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ORIGINAL RESEARCH

Acquisition of tumor cell phenotypic diversity along the EMT spectrum under hypoxic pressure: Consequences on susceptibility to cell-mediated cytotoxicity

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ABSTRACT

Tumor escape to immunosurveillance and resistance to immune attacks present a major hurdle in cancer therapy, especially in the current era of new cancer immunotherapies. We report here that hypoxia, a hallmark of most solid tumors, orchestrates carcinoma cell heterogeneity through the induction of phenotypic diversity and the acquisition of distinct epithelial–mesenchymal transition (EMT) states. Using lung adenocarcinoma cells derived from a non-metastatic patient, we demonstrated that hypoxic stress induced phenotypic diversity along the EMT spectrum, with induction of EMT transcription factors (EMT-TFs) SNAI1, SNAI2, TWIST1, and ZEB2 in a hypoxia-inducible factor-1 α (HIF1A)-dependent or -independent manner. Analysis of hypoxia-exposed tumor subclones, with pronounced epithelial or mesenchymal phenotypes, revealed that mesenchymal subclones exhibited an increased propensity to resist cytotoxic T lymphocytes (CTL), and natural killer (NK) cell-mediated lysis by a mechanism involving defective immune synapse signaling. Additionally, targeting EMT-TFs, or inhibition of TGF- β signaling, attenuated mesenchymal subclone susceptibility to immune attack. Together, these findings uncover hypoxia-induced EMT and heterogeneity as a novel driving escape mechanism to lymphocyte-mediated cytotoxicity, with the potential to provide new therapeutic opportunities for cancer patients.

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Antitumor cytotoxic response; CTL; EMT; hypoxia; HIF; NSCLC; NK cells; TGF- β

Introduction

The tumor microenvironment is a complex system that contains numerous cell types playing important roles in tumor development and progression. Besides stromal cells, metabolic mediators are also involved, such as hypoxia, an essential metabolic element of the tumor microenvironment that may help to shape cellular plasticity and tumor heterogeneity.^{1,2} Hypoxic stress is predominantly caused by an abnormal formation of the vasculature of the rapidly growing tumor mass, and the net result is heterogeneously distributed areas of low oxygen pressure.³ In this context, cancer cell adaptation allows for their survival and may give rise to heterogeneity and the emergence of therapy-resistant phenotypes. Accumulating evidence also points to hypoxia as an important trigger for cancer cell invasion or metastases via the activation of hypoxic cascades and hypoxia-inducible factor (HIF)-1.⁴ Hypoxic stress also has clinical implications, with the severity of tumor hypoxia known to correlate with tumor progression and therapeutic resistance, particularly in breast cancer.^{5,6} Although the advent of high-throughput analyses coupled with single-cell-based approaches has revealed considerable inter-tumor (between tumors of the same type) and intra-tumor (within tumors, different cancer cell subclones) heterogeneities,^{7,8} the role of hypoxia in driving

or generating heterogeneous cancer cell populations warrants further investigation.

The epithelial–mesenchymal transition (EMT) process involves the conversion of epithelial cells into migratory and invasive cells, presumably in a transient and reversible manner, and represents at least one of the crucial steps required for tumor progression via invasion and metastatic spread.⁹ It may result from a combination of oncogenic mutations, epigenetic regulation, and microenvironmental control. Recently, there has been an expanding body of research linking EMT and the mesenchymal-like phenotypic states of cells to therapy resistance and the emergence of cancer stem cell (CSC) properties.⁹ We and others have provided evidence to indicate that the acquisition of the EMT phenotype can control immunosuppression,^{10,11} and confer resistance to T-cell-mediated cytotoxicity.^{12,13} Intriguingly, despite the growing list of EMT inducers being discovered and an understanding of their underlying mechanisms of action,⁹ the role of hypoxia in triggering this process remains relatively unclear. Contradicting results exist to support both the promoting and suppressive roles of hypoxia, and findings indicate differences in terms of the mechanisms at play in human and in mouse.^{14–17} Given that hypoxia-mediated changes seem to have a pivotal role in cancer cell

plasticity and in clinical outcomes, it would be paramount to explore the role of EMT in hypoxia-induced cancer cell heterogeneity and its impact on the quality of cell-mediated cytotoxicity. In this study, we used non-metastatic NSCLC cells to elucidate how hypoxic stress is involved in shaping tumor heterogeneity and the subsequent regulation of the antitumor cytotoxic response.

Results

NSCLC IGR-Heu cell heterogeneity and EMT phenotypes in response to hypoxic stress

NSCLC IGR-Heu carcinoma cells in standard 2 D cultures exhibited an intermediate epithelial state, with relatively homogenous, epithelial-like features and often growing as clusters with cell-cell junctions (Fig. 1A). Exposure to hypoxia (1% O₂) for 72 h caused increased phenotypic heterogeneity with distinct morphologies, with individual, elongated cells that were variably spread or situated in clusters (Fig. 1A). A gene set enrichment analysis (GSEA) revealed enrichment of and alterations to various biological processes under short-term hypoxia (Fig. 1B). Expectedly, a hypoxia hallmark signature was the most significantly enriched process, and pathways involved in stress response and cell death, such as p53 and tumor necrosis factor- α , were upregulated. EMT was also enriched, whereas several proliferation and mitosis-related signatures had negative enrichment scores. We then asked the extent to which EMT transcription factors (EMT-TFs) could be influenced by hypoxia in primary NSCLC IGR-Heu cells, and evaluated their dependency on HIF-1 α . The cells were exposed to normoxic or hypoxic conditions for 72 h in the presence of HIF1A or non-targeting siRNA. qRT-PCR analysis revealed induction of EMT-TFs (SNAI1, SNAI2, TWIST1, and ZEB2) under hypoxia, as well as related markers, such as TGFB1 and VIM (Fig. 1C). siRNA targeting of HIF1A strongly diminished the induction of ZEB2, VIM, and TGFB1, implying an hypoxia/HIF1A-dependency for their gene expression. Well-known HIF targets CA9, PDK1, LOXL2, VEGFA, and NDRG1 were also affected by HIF1A knockdown under hypoxia. Moreover, differences in cell morphology were observed between siHIF1A and siNT-treated cells (Fig. S1). SNAI1 and SNAI2 remained unchanged after HIF1A knockdown, whereas TWIST1 expression was only slightly reduced, indicating that numerous factors, rather than HIF1A alone, may drive or promote EMT states, presumably via their propensity to co-activate EMT-TFs in carcinoma cells.

To further assess a potential link between hypoxia and EMT in human lung tumors, we explored the samples from The Cancer Genome Atlas lung adenocarcinoma (TCGA-LUAD) dataset¹⁸ corresponding to primary surgically resected tumors (i.e., early stage disease). Markedly, tumors with high HIF1A levels showed an elevated expression of known HIF targets and EMT-TFs, suggesting enrichment of these genes in hypoxic lung adenocarcinomas included in the TCGA-LUAD project (Fig. 1D). It is also noteworthy that patients with high HIF1A expression in their tumors showed a higher risk of recurrence after primary management; this denotes a relative aggressiveness for these tumors, regardless of secondary treatments (Fig. S2). Correlation scores between each EMT-TF and

hypoxia signatures (hallmark_Hypoxia, Manolo_hypoxia_UP) or HIF1A expression consistently found significant and positive correlations for hypoxia signatures and HIF1A expression with EMT-TF expression (Fig. 1E). These findings again underscore the link between hypoxia and EMT in human TCGA lung adenocarcinoma tumors.

Prolonged hypoxia potentiates EMT in primary NSCLC IGR-Heu cells

To better recapitulate the chronicity of hypoxia in human tumors, we exposed IGR-Heu cells to prolonged hypoxic stress. We found that distinct carcinoma cell morphologies were more pronounced following this treatment condition, with easily detectable clusters of compact cells contrasting with cells displaying dendritic and spindle shapes, or areas with mixed morphologies (Fig. 2A). Gene expression profiles analyzed using GSEA showed that EMT was the most enriched process under sustained hypoxia compared to normoxia, suggesting potentiation of the EMT process (Fig. 2B). Accordingly, Hallmark_EMT signature was upregulated in prolonged (45 d) compared to short (72 h) hypoxic conditions as shown by a positive enrichment score. An analysis of protein extracts from cells cultured under short-term or prolonged hypoxia further indicated that maintenance of a hypoxic state accentuates the expression of EMT-TFs and markers including vimentin (Fig. 2C). However, maintenance of hypoxia for over 6 mo did not further accentuate the mesenchymal phenotype; instead, it led to a persistent perhaps increased epithelial state, as measured by E-cadherin expression. This suggests that hypoxia induces EMT to different extents in subsets of primary NSCLC IGR-Heu cells, with some cells retaining an epithelial-like phenotype. These findings were corroborated by dual-immunofluorescence analysis of hypoxia-treated IGR-Heu carcinoma cells as compared with parental cells (Fig. 2D).

Lung adenocarcinoma subclones with distinct EMT phenotypes following prolonged hypoxic stress display distinct susceptibility to lymphocyte-mediated lysis

To gain further insight into this heterogeneity from prolonged hypoxic conditions, we isolated a panel of carcinoma clones from the bulk of hypoxic-maintained IGR-Heu cells. Although a spectrum of phenotypes was observed, two predominant populations were distinguishable (Fig. 3A): those with a pronounced epithelial (Epi) phenotype (CDH1(E-cad)^{high}, EPCAM^{high}, VIM^{low}) and those with pronounced a mesenchymal (Mes) phenotype (CDH1(E-cad)^{low}, EPCAM^{low}, VIM^{high}); the latter also showed a high expression of EMT-TFs and EMT-associated genes. Representative Mes and Epi tumor subclones are depicted in Fig. 3B. We next investigated whether the EMT status of carcinoma cells correlated with differences in susceptibility to lymphocyte-mediated killing. To this end, we evaluated the cytotoxicity of human NK92 cells and the autologous cytotoxic T lymphocytes (CTL) clone Heu171¹⁹ toward hypoxia-exposed Epi and Mes IGR-Heu derivatives. We found that CTL-mediated and natural killer (NK) cell-mediated lyses of the Mes IGR-Heu variant were lower than that of the Epi IGR-Heu variant (Fig. 3C). The resistance to

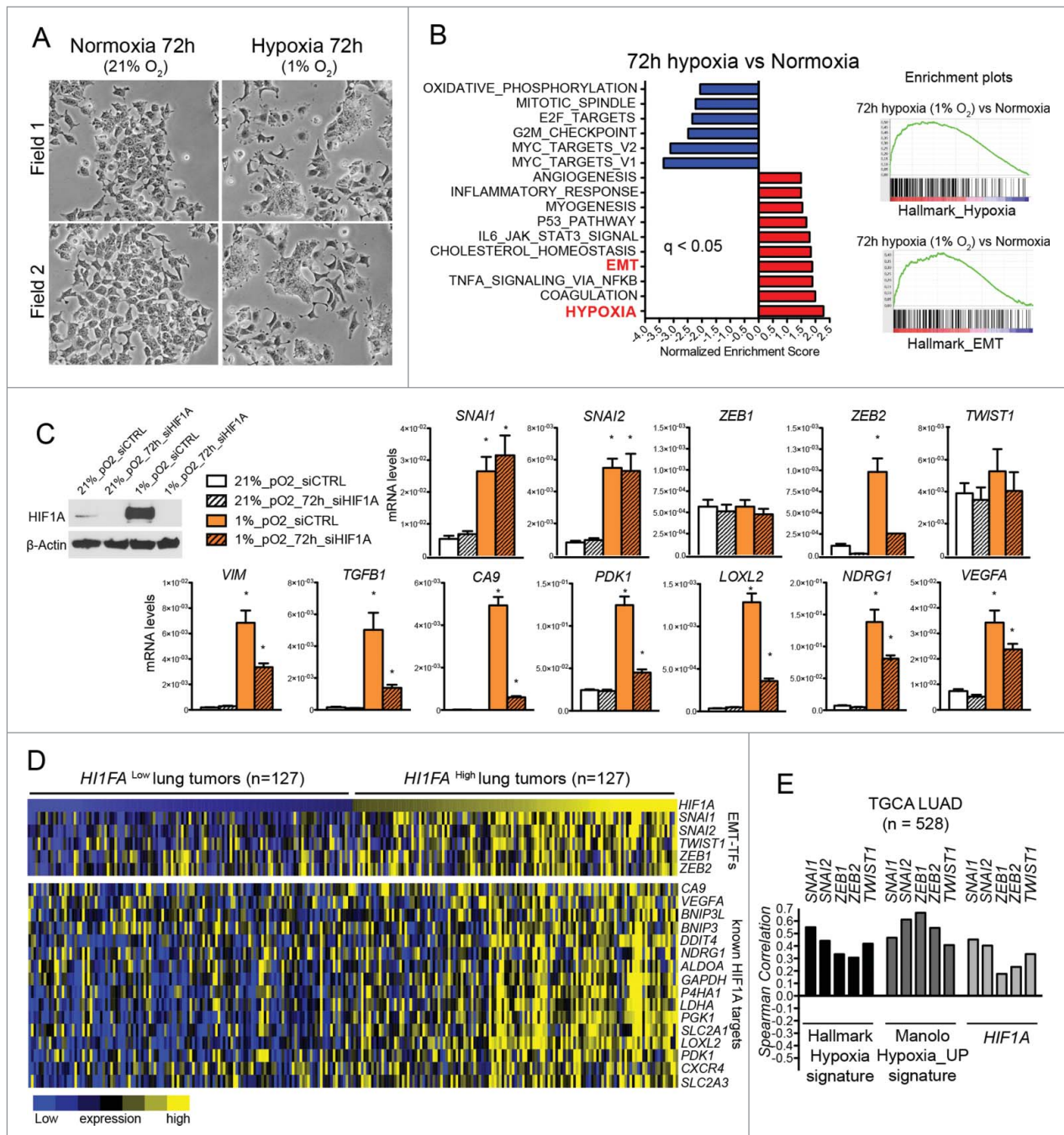


Figure 1. Hypoxia increases tumor heterogeneity and EMT phenotype in NSCLC IGR-Heu cells and in human lung adenocarcinomas. (A) Morphology of IGR-Heu cells under normoxia (left) and hypoxia (1% O₂ for 72 h). Scale bar, 40 μm. (B) Effect of 72 h hypoxia on gene expression profile in IGR-Heu cells. GSEA analyses for hallmark gene signatures. NES, normalized enrichment score, *q* value, false discovery rate. Positive scores (red bars) indicate gene set enrichment with hypoxia, whereas negative scores indicate downregulation (blue bars). Enrichment plots for Hypoxia and EMT gene sets. (C) IGR-Heu cells were transfected with HIF1A and/or non-targeting siRNAs and cultivated for 72 h under normoxic or hypoxic conditions. Immunoblots were probed with anti-HIF1A. Quantitative RT-PCR analysis of EMT-TFs (SNAI1, SNAI2, ZEB1, ZEB2, TWIST1), EMT-related genes (VIM, TGFB1), and HIF targets (CA9, LOXL2, PDK1, VEGFA, NDRG1). Results are from two independent experiments performed in triplicate. Bars; error bars, ± SEM. (D) Heatmap of mRNA expression levels of EMT-TFs and HIF1A targets in tumors samples with low (left) vs. high (right) HIF1A expression from lung adenocarcinoma TCGA cohort. Cases with gene expression ≥ highest 1/4 were recorded as high, ≤ lowest 1/4 as low. Samples are ordered by HIF1A expression. (E) Correlations for EMT-TF, HIF1A and hallmark hypoxia signature in TCGA lung adenocarcinoma.

CTL Heu171 attack is presumably due to the absence of detectable E-cadherin in the hypoxia-induced Mes IGR-Heu cells since the cytolytic function of autologous CTL Heu171 relies on integrin CD103 and its interactions with its preferred ligand,

E-cadherin.²⁰ Regarding the impairment of Mes subclone susceptibility to NK92 cells, we asked whether this impaired susceptibility was due to defects in the recognition of target cells by the NK cells. We therefore examined conjugate formation

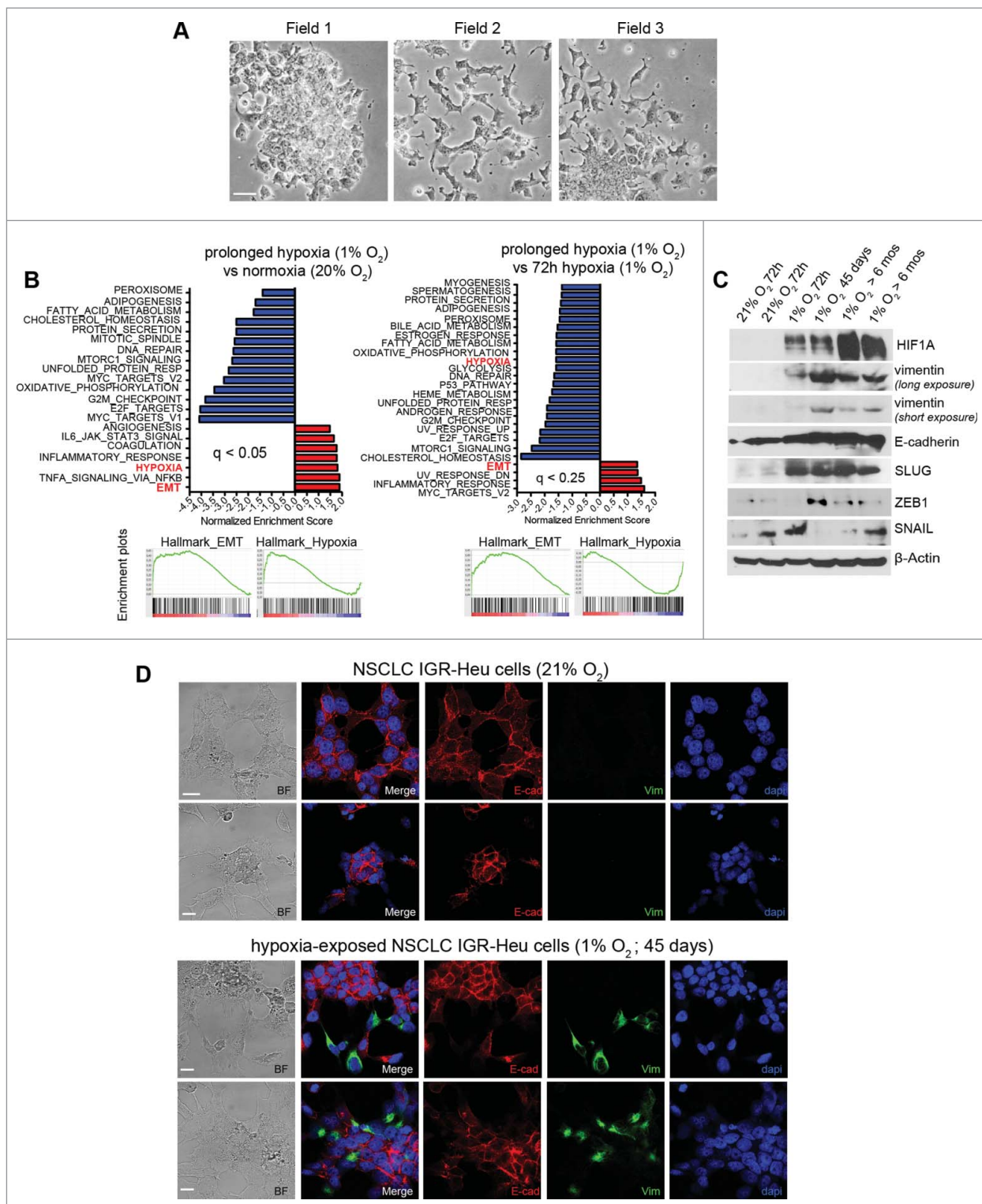


Figure 2. Prolonged hypoxia induces various EMT states. (A) Marked heterogeneity of IGR-Heu cells maintained for 45 d under 1% O₂. Fields 1, 2 and 3 show areas from the same culture flask: field 1, more epithelial-like, clustered cells; field 2, more mesenchymal-like cells; and field 3, mixed populations. Scale bar, 20 μm. (B) Effect of prolonged (45 d) hypoxia on gene expression in IGR-Heu cells. Prolonged hypoxia vs. normoxic condition (left), and prolonged hypoxia vs. short-term hypoxia (right). EMT_hallmark was enriched in the two settings. (C) Western blotting for extracts of IGR-Heu cells maintained under 1% O₂ hypoxia for 72 h, 45 d, or >6 mo (passages 12 and 14). (D) Immunofluorescence staining of E-cadherin (red) and vimentin (green) in IGR-Heu cells maintained under normoxia or hypoxic stress for 45 d. Nuclei were stained blue (DAPI). Observations were carried out using confocal microscopy. Scale bar, 10 μm.

after co-culturing NK cells with either Epi IGR-Heu or Mes IGR-Heu subclones for 30 min. Confocal microscopy indicated no obvious differences in the capacity of NK cells to form

conjugates with Epi or Mes target cells (Fig. 3D). However, a significant decrease in tyrosine phosphorylation at the contact area of NK cells was observed when grown in co-cultures with

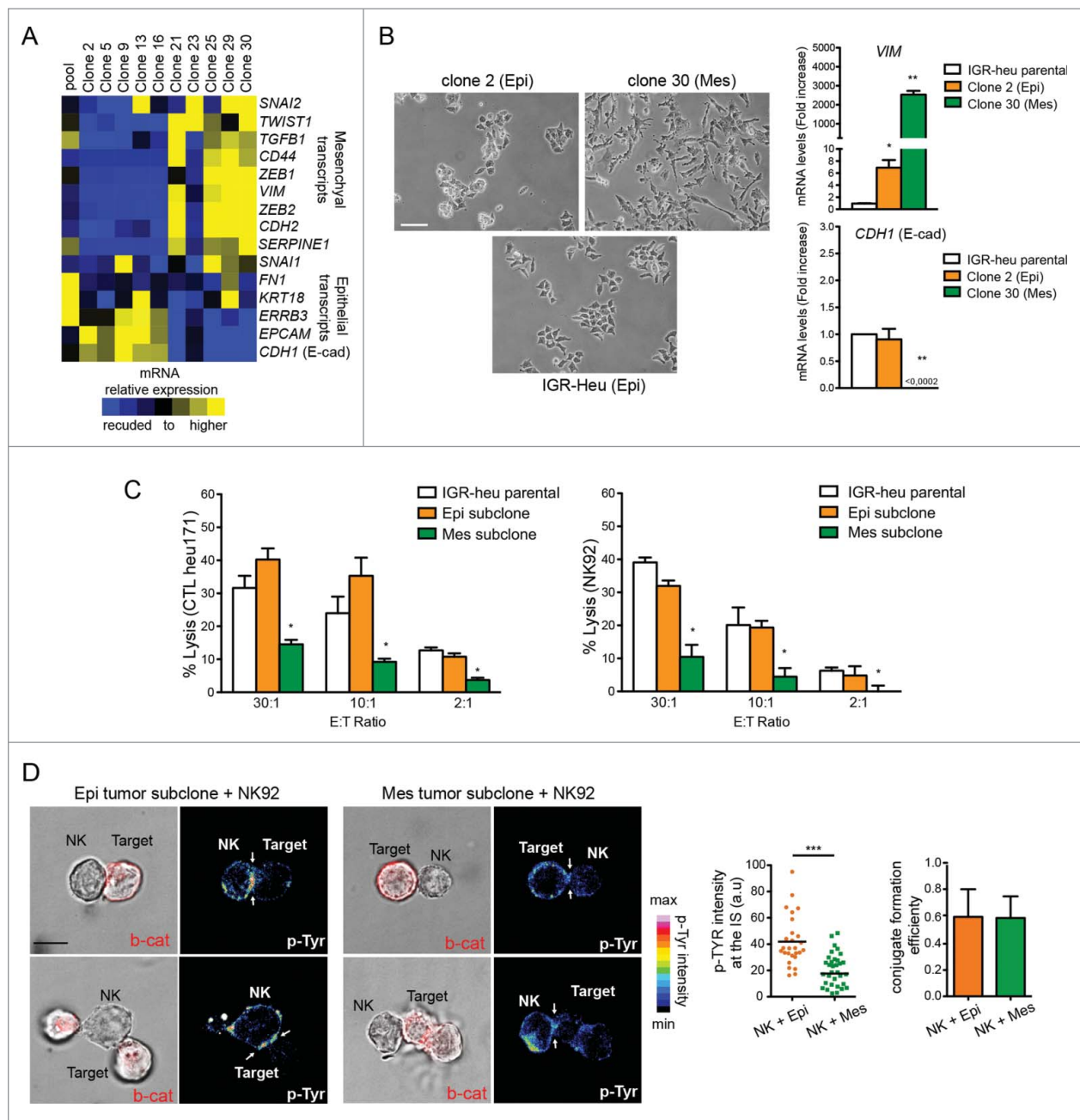


Figure 3. IGR-Heu tumor variants with Epi and Mes phenotypes following prolonged hypoxic stress displayed various susceptibility to lymphocyte-mediated lysis. (A) Heatmap of qRT-PCR analysis showing the expression of a panel of epithelial- and mesenchymal-associated genes in isolated tumor clones. (B) Left: Morphology of an Epi (more epithelial-like) and Mes (more mesenchymal-like) clones compared with IGR-Heu parental cells. Scale bar, 40 μ m. Right: Quantitative RT-PCR analysis of *VIM* and *CDH1*(E-cad) expression in tumor IGR-Heu parental, Epi and Mes clones. Bars represent the means of two experiments \pm SEM performed in triplicate. (C) NK-mediated (left) and CTL-mediated (right) lysis of IGR-Heu tumor variants with more epithelial (Epi subclone, parental cells), or more mesenchymal (Mes subclone) phenotypes, at different effector:target (E:T) ratios. Heu171 CTL clone (left) and NK92 clone (right) were used as effectors. Bars represent the means of three experiments \pm SEM performed in triplicate. (D) Confocal microscopy of immunological synapses (arrows) after culturing Epi or Mes clones (targets) in the presence NK92 clone for 30 min. Fixed cells were stained for anti-phosphorylated-Tyrosine (p-Tyr). Efficiency of conjugate formation between NK92 and target cells; MFI \pm SD derived from p-Tyr signals from a minimum of 25 cell conjugates. * $p \leq 0.05$ (Mann-Whitney test).

the Mes subclones as compared with Epi subclones (Fig. 3D), suggesting for less effective immune synapse signaling in NK cells interacting with Mes hypoxia-derived tumor cells.

Targeting EMT status increases susceptibility of hypoxia-derived Mes carcinoma cells to cell-mediated cytotoxicity

To ascertain the extent to which the hypoxia-induced EMT status impairs carcinoma cell susceptibility to NK-mediated

killing, we thought to disrupt the EMT status of Mes IGR-Heu cells using siRNAs directed against *SNAI1*, *SNAI2*, *ZEB1*, *ZEB2*, and *TWIST1*. Data depicted in Fig. 4A indicated that the EMT status was affected by each treatment (Fig. 4A). For instance, whereas reducing *SNAI1* or *SNAI2* expression only had a minor effect on E-cadherin expression, their combined silencing led to a net gain in epithelial markers, such as EPCAM and keratin-18. A knockdown of *TWIST1* also resulted in an increase in keratin-18, whereas

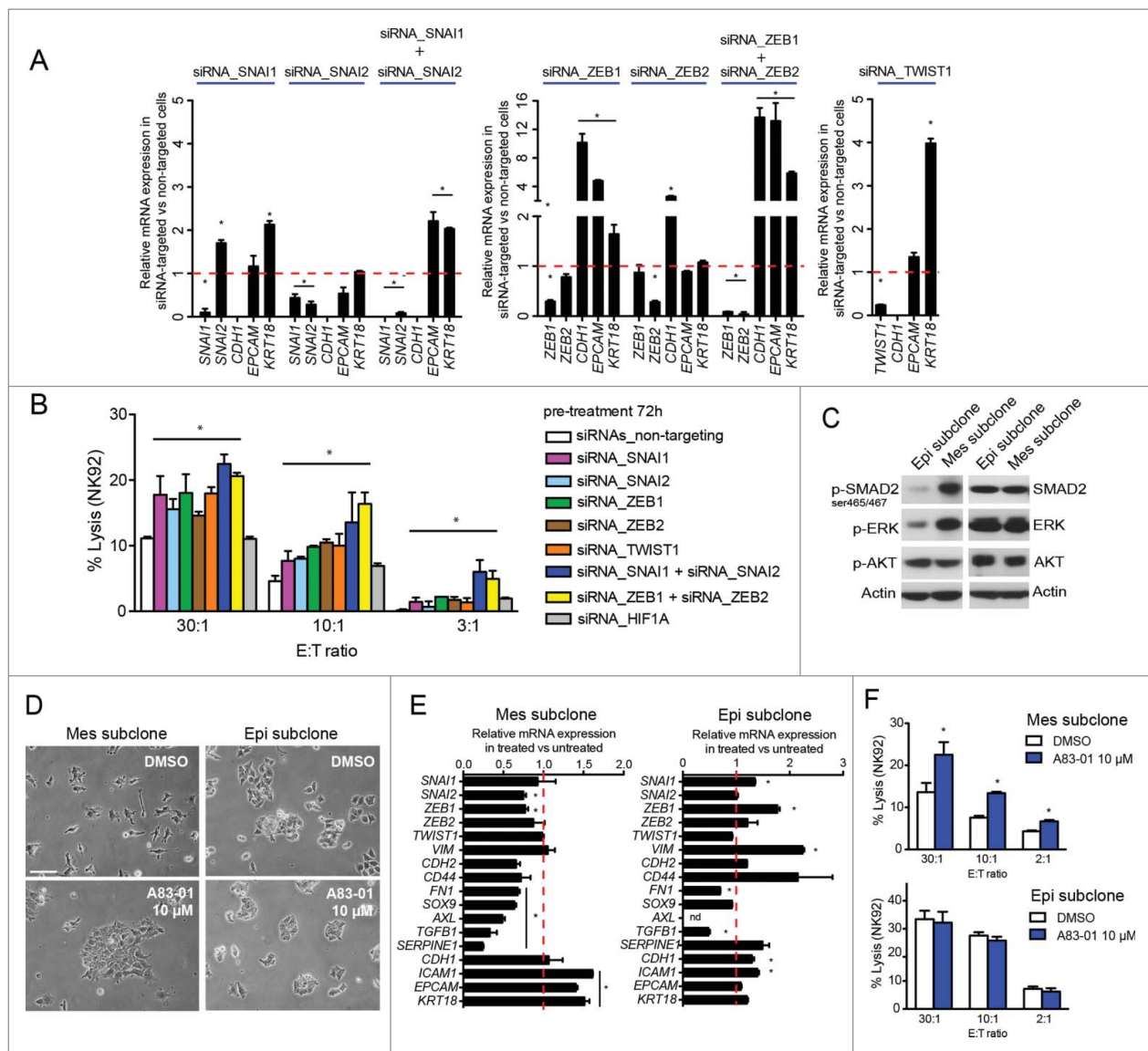


Figure 4. Neutralizing EMT-TF, endogenous TGF- β signaling in IGR-Heu hypoxia-derived Mes subclone increases susceptibility to NK-mediated lysis. (A) Quantitative RT-PCR results from the Mes subclone pretreated for 72 h with siRNA against the indicated EMT-TF factors, and effects of epithelial-related genes expression. (B) Cytotoxicity assay for NK-mediated lysis of IGR-Heu Mes subclone after siRNA targeting of EMT-TF. Bars represent the means of three experiments \pm SEM performed in triplicate. (C) Immunoblots comparing expression of phosphorylated ERK, SMAD2 and AKT in IGR-Heu Epi and Mes subclones. (D–F) Effects of pharmacological inhibition of TGF- β signaling with 10 μ M A83-01 in Epi and Mes tumor subclones cells treated for 72 h. Results are from two experiments performed in triplicate. Bars; error bars, \pm SEM.

siZEB1, siZEB2, or the combination of siZEB1/siZEB2 potentially upregulated E-cadherin, or the expression all three epithelial markers, respectively.

We next performed cytolytic assays following the knock-down of EMT-TFs in Mes IGR-Heu variants through co-culturing with NK cells. We found that the attenuation of the EMT state increased the susceptibility of Mes subclones to NK-mediated lysis at different effector–target ratios (Fig. 4B). As noted above, the combined targeting of SNAI1 and SNAI2 or ZEB1 and ZEB2 was more effective at increasing cell lysis than targeting any of these genes alone. Moreover, those effects were not detectable when similar treatments were applied to Epi subclones (Fig. S3). These observations suggest that EMT induction in some cells after an hypoxic stress could protect them from immune attacks.

Targeting EMT-associated factors increases cell lysis susceptibility of hypoxia-derived Mes carcinoma cells

Compared with Epi cells, Mes cells were found to have increased phosphorylation of ERK and SMAD2, and a slight reduction in AKT phosphorylation (Fig. 4C). Moreover, we noted that TGF- β expression was induced under hypoxia in these cells (Fig. S4) and strongly diminished after neutralization of EMT-TFs with siRNAs (Fig. S5). We thus assessed whether inhibiting TGF- β signaling could sensitize Mes IGR-Heu cells to NK-mediated lysis. In the Mes subclone, 10 μ M A83-01 treatment for 72 h resulted in substantial morphological changes, a lowered expression of EMT/TGF- β -related genes and phospho-SMAD2 (Figs. 4D, E and S6). Additionally, in cytotoxicity assays, we found that these Mes-like cells were

more susceptible to both NK cell- (Fig. 4F) and CTL-mediated lysis under these culture conditions (Fig. S7), whereas Epi cells were marginally affected by such treatment. Similar results were obtained with a different TGF- β signaling inhibitor LY2157299 (25 μ M), which is currently in clinical development (Fig. S8). These data are in support of a role for TGF- β signaling in conferring resistance to lymphocyte-mediated lysis.

Discussion

Tissue hypoxia has been regarded as a central factor for tumor aggressiveness and metastasis. Notably, we found that hypoxia promotes cellular and functional heterogeneity in NSCLC cells in association with the emergence of distinct EMT phenotypes and various susceptibilities to lymphocyte-mediated killing. Intriguingly, the observed phenotypic changes were particularly exacerbated when hypoxic stress was maintained for a prolonged period, a situation presumably transposable to most solid tumors. Our results indicate that the shift toward a mesenchymal-like phenotype is only observed in a fraction of stressed cells (Fig. 5), as demonstrated by our analysis of both carcinoma cell populations and isolated clones. While some cells undergo EMT, others do not move along the EMT spectrum. Because the reduction in HIF1A levels in cells was able to restrain the switch to a mesenchymal-like phenotype, we propose that the observed changes in the carcinoma cell population are mainly due to adaptive phenotypic plasticity of certain epithelial-like cells or hybrid/intermediate EMT cells. However, we cannot totally exclude the contribution of a parallel selection of preexisting cells of a more advanced mesenchymal state. A thorough assessment of such hypotheses will require systematic and multiparametric approaches. Further investigations are also needed to unravel the detailed mechanisms as to why some carcinoma cells are more prone than others to hypoxia-induced EMT.

Focusing on hypoxia-exposed carcinoma subclones with Epi- and Mes-like phenotypes, we found that carcinoma Mes-like cells, associating with expression of EMT-related factors expression, were functionally primed to resist CTL- and NK cell-mediated lysis, which may be a prerequisite for the

establishment of tumor resistance and escape (Fig. 5). Our data therefore provide new evidence connecting hypoxia, EMT and therapy resistance, and point to a potential role of microenvironmental hypoxia in promoting carcinoma cell heterogeneity and plasticity, which could subsequently, through the emergence of immunoresistant variants, limit the effectiveness of solid tumor immunotherapies.²¹ As corollary, one could speculate that the degree of both EMT and hypoxic targets may be predictive for cell-mediated cytotoxicity responses. Indeed, the existence of a spectrum of EMT states,⁹ yet to be fully characterized, could presage a greater variability of responses against immune effector killer cells. Notwithstanding, there remains a possibility of active, cooperative events between mesenchymal-like and epithelial-like carcinoma cells in patients.

We previously reported the potential utility of EMT scoring to estimate the EMT content in a given tumor and to help guide clinical decisions²²; however, this may provide only part of the information required, especially in the context of heterogeneous tumor populations. Scoring EMT *in situ* in individual carcinoma cells should allow us to refine our analysis of the role of hypoxia in generating refractoriness in targeted therapies in tumor microdomains, such an *in situ* scoring technique is currently in development.^{23,24}

Recent studies using human cancer cohorts have revealed that human tumors with high EMT also display higher immune activation marks, increased infiltration by immune cells, and an elevated expression of immune-checkpoint molecules.^{25,26} Nevertheless, the clinical impact of these findings remains unclear. Our data, which points to hypoxia as an additional important parameter for consideration, could shed new light on these earlier works.

From both functional and therapeutic standpoints, we show that targeting EMT through the inactivation of EMT-TFs or through pharmacological inhibition of TGF- β , sensitizes mesenchymal cells to NK cell- or CTL-mediated killing. Our observations suggest that a TGF- β signaling mechanism, or the actions of TGF- β -associated factors, may be required in Mes subclones for their commitment to a resistant phenotype; albeit, other important components of this resistance mechanism likely exist and will need to be investigated. Aside from our observations, it is worth noting that a previous study reported that transfection of *Snail1* in murine B16 melanoma cells induced regulatory T cells along with an expansion of CD4⁺ Foxp3⁺ cells.¹⁰ The proposed mechanism involved reduced dendritic cells (DC) maturation and production of thrombospondin-1 (TSP1), a known activator of TGF- β . By extension, it is tempting to speculate that carcinoma cells moving along the EMT spectrum, could also produce various levels of cytokines and immunosuppressive substances, thus, affecting the activity and the recruitment of T Reg, and other immunosuppressive populations, such as myeloid-derived suppressor cells (MDSC) promoting immune tolerance.

The results of the present study provide new evidence that hypoxia, through EMT induction, may also contribute to a key phase of cancer immunoediting²⁷ since besides conferring resistance to cell death, EMT can reduce immune recognition through altering the immune synapse. We propose that hypoxia-induced EMT and the subsequent tumor heterogeneity is a novel mechanism for tumor immunoediting. We envision

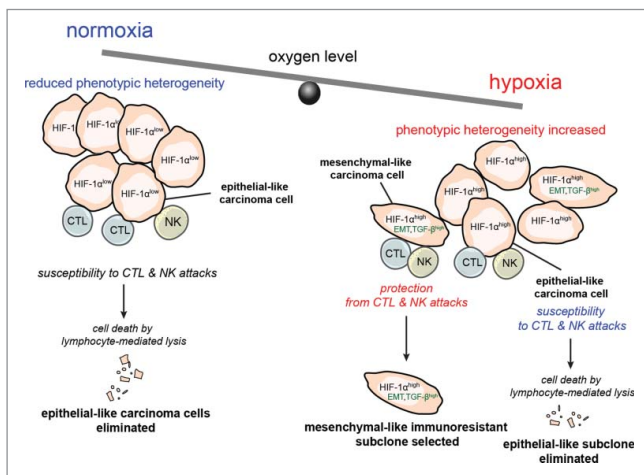


Figure 5. Working model depicting how hypoxic conditions could foster the emergence of mesenchymal-like carcinoma cells resistant to lymphocyte-mediated killing.

such altered interaction as a mechanism that may allow the selection of tumor cell variants capable of escaping detection and destruction by the innate and adaptive arms of the immune system. Full characterization of the underlying mechanism will need further investigation.

In conclusion, hypoxia clearly plays a crucial role in shaping carcinoma cell plasticity, in the induction of cell heterogeneity, and in the subsequent emergence of immunoresistant variants harboring a mesenchymal phenotype.

Materials and methods

Cell culture and reagents

Primary NSCLC IGR-Heu cells were established in our laboratory from a resection of a non-metastatic NSCLC patient and grown as previously.²⁸ For hypoxic conditioning, cells were maintained under hypoxia (1% pO_2 with 5% CO_2) for the indicated time frames in a hypoxia chamber (InVivo2 400 Hypoxia Workstation; Ruskinn Technology, UK). The CTL Heu 171 cytotoxic T lymphocyte (CTL) clone originated from the same patient and tumor piece as that used to derive IGR-Heu tumor cells. The clone was cultured and stimulated as described previously.²⁸ The NK-92 cell line²⁹ was obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI-1640 medium supplemented with 10% FBS, 2.5% of human AB serum, and 200 U/mL IL-2. Unless indicated, cells were maintained at 37°C in a 5% CO_2 and 95% air (21% O_2) incubator. LY2157299 from Selleckchem (Houston, TX), PD0325901 from Sigma-Aldrich (St. Louis, MO), and A83-01 from Tocris Bioscience (Avonmouth, UK).

Western blot analysis

Western blotting was performed as described previously.¹² Briefly, adherent cells were lysed on ice with lysis buffer (62.5 mmol/L Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol) containing a cocktail of protease (Thermo Fisher Scientific, Waltham, MA, USA) and phosphatase inhibitors (Roche Life Science, Penzberg, Germany). Antibodies from an EMT sampler kit (Cell Signaling Technology; Danvers, MA) were used to probe for ZEB-1, SLUG, SNAIL, E-CADHERIN, and VIMENTIN. Antibodies to p-SMAD2(Ser465/467), SMAD2, phospho-ERK1/2(Thr202/Tyr204), ERK1/2, p-AKT (Ser473), AKT were from Cell Signaling Technology. β -Actin was purchased from Sigma-Aldrich, and anti-human HIF-1 α /Clone 54 was from BD Biosciences (San Jose, CA, USA).

Gene silencing by RNA interference

The functionally validated siRNAs used in this study were from Qiagen (FlexiTube) and Thermo Fisher Scientific (Silencer Select) and directed against human SNAI1, SNAI2, ZEB1, ZEB2, TWIST1, TGF β 1. siRNAs were transfected using Lipofectamine RNAiMAX Transfection reagent (Thermo Fisher Scientific), with appropriate controls.

Cytotoxicity assays

The cytotoxic activities of NK and CTL clones were measured by a conventional 4-h ^{51}Cr release assay. ^{51}Cr was purchased from PerkinElmer (Waltham, MA, USA). For some cytotoxic assays, tumor cells were incubated with compounds vs. DMSO, or siRNAs for 48–72 h before their use as targets. Target tumor cells were labeled with ^{51}Cr for 1 h, and then co-cultured in one of several effector:target (E:T) ratios for 4 h in round-bottomed 96-well plates using 2,000 target cells per well in a final volume of 200 μ L. Cells were then pelleted by centrifugation and 60 μ L of supernatant was transferred to a 96-well LumaPlate (PerkinElmer), dried at 46°C overnight, and counted on a Packard TopCount NXT (PerkinElmer). The percentage of specific lysis was calculated using the standard formula [(experimental cpm – spontaneous cpm)/total cpm incorporated] \times 100, and the results are expressed as the mean of triplicate samples.

Confocal microscopy

Conjugate formation between tumor cells (target) and NK cells (effector) was measured by co-culture on poly-L-lysine-coated slides at 37°C for 30 min at an E/T 3:1 ratio, respectively. Cells were then fixed with freshly prepared 3% paraformaldehyde for 10 min, incubated in a 50-mM NH $_4$ Cl quenching solution (in PBS), permeabilized with 0.25% Triton X-100 in PBS for 10 min, and blocked with 10% FCS (v/v) in PBS for 20 min. Fixed cells were stained with anti-phosphotyrosine antibody, 4G10 clone (BD Biosciences), or anti- β -catenin (D10A8, Cell Signaling Technology) for 2 h or overnight at 4°C, and then incubated for 1 h with secondary anti-mouse Alexa Fluor 488 or anti-rabbit Alexa Fluor 555 (Thermo Fisher Scientific). All antibodies were diluted in PBS containing 1 mg/mL BSA. Cell nuclei were stained using ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Conjugates were imaged on a Leica TCS SPE laser scanning confocal microscope using sequential scanning and LASAF software. The efficiency of conjugate formation between effector and tumor cells was estimated by visual counting and calculating the ratio of effector cells able to form conjugates with target cells to target cells \times 100 in 10 different fields per condition. Phospho-tyrosine intensity was assessed using ImageJ software (NIH, Bethesda, MD, USA) by computing the mean fluorescence intensities (MFI) of the contact regions on the effector cells after background subtractions. Tumor heterogeneity in IGR-Heu and hypoxia-exposed derivatives was determined using fluorescence. Cells were trypsinized, plated onto poly-L-lysine-coated slides, incubated overnight at 37°C, fixed and permeabilized as above, and incubated with anti-vimentin (Clone V9, Dako; Trappes, France) or anti-E-cadherin (24E10; Cell Signaling Technology) antibodies, using appropriate Alexa Fluor secondary antibodies to counterstain.

RNA preparation, cDNA synthesis and quantitative real-time PCR

Total RNA extraction was performed using Trizol reagent and the samples subjected to DNase treatment (DNA-free kit). Reverse transcription was performed using Maxima Reverse

Transcriptase followed by qPCR using real-time PCR Master SYBR Green on an StepOnePlus Real Time PCR system. All products were from Thermo Fisher Scientific. The majority of oligonucleotide sequences used were designed previously and are published elsewhere.³⁰ The full list is available upon request.

RNA-Seq and microarray gene expression analysis

To investigate the hypoxic responses of human primary IGR-Heu NSCLC cells, gene expression profiles were studied by GeneChip Human Gene 2.0 ST Array and the data processed as described previously.³¹ Gene set enrichment analyses (GSEA) were performed with the GSEA platform of the Broad Institute (<http://www.broadinstitute.org/gsea/index.jsp>). Human samples consisted of early-stage specimens of lung adenocarcinomas from The Cancer Genome Atlas (TCGA) project collection (<http://cancergenome.nih.gov/>). TCGA RNA-Seq expression data and sample information were accessed before June 2016 from cBioPortal³² and the TCGA public access data (<http://tcga-data.nci.nih.gov/>).

Statistical analysis

Data analyses were performed using GraphPad (GraphPad Prism, La Jolla, CA, USA) and Excel (Microsoft Corp., Redmond, WA, USA). Statistical tests were performed using a two-tailed $\alpha = 0.05$ level of significance; * $p \leq 0.05$. Spearman coefficients were used for correlations between variables. Comparisons between groups were performed using the Wilcoxon, Mann–Whitney, and ANOVA tests, as appropriate. Recurrence free survival and overall survival curves were generated by the Kaplan–Meier method and compared using the log-rank test.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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