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RAPID COMMUNICATION

OXCT1 regulates NF-κB signaling pathway through β-hydroxybutyrate-mediated ketone body homeostasis in lung cancer

Metabolic reprogramming is one of the hallmarks of cancer.[1] Ketone bodies behave as alternative fuel for cancer cells to support survival and proliferation.[2] 3-Oxoacid CoA-transferase 1 (OXCT1) is a key enzyme that catalyzes the first and rate-limiting step of ketolysis. Recently, several studies have revealed the significance of OXCT1 in cancer development, though the underlying mechanisms remain largely unknown.[3] In this study, we revealed a novel regulatory mechanism for tumorigenesis that OXCT1 regulated SREBP1-TRIM21-p65 axis through ketone body homeostasis in non-small cell lung cancer (NSCLC). In terms of mechanism, we found that OXCT1 could activate NF-κB signaling pathway by suppressing transcriptional activity of the sterol regulatory element binding protein 1 (SREBP1). As a transcription factor, SREBP1 could bind to the promoter of E3 ubiquitin ligase TRIM21, which mediated the ubiquitination of p65. Furthermore, we demonstrated that OXCT1 could maintain the homeostasis of β-hydroxybutyrate (β-HB), which acted as a signaling metabolite to activate SREBP1. Thus, β-HB connected OXCT1 with SREBP1 to activate NF-κB signaling pathway and promoted tumor initiation and progression. Taken together, these findings highlight a previously unappreciated mechanism for activation of NF-κB signaling by OXCT1 and ketone body, and demonstrate that targeting OXCT1 can inhibit NSCLC tumorigenesis.

The growth of NSCLC cells was inhibited in the serum-starved medium. Supplementation of β-HB rescued the growth of NSCLC cells in a dose-dependent manner (Fig. 1A; Fig. S1B, C). However, supplementation of β-HB could not rescue the growth of HBE cells in serum-starved medium (Fig. S1A). OXCT1 is a rate-limiting enzyme in the utilization of ketone bodies and plays a critical role in ketolysis. Compared with HBE cells, the mRNA and protein levels of OXCT1 were higher in NSCLC cells (Fig. 1B; Fig. S1H). OXCT1 knockdown dramatically decreased the growth of NSCLC cells, but only slightly inhibited the growth of HBE cells (Fig. 1C; Fig. S1I–K). We also found that cell migration was significantly inhibited in NSCLC cells following OXCT1 knockdown (Fig. S2B, C).

OXCT1 knockdown decreased the expression of MMP2, MMP9, β1-intgrin and u-PA (Fig. S3A), which were involved in cell proliferation and migration and were transcribed by p65. Next, we investigated the role of OXCT1 in the regulation of p65 expression. Following OXCT1 knockdown and TNFα treatment for indicted time, expression of p65 and phosphorylated p65 were decreased, which could be rescued by TRIM21 knockdown (Fig. 1D, F). TRIM21 is an E3 ubiquitin ligase and belongs to the tripartite motif (TRIM) family. The protein and mRNA levels of TRIM21 increased when OXCT1 was knocked down in H1299 cells (Fig. 1E). TRIM21 overexpression increased the ubiquitination of p65 (Fig. S4F). All these results indicated that OXCT1 could negatively regulate the ubiquitination of p65 and activate NF-κB signaling pathway by down-regulating TRIM21 in NSCLC cells.

Since both the protein and mRNA expression of TIRM21 increased following OXCT1 knockdown, we speculated that OXCT1 might regulate TRIM21 expression on transcriptional level. RNA-seq assay results showed that SREBP1 was a putative transcription factor of *Trim21*, which was significantly up-regulated when OXCT1 was knocked down (Fig. S5A). Sterol regulatory element binding protein 1 (SREBP1) belongs to the basic helix-loop-helix-leucinezipper (bHLH-Zip) family. SREBP1 overexpression increased both the protein and mRNA levels of TRIM21 in H1299 cells (Fig. 1G; Fig. S5B). To determine whether SREBP1 could initiate TRIM21 transcription, different genomic fragments of *Trim21* promoter were cloned into the pGL3-enhancer vector, and luciferase reporter assays showed that SREBP1 could promote transcriptional activity of promoter fragment P4 (Fig. 1H; Fig. S5E). SREBP1 knockdown decreased the activity of

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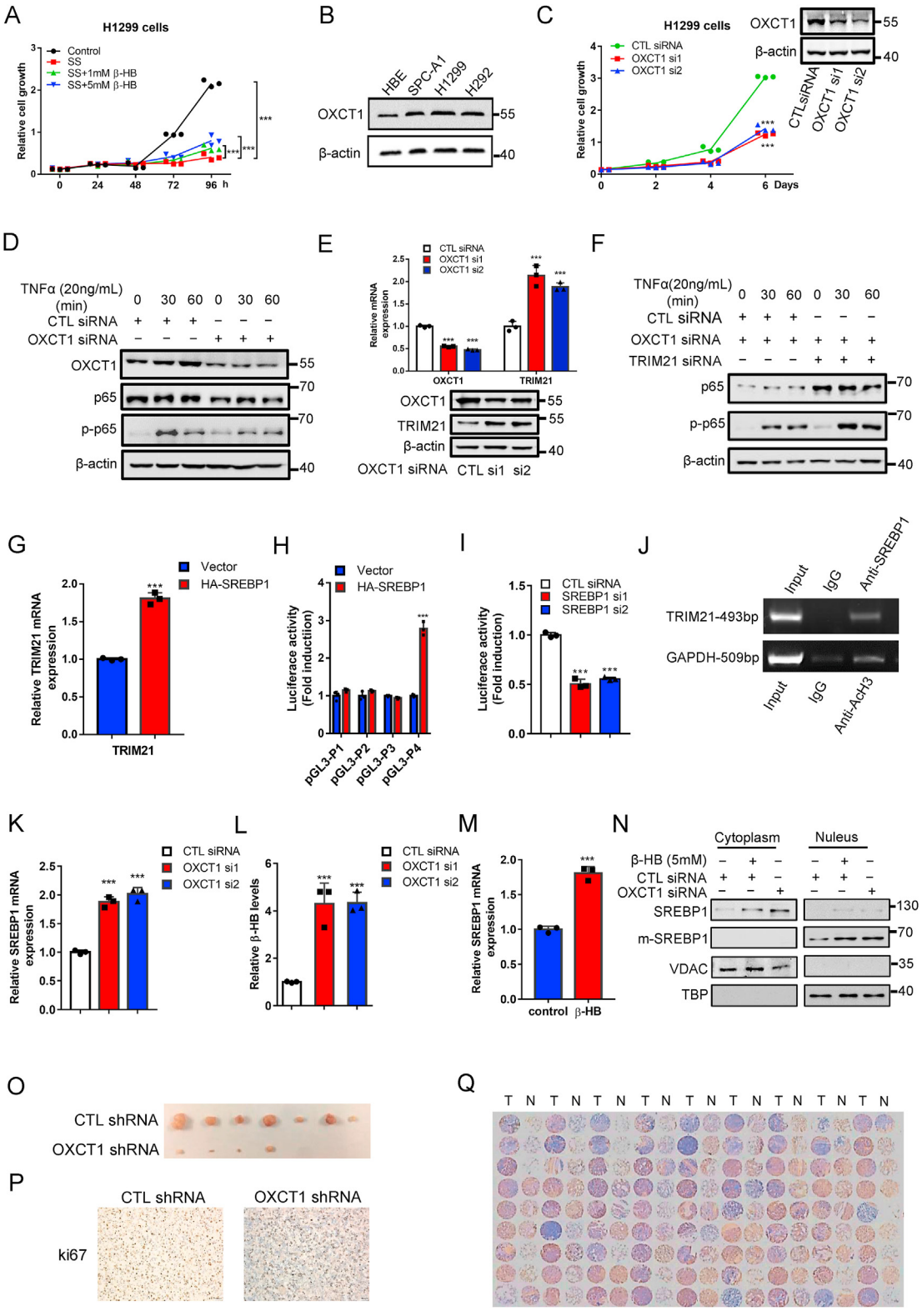

Figure 1 OXCT1 promotes the proliferation and migration of NSCLC, through SREBP1-TRIM21-p65 axis. **(A)** H1299 were cultured in normal condition or in serum starved (SS) condition in the presence or absence of β-HB (1 mM or 5 mM) and cell growth assay was performed. **(B)** The protein expressions of OXCT1 were detected by Western blot. **(C)** OXCT1 siRNAs were transfected into H1299 cells and cell growth assay was performed. Western blot assay was performed to confirm the transfection efficiency (right panel). **(D)** OXCT1 siRNAs were transfected into H1299 cells followed by treatment with TNFα for 0, 30, and 60 min. The protein expressions were determined by Western blot. **(E)** OXCT1 siRNAs were transfected into H1299 cells and the mRNA levels and protein

TRIM21 promoter (Fig. 1I). Next, the P4 genomic fragments of the *Trim21* were analyzed and putative SREBP1 binding sequence ATCACTCCAC was identified (Fig. S5F). Chromatin immunoprecipitation experiment and EMSA results indicated that SREBP1 could bind to the *Trim21* promoter (Fig. 1J; Fig. S5G). The above results indicated that SREBP1 acted as a transcription factor of TRIM21 in NSCLC cells.

Our study demonstrated that protein and mRNA levels of SREBP1 were increased following OXCT1 knockdown (Fig. 1K; Fig. S6A). Next, we tried to explore its underlying mechanism. OXCT1 did not interact with SREBP1 in H1299 cells (Fig. S6B). OXCT1 knockdown induced accumulation of β-HB (Fig. 1L) and activation of luciferase activity of TRIM21 promoter (Fig. S6B). Previous study indicated that β-HB could act as a "non-canonical" signaling mediator.[4,5] Therefore, we speculated that OXCT1 regulated SREBP1-mediated transcription of TRIM21 through β-HB in NSCLC cells. Both mRNA and protein expression of SREBP1 were increased when H1299 cells were treated with β-HB (Fig. 1M; Fig. S6C). Moreover, the mature form of SREBP1 increased in cell nucleus following OXCT1 knockdown or β-HB treatment (Fig. 1N). Meanwhile, *Trim21* promoter activity was increased when H1299 cells were treated with β-HB (Fig. S6D). These results highlighted a novel molecular mechanism that OXCT1 mediated SREBP1 transcriptional activity by maintaining β-HB homeostasis.

OXCT1 knockdown H1299 stable cell line (H1299-shOXCT1) was constructed using OXCT1 shRNA (Fig. S7A). Soft agar assay showed that H1299-shOXCT1 cells formed smaller colonies compared with H1299-shCTL cells (Fig. S7D). Furthermore, a reduction in tumor size and weight was observed in tumors formed by H1299-shOXCT1 cells compared with those in control group following xenograft assay (Fig. 1O, P). We evaluated the expression of Ki67 and TTF-1 in xenograft tumor. Ki67 expression was lower in tumors formed by H1299-shOXCT1 cells than in tumors formed by H1299-shCTL stable cells (Fig. 1P). TTF-1 expression was higher in tumors derived from H1299-shOXCT1 cells than in tumors formed by H1299-shCTL stable cells (Fig. S7I). The above results indicated that OXCT1 deficiency inhibited tumor progression in NSCLC.

We extracted total proteins from the adjacent normal tissue and lung cancer tissue of 13 pairs of NSCLC patients, and Western blot results showed that the expression of OXCT1 were higher in NSCLC tissues (Fig. S10A). Meanwhile, similar results were observed when the expression levels of OXCT1 were examined by immunohistochemistry in NSCLC tissue array (Fig. 1Q; S10B). Patients with high expression levels of OXCT1 exhibited worse survival rate than patients with low levels of OXCT1 (Fig. S10D).

Generally, ketone bodies provide an energy source for tissues during fasting or prolonged exercise. OXCT1 acts as a key enzyme in ketone utilization process. Here, we found that OXCT1 knockdown resulted in the accumulation of β-HB. Accumulated β-HB increased the expression of transcription factor SREBP1. SREBP1 could bind to the promoter of *Trim21* and mediated its transcription in NSCLC cells. As an E3 ubiquitin ligases, TRIM21 promoted the ubiquitination and degradation of p65, and inhibited the tumorigenesis of NSCLC. This study highlights a previously unappreciated mechanism for activation of NF-κB signaling by OXCT1 through β-HB-SREBP1-TRIM21 axis, and demonstrates that targeting OXCT1 can inhibit tumorigenesis.

Author contributions

Jian-Bin Wang, Bentong Yu and Caifeng Xie designed the experiments and interpreted data. Zhuo Lu, Tianyu Han, Tao Wang, Mingxi Gan performed the experiments. Jian-Bin Wang and Caifeng Xie analyzed data. Jian-Bin Wang, Wei Zhang and Caifeng Xie wrote the paper.

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expressions were analyzed. **(F)** H1299 cells were co-transfected with OXCT1 siRNA and control (CTL) siRNA, or OXCT1 siRNA and TRIM21 siRNA for 48 h, then treated with TNFα. Proteins were collected and all proteins in NF-κB signaling pathway were detected by Western blot. **(G)** HA-SREBP1 plasmids were transfected into H1299 cells, and qPCR was performed. **(H)** Indicated plasmids were transfected into H1299 cells and dual luciferase reporter assay was performed to detected the transcription activity of TRIM21 promoter. **(I)** Indicated plasmids and SREBP1 siRNAs were transfected into H1299 cells and dual luciferase reporter assay was performed to detected the transcription activity of TRIM21 promoter. **(J)** ChIP assay was performed with indicated antibodies, followed by PCR using indicated primers. **(K)** OXCT1 siRNAs were transfected into H1299 cells, and qPCR was performed to detected the mRNA levels of SREBP1. **(L)** OXCT1 siRNAs were transfected into H1299 cells and intracellular β-HB levels were measured. **(M)** H1299 cells were treated with 5 mM exogenous β-HB for 24 h and qPCR was performed. **(N)** H1299 were transfected with indicated siRNAs or treatment with β-HB. The cytoplasmic and nuclear proteins were separated; the protein levels were determined by Western blot. **(O)** Xenograft tumorigenesis. Nude mice were subcutaneously injected with H1299-shCTL stable cells and H1299-shOXCT1 stable cells (1×10^7). Tumors were dissected out and photographed. **(P)** Immunohistochemical staining of tumors formed by H1299-shCTL cells and H1299-shOXCT1cells for Ki67 and TTF1. The scale bars are 20 μm. **(Q)** Immunohistochemical staining of a lung adenocarcinoma tissue microarray using anti-OXCT1 antibody. T: tumor tissue; N: adjacent normal tissues. Data represents the average of three independent experiments (mean ± SD). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Conflict of interests

The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.04.020.

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