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Inhibition of Semaphorin 7A leads to decreased tumor growth and metastasis of mammary tumor cells

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Keywords:	Semaphorin; Metastasis; MMPs; hypoxia; TGF-b; tumor growth; mammary tumor
Abstract:	BACKGROUND: Solid tumors can hijack many of the same programs used in neurogenesis to enhance tumor growth and metastasis, thereby generating a plethora of neurogenesis-related molecules including semaphorins. Among them, we have identified Semaphorin7A (SEMA7A) in breast cancer. In this study we investigated effects of inhibiting Semaphorin 7A in a model of metastatic breast cancer. METHODS: SEMA7A expression was analyzed by qRT-PCR in breast cancer and mammary cells. We performed SEMA7A shRNA knock-down in the 4T1 tumor cells and cells were analyzed for gene expression, migration and ki67 proliferation. Stiffness of cells was measured by Atomic Force Microscopy. Wild-type and SEMA7A deficient mice were inoculated with wild-type or SEMA7A silenced 4T1 cells and assayed for tumor growth and metastasis. RESULTS: SEMA7A is highly expressed in both metastatic human and murine breast cancer cells. We show that both TGF-1 and hypoxia elicits the production of SEMA7A in mammary cells. Further, PI3K/AKT and HIF-1 inhibitors decreased SEMA7A expression in mammary tumor cells. SEMA7A shRNA silencing in 4T1 cells resulted in decreased mesenchymal markers MMP-3, MMP-13, Vimentin and TGF-1. SEMA7A silenced cells show increased stiffness with reduced migratory and proliferative potential. In vivo, SEMA7A silenced 4T1 tumor bearing mice showed decreased tumor growth and metastasis. Genetic ablation of host-derived SEMA7A synergized to further decrease the growth and metastasis of 4T1 cells. CONCLUSION: Our findings suggest novel functional roles for SEMA7A in breast cancer and that SEMA7A could be a novel therapeutic target to limit tumor growth and metastasis.
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October 4, 2016

Meghan Byrne
Senior Editor, PlosOne
1160 Battery Street
Koshland Building East, Suite 100
San Francisco, CA 94111

Dear Dr. Byrne

We would like to submit the manuscript entitled, "Inhibition of Semaphorin 7A leads to decreased tumor growth and metastasis of mammary tumor cells" for consideration publication in the PLOS One Journal. All authors concur with this submission. The contents of this manuscript have not been publicly disclosed, nor has this manuscript been submitted elsewhere.

The results of the present study are exciting because they are first to show a functional role for Semaphorin 7A (SEMA7A) in promoting breast cancer tumor growth and metastasis. We also are the first to describe the role of both tumor-derived and host-derived SEMA7A in promoting mammary tumor growth and metastasis. Inhibition of SEMA7A significantly increased the survival of mammary tumor-bearing mice and hence could pose SEMA7A as an attractive therapeutic target for breast cancer and of interest to the readers of PLOS One. We believe that these studies increase the scope of knowledge in the role of Semaphorin 7A in contributing towards metastasis.

We would like to suggest Dr. Eli Gilboa, University of Miami, Dr. Eduardo Sotomayor, George Washington University School of Medicine and Dr. Miki Rahat, Carmel Medical Center in Haifa as three of the reviewers of this manuscript since they have extensive knowledge in cancer biology and therapies for cancer.

Thank you for your consideration.

Sincerely,

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2 metastasis of mammary tumor cells
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Abstract

BACKGROUND:

Solid tumors can hijack many of the same programs used in neurogenesis to enhance tumor growth and metastasis, thereby generating a plethora of neurogenesis-related molecules including semaphorins. Among them, we have identified Semaphorin7A (SEMA7A) in breast cancer. In this study we investigated effects of inhibiting Semaphorin 7A in a model of metastatic breast cancer.

METHODS:

SEMA7A expression was analyzed by qRT-PCR in breast cancer and mammary cells. We performed SEMA7A shRNA knock-down in the 4T1 tumor cells and cells were analyzed for gene expression, migration and ki67 proliferation. Stiffness of cells was measured by Atomic Force Microscopy. Wild-type and SEMA7A deficient mice were inoculated with wild-type or SEMA7A silenced 4T1 cells and assayed for tumor growth and metastasis.

RESULTS:

SEMA7A is highly expressed in both metastatic human and murine breast cancer cells. We show that both TGF-β1 and hypoxia elicits the production of SEMA7A in mammary cells. Further, PI3K/AKT and HIF-1α inhibitors decreased SEMA7A expression in mammary tumor cells. SEMA7A shRNA silencing in 4T1 cells resulted in decreased mesenchymal markers MMP-3, MMP-13, Vimentin and TGF-β1. SEMA7A silenced cells show increased stiffness with reduced migratory and proliferative potential. *In vivo*,

59 SEMA7A silenced 4T1 tumor bearing mice showed decreased tumor growth and
60 metastasis. Genetic ablation of host-derived SEMA7A synergized to further decrease the
61 growth and metastasis of 4T1 cells.
62
63 CONCLUSION
64 Our findings suggest novel functional roles for SEMA7A in breast cancer and that
65 SEMA7A could be a novel therapeutic target to limit tumor growth and metastasis.
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INTRODUCTION

Semaphorins are a large family of conserved proteins originally characterized as directional cues in axonal guidance and neurite outgrowth in neurogenesis [1-3]. Subsequently, it has been revealed that semaphorins and their receptors carry out roles beyond neurogenesis and serve functions in immune regulation, extracellular matrix remodeling, organogenesis, and angiogenesis [3-7]. Studies have identified the expression of Semaphorin 7A (SEMA7A) in tumor cells, however, few have described a functional role for SEMA7A in tumor progression [8-11]. Hence, its contribution to tumor progression remains relatively unclear in comparison to other vertebrate semaphorins.

SEMA7A, or CD108w, is a ~80 kDa GPI-anchored transmembrane protein expressed by multiple cell types including: neurons, immune cells, melanocytes, fibroblasts, bone cells, and tumor cells [12]. This protein can be shed from the cellular membrane by action of ADAM-17(TACE) [13]. Both anchored and soluble forms of SEMA7A have been shown to bind to PlexinC1 and beta-1 integrin (CD29) [14-17]. The latter activates the MAPK and FAK pathways in a model of experimental autoimmune encephalomyelitis causing an increase in proinflammatory cytokine gene transcripts and proteins. Our group has demonstrated that DA-3 murine mammary tumor cells exhibit high levels of SEMA7A at both the transcript and protein level. Inhibition of DA-3-derived SEMA7A resulted in decreased angiogenesis, causing the retardation of tumor growth *in vivo [15]*. However, the role of SEMA7A in the metastasis of solid tumors is unclear.

It has been shown that activation of the PI3K/AKT pathway leads to the expression of SEMA7A [17, 18]. Given that the PI3K/AKT pathway plays an important role in tumor cell survival and metastasis [19, 20], we investigated the connection between SEMA7A and

106 the PI3K/AKT pathway in breast cancer[17]. It is well established that TGF-β plays an
107 important role in the Epithelial to Mesenchymal Transition (EMT), which has become a
108 hallmark for the de-differentiation of the normal mammary epithelium into tumor cells [21,
109 22]. However, within the context of breast cancer, it is largely unknown if TGF-β is
110 involved in the expression of SEMA7A. In addition to TGF-β mediated PI3K/AKT
111 activation, Morote *et al.* have shown in endothelial cells that hypoxia can induce the
112 expression of SEMA7A through the Hypoxia Responsive Elements (HREs) in its promoter
113 [23].

114 In this study, we characterized SEMA7A expression in both human and mouse breast
115 cancer cell lines and found that TGF-β and hypoxia can increase SEMA7A expression in
116 these cells. Utilizing shRNA and genetic deletions of SEMA7A, we determined that
117 inhibition *of* tumor-derived and host derived SEMA7A in 4T1 breast cancer model results
118 in decreased tumor growth and metastasis. Our study shows that SEMA7A plays a critical
119 functional role in the growth and metastasis of highly aggressive mammary tumor cells.

120 **MATERIALS AND METHODS**

121 **Mice and cell lines**

122 Female BALB/c mice (8-12 week-olds) were obtained from Charles River Laboratories,
123 and SEMA7A$^{-/-}$ mice generated by Dr. A.L. Kolodkin (Johns Hopkins University,
124 Baltimore, MD), were purchased from Jackson Laboratories. Using a speed congenic
125 approach [24, 25] SEMA7A$^{-/-}$ mice were backcrossed to a BALB/c background, reaching
126 99.9% of desired BALB/c background. Mice were housed and used according to the
127 National Institutes of Health guidelines, under protocols approved by Florida Atlantic

128 University Institutional Animal Care and Use Committee. EpH4 mammary cells were
129 provided by Dr. Jenifer Prosperi, Indiana University School of Medicine-South Bend, IN.
130 EpH4 cells, 4T1 and 4T1-LUC (Perkin Elmer) cells were grown in complete DMEM media
131 (DMEM with 10% FBS). MCF-10A, BT-474, T-47D, MCF-7, MDA-MB468, BT-20,
132 HC1937, CA1a and MDA-MB231 (American Type Culture Collection, Manassas, VA,
133 USA) were grown and maintained in DMEM containing 5% FBS as described previously
134 [26, 27]. Female BALB/c or SEMA7A$^{-/-}$ BALB/c mice were inoculated in the lower right
135 ventral quadrant with 5 x 10^5 luciferase transfected 4T1 mammary tumor cells.
136 Bioluminescent Imaging studies of the 4T1-LUC were done up to 3-weeks post-tumor cell
137 implantation. For 4T1 and 4T1-LUC tumor bearing mice, tissues were collected at 42-
138 days post-tumor cell implantation.

139 **RNA isolation and real-time reverse transcriptase-polymerase chain reaction**

140 Total RNA was extracted from murine or human tumor cells, using the RNeasy Protect
141 Mini Kit (Qiagen) according to manufacturer's instructions. Briefly, cDNA was synthesized
142 using Quantitech Reverse Transcription Kit (Qiagen) and gene expression was detected
143 by SYBR Green real-time PCR analysis using SYBR RT^2 qPCR primers (Qiagen,
144 proprietary primers, sequence not disclosed) from SABioscience (Qiagen). The mRNA
145 levels of gene of interest were normalized to β-actin, GAPDH or HSP90ab mRNA levels.
146 PCR cycles followed the sequence: 10 min at 95°C of initial denaturation; 15 secs at
147 95°C; and 40 cycles of 1 min each at 60°C for annealing. The samples were amplified
148 using the Stratagene Mx3005O cycler.

149 **Flow cytometry studies**

150 Ki67 antibodies (Biolegend) were used to determine cellular proliferation by flow
151 cytometry as per manufacturer's protocol. Cell signaling pathways were also determined
152 by flow cytometry. 4T1 mammary tumor cells were treated with Wortmannin for PI3K/AKT
153 inhibition, and total and phosphoAKT (Cell Signaling) were assessed by flow cytometry
154 using manufacturer's Methanol Intracellular Staining protocol. 50,000 cells were acquired
155 and total and phophoPI3/AKT were determined, using a FACS Calibur (BD) flow
156 cytometer, followed by analysis using FloJo software (Tree Star, Inc.).

157 **Silencing of SEMA7A in 4T1 murine mammary tumor cells**

158 Semaphorin 7A gene silencing in 4T1-LUC mammary tumor cells was achieved using
159 RNA interference via short hairpin RNA (Qiagen). To confirm gene knockdown, real time
160 quantitative polymerase chain reaction (q-PCR) (Qiagen) was performed using the
161 SEMA7A specific primers according to manufacturer's protocol. Cells were passaged
162 and selected until at least a 5-fold decrease in the SEMA7A gene expression was
163 achieved when compared to the scramble control. In non-luciferase 4T1 cells, SEMA7A
164 gene silencing in 4T1 mammary tumor cells was achieved using RNA interference via
165 short hairpin RNA (Mirimus). Cells were transfected with an shRNA plasmid system using
166 Avalanche transfection reagent (EZ-Biosystems). An optimized short hairpin RNA
167 algorithm was used to select the top three shRNA targeting sequences. The shRNA
168 vector also expressed a GFP reporter protein [28]. The vectors allow for direct visual
169 confirmation of shRNA-mirE expression as they constitutively express the shRNA,
170 fluorescent marker and puromycin selection marker from a single transcript that is driven
171 by the CMV promoter. As a negative control, we used multiple control clones with the
172 shRNA against Renilla Firefly Luciferase. Gene knockdown was confirmed by q-PCR

173 (Qiagen) using the SEMA7A specific primers according to manufacturer's protocol. The
174 results of gene expression were then confirmed by determination for the SEMA7A protein.

175 **AFM Cell Stiffness Measurements**

176 Cell stiffness measurements were acquired on living 4T1 cells. The bare AFM tip was
177 lowered onto the cell surface at 4 μm/s [29]. The acquired force-indentation curves of the
178 cells were fit to a model initially proposed by Hertz to estimate the Young's modulus
179 assuming that the cell is an isotropic elastic solid and the AFM tip is a rigid cone [30] .
180 The model is as follows:

181

182
$$F = \frac{K}{2(1-\nu^2)} \frac{4}{\pi \tan\theta} \alpha^2$$

183

184 where F is the applied force, α the indentation, K the Young's modulus, θ the angle
185 formed by the indenter and the plane of the surface (55°) and ν, Poisson ratio (0.5).
186 Young's modulus was obtained by least square analysis of the force-indentation curves
187 using Igor Pro software.

188 **Wounding assay**

189 4T1 LUC shRNA control or 4T1-LUC SEMA7A shRNA KD mammary tumor cells were
190 cultured under optimal conditions using DMEM culture media with 10% FBS until ~80%
191 confluency was achieved. Wound was generated, morphology and migration was
192 assessed at 0, 6 and 12 hours post-wounding.

193 **Statistical Analysis**

194 Results are expressed as means ± standard deviation. Statistical analyses were
195 performed using GraphPad Prism 6 software (LaJolla, CA). Statistical comparisons were
196 performed using an unpaired 2-tailed Student ***t*** test, with significance at $p < 0.05$.
197 Nonparametric analysis was used to determine tumor growth and metastasis. For the
198 survival analysis, the Kaplan-Meier method was used.
199

200 **RESULTS**

201 **SEMA7A expression is increased in metastatic breast cancer cells**

202 To determine the expression of SEMA7A in breast cancer, quantitative RT-PCR was
203 performed in human breast cancer cell lines with varying potential for metastasis. Highly
204 metastatic human breast cancer cell lines, MDA-MB-231and CA1a, expressed the
205 highest levels of SEMA7A with lower levels of expression in MCF-7A, a cell line that
206 exhibits decreased metastatic potential (Fig. 1A). In contrast, non-tumorigenic breast
207 cells, MCF10A, expressed the lowest levels of SEMA7A (Fig. 1A). Furthermore, we
208 characterized expression of SEMA7A in murine breast cancer cell lines with varying
209 degrees of metastatic potential. We found that EpH4 cells, a non-tumorigenic cell line
210 derived from spontaneously immortalized mouse mammary gland epithelial cells [31],
211 expressed the least SEMA7A at both mRNA and protein levels (Fig. 1B-C). In contrast,
212 a cell line comparable to the human MDA-MB231 cells, murine 4T1 mammary tumor cells,
213 expressed the highest levels of SEMA7A. Non-and poorly-metastatic murine cell lines,
214 67NR and 4T07, expressed intermediate amounts of SEMA7A at both mRNA and protein
215 levels (Fig. 1B-C). We found that 4T1 cells had heterogeneous expression of SEMA7A
216 and it was observed that non-adherent 4T1 cells had very high expression of SEMA7A

217 relative to adherent cells (Supplemental Fig. 1). Given that PI3K/AKT has been shown
218 to modulate SEMA7A expression, we treated human MCF10A cells with either 0.5 μM or
219 1 μM Wortmannin, an irreversible PI3/AKT inhibitor, prior to stimulation with TGF-β
220 (5ng/ml). This resulted in a decreased expression of SEMA7A in the treated groups as
221 determined by qRT-PCR (Fig. 1D). Similarly, pretreatment of murine EpH4 cells with
222 Wortmannin blocked the induction of SEMA7A expression in a dose-dependent manner
223 (Fig. 1E). We next examined the effect of TGF-β on the phosphorylation of PI3K/AKT
224 proteins in the presence/absence of Wortmannin by flow cytometry using the 4T1
225 mammary tumor cells. While there were no significant differences in total AKT expression
226 in cultures between untreated and those treated with Wortmannin, a decrease in mean
227 fluorescence intensity could be seen when serine is phosphorylated at position 473 (Fig.
228 1F) resulting in decreased SEMA7A production. Our results demonstrate that SEMA7A
229 is highly expressed in both human and murine breast cancer cells. Further, we show that
230 TGF-β signaling via the PI3K/AKT pathway can elicit the expression of SEMA7A in
231 mammary cells.

232 **Figure 1. SEMA7A is highly expressed in metastatic human and murine cell lines**
233 **and TGF-β induces SEMA7A expression via AKT signaling.** A-B) $1x10^6$ cells were
234 grown to ~75% confluency, trypsinized and lysed for RNA extraction then assayed for
235 SEMA7A gene expression by quantitative real time PCR. C) Cell free supernatants were
236 collected from murine breast cell lines and assayed by ELISA. D-E) $1x10^5$ MCF-10A or
237 EpH4 cells were pre-treated for 12 hours in serum-free media with Wortmannin (0.5 or 1
238 uM) or DMSO vehicle, 5% FBS was then added and then cultured in 1 ml of complete
239 media with 5ng/mL of rmTGF-beta-1 or vehicle for 24 hours. F-G) 4T1 cells were treated

240 with a PI3K/AKT inhibitor, Wortmannin for one hour. PI3K/ AKT phosphorylation was
241 assayed one hour later by flow cytometry and cell-free supernatants were assayed for
242 SEMA7A at 24 hrs. *p*-value (**) ≤ 0.01, (***) <0.001.

243 **Supplemental Figure 1. Non-adherent 4T1 cells expressed high levels of SEMA7A**
244 **compared to very-adherent 4T1 cell.** A) 4T1 tumor cells were grown in optimal
245 conditions to ~75% confluency and stained for SEMA7A mRNA expression using RNAish.
246 B) 4T1 cells were selected for 10 passages based on detachment time upon
247 TrypleExpress disassociation from standard cell cultured coated polystyrene plates. C)
248 4T1 sublines cells were then lysed and analyzed for SEMA7A expression. *p*-value ≤ (**)
249 <0.01.

250

251 **Hypoxia induces expression of SEMA7A in murine mammary cell lines and**
252 **inhibition of HIF-1 α decreases SEMA7A**

253 Hypoxia is a common hallmark of solid tumors such as breast tumors [32, 33]. It is
254 characterized as being an imbalance between intracellular oxygen delivery and oxygen
255 consumption[34]. Morote-Garcia et al., reported that hypoxia induction of HIF-1α induces
256 SEMA7A expression in endothelial cells [23]. However, it was still unknown what
257 physiological processes can promote the aberrant upregulation of SEMA7A in tumor cells.
258 Here we investigated the role of hypoxia in driving the expression of SEMA7A in
259 mammary cells. EpH4 cells and 4T1 were grown in optimal conditions and then placed in
260 a hypoxic chamber containing less than 1% O_2 using 5% CO_2 – Nitrogen gas or normoxic
261 conditions for up to 24 hours. Cell-free supernatants were then analyzed for SEMA7A
262 protein by ELISA. In a time-dependent manner, both EpH4 and 4T1 cells showed

263 increased SEMA7A production compared to normoxic controls (Fig. 2A). PI3K/AKT
264 signaling has been shown to regulate HIF1-α expression during hypoxic conditions [35].
265 This occurs when AKT is phosphorylated at Ser473 and its downstream target, HIF-1α,
266 is activated. To understand the role of the PI3K/AKT pathway in the upregulation of
267 SEMA7A during hypoxia, we pretreated 4T1 cells with PI3K inhibitor LY294002 (25 μM)
268 prior to exposure to hypoxic conditions. The PI3K inhibitor significantly ($p \leq 0.001$) blocked
269 the induction of SEMA7A by hypoxia in 4T1 cells by 70% (Fig. 2B). To further delineate
270 the specific role of HIF-1 α in inducing SEMA7A, 4T1 cells were treated with the inhibitor
271 Chetomin, which can disrupt the binding of HIF1- α to transcriptional coactivator p300.
272 Pre-treatment of 4T1 cells with Chetomin significantly ($p \leq 0.001$) blocked production of
273 SEMA7A in hypoxic conditions (Fig. 2C). Using an intermediate-expressing murine
274 breast tumor cell line, 4T07, we induced activation of HIF1- α using a hypoxia mimic,
275 Cobalt (II) Chloride ($CoCl_2$). We analyzed the expression of HIF1- α by flow cytometry
276 after treating 4T07 cells with $CoCl_2$ (100 μM) and found a significant increase in the levels
277 of HIF1- α (Fig. 3A), with a two-fold increase in SEMA7A expression (Fig. 3B). Treatment
278 with Chetomin reduced expression of SEMA7A in murine 4T07 tumor cells stimulated
279 with $CoCl_2$ (Fig. 3C). These results indicate that SEMA7A expression in mammary cells
280 can be induced through a hypoxic stimuli and inhibition of the PI3K/AKT pathway blocks
281 this induction.

282 **Figure 2. Hypoxia induces SEMA7A expression in mammary cells.** A) 1×10^6 4T1 or
283 EpH4 cells were grown to confluency, then incubated under 1% oxygen hypoxic
284 conditions or normoxic conditions. B) 4T1 cells were grown to 100% confluency in
285 reduced serum condition, treated with LY294002 for 4 hours, incubated under 1% oxygen

286 hypoxic conditions or normoxic conditions. C) $1x10^6$ 4T1 cells were treated with Chetomin
287 or control for 6 hours and then incubated under 1% oxygen hypoxic conditions or
288 normoxic conditions for 24 hours. Cell-free supernatants were collected at specific
289 timepoints and assayed for SEMA7A by ELISA. *p*-value (***) <0.001.

290 **Figure 3. HIF-1α production correlates with SEMA7A expression in 4T07 cells.** A)
291 4T07 cells were grown to 100% confluency in reduced serum conditions for 12 hours,
292 then stimulated with $CoCl_2$ or control for 24 hours, and cells were harvested for HIF-
293 1α determination by intracellular flow cytometry staining. B) 4T07 cells were grown to
294 100% confluency in reduced serum condition, treated with $CoCl_2$ or vehicle for 24 hours,
295 cells were then lysed and analyzed for SEMA7A expression by qPCR. C) $1x10^6$ 4T07
296 cells were treated with or without Chetomin for 6 hours and then stimulated by $CoCL_2$ for
297 24 hours, cells were then lysed and analyzed for SEMA7A by qPCR. *p*-value (**) ≤ 0.01,
298 (***) ≤0.001.

299

300 **SEMA7A gene knock down in 4T1 mammary tumor cells results in morphologic**
301 **and functional changes**

302 We have previously reported that tumor-derived SEMA7A induces the production of
303 angiogenic molecules in macrophages [15]. To understand the role of SEMA7A in tumor
304 cells, SEMA7A gene was knocked down in 4T1 mammary tumor cells. The effectiveness
305 of gene knockdown was assessed by qRT-PCR. Greater than 6-fold gene knockdown
306 was observed in shRNA transfected 4T1-LUC mammary tumor cells (Fig. 4A).
307 Suppression of SEMA7A gene in mammary tumor cells resulted in increased cell-to-cell
308 contact in 4T1-LUC mammary tumor cells compared to 4T1 scramble control (Fig. 4B).

309 Silencing of SEMA7A induced an epithelial-like morphology in 4T1-LUC cells with growth
310 of tumor cells in compact clusters. Semaphorins are known to regulate cell migration and
311 tumor cell migration is dependent upon morphological changes and cell association with
312 extracellular matrix, and release of metalloproteases [36]. The expression of vimentin and
313 CD44 was decreased in 4T1-LUC-shRNA-SEMA7A knock-down (kd) cells. Fibronectin
314 and E-cadherin expression remained unchanged, while desmoplakin was increased
315 (Fig.4C). Silencing of the SEMA7A gene decreased the expression of TGF-β1 (Fig. 4D).
316 Matrix metalloproteinases MMP-2, -9, -10 and -13 were also significantly ($p \leq 0.001$)
317 decreased in SEMA7A silenced 4T1 mammary tumor cells (Fig. 4E). We next transfected
318 murine 4T1 tumor cells with a plasmid encoding for the rat SEMA7A gene, which has
319 90% homology with the murine SEMA7A. We achieved an 18-fold increase in rat
320 SEMA7A expression, which correlated with a doubling in expression of only
321 mesenchymal MMPs: MMP3 and MMP13 (Supplemental Fig. 2). As cell migration may
322 also be dependent on integrins, we explored the effect of SEMA7A gene silencing on
323 integrin expression. There was a significant ($p \leq 0.001$) decrease in the expression of its
324 receptor integrin β1. Also decreased was integrin β3, integrins α5 and α7 (Fig. 4F). Our
325 results show that a strong linkage between the expression of SEMA7A and the expression
326 of mesenchymal markers and MMPs. We continued our study with the gene-silencing
327 approach to evaluate the role of SEMA7A in tumor cell function.
328 (***) ≤0.001

329 **Figure 4. Gene silencing of SEMA7A in 4T1-LUC cells decreases expression of**
330 **mesenchymal and pro-metastatic genes**. A) Gene expression of 4T1-LUC scramble
331 control cells and 4T1-LUC SEMA7A shRNA silenced cells was assayed by qPCR. (B)

332 Morphology was observed by phase contrast imaging. (C-F) Gene expression of 4T1-
333 LUC sublines was analyzed for differential gene expression by qPCR. *p*-value (**) ≤ 0.01,
334 (***) ≤0.001.

335 **Supplemental Figure 2. Exogenous overexpression of SEMA7A in 4T1 cells.** A)
336 4T1 cells were transfected with a plasmid encoding for full-length rat SEMA7A using
337 Avalanche transfection reagent, $1x10^6$ cells were grown to ~75% confluency, trypsinized,
338 lysed for RNA extraction and then assayed for exogenous SEMA7A. B-C) Endogenous
339 MMP-13 and MMP-3 gene expression was assayed by qPCR. *p*-value (**) ≤0.01, (***) ≤
340 0.001.

341

342 **SEMA7A gene silencing increases stiffness of 4T1 cells**

343 It is known that cancerous cells are less stiff compared to normal cells [37, 38]. Atomic
344 force microscopy (AFM) measurements were acquired to determine the role of SEMA7A
345 in mediating cell stiffness. In these studies, the AFM cantilever was used as a
346 microindenter, probing the cell less than 1 mm using applied forces of less than 1 nN as
347 to not damage the cell. Figure 5A shows representative force-indentation curves acquired
348 for 4T1 shRNA control and 4T1-LUC-shRNA-SEMA7A knock-down cells. Following
349 SEMA7A knockdown, 4T1 cells indented less for equivalent applied forces. Each force-
350 indentation curve was fitted to the Hertz's model. Histograms in Figure 5B reveal the data
351 distribution of the Young's modulus values for both cell types. The average Young's
352 modulus value calculated for the 4T1 mammary tumor cells was 3.7±0.3 kPa (Fig. 5C).
353 Following SEMA7A knockdown, this value increased to 7.5±1 kPa, indicating an increase
354 in the measured cell stiffness.

355 **Figure 5. SEMA7A alters tumor cell stiffness.** A) Representative force-indentation
356 curves from AFM cell stiffness measurements acquired for 4T1-LUC scramble shRNA
357 control cells and 4T1-LUC 6-fold shRNA SEMA7A gene knockdown. Fitted curves
358 derived from the Hertz model are overlaid on the raw data. AFM measurements were
359 acquired at 37°C at a constant cantilever retraction rate, applied force and contact
360 time. B) Data distribution of Young's modulus values for 4T1-LUC scramble shRNA
361 control cells (white; N=35) and 4T1-LUC 6-fold shRNA SEMA7A gene knockdown cells
362 (blue; N=29). C) Average of Young's modulus values for stiffness measurements from B.
363 The error is the SEM. *p*-value (**) ≤ 0.01.

364

365 **SEMA7A gene silencing decreases proliferation and migration of 4T1 cells**

366 Given that tumor cell proliferation is a critical factor in determining patient outcomes, we
367 assayed the expression of a commonly used clinical proliferation marker, ki67. We found
368 a decrease of nearly half the expression of ki67 in 4T1-LUC cells that had been silenced
369 for the SEMA7A gene (Fig. 6A-B). To test the effect of SEMA7A gene silencing on cell
370 motility, wound healing assay was employed. Wounding assay revealed decreased tumor
371 cell migration by SEMA7A silenced tumor cells at both 6 and 12 hours post-wounding
372 (Fig. 6C-D). These findings suggest that SEMA7A may also play a role in tumor cell
373 motility.

374 **Figure 6. Silencing of SEMA7A gene in 4T1-LUC mammary tumor decreases cell**
375 **motility and proliferation.** A-B) Proliferation of SEMA7A expressing and shRNA
376 silenced cells were measured by Ki67 intracellular staining. C-D) Motility of 4T1-LUC

377 scramble control cells and 4T1-luc SEMA7A shRNA was assayed using a wound healing
378 assay and measured as percentage wound closure. *p*-value (***) ≤ <0.001.

379

380 **SEMA7A gene silencing reduces tumor growth and metastasis and increases**
381 **survival**

382 Since SEMA7A gene silencing resulted in decreased tumor cell proliferation and
383 migration, we wanted to further investigate if decreased SEMA7A would retard tumor
384 growth in mammary tumor bearing mice. Mice injected with either 4T1-LUC scramble
385 control or 4T1-LUC-SEMA7A-shRNA-kd mammary tumor cells were imaged for luciferase
386 bioluminescence at days 4, 11 and 18. At day 4 post tumor cell implantation, there was
387 no significant difference in tumor growth between the two groups. However, by days 11
388 and 18 post-tumor cell implantation, there was a reduction (*p=.00001*) in tumor growth in
389 mice injected with 4T1-LUC-SEMA7A-shRNA-kd tumor cells (Fig. 7A). This was
390 quantified by luciferase signal detected by photons/sec (Fig. 7B). More importantly, an
391 increase in survival (*p=0.0001*) was observed in mice bearing SEMA7A gene silenced
392 mammary tumors (Fig. 7C). Since we observed increased survival in mice bearing
393 SEMA7A gene silenced tumors, we next determined if this translated into decreased
394 metastasis. Lungs are one of the first organs that are infiltrated and colonized by
395 metastatic breast tumors. Thus, lungs from 4T1-LUC scramble control or 4T1-LUC-7A-
396 shRNA-kd mammary tumor bearers were analyzed for metastasis by luciferase signal
397 detection, India Ink and H&E staining. Detection of the luciferase signals revealed a
398 steep reduction of tumor cell signal in the lungs of 4T1-LUC SEMA7A-shRNA mammary
399 tumor bearers (Fig. 7D). Gross morphologic and histological examination of lungs stained

400 by India Ink from the two groups revealed very few metastatic foci in 4T1-LUC 7A-shRNA
401 mammary tumor bearers (Fig. 7E). Enumeration of the metastatic foci in the lungs
402 revealed >30 metastatic foci in 4T1-LUC scramble control compared to <5 metastatic foci
403 in lungs of 4T1-LUC 7A-shRNA-kd mammary tumor bearers (Fig. 7E). We determined
404 that inhibition of tumor-derived SEMA7A in 4T1 cells decreased tumor growth and
405 metastasis.

406 **Figure 7. Inhibition of tumor-derived SEMA7A decreases tumor growth rate and**
407 **metastasis in 4T1-LUC tumor-bearing mice.** A-B) Mice representative of 14 mice/group
408 at 4 different time points are shown with quantification of tumor-specific bioluminescence.
409 C) Kaplan-Meier survival curve of tumor bearing mice. D) At day 42 post tumor
410 implantation, animals inoculated with SEMA7A shRNA silenced 4T1 cells showed
411 decrease metastasis compared to the scramble shRNA control 4T1 cells as determined
412 by quantification of bioluminescent signal and E) India Black staining of the lungs. N=14;
413 *p*-value (***) ≤ 0.001, (****) ≤0.0001.

414

415 **Ablation of host-derived SEMA7A decreases rate of tumor growth and metastasis**
416 **and increases survival**

417 Given that host cells, such as immune cells, also express SEMA7A we questioned if host-
418 derived SEMA7A could also contribute to tumor progression. Wild-type or SEMA7A
419 deficient female BALB/c mice were inoculated with 4T1-LUC cells and bioluminescent
420 imaging was performed for 28 days. 4T1-LUC tumor-bearing mice have a decrease in
421 tumor growth rate (Fig. 8A-B) and increased survival (Fig. 8C), but more importantly, a
422 decrease in metastasis to the lungs was also observed (Fig. 8D).

423 **Figure 8. Genetic ablation of host-derived SEMA7A decreases tumor growth rate**
424 **and metastasis in 4T1-LUC tumor-bearing mice.** A-B) Mice representative of 15
425 mice/group at 4 different time points are shown with quantification of tumor-specific
426 bioluminescence. C) Kaplan-Meier survival curve of tumor bearing mice. D) At day 44
427 post tumor implantation, metastasis was determined by quantification of bioluminescent
428 signal from the lungs. N=15; *p*-value (***) ≤ 0.001.

429

430 We proceeded to test if ablation of host derived SEMA7A could synergize with gene
431 silencing of tumor-derived SEMA7A. In this approach, we used the wild-type 4T1 cells
432 from early passage that maintained heterogeneous populations. We achieved an 80%
433 reduction in SEMA7A expression (Fig. 9A) in two subsets of 4T1 cells using an optimized
434 microRNA backbone targeting the 5' end of the SEMA7A mRNA (4T1-SEMA7A-shRNA1)
435 and another targeting the 3' end of the SEMA7A mRNA (4T1-SEMA7A-shRNA2.) As a
436 control, we used cells expressing shRNA targeting the Renilla luciferase gene (4T1-
437 Renilla-shRNA), which maintained equal expression to the 4T1 wild-type cells (data not
438 shown). Subsequently, the SEMA7A shRNA cells or Renilla shRNA control cells were
439 implanted into either wild-type or SEMA7A$^{-/-}$ female BALB/C mice. Silencing of SEMA7A
440 in 4T1 cells resulted in reduced tumor growth and ablation of host-derived SEMA7A
441 further synergized to reduce tumor growth rate (Fig. 9B), and metastasis to the lung (Fig.
442 9C-D). Our results show that ablation of host-derived SEMA7A and tumor-derived
443 SEMA7A can significantly improve outcomes in our murine breast cancer models.

444 **Figure 9. Inhibition of host-derived and tumor-derived SEMA7A decreases tumor**
445 **growth rate and metastasis in 4T1 tumor-bearing mice.** A) Gene silencing of SEMA7A
446 in 4T1 cells. B) Caliper measurements of tumor volume at 5 different time points. C-D) At
447 day 42 post tumor implantation, lungs were excised and metastatic foci were quantified.
448 N=15); *p*-value (**) ≤0.01, (***) ≤ 0.001.

449

450 **DISCUSSION**

451 The objective of this study was to delineate the role of SEMA7A in breast cancer. We
452 accomplished this objective by: 1) characterizing the expression of SEMA7A in murine
453 and human cell lines, 2) determining the effect of TGF-β, the PI3K/AKT axis and hypoxia
454 in modulating SEMA7A expression, and 3) assessing both *in vitro* and *in vivo* effects of
455 SEMA7A inhibition in mammary tumors.

456 We first determined the range of SEMA7A expression in various human and murine
457 breast cell lines. The non-tumorigenic line, MCF10A, had the lowest expression of
458 SEMA7A (Fig. 1A) whereas the aggressive MCF10CA1a cell line had 1000 times greater
459 expression. So et al., [39] reported that MCF10CA1a cells have increased
460 phosphorylation levels pErk, pAkt, Stat3 and Pak4, and we show concurrently there was
461 an increase in SEMA7A expression. We also showed that the murine counterparts of
462 MCF10A, the EpH4 cells, had very low expression of SEMA7A. Allegra et al., [9] showed
463 that EpRAS cells, generated via a RAS mutation, expressed high levels of SEMA7A.
464 These findings show that both pAKT and RAS/Erk activation can induce the expression

465 of SEMA7A. It would be beneficial to determine if specific mutations in these pathways
466 lead to differential SEMA7A expression.

467 Morote-Garcia et al. showed the presence of HREs in the SEMA7A promoter [23]. We
468 are the first to show that hypoxia can also induce the expression of SEMA7A in mammary
469 cells. The frequency of these HREs could also be a determining factor in the disparate
470 expression of SEMA7A between EpH4 cells and 4T1. We continued by exploring the link
471 between hypoxic stimuli and PI3K/AKT activation given that it's been shown that AKT
472 activation increases HIF-1α activity[35]. By blocking phosphorylation of AKT we were able
473 to reduce the induction of SEMA7A by hypoxia (Fig. 2B), showing that the cross talk
474 between hypoxia-derived signals and AKT are important for SEMA7A expression. To
475 verify to what extent HIF-1α plays a role in this pathway, we used the inhibitor Chetomin.
476 In both 4T1 and 4T07 cells, Chetomin was very efficient at blocking the expression of
477 SEMA7A. Our findings demonstrate that hypoxic stimuli are a critical factor in the
478 induction of SEMA7A in mammary cells.

479 Inhibition of tumor-derived SEMA7A lessened the malignant potential of 4T1 cells. In
480 accordance with our studies, Saito et al.,[40] has shown that shRNA inhibition of SEMA7A
481 in oral squamous cell carcinoma (OSCC) cells resulted in decreased cellular proliferation,
482 which was attributed to down-regulation of cyclins. Additionally, Saito et al., [40] showed
483 that inhibition of SEMA7A decreased invasiveness of oral squamous cell carcinoma cells
484 and secretion of matrix metalloproteases. Our biophysical studies conducted on the 4T1
485 cells revealed an increase in cell stiffness when the SEMA7A gene is silenced. A softer
486 cell can spread more easily on a substrate, thus facilitating migration. Thus, the stiffening
487 following SEMA7A knockdown is likely to promote a less migratory cell phenotype. This

488 indeed was observed in our *in vivo* model. *In vivo*, BALB/c mice inoculated with SEMA7A
489 silenced 4T1-LUC cells showed a decrease tumor growth rates and delayed onset of
490 metastatic disease compared to mice bearing 4T1-LUC cells.

491 We also evaluated the contribution of host-derived SEMA7A using genetic deletion of
492 SEMA7A in BALB/c mice. We found that tumor growth was reduced in SEMA7A$^{-/-}$ 4T1-
493 LUC tumor bearing mice (Fig. 8A-B). There was also a decrease in metastases to the
494 lung at day 42 post-tumor implantation (Fig. 8D-E). Ma et al. similarly showed in a
495 syngeneic model of melanoma, that ablation of host-derived SEMA7A was sufficient to
496 decrease the rate of metastasis [8]. They also reported that antibody mediated
497 neutralization of SEMA7A had similar effects. The heterogeneous 4T1 model, SEMA7A^{-}
498 $^{/-}$ tumor-bearing also held a difference until day 28 but that difference was negated at day
499 35. It is possible that the 4T1 cells started to produce levels of SEMA7A that overpowered
500 the effects of ablating host-derived SEMA7A. When combined, inhibition of tumor-derived
501 SEMA7A and host-derived SEMA7A resulted in the reduced tumor growth and
502 metastasis.

503 Recently, Black et al. corroborated the necessity of studying SEMA7A in the context of
504 breast cancer as a prognostic parameter of metastasis and poor survival in breast cancer
505 patients [10]. Using two models, we have elucidated a role for SEMA7A in regulating
506 mammary tumor progression, warranting subsequent studies to survey the downstream
507 molecular effects of SEMA7A that lead to tumor growth and metastasis. Overall, our
508 collective results support our hypothesis that SEMA7A expression plays a functional role
509 in promoting breast cancer growth and metastasis. Our findings postulate a novel role for

510 SEMA7A in breast cancer that may lead to further findings of prognostic and therapeutic
511 value.
512
513
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532 **REFERENCES**

533 [1] Pasterkamp RJ, Kolodkin AL. Semaphorin junction: making tracks toward neural
534 connectivity. Curr Opin Neurobiol. 2003;13:79-89.
535 [2] Zhou Y, Gunput RA, Pasterkamp RJ. Semaphorin signaling: progress made and promises
536 ahead. Trends Biochem Sci. 2008;33:161-70.
537 [3] Műzes G, Sipos F. Relation of immune semaphorin/plexin signaling to carcinogenesis. Eur J
538 Cancer Prev. 2014;23:469-76.
539 [4] Epstein JA, Aghajanian H, Singh MK. Semaphorin signaling in cardiovascular development.
540 Cell Metab. 2015;21:163-73.
541 [5] Garcia-Areas R, Libreros S, Iragavarapu-Charyulu V. Semaphorin7A: branching beyond
542 axonal guidance and into immunity. Immunol Res. 2013;57:81-5.
543 [6] Ito D, Nojima S, Kumanogoh A. [The role of semaphorin family in immune systems]. Nihon
544 Rinsho Meneki Gakkai Kaishi. 2014;37:1-10.
545 [7] Morihana T, Kumanogoh A. [Immune semaphorins and allergic diseases]. Arerugi.
546 2013;62:155-62.
547 [8] Ma B, Herzog EL, Lee CG, Peng X, Lee CM, Chen X, et al. Role of chitinase 3-like-1 and
548 semaphorin 7a in pulmonary melanoma metastasis. Cancer Res. 2015;75:487-96.
549 [9] Allegra M, Zaragkoulias A, Vorgia E, Ioannou M, Litos G, Beug H, et al. Semaphorin-7a
550 reverses the ERF-induced inhibition of EMT in Ras-dependent mouse mammary epithelial cells.
551 Mol Biol Cell. 2012;23:3873-81.
552 [10] Black SA, Nelson AC, Gurule NJ, Futscher BW, Lyons TR. Semaphorin 7a exerts
553 pleiotropic effects to promote breast tumor progression. Oncogene. 2016.
554 [11] Formolo CA, Williams R, Gordish-Dressman H, MacDonald TJ, Lee NH, Hathout Y.
555 Secretome signature of invasive glioblastoma multiforme. J Proteome Res. 2011;10:3149-59.
556 [12] Jongbloets BC, Ramakers GM, Pasterkamp RJ. Semaphorin7A and its receptors:
557 pleiotropic regulators of immune cell function, bone homeostasis, and neural development.
558 Semin Cell Dev Biol. 2013;24:129-38.
559 [13] Fong KP, Barry C, Tran AN, Traxler EA, Wannemacher KM, Tang HY, et al. Deciphering
560 the human platelet sheddome. Blood. 2011;117:e15-26.
561 [14] Holmes S, Downs AM, Fosberry A, Hayes PD, Michalovich D, Murdoch P, et al. Sema7A is
562 a potent monocyte stimulator. Scand J Immunol. 2002;56:270-5.
563 [15] Garcia-Areas R, Libreros S, Amat S, Keating P, Carrio R, Robinson P, et al. Semaphorin7A
564 promotes tumor growth and exerts a pro-angiogenic effect in macrophages of mammary tumor-
565 bearing mice. Front Physiol. 2014;5:17.
566 [16] Liu H, Juo ZS, Shim AH, Focia PJ, Chen X, Garcia KC, et al. Structural basis of
567 semaphorin-plexin recognition and viral mimicry from Sema7A and A39R complexes with
568 PlexinC1. Cell. 2010;142:749-61.
569 [17] Kang HR, Lee CG, Homer RJ, Elias JA. Semaphorin 7A plays a critical role in TGF-beta1-
570 induced pulmonary fibrosis. J Exp Med. 2007;204:1083-93.

571 [18] Gan Y, Reilkoff R, Peng X, Russell T, Chen Q, Mathai SK, et al. Role of semaphorin 7a
572 signaling in transforming growth factor β1-induced lung fibrosis and scleroderma-related
573 interstitial lung disease. Arthritis Rheum. 2011;63:2484-94.
574 [19] Brotelle T, Bay JO. [PI3K-AKT-mTOR pathway: Description, therapeutic development,
575 resistance, predictive/prognostic biomarkers and therapeutic applications for cancer]. Bull
576 Cancer. 2016;103:18-29.
577 [20] Ciruelos Gil EM. Targeting the PI3K/AKT/mTOR pathway in estrogen receptor-positive
578 breast cancer. Cancer Treat Rev. 2014;40:862-71.
579 [21] Morrison CD, Parvani JG, Schiemann WP. The relevance of the TGF-β Paradox to EMT-
580 MET programs. Cancer Lett. 2013;341:30-40.
581 [22] Imamura T, Hikita A, Inoue Y. The roles of TGF-β signaling in carcinogenesis and breast
582 cancer metastasis. Breast Cancer. 2012;19:118-24.
583 [23] Morote-Garcia JC, Napiwotzky D, Köhler D, Rosenberger P. Endothelial Semaphorin 7A
584 promotes neutrophil migration during hypoxia. Proc Natl Acad Sci U S A. 2012;109:14146-51.
585 [24] Markel P, Shu P, Ebeling C, Carlson GA, Nagle DL, Smutko JS, et al. Theoretical and
586 empirical issues for marker-assisted breeding of congenic mouse strains. Nat Genet.
587 1997;17:280-4.
588 [25] Wakeland E, Morel L, Achey K, Yui M, Longmate J. Speed congenics: a classic technique
589 in the fast lane (relatively speaking). Immunol Today. 1997;18:472-7.
590 [26] Nishiyama A, Tsuji S, Yamashita M, Henriksen RA, Myrvik QN, Shibata Y. Phagocytosis of
591 N-acetyl-D-glucosamine particles, a Th1 adjuvant, by RAW 264.7 cells results in MAPK
592 activation and TNF-alpha, but not IL-10, production. Cell Immunol. 2006;239:103-12.
593 [27] Nishiyama A, Shinohara T, Pantuso T, Tsuji S, Yamashita M, Shinohara S, et al. Depletion
594 of cellular cholesterol enhances macrophage MAPK activation by chitin microparticles but not by
595 heat-killed Mycobacterium bovis BCG. Am J Physiol Cell Physiol. 2008;295:C341-9.
596 [28] Fellmann C, Hoffmann T, Sridhar V, Hopfgartner B, Muhar M, Roth M, et al. An optimized
597 microRNA backbone for effective single-copy RNAi. Cell Rep. 2013;5:1704-13.
598 [29] Wojcikiewicz EP, Zhang X, Chen A, Moy VT. Contributions of molecular binding events and
599 cellular compliance to the modulation of leukocyte adhesion. J Cell Sci. 2003;116:2531-9.
600 [30] Hoh JH, Schoenenberger CA. Surface morphology and mechanical properties of MDCK
601 monolayers by atomic force microscopy. J Cell Sci. 1994;107 (Pt 5):1105-14.
602 [31] Fialka I, Schwarz H, Reichmann E, Oft M, Busslinger M, Beug H. The estrogen-dependent
603 c-JunER protein causes a reversible loss of mammary epithelial cell polarity involving a
604 destabilization of adherens junctions. J Cell Biol. 1996;132:1115-32.
605 [32] Gilkes DM, Semenza GL. Role of hypoxia-inducible factors in breast cancer metastasis.
606 Future Oncol. 2013;9:1623-36.
607 [33] Milani M, Harris AL. Targeting tumour hypoxia in breast cancer. Eur J Cancer.
608 2008;44:2766-73.
609 [34] Haase VH. The sweet side of HIF. Kidney Int. 2010;78:10-3.
610 [35] Pore N, Jiang Z, Shu HK, Bernhard E, Kao GD, Maity A. Akt1 activation can augment
611 hypoxia-inducible factor-1alpha expression by increasing protein translation through a
612 mammalian target of rapamycin-independent pathway. Mol Cancer Res. 2006;4:471-9.
613 [36] Capparuccia L, Tamagnone L. Semaphorin signaling in cancer cells and in cells of the
614 tumor microenvironment--two sides of a coin. J Cell Sci. 2009;122:1723-36.
615 [37] Lekka M, Laidler P, Gil D, Lekki J, Stachura Z, Hrynkiewicz AZ. Elasticity of normal and
616 cancerous human bladder cells studied by scanning force microscopy. Eur Biophys J.
617 1999;28:312-6.
618 [38] Cross SE, Jin YS, Rao J, Gimzewski JK. Nanomechanical analysis of cells from cancer
619 patients. Nat Nanotechnol. 2007;2:780-3.

620 [39] So JY, Lee HJ, Kramata P, Minden A, Suh N. Differential Expression of Key Signaling
621 Proteins in MCF10 Cell Lines, a Human Breast Cancer Progression Model. Mol Cell Pharmacol.
622 2012;4:31-40.
623 [40] Saito T, Kasamatsu A, Ogawara K, Miyamoto I, Saito K, Iyoda M, et al. Semaphorin7A
624 Promotion of Tumoral Growth and Metastasis in Human Oral Cancer by Regulation of G1 Cell
625 Cycle and Matrix Metalloproteases: Possible Contribution to Tumoral Angiogenesis. PLoS One.
626 2015;10:e0137923.

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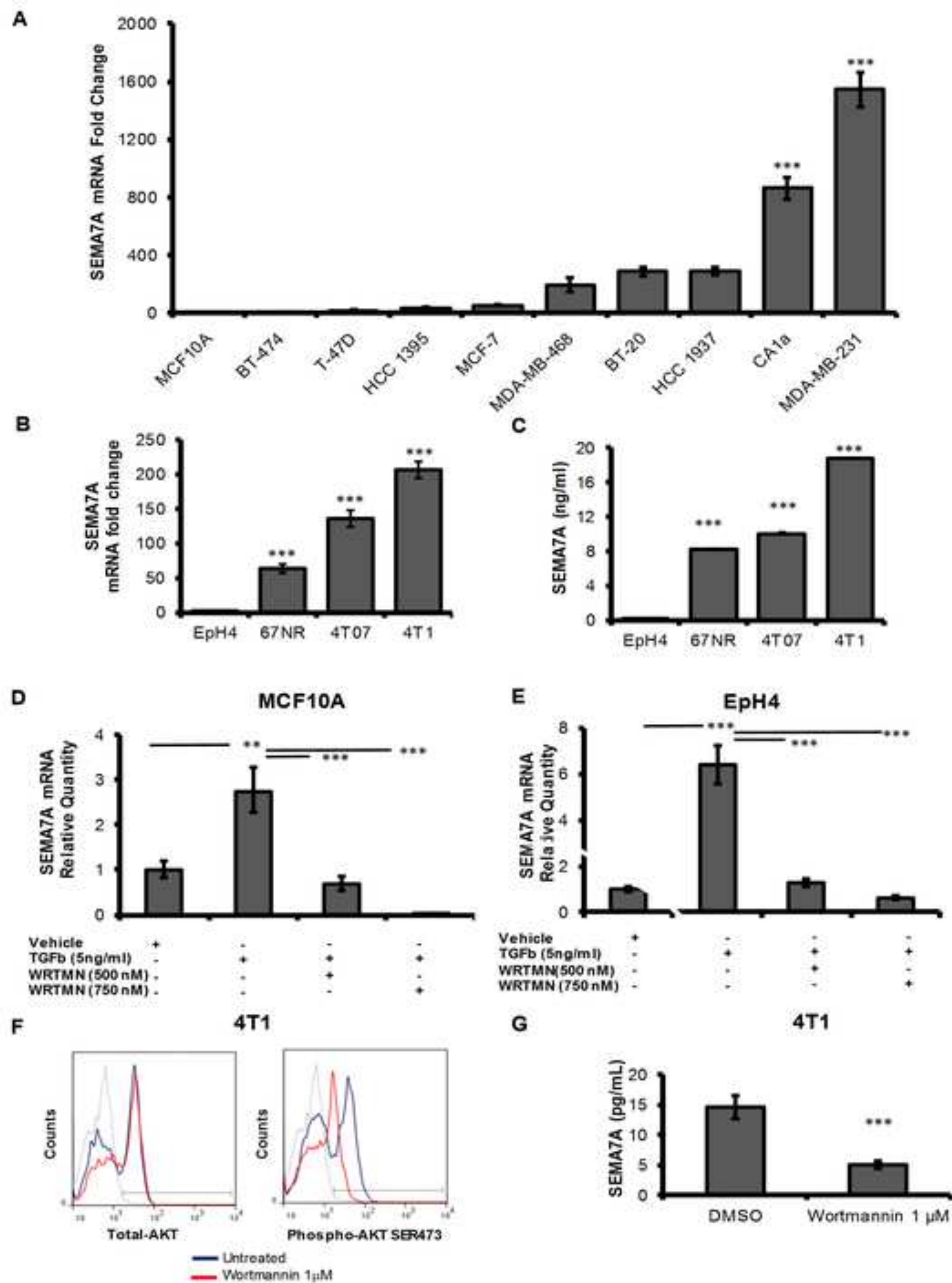

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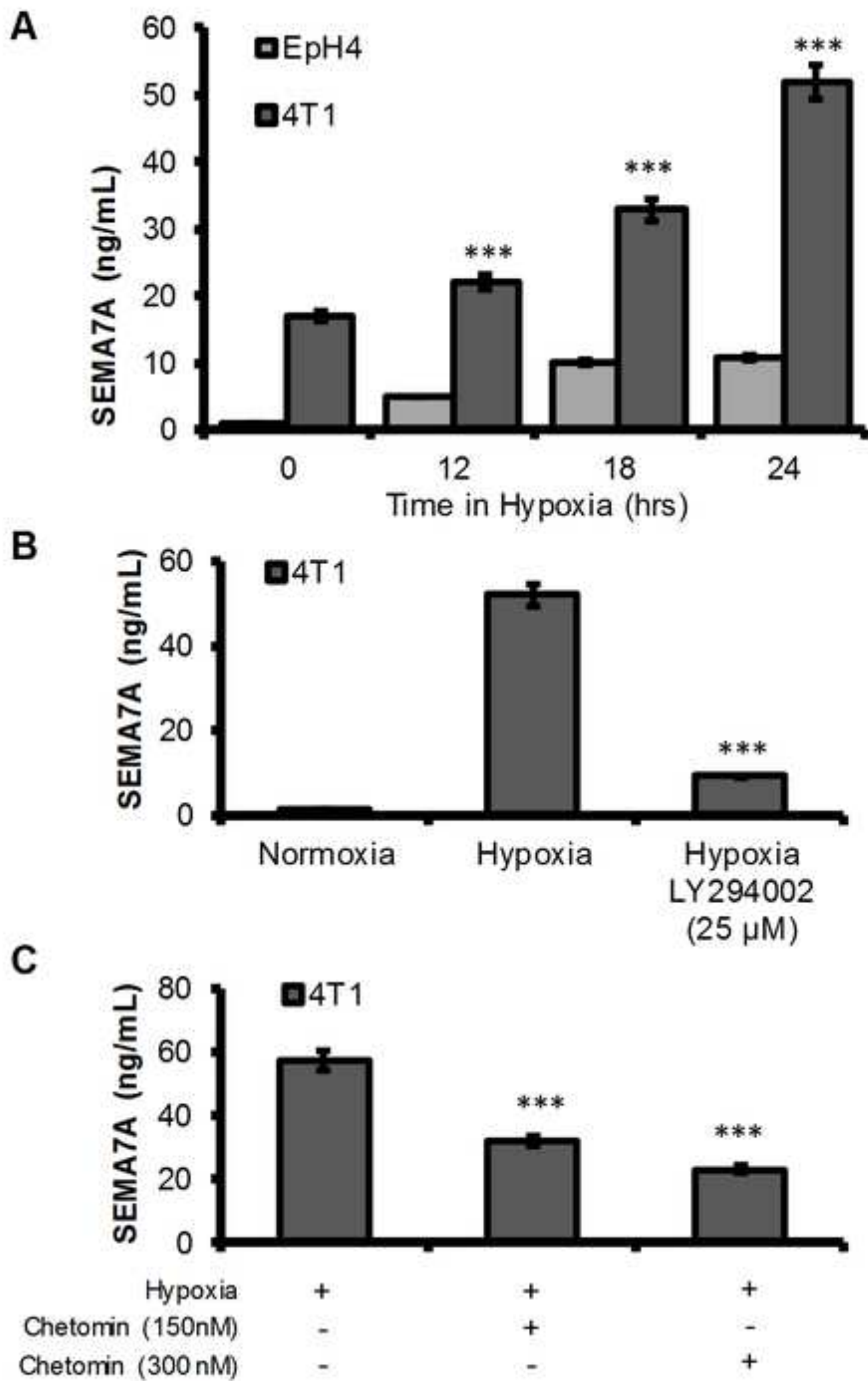

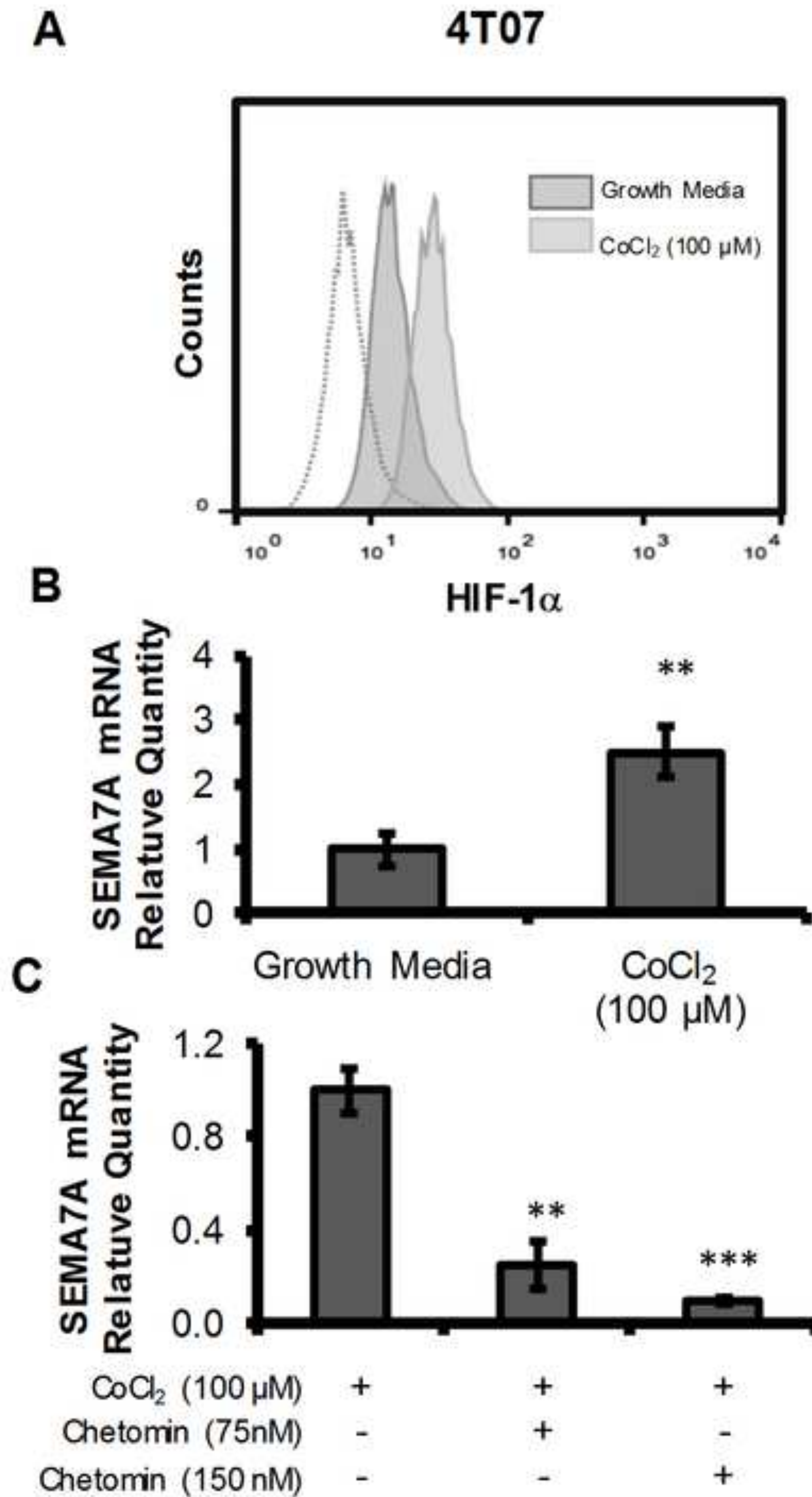
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Growth Media
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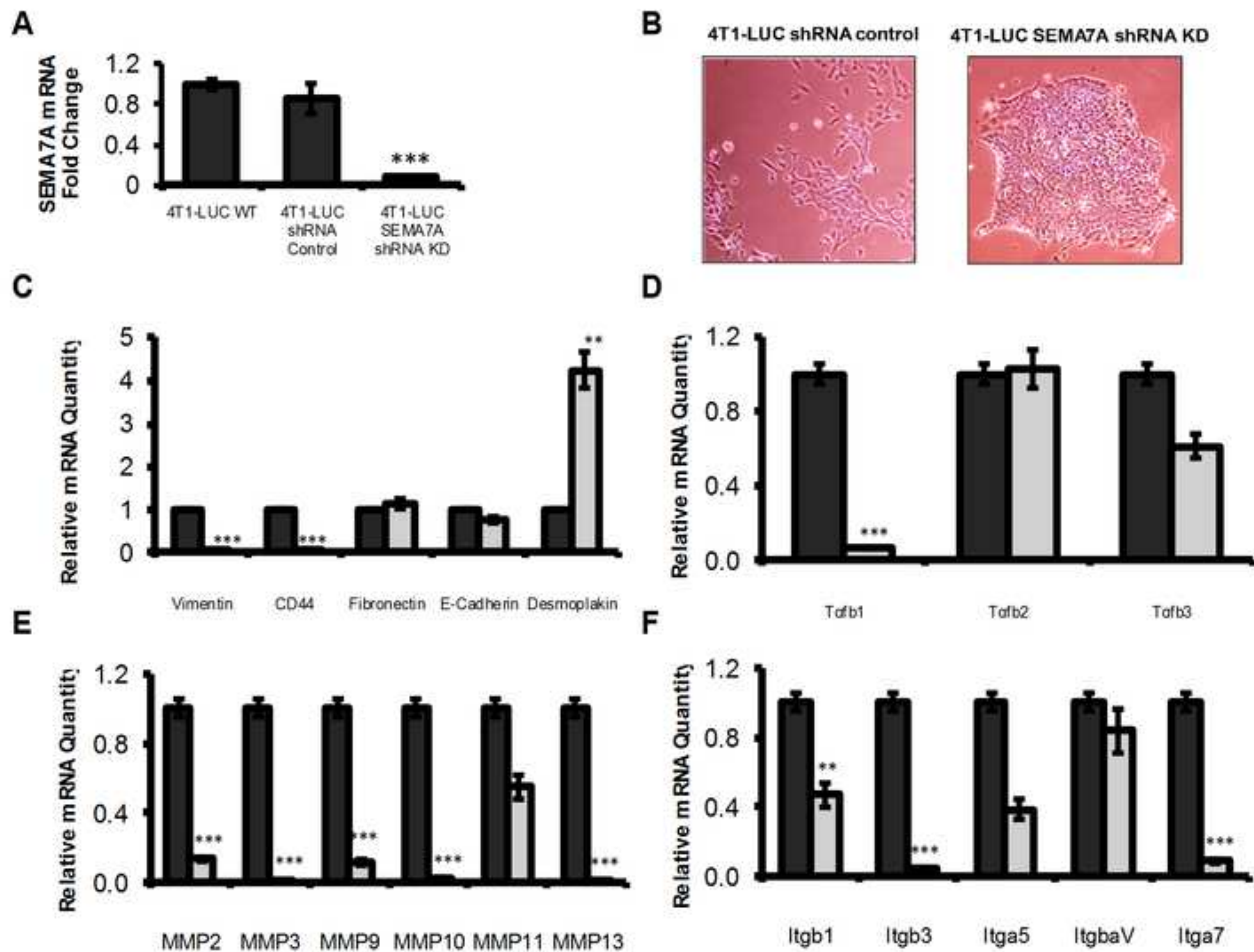

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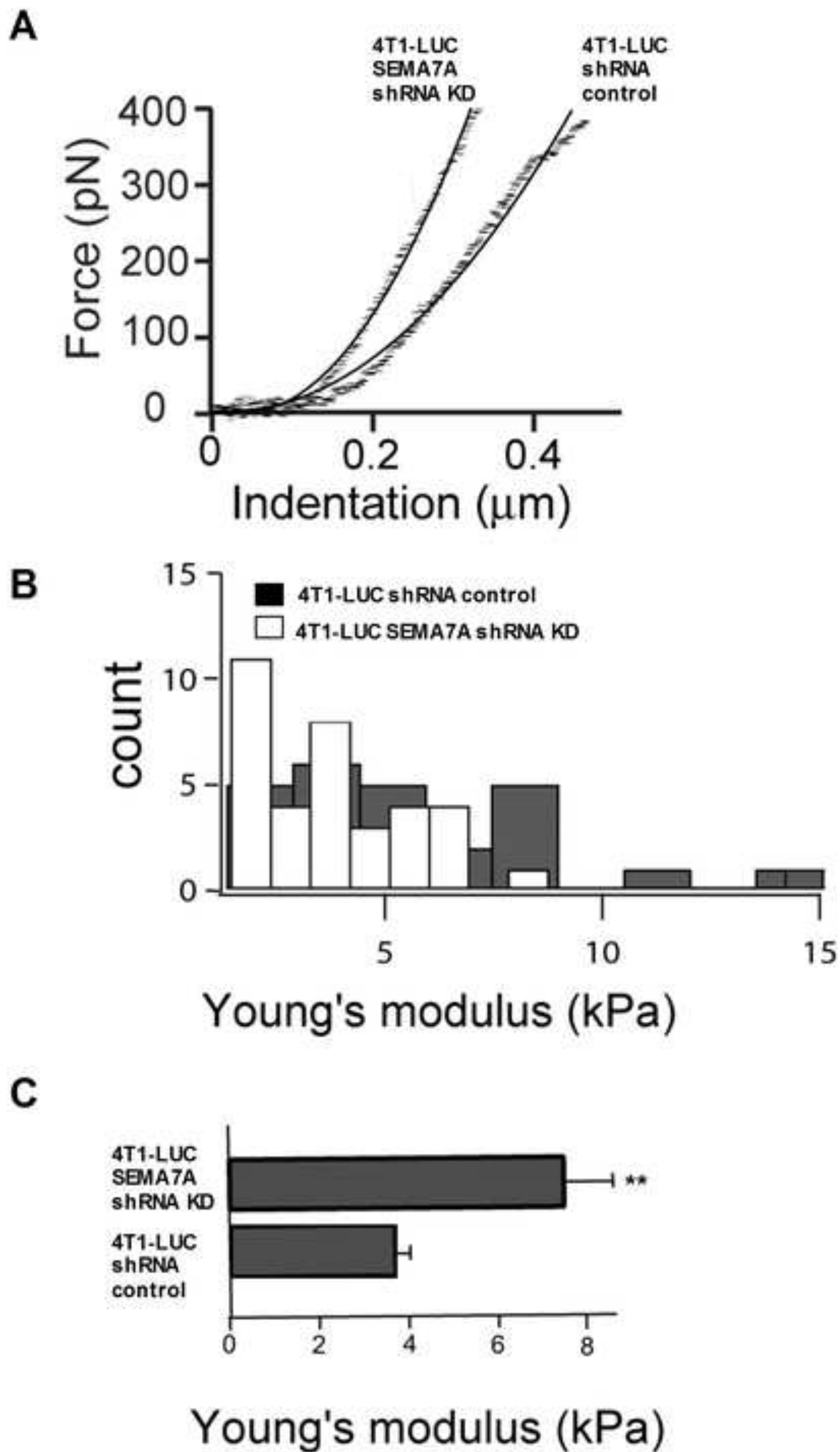

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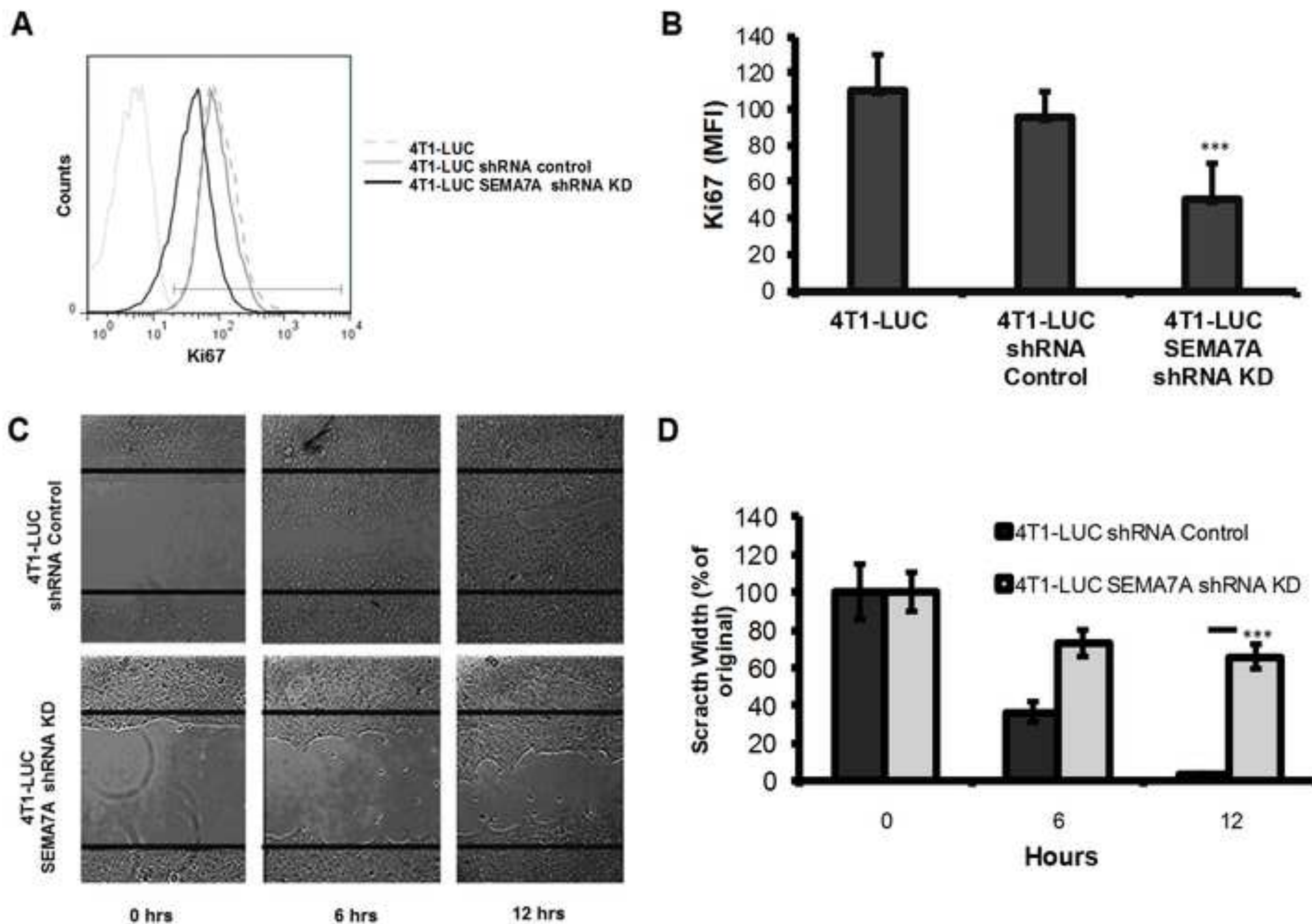

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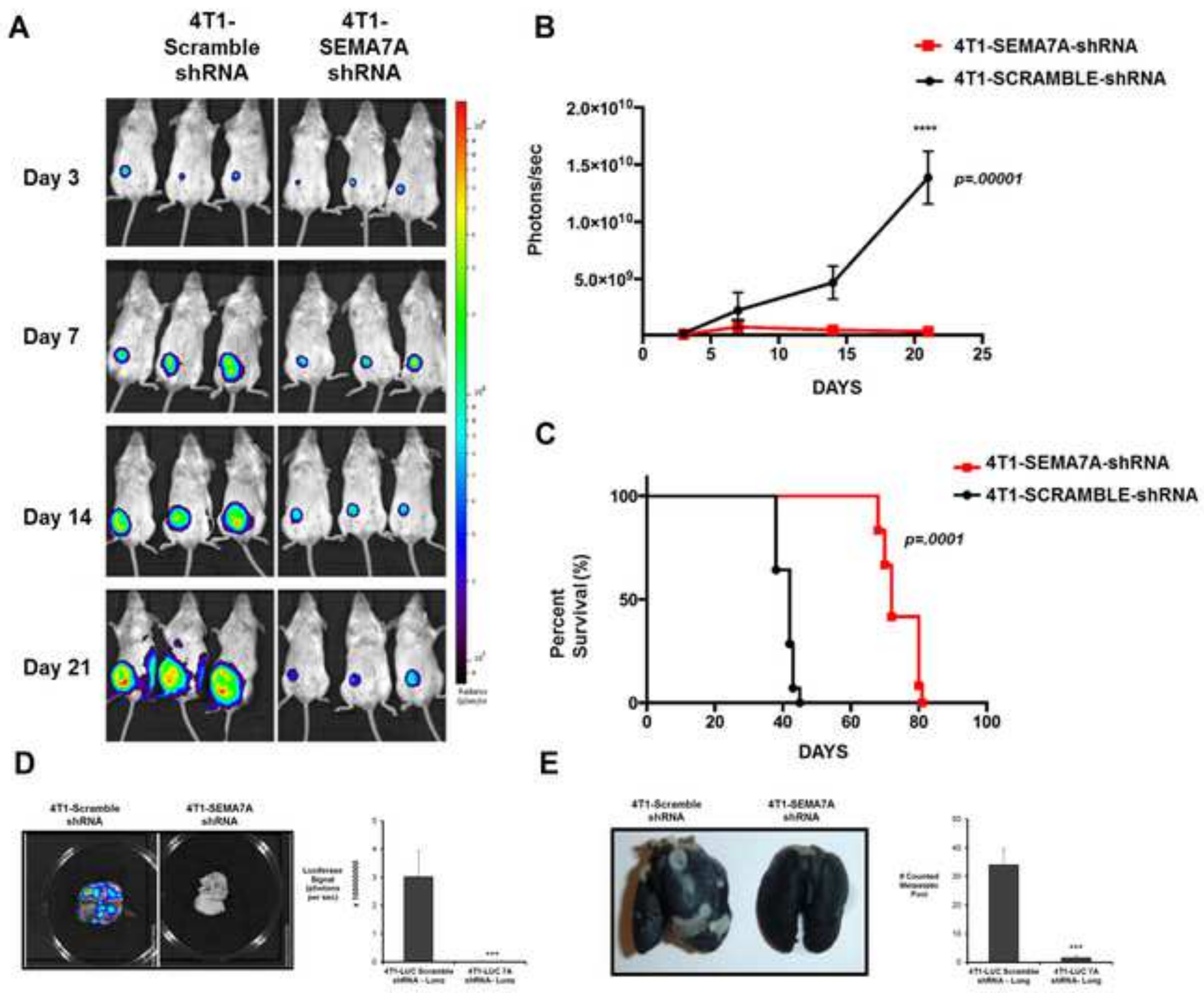

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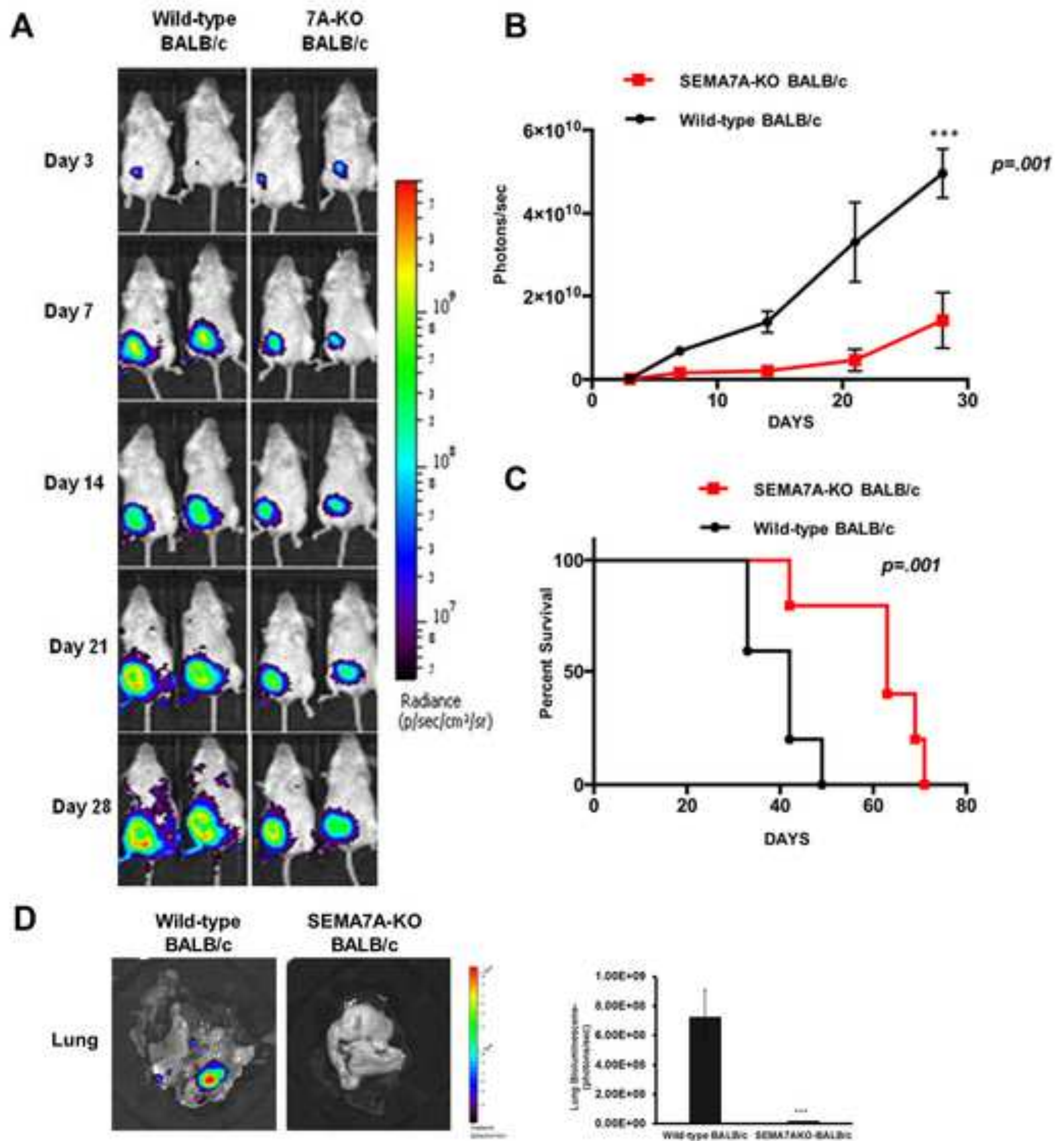

Figure 9

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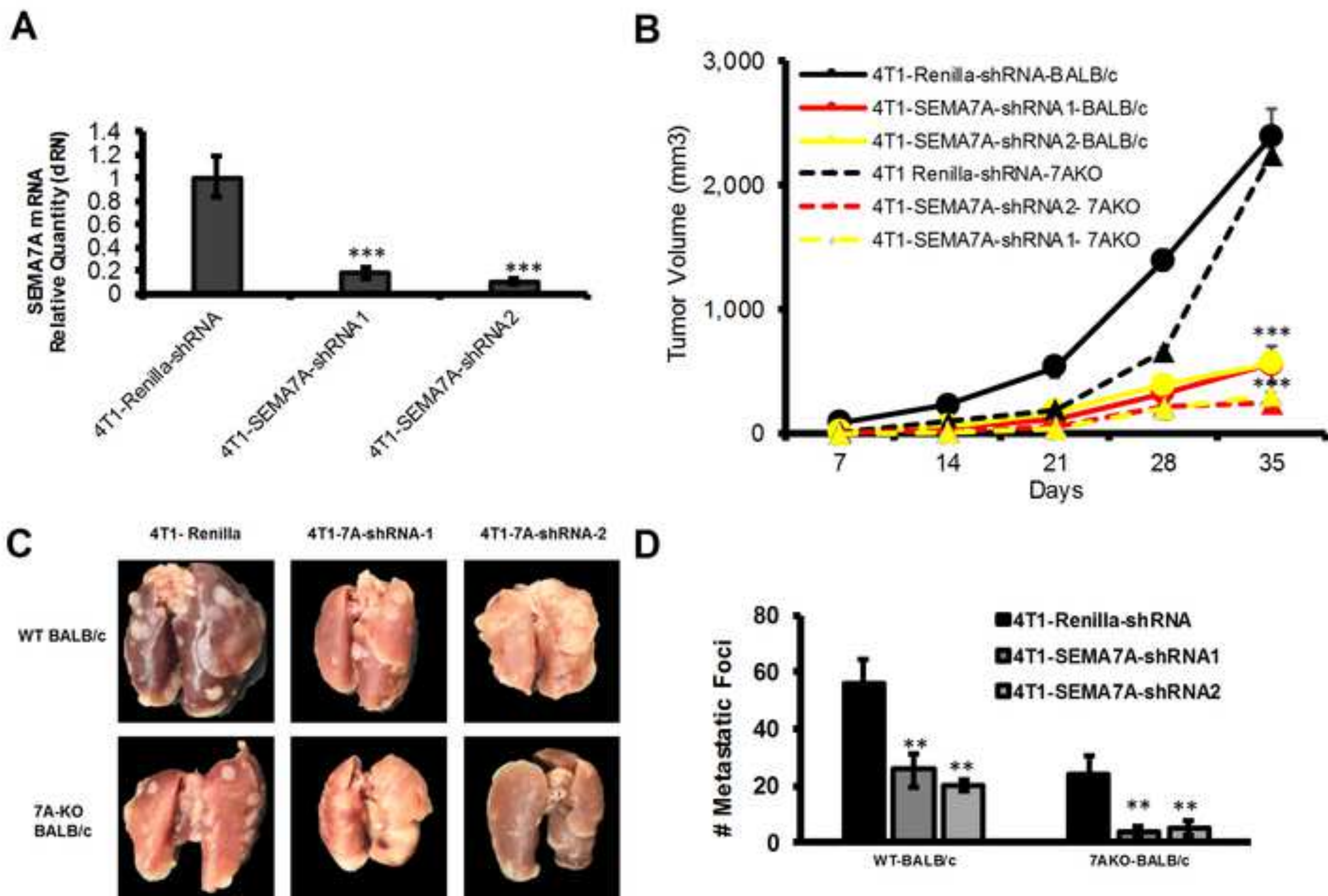
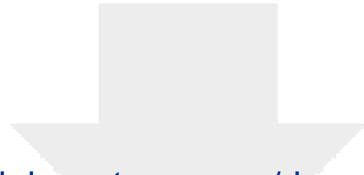


Supplemental 1



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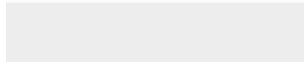
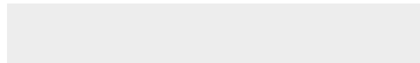
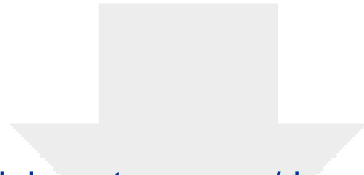




Supplemental 2



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