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Stromal extracellular matrix is a microenvironmental cue promoting resistance to EGFR tyrosine kinase inhibitors in lung cancer cells

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ABSTRACT

The acquisition of resistance to EGFR tyrosine kinase inhibitors (TKIs) remains a critical problem in lung cancer clinic, but the underlying mechanisms have remained incompletely understood. Although the TKI-induced or –selected genetic changes are known to drive resistance, resistance also occurs in tumor cells without genetic changes through poorly-characterized processes. Here, we show that the extracellular matrix (ECM) from various components of the tumor microenvironment, including neighboring tumor cells and fibroblasts, may be the driver of resistance in the absence of genetic changes. Unlike genetic changes, which may evolve during relatively long time of chronic EGFR TKI treatment to drive resistance, briefly culturing on de-cellularized ECM, or co-culturing with the ECM donor cells, immediately confers resistance to tumor cells that are otherwise sensitive to EGFR TKIs. We show evidence that collagen in the ECM may be its primary constituent driving resistance, at least partly through the collagen receptor Integrin-β1. Intriguingly, such effect of ECM and collagen is dose-dependent and reversible, suggesting a potential clinic-relevant application for targeting this effect. Collectively, our results reveal that the stromal ECM acts as a microenvironmental cue promoting EGFR TKI resistance in lung cancer cells, and targeting collagen and Integrin-β1 may be useful for treating resistance, especially the resistance without clearly-defined genetic changes, for which effective therapeutics are lacking.

1. Introduction

Lung cancer is the deadliest human malignancy worldwide and causes ~1.5 million deaths each year (according to World Health Organization statistics). About 10–30% of lung cancers express mutations of EGFR, a receptor tyrosine kinase gene and one of the most frequently mutated in lung cancer. About 90% of EGFR mutant patients express $EGFR^{L858R}$ or exon 19 deletion EGFR mutations, both of which result in constitutive activation of EGFR and downstream oncogenic signaling to promote tumorigenesis (Arteaga and Engelman, 2014; Tan et al., 2016; Engelman and Jänne, 2008; Camidge et al., 2014; Blakely and Bivona, 2012; Wang et al., 2016; Campo et al., 2016; Levy et al., 2016; Shi et al., 2017). Since lung cancer cells expressing these mutations are addictive to EGFR receptor tyrosine kinase activity, EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib, erlotinib, and osimertinib are clinically effective in treating EGFR mutant lung cancer. However, the efficacy of these drugs is transient, and the acquired resistance inevitably and quickly develops.

In some patients, resistance is driven by new EGFR mutations: about 50–60% of patients who acquire resistance to older generation EGFR TKIs (e.g. erlotinib and gefitinib) have T790 M mutation, which drives resistance by affecting the EGFR ATP binding pocket (Arteaga and Engelman, 2014; Tan et al., 2016; Engelman and Jänne, 2008; Camidge et al., 2014; Blakely and Bivona, 2012; Wang et al., 2016; Campo et al., 2016; Levy et al., 2016; Shi et al., 2017); and 5–30% of acquired resistance to new generation EGFR TKIs (e.g. osimertinib) is driven by C797S mutation (Shi et al., 2017; Jia et al., 2016; Patel et al., 2017; Wang et al., 2017). Such “new EGFR mutations”-driven resistance may be treated with newer TKIs that selectively inhibit T790M or C797S. For instance, osimertinib selectively inhibits T790M and has been approved by FDA for treating T790M-positive resistance; and TKIs that selectively inhibit C797S are under development for treating C797S-positive

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resistance (Jia et al., 2016; Patel et al., 2017; Wang et al., 2017).

For patients who have acquired resistance but do not have new EGFR mutations, the underlying mechanism is not fully understood and curative therapeutics are lacking. Notably, recent studies have shown an emerging role for the extracellular matrix (ECM) in cancer malignant progression and resistance to targeted therapies (Davies and Albeck, 2018; Belli et al., 2018; Theocharis and Karamanos, 2017; Senthebane et al., 2017). In the present study, we have investigated a novel effect for ECM acting as a microenvironmental cue driving the acquisition of resistance to EGFR TKIs. Our results suggest that this pro-oncogenic effect of ECM is at least partly mediated by collagen, the major ECM constituent, and the collagen receptor Integrin-β1. Taken together, these results warrant future studies testing whether this new resistance-driving mechanism is useful for developing more effective interventional strategies for treating EGFR mutant lung cancer patients who acquire EGFR TKI resistance in the absence of new EGFR mutations.

2. Results

2.1. ECM confers reversible EGFR TKI resistance to EGFR mutant lung cancer cells

To explore a potential role for ECM in the acquisition of resistance to EGFR TKIs, we prepared de-cellularized ECM monolayers from EGFR mutant HCC827 lung cancer cells using a methodology described previously (Beacham et al., 2007; Liu et al., 2015). Then, we seeded a TKI-sensitive EGFR mutant lung cancer cell line, HCC4006 (Zhang et al., 2016), on these monolayers, followed by treating the cells with various concentrations of gefitinib (Fig. 1A). Surprisingly, such brief culture on the ECM immediately confers resistance to HCC4006 cells to gefitinib to an extent comparable to that of HCC4006GR cells (Fig. 1B), an EGFR TKI-resistant sub-clone we generated by chronically treating parental HCC4006 cells with high concentrations (0.1–1 μM) of gefitinib for months (Zhang et al., 2016). Notably, stronger resistance-driving ability of ECM could be detected when it was prepared from more cells (Fig. 1C), exhibiting a dose-dependent effect.

To examine whether this observation is specific to the ECM from HCC827 cells, we also prepared ECM from other lung cancer cell lines, including H1975 and 393 P (Zhang et al., 2016). Similar to the effect of HCC827 ECM, ECM from both H1975 and 393 P cells also promoted resistance (Fig. 1D, E), suggesting that common component(s) of the ECM from distinct donor cells drives resistance. When HCC4006 cells were trypsinized after culturing on the ECM and re-seeded on plastic plates without ECM, they completely regained the sensitivity to gefitinib (Fig. 1F), suggesting that the ECM-driven resistance is reversible.

2.2. Cell density does not affect resistance

Under certain circumstances, it is known that cell growth can be affected when cells are cultured on ECM components, such as collagen (Tong et al., 2018; Senoo and Hata, 1994; Nakanuma et al., 1997). To preclude a possibility that changes in cell growth regulate the acquisition of resistance, we grew HCC4006 and H1975 cells to various densities and treated them with gefitinib or osimertinib, respectively. The results show that the densities of the cells had no effect on their responsiveness to the drugs in both cases (Fig. 2A–B). In addition, distinct sizes of HCC4006 cell spheres formed on matrigel also responded to gefitinib treatment similarly (Fig. 2C–D), further supporting that the cell density or relative growth status does not affect resistance.

It should be noted that higher cell density in above experiments should associate with the presence of more ECM in the cell culture. This raises an interesting question - why such increase of ECM did not promote resistance? Given that ECM is detached and degraded during cell death (Mason et al., 2017; Lei et al., 1996; Meredith and Schwartz, 1997), we suspect that the resistance-driving function of ECM depends on the viability of the cells. In other words, if the cells are sensitive to the TKIs, it is likely that their ECM will not generate resistance to the acute EGFR TKI treatment in our experiments due to the cell death-associated quick loss of function (detached or degraded). If this is true, then co-culturing with cells that are not responding to the TKIs, therefore can provide functional ECM, should be able to promote resistance in tumor cells that are otherwise sensitive to the TKIs.

2.3. Fibroblasts, or tumor cells that are resistant to EGFR TKIs, promote resistance in neighboring tumor cells

To test this bold hypothesis, we labeled the TKI-sensitive HCC827 cells with GFP and co-cultured them with parental HCC827 cells (as the control), or with H1975 and 393 P cells, which are resistant to gefitinib (Fig. 3A) due to the expression of $EGFR^{T790M}$ or $KRAS^{G12D}$ (Zhang et al., 2016; Gibbons et al., 2009), respectively. Then, we treated these co-cultured cells with various concentrations of gefitinib (Fig. 3B). In support of our hypothesis, co-culturing with the parental HCC827 cells failed, but co-culturing with H1975 or 393 P cells significantly promoted, resistance of the GFP-labeled HCC827 cells (Fig. 3C).

To further validate our hypothesis, we also labeled H1975 cells with GFP and examined the effects of fibroblasts, which have been shown to be part of the tumor stroma, be resistant to EGFR TKIs, and promote the acquisition of EGFR TKI resistance (Yi et al., 2018; Ishibashi et al., 2017; Choe et al., 2015). Consistent with these reports and our above results, co-culturing H1975 with fibroblasts (Swiss3T3, IMR-90, and MRC5, Andrianifahanana et al., 2016; Kang et al., 2017), or briefly culturing H1975 cells on de-cellularized ECM from these fibroblasts dramatically promoted resistance to osimertinib treatment (Fig. 4A–C).

2.4. Collagen mediates the ECM-driven resistance through Integrin- β1

Collagen is the major constituent of ECM and has been shown to associate with malignant progression and resistance to cancer therapies (Naci et al., 2015; Kharaishvili et al., 2014; Chen et al., 2013). In support of a role for collagen mediating the ECM-driven resistance, culturing the cells on purified collagen I was sufficient to promote the acquisition of resistance to EGFR TKIs (Fig. 5A–B). Similar to the effect of ECM, collagen I also dose-dependently promoted resistance (Fig. 5C). Remarkably, knockdown of Integrin- β1, a subunit of Integrin complexes that serve as collagen receptors, partially but significantly suppressed the resistance driven by both collagen I and de-cellularized ECM (Fig. 5D–F), suggesting that collagen and Integrin- β1 at least partly mediate the resistance-driving function of ECM and may be useful targets for developing interventional strategies. In further support of this idea, a collagen synthesis inhibitor, CHP (cis-4-Hydroxy-L-proline), effectively inhibited collagen production (Fig. 6A) and synergized with osimertinib to suppress the growth of GFP-labeled H1975 cells co-cultured with parental H1975 cells or fibroblasts (Fig. 6B).

To explore a potential role for the basal Integrin- β1 expression in the regulation of resistance, we have generated multiple EGFR inhibitor-resistant sublines from parental EGFR mutant lung cancer cell lines, including HCC827GR, HCC827ER, HCC4006GR, and H1975OR. The suffix “GR”, “ER”, and “OR” indicate that the sublines were generated by chronically treating the parental cells with gefitinib, erlotinib, or osimertinib, respectively. By performing Western blotting, we found that the Integrin- β1 expression levels were not associated with resistance to EGFR inhibitors (Fig. 5G). For instance, HCC4006GR cells expressed more Integrin- β1 compared to parental HCC4006 cells, but H1975OR cells expressed less Integrin- β1 compared to parental H1975 cells, suggesting that the interaction between ECM/collagen and Integrin- β1 plays a more critical role in the acquisition of resistance than the basal expression of Integrin- β1 does. As such, it would be interesting for future studies to determine whether common signaling pathway(s) downstream of Integrin beta 1 is activated in response to ECM/collagen to drive the resistance phenotype.

To validate our findings from cell culture experiments, we

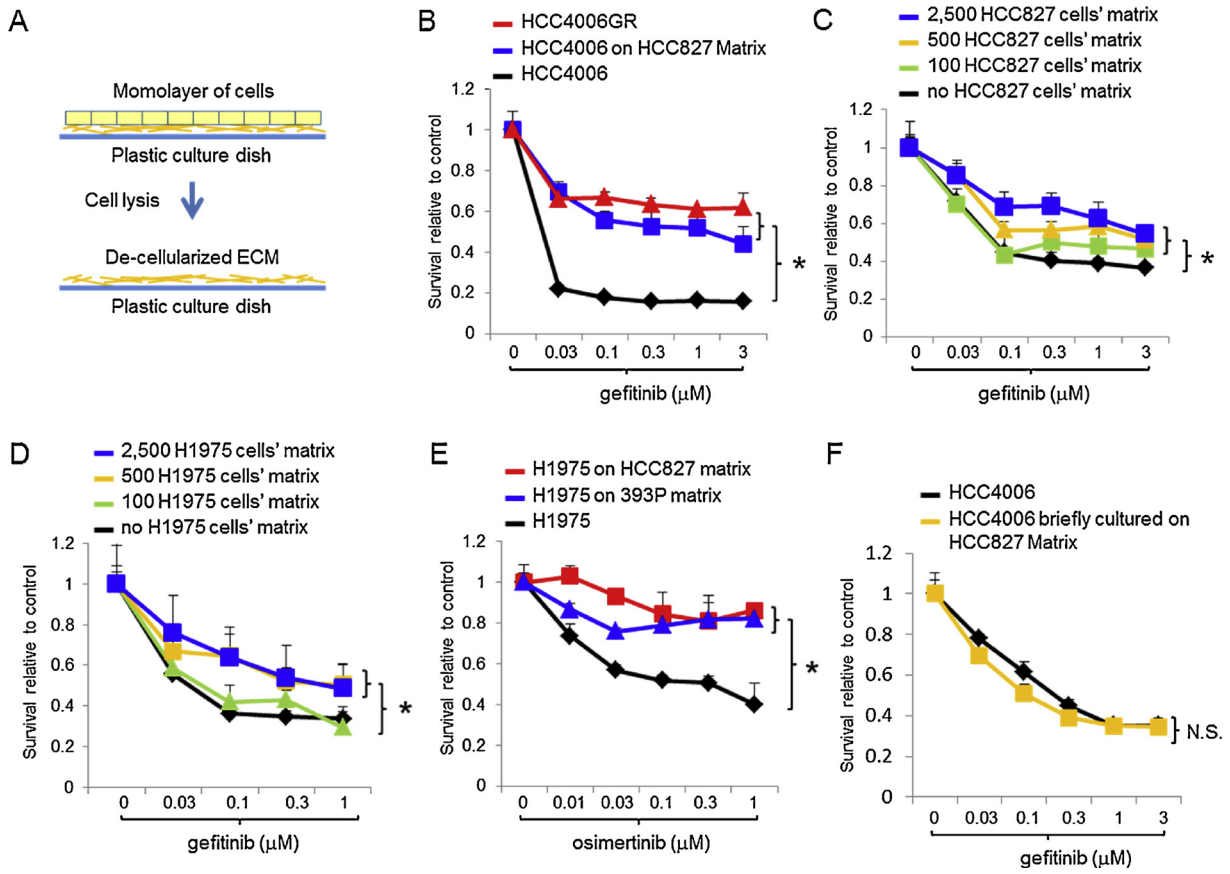

Fig. 1. ECM promotes reversible resistance to EGFR TKIs.
(A) Schemic for the preparation of de-cellularized ECM from tumor cell monolayer grown on plastic culture dish. (B) HCC4006 cells grown on plastic culture dish or de-cellularized HCC827 ECM, or HCC4006GR cells grown on plastic culture dish, were treated with various concentrations of gefitinib as indicated for 3 days. Cell survival was quantitated by MTT assay and normalized to that of vehicle (DMSO)-treated cells (set as 1.0). (C, D) HCC4006 cells grown on de-cellularized ECM from various numbers of HCC827 (C) or H1975 cells (D) were treated with various concentrations of gefitinib as indicated for 3 days. Cell survival was quantitated by MTT assay and normalized to that of vehicle (DMSO)-treated cells (set as 1.0). (E) H1975 cells grown on plastic culture dish or de-cullularized ECM from HCC827 or 393 P cells were treated with various concentrations of osimertinib as indicated for three days. Cell survival was quantitated by MTT assay and normalized to that of vehicle (DMSO)-treated cells (set as 1.0). (F) HCC4006 cells were, or were not, briefly cultured on de-cellularized ECM from HCC827 cells, then trypsinized and re-plated on plastic culture dishes, followed by treatment with various concentrations of gefitinib as indicated for three days. Cell survival was quantitated by MTT assay and normalized to that of vehicle (DMSO)-treated cells (set as 1.0). Note: * indicates *t*-test $p < 0.05$ in all figures.

continuously treated HCC827 subcutaneous xenograft tumors with erlotinib for 6 weeks until they acquired resistance. By performing sirius red/fast green staining and quantitation, we found that collagen was significantly increased in the erlotinib-resistant tumors compared to the treatment naïve tumors (Fig. 7A–B), suggesting that the increased collagen in the tumor stroma contributes to the acquisition of resistance in vivo.

2.5. Collagen and ECM promote resistance independently of cell adhesion

Notably, our above results show that seeding cells on collagen or ECM from a variety of cell lines similarly promotes resistance, leading us to test whether the collagen and ECM coating commonly regulate the sensitivity of cells to EGFR inhibitor by providing an attachment support to cells. Our results show that seeding cells on both collagen and laminin, another critical ECM constituent, increased cell adhesion/attachment capacities (Fig. 8A–C). However, while collagen coating consistently promoted resistance (Fig. 8D), laminin coating completely had no effect (Fig. 8E). Therefore, it is unlikely that ECM or collagen promote resistance by providing an attachment support to cells.

Moreover, we tested the cell adhesion capacities under both non-coated condition and ECM/collagen/laminin-coated conditions. Compared to the non-coated condition (culturing cells on regular plastic cell culture plate), our results show that seeding on ECM from fibroblasts (Swiss3T3) or some of the tumor cells (HCC827 and HCC4006) increased the cell adhesion capacity (Fig. 9). However, seeding on ECM from H1975 tumor cells did not affect the adhesion capacity (Fig. 9). Because ECM from all of these cells promoted resistance (Figs. 1 and 4), these results suggest that ECM does not promote cell adhesion capacities to drive resistance. Interestingly, although seeding on either collagen or laminin enhanced cell adhesion capacities (Fig. 8A–C), only collagen but not laminin promoted resistance (Fig. 8D and E), again suggesting that the resistance-driving function of ECM is likely independent of cell adhesion.

3. Discussion

Acquired resistance to EGFR TKIs in EGFR-mutated patients remains a critical problem in lung cancer clinic. The identification of new (or call secondary) EGFR mutations that drive the acquisition of resistance, such as T790M and C797S mutations, have had significant impact on both our understanding of the biology of resistance and the clinical practice. However, for patients who do not have these new mutations, effective treatment options are limited. Therefore, there is a need for identifying resistance drivers of such resistance to develop interventional strategies. In the present study, we provide evidence that ECM acts through its component collagen and Integrin-β1 to drive such resistance (Figs. 1 and 5), that inhibiting collagen synthesis reverses

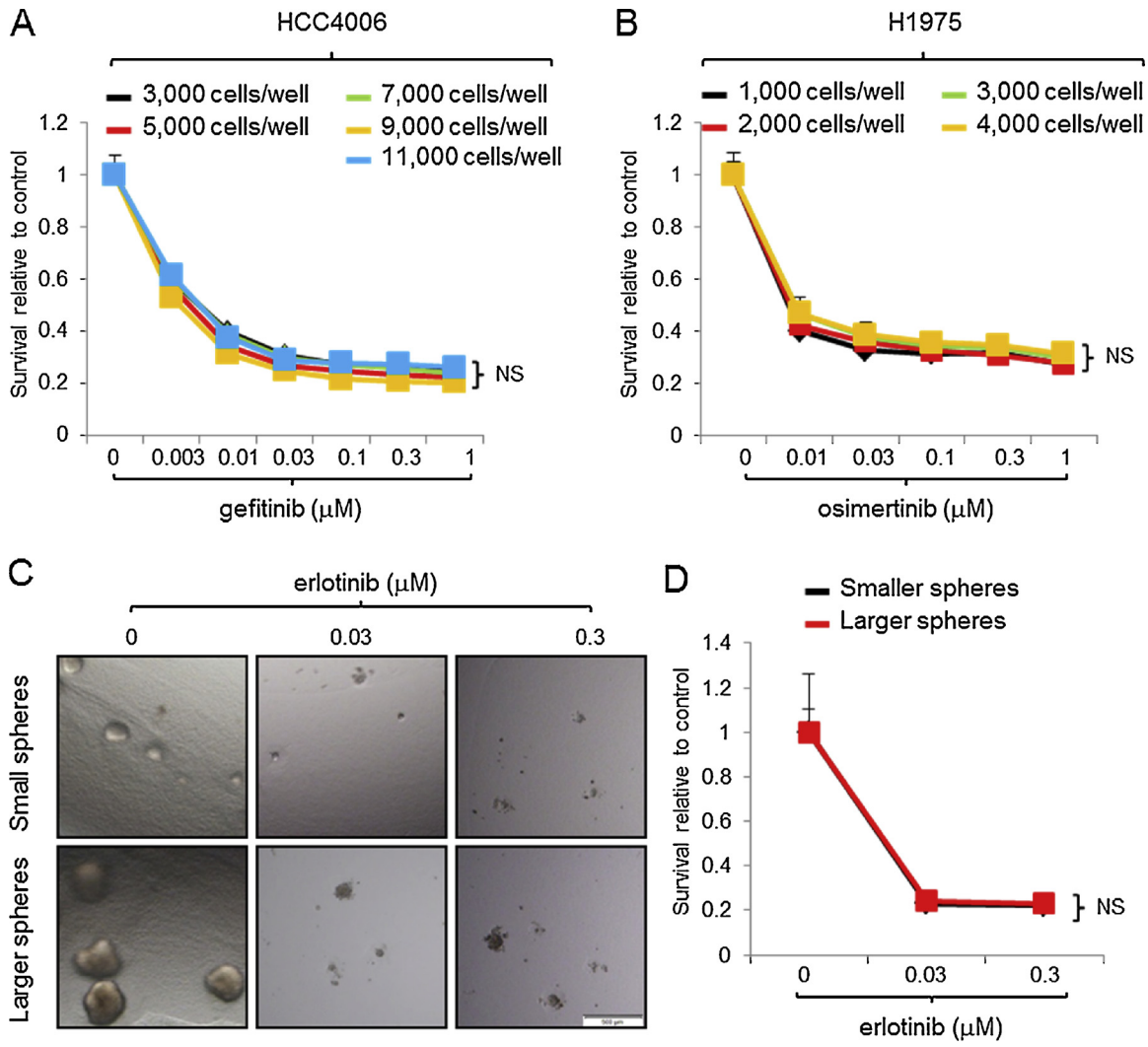

Fig. 2. Cell density does not affect resistance.
(A) HCC4006 cells were plated at increasing densities as indicated on plastic culture dishes and treated with various concentrations of gefitinib as indicated for three days. Cell survival was quantitated by MTT assay and normalized to that of vehicle (DMSO)-treated cells (set as 1.0). (B) H1975 cells were plated at increasing densities as indicated on plastic culture dishes and treated with various concentrations of osimertinib as indicated for three days. Cell survival was quantitated by MTT assay and normalized to that of vehicle (DMSO)-treated cells (set as 1.0). (C) Light microscopic photos for distinct sizes of HCC827 spheres grown on matrigel and treated with DMSO or erlotinib (0.03 and 0.3 μm) as indicated for five days. Bar: 200 μm. (D) Cell survival in (C) were quantitated by MTT assay and normalized to that of vehicle (DMSO)-treated cells (set as 1.0). Note: In all figures, * indicates *t*-test $p < 0.05$, and "NS" indicates *t*-test $p > 0.05$ (not statistically significant).

resistance (Fig. 6), and that collagen production is increased in EGFR TKI-resistant tumors (Fig. 7), providing a rationale for future studies testing the clinical efficacy of collagen/Integrin inhibitors.

For resistance without new EGFR mutations, previous studies have identified several candidate resistance-driving mechanisms. For instance, a list of non-EGFR gene mutations, overexpression, activation, or amplification (e.g. PI3K, BRAF, ERBB2, c-MET, AXL, TGF-β, and etc.) have been identified in patient tissues as well as in preclinical models, where these changes may drive EGFR bypassing signaling to enable the cells to escape from EGFR TKI treatment; epithelial-mesenchymal transition (EMT) and non-small-cell to small-cell cell lung cancer histology transition have also been shown to cause resistance in a subset of patients (Arteaga and Engelman, 2014; Tan et al., 2016; Engelman and Jänne, 2008; Camidge et al., 2014; Blakely and Bivona, 2012; Wang et al., 2016; Campo et al., 2016; Levy et al., 2016; Shi et al., 2017). Notably, ECM and collagen are known to regulate, and/or be regulated by, pathways associated with most if not all of the above changes. For instance, PI3K, AXL, c-MET, BRAF, and TGF-β signaling has been implicated in regulating the expression and production of collagen (Dooley et al., 2012; Sun et al., 2018; Ishikura-Kinoshita et al., 2012; Chen et al., 2016; Hutchison et al., 2010; Ghatak et al., 2014; Jolly et al., 2016); EMT has been shown to promote ECM remodeling through mechanisms involving the switch of expression patterns of collagen subtypes (Rajesh et al., 2017; Venning et al., 2015); on the other hand, collagen and ECM have also been reported to regulate ERBB2, PI3K, c-MET, AXL, and TGF-β (Cheng et al., 2016; Liu et al., 2018; Jokela et al., 2018; Liu et al., 2018 May; McCall-Culbreath et al., 2008). Therefore, it is highly likely that the resistance-driving role for ECM and collagen presented in this study is not completely independent of previous identified mechanisms in the field. Rather, these molecules should be part of an integrated signaling network that drives resistance. It would be interesting for future studies to determine whether they commonly associate with the acquisition of resistance, or specifically with resistance driven by certain types of gene mutation/expression/activity changes.

ECM is a significant component of tumor stroma and can be deposited by essentially all cell types in the tumor microenvironment. Our results (Figs. 1, 3 and 4) show that ECM from both tumor cells and fibroblasts similarly promotes resistance in neighboring tumor cells that are otherwise sensitive to EGFR TKIs, suggesting that the stromal ECM

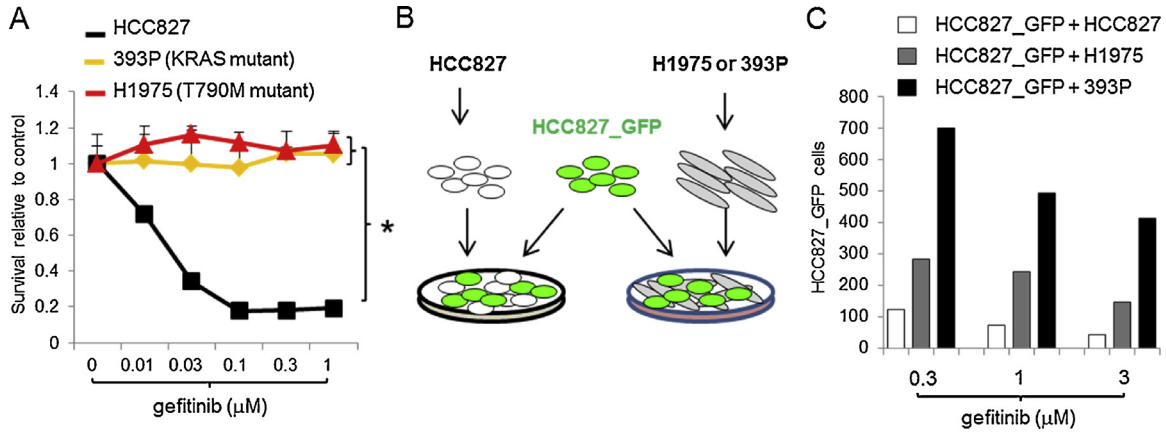

Fig. 3. EGFR TKI-resistant tumor cells promote resistance in neighboring tumor cells.
(A) HCC827, 393 P, and H1975 cells were plated on plastic culture dishes and treated with various concentrations of gefitinib as indicated. Cell survival was quantitated by MTT assay and normalized to that of vehicle (DMSO)-treated cells (set as 1.0). Note: * indicates *t*-test $p < 0.05$. (B) Schematic for co-culture experiment. GFP-transfected HCC827 cells (HCC827_GFP) were mixed with parental HCC827, 1975, or 393 P (1:1 ratio in all cases) and plated on plastic culture dishes. (C) Cell co-cultures shown in (B) were treated with various concentrations of gefitinib as indicated for five days, and survived HCC827_GFP cells were quantitated by flow cytometry analyses.

from distinct donor cell types collectively functions as a micro-environmental cue to promote resistance. Remarkably, our results show that the responsiveness to EGFR TKIs correlates with the availability of functional ECM in the presence of the TKIs (Figs. 2–4), suggesting a model in which the TKI-resistant cells, such as fibroblasts and tumor cells that have acquired resistance, provide ECM to promote resistance in the neighboring tumor cells through the collagen-Integrin interactions (Fig. 10). It should be noted that our results do not preclude the possibility that changes in the ECM may also promote the resistance in the TKI-sensitive cells, which would be an interesting direction for future studies.

4. Materials and methods

4.1. Cell lines, materials, and reagents

Human lung cancer cell lines, including HCC4006, HCC827, and H1975 were from ATCC. To generate EGFR TKI-resistant sublines, including HCC4006GR, HCC827GR, HCC827ER, and H1975OR, the parental cells were continuously treated with 1 μM gefitinb or erlotinib or osimertinib for 10–12 weeks. The suffix "GR", "ER", or "OR" indicates the sublines were generated by treaing with gefitinib, erlotinib, or osimertinib, respectively. Mouse 393 P lung cancer cell line was a gift from Jonathan Kurie MD (University of Texas MD Anderson Cancer Center). Swiss3T3, IMR-90, and MRC5 fibroblast cell lines were gifts from Edward Leof Ph.D. (Mayo Clinic). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was from Sigma. Gefitinib was from SelleckChem. Osimertinib was from LC laboratories. Erlotinib was a gift from Julian Molina MD (Mayo Clinic). CHP (cis-4-Hydroxy-L-proline) was from Sigma. Sirius red collagen detection kit and Sirius red/fast green collagen staining kit were from Chondrex. Plasticwares and lab consumables were from Fisher Scientific. Pathogen-free and growth factor-reduced matrigel solution was from BD Biosciences. Gibco-branded fetal bovine serum (FBS) was from Invitrogen. SiRNAs were from Santa Cruz. Rabbit monoclonal Integrin-β1 and Tubulin antibodies were from Cell Signaling. All other chemicals and reagents were from Sigma unless specifically indicated.

4.2. Cell culture and transfection

Lung cancer cell lines and fibroblasts were cultured in RPMI-1640 or DMEM medium (both from MediaTech) supplemented with 10% FBS, respectively. All cells were maintained at 37° in 5% CO2 humidified incubators. Transient transfection of siRNAs or stable transfection of GFP (pGIPZ GFP vector from Open Biosystems) was performed by using RNAiMAX or Lipofectamine2000 transfection reagents (Invitrogen), respectively, as described by the manufacturer.

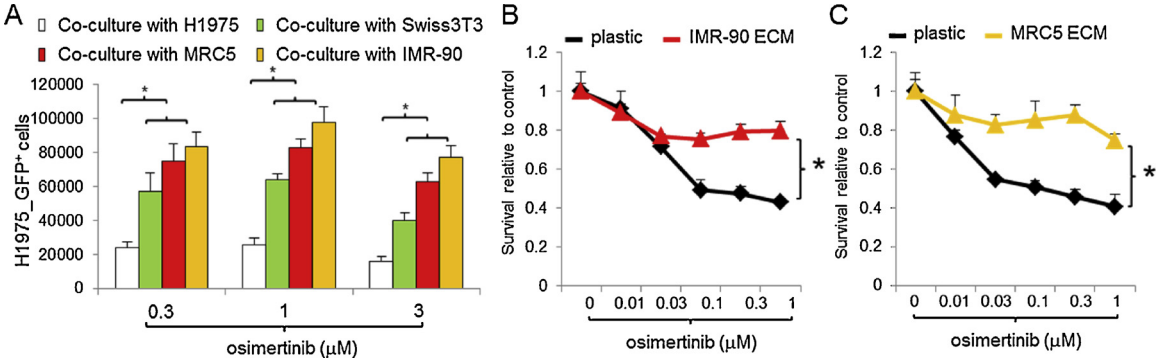

Fig. 4. Fibroblasts and their ECM promote resistance in neighboring tumor cells.
(A) GFP-transfected H1975 (H1975_GFP) cells were co-cultured with parental H1975 cells or with fibroblasts (Swiss3T3, MRC5, and IMR-90) and treated with various concentrations of osimertinib as indicated for five days. Survived H1975_GFP cells were quantitated by flow cytometry analyses. (B, C) H1975 cells cultured on plastic dishes or de-cellularized ECM from fibroblasts and treated with various concentrations of osimertinib for three days. Cell survival was quantitated by MTT assay and normalized to that of vehicle (DMSO)-treated cells (set as 1.0). Note: * indicates *t*-test $p < 0.05$ in all figures.

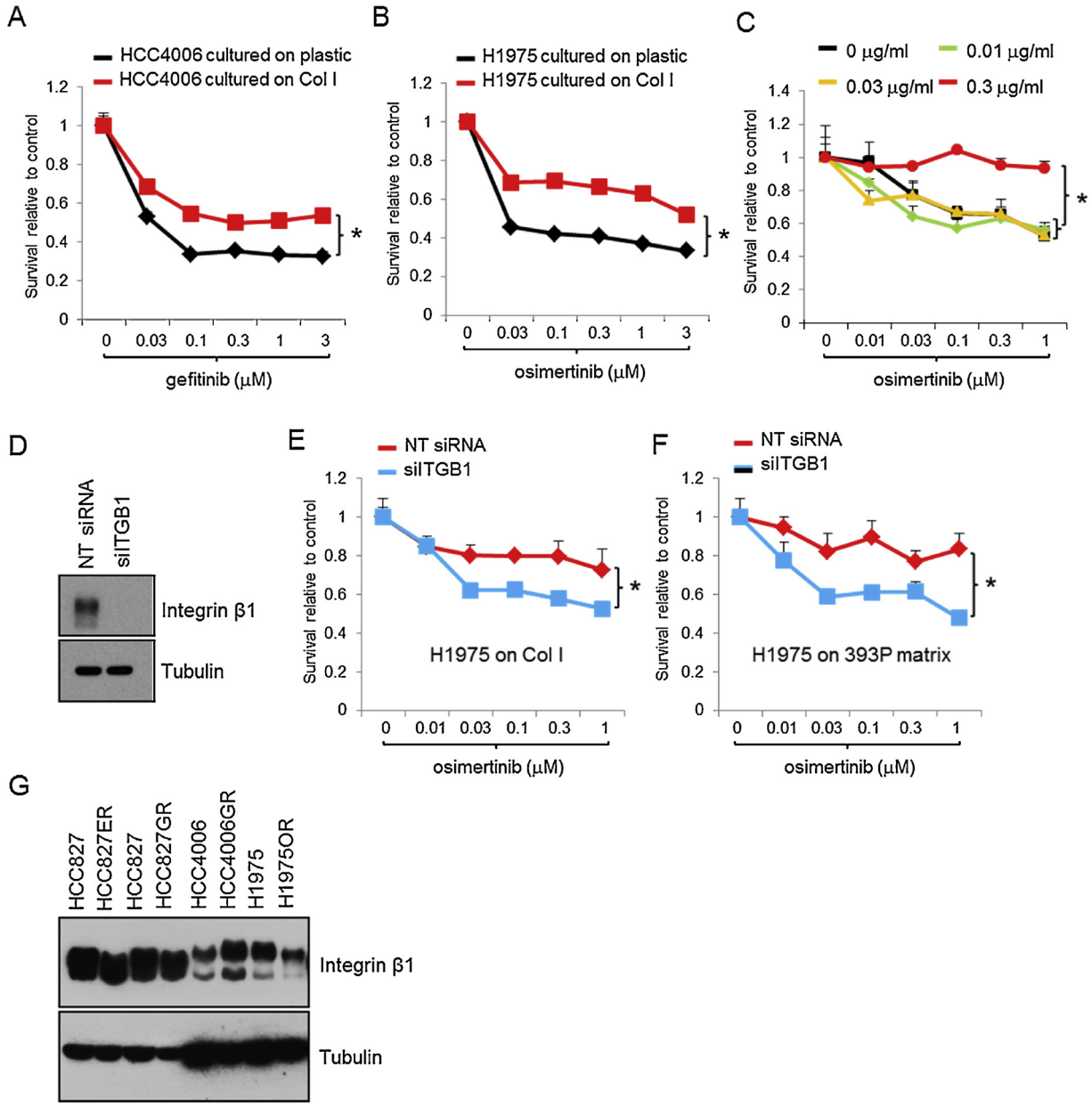

Fig. 5. Collagen and Integrin-β1 promote resistance.
(A, B) HCC4006 (A) or H1975 (B) cells were cultured on plastic dishes coated with or without collagen I (Col I, 0.1 μg/ml) and treated with various concentrations of gefitinib (A) or osimertinib (B) as indicated for three days. Cell survival was quantitated by MTT assay and normalized to that of vehicle (DMSO)-treated cells (set as 1.0). (C) H1975 cells were plated on plastic dishes coated with increasing concentrations of Col I and treated with various concentrations of osimertinib as indicated for three days. Cell survival was quantitated by MTT assay and normalized to that of vehicle (DMSO)-treated cells (set as 1.0). (D) Western blots for H1975 cells transfected with non-targeting (NT) control siRNA or Integrin-β1 (ITGB1) siRNA. (E, F) H1975 cells were transfected with NT or ITGB1 siRNAs and cultured on Col I-coated plastic dishes or de-cellularized ECM from 393 P cells. Then, the cells were treated with various concentrations of osimertinib as indicated for three days. Cell survival was quantitated by MTT assay and normalized to that of vehicle (DMSO)-treated cells (set as 1.0). (G) Western blots for EGFR mutant lung cancer cell lines and their sublines that are resistant to EGFR TKIs. Note: * indicates *t*-test $p < 0.05$ in all figures.

4.3. Matrigel tumor cell sphere culture

Matrigel monolayers were prepared by evenly coating the chamber of 96-well plate with 10ul cold phenol red free and grow factor reduced matrigel solution. The coated plates were covered and incubated at 37° for 30 min to allow the gel formation, followed by covering each chamber with 50 μl warm RPMI-1640 culture medium containing 10% FBS and 2% matrigel. Lastly, 400 tumor cells suspended in 50 μl RPMI-1640 culture medium containing 10% FBS were plated on the top in each chamber. The light photos for matrigel tumor cell spheres were captured by using an Olympus IX-81 inverted microscope.

4.4. Preparation of de-cellularized ECM

The de-cellularized ECM monolayers were prepared according to the published protocols (Beacham et al., 2007; Liu et al., 2015) with minor modifications. Briefly, the cell monolayers growing on the plastic culture dishes were rinsed by phosphate saline buffer (PBS) thoroughly and exposed to de-cellularization buffer containing PBS supplemented with 2% NH4OH and 0.5% Triton X-100 at 37 °C for at least 30 min until no intact cells were visible under a microscope. Then, the de-cellularization buffer was vacuumed and the resulted ECM was rinsed with PBS for three times and incubated at 4 ° overnight before being

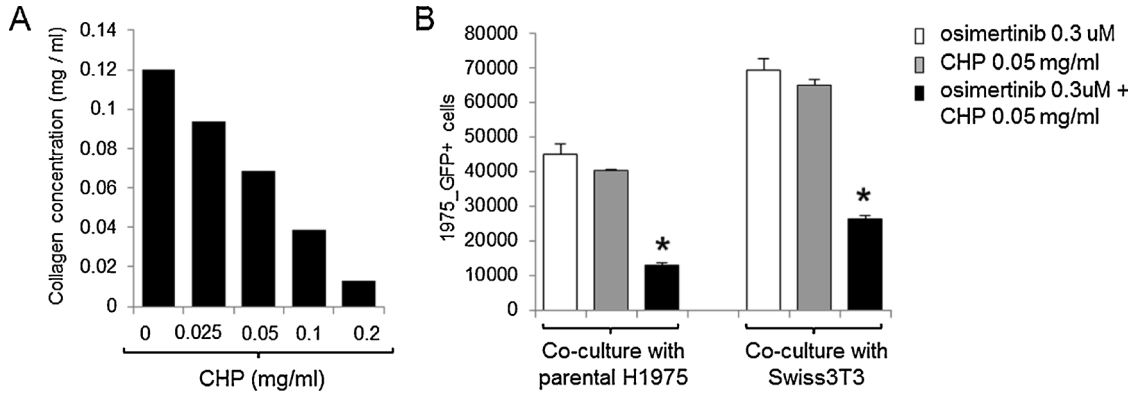

Fig. 6. Collagen synthesis inhibitor suppresses resistance.
(A) Quantitation of collagen amounts in the cell lysates from Swiss3T3 cells treated with various concentrations of CHP for three days. (B) GFP-transfected H1975 cells (H1975_GFP) were co-cultured with parental H1975 cells or Swiss3T3 fibroblasts and treated with osimertinib and CHP, individually or in combination as indicated, for five days. Survived H1975_GFP cells were quantitated by flow cytometry analyses.

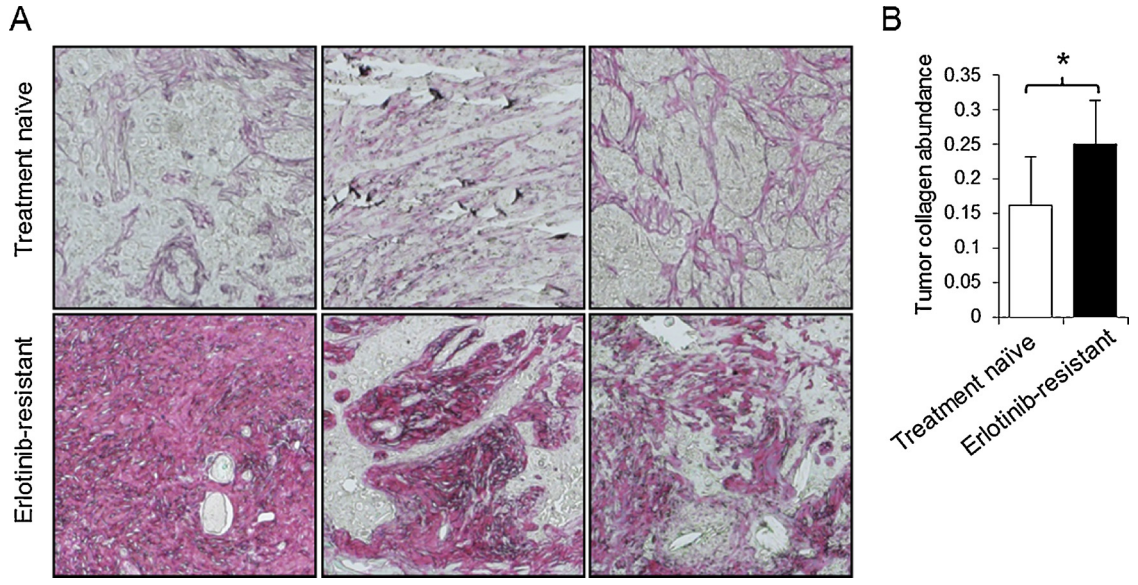

Fig. 7. Increased collagen production associates with the acquisition of resistance to erlotinib.
(A) Representative images for Sirius red/fast green staining of HCC827 subcutaneous xenograft tumors that were not treated (treatment naïve, n = 3) or treated with 50 mg/kg/day erlotinib for 6 weeks and had acquired resistance (erlotinib-resistant, n = 6). (B) Quantitation of collagen in treatment naïve and erlotinib-resistant tumors as described in (A). * indicates *t*-test $p < 0.05$.

used for cell culture experiments.

4.5. MTT assay

Cells were seeded on 96-well plates and treated as indicated in the figures. At the end of treatment, cells were incubated with 1 mg/ml MTT at 37° for two hours, and cell viability was determined by measuring the optical absorbance at 570 nm.

4.6. Flow cytometry

Flow cytometry was performed to determine the viability of GFP-labeled tumor cells in co-culture with other tumor cells (not GFP-labeled) or fibroblasts. Briefly, cell co-cultures were first treated as indicated in figures. AT the end of treatment, cells were trypsinized into single cells, washed, and suspended in PBS. GFP positive cells were quantitated by using the Attune NxT flow cytometer (Thermo Fisher Scientific) and analyzed by flowjo v10 software (FLOWJO, LLC).

4.7. Collagen assay

For cultured cells, total collagen was quantitated by using a Sirius Red Collagen Detection Kit (Chondrex) as described by the manufacturer. Briefly, cells were plated on 6-well plates were treated with various concentrations of CHP as indicated in Fig. 6 for three days. At the end of treatment, cells were rinsed with ice cold distilled water, scraped into 0.05 M acetic acid solution, and pipetted thoroughly. The resulted lysates were then mixed with Sirius red solution, and the concentration of total collagen was determined by measuring the optical absorbance at 530 nm.

To generate erlotinib resistant subcutaneous xenograft tumors, HCC827 cells were subcutaneously injected into the flanks of 10 weeks old nude mice (1 million of cells were suspended in 100 ul of PBS for each injection). One week after the injection, the tumor bearing mice were treated with erlotinib (50 mg/kg/day, 5 times a week) by gavage for another 6 weeks. Then, the mice were sacrificed and subcutaneous tumors were formalin-fixed and paraffin-embedded, and cut into 5-uM thick sections for subsequent collagen staining and quantitation by using the Sirius red/fast green collagen staining kit (Chondrex). All

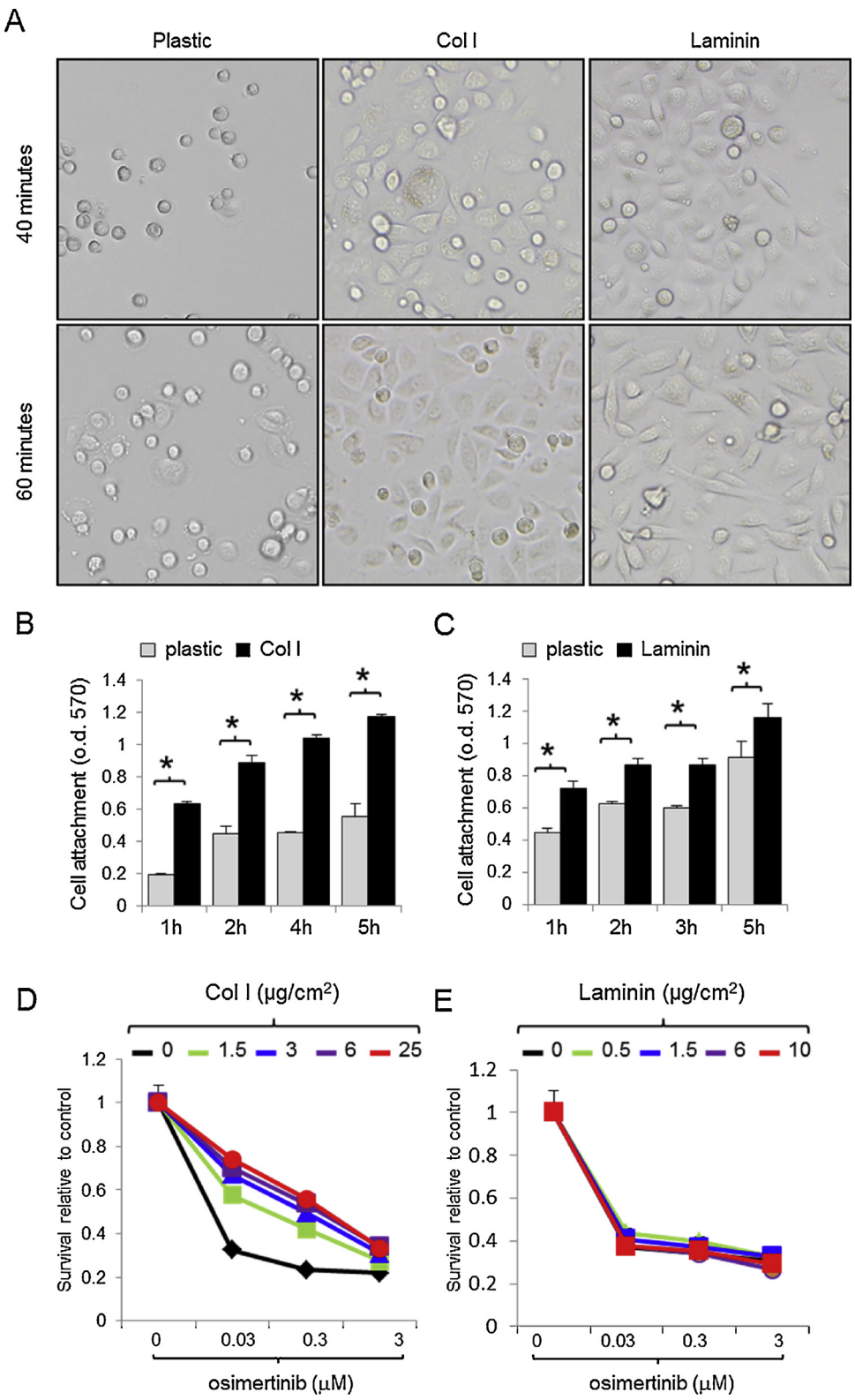

Fig. 8. Culturing on collagen but not laminin promotes resistance to EGFR TKI.
(A) Light microscope images for H1975 cells seeding on non-coated cell culture plate (plastic), collagen I (Col I), or Laminin for the indicated times. Note that most cells cultured on plastic plate are not attached to the plate and exhibit round morphology within 60 min after seeding. (B, C) Cell adhesion assays for HCC827 cells seeding on non-coated cell culture plate (plastic), collagen I (Col I), or Laminin for the indicated times. * indicates *t*-test $p < 0.05$. (D, E) H1975 cells were seeded on various concentrations of collagen I (Col I; D) or Laminin (E) and treated with osimertinib as indicated for three days. Cell survival was quantitated by MTT assay and normalized to that of vehicle (DMSO)-treated cells (set as 1.0).

mouse experiments were approved by the institutional IACUC and performed under the institutional guidelines and policies. Briefly, paraffin-embedded tissue sections were deparaffinized and hydrated. The sections were then incubated with the dye solution for 30 min at room temperature. After washing, the dye extraction buffer was loaded on each sample and gently mixed by pipetting until the color is eluted from the tissue section. Finally, the eluted dye solution was collected and the OD values at 540 nm and 605 nm were measured with a spectrophotometer. The amount of collagen was calculated according to the following formula: Collagen (μg/section) = OD 540 value - (OD 605 value x 0.291)/0.0378.

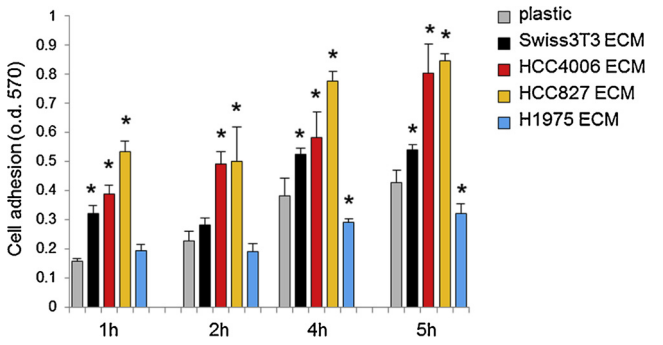

Fig. 9. The effect of ECM on cell adhesion.
Cell adhesion assays were performed for HCC827 cells seeding on non-coated cell culture plate (plastic) or the cell-free ECM from Swiss3T3, HCC4006, HCC827, and H1975 cells. Note that the ECM from distinct cells does not consistently promote cell adhesion compared to non-coated plate. * indicates *t*-test $p < 0.05$.

4.8. Cell attachment assay

Cell attachment was performed in 24-well, flat-bottom plastic plates coated with different proteins or cell-free ECM. Briefly, collagen I (BD Biosciences), fibronectin (Millipore), and laminin (Sigma) at different concentration were coated onto 24-well plates overnight at 4 °C. Cell-free ECM was prepared from various cell lines respectively. 1 × 105 tumor cells were then seeded to the collagen I-, fibronectin-, laminin and cell-free ECM-coated 24-well plates and incubated at 37 °C in humidified 5% CO2 conditions for 1–6 hours. The plate was washed twice with PBS to remove unbound cells. Then, cells remaining attached to the plate were analyzed using MTT. This assay was repeated at least three times.

4.9. Western blotting

For western blotting experiments, the whole cell protein lysates were prepared by using a RIPA lysis buffer kit (Santa Cruz) as described

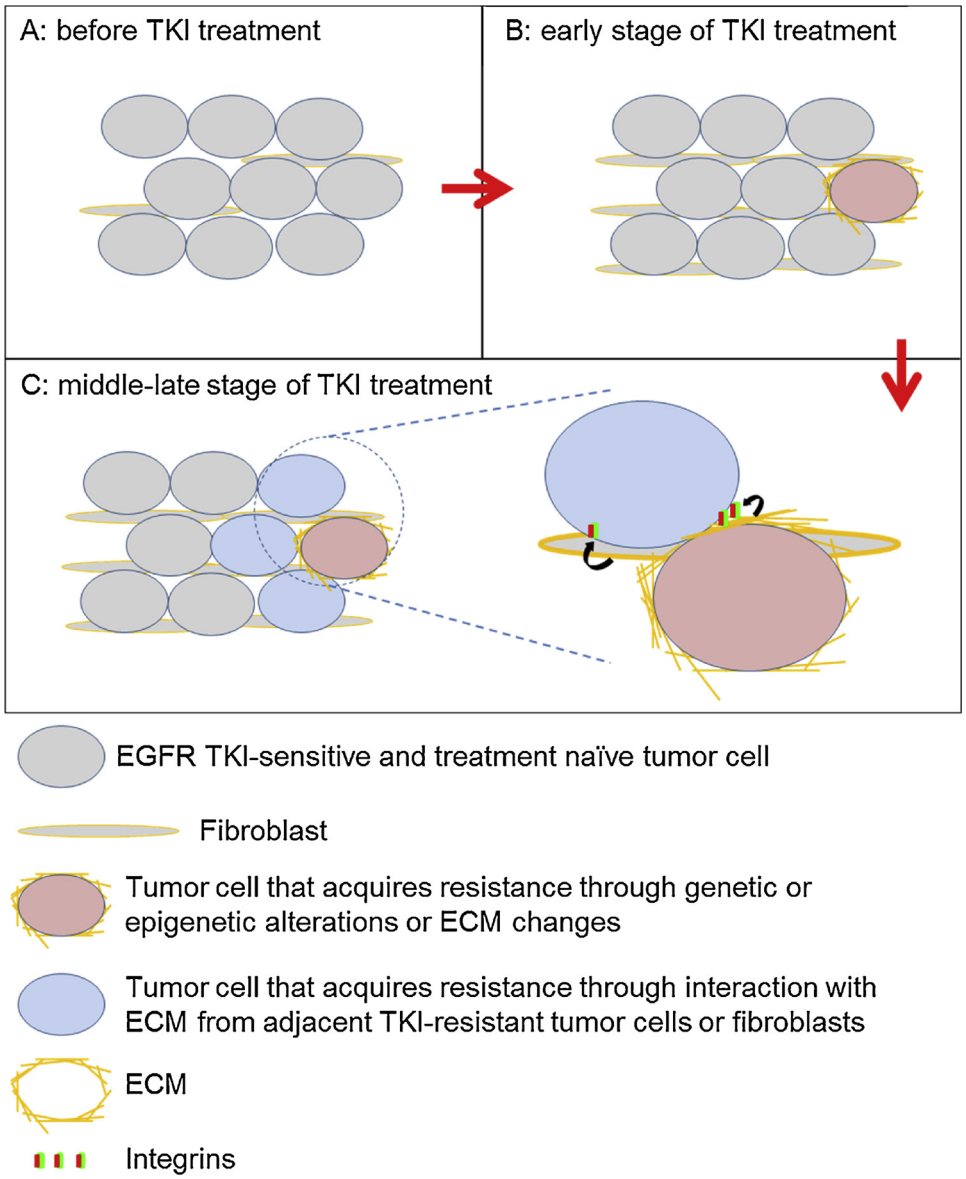

Fig. 10. A hypothetical model for the stromal ECM acting as a microenvironmental cue to drive EGFR TKI resistance. (A) A treatment naïve EGFR mutant lung tumor contains TKI-sensitive tumor cells. (B) During the early stage of TKI treatment, more fibroblasts may be recruited to the tumor and some tumor cells may acquire TKI resistance through genetic or epigenetic alterations or ECM changes. (C) At the middle to late stages of TKI treatment, the ECM from TKI-resistant tumor cells or fibroblasts may promote resistance through Integrins in neighboring tumor cells that are otherwise sensitive to the EGFR TKIs.

by the manufacturer. 10–30 μg proteins were separated by SDS-PAGE electrophoresis and transferred onto PVDF membranes (Bio-Rad), which were incubated in 5% milk at room temperature for an hour to block non-specific protein binding. After incubation with primary antibodies at 4 °C overnight, the membranes were washed and incubated with appropriate HRP-conjugated secondary antibodies for 1–2 h at room temperature. The protein bands were visualized by using a chemiluminescence substrates kit (Pierce).

4.10. Statistical analysis

T-tests were performed by using the Microsoft office excel software. A two-sided p value of less than 0.05 was considered statistically significant.

Author contributions

Y.W., T.Z., and L.G. performed experiments and collected and analyzed data. T.R. and Y.Y. conceived and supervised the project, and provided funding and administrative supports. All authors wrote and approved the paper.

Competing interest statement

The authors have declared that no competing interest exists.

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