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The receptor for advanced glycation end products (RAGE) sustains autophagy and limits apoptosis, promoting pancreatic tumor cell survival

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Activation of the induced receptor for advanced glycation end products (RAGE) leads to initiation of NF-κB and MAP kinase signaling pathways, resulting in propagation and perpetuation of inflammation. RAGE-knockout animals are less susceptible to acute inflammation and carcinogen-induced tumor development. We have reported that most forms of tumor cell death result in release of the RAGE ligand, high-mobility group protein 1 (HMGB1). We now report a novel role for RAGE in the tumor cell response to stress. Targeted knockdown of RAGE in the tumor cell, leads to increased apoptosis, diminished autophagy and decreased tumor cell survival . In contrast, overexpression of RAGE is associated with enhanced autophagy, diminished apoptosis and greater tumor cell viability. RAGE limits apoptosis through a p53-dependent mitochondrial pathway. Moreover, RAGE-sustained autophagy is associated with decreased phosphorylation of mammalian target of rapamycin (mTOR) and increased Beclin-1/VPS34 autophagosome formation. These findings show that the inflammatory receptor, RAGE, has a heretofore unrecognized role in the tumor cell response to stress. Furthermore, these studies establish a direct link between inflammatory mediators in the tumor microenvironment and resistance to programmed cell death. Our data suggest that targeted inhibition of RAGE or its ligands may serve as novel targets to enhance current cancer therapies.
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A growing body of literature supports the notion that many of the phenotypic alterations observed in cancer cells are a result of inflammatory signals found within the tumor microenvironment. Recent efforts have focused on identifying the responsible molecular pathways in cancer-associated inflammation that result in promotion of carcinogenesis and resistance to therapy.[1,2] One such molecular target is the inflammatory receptor, receptor for advanced glycation end products (RAGE). The expression of RAGE enhances tumor formation in an inflammatory carcinogen model of cancer and has been shown to be associated with increased resistance to chemotherapy.[3–5]

The receptor for advanced glycation end products, an evolutionarily recent type I transmembrane receptor, is a member of the immunoglobulin superfamily. It is encoded within the gene-dense major histocompatibility class III region, adjacent to TNF-α and several complement components. The ligands identified for RAGE include high-mobility group protein 1 (HMGB1), advanced glycation end products and some but not all members of the S100 protein family.[6] RAGE/RAGE ligand interactions are associated with survival of cells expressing this receptor, inflammation, increased levels of phosphorylated extracellular signal-regulated kinase and increased levels of NF-κB p65.[6,7] Stressed cells markedly enhance RAGE expression, which has been implicated in the pathogenesis of a variety of inflammatory diseases, including sepsis, diabetes, atherosclerosis, and Alzheimer's disease.[6–9] RAGE has also been linked to the development and progression of cancer.[10] Mice deficient for RAGE expression are resistant to DMBA/TPA-induced skin carcinogenesis showing increased apoptosis in the tumor microenvironment and diminished inflammatory responses;[11] however, the mechanism by which this occurs was not previously delineated. Inhibition of RAGE and its ligands with antibodies or sRAGE expression causes decreased growth and metastases of both implanted tumors and tumors developing spontaneously in susceptible mice.[12] RAGE mediates resistance to hypoxia and consequently, RAGE knockdown in murine hepatomas slowed tumor growth.[13] Inhibition of RAGE interaction with S100p leads to enhanced antitumor activity of conventional chemotherapy in a xenograft model of pancreatic cancer.[14] In humans, RAGE and RAGE splice variants are overexpressed in adenocarcinomas of the pancreas and correlate with proliferation and invasiveness.[15] Polymorphisms in the RAGE gene have also been linked to increased risk of gastric cancer.[16]

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Abbreviations: DAMPs, damage-associated molecular pattern molecules; HMGB1, high-mobility group protein 1; RAGE, receptor for advanced glycation end products; shRNA, short hairpin RNA
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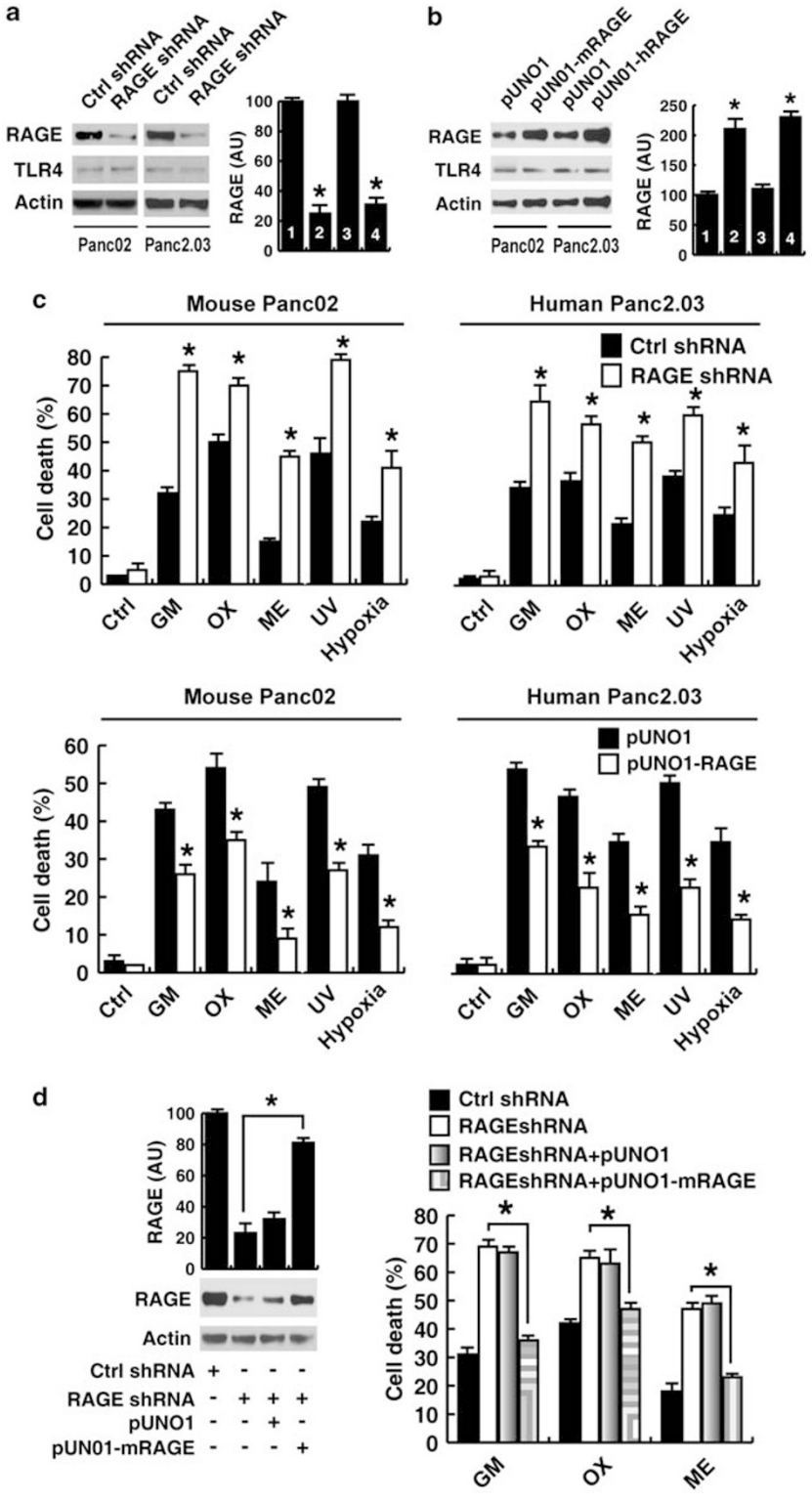
We have previously shown that one of the principle ligands for RAGE, the inflammatory cytokine HMGB1, is released following most forms of pancreatic tumor cell death.[17,18] We show here that the expression of RAGE correlates with tumor cell survival following cytotoxic insult. RAGE-mediated tumor cell survival is associated with increased 'programmed' cell survival (autophagy) and decreased programmed cell death (apoptosis). RAGE limits apoptosis through a p53-dependent mitochondrial pathway. Moreover, RAGE-sustained autophagy is associated with decreased phosphorylation of mammalian target of rapamycin (mTOR) and increased Beclin-1/VPS34 autophagosome formation. These findings suggest that RAGE promotes tumor cell survival in stressed cells and provides the framework for the development of novel clinical approaches targeting the RAGE pathway.

Results

RAGE expression promotes tumor cell survival following genotoxic or metabolic stress. We analyzed RAGE expression in several cultured tumor cell lines. RAGE was expressed in a variety of human and mouse tumors under *in vitro* growth conditions, including pancreatic (Panc1.28, Panc2.03, Panc3.27 and Panc02), breast (4TI) and colonic (MC38, CT26) carcinomas, a fibrosarcoma (MCA205) and melanoma (B16V1, B16M05) (Supplementary Figure 1a). Interestingly, RAGE expression was greatest in the tumor cell lines derived from murine and human adenocarcinoma of the pancreas. RAGE and its ligand, HMGB1, were also expressed in murine pancreatic tumor cell lines growing *in vivo* (Supplementary Figure 1b). We chose to focus on these pancreatic tumors for our subsequent studies. As RAGE expression is associated with increased nuclear translocation of NF-κB, a known tumor survival factor, we sought to better understand the effect of RAGE expression on tumor cell response to stress. We used a target-specific short hairpin RNA (shRNA) against RAGE to knockdown the expression of RAGE and a full-length plasmid expressing the murine or human RAGE gene (pUNO1-RAGE) to force overexpression in murine (Panc02) and human (Panc2.03) pancreatic tumor cell lines. Transfection of RAGE-shRNA decreased RAGE protein levels by nearly 80% (Figure 1a). Similarly, transfection with a full-length RAGE plasmid increased the RAGE protein levels by twofold in our cell lines (Figure 1b). One of the earliest events following the activation of RAGE is phosphorylation of the extracellular signal-regulated kinase (ERK).[19] Consistent with this notion, we observed that knockdown of RAGE almost completely abrogated the ability of our tumor cell lines to phosphorylate ERK in response to chemotherapy-induced stress (Supplementary Figure 2). Next, we measured the effect of RAGE knockdown on cell viability following exposure to genotoxic or metabolic stress. We observed that depletion of RAGE significantly increased cell death of both murine and human pancreatic tumor cell lines following exposure to cytotoxic chemotherapy, UV radiation and hypoxia (Figure 1c and Supplementary Figure 3). In contrast, forced overexpression of RAGE promoted survival of tumor cells following exposure to these stressors (Figure 1c). Measurement of long-term cell viability by colony formation assays confirmed that RAGE depletion decreased tumor cell viability and RAGE overexpression increased tumor cell survival following exposure to cytotoxic chemotherapy (Supplementary Figure 4). Increased cytotoxicity in RAGE knockdowns was observed with several chemotherapeutic agents that use varying mechanisms of action, including oxaliplatin (platinating/DNA-crosslinking agent), melphalan (alkylating agent) and gemcitabine (nucleoside analog). Re-expression of RAGE with delivery of a full-length plasmid vector restored a 'protective' phenotype confirming the specificity of RAGE in promoting tumor cell survival (Figure 1d).

RAGE promotes tumor cell survival by enhancing autophagy and limiting apoptosis. To better understand the mechanisms of RAGE-mediated pro-survival signals, we measured caspase-3 activity as a measure of the terminal effector phase of apoptotic cell death. Knockdown of RAGE led to a significantly greater induction of cleaved caspase-3 in these murine tumor cell lines following treatment with chemotherapeutic agents (Figures 2a and b). Consistent with this observation, addition of the pan-caspase inhibitor ZVAD-FMK reversed the increased caspase-3 activity and enhanced tumor cell death observed with RAGE knockdown (Figure 2a). This suggests that depletion of RAGE enhances apoptotic cell death following cytotoxic or metabolic stress. As autophagy is an increasingly recognized mechanism for tumor cell survival and resistance to apoptosis in response to stress,[20] we examined levels of autophagy in murine pancreatic tumor cell lines treated with chemotherapeutic agents. Detection of LC3-II by immunoblotting or immunofluorescence is a widely used method for showing increased autophagy.[21] We observed a significant increase in LC3-II levels and an aggregation of LC3 into punctae in both human and murine pancreatic tumor cells treated with the LD50 of our chemotherapeutic agents (Figures 2b and c). Knockdown of RAGE limited this increase in markers of autophagy (Figures 2b and c). An alternative method for detecting autophagy is measuring enhanced degradation of p62 (SQSTM1/sequestosome), a long-lived scaffolding protein involved in the transport of ubiquitinated proteins destined for proteosomal digestion.[22,23] Indeed, knockdown of RAGE limited degradation of p62 induced by chemotherapeutic agents (Figure 2b). Consistent with the decreased autophagy observed in RAGE-knockdown tumor cells lines, we found an increase in markers of apoptosis (cleaved PARP and cleaved caspase-3). Wortmannin, a class III phosphoinositol-3 kinase (PI3K) inhibitor that blocks autophagy, significantly diminished LC3 punctae formation in both control shRNA- and RAGE shRNA-treated Panc02 cells (Figure 2c). Consistent with these findings, we observed that forced expression of RAGE led to decreased levels of cleaved caspase-3 and LC3 punctae in tumor cells treated with chemotherapeutic agents (Supplementary Figure 5). To confirm that this observation was due to targeted knockdown of RAGE rather than nonspecific factors following transfection with shRNA vectors, we examined re-expression of RAGE through transfection with the

pUNO1-mRAGE construct. Re-expression of RAGE in the knockdown tumor cell lines restored apoptosis and autophagy to basal levels (Figure 2d). We also examined markers of autophagy and apoptosis in fibroblasts from RAGE-knockout animals. Primary short-term cultures of lung fibroblasts from wild-type and RAGE-knockout controls were propagated in short-term cultures. We observed a significant reduction in the levels of LC3-II protein expression in the RAGE KO fibroblasts following exposure to chemotherapeutic agents compared with their wild-type



counterparts (Supplementary Figure 6). The RAGE ligand, HMGB1, is released following several methods of inducing tumor cell death including chemotherapy.[17,18] We hypothesize that HMGB1 released upon tumor cell death interacts with RAGE leading to increased resistance to programmed cell death. Consistent with this hypothesis, we observed that depletion of the pool HMGB1 by shRNA in panc02 cells rendered them significantly more sensitive to melphalan-induced apoptotic cell death as shown by increased cleaved PARP. We also observed decreased LC3-II, consistent with the lower levels of autophagy (Figure 2e). We have also observed that exogenous provision of rHMGB1 can promote autophagy in pancreatic tumor cell lines through a RAGE-dependent pathway (Tang DL *et al*., unpublished data). These findings strongly support a critical role for HMGB1/RAGE pathway in the regulation of apoptosis and autophagy in stressed or dying tumor cells.

Increased apoptosis in RAGE-knockdown tumor cells is associated with phosphorylation of p53 and its translocation to the mitochondria. To further characterize the molecular events associated with the regulation of apoptosis by RAGE, we examined a panel of apoptotic markers. Depletion of RAGE by shRNA increased phosphorylation of p53 at Ser392, but not Ser6 and Ser 15 (Figure 3a and Supplementary Figure 7), following treatment with chemotherapeutic agents. At the same time, RAGE shRNA had no effect on the level of acetylation of p53 at Lys379 (Figure 3a). The expression levels of anti-apoptotic members of the mitochondrial pathway, including Bcl-2 and Bcl-XL, were reduced by $\sim$40–70% in RAGE-knockdown Panc02 cells when compared with control cells in response to chemotherapy treatment. However, there was no significant difference in expression levels of the cytosolic proapoptotic Bcl-2 family members, Bax and PUMA, when comparing control shRNA- and RAGE shRNA-treated Panc02 cells. This suggests that RAGE expression limits the p53-dependent mitochondrial pathway of apoptosis. To confirm this, we also used pifithrin alpha (PFT-α), a pharmacological antagonist of p53,[24] to block p53 expression and function. Pretreatment with PFT-α blocked phosphorylation of p53 at Ser392 and cleavage of caspase-3 in RAGE-knockdown Panc02 cells following oxaliplatin treatment (Figure 3b). Furthermore, inhibition of p53 by PFT-α decreased the oxaliplatin-induced activation of caspase-3 and cell death in RAGE-knockdown Panc02 cells (Figure 3b). Similarly, we observed that knockdown of RAGE had no effect on oxaliplatin-induced tumor cell death in p53-knockout HCT116 cell lines (Figure 3c). Next, we observed that the depletion of RAGE increased translocation of p53 to the mitochondria and promoted cytochrome c release from the mitochondria to the cytosol following oxaliplatin treatment (Figure 3d). RAGE shRNA, however, did not influence the p53 reporter activity induced by chemotherapeutic drugs, such as oxaliplatin and melphalan (Figure 3e). Together, these results suggest that when RAGE is present, it limits translocation of p53 to the mitochondria, in turn inhibiting apoptosis and enhancing survival following treatment with chemotherapy.

Decreased autophagy in RAGE-knockdown tumor cells is associated with increased levels of phosphorylated mTOR and decreased autophagosome formation. Autophagy is controlled by several kinases including mTOR, which promotes active protein translation and suppresses autophagy.[25] To gain insight into the molecular mechanism by which RAGE regulates autophagy in pancreatic cancer cells, we examined the effect of RAGE expression on mTOR. mTOR is autophosphorylated at Ser2481 and phosphorylated at Ser2448 through the class I PI3 kinase/Akt signaling pathway.[26] Phosphorylation of mTOR was increased in RAGE shRNA-treated Panc02 cells when compared with control shRNA-treated cells (Figure 4a). Unexpectedly, the phosphorylation of the mTOR downstream target 4E-BP1 (p-4E-BP1) was inhibited following depletion of RAGE (Figure 4a). This discrepancy suggests that a more complex signaling pathway regulates p-4E-BP1. Furthermore, p-4E-BP1 is also positively regulated by the ERK pathway in lung endothelial cells.[27] Consistent with this finding, we observed that knockdown of RAGE almost completely abrogated the ability of our tumor cell lines to phosphorylate ERK in response to chemotherapy-induced stress (Supplementary Figure 2). At the same time, RAGE shRNA had no effect on the expression level of Raptor and Rictor, two molecular components of the mTORC1 and mTORC2 complexes[28] (Figure 4a).

Treatment with rapamycin, a specific inhibitor of mTOR,[29] limited phosphorylation of mTOR and increased autophagy as shown by increased LC3-II in RAGE-knockdown tumor cell lines treated with oxaliplatin (Supplementary Figure 8). These observations suggest that targeted knockdown of RAGE limits autophagy and tumor cell survival following treatment with chemotherapy through an mTOR-dependent pathway.

The Beclin-1/VPS34 (the yeast homolog of the class III PI3K) complex is important in mediating the localization of other autophagy-related proteins to the preautophagosomal membrane.[30] Thus, we evaluated the effects of RAGE shRNA

Figure 1 RAGE promotes tumor cell survival following genotoxic or metabolic stress. (**a**) Targeted knockdown of RAGE protein following transfection with RAGE shRNA. RAGE, TLR4 and actin protein levels in Panc02 (murine) or Panc2.03 9 (human) cells transfected with nonspecific shRNA (ctrl shRNA, '1, 3') or RAGE shRNA ('2, 4') at 48 h after transfection (*$P<0.05$ *versus* ctrl shRNA). (**b**) RAGE protein was overexpressed following transfection with a RAGE plasmid. RAGE, TLR4 and actin protein levels in Panc02 or Panc2.03 cells transfected with empty vector (pUNO1, '1, 3') or RAGE plasmids (pUNO1-RAGE, '2, 4') at 48 h after transfection (*$P<0.05$ *versus* pUNO1). (**c**) RAGE expression correlates with tumor cell survival. Knockdown of RAGE or overexpression of RAGE in pancreatic cancer cell lines is indicated. These cells were then treated with the LD50 of individual chemotherapeutic agents (oxaliplatin, 'OX', 160 μg/ml; melphalan, 'ME', 320 μg/ml; or gemcitabine, 'GM', 100 nM), UV irradiation (5 min after 50 mJ/cm^2) or hypoxia (1% O_2). Cell viability was examined at 24 h ($n=3$, *$P<0.05$ RAGE shRNA *versus* ctrl shRNA or pUNO *versus* pUNO1-RAGE). (**d**) Re-expression of RAGE with full-length plasmid restored the chemotherapy-protective phenotype. RAGE was overexpressed in RAGE-knockdown Panc02 tumor cells as indicated and then treated with chemotherapeutic agents (oxaliplatin, 'OX', 160 μg/ml; melphalan, 'ME', 320 μg/ml; and gemcitabine, 'GM', 100 nM). Cell viability was examined at 24 h ($n=3$, *$P<0.05$)

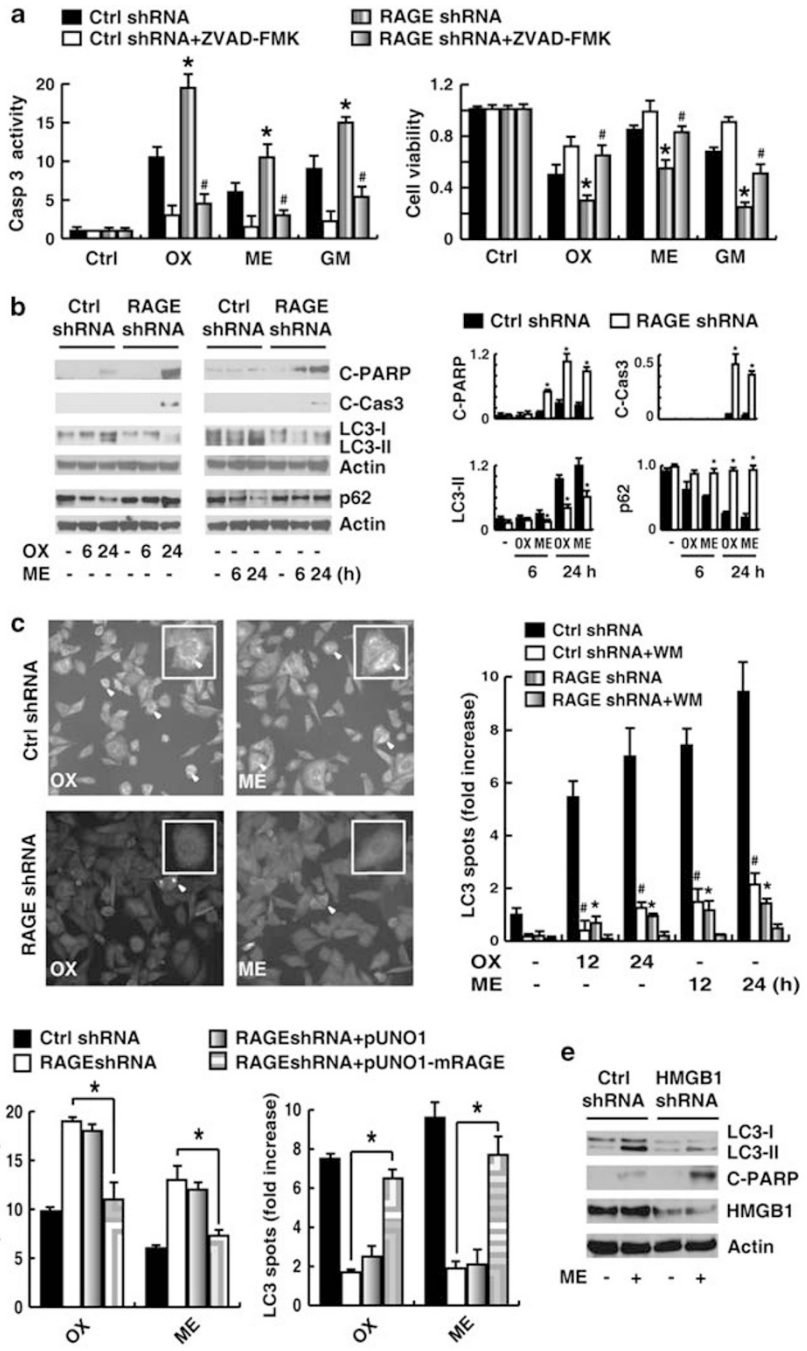


Figure 2 Targeted knockdown of RAGE in murine and human pancreatic tumor cell lines results in increased apoptosis and decreased autophagy following treatment with chemotherapeutic agents. (**a**) A pan-caspase inhibitor reverses the increased caspase-3 activity and tumor cell death observed in RAGE-knockdown cells. Panc02 tumor cells were pretreated with the pan-caspase inhibitor (ZVAD-FMK, 20 μM) for 1 h and then treated with chemotherapeutic agents (oxaliplatin, 'OX', 160 μg/ml; melphalan, 'ME', 320 μg/ml; and gemcitabine, 'GM', 100 nM). Caspase-3 activity and cell viability were examined at 24 h ($n=3$, $^{*}P<0.05$ RAGE shRNA *versus* ctrl shRNA, $^{\#}P<0.05$ RAGE shRNA + ZVAD-FMK *versus* RAGE shRNA). (**b**) Knockdown of RAGE decreases markers of autophagy and increases markers of apoptosis. Panc02 tumor cells were treated with chemotherapeutic agents (oxaliplatin, 'OX', 160 μg/ml; melphalan, 'ME', 320 μg/ml) for the indicated time. Western blot analysis of protein levels is indicated. Left panel is a representative picture of western blots ($n=3$, $^{*}P<0.05$ RAGE shRNA *versus* ctrl shRNA). (**c**) An autophagy inhibitor limits LC3 punctae in control and RAGE-knockdown cells. Panc02 tumor cells were pretreated with the PI3K/autophagy inhibitor, wortmannin (WM) 100 nM for 1 h and then treated with chemotherapeutic agents (oxaliplatin, 'OX', 160 μg/ml; melphalan, 'ME', 320 μg/ml) for the indicated time. Cells were immunostained with LC3-specific antibodies. Images of 1000 cells were analyzed to obtain the average LC3 spots by imaging cytometric (arrayscan) analysis ($n=3$, $^{*}P<0.05$ RAGE shRNA *versus* ctrl shRNA; $^{\#}P<0.05$ ctrl shRNA + WM versus ctrl shRNA). Left panel is a representative picture of each condition. Inset shows a higher magnification of LC3 stain. (**d**) Re-expression of RAGE with full-length plasmid restores relative autophagy and apoptosis levels to baseline. RAGE was overexpressed in RAGE-knockdown Panc02 tumor cells and cells were treated with chemotherapeutic agents (oxaliplatin, 'OX', 160 μg/ml; melphalan, 'ME', 320 μg/ml). Caspase-3 activity and LC3 spot formation were examined at 24 h ($n=3$, $^{*}P<0.05$). (**e**) HMGB1-knockdown cells show increased resistance to melphalan-induced apoptotic cell death. Panc02 cells were knocked down by HMGB1 shRNA and ctrl shRNA for 48 h and then stimulated with melphalan ('ME', 320 μg/ml) for 24 h. Representative western blot analysis of protein levels is presented

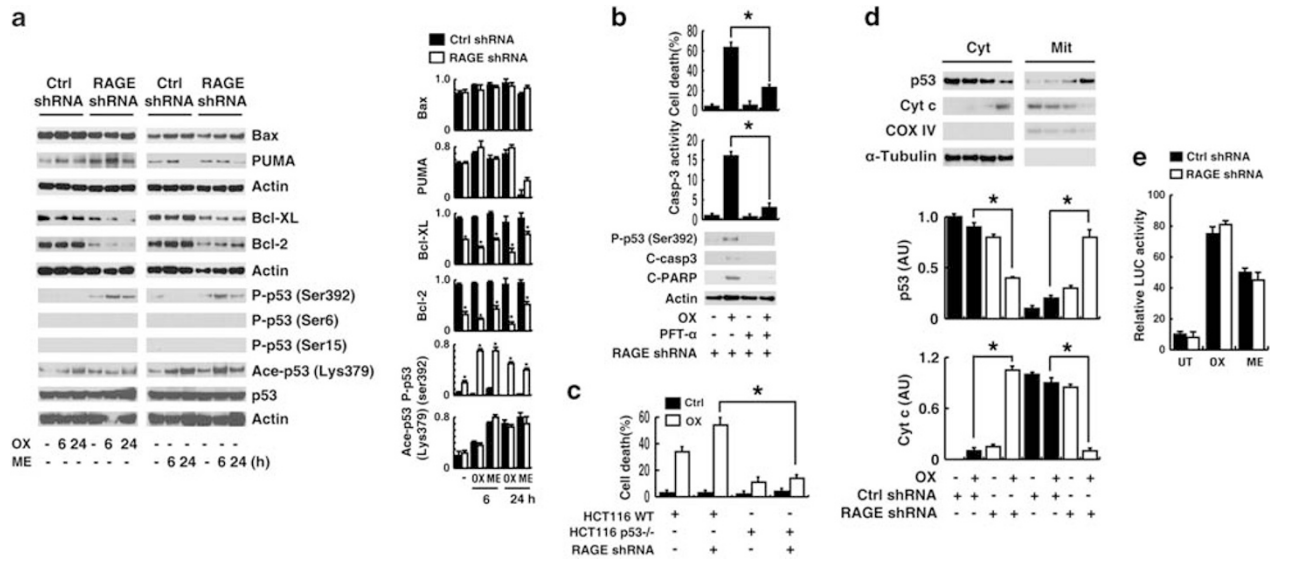


Figure 3 Increased apoptosis in RAGE-knockdown tumor cells is p53 dependent. (**a**) RAGE knockdown significantly increases phosphorylation of p53 at Ser392 and decreases anti-apoptotic Bcl-2 family proteins following treatment with anticancer agents. Panc02 cells were treated with chemotherapeutic agents (oxaliplatin, 'OX', 160 μg/ml; melphalan, 'ME', 320 μg/ml) for the indicated time. Western blot analysis of protein levels is presented. Left panel is a representative picture of western blots ($n=3$, $^*P<0.05$ RAGE shRNA *versus* ctrl shRNA group). (**b**) A small molecule inhibitor of p53 reverses chemotherapy-induced apoptosis in RAGE-knockdown cells. RAGE-knockdown Panc02 cells were pretreated with a p53 inhibitor (PFT-α, 20 μM) for 1 h and then treated with oxaliplatin ('OX', 160 μg/ml) for 24 h. Western blot analysis of protein levels are indicated. Caspase-3 activity and cell death were examined at 24 h ($n=3$, $^*P<0.05$). (**c**) p53–/– reverses anticancer drug-induced cell death in RAGE-knockdown cells. RAGE was knocked down by shRNA transfection in p53 WT HCT116 cell or p53–/– HCT116 cells, and then these cells were treated with oxaliplatin ('OX', 160 μg/ml). Cell death was assayed at 24 h ($n=3$, $^*P<0.05$). (**d**) RAGE knockdown increases mitochondrial translocation of p53 in cells following treatment with chemotherapeutic agents ($n=3$, $^*P<0.05$). RAGE-knockdown Panc02 cells were treated with oxaliplatin ('OX', 160 μg/ml) for 12 h. Western blot analysis of p53 and cytochrome c (Cyt c) was then assessed in the cytoplasmic and mitochondrial fractions of these cells. The successful separation of cytoplasmic ('Cyt') and mitochondrial ('Mit') fraction was confirmed by western blot analysis of each fraction for known cytoplasmic (tubulin) and mitochondrial (COX IV) proteins. (**e**) RAGE shRNA does not influence chemotherapy drug-induced p53 reporter activity. The indicated Panc02 cells were transfected with a p53 reporter. After 24 h of transfection, cells were treated separately with the chemotherapeutic agents (oxaliplatin, 'OX', 160 μg/ml; melphalan, 'ME', 320 μg/ml). A luciferase assay was carried out at 12 hours after treatment

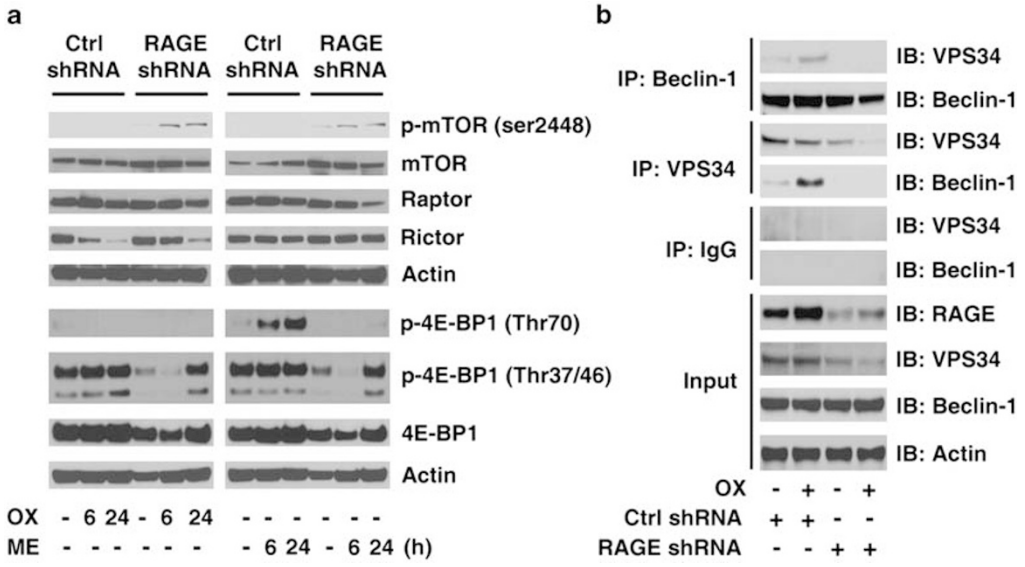


Figure 4 Decreased autophagy in RAGE-knockdown tumor cells is associated with increased levels of phosphorylated mTOR and decreased autophagosome formation. (**a**) RAGE knockdown results in increased phosphorylation of mTOR and decreased phosphorylation of 4E-BP1. The indicated Panc02 cells were treated with anticancer drugs (oxaliplatin, 'OX', 160 μg/ml; melphalan, 'ME', 320 μg/ml) for the given times. Western blot analysis of protein levels is shown. (**b**) RAGE knockdown blocks Beclin-1/VPS34 complex formation during autophagy. RAGE-knockdown Panc02 cells were treated with oxaliplatin ('OX', 160 μg/ml) for 3 h. Cell lysates were prepared for IP with anti-Beclin-1 or anti-VPS34 or IgG. The resulting immune complexes were analyzed by western blotting using antibodies to Beclin-1 or VPS34

on Beclin-1/VPS34 complex formation and activity in Panc02 cells. Panc02 RAGE-knockdown cells expressed similar levels of Beclin-1, but less VPS34 when compared with control shRNA-treated cells (Figure 4b). It is noted that less Beclin-1 coimmunoprecipitated with VPS34 in RAGE-knockdown Panc02 cells when compared with control shRNA-treated Panc02 cells (Figure 4b). This suggests that in addition to inhibiting autophagy through phosphorylation of mTOR, knockdown of RAGE also limits autophagy by limiting the formation of the Beclin-1/VPS34 complex.

Increased sensitivity to chemotherapy, increased apoptosis and decreased autophagy in RAGE-knockdown tumor cells is dependent on ATG5. ATG5 is a critical link between autophagy and apoptosis.[31] As we have shown that RAGE-knockdown cells exhibit a shift from autophagy to apoptosis following treatment with chemotherapy, we examined the role of ATG5 in this pathway. We transfected pancreatic tumor cell lines with shRNA specific for RAGE, ATG5 or Beclin-1 (Figure 5a). Consistent with a role for ATG5 in both apoptotic and

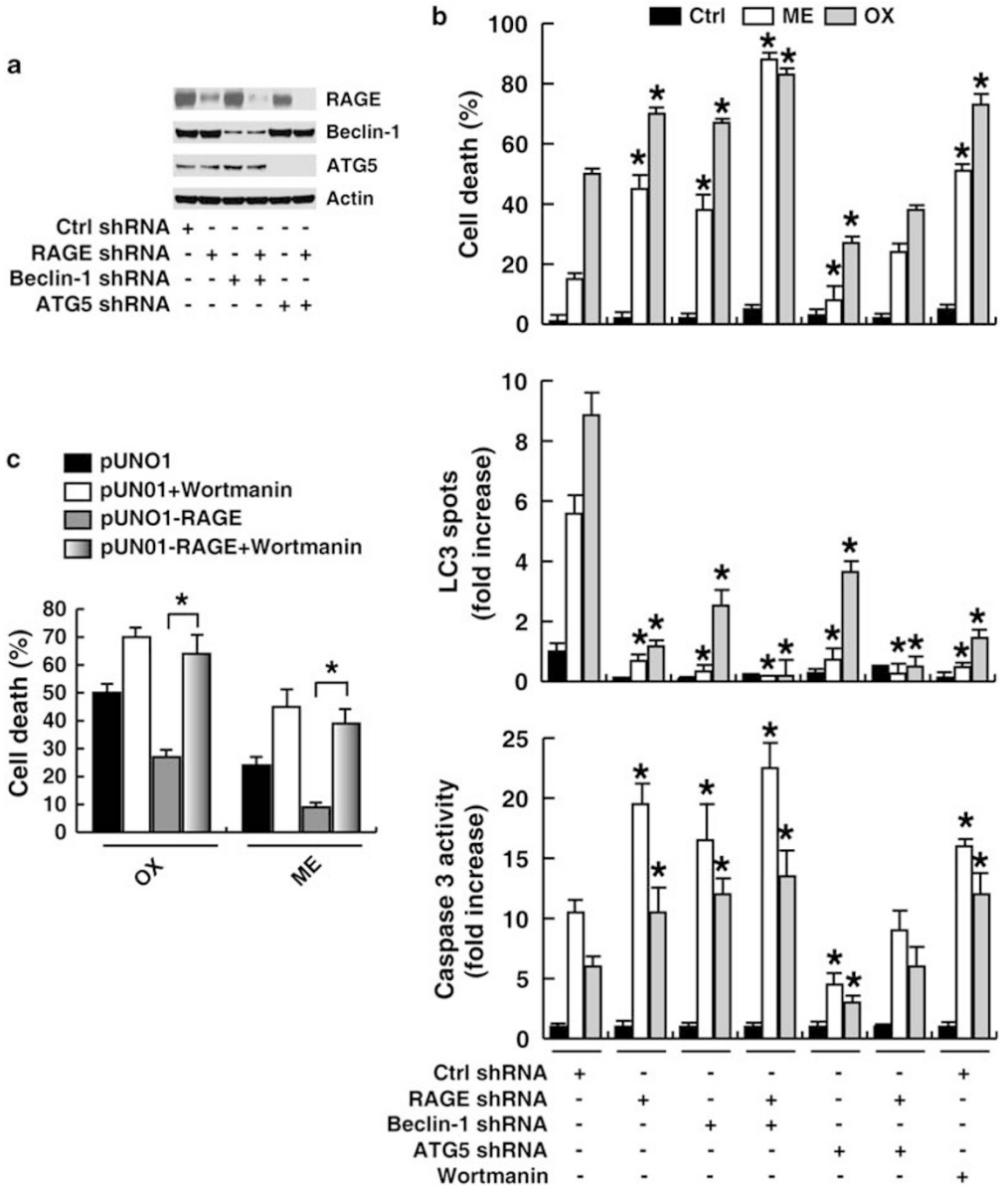


Figure 5 Increased sensitivity to chemotherapeutic agents, increased apoptosis and decreased autophagy in RAGE-knockdown tumor cells is dependent on ATG5. (**a**) Western blot analysis of protein levels in Panc02 cells as indicated after knockdown of RAGE, Beclin-1 and ATG5 by shRNA. (**b**) Depletion of ATG5, but not Beclin-1, reverses the increased chemosensitivity in RAGE-knockdown cells. The indicated Panc02 cells were treated with chemotherapeutic drugs (oxaliplatin, 'OX', 160 μg/ml; melphalan, 'ME', 320 μg/ml) with or without wortmannin (100 nM) and then assayed for cell death, caspase-3 activity and LC3 punctae formation ($n=3$, $^*P<0.05$ *versus* ctrl shRNA group). (**c**) An autophagy inhibitor increases cell death following chemotherapy in cells overexpressing RAGE. The indicated Panc02 tumor cells were pretreated with wortmannin (100 nM) for 1 h and then treated with chemotherapeutic agents (oxaliplatin, 'OX', 160 μg/ml; melphalan, 'ME', 320 μg/ml). Cell viability was examined at 24 h ($n=3$, $^*P<0.05$)

autophagic pathways, we observed that its knockdown decreased both LC3 punctae formation and caspase-3 activity in pancreatic tumor cells following treatment with chemotherapeutic agents. Interestingly, depletion of ATG5 but not Beclin-1 reversed the increased chemosensitivity of RAGE-knockdown tumor cell lines (Figure 5b). Furthermore, depletion of ATG5 reversed the proapoptotic effects of RAGE knockdown as shown by decreased caspase-3 activity. As expected, knockdown of Beclin-1 and RAGE resulted in marked decreases in apparent autophagy. Wortmanin, an autophagic inhibitor, increased or restored apoptosis and cell death following treatment with chemotherapy in both wild-type cells and cells overexpressing RAGE (Figures 5b and c). Together, these data support the notion that RAGE, through ATG5, has an important role in the balance between autophagy and apoptosis and ultimately tumor cell survival following treatment with chemotherapy.

Targeted knockdown of RAGE increases sensitivity to chemotherapy *in vivo* and is associated with increased apoptosis and decreased autophagy. To test whether targeted knockdown of RAGE also increased sensitivity to chemotherapy *in vivo*, we inoculated C57/BL6 mice with 10^6 Panc02 tumor cells with stable transfection of control or RAGE-specific shRNA and treated with oxaliplatin. *In vivo*, growth of the RAGE-knockdown tumor cells was significantly slower than the controls at all tumor cell injection concentrations (range 10^5–10^6; data not shown). Growth of RAGE-knockdown tumor cells was significantly inhibited and in some cases completely cleared at a dose of oxaliplatin (7 mg/kg) that was clinically ineffective on control shRNA-transfected tumors (Figure 6a). We observed that tumor cells transfected with RAGE-specific shRNA showed decreased autophagy and increased apoptosis *in vivo* when treated with oxaliplatin as compared with control shRNA-transfected tumor cells (Figures 6b and c). Together, these results show that RAGE is critical in modulating apoptosis and autophagy in stressed cancer cells *in vivo.*

Discussion

The receptor for advanced glycation end products is an induced receptor on both inflammatory and endothelial cells. We and others have now shown that this receptor can also be constitutively expressed on many murine and human epithelial tumor cell lines.[4,32–34] Interestingly, we observed the highest levels of RAGE expression in murine and human pancreatic adenocarcinoma tumors, a tumor type notoriously resistant to conventional therapies. Genotoxic and/or metabolic stress led to modest but reproducible increases in overall expression of RAGE on murine pancreatic and colorectal adenocarcinoma cell lines. We observed minimal RAGE expression in murine lung and renal tumor cell lines. This is consistent with recent reports showing that human lung carcinomas, unlike most other cancer types, often downregulate RAGE expression (both at the mRNA and protein level).[27]

Remarkably, we observed that RAGE expression correlated directly with the ability of both murine and human pancreatic tumor cell lines to survive cytotoxic insult. Targeted knockdown of RAGE significantly increased cell death, whereas forced overexpression promoted survival. The enhanced sensitivity to cell death in the setting of RAGE knockdown was associated with increased apoptosis and could be reversed in part by treatment with pan-caspase inhibitors. In cells treated with a pharmacological inhibitor of p53, PFT-α, and p53-knockout tumor cell lines, we observed abrogation of the increased cell death observed with RAGE knockdown. p53 induces apoptosis through a mechanism that

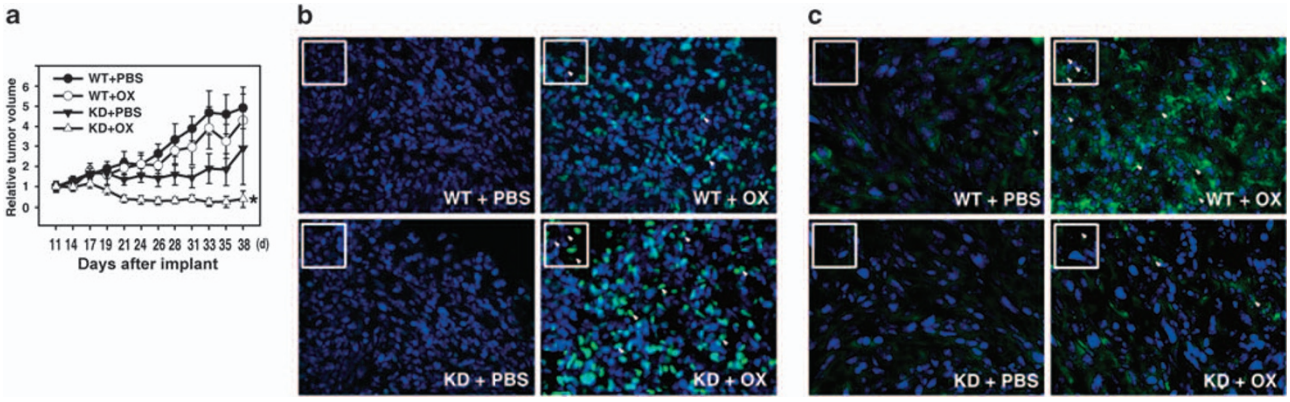


Figure 6 Expression of RAGE mediates chemoresistance *in vivo* and is associated with decreased apoptosis and increased autophagy. (**a**) RAGE-knockdown tumor cells are more sensitive to oxaliplatin *in vivo*. C57/BLl6 mice were inoculated with 10^6 Panc02 tumor cells following stable transfection of control (WT) or RAGE-specific shRNA (KD) and treated with oxaliplatin ('OX', 7 mg/kg) or PBS beginning at day 11 (day 11 normalized to 1). Tumors were measured twice weekly, and volumes were calculated for 38 days (*$P<0.05$ KD + OX *versus* WT + OX). (**b**) RAGE knockdown increases apoptosis following treatment with oxaliplatin (7 mg/kg) *in vivo*. Included here are representative images of implanted WT (control shRNA) and KD (RAGE shRNA) pancreatic tumors sections that were analyzed by TUNEL assay (green signal). Nuclear staining was performed with Hoechst 33342 (blue signal). Inset shows a higher magnification of TUNEL stain. (**c**) RAGE knockdown decreases autophagy after treatment with oxaliplatin (7 mg/kg) *in vivo*. Included here are representative images of implanted WT (control shRNA) and KD (RAGE shRNA) pancreatic tumors sections that were analyzed by indirect IF staining of LC3 protein (green signal). Nuclear staining was performed with Hoechst 33342 (blue signal). Inset shows a higher magnification of cells following staining with LC3. The color reproduction of the figure is available on the html full text version of the paper

does not depend solely on its nuclear role, but rather involves translocation of p53 to the mitochondria and induction of mitochondrial outer membrane permeability.[35] Stressed tumor cells with RAGE knockdown showed increased phosphorylation of p53. This was associated with a decrease in cytosolic and increase in mitochondrial translocation, rather than transactivation. This suggests that when present, RAGE protects tumor cells from cytotoxic insult, in part, by limiting progression through a mitochondrial, p53-mediated pathway of programmed cell death.

Our observations that the proapoptoic effects of RAGE knockdown were p53 dependent led us to examine the role of autophagy in the setting of RAGE knockdown or overexpression. It has been reported that p53 regulates autophagy in a dual manner. The pool of cytoplasmic p53 protein represses autophagy in a transcription-independent manner,[36,37] whereas the pool of nuclear p53 stimulates autophagy through the transactivation of specific genes.[38] Although autophagy was initially described as a non-apoptotic pathway of programmed cell death, it now appears that in most instances it serves as a survival strategy by which stressed cells limit apoptosis.[39,40] Autophagy is increased in pancreatic cancer cells in resected tumors and correlates with poor patient outcome.[41] Both murine and human pancreatic tumor cell lines showed clear evidence of enhanced autophagy following treatment with chemotherapeutic agents. Knockdown of RAGE limited induction of this autophagic response. Similarly, overexpression of RAGE led to greater induction of autophagy and improved tumor cell survival. Autophagy in mammalian cells is predominately under the control of the serine kinase, mTOR, which suppresses autophagy and enhances transcriptional activities in response to nutrient availability.[29,39,40] Phosphorylation of mTOR released the suppression of autophagy allowing for formation of a multiprotein complex that includes PI3K, Beclin-1 and vacuolar protein-sorting factor protein 15 (VPS15).[42] We showed that knockdown of RAGE enhanced mTOR phosphorylation in response to chemotherapy, thus preventing induction of a survival response. RAGE knockdown also prevented stabilization of the Beclin-1/VPS34 complex, further limiting the autophagic response. The induction of autophagy in RAGE-knockdown tumor cell lines was also partly dependent on the activity of ATG5. ATG5 has been shown to be an important molecule in both apoptosis and autophagy.[43] Out data suggest that under stress, the cell balances apoptotic and autophagic responses through ATG5. Autophagy has become a dominant theme in cancer research and target for emergent therapies as it enables survival within the tumor microenvironment under stress.[20] An understanding of how various inflammatory signals promote these survival pathways is increasingly being explored. Our findings suggest that RAGE is a potential mediator of enhanced autophagy in the tumor microenvironment.

The endogenous ligand for RAGE in the setting of cancer is not known, although it is likely that there are multiple ligands for this promiscuous receptor. The RAGE ligand S100p has been shown to promote pancreatic and colorectal tumor cell survival, invasiveness and resistance to chemotherapy through RAGE interactions.[32] We have previously shown that one of the RAGE ligands, HMGB1, is released following several methods of inducing tumor cell death including chemotherapy.[17,18] We have also observed that exogenous provision of rHMGB1 can promote autophagy in pancreatic tumor cell lines through a RAGE-dependent pathway (Tang DL *et al*., submitted). Here, we now report that reduction of the intracellular pool of HMGB1 through targeted knockdown, enhanced tumor cell death by increasing programmed cell death (apoptosis) and diminishing program cell survival (autophagy). Together, these observations suggest a new paradigm in tumor biology, whereby stressed or dying tumor cells release pro-inflammatory factors that in aggregate are termed DAMPs. When released, DAMPs interact with receptors (DAMP-R) such as RAGE on surviving, stressed cells in the tumor microenvironment. This favors tumor survival in part by increasing programmed cell survival (autophagy) and decreasing programmed cell death. On the basis of our findings and an emerging literature regarding the effects of DAMPs in the tumor microenvironment, we hypothesize that cancer is fundamentally a disorder of cell death.[44] The findings presented here are consistent with this hypothesis.

In conclusion, we have shown a novel role for RAGE in enhancing tumor cell survival through increased autophagy and diminished programmed cell death in pancreatic tumor cells both *in vitro* and *in vivo*. These data support the development of novel clinical therapies targeting the RAGE pathway.

Materials and Methods

Reagents. The antibodies to cleaved –caspase-3, cleaved PARP, Bax, PUMA, Bcl-XL, Bcl-2, p-p53 (Ser392), p-p53 (Ser6), p-p53 (Ser15), Ace-p53 (Lys379), p53, p-4E-BP1 (Thr70), p-4E-BP1 (Thr37/46), 4E-BP1, p-mTOR (Ser2448), mTOR, Raptor, Rictor, VPS34, p-ERK (Thr202/Tyr204) and ERK were obtained from Cell Signaling Technology (Danvers, MA, USA). The antibodies to α-tubulin and actin were from Sigma (St. Louis, MO, USA). The antibody to TLR4 was from Abcam (Cambridge, MA, USA). The antibodies to LC3, ATG5 and p62 were from Novus (Littleton, CO, USA). Anti-RAGE antibody was from Sigma or Abcam. Anti-Beclin-1 antibody was from Cell Signaling Technology or Novus. Anti-cytochrome c antibody was from Santa Cruz Technology (Santa Cruz, CA, USA). Alexa Fluor 488 or 647-conjugated anti-rabbit or anti-mouse Ig were from Invitrogen (Carlsbad, CA, USA). ZVAD-FMK was purchased from Calbiochem (Gibbstown, NJ, USA). Gemcitabine was from Eli Lilly (Indianapolis, Indiana, USA). Oxaliplatin, melphalan and wortmannin were obtained from Sigma.

Cell culture. Cell lines were derived from the Hillman Cancer Institute. All cell lines were cultured in RPMI or DMEM medium 1640 supplemented with 10% heat-inactivated FBS, 2 mM glutamine and antibiotic-antimycotic mix in a humidified incubator with 5% CO_2 and 95% air.

Cell viability assay. Cells were plated at a density of 10^4 cells per well in 96-well plates in 100 μl RPMI. Cell viability was evaluated using the CCK8 assay kit (Dojindo Laboratories, Tokyo, Japan) according to the manufacturer's instructions. Long-term cell viability by colony formation assay was also carried out. Cells were treated for 24 h with chemotherapeutic agents (oxaliplatin, 'OX', 160 μg/ml; melphalan, 'ME', 320 μg/ml; and gemcitabine, 'GM', 100 nM) and plated at a density of 2×10^3 cells per well in 24-well culture plates. Colonies were visualized by crystal violet staining 3 weeks later.

Subcutaneous tumor models. To generate murine subcutaneous tumors, 10^6 Panc02 WT or RAGE-knockdown cells were injected subcutaneously to the right of the dorsal midline in C57/Bl6 mice. Tumors were measured twice weekly, and volumes were calculated using the formula length $\times$ width$^2 \times \pi/6$. The procedures for performing animal experiments were approved and in accordance

with the principles and guidelines of the University of Pittsburgh Institutional Animal Care and Use Committee.

Western blotting. Proteins in the whole-cell lysate were resolved on 4–12% Criterion XT Bis–Tris gels (Bio-Rad, Hercules, CA, USA) and transferred to a nitrocellulose membrane as previously described.[45,46] After blocking, the membrane was incubated for 2 h at 25 °C or overnight at 4 °C with various primary antibodies. After incubation with peroxidase-conjugated secondary antibodies for 1 h at 25 °C, the signals were visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA) according to the manufacturer's instruction. The relative band intensity was quantified using the Gel-pro Analyzer® software (Media Cybernetics, Bethesda, MD, USA).

Gene transfection and shRNA. The RAGE-shRNA, Beclin-1-shRNA, ATG5-shRNA, HMGB1-shRNA (Sigma) or pUNO1-RAGE (Invivogen, San Diego, CA, USA) were transfected into cells using Lipofectamine 2000 reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. At the end of the shRNA treatment (48 h), the medium was changed before the addition of the chemotherapy agent.

Immunofluorescence analysis. Cells were cultured on glass coverslips and fixed in 3% formaldehyde for 30 min at room temperature before detergent extraction with 0.1% Triton X-100 for 10 min at 25 °C. Coverslips were saturated with 2% BSA in PBS for 1 h at room temperature and processed for immunofluorescence with primary antibodies followed by Alexa Fluor 488 or 647-conjugated anti-rabbit or anti-mouse Ig, respectively. Nuclear morphology was analyzed with the fluorescent dye Hoechst 33342 (Sigma). Between all incubation steps, cells were washed three times for 3 min with 0.5% BSA in PBS. Images were taken with a fluorescence microscope.

For tissue immunofluorescence analysis, tissues were embedded in optimum cutting temperature cryomedium (Sakura, Zoeterwoude, The Netherlands) and subsequently, cut into 8-μm sections as described previously.[11] Tissue sections were stained with LC3, RAGE or HMGB1 antibody followed by Alexa Fluor 488-conjugated anti-rabbit Ig. Nuclear morphology was analyzed with the fluorescent dye Hoechst 33342 (Sigma). Actin was imaged with phalloidin binding (Sigma). TUNEL assay was carried out using the *In Situ* Cell Death Detection Kit, according to the manufacturer's recommendations (Roche, Stockholm, Sweden).

Immunoprecipitation analysis. Cells were lysed at 4 °C in ice-cold RIPA lysis buffer (Millipore, Bedford, MA, USA), and cell lysates were cleared by centrifugation (12000 $\times$ *g*, 10 min). The concentrations of proteins in the supernatant were determined by BCA assay. Before immunoprecipitation, samples containing equal amount of proteins were pre-cleared with protein A or protein G agarose/sepharose (Millipore) (4 °C, 3 h), and subsequently incubated with various irrelevant IgG or specific antibodies (5 μg/ml) in the presence of protein A or G agarose/sepharose beads for 2 h or overnight at 4 °C with gentle shaking.[47] After incubation, agarose/sepharose beads were washed extensively with PBS, and proteins were eluted by boiling in 2 $\times$ SDS sample buffer before SDS-PAGE.

Caspase-3 activity assay. Caspase-3 activity was assayed using the Caspase-3 Colorimetric Assay Kit (Calbiochem) according to manufacturer's instructions.

p53 reporter assay. p53 reporter activity was assayed using the p53 reporter (luc) kit (SABiosciences Corporation, Frederick, USA) according to the manufacturer's instructions.

Analysis of autophagy by imaging cytometry. Cells were seeded in 96-well plates and cultured in the presence of stimulus for given time, then fixed with 3% paraformaldehyde and stained with LC3 antibody. Secondary antibodies were Alexa Fluor 488-conjugated anti-rabbit Ig. Nuclear morphology was analyzed with the fluorescent dye Hoechst 33342 (Sigma). Images data were collected with an ArrayScan HCS 4.0 imaging cytometer with a $\times$ 20 objective (Cellomics, Pittsburgh, PA, USA). Arrayscan is an automated fluorescent-imaging microscope that collects information about the spatial distribution of fluorescently labeled components in cells placed in 96-well microtiter plates. The Spot Detector BioApplication (Cellomics, Pittsburgh, PA, USA) was used to acquire and analyze the images after optimization. Images of 1000 cells for each treatment group were analyzed to obtain LC3 fluorescence spot number per cell as previously described.[45,48]

Statistical analysis. Data are expressed as means $\pm$ S.E.M. of three independent experiments carried out in triplicate. One-way ANOVA was used for comparison among the different groups. When the ANOVA was significant, *post hoc* testing of differences between groups was performed using LSD test. A *P*-value <0.05 was considered significant.

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