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High-mobility Group Box 1 Induces the Epithelial-mesenchymal Transition, Glycolytic Switch, and Mitochondrial Repression via Snail Activation

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Cancer cells undergo the epithelial-mesenchymal transition (EMT) and show unique oncogenic metabolic phenotypes such as the glycolytic switch (Warburg effect) which are important for tumor development and progression. The EMT is a critical process for tumor invasion and metastasis. High-mobility group box 1 (HMGB1) is a chromatin-associated nuclear protein, but it acts as a damage-associated molecular pattern molecule when released from dying cells and immune cells. HMGB1 induces the EMT, as well as invasion and metastasis, thereby contributing to tumor progression. Here, we show that HMGB1 induced the EMT by activating Snail. In addition, the HMGB1/Snail cascade was found induce a glycolytic switch. HMGB1 also suppressed mitochondrial respiration and cytochrome c oxidase (COX) activity by a Snail-dependent reduction in the expression of the COX subunits COXVIIa and COXVIIc. HMGB1 also upregulated the expression of several key glycolytic enzymes, including hexokinase 2 (HK2), phosphofructokinase-2/fructose-2,6-bisphosphatase 2 (PFKFB2), and phosphoglycerate mutase 1 (PGAM1), in a Snail-dependent manner. However, HMGB1 was found to regulate some other glycolytic enzymes including lactate dehydrogenases A and B (LDHA and LDHB), glucose transporter 1 (GLUT1), and monocarboxylate transporters 1 and 4 (MCT1 and 4) in a Snail-independent manner. Transfection with short hairpin RNAs against HK2, PFKFB2, and PGAM1 prevented the HMGB1-induced EMT, indicating that glycolysis is associated with HMGB1-induced EMT. These findings demonstrate that HMGB1 signaling induces the EMT, glycolytic switch, and mitochondrial repression via Snail activation.

Key words : Epithelial-mesenchymal transition, glycolytic switch, HMGB1, snail

Introduction

Cancer cells exhibit epithelial-mesenchymal transition (EMT) and distinct oncogenic metabolic phenotypes, which are important for tumor development and progression [14, 33]. EMT, which normally occurs during embryogenesis, is a critical process for tumor invasion and metastasis [7, 39, 54]. During EMT, cells lose their epithelial morphology and acquire mesenchymal traits in addition to downregulating epithelial markers (such as E-cadherin) and upregulating mesenchymal markers (such as vimentin and fibronectin) [7, 39]. Snail is known to be a major regulator of EMT in various cancers and acts by directly repressing E-cadherin expression [35, 47].

Metabolic reprogramming is another hallmark of cancer [14, 33]. Oncogenic metabolism (including the glycolytic switch) has been shown to promote cell proliferation and growth [3, 6, 8]. Most cancer cells exhibit increased glycolysis. Cancer cells produce most of their ATP through glycolysis, even under aerobic conditions, a phenomenon termed "Warburg effect" (the glycolytic switch) [18, 46]. Enhanced glycolysis leads to the accumulation of glycolytic intermediates, which are used for macromolecular biosynthesis by cancer cells to support their rapid proliferation. Mitochondrial dysfunction has been frequently observed in many human tumors and is linked with the glycolytic switch [12, 23]. Several

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proteins such as HIF-1, Myc, p53, STAT3 and Akt, have been implicated in the glycolytic switch and mitochondrial dysfunction [3]. Recently, Snail was been shown to induce the glycolytic switch and suppress mitochondrial respiration and cytochrome c oxidase (COX) activity [27]. COX is the terminal enzyme of the mitochondrial respiratory chain and decreased mitochondrial respiratory activity is closely associated with COX activity. Recent findings have shown that the glycolytic switch is closely associated with EMT in cancer cells [19, 25]. In addition, the increased production of lactic acid leads to microenvironmental acidosis and contributes to local invasion and metastasis [3, 18]. Mitochondrial dysfunction is also linked to enhanced metastatic potential in cancer cells [38].

HMGB1 is a highly conserved chromosomal protein that functions as a transcriptional regulator in the nucleus [21]. However, HMGB1 can be passively released from dying cells during necrosis, apoptosis, and autophagy or actively secreted by immune cells in response to exogenous and endogenous stimuli [21]. When released from dying cells and immune cells, HMGB1 acts as a damage-associated molecular pattern (DAMP), which induce inflammation and promotes tumor progression. In addition, HMGB1 expression is increased in various types of tumors and HMGB1 levels are also elevated in the serum of patients with cancer [21]. Therefore, high HMGB1 serum levels or expression is associated with poor prognosis, indicating that HMGB1 plays a critical role in tumor formation, invasion, and metastasis [21]. In fact, HMGB1 is known to contribute to the malignant cancer phenotype by inducing tumor growth, EMT, invasion, metastasis, energy metabolism, and angiogenesis [21, 32, 44]. HMGB1 binds with high affinity to several receptors, including the receptor for advanced glycation end products (RAGE; a member of the immunoglobulin gene superfamily of cell surface molecules), Toll-like receptor (TLR) 2, and TLR4, and activates cell signaling pathways, such as the MAP kinase, NF-κB, and phosphoinositide 3-kinase-AKT pathways, thereby promoting inflammatory responses, cell migration, and angiogenesis [21, 44].

In this study, we examined the effects of HMGB1 on the MCF-7 cell line, which is a less aggressive and a non-invasive luminal A subtype breast cancer cell line. First, we observed that HMGB1 induces EMT by activating Snail. Because Snail induces a glycolytic switch, we examined the induction of the glycolytic switch by HMGB1. In fact, we have previously reported that HMGB1 induces the glycolytic switch through Snail. In addition, HMGB1 suppresses mitochondrial respiration and cytochrome c oxidase (COX) activity by reducing the expression of COX subunits in a Snail-dependent manner. We examined the effects of HMGB1 and Snail on the expression of several key glycolytic enzymes, and found that HMGB1 induces hexokinase 2 (HK2), phosphofructokinase-2/fructose-2,6-bisphosphatase 2 (PFKFB2), and phosphoglycerate mutase 1 (PGAM1) in a Snail-dependent manner. We studied the impact of HK2, PFKFB2, and PGAM1 on HMGB1-induced EMT. We observed that HK2/PFKFB2/PGAM1 shRNA suppressed HMGB1- induced EMT and Snail expression, indicating that glycolytic enzymes, such as HK2, PFKFB2, and PGAM1 may contribute to Snail expression in a reciprocal manner. Our experiments clarified the role of Snail in HMGB1-induced EMT, glycolytic switch, and mitochondrial repression, and COX activity inhibition.

Materials and Methods

Cell culture

MCF-7, MDA-MB231, and Madin Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection. HCT116 cells were provided by Dr. Kim YJ (Pusan National University, Pusan, Korea). The cell lines were passaged twice per week after receipt, and low-passage cultures (passage 5-25) were used for experiments. The cells were routinely tested for mycoplasma contamination using the Mycoplasma PCR Detection Kit (iNtRON Biotechnology, Korea). MCF-7 and MDCK cells were cultured in Eagle's minimal essential medium (Hyclone, Logan, UT, USA); MDA-MB231 and HCT116 cells were cultured RPMI supplemented with 10%(v/v) heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin-streptomycin (Hyclone, Logan, UT, USA) in a 37℃ humidified incubator with 5% CO_2. Cells were treated with 1 μg/mL recombinant HMGB1 (HMGBiotech, Milano, Italy).

Transfection and short hairpin RNA (shRNA) interference

The expression vector pCR3.1-Snail-Flg (provided by Dr. Yook JI, Yonsei University) was transfected into MCF-7 cells using jetPEI (Polyplus-transfection, SA, USA). pSUPER vectors for shRNAs against control, Snail, HK2, PFKFB2, and PGAM1 were produced and transfected into MCF-7, MDA-MB231, HCT116, and MDCK cells as described previously

Table 1. shRNA target sequences used in this study

Genes	Target sequence 5′ to 3′
Con shRNA	AATTCTCCGAACGTGTCACGT
Snail shRNA	GCGAGCTGCAGGACTCTAA
HK shRNA	GCAGAAGGTTGACCAGTATCT
PFKFB2 shRNA	TCAGGAAGGCTCCTTACCCTGTAGCAG
PGAM1 shRNA	GGCGGTTGAAATAGTCCCACATGTGGT

[27]. For shRNA interference, we used the shRNA target sequences listed in Table 1.

Immunoblotting, nuclear fractionation, and immunofluorescence (IF) microscopy

Immunoblotting was performed as described previously [22, 27], using antibodies against: Snail (Abgent, San Diego, CA, USA), E-cadherin, HDAC1, COXVIIc (Santa Cruz, CA, USA), COXVIIa (Mitoscience, Eugene, OR, USA), and α-tubulin (Biogenex, CA, USA). Nuclear extracts were prepared using the NucBuster Protein Extraction Kit (Novagen, Germany) [27]. For immunofluorescence (IF) microscopy, MCF-7 cells were fixed for 10 min in 3.7% formaldehyde in phosphate buffered saline (PBS), permeabilized in PBS containing 0.2% Triton X-100 for 30 min, and blocked with 2% bovine serum albumin in 0.1% PBST for 3 hr. For E-cadherin staining, cells were incubated with mouse anti-E-cadherin antibody (Santa Cruz, CA, USA) overnight at 4℃ and immunostained with Alexa Fluor 488-labeled anti-mouse secondary antibody (Molecular Probes, NY, USA). Hoechst 33342 (Molecular Probes, NY, USA) was used to stain the cell nuclei. The cells were finally visualized under a fluorescence microscope.

Quantitative real-time polymerase chain reaction (qRT-PCR) and chromatin immunoprecipitation (ChIP) assay

qRT-PCR was performed as described previously [22, 27]. Total mRNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Transcript levels were assessed by qRT-PCR using primers targeted toward genes encoding Snail, E-cadherin, COX subunits and assembly factors, glycolytic enzymes, and β-actin. Previously described primer sequences were used [26, 27, 30], along with the following additional primers: HK2 sense, 5′-ATCCCTGAGGACATCATG CGA-3′; HK2 antisense, 5′-ACCCAGTGGGAGCTTCTTGT-3′; PFKFB2 sense, 5′-GCGTCCCTCATGTTGCCTTGCT-3′; PFKFB2 antisense, 5′-TCCAGGCCTCGTTTTGGTGGC-3′; PGAM1 sense, 5′-TCAGCTACCCTCCTGTGAGA-3′; PGAM1 antisense, 5′-CAGATGCTTGACAATGCCCC-3′; PKM2 sense, 5′-TTGCCCGTGAGGCAGAGGCT-3′; PKM2 antisense, 5′-GGCCCCACTGCAGCACTTGA-3′; LDHA sense, 5′-GGCCT GTGCCATCAGTATCT-3′; LDHA antisense, 5′-GCCGTGAT AATGACCAGCTT-3′; LDHB sense, 5′-AGGATTCATCCCG TGTCAAC-3′; LDHB antisense, 5′-CCCACAGGGTATCTGC ACTT-3′; GLUT1 sense, 5′-TCATCGTGGCTGAACTCTTC-3′; GLUT1 antisense, 5′-GATGAAGACGTAGGGACCAC-3′; MCT1 sense, 5′-GTGGCTCAGCTCCGTATTGT-3′; MCT1 antisense, 5′-GAGCCGACCTAAAAGTGGTG-3′; MCT2 sense, 5′-TGCTGCTGGGCAACTTCTTCTG-3′; MCT2 antisense, 5′-CTCCCCGTTTTTCTCAGGCTC-3′. Values were normalized to those of β-actin.

The ChIP assay was performed using a ChIP assay kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Isotype control IgG and anti-Snail antibodies (Santa Cruz, CA, USA) were used to immunoprecipitate DNA-containing complexes. ChIP-enriched DNA was analyzed by PCR using primers complementary to sequences containing putative Snail binding sites in the HK2 and PGAM1 promoters (Table 2). Putative Snail binding sites in the HK2 and PGAM1 promoter regions are listed in Table 3.

Assays for mitochondrial respiration, glucose consumption, lactate production, ATP production, and COX activity

Mitochondrial respiration was measured as described previously [27, 50]. For mitochondrial respiration assay, ex-

Table 2. Primers used in this study for ChIP assay

Genes		Target sequence 5′ to 3′
HK2 #1 (for E2)	sense	CCCCAGTCCCTTTTTCCCTG
	antisense	GGAACTCGCGCTGCAGAA
HK2 #2 (for E3)	sense	GAGCACGTGGAGAGAATCGT
	antisense	GAACCGCTCGTCTCCTACAC
PGAM1 #1 (for E1)	sense	GCGCACACCTGGAGGG
	antisense	CGCTCGCACCGCTCAG

Table 3. Putative Snail binding sites in the gene promoters

Gene	Snail binding sites	Positions form transcription start site
HK2	E1	$^{-469}$ CAGGTG $^{-464}$
	E2	$^{+996}$ CACGTG $^{+1001}$
	E3	$^{+1041}$ CAGCTG $^{+1046}$
PGAM1	E1	$^{-15}$ CACCTG $^{-10}$

ponentially growing cells (1.5×10^6) were washed with TD buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 25 mM Tris-HCl, pH 7.4) and collected and resuspended in complete medium without phenol red. The cells (5×10^5) were, then transferred to a Mitocell chamber equipped with a Clark oxygen electrode (782 Oxygen Meter; Strathkelvin Instruments, Glasgow, UK). Oxygen consumption rates were measured after adding 30 μM DNP to obtain the maximum respiration rate, and its specificity for mitochondrial respiration was confirmed by adding 5 mM KCN [50]. COX activity was determined by measuring the KCN-sensitive COX-dependent O_2 consumption rate by adding 3 mM TMPD in the presence of 30 μM DNP and 20 μM antimycin A. Glucose, lactate, and intracellular ATP levels were determined using a Glucose Oxidation Assay Kit (Sigma, MO, USA), a colorimetric- and fluorescence-based Lactate Assay Kit (BioVision, CA, USA), and an ATP Bioluminescence Assay Kit (Roche, Switzerland), respectively. Levels of oxygen consumption, glucose, lactate, and intracellular ATP were normalized to protein concentrations. The level of ATP produced by aerobic respiration and glycolysis was determined by measuring lactate production and oxygen consumption [27, 41].

Statistical analysis

qRT-PCR and assays for mitochondrial respiration, glucose consumption, lactate production, ATP production, and COX activity were performed at least in triplicate, and most experiments were repeated more than three times. Data were analyzed by Student's *t*-test (unpaired, two-tailed), and the results are expressed as the mean ± standard error (SE). $P<0.05$ was considered statistically significant.

Results

HMGB1 induces EMT through Snail activation

We examined the effects of recombinant HMGB1 protein on the non-invasive breast cancer cell line MCF-7. We used fully reduced HMGB1 in this study. Several different concentrations of HMGB1 have been shown to stimulate the migration and EMT of many types of cells including D16 cells (10-100 ng/ml) [32], human airway epithelial cells (10-300 ng/ml) [4], HCT116 and LoVo cells (0.5-2 μg/ml) [55], and prostate cancer PC3 cells (0.5-2 μg/ml) [53]. Therefore, we examined the effects of HMGB1 at concentrations of 1-1,000 ng/ml. HMGB1 treatment induced EMT in a dose-dependent manner (data not shown). Maximal EMT induction was observed at an HMGB1 concentration of 1 μg/ml. EMT was assessed by observing morphological changes in mesenchymal cells, including loss of intercellular adhesion and formation of a spindle-like cell shape and pseudopodia (Fig. 1A). The phenotypic changes were accompanied by the reduced expression of E-cadherin, one of the hallmarks of EMT, as revealed by IF (Fig. 1A).

We investigated whether EMT-inducing transcription factors (including Snail, Slug, ZEB1. ZEB2, Twist1, Twist2, and E12/E47) are involved in HMGB1-induced EMT using real-time qRT-PCR. Among several EMT-inducing transcription factors, the levels of Snail, Twist1, ZEB1, and ZEB2 were increased by HMGB1 treatment (Table 4). Because Snail acts as an upstream regulator of Twist1 and ZEB1/2 and is considered a major mediator in EMT [40], we examined whether Snail affects the HMGB1-mediated levels of EMT-inducing transcription factors. Snail shRNA prevented the expression of Twist, ZEB1 and ZEB2 induced by HMGB1 (Table 4). Therefore, we focused on the role of Snail in HMGB1-induced EMT.

Snail is a typical E-cadherin repressor of EMT [35, 47]. We examined the effects of Snail expression on HMGB1-induced EMT. HMGB1 treatment increased Snail expression in a dose-dependent manner (data not shown) and induced the nuclear accumulation of Snail proteins (Fig. 1B - Fig. 1C). Thus, we investigated the effects of Snail knockdown on HMGB1-induced EMT in MCF-7 cells. Transfection with Snail shRNA prevented HMGB1-induced EMT-like morphological changes and E-cadherin downregulation, as revealed by IF (Fig. 1D). In addition, Snail shRNA suppressed

Table 4. Regulation of EMT-inducing transcription factors by HMGB1

Genes	HMGB1 3 d	
	shCon	shSnail
Snail	1.862**	0.994##
Slug	1.014	1.081
Twist1	1.928**	1.038##
Twist2	0.953	0.995
ZEB1	1.509**	1.050##
ZEB2	1.815**	1.084##
E12/E47	1.031	1.018

MCF-7 cells were transfected with shSnail and then treated with HMGB1. mRNA expression was analyzed by qRT-PCR using the indicated primers.
**$p<0.01$ versus control.
##$p<0.01$ versus shCon.

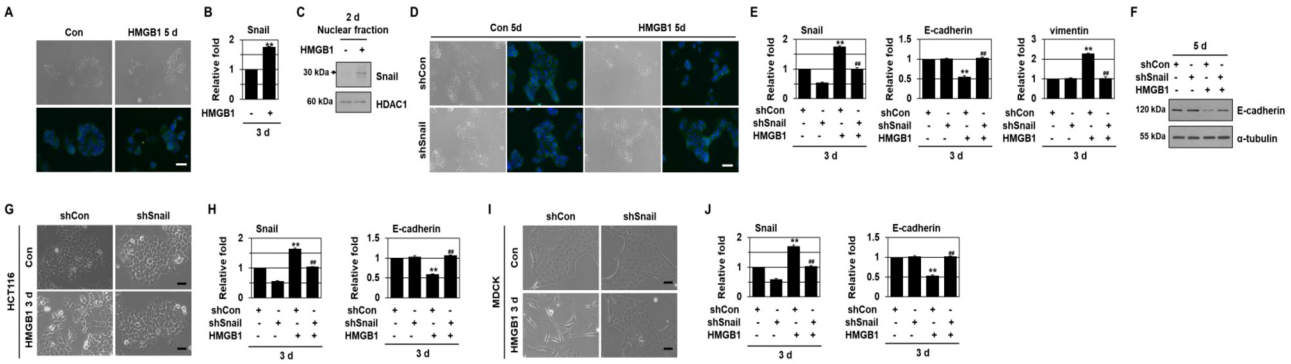

Fig. 1. HMGB1 induces EMT via Snail activation. (A) MCF-7 cells were treated with HMGB1. The cells were analyzed by phase-contrast and fluorescence microscopy to assess cell morphology and E-cadherin expression (fluorescence in green). Nuclei were stained with Hoechst 33342 (blue). (B) and (C) MCF-7 cells were treated with recombinant HMGB1. Snail mRNA expression was analyzed by qRT-PCR (B). The nuclear fractions were analyzed by immunoblotting to assess Snail and HDAC1 expression (C). $^{**}P<0.01$ versus untreated. (D)-(F) MCF-7 cells were transiently transfected with Snail shRNA and then treated with recombinant HMGB1. The cells were analyzed by phase-contrast and fluorescence microscopy to assess cell morphology and E-cadherin expression (fluorescence in green). Nuclei were stained with Hoechst 33342 (blue) (D). mRNA expression was analyzed by qRT-PCR (E), protein level was evaluated by immunoblotting (F) using the indicated primers and antibodies. $^{**}P<0.01$ versus untreated, $^{\#\#}P<0.01$ versus control shRNA. (G)-(J) HCT116 [(G) and (H)], and MDCK [(I) and (J)] cells were transiently transfected with Snail shRNA and then treated with HMGB1. The cells were analyzed by phase-contrast microscopy to assess cell morphology [(G) and (I)]. mRNA expression was analyzed by qRT-PCR [(H) and (J)], using the indicated primers. $^{**}P<0.01$ versus untreated, $^{\#\#}P<0.01$ versus control shRNA.

HMGB1-induced E-cadherin downregulation and vimentin upregulation at the mRNA or protein level (Fig. 1D - Fig. 1F). E-cadherin levels were high in MCF-7 control cells because the basal levels of Snail are very low; thus, Snail shRNA by itself had no effect on E-cadherin expression in the control cells (Fig. 1E - Fig. 1F).

Further, we examined the effects of HMGB1 on EMT in HCT116 and MDCK cells. HMGB1 treatment induced EMT in these cells (Fig. 1G - Fig. 1J). In addition, treatment with Snail shRNA suppressed HMGB1-induced EMT and E-cadherin downregulation in HCT116 and MDCK cells (Fig. 1G - Fig. 1J). The levels of Snail and E-cadherin expression did not differ between cell lines such as MCF-7, HCT116 and MDCK (Fig. 1E, Fig. 1H, Fig. 1J). These results indicate that HMGB1 regulates EMT through the induction of Snail expression not only in MCF-7 cells but also in HCT116 and MDCK cells.

HMGB1 induces glycolytic switch, mitochondrial repression, and COX activity inhibition via Snail

Because Snail has been shown to induce glycolytic switch and mitochondrial repression [25-27], we assessed whether HMGB1 induces glycolytic switch and mitochondrial repression through Snail. HMGB1 treatment significantly increased glucose consumption and lactate production and decreased O_2 consumption (Fig. 2). Although the effects of HMGB1 on glucose consumption, lactate concentration and O2 consumption were slight, HMGB1 has the potential to significantly induce glycolytic switch and mitochondrial repression. It is noteworthy that the knockdown of Snail by shRNA alter basal metabolism under normal conditions, even though the basal levels of Snail are extremely low in MCF-7 cells [27]. Thus, these results indicate that basal levels of Snail have an important effect, particularly on metabolism (glucose consumption, lactate production, O2 consumption) and COX activity. ATP levels were similar between control and HMGB1-treated cells (Fig. 2). HMGB1 treatment increased the ratio of ATP produced by glycolysis versus aerobic respiration (Fig. 2), indicating that HMGB1 induces the glycolytic switch.

Treatment with Snail shRNA prevented HMGB1-induced glycolytic switch and mitochondrial repression in MCF-7 cells (Fig. 2A). Similar inhibitory effects of Snail shRNA on HMGB1-induced glycolytic switch and mitochondrial repression were observed in HCT116 and MDCK cells (Fig. 2B, Fig. 2C). These results indicate that HMGB1 induces glycolytic switch and mitochondrial repression via Snail activation in many cell lines, including MCF-7, HCT116, and MDCK.

Because the activity of COX, the terminal enzyme in the

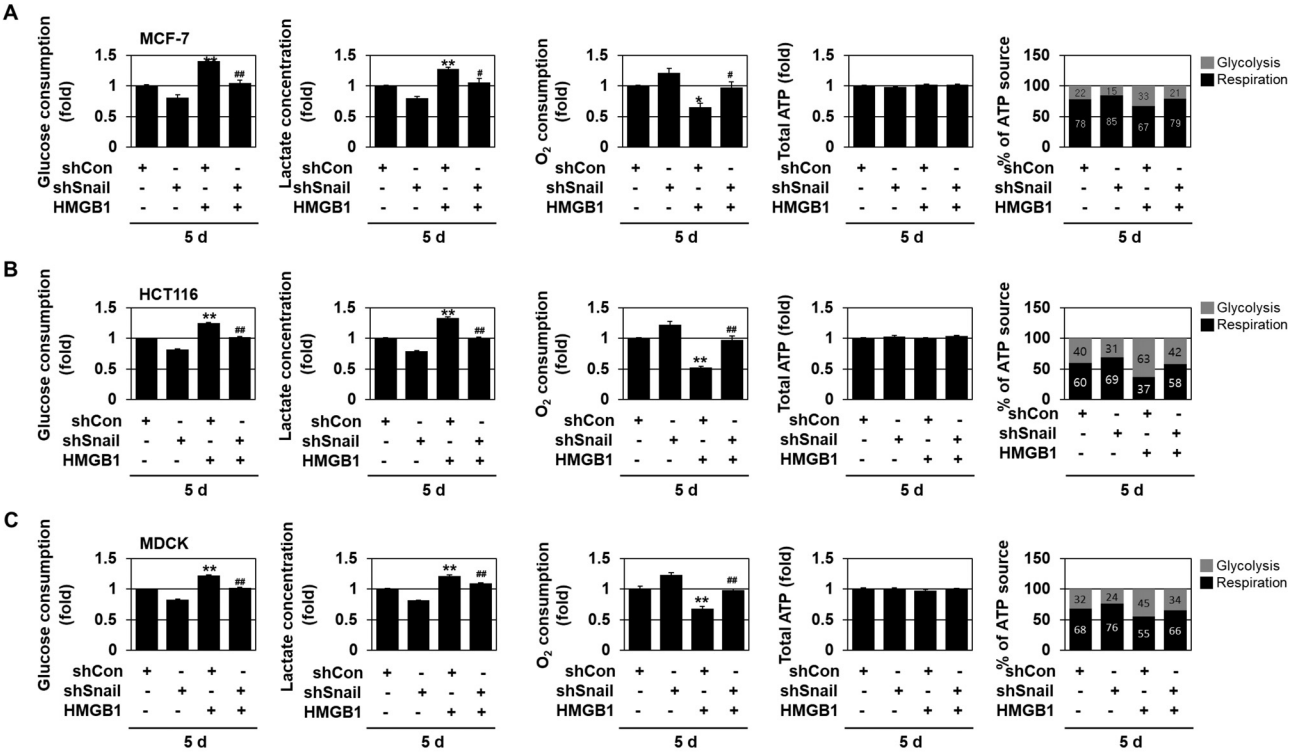

Fig. 2. Snail expression is linked to HMGB1-induced glycolytic switch and mitochondrial repression. (A) – (C) Glucose consumption, lactate production, mitochondrial respiration, and total ATP concentration were measured in MCF-7 (A), HCT116 (B), and MDCK (C) cells transiently transfected with Snail shRNA and then treated with recombinant HMGB1. *P<0.05, **P<0.01 versus untreated; $^{\#}P$<0.05, $^{\#\#}P$<0.01 versus control shRNA. The amount of ATP produced by aerobic respiration (black bars) and glycolysis (gray bars) was calculated by measuring oxygen consumption and lactate production in the cells [right panels in (A) – (C)]. All error bars represent SE.

mitochondrial respiratory chain, is closely associated with mitochondrial respiratory activity, we examined the effects of HMGB1 on COX activity. HMGB1 treatment reduced COX enzymatic activity (Fig. 3A). Transfection with Snail shRNA suppressed HMGB1-induced COX inhibition in MCF-7 cells (Fig. 3A). Similar results were obtained for HCT116 and MDCK cells (Fig. 3A), indicating that HMGB1 induces COX inhibition via Snail activation.

Eukaryotic COX is composed of 13 different subunits, and their assembly is regulated by the sequential action of several nucleus-encoded assembly factors [17, 34]. Previously, we showed that Snail downregulates the expression of COX subunits, including COXVIc, COXVIIa, and COXVIIc [27]. We, therefore, examined here, the effects of HMGB1 on the expression of COX subunits and assembly factors (Table 5). HMGB1 treatment appeared to downregulate the expression

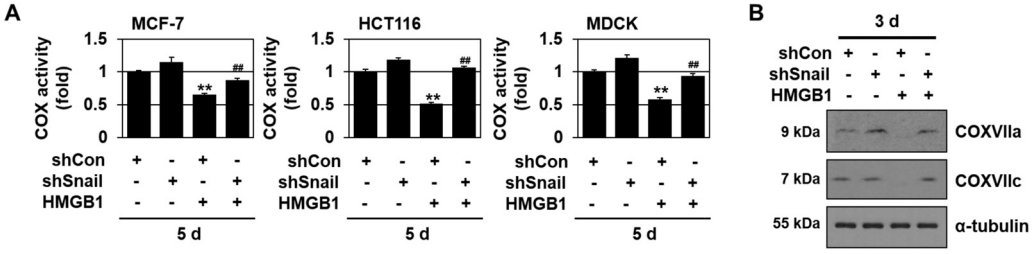

Fig. 3. Snail is involved in HMGB1-induced COX inhibition by downregulating the expression of multiple COX subunits. (A) MCF-7, HCT116, and MDCK cells were transfected with Snail shRNA and then treated with HMGB1. The cells were analyzed for COX activity. **P<0.01 versus untreated, $^{\#\#}P$<0.01 versus control shRNA. (B) MCF-7 cells were transfected with Snail shRNA and then treated with HMGB1. Protein levels of COXVIIa, COXVIIc, and α-tubulin were analyzed by immunoblotting. All error bars represent SE. For all immunoblotting images, cropped blots are shown.

of COXIV, COXVb, COXVIIa, COXVIIc, COX11, and SCO1 (Table 5). Snail shRNA transfection suppressed the HMGB1-induced reduction of the levels of COXVIIa and COXVIIc (Fig. 3B, Table 5) but not those of COXIV, COXVb, COX11, and SCO1 (Table 5). Notably, Snail-mediated COXVIc repression was not observed in HMGB1-treated cells (Table 5). These results suggest that Snail is likely involved in HMGB1-mediated COXVIIa and COXVIIc gene repression.

HMGB1 induces glycolytic enzymes in a Snail-dependent manner

To drive the switch to aerobic glycolysis and lactate production, cancer cells induce the activation and expression of several glycolytic enzymes, including HK2, PFKFB, PGAM1, the M2 isoform of the pyruvate kinase muscle isozyme (PKM2), lactate dehydrogenases A and B (LDHA and LDHB), and several transporters, including glucose transporter 1 (GLUT1) and monocarboxylate transporters 1 and 4 (MCT1 and 4) [2, 42, 45].

Table 5. Regulation of gene expression of COX subunits and assembly factors by HMGB1

	Genes	HMGB1 3 d	
		shCon	shSnail
COX subunits	COXIV	0.670**	0.658
	COXVa	1.065	1.000
	COXVb	0.556**	0.580
	COXVIa	1.190	1.179
	COXVIb	1.017	0.980
	COXVIc	1.072	1.040
	COXVIIa	0.586**	1.012##
	COXVIIb	1.000	1.059
	COXVIIc	0.533**	1.084##
	COXVIII	0.971	1.032
Assembly factors	COX10	1.102	1.012
	COX11	0.574**	0.559
	COX15	1.059	1.045
	COX17	1.055	1.034
	COX18	1.079	1.033
	COX19	0.881	1.053
	LRPPRC	1.031	1.074
	SURF1	1.019	0.996
	SCO1	0.536**	0.611
	SCO2	1.066	1.060

HMGB1, high-mobility group box 1; COX, cytochrome c oxidase. MCF-7 cells were transiently transfected with shSnail and then treated with HMGB1. mRNA expression was analyzed by qRT-PCR using the indicated primers.
**$p<0.01$ versus untreated.
##$p<0.01$ versus shCon.

Because HMGB1 is known to induce the glycolytic switch, we examined the effects of HMGB1 on the expression of key glycolytic enzymes, including HK2 (converting glucose to glucose-6-phosphate), PFKFB2 (producing fructose-2,6-bisphosphate (F2,6BP)), PGAM1 (converting 3-phosphoglycerate (3-PG) to 2-PG), PKM2 (dephosphorylating phosphoenolpyruvate to pyruvate), LDHA/B (converting pyruvate to lactate and back), GLUT1 (mediating glucose uptake), and MCT1 and MCT4 (transporting lactate into and out of cells respectively) [45].

HMGB1 treatment upregulated HK2, PFKFB2, PGAM1, LDHB, and MCT1 expression and downregulated LDHA, GLUT1, and MCT4 expression. It had no effects on PKM2 expression (Table 6). Next, we determined whether Snail regulates the expression of HMGB1-induced enzymes. Snail increased the expression of HK2, PFKFB2, PGAM1, MCT1 and MCT4 (Table 6), suggesting that Snail may be involved in regulating these HMGB1-induced enzymes. Transfection with Snail shRNA suppressed HMGB1-induced HK2, PFKFB2, and PGAM1 expression (Table 6), indicating that HMGB1 increases the expression of HK2, PFKFB2, and PGAM1 in a Snail-dependent manner. Notably, Snail shRNA did not inhibit HMGB1-mediated alteration of LDHA, LDHB, GLUT1, MCT1, and MCT4 expression (Table 6), suggesting that HMGB1 regulates LDHA, LDHB, GLUT1, MCT1, and MCT4

Table 6. Regulation of gene expression of glycolytic enzymes by HMGB1

	Genes	Snail TF 3 d	HMGB1 3 d	
			shCon	shSnail
Glycolytic enzymes	HK2	2.254**	1.948**	1.036##
	PFKFB2	2.552**	2.321**	1.057##
	PGAM1	1.463**	3.242**	1.035##
	PKM2	1.006	1.074	0.980
	LDHA	1.099	0.707**	0.711
	LDHB	1.049	46.972**	47.565
	GLUT1	0.976	0.564**	0.751
	MCT1	2.021**	1.499**	1.543
	MCT4	2.046**	0.552**	0.575

HK2, hexokinase 2; PFKFB2, 6-phosphofructo-2-kinse/fructose-2,6-biphosatases 2; PGAM1, phosphoglycerate mutase 1; PKM2, M2 isoform of pyruvate kinase, muscle; LDHA and LDHB, lactate dehydrogenase A and B; GLUT1, glucose transporter 1; MCT1 and MCT4, monocarboxylate transporters 1 and 4.
MCF-7 cells were transiently transfected with Snail. MCF-7 cells were transiently transfected with shSnail and then treated with HMGB1. mRNA expression was analyzed by qRT-PCR using the indicated primers.
**$p<0.01$ versus control (Mock or untreated).
##$p<0.01$ versus shCon.

expression in a Snail-independent manner. Thus, we focused on studying the role(s) of HK2, PFKFB2, and PGAM1 in the HMGB1/Snail axis-induced EMT.

Therefore, we examined the effects of HK2, PFKFB2, and PGAM1 on HMGB1-induced EMT. Treatment with shRNAs against HK2, PFKFB2, and PGAM1 suppressed HMGB1-induced EMT and E-cadherin downregulation (Fig. 4A, Fig. 4B), indicating that glycolysis is linked with HMGB1-induced EMT, and also suppresses HMGB1-induced Snail expression (Fig. 4B).

Then, we examined HK2, PFKFB2, and PGAM1 expression in the breast cancer cell line MDA-MB231. Unlike MCF-7, MDA-MB231, an invasive human breast carcinoma cell line, exhibits autocrine Wnt signaling activity and expresses high levels of Snail and low levels of E-cadherin [36, 49]. In addition, compared with MCF-7 cells, MDA-MB231 cells have higher glycolytic activity and lower respiration activity [11].

MDA-MB231 cells expressing higher levels of Snail than MCF-7 cells exhibited higher of HK2 and PGAM1 expression levels, whereas MCF-7 cells exhibited lower levels of these enzymes (Fig. 4C). When Snail was overexpressed in MCF-7 cells, HK2 and PGAM1 expression also increased (Fig. 4C). Transfection with Snail shRNA suppressed HK2 and PGAM1 expression in MDA-MB231 cells (Fig. 4C). In the case of PFKFB2, we recently reported that PFKFB2 expression is higher in MDA-MB231 cells than in MCF-7 cells, and is increased by Snail overexpression in MCF-7 cells. Snail shRNA decreased PFKFB2 expression in MDA-MB231 cells [29]. This indicates that HK2, PFKFB2, and PGAM1 expression is regulated by Snail levels in MCF-7 and MDA-MB231 cells.

Recently, we showed that Snail binds to the PFKFB2 promoter [29]. We further investigated whether Snail binds to the HK2 and PGAM1 promoter using a ChIP assay. We found three E-boxes for Snail that bound between −500 and +1,500 from the transcription start site (TSS) in the promoter region of HK2 (Fig. 4D). The three predicted Snail binding sites were numbered E1, E2, and E3. The ChIP-enriched DNA was amplified using primer set #1 or #2, which target the E2 or E3 binding sites in the HK2 promoter, respectively. We also found one E-box for Snail that bound between −1,000 and +1,000 of the TSS in the promoter region of PGAM1 (Fig. 4D). The ChIP assay showed that Snail binds to the HK2 and PGAM1 promoters in Snail-overexpressing MCF-7 cells (Fig. 4D). Snail also bound to the HK2 and PGAM1 promoters in MDA-MB231 cells (Fig. 4D). Treatment with Snail shRNA inhibited Snail binding to the HK2 and PGAM1 promoters in MDA-MB231 cells (Fig. 4D), indicating that Snail may induce the expression of HK2 and PGAM1.

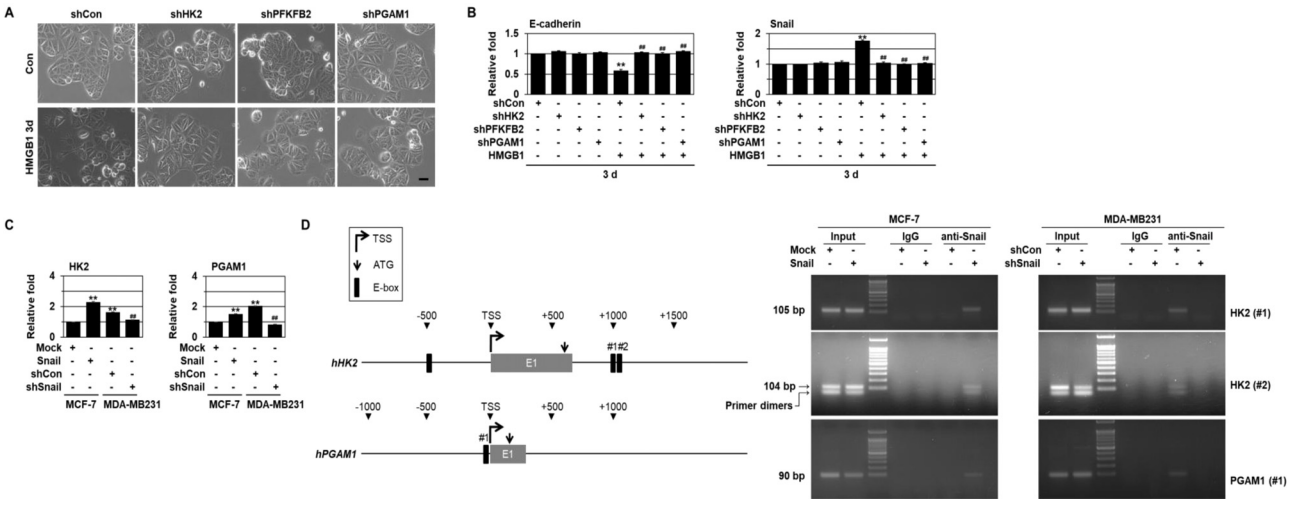

Fig. 4. Inhibition of HK2, PFKFB2, and PGAM1 expression attenuates HMGB1-induced EMT. (A) and (B) MCF-7 cells transiently transfected with shRNAs against HK2, PFKFB2, and PGAM1 and then treated with HMGB1 were analyzed by phase-contrast microscopy to assess cell morphology (A). mRNA expression was analyzed by qRT-PCR using the indicated primers (B). $^{**}P<0.01$ versus untreated, $^{\#\#}P<0.01$ versus control shRNA. (C) and (D) MCF-7 and MDA-MB231 cells were transiently transfected with Snail shRNA for 3 days. Snail mRNA expression was analyzed by qRT-PCR using the indicated primers (C). $^{**}P<0.01$ versus Mock, $^{\#\#}P<0.01$ versus control shRNA. A schematic diagram of the human HK2 and PGAM1 proximal promoter regions is shown in the left panel. ChIP assay was performed using IgG or anti-Snail antibodies, and ChIP-enriched DNA was analyzed by PCR using primers complementary to the Snail binding region (right panel) (D). All error bars represent SE. Scale bars = 100 μm.

Discussion

HMGB1 is highly expressed in various types of cancers and is also known to contribute to the malignant phenotype of cancer cells by inducing tumor growth, EMT, invasion, metastasis, energy metabolism, and angiogenesis [21, 32, 44].

HMGB1 induces EMT in several cancer cells, thereby enhancing invasion and metastasis. [4, 31, 55]. HMGB1-induced EMT is mediated through RAGE accompanied by the increased production of TGF-β [16]. In another study, HMGB1 induced EMT by the PI3K/AKT/GSK3β/β-catenin signaling pathway in a RAGE-dependent manner without inducing TGF-β production in human airway epithelial cells [4]. The interaction between HMGB1 and RAGE also activated Snail/NF-κB signaling pathways, leading to EMT by activation of MMP7 in colorectal carcinoma [55]. In addition, HMGB1 was found to not induce EMT in alveolar type II cells derived from $RAGE^{-/-}$ mice [16]. HMGB1 contributes to tumor metastasis by activating TLR2/4 [5, 51]. Through activation of TLR4, HMGB1 increases NF-κB and MAPK expression levels, which are involved in the proliferation, invasion and metastasis of tumor cells [13]. Interaction between tumor cell-derived HMGB1and TLR4 on platelets contribute to promotion of metastasis in Lewis lung carcinoma tumor cells [51]. Furthermore, HMGB1 has been shown to regulate a common signaling pathway (p38/p42/44 MAPK, JNK, and PI3K and AKT1/2/3) through RAGE and TLR4 [10]. Thus, HMGB1 interaction with RAGE and TLR4 may be regulated in a reciprocal manner.

While these findings have implicated HMGB1 in EMT induction, we found that HMGB1 not only induces EMT, but also induces the glycolytic switch and mitochondrial repression in a Snail-dependent manner, which may contribute to tumor progression. We showed that HMGB1 induces EMT and upregulates Snail expression in several cancer cell lines, including MCF-7, HCT116, and MDCK (Fig. 1). Transfection with Snail shRNA prevented HMGB1-induced EMT (Fig. 1D - Fig. 1J), suggesting that Snail may mediate HMGB1-induced EMT.

As a major transcription factor for EMT, Snail triggers metastasis in response to several oncogenic signaling pathways [35, 47]. Additionally, Snail plays a key role in inducing the glycolytic switch and suppressing mitochondrial respiration and COX activity [27]. Snail has been also shown to repress the expression of fructose-1,6-bisphosphatase (FBP1), a rate-limiting enzyme in gluconeogenesis, in basal-like breast cancer, and this metabolic reprogramming results in increased CSC-like properties and tumorigenicity by enhancing the interaction between β-catenin and T-cell factor [9]. Therefore, HMGB1 may contribute to glycolytic switch and mitochondrial repression through the regulation of Snail. We also found that HMGB1 induced the glycolytic switch and mitochondrial repression (Fig. 2). Snail shRNA prevented HMGB1-induced glycolytic switch and mitochondrial repression (Fig. 2). These results indicate that the Snail axis is closely linked with the HMGB1-induced glycolytic switch and mitochondrial repression, thereby contributing to HMGB1-mediated tumor progression. It was found that ATP levels in cells treated with 1 μg/ml HMGB1 were similar to those in control cells (Fig. 2); however, ATP levels were increased in MCF-7 cells treated with 10 μg/ml HMGB1 (data not shown). Recently, it was reported that HMGB1 (10 μg/ml) increases ATP production in human and murine pancreatic tumor cell lines in a time- and dose-dependent manner and enhances mitochondrial complex I activity through the mitochondria-targeted accumulation of RAGE [20]. Thus, although high concentrations of HMGB1 increased ATP levels, low levels of HMGB1 induced the glycolytic switch without affecting total ATP production. HMGB1 increased the ratio of ATP produced by glycolysis versus aerobic respiration (Fig. 2). The precise mechanism for the dose-dependent differential effect of HMGB1 on ATP production is unknown and requires further investigation.

Treatment with Snail shRNA prevented HMGB1-induced COX inhibition (Fig. 3A), indicating that HMGB1 induces COX inhibition through the Snail axis. Snail has been shown to suppress the expression of COXVIc, COXVIIa, and COXVIIc, and thereby inhibit mitochondrial respiration and COX activity [27]. HMGB1 also suppressed the expression of COXIV, COXVb, COXVIIa, COXVIIc, COX11, and SCO1 (Table 5). Transfection with Snail shRNA attenuated the HMGB1-induced downregulation of COXVIIa and COXVIIc, but not that of COXIV, COXVb, COX11, or SCO1 (Fig. 3B, Table 5).

Thus, HMGB1-induced mitochondrial repression and COX inhibition are likely to be mediated by COXVIIa and COXVIIc inhibition via Snail activation. Our results suggested that HMGB1 might promote tumor progression by enhancing the Snail-mediated glycolytic switch, mitochondrial repression, and COX inhibition.

Oncogenic metabolism (including glycolytic switch) has been shown to support cell proliferation and growth [3, 6,

8]; however, recent findings suggest that oncogenic metabolism is required for tumor progression (including EMT and metastasis) [25, 28, 33]. We previously reported that glutaminase and glutamine metabolism play an important role in Dlx-2-, TGF-β-, Wnt-, and Snail-induced EMT [25], indicating that metabolic alterations in cancer cells are closely associated with EMT. This suggests that the glycolytic switch may be involved in HMGB1-induced EMT. We found that HMGB1 induced the glycolytic switch and regulated the expression of several key glycolytic enzymes, including HK2, PFKFB2, PGAM1, LDHA, LDHB, GLUT1, MCT1 and MCT4 (Fig. 2, Table 6). It was previously reported that the expression of the key enzymes involved in glycolytic metabolism was remarkably increased in pancreatic tumor cells compared with that in normal pancreatic cells, leading to a significant induction of aerobic glycolytic metabolism [42]. We showed that HMGB1 upregulated HK2, PFKFB2, PGAM1, LDHB and MCT1 expression and downregulated LDHA, GLUT1, and MCT4 expression. It had no effects on PKM2 expression (Table 6). Among these genes regulated by HMGB1, only the expression of HK2, PFKFB2, and PGAM1 was induced by Snail (Table 6).

HK2 is a first step enzyme in glycolysis and converts glucose to glucose-6-phosphate [15]. PFKFB2 synthesizes and degrades fructose-2,6-bisphosphate (F2,6BP), which is one of the most potent allosteric activators of 6-phosphofructo-1-kinase (PFK1; also known as PFKM), a rate-limiting enzyme in glycolysis. PFKFB2 controls a key regulatory step in glycolysis [15]. PGAM1 converts 3-PG to 2-PG [15]. HK2, PFKFB2, and PGAM1 are highly expressed in various cancer types and play critical roles in tumor progression [15]. Previously, Snail has been shown to induce PFKFB2 expression. Furthermore, we showed that PFKFB2 contributes to Wnt/Snail-induced EMT and metastasis [29]. We also showed that HK2, PFKFB2, and PGAM1 expression was upregulated by HMGB1 treatment in a Snail-dependent manner (Table 6). Treatment with shRNAs targeted towards HK2, PFKFB2, and PGAM1 attenuated HMGB1-induced EMT and Snail expression, suggesting that HK2/PFKFB2/PGAM1 may be involved in HMGB1/Snail-induced EMT (Fig. 4).

MCF-7 cells represent the luminal A subtype of breast cancer, which is known to be non-invasive, highly differentiated, and positive for estrogen receptor and progesterone receptor, whereas MDA-MB231 cells represent basal-like subtype, which is associated with poor prognosis and is difficult to treat [1, 37, 43]. MDA-MB231 cells also have higher glycolytic activity and a lower respiration activity than those of MCF-7 cells. Moreover, Snail levels are low in MCF-7 cells, but high in MDA-MB231 cells [36, 49]. We recently showed that PFKFB2 expression is regulated by Snail levels in MCF-7 and MDA-MB231 cells. Snail also binds to the PFKFB2 promoter [29]. Thus, we further compared the Snail-mediated effect on HK2 and PGAM1 expression in MCF-7 and MDA-MB231 cells. HK2 and PGAM1 expression were increased in Snail-overexpressing MCF-7 cells (Fig. 4C, Table 6). HK2 and PGAM1 expression were also increased in MDA-MB231 cells which exhibit elevated levels of Snail (Fig. 4C). Treatment with Snail shRNA suppressed HK2 and PGAM1 expression in MDA-MB231 cells (Fig. 4C). In addition, Snail bound to the HK2 and PGAM1 promoters in Snail-overexpressing MCF-7 cells (Fig. 4D). In MDA-MB231 cells, Snail bound to the HK2 and PGAM1 promoters, and treatment with Snail shRNA inhibited Snail binding to these promoters (Fig. 4D). These results indicate that Snail may induce the expression of not only PFKFB2 [29] but also HK2 and PGAM1. Thus, glycolysis is thought to be involved in HMGB1/Snail-induced EMT. Silencing HK2, PGAM1, and PFKFB2 by shRNA abrogates the effect of HMGB1 on EMT and the effects of these glycolytic enzymes are almost identical. In addition, the knockdown of glycolytic enzymes decreases HMGB1-induced Snail expression. These results suggest that the effect of Snail and the abovementioned glycolytic enzymes on each other are reciprocal. This study shows a reciprocal regulation between these molecules.

Therefore, we propose that all oncogenic metabolic enzymes are reciprocally regulated upon metabolic alterations in cancer cell's. Therefore, inhibiting any of these enzymes may lead to the suppression of the associated pathway, and thereby induce altered metabolism in tumors and, in turn, affect tumorigenesis.

Recently, the reciprocal interaction of cellular metabolism with epigenetic modifications was reported to affect tumorigenesis. Metabolic enzymes and metabolites regulate epigenetic modifications. Epigenetic changes in genes involved in metabolism also regulate their transcription [52]. PKM2 phosphorylates histone H3 on threonine 11 upon EGF receptor activation, which leads to increased H3K9 acetylation and induced MYC transcription. Acetyl-CoA, a metabolite of glycolysis, is required for histone acetylation [48].

These findings suggest that glycolytic enzymes, such as HK2, PGAM1, and PFKFB2, may regulate Snail expression through epigenetic modifications.

We previously found that inhibiting glutamine (Gln) metabolism leads to increased p53 expression, which decreases Snail mRNA stability by inducing the p53-dependent upregulation of Snail-targeting miRNAs [25]. This suggests that Gln metabolism stabilizes Snail mRNA by inhibiting the p53-dependent regulation of Snail-targeting miRNAs. Furthermore, this investigation demonstrates the possibility that glycolysis and glycolytic enzymes, such as HK2, PGAM1, and PFKFB2, may regulate the p53-dependent modulation of Snail-targeting miRNAs to affect Snail expression .

The mechanism of reciprocal regulation between Snail and glycolytic enzymes requires further investigation.

Taken together, our results suggest that the HMGB1/Snail axis plays a crucial role in EMT and the glycolytic switch by regulating the expression of key glycolytic enzymes, including HK2, PFKFB2, and PGAM1. Furthermore, the glycolytic switch closely associates with EMT to promote tumor progression. The serum HMGB1 level has been shown to increase in patients with invasion and lymph node metastasis in lung, cervical, gastric, and hepatocellular cancers compared to healthy controls; thus, HMGB1 appears to be a useful serological biomarker for early cancer diagnosis as well as for evaluating tumorigenesis, stage, and prognosis of cancer [21, 24].

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초록 : HMGB1/Snail cascade에 의한 epithelial-mesenchymal transition 및 glycolytic switch, mitochondrial repression 유도

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암세포는 epithelial mesenchymal transition (EMT)를 통해 tumor invasion과 metastasis가 일어나며, 또한 정상세포와 다른 oncogenic metabolic phenotypes 획득 즉, glycolytic switch 등이 암 발생과 진행에 깊이 연관되어 있음이 잘 알려져 있다. High-mobility group box 1 (HMGB1)은 chromatin-associated nuclear protein으로 알려져 있으나, dying cells 또는 immune cells로부터 방출되기도 한다. 방출된 HMGB1은 damage-associated molecular pattern (DAMP)로서 작용하여 EMT 및 invasion, metastasis를 유도함으로서 tumor progression에 기여한다고 알려졌다. 본 연구에서 HMGB1에 의해 EMT와 glycolytic switch 유도되며, 이 과정은 Snail 의존적임을 확인하였다. 또한 HMGB1/Snail cascade는 COX subunits인 COXVIIa와 COXVIIc의 발현 억제를 통해 mitochondrial repression과 cytochrome c oxidase (COX) inhibition을 유도하였다. HMGB1은 Snail를 통해 glycolytic switch의 주요 효소인 hexokinase 2 (HK2), phosphofructokinase-2/fructose-2,6-bisphosphatase 2 (PFKFB2), phosphoglycerate mutase 1 (PGAM1)의 발현을 증가시켰다. 이들 효소는 glycolytic switch에 중요하게 관여하는 것으로 알려져 있다. 이들 해당과정의 효소들을 knockdown한 결과 HMGB1에 의한 EMT를 억제함으로써 glycolysis와 HMGB1-induced EMT가 밀접하게 연관되어 있을 제시하였다. 이상의 연구 결과들은 HMGB1/Snail cascade가 EMT 및 glycolytic switch, mitochondrial repression에 중요하게 작용할 것임을 시사한다.