<0.05); and also in EAC cell line FLO-1 and JHU-ad1 although the change was not statistically significant. These results indicate the proliferative effect of miR-196b in EC especially in ESCC. To test the impact of miR-196b on invasion, we performed Matrigel invasion assays. Consistent with the effect of miR-196b on proliferation, a significantly increased invasive capability was observed in miR-196b

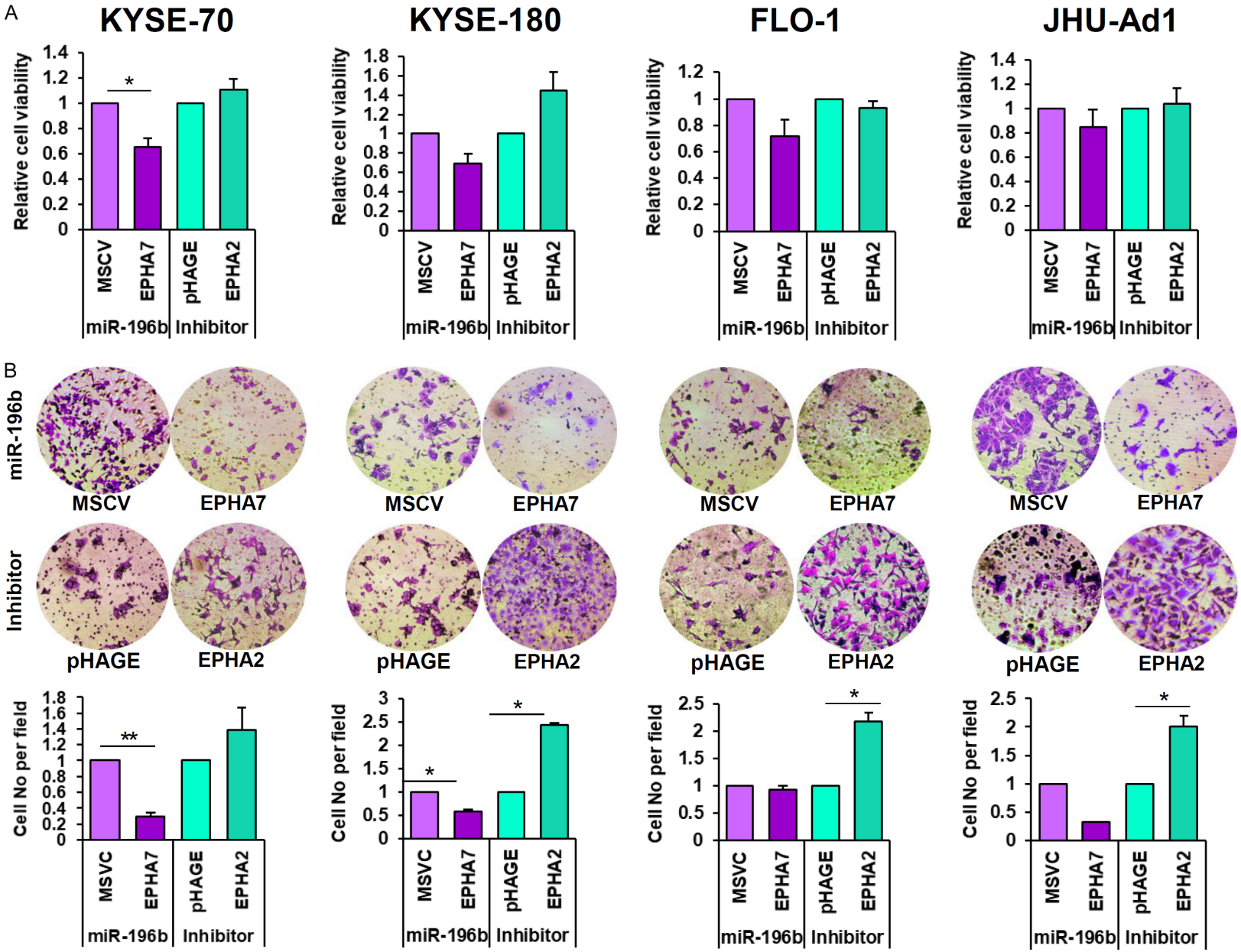
A
KYSE-70
KYSE-180
FLO-1
JHU-Ad1
Relative cell viability
MSCV
EPHA7
pHAGE
EPHA2
miR-196b
Inhibitor
B
miR-196b
Inhibitor
MSCV
EPHA7
pHAGE
EPHA2
Cell No per field
MSVC

Figure 4. EPHA7 and EPHA2 rescue experiment showed that the oncogenic effects of miR-196b are partially reversed by EPHA7 restoration and enhanced by EPHA2 transfection. The MSCV-EPHA7 or pCLXSN-EPHA2 cDNA expression vectors were transfected into EC cells after 48 h miR-196b mimic or inhibitor transfection. A. The effects of EPHA7 and EPHA2 restoration on cell proliferation in miR-196b transfected cell lines were measured by MTT. The cell proliferation was significantly decreased after transfection of EPHA7 into miR-196b transfected cells compared to the transfection of MSCV empty control in KYSE-70 and slightly decreased in KYSE-180, FLO-1 and JHU-ad1 cells. The cell proliferation was significantly increased after transfection of EPHA2 into miR-196b inhibitor transfected cells compared to the transfection of pCLXSN empty in KYSE-70, KYSE-180 but not in EAC cell line FLO-1 and JHU-ad1 cells. B. The effects of EPHA2 restoration on cell invasion measured by transwell assays. The cell invasion was significantly decreased after transfection of EPHA7 into miR-196b transfected cells compared to the transfection of MSCV empty control one in KYSE-70, KYSE-180 and JHU-ad1 cells. The cell invasion was significantly increased after transfection of EPHA2 into miR-196b inhibitor transfected cells compared to the transfection of pCLXSN empty in KYSE-180, FLO-1 and JHU-ad1 cells. Values represent the mean ± S.D. for three independent experiments. (**P<0.01, *P<0.05).

transfected ESCC cell lines KYSE-70 and KYSE-180 and EAC cell line FLO-1 (P<0.01) compared to the mock transfected ones; while also increased in EAC cell line JHU-ad1 although the change was not statistically. Conversely, transfection of miR-196b inhibitor significantly decreased invasive capability compared to the transfection of inhibitor mock in ESCC cell lines KYSE-70, KYSE-180 (P<0.01 and P<0.05) and in EAC cell line FLO-1 (P<0.01) and slightly decreased the invasive capability in JHU-ad1 although the change was not statistically significant (**Figure 3B**). These results suggest that overexpression of miR-196b plays an important role not only in promoting cell proliferation but also in cell invasion in EC specifically in ESCC development.

To demonstrate whether miR-196b oncogenic function is involved in the EPH-related pathways, we performed a rescue experiment by transiently transfecting MSCV-EPHA7 plasmid containing human full length EPHA7 cDNA into the miR-196b transfected cell lines. This resulted in a significantly decreased proliferation compared to the MSCV empty vector control in KYSE-70 and a modest decrease in KYSE-180. We observed a similar decrease in EAC cell lines, FLO-1 and JHU-ad1 although not statistically significant (**Figure 4A**). In addition, MSCV-EPHA7 transfection into miR-196b transfected cells also resulted in significant reduced invasion capability in ESCC cell lines KYSE-70, KYSE-180 and EAC cell line JHU-ad1, but not in FLO-1 cells (**Figure 4B**). These results suggest that the oncogenic effect of miR-196b at least in part, is due to inhibition of EPHA7. To further determine whether miR-196b functions as an oncomiR by suppressing EPHA7, which in turn activates or restores EPHA2 function, the cell proliferation was significantly decreased after transfection of EPHA7 into miR-196b transfected cells compared to the transfection of MSCV empty control in KYSE-70 and slightly decreased in KYSE-180, FLO-1 and JHU-ad1 cells. The cell proliferation was significantly increased after transfection of EPHA2 into miR-196b inhibitor transfected cells compared to the transfection of pCLXSN empty in KYSE-70, KYSE-180 but not in EAC cell line FLO-1 and JHU-ad1 cells (**Figure 4A**). The cell invasion was significantly decreased after transfection of EPHA7 into miR-196b transfected cells compared to the transfection of MSCV empty control one in KYSE-70, KYSE-180 and JHU-ad1 cells. The cell invasion was significantly increased after transfection of EPHA2 into miR-196b inhibitor transfected cells compared to the transfection of pCLXSN empty in KYSE-180, FLO-1 and JHU-ad1 cells (**Figure 4B**). Values represent the mean ± S.D. for three independent experiments (**P<0.01, *P<0.05). These results suggest that miR-196b promotes cell proliferation and invasion by suppressing EPHA7, which leads to EPHA2 activation in EC oncogenesis.

miR-196b promotes epithelial-to-mesenchymal transition (EMT) by abrogated blocking of EPHA7 and activating EPHA2

During malignant development, tumor cells can undergo EMT and adopt fibroblast-like cell migration or amoeboid movement, by which they migrate as individual cells. Growing evidence indicates that EPHA2 promotes EMT [28, 32, 33]. We asked if miR-196b promotes EMT by relieving EPHA7-mediated EPHA2 blocking. We observed significant cell morphological changes reminiscent of EMT as the cells lost their epithelial cobblestone-like morphology to acquire a more elongated fibroblast-like spindle-shape following miR-196b transfection in

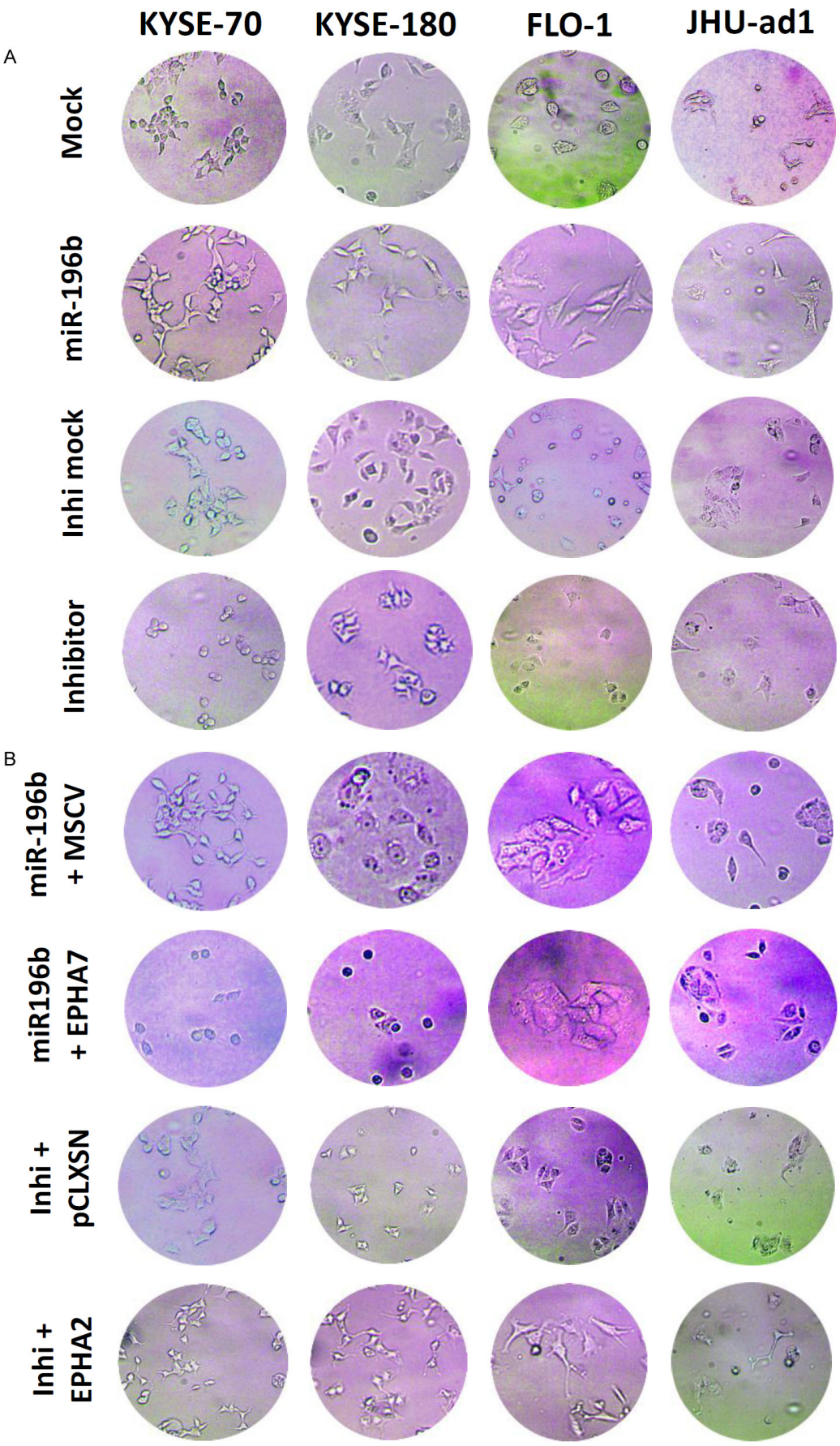
KYSE-70
KYSE-180
FLO-1
JHU-ad1
A
Mock
miR-196b
Inhi mock
Inhibitor
B
miR-196b + MSCV
miR196b + EPHA7
Inhi + pCLXSN
Inhi + EPHA2

Figure 5. miR-196b induced EMT via EPHA7 and EPHA2 regulation. Cell morphology was observed by microscopy in EC cell lines 72 h after miRNA transfection. A. miR-196b induced EMT. Overexpression of miR-196b caused more elongated, irregular fibroblastoid shape (EMT phenotype) compared to the mock-transfected cells in all EC cell lines (top panel). Conversely, transfection of miR-196b inhibitor reversed the ESCC cell lines from EMT to MET morphology (bottom panel). B. Rescue experiment of EPHA7 and EPHA2 showed that the miR-196b induces EMT that are partially reversed by EPHA7 restoration and enhanced by EPHA2 transfection. Re-expression of EPHA7 in miR-196b transfected cells reversed EC cell lines from EMT to epithelioid appearance MET morphology while re-expression of EPHA2 into miR-196b inhibitor transfected cells resulted in more EMT morphology compared to their empty control transfected cells, respectively.

ESCC cells, but not in EAC cells. Transfection of miR-196b inhibitor or re-expression of EPHA7 reversed the ESCC cell lines from EMT to MET (**Figure 5A**). Consistent with the morphological changes, Western blot analyses revealed a downregulation of epithelial marker E-cadherin, and concomitant upregulation of EMT marker, vimentin in miR-196b transfected cells when compared to the mock control. Reversed results were observed in miR-196b inhibitor transfected ESCC cell lines compared to that of the inhibitor mock transfection (**Figure 1B**). The rescue experiment showed that transfection of EPAH7 into miR-196b transfected cells abrogated the miR-196b-induced EMT morphological changes and induced cells from elongated fibroblast-like spindle-shaped to round-shaped. Conversely, the opposite result was observed when transfecting EPAH2 into miR-196b inhibitor transfected cells (**Figure 5B**). These results indicate that miR-196b promotes ESCC development via abrogated blocking of EPHA7 to EPHA2-mediated EMT.

miR-196b mediates UV and chemotherapy resistance in EC cell lines

Having demonstrated that miR-196b promotes EMT, which is implicated in the development of therapeutic resistance [34], we next addressed whether miR-196b is involved in therapeutic resistance by promoting EPHA2 mediated EMT. To evaluate this hypothesis, we treated miR-196b transfected cells with UV exposure and chemotherapeutic agents after 48 h following miR-196b transfection. miR-196b expression significantly reduced sensitivity to UV in all four cell lines in a dose-dependent manner. miR-196b expression significantly reduced sensitivity to paclitaxel and cisplatin in all cell lines assayed except JHU-ad1. Conversely, miR-196b knockdown by miR-196b inhibitor increased cell sensitivity in both UV treatment and chemo agents, paclitaxel and cisplatin. A significant reduced sensitivity was observed in both ESCC lines when 5-FU (2.5 uM) and epirubicin treatment was done in low concentration after miR-196b transfection but not in EAC cell lines. Furthermore, miR-196b transfection reduced the sensitivity of both ESCC cell lines and FLO-1 but not in JHU-ad1 when treated with epirubicin by medium dose (100 nM) (**Figure 6**). These data suggest that miR-196b is involved in UV and chemotherapy resistance in EC.

Discussion

The miR-196b gene is located in a highly conserved region on Chromosome 7p15. It has been emerging as a strong oncomiR in multiple cancers. Previous work has established that miR-196b was one of the most overexpressed miRNAs in most cancers [35-37] including EC [38, 39]. To the best of our knowledge, our study has successfully established that miR-196 directly targets the EPHA7/EPHA2 pathways in EC oncogenesis for the first time. We demonstrated that miR-196b promoted cellular proliferation, invasion and chemoresistance by abrogating the blocking function of EPHA7 to EPHA2 oncogenic signaling (**Figure 5**). Interestingly, in clinical samples, we detected higher frequent upregulation of miR-196b in dysplastic esophageal epithelial tissue (93%) than histologically normal esophageal epithelial tissue (73%), suggesting dysplastic esophageal epithelium presented more miR-196b alterations than normal the same didcordant genetic changes have been reported in esophageal epithelium during the esophageal carcinogenesis. Consistent with our finding, same discordant genetic changes were reported in lung cancer in which histologically normal bronchial epithelial tissues had genetic changes more similar to those in the SCCs than in dysplastic lesions [40]. These divergent findings have implications for understanding the steps involving ESCC and EC prevention.

Consistent with the reports that miR-196b overexpression is associated with metastasis [35, 41], we found miR-196b promoted EC cell

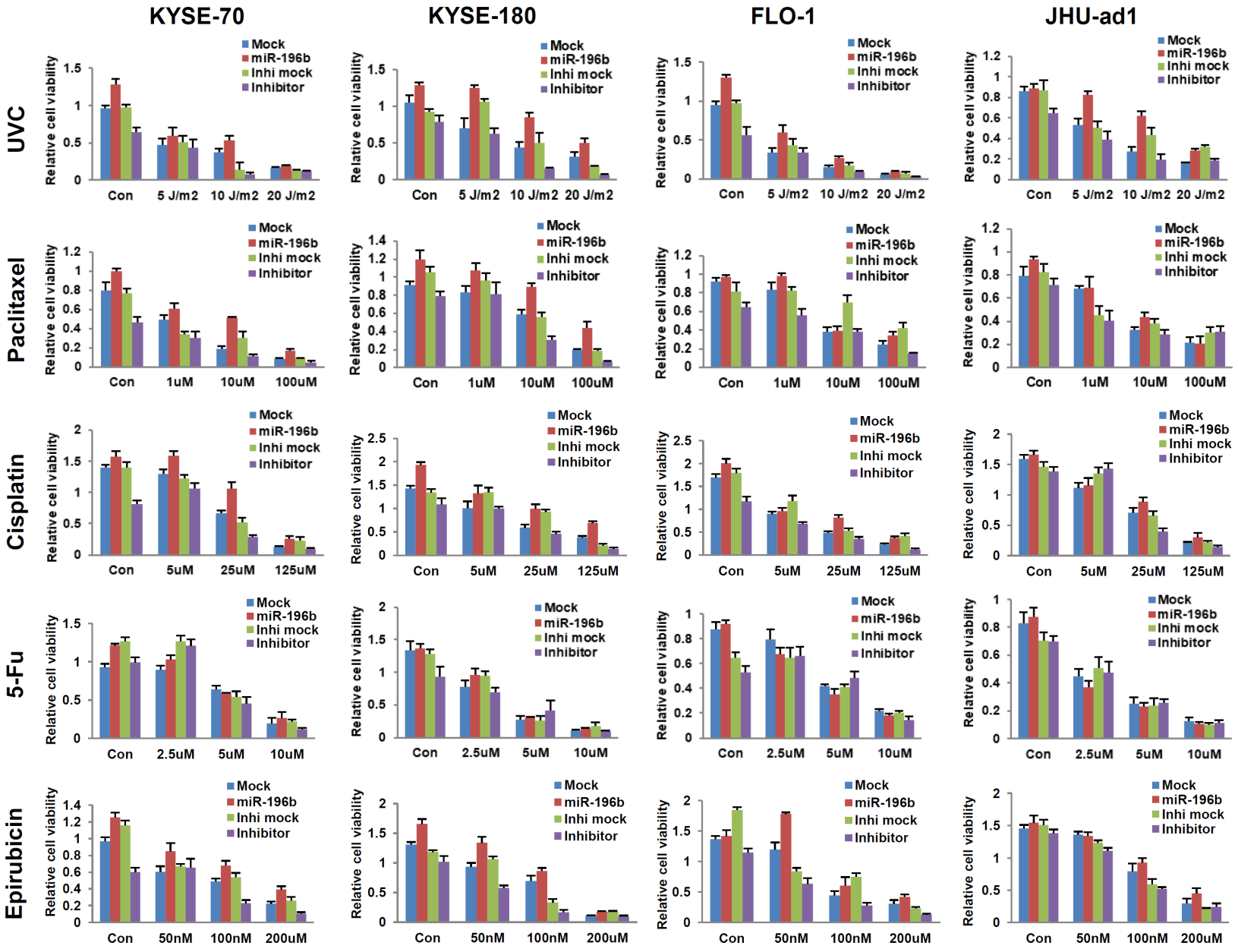
KYSE-70
KYSE-180
FLO-1
JHU-ad1
UVC
Paclitaxel
Cisplatin
5-Fu
Epirubicin
Relative cell viability
Mock
miR-196b
Inhi mock
Inhibitor
Con
5 J/m2
10 J/m2
20 J/m2
1uM
10uM
100uM
5uM
25uM
125uM
2.5uM
50nM
100nM
200uM

Figure 6. Effect of miR-196b on sensitivity of EC cell lines to UV/Chemosensitivity. miR-196b mimic, inhibitor or their mocks were transfected into EC cell lines. Cells were treated with UVC, paclitaxel, cisplatin, 5-FU, or epirubicin at indicated conditions after 48 h miRNAs transfection. Cell sensitivity was measured by MTT assay after 48 h treatment. miR-196b overexpression either significantly or partially decreased cell sensitivity to UV, paclitaxel, cisplatin, 5-FU and epirubicin in ESCC cell lines compared to mock-transfected cells. Inhibition of miR-196b resulted in an inverse effect. Results are displayed as mean data ± SE. **P<0.01, *P<0.05, with comparison to the mock.

proliferation and invasion. In addition, we found that miR-196b overexpression is more prevalent in metastatic versus non-metastatic ESCC in clinical EC tissues although we did not observe a significant difference between metastatic and non-metastatic ESCC groups (data not shown). A larger sample size of the clinical samples is needed for further analysis.

We provide insight into the molecular pathogenesis of miR-196b regulating EPH receptors and the downstream pathway in EC. The EPH receptors were first discovered in a human carcinoma cell line [42, 43] which represent the biggest subfamily of receptor tyrosine kinases (RTKs), The EPH receptors play an essential role in a variety of cellular processes during development and adult tissue homeostasis [44-46]. The EPH family of receptors are divided into two classes that consist of nine EPHA members and five EPHB members according to their sequence homology [47]. The EPH family of receptors function through interactions with membrane-bound Eph receptor-interacting protein (ephrin) ligands to mediate cellular morphology change, motility, migration, and proliferation [44, 48-50]. EPHA and EPHB receptors bind promiscuously to ephrin-A (5 members) and B ligands (3 members) with some potential cross-talk between groups [51]. The complexity of interactions conveyed by this promiscuous binding leads to considerable diversity in functional output. The EPHA7 receptor, which is highly conserved in vertebrates, has attracted growing attention in cancer research [52]. Recent studies have demonstrated that downregulation of EPHA7 is correlated with ESCC metastasis and poor prognosis. However, the mechanism of EPHA7 in ESCC is still unclear. EPHA2 is usually downregulated in normal epithelial cells [53]. Overexpression of EPHA2 has been widely detected in numerous cancers, including ESCC [29, 54-59] and associated with increased malignancy, poor prognosis and chemo-resistance [60-62]. We found an inverse correlation between EPHA7 and EPHA2 expression in EC cell lines, as shown in lymphoma [30]. We further demonstrated that downregulation of EPHA7 by miR-196b can restore EPHA2 oncogenic signals in EC, which is consistent with the observation that EPHA7 can block EPHA2-mediated oncogenic signaling in lymphoma [30].

The detailed mechanism by which miR-196b regulates EPHA2 is still unclear. In the context of this study, a computer-assisted search of the PubMed database revealed several relevant potential pathways. Specifically, EPHA2 has been demonstrated to be directly activated by Ras/Raf/MAPK pathways [63] that were inhibited by EPHA7 [64, 65]. To expand on this, EPHA7 could attenuate the activation of EPHA2 by inhibiting the Ras/Raf/MAPK cascade. In addition, it has been demonstrated that the tumor suppressor gene hypermethylated in cancer 1 (HIC1), is a transcriptional repressor of EPHA2. miR-196b target gene prediction by microrna.org detected a binding site in HIC1 3'UTR, suggesting an alternative possibility that miR-196b promotes EPHA2 expression by directly targeting its transcriptional repressor HIC1. These data support that miR-196b is involved in a complex transcriptional and epigenetic mechanistic network in EC development.

miR-196b has been associated with EMT and radiochemoresistance [35, 66-68]. However, the link between miR-196b and EPHA7/EPHA2 on chemo-resistance in EC cell lines is still unreported. EPHA2 promotes EMT, a latent developmental process leading to radiochemoresistance [69]. In this study, we found that forced expression of miR-196b significantly induced the resistance to UV, cisplatin, and paclitaxel treatment and slightly to 5-FU and epirubicin which is consistent with the predicted function of miR-196b in EMT [70]. We propose that miR-196b induced radiochemoresistance via inhibition of EPHA7, resulting in activation of EPHA2 mediated EMT. In addition, evidence exists for Ras/Raf/MAPK pathway dependent regulation of EPHA2 in UV radiated

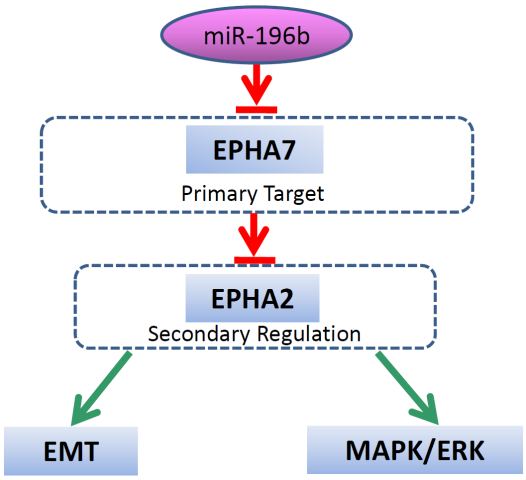

Figure 7. A schematic model for the Regulation of miR-196b. miR-196b directly targets EPHA7. Down-regulation of EPHA7 could release the inhibition of EPHA2 mediated EMT and radiochemoresistance.

induced apoptosis [66], which support our data on our UV and chemo-treatment assays.

Conclusions

We discovered a novel mechanism of oncogenic function of miR-196b in EC. miR-196b leads to the activation of EPHA2-mediated EMT progression, resulting in an aggressive molecular event in the development of radio/chemoresistance in EC (**Figure 7**). Therefore, miR-196b may serve as a potential biomarker for EC detection and therapy.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. A list of representative miRNAs that are dysregulated in EC

Upregulated miRNA	Downregulated miRNA
miR-196b [1-6]	miR-375 [1, 7]
miR-196a [8, 9]	miR-141-3p [1]
miR-135b-5p [10]	miR-200a-5p [1]
miR-15b-5p [10]	miR-200b-3p [1]
miR-18a [11]	miR-429 [1]
miR-21 [11]	miR-203 [1, 12, 7]
miR-223 [7]	miR-202 [1, 7]
miR-93 [8]	miR-205 [1, 7]
miR-4746 [8]	miR-1 [1]
miR-145 [13]	miR-133b [1]
miR-675-3p [14]	miR-95 [1]
miR-105 [15]	miR-381 [1]
miR-34a [16]	miR-485-5p [17]
miR-502 [18]	miR-124 [19]
miR-28-5p [20]	miR-195-5p [10]
miR-224 [21]	miR-5095 [22]
miR-93 [23]	miR-139-5p [1]
miR-301b [24]	miR-409-3p [1]
miR-487a [25]	miR-495 [1]
miR-139-5p [26]	miR-148a-3p [27]
miR-183 [28]	miR-29c [29]
miR-877-3p [1]	miR-542-3p [30]
miR-718 [1]	miR-4261 [31]
miR-665 [1]	miR-140 [32]
miR-374a [33]	miR-338-5p [34]
miR-506 [35]	miR-299-5p
miR-27a [40, 36]	miR-143 [23]
miR-24-2 [40, 36]	miR-149-5p [37]
miR-141 [38]	miR-4328 [1]
miR-556-3p [39]	miR-199b-5p [1]
miR-126 [40]	miR-379-5p [1]

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