NADPH Oxidase NOX5-S and Nuclear Factor κ B1 Mediate Acid-Induced Microsomal Prostaglandin E Synthase-1 Expression in Barrett's Esophageal Adenocarcinoma Cells

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ABSTRACT

The mechanisms of progression from Barrett's esophagus (BE) to esophageal adenocarcinoma (EA) are not known. Cyclooxygenase-2 (COX-2)-derived prostaglandin E₂ (PGE₂) has been shown to be important in esophageal tumorigenesis. We have shown that COX-2 mediates acid-induced PGE₂ production. The prostaglandin E synthase (PGES) responsible for acid-induced PGE₂ production in BE, however, is not known. We found that microsomal PGES1 (mPGES1), mPGES2, and cytosolic PGES (cPGES) were present in FLO EA cells. Pulsed acid treatment significantly increased mPGES1 mRNA and protein levels but had little or no effect on mPGES2 or cPGES mRNA. Knockdown of mPGES1 by mPGES1 small interfering RNA (siRNA) blocked acid-induced increase in PGE₂ production and thymidine incorporation. Knockdown of NADPH oxidase, NOX5-S, a variant lacking calcium-binding

domains, by NOX5 siRNA significantly inhibited acid-induced increase in mPGES1 expression, thymidine incorporation, and PGE₂ production. Overexpression of NOX5-S significantly increased the luciferase activity in FLO cells transfected with a nuclear factor κ B (NF- κ B) in vivo activation reporter plasmid pNF- κ B-Luc. Knockdown of NF- κ B1 p50 by p50 siRNA significantly decreased acid-induced increase in mPGES1 expression, thymidine incorporation, and PGE₂ production. Two novel NF- κ B binding elements, GGAGTCTCCC and CGGGACACCC, were identified in the mPGES1 gene promoter. We conclude that mPGES1 mediates acid-induced increase in PGE₂ production and cell proliferation. Acid-induced mPGES1 expression depends on activation of NOX5-S and NF- κ B1 p50. Microsomal PGES1 may be a potential target to prevent or treat EA.

Introduction

Esophageal adenocarcinoma (EA) has increased in incidence at a rate exceeding that of any other cancer (Blot and McLaughlin, 1999; Howe et al., 2001; Pohl and Welch, 2005). The major risk factor for esophageal adenocarcinoma is gastroesophageal reflux disease (GERD) complicated by Barrett's esophagus (BE) (Lagergren et al., 1999). However, the mechanisms of progression from BE to EA are not fully understood.

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Reactive oxygen species (ROS) may be an important factor mediating this progression from BE to EA (Farhadi et al., 2002; Sihvo et al., 2003). Overproduction of ROS, derived from upregulation of NADPH oxidase, EF-hand calcium binding domain 5-S (NOX5-S), increases cell proliferation and decreases apoptosis, possibly contributing to progression from BE to dysplasia and to adenocarcinoma (Fu et al., 2006). We have found that overexpression of NOX5-S significantly increases cyclooxygenase 2 (COX-2) expression as well as prostaglandin E₂ (PGE₂) production in BE (Si et al., 2007), suggesting that the NOX5-S-mediated increase in cell proliferation is partially dependent on activation of COX-2.

COX-2 has been shown to play a role in the progression from BE to EA since 1) COX-2 overexpression has been demonstrated in both Barrett's metaplastic and adenocarcinoma cells (Shirvani et al., 2000); 2) COX-2 expression increases significantly in ex vivo BE tissues pulsed with acid or bile salts, and this effect is attenuated by a selective COX-2 inhibitor NS-398 [*N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide]

ABBREVIATIONS: ANOVA, analysis of variance; BE, Barrett's esophagus; ChIP, chromatin immunoprecipitation; COX, cyclooxygenase; cPGES, cytosolic prostaglandin E synthase; Ct, threshold cycle; DMEM, Dulbecco's modified Eagle's medium; EA, esophageal adenocarcinoma; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GERD, gastroesophageal reflux disease; NADPH, nicotinamide adenine dinucleotide 3'-phosphate reduced form; NF- κ B, nuclear factor κ B; NOX5-S, NADPH oxidase, a variant lacking calcium-binding domains; PG, prostaglandin; PGE₂, prostaglandin E₂; PGES, prostaglandin E synthase; mPGES, microsomal prostaglandin E synthase; NS-398, *N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide; ROS, reactive oxygen species; RT-PCR, reverse-transcription polymerase chain reaction; siRNA, small interfering RNA.

(Shirvani et al., 2000); 3) selective COX-2 inhibitors significantly decrease the development of esophageal adenocarcinoma in a rat model of BE (Buttar et al., 2002); and 4) in EA cells, selective COX-2 inhibitors significantly decrease proliferation and increase apoptosis (Zimmermann et al., 1999; Souza et al., 2000). However, COX-2 inhibitors have severe cardiovascular side effects (Solomon et al., 2005), limiting their usefulness in the treatment of cancer. Therefore, it is important to explore COX-2's downstream targets.

PGE₂ is known to increase cell proliferation and has been shown to increase promoter methylation and to enhance tumor growth (Xia et al., 2012). PGE₂ is synthesized from arachidonic acid by PGE synthase (PGES). Thus far, three isoforms of PGES have been identified: a cytosolic (cPGES) and two microsomal (mPGES) isomerases (Jakobsson et al., 1999; Tanioka et al., 2000; Tanikawa et al., 2002). The cPGES (PTGES3) is a 26-kDa protein constitutively expressed in various homeostatic cells and selectively coupled with COX-1 (Tanioka et al., 2000). The mPGES2 (PTGES2) is a 33-kDa protein overexpressed in colorectal adenocarcinoma (Murakami et al., 2003; Schroder et al., 2006). Microsomal PGES1 (PTGES1) is a 16-kDa membrane-associated protein coupled with COX-2, and it may be induced in a variety of pathologic conditions, including esophageal cancer (Soma et al., 2007; von Rahden et al., 2008). Microsomal PGES1 has been shown to be elevated in an animal model of BE (Jang et al., 2004) and in human EA (von Rahden et al., 2008). Microsomal PGES1 is also expressed in the stromal cells of rat BE (Jang et al., 2004). Interestingly, normal esophageal tissues do not express mPGES1 (Jang et al., 2004). These data suggest that mPGES1 may be important in the development of esophageal adenocarcinoma and may provide a preventive or therapeutic approach. Whether PGESs mediate acid-induced increase in PGE₂ production and cell proliferation in BE is not known. Moreover, the mechanisms of mPGES1 upregulation in EA are not fully understood.

In this study, we discovered that mPGES1 mediates acid-induced increase in PGE₂ production and cell proliferation. To our knowledge, we are the first to report that acid-induced mPGES1 expression depends on activation of NOX5-S and NF- κ B p50. We also identified two NF- κ B binding sites in the promoter region of mPGES1, responsible for the regulation of mPGES1 expression.

Materials and Methods

Cell Culture and Acid Treatment. The human Barrett's adenocarcinoma cell line FLO, derived from human Barrett's esophageal adenocarcinoma (Hughes et al., 1997), was generously provided by Dr. David Beer (University of Michigan). The FLO cells and human EA cell line OE33 (Sigma-Aldrich, St. Louis, MO) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics.

The human esophageal squamous epithelial cell line HET-1A (American Type Culture Collection, Manassas, VA) was cultured in the bronchial epithelial cell medium (BEGM BulletKit; Lonza, Walkersville, MD) containing a basal medium (BEBM) plus additives (BEGM SingleQuots; Lonza) in wells precoated with a mixture of 0.01 mg/ml fibronectin and 0.03 mg/ml vitronectin 100 collagen (Cohesion, Palo Alto, CA). The cells were cultured at 37°C in a 5% CO₂-humidified atmosphere.

For acid treatment, FLO cells were exposed to acidic DMEM (pH 4.0, 250 μ l) or normal DMEM (control) added to each well in a 12-well

plate for 1 hour; the final pH was approximately 4.9 after a 1-hour incubation. They then were washed and cultured in fresh medium (pH 7.2) for an additional 24 hours. Finally, the culture medium and cells were collected for measurement of PGE₂ and mPGES1 mRNA and protein level.

Small Interfering RNA and Plasmid Transfection. For small interfering RNA (siRNA) transfection, at 40–50% confluence, cells were trypsinized and diluted 1:5 with fresh medium without antibiotics (1–3 $\times$ 10⁵ cells/ml) and transferred to 12-well plates (1 ml per well). Transfection of siRNAs was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For each well, 60 pmol of siRNA duplex of mPGES1, mPGES2, cPGES, p50, NOX5, or control siRNAs formulated into liposomes were applied; the final volume was 1.2 ml per well. Twenty-four hours later, the transfectants were exposed to acidic DMEM (pH 4.0) for 1 hour, washed, and cultured in fresh medium (pH 7.2) for an additional 24 hours. Finally, the culture medium and the transfectants were collected for measuring the mPGES1 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 hours. Control siRNA is a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA.

For plasmid transfection, 0.5 μ g of plasmids (NOX5-S, mPGES1, p50, p65, pCMV) or 0.1 μ g of *Renilla luciferase* plasmid formulated into liposomes were applied. All other procedures were similar to those described previously.

Reverse-Transcription Polymerase Chain Reaction. Total RNA was extracted by TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Subsequently, 1.0 μ g of total RNAs were reversely transcribed by using a kit SUPERScript First-Strand Synthesis System for reverse-transcription polymerase chain reaction (RT-PCR) (Invitrogen).

Quantitative Real-Time PCR. The mRNA expression was measured using real-time PCR analysis. Real-time PCR analysis was performed in a 15- μ l reaction on 96-well clear plate using Power SYBR Green RT-PCR Reagents Kit (Applied Biosystems, Foster City, CA). The primers used were: mPGES1 sense (5'-GGGGTCTTGGGTCTCTG TAT-3'), mPGES1 antisense (5'-GACTGCAGCAAAGACAT-CCA-3'), mPGES2 sense (5'-CTGTCCCATGGCTACATCTCT-3'), mPGES2 antisense (5'-CGCCACAACCTTTCTTTTA-3'), cPGES sense (5'-AAGTC-GACTCCCTAGCAGCA-3'), cPGES antisense (5'-CGTACCACCTTGC AGAAGCA-3'), 18S sense (5'-CGGACAGGATTGACAGATTGATAGC-3'), and 18S antisense (5'-TGCCAGAGTCT CGTTCGTTATCG-3'). Reactions were performed in an Applied Biosystems StepOnePlus Real-Time PCR System for one cycle at 94°C for 5 minutes; 40 cycles at 94°C for 30 seconds, 59°C for 30 seconds, and 72°C for 30 seconds; one cycle at 94°C for 1 minute; and one cycle at 55°C for 30 seconds. 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 the 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.

Plasmids Construct. The primers used for construction of the mPGES-1 plasmid were as follows: 5'-CCGGGTACCGGAGTCTC CCTATGTTGCCC-3' (1F), 5'-CCGGGTACCGGAGGAGCA GGTAG CGTCTC-3' (3F), 5'-ACAACTGCGCTGGGGAGTGAC-3' (4F), 5'-CC GGGTACCCTGGGAATACAGGCGAGCAC-3' (5F), 5'-CTGGGTCTT TGACATTCGGAGAC-3' (791F), 5'-CGTAGTCTCACTATGTTGCC-CAGGC-3' (1318F), 5'-CCGCTCGAGCATCTTGATGACCAGCAGCGT-3' (1R), 5'-CCGCTCGAGCGCAGCTCAACTGTGGG TG-3' (5R), 5'-GTCTC CGAATGTCAAGGACCCAG-3' (791R), and 5'-GCCTGGGCAAC AT-AGTGAGACTACG-3' (1318R).

Four primers (5F and 5R, 4F and 1R for nested PCR) were used to construct the mPGES1318 plasmid containing the mPGES1 promoter fragment -1328 to -10 (position from ATG). The mPGES1318 reporter plasmid contains three potential binding sites: 1) GGAGTCTCCC (from -1269 to -1260), 2) GGGCTCACCC (from -656 to -647), and 3) CGGGACACCC (from -211 to -202). The second plasmid mPGES791 was constructed by using four primers (3F and 1R, 1F and 2R for nested PCR) and contained the mPGES1 promoter fragment -791 to 0 (position from ATG). Plasmid mPGES791 includes potential binding sites 2 and 3 (Fig. 1). Human genomic DNA was used as a template.

Other three plasmids—mPGES1318M1, mPGES1318M2, and mPGES791M—were constructed by using overlap extension PCR (Fig. 1). The mPGES1318M1 was constructed by three PCRs using three paired primers: 5F-791R, 791F-5R, and 5F-5R, whereas mPGES1318 was used as a template. Similarly, the mPGES1318M2 was constructed by three PCRs using three paired primers: 5F-1318R, 1318F-5R, and 5F-5R, whereas mPGES1318M1 was used as a template. The mPGES791M was constructed by three PCRs using three paired primers (3F-791R, 791F-1R, and 3F-1R), and mPGES791 was used as a template.

The PCR products were inserted into pGL3-Basic plasmid (Promega, Madison, WI) between the Kpn I (Invitrogen) and Xho I (Invitrogen) restriction sites. The constructed plasmids were verified by sequencing at GENEWIZ, Inc. (South Plainfield, NJ).

Luciferase Assay. The FLO EA cells were seeded in 12-well plates for 24 hours. We used 0.1 μ g of *R. luciferase* and 0.5 μ g of mPGES1318, mPGES791, mPGES1318M1, mPGES1318M2, mPGES791M, or NOX5-S reporter plasmids with or without pCMV plasmid (control), NOX5-S, p50, or p65 expression plasmids and transfected them by using Lipofectamine 2000 (Invitrogen). Luciferase activity was assayed 24 hours after transfection. Cell extracts were prepared by lysing the cells with lysis buffer (Promega). The lysate was centrifuged at 13,000g for 10 minutes to pellet the cell debris. The luciferase activities in the cell lysates were measured by use of a TopCount-NXT Microplate Scintillation and Luminescence Counter (PerkinElmer Life and Analytical Sciences, Waltham, MA) according to the protocol (Promega) and were normalized to *R. luciferase*. The number of experiments is indicated in figure legends; each experiment was performed in triplicate.

Western Blot Analysis. Cells were lysed in a Triton X lysis buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM sodium chloride, 50 mM sodium fluoride, 5 mM EDTA, 1% (v/v) Triton X-100, 40 mM β -glycerol phosphate, 40 mM *p*-nitrophenylphosphate, 200 μ M sodium orthovanadate, 100 μ M phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A, and 1 μ g/ml aprotinin. The suspension was centrifuged at 15,000g for 5 minutes, and the protein concentration in the supernatant was determined. Western blot analysis was performed as described previously elsewhere (Cao et al., 2003; Fu et al., 2006). In brief, after these supernatants were subjected to SDS-PAGE, the separated proteins were transferred electrophoretically to a nitrocellulose membrane at 100 V for 1 to 2 hours. The nitrocellulose membranes were blocked in 5% nonfat dry milk and then incubated with appropriate primary antibodies followed by a 60-minute

incubation in horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). Detection was achieved with an enhanced chemiluminescence agent (GE Healthcare, Piscataway, NJ).

The primary antibodies used were human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:1000), mPGES1 antibody (1:1000), mPGES2 antibody (1:1000), and cPGES antibody (1:1000).

Protein Measurement. The amount of protein was determined by a colorimetric assay using the protein assay kit from Bio-Rad Laboratories (Richmond, CA) according to the Bradford method (Bradford, 1976).

[³H]Thymidine Incorporation. For acid treatment, FLO cells were incubated with acidic DMEM (pH 4.0) for 1 hour, washed, and cultured in fresh medium (pH 7.2) for an additional 24 hours. For siRNA transfection, 24 hours after siRNAs of mPGES1, mPGES2, cPGES, or control were introduced, the cells were treated without or with acidic medium and were then incubated with methyl-[³H]thymidine (0.05 μ Ci/ml) for 4 hours. After being washed 3 times with phosphate-buffered saline to remove any unincorporated radioactivity, the cells were collected and homogenized with a lysis buffer containing 50 mM HEPES, 50 mM sodium chloride, 1% Triton X-100, 1% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol (pH 7.4). The methyl-[³H]thymidine uptake was measured in a Topcount-NXT Microplate Scintillation and Luminescence Counter (PerkinElmer Life and Analytical Sciences). The level of protein in the homogenates was also determined, and the level of methyl-[³H]thymidine incorporation was normalized to the protein content.

PGE₂ Measurement. PGE₂ in culture medium was quantified by using a PGE₂ enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI).

Chromatin Immunoprecipitation Assay. A chromatin immunoprecipitation (ChIP) assay was performed using a ChIP assay kit (Upstate Biotechnology, Charlottesville, VA) following the manufacturer's protocol. In brief, FLO cells grown on plastic dishes for 2 days (1×10^6 cells) were treated with acidic medium (pH 4.0) for 1 hour and then treated with 1% formaldehyde for 10 minutes to cross-link p50 to DNA. After removal of the formaldehyde, cells were washed with ice-cold phosphate-buffered saline containing 0.1% EDTA and protease inhibitors (1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1% protease inhibitor mixture), gently scraped into a conical tube, and centrifuged for 5 minutes at 700g at 4°C. Pelleted cells were resuspended in 400 μ l of lysis buffer [10 mM HEPES, pH 7.9, 60 mM KCl, 0.5% (v/v) Nonidet P-40] with protease inhibitors and incubated on ice for 10 minutes. Nuclei were recovered by centrifugation at 1000g for 10 minutes and resuspended in 400 μ l of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) containing protease inhibitors. The mixture was incubated on ice for 10 minutes, and the lysate was sonicated eight times for 10 seconds each time on ice to shear the genomic DNA to lengths of 0.2–1.0 kb.

The debris was removed by centrifugation, and the supernatant was then diluted 10 times with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 16.7 mM NaCl, and protease inhibitors, pH 8.0). We kept 500 μ l of the diluted lysate for

Fig. 1. Construct diagram. Boldface characters indicate mutation sites.

input control. The chromatin solution was precleared with salmon sperm DNA/protein A-agarose for 1 hour at 4°C. Anti-p50 antibody (Upstate Biotechnology) was added to the supernatant fraction and incubated overnight at 4°C with rotation. The mixture was then incubated with 60 μ l of salmon sperm DNA/protein A-agarose slurry at 4°C with rotation for 1 hour. Normal rabbit IgG antibody (SeroTec, Raleigh, NC) was used for a negative control.

The protein A-agarose-antibody histone complex was pelleted by a gentle centrifugation (1000g at 4°C for 1 minute). The pellet was washed sequentially (3–5 minutes per wash) on a rotating platform with 1 ml each of low-salt washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl, pH 8.0), high-salt washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl, pH 8.0), LiCl washing buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0), and 1 $\times$ TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

After the final wash, the pellet was eluted by two 15-minute incubations with 250 μ l of freshly made elution buffer (1% SDS and 50 mM NaHCO₃). Two fractions of elutes were combined, and 20 μ l of 5 M NaCl was added to the supernatant. Cross-linking was reversed by heating samples at 65°C for 4 hour, followed by addition of 10 μ l of 0.5 mM EDTA, 20 μ l of 1 M Tris-HCl (pH 6.5), and 2 μ l of 10 mg/ml proteinase K.

The samples were incubated at 45°C for 2 hours, and DNA was then extracted by phenol chloroform extraction, followed by ethanol precipitation. DNA pellets were resuspended in 50 μ l of H₂O, and 5 μ l of them were used for PCR analysis. The PCR was performed by using three pairs of primers (covering three potential NF- κ B binding sites) at 94°C for 5 minutes, 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds for 35 cycles, followed by a 7-minute extension at 72°C. The first pair of primers targeted the –313 to –156 region of human mPGES1 promoter (sense 5'-CCAC ATGCTCCACACTGTAGAT-3' and antisense 5'-AGAAAGACAAGCAGTTGCCGGA-3'); The second pair targeted the –717 to –554 region of human mPGES1 promoter (sense 5'-CTCCTGTCTGACCCCTATTCTGTG-3' and antisense 5'-TAGAACCCGTGACTGTGACTATGTG-3'); and the third targeted the –1364 to –1062 region of human mPGES1 promoter (sense 5'-GAATACAGGCGAGCACCACCATG-3' and antisense 5'-TCCTGGAACCTCTTCCCCAGTCA-3').

Gel Mobility Shift Assay. A gel-shift assay was performed using an Odyssey Infrared EMSA Kit following the manufacturer's protocol. Two oligonucleotides derived from human mPGES-1 promoter (–1277 to –1253 and –220 to –196) were synthesized and labeled with IRDye 700 by Integrated DNA Technologies, Inc. (Coralville, IA). The gel-shift assay was performed by incubating 0.5 μ g of recombinant NF- κ B p50 protein (Cayman Chemical Co.) with 1 μ l of 50 nM IRDye700 labeled

Fig. 2. Microsomal PGES1 is involved in the acid-induced increase in cell proliferation and PGE₂ production. (A) mPGES1, mPGES2, and cPGES were detectable in FLO cells by PCR. A typical image of three Western blot analyses (B) and summarized data (C) show that mPGES1 siRNA effectively knocked down mPGES1. (D) Knockdown of mPGES1 significantly decreased the thymidine incorporation. FLO cells were treated with acid (pH 4.0, 1 hour), washed, and incubated with regular medium 24 hours after mPGES1 siRNA and control siRNA were introduced into FLO cells by Lipofectamine 2000. (E) Knockdown of mPGES1 significantly decreased PGE₂ levels in response to acid treatment. *N* = 3, ANOVA, **P* < 0.05; ***P* < 0.001, compared with control siRNA group; ANOVA, ##*P* < 0.01, compared with control siRNA group plus acid group.

probe in a 20 μ l of reaction buffer for 20 minutes at room temperature. For a supershift assay with an NF- κ B p50 antibody, the antibody was preincubated with the NF- κ B p50 protein for 20 minutes at room temperature before the addition of the IRDye700-labeled probes.

The oligonucleotides used were as follows: wild-type competitor 1 (–1277 to –1253, mPGES1-WT1): 5'-GTAGAGACGGAGTC TCCC TATGTT-3' and 3'-AACATAGGGAGACTCCGTCTCTAC-5', containing potential NF- κ B binding site 1; mutant competitor 1 (–1277 to –1253, mPGES1-MUT1): 5'-GTAGAGACGTAGTCTCACTATGTT-3' and 3'-AACATAGTGAGACTACGTCTCTAC-5'; wild-type competitor 3 (–220 to –196, mPGES1-WT3): 5'-GACTGGG TCCGGGACACCCG GAGA-3' and 3'-TCTCCG GGTGTCCCGGACCCAGTC-5', containing potential NF- κ B binding site 3; or mutant competitor 3 (–220 to –196, mPGES1-MUT3): 5'-GACTGGGTCCTTGACATTCCGAGA-3' and 3'-TCTCCGAATGTCAAGGACCCAGTC-5'. The DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel with 2.5% glycerol in 0.5 $\times$ TBE buffer for 2 hours at 70 V.

Materials. Human NOX5 siRNA was purchased from Applied Biosystems; GAPDH antibody, control siRNA, mPGES1 siRNA, mPGES2 siRNA, cPGES siRNA, and p50 siRNA were from Santa Cruz Biotechnology; p50 plasmid (Ballard et al., 1992) was from AddGene (Plasmid 21965; Cambridge, MA); The pCMV-tag5a-NOX5-S plasmid was generously provided to us by Dr. David Lambeth (Emory University School of Medicine, Atlanta, GA). The p65 plasmid was generously provided to us by Dr. Eugene Chin (Brown University, Providence, RI). The p50 antibody was purchased from Cell Signaling Technology (Danvers, MA); mPGES1 antibody, mPGES2 antibody, and cPGES antibody were from Cayman Chemical Co.; and Triton X-100, phenylmethylsulfonyl fluoride, DMEM, antibiotics, and other reagents were purchased from Sigma-Aldrich.

Statistical Analysis. Data were expressed as the means $\pm$ S.E. Statistically significant differences between two groups were determined by Student's *t* test. Differences among multiple groups were tested by analysis of variance (ANOVA) and were checked for significance by Fisher's protected least significant difference test. The percentage increase was calculated as the following:

$$\% \text{ Increase} = (\text{acid treatment group} - \text{without-acid treatment group}) \times 100 / \text{without-acid treatment group}.$$

Results

Acid Upregulates mPGES1 Expression in FLO EA Cells. We have shown that acid-induced increase in cell proliferation is mediated by COX-2-derived production of PGE₂. However, PGE synthases involved in acid-induced PGE₂ production are not known. First, we determined whether PGES(s) is/are present in FLO cells by use of RT-PCR. Figure 2A shows that mPGES1, mPGES2, and cPGES were detectable in the FLO EA cells.

Next, we examined which PGESs mediate an acid-induced increase in cell proliferation. Figure 2, B and C, shows that mPGES1 siRNA effectively knocked down the mPGES1 protein. Acid statistically significantly increased cell proliferation by $67.7 \pm 15.6\%$ (Fig. 2D, ANOVA, $P < 0.01$) and PGE₂ production by $52.6 \pm 8.7\%$ (Fig. 2E, ANOVA, $P < 0.01$). More importantly, the acid-induced increase in thymidine incorporation and PGE₂ production was blocked by knockdown of mPGES1 (Fig. 2, D and E). Figures 3, A and B, and Fig. 4, A and B, show that mPGES2 siRNA and cPGES siRNA effectively knocked down mPGES2 and cPGES, respectively. However, knockdown of mPGES2 and cPGES did not have any effect on acid-induced increase in thymidine incorporation (Figs. 3C and 4C). These data suggest that the acid-induced increase in cell proliferation and PGE₂ production may be mediated by activation of mPGES1 rather than mPGES2 or cPGES.

We further examined the expression of mPGES1 in different cell lines. We found that mPGES1 protein levels in

Fig. 3. Microsomal PGES2 is not involved in the acid-induced increase in cell proliferation. A typical image of three Western blot analyses (A) and summarized data (B) show that mPGES2 siRNA effectively knocked down mPGES2. (C) Knockdown of mPGES2 did not have a statistically significant effect on acid-induced increase in thymidine incorporation. FLO cells were treated with acid (pH 4.0, 1 hour), washed, and incubated with regular medium for an additional 24 hours after mPGES2 siRNA and control siRNA were introduced into FLO cells for 24 hours by Lipofectamine 2000. $N = 3$, ANOVA, * $P < 0.05$; ** $P < 0.01$, compared with control siRNA group; ANOVA, # $P < 0.05$, compared with mPGES2 siRNA group.

Fig. 4. Cytoplasmic PGES is not involved in the acid-induced increase in cell proliferation. A typical image of three Western blot analyses (A) and summarized data (B) show that cPGES siRNA effectively knocked down cPGES. (C) Knockdown of cPGES did not have a statistically significant effect on acid-induced increase in thymidine incorporation. FLO cells were treated with acid (pH 4.0, 1 hour), washed, and incubated with regular medium for an additional 24 hours after cPGES and control siRNAs were introduced into FLO cells for 24 hours by Lipofectamine 2000. $N = 3$, ANOVA, $*P < 0.05$; $**P < 0.01$, compared with control siRNA group; ANOVA, $\#P < 0.05$, compared with cPGES siRNA group.

EA cell lines FLO and OE33 were significantly higher than in the normal squamous epithelial cell line HET-1A (Fig. 5, A and B). We also found that acid statistically significantly increased the mPGES1 mRNA levels by $109.6 \pm 28.5\%$ ($P < 0.05$, ANOVA) (Fig. 5C) in FLO cells. The data indicate that mPGES1 may be overexpressed in EA cells and that acid may upregulate mPGES1 expression.

Role of NOX5-S in Acid-Induced mPGES1 Expression. We have shown that NOX5-S is involved in the acid-induced increase in cell proliferation. Thus, we also examined the role of NOX5-S in acid-induced mPGES1 expression by knockdown of NOX5-S with NOX5 siRNA, which has been shown by us to effectively knock down NOX5-S (Fu et al., 2006). Figure 6, B, C, and E, shows that knockdown of NOX5-

Fig. 5. mPGES1 expression in EA cells. A typical image of three Western blot analyses (A) and summarized data (B) show that mPGES1 protein expression in FLO and OE33 cells was significantly increased when compared with HET-1A cells. The mPGES1 protein was nearly undetectable in HET-1A cells. (C) Acid treatment significantly increased mPGES1 mRNA levels in FLO cells. FLO cells were treated with acid (pH 4.0, 1 hour), washed, and incubated with regular medium for an additional 24 hours. $N = 3$, ANOVA, $*P < 0.05$, compared with HET-1A group; t test, $\#P < 0.05$, compared with control group.

S statistically significantly decreased the acid-induced increase in mPGES1 mRNA levels from $81.5 \pm 26.5\%$ to $32.7 \pm 9.3\%$ ($N = 6$, $P < 0.05$, Student's t test) and the mPGES1 protein levels from $126.9 \pm 22.4\%$ to $66.2 \pm 12.8\%$ ($N = 3$, $P < 0.05$, Student's t test) in FLO cells. In addition, knockdown of NOX5-S significantly decreased the acid-induced increase in thymidine incorporation from $90.6 \pm 11.4\%$ to $50.1 \pm 5.8\%$ ($P < 0.05$, Student's t test) (Fig. 6G) and in PGE₂ production from $75.2 \pm 6.3\%$ to $37.6 \pm 18.0\%$ ($P < 0.05$, Student's t test) (Fig. 6I).

The data suggest that NOX5-S may be involved in acid-induced mPGES1 expression and PGE₂ production. Consistent with our previous finding (Fu et al., 2006), knockdown of NOX5-S statistically significantly decreased thymidine incorporation by $22.2 \pm 6.1\%$ compared with control at the basal condition ($N = 6$, $P < 0.02$, Student's t test) (Fig. 6F), suggesting that NOX5-S may be involved in the increased cell

proliferation of EA cells at the basal condition. Knockdown of NOX5-S did not have any statistically significant effect on mPGES1 mRNA, mPGES1 protein, or PGE₂ production at the basal condition (Fig. 6, A, D, and H).

To further confirm the role of NOX5-S in acid-induced mPGES1 expression, we examined whether NOX5-S activates mPGES1 promoter. We constructed an mPGES1 reporter plasmid by ligating the mPGES1 promoter region (−1328 to −10, position from ATG) into a PGL3-basic plasmid we termed mPGES1318. Cotransfection of an mPGES1318 reporter plasmid and an NOX5-S expression plasmid statistically significantly increased the luciferase activity by $77.4 \pm 7.4\%$ ($P < 0.05$, ANOVA) (Fig. 6J), which suggests that NOX5-S derived ROS may activate mPGES1 promoter.

Role of NF- κ B in Acid-Induced mPGES1 Expression. We have shown that NOX5-S-derived ROS activate NF- κ B. After analyzing mPGES1 promoter region, we found three

Fig. 6. NOX5-S may be involved in acid-induced mPGES1 expression. Transfection of NOX5 siRNA and control siRNA was performed with Lipofectamine 2000. After 4-hour transfection, the transfection medium was replaced with regular medium. Then, 24 hours later, the transfectants were exposed to acid (pH 4.0, 1 hour), washed, and incubated with regular medium for an additional 24 hours. (A) At the basal condition, knockdown of NOX5 slightly decreased mPGES1 mRNA expression, but the decrease was not statistically significant ($N = 9$, t test $P = 0.0653$). (B) Acid treatment-induced increase in mPGES1 mRNA expression was significantly decreased by knockdown of NOX5 in FLO cells ($N = 6$). A typical image of three Western blot analyses (C) and summarized data (D) show that at the basal condition knockdown of NOX5 slightly decreased mPGES1 protein expression, but this decrease was not statistically significant ($N = 3$, t test $P = 0.0571$). (E) Summarized data from C show that the acid treatment-induced increase in mPGES1 protein expression was significantly decreased by knockdown of NOX5 in FLO cells ($N = 3$). (F) At the basal condition, knockdown of NOX5-S significantly decreased thymidine incorporation ($N = 6$), suggesting that NOX5-S may be involved in the increased cell proliferation of esophageal adenocarcinoma cells at the basal condition. (G) Acid treatment-induced increase in thymidine incorporation was significantly decreased by knockdown of NOX5 in FLO cells ($N = 7-17$). (H) At the basal condition, knockdown of NOX5-S did not have a significant effect on PGE₂ levels ($N = 9$). (I) Acid treatment-induced increase in PGE₂ production was significantly decreased by knockdown of NOX5 in FLO cells ($N = 4-7$). (J) FLO cells were transfected with $0.1 \mu\text{g}$ of *R. luciferase* and $0.5 \mu\text{g}$ of mPGES1318 reporter plasmid with NOX5-S or pCMV plasmid by the use of Lipofectamine 2000. NOX5-S overexpression significantly increased the luciferase activity, suggesting that NOX5-S may be involved in mPGES1 promoter activation. These data suggest that NOX5-S may mediate the acid-induced increase in mPGES1 expression. The percentage increase was calculated as follows: Percentage increase = (Acid-treatment group – Without-acid treatment group) $\times$ 100/Without-acid treatment group. Student's t test * $P < 0.05$; ** $P < 0.02$.

potential NF- κ B binding sites: 1) GGAGTCTCCC (from -1269 to -1260), 2) GGGCTCACCC (from -656 to -647), and 3) CGGGACACCC (from -211 to -202, position from ATG). Thus, we examined the role of NF- κ B in acid-induced mPGES1 expression by knockdown of NF- κ B p50 with p50 siRNA, which has been shown by us to effectively knock down p50 (Hong et al., 2010). Figure 7B, C, and E shows that the knockdown of p50 with p50 siRNA statistically significantly decreased the acid-induced increase in mPGES1 mRNA levels from $68.4 \pm 10.6\%$ to $20.5 \pm 20.5\%$ ($P < 0.05$, Student's *t* test) and in mPGES1 protein levels from $161.5 \pm 22.5\%$ to $68.9 \pm 42.2\%$ ($P < 0.05$, Student's *t* test). In addition, the knockdown of p50 statistically significantly reduced the acid-induced increase in thymidine incorporation from $64.2 \pm 13.3\%$ to $29.2 \pm 11.6\%$ ($P < 0.05$, Student's *t* test) (Fig. 7G) and in PGE₂

production from $113.5 \pm 19.0\%$ to $48.9 \pm 13.7\%$ ($P < 0.05$, Student's *t* test) (Fig. 7I).

The data suggest that NF- κ B1 p50 may be involved in acid-induced mPGES1 expression. The knockdown of p50 statistically significantly decreased thymidine incorporation by $18.4 \pm 6.9\%$ compared with control at the basal condition ($N = 9$, $P < 0.05$, Student's *t* test) (Fig. 7F), suggesting that p50 may also be involved in the increased cell proliferation of EA cells at the basal condition. Knockdown of p50 did not have any statistically significant effect on mPGES1 mRNA, mPGES1 protein, or PGE₂ production at the basal condition (Fig. 7, A, D, and H).

To confirm the role of p50 in acid-induced mPGES1 expression, we examined whether NF- κ B activates mPGES1 promoter. The mPGES1318 reporter plasmid contains the

Fig. 7. NF- κ B may be involved in acid-induced mPGES1 expression. Transfections of p50 siRNA and control siRNA were performed with Lipofectamine 2000 in FLO cells. After 4-hour transfection, the transfection medium was replaced with regular medium. Then, 24 hours later, the cells were exposed to acid (pH 4.0, 1 hour), washed, and incubated with regular medium for an additional 24 hours. (A) At the basal condition, knockdown of p50 did not have any significant effect on mPGES1 mRNA expression ($N = 6$). (B) The acid treatment-induced increase in mPGES1 mRNA expression was significantly decreased by knockdown of p50 in FLO cells ($N = 5-8$). (C) A typical image of three Western blot analyses and (D) summarized data show that at the basal condition knockdown of p50 did not have any significant effect on mPGES1 protein expression. (E) Summarized data from figure C show that the acid treatment-induced increase in mPGES1 protein expression was significantly decreased by knockdown of p50 in FLO cells ($N = 3$). (F) At the basal condition, knockdown of p50 significantly decreased thymidine incorporation ($N = 9$), suggesting that p50 may be involved in the increased cell proliferation of esophageal adenocarcinoma cells at the basal condition. (G) Acid treatment-induced increase in thymidine incorporation was significantly decreased by knockdown of p50 in FLO cells ($N = 12$). (H) At the basal condition, knockdown of p50 slightly decreased PGE₂ production, but the decrease was not statistically significant ($N = 4$). (I) Acid treatment-induced increase in PGE₂ production was significantly decreased by knockdown of p50 in FLO cells ($N = 4-7$). (J) FLO cells were transfected with 0.1 μ g of *R. luciferase* and 0.5 μ g of mPGES1318 plasmid with p50, p65, p50 plus p65, or pCMV plasmid by the use of Lipofectamine 2000. Overexpression of p50, p65 or p50, and p65 in combination significantly increased the luciferase activity, suggesting that NF- κ B1 may activate mPGES1 promoter ($N = 3$). The data suggest that NF- κ B may mediate the acid-induced increase in mPGES1 expression. The percentage increase was calculated as follows: percentage increase = (acid-treatment group - without-acid treatment group) $\times$ 100/without-acid treatment group. ANOVA, ** $P < 0.01$, compared with mPGES1318 plus pCMV group; *t* test, * $P < 0.05$, compared with control siRNA.

three potential binding sites already described. Cotransfection of FLO cells with mPGES1318 reporter plasmid and p50 expression plasmid, p65 expression plasmid, or p50 plus p65 statistically significantly increased luciferase activity ($P < 0.05$, ANOVA) (Fig. 7F), suggesting that NF- κ B1 p50 may activate mPGES1 promoter.

NF- κ B Binding Sites in mPGES1 Promoter. We examined NF- κ B1 p50 binding to three possible binding sites in the mPGES1 promoter by use of a ChIP assay. In the ChIP assay, mPGES1 DNA of the mPGES1 promoter-containing binding sites 1 and 3 were detectable in the immunoprecipitated chromatin samples of the FLO cell lysate via use of PCR with two pairs of primers targeting the -1364 to -1062 (containing binding site 1) and -313 to -156 (position from ATG, containing binding site 3) regions of mPGES1 promoter (Fig. 8, A and C). However, no cDNA product was detected via PCR with a pair of primers targeting the -771 to -554 (containing binding site 2) (Fig. 8B). The PCR products were sequenced and were specific for mPGES1 promoter. The data suggest that NF- κ B1 p50 binds to binding sites 1 and 3 in the mPGES1 promoter.

To further confirm these results, we performed gel-mobility supershift assays. In the gel-shift assay, two prominent complexes were detectable with either the oligonucleotide mPGES1-WT1 (containing binding site 1 GGAGTCTCCC [from -1269 to -1260], position from ATG) (Fig. 9A) or mPGES1WT3 (containing binding site 3 CGGGACACCC [from -211 to -202], position from ATG) (Fig. 9B). Competition experiments with unlabeled (cold) oligonucleotides (mPGES1-WT1 or mPGES1-WT3) significantly reduced the bindings. The addition of the mutant oligonucleotides (mPGES1-MUT1 or mPGES1-MUT3) had less effect on binding (Fig. 9, A and B). In the supershift assays, the bands were supershifted with a p50 antibody (Fig. 9, A and B). These data suggest that p50 binds to two sites—binding sites 1 and 3—in the mPGES1 promoter.

To examine whether p50 activates binding sites 1 and 3, we constructed an additional mPGES1 reporter plasmid mPGES791 (containing the mPGES1 promoter fragment -791 to 0, position from ATG, and containing binding sites 2 and 3), and three mutant plasmids as follows: mPGES791M, mPGES1318M1, and mPGES1318M2. Binding site 3 was mutated in the mPGES791M plasmid and mPGES1318M1 plasmid. Both binding sites 1 and 3 were mutated in mPGES1318M2. Cotransfection of FLO cells with mPGES1318M1 plasmid and p50 expression plasmid, p65 expression plasmid, or p50 plus p65 statistically significantly increased the luciferase activity ($P < 0.05$, ANOVA) (Fig. 10A). In contrast, cotransfection of FLO cells with mPGES1318M2 plasmid and p50 expression plasmid, p65 expression plasmid, or p50 plus p65 had no effect on the luciferase activity (Fig. 10B). These data suggest that NF- κ B1 p50 may activate binding site 1.

In addition, cotransfection of FLO cells with mPGES791 reporter plasmid and p50 expression plasmid, p65 expression plasmid, or p50 plus p65 in combination statistically significantly increased the luciferase activity ($P < 0.01$, ANOVA) (Fig. 10C). However, cotransfection of FLO cells with mPGES791M plasmid and p50 expression plasmid, p65 expression plasmid, or p50 plus p65 in combination had no effect on the luciferase activity (Fig. 10D), although binding site 2 was not mutated. These data suggest that NF- κ B1 p50

Fig. 8. NF- κ B binding sites in the mPGES1 promoter. (A) A typical example of three experiments shows that mPGES1 DNA was detectable by PCR using primers covering the potential NF- κ B binding site 1 in the immunoprecipitated chromatin sample of FLO cell lysate with an antibody against p50. (B) A typical example of three experiments shows that mPGES1 DNA was undetectable by PCR using primers covering the potential NF- κ B binding site 2 in the immunoprecipitated chromatin sample of FLO cell lysate with an antibody against p50. (C) A typical example of three experiments shows that mPGES1 DNA was detectable by PCR using primers covering the potential NF- κ B binding site 3 in the immunoprecipitated chromatin sample of FLO cell lysate with an antibody against p50. These data suggest that p50 binds to binding sites 1 and 3 in the mPGES1 promoter. The PCR products were sequenced and specific for the mPGES1 promoter. Positive, genomic DNA was used as a positive control; rabbit IgG or buffer was used as the negative control.

may activate binding site 3, but not binding site 2, of mPGES1 promoter.

Discussion

GERD complicated by BE is a major risk factor for EA (Lagergren et al., 1999). The mechanisms of acid reflux-induced progression from BE to EA are not fully understood. We have shown that NOX5-derived ROS may be important for this progression (Si et al., 2007) as overexpression of NOX5-S in Barrett's cells significantly increases COX-2 expression and PGE₂ production, an increase in PGE₂ that mediates the acid-induced increase in cell proliferation (Si et al., 2007). PGES has been shown to mediate COX-2-derived PGE₂ production (Murakami et al., 2002; Park et al., 2006) and is expressed in EA (von Rahden et al., 2008). Thus far, three isoforms of PGES have been identified as follows: cPGES, mPGES1, and mPGES-2 (Tanioka et al., 2000; Murakami et al., 2003). Which PGE synthases are involved in acid-induced PGE₂ production is not known.

We found that all isoforms of PGES were present in FLO EA cells. Only mPGES1, however, appeared to mediate the acid-induced increase in cell proliferation and PGE₂ production, because the acid-induced increase in cell proliferation and PGE₂ production was significantly reduced by knockdown of mPGES1 but not by that of mPGES2 or cPGES. In addition, mPGES1 protein levels were significantly increased in EA cells, and mPGES1 mRNA expression was upregulated in response to acid treatment in FLO cells. The mPGES1 protein was nearly undetectable by Western blot analysis in the human squamous epithelial cells HET-1A. This result is consistent with the literature (Jang et al., 2004).

Human mPGES1 was identified in 1999 and is a member of the membrane-associated proteins belonging to the

Fig. 9. NF- κ B binding sites in mPGES1 promoter in a gel-shift assay. (A) A prominent complex was detectable after the labeled oligonucleotide mPGES1-WT1 (containing binding site 1 GGAGTCTCCC [from -1269 to -1260], position from ATG) was incubated with the recombinant protein p50. The competition experiments with unlabeled (cold) mPGES1-WT1 oligonucleotide significantly reduced binding. Addition of the mutant oligonucleotides mPGES-MUT1 had less effect on binding. In a supershift assay, the supershifted band was detected with a p50 antibody. (B) A prominent complex was detectable after the labeled oligonucleotide mPGES1WT3 [containing binding site 3 CGGGACACCC (from -211 to -202), position from ATG] was incubated with the recombinant protein p50. Competition experiments with unlabeled (cold) mPGES1-WT3 oligonucleotide significantly reduced binding. Addition of the mutant oligonucleotides mPGES-MUT3 had less effect on binding. In a supershift assay, the supershifted band was detected with a p50 antibody.

eicosanoid and glutathione metabolism family (Jakobsson et al., 1999). Human mPGES1 is overexpressed in varieties of inflammatory conditions, such as arthritis (Murakami et al., 2003; Westman et al., 2004), inflammatory bowel disease (Subbaramaiah et al., 2004), and hepatitis (Murakami et al., 2003). Microsomal PGES1 is also upregulated in human tumors, such as colon (Yoshimatsu et al., 2001), stomach (van Rees et al., 2003; Jang, 2004; Gudis et al., 2007), larynx (Kawata et al., 2006), lung (Jakobsson et al., 1999; Yoshimatsu et al., 2001), liver (Takii et al., 2007; Breinig et al., 2008), pancreas (Hasan et al., 2008), breast (Mehrotra et al., 2006), ovary (Rask et al., 2006), and brain (Payner et al., 2006; Lalier et al., 2007; Baryawno et al., 2008; Barbieri et al., 2011). These data indicate a crucial role of mPGES1 in tumorigenesis.

The mechanisms of acid-induced mPGES1 expression are not known. Because NOX5-S is involved in the acid-induced increase in cell proliferation and PGE₂ production (Si et al., 2007), we examined the role of NOX5-S in acid-induced mPGES1 expression. We found that NOX5-S may be involved in acid-induced mPGES1 expression because 1) knockdown of NOX5-S significantly decreased the acid-induced increase in mPGES1 mRNA and protein expression, thymidine incorporation, and PGE₂ production in FLO cells; and 2) cotransfection of mPGES1 reporter plasmid and NOX5-S expression plasmid significantly increased the luciferase activity, indicating that activation of mPGES1 promoter may be mediated by NOX5-S. Our data are also consistent with the literature that ROS increase mPGES1 expression in mouse cardiac endothelial cells (Barbieri et al., 2011).

ROS have been reported to activate NF- κ B (Flohe et al., 1997). We have also shown that NOX5-S-derived ROS activate NF- κ B in the BE cell line BAR-T (Si et al., 2007). NF- κ B, a transcription factor, is known to function as a 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- κ B 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- κ B1 (p105/p50), and NF- κ B2 (p100/p52). p105 and p100 are larger precursor proteins containing I κ B (an inhibitor of κ B)-like ankyrin repeat sequences in their carboxyl termini. Because of their I κ B-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 (Karin et al., 2002).

In the cytoplasm, NF- κ B is in an inactive state, and its activity is regulated by three pathways. In the first pathway, a heterotrimer composed of p50 and p65, and I κ B 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 I κ B-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- κ B activates gene transcription (Karin et al., 2002; De Bosscher et al., 2006). p50 plays an important role in lymphoid organogenesis and inflammation, whereas

Fig. 10. NF- κ B may activate mPGES1 promoter via binding sites 1 and 3. (A) In FLO cells transfected with mPGES1318M1, overexpression of p50, p65 or p50, and p65 in combination significantly increased the luciferase activity, suggesting that NF- κ B1 may activate mPGES1 promoter via binding site 1. The mPGES1318M1 plasmid contains mutant binding site 3. FLO cells were transfected with 0.1 μ g of *R. luciferase* and 0.5 μ g of mPGES1318M1 plasmid with p50, p65, p50 plus p65, or pCMV plasmid by use of Lipofectamine 2000. (B) Cotransfection of FLO cells with mPGES1318M2 plasmid and p50, p65, or p50 and p65 plasmids in combination had no effect on the luciferase activity, suggesting that mutation of binding sites 1 and 3 almost abolishes p50-induced activation of mPGES1 promoter. Both binding sites 1 and 3 were mutated in mPGES1318M2. The data also suggest that p50 may not activate mPGES1 promoter via binding site 2 because binding site 2 is not mutated in the plasmid mPGES1318M2. (C) In FLO cells transfected with mPGES791 plasmid, overexpression of p50, p65 or p50, and p65 in combination significantly increased the luciferase activity, suggesting that NF- κ B1 may activate mPGES1 promoter via binding site 3. The mPGES791 was generated by inserting the mPGES1 promoter fragment -791 to 0 (position from ATG) into a pGL3 basic plasmid and contains binding sites 2 and 3. (D) Cotransfection of FLO cells with mPGES791M plasmid and p50, p65, or p50 plus p65 plasmids had no effect on the luciferase activity, suggesting that mutation of binding site 3 almost abolishes p50-induced activation of mPGES1 promoter. Binding site 3 was mutated in plasmid mPGES791M. The data also suggest that p50 may not activate mPGES1 promoter via binding site 2 because binding site 2 is not mutated in the plasmid mPGES791M. $N = 3$, ANOVA, $^{**}P < 0.01$, compared with control mPGES1318M1 plus pCMV group or mPGES791 plus pCMV group.

p52 is mainly involved in lymphoid organogenesis (Shih et al., 2011). Therefore, we focused on the role of p50 in acid-induced mPGES1 expression.

We found that NF- κ B p50 may mediate acid-induced mPGES1 expression because knockdown of NF- κ B1 p50 significantly decreased the acid-induced mPGES1 protein and mRNA expression, thymidine incorporation, and PGE₂ production. In addition, NF- κ B may activate mPGES1 promoter; cotransfection of mPGES1 reporter plasmids with p50, p65, or p50 plus p65 plasmids significantly increased the luciferase activity in FLO cells. This result is consistent with the findings in the mouse macrophage cell line RAW 264.7 (Diaz-Munoz et al., 2010), rat chondrocytes, and pulmonary A549 cells (Catley et al., 2003; Moon et al., 2005).

In addition, knockdown of NOX5-S and p50 significantly decreased the thymidine incorporation under the basal condition, suggesting that NOX5-S and p50 may be needed tonically for cell proliferation.

NF- κ B binding sites in human mPGES1 are not known. After analyzing the mPGES1 promoter region, we found three potential NF- κ B binding sites as follows: 1) GGAGTCTCCC (from -1269 to -1260), 2) GGGCTCACCC (from -656 to -647), and 3) CGGGACACCC (from -211 to -202, position from ATG). Our data indicate that NF- κ B1 p50 only binds to the potential binding sites 1 and 3 because 1) in the ChIP assay, the mPGES1 DNA of the mPGES1 promoter covering binding sites 1 and 3 was detectable in the immunoprecipitated chromatin samples of the FLO cell lysate by PCR; 2) in the

gel-shift assays, two prominent complexes were detectable with either the oligonucleotide mPGES1-WT1 (containing binding site 1) or mPGES1-WT3 (containing binding site 3); 3) competition experiments with unlabeled (cold) mPGES1 oligonucleotides significantly reduced the bindings, and addition of the mutant oligonucleotides had less effect on binding; 4) in the supershift assays, the bands were supershifted with a p50 antibody; 5) p50 activated the third binding site in a cotransfection experiment with mPGES791 reporter plasmid, and mutation of binding site 3 completely blocked this activation; and 6) p50 activated the binding site 1 in a cotransfection experiment with mPGES1318M1 plasmid where binding site 3 was mutated and mutation of both binding sites 1 and 3 completely blocked the activation.

Our data suggest that acid-induced mPGES1 expression may be mediated by activation of NOX5-S and NF- κ B p50. However, our data cannot exclude other mechanisms involved in acid-induced mPGES1 expression. Microsomal PGES1 is present in FLO EA cells and mediates acid-induced cell proliferation and PGE₂ production. Acid-induced mPGES1 expression depends in part on activation of NOX5-S and NF- κ B. It is possible that in BE the acid reflux upregulates NOX5-S, increases ROS production, and activates NF- κ B. Activation of NF- κ B in turn upregulates mPGES1 expression and increases PGE₂ production, thereby enhancing cell proliferation and contributing to the esophageal tumorigenesis.

Authorship Contributions

Participated in research design: Zhou, Li, Cao.

Conducted experiments: Zhou, Li.

Performed data analysis: Zhou, Li, Cao.

Wrote or contributed to the writing of the manuscript: Zhou, Li, Resnick, Wands, Cao.

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