

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Increased Lactate Secretion by Cancer Cells Sustains Non-cell-autonomous Adaptive Resistance to MET and EGFR Targeted Therapies

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1691738> since 2019-04-05T16:13:17Z

Published version:

DOI:10.1016/j.cmet.2018.08.006

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

Increased Lactate Secretion by Cancer Cells Sustains Non-cell-autonomous Adaptive Resistance to MET and EGFR Targeted Therapies

Maria Apicella¹, Elisa Giannoni², Stephany Fiore^{1, 3, 11}, Karin Johanna Ferrari⁴, Daniel Fernández-Pérez⁴, Claudio Isella¹, Carlotta Granchi⁵, Filippo Minutolo⁵, Antonino Sottile¹, Paolo Maria Comoglio¹, Enzo Medico^{1, 3}, Filippo Pietrantonio^{6, 7}, Marco Volante^{3, 8}, Diego Pasini^{4, 9}, Paola Chiarugi^{2, 10}, Silvia Giordano^{1, 3, 12}, *silvia.giordano@unito.it Simona Corso^{1, 3, 12, 13}, **simona.corso@unito.it

¹Candiolo Cancer Institute - FPO, IRCCS, Strada Provinciale 142, Torino, Candiolo 10060, Italy

²Department of Experimental and Clinical Biomedical Sciences, University of Florence, Florence 50134, Italy

³University of Torino, Department of Oncology, 10060, Torino, Candiolo, Italy

⁴Department of Experimental Oncology, European Institute of Oncology, 20139 Milan, Italy

⁵Department of Pharmacy, University of Pisa, 56126 Pisa, Italy

⁶Medical Oncology Department, Fondazione IRCCS Istituto Nazionale dei Tumori, 20133 Milan, Italy

⁷Department of Oncology and Hemato-oncology, University of Milan, Milan, Italy

Summary

The microenvironment influences cancer drug response and sustains resistance to therapies targeting receptor-tyrosine kinases. However, if and how the tumor microenvironment can be altered during treatment, contributing to resistance onset, is not known. We show that, under prolonged treatment with tyrosine kinase inhibitors (TKIs), EGFR- or MET-addicted cancer cells displayed a metabolic shift toward increased glycolysis and lactate production. We identified secreted lactate as the key molecule instructing cancer-associated fibroblasts to produce hepatocyte growth factor (HGF) in a nuclear factor κ B-dependent manner. Increased HGF, activating MET-dependent signaling in cancer cells, sustained resistance to TKIs. Functional or pharmacological targeting of molecules involved in the lactate axis abrogated *in vivo* resistance, demonstrating the crucial role of this metabolite in the adaptive process. This adaptive resistance mechanism was observed in lung cancer patients progressed on EGFR TKIs, demonstrating the clinical relevance of our findings and opening novel scenarios in the challenge to drug resistance.

The tumor microenvironment shapes cancer progression. Apicella et al. now show that cancer-associated fibroblasts play an active metabolic role in adaptive cancer drug resistance to tyrosine kinase inhibitors.

Introduction

The clinical efficacy of anti-cancer targeted therapies is strongly limited by the rapid and inexorable development of acquired drug resistance. Therefore, understanding resistance-causing mechanisms is a critical challenge to improve the outcome of candidates for such treatment strategies. In the last decade, the use of *in vitro* models allowed the identification of a number of molecular mechanisms responsible for acquired resistance to tyrosine kinase inhibitors (TKIs). The clinical importance of these resistance models is unquestionable, as many mechanisms of resistance identified *in vitro* have been subsequently translated in the clinic ([Camidge et al., 2014](#); [Engelman and Settleman, 2008](#); [Lovly and Shaw, 2014](#)). However, lacking the epithelial-stromal interactions typically occurring *in vivo*, *in vitro* models preclude the identification of possible non-cell-autonomous mechanisms of resistance. It is now firmly established that the tumor microenvironment may affect important tumor functions, such as proliferation and metastatic ability, and can also influence treatment responses ([Bhowmick et al., 2004](#); [Shiao et al., 2011](#)). Indeed, recent reports suggest an important role of tumor stroma in sustaining drug resistance mediated by pro-inflammatory pathways or by the secretion of growth factors that can activate, in cancer cells, signaling pathways compensatory to those inhibited pharmacologically ([Carbone et al., 2011](#); [Meads et al., 2009](#); [Straussman et al., 2012](#); [Wilson et al., 2012](#)). In this context, a key role is played by the hepatocyte growth factor (HGF), whose secretion by mesenchymal cells elicits activation of the MET tyrosine kinase receptor ([Bottaro et al., 1991](#); [Giordano et al., 1989](#)). Several studies have shown that MET activation can broadly behave as a bypass mechanism, compensating for loss of drug-inhibited signaling pathways in several tumor contexts ([Harbinski et al., 2012](#); [Straussman et al., 2012](#); [Wilson et al., 2012](#)). However, whether and how cancer cells can instruct their microenvironment to activate resistance-mediating pathways is poorly known.

In order to identify and study non-cell-autonomous mechanisms of acquired resistance to molecular therapies, we generated *in vivo* models of acquired resistance to MET or epidermal growth factor receptor (EGFR) TKIs. MET has been studied extensively as a target for therapeutic intervention ([Comoglio et al., 2018](#); [Peters and Adjei, 2012](#)), and evidence accumulated in the last decade suggests that, in selected contexts, MET-targeting drugs displayed clinical efficacy in patients with tumors harboring MET oncogenic alterations ([Cui, 2014](#); [Gherardi et al., 2012](#)). EGFR is an established therapeutic target in oncology. EGFR TKIs are the standard first-line therapy for patients with EGFR-mutation-positive advanced non-small-cell lung cancer (NSCLC) ([Maemondo et al., 2010](#); [Mok et al., 2009](#); [Rosell et al., 2012](#); [Zhou et al., 2011](#)) and are also approved for pancreatic cancer ([Moore et al., 2007](#)). Unfortunately, as usually reported for molecular therapies, patients initially responsive to MET or EGFR inhibitors invariably become resistant and progress ([Camidge et al., 2014](#); [Lennerz et al., 2011](#); [Sequist et al., 2011](#); [Yu et al., 2013](#)). Even if preclinical studies led to the identification of several cell-autonomous mechanisms of acquired resistance ([Cepero et al., 2010](#); [Chong and Jänne, 2013](#); [Corso et al., 2010](#); [Dillon et al., 2011](#); [Ohashi et al., 2013](#); [Qi et al., 2011](#)), we are far from having a comprehensive view of resistance causes. Indeed, for example, resistance to EGFR TKIs remains unexplained in about one-third of NSCLCs. In particular, non-cell-autonomous mechanisms have not been adequately explored in preclinical studies and in the clinics.

The *in vivo* models of adaptive resistance to MET or EGFR TKIs we generated demonstrate that non-cell-autonomous factors can play a pivotal role in resistance onset. Notably, we identified for the first time a metabolism based mechanism of adaptive resistance, whereby TKI-induced metabolic switch of tumor cells, with increased lactate production, instructs cancer-associated fibroblasts (CAFs) to overproduce HGF, eventually enforcing drug resistance and tumor progression. Increased production of tumor lactate and stromal HGF, which are potentially targetable adaptive alterations, were detected in advanced NSCLC patients upon emergence of resistance to EGFR TKIs, thus confirming the clinical relevance of our findings.

Results

In Vivo-Generated Tumor Adaptive Resistance to a MET TKI, JNJ-605, Is Mediated by Non-cell-autonomous Mechanisms

To investigate in vivo mechanisms of acquired resistance to MET targeted therapies, we continuously treated mice bearing EBC1 (a MET-addicted NSCLC cell line; [Cepero et al., 2010](#)) xenograft tumors with a specific anti-MET compound (JNJ-605) ([Cepero et al., 2010](#)), until resistance onset (RES-J tumors; [Figure 1A](#)) (see [STAR Methods](#) for details). Tumors grown in untreated animals represented matched controls (wild-type [WT] tumors). From each resistant and control tumor we derived cancer cell lines ex vivo. After a few passages in culture, thanks to their faster growth rate, EBC1 tumor cells overwhelmed the fibroblasts present in the dish; this allowed us to obtain a homogeneous cancer cell population ([Figure S1A](#)). Surprisingly, we observed that 4 of 5 EBC1 cell lines derived from JNJ-605-resistant tumors (RES-J EBC1) were not resistant to the MET TKI in vitro, showing a half maximal inhibitory concentration (IC50) similar to that of control cells ([Figure 1B](#)). Molecular analysis ruled out the presence of the most frequent genetic alterations known to confer resistance to MET TKIs (data not shown). However, when subcutaneously re-injected in mice, RES-J EBC1 cells re-originated drug-resistant tumors; as expected, re-injected WT EBC1 cells gave rise to drug-sensitive tumors ([Figure 1C](#)). This result suggests that RES-J EBC1, although not autonomously drug resistant, had acquired the permanent ability to generate a drug-resistant tumor mass.

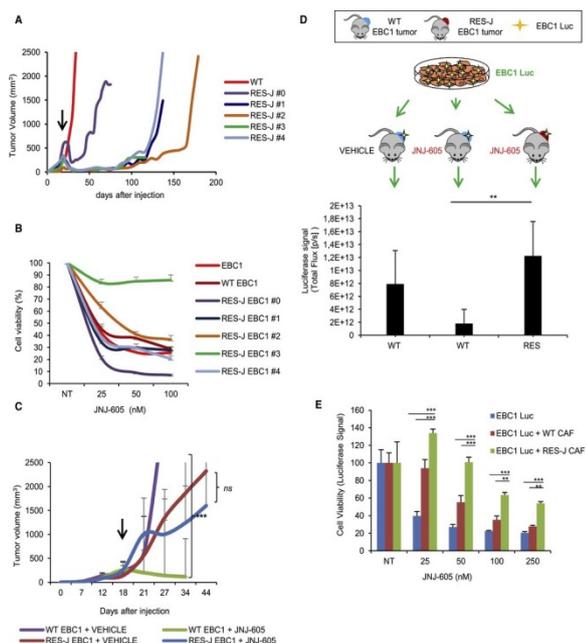


Figure 1 In Vivo-Generated Tumor Adaptive Resistance to JNJ-605 Is Mediated by Non-cell-autonomous Mechanisms

(A) The NSCLC cell line EBC-1 was subcutaneously injected into NOD-SCID mice. When tumors reached a volume >300 mm³, five mice started a daily treatment with the maximum tolerated dose of the MET inhibitor JNJ-605 until resistance onset (RES-J tumors). The growth curves of the resistant tumors and of one representative untreated tumor (WT) are shown. The arrow indicates treatment start.

(B) Cell viability assay (based on ATP content) of EBC1 cells derived from either WT or RES-J tumors, treated in vitro for 3 days with the indicated increasing concentrations of JNJ-605.

(C) EBC1 cells derived either from a WT or a representative RES-J EBC1 tumor were subcutaneously re-injected in NOD-SCID mice; upon tumor growth, mice were treated either with vehicle or with JNJ-605 (n = 5). The arrow indicates treatment start. Tumor growth curves are reported in the graph.

(D) Parental EBC1 cells (sensitive to MET inhibitors) were transduced with a lentiviral vector containing the luciferase cDNA (EBC1 Luc), and 1 × 10⁶ cells were injected into preexisting WT or RES-J EBC1 tumors. Mice were treated with JNJ-605 (or with vehicle) for 8 weeks, as indicated (n = 5). The graph represents the luminescence signal of explanted tumors analyzed by IVIS imaging as a readout of viable EBC1 Luc cells.

(E) Cell viability assay (based on luciferase signal) performed on co-culturing parental EBC1 Luc cells with tumor-derived (murine) CAFs isolated from WT or resistant tumors (WT or RES-J CAFs), in the absence or in the presence of JNJ-605 at the indicated doses. In (B)–(E), data are represented as means ± SD. **p < 0.01; ***p < 0.001; ns, not significant. One-way ANOVA followed by Bonferroni multiple comparisons test was used for (D); two-way ANOVA followed by Bonferroni. In order to unravel the role of the tumor microenvironment in the adaptive resistance to the MET TKI, we performed in vivo co-culture experiments. Luciferase-transduced parental EBC1 cells (EBC1 Luc), sensitive to MET TKIs, were injected in preformed WT or RES-J tumors, and mice were randomized into three experimental groups: (1) WT untreated tumors, (2) WT tumors treated with JNJ-605, and (3) RES-J tumors treated with JNJ-605. After 6 weeks of treatment, the animals were sacrificed and the level of the luciferase signal (proportional to the number of living EBC1 Luc cells) was evaluated by IVIS imaging. Interestingly, EBC1 Luc cells were not growth inhibited by the MET TKI when injected into preformed drug-resistant tumors, while they responded to drug treatment when injected into drug-sensitive (WT) tumors ([Figure 1D](#)). To identify the cellular component of RES-J tumors critical for conferring resistance to parental cancer cells, we first evaluated the role of RES-J EBC1 cells. EBC1 Luc cells were co-cultured in vitro with tumor-derived RES-J EBC1 cells or WT EBC1 cells, in the presence of increasing

605. As shown, neither RES-J EBC1 cells nor WT EBC1 cells affected EBC1 Luc sensitivity to the MET inhibitor ([Figure S1B](#)), reinforcing the hypothesis that stromal populations could be responsible for the conferred resistance.

CAFs are the principal component of the tumor stroma and regulate tumor cell functions by secreting growth factors, chemokines, and extracellular matrix ([Zhang and Liu, 2013](#)). Several studies have previously reported tumor stroma interactions capable of mediating chemo-resistance ([Hu et al.,](#)

2015). Moreover, recent findings demonstrate that CAF-secreted factors can modulate responsiveness to targeted agents in different tumor contexts (Straussman et al., 2012). We obtained pure mouse fibroblast cultures from resistant or control tumors (RES-J CAF or WT CAF) (Figure S1A) by treating the collagenase-dissociated tumors with diphtheria toxin, which selectively kills human (EBC1) but not mouse (stroma) cells (Arbiser et al., 1999). When tested in in vitro co-culture assays, CAFs derived from resistant tumors rendered WT EBC1 cells resistant to the MET TKI, even at high drug concentrations, while CAFs derived from control tumors induced resistance to low TKI doses only (Figure 1E). These results suggest that, in this model, the adaptive resistance to MET TKI treatment is not intrinsic to cancer cells, but it is due to the interplay with tumor stroma, in particular with CAFs.

Increased HGF Production by CAFs Is Responsible for Adaptive Resistance to JNJ-605

To understand if CAFs fuel resistance to JNJ-605 through secretion of soluble molecule(s), we treated EBC1 cells with increasing concentrations of JNJ-605 in the presence or absence of RES-J or WT CAF conditioned media.

Media obtained from different RES-J CAF populations strongly impaired EBC1 sensitivity to JNJ-605, while WT CAF media did not alter the drug response (Figure 2A). This shows that the adaptive resistance to MET inhibitors was mediated by soluble factor(s) secreted by resistant CAFs. Interestingly, western blot analysis revealed that the presence of RES-J CAF conditioned media (but not of WT CAFs conditioned media) preserved MET phosphorylation and activation of MET downstream pathways in tumor cells, despite treatment with the MET TKI (Figure 2B). As it was previously shown that HGF, the MET ligand, causes a dose-dependent increase in the IC50 of MET TKIs, stabilizing MET homodimers (less sensitive to MET TKIs) and activating TKI-free receptors (Pennacchietti et al., 2014), we considered HGF involvement in resistance onset. Indeed, quantitative real-time PCR and ELISA experiments revealed that in vitro RES-J CAFs expressed and secreted HGF at significantly higher levels than WT CAFs (Figures 2C and 2D). An important stromal HGF mRNA increase was also confirmed in vivo through RNA in situ hybridization on formalin-fixed and paraffin-embedded (FFPE) slides of the original resistant tumors, as well as on slides of resistant tumors generated upon RES-J EBC1 re-injection in mice (Figure 2E).

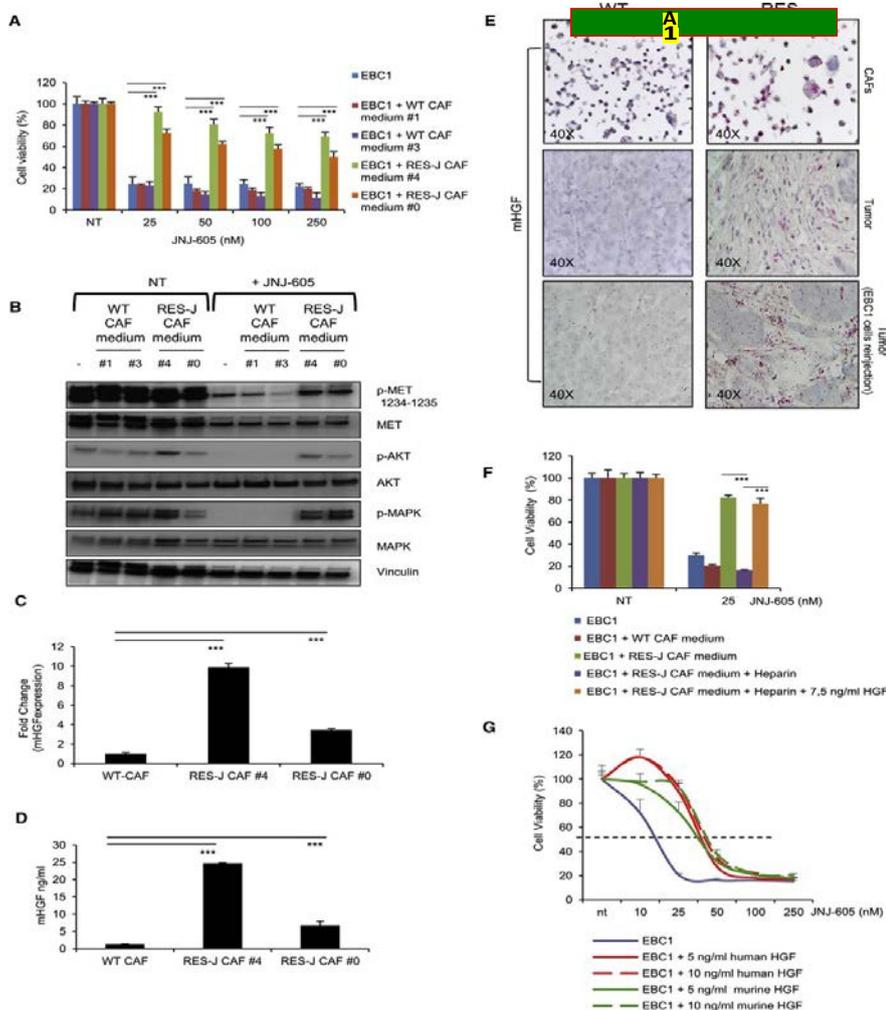


Figure 2 CAF-Increased HGF Production Is Responsible for Adaptive Resistance to JNJ-605

(A) Cell viability assay (based on cell ATP-content) on parental EBC1 cells treated for 3 days with increasing JNJ-605 concentrations in the absence or presence of the conditioned media of WT or RES-J CAFs (derived from two different WT or RES-J tumors).

(B) Western blot analysis of parental EBC1 cells treated with JNJ-605 (100 nM) in the absence or presence of the conditioned media of WT or RES-J CAFs (derived from two different WT or RES-J tumors). Blots have been probed with the indicated antibodies. Vinculin was used as loading control.

(C) Quantitative real-time PCR analysis of mouse HGF (mHGF) mRNA levels in RES-J CAFs compared with WT CAFs.
 (D) ELISA assay quantifying the concentration of mouse HGF (mHGF) in the conditioned media of representative WT and RES-J CAFs.
 (E) Detection of mouse HGF mRNA by RNA in situ hybridization in representative WT and RES-J CAFs derived in culture (top), in the corresponding original WT and RES-J EBC1 tumor FFPE slides (middle) and in FFPE slides of tumor masses generated upon reinjection in mice of WT and RES-J EBC1 cells (bottom).
 F) Cell viability assay on parental EBC1 cells treated for 3 days with JNJ-605 (25 nM) in the absence or in the presence of (1) WT CAF conditioned medium; (2) RES-J CAF conditioned medium; (3) RES-J CAF conditioned medium upon heparin-mediated HGF depletion; (4) RES-J CAF conditioned medium upon heparin-mediated HGF depletion and re-introduction of purified mHGF.
 (G) Cell viability assay (based on cell ATP content) performed on culturing parental EBC1 cells with increasing concentrations of JNJ-605 for 3 days in the absence of any exogenous ligand (blue line) or in the presence of either purified human HGF (red lines) or murine HGF (green lines) at two different doses. The dashed black line indicates the 50% decrease in cell viability.
 In (A), (C), (D), (F), and (G), data are represented as means \pm SD. ***p < 0.001. One-way ANOVA followed by Bonferroni multiple comparisons test was used for (C) and (D). Two-way ANOVA followed by Bonferroni multiple comparisons test was used for (A) and (F).

To prove that HGF was critical to mediate CAF-induced resistance, the conditioned medium of RES-J CAFs was depleted from HGF through heparin-binding; as shown in Figure 2F, upon depletion, RES-J CAF conditioned medium lost the ability to induce resistance, while re-introduction of purified murine HGF, at the concentration range found in the RES-J CAF medium, restored resistance to JNJ-605. These results demonstrate the causative role of CAF-produced HGF in this model of resistance. Notably, while mouse HGF was reported to activate only some of the MET-driven biological activities in human cells (Cecchi et al., 2015), we clearly observed that, in the specific context of resistance to MET TKIs, mouse and human purified HGF exerted superimposable effects on human MET-addicted cells, inducing a similar shift of the TKI IC50 (Figure 2G).

Lactate Is the Key Metabolite Driving HGF Upregulation in CAFs

We demonstrated that increased production of stromal HGF is responsible for the tumor adaptive resistance to the MET TKI. Moreover, as shown in Figures 1C and 2E, RES-J EBC1 cells, upon in vivo re-injection, were able to recreate a resistant tumor mass displaying stromal HGF overexpression. Trying to reproduce this phenomenon in vitro, we co-cultured WT CAFs with WT or RES-J EBC1 cells in the presence of the MET inhibitor and monitored mouse HGF production. Interestingly, after 3 weeks of co-culture with RES-J EBC1 cells (but not with WT EBC1), WT CAFs significantly increased their HGF production (Figure 3A). This demonstrates that RES-J EBC1 cells were directly able to instruct fibroblasts to overproduce the MET ligand.

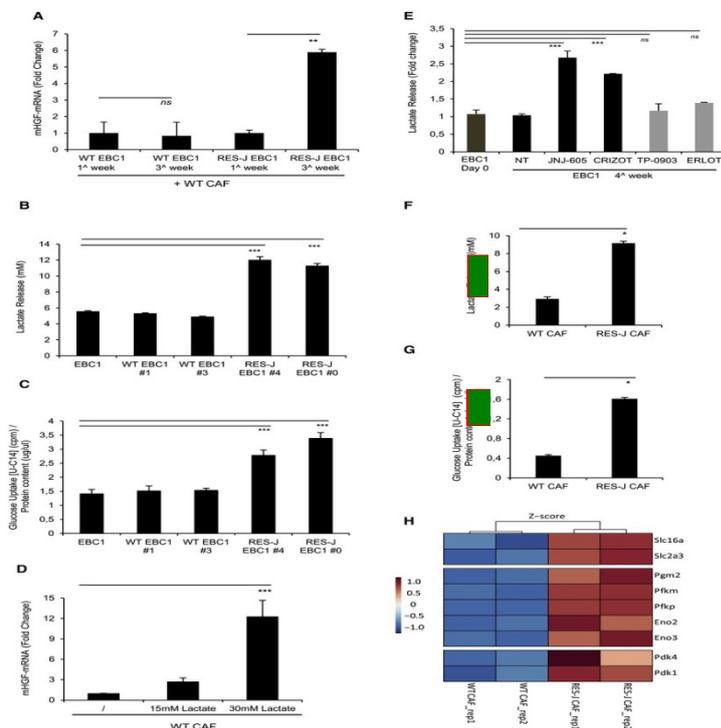


Figure 3 Lactate-Induced Metabolic Shift Drives HGF Upregulation in CAFs

(A) Quantitative real-time PCR analysis of mouse HGF mRNA levels in WT CAFs co-cultured with WT EBC1 or with RES-J EBC1 cells for 1 or 3 weeks.
 (B) Enzymatic assay quantifying lactate concentration in the conditioned media of parental EBC1 cells or of two representative WT and RES-J EBC1 cells.
 (C) Analysis of [U-14C]glucose uptake in parental, WT, or RES-J EBC1 cells.
 (D) Quantitative real-time PCR analysis of mouse HGF mRNA levels in WT CAFs cultured in the presence of increasing concentrations of lactate, up to levels present in RES-J EBC1 media.

(E) Enzymatic assay quantifying lactate concentration in the conditioned media of parental EBC1 cells treated for 4 weeks with two different MET TKIs (JNJ-605 or crizotinib), with a specific AXL inhibitor (TP-0903) or with a specific EGFR TKI (erlotinib).

(F) Enzymatic assay quantifying lactate concentration in the conditioned media of representative WT and RES-J CAFs.

(G) Analysis of [U-14C]glucose uptake in WT or RES-J CAFs.

(H) Heatmap of Z score transformed normalized counts of selected genes with gene ontology terms related to glucose metabolism in WT and RES-J CAFs.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns not significant. In (A), (C)–(F), and (G), data are represented as means \pm SD. One-way ANOVA followed by Bonferroni multiple comparisons test was used for (A)–(E). Two tail Student t test was used for (EF) and (FG).

Next, we wondered which molecular mechanism enables RES-J EBC1 cells to instruct the microenvironment, promoting HGF expression in CAFs. It is known that tumor cells and CAFs are “metabolically coupled” in a symbiotic relationship (Fiaschi et al., 2012; Salem et al., 2012). Moreover, resistant cells often present a marked Warburg metabolism, which leads to production and secretion of high amounts of lactate (Bacci et al., 2016; Curtarello et al., 2015; Lai et al., 2014; Zhou et al., 2010). We thus wondered if RES-J EBC1 cells were metabolically different from their WT counterpart. An enzymatic assay performed on the supernatant of two different WT and RES-J EBC1 and of parental EBC1 cells revealed a significant increase in lactate release in RES-J cells compared with controls (Figure 3B); in parallel, analysis of [U-14C]glucose uptake showed a significant increase in glucose consumption in RES-J EBC1 cells, suggesting a higher exploitation of the glycolytic metabolism compared with control cells (Figure 3C). Moreover, increased expression of acknowledged markers of Warburg metabolism (hexokinase 2 [HK2], Glucose Transporter 1 [GLUT1], Monocarboxylate transporter 4 [MCT4]) confirmed the metabolic switch of RES-J EBC1 toward aerobic glycolysis (Figure S1C).

We hypothesized that the metabolic reprogramming of resistant cells could promote HGF transcription in CAFs; indeed, HGF is a molecule often produced in response to extracellular stimuli that can modify cellular homeostasis (Carrolo et al., 2003; Pennacchietti et al., 2003; Zeng et al., 2002). Interestingly, exposure to lactate induced HGF transcriptional upregulation in WT CAFs (Figure 3D), suggesting a key role of this metabolite in the observed adaptive resistance. Lactate release can induce media acidification; however, we proved that medium acidification did not cause any significant increase of HGF production in CAFs (data not shown), ruling out the role of pH in the acquisition of resistance.

The metabolic switch observed in tumor cells could be reproduced in vitro by prolonged treatment of parental EBC1 cells with MET TKIs, but not by treatment with TKIs targeting kinases other than MET. Indeed EBC1 cells, cultured for 3 weeks in the presence of suboptimal doses of JNJ-605 or crizotinib (a multikinase MET inhibitor) increased their lactate release up to the levels observed in RES-J EBC1. No significant lactate increase was observed in EBC1 cells treated with TKIs targeting other kinases, such as erlotinib or TP-0903 (EGFR and AXL inhibitors, respectively) (Figure 3E). To understand if the metabolic switch was due to the selection of a preexisting high-Warburg population or to the metabolic adaptation of tumor cells, we measured glucose uptake in parental EBC1 cells using a fluorescent glucose analog (2-NBDG [2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose]). Flow cytometry analysis revealed a single 2-NBDG incorporation peak (Figure S1D, left panel). Moreover, when the two curve tails (corresponding to the most and least glycolytic cells) were sorted, cultured for 1 week, and analyzed for glucose uptake, they showed the same uptake profile of parental cells (Figure S1D, right panels). These results suggest that the metabolic switch observed in resistant EBC1 cells is not the result of the selection of a preexisting subpopulation displaying a higher Warburg metabolism.

Like RES-J EBC1, RES-J CAFs also showed an increased lactate release (Figure 3F) and glucose uptake (Figure 3G) compared with WT CAFs, suggesting that CAFs themselves had undergone a metabolic switch. RNA sequencing data confirmed that RES-J CAFs showed features of enhanced glycolytic metabolism. Glucose and lactate transporters (MCT4, GLUT3), several glycolytic enzymes (PGM, PFK, and ENO), and negative regulators of the tricarboxylic acid cycle (PDKs) were indeed overexpressed in RES CAFs (Figure 3H) compared with WT CAFs.

HGF overexpression in RES-J CAFs was indefinitely maintained in culture in the absence of RES-J EBC1 cells. However, pharmacologic or genetic inhibition in RES-J CAFs of either lactate dehydrogenase (LDH) or the lactate importer MCT1 significantly decreased HGF mRNA levels in these cells (Figures S1E–S1G). These results suggest a crucial role of the glycolytic switch in CAFs for their indefinite HGF overexpression in culture.

To understand if the constitutive HGF overexpression in RES-J CAFs relies on epigenetic changes at the Hgf locus, we performed chromatin immunoprecipitation (ChIP) experiments for different regulatory histone modifications. As shown in Figures S2A and S2B, histone marks of active transcription were differentially accumulated at the transcription start site of the Hgf gene in RES-J CAFs compared with WT CAFs. Moreover, H3K36me3, a modification linked to gene transcriptional elongation, was notably increased within the Hgf gene body, whereas repressive marks were not detectable at Hgf promoter in both CAF cell lines.

Lactate-Induced HGF Transcriptional Upregulation in CAFs Is Mediated by NF- κ B

It is known that the nuclear factor κ B (NF- κ B) transcription factor can promote HGF gene transcription (Harrison and Farzaneh, 2000; Yin et al., 2014). Since it was previously reported that lactate stimulates the NF- κ B pathway by triggering I κ B α degradation (Végran et al., 2011), we explored if lactate-induced HGF overexpression in RES-J CAFs was mediated by this transcription factor. Indeed, western blot analysis showed a decrease of I κ B α , as well as an increase of NF- κ B levels, in RES-J CAFs compared with WT CAFs (Figure 4A, left panel). Exposure to lactate concentrations, in the range of those released by RES-J CAFs, induced a reduction of I κ B α levels in WT CAFs and a concomitant increase in NF- κ B expression, similar to that observed in RES-J CAFs (Figure 4A, left panel); this demonstrates that exposure to lactate is sufficient to induce CAF NF- κ B upregulation. Importantly, we showed that NF- κ B is required for HGF overexpression since CAF treatment with two different NF- κ B pathway inhibitors (IKK-16 and BMS-345541), significantly reduced both lactate-induced HGF upregulation in WT CAFs and basal HGF overexpression in

RES-J CAF (Figure 4A, central and right panels, and Figure 4B). Importantly, the conditioned medium of RES-J CAFs grown in the presence of NF- κ B pathway inhibitors lost the ability to confer resistance to JNJ-605 (Figure 4C), as it contained HGF levels similar to WT CAF medium (Figure S2C). These data prove the requirement of NF- κ B activity in the lactate-HGF axis. Végran and colleagues previously showed that lactate induces transcriptional changes in endothelial cells by activating NF- κ B, and that this activation requires the production of reactive oxygen species (ROS) (Végran et al., 2011). Interestingly, we observed that also in WT CAFs, lactate was able to induce ROS upsurge, I κ B α degradation, and the consequent NF- κ B activation (Figures S2D and S2E). The crucial role played by ROS was demonstrated by the inactivation of the lactate/NF- κ B axis in the presence of N-acetylcysteine, inhibiting ROS production (Figures S2D and S2E). Thus, the lactate/NF- κ B/HGF axis described in our work presents several similarities with what is observed in endothelial cells. In both cases, lactate released by cancer cells induced NF- κ B activation in non tumor cells.

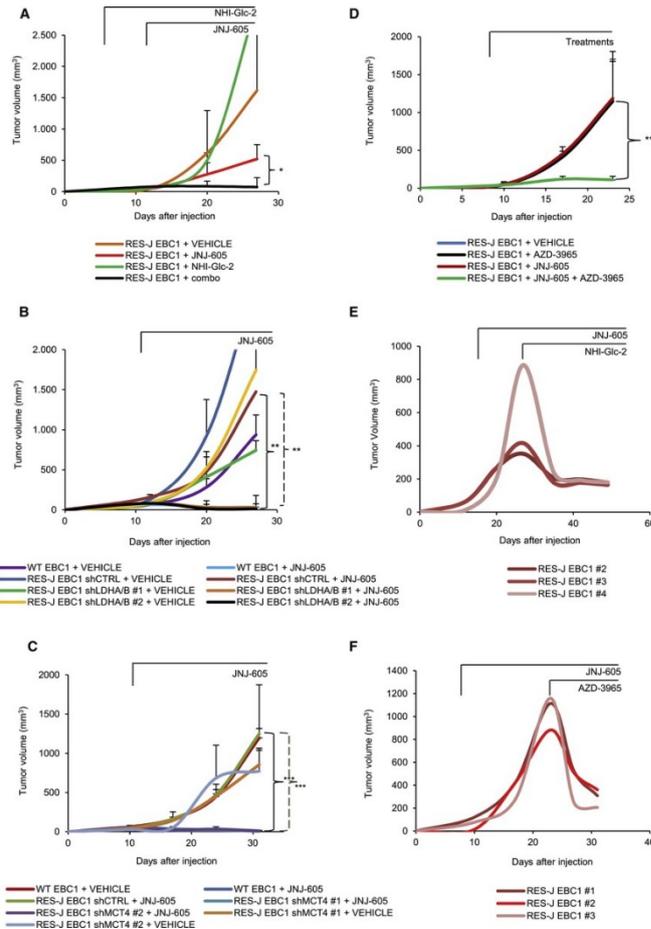


Figure 4 HGF Transcriptional Upregulation in CAFs Is NF-κB Dependent

(A) Western blot analysis for IκBα and NF-κB levels in WT CAFs stimulated with increasing doses of lactate and in RES-J CAFs in the absence or in the presence of NF-κB pathway inhibitors (IKK-16 and BMS-345541). Actin was used as loading control.
 (B) ELISA assay quantifying the concentration of mouse HGF (mHGF) in conditioned media of WT CAFs stimulated with increasing doses of lactate and of RES-J CAFs treated with two different NF-κB pathway inhibitors (IKK-16 and BMS-345541).
 (C) Cell viability assay (based on cell ATP content) on parental EBC1 cells treated for 3 days with the IC50 concentration of JNJ-605, in the presence of conditioned media derived from: (1) WT CAFs; (2) RES-J CAFs; (3) RES-J CAFs pretreated for 72 hr with the NF-κB pathway inhibitors IKK-16 or BMS-345541; (4) EBC1 pretreated for 72 hr with the NF-κB pathway inhibitors IKK-16 or BMS-345541 as control.
 *p < 0.05; **p < 0.01; ***p < 0.001. In (B) and (C), data are represented as means ± SD. One-way ANOVA followed by Bonferroni multiple comparisons test was used for (B) and two-way ANOVA followed by Bonferroni multiple comparisons test was used for (C).

Pharmacologic Inhibition and Genetic Interference of Lactate Metabolism Bypass Adaptive Resistance to MET Inhibition in different MET-Addicted Models

Since the adaptive resistance to MET TKIs in EBC1 tumors was strongly linked to a metabolic tumor rewiring and, in particular, to increased lactate production and efflux from tumor cells, and influx in CAFs, we explored the possible therapeutic potential of these findings. We subcutaneously re-injected RES-J EBC1 cells in mice that were treated with either vehicle or the LDH inhibitor NHI-Glc-2 (Calvaresi et al., 2013), in the absence or presence of JNJ-605. While NHI-Glc-2 treatment (50 mg/kg) significantly decreased the NAD⁺/NADH ratio inside the tumors (Figure S3A), it did not impair tumor growth, but it completely prevented the onset of resistance to JNJ-605 (Figure 5A). The combo treatment was well tolerated by mice, which did not show significant alterations in hepatic or renal function (Figure S3B). Similar results were obtained through genetic LDH inhibition in tumor cells as well. RES-J EBC1 cells were stably transduced with lentiviral vector particles encoding for two different couples of short hairpin RNAs targeting the two LDH isoforms expressed in these tumors, LDH A and B (shLDHA/B, Figure S3C), or for a control short hairpin RNA (shCTRL). When transduced cells were re-injected in mice, LDH silencing completely restored the response to JNJ-605 (Figure 5B). As for NHI-Glc-2 treatment, silencing per se did not affect tumor growth.

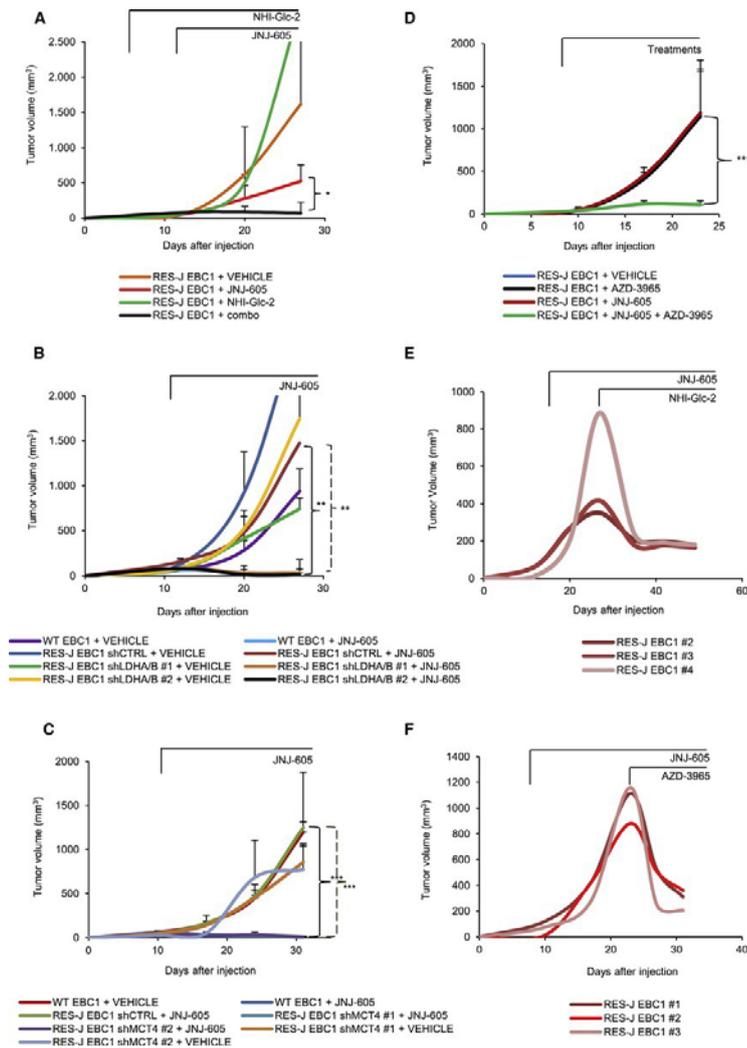


Figure 5 Pharmacologic Inhibition or Genetic Interference of Lactate Metabolism Prevents JNJ-605 Resistance Onset and Overcomes Already Established Resistance In Vivo

(A) Tumor growth curves of RES-J EBC1 cells subcutaneously re-injected in NOD-SCID mice. Animals ($n = 5/\text{group}$) were treated with either vehicle, JNJ-605 (50 mg/kg), or the LDH inhibitor NHI-Glc-2 (50mg/kg), alone or in combination (combo). The lines indicate JNJ-605 or NHI-Glc-2 treatment start.

(B) RES-J EBC1 cells transduced either with lentiviral shRNAs targeting the LDH A and LDH B isoforms (two different shRNA pairs) or with a control shRNA (shCTRL) were subcutaneously re-injected in NOD-SCID mice and treated with vehicle or JNJ-605 (50 mg/kg) ($n = 5/\text{group}$). As control, EBC1 cells obtained from WT tumors were re-injected in mice and treated with vehicle or JNJ-605 ($n = 5/\text{group}$).

(C) RES-J EBC1 cells transduced either with lentiviral shRNAs targeting the lactate transporter MCT4 (two different shRNAs: #1 and #2) or with a control shRNA (shCTRL) were subcutaneously re-injected in NOD-SCID mice and treated with vehicle or JNJ-605 (50 mg/kg) ($n = 6/\text{group}$). As control, EBC1 cells obtained from WT tumors were re-injected in mice and treated with vehicle or JNJ-605 ($n = 3/\text{group}$). The line indicates treatment start.

(D) Tumor growth curves of RES-J EBC1 cells subcutaneously re-injected in NOD-SCID mice. Animals were treated with either JNJ-605 (50 mg/kg) or with JNJ-605 plus the MCT1 inhibitor AZD-3965 (100 mg/kg) ($n = 6$). Vehicle or AZD-3965 alone were used as control ($n = 3$). The line indicates treatment start.

(E) Three mice shown in (A) (red line), bearing RES-J EBC1 tumors already under treatment with JNJ-605, on day 27 started NHI-Glc-2 treatment as well. The graph shows the tumor growth curve of each mouse. The lines indicate JNJ-605 or NHI-Glc-2 treatment start.

(F) Three mice shown in (D) (red line), bearing RES-J EBC1 tumors already under treatment with JNJ-605, on day 23 started AZD-3965 treatment as well. The graph shows the tumor growth curve of each mouse. The lines indicate JNJ-605 or AZD-3965 treatment start.

In (A)–(D), data are represented as means \pm SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Two-way ANOVA followed by Bonferroni multiple comparisons test was used.

Since lactate production in tumor cells was required to establish resistance, we then explored the effect of blocking lactate export from tumor cells into the microenvironment. We performed in vivo experiments by injecting mice with RES-J EBC1 cells stably transduced with two different MCT4 shRNAs (Figure S3D). As shown in Figure 5C, MCT4 abrogation in resistant tumor cells completely restored the response to MET TKIs. Moreover, as in vitro MCT1 inhibition in CAF was sufficient to revert their HGF overexpression (Figures S1E–S1G), we explored the effect of blocking lactate cell import during the in vivo establishment of resistance. Mice injected with RES-J EBC1 cells were treated with the AZD3965 MCT1 inhibitor (already in clinical investigation in humans, NCT01791595). As shown in Figure 5D, MCT1 inhibition significantly restored cancer cell sensitivity to TKIs. Finally,

pharmacologic inhibition of either LDH or MCT1 was able not only to prevent resistance onset but also to overcome established resistance, as both NHI-Glc-2 and AZD3965 induced regression of already resistant tumors, re-sensitizing tumor cells to MET TKIs (Figures 5E and 5F).

Overall, these results demonstrate a crucial role of lactate metabolism in the adaptive resistance to MET TKIs, and suggest LDH, MCT4, and MCT1 as possible therapeutic targets. Moreover, since in the silencing experiments LDH and MCT4 inhibition was restricted to the cancer cells, this formally demonstrates that lactate overproduction and oversecretion by tumor cells can be considered the *primum movens* for the development of resistance. This metabolism-driven, non-cell-autonomous mechanism of adaptive resistance to MET inhibition is not unique to the EBC1 model. In fact, also in the case of another MET-addicted cell line, GTL16 (derived from a gastric cancer; Smolen et al., 2006), tumor cells obtained from resistant tumors were not resistant in vitro to JNJ-605 but were able to promote HGF overexpression in CAFs, with the same lactate-based mechanism described for EBC1 cells (Figures S4A–S4G). Indeed, in vivo treatment with the LDH inhibitor NHI-Glc-2 restored the response to JNJ-605 also in RES-J GTL16 tumors (Figure S4H). Moreover, similar results were observed in EBC1 tumors upon acquired resistance to crizotinib (Figures S5A–S5F), proving that this mechanism of resistance is not restricted to the JNJ-605 inhibitor.

The Lactate-HGF Axis Mediates Adaptive Resistance to EGFR TKIs

In order to understand if the identified non-cell-autonomous mechanism of adaptive resistance could have a wider occurrence, we evaluated if it could mediate TKI resistance in tumors addicted to oncogenes other than MET, and, specifically, in EGFR-addicted lung cancers. The reasons for this choice were many: (1) EGFR and MET are biochemically and functionally linked and can frequently compensate for each other (Engelman and Settleman, 2008; Puri and Salgia, 2008); (2) in vitro screenings previously demonstrated that HGF is able to confer resistance to EGFR TKIs (Straussman et al., 2012; Wilson et al., 2012), even if the mechanisms underpinning HGF upregulation are unknown; (3) differently from MET inhibitors, EGFR TKIs (erlotinib and gefitinib) are currently used in clinical practice (Maemondo et al., 2010; Mok et al., 2009; Rosell et al., 2012; Zhou et al., 2011), allowing evaluation of whether this mechanism of adaptive resistance is relevant in cancer patients who are currently candidates for targeted therapies. To generate in vivo tumor models of resistance to erlotinib, mice subcutaneously injected with the human NSCLC HCC827 cell line, bearing deletion of EGFR exon 19 and sensitive to EGFR TKIs, were continuously treated with erlotinib until resistance onset (RES-E HCC827, Figure 6A). Mice grown without treatment were used as controls (WT HCC827). As observed in the MET-addicted models, tumor cells derived from RES-E HCC827 tumors were not resistant in vitro to erlotinib (Figure 6B), but resistance was conferred by the conditioned media of CAFs derived from RES-E HCC827 tumors (Figure 6C). These CAFs expressed and released significantly higher levels of HGF than WT CAFs (Figures 6D and 6E). In line with the MET models, RES-E HCC827 tumors relied on a higher glycolytic metabolism (Figure S6A), with increased lactate production compared with WT cells (Figure 6F); moreover, RES-E CAFs showed increased NF- κ B levels (Figure 6G). Importantly, LDH A/B silencing (Figures 6H and S6B), MCT4 silencing (Figures 6I and S6C), or concomitant MET-EGFR inhibition (Figure 6J) effectively restored in vivo response to erlotinib in RES-E HCC827 tumors, proving the causative role of the lactate/HGF-induced MET activation in this model of resistance as well.

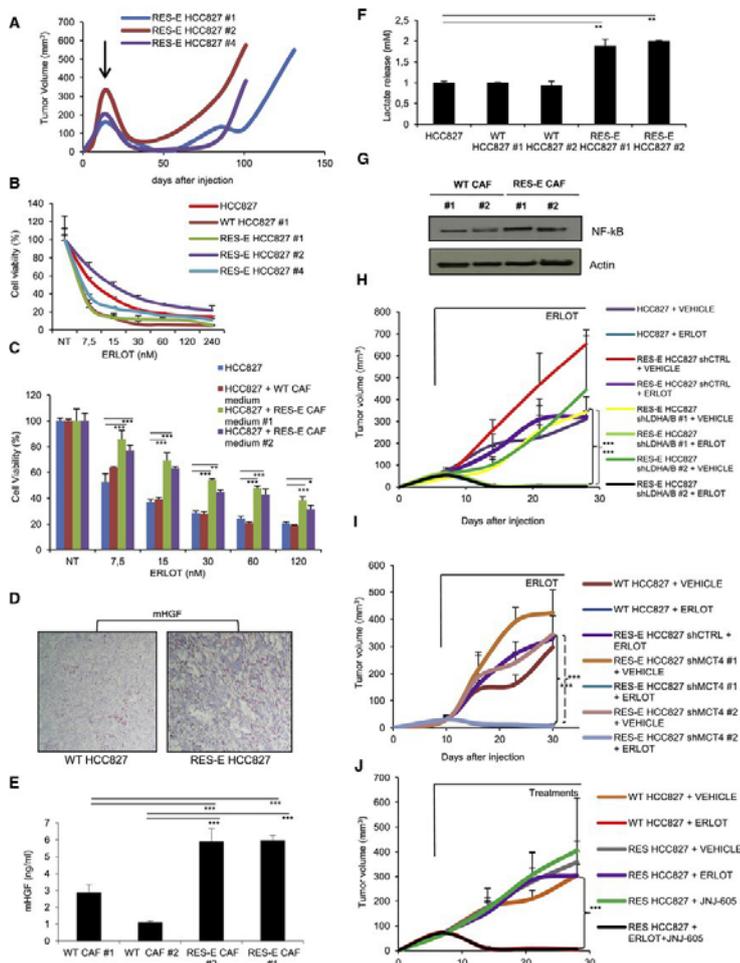


Figure 6 A Metabolism-Driven, Non-cell-autonomous Mechanism Sustains In Vivo Adaptive Resistance to EGFR Inhibition in HCC827 Lung Carcinoma Cells

(A) The NSCLC cell line HCC827 was subcutaneously injected in NOD-SCID mice. When tumors reached a volume >300 mm³, three mice started a daily treatment with the maximum tolerated dose of the EGFR inhibitor erlotinib until the tumors became resistant and grew in the presence of the inhibitor (RES-E HCC827 tumors). The graph shows the growth curves of the resistant tumors. The arrow indicates treatment start.

(B) In vitro cell viability assay of HCC827 cells derived from either WT or RES-E HCC827 tumors, treated for 3 days with increasing concentrations of erlotinib.

(C) Cell viability assay on parental HCC827 cells treated for 3 days with increasing concentrations of erlotinib in the absence or presence of conditioned media of representative WT or RES-E CAFs (derived from a WT or two different RES-E tumors).

(D) Detection of mouse HGF (mHGF) mRNA by RNA in situ hybridization in representative WT and RES-E HCC827 tumor FFPE sections.

(E) ELISA assay quantifying the concentration of mouse HGF (mHGF) in conditioned media of two different WT and RES-E CAFs.

(F) Lactate concentration in the conditioned media of two representative WT and RES-E HCC827 cells.

(G) Western blot analysis for NF- κ B levels in WT and RES-E HCC827 CAFs obtained from HCC827 tumors. Actin was used as loading control.

(H) RES-E HCC827 cells transduced either with lentiviral shRNAs targeting the LDH A and LDH B isoforms (two different shRNA pairs) or with a control shRNA (shCTRL) were subcutaneously re-injected in NOD-SCID mice and treated with vehicle or erlotinib (50 mg/kg) (n = 5/group). As control, HCC827 cells obtained from WT tumors were re-injected in mice and treated with vehicle or erlotinib (n = 3/group).

(I) RES-E HCC827 cells transduced either with lentiviral shRNAs targeting the lactate transporter MCT4 (two different shRNA, #1 and #2) or with a control shRNA (shCTRL) were subcutaneously re-injected in NOD-SCID mice and treated with vehicle or erlotinib (50 mg/kg) (n = 6/group). As control, HCC827 cells obtained from WT tumors were re-injected in mice and treated with vehicle or erlotinib (n = 3/group).

(J) Tumor growth curves of HCC827 cells derived from RES-E HCC827 tumors and subcutaneously re-injected in NOD-SCID mice. Animals (n = 5/group) were treated with either vehicle, or erlotinib (50 mg/kg), or JNJ-605 (50 mg/kg), alone or in combination (combo). The lines indicate treatment start. n = 5/group.

In (B), (C), (E), (F), (H)–(J), data are represented as means \pm SD. *p < 0.05; **p < 0.01; ***p < 0.001. One-way ANOVA with Bonferroni multiple comparisons test was used for (E) and (F). Two-way ANOVA followed by Bonferroni multiple comparisons test was used for (C) and (H)–(J).

As observed in the EBC1 model, the metabolic switch in tumor cells could be mimicked in vitro by prolonged treatment of HCC827 parental cells with EGFR TKIs but not by treatment with TKIs targeting other kinases (Figure S6D). The metabolic switch was not due to the selection of a preexisting metabolically different subpopulation, since no population with a stably different glucose uptake was detected by fluorescence-activated cell sorting analysis on HCC827 parental cells (data not shown).

In order to verify whether our results are extendable to other oncogene-addicted lung cancer models, we executed the in vitro prolonged treatment in other three lung cancer cell lines, namely HCC4006, PC9, and HCC78. As shown in Figure S6E, in all these cell lines, we observed a significant increase of lactate release in response to TKIs targeting the “driver” oncogene but not to TKIs targeting unrelated oncogenes. Notably, this was true not only in EGFR-addicted tumor cells (HCC-4006 and PC-9) but also in the ROS1-addicted lung cancer model (HCC-78). Lactate increase was also observed in HER2-addicted breast cancer cells upon prolonged lapatinib treatment (Figure S6E), further suggesting that this mechanism of resistance could be extendable beyond MET and EGFR addiction.

Stromal HGF and Tumor MCT4 Are Overexpressed in Patients Progressed on EGFR TKI Therapy

In order to evaluate the clinical relevance of our findings, we investigated whether activation of the lactate/HGF axis may enforce clinical resistance to TKIs. As MET inhibitors are not yet approved for routine clinical use, we focused on patients with EGFR-mutation-positive NSCLC and acquired resistance to EGFR TKIs. We collected paired FFPE tumor samples, obtained at diagnosis and upon resistance onset, of six advanced NSCLC patients with acquired resistance to erlotinib or gefitinib; none of them presented the T790M mutation at disease progression. In five patients, no classic mechanism of resistance was identified, while in one of them a low-grade MET gene amplification had been detected upon progression (Table S1 Figure S7A). The samples were analyzed through in situ hybridization for human HGF mRNA levels, followed by pan-cytokeratin immunohistochemistry (IHC) to differentiate cancer/stroma signals. In 3 of 6 pairs, we detected a >2-fold increase in the HGF mRNA signal in the tumor stroma upon resistance acquisition (Figures 7A and 7B). In all the analyzed samples, the tumor cells expressed the HGF receptor MET (data not shown).

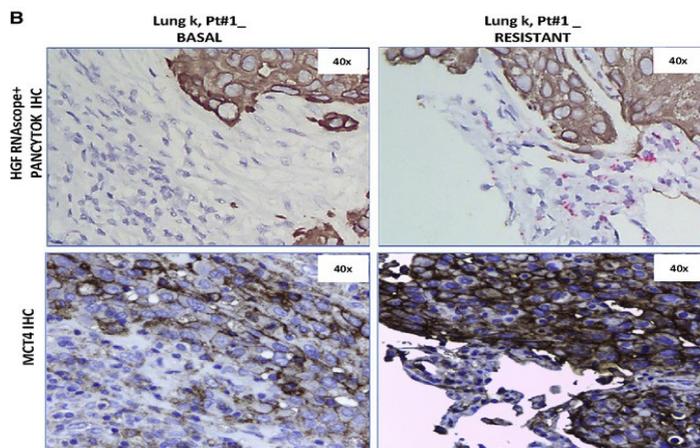
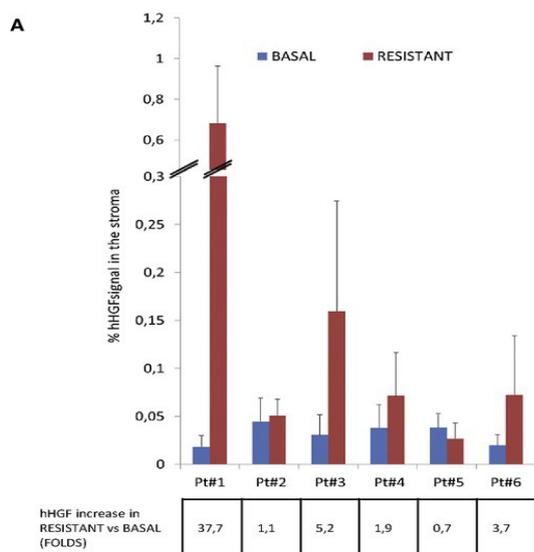


Figure 7 Stromal HGF and Tumor MCT4 Are Increased in NSCLC Patients Progressed upon EGFR TKI Therapy

(A) Paired FFPE biopsies of six NSCLC patients (Pt), progressed after EGFR TKI treatment, were analyzed through in situ hybridization (RNAscope) for human HGF (hHGF), followed by pan-cytokeratin staining (IHC) to mark tumor cells. The first biopsy was taken at diagnosis (BASAL), the second after EGFR TKI resistance onset (RESISTANT). The graph shows the percentage of hHGF RNAscope signal in the stroma (data are represented as means \pm S D). The fold changes of hHGF signal in RESISTANT versus BASAL biopsies are reported below the graph.

(B) Representative images of paired biopsies of Pt#1 (BASAL and RESISTANT). Upper panels: In situ hybridization (RNAscope) for human HGF (red spots), followed by pan-cytokeratin staining (IHC) to mark the tumor area (brown). Lower panels: IHC staining for the lactate transporter MCT4. The same paired samples were also analyzed by IHC for MCT-4 expression. In both the EBC1- and the HCC827-resistant models, the level of this transporter was increased in resistant cells compared with controls (Figures S2 and S6). In the analyzed NSCLC tumors, MCT4 and HGF expression showed a concordant change upon relapse in 4 of 6 tumors (Figures 7 and S7A). To define if MCT4-positive cells were localized within the hypoxic areas of resistant tumors, we performed IHC staining for MCT4 and carbonic anhydrase IX (CAIX, a known marker of cell hypoxia) on consecutive slides of the patients' paired biopsy samples. In the analyzed tumors, the MCT4-positive areas only rarely overlapped with CAIX-positive ones (Figure S7B), indicating that the upregulation of MCT4 is not linked to hypoxia in this context.

Discussion

Acquired resistance is one of the major limitations to the efficacy of targeted therapies. For this reason, in the last decade, enormous efforts have been made to identify the molecular mechanisms sustaining resistance. Most of the preclinical studies performed so far relied on in vitro experiments carried out in tumor cell lines. In these models, resistance is due to cell-autonomous mechanisms as the therapeutic treatment often selects preexisting clones bearing genetic alterations either in the target itself or in downstream or parallel signaling pathways, eventually compensating for the drug-inhibited kinase. Works performed in these in vitro models identified several molecular mechanisms responsible for acquired resistance to TKIs, many of which have been validated in patients (Camidge et al., 2014; Engelman and Settleman, 2008; Lovly and Shaw, 2014) and even clinically exploited as therapeutic targets, as successfully shown for osimertinib in the treatment of NSCLC patients with EGFR T790M-driven acquired resistance to first-/second-generation EGFR TKIs (Mok et al., 2017). However, in vitro models have clear limitations, such as the artificial growing conditions and the lack of epithelial-stromal interactions, precluding the identification of non-cell-autonomous mechanisms of resistance. Tumor microenvironment has recently emerged as an important player in sustaining resistance to targeted therapies, for example, through production of ligands that, in a paracrine manner, activate signals able to compensate for the drug-inhibited pathways in tumor cells (Harbinski et al., 2012; Straussman et al., 2012; Wilson et al., 2012). In particular, HGF production by stromal cells has been shown to be a mechanism of primary (innate) resistance to TKIs targeting different oncogenes (Straussman et al., 2012; Wilson et al., 2012). In their pivotal work, Straussman and colleagues also reported an increased HGF expression in 5 of 10 melanoma patients during treatment with BRAF inhibitors (Straussman et al., 2012). However, the mechanism(s) leading to HGF

overexpression or its possible occurrence in other tumor types upon different molecular treatments are not known. In general, our knowledge of non-cell-autonomous mechanisms of resistance is largely incomplete; e.g., it is entirely unknown how tumor cells undergoing drug treatment can instruct their microenvironment to activate “rescue pathways.”

Here, we report for the first time that increased lactate production, occurring in cancer cells under TKI treatment, instructs the tumor microenvironment to sustain an adaptive resistance to targeted therapies. In particular, continuous and prolonged treatment with MET or EGFR TKIs induced an exacerbation of the Warburg metabolism in tumor cells, with increased lactate release. This metabolite led CAFs to increase HGF production, which, in turn, activated MET in cancer cells, overcoming the inhibitory effect of TKIs. Targeting tumor lactate metabolism was sufficient to overcome resistance, thus demonstrating the causative role of the lactate/HGF axis. Importantly, overexpression of stromal HGF and tumor MCT4 was identified in NSCLC patients who acquired resistance to EGFR TKIs, giving a proof of concept of the clinical relevance of our preclinical findings.

While the ability of stromal HGF overexpression to sustain resistance to TKIs targeting kinases other than MET (through the activation of a parallel signaling pathway) is expected, the observation that HGF can overrule the activity of MET TKIs in MET-addicted cells is somehow counterintuitive. Indeed, Pennacchietti and colleagues showed that stromal-derived HGF caused dose-dependent increase of the IC50 of MET TKIs without affecting the fraction of viable cells at the highest drug concentration (i.e., it reduced their potency but not their efficacy). We observed the same effect described by Pennacchietti et al. with both human and murine HGF (Figure 2G). Interestingly, vertical inhibition of both MET (with a TKI) and its ligand (with an HGF neutralizing antibody) was able to overcome resistance to MET TKIs (Pennacchietti et al., 2014).

A critical concept emerging from this work is that, during the acquisition of drug resistance, a causative expression rewiring may occur not only in cancer cells but also in the tumor microenvironment. We showed that increased lactate release, due to a metabolic switch induced by drug treatment in cancer cells, is able to increase the expression of lactate transporters, glycolytic enzymes, and negative regulators of the tricarboxylic acid cycle in CAFs and to stably instruct them to overexpress HGF, a cytokine known to protect both normal and cancer cells from apoptosis induced by many stimuli (Carrolo et al., 2003; Pennacchietti et al., 2003; Zeng et al., 2002). It is well established that metabolic plasticity is an effective strategy exploited by cancer cells to counteract environmental- or drug-induced stresses (Martinez-Outschoorn et al., 2017). CAFs, one of the major components of the tumor microenvironment, profoundly affect metabolic energetics of cancer cells, engaging a symbiotic relationship with them (Bonuccelli et al., 2010; Fiaschi et al., 2012; Martinez-Outschoorn et al., 2014; Salem et al., 2012; Sousa et al., 2016). As recently reported, metabolic coupling between different cell populations of the tumor, with complementary metabolic profiles, can also sustain the acquisition of resistance to anti-angiogenic treatments (Allen et al., 2016; Jiménez-Valerio et al., 2016; Pisarsky et al., 2016).

An important open question is whether any condition causing a glycolytic switch in tumors (such as hypoxia or treatment with drugs such as metformin) could lead to acquisition of resistance to TKIs in solid tumors. In preliminary *in vivo* experiments, we observed that a few weeks of treatment with metformin was not sufficient per se to modify the response to TKIs (Methods S1). Moreover, in the human resistant cancer samples analyzed, we did not find any correlation between HGF production and local hypoxia (Figure S7B), suggesting that hypoxia is not playing a crucial role in this model of resistance. Future studies are required to comprehensively answer all these questions.

Another key point is that the identified mechanism of adaptive resistance is not based on genetic alterations preexisting in the tumor cells. Recently, it has been demonstrated that drug-tolerant cells without bona fide resistance mechanisms may survive initial drug treatment by epigenetic adaptation and further evolve by acquiring genetic resistance mechanisms (Hata et al., 2016). In line with this, lactate/HGF adaptive resistance may represent an intermediate (and possibly reversible) step toward acquisition of a cell-autonomous resistant phenotype, allowing cancer cells to survive initial therapy to generate a reservoir from which the onset of genetic lesions can lead to an irreversible condition of resistance.

Currently, acquired resistance to EGFR TKIs remains unexplained in about one-third of EGFR-mutation-positive NSCLC patients and in a similar proportion of RAS and BRAF WT metastatic colorectal cancer patients receiving anti-EGFR monoclonal antibodies (Pietrantonio et al., 2017). How many of these tumors do rely on the lactate/HGF axis for their survival? The currently analyzed patient series is too small to derive any robust conclusion on its incidence in resistant patients. Remarkably, stromal HGF was reported to induce innate resistance to inhibitors of a range of kinases other than MET and EGFR, such as HER2, FGFR, ALK, PDGFR, and BRAF (Straussman et al., 2012;

Wilson et al., 2012). Interestingly, we observed lactate increase upon prolonged *in vitro* treatments with ROS1 TKIs in ROS1-addicted cells or HER2 TKIs in HER2-addicted cancer cells (Figure S6E). These results suggest that the described model of resistance could develop in a broad range of tumors and that it could be extended beyond EGFR and MET addiction, further expanding the possible clinical usefulness of our findings.

The fact that increased lactate release can indirectly sustain resistance to different targeted therapies might have profound therapeutic implications. Since tumor lactate levels also correlate with increased metastasis, tumor recurrence, and poor outcome, drugs targeting lactate metabolism have been studied in numerous preclinical trials and are now in clinical development (reviewed in Martinez-Outschoorn et al., 2017). In particular, LDH A is considered a safe therapeutic target in humans since hereditary loss of LDH A results in mild symptoms of exertional myopathy (Kanno et al., 1988); moreover, MCT1 inhibitors have already entered clinical investigation (NCT01791595). In our work, we demonstrated that different players of the lactate axis (both lactate producer and lactate transporters) are crucial for the establishment and maintenance of non-cell-autonomous resistance. These data open new possible applications for LDH, MCT4, and MCT1 inhibitors in preventing/overcoming resistance onset. Alternatively, our data suggest the benefit of combining EGFR TKIs with anti-MET treatments in order to overcome or even prevent resistance onset in a subset of patients not showing MET genetic alterations. In conclusion, our results pave the way for novel combinatorial treatments, simultaneously targeting lactate metabolism—at different levels—and a driver oncogene. Future work is needed to test these therapeutic options, which might show higher long-term efficacy and lower probability of resistance onset compared with classic TKI associations.

Limitations of the Study

Using *in vivo* xenograft models, we identified a metabolism-based mechanism of adaptive resistance, in which increased lactate production induced by MET/EGFR TKI treatment instructs CAFs to overproduce HGF, eventually enforcing drug resistance and tumor progression. Increased production of tumor lactate and stromal HGF was detected not only in the analyzed experimental models but also in advanced NSCLC patients upon emergence of resistance to EGFR TKIs, thus confirming the clinical relevance of our findings. Although *in vitro* results suggest that the model of resistance described could develop in a broad range of tumors, future experiments are needed to assess how widely it takes place in patients. Moreover, it is not known if, under the same conditions, this adaptive resistance can be only transient, allowing the selection of genetically based mechanisms of resistance.

It also needs to be considered that all the *in vivo* experiments shown were performed in immunocompromised mice. As cells of the immune system can respond to HGF, the effect of the increased amount of this ligand in the microenvironment could modulate and possibly affect the activity of the immune system. This aspect could not be analyzed in the present work.

Acknowledgments

We thank all our colleagues for helpful scientific discussion; Barbara Martinoglio and Roberta Porporato for providing technical support with real-time PCR and in situ hybridization; Paola Bernabei for technical support with FACS and sorting experiments; Stefania Giove for support with histological preparations; Francesco Fesi for histochemical analysis; Alberto Puliafito for RNAscope quantification; employees of the animal facility; Dr. Carlos Sebastian for help with metabolic experiments; Dr. Annalisa Petrelli for support in RNAi experiments; Dr. Natale for critical reading of the manuscript. JNJ-605 was kindly provided by Janssen. This work was funded by the University of Torino, Fondo Ricerca Locale 2013 to S.C.; PROGETTO ATENEO/Compagnia San Paolo 2016, University of Torino to S.C.; the Italian Association for Cancer Research (AIRC), IG grant 20210 to S.G.; Fondazione Piemontese per la Ricerca sul Cancro (ONLUS) 5 X 1000 Fondi Ministero della Salute 2013 to A.S. and 2014 to S.G.; M.A. is a recipient of a Fondazione Veronesi Research Fellowship.

Author Contributions

Conceptualization, S.G., M.A., S.C., P.C., and D.P.; Methodology, M.A., E.G., S.F., and K.J.F.; Investigation and Validation, M.A., E.G., S.F., and K.J.F.; Formal Analysis, M.A., E.G., S.F., P.C., E.M., C.I., and D.F.-P.; Writing – Original Draft, S.G., M.A., and S.C.; Writing – Review & Editing, S.G., M.A., S.C., E.G., S.F., C.I., C.G., F.M., A.S., P.M.C., E.M., F.P., M.V., P.C., K.J.F., D.P., D.F.-P.; Supervision, S.G. and S.C.; Resources, C.G., F.M., F.P., M.V., and A.S.; Project Administration, S.G. and S.C.; Funding Acquisition: S.G., S.C., and A.S.

Declaration of Interests

C.G. and F.M. are co-inventors of patents owned by the University of Pisa covering inhibitors of LDH and of cellular lactate production.

Supplemental Information

Supplemental Information includes seven figures and one method and can be found with this article online at <https://doi.org/10.1016/j.cmet.2018.08.006>.

References

- Allen E., Miéville P., Warren C.M., Saghafinia S., Li L., Peng M.W. and Hanahan D., Metabolic symbiosis enables adaptive resistance to anti-angiogenic therapy that is dependent on mTOR signaling, *Cell Rep.* **15**, 2016, 1144–1160.
- Arbiser J.L., Raab G., Rohan R.M., Paul S., Hirschi K., Flynn E., Price E.R., Fisher D.E., Cohen C. and Klagsbrun M., Isolation of mouse stromal cells associated with a human tumor using differential diphtheria toxin sensitivity, *Am. J. Pathol.* **155**, 1999, 723–729.
- Bacci M., Giannoni E., Fearn A., Ribas R., Gao Q., Taddei M.L., Pintus G., Dowsett M., Isacke C.M., Martin L.A., et al., miR-155 drives metabolic reprogramming of ER+ breast cancer cells following long-term estrogen deprivation and predicts clinical response to aromatase inhibitors, *Cancer Res.* **76**, 2016, 1615–1626.
- Bhowmick N.A., Neilson E.G. and Moses H.L., Stromal fibroblasts in cancer initiation and progression, *Nature* **432**, 2004, 332–337.
- Bonuccelli G., Tsigos A., Whitaker-Menezes D., Pavlides S., Pestell R.G., Chiavarina B., Frank P.G., Flomenberg N., Howell A., Martinez-Outschoorn U.E., et al., Ketones and lactate "fuel" tumor growth and metastasis: evidence that epithelial cancer cells use oxidative mitochondrial metabolism, *Cell Cycle* **9**, 2010, 3506–3514.
- Bottaro D.P., Rubin J.S., Faletto D.L., Chan A.M., Kmieciak T.E., Vande Woude G.F. and Aaronson S.A., Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product, *Science* **251**, 1991, 802–804.
- Calvaresi E.C., Granchi C., Tuccinardi T., Di Bussolo V., Huigens R.W., Lee H.Y., Palchaudhuri R., Macchia M., Martinelli A., Minutolo F., et al., Dual targeting of the Warburg effect with a glucose-conjugated lactate dehydrogenase inhibitor, *ChemBioChem* **14**, 2013, 2263–2267.
- Camidge D.R., Pao W. and Sequist L.V., Acquired resistance to TKIs in solid tumours: learning from lung cancer, *Nat. Rev. Clin. Oncol.* **11**, 2014, 473–481.
- Carbone C., Moccia T., Zhu C., Paradiso G., Budillon A., Chiao P.J., Abbruzzese J.L. and Melisi D., Anti-VEGF treatment-resistant pancreatic cancers secrete proinflammatory factors that contribute to malignant progression by inducing an EMT cell phenotype, *Clin. Cancer Res.* **17**, 2011, 5822–5832.
- Carrolo M., Giordano S., Cabrita-Santos L., Corso S., Vigário A.M., Silva S., Leirião P., Carapau D., Armas-Portela R., Comoglio P.M., et al., Hepatocyte growth factor and its receptor are required for malaria infection, *Nat. Med.* **9**, 2003, 1363–1369.
- Cecchi F., Lih C.J., Lee Y.H., Walsh W., Rabe D.C., Williams P.M. and Bottaro D.P., Expression array analysis of the hepatocyte growth factor invasive program, *Clin. Exp. Metastasis* **32**, 2015, 659–676.
- Cepero V., Sierra J.R., Corso S., Ghiso E., Casorzo L., Perera T., Comoglio P.M. and Giordano S., MET and KRAS gene amplification mediates acquired resistance to MET tyrosine kinase inhibitors, *Cancer Res.* **70**, 2010, 7580–7590.
- Chong C.R. and Jänne P.A., The quest to overcome resistance to EGFR-targeted therapies in cancer, *Nat. Med.* **19**, 2013, 1389–1400.
- Comoglio P.M., Trusolino L. and Boccaccio C., Known and novel roles of the MET oncogene in cancer: a coherent approach to targeted therapy, *Nat. Rev. Cancer* **18**, 2018, 341–358.
- Corso S., Ghiso E., Cepero V., Sierra J.R., Migliore C., Bertotti A., Trusolino L., Comoglio P.M. and Giordano S., Activation of HER family members in gastric carcinoma cells mediates resistance to MET inhibition, *Mol. Cancer* **9**, 2010, 121.
- Cross D.A., Ashton S.E., Ghorghiu S., Eberlein C., Nebhan C.A., Spitzler P.J., Orme J.P., Finlay M.R., Ward R.A., Mellor M.J., et al., AZD9291, an irreversible EGFR TKI, overcomes T790M-mediated resistance to EGFR inhibitors in lung cancer, *Cancer Discov.* **4**, 2014, 1046–1061.
- Cui J.J., Targeting receptor tyrosine kinase MET in cancer: small molecule inhibitors and clinical progress, *J. Med. Chem.* **57**, 2014, 4427–4453.
- Curtarello M., Zulato E., Nardo G., Valtorta S., Guzzo G., Rossi E., Esposito G., Msaki A., Pastò A., Rasola A., et al., VEGF-targeted therapy stably modulates the glycolytic phenotype of tumor cells, *Cancer Res.* **75**, 2015, 120–133.
- Dillon R., Nilsson C.L., Shi S.D., Lee N.V., Krastins B. and Greig M.J., Discovery of a novel B-Raf fusion protein related to c-Met drug resistance, *J. Proteome Res.* **10**, 2011, 5084–5094.
- Engelman J.A. and Settleman J., Acquired resistance to tyrosine kinase inhibitors during cancer therapy, *Curr. Opin. Genet. Dev.* **18**, 2008, 73–79.
- Fiaschi T., Marini A., Giannoni E., Taddei M.L., Gandellini P., De Donatis A., Lanciotti M., Serni S., Cirri P. and Chiarugi P., Reciprocal metabolic reprogramming through lactate shuttle coordinately influences tumor-stroma interplay, *Cancer Res.* **72**, 2012, 5130–5140.
- Gherardi E., Birchmeier W., Birchmeier C. and Vande Woude G., Targeting MET in cancer: rationale and progress, *Nat. Rev. Cancer* **12**, 2012, 89–103.

Giordano S., Ponzetto C., Di Renzo M.F., Cooper C.S. and Comoglio P.M., Tyrosine kinase receptor indistinguishable from the c-met protein, *Nature* **339**, 1989, 155–156.

Harbinski F., Craig V.J., Sanghavi S., Jeffery D., Liu L., Sheppard K.A., Wagner S., Stamm C., Bunes A., Chatenay-Rivauday C., et al., Rescue screens with secreted proteins reveal compensatory potential of receptor tyrosine kinases in driving cancer growth, *Cancer Discov.* **2**, 2012, 948–959.

Harrison P.M. and Farzaneh F., Regulation of HGF/SF gene expression in MRC-5 cells by N-acetylcysteine, *Biochem. Biophys. Res. Commun.* **279**, 2000, 108–115.

Hata A.N., Niederst M.J., Archibald H.L., Gomez-Caraballo M., Siddiqui F.M., Mulvey H.E., Maruvka Y.E., Ji F., Bhang H.E., Krishnamurthy Radhakrishna V., et al., Tumor cells can follow distinct evolutionary paths to become resistant to epidermal growth factor receptor inhibition, *Nat. Med.* **22**, 2016, 262–269.

Hu Y., Yan C., Mu L., Huang K., Li X., Tao D., Wu Y. and Qin J., Fibroblast-derived exosomes contribute to chemoresistance through priming cancer stem cells in colorectal cancer, *PLoS One* **10**, 2015, e0125625.

Jiménez-Valerio G., Martínez-Lozano M., Bassani N., Vidal A., Ochoa-de-Olza M., Suárez C., García-Del-Muro X., Carles J., Viñals F., Graupera M., et al., Resistance to antiangiogenic therapies by metabolic symbiosis in renal cell carcinoma PDX models and patients, *Cell Rep.* **15**, 2016, 1134–1143.

Kanno T., Sudo K., Maekawa M., Nishimura Y., Ukita M. and Fukutake K., Lactate dehydrogenase M-subunit deficiency: a new type of hereditary exertional myopathy, *Clin. Chim. Acta* **173**, 1988, 89–98.

Lai I.L., Chou C.C., Lai P.T., Fang C.S., Shirley L.A., Yan R., Mo X., Bloomston M., Kulp S.K., Bekaii-Saab T., et al., Targeting the Warburg effect with a novel glucose transporter inhibitor to overcome gemcitabine resistance in pancreatic cancer cells, *Carcinogenesis* **35**, 2014, 2203–2213.

Lennerz J.K., Kwak E.L., Ackerman A., Michael M., Fox S.B., Bergethon K., Lauwers G.Y., Christensen J.G., Wilner K.D., Haber D.A., et al., MET amplification identifies a small and aggressive subgroup of esophagogastric adenocarcinoma with evidence of responsiveness to crizotinib, *J. Clin. Oncol.* **29**, 2011, 4803–4810.

Lovly C.M. and Shaw A.T., Molecular pathways: resistance to kinase inhibitors and implications for therapeutic strategies, *Clin. Cancer Res.* **20**, 2014, 2249–2256.

Maemondo M., Inoue A., Kobayashi K., Sugawara S., Oizumi S., Isobe H., Gemma A., Harada M., Yoshizawa H., Kinoshita I., et al., Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR, *N. Engl. J. Med.* **362**, 2010, 2380–2388.

Martinez-Outschoorn U.E., Lisanti M.P. and Sotgia F., Catabolic cancer-associated fibroblasts transfer energy and biomass to anabolic cancer cells, fueling tumor growth, *Semin. Cancer Biol.* **25**, 2014, 47–60.

Martinez-Outschoorn U.E., Peiris-Pagés M., Pestell R.G., Sotgia F. and Lisanti M.P., Cancer metabolism: a therapeutic perspective, *Nat. Rev. Clin. Oncol.* **14**, 2017, 113.

Meads M.B., Gatenby R.A. and Dalton W.S., Environment-mediated drug resistance: a major contributor to minimal residual disease, *Nat. Rev. Cancer* **9**, 2009, 665–674.

Mok T.S., Wu Y.L., Ahn M.J., Garassino M.C., Kim H.R., Ramalingam S.S., Shepherd F.A., He Y., Akamatsu H., Theelen W.S., et al., Osimertinib or platinum-pemetrexed in EGFR T790M-positive lung cancer, *N. Engl. J. Med.* **376**, 2017, 629–640.

Mok T.S., Wu Y.L., Thongprasert S., Yang C.H., Chu D.T., Saijo N., Sunpaweeavong P., Han B., Margono B., Ichinose Y., et al., Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma, *N. Engl. J. Med.* **361**, 2009, 947–957.

Moore M.J., Goldstein D., Hamm J., Figer A., Hecht J.R., Gallinger S., Au H.J., Murawa P., Walde D., Wolff R.A., et al., Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group, *J. Clin. Oncol.* **25**, 2007, 1960–1966.

Ohashi K., Maruvka Y.E., Michor F. and Pao W., Epidermal growth factor receptor tyrosine kinase inhibitor-resistant disease, *J. Clin. Oncol.* **31**, 2013, 1070–1080.

Pasini D., Malatesta M., Jung H.R., Walfridsson J., Willer A., Olsson L., Skotte J., Wutz A., Porse B., Jensen O.N., et al., Characterization of an antagonistic switch between histone H3 lysine 27 methylation and acetylation in the transcriptional regulation of Polycomb group target genes, *Nucleic Acids Res.* **38**, 2010, 4958–4969.

Pennacchietti S., Cazzanti M., Bertotti A., Rideout W.M., Han M., Gyuris J., Perera T., Comoglio P.M., Trusolino L. and Michieli P., Microenvironment-derived HGF overcomes genetically determined sensitivity to anti-MET drugs, *Cancer Res.* **74**, 2014, 6598–6609.

Pennacchietti S., Michieli P., Galluzzo M., Mazzone M., Giordano S. and Comoglio P.M., Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene, *Cancer Cell* **3**, 2003, 347–361.

Peters S. and Adjei A.A., MET: a promising anticancer therapeutic target, *Nat. Rev. Clin. Oncol.* **9**, 2012, 314–326.

Pietrantonio F., Vernieri C., Siravegna G., Mennitto A., Berenato R., Perrone F., Gloghini A., Tamborini E., Lonardi S., Morano F., et al., Heterogeneity of acquired resistance to anti-EGFR monoclonal antibodies in patients with metastatic colorectal cancer, *Clin. Cancer Res.* **23**, 2017, 2414–2422.

Pisarsky L., Bill R., Fagiani E., Dimeloe S., Goosen R.W., Hagmann J., Hess C. and Christofori G., Targeting metabolic symbiosis to overcome resistance to anti-angiogenic therapy, *Cell Rep.* **15**, 2016, 1161–1174.

Puri N. and Salgia R., Synergism of EGFR and c-Met pathways, cross-talk and inhibition, in non-small cell lung cancer, *J. Carcinog.* **7**, 2008, 9.

Qi J., McTigue M.A., Rogers A., Lifshits E., Christensen J.G., Jänne P.A. and Engelman J.A., Multiple mutations and bypass mechanisms can contribute to development of acquired resistance to MET inhibitors, *Cancer Res.* **71**, 2011, 1081–1091.

Rosell R., Carcereny E., Gervais R., Vergnenegre A., Massuti B., Felip E., Palmero R., Garcia-Gomez R., Pallares C., Sanchez J.M., et al., Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial, *Lancet Oncol.* **13**, 2012, 239–246.

Salem A.F., Whitaker-Menezes D., Lin Z., Martinez-Outschoorn U.E., Tanowitz H.B., Al-Zoubi M.S., Howell A., Pestell R.G., Sotgia F. and Lisanti M.P., Two-compartment tumor metabolism: autophagy in the tumor microenvironment and oxidative mitochondrial metabolism (OXPHOS) in cancer cells, *Cell Cycle* **11**, 2012, 2545–2556.

Sequist L.V., Waltman B.A., Dias-Santagata D., Digumarthy S., Turke A.B., Fidias P., Bergethon K., Shaw A.T., Gettinger S., Cospser A.K., et al., Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors, *Sci. Transl. Med.* **3**, 2011, 75ra26.

Shiao S.L., Ganesan A.P., Rugo H.S. and Coussens L.M., Immune microenvironments in solid tumors: new targets for therapy, *Genes Dev.* **25**, 2011, 2559–2572.

Smolen G.A., Sordella R., Muir B., Mohapatra G., Barmettler A., Archibald H., Kim W.J., Okimoto R.A., Bell D.W., Sgroi D.C., et al., Amplification of MET may identify a subset of cancers with extreme sensitivity to the selective tyrosine kinase inhibitor PHA-665752, *Proc. Natl. Acad. Sci. USA* **103**, 2006, 2316–2321.

Sommer C., Strähle C., Köthe U., and Hamprecht F.A. (2011). *ilastik: interactive learning and segmentation toolkit*. Proceedings of the Eighth IEEE International Symposium on Biomedical Imaging (ISBI). IEEE. 230–233.

Sousa C.M., Biancur D.E., Wang X., Halbrook C.J., Sherman M.H., Zhang L., Kremer D., Hwang R.F., Witkiewicz A.K., Ying H., et al., Pancreatic stellate cells support tumour metabolism through autophagic alanine secretion, *Nature* **536**, 2016, 479–483.

Straussman R., Morikawa T., Shee K., Barzily-Rokni M., Qian Z.R., Du J., Davis A., Mongare M.M., Gould J., Frederick D.T., et al., Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion, *Nature* **487**, 2012, 500–504.

Végran F., Boidot R., Michiels C., Sonveaux P. and Feron O., Lactate influx through the endothelial cell monocarboxylate transporter MCT1 supports an NF- κ B/IL-8 pathway that drives tumor angiogenesis, *Cancer Res.* **71** 2011, 2550–2560.

Wilson T.R., Fridlyand J., Yan Y., Penuel E., Burton L., Chan E., Peng J., Lin E., Wang Y., Sosman J., et al., Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors, *Nature* **487**, 2012, 505–509.

Yin J., Lee J.H., Zhang J., Gao Z., Polotsky V.Y. and Ye J., Regulation of hepatocyte growth factor expression by NF- κ B and PPAR γ in adipose tissue, *Am. J. Physiol. Endocrinol. Metab.* **306**, 2014, E929–E936.

Yu H.A., Arcila M.E., Rekhtman N., Sima C.S., Zakowski M.F., Pao W., Kris M.G., Miller V.A., Ladanyi M. and Riely G.J., Analysis of tumor specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with EGFR-mutant lung cancers, *Clin. Cancer Res.* **19**, 2013, 2240–2247.

Zeng Q., Chen S., You Z., Yang F., Carey T.E., Saims D. and Wang C.Y., Hepatocyte growth factor inhibits anoikis in head and neck squamous cell carcinoma cells by activation of ERK and Akt signaling independent of NF κ B, *J. Biol. Chem.* **277**, 2002, 25203–25208.

Zhang J. and Liu J., Tumor stroma as targets for cancer therapy, *Pharmacol. Ther.* **137**, 2013, 200–215.

Zhou C., Wu Y.L., Chen G., Feng J., Liu X.Q., Wang C., Zhang S., Wang J., Zhou S., Ren S., et al., Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): a multicentre, open-label, randomised, phase 3 study, *Lancet Oncol.* **12**, 2011, 735–742.

Zhou M., Zhao Y., Ding Y., Liu H., Liu Z., Fodstad O., Riker A.I., Kamarajugadda S., Lu J., Owen L.B., et al., Warburg effect in chemosensitivity: targeting lactate dehydrogenase-A re-sensitizes taxol-resistant cancer cells to taxol, *Mol. Cancer* **9**, 2010, 33.

E-Extra

Keywords: HGF/MET; EGFR; targeted therapy; resistance; lactate; LDH; MCT1/4; tumor metabolism; CAFs; tumor microenvironment

Experimental Model and Subject Details

Animal Studies

Animal handling and experimentation was performed in accordance with the European Union directives and the Italian Guidelines for Care and Use of Laboratory Animals. All animal procedures were approved by the Ethical Commission of the IRCC in Candiolo and the Italian Ministry of Health. All experiments were performed in 7-8 weeks old female mice NOD SCID mice purchased by Charles River (Milan, Italy) maintained on a 12h light/dark cycle at 22°C. In each animal experiment, mice were randomly assigned to each group.

Cell Culture

The EBC1 (derived from a lung squamous cell carcinoma and purchased from the Japan Cancer Resource Bank), GTL16 (Smolen et al., 2006), HCC827 (Lung Adenocarcinoma cell line, from ATCC, carrying delE746-A750 and EGFR amplification) cell lines were cultured in RPMI medium 1640 (Sigma-Aldrich; Saint Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μ g/mL streptomycin. HCC78 cells (carrying SLC34A2-ROS1 gene rearrangement), were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) cell bank. The EGFR mutant NSCLC cell line HCC-4006 (carrying delE746-A750) and the breast cancer cell line SKBR3 were obtained from ATCC. An internal batch of PC-9 lung adenocarcinoma cells (kindly given by Prof. W. Pao (Cross et al., 2014)) cells was re-authenticated soon before experimental application.

The genetic identity of the cells has been verified in 2015, 2017 and 2018 by short tandem repeat profiling (Cell ID, Promega, Madison, WI, USA). Mycoplasma testing is routinely performed using the PCR Mycoplasma Detection Kit (Applied Biological Materials Inc., Richmond, BC, Canada). Verified cell lines are generally thawed few weeks before the experiments and kept in culture for 3-6 months. Tumor and CAF cells derived ex-vivo from EBC1, GTL16 and HCC827 xenografts were cultured in RPMI-1640, supplemented as described before.

Patients' Tumor FFPE Analysis

The study was conducted according to guidelines and regulations by the Research Ethics Committee of the AOU San Luigi/University of Turin and of Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, as explicated by formal approval to M.V and to F.P. of current projects regulating the use of retrospective solid tumor tissues (see Ethics Committee Approvals n.167/2015, prot.17975, 14/11/2015 and n.204/2016, prot.20840, 22/12/2016; protocol INT 117/12). Before the analysis, the samples have been anonymized by staff members of the two hospitals, not involved in the project. No references to the patients can be inferred from the characterization presented in the work.

Methods S1 presents the in vivo treatment with Metformin and MET/EGFR TKIs (related to the Discussion section). (Please introduce a line-spacing to better separate this paragraph from the previous one.)

Method Details

Animal Tumor Models and Compounds

1x10⁶ EBC1/GTL16/HCC827 cells were injected subcutaneously into the right posterior flanks of six week old immunodeficient NOD SCID female mice. When tumors reached a volume of around 200-500 mm³, mice were treated with JNJ-605 or Crizotinib or Erlotinib at the maximum tolerated dose (50 mg/kg). Both drugs were administered daily by oral gavages. Tumor size was evaluated weekly by caliper measurements and approximate volume of the mass was calculated using the formula $\frac{4}{3} \pi (D/2) (d/2)^2$, where d is the minor tumour axis and D is the major tumour axis. The MET inhibitor JNJ-605 was provided by Janssen Pharmaceutica NV. Crizotinib (PF-02341066), LAPATINIB (FC12433) and Erlotinib Mesylate (FE65018) were purchased from Carbosynth (Compton, Berkshire, UK).

For reinjection experiments, 1x10⁶ EBC1 cells (WT, RES-J, RES-J shCTRL, RES-J shLDHA/B #1, RES-J shLDHA/B #2) and 1x10⁶ GTL16 cells (WT and RES-J), 1x10⁶ HCC827 cells (WT, RES-E, RES-E shCTRL, RES-E shLDHA/B #1, RES-E shLDHA/B #2) were subcutaneously injected into the right posterior flanks of six week old immunodeficient NOD SCID female mice. Mice were treated for the indicated days with the following regimens, (either single agent or in combination): LDH-inhibitor treatment (50 mg/kg) three times a week, by intraperitoneal injection; JNJ-605 (50 mg/kg) daily, ERLOTINIB (50 mg/kg) daily, AZD3965 (100 mg/kg) daily, Metformin (50mg/kg) daily, all of them by oral gavages. Tumour size was evaluated weekly by caliper measurements and approximate volume of the mass was calculated as described before. Experiments were not performed in blind.

Ex-Vivo Cell Cultures

WT and RES-J/C/E EBC1/GTL16/HCC827 cells were derived from tumours after digestion with collagenase 1 (Sigma). After one hour incubation at 37° in a shaking incubator, cells were centrifuged. The pellet was resuspended in L-15 medium (Sigma) supplemented with 10% FBS (Sigma) and

cells were incubated for 5 minutes with DNase (Sigma). Cells were washed in L-15 medium, centrifuged and the pellet was resuspended in 5mL red blood cells lysis buffer.

After 5 minutes incubation, cells were centrifuged, plated in a cell culture plate and cultured in RPMI-1640, supplemented as described before. WT and RES-CAF were derived from EBC1, GTL16 and HCC827 tumors as previously described. To obtain pure fibroblast cultures, cells were treated with 10 ng/ml diphtheria toxin (Sigma) for 1 week. Fibroblasts were cultured in RPMI-1640 supplemented as described before.

Cell Viability Assays

For growth curve and cell viability assay, cells were seeded in triplicates in 96-well culture plates (1500-2000 cells/well), in the presence of drugs. In each well, cells were seeded in 50µl 10% FBS medium and either 100µl of fibroblasts conditioned medium or serum free medium were added. 50 µl of serum free medium containing the drug at the indicated concentrations were added in each well. After 72 hours, cell viability was measured by using Cell Titer-Glo Luminescent Cell Viability Assay (Promega), which directly measures the intracellular ATP content, resulting in quantification of the number of healthy cells in culture.

In Vitro Co-culture Experiments

For the in vitro co-culture experiments parental EBC1 cells transduced with a lentiviral vector containing the luciferase cDNA and CAFs derived from wild type or resistant tumors were plated in a 96-well culture plates at 1:3 ratio. Cells were treated for 72h with JNJ-605 at the indicated doses and cell viability was assessed by adding luciferin. The bioluminescent reaction was quantified using VICTOR X Multilabel Plate Readers (PerkinElmer).

In Vivo Co-culture Experiments

Wild type tumors and tumors resistant to treatment with JNJ-605 were generated as described before. When tumors reached a volume of 400 mm³, 1x10⁶ EBC1 cells transduced with a lentiviral vector containing the luciferase cDNA were inoculated into preformed tumors. Five resistant tumors and five wild type tumors were treated with JNJ-605 at the maximum tolerated dose, while other five wild type tumors were treated with the vehicle, as controls. JNJ-605 was administered daily by oral gavages and luciferase signal (from parental sensitive cells) was measured at the end of the experiment (6 weeks) using IVIS Spectrum (PerkinElmer). Experiments were not performed in blind.

CAF Treatment with Exogenous Lactate

WT CAFs (1,5x10⁶ cells/60cm² culture dishes) were grown in complete medium for 24h and then serum-starved and treated with increasing concentrations of sodium L-Lactate (#71718 Sigma-Aldrich) for 72 h.

Treatment with NF-κB Inhibitors

WT and RES-J CAFs were treated with 1µM of IKK-16 (Santa Cruz) and 1µM BMS-345541 (SIGMA) for 48h, in presence or in absence of Lactate as described before. After that, murine-HGF concentration in cell culture supernatants was quantified through the ELISA assay. For cell viability assay on EBC1 cells confluent RES-J CAFs or EBC1 cells were treated with 1µM of NF-κB inhibitors for 72h and then the media were collected and used on the parental EBC1 for the cell viability assay.

Protein Extraction and Western Blot

For Western blot analysis, cells were plated in 6-well plates, allowing them to reach 50-60% confluence. Cells were grown in the presence of 10% FBS RPMI medium, conditioned medium of wild type fibroblasts or supernatant of resistant

Animal Tumor Models and Compounds

1x10⁶ EBC1/GTL16/HCC827 cells were injected subcutaneously into the right posterior flanks of six week old immunodeficient NOD SCID female mice. When tumors reached a volume of around 200-500 mm³, mice were treated with JNJ-605 or Crizotinib or Erlotinib at the maximum tolerated dose (50 mg/kg). Both drugs were administered daily by oral gavages. Tumor size was evaluated weekly by caliper measurements and approximate volume of the mass was calculated using the formula $\frac{4}{3} \pi (D/2) (d/2)^2$, where d is the minor tumour axis and D is the major tumour axis. The MET inhibitor JNJ-605 was provided by Janssen Pharmaceutica NV. Crizotinib (PF-02341066), LAPATINIB (FC12433) and Erlotinib Mesylate (FE65018) were purchased from Carbosynth (Compton, Berkshire, UK).

For reinjection experiments, 1x10⁶ EBC1 cells (WT, RES-J, RES-J shCRTL, RES-J shLDHA/B #1, RES-J shLDHA/B #2) and 1x10⁶ GTL16 cells (WT and RES-J), 1x10⁶ HCC827 cells (WT, RES-E, RES-E shCRTL, RES-E shLDHA/B #1, RES-E shLDHA/B #2) were subcutaneously injected into the right posterior flanks of six week old immunodeficient NOD SCID female mice. Mice were treated for the indicated days with the following regimens, (either single agent or in combination): LDH-inhibitor treatment (50 mg/kg) three times a week, by intraperitoneal injection; JNJ-605 (50 mg/kg) daily, ERLOTINIB (50 mg/kg) daily, AZD3965 (100 mg/kg) daily, Metformin (50mg/kg) daily, all of them by oral gavages. Tumour size was evaluated weekly by caliper measurements and approximate volume of the mass was calculated as described before. Experiments were not performed in blind.

Ex-Vivo Cell Cultures

WT and RES-J/C/E EBC1/GTL16/HCC827 cells were derived from tumours after digestion with collagenase 1 (Sigma). After one hour incubation at 37° in a shaking incubator, cells were centrifuged. The pellet was resuspended in L-15 medium (Sigma) supplemented with 10% FBS (Sigma) and cells were incubated for 5 minutes with DNase (Sigma). Cells were washed in L-15 medium, centrifuged and the pellet was resuspended in 5mL red blood cells lysis buffer. After 5 minutes incubation, cells were centrifuged, plated in a cell culture plate and cultured in RPMI-1640, supplemented as described before. WT and RES-CAF were derived from EBC1, GTL16 and HCC827 tumors as previously described. To obtain pure fibroblast cultures, cells were treated with 10 ng/ml diphtheria toxin (Sigma) for 1 week. Fibroblasts were cultured in RPMI-1640 supplemented as described before.

Cell Viability Assays

For growth curve and cell viability assay, cells were seeded in triplicates in 96-well culture plates (1500-2000 cells/well), in the presence of drugs. In each well, cells were seeded in 50µl 10% FBS medium and either 100µl of fibroblasts conditioned medium or serum free medium were added. 50 µl of serum free medium containing the drug at the indicated concentrations were added in each well. After 72 hours, cell viability was measured by using Cell Titer-Glo Luminescent Cell Viability Assay (Promega), which directly measures the intracellular ATP content, resulting in quantification of the number of healthy cells in culture.

In Vitro Co-culture Experiments

For the in vitro co-culture experiments parental EBC1 cells transduced with a lentiviral vector containing the luciferase cDNA and CAFs derived from wild type or resistant tumors were plated in a 96-well culture plates at 1:3 ratio. Cells were treated for 72h with JNJ-605 at the indicated doses and cell viability was assessed by adding luciferin. The bioluminescent reaction was quantified using VICTOR X Multilabel Plate Readers (PerkinElmer).

In Vivo Co-culture Experiments

Wild type tumors and tumors resistant to treatment with JNJ-605 were generated as described before. When tumors reached a volume of 400 mm³, 1x10⁶ EBC1 cells transduced with a lentiviral vector containing the luciferase cDNA were inoculated into preformed tumors. Five resistant tumors and five wild type tumors were treated with JNJ-605 at the maximum tolerated dose, while other five wild type tumors were treated with the vehicle, as controls. JNJ-605 was administered daily by oral gavages and luciferase signal (from parental sensitive cells) was measured at the end of the experiment (6 weeks) using IVIS Spectrum (PerkinElmer). Experiments were not performed in blind.

CAF Treatment with Exogenous Lactate

WT CAFs (1.5x10⁶ cells/60cm² culture dishes) were grown in complete medium for 24h and then serum-starved and treated with increasing concentrations of sodium L-Lactate (#71718 Sigma-Aldrich) for 72 h.

Treatment with NF- κ B Inhibitors

WT and RES-J CAFs were treated with 1 μ M of IKK-16 (Santa Cruz) and 1 μ M BMS-345541 (SIGMA) for 48h, in presence or in absence of Lactate as described before. After that, murine-HGF concentration in cell culture supernatants was quantified through the ELISA assay. For cell viability assay on EBC1 cells confluent RES-J CAFs or EBC1 cells were treated with 1 μ M of NF- κ B inhibitors for 72h and then the media were collected and used on the parental EBC1 for the cell viability assay.

Protein Extraction and Western Blot

For Western blot analysis, cells were plated in 6-well plates, allowing them to reach 50-60% confluence. Cells were grown in the presence of 10% FBS RPMI medium, conditioned medium of wild type fibroblasts or supernatant of resistant coupling reaction with phenylboronic acid. The following biphenyl derivative was transformed into a ketoester intermediate in the presence of potassium tert-butoxide and dimethyl oxalate. A subsequent reductive cyclization step with SnCl₂ in DME produced the N-hydroxyindole methyl ester. This compound was then condensed with a tetra-acetylated glucose moiety. Final deprotection of the hydroxyl groups with sodium methoxide in methanol produced NHI-Glc-2.

Chromatin Immunoprecipitation (ChIP) and Antibodies

Chromatin immunoprecipitation (ChIP) analyses were performed with the following antibodies: rabbit anti-H3 (Abcam, Cat. 1791), rabbit anti-H3K4me1 (Abcam, Cat. 8895), rabbit anti-H3K4me3 (Active Motif, Cat. 39159), rabbit anti-H3K27me3 (Cell Signaling, Cat. 9733), rabbit anti-H3K27ac (Active Motif, Cat. 39133), rabbit anti-H3K36me3 (Cell Signaling, Cat. 4909), rabbit anti-H3K9me3 (Abcam, Cat. 8898). Rabbit IgG (Sigma, Cat. I5006) was used as negative control in ChIP. ChIP assays were carried out as described previously (Pasini et al., 2010). Briefly, 1% formaldehyde cross-linked chromatin was fragmented by sonication to an average size of 300–500bp and incubated overnight in IP Buffer (33 mM Tris/HCl, pH 8, 100 mM NaCl, 5 mM EDTA, 0.2% Na₃N, 0.33% SDS, 1.66% Triton X-100) at 4°C with 3 μ g of the indicated antibodies, followed by incubation for 2 hr with protein A sepharose beads (GE Healthcare). Beads were washed three times with 150 washing buffer (20 mM Tris/HCl, pH 8, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1 % Triton X-100), once with 500 washing buffer (20 mM Tris/HCl, pH 8, 500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100), and finally re-suspended in 120 μ l of de-crosslinking solution (0.1 M NaHCO₃, 1% SDS).

RNA Sequencing mRNA-seq library preparation from 4 μ g of total RNA was performed with TruSeq RNA Sample Prep Kit V2 (Illumina) according to the manufacturer's instructions.

RNA-seq was carried out using the SMART-seq2 protocol (PMID:24385147) with minor modifications. Briefly, the poly-A containing mRNA molecules from 5 ng of total RNA were copied into first strand cDNA by reverse transcription and templateswitching using oligo(dT) primers and an LNA-containing template-switching oligo (TSO). The resulting cDNA was pre-amplified, purified and tagged with Tn5 transposase produced in-house using a described protocol (PMID:25079858). cDNA fragments generated after tagmentation were gap-repaired, enriched by PCR and purified to create the final cDNA library. Sequencing was carried out in a HiSeq 2000, single end mode, 50bp.

RNA-seq Analysis Demultiplexing was performed using bcl2fastq v2.19. Reads were aligned to mm9 genome using tophat (v2.1.1) with parameters --no-coverage-search --library-type fr-unstranded. Then, PCR duplicates were removed using picardTools (v1.62) (<http://broadinstitute.github.io/picard/>) and read counts per gene were calculated using Htseq-count (v0.7.2) (PMID:25260700) with parameters -q -t exon --stranded=no --mode=intersection-nonempty. Genes with 0 counts in all samples were removed and differential expression analysis was performed using DESeq2 (v3.7) (PMID:25516281) with default parameters. Genes with an absolute log₂FC \geq 2 and FDR \leq 0.1 were considered as differentially expressed. The list of upregulated and downregulated genes were loaded into Enrichr (PMID:27141961) to perform gene set enrichment analysis. Finally, read counts were transformed using rlog function (regularized logarithmic transformation) from DESeq2 and then transformed to Z-score for heatmap representation.

Quantification and Statistical Analysis

Stromal hHGF RNAscope Signal Quantification

To quantify the amount of positive RNAscope regions in the stromal (pan-cytokeratin negative) areas, 5 digital images/slide have been captured at 20x magnification. We performed image segmentation by means of Ilastik (Sommer et al., 2011). Briefly Ilastik was trained to recognize RNAscope positive regions (red hue regions), nuclei (blue hue stained for with hematoxylin), tumor cells (brown hue stained for pan-cytokeratin) and background. Once trained, the algorithm was applied to all images in the dataset. The output of Ilastik was a set of labeled images, which were post-processed as follows: holes in nuclei regions were filled; tumor and nuclei boundaries were smoothed by morphological closing. Segmented nuclei were filtered for size, in order to remove small segmentation leftovers. RNAscope positive regions enclosed within tumor regions were excluded from the computation and nuclei enclosed into brown regions were considered as tumor and therefore excluded from the analysis. The total image area corresponding to nuclei or RNAscope positive regions was computed. The amount of RNAscope positive divided by the total stromal nuclei area blue plus red area was used as a proxy of the amount of stromal HGF. Postprocessing and area fraction calculation was performed thanks to a custom-written Matlab (The Mathworks) algorithm. Both images capture and their analysis have been performed blind by two different people.

Images were captured with the AxiovisionLe software (Zeiss, Gottingen, Germany) using an Axio Zeiss Imager 2 microscope (Zeiss, Gottingen, Germany). MCT4 staining was scored blind by the pathologist using the H-score which is based on the intensity of the signal and on the percentage of cells stained, and results in a score ranging from 0 to 300.

Data and Software Availability

The accession number for the RNA-seq data reported in this paper is GSE115172 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE115172>). Original data are posted on Mendeley data at <https://data.mendeley.com/datasets/hx5jtytxr/draft?a=31cf116c-5e02-44ce-9bee-e19ca022e6e4>. <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE115172>; <https://data.mendeley.com/datasets/hx5jtytxr/draft?a=31cf116c-5e02-44ce-9bee-e19ca022e6e4>.

Statistical Analysis

Statistical testing was performed with GraphPAD PRISM Software 7.02, using: T-TEST, One-Way ANOVA or Two-way ANOVA (both followed by Bonferroni multiple comparisons experiments), as indicated in Figure Legends. Error bars in the figures represent the SD. Statistical significance: *p < 0,05; **p < 0,01; ***p < 0,001.

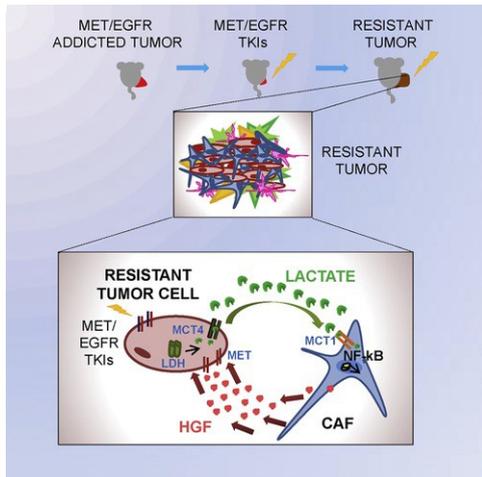
Supplemental Information

[Multimedia Component 1](#)

Document S1. Figures S1–S7 and Methods S1

[Multimedia Component 2](#)

Document S2. Article plus Supplemental Information



Highlights

- Lactate production is increased in MET/EGFR TKI-resistant cancer cells
- CAF lactate uptake stimulates HGF overexpression, driving adaptive resistance
- LDH, MCT4, and MCT1 inhibition abrogates in vivo adaptive resistance to MET/EGFR TKIs
- Stromal HGF and tumor cell MCT4 are increased in some EGFR TKI-resistant patients