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# NRF2/SHH signaling cascade promotes tumor-initiating cell lineage and drug resistance in hepatocellular carcinoma

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Running title: NRF2/SHH signaling cascade regulates liver T-ICs

**Abstract**

Solid evidence shows that tumor-initiating cells (T-ICs) are the root of tumor relapse and drug resistance, which lead to a poor prognosis in patients with hepatocellular carcinoma (HCC). Through an *in vitro* liver T-IC enrichment approach, we identified nuclear factor (erythroid-derived 2)-like 2 (NRF2) as a transcription regulator that is significantly activated in enriched liver T-IC populations. In human HCCs, NRF2 was found to be overexpressed, which was associated with poor patient survival. Through a lentiviral based knockdown approach, NRF2 was found to be critical for regulating liver T-IC properties, including self-renewal, tumorigenicity, drug resistance and expression of liver T-IC markers. Furthermore, we found that ROS-induced NRF2 activation regulates sorafenib resistance in HCC cells. Mechanistically, NRF2 was found to physically bind to the promoter of sonic hedgehog homolog (SHH), which triggers activation of the sonic hedgehog pathway. The effect of NRF2 knockdown was eliminated upon administration of recombinant SHH, demonstrating that NRF2 mediated T-IC function via upregulation of *SHH* expression. Our study suggested a novel regulatory mechanism for the canonical sonic hedgehog pathway that may function through the NRF2/SHH/GLI signaling axis, thus mediating T-IC phenotypes.

Keywords: drug resistance, hepatocellular carcinoma, sonic hedgehog, sorafenib, tumor initiating cells

**Abbreviations:** cancer stem cells, CSCs; chromatin immunoprecipitation, ChIP; glioma-associated oncogene homolog 1, GLI1; hepatocellular carcinoma, HCC; immunofluorescence, IF; kelch-like ECH-associated protein 1, KEAP; nuclear factor (erythroid-derived 2)-like 2, NRF2; quantitative reverse transcription polymerase chain reaction, qPCR; reactive oxygen species, ROS; sonic hedgehog homolog, SHH; tumor-initiating cells, T-ICs

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## 1. Introduction

Hepatocellular carcinoma (HCC) is a very deadly disease, ranking as the fifth most commonly diagnosed cancer and the second leading cause of cancer-related mortality in the world (1). The poor prognosis of HCC patients is mainly attributed to late diagnosis and frequent relapse and to the refractory nature of HCC to chemotherapy. Sorafenib is the first small molecular inhibitor approved by the FDA for HCC treatment; however, its survival benefits are modest (2). Recently, regorafenib was approved by the FDA as a second-line treatment for advanced HCC patients, and the drug provides some, though still very minimal, survival benefit to HCC patients who progress on sorafenib (3). With these unmet clinical needs, there is an urgent need to identify mechanisms of sorafenib resistance to improve the survival of HCC patients. Compelling evidence has emerged in support of a cancer stem cell (CSC)/tumor-initiating cell (T-IC) model in HCC, which is believed to play a crucial role in tumor recurrence and therapeutic resistance (4-7). To identify the crucial transcription factors that regulate the function of liver T-ICs, we previously enriched the liver T-IC population by serially passaging hepatospheres in the presence of chemotherapeutic drugs (8). We compared the expression profiles of enriched T-IC populations and their differentiated progenies and found that nuclear factor (erythroid-derived 2)-like 2 (NRF2) was markedly activated in T-IC-enriched hepatospheres compared to their differentiated progenies, suggesting that NRF2 plays a role in maintaining liver cancer stemness.

NRF2 is a redox-sensitive basic leucine zipper protein that regulates the cellular response to oxidative and electrophilic insults (9). Under homeostatic conditions, NRF2 is localized in the cytoplasm, where it is sequestered by its inhibitor, kelch-like ECH-associated protein 1 (KEAP1) (10). The role of NRF2 in cancer remains controversial, and it is still unclear whether NRF2 acts as a tumor suppressor gene or oncogene. Genetic manipulation studies have shown that enhancing the activity of NRF2 inhibits carcinogenesis (11). In particular, the efficacy of chemopreventive drugs has been shown to be abolished in NRF2-null mice (12). Indeed, a number of studies have shown that this transcription factor is oncogenic and important for conferring resistance to chemotherapy (13). In HCC, activating mutations of the NRF2 gene were found in 6.4% of tumor samples, further supporting its oncogenic role in this disease (14). In HCC, NRF2 was reported to play a role in the preneoplastic stages of hepatocarcinogenesis (15-16). Recently, the role of NRF2 in T-ICs was also

reported. In a subset of enriched colon T-ICs, the secretome was characterized by an NRF2 antioxidant signature (17). Lentiviral-based mediated knockdown experiments revealed that the suppression of NRF2 inhibited the self-renewal ability of glioma stem cells (18). Although these findings suggest a potential role of NRF2 in regulating T-ICs, the molecular mechanism by which NRF2 regulates T-IC properties is not fully understood.

In this study, we examined whether NRF2 plays a crucial role in the regulation of liver T-IC function. First, we found that overexpression of NRF2 protein was associated with advanced tumor stage and patient survival in HCC. NRF2 was found to regulate liver T-IC phenotypes, including tumorigenicity, self-renewal, drug resistance and stemness marker expression. Specifically, reactive oxygen species (ROS)-induced NRF2 upregulation regulated resistance to sorafenib. Mechanistically, NRF2 was found to physically bind to the promoter of sonic hedgehog (SHH), which triggers activation of the SHH pathway. Targeting the NRF2/SHH signaling cascade is a new potential therapeutic approach for the treatment of HCC.

## 2. Materials and Methods

### 2.1. Cell lines and cell culture

The human HCC cell lines Huh7 (Japan Cancer Research Bank), Bel7402 (Shanghai Institute of Cell Biology, Chinese Academy of Sciences), and 293FT (Invitrogen) were maintained in high-glucose DMEM (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL), 100 mg/mL penicillin G, and 50 μg/mL streptomycin (Gibco BRL) at 37°C in a humidified atmosphere containing 5% $CO_2$. Cells were used within 20 passages after thawing.

### 2.2. Human tissue specimens for mRNA expression analysis

Paired patient HCC and adjacent noncancerous liver tissue specimens were collected at the time of surgical resection with informed consent from patients at Queen Mary Hospital, Hong Kong, from 1991 to 2013. The use of human clinical specimens was approved by the Institutional Review Board (IRB) of the University of Hong Kong/Hospital Authority Hong Kong West Cluster.

### 2.3. Sphere formation assay

A total of 200 single HCC cells were plated onto 24-well polyHEMA (Sigma-Aldrich)-coated plates. Cells were grown in DMEM/F12 medium (Invitrogen) for 10 days supplemented with 4 μg/mL insulin (Sigma-Aldrich), B27 (Invitrogen), 20 ng/mL EGF (Sigma-Aldrich), and 20 ng/mL basic FGF (Invitrogen).

### 2.4. Flow cytometric analysis

Phycoerythrin (PE)-conjugated CD24 or CD47 (BD Biosciences) was incubated in phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS) for 30 minutes at 4°C. Isotype-matched mouse immunoglobulins served as controls. The samples were analyzed using a BD Accuri C6 flow cytometer and FACSDiva software (BD Biosciences).

### 2.5. Annexin V apoptosis assay

Cells were stained in binding buffer (BD Biosciences), propidium iodide (PI, Invitrogen) and FITC-conjugated Annexin V (BioVision) for 30 minutes at room temperature. The apoptosis percentage was determined using a BD Accuri C6 flow cytometer and FACSDiva software (BD Biosciences).

### 2.6. Tumorigenicity assay

*In vivo* evaluation of tumorigenicity was performed with NOD-SCID mice by induction of tumor xenografts. Cells were suspended in 1:1 culture medium and BD Matrigel Matrix (BD Biosciences) and subcutaneously injected into the flanks of the NOD-SCID mice, which were kept under observation. Briefly, each mouse received two injections of cells in both flanks, and cells from each experimental group (NTC vs shNRF2) were injected into different mice. Tumors were harvested at the end of the experiment for documentation. Tumor-initiating cell frequency was calculated using Extreme Limiting Dilution Analysis (ELDA) software. The study protocol was approved by and performed in accordance with the guidelines for the Use of Live Animals in Teaching and Research at the University of Hong Kong and the Hong Kong Polytechnic University.

### 2.7. Statistical analysis

The statistical significance of the qPCR, spheroid formation assay, flow cytometry analysis, invasion assay and migration assay results was determined by Student's t test using Microsoft Office Excel software (Microsoft Corporation). The results are shown as the means and standard deviations, and $p$ values less than 0.05 were considered statistically significant (* $p$<0.05, ** $p$<0.01, *** $p$<0.001, **** $p$<0.0001). A chi-square test was used to assess the correlations between clinicopathological parameters and NRF2 expression. Kaplan-Meier survival analysis was used to analyze disease-free survival and overall survival, and statistical significance was calculated by the log-rank test; these analyses were carried out using SPSS 20.

Additional experimental procedures are provided in the Supplementary Information.

## 3. Results

### 3.1. NRF2 is activated in enriched liver T-IC populations and overexpressed in human HCC specimens

In our previous study, we established drug-resistant hepatospheres derived from PLC/PRF/5 cells via the administration of chemotherapeutic drugs (cisplatin and doxorubicin) over 16 serial passages. These hepatospheres showed enhanced capabilities for self-renewal and tumorigenicity compared with those in their differentiated counterparts (8). Upon further gene expression analysis of the drug-sensitive versus drug-resistant hepatospheres, we found that the NRF2 inhibitor KEAP1 was downregulated by ~2.5-fold in drug-resistant hepatospheres (GEO accession number GSE47563). Consistent with this finding, we observed increased cytoplasmic and nuclear NRF2 protein expression in hepatospheres derived from PLC/PRF/5 cells by western blotting and immunofluorescence (IF) staining analyses **(Figure 1A-B)**. Quantitative reverse transcription polymerase chain reaction (qPCR) confirmed this observation, showing elevated expression of NRF2 downstream targets, including NQO1, GSAT4, HMOX1 and GCLC, in hepatospheres derived from PLC/PRF/5 cells **(Figure 1C)**. Upon analysis of the publicly available dataset GSE25097, we found that NQO1 and GSTA4 were significantly upregulated in HCC compared with adjacent nontumor liver tissue, suggesting an oncogenic role of NRF2 in liver carcinogenesis **(Figure 1D)**. To further confirm NRF2 overexpression in HCC, we utilized a tissue microarray consisting of 100 HCC samples and corresponding matched nontumor liver tissues to evaluate NRF2 expression by immunohistochemical staining. A quick scoring method was employed to quantitate the expression of NRF2 (please refer to Supplementary Information for details). Approximately 51% of the patients showed an upregulation of NRF2 in tumor tissue compared with nontumor tissue **(Figure 1E)**. Statistical analysis revealed that the upregulation of NRF2 was significantly associated with elevated AFP levels ($p$=0.016, chi-square test) and vascular invasion ($p$=0.022, chi-square test) **(Table 1)**. Patients with high NRF2 expression had shorter overall and disease-free survival ($p$=0.008 and $p$=0.023, respectively; log-rank test) **(Figure 1F)**. Interestingly, there was no significant increase in NRF2 mRNA levels in 41 randomly selected HCC patient specimens by qPCR analysis **(Figure S1)**. This result suggests that NRF2 may play an oncogenic role at the protein level.

### 3.2. NRF2 regulates the T-IC properties of HCC cells

To further examine whether NRF2 functionally contributes to liver T-IC phenotypes, we suppressed NRF2 expression in high NRF2-expressing Huh7 and Bel7402 cells using a lentiviral-based approach. Western blotting showed successful establishment of knockdown clones **(Figure 2A)**. Knockdown of NRF2 significantly reduced the spheroid-forming ability of both Huh7 and Bel7402 HCC cells **(Figure 2B)**. Next, flow cytometry was used to assess the expression levels of two known liver T-IC markers, CD24 and CD47. We found that shNRF2-transfected cells exhibited decreased expression of these markers **(Figure 2C)**. Finally, we determined how NRF2 may affect the tumorigenicity of HCC cells by an *in vivo* tumorigenicity assay using limiting dilution analysis. In both Huh7 and Bel7402 cells, knockdown of NRF2 dramatically reduced the size and number of tumors formed **(Figure 2D, Table S1)**.

### 3.3. NRF2 regulates the migration, invasion and chemoresistance of HCC cells

NRF2 activation was found to promote the metastatic phenotypes of cancer cells (19). Consistent with the above findings, Matrigel-coated (for invasion) and uncoated (for migration) Transwell assays showed that both Huh7 and Bel7402 shNRF2 cells had significantly lower migration and invasion efficiencies **(Figure 3A-B)**. Furthermore, suppression of NRF2 also resulted in the sensitization of HCC cells to the commonly used chemotherapeutic drug 5-fluorouracil (5-FU) **(Figure 3C)**. Annexin V staining demonstrated decreased survival and reduced resistance to 5-FU following NRF2 knockdown. The chemoprotective effect of NRF2 was not limited to 5-FU, as we obtained similar findings in shNRF2 cells upon treatment with doxorubicin **(Figure 3D)**. Collectively, we found NRF2 to play a critical role in HCC metastasis and the refractory nature of HCC to chemotherapy.

### 3.4. ROS-induced NRF2 activation regulates sorafenib resistance in HCC cells

In our previous study, we found that sorafenib-resistant HCC cells demonstrated enhanced T-IC traits (20). Since we showed that NRF2 plays a crucial role in the regulation of liver T-ICs, we hypothesize that NRF2 suppression sensitizes cells to sorafenib through the regulation of liver T-ICs. To test this hypothesis, we first examined the expression of NRF2 in our established sorafenib-resistant Bel7402 and Huh7 cells (20). By western blot analysis, we found upregulated nuclear NRF2 expression in sorafenib-resistant HCC cells compared with that in mock control cells **(Figure 4A)**. To further confirm this observation, we examined ARE/NRF2 luciferase activity in sorafenib-resistant

HCC cells. Consistently, we found enhanced NRF2 activity in these cells compared to that in mock control cells **(Figure 4B)**. ROS are known to play a crucial role in liver carcinogenesis (21). NRF2 has been reported to act as a major regulator of cellular redox levels (22). In view of this, we examined whether sorafenib modulates NRF2 activity via an increase in ROS flux. Upon the addition of $H_2O_2$ at doses of 25 μM and 100 μM, we found a corresponding increase in NRF2 protein and activity levels but not its mRNA levels **(Figure 4C&D, Figure S2)**. Consistently, sorafenib at doses of 5 μM and 10 μM also induced ROS in both Bel7402 and Huh7 cells **(Figure 4E)**. The increase in ROS levels was observed in cisplatin- and doxorubicin-treated HCC cells (**Figure S3**). Finally, we examined the role of NRF2 in the regulation of sorafenib resistance by ascertaining the effect of NRF2 knockdown on the sensitivity of HCC cells to sorafenib. By Annexin V staining, we found that the suppression of NRF2 in both Bel7402 and Huh7 cells led to decreased survival and reduced resistance to sorafenib **(Figure 4F)**.

### 3.5. NRF2 regulates the traits of liver T-ICs via the SHH/GLI signaling cascade

To determine the major downstream mediator of NRF2 pertaining to liver T-IC function, we performed bioinformatics analysis using the UCSC Genome Browser and found one putative NRF2 binding site downstream of the transcription start site in the SHH promoter **(Figure 5A)**. Using a chromatin immunoprecipitation (ChIP) assay, we demonstrated for the first time that NRF2 directly binds to the promoter of SHH. NRF2 suppression led to a decreased fold enrichment at this site **(Figure 5A)**. As shown in **Figure 5B**, both SHH mRNA and protein levels were consistently downregulated upon NRF2 knockdown in shNRF2 clones of Bel7402 and Huh7 cells. Likewise, downstream signaling molecules, including GLI1 and SUFU, were repressed upon NRF2 knockdown **(Figure 5C)**. According to The Cancer Genome Atlas - Liver Hepatocellular Carcinoma (TCGA-LIHC) dataset, there is a significant correlation between the expression of SHH and NRF2 in HCC specimens ($p$<0.0001) **(Figure 5D)**. The correlation between NRF2 and SHH/GLI signaling was further reinforced by the positive correlation between NRF2 and GLI1 expression in a cohort of 51 HCC samples (p=0.0198) **(Figure 5E).** To further confirm the role of SHH as the downstream effector of NRF2 in mediating T-IC function, we treated NRF2 knockdown HCC cells with recombinant SHH at a dose of 200 ng/mL to investigate whether the effects of NRF2 suppression can be eliminated by recombinant SHH. We compared sphere-forming ability, sorafenib sensitivity and T-IC marker

expression of shNRF2 HCC cells with or without recombinant SHH. We found that the addition of recombinant SHH recovered the inhibitory effects of NRF2 knockdown on sphere formation and T-IC marker expression and reversed the effects on sorafenib sensitization **(Figure 6A-C)**. This result demonstrated that NRF2 may regulate liver T-IC function, at least in part through the SHH/GLI signaling cascade.

## 4. Discussion

Using an *in vitro* liver T-IC enrichment approach by serial passaging of hepatospheres in the presence of anticancer chemotherapeutic drugs, we found that NRF2 represents a crucial transcription factor regulating the stemness properties of HCC. NRF2 was first identified to play a critical role in regulating homeostasis in hematopoietic stem cells through interaction with CXCR4 (23). In response to dynamic changes in intracellular ROS levels, NRF2 regulates airway basal stem cell homeostasis through the Notch signaling pathway (24). Recently, NRF2 was reported to play a crucial role in induced pluripotent stem cell reprogramming (25). Although previous reports support the role of NRF2 in the regulation of T-ICs in a variety of cancer types, including colon cancer, glioma and breast cancer (17-19), the regulatory mechanism of NRF2 in T-ICs is not well characterized.

In HCC, two recent studies using whole-exome sequencing reported mutations of either *NRF2* (6.4%) or *KEAP1* (8%) (14, 26), suggesting the significance of NRF2 activation in a subset of HCC samples. By analyzing publicly available datasets, we found a significant increase in the expression of NRF2-associated antioxidant genes, including NQO1 and GSTA4, in HCC, suggesting an oncogenic role of NRF2 in liver carcinogenesis. However, we did not observe an increase in NRF2 mRNA expression in this dataset or in our qPCR analysis of 41 in house HCC clinical samples. This result is contrary to a previous report showing increased levels of *NRF2* mRNA in HCC to be significantly correlated with poor prognosis (27). By immunohistochemical staining analysis, we found that NRF2 protein was upregulated in HCC compared with adjacent nontumor liver samples. Furthermore, we found that overexpression of NRF2 was significantly correlated with an aggressive phenotype and poor patient survival in HCC. This result suggests that NRF2 may play an oncogenic role upon translational regulation. This clinical observation is in line with the results of an NRF2 knockout rat model showing that NRF2 plays a crucial role in the early stages of hepatocarcinogenesis (16).

NRF2 expression levels are positively correlated with the expression levels of liver T-IC markers, including CD24 (5) and CD47 (8). Using a knockdown approach, we found that suppression of NRF2 inhibited both the sphere-forming ability and the tumor-forming ability of HCC cells. Consistently, NRF2 has been found to play a regulatory role in mammosphere and tumor formation in breast and brain cancer models (18, 28). Some earlier studies suggested a role of NRF2 activation in

chemoresistance. In colon cancer, NRF2 signaling was found to be linked to doxorubicin resistance in T-IC-enriched colon spheres (29). In addition, silencing NRF2 led to sensitization of pancreatic cancer cells to 5-FU (30). Consistent with previous findings, we found that NRF2 regulates the sensitivity of HCC cells to 5-FU and doxorubicin. Specifically, we found that NRF2 was activated in our established sorafenib-resistant HCC cells, and suppression of NRF2 sensitized these cells to sorafenib. This finding indicates that the activation of NRF2 contributes to the acquisition of resistance to sorafenib. This result is consistent with previous reports showing a potential role for the p62-KEAP-NRF2 pathway in sorafenib resistance in HCC cells (31). Our study further provides evidence that NRF2 regulates sorafenib resistance through modulation of liver T-ICs. Apart from this mechanism, regulation of ROS levels is also crucial for determining the sensitivity of HCC cells to sorafenib (32). Consistently, we showed that sorafenib modulates NRF2 activity via an increase in ROS flux. Administration of $H_2O_2$ at doses of 25 μM and 100 μM led to a corresponding increase in NRF2 protein levels and activity. However, there was no change in NRF2 mRNA levels. Based on these findings, we believe that ROS regulate NRF2 in a transcription-independent manner. Similar to previous findings (31,33), we believe that ROS leads to dissociation of KEAP1 and NRF2, resulting in an increase in both NRF2 activity and its protein levels. In Figure 4C, we found that ROS-induced activation of NRF2 leads to resistance to sorafenib. In Figure S3, we found that the increase in ROS levels was also observed in cisplatin- and doxorubicin-treated HCC cells. Therefore, ROS-induced NRF2 activation may regulate resistance not only to sorafenib but also to chemotherapeutic drugs.

Through bioinformatics analysis, we found one putative NRF2 binding site upstream of the transcription start site in the SHH promoter. Using a ChIP assay, we found a physical interaction between NRF2 and the SHH promoter. Upon knockdown of NRF2, we found a consistent decrease in downstream signaling molecules, including GLI1 and SUFU. Furthermore, NRF2 and SHH were also positively correlated in an expanded cohort of clinical HCC samples, strongly indicating that SHH is likely regulated by NRF2. In this study, we confirmed that SHH is a downstream effector of NRF2, and recombinant SHH was employed to rescue the T-IC phenotypes in shNRF2-transfected cells. Interestingly, recombinant SHH reversed the suppressive effect of NRF2 knockdown on self-renewal, sorafenib resistance and T-IC marker expression in HCC cells. Using a transgenic mouse model, SHH was found to induce liver fibrosis and promote hepatocarcinogenesis by activating the sonic

hedgehog pathway (34). Clinically, the expression level of SHH is significantly upregulated in HCC (35, 36). Although the genes involved in sonic hedgehog signaling have been identified as therapeutic targets for different types of cancer, including HCC (37), its upstream regulator remains unidentified. Our study suggests a novel regulatory mechanism for the canonical sonic hedgehog pathway that may function through the NRF2/SHH/GLI signaling axis, thus mediating T-IC phenotypes.

In conclusion, we have demonstrated that NRF2 regulates liver T-ICs and sorafenib resistance in HCC cells through regulation of the SHH/GLI signaling cascade (**Figure S4**). Targeting NRF2 alone or in combination with other targets may be a novel therapeutic strategy for the treatment of HCC.

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**Figure Legends**

**Figure 1. NRF2 is activated in the enriched T-IC population and contributes to poor patient survival. (A-B)** Western blot and immunofluorescence analyses show the expression of cytoplasmic and nuclear NRF2 in hepatospheres and their corresponding differentiated counterparts. Scale bar represents 25 μm. **(C)** qPCR analysis confirmed the upregulation of genes downstream of NRF2, including NQO1, GSTA4, HMOX1, and GCLC (*$p$<0.05, ** $p$<0.01, $t$ test, ns: not significant). **(D)** By analysis of the publicly available dataset GSE25097, we found upregulation of NQO1 and GSTA4 mRNA in a cohort of 243 paired HCC and nontumor liver samples (***$p$<0.001, ****$p$<0.0001, $t$ test). In addition, NQO1 and GSTA4 were upregulated in cirrhotic samples compared with healthy donor samples (**$p$<0.01, **** $p$<0.0001, $t$ test). **(E)** A tissue microarray consisting of 100 tumor tissues and corresponding nontumor liver tissues was subjected to IHC analysis. Representative IHC images show NRF2 expression in HCC and its corresponding nontumor counterpart (case 20). Two HCC cases, one with low NRF2 expression (case 23) and the other with high NRF2 expression (case 63), are shown. Scale bar represents 20 μm. **(F)** The overall and disease-free survival rates of HCC patients with high NRF2 overexpression were significantly lower than those of patients with low NRF2 expression ($p$=0.008 and $p$=0.023, respectively; log-rank test).

**Figure 2. NRF2 regulates the liver T-IC characteristics of HCC cells. (A)** Two different shNRF2 sequences were used (shNRF2-1 and shNRF2-2) for lentivirus-based knockdown. Western blotting shows the successful knockdown of NRF2 in Bel7402 and Huh7 cells. **(B)** Knockdown of NRF2 reduced the size and number of hepatospheres formed by Bel7402 and Huh7 cells (**$p$<0.01, ***$p$<0.001, $t$ test). The scale bar represents 100 μm. **(C)** Knockdown of NRF2 in Bel7402 and Huh7 cells also decreased the expression of CD24 and CD47 compared with NTC cells (*$p$<0.05, ***$p$<0.001, $t$ test). **(D)** Knockdown of NRF2 in Bel7402 and Huh7 cell lines suppressed tumorigenicity compared with that in NTC cells. Representative photos show tumor formation after the injection of 10000 and 500000 Bel7402 and Huh7 cells.

**Figure 3. NRF2 regulates the migration, invasion and chemoresistance of HCC cells.** Knockdown of NRF2 reduced the number of **(A)** migratory and **(B)** invasive HCC cells in uncoated and Matrigel-

coated Transwell assays, respectively (**$p$<0.01, ***$p$<0.001, $t$ test). Scale bars represent 100 μm. Bel7402 and Huh7 shNRF2 cells showed a higher percentage of annexin V positivity in response to **(C)** 5-fluorouracil (5-FU) and **(D)** doxorubicin (*$p$<0.05, **$p$<0.01, $t$ test).

**Figure 4. ROS-induced NRF2 activation regulates sorafenib resistance in HCC cells. (A)** Sorafenib-resistant clones derived from Bel7402 and Huh7 cells showed increased nuclear NRF2 protein expression compared with that in their corresponding mock counterparts by western blot analysis. **(B)** Histograms show an increase in the relative luciferase activity of the antioxidant responsive element (ARE), presented as the ratio of firefly luciferase to Renilla luciferase activity, in sorafenib-resistant HCC cells compared with their corresponding mock counterparts (*$p$<0.05, $t$ test). **(C)** Flow cytometry analysis revealed that 25 and 100 μM $H_2O_2$ increased the levels of reactive oxygen species (ROS) in Bel7402 and Huh7 cells (*$p$<0.05, $t$ test). Following the addition of 25 and 100 μM $H_2O_2$, NRF2 levels in Bel7402 and Huh7 cells were increased, as shown by western blot analysis. **(D)** $H_2O_2$ at 25 and 100 μM increased the luciferase activity of the antioxidant responsive element (ARE) in Bel7402 and Huh7 cells (*$p$<0.05, **$p$<0.01, $t$ test). **(E)** Sorafenib at doses of 5 and 10 μM increased ROS levels in a dose-dependent manner (*$p$<0.05, $t$ test). **(F)** Knockdown of NRF2 in Bel7402 and Huh7 cells led to a higher percentage of Annexin V-positive cells in response to sorafenib (*$p$<0.05, **$p$<0.01, ***$p$<0.001, $t$ test).

**Figure 5. NRF2 directly binds to the SHH promoter and regulates its expression. (A)** Schematic diagram showing one NRF-2 binding site (+703 bp) on the promoter of SHH. Knockdown of NRF2 in Bel7402 and Huh7 cells reduced the fold enrichment of binding to the SHH promoter, as shown by ChIP assays (*$p$<0.05, ***$p$<0.001, $t$ test). **(B)** Bel7402 and Huh7 shNRF2 cells showed reduced protein and gene expression of SHH by western blotting and qPCR, respectively (*$p$<0.05, **$p$<0.01, $t$ test). **(C)** Western blot analysis shows decreased expression of GLI1 and SUFU in Bel7402 and Huh7 cells upon knockdown of NRF2. **(D)** A significant correlation between the expression of SHH and NRF2 in HCC tumors was identified according to The Cancer Genome Atlas - Liver Hepatocellular Carcinoma (TCGA-LIHC) database ($p$<0.0001). **(E)** Expression of NRF2 was significantly correlated with GLI1 in a cohort of 51 HCC clinical samples ($p$=0.0198; Fisher's exact test). Case #10 showed high expression of both NRF2 and GLI1, while case #14 showed low expression of these proteins.

**Figure 6. NRF2 directly regulates the SHH/GLI signaling cascade. (A)** The addition of 200 ng/mL recombinant SHH protein abolished the inhibitory effect of NRF2 knockdown on the size and number of spheres formed from Bel7402 and Huh7 cells (*$p$<0.05, **$p$<0.01, *t* test). **(B)** The addition of 200 ng/mL recombinant SHH also reduced the percentage of Annexin V-positive Bel7402 and Huh7 cells in response to sorafenib treatment (*$p$<0.05, **$p$<0.01, *t* test). **(C)** The addition of 200 ng/mL recombinant SHH increased the expression of CD47 and CD24 upon NRF2 knockdown in Bel7402 and Huh7 cells (*$p$<0.05, **$p$<0.01, ***$p$<0.001, *t* test).