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Research Article

NRF2/ACSS2 axis mediates the metabolic effect of alcohol drinking on esophageal squamous cell carcinoma

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Alcohol drinking is a leading risk factor for the development of esophageal squamous cell carcinoma (ESCC). However, the molecular mechanisms of alcohol-associated ESCC remain poorly understood. One of the most commonly mutated genes in ESCC is nuclear factor erythroid 2 like 2 (*NFE2L2* or *NRF2*), which is a critical transcription factor regulating oxidative stress response and drug detoxification. When NRF2 is hyperactive in cancer cells, however, it leads to metabolic reprogramming, cell proliferation, chemoradioresistance, and poor prognosis. In this study, hyperactive NRF2 was found to up-regulate acetyl-CoA synthetase short-chain family members 2 (ACSS2), an enzyme that converts acetate to acetyl-CoA, in ESCC cells and mouse esophagus. We also showed that knockdown of *NRF2 or ACSS2* led to decreased ACSS2 expression, which in turn reduced the levels of acetyl-CoA and ATP with or without ethanol exposure. In addition, ethanol exposure enhanced lipid synthesis in ESCC cells. Moreover, we observed a change in the metabolic profile of ESCC cells exposed to ethanol as a result of their NRF2 or ACSS2 status. We further showed that ACSS2 contributed to the invasive capability of NRF2^{high} ESCC cells exposed to ethanol. In conclusion, the NRF2/ACSS2 axis mediates the metabolic effect of alcohol drinking on ESCC.

Introduction

Esophageal squamous cell carcinoma (ESCC) is the 6th most common cancer worldwide [1]. Globally, the highest incidences of ESCC are found in the African esophageal cancer corridor (Eastern and Southern Africa) and the Asian esophageal cancer belt (East and Central Asia) [1,2]. Alcohol drinking is an established risk factor for ESCC [3–6]. The risk of ESCC development is strongly associated with an individual's daily average alcohol consumption, thus, individuals who drink ≥ 70 g/day have the highest risk of developing ESCC [7–10]. Current molecular mechanisms of alcohol-associated ESCC focus primarily on the effects of acetaldehyde. Ethanol is metabolized to acetaldehyde by alcohol dehydrogenase (ADH), cytochrome P450 2E1, and, to a much lesser extent by catalase, and is further oxidized from acetaldehyde to acetate by acetaldehyde dehydrogenase 2 [11]. Acetaldehyde is a highly reactive compound that causes various damages to DNA [12–16] and proteins [17,18].

Recently, studies on human ESCC samples from China, Japan, and Malawi with next-generation sequencing have identified driver mutations of ESCC [19–22]. One of the most common mutated genes is nuclear factor (erythroid-derived 2)-like 2 (*NFE2L2* or *NRF2*), which is a transcription factor regulating oxidative stress response and drug detoxification genes [23–28]. Under normal conditions, NRF2 is bound to Kelch-like ECH-associated protein 1 (KEAP1) dimers, for ubiquitinylation by the

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CUL3 E3 ubiquitin-protein ligase, and subsequent proteasomal degradation. In the normal esophagus, NRF2 interacts with the Wnt pathway, Pparβ/δ, and PI3K/Akt pathway, all of which are involved in the development of esophageal epithelium [29]. Moreover, NRF2 is important in normal squamous differentiation of basal progenitor cells [30], and maintaining esophageal barrier function through its regulation of energy-dependent tight junction [31]. As a result, NRF2 is involved in the pathogenesis of eosinophilic esophagitis and gastroesophageal reflux disease [30,31].

Somatic mutations of *NRF2*, *KEAP1*, or *CUL3* result in the constitutive transcription of NRF2 cytoprotective genes in cancer cells, which leads to increased metabolism, proliferation, and chemoradioresistance in these cells [32,33]. *NRF2* is mutated in ∼10% of ESCC cases, while genes encoding its regulators *CUL3* and *KEAP1* are mutated in ∼3% and 4% of ESCC cases, respectively [34]. *KEAP1* and *CUL3* mutations may be found all over the length of the protein. *NRF2* mutations are mostly located in the KEAP1 binding domain at the N-terminus of the NRF2 protein, and therefore decrease the binding affinity of KEAP1 and subsequent degradation of NRF2 [35–37]. More recently, it has been reported that ESCC patients with high nuclear NRF2 expression have significantly poorer prognosis [38]. Through NRF2 ChIP-seq of mouse esophageal samples, we previously showed that hyperactive NRF2 bound to the promoter regions of many metabolic genes, one of which was acetyl-CoA synthetase short-chain family member 2 (*Acss2*). Moreover, microarray analysis of differential gene expression in *Keap1*$^{-/-}$ esophagus in comparison with *Nrf2*$^{-/-}$;*Keap1*$^{-/-}$ esophagus identified *Acss2* as one of the genes up-regulated due to NRF2 hyperactivation [39].

ACSS2 belongs to a family of acetyl-CoA synthetase short-chain enzymes involved in metabolizing acetate to acetyl-CoA [40–42]. ACSS1 and ACSS3 are located in the mitochondria, while ACSS2 is cytosolic and nuclear [42–46]. ACSS2 is critical for tumor metabolism in hypoxic and glucose-limited environments as cancer cells utilize acetate as a carbon source, leading to a metabolic switch from aerobic glycolysis to oxidative phosphorylation (OXPHOS) [40,41,45,47]. ACSS2 controls acetate's contribution to fatty acid synthesis and supports the biosynthesis of membrane phospholipids in breast cancer [47]. It helps cancer cells survive in a hypoxic environment through lipogenesis [45]. It also promotes the transcription of lipid synthesis and cell proliferation genes in breast cancer and hepatocellular carcinoma cells [40,48,49].

In this study, we showed that NRF2 regulated ACSS2 expression in esophageal squamous epithelial cells *in vitro* and *in vivo*. The NRF2/ACSS2 axis and ethanol exposure promoted OXPHOS and lipid synthesis, led to metabolic reprogramming, and enhanced invasion, in ESCC cells.

Materials and methods

Cell culture

ESCC cell lines were selected based on NRF2 expression. KYSE410 cells carry wild-type *NRF2* and express a low level of NRF2, thus are defined as NRF2^{low}. KYSE70 cells carry a homozygous point mutation (*NRF2*^{W24C}) and express a high level of NRF2, and thus are defined as NRF2^{high} [19,50]. When *NRF2* was knockdown by siRNA in KYSE70 cells, these cells were defined as NRF2^{low}-KYSE70 cells. When *KEAP1* was knockdown by siRNA in KYSE410 cells, these cells were defined as NRF2^{high}-KYSE410 cells.

RPMI 1640 Glutamax media (Gibco, Gaithersburg, MD) supplemented with 10% FBS and 0.1% penicillin/streptomycin was used to culture cells under normal conditions. For cell-based assays where starvation media was used, cells were either cultured in nutrient-free DMEM media (Gibco) for 4 h or RPMI 1640 without glucose (Gibco) supplemented with 10% dFBS, 5 mM glucose and 300 μM acetate for assays that run for 24 or 72 h. In these long-term ethanol exposure studies, 5 mM glucose rather than 10 mM glucose was used as heavy alcohol drinkers have been shown to consume less dietary glucose, and absorb less glucose from dietary sources [51–54]. After a dose–response experiment with ethanol, 50 mM ethanol was chosen for subsequent experiments that required ethanol exposure.

siRNA transfection

siRNA transfection was done using Lipofectamine RNAiMax (Invitrogen, Waltham, MA), Optimem limited serum media (Gibco), *ACSS2* siRNA (AM16708, ID177990, Invitrogen), *NFE2L2* siRNA (4392421, IDs9491, Invitrogen), or *KEAP1* siRNA (4392420, IDs18982, Invitrogen). Transfection was conducted according to the manufacturer's protocol. Gene knockdown was achieved 48–72 h after transfection.

Biochemical Journal (2020) **477** 3075–3089
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CRISPR Cas9 knockdown

CRISPR Cas9 knockdown was done by Synthego (Redwood City, CA). The sequence targeted was 482 bp from the UTR on exon 2 of *NRF2*. The guide RNA sequence used was UAUUUGACUUCAGUCAGCGA. The guide target was found near a CGG PAM sequence. Results were analyzed through an ICE analysis tool. CRISPR generated a mixed population of cells (Supplementary Figure S3). Western blotting confirmed the knockdown result (Figure 1C,D).

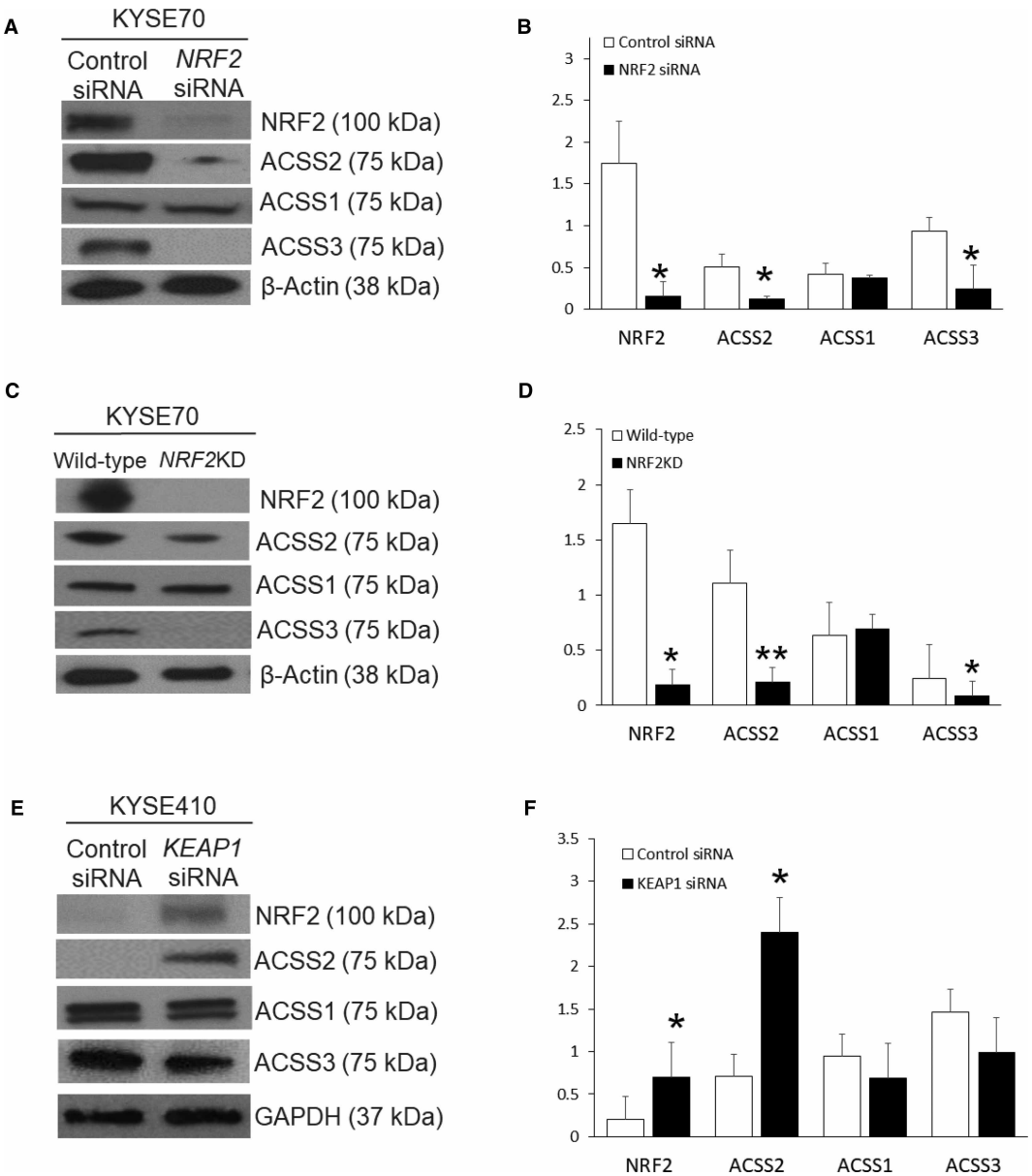

Figure 1. NRF2 regulates the expression of ACSS2 in ESCC cells.

(**A**,**B**) *NRF2KD* in KYSE70 cells through *NRF2* siRNA transfection led to a significant decrease in ACSS2 and ACSS3. (**C**,**D**) *NRF2KD* in KYSE70 cells through CRISPR–Cas9 also led to a significant decrease in ACSS2 and ACSS3. (**E**,**F**) A significant increase in NRF2 and ACSS2 expression was observed in NRF2^{high} KYSE410 cells due to *KEAP1* siRNA transfection as compared with control.

Biochemical Journal (2020) 477 3075–3089
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Western blotting

Total protein was isolated from human ESCC cells and mouse tissues using a standard method. Antibodies to ACSS1 and ACSS3 were purchased from Proteintech (Rosemont, IL) and were used at a concentration of 1 : 3000 and 1 : 600, respectively. ACSS2 antibody (Cell Signaling, Danvers, MA) was used at a concentration of 1 : 3000. NRF2 antibody (Abcam, Cambridge, MA) was used at a 1 : 2000 concentration, while GAPDH (Genetex, Irvine, CA) and β-actin (Cell Signaling) were used as loading controls at 1 : 10 000 and 1 : 8000, respectively. All antibodies were diluted using SuperBlock T20 blocking buffer (Thermofisher Scientific, Waltham, MA). Data were based on biologic triplicates.

Cell-based assays

Acetate was measured in KYSE70 and KYSE410 cell lysates using acetate colorimetric assay kit (ab204719, Abcam). Cells were seeded at a 2.0×10^6 density in three 75 cm^2 flasks per group, after which siRNA transfection was conducted 24 h after seeding. Cells were cultured in a normal medium.

An acetyl-CoA fluorometric assay kit (ab87546, Abcam) and an ATP assay kit (MAK190, Sigma–Aldrich, St. Louis, MO) were used for acetyl-CoA and ATP measurements. Cells were either cultured in nutrient-free DMEM media (Gibco) for 4 h (Figure 4C–H) or with RPMI 1640 with no glucose (Gibco) supplemented with 10% dFBS, 5 mM glucose, and 300 μM acetate with or without 50 mM ethanol for 72 h, with media and ethanol replaced every 24 h (Figure 4I,L). 1-(2,3-di(Thiophen-2-yl)quinoxalin-6-yl)-3-(2-methoxyethyl)urea, an ACSS2 inhibitor (ACSS2i), was purchased from Millipore-Sigma (Burlington, MA).

To determine the effects of ACSS2 and ethanol on fatty acid synthesis in KYSE70 cells, cells were first transfected with siRNA in 75 cm^2 flasks under normal cell culture conditions for 72 h. Afterwards, these transfected cells were seeded in 100 mm plates at a concentration of 6.0×10^6 cells per plate. Cells were cultured in RPMI 1640 media with no glucose (Gibco) supplemented with 10% dFBS, 5 mM glucose and 300 μM acetate with or without 50 mM ethanol for 24 h. At the end of the 24 h period, cells were collected using trypsin and washed with cold 1X PBS before running the assay. The fatty acid synthesis was measured using a free fatty acid fluorometric assay kit (ab65341, Abcam). Data were based on biologic triplicates to ensure reproducibility.

Immunohistochemistry (IHC) staining

Deparaffinized sections were pre-treated to retrieve antigens before detection with a rabbit polyclonal anti-NRF2 (Invitrogen) at a 1 : 200 dilution, and a rabbit polyclonal anti-ACSS2 (Sigma–Aldrich) at a 1 : 25 dilution. Cytokeratin 5 IHC with anti-CK5 (Invitrogen) was used to validate the antigenicity of human tissue sections.

Human ESCC formalin-fixed paraffin-embedded tissue sections were decoded without patient information. IHC staining was scored based on a positive staining area and staining intensity. Numeric scores were provided for staining area and staining intensity: 3 for high intensity or a high percentage of positive staining (>75% cancer cells); 2 for moderate staining or moderate percentage (50-75%); 1 for low staining intensity or low percentage of positive staining (25-50%); and 0 for no staining. A combined numerical score for each antigen was generated by adding the numerical score of the percentage of positive staining to that of the staining intensity.

NRF2 ChIP-PCR assay

These assays were performed using the EZ-ChIP PCR kit (17-371, Millipore-Sigma) as previously described [31]. Immunoprecipitations were performed using control mouse IgG or 2 μg/ml rabbit anti-NRF2 polyclonal antibody (Santa-Cruz Biotech, Santa Cruz, CA). The NRF2 binding region — antioxidant response element (ARE) in mouse *Acss2* gene was identified from our previous ChIP-seq data [39]. The PCR control used was a *Gapdh* primer provided with the kit. The *Nqo1* primer sequences were based on a previous publication and served as a positive control [55]. The PCR primer sequences used were 5′-TACACCCTCACCAGCACATT-3′, and 5′-TTTCTGCTGGATGTGGTGG-3′. The predicted sizes of PCR products of *Gapdh*, *Nqo1*, and *Acss2* were 166 bp, 186 bp, and 595 bp, respectively.

Seahorse cell mito-stress assay

The Seahorse cell mito-stress assay was used to characterize the effect of NRF2, ACSS2, and ethanol on cell energy phenotype and OXPHOS in ESCC. KYSE70 cells were seeded at a density of 2×10^4 cells/well, and KYSE410 cells were seeded at a density of 2.5×10^4 cells/well in XFp plates and allowed to stabilize overnight. XF media was supplemented with 5 mM D-glucose and 2 mM L-glutamine as described in the manufacturer's

instructions for the XF Cell Mito-Stress assay with or without 20 mM ethanol. Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were next measured using a Seahorse XFp analyzer (Agilent Inc., Santa Clara, CA). Cells were then treated with or without ethanol for 1.5 h. For the cells treated with ethanol, ethanol was added not only to the media but also to the surrounding moat. Time 0 in the Seahorse graphs (Figure 5) indicated the beginning of Seahorse measurement (i.e. once the plates were placed in the instrument). For KYSE70 cells, 1 μM oligomycin, 0.5 μM FCCP, and 0.5 μM rotenone were simultaneously applied to measure the glycolysis and OXPHOS in the cells. Injections of 1.5 μM oligomycin, 3.0 μM FCCP, and 0.5 μM rotenone were simultaneously applied to measure the same energy-producing pathways in KYSE410 cells. Injections were determined by cell characterization methods stated in the manufacturer's instructions for the XF Cell Mito-Stress assay. Incucyte® (Sartorius, Göttingen, Germany) was used to determine cell number in each well. The results were normalized to cell number and analyzed using Wave software (Agilent Inc.). Data were based on biological triplicates to ensure reproducibility.

Cell invasion assay

The assay was done according to the Corning cell invasion assay protocol. Control, *ACSS2*KD, *NRF2*KD, and ACSS2i treated KYSE70 cells were loaded at a density of 2×10^4 cells/insert in growth factor reduced Matrigel invasion chamber cell culture inserts (8 μm pore size; Corning; Corning, NY). Cells were then incubated for 36 h with either control or 50 mM ethanol containing serum-free medium in the upper chamber. The bottom chamber was filled with 750 μl of 10% FBS-containing RPMI medium. Cells at the bottom of the plate were stained with 1 μg/ml Hoechst, and analysis was done using Pico ImageXpress imaging (Molecular Devices, San Jose, CA). Data were based on biological triplicates to ensure reproducibility.

Animal experiments

Animal experiments were approved by the Institutional Animal Care and Use Committees at the North Carolina Central University (protocol number XC-12-03-2008). Animals in these experiments were bred and maintained in our animal facility. Wild-type, $Nrf2^{-/-}$ and $Keap1^{-/-}$ esophageal tissues were obtained as previously reported [39]. *Sox2CreER* mice were obtained from the Jackson Laboratory (Bar Harbor, ME), and $LSL\text{-}Nrf2^{E79Q/+}$ mice were obtained from Dr. Bernard Weissman's lab at the University of North Carolina at Chapel Hill, Chapel Hill, NC [56]. Cre recombinase was activated by intraperitoneal injection of tamoxifen (75 mg/kg in corn oil, once per day, 5 days). Mice were then sacrificed by CO_2 asphyxiation at 5 weeks after tamoxifen induction. No anesthetics were used for mice.

Statistical analyses

Student's *t*-test was used to analyze Western blot, cell-based assay, Seahorse, and cell invasion results from biologic triplicates. A Pearson correlation test was used to analyze the relationship between NRF2 and ACSS2 expression in human samples.

Results

NRF2 regulates expression of ACSS2 *in vitro*

To determine whether NRF2 regulates ACSS2 *in vitro*, we selected two human ESCC cell lines, $NRF2^{high}$-KYSE70 and $NRF2^{low}$-KYSE410, based on the level of NRF2 expression [22,50,57]. When KYSE70 cells were transfected with *NRF2* siRNA, there was a significant decrease in the expression of NRF2, ACSS2, and ACSS3 (Figure 1A). A similar result was observed when *NRF2* was knocked down using CRISPR–Cas9 in KYSE70 cells (Figure 1B). On the contrary, *KEAP1* siRNA led to an increase in NRF2 and ACSS2 expression in KYSE410 cells (Figure 1C). These results indicate that NRF2 regulates ACSS2 protein expression *in vitro*.

NRF2 regulates ACSS2 expression and binds to the promoter of Acss2 *in vivo*

We next determined whether NRF2 regulated ACSS2 expression *in vivo*. Using IHC, we examined the expression of NRF2 and ACSS2 in esophageal squamous epithelial cells of $Nrf2^{-/-}$, wild-type, and $Keap1^{-/-}$ mice. As expected, when NRF2 was constitutively hyperactive in $Keap1^{-/-}$ esophagus, ACSS2 expression was up-regulated as compared with wild-type and $Nrf2^{-/-}$ esophagi (Figure 2A). Similar results were also observed in the lung, liver, and kidney tissues (Supplementary Figure S1). We also used $Sox2CreER;LSL\text{-}Nrf2^{E79Q/+}$ mice to determine whether induced hyperactivation of NRF2 in the adult esophagus may up-regulate ACSS2

Biochemical Journal (2020) 477 3075–3089
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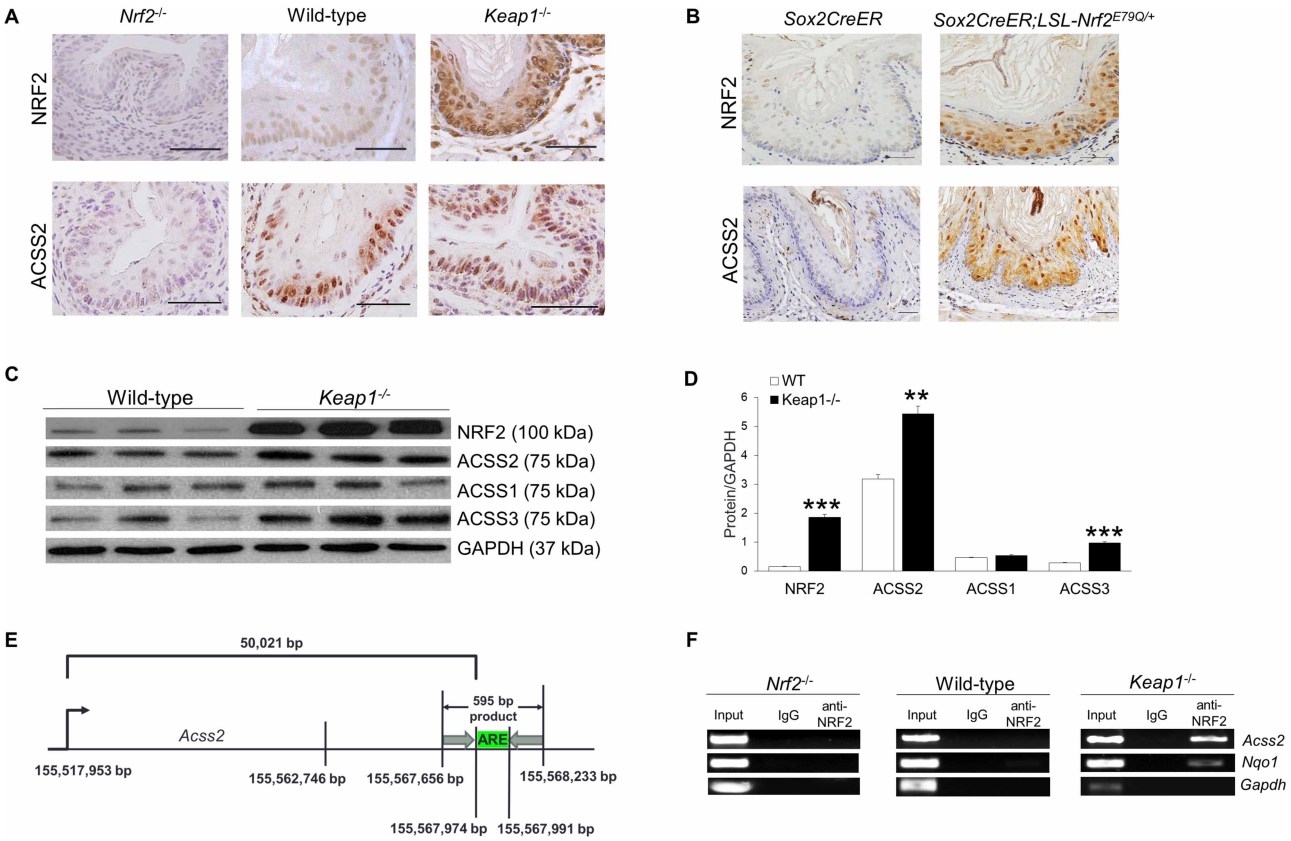

Figure 2. NRF2 regulates ACSS2 expression and binds to the promoter of *Acss2 in vivo*.

(**A**) Immunohistochemistry of mouse esophagus showed that NRF2 and ACSS2 expression increased when NRF2 expression was activated (*Keap1*$^{-/-}$) and decreased when NRF2 was deficient (*Nrf2*$^{-/-}$); scale bar = 50 μm. (**B**) Immunohistochemistry also showed ACSS2 up-regulation due to NRF2 hyperactivation in the esophagus that expressed a constitutively active mutant *Nrf2* (*Sox2CreER;LSL-Nrf2*$^{E79Q+}$) as compared with control (*Sox2CreER*); scale bar = 50 μm. (**C**) Western blot of wild-type and *Keap1*$^{-/-}$ mouse esophagi showed increased NRF2 expression and overexpression of ACSS2 and ACSS3. (**D**) Statistical analysis of the Western blot revealed significant increases in ACSS2 and ACSS3 expression in *Keap1*$^{-/-}$ mouse esophagi. (**E**) Schematic diagram showing ARE region in mouse *Acss2* gene, and the ChIP-PCR primer target (595 bp). (**F**) NRF2 ChIP-PCR of wild-type, *Keap1*$^{-/-}$ and *Nrf2*$^{-/-}$ mouse esophagi identified *Acss2* as a target gene of hyperactive NRF2. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

expression. *Sox2CreER;LSL-Nrf2*$^{E79Q/+}$ mouse expressed a high level of NRF2 in the esophagus due to the expression of a mutant allele (E79Q) after tamoxifen induction. Indeed, ACSS2 was overexpressed in the *Sox2CreER;LSL-Nrf2*$^{E79Q/+}$ esophagus (Figure 2B).

Using Western blotting, we confirmed ACSS2 and ACSS3 up-regulation in *Keap1*$^{-/-}$ esophagi as compared with wild-type esophagi (Figure 2C,D). Our previous NRF2 ChIP-seq data using mouse esophagus indicated that hyperactive NRF2 bound to mouse *Acss2* gene [39]. To further validate this observation, we performed NRF2 ChIP-PCR using mouse esophageal tissues. Consistent with our ChIP-seq data, NRF2 ChIP-PCR revealed NRF2 binding to mouse *Acss2* gene in *Keap1*$^{-/-}$ esophagus, but not in *Nrf2*$^{-/-}$ and wild-type esophagi (Figure 2E,F). The regulation of ACSS2 expression by NRF2 was further supported through IHC of human ESCC samples. A significantly positive correlation between NRF2 and ACSS2 expression was observed in our human samples (Figure 3). These results using the mouse and human tissue samples indicate that NRF2 regulates ACSS2 expression *in vivo*.

NRF2/ACSS2 axis mediates ethanol metabolism in ESCC cells

To gain insight into the relationship between NRF2, ACSS2, and ethanol metabolism in ESCC, we first investigated the roles of NRF2 and ACSS2 in acetate metabolism. We measured cellular acetate and acetyl-CoA in

Biochemical Journal (2020) **477** 3075–3089
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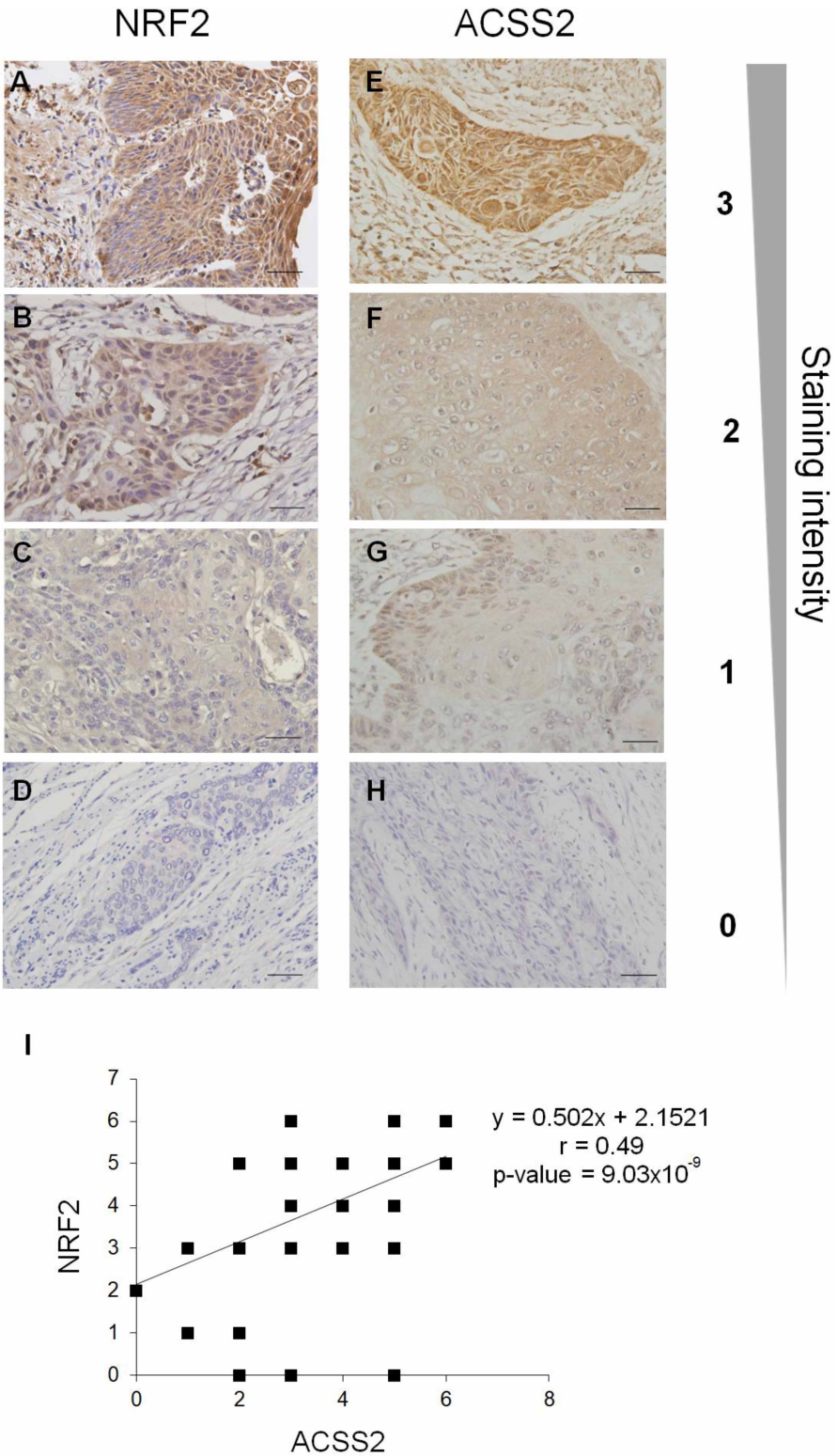

Figure 3. NRF2 and ACSS2 expression are positively correlated in human ESCC tissue samples.

ESCC samples were categorized based on the staining intensity (Scale bar = 50 μm). Images in panel (**A**–**H**) came from different patients. (**A**) High NRF2 staining intensity; (**B**) moderate NRF2 staining intensity; (**C**) low NRF2 staining intensity; (**D**) no NRF2 staining; (**E**) high ACSS2 staining intensity; (**F**) moderate ACSS22 staining intensity; (**G**) low ACSS2 staining intensity; (**H**) no ACSS2 staining. (**I**) IHC of human samples revealed a significantly positive correlation between NRF2 and ACSS2 expression in these samples.

Biochemical Journal (2020) **477** 3075–3089
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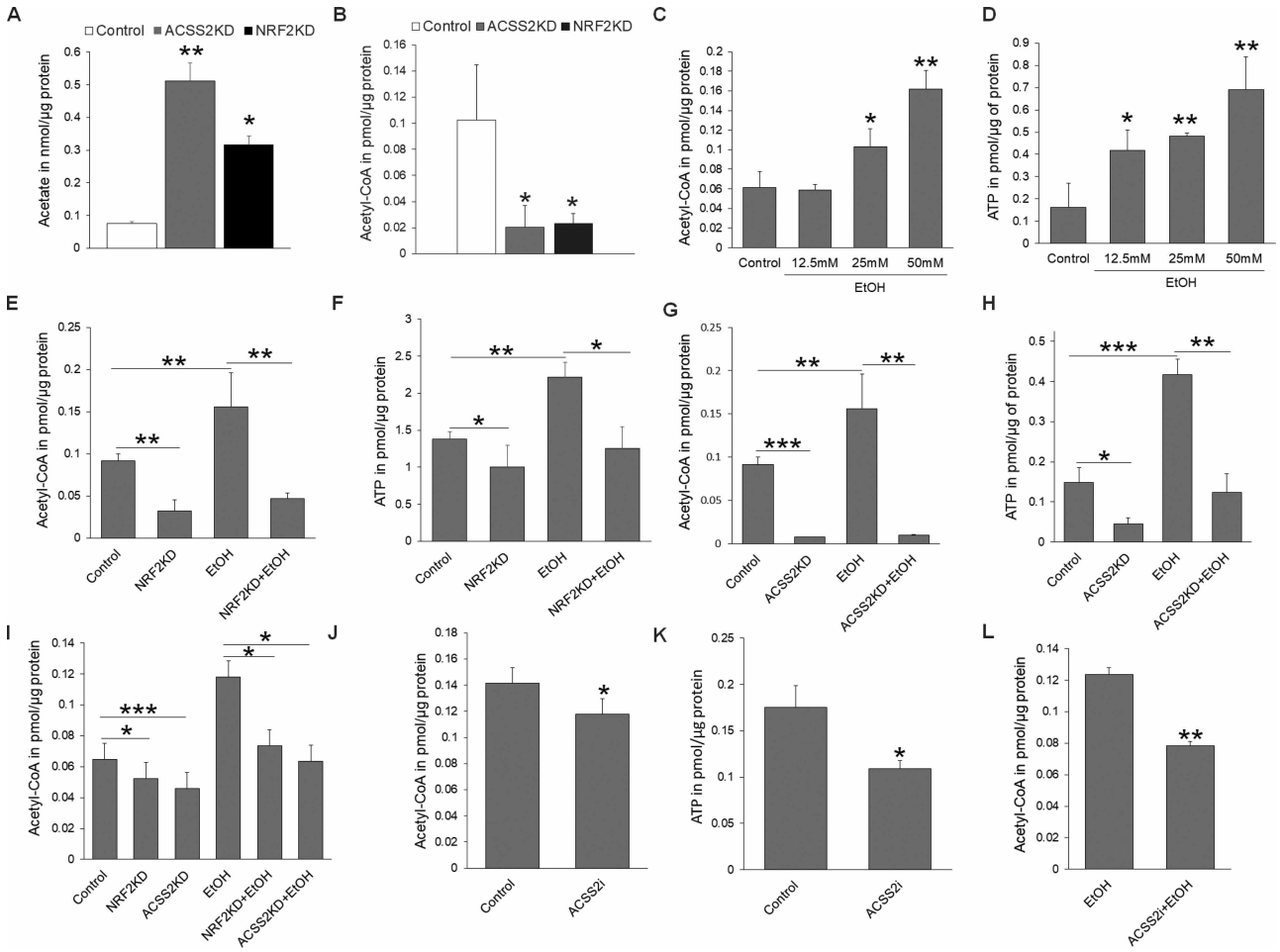

Figure 4. NRF2/ACSS2 axis regulates acetate metabolism in NRF2^{high} KYSE70 ESCC cells.

(**A**) Acetate metabolism was significantly inhibited when *ACCS2* or *NRF2* was knocked down. (**B**) Acetyl-CoA was significantly decreased when *ACSS2* or *NRF2* was knocked down. (**C**,**D**) Ethanol exposure significantly increased acetyl-CoA and ATP in a dose-dependent manner. Based on these results, 50 mM ethanol was used to treat cells in the subsequent experiments. (**E**,**F**) 50 mM ethanol exposure significantly increased acetyl-CoA and ATP, whereas *NRF2* knockdown counteracted such effects. (**G**,**H**) *ACSS2* knockdown inhibited acetyl-CoA and ATP. (**I**) Knockdown of *NRF2* or *ACSS2* led to a significant decrease in acetyl-CoA in ESCC cells in the presence or absence of ethanol exposure. (**J**,**K**) ESCC cells treated with an ACSS2 inhibitor had significantly decreased acetyl-CoA and ATP as compared with control. (**L**) ACSS2 inhibitor significantly decreased cellular acetyl-CoA when cells were exposed to ethanol over 72 h. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

KYSE70 cells transfected with *ACSS2* or *NRF2* siRNA, and KYSE410 cells transfected with *KEAP1* siRNA (Supplementary Figure S2A,B). We did not further investigate ACSS3 because it has a low affinity for acetate but a higher affinity for propionate. Hence, ACSS3 is not a major enzyme in the acetate metabolism pathway [58]. As observed in Figure 4, *ACSS2*KD led to a significant increase in acetate and a significant decrease in acetyl-CoA. Similar findings were also observed in *NRF2*KD KYSE70 cells (Figure 4A,B). When NRF2 was up-regulated in KYSE410 cells, cellular acetate decreased, while acetyl-CoA production increased (Supplementary Figure S2C,D). These results indicate that NRF2 and ACSS2 regulate acetate metabolism in ESCC cells.

We then examined the role of the NRF2/ACSS2 axis in ethanol metabolism. We first determined the dose-dependent effects of ethanol on acetyl-CoA and ATP in KYSE70 cells (Figure 4C,D). We observed that ethanol increased acetyl-CoA and ATP production in a dose-dependent manner (Figure 4C,D). When KYSE70 cells were transfected with either *NRF2* or *ACSS2* siRNA, significant decreases in acetyl-CoA and ATP were observed in the presence or absence of ethanol exposure (Figure 4E–H). A significant decrease in acetyl-CoA could still

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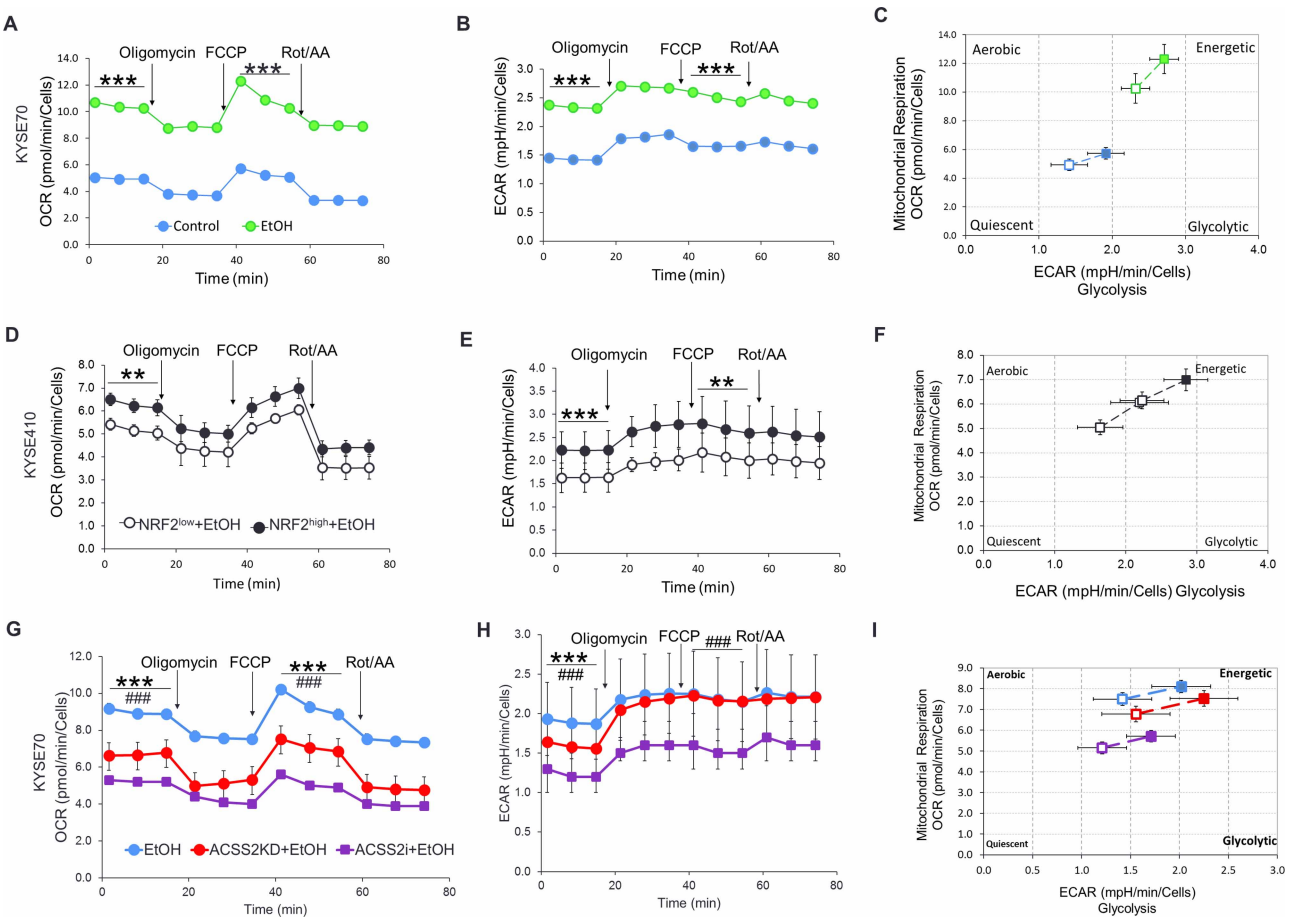

Figure 5. ACSS2 and ethanol induce a metabolic shift in NRF2^{high} ESCC cells.

(**A**,**B**) A significant increase in OCR and ECAR were observed when KYSE70 cells were exposed to ethanol. (**C**) KYSE70 cells became more energetic when exposed to ethanol. (**D**,**E**) Increased OCR and ECAR in NRF2^{high}-KYSE410 cells were observed after ethanol exposure as compared with control NRF2^{low}-KYSE410 cells. (**F**) Hyperactive NRF2 led to a more energetic phenotype in KYSE410 cells. (**G**,**H**) Down-regulation or inhibition of ACSS2 decreased OCR and ECAR in KYSE70 cells. The statistical differences between wild-type and *ACSS2KD* were indicated by asterisks (*), while those between control and ACSS2i treatment was indicated by hashtags (#). (**I**) ACSS2 inhibition led to a less energetic phenotype in KYSE70 cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. $^{\#\#\#}P < 0.001$.

be observed after 72 h (Figure 4I). Similarly, when ACSS2 was inhibited with a chemical inhibitor in KYSE70 cells, significant decreases in acetyl-CoA and ATP were also observed (Figure 4J–L). On the contrary, when KYSE410 cells were transfected with *KEAP1* siRNA and exposed to ethanol over 72 h, there was a significant increase in acetyl-CoA compared with control KYSE410 cells (Supplementary Figure S2E). These results demonstrate that ethanol is used as an energy source in NRF2^{high} ESCC cells and that the NRF2/ACSS2 axis contributes to ethanol metabolism in ESCC cells.

Ethanol exposure causes a metabolic shift in NRF2^{high} ESCC cells

Our previous report showed that hyperactive NRF2 caused metabolic reprogramming in the esophagus [39]. Together with our current findings, we hypothesized that ethanol exposure promoted OXPHOS in NRF2^{high} ESCC cells. To test this hypothesis, we ran a Seahorse Mito-stress assay on KYSE70 cells exposed to ethanol. As shown in Figure 5A, KYSE70 cells exposed to ethanol up-regulated OXPHOS at both the baseline and stressed levels as compared with control. ECAR, a measure of glycolysis, was also up-regulated during ethanol exposure (Figure 5B). Additionally, ethanol exposure of these NRF2^{high} cells made the cells adopt a more energetic phenotype (Figure 5C).

Biochemical Journal (2020) 477 3075–3089
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We then determined whether the NRF2/ACSS2 axis played a role in this metabolic shift. We first tested the impact of NRF2 on OXPHOS in NRF2^{high} and NRF2^{low} ESCC cells exposed to ethanol. As shown in Figure 5D, NRF2 hyperactivation led to a significant increase in baseline OXPHOS in KYSE410 cells. A much greater change in OXPHOS was observed in KYSE70 cells when *NRF2* was knocked down using a CRISPR–Cas9 approach (Supplementary Figure S4A). *NRF2*KD in KYSE70 cells led to a significant decrease in OXPHOS at both the baseline and stressed levels. NRF2 hyperactivation led to increased ECAR, while its down-regulation led to decreased ECAR (Figure 5E, Supplementary Figure S4B). These results were in agreement with our previous work, in which hyperactive NRF2 up-regulated glycolysis in the mouse esophagus [39].

NRF2 hyperactivation also produced a more energetic phenotype in KYSE410 cells (Figure 5F). Conversely, down-regulation of NRF2 in KYSE70 cells decreased their mitochondrial and glycolytic activity (Supplementary Figure S4C). We also investigated the role of ACSS2 on OXPHOS and ECAR in ESCC cells exposed to ethanol. *ACSS2*KD or ACCS2 inhibition led to a significant decrease in mitochondrial respiration in KYSE70 cells (Figure 5G). Baseline ECAR was significantly decreased when *ACSS2* was either knocked down or when the ACSS2 enzyme was inhibited (Figure 5G). Finally, metabolic phenotype analysis showed that *ACSS2*KD or ACSS2 inhibition led to a decrease in mitochondrial respiration (Figure 5I). These results show that ACSS2 and ethanol up-regulate OXPHOS in NRF2^{high} ESCC

Ethanol exposure promotes lipid synthesis in NRF2^{high} ESCC cells

Acetate metabolism via ACSS2 has been shown to promote lipid synthesis in different cancers [40,47]. We hypothesized that ESCC cells would increase lipid synthesis when exposed to ethanol and that this process would be dependent on the NRF2/ACSS2 axis. We exposed KYSE70 and KYSE410 cells to 50 mM ethanol over 24 h, and then measured the amount of cellular free fatty acid. As shown in Figure 6A,B, there was an increase in free fatty acids when ESCC cells were exposed to ethanol. The increase in free fatty acid was significantly decreased when either *ACSS2* or *NRF2* was knocked down (Figure 6A), or when ACSS2 was chemically inhibited (Figure 6A,B). However, NRF2 hyperactivation led to significantly increased lipid synthesis after ethanol exposure (Figure 6B). This increase in lipid synthesis, however, was significantly suppressed when ACSS2 was chemically inhibited in *KEAP1*KD KYSE410 cells (Figure 6B). These data suggest that ethanol can be used for lipid synthesis via the NRF2/ACSS2 axis in ESCC cells.

Ethanol exposure facilitates invasion of NRF2^{high} ESCC cells

Previous reports have indicated that ethanol exposure increased cancer cell invasion [59–62]. Acetate metabolism by ACSS2 has been implicated in vascular invasion and metastasis of glioblastoma [61]. We hypothesized that ethanol-induced metabolic reprogramming mediated by the NRF2/ACSS2 axis in ESCC cells would impact cell invasion. As shown in Figure 6C, NRF2^{high} cells exposed to ethanol had the highest percentage of invasive cells compared with control. However, cell invasion significantly decreased in ethanol-exposed cells when ACSS2 was chemically inhibited, or when *NRF2* or *ACSS2* was knocked down by siRNA. These results suggest that ethanol exposure facilitates invasion of NRF2^{high} ESCC cells through the NRF2/ACSS2 axis.

Discussion

In this study, we show a novel mechanism of alcohol-associated ESCC. Previous studies on alcohol-associated cancer have mainly focused on acetaldehyde due to its reactive nature and its ability to induce genetic mutations as well as protein aberrations [17,18,63-65]. Our data indicate that the NRF2/ACSS2 axis mediates the metabolic effects of ethanol exposure, i.e. increased OXPHOS and lipid synthesis, on NRF2^{high} ESCC. This discovery is consistent with previous studies showing that acetate supports cell proliferation, invasion, and metastases in other cancers [40,41,47,66].

Inside epithelial cells, ethanol is metabolized to acetaldehyde by alcohol dehydrogenase, cytochrome P450 2E1 and, to a much lesser extent by catalase, and is further oxidized to acetate by acetaldehyde dehydrogenase. ADH-mediated ethanol metabolism results in the generation of reducing equivalents in the form of reduced nicotinamide adenine dinucleotide and acetaldehyde, whereas ethanol oxidation by CYP2E1 leads to the production of acetaldehyde, but also the generation of reactive oxygen species [67]. Systemic metabolism of ethanol causes the reduction in retinoid, zinc and methyl groups, and the accumulation of iron. Through the bloodstream, ethanol is circulated to the esophageal epithelium and salivary glands. In the saliva, ethanol is oxidized by microbes to acetaldehyde. When ethanol and acetaldehyde contact esophageal epithelial cells, ethanol perturbs the lipid bilayer of the cell membrane and interferes with the function of intrinsic membrane proteins,

Biochemical Journal (2020) **477** 3075–3089
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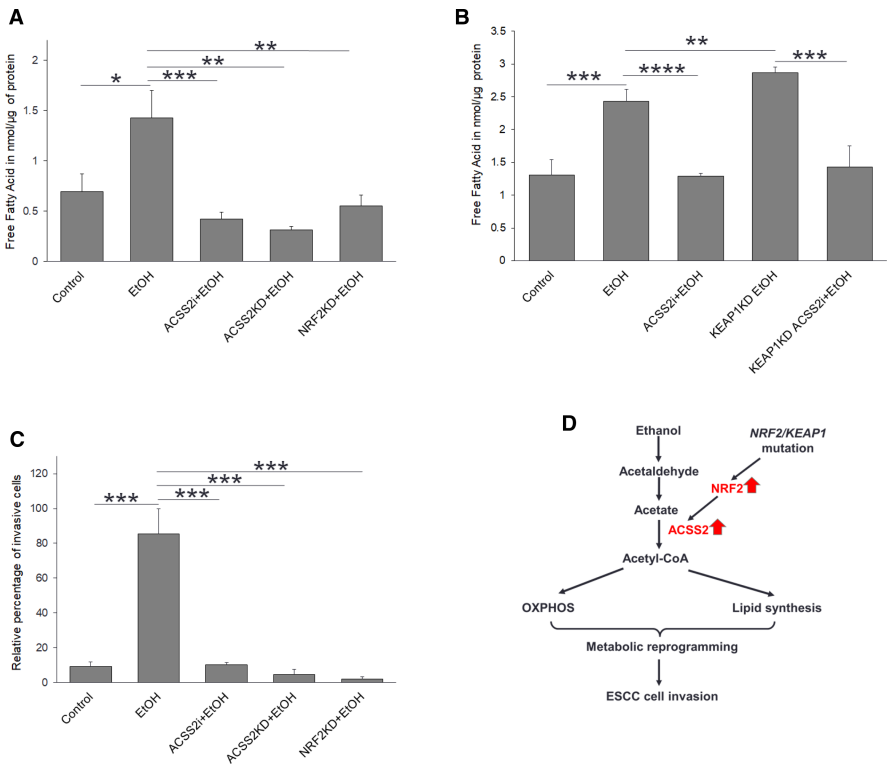

Figure 6. NRF2/ACSS2 axis facilitates lipid biosynthesis and cell invasion in ESCC cells exposed to ethanol.
(**A**) Ethanol exposure increased free fatty acid in KYSE70 cells. Lipid synthesis decreased in KYSE70 cells when NRF2 or ACCS2 was knocked down or when ACSS2 was inhibited. (**B**) Ethanol exposure increased lipid synthesis in KYSE410 cells, ACSS2 inhibition during ethanol exposure led to a significant decrease in lipid synthesis. Up-regulation of NRF2 during ethanol exposure led to a significant increase in lipid synthesis compared with control KYSE410 cells exposed to ethanol. Inhibition of ACSS2, however, after NRF2 up-regulation led to a significant decrease in lipid synthesis. (**C**) Ethanol exposure led to an increase in the KYSE70 cell invasion. *NRF2*KD, *ACSS2*KD, and ACSS2i led to a significant decrease in cell invasion. (**D**) A schematic showing that the NRF2/ACSS2 axis mediates metabolic reprogramming and promotes invasion of ESCC cells when exposed to ethanol. *NRF2/KEAP1* mutations result in NRF2 hyperactivation, which further up-regulates ACSS2. ACSS2 overexpression enhances acetyl-CoA turnover, which is used for lipid synthesis and energy production through OXPHOS. These metabolic changes support ESCC cell invasion. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

such as TLR4, Notch, Shh, and Wnt pathway receptors, and thus activates or inhibits downstream signaling. Inside epithelial cells, ethanol metabolism causes oxidative damages to DNA, proteins, and lipids, and modulates fatty acid metabolism as well. These systemic and local effects of ethanol and acetaldehyde stimulate cell proliferation, inflammation, and angiogenesis, and suppress squamous cell differentiation, and therefore promote ESCC [11]. Our previous study demonstrated PAX9 inhibition as a mechanism of alcohol-associated ESCC [68].

Our current study is the first to directly link alcohol drinking, acetate metabolism, and ESCC. Ethanol metabolism has also been shown to generate oxidative stress and thus activates NRF2. This relationship between ethanol exposure and NRF2, however, remains complex, as ethanol has been shown to suppress NRF2 expression in alveolar epithelial cells and an NRF2 activator (sulforaphane) blocks the effects of ethanol [69]. Activation of NRF2 prevents ethanol-induced oxidative stress, lipid accumulation, and accelerated acetaldehyde metabolism [70,71]. Overall, the effect of ethanol on NRF2 expression depends on the concentration of ethanol, duration of ethanol exposure, cellular context, and potentially some other factors. When we exposed human ESCC KYSE510 cells (*NRF2* wild-type) to 100 mM ethanol for 24 h, we observed significant activation of NRF2 transcriptional activity using a luciferase reporter assay (unpublished data). However, it remains to be determined whether ethanol exposure can act in the same way in normal esophageal squamous epithelial cells in which NRF2 activity can be activated, but not to the level in *NRF2*-mutant ESCC cells.

Biochemical Journal (2020) 477 3075–3089
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Our previous microarray analysis comparing *Keap1*$^{-/-}$ to *Nrf2*$^{-/-}$;*Keap1*$^{-/-}$ mouse esophagi identified *Acss2* as one of the genes differentially expressed due to NRF2 hyperactivation. In this study, we further established ACSS2 as a transcriptional target of NRF2 in the esophagus *in vitro* and *in vivo*. Additionally, our prior ChIP-seq data and our current ChIP PCR data, indicate that NRF2 is a transcriptional regulator of ACSS2 [39].

The NRF2/ACSS2 axis allows ESCC cells to employ ethanol-derived acetate as an energy source and may thus facilitate alcohol-associated ESCC. Acetate metabolism is most prevalent in cancers when there is a lack of glucose or oxygen required for aerobic glycolysis [40,47]. It has been proposed that cancer cells consume acetate from carbohydrate fermentation in the human gut. In carbohydrate fermentation studies, the highest acetate concentration recorded was 181.3 ± 23.9 μM [41,72]. More recently, it has been established that acetate may be synthesized from glucose metabolism and mitochondrial coupling. This process involves the conversion of pyruvate to acetate by coupling either reactive oxygen species to pyruvate decarboxylation or by neomorphic enzyme activity from keto acid dehydrogenases that enable them to function as pyruvate decarboxylases [73]. Such production of acetate can only occur if there is a sufficient amount of glucose. The normal concentration of acetate in the blood is within 50–200 μM [72,74-76]. Ethanol intake from alcohol drinking would produce a high level of acetate in the blood. Ethanol consumption has been reported to increase plasma acetate to 750 μM. Moreover, this elevated concentration of plasma acetate may be persistent over 24 h, unlike endogenous acetate produced from carbohydrate fermentation which remains elevated at 200 μM for <1 h [72,77]. Furthermore, alcoholics and heavy drinkers oxidize ethanol at much faster rates than occasional drinkers. The fast oxidation of ethanol led to significantly elevated blood acetate levels of up to 1 mM [74,75].

Our schematic model (Figure 6D) explains how ESCC cells utilize acetate as an energy source originally derived from ethanol. According to our data, the concentration of acetyl-CoA and ATP in ESCC cells significantly increased after ethanol exposure. Acetyl-CoA and ATP production from endogenous acetate and exogenous acetate (via ethanol) was shown to be dependent on the NRF2/ACSS2 axis (Supplementary Figure S2 and Figure 6). Elevated acetyl-CoA production from ethanol exposure was used for OXPHOS leading to a significant metabolic shift (Figure 4A–C). NRF2/ACSS2 axis was involved in the up-regulation of OXPHOS after ethanol exposure (Supplementary Figure S4 and Figure 5D–I). Increased acetate metabolism from ethanol exposure also led to increased lipid synthesis in both NRF2^{high} and NRF2^{low} ESCC cells (Figure 6A,B). This up-regulated lipid synthesis in KYSE70 cells was dependent on the NRF2/ACSS2 axis as shown by the significant decrease in lipid synthesis due to *NRF2*KD, *ACSS2*KD, or ACSS2 inhibition (Figure 6A). Additionally, the significant increase in lipid synthesis when NRF2 was up-regulated in KYSE410 cells, as well as the significant decrease when ACSS2 was chemically inhibited during ethanol exposure, provide further support for the important role of the NRF2/ACSS2 axis in ethanol-induced lipid synthesis in ESCC cells (Figure 6B). These findings are in agreement with our prior metabolomic analysis of *Keap1*$^{-/-}$ versus wild-type mouse esophagi [39]. Taken together, NRF2 hyperactivity co-operates with alcohol drinking to facilitate metabolic changes in ESCC cells.

Metabolic reprogramming of cancer cells is expected to impact cancer cell behaviors [78]. An increase in OXPHOS has been reported to be important for cancer cell invasion and migration by providing necessary energy for microtubule motility [79,80]. In this study, *NRF2*KD, *ACSS2*KD, and ACSS2 inhibition in KYSE70 cells exposed to ethanol, not only led to a significant decrease in OXPHOS but also a significant decrease in cell invasion (Supplementary Figure S4, Figures 5G and 6C). Lipid synthesis has also been shown to serve important roles in cancer cell invasion and metastasis including structural support, and synthesis of chemical signals in the tumor microenvironment [70–74]. Our data show that ethanol exposure fuels lipid synthesis. This is in agreement with other reports that increased acetate metabolism resulted in significant increases in cholesterol and phospholipid in breast and hepatocellular carcinoma leading to more severe disease [40,47]. In our data, both ethanol-induced lipid synthesis and cancer cell invasion were facilitated by the NRF2/ACSS2 axis. Nevertheless, our data using the fatty acid fluorometric assay does not provide direct evidence to support acetate as a carbon source of fatty acid synthesis. A carbon tracing experiment is warranted in the future to further validate ethanol and acetate as carbon sources for ESCC cells.

In summary, the NRF2/ACSS2 axis regulates downstream ethanol metabolism and metabolic reprogramming in ESCC, which may promote alcohol-associated ESCC (Figure 6D).

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contributions

J.O.O. conducted the experiments and analyzed the data. Z.X., C.H., and N.G. helped with the experiments. W.Y., J.G., E.O. and C.P. helped with the analysis of human samples. J.O. and X.C. designed the experiments, wrote and revised the manuscript. X.C. supervised the whole process.

Abbreviations

ARE, antioxidant response element; ECAR, extracellular acidification rate; ESCC, esophageal squamous cell carcinoma; H&E, hematoxylin and eosin; IHC, immunohistochemistry; KD, knockdown; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation.

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Footnote

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