1 **Title:** Endoplasmic reticulum-localized ECM1b suppresses tumor growth and regulates
2 MYC and MTORC1 through modulating MTORC2 activation in esophageal squamous
3 cell carcinoma
4
5 **Short Title:** ECM1b as tumor suppressor in ESCC
6
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13 **Key words**
14 Signal transduction; Protein translation; Esophageal cancer
15
16 **Significance of the results outlined in no more than 5 sentences:**
17 We demonstrate a novel tumor-suppressive role of *ECM1* in ESCC, as opposed to the
18 previously well-established oncogenic role in other cancer types. *ECM1b*, the dominant
19 splicing variant in the esophagus, encodes an endoplasmic reticulum-localized protein.
20 *ECM1b* suppresses MTORC2 activation by inhibiting MTORC2/ribosome association.
21 By regulating MTORC2/MYC/MTORC1 signaling, *ECM1b* suppresses *in vivo* tumor
22 growth and general protein translation and enhances chemosensitivity. *ECM1b* is a
23 tumor suppressor that may be useful as a biomarker in therapeutic management of
24 ESCC.

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

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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.

(185/185 words)

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

63 there is no available targeted therapy for ESCC management. Identifying suitable drug
64 targets is needed. More detailed molecular and functional analyses of genes involved
65 in ESCC tumorigenesis are urged.
66 We performed transcriptomic analysis on normal esophageal/ESCC paired tissue
67 samples and identified *Extracellular matrix protein 1* (*ECM1*) as one of the most
68 downregulated genes. *ECM1* encodes a secreted protein that is involved in
69 endochondral bone formation and angiogenesis [3], lipoid proteinosis [4], T-cell
70 development [5] and tumor development in which *ECM1* plays critical oncogenic roles
71 [6-8] . Three variants resulting from alternative splicing exist, resulting in three protein
72 isoforms, ECM1a/b/c [3]. ECM1a is the most studied isoform, while there are very
73 limited studies on ECM1b/c.
74 We examined the expression of *ECM1* in ESCC and several other squamous cell
75 carcinomas (SCCs) and found that *ECM1* RNA expression is significantly
76 downregulated in tumors, as compared to normal tissue samples. We employed various
77 *in vivo* and *in vitro* assays to functionally characterize *ECM1*. We demonstrate that
78 cellular *ECM1b* is a novel tumor suppressor in ESCC. It encodes an endoplasmic
79 reticulum (ER)-localized protein and acts as a MTORC2 regulator that suppresses
80 MTORC2 activation by modulating MTORC2/ribosome association, which leads to
81 suppressed protein translation and enhanced chemosensitivity. The results suggest
82 *ECM1b* as a tumor suppressor in ESCC and a biomarker for ESCC therapeutics.
83
84
85 **2. Materials and methods**
86 *2.1. ESCC patient tissue samples*
87 ESCC tissue specimens were collected from the Queen Mary Hospital from 2001 to
88 2006, as previously reported [9]. Approval for this study was obtained from the Hospital
89 Institutional Review Board at the University of Hong Kong.
90
91 2.2. *Transcriptomic analysis*
92 We sequenced the RNA from four paired adjacent normal esophageal/ESCC tissue
93 samples using the Illumina HiSeq 2000 (2x100bp paired reads). The raw RNA-seq data
94 was cleaned and aligned to the hg19 reference genome using Tophat [10] (version
95 2.0.14, bowtie version 2.2.4) with library-type fr-firststrand parameter. The gene
96 expression profile was calculated by Cufflinks [11] (version 2.2.1) with the Ensemble
97 gene annotation file. The differentially expressed genes were analyzed using Cuffdiff
98 [11] between each normal and tumor paired sample and between the pooled normal and
99 tumor samples. MISO [12] was used to identify alternative splicing events; the
100 alternative splicing events were then visualized using Integrative Genomics Viewer

101 [13].

102

103 *2.3. Chemical reagents*

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

105

106 *2.4. Cell lines*

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

119

120 2.5. *Plasmids and lentivirus preparation and infection*

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

137

138 2.6. *Conditioned medium preparation*

139 Conditioned media were collected as described [17].

140

141 *2.7. In vivo tumorigenicity assay*

142 Subcutaneous injection of cancer cells in nude mouse was performed as described [14].

143 For KYSE180TS, $3x10^6$ of cells were injected per site.

144

145 *2.8. Cell proliferation assay*

146 The proliferation and viability of cells were determined by the 3-(4,5-dimethylthiazol-

147 2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as we previously described [9].

148

149 2.9. *Cell size determination*

150 Cells were harvested by trypsinization and subjected to flow cytometry analysis using

151 the BD FACSCanto II (BD Biosciences, San Jose, CA). Cell size was indicated by the

152 readings of forward scatter area.

153

154 *2.10. Western blotting analysis*

155 Western blotting analysis was performed as previously described [14]. Antibodies used

156 are listed in Supplementary Materials and methods.

157

158 *2.11. In vitro translation assay*

159 Cells labeled with pLVX-EF1a-RLuc-POLIRES-FLuc were utilized. Cells were treated

160 with 30uM Enduren (Promega Corporation, Madison, WI) or 3mg/mL D-Luciferin

161 potassium salt (Biovision, Inc., Milpitas, CA) for 15 minutes and subjected to

162 bioluminescence imaging using the PE-IVIS Spectrum imaging system (PerkinElmer,

163 Waltham, MA).

164

165 *2.12. Subcellular fractionation*

166 Subcellular fractionation was performed using the Subcellular Protein Fractionation Kit

167 for Cultured Cells (Thermo Fisher Scientific, Waltham, MA) according to

168 manufacturer's protocol.

169

170 *2.13. Immunofluorescence confocal microscopic co-localization analysis*

171 Immunofluorescence staining was performed as described [9]. ECM1 antibody

172 (#HPA027241, Sigma-Aldrich Corporation, St. Louis, MO) was used to target ECM1b,

173 followed by incubation with Alexa Fluor™ 488 secondary antibody (Thermo Fisher

174 Scientific). Endoplasmic reticulum was labeled using Concanavalin A, Alexa Fluor™

175 594 Conjugate (Thermo Fisher Scientific). DAPI was used to label the nucleus.

176 Confocal imaging was performed using the Carl Zeiss LSM 800 (Carl Zeiss AG,

Oberkochen, Germany) with a 63x 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 hours before cisplatin treatment (10 uM for 72 hours). 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 (Figure 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) (Figure 1A). Interestingly, *ECM1* RNA expression is also downregulated in cervical cancer, head and neck squamous carcinoma (HNSCC), and oral squamous cell carcinoma (OSCC) (Figure 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 Figure 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 (Figure 1C and Supplementary Figure 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 (Figure 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] (Figure 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 (Figure 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

253 KYSE30TSI/150/180TS/450, and cells were injected subcutaneously into the mice and
254 compared with cells expressing green fluorescent protein (GFP) as controls. *ECM1b*
255 overexpression suppresses *in vivo* tumor formation, while *ECM1a* overexpression does
256 not significantly alter tumor size in all the cell lines tested, regardless of the endogenous
257 *ECM1* expression level. (Figure 2A). These data collectively showed that *ECM1b*, but
258 not *ECM1a*, acts as a tumor suppressor in ESCC. Two cell lines, KYSE30TSI (K30)
259 and KYSE180TS (K180), showing greater tumor-suppressive effects by *ECM1b*
260 overexpression, were used for the following functional and mechanistic analyses.
261 *In vitro* cell proliferation was examined. *ECM1b* overexpression causes proliferation
262 suppression in KYSE30TSI and KYSE180TS cell lines (Figure 2B). We further
263 examined cell growth by determining cell size through flow cytometry. *ECM1b*
264 overexpression evidently reduces cell size in both cell lines (Figure 2C). These data
265 showed the inhibitory effects of *ECM1b* on cell proliferation and cell growth in ESCC
266 cell lines.
267

268 *3.6. MYC protein expression and MTORC1 signaling are downregulated in ECM1b-*
269 *overexpressing cells*

270 Major signaling pathways that regulate cell proliferation and cell growth include the
271 MYC signaling and MTORC1 signaling pathways [21, 22]. Therefore, we examined
272 MYC protein expression and phosphorylation status of p70S6K, one of the major
273 downstream effectors of MTORC1 signaling, in *ECM1b*-overexpressing ESCC cell
274 lines. MYC protein expression and phosphorylated p70S6K both showed
275 downregulation in the two cell lines tested (Figure 3A).
276 We further examined the public datasets for *ECM1* co-expression profiles and
277 performed enrichment analysis with the molecular signatures database hallmark gene
278 set collection [23]. *ECM1* expression was shown to significantly inversely correlate
279 with three hallmark gene sets related to activated MYC and MTORC1 signaling in
280 ESCC, cervical SCC, HNSCC, and lung SCC (Supplementary Table 2). These data
281 suggest a potential role of ECM1b in MYC/MTORC1 signaling regulation across
282 different SCCs.
283

284 *3.7. ECM1b suppresses MYC protein translation*

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

(Figure 3B). However, MYC protein expression downregulation by *ECM1b* was significantly diminished, when the protein translation machinery was suppressed by puromycin treatment (Figure 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 (Figure 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 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 (Figure 3E), while MTORC1 inhibition by rapamycin treatment did not downregulate MYC protein expression (Figure 3F) in both ESCC cell lines.

We observed suppressed AKT phosphorylation on serine 473 in ECM1b-overexpressing cells (Figure 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 (Figure 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

329 leads to suppression of both MYC protein expression and p70S6K phosphorylation in
330 ESCC cells (Figure 4A), demonstrating that MTORC2 acts upstream of both MYC and
331 MTORC1 signaling We further confirmed that HDAC inhibition by vorinostat also
332 suppressed MYC protein expression in ESCC cells (Figure 4B). These data suggest a
333 functional MTORC2/HDAC/MYC signaling axis in ESCC, possibly contributing to
334 *ECM1b*-induced MYC downregulation.
335 To examine the contribution of the suppressed MTORC2/AKT/MTORC1 signaling to
336 *ECM1b*-induced tumor suppression, we expressed a constitutively active AKT mutant
337 (caAKT) in ECM1b-overexpressing cells and performed the nude mouse subcutaneous
338 tumorigenicity assay. Consistent with our hypothesis, compensation of AKT/MTORC1
339 rescued the down-regulation of p-p70S6K only, but not the down-regulation of MYC
340 *in vitro* (Figure 4C), and partially rescued *in vivo* tumor growth in both cell lines tested
341 (Figure 4D), suggesting that inhibitions of other signaling pathways, likely the
342 MTORC2/HDAC/MYC signaling also contribute to tumor suppression by *ECM1b*.
343
344 *3.10. ER-localized ECM1b modulates MTORC2 activation by regulating MTORC2-*
345 *ribosome association*
346 We analyzed the detailed molecular mechanisms of MTORC2 regulation by ECM1b in
347 ESCC. Since ECM1b was shown to be a cellular protein (Figure 1E), we first examined
348 the subcellular localization of ECM1b by subcellular fractionation. ECM1b was found
349 to be mainly localized in the membranous fraction, together with markers for ER and
350 mitochondria (Figure 5A). We then performed immunofluorescence staining followed
351 by confocal microscopy to further localize the ECM1b protein. The ECM1b protein co-
352 localized with fluorescent signals of an ER-interacting protein concanavalin A in fixed
353 ESCC cells (Figure 5B and C).
354 MTORC2 activation requires association with ribosomes in ER [18]. Given the
355 evidence that ECM1b localizes in the ER, we hypothesized that the ECM1b regulates
356 MTORC2/ribosome association. Ribosome pull-down [18] was performed in *ECM1b*-
357 overexpressing cells, as compared to GFP-overexpressing cells. We found that the
358 ECM1b protein expression reduces ribosome-associated RICTOR expression (Figure
359 5D). These data suggested that ECM1b regulates activation of MTORC2 through
360 modulating MTORC2-ribosome association in ER.
361
362 *3.11. ECM1b regulates general protein translation mediated through MTORC2*
363 We showed that *ECM1b* suppressed general protein translation in ESCC cell lines
364 (Figure 3D). We further examined whether MTORC2 mediated such suppression. We
365 first showed that MTORC2 inactivation by RICTOR-fKO significantly suppressed both
366 cap-dependent and IRES-mediated protein translation (Figure 5E and F). Further

367 overexpression of ECM1b in RICTOR-fKO cells did not enhance protein translation
368 suppression, suggesting that *ECM1b*-induced protein translation suppression was
369 mediated through MTORC2 regulation.
370
371 *3.12. ECM1b modulates chemosensitivity*
372 The mTOR signaling pathway confers chemoresistance in cancer [32]. Given the
373 evidence that *ECM1b* regulates both MTORC2/MTORC1, we hypothesized that
374 *ECM1b* overexpression enhances chemosensitivity in ESCC. We applied cisplatin
375 treatment, one of the most commonly used chemotherapeutic agents in ESCC disease
376 management, in *ECM1b*-overexpressing cells and determined cell viability. Consistent
377 with our hypothesis, *ECM1b*-overexpressing cells showed decreased viability after
378 cisplatin treatment (Figure 6A). We also investigated whether MTORC2 mediates
379 regulation of chemosensitivity by *ECM1b*. We showed that MTORC2 inactivation by
380 RICTOR-fKO enhanced chemosensitivity to cisplatin treatment, while overexpression
381 of *ECM1b* in RICTOR-fKO cells did not further enhance chemosensitivity. These data
382 suggested that *ECM1b* enhances chemosensitivity mediated by MTORC2.
383
384
385 **4. Discussion**
386 This study provides evidence of a tumor-suppressive role of *ECM1* in ESCC. We
387 performed transcriptomic profiling on a small number of ESCC tumors. *ECM1* was
388 identified as a top downregulated gene. We verified that *ECM1* RNA expression is
389 significantly downregulated in ESCC, as well as in several other SCCs. Two *ECM1*
390 variants are expressed in esophageal tissues and ESCC; both were significantly
391 downregulated in ESCC tumor samples and cell lines. We showed that only the cellular
392 ECM1b, but not the secreted ECM1a, confers tumor suppression in our cell line-based
393 nude mouse tumorigenicity assay. Interestingly, *ECM1a* has been extensively studied
394 in breast, liver, and thyroid cancers for its oncogenic role[6-8, 33, 34]. *ECM1b* has not
395 been functionally characterized before. Our data now suggests a novel and highly
396 tissue-specific tumor suppressive role of *ECM1* in ESCC.
397
398 Across a panel of normal human tissues, the esophagus shows the top *ECM1* RNA
399 expression levels[35, 36] (Supplementary Figure 5). In skin development, *ECM1b*
400 expression is induced by differentiation and persists in differentiated keratinocytes [37].
401 This pattern was also observed in our analysis (Figure 1A; GSE29001), in which normal
402 differentiated cells showed the top *ECM1* expression followed by normal basal cells,
403 while cancer cells exhibited the lowest *ECM1* expression. Whether ESCC arises from
404 undifferentiated basal cells or differentiated suprabasal cells of the esophagus remains

405 unresolved. Generally, suppression of *ECM1* expression may be involved in de-
406 differentiation and offer advantages in cancer development.
407
408 We showed that MYC protein expression and MTORC1/p70S6K signaling are
409 downregulated in *ECM1b*-overexpressing cells, which is mediated by MTORC2/AKT
410 signaling (Figure 6B). Interestingly, we also found that *ECM1* RNA expression is
411 significantly inversely correlated with activated MYC and MTORC1 signaling
412 signatures in ESCC, HNSCC, lung SCC, and cervical SCC. Both MYC and
413 AKT/MTORC1 signaling pathways are well-characterized critical oncogenic players in
414 ESCC [38], HNSCC [39], lung SCC [40], and cervical SCC [41]. These data imply a
415 general tumor-suppressive role of *ECM1b* in SCCs. Further studies are needed to verify
416 and determine the role of *ECM1b* in other SCCs.
417
418 *ECM1b* regulates activation of MTORC2 by modulating MTORC2/ribosome
419 association in ER. ECM1b localizes to the ER, as demonstrated by subcellular
420 fractionation and confocal microscopic co-localization analysis. ECM1b protein
421 expression cannot be detected in conditioned medium, indicating it is retained in ER.
422 ECM1b protein does not possess canonical ER localization peptides [42], suggesting
423 that protein-protein interactions may be involved in ECM1b retention in ER.
424 Interestingly, ECM1a protein, possessing the same N- and C-termini as ECM1b (Figure
425 1E), does not specifically localize to ER (Supplementary Figure 6), further supporting
426 the key role of protein-protein interactions in ER localization. Detailed mechanism of
427 ER-localization of ECM1b requires further investigation. Ribosome pulldown also
428 showed that RICTOR/ribosome interaction is reduced in *ECM1b*-overexpressing cells.
429 MTORC2/ribosome association is a critical step in MTORC2 activation [18]. We
430 provide evidence that *ECM1b* plays a role in modulating such association and activation
431 of MTORC2. Whether a cellular, ER-targeted ECM1a construct confers similar tumor
432 suppressive role as ECM1b also requires further detailed functional and molecular
433 analyses.
434
435 Currently chemoradiotherapy remains the only treatment scheme besides surgery for
436 ESCC patients worldwide [43]. Therefore, prognostic biomarkers for
437 chemoradiotherapy provide critical clinical information for disease management. We
438 showed that *ECM1b* expression sensitizes ESCC cells to cisplatin, which is commonly
439 used in ESCC patient management. Further studies are needed to examine the
440 prognostic role of ECM1b protein expression in ESCC patient samples by
441 immunohistochemical staining. The lack of molecular targeted therapy in ESCC
442 treatment emphasizes the need for identification and verification of novel suitable drug

443 targets. Given the evidence that MTORC2 demonstrates critical roles in
444 MYC/MTORC1 regulation, general protein translation regulation, and
445 chemosensitivity, it serves as a suitable drug target in ESCC. Several dual-
446 MTORC1/MTORC2 inhibitors have been identified and tested [44-46]. In ESCC,
447 targeting MTORC1/MTORC2 shows promising results in preclinical studies [47-49].
448 Consistent with a recent study focusing on the role of RICTOR in ESCC [50], the
449 present study further provides novel data supporting anti-RICTOR/MTORC2 in ESCC
450 treatment.
451
452 This study shows that ER-localized ECM1b is a tumor suppressor in ESCC and
453 provides new insights into the regulation of MYC and MTORC1 signaling pathways
454 by MTORC2, as well as showing the potential usefulness of *ECM1* in clinical
455 management of ESCC.
456
457
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463 Medicine Core Facility for providing facilities for flow cytometry, confocal microscopy
464 imaging, and live-cell bioluminescence imaging.

465 Figure 1. *ECM1* expression and clinical significance in SCCs. (A) *ECM1* RNA
466 expression is significantly downregulated in ESCC. (B) *ECM1* RNA expression is
467 significantly downregulated in HNSCC, cervical cancer, and OSCC. (C) RNA
468 sequencing analysis revealed differential expression of the three *ECM1* variants in
469 esophageal tissues and ESCC. (D) *ECM1*a and *ECM1b* RNA expression is
470 downregulated in a panel of ESCC paired normal/tumor tissue samples, and a panel of
471 ESCC cell lines using immortalized human esophageal epithelial cell line NE1 as
472 reference. (E) ECM1a and ECM1b differ in a single internal domain. The shaded area
473 indicates ECM1a-specific region. (F) ECM1a and ECM1b protein expression exhibit
474 distinct secreted/cellular localization. NB: Normal basal epithelial cells; ND: normal
475 differentiated epithelial cells; *: p-value < 0.01; **: p-value < 0.001; ***: p-value <
476 0.0001; #: Adjusted p-value < 0.1; ###: Adjusted p-value < 0.001. Datasets SRP008496
477 and GSE3524 were analyzed by independent samples t-test; dataset GSE9750 was
478 analyzed by Wilcoxon rank-sum test due to the skewed distribution of the data; other
479 datasets were analyzed by paired samples t-test. CL: cell lysate; CM: conditioned
480 medium. α: corresponding bands for ECM1a; β: corresponding bands for ECM1b. The
481 difference of ECM1a protein migration on SDS-PAGE gel between CL and CM is likely
482 due to N-glycosylation [20].
483
484 Figure 2. ECM1 protein expression regulates *in vivo* tumor growth and *in vitro* cell
485 proliferation and cell growth. (A) ECM1b expression suppresses subcutaneous
486 xenograft tumor growth in four ESCC cell lines, while ECM1a expression does not
487 significantly alter tumor growth. Representative xenograft tumor images are shown.
488 Scale bar = 5mm. (B) ECM1b expression suppresses *in vitro* proliferation in two ESCC
489 cell lines. (C) Representative images showing that ECM1b expression reduces *in vitro*
490 cell size in two ESCC cell lines. Statistical significance was determined by comparing
491 the data from different groups of the last time point. n.s.: not statistically significant; #:
492 Adjusted p-value < 0.1; ###: Adjusted p-value < 0.001; *: p-value < 0.05; **: p-value
493 < 0.01.
494
495 Figure 3. MYC protein expression and MTORC1 signaling are downregulated in
496 ECM1b-overexpressing cells. (A) Western blotting shows suppressed MYC protein
497 expression, phosphorylation of p70S6K threonine 389, phosphorylation of AKT serine
498 473, and phosphorylation of PRAS40 threonine 246 in ECM1b-expressing cells. (B)
499 Western blotting shows that MG-132 treatment did not diminish suppression of MYC
500 protein expression by ECM1b. Cells were treated with 20uM MG-132 for 3 hours
501 before cell lysate collections. (C) Western blotting shows that puromycin treatment
502 diminished suppression of MYC protein expression by ECM1b. Cells were treated with

503 50ug/mL puromycin for 1 hour before cell lysate collections. (D) *In vitro* live-cell
504 bioluminescence-based protein translation assay showed reduced cap-dependent (Cap)
505 and IRES-mediated (IRES) protein translations in ECM1b-overexpressing cells
506 compared to control cells. (E) Western blotting reveals that MYC fKO did not suppress
507 phosphorylation of p70S6K. (F) Western blotting reveals that MTORC1 inhibition by
508 rapamycin did not suppress MYC protein expression. Cells were treated with 10nM
509 rapamycin for 48 hours before cell lysate collections. G:GFP control; Eb: ECM1b; p-:
510 phosphorylated; t-: total form; D: DMSO control; MG: MG-132; Puro: puromycin; **:
511 *p*-value < 0.05; ***: *p*-value < 0.01; C: scrambled sgRNA control; sg1: MYC sgRNA1;
512 sg2: MYC sgRNA2; RA: rapamycin. Vinculin was used as a loading control.
513
514 Figure 4. MTORC2-mediated regulations of MYC protein expression and MTORC1
515 signaling by ECM1b in ESCC. (A) Western blotting shows suppressed MYC protein
516 expression, phosphorylation of p70S6K, and phosphorylation of AKT serine 473 in
517 RICTOR-fKO cells. (B) Western blotting shows suppressed MYC protein expression
518 by HDAC inhibition. Cells were treated with 20uM vorinostat for 3 hours before cell
519 lysate collections. (C) Western blotting shows that expression of caAKT rescued the
520 down-regulation of p-p70S6K only, but not the down-regulation of MYC protein
521 expression. (D) Expression of caAKT partially rescued ECM1b-induced subcutaneous
522 tumor suppression. Representative xenograft tumor images are shown. Scale bar = 5mm.
523 C: scrambled sgRNA control; sg1: RICTOR sgRNA1; D: DMSO control; vori:
524 vorinostat; #: Adjusted *p*-value < 0.1; ##: Adjusted *p*-value < 0.05; n.s.: not statistically
525 significant. Vinculin was used as a loading control.
526
527 Figure 5. ER-localized ECM1b regulates MTORC2-ribosome association. (A)
528 Subcellular fractionation followed by Western blotting shows that ECM1b localized in
529 the membranous fraction. Vinculin: cytosolic marker; ERp72: ER marker; COX IV:
530 mitochondria marker; p84: nuclear marker. (B and C) Immunofluorescence confocal
531 analysis showed ECM1b co-localized with fluorescence signals of ER-specific
532 AlexaFluor-conjugated concanavalin A in ECM1b-overexpressing cells. Correlation
533 coefficient (R) between ECM1b and concanavalin A signals are shown. Corresponding
534 representative confocal fluorescence images are shown in Supplementary Figure 4. (D)
535 Ribosome-pulldown followed by Western blotting showed reduced RICTOR protein
536 expression in ribosome pulldown eluates in ECM1b-overexpressing cells, as compared
537 to GFP-overexpressing cells. RPL26 was used a loading control for ribosome. (E and
538 F) *In vitro* protein translation assay showed that RICTOR fKO significantly suppressed
539 both cap-dependent and IRES-mediated protein translations. Overexpression of
540 ECM1b in RICTOR-fKO cells did not further enhance protein translation suppression.

541 The protein translation index profiles of GFP and ECM1b in K180 cells were duplicated
542 from Figure 3D, as these data were generated from the same batches of samples. G:
543 GFP control; Eb: ECM1b; ##: Adjusted *p*-value < 0.01; ###: Adjusted *p*-value <
544 0.001;####: Adjusted *p*-value < 0.0001; n.s.: not statistically significant.
545

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

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