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TITLE: Targeting Master Regulators of the Breast Cancer Metastasis Transcriptome

PRINCIPAL INVESTIGATOR: Timothy A. Chan MD, PhD

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15. ABSTRACT

The goal of our work is to characterize and target the master regulators of the breast cancer metastasis transcriptome. We seek to elucidate the key factors controlling large scale genetic programs that bring about the metastasis phenotype and to discover ways to target these regulators. To this end, this year, we have made substantial progress. We have comprehensively completed transcriptome profiling of the largest set of breast cancer primary/metastasis matched pairs that we know of. We have characterized the gene expression programs and the corresponding master regulators of these programs. The first major program we have worked out is that of neoangiogenesis. We showed that our top regulators control metastasis vasculogenesis by controlling RECK, which in turn, orchestrates a highly coordinated neoangiogenic switch. We have also developed in vitro and genomic platforms that will enable us to work out the blueprint of the hierarchy of regulators controlling the metastasis gene program.

16. SUPPLEMENTARY NOTES

metastasis, transcriptome, breast cancer, angiogenesis
Introduction

The vast majority of breast cancer deaths are due to metastatic progression. The central tenet underlying our work is that master regulators of the metastasis transcriptome are fundamentally important for the metastatic process and that systematic understanding of these changes will be of tremendous clinical diagnostic value and help lay the foundation for effective treatments to eradicate breast cancer. Understanding the ultimate drivers of metastasis is a crucial step in achieving the goal of a cure for metastatic disease. Through a systematic approach employing global, large-scale analysis of primary tumors and metastases, rigorous state-of-the-art bioinformatic analysis, and functional models using isogenic human breast cancer cells with varying metastatic potential, we will work to elucidate the mechanisms underlying our candidates as master regulators of breast cancer metastasis. We are developing methods to target these candidates using small molecules. Our specific research goals are the following. (Aim 1) First, we will elucidate the effects of our top candidates on the breast cancer metastasis transcriptome and epigenome. These studies will utilize microarray and next-generation sequencing technologies. We will define how these candidates remodel the transcriptome and epigenome to promote metastasis. (Aim 2) Second, we will determine the dependence of breast cancer metastasis on our candidates in both hormone positive and negative breast cancers. We will study the physiologic basis of how these genes regulate the metastatic process using both in vitro models and multiple mouse models of metastasis. And, we will apply our results to the clinic by determining the prognostic value of varying levels of our candidates in primary tumors. (Aim 3) Third, we will develop an approach to target our candidates to inhibit breast cancer metastasis using small molecules.

Keywords

metastasis, regulator, breast cancer, transcriptome, epigenetic

Overall Project Summary

For the first funding year, we have concentrated on Tasks 1 and 2 in the Statement of Work. This was to (1) elucidate the effects of our candidate master regulators of metastasis on the breast cancer metastasis transcriptome and (2) to determine the dependence of breast cancer metastasis on master regulators such as Trim25 and BRD8). To the end, we have made significant progress and are on track.
A primary effort this year was to evaluate the effects of expression of master regulators BRD8 and TRIM25 using global approaches. We have performed many of these analyses. Using both overexpression and knockout systems, examined the individual effector pathways which are regulated by these proteins. We have also completed our analysis of primary breast tumors and matched metastases using expression arrays and compared these results to the in vitro expression models. We compared these results to that of the in vitro cellular systems using GSEA and transcriptional module mapping.

Our data shows that one of the top GSEA enriched pathways regulated by TRIM25 and BRD8 is the neoangiogenesis pathway. These two genes converge to regulate a protein called RECK. We showed that RECK is shut off selectively in metastases and this change in expression also is associated with markedly increased likelihood of metastasis.

We went further and dissected the mechanism by which this happened. We showed that during development of metastasis, RECK controls a Stat3-dependent neoangiogenic switch. Its does this by associating with gp130 and multiple Stat3-dependent receptors on the cell membrane. Therefore, BRD8/TRIM25 is able to control angiogenesis by downregulating RECK, which controls a large angiogenic program. We showed that the effectors of this program includes multiple terminal effectors of angiogenesis, such as uPAR, VEGF, and others. Interestingly, this program is highly dependent in vivo on Stat3, as Stat3 can rescue a RECK loss phenotype. These results were published in *Oncogene* in 2014. This report puts RECK and the RECK-controlled angiogenic switch firmly within the regulomes governed by the metastasis master regulator candidates. It also answers the long sought question of what the root drivers of the neoangiogenic cascade in metastases are.

We are also actively working to develop systems to analyze the genome-wide targets of TRIM25 and BRD8 using CHIP-seq. So far, we have optimized antibodies for CHIP. Using CHIP followed by DNA bioanalysis, we have confirmed that TRIM25 and BRD8 associate with chromatin. These results are required as a required step to our eventual goal of characterizing how TRIM25 and BRD8 modulates the epigenome.

Lastly, this year, we have also made progress in our work on generating a blueprint of the master regulator hierarchy governing metastasis. As noted in our preliminary data, we have identified a collection of master regulators that control the metastasis transcriptome. TRIM25 and BRD8 are among the top ranking transcription factors among a set of 20 which control various arms of the metastasis program. We have now knocked down all 20 in pairwise combinations so that we can detect cooperativity. These will be analyzed using RNAseq this coming year. This critical experiment will be one of the foundations of Task 1 and it is progressing nicely.

In summary, we have made good progress. We are looking forward to continuing this work with the DOD.
**Key Research Accomplishments**

- Established overexpression and knockdown systems for TRIM25, BRD8 and multiple other top candidate master regulators required for the study of genomic changes leading to metastasis. These systems are a significant resource for the metastasis field.
- Characterization of the neoangiogenic genetic program of BRD8 and TRIM25 as being directed by silencing of RECK in metastatic lesions.
- RECK coordinately controls an orchestrated neoangiogenic switch during development of breast cancer metastases and is responsible for the angiogenic phenotype in metastases.

**Conclusions**

In summary, we have made substantial progress toward our aims during year 1. The aims have not changed. This year, we have completed metastasis transcriptome profiling and characterization. We have characterized a major arm of this program – the neoangiogenic arm – controlled by the metastasis regulators as involving the metastasis suppressor RECK. We have comprehensively worked out how RECK causes the vascularization that is required for metastases to take root. We have also established innovative reagents to study the master regulators, which will benefit the field as a whole. Plans for this coming year are to proceed with the aims as outlined in the grant. We plan to use transcriptome profiling of TRIM25/BRD8 and other master regulators to work out the regulatory hierarchy. We also plan to begin the work with mice to model the effects of the master regulators on breast cancer metastasis.

**Publications/References**


**Inventions, Patents, and Licenses**

Nothing to report

**Reportable Outcomes**

Nothing to report

**Other Achievements**

Nothing to report
BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person.

DO NOT EXCEED FOUR PAGES.

NAME
Timothy A. Chan

POSITION TITLE
Associate Member/ Associate Professor

Human Oncology and Pathogenesis Program

Memorial Sloan-Kettering Cancer Center

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)

<table>
<thead>
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<th>INSTITUTION AND LOCATION</th>
<th>DEGREE (if applicable)</th>
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<td>AB</td>
<td>1991-1995</td>
<td>Biochemical Sciences (Summa cum laude)</td>
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<td>Residency</td>
<td>2003-2007</td>
<td>Radiation Oncology</td>
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A. Personal Statement

Timothy A. Chan MD, PhD, is an Associate Member and Attending Physician at the Memorial Sloan-Kettering Cancer Center (MSKCC), Vice Chair of the Dept. of Radiation Oncology, and Associate Professor at the Weill Cornell School of Medicine. He is PI of a cancer genetics laboratory and is an expert in the study of tumor suppressor genes. Dr. Chan received an MD, a PhD in cancer genetics, completed a residency in Radiation Oncology, and a fellowship in cancer genomics and epigenomics. The Chan laboratory specializes in using genomic analysis to characterize the role of tumor suppressors in oncogenesis and in using multiple approaches for the study of cancer genes. These approaches include large-scale analyses, functional genomics, epigenomics, biochemical and molecular analyses, and mouse modeling. The lab has a special interest in deciphering the mechanisms underlying treatment response. He is also an integral part of the clinical teams at MSKCC and thus is in a unique position to effectively integrate his studies in the laboratory and efforts involving clinical treatments and samples. Dr. Chan is part of a multidisciplinary, collaborative research team that has the scope and breadth of expertise needed to obtain definitive outcomes for the studies proposed, and is dedicated to the success of this project.

B. Positions and Honors

Positions and Employment
1993, summer Cell cycle regulation of histone gene expression. Post-transcriptional regulation of tumor


7/06-6/07  Post-doctoral fellow, Epigenetic mechanisms of carcinogenesis. Advisor: Prof. Steve Baylin. Dept. of Oncology, Johns Hopkins School of Medicine, Baltimore, MD

8/2007-present  Assistant Member, Dept. of Radiation Oncology and Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, New York, NY

8/2008-present  Assistant Professor, Gerstner Sloan-Kettering Graduate School of Biomedical Sciences, Memorial Sloan-Kettering Cancer Center, NY

5/2009-present  Beene Translational Oncology Core, Faculty advisor, Memorial Sloan-Kettering Cancer Center

5/2010-present  The Cancer Genome Atlas, Colorectal Disease, MSKCC, Principal Investigator

9/2009-present  Co-director, MSKCC Acoustic Neuroma Service

2010-present  TCGA Colorectal Leadership, Analysis Working Group and Writing Committee

2010-present  Assistant Professor, Weill Cornell School of Medicine

2012-present  Associate Member, Human Oncology and Pathogenesis Program and Dept of Radiation Oncology, Memorial Sloan-Kettering Cancer Center, New York, NY

2012-present  Associate Professor, Gerstner Sloan-Kettering Graduate School of Biomedical Sciences, Memorial Sloan-Kettering Cancer Center, NY

2013-present  Vice Chair, Dept. of Radiation Oncology

2013-present  Director of Translational Oncology, Dept. of Radiation Oncology

Other Experience and Professional Memberships

2007-present  American Association for Cancer Research (AACR)

2003-present  American Society for Therapeutic Radiology and Oncology (ASTRO)

2006-present  American Society of Clinical Oncology (ASCO)


2007-present  Scientific Review Committee, American Society for Therapeutic Radiology and Oncology

5/2010-present  The Cancer Genome Atlas Project Analysis Working Group, member

2010-present  Scientific Advisory Board, Cancer Genetics, Inc. (New York)

2011-present  Scientific Advisory Board, QuanDx, Inc.

Honors

1994  Barry Goldwater National Science Prize

1994  Cold Spring Harbor Laboratory Undergraduate Research Program

1995  Phi Beta Kappa (top 24 of class of 1995 at Harvard College)

1995-2002  NIH Medical Scientist Training Program

1996  Howard Hughes Medical Institute Research Fellowship

2001  Paul Erlich Prize: awarded for excellence in research

2002  Johns Hopkins Fellows Research Award: for outstanding research

2005  ASTRO Young Investigator Travel Award

2005  ASTRO Translational Research Travel Award

2005  ASTRO Award of Merit: for outstanding research

2006  Young Investigator Award, American Society of Clinical Oncology

2008  FAMRI Clinical Scholar

2008  Louis Gerstner Investigator Award
C. Selected Peer-reviewed Publications (15 selected out of 77, in chronological order)


D. Research Support

**Ongoing Research Support** *(selected due to space)*

**NIH/NCI RO1-154767A1, Chan (PI)***

Elucidating the function of the widely inactivated phosphatase PTPRD in the molecular pathogenesis of glioblastoma

Role: PI

Aim is to determine the molecular mechanisms of PTPRD tumor suppression in GBM.

**NIH R21DE23229-01, Chan (PI)***

The mutational landscapes underlying aggressiveness in adenoid cystic carcinoma.

Role: PI

Aim is to determine the exonic mutations underlying tumor aggressiveness in adenoid cystic carcinoma.

**2010TC, Chan (PI)***

Dissecting and Targeting the PTPRD/STAT3 Axis in Glioma

Role: PI

Aim is to evaluate the STAT3 signaling cascade in GBM and to develop strategies to target this in gliomas.

**Era of Hope Award, Chan (PI)***

Congressionally Directed Medical Research Programs

Department of Defense (DOD)

Role: PI

Aim is to characterize master regulators of breast cancer metastasis.

**Completed Research Support**

**AVON Foundation Research Grant, Chan (PI)***

Clinical impact of genomic biomarkers for recurrence.

Role: PI

Aim is to identify genomic biomarkers for recurrence in breast cancer patients.

**Metastasis Research Center Grant, Chan (PI)***

Large-scale analysis of the breast cancer epigenome

Role: PI

Aim is to use next-generation sequencing technologies to identify biomarkers for breast cancer metastasis.
RECK controls breast cancer metastasis by modulating a convergent, STAT3-dependent neoangiogenic switch

LA Walsh1, DM Roy1,2, M Reyngold3, D Giri4, A Snyder1,5, S Turcan1, CR Badwe6, J Lyman7, J Bromberg5, TA King7 and TA Chan1,3

Metastasis is the primary cause of cancer-related death in oncology patients. A comprehensive understanding of the molecular mechanisms that cancer cells usurp to promote metastatic dissemination is critical for the development and implementation of novel diagnostic and treatment strategies. Here we show that the membrane protein RECK (Reversion-inducing cysteine-rich protein with kazal motifs) controls breast cancer metastasis by modulating a novel, non-canonical and convergent signal transducer and activator of transcription factor 3 (STAT3)-dependent angiogenic program. Neoangiogenesis and STAT3 hyperactivation are known to be fundamentally important for metastasis, but the root molecular initiators of these phenotypes are poorly understood. Our study identifies loss of RECK as a critical and previously unknown trigger for these hallmarks of metastasis. Using multiple xenograft mouse models, we comprehensively show that RECK inhibits metastasis, concomitant with a suppression of neoangiogenesis at secondary sites, while leaving primary tumor growth unaffected. Further, with functional genomics and biochemical dissection we demonstrate that RECK controls this angiogenic rheostat through a novel complex with cell surface receptors to regulate STAT3 activation, cytokine signaling, and the induction of both vascular endothelial growth factor and urokinase plasminogen activator. In accordance with these findings, inhibition of STAT3 can rescue this phenotype both in vitro and in vivo. Taken together, our study uncovers, for the first time, that RECK is a novel regulator of multiple well-established and robust mediators of metastasis; thus, RECK is a keystone protein that may be exploited in a clinical setting to target metastatic disease from multiple angles.

INTRODUCTION

Breast cancer is one of the most common cancers among women, causing over 400,000 deaths annually worldwide.1 Metastatic disease is a major cause of morbidity and mortality and is currently incurable, making it a primary obstacle to improving breast cancer outcomes.2 The molecular basis of metastasis remains incompletely understood. In recent years, a number of metastasis-suppressor genes (MSGs) have been discovered. These genes are defined by their specific ability to inhibit metastasis without altering primary tumor growth. Both preclinical models and retrospective human studies suggest that MSGs have key roles in controlling the development of metastasis.3 Elucidating the molecular mechanisms by which these genes control the metastatic process offers valuable insight into tumor biology and may lead to new therapeutic options.

Reversion-inducing cysteine-rich protein with kazal motifs (RECK) is a putative MSG that is implicated in tumor progression.4 RECK is a membrane-anchored protein that is involved in several physiological processes, including regulation of extracellular matrix (ECM) integrity, vascular growth during development and stabilization of tissue architecture.5,6 In some cell systems, RECK can influence matrix metalloproteinase (MMP) function.7 RECK expression is frequently silenced in tumor cells.8,9 Interestingly, a recent study focusing on genome-wide DNA methylation profiling of CpG islands in breast cancer identified the RECK gene as a common target of promoter hypermethylation.10 Despite documented effects of RECK on the behavior of tumor cells, the signaling pathways targeted by RECK and the specific mechanism by which RECK modulates metastasis remain elusive. Furthermore, while RECK-mediated invasion has been largely attributed to changes in MMP expression in vitro, most tumors and tumor cell lines express extremely low levels of MMPs, suggesting that additional mechanisms are likely at play.11,12

In this study, we apply a systematic, multifaceted strategy using human tumor samples, in vivo metastasis models and multiple, unbiased high throughput analyses to provide a comprehensive analysis of the role of RECK during breast cancer metastasis. We generated new data sets that include large number of patient samples and used them to illustrate a relationship between RECK expression and disease-specific survival. Furthermore, analyses of matched pairs of primary human breast tumors and distant metastases reveal that RECK expression is further silenced during metastatic progression. We demonstrate using multiple mouse models that RECK reconstitution in vivo suppresses tumor metastases. Using several unbiased screens, we have identified novel pathways and binding partners that directly mediate the effects of RECK on metastasis. Finally, we show that RECK controls these phenotypes through signal transducer and activator of transcription factor 3 (STAT3)-dependent regulation of an angiogenic program.

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RESULTS

Analysis of matched lymph node and distant metastases demonstrates that RECK expression is downregulated during metastatic breast cancer progression.

We first sought to explore whether RECK expression was lost specifically during metastatic progression or whether the process of RECK silencing was already completed in the primary tumor. To answer this question, we first evaluated RECK gene expression in 36 primary tumors and lymph node metastasis pairs. Microarray analysis of these tumors revealed a significant decrease in RECK expression in metastatic lymph nodes compared with matched primary tumors in luminal A, luminal B and Her 2 subtypes (P = 0.0009, Figure 1A). These results were confirmed by quantitative reverse transcriptase–PCR (qRT–PCR; P < 0.01, Supplementary Figure S1A). Interestingly, we did not see a significant difference in RECK expression in the basal subtype (Figure 1A); however, the baseline expression of RECK in our basal primary tumors was already considerably lower than other subtypes (Supplementary Figure S1B). This is not seen with other larger patient cohorts, suggesting that there may be significant diversity in RECK expression between different basal tumors.

We next wanted to determine whether the RECK silencing was also evident in distant metastases. We performed immunohistochemistry (IHC) for RECK on 43 matched primary breast tumors and distant metastatic lesions (including the lung, liver and bone) and scored the staining intensity. Distant metastases had significantly decreased RECK expression compared with matched primary tumors (P = 0.001; Figure 1B, Supplementary Figure S1C and D). Together, these data show that RECK expression is lost in many tumors during progression from primary tumor to metastasis, consistent with a role for RECK in protecting against metastatic breast cancer.

RECK does not alter cell proliferation and loss of RECK alone is not sufficient to achieve cellular transformation. As RECK expression is low in most solid tumor cell lines, we next investigated the effects of reconstituting RECK expression (by constitutively expressing RECK via lentiviral transduction) in three highly invasive breast cancer cell lines (MDA-MB-231, LM2-4175 and BOM1-1833). RECK expression was confirmed by western blotting (Figure 2a), and proper membrane localization was visualized by immunocytochemistry and confocal microscopy (Figure 2b). Altered RECK expression did not change the rate of cell growth in vitro (Figures 2c–e) nor did it result in cell cycle changes (Figures 2f–h). Given that RECK expression is lower in clinical tumor specimens compared with normal breast tissue, we next sought to examine whether loss of RECK is sufficient for malignant transformation. Stable knockdown of RECK did not transform normal human mammary epithelial cells (HMECs) in xenograft assays (Figures 2i–k). These results suggest that RECK loss is not sufficient to result in transformation or altered cell division but instead may act via other mechanisms.

RECK suppresses vascular endothelial growth factor (VEGF) expression, endothelial recruitment in metastatic lesions and metastatic burden in vivo

We next examined whether reconstituting RECK could reduce metastatic burden in an in vivo model of metastasis. Accordingly, we stably expressed RECK in LM2-4175 cells (an in vivo selected and highly lung metastatic cell line)3,14 and injected them into the lateral tail vein of athymic nude mice. Bioluminescent imaging (BLI) of mice re-expressing RECK revealed a significant decrease in metastatic tumor burden compared with empty vector (EV) control (Figures 3a and b). Given that a significant difference in metastatic burden was first evident via BLI at 4 weeks post-intravenous injection (Figure 3b), we harvested lung tissue from each group at this time point for detailed analysis. Consistent with the BLI data at 4 weeks, hematoxylin and eosin (H&E) staining of lung tissue (Figure 3c) revealed a significant decrease in tumor burden (Figure 3d) and the number of metastatic foci (Figure 3e) in mice injected with cells re-expressing RECK.

RECK has been previously suggested to have a role in vascular organization.2 Therefore, we stained lung tissue for CD34 and VEGF. RECK-expressing metastatic foci had significantly less CD34 staining (Figures 3c and f), suggesting that RECK suppresses de novo blood vessel formation or recruitment. This phenotype was concomitant with decreased VEGF (Figures 3c and g) and Ki67 expression (Figures 3c and h), demonstrating that RECK may interfere with metastatic outgrowth by reducing vascularization. A decrease in metastasis in vivo was also observed after intracardiac injection of BOM1-1833 cells (an in vivo selected and highly bone metastatic cell line)13 re-expressing RECK (Figures 3i and j).

RECK has been hypothesized to act in part through MMP-2, MMP-9 and MT1-MMP in some cell systems.15–17 Interestingly, in our breast metastatic model, immunostaining of metastatic nodules for MMP-2, MMP-9 and MT1-MMP revealed no significant changes in expression (Supplementary Figure S2A–C). However, as immunostaining does not discriminate between latent and active MMP-2 and MMP-9, we cannot discount the possibility that there may be differences in MMP activity. Nevertheless, MMP-2 and MMP-9 were undetectable in conditioned media from MDA-MB-231, LM2-4175 and BOM1-1833 by enzyme-linked immunosorbent assay (ELISA; data not shown), suggesting that any differences seen in vivo are likely from changes in secretion of these proteins from surrounding stroma and not from the tumor cells themselves. Together, these data suggest that the phenotypic effects of RECK expression in breast cancer metastases may involve novel mechanisms independent of MMPs.

Coordinate control of a cooperative neovascularization program by RECK via regulation of endothelial remodeling and multiple secreted regulators of angiogenesis

In order to gain further insight into the molecular changes associated with RECK expression, we analyzed the transcriptomes of MDA-MB-231 and LM2-4175 cells overexpressing RECK. Ingenuity pathway analysis of differentially expressed genes between control and RECK-overexpressing cells (Supplementary Tables S1 and S2) revealed significant alterations in IL-6, IL-8 and IL-10 signaling pathways in both cell lines (Figures 4a and b, Supplementary Tables S5 and S6). In addition, VEGF and hypoxia-inducible factor-1α signaling pathways were also altered in MDA-MB-231 and LM2-4175 cells, respectively. All of these pathways are intimately involved in the regulation of angiogenesis, consistent with our hypothesis that RECK can control transcriptional programs that influence tumor neovascularization.18–20

We next asked whether reconstituting RECK was sufficient to alter VEGF secretion or endothelial cell phenotypes in vitro. ELISAs were performed on conditioned media from MDA-MB-231, LM2-4175 and BOM1-1833 cells re-expressing RECK, and all were found to secrete significantly less VEGF compared with their respective controls (Figure 4c). In addition, conditioned media from MDA-MB-231 and LM2-4175 cells re-expressing RECK inhibited chemotaxis of endothelial cells using Boyden transwell assays (Figures 4d and e). Importantly, this phenotype was rescued with the addition of recombinant VEGF. Furthermore, conditioned media from MDA-MB-231 and LM2-4175 cells re-expressing RECK significantly reduced endothelial cell tube formation in vitro (Figures 4f and g), showing that RECK can regulate endothelial organization. These data suggest that RECK may be an important modulator of tumor-mediated angiogenesis, consistent with the changes observed in vivo.

To determine whether there are other potential angiogenic factors besides VEGF that are altered by RECK, we performed an
antibody array screen of 55 known pro- or anti-angiogenic factors, including various ECM components, proteases, growth factors and signaling molecules. LM2-4175 cells expressing either RECK or EV were subject to a low oxygen challenge (0.5% O₂) to mimic the hypoxic tumor environment and stimulate release of angiogenic factors. After 24 h, conditioned media was isolated from both cell lines and screened with the array. Strikingly, the array revealed that reconstituting RECK coordinately suppressed many pro-angiogenic factors and increased the secretion of anti-angiogenic factors (Figures 4h and i). For example, reconstituting RECK potently suppressed the secretion of urokinase plasminogen activator (uPA), a major modulator of extracellular proteolysis and angiogenesis and a known mediator of breast cancer metastasis.21

We confirmed RECK-mediated suppression of uPA secretion by ELISA in three breast cancer cell lines (Figure 4j). Together these data demonstrate an important role for RECK in coordinately...
modulating multiple angiogenic factors and for the first time reveal a link between RECK and regulation of uPA.

A spontaneous metastasis assay reveals that RECK suppresses metastasis in part through negative regulation of uPA. Given our finding that RECK modulates the secretion of uPA, a well-documented protein involved in ECM proteolysis and breast cancer metastasis, we next wanted to determine whether reconstituting RECK in breast cancer cells could suppress spontaneous metastasis in vivo. Metastasis is a multistep process, including initial invasion of surrounding tissue, intravasation/extravasation and survival and outgrowth at secondary sites. Although frequently modeled using tail vein injection, metastasis modeled via orthotopic injection may more accurately recapitulate the steps necessary for metastasis. MDA-MB-231 cells expressing either EV or RECK were orthotopically injected into the mammary fat pad of NOD/SCID/IL2Rγ-deficient (NSG) mice. Consistent with our in vitro proliferation results, primary tumor volume was unchanged following RECK re-expression (Figure 5a). BLI of mice at day 40 revealed a significant decrease in spontaneous metastasis from tumors expressing high RECK vs control (Figures 5b and c), which was confirmed by H&E staining and quantification of tumor burden in the lung and liver (Figures 5d and e). IHC was used to confirm that RECK expression was high in primary tumors with reconstituted RECK at day 40 (Figure 5f). Furthermore, IHC for uPA in primary tumors re-expressing RECK revealed a significant decrease in uPA staining intensity compared with controls (Figure 5g), again suggesting a strong relationship between RECK, uPA and metastasis.

**Figure 2.** RECK does not affect cell cycle or growth rate in invasive breast cancer cells and loss of RECK alone is insufficient to transform cells. (a) Overexpression of RECK in breast cancer cell lines. Representative western blotting analysis of MDA-MB-231, LM2-4175 and BOM1-1833 cells stably transduced with the lentiviral constructs indicated. β-Actin was used as a loading control. (b) Representative confocal immunofluorescent images of MDA-MB-231, LM2-4175 and BOM1-1833 cells stably transduced with lentiviral constructs indicated. IgG was used as a staining control. Scale bar represents 20 μM. Growth curve of (c) MDA-MB-231 (d) LM2-4175 and (e) BOM1-1833 cells stably transduced with lentiviral constructs indicated. Data are presented as mean ± s.e.m. (n = 3). Flow cytometric cell cycle analysis of (f) MDA-MB-231 (g) LM2-4175 and (h) BOM1-1833 cells stably transduced with lentiviral constructs indicated. Cell cycle curves and quantification of stages of cell cycle was based on the Watson (pragmatic) model (n > 10,000 events). (i) Representative immunoblot of HMEC-hTERT stably infected with the lentivirus indicated. HMEC-hTERT stably infected with the indicated lentiviral constructs were injected into the (j) lateral tail vein of athymic nude mice (n = 5) or (k) the mammary fat pad of NSG mice (n = 5). Representative BLI images at 15 weeks postinjection are shown.
Depletion of RECK promotes a uPA-dependent invasion and pro-angiogenic program

To evaluate the effect of RECK perturbation on VEGF and uPA, we knocked down RECK in the breast cancer cell lines Hs343T and Hs606T. We chose these cell lines as they have significant endogenous levels of RECK expression (Cancer Cell Line Encyclopedia, Broad, Supplementary Figure S3). Hs343T and Hs606T cells were transfected with scramble (control) or RECK small interfering RNA (siRNA). RECK knockdown was confirmed by western blotting (Figure 6a). Consistent with our RECK re-expression experiments, RECK knockdown had no significant effect on the growth of cells. Representative growth curves from Hs606T cells are shown in Figure 6b. ELISA performed on conditioned media isolated from these cells demonstrated that RECK knockdown results in a significant increase in VEGF and uPA secretion (Figures 6c and e). These changes were concurrent with changes in VEGF and PLAU gene expression (Figures 6d and f). Furthermore, using a Matrigel transwell invasion assay, we revealed that RECK knockdown significantly increased invasion of both Hs343T and Hs606T lines and that this was rescued by concomitant knockdown of uPA (siPLAU Figures 6g and h). This suggests that the increased invasive potential associated with the loss of RECK could be, in part, due to increases in uPA secretion. Finally, to test the effects of RECK loss on angiogenic markers, we repeated the angiogenesis protein array using conditioned media from Hs606T cells transfected with either scramble (control) or RECK siRNA. Consistent with previous results, RECK knockdown resulted in high levels of pro-angiogenic factors in conditioned media, including VEGF and uPA, and reduced levels of anti-angiogenic factors (Figures 6i and j).
RECK associates with multiple cell surface receptors, modulates cytokine signaling and activates STAT3 signaling.

To further dissect the details of the signaling pathways that underlie the effects of RECK, we tested the results of RECK knockdown on global gene expression in Hs343T and Hs606T cells (Supplementary Tables S3 and S4). Microarray and ingenuity pathway analysis analyses revealed that IL-6, IL-8, L10 and Janus-activated kinase/STAT signaling pathways were significantly altered in response to RECK knockdown in both the cell lines tested (Figures 7a and b, Supplementary Tables S7 and S8).

Figure 4. RECK regulates angiogenic and cytokine transcriptional programs, endothelial reorganization and a neovascular switch. RECK expression resulted in significant changes in IL-6, IL-10, IL-8, HIF1α and VEGF pathway genes. U133A 2.0 expression arrays were used to compare the transcriptomes of (a) MDA-MB-231+EV and MDA-MB-231+RECK cells or (b) LM2-4175+EV and LM2-4175+RECK cells. Differentially expressed genes were analyzed using the IPA software. (c) ELISA was used to determine VEGF concentrations in conditioned media from MDA-MB-231, LM2-4175 and BOM1-1833 cells stably transduced with lentiviral constructs indicated. Data are presented as mean ± s.e.m. (***P < 0.001, n = 3). (d) RECK suppresses endothelial cell migration, which is rescued by VEGF. Conditioned media from MDA-MB-231+EV, MDA-MB-231+RECK or MDA-MB-231+RECK+10 μM recombinant VEGF was used as a chemoattractant for HUVEC cells in a Boyden transwell migration assay. Data are presented as mean ± s.e.m. (*P < 0.05, **P < 0.01, n = 4). (e) Same as in panel (d) but with LM2-4175 cells (*P < 0.05, **P < 0.01, n = 4). (f) RECK regulates endothelial cell reorganization. Conditioned media from MDA-MB-231 or LM2-4175 cells stably transduced with the indicated lentiviral constructs was used to resuspend HUVEC cells in an in vitro tube formation assay. 5% BSA and 5% BSA +10 μM recombinant VEGF were used as negative and positive controls, respectively. Representative images are shown in panel (f). Scale bars represent 200 μm. (g) Quantification of tube formation data are presented as mean ± s.e.m. (***P < 0.001, n = 3). (h) ELISA was used to determine uPA concentrations in conditioned media from MDA-MB-231, LM2-4175 and BOM1-1833 cells stably transduced with the indicated lentiviral constructs. Data are presented as mean ± s.e.m. (***P < 0.001, n = 3).

RECK regulates metastasis and angiogenesis via STAT3

LA Walsh et al

Importantly, these data are consistent with pathways altered by reconstituting RECK in previous experiments (Figures 4a and b, Supplementary Tables S1).

Given that RECK is a glycosylphosphatidylinositol-anchored membrane protein, we hypothesized that RECK may be altering signaling pathways by directly binding other membrane proteins, extracellular cytokines or proteases. To test this, we modified a receptor antibody array to screen for potential RECK binding partners. RECK-immunoprecipitated lysate from Hs606T cells was used to probe a panel of 119 soluble extracellular or membrane-bound proteins. Immunoprecipitation revealed that RECK bound to IL-8, β1-integrin and galectin-1 (Figure 7c). As a positive control, we confirmed RECK binding in this modified system and by conventional western blotting (Figures 7c and d). We validated the interaction screen by immunoblot for β1-integrin and galectin-1 (Figure 7d). As one of the most significantly altered pathways common to all microarray analysis was IL-6 signaling, we next determined that RECK also directly complexes with IL-6RA and gp130, the principle mechanism of IL-6 signaling in breast cancer (Figure 7d). Finally, we identified that RECK also binds to the uPA receptor uPAR (Figure 7d), providing a possible additional mechanism for RECK-mediated suppression of uPA activity.

To characterize RECK-induced signaling events in the cytoplasm, we used a phospho-protein array and compared lysate from MDA-MB-231+EV and MDA-MB-231+RECK cells. RECK expression results in decreased phosphorylation of a number of growth-related oncoproteins. Among the most significantly downregulated phospho-proteins was pSTAT3. RECK expression resulted in a loss of phosphorylation on the activating Y705 downstream effector of IL-6 and IL-8 and is capable of inducing both VEGF and uPA.24-27 We confirmed that pSTAT3 is regulated by RECK using a doxycycline-inducible construct, showing that RECK induction resulted in significantly decreased pSTAT3 levels (Figure 7e). Furthermore, even under hypoxic conditions, which are known to potently activate STAT3 via phosphorylation, pSTAT3 was suppressed upon RECK reconstitution in LM2-4175 cells (Figure 7f). Consistent with these data, knockdown of RECK with siRNA in Hs343T and Hs606T cells resulted in increased pSTAT3 (Figures 7g and h).

**Figure 5.** Orthotopic metastasis model demonstrates that RECK decreases lung and liver metastasis but does not significantly alter growth of the primary tumor. MDA-MB-231+EV and MDA-MB-231+RECK cells were injected into the mammary fat pad of NSG mice. (a) Quantification of primary tumor volume over time. Data are presented as mean ± s.e.m. (NS = not significant, n = 7). (b) RECK suppresses metastatic dissemination in an orthotopic model. Representative BLI of mice at days 15 and 40. (c) Quantification of BLI on the contralateral side from primary tumor at day 40. Data are presented as whiskers min. to max. (P = 0.003, n = 7). Lung and liver tissue was isolated from mice at day 40 and were sectioned and stained with H&E. Representative H&E sections and quantification of metastatic tumor burden are shown for both (d) lung and (e) liver. Scale bars represent 300 μm. Data are presented as mean ± s.e.m. (n = 7). (f) Representative image of primary tumors stained with H&E and IHC for RECK. Data are presented as mean ± s.e.m. Scale bars represent 200 μm. (g) RECK expression suppresses uPA. Representative IHC of primary tumors for uPA and quantification of staining intensity. Scale bars represent 150 μm. Quantification was performed using the MetaMorph software. Data are presented as mean ± s.e.m. (*P < 0.05, n ≥ 4).

Figure 6. RECK depletion elevates VEGF and uPA secretion, activation of a coordinate pro-angiogenic program, and increases invasive potential but does not affect cell growth. (a) Representative immunoblot of Hs343T and Hs606T cells 48 h after transfection with SCR (scramble) or RECK siRNA. (b) Growth curve of Hs606T cells transfected with the indicated siRNA. Data are presented as mean ± s.e.m. (NS = not significant, n = 3). (c) VEGF ELISA on conditioned media isolated from Hs343T and Hs606T transfected with the indicated siRNA. Data are presented as mean ± s.e.m. (***P < 0.001, n = 3). (d) qRT-PCR of VEGF gene expression after transfection with the indicated siRNA. Data are presented as mean ± s.e.m. (***P < 0.001, n = 3). (e) uPA ELISA on conditioned media isolated from Hs343T and Hs606T transfected with the indicated siRNA. Data are presented as mean ± s.e.m. (***P < 0.001, n = 3). (f) qRT-PCR of PLAU gene expression after transfection with the indicated siRNA. Data are presented as mean ± s.e.m. (*P < 0.05, **P < 0.01, ***P < 0.001, n = 3). Quantification of Boyden transwell invasion of (g) Hs343T and (h) Hs606T cells transfected with the indicated siRNAs. Data are presented as mean ± s.e.m. (***P < 0.001, n ≥ 3). Conditioned media from Hs606T cells transfected with the indicated siRNA was used to probe an angiogenesis antibody array. (i) Image of array was used to quantify protein expression by densitometry, and altered candidates are shown in heat map (j).

Figure 7. RECK binds to multiple cell surface receptors and regulates cytokine and STAT3 signaling to modulate metastasis and survival. RECK knockdown resulted in significant changes in cytokine and angiogenesis pathways, including IL-6, IL-10, IL-8, Jak/Stat and VEGF. Human Genome U133A 2.0 expression arrays were used to compare the transcriptomes of (a) scramble vs siRECK in Hs343T cells and (b) scramble vs siRECK in Hs606T cells. Differentially expressed genes were analyzed using the IPA software. Immunoprecipitation using anti-RECK antibody in Hs606T cell lysate was used to probe a human soluble receptor antibody array. (c) Image of array highlighting enhanced RECK, IL-8, ITGB1 and GAL1 signal in IP-RECK compared with IgG (control). In the lower panel, data for each protein were taken at different exposures. (d) RECK binds to novel receptors that signal through STAT3. RECK co-IP demonstrates that RECK binds to β1-integrin, Galectin-1, IL-6Ra, gp130 and uPAR. (e) RECK decreases STAT3 activation. Representative immunoblot of lysates from MDA-MB-231 cells stably infected with a doxycyclin (dox)-inducible RECK plasmid. Cells were treated with 1 μg/ml dox for 18 h before harvesting lysate. Whole-cell lysate was used to probe for RECK, STAT3 and pSTAT3. β-Actin was used as a loading control. (f) RECK decreases STAT3 activation in hypoxic conditions. Representative immunoblot of LM2-4175 cells stably infected with lentiviral constructs indicated. Cells were placed in hypoxic conditions (0.5% O2) for 24 h before harvesting lysates. RECK depletion increases pSTAT3 levels. Representative immunoblot analysis of (g) Hs343T and (h) Hs606T cells transfected with the indicated siRNA. (i) STAT3 knockdown reverses the increased invasion resulting from RECK depletion. Quantification of invasion of Hs606T cells transfected with indicated siRNAs. Data are presented as mean ± s.e.m. (P < 0.05, n = 3). (j) STAT3 expression reverses increases in VEGF secretion post-RECK depletion. VEGF ELISA on conditioned media isolated from Hs606T cells transfected with indicated siRNA. Data are presented as mean ± s.e.m. (***P < 0.001, n = 3). (k) STAT3 reverses RECK-dependent suppression of metastasis. LM2-4175+RECK, LM2-4175+STAT3 and LM2-4175+RECK+STAT3 cells were injected into the mammary fat pad of NSG mice. Quantification of BLI on the contralateral side of the primary tumor was performed on day 30. Data are presented as whisker box plots, with the line depicting the mean and the boxes showing 1 s.d. (n = 5). (l) Lungs were isolated, sectioned and stained with H&E, and the metastatic tumor burden was quantified out of 10. (m) Kaplan–Meier of mice infected via tail vein with cells described in panel (k). Increased survival conferred by RECK expression is reversed by concomitant overexpression of STAT3. P = 0.023 (n = 5, log-rank).
RECK regulates metastasis and angiogenesis via STAT3

LA Walsh et al.

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Modulation of metastasis and angiogenesis by RECK is dependent on STAT3 signaling

We next investigated whether the effects of RECK on angiogenesis, invasion and metastasis are STAT3 dependent. First, we determined that the increase in HS606T invasion caused by RECK knockdown was attenuated with the concurrent knockdown of STAT3, demonstrating that RECK-mediated invasiveness is, in part, mediated by STAT3 (Figure 7i, Supplementary Figure S4B). In addition, increased VEGF secretion conferred by RECK knockdown was also attenuated following concurrent knockdown of STAT3 (Figure 7j). This shows that, like the invasion phenotype, RECK-mediated VEGF secretion is also due, at least in part, to STAT3 activation. Finally, we determined whether exogenously expressing STAT3 can reverse the ability of RECK to suppress metastasis. For this, we again made use of our in vivo metastasis model. LM2-4175 cells that stably overexpressed RECK were transduced with STAT3 and injected into the mammary fat pad of NSG mice (spontaneous metastasis model). BLI and histological analysis of lung metastasis at day 30 revealed decreased metastasis in mice with cells expressing RECK alone, as expected (Figures 7k and l). This phenotype was attenuated with the concomitant expression of STAT3, suggesting that STAT3 is downstream of RECK and that metastatic suppressive activity of RECK is mediated by STAT3. Using our tail vein in vivo metastasis assay and these cells, we found that mice injected with cells expressing RECK has a significant survival advantage that was abrogated by co-expression of STAT3 and RECK (P = 0.023, Figure 7m). Together, these results demonstrate that the invasive and angiogenic phenotypes that result from RECK suppression are mediated through STAT3 signaling.

Suppression of RECK expression is associated with poor survival in human breast cancer

Finally, we sought to validate the association of RECK expression with breast cancer survival outcomes and to determine the relationship of RECK loss with pathological covariates. Previous studies linking RECK with clinical outcomes used relatively small numbers of samples. We dichotomized 1586 breast cancer patients from the Curtis data set into ‘RECK high’ and ‘RECK low’ groups based on median RECK expression and analyzed disease-specific survival. Here, high RECK expression conferred a significant survival advantage (P = 0.025, Figure 8a). This was confirmed in two independent, large breast cancer data sets using identical methodology (P = 0.006, Figure 8b, Supplementary Figure SSA). Due to the unique size of the Curtis data set, we were able to further categorize RECK expression as ‘normal’ or ‘low’, based on values in normal breast tissue (Supplementary Figure SSB). Here, ‘low’ is defined as having an outlier level of RECK expression below the 10th percentile of RECK expression in normal breast tissue. Patients with primary tumors harboring normal RECK expression experienced significantly superior survival compared with those with tumors bearing low RECK (P = 0.000083, Figure 8c).

In addition to poorer survival, we found that higher primary tumor grade, cellularity and size were also significantly associated with low RECK expression (Figures 8d–f). RECK expression was not significantly associated with estrogen receptor, progesterone receptor or HER2 status in the Curtis or TCGA breast cancer cohorts (Figures 8g–i, Supplementary Figure SSC–E). Notably, the expression of RECK was significantly decreased in all breast cancer subtypes compared with normal breast tissue (Figures 8j and k, Supplementary FiguresSSF and Gj), suggesting that loss of RECK expression can occur across all breast cancer types. Together these results demonstrate that RECK expression is an important determinant of breast cancer survival, that decreased RECK expression is associated with aggressive tumor characteristics and that RECK alteration is likely selected for in cancers.

**DISCUSSION**

RECK is recognized as a frequently inactivated metastasis suppressor in a number of human malignancies. Although loss of RECK has been linked to aggressive tumor behavior and metastasis, the molecular mechanisms by which RECK affects these processes is incompletely understood. The effect of RECK on tumor cells has been thought to be attributable to changes in MMP activity. However, in most tumors, these proteases are not predominantly produced by the malignant cells themselves but instead by adjacent stroma.31 Furthermore, MMP levels in our *in vivo* tumor models do not significantly associate with RECK expression. Accordingly, our data here suggest that, at least in metastatic progression, RECK functions by associating with a number of receptors that signal through STAT3, modulating STAT3 signaling and promoting the coordinated expression of a multifaceted invasion and angiogenic program. As RECK is known to have key roles in normal development,12,33 we reveal a molecular framework for how RECK regulates metastasis and highlight how metastatic cells can usurp RECK-dependent processes to promote tumor cell survival at distant sites.

We established a significant relationship between RECK expression and disease-specific survival in breast cancer. Through the use of matched pairs of primary human breast tumors and both distant and lymph node metastasis, we show that RECK expression is suppressed in metastatic lesions compared with primary tumors. This is critical as it demonstrates that loss of RECK in metastases is not simply due to sporadic differences between primary tumors but occurs during metastatic progression. Furthermore, as tumor heterogeneity often exists within breast tumors, it would be interesting to determine whether cells with the lowest levels of RECK expression within an individual primary tumor are in fact the founder cells in distant metastases.

Preclinical data and retrospective human studies suggest that MSGs have the potential to improve patient selection for therapy and may predict response to treatment.34 For example, mechanistic insights into the function of the MSG NM23 have led to the development of novel treatment strategies both exploiting aberrant NM23-associated cell signaling and reconstituting NM23 itself. Clinical trials using NM23 as an anti-metastasis therapy are currently underway.29 Notably, it has been shown *in vitro* that treating cell lines harboring epigenetically silenced RECK with HDAC or DNA methyltransferase inhibitors can lead to the re-expression of RECK.35,36 In our study, RECK reconstitution in breast cancer cells significantly decreased metastatic burden in multiple *in vivo* models of metastasis. As such, it may be reasonable to explore new treatment strategies aimed at treating tumors with RECK silencing with epigenetically targeted therapies.

RECK not only affects invasive potential of cancer cells but its loss also supports the survival and expansion of tumor cells in distant organs and tissues. Angiogenesis is a hallmark of cancer, wound healing and various ischemic and inflammatory diseases.37,38 VEGF is a potent inducer of angiogenesis and lymphangiogenesis and is a highly specific mitogen for endothelial cells.39 Here, we reveal that RECK directly modulates VEGF secretion. Furthermore, RECK convergently regulates multiple pro-and anti-angiogenic factors in a coordinated fashion. These data therefore provide detailed mechanistic insights into the RECK-mediated angiogenic phenotype.40

Our data reveal that RECK functions by regulating STAT3 activity. STAT3 activation has been linked to VEGF expression and oncogenes, but the mechanisms underlying this hyperactivity are not well understood.41,42 Two primary transcription factors that directly regulate VEGF expression are STAT3 and hypoxia-inducible factor-1α.43 Induction of VEGF can be achieved by these transcription factors through diverse stimuli, including hypoxia and cytokines. Our data show that RECK binds to many receptors
**Figure 8.** Clinical and pathological correlates of RECK loss in breast cancer. (a) Loss of RECK expression is associated with poor clinical outcome. Kaplan–Meier survival plot of 1586 breast cancer patients from the Curtis breast cancer data set separated into ‘RECK High’ and ‘RECK Low’ groups based on the median RECK expression ($P=0.025$, log-rank). (b) Kaplan–Meier survival plot of 295 breast cancer patients from the van de Vijver breast cancer data set separated into ‘RECK High’ and ‘RECK Low’ groups based on the median RECK expression ($P=0.006$, log-rank). (c) Kaplan–Meier survival plot of 1586 breast cancer patients from the Curtis breast cancer data set separated into ‘RECK Normal’ and ‘RECK Low’ groups based on the RECK expression levels in normal breast tissue ($P=0.000083$, log-rank). See text for rationale. RECK expression from the Curtis breast cancer data set by (d) tumor grade in 1902 tumors, (e) cellularity in 1927 tumors and (f) tumor size in 1972 tumors. RECK expression in tumor sets segregated by (g) ER status in 1948 tumors, (h) PR status and (i) HER2 status in 1992 tumors. All data are presented as mean ± s.e.m. ($^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.0001$, NS = not significant). (j) RECK expression based on histological subtype in 2037 breast cancer tumors. Data are presented as Tukey’s box plot. (k) RECK expression based on PAM50 subtype in 1986 breast cancer tumors. Data are presented as Tukey’s box plot.
that are known activators of STAT3 signaling and angiogenesis, including IL-8, galectin-1, B1-integrin, uPAR and the primary activators of STAT3 signaling in breast cancer, IL-6RA and gp130.\textsuperscript{23,44–48} Interestingly, it has previously been reported that the inhibition of gp130 signaling in breast cancer blocks STAT3 activation, inhibiting malignancy \textit{in vivo}.\textsuperscript{59} It seems that RECK has a central role in coordinating and downregulating the extent of STAT3 activation downstream of these various receptors. This activity, in turn, regulates neovascular programs that are important for metastases. Of note, STAT3 can be activated by phosphorylation at Y705 and/or S727. pSTAT3-Y705, which was assessed in this study, is the STAT3 phosphorylation site that is associated with IL-6 and IL-8 signaling.\textsuperscript{50,51} In contrast, STAT3-S727 phosphorylation is associated with cellular proliferation via extracellular signal–regulated kinase signaling and has been shown to inversely correlate with pSTAT3-Y705 levels.\textsuperscript{52–55} Here we show that RECK knockdown results in increased pSTAT3-Y705 and decreased pSTAT3-S727 (Supplementary Figures S6A and B). This further supports our model of RECK-mediated STAT3 regulation of metastatic processes via inhibition of IL-mediated STAT3 phosphorylation.

Interestingly, studies focusing on microRNA expression and metastasis have revealed that STAT3 can control microRNAs, which in turn regulates RECK expression.\textsuperscript{56} These data combined with the findings in our study point to a feedback loop between RECK, STAT3 and microRNAs. Of note, our study did not see changes in tumorigenicity or primary tumor growth by directly modulating RECK expression. In contrast, studies on microRNAs that modulate RECK do report changes in tumorigenicity, suggesting that RECK expression is just one of the many potential targets of microRNAs.\textsuperscript{57}

In our study, we showed that RECK mediated suppression of spontaneous metastasis \textit{in vivo}, which suggests that RECK can affect multiple steps in the metastatic process. Interestingly, RECK suppression was concomitant with increased expression of uPA, a known inducer of breast cancer metastasis. \textit{In vitro}, it has been shown that uPA itself has the capacity to increase the invasiveness of tumor cells,\textsuperscript{58} which we have confirmed here using breast cancer cell lines. The uPA system is a serine protease family that includes uPA, uPAR and plasminogen activator inhibitors.\textsuperscript{59} uPA catalyzes the activation of plasminogen to plasmin, which degrades the ECM and basement membranes either directly or indirectly through activating pro-MMPs (as most MMPs are secreted as latent zymogens).\textsuperscript{50,60,61} This uPA–RECK relationship may partially explain how most tumors and tumor cell lines secrete very low levels of MMPs, yet retain strong invasive potential. Although MMPs have a clear role in metastasis, \textit{in vivo} studies are complicated owing to the presence of both inactive and active MMPs, whose conversion is catalyzed by other proteases. Further, the cellular origin of secreted MMPs \textit{in vivo} is difficult to ascertain. As a result, we cannot exclude the importance of MMPs in our study, though our discovery of RECK-mediated uPA secretion provides both complimentary (uPA-induced MMP activation) and alternative (direct ECM proteolysis) mechanisms of RECK-associated suppression of metastasis.

In summary, RECK is a multifunctional and broadly important MSG that is frequently suppressed in cancer. Our observations address several ill-defined issues central to the development of metastasis: how a frequently inactivated MSG suppresses metastases, how metastases activate neovascular programs, what signaling mechanisms underlie RECK’s control of angiogenesis, and the molecular causes of STAT3 hyperactivity in metastatic lesions. Our work provides critical insights into our understanding of the metastatic process.

**Materials and Methods**

**Expression data sets**

The van de Vijver\textsuperscript{62} and Stockholm\textsuperscript{63} breast cancer data sets were downloaded from the Gene Expression Omnibus (GEO [http://www.ncbi.nlm.nih.gov/geo]). The Curtis data set\textsuperscript{64} and the TCGA breast cancer data set\textsuperscript{64} were downloaded from [http://www.oncomine.org](http://www.oncomine.org).

**Primary human breast cancer and matched lymph node metastases**

Paired primary tumor and lymph node metastases were collected during surgical resection. All tissue was used with informed consent and banked at Memorial Sloan-Kettering Cancer Center (MSKCC) in accordance with IRB approval. All samples were snap-frozen and stored at \(-80°C\) until use. All samples were H&E stained and independently reviewed by a breast cancer pathologist. Tumors were microdissected to obtain >70% purity. RNA was isolated from fresh frozen samples using the RNeasy Plus mini prep kit (Qiagen, Valencia, CA, USA). Nucleic acid quality was determined with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Primary tumor and matched metastasis pairs, for which RNA of sufficient quantity and quality was available, were analyzed on Agilent Human Genome U133 2.0 Array (Agaffymetrix, Santa Clara, CA, USA) at the genomics core at Sloan-Kettering Institute according to the manufacturer’s protocol.

**Primary human breast cancer and matched distant metastases**

Paired primary tumor and distant metastases were collected with informed consent and banked at MSKCC in accordance with IRB approval. All samples were formalin fixed and paraffin embedded until use. All samples were H&E stained and independently reviewed by a breast cancer pathologist. Samples were also sectioned (5 μm) and immunostained with a RECK antibody and blindly scored.

**Microarray analysis of cell lines**

Total RNA was isolated from cell lines using the RNeasy Plus mini prep kit (Qiagen). Nucleic acid quality was determined with the Agilent 2100 Bioanalyzer. Samples were analyzed on Agilent Human Genome U133 2.0 Array at the genomics core at Sloan-Kettering Institute according to the manufacturer’s protocol.

**Gene expression microarray analysis**

Gene expression microarray analysis in cell lines was performed with the Human Genome U133A 2.0 microarray (Agffymetrix). CEL files were imported into the Partek Genomics Suite software and normalized using robust multarray average quartile normalization and log probe summarization. Differentially expressed genes were identified by analysis of variance. Genes that were differentially expressed at \(P < 0.05\) and had at least an absolute value of 1.2× fold change (1.5-fold change for Hs343T) were considered significant. Enriched pathways were identified in the differentially expressed genes in Ingenuity pathway analysis (Ingenuity Systems, Valencia, CA, USA) and determined significant at \(-\log2(P\text{-value}) > 1.3\) (Supplementary Tables S5–S8).

**Animal studies**

All animal work was done in accordance with the Institutional Animal Care and Use Committee at MSKCC. Athymic Nu/Nu mice were obtained from Harlan Laboratories (Indianapolis, IN, USA) and NSG mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). All animals were injected between 5 and 7 weeks of age. Intracardiac injections of BOM1-1833 cells and intravenous injections of LM2-4175 cells were performed as previously described.\textsuperscript{1.14} Single-cell suspensions of the indicated cell lines (1 × 10\(^6\) cells) in 1.1 serum-free media/Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were injected orthotopically into the mammary fat pad of 6-week-old female NSG mice. Animals were imaged weekly via BLI (IVIS, Xenogen, Alameda, CA, USA).

**Cell lines**

Hs606T, Hs343T, human umbilical vein endothelial cells (HUVEC), HEK293T and HMEC-H1ERT were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured according to ATCC’s recommended conditions. MDA-MB-231, LM2-4175 and BOM1-1833 were cultured in 1:1 serum-free media/Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and determined signifi- cant at \(-\log2(P\text{-value}) > 1.3\) (Supplementary Tables S5–S8).
all generous gifts from Dr Joan Massague (Memorial Sloan-Kettering Cancer Center, New York, NY, USA) and were cultured as described previously.13,14

Cell proliferation assay
In all, 1 x 10^5 cells were plated in 6-cm plates in triplicate, and cell numbers were counted at the indicated days using a VICELL cell counter (Beckman, Indianapolis, IN, USA).

Cell cycle analysis
Cells were trypsinized, fixed with ice cold 70% ethanol and stained using 7-AAD. Cell cycle analysis was performed on a FACS Calibur (BD Biosciences). Analysis was performed using the FlowJo software (FlowJo, Ashland, OR, USA), and cell cycle quantification was performed using the Watson (Pragmatic) model.

Immunoblotting
Cell lysates were prepared with Celllytic M (Sigma, St Louis, MO, USA) containing protease and phosphatase inhibitor (Thermo Scientific, Pittsburgh, PA, USA). Debris was removed via centrifugation for 10 min at 14 000 r.p.m. in an Eppendorf Centrifuge 5424C (Eppendorf, Hauppaug, NY, USA) at 4 °C. Protein concentrations were determined by BCA protein assay kit (Thermo Scientific), and equal amounts of protein samples were loaded on NuPAGE Bis-Tris 4–12% sodium dodecyl sulfate gels (Invitrogen, Grand Island, NY, USA) and blotted onto polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Imaging was performed on a FujiFilm LAS4000 (GE Healthcare, Pittsburgh, PA, USA). All blots were repeated three times, and representative images were chosen for publication.

Chemotaxis assay
Conditioned media from the indicated cell types was placed in the bottom chamber of a 12-well plate. HUVEC cells were seeded at a density of 5 x 10^4/well in transwell inserts placed in the upper chamber (8 μm, BD Biosciences). After incubation for 12 h, migrated cells were quantified by counting on a Nikon TE2000E brightfield microscope, Nikon Instruments, Melville, NY, USA).

ELISA
All ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA) and performed according to manufacturer’s instructions. For isolation of conditioned media, equal number of cells was seeded, and media was conditioned overnight. To remove cellular contaminant, conditioned media was collected and centrifuged at 300 g for 10 min in an Eppendorf Centrifuge 5424C (Eppendorf) at 4 °C.

Antibody arrays
Proteome Profiler antibody arrays were purchased from R&D Systems and used according to the manufacturer’s instructions. Conditioned media was generated as described above (ELISA). Imaging was performed on a FujiFilm LAS4000. Quantification of densitometry was achieved using the ImageQuantTL software (GE Healthcare).

Virus production and generation of stable cell lines
HEK 293T cells were transfected with lentiviral expression constructs using FuGENE 6 (Promega) in combination with pCMV-dR8.2 (gag/pol) and VSV-G (env) expression vectors using FuGENE 6 (Promega). Viral stocks were collected 48 h posttransfection, filtered (0.45 μm) and placed on target cells for 8 h in the presence of 8 μg/ml polybrene. Forty-eight hours postinfection, cells were selected in the presence of puromycin (2 μg/ml).

RNA interference
Knockdown using scramble and/or gene-specific siRNA (ON-TARGET SMARTpool, Thermo Scientific) was performed using Lipofectamine RNAiMAX (Invitrogen). Stable knockdown was achieved using scramble and RECK-specific shRNA constructs (Thermo Scientific) to generate virus as above (see ‘Virus production and generation of stable cell lines’).
Immunoprecipitation
For immunoprecipitation, 50 μl of protein G magnetic beads (Pure-Proteome, Millipore) were crosslinked with 10 μg anti-RECK goat polyclonal antibody (R&D Systems) or goat polyclonal IgG antibody (R&D Systems) using 5 mM BS3 according to the manufacturer’s instructions (protocol guide, Millipore). Confluent Hs606T whole-cell lysates (500 μg total protein) were incubated with crosslinked beads at 4°C overnight with gentle agitation. The immunocomplexes were washed three times with TBS-T (0.1%), bound protein eluted in 50 μl 0.2% glycine-HCl (pH 2.5) and neutralized using 5 μl of 1 M Tris (pH 8.5). Eluates were then used to probe an antibody array (Human Soluble Receptor Antibody Array, Non-Hematopoietic Panel, R&D Systems). This array was modified by using immunoprecipitated eluate instead of conditioned media or cell lysate. Eluates were also subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting analysis for RECK (goat antibody, R&D Systems), uPAR (goat antibody, R&D Systems), galectin-1 (rabbit antibody, Cell Signaling, Beverly, MA, USA), and integrin β1 (rabbit antibody, Cell Signaling).

Statistical analysis
For survival analysis, survival curves were analyzed using the Kaplan–Meier method and log rank test with the IBM Statistica 20 software (IBM, Road Armonk, NY, USA). All other statistical analysis was performed using the Prism 6 Software (GraphPad Software, La Jolla, CA, USA).

Kinexus antibody microarray
Protein lysates were sent to Kinexus Bioinformatics Corporation (Vancouver, BC, Canada) and profiled using their Kinex 850 Antibody Microarray (KAM). All samples were normalized according to the Kinexus normalization guidelines, and a list of downregulated candidate proteins was generated.

Antibodies

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Microarray accession numbers
Gene Expression Omnibus (GEO)—accession number GSE56898.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS
LAW and DMR designed, performed and analyzed the experiments and aided in writing the manuscript. MR, DG, AS, ST, CAB, JL, JB and TAK contributed to the completion of various experiments. LAW and TAC conceived the study, supervised the research and contributed to writing the manuscript.

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