Role of NADH Dehydrogenase (Ubiquinone) 1 Alpha Subcomplex 4-Like 2 in Clear Cell Renal Cell Carcinoma

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Abstract

Purpose: We delineated the functions of the hypoxia-inducible factor-1α (HIF1α) target NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4-like 2 (NDUFA4L2) in clear cell renal cell carcinoma (ccRCC) and characterized NDUFA4L2 as a novel molecular target for ccRCC treatment.

Experimental Design: We evaluated normal kidney and ccRCC patient microarray and RNAseq data from Oncomine and The Cancer Genome Atlas for NDUFA4L2 mRNA levels and the clinical implications of high NDUFA4L2 expression. In addition, we examined normal kidney and ccRCC patient tissue samples, human ccRCC cell lines, and murine models of ccRCC for NDUFA4L2 mRNA and protein expression. Utilizing short hairpin RNA, we performed NDUFA4L2 knockdown experiments and analyzed the proliferation, clonogenicity, metabolite levels, cell structure, and autophagy in ccRCC cell lines in culture.

Results: We found that NDUFA4L2 mRNA and protein are highly expressed in ccRCC samples but undetectable in normal kidney tissue samples, and that NDUFA4L2 mRNA expression correlates with tumor stage and lower overall survival. In addition, we demonstrated that NDUFA4L2 is an HIF1α target in ccRCC and that NDUFA4L2 knockdown has a profound antiproliferative effect, alters metabolic pathways, and causes major stress in cultured RCC cells.

Conclusions: Collectively, our data show that NDUFA4L2 is a novel molecular target for ccRCC treatment. Clin Cancer Res; 22(11); 2791–801. ©2016 AACR.

Introduction

Clear cell renal cell carcinoma (ccRCC) is the most common form of kidney cancer, representing more than 80% of all diagnoses (1). ccRCC is a disease of altered cell metabolism (2), and many genes known to contribute to the development and/or progression of ccRCC are involved in sensing oxygen, iron, nutrients, and energy (3). Altered metabolism is now a widely accepted hallmark of cancer (4), and metabolic alterations greatly influence tumor development and progression (5). Thus, targeting metabolic abnormalities in ccRCC may provide novel opportunities for developing more effective treatments (2).

The majority of ccRCCs possess an inactivating mutation in the von Hippel–Lindau (VHL) tumor suppressor gene, which leads to the stabilization of hypoxia-inducible factors (HIF; refs. 6, 7). HIF1α and HIF2α are transcription factors that influence expression of many genes involved in altered metabolic pathways in cancer, including glucose uptake, enhanced glycolytic metabolism, and decreased mitochondrial respiration (8). We previously demonstrated that expression of a constitutively active HIF1α mutant protein exclusively in kidney proximal tubule cells is sufficient to drive a program of early tumorigenesis in a murine model of ccRCC called the TRACK (TRANsgenic model of Cancer of the Kidney) model (9). TRACK mice exhibit increased expression of HIF1α target genes that are associated with a shift in metabolism from mitochondrial oxidative phosphorylation to increased aerobic glycolysis and lactate production, similar to what is observed in human ccRCC (10). In addition, our metabolomics data show that TRACK kidneys and human ccRCC samples have increased levels of glycolytic intermediates and lactate, and decreased levels of metabolites of the tricarboxylic acid (TCA) cycle (10). Similar to our findings in the TRACK kidneys, Areola and colleagues found that stable expression of HIF1α in primary kidney epithelial cells increased expression of key glycolytic enzymes and favored pyruvate conversion into lactate under nutrient poor conditions (11). In contrast, stable expression of HIF2α in primary kidney epithelial cells did not appear to mediate glycolysis or inhibit oxidative phosphorylation (11). Indeed, HIF2α had minimal influence on glycolysis and was found to support oxidative phosphorylation in a nutrient rich environment (11).

In the current study, we evaluated the levels and role of the gene NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4-like 2
In this study, we show that NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4-like 2 (NDUFA4L2) is a novel molecular target for clear cell renal cell carcinoma (ccRCC) treatment. We demonstrate that NDUFA4L2 is highly expressed at the mRNA and protein levels in human ccRCC, yet is not expressed in normal, healthy kidney tissue. We also show that NDUFA4L2 mRNA expression is highly expressed in almost all ccRCC samples compared with normal kidney tissue, with an even greater expression in higher-stage ccRCC. In addition, patients with the highest expression of NDUFA4L2 in their tumors exhibit a lower overall survival. Knockdown of NDUFA4L2 in cell culture models of ccRCC greatly impairs proliferation, perturbs metabolic pathways, and causes major stress on RCC cells.

**Materials and Methods**

**Analysis of published data**

The Oncomine database was used to compare microarray data from human ccRCC samples and matched normal kidney tissue (19). We analyzed the most highly expressed transcripts in tumors compared with normal kidney tissue in four studies (12–15). Clinical parameters and next-generation RNA sequencing data from the Oncomine database (12–15) and RNA sequencing (RNAseqv2) data from The Cancer Genome Atlas (TCGA; refs. 16, 17), and NDUFA4L2 mRNA expression is similarly increased by >50-fold in TRACK kidneys (10, 16). Hypoxia induces NDUFA4L2 expression via a mechanism involving HIF1α, but not HIF2α (18). Blocking HIF1α by RNA interference abrogated NDUFA4L2 expression in a VHL-negative ccRCC cell line, whereas blocking HIF2α expression by RNA interference did not affect the expression of NDUFA4L2 (18). Under hypoxic conditions, NDUFA4L2 is involved in lowering mitochondrial oxygen consumption and complex I mitochondrial activity, which in turn causes a shift from mitochondrial respiration to anaerobic glycolysis and reduces reactive oxygen species production (18). Thus, NDUFA4L2 plays a role in altering cellular metabolism when HIF1α is stabilized and active within a cell. In the current study, we analyzed the functions of NDUFA4L2 in ccRCC.

**Cell proliferation and colony formation assays**

RCC4 (ATCC) and SKRC48 (Memorial Sloan Kettering Cancer Center, New York, NY; cell lines were not authenticated) cell lines were cultured in DMEM (MP Biomedicals; 1033122) containing 10% FBS (Gibco, Life Technologies; 10439-016). Caki1 cells (ATCC) were grown in McCoy 5a medium modified (Gibco; 16600-082) containing 10% FBS, and 786-O and HK2 cells (ATCC) were grown in RPMI1640 medium (Gibco; 11875-093) containing 10% FBS. Cells were cultured in a humidified tissue culture incubator at 37°C in a 10% CO2 atmosphere.

For proliferation assays, cells were plated at 2.5 × 10^4 per well in multiwell culture plates. Cell numbers were counted in triplicate using an electron particle counter (Coulter; Beckman Coulter) after 1, 3, 5, and 7 days of culture. For colony formation assays, cells were plated at 500 per 6-well plate. After approximately 14 days, cells were stained with crystal violet. The number of colonies was quantified using ImageJ software (NIH, Bethesda, MD).

**Knockdown of NDUFA4L2 using shRNA**

We produced short hairpin RNA (shRNA) containing lentiviral particles as described previously (23). We used two pLKO shNDUFA4L2 vectors directed specifically toward human NDUFA4L2 (Sigma Aldrich; TRCN0000046592 and TRCN0000046589) or a pLKO nontargeting shRNA plasmid (control; Supplementary Table S1). RCC4, SKRC48, Caki1, 786-O, and HK2 cell lines were infected with shRNA lentiviral particles, and 4 μg/mL puromycin (Sigma; Aldrich; 107689) for 24 hours. After infections, cells were treated with 2 μg/mL (SKRC48, Caki1, and HK2) or 4 μg/mL (RCC4 and 786-O) puromycin (Sigma; P8833) for 4 days and knockdown was assessed by Western blotting.

**Western blot analysis**

Proteins were extracted in SDS final sample buffer, boiled, separated on SDS–PAGE gels, and transferred onto nitrocellulose membranes. Membranes were probed with primary antibodies (Supplementary Table S2) overnight at 4°C, and further incubated with secondary antibody at 22°C for 1 hour. Chemiluminescence was recorded with a quantitative gel imaging station (Bio-Rad ChemiDoc) and data were analyzed using Image Lab software (Bio-Rad).

**RNA isolation and PCR reactions**

Total RNA was isolated using TRizol reagent (Ambion, Life Technologies; 15596018). mRNA (5 μg) was reverse transcribed using the qScript cDNA SuperMix (Quanta Biosciences; 95048;

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**Translational Relevance**

In this study, we show that NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4-like 2 (NDUFA4L2) is a novel molecular target for clear cell renal cell carcinoma (ccRCC) treatment. We demonstrate that NDUFA4L2 is highly expressed at the mRNA and protein levels in human ccRCC, yet is not expressed in normal, healthy kidney tissue. We also show that NDUFA4L2 mRNA expression is highly expressed in almost all ccRCC samples compared with normal kidney tissue, with an even greater expression in higher-stage ccRCC. In addition, patients with the highest expression of NDUFA4L2 in their tumors exhibit a lower overall survival. Knockdown of NDUFA4L2 in cell culture models of ccRCC greatly impairs proliferation, perturbs metabolic pathways, and causes major stress on RCC cells.
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Results

Semiquantitative RT-PCR reactions were performed with 2μg CDNA as template using a Bio-Rad iCycler (25). All primers were designed around introns (Supplementary Table S3).

Metabolomics analysis

Cells were plated on 10-cm plates and metabolites were extracted following established protocols using 80% methanol (cooled to ~80°C; refs. 10, 26). Metabolites were dried using a SpeedVac (Savant) and stored at ~80°C until being shipped on dry ice to the Metabolomics Core Facility at Beth Israel Deaconess Medical Center (BIDMC). Targeted mass spectrometry was performed by the BIDMC core facility to analyze metabolites (26).

Metabolomics data were processed, normalized to protein, and analyzed using the MetaboAnalyst program (27). For statistical analyses, one-way ANOVA was performed on parental RCC4 versus shNDUFA4L2.1-treated cells and on shCTL-treated versus shNDUFA4L2.1-treated cells. Metabolites that were significantly altered (P < 0.05) in shNDUFA4L2.1-treated cells compared with both parental and shCTL-treated cells were evaluated and used for pathway analysis.

Electron microscopy

Cells were plated on a 6-well plate and grown to approximately 90% confluence. Cells were fixed in 2.5% glutaraldehyde, 4% paraformaldehyde, and 0.02% picric acid in 0.1 mol/L sodium cacodylate buffer, pH 7.3 for at least 1 hour at 4°C (28). The plates were processed and embedded in EMbed 812 resin (Electron Microscopy Sciences) as described previously (29). Images were taken by the WCMC Electron Microscopy & Histology core facility on a JSM 100-CX transmission electron microscope (JEOL USA, Inc.).

For each cell type, three images were taken of five different cells (15 images total/cell type). Analyses of images were performed in a blinded manner with respect to sample identity. Swollen mitochondria were defined as large, round in shape with few, disorganized cristae. Improved mitochondria were contracted and regular in shape, with more organized cristae. Autophagosomes were defined as vacuoles with double membranes and/or undigested materials, and autolysosomes were defined as vacuoles with single membranes and amorphous electron-dense regions (30).

Statistical analyses

Student t-test was performed on at least three separate, independent experiments (n = 3 or >3) using the Graph Pad Prism 6.0 software. Metabolomics data were analyzed with MetaboAnalyst (27) and RNAseq data were analyzed with EdgeR software (Bioconductor; ref. 20).

Results

NDUFA4L2 mRNA and protein levels are increased in human ccRCC

To identify potentially important genes that contribute to renal carcinogenesis, we first searched the Oncomine database of published microarray data from matched malignant and nonmalignant samples (19). We analyzed four studies that in total compared gene expression data from 69 ccRCC samples and 45 adjacent, nontumor kidney samples (12-15; Table 1). In addition, we collected TCGA RNAseqV2 data from 530 ccRCC samples and 72 adjacent nontumor samples (Table 1; ref. 17). In each of these studies, one gene, in particular, NDUFA4L2, stood out as having a strikingly large fold increase in ccRCC. Indeed, Yusenko and colleagues (14) found that NDUFA4L2 transcripts are increased by nearly 90-fold in ccRCC compared with nontumor kidney samples (Table 1). In addition, approximately 90% of the 530 ccRCC samples for which RNAseq data is available in TCGA possessed elevated NDUFA4L2 expression (17). Although NDUFA4L2 mRNA is considerably increased in ccRCC compared with normal kidney tissue according to microarray and RNAseq data, this gene has not been well characterized and its role in ccRCC has not been thoroughly defined.

Following this analysis of published mRNA expression data, we evaluated both NDUFA4L2 mRNA and protein levels in human ccRCC tumors and matched nontumor kidney tissue by performing semiquantitative PCR and Western blot analyses, respectively. We found that NDUFA4L2 transcripts were highly elevated in tumors compared with normal, adjacent kidney tissue (Fig. 1A, left). Tumor 1 has an approximately 20-fold and tumor 2 has an approximately 30-fold increase in NDUFA4L2 transcripts compared with nonmalignant kidney tissue samples from the same patients. In addition, we show that NDUFA4L2 protein (~13 kDa) is highly expressed in tumor samples from patients 3, 4, and 5 (Fig. 1A, right). Quantitation of bands indicates that NDUFA4L2 protein is increased by 10- to 30-fold in tumors compared with adjacent nonmalignant kidney tissue. These mRNA and protein data from human patient samples are striking, as NDUFA4L2 is highly expressed in each of the five tumor samples, and is barely detectable in each of the nonmalignant renal tissue samples under our conditions for PCR and Western blotting (Fig. 1A). These data also suggest that elevated expression of NDUFA4L2 is a common event in ccRCC and that expression is restricted to the tumors.

To identify relevant cell line models to utilize in further experimentation, we evaluated NDUFA4L2 at the mRNA and protein levels in various human ccRCC cell lines and a normal, nontransformed human proximal tubule cell line, HK2 (Fig. 1B). RCC4 and SKRC48 ccRCC cell lines have activated HIF1α and thus we detected NDUFA4L2 mRNA and protein in these cells (Fig. 1B). We did not detect NDUFA4L2 mRNA in the ccRCC cell lines Caki1 and 786-O, in which HIF1α is not active, nor in the normal proximal tubule cell line HK2 (Fig. 1B). On the basis of high NDUFA4L2 expression, we chose to use the RCC4 and SKRC48 cell lines as in vitro ccRCC models for further studies.

The commercially available antibodies targeting NDUFA4L2 (Supplementary Table S1) also detect NDUFA4 (~10 kDa; Fig. 1A and B, right). NDUFA4L2 and NDUFA4 have high homology at the C-terminus, the region of the NDUFA4L2 protein that the antibodies recognize. NDUFA4 was recently shown to be a subunit of mitochondrial complex IV (cytochrome c oxidase; ref. 31). NDUFA4 protein is decreased in the tumor samples by approximately 10- to 30-fold (Fig. 1A, right) and by approximately 5-fold in RCC4 and SKRC48 cells compared with Caki1 cells (Fig. 1B, right).

Increased NDUFA4L2 expression correlates with more advanced human ccRCC stage, grade, and reduced survival

To assess if increased NDUFA4L2 expression in ccRCC had any clinical relevance, we evaluated TCGA RNAseq data and corresponding clinical parameters (17). For our study, level 2 RSEM (RNA-seq by Expectation-Minimization) normalized expression count data were obtained from the TCGA. With regard
to available clinical information, we prioritized tumor AJCC stage, Fuhrman grade, and survival data from patients with histologically confirmed ccRCC. AJCC staging is the most important factor predicting prognosis and it is based on tumor size and metastatic involvement (32). Patient:stage distributions were stage I \( (n = 253) \), stage II \( (n = 56) \), stage III \( (n = 125) \), and stage IV \( (n = 81) \). Stage III ccRCCs have an approximate 25% increase in NDUFA4L2 mRNA expression compared with stages I and II (Fig. 1C). Fuhrman grading, which refers to how closely the cancer cells look like normal kidney cells under...
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A.

NDUFA4L2 expression is mediated specifically by HIF1α in TRACK kidneys. A, expression of NDUFA4L2 mRNA (left) and protein (right) in kidney tissue from the TRACK model, γ-HIF2αm3 TG+ mice, and TG− mice. B, NDUFA4L2 protein expression in various tissues of the TRACK model. For semiquantitative RT-PCR, 36B4 mRNA is used as a loading control. For Western blot analysis β-actin is the loading control. Western blot and RT-PCR were performed on three or more mice (n ≥ 3) and results are representative of triplicate experiments with similar results.

a microscope, is another important prognosis factor. Patient: grade distributions were grade I (n = 11), grade II (n = 229), grade III (n = 203), and grade IV (n = 75). Grade III ccRCCs have an approximately 35% increase in NDUFA4L2 mRNA expression compared with stage II (Fig. 1D).

Next, we evaluated overall survival based on NDUFA4L2 mRNA levels (Fig. 1E). Notably, nearly all ccRCC samples (~90%) have high NDUFA4L2. Consequently, we defined two patient subgroups based on normalized NDUFA4L2 expression, (i) patients with lower NDUFA4L2 expression (counts < 60,000; n = 448), and (ii) patients with higher NDUFA4L2 expression (normalized count > 60,000; n = 60), which is >90-fold higher NDUFA4L2 expression than in normal kidney tissue. We found that patients with the highest NDUFA4L2 mRNA levels exhibited lower overall survival compared with patients with lower NDUFA4L2 mRNA (log-rank test; P = 0.0011; Fig. 1E). For patients with lower NDUFA4L2 expression, the median survival is 2,454 days, and for patients with the highest NDUFA4L2 expression the median survival is 1,111 days (Fig. 1E).

NDUFA4L2 is HIF1α regulated in ccRCC

It was previously reported that NDUFA4L2 is a direct HIF1α target under hypoxic conditions (18). To validate HIF1α regulation of NDUFA4L2 in ccRCC, we assessed NDUFA4L2 expression in the kidneys of the TRACK murine model of ccRCC, as well as in HIF2αm3 transgenic (TG+) and transgenic negative (TG−) mice. TRACK mice express a constitutively active form of HIF1α specifically in proximal tubule cells and HIF2αm3 TG+ mice express a constitutively active form of HIF2α specifically in proximal tubule cells (9, 33). We previously reported that TRACK mice display a tumorigenesis program that more closely resembles early human ccRCC than HIF2αm3 TG− mice (9, 10, 16, 33, 34). We found that NDUFA4L2 expression is regulated by HIF1α and not HIF2α, as NDUFA4L2 transcripts and protein are highly expressed in the TRACK kidneys, but not in HIF2αm3 TG− or TG− mouse kidneys (Fig. 2A). In addition, Western blot analyses of different tissues from TRACK mice indicate that NDUFA4L2 expression is unique to the kidneys where constitutively active HIF1α is specifically expressed (Fig. 2B). NDUFA4L2 protein cannot be detected in the normal pancreas, liver, heart, lung, testes, or intestines of TRACK mice (Fig. 2B).

To determine whether HIF1α plays a role in direct regulation of NDUFA4L2, we used the integrated genome viewer to analyze a published HIF1α ChIPseq dataset (GEO: GSE39089; ref. 22). These data indicate that HIF1α is recruited to the NDUFA4L2 locus in hypoxic human umbilical vein endothelial cells (Supplementary Fig. S1A). It is therefore likely that HIF1α plays a similar role in kidney proximal tubule epithelial cells. In addition, hierarchical clustering analysis of the TCGA ccRCC dataset shows that NDUFA4L2 clusters with other known HIF1α target genes in ccRCC, such as VEGFA, HK2, and SLC16A3 (Supplementary Fig. S1B).

NDUFA4L2 knockdown impairs cell proliferation and colony formation in ccRCC cell lines

To assess the functional significance of NDUFA4L2 in ccRCC, we performed knockdown of NDUFA4L2 with two independent shRNAs via lentivirus-mediated infection in RCC4 and SKRCA4 ccRCC cells that express NDUFA4L2 (Fig. 1B). We confirmed NDUFA4L2 knockdown by >90% at the protein level with both NDUFA4L2 shRNA targeting constructs, shNDUFA4L2.1 and shNDUFA4L2.2, by Western blotting. No changes in NDUFA4L2 levels were observed when RCC4 and SKRCA4 cells were infected with a nontargeted control shRNA (shCTL; Fig. 1A).

We next analyzed the growth characteristics of ccRCC cells that lack NDUFA4L2 expression. Both RCC4 and SKRCA4 cells infected with shNDUFA4L2 constructs showed reduced proliferation over 7 days compared with the parental or shCTL-treated cells (Fig. 3B). In both RCC4 and SKRCA4 cell lines, shNDUFA4L2.1 treatment reduced cell proliferation by more than 60% and shNDUFA4L2.2 treatment reduced cell proliferation by approximately 90% (Fig. 3B).

As further confirmation of the importance of NDUFA4L2 for cell proliferation, we assayed for clonogenic growth in RCC4 and SKRCA4 cells lacking NDUFA4L2 expression. Consistent with the proliferation assays, NDUFA4L2 knockdown greatly diminished the capacity of cells to form colonies (Fig. 3C), reducing the numbers of colonies by >90% (Fig. 3D). Collectively, the proliferation and colony formation assays show that NDUFA4L2 expression promotes cell proliferation and clonogenic growth of VHL-negative ccRCC cells.

We also performed viral infection with the shCTL, shNDUFA4L2.1, and shNDUFA4L2.2 constructs in Caki1 and 786-O ccRCC cell lines and in HK2, a nontransformed proximal tubule cell line which does not express NDUFA4L2 (Fig. 1B). We found that treatment with the shCTL and shNDUFA4L2.1 constructs had no effect on proliferation and clonogenicity (Supplementary Fig. S2A and S2B). Treatment with the shNDUFA4L2.2 construct impaired proliferation by approximately 30% to 50% in the Caki1 and 786-O cell lines and impaired clonogenicity by approximately 80% in Caki1 cells (Supplementary Fig. S2A and S2B). However, the shNDUFA4L2.2 did not impair proliferation in the HK2 normal proximal tubule cell line (Supplementary Fig. S2A). These data suggest that the Caki1 and 786-O cell lines have low levels of NDUFA4L2 not detectable in our assays or that shNDUFA4L2.2 has an additional off-target effect.
NDUFA4L2 knockdown alters metabolic pathways

To evaluate why NDUFA4L2 is critical for proliferation and clonogenic growth of RCC4 and SKRC48 cells, we next evaluated changes in metabolite levels upon NDUFA4L2 knockdown. Under hypoxic conditions, NDUFA4L2 inhibits complex I of oxidative phosphorylation and thus mediates a cellular shift to glycolysis for generating ATP (18). ccRCC is widely characterized as a metabolic cancer that relies on glycolysis instead of oxidative phosphorylation to produce ATP (3), which is an adaptive measure to facilitate the uptake and incorporation of nutrients into biomass to produce new cells (35).

A metabolomics analysis demonstrated that RCC4 cells treated with shNDUFA4L2.1 showed increases in the levels of several TCA cycle intermediates and decreases in the levels of several pentose phosphate pathway intermediates compared with parental and shCTL-treated cells (Fig. 4A and B). With respect to the TCA cycle, we observed 2-fold or greater increases in the levels of citrate/isocitrate, a-ketoglutarate, succinate, and oxaloacetate (Fig. 4A). In contrast, we saw marked decreases in the pentose phosphate pathway intermediates ribose 5-phosphate, sedoheptulose 1,7-bisphosphate, glyceraldehyde 3-phosphate, and fructose 6-phosphate (Fig. 4B). These data suggest that upon NDUFA4L2 knockdown, pyruvate generated by the glycolysis pathway is converted to acetyl-CoA to fuel the TCA cycle and as such, glycolytic intermediates are not shunted into the pentose phosphate pathway (Fig. 4C). This is important because proliferating cancer cells shunt glycolytic metabolites into the pentose phosphate pathway to make ribose-5-phosphate, which is needed for nucleic acid
Knockdown of NDUFA4L2 causes metabolic alterations. A, comparison of the levels of the TCA cycle intermediates citrate/isocitrate, \( \alpha \)-ketoglutarate, succinate, and oxaloacetate in parental RCC4 cells and cells infected with the indicated shRNA constructs for 24 hours followed by puromycin for 96 hours. B, comparison of the levels of the pentose phosphate pathway intermediates ribose 5-phosphate, sedoheptulose 7-phosphate, glyceraldehyde 3-phosphate, and fructose 6-phosphate in parental RCC4 cells and cells infected with the indicated shRNA constructs for 24 hours followed by puromycin for 96 hours. C, schematic overview of the changes in the TCA cycle and pentose phosphate pathway upon treatment with \( \text{shNDUFA4L2.1} \) in RCC4 cells. Arrows next to metabolites indicate that the metabolite levels are changed in \( \text{shNDUFA4L2.1} \)-treated cells compared with parental and \( \text{shCTL} \)-treated cells. Metabolomics analysis was performed on samples in triplicate (\( n = 3 \) for each group) and data are graphed as the mean \( \pm \) SEM. **, \( P < 0.01 \); ****, \( P < 0.0001 \).
production (35). Ribose 5-phosphate is 1.6-fold lower in shNDUFA4L2.1-treated RCC4 cells compared with shCTL-treated cells, and 2.2-fold lower compared with parental RCC4s (Fig. 4B). In addition, a principal component analysis showed that RCC4 parental and shCTL-treated cells cluster distinctly from RCC4 cells treated with shNDUFA4L2.1 (Supplementary Fig. S3A), and pathway analysis of significantly altered metabolites in the RCC4 cells treated with shNDUFA4L2.1 compared with the parental and shCTL-treated cells demonstrated that two of the most significantly altered metabolic pathways were metabolism of pyrimidine and purine nucleotides (Supplementary Fig. S3B). Thus, the metabolomics data demonstrate that NDUFA4L2 knockdown alters metabolism in a manner such that the pentose phosphate pathway is downregulated and less ribose 5-phosphate is being produced, which in turn likely reduces purine and pyrimidine synthesis.

**NDUFA4L2 knockdown improves mitochondrial morphology and induces autophagy**

Cancer cells that have a reduced capacity for aerobic respiration also exhibit abnormalities in both the content and composition of their mitochondria (36). We hypothesized that NDUFA4L2 inhibits aerobic respiration in ccRCC, and that elevated expression of NDUFA4L2 contributes to abnormal mitochondrial structure. When we analyzed RCC4 parental, shCTL-treated, and NDUFA4L2 knockdown cells by transmission electron microscopy, we found that RCC4 parental and shCTL-treated cells had many swollen mitochondria with disorganized cristae (Fig. 5A, filled arrows). However, RCC4 cells treated with both shNDUFA4L2 constructs had more contracted mitochondria with improved cristae (Fig. 5A, open arrows). Indeed, approximately 50% of the mitochondria in parental and shCTL-treated RCC4 cells are swollen with disorganized cristae, whereas less than 10% display this morphology in cells treated with shNDUFA4L2.1 and shNDUFA4L2.2 (Fig. 5B). Though the morphology of the mitochondria is changed when NDUFA4L2 levels are reduced, the numbers of mitochondria remain the same (Fig. 5C).

NDUFA4L2 knockdown also promotes autophagy, a process by which cytotoxic components are engulfed in double-membrane autophagosomes that fuse with lysosomes for degradation (37), and mitochondrial turnover is dependent on autophagy (38). In RCC4 cells treated with both shNDUFA4L2 constructs, we visualized many autophagosomes (Fig. 5A, filled stars) as defined by vacuoles with double membranes and/or undigested materials, and autolysosomes (Fig. 5A, open stars) as defined by vacuoles with single membranes and amorphous electron-dense regions (30). We detected a 3.8 (±1.3) -fold increase in autophagosomal vesicles in RCC4 cells treated with shNDUFA4L2.1 and a 5.1 (±1.6) -fold increase in shNDUFA4L2.2-treated cells compared with parental cells (Fig. 5D). In addition, we counted more autolysosomes than autophagosomes in RCC4 cells treated with shNDUFA4L2.1 and shNDUFA4L2.2 (Fig. 5D).

LC3, or tubule-associated protein 1A/1B-light chain 3, is a biomarker of autophagy. During autophagy, the cytotoxic form of LC3 (LC3-I) is converted to its lipidated form, LC3-II, which is recruited to autophagosomal membranes. We detected a >8-fold induction of LC3-II in both RCC4 and SKRC48 lines treated with shNDUFA4L2.1 and shNDUFA4L2.2 compared with the parental and shCTL-treated cells (Fig. 5E). Together, the improved mitochondrial structures and the induction of autophagy upon NDUFA4L2 knockdown suggest that NDUFA4L2 knockdown promotes mitochondrial turnover via autophagy.

We also analyzed Caki1 cells, which do not have elevated expression of NDUFA4L2 (Fig. 1B), to determine whether shRNA treatment improves mitochondria morphology and/or induces autophagy. We found that mitochondria in Caki1 parental and Caki1 cells treated with shCTL, shNDUFA4L2.1, and shNDUFA4L2.2 have contracted mitochondria with typical cristae (Supplementary Fig. S4A). There was no improvement in mitochondrial morphology in Caki1 upon treatment with either shNDUFA4L2 construct (Supplementary Fig. S4A).

Next, we evaluated the numbers of autophagic vacuoles in Caki1 parental and shRNA-treated cells. We found that Caki1 cells treated with shNDUFA4L2.2 had more autophagic vacuoles than Caki1 parental cells and cells treated with shCTL and shNDUFA4L2.1 (Supplementary Fig. S4B). However, the increase in autophagic vacuoles seen in Caki1 cells treated with shNDUFA4L2.2 was not as high as in RCC4 cells treated with shNDUFA4L2.1 or shNDUFA4L2.2 (Fig. 5D and Supplementary Fig. S4B). In addition, we did not detect an increase in LC3-II levels in Caki1, 786-O, or HK2 cells treated with either shNDUFA4L2 construct (Supplementary Fig. S5).

**Discussion**

Altered metabolism is a major characteristic of ccRCC. We recently reported on the importance of HIF1α in mediating altered tumor metabolism in the TRACK model of ccRCC, similar to the changes observed in human ccRCC (10, 16). Targeting pathways that mediate altered metabolism may provide novel and more effective treatments (2, 17). In this study, we demonstrated that an HIF1α target gene involved in mediating glycolysis, NDUFA4L2, is highly expressed at the mRNA and protein levels in human ccRCC, yet is not expressed in normal kidney tissue (Table 1 and Fig. 1). We also showed that NDUFA4L2 mRNA levels are greater in the more advanced staged and graded ccRCCs (Fig. 1C and D), and that patients with the highest expression of NDUFA4L2 in their tumors exhibit a lower overall survival rate (Fig. 1E). Moreover, we found that NDUFA4L2 knockdown leads to a profound decrease in proliferation and colony formation in HIF1α positive cell lines (Fig. 3). Collectively, these data demonstrate that NDUFA4L2 is an attractive therapeutic target to treat ccRCC.

Previous work on NDUFA4L2 is limited. In the one major study characterizing NDUFA4L2, Tello and colleagues demonstrated that NDUFA4L2 is a direct HIF1α target gene (18). By assessing NDUFA4L2 expression in our various murine models, we confirmed that NDUFA4L2 is regulated by HIF1α, but not HIF2α (Fig. 2). Although deletion of HIF1α has previously been reported in ccRCC (39, 40), the results shown here and available in the TCGA-KIRC RNAseq expression data support an important role for HIF1α, acting in part via NDUFA4L2, in the majority of human ccRCCs (34).

We and others have shown that HIF1α mediates the Warburg effect in ccRCC, a phenomenon in which cancer cells rely on aerobic glycolysis to generate ATP instead of the TCA cycle and oxidative phosphorylation (10, 16, 41). Tello and colleagues showed that NDUFA4L2 inhibits complex I of oxidative phosphorylation to mediate a shift to glycolysis (18). Thus, it is likely that NDUFA4L2 has a role in mediating the Warburg effect in ccRCC. Interestingly, aerobic glycolysis is a less efficient
method for producing ATP. One explanation for why aerobic respiration is favored is that proliferating cancer cells not only require ATP, but also need nucleotides, fatty acids, and proteins (35). Reprogrammed metabolism supports the synthesis of these macromolecules required for rapid proliferation (35).

We demonstrated that NDUFA4L2 is important for supporting ccRCC metabolism to generate nucleic acids (Fig. 4). Nucleic acids, which include DNA and RNA, are vital building blocks for cell growth and division. Thus, it is likely that NDUFA4L2 knockdown impairs proliferation in ccRCC cells, at least in part, by disrupting the synthesis of critical macromolecules, DNA and RNA.

Cancer cells that have reduced aerobic respiration frequently display abnormalities in both the content and composition of their mitochondria (36). In parental RCC4 and RCC4 shCTLe-treated cells, we visualized swollen mitochondria with abnormal, disorganized cristae (Fig. 5). As mitochondrial morphology and function are closely linked (42), these data suggest that these cells have diminished mitochondrial capacity. However, when NDUFA4L2 levels are reduced, we found that the...
mitochondrial structures improve and the cristae become less disorganized (Fig. 5). These findings, and the metabolomics data indicating that TCA cycle intermediates are increased upon NDUFA4L2 knockdown (Fig. 3), suggest that mitochondrial bioenergetics are improved when NDUFA4L2 levels are reduced. The restoration of mitochondrial bioenergetics is currently being pursued as an approach for treating a variety of different diseases, including aging-related diseases. SS-31, a mitochondrial-targeted peptide, is one example of a compound that protects mitochondrial cristae and promotes ATP synthesis by interacting with cardiolipin, an inner mitochondrial membrane phospholipid that has a central role in the structure and organization of cristae (43). Interestingly, SS-31 was shown to protect the kidneys of rats after ischemia (44). Similar to our data, treatment with SS-31 resulted in more normal and elongated mitochondria with finely stacked cristae membranes (44). Targeting mitochondrial biogenesis for the treatment of ccRCC has not been well studied, and SS-31 has not been tested in the context of this disease. However, based on our data from knocking down NDUFA4L2 in ccRCC cells and the success of SS-31 in protecting rat kidneys from ischemia, further research into improving mitochondria bioenergetics in the context of active HIF1α as a treatment for ccRCC is warranted.

Mitochondrial turnover is dependent on the intracellular degradation process autophagy (38), which we detected in the ccRCC cells upon NDUFA4L2 knockdown (Fig. 5). Thus, it is possible that autophagy is induced in NDUFA4L2 knockdown cells to remove the damaged mitochondria to generate more functional mitochondria. Another potential explanation for the induction of autophagy in the RCC4 and SKRC48 NDUFA4L2 knockdown cells is that the cells are nutrient-deprived when NDUFA4L2 levels are diminished. It is well established that autophagy is induced under starvation or serum deprivation (38), and although nutrients are readily available to NDUFA4L2 knockdown cells in culture, their ability to metabolize nutrients is altered (Fig. 4). Although further experiments are required to delineate the exact influence of NDUFA4L2 knockdown on autophagy, it is clear that NDUFA4L2 knockdown causes major stress on RCC4 and SKRC48 cells.

ccRCC is characterized as a highly metabolic disease and alterations in metabolism are likely to be fundamental to the development of advanced ccRCC (3). Thus, targeting the metabolic basis of ccRCC could be an effective strategy for treating the disease (17, 45–47).

Targeting NDUFA4L2 for inhibition to treat ccRCC has several advantages. To start, NDUFA4L2 is an appealing target because it is so frequently and highly expressed in ccRCC but is not normally expressed in kidney tissue or other tissues of the body. In addition, targeting NDUFA4L2 should not disrupt glycolysis in normal cells. Many of the other genes involved in increasing the rate of glycolysis in cancer cells encode enzymes that also play critical roles in nontransformed cells, and thus inhibiting them could harm healthy, normal cells. For these reasons, and because of our data showing that NDUFA4L2 knockdown profoundly inhibits ccRCC proliferation, we propose that NDUFA4L2 is a novel molecular target for inhibition to be pursued for the treatment of ccRCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: D.R. Minton, L. Fu, L.J. Gudas
Development of methodology: D.R. Minton, L. Fu, L.J. Gudas
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.R. Minton, L. Fu, L.J. Gudas
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.R. Minton, L. Fu, N.P. Mongan, M.M. Shevchuk, D.M. Nanus, L.J. Gudas
Writing, reviews, and/or revision of the manuscript: D.R. Minton, L. Fu, N.P. Mongan, M.M. Shevchuk, D.M. Nanus, L.J. Gudas
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.R. Minton
Study supervision: L.J. Gudas

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Role of NDUFA4L2 in Clear Cell Renal Cell Carcinoma


Role of NADH Dehydrogenase (Ubiquinone) 1 Alpha Subcomplex 4-Like 2 in Clear Cell Renal Cell Carcinoma

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