

NADPH oxidase NOX5-S and NF- κ B1 Mediate Acid-Induced microsomal prostaglandin synthase-1 (mPGES1) Expression In Barrett's Esophageal Adenocarcinoma (EA) Cells

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Abbreviations:

BE: Barrett's esophagus; COX: cyclooxygenase; EA: esophageal adenocarcinoma; EDTA: ethylenediaminetetraacetic acid; FBS: fetal bovine serum; GAPDH: glyceraldehydes-3-phosphate dehydrogenase; GERD: gastroesophageal reflux disease; NADPH: Nicotinamide adenine dinucleotide 3'-phosphate reduced form; NOX: NADPH oxidase; PG: prostaglandin; PGES: prostaglandin E synthase; mPGES: microsomal PGES; cPGES: cytosolic PGES; ROS: reactive oxygen species; RT-PCR: reverse transcription polymerase chain reaction; SDS: sodium dodecyl sulfate; siRNA: small interfering RNA.

Abstract

Mechanisms of progression from Barrett's esophagus (BE) to esophageal adenocarcinoma (EA) are not known. Cyclooxygenase-2 (COX2)-derived prostaglandin E₂ (PGE₂) has been shown to be important in esophageal tumorigenesis. We have shown that COX2 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, respectively. Knockdown of mPGES1 by mPGES1 siRNA blocked acid-induced increase in PGE₂ production and thymidine incorporation. Knockdown of NOX5-S 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 an NF-κB in vivo activation reporter plasmid pNF-κB-Luc. Knockdown of NF-κB1 p50 by p50 siRNA significantly decreased acid-induced increase in mPGES1 expression, thymidine incorporation and PGE₂ production. Two novel NF-κB 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-κB1 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, mechanisms of progression from BE to EA are not fully understood.

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 up-regulation of 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 (COX2) expression as well as prostaglandin E₂ (PGE₂) production in BE (Si et al., 2007), suggesting that 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 (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); 4) in EA cells selective COX-2 inhibitors significantly decrease proliferation and increase apoptosis (Souza et al., 2000; Zimmermann et al., 1999). However, COX-2 inhibitors have severe cardiovascular side effects (Solomon et al., 2005), limiting their usefulness in treatment of cancer. Therefore, it is important to explore its 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). So far three isoforms of PGES have been identified: a cytosolic (cPGES) and two microsomal (mPGES) isomerases (Jakobsson et al., 1999; Tanikawa et al., 2002; Tanioka et al., 2000). 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). The mPGES1 (PTGES1) is a 16-kDa membrane-associated protein, coupled with COX-2 and may be induced in a variety of pathological 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, mechanisms of mPGES1 upregulation in EA are not fully understood.

In the present study, we find 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-κB1 p50. We also identified two NF-κB binding sites in the promoter region of mPGES1, responsible for regulation of the mPGES1 expression.

Materials and Methods

Cell culture and acid 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). FLO cells and Human EA cell line OE33 (Sigma, St. Louis, MO) were cultured in DMEM containing 10% fetal bovine serum and antibiotics.

Human esophageal squamous epithelial cell line HET-1A (ATCC, 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, Walkersville, MD) in wells precoated with a mixture of 0.01 mg/ml fibronectin and 0.03 mg/ml vitrogen 100 (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, 250µl) or normal DMEM (control) added to each well in a 12-well plate for 1 h, and the final pH was about 4.9 after a 1-h incubation, washed, and cultured in fresh medium (pH 7.2) for an additional 24 h. Finally, the culture medium and cells are collected for measurements of PGE₂ and mPGES1 mRNA and protein level.

Small Interfering RNA (siRNA) and Plasmid Transfection- For siRNA transfection, at 40–50% confluence cells were trypsinized and diluted 1:5 with fresh medium without antibiotics (1–3 × 10⁵ cells/ml) and transferred to 12-well plates (1 ml per 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 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 h,

washed, and cultured in fresh medium (pH 7.2) for an additional 24 h. Finally, the culture medium and the transfectants were collected for measuring mPGES1 mRNA and protein level. Transfection efficiencies were determined by fluorescence microscopy after transfection of Block-it fluorescent oligo (Invitrogen, Carlsbad, CA) and were about 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, 0.5µg plasmids (NOX5-S, mPGES1, p50, p65, pCMV) or 0.1µg renilla 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, Carlsbad, CA) according to the manufacturer's protocol. 1.0 µg of total RNAs were reversely transcribed by using a kit SUPERSCRIPT First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA).

Quantitative real-time PCR-The mRNA expression was measured using real-time PCR analysis. Real-time PCR analysis was performed in a 15 µl 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'- GGGGTCTTGGGTTCTGTAT-3'), mPGES1 antisense (5'- GACTGCAGCAAAGACATCCA-3'), mPGES2 sense (5'-CTGTCCCATGGCTAC ATCCT-3'), mPGES2 antisense (5'- CGCCAC AAACCTTTCCTTTA-3'), cPGES sense (5'-AAG TCGACTCCCTAGCAGCA-3'), cPGES antisense (5'-CGTACCACTTTGCAGAAGCA-3'), 18S sense (5'-CGGACAGGATTGACAGATTGAT AGC-3') and 18S antisense (5'- TGCCAGAGTCT CGTTCGTTATCG-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; one cycle at 94 °C for 1 min; and one 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 Ct, 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.

Plasmids Construct - The primers used for construction of mPGES-1 plasmid were as follows: 5'-CCGGGTACCGGAGTCTCCCTATG TTGCCC-3' (1F), 5'-CCGGGTACCAGGCCAG CAGGTTAGCGTCTC-3' (3F), 5'-ACAAC TGC GCTGGGGAGTGAC-3' (4F), 5'-CCGGGTA CCCTGGGAATACAGGCGAGCAC-3' (5F), 5'-CTGGGTCCTTGACATTCGGAGAC-3' (791F), 5'-CGTAGTCTCACTATGTTGCCAG GC-3' (1318F), 5'-CCGCTCGAGCTCTGGCC AGCGCAGCTCAA-3' (1R), 5'-CCGCTCGAG ACATCTTGATGACCAGCAGCGT-3' (2R), 5'-CCGCTCGAGCGCAGCTCAACTGTGGG TG-3' (5R), 5'-GTCTCCGAATGTCAA GGACCCAG-3' (791R), 5'-GCCTGGGCAAC ATAGTGAGACTACG-3' (1318R).

Four primers (5F and 5R, 4F and 1R for nested PCR) were used to construct mPGES1318 plasmid, containing the mPGES1 promoter fragment -1328 to -10 (position from ATG). 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 site 2 & 3 (figure 1). Human genomic DNA was used as a template.

Other three plasmids mPGES1318M1, mPGES1318M2, and mPGES791M were constructed by using the over-lap extension PCR (figure 1). The mPGES1318M1 was constructed by three PCRs using three paired primers: 5F - 791R, 791F - 5R, and 5F - 5R, while 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, while 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 Kpn I (Invitrogen, Carlsbad, CA) and Xho I (Invitrogen, Carlsbad, CA) restriction site. The constructed plasmids were verified by sequencing at GENEWIZ, Inc. (South Plainfield, NJ).

Luciferase assay- FLO EA cells were seeded in 12-well plates for 24 h. 0.1 µg renilla and 0.5 µg of mPGES1318, mPGES791, mPGES1318M1, mPGES1318M2, mPGES791M, or NOX5-S reporter plasmids with or without pCMV plasmid (control), NOX5-S, p50 or p65 expression plasmids were transfected by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). 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 Topcount-NXT™ Microplate Scintillation and Luminescence Counter (Packard Bioscience company) according to the protocol (Promega, Madison, WI) and normalized to renilla. The

number of experiments was indicated in figure legends and each experiment was performed in triplicate.

Western blot analysis- Cells were lysed in a Triton X lysis buffer containing 50 mM Tris-hydrochloride (pH 7.5), 100 mM sodium chloride, 50mM sodium fluoride, 5mM EDTA, 1% (v/v) Triton X-100, 40mM 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 15,000 \times *g* for 5 min, and the protein concentration in the supernatant was determined. Western blot analysis was done as described previously (Cao et al., 2003; Fu et al., 2006). Briefly, after these supernatants were subjected to SDS-PAGE, the separated proteins were electrophoretically transferred to a nitrocellulose membrane at 100 V for 1-2 hours. 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). Detection was achieved with an enhanced chemiluminescence agent (Amersham Biosciences).

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 method of Bradford (Bradford, 1976).

[³H]-thymidine incorporation- For acid treatment, FLO cells were incubated with acidic DMEM (pH 4.0) for 1 h, washed, and cultured in fresh medium (pH 7.2) for an additional 24 h. For siRNA transfection, 24 hours after siRNAs of mPGES1, mPGES2, cPGES, or control were introduced, cells were treated without or with acidic medium, and then incubated with methyl-[³H] thymidine (0.05 μ Ci/ml) for 4 h. After being washed three times with PBS to remove unincorporated radioactivity, cells were collected and homogenized with a lysis buffer containing (pH7.4): 50 mM HEPES, 50 mM sodium chloride, 1% Triton X-100, 1% Non-idet P-40, 0.1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol. Methyl-[³H]thymidine uptake was measured in a Topcount-NXTTM 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.

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

Chromatin Immunoprecipitation (ChIP) Assay- A ChIP assay was performed using the ChIP assay kit (Upstate, Charlottesville, VA) following manufacturer's protocol. Briefly, FLO cells grown on plastic dishes for 2 days (1×10^6 cells) were treated with acidic medium (pH 4.0) for 1 h and then treated with 1% formaldehyde for 10 min 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 min at 700 \times g 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 min. Nuclei were recovered by centrifugation at 1000×g for 10 min and resuspended in 400 µl of SDS lysis buffer (1% SDS, 10 mM EDTA, 50mM Tris-HCl, pH 8.1) containing protease inhibitors. The mixture was incubated on ice for 10 min, and the lysate was sonicated eight times for 10 s each time on ice to shear the genomic DNA to lengths of 0.2–1 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). Five hundred micro liters of the diluted lysate were kept for input control. The chromatin solution was precleared with salmon sperm DNA/protein A-agarose for 1 h at 4°C. Anti-p50 antibody (Upstate, Charlottesville, VA) was added to the supernatant fraction and incubated overnight at 4°C with rotation. The mixture was then incubated with 60µl of salmon sperm DNA/protein A-agarose slurry at 4°C with rotation for 1 h. 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 (1000×g at 4°C for 1 min). The pellet was washed sequentially (3–5 min 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, 20mM Tris-HCl, 150 mM NaCl, pH 8.0), high salt washing buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, 500mM NaCl,pH8.0), LiCl washing buffer (0.25M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0), and 1×TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH8.0). After the final wash, the pellet was eluted by two 15-min incubations with 250µl of freshly made elution buffer (1% SDS and 50 mM NaHCO₃). Two fractions of elutes were combined, and 20µl of 5 M NaCl was added to the supernatant. Crosslinking was reversed by heating samples at 65°C for 4 h, followed by addition of 10µl of 0.5mM EDTA, 20µl of 1M Tris-HCl, pH6.5, and 2µl of 10 mg/ml proteinase K. Samples were incubated at 45°C for

2 h, and DNAs were then extracted by phenol chloroform extraction, followed by ethanol precipitation. DNA pellets were resuspended in 50µl of H₂O, and 5µl of them were used for PCR analysis. PCR was carried by using three pairs of primers (covering three potential NF-κB binding sites) at 94°C for 5 min, 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s for 35 cycles, followed by a 7-min extension at 72°C. The first pair of primers targeted the -313 to -156 region of human mPGES1 promoter (sense 5'-CCAC ATGCTCCCACACTGTAGAT-3' and antisense 5'-AGAAAGACAAGCAGTTGCCGGA-3'); The second pair targeted -717 to -554 region of human mPGES1 promoter (sense 5'-CTCCTGTC TGACCCCTATTCTGTC-3' and antisense 5'-TAGAACCCGTGACTGTGACTATGTG-3'); and the third targeted -1364 to -1062 region of human mPGES1 promoter (sense 5'-GAATACA GGCGAGCACCACCATG-3' and antisense 5'-TCCTGGA ACTCTTTCCCCAGTCA-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 (IDT). The gel shift assay was performed by incubating 0.5µg of recombinant NF-κB p50 protein (Cayman Inc., Ann Arbor, MI) with 1 µl of 50 nM IRDye700 labeled probe in a 20µl reaction buffer for 20 min at room temperature. For a supershift assay with an NF-κB p50 antibody, the antibody was preincubated with the NF-κB p50 protein for 20 min at room temperature before addition of the IRDye700 labeled probes. Oligonucleotides used are the following: wild type competitor 1 (-1277 to -1253, mPGES1-WT1): 5'-GTAGAGACGGAGTC TCCCTATGTT-3' and 3'-AACATAGGGAGAC TCCGTCTCTAC-5', containing potential NF-κB binding site 1; mutant competitor 1 (-1277 to -1253, mPGES1-MUT1): 5'-GTAGAGACGTAG TCTCACTATGTT-3' and 3'-AACATAGTGAG

ACTACGTCTCTAC-5'; wild type competitor 3 (-220 to -196, mPGES1-WT3): 5'-GACTGGG TCCGGGACACCCGGAGA-3' and 3'-TCTCCG GGTGTCCCGGACCCAGTC-5', containing potential NF- κ B binding site 3; or mutant competitor 3 (-220 to -196, mPGES1-MUT3): 5'-GACTGGGTCCTTGACATTCGGAGA-3' and 3'-TCTCCGAATGTCAAGGACCCAGTC-5'. The DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel with 2.5% glycerol in 0.5 x TBE buffer for 2 h at 70 V.

Materials- Human NOX5 siRNA was purchased from Applied Biosystems (Foster City, CA); 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). P50 antibody was purchased from Cell Signaling technology (Danvers, MA); mPGES1 antibody, mPGES2 antibody and cPGES antibody were from Cayman (Ann Arbor, MI); Triton X-100, phenylmethylsulfonyl fluoride, DMEM, antibiotics, and other reagents were purchased from Sigma (St. Louis, MO).

Statistical analysis- Data were expressed as means \pm SE. Statistical differences between two groups were determined by Student's *t*-test. Differences among multiple groups were tested by ANOVA and checked for significance by Fisher's protected least significant difference test. % increase was calculated as the following: % increase = (acid-treatment group – without-acid-treatment group) * 100/ without-acid-treatment group.

Results

Acid up-regulates mPGES1 expression in FLO EA cells

We have shown that acid-induced increase in cell proliferation is mediated by COX2-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 RT-PCR. Figure 2A showed that mPGES1, mPGES2 and cPGES were detectable in FLO EA cells.

Next we examined which PGESs mediate acid-induced increase in cell proliferation. Figure 2B & 2C showed that mPGES1 siRNA effectively knocked down mPGES1 protein. Acid significantly increased cell proliferation by 67.7±15.6% (figure 2D, ANOVA, P<0.01) and PGE₂ production by 52.6±8.7% (figure 2E, ANOVA, P<0.01). More importantly, acid-induced increase in thymidine incorporation and PGE₂ production was blocked by knockdown of mPGES1 (Figure 2D and 2E). Figure 3A, 3B, 4A and 4B showed 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 (figure 3C & 4C). These data suggest that 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 EA cell lines FLO and OE33 were significantly higher than in normal squamous epithelial cell line HET-1A (Figure 5A & 5B). We also found that acid significantly increased the mPGES1 mRNA levels by 109.6±28.5% (ANOVA, P<0.05) (Figure 5C) in FLO

cells. The data indicate that mPGES1 may be over-expressed 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 acid-induced increase in cell proliferation. Therefore we 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 6B, 6C and 6E showed that knockdown of NOX5-S significantly decreased acid-induced increase in mPGES1 mRNA levels from $81.5 \pm 26.5\%$ to $32.7 \pm 9.3\%$ increase (N=6, t test, $P < 0.05$) and mPGES1 protein levels from $126.9 \pm 22.4\%$ to $66.2 \pm 12.8\%$ increase (N=3, t test, $P < 0.05$) in FLO cells. In addition, knockdown of NOX5-S significantly decreased acid-induced increase in thymidine incorporation from $90.6 \pm 11.4\%$ to $50.1 \pm 5.8\%$ (t test, $P < 0.05$, figure 6G) and in PGE₂ production from $75.2 \pm 6.3\%$ to $37.6 \pm 18.0\%$ increase (t test, $P < 0.05$, figure 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 significantly decreased thymidine incorporation by $22.2 \pm 6.1\%$ control at the basal condition (figure 6F, N=6, $P < 0.02$, t test), suggesting that NOX5-S may be involved in the increased cell proliferation of esophageal adenocarcinoma cells at the basal condition. Knockdown of NOX5-S did not have any statistically significant effect on mPGES1 mRNA, mPGES1 protein and PGE₂ production at the basal condition (figure 6A, 6D, and 6H).

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 mPGES1 promoter region (-1328 to -10, position from ATG) into a PGL3-basic plasmid, named as mPGES1318. Co-transfection of an mPGES1318 reporter plasmid and an

NOX5-S expression plasmid significantly increased the luciferase activity by 77.4 ± 7.4 % (ANOVA, $P < 0.05$, figure 6J), suggesting 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 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 B1 p50 with p50 siRNA, which has been shown by us to effectively knock down p50 (Hong et al., 2010). Figure 7B, 7C and 7E showed that knockdown of p50 with p50 siRNA significantly decreased acid-induced increase in mPGES1 mRNA levels from 68.4 ± 10.6 % to 20.5 ± 20.5 % increase (t test, $P < 0.05$) and in mPGES1 protein levels from 161.5 ± 22.5 % to 68.9 ± 42.2 % increase (t test, $P < 0.05$). In addition, knockdown of p50 significantly reduced acid-induced increase in thymidine incorporation from 64.2 ± 13.3 % to 29.2 ± 11.6 % (t test, $P < 0.05$, figure 7G) and in PGE₂ production from 113.5 ± 19.0 % to 48.9 ± 13.7 % increase (t test, $P < 0.05$, figure 7I). The data suggest that NF- κ B1 p50 may be involved in acid-induced mPGES1 expression. Knockdown of p50 significantly decreased thymidine incorporation by 18.4 ± 6.9 % control (figure 7F, N=9, $P < 0.05$, t test) at the basal condition, suggesting that p50 may also be involved in the increased cell proliferation of esophageal adenocarcinoma cells at the basal condition. Knockdown of p50 did not have any statistically significant effect on mPGES1 mRNA, mPGES1 protein and PGE₂ production at the basal condition (figure 7A, 7D, and 7H).

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 above three potential binding sites. Co-transfection of FLO cells with mPGES1318 reporter plasmid and p50 expression plasmid, p65 expression plasmid, or p50 plus p65 significantly increased luciferase activity (ANOVA, $P < 0.05$, figure 7F), suggesting that NF- κ B p50 may activate mPGES1 promoter.

NF- κ B binding sites in mPGES1 promoter

We examined NF- κ B p50 binding to three possible binding sites in the mPGES1 promoter by a chromatin immunoprecipitation (ChIP) assay. In the ChIP assay, mPGES1 DNAs of the mPGES1 promoter containing binding site 1 and 3 were detectable in the immunoprecipitated chromatin samples of the FLO cell lysate by PCR using 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 (Figure 8A & 8C). However, no cDNA product was detected by PCR using a pair of primers targeting the -771 to -554 (containing binding site 2) (figure 8B). The PCR products were sequenced and were specific for mPGES1 promoter. The data suggest that NF- κ B p50 binds to binding site 1 and 3 in the mPGES1 promoter.

To further confirm the above results, we did 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, figure 9A) or mPGES1WT3 (containing binding site 3 CGGGACACCC (from -211 to -202), position from ATG, figure 9B). Competition experiments with unlabelled (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 (figure

9A & 9B). In the supershift assays, the bands were super-shifted with a p50 antibody (figure 9A & 9B). These data suggest that p50 binds to the two sites: binding site 1 and 3 in the mPGES1 promoter.

To examine whether p50 activates binding site 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 site 2 & 3), and three mutant plasmids: mPGES791M, mPGES1318M1, and mPGES1318M2. Binding site 3 was mutated in mPGES791M plasmid and mPGES1318M1 plasmid. Both binding site 1 and 3 were mutated in mPGES1318M2. Co-transfection of FLO cells with mPGES1318M1 plasmid and p50 expression plasmid, p65 expression plasmid, or p50 plus p65 significantly increased luciferase activity (ANOVA, $P < 0.05$, figure 10A). In contrast, co-transfection of FLO cells with mPGES1318M2 plasmid and p50 expression plasmid, p65 expression plasmid, or p50 plus p65 did not have any effect on luciferase activity (figure 10B). The data suggest that NF- κ B1 p50 may activate binding site 1.

In addition, co-transfection of FLO cells with mPGES791 reporter plasmid and p50 expression plasmid, p65 expression plasmid, or p50 plus p65 in combination significantly increased luciferase activity (ANOVA, $P < 0.01$, figure 10C). However, co-transfection of FLO cells with mPGES791M plasmid and p50 expression plasmid, p65 expression plasmid, or p50 plus p65 in combination did not have any effect on luciferase activity (figure 10D) although binding site 2 was not mutated. The data suggest that NF- κ B1 p50 may activate binding site 3, but not binding site 2 of mPGES1 promoter.

Discussion

Gastro-esophageal reflux disease complicated by Barrett's esophagus (BE) is a major risk factor for esophageal adenocarcinoma (EA) (Lagergren et al., 1999). 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) since overexpression of NOX5-S in Barrett's cells significantly increases COX2 expression and PGE₂ production, an increase in PGE₂ which mediates acid-induced increase in cell proliferation (Si et al., 2007). PGES has been shown to mediate COX2-derived PGE₂ production (Murakami et al., 2002; Park et al., 2006) and is expressed in esophageal adenocarcinomas (von Rahden et al., 2008). So far three isoforms of PGES have been identified: cPGES, mPGES1 and mPGES-2 (Murakami et al., 2003; Tanioka et al., 2000). 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 acid-induced increase in cell proliferation and PGE₂ production since 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 up-regulated in response to acid treatment in FLO cells. The mPGES1 protein was nearly undetectable by Western blot analysis in 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 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 (Gudis et al., 2007; Jang, 2004; van Rees et al., 2003), larynx (Kawata et al., 2006), lung (Jakobsson et al., 1999; Yoshimatsu et al., 2001), liver (Breinig et al., 2008; Takii et al., 2007), pancreas (Hasan et al., 2008), breast (Mehrotra et al., 2006), ovary (Rask et al., 2006), and brain (Barbieri et al., 2011; Baryawno et al., 2008; Lalier et al., 2007; Payner et al., 2006). These data indicate a crucial role of mPGES1 in tumorigenesis.

Mechanisms of acid-induced mPGES1 expression are not known. Since NOX5-S is involved in 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 since 1) knockdown of NOX5-S significantly decreased acid-induced increase in mPGES1 mRNA and protein expression, thymidine incorporation and PGE₂ production in FLO cells; 2) co-transfection of mPGES1 reporter plasmid and NOX5-S expression plasmid significantly increased luciferase activity, indicating 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 BE cell line BAR-T (Si et al., 2007). NF- κ B, a transcription factor, is known to function as a tumor promoter (Karin, 2006; Pikarsky et al., 2004) 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, respectively (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, 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 (De Bosscher et al., 2006; Karin et al., 2002). 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 acid-induced mPGES1 expression.

We found that NF- κ B1 p50 may mediate acid-induced mPGES1 expression because knockdown of NF- κ B1 p50 significantly decreased acid-induced mPGES1 protein and mRNA expression, thymidine incorporation and PGE₂ production. In addition, NF- κ B may activate mPGES1 promoter as co-transfection 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 a 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 also 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 mPGES1 promoter region, we found three 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). Our data indicate that NF- κ B1 p50 only binds to the potential binding sites 1 and 3 because 1) in the ChIP assay, mPGES1 DNAs of the mPGES1 promoter covering binding sites 1 and 3 were 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 unlabelled (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 super-shifted 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; 6) p50 activated binding site 1 in a cotransfection experiment with mPGES1318M1 plasmid where binding site 3 was mutated and mutation of both binding site 1 & 3 completely blocked the activation.

Our data suggest that acid-induced mPGES1 expression may be mediated by activation of NOX5-S and NF- κ B1 p50. However, our data cannot exclude other mechanisms involving in acid-induced mPGES1 expression.

In conclusion, mPGES1 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 Barrett's esophagus 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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Footnote

Dr. Xiaoxu Zhou and Dr. Dan Li contributed equally.

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Figure legends

Figure 1. Construct diagram. Bold characters indicate mutation sites.

Figure 2. Microsomal PGES1 is involved in acid induced increase in cell proliferation and PGE₂ production. A) mPGES1, mPGES2 and cPGES were detectable in FLO cells by PCR. B) A typical image of three Western blot analyses and C) summarized data 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, 1h), washed, 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 under basal condition as well as 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.

Figure 3. Microsomal PGES2 is not involved in acid-induced increase in cell proliferation.

A) A typical image of three Western blot analyses and B) summarized data 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, 1h), 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.

Figure 4. Cytoplasmic PGES is not involved in acid-induced increase in cell proliferation.

A) A typical image of three Western blot analyses and B) summarized data 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, 1h), washed, and incubated with regular medium for an additional 24 hours after cPGES and control siRNAs were introduced into FLO cells for 24 h 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.

Figure 5. mPGES1 expression in EA cells. A) A typical image of three Western blot analyses and B) summarized data show that mPGES1 protein expression in FLO and OE33 cells was significantly increased, when compared with HET-1A cells. 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, 1h), washed, 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.

Figure 6. NOX5-S may be involved in acid-induced mPGES1 expression. Transfection of NOX5 siRNA and control siRNA was carried out with Lipofectamine 2000. After 4-h transfection, the transfection medium was replaced with regular medium. 24 hours later, the transfectants were exposed to acid (PH4.0, 1h), washed, and incubated with regular medium for an additional 24 h. A) At the basal condition knockdown of NOX5 slightly decreased mPGES1 mRNA expression, but the decrease did not have statistical significance (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). C) A typical image of three Western blot analyses and D) summarized data show that at the basal condition knockdown of NOX5 slightly decreased mPGES1 protein expression, but the decrease did not have statistical significance (N=3, t test P=0.0571). E) Summarized data from figure C show that 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 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 µg renilla and 0.5µg mPGES1318 reporter plasmid with NOX5-S or pCMV plasmid by using 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 acid-induced increase in mPGES1 expression. % increase was calculated as the following: % increase = (acid-treatment group – without-acid-treatment group) * 100/ without-acid-treatment group. t test * P < 0.05, ** P<0.02.

Figure 7. NF-κB may be involved in acid induced mPGES1 expression. Transfections of p50 siRNA and control siRNA were carried out with Lipofectamine 2000 in FLO cells. After 4-h transfection, the transfection medium was replaced with regular medium. 24 hours later, the cells

were exposed to acid (pH 4.0, 1h), washed, and incubated with regular medium for an additional 24 h. A) At the basal condition knockdown of p50 did not have any significant effect on mPGES1 mRNA expression (N=6). B) 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 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 did not have statistical significance (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 μg renilla and 0.5 μg mPGES1318 plasmid with p50, p65, p50 plus p65 or pCMV plasmid by using Lipofectamine 2000. Over-expression of P50, p65 or p50 and p65 in combination significantly increased the luciferase activity, suggesting that NF-κB1 may activate mPGES1 promoter (N=3). The data suggest that NF-κB may mediate acid-induced increase in mPGES1 expression. % increase was calculated as the following: % increase = (acid-treatment group – without-acid-treatment group) * 100/ without-acid-treatment group. ANOVA, ** P < 0.01, compared with mPGES1318 plus pCMV group; t test, * P < 0.05, compared with control siRNA.

Figure 8. NF- κ B binding sites in mPGES1 promoter. A) A typical example of three experiments showed that mPGES1 DNA was detectable by PCR using primers covering 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 showed that mPGES1 DNA was undetectable by PCR using primers covering 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 showed that mPGES1 DNA was detectable by PCR using primers covering 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 site 1 and 3 in the mPGES1 promoter. The PCR products were sequenced and specific for the mPGES1 promoter. Positive, genomic DNA used as a positive control; rabbit IgG or buffer was used as negative controls.

Figure 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. Competition experiments with unlabelled (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 unlabelled (cold) mPGES1-WT3 oligonucleotide significantly reduced binding. Addition of the mutant

oligonucleotides mPGES-MUT3 had less effect on binding. In a super-shift assay, the super-shifted band was detected with a p50 antibody.

Figure 10. NF- κ B may activate mPGES1 promoter via binding site 1 & 3. A) In FLO cells transfected with mPGES1318M1, over-expression 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. mPGES1318M1 plasmid contains mutant binding site 3. FLO cells were transfected with 0.1 μ g renilla and 0.5 μ g mPGES1318M1 plasmid with p50, p65, p50 plus p65 or pCMV plasmid by using Lipofectamine 2000. B. Co-transfection 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 site 1 & 3 almost abolishes p50-induced activation of mPGES1 promoter. Both binding site 1 and 3 were mutated in mPGES1318M2. The data also suggest that p50 may not activate mPGES1 promoter via binding site 2 since binding site 2 is not mutated in plasmid mPGES1318M2. C) In FLO cells transfected with mPGES791 plasmid, over-expression 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 site 2 & 3. D. Co-transfection 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 since binding site 2 is not mutated in plasmid mPGES791M. N=3, ANOVA, ** P < 0.01, compared with control mPGES1318M1 plus pCMV group or mPGES791 plus pCMV

group.

	Binding site 1	Binding site 2	Binding site 3			
	-1269	-1260 -656	-647 -211	-202		
	↓	↓	↓	↓		
-1328	-----GGAGTCTCCC	-----GGGCTCACCC	-----CGGGACACCC	----	-10 MPGES1318	
-1328	-----GGAGTCTCCC	-----GGGCTCACCC	-----CTTGACATTC	----	-10 MPGES1318M1	
-1328	-----GTAGTCTCAC	-----GGGCTCACCC	-----CTTGACATTC	----	-10 MPGES1318M2	
		-791	-----GGGCTCACCC	-----CGGGACACCC	-----	-1 MPGES791
		-791	-----GGGCTCACCC	-----CTTGACATTC	-----	-1 MPGES791M

Figure 1

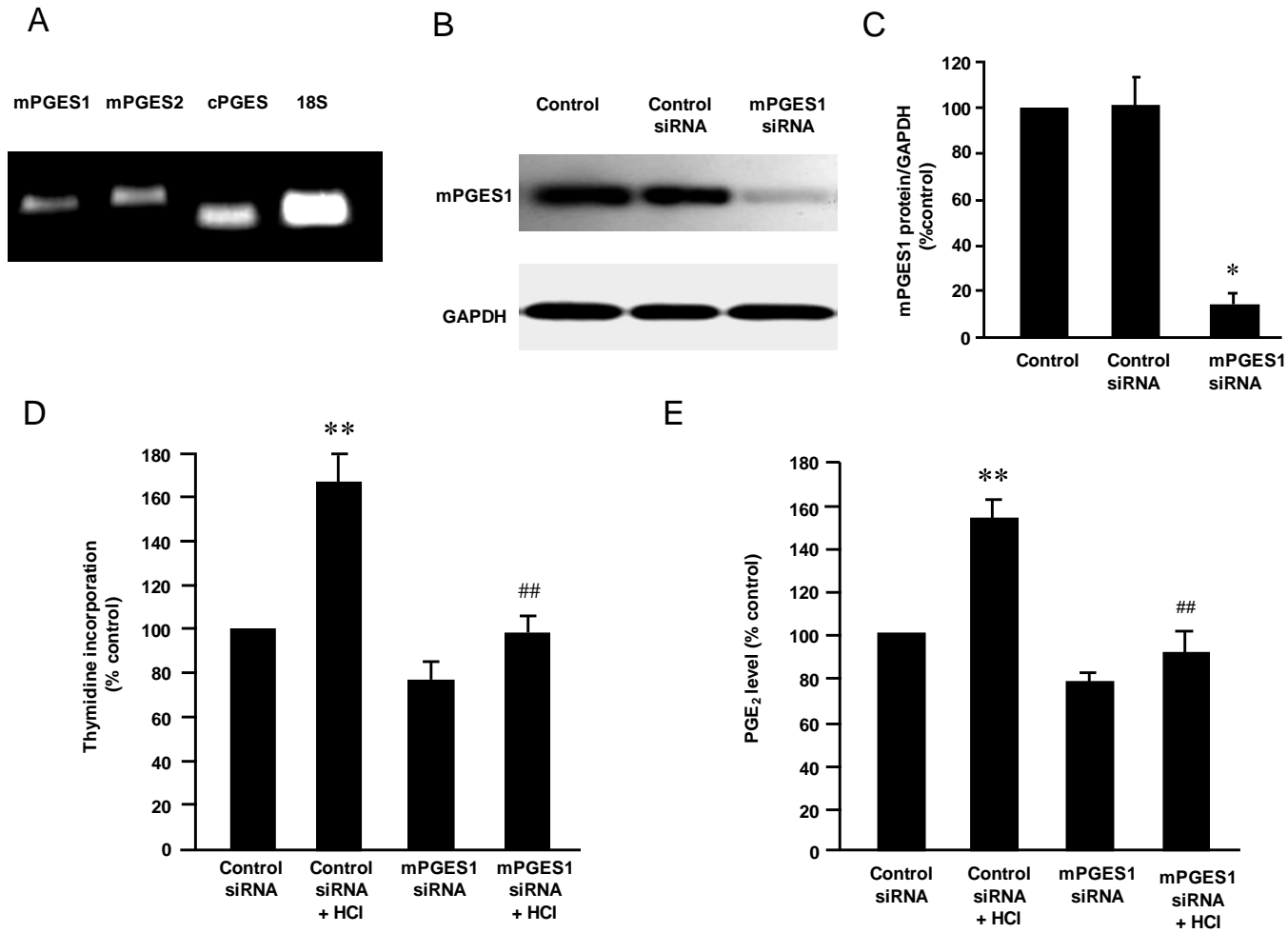


Figure 2

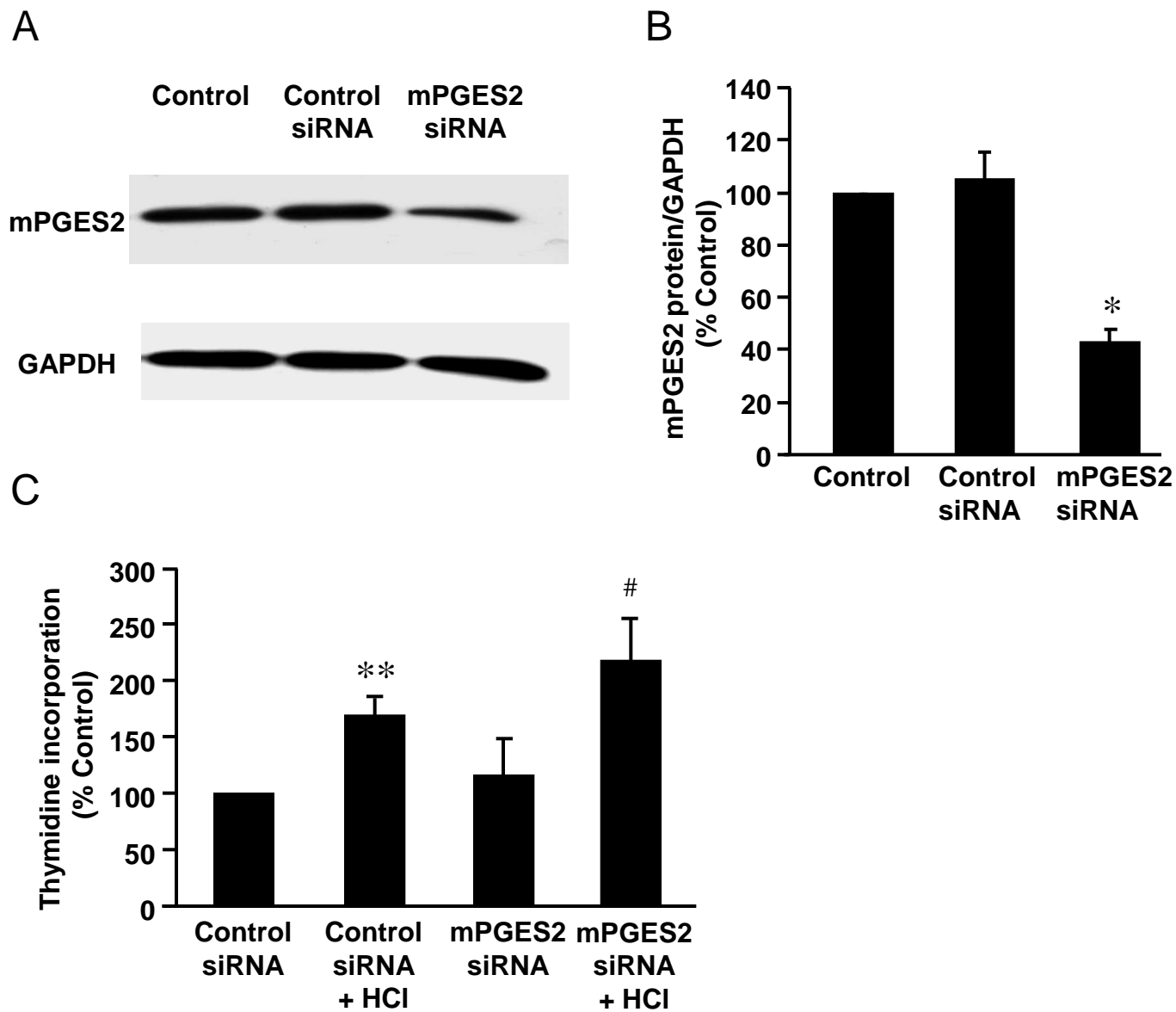


Figure 3

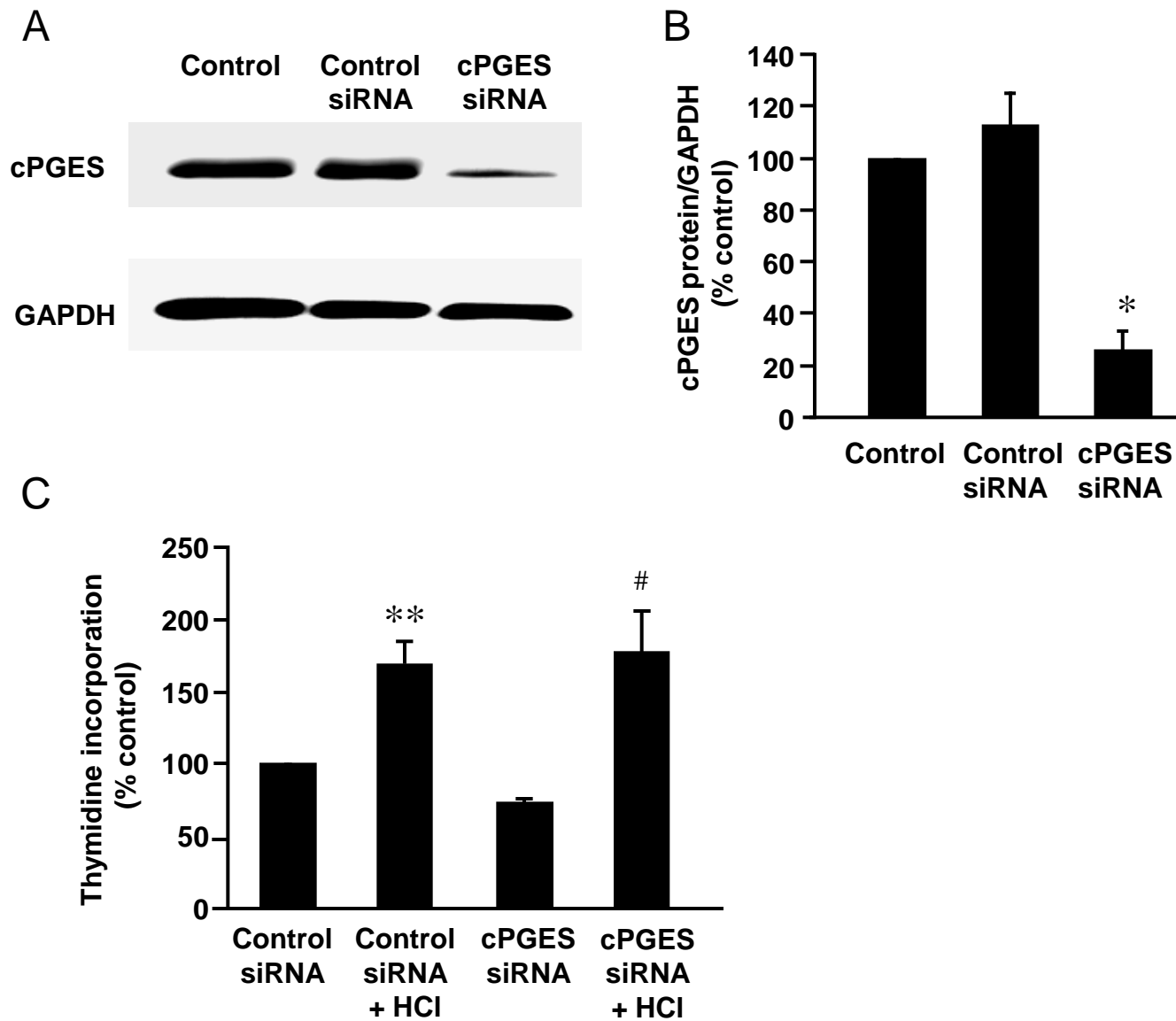


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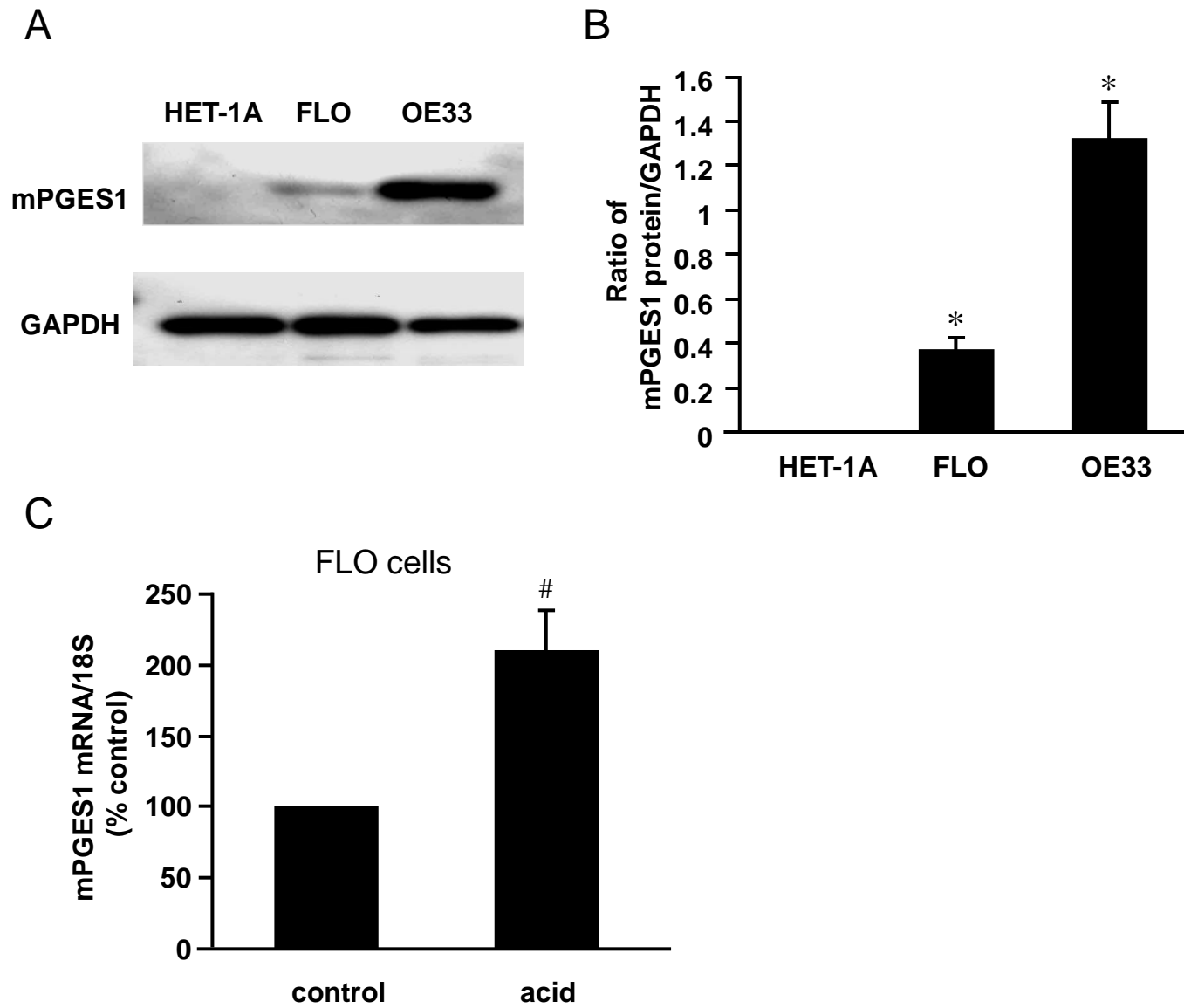


Figure 5

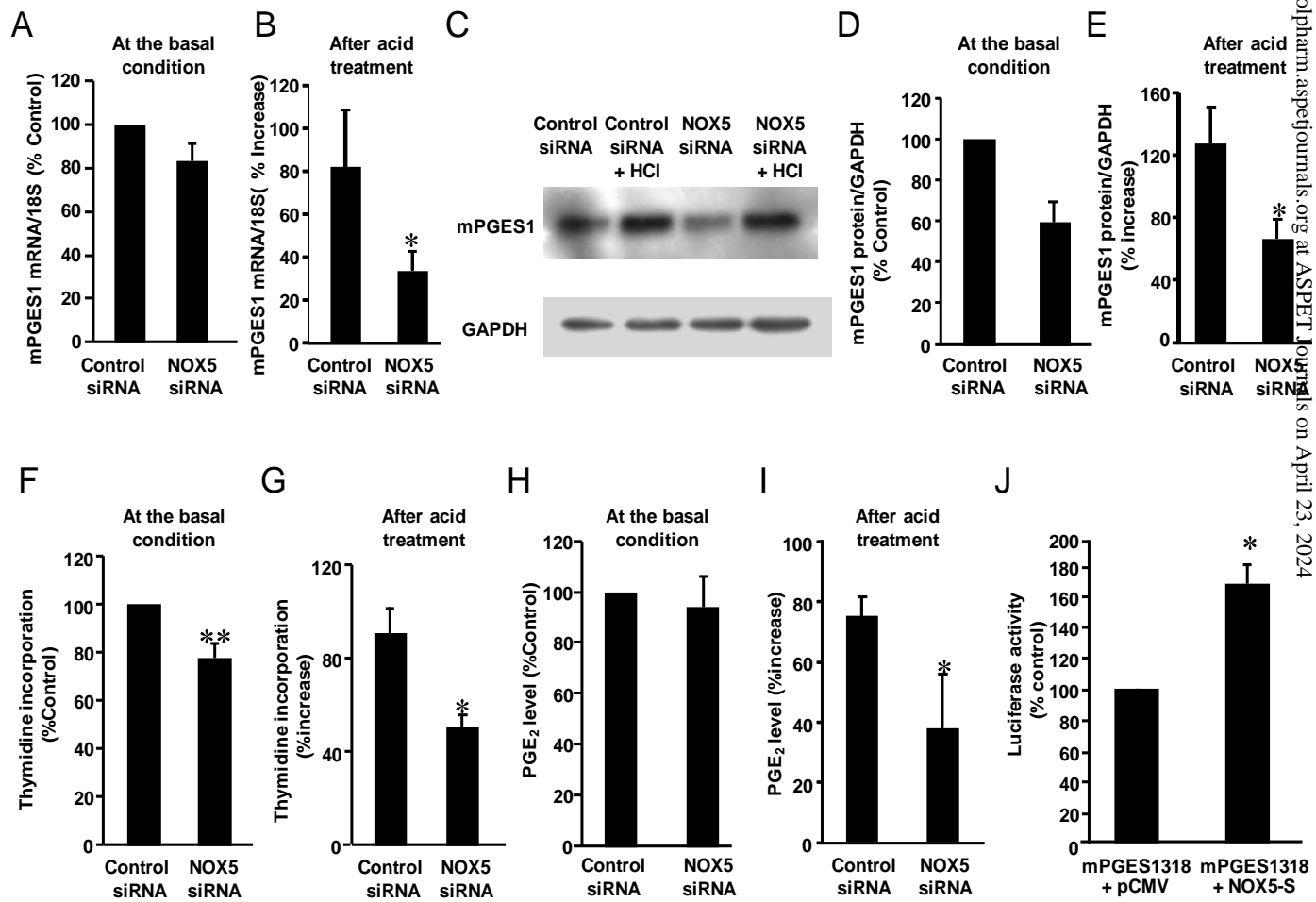


Figure 6

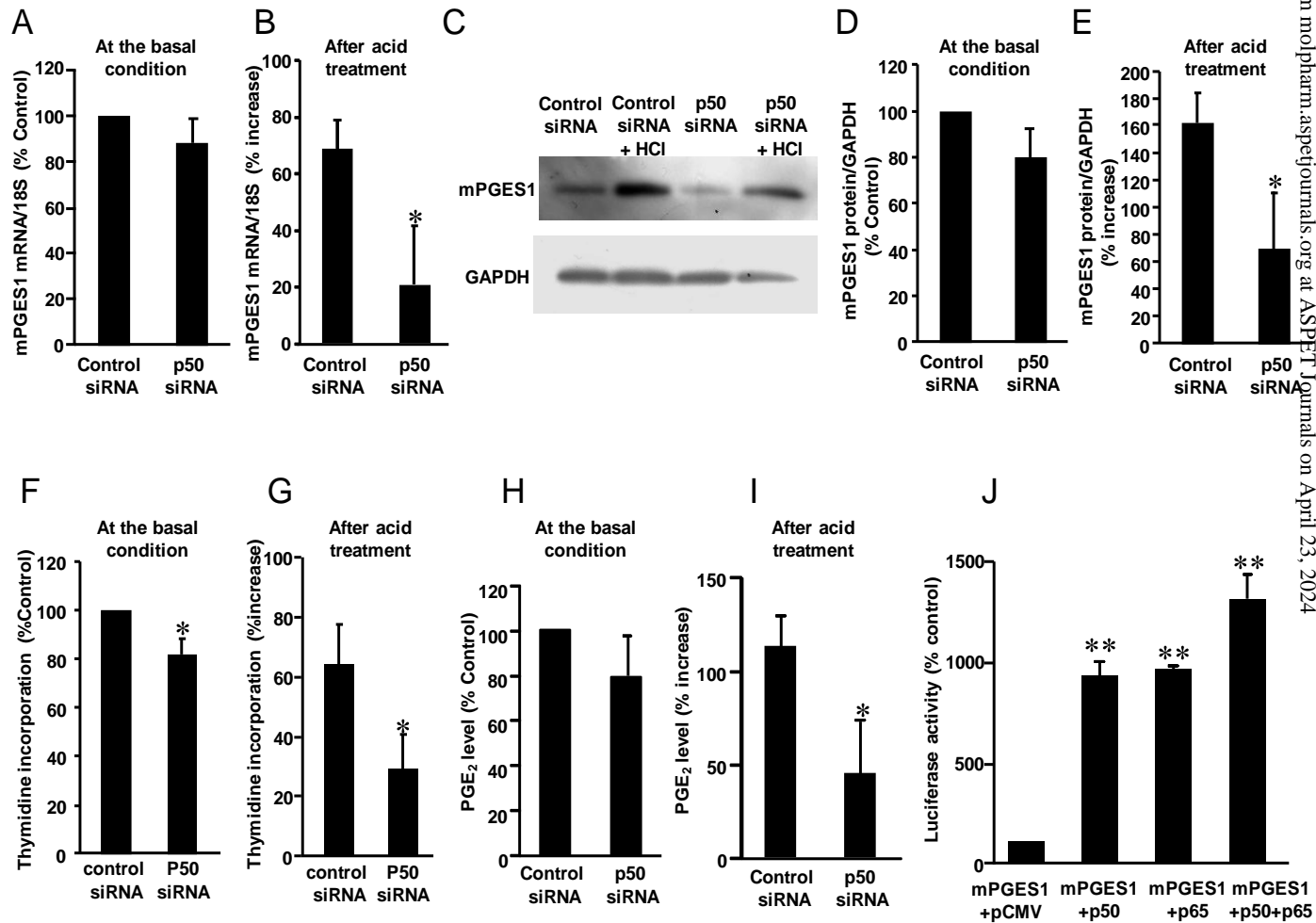


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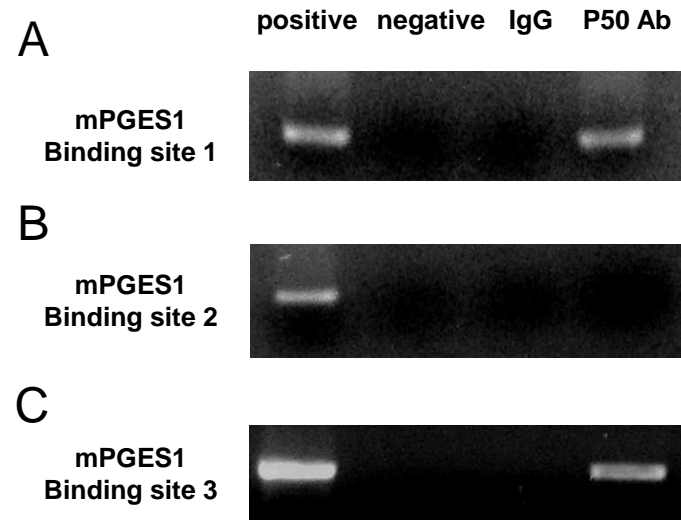
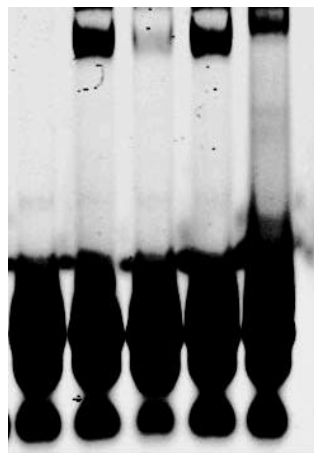


Figure 8

A

	lane	1	2	3	4	5
IRDye 700-mPGES1-WT1	+	+	+	+	+	+
p50 protein	-	+	+	+	+	+
Cold mPGES1WT1(200×)	-	-	+	-	-	-
Cold mPGES1-MUT1(200×)	-	-	-	+	-	-
P50 antibody	-	-	-	-	+	+

**B**

	lane	1	2	3	4	5
IRDye 700-mPGES1-WT3	+	+	+	+	+	+
p50 protein	-	+	+	+	+	+
Cold mPGES1-WT3(200×)	-	-	+	-	-	-
Cold mPGES1-MUT3(200×)	-	-	-	+	-	-
P50 antibody	-	-	-	-	-	+

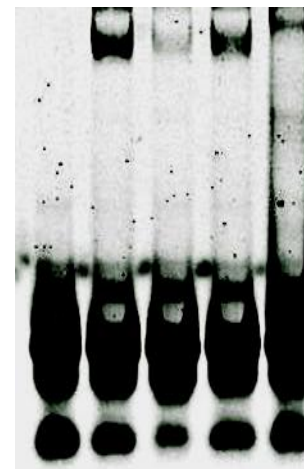
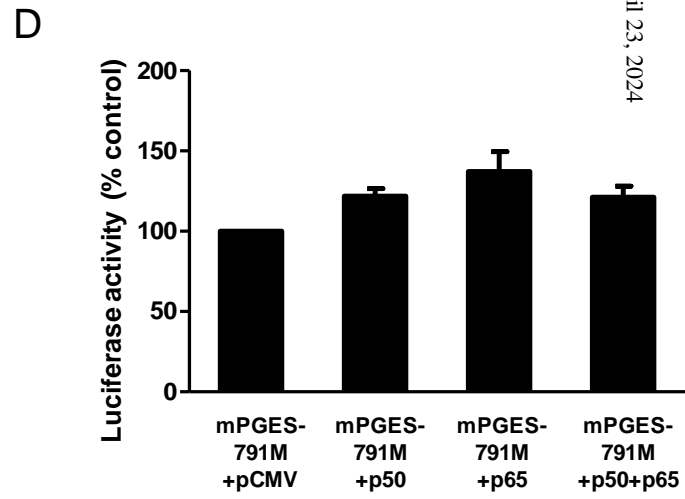
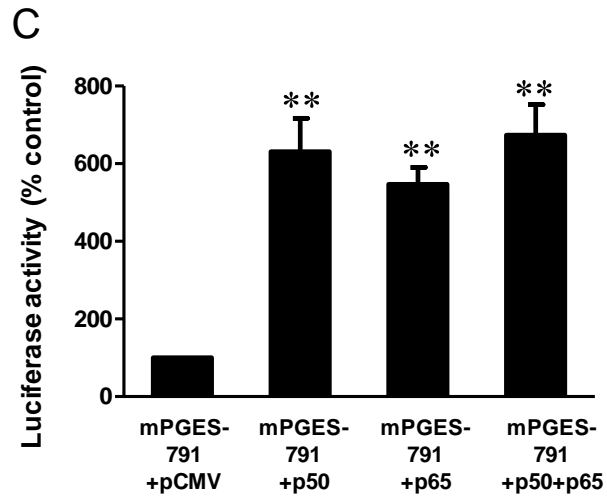
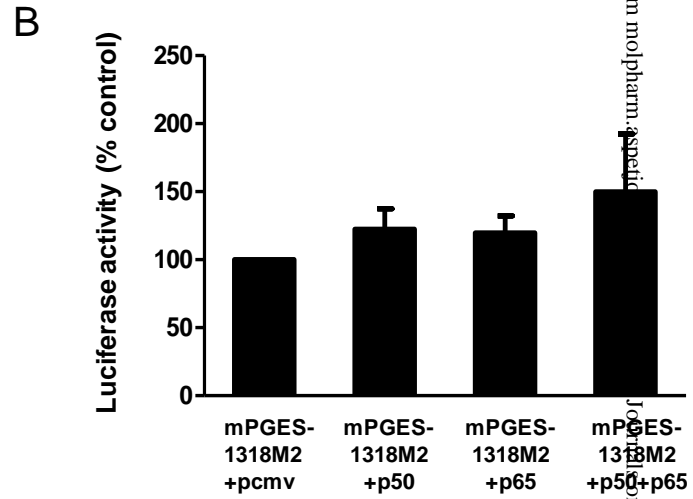
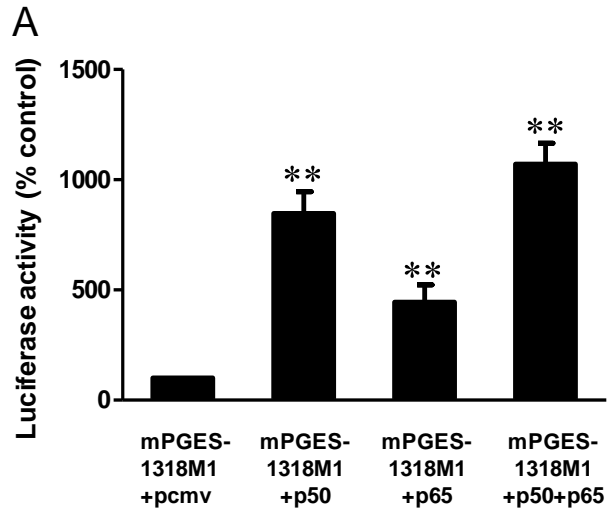


Figure 9



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Figure 10