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ORIGINAL ARTICLE

The positive feedback loop between Nrf2 and phosphogluconate dehydrogenase stimulates proliferation and clonogenicity of human hepatoma cells

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ABSTRACT

Recent studies report that nuclear factor-erythroid-2-related factor 2 (Nrf2) facilitates tumor progression through metabolic reprogramming in cancer cells. However, the molecular mechanism underlying the oncogenic functions of Nrf2 is not yet well understood. Some of the pentose phosphate pathway (PPP) enzymes are considered to play a role in the cancer progression. The present study was intended to explore the potential role of phosphogluconate dehydrogenase (PGD), one of the PPP enzymes, in the proliferation and migration of human hepatoma HepG2 cells. Genetic ablation of Nrf2 attenuated the expression of PGD at both transcriptional and translational levels. Notably, Nrf2 regulates the transcription of *PGD* through direct binding to the antioxidant response element in its promoter region. Nrf2 overexpression in HepG2 cells led to increased proliferation, survival, and migration, and these events were suppressed by silencing *PGD*. Interestingly, knockdown of the gene encoding this enzyme not only attenuated the proliferation and clonogenicity of HepG2 cells but also downregulated the expression of Nrf2. Thus, there seems to exist a positive feedback loop between Nrf2 and PGD which is exploited by hepatoma cells for their proliferation and survival. Treatment of HepG2 cells with ribulose-5-phosphate, a catalytic product of PGD, gave rise to a concentration-dependent upregulation of Nrf2. Collectively, the current study shows that Nrf2 promotes hepatoma cell growth and progression, partly through induction of *PGD* transcription.

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Nrf2; phosphogluconate dehydrogenase; pentose phosphate pathway; ribulose-5-phosphate; human hepatoma HepG2 cells

Introduction

Nuclear factor-erythroid-2-related factor 2 (Nrf2) is a transcription factorinvolved in the cellular defense against oxidative stress and carcinogenic insults by inducing the expression of genes responsible for encoding antioxidant/ carcinogen detoxifying enzymes and related cytoprotective proteins [1,2]. Due to such roles, Nrf2 has been considered as one of the prime targets for cancer chemoprevention [3]. However, recent studies have shown that cancer cells also harness Nrf2 for their survival advantage, invasiveness, and chemoresistance [4]. Many different types of cancer have mutations in Nrf2 or its inhibitor Kelch-like ECH-associated protein 1 (Keap1), which result in constitutive overactivation of Nrf2 signaling [4–6]. However, the molecular mechanisms by which Nrf2 promotes cancer plasticity have not yet been fully elucidated. Nrf2 upregulates transcription of target genes by binding to the antioxidant response element (ARE) present in the 5′-upstream of their promoter region [7]. ARE, also known as electrophile response element (EpRE), has a core sequence of 5′-TGAG/CNNNGC-3′ [8]. An oligonucleotide microarray analysis identified Nrf2-regulated genes, and some of them were found to encode a series of enzymes involved in intermediary metabolism [9].

Metabolic reprogramming is one of the hallmarks of cancer defined by Hanahan and Weinberg in their updated seminal review paper published in 2011 [10]. A growing body of evidence supports that metabolic reprogramming is necessary to support cancer cell

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proliferation and survival [11–13]. The Warburg effect describes the paradoxical phenomenon (the so-called "aerobic glycolysis") in which cancer cells, even in the presence of oxygen, prefer glycolysis to the energetically more efficient mitochondrial oxidative phosphorylation [14]. In addition, this shift provides cancer cells with important macromolecular precursors needed to support their rapid proliferation and growth [15].

Phosphogluconate dehydrogenase (PGD) is one of the key enzymes in the pentose phosphate pathway (PPP) which converts 6-phosphogluconate to ribulose-5-phosphate (Ru-5-P) through oxidative decarboxylation. During this process, one molecule of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) is also produced. NADPH is essential for both cellular defense against oxidative stress and for reductive biosynthesis, such as lipogenesis [16]. Ru-5-P is converted by ribose-5-phosphate isomerase to ribose-5-phosphate which serves as a precursor of nucleotide biosynthesis [17]. Increased expression/activity of PGD has been reported in several malignancies including lung cancer [18,19], thyroid cancer [20], and cervical intraepithelial neoplasia [21]. Suppression of PGD was shown to attenuate cancer cell proliferation through reduction of lipogenesis and RNA biosynthesis [22]. Likewise, *PGD* knockdown suppresses growth of lung cancer cells by inducing senescence [19]. Additionally, PGD was found to be necessary for c-Met phosphorylation which was associated with invasiveness of lung cancer cells [18].

In this study, the role of PGD in cancer cell proliferation and migration induced by Nrf2 was investigated in human hepatoma HepG2 cells. Here we report that Nrf2 induces *PGD* transcription by directly binding to the ARE present in the promoter region of this gene.

Materials and methods

Materials

Primary antibodies against PGD and Keap1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The primary antibody for heme oxygenase-1 (HO-1) was bought from Enzo Life Sciences (Farmingdale, NY). The primary antibody for actin was obtained from AbClon Inc. (Seoul, South Korea). Antibodies against Nrf2 and p62 were purchased from Abcam (Cambridge, MA). The secondary anti-rabbit IgG, horseradish peroxidase-linked antibody was bought from Cell Signalling Technology (Beverly, MA). Ru-5-P was purchased from Sigma-Aldrich Co. (St. Louis, MO).

Cell culture

HepG2 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v fetal bovine serum (FBS) and 100 units/ml antibiotic–antimycotic (Thermo Fisher Scientific; Rockford, IL).

siRNA transient transfection

HepG2 cells were reverse transfected with corresponding siRNA. The following siRNAs were used for transfection: human negative-siRNA (5′-CCUCGUG CCGUUCCA UCAGGUAGUU-3′), siNrf2 (5′-AAGAGUAUGA GCUGGAA AAACTT-3′, Invitrogen), siPGD (5′-CUCACACCUAUGAAC UCUU(dTdT)-3′, Bioneer). siRNA was diluted in 1 ml of Opti-MEM and vortex-mixed. Lipofectamine® RNAiMAX (Invitrogen;Carlsbad, CA) reagent was then added to the mixture followed by incubation for at least 20 min prior to addition to the freshly seeded cells in media without antibiotics and FBS. After 24 h transfection, the medium was changed to full media (with 10% FBS and 1% antibiotic–antimycotic). After incubation for additional 48 h, cells were harvested for either mRNA extraction or protein analysis.

Plasmid transfection

HepG2 cells were transfected with pcDNA3 plasmid carrying cDNA for Nrf2 and a Myc tag (Plasmid #21555 from Addgene; Cambridge, UK) using Lipofectamine®2000 (Invitrogen; Carlsbad, CA) reagent. The transfection was done according to the manufacturer's instructions. A pCMV3-N-Myc plasmid vector carrying cDNA for PGD (HG10393-NM from Sino Biological; Wayne, PA) was used for the overexpression of PGD. An empty vector (pcDNA 3.1 plasmid) was used as a control.

Thiazolyl blue tetrazolium bromide (MTT) assay

Transfected cells incubated for 72 h were trypsinized and seeded at a density of 3.5×10^3 cells per well in a 96-well plate. After 48 h of incubation, the medium was replaced by 0.5 mg/ml of MTT in DMEM. After incubation for 3 h, the supernatant was removed, and the formazan crystals formed was dissolved in dimethyl sulfoxide (DMSO). The absorbance at 570 nm was read using a micro-plate reader (Bio-Rad Laboratories; Hercules, CA).

Colony forming assay

The cells were transfected with the indicated expression vectors or siRNAs in a 60 mm plate as described above. After 72 h of transfection, the cells were washed, counted and plated at a density of 500 cells per well in a 6-well plate. Cells were further cultured for 10 days in standard conditions. The plates were washed with phosphate-buffered saline (PBS), fixed with chilled methanol for 10 min, and stained with 0.1% crystal violet solution. Plates were imaged by using the LAS-4000 image reader (Fuji film), and colonies having more than 50 cells were counted.

Migration assay

Two-well Culture-Inserts (Ibidi®) were attached to 12-well plates. HepG2 cells transfected with appropriate vectors were seeded at a density of 2.5×10^4 cells on each well of the Culture-Insert. After incubation for 24 h, the silicon inserts were removed, and cells were photographed under the microscope. The cells were again photographed after 48 h to assess the closure of the wound gap.

Western blot analysis

Standard SDS-PAGE and Western blotting procedures were used to analyze the expression of various proteins. Protein samples were prepared using SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.02% bromophenol blue) . All proteins were visualized using a horseradish peroxidase-conjugated secondary antibody and Amersham ECL™ Western Blotting Detection Reagents (GE Healthcare Life Sciences; Marlborough, MA).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from HepG2 cells using TRIzol® (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RT-PCR was performed following standard procedures. One microgram of the extracted RNA for each sample was reverse transcribed to complementary DNA with M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase at 42 °C for 50 min and 72 °C for 15 min. One μL of cDNA was used for further amplification in sequential reactions using Solg™ 2X Taq PCR Smart mix 1 (SolGent, Seoul, Korea). The sequences of the primer pairs used are as follows (forward and reverse, respectively): *PGD*, 5′-GCTCTTCGGTTCTG CTCTGT-3′ and 5′-CCAGTTCCCACTTTTGCAGC-3′; *Nrf2,* 5′-TTCAAAGCGTCCGAACTCCA-3′ and 5′-AATGTCTGCGC CAAAAGCTG-3′; *Keap1,* 5′-CAGAGGTGGTGGTGTTGCTTA-3′ and 5′-AGCTCGTTCATGATGCCAAAG-3′; *HO-1,* 5′-CCAGGCAGAGAATGCTGAGTTC-3′ and 5′-TCTTCTATCA CCCTCTGCCTGAC-3′; *GCLC*, 5′-ACCA GGACAGCC-3′ and 5′-ATG ATG CCA AAG-3′; *GAPDH,* 5′-ACCACAGTCCAT GCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′.

Immunocytochemical analysis

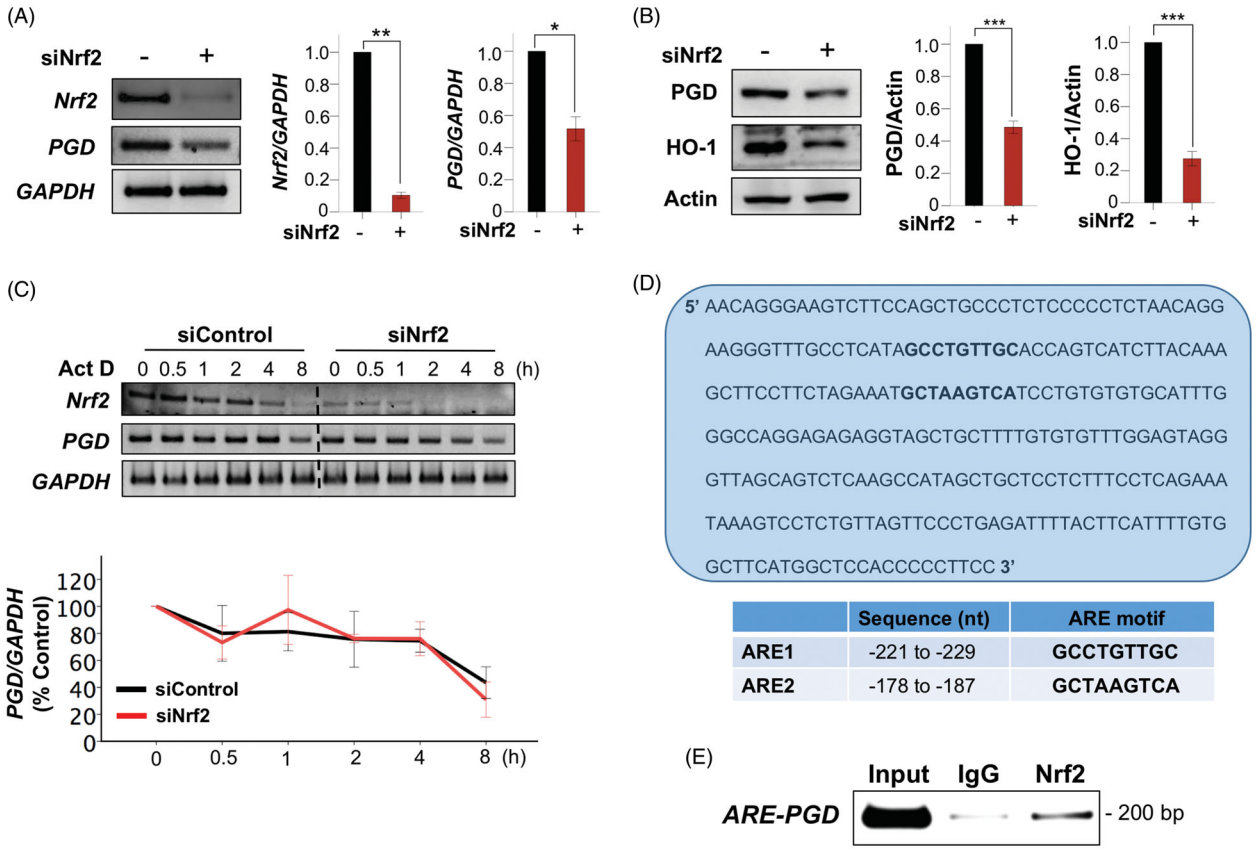
HepG2 cells transfected with either siControl or siPGD RNA were plated on 8-well chamber slides at a density of 2×10^4 per well. After fixation with 4% formaldehyde for 15 min at 37 °C, the cells were rinsed with PBST (PBS containing 0.1% Tween-20) and incubated with 0.1% Triton X-100 in PBS for 5 min. Then, the cells were washed and blocked with 0.05% Triton X-100 in PBS containing 5% bovine serum albumin (BSA) at room temperature for 1 h. The cells were then washed with PBST and incubated with the Nrf2 primary antibody overnight at 4 °C. After washing with PBST, the cells were incubated with FITC-conjugated anti-rabbit IgG secondary antibody in PBST containing 5% BSA at room temperature for 1 h. After washing with PBST, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) in PBS for 15 min. The cells were then washed with PBST and examined under a fluorescent microscope.

Chromatin immunoprecipitation (ChIP) assay

For the ChIP assay, cells were washed with PBS and cross-linked with a 1% formaldehyde solution for 30 min at room temperature. The crosslinking reaction was stopped by the addition of glycine to 125 mM final concentration. Cell lysates were sonicated to generate DNA fragments with the average size of 300–600 base pairs. This was followed by immunoprecipitation with indicated antibodies, which were bound to Protein Agarose A/G (Santa Cruz, CA) by an overnight incubation prior to use. Bound DNA fragments were eluted and purified using the Biomedic® Plasmid DNA Miniprep Kit (Seoul, South Korea). The collected DNA was then amplified by PCR. The primers for the *PGD-ARE* region are 5′-CCCCCTCTAACAGGAAGGGT-3′ and 5′-ACCACTTTTTCCCCCATAGACAA-3′.

Statistical analysis

Results were expressed as the means ± SEM of at least three independent experiments. The statistical significance of the difference between two groups was evaluated using Student's *t* test. Analysis was performed



	Sequence (nt)	ARE motif
ARE1	-221 to -229	GCCTGTTGC
ARE2	-178 to -187	GCTAAGTCA

Figure 1. Nrf2 regulates the transcription of *PGD* by binding to the ARE in the promoter region. HepG2 cells were transfected with either a non-targeting siRNA or siNrf2 RNA and incubated for 72 h. (A) The expression of *PGD* as well as *Nrf2* was determined by PCR. Relative mRNA levels were normalized to *GAPDH*. (B) Total cell lysates were subjected to Western blot analysis. Band intensities were normalized to actin. (C) Transfected cells were treated with actinomycin D (Act; 10 μg/ml) for indicated time periods. Mean band intensities normalized to *GAPDH* were divided by the 0 h control multiplied by 100. (D) The *PGD* gene promoter sequence harboring the ARE consensus motif for Nrf2 binding. The nucleotides of the ARE consensus motif (GCNNNNTCA) are indicated in bold. (E) A ChIP assay was performed on HepG2 cells to detect the binding of Nrf2 to the ARE in the promoter region of *PGD*. Protein-DNA complexes were stabilized by crosslinking. The cell lysate was immunoprecipitated with a Nrf2 antibody. Reverse crosslinking was performed, and DNA was extracted and purified. DNA samples were amplified by PCR. For all panels, each bar shows the mean ± SEM of three independent experiments. The *p* values were determined by independent *t*-test (*, $0.05 > p > 0.01$; **, $0.01 > p > 0.001$; ***, $0.001 > p$ versus control).

using IBM SPSS Statistics (Version 23, IBM SPSS Statistics, Armonk, NY). Statistical significance was accepted at $p < 0.05$, unless otherwise indicated.

Results

Nrf2 directly regulates PGD transcription in HepG2 cells

Nrf2 has been shown to regulate the expression of metabolic enzymes in various cancer cell lines [9,23]. Knockdown of *Nrf2* with siRNA resulted in the inhibition of *PGD* mRNA expression (Figure 1(A)) and its protein product (Figure 1(B)). Under the same experimental conditions, expression of HO-1, a well-known target of Nrf2 [24], was also significantly suppressed (Figure 1(B)). To explore whether Nrf2 is associated with the stability of *PGD* mRNA, HepG2 cells were treated with the transcription inhibitor actinomycin D (10 μg/ml). However, the levels of remaining *PGD* mRNA did not significantly differ between siControl and siNrf2 transfected cells (Figure 1(C)), suggesting that Nrf2 may induce PGD expression through a transcriptional rather than post-transcriptional mechanism.

Upon activation, the heterodimer Nrf2 bound to a small Maf protein interacts with ARE in the regulatory region of its target genes [25,26]. Nrf2 has been found to directly bind to the ARE-region of *PGD* in A549 cells [23]. The promoter region of *PGD* harbors an Nrf2 binding site (Figure 1(D)). To confirm Nrf2 interaction with the ARE consensus sequence in the PGD regulatory region in HepG2 cells, a ChIP assay was performed. As

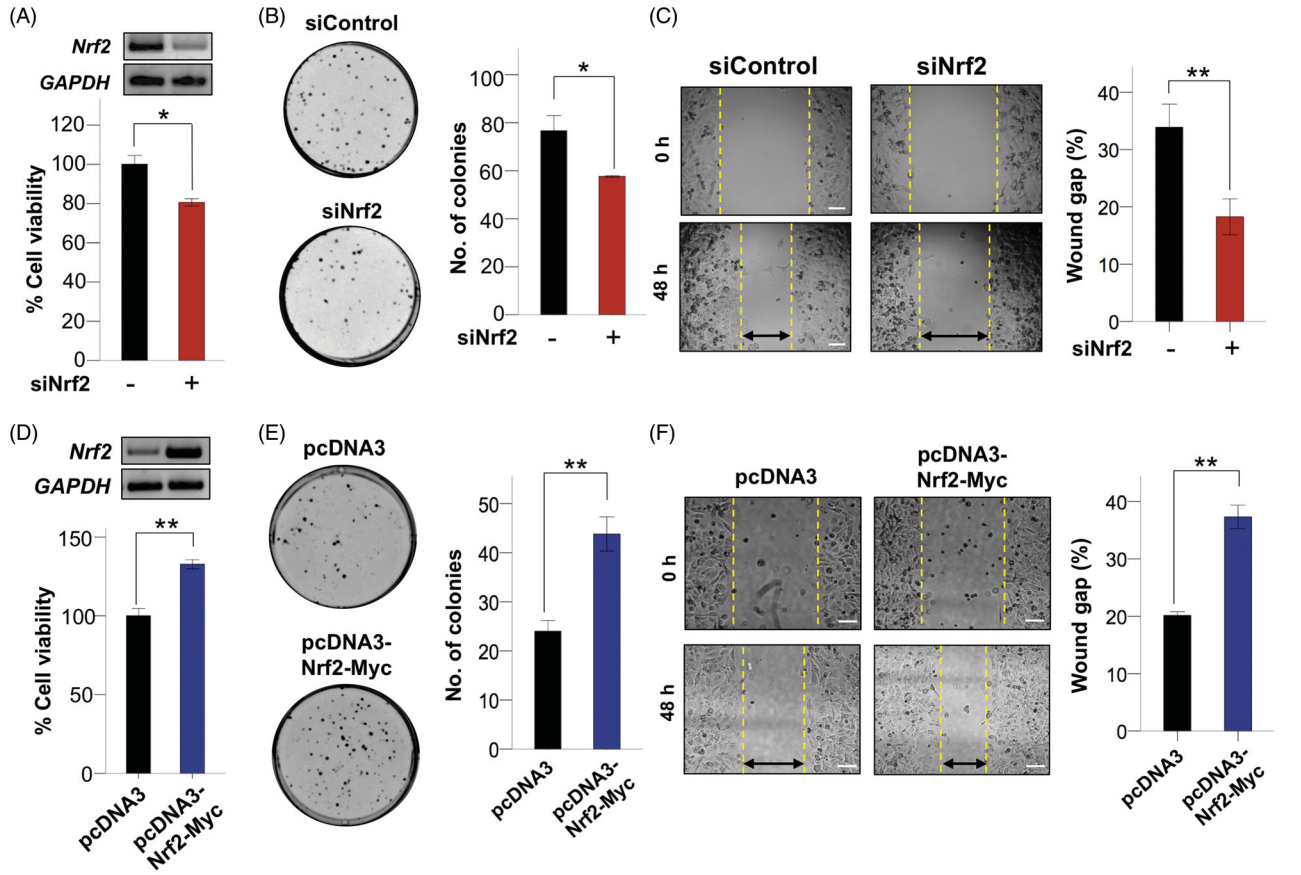


Figure 2. Nrf2 mediates proliferation and survival of HepG2 cells. (A and B) HepG2 cells were transfected with either a non-targeting siRNA or siNrf2 RNA. (A) After 48 h of incubation, cells were seeded in a 96-well plate. Cell viability was measured by the MTT assay 48 h after seeding. (B) After 48 h of incubation, cells were seeded in a 6-well plate. Colonies formed were counted after incubation for 10 days. (C) Cells were seeded into the two wells of an insert. After 24 h of incubation, the insert was removed and the cells were incubated for additional 48 h. The wound gap was measured under a light microscope as described in Materials and methods. (D and E) HepG2 cells were transfected with pcDNA3 (Control) or pcDNA3-Nrf2-Myc (Nrf2-expressing plasmid). After 24 h of incubation, cells were seeded in a 96-well plate. Cell viability was measured by the MTT assay 48 h after seeding (D). The remaining proportion of cells was seeded in a 6-well plate. Colonies were counted after incubation for 10 days (E). (F) The migrative capability of HepG2 cells transfected with an empty or an Nrf2 overexpressing vector was measured as described in legend to (C). For all panels, each bar shows the mean of three independent experiments ± SEM. The p values were determined by independent t-test (*, $0.05 > p > 0.01$; **, $0.01 > p > 0.001$ versus control).

illustrated in Figure 1(E), Nrf2 binds to ARE present in the PGD gene promoter. This finding is consistent with that reported by Mitsuishi and colleagues who identified *PGD* as one of the Nrf2 target genes [23].

Nrf2 promotes proliferation and migration of HepG2 cells

Previous studies have shown that Nrf2 promotes proliferation and survival of several types of cancer cells [4–6,23,24,27,28]. To validate this in HepG2 cells, the MTT assay and the clonogenic assay were performed. The silencing of *Nrf2* by siRNA transfection attenuated proliferation (Figure 2(A)) and survival (Figure 2(B)) of HepG2 cells. Further, Nrf2 gene knockdown also dampened the migrative capability of these cells (Figure 2(C)). In contrast, Nrf2 overexpression with pcDNA3-Nrf2-Myc plasmid provoked opposite effects on all these events (Figure 2(D, E, and F)).

PGD is important for proliferation of HepG2 cells

Rapid proliferation of cancer cells requires not only ATP but also building blocks necessary for the synthesis of nucleotides, fatty acids and amino acids [11]. Ru-5-P, a byproduct of PGD, is utilized in nucleic acid biosynthesis [22]. HepG2 cells were transfected with siControl RNA or siPGD RNA, and cell proliferation and survival were assessed. *PGD* silencing resulted in a decreased viability (Figure 3(A)) and clonogenicity (Figure 3(B)) of

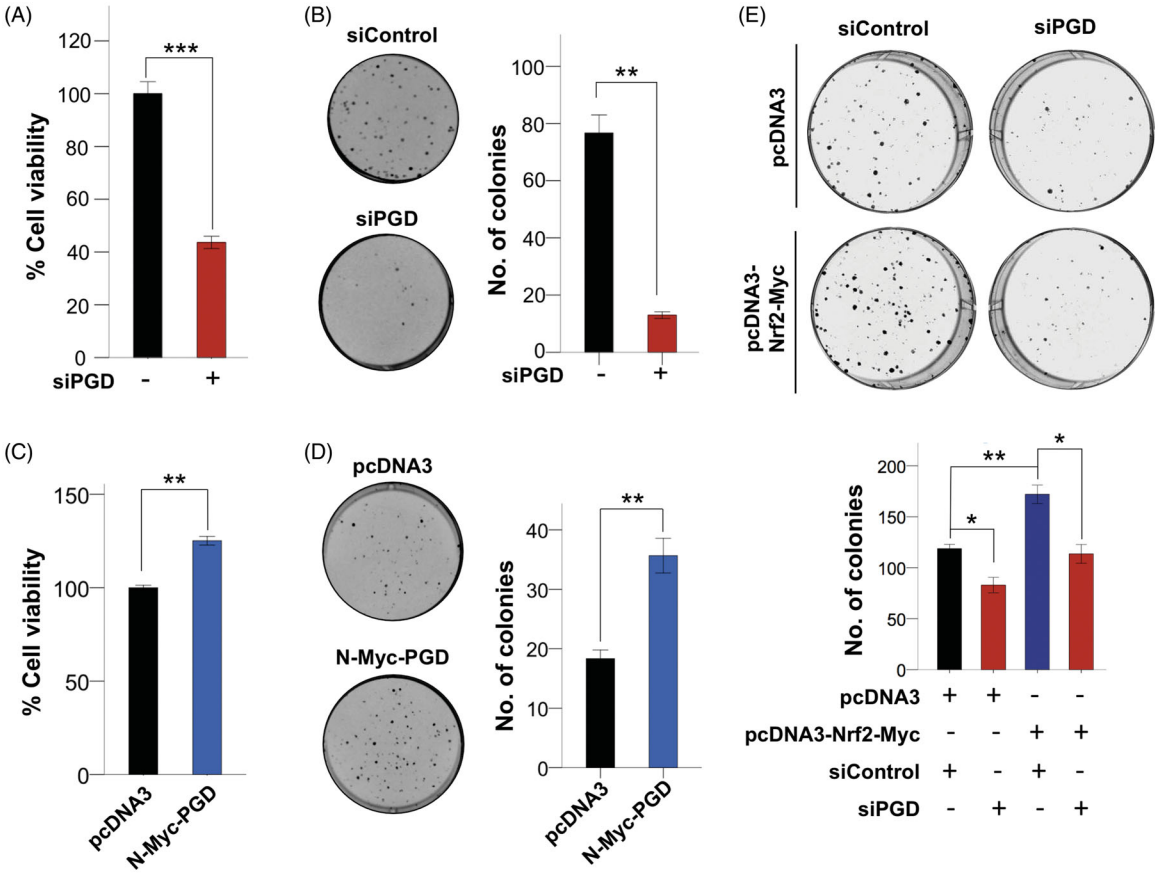


Figure 3. PGD is involved in HepG2 cell proliferation. (A and B) HepG2 cells were transfected with either a non-targeting siRNA or siPGD RNA. After 48 h of incubation, cells were seeded in a 96-well plate and a 6-well plate to measure the viability and clonogenicity, respectively after additional incubation for 48 h and 10 days . (C and D) HepG2 cells transfected with pcDNA3 (Control) or N-Myc-PGD plasmid. The viability and colony forming ability of cells were determined by the MTT (C) and the clonogenicity (D) assays, respectively as described above. (E) HepG2 cells were transfected with either a non-targeting siRNA or siPGD RNA and incubated for 24 h. Each group was then subsequently co-transfected with either pcDNA3 (Control) or pcDNA3-Nrf2-Myc (Nrf2-expressing plasmid). After 48 h of incubation, cells were seeded in a 6-well plate. Colonies were counted after 10 days of incubation. For all panels, each bar shows the mean ± SEM of three independent experiments. The *p* values were determined by independent *t*-test (*, $0.05 > p > 0.01$; **, $0.01 > p > 0.001$; ***, $0.001 > p$ versus control).

HepG2 cells whereas overexpression of PGD enhanced cell viability (Figure 3(C)) and colony formation (Figure 3(D)).

PGD mediates Nrf2-induced HepG2 cell proliferation and survival

HepG2 cells transfected with pcDNA 3.1 (Mock) or pcDNA 3.1-Nrf2-Myc plasmid (Nrf2 plasmid) were co-transfected with either siControl or siPGD RNA. As shown in Figure 3(E), both Nrf2 plasmid and siControl RNA co-transfected cells had an increased clonogenicty compared to the cells transfected with mock vector and siControl RNA. However, this was repressed by the knockdown of *PGD* in the Nrf2 overexpressing cells (Figure 3(E)). Given that the increase in cell proliferation as a consequence of Nrf2 overexpression was hindered by silencing *PGD*, this finding suggests that PGD can contribute to the Nrf2-mediated proliferation and survival of HepG2 cells.

PGD regulates Nrf2 via a positive feedback loop

A previous study provided evidence that glucose-6-phosphate dehydrogenase, the first enzyme in the PPP, and NADPH are important in the Nrf2-induced expression of antioxidant proteins, such as HO-1 and glutathione reductase [29]. Since PGD is also a part of the PPP, we examined its effect on expression of Nrf2 and its target proteins. Notably, silencing of *PGD* dampened the endogenous expression of Nrf2 (Figure 4(A)) in HepG2 cells. In addition, the ectopic expression of Nrf2 using the mammalian expression vector, pcDNA3 was also attenuated upon siPGD RNA co-transfection (Figure

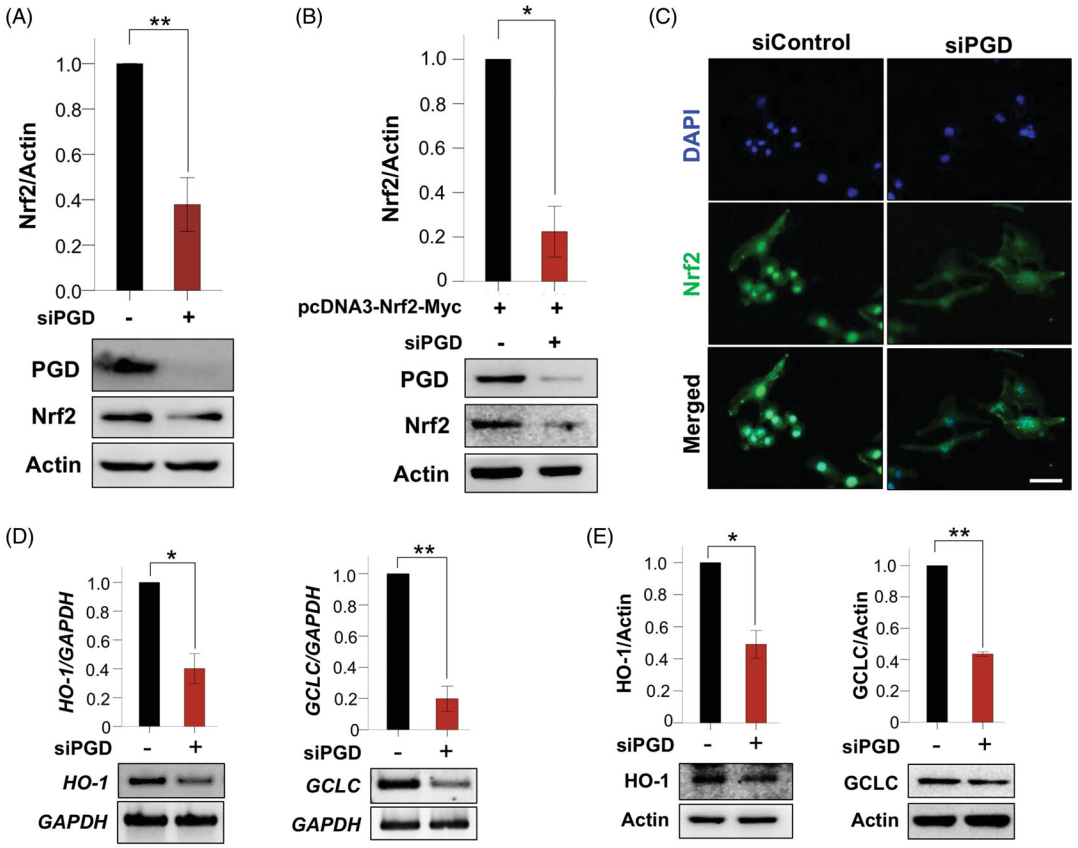


Figure 4. PGD regulates Nrf2 expression in HepG2 cells. The cells were transfected with either a non-targeting siRNA or siPGD RNA and incubated for 72 h. (A) The protein level of Nrf2 in total cell lysates was determined by the Western blot analysis. (B) Cells transfected with pcDNA 3.1-Nrf2-Myc were co-transfected with either non-targeting siRNA or siPGD. Total lysates were subjected to Western blot analysis. Band intensities were normalized to actin. (C) Cells were fixed, and the localization of Nrf2 was visualized by immunofluorescence staining as described in Materials and methods. Scale bar: 10 μm. (D) mRNA levels of Nrf2 target genes were measured by RT-PCR. Band intensities were normalized to *GAPDH*. (E) Protein levels of Nrf2 target genes were measured by Western blot analysis. Band intensities were normalized to actin. For all panels, each bar shows the mean ± SEM of three independent experiments. The *p* values were determined by independent *t*-test (*, $0.05 > p > 0.01$; **, $0.01 > p > 0.001$ versus control).

4(B)). Furthermore, immunocytochemical analysis showed that genetic ablation of *PGD* decreased the nuclear localization of Nrf2 (Figure 4(C)). The expression of HO-1 and GCLC, two representative Nrf2 target molecules, was also blunted by *PGD* silencing at both transcriptional (Figure 4(D)) and translational (Figure 4(E)) levels.

PGD promotes the degradation of Keap1, a negative regulator of Nrf2

Keap1 is a negative regulator of Nrf2 [1–3,30]. It interacts with the N-terminal Neh2 domain of Nrf2 to facilitate proteolytic degradation of this transcription factor [1–3,31]. *PGD* silencing did not affect the mRNA expression (Figure 5(A)), but increased the protein expression of Keap1 (Figure 5(B)). Thus, it is likely that PGD may promote degradation of Keap1 protein primarily *via* post-translational mechanisms. The sequestosome 1 (p62/SQSTM) is a scaffold protein that binds to polyubiquitinated proteins and targets protein aggregates and damaged organelles for degradation *via* the autophagy pathway [32]. Previous studies have shown the degradation of Keap1 through p62-dependent autophagy [28,32]. The p62-mediated sequestration of Keap1 into autophagosomes resulted in a decrease in Nrf2 ubiquitination and consequently an increase in Nrf2 stability [33]. Of note, knockdown of *PGD* also reduced p62 protein levels (Figure 5(C)). Likewise, a pharmacologic inhibition of PGD with Physcion [22] suppressed p62 expression with concurrent restoration of Keap1 accumulation (Figure 5(D)), suggesting that p62-dependent degradation of Keap1 may account for PGD-induced stabilization of Nrf2.

PGD converts 6-phosphogluconate to Ru-5-P which then enters the non-oxidative arm of the PPP and

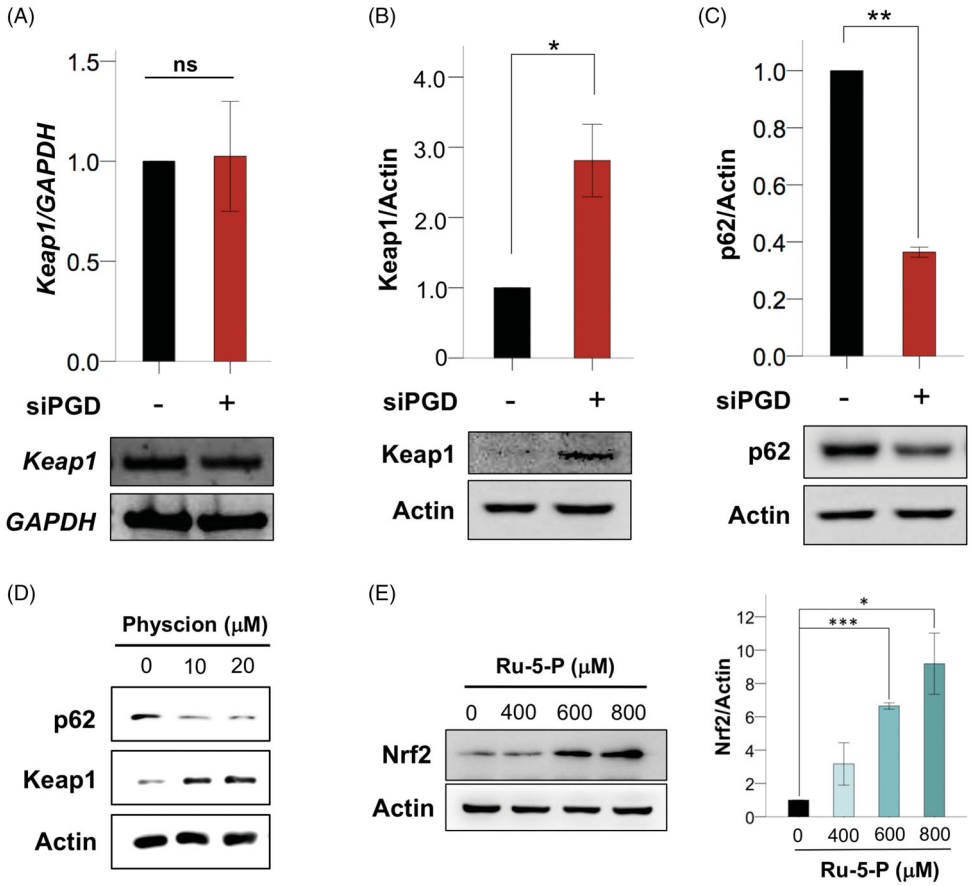


Figure 5. PGD promotes the degradation of Keap1 protein. (A to C) HepG2 cells were transfected with either non-targeting siRNA or siPGD RNA. (A) The expression of *Keap1* mRNA was determined by RT-PCR. Relative mRNA levels were normalized to *GAPDH*. (B and C) Total cell lysates were subjected to Western blot analysis of Keap1 (B) and p62 (C). The band intensities of both proteins were normalized to actin. (D and E) HepG2 cells were treated with indicated concentrations of Physcion or Ru-5-P for 24 h. The expression levels of p62, Keap1 (D) and Nrf2 (E) were measured by Western blot analysis. For all panels, each bar shows the mean ± SEM of three independent experiments. The p values were determined by independent t-test (*, $0.05 > p > 0.01$; **, $0.01 > p > 0.001$; ***, $0.001 > p$ versus control). ns: not significant.

proceeds to purine biosynthesis [34]. To determine whether this particular metabolite could mediate the PGD-induced Nrf2 accumulation, HepG2 cells were treated directly with increasing concentrations (400, 600, and 800 μM) of Ru-5-P for 24 h. Ru-5-P treatment increased expression of Nrf2 in a concentration-dependent manner (Figure 5(E)).

Discussion

Nrf2 functions as a master regulator of antioxidant response and hence is a protector of oxidative stress and other environmental insults [1,2,35]. Damage to DNA, proteins, or lipids due to oxidative stress has been linked to inflammation and cancer [36]. *Nrf2*-knockout mice were found to be more susceptible to experimentally induced carcinogenesis [37,38]. Furthermore, sensitivity to the chemoprotective and chemopreventive agents was markedly diminished in *Nrf2* knockout mice [39,40]. However, recent studies have revealed an opposite effect of Nrf2 in cancer. Mutations to either Nrf2 or its regulatory protein, Keap1 cause aberrant overactivation of this transcription factor in several malignancies, such as esophageal [41], skin [41], renal [42], lung [43,44], gastric [44], colorectal [44], and liver [44,45] cancer. Nrf2 confers optimal reducing power on cancer cells to promote their growth and survival [46]. Nonetheless, the function of Nrf2, either cytoprotective [1–3,9,25,26,35–40,47–49] or oncogenic [4–6,23,24,27,28,41–46,50–52], is still controversial. Thus, there is a need to conduct additional research in order to more accurately predict the function as well as regulation of Nrf2 in each step of the multi-stage carcinogenesis. In addition, identification and characterization of the target genes of Nrf2 are needed in order to better understand its differential roles in carcinogenesis.

There are multiple lines of compelling evidence supporting that cancer is a metabolic disease [53].

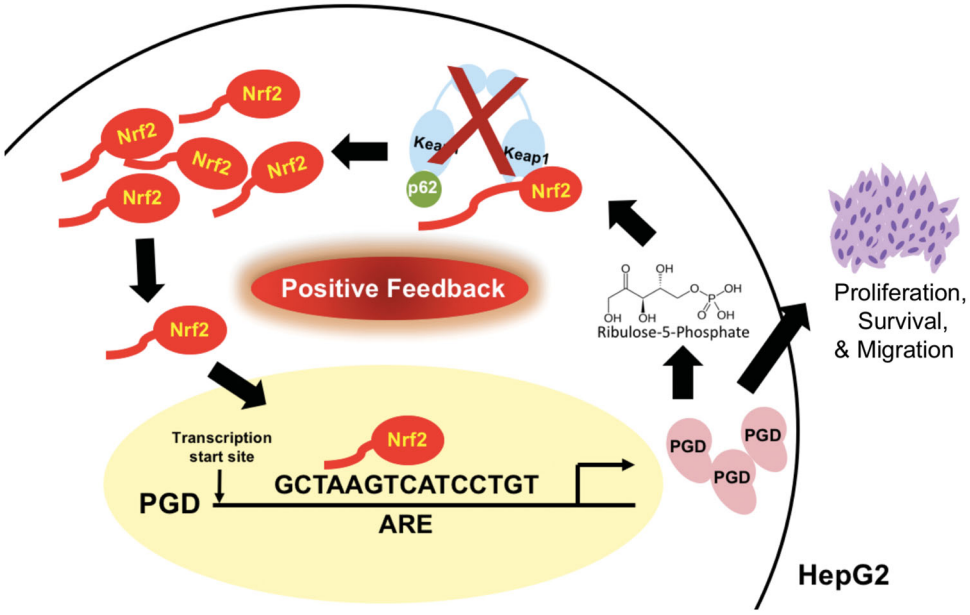


Figure 6. A schematic representation of the proposed positive feedback loop between Nrf2 and PGD. Nrf2 binds to the ARE consensus sequence present in the *PGD* regulatory region, thereby promoting its transcription in HepG2 cells. The increased PGD expression leads to stimulation of hepatoma cell proliferation, survival and migration. Moreover, the increase in the accumulation of PGD product Ru-5-P induces the degradation of Keap1 through p62 upregulation to create a positive feedback loop between PGD and Nrf2.

Metabolic reprogramming in cancer cells supports bioenergetics and the macromolecular synthesis required for their proliferation and survival. This is achieved through activation of distinct signal transduction pathways and reorganization of transcriptional network [10–16,54]. The high catalytic activity of PGD has been associated with poor relapse-free survival times in breast cancer [55]. Elevated PGD expression levels also correlate with an advancing stage of lung carcinoma [18]. On the other hand, cancer cell proliferation was found to be inhibited upon *PGD* silencing [18,19]. We have previously reported the elevated expression of *PGD* and some other PPP-related genes in the chemically induced hepatocarcinogenesis [56]. Our present study demonstrates that Nrf2 regulates PGD expression by binding to the ARE located in the regulatory region of *PGD* in cultured human hepatoma cells. This finding is consistent with the result of a previous study done with lung cancer cells [23].

Some of Nrf2 target proteins have been shown to take part in cancer cell proliferation and survival. HO-1, for instance, was shown to be involved in the metastasis [57], invasion [58], and resistance to apoptosis [59] in various cancer types [reviewed in 45 and more references therein]. Similarly, NAD(P)H:quinone oxidoreductase is found to be elevated in several solid tumors [60,61], and plays a role in the growth and survival of tumors [62–64]. In addition, higher protein levels of GCLC were detected in colorectal tumor tissue than in adjacent normal tissue [65], and this was implicated in the development of tamoxifen-resistant breast cancer [66]. Unlike these Nrf2-regulated antioxidant enzymes, there is paucity of data describing the signals related to PGD as a modulator of the tumorigenic processes mediated by Nrf2. The present study provides convincing evidence supporting the involvement of PGD in Nrf2-induced proliferation in human hepatoma cells.

Keap1 is a negative regulator of Nrf2 [1-3,30]. In normal conditions, Keap1 sequesters Nrf2 in the cytoplasm, thereby inhibiting its translocation to the nucleus [1–3,30,31]. It also facilitates the degradation of Nrf2 by serving as an adaptor between Nrf2 and the Cullin3-based E3-ligase ubiquitylation complex [67]. Electrophiles, ROS, and reacrive nitrogen species can modify the reactive 'sensor' cysteine residues of Keap1, leading to its inactivation [2,7,68]. Consequently, Nrf2 is stabilized, and translocates to the nucleus where it induces the transcription of numerous cytoprotective genes through binding to ARE/EpRE located in their promoter region [8]. Besides this canonical pathway, the ubiquitin binding autophagic adaptor protein p62 interacts with the Nrf2-binding site (Kelch domain) of Keap1, thereby protecting Nrf2 from proteasome degradation [33,69]. In hepatocytes, one mechanism underlying Keap1 degradation involves p62-dependent autophagy [32]. In this study, we have shown that genetic or pharmacologic inhibition of PGD enhanced Keap1 expression

levels with concomitant decreases in p62 accumulation. We speculate that p62-dependent autophagic degradation of Keap1 is likely to be involved in the regulation of Nrf2 by PGD.

Oncometabolites are endogenous substances whose abnormal accumulation contributes to the growth and metastasis of tumors [70]. Numerous oncometabolites have already been identified, and some of them play a role in the control of cell division processes [71–73]. PGD activity produces Ru-5-P and NADPH as byproducts. Interestingly, Ru-5-P was found to inhibit AMPK activation and to activate lipogenesis through disruption of the active LKB1 complex [22]. In leukemia cells, targeting PGD was proven to be selective and non-toxic to normal cells [22]. We found that *PGD*, as a Nrf2 target gene, upregulates Nrf2 expression, and hence comprises a positive feedback loop with Nrf2. This opens an exciting possibility of attenuating Nrf2 signaling by targeting PGD in cancer cells, especially those which exhibit chemoresistance due to Nrf2 overactivation.

In summary, Nrf2 regulates the expression of PGD by directly binding to the ARE in its regulatory region. The upregulation of PGD by Nrf2 plays an important role in Nrf2-mediated tumor plasticity through stimulation of cell proliferation, survival, and migration (Figure 6). Taken together, these results suggest PGD as a potential target in inhibiting growth and progression of hepatocellular carcinoma with hyperactivated Nrf2 signaling.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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