Dual inhibition of cMET and EGFR by microRNA-338-5p suppresses metastasis of esophageal squamous cell carcinoma

Di CUI[1], Yun ZHU[1], Dong Dong YAN[1], Nikki P.Y. LEE[2], Liang HAN[1], Simon LAW[2], George S.W. TSAO[1], Annie L.M. CHEUNG[1,*]

1 School of Biomedical Sciences, Li Ka Shing Faculty of Medicine, University of Hong Kong, Hong Kong SAR, China

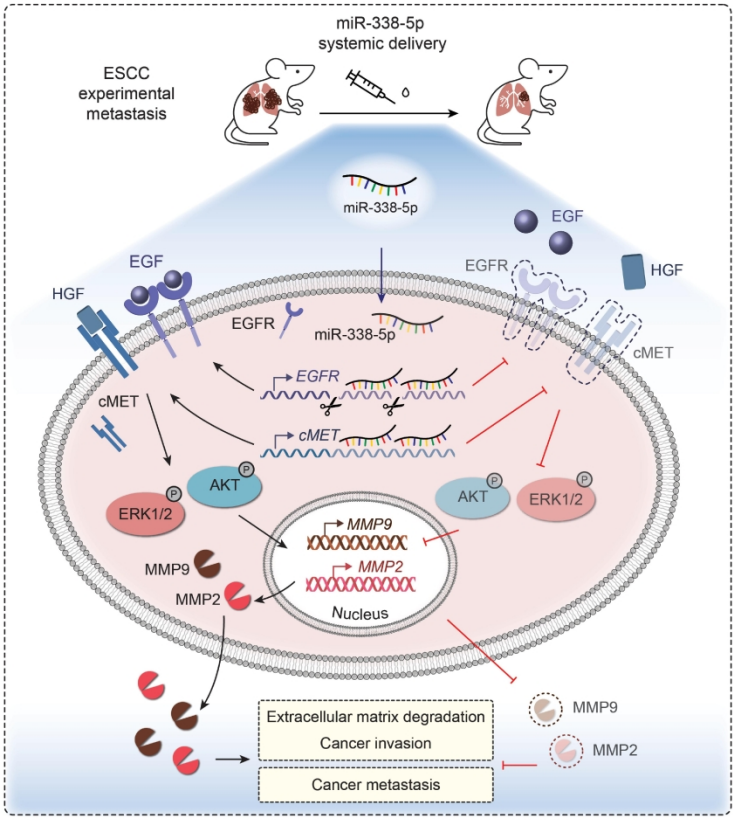
2 Department of Surgery, Li Ka Shing Faculty of Medicine, University of Hong Kong, Hong Kong SAR, China

*** Corresponding Author:** Annie L.M. CHEUNG, School of Biomedical Sciences, Li Ka Shing Faculty of Medicine, University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong SAR, China. Phone: 852-3917-9293; Fax: 852-2817-0857; email: lmcheung@hku.hk

Abstract

MicroRNAs, as a group of post-transcriptional regulators, regulate multiple pathological processes including metastasis during tumor development. Here, we demonstrated the metastasis-suppressive function of microRNA (miR)-338-5p in esophageal squamous cell carcinoma (ESCC). Overexpression of miR-338-5p had inhibitory effect on invasive ability of ESCC cells and extracellular matrix degradation, while silencing miR-338-5p had opposite effects. Mechanistically, miR-338-5p directly targeted the 3' untranslated regions of hepatocellular growth factor receptor cMet (cMET) and epidermal growth factor receptor (EGFR). As a result, miR-338-5p inhibited the downstream signaling cascades of cMET and EGFR, and repressed cMET- and EGFR-mediated ESCC cell invasion. Re-expression of cMET or EGFR in miR-338-5p overexpressing ESCC cells was sufficient to derepress ESCC cell invasion both *in vitro* and *in vivo*. We further showed that such manipulation downregulated the expression and secretion of matrix metalloproteinases 2 and 9, which resulted in impaired extracellular matrix degradation and cell invasion. Most importantly, systemic delivery of miR-338-5p mimic significantly inhibited metastasis of ESCC cells in nude mice. Taken together, our results uncovered a previously unknown mechanism through which miR-338-5p suppresses ESCC invasion and metastasis by regulating cMET/EGFR-MMP2/9 axis, and highlighted the potential significance of miR-338-5p-based therapy in treating patients with metastatic ESCC.

Key words: Metastasis, esophageal squamous cell carcinoma, miR-338-5p, cMET signaling, EGFR signaling



Graphic abstract

167 × 187 mm

Summary

In this study, miR-338-5p was found to suppress ESCC metastasis by inhibiting cMET/EGFR-MMP2/9 axis. Moreover, the therapeutic potential of systemically administered miR-338-5p in inhibiting ESCC metastasis was evaluated using *in vivo* metastasis assay.

Introduction

Esophageal cancer is one of the deadliest cancers globally, being the seventh most common cancer and the sixth ranking cancer in terms of mortality (1). As the major subtype of esophageal cancer, esophageal squamous cell carcinoma (ESCC) constitutes more than 80% of esophageal cancer cases worldwide (2,3). Despite multidisciplinary therapeutic strategies, the outcome of ESCC patients is still dismal with a 5-year survival rate of less than 20% (4). The malignancy of ESCC mainly manifests as high incidence of local invasion and distant metastasis (5-7). Therefore, a fundamental understanding of molecular pathways driving cancer cell invasion and metastasis is essential to the development of new therapies for ESCC.

MicroRNAs (miRNAs) are a group of non-coding RNAs with a length of 21-24 nt, which are endogenously transcribed from introns or "junk" DNA sequences in genome (8,9). MicroRNAs are known to exert biological functions mainly through interfering with gene translation (8). Increasing studies revealed the involvement of miRNAs, either as oncogenic miRNAs or tumor-suppressive miRNAs, in local invasion and distant metastasis of ESCC (10,11). In addition, recent studies indicated the benefits of miRNAs as potential diagnostic biomarkers and novel anti-tumor agents (12-14). We previously found a significant correlation between reduced circulating miR-338-5p level and advanced pathologic stages of ESCC (15), which suggested that miR-338-5p might functionally suppress ESCC development. Up to now, the studies on miR-338-5p in association with ESCC are mainly focused on it role in suppressing chemo- or radio-resistance of ESCC cells (15-17). Whether miR-338-5p is also involved in metastasis of ESCC remains elusive. In this study, we investigated the role of miR-338-5p in ESCC metastasis. We present *in vitro* and *in vivo* data

to show miR-338-5p is a metastasis-suppressor in ESCC via targeting cMET/EGFR-MMP2/9 axis. The potential value of miR-338-5p in miRNA replacement therapy was also explored.

Materials and Methods

Cell lines, clinical samples, and recombinant growth factors

ESCC cell lines including KYSE30, KYSE150, KYSE270, KYSE410, KYSE450, KYSE510 (18) were obtained from DSMZ (Braunschweig, Germany) and cultured in RPMI1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Luciferase-expressing KYSE150 cell line (KYSE150-luc) was used in *in vivo* metastasis experiments as previously described (19). The ESCC cell line T.Tn (20) was maintained in Dulbecco's Modified Eagle Medium (DMEM)/F12 (Sigma-Aldrich) supplemented with 10% FBS. All cell lines which were authenticated using short tandem repeat analysis, were cultured at 37°C in 5% CO_2 and routinely checked for mycoplasma contamination. Human ESCC primary tumor samples (n = 45) were collected with informed consent from patients who were treated at Queen Mary Hospital, Hong Kong (IRB#UW13-300). Recombinant human epidermal growth factor (EGF, #PHG0313) and hepatocyte growth factor (HGF, #PHG0324) proteins were obtained from Gibco (Thermo Fisher Scientific) and used for cell treatment according to manufacturer's instructions.

Plasmids and transfection

Vectors for transient and stable overexpression of scrambled-miR control or miR-338-5p (i.e. pcDNA6.2-miR-Ctrl/miR-338-5p and pLenti-CMV-miR-Ctrl/miR-338-5p, respectively) were constructed as previously described (15). Vectors for stable knockdown of miR-338-5p (pmiRZip-338-5p) and scrambled control (pmiRZip-Ctrl) were purchased from System

Biosciences (Mountain View, CA, USA). Luciferase reporter vector (psiCHECK2) utilized in dual-luciferase assay was given by Prof. Kwanghee Baek (Kyung Hee University, South Korea). For stable overexpression of cMET and EGFR, pLVU/RED-cMET and pLVU/RED-EGFR were reconstructed using Gateway system (Invitrogen, Thermo Fisher Scientific). The cMET and EGFR entry clones, pDONR223- cMET and pDONR223- cMET, as well as backbone vector pLVU/RED, were obtained from Addgene (Watertown, MA, USA). For stable knockdown of endogenous cMET and EGFR, pLKO.1-shRNA clones for lentivirus packaging were purchased from Sigma-Aldrich (**Supplementary Table 1**). MirVana miRNA mimic of miR-338-5p and the corresponding miRNA control utilized for transient expression of miR-338-5p were purchased from Ambion (Thermo Fisher Scientific).

Lipofectamine® 2000 and Lipofectamine RNAiMAX reagent (Invitrogen, Thermo Fisher Scientific) were used as transfection reagents for plasmid and miRNA mimic, respectively, according to manufacturer's instructions.

Cell viability assay

Cell viability was assessed using Resazurin assay (Sigma-Aldrich) according to manufacturer's instructions. Relative proliferation was calculated by normalizing to the corresponding miR-Ctrl or miRZip-Ctrl cells.

Quantitative real-time PCR (qPCR)

MicroRNAs were extracted from clinical samples using mirVana miRNA isolation kit (Invitrogen, Thermo Fisher Scientific) and reverse-transcribed using TaqMan™ microRNA reverse transcription kit (Applied Biosystems, Thermo Fisher Scientific). MicroRNA expression was detected according to instruction of TaqMan miRNA assays (Applied Biosystems, Thermo Fisher Scientific) for miR-338-5p (ID: 002658) with U6 snRNA (ID:

001973) as internal control. Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized using high-capacity cDNA reverse transcription kit (Applied Biosystems, Thermo Fisher Scientific). Quantitative real-time PCR was performed using iTaq universal SYBR green supermix (Bio-Rad Laboratories, Inc., Hercules, California, USA). The specific primer sequences for gene are listed in **Supplementary Table 2**. All qPCR experiments were performed on BioRad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) Expression of 18S ribosomal RNA was utilized as internal control for calibration. Relative gene expression values were shown as $2^{-\Delta\Delta Ct}$ representing its fold change compared with experimental control. The absolute expression value of miR-338-5p in clinical samples was expressed as $2^{-\Delta Ct}$.

Western blot analysis

Western blot was performed as previous described [18]. The information of antibodies were listed in **Supplementary Table 3.** Protein bands from chemiluminescent signals were visualized by exposure to Fuji medical x-ray film (Fujifilm, Tokyo, Japan) or ChemiDoc™ imaging system (Bio-Rad Laboratories, Inc.). Protein bands from Western blot films were quantified using ImageJ according to intensity, and normalized to that of β-actin.

Prediction of potential miR-338-5p target genes

Web-based bioinformatic tools including DIANA Tools (http://diana.imis.athena-innovation.gr), TargetScan (http://www.targetscan.org), miRanda (http://www.microrna.org), and Microcosm (https://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) were utilized according to different algorithms to predict novel potential targets of miR-338-5p.

Dual luciferase reporter assay

The 3'UTR region of cMET and EGFR were cloned and ligated into psiCHECK2 vectors (psiCHECK2-cMET-3'UTR-WT and psiCHECK2-EGFR-3'UTR-WT). Site-directed mutagenesis was performed using QuikChange Lightning Site-directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) to alter the predicted miRNA-binding site in the 3'UTR. The primers used for generating psiCHECK-2-cMET-3'UTR-WTΔ1/Δ2/Δ1Δ2 and psiCHECK-2-EGFR-3'UTR-WTΔ1/Δ2/Δ1Δ2 vectors were listed in **Supplementary Table 4**. Luciferase activity was detected using Promega Dual-Luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Cell invasion and migration assay

Transwell migration and invasion assays were carried out as described previously (21). Cell invasion was also detected by QCM™ gelatin invadopodia assay (Sigma-Aldrich) according to the manufacturer's instructions. Florescent microscopic images were taken under Carl Zeiss LSM 880 confocal system (ZEISS Microscopy, Jena, Germany) and analyzed by ImageJ software. At least 200 cells were analyzed in triplicates for each sample. Cell invasion was quantified as percent degradation area of total cell area and normalized to corresponding control.

Experimental metastasis in nude mice and *in vivo* delivery of miR-338-5p

Experimental metastasis in nude mice was performed as previously described (19). To investigate whether miR-338-5p inhibits tumor metastasis *in vivo* and whether such function is mediated through targeting cMET and EGFR, four luciferase (luc)-expressing KYSE150 sublines with stable overexpression of the following were generated: (i) scrambled miRNA control; (ii) miR-338-5p; (iii) miR-338-5p and cMET; (iv) miR-338-5p and EGFR. About

5×10^5 ESCC cells were suspended in 100 µl PBS and injected into 4 different groups of nude mice (n = 7/group) through the lateral tail vein for comparison of metastatic activity. The condition and well-being of the mice were monitored closely. Bioluminescence signal was monitored and detected by the IVIS1 Imaging System (PerkinElmer, Hopkinton, MA, USA) to assess lung metastasis. The average radiance values (p/s/cm/sr) were collected for further statistical analysis.

To evaluate the therapeutic potential of systemically administered miR-338-5p in inhibiting tumor metastasis, 5×10^5 KYSE150-luc cells were injected intravenously into 6-week-old nude mice via the tail vein. After 1 week when established lung metastasis became detectable, the mice were randomly separated into 4 treatment groups (n = 6/group) and injected intravenously (0.1 ml/animal) every 5 days with: (i) 20 µg miRIDIAN microRNA human hsa-miR-338-5p mimic (Horizon Discovery Group plc, Cambridge, United Kingdom), (ii) 20 µg microRNA mimic negative control #1 (miR-Ctrl mimic, Horizon Discovery Group plc), (iii) vehicle (*In vivo*-jetPEI®, Polyplus transfection, Illkirch, France), or (*iv*) saline. System delivery of miRNA mimic. Each mouse received a total of 5 injections. Metastasis was monitored once a week by bioluminescent imaging. The protocols of *in vivo* metastasis in nude mice were approved by The Committee on the Use of Live Animals on Teaching and Research, the University of Hong Kong (CULATR No. 4795-18).

Statistical analysis of experimental and clinical data

The experimental data were analyzed with PRISM 5.0 software (GraphPad Software Inc., San Diego, CA, USA). All *in vitro* experiments and assays were repeated at least 3 times, and the data were expressed as mean ± SEM and compared by Student's *t*-test. The RNAseq data of esophageal cancer (ESCA) cohort in The Cancer Genome Atlas (TCGA) database were acquired from Genomic Data Commons Data Portal, NCI (https://portal.gdc.cancer.gov/).

Pathway activities (shown as z score of PARADIGM pathways) were obtained from University of California Santa Cruz's Xena browser (https://xenabrowser.net/). Pearson's correlation analysis was used to analyze the correlation between (*i*) miR-338-5p expression and protein levels of cMET and EGFR in ESCC; (*ii*) miR-338-5p expression and RNA expressions of cMET, EGFR, MMP2, and MMP9, in TCGA-ESCA data corhort; and (*iii*) miR-338-5p expression and pathway activities of EGFR-involved signaling events. Significance was considered when *p* values were $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Results

MicroRNA-338-5p suppressed ESCC cell invasion *in vitro*

To investigate the role of miR-338-5p in metastasis of ESCC, we firstly established several ESCC cell lines with stable over-expression of miR-338-5p (**Figure 1A upper panel**) and and then performed transwell assays to evaluate the metastasis potential of these cells. The results showed that ectopic expression of miR-338-5p significantly inhibited cell invasion and migration abilities in ESCC cell lines, compared with cells expressing miR-Ctrl (**Figure 1B**). On the other hand, ESCC cells with miR-338-5p stable knockdown (**Figure 1A lower panel**) exhibited enhanced invasive ability, but the cell migration ability of these cells were unaffected. (**Figure 1C**). Notably, neither overexpressing nor inhibiting miR-338-5p had any effect on cell viability (**Figure S1**), indicating that the changes in metastatic potential observed above were unlikely to be due to changes in cell proliferation. Together, these results showed that miR-338-5p can suppress the invasiveness of ESCC cell *in vitro*.

We next explored the mechanism underlying the inhibition of ESCC metastasis by miR-338-5p. Epithelial-mesenchymal transition (EMT) and extracellular matrix (ECM) remodeling are

two critical processes during tumor metastasis (22). Firstly, to investigate whether miR-338-5p suppressed ESCC cell invasion through reversing EMT, the expressions of epithelial and mesenchymal markers were studied in miR-Ctrl and miR-338-5p overexpressing ESCC cell lines. The results showed that, the expression of E-cadherin was not obviously upregulated upon miR-338-5p overexpression, and that there was no decrease in expression of N-cadherin, indicating that miR-338-5p did not affect EMT in ESCC cells (**Figure S2**). We then examined whether miR-338-5p overexpression could affect ECM degradation by ESCC cells. As shown in **Figure 1D**, miR-338-5p overexpression significantly reduced gelatin degradation by ESCC cells, indicating that miR-338-5p hindered ECM remodeling during cell invasion in ESCC. Altogether, these results suggested that miR-338-5p could repress ESCC cell invasion, possibly through suppressing ECM degradation by ESCC cells.

MicroRNA-338-5p directly targeted cMET and EGFR in ESCC

MicroRNAs exert their functions mainly by targeting certain genes and regulating protein translation. To identify the target genes of miR-338-5p that meditated its anti-invasive effect, multiple miRNA target prediction tools including miRanda, TargetScan, microcosm, and DIANA were utilized. The results unanimously indicated that cMET and EGFR, which are two well-known oncogenes with metastasis-promoting functions in ESCC (23), were potential targets of miR-338-5p (**Figure 2A**). The predicted binding sites of miR-338-5p on 3'UTRs of cMET and EGFR are illustrated in **Figure 2B**. To evaluate whether miR-338-5p regulated cMET and EGFR by directly targeting their 3'UTRs, a series of dual-luciferase assays were then performed. Vector constructs containing wild type 3'UTR of cMET and EGFR, or site-directed mutants (Δ1, Δ2, and Δ1Δ2) with deletion of either or both binding sites of miR-338-5p, were co-transfected into KYSE150 cells together with miR-338-5p

overexpression plasmid (i.e. pcDNA6.2-miR-338-5p) or miR-338-5p mimicking oligonucleotides. The results showed that miR-338-5p overexpression significantly reduced luciferase activity of wild type 3'UTR of cMET (**Figure 2C**). Notably, mutation of either one of the binding sites could only partially eliminate the inhibitory effect of miR-338-5p, whereas mutation of both binding sites completely abolished the inhibitory effect (**Figure 2C**). This indicated that both binding sites are required for the suppressive effect of miR-338-5p on cMET expression. As for EGFR, expression of miR-338-5p also significantly downregulated the luciferase activity of wild type EGFR 3'UTR. However, mutation of binding site 1 only partially abrogated the inhibitory effect of miR-338-5p, whereas mutation of binding site 2 alone or both two binding sites was sufficient to completely eliminate the inhibitory effect of miR-338-5p (**Figure 2D**). This suggested that the second binding site within its 3'UTR region was especially crucial for miR-338-5p to exert suppressive effect on EGFR expression.

The ability of miR-338-5p to negatively regulate expressions of cMET and EGFR was confirmed by western blot and qPCR. Overexpression of miR-338-5p resulted in decreased protein levels of cMET and EGFR in ESCC cell lines (**Figure 2E**), while silencing miR-338-5p augmented cMET and EGFR protein expression (**Figure 2F**). Consistently, transient overexpression of miR-338-5p using pcDNA6.2-miR-338-5p plasmid or miR-338-5p mimic resulted in reduction of cMET and EGFR protein levels in a dose-dependent manner (**Figure 2G**). Moreover, results from qPCR analysis showed that overexpression of miR-338-5p significantly reduced expression of EGFR in all tested ESCC cell lines, whereas the reduced expression of cMET mRNA was only observed in T.Tn ESCC cells (**Figure 2H**). Likewise, stable silencing miR-338-5p resulted in consistent upregulation of EGFR in ESCC cell lines, but not cMET (**Figure 2I**). These results suggested miR-338-5p regulated cMET and EGFR via different mechanisms, through disrupting mRNA translation of cMET and promoting

mRNA degradation of EGFR. In addition, we also analyzed the expression of miR-338-5p, cMET and EGFR in clinical ESCC tumor samples and esophageal cancer (ESCA) dataset from TCGA database. Data from 45 cases of ESCC primary tumors showed that the expression of miR-338-5p was negatively correlated with protein levels of cMET and EGFR (**Figure 2J**). Consistent with our qPCR results, analysis of RNA sequencing data from TCGA/ESCA data cohort showed a negative correlation between miR-338-5p and EGFR mRNA, but such negative correlation was not found between miR-338-5p and cMET mRNA expression (**Figure 2K**). Taken together, these data suggested that miR-338-5p directly targets cMET and EGFR in ESCC with distinct regulatory mechanisms.

cMET and EGFR mediated the inhibitory effect of miR-338-5p on ESCC metastasis *in vitro* and *in vivo*

Given that miR-338-5p directly repressed the expression of cMET and EGFR, we next asked whether cMET and EGFR indeed mediate the inhibitory effect of miR-338-5p on invasion of ESCC cells. To answer this question, we re-introduced cMET and EGFR, respectively, back into the ESCC cell lines with stable expression of miR-338-5p (**Figure S3A**) and performed transwell assay to check their metastatic potential. The results showed that re-overexpressing either cMET or EGFR was sufficient to abolish the anti-invasive effect of miR-338-5p in ESCC cells (**Figure 3A**). On the other hand, silencing cMET or EGFR by shRNAs in miR-338-5p stable knockdown ESCC subclones (**Figure S3B**) reversed the upregulated cell invasion ability (**Figures 3B and C**). To consolidate our observations in cell lines, a luciferase labeled ESCC cell line KYSE150 (KYSE150-luc) was intravenously injected into nude mice for *in vivo* study. Bioluminescent imaging (**Figure 3D**) and histological examination (**Figure 3E**) of the mouse lungs showed that overexpression of miR-338-5p

subdued metastasis of KYSE150-luc cells to the lungs. Such inhibitory effect was abolished upon re-expression of cMET or EGFR (**Figures 3D and E**). There was no significant difference in body weight among the experiment groups throughout the experiment (**Figure S4**). Together, these results suggested that miR-338-5p exerted its suppressive effect on ESCC metastasis at least partially through targeting cMET and EGFR.

miR-338-5p abolished cMET and EGFR-mediated signaling cascade and cell invasion in ESCC

Both cMET and EGFR belong to the receptor tyrosine kinases (RTKs) superfamily which mediate intracellular signaling transduction upon sensing extracellular stimulation by ligand binding (24). Since cMET and EGFR were found to mediate the metastasis-suppressive effect of miR-338-5p, we next explored whether miR-338-5p affected the downstream signaling cascades of cMET and EGFR in ESCC cells. The activation status of multiple downstream effectors of the cMET and EGFR signaling pathway was analyzed using western blots after treatment with recombinant human HGF and EGF, respectively. The results showed that miR-338-5p overexpression suppressed activation of Gab1, AKT and ERK in ESCC cells, indicating attenuation of cMET and EGFR signaling in comparison with control cells (**Figure 4A and B**). Moreover, we noted that miR-338-5p expression had a significant inverse correlation with the activity of EGFR signaling (**Figure S5A**) and a positive correlation with pathway activity of EGFR downregulation (**Figure S5B**) in the TCGA-ESCA data cohort, which further confirmed that miR-338-5p was able to inhibit EGFR-mediated signaling. Notably, miR-338-5p was also found to have significant negative correlations with EGFR signaling events mediated by phospholipase C-γ (PLCγ) and Shc transforming protein (Shc)

(**Figures S5C** and **D**). This is consistent with our results in **Figure 4B** showing reduced activation of PLCγ and Shc in miR-338-5p overexpressing ESCC cells.

Since miR-338-5p overexpression could suppress cMET and EGFR downstream signaling in ESCC cells, we next asked whether miR-338-5p could also inhibit cMET- or EGFR-mediated ESCC cell invasion. Transwell invasion assays were performed using ESCC cell lines expressing either miR-Ctrl or miR-338-5p in the presence of HGF or EGF to induce cMET or EGFR dependent cell invasion (**Figure 4C)**. As shown in **Figures 4D** and **E**, overexpression of miR-338-5p could significantly ameliorate the HGF- and EGF-induced cell invasion. Collectively, these results suggested that miR-338-5p can suppress cMET and EGFR-mediated signaling cascade and cancer cell invasion in ESCC.

miR-338-5p negatively regulated the expression of matrix metalloproteinases 2 and 9

Matrix metalloproteinases (MMPs) play essential roles in ECM remodeling during cancer cell invasion and metastasis (22). As ECM degradation ability of ESCC cells was impaired upon overexpression of miR-338-5p (**Figure 1D**), we then explored the possible role of miR-338-5p in regulating the expression of MMPs. To this end, the mRNA expressions of several MMP family members in ESCC cell lines expressing either miR-Ctrl or miR-338-5p were determined by qPCR. The results showed that the mRNA levels of two well-known gelatinases, namely MMP2 and MMP9, were consistently downregulated in all tested ESCC cell lines (**Figure 5A**). Conversely, silencing miR-338-5p augmented MMP2 and MMP9 mRNA expressions (**Figure 5B**). Supporting this observation, we found that miR-338-5p expression was negatively correlated with MMP2 and MMP9 in the TCGA-ESCA data cohort (**Figure 5C**). Previous studies reported that cMET and EGFR signaling could promote MMP2 and MMP9 expression in multiple cancer types (25-28). To determine whether miR-338-5p modulates MMP2 and MMP9 expression through regulating cMET and EGFR

signaling, we examined the MMP2 and MMP9 levels in miR-338-5p overexpressing ESCC cells with re-expression of cMET or EGFR. Interestingly, re-expressing either cMET or EGFR was sufficient to restore the expression of MMP2 and MMP9 in ESCC cells at both mRNA and protein levels (**Figure 5D and 5E**). In addition, re-expression of cMET or EGFR also restored the secretion of functional form of MMP2 and MMP9 (**Figure 5E lower panel**). Taken together, these data indicated that miR-338-5p repressed expression of MMP2 and MMP9 to inhibit ECM degradation ability of ESCC cells, possibly via targeting cMET and EGFR signaling.

Systemic delivery of miR-338-5p suppressed ESCC metastasis *in vivo*

The suppressive effect of genetically overexpressed miR-338-5p on ESCC metastasis (**Figures 3D** and **E**) suggested that exogenous miR-338-5p might have therapeutic application in treatment of ESCC, especially in suppressing metastasis. To test this possibility, we evaluated the potential of systemically delivered miR-338-5p mimicking oligonucleotides in inhibiting metastasis of ESCC cells *in vivo*. Bioluminescent imaging and histological examination of the lungs at the end of the experiment showed that mice receiving systemically administered miR-338-5p displayed significantly reduced lung metastasis of ESCC, compared with control mice receiving non-target miR-Ctrl mimic (**Figure 6A and B**). Notably, miR-338-5p mimic suppressed aggravation of lung metastasis during the entire treatment period, with the effect becoming statistically significant from Week 4 onwards (**Figure 6C**). There was no significant loss of body weight in the mice throughout the experiment (**Figure S6**), which indicated that *in vivo* delivery of miR-338-5p did not have any obvious toxic effect. Altogether, these results demonstrated the potential therapeutic benefit of administrating miR-338-5p for treating metastatic ESCC.

Discussion

Non-coding RNAs, as a large pool of post-transcriptional regulators, are widely involved in cancer biology. Several studies emphasized the clinical correlation between aberrant expression of miR-338-5p and pathological processes of multiple cancer types (29-32). Up to now, validated target genes of miR-338-5p in ESCC include Id-1 (15), survivin (16), and FERMT2 (17). Through repressing the expressions of these target genes, miR-338-5p can attenuate chemo- and radio-resistance by enhancing cell apoptosis (15,16). In this study, *in vitro* and *in vivo* data showed that miR-338-5p overexpressing ESCC cells were less metastatic. We observed that miR-338-5p overexpression or knockdown had no obvious effects on cancer cell viability and EMT, whereas miR-338-5p stable expression reduced the ability of ESCC cells to degrade ECM, indicating that miR-338-5p overexpression in ESCC cells can suppress ECM remodeling. Increased ECM degradation activity is well established as an important mechanism underlying tumor metastasis. This process requires localized secretion of specialized proteases, such as MMPs (33-35). In this study, we found that both the mRNA levels and functional forms of MMP2 and MMP9 were reduced by miR-338-5p in ESCC cells. Bioinformatic analysis also showed that miR-338-5p expression was negatively correlated with MMP2 and MMP9 in esophageal cancer. Our *in vitro* experiments showed that MMP2 and MMP9 expressions were negatively regulated by miR-338-5p in ESCC cells. Thus, diminished MMP2- and MMP9-dependent ECM degradation might be the underlying mechanism by which miR-338-5p suppresses ESCC cell invasion.

Genomic analyses have uncovered aberrant activation of RTK pathways in ESCC (36,37). Among different types of RTKs, cMET and EGFR are of great significance in ESCC because of their frequent genomic alterations (37). Abnormal increase in HGF or cMET expression

has been reported to be associated with poor prognosis of ESCC (38,39). Functional studies also support the involvement of HGF/cMET signaling in regulating ESCC tumor metastasis (39,40). Here, we for the first time identified cMET as a direct downstream target of miR-338-5p and provided experimental evidence characterizing miR-338-5p/cMET as a novel regulatory axis in ESCC metastasis. Re-introduction of cMET was able to abrogate the inhibitory effect of miR-338-5p on metastasis-related processes in ESCC cells both *in vitro* and *in vivo*. Moreover, overexpression of miR-338-5p was sufficient to repress the HGF-activated cMET downstream signaling and cMET-mediated ESCC cell invasion. These data therefore revealed a novel mechanism through which miR-338-5p suppresses ESCC metastasis via targeting cMET-mediated signaling.

Several studies have demonstrated that abnormal EGFR expression and overactive EGF signaling are involved in the development of cancer including ESCC (37,41-43). In this study, we reported a robust miR-338-5p/EGFR targeting axis in ESCC. We found that miR-338-5p directly targeted and suppressed EGFR expression, as well as attenuated EGFR-mediated signaling transduction and cell invasion. In support of this, there are significant negative correlations between miR-338-5p and EGFR expression, and between miR-338-5p and EGF signaling activities in human esophageal cancer. So far, the miR-338-5p/EGFR regulatory axis has been reported to inhibit proliferation and cloning formation of hepatocellular carcinoma cells *in vitro* (44). However, we did not observe significant changes in cell proliferation rates of ESCC cells upon miR-338-5p expression. This suggests that the miR-338-5p/EGFR regulatory axis may have distinct regulatory effects in different cancer types and cellular contexts.

The oncogenic functions of cMET and EGFR have been studied for over a decade. As cMET and EGFR share most of the same downstream effectors (such as Gab1, AKT, and ERK1/2),

it is not surprising that they cooperate and crosstalk with each other to induce similar functional effects (45-47). Dual-inhibition of cMET and EGFR by miRNAs has been reported to have an enhanced tumor-suppressive effect in head and neck squamous cell carcinoma and non-small cell lung cancer (48,49). As for ESCC, although the oncogenic effects of cMET and EGFR have long been emphasized, the benefits and therapeutic impact of cMET/EGFR dual inhibition are still underexplored. In the present study, we showed for the first time that dual inhibition of cMET/EGFR by miR-338-5p had significant anti-metastatic effect in ESCC. Furthermore, dual inhibition of cMET/EGFR by miR-338-5p resulted in reduced downstream gene expression including MMP2 and MMP9. Thus, our results collectively support a model in which the miR-338-5p-cMET/EGFR-MMP2/9 axis plays a role in suppressing ESCC cell invasion.

MicroRNAs are increasingly regarded as ideal therapeutic targets for treatment of human diseases. Replacement of tumor-suppressive miRNAs, for example, is an attractive approach in cancer therapy. One of the advantages is that miRNAs act as regulators of signaling networks and can mediate silencing of multiple target genes simultaneously. This attribute is believed to enhance anti-cancer effects, compared with conventional therapies such as chemoradiotherapy and single-targeting inhibitors (50). In this study, we showed that systemic delivery of miR-338-5p mimicking oligos could significantly inhibit ESCC metastasis, indicating a potential application of administrating miR-338-5p for treating metastatic ESCC.

In summary, this study demonstrated the function of miR-338-5p in suppressing ESCC cell invasion and metastasis. Mechanistically, miR-338-5p directly targets cMET and EGFR, and represses their downstream signaling to decrease the expression and secretion of MMP2 and MMP9, thereby reducing the ECM degradation ability of ESCC cells (**Figure 6D**). Our

results provide novel insights into the mechanism of ESCC tumor invasion and metastasis, and highlight the potential therapeutic benefits of using miR-338-5p replacement therapy in treating patients with advanced ESCC.

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Authors' Contribution

D.C. and A.L.M.C. conceived this study, designed the experiments and drafted the manuscript; D.C. performed the experiments under supervision of A.L.M.C.; Y.Z. and D.D.Y. were involved in *in vivo* experiments and data analysis; N.P.Y.L., L.H., S.L. and G.S.W.Y, provided technical advice and/or material support; Z.Y., D.D.Y., N.P.Y.L., S.L, G.S.W.Y, and A.L.M.C. reviewed the manuscript for intellectual content; A.L.M.C. obtained funding for this work. All authors read and approved the manuscript before its submission.

Competing interests

The authors declare no competing interests.

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Figure legends

Figure 1. miR-338-5p suppressed invasion and ECM degradation ability of ESCC cells. (A) Efficiency test of miR-338-5p stable overexpression (upper panel) and knockdown (lower panel) in ESCC cell lines by qPCR. (B-C) Transwell invasion and migration assays of ESCC cells with miR-Ctrl/338-5p stable overexpression (B) and knockdown (C). Scale bar = 200 μm. (D) Cy3-labeled gelatin degradation assay of ESCC cells with miR-Ctrl/338-5p stable overexpression. Scale bar = 50 μm. Data are presented as mean ± S.E.M. n = 3. ***p < 0.001, Student's t-test.

Figure 2. EGFR and cMET are direct targets of miR-338-5p. (A) Venn diagram shows EGFR and cMET were predicted as target genes of miR-338-5p by miRNA target predicting tools, including DIANA, Microcosm, TargetScan, and MiRanda. (B) Predicted miR-338-5p binding sites on 3'UTR of cMET and EGFR mRNAs. (C-D) Dual-luciferase assays for KYSE150 cells transfected with psiCHECK2 vectors reconstructed with wildtype (WT) and mutant constructs (i.e. Δ1, Δ2 and Δ1Δ2) of cMET (C) or EGFR (D) 3'UTRs, together with ectopic expression of miR-338-5p by pcDNA6.2 vector (left panel) or miRNA mimics (right panel). (E) Western blot analysis of EGFR and cMET in miR-338-5p stable overexpressing ESCC cells. (F) Western blot analysis of EGFR and cMET in miR-338-5p stable knockdown ESCC cells. Relative protein expression levels were calculated using the intensities of immunoblots and normalized to β-actin. (G) Expressions of cMET and EGFR upon miR-338-5p transient expression. (H-I) qPCR analysis of EGFR and cMET in ESCC cells with miR-338-5p stable overexpression (H) and silencing (I), respectively. (J) Correlation between expression of miR-338-5p and protein levels of cMET and EGFR in 45 ESCC tumor samples. (K) Correlation between expression of miR-338-5p and mRNA levels of cMET and EGFR in ESCA data cohort from TCGA database. (C, D, H and I) Results are normalized to

corresponding control cells. Data are presented in mean value ± S.E.M. n = 3. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Student's *t*-test. (J-K) Statistics are performed with Pearson's correlation analysis.

Figure 3. cMET and EGFR abolished the invasion-suppressive and anti-metastatic effects of miR-338-5p *in vitro* and *in vivo*. (A) Transwell invasion assays of ESCC cells co-expressing miR-338-5p and empty vector control (EV), cMET or EGFR. (B-C) Transwell invasion assays of ESCC cells with co-knockdown of miR-338-5p and cMET (B) or EGFR (C). (D) Lung metastasis in nude mice 8 weeks after intravenous injection of KYSE150-luc cells expressing miR-Ctrl, or miR-338-5p together with EV, cMET or EGFR. Comparisons were made with the EV group using Student's *t*-test (n = 7). (E) Representative H&E stained sections of mouse lungs. (A-C) Scale bar = 100 μm. (E) Scale bar = 200 μm.

Figure 4. miR-338-5p abolished cMET- and EGFR-mediated cell invasion and signaling cascade in ESCC cells. (A-B) Western blots testing cMET- and EGFR-mediated signaling cascades upon HGF and EGF stimulation, respectively, in miR-Ctrl/338-5p overexpressing ESCC cells. (C) Schematic representation of EGF/HGF-induced cell invasion. (D-E) Transwell invasion assays of ESCC cells with miR-Ctrl/338-5p stable overexpression using HGF and EGF as chemo-attractants, respectively. Scale bar = 200 μm. Results are normalized to corresponding cells expressing miR-Ctrl. Data are presented in mean value ± S.E.M. n = 3. ***$p < 0.001$, Student's *t*-test.

Figure 5. miR-338-5p negatively regulated the expression of MMP2 and MMP9. (A) heatmap of mRNA levels of MMPs in miR-Ctrl/338-5p stable overexpressing ESCC cells. (B) qPCR analysis of MMP2 and MMP9 in stable miR-338-5p-silenced ESCC cells. (C) Correlation between miR-338-5p and MMP2/MMP9 gelatinases in TCGA esophageal carcinoma (ESCA) data cohort as determined using Pearson's correlation analysis. (D-E)

qPCR (D) and Western blot (E) analysis of MMP2 and MMP9 expression in ESCC cells co-expressing miR-338-5p and empty vector control (EV), cMET or EGFR. Data are presented as mean ± S.E.M. n = 3. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Student's *t*-test.

Figure 6. Systemic delivery of miR-338-5p mimic suppressed ESCC metastasis *in vivo*. (A) Lung metastasis in nude mice 5 weeks after intravenous injection of KYSE150-luc cells. Effects of intravenous injections of miRNA mimic negative control, miR-338-5p mimic, vehicle and saline were shown in the bioluminescent images and statistical quantification. (B) Representative H&E stained sections of lungs at the end of the experiment. Scale bar = 200 μm. (C) Quantification of bioluminescent intensities in the lung areas of nude mice during experimental period. Data are presented in mean value ± S.E.M. miR-Ctrl vs miR-338-5p mimic: *$p < 0.05$, **$p < 0.01$, Student's *t*-test. (D) Schematic illustration of the mechanism by which miR-338-5p suppresses ESCC cell invasion and metastasis.

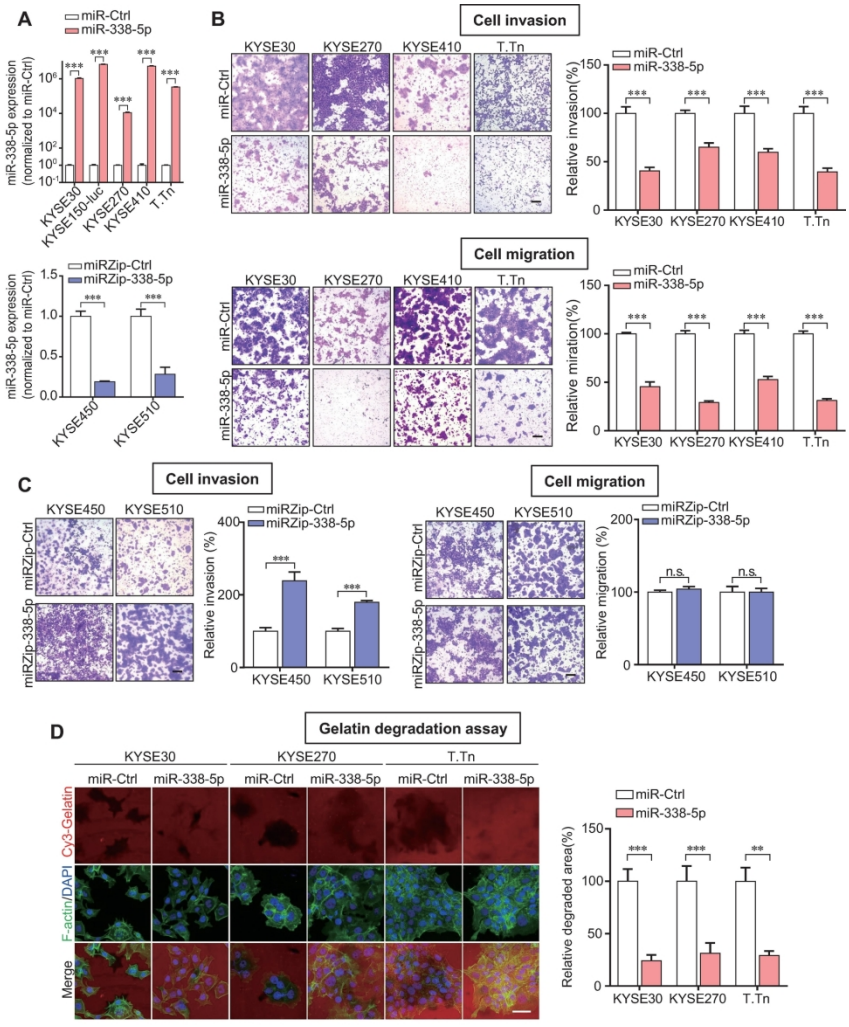

Figure 1

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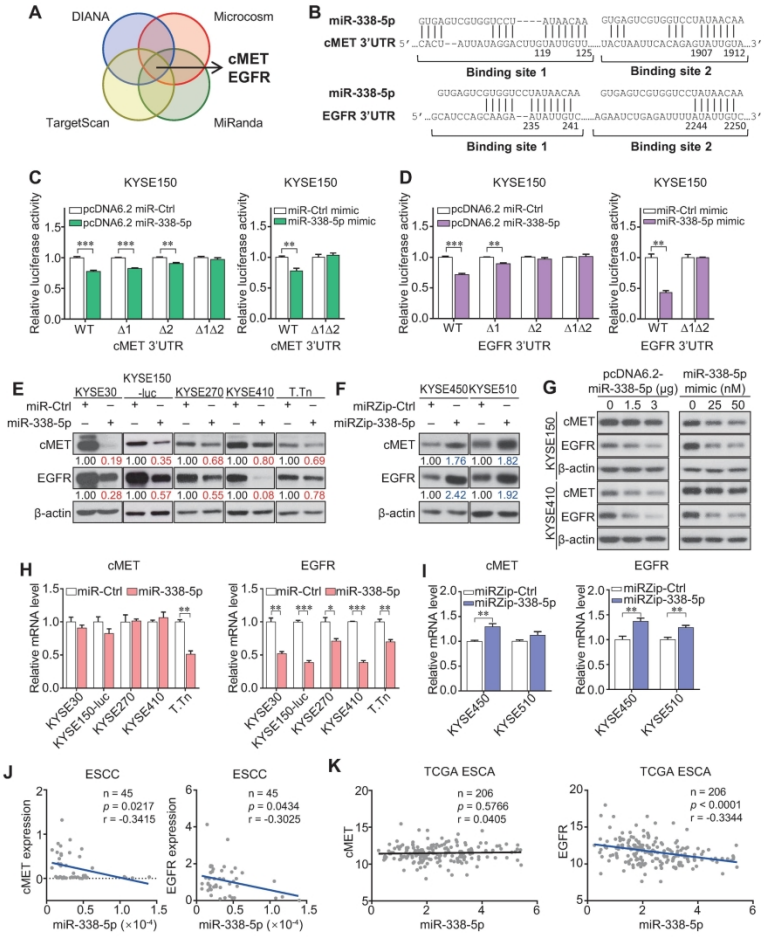

Figure 2

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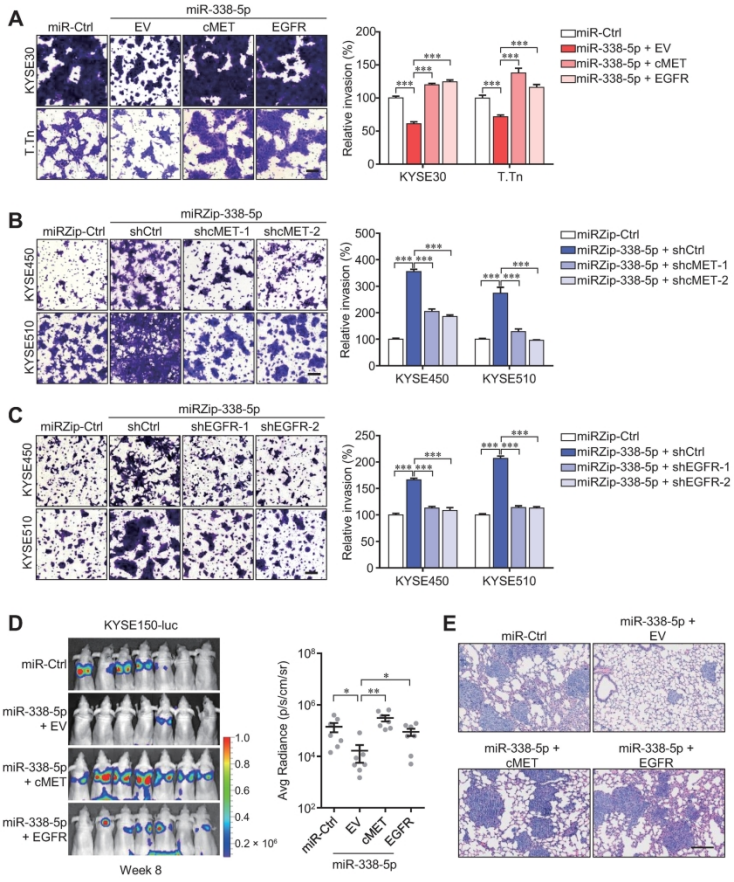

Figure 3

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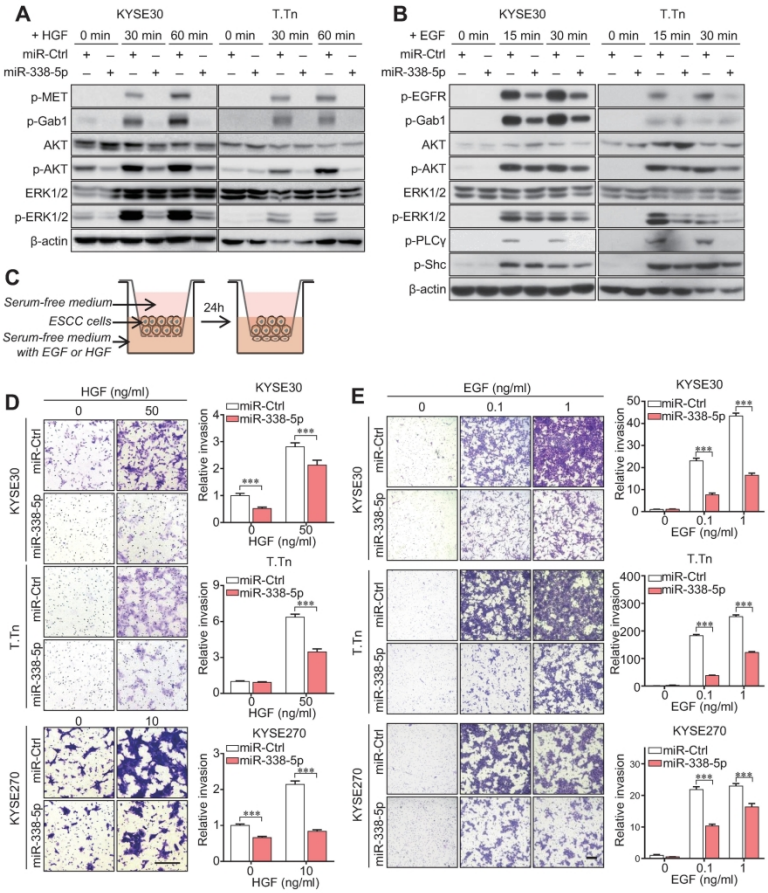

Figure 4

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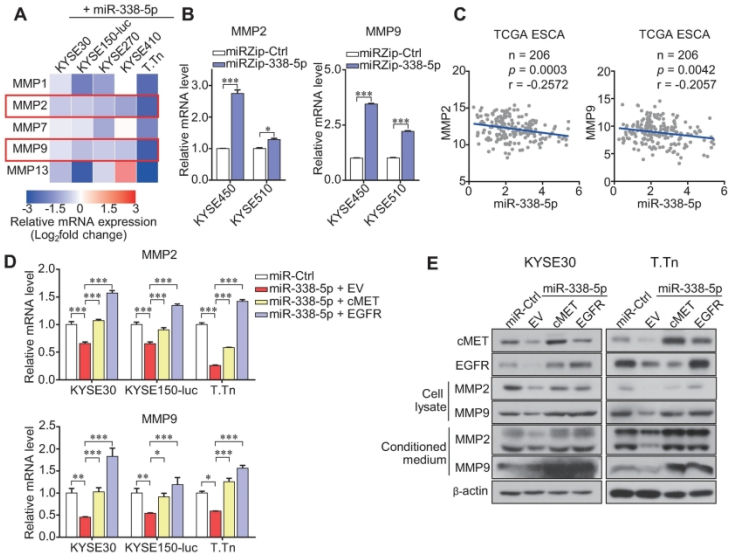

Figure 5

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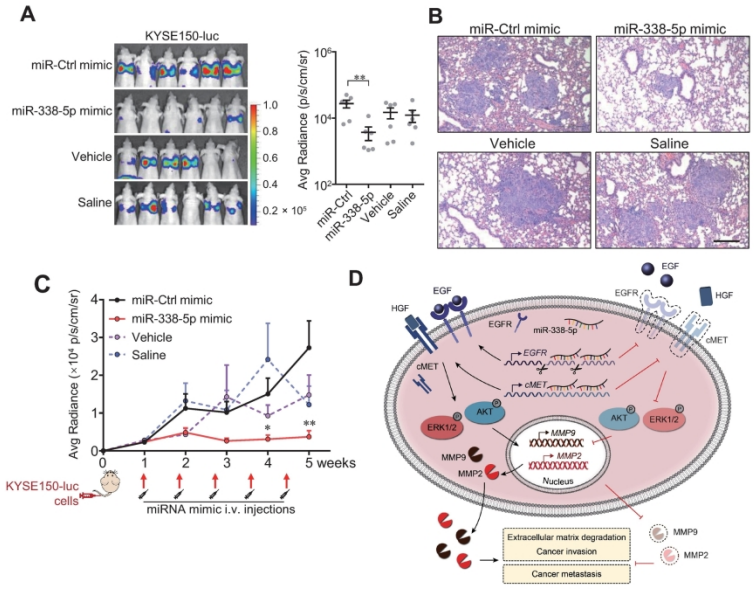

Figure 6

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