

## Oxidative Stress Impairs the Stimulatory Effect of S100 Proteins on Protein Phosphatase 5 Activity

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Oxidative stress is the consequence of an imbalance between the production of harmful reactive oxygen species and the cellular antioxidant system for neutralization, and it activates multiple intracellular signaling pathways, including apoptosis signal-regulating kinase 1 (ASK1). Protein phosphatase 5 (PP5) is a serine/threonine phosphatase involved in oxidative stress responses. Previously, we reported that S100 proteins activate PP5 in a calcium-dependent manner. S100 proteins belong to a family of small EF-hand calcium-binding proteins involved in many processes such as cell proliferation, differentiation, apoptosis, and inflammation. Therefore, we investigated the effects of oxidative stress on S100 proteins, their interaction with PP5, and PP5 enzyme activity. Recombinant S100A2 was easily air-oxidized or Cu-oxidized, and oxidized S100A2 formed cross-linked dimers and higher molecular-mass complexes. The binding of oxidized S100A2 to PP5 was reduced, resulting in decreased PP5 activation *in vitro*. Oxidation also impaired S100A1, S100A6, S100B, and S100P to activate PP5, although the low dose of oxidized S100 proteins still activated PP5. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) induced S100A2 oxidation in human keratinocytes (HaCaT) and human hepatocellular carcinoma (Huh-7) cells. Furthermore, H<sub>2</sub>O<sub>2</sub> reduced the binding of S100A2 to PP5 and decreased PP5 activation in HaCaT and Huh-7 cells. Importantly, even the low dose of S100A2 achieved by knocking down increased dephosphorylation of ASK1 and reduced caspase 3/7 activity in Huh-7 cells treated with H<sub>2</sub>O<sub>2</sub>. These results indicate that oxidative stress impairs the ability of S100 proteins to bind and activate PP5, which in turn modulates the ASK1-mediated signaling cascades involved in apoptosis.

**Keywords:** HaCaT; Huh-7; oxidative stress; protein phosphatase 5; S100 protein

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### Introduction

The S100 family consists of small, acidic proteins with EF-hand Ca<sup>2+</sup>-binding abilities. Of the known S100 proteins, more than 25 members are present in humans (Schafer and Heizmann 1996). Each S100 monomer contains two EF-hand Ca<sup>2+</sup> binding sites that, when stimulated by Ca<sup>2+</sup>, trigger a conformational change that exposes a hydrophobic surface, thereby allowing the monomer to interact with target proteins (Santamaria-Kisiel et al. 2006). S100 proteins are expressed in a tissue- and cell-specific manner and regulate numerous physiological functions such as cell growth, differentiation, and the cell cycle (Donato et al. 2013). S100 proteins are involved in many diseases such as Alzheimer's disease, Down's syndrome, cardiomyopathy, and cancer (Heizmann 2002; Heizmann et al. 2002).

The physiological function of each S100 protein is determined by its tissue distribution and target specificity, and is affected by its affinity for metal ions, oligomerization properties, and post-translational modifications (Zimmer et al. 2003). Among the S100 family members, S100A2 was first isolated from bovine lung tissue (Glenny et al. 1989), and is expressed in many tissues such as kidney, lung, prostate, skin, and mammary gland (Ilg et al. 1996; Maelandsmo et al. 1997). Functionally, S100A2 interacts with the C-terminal region of p53 (Mueller et al. 2005) and is involved in carcinogenesis and responses to oxidative stress (Zhang et al. 2002; Wolf et al. 2011). S100A2 is readily oxidized in keratinocytes, and oxidation promotes its oligomerization (Zhang et al. 2002). In turn, oxidized S100A2 plays a role in cellular protection against oxidative stress by participating in the proof-reading response

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(Deshpande et al. 2000).

Reactive oxygen species (ROS) consist of radical and non-radical oxygen species such as hydrogen peroxide and superoxide anion. ROS are derived from endogenous mitochondrial oxidative phosphorylation or interactions with and exposures to exogenous sources. Oxidative stress can result in ROS-mediated cell damage, but is also involved in regulating signaling pathways such as the mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase, and Nrf2- and Ref1-mediated signaling pathways (Ray et al. 2012). In the MAPK pathway, apoptosis signal-regulating kinase 1 (ASK1) is an upstream MAP kinase kinase kinase (MAPKKK) regulating the c-Jun N-terminal kinase (JNK) and p38 MAPK pathways, which leads to apoptosis (Ichijo et al. 1997; Takeda et al. 2008). As a modulator of ASK1 activity, protein phosphatase 5 (PP5) is a key molecule in oxidative stress responses (Morita et al. 2001).

PP5 is a member of the phosphoprotein phosphatase (PPP) family of serine/threonine phosphatases that includes PP1, PP2A, and PP2B (Hinds and Sanchez 2008). PP5 contains a C-terminal catalytic domain and three N-terminal tetratricopeptide repeat (TPR) motifs that are unique within the PPP family (Becker et al. 1994; Chen et al. 1994). The TPR motif consists of a sequence of 34 amino acids, with three to sixteen copies of the motif arranged in tandem (D'Andrea and Regan 2003; Zeytuni and Zarivach 2012). These motifs can act as interaction scaffolds for protein complex formation. In addition to participating in the oxidative stress response, PP5 plays a role in cell cycle regulation and nuclear receptor signaling (Chinkers 2001).

Previously, we reported that PP5 is a novel target of S100 proteins (Yamaguchi et al. 2012). S100A1, A2, A6, and S100B interact with the TPR domains of PP5 in a  $\text{Ca}^{2+}$ -dependent manner and significantly activate its phosphatase activity (Yamaguchi et al. 2012). As S100A2 and PP5 are both involved in the oxidative stress signaling pathway, in this study we examined the effects of oxidation on the structure of S100A2 and its ability to activate PP5. Our study showed that oxidation of S100 proteins dramatically reduced their ability to interact with PP5 and, consequently, their ability to fully activate PP5. Oxidation of S100A2 was also observed in HaCaT human keratinocytes and Huh-7 hepatocellular carcinoma cells. In these cell lines, we observed decreased interaction between oxidized S100A2 and PP5 and the attenuation of the enzyme activation, suggesting that S100 proteins may regulate PP5 under oxidative stress.

## Materials and Methods

### Materials

Anti-PP5 and anti-S100A2 antibodies were purchased from BD Biosciences (San Jose, CA, USA) and from Sigma (St. Louis, MO, USA), respectively. An anti- $\beta$ -actin antibody was obtained from Sigma. An anti-phospho-ASK1 (Thr845) antibody and Horseradish Peroxidase (HRP)-conjugated antibody were obtained from Cell Signaling (Beverly, MA, USA). Nickel-nitrilotriacetic acid-agarose

was purchased from Qiagen (Hilden, Germany). All other chemicals were obtained from Sigma.

### Plasmids and recombinant proteins

The pET11a-S100 plasmids were prepared as previously reported (Okada et al. 2002). The sequence of each construct was confirmed using ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Human PP5 cDNA was amplified by PCR from a human cDNA library, and the PCR product was cloned into the pET16a vector. The human S100A2 siRNA and scramble (control) vectors were constructed as reported previously (Bulk et al. 2009) using the following oligonucleotides: S100A2 siRNA sense, 5'-GATGAGAACAGTGACCAGCAG-3'; S100A2 siRNA antisense, 5'-CTGCTGGTCACTGTTCTCATC-3'; scramble sense, 5'-AGATCCGTATAGTGTACCTTA-3'; scramble antisense, 5'-TAAGGTACACTATACGGATCT-3'. These fragments were cloned into a pBasi-hU6 vector (Takara Bio, Otsu, Japan). Mouse ASK1 was cloned into a pME18S-cMyc plasmid as reported previously (Yamaguchi et al. 2012). Histidine-tagged PP5 (His-PP5) protein was expressed and purified according to the manufacturer's protocol. S100 proteins (S100A1, S100A2, S100A6, S100B, and S100P) were expressed and purified as described previously (Yamashita et al. 1999; Okada et al. 2004). Air-oxidized S100 proteins were prepared by storing the recombinant S100 proteins (0.5 mg/mL) at  $-30^{\circ}\text{C}$  for 3 months. Cu-oxidized S100 proteins were prepared as described previously (Matsui Lee et al. 2000). In brief, the S100 proteins (10  $\mu\text{M}$ ) were incubated with 80  $\mu\text{M}$  of  $\text{CuCl}_2$  in 20 mM Tris-HCl buffer (pH 7.6) for 2 h at  $37^{\circ}\text{C}$ . The copper chelator diethylenetriamine- $\text{N,N,N',N',N'}$ -pentaacetic acid was then added to stop the reaction. Samples were desalted with a Centriprep-3 filter unit (Amicon Inc., Beverly, MA, USA) and then dialyzed in 20 mM Tris-HCl buffer (pH 7.6). Samples were subjected to reducing conditions (treatment with 5 mM dithiothreitol [DTT]) or non-reducing conditions (no DTT) and analyzed with Tricine SDS-PAGE electrophoresis.

### Plasmid transfection

The siRNA expression vector (S100A2 or scramble) and mouse ASK1-cMyc expression vector were transfected into Huh-7 cells with the X-tremeGENE HP transfection reagent (Sigma) according to the manufacturer's protocol.

### Cell culture and $\text{H}_2\text{O}_2$ treatment

HaCaT and Huh-7 cells were purchased from the Japanese Collection of Research Bioresources (Osaka, Japan). Cell lines were maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified  $\text{CO}_2$  incubator (5%  $\text{CO}_2$ /95%  $\text{O}_2$ ).

Approximately  $5.5 \times 10^5$  cells were seeded on a 10-cm dish and cultured for 3 days. Then, the medium was removed and the cells were washed with phosphate-buffered saline (PBS). The cells were then incubated for 90 min in serum-free medium with  $\text{H}_2\text{O}_2$  (0-2 mM). Following  $\text{H}_2\text{O}_2$  treatment, the cells were washed twice with ice-cold PBS and lysed in a sample buffer containing 10% glycerol, 4% SDS, and 0.01% bromophenol blue in 50 mM Tris-HCl (pH 6.8). To reduce the formation of disulfide links, the samples were incubated with DTT (50 mM). These sample proteins were separated using Tricine SDS-PAGE electrophoresis.

### Surface plasmon resonance (SPR)

Protein binding interactions were analyzed using an SPR Biacore 2000 system (GE Healthcare, Waukesha, WI, USA). Amine coupling of PP5 to the dextran surface of a CM5 chip was carried out using *N*-ethyl-*N'*-(3-diethylaminopropyl) carbodiimide, *N*-hydroxysuccinimide, and ethanolamine-HCl. His-PP5 (100  $\mu\text{g}/\text{mL}$ , 140  $\mu\text{L}$ ) was immobilized in 20 mM ammonium acetate (pH 4.2) until 1,980 (0.23 pmol) or 2,448 (0.28 pmol) response units were bound and a stable baseline was obtained. A solution of 20 mM HEPES (pH 7.4), 150 mM NaCl, 0.005% Tween 20, and 1 mM  $\text{CaCl}_2$  was used at a flow rate of 20  $\mu\text{L}/\text{min}$ . Varying concentrations of the recombinant S100 proteins (10–1,250 nM each) were injected. The His-PP5-coupled sensor chip was regenerated between protein injections with a 60-s wash with 50 mM NaOH. The response curves were prepared by subtracting the signal generated simultaneously on a control flow cell. Biacore sensorgram curves were analyzed in BIAevaluation 3.0 software (Biacore, Uppsala, Sweden) using a 1:1 Langmuir model.

### Tricine SDS-PAGE and Western blotting analysis

Samples were separated with Tricine SDS-PAGE gel electrophoresis under reducing and non-reducing conditions. Following sample separation, the gels were either stained with Coomassie Brilliant Blue (CBB) or subjected to Western blotting. Western blotting was performed by transferring proteins onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.05% Tween 20 (TTBS), and then incubated with an anti-S100A2, anti-PP5, anti-phospho-ASK1 (Thr845), or anti- $\beta$ -actin antibody in the same buffer overnight. After washing with TTBS, membranes were incubated with an HRP-labeled secondary antibody for 2 h. Signals were visualized with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA).

### Immunoprecipitation of PP5

Huh-7 and HaCaT cells were briefly washed with cold PBS and then lysed with lysis buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.5% Triton-X100, 0.5% Nonidet P-40) containing protease inhibitors (Sigma). Lysed samples were sonicated and centrifuged at 15,000 rpm for 10 min at 4°C. Supernatants were collected and protein concentration determined with the Bio-Rad assay (Bio-Rad, Hercules, CA, USA). Immune complexes were immunoprecipitated from the cleared lysate (400  $\mu\text{g}$  for Western blotting and 700  $\mu\text{g}$  for the PP5 assay) by incubating with anti-PP5 antibody in a buffer (20 mM Tris-HCl at pH 7.5, 100 mM KCl, and 1 mM  $\text{CaCl}_2$ ) for 16 h at 4°C. Subsequently, 30  $\mu\text{L}$  of protein G-Sepharose beads were added and the samples incubated for an additional 3 h. The beads were then washed extensively and used for Western blotting or the PP5 assay.

### In vitro phosphatase assay

The Ser/Thr Phosphatase Assay kit (Upstate Biotechnology, Lake Placid, NY, USA) was used to measure the activity of PP5 phosphatase according to the manufacturer's protocol. In brief, His-PP5 protein (250 ng) and different amounts of S100 proteins (0–10  $\mu\text{g}$ ) were incubated with the phosphopeptide substrate (KRpTIRR; 100  $\mu\text{M}$ ) in 50  $\mu\text{L}$  of a buffer (20 mM Tris-HCl at pH 7.5, 20 mM  $\text{MgCl}_2$ , 0.01% Tween 20, and 1 mM  $\text{CaCl}_2$ ) for 10 min at 37°C. Following the incubation, 100  $\mu\text{L}$  of malachite green solution (0.034% malachite green, 10 mM ammonium molybdate, 1 M HCl, 3.4% ethanol, and

0.01% Tween 20) was added to the sample and its absorbance at 630 nm was measured with a microplate reader. A phosphate standard curve was prepared and used to calculate the amount of released phosphate. Endogenous PP5 activity was measured in triplicate samples of Huh-7 and HaCaT cell lysates incubated with 10  $\mu\text{L}$  of prepared beads and 1 mM  $\text{CaCl}_2$  for 1 h at 37°C. After centrifugation, the supernatants were assessed with the phosphatase assay.

### Caspase activity assay

Caspase-3 activity was measured with the Caspase-Glo 3/7 Assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Approximately  $1 \times 10^4$  Huh-7 cells/well were seeded onto a 96-well plate and then transfected with S100A2 siRNA or scramble vector. After 48 h of transfection, the cells were treated with or without  $\text{H}_2\text{O}_2$  (2 mM) and ionomycin (1  $\mu\text{M}$ ) for 2 h. Caspase-Glo 3/7 reagent was added and the plate was shaken for 1 h. The contents were transferred to a white-walled plate and the luminescence from each well was measured with an SH-9000 microplate reader (Corona Electric, Ibaraki, Japan).

### Statistical analysis

Data were analyzed with Student's *t*-test, with statistical significance set at  $P < 0.01$  and  $P < 0.05$ . Data are expressed as the mean  $\pm$  standard deviation (SD).

## Results

### *Oxidation of S100A2 promotes oligomerization and attenuates its ability to activate PP5*

To study the effects of oxidation on S100A2 formation, recombinant S100A2 was expressed and purified from *Escherichia coli*, and then subjected to one of three sample preparation methods. For the freshly-prepared samples, the protein was immediately used. For the air-oxidized samples, a portion of the protein sample was diluted to 0.5 mg/mL and kept at  $-30^\circ\text{C}$  for 3 months to promote oxidization. For the Cu-oxidized samples, freshly-prepared S100A2 was treated with Cu as previously described (Matsui Lee et al. 2000). Freshly-prepared, air-oxidized, and Cu-oxidized samples were each separated by Tricine SDS-PAGE gel electrophoresis under reducing (with DTT) or non-reducing (without DTT) conditions, and the gels subsequently stained with CBB (Fig. 1A). Under reducing conditions, most proteins were in the monomeric form, with a minority dimeric. Faint, smeared bands were observed above the dimer band in the air- and Cu-oxidized samples, and may represent higher molecular mass (HMM) forms. Air oxidation dramatically decreased the amount of the monomeric form and increased the amount of dimeric and HMM forms. Furthermore, Cu oxidation completely depleted monomeric S100A2, which was complemented by an increase in the amount of dimeric and HMM forms. These results indicated that oxidation of S100A2 promoted oligomerization via intermolecular disulfide bridge formation.

We reported previously that S100A2 is a natural activator of PP5, a key enzyme in the oxidative stress response (Yamaguchi et al. 2012). The binding of S100A2 to the TPR domain of PP5 activates its phosphatase activity in a

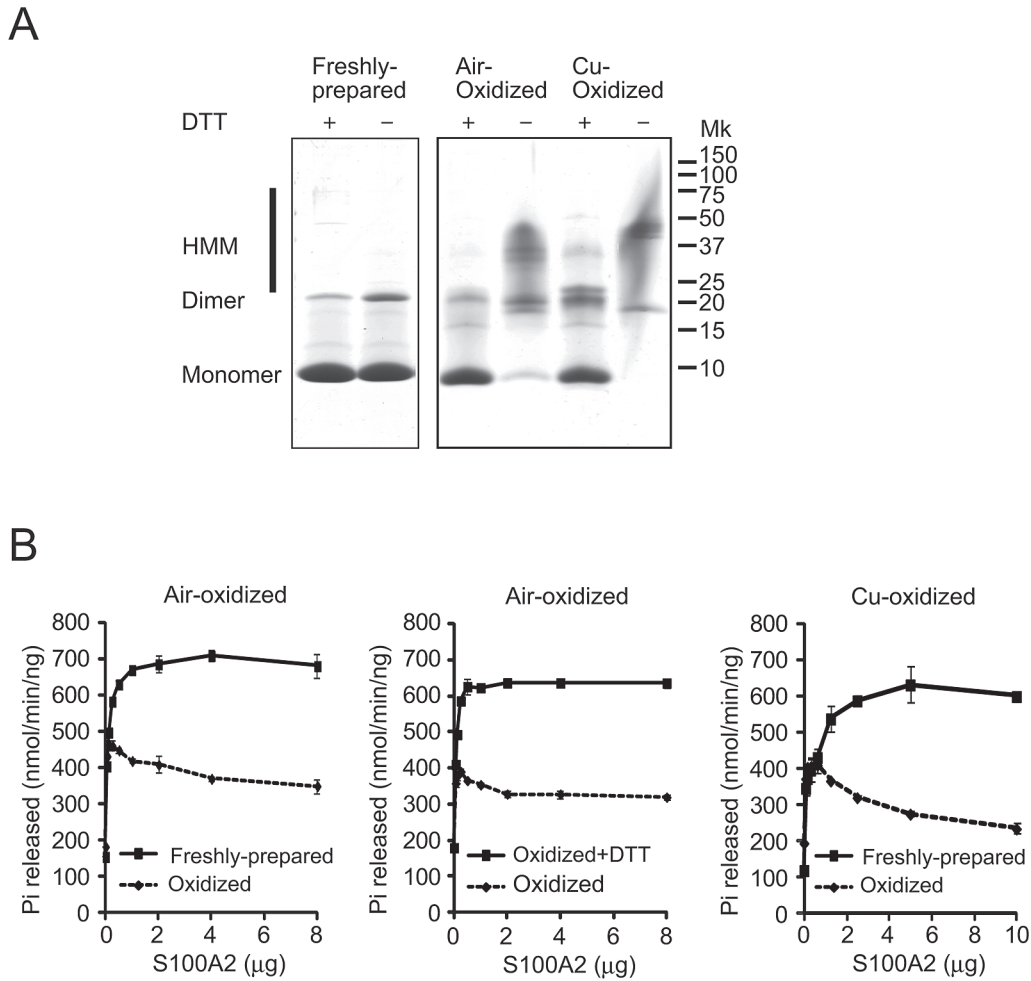


Fig. 1. Effects of oxidation on S100A2 and its activation of PP5.

(A) Freshly-prepared, air-oxidized, and Cu-oxidized S100A2 were separated on 15% Tricine SDS-PAGE gels under reducing (DTT+) or non-reducing (DTT-) conditions and stained with CBB. HMM: high molecular mass complex. Molecular weight markers (Mk) are shown on the right. (B) PP5 activation assay. His-PP5 was incubated with a synthetic phosphopeptide (KRpTIRR) and varying amounts of S100 proteins in the presence of 1 mM  $\text{CaCl}_2$ . After addition of malachite green solution with 0.01% Tween 20, the absorbance at 630 nm was measured. A standard curve calculated from a known amount of phosphate was used to determine the amount of released phosphate. Each data point represents the mean  $\pm$  SD of triplicate determinants.

$\text{Ca}^{2+}$ -dependent manner. Therefore, we examined the effects of freshly-prepared and oxidized S100A2 on PP5 activation. S100 proteins were incubated with His-PP5 and a phosphopeptide substrate in the presence of  $\text{Ca}^{2+}$ , and the amount of released phosphate was measured (Fig. 1B). Freshly-prepared S100A2 significantly activated PP5 ( $709.4 \pm 14.0$  nmol/min/ng protein with  $4 \mu\text{g}$  of S100A2), but air oxidation attenuated PP5 activation ( $369.2 \pm 5.2$  nmol/min/ng protein with  $4 \mu\text{g}$  of oxidized S100A2). This attenuation was ameliorated by reducing oxidized S100A2 with DTT (Fig. 1B, middle panel). Cu oxidation of S100A2 also dramatically attenuated its ability to activate PP5 (Fig. 1B, right panel). Interestingly, both oxidized and freshly-prepared S100A2 could similarly activate PP5 at low concentrations.

#### *Oxidation of S100A2 attenuates interaction of S100A2 with PP5*

To determine how oxidation attenuates PP5 activation by S100A2, we examined the interactions between S100A2 and PP5 using SPR (Fig. 2). His-PP5 was immobilized on the dextran surface of a CM5 chip. Varying amounts of S100A2 were added in the presence of  $\text{Ca}^{2+}$ , and S100A2 binding was assessed. As shown in Fig. 2A (top row), freshly-prepared S100A2 bound to PP5 in a dose-dependent manner (approximately 290 response units [RU] with 1250 nM S100A2 at 150 s). Treatment of freshly-prepared S100A2 with a reducing agent (DTT) slightly increased its binding to PP5 (372 RU with 156 nM S100A2 at 150 s). Air-oxidized S100A2 clearly lost its ability to interact with PP5 (fewer than 23 RU with 1250 nM S100A2 at 150 s), but a small amount of binding was still observed at a low concentration (78 nM). The reduction in binding was coun-

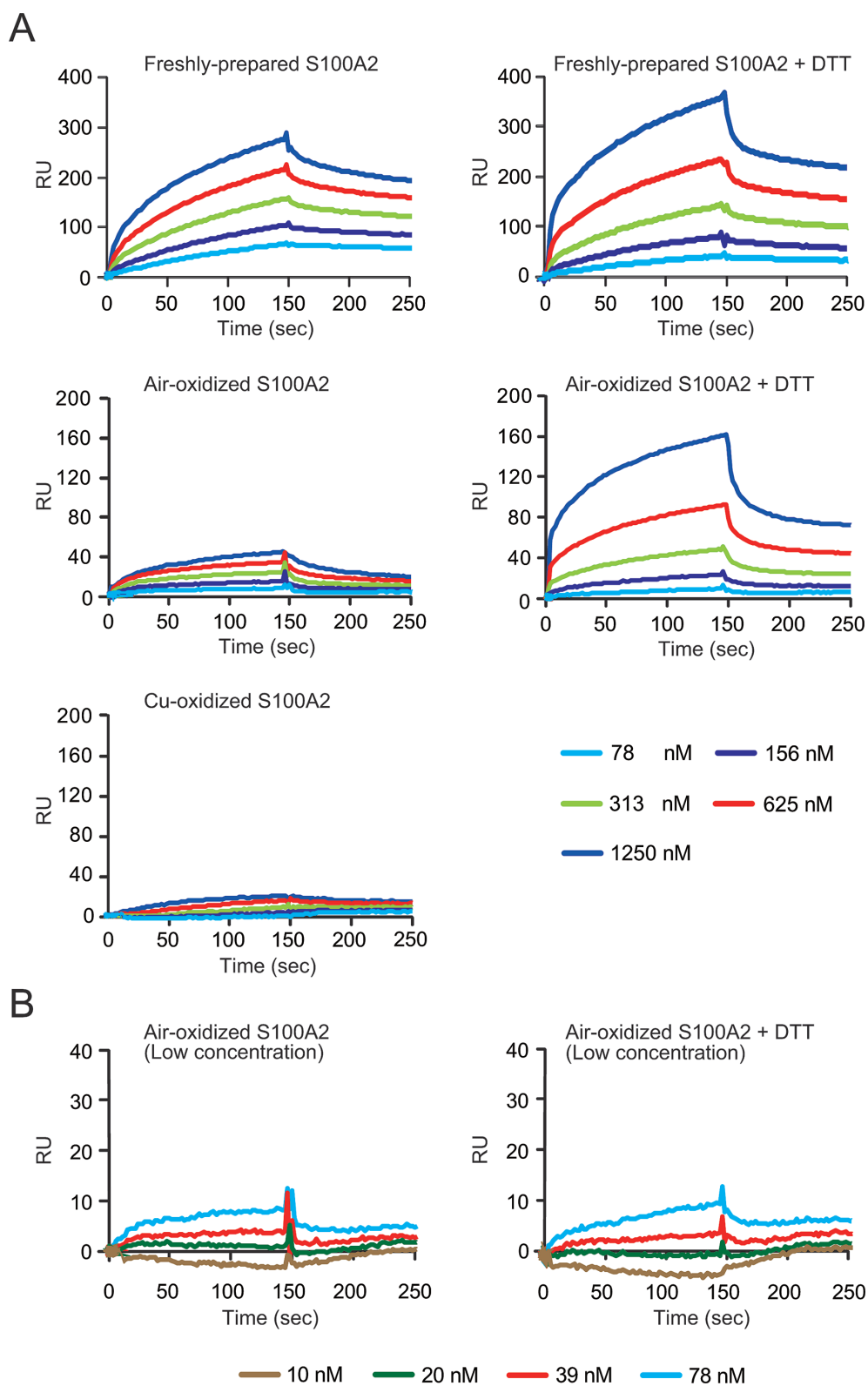


Fig. 2. Interaction of PP5 and freshly-prepared or oxidized S100A2.

Protein binding analyses were performed using an SPR Biacore 2000 system. His-PP5 was immobilized on the dextran surface of a CM5 chip and varying concentrations of recombinant S100 proteins (10-1,250 nM each) were injected. Biacore sensorgram curves were evaluated with BIAevaluation 3.0 software using a 1:1 Langmuir model. (A) Sensorgram curves collected for higher concentrations of S100A2 (78-1,250 nM). (B) Sensorgram curves collected for lower concentrations of S100A2 (10-78 nM). Note that low concentrations of oxidized S100A2 (39 and 78 nM) bind to PP5. RU: response units.

teracted by DTT treatment (Fig. 2A, middle row). A similar reduction in PP5 binding was observed with Cu-oxidized S100A2 (Fig. 2A, bottom row). Altogether, these results indicated that oxidation attenuated the ability of S100A2 to interact with PP5.

As shown in Fig. 1B, low concentrations of oxidized and freshly-prepared S100A2 had comparable effects on PP5 activity. To clarify the mechanism behind these observations, we conducted SPR experiments using lower concentrations (10-78 nM) of air-oxidized S100A2 (Fig. 2B). Both air-oxidized and reduced (air-oxidized + DTT) S100A2 bound to PP5 to a similar degree. Although the details of this mechanism remain to be determined, this observation could explain the activation of PP5 activation by low concentrations of oxidized S100A2 (Fig. 1B).

#### *Oxidation attenuates the ability of other S100 proteins to activate PP5*

Oxidation of S100A2 resulted in the formation of disulfide bridges connecting cysteine residues and attenuated the ability of S100A2 to interact with and activate PP5. As these cysteine residues are conserved among S100 proteins, we examined the effects of oxidizing other S100 proteins that are known to activate PP5 (Yamaguchi et al. 2012). Each S100 protein was mixed with His-PP5 and a phosphopeptide substrate in the presence of  $\text{Ca}^{2+}$ , and the amount of released phosphate was measured. Freshly-prepared S100 proteins (S100A1, S100A6, S100P, and S100B) activated PP5 in a dose-dependent manner (Fig. 3). Although the degree of PP5 activation varied among the S100 proteins, all oxidized S100 proteins were unable to

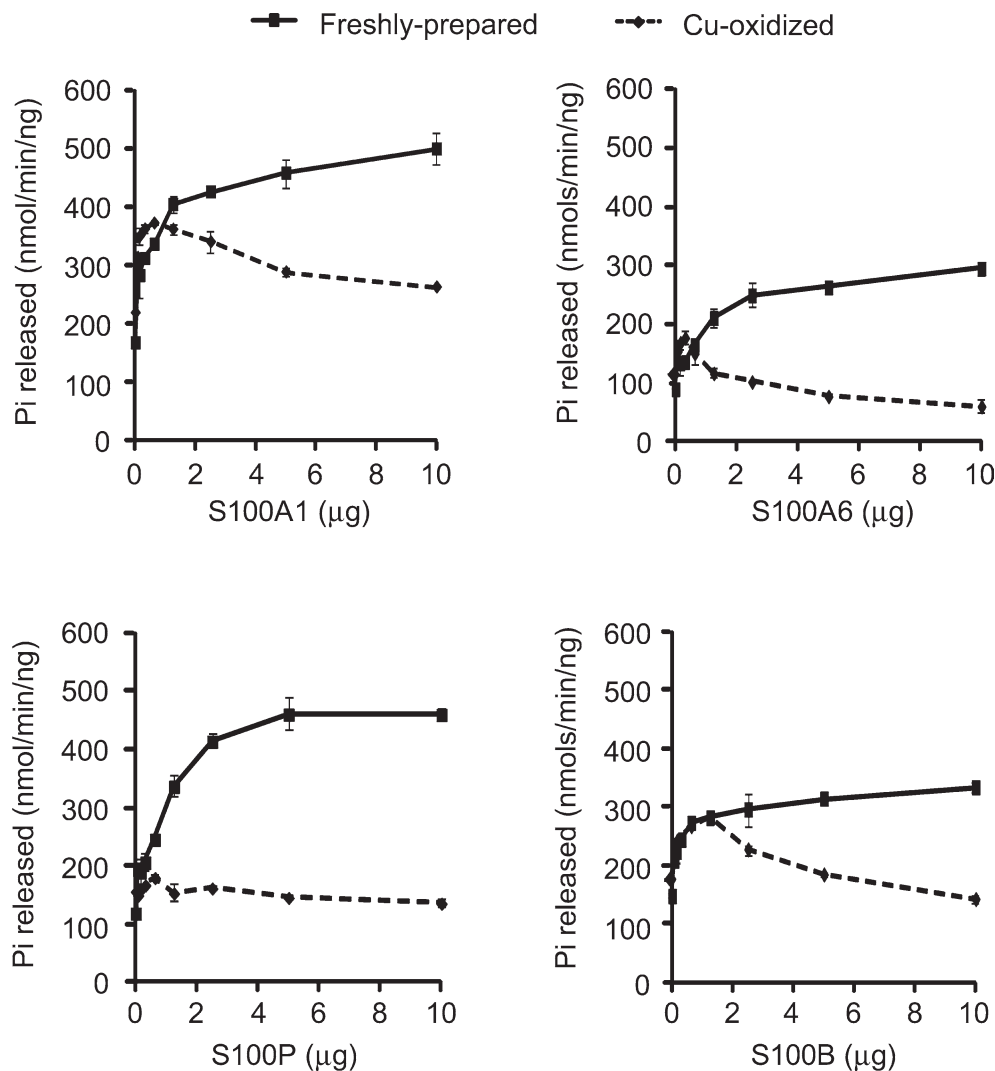


Fig. 3. Effects of oxidation of S100 proteins on PP5 activation.

S100A1, S100A6, S100P, and S100B proteins were freshly prepared or Cu oxidized, and then assayed for PP5 activation ability. The phosphatase activity of PP5 was measured with a Ser/Thr Phosphatase Assay kit. Varying amounts (0-10  $\mu\text{g}$ ) of S100 proteins were incubated with His-PP5 for 10 min at 37°C in the presence of 1 mM  $\text{CaCl}_2$ . After addition of malachite green solution, sample absorbance at 630 nm was measured. The amount of released phosphate was calculated using a phosphate standard curve prepared from a known amount of phosphate. Each data point represents the mean  $\pm$  SD of triplicate determinants.

activate PP5 to the extent of freshly-prepared samples. As was observed with S100A2, both the oxidized and freshly-prepared forms of these other S100 proteins had comparable effects on PP5 activity at low concentrations. These results indicated that oxidation-associated attenuation of the ability to activate PP5 was not limited to S100A2, but was shared by other S100 proteins as well.

*Oxidative stress promotes oligomerization of S100A2 in HaCaT and Huh-7 cells*

We further examined whether oxidative stress promotes oligomerization of S100A2 in intact cells. The

immortalized human keratinocyte HaCaT cell line and the human hepatocellular carcinoma Huh-7 cell line were used because expression of S100A2 was observed in these cell lines (Zhang et al. 2002; Shimamoto et al. 2013). Cells were treated with varying concentrations of H<sub>2</sub>O<sub>2</sub> (0-2 mM) and proteins were extracted. Protein samples were treated with or without DTT and separated with Tricine SDS-PAGE electrophoresis. Endogenous S100A2 was detected by Western blotting with an anti-S100A2 antibody (Fig. 4). In HaCaT cells, H<sub>2</sub>O<sub>2</sub> treatment increased the formation of dimeric S100A2 and HMM complexes in a dose-dependent manner (Fig. 4, upper left). The addition of DTT clearly

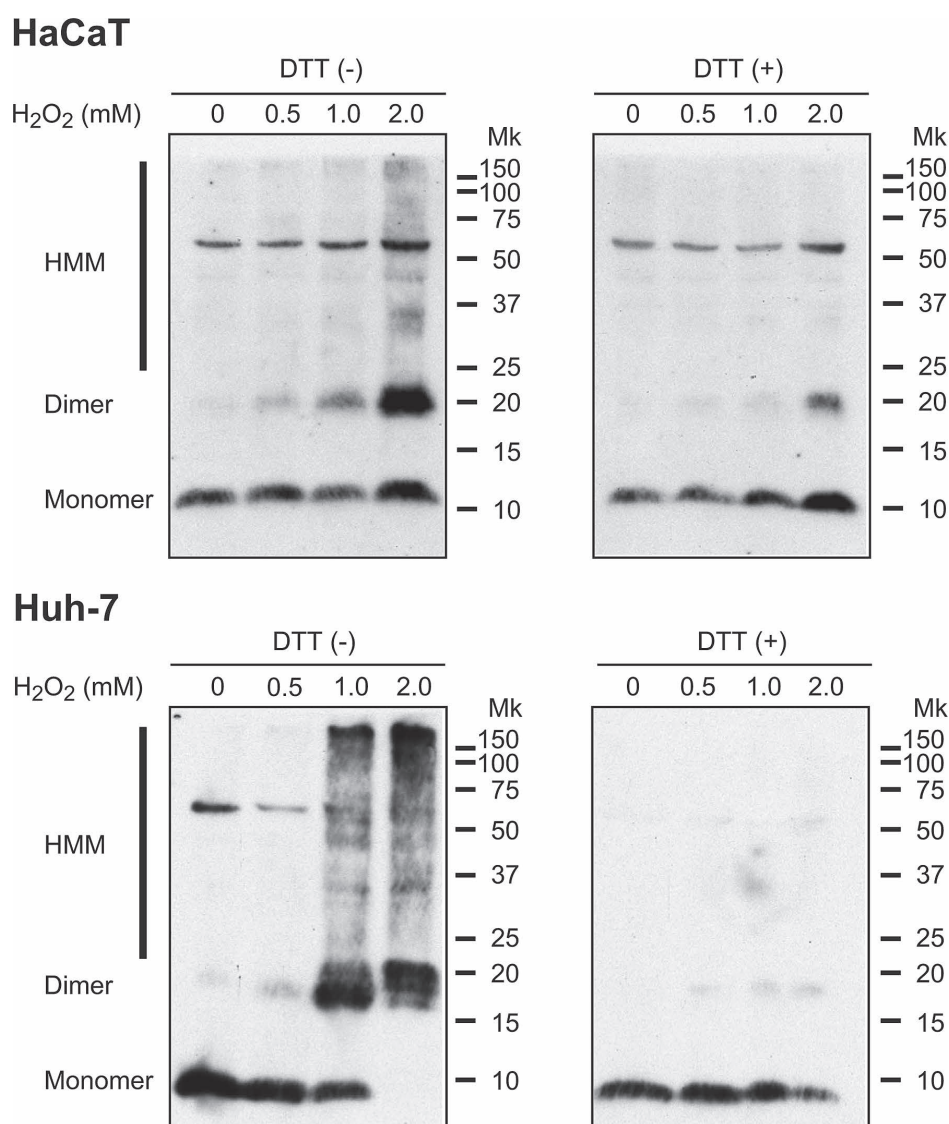


Fig. 4. Effect of H<sub>2</sub>O<sub>2</sub>-induced oxidative stress on S100A2 formation in HaCaT and Huh-7 cells.

For these experiments,  $5.5 \times 10^5$  cells were plated on a 10-cm dish and cultured for 3 days in standard medium. Medium was aspirated and the cells were washed with PBS prior to being incubated with H<sub>2</sub>O<sub>2</sub> (0-2 mM) in serum-free medium for 90 min. After the exposure, cells were washed with ice-cold PBS and lysed in sample buffer (50 mM Tris-HCl at pH 6.8, 10% glycerol, 4% SDS, and 0.01% bromophenol blue). To reduce disulfide linkages, 50 mM DTT was added to the sample before electrophoresis. Proteins were separated on 15% Tricine SDS-PAGE gels, blotted onto nitrocellulose membranes, and subjected to Western blotting with an anti-S100A2 antibody. The band migrating with an apparent MW of 60 kDa is a non-specific HRP-labeled secondary antibody-reactive band. HMM, high molecular mass complex; Mk, molecular weight marker.

reduced the formation of these dimers and HMM complexes (Fig. 4, upper right). In Huh-7 cells, the formation of dimeric and HMM complexes following  $H_2O_2$  treatment was prominent, and DTT treatment greatly attenuated the formation of these complexes (Fig. 4, bottom). These results suggested that  $H_2O_2$  treatment promoted intermolecular disulfide bridge formation within S100A2 in cells.

*Oxidative stress inhibits the interaction between S100A2 and PP5 and results in attenuation of PP5 activation in Huh-7 cells*

As endogenous S100A2 was observed to be oxidized in intact cells, the interaction between PP5 and S100A2, as well as PP5 activity, were measured under oxidative conditions. After confirming that Huh-7 and HaCaT cells express endogenous S100A2 and PP5 (Fig. 5A), the cells were

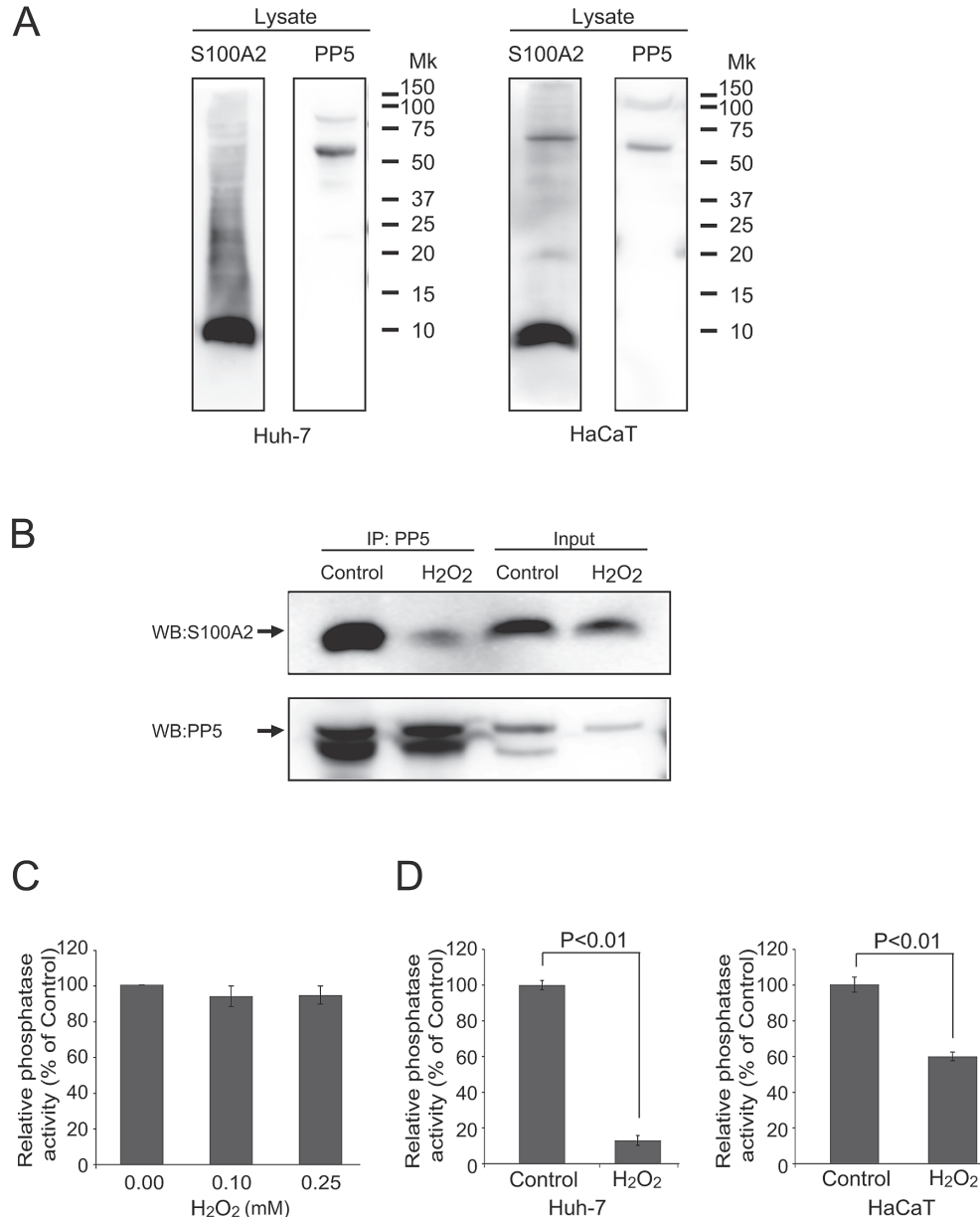


Fig. 5. Effect of  $H_2O_2$ -induced oxidative stress on the interaction between S100A2 and PP5 and on PP5 activation. (A) The expression of S100A2 and PP5 in Huh-7 and HaCaT cells was confirmed by Western blot analysis ( $20 \mu\text{g}$  cell lysate/lane). (B) Huh-7 cells were treated with or without  $2 \text{ mM } H_2O_2$  in the presence of  $1 \mu\text{M}$  ionomycin for 90 min at  $37^\circ\text{C}$ . Immune complexes were precipitated using an anti-PP5 antibody and protein G-Sepharose beads. After extensive washing, beads were used for Western blotting with anti-S100A2 or anti-PP5 antibodies. Approximately  $20 \mu\text{g}$  of protein was loaded per lane. (C) The effect of  $H_2O_2$  on PP5 activity was analyzed *in vitro*. Up to  $0.25 \text{ mM}$  of  $H_2O_2$  was added to the reaction buffer and incubated for 1 h at RT. A synthetic phosphopeptide substrate was added and phosphatase activity was measured ( $N = 3$ ). (D) Phosphatase activity of endogenous PP5 bound to beads was measured, and relative phosphatase activity was calculated. Data are expressed as the mean  $\pm$  SD of at least two independent experiments performed in triplicate.



treated with 1  $\mu$ M ionomycin with or without 2 mM  $H_2O_2$  for 90 min. Cell lysate was then collected for an immunoprecipitation assay using an anti-PP5 antibody.

Western blotting showed that  $H_2O_2$  treatment decreased the binding of S100A2 to PP5 (Fig. 5B). To examine the effects of oxidative conditions on PP5 activity in the absence of S100 proteins, we used an *in vitro* phosphatase assay system.  $H_2O_2$  was added to the reaction buffer and incubated for 1 h at RT. Then, a phosphopeptide substrate was added and phosphatase activity was measured (Fig. 5C). The results showed that the addition of  $H_2O_2$  up to 0.25 mM had little effect on PP5 activity in the absence of S100 proteins (activity with 0.25 mM of  $H_2O_2$  was calculated as  $94.3 \pm 5.0\%$  of control). In living cells, PP5 activity was reduced by  $H_2O_2$  treatment in Huh-7 cells ( $13.0 \pm 2.7\%$  of control,  $P < 0.01$ ) and in HaCaT cells ( $59.8 \pm 2.4\%$  of control,  $P < 0.01$ ; Fig. 5D). These observations were

consistent with the results of our other *in vitro* experiments (Figs. 1 and 2).

#### Knockdown of S100A2 in Huh-7 cells decreases phosphorylated ASK1 and caspase activity under oxidative stress.

Under oxidative conditions, ASK1 is activated by the oligomerization-dependent autophosphorylation of Thr845 (Hattori et al. 2009). The activated ASK1 induces apoptosis by increasing the activity of caspases such as caspase 3 and 9 (Hatai et al. 2000). Given that PP5 modulates ASK1 activity by dephosphorylating ASK1, we examined whether a decrease in S100A2 levels affects ASK1 activity under oxidative conditions. Accordingly, Huh-7 cells were transfected with the mouse ASK1-cMyc expression vector with S100A2 siRNA or the corresponding scramble vector. We thus confirmed knockdown of S100A2 expression (Fig. 6A). Western blotting analysis with the anti-phospho-

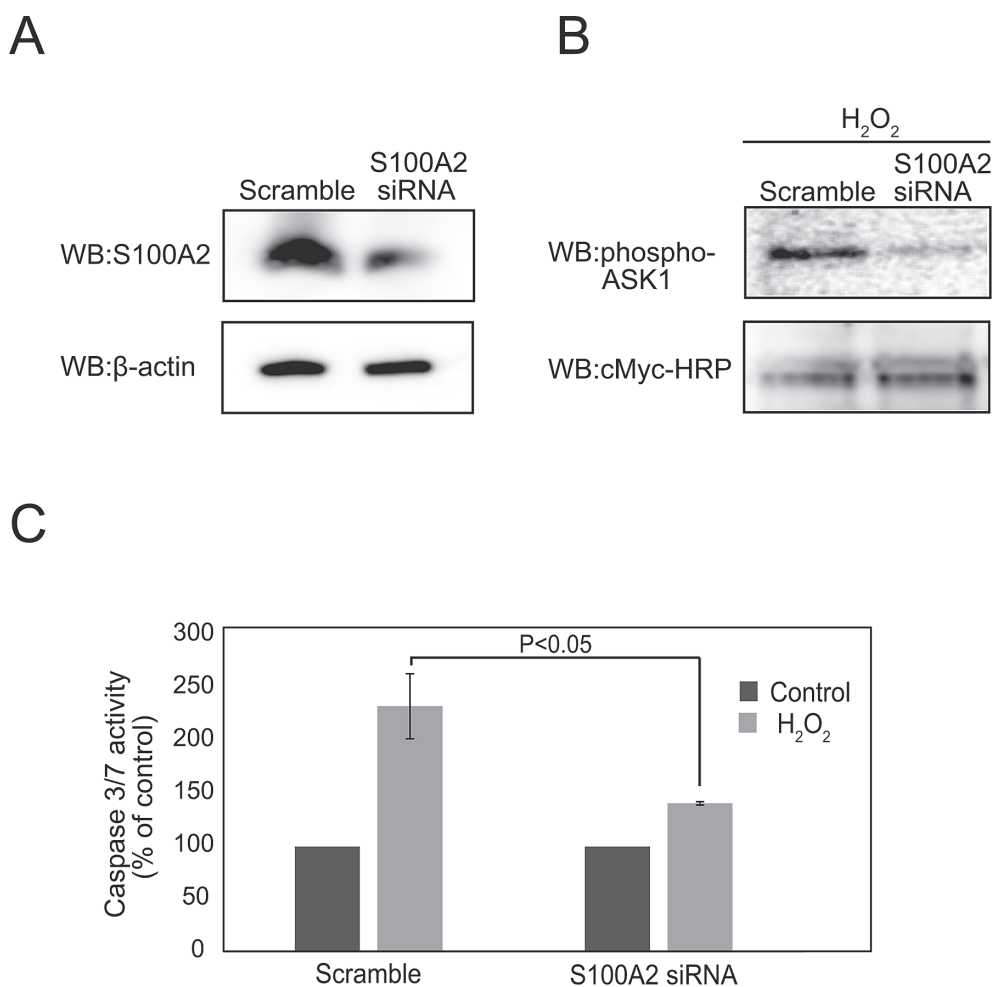


Fig. 6. Knockdown of S100A2 expression in Huh-7 cells modulates ASK1 and caspase activity under oxidative stress.

(A) Huh-7 cells were transfected with S100A2 siRNA or scramble (control) expression vector using the X-tremeGENE HP transfection reagent. After 48 h, Western blotting was performed to confirm S100A2 knockdown. (B) Huh-7 cells were transfected with S100A2 siRNA or scramble in mouse ASK1-cMyc expression vector. After treatment with  $H_2O_2$  (2 mM) and ionomycin (1  $\mu$ M) for 1 h, Western blotting analysis was performed with anti-phospho-ASK1 or anti-cMyc-HRP antibody. (C) Huh-7 cells were transfected with S100A2 siRNA or scramble (control) expression vector. After treatment with  $H_2O_2$  (2 mM) and ionomycin (1  $\mu$ M) for 2 h, caspase 3/7 activity was measured using the Caspase-Glo 3/7 assay kit. Activity is represented as % of control (N = 3).

ASK1 antibody showed that, following treatment with H<sub>2</sub>O<sub>2</sub> (2 mM) and ionomycin (1  $\mu$ M), S100A2 knockdown decreased phosphorylated ASK1 levels compared to control (Fig. 6B). Under the conditions used, the anti-phospho-ASK1 antibody only detected the phosphorylation of transfected ASK1. These results coincided with the result that at low doses S100A2 could activate PP5 in oxidative conditions, whereas high doses inhibited PP5 (Fig. 1B). Furthermore, H<sub>2</sub>O<sub>2</sub> and ionomycin treatment increased the caspase 3/7 activity to  $232.8 \pm 30.9\%$  compared to untreated cells; S100A2 knockdown significantly reduced this treatment-induced activity to  $140.9 \pm 1.6\%$  ( $P < 0.05$ ) (Fig. 6C). Taken together, we propose that the low dose of S100A2 activates PP5 and decreases ASK1 activity by dephosphorylating ASK1, which in turn lowers the caspase 3/7 activity.

### Discussion

In the normal intracellular reducing environment, S100 proteins form antiparallel, non-covalently linked homodimers or heterodimers (Potts et al. 1995; Kilby et al. 1996) and are involved in numerous signaling pathways. Under oxidizing conditions, the oxidation of cysteine residues causes cross-linking of S100 proteins by interchain disulfide bond formation and modifies their intra- and extracellular functions (Lim et al. 2009). For example, Cu-mediated cross-linking of S100A4 increased activation of NF- $\kappa$ B and secretion of tumor necrosis factor- $\alpha$  from human A375 melanoma cells—effects that were even more pronounced in cells transfected with the receptor for advanced glycation end products (Haase-Kohn et al. 2011). Disulfide-linked S100B dimers markedly stimulated nitric oxide production from the microglial cell line BV2 (Matsui Lee et al. 2000). Among the S100 proteins, S100A2 readily undergoes oxidization in both test tubes and intact cells. Our study demonstrated that air oxidation of S100A2 occurred during several months in storage at  $-30^{\circ}\text{C}$  and promoted the formation of dimers or HMM complexes. Furthermore, Cu oxidation increased the relative amounts of these forms (Fig. 1A). The structure of the HMM complexes is not known, but trimers and tetramers of S100A8 and S100A9 have been reported following cross-linking (Teigelkamp et al. 1991), and multimers including tetramers, hexamers, and octamers of S100B have been observed in human brain extract (Ostendorp et al. 2007). These HMM complexes may therefore represent disulfide cross-linked forms of multimeric S100A2 (Deshpande et al. 2000).

We previously showed that S100 proteins significantly activate PP5 phosphatase activity (Yamaguchi et al. 2012). The activation of PP5 by S100 proteins was also validated with a different assay substrate, 6,8-difluoro-4-methylumbelliferyl phosphate (Mazaloukas et al. 2014). PP5 is one of the targets of S100 proteins and is important for oxidative stress responses (Morita et al. 2001). Interestingly, we found that higher concentrations of oxidized S100A2 dra-

matically reduced its ability to activate PP5, whereas lower concentrations of oxidized and freshly-prepared S100A2 could activate PP5 to a similar extent. This phenomenon could be explained by the results from our SPR analysis, which showed that although higher concentrations of oxidized S100A2 lost the ability to bind PP5, oxidized S100A2 could still bind to PP5 at lower concentrations (10–78 nM).

The cysteine residues involved in disulfide bridge formation are conserved among S100 proteins (Marenholz et al. 2004). In addition to the S100A2, the oxidization of other S100 proteins both attenuated their binding to PP5 and PP5 activation (Fig. 3). These results suggest that the modulation of PP5 activity by S100 proteins is not limited to S100A2, but also could encompass other S100 proteins, including S100A1, S100A6, S100P, and S100B. We have previously reported that while oxidized S100A4 fails to bind PP5, this protein nonetheless prevents S100A1-mediated PP5 activation by directly interacting with S100A1 (Tsuchiya et al. 2014). PP5 is ubiquitously expressed in many tissues, with particularly high levels in the brain and neurons (Hinds and Sanchez 2008), and is localized in both the nucleus and cytoplasm. By contrast, the localization of S100 proteins is limited and differs among tissues (Zimmer et al. 1995). It was reported that S100B protects L6 myoblasts from apoptosis under oxidative conditions (Tubaro et al. 2011) and promotes proliferation and dampens NGF-dependent differentiation of PC12 neuronal cells (Arcuri et al. 2005). In pancreatic ductal adenocarcinoma, S100A6 promotes cell migration and invasion (Chen et al. 2015). Our results showed that high concentrations of S100 proteins inhibit PP5 activity, whereas low concentrations still activate PP5 under oxidative conditions. The different cell fate under oxidative conditions could therefore be explained by the amount of S100 proteins in different cell types. When combined with the observation that the degree of PP5 activation varies among S100 proteins, these observations indicate that the activity of PP5 in each tissue could be differentially regulated by S100 proteins under normal versus oxidative stress conditions.

Based on the results of our *in vitro* experiments, we next analyzed and compared the effects of oxidative stress on S100A2 in intact HaCaT and Huh-7 cells. A larger amount of the dimeric form and HMM complexes of S100A2 was observed in Huh-7 cells treated with H<sub>2</sub>O<sub>2</sub>, compared with the amount in HaCaT cells (Fig. 4). In turn, DTT treatment reversed the formation of these structures. These results are consistent with the report that HaCaT cells are resistant to oxidative dimerization of S100A2 (Zhang et al. 2002), but the mechanism responsible for this resistance remains to be determined. Although oxidation of S100A2 and attenuation of PP5 activation were observed in intact cells (Fig. 5), the physiological ramifications are still unclear. Following its binding with S100A2, the transcriptional activity of p53 increases (Mueller et al. 2005). Because p53 contains cysteine residues in its central DNA

binding region, it was proposed that oxidized S100A2 may serve as an electron donor to p53 to maintain its integrity under conditions of oxidative stress (Deshpande et al. 2000). PP5 influences both the phosphorylation state and the ability of p53 to bind DNA. In addition to regulating p53, PP5 functions upstream of p53 to regulate p21<sup>cip1</sup>-mediated G1 cell cycle arrest (Zuo et al. 1998). Inhibition of PP5 induces p21<sup>cip1</sup> expression and marked antiproliferative effects (Zuo et al. 1998). Oxidative stress may therefore regulate p53 function via direct modulation of PP5 activity through its interaction with S100A2.

In addition to these targets, PP5 negatively regulates ASK1 (Morita et al. 2001). ASK1 is a member of the MAPKKK family, and it activates the JNK and p38 MAPK pathways in response to oxidative stress and other pathophysiological stressors (Ichijo et al. 1997; Takeda et al. 2008). ASK1 is involved in ROS-induced cell death and oxidative stress-related diseases such as cancer, cardiovascular disease, and neurodegenerative disease (Tobiume et al. 2001; Nagai et al. 2007). Oxidative stress associated with ROS such as H<sub>2</sub>O<sub>2</sub> increases both the interaction of PP5 with ASK1, and the dephosphorylation of the essential phosphothreonine residue Thr845 of ASK1 by PP5 (Morita et al. 2001). Dephosphorylated ASK1 loses its kinase activity, and ASK1-dependent apoptosis is inhibited (Morita et al. 2001). Given that S100 proteins activate PP5, the increased ASK1 activity following oxidation may be linked to the attenuation of PP5 activation by S100 proteins. Further studies are warranted to improve understanding of the mechanism by which PP5 is regulated by S100 proteins, and the associated physiological effects of this regulation under oxidative stress.

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### Conflict of Interest

The authors declare no conflict of interest.

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