Signaling in H$_2$O$_2$-Induced Increase in Cell Proliferation in Barrett's Esophageal Adenocarcinoma Cells

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ABSTRACT

Mechanisms whereby acid reflux may accelerate the progression from Barrett's esophagus (BE) to esophageal adenocarcinoma (EA) are not fully understood. We have previously shown that NADPH oxidase NOX5-S generates reactive oxygen species (ROS) when Barrett's metaplastic cells are exposed to acid. Besides metaplastic cells, other H$_2$O$_2$-producing cells (e.g., inflammatory cells) present in BE mucosa may produce additional ROS, which may also affect metaplastic cells contributing to esophageal tumorigenesis. In this study, we investigate whether exogenous H$_2$O$_2$ stimulates cell proliferation by increasing NOX5-S expression. Low dose (10^{-13} M) of H$_2$O$_2$ significantly increased thymidine incorporation, NOX5-S mRNA, and protein expression in a Barrett's EA cell line FLO. H$_2$O$_2$-induced increase in NOX5-S expression was significantly inhibited by knockdown of nuclear factor (NF)-κB1 p50 with p50 small interfering RNA (siRNA) in EA cell lines FLO and OE33. H$_2$O$_2$ significantly increased p65 phosphorylation and the luciferase activity in FLO cells transfected with a NF-κB activation reporter plasmid pNF-κB-Luc. H$_2$O$_2$-induced increase in luciferase activity in FLO cells was significantly decreased by knockdown of extracellular signal-regulated kinase 2 (ERK2) mitogen-activated protein kinase (MAPK). Overexpression of p50 and p65 remarkably increased the luciferase activity in FLO cells transfected with a NOX5-S reporter plasmid NOX5-LP. In addition, H$_2$O$_2$-induced thymidine incorporation in FLO cells was significantly decreased by the MAPK kinase 1/2 inhibitor 2′-amino-3′methoxyflavone (PD98059) and ERK2 siRNA but not by ERK1 siRNA. Likewise, H$_2$O$_2$-induced increase in NOX5-S expression was significantly decreased by ERK2 siRNA in FLO and OE33 cells. We conclude that a low dose of H$_2$O$_2$ increases cell proliferation. H$_2$O$_2$-induced increase in cell proliferation may depend on sequential activation of ERK2 MAPK, NF-κB1 p50, and NOX5-S.

Introduction

Esophageal adenocarcinoma has increased in incidence at a rate exceeding that of any other cancers (Blot and McLaughlin, 1999; Howe et al., 2001; Pohl and Welch, 2005). The major risk factor for esophageal adenocarcinoma is gastroesophageal reflux disease complicated by Barrett's esophagus (BE) (Lagergren et al., 1999). Approximately 10% of gastroesophageal reflux disease patients develop BE where esophageal squamous epithelium damaged by acid reflux is replaced by a metaplastic, intestinal-type epithelium. The specialized intestinal metaplasia of BE is associated with a 30- to 125-fold increased risk for the development of esophageal adenocarcinoma (Haggitt, 1994; Kim et al., 1997; Wild and Hardie, 2003). However, mechanisms of the progression from metaplasia (BE) to adenocarcinoma are not fully understood.

Reactive oxygen species (ROS) may be an important factor mediating this progression because 1) high levels of ROS are present in BE (Olyaee et al., 1995; Wetscher et al., 1997) and in esophageal adenocarcinoma (Farhadi et al., 2002; Sihvo et al., 2003) and 2) ROS may damage DNA, RNA, lipids, and proteins, leading to increased mutation and altered functions of enzymes and proteins (e.g., activation of oncogene products and/or inhibition of tumor suppressor proteins) (Farhadi et al., 2002; Ohshima et al., 2003). Besides metaplastic cells, other cells (e.g., inflammatory cells) in BE mucosa may also produce ROS and affect metaplastic cells.

Lower levels of ROS, seen in nonphagocytic cells, were thought to be byproducts of aerobic metabolism. More recently, superoxide-generating homologs of phagocytic NADPH oxidase-catalytic subunit gp91phox (NOX1, NOX3–NOX5, DUOX1, and

ABBREVIATIONS: BE, Barrett's esophagus; NOX, NADPH oxidase; ROS, reactive oxygen species; EA, esophageal adenocarcinoma; NF-κB, nuclear factor-κB; Luc, luciferase; MAPK, mitogen-activated protein kinase; siRNA, small interfering RNA; ERK1/2, extracellular signal-regulated kinase 1/2; MEK, mitogen-activated protein kinase kinase; PD98059, 2′-amino-3′methoxyflavone; DMEM, Dulbecco's modified Eagle's medium; SN50, AAVALLPAVLLALLAPVQRKRQKLMP; RT-PCR, reverse transcription-polymerase chain reaction; ANOVA, analysis of variance.

DUOX2) and homologs of other subunits (p41phox or NOXO1, p51phox, or NOXA1) have been found in several cell types (Suh et al., 1999; Bánfi et al., 2000; Lambeth, 2004), suggesting that ROS generated in these cells may have distinctive cellular functions. We have shown that NOX5-S is the major isoform of NADPH oxidase in FLO EA cells (Hong et al., 2010b) and that the expression of NOX5-S is significantly higher in BE with high-grade dysplasia than in BE without dysplasia (Fu et al., 2006). The expression of NOX5-S is also significantly higher in FLO cells than in esophageal squamous epithelial cells (Hong et al., 2011). We have also shown that acid-induced H_2O_2 production is mediated by the NADPH oxidase NOX5-S (Hong et al., 2010c). Overproduction of ROS, derived from up-regulation of NOX5-S, increases cycloxygenase-2-derived prostaglandin E_2 production (Fu et al., 2006) and down-regulates a tumor suppressor gene p16 (Hong et al., 2010c), thus increasing cell proliferation and decreasing apoptosis. These changes may contribute to progression from BE to dysplasia and to adenocarcinoma. However, whether exogenous ROS increase cell proliferation via up-regulation of NOX5-S in EA cells is not known. In the present study, we find that H_2O_2 increases cell proliferation by sequential activation of mitogen-activated protein kinase (MAPK), NF-κB, and NOX5-S.

Materials and Methods

Cell Culture and H_2O_2 Treatment. Human Barrett's adenocarcinoma cell line FLO was derived from human Barrett's esophageal adenocarcinoma (Hughes et al., 1997) and generously provided by Dr. David Beer (University of Michigan, Ann Arbor, MI). These cells were cultured in DMEM containing 10% fetal bovine serum and antibiotics at 37°C in a 5% CO_2 humidified atmosphere. Human EA cell line OE33 was purchased from Sigma-Aldrich (St. Louis, MO) and cultured in DMEM containing 10% fetal bovine serum and antibiotics. The cell lines were cultured at 37°C in a 5% CO_2-humidified atmosphere.

For H_2O_2 treatment, FLO cells were incubated with different concentrations of H_2O_2 (10^{-5}, 10^{-7}, 10^{-9}, 10^{-11}, 10^{-13}, 10^{-14}, and 10^{-15}M) for 48 h. For inhibitor treatment, FLO cells were exposed to DMEM plus H_2O_2 (10^{-13} M) in the absence or presence of MEK1 kinase inhibitor PD98059 (2′-amino-3′-methoxyflavone, 10^{-5} M) or cell-permeable NF-κB inhibitor SN50 (AAVALLPAVLLALLAPVQRKRQKLMP, 10^{-5} M) for 24 h. The culture medium and cells were then collected for measurement of H_2O_2 and NOX5-S mRNA level.

Small Interfering RNA and Plasmid Transfection. For small interfering RNA (siRNA) transfection, at 40 to 50% confluence, cells were trypsinized and diluted 1:5 with fresh medium without antibiotics (1–3 × 10^5 cells/ml) and transferred to 12-well plates (1 ml/well). Transfection of siRNAs was carried out with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For each well, 60 pmol of siRNA duplex of NOX5, p50, ERK2, ERK1, or control siRNAs formulated into liposomes were applied; the final volume was 1.2 ml/well. Twenty-four hours later, the transfectants were exposed to H_2O_2 (10^{-13} M) in fresh medium for an additional 48 h. Finally, the culture medium and the transfectants were collected for measuring NOX5-S mRNA and protein level. Transfection efficiencies were determined by fluorescence microscopy after transfection of Block-it fluorescent oligo (Invitrogen) and were approximately 90% at 48 h. Control siRNA is a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA. For plasmid transfection, 0.5-μg plasmids (NOX5-LP, pNF-κB-Luc, or pCDNA3.0) or 0.1-μg *Renilla reniformis* luciferase plasmid formulated into liposomes were applied. All other procedures were similar to those described above.

RT-PCR. Total RNA was extracted by TRIzol reagent (Invitrogen) according to the protocol of the manufacturer, and 1.0 μg of total RNAs were reversely transcribed by use of the kit SUPERSCRIPT First-Strand Synthesis System for RT-PCR (Invitrogen).

Quantitative Real-Time PCR. Gene expression and regulation were measured using real-time PCR analysis. Random hexamers were used for real-time PCR analysis performed in a 15-μl reaction on a 96-well clear plate using Power SYBR Green RT-PCR reagents kit (Applied Biosystems). The primers used were as follows: NOX5-S sense (5′-AAGACTCCATCACGGGGCTGCA-3′), NOX5-S antisense (5′-CCTTCAGCACCTTGGCCAGA-3′), 18S sense (5′-CGGACAGGATTGACAGATTGATAGC-3′), and 18S antisense (5′-TGCCAGAGTCTCGTTCGTTATCG-3′). Reactions were carried out in an Applied Biosystems StepOnePlus real-time PCR system for one cycle at 94°C for 5 min; 40 cycles at 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s; 1 cycle at 94°C for 1 min; and 1 cycle at 55°C for 30 s. Fluorescence values of SYBR Green I dye, representing the amount of product amplified at that point in the reaction, were recorded in real time at both the annealing step and the extension step of each cycle. The C_t, defined as the point at which the fluorescence signal was statistically significant above background, was calculated for each amplicon in each experimental sample using a StepOne software. This value was then used to determine the relative amount of amplification in each sample by interpolating from the standard curve. The transcript level of each specific gene was normalized to 18S amplification.

Luciferase Assay. FLO EA cells were seeded in 12-well plates for 24 h. *R. reniformis* luciferase (0.1 μg) and 0.5 μg of pCDNA3.0 (control) or NOX5-S luciferase reporter plasmid NOX5-LP containing the NOX5-S promoter fragment (−2501 to −1 from ATG) (Si et al., 2007) or pNF-κB-Luc plasmid were transfected by using Lipofectamine 2000 (Invitrogen). Luciferase activity was assayed 24 h after transfection. Cell extracts were prepared by lysing the cells with lysis buffer (Promega, Madison, WI). The lysate was centrifuged at 13,000 rpm for 10 min to pellet the cell debris. The luciferase activities in the cell lysates were measured by using a Packard TopCount-NXT microplate scintillation and luminescence counter (PerkinElmer Life and Analytical Sciences, Waltham, MA) according to the protocol (Promega) and normalized to *R. reniformis* luciferase. The number of experiments was indicated in figure legends, and each experiment was performed in triplicate.

Western Blot Analysis. Cells was lysed in Triton X-100 lysis buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% (v/v) Triton X-100, 40 mM β-glycerol phosphate, 40 mM *p*-nitrophenylphosphate, 200 μM sodium orthovanadate, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml aprotinin. The suspension was centrifuged at 15,000*g* for 5 min, and the protein concentration in the supernatant was determined. Western blot was done as described previously (Cao et al., 2003; Fu et al., 2006). In brief, after these supernatants were subjected to SDS-polyacrylamide gel electrophoresis, the separated proteins were electrophoretically transferred to a nitrocellulose membrane at 30 V overnight. The nitrocellulose membranes were blocked in 5% nonfat dry milk and then incubated with appropriate primary antibodies followed by a 60-min incubation in horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). Detection was achieved with an enhanced chemiluminescence agent (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Primary antibodies used were human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:1000), NOX5-S antibody (1:1000), p-ERK1/2 antibody (1:1000), ERK2 antibody (1:1000), p-p65 antibody (1:1000), and p65 antibody (1:1000). NOX5 antibody against a unique NOX5 peptide (NH₂-CLQTRTQPGRPDWSKV-COOH) was prepared by Sigma-Genosys (The Woodlands, TX) and used at a dilution of 1:1000.

Protein Measurement. The amount of protein was determined by colorimetric analysis using the protein assay kit from Bio-Rad

Laboratories (Hercules, CA) according to the Bradford method (Bradford, 1976).

[³H]Thymidine Incorporation. For PD98059 treatment, cells were preincubated with PD98059 10^{-5} M for 1 h and then treated without (control) or with H_2O_2 (10^{-13} M) in the absence (control) or presence of PD98059 for 48 h. For siRNA transfection 24 h after siRNAs of NOX5, p50, ERK2, ERK1, or control was introduced, cells were treated without or with H_2O_2 (10^{-13} M) for 48 h, and then incubated with *methyl*-[³H]thymidine (0.05 μCi/ml) for 4 h. After being washed three times with phosphate-buffered saline to remove unincorporated radioactivity, cells were collected and homogenized with a lysis buffer containing 50 mM HEPES, 50 mM NaCl, 1% Triton X-100, 1% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol, pH 7.4. [*methyl*-³H]Thymidine uptake was measured in a TopCount-NXT microplate scintillation and luminescence counter (Packard Bioscience Company). The level of protein in the homogenates was also determined, and the level of [*methyl*-³H]thymidine incorporation was normalized to protein content.

Amplex Red Hydrogen Peroxide Fluorescent Assay. Levels of H_2O_2 in culture medium were determined by the Amplex Red H_2O_2 assay kit (Invitrogen), according to the manufacturer's instruc-

tion. This assay uses the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) to detect H_2O_2. In the presence of peroxidase, the Amplex Red reagent reacts with H_2O_2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product resorufin. Fluorescence is then measured with a fluorescence microplate reader with an excitation at 540 nm and emission detection at 590 nm.

Materials. Human NOX5 siRNA was purchased from Applied Biosystems (Foster City, CA). PD98059 and SN50 were bought from Calbiochem (San Diego, CA); ERK2 antibody, GAPDH antibody, control siRNA, ERK2 siRNA, ERK1 siRNA, and p50 siRNA were from Santa Cruz Biotechnology; and phosphorylated MAPK antibody, phosphorylated p65 antibody, and p65 antibody were from Cell Signaling Technology (Danvers, MA). pNF-κB-Luc vector was purchased from Clontech Laboratories, Inc. (Mountain View, CA). P50 plasmid (Ballard et al., 1992) was from Addgene (Cambridge, MA). Triton X-100, phenylmethylsulfonyl fluoride, DMEM, antibiotics, and other reagents were purchased from Sigma-Aldrich.

Statistical Analysis. Data were expressed as means ± S.E. Statistical differences between two groups were determined by Student's *t* test. Differences among multiple groups were tested by
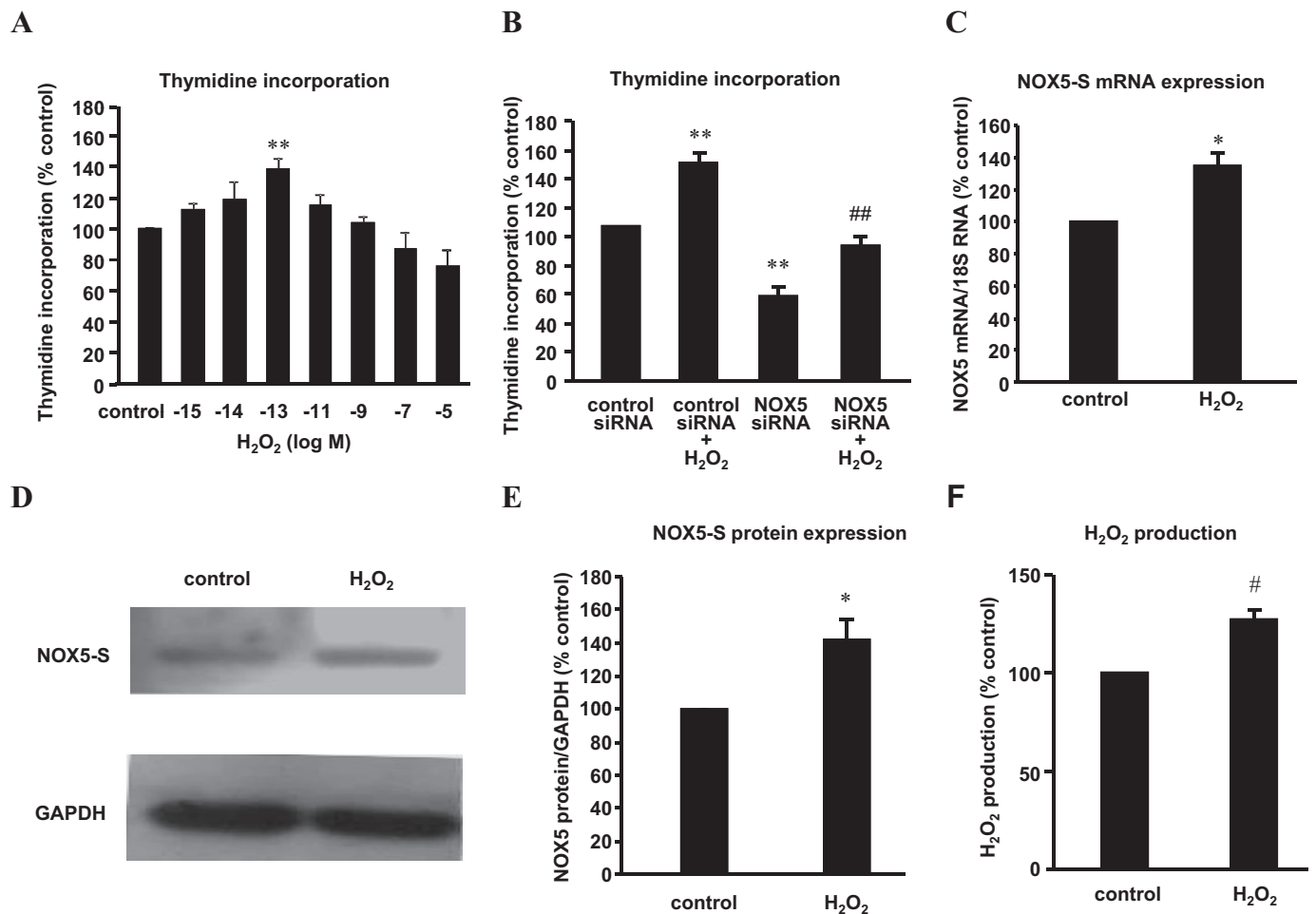

Fig. 1. H_2O_2 up-regulates NOX5-S expression in FLO cells. A, FLO cells were incubated with different concentrations of H_2O_2 (10^{-5}, 10^{-7}, 10^{-9}, 10^{-11}, 10^{-13}, 10^{-14}, 10^{-15} M) for 48 h and then incubated with *methyl*-[³H]thymidine (0.05 μCi/ml) for 4 h. H_2O_2 (10^{-13} M) significantly increased thymidine incorporation, whereas higher doses (10^{-5} and 10^{-7} M) slightly decreased thymidine incorporation. B, FLO cells were treated with H_2O_2 (10^{-13} M, 48 h) 24 h after NOX5 siRNA and control siRNA were introduced into FLO cells by Lipofectamine 2000. Knockdown of NOX5-S significantly decreased thymidine incorporation at basal condition and in response to H_2O_2 treatment. C, FLO cells were treated with 10^{-13} M H_2O_2 for 48 h, and then NOX5-S mRNA levels were measured by real-time PCR. H_2O_2 (10^{-13} M) significantly increased NOX5-S mRNA levels. D and E, a typical image of three Western blot analyses (D) and summarized data (E) show that 10^{-13} M H_2O_2 significantly increased NOX5-S protein level. FLO cells were treated with 10^{-13} M H_2O_2 for 48 h. F, FLO cells were treated with 10^{-13} M H_2O_2 for 48 h, washed, and cultured for an additional 24 h. H_2O_2 levels in culture medium were measured by using an Amplex Red H_2O_2 assay kit. A 48-h H_2O_2 treatment significantly increased H_2O_2 production ($n = 3$). *t* test, ∗, $P < 0.05$, #, $P < 0.01$; ANOVA, ∗∗, $P < 0.01$, compared with control or control siRNA group; ANOVA, ##, $P < 0.01$, compared with control siRNA plus H_2O_2 group.

ANOVA and checked for significance by Fisher's protected least significant difference test.

Results

H$_2$O$_2$ Up-regulates NOX5-S Expression. To investigate whether H$_2$O$_2$ affects cell proliferation, we obtained a H$_2$O$_2$ dose-response curve. Figure 1A shows that 10^{-13} M H$_2$O$_2$ significantly increased thymidine incorporation by $39.3 \pm 6.6\%$ in FLO cells, whereas higher doses (10^{-5} and 10^{-7} M) slightly decreased thymidine incorporation. The data suggest that low dose of H$_2$O$_2$ may increase cell proliferation in FLO cells.

We have shown that NOX5-S mediates acid-induced increase in cell proliferation. Therefore, we studied whether NOX5-S mediates exogenous H$_2$O$_2$-induced increase in cell proliferation. We found that knockdown of NOX5-S with NOX5 siRNA, which has been shown by us to effectively knock down NOX5-S (Fu et al., 2006), significantly decreased thymidine incorporation at basal condition as well as in response to H$_2$O$_2$ treatment in FLO cells (Fig. 1B), suggesting

that NOX5-S may mediate H$_2$O$_2$-induced increase in cell proliferation.

Next, we examined whether H$_2$O$_2$ up-regulates NOX5-S. NOX5-S mRNA was measured by real-time PCR. H$_2$O$_2$ (10^{-13} M; 48-h treatment) significantly increased NOX5-S mRNA level by $34.9 \pm 7.8\%$ (ANOVA, $P < 0.05$; Fig. 1C) and protein level by $42.5 \pm 11.6\%$ (ANOVA, $P < 0.05$; Fig. 1, D–E) in FLO cells. Likewise, H$_2$O$_2$ significantly increased the NOX5-S mRNA level by $93.9 \pm 13.5\%$ in an EA cell line OE33 (ANOVA, $P < 0.01$; Fig. 2D). To examine whether up-regulation of NOX5-S enhances H$_2$O$_2$ production, FLO cells were incubated with 10^{-13} M H$_2$O$_2$ for 48 h, washed, and then cultured in regular culture medium for additional 24 h. Culture medium was collected for measurement. Forty-eight-hour treatment of H$_2$O$_2$ significantly increased H$_2$O$_2$ production by $27.6 \pm 4.9\%$ (t test, $P < 0.01$; Fig. 1F).

Role of NF-κB in H$_2$O$_2$ Induced NOX5-S Expression. To examine the role of NF-κB in H$_2$O$_2$-induced NOX5-S expression, we used p50 siRNA, which has been shown by us to
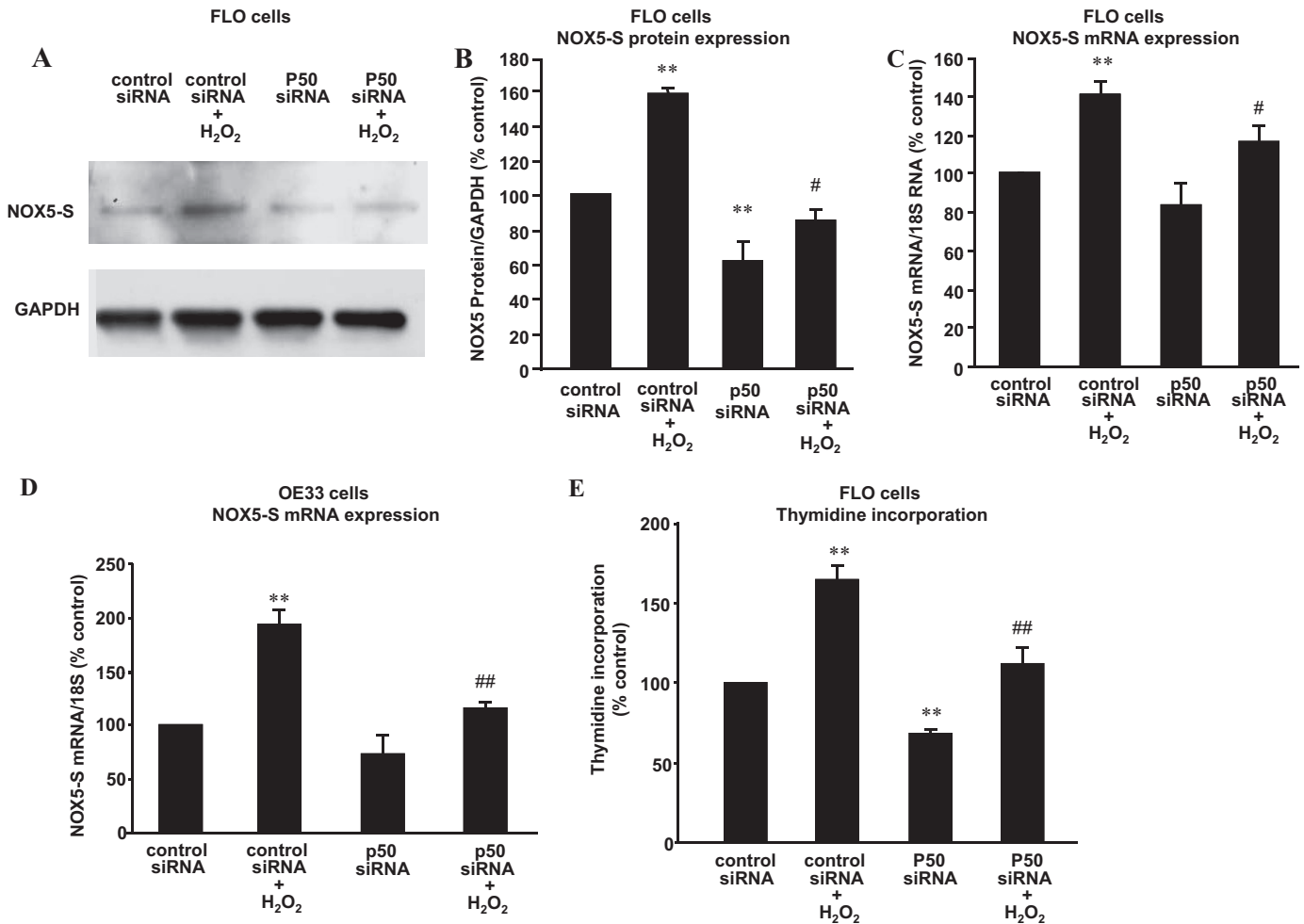

Fig. 2. Role of NF-κB in H$_2$O$_2$-induced NOX5-S expression. Transfection of p50 siRNA and control siRNA was carried out with Lipofectamine 2000. After a 4-h transfection, the transfection medium was replaced with regular medium. Twenty-four hours later, the transfectants were exposed to H$_2$O$_2$ (10^{-13} M) in fresh medium for an additional 48 h. A and B, a typical image of three Western blot analyses (A) and summarized data (B) show that knockdown of p50 significantly decreased NOX5-S protein expression at basal condition and in response to H$_2$O$_2$ treatment in FLO cells. C, knockdown of p50 significantly decreased NOX5-S mRNA levels in response to H$_2$O$_2$ treatment in FLO cells. D, knockdown of p50 significantly decreased NOX5 mRNA levels in response to H$_2$O$_2$ treatment in OE33 cells. E, knockdown of p50 significantly decreased not only thymidine incorporation at basal condition but also H$_2$O$_2$-induced increase in thymidine incorporation in FLO cells. The data suggest that p50 mediates H$_2$O$_2$-induced increase in NOX5-S expression and in cell proliferation. $n = 3$, ANOVA, ∗, $P < 0.05$, ∗∗, $P < 0.01$, compared with control siRNA group; ANOVA. #, $P < 0.05$, compared with control siRNA + H$_2$O$_2$ group; ##, $P < 0.01$, compared with control siRNA group plus H$_2$O$_2$ group.

effectively knock down p50 (Hong et al., 2010a). Figure 2, A, B, and C, shows that knockdown of p50 significantly decreased NOX5-S mRNA levels from 140.7 ± 6.8 to $116.6 \pm 7.2\%$ (ANOVA, $P < 0.05$) and NOX5-S protein expression from 158.0 ± 2.2 to $85.0 \pm 5.8\%$ (ANOVA, $P < 0.05$) in response to H_2O_2 treatment in FLO cells. Likewise, the knockdown of p50 significantly decreased NOX5-S mRNA levels from 193.9 ± 13.5 to $116.6 \pm 6.4\%$ in OE33 cells (ANOVA, $P < 0.01$; Fig. 2D). In addition, knockdown of p50 significantly decreased H_2O_2-induced thymidine incorporation from 164.9 ± 8.42 to $112.3 \pm 9.2\%$ (ANOVA, $P < 0.05$; Fig. 2E).

The data suggest that NF-κB1 p50 may be involved in H_2O_2-induced NOX5-S expression. To further confirm this result, we transfected FLO cells with a NF-κB reporter plasmid pNF-κB-Luc. H_2O_2 (10^{-13} M) significantly increased the luciferase activity by $36.0 \pm 10.0\%$ (t test, $P < 0.05$; Fig. 3A). In addition, 10^{-13} M H_2O_2 (48-h treatment) significantly increased p65 phosphorylation by $59.4 \pm 19.6\%$ (ANOVA, $P < 0.05$; Fig. 3, D and E). These data suggest that H_2O_2 may activate NF-κB.

To examine whether NF-κB activates NOX5-S promoter, we transfected FLO cells with a NOX5-S reporter plasmid, NOX5LP. Transfection with p65 or p50 expression plasmid

significantly increased luciferase activity to $124.1 \pm 3.6\%$ (Fig. 3B) and to $140.2 \pm 8.3\%$ (Fig. 3C), respectively, suggesting that NF-κB may activate NOX5-S promoter.

Role of MAPK in H_2O_2-Induced NOX5-S Expression. To examine the role of MAPK in H_2O_2-induced NOX5-S expression, we used the MAPK kinase 1 (MEK1) inhibitor PD98059. Figure 4, A–C, shows that PD98059 significantly decreased H_2O_2-induced NOX5 protein levels from 298.2 ± 33.4 to $122.2 \pm 35.83\%$ (ANOVA, $P < 0.05$; Fig. 4, A and B) and NOX5 mRNA levels from 186.5 ± 26.8 to $28.3 \pm 12.2\%$ control (ANOVA, $P < 0.05$; Fig. 4C). In addition, PD98059 significantly reduced thymidine incorporation from 163 ± 9.9 to $94.7 \pm 2.1\%$ control (ANOVA, $P < 0.01$; Fig. 4D).

To determine which MAPKs mediate H_2O_2-induced NOX5-S expression, we used ERK1 and ERK2 siRNAs that have been shown by us to effectively knock down ERK1 and ERK2 (Hong et al., 2010a), respectively. Figure 5, A–C, shows that knockdown of ERK2 with ERK2 siRNA significantly decreased NOX5 protein expression from 160.1 ± 19.4 to $122.9 \pm 5.2\%$ control (ANOVA, $P < 0.01$; Fig. 5, A and B) and NOX5 mRNA levels from 153.9 ± 12 to $116.2 \pm 3.5\%$ control (ANOVA $P < 0.01$; Fig. 5C) in FLO cells. Likewise, in OE33 cells knockdown of ERK2 significantly reduced NOX5 mRNA levels from 169.4 ± 5.7 to $114.3 \pm 4.6\%$ control
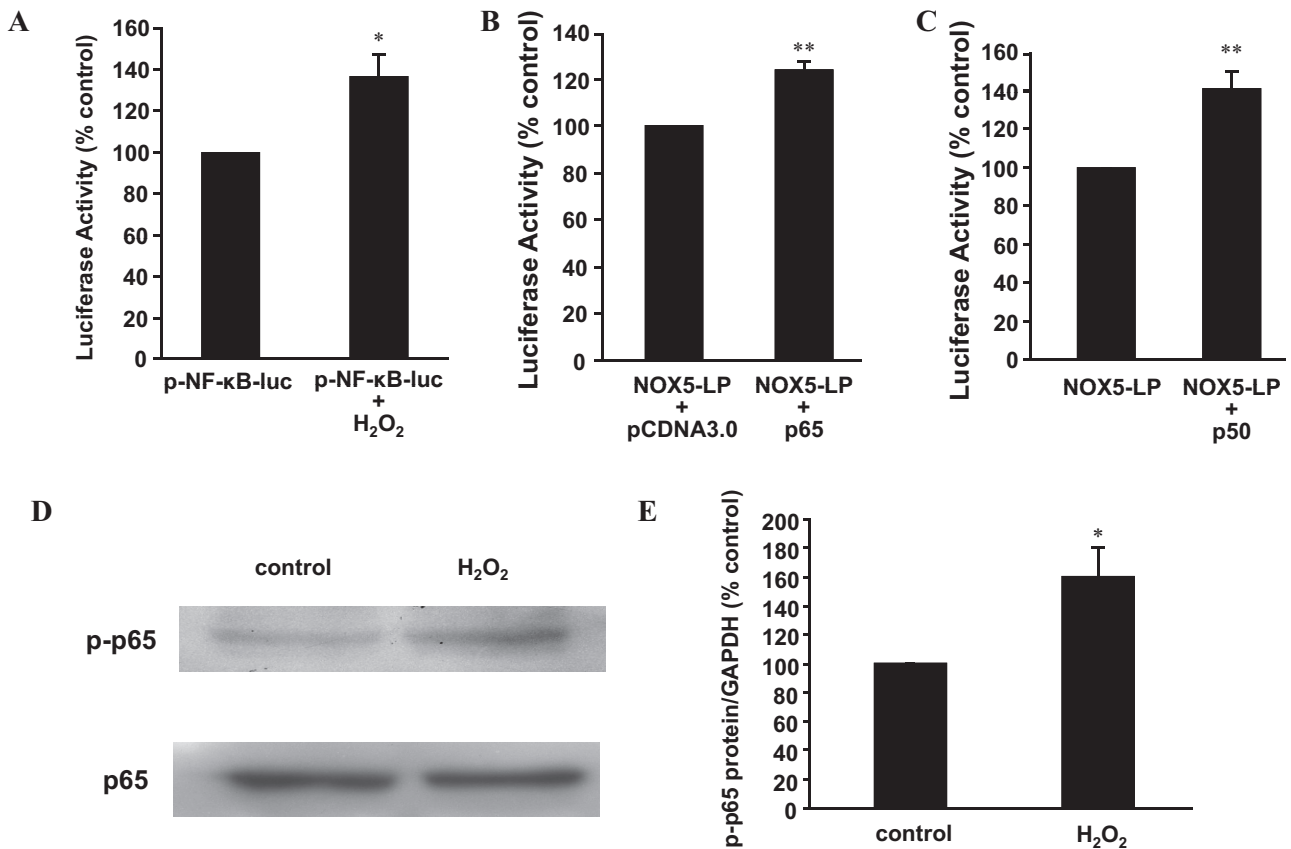

Fig. 3. NF-κB1 p50 may be involved in H_2O_2-induced NOX5-S. A, twenty-four hours after transfection with pNF-κB-Luc plasmid and *R. reniformis* luciferase plasmid, FLO cells were treated with 10^{-13} M H_2O_2 for 48 h, and then luciferase activity was measured. H_2O_2 (10^{-13} M) significantly increased the luciferase activity in FLO cells, suggesting that H_2O_2 may activate NF-κB. B, twenty-four hours after transfection with NOX5-LP and *R. reniformis* luciferase plasmid plus p65 or pCDNA3.0 (control), luciferase activity was measured. Transfection with p65 expression plasmid significantly increased luciferase activity, suggesting that p65 may activate NOX5-S promoter. C, twenty-four hours after transfection with NOX5-LP and *R. reniformis* luciferase plasmid plus p50 or pCMV4 (control), luciferase activity was measured. Transfection with p50 expression plasmid significantly increased luciferase activity, suggesting that p50 may activate NOX5-S promoter. D and E, a typical image of three Western blot analyses (D) and summarized data (E) show that 10^{-13} M H_2O_2 significantly increased p65 phosphorylation. FLO cells were treated with 10^{-13} M H_2O_2 for 48 h. $n = 3$, t test, ∗, $P < 0.05$, compared with pNF-κB-Luc group or NOX5-LP plus pCDNA3.0 group; ∗∗, $P < 0.01$, compared with NOX5-LP or NOX5-LP + pCDNA3.0.

A

control   H$_2$O$_2$ H$_2$O$_2$ + Vehicle   H$_2$O$_2$ + PD98059

NOX5-S

GAPDH

B

NOX5-S protein expression

C

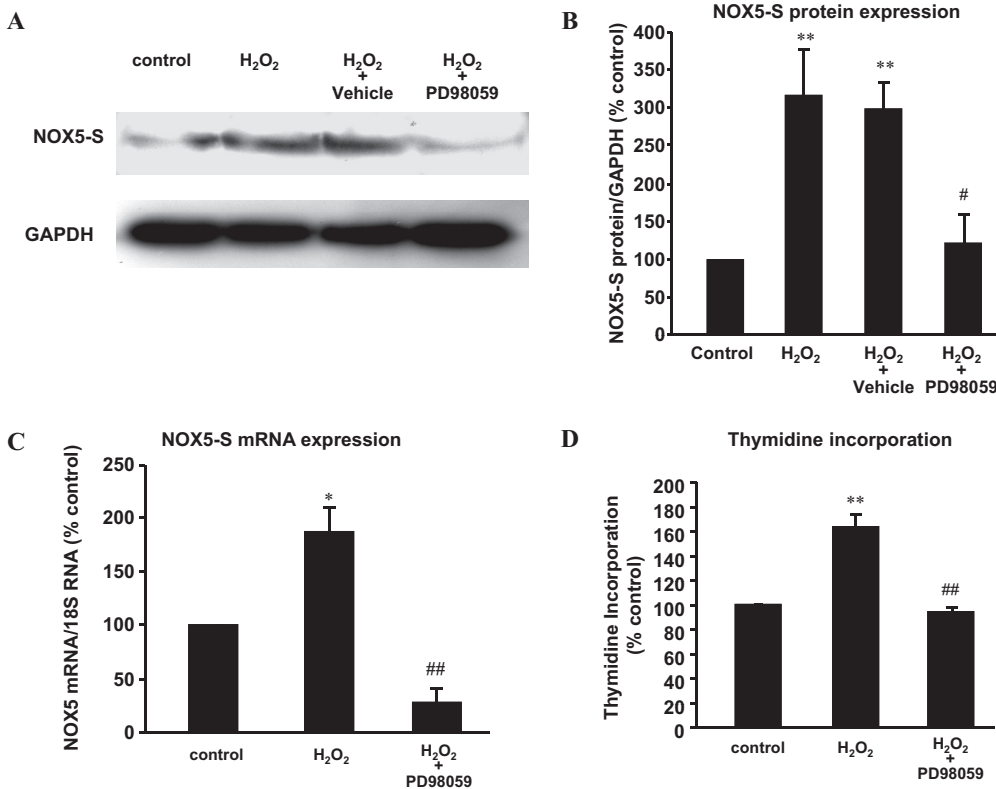NOX5-S mRNA expression

D

Thymidine incorporation

Fig. 4. Role of MAPK in H$_2$O$_2$-induced NOX5-S expression. A and B, a typical image of three Western blot analyses (A) and summarized data (B) show that H$_2$O$_2$ significantly increased NOX5-S protein expression in FLO cells, an increase that was significantly decreased by PD98059. C, H$_2$O$_2$ significantly increased NOX5-S mRNA levels in FLO cells, an increase that was significantly decreased by PD98059, suggesting that MAPK may be involved in H$_2$O$_2$-induced NOX5-S expression. D, H$_2$O$_2$ significantly increased thymidine incorporation in FLO cells, an increase that was significantly decreased by PD98059, suggesting that MAPK may be involved in H$_2$O$_2$-induced increase in cell proliferation. $n = 3$, ANOVA, ∗, $P < 0.05$, ∗∗, $P < 0.01$, compared with control group; #, $P < 0.05$, compared with H$_2$O$_2$ + vehicle group; ##, $P < 0.01$, compared with H$_2$O$_2$ group.

(ANOVA $P < 0.01$; Fig. 5D). In addition, the knockdown of ERK2 significantly reduced thymidine incorporation from 149.3 ± 12.4 to 99.8 ± 14.9% control in FLO cells (ANOVA, $P < 0.01$; Fig. 5E). In contrast, the knockdown of ERK1 with ERK1 siRNA had no significant effect on NOX5 mRNA levels (Fig. 6A) and thymidine incorporation (Fig. 6B) in FLO cells. The data suggest that ERK2 MAPK may be involved in H$_2$O$_2$-induced NOX5-S expression. To investigate whether H$_2$O$_2$ activates ERK2 MAPK, we studied ERK2 MAPK phosphorylation. Figure 7, A and B, shows that H$_2$O$_2$ significantly increased ERK2 MAPK phosphorylation, suggesting that H$_2$O$_2$ may activate ERK2 MAPK. PD98059 significantly decreased ERK2 phosphorylation, suggesting that PD98059 effectively blocks MAPK pathway. However, NF-κB inhibitor SN50 had no significant effect on ERK2 phosphorylation. The knockdown of ERK2 with ERK2 siRNA significantly reduced H$_2$O$_2$-induced increase in luciferase activity in FLO cells transfected with pNF-κB-Luc (Fig. 7C), suggesting that ERK2 MAPK may mediate H$_2$O$_2$-induced activation of NF-κB.

Discussion

Gastroesophageal reflux disease complicated by BE is a major risk factor for EA (Lagergren et al., 1999). Mechanisms whereby acid reflux may accelerate the progression from BE to EA are not fully understood. ROS have been reported to be increased both in BE (Olyaee et al., 1995; Wetscher et al., 1997) and esophageal adenocarcinoma (Farhadi et al., 2002; Sihvo et al., 2003). We have previously shown that Barrett's metaplastic cells may be a source of ROS and that NADPH oxidase NOX5-S is responsible for acid-induced H$_2$O$_2$ production in Barrett's and EA cells (Hong et al., 2010c). Besides metaplastic cells, other cells in BE mucosa (e.g., inflam-

matory cells) may also produce ROS, which may affect metaplastic cells and thereby contribute to esophageal tumorigenesis.

We have shown that H$_2$O$_2$ increases cell proliferation in Barrett's cell line BAR-T and EA cell line OE33 (Hong et al., 2010c). Consistent with our previous findings, we found that a low dose (10^{-13} M) of H$_2$O$_2$ significantly increased thymidine incorporation, whereas higher doses (10^{-5} and 10^{-7} M) slightly decreased thymidine incorporation in FLO cells. The data suggest that low dose of H$_2$O$_2$ may increase cell proliferation in FLO cells. However, mechanisms whereby ROS increase cell proliferation are not fully understood.

In this study, we investigated whether exogenous H$_2$O$_2$ affects cell proliferation in a Barrett's EA cell line FLO by stimulating NOX5-S expression. We found that NOX5-S may mediate H$_2$O$_2$-induced increase in cell proliferation because 10^{-13} M H$_2$O$_2$ significantly increased NOX5-S mRNA level in FLO and OE33 cells as well as protein level and H$_2$O$_2$ production in FLO cells, whereas knockdown of NOX5-S blocked H$_2$O$_2$-induced increase in thymidine incorporation in FLO cells.

ROS have been reported to activate NF-κB (Flohé et al., 1997). We have also shown that NOX5-S-derived ROS activates NF-κB in BE cell line BAR-T (Si et al., 2007). NF-κB, a transcription factor, is known to function as tumor promoter (Pikarsky et al., 2004; Karin, 2006) and plays a key role in the development of colitis-associated cancer (Greten et al., 2004) and cholestatic hepatitis-associated hepatocellular carcinoma (Pikarsky et al., 2004). NF-κB is thought to be a member of a family of Rel domain-containing proteins, including Rel A (also called p65), Rel B, c-Rel, NF-κB1 (p105/p50), and NF-κB2 (p100/p52). p105 and p100 are larger precursor proteins containing IκB (an inhibitor of κB)-like
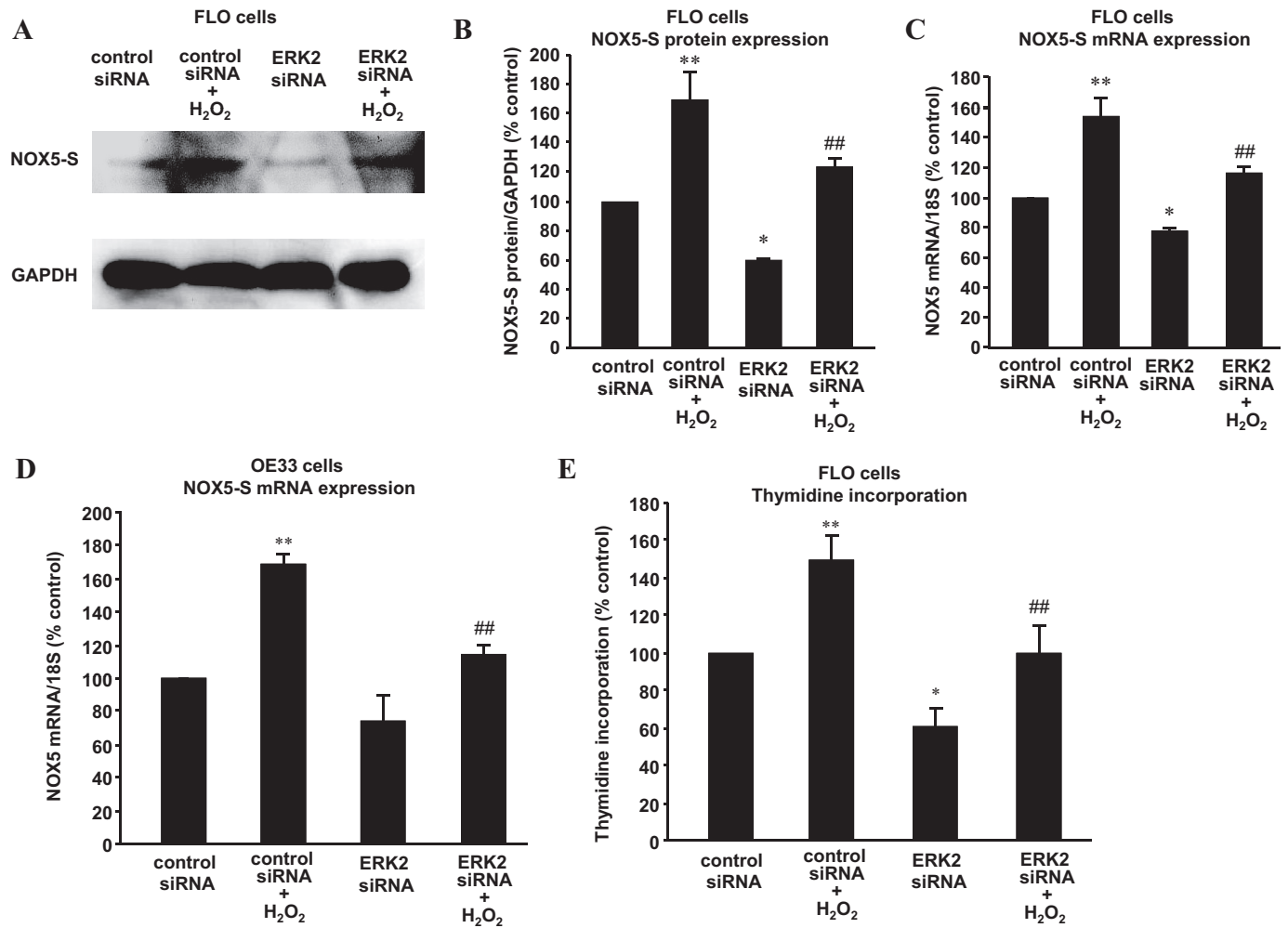
A FLO cells

NOX5-S

GAPDH

B FLO cells
NOX5-S protein expression

C FLO cells
NOX5-S mRNA expression

D OE33 cells
NOX5-S mRNA expression

E FLO cells
Thymidine incorporation

Fig. 5. ERK2 MAPK may be involved in H_2O_2-induced NOX5-S expression. Transfection of ERK2 siRNA and control siRNA was carried out with Lipofectamine 2000. After a 4-h transfection, the transfection medium was replaced with regular medium. Twenty-four hours later, the transfectants were exposed to H_2O_2 (10^{-3} M) in fresh medium for an additional 48 h. A and B, a typical image of three Western blot analyses (A) and summarized data (B) show that knockdown of ERK2 significantly decreased NOX5-S protein expression at basal condition and in response to H_2O_2 treatment in FLO cells. C, knockdown of ERK2 significantly decreased NOX5 mRNA levels at basal condition and in response to H_2O_2 treatment in FLO cells. D, knockdown of ERK2 significantly decreased NOX5 mRNA levels in response to H_2O_2 treatment in OE33 cells. E, knockdown of ERK2 significantly decreased thymidine incorporation at basal condition and in response to H_2O_2 treatment in FLO cells. The data suggest that ERK2 may mediate H_2O_2-induced increase in NOX5-S expression and in cell proliferation. $n = 3$, ANOVA, $*$, $P < 0.05$, $**$, $P < 0.01$, compared with control siRNA group; #, $P < 0.05$, ##, $P < 0.01$, compared with control siRNA plus H_2O_2 group.

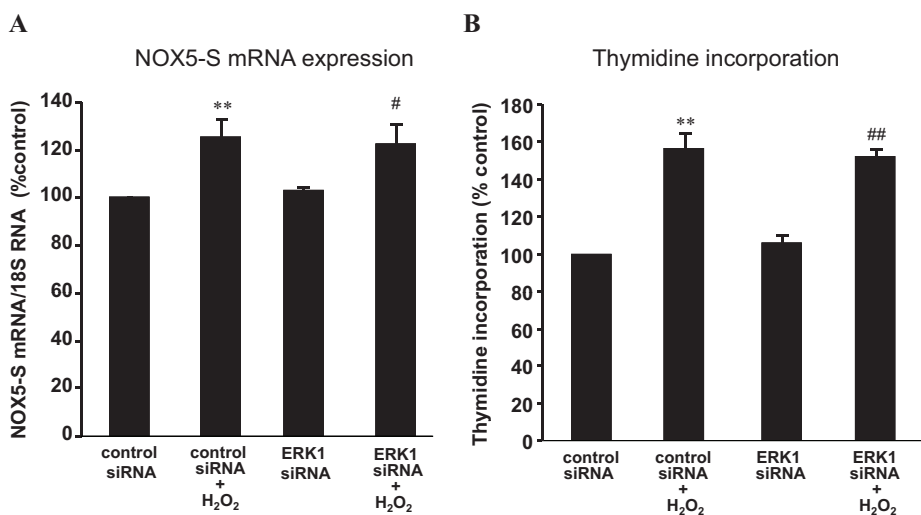**A** NOX5-S mRNA expression

B Thymidine incorporation

Fig. 6. ERK1 MAPK may be not involved in H_2O_2-induced NOX5-S expression. Transfection of ERK1 siRNA and control siRNA was carried out with Lipofectamine 2000. After a 4-h transfection, the transfection medium was replaced with regular medium. Twenty-four hours later, the transfectants were exposed to H_2O_2 (10^{-13} M) in fresh medium for an additional 48 h. A, knockdown of ERK1 had no effect on H_2O_2-induced NOX5 mRNA levels. B, knockdown of ERK1 had no effect on H_2O_2-induced increase in thymidine incorporation. The data suggest that ERK1 may not be involved in H_2O_2-induced increase in NOX5-S expression and in cell proliferation. $n = 3$, ANOVA, $**$, $P < 0.01$, compared with control siRNA group; #, $P < 0.05$, ##, $P < 0.01$, compared with control siRNA plus H_2O_2 group.

A

B

ERK2 phosphorylation

C

NF-κB activation

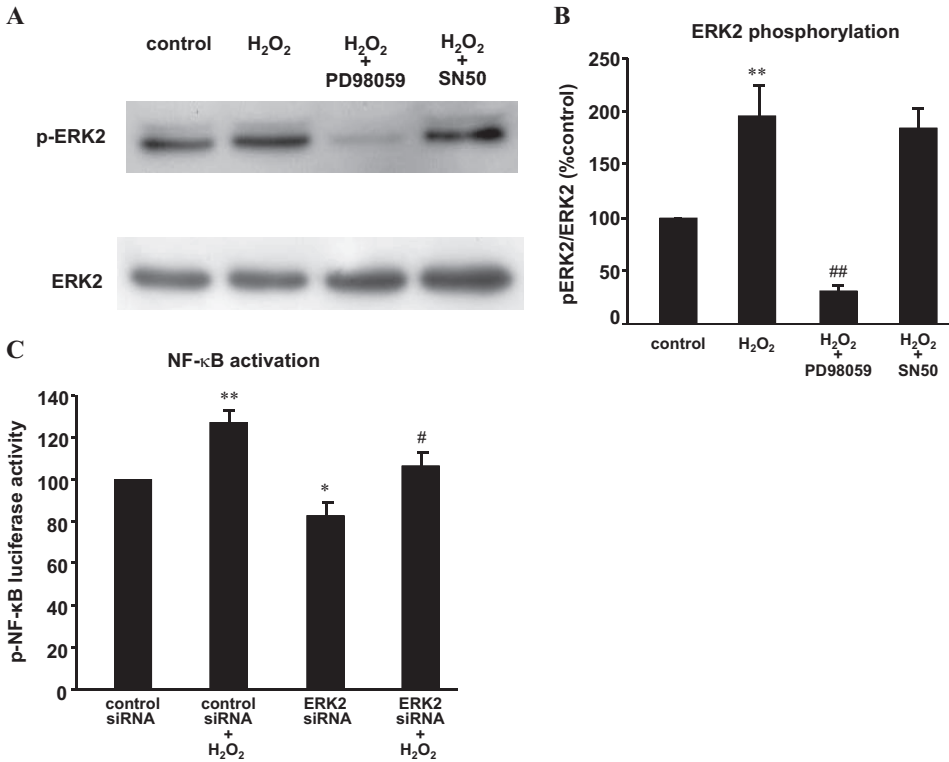**Fig. 7.** H_2O_2 may activate ERK2 MAPK. A and B, a typical image of three Western blot analyses (A) and summarized data (B) show that H_2O_2 significantly increased ERK2 phosphorylation, an increase that was significantly decreased by PD98059 (10^{-5} M) but not by SN50 (10^{-5} M). FLO cells were exposed to 10^{-5} M H_2O_2 for 15 min in the absence or presence of PD98059 (10^{-5} M) or SN50 (10^{-5} M). C, FLO cells were transfected with 0.1 μg of *R. reniformis* luciferase plasmid and 0.5 μg of pNF-κB-Luc plasmid by using Lipofectamine 2000. Twenty-four hours after transfection with plasmids, 60 pmol of ERK2 or control siRNA per well were transfected. Twenty-four hours later, FLO cells were treated with 10^{-13} M H_2O_2 for additional 48 h. Finally, the luciferase activity was measured. H_2O_2 significantly increased luciferase activity in FLO cells, an increase that was significantly reduced by knockdown of ERK2, suggesting that activation of NF-κB may depend on activation of ERK2. $n = 3$. ∗∗, $P < 0.01$, ANOVA, compared with control siRNA group; ∗, $P < 0.05$, ANOVA, compared with control siRNA group; #, $P < 0.05$, ANOVA, compared with control siRNA plus H_2O_2 group; ##, $P < 0.01$, ANOVA, compared with H_2O_2 group.

ankyrin repeat sequences in their carboxyl termini. Because of their IκB-like ankyrin repeat sequences, these precursors are retained in the cytoplasm and require proteolytic processing to generate their mature DNA-binding proteins p50 and p52, respectively (Karin et al., 2002). In the cytoplasm, NF-κB is in an inactive state, and three pathways regulate its activity. In the first pathway, a heterotrimer composed of p50, p65, and IκB is degraded in a ubiquitin-dependent reaction, leading to the translocation of the p65-p50 dimers to the nucleus (Karin et al., 2002). In the second pathway, the dimers consisting of p100 and Rel B undergo proteolytic removal of the IκB-like C-terminal domain of p100, allowing Rel B-p52 dimers to translocate to nucleus. In the third pathway, p50 (or p52), homodimers enter the nucleus where NF-κB activates gene transcription (Karin et al., 2002; De Bosscher et al., 2006). p50 plays an important role in lymphoid organogenesis and inflammation, whereas p52 is mainly involved in lymphoid organogenesis (Shih et al., 2011). Therefore, we focused on the role of p50 in NOX5-S expression.

We found that knockdown of p50 with p50 siRNA significantly decreased cell proliferation in FLO cells and inhibited H_2O_2-induced increase in NOX5-S expression in OE33 and FLO cells, suggesting that NF-κB1 p50 may be involved in H_2O_2-induced NOX5-S expression in FLO and OE33 cells. This result is further supported by the following findings: 1) H_2O_2 significantly increased p65 phosphorylation and the luciferase activity in FLO cells transfected with a reporter plasmid pNF-κB-Luc, suggesting that H_2O_2 may activate NF-κB; 2) transfection with p50 or p65 expression plasmid significantly increased a NOX5-S reporter plasmid NOX5-LP, suggesting that NF-κB may activate NOX5-S promoter.

ROS have been shown to activate MAPKs (Kumar et al.,

2008). ERK1 and ERK2 are isoforms of the "classic" MAPK. Both ERK1 and ERK2 are activated by MAPK kinase (MEK) 1/2. MEK1/2 phosphorylates threonine and tyrosine residues in the Thr–Glu–Tyr (TEY) sequence of ERK1/2, resulting in the activation of ERK1/2. The ERK1/2 MAPK pathway is involved in various cellular functions, including cell proliferation, differentiation, and migration (Nishimoto and Nishida, 2006). This pathway is constitutively active in human tumors (Gioeli et al., 1999; Hoshino et al., 1999; Tanaka et al., 2003) and is known to be involved in acid-induced increase in cell proliferation in EA cells (Souza et al., 2002, 2004). It is also known to be involved in acid-induced increase in cell proliferation and in MAPK phosphorylation in SEG1 cells (Souza et al., 2002, 2004), suggesting that ERK1/2 MAPKs are activated by acid treatment. However, whether MAPKs contribute to H_2O_2-induced expression of NOX5-S by up-regulation of NF-κB and increase in cell pro-
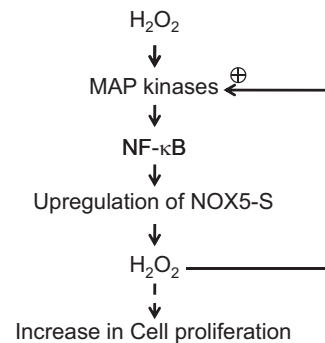

Fig. 8. Signaling in H_2O_2-induced increase in cell proliferation. Low doses of H_2O_2 increase cell proliferation. H_2O_2-induced increase in cell proliferation may depend on sequential activation of ERK2 MAPK and NF-κB and thus up-regulate NOX5-S expression, which further enhances production of ROS (a positive feedback).

liferation is not known. We found that MEK1 kinase inhibitor PD98059 and knockdown of ERK2 significantly decreased H_2O_2-induced increase in NOX5-S protein and mRNA levels and in thymidine incorporation in EA cells, whereas knockdown of ERK1 had no effect. In addition, H_2O_2 significantly increased ERK2 MAPK phosphorylation, suggesting that H_2O_2 may activate ERK2 MAPK. These data suggest that ERK2 MAPK, but not ERK1, may be involved in H_2O_2-induced NOX5-S expression.

We examined the relationship next between ERK2 MAPK and NF-κB. We found that ERK2 MAPK activates NF-κB because 1) knockdown of ERK2 significantly reduced H_2O_2-induced increase in luciferase activity in FLO cells transfected with pNF-κB-Luc and 2) NF-κB inhibitor SN50 had no significant effect on ERK2 phosphorylation. This result is consistent with the findings in a *Citrobacter rodentium*-induced transmissible murine colonic hyperplasia model (Chandrakesan et al., 2010).

Mechanisms of NOX5-S-mediated increase in cell proliferation are not fully understood. We have shown two possible mechanisms: 1) NOX5-S-derived ROS activate cyclooxygenase 2, increase prostaglandin E_2 production, and thereby enhance cell proliferation (Si et al., 2007) and 2) NOX5-S-derived ROS also cause p16 promoter hypermethylation, down-regulate p16 expression, and thus, stimulate cell proliferation (Hong et al., 2010c).

In conclusion, low doses of H_2O_2 increase cell proliferation. H_2O_2-induced increase in cell proliferation may depend on sequential activation of ERK2 MAPK and NF-κB and thus up-regulate NOX5-S expression (Fig. 8). It is possible that in Barrett's esophagus ROS produced by Barrett's cells, inflammatory cells, and other parenchymal cells may activate ERK2 MAPK and NF-κB and cause up-regulation of NOX5-S, which further enhances production of ROS (a positive feedback) and increases cell proliferation and DNA damage, thereby contributing to the esophageal tumorigenesis.

Authorship Contributions

Participated in research design: Zhou and Cao.

Conducted experiments: Zhou and Li.

Contributed new reagents or analytic tools: Resnick, Wands, and Cao.

Performed data analysis: Zhou and Cao.

Wrote or contributed to the writing of the manuscript: Zhou, Resnick, Behar, and Cao.

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