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RESEARCH PAPER

DFNA5 inhibits colorectal cancer proliferation by suppressing the mTORC1/2 signaling pathways via upregulation of DEPTOR

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ABSTRACT

The human deafness, autosomal dominant 5 gene (DFNA5), a newly discovered executor of pyroptosis, has been strongly implicated in the tumorigenesis of several human cancers. However, an understanding of the functional role of DFNA5 in the development and progression of colorectal cancer (CRC) is limited. In this study, we demonstrated that DFNA5 was downregulated in CRC tissues. Ectopic expression of DFNA5 inhibited tumor cell growth in vitro, retarded tumor formation in vivo, and blocked a cell-cycle transition from the G0/G1 to the S phase, whereas a DFNA5 knockdown promoted cell proliferation. Western blotting showed that the levels of cell cycle-related proteins, including cyclin D1, cyclin E, CDK2, and p21, were accordingly altered upon DFNA5 overexpression or DFNA5 knockdown. Mechanistic studies indicated that DFNA5 exerted its tumor suppressor functions by antagonizing mTORC1/2 signaling via upregulation of DEPTOR. In addition, blockage of mTORC1/2 signaling by Torin-1 abolished the accelerative proliferation by DFNA5 knockdown. In conclusion, these results indicated that DFNA5 inhibits the proliferation and tumor formation of colon cancer cells by suppressing mTORC1/2 signaling.

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Introduction

Colorectal cancer (CRC) is one of the most common cancers and the second leading cause of cancer death worldwide[1]. Treatment by surgery, chemotherapy and radiotherapy is effective for a proportion of CRC patients. However, many patients still die from a recurrence and metastasis of tumors [2]. A fast-growing number of genetic and molecular alterations have been found in CRC, including genetic mutations, microsatellite instability, and DNA hypermethylation [3]. Thus, a better understanding of the mechanisms underlying the genesis and progression of CRC is in high demand. Such an understanding will enable the development of new diagnostic and therapeutic strategies to prevent and treat CRC.

The human deafness, autosomal dominant 5 gene (DFNA5) at chromosome 7p15, which is also known as GSDME, belongs to the gasdermin superfamily [4]. Mutations in DFNA5 can cause autosomal dominant sensorineural hearing loss [5,6]. Pyroptosis is a kind of the inflammasomes-induced programmed cell death mediated by gasdermin proteins [7]. Recently, a number of studies have identified that DFNA5 is a newly discovered executor of cell pyroptosis [8,9]. In our previous studies, we have demonstrated that lobaplatin and 5-FU could trigger DFNA5-mediated pyroptosis in CRC and gastric cancer, respectively [10,11]. Chemotherapy-related pyroptosis is a new approach to inhibit the progression of cancer. Moreover, Liu and Lu found that molecular-targeted and immunologic therapies could also elicit tumor cell death via DFNA5-related pyroptosis [12,13]. However, studies on the function of DFNA5 in the development and progression of cancer are still limited. Only a few studies have shown that DFNA5 is downregulated in tumor tissue because of promoter hypermethylation. DFNA5 was considered as a tumor suppressor in

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several cancers, including breast cancer, gastric cancer and CRC [14–16]. Thus, the precise function and molecular mechanism of DFNA5 in cancer need to be elucidated further.

Mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase that belongs to the PI3K/AKT/mTOR pathway [17,18]. mTOR exists in two complexes (mTORC1 and mTORC2) and plays vital roles in connecting both intracellular and extracellular signals, thus regulating cell proliferation, growth, survival, metabolism, and autophagy [19,20]. mTORC1 regulates protein biosynthesis via phosphorylation of ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). As an important hydrophobic kinase, mTORC2 is related to activating AKT by phosphorylation at serine 473 [21,22]. A previous study found that activation of the mTOR pathway could elevate normal intestinal proliferation [23]. Downregulation of this signaling pathway could inhibit proliferation and enhance apoptosis of colon cancer cells [24]. Therefore, molecular inhibitors of the mTOR signaling pathway have been suggested as potential therapeutic targets in CRC [25,26].

Here, we reported that DFNA5 was downregulated in CRC tissues. Ectopic expression of DFNA5 inhibited tumor cell growth in vitro, retarded tumor formation in vivo, and blocked a cell-cycle transition from the G0/G1 to the S phase. Mechanistic studies indicated that DFNA5 exerted its tumor suppressor functions by antagonizing mTORC1/2 signaling via upregulation of DEPTOR. Taken together, these results indicated that DFNA5 inhibits the proliferation and tumor formation of colon cancer cells by suppressing mTORC1/2 signaling.

Methods

Clinical samples and cell cultures

HT-29, SW480, HCT116, Caco-2 and RKO cells (Shanghai Institute of Cell Biology, Chinese Academy of Sciences) were maintained in DMEM medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C in 5% CO2.

Lentiviral vectors and transfection

The lentiviral vectors were constructed by GeneChem Co., Ltd. (Shanghai, China). The pUbi-EGFP-DFNA5 lentiviral vectors and their control vectors were prepared and used to increase DFNA5 expression. The phU6-EGFP-shRNA-DFNA5 lentiviral vectors and their control vectors were prepared and used to inhibit DFNA5 expression. The target shRNA sequence was 5′-CCAGTTCGAGTTCTGCCTT-3′. According to the manufacturer's protocol, we performed all transfections.

Cell growth and cell viability assays

HCT-116, SW480, HT-29, Caco-2 cells were transfected with lentiviral vectors. For cell growth, cells were seeded into 35-mm culture dishes for 7 days. The cells were counted using a hemocytometer under a light microscope every 2 days. Cells were seeded into 96-well culture plates at 5000 cells/well for 24 hours. Cell viability was examined using the MTT assay (Dojindo, Tokyo, Japan) every day according to the manufacturer's instructions.

Cell cycle assay

Cell cycle assay were performed as described previously [10]. Cells were collected and fixed in 75% cold ethanol at 4°C overnight. After treating with RNase A at 37°C for 30 min, cells were stained with propidium iodide (PI) in the dark for 30 min. Cell cycle were assessed using flow cytometry (BD Biosciences). Flowjo 10.0 software (FlowJo LLC, Ashland, OR, USA) was used to analyze the data.

RNA-sequencing

Total RNA was extracted from the tissues using Trizol (Invitrogen, Carlsbad, CA, USA) according to manual instruction. Total RNA was qualified and quantified using a Nano Drop and Agilent 2100 bioanalyzer (Thermo Fisher Scientific, MA, USA). RNA-seq libraries were prepared from three biological replicates for each experimental condition and sequenced on an Illumina HiSeq 4000 or X-ten platform (BGI-Shenzhen, China). The transcriptome annotation version was presented in the

present study. Differential expression analysis was performed using the DESeq2 (v1.4.5) with Q value ≤ 0.05). Differentially expressed genes were identified by the false discovery rate (FDR) value of ≤0.001 and Fold Change ≤2, for further analysis. The heatmap was drawn by pheatmap (v1.0.8) according to the gene expression in different samples ((https://cran.r-project.org/web/packages/pheatmap/index.html). To take insight to the change of phenotype, KEGG (https://www.kegg.jp/) enrichment analysis of annotated different expressed gene was performed by Phyper (https://en.wikipedia.org/wiki/Hypergeometric_distribution) based on Hypergeometric test. The significant levels of terms and pathways were corrected by Q value with a rigorous threshold (Q value ≤ 0.05) by Bonferroni.

Nude mouse xenograft assay

All animals were purchased by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Xi'an Jiaotong University in this study. Subsequently, HT-29 cells (5×10^6) in logarithmic growth phase were subcutaneously injected into the flanks of 4-week-old female BALB/c-nude mice (Shanghai SLAC Laboratory Animal Co. Ltd., Shanghai, China). After 1-week injection, the tumor width (b) and length (a) were measured using the callipers every 3 days. The tumor volume (V) was obtained as follows: $V = ab^2/2$., The animals were sacrificed and the xenograft tumors were measured at the end of the experiment.

Immunofluorescence (IF) and immunohistochemistry (IHC)

The IF assay was carried out as described previously [27]. The sample was observed using a fluorescence microscope to measure EdU labeling. For IHC, the staining procedure was performed using the standard avidin–biotin complex method. The extent of positively stained cells was scored on a scale from 0 to 4: 0–5% (0), 6–25% (1), 26–50% (2), 51–75% (3), and 76–100% (4). The staining intensity was divided into 4 score ranks: negative (0), light brown (1), brown (2) and dark brown (3). The immunoreactivity scores (IRSs) = extent score × intensity score. An IRS of ≤3 was defined as negative, and a score of >3 was defined as positive.

Protein extraction and Western blotting

RIPA buffer (Heart, Xi'an, China) was used to lyse cells and fresh tissue. Tissue or cell lysates containing 50 μg of total protein were then loaded and separated to SDS–PAGE (Beyotime, Shanghai, China) and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% fat-free dry milk at room temperature for 1 h and incubated with appropriate primary antibodies at 4°C overnight (anti-DFNA5, cyclin A, cyclin B, cyclin D1, cyclin E, CDK2, CDK4, p21, p27, Deptor, AKT, p-AKT, S6K1, p-S6K1 or GAPDH, 1:1000 dilution). The membrane was then washed four times with TBS-Tween-20 buffer for 8 min each and incubated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Chemiluminescent HRP substrate (Millipore, Billerica, MA, USA) was purchased and used to visualize the protein bands. The antibodies against GAPDH was purchased from Santa Cruz (Dallas, TX, USA), the antibodies against DFNA5, cyclin A, cyclin B, cyclin D1, cyclin E, CDK2, CDK4, p27, AKT, S6K1 and p-AKT were purchased from Abcam (Cambridge, MA, USA), and the antibodies against p21, p-S6K1 and DEPTOR were purchased from Cell Signaling Technology (Danvers, MA, USA).

Statistical analysis

Every experiment was repeated three times. Data are showed as the means ± SD. Student's t-test or $\chi 2$ test was used to compare the differences among the groups. Statistical analyses were performed with SPSS 20.0 software (SPSS Inc, Chicago, IL, USA). A P value less than 0.05 was considered statistically significant.

Results

DFNA5 inhibits colon cancer cell proliferation in vitro and in vivo

First, we measured the expression levels of DFNA5 in five colon cancer cell lines and found that HT-

29 and HCT116 cells had high expression levels of DFNA5 and that SW480, CACO-2 and RKO cells did not express DFNA5 (Figure 1(a)). To further gain insight into the impact of DFNA5 in colorectal carcinogenesis, a series of in vitro experiments were performed in colon cancer cells with the loss-of-function and the gain-of-function of DFNA5. The knockdown of DFNA5 in HT-29 and HCT116 cells and the ectopic expression of DFNA5 in HT-29, HCT116, SW480 and CACO-2 cells were validated by western blotting analysis (Figure 1(b)).

We next investigated the role of DFNA5 in the proliferation and colony formation of colon cancer cells. Cell growth assays were performed to assess the effect of different DFNA5 expression levels on the proliferation of colon cancer cells. In the present study, we found that enhancing DFNA5 expression in colon cancer cells resulted in a decreased cell proliferation. However, depleting DFNA5 expression in HT-29 and HCT116 cells had the opposite result (Figure 1(c)). We subsequently assessed the effect of DFNA5 on colony formation. As expected, we found that knockdown of DFNA5 significantly promoted the ability of colony formation in both shDFNA5 colon cancer cell lines compared with that in the shControl cell lines. The opposite results were observed in the DFNA5-overexpression cells (Figure 1(d)).

To investigate whether DFNA5 plays a role in CRC proliferation in vivo, a subcutaneous xenograft model of HT-29 cells was established in Balb/c nude mice. The DFNA5-overexpresion group showed a lower increase in tumor volume and weight than the control group (Figure 2(a)). To investigate whether the tumor suppression potential of DFNA5 in xenograft tumor tissues could be due to its cell proliferation inhibition, the expression levels of the universal proliferation biomarker Ki67 were determined by IHC. The results showed the xenograft tumor tissues formed by the DFNA5-overexpression cells demonstrated a much lower Ki67-staining score than those formed by the control cells (Figure 2(c)). Changes in Ki67 and tumor volume and weight were opposite of the control groups in DFNA5-knockdown HT-29 cells (Figure 2(b,c)).

DFNA5 arrests the transition of cell cycle from the G0/G1 to the S phase in colon cancer cells

To understand the mechanism of DFNA5 in the inhibition of cell proliferation, we performed cell cycle analysis using a flow cytometry assay. The results showed that overexpression of DFNA5 remarkably induced cell cycle arrest at the G0/G1 phase in colon cancer cells. Additionally, changes in the DFNA5 expression level had no significant impact on the G2 phase of colon cancer cells (Figure 3(a) and Fig S1A). 5-Ethynyl-2′-deoxyuridine (EdU) assays demonstrated that overexpression of DFNA5 significantly reversed colon cell proliferation (Figure 3(b) and Fig S1B). Conversely, the DFNA5 knockdown in colon cancer cells increased the proportion of cells in the S phase, decreased the proportion in the G0/G1 phase and promoted the proliferation measured by an EdU assay (Figure 3(a,b)). Our results demonstrated that DFNA5 inhibits colon cancer cell proliferation by arresting the transition from the G0/G1 to the S phase.

To further understand the molecular mechanisms of DFNA5 in inhibiting cell proliferation and the cell cycle, gene expression profiles were analyzed by RNA sequencing in DFNA5-overexpression SW480 cell lines compared with their control counterparts. First, we performed an enrichment analysis of GO biological processes. The results of the GO enrichment showed that DFNA5 overexpression had significantly modulated the biological processes of phosphorylation, cell cycle, cellular response to DNA damage stimulus, chromatin organization, protein ubiquitination and others (Figure 4(a)). Indeed, the results of the RNA sequencing demonstrated that a number of biomarkers related to the cell cycle were markedly changed after DFNA5 overexpression (Figure 4(b)). The results of western blotting were consistent with the flow cytometry and RNA sequencing analyses. The expression of proteins involved in the G1 to S transition, including cyclins D1 and E, as well as cyclin-dependent kinase (CDK) 2, but not the G2 to M transition, including cyclins A and B, were decreased in the DFNA5-overexpression colon cancer cells. In addition, the cell cycle inhibitor p21 and G0 marker p130 was significantly upregulated (Figure 4(c) and Fig. S2). By contrast, the knockdown of

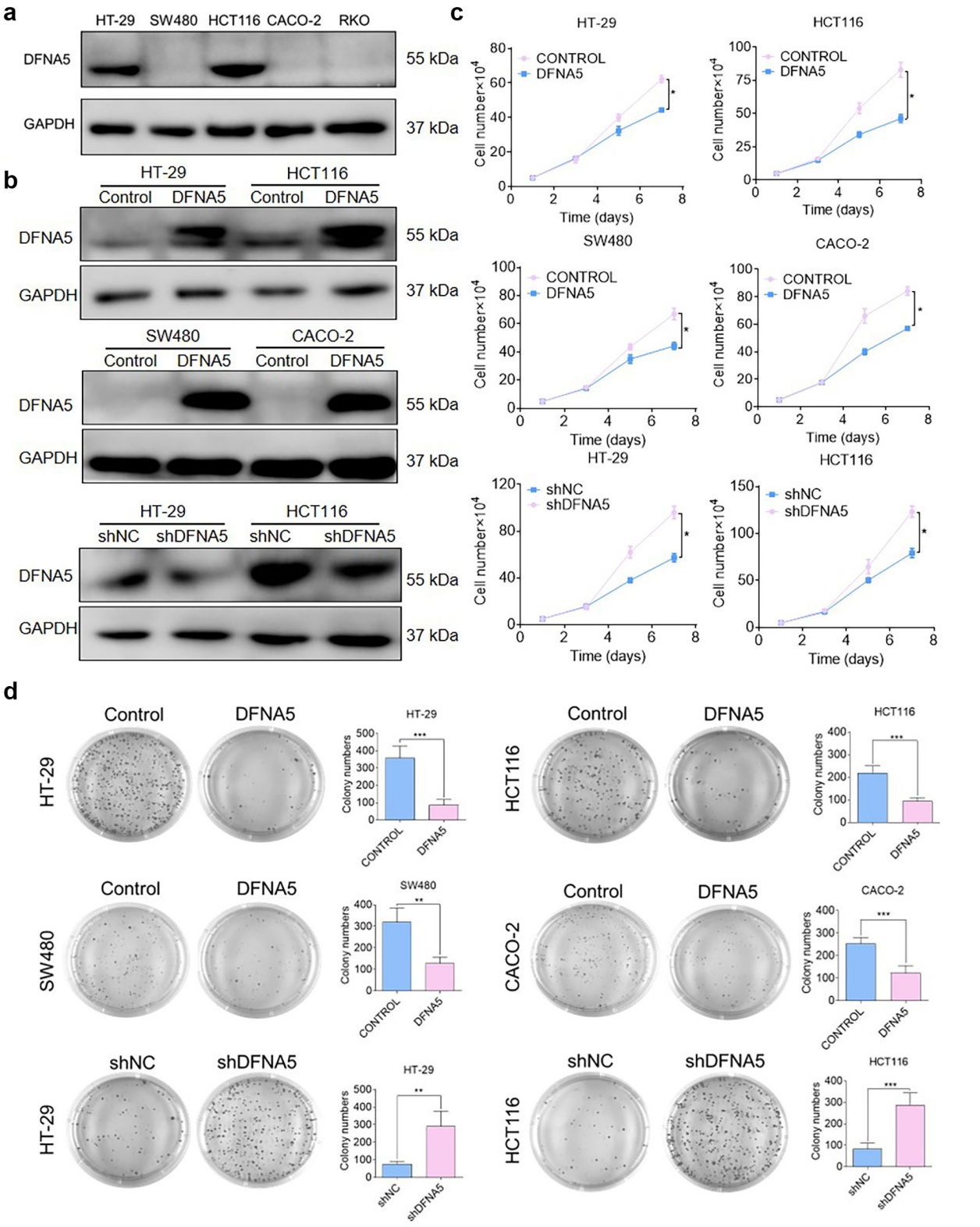

Figure 1. DFNA5 inhibited the proliferation of colon cancer cells. (a) DFNA5 expression was analyzed by western blotting in 5 colon cancer cell lines. (b) Overexpression and knockdown of DFNA5 in colon cells was confirmed by Western blotting. (c) Cell growth assay was performed using DFNA5-overexpressing and DFNA5-knockdown colon cancer cells. (d) DFNA5 significantly inhibited colony formation of colon cancer cells.

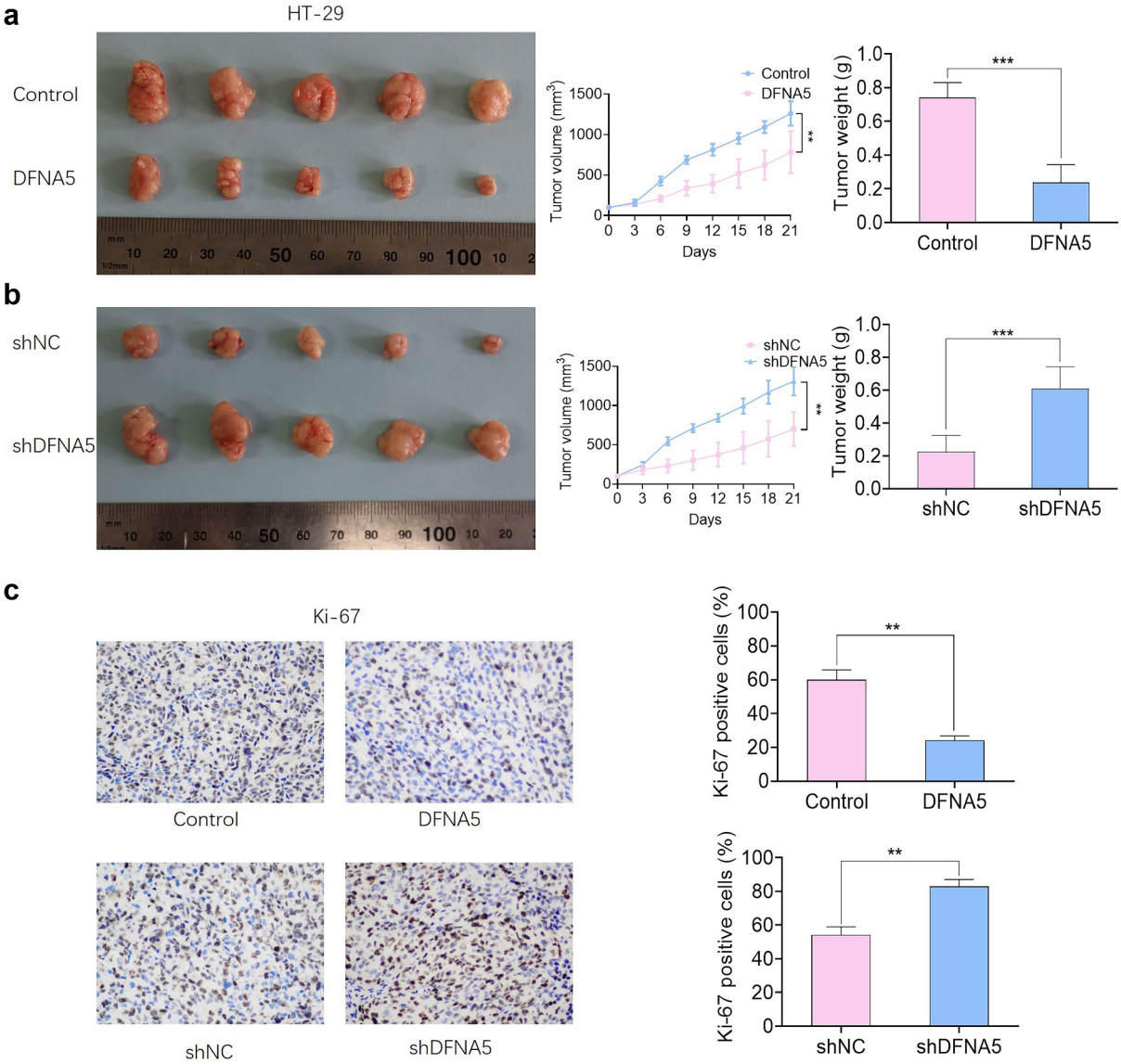

Figure 2. DFNA5 inhibits the proliferation of colon cancer cells in vivo. (a) Overexpression of DFNA5 in HT-29 cell line inhibited the tumor formation in vivo. (b) Knockdown of DFNA5 in HT-29 cell line promoted the tumor formation in vivo. (c) Immunohistochemical staining for Ki67 is shown in tumor xenografts of DFNA5 knockdown and overexpressing HT-29 cell.

DFNA5 in HT-29 and HCT116 cells had the opposite results. Moreover, the results were verified by the correlation analysis of the TCGA database (Figure 4(d)).

DFNA5 suppresses mTORC1/2 signaling via upregulation of DEPTOR

To further identify the molecular mechanisms underlying the tumor suppressive action of DFNA5, KEGG pathway enrichment analysis was performed on the differentially expressed genes detected. The result showed that DFNA5 overexpression significantly dysregulated various pathways, including mTOR signaling, cell cycle, proteoglycans in cancer, HIF-1 signaling, regulation of actin cytoskeleton, colorectal cancer, TGF-beta signaling, Wnt signaling, AMPK signaling pathways and others (Figure 5(a)). Among these, mTOR signaling was the most enriched pathway. Interestingly, we found DEPTOR, a naturally inhibitor of the mTORC1 and mTORC2 complexes [28], was markedly upregulated after DFNA5 overexpression.

The phosphorylation of S6K1 (Thr389) and AKT (Ser473) are commonly used as surrogate

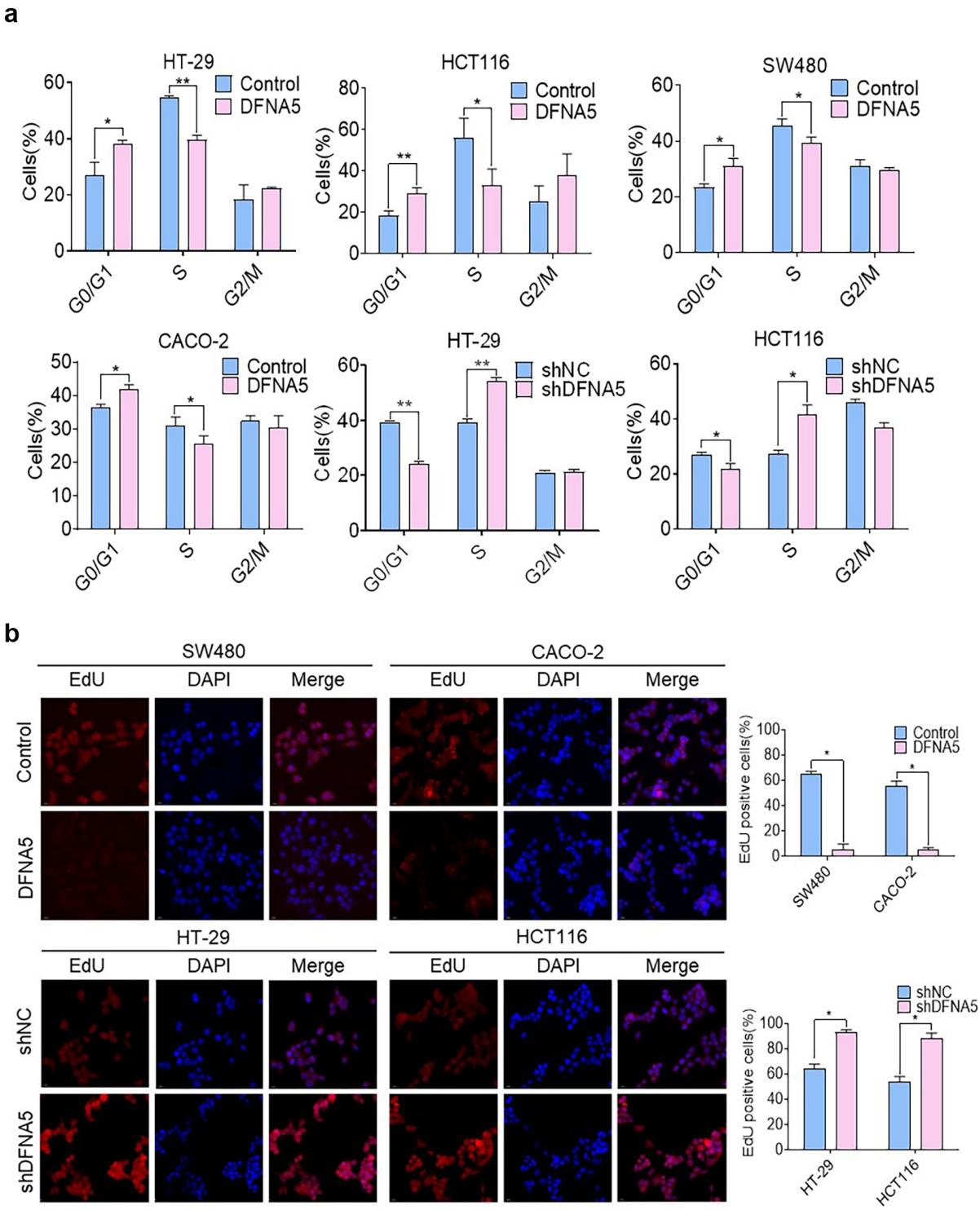

Figure 3. DFNA5 arrested the cell cycle at the G0/G1 phase. (a) DFNA5 induced remarkably cell cycle arrest at the G0/G1 phase. (b) EdU assay was performed using DFNA5-overexpressing and DFNA5-knockdown colon cancer cells.

markers of mTORC1 and mTORC2 activity [29]. Consistently, western blotting showed that ectopic expression of DFNA5 significantly decreased the mTORC1 and mTORC2 activities in colon cancer cells, accompanied with an increased protein expression of DEPTOR and a decreased protein expression of p-S6K1 and p-AKT (Ser473). Changes in the protein expression levels of several key mTORC1/2 signaling genes, including DEPTOR, p-S6K1 and p-AKT, were found to be

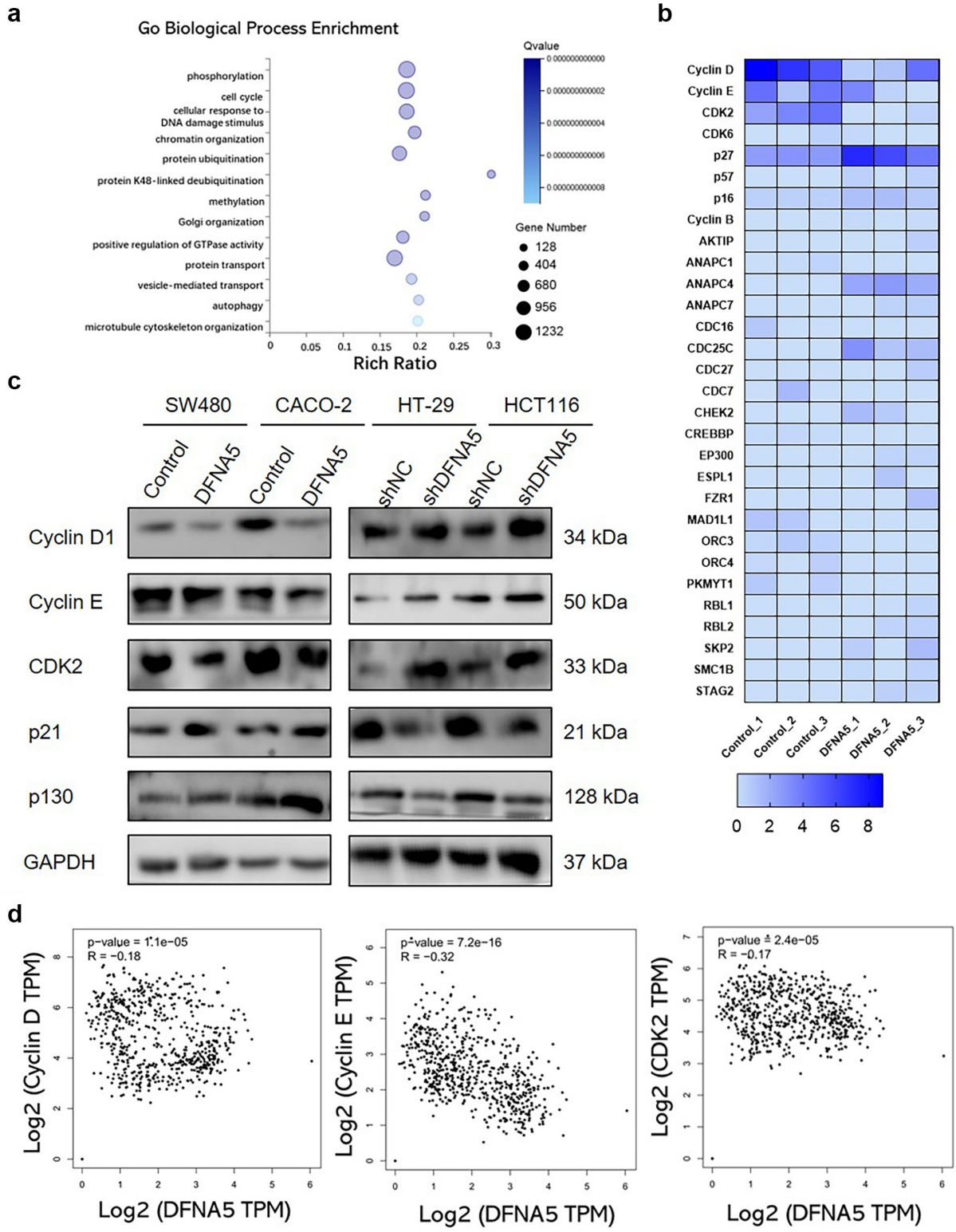

Figure 4. The molecular change of cell cycle when manipulating DFNA5 in colon cancer cells. (a) Enrichment analysis of the GO biological process using the data of RNA sequence was performed after DFNA5 overexpression. (b) RNA sequence results showed many biomarkers related to cell cycle were changed markedly after DFNA5 overexpression. (c) Western blot analyses of various cell cycle regulatory proteins with abundance change in colon cancer cells. (d) Correlation analyses of DFNA5 and Cyclin D/Cyclin E/CDK2 in TCGA database.

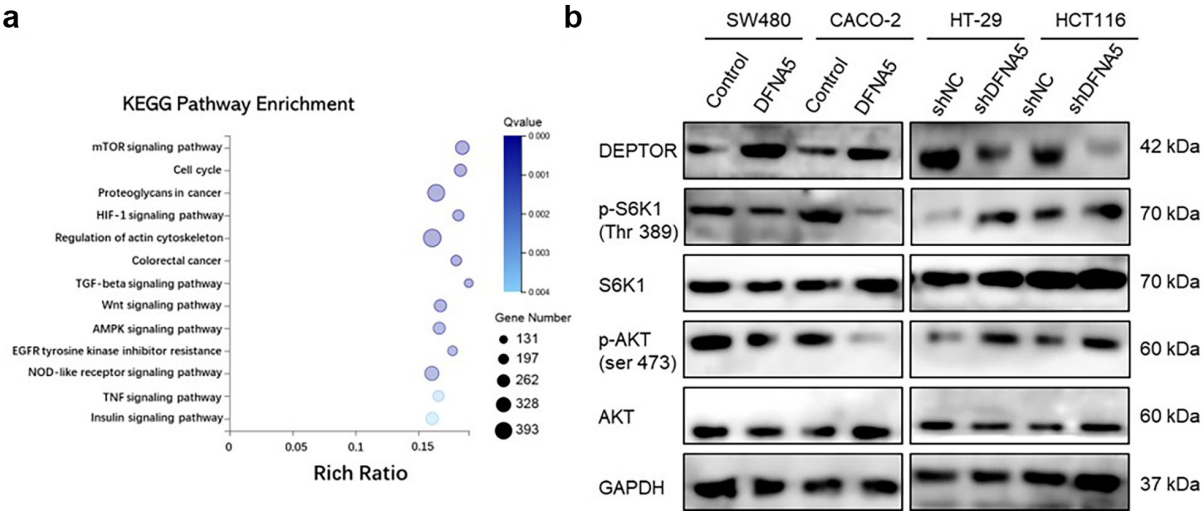

Figure 5. DFNA5 attenuated the activity of the mTORC1/2 pathway in colorectal cancer. (a) KEGG pathway enrichment analyses were performed by over-expression genes affected by DFNA5. (b) DFNA5 inhibited the mTORC1/2 pathway as evidenced by protein expression of factors in the mTORC1/2 signaling pathway in colon cancer cells by Western blotting.

the opposite in DFNA5-knockdown HT-29 and HCT116 cells compared to the control groups (Figure 5(b) and Fig. S3).

Blockage of mTORC1/2 signaling by Torin-1 abolishes the accelerative proliferation by DFNA5 knockdown

We next investigated whether the activation of mTORC1/2 signals plays a critical role in enhanced cell proliferation and survival by DFNA5 downregulation. We then treated shDFNA5 HT-29 and HCT116 cells with Torin-1 (100 nM), which is a dual small molecule inhibitor of mTORC1 and mTORC2 [30,31]. The western blotting results demonstrated that Torin-1 inactivated mTORC1 and mTORC2, as shown by the decreased phosphorylation of S6K1 and AKT (Figure 6(a)). Furthermore, Torin-1 could significantly inhibit cell proliferation and colony-formation ability (Figure 6(b,c,d). The expression level of cell cycle-related proteins, including cyclin D1, cyclin E and CDK2, were downregulated. Conversely, the cell cycle inhibitor p21 was upregulated. These results indicated a rescue effect of Torin-1 (Figure 6(a)).

Given the variety of downstream cell cycle-related proteins regulated by AKT, we next used MK-2206 (1 μM), an allosteric inhibitor of AKT, to investigate the effect of AKT on cell growth upon downregulating DFNA5 [32]. The results of western blotting showed that the protein expression of p-AKT (Ser 473) was decreased by the AKT inhibitor (Fig. S4A). As expected, MK-2206 suppressed the elevation of proliferation and colony formation in the DFNA5-knockdown HT-29 and HCT116 cells (Fig. S4B and Fig. S4C). In addition, cell cycle-related markers, including cyclin D1, cyclin E, CDK2, and p21, were also rescued by MK-2206 (Fig. S4A).

DFNA5 is downregulated in primary CRC tissues and positively correlates with DEPTOR

To determine the protein expression level of DFNA5 in CRC, we performed IHC to examine its level in 90 pairs of randomly selected tumor and corresponding adjacent tissues. DFNA5 immunostaining was primarily observed in the cytoplasm of the colon cancer cells (Figure 7(a)). We found that the DFNA5 expression was significantly decreased in CRC tissues compared to normal adjacent tissues ($P < 0.05$, Figure 7(b, c)). Moreover, we randomly selected 30 CRC tissues and examined the protein expression level of DEPTOR (Figure 7(d)). We demonstrated that the expression levels of DFNA5 and DEPTOR were positively correlated, which was consistent with our previous results (Figure 7(e)). The correlation between DFNA5 and the

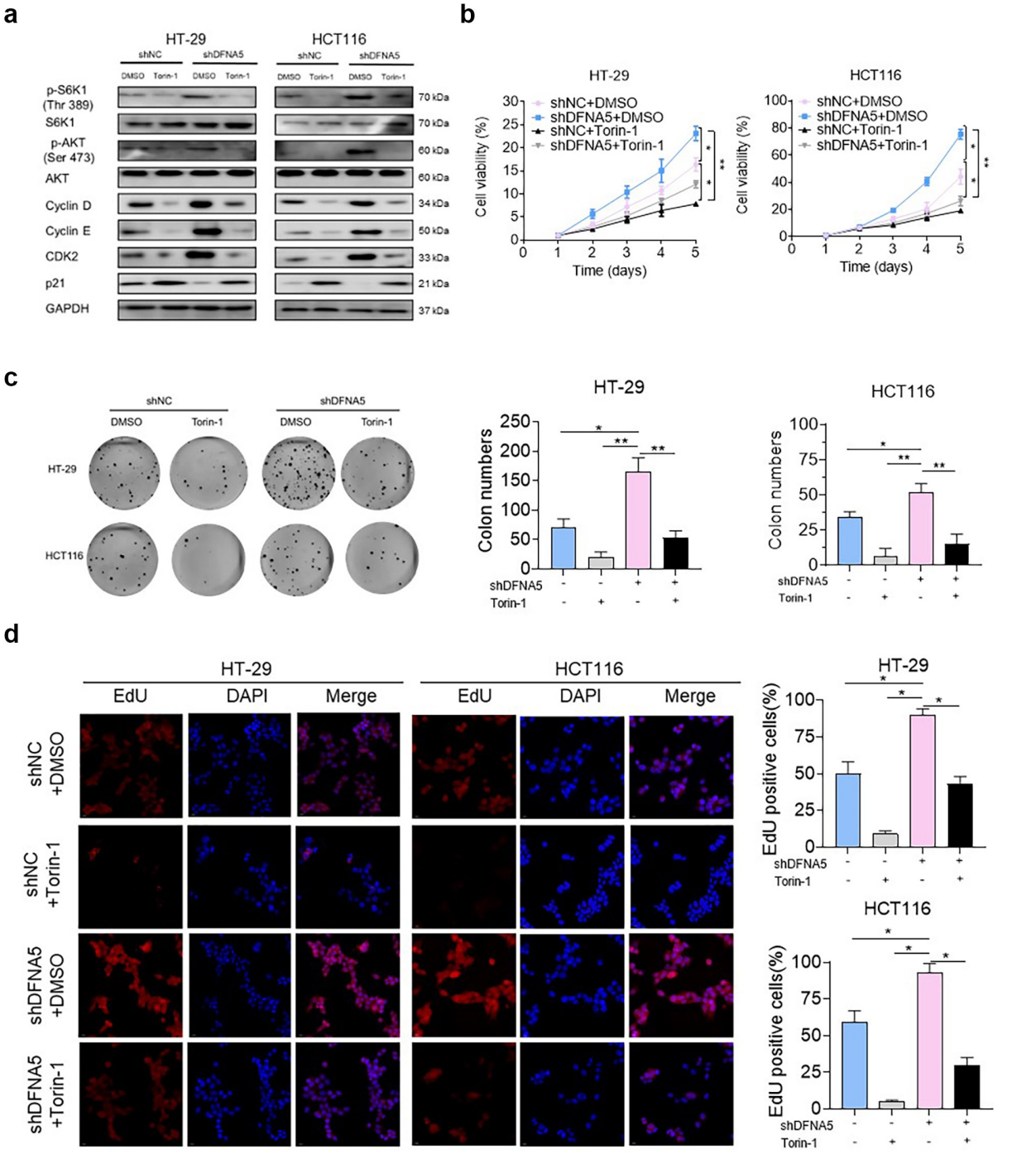

Figure 6. Effect of knock-down DFNA5 on colon cancer cell growth in the presence of mTORC1/2 inhibitor (Torin-1). (a) Inhibition of S6K1 and AKT in HT-29 and HCT116 cells was confirmed by Western blotting. The levels of cell cycle-related markers, including cyclin D1, cyclin E, CDK2, and p21, were rescued by Torin-1. (b) Torin-1 inhibited the proliferation of HT-29 and HCT116 cells analyzed by the MTT assay. (c) Torin-1 inhibited colony formation of HT-29 and HCT116 cells. (d) Torin-1 inhibited proliferation of HT-29 and HCT116 cells analyzed by the EdU assay.

clinicopathological characteristics of the patients was analyzed. However, no significant differences were found in the present analysis (Table S1).

Furthermore, a western blotting analysis was performed to evaluate the expression level of DFNA5 and DEPTOR in eight CRC tissues and paired normal tissues (figure 7(f)). The results showed that the DFNA5 expression levels were decreased in the tumor tissues compared with the normal tissues and had positive correlation with

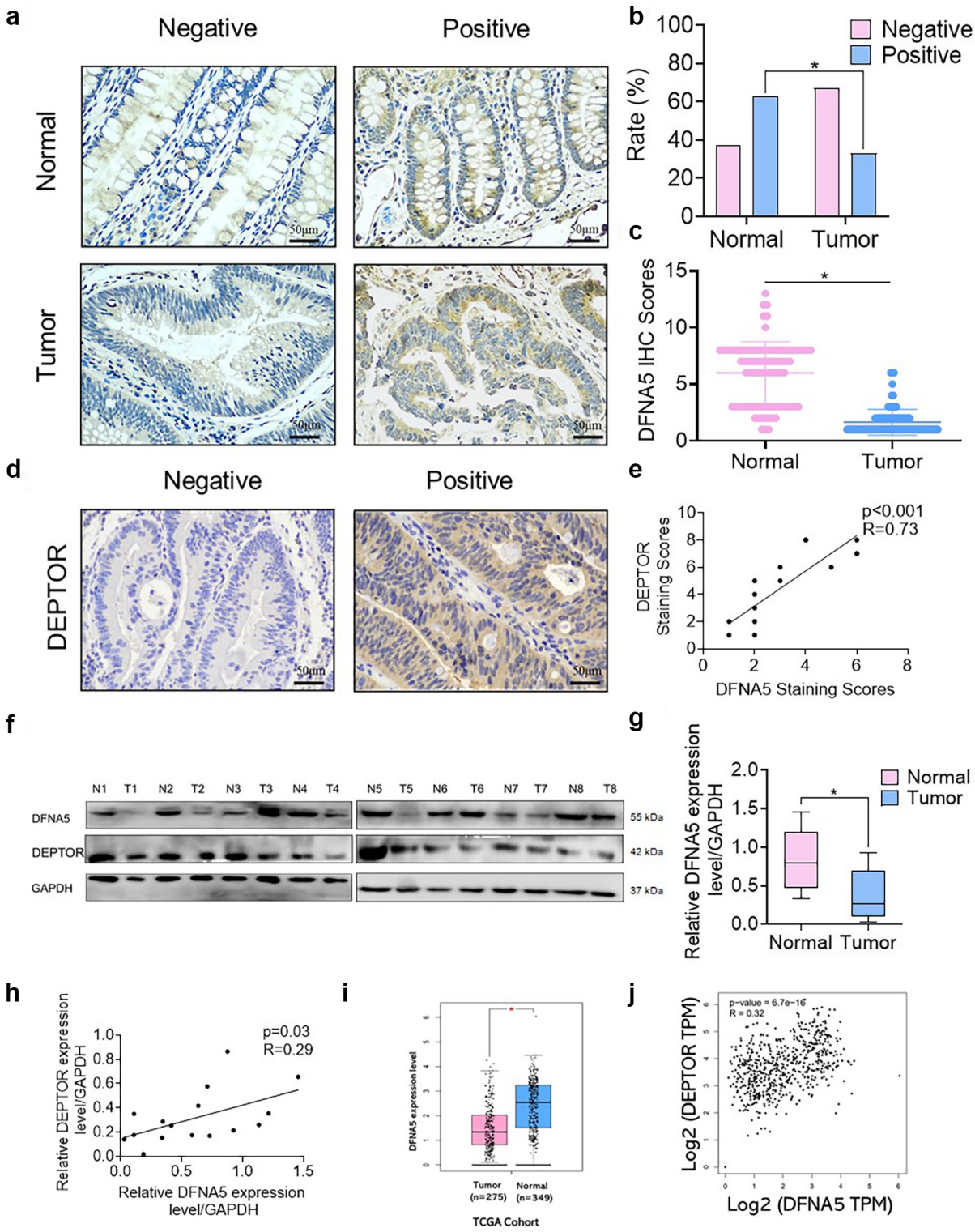

Figure 7. The expression of DFNA5 in colorectal cancer (CRC) is lower than normal tissue samples. (a) The expression of DFNA5 in CRC and normal tissue samples by the immunohistochemistry (IHC) staining. (b) The positivity of DFNA5 staining in CRC and normal tissue samples. (c) The IHC score of DFNA5 staining in CRC and normal tissue samples. (d) The expression of DEPTOR in CRC tissue samples by the IHC staining. (e) DFNA5 positively correlates with DEPTOR. (F, G, H). DFNA5 and DEPTOR expression level was analyzed by western blotting in paired normal colorectal (n) and tumor tissues (t) collected from 8 patients. (i, j) Data from the TCGA dataset showed that the expression of DFNA5 in CRC tissues was significantly lower than that in normal tissues (i) and positively correlates with DEPTOR (j).

DEPTOR, which was consistent with the results of the IHC (Figure 7(g,h)). In addition, analysis of the data from the TCGA database also verified our result that DFNA5 expression was downregulated in the tumor tissue of CRC patients (Figure 7(i)) and positively correlated with DEPTOR (Figure 7(j)).

Discussion

Currently, studies on the involvement of DFNA5 in CRC development and progression are limited and disputed. Therefore, it is important to explore the function of DFNA5 in CRC further. In the present study, we demonstrate that DFNA5 plays a vital role in the inhibition of cell growth by inhibiting the mTORC1/2 signaling pathway in CRC. This conclusion is based on several observations: (1) the DFNA5 protein was significantly downregulated in the primary CRC tissue; (2) overexpression of DFNA5 inhibited the cell cycle, proliferation and colony formation, with the opposite results observed in the DFNA5-knockdown cells; (3) the results of RNA sequencing, TCGA and western blotting demonstrated that the modulation of DFNA5 affected the mTORC1/2 pathway in colon cancer cells, including DEPTOR, p-S6K1 and p-AKT; and (4) the mTORC1/2 inhibitor Torin-1 abrogated enhanced cell proliferation and colony formation after DFNA5 silencing.

Mechanistically, epigenetic silencing through promoter methylation is supposed to contribute to inhibition of the expression of DFNA5, which was previously found in colorectal, breast, and gastric cancer [14,15,33,34]. However, recently, Lu et al. found that the DFNA5 protein was pervasively detected in most lung cancer cell lines and in lung cancer tissue from patients, which suggests that it might act as an oncogenic driver in lung cancer. These authors presumed that several previous studies primarily used in vitro system or invalidated antibodies, which leaded to the epigenetic gene silencing of DFNA5 [13]. Thus, the expression level of DFNA5 in CRC needed to be demonstrated further. In this study, we proved that the expression of DFNA5 was downregulated in primary CRC tissue using a larger sample size compared with a previous study, which was verified with the TCGA database [14]. In addition, Yokomizo et al. demonstrated that the downregulation of DFNA5 was observed in tumors lesions with more lymphatic vessel invasion and a high TNM stage in the CRC samples [14]. Unlike the results of Yokomizo, we did not find a significant relationship between DFNA5 expression and tumor stage in the present study.

Subsequently, the function of DFNA5 in CRC is a controversial problem. In this study, we found that DFNA5 inhibited the cell cycle, colony formation and the proliferation of colon cancer cells. Consistent with our results, Kim et al. found an increase in colony numbers, colony size, and cell growth in DFNA5-knockdown colon cancer cells [5]. However, Liu et al. knocked out DFNA5 in lymphoma, breast cancer, and gastric cancer cell lines, and further found that the DFNA5 deficiency did not affect cancer cell growth [12]. In vivo, Croes et al. did not find any significant differences between the DFNA5 KO and WT mice, neither in the number of cancer-affected mice nor in the multiplicity of proliferative tumor lesions per mouse. These authors only found that there was a trend toward more severe inflammation in the DFNA5 WT mice compared to the DFNA5 KO mice, which revealed a complexity in the molecular mechanism that is likely amplified in vivo [35]. Wang et al. indicated that the overexpression of DFNA5 inhibited cell growth through arresting G2/M phase in human hepatocellular carcinoma, which was inconsistent with our result [36]. Nevertheless, in our study, DFNA5 primarily regulated the G1/S phase transition of colon cancer cells. We think the reason may be related to different cell- and context-specific variables.

The mTORC1/2 signaling pathways, which are related to the promotion of tumor cell cycle, proliferation and survival, are crucial hallmarks of various tumor types [19,37,38]. Cell cycle progression, driven forward by cyclins and CDKs, is often affected by the activity of AKT and S6K1 in cancer development. AKT is activated by phosphorylation on Ser473 or Thr308. Subsequently, it regulates a variety of downstream cell cycle-related protein substrates, including GSK3β, cyclin D and P21 [39,40]. Previously, Kim et al. found that colon cancer cell lines with silenced DFNA5 could increase the phosphorylation of AKT and some

cyclin proteins [5]. To the best of our knowledge, this was the first study to demonstrate the connection between DFNA5 and the mTOR signaling pathway. DFNA5 inhibited the tumor growth of colon cancer cells by suppressing mTORC1/2 signaling via upregulation of DEPTOR. DEPTOR, which binds mTOR, associates with both mTORC1 and mTORC2 and suppresses their activity [41]. As a tumor suppressor, DEPTOR is correlated with inhibiting protein synthesis, cell proliferation and survival. Previous studies reported a low expression of DEPTOR in many human cancers [41–45]. However, the role of DEPTOR is controversial in CRC. Wang et al. found that as a downstream target of the Wnt/b-catenin/c-Myc signaling pathway, DEPTOR was overexpressed and required for colon cancer cell proliferation [46]. In contrast, Lai et al. demonstrated that there was no significant difference in DEPTOR expression between CRC and adjacent normal tissue. DEPTOR might play a role as tumor suppressor, as the upregulation of DEPTOR was associated with less nodal metastasis and a lower histological grade [47]. Consistently, our result revealed that the overexpression of DFNA5 upregulated DEPTOR remarkedly, which resulted in an inactivity of the mTORC1/2 signaling pathway and the inhibition of colon cancer cell growth.

In conclusion, our study demonstrated that DFNA5, which was downregulated in CRC, inhibited tumor cell growth in vivo, and retarded cell proliferation, cell cycle, and colony formation in vitro. Our results suggested that DFNA5 inhibits colon cancer cell growth by suppressing mTORC1/2 signaling via upregulation of DEPTOR.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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