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High Mobility Group Box 1 (HMGB1) Activates an Autophagic Response to Oxidative Stress

Daolin Tang,* Rui Kang,* Kristen M. Livesey, Herbert J. Zeh III, and Michael T. Lotze

Abstract

Aims: Autophagy, the process by which cells break down spent biochemical and damaged components, plays an important role in cell survival following stress. High mobility group box 1 (HMGB1) regulates autophagy in response to oxidative stress. ***Results:*** Exogenous hydrogen peroxide (H_2O_2) treatment or knockdown of the major superoxide scavenger enzyme, superoxide dismutase 1 (SOD1), by small interfering RNA (siRNA) increases autophagy in mouse and human cell lines. Addition of either SOD1 siRNA or H_2O_2 promotes cytosolic HMGB1 expression and extracellular release. Importantly, inhibition of HMGB1 release or loss of HMGB1 decreases the number of autophagolysosomes and autophagic flux under oxidative stress *in vivo* and *in vitro*. ***Innovation:*** HMGB1 release may be a common mediator of response to oxidative stress. ***Conclusion:*** HMGB1 is important for oxidative stress-mediated autophagy and serves as a new target for the treatment of stress-associated disorders. *Antioxid. Redox Signal.* 15, 2185–2195.

Introduction

Oxidative stress occurs when the generation of reactive oxygen species (ROS) in a system exceeds its ability to neutralize and eliminate them (19, 28). There are many pathological conditions associated with oxidative stress, including ischemia-reperfusion injury, atherosclerosis, cancer, obesity, and aging (19, 28). Dependent upon the concentration, location, and intracellular conditions, ROS such as superoxide anion, hydroxyl radicals, and hydrogen peroxide (H_2O_2) can cause toxicity or act as signaling molecules in various pathways that regulate both cell survival and cell death. The cellular levels of ROS are controlled by antioxidant enzymes, including the thioredoxin–thioredoxin reductase and glutaredoxin–glutathione systems. Intracellular nonprotein thiols, such as the small cysteine-containing tripeptide glutathione and the amino acid cysteine, which assist thiol-regulating enzymes, are mostly reduced to the disulfide form intracellularly and outside of cells, serving as small-molecule antioxidants. As the major antioxidant enzymes, superoxide dismutases (SOD) play a crucial role in scavenging ROS (28). ROS can induce autophagy (1) through several distinct mechanisms involving autophagy-related gene 4 (ATG4) (36), catalase (50), and the mitochondrial electron transport chain (7). However, the precise mechanisms mediating this form of autophagy remain unclear.

Autophagy is a tightly regulated sequestration process whereby a cell either degrades its own components through the lysosomal machinery (21) or expels it through exocytosis. By maintaining a balance between the synthesis, degradation, and recycling of cellular components, autophagy plays a critical role in normal cell growth, development, and homeostasis (22). In most situations, autophagy promotes survival in response to stress such as starvation and exposure to anticancer drugs (25). Defective autophagy is implicated in tumorigenesis, as the essential autophagy regulator beclin 1 is monoallelically deleted in human breast, ovarian, and prostate cancers, and Beclin $1^{+/-}$ mice are tumor-prone.

High mobility group box 1 (HMGB1) was first purified from nuclei approximately 40 years ago and termed one of the HMG proteins because of its rapid mobility on electrophoresis gels

Innovation

The precise mechanisms mediating autophagy in oxidative stress remain unclear. We demonstrated that HMGB1 is an autophagy sensor in the setting of oxidative stress. These findings provide insight into how HMGB1, a damage-associated molecular pattern (DAMP) molecule, triggers autophagy as a defense mechanism under conditions of cellular stress.

Departments of Surgery, Hillman Cancer Center, University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania.
*These authors contributed equally to this article.

(9). HMGB1 is a highly conserved nuclear protein, acting as a chromatin-binding factor that promotes access of the transcriptional machinery to specific DNA targets by binding/bending DNA (30). In addition to its nuclear role, HMGB1 also functions as an extracellular signaling molecule during inflammation, cell differentiation, cell migration, and tumor metastasis (26, 30). HMGB1 is passively released by necrotic cells and is also actively secreted by immunostimulated macrophages, dendritic cells, and enterocytes (26). Oxidative stress such as H_2O_2 induces HMGB1 secretion and release in macrophages and monocytes (43). Moreover, antioxidants such as ethyl pyruvate (47), quercetin (40), and green tea (23) are protective in the setting of experimental inflammation, partially through attenuating systemic HMGB1 accumulation. HMGB1 plays important roles in oxidative stress-associated diseases (42). Recent work has shown that autophagy regulates selective HMGB1 release in tumor cells that are destined to die (45). In contrast, exogenous HMGB1-induced autophagy promotes chemotherapeutic resistance in tumor cells (24, 38). The role of HMGB1, however, in regulating autophagy induced by oxidative stress remains unclear.

Here, we demonstrate that HMGB1 mediates oxidative stress-induced autophagy. Addition of exogenous H_2O_2 or suppression of SOD1 expression by RNA interference (RNAi) induced autophagy, which was associated with HMGB1 translocation and release in mouse embryonic fibroblasts (MEFs). In contrast, administration of the antioxidant, N-acetyl cysteine (NAC) or knockout of HMGB1 inhibited oxidative stress-induced autophagy in MEFs. Furthermore, our experimental data suggest a role for HMGB1 in the regulation of autophagic flux partially through regulating microtubule-associated protein light chain 3 (LC3) turnover, degradation of the SQSTM1/sequestosome (p62), and maturation of autophagosomes. Moreover, targeted knockdown of HMGB1 increases sensitivity to chemotherapy *in vivo* and is associated with increased apoptosis and decreased autophagy. Overall, these data support a novel role for HMGB1 as an activator of the autophagic response to oxidative stress.

Results

Knockdown of SOD1 by siRNA induces autophagy

Superoxide dismutase 1 (SOD1) is an abundant copper/zinc enzyme found in the cytoplasm that converts superoxide into hydrogen peroxide and molecular oxygen. To explore the potential role for SOD1 in the regulation of autophagy, a target-specific siRNA against SOD1 was transfected into mouse embryonic fibroblasts (MEFs). Transfection of SOD1-siRNA led to a significant decrease in SOD1 protein at 24–48 h (Fig. 1A).

We then investigated whether knockdown of SOD1 by siRNA had an effect on autophagy. Throughout the process of autophagy, a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form the LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to the autophagosomal membrane (12). Detection of LC3 has become a widely used method for monitoring autophagy and autophagy-related processes (29). Attenuation of SOD1 expression in MEFs increased LC3 expression and accumulation of LC3-II by Western blot assay (Fig. 1A). Furthermore, there was an increase in endogenous LC3 punctae formation following knockdown of SOD1 at 48 h by immunofluorescence analysis (Fig. 1B).

An alternative method for detecting autophagic flux is measuring enhanced degradation of p62, a long-lived scaffolding protein involved in the transport of ubiquitinylated proteins destined for proteosomal digestion (4, 31). p62 has LC3 binding domains, which target this protein for incorporation into the autophagosome, therefore serving as a selective substrate of autophagy (31). Consistent with increased autophagic flux, the levels of p62 are downregulated in SOD1 knockdown MEFs by immunoblotting (Fig. 1A) and immunofluorescence analysis (Fig. 1C). Together, these findings demonstrate that the level of SOD1 is strongly correlated with levels of autophagy.

The antioxidant NAC inhibits oxidative stress-induced autophagy

To determine whether increase in ROS generation is responsible for the increased autophagy observed in SOD1 knockdown cells, we evaluated the effects of the antioxidant N-acetyl cysteine (NAC) on autophagy in MEFs. We found that treatment with NAC significantly blunted the increased LC3-II expression (Fig. 2A) and LC3 punctae formation (Fig. 2B) observed in SOD1 knockdown MEFs.

Hydrogen peroxide (H_2O_2) is an ROS produced by a wide variety of enzymes, including SOD1 and several NADPH oxidases. Therefore, we investigated the effects of H_2O_2 on autophagy in the presence or absence of NAC. Consistent with previous studies (8), exogenous H_2O_2 led to an increase in LC3-II expression (Fig. 2C) and LC3 punctae formation (Fig. 2D) in MEFs. In contrast, NAC pretreatment inhibited H_2O_2-induced LC3-II expression and LC3 punctae formation, suggesting a potential role for ROS in the regulation of autophagy.

Treatment with H_2O_2 or knockdown of SOD1 increases cytoplasmic translocation and release of HMGB1

HMGB1 is an abundant nuclear protein with proinflammatory activity dependent upon its extranuclear function (3, 26). To investigate the distribution of HMGB1 under conditions of oxidative stress, cells were stained with specific anti-HMGB1 antibodies. Untreated MEFs displayed primarily nuclear localization of HMGB1 (Fig. 3A). However, in cells transfected with SOD1 siRNA or those treated with exogenous H_2O_2, there was an increase in the percentage of HMGB1 localized to the cytoplasm (Fig. 3A). Moreover, Western blot analysis demonstrated that mitochondrial HMGB1 expression is increased following H_2O_2 treatment (Fig. 3B). Furthermore, the levels of HMGB1 released into the culture medium were subsequently measured by Western blot analysis. HMGB1 was not observed in the culture medium in the absence of treatment, but HMGB1 release was detected following SOD1 siRNA transfection or exogenous H_2O_2 treatment unaccompanied by measurable lactate dehydrogenase (LDH) release (Fig. 3C). Consistent with the notion that ROS regulate these changes in HMGB1 localization, NAC inhibited oxidative stress-induced HMGB1 release (Fig. 3C). These data suggest that HMGB1 is involved in the response to oxidative stress.

Furthermore, pretreatment of the MEF and HCT116 cell lines with inhibitors of PI3K which inhibit autophagy [*e.g.*, 3-methyladenine (3-MA)] blocked SOD1 siRNA or exogenous H_2O_2 treatment induced HMGB1 release (Fig. 3D). Moreover, knockout of Atg5, a gene product required for the formation of autophagosomes, significantly inhibited H_2O_2-induced

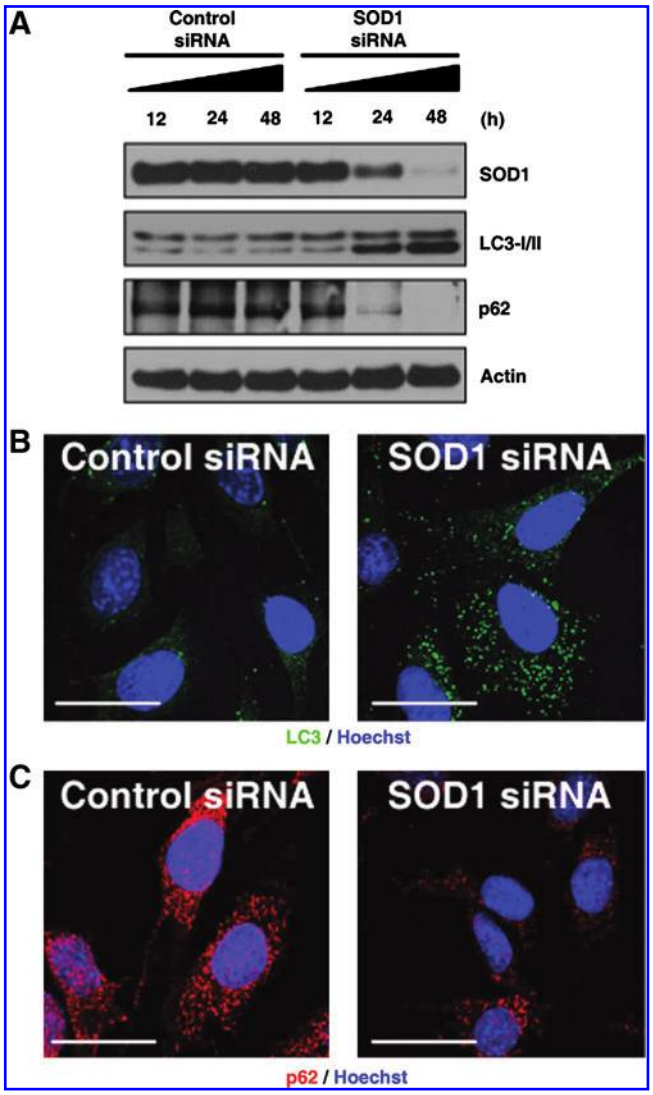

FIG. 1. Knockdown of SOD1 by siRNA induces autophagy. (A) Time-dependent effects of SOD1 downregulation. MEFs were transfected with SOD siRNA or control siRNA. After transfection for the indicated time, total protein extracts were used for Western blot analysis. Actin was used as a loading control. Immunoblot shown is representative of three experiments with similar results. **(B, C)** Confocal microscopic analysis of LC3 (*green*) and p62 (*red*) using specific antibodies after transfection with SOD1 siRNA and control siRNA for 48 h in MEFs. Images are representative of 10 random fields. *Bar* = 30 μm. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

HMGB1 translocation and release (Fig. 3E). This suggests that autophagic stimuli regulate HMGB1 cytoplasmic translocation and release in response to oxidative stress.

DNA damage from genetic or metabolism stress induces HMGB1 release (2, 24, 38). Activation of the nuclear enzyme poly (ADP-ribose) polymerase (PARP) by oxidant-mediated DNA damage is an important pathway of cell dysfunction and tissue injury under conditions associated with oxidative stress. To explore whether DNA damage from oxidative stress induces the release of HMGB1, we treated cells with the PARP inhibitor 1,5-isoquinolinediol (DHIQ). DHIQ inhibited H_2O_2 (0.25 m*M*) -induced HMGB1 release (Fig. 3F), suggesting that HMGB1 release during oxidant-mediated DNA damage is PARP dependent.

To explore the role of HMGB1 release in oxidative stress-mediated autophagy, MEFs and HCT116 cells were treated with a specific neutralizing antibody against HMGB1. HMGB1-neutralizing antibodies significantly inhibited SOD1 siRNA and H_2O_2-induced LC3 punctae formation compared with the IgG control (Fig. 3G). Moreover, knockout of HMGB1 in MEFs increased H_2O_2-induced oxidative injury (Fig. 3H). These findings suggest that there is a positive feedback loop between HMGB1 release and autophagy during oxidative stress.

HMGB1 mediates oxidative stress induced autophagy in vitro

To determine whether HMGB1 affects the levels of autophagy in response to oxidative stress, we assayed autophagic flux and autophagosome number in HMGB1 wildtype ("+/+") and HMGB1 knockout ("−/−") MEFs. We found that the loss of HMGB1 inhibited SOD1 knockdown and exogenous H_2O_2-induced LC3-II expression and LC3 punctae formation, suggesting a potential role for HMGB1 in the regulation of oxidative stress-induced autophagy (Figs. 4A and 4B). Moreover, loss of HMGB1 increased p62 protein levels under conditions of oxidative stress (Fig. 4A), indicating that its degradation is dependent on HMGB1-mediated autophagy. Furthermore, knockdown of HMGB1 in HCT116 colon cancer cells and Panc02 pancreatic cancer cells inhibited SOD1 knockdown and exogenous H_2O_2-induced LC3 punctae formation, suggesting a widespread role for HMGB1 in the regulation of oxidative stress-induced autophagy (Fig. 4C).

To further confirm that HMGB1 regulates autophagic flux in response to oxidative stress, we analyzed the co-localization of lysosomal-associated membrane protein 2 (LAMP2) and LC3 in the presence or absence of bafilomycin A1, an inhibitor of autophagic vacuole and lysosome fusion (44). Similar to HMGB1 wild-type cells treated with bafilomycin A1 under conditions of oxidative stress, loss of HMGB1 expression decreased LAMP2/LC3 co-localization in MEFs (Fig. 4D). Moreover, treatment with bafilomycin A1 induced further increases in LC3-II expression in $Hmgb1^{+/+}$ MEFs compared with $Hmgb1^{-/-}$ MEFs (Fig. 4D). Collectively, these findings demonstrate that HMGB1 is necessary for oxidative stress induced autophagy.

HMGB1 contains three cysteines, Cys23, 45, and 106. Under mild oxidative conditions, the Cys23 and Cys45 readily form an intramolecular disulfide bridge, whereas Cys106 remains in the reduced form. The Cys106-to-Ser mutation (C106S) impairs the nuclear localization of HMGB1 and increases levels of cytoplasmic HMGB1 (10). Our recent study demonstrated that mutation of cysteine 106, but not the vicinal cysteines 23 and 45, of HMGB1 promotes cytosolic localization and sustained starvation-induced autophagy (39). Moreover, the intramolecular disulfide bridge (C23/45) of HMGB1 is required for binding to Beclin1 and sustaining autophagy during starvation (39). Similarly, transfection of wild-type HMGB1 and the C106S variant, but not the mutant C23S or C45S cDNA, restored autophagy in $Hmgb1^{-/-}$ cells following H_2O_2 treatment (Fig. 4F). Thus, the cysteines within HMGB1 are necessary for the regulation of autophagy.

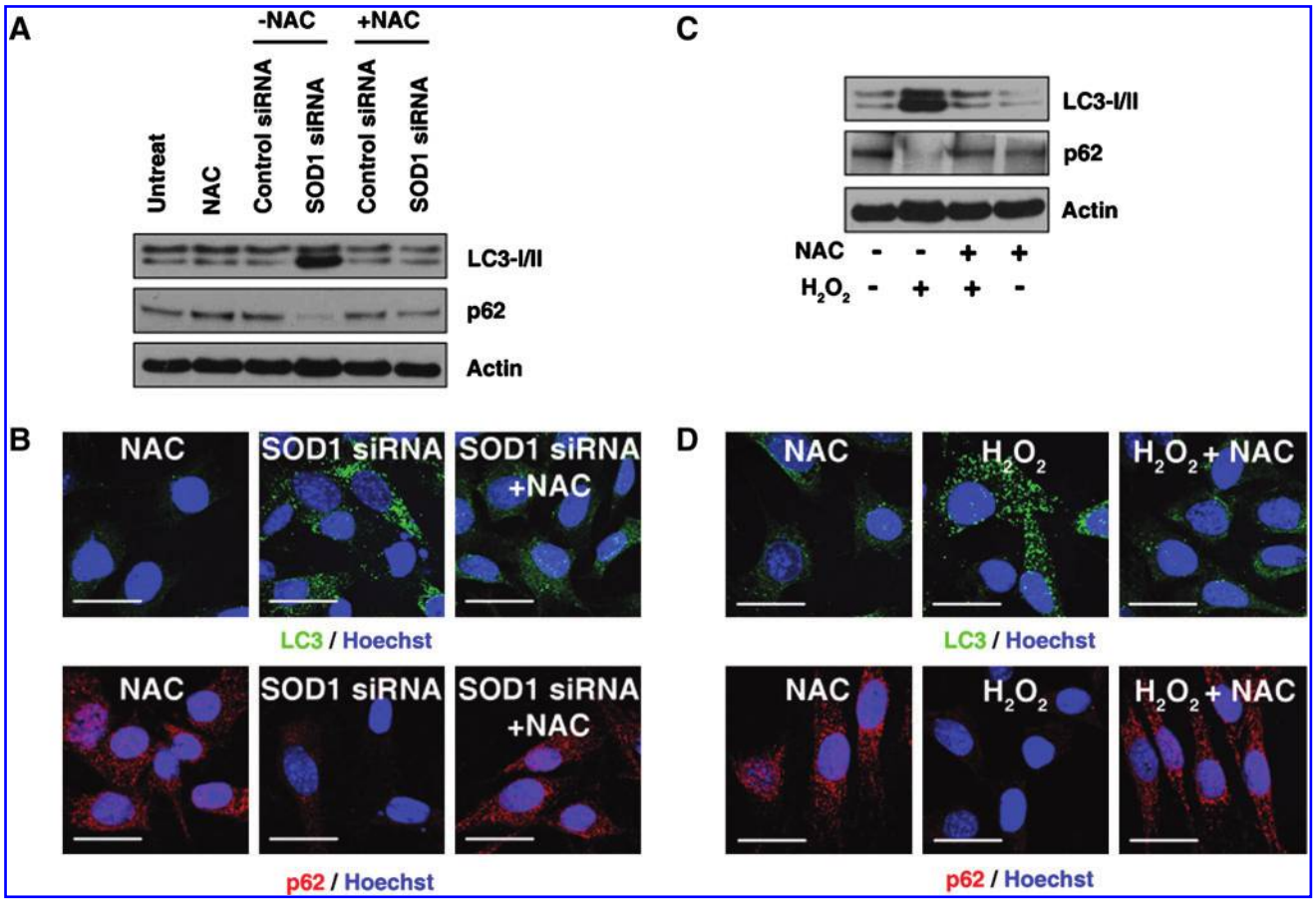

FIG. 2. The antioxidant NAC inhibits oxidative stress-induced autophagy. (A, B) NAC inhibits SOD1 siRNA-induced autophagy. MEFs were transfected with SOD siRNA or control siRNA for 48 h, and then treated with NAC (50 m*M*) for 12 h. The total protein extracts were used for Western blot analysis. Data are representative of three experiments with similar results **(A)**. In parallel, LC3 punctae formation (*green*) and p62 (*red*) were assayed by confocal microscopic analysis. Images are representative of 10 random fields. *Bar* = 30 μm **(B). (C, D)** NAC inhibits H_2O_2-induced autophagy. MEFs were treated with H_2O_2 (0.05 m*M*) for 12 h with or without NAC (50 m*M*). The total protein extracts were used for Western blot analysis. Data are representative of three experiments with similar results **(C)**. In parallel, LC3 punctae formation (*green*) and p62 (*red*) were assayed by confocal microscopy analysis. Images are representative of 10 random fields. *Bar* = 30 μm **(D)**. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

Targeted knockdown of HMGB1 increases sensitivity to chemotherapy in vivo *and is associated with increased apoptosis and decreased autophagy in the setting of oxidative stress*

It is well established that cancer interventions with some chemotherapeutic agents and radiation therapies generate ROS. The receptor for advanced glycation end products (RAGE) is one of the major receptors for HMGB1. Our previous studies demonstrated that RAGE sustains autophagy and limits apoptosis, promoting pancreatic tumor cell survival *in vivo* and *in vitro* (17). Moreover, RAGE is an important regulator of autophagy during oxidative stress (14, 16). To test if targeted knock down of HMGB1 also increased sensitivity to chemotherapy *in vivo,* we inoculated C57/BL6 mice subcutaneously with 10^6 Panc02 tumor cells with transfection of control or HMGB1 specific shRNA and treated with gemcitabine. *In vivo,* growth of the HMGB1 knockdown tumor cells was significantly slower than controls. Growth of HMGB1 knockdown tumor cells was significantly inhibited and in some cases completely abrogated at a dose of gemcitabine that was clinically ineffective in control shRNA-transfected tumors (Fig. 5A). Moreover, we observed that knockdown of HMGB1 decreased autophagy and increased apoptosis in tumors treated *in vivo* with gemcitabine as compared with the control group (Fig. 5B). 4-hydroxy-2-nonenal (HNE) is a highly reactive aldehyde generated by the exposure of polyunsaturated fatty acids to peroxides and ROS. It nonenzymatically forms stable protein adducts with histidine, lysine, and cysteine side chains that have been used as biomarkers for oxidative stress in tissue (46). Gemcitabine administration increased ROS production in both HMGB1 wild-type and knockdown pancreatic tumors by monitoring HNE binding (Fig. 5B). These results suggest that HMGB1 regulates downstream events (*e.g.,* apoptosis and autophagy) in chemotherapy-mediated oxidative stress *in vivo.*

Discussion

The primary function of autophagy is to recycle cellular components to sustain metabolism during nutrient deprivation and to prevent the accumulation of damaged toxic proteins and organelles (22, 25). ROS, the partially reduced or

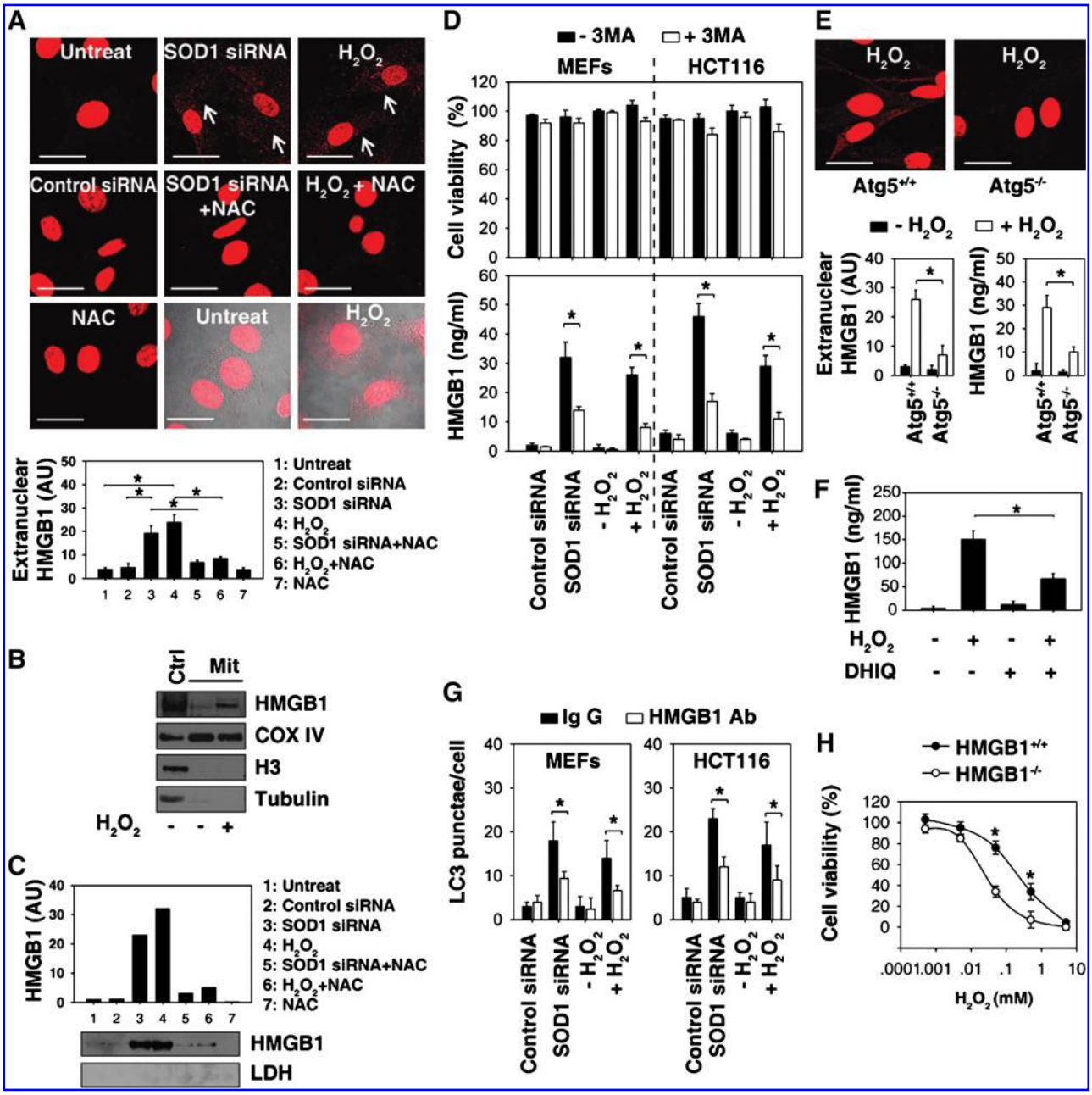

FIG. 3. Knockdown of SOD1 or H_2O_2 increases cytoplasmic translocation and release of HMGB1. (A) Confocal microscopic analysis of HMGB1 (*red*) using specific antibodies following transfection with SOD1 siRNA or control siRNA for 48 h or treatment with H_2O_2 (0.05 m*M*) for 12 h with or without NAC (50 m*M*) in MEFs. *Bar* = 30 μm. Relative quantitative analysis of extranuclear HMGB1 fluorescent intensity shown as means ± SD from 10 random fields (*$p < 0.05$. One-way ANOVA, followed by LSD). AU: Arbitrary Units. Representative images of HMGB1 overlaid on the differential interference contrast (DIC) images are shown in the *lower panel*. **(B)** Western blot analysis of HMGB1 levels in isolated mitochondria (Mit) with or without H_2O_2 (0.05 m*M*) for 12 h in MEFs. Untreated whole cell lysate was used as a positive control (Ctrl) for nonmitochondrial protein. To confirm that these were the appropriate fractions, the Western blots were probed for COX IV as a mitochondrial marker, tubulin as a cytoplasmic marker, and histone H3 as a nuclear marker. **(C)** Conditions as indicated in **(A)**, the level of HMGB1 and LDH released into the cell culture medium was assayed by Western blot analysis. Relative quantitative analysis of HMGB1 band density shown in *top panel* (AU: arbitrary units). **(D)** Analysis of HMGB1 release by ELISA in the presence or absence of 3-methyladenine ("3-MA", 10 m*M*) after SOD1 siRNA or control siRNA for 48 h or treatment with H_2O_2 (0.05 m*M*) for 12 h. In parallel, the cell viability was analyzed using a CCK-8 kit ($n = 3$, *$p < 0.05$). **(E)** Analysis of HMGB1 translocation by confocal microscopy in $Atg5^{+/+}$ and $Atg5^{-/-}$ MEFs after treatment with H_2O_2 (0.05 m*M*) for 12 h. Representative images of HMGB1 location (*red*) is shown in *left panel*. *Bar* = 30 μm. In parallel, HMGB1 release was assessed by ELISA ($n = 3$, *$p < 0.05$). **(F)** MEFs were treated with H_2O_2 (0.25 m*M*) in the presence or absence of the PARP inhibitor DHIQ (300 μM). At 12 h following treatment, the cell viability was analyzed using a CCK-8 kit ($n = 3$, *$p < 0.05$). **(G)** LC3 punctae formation was determined by confocal microscopic analysis in the presence or absence of HMGB1-neutralizing antibody (10 μg/ml) after SOD1 siRNA or control siRNA for 48 h or treatment with H_2O_2 (0.05 m*M*) for 12 h ($n = 10$ random fields, *$p < 0.05$). **(H)** Loss of RAGE expression increases H_2O_2-induced oxidative cytotoxicity. $HMGB1^{+/+}$ and $HMGB1^{-/-}$ MEFs were treated with H_2O_2 at indicated dose for 24 h and then the cell viability was analyzed ($n = 3$, *$p < 0.05$ versus $HMGB1^{-/-}$ MEFs). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

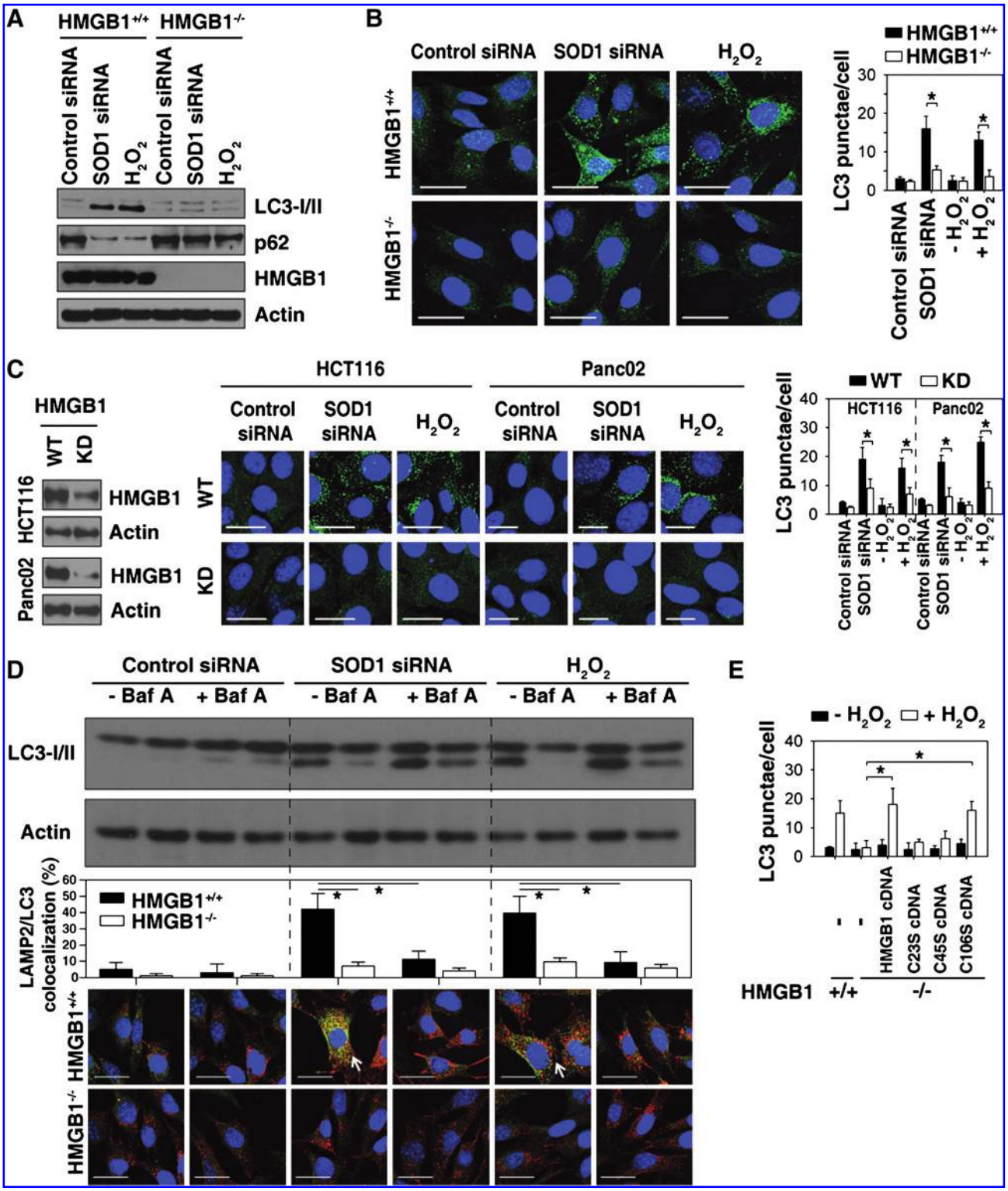

FIG. 4. Autophagy induced by oxidative stress is mediated by HMGB1. (A) Western blot analysis of indicated protein levels in HMGB1$^{+/+}$ and HMGB1$^{-/-}$ MEFs after transfection with SOD1 siRNA or control siRNA for 48 h or treatment with H_2O_2 (0.05 m*M*) for 12 h. Immunoblot shown is representative of three experiments with similar results. **(B, C)** In parallel, LC3 punctae formation was assayed by confocal microscopic analysis in MEFs or HMGB1 knockdown HCT116 and Panc02 cells (*$p < 0.05$. One-way ANOVA, followed by LSD). KD: HMGB1 knockdown, WT: HMGB1 wild type. Images are representative of 10 random fields. *Bar* = 30 μm (MEFs); *Bar* = 15 μm (HCT116 and Panc02). **(D)** Co-localization of LC3 (*green*)/LAMP2 (*red*) was assayed by confocal microscopic analysis (*$p < 0.05$. One-way ANOVA, followed by LSD) using the conditions indicated in **(A)**. Images are representative of 10 random fields. Baf A: Bafilomycin A1 (100 n*M*); *Bar* = 30 μm. In parallel, LC3 expression was assayed by Western blot analysis (*top panel*). **(E)** HMGB1$^{-/-}$ MEFs were transfected with wild-type or cysteine mutant HMGB1 cDNA as indicated and then were treated with H_2O_2 (0.05 m*M*) for 12 h. The LC3 punctae formation was assayed by confocal microscopic analysis (*$p < 0.05$. One-way ANOVA, followed by LSD, $n = 10$ random fields). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

activated derivatives of oxygen, are highly reactive and toxic and can lead to cell death by damaging proteins, lipids, carbohydrates, and DNA (19, 37). The role of ROS such as superoxide and H_2O_2 in autophagy has been demonstrated in many studies (6–8, 11, 27, 33, 50). However, the mechanism by which autophagy is induced in response to oxidative stress remains unclear. In this study, we demonstrated that HMGB1 is necessary for activation of autophagy in response to oxidative stress, acting as a Redox sensor in the cell.

HMGB1 is one of the best characterized DAMPs (3, 34). A rapidly growing body of literature supports the function of the DNA-binding protein, HMGB1, as both a regulator of intracellular transcription and as an extracellular cytokine/inflammatory mediator (3, 34, 35, 49). To perform its role as a DAMP, HMGB1 must transit from the nucleus, through the cytoplasm, to the extracellular environment (3, 26). This process can occur during cell activation as well as cell death. Consistent with this notion, we found that SOD1 siRNA or exogenous H_2O_2 promoted HMGB1 release in MEFs, suggesting that ROS are important signals inducing HMGB1 translocation and release. Our previous studies demonstrated that oxidative stress induces the release of HMGB1 potentially through MAPK- and chromosome region maintenance (CRM1)-dependent mechanisms (43). In the current study, we confirmed that PARP signaling is also involved in the release of HMGB1 during oxidative injury.

SOD are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide (28). As such, they are an important antioxidant defense in nearly all cells exposed to oxygen. In humans, three forms of superoxide dismutase are present in various compartments. SOD1, SOD2, and SOD3 are located in the cytoplasm, mitochondria, and extracellular environment respectively (28). A recent study demonstrated that overexpression of SOD2 inhibits starvation-induced autophagy in HeLa cells (6). Moreover, others have shown that late autophagic vacuoles contain higher SOD1 concentrations and are larger in hepatocytes, indicating that SOD1 is highly resistant to lysosomal degradation (32). We found that suppression of SOD1 expression by RNAi increased HMGB1 translocation and release as well as autophagy in MEFs. Interestingly, the protective effects of hydrogen gas on murine polymicrobial sepsis were associated with an increase in the activities of SOD and a reduction in the levels of HMGB1 in serum and tissue (48). Thus, the activity of SOD that we observed *in vitro* may be relevant to HMGB1 release *in vivo*.

Notably, our experimental data suggest that HMGB1 may function as a regulator of ROS-induced autophagy, because: *1) HMGB1 regulates the turnover of LC3.* LC3 is specifically localized to autophagic structures, including the autophagosome and its precursor structure, the isolation membrane, and its derivative, the autolysosome (20). Under conditions in which autophagy is enhanced, such as exposure to ROS and starvation, LC3-positive punctae are apparent. In contrast, loss of HMGB1 inhibited ROS-induced LC3-positive punctae. At the new cleaved amino terminal glycine residue, LC3-I is modified with phosphatidylethanolamine, and this is referred to as LC3-II, the form that becomes incorporated into the autophagosome membranes. This step involves a ubiquitination-like reaction mediated by Atg7 (E1-like activating enzyme), Atg3 (E2-like conjugating enzyme), and the Atg16L complex (E3-like ligase enzyme) (20). Loss of HMGB1 inhibited LC3-II formation under oxidative stress indicating that HMGB1 may regulate the LC3 ubiquitination-like reaction. *2) HMGB1 regulates the autophagic degradation of p62.* Since p62 accumulates when autophagy is inhibited, and decreased levels are observed when autophagy is induced, p62 may be used as a marker of autophagic flux (5). Accumulation of p62 has been observed in HMGB1 deficient cells following oxidative stress, indicating that there is an inhibition of autophagy or defects in autophagic degradation in HMGB1-deficient cells. Moreover, others have demonstrated that suppressing ROS or p62 accumulation prevents damage resulting from autophagy defects, indicating that failure to regulate p62 causes oxidative stress (27). Importantly, sustained p62 expression resulting from autophagy defects was sufficient to alter NF-κB regulation and gene expression and ultimately promote tumorigenesis (27). Indeed, overexpression of HMGB1 is associated with all of the central hallmarks of cancer (41). *3) HMGB1 regulates autophagolysosome formation.* Bafilomycin A1 is a specific inhibitor of the vacuolar type H^+-ATPase (V-ATPase) in cells (44) and inhibits the acidification of organelles containing this enzyme, such as lysosomes and endosomes. Similar to treatment with bafilomycin A1, HMGB1 depletion results in a significant decrease in the number of mature autophagosomes (autophagolysosomes) detected by colocalization of the autophagosome marker, LC3 and the lysosome marker, LAMP-2. *4) Loss of HMGB1 in tumors placed subcutaneously promotes the response to chemotherapy.* Targeted knockdown of HMGB1 increases sensitivity to chemotherapy *in vivo,* associated with increased apoptosis and decreased autophagy with oxidative stress. 4-hydroxy-2-nonenal (HNE) staining as a measure of oxidative stress in tissue (46), demonstrated increased ROS production in both HMGB1 wild-type and knockdown pancreatic tumors treated with chemotherapy, suggesting that HMGB1 effects are downstream to oxidative stress.

We have found that deletion, depletion, or inhibition of HMGB1 blocks starvation and rapamycin-induced autophagy in human and mouse cells subjected to knockout, knockdown or pharmacological inhibition of HMGB1 (39). Moreover, HMGB1 regulates autophagy by directly interacting with the autophagy protein Beclin1, without altering its expression (13). HMGB1 may be involved in the regulation of Bcl-2 phosphorylation during autophagy by the extracellular signal regulated kinase (ERK) and the MAPK pathway, since ablation of HMGB1 diminishes starvation-induced phosphorylation of both ERK1/2 and Bcl-2 (39). In addition, HMGB1 release and the cellular redox state regulate autophagy and apoptosis in cancer cells through a RAGE-dependent mechanism (15, 38). Importantly, targeted knockdown of HMGB1 increases sensitivity to gemcitabine in a homograft mouse pancreatic tumor model and is associated with decreased autophagy in the setting of treatment-induced oxidative stress. These results provide evidence for a critical signaling pathway that links autophagy to the cancer-associated dysregulation of HMGB1.

In summary, we demonstrate here that HMGB1 is an activator of the autophagic response to oxidative stress. However, the exact downstream signaling events regulated by HMGB1 leading to autophagy are unknown and will be the focus for future investigations. Since oxidative stress is involved in many human diseases such as atherosclerosis, Parkinson's disease, heart failure, myocardial infarction, Alzheimer's disease, obesity, and cancer (19, 28), our studies of autophagy regulation could provide insights useful in the

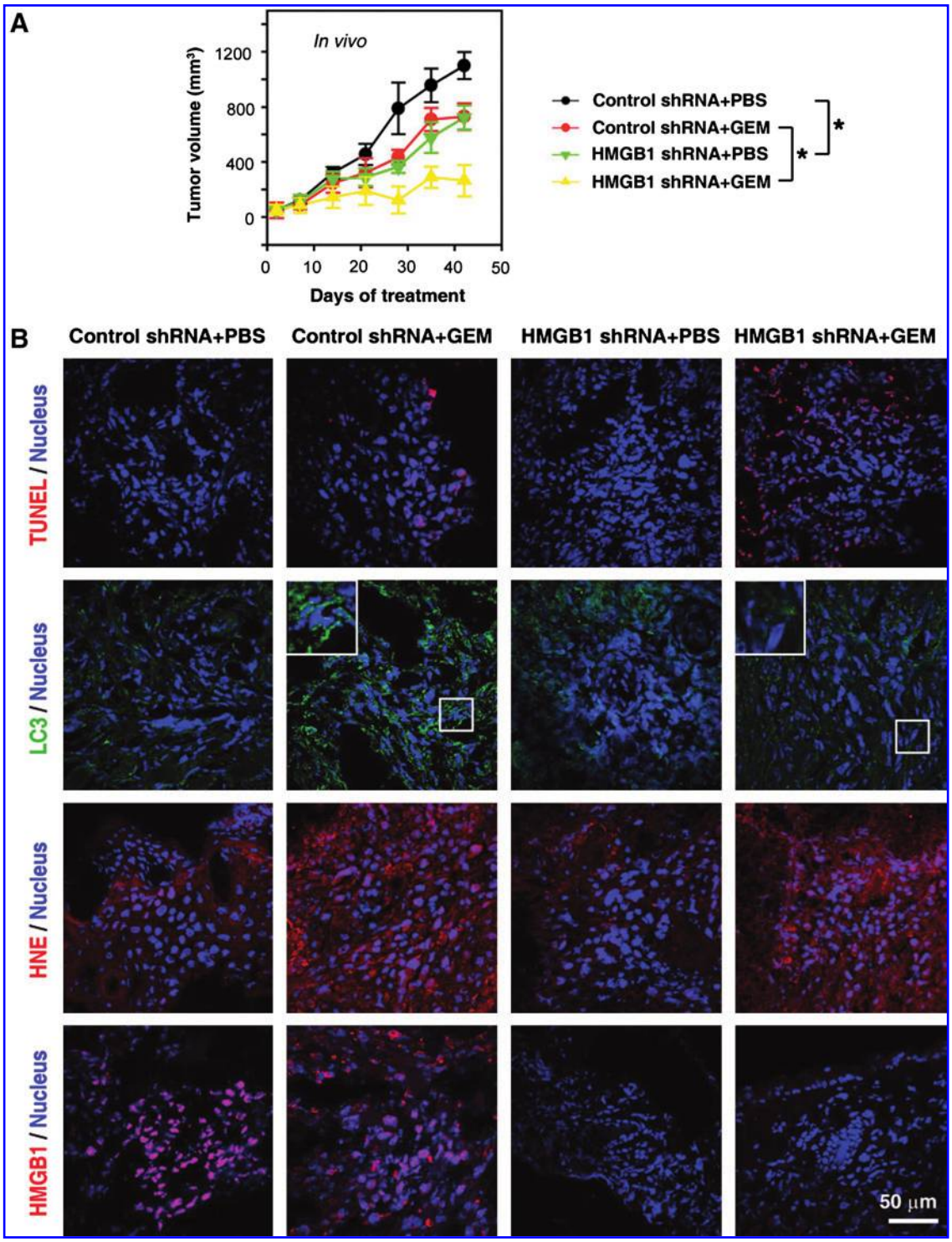

FIG. 5. Expression of HMGB1 mediates chemoresistance *in vivo* by reducing apoptosis and enhancing autophagic flux during oxidative stress. (A) HMGB1 knockdown tumor cells are more sensitive to gemcitabine *in vivo*. C57/BLl6 mice were inoculated with 10^6 Panc02 tumor cell following transfection of control or HMGB1-specific shRNA and treated with gemcitabine (GEM, 15 mg/kg, twice/week) or PBS beginning on day 10. Tumors were measured twice weekly, and volumes were calculated for 42 day ($n=5$ mice/group and expressed as mean ± SD, $^*p<0.05$). **(B)** On day 42, HMGB1 expression, apoptosis (TUNEL), autophagy (LC3), and oxidative stress (HNE) in tumor samples were assayed by immunofluorescence. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

development of novel drugs or approaches for controlling autophagy through HMGB1 regulation in these human diseases.

Materials and Methods

Reagents

The antibodies to actin and β-tubulin were obtained from Sigma (St. Louis, MO). The antibodies to SOD1 and 4 hydroxynonenal (HNE) were obtained from Abcam (Cambridge, MA). The antibodies to HMGB1, LC3, and LAMP2 were obtained from Novus (Littleton, CO). The antibodies to p62 and LDH were from Santa Cruz Technology (Santa Cruz, CA). The antibodies to mitochondrial cytochrome oxidase (COX) IV and histone H3 were obtained from Cell Signaling Technology (Danvers, MA). The HMGB1 neutralizing antibody was obtained from Novus (Cat #H00003146-M08). H_2O_2, NAC, DHIQ, and 3MA were obtained from Sigma. HMGB1 full length or mutant (C23S, C45S, C106S) cDNA were kind gifts from Dr. George Hoppe (Cole Eye Institute, Cleveland Clinic, Cleveland, OH) (10).

Cell culture and treatment

$HMGB1^{+/+}$ and $HMGB1^{-/-}$ immortalized mouse embryonic fibroblasts (MEFs) were a kind gift from Dr. Marco E. Bianchi (San Raffaele Institute, Italy). $Atg5^{+/+}$ and $Atg5^{-/-}$ MEFs were a kind gift from Dr. Noboru Mizushima (Tokyo Medical and Dental University, Tokyo, Japan). The HCT116 colon cancer cells and Panc02 pancreatic cancer cells were derived from the American Type Culture Collection (Manassas, VA). Cells were cultured in medium supplemented with 10% heat-inactivated fetal bovine serum, 2 m*M* glutamine and penicillin-streptomycin mix (Invitrogen, Carlsbad, CA; final concentration 50ug/ml each) in a humidified incubator with 5% CO_2 and 95% air. Cells were pretreated with N-acetyl cysteine (NAC, 50 m*M*), 3-methyladenine (3MA, 10 m*M*), or bafilomycin A1 (Baf A, 100 n*M*) for 1 h and then treated with H_2O_2 (0.05 m*M*) for 12 h, as indicated in the figure legend. In the oxidative injury model, MEFs were pretreated with 1,5-isoquinolinediol (DHIQ, 300 μM) for 1 h and then treated with H_2O_2 (0.25 m*M*) for 12 h. To explore the effect of HMGB1 on oxidative injury-induced cell viability, $Hmgb1^{+/+}$ and $Hmgb1^{-/-}$ MEFs were treated with various concentrations of H_2O_2 ranging from 0.0005 m*M* to 5 m*M* for 24 h.

RNAi

SOD1 small interfering RNA (siRNA), control siRNA (Santa Cruz Technology), HMGB1 short hairpin RNA (shRNA), and control shRNA (Sigma) were transfected into cells using the X-tremeGENE siRNA reagent (Roche Applied Science, Basel, Switzerland) or lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions (18).

Western blot analysis

Proteins from cell lysates were resolved on 4%–12% Criterion XT Bis-Tris gels (Bio-Rad, Hercules, CA) and transferred to a nitrocellulose membrane. 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) according to the manufacturer's instruction. The relative band intensity was quantified using the Gel-pro Analyzer® software (Media Cybernetics, Bethesda, MD).

HMGB1 release assay

HMGB1 released into cell culture supernatants was evaluated by Western blot analysis or enzyme-linked immunoabsorbent assay kits from the Shino-Test Corporation (Sagamihara-shi, Kanagawa, Japan), according to the manufacturer's instructions. The relative band intensity of Western blots was quantified using the Gel-pro Analyzer® software (Media Cybernetics).

Cell viability assay

Following treatment, cell viability was evaluated using the Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Tokyo, Japan), according to the manufacturer's instructions. CCK-8 allows for convenient assays by utilizing Dojindo's highly water-soluble tetrazolium salt. WST-8 [2-(2-methoxy-4-nitrophenyl)-3- (4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] produces a water-soluble formazan dye upon reduction in the presence of an electron carrier. The detection sensitivity of CCK-8 is higher than other tetrazolium salts such as MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]. In parallel, analysis of cell viability by trypan blue exclusion assay was performed and yielded similar results.

Isolation and subcellular fractionation of mitochondria

Subcellular fractionations of cells were carried out using a mitochondria isolation kit obtained from Pierce, according to the manufacturer's instructions.

Immunofluorescence analysis

Cells were cultured on glass cover-slips and fixed in 3% formaldehyde for 30 min at room temperature prior to detergent extraction with 0.1% Triton X-100 for 10 min at 25°C. Cover slips were saturated with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at room temperature, and processed for immunofluorescence with primary antibodies followed by Alexa Fluor 488 or Cy3-conjugated IgG (Invitrogen, San Diego, CA). Nuclear morphology was analyzed with the fluorescent dye Hoechst 33342. Between all incubation steps, cells were washed three times for 3 min with 0.5% BSA in PBS. In brief, images were collected using a laser-scanning confocal microscope (Fluoview FV-1000; Olympus) using a 60x Plan Apo/1.45 oil immersion objective and Fluoview software (FV10-ASW 1.6; Olympus). Images were subsequently analyzed for fluorescent intensity levels and co-localization of various stains by Image-Pro Plus 5.1 software (Media Cybernetics).

For tissue immunofluorescence analysis, tissues were embedded in optimum cutting temperature cryomedium (Sakura, the Netherlands) and, subsequently, cut into 8 μm sections as described previously (17). Tissue sections were stained with LC3, 4-hydroxy-2-nonenal (HNE), or HMGB1 antibody, followed by Alexa Fluor 488 or Cy3-conjugated Ig. Nuclear morphology was analyzed with the fluorescent dye Hoechst 33342 (Sigma). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed

using the In Situ Cell Death Detection Kit, according to the manufacturer's recommendations (Roche, Sweden).

Autophagy assays

Autophagy was evaluated by Western blotting for LC3-I/II and quantifying the percentage of cells with LC3 punctae (17). Autophagic flux assays were performed by Western blotting for p62, quantifying the percentage of cells with p62 punctae, and determining the percentage co-localization of LAMP2 and LC3.

Subcutaneous tumor models

For generation of murine subcutaneous tumors, 10^6 Panc02 wild-type or HMGB1 knockdown cells were injected subcutaneously to the right of the dorsal midline on C57/Bl6 mice. Tumors were measured twice weekly, and volumes were calculated using the formula $\text{length} \times \text{width}^2 \times \pi/6$ (17). 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.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Dr. Daolin Tang
Department of Surgery
University of Pittsburgh School of Medicine
5117 Centre Avenue
Pittsburgh, PA 15213

E-mail: tangd2@upmc.edu

or

Prof. Michael T. Lotze
Department of Surgery
University of Pittsburgh Cancer Institute
G.27A Hillman Cancer Center
5117 Centre Avenue
Pittsburgh, PA 15213

E-mail: lotzemt@upmc.edu

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Abbreviations Used

3-MA = 3-methyladenine
ATG = autophagy-related gene
BSA = bovine serum albumin
COX IV = mitochondrial cytochrome oxidase IV
DAMP = damage-associated molecular pattern molecule
DHIQ = 1,5-isoquinolinediol
HMGB1 = high mobility group box 1
HNE = 4-hydroxy-2-nonenal
H_2O_2 = hydrogen peroxide
LAMP2 = lysosomal-associated membrane protein 2
LC3 = microtubule-associated protein light chain 3
MEFs = mouse embryonic fibroblasts
NAC = N-acetyl cysteine
PBS = phosphate buffered saline
RNAi = RNA interference
ROS = reactive oxygen species
shRNA = short hairpin RNA
siRNA = small interfering RNA
SOD = superoxide dismutase

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