1. Introduction

Tumor metabolism is closely related to tumorigenesis and tumor progression (Burragge et al., 2014; Gill et al., 2016; Nelson et al., 2013; Pike et al., 2011; Sonnet et al., 2016; Watanabe et al., 1984). Gill et al. (2016) presented that the increased rate of glycolysis can support cancer cells rapid proliferation through fulfilling cells biosynthetic demands. Pike et al. (2011) showed fatty acid oxidation promotes cancer cells growth and survival through providing bioenergetic fuel for cells. Nelson et al. (2013) demonstrated a primary metabolite of cholesterol, 27-hydroxycholesterol (27HC), promotes tumor cells growth and metastasis through signal transduction. The above studies show that the metabolism effects tumor growth in 3 ways: providing biosynthetic materials, providing energy, or through signal transduction. Branched-chain amino acids (BCAA) catabolism is abnormal in many human diseases (Burragge et al., 2014; Sonnet et al., 2016; Watanabe et al., 1984). Branched-chain amino transferase (BCAT) is the first enzyme in BCAA catabolism which has a higher activity in tumors and has been shown to be a useful marker for grading and genetic characterization in glioma, colorectal cancer (CRC) and medulloblastoma (Conway et al., 2016; de Bont et al., 2008; Mitchell et al., 2014). However, healthy human prostate tissue was found to have elevated BCAT levels relative to malignant tissue (Billingsley et al., 2014). Therefore, the role of BCAA catabolism in tumors need to be further understood.

BCKDK is a key negative regulation enzyme in BCAA catabolism that inhibits the dehydrogenase activity of the branched-chain α-keto acid dehydrogenase (BCKDH) complex by dephosphorylating the E1 component of the complex (Chuang et al., 2002). BCKDK plays an important role in many serious human diseases, including Huntington’s disease (Mochel et al., 2007), Maple syrup urine disease (Beaudet, 2012; Mochel et al., 2007), Maple syrup urine disease (Beaudet, 2012; Mochel et al., 2007), and Alzheimer’s disease (Mochel et al., 2007). BCKDK plays an important role in many serious human diseases, including Huntington’s disease (Mochel et al., 2007), Maple syrup urine disease (Beaudet, 2012; Mochel et al., 2007), and Alzheimer’s disease (Mochel et al., 2007).
Sonnet et al., 2016), human autism with Epilepsy (Novario et al., 2012), and Obesity (Burrage et al., 2014). However, the relationship between BCKDK and cancer is unknown.

CRC is the third most commonly diagnosed cancer in males and the second in females, and the new global incidence is over 1.2 million cases (Torre et al., 2015). Therefore, the mechanism of CRC tumorigenesis need to be further elucidated. Watanabe et al. (1984) reported that BCAA levels in the tumor tissue of colon cancers were generally higher than those in the respective nontumorous tissue. On the other hand, Wubetu et al. (2014) showed BCAA suppressed insulin-initiated proliferation of human Colon tumor cells through induction of autophagy. Yoshikawa et al. (2006) demonstrated BCAT1 over-expression is a sensitive marker of metastasis in CRC. Therefore, the role of BCAA catabolism in CRC is controversial. BCAA is a biosynthetic material, however, whether BCAA is a potential carcinogen is unclear.

In this study, we identify BCKDK as an upstream kinase of MEK. We show that the BCKDK positively regulates MEK/ERK signaling by directly phosphorylating MEK at Ser221. BCKDK has a higher expression in the colorectal tissues of CRC patients versus normal tissues. The higher that BCKDK is expressed, the shorter survival time is in CRC patients. Over-expression of BCKDK increases the clone formation of JB6 C141 and WiDr cells ex vivo. Knockdown of BCKDK inhibits colorectal tumor progression ex vivo and in vivo. The above suggests that BCAAs catabolism may crossstalk with MAPK signaling pathway, whereby BCKDK plays a crucial role in human CRC tumorigenesis through phosphorylating MEK at ser221.

2. Materials and Methods

2.1. Plasmids, shRNA, Antibodies, and Other Reagents

The plasmids of pBABEpuro-HA-MEK1 (catalog: 53195) and pDONR223-BCKDK (catalog: 23794) were purchased from Addgene (Cambridge, MA, USA). The plasmid of pCMV-C-Flag (catalog: D2632) was purchased from Beyotime Biotechnology (Shanghai, China). The plasmid of pCMV-Myc (catalog: 631604) was purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA). The plasmid of pET23a-His (catalog: 69745-3) was purchased from Novagen (Darmstadt, Hessen, Germany). The plasmids of pCMV-BCKDK-Flag, pCMV-Myc-MEK1, pET23a-MEK1 (residues 62–393)-His, and pLKO.1-shBCKDK were constructed by our laboratories. The sense template sequence for BCKDK amplification was 5′-CGGAGAACCACTGATGATC-3′.

CGTCCGTGCTGAGG-3′ (EcoRI end underlined) and the antisense template sequence was 5′-CGCCGCGAGGATCATGATCAGATCCTTTTCCCCGCC-3′ (XhoI end underlined). The amplified BCKDK and pCMV-C-Flag vector were digested with EcoRI and XhoI, ligation, and colony screening for pCMV-BCKDK-Flag. The sense template sequence for MEK1 amplification was 5′-GCCGCTGACGGCCCAAGAACAG GACGCGACG-3′ (XhoI end underlined) and the antisense template sequence was 5′-ATAAAGTTGCCGCCTTAAACAAATGGTGGAGCCGACGACG-3′ (NotI end underlined). The amplified MEK1 was cloned into pCMV-Myc vector at XhoI and NotI sites. The sense template sequence for MEK1 (residues 62–393) amplification was 5′-CGGAGATCCGGGAAGCGCCT TGGACCTTCGTAGC-3′ (BamHI end underlined) and the antisense template sequence was 5′-CGGGTGAGACGAAATGCTGAGCCGATGATC-3′ (XhoI end underlined). The amplified MEK1 (residues 62–393) was cloned into pet23a his vector at BamHI and XhoI sites. 5 sequences were designed to knock down BCKDK, and the sequences are: 1. 5′-CCCGGCGTCCGACTTCTGGAGAACACTGACCTTGGCACAAGAATGGACGCA CTGTTTTG-3′; 2. 5′-CGCCGCGAGACGAAATGCTGAGCCGATGATC TC TGACCTGAGACCTGACCTGATGATC TGGACCTTCGTAGC-3′; 3. 5′-CCGGGATCCGGGAAGCGCCT TGGACCTTCGTAGC-3′; 4. 5′-CCGGGATCCGGGAAGCGCCT TGGACCTTCGTAGC-3′; 5. 5′-CCGGGATCCGGGAAGCGCCT TGGACCTTCGTAGC-3′. A mock shRNA with a sequence lacking significant homology to the human genome database was used as the mock shRNA. The sequence was: 5′-CCGG

CCTAAGGTATAGCGCCCTCGCAGCAGCGCGACTTACAGGTTTTGG-3′. The sense and anti-sense oligonucleotides were synthesized, annealed and cloned into the pLKO.1-TRC cloning vector at the EcoRI and AgeI sites as described by the manufacturer (Moffat et al., 2006).

Anti-mouse BCKDK antibody (catalog: sc-374425) and anti-β-actin antibody (catalog: sc-130656) were purchased from Santa Cruz Technology, Inc. (Santa Cruz, CA, USA). Anti-p-MEK1/2 (ser221) (catalog: 2338), phospho-p44/42MAPK (Erk1/2) (T202/Y204) (D13.14.4E) (catalog: 4370), t-MEK (catalog: 8727), and t-ERK (catalog: 4695) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-Flag antibody (catalog: F1804, catalog: F7425) was purchased from Sigma-Aldrich (St. Louis, MO, USA), HRP-conjugated anti-mouse antibody (catalog: E030110) and HRP-conjugated anti-rabbit antibody (catalog: E030120) antibody were purchased from EarthOx Life Sciences (San Francisco, CA, USA). Simple-lect (catalog: Project-01) was purchased from Signaling Dawn Biotech (Wuhan, Hubei, China) for transfection. G418 (catalog: A1720) and puromycin were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used for setting up stable cell lines.

2.2. Cell Culture

The human colon cancer cell lines (DLD1, HCT8, SW480, WiDr, HCT116, and HCT15) and the normal cell lines (HIEC-6, JB6 C141, and HEK293T) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA).

2.3. Western Blot

Cells (0.8–2 × 10⁶) were cultured in 10 cm diameter dishes to 70–80% confluence, and then starved no serum for either 12 h (JB6 C141 cell lines) or 24 h (colon cancer cell lines). The cells were then treated with 20 ng/mL epidermal growth factor (EGF) (R&D catalog: 236-EG-200) for 15 min. EGF is a well-known tumor promotion agent used to study malignant cell transformation in animal and cell models of cancer (Hunter and Karin, 1992; Geschmenaier et al., 1994; Sassone-Corsi et al., 1999; Scadini et al., 2014). After this, cells were washed once in PBS before being lysed in RIP buffer (1 × PBS, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mmol/L Na3VO4, 1 mmol/L aprotinin and 1 mmol/L phenylmethylsulfonyl fluoride). In addition, HCT116 cells were treated with 0–3200 μM BCKDK inhibitor for 48 h, with the later 24 h together with no serum. Then, samples were sonicated in 15 second intervals three times, and insoluble debris was removed by centrifugation at 13000 rpm for 15 min. Protein content was determined by Bradford method (Bradford, 1976). 30–120 μg of protein was separated by 10% SDS-PAGE and visualized by chemiluminescence (Bio-RAD, USA) in triplicate.

2.4. Growth Curve Analysis and MTS Assays

2 × 10⁶ cells were plated in each dish and counted at different times in triplicate, using a hemocytometer to generate a growth curve. For inhibition assays, 1 × 10⁶ HCT116 cells were seeded in 96-well plates for 24 h, and then cells were fed with fresh medium containing different concentrations of BCKDK inhibitor, phenyl butyrate (PB), for 48 h. The cytotoxicity of inhibitors was measured with MTS assay kit (Promega catalog: G3580) according to the manufacturer’s instructions in triplicate, with the results expressed as the percentage of inhibition.

2.5. Bacterial Expression and Purification of the MEK1-His

The pET23a-mek1 (residues 62–393)-his plasmid was expressed in E. coli BL21 bacteria (Novagen; Darmstadt, Hessen, Germany). Bacteria were grown at 37 °C overnight. Then, bacteria were harvested by
centrifugation at 3000 rpm. The pellets were washed with PBS 5 times, and were disrupted by sonication. The lysate was again centrifuged, and the new pellets were washed by PBS for another 5 times. The clean pellets were then dissolved with a minimum volume of 8 M Urea buffer (8 M Urea, 50 mM Tris, 0.5 M NaCl, 0.5% Triton-100, pH 8.0). The protein in supernatant was saved, and diluted to 1 M Urea buffer for use.

2.6. Immunoprecipitation and IP Kinase Assay

HEK293T cells were transfected with different plasmids for 48 h and HCT116 were seeded in 10 cm dishes for 24 h. Then, cells were harvested in IP buffer (50 mM tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP40, and 1 mM DTT). 2 mg proteins were subjected to immunoprecipitation following the manufacturer’s instructions. (http://www.scbt.com/protocols.html?protocol = immunoprecipitation). The mouse source antibody was used for IP and the rabbit source antibody was used for western blotting.

The BCKDK-Flag kinase was prepared with same method of Immunoprecipitation except BCKDK-Flag was diluted in 1× kinase buffer (Billerica, MA, USA) instead of 2× loading buffer (Santa Cruz, CA, USA). Likewise, MEK1 (residues 62-393)-his was prepared as above. 2 mg MEK1 (residues 62-393)-his was used for IP Kinase. The kinase and substrate were incubated at 37 °C for 70 min in 1× kinase buffer containing 100 μmol/L unlabeled ATP. If a kinase inhibitor was used, the kinase was first incubated with the inhibitor (0–3200 μM) at 32 °C for 20 min in 1× kinase buffer. The appropriate substrates were then added to the reactions and incubated at 37 °C for another 70 min. Samples were treated with 5× loading buffer and analyzed by western blotting.

2.7. Anchorage-independent Cell Transformation Assay

Different cell lines (8 × 10^5/well) were seeded in 6-well plates, and exposed or not exposed to EGF (20 ng/mL). BCAA (180–5670 μM) or inhibitor (0–3200 μM). The cells were then cultured in 1 mL of 0.33% BME (Eagle basal medium, Sigma-Aldrich Corp.) Agar (Sigma-Aldrich Corp.) containing 10% FBS, 2 mM l-glutamine, and 25 μg/ml gentamicin, with an additional 3 mL of 0.5% BME agar containing 10% FBS, 2 mM l-glutamine, and 25 μg/ml gentamicin being below. Then the cells were maintained in a 37 °C, 5% CO2 incubator for 4–7 days and the colonies were observed and assessed by microscopy.

2.8. Tumor Xenografts and Immunohistochemistry

Male athymic Balb/c nude mice (4–6-week-old) were purchased from Beijing HFK Bioscience Co, Ltd. (Beijing, China). The mice were housed and maintained with the guide for care and use of laboratory animals which were approved by the Fourth Military Medical University. Mice were divided and randomized into two groups. Each of the different cell lines (3 × 10^6 in 200 μl PBS) was injected subcutaneously into the right flank. The tumor volumes were measured every other day and were calculated with the formula: V = 0.52 (length × width × height). The tumor tissues were prepared with paraffin sections after fixation with formalin, and then stained with hematoxylin and eosin (H&E) or p-MEK1/2(ser221) (1:50) and p-ERK1/2 (Tyr202/Tyr204) (1:50).

2.9. BCAA Assay and Tissue Microarray

The sera were obtained from the department of State Key Laboratory of Cancer Biology, Xijing hospital of the Fourth Military Medical University. The tissues used for BCAA assay and tissue microarray (TMA) underwent CRC were obtained from the department of Urology, Xijing hospital of the Fourth Military Medical University. This study was approved by the ethical committee of the Fourth Military Medical University. Samples were obtained with informed consent. The BCAA assay was following the manufacturer’s instructions (http://www.sigmaaldrich.com/catalog/product/sigma/mak003?lang=en&region=GB). The TMA was stained with anti-BCKDHA, anti-BCKDK or anti-p-BCKDK antibody. The immuno-scores were calculated following the Remmele score method (Regitnig et al., 2002), and the scores > 3 were used as positive group, the others were used as negative group.

2.10. Statistical Analysis

All quantitative data in the present study were performed at least in triplicate. The results are expressed as the mean ± standard deviation. A two-tailed ANOVA or Student’s t-test was used to evaluate the data, and p < 0.05 was considered significant (**p < 0.01, ***p < 0.001).

3. Results

3.1. BCAA Cannot Promote CRC Tumorigenesis

BCAA is catabolized to R-CoA in normal cells. This process can be limited when BCKDH is phosphorylated by BCKDK at the Ser293 (Fig. S1a) (Chuang et al., 2002). The concentration of BCAA is higher in tumor tissues compared with normal tissues (Nezami Ranjbar et al., 2015). Metabolites can promote cell transformation directly (Liu et al., 2015; Nelson et al., 2013). Although the level of BCAA is higher in tumor, its role on cell transformation remains unrevealed. The JB6 C141 cell line represented a unique model for studying neoplastic transformation (Bernstein and Colburn, 1989); and therefore BCAA was added to JB6 C141 cells to assess its effect on anchorage-independent cell transformation.

The result indicated that BCAA could not promote cell transformation, even at high concentrations of BCAA (16 times higher than normal concentration, Fig. S1b) (Areces et al., 2015; Kooman et al., 1997; Szpehter et al., 2014). Next, we detected the concentration of BCAA in the sera from 31 normal people and CRC patients or in the tissues from 10 CRC patients paired tumor and corresponding tumor adjacent tissues. The result showed that the concentration of BCAA had no significant change in CRC patient sera or tissues compared with normal people sera (p = 0.837) or adjacent tissues (p = 0.661) (Fig. S1c). Therefore, BCAA could not promote CRC tumorigenesis. It has been widely reported that BCAA catabolism is abnormal in cancer patients; especially BCAAT1 plays an important role in glioma (Tönjes et al., 2013). However, there are two more key limiting enzymes, BCKDH and BCKDK in BCAA catabolism, and whether they are related to cancer, like BCAAT1, has never been reported. Therefore, we decided to explore the role of BCKDH and BCKDK in CRC.

3.2. BCKDK Over-expression is Associated With Poor Prognosis in CRC Patients

Next, the expression levels of BCKDHA and BCKDK were analyzed in CRC tissue and corresponding tumor adjacent tissue samples. The results indicated that expression level of BCKDHA was higher in CRC tissue than that of corresponding tumor adjacent tissue samples from 117 cancer patients (Fig. 1a upper and middle panel), although, it had no effect on survival time (p = 0.112, Fig. 1a lower panel). The expression level of BCKDK was also higher in cancer tissue than adjacent tissue (Fig. 1b upper and middle panel), and it had a significant effect on survival time (p = 0.011, Fig. 1b lower panel). BCKDHA is the substrate of BCKDK, so next the expression levels of p-BCKDHA were assessed in the same way. While p-BCKDK, was higher in cancer tissue than adjacent tissue (Fig. 1c upper and middle panel), it had no significant effect on survival time (p = 0.368, Fig. 1c lower panel). The above data suggest that BCKDK may be more important than BCKDHA in CRC patients. The higher level of BCKD can result in higher level of BCAA; however, BCAA has no effect on cell transformation (Fig. S1b). Since BCAA catabolism is abnormal in cancer patients, BCKDK is one of the three
BCKD overexpression is associated with poor prognosis in CRC patients. (a) Immunohistochemical examination for the expression of BCKDHA in 117 cases of human colon cancer tissues and matching adjacent tissues. Pictures from 1 representative case are shown in the upper panel, and the 2 scale bars from left to right in each group correspond to 50 and 25 μm respectively. The table summarizes the results of the immunohistochemical examination (middle panel). The median survival time is no different between BCKDHA positive CRC patients and BCKDHA negative CRC patients (lower panel). The survival time table was analyzed via the Kaplan-Meier method. (b) Immunohistochemical examination for the expression of BCKDK in 113 cases of human colon cancer tissues and matching adjacent tissues. Pictures from 1 representative case are shown in the upper panel, and the 2 scale bars from left to right in each group correspond to 50 and 25 μm, respectively. The table summarizes the results of the immunohistochemical examination (middle panel). The median survival time is shorter for BCKDK-positive CRC patients than for BCKDK-negative CRC patients (lower panel). The survival time table was analyzed via the Kaplan-Meier method. (c) Immunohistochemical examination for the expression of p-BCKDHA in 118 cases of human colon cancer tissues and matching adjacent tissues. Pictures from 1 representative case are shown in the upper panel, and the 2 scale bars from left to right in each group correspond to 50 and 25 μm, respectively. The table summarizes the results of the immunohistochemical examination (middle panel). The median survival time is not different between p-BCKDHA-positive CRC patients and p-BCKDHA-negative CRC patients (lower panel). The survival time table was analyzed via the Kaplan-Meier method.
key molecules in BCAA catabolism (Fig. S1a), and there is no report about the role of BCKDK in cell transformation, we therefore further explored the possible role of BCKDK in CRC, described below.

3.3. BCKDK Promotes Tumorigenesis

First, BCKDK expression level was analyzed in 6 CRC cell lines and 1 normal intestinal epithelial cell line (Fig. 2a). The results showed that BCKDK was highly expressed in DLD1 and HCT116 cells, moderately expressed in HCT8 and HCT15 cells, and poorly expressed in SW480 and WiDr cells. The BCKDK level of normal HIEC-6 cells was the lowest. This result was consistent with that in CRC tissues from patients. To test whether BCKDK can promote cell transformation, BCKDK was overexpressed in JB6 CI41 cells or WiDr cells which poorly expressed BCKDK. JB6 CI41 or WiDr stable cell lines that overexpressed the pcMV-c-Flag or pcMV-BCKDK-Flag were generated and the growth curves of JB6-Mock and JB6-BCKDK cells, or WiDr-Mock and WiDr-BCKDK cells were compared. The results showed that JB6-BCKDK cells grew faster than JB6-Mock cells (Fig. 2b upper left panel, inner session of left panel indicating BCKDK overexpression). Next, the anchorage-independent growth of JB6-Mock or JB6-BCKDK was compared, and the result indicated that the number of colonies in JB6-BCKDK cell cultures was much more than that in JB6-Mock cell cultures (Fig. 2b upper right panel). The corresponding statistical significance is shown in the right panel of Fig. 2b. Similar results were observed in the cultures of WiDr-Mock or WiDr-BCKDK stable cells (Fig. 2b lower panel). These results indicate that BCKDK promotes cell transformation. To verify this idea further, BCKDK was knocked down in HCT116 or DLD1 CRC cells to generate two stable shBCKDK cell lines and two stable shMock cell lines (HCT116-shBCKDK, HCT116-shMock or DLD1-shBCKDK, DLD1-shMock). As shown in Fig. 2c inner session of left panel by the result of Western blot, BCKDK was knocked down by shRNA sequence for lines 4 and 5. Next, growth curves of HCT116-shMock, -shBCKDK4, or -shBCKDK5 cells were tested, and the results demonstrated that HCT116-shBCKDK cells grew dramatically slower than HCT116-shMock cells (p < 0.05, Fig. 2c upper left panel). Next, the anchorage-independent growth of the HCT116-shMock or HCT116-shBCKDK cell lines was evaluated, and the results indicated that the number of colonies in HCT116-shBCKDK cell cultures was much less than in HCT116-shMock cell cultures (Fig. 2c upper right panel). Similar results were observed in the DLD1-shMock or DLD1-shBCKDK cell lines (Fig. 2c lower panels). Therefore, these results indicated that knockdown of BCKDK in colorectal tumor cells inhibited tumorigenesis ex vivo. Next, tumor xenograph assays were performed in male athymic Balb/c nude mice. We injected HCT116-shMock or HCT116-shBCKDK4 cells (3 × 10^6) subcutaneously into the right flank, with tumor size assessed over time. Tumors in HCT116-shMock-inoculated mice grew to a much larger size compared to those in HCT116-shBCKDK-inoculated mice (Fig. 2d left panel). The tumor growth curve is shown in the right panel of Fig. 2d. These data demonstrated that blocking BCKDK expression in CRC cells significantly reduces their tumorigenic properties ex vivo and in vivo, and further confirmed that BCKDK promotes cell transformation.

3.4. BCKDK Promotes Tumorigenesis Through Up-regulating the MEK-ERK Signaling Pathway

We confirmed that BCKDK promoted cell transformation ex vivo and in vivo. Next, we wanted to know which signaling pathway was involved in this process. The MAPK signaling pathway has been widely reported to link with carcinogenesis, with MEK and ERK being two critical kinases in this pathway (Rauch et al., 2016; Zhu et al., 2007). Therefore, p-MEK1/2 (ser221) and p-ERK1/2 (T202/Y204) levels were tested in JB6 CI41 and WiDr BCKDK stable cell lines, and the results indicated that the level of p-MEK and p-ERK were up-regulated when BCKDK was overexpressed (Fig. 3a). These results suggest that BCKDK promotes CRC through up-regulating MEK-ERK activity. Next, the level of p-MEK and p-ERK were tested in the HCT116 or DLD1 shBCKDK cell lines. Both p-MEK and p-ERK were decreased significantly when BCKDK was knocked down in each of these cell lines (Fig. 3b). Next, the expression level of p-MEK1/2 (Ser221), p-ERK1/2 (T202/Y204), and BCKDK were detected in tumor tissues dissected from the xenografts in the study by Western blotting or immunohistochemical analysis. The results confirmed that the level of p-MEK1/2 (Ser221), p-ERK1/2 (T202/Y204), and BCKDK were higher in the tumor tissue of HCT116-shMock mice than in the tumor tissue of HCT116-shBCKDK mice (Fig. 3c). And the dissected tissues were also stained with hematoxylin & eosin (H&E) to confirm these tissues belong to tumor tissues (Fig. 3c right lower panel). These data confirmed that BCKDK promoted colorectal tumorigenesis through up-regulating the MEK-ERK signaling pathway.

3.5. BCKDK Directly Phosphorylates MEK1 at ser221

We elucidated that the levels of p-MEK1/2 (ser221) and p-ERK1/2 (T202/Y204) could be controlled by BCKDK, which implied that BCKDK might interact with MEK and phosphorylate it at Ser221 directly. First, whether BCKDK can interact with MEK was tested by co-immunoprecipitation experiments utilizing HEK293T cells. MEK1 and MEK2 share 79% amino acid similarity and are able to phosphorylate ERK substrates with equivalent capacity (Scholl et al., 2005). However, MEK1 plays a more important role in the growth and development of mice (Bélanger et al., 2003; Giroux et al., 1999). So pcMV-BCKDK-Flag or pcMV-Myc-MEK1 construct was transfected or co-transfected into HEK293T cells, then immunoprecipitated the tagged proteins with the Flag or Myc antibody, and then probed with anti-Flag or anti-Myc, respectively, in Western Blot assays. The results demonstrated that BCKDK could co-immunoprecipitate with MEK1 (Fig. 4a). However, since the detected interaction was performed in an overexpression system, we next assessed whether endogenous BCKDK was immunoprecipitated from HCT116 cells, as detected by Western blotting with a MEK antibody. The results indicated that BCKDK could co-immunoprecipitates with MEK1 in HCT116 cells (Fig. 4b). BCKDK is a protein kinase, so we next determined if it can phosphorylate MEK directly. An in vitro kinase assay was set up to test this idea. For this, we first obtained BCKDK by immunoprecipitation from overexpressing HEK293T cells and used it as the kinase. Inactive MEK1 (residues 62–393) was used as the substrate. The kinase assay was performed in the presence of cold ATP, with p-MEK1/2(Ser221) subsequently detected by Western blotting. The results indicated that BCKDK could phosphorylate MEK1 at Ser221 directly (Fig. 4c). Next, BCKDK was overexpressed in HEK293T cells, the cells were stimulated with EGF, and the endogenous phosphorylation of MEK was subsequently analyzed. These data indicated that increasing the amount of BCKDK leads to increased phosphorylation of endogenous MEK1/2 (Fig. 4d), further suggesting that BCKDK can phosphorylate MEK1/2 at Ser221 directly.

3.6. BCKDK Inhibitor Reduces Tumorigenic Properties

As shown above, BCKDK can promote tumorigenesis in CRC. Therefore, compounds may reduce tumorigenic properties if they can inhibit BCKDK. PB is an inhibitor of BCKDK which has been used to modulate BCAA catabolism (Tso et al., 2013). However, whether PB can block CRC is not known. Here, we used this inhibitor to further confirm the hypothesis that BCKDK promotes colorectal tumorigenesis via phosphorylating MEK1 at Ser221 and then activating the MEK-ERK signal pathway. First, MTS assays were performed to assess any potential cytotoxicity of this inhibitor in HCT116 cells that expressed a high level BCKDK. The results indicated that PB had no cytotoxicity in HCT116 cells at 48 h, even at a very high (3200 M) concentration (Fig. 5a). Second, PB was used directly in an in vitro kinase assays to test if it can inhibit p-MEK1 by reducing BCKDK activity. The results indicated that PB can inhibit the phosphorylation level of MEK1 at Ser221 in a dose dependent manner (Fig. 5b). Third, the expression of p-MEK1/2 (ser221), p-ERK1/2 (T202/Y204), and p-BCKDKHA (Ser393) were detected in the HCT116 cells treated with different concentrations of PB. The results demonstrated that PB inhibited the level...
Fig. 2. BCKDK promotes tumorigenesis. (a) Expression of BCKDK in 7 different colorectal cell lines. (b) Growth curves of vector control cells (JB6-Mock) and BCKDK-overexpressing cells (JB6-BCKDK) (upper left panel). Insert shows verification of the cell lines identified by Western blot. Data are represented as mean ± standard deviation from triplicate experiments. The asterisk indicates a significant increase in cell number in JB6-BCKDK cells compared with JB6-Mock cells (*, \( P < 0.05 \)). BCKDK can transform JB6 C141 cells ex vivo as illustrated by growth of BCKDK-transformed cells in soft agar. Photomicrograph of representative colony formation in soft agar of vector control cells (JB6-Mock) compared with BCKDK-overexpression cells (JB6-BCKDK) is shown (upper right panel). Growth curves of vector control cells (WiDr-Mock) and BCKDK-overexpressing cells (WiDr-BCKDK) (lower left panel). Insert shows verification of the cell lines identified by Western blot. Data are represented as mean ± standard deviation from triplicate experiments. The asterisk indicates a significant increase in cell number in WiDr-BCKDK cells compared with WiDr-Mock cells (*, \( P < 0.05 \)). BCKDK can enhance the transformation of WiDr cells ex vivo as illustrated by growth of BCKDK transformed cells in soft agar. Photomicrograph of representative colony formation in soft agar of vector control cells (WiDr-Mock) compared with BCKDK-overexpressing cells (WiDr-BCKDK) is shown (lower right panel). (c) Growth curves of HCT116/DLD1-shMock, HCT116/DLD1-shBCKDK4, and HCT116/DLD1-shBCKDK5 cells (left panel). Insert shows verification of the knockdown cell lines identified by Western blot. Data are represented as mean ± standard deviation from triplicate experiments. The asterisks indicate a significant increase compared with shMock cells (*, \( P < 0.05 \)). Knockdown of BCKDK reduces tumorigenic properties of HCT116/DLD1 CRC cells ex vivo. Representative photomicrograph of colony formation in soft agar of vector control cells (shMock) compared with BCKDK-knockdown cells (shBCKDK4 or shBCKDK5) is shown (right panel). Data are represented as mean ± standard deviation from triplicate experiments (right panel). (d) Knockdown of BCKDK reduces tumorigenic properties of HCT116 CRC cells in vivo. Tumors dissected from each group are shown (left panel). Final average tumor growth curve of mice injected with HCT116-shMock or HCT116-shBCKDK cells is shown (right panel). Data are shown as means ± standard deviation of measurements. The asterisk indicates a significant decrease in tumor size in HCT116-shBCKDK4-injected mice compared with HCT116-shMock-injected mice (*, \( P < 0.05 \)). All Western blot data are representatives of results from triplicate experiments.
of p-MEK1/2 (ser221), p-ERK1/2 (T202/Y204), or p-BCKDHA at 200 μM and 800 μM (Fig. 5c). Last, soft agar assays were used to test if PB could block anchorage-independent growth of HCT116 cells. The data indicated that PB inhibited anchorage-independent growth of HCT116 cells from the concentration of 800 μM, and corresponding statistical significance was showed in the lower right panel (Fig. 5d).

Taken together, our study indicates that BCKDK promotes colorectal tumorigenesis through up-regulation of the MAPK signaling pathway by phosphorylating MEK at Ser221.

4. Discussion

CRC is the fourth most common cause of cancer deaths, with 600,000 deaths is reported in the world annually (Torre et al., 2015). Significant progress has been achieved in understanding the molecular pathways involved in the carcinogenesis of CRC during the past three decades, which brings great benefits to CRC patients by targeted drugs (He et al., 2016; Kuo et al., 2016; Manchado et al., 2016; van der Velden et al., 2016). As the effectiveness of those targeted drugs is limited by intrinsic drug resistance (Tong et al., 2017; Zhang et al., 2016), there are some patients who are remaining “undruggable”. About 40% of CRC patients with wild type EGFR, KRAS, or BRAF are still resistant to anti-EGFR treatment (Bardelli and Siena, 2010; De Roock et al., 2010; Douillard et al., 2013). MEK has unique characteristics among the components of the MAPK pathways and has been considered as an ideal therapeutic target, because it can integrate many mitogenic signals into the ERKs, its only substrates. The expression level of MEK is higher in renal cell carcinoma resistant tissues than in renal cell carcinoma tissue when treated with...
tyrosine kinase inhibitors (TKIs) (Li et al., 2015; Qin et al., 2016), suggesting an alternative activation signaling pathway of MEK may exist besides the RAF-MEK pathway, and therefore contribute to drug resistance. MEK is a downstream key kinase of the EGFR signaling pathway and is a potential therapeutical target (McCubrey et al., 2010). In our research, BCKDK was demonstrated as an upstream kinase of MEK, phosphorylating MEK at serine 221 (Fig. 4). Therefore, blocking the BCKDK-MEK signaling pathway might be a potential therapeutic strategy for CRC.

PB is a well-known BCKDK inhibitor which can inhibit phosphorylation of BCKDHA. It also acts as a histone deacetylase inhibitor (Lea and Tulsyan, 1995) which had been shown to inhibit tumor growth in a wide variety of cancer cell lines, such as, colorectal cancer, glioblastoma, prostate cancer, and melanoma (Huang et al., 2000; Kusaczuk et al., 2016; Carducci et al., 1996; Liu et al., 1994). Furthermore, Sung and Waxman (2007) demonstrated that Weekly infusions of PB combined with FUra were fairly well tolerated with disease stabilization in 3/4 (75%) of colorectal cancer patients. However, Brinkmann et al. (2001) showed that hyperacetylated histones accumulation induced by histone deacetylase inhibitors was not sufficient to cause growth inhibition in all cell types. And the dose limiting neurocortical toxicity of confusion and excessive somnolence occurred in the patients of colorectal cancer, glioblastoma or prostate cancer who were treated with PB (Sung and Waxman, 2007; Kusaczuk et al., 2016; Carducci et al., 2001). High concentrations of PB are required to block BCKDK activity ex vivo (Brunetti-Pierri et al., 2011) or in vivo (Fig. 5b). Therefore, more efficient inhibitors than PB for targeting BCKDK should be identified to overcome drug resistance of CRC caused by consistent MEK activation.

Many biomarkers have been used for CRC screening. Stool-Based Biomarker, Hemoglobin is widely used despite the low specificity (80%–90%) and relatively low sensitivity (30%–50%) of this assay for the detection of advanced polyps and cancers (Zhu et al., 2010). Other types of fecal biomarkers, such as DNA biomarkers, have also been extensively tested and approved for clinical use, including the chromosomal instability pathway (CIN), the microsatellite instability (MSI) pathway, and the serrated polyp or CpG island methylator phenotype (CIMP) pathway. APC, EGFR, KRAS, BRAF, TP53, PIK3CA, ERBB2 detection have likewise been used in CRC screening (Dickinson et al., 2015). Our data shows that BCKDK is expressed at higher levels in colorectal tissues versus adjacent normal tissues, and patients who have higher BCKDK immuno-scores in their local focus tissues have a shorter survival time (Fig. 1b). This suggests that BCKDK can be another potential and valuable biomarker for the diagnosis of CRC.

BCAA level increases when BCKDK is over-expressed (Chuang et al., 2004; Garcia-Cazorla et al., 2014; Nellis et al., 2003), but the role of...
BCAA in colorectal tumorigenesis or cancer growth has remained undetermined, whether the concentration of BCAA in CRC patient serums or tumor tissues is also higher compared with the normal people serums or adjacent tissues is still unknown. First, our data indicated that BCAA could not promote cell transformation directly (Fig. S1b). Second, the concentration of BCAA has no significant change in CRC patient serums or tumor tissues compared with the normal people serums or adjacent tissues (Fig. S1c). BCAA in cancer (three essential amino acids) may be used for protein synthesis (Chiarla et al., 1997). In summary, BCAA could not promote CRC tumorigenesis, and these three essential amino acids might provide raw materials for biosynthesis to maintain tumor cells’ growth and survival (Fig. 5e).

Taken together, our data revealed that BCKDK is highly expressed in CRC tissue, may be a valuable biomarker for CRC, and may promote transformation or oncogenesis by phosphorylating MEK directly at Ser221 (Fig. 5e) rather than through BCAA catabolism. Crosstalk between BCKDK and MEK may therefore serve as therapeutic target to overcome drug resistance of CRC.

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**Conflict of Interest**

The authors have no conflict of interest.
Author Contributions

Peipei Xue, Juanjuan Xiao, Linni Fan, Huimin Sun, Malyarenko Olesya, and Jianming Zhan were involved in the acquisition of experimental data. Wei Yan and Zhe Wang performed the tissue microarray analysis. Shaoqing Liu analyzed the clinical data. Jianyong Zheng and Ruijuan Xiu provided advice on the manuscript. Peipei Xue, Fanfan Zeng, Qihong Duan and Feng Zhu designed the study, analyzed the data and drafted the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found at http://dx.doi.org/10.1016/j.ebiom.2017.05.001.

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