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Original Articles

Endoplasmic reticulum-localized ECM1b suppresses tumor growth and regulates MYC and MTORC1 through modulating MTORC2 activation in esophageal squamous cell carcinoma

Valen Zhuoyou Yu[a,1], Josephine Mun Yee Ko[a,1], Lvwen Ning[a], Wei Dai[a], Simon Law[b], Maria Li Lung[a,*]

[a] Department of Clinical Oncology, University of Hong Kong Li Ka Shing Faculty of Medicine, Pokfulam, Hong Kong
[b] Department of Surgery, University of Hong Kong Li Ka Shing Faculty of Medicine, Pokfulam, Hong Kong

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ABSTRACT

Esophageal squamous cell carcinoma (ESCC) is a deadly disease with dismal 5-year survival. *Extracellular matrix protein 1* (*ECM1*) was identified as one of the most downregulated genes by transcriptomic analysis of normal esophageal/ESCC paired tissue samples. *ECM1* plays oncogenic roles in cancer development in various cancer types. However, little is known about its role in ESCC. *In vivo* and *in vitro* functional assays coupled with analyses on public datasets and detailed molecular and mechanistic analyses were used to study the gene. We demonstrate that as opposed to the previously identified oncogenic role of *ECM1a*, *ECM1b* is a novel tumor suppressor in ESCC. *ECM1* is significantly downregulated in ESCC and several other squamous cell carcinomas. *ECM1b* encodes a cellular protein that suppresses MYC protein expression and MTORC1 signaling activity. MTORC2 inactivation leads to suppressed MYC expression and MTORC1 signaling. ECM1b localizes to the endoplasmic reticulum and suppresses MTORC2 activation by inhibiting MTORC2/ribosome association. By regulating MTORC2/MYC/MTORC1 signaling, *ECM1b* suppresses general protein translation and enhances chemosensitivity. We provide evidence establishing a novel role of *ECM1* in cancer that suggests *ECM1b* as a biomarker for ESCC disease management.

1. Introduction

Esophageal carcinoma ranks as the seventh most frequent and sixth most deadly cancer worldwide, with an estimated 572,000 new cases and 509,000 deaths in 2018 [1]. There are two major histologic forms including esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma. The latter is more prevalent in developed countries, and ESCC is the dominant histologic type in Asia [2]. The molecular events leading to initiation, development and metastasis of ESCC are still largely unknown. Currently there is no available targeted therapy for ESCC management. Identifying suitable drug targets is needed. More detailed molecular and functional analyses of genes involved in ESCC tumorigenesis are urged.

We performed transcriptomic analysis on normal esophageal/ESCC paired tissue samples and identified *Extracellular matrix protein 1* (*ECM1*) as one of the most downregulated genes. *ECM1* encodes a secreted protein that is involved in endochondral bone formation and angiogenesis [3], lipoid proteinosis [4], T-cell development [5] and tumor development in which *ECM1* plays critical oncogenic roles [6–8]. Three variants resulting from alternative splicing exist, resulting in three protein isoforms, ECM1a/b/c [3]. ECM1a is the most studied isoform, while there are very limited studies on ECM1b/c.

We examined the expression of *ECM1* in ESCC and several other squamous cell carcinomas (SCCs) and found that *ECM1* RNA expression is significantly downregulated in tumors, as compared to normal tissue samples. We employed various *in vivo* and *in vitro* assays to functionally characterize *ECM1*. We demonstrate that cellular *ECM1b* is a novel tumor suppressor in ESCC. It encodes an endoplasmic reticulum (ER)-localized protein and acts as a MTORC2 regulator that suppresses MTORC2 activation by modulating MTORC2/ribosome association, which leads to suppressed protein translation and enhanced chemosensitivity. The results suggest *ECM1b* as a tumor suppressor in ESCC and a biomarker for ESCC therapeutics.

* Corresponding author. L6-43, 6/F, Laboratory Block, 21 Sassoon Road, Pokfulam, Hong Kong.
E-mail address: mlilung@hku.hk (M.L. Lung).
[1] Equal contribution.

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2. Materials and methods

2.1. ESCC patient tissue samples

ESCC tissue specimens were collected from the Queen Mary Hospital from 2001 to 2006, as previously reported [9]. Approval for this study was obtained from the Hospital Institutional Review Board at the University of Hong Kong.

2.2. Transcriptomic analysis

We sequenced the RNA from four paired adjacent normal esophageal/ESCC tissue samples using the Illumina HiSeq 2000 (2x100bp paired reads). The raw RNA-seq data was cleaned and aligned to the hg19 reference genome using Tophat [10] (version 2.0.14, bowtie version 2.2.4) with library-type fr-firststrand parameter. The gene expression profile was calculated by Cufflinks [11] (version 2.2.1) with the Ensemble gene annotation file. The differentially expressed genes were analyzed using Cuffdiff [11] between each normal and tumor paired sample and between the pooled normal and tumor samples. MISO [12] was used to identify alternative splicing events; the alternative splicing events were then visualized using Integrative Genomics Viewer [13].

2.3. Chemical reagents

All inhibitors used in this study were purchased from Selleckchem (Houston, TX).

2.4. Cell lines

Immortalized human esophageal epithelial cell line NE1 (Research resource identifier:CVCL_E306) and ESCC cell lines including 81T (CVCL_Y011), EC1 (CVCL_5V05), HKESC-2 (CVCL_D571), KYSE30 (CVCL_1351), KYSE70 (CVCL_1356), KYSE150 (CVCL_1348), KYSE180 (CVCL_1349), KYSE270 (CVCL_1350), KYSE450 (CVCL_1353), KYSE510 (CVCL_1354), KYSE520 (CVCL_1355), SLMT (CVCL_E305), and T.Tn (CVCL_3175) were cultured as described [14]. KYSE30TSI was established through two rounds of nude mouse subcutaneous xenograft tumor segregant establishments from KYSE30 cell line [14]. KYSE180TS was established from a KYSE180 nude mouse subcutaneous xenograft tumor segregant. These two derived cell lines are used in *in vivo* tumorigenicity assay. Cell line authentication by STR DNA profiling and mycoplasma test by PCR amplification of mycoplasma DNA were performed in all cell lines used.

2.5. Plasmids and lentivirus preparation and infection

The protein coding sequences of *ECM1a* and *ECM1b* were amplified from NE1 and cloned into pLVX-EF1a lentiviral vector [14]. The GFP-encoding control plasmid pLVX-EF1a-GFP was used [15]. Oligonucleotides encoding MYC- (sgRNA1: CTTCGGGGAGACAACGACGG; sgRNA2: AGAGTGCATCGACCCCTCGG) and RICTOR-targeted sgRNAs (sgRNA1: GTGCCAAATAATTATCCATG) were designed using sgRNA Design Tool (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design) and cloned into lentiCRISPRv2 vector (Addgene plasmid # 52961; http://n2t.net/addgene:52961; RRID:Addgene_52961). Non-targeting sgRNA (sequence: GTTCCGCGTTACATAACTTA) was used as a negative control [16]. A plasmid encoding myr-tagged AKT1 (Addgene plasmid # 46969; http://n2t.net/addgene:46969; RRID:Addgene_46969) was used to express constitutively active AKT1 in ECM1b over-expressing cells. The Renilla luciferase-POLIRES-Firefly luciferase cassette was amplified from pcDNA3 RLUC POLIRES FLUC (Addgene plasmid # 45642; http://n2t.net/addgene:45642; RRID:Addgene_45642) and cloned into pLVX-EF1a. Lentivirus preparation and infection were performed as described [14].

2.6. Conditioned medium preparation

Conditioned media were collected as described [17].

2.7. In vivo tumorigenicity assay

Subcutaneous injection of cancer cells in nude mouse was performed as described [14]. For KYSE180TS, 3×10^6 of cells were injected per site.

2.8. Cell proliferation assay

The proliferation and viability of cells were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as we previously described [9].

2.9. Cell size determination

Cells were harvested by trypsinization and subjected to flow cytometry analysis using the BD FACSCanto II (BD Biosciences, San Jose, CA). Cell size was indicated by the readings of forward scatter area.

2.10. Western blotting analysis

Western blotting analysis was performed as previously described [14]. Antibodies used are listed in Supplementary Materials and methods.

2.11. In vitro translation assay

Cells labeled with pLVX-EF1a-RLuc-POLIRES-FLuc were utilized. Cells were treated with 30 μM Enduren (Promega Corporation, Madison, WI) or 3 mg/mL D-Luciferin potassium salt (Biovision, Inc., Milpitas, CA) for 15 min and subjected to bioluminescence imaging using the PE-IVIS Spectrum imaging system (PerkinElmer, Waltham, MA).

2.12. Subcellular fractionation

Subcellular fractionation was performed using the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's protocol.

2.13. Immunofluorescence confocal microscopic co-localization analysis

Immunofluorescence staining was performed as described [9]. ECM1 antibody (#HPA027241, Sigma-Aldrich Corporation, St. Louis, MO) was used to target ECM1b, followed by incubation with Alexa Fluor™ 488 secondary antibody (Thermo Fisher Scientific). Endoplasmic reticulum was labeled using Concanavalin A, Alexa Fluor™ 594 Conjugate (Thermo Fisher Scientific). DAPI was used to label the nucleus. Confocal imaging was performed using the Carl Zeiss LSM 800 (Carl Zeiss AG, Oberkochen, Germany) with a 63× objective. Co-localization analysis was performed using the Zen blue edition (Carl Zeiss AG).

2.14. Ribosome pulldown

Ribosome pulldown was performed as described [18].

2.15. Chemosensitivity assay

Cells were seeded and incubated for 48 h before cisplatin treatment (10 μM for 72 h). End-point cell survival was determined by MTT assay.

Viability index was calculated as $MTT^{cisplatin}/MTT^{PBS}$, therefore minimizing the confounding effect of proliferation rate of the cells.

2.16. Statistical analysis

Independent samples *t*-test was applied unless indicated otherwise. A *p*-value less than 0.05 was considered statistically significant. All tests of significance were 2-sided. The error bars shown in the figures represent the 95% confidence interval. For multiple-test comparisons, the *p*-value was adjusted as described [14]. An adjusted *p*-value less than 0.05 is considered significant. An adjusted *p*-value less than 0.1 is considered marginally significant.

3. Results

3.1. RNA sequencing analysis using four sets of esophageal normal/tumor paired tissue samples revealed differentially expressed genes in ESCC

We performed transcriptomic analysis by sequencing the RNA of four sets of normal esophageal/ESCC paired tissue samples from Hong Kong ESCC patients with advanced disease. In total, of 57815 protein-coding and non-protein-coding genes, 15354 genes are expressed with Fragments Per Kilobase of transcript per Million (FPKM) > 1 in the grouped analysis. There were 117 genes significantly differentially expressed (23 downregulated genes and 94 upregulated genes; false discovery rate < 0.05; FPKM > 1 in either normal or tumor group) between normal and tumor samples (Supplementary Table 1).

3.2. ECM1 is downregulated in ESCC

Among the significantly downregulated genes, *ECM1* is of specific interest (Fig. 1A). *ECM1* has been shown to play oncogenic roles in various types of cancer. We further verified the expression of *ECM1* in ESCC. *ECM1* RNA expression is downregulated significantly in three sets of normal esophageal/tumor paired tissue samples by RNA sequencing (SRP007169, SRP008496, and SRP064894) and two sets of samples by microarray analysis (GSE20347 and GSE29001; probe 209365_s_at) (Fig. 1A). Interestingly, *ECM1* RNA expression is also downregulated in cervical cancer, head and neck squamous carcinoma (HNSCC), and oral squamous cell carcinoma (OSCC) (Fig. 1B), potentially suggesting a general role of *ECM1* in SCC. *ECM1* RNA also shows significant differential expressions across different pathologic stages in ESCC patients, with stage I patient samples having the top expression level in an ESCC dataset (*p*-value = 0.0149, n = 98, Supplementary Fig. 1) [19], further suggesting that *ECM1* plays a role in tumor progression in ESCC.

3.3. ECM1a and ECM1b are expressed in esophageal tissue and ESCC

The *ECM1* locus has three variants produced by alternative splicing, namely *ECM1a* (NCBI reference sequence: NM_004425), *ECM1b* (NM_022664), and *ECM1c* (NM_001202858), each encoding for the corresponding protein isoforms [3]. Our RNA sequencing analysis revealed that only *ECM1a* and *ECM1b* are expressed in esophageal tissues and ESCC (FPMK > 1), with *ECM1b* being the dominant splicing variant in normal esophageal tissue (Fig. 1C and Supplementary Fig. 2). Therefore, we focused on these two variants. RNA expression of the two *ECM1* variants in panels of normal esophageal/tumor paired tissue samples and ESCC cell lines was examined by quantitative real-time PCR. Consistent with the above RNA sequencing/microarray analysis results, *ECM1a* is downregulated in 75% (6/8) of ESCC tissue samples and 69% (9/13) of ESCC cell lines tested, while *ECM1b* is downregulated in 100% (8/8) of ESCC tissue samples and 69% (9/13) of ESCC cell lines tested (Fig. 1D).

3.4. ECM1a and ECM1b show different secreted/cellular protein localizations

ECM1a protein is well-recognized as a secreted protein with a signal peptide [20] (Fig. 1E). Although bearing the identical N/C-terminus as ECM1a, no clear evidence of the cellular localization of ECM1b has been shown. Therefore, we examined the cellular localization of both isoforms in ESCC cell lines. We expressed exogenous ECM1a and ECM1b protein by lentiviral transduction in two ESCC cell lines. We observed ECM1a protein expression in conditioned medium of ESCC cell lines, but surprisingly, no ECM1b protein expression was observed in conditioned medium (Fig. 1F), suggesting that ECM1b is not secreted.

3.5. ECM1b expression level affects tumorigenesis in the mouse model and in vitro cell proliferation and cell growth

ECM1a and ECM1b protein expression was restored by overexpression in four tumorigenic cell lines with downregulated endogenous *ECM1* expression, namely KYSE30TSI/150/180TS/450, and cells were injected subcutaneously into the mice and compared with cells expressing green fluorescent protein (GFP) as controls. *ECM1b* overexpression suppresses *in vivo* tumor formation, while *ECM1a* overexpression does not significantly alter tumor size in all the cell lines tested, regardless of the endogenous *ECM1* expression level. (Fig. 2A). These data collectively showed that *ECM1b*, but not *ECM1a*, acts as a tumor suppressor in ESCC. Two cell lines, KYSE30TSI (K30) and KYSE180TS (K180), showing greater tumor-suppressive effects by *ECM1b* overexpression, were used for the following functional and mechanistic analyses.

In vitro cell proliferation was examined. *ECM1b* overexpression causes proliferation suppression in KYSE30TSI and KYSE180TS cell lines (Fig. 2B). We further examined cell growth by determining cell size through flow cytometry. *ECM1b* overexpression evidently reduces cell size in both cell lines (Fig. 2C). These data showed the inhibitory effects of *ECM1b* on cell proliferation and cell growth in ESCC cell lines.

3.6. MYC protein expression and MTORC1 signaling are downregulated in ECM1b-overexpressing cells

Major signaling pathways that regulate cell proliferation and cell growth include the MYC signaling and MTORC1 signaling pathways [21,22]. Therefore, we examined MYC protein expression and phosphorylation status of p70S6K, one of the major downstream effectors of MTORC1 signaling, in *ECM1b*-overexpressing ESCC cell lines. MYC protein expression and phosphorylated p70S6K both showed downregulation in the two cell lines tested (Fig. 3A).

We further examined the public datasets for *ECM1* co-expression profiles and performed enrichment analysis with the molecular signatures database hallmark gene set collection [23]. *ECM1* expression was shown to significantly inversely correlate with three hallmark gene sets related to activated MYC and MTORC1 signaling in ESCC, cervical SCC, HNSCC, and lung SCC (Supplementary Table 2). These data suggest a potential role of ECM1b in MYC/MTORC1 signaling regulation across different SCCs.

3.7. ECM1b suppresses MYC protein translation

We further investigated the mechanism of MYC protein expression regulation by *ECM1b* overexpression. Quantitative real-time PCR showed that *MYC* RNA expression is not altered by *ECM1b* overexpression (Supplementary Fig. 3). Ubiquitination-mediated protein degradation is a well-known mechanism of MYC protein expression regulation [24]. Surprisingly, inhibition of the ubiquitin-proteasome system by MG-132 does not significantly mitigate MYC protein expression downregulation by *ECM1b* (Fig. 3B). However, MYC protein

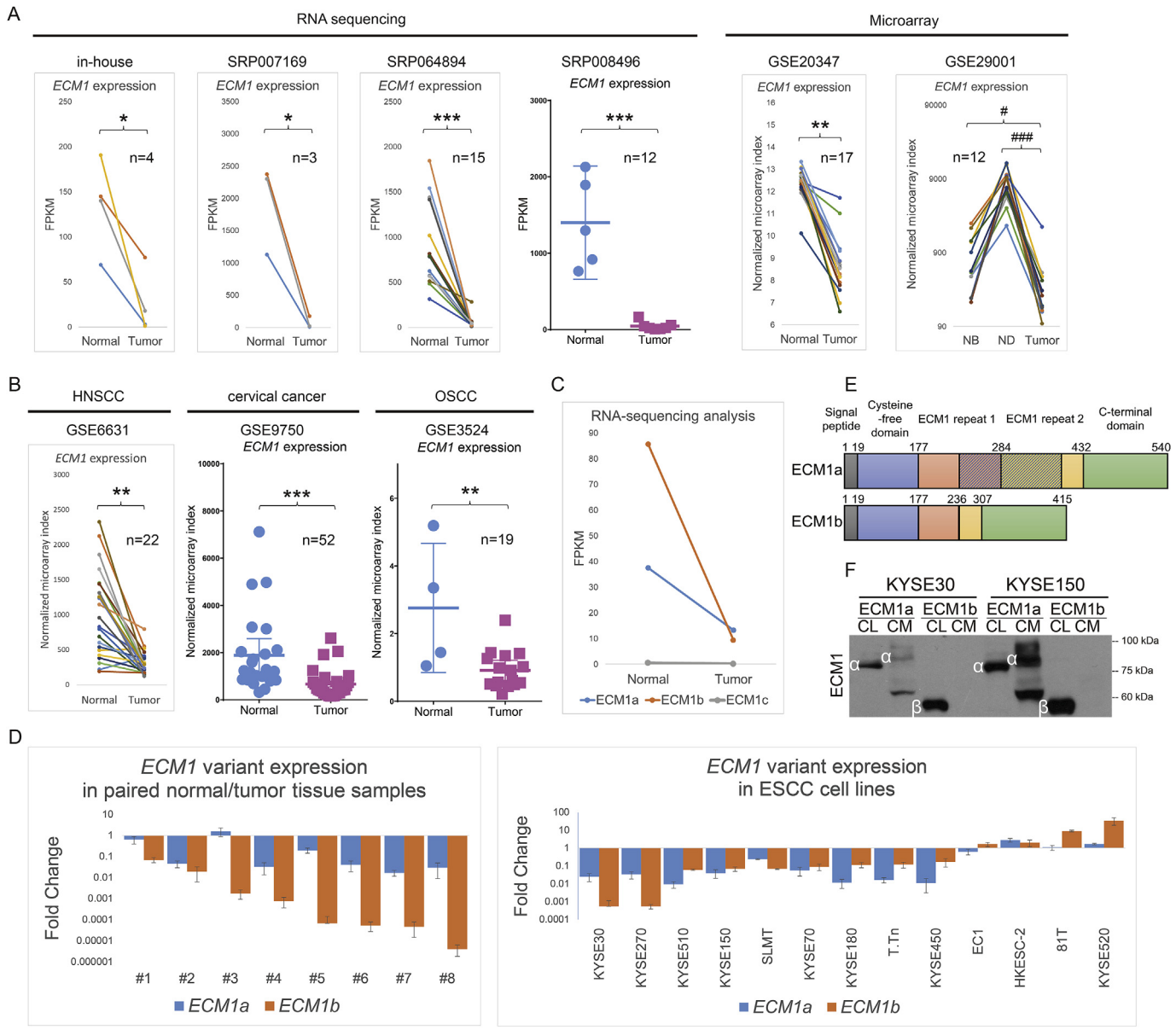

Fig. 1. *ECM1* expression and clinical significance in SCCs. (A) *ECM1* RNA expression is significantly downregulated in ESCC. (B) *ECM1* RNA expression is significantly downregulated in HNSCC, cervical cancer, and OSCC. (C) RNA sequencing analysis revealed differential expression of the three *ECM1* variants in esophageal tissues and ESCC. (D) *ECM1*a and *ECM1b* RNA expression is downregulated in a panel of ESCC paired normal/tumor tissue samples, and a panel of ESCC cell lines using immortalized human esophageal epithelial cell line NE1 as reference. (E) ECM1a and ECM1b differ in a single internal domain. The shaded area indicates ECM1a-specific region. (F) ECM1a and ECM1b protein expression exhibit distinct secreted/cellular localization. NB: Normal basal epithelial cells; ND: normal differentiated epithelial cells; *: p-value < 0.01; **: p-value < 0.001; ***: p-value < 0.0001; #: Adjusted p-value < 0.1; ###: Adjusted p-value < 0.001. Datasets SRP008496 and GSE3524 were analyzed by independent samples t-test; dataset GSE9750 was analyzed by Wilcoxon rank-sum test due to the skewed distribution of the data; other datasets were analyzed by paired samples t-test. CL: cell lysate; CM: conditioned medium. α: corresponding bands for ECM1a; β: corresponding bands for ECM1b. The difference of ECM1a protein migration on SDS-PAGE gel between CL and CM is likely due to N-glycosylation [20].

expression downregulation by *ECM1b* was significantly diminished, when the protein translation machinery was suppressed by puromycin treatment (Fig. 3C). These data suggested that suppressed protein translation regulation significantly contributes to the downregulated MYC protein expression.

3.8. *ECM1b* suppresses general protein translation

MYC and MTORC1 signaling pathways have been implicated in cellular protein translation regulation [25,26]. We then examined the regulation of general protein translation regulation by *ECM1b* using a live cell bioluminescence-based protein translation reporter [27]. We observed both reduced cap-dependent and internal ribosome entry site (IRES)-mediated protein translations in *ECM1b*-overexpressing cells compared to GFP-overexpressing cells (Fig. 3D).

3.9. MTORC2 mediates regulation of MYC protein expression and MTORC1 signaling by ECM1b

The MYC and MTORC1 signaling pathways have been shown to interact with each other [25,28]. In order to further dissect the regulation of these two signaling pathways by *ECM1b* overexpression, we first investigated the interaction between MYC and MTORC1 signaling pathways in ESCC. We interfered with MYC and MTORC1 signaling

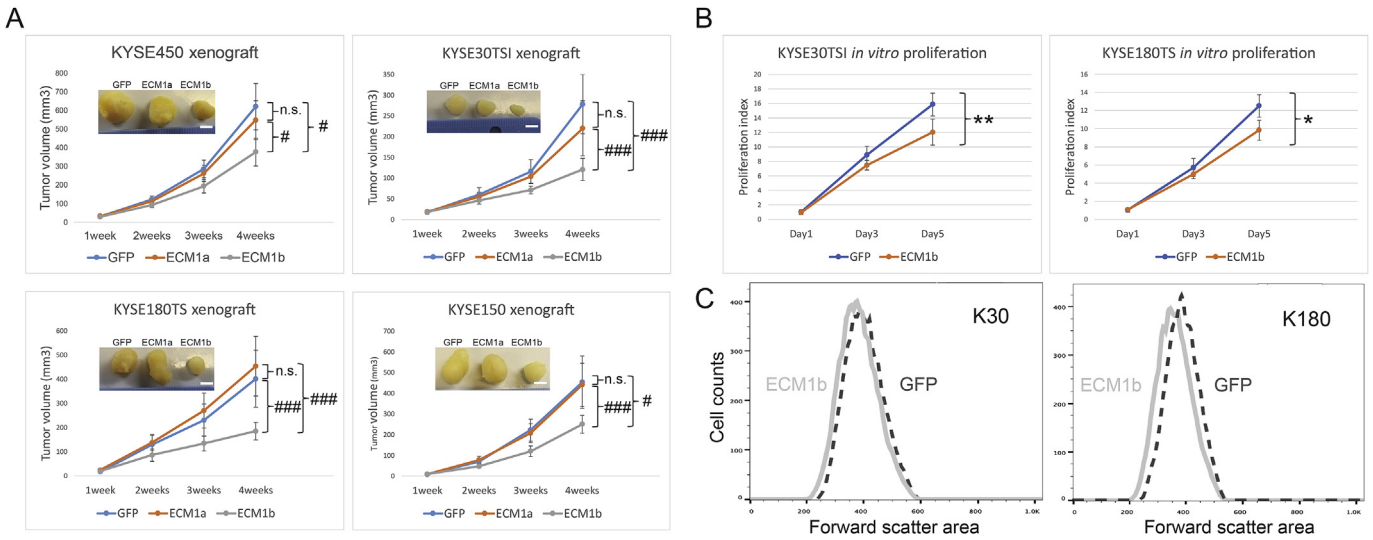

Fig. 2. ECM1 protein expression regulates *in vivo* tumor growth and *in vitro* cell proliferation and cell growth. (A) ECM1b expression suppresses subcutaneous xenograft tumor growth in four ESCC cell lines, while ECM1a expression does not significantly alter tumor growth. Representative xenograft tumor images are shown. Scale bar = 5 mm. (B) ECM1b expression suppresses *in vitro* proliferation in two ESCC cell lines. (C) Representative images showing that ECM1b expression reduces *in vitro* cell size in two ESCC cell lines. Statistical significance was determined by comparing the data from different groups of the last time point. n.s.: not statistically significant; #: Adjusted *p*-value < 0.1; ###: Adjusted *p*-value < 0.001; *: *p*-value < 0.05; **: *p*-value < 0.01.

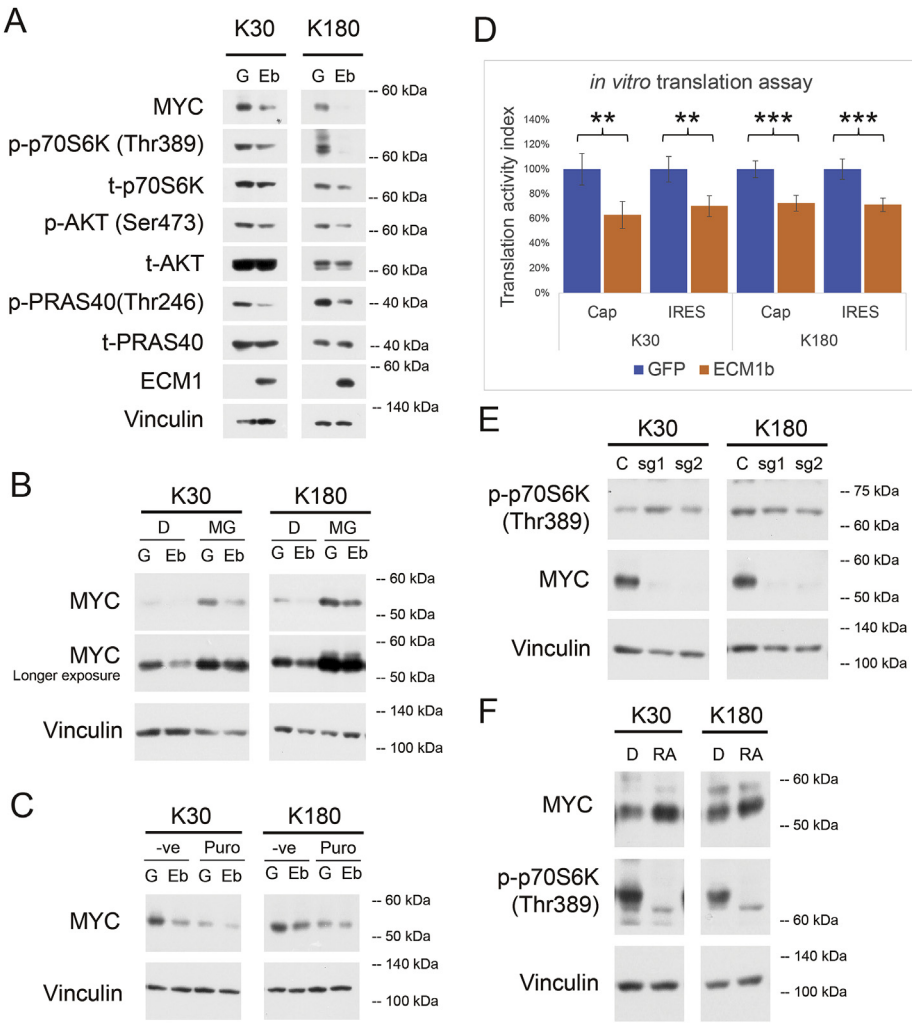

Fig. 3. MYC protein expression and MTORC1 signaling are downregulated in ECM1b-overexpressing cells. (A) Western blotting shows suppressed MYC protein expression, phosphorylation of p70S6K threonine 389, phosphorylation of AKT serine 473, and phosphorylation of PRAS40 threonine 246 in ECM1b-expressing cells. (B) Western blotting shows that MG-132 treatment did not diminish suppression of MYC protein expression by ECM1b. Cells were treated with 20 μM MG-132 for 3 h before cell lysate collections. (C) Western blotting shows that puromycin treatment diminished suppression of MYC protein expression by ECM1b. Cells were treated with 50 μg/mL puromycin for 1 h before cell lysate collections. (D) *In vitro* live-cell bioluminescence-based protein translation assay showed reduced cap-dependent (Cap) and IRES-mediated (IRES) protein translations in ECM1b-overexpressing cells compared to control cells. (E) Western blotting reveals that MYC fKO did not suppress phosphorylation of p70S6K. (F) Western blotting reveals that MTORC1 inhibition by rapamycin did not suppress MYC protein expression. Cells were treated with 10 nM rapamycin for 48 h before cell lysate collections. G:GFP control; Eb: ECM1b; p-: phosphorylated; t-: total form; D: DMSO control; MG: MG-132; Puro: puromycin; **: *p*-value < 0.05; ***: *p*-value < 0.01; C: scrambled sgRNA control; sg1: MYC sgRNA1; sg2: MYC sgRNA2; RA: rapamycin. Vinculin was used as a loading control.

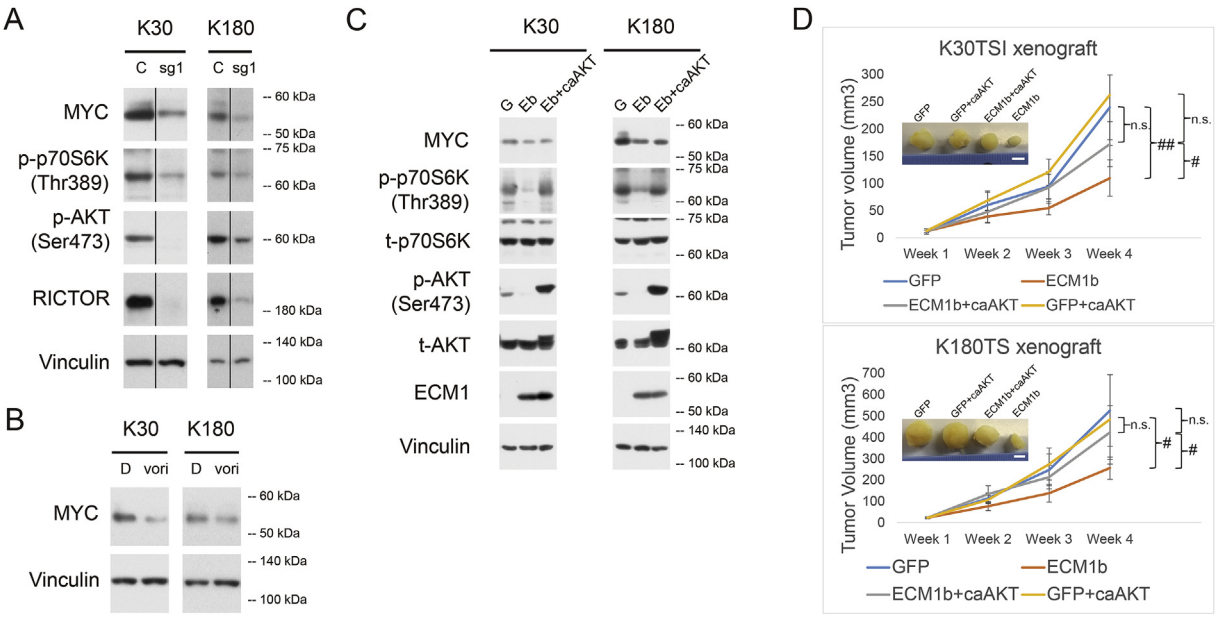

Fig. 4. MTORC2-mediated regulations of MYC protein expression and MTORC1 signaling by ECM1b in ESCC. (A) Western blotting shows suppressed MYC protein expression, phosphorylation of p70S6K, and phosphorylation of AKT serine 473 in RICTOR-fKO cells. (B) Western blotting shows suppressed MYC protein expression by HDAC inhibition. Cells were treated with 20 μM vorinostat for 3 h before cell lysate collections. (C) Western blotting shows that expression of caAKT rescued the down-regulation of p-p70S6K only, but not the down-regulation of MYC protein expression. (D) Expression of caAKT partially rescued ECM1b-induced subcutaneous tumor suppression. Representative xenograft tumor images are shown. Scale bar = 5 mm. C: scrambled sgRNA control; sg1: RICTOR sgRNA1; D: DMSO control; vori: vorinostat; #: Adjusted *p*-value < 0.1; ##: Adjusted *p*-value < 0.05; n.s.: not statistically significant. Vinculin was used as a loading control.

pathways by clustered regularly interspaced short palindromic repeats (CRISPR)-mediated functional knockout (fKO) and by rapamycin treatment, respectively. Interestingly, MYC fKO did not suppress phosphorylation of p70S6K (Fig. 3E), while MTORC1 inhibition by rapamycin treatment did not downregulate MYC protein expression (Fig. 3F) in both ESCC cell lines.

We observed suppressed AKT phosphorylation on serine 473 in ECM1b-overexpressing cells (Fig. 3A), indicating there is a hypoactivated MTORC2, the kinase complex specifically phosphorylating AKT serine 473 [29]. AKT is a well-known critical upstream regulator of MTORC1 [30]. We then examined the phosphorylation status of PRAS40, one of the main AKT downstream players mediating the AKT/MTORC1 signaling. We observed suppressed phosphorylation of PRAS40 (Fig. 3A), indicating that AKT/PRAS40/MTORC1 signaling is indeed involved in the regulation of MTORC1 in ECM1b-expressing cells. MTORC2 has also been shown to regulate MYC protein expression through histone deacetylase (HDAC) independent of AKT/MTORC1 [31]. Therefore, we hypothesized that ECM1b regulated MTORC2/HDAC/MYC and MTORC2/AKT/MTORC1 signaling in parallel in ESCC. To verify our hypothesis, we firstly applied CRISPR-fKO to target RICTOR, the key component specific to MTORC2 [29]. MTORC2 inactivation by RICTOR-fKO leads to suppression of both MYC protein expression and p70S6K phosphorylation in ESCC cells (Fig. 4A), demonstrating that MTORC2 acts upstream of both MYC and MTORC1 signaling We further confirmed that HDAC inhibition by vorinostat also suppressed MYC protein expression in ESCC cells (Fig. 4B). These data suggest a functional MTORC2/HDAC/MYC signaling axis in ESCC, possibly contributing to *ECM1b*-induced MYC downregulation.

To examine the contribution of the suppressed MTORC2/AKT/MTORC1 signaling to *ECM1b*-induced tumor suppression, we expressed a constitutively active AKT mutant (caAKT) in ECM1b-overexpressing cells and performed the nude mouse subcutaneous tumorigenicity assay. Consistent with our hypothesis, compensation of AKT/MTORC1 rescued the down-regulation of p-p70S6K only, but not the down-regulation of MYC *in vitro* (Fig. 4C), and partially rescued *in vivo* tumor growth in both cell lines tested (Fig. 4D), suggesting that inhibitions of other signaling pathways, likely the MTORC2/HDAC/MYC signaling also contribute to tumor suppression by *ECM1b*.

3.10. ER-localized ECM1b modulates MTORC2 activation by regulating MTORC2-ribosome association

We analyzed the detailed molecular mechanisms of MTORC2 regulation by ECM1b in ESCC. Since ECM1b was shown to be a cellular protein (Fig. 1E), we first examined the subcellular localization of ECM1b by subcellular fractionation. ECM1b was found to be mainly localized in the membranous fraction, together with markers for ER and mitochondria (Fig. 5A). We then performed immunofluorescence staining followed by confocal microscopy to further localize the ECM1b protein. The ECM1b protein co-localized with fluorescent signals of an ER-interacting protein concanavalin A in fixed ESCC cells (Fig. 5B and C).

MTORC2 activation requires association with ribosomes in ER [18]. Given the evidence that ECM1b localizes in the ER, we hypothesized that the ECM1b regulates MTORC2/ribosome association. Ribosome pull-down [18] was performed in *ECM1b*-overexpressing cells, as compared to GFP-overexpressing cells. We found that the ECM1b protein expression reduces ribosome-associated RICTOR expression (Fig. 5D). These data suggested that ECM1b regulates activation of MTORC2 through modulating MTORC2-ribosome association in ER.

3.11. ECM1b regulates general protein translation mediated through MTORC2

We showed that *ECM1b* suppressed general protein translation in ESCC cell lines (Fig. 3D). We further examined whether MTORC2 mediated such suppression. We first showed that MTORC2 inactivation by RICTOR-fKO significantly suppressed both cap-dependent and IRES-mediated protein translation (Fig. 5E and F). Further overexpression of ECM1b in RICTOR-fKO cells did not enhance protein translation suppression, suggesting that *ECM1b*-induced protein translation suppression was mediated through MTORC2 regulation.

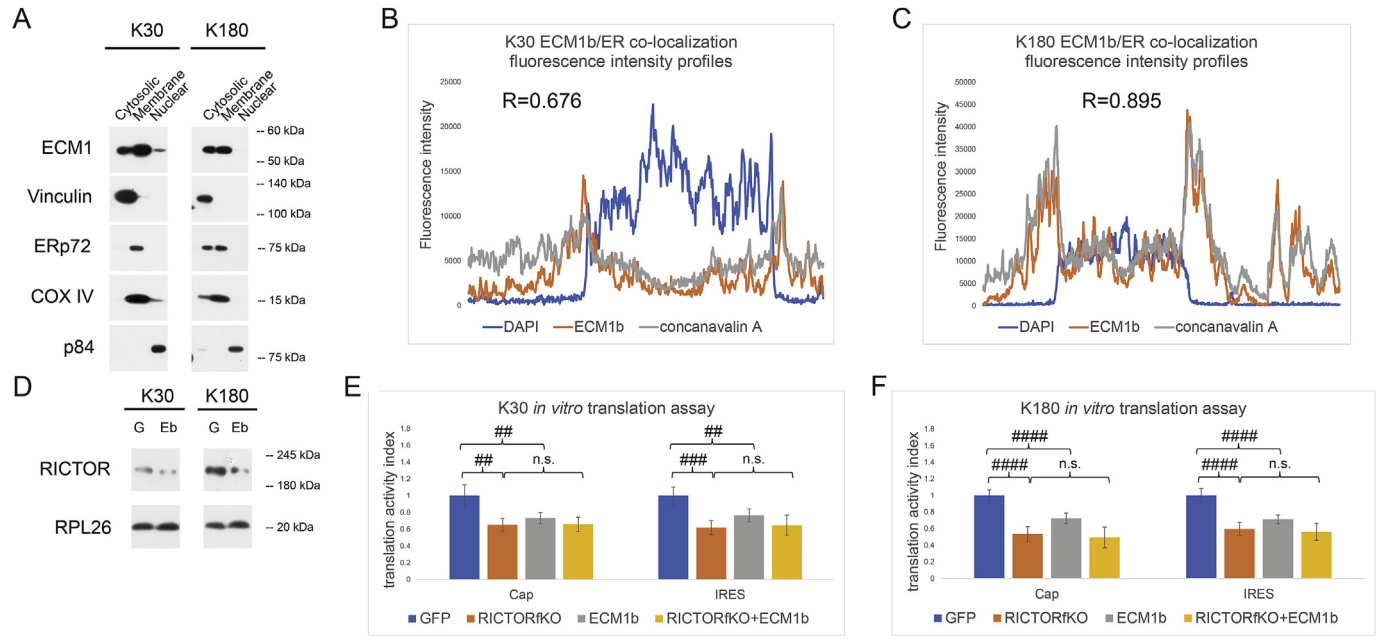

Fig. 5. ER-localized ECM1b regulates MTORC2-ribosome association. (A) Subcellular fractionation followed by Western blotting shows that ECM1b localized in the membranous fraction. Vinculin: cytosolic marker; ERp72: ER marker; COX IV: mitochondria marker; p84: nuclear marker. (B and C) Immunofluorescence confocal analysis showed ECM1b co-localized with fluorescence signals of ER-specific AlexaFluor-conjugated concanavalin A in ECM1b-overexpressing cells. Correlation coefficient (R) between ECM1b and concanavalin A signals are shown. Corresponding representative confocal fluorescence images are shown in Supplementary Fig. 4 (D) Ribosome-pulldown followed by Western blotting showed reduced RICTOR protein expression in ribosome pulldown eluates in ECM1b-overexpressing cells, as compared to GFP-overexpressing cells. RPL26 was used a loading control for ribosome. (E and F) *In vitro* protein translation assay showed that RICTOR fKO significantly suppressed both cap-dependent and IRES-mediated protein translations. Overexpression of ECM1b in RICTOR-fKO cells did not further enhance protein translation suppression. The protein translation index profiles of GFP and ECM1b in K180 cells were duplicated from Fig. 3D, as these data were generated from the same batches of samples. G: GFP control; Eb: ECM1b; ##: Adjusted *p*-value < 0.01; ###: Adjusted *p*-value < 0.001; ####: Adjusted *p*-value < 0.0001; n.s.: not statistically significant.

3.12. ECM1b modulates chemosensitivity

The mTOR signaling pathway confers chemoresistance in cancer [32]. Given the evidence that *ECM1b* regulates both MTORC2/MTORC1, we hypothesized that *ECM1b* overexpression enhances chemosensitivity in ESCC. We applied cisplatin treatment, one of the most commonly used chemotherapeutic agents in ESCC disease management, in *ECM1b*-overexpressing cells and determined cell viability. Consistent with our hypothesis, *ECM1b*-overexpressing cells showed decreased viability after cisplatin treatment (Fig. 6A). We also investigated whether MTORC2 mediates regulation of chemosensitivity by *ECM1b*. We showed that MTORC2 inactivation by RICTOR-fKO enhanced chemosensitivity to cisplatin treatment, while overexpression of *ECM1b* in RICTOR-fKO cells did not further enhance chemosensitivity. These data suggested that *ECM1b* enhances chemosensitivity mediated by MTORC2.

4. Discussion

This study provides evidence of a tumor-suppressive role of *ECM1* in ESCC. We performed transcriptomic profiling on a small number of ESCC tumors. *ECM1* was identified as a top downregulated gene. We verified that *ECM1* RNA expression is significantly downregulated in ESCC, as well as in several other SCCs. Two *ECM1* variants are expressed in esophageal tissues and ESCC; both were significantly downregulated in ESCC tumor samples and cell lines. We showed that only the cellular ECM1b, but not the secreted ECM1a, confers tumor suppression in our cell line-based nude mouse tumorigenicity assay. Interestingly, *ECM1a* has been extensively studied in breast, liver, and thyroid cancers for its oncogenic role [6–8,33,34]. *ECM1b* has not been functionally characterized before. Our data now suggests a novel and highly tissue-specific tumor suppressive role of *ECM1* in ESCC.

Across a panel of normal human tissues, the esophagus shows the top *ECM1* RNA expression levels [35,36] (Supplementary Fig. 5). In skin development, *ECM1b* expression is induced by differentiation and persists in differentiated keratinocytes [37]. This pattern was also observed in our analysis (Fig. 1A; GSE29001), in which normal differentiated cells showed the top *ECM1* expression followed by normal basal cells, while cancer cells exhibited the lowest *ECM1* expression. Whether ESCC arises from undifferentiated basal cells or differentiated suprabasal cells of the esophagus remains unresolved. Generally, suppression of *ECM1* expression may be involved in de-differentiation and offer advantages in cancer development.

We showed that MYC protein expression and MTORC1/p70S6K signaling are downregulated in *ECM1b*-overexpressing cells, which is mediated by MTORC2/AKT signaling (Fig. 6B). Interestingly, we also found that *ECM1* RNA expression is significantly inversely correlated with activated MYC and MTORC1 signaling signatures in ESCC, HNSCC, lung SCC, and cervical SCC. Both MYC and AKT/MTORC1 signaling pathways are well-characterized critical oncogenic players in ESCC [38], HNSCC [39], lung SCC [40], and cervical SCC [41]. These data imply a general tumor-suppressive role of *ECM1b* in SCCs. Further studies are needed to verify and determine the role of *ECM1b* in other SCCs.

ECM1b regulates activation of MTORC2 by modulating MTORC2/ribosome association in ER. ECM1b localizes to the ER, as demonstrated by subcellular fractionation and confocal microscopic co-localization analysis. ECM1b protein expression cannot be detected in conditioned medium, indicating it is retained in ER. ECM1b protein does not possess canonical ER localization peptides [42], suggesting that protein-protein interactions may be involved in ECM1b retention in ER. Interestingly, ECM1a protein, possessing the same N- and C-termini as ECM1b (Fig. 1E), does not specifically localize to ER (Supplementary Fig. 6), further supporting the key role of protein-protein interactions in ER

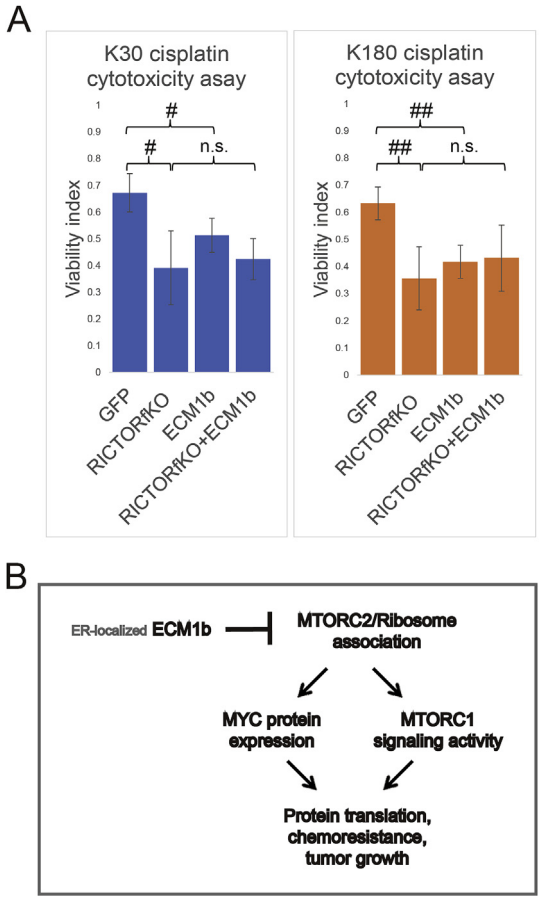

Fig. 6. ECM1b modulated chemosensitivity in ESCC. (A). *In vitro* cisplatin cytotoxicity assay showed that ECM1b overexpression and RICTOR-fKO both enhanced chemosensitivity in two ESCC cell lines tested. ECM1b-overexpression and RICTOR-fKO did not show synergistic effects on chemosensitivity modulation. (B) Proposed model illustrating the mechanism of tumor suppression of *ECM1b* in ESCC. #: Adjusted *p*-value < 0.1; ##: Adjusted *p*-value < 0.01; n.s.: not statistically significant.

localization. Detailed mechanism of ER-localization of ECM1b requires further investigation. Ribosome pulldown also showed that RICTOR/ribosome interaction is reduced in *ECM1b*-overexpressing cells. MTORC2/ribosome association is a critical step in MTORC2 activation [18]. We provide evidence that *ECM1b* plays a role in modulating such association and activation of MTORC2. Whether a cellular, ER-targeted ECM1a construct confers similar tumor suppressive role as ECM1b also requires further detailed functional and molecular analyses.

Currently chemoradiotherapy remains the only treatment scheme besides surgery for ESCC patients worldwide [43]. Therefore, prognostic biomarkers for chemoradiotherapy provide critical clinical information for disease management. We showed that *ECM1b* expression sensitizes ESCC cells to cisplatin, which is commonly used in ESCC patient management. Further studies are needed to examine the prognostic role of ECM1b protein expression in ESCC patient samples by immunohistochemical staining. The lack of molecular targeted therapy in ESCC treatment emphasizes the need for identification and verification of novel suitable drug targets. Given the evidence that MTORC2 demonstrates critical roles in MYC/MTORC1 regulation, general protein translation regulation, and chemosensitivity, it serves as a suitable drug target in ESCC. Several dual-MTORC1/MTORC2 inhibitors have been identified and tested [44–46]. In ESCC, targeting MTORC1/MTORC2 shows promising results in preclinical studies [47–49]. Consistent with a recent study focusing on the role of RICTOR in ESCC [50], the present study further provides novel data supporting anti-RICTOR/MTORC2 in ESCC treatment.

This study shows that ER-localized ECM1b is a tumor suppressor in ESCC and provides new insights into the regulation of MYC and MTORC1 signaling pathways by MTORC2, as well as showing the potential usefulness of *ECM1* in clinical management of ESCC.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.07.005.

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