NDUFA4L2 fine-tunes oxidative stress in hepatocellular carcinoma

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TRANSLATIONAL RELEVANCE

Hepatocellular carcinoma (HCC) frequently experiences hypoxia. Hypoxia results in an inefficient transfer of electrons during oxidative phosphorylation leading to increased oxidative stress. In this study, we demonstrated that a less active complex I subunit in the electron transport chain, NDUFA4L2, was significantly over-expressed in HCC and other human cancer types. Over-expression of NDUFA4L2 was associated with aggressive clinical features in human HCC and shorter overall survival in HCC patients. A series of *in vitro* and *in vivo* assays converged to show that NDUFA4L2 reduced ROS-mediated apoptosis to confer HCC cells growth advantages. As NDUFA4L2 is a direct transcriptional target of HIF, we found that HIF inhibitor, digoxin, profoundly inhibited growth of tumors that expressed high level of NDUFA4L2 in orthotopic model. Our findings suggested that cancer patients with NDUFA4L2 over-expression may be suitable candidates for HIF inhibitor treatment.
ABSTRACT

Purpose: Hepatocellular carcinoma (HCC) lacks effective curative therapy. Hypoxia is commonly found in HCC. Hypoxia elicits a series of pro-tumorigenic responses through hypoxia-inducible factor-1 (HIF-1). Better understanding of the metabolic adaptations of HCC cells during hypoxia is essential to the design of new therapeutic regimen.

Experimental Design: Expressions of genes involved in the electron transport chain (ETC) in HCC cell lines (20% and 1% O₂) and human HCC samples were analyzed by transcriptome sequencing. Expression of NDUFA4L2, a less active subunit in complex I of the ETC, in 100 pairs of HCC and non-tumorous liver tissues were analyzed by qRT-PCR. Student’s t test and Kaplan-Meier analysis were used for clinicopathological correlation and survival studies. Orthotopic HCC implantation model was used to evaluate the efficiency of HIF inhibitor.

Results: NDUFA4L2 was drastically over-expressed in human HCC and induced by hypoxia. NDUFA4L2 over-expression was closely associated with tumor microsatellite formation, absence of tumor encapsulation, and poor overall survival in HCC patients. We confirmed that NDUFA4L2 was HIF-1-regulated in HCC cells. Inactivation of HIF-1/NDUFA4L2 increased mitochondrial activity and oxygen consumption, resulting in ROS accumulation and apoptosis. Knockdown of NDUFA4L2 markedly suppressed HCC growth and metastasis in vivo. HIF inhibitor, digoxin, significantly suppressed growth of tumors that expressed high level of NDUFA4L2. Conclusions: Our study has provided the first clinical relevance of NDUFA4L2
in human cancer and suggested that HCC patients with NDUFA4L2 over-expression may be suitable candidates for HIF inhibitor treatment.
INTRODUCTION

Hepatocellular carcinoma (HCC), a malignancy derived from hepatocytes, accounts for 90% of primary liver cancer. It is the fifth most common cancer and the third leading cause of cancer deaths in the world (1). Majority of deaths in HCC is attributed to its asymptomatic nature which delays diagnosis and treatment. Most HCC patients are not suitable for the only promising curative therapies, surgical resection and liver transplantation. Sorafenib, an oral multi-kinase inhibitor and the only FDA approved drug for advanced HCC patients, could modestly prolong the survival of patients for 3 months (2, 3). Liver is an organ responsible for many important metabolic functions in the body such as the Cori-cycle, glycogen metabolism, and blood glucose homeostasis. Hepatocarcinogenesis is accompanied by the loss of normal metabolic functions in the liver and the acquisition of new metabolic functions which favor cancer growth. Exploration on the molecular contexts associated with these metabolic changes will help to identify novel targets for HCC treatment.

Hypoxia, or oxygen (O₂) deprivation, is frequently found in regions of HCC that are distant from functional vasculature. Palliative therapies such as transcatheter arterial (chemo)embolization (TAE/TACE) and hepatic artery ligation that involve restriction of blood supply to the tumors adversely induce hypoxia (4). To overcome the shortage of O₂, cells adapt to hypoxia through HIFs which are composed of the constitutively expressed
HIF-1β subunit and the oxygen labile subunit HIF-1/2α (5). In the presence of O₂, HIF-1/2α is hydroxylated by prolyl hydroxylases (PHDs) (6), facilitating binding of von Hippel-Lindau (VHL), which poly-ubiquitinates HIF-1/2α for proteosomal degradation (7). In the absence of O₂ stabilized HIF-1/2α dimerizes with HIF-1β to initiate transcription of genes related to hypoxia adaptive responses that advantage cancer development (8). While it is known that upregulation of HIF-1 is closely associated with poor clinical outcome in HCC patients (4), the detailed molecular mechanisms by which HIF-1 promotes HCC progression remain poorly understood.

HIF-1α transcriptionally activates many metabolic genes, allowing the cells to adapt to hypoxia, by shunting the glucose intermediates into glycolysis instead of the TCA cycle (9). These metabolic genes include glucose transporter (GLUT1), hexokinase 2 (HK2), lactate dehydrogenase A (LDHA), and pyruvate dehydrogenase kinase 1 (PDK1). GLUT1 facilitates glucose uptake (10). HK2, hexokinase 2 catalyzes the phosphorylation of glucose to glucose-6-phosphate, the first step of glycolysis (11, 12). LDHA converts pyruvate to lactate (13, 14). PDK1 inactivates pyruvate dehydrogenase (PDH) to prevent pyruvate conversion to acetyl CoA. These processes restrict the entrance of glucose intermediates into the TCA cycle, thereby reducing the activity of oxidative phosphorylation (OXPHOS) in the mitochondria (9). Interestingly, all these metabolic genes are involved in cancer progression.
The OXPHOS system comprises 4 electron transport chain (ETC) complexes including complex I (NADH-ubiquinone oxidoreductase), complex II (succinate:ubiquinone oxidoreductase), complex III (ubiquinol-cytochrome c reductase), and complex IV (cytochrome c oxidase). Most ATP in the cells is produced when electrons transfer through these complexes to the ultimate electron acceptor, O2. Complex I, a 1MDa complex of 45 subunits, is the first step where OXPHOS takes place. Complex I catalyzes the transfer of electrons from NADH to flavoprotein through eight iron-sulfur clusters, and finally to ubiquinone. Complex I is a major ROS-producing site. Low level of ROS has been shown to activate signaling pathways such as MAPK-ERK, JNK, p38 MAPK, and FAK to promote cancer cell survival and metastasis (15), while excessive ROS accumulation suppresses cancer cell growth through induction of G2/M cell cycle arrest and apoptosis (16-18). Most of the chemotherapeutic and radiotherapeutic strategies against cancer cells are mediated through ROS induction (19, 20).

HIF-1α regulates several ETC components to minimize ROS production and optimize mitochondrial respiration. HIF-1α induces the switching of COX subunit 4 isoforms from COX4-1 to COX4-2 in complex IV to maximize the efficiency of OXPHOS under hypoxia (21). HIF-1α induces expression of a microRNA, miR-210, which suppresses iron-sulfur
cluster assembly proteins (ISCU1/2) in complex I and III to reduce oxygen consumption, ROS production, and apoptosis during hypoxic stress (22). Mainly demonstrated in mouse embryonic fibroblasts, HIF-1α induced ETC complex I subunit, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4-like 2 (NDUFA4L2), to reduce complex I activity and ROS production (23).

Currently, knowledge about NDUFA4L2, particularly on its roles in cancer development is scarce. NDUFA4L2 was found to be one of the seven biomarkers that distinguish medullary thyroid carcinoma (MTC) from head and neck paraganglioma (PGL) (24). Earlier study showed that NDUFA4L2 is overexpressed in PGL tumors that lack VHL (25). Nonetheless, its clinical implications and in vivo roles in cancers, particularly in HCC, have never been thoroughly studied. This study uncovers the clinical relevance and roles of NDUAF4L2 in REDOX homeostasis in HCC. Furthermore, we have successfully demonstrated that targeting HIF-1/NDUFA4L2 pathway by HIF inhibitor represent a novel therapeutic strategy for HCC.
MATERIALS AND METHODS

Patient samples

Human HCC and the corresponding paired non-tumorous liver tissues were collected at Queen Mary Hospital, the University of Hong Kong during surgical resection. Human lung SCC and the corresponding paired non-tumorous lung tissues samples were kindly provided by Dr. Maria P. Wong (the University of Hong Kong). Use of human samples was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster.

Cell lines

Human HCC cell line, PLC/PRF/5, and human cervical cancer cell line, HeLa, were obtained from American Type Culture Collect (ATCC) and cultured according to ATCC recommendations. Metastatic human HCC cell line, MHCC97L, was a gift from Dr. Z. Y. Tang (Fudan University of Shanghai). All the cell lines used were authenticated by the AuthentiFiler™ PCR Amplification Kit (Applied Biosystems) on September 1st, 2014. All cell lines used in this manuscript were thawed from the authenticated cell stock and used within 4 passages.

Clinicopathological correlation and patients’ survival analysis

The clinicopathological features of HCC patients were analysed by pathologist as we
previously described (26). The parameters included age, gender, tumor size, cellular differentiation by Edmondson grading, direct liver invasion, absence of tumor encapsulation, presence of tumor microsatellite formation, venous invasion and background liver disease. Overall survival was calculated from the date of surgical resection to the date of death or last follow-up. The prognostic significance of NDUFA4L2 over-expression was determined by the Kaplan-Meier method followed by the log-rank test, as we previously described (27, 28). All statistical tests were performed by SPSS20.0. The demographic data of HCC patients were summarized in Supplementary Table S1.

**Fluorescence imaging and flow cytometry analysis**

Cells were grown on glass coverslips in 6-well culture plate and cultured in 20% and 1% O₂ for 24 hrs. Cells were stained with 2 μM 5,5′,6,6′-tetrachloro-1,1′,3,3′ -tetaethyl-benzimidazolylcarbocyanine chloride (JC-1) (Invitrogen). Images were captured and scanned under 20X magnification with LSM 510 Meta laser scanning microscope (Carl Zeiss) connected to Axiocam microscope camera (Carl Zeiss). For flow cytometry analysis, cells were stained with 2 μM JC-1.

**Oxygen consumption assay**

Cells were grown on 96-well culture plate and cultured in 20% and 1% O₂ for 24 hrs. Cells
were stained with oxygen sensitive probe MitoXpress\textsuperscript{®}-Intra (NanO\textsubscript{2}) and washed according
to the manufacturer’s instruction (Luxcel Bioscience). Phosphorescence was measured by a
multilabel reader Victor\textsuperscript{2} (Perkin-Elmer Life Sciences) with 340 nm excitation and 642 nm
emission filters.

**ROS measurement**

Trypsinized cells were washed with PBS and stained with 2 μM general ROS indicator
chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate (CM-H\textsubscript{2}DCFDA) (Life Technologies)
or 5 μM mitochondrial ROS indicator MitoSOX\textsuperscript{TM} (Molecular Probes) for 5 and 10 mins
respectively and analyzed by flow cytometer FACSCanto II Analyzer (BD Biosciences). For
MitoSOX\textsuperscript{TM} staining, cells were washed with staining buffer 3 times before flow cytometry
analysis. Data from flow cytometry studies were analyzed by FlowJo software. For
antioxidant treatment, 0.5, 2, or 5 mM of N-acetyl-L-cysteine (NAC) or L-ascorbic acid
(Sigma Aldrich) were incubated at 37°C for 24 hrs prior to ROS measurement. For *in vivo*
ROS measurement, tumors harvested from mice were dissociated with gentleMACS\textsuperscript{™}
dissociator and single cell suspensions were stained with 2μM CM-H\textsubscript{2}DCFDA for flow
cytometry analysis.

**Apoptosis and cell proliferation assays**
For apoptosis measurement, 2.5 x 10^5 cells were cultured in 20% and 1% O_2 for 24 hrs. Apoptosis was determined by flow cytometry after staining with propidium iodide (Calbiochem) and annexin V (MBL International Corporation). For anti-oxidant treatment, 0.1 mM glutathione ethyl ester (GSH-EE) (Cayman Chemical) was added to MHCC97L-shL2 cells and cultured in 1% O_2 for 40 hrs. For cell proliferation measured by cell counting, 2 x 10^4 MHCC97L-NTC and -shL2 cells were grown on 12-well culture plate and cultured in 20% and 1% O_2 for 4 days. Cells were trypsinized and counted with Z1 coulter particle counter (Beckman Coulter) every 24 hrs. For cell proliferation measured by 5-bromo-2’-deoxyuridine (BrdU) assay, 2.5 x 10^4 MHCC97L-NTC and -shL2 cells were grown on 96-well culture plate and cultured in 20% and 1% O_2 for 24 hrs. BrdU labelling and colorimetric measurement were performed according to the manufacturer’s instructions (Roche Life Science).

**Animal studies**

All animal studies were approved by the Committee on the Use of Live Animals in Teaching and Research, the University of Hong Kong and followed under the Animals (Control of Experiments) Ordinance of Hong Kong. For orthotopic liver tumor injection in nude mice, 1 x 10^6 luciferase-labeled MHCC97L cells were injected into the left lobes of the livers of 6 to 8 week-old BALB/c nude mice. Six weeks after injection, mice were administered with 100
mg/kg D-luciferin by peritoneal injection 5 mins before bioluminescent imaging (IVIS™ 100 Imaging System, Xenogen). Livers and lungs were harvested for ex-vivo imaging and histological analysis. Livers were harvested for ex-vivo imaging and histological analysis as described above. For subcutaneous tumor model, 1 x 10⁶ MHCC97L cells were injected subcutaneously to the flanks of nude mice. Tumor volume was calculated with the formula: length (mm) x width (mm) x depth (mm) x 0.52 (mm³). For pharmacological studies, drug administration began a week after orthotopic or subcutaneous injection. Mice were administered with 1.2 mg/kg/day digoxin or vehicles (saline) by intraperitoneal injection for 21 consecutive days.

Transcriptome sequencing

Transcriptome sequencing was performed in 16 pairs of HCC tissues and corresponding NT liver tissues or MHCC97L cells exposed to 20% and 1% O₂ for 24 hrs. TruSeq standard mRNA sample Prep kit (Illumina) was used for polyA + mRNA library preparation. Illumina HiSeq2000 was employed for 100bp paired-end sequencing (Axeq Technologies) and data were analyzed by TopHat-Cufflinks pipeline (29). Values were indicated by FPKM (fragments per kilobase transcript sequence per million mapped reads). Pathway analysis was performed by the Database for Annotation, Visualization and Integrated Discovery (DAVID).
**Statistics**

Statistical analysis was performed by 2-tailed Student *t* test or Wilcoxon’s signed rank test using GraphPad Prism 5.0 (GraphPad Software Inc.). All functional assays are representative of ≥ 3 independent experiments and expressed as mean ± SEM. *P* value less than 0.05 was considered to be statistically significant.

**Cell lines, clinicopathological study, quantitative real-time PCR, establishment of knockdown and knockout HCC cells, in vitro assays and TCGA/Oncomine data**

The following are described in supplementary materials and methods: cell lines, clinicopathological study, quantitative real-time PCR (qRT-PCR), chromatin immunoprecipitation (ChIP) assay, establishment of HIF or NDUFA4L2 knockdown and HIF knockout HCC cells, mitochondrial membrane potential, mitochondrial mass, migration assay, histology and TCGA/Oncomine data.

**RESULTS**

*Mitochondrial NDUFA4L2 is frequently over-expressed in human HCC and other human*
solid cancers.

To better understand the hypoxia-adaptation system in HCC, we performed transcriptome sequencing to compare the gene expression profiles of a HCC cell line, MHCC97L, that were exposed to normoxia (20% O₂) and hypoxia (0.1% and 1% O₂). Among the up-regulated genes, mitochondrial NDUFA4L2 was one of the five most abundant genes in hypoxic conditions indicating the biological relevance of this gene in human HCC. Other well characterized HIF target genes including ANPTGL4 (30), PLOD2 (31), VEGF (32), CA9 (33) and P4HA2 (34), were on the top 20 of the list. Intriguingly, when we interrogated the expression of genes that are involved in the mitochondrial complex I, only the subunit NDUFA4L2 was distinctly and dramatically up-regulated in hypoxia (both 0.1% and 1% O₂) while all the other subunits remained unaffected or down-regulated (Fig. 1A). We further examined the transcriptome sequencing data on 16 primary HCCs and their corresponding non-tumorous (NT) livers and found that NDUFA4L2 was substantially over-expressed in HCC tissues (Fig. 1A). Moreover, over-expression of NDUFA4L2 mRNA was validated by quantitative real-time PCR (qRT-PCR) in a separate, larger cohort of 100 pairs of HCC samples (Fig. 1B). Furthermore, over-expression of NDUFA4L2 in HCC tumors was significantly associated with aggressive pathological features including presence of tumor microsatellite formation (Student $t$ test; $P=0.002$) and absence of tumor encapsulation (Student $t$ test; $P=0.039$) (Fig. 1C and Supplementary Table S4). More importantly,
over-expression of NDUFA4L2 tended to be associated with poorer overall survival of HCC patients (Student $t$ test; $P=0.086$) (Fig. 1C). The Cancer Genome Atlas (TCGA) database which includes transcriptome sequencing data of 49 pairs of HCC and NT liver tissues from an independent cohort echoed with our in-house data (Fig. 1D). Intriguingly, when we studied the microarray data available in the Oncomine database, we noticed that NDUFA4L2 was also over-expressed in other human solid cancers including renal cell carcinoma (RCC), lung squamous cell carcinoma (lung SCC), as well as colorectal carcinoma (Fig. S1). The over-expression of NDUFA4L2 mRNA in lung SCC was further confirmed in our 31 pairs of lung SCC tissues and their normal counterparts by qRT-PCR (Fig. 1E). Collectively, these results demonstrated the clinical relevance of NDUFA4L2 in human HCC and other human solid cancers.

**HIF-1α, but not HIF-2α, positively regulates NDUFA4L2 in hypoxia.**

To investigate whether NDUFA4L2 expression was mediated by HIFs under hypoxia, we generated HCC cells, MHCC97L, that stably expressed short hairpin RNA (shRNA) against HIF-1α (-shHIF-1α) or HIF-2α (-shHIF-2α) (Fig. S2A). The hypoxia-induced NDUFA4L2 expression was markedly abolished when HIF-1α, but not HIF-2α was knocked down (Fig. 2A). We further established HIF-1α knockout cells by Transcription Activator-like Effector Nuclease (TALEN) approach and found that hypoxia-induced NDUFA4L2 expression was
drastically abrogated (Fig. 2B and S2B). Digoxin inhibited HIF-1 protein synthesis and profoundly inhibited hypoxia-induced NDUFA4L2 expression in a dose dependent manner (Fig. 2C). In addition, by in silico analysis, we identified two potential hypoxia response elements (HREs) containing the core HIF binding sequence motif 5’-RCGTG-3’ at the promoter of NDUFA4L2 (Fig. 2D). ChIP assay in cell lines of different origins, MHCC97L and HeLa cells, indicated that DNA was enriched when HIF-1α antibody was used, as compared to the IgG control (Fig. 2E and S3). Intriguingly, we consistently detected a band beneath the expected protein size of NDUFA4L2. This protein was only expressed in normoxia but not in hypoxia (Fig. 2A, 2B, 2C, 3A and 3B). NDUFA4L2 shared 67% amino acid sequence with its paralogue NDUFA4, which is 6 amino acids shorter than NDUFA4L2. To confirm the identity of the unknown protein beneath NDUFA4L2, we knocked down NDUFA4 (-shA4-30 and -shA4-88) in MHCC97L cells and confirmed that the lower band was NDUFA4 (Fig. S4A and S4B). These results demonstrated that NDUFA4L2 was preferentially expressed under hypoxia solely through HIF-1α while NDUFA4 protein was preferentially expressed under normoxia.

NDUFA4L2 reduces mitochondrial activity by decreasing oxygen consumption and prevents excessive ROS production under hypoxia.
To investigate the functions of hypoxia-induced NDUFA4L2 in mitochondria in vitro, we generated NDUFA4L2 loss-of-function and gain-of-function HCC cell models. For NDUFA4L2 loss-of-function HCC cell model, shRNA against NDUFA4L2 (shL2) or non-target control (NTC) was stably expressed in MHCC97L cells by lenti-viral transfection approach (Fig. 3A). For NDUFA4L2 gain-of-function HCC cell model, NDUFA4L2 and empty vector (EV) were stably expressed in another HCC cell line which expressed a lower level of NDUFA4L2, PLC/PRF/5 (PLC) (Fig. 3B). As NDUFA4L2 is located in the first complex of the ETC, we asked if NDUFA4L2 would affect the mitochondrial activity by staining the cells with a probe, JC-1, which accumulates in active mitochondria to indicate mitochondrial potential. By fluorescence imaging, we showed that the mitochondrial membrane potential was decreased in hypoxia but was elevated when NDUFA4L2 was knocked down (Fig. 3C). Flow cytometry analysis further confirmed the result (Fig. 3C). Similar observation was obtained with tetramethylrhodamine ethyl ester (TMRE), another fluorescent probe that measures the mitochondrial membrane potential (Fig. S5A). Consistently, mitochondrial mass was increased in NDUFA4L2 knockdown cells as indicated by the nonyl acridine orange (NAO) staining (Fig. 3D). Consistently, we found that oxygen consumption in the mitochondria was significantly elevated when NDUFA4L2 was knocked down under hypoxia (Fig. 3E). HIF-1α knockout mirrored the effect of NDUFA4L2 knockdown in oxygen consumption (Fig. 3E), further supporting the regulatory role of
HIF-1α on NDUFA4L2. Hypoxia triggers an imbalanced electron flow through the ETC leading to generation of ROS in the mitochondria. Hence, we evaluated the intracellular ROS level by the general ROS indicator, chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) and mitochondrial ROS indicator, MitoSOX™. ROS level was elevated when cells were exposed to hypoxia and was further induced when NDUFA4L2 was knocked down especially under hypoxic condition (Fig. 3F). Reversely, a significant reduction of ROS was found in NDUFA4L2 overexpressing HCC cells (Fig. 3F). Similar trend was observed in HCC cells that expressed the constitutively active form of HIF-1α (CA5) (Fig. 3F). To eliminate off-target effect, small interfering RNAs (siRNAs) targeting different sequences of NDUFA4L2 were transfected into MHCC97L cells (Fig. S5B) and similar metabolic consequences were found as compared to stable knockdown of NDUFA4L2 (Fig. S5C and S5D).

NDUFA4L2 maintains redox homeostasis and promotes cell proliferation and survival.

Knockdown of NDUFA4L2 resulted in a dramatic increase of ROS, which we speculated would be harmful to HCC cells. To demonstrate the roles of ROS in HCC cells, we employed two antioxidants, N-acetyl-L-cysteine (NAC) and L-ascorbic acid. ROS induced by NDUFA4L2 knockdown was rescued dose dependently by both antioxidants (Fig. 4A). To further demonstrate the regulation of HIF-1α on NDUFA4L2 in ROS scavenging, we
re-expressed NDUFA4L2 in HIF-1α knockdown PLC cells. ROS was dramatically induced in HIF-1α knockdown cells under hypoxia and was significantly rescued upon re-expression of NDUFA4L2 (Fig. 4B). Excessive accumulation of ROS can cause cell senescence and apoptosis, eventually leading to cell death (16-18). We therefore evaluated apoptosis by annexin V-propidium iodide (PI) staining in MHCC97L-NTC and -shL2 cells. MHCC97L-NTC cells showed higher annexin V and PI staining under hypoxic than normoxic condition, while knockdown of NDUFA4L2 further increased apoptosis (Fig. 4C), which could be rescuated by extracellular glutathione ethyl ester (GSH-EE), a modified and more permeable form of glutathione, an antioxidant that counteracts mitochondrial ROS (Fig. S6A and S6B). To further confirm that increased apoptosis was caused by ROS, we exogenously treated the cells with H$_2$O$_2$, a common source of ROS (35). Apoptosis was elicited by H$_2$O$_2$ drastically in MHCC97L-NTC cells and was further elevated in -shL2 cells (Fig. S6C).

Consistently, both the cell counting and 5-bromo-2’-deoxyuridine (BrdU) assays showed that cell proliferation was substantially reduced when NDUFA4L2 was knocked down especially under hypoxic condition (Fig. 4D and S6D). More interestingly, cell proliferation was restored by NAC in the NDUFA4L2 knockdown HCC cells (Fig. 4E), suggesting that impairment of cell proliferation was associated with ROS. All these data converged to show that NDUFA4L2 was responsible to reduce ROS under hypoxia, thereby maintaining cell survival and promoting cell proliferation.
**NDUFA4L2 promotes tumor growth, reduces oxidative stress and enhances lung metastasis.**

To demonstrate the functions of NDUFA4L2 in tumor growth *in vivo*, we orthotopically injected the luciferase-labeled MHCC97L-NTC and -shL2 cells into nude mice. MHCC97L-NTC tumors grew substantially larger than the MHCC97L-shL2 tumors (Fig. 5A and 5B). To evaluate the proliferative output of MHCC97L-NTC and -shL2 cells *in vivo*, we stained the tumors with proliferative marker (Ki67). We found that knockdown of NDUFA4L2 markedly reduced Ki67 staining (Fig. 5C). We further dissociated the tumors and measured the ROS level of the tumors derived from MHCC97L-NTC and -shL2 cell lines. Consistent with our *in vitro* data, knockdown of NDUFA4L2 elevated ROS *in vivo* (Fig. 5D). Moreover, knockdown of NDUFA4L2 markedly repressed growth of metastatic lesions in the lungs of the tumor-bearing mice as indicated by Xenogen imaging (4 out of 6 in MHCC97L-NTC and 1 out of 6 in -shL2 group) (Fig. 5E). We further found that knockdown of NDUFA4L2 significantly reduced cell migratory ability of HCC cells in Transwell assay. Interestingly, the migratory ability of HCC cells was inversely correlated with the amount of intracellular ROS (Fig. S7A and 3E), suggesting that ROS may also affect cell motility.

**Pharmacologic inhibition of HIF suppresses progression of tumors that express high level**
of NDUFA4L2.

So far, we found that hypoxia-induced NDUFA4L2 tightly regulate REDOX homeostasis and survival in HCC cells, and HIF-1α is the central regulator of NDUFA4L2. We therefore reasoned that HIF inhibitor could repress the tumors that express high levels of NDUFA4L2 by damaging HCC cells through elevating ROS. We evaluated the ROS levels of MHCC97L-NTC and -shL2 cells treated with a HIF inhibitor, digoxin, under hypoxic condition. We observed that digoxin was more efficient in elevating the ROS level in MHCC97L-NTC cells than -shL2 cells (Fig. 6A). Next we evaluated the effect of digoxin in vivo. We performed orthotopic implantation of the luciferase-labeled MHCC97L-NTC and -shL2 cells in nude mice. One week after implantation, mice were administered with 1.2 mg/kg/day digoxin or vehicle control (saline) through intraperitoneal (i.p.) injection for 21 days. Digoxin markedly repressed growth of tumors with a more prominent effect on the MHCC97L-NTC group as compared to MHCC97L-shL2 group (Fig. 6B). Similarly, digoxin decreased the growth of subcutaneous tumors derived from MHCC97L-EV cells more significantly as compared to MHCC97L-shHIF-1α cells (Fig. S7B). Meanwhile, body weights of the animals were not affected by digoxin (Fig. S7C). These results suggested that HIF inhibitor blocks HCC progression at least partially through HIF-1α/NDUFA4L2.

Potential gene targets of NDUFA4L2
To explore novel functions of NDUFA4L2, we performed transcriptome sequencing to compare the global gene expression profiles of MHCC97L-NTC and MHCC97L-shL2 cells. The expressions of 629 genes were found to be decreased in MHCC97L-shL2 cells for more than 0.5 fold as compared to MHCC97L-NTC (Supplementary Table S5). The expressions of 683 genes were found to be increased for more than 2 fold in MHCC97L-shL2 cells relative to MHCC97L-NTC (Supplementary Table S6). Intriguingly, pathway analysis by DAVID suggested that NDUFA4L2 may regulate multiple biological processes including membrane potential and membrane depolarization (Supplementary Table S7). Of note, NRF2, a transcription factor that activates anti-oxidant genes, was found to be slightly induced (FPKM 29.246 in MHCC97L-NTC Vs FPKM 34.151 in -shL2), suggesting that a compensatory anti-oxidant producing mechanism might be involved in response to the high ROS level upon NDUFA4L2 knockdown.

**DISCUSSION**

ROS are fundamentally produced from O$_2$ when it is consumed in different metabolic reactions. These reactions take place in the mitochondria at the ETC for ATP generation, endoplasmic reticulum during disulfide bond formation for protein folding, and peroxisomes during β-oxidation of fatty acids. Under hypoxia, these metabolic events cannot be carried out properly, leading to the generation of O$_2$-containing free radicals. In the mitochondria that
experiences shortage of the ultimate electron acceptor, O₂, electrons cannot be efficiently pass
through the ETC, leading to rapid accumulation of ROS in complex I and III. Hypoxia is a
common finding in HCC and our study revealed the molecular mechanisms by which hypoxic
HCC cells modulate ROS level in the mitochondria. Our study showed that HCC cells, by
utilizing NDUFA4L2 in the complex I of the ETC, reduced the activity of the mitochondria to
prevent ROS accumulation and apoptosis (Fig. 6C). Not only have we confirmed that this was
dependent on HIF-1α in HCC cells, more importantly, our study unprecedentedly revealed the
clinical relevance of NDUFA4L2 in human HCC. Apart from HCC, we also showed that
NDUFA4L2 was over-expressed in multiple solid cancers, suggesting that our findings do not
limit to HCC but applies to other solid cancer models that frequently experience hypoxia.
Notably, we and Tello et al. consistently found that NDUFA4 and NDUFA4L2 have opposite
expression patterns at normoxia and hypoxia in different cell models (23). Studies have
documented that HIF-1α could induce expression of siah E3 ubiquitin protein ligase 2
(SIAH2) and mitochondrial LON peptidase to degrade mitochondrial proteins such as
α-ketoglutarate dehydrogenase and COX4-1 to reduce mitochondrial flux under hypoxia,
respectively (21, 36). Whether NDUFA4 is degraded in a similar fashion remains an
interesting topic to be addressed.

Prenatal lethality in NDUFA4L2 knockout mice has greatly limited the expansion of
knowledge on the \textit{in vivo} functions of this complex I subunit (23). Our study, using orthotopic liver cancer model, has provided the first \textit{in vivo} evidence to confirm the pro-tumorigenic functions of NDUFA4L2. Our study also demonstrated that digoxin profoundly blocked growth of orthotopic tumors that expressed high level of HIF-1α/NDUFA4L2. Digoxin has been shown to be able to inhibit HIF-1α synthesis and is well-tolerated in patients (30). Digoxin has been used for the treatment of congestive heart failure and is currently under clinical trial for the treatment of breast cancer (ClinicalTrials.gov identifier: NCT01763931). Our study suggested that digoxin may be the most suitable candidate for patients whose primary HCCs express high levels of HIF-1α or NDUFA4L2.

Worth mentioning, complex I inhibitors including metformin has promising anti-tumor effects in the clinical setting. Metformin has been the most frequent prescribed antidiabetic medication in the world. By reducing the ATP production in the ETC, metformin activates tumor suppressor 5′-AMP-activated protein kinase (AMPK) (37). Reports have indicated that metformin reduced cancer incidence (38, 39). Metformin also reduced proliferative output of breast cancer cells in non-diabetic cancer patients (40). These interesting findings highlight an interesting dilemma – is energy or REDOX homeostasis more important for cancer survival? Accumulating studies suggested that ATP is not the limiting factor while excessive ROS is cell destructive. Unquestionably, cancer cells could not survive without energy; therefore,
cancer cells need to acquire a tightly controlled homeostatic balance for the ETC, a place that generates both ATP and ROS, to sustain tumor growth. Drugs that could tip this balance merits exploration for their efficiency in cancer treatment.

REDOX homeostasis can be achieved by two mechanisms: (1) reduction of ROS production, and (2) elevation of anti-oxidant production. Our study showed that NDUFA4L2 directly decreased the generation of ROS in the mitochondria of HCC cells. Meanwhile, genes that are associated with the production and utilization of anti-oxidants including NADPH, glutathione and thioredoxin have been implicated in HCC. Glucose 6 phosphate dehydrogenase (G6PD), a critical enzyme in the pentose phosphate pathway generating NADPH, has been shown to be over-expressed in HCC in a PTEN-dependent manner (41). Over-expression of thioredoxin (TXN) and thioredoxin-related REDOX molecules, glutaredoxin (GLRX) and peroxiredoxin (PRDX), was documented in HCC and was associated with increased proliferative output, aggressive clinicopathological parameters, presence of metastasis, and poor prognosis in HCC patients (42, 43). Elevation of cysteine/glutamate transporter, xCT, which is important for glutathione production, was found to be associated with poor clinical outcome in HCC and disruption of xCT in HCC cells increased ROS-mediated autophagic cell death (44). A recent study has elegantly demonstrated that co-treatment of inhibitors blocking different pathways that synthesize antioxidants could synergistically prevent tumor growth (45). Along similar
lines, our current study suggested that raising ROS level through HIF inhibitor which suppressed HIF-1α/NDUFA4L2 pathway represents an attractive therapeutic strategy for HCC treatment. In the coming decade, combination therapies targeting multiple machineries that buffer ROS represent an exciting translation research direction.

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Figure 1. NDUFA4L2 is over-expressed in human HCC and other human solid cancers.

(A) Transcriptional sequencing data comparing FPKM (fragments per kilobase transcript sequence per million mapped reads) values of genes encoding mitochondrial complex I subunits in HCC cells cultured in (left) 0.1% O2 or (middle) 1% O2 relative to 20% O2, and (right) 16 pairs of human HCC and the corresponding non-tumorous (NT) tissues. (B) mRNA expression levels of NDUFA4L2 normalized with hypoxanthine-guanine phosphoribosyltransferase (HPRT) in an expanded cohort of 100 cases of paired human HCC and corresponding NT tissues were determined by qRT-PCR. Waterfall plot shows that NDUFA4L2 is over-expressed in 71% of human HCC samples by at least 2 fold. (C) Clinico-pathological analysis shows that NDUFA4L2 over-expression is closely associated with more aggressive features in HCC including the presence of tumor microsatellite formation and the absence of tumor encapsulation. HCC patients with high NDUFA4L2 expression (2 median) tend to have lower 5-year overall survival rate (Student t test, P=0.086). (D) mRNA expression levels of NDUFA4L2 obtained from The Cancer Genome Atlas (TCGA) database of 49 paired human HCC and corresponding NT tissues. (E) mRNA expression levels of NDUFA4L2 normalized with β-actin in 31 cases of paired human lung squamous cell carcinoma (SCC) and corresponding NT tissues were determined by qRT-PCR. Waterfall plot shows that NDUFA4L2 is over-expressed in 63% of human lung SCC samples by at least 2 fold.
Figure 2. NDUF4L2 is regulated by HIF-1α but not HIF-2α in HCC.

(A) mRNA and protein expression of NDUF4L2 in MHCC97L-EV, shHIF-1α and shHIF-2α cells cultured in 20% and 1% O₂ for 24 and 48 hrs, respectively. (B) NDUF4L2 protein expression in MHCC97L-WT (parental) and -HIF-1α/− (KO) cells cultured in 20% and 1% O₂ for 48 hrs. (C) NDUF4L2 mRNA and protein expression in MHCC97L cells treated with HIF inhibitor digoxin at the indicated doses. (D) Putative hypoxia response elements (HREs) are mapped to the promoter of NDUF4L2. Core HIF binding sequences (5’-RCGCTG-3’) are highlighted as bold with underlines while ancillary sequence is highlighted as bold. (E) ChIP assay was performed with IgG and HIF-1α antibodies in MHCC97L cells cultured in 20% and 1% O₂; qRT-PCR was performed after immuno-precipitation with anti-IgG or anti-HIF-1α antibodies, represented as fold enrichment normalized to 20% O₂. (Results represent mean ± SEM. Student t test; n = 3 independent experiments: *P<0.05; **P<0.01; ***P<0.001 as indicated).
Figure 3. NDUFA4L2 reduces mitochondrial activity, oxygen consumption and ROS production in hypoxia.

(A) mRNA and protein expression of NDUFA4L2 in MHCC97L-NTC and -shL2 cells exposed to 20% and 1% O₂ for 24 and 48 hrs, respectively (Results represent mean ± SEM. Student t test; n = 3 independent experiments: ***p<0.001 as indicated). (B) mRNA and protein expression of NDUFA4L2 in PLC-EV and -NDUFA4L2 cells cultured in 20% O₂ for 24 and 48 hrs, respectively (Results represent mean ± SEM. Student t test; n = 3 independent experiments: ***p<0.001 as indicated). (C) (Left) Representative fluorescent images of JC-1 stained MHCC97L-NTC and -shL2 cells that were exposed to 20% and 1% O₂. (Right) Mitochondrial membrane potential was further determined with JC-1 staining by flow cytometry analysis (Results represent mean ± SEM. Student t test; n = 5 independent experiments: ***p<0.001 as indicated). Scale, 50 μm. (D) Mitochondrial mass was measured with NAO staining by flow cytometry analysis (Results represent mean ± SEM. Student t test; n = 3 independent experiments: ***p<0.001 as indicated). (E) Oxygen consumption was measured with MitoXpress®-Intra (NanoO2) in MHCC97L-NTC and -shL2 cells or MHCC97L-WT and -HIF-1α/- (-KO) cells exposed to 20% and 1% O₂ (Results represent mean ± SEM. Student t test; n = 3 independent experiments: *p<0.05; **p<0.01 as indicated). (F) ROS level was measured with CM-H₂DCFDA or MitoSOX staining by flow cytometry analysis in (i and ii) MHCC97L-NTC and -shL2 cells cultured in 20% and 1% O₂, (iii) PLC-EV and -NDUFA4L2 cells cultured in 20% O₂ and (iv) PLC-EV and -CA5 cells cultured in 20% O₂ (Results represent mean ± SEM. Student t test; n = 3 independent experiments: *p<0.05; **p<0.01 as indicated). Values obtained in mitochondrial membrane potential, mitochondrial mass, oxygen consumption, ROS and mitochondrial ROS were normalized to 20% O₂ MHCC97L-NTC or PLC-EV.
Figure 4. NDUF4L2 reduces intracellular ROS to enhance HCC cell survival.

(A) CM-H2DCFDA staining showing the ROS levels of MHCC97L-NTC and -shL2 cells exposed to 1% O2 for 24 hrs in the presence of indicated concentrations of NAC and ascorbic acid. (Top) Representative flow cytometry analysis showing the ROS levels in the indicated subclones. (Bottom) Histograms summarizing the ROS levels in the indicated subclones. The ROS level was relative to 1% O2 MHCC97L-NTC. (B) CM-H2DCFDA staining showing the ROS levels of PLC-EV, -shHIF-1α and HIF-1α knockdown with NDUF4L2 overexpressing (-shHIF-1α + NDUF4L2) cells exposed to 1% O2 for 24 hrs. The ROS level was relative to 20% O2 PLC-EV. (Top) Representative flow cytometry analysis showing the ROS levels in the indicated subclones. (Bottom) Histograms summarizing the ROS levels in the indicated subclones. (C) Annexin V and PI staining showing the percentage of apoptotic cells in MHCC97L-NTC and -shL2 cells exposed to 20% and 1% O2 for 24 hrs. (D) Cell proliferation of MHCC97L-NTC and -shL2 cells exposed to 20% and 1% O2 for 96 hours. (E) BrdU assay of MHCC97L-NTC and -shL2 cells treated with indicated concentrations of NAC were exposed to 1% O2 for 24 hrs (Results represent mean ± SEM. Student t test; n = 3 independent experiments: *P<0.05; **P<0.01; ***P<0.001 as indicated).
Figure 5. Knockdown of NDUFA4L2 suppresses tumor growth and induces oxidative stress in vivo.

(A) Bioluminescence and (B) tumor volume of the orthotopic tumors derived from MHCC97L-NTC and -shL2 cells. Scale, 1 cm. (C) (Left) Representative IHC pictures of Ki67 positivity in tumors. Cells were scored from 0 to 3 based on the staining intensity as represented by the arrows. Scale, 100 μm. (Right) More than 1,000 cells were scored in each animal and average percentage was calculated for each score. (D) Workflow of the measurement of intracellular ROS level in mouse model. Nude mice were orthotopically injected with MHCC97L-NTC and -shL2 cells. CM-H₂DCFDA staining showing the ROS levels of the dissociated orthotopic tumors derived from MHCC97L-NTC and -shL2. (E) Bioluminescence of the lungs harvested from orthotopic tumor-bearing mice implanted with MHCC97L-NTC and -shL2 cells (Student t test; n = 6 per group: *P<0.05; **P<0.01).
Figure 6. HIF inhibitors suppress growth of tumors which express high level of NDUF4L2.
(A) CM-H$_2$DCFDA staining showing the ROS levels of MHCC97L-NTC and -shL2 cells exposed to 1% $O_2$ for 24 hrs in the presence of indicated concentrations of HIF inhibitor digoxin (Results represent mean ± SEM. Student t test; n = 3 independent experiments; **$P<0.01$; ***$P<0.001$ as indicated). (B) Tumor volumes of orthotopic tumors derived from MHCC97L-NTC and -shL2 cells in mice that were administered with digoxin (1.2 mg/kg/day, IP) or vehicle (saline) for 21 consecutive days (Student t test; n = 6 per group: *$P<0.05$; ***$P<0.001$ as indicated). Scale, 1 cm. (C) Regulation and role of NDUF4L2 in human HCC. (i) Hypoxia elicits an imbalanced electron flow through the electron transport chain (ETC), leading to excessive ROS accumulation which is detrimental to HCC cells. (ii) HIF-1α overcome by transcriptionally activating NDUF4L2 in the mitochondrial complex I selectively to reduce the mitochondrial activity and the electron flux through ETC, thereby reducing ROS and ROS-induced apoptosis. This metabolic adaptation of oxidative stress mediated by HIF-1α under hypoxia conferred survival advantage to the rapidly growing HCC cells which frequently experience hypoxia.
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