Hyperactivity of the transcription factor Nrf2 causes metabolic reprogramming in mouse esophagus

Mutations in the genes encoding nuclear factor (erythroid-derived 2)-like 2 (NRF2), Kelch-like ECH-associated protein 1 (KEAP1), and cullin 3 (CUL3) are commonly observed in human esophageal squamous cell carcinoma (ESCC) and result in activation of the NRF2 signaling pathway. Moreover, hyperactivity of the transcription factor NRF2 has been found to cause esophageal hyperproliferation and hyperkeratosis in mice. However, the underlying mechanism is unclear. In this study, we aimed to understand the molecular mechanisms of esophageal hyperproliferation in mice due to hyperactive NRF2.


diseases were obtained from genetically modified mice that differed in the status of the Nrf2 gene and genes in the same pathway (Nrf2−/−, Keap1−/−, K5Cre;Pkm2flo/flo;Keap1−/−, and WT) and analyzed for metabolic profiles, Nrf2 ChIP-seq, and gene expression. We found that hyperactive NRF2 causes metabolic reprogramming and up-regulation of metabolic genes in the mouse esophagus. One of the glycolysis genes encoding pyruvate kinase M2 (Pkm2) was not only differentially up-regulated, but also glycosylated and oligomerized, resulting in increased ATP biosynthesis.

Furthermore, constitutive knockout of Pkm2 failed to inhibit this esophageal phenotype in vivo, and this failure may have been due to compensation by Pkm1 up-regulation. Transient inhibition of NRF2 or glycolysis inhibited the growth of human ESCC cells in which NRF2 is hyperactive in vitro. In summary, hyperactive NRF2 causes metabolic reprogramming in the mouse esophagus through its transcriptional regulation of metabolic genes. Blocking glycolysis transiently inhibits cell proliferation and may therefore have therapeutically beneficial effects on NRF2-high ESCC in humans.

Esophageal cancer affects 16,940 adults in the United States, and the 5-year survival rate is 18% (1). There are mainly two histological types of esophageal cancer, squamous cell carcinoma (ESCC) and adenocarcinoma, each having a distinct etiology. Low income, moderate/heavy alcohol intake, tobacco use, and infrequent consumption of raw fruits and vegetables account for almost all cases of ESCC (2). With the recent technological advances in NextGen sequencing, human ESCC samples from North and South America, China, Japan, Vietnam, and Malawi have been sequenced. Among many gene mutations, nuclear factor (erythroid-derived 2)-like 2 (NRF2 or NFE2L2) mutations are commonly seen with a frequency over 5%, even up to ~20% in certain reports. Mutations in other genes of the NRF2 signaling pathway, Kelch-like ECH-associated protein 1 (KEAP1) and cullin 3 (CUL3), are relatively less common. NRF2 mutations are mostly located in the DLG and ETGE motifs (KEAP1-binding domain) and the DNA-binding domain, whereas KEAP1 mutations tend to be scattered across the whole gene. NRF2 mutations and KEAP1 mutations tend to be mutually exclusive (3, 4).

As a major cellular defense mechanism, the NRF2 signaling pathway is known to regulate expression of enzymes involved in detoxification and anti-oxidative stress response. NRF2 forms heterodimers with small MAF proteins and binds to the anti-oxidant-response elements of target genes when cells are exposed to oxidative stress.
exposed to oxidative stress or xenobiotics. KEAP1 inhibits the function of NRF2 by retaining NRF2 in the cytoplasm under normal physiological conditions and by allowing nuclear translocation of NRF2 under stress conditions (5). In fact, NRF2 and KEAP1 have been classified among 291 high-confidence cancer driver genes (6). NRF2 signaling pathway has long been recognized as a double-edged sword in carcinogenesis (7, 8). On the one hand, chemical or genetic activation of NRF2 induced cytoprotective enzymes and thus provided protection against chemical carcinogenesis in multiple models (9). Nrf2<sup>-/-</sup> mice were more susceptible to chemical carcinogenesis than WT counterparts (10, 11). On the other hand, cancer cells can hijack the NRF2 signaling pathway for their survival through mechanisms that lead to constitutive activation of NRF2 signaling, such as somatic mutations of KEAP1/NRF2/CUL3, accumulation of disruptor proteins, skipping of NRF2 exon 2, KEAP1 succinylation, KEAP1 hypermethylation, increased NRF2 expression, and electrophilic attack by oncometabolites. As a consequence, NRF2 hyperactivation promoted cell proliferation, conferred radiochemoresistance to cancer cells, promoted metabolic reprogramming, and accelerated distant metastases (12–16). Therefore, NRF2 signaling has been regarded as a molecular target for cancer therapy (17).

The important role of the NRF2 signaling pathway in the esophagus was first revealed in a mouse study. Genetic activation of Nrf2 in Keap1<sup>-/-</sup> mice resulted in esophageal hyperproliferation and hyperkeratosis (18). To our best knowledge, all transcriptionally impacted genes downstream of Keap1 knockout are Nrf2-responsive, although it is still possible that these KEAP1 substrates may still impact NRF2-independent transcription. Among the KEAP1 substrates other than NRF2 (19–26), WTX, PALB2, SQSTM1/P62, DPP3, and CDK20 proteins bind KEAP1 via an “ETGE” motif to displace NRF2, thus inhib-

**Figure 1. Hyperactive NRF2 was associated with up-regulation of metabolic genes.** Fifty three cases of ESCC were clustered into two groups, NRF2<sup>high</sup> (n = 17) and NRF2<sup>low</sup> (n = 36), based on microarray data of esophagus-specific NRF2 target genes (GSE23400 dataset) (A). After two-class SAM analysis, the volcano graph showed that multiple metabolic genes were significantly up-regulated enriched in NRF2<sup>high</sup> ESCC compared with NRF2<sup>low</sup> ESCC (B).
Nrf2 ubiquitination and driving NRF2-dependent transcription. In fact, Keap1/H11002/H11002/H11002 mice died within 3 weeks, and Nrf2/H11002/H11002/H11002;Keap1/H11002/H11002/H11002 completely rescued the phenotype (18). K5Cre;Nrf2fl/fl;Keap1/H11002/H11002/H11002 mice did not develop any esophageal phenotype of Keap1/H11002/H11002/H11002 mice (27). We also found that Nrf2 target genes and gene sets associated with oxidoreductase activity, mitochondrial biogenesis, and energy metabolism were down-regulated in the Nrf2/H11002/H11002/H11002 esophagus. Consistent with these observations, ATP biogenesis and CoxIV (a mitochondrial marker) were also down-regulated (28). Previous ChIP-seq experiments have shown that NRF2 as a transcription factor regulates transcription of hundreds of genes in human lymphoblastoid cells (29), mouse macrophages (30), mouse embryonic fibroblasts (31), and mouse hepatoma cells (32).

Therefore, we hypothesized that hyperactive Nrf2 caused esophageal hyperproliferation and hyperkeratosis through transcriptional regulation of gene expression. Using genetically modified mice with various Nrf2 status (Nrf2/H11002/H11002/H11002, WT, and Keap1/H11002/H11002/H11002), here we aimed to understand how hyperactive Nrf2 causes the esophageal phenotype in mice.

Results

Hyperactive Nrf2 in human ESCC was associated with overexpression of metabolic genes

Using gene microarray data of 53 cases of human ESCC (33) (GEO23400), we performed clustering analysis with a list of esophagus-specific Nrf2 target genes (34), and we found two clusters, Nrf2/H11002/H11002/H11002 (n = 17) and Nrf2/H11002/H11002/H11002 (n = 36) (Fig. 1A). By comparing gene expression between these two groups, we found that a group of metabolic genes involved in glycolysis, pentose phosphate pathway (PPP), fatty acid metabolism, and GSH metabolism was overexpressed in Nrf2/H11002/H11002/H11002 ESCC (Fig. 1B; File S1). This observation suggested that Nrf2 regulates the expression of metabolic genes in the esophagus.

Hyperactive Nrf2 resulted in metabolic reprogramming in mouse esophagus

833 metabolites on 93 subpathways were analyzed by a biochemical profiling platform. With p < 0.01 as the cutoff, 10 subpathways were significantly different between WT esophagus and Nrf2/H11002/H11002/H11002 esophagus (Fig. 2A), and 47 subpathways between WT esophagus and Keap1/H11002/H11002/H11002 esophagus (Fig. 2B). Among these subpathways, GSH metabolism and γ-glutamyl amino acid are known to be subject to Nrf2 regulation. Interestingly, multiple subpathways of carbohydrate metabolism and fatty acid metabolism were identified, for example, PPP, TCA cycle, and glycogen metabolism in the Keap1/H11002/H11002/H11002 esophagus (File S2 and S3). Heatmaps of glycolysis metabolites, TCA cycle metabolites, PPP metabolites, pentose metabolites, and nucleotide-sugar and amino-sugar metabolism showed an overall tendency of enhanced metabolism in Keap1/H11002/H11002/H11002 esophagus as compared with WT esophagus. Enhancement of GSH
Hyperactive Nrf2 in the esophagus

Figure 3. Differential expression of energy metabolism genes in Keap1<sup>−/−</sup> esophagus. GSA analysis showed enrichment of four metabolic pathways in addition to classical Nrf2 pathways in Keap1<sup>−/−</sup> esophagus (n = 3) as compared with Keap1<sup>−/−</sup> esophagus (n = 3) (A). Many metabolic genes were up-regulated as shown on the volcano graph (thresholds set at p < 0.05 and fold change > 2) after two-class SAM analysis. p values were determined using two-way ANOVA analysis (B). Differential expression of two canonical Nrf2 target genes in Keap1<sup>−/−</sup> esophagus was validated by Western blotting (C) and IHC (D).

metabolism in Keap1<sup>−/−</sup> esophagus suggested a good quality of our metabolomics data (Fig. 2C).

In addition, significant changes in heme metabolism were consistent with the known anti-oxidative function of Nrf2. Significant changes in lipid biosynthesis, β-oxidation, eicosanoids and endocannabinoids, and nucleotide biosynthesis in Keap1<sup>−/−</sup> esophagus were consistent with the phenotype of esophageal hyperproliferation (File S3). These data demonstrated that hyperactive Nrf2 resulted in metabolic reprogramming in Keap1<sup>−/−</sup> esophagus.

**Hyperactive Nrf2 up-regulated metabolic genes in mouse esophagus**

Gene microarray data from our previous experiment using Keap1<sup>−/−</sup> and Nrf2<sup>−/−</sup>;Keap1<sup>−/−</sup> esophagus at P7 were further analyzed for differential gene expression (34). Gene set analysis showed enrichment of 41 gene sets in Keap1<sup>−/−</sup> esophagus among which four gene sets were metabolism pathways and nine were known Nrf2-associated pathways (Fig. 3A; File S4). Consistent with this observation, multiple metabolic genes involved in glycolysis (Hk1 and Pkm2), PPP (G6pd), fatty acid metabolism (Elov7, Acss2, and Acot4), and GSH metabolism (Gsts, Gclm, Gclc, xCT, Gss, and Gpx2), were up-regulated in Keap1<sup>−/−</sup> esophagus (Fig. 3B; File S5). Using two canonical Nrf2 target genes (xCT and Gclm), we confirmed overexpression of these genes in Keap1<sup>−/−</sup> esophagus by Western blotting (Fig. 3C) and IHC (Fig. 3D).

Using Nrf2 ChIP-seq, we identified 1940 genes as potential Nrf2 target genes in mouse esophagus (File S6). Among these genes, 10 participated in glycolysis, 4 in PPP, 3 in glycolenogen, 6 in fatty acid metabolism, 3 in glutaminolysis, 1 in NADPH synthesis, and 21 in GSH metabolism (Table 1). Using IHC and Western blotting, we confirmed overexpression of glycolysis genes (Hk1, Gpi1, Pfkfb2, Aldoa, Enol1, and Enol4), PPP genes (G6pd, Pgd, Tkt, and Tald1), and NADPH synthesis genes (Idh1 and Me2) in Keap1<sup>−/−</sup> esophagus as compared with WT esophagus (Fig. 4, A and B). Consistent with differential expression of individual genes, gene set analysis also showed enrichment of many metabolic gene sets, such as glycolysis and PPP (File S7; Fig. 5A). We further analyzed activities of the rate-limiting enzymes of glycolysis (HK and PK) and PPP (G6PD). Significantly higher activities of these enzymes were found in Keap1<sup>−/−</sup> esophagus as compared with WT esophagus (Fig. 5, B–D). All these data suggested that hyperactive Nrf2 caused metabolic reprogramming through transcriptional regulation of metabolic genes, and metabolic reprogramming may potentially play a critical role in the esophageal phenotype (hyperproliferation and hyperkeratosis) of Keap1<sup>−/−</sup> esophagus.

**Nrf2 regulated the expression and function of Pkm2 in mouse esophagus**

Because PK is a rate-limiting enzyme of glycolysis and Pkm was identified by Nrf2 ChIP-seq. ChIP-PCR was performed to validate binding of Nrf2 to the promoter of the mouse Pkm gene (Fig. 6A). The PKM gene is known to be alternatively spliced to generate transcripts encoding either PKM1 or PKM2, with PKM1 expressed in tissues with high catabolic demand such as...
muscle and PKM2 in most adult tissues and cancer. Using Western blotting, we confirmed overexpression of Pkm2, but not Pkm1, in Keap1−/− esophagus (Fig. 6B). IHC showed that Pkm2 was overexpressed in squamous epithelial cells, whereas Pkm1 was expressed in smooth muscle cells (Fig. 6C).

To explain why hyperactive Nrf2 selectively up-regulated Pkm2 expression, we examined the expression of hnRNPA1, hnRNPA2, RBM4, and PTB, which were known to regulate alternative splicing of the Pkm gene (35, 36). As expected, the Keap1−/− esophagus expressed higher levels of hnRNPA1, hnRNPA2, and PTB and a lower level of RBM4 than WT esophagus (Fig. 6D).

An overall increase in nucleotide-sugar and amino-sugar metabolism in Keap1−/− esophagus suggested that glycosylation may be enhanced by hyperactive Nrf2. Indeed, there was an increase in protein glycosylation in the Keap1−/− esophagus. Pkm2 glycosylation was also enhanced as detected by Pkm2 Western blotting after immunoprecipitation with anti-O-GlcNAc (Fig. 6E). Pkm2 exists in a mixture of monomer, dimer, and tetramer with the tetramer being the most enzymatically active form. Although all three forms of Pkm2 were increased in Keap1−/− esophagus, a dramatic increase of tetrameric Pkm2 suggested an increase of glycolysis and subsequent increase in ATP production in the mitochondria (Fig. 6F). As expected, ATP biosynthesis was increased as well (Fig. 6G).

**Pkm2 knockout failed to inhibit esophageal phenotype in Keap1−/− mice**

To further test the role of Pkm2 in hyperactive Nrf2-mediated esophageal phenotype in Keap1−/− esophagus, we generated K5Cre;Pkm2fl/fl;Keap1−/− mice to constitutively knock out Pkm2 in esophageal squamous epithelial cells in vivo. To our surprise, Pkm2 knockout did not significantly modify PCNA expression, BrdU incorporation, and the expression of squamous differentiation markers (Krt5, Krt1, and loricrin) (Fig. 7A). Instead, Pkm1 was overexpressed in the esophageal squamous epithelial cells of K5Cre;Pkm2fl/fl;Keap1−/− esophagus as a result of Pkm2 knockout.

**Nrf2 knockdown, Pkm2 knockdown, and glycolysis inhibitors suppressed the proliferation of NRF2high human ESCC cells in vitro**

We next sought to determine the transient effects of NRF2 or PKM2 inhibition on the proliferation of esophageal squamous epithelial cells. Human ESCC cells, KYSE510 and KYSE450, both express a low level of NRF2 (NRF2low cells), and KYSE70 expresses a high level of NRF2 due to a point mutation of the NRF2 gene (NRF2high cells) (37–39). KYSE450 cells (NRF2low) and KYSE70 cells (NRF2high) were transfected with siRNAs and/or treated with glycolysis inhibitors, 2DG (HK inhibitor) (40) and shikonin (PKM2 inhibitor) (41). NRF2low cells were treated with KEAP1 siRNA to activate NRF2. Transfection effi-

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**Table 1**

| Pathway | Gene | Full Description | Distance to Start | Position | Wide-type | Keap1−/− |
|---------|------|------------------|------------------|----------|-----------|-----------|
| Glycolysis | Glut1 | glucose transporter 1 | -2940 | upstream, in gene | 3.50 | 21.00 |
|         | HK1  | hexokinase 1     | -1000 | in gene, in gene | 4.33 | 27.00 |
|         | HK2  | hexokinase 2     | -56070 | upstream, downstream | 1.40 | 10.00 |
|         | G6P  | glucose phosphate isomerase 1 | -3711 | upstream, upstream | 4.77 | 16.00 |
|         | G6P2 | 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2 | -13013, 12197, 11621 | in gene, in gene | 5.40 | 16.00 |
|         | PKM2 | aldolase A, fructose-bisphosphate | -17594 | in gene, in gene | 10.10 | 28.50 |
|         | PKM3 | 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4 | 1580, 831 | in gene, in gene | 6.00 | 9.00 |
|         | Pkm1 | enolase 1, alpha non-neuron | 2351 | upstream, downstream | 3.00 | 14.00 |
|         | Pkm2 | enolase 4 | 3551 | upstream, in gene | 5.50 | 24.50 |
| PPP pathway | Tt | transketolase | -2357 | in gene, in gene | 5.00 | 8.00 |
|         | Tm | transaldolase | -109, -4205 | upstream, upstream | 5.70 | 32.50 |
|         | Td | triokinase | -467, 3817 | in gene, in gene | 5.00 | 27.50 |
|         | Tl | transaldolase 1 | 896 | in gene | 8.00 | 24.00 |

**Glycogenolysis**

- **Glycogen** | Phosphoglucomutase 5 | -175282 |
- **Glu** | Glucose-6-phosphate dehydrogenase | -583, 1697 |
- **Gnu** | Glucose-6-phosphate dehydrogenase X-linked | -401 |

**Fatty acid metabolism**

- Esr1 | ELAVL1, family member 1 | 14900 |
- Ac | fatty acid desaturase 1 | -9256 |
- Aco2 | acyl-CoA synthetase chain-short family member 2 | 50021 |
- Aco7 | acyl-CoA thioesterase 7 | 12348, 14692, 8938 |
- Aco12 | acyl-CoA dehydrogenase family member, 10 | 41246 |
- Aco12 | acyl-CoA dehydrogenase family member, 12 | -326 |

**Glutaminolysis**

- X Caf1 | glutamine/glutamate neutral amino acid transporter, member 4 | -7401 |
- Glu2 | glutaminase 2 (liver, mitochondria) | -8891 |
- Gpu2 | glutaminase pyruvate transaminase (alanineaminotransferase) | -40295 |

**NADEPH synthesis**

| NADPH synthesis | Me | malic enzyme 1, NAD(P)+-dependent, cytosolic | 490 |
|                 | Glut1 | glutathione S-transferase, alpha 4 | -185 |
|                 | Glut4 | glutathione S-transferase, alpha 3 | -175 |
|                 | Glut1 | glutathione S-transferase a (malealnuclease) | 131 |
|                 | Gsp1 | glutathione S-transferase, p 1 | -8223, 8, -8209 |
|                 | Gsp2 | glutathione S-transferase, p2 | -3923, 4301, -3891 |
|                 | Gom2 | glutathione S-transferase omega 2 | 19191 |
|                 | Mgt1 | microsomal glutathione S-transferase 2 | 9687 |
|                 | Gom7 | glutathione S-transferase, mu 7 | -7953 |
|                 | Gom8 | glutathione S-transferase, mu 8 | 4066 |
|                 | Gom9 | glutathione S-transferase, mu 9 | 4230 |
|                 | Gom2 | glutathione S-transferase, mu 2 | 271 |
|                 | Gom1 | glutathione S-transferase, mu 1 | 4469, 4556 |
|                 | Gcm | glutamylcysteine ligase, modifier subunit | -9112 |
|                 | Gcl | glutamylcysteine ligase, catalytic subunit | -3805, -3493, -3911 |
|                 | xCaf1 | glutathione S-transferase 7 (cystein amino acid transporter, y system), member 11 | 6941, 5373, -83 |
|                 | Gsp2 | glutathione S-transferase 2 | -26462, 7170, 1698, 130 |
|                 | Gsp4 | glutathione S-transferase 4 | -36 |
|                 | Gsp4 | glutathione S-transferase 4 | -350 |
|                 | Gsp4 | glutathione S-transferase 4 | 122, 714 |
|                 | Sol | superoxide dismutase 1, soluble | -38, 634 |
|                 | Org | catalase | 4737 |

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**Pkm2 knockout failed to inhibit esophageal phenotype in Keap1−/− mice**

**Nrf2 knockdown, Pkm2 knockdown, and glycolysis inhibitors suppressed the proliferation of NRF2high human ESCC cells in vitro**

We next sought to determine the transient effects of NRF2 or PKM2 inhibition on the proliferation of esophageal squamous epithelial cells. Human ESCC cells, KYSE510 and KYSE450, both express a low level of NRF2 (NRF2low cells), and KYSE70 expresses a high level of NRF2 due to a point mutation of the NRF2 gene (NRF2high cells) (37–39). KYSE450 cells (NRF2low) and KYSE70 cells (NRF2high) were transfected with siRNAs and/or treated with glycolysis inhibitors, 2DG (HK inhibitor) (40) and shikonin (PKM2 inhibitor) (41). NRF2low cells were treated with KEAP1 siRNA to activate NRF2. Transfection effi-
ciency was determined by Western blotting (Fig. 8, A and E), cell proliferation by colony formation assay (Fig. 8, B, F, D, and H), and ATP biosynthesis (Fig. 8, C and G). Nrf2 knockdown, PKM2 knockdown, and glycolysis inhibitors suppressed cell proliferation and ATP biosynthesis in both cells in vitro. Similar results were found in KYSE510 cells (data not shown). Consistent with the in vivo data, PKM2 siRNA knockdown resulted in up-regulation of PKM1 (Fig. 8A, lane 3 versus lane 1, lane 5 versus lane 2; and E, lane 2 versus lane 1).

Discussion

This study clearly demonstrated that hyperactive Nrf2 causes metabolic reprogramming through transcriptional regulation of metabolic genes in mouse esophagus. To our knowledge, it is the first study to demonstrate that hyperactive Nrf2 differentially up-regulated the Pkm2 isoform in the esophageal squamous epithelial cells in vivo. However, constitutive Pkm2 knockout in the mouse esophagus failed to inhibit the esophageal phenotype in Keap1−/− mice. Nrf2 signaling pathway has been reported to regulate energy metabolism. In A549 cells (a lung cancer cell line with hyperactive Nrf2), Nrf2 was found to regulate the expression of metabolic genes involved in PPP, nucleotide synthesis, and NADPH synthesis. Metabolic pathways such as glycolysis, PPP, glutaminolysis were subject to Nrf2 regulation as well (42). Similar results were observed in mouse forestomach when Nrf2 became hyperactive due to Keap1 knockout (42). In a transgenic mouse line with constitutively active Nrf2, Nrf2 activation in mouse skin was found to cause significant alterations in metabolic pathways of GSH, purine, and amino acid, PPP, citric acid cycle, and glycolysis (43). Nrf2 activation by phosphomimetic P62 up-regulated metabolic genes and thus enhanced GSH production and several metabolic pathways in human liver cells (44). In addition, Nrf2 also regulated serine biosynthesis (45) and lipid metabolism (46). However, careful examination of the metabolomics data of ours and others showed that metabolic pathways regulated by hyperactive Nrf2 depended on the cellular or tissue context (44, 47). For example, a Keap1 knockout in skeletal muscle cells in vivo up-regulated metabolic genes of glycogenolysis (Gbe1 and Phka1). However, its regulation of glycogen metabolism was different in the liver potentially because of the different enzyme makeup in these tissues (47). Metabolism in cul-

Figure 4. Hyperactive Nrf2 up-regulated the expression of metabolic genes in mouse esophagus. IHC (A) and Western blotting (B) confirmed overexpression of glycolysis genes, PPP genes, and NADPH synthesis genes in Keap1−/− esophagus as compared with WT esophagus. Scale bar, 50 μm.
tured cells and live tissues responded to genetic or pharmaco-
logical manipulation of NRF2 signaling differently because of their differential nutrient use (48). Thus, there is a need to understand the role of NRF2 in metabolism in specific cellular or tissue context. Our data clearly showed that many metabolic pathways were impacted by hyperactive Nrf2 in mouse esophagus (Fig. 2). Consistent with metabolic changes, the expression of many metabolic genes was up-regulated (Fig. 4), and the activities of three enzymes (HK, PK, and G6PD) were enhanced (Fig. 5). In addition to the classical Warburg effect (aerobic glycolysis), hyperactive Nrf2, in fact, impacted oxidative phosphorylation in the esophagus as well, consistent with a current model of cancer metabolism (49). Our results support the notion that Nrf2 regulates mitochondrial fatty acid oxidation, respiration, and ATP biogenesis (50). In addition to its nonmetabolic functions, PKM2 has also been regarded as an important player in aerobic glycolysis and the growth of cancer cells (48, 55). Almost universal expression of PKM2 in rapidly proliferating cells suggests that PKM2 expression is permissive to the metabolic requirements of proliferation (56). In addition to its transcriptional regulation, PKM2 was also subject to regulation by O-GlcNAcylation and polymerization (57). In this study, hyperactive Nrf2 was found to up-regulate Pkm2 expression through transcriptional regulation and alternative splicing of Pkm gene in mouse esophagus (Fig. 6).
The net effect of PKM2 inhibition may depend on the cellular context. Fast-growing cells may rely more on PKM2 to regulate their PK activity to support anabolic metabolism. Replacing PKM2 with PKM1 results in loss of flexibility to tune PK activity that is critical for such fast-growing cells. Slow-growing cells may not have such a dependence (58). Our data showed that constitutive Pkm2 knockout had no effect on the esophageal phenotype in vivo. This may be explained by several possibilities. 1) Permanent Pkm2 knockout can be compensated by Pkm1 overexpression (Fig. 7A). In fact, Pkm1 overexpression has been observed in Pkm2 knockout cells or tissues in multiple studies in vitro (58, 59) and in vivo (60, 61). Pkm1 overexpression is due to transcriptional up-regulation, although the possibility of post-transcriptional regulation cannot be excluded. It has been shown in the literature that germline or conditional loss of Pkm2 in vivo not only reduced the mRNA levels of Pkm2 and total Pkm, but also increased the mRNA level of Pkm1 in multiple mouse tissues, bone marrow hematopoietic cells, mammary tumor cells, and sarcoma cells (2, 58, 60, 62, 63). Blocking glycolysis may be compensated by increased serine and glycine biosynthesis from both glucose and glutamine (64).

Two recent studies have shown that hyperactive NRF2 in cancer cells resulted in energy dependence on glutaminolysis and inhibition of glutaminase suppressed cancer cell growth (65, 66). Indeed, glutamate level was significantly higher in the Keap1/−/− esophagus than the WT esophagus. Consistent with this, glutaminase 2 was overexpressed in the Keap1/−/− esophagus as compared with the WT esophagus (n = 4/group) (3). Many factors other than oncogenes, for example tissue type (e.g. cell lineage and environment) and interactions with benign cells, contribute to the metabolic phenotype of cancers (48, 67).

Nevertheless, transient inhibition of PKM2 by siRNA knockdown and shikonin in vitro suppressed cell proliferation and ATP biosynthesis in human ESCC cells, albeit compensative overexpression of PKM1 (Fig. 8). These data suggest that in the clinical setting where transient inhibition is desirable, PKM2 inhibition remains a promising approach for therapy of NRF2high ESCC. To fully elucidate the functional role of PKM2 in NRF2high ESCC, we have developed a mouse line carrying a knockin floxed mutant Nrf2 allele, which allows conditional activation of Nrf2 signaling when crossed with an esophageal epithelium-specific Cre line. Strong esophageal phenotypes, hyperproliferation and hyperkeratinization, developed at 4

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5 C. Paiboonrungruan and X. Chen, unpublished data.
weeks after tamoxifen induction. (unpublished preliminary
data). This line will be crossed with
Pkm2fl/fl
mice to determine
whether conditional blockage of Pkm2 may inhibit the esoph-
ageal phenotype due to hyperactive Nrf2, at least in a short
term.

It should be noted that our study has one limitation. The
whole esophagus (epithelium and muscle) instead of the epithe-
ilium alone was used for metabolomic profiling, Western blot-
ting, and enzyme activity assays in this study. This is due to a
practical limitation that all Keap1/H11002/H11002 mice die within 3 weeks
after birth (18). We harvested mouse esophagus from P7 mice
when mice are healthy yet the esophagus is tiny. At this age, it is
impractical to harvest enough epithelium from
Keap1/H11002/H11002 mice.

To overcome this limitation, in addition to experiments using
the whole esophagus, we used immunohistochemistry to vali-
date differential expression of metabolic genes in esophageal
squamous epithelial cells in vivo (Figs. 3, 4, and 6).

In summary, we characterized metabolic reprogramming
and differential gene expression in mouse esophagus due to
hyperactive Nrf2. Our data pointed out metabolic pathways (e.g. glycolysis and oxidative phosphorylation) and genes (e.g. Pkm2) as downstream targets. Further studies are warranted to find out how to inhibit the esophageal phenotype in the
NRF2high esophagus and translate the discovery for targeted
therapy of NRF2high ESCC.

Experimental procedures
Animal experiment
Animal experiments were approved by the Institutional Ani-
mal Care and Use Committees at the North Carolina Central
University (protocol number XC-12-03-2008). Animals in
these experiments were bred and maintained at the local animal
facility according to local legislation. WT C57BL/6J mice were
purchased from the The Jackson Laboratory (Bar Harbor, ME).
Nrf2−/− and Keap1−/− mice on the C57BL background were
obtained from the Experimental Animal Division, RIKEN Bio-
Source Center (Tsukuba, Japan) (18). K5Cre mice (68), Pkm2fl/fl
mice (60) (The Jackson Laboratory), and Keap1−/− mice were
crossed to generate K5Cre;Pkm2fl/fl;Keap1−/− mice. Mouse
esophagus at P7 was harvested for analysis of metabolomics,
Nrf2 ChIP-seq, morphology, and gene expression. BrdU (50
mg/kg, i.p.) was given to some mice 2 h before sacrifice.

Metabolic profiling
Three groups of mouse esophageal tissue samples, six of each
genotype (Nrf2−/−, WT, and Keap1−/−), were analyzed by a
biochemical profiling platform (Metabolon Inc, Durham, NC) with ultrahigh performance LC-tandem MS (File S2 and S3). Keap1/H11002 esophagus was much smaller than others and two esophagi were pooled together to make one sample for the analysis. Raw data were extracted, peak-identified and QC processed by Metabolon. Refined data were analyzed by two-way ANOVA with SPSS and plotted with GraphPad. Heatmap was generated using R (gplots package).

Histochemical staining and IHC

Hematoxylin and eosin staining was conducted based on a routine protocol. For IHC staining, deparaffinized sections were pre-treated to retrieve antigens using a Tris-based Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA), prior to incubation with an antibody overnight at 4 °C. Detection of the antibody complex was done using the streptavidin-peroxidase reaction kit with 3,3-diaminobenzidine as a chromogen (ABC kit, Vector Labs) (Table 2). BrdU-labeling index was calculated by dividing the number of BrdU+ epithelial cells with the total number of epithelial cells in the esophagus. Three cross-sections of each mouse esophagus were stained for BrdU and counted.

Western blotting

Protein was isolated from mouse tissues using a standard method. In brief, tissue lysates were prepared by homogenizing tissue with the TissueLyser bead mill (Qiagen, Valencia, CA) in RIPA buffer. Cell debris was removed by a short centrifugation, and an aliquot of cleared lysate was kept for protein quantitation using the BCA protein assay kit (Bio-Rad). Protein samples were separated by SDS-PAGE, and transferred to a nitrocellulose membrane (Millipore, Billerica, MA). After blocking with 5% nonfat dry milk, the membrane was probed with an antibody at 4 °C overnight (Table 2). Immunoreactivity was visualized by applying HRP ECL substrate and immediately exposing the membranes to film. The blots were stripped and re-probed for GAPDH.

ChIP-seq and ChIP-PCR

Nrf2 ChIP-seq was conducted by Active Motif (Carlsbad, CA) using a ChIP-seq validated antibody (sc-13032, Santa Cruz Biotechnology) and three mouse esophageal tissue samples, one of each genotype (Nrf2<sup>−/−</sup>, WT, and Keap1<sup>−/−</sup>) (GSE122504). The quality of ChIP DNA was controlled by quantitative PCR of two positive control genes (Nqo1 and Txnr6l1) and a negative control (Cnt1). Enriched genes in Keap1<sup>−/−</sup> esophagus as compared with Nrf2<sup>−/−</sup> and WT samples were regarded as potential Nrf2 target genes. This gene list was further analyzed for gene sets using an on-line tool (http://cpdb.molgen.mpg.de)⁶ (70, 71).

Figure 8. Transient inhibition of glycolysis suppressed colony formation and ATP biosynthesis of ESCC cells in vitro. KYSE450 cells (NRF2<sup>low</sup>) and KYSE70 cells (NRF2<sup>high</sup>) were transfected with siRNA or treated with glycolysis inhibitors, 2DG (HK inhibitor) and shikonin (PKM2 inhibitor). Transfection efficiency and PKM1/GAPDH were determined by Western blotting (A and E), cell proliferation by colony formation assay (B, F, D, and H), and ATP biosynthesis by a bioluminescent assay (C and G) (n = 3). *, p < 0.05; **, p < 0.01; ***, p < 0.001. P values were determined using Student’s t-test. ⁶ Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.
Nrf2-binding sites within the DNA sequence of mouse Pkm gene (ENSMUSG00000032294) were predicted using Anchored Combination TFBS Cluster Analysis (oPOSSUM version 3.0) (69). ChIP analysis was performed using an EZ-ChIP kit (Millipore, Billerica, MA) according to the manufacturer’s instructions with the anti-Nrf2 used for ChIP-seq. Immunoprecipitated DNA or input was PCR-amplified with the following primer pairs:

- Pkm (5'-H11032-CTT-CCTATCCGTAGTTTTCCAT-3' and 5'-H11032-CAGAATAATTC-TGTGATCCGTGAGA-3'; predicted size 177 bp);
- Negative control (5'-H11032-GGTATGATGACAACCCTTTACG-3' and 5'-H11032-AAA-CTCAGTCGCTGGACCAAGAC-3'; predicted size 155 bp).

**Analysis of gene microarray data**

Microarray data of two GEO datasets, GSE34278 (Nrf2+/−; Keap1+/− mouse esophagus versus Keap1+/− mouse esophagus) and GSE23400 (53 cases of human ESCC), were analyzed. Differentially expressed genes were obtained from two-class significance analysis of microarrays (SAM) in Excel with the median number of false positives less than 1 (34). GSA was carried out using R (GSA package). Mouse data were obtained from our previous study on mouse esophagus at P7 (34). Hierarchical clustering analysis was performed using the R package. Volcano graph was generated with Origin software (VolcanoPlot package, OriginLab, Northampton, MA).

**Enzymatic activity and ATP**

HK activity (MAK091, Sigma), PK activity (MAK072, Sigma), G6PD activity (12581, Cell Signaling), and ATP (MAK190, Sigma) were analyzed according to the manufacturer’s instructions of the kits.

**Cell culture and treatment**

Human ESCC cells, KYSE510 (NRF2low), KYSE450 (NRF2low), and KYSE70 (NRF2high), were obtained from the ATCC (Manassas, VA) and ECACC (Porton Down, Salisbury, UK). NRF2 hyperactivation in KYSE70 cells was due to a homozygous point mutation of the NRF2 gene (W24C) (37–39). KYSE510, KYSE450, and KYSE70 cells were exposed to 2DG (Sigma), shikonin (Sigma), or transfected with siRNAs (ThermoFisher Scientific, Waltham, MA) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. All cell culture experiments were triplicated.

**Statistical analyses**

GraphPad Prism 6 (GraphPad Software, La Jolla, CA) was used for Student’s t test. SPSS was used for two-way ANOVA analysis. \( p < 0.05 \) was considered statistically significant.
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