Inhibition of Integrin-mediated Crosstalk with Epidermal Growth Factor Receptor/Erk or Src Signaling Pathways in Autophagic Prostate Epithelial Cells Induces Caspase-independent Death

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In vivo in the prostate gland, basal epithelial cells adhere to laminin 5 (LM5) via $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins. When placed in culture primary prostate basal epithelial cells secrete and adhere to their own LM5-rich matrix. Adhesion to LM5 is required for cell survival that is dependent on integrin-mediated, ligand-independent activation of the epidermal growth factor receptor (EGFR) and the cytoplasmic tyrosine kinase Src, but not PI-3K. Integrin-mediated adhesion via $\alpha 3\beta 1$, but not $\alpha 6\beta 4$ integrin, supports cell survival through EGFR by signaling downstream to Erk. PC3 cells, which do not activate EGFR or Erk on LM5-rich matrices, are not dependent on this pathway for survival. PC3 cells are dependent on PI-3K for survival and undergo caspase-dependent death when PI-3K is inhibited. The death induced by inhibition of EGFR or Src in normal primary prostate cells is not mediated through or dependent on caspase activation, but depends on the induction of reactive oxygen species. In addition the presence of an autophagic pathway, maintained by adhesion to matrix through $\alpha 3\beta 1$ and $\alpha 6\beta 4$, prevents the induction of caspases when EGFR or Src is inhibited. Suppression of autophagy is sufficient to induce caspase activation and apoptosis in LM5-adherent primary prostate epithelial cells.

INTRODUCTION

In vivo, the precise regulation of epithelial cell homeostasis involves interactions between cells and their microenvironment. Cells receive signals from both the extracellular matrix in the basement membrane and soluble factors secreted by the stroma that precisely control the timing of cell division, growth arrest, differentiation, and survival. Integrins on the cell surface that interact with laminin 5 (LM5) in the extracellular matrix, such as $\alpha 3\beta 1$ and $\alpha 6\beta 4$, are critically involved in mediating survival. Genetic loss of LM5, or its receptors $\alpha 3$, $\alpha 6$, or $\beta 4$ integrins, in vivo results in cell detachment and induction of caspase-mediated apoptosis, even in the presence of soluble factors (Ryan *et al.*, 1999; DiPersio *et al.*, 2000). This detachment-induced form of apoptosis has been termed anoikis (Frisch and Screaton, 2001). In vitro anoikis can be rescued by expression of an activated form of FAK, Rac, or Akt (Frisch *et al.*, 1996; Rytomaa *et al.*, 2000; Coniglio *et al.*, 2001), suggesting that integrin-mediated

signaling through these molecules is required to maintain cell survival. However, studies in which specific signaling pathways are inhibited while integrins are still engaged suggest alternative pathways, such as Ras/Erk or Jnk, are required for integrin-mediated survival (Almeida *et al.*, 2000; Manohar *et al.*, 2004). Whether signaling from multiple pathways is involved in mediating integrin-dependent survival and whether different pathways are unique to specific cell types have not been extensively investigated.

In addition to classical caspase-mediated apoptosis, such as that observed during anoikis, several other mechanisms of cell death have been described (Melino *et al.*, 2005). Other forms of cell death include caspase-independent cell death, autophagy, or cornification. The role of integrins in regulating cell survival through suppression of these other death pathways is unknown. However, some of the same integrin-induced signal transduction pathways that have been linked to survival are also important for regulating these alternative cell death pathways. For example the Ras/Erk and PI-3K pathways act as positive and negative regulators, respectively, of autophagy in several cell types (Kondo *et al.*, 2005). Additionally, epithelial cells have been shown to undergo death by cornification in response to inhibition of Erk and Jnk, but not PI-3K (Uzgare and Isaacs, 2004). Finally, death induced by over expression of Ras, or suppression of Raf in melanoma cells leads to caspase-independent cell death (Chi *et al.*, 1999; Panka *et al.*, 2006). Whether integrin-induced activation of specific signaling pathways plays a role in regulating any of these cell death mechanisms has not been determined.

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Abbreviations used: EGFR, epidermal growth factor receptor; PEC, prostate epithelial cells; LM, laminin; PI-3K, phosphatidylinositol 3-kinase.

Although studies with various established cell lines have been extremely useful for elucidating potential signaling pathways involved in integrin-mediated survival, it is important to place the findings in the context of a defined organ system where the specific cell type, the integrins expressed, and the matrix being studied are better defined. Basal epithelial cells in the prostate gland express $\alpha 6 \beta 4$ and $\alpha 3 \beta 1$ integrins and adhere to a basement membrane rich in LM5 (Knox *et al.*, 1994). When these cells are placed in culture they retain in vitro a majority of the properties seen in vivo, including the ability to secrete and organize their own LM5-rich matrix (Gmyrek *et al.*, 2001; Yu *et al.*, 2004).

Our work and that of others have demonstrated that integrin engagement is sufficient to activate receptor tyrosine kinases (Plopper *et al.*, 1995; Miyamoto *et al.*, 1996; Wang *et al.*, 1996; Moro *et al.*, 1998; Danilkovitch-Miagkova *et al.*, 2000; Kuwada and Li, 2000; Marcoux and Vuori, 2003; Bill *et al.*, 2004). We demonstrated that adhesion of normal epithelial cells to matrix is sufficient to induce activation of the epidermal growth factor receptor (EGFR), independently of ligand (Bill *et al.*, 2004). In addition, we demonstrated that integrin-mediated activation of a subset of signaling pathways, namely the Ras/Erk and PI-3K/Akt pathways, are dependent on integrin-induced EGFR activation. Because both of these pathways have been implicated in regulating integrin-mediated survival, we hypothesized that integrin-mediated survival of epithelial cells via Ras/Erk or PI-3K/Akt pathways could be mediated through integrin-dependent activation of EGFR. To test this hypothesis, we assessed the ability of primary prostate epithelial cells (PECs) adherent to their endogenous LM5-rich matrix to survive in the context of EGFR and downstream signaling inhibitors.

MATERIALS AND METHODS

Antibodies

EGFR immunoprecipitating and blocking monoclonal antibodies were purified in the Monoclonal Antibody Core at VARI from hybridoma cells obtained from American Type Culture Collection (ATCC; Manassas, VA; HB-8508). EGFR (Ab12) immunoblotting antibodies were purchased from NeoMarkers (Fremont, CA). Erk and p130Cas antibodies were purchased from Becton-Dickinson Transduction Labs (Lincoln Park, NJ). Phospho-specific antibodies against Erk1/2 (T202/Y204) and Akt (S473) and antibodies to Bcl-2 and Bcl-XL were purchased from Cell Signaling (Beverly, MA). The anti-phosphotyrosine mAb 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY). The Akt antibody was described previously (Bill *et al.*, 2004). Blocking antibodies for $\beta 4$ integrin (ASC-8) and $\alpha 3$ integrin (P1B5) were purchased from Chemicon (Temecula, CA) and GoH3 $\alpha 6$ integrin antibody was obtained from Becton-Dickinson.

Cell Culture

Primary cultures of human PECs were derived from normal human prostatic tissue and cultured as described previously (Gmyrek *et al.*, 2001). Human samples were obtained after institutional IRB approval. PECs were maintained in Keratinocyte-SFM medium (Invitrogen, Carlsbad, CA) supplemented with bovine pituitary extract and epidermal growth factor (EGF). All experiments were conducted on cells between passages 3 and 5. PC3 cells were obtained from ATCC. PC3 cells were maintained in F12K medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 U of penicillin, and 50 mg of streptomycin/ml.

Integrin Signaling

Preparation of cells for adhesion to extracellular matrices was carried out as described in Miranti (2002). Briefly, cells were growth factor-starved for 48 h, trypsinized, treated with soybean trypsin inhibitor (Invitrogen), washed in PBS, and placed in suspension in growth factor-free medium for 30–60 min. Cells were then either plated on tissue culture plates blocked with 1% BSA (Sigma, St. Louis, MO) to allow deposition of endogenous LM5-rich matrix or directly replated on LM5-coated plates obtained from culturing PECs as described previously for LM5-secreting cells (Xia *et al.*, 1996). In some cases, PC3 cells were also plated on laminin 1 (LM1; Invitrogen). Similar results were obtained in PC3 cells on LM1 as on LM5. Occasionally cells were also treated with 2–10 ng/ml EGF (Upstate Biotechnology) or 50 ng/ml HGF

(Calbiochem, La Jolla, CA) for 10 min. A suspension control was maintained at 37°C. Two hours after plating on the matrix cells were lysed either in Triton X-100 (50 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 50 mM NaF, 50 mM β -glycerophosphate, 5 mM sodium pyrophosphate, 1 mM Na_3VO_4 , 1 mM PMSF, 100 U/ml aprotinin, 10 $\mu\text{g}/\text{ml}$ pepstatin, and 10 $\mu\text{g}/\text{ml}$ leupeptin) or RIPA (10 mM Tris, pH 7.2, 158 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% NaDOC, 1% Triton X-100, 1 mM Na_3VO_4 , 1 mM PMSF, 100 U/ml aprotinin, 10 $\mu\text{g}/\text{ml}$ pepstatin, and 10 $\mu\text{g}/\text{ml}$ leupeptin) buffers. Pharmacological inhibitors, PD168393, AG1478, LY294002, SU6656, or PP2, purchased from Calbiochem, were added to suspension cells 20 min before plating on matrix; except for SU6656, which was added 16 h before placing cells in suspension. All working concentrations of the pharmacological inhibitors were determined by titrating to the minimum inhibitor concentration that effectively blocked the target of the pharmacological inhibitor for the duration of our experiments. Inhibitor effectiveness was monitored by Western blotting. Specifically, PD168393 and AG1478 were tested for their ability to inhibit EGFR tyrosine phosphorylation, p130Cas tyrosine phosphorylation (a Src substrate) was used to test SU6656 and PP2, phosphorylation of Akt was used for LY294002, and U0126 was tested against phosphorylated Erk. Titrations were performed for each drug in each cell type.

Antibody Blocking Assays

Blocking Integrins. For integrin blocking studies, PECs were starved and placed in suspension and then plated on 1% bovine serum albumin (BSA)-blocked eight-chamber slides in the presence of 10 $\mu\text{g}/\text{ml}$ blocking anti- $\beta 4$ integrin antibody (ASC-8), anti- $\alpha 3$ integrin antibody (P1B5), anti- $\alpha 6$ integrin antibody (GoH3), or IgG. Cells were allowed to adhere to endogenous LM5-rich matrix for 48–72 h in the presence of the indicated antibodies. Cells were monitored for viability by Annexin V staining, caspase activation, autophagy induction with LC3-GFP, or for Erk activation by immunoblotting.

Blocking EGF Binding. Thirty minutes before plating, growth-factor-starved PEC suspension cells were pretreated with 0–10 $\mu\text{g}/\text{ml}$ mAb to EGFR (AB225 [HB-8508, ATCC]) or 10 $\mu\text{g}/\text{ml}$ nonspecific mouse IgG for 30 min with occasional mixing. Cells were allowed to adhere to LM5-rich matrix in the presence or absence of 2 ng/ml EGF for 2 h. Cells were lysed, and EGFR tyrosine phosphorylation and Erk activation were monitored in immunoprecipitates or cell lysates, respectively.

Cell Survival Assays. PECs were starved and placed in suspension as described above and then plated on 1% BSA-blocked tissue culture plates to allow deposition and adhesion to endogenous LM5 matrix. Serum-starved PC3 cells were plated on 1% BSA-blocked tissue culture plates precoated with 10 $\mu\text{g}/\text{ml}$ laminin. Pharmacological inhibitors, 1 μM staurosporine (Promega, Madison, WI), 0.5 μM PD168393, 1 μM AG1478, 10 μM U0126, 10 μM LY294002, 0.5–2 μM SU6656, 10 μM PP2, 50 μM butylated hydroxyanisole (BHA), 1.25 mM N-acetylcysteine (NAC), or 10 mM 3-methyladenine (3MA), were then added. Cells were allowed to adhere for 4 h and then nonadherent cells were removed and drugs were replaced. Cells were incubated for an additional 72 h. LY294002 was replenished 48 h after plating.

To assess cell death, cells were stained with Annexin V using a kit obtained from Molecular Probes (Invitrogen). Staining was carried out according to the supplied protocols. For all staining procedures both attached and floating cells were collected. Attached cells were removed by trypsinization and pooled with floating cells, and all cells were washed one time. For Annexin V staining, cells were resuspended in Annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4) containing Alexa-fluor-conjugated Annexin V and incubated in the dark for 15 min. Samples were put on ice and immediately analyzed. Extent of staining was monitored by fluorescence-activated cell sorting (FACS) using a FACS Caliber (Becton-Dickinson) and CellQuest version 3.1.3 acquisition and analysis software (Becton-Dickinson) immediately after staining. On several occasions Annexin V staining was also monitored in adherent cells (without trypsinization) by microscopy using a Nikon Eclipse TE300 fluorescence microscope (Melville, NY) and OpenLab version 3.1.7 image analysis software (Improvision, Lexington, MA).

Caspase Activity Assays. Caspase 3 and 7 activity in PEC and PC3 cells was directly measured using a CaspaseGlo 3/7 kit (Promega) following the manufacturer's suggested protocol. For PECs 10,000 cells/well were plated on endogenous LM5 in BSA-coated 96-well plates in the presence of DMSO, 1 μM staurosporine (Promega), 0.5 μM PD168393, 10 μM PP2, or 10 mM 3MA, with or without 20 μM zVAD (Promega). For PC3 cells, 10,000 cells/well were plated on 1% BSA blocked 96-well plates precoated with 10 $\mu\text{g}/\text{ml}$ laminin, respectively. Cells were plated in the presence of DMSO, 1 μM staurosporine, 0.5 μM PD168393, 10 μM LY294002, 2 μM SU6656, and 10 μM PP2. CaspaseGlo reagent was added at various times after inhibitor treatment and incubated for 1 h at room temperature in the dark. Relative light intensity was measured in each well using a Fluoroskan Assent FL fluorometer and software (Labsystems, Franklin, MA).

Immunoprecipitation and Immunoblotting. Immunoprecipitation mixtures containing 500–1000 μg protein were incubated with the appropriate anti-

bodies for 3 h at 4°C with either protein A- or protein G-conjugated agarose beads (Pierce, Rockford, IL) to capture the complexes. All immunoprecipitated complexes were washed three times with their respective lysis buffer. Immunoprecipitated samples from adhesion assays were resuspended in 2× SDS sample buffer. In some cases 50–75 µg of total cell lysates were placed directly in 2× SDS sample buffer. All resuspended samples were boiled and subjected to SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were blocked with 5% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 2 h, followed by a 2-h incubation with the appropriate primary antibodies in 5% BSA/TBST. After several washes, blots were incubated with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Richmond, CA) for 1 h in 5% BSA/TBST and visualized with a chemiluminescence reagent and captured by a CCD camera in a Bio-Rad Chemi-Doc Imaging System. Levels of activation, relative to total levels of protein, from blots captured by CCD camera were quantified using Quantity One software (Bio-Rad). Blots were stripped in low-pH 2% SDS at 65°C for 60 min, rinsed, and reprobed for total levels of protein in the immunoprecipitates or cell lysates.

Autophagy Assay. LC3-GFP in the pBABE expression vector was kindly provided by Dr. Jay Debnath (University of California, San Francisco, CA). The LC3-GFP cDNA BamHI/Sall restriction fragment was subcloned into the BglIII and Sall restriction sites of pShuttle-CMV (Stratagene, La Jolla, CA). pAd-Easy (Stratagene) adenoviral recombinants containing LC3-green fluorescent protein (GFP) were generated in BJ5183-AD1 bacteria. HEK293 cells were transfected with adenoviral recombinant DNA and adenoviruses purified using a kit from Clontech and titrated by GFP expression in PECs.

PECs were infected at an moi of 2 with adenoviruses expressing LC3-GFP fusion protein. Twenty-four hours later, cells were growth factor-starved or left in complete medium and allowed to adhere to their own LM5. For antibody blocking experiments, antibodies were added at the time plating on matrix. Localization of LC3-GFP was monitored by standard fluorescence microscopy at 24 and 48 h after plating using a Nikon Eclipse TE300 fluorescence microscope and OpenLab version 3.1.7 image analysis software (Improvision).

RESULTS

Integrin Engagement in PECs Activates EGFR Independent of Ligand

Primary PECs derived from prostatectomy tissues, when placed in serum-free culture, secrete and organize an extracellular matrix containing LM5 within 2–3 h after plating (Yu *et al.*, 2004). Adhesion of PECs to this LM5-rich matrix induces activation of the ErbB family receptor tyrosine kinase EGFR (Figure 1A) and its downstream target Erk (Figure 1B). Adhesion to the LM5-rich matrix failed to stimulate detectable levels of Akt activation (Figure 1C). However, Akt was activated by treatment with EGF or human growth factor (HGF), demonstrating that the PI-3K/Akt pathway is intact in these cells. Inhibition of EGFR activity with the EGFR-specific inhibitors AG1478 (not shown) or PD168393 blocked integrin-induced EGFR tyrosine phosphorylation (Figure 1A) and downstream signaling to Erk (Figure 1B). Thus integrin-mediated activation of Erk on LM5-rich matrix is dependent on EGFR.

Src kinases have been implicated in regulating integrin-mediated activation of receptor tyrosine kinases (Danilkovitch-Miagkova *et al.*, 2000; Moro *et al.*, 2002); however, in PECs, inhibition of Src activation by the Src kinase-specific inhibitor SU6656 (Blake *et al.*, 2000) had no effect on EGFR activation or its downstream target Erk (Figure 1, A and B). Inhibition of Src did block integrin-mediated activation of its downstream substrate p130Cas (Figure 1D). Reciprocally inhibition of EGFR did not block integrin-mediated tyrosine phosphorylation of the Src substrate p130Cas. These data demonstrate that adhesion of PECs to LM5-rich matrix regulates two independent signaling pathways: one that activates EGFR, independent of ligand, and signals to Erk and one that activates Src and its downstream target p130Cas, independently of EGFR.

To rule out the possibility that residual EGF was responsible for activation of EGFR and Erk, we used a blocking

Figure 1. Integrin-mediated signaling on LM5 in PECs. (A–E) Growth factor-starved PECs were placed in suspension (S) and treated with DMSO (–), 0.5 µM PD168393 (PD), or 0.5 µM SU6656 (SU) before plating on LM5 (LM) for 2 h. (A) EGFR or (D) p130Cas were immunoprecipitated (IP) and levels of tyrosine phosphorylation were monitored by immunoblotting with anti-phosphotyrosine antibodies (P-Tyr Blot). (B) Erk and (C) Akt activation were monitored by immunoblotting of whole cell extracts with the anti-Erk (T202/Y204) and anti-Akt (S473) phospho-specific antibodies (P-Erk Blot, P-Akt Blot). Some cells were treated with 10 ng/ml EGF or 50 ng/ml HGF for 10 min. (E) PECs placed in suspension were left untreated (0), treated with 2–10 µg anti-EGFR antibody (µg Ab), or treated with nonspecific IgG (Ig) for 30 min before plating on LM5. At the time of plating 2 ng/ml EGF was added (+EGF) to some samples. EGFR activation was monitored in immunoprecipitates by immunoblotting with anti-phosphotyrosine antibodies (P-Tyr Blot). Erk activation was monitored by immunoblotting of whole cell extracts with anti-Erk phospho-specific antibodies (P-Erk Blot). Total levels of each protein in the immunoprecipitates and cell lysates were measured by immunoblotting of stripped blots with the indicated antibodies. Levels of EGFR and Erk activation relative to total protein levels were quantified using Quantity One software (Bio-Rad) and are displayed to the right of the respective blots. (F) Fifty micrograms of whole cell extracts from PEC or PC3 cells were analyzed for EGFR levels by immunoblotting. (G–I) PC3 cells were serum-starved and placed in suspension and treated with DMSO (–), 0.5 µM PD168393, or 1 µM AG1478 before plating on LM5 (LM) for 1 h. Some cells were also treated with 2 ng/ml EGF (+EGF) for 10 min. (G) EGFR was immunoprecipitated (IP) and levels of tyrosine phosphorylation monitored by immunoblotting (P-Tyr Blot). (H) Erk and (I) Akt activation were monitored by immunoblotting of whole cell extracts with anti-Erk and anti-Akt phospho-specific antibodies (P-Erk Blot, P-Akt Blot).

antibody that prevents EGF binding to EGFR, and thus EGFR activation by EGF. Stimulation of PECs with EGF

effectively activates EGFR and Erk in PECs. Pretreatment of PECs with the EGFR blocking antibody did not significantly inhibit integrin-induced activation of EGFR or Erk (Figure 1E). In contrast, the same EGFR-blocking antibody blocked EGF-induced activation of EGFR and Erk, thereby reducing its activation to similar levels seen on matrix alone (Figure 1E). These data indicate that the ability of integrins to activate EGFR occurs independently of EGFR ligand.

The ability of integrins to activate Erk in epithelial cells is dependent on EGFR activation and is regulated in part by the level of EGFR expression (Moro *et al.*, 1998; Bill *et al.*, 2004). For instance, over expression of EGFR in fibroblasts (where Erk activation is not dependent on EGFR) leads to EGFR-dependent Erk activation in fibroblasts (Moro *et al.*, 1998). Adhesion of the epithelial cell line PC3, which expresses threefold less EGFR than PECs (Figure 1F), to the LM5-rich matrix produced by PECs fails to activate EGFR or Erk (Figure 1, G and H). EGFR is active in PC3 cells because stimulation with EGF activates both EGFR and Erk. In contrast, adhesion to matrix does not increase Akt activation, which is partially constitutively activated (Figure 1I). This is likely due to the loss of Pten expression in these cells (Vlietstra *et al.*, 1998). Consequently, constitutively active Akt is not blocked by EGFR inhibition. Thus, we predict that survival of these two cell lines on LM5-rich matrix is likely to be mediated by different signaling pathways.

EGFR and Src Independently Regulate Integrin-mediated Cell Survival in PECs

Treatment of LM5-rich adherent PECs with two EGFR-specific inhibitors, AG1478 or PD168393, results in the induction of cell death as measured by Annexin V staining (Figure 2, A and B). Maximal Annexin V staining is observed 72 h after drug treatment and occurs in over 85% of the cells (Figure 2A). Inhibition of Erk activation, the downstream target of EGFR, with U0126, but not inhibition of PI-3K with LY294002, induced cell death to the same extent as loss of EGFR signaling (Figure 2C). Together with the signaling

data shown in Figure 1 these findings indicate that Erk signaling downstream of EGFR is required for PEC survival on LM5-rich matrix. Inhibition of Src by SU6656 or PP2 also induced cell death (Figure 2, B and C), with a time course and effectiveness that is similar to that seen with EGFR or Erk inhibition. Simultaneous inhibition of EGFR and Src did not increase the amount of cell death observed (Figure 2C), suggesting that although these molecules lie on separate signaling pathways (see Figure 1), they may regulate cell survival through a similar downstream mechanism. All drugs were effective at inhibiting their respective signaling pathways at the concentrations used (see *Methods and Materials*).

Integrin-mediated Survival in PC3 Cells Is Not EGFR-dependent

In our studies we observed that adhesion of PC3 cells to LM5 (or LM1), does not induce the activation of EGFR or signal downstream to activate Erk (see Figure 1, G and H). Accordingly, inhibition of EGFR does not induce significant cell death in PC3 cells as measured by Annexin V staining (Figure 2D).

PC3 cells do not express the PI-3K/Akt inhibitor Pten, and consequently Akt is activated independent of matrix in PC3 cells and is not blocked when EGFR signaling is inhibited (Figure 1H). However, inhibition of the PI-3K pathway with LY294002 induced cell death in LM-adherent PC3 cells (Figure 2D). The extent of cell death induced by inhibition of PI-3K in PC3 cells was only twofold compared with the fourfold increase observed with inhibition of EGFR in PECs, suggesting that other mechanisms might be involved in regulating PC3 cell survival on matrix. We blocked Src signaling in PC3 cells with either SU6656 or PP2 and found that integrin-mediated survival in PC3 cells, like PECs, is also dependent on Src (Figure 2D). Inhibition of Src resulted in a three to fourfold increase in cell death. Thus cells that are able to activate EGFR/Erk signaling on LM5 are dependent on this pathway for survival. Cells unable to activate the

Figure 2. Integrin-induced activation of EGFR and Src is required for LM5-mediated survival. PECs were growth factor-starved for 48 h and placed in suspension for 30 min. Cells were allowed to adhere to LM5 in the absence or presence of 1 μ M AG1478 (AG), 0.5 μ M PD168393 (PD), 10 μ M U0126 (UO), 10 μ M LY294002 (LY), 0.5 μ M SU6656 (SU), or 10 μ M PP2 (PP2) for 4 h and then nonadherent cells were washed away, and drugs were replaced. (A) Percent Annexin V staining was quantified by FACS at 0, 24, 48, or 72 h after inhibition of EGFR with AG1478 (AG) or (B) visualized by phase-contrast and fluorescence microscopy after 72 h of treatment with PD168393 (PD) or SU6656 (SU). (C) Percent Annexin V staining 72 h after treatment of PECs with PD168393 (PD), AG1478 (AG), U0126 (UO), SU6656 (SU), PP2 (PP2), LY294002 (LY), or both PD and PP2 was quantified by FACS. (D) Growth factor-starved PC3 cells were placed in suspension and pretreated with DMSO, 0.5 μ M PD168393 (PD), 10 μ M LY294002 (LY), 2 μ M SU6656 (SU), or 10 μ M PP2 (PP2). Cells were analyzed for percent Annexin V staining by FACS 72 h after plating on laminin. Error bars, SD; n = 4; *p < 0.001 compared with DMSO control.

Figure 3. $\alpha 3$ integrin regulates survival in PECs. (A) PECs were kept in suspension in complete medium for 8 h. At 0, 1, 2, 3, 6, and 8 h after being suspended, a sample of cells was removed and analyzed for Annexin V positivity by FACS. (B–D) PECs were growth factor-starved for 48 h and placed in suspension for 30 min. At the time of plating on LM5, PECs were treated with anti-integrin antibodies to $\beta 4$ ($\beta 4$), $\alpha 3$ ($\alpha 3$), or IgG (IgG). (B) Annexin V staining was monitored 72 h after plating and visualized by fluorescence microscopy. DAPI was used to stain the nuclei (DAPI). (C) Quantification of percent Annexin V staining from B. (D) Cells were pretreated with anti-integrin antibodies ($\beta 4$, $\alpha 3$), or U0126 (U0) before adhesion to LM5. The levels of Erk activation were measured by immunoblotting of cell extracts with anti-phospho Erk antibodies (P-Erk Blot). Total levels of Erk were monitored by immunoblotting with anti-Erk antibodies (Erk Blot). Error bars, SD.

EGFR/Erk pathway use other signaling pathways, such as PI-3K, for survival.

LM5-dependent Survival through EGFR Is Mediated by Engagement of $\alpha 3$ Integrin in PECs

PECs express $\alpha 3\beta 1$ and $\alpha 6\beta 4$, both of which mediate adhesion to LM5 (Delwel *et al.*, 1994; Niessen *et al.*, 1994). As expected, placing PECs in suspension rapidly induces cell death, with 80–90% of cells displaying Annexin V positivity within 6 h (Figure 3A). To determine which of these integrin receptors, $\alpha 3\beta 1$ or $\alpha 6\beta 4$, is mediating survival, we used specific blocking antibodies raised against $\alpha 3$ or $\beta 4$ integrin. PECs treated with either $\alpha 3$ or $\beta 4$ blocking antibodies were still able to adhere to LM5-rich matrix; however, treatment with anti- $\alpha 3$ antibody blocked cell spreading (not shown). Blocking antibody to $\alpha 3$ integrin induced cell death to a similar extent as that seen with inhibition of EGFR or Src (Figure 3, B and C), i.e., 70–80%. Blocking $\beta 4$ integrin on the other hand did not compromise survival. Furthermore, cells treated with blocking antibodies to $\alpha 3$ integrin, but not $\beta 4$, were also defective in activating Erk (Figure 3D) and EGFR (not shown). These data indicate that signaling through $\alpha 3$ integrin regulates LM5-mediated activation of EGFR and its subsequent activation of Erk. Thus signaling from LM5 through $\alpha 3\beta 1$ to EGFR and downstream to Erk is critical for regulating survival.

Cell Death in PECs Is Not Caspase-dependent

Loss of adhesion has been associated with a specialized form of caspase-dependent apoptosis known as anoikis. Despite the fact that 85–90% of our cells were Annexin V positive, only 30% were positive by TUNEL and only 14% displayed sub-G0 content based on PI staining (not shown). This suggested that cell death due to loss of integrin signaling, as opposed to loss of adhesion, may proceed via a different mechanism. To determine if PECs were dying via caspase-dependent apoptosis, we first monitored caspase 3 cleavage and loss of Bcl-XL or Bcl-2. Neither loss of Bcl-XL or Bcl-2,

nor activation of caspase 3 was detectable by immunoblotting (Figure 4A). We then used an enzyme assay to directly measure caspase activity in dying PECs. Interestingly, in PECs loss of integrin-mediated signaling through EGFR, Src, or both simultaneously, failed to induce significant caspase 3/7 activity throughout a 72-h time course (Figure 4, B and C). Staurosporine is an efficient activator of classical apoptosis and caspases. To demonstrate that PECs were capable of activating caspases, we measured caspase 3/7 activity in staurosporine-treated PECs. A 16-fold increase in caspase 3/7 activity was observed in staurosporine-treated PECs (Figure 4B) as well as a loss of full-length caspase 3 as measured by immunoblotting (Figure 4A). This activity was inhibited by the caspase inhibitor zVAD. Furthermore, inhibiting caspase activity with zVAD was not sufficient to rescue cells from death upon EGFR or Src inhibition (Figure 4, D and E). One possibility is that the Annexin V positive cells are not dying. However, counting the number of remaining live cells after 72 h, as determined by trypan blue exclusion, indicated that 90% of the remaining cells were indeed dead (Figure 4E).

In contrast, induction of apoptosis in PC3 cells by treatment with LY294002 to inhibit PI-3K activity, or SU6656 or PP2 to inhibit Src activity, induced a 2.5-fold increase in caspase activity (Figure 4F).

Cell Death in PECs Is Not Due To Autophagy

Nutrient and serum deprivation can induce cells to enter a state of survival termed autophagy (Lum *et al.*, 2005). Knowing that our experiments were conducted under growth factor starvation conditions, we suspected that the cell death we were observing could be autophagic in nature (Baehrecke, 2005). To address this possibility, we first determined whether placing PECs under starvation conditions was sufficient to induce autophagy. LC3 protein is generally present throughout the cell, and upon induction of autophagy it is processed and incorporated into autophagic vacuoles. PECs were infected with an adenovirus that expresses an LC3-GFP fusion

Figure 4. Cell death induced in PECs is caspase-independent. (A) PECs were pretreated with DMSO (c), Staurosporine (Str), PD168393 (PD), U0126 (U0), or SU6656 (SU) and allowed to adhere to laminin for 72 h. The levels of Bcl-XL, Bcl-2, and caspase 3 were monitored by immunoblotting of cell lysates. Total levels of protein in the lysates were monitored by immunoblotting with anti-tubulin. (B) PECs were plated on LM5 and treated with DMSO, 1 μ M staurosporine (STR) for 24 h or 0.5 μ M PD168393 (PD), 10 μ M PP2 (PP2), or both (PD+PP2) for 72 h. The caspase inhibitor z-VAD at 20 μ M was added at the time of plating as indicated (zVAD). Caspase 3/7 activity was monitored every 1–4 h. Caspase data are expressed as fold increase in caspase activity over that observed in untreated cells. (C) PECs were drug treated as in B. Caspase 3/7 activity was monitored 72 h after drug treatment. (D) PECs were plated on LM5 and treated with DMSO (untreated), 0.5 μ M PD168393 (PD), or 10 μ M PP2 (PP2). z-VAD at 20 μ M was added at the time of plating as indicated (zVAD). Treated cells were sampled every 4–12 h over a 72-h time course and analyzed for percent Annexin V staining by FACS. (E) PECs were treated as in D. Cells were counted in the absence (total cell count) or presence (live cell count) of trypan blue to determine the total number of live versus dead cells. (F) PC3 cells were plated on laminin, treated with DMSO, 10 μ M LY294002 (LY), 2 μ M SU6656 (SU), or 10 μ M PP2 (PP2). Thirty-six hours later caspase 3/7 activity was measured. Error bars, SDs.

protein. Induction of autophagy is indicated by a shift from very diffuse LC3-GFP fluorescence throughout the cell to punctate fluorescence within the cytoplasm (Boya *et al.*, 2005). As early as 24 h after plating growth factor-starved LC3-GFP expressing PECs on LM5, punctate fluorescence was evident. By 48 h, multiple punctate fluorescent areas were observed in over 90% of cells under growth factor starvation conditions (Figure 5, A and B). Punctate fluorescence was rarely observed (in <10% in cells) in normal growth media at 24 or 48 h after plating on LM5 (Figure 5, A and B). Thus adhesion to LM5-rich matrix in growth factor-deprived cells leads to induction of autophagy. Furthermore, adhesion of growth factor-deprived PECs to their LM5-rich matrix is sufficient to mediate cell survival for at least 8 d. However, removal from matrix and placement in suspension results in maximum Annexin V positivity within 6 h (Figure 3A). Pretreatment of GFP-LC3 expressing cells with integrin-blocking antibodies to α 3 integrin resulted in a threefold reduction in LC3 punctate staining versus a 1.5-fold reduction with β 4 or α 6 blocking antibodies (Figure 5C). Thus adhesion of growth-factor deprived PECs to LM5-rich matrix primarily via α 3 β 1, but also to some extent through α 6 β 4, is required to maintain autophagy.

The autophagy inhibitor 3MA is a type-III PI3K-inhibitor that blocks the formation of autophagic vacuoles. We expected that if cell death was due to autophagy, treatment

with 3MA would rescue the cells from death. However, inhibiting autophagy in PECs by treatment with 3MA, in the presence of EGFR or Src inhibitors, does not rescue cells from death (Figure 6A). In fact, treatment of starved PECs with 3MA is sufficient to induce cell death. Cell death under autophagic inhibitory conditions is accompanied by caspase activation (Figure 6B). These data indicate that autophagy is acting as an integrin-mediated survival mechanism in LM5-rich matrix adherent PECs. Simultaneous inhibition of autophagy and EGFR/Erk signaling increases caspase activity. Similar results were obtained using bafilomycin (not shown), which inhibits autophagy by inhibiting fusion between autophagosomes and lysosomes by blocking vacuolar H⁺ ATPase (Yamamoto *et al.*, 1998).

If α 3 β 1 integrin regulates cell survival by maintenance of autophagy and signaling through EGFR/Erk and if blocking autophagy and EGFR induces caspase-mediated death, then blocking α 3 β 1 integrin should also induce caspase activation. Cells pretreated with α 3 blocking antibodies, but not α 6 or β 4 blocking antibodies, induced a fivefold increase in caspase 3/7 activity (Figure 6C).

Caspase-independent death has been linked to the generation of reactive oxygen species (ROS) in several cell systems. To determine if the mechanism by which inhibition of EGFR signaling induces cell death is due to the generation of ROS, PECs were pretreated with two different ROS inhibi-

Figure 5. Growth factor starvation of PECs induces autophagy. PECs were infected with an adenovirus to express the LC3-GFP fusion protein, and cells were placed in either normal growth media (growth) or starvation media (starvation). (A) Cells were observed at 20 $\times$ magnification by fluorescence microscopy to evaluate the pattern of LC3-GFP localization 48 h after plating. Some GFP is seen accumulating in the nucleus under both growth conditions. (B) To rule out the possibility of nonspecific staining artifacts cells displaying at least 10 punctate spots or more in the serum-starved (SM) conditions were scored as positive for LC3 staining. Over 90% of the starved cells met this criterion. To account for any low levels of autophagy in normal growth media (NGM), cells displaying three or more punctate spots were scored as positive for LC3 staining. Less than 10% of the cells in growth medium had three or more punctate spots. (C) Before plating on LM5, LC3-GFP-infected cells were pretreated with anti-integrin antibodies to $\alpha 3$ (alpha3), $\alpha 6$ (alpha6), or $\beta 4$ (beta4), or IgG (IgG). After 48 h the number of cells containing 10 or more LC3 punctate spots was counted.

tors, 50 μ M butylated hydroxyanisole (BHA) or 1.25 mM *N*-acetylcysteine (NAC). Treatment of PECs with NAC or BHA alone did not significantly increase or decrease the basal level of Annexin V staining. However, pretreatment with either NAC or BHA prevented the induction of Annexin V staining in cells treated with the EGFR inhibitor PD168393 (Figure 7). Thus loss of integrin-mediated signaling through EGFR results in an increase in ROS, which is required for the subsequent induction of caspase-independent death.

DISCUSSION

Using primary cultures of epithelial cells isolated from human PECs, we have identified at least three integrin-mediated signaling pathways whereby adhesion of PECs to their native LM5-rich matrix mediates cell survival (Figure 8). Adhesion of growth factor starved PECs to LM5-rich matrix is required to maintain autophagy. Signaling through $\alpha 3\beta 1$, and to a lesser extent $\alpha 6\beta 4$, is required for autophagy. Under starvation conditions cell survival is also dependent on at least two additional independent integrin signaling pathways: 1) integrin-mediated activation of EGFR and subsequent signaling to Erk and 2) integrin-mediated activation of Src, the former being dependent on $\alpha 3\beta 1$ integrin. Interestingly, there was no activation of the PI-3K/Akt signaling pathway in PECs on LM5; consequently there was no dependence on this pathway for survival in normal PECs. In the presence of an intact autophagy pathway, inhibition of EGFR/Erk or Src is sufficient to induce cell death, but this death is mediated through a caspase-independent mechanism that is dependent on the generation of reactive oxygen species. On the other hand, disruption of autophagy, pharmacologically or by blocking $\alpha 3\beta 1$, leads to caspase activation and death.

Integrin-mediated transactivation of receptor tyrosine kinases has been widely reported, however, the biological significance of this cross-talk is largely unknown. In this study we demonstrate that EGFR and its ability to activate Erk is a critical pathway for integrin-mediated survival in primary PECs adherent to their native matrix. Previous studies demonstrated that survival of EGFR over expressing

Figure 6. Blocking autophagy induces death and caspases in PECs. PECs were plated on LM5 and treated with DMSO, 0.5 μ M PD168393 (PD), 10 mM 3-methyladenine (3MA), or both (PD + 3MA) for 72 h. The caspase inhibitor z-VAD at 20 μ M was added at the time of plating as indicated (zVAD). Cells were analyzed for (A) Annexin V staining by fluorescence microscopy and (B) fold increase in caspase activity relative to DMSO-treated cells. (C) PECs plated on LM5 were treated with DMSO, 0.5 μ M PD168393 (PD), 1 μ M staurosporine, 10 μ g/ml $\alpha 3$ (a3), $\alpha 6$ (a6), $\beta 4$ (b4) blocking integrin antibodies or IgG (Ig) and the fold increase in caspase activity was measured 48 h later.

Figure 7. Generation of ROS is required for caspase-independent death induced by EGFR inhibition. PECs were growth factor-starved for 48 h and placed in suspension for 30 min. Cells were allowed to adhere to LM5 in the absence or presence of 50 μ M butylated hydroxyanisole (BHA) or 1.25 mM *N*-acetylcysteine (NAC) with or without 0.5 μ M PD168393 (PD). Percent Annexin V staining was quantified 72 h later by fluorescence microscopy.

NIH-3T3 cells on fibronectin required integrin-mediated activation of EGFR, but did not involve signaling to Erk, but rather PI-3K (Moro *et al.*, 1998). These differences may reflect the cell type, fibroblasts versus epithelial cells, or the matrix that was used, fibronectin versus laminin. Although LM5 is the predominant matrix secreted by PECs, we cannot rule out the possibility that additional matrix materials may also be present that could be contributing to PEC survival. Nonetheless, we have demonstrated that integrin-mediated signaling through $\alpha 3\beta 1$ is required for survival mediated by both autophagy and EGFR/Erk signaling on this PEC-generated matrix.

Because PECs rapidly secrete their own LM5-rich matrix, it has not been possible to determine the role of different matrices in regulating long-term survival of PECs. However,

Figure 8. Model for LM5-mediated survival. Adhesion of growth factor-deprived PECs to LM5 via $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrin mediates cell survival by maintaining starvation-induced autophagy. Signaling via $\alpha 3\beta 1$ to Erk through EGFR or through Src is also required for cell survival. The PI-3K/Akt pathway is not activated on LM5 and not required for survival. Disruption of either the EGFR/Erk or Src pathway leads to caspase-independent cell death, due to the generation of reactive oxygen species (ROS), whereas disruption of autophagy leads to caspase activation and death.

in short-term 1-h adhesion assays we have observed that adhesion of PECs to LM1, but not the LM5-rich matrix, is sufficient to activate Akt, suggesting signaling pathways on other matrices may be important in survival. We are currently developing siRNA-based methods for eliminating LM5 from the PEC matrix, which will allow us to investigate the role of EGFR and other signaling pathways in mediating survival on other matrices.

Integrin-mediated survival of primary keratinocytes on LM5 has been shown to involve signaling to Erk (Manohar *et al.*, 2004). As in our studies, keratinocyte survival on LM5 was dependent on $\alpha 3\beta 1$ integrin. Whether Erk activation in keratinocytes is dependent on integrin signaling through EGFR has not been reported. However, autocrine ligand-mediated signaling through EGFR to Erk was shown to contribute to cell survival of keratinocytes in suspension (Jost *et al.*, 2001). We have ruled out a role for autocrine ligand involvement in PECs, because ligand binding blocking antibodies do not block integrin-mediated EGFR activation or downstream signaling to Erk. Given the similar findings in primary prostate epithelial cells and keratinocytes, we predict that keratinocyte survival on LM5 should also involve integrin-mediated activation of EGFR and subsequent downstream signaling to Erk.

Whether integrin-mediated activation of other receptor tyrosine kinases is involved in regulating survival on matrix is not known. However, it was recently demonstrated that ligand-independent activation of c-Met in PC3 cells was required for cell survival (Shinomiya *et al.*, 2004). The specific integrins, matrix, and signaling pathways involved in c-Met-mediated survival are currently unknown. Furthermore, whether c-Met regulates integrin-mediated survival in normal primary PECs is also unknown. Our data indicate that PC3 cells, which express low levels of EGFR relative to PECs, do not activate EGFR or Erk upon integrin engagement and do not depend on this pathway for integrin-mediated survival. Instead survival of PC3 cells requires PI-3K and Src. c-Met is known to activate these signaling pathways in response to HGF, but whether c-Met participates in integrin-mediated signaling to PI-3K or Src in PC3 cells has not been determined.

Surprisingly, interference with integrin signaling through EGFR/Erk or Src leads to caspase-independent death. This was unexpected, because cell death due to anoikis has been reported to be caspase-dependent. Our results suggest that the mechanism of cell death induced during complete loss of cell adhesion through integrins is different from the mechanism of cell death induced by interfering with specific signaling downstream of integrin engagement. One possible explanation is that loss of attachment is likely to interfere with several different signaling pathways simultaneously, whereas our studies individually dissected distinct pathways. In fact, blocking $\alpha 3\beta 1$ alone was sufficient to induce caspase activity in PECs, similar to what has previously been observed in keratinocytes derived from integrin $\alpha 3$ null mice (Manohar *et al.*, 2004). We have demonstrated that at least one of the signaling pathways activated by $\alpha 3\beta 1$ is EGFR/Erk. Src activation is also important for integrin-mediated survival, but inhibition of both EGFR and Src was also not sufficient to induce caspases, indicating additional survival pathways are involved.

Previous studies in mammary epithelial cells demonstrated that cells in the center of acinar structures undergo autophagy and die during morphogenesis (Melino *et al.*, 2005). However, the current prevailing theory on the primary role of autophagy is to promote temporary survival under growth factor and nutrient deprivation conditions,

rather than to be a direct mechanism for programmed cell death (Lum *et al.*, 2005). Interestingly, inhibiting autophagy in LM5-adherent PECs induced caspase activation and cell death and did not rescue cells induced to die by inhibition of EGFR/Erk, suggesting that cell death was not dependent on autophagy. Thus adhesion of starved PECs to a LM5-rich matrix induces an autophagic state that permits survival, but further assault by inhibiting EGFR/Erk activation leads to caspase-independent death.

Blocking $\alpha 3\beta 1$ integrin significantly reduced the extent of autophagy induced under starvation conditions. Thus, in addition to regulating EGFR/Erk, $\alpha 3\beta 1$ integrin is also required to maintain autophagy. Although blocking $\alpha 6\beta 4$ integrin also reduced autophagy, the effect was not as dramatic as blocking $\alpha 3\beta 1$. Furthermore, $\alpha 6\beta 4$ was not required for EGFR/Erk activation and disruption of this integrin alone failed to induce caspase activation or cell death. Therefore, the small reduction in autophagy seen by blocking $\alpha 6\beta 4$ may not be sufficient enough to overcome other survival pathways that are active in the cell, such as EGFR/Erk and/or Src. Interestingly, inhibition of both autophagy and EGFR signaling lead to a small (although not statistically significant in the four assays examined), but consistent increase in caspase activation, suggesting that in the absence of autophagy signaling through EGFR/Erk may still contribute to cell survival. One possibility is that signaling through EGFR/Erk helps to maintain autophagy. However, if this is true, then there must be other pathways involved, because inhibition of EGFR/Erk alone is not sufficient to induce caspase-mediated death.

It is interesting to note that signaling through PI-3K is actually inhibitory to the development of autophagy (Rusten *et al.*, 2004). Adhesion of PECs to LM5 does not activate the PI-3K pathway. This suggests that the absence of strong PI-3K signaling permits the survival of PECs on LM5 through autophagy. It is striking that cell death induced by loss of EGFR/Erk or Src signaling is not sufficient to activate caspases. This suggests the existence of a strong anticaspase mechanism present in PECs. Recent studies have suggested that the presence of an autophagic state can be inhibitory to the activation of caspases (Degenhardt *et al.*, 2006; Abedin *et al.*, 2007). One model proposes that the autophagy pathway selectively targets damaged mitochondria for destruction by walling them off from the cytoplasm and thus preventing the release of enzymes required for the induction of caspases. Therefore, it is possible that the absence of a PI-3K pathway may allow this shift to a caspase inhibitory state. Thus in the EGFR/Erk inhibited cells, caspase-mediated death is dominantly inhibited, forcing other death mechanisms to be activated when this level of stress is induced.

Many human prostate cancers have reduced levels of the negative PI-3K regulator, Pten, and the PI-3K/Akt pathway is constitutively activated in those tumors (McMenamin *et al.*, 1999). Furthermore, the development of prostate cancer is accompanied by the loss of LM5 in the basement membrane (Davis *et al.*, 2001). Therefore, given that autophagy is driven by LM5-mediated adhesion and suppression of PI-3K signaling, we would predict that loss of LM5 and increased PI-3K signaling would prevent the induction of an autophagy survival pathway in tumor cells and make them more sensitive to caspase-mediated death.

Several caspase-independent mechanisms of cell death have been described, including activation of cathepsins, calpeptins, ROS, and release of numerous destructive enzymes from the mitochondria (Kroemer and Martin, 2005). To date we have been unable to detect release of cytochrome C from mitochondria in PECs treated with EGFR inhibitors, and

inhibition of calpeptin did not rescue the death induced by inhibiting EGFR/Erk signaling (not shown). However, by blocking the generation of ROS with two different inhibitors, we were able to prevent the cell death induced by inhibition of EGFR/Erk. Thus adhesion to matrix and signaling through EGFR/Erk may act to limit ROS production. Integrin $\alpha 1$ null mesangial cells have been reported to have enhanced ligand-independent EGFR activation and excessive ROS production, suggesting that integrin signaling can help to modulate EGFR activation and limit ROS production (Chen *et al.*, 2007). Another report suggests that $\alpha 1$ negatively regulates EGFR activation by stimulating the activity of TC-PTP (Mattila *et al.*, 2005). However, if this same mechanism is acting in PECs, then loss of EGFR/Erk signaling would lead to reduced ROS production rather than its increase. Thus the mechanism by which loss of EGFR/Erk signaling leads to enhanced ROS production is not clear. Interestingly, a recent report indicates that high levels of ROS are required for starvation-induced autophagy (Scherz-Shouval *et al.*, 2007). Therefore it is possible that one side effect of EGFR/Erk inhibition may be to further enhance autophagy through increased generation of ROS.

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