ABSTRACT

The tumor-promoting functions of autophagy are primarily attributed to its ability to promote cancer cell survival. However, emerging evidence suggests that autophagy plays other roles during tumorigenesis. Here, we uncover that autophagy promotes oncogenic RAS-driven invasion. In epithelial cells transformed with oncogenic RAS, depletion of autophagy-related genes suppresses invasion in three-dimensional culture, decreases cell motility, and reduces pulmonary metastases in vivo. Treatment with conditioned media from autophagy-competent cells rescues the invasive capacity of autophagy-deficient cells, indicating that these cells fail to secrete factors required for RAS-driven invasion. Reduced autophagy diminishes the secretion of the promigratory cytokine interleukin-6 (IL-6), which is necessary to restore invasion of autophagy-deficient cells. Moreover, autophagy-deficient cells exhibit reduced levels of matrix metalloproteinase 2 and WNT5A. These results support a previously unrecognized function for autophagy in promoting cancer cell invasion via the coordinate production of multiple secreted factors.

SIGNIFICANCE: Our results delineate a previously unrecognized function for autophagy in facilitating oncogenic RAS-driven invasion. We demonstrate that an intact autophagy pathway is required for the elaboration of multiple secreted factors favoring invasion, including IL-6. Cancer Discov; 4(4): 466–79. © 2014 AACR.

INTRODUCTION

The RAS proteins are members of a family of small GTPases critical in mediating cellular responses following activation by upstream extracellular signals, such as growth factors. Oncogenic mutations in RAS, which result in constitutive activation, are found in approximately 30% of human cancers; they are highly prevalent in several carcinomas, including lung, pancreas, and colon (1, 2). Notably, oncogenic RAS drives diverse cellular programs—proliferation, cell survival, migration, invasion, and alterations in differentiation—that support tumor initiation and progression. Such mutations present a formidable therapeutic obstacle, because patients harboring mutant KRAS are refractory to most available systemic therapies and exhibit extremely poor survival (2). Hence, identifying new processes to target cancer cells with...
hyperactive RAS remains a question of immense clinical significance. One such pathway may be macroautophagy (autophagy), a tightly controlled lysosomal degradation process that promotes cell survival during nutrient starvation and stress. Recent evidence indicates that basal autophagy levels are enhanced upon oncogenic RAS activation and support RAS-driven transformation and tumorigenesis (3–7).

The tumor-promoting functions of autophagy are largely ascribed to its importance as a survival pathway in response to diverse environmental stresses (8, 9). For example, enhanced autophagy is observed in poorly perfused, hypoxic tumor regions, and loss of autophagy is associated with increased necrosis (10). Autophagy also promotes tumor cell survival in response to various cytotoxic and targeted chemotherapies (11). Importantly, studies of oncogenic RAS transformation have revealed that the protumoral effects of autophagy are not limited to increased survival of cancer cells under duress; rather, autophagy contributes to the metabolic fitness of the entire tumor population (3–6). Because strong oncogenic insults, such as RAS activation, are marked by profound metabolic alterations that drive both energy production and biosynthetic capacity in rapidly proliferating cells, it has been hypothesized that autophagy maintains key metabolic pathways in RAS-transformed cells. In support, a growing body of work has unveiled a requirement for autophagy in driving proliferation as well as sustaining multiple core metabolic functions in RAS-transformed cells (3–7). These results are not unique to oncogenic RAS activation, as deletion of RB1CC1/FIP200, a mediator of autophagosome initiation, are not unique to oncogenic RAS activation, as deletion of RB1CC1/FIP200, a mediator of autophagosome initiation, inhibits polyoma middle T-driven mammary cancer, due to reduced proliferation and glucose metabolism (12).

In addition to its effects on proliferation and metabolism, oncogenic RAS drives diverse aggressive cellular behaviors that support tumor progression and metastasis; importantly, RAS-transformed epithelial cells exhibit highly invasive behavior associated with an epithelial-to-mesenchymal transition (EMT; ref. 13). Here, in epithelial cells transformed with oncogenic RAS, we demonstrate that autophagy facilitates extracellular matrix (ECM) invasion, tumor cell motility, and pulmonary metastasis in vivo. Using a three-dimensional (3D) culture system, we uncover that autophagy inhibition restricts RAS-driven cell invasion and restores several aspects of normal epithelial architecture, including the polarized deposition of basement membrane and cell–cell junctional integrity. Furthermore, autophagy is required for the production of multiple secreted factors in RAS-transformed cells, including interleukin-6 (IL-6), matrix metalloproteinase 2 (MMP2), and WNT5A, which altogether facilitate cancer cell invasion.

RESULTS

Autophagy Promotes Invasion Driven by Oncogenic RAS in 3D Culture

To elucidate how autophagy affects the cellular behavior of RAS-transformed epithelial cells, we used the MCF10A 3D epithelial culture system to interrogate how autophagy affects the growth and morphogenesis of cells expressing oncogenic RAS (14). We generated stable pools of MCF10A human mammary epithelial cells expressing a control vector (BABE) or an oncogenic form of HRAS (HRASV12) that enhances basal autophagy and elicits robust anchorage-independent transformation (3). When cultured on laminin-rich ECM, control MCF10A cells formed hollow, spherical acini (Supplementary Fig. S1A; ref. 15). In contrast, HRASV12-transformed cells produced grossly aberrant structures notable for extensive protrusions that invaded the surrounding ECM. Individual HRASV12 structures formed these invasive protrusions in as early as 3 to 5 days, ultimately producing disorganized networks of cells intermingled with large cell clusters after 8 days in 3D culture (Fig. 1A and B, left). The 3D morphology we observed using HRASV12 MCF10A cells resembles that reported for mouse mammary cells expressing oncogenic RAS and grown in a 3D collagen matrix (16).

To inhibit autophagy in this experimental system, we stably expressed unique short hairpin RNAs (shRNA) against two autophagy genes (ATG)—ATG7 (shATG7-1 and shATG7-2) or ATG12 (shATG12)—in MCF10A cells expressing HRASV12. ATG7 or ATG12 knockdown decreased target protein levels, reduced basal- and starvation-induced autophagy in Hank’s Buffed Saline Solution (HBSS), and increased protein levels of the autophagy substrate p62/SQSTM1 (Supplementary Fig. S1B–S1E). In 3D culture, the invasive protrusions observed with oncogenic RAS activation were profoundly attenuated in ATG-deficient cells. Instead, HRASV12 shATG (shRNAs against autophagy genes) structures were spherical in morphology, similar to nontransformed BABE controls (Fig. 1A and B). Decreased invasive protrusions following autophagy inhibition were also observed upon stable ATG3 knockdown (shATG3), and upon treatment with chloroquine or bafilomycin A, two lysosomal inhibitors that block the late steps of autophagy (Supplementary Fig. S1F). Importantly, ATG knockdown in HRASV12 cells did not affect RAS expression or–activation–associated phosphorylation of the major downstream effector MAPK–ERK (Supplementary Fig. S1G). Thus, the reduction in 3D invasive protrusions following ATG knockdown is not due to decreased expression or activity of oncogenic RAS.

The disruption of basement membrane integrity is a hallmark of carcinoma invasion in vivo (14). To corroborate whether the protrusions we observed in HRASV12-transformed 3D cultures represented invasive behavior, we first evaluated basement membrane integrity by examining the expression and localization of the basement membrane protein LAMA5 (laminin 5) in HRASV12-derived acini. Consistent with previous reports, control (BABE) nontransformed MCF10A acini displayed polarized deposition of LAMA5 onto the basal surface (Fig. 2A, left; ref. 15). In contrast, the expression of HRASV12 resulted in cytosolic accumulation of LAMAS, with no evidence of polarized deposition at the cell–ECM interface. Notably, this aberrant cytosolic staining pattern was especially significant in response to various cytotoxic and targeted chemotherapies.
Figure 1. Autophagy is required for the formation of invasive protrusions mediated by HRASV12 in 3D culture. A and B, HRASV12 MCF10A cells stably expressing shCNT or shATGs were 3D cultured on Matrigel for the indicated number of days. Representative phase contrast images at the indicated magnifications are shown. Scale bar, 100 μm.
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To extend these results, we evaluated ECM proteolytic activity in control and autophagy-deficient HRASV12 cultures by assessing fluorescence emanating from the proteolytic cleavage of dye-quenched collagen IV (DQ-COL4). In control (BABE) nontransformed acini, we observed a faint ring of fluorescence surrounding each structure, corresponding to COL4 degradation due to the normal outgrowth of acini during 3D morphogenesis. On the other hand, HRASV12 shCNT-expressing structures exhibited high levels of fluorescence that extended well beyond the immediate vicinity of individual structures (Fig. 2B). Notably, streaks of fluorescence connecting adjacent structures were frequently observed in HRASV12 shCNT cultures (Fig. 2B), which resembled the networks of invasive protrusions (Fig. 1B). In contrast, HRASV12 shATG-derived structures exhibited a ring-like COL4 degradation pattern that was restricted to the cell–ECM interface, similar to that observed in nontransformed controls (Fig. 2B). Thus, the absence of morphologic protrusions in ATG-deficient HRASV12 cultures was associated with the restoration of basement membrane integrity and reduced ECM proteolytic activity. Together, these findings corroborate that autophagy supports RAS-driven invasion in 3D culture.

ATG Depletion in HRASV12 Structures Does Not Promote Apoptosis or Proliferation Arrest in 3D Culture

We next evaluated the impact of autophagy inhibition on oncogenic RAS-driven proliferation and cell survival. During normal MCF10A acinar morphogenesis, autophagy inhibition results in the enhanced apoptosis of cells occupying the luminal space (17). To test whether autophagy deficiency similarly affected apoptosis in HRASV12 structures, we immunostained structures with an antibody against cleaved CASP3 (caspase-3). In contrast to the robust luminal apoptosis observed in control acini (BABE), only isolated cleaved CASP3-positive cells were observed in HRASV12 shCNT structures, consistent with the ability of oncogenic RAS to promote cell survival in 3D culture (Fig. 3A).
Figure 3. Autophagy inhibition in HRAS\textsuperscript{V12} MCF10A structures does not promote apoptosis or proliferation arrest. **A**, left, two representative images of day 8 3D cultures of BABE and HRAS\textsuperscript{V12} MCF10A cells expressing shCNT or shATG\textsubscript{7} immunostained with an antibody against cleaved CASP3 and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to detect nuclei. Scale bar, 50 μm. Right, quantification of cleaved CASP3-positive cells present within 3D cultures of each indicated cell type (mean ± SD, Student t test). **B**, representative phase (top) and corresponding wide-field fluorescence (bottom) images of BABE and HRAS\textsuperscript{V12} cells expressing shCNT or shATG\textsubscript{7}s stained with the intravital dye EtBr. Scale bar, 100 μm. **C**, left, two representative images of day 8 3D cultures of BABE and HRAS\textsuperscript{V12} cells expressing shCNT or shATG\textsubscript{12}s stained with the intravital dye EtBr. Scale bar, 100 μm. Right, quantification of Ki67-positive nuclei present within 3D cultures of each indicated cell type (mean ± SD, Student t test).
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Upon enumerating cleaved CASP3-positive cells from these 3D cultures, we found that ATG knockdown did not significantly affect apoptosis in comparison with shCNT cultures (Fig. 3A). To assess whether autophagy inhibition potentially affected nonapoptotic death processes, we also stained day 8 3D cultures with ethidium bromide (EtBr), an intravital dye that is incorporated into all dying cells. Although acini derived from nontransformed (BABE) cultures displayed high levels of EtBr staining corresponding to luminal cell death (Fig. 3B), HRASV12 structures displayed only occasional EtBr cells scattered throughout the structures. Although ATG knockdown in HRASV12 cultures resulted in spherical structures that lacked invasive protrusions, we did not observe any increase in EtBr staining in these cultures (Fig. 3B). Thus, in contrast to normal and oncogenic PIK3CA MCF10A acinar morphogenesis, autophagy inhibition does not promote apoptosis in RAS-transformed 3D structures (17, 18).

To evaluate the effects of autophagy inhibition on the proliferative capacity of HRASV12 structures, we immunostained cultures with the proliferation marker Ki67 on day 8, a time point at which normal MCF10A acini exhibited reduced proliferation (19). As expected, low levels of Ki67-positive cells were observed in BABE structures (Fig. 3C, left). However, both control and autophagy-deficient HRASV12 structures displayed high levels of Ki67-positive cells (Fig. 3C). Overall, these results indicate that although autophagy deficiency potently restricts HRASV12-driven invasion, it does not universally suppress the diverse oncogenic effects of HRASV12 in 3D culture, including the ability of activated RAS to inhibit apoptosis and sustain proliferation.

Autophagy Supports Oncogenic RAS-Driven Cell Migration In Vitro and Pulmonary Metastasis In Vivo

Because defects in invasive capacity are often associated with diminished cell motility, we next measured cell migration in autophagy-competent and -deficient epithelial cells. Upon ATG depletion, HRASV12 MCF10A cells demonstrated an approximately 30% reduction in migratory capacity in a monolayer wound-healing assay of cell migration (Fig. 4A). Similar results were obtained using a Transwell migration assay, which demonstrated a significant decrease in migration of ATG knockdown cells (Fig. 4B). We further corroborated these results using MDA-MB-231 cells, a highly migratory, KRAS-mutant breast cancer cell line. siRNA-mediated knockdown of either ATG7 or ATG12 in MDA-MB-231 cells resulted in reduced LC3-II formation (Supplementary Fig. S1H) as well as decreased wound closure (Fig. 4C, left). A similar decrease in MDA-MB-231 migration was also observed in the presence of the lysosomal inhibitor bafilomycin A (Fig. 4C, right). Therefore, in addition to supporting invasion of HRASV12 MCF10A cells in 3D culture, autophagy facilitates the migration of cells expressing oncogenic RAS in monolayer culture. Finally, we used an experimental metastasis assay to evaluate whether the effects of autophagy inhibition on invasion and migration correlated with changes in metastatic capacity in vivo; in support, the ability of HRASV12 MCF10A cells to produce pulmonary metastases was reduced upon ATG knockdown (Fig. 4D).

Altered Differentiation of HRASV12 MCF10A Cells upon Autophagy Inhibition

Constitutive RAS activation alters epithelial differentiation by driving an EMT (20, 21), a process associated with increased invasive and migratory capacity in vitro and with metastatic capacity in vivo (13). Therefore, we evaluated how autophagy inhibition affects protein expression changes associated with RAS-induced EMT. We isolated BABE-, HRASV12 shCNT-, and HRASV12 shATG-expressing cells from day 8 3D cultures and determined the protein expression of a panel of EMT-associated genes by immunoblotting. In comparison with nontransformed BABE acini, HRASV12 shCNT structures displayed decreased keratin 14 (KRT14), an epithelial marker, and a corresponding increase in the mesenchymal protein vimentin (VIM; Supplementary Fig. S2A). ATG knockdown reversed these HRASV12-driven changes in differentiation, resulting in an increase in KRT14 protein levels and a corresponding decrease in VIM levels compared with HRASV12 shCNT cells isolated from 3D culture (Supplementary Fig. S2A). However, autophagy inhibition had minimal effects on other EMT markers that were altered by oncogenic RAS expression. Only a slight increase in E-cadherin (CDH1) was observed in shATG cells, decreased fibronectin (FN1) was only observed in shATG7-1-expressing cells, and N-cadherin (CDH2) levels were unchanged following ATG knockdown (Supplementary Fig. S2A).

During EMT, cells commonly lose the ability to form cell–cell junctions (22). Therefore, we analyzed the effects of autophagy inhibition on cell–cell junctional integrity in HRASV12 3D structures by immunostaining for β-catenin (CTNNB1). Normal MCF10A acini (BABE) displayed strong β-catenin staining at cell–cell contacts, indicating intact adherens junctions, whereas the expression of HRASV12 resulted in a near-complete loss of β-catenin junctional staining; in these cultures, only isolated focal areas of junctional β-catenin staining were observed (Supplementary Fig. S2B). Upon ATG knockdown in HRASV12 structures, both the expression and junctional localization of β-catenin were significantly restored (Supplementary Fig. S2B).

On the basis of these results, we conclude that autophagy inhibition modulates certain aspects of mesenchymal differentiation in RAS-transformed cells in 3D culture, most notably the suppression of VIM, as well as the restoration of KRT14 expression and epithelial cell–cell contacts. Nonetheless, autophagy deficiency does not broadly suppress RAS-driven EMT.

ATG Knockdown in HRASV12 Cells Inhibits the Production of Proinvasive Secreted Factors in 3D Culture

Cell migration and invasion involves the secretion of multiple factors that cooperate to promote motility and to degrade the surrounding ECM (23, 24). To ascertain whether defects in RAS-driven invasion observed following autophagy suppression were the result of decreased production of proinvasive factors, we performed a coculture assay in which HRASV12 shATG7-1 cells (coexpressing GFP for tracking purposes) were combined with HRASV12 shCNT cells at a ratio of 3:1, respectively. Although HRASV12 shATG7-1–GFP cells cultured alone grew as spherical structures (Fig. 5A, left), upon coculture with HRASV12 shCNT cells, HRASV12 shATG7-1–GFP structures became dispersed and formed invasive protrusions
Figure 4. ATG knockdown suppresses the motility and reduces the metastatic potential of cells expressing oncogenic RAS. **A,** representative images (left) and quantification (right) of wound-healing assay on HRASV12 MCF10A cells expressing shCNT or shATGs. Confluent monolayers were scratched and wound width was measured at 0 and 6 hours after initial wounding to quantify the decrease in scratch width (mean ± SD, Student t test; shCNT, n = 16; shATG7-2, n = 8; shATG12, n = 14). Scale bar, 100 μm. **B,** Transwell migration of HRASV12 MCF10A cells expressing shCNT or shATGs. Twenty-four hours after plating, cells that migrated to the bottom of the filter were stained with crystal violet. Results are expressed as the mean crystal violet extracted from stained cells (mean ± SD, Student t test, n = 9). **C,** wound-healing assays of MDA-MB-231 cells expressing siATGs or in the presence of 10 nmol/L bafilomycin A (BafA). Graphs represent the decrease in scratch width at 10 and 9 hours after initial wounding, respectively (mean ± SD, Student t test; siCNT, n = 16; siATG7, n = 16; siATG12, n = 10; dimethyl sulfoxide (DMSO), n = 6; BafA, n = 6). **D,** representative images (left) and quantification (right) of ZsGreen-positive metastatic foci following tail vein injection of ZsGreen-expressing HRASV12 shCNT, shATG7-1, or shATG12 cells (mean ± SEM; shCNT, n = 7; shATG7-1, n = 7; shATG12, n = 8).
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**Figure 5.** ATG knockdown in HRAS<sup>V12</sup> cells inhibits the production of proinvasive secreted factors in 3D culture. **A**, 3D coculture of HRAS<sup>V12</sup> shATG-7-1 with HRAS<sup>V12</sup> shCNT cells rescues invasion of HRAS<sup>V12</sup> shATG-7-1 cells. HRAS<sup>V12</sup> shATG-7-1 cells expressing GFP were cultured for 8 days in 3D either alone (left) or together with HRAS<sup>V12</sup> shCNT cells expressing an empty vector (BABE). Structures were imaged by phase contrast and wide-field fluorescence microscopy or fixed, counterstained with phalloidin (to visualize F-actin) and 4′,6-diamidino-2-phenylindole (DAPI), and imaged by confocal microscopy. Phase: scale bar, 100 μm. Confocal: scale bar, 50 μm. **B**, HRAS<sup>V12</sup> MCF10A cells expressing shATGs were cultured in 3D for 3 days and subsequently treated with BABE or HRAS<sup>V12</sup> shCNT conditioned media (CM). Representative phase contrast images at 24 and 72 hours following the addition of conditioned media. Scale bar, 100 μm. **C**, 3D cultures of HRAS<sup>V12</sup> MCF10A cells expressing shATGs were treated with BABE or HRAS<sup>V12</sup> shCNT conditioned media for 72 hours; thereafter, cultures were fixed and immunostained with an antibody against LAMAS (human specific) to detect basement membrane and DAPI counterstained. Two representative images per condition are shown. Scale bar, 50 μm.
(Fig. 5A, right). Hence, we hypothesized that factors from neighboring HRASV12 shCNT cells are sufficient to rescue in trans the invasion defect in HRASV12 shATG7-1 cells. To further test this prediction, we grew HRASV12 shATG cells in 3D culture for 3 days and subsequently treated these structures with conditioned media produced from either BABE or HRASV12 shCNT cultures. HRASV12 shATG structures remained as compact spheres following treatment with BABE conditioned media (Fig. 5B and Supplementary Fig. S3A). In contrast, conditioned medium from HRASV12 shCNT cultures elicited invasive protrusions at 24 hours following treatment, which became fully evident by 72 hours (Fig. 5B and Supplementary Fig. S3A); notably, conditioned medium addition did not induce invasion in nontransformed BABE acini (Supplementary Fig. S3B). Furthermore, basement membrane integrity was lost in HRASV12 shATG cells treated with HRASV12 conditioned medium (Fig. 5C). These findings demonstrate that autophagy inhibition in HRASV12 cells inhibits the production of secreted factors required for RAS-driven invasion in 3D culture.

Diminished Secretion of IL-6 Contributes to Reduced Invasion in Autophagy-Deficient HRASV12 Cells

During RAS-induced senescence, ATG depletion inhibits IL-6 production following acute oncogenic RAS activation in IMR90 fibroblasts, indicating that autophagy supports the production of IL-6 in response to oncogenic RAS activation (25). Because IL-6 has been demonstrated to support RAS-driven tumorigenesis, promote migration, and invasion, and also drive EMT (26–28), we tested whether IL-6 levels were altered in HRASV12 shATG 3D cultures. Analysis of IL-6 in conditioned media collected from 3D cultures by ELISA indicated a significant reduction in secreted IL-6 levels in HRASV12 shATG-expressing cultures compared with HRASV12 shCNT cultures (Fig. 6A). Furthermore, this decrease in

Figure 6. Autophagy supports IL-6 secretion necessary for oncogenic RAS-driven invasion in 3D culture A, levels of IL-6 in conditioned media (CM) collected on day 6 from 3D cultures of the indicated cell types (mean ± SD, ANOVA; BABE, n = 3; HRASV12, n = 5). B, IL-6 expression levels normalized to GAPDH in cells collected from day 8 3D cultures (mean relative to BABE ± SD, Student t test, n = 3). qPCR, quantitative PCR. C, IL-6 protein levels in day 8 3D cultures from the indicated cell types. D, representative phase contrast images of HRASV12 shATG 3D cultures treated for 48 hours with BABE conditioned media (top) or with HRASV12 shCNT conditioned media containing an IL-6 function-blocking antibody (Ab; bottom) or IgG control antibody (middle). Scale bar, 100 μm. E, representative phase contrast images of HRASV12 shATG 3D cultures grown in the presence or absence of 200 ng/mL recombinant human IL-6 (rhIL-6) for 7 days. Scale bar, 100 μm.
secreted IL-6 was not the result of reduced IL6 gene expression; in fact, quantitative PCR (qPCR) analysis revealed that IL6 transcript levels in HRASV12 shATG cells were increased, rather than decreased, in comparison with HRASV12 shCNT cells (Fig. 6B). Notably, studies of RAS-induced senescence similarly demonstrated that autophagy-deficient cells exhibit reduced IL-6 protein levels due to impaired translation, rather than transcription (25, 29). In contrast, we uncovered that ATG depletion did not attenuate IL-6 protein levels in RAS-transformed cells grown in 3D culture (Fig. 6C). These results suggest that autophagy facilitates IL-6 secretion during HRASV12 3D morphogenesis.

To ascertain the functional significance of these results, we interrogated whether IL-6 was necessary for HRASV12-driven invasion in 3D culture. First, we treated HRASV12 shATG structures with HRASV12 shCNT conditioned media in the presence versus absence of an IL-6 function-blocking antibody. The addition of IL-6 function-blocking antibody attenuated the ability of HRASV12 shCNT conditioned media to promote invasive protrusions in HRASV12 shATG cultures, whereas an immunoglobulin G (IgG) isotype control had no effect (Fig. 6D). In parallel, we tested how exogenous recombinant human IL-6 (rhIL-6) treatment affected HRASV12 shATG cells during 3D morphogenesis. rhIL-6 addition did not affect nontransformed BABE acini (Supplementary Fig. S3C) but partly restored invasion in HRASV12 shATG cultures, resulting in large globular structures, increased invasive protrusions, and loss of basement membrane integrity (Fig. 6E and Supplementary S3D–S3E). Also, rhIL-6 addition partially reversed the effects of autophagy inhibition on KRT14 and VIM expression in HRASV12 shATG7 cells (Supplementary Fig. S3F). Hence, our results suggest that autophagy promotes efficient IL-6 secretion by HRASV12 cells in 3D culture, which is necessary for invasion.

**Autophagy Facilitates MMP2 and WNT5A Expression by HRASV12 Cells in 3D Culture**

In addition to identifying a defect in IL-6 production following ATG knockdown, we performed a qPCR array to measure the expression levels of genes involved in EMT and invasion, and identified WNT5A and MMP2 as two candidate factors whose expression was upregulated in HRASV12 cells relative to BABE cells but potently suppressed upon autophagy inhibition. qPCR analysis of cells collected from 3D cultures confirmed a 2-fold decrease in MMP2 and WNT5A expression in HRASV12 shATG cells compared with HRASV12 shCNT cells (Fig. 7A and B). Notably, we also evaluated the effects of rhIL-6 treatment on MMP2 and WNT5A expression in shATG7-1 cultures and found that this was not sufficient to rescue expression, indicating that regulation of these factors was independent of IL-6 (Supplementary Fig. S3G).

Because these secreted factors have been implicated in cell migration and invasion, we further evaluated whether their decreased expression following ATG knockdown also contributed to the reduced invasive potential of HRASV12 shATG cells. First, we used gelatin zymography to assess MMP2 activity in conditioned media from 3D cultures. MMP2 activity was enhanced in HRASV12 cells compared with nontransformed (BABE) controls, and upon ATG knockdown in HRASV12 cells, this activity was reduced (Fig. 7C). The increase in MMP2 expression and secretion following constitutive RAS activation was necessary for RAS-driven invasion, as addition of an MMP2 inhibitor, Arp-100, was sufficient to inhibit the formation of invasive protrusions in HRASV12 3D cultures (Fig. 7D). Furthermore, the decrease in WNT5A expression correlated with a decrease in WNT5A protein levels in HRASV12 shATG cells isolated from 3D culture (Fig. 7E). Moreover, the addition of recombinant WNT5A to HRASV12 shATG7-1 3D cultures promoted the dissociation of cells within the structures and enhanced the formation of invasive protrusions (Fig. 7F). Thus, in addition to IL-6, autophagy facilitates the production of multiple secreted promigratory and invasive factors that support RAS-driven invasion in 3D culture.

**DISCUSSION**

Our results delineate a previously unrecognized function for autophagy in facilitating oncogenic RAS-driven invasion and migration. Using a 3D culture system, we demonstrate that suppression of autophagy in HRASV12 MCF10A cells restricts the formation of invasive protrusions, restores basement membrane integrity, and attenuates ECM proteolysis. In addition, autophagy inhibition diminishes cell migration in vitro and pulmonary metastasis in vivo. Upon treatment with conditioned media produced from autophagy-competent HRASV12 cells, invasion is completely restored in autophagy-deficient HRASV12 cultures, indicating that autophagy mediates the production of secreted factors that drive invasion in oncogenic cells. In further support, we uncover that autophagy inhibition elicits the coordinate reduction of multiple molecules favoring invasion. Overall, these findings expand our understanding of how autophagy supports cancer progression.

Although autophagy inhibition suppresses invasion in 3D culture, it does not ubiquitously revert oncogenic RAS-driven changes in cell behavior. Indeed, MAPK activation remains unaltered following autophagy inhibition in this 3D culture model, and, moreover, the oncogenic activation of RAS continues to disrupt fundamental aspects of 3D morphogenesis in autophagy-deficient cells. First, autophagy inhibition does not alter the ability of HRASV12 to suppress apoptosis in 3D culture. Moreover, autophagy inhibition does not suppress proliferation in HRASV12 3D cultures; rather, the spherical structures from HRASV12 ATG knockout cells remain highly proliferative over extended periods. Remarkably, both others and we have shown that ATG depletion reduces soft-agar growth and attenuates the proliferation of RAS-transformed cells grown in monolayer (3–5, 30); hence, the absence of proliferative suppression in this 3D culture model may be context dependent. These results also differ from those obtained in KRAS-mutant mouse cancer models in which genetic ATG deletion impairs proliferation and, in certain cases, enhances apoptosis (4, 6, 7). Certain reasons may explain these differences. First, we have only reduced ATGs using RNA interference (RNAs), rather than genetically eliminating these proteins. Second, the experiments here are of significantly shorter duration in comparison with autophagy-deficient KRAS-mutant tumor growth in vivo.

Although previous studies have demonstrated that autophagy supports the invasion of glioblastoma cells, the mechanistic underpinnings remain unclear (31, 32). Cell
Figure 7. WNT5A and MMP2 are reduced following autophagy inhibition in 3D culture. 

A and B, RNA was isolated from BABE, HRASV12 shCNT, and HRASV12 shATG cells cultured in 3D for 8 days. Expression levels of MMP2 and WNT5A were determined by qPCR and normalized to an internal control GAPDH. Results represent the mean relative to BABE ± SD (MMP2, n = 4; WNT5A, n = 3; Student t test).

C, conditioned medium (CM) was collected from BABE, HRASV12 shCNT, and HRASV12 shATG cells grown in 3D culture. Activity levels of MMP9 and MMP2 in the conditioned media were determined by zymography.

D, HRASV12 shCNT cells were grown in the absence (top) or presence (bottom) of 25 μmol/L Arp-100. Left, structures were imaged on day 8 by phase contrast microscopy. Right, representative confocal images of structures immunostained with anti–phospho-Ezrin/Radixin/Moesin (p-ERM) to detect cell borders and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Scale bars, 100 μm.

E, BABE, HRASV12 shCNT, and HRASV12 shATG cells were collected from 3D culture on day 8, lysed, and protein levels of WNT5A were determined by immunoblot analysis.

F, HRASV12 shATG7-1 cells were grown in 3D for 8 days in the absence (top) or presence (bottom) of 500 ng/mL WNT5A. Left, representative phase contrast images. Right, representative confocal images of structures immunostained with anti-phospho-ERM to detect cell borders and counterstained with DAPI. Scale bars, 100 μm.
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invasion requires the production and secretion of factors that stimulate migration and degrade the surrounding ECM (24). Upon treatment of autophagy-depleted HRAS V12 cells with conditioned media produced from their autophagy-competent counterparts, the ability to form invasive protrusions is completely restored, suggesting that autophagy is required for the efficient production of secreted factors that promote invasion and migration of HRAS V12 cells. Notably, conditioned media treatment does not promote invasion in nontransformed BABE cells, indicating that oncogenic RAS pathway activation is still required for invasion.

Importantly, we identify IL-6 as one critical factor whose secretion is ATG dependent; our results substantiate that this proinvasive cytokine is necessary to restore invasion in autophagy-deficient HRAS V12 cells. They also point to a specific role for autophagy in facilitating IL-6 secretion; upon ATG knockdown, RAS-transformed cells fail to secrete IL-6 into the conditioned media, yet both IL-6 transcription and translation remain intact. These results differ from recent studies of oncogenic RAS-mediated senescence, in which reduced IL-6 secretion in autophagy-deficient cells is proposed to be secondary to decreased protein synthesis (25, 29).

Although it has been traditionally viewed as an autodigestive process, growing evidence suggests new roles for autophagy in both conventional and unconventional secretion (33). Indeed, a genetic role for ATGs has been implicated in (i) unconventional secretion of proteins lacking N-terminal endoplasmic reticulum signal sequences (34–37), (ii) efficient egress of secretory lysosomes (38, 39), and (iii) conventional secretion of growth factors (40, 41). Further dissection of how autophagy directs secretion of proteins lacking N-terminal endoplasmic reticulum signal sequences is completely restored, suggesting that autophagy is required for the efficient production of secreted factors that promote invasion and migration of HRAS V12 cells. Notably, conditioned media treatment does not promote invasion in nontransformed BABE cells, indicating that oncogenic RAS pathway activation is still required for invasion.

Importantly, we identify IL-6 as one critical factor whose secretion is ATG dependent; our results substantiate that this proinvasive cytokine is necessary to restore invasion in autophagy-deficient HRAS V12 cells. They also point to a specific role for autophagy in facilitating IL-6 secretion; upon ATG knockdown, RAS-transformed cells fail to secrete IL-6 into the conditioned media, yet both IL-6 transcription and translation remain intact. These results differ from recent studies of oncogenic RAS-mediated senescence, in which reduced IL-6 secretion in autophagy-deficient cells is proposed to be secondary to decreased protein synthesis (25, 29).

Although it has been traditionally viewed as an autodigestive process, growing evidence suggests new roles for autophagy in both conventional and unconventional secretion (33). Indeed, a genetic role for ATGs has been implicated in (i) unconventional secretion of proteins lacking N-terminal endoplasmic reticulum signal sequences (34–37), (ii) efficient egress of secretory lysosomes (38, 39), and (iii) conventional secretion of growth factors (40, 41). Further dissection of how autophagy directs the secretion of IL-6 and other factors during RAS transformation remains an important topic for future study. Remarkably, IL-6 re-addition only partially restores invasion and mesenchymal differentiation in HRAS V12 autophagy-deficient cultures, indicating that other factors promote invasion. In support of this possibility, these cells exhibit reduced levels of other proinvasive molecules, including WNT5A and MMP2. In contrast to reduced IL-6 secretion, which is likely a proximal event following ATG knockdown, these changes in WNT5A and MMP2 result from decreased gene expression, indicating that autophagy inhibition produces broader transcriptional changes contributing to reduced invasion by HRAS V12 cells.

Recently, the deletion of RB1CC1/FIP200, a gene mediating autophagosome initiation, was demonstrated to reduce lung metastases in the MMTV-PyMT breast cancer model. However, as RB1CC1 deletion profoundly restricted primary tumor growth, it was unclear whether decreased metastasis was secondary to reduced primary tumor burden (12). In addition, although liver-specific deletion of ATG7 or ATG5 initiates the development of benign adenomas, these tumors are unable to progress to adenocarcinomas, suggesting that autophagy is required for advanced tumor progression (42, 43). Here, in epithelial cells transformed with oncogenic RAS, we demonstrate that defective autophagy results in decreased invasion and migration, which correlates with the reduced ability to metastasize in vivo. Although our results do not rule out potentially important functions for autophagy in disseminated cell survival or outgrowth at foreign tissue sites, they delineate new roles for autophagy in the control of secretion during carcinoma progression.

METHODS

Cell Lines

MCF10A cells were obtained from the American Type Culture Collection (ATCC) and cultured as previously described (44). MDA-MB-231 cells were obtained from the ATCC and cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS, penicillin, and streptomycin. Cell lines were passaged for less than 6 months following resuscitation and were not authenticated.

3D Culture Assays

MCF10A overlay 3D culture was performed as previously described (44). As indicated, the following reagents were added to cultures: 500 ng/mL WNT5A (R&D Systems), 200 ng/mL IL-6 (PeproTech), 25 μg/mL anti-IL-6 function-blocking antibody (R&D Systems), 25 μg/mL IgG control antibody (BD Biosciences), 25 μg/mL Arp100 (Santa Cruz Biotechnology), 5 μmol/L chloroquine diphosphate salt (Sigma), and 5 nmol/L bafilomycin A (Sigma). For the 3D ECM degradation assay, human DG-COL4 (Invitrogen) was mixed with Matrigel to a final concentration of 25 μg/mL before plating. To collect cells for immunoblotting and RNA isolation, cultures were incubated with 0.25% Trypsin/EDTA at 37°C for 10 minutes to dissociate cells from surrounding matrix and create a single-cell suspension. Cells were resuspended in media containing 20% serum and washed twice with PBS to remove residual Matrigel.

For coculture assays, shATG7-1 was expressed in HRAS V12 cells stably expressing pBabeHygro-GFP. This GFP-labeled “target” cell line was then cultured in isolation or combined with unlabeled (pBabeHygro) HRAS V12 shCNT cells at a ratio of 3:1, with total cell number kept constant at 7,500 cells per well. For conditioned media experiments, HRAS V12 shATG7-expressing cells were grown in 3D culture for 3 days; subsequently, the media was replaced with conditioned media harvested from BABE or HRAS V12 shCNT MCF10A cells grown in 3D culture for 6 to 8 days. When indicated, 25 μg/mL anti-IL-6 function-blocking or IgG isotype control antibody was added to the conditioned media.

Wound-Healing Assay

Cells were grown to confluence in 3.5-cm dishes and incubated overnight in assay media lacking EGF for MCF10A cells or DMEM + 2% FBS for MDA-MB-231 cells. Wound healing was performed in the presence of 2 μg/mL mitomycin C (Sigma). Cells were wounded with a 200-μL pipette tip and imaged at the time of wounding (0 hours) and the indicated time points. Average wound widths were measured at each time point, and decreases in wound width were calculated by subtracting the average width at the final time point from the average width at 0 hours using MetaMorph Software (v6.0).

Transwell Assay

Cells were starved overnight in assay media lacking EGF and then plated at 1.0 × 104 in the top chamber of an 8-μm Transwell filter in assay media lacking EGF. The bottom chamber was filled with assay medium containing 5 ng/mL EGF. Cells were allowed to migrate for 24 hours, after which the top of each filter was cleared of cells. Cells attached to the bottom of the filter were fixed and stained with crystal violet. Crystal violet was extracted with 10% acetic acid and the absorbance was measured at 600 nm.

Experimental Metastasis Assay

For experimental metastasis assays, cells were infected with pHIV-ZsGreen (Addgene, plasmid 18121). A total of 1.0 × 106 HRAS V12 shCNT, shATG7-1, and shATG72 cells stably expressing ZsGreen were injected into the tail vein of nude mice (B6C3F1) under isoflurane anesthesia. After 10 days, whole lungs were fixed and imaged to detect the number of ZsGreen-positive foci per lung. All animal experiments were conducted in accordance with approved institutional guidelines.

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protocols of the University of California, San Francisco (UCSF; San Francisco, CA) Institutional Animal Care and Use Committee (IACUC).

IL-6 ELISA

Day 5 3D cultures were washed twice with PBS and cultured for 18 hours in serum-free media. Conditioned medium was collected, and total protein levels were determined by BCA assay (Thermo Scientific) to normalize samples. IL-6 levels were measured using the Quantikine High Sensitivity ELISA Kit (R&D Systems).

Statistical Analyses

Each experiment was repeated at least three independent times. GraphPad Prism software (v5.0b) was used for generation of graphs and statistical analyses. P values were determined by a Student t test or ANOVA as stated.

Disclosure of Potential Conflicts of Interest

J. Debnath has received honoraria from the Speakers’ Bureaus of Amgen and Novartis. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: R. Lock, C.M. Kenific, J. Debnath
Development of methodology: R. Lock, C.M. Kenific, J. Debnath
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Lock, C.M. Kenific, A.M. Leidal, E. Salas, J. Debnath
Analysis and interpretation of data (e.g., statistical analysis, biosatistics, computational analysis): R. Lock, C.M. Kenific, A.M. Leidal, E. Salas, J. Debnath
Writing, review, and/or revision of the manuscript: R. Lock, A.M. Leidal, J. Debnath
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Lock
Study supervision: J. Debnath

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