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Inhibition of Mitochondrial Complex II Induces Mitophagy and Attenuates Cell Proliferation*

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Abstract Mitochondrial complex II, or succinate dehydrogenase (SDH), is regarded as a central regulator of respiratory adaptation and metabolic reprogramming in various stimuli and abnormalities. Four subunits of complex II are considered as tumor suppressors, whose mutations are associated with various type of cancer. However, little is known how complex II regulates cell proliferation. 2-Thenoyltrifluoroacetone (TTFA), an inhibitor of mitochondrial complex II, and SDHB shRNA were used to abolish the activity of complex II in cell lines. Inhibition the activity of complex II by TTFA treatment or knockdown of SDHB could trigger mitochondrial fragmentation and subsequently mitophagy. We also found that inhibition of complex II also increased the glucose consumption and the lactate production which termed as Warburg effect. Despite of these, complex II dysfunction showed negative regulation to cell proliferation. Collectively, complex II is a potential target to induce mitophagy and inhibit cell proliferation.

Key words mitochondrial complex II, SDH, SDHB, mitophagy, cell proliferation
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Mitochondrial complex II locates on the inner membrane of mitochondria and plays critical roles in metabolic reprogramming and respiratory adaptation in various stimuli and abnormalities[1-2]. Complex II composes four subunits, succinate dehydrogenase (SDH)A, SDHB, SDHC and SDHD, which are assembled under the assistance from three nuclear encoded assembly factors, SDHAF1, SDHAF2 and SDHAF3[3]. Germline mutations of these genes are associated with paraganglioma[4-6], pheochromocytoma[7-8], renal cell carcinoma[9], colorectal cancer[10] and gastrointestinal stromal tumors[11-12], which may be contributed by the pivotal roles of these genes in energy production and metabolism[13]. There are three main theories regarding the role of SDH mutation in tumorigenesis. The first one, also the widely accepted hypothesis, pseudohypoxia theory[14], in which, accumulated succinate, the consequence of complex II deficiency, inhibits the activity of HIF1-α-proyl hydroxylases (HPHs or PHDs) by competitive inhibitory mechanism. This leads to the stabilization of hypoxia inducing factors (HIFs), which increases glycolysis under normoxia and promotes tumorigenesis[15]. The second one is that complex II deficiency increases ROS, which may generate gene mutation and thus promote tumorigenesis[16-17]. The last one is that the metabolic intermediate by SDH mutation may play a role as metabolic signal[18] that regulates cellular behaviors[19-21]. In this study, we found that inhibition of complex II either by complex II inhibitor TTFA or SDHB knockdown induced mitochondrial fragmentation and subsequently mitophagy. We also found that complex II dysfunction showed negative regulation to cell proliferation.

1 Materials and Methods

1.1 Plasmids and other reagents

All chemicals used in this study were of

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analytical grade or higher. Plasmids: p-EGFP-LC3 (plasmid#21073), pDsRed2-Mito (plasmid#632421), pBABE-puro vector (plasmid #1764) were obtained from Addgene (USA). shSDHB-pBABE-puro plasmids were constructed by cloning oligonucleotides into pBABE-puro vector. ShSDHB primers are presented in Table 1. TTFA (ACM326910) was bought from Alfa Aesar (USA); Lactic Acid assay kit (Cat#A019-1-1) and ATP kit (Cat#A095-1-1) were bought form Nanjin Jiancheng (China); Polythylenimine (PEI, 9002-98-6, Polysciences. Inc, USA) transfection reagents were used for plasmid transfection.

1.2 Cells and culture

Cell lines were authenticated by short tandem repeats (STR) DNA profiling and mycoplasma contamination has been tested. HeLa cells, HEK293T cells were obtained from American Type Culture Collection (ATCC). All cell lines were cultured at 37℃ with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 100 mg/L penicillin and 100 mg/L streptomycin.

1.3 Western−blotting

Cells were harvested in lysis buffer (150 mmol/L NaCl, 20 mmol/L Tris, pH 7.4, 1 mmol/L EGTA, 1 mmol/L EDTA, 1% SDS, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L Na_3VO_4, and protease inhibitors (Roche)). After boiled with 6×loading buffer, samples were loaded for SDS-PAGE, proteins are separated by electrophoresis at 100 V for 1.5 h followed by membranes transfers. The membranes were then blocked with 5% fat-free milk for 2 h at room temperature. Membranes were then incubated with the indicated primary antibodies, followed by the appropriate HRP-conjugated Goat anti-mouse (RRID: AB_2341088, 1∶3 000) HRP-conjugated Goat anti-rabbit (RRID: AB_2721169, 1∶3 000) secondary antibodies for subsequent enhanced chemiluminescent (ECL) (EMD Millipore, USA) exposure. The following antibodies were used: anti-SDHB antibody (RRID: AB_301432, 1∶5 000), CoxIV antibody (RRID: AB_2085278, 1∶1 000), Timm23 (RRID: AB_398754, 1∶1 000), Tomm20 (RRID: AB_399595, 1∶1 000), LC3 (RRID: AB_2274121, 1∶1 000), Actin (RRID: AB_476744, 1∶10 000).

1.4 Immunofluorescence

HeLa cells were seeded on glass coverslips were transfected with plasmids or treated with TTFA as indicated in the figures. 4% paraformaldehyde was used for cell fixation (Dingguo Changsheng Biotechnology, China), after 15 min fixation at room temperature, cells were permeabilized with PBS containing 0.1% Triton X-100 at 4℃ for 10 min. After 1 h goat serum blockage, cells were incubated with primary antibody Tomm20 antibody (RRID: AB_399595, 1∶200) and CY3-conjugated secondary antibodies (RRID: AB_2534030, 1∶1 000). After washed three times in PBS, a confocal microscope was used for images were acquiring (Leica).

1.5 Statistical analysis

Three independent technical repeats were done for all experiments. GraphPad Prim software 8.0 (GraphPad, USA) to analyze the data and the value were represented as $\bar{x}\pm s$ Student's *t*-test $P\leqslant 0.05$ was regarded as significant.

2 Results

2.1 Inhibition of complex II induces mitochondria fragmentation and reduces mitochondrial proteins

Complex II locates on the inner membrane of mitochondrion, the loss of which may affect mitochondrial function. To test whether complex II inhibition would affect the mitochondrial morphology, we treated cells with TTFA, which is an inhibitor of complex II, and then mitochondrial morphology was observed. It showed that TTFA treatment induced mitochondrial fragmentation in both HeLa and MEF cells (Figure 1a). The protein level of VDAC1 (voltage-dependent anion channel 1) and Timm23 protein on the mitochondrial outer membrane were decreased in a dosage-dependent manner with TTFA along with the increase of LC3-II which indicates the level of autophagy (Figure 1b). Furthermore, the decreased level of MFN1 may explain the fragmentation of mitochondria. In coincidence with decreased mitochondrial membrane proteins, we also found the FunDC1, the receptor of mitophagy, was also decreased. These data indicate that mitochondrial complex II inhibition affects mitochondrial morphology and reduces mitochondrial protein through autophagy. Consistently, ATP levels were also found decreased when cells treated with TTFA (Figure 1c).

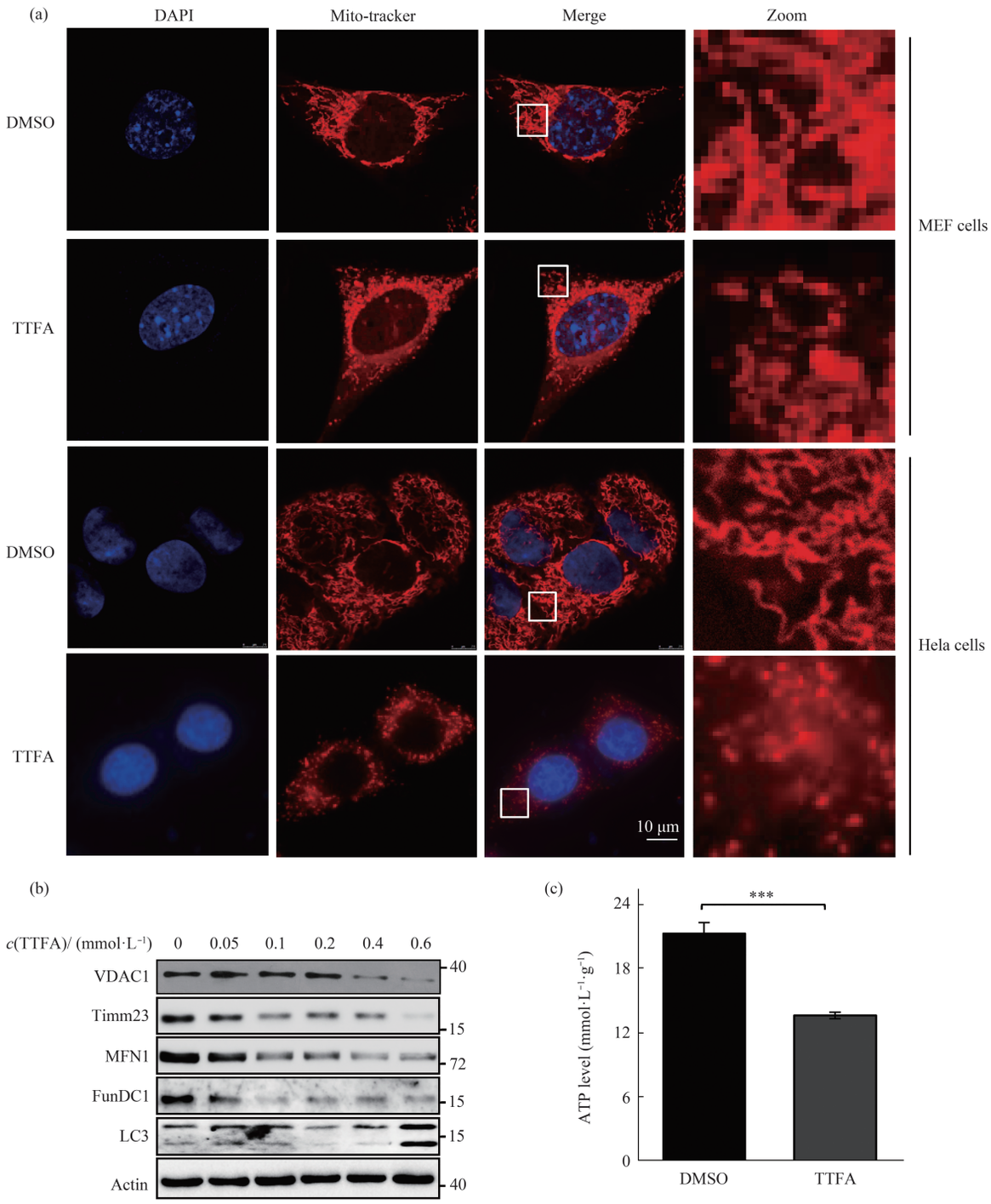

Fig. 1　Complex II inhibition induces fragmentation of mitochondria

(a) Fluorescence microscopic analysis of mitochondrial morphology of TTFA-treated HeLa and MEF cells. Mito-tracker-Red (red) was used as a probe for mitochondria, DAPI was used to stain the nuclei (blue). (b) Western-blotting for mitochondrial protein VDAC1, Timm23, MFN1, FunDC1, LC3 and Actin as loading control in HeLa cells treated with TTFA. (c) ATP levels in HeLa cells treated with DMSO and TTFA. Student's *t*-test was performed to analysis the cell numbers and the values were average of 3 wells, error bars represented in $\bar{x}\pm s$, ***$P\leqslant 0.001$.

2.2　Inhibition of complex II induces mitophagy

Mitophagy is a type of autophagy which targets to damaged mitochondria selectively and plays critical roles in mitochondrial quality control[22]. We found that TTFA induced mitochondrial protein reduction and autophagy marker LC3-II (Figure 1b), which

indicates that the protein reduction may be contributed by mitophagy. In order to verify this, we overexpressed GFP-LC3 and found that, after TTFA treatment, GFP-LC3 co-localized with mitochondria, indicating that autophagy machinery can be activated by TTFA and targeted to mitochondria (Figure 2a). Consistently, the time and dosage-dependent activation of type II LC3 together with the reduction of mitochondrial protein markers Timm23 and CoxIV and autophagy marker P62, in both HeLa cells and

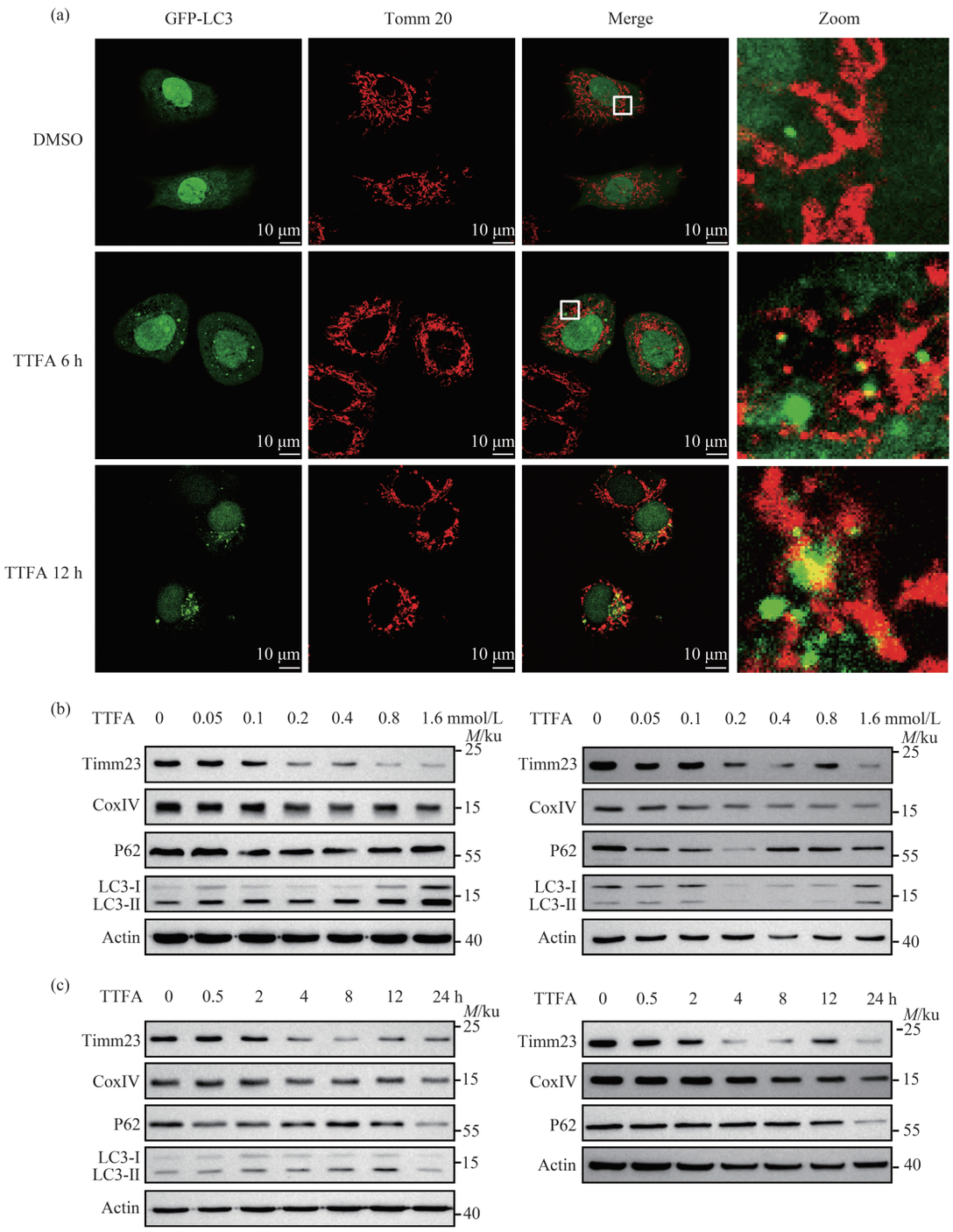

Fig. 2 TTFA induces mitophagy in HeLa and HEK293T cells

(a) Fluorescence microscopic analysis of mitochondrial morphology in TTFA treated HeLa cells. GFP-LC3 (Green) was transfected in HeLa cells followed by 0.4 mmol/L TTFA treatment for 6 (middle) or 12 (down) hours, Tomm20 was used as a mitochondrial marker (red). (b) Western-blotting for Timm23, CoxIV as mitochondrial markers, P62 and LC3 as autophagy markers and Actin as loading control. HeLa (left) and HEK293T cells (right) treated with TTFA in indicated concentration gradient. (c) Western-blotting of mitochondria protein Timm23, CoxIV as mitochondrial markers, P62 for autophagy and Actin as a loading control in HeLa (left) and HEK293T cells (right) treated with 0.4 mmol/L TTFA in indicated time course.

HEK293T cells, indicate that TTFA-medicated mitochondrial protein reduction is mediated by mitophagy (Figure2 b, c).

2.3 *SDHB* knockdown regulates cell proliferation

SDHB is the core subunit of complex II that tethers SDHA to SDHC and SDHD, which integrity is critical for complex II activity. In order to evaluate the role of complex II in cell proliferation regulation, we first generated S*DHB* knocked-down stable cell line with specific shRNA in HeLa cells and obtained polyclonal cells (named *SDHB*KD) (Figure 3a). Mitochondrial morphological analysis in *SDHB* knockdown cells showed that mitochondria were fragmented (Figure 3b), which was similar to the phenotypes of TTFA-treated HeLa or MEF cells. Complex II inhibition-induced mitochondrial abnormalities may induce metabolic change in cells. We found that the culture medium in *SDHB* knockdown cells was significantly acidified (Figure 3c), suggesting that *SDHB* knockdown cells may produce more acids. Indeed, lactate production was dramatically increased in *SDHB* knockdown cells, indicating that complex II dysfunction affects the cellular respiration (Figure 3d). In addition, consumption of glucose was increased in *SDHB* knockdown cells (Figure 3e). These results indicate that the normal respiration is affected in *SDHB* knockdown cells and shows a Warburg effect, which relies on glycolysis. Warburg effect is normally seen in solid tumors and believed to be benefit for the tumor proliferation. However, cell proliferation analysis showed that inhibition of complex II significantly inhibits cell proliferation (Figure 3f). This probably because of the mitochondrial complex II function is still required for maintaining cellular function and promoting cell proliferation.

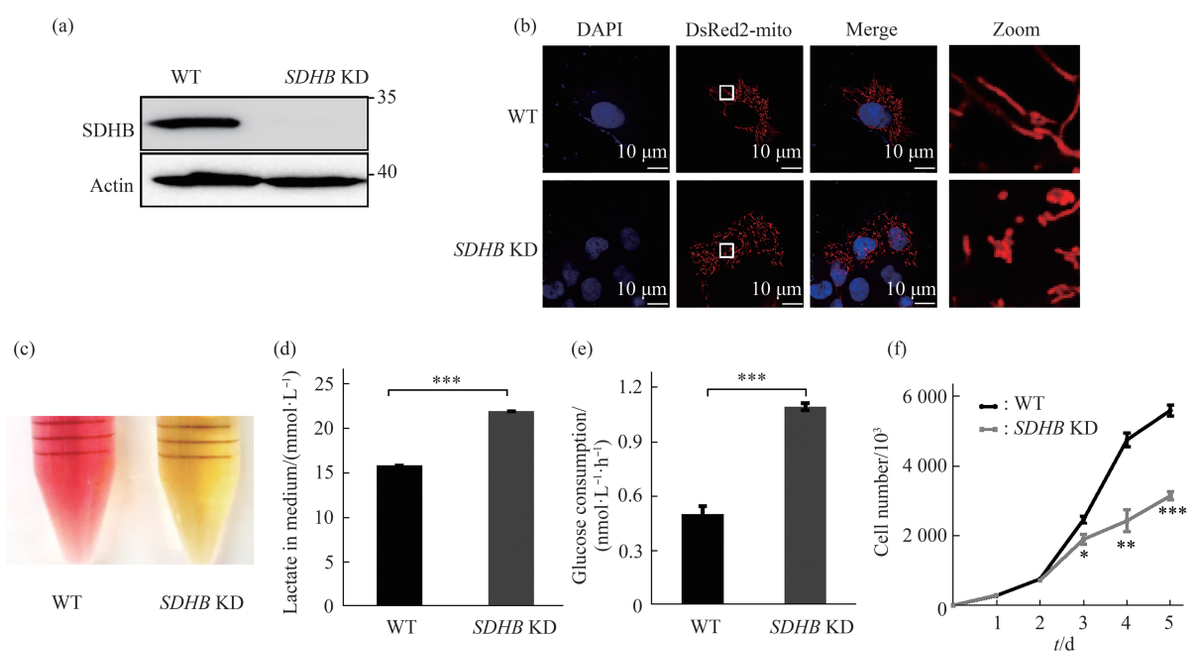

Fig. 3　*SDHB* knockdown attenuates the growth of HeLa cells

(a) Western-blotting for protein levels of *SDHB* in wildtype (WT) and *SDHB* knockdown (*SDHB* KD) polyclonal cell lines, Actin as loading control. (b) Fluorescence microscopic analysis of mitochondrial morphology in WT and *SDHB* KD cells. DsRed2-mito plasmids were transfected to track mitochondria (red), DAPI was used to stain the nuclei (blue). (c) Image of the culture medium from WT and *SDHB* KD wells. (d) Lactate levels in the medium of WT and *SDHB* KD wells. (e) Glucose consumption rate of WT and *SDHB* KD cells. (f) Growth rate of WT and *SDHB* KD HeLa cells. Student's *t*-test was performed to analysis data and the values were average of 3 wells, error bars represented in $\bar{x}\pm s$, **P*≤0.05, ***P*≤0.01, ****P*≤0.001.

3 Discussion

Mitochondrion is the central hub of intercellular signaling transductions, which regulate multiple cellular behaviors such as inflammatory response[23] and cell proliferation[24]. It is also a highly dynamic organelle, which regulated through its morphology and homeostasis, such as elongation, fragmentation and mitophagy[22]. Complex II is a part of electron transport chain that relatively independent of complex I, III and IV. It is important to see that complex II also plays a role in regulation of mitochondrial morphology and mitophagy. Considering mutations in complex II components are associated with multiple types of cancer[25-27]. Our study may have provided an insight of how aberrant complex II contributes to tumorigenesis. In this study, we demonstrated that inhibition of complex II induced mitochondrial fragmentation and mitophagy. Even though the *SDHB* knockdown can induce Warburg effect, which is normally required for solid tumor proliferation, it inhibited cell proliferation. This seems to be a paradox and there are still more research work need to be done to further elucidate the link between *SDHB* mutation-associated tumors and SDHB-deficiency-induced cell proliferation retardation. Since complex II is an important component of mitochondria, which function is essential for cell proliferation. However, Complex II dysfunction may change the metabolites, which may regulate the innate immune response and dictate pro-tumor immune response *in vivo*[19-21]. For instance, lactate and succinate are reported as pro-tumor metabolites that regulate the polarization of tumor associated macrophages, which is a common feature in malignant solid tumors. Indeed, we observed significant increased secretion of lactate in *SDHB* knockdown cells. It is possible that in complex II dysfunction cells, the alterations in metabolites contribute to the divergent from anti-tumor response to pro-tumor response, which overrides the drawback of complex II dysfunction in cell proliferation. Hence, it is worthy to test how the tumor proliferation in the context of complex II dysfunction model.

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抑制线粒体复合体II诱导线粒体自噬影响细胞增殖的研究*

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摘要 线粒体复合体II，也被称为琥珀酸脱氢酶，参与线粒体呼吸作用及代谢重编程的调控过程. 复合体II由4个亚基构成，其突变与肿瘤的发生密切相关. 本文探讨复合体II与线粒体自噬调控及细胞增殖之间的关系. 实验采用复合体II的特异性抑制剂TTFA或敲除复合体II的B亚基（SDHB）使其功能缺失. 结果发现，复合体II功能的缺失显著引起线粒体形态的片段化进而发生线粒体自噬，导致线粒体蛋白水平减少，抑制ATP生成；由于线粒体功能受到抑制，细胞葡萄糖消耗及乳酸产生水平增加，并显著抑制细胞的细胞的增殖. 综上所述，复合体II功能缺失可能通过调控线粒体自噬而影响细胞增殖，从而在肿瘤发生中起重要作用.

关键词 线粒体复合体II，琥珀酸脱氢酶（SDH），琥珀酸脱氢酶B亚基（SDHB），线粒体自噬，细胞增殖

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