The miR-195 Axis Regulates Chemoresistance through TUBB and Lung Cancer Progression through BIRC5

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Chemoresistance and metastasis are the major reasons for non-small cell lung cancer (NSCLC) treatment failure and patient deaths. We and others have shown that miR-195 regulates the sensitivity of NSCLC to microtubule-targeting agents (MTAs) in vitro and in vivo and that miR-195 represses the migration and invasion of NSCLC cells in vitro. However, the relationship between miR-195 and microtubule structure and function and whether miR-195 represses NSCLC metastasis in vivo remain unknown. We assessed the correlation between tumor levels of TUBB and patient survival, the effect of TUBB on drug response, and the effect of miR-195 on migration, invasion, and metastasis in vitro and in vivo. We found that miR-195 directly targets TUBB; knockdown of TUBB sensitizes cells to MTAs, while overexpression confers resistance; high expression of TUBB is correlated with worse survival of lung adenocarcinoma; TUBB is also regulated by CHEK1, which has been shown to regulate chemoresistance; and miR-195 targets BIRC5 to repress migration and invasion in vitro and metastasis in vivo. Our findings highlight the relevance of the miR-195/TUBB axis in regulating the response of NSCLC to MTAs and the importance of the miR-195/BIRC5 axis in regulating NSCLC metastasis.

INTRODUCTION
Lung cancer is the leading cause of cancer deaths worldwide.1 Around 85% of lung cancer is non-small cell lung cancer (NSCLC), which is histologically classified into lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), and large cell lung carcinoma. For decades, microtubule-targeting agents (MTAs) have been used in the treatment of NSCLC. Although MTAs have achieved promising results in certain patients, the response rate in NSCLC is very low (~10%–40%), which poses a significant barrier to improving long-term outcomes of NSCLC patients.2–5 Even in responsive NSCLC patients, resistance usually develops over time, resulting in disease recurrence and metastasis, the latter of which is responsible for 90% of lung cancer deaths.5 Therefore, it is critical to understand the mechanisms underlying drug resistance and metastasis and the relationships between them.

Multiple mechanisms have been implicated in drug resistance. One common mechanism is selective efflux mediated by membrane-bound ATP-dependent pumps, which pump substrates—toxic metabolites and xenobiotics—into the blood, reducing effective drug concentrations in tumor cells and conferring resistance to a large number of structurally unrelated cytotoxic drugs, including the MTAs.7–10 Other mechanisms include tubulin mutations, altered microtubule binding dynamics, and impaired centrosome clustering.11 Changes in tubulin expression have also been shown to contribute to resistance to MTAs.7,10,12–14 Specifically, overexpression of class III beta-tubulin (TUBB3) has been implicated in resistance to paclitaxel in several cancer types, including pancreatic ductal adenocarcinoma, breast cancer, and lung cancer.15,17–19 In addition, there has been some debate about the role of tubulin beta class I (TUBB) in paclitaxel resistance in NSCLC. TUBB mutations were first reported to correlate with paclitaxel resistance in NSCLC patients.20 It was later argued, however, that TUBB mutations are not common in NSCLC and that the reported TUBB mutations likely occur in TUBB pseudogenes.21–23 No correlation of TUBB expression with paclitaxel resistance was found.21 To date, whether or not TUBB plays a role in chemoresistance has not been experimentally determined.

Metastasis is the main cause of cancer deaths and is largely driven by invasion of tumor cells into surrounding tissues and migration of tumor cells to distant sites. Survivin, encoded by the BIRC5 gene, has been shown to regulate the migration and invasion of various cancer cells, including melanoma, colorectal cancer, oral squamous cell carcinoma, cervical cancer, and breast cancer.24–29 BIRC5 is overexpressed in various tumors, with high expression correlating with worse survival.30,31 Repression of survivin has been proposed as a potential cancer therapy.32

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Table 1. Expression of Tubulin Isotypes in miR-195-Transfected Cells Relative to Control Cells

| Gene   | H358     | H1993    |
|--------|----------|----------|
| TUBB   | 0.58     | 0.64     |
| TUBA1A | 0.64     | 0.64     |
| TUBB4B | 0.66     | 0.72     |
| TUBA3D | 0.74     | 0.79     |
| TUBB4Q | 0.81     | 0.69     |
| TUBG2  | 0.82     | 1.08     |
| TUBG1  | 0.83     | 0.86     |
| TUBD1  | 0.84     | 0.94     |
| TUBA3E | 0.85     | 0.92     |
| TUBA1B | 0.86     | 1.04     |
| TUBA1C | 0.87     | 0.91     |
| TUBA4  | 0.92     | 0.85     |
| TUBB1  | 0.92     | 1.09     |
| TUBB3  | 0.95     | 1.05     |
| TUBB8  | 0.97     | 0.96     |
| TUBA8  | 0.99     | 0.97     |
| TUBB6  | 1.00     | 0.99     |
| TUBB4  | 1.00     | 1.08     |
| TUBA3C | 1.03     | 0.97     |
| TUB1E  | 1.08     | 1.03     |
| TUBR2B | 1.12     | 0.90     |
| TUBA4A | 1.20     | 0.82     |
| TUBR2A | 1.33     | 1.04     |

*These isotypes were down-regulated by miR-195 by at least 20% in both cell lines.

We have recently shown that survivin regulates both apoptosis and senescence in NSCLC cells. However, the role of survivin in regulating the migration and invasion of NSCLC cells has not been fully studied.

microRNAs (miRNAs) are a family of small non-coding RNAs that post-transcriptionally repress gene expression. Among them, miR-195 has been shown to regulate various aspects of cancer, including chemoresistance and metastasis. Specifically, miR-195 increases chemosensitivity of colon cancer and cervical cancer, but correlates with acquired chemoresistance in glioblastoma. We have recently shown that miR-195 targets CHEK1 to sensitize NSCLC to MTAs. miR-195 has also been implicated as a regulator of cancer metastasis through a few reports showing its regulation of migration and invasion in vitro. For example, miR-195 represses cancer cell migration and invasion in esophageal squamous cell carcinoma, breast cancer, osteosarcoma, and hepatocellular carcinoma. In contrast, miR-195 promotes the migration and invasion of melanoma. The actual effects of miR-195 on metastasis in vivo have not been established.

Our investigation of the function of miR-195 in NSCLC revealed that TUBB is a direct target of miR-195, regulating the response of NSCLC cells to MTAs and that expression of TUBB correlates with the prognosis of NSCLC patients. We also found that miR-195 and TUBB had no effect on microtubule structure. Interestingly, we show that TUBB expression can be regulated by another target of miR-195, CHEK1, which had previously been shown to regulate chemoresistance in NSCLC. Finally, we report that miR-195 represses the metastasis of NSCLC in vivo and that its target, BIRC5, regulates the migration and invasion of NSCLC cells. Our results not only advance our understanding of the mechanisms of drug resistance and metastasis in NSCLC, but also shed light on strategies for addressing both problems by exploiting a single vulnerability in cancer cells.

RESULTS

TUBB Expression Correlates with Prognosis in Lung Adenocarcinoma Patients

We recently demonstrated that miR-195 synergizes with MTAs to repress the growth of NSCLC cells. We subsequently asked whether miR-195 had an effect on microtubules. We re-visited the gene expression profiling of two NSCLC cell lines, H1993 and H358, transfected with miR-195 as we previously reported. We focused on the expression of tubulin isotypes and found that none were down-regulated by at least 2-fold (or 50%) by miR-195 (Table 1). However, four isotypes (TUBB, TUBA1A, TUBB4B, and TUBA3D) were down-regulated by miR-195 by at least 20% in both cell lines, encouraging us to further investigate whether these genes are direct targets of miR-195 or participate in the regulation of cellular response to MTAs. We found that high expression of TUBB, TUBA3D, or TUBB4B is correlated with worse overall survival and worse chemotherapy response in lung adenocarcinoma patients (Figures 1A and 1B; Figure S1), as assessed by Kaplan-Meier survival analysis and log-rank test. However, the expression of these genes is not significantly correlated with overall survival or chemotherapy response in lung squamous cell carcinoma patients (Figures 1C and 1D; Figure S2). Among these three genes, we identified a binding site for miR-195 only in the 3' UTR of TUBB (Figure 2G). In similar analysis of data from The Cancer Genome Atlas (TCGA), we found that high expression of TUBB is correlated with worse overall survival and recurrence-free survival of lung adenocarcinoma, but not squamous cell carcinoma (Figures 1E–1H).

TUBB Is Targeted by miR-195 and Regulates the Response of Lung Adenocarcinoma Cells to MTAs

TUBB is expressed at higher levels in tumor tissues compared to adjacent normal tissues in both lung adenocarcinoma and squamous cell carcinoma patients (Figures 2A and 2B), as shown by analysis of gene expression in TCGA datasets. Specifically, TUBB is upregulated in tumor tissues in 51 of 57 lung adenocarcinoma patients and in 49 of 51 lung squamous cell carcinoma patients. Expression of TUBB is negatively correlated with miR-195 in lung adenocarcinoma, but not squamous cell carcinoma (Figures 2C and 2D). We demonstrated that this relationship is causal—miR-195 inhibits both the mRNA and protein levels of TUBB (Figures 2E and 2F)—and confirmed the direct and specific binding of miR-195 to the 3' UTR of TUBB by luciferase assay (Figure 2G). To functionally validate that TUBB
regulates the response of lung adenocarcinoma cells to MTAs, we knocked down TUBB with a validated siRNA pool designed against TUBB (siTUBB)\(^{19}\) (Figure 3A). We found that knockdown of TUBB sensitizes H1299 cells to eribulin, but not paclitaxel, and sensitizes H1993 and A549 cells to both paclitaxel and eribulin (Figures 3B and 3C). Importantly, we demonstrated that overexpression of TUBB slightly, but significantly, contributes to the resistance of lung adenocarcinoma cells to paclitaxel and eribulin (Figure 4). Collectively, these results support that TUBB is a direct target of miR-195 and regulates the response of lung adenocarcinoma cells to MTAs.

Since we previously demonstrated that miR-195 targets the cell-cycle checkpoint kinase CHEK1 to sensitize NSCLC cells to MTAs,\(^{38}\) we next asked whether CHEK1 could regulate the expression of TUBB. Intriguingly, the expression of TUBB is significantly correlated with CHEK1 expression in both lung adenocarcinoma and squamous cell carcinoma patients (Figures 3B and 3C). Importantly, we demonstrated that overexpression of TUBB slightly, but significantly, contributes to the resistance of lung adenocarcinoma cells to paclitaxel and eribulin (Figure 4). Collectively, these results support that TUBB is a direct target of miR-195 and regulates the response of lung adenocarcinoma cells to MTAs.

miR-195 Represses the Metastasis of Lung Adenocarcinoma

Hallmarks of cancer, migration, and invasion greatly promote tumor progression. miR-195 has been demonstrated to repress the growth, migration, and invasion of lung cancer cells.\(^{41,46}\) However, the observed repression of migration and invasion could not be distinguished from inhibitory effects on cell growth. To control for the cytotoxicity of miR-195, we assessed cell viability while performing transwell migration and Matrigel invasion assays. The number of cells migrating or invading to the other side of membrane was normalized to cell viability, yielding a "relative cell count" for migration and invasion. The results confirmed that miR-195 inhibits the migration and invasion of lung adenocarcinoma cells independent of its effect on cell viability and that miR-195 inhibitors promote migration and invasion of cancer cells, albeit slightly (Figures 7A–7D; Figure S4). In addition, we exploited a chemotaxis assay designed for real-time visualization and automated analysis of cell migration and invasion to show that miR-195 inhibits the migration and invasion of cancer cells (Figures 8A and 8B).

We generated cells stably overexpressing miR-195 (H1299/luc-miR-195 #116 and #126) and demonstrated that constitutive overexpression of miR-195 inhibits cancer cell migration and...
invasion in vitro (Figures S5A–S5E). To assess the effect of miR-195 on experimental metastasis in vivo, we injected control cells (H1299/luc-EV) and miR-195 overexpressing cells (H1299/luc-miR-195 #116) intravenously into nude mice. Because these cells express luciferase, we were able to use bioluminescence to visualize cell localization. After 10 weeks, we found lung metastases in 1 of 7 mice in the control group and no lung metastases in the miR-195 overexpressing group (Figures S5F and S5G). It is not clear why metastasis occurred in only one mouse in the control group. We believe this is due to experimental variance with H1299 cells, supported by one report showing that H1299 cells inoculated intravenously did not show lung or other organ metastasis for up to 4 months47 and another report demonstrating lung metastasis with H1299 cells in only 6 weeks.48

Leveraging an experimental model of lung metastasis, mouse lung adenocarcinoma cell line 344SQ, which has been demonstrated to form lung metastases when inoculated subcutaneously into syngeneic mice,49 we subcutaneously injected 344SQ/control cells and 344SQ cells overexpressing miR-195 (344SQ/miR-195) into syngeneic mice (4 mice in the control group and 5 mice in the miR-195 group) (Figure S6A). Due to the small number of mice and large variance in tumor size, tumor weight and size in the miR-195 group were not significantly smaller than those in the control group (Figures S6B–S6E). The body weights of the mice were not significantly different between two groups either (Figure S6F). However, we observed lung metastases in 4 of 4 mice in the control group, and in only 1 of 5 mice in the miR-195 group (Figure 7E). Quantification of lung metastasis demonstrated that the number of metastases is significantly smaller in the miR-195 group than in the control group, implying that miR-195 represses lung metastasis in vivo (Figures 7F–7H).

Survivin Mediates the Role of miR-195 to Regulate the Migration and Invasion of Lung Adenocarcinoma Cells

BIRC5 has been shown to regulate apoptosis, senescence, migration, and invasion of cancer cells.33,50,51 We recently demonstrated that miR-195 targets BIRC5 to induce apoptosis and senescence in NSCLC cells.33 We were curious whether BIRC5 also mediates the function of miR-195 to regulate cell migration and invasion. We found that overexpression of BIRC5 indeed reverses the effects of miR-195 and that knockdown of BIRC5 inhibits the migration and invasion of cancer...
cells, confirming that miR-195 targets BIRC5 to regulate the migration and invasion of lung cancer cells (Figures 8A–8D). However, we did not find a correlation between miR-195/BIRC5 expression and tumor stage in TCGA data (Figure S7). We also found that miR-195 has no influence on vimentin and E-cadherin protein levels, indicating that miR-195 probably does not regulate the epithelial-mesenchymal transition of NSCLC cells (Figure S8). Collectively, our data indicate that miR-195/BIRC5 dysregulation is not a driver of lung metastasis, but a contributing factor.

DISCUSSION
The capacity to simultaneously target multiple oncogenic pathways potentially enables a tumor suppressor miRNA to therapeutically address cancer heterogeneity. However, concerns have been raised over potential off-target effects, given that any gene possessing the target sequence of a miRNA in its 3′ UTR can theoretically be repressed by that miRNA. A complete understanding of the functions and targets of a miRNA in the context of cancer is therefore required for proper evaluation of its clinical potential. miR-195 has been shown to target various cancer-related genes to regulate lung cancer cell growth and chemosensitivity.38,41 Our investigation demonstrated that miR-195 targets TUBB to regulate the chemosensitivity of NSCLC cells. Interestingly, although TUBB is the most abundant among tubulin proteins, miR-195 regulation of TUBB does not alter microtubule structure. In parallel, we found that miR-195 represses lung metastasis in vivo, likely by targeting BIRC5 to regulate the migration and invasion of NSCLC cells. These findings enhance our understanding of the downstream targets of miR-195 and support the application of miR-195 replacement as a therapeutic approach to address both chemoresistance and metastasis.

The correlation between TUBB mutations and paclitaxel resistance in NSCLC was debated almost two decades ago.20–23 Similarly, the correlation between TUBB expression and paclitaxel resistance has also been discussed.17 However, neither TUBB mutations nor expression were found to correlate with NSCLC resistance to paclitaxel, and the role of TUBB in chemoresistance has never been experimentally validated. Here, we demonstrate that TUBB expression is increased in tumor tissues compared to adjacent normal tissues in lung adenocarcinoma and squamous cell carcinoma patients and that higher expression of TUBB is correlated with worse overall survival and poor response to chemotherapy in lung adenocarcinoma patients. Importantly, overexpression of TUBB confers resistance to MTAs, while knockdown of TUBB sensitizes NSCLC cells to MTAs. These findings demonstrate the oncogenic potential of TUBB and support the therapeutic value of targeting TUBB to chemosensitize NSCLC.

Additionally, we show that TUBB is a direct target of miR-195 and can be regulated by another target of miR-195, CHEK1. Consistent with this, we found that the expression of TUBB is positively associated with the expression of CHEK1 in lung adenocarcinoma and squamous cell carcinoma tissues. BIRC5, a well characterized oncogene, has been shown to regulate the growth, migration, and invasion of various cancer cells.25,50,52,53 Strategies to repress survivin have been proposed as attractive treatment options.54,55 We have recently shown that miR-195 directly targets BIRC5 to regulate the apoptosis and senescence of NSCLC cells.33 Here, we report that miR-195 directly targets BIRC5 to regulate the migration and invasion of NSCLC cells. Our results indicate that the miR-195/survivin axis is one of the major regulators of NSCLC migration and invasion and therefore a candidate target for treatment of NSCLC.

In conclusion, this report demonstrates that miR-195 targets TUBB to regulate the response of NSCLC cells to MTAs and that the expression of TUBB can be a prognostic factor in lung adenocarcinoma. It reveals
that BIRC5 mediates the function of miR-195 to regulate the migration and invasion of NSCLC cells. It also establishes the relevance of the miR-195/TUBB and miR-195/BIRC5 axis as a focal therapeutic target to simultaneously overcome drug resistance and repress lung cancer metastasis.

MATERIALS AND METHODS

Reagents and Cell Lines

Paclitaxel was obtained from Teva Pharmaceutical Industries (PA, USA). Eribulin mesylate (Eisai) was kindly provided by Dr. Peter Houghton. miR-195 mimics were purchased from Dharmacon (CO, USA) and IDT (IA, USA). Two miR-195 inhibitors, IH-300643-05-0005 (miR-195 inh #1) and HSTUD0320 (miR-195 inh #2), were obtained from Dharmacon and Sigma-Aldrich (MO, USA). Two negative control oligomers (oligos) (D-001810-10-05 and CN-001000-01-20) were purchased from Dharmacon. Lipofectamine RNAiMAX and 2000 transfection reagents were purchased from Thermo Fisher Scientific (MA, USA). Oligos were transfected into cells at 25 nM unless otherwise specified.

ON-TARGETplus TUBB SMARTpool siRNAs were purchased from GE Dharmacon (L-010325-00-0005). The targeted sequences include:

- 5’-GGAAUGGGCACUCUCCUUAA-3’, 5’-AAGAAUACC CUGAUCCGAU-3’, 5’-UCCAUCAGUUGGUAGAGAA-3’, and 5’-CCGCAUCUGUGUAUCA-3’.

Two siRNAs designed against different regions of CHEK1 were purchased from Sigma-Aldrich: SASI_Hs02_00326304 was designed to target 5’-TAGATATG AAGCGTGCCG-3’.

Four NSCLC cell lines—H1299 (large cell carcinoma), H1993, A549, and H358 (lung adenocarcinoma)—were established at the National Cancer Institute and obtained from Dr. John Minna and Adi Gazdar at the Hamon Center for Therapeutic Oncology Research at University of Texas (UT) Southwestern Medical Center in Dallas, TX. All cell lines were grown in RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cell lines were grown in a humidiﬁed atmosphere with 5% CO2 at 37°C, authenticated using short tandem repeat proﬁling, and conﬁrmed to be mycoplasma-free through PCR. Cells were discarded when they were close to passage 20.

H1299/control, H1299/miR-195, 344SQ/control, and 344SQ/miR-195 cells were generated using luciferase-pcDNA3, which was a gift from William Kaelin (Addgene plasmid #18964). TUBB cDNA was cloned from pLX304-TUBB (DNASU, HsCD00435210) and inserted into pIRESpuro3 (Catalog #631669, Clontech, CA, USA).

Cell Viability Assays

Cells were plated in 96-well format and/or transfected with oligos in different concentrations. After incubating for 24–48 h, cells were further treated with medium or different concentrations of drugs. After a total incubation of 120 h, cell viability was determined using the CellTiter-Glo cell viability assay (Promega). Dose-response curves were generated using GraphPad Prism 7 (CA, USA).
RNA Extraction and qRT-PCR
Total RNA was prepared using the mirVana miRNA Isolation Kit (Ambion) according to manufacturer’s instructions. Intracellular miRNA levels were assessed by qRT-PCR on an ABI ViiA7 System using miRNA expression assay primer and probe sets (Applied Biosystems). RNU44, RNU66, or U6 were used as controls for normalization of miRNA expression. mRNA levels were assessed by qRT-PCR on an ABI ViiA7 System using SYBR Green (Thermo Fisher) and primers designed by IDT and purchased from Sigma. GAPDH expression was used as a control for normalization of mRNA expression. Threshold cycle times (Ct) were obtained, and relative gene expression was calculated using the comparative cycle time method. Primers used for GAPDH: forward-5'-GAAGGTGAAGGTCGGAGTC-3' and reverse-5'-GAAGATGGTGATGGGATTTC-3'. Primers used for TUBB: forward-5'-AGGTTAGGGTAGAAA-3' and reverse-5'-GAGGTAAGTTGGAAGGAAG-3'.

Luciferase Reporter Assay
Luciferase reporter assays were performed as previously reported.56 Mutant constructs were generated with the seed target sites mutated or deleted as specified in Results. H1299 cells were maintained in 96-well plates and co-transfected with luciferase reporters (0.05 μg/well) and miRNA mimics or control oligo (15 nM). Luciferase activities were measured 72 h later using the Dual-Glo Luciferase Assay System (Promega). Firefly luciferase was normalized to sea pansy luciferase activity to evaluate the regulatory effect of miR-195 on its putative targets. Primers used for TUBB 3' UTR: forward-5'-GTATCAGACGCTCGACCAGCCTTGGTCCCTAAGCC-3' and reverse-5'-GTTATCCGGTGCCAGCTTTTTTCTTTTTTTTTTTTACATTTCCATTTATTTATTTGG-3'.

Figure 6. miR-195 Has No Effect on Microtubule Structure
Cells were treated with different conditions (20 nM oligos and/or 10 nM paclitaxel/eribulin) and stained with antibodies against tubulin (green) and DAPI (blue). Pictures were taken under 60× magnification, and scale bars represent 20 μm. Experiments were repeated at least twice.
Primers used for TUBB 3’ UTR with mutations in the binding site of miR-195: forward-5’-GTTGAACTTGAGAGTTTTTTTCATAT TGAAAAGATGACATCGCC-3’ and reverse-5’-AAAAACTCTCAA GTTCAACATAAAATAGAAATCTCAAATGTAGGATAGAA-3’.

Western Blots
Cell lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer. Equal amounts of lysate were resolved by SDS-PAGE and transferred to Immobilon polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked and probed with specific primary antibodies. Anti-Chk1 (A300-161A-T) was purchased from Bethyl Laboratories (TX, USA). Antibodies to calnexin (sc-11397) and GAPDH (sc-25778) were purchased from Santa Cruz Biotechnology (TX, USA). Anti-actin (4970P) was purchased from Cell Signaling Technology (MA, USA). Anti-TUBB (T7816-100UL) was purchased from Sigma (MO, USA). Bound antibodies were detected with secondary antibodies conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotechnology) and visualized by enhanced chemiluminescent (ECL) substrate (Pierce/Thermo Fisher Scientific) on an Odyssey Fc Imaging System (LI-COR). All western blots were repeated at least twice.

Migration and Invasion Assay
Cell migration was assessed using the Boyden chamber assay. After being transfected with oligos as specified, cells in serum-free medium were seeded into the upper chamber of 8-μm pore size inserts (Corning). The inserts were placed into the wells of a 24-well plate with media containing 10% FBS. The invasion assay was similarly set up, except that the inserts were coated with Matrigel. After 24 h incubation, cells that migrated through the membrane or invaded through the Matrigel into the lower chamber of the inserts were fixed with ethanol and stained with crystal violet. Pictures were taken from five randomly selected visual fields, and cells were counted using ImageJ (NIH). Chemotaxis was assayed in a similar manner. After seeding cells into a ClearView plate (Essen), cells migrating or invading to the lower chamber of the inserts were recorded using an IncuCyte Zoom live cell imaging system (Essen BioScience).

Animal Experiments
Animal protocols were approved by the Institutional Animal Care and Use Committee of the UT Health Science Center at San Antonio. Six- to seven-week-old female athymic nude Foxn1nu (nu/nu) mice were purchased from Envigo. Five- to seven-week-old female 129/ SV mice were purchase from Charles River. 1 × 10⁶ cells in 200 μL PBS/Matrigel (v/v, 1:1) were injected subcutaneously into the flanks of the mice. Mice were randomized into different groups without blinding. Tumor volumes were measured using calipers.
and calculated by the formula volume = \( \frac{1}{2} \times \text{width}^2 \times \text{length} \). For tail vein injections, \( 1 \times 10^6 \) cells were injected via the lateral tail vein. Growth of lung metastases was monitored via IVIS Spectrum In Vivo Imaging System (CA, USA).

### Statistical Analysis

Mean values between groups were compared by Student’s two-sided t test as implemented in GraphPad Prism 7, with \( p < 0.05 \) considered statistically significant. Results are representative of 2 to 4 individual experiments or as indicated in the figures. Differences in mouse body weight over time were compared by two-way ANOVA.

### SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omto.2019.07.004.

### AUTHOR CONTRIBUTIONS

X.Y. and A.P. were involved in study concept and design; acquisition, analysis, and interpretation of data; and drafting of the manuscript. Y.Z. and B.W. were involved in acquisition and analysis of data. J.M.K. provided materials and was involved in critical revision of the manuscript for important intellectual content. A.P. supervised the study, obtained funding, provided material support, and was involved in study concept and design, drafting of the manuscript, critical revision of the manuscript for important intellectual content, and statistical analysis. All authors read and approved the final manuscript.

### CONFLICTS OF INTEREST

The authors declare no competing interests.

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