Master of Science

Branched-chain amino acids sustains pancreatic cancer growth by regulating lipid metabolism

췌장암 지질 대사에서 BCAA 의 역할 규명

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Branched-chain amino acids sustains pancreatic cancer growth by regulating lipid metabolism

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ABSTRACTS

Branched-chain amino acids (BCAAs) catabolism and high levels of enzymes in the BCAAs pathway have recently been linked to cancer growth and survival. However, the specific roles of BCAAs metabolism in cancer growth and survival remains unclear. Here, I find that BCAAs metabolism plays an important role in the growth of human pancreatic duct adenocarcinoma (PDAC) through regulation of lipogenesis. PDAC cells exhibited significantly increased uptake of BCAAs through upregulated Branched-chain amino acid aminotransferase 2 (BCAT2) levels compared to non-transformed human pancreatic ductal cells (HPDEs). Knockdown of BCAT2 dramatically impaired PDAC growth without significant changes in glutamate and ROS levels, but did not impair HDPE growth. Furthermore, PDAC growth was strongly inhibited upon Branched-chain α-keto acid dehydrogenase a (BCKDHA) knockdown. Surprisingly, BCKDHA knockdown had no significant effect on mitochondrial metabolism, including tricarboxylic acid (TCA) cycle intermediates and oxygen consumption rate, but significantly reduced fatty acid synthesis, indicating that PDAC can supply carbon fuel to fatty acids using BCAAs. Thus, our data shows that BCAAs pathway may provide novel therapeutic targets for pancreatic cancer treatment.

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Introduction

Pancreatic cancer is a highly aggressive tumor and poor prognosis[1]. Pancreatic ductal adenocarcinoma (PDAC) is the most common malignant pancreatic tumor and an expected 5-year survival rate of ~8%[2]. An unfortunate fact is that PDAC is estimated to become the second leading cause of cancer-related death by 2020[3]. PDAC is commonly detected after it has already spread beyond the pancreas because it shows no signs or symptoms in the early stages, making it hard to diagnose early[4]. So, the best treatment is surgery, but very few patients can be treated with surgery. Therefore, there is an urgent need new therapeutic strategy for pancreatic cancer treatment.

Cancer metabolism is one of the oldest fields of research in cancer biology. A common feature of cancer cells is that they induce changes in metabolism to obtain the nutrients they need in a nutrient-free environment in order to make new biomass necessary for the rapid growth and survival of cancer cells. To induce these changes, cancer cells reprogram directly and indirectly their metabolic process[5]. Metabolic reprogramming, as known as the Warburg effect, explains how and why cancer cells use glucose as an energy source for tumorigenesis through aerobic glycolysis[6]. Cancer metabolism based on the elements that metabolic activities are altered in cancer cells relative to normal cells[7]. Therefore, studying cancer cell metabolism is an attractive strategy for treating cancer cells.

BCAAs are essential amino acids containing leucine, isoleucine, and valine. BCAAs is essential for cancer growth and tumors and cancer cells increase BCAAs uptake[8]. BCAAs presents a number of novel features. One of the features, it supplies the energy needed for cancer growth and survival and can be oxidized for protein synthesis[9]. Recently studies have shown that high levels of enzyme in BCAA catabolism and BCAA pathway are associated with cancer growth and survival [9]. Also it has become evident that the enzymes catalyzing

the first step in BCAA degradation are overexpressed in many cancers[10-12]. The first step in their catabolism is the conversion of BCAAs to BCKAs by Branched-chain amino acid aminotransferase (BCAT) enzymes. Another enzyme, the branched chain α-keto acid dehydrogenase (BCKDH) is present in mitochondria and catabolized BCKAs to Acetyl coA[13]. Finally, these BCAA metabolites are metabolized by a series of enzymatic reactions to the end products (acetyl-CoA of leucine, acetyl-CoA of valine and acetyl-CoA and succinyl-CoA of isoleucine), which enter the TCA cycle[14]. Interestingly, several studies have reported that supplying animals with a BCAA deficient diet increased lipolysis[15]. Similarly, in female broiler chickens, low dietary BCAA levels reduced fatty acid synthesis[16]. Thus, the intimate relationship between BCAA metabolism and fatty acid metabolism cannot be denied. And studies were conducted to explore this relationship in cancer metabolism.

Lipids promote cell growth and proliferation as well as energy supply. Recent cancer studies have shown that energy metabolism, especially lipid metabolism, is considerably increased during carcinogenesis[17,18]. Disorders of lipid metabolism are characterized not only by congenital or acquired factors, but also by abnormalities of lipids and lipid metabolites. Generally, lipids are mainly classified as lipids (such as phospholipids, glycolipids, and sterols) and fats (such as triglycerides). Triglyceride (TG) is an important energy storage material and plays an important role in energy supply[19], temperature control and organism protection as well as in assisting absorption of fat-soluble vitamins. Phospholipids are also a major component, preventing cell membrane damage and promoting TG metabolism and reduced cholesterol deposition. Lipid levels are regulated by a variety of related genes, hormones and enzymes. Thus, targeting lipid metabolism could be a novel strategy for cancer prevention and treatment[17,18,20].

In this study, I demonstrate that PDAC cells require BCAAs for growth as a source of carbon fuel for lipid biosynthesis. BCAT2 or BCKDHA knockdown, the key enzymes in BCAA

catabolism resulted in significant suppression of PDAC growth, whereas HPDE growth was not impaired upon BCAT2 or BCKDHA knockdown Interestingly, BCAT2 knockdown had no effects on both glutamate and ROS levels. Furthermore, levels of TCA cycle intermediates and oxygen consumption rate were not altered upon BCKDHA knockdown, but it led to dramatic reduction of levels of both fatty acids and triglycerides. Thus, our data suggest that targeting BCAA metabolism may be an effective target for pancreatic cancer treatment.

Material and Methods

Cell culture

Cell lines were acquired from the American Type Culture Collection (Manassas, VA, USA) and were tested regularly for mycoplasma contamination. All cells were cultured in Dulbecco's modified Eagle's Medium (DMEM; Thermo Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (Thermo Scientific, Waltham, MA, USA) and maintained at 37°C in humidified air with 5% CO_2 and in. HPDE cells were cultured as described previously[21].

Reagents and antibodies

Primary antibodies against BCKDHA(#271538), BCKDHB(#374630), ACTIN(#47778) were purchased from Santa Cruz Biotechnology and BCAT2(#9432) were purchased from Cell Signaling Technology. The secondary antibodies, horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse antibodies were from Bethyl Laboratories. N-acetyl-l-cysteine (NAC; A9165) and Adipored (#PT-7009) were obtained Sigma-Aldrich and Lonza

Metabolomic analysis

Cells were grown to about 60% confluence in complete media (DMEM, 10% FBS) on 10cm dishes in biological triplicate. After 24 h, the cells were then harvested using 1.4 mL of cold methanol/H_2O (80/20, v/v) after sequential wash with PBS and H_2O and lysed by vigorous vortexing and 100 μL of 5 μM of internal standard was added. Metabolites were extracted from the aqueous phase by liquid-liquid extraction after adding chloroform. The

aqueous phase was dried using vacuum centrifuge and the sample was reconstituted with 50 μL of 50% methanol prior to LC-MS/MS analysis. The LC/MS/MS system was equipped with an Agilent 1290 HPLC (Agilent), Qtrap 5500 (ABSciex), and reverse phase column (Synergi fusion RP 50 × 2 mm). Three microliters were injected into the LC-MS/MS system and ionized with a turbo spray ionization source. Multiple reaction monitoring (MRM) was used in the negative ion mode and the extracted ion chromatogram (EIC) corresponding to the specific transition for each metabolite was used for quantitation. Area under the curve of each EIC was normalized to that of EIC of internal standard. The peak area ratio of each metabolite to that of the internal standard was normalized using the protein amount in each sample and was then used for relative comparison.

Lentiviral-mediated shRNA targets

The RNAi Consortium clone IDs for the RNAs used in this study are as follows: shBCAT2-1 (TRCN00000286203), shBCAT2-2 (TRCN00000286266), shBCKDHA-1 (TRCN0000028456) and shBCKDHA-2 (TRCN0000028398). For the PEI-based method, 293T cells were seeded at 1×10^7 cells per 10cm dish about 24 h before transfection. Cells were rinsed and supplemented with a fresh serum-free culture medium just before transfection. The plasmid DNA mix (8 μg per dish) and 1ug/ml of PEI stock solution were mixed and placed on a vortex stirrer for 1 min. After 30 min, the transfection mixture was completed up to 1ml with serum-free culture medium and added to the cells. 6h incubation after, the media was changed to complete media.

Cell proliferation assay

Cells were plated in 24-well plates at 2,000cells per well in 0.5ml of media. Media was not changed during the course of the experiment and cells were fixed in 10% formalin and stained with 0.1% crystal violet (Sigma-Aldrich, St Louis, MO, USA) on indicated time points. Dye was extracted 10% acetic acid and the relative proliferation was quantified by OD at 595nm

Quantitative real-time PCR

RNA was isolated with TRIzol (QIAGEN) and cDNA was synthesized with MMLV HP reverse transcriptase (Epicentre, Madison, WI, USA). Quantitative real-time PCR was performed with SYBR Green dye using an ArizMx Real-Time PCR system. The primer sequences were designed as follows; SLC1A5 (Forward; CCCTCATCTACTTCCTCTTCAC, Reverse; TTATTCTCCTCCACGCACTTC), SLC3A2 (Forward; TGCTCAGGCTGACATTGTAGC, Reverse; TCAGCCAAGTACAAGGGTGC), SLC7A5 (Forward; CCGTGAACTGCTACAGCGT, Reverse; CTTCCCGATCTGGACGAAGC)

Colony formation

Cells were plated in 6-cm dishes at 250 cells per well in growth medium with 10% FBS and 1% antibiotics. And cells were treated with NAC the day after seeding. After 10~12 days, cells were stained with 0.2% crystal violet and colonies were counted.

Western blot analysis

For western blotting, Cells were lysed in ice-cold lysis buffer (RIPA buffer) containing protein inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Samples were then

incubated in RIPA buffer for 30 min and soluble lysate fractions were isolated by centrifugation at 32,000 ×g for 15 min. Protein concentrations were analyzed with the Pierce BCA Protein Assay (Thermo Scientific, Waltham, MA, USA) Equal amounts of lysates were mixed with DW and Laemmli loading dye and boiled for 5 min. The lysates were loaded for SDS-PAGE and the proteins were transferred to PVDF membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat skim milk in Tris-buffered saline and 0.1% Tween 20 (TBS-T), and incubated with primary antibody at 4℃ for overnight. After washing with TBS-T, membranes were exposed to the appropriate horseradish peroxidase-conjugated secondary antibody for 1hr at Room temperature. The protein-antibodies complexes were visualized on LAS 2000 using an enhanced chemiluminescence (ECL) detection system (Advansta Inc., Menlo Park, CA, USA).

Detection of reactive oxygen species

To analyze cytoplasmic ROS, cells were incubated with 2',7'-dichlorodihydrofluorescein diacetate (DCFDA, 5 μM; Invitrogen, Carlsbad, CA, USA) for 30 min. The cells were washed twice with PBS at room temperature to remove excess DCFDA; labeled cells were trypsinized, rinsed and resuspended in PBS. Oxidation of DCFDA to high fluorescence 2 ', 7'-dichloro-fluorescein (DCF) proportional to the occurrence of ROS was analyzed by flow cytometry. To measure mitochondrial ROS, cells were incubated with 5 μM MitoSOX ™ reagent (Thermo Scientific, Waltham, MA, USA) for 10 min at 37 ° C, then trypsinized, washed with PBS and resuspended in 200 μL of PBS. The stained cells were then quantitatively analyzed and analyzed with a flow cytometer (BeckmanCoulter, Brea, CA, USA). Excitation wavelength was 510 nm, and emission wavelength was 580 nm.

Quantitation of intracellular ATP

Intracellular ATP concentrations were determined using an ATP Colorimetric/Fluorometric Assay Kit (Biovision Incorporated, Milpitas, CA, USA) according to the manufacturer's instructions. Cells were lysed in 100μl of ATP assay buffer and 50ul of the supernatant was collected and added to a 96well plate. To each well was added 50ul of ATP assay buffer containing ATP probe, ATP converter and developer. Absorbance was analyzed at 570nm.

OCR measurement

Oxygen consumption rate were analyzed as previously described[22]. Briefly, cells were plated in a 24-well Seahorse plate and cultured at 37ºC and 5% CO2. The medium was replaced the following day with unbuffered DMEM, and cells were incubated at 37ºC without CO2 for 1 h. Oligomycin, FCCP, and rotenone were added to final concentrations of 2 μM, 5 μM, and 2 μM, respectively.

Aipored staining

For Aipored staining, PDAC cells, infected with lentiviral shRNAs targeting BCKDHA were plated in a 35-mm confocal dish then incubated for another 24 h. The cells were then stained with Aipored (Lonza, #PT-7009) for 10min at 37 °C. The images were captured using an LSM780 confocal fluorescent microscope (ZEISS).

Transcript analysis of clinical mRNA microarrays

The transcription levels of transporters of BCAAs across normal and pancreatic cancer tissue was retrieved from the Oncomine platform (https://www.oncomine.org/), an online cancer microarray database. Using the default settings for various groups of filters, each query for accessing mRNA expression data was executed using threshold parameters of p-value 1e-4, fold-change 2, and gene ranking in the top 10%. The gene expression profiling interactive analysis (GEPIA) (http://gepia.cancer-pku.cn/) was used for analyzing RNA sequencing expression levels of transporters of BCAAs between normal and pancreatic cancer tissue.

Xenograft studies

Female severe combined immunodeficiency mice were bought from Charles River Laboratories. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Asan Institute for Life Sciences (protocol 2018-02-304). For subcutaneous xenografts, 8988T cells were infected with lentiviral shRNAs targeting BCKDHA (n=2) and GFP (control hairpin, n=1) and received short puromycin selection (2 μg/mL). $1x10^6$ cells, suspended in 100 μL Hanks Buffered Saline Solution (HBSS) were injected on the lower flank of the subcutaneous area into each mouse (4 per group). The length and width of the tumor were measured three times per week and the volume was calculated according to the formula (length x width 2) / 2.

Statistics

Data are presented as mean ± standard deviation. All comparisons were analyzed using unpaired Student's t-test.

Results

1. PDAC cells display increased BCAAs uptake

Previous studies have shown a clear positive association between BCAAs levels and future development of pancreatic cancer[23,24]. However, the roles of BCAA in cancer metabolism is not yet clear. To investigate whether BCAA related to cancer development, I first measured the uptake of BCAAs in PDAC cell lines, 8988T, Miapaca2 and non-transformed human pancreatic ductal cell (HPDE) used as control. I observed that BCAAs uptake in PDAC cells is higher than HPDE (Fig. 1A, B and C). Given that BCAA transporters are over-expressed in many cancers and this transporter often plays a key role in oncogenesis[25], I further analyzed the expression of BCAA transporters in PDAC using the Oncomine database (available at http://www.oncomine.org). The expression of BCAA transporters was higher in PDAC than in normal pancreatic cancer samples (Figure 1D, E and F), suggesting that BCAA transporters may increase BCAAs uptake in PDAC. Additionally, the expression of BCAA transporters was validated using the GEPIA database (available at http://gepia.cancer-pku.cn/) and was observed to present a higher expression in 179 pancreatic cancer tissues than in 171 normal tissues. To confirm these findings, mRNA expression levels of SLC1A5, SLC3A2 and SLC7A5, known as BCAA transporters, were determined. As shown in Fig. 1G, mRNA expression level was significantly increased in PDAC cell compared to HPDE cell. Together, these results indicate that PDAC cells exhibit increased levels of BCAAs uptake.

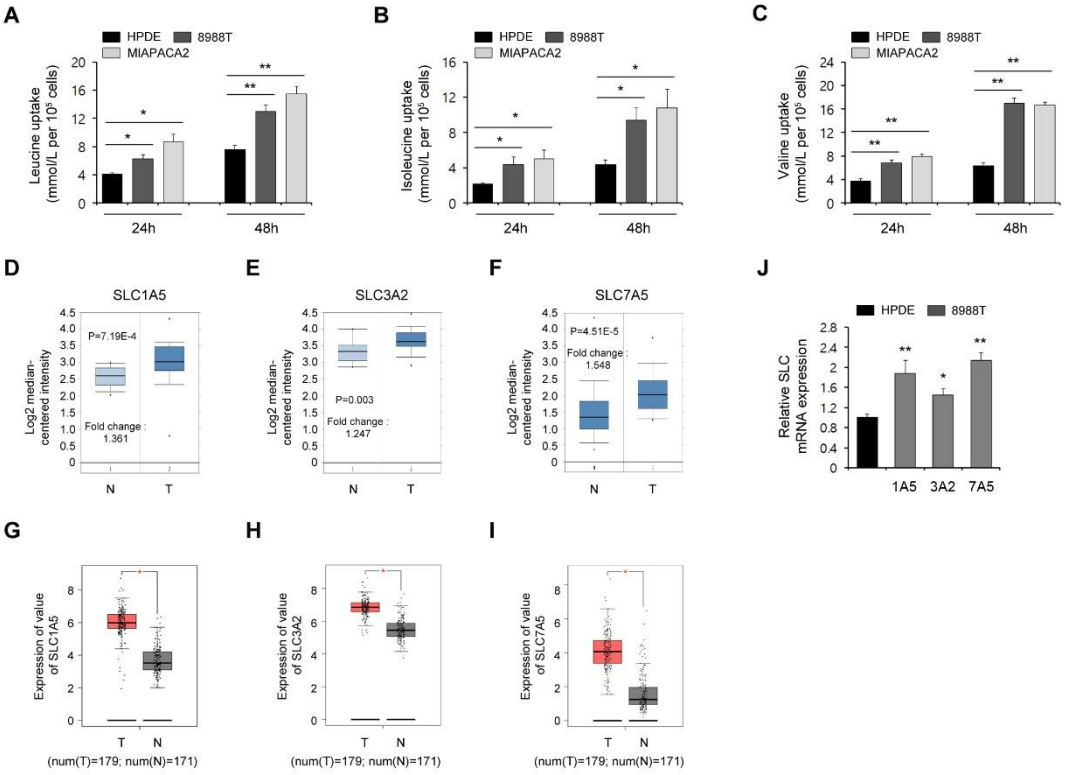

Fig. 1 BCAAs uptake increases in PDAC. A-C BCAAs were analyzed at the indicated time points via LC-MS/MS. **D-F** Box plots derived from gene expression data in Oncomine comparing expression of a specific BCAA transporter genes in normal pancreatic tissue (N) and PDAC (T). **G-I** The expression of BCAA transporter genes were detected in 179 pancreatic cancer tissues (T) and 171 normal pancreatic tissues (N) from GEPIA. Axis units are Log2 (TPM+1). **J** mRNA expression of SLC1A5, SLC3A2, SLC7A5 was measured by Quantitative real time PCR analysis. Error bars represent the standard deviations (s.d.) of triplicate wells from a representative experiment. *, $p < 0.05$; **, $p < 0.01$.

2. BCAT2 is critical for PDAC growth

To confirm the importance of BCAAs metabolism in PDAC growth, I first performed western blotting to detect the protein expression of BCAT1 and BCAT2 (branched amino acid transaminases 1 and 2) required for the first step in BCAA metabolism. As shown in Fig. 2A, PDAC cells exhibited increased expression of BCAT2, but not BCAT1 compared with HPDE cells. To elucidate whether BCAT2 is essential for cancer metabolism, I measured the growth of PDAC cells upon knockdown of BCAT2. Knockdown of BCAT2 inhibited cell proliferation in 8988T and Miapaca2 cells (Fig. 2B,2C). To confirm these results, MTT assay was performed to assess cell viability. As shown in Figure 2D and 2F, knockdown of BCAT2 significantly reduced cell proliferation. BCAT2 knockdown also markedly reduced colony formation. (Fig. 2F,2G). These results suggest that BCAT2 is important for pancreatic cancer cell proliferation. I observed that inhibition of BCAA had no effect on HPDE cells. In contrast to PDAC cells, HPDE cells were not affected growth by BCAT2 knockdown (Fig. 2H). These results demonstrate that increase in BCAT2 expression level is required for the pancreatic cancer cell growth.

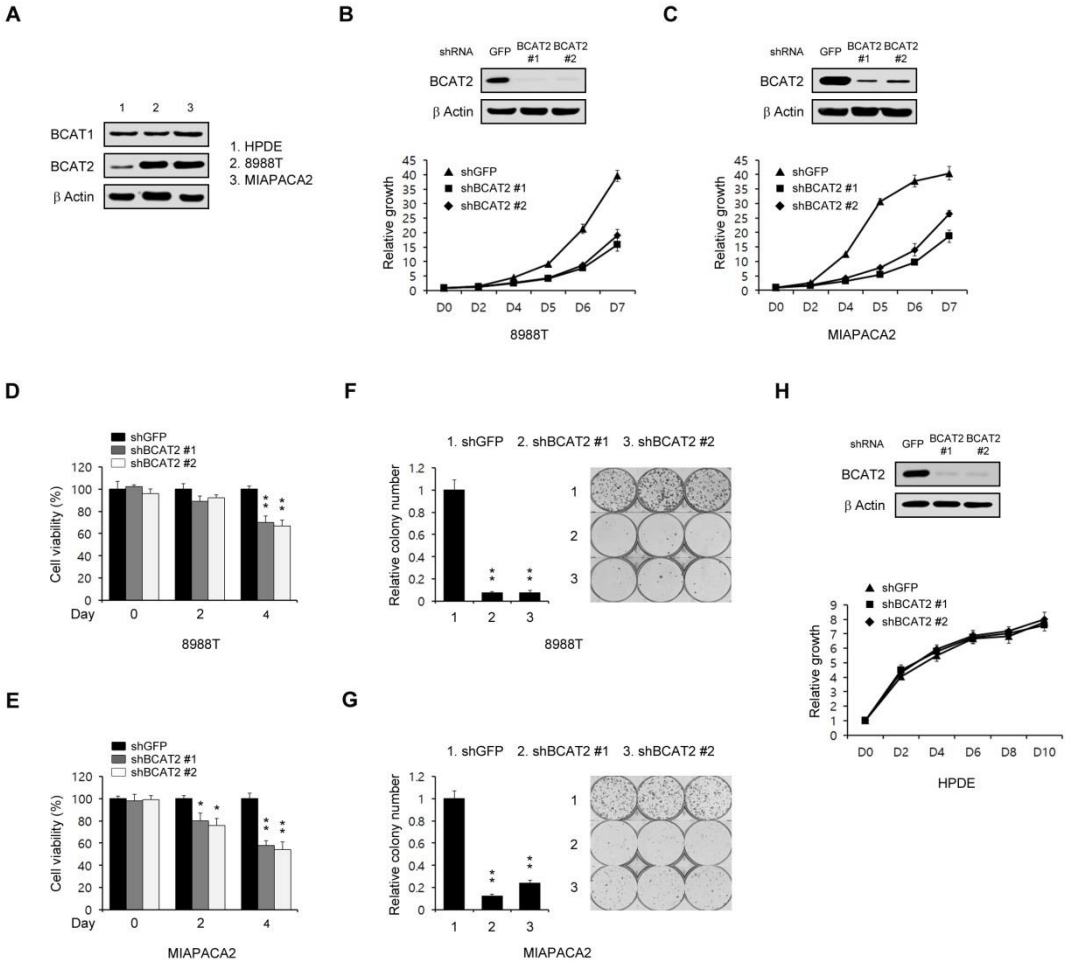

Fig. 2 Pancreatic cancers require BCAT2 for growth. A Immunoblots of BCAT1 and 2 in HPDE and PDAC cells. **B-C** PDAC cells expressing control shRNA (shRNA) or two independent shRNAs to BCAT2 were assayed for cell growth. **D-E** PDAC cells expressing control shRNA (shRNA) or two independent shRNAs to BCATs were assayed for cell viability. **F-G** ex Relative clonogenic growth of PDAC cells pressing a control shRNA(shGFP) or two independent shRNAs targeting BCAT. Error bars represent the s.d. of triplicate wells from a representative experiment. **H** HPDE cells expressing control shRNA (shRNA) or shRNA targeting BCAT2 were assayed for cell growth. Error bars represent the

standard deviations (s.d.) of triplicate wells from a representative experiment. *, $p < 0.05$; **, $p < 0.01$.

3. BCAT2 suppression does not affect redox homeostasis

BCAA catabolism is proceeded in two steps. The first step is trans-aminated with BCKA by BCAT isoenzymes, in which BCAA amino groups are converted to α-ketoglutarate to produce glutamate. The second step transforms BCAT into Acetyl CoA by BCKDH catalysis (Fig. 3A)[25,26] . In other carcinomas, BCAT2 regulates glutamate level[27]. To confirm the preceding results, I measured glutamate level in PDAC cells. The reduction of BCAT2 had no effect on glutamate levels in PDAC cells. (Fig. 3B). In BCAA metabolism, bcat2 is an enzyme that converts a-KG to glutamate. Thus, I experimented whether BCAT2 effect to redox homeostasis. As shown in Figure 3C and 3D, I measured ROS in cytoplasm and mitochondria by use DCFDA and MitoSOX. As a result, the knockdown of BCAT2 had no effect on both DCFDA and MitoSOX. To confirm these results, the antioxidant NAC was used in rescue experiments. NAC had no rescue effect on PDAC growth upon BCAT2 knockdown (Fig. 3E,3F). Taken together, these data demonstrate that inhibition of PDAC growth by BCAT2 suppression does not affect glutamate and ROS levels.

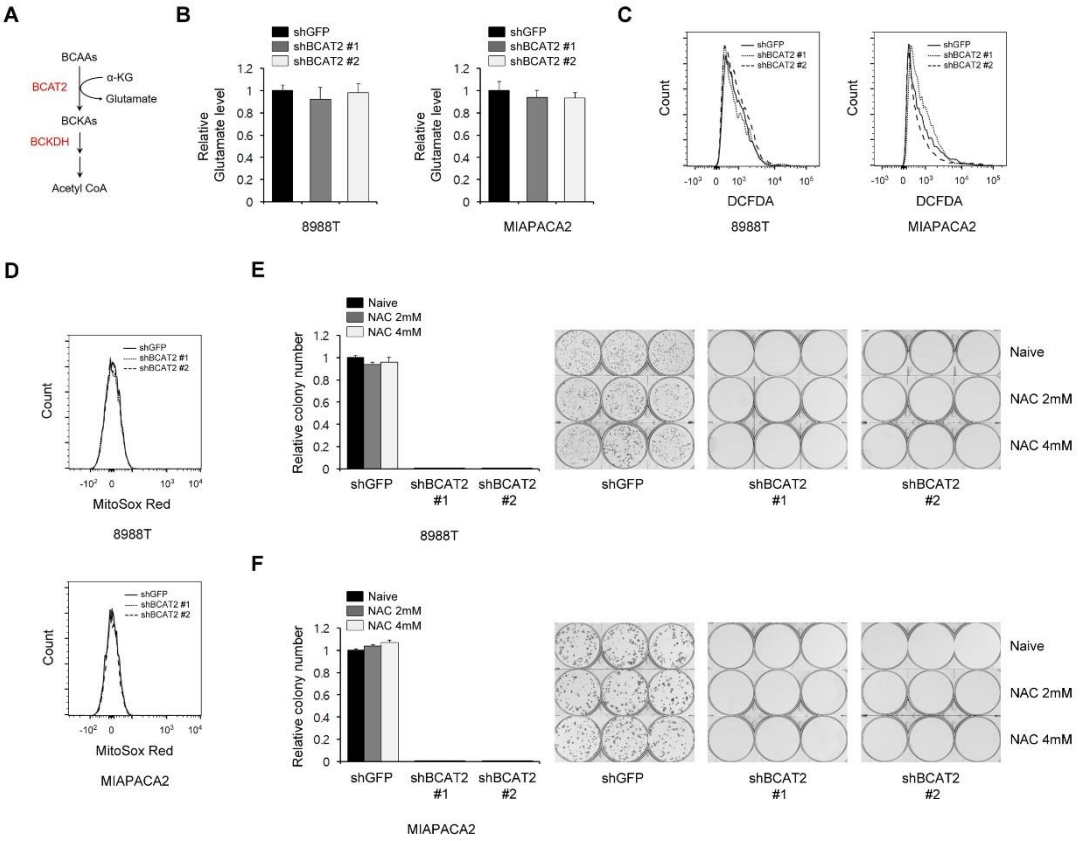

Fig. 3 BCAT2 has no effect on redox homeostasis. A Schematic depiction of the cytoplasmic reactions that convert BCAAs into Acetyl-CoA. **B** Relative glutamine level of PDAC cells expressing a control shRNA (shGFP) or a shRNA targeting BCAT2. **C-D** PDAC cells expressing a control shRNA (shGFP) or a shRNA targeting BCAT2 were subjected to DCFDA assay (C) or MitoSox Red assay (D). **E-F** Relative clonogenic growth of PDAC cells a control shRNA (shGFP) or a shRNA targeting BCAT2 with or without NAC (4mM).

4. BCKDH suppression impairs PDAC growth

Next, I investigated the importance of, the enzyme of the second step of BCAA metabolism, on PDAC growth. To determine whether BCKDH affects cancer metabolism, growth of pancreatic cancer was measured upon BCKDHA knockdown. Knockdown of BCKDHA reduced cell proliferation in PDAC cells (Fig. 4A, 4B). To confirm these results, MTT assay was performed to assess cell viability. As shown in Figure 4C and 4D, knockdown of BCKDHA dramatically reduced cell proliferation. BCKDHA knockdown also significantly inhibited colony formation (Fig. 4E,4F). Interestingly, I observed that inhibition of BCAA does not affect HPDE cells. Consistent with the results of BCAT2, the BCKDHA minimum also had no effect on HPDE growth (Fig. 4G). These results suggest that inhibition of BCKDHA impairs with the proliferation of PDAC, but not HPDE

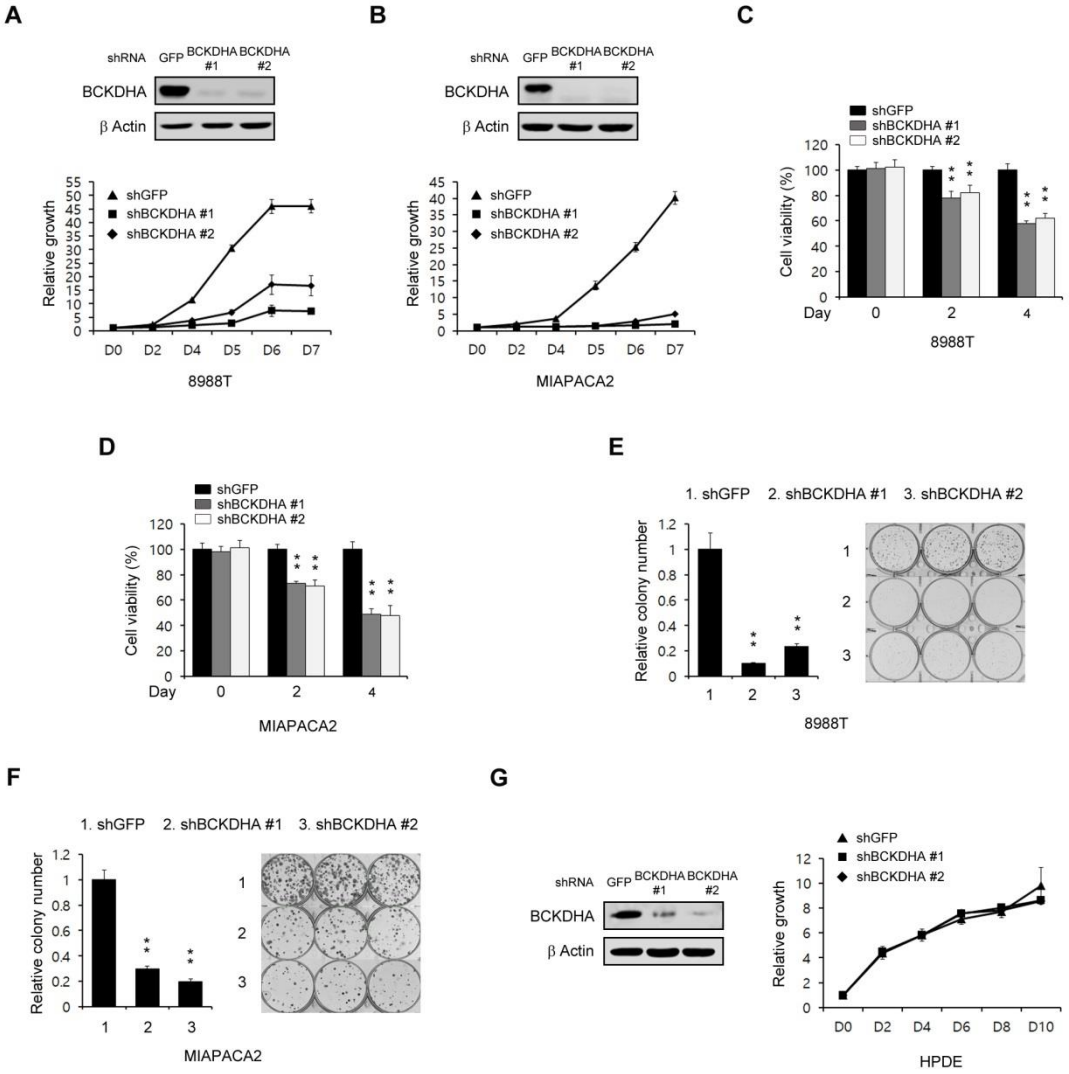

Fig. 4 Pancreatic cancers require BCKDHA for growth. A-B PDAC cells expressing control shRNA (shRNA) or shRNA targeting BCKDHA were assayed for cell growth. **C-D** PDAC cells expressing control shRNA (shRNA) or shRNA targeting BCKDHA were assayed for cell viability. **E-F** Relative clonogenic growth of PDAC cells expressing a control shRNA(shGFP) or shRNA targeting BCKDHA. Error bars represent the s.d. of triplicate wells from a representative experiment. **G** HPDE cells expressing control shRNA

(shRNA) or shRNA targeting BCKDHA were assayed for cell growth. Error bars represent the standard deviations (s.d.) of triplicate wells from a representative experiment. **, $p < 0.01$.

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5. BCAAs metabolism has no effect on mitochondrial metabolism

To examine the mitochondrial function of BCAA metabolism in PDAC, I first attempted to measure oxygen consumption rate and ATP level. ATP levels were no effected by BCKDHA knockdown (Fig. 5A). As shown in Fig. 5B, oxygen consumption rate was also not changed upon knockdown of BCKDHA. I next examined the TCA cycle metabolites. when BCKDHA activity was impaired, TCA cycle metabolites were not altered, but interestingly citrate levels were significantly reduced. (Fig. 5C). Fatty acid synthesis requires acetyl-CoA generated from citrate produced by the TCA cycle. Thus, I confirmed to determine if the decrease in citrate levels affects Fatty Acid Synthesis, and as a result, I found that fatty acid levels were markedly decreased (Fig. 5D). I next confirmed the intracellular TG accumulation by using an Adipored signal. As shown in Fig. 5E, the Adipored signal was highly reduced, indicating a decrease in triglyceride. Taken together, these data demonstrate that BCKDHA suppression did not significantly affect mitochondrial metabolism, but it inhibited fatty acid synthesis and confirmed that PDAC can supply carbon fuel to fatty acids using BCAAs.

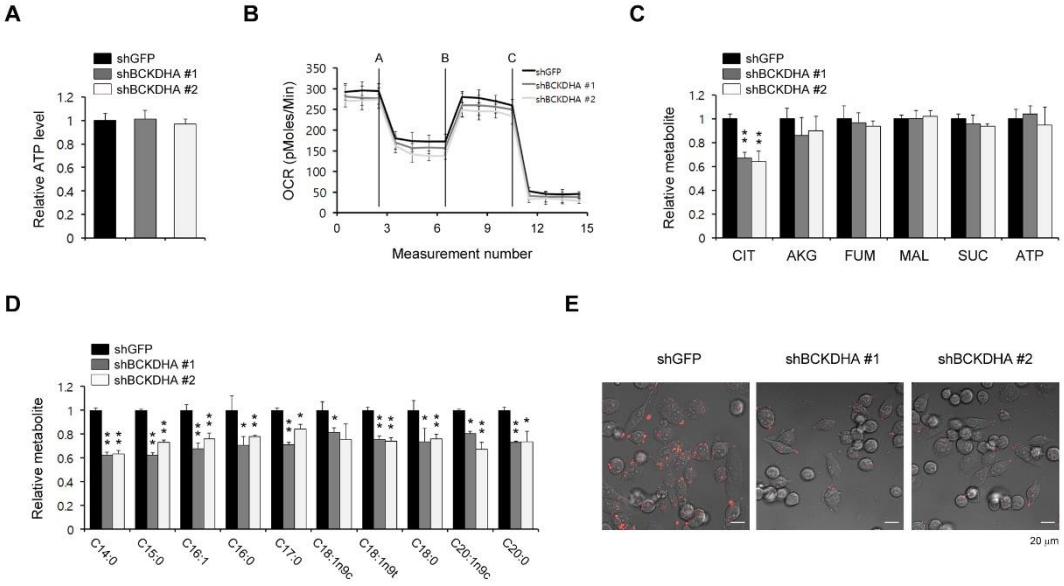

Fig. 5 Lipogenesis requires BCAAs metabolism. A PDAC cells were plated in a complete media that was replaced the following day with BCKDHA knockdown, incubated for another 24 hours, and assayed for intracellular ATP. Error bars, SD of triplicate wells from a representative experiment. **B** Oxygen consumption rates were measured with an extracellular flux analyzer. PDAC cells were plated in a complete media that was replaced the following day with BCKDHA knockdown. Error bars, SD of triplicate wells from a representative experiment. **C** TCA metabolite pools in PDAC cells expressing control (shGFP) or BCADHA shRNAs (shBCKDHA) were analyzed via LC-MS/MS. Error bars, SD of triplicate wells from a representative experiment. **D** FFA metabolite pools in PDAC cells expressing control (shGFP) or BCADHA shRNAs (shBCKDHA) were analyzed via LC-MS/MS. Error bars, SD of triplicate wells from a representative experiment. **E** Adipored staining was performed on PDAC cells with BCKDHA knockdown. Error bars represent the

standard deviations (s.d.) of triplicate wells from a representative experiment. *, $p < 0.05$; **, $p < 0.01$.

6. BCKDHA knockdown inhibits tumor formation.

To clarify the relationship between BCKDHA expression and tumorigenic capacity in mice, I subcutaneously injected 8988T cells expressing control (shGFP) or BCKDHA shRNAs (shBCKDHA) into mice. Tumor growth was evaluated 14 days after injection. As shown in Fig, 6A, BCKDHA knockdown had a significant inhibitory effect on tumor growth. Xenograft tumors generated from human PDAC cells expressing BCKDHA shRNAs were blotted for BCKDHA (Fig. 6B). Moreover, I was concerned about toxicity after BCKDHA knockdown, but there were no health and body weight problems of the mice (Fig. 6C). I also observed that BCKDHA knockdown significantly reduced the size of tumor xenografts (Fig. 6D). These observations demonstrated BCKHDA knockdown led to a significant inhibition of tumor growth *in vivo*.

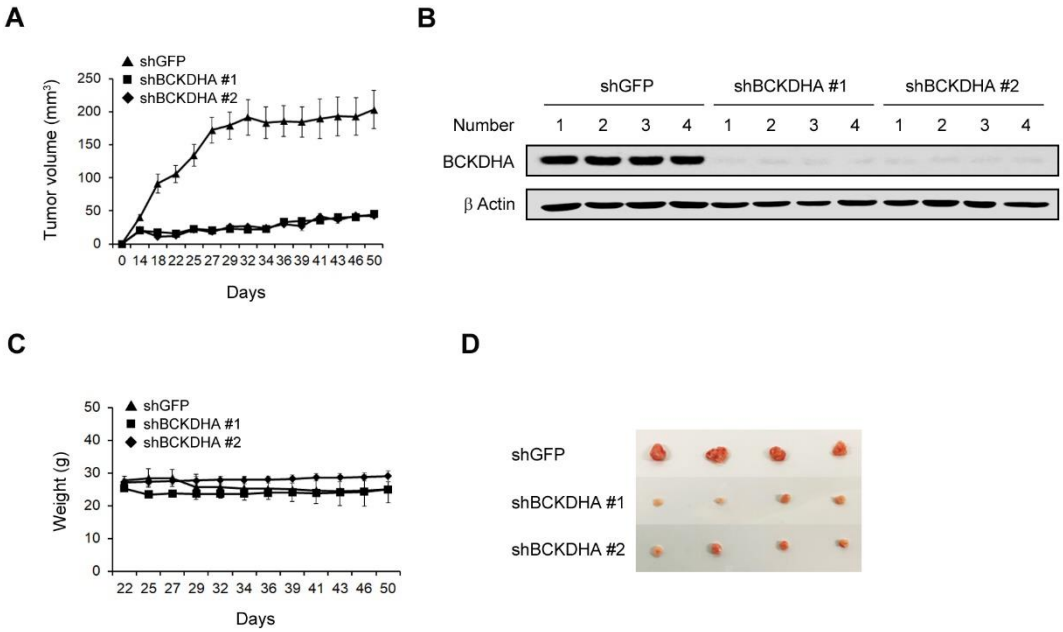

Fig. 6 Knockdown of BCKDHA impaired tumor growth in a xenograft mouse model. A 4 mice per group were used in the xenograft experiment, and the tumor volumes of mice were measured. **B** Immunoblots of BCKDHA in BCKDHA knockdown tumor. **C** body weight for BCKDHA knockdown mice model. **D** Photograph of xenograft tumors derived from mice.

Discussion

It is well known that elevated plasma levels of branched chain amino acids (BCAA) are associated with pancreatic cancer attack rate[23,24]. However, the roles of BCAA metabolism in PDAC growth are still unclear. Thus, I examine to determine the mechanism by which BCAA metabolism regulates PDAC growth. Our study showed that BCAA uptake increased in PDAC compared to HPDE. To confirm the importance of BCAAs, I suppressed BCAT2, the first enzyme in BCAAs metabolism, and found that BCAT2 is important for PDAC growth. Previous studies have shown that BCAT2 regulates and maintains intracellular glutamate levels and ROS in other carcinomas[27]. In PDAC, it was confirmed that the intracellular glutamate level and ROS were not affected by the decrease of BCAT2. So, I investigated the functions of BCKDHA, the second enzyme of the BCAA mechanism in PDAC growth. Like bcat2, BCKDHA inhibition also significantly reduced PDAC cell growth. Interestingly, BCAT2 and BCKDHA inhibition did not affect HPDE cell growth. Then, I confirmed mitochondrial function by inhibition of BCKDHA. As a result, oxygen consumption rate (OCR) and ATP levels did not affect mitochondrial function. In addition, BCKDHA knockdown had no significant effect on the level of TCA cycle intermediates except for Citrate, indicating that reduced level of intracellular citrate may inhibit PDAC growth. Citrate, the first intermediate of the Krebs cycle, is important for tumor growth, as an important carbon source for various metabolites[28]. In particular, citrate is required for fatty acid biosynthesis[29]. In this study, inhibition of BCKDHA has been shown to dramatically reduce fatty acid synthesis, and it was confirmed that BCAAs provide carbon fuel to fatty acids. These results suggest that the BCAAs metabolism delivery the nutrient to the synthesis of fatty acids, which is critical for PDAC growth and may be targets for

treatment of pancreatic cancer. Therefore, the major enzymes in the metabolism of BCAAs can be the target of pancreatic cancer treatment.

Conclusion

PDACs are a major cause of cancer-related deaths worldwide, and patients can not be treated at diagnosis. BCAAs are associated with the incidence of pancreatic cancer. BCAAs, essential amino acids containing leucine, isoleucine, and valine, are essential for cancer growth. However, the role of BCAA metabolism in PDAC growth remains unclear. I study to determine the therapeutic potential of targeting BCAA metabolism in PDAC. This data have shown that PDAC growth is robustly reduced upon both BCAT2 and BCKDHA knockdown, the major enzymes of BCAAs metabolism. Interestingly, inhibition of BCKDHA reduces fatty acid synthesis and BCAA provides carbon fuel to fatty acids. These data suggest that the BCAA metabolism transfer the nutrient to the fatty acids synthesis, which is essential for PDAC growth. Clearly, there is a strong impetus to identify new therapeutic targets, and an overwhelming need for new agents, to treat pancreatic cancer. Based on our finding, targeting the PDAC-specific reliance on BCAA metabolism may provide an appreciate therapeutic rationale for pancreatic cancer.

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Abstract in Korea

BCAA 대사에서의 BCAAs (Branched-chain amino acids) 이화 작용과 중요 효소들의 높은 발현은 최근 암의 성장, 생존과 밀접한 관련이 있다고 보고되고 있다. 그러나 췌장암 세포의 성장과 생존에 있어서 BCAAs 대사의 정확한 역할은 여전히 불확실하다. 따라서, 본 연구에서 BCAAs 대사가 지방산 생성 조절을 통한 PDAC (pancreatic ductal adenocarcinoma) 성장에 중요한 역할을 한다는 것을 규명하였다. PDAC 세포는 췌장 정상 세포인 HPDE (human pancreatic ductal cells)보다 훨씬 많은 BCAAs 를 흡수하는 것을 확인하였으며, 임상 환자 샘플에서 정상 환자 샘플과 비교해서 BCAAs transporter 인 SLC1A5, SLC3A2 그리고 SLC7A5 의 mRNA 발현이 확연히 증가되었음을 확인하였다. PDAC 성장에서 BCAAs 대사의 중요성을 확인하기 위해 BCAAs 이화 작용에 첫번째 효소인 BCAT2 를 억제하였으며, 그 결과 PDAC 세포 성장을 심각하게 저해하는 것을 관찰하였다. 다른 암종에서 BCAT2 가 세포내 glutamate level 과 ROS 를 조절하고 유지한다는 보고되었으나, 췌장암 세포에서는 동일한 결과를 관찰하지 못했음. PDAC 성장에서 BCAAs 대사의 중요성을 확인하기 위해 BCAAs 이화 작용의 두번째 효소인 Branched-chain α-keto acid dehydrogenase A (BCKDHA)를 억제하였을 경우에 BCAT2 를 억제했을 경우와 동일하게 PDAC 세포 성장이 현저히 감소되는 것을 확인하였다. 흥미롭게도 BCAT2 나 BCKDHA 억제는 HPDE 세포 성장에는 별다른 영향을 주지 않았다. BCKDHA 억제는 tricarboxylic acid (TCA) cycle 중간산물과 산소 소비율을 포함하여 mitochondrial metabolism 에 유의한 영향을 미치지 않았지만, 지방산 합성을 크게 억제하여 PDAC 가 BCAAs 를 사용하여 지방산에 탄소 연료를 공급할 수 있음을 확인하였다. 그러므로, 이 데이터는 BCAAs 대사가 췌장암 성장에 중요한 지방산 합성에 영양분을 공급하는 역할을 한다고 제시할 수 있다고 사료되며, 이 결과들은 BCAAs 대사의 중요 효소들은 췌장암 치료를 위한 타깃이 될 수 있다고 사료된다.

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