Biochemical and Biophysical Research Communications 435 (2013) 634–639

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Hydrogen peroxide inhibits transforming growth factor-β1-induced cell cycle arrest by promoting Smad3 linker phosphorylation through activation of Akt-ERK1/2-linked signaling pathway

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ARTICLE INFO

*Article history:*
Received 4 May 2013
Available online 17 May 2013

*Keywords:*
$H_2O_2$
TGF-β1
Growth inhibition
Smad3 linker phosphorylation

ABSTRACT

Hydrogen peroxide ($H_2O_2$) functions as a second messenger in growth factor receptor-mediated intracellular signaling cascade and is tumorigenic by virtue of its ability to promote cell proliferation; however, the mechanisms underlying the growth stimulatory action of $H_2O_2$ are less understood. Here we report an important mechanism for antagonistic effects of $H_2O_2$ on growth inhibitory response to transforming growth factor-β1 (TGF-β1). In Mv1Lu and HepG2 cells, pretreatment of $H_2O_2$ (0.05–0.2 mM) completely blocked TGF-β1-mediated induction of $p15^{INK4B}$ expression and increase of its promoter activity. Interestingly, $H_2O_2$ selectively suppressed the transcriptional activation potential of Smad3, not Smad2, in the absence of effects on TGF-β1-induced phosphorylation of the COOH-tail SSXS motif of Smad3 and its nuclear translocation. Mechanism studies showed that $H_2O_2$ increases the phosphorylation of Smad3 at the middle linker region in a concentration- and time-dependent manner and this effect is mediated by activation of extracellular signal-activated kinase 1/2 through Akt. Furthermore, expression of a mutant Smad3 in which linker phosphorylation sites were ablated significantly abrogated the inhibitory effects of $H_2O_2$ on TGF-β1-induced increase of $p15^{INK4B}$-Luc reporter activity and blockade of cell cycle progression from G1 to S phase. These findings for the first time define $H_2O_2$ as a signaling molecule that modulate Smad3 linker phosphorylation and its transcriptional activity, thus providing a potential mechanism whereby $H_2O_2$ antagonizes the cytostatic function of TGF-β1.



## 1. Introduction

Transforming growth factor-β1 (TGF-β1) is a multifunctional cytokine and its signaling controls a diverse set of cellular processes, such as cell growth, apoptosis, differentiation, and cell adhesion [1]. One of the most extensively studied TGF-β1-medited cellular responses is growth inhibition. In normal epithelial cells, TGF-β1 causes cell cycle arrest at $G_1$ stage by up-regulating expression of potent negative regulators of the cell cycle, such as $p15^{INK4B}$ and $p21^{WAF1}$, [2,3] or by down-regulating expression of growth promoting factors, such as c-Myc and Id1 [4,5]. Recent studies also suggest that up-regulation of TGF-β signaling triggered by PTEN loss serves as a growth barrier to constraint proliferation of cancer cells [6,7]. The maintenance of cytostatic function of TGF-β1 thus seems to be crucial for prevention of early-stage carcinogenesis, acting to maintain normal epithelial cell homeostasis.

TGF-β elicits its cellular responses by stimulating Smad pathway through a heteromeric complex of type I (TβRI) and type II (TβRII) transmembrane serine and threonine kinase receptors [8]. Smad3, one component of Smad pathway, plays a central role in conveying anti-proliferative TGF-β1 signal from receptors to the nucleus. In epithelial cells stimulated with TGF-β1, Smad3 mediate transcriptional activation of cyclin-dependent kinase (CDK) inhibitors as well as transcriptional repression of the growth promoting gene c-myc [2–4]. The mouse embryonic fibroblast (MEF) cells derived from Smad3 knockout mouse show resistance to TGF-β1-induced growth inhibition. The Smad3 contains two conserved polypeptide segments, the MH1 and MH2 domains, which are joined together by a less conserved linker region [9]. A growing body of evidences suggest that phosphorylation of not only the C-terminal SXSS motif but also the linker regions of Smad3 appears to have an important role in regulating its transcriptional activity under physiologic and pathologic conditions [10,11]. Several Ser/Thr kinases, including

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http://dx.doi.org/10.1016/j.bbrc.2013.05.035

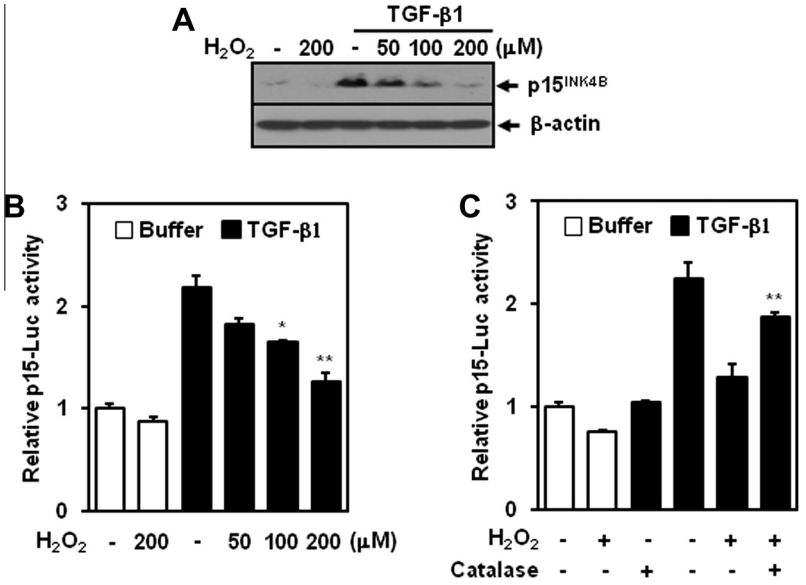


**Fig. 1.** $H_2O_2$ inhibits the stimulatory effect of TGF-β1 on expression of cell cycle inhibitor, $p15^{INK4B}$. (A) Eph4 cells were pretreated with $H_2O_2$ at indicated concentrations for 30 min, and then incubated with TGF-β1 (5 ng/ml) for 24 h. Expression of endogenous $p15^{INK4B}$ and β-actin were observed by immunoblot analysis. (B) HepG2 cells were transiently transfected with $p15^{INK4B}$-Luc reporter (p15-Luc). (C) HepG2 cells were co-transfected with p15-Luc and pCR3.1 or pCR3.1-catalase. After 24 h of transfection, cells were preincubated with $H_2O_2$ at the indicated concentrations for 30 min before stimulation with TGF-β1 (5 ng/ml) for 24 h. Luciferase activities were normalized on the basis of β-galactosidase expression to adjust for variation in transfection efficiency.

extracellular signal-regulated kinase (ERK), cyclin-dependent kinase (CDK), glycogen synthase kinase 3-β (GSK3β), and $Ca^{2+}$-calmodulin-depedent kinase II (CaMKII), have been known to modulate TGF-β signaling through Smad3 linker phosphorylation [12–15], but the biological significance of linker phosphorylated Smad3 pathway remains to be further addressed.

Reactive oxygen species (ROS) including superoxide ($O_2$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical (HO) are constantly produced in aerobic organism during intracellular metabolism [16]. In normal physiological condition, cells maintain the intracellular ROS levels within a non-toxic range by balancing the ROS generation and scavenging systems [16]. Under pathological conditions, excessive ROS generate an array of oxidative lesions within cells [17,18]. However, recent studies also indicate that increased ROS at low-to-moderate levels may function as key signaling molecules that mediate various growth-related responses. For example, locally and transiently increased $H_2O_2$ is required for platelet-derived growth factor-induced cell proliferation [19]. Increased $H_2O_2$ production in the context of oncogene activation promotes growth factor independent-proliferation of cancer cells by increasing cyclin D expression [20]. Despite this association of ROS with cell proliferation, however, the molecular mechanisms underlying action of the growth promoting ROS has remained largely unknown.

In the present study, we provide evidences that $H_2O_2$ serves as a negative signaling molecule to inhibit transcriptional activation potential of Smad3, and consequently override TGF-β1-dependent growth inhibition for epithelial cells. In addition, our data for the first time demonstrate that this antagonistic effect of $H_2O_2$ is mediated mainly via the phosphorylation of Smad3 linker region through activation of Akt-ERK1/2 signaling pathway.

## 2. Materials and methods

### 2.1. Reagents

The recombinant TGF-β1 and XMD8-92, a specific inhibitor of ERK5, were purchased from R&D systems (Minneapolis, MA). Specific inhibitors for MAPK kinase (U0126), p38 MAP kinase (PD169316), JNK1/2 (SP600125), Akt (AktIV), c-Src (SU6656), PKC (Calphostin C), and CaMKIV (KN-62) were obtained from Calbiochem (La Jolla, CA). $H_2O_2$ and N-acetylcysteine (NAC) were purchased from Sigma–Aldrich (St. Louis, MO).

### 2.2. Cell culture

The HepG2 human hepatoblastoma cell lines and Mv1Lu mink lung epithelial cell line were purchased from the American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) or minimum essential medium (Invitrogen, Carlsbad) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C under a humidified 95.5% (v/v) mixture of air and $CO_2$. MCF10A cells were cultured in DMEM/F12 supplemented with 5% horse serum, 10 μg/ml insulin (Biofluids, Rockville, MD), 20 ng/ml EGF (Biofluids, Rockville, MD), 0.5 μg/ml hydrocortisone and 100 ng/ml cholera toxin (both from Sigma, St. Louis, MO).

### 2.3. DNA transfection and luciferase assay

The expression plasmid for human catalase, pCMV-catalase, was obtained from Dr. Jae-Hong Kim (Korea University, Korea). The pCMV-Myc-Smad3 (EPSM) plasmid was gift from Dr. Fang Liu (The State University of New Jersey, USA). Cells were seeded on 24-well plates ($1 \times 10^5$ cells per plate), and co-transfected with 0.5 μg of pGL3-p15 luciferase or pG5E1b luciferase reporter plasmid, together with expression plasmid with FuGENE 6 (Roche, Mannheim) according to the manufacturer's instructions. All the plasmids were co-transfected with 0.2 μg of CMV-β-GAL, a eukaryotic expression vector in which *Escherichia coli* β-galactosidase (Lac Z) structural gene is under the transcriptional control of the CMV promoter. Luciferase reporter activity was assessed on a luminometer with a dual-luciferase Reporter Assay System (Promega, Madison, WI) and the activity was normalized against the CMV-β-GAL gene. Transfection experiments were performed in duplicate with two independently isolated sets, and these results were averaged.

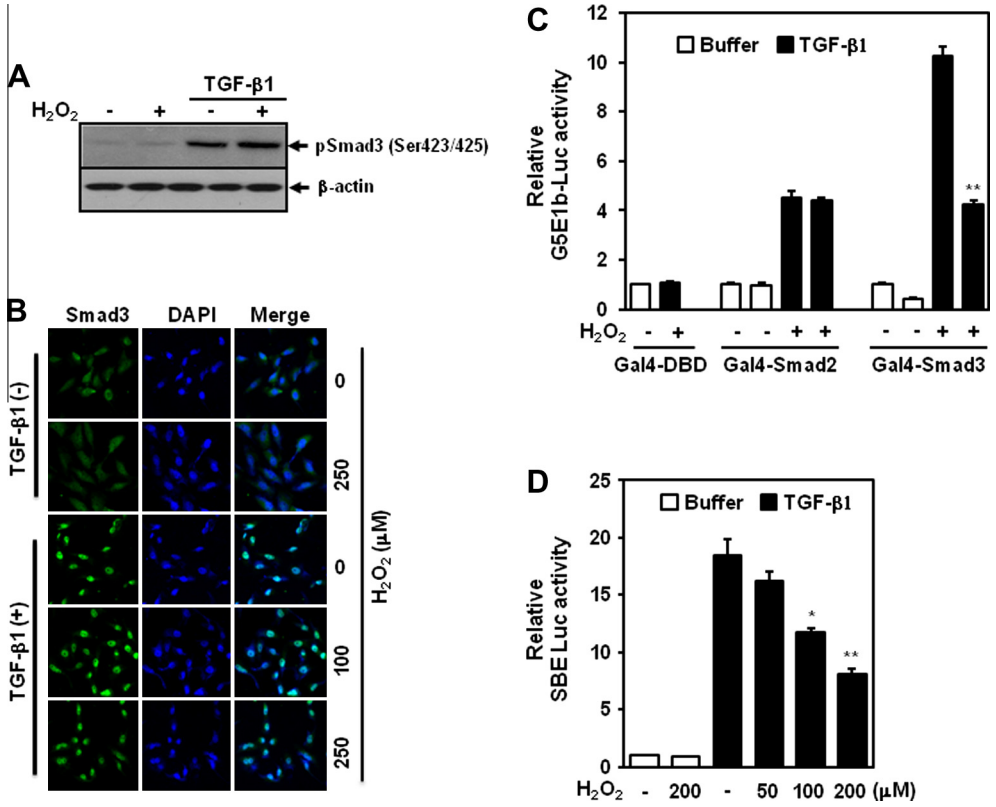


**Fig. 2.** $H_2O_2$ inhibits the TGF-β1-induced Smad3-dependent SBE-Luc reporter activity. (A) Ep4 cells were preincubated with $H_2O_2$ (200 μM) for 30 min before stimulation with TGF-β1 (5 ng/ml) for 30 min. Expression of endogenous phosphor-Smad3 (Ser423/425) and β-actin were observed by immunoblot analysis. (B) Mv1Lu cells were preincubated with $H_2O_2$ (0, 100, and 200 μM) for 30 min before stimulation with TGF-β1 (5 ng/ml) for 60 min. Cells were fixed in 3.5% paraformaldehyde, permeabilized, and immunostained for Smad3 (Alexa488; green). The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; blue). The merger of Alexa488 and DAPI is shown in the right panel. Original magnification, 40×. The images presented here are representative of multiple fields from three independent experiments. (C) HepG2 cells were transiently co-transfected with G5E1b-Luc reporter and GAL4-DBD, GAL4-Smad2 or GAL4-Smad3. After 24 h of transfection, the cells were pre-treated with $H_2O_2$ (200 μM) for 30 min before stimulation with TGF-β1 (5 ng/ml) for 24 h. (D) HepG2 cells were transiently transfected with SBE-Luc reporter. After 24 h of transfection, the cells were pre-treated with the indicated concentrations of $H_2O_2$ for 30 min before stimulation with TGF-β1 (5 ng/ml) for 24 h. Luciferase activities were normalized on the basis of β-galactosidase expression to adjust for variation in transfection efficiency. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

### *2.4. Lentiviral vector production and infection*

The lentiviral vector carrying Smad3 (EPSM) was cloned from pCMV-Myc-Smad3 (EPSM). The lentiviral vector pCAG was digested with Mlu5 and Nhe3, and the two primers used are as follows: Forward, 5′-GATCACGCGTGGATCCCATCGATTTAAAGCT-3′ Reward, 5′-GATCGCTAGCCCCTCTAGATGCATG-3′. For the production of lentivirus, 293T cells were co-transfected with pCAG-GFP or pCAG-Smad3 (EPSM) together with pCMV-△8.91 and pMDM-G (Addgene, Cambridge, MA) by FuGENE6. The virus-containing supernatant was collected 72 h after transfection, cleared by centrifugation (2000 rpm/min, 10 min, and 4 °C), and then filtered through a 0.45 μm filter (Millipore, Billerica, MA). Target cells ($1 \times 10^5$/well) were seeded in 6-well plate, and after incubation at 37 °C for 24 h, the medium of each well was replaced with 1 ml of viral suspension supplemented with 8 mg/ml polybrene (Sigma–Aldrich, St. Louis, MO). Then, the plates were centrifuged at 1200 rpm for 30 min at room temperature, followed by 12 h incubation in standard cell culture condition, and media were replaced with fresh DMEM. After 48 h of additional incubation, the expression of Smad3 (EPSM) was confirmed by immunoblot analysis with anti-myc antibody.

### *2.5. Statistical analysis*

Statistical analyses were performed using SigmaPlot 2001 (Systat Software, Inc., Richmond, CA). Statistical significance was assessed by comparing the means values (±SD) using a Student's *t*-test for paired data.

Details of materials and methods used in this study are provided in Supplementary materials and methods. Western blot analysis, indirect immunofluorescence microscopy, and flow cytometry analysis were performed using standard methods.

## 3. Results and discussion

### *3.1. $H_2O_2$ inhibits TGF-β1-induced $p15^{INK4B}$ expression and its promoter activity*

Previous studies indicated that $H_2O_2$ functions as a second messenger to mediate intracellular signaling cascade leading to growth factor-stimulated cell proliferation. Because growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) are well known to antagonize cytostatic function of TGF-β1, we examined whether $H_2O_2$ can functions as a negative signaling molecule of the TGF-β1 pathway in regulation of the growth inhibition of epithelial cells. Pretreatment of Mv1Lu mink lung epithelial cells with $H_2O_2$ dose-dependently inhibited the expression of cell cycle inhibitor $p15^{INK4B}$ induced by TGF-β1 (Fig. 1A). Concentrations of $H_2O_2$ as low as 50 μM were capable of inhibiting $p15^{INK4B}$ expression. Consistently, reporter gene analysis also showed that TGF-β1-induced $p15^{INK4B}$ promoter activity was dose-dependently decreased by treatment with $H_2O_2$ (Fig. 1B). The inhibitory effect of $H_2O_2$ in the anti-proliferative action of

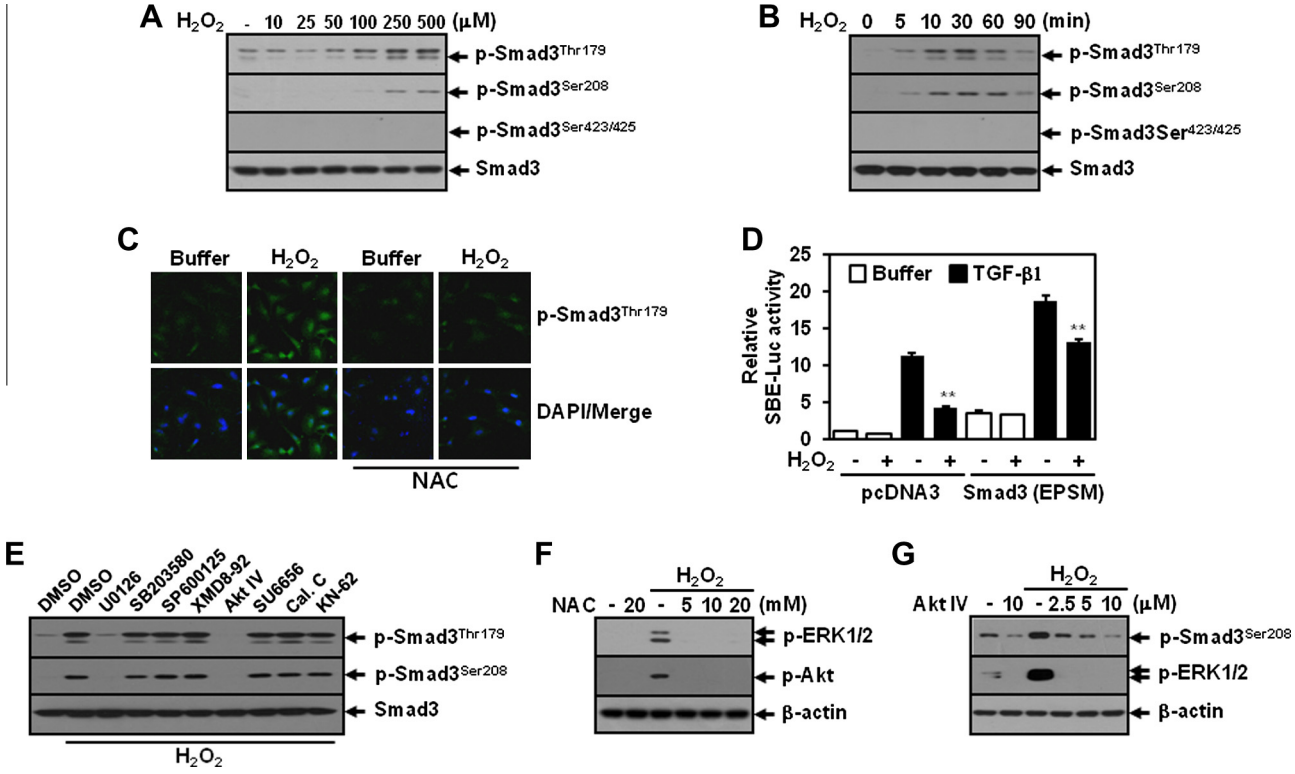


**Fig. 3.** $H_2O_2$ represses Smad3 activity by inducing phosphorylation of its linker region through activation of Akt-ERK1/2 pathway. (A) MCF10A cells were stimulated with the indicated concentrations of $H_2O_2$ for 30 min. (B) Mv1Lu cells were stimulated with $H_2O_2$ (200 μM) for the indicated times. The extent of Smad3 phosphorylation was analyzed by Western blot with phosphospecific Smad3 antibodies against $Thr^{179}$, $Ser^{208}$, and $Ser^{423/425}$. (C) Mv1Lu cells were pre-treated with NAC (20 mM) for 30 min, and then stimulated with $H_2O_2$ (200 μM) for 30 min. Cells were fixed in 3.5% paraformaldehyde, permeabilized, and immunostained for phosphor-Smad3$Thr^{179}$ (Alexa488; green). The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; blue). The merger of Alexa488 and DAPI is shown in the lower panel. Original magnification, 40×. The images presented here are representative of multiple fields from three independent experiments. (D) HepG2 cells were co-transfected with SBE-Luc reporter and pCDNA3 or pCDNA3-Smad3 (EPSM). After 24 h of transfection, cells were preincubated with $H_2O_2$ at the indicated concentrations for 30 min before stimulation with TGF-β1 (5 ng/ml) for 24 h. Luciferase activities were normalized on the basis of β-galactosidase expression to adjust for variation in transfection efficiency. (E) MCF10A cells were pre-incubated with U0126 (10 μM), SB203580 (5 μM), SP600125 (25 μM), XMD8-92 (5 μM), Akt IV (5 μM), SU6656 (10 μM), Calphostin C (Cal. C, 50 nM), and KN-62 (20 μM) for 30 min, respectively, and then treated with $H_2O_2$ (200 μM) for 30 min. MCF10A cells were pre-incubated with the indicated concentrations of (F) NAC or (G) Akt IV, and then treated with $H_2O_2$ (200 μM) for 30 min. Western blot analysis with phosphospecific Smad3 antibodies against $Thr^{179}$ (E) or against $Ser^{208}$ (E and G) or with phosphospecific antibody to ERK1/2 (F and G) or Akt (F). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

TGF-β1 was confirmed by co-transfection of cells with plasmid encoding catalase, a $H_2O_2$ scavenging enzyme, which significantly blocked $H_2O_2$-mediated inhibition of TGF-β1-induced $p15^{INK4B}$ promoter activity (Fig. 1C). These results suggest that $H_2O_2$ acts as a negative regulator of anti-proliferative function of TGF-β1.

### 3.2. Effects of $H_2O_2$ on TGF-β1-mediated Smad3 activation

In order to ascertain a mechanism underlying inhibition of TGF-β1-mediated cell cycle arrest by $H_2O_2$, we investigated effects of $H_2O_2$ on TGF-β1-mediated activation of Smad3, which is a central mediator for conveying TGF-β growth inhibitory signal from receptors to the nucleus. A key event in Smad3 activation by TGF-β1 is its phosphorylation at carboxyl (C)-terminal SSXS motif, which triggers accumulation of Smad3 into the nucleus. In Eph4 mouse mammary epithelial cells pretreated with $H_2O_2$, a little difference on its C-terminus phosphorylation was seen with TGF-β1 treatment compared to $H_2O_2$-untreated cells (Fig. 2A). We also found that TGF-β1 induces the nuclear translocation of Smad3 in $H_2O_2$-pretreated cells without remarkable difference to that seen in the control cells (Fig. 2B). To determine whether $H_2O_2$ can directly suppress transcriptional activities of R-Smads, Smad2 and Smad3, we used a heterologous reporter assay in which GAL4 DNA-binding domain was fused to the Smad2 or Smad3 protein. GAL4-Smad2 or GAL4-Smad3 construct was co-transfected with a luciferase reporter construct (G5E1b-Luc), which contained five GAL4-binding sites upstream of the AdE1B TATA box. As shown in Fig. 2C, TGF-β1 treatment did not affect transcription by the minimal DNA-binding domain (GAL4-DBD) while it strongly induced transcriptional activity of GAL4-Smad2 or GAL4-Smad3 fusion protein. Interestingly, TGF-β1-mediated stimulation of transcriptional activity of GAL4-Smad2 did not affected by $H_2O_2$ whereas $H_2O_2$ strongly suppressed the transcriptional activity of GAL4-Smad3 stimulated by TGF-β1 (Fig. 2C). The basal activity of GAL4-Smad3 was also significantly suppressed by $H_2O_2$. To further confirm the inhibitory effect of $H_2O_2$ on TGF-β1-mediated Smad3-dependent transcriptional activation, HepG2 cells were transiently transfected with an artificial $SBE_4$-Luc reporter construct, which comprises four tandem repeats of Smad-binding elements and measures a Smad3/4-specific response. The TGF-β1-mediated increase in $SBE_4$-Luc reporter activity was strongly abolished by $H_2O_2$ in a dose-dependent manner (Fig. 2D). These results indicate that $H_2O_2$ can directly suppress transcriptional activity of Smad3 without affecting its canonical activation mode such as COOH-tail phosphorylation and nuclear transport.

### 3.3. $H_2O_2$ inhibits transcriptional activation potential of Smad3 by inducing its linker phosphorylation through activation of Akt-ERK1/2 pathway

Since it has become increasingly clear that phosphorylation status of Smad3 in the linker region connecting conserved two MH1 and MH2 domains has an important regulatory influence on its transcriptional activity [9–11], we examined whether $H_2O_2$ affects

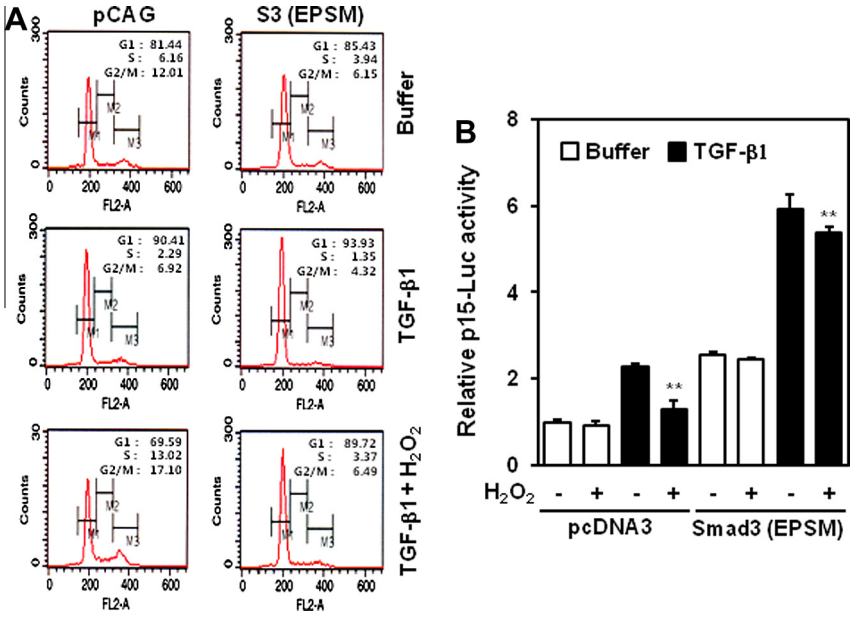


**Fig. 4.** The linker phosphorylated Smad3 mediates $H_2O_2$-induced suppression of TGF-β1-induced cell cycle arrest and p15-Luc reporter activity. (A) MCF10A cells were infected with lentiviral particles carrying a Smad3 EPSM gene or a control vector, pCAG. After 36 h of infection, the cells were pre-incubated with $H_2O_2$ (200 μM) for 30 min, and then treated with TGF-β1 (5 ng/ml) for 24 h. FACScan analysis show the percentages of cells at G1, S/G2, and M phases. (B) HepG2 cells were transiently co-transfected with p15-Luc and pcDNA3 or pcDNA3-Smad3 (EPSM). After transfection, the cells were pre-incubated with indicated concentrations of $H_2O_2$ for 30 min before stimulation with TGF-β1 (5 ng/ml) for 24 h. Luciferase activities were normalized on the basis of β-galactosidase expression to adjust for variation in transfection efficiency.

Smad3 linker phosphorylation. Treatment of MCF10A cells with various concentrations of $H_2O_2$ resulted in dose-dependent increases in phosphorylation of Smad3 at Thr-179 and Ser-208 (Fig. 3A). Similar finding was seen for Mv1Lu cells treated with $H_2O_2$ (100 μM) at various time periods (Fig. 3B). The Smad3 linker phosphorylation reached a peak at 10 min and then declined to baseline within 90 min. Immunofluorescence analysis also showed that $H_2O_2$-induced linker-phosphorylated Smad3 at Thr-179 is substantially localized to cell nuclei and this significant increase of linker phosphorylated Smad3 in the nucleus was completely abolished in Mv1Lu cells co-treated with $H_2O_2$ and *N*-acetyl-L-cysteine (NAC), a ROS scavenger (Fig. 3C), implying Smad3 linker region as a potential target of $H_2O_2$ to modulate Smad3 transcriptional activity. We thus determined whether phosphorylation of Smad3 in the linker region plays an important role in the suppressive effect of $H_2O_2$ on the transcription activation potential of Smad3 stimulated by TGF-β1. As shown in Fig. 3D, inhibition of TGF-β1-stimulated $SBE_4$-Luc reporter activity by $H_2O_2$ was substantially reduced by co-transfection with a Smad3 EPSM (ERK-/Pro-directed kinase site mutant) plasmid which lacking four phosphorylation sites in the linker region.

Because several intracellular kinases, including mitogen-activated protein kinases, cyclin-dependent kinase, GSK 3β and $Ca^{2+}$/calmodulin-dependent protein kinase II, have been implicated in the induction of Smad3 linker phosphorylation [12–15], we next investigated pathways responsible for $H_2O_2$-induced phosphorylation of Smad3 linker region. To do this, we employed specific inhibitors for each of pathways together with $H_2O_2$. The $H_2O_2$-mediated increase in linker phosphorylated Smad3 level was strongly blocked by pretreatment of either U0126, a specific inhibitor of MEK1/2, or Akt IV, a specific inhibitor of Akt, whereas other inhibitors had no significant effect (Fig. 3E). Consistent with this result, treatment with $H_2O_2$ resulted in an enhanced phosphorylation of ERK1/2 and Akt and this effect was completely abolished by pretreatment with NAC (Fig. 3F). In addition, the Akt inhibitor Akt IV perfectly blocked ERK1/2 phosphorylation induced by $H_2O_2$ (Fig. 3G), suggesting that Akt acts upstream of ERK1/2 kinase in $H_2O_2$ signaling to Smad3 linker phosphorylation. These results indicate that $H_2O_2$ directly suppressed Smad3 transcriptional activity by inducing Smad3 linker phosphorylation through the activation of Akt-ERK1/2 signaling cascade.

### *3.4. Linker phosphorylation of Smad3 is critical event for the $H_2O_2$-mediated inhibition of anti-proliferative TGF-β1 signaling*

To determine the effect of linker phosphorylated Smad3 on inhibition of TGF-β1-induced cell cycle arrest by $H_2O_2$, we infected MCF10A cells with lentiviral particles encoding Smad3 EPSM gene. FACS analysis showed that overexpression of Smad3 EPSM resulted in a significant rescue against disruption of TGF-β1-induced $G_1$ cell cycle arrest by $H_2O_2$ (Fig. 4A). In parallel, $H_2O_2$-mediated suppression of TGF-β1-induced $p15^{INK4B}$ promoter reporter activity was also significantly attenuated by co-transfection with Smad3 EPSM (Fig. 4B). These results indicate that the ablation of Smad3 linker phosphorylation is responsible for the suppression of $H_2O_2$ effect by the Smad3 EPSM.

The coordinated and properly balanced regulation between two opposite growth inhibition and stimulation signaling pathways is important for maintaining normal physiological conditions and preventing tumor occurrence. Accumulating evidences indicate that increased ROS at low-to-moderate levels function as important signaling molecules that mediate cell proliferation response [19,20]. In addition, elevated levels of ROS observed in cancer cells are known to derive aberrant cell growth [21]. These various observations thus support the notion that maintenance of a high level of intracellular ROS may serve as a major contributing factor in tipping the balance of the growth regulation toward proliferation. However, so far the interplay between ROS and growth inhibition pathway has not been well established.

The transiently and locally generated $H_2O_2$ in response to growth factors is generally considered as a second messenger to activate receptor tyrosine kinase pathway via an oxidative inhibition of protein tyrosine phosphatase [22]. Recent studies show that cytostatic effects of TGF-β1 can be negatively modulated

by activation of survival and proliferation signals, such as the Akt/mTOR or the Ras/Erk pathway [23,24]. In addition, Ortiz et al. demonstrate that deficiency of protein-tyrosine phosphatase 1B confers resistance to anti-proliferative TGF-β1 response [25]. These observations raise a possibility that $H_2O_2$ can serve as a critical signaling molecule in negative regulation of the cytostatic function of TGF-β1. Our finding that $H_2O_2$ strongly blocks the stimulatory effect of TGF-β1 on expression of cell cycle inhibitor, $p15^{INK4B}$, supports this speculation.

Smad3 plays a central role in conducting anti-proliferative TGF-β1 signal into cells. A growing body of evidences indicates that phosphorylation of Smad3 in the linker region appears to have an important role in regulating its transcriptional activity [10,11], although the biological significance of linker phosphorylated Smad3 pathway remains to be further determined. Our mechanism study clearly demonstrate that $H_2O_2$ represses TGF-β1-induced transcriptional activation potential of Smad3, but not Smad2, without affecting its COOH-tail phosphorylation and nuclear translocation, and this repression is accompanied with Akt-mediated ERK1/2-dependent phosphorylation of Smad3 in the linker region. Furthermore, we provide clear evidence that the ablation of Smad3 linker phosphorylation is responsible for the suppression of $H_2O_2$ effect on TGF-β1-induced cell cycle arrest. Consequently, Smad3 linker phosphorylaion through Akt-ERK1/2-linked pathway represents a straightforward mechanism by which $H_2O_2$ can negatively regulate anti-proliferative function of TGF-β1. Thus, the data presented here for the first time define a potential role of $H_2O_2$ as an intracellular signaling molecule to antagonize growth inhibitory function of TGF-β1.

## Acknowledgments

We thank Dr. Fang Liu (The State University of New Jersey, USA) for providing phospho-Smad3 (Ser-208) and phospho-Smad3 (Thr-179) antibodies. This work was supported by a grant from the National R&D Program for Cancer Control, Ministry for Health and Welfare, Republic of Korea (1020420) and by the National Research Foundation Grant of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2009-0081 756).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.035.

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