Downregulation of PHF10 Promotes Proliferation, Migration and Epithelial-Mesenchymal Transition of Colorectal Cancer Cells by Activating mTOR Pathway

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Received April 27, 2024
Accepted July 18, 2024
Electronic access August 15, 2024

Many studies tried to find the genes that are associated with colorectal cancer patients' survival. Recent studies have focused on PHF10, which is known to regulate chromatin remodeling. However, the mechanism by which PHF10 suppresses colon cancer formation and proliferation has not yet been studied. Lack of information about the downstream targets of PHF10 and the mechanisms that inhibit its cancer progression has limited effective treatments for colorectal cancer patients, so breakthrough is needed. Through this study, we used the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) knock out system to reveal the role of PHF10 in suppressing cancer progression. Knocking out PHF10 was found to promote cancer progression by increasing epithelial to mesenchymal transition. Epithelial to mesenchymal transition occurs during normal embryonic development, but it is also involved in tuner progression through metastatic expansion. We aimed to reveal that loss-of-function of PHF10 mediates the mTOR pathway that promotes tumorigenesis. We confirmed that PHF10 downregulation is associated with poor prognosis in colorectal cancer, and our results suggest that knocking out PHF10 promotes tumorigenesis by upregulating the mTOR pathway. This is the first study to discover that PHF10 can regulate the mTOR pathway, and it suggests that using drugs targeting the mTOR pathway can be an effective treatment strategy for colon cancer patients with downregulated PHF10.

Keywords: colorectal cancer, PHF10, CRISPR, mTOR pathway, epithelial to mesenchymal transition

Introduction

Colorectal cancer (CRC) remains one of the most prevalent and deadly types of cancer globally[1]. Current treatment strategies for CRC are often insufficient due to issues such as metastasis, drug resistance, and high recurrence rates. The metastasis (CRC in particular) involves a series of sequential steps where tumor cells detach from the primary tumor, invade surrounding tissues, enter the systemic circulation, and eventually metastasize to distant organs, predominantly spreading along the mesenteric circulation to the liver and less frequently to the lungs and bones.

Epithelial-mesenchymal transition (EMT) has been proposed as a critical mechanism regulating the early stages of metastasis. EMT is a molecular program where epithelial cells, initially polarized and differentiated with numerous cell-to-cell junctions, undergo reprogramming to acquire a mesenchymal phenotype[2]. This phenotypic transformation is a key molecular characteristic distinguishing metastatic from non-metastatic cancer cells,[3] making increased EMT a marker of tumorigenesis. Genes mediating EMT are expected to play a crucial role in tumorigenesis.

In this study, PHF10 downregulation's effects on the mTOR pathway, CRC cell invasion, and migration were examined. Previous studies identified PHF10 primarily as a chromatin remodeler. Wei et al. (2010) found that PHF10 inhibits the expression of caspase-3 and impairs programmed cell death pathways in human GCs.[4] Also, PHF10 expression was correlated with the advancement of the illness, as evidenced by a rise in PHF10 mRNA levels from primary melanoma foci to regional cutaneous metastases and beyond to distant metastatic nodes; depletion of PHF10 induced G1 accumulation and an aging-like phenotype in melanoma cells[5]. But little is known about its role in the development and progression of CRC[6].

The data shows that knocking out PHF10 increased CRC cell proliferation, suggesting a regulatory role in cell migration and invasion. This also confirmed that the effect of PHF10 knockout promoted cell migration and invasion. Furthermore, consistent with existing research linking cell migration and invasion with upregulation of the mTOR pathway,[7] we observed that PHF10 knockout samples showed activation of the mTOR pathway.

Our findings indicate that in the HCT116 colon cancer cell line, PHF10 plays a tumor-suppressive role in tumor progression, and PHF10 depletion promotes cell migration and invasion. Additionally, this promotes activation of the mTOR pathway, making cells sensitive to rapamycin. Our study suggests that patients with low PHF10 expression may benefit from targeting this pathway as a potential therapeutic approach.

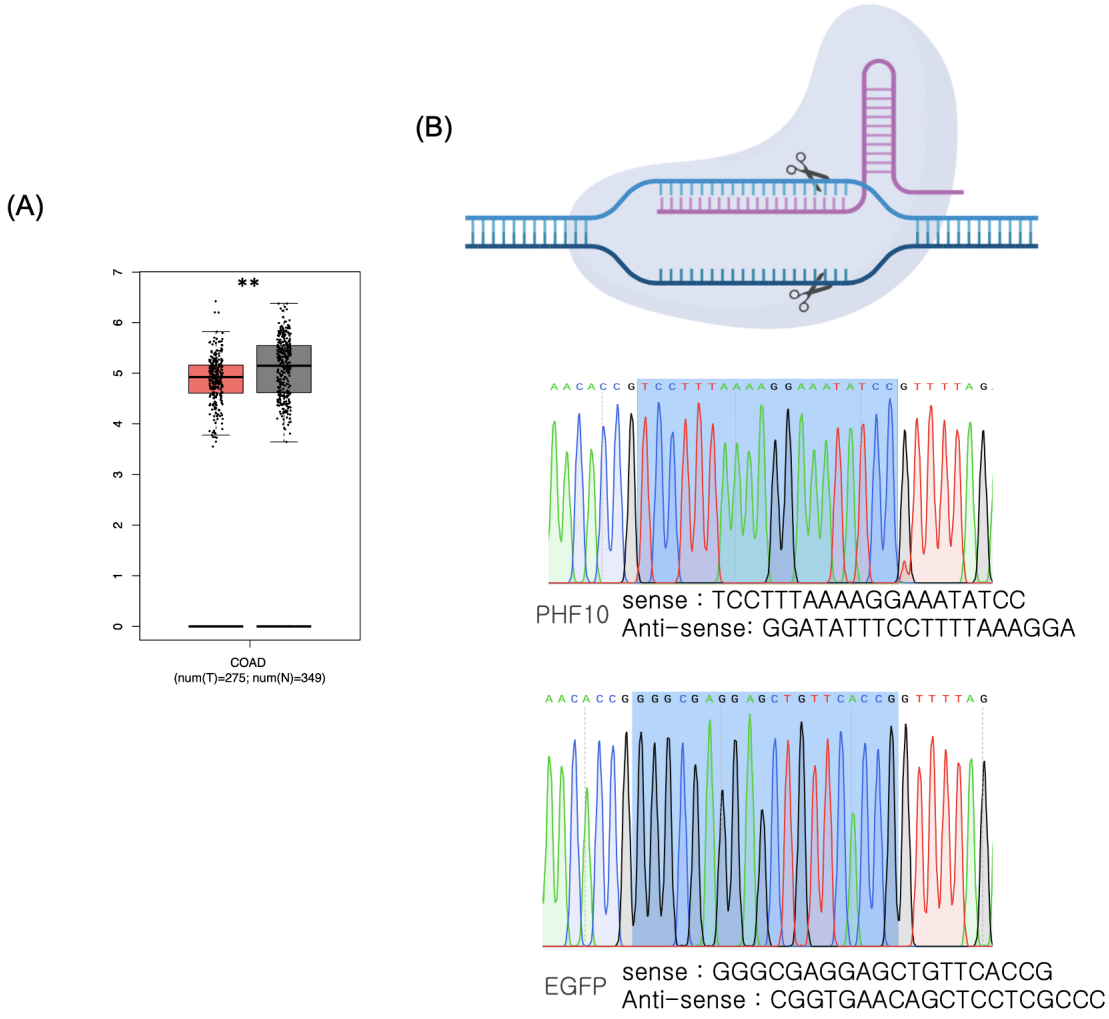

Fig. 1 PHF10 expression is downregulated in CRC patients
(A) Expression of PHF10 in colorectal cancer (pink column) and normal colon (grey column) tissue (**P<0.01)
(B) Schematic diagram of CRISPR-cas9 system and results of sanger sequencing of CRISPR-PHF10 and CRISPR-EGFP (control) vector

Results

Analysis of public patient databases, which included 624 normal colon and colon cancer samples, revealed a marked reduction in PHF10 expression in 349 colon cancer patients (Figure 1a). This suggests that PHF10 may have a tumor suppressive role in the context of colon cancer.

To further explore the function of PHF10 in colon cancer, we employed CRISPR technology using an sgPHF10 vector to knock out PHF10 in the HCT116 colon cancer cell line (Figure 1b). EGFP was used as a control for PHF10. It can be used as a control because it does not target any endogenous genes (Figure 1b). This approach aimed to elucidate the potential mechanisms by which PHF10 influences colon cancer progression and patient outcomes.

Given our observations of reduced PHF10 expression in CRC patients, we hypothesized that PHF10 plays a tumor suppressive role, and its knockout could enhance the aggressive behavior of tumors.

To test this hypothesis, we transfected HCT116 colon cancer cells with a CRISPR sgPHF10 vector. Subsequent analyses confirmed significant reductions in both PHF10 protein expression (Figure 2a) and mRNA levels (Figure 2b) following transfection.

Furthermore, depletion of PHF10 was found to increase the proliferation of CRC cells (Figure 2c). These findings support the notion that PHF10 serves a tumor suppressive function and is involved in the regulation of CRC proliferation. This implies that the downregulation of PHF10 could be a critical factor in

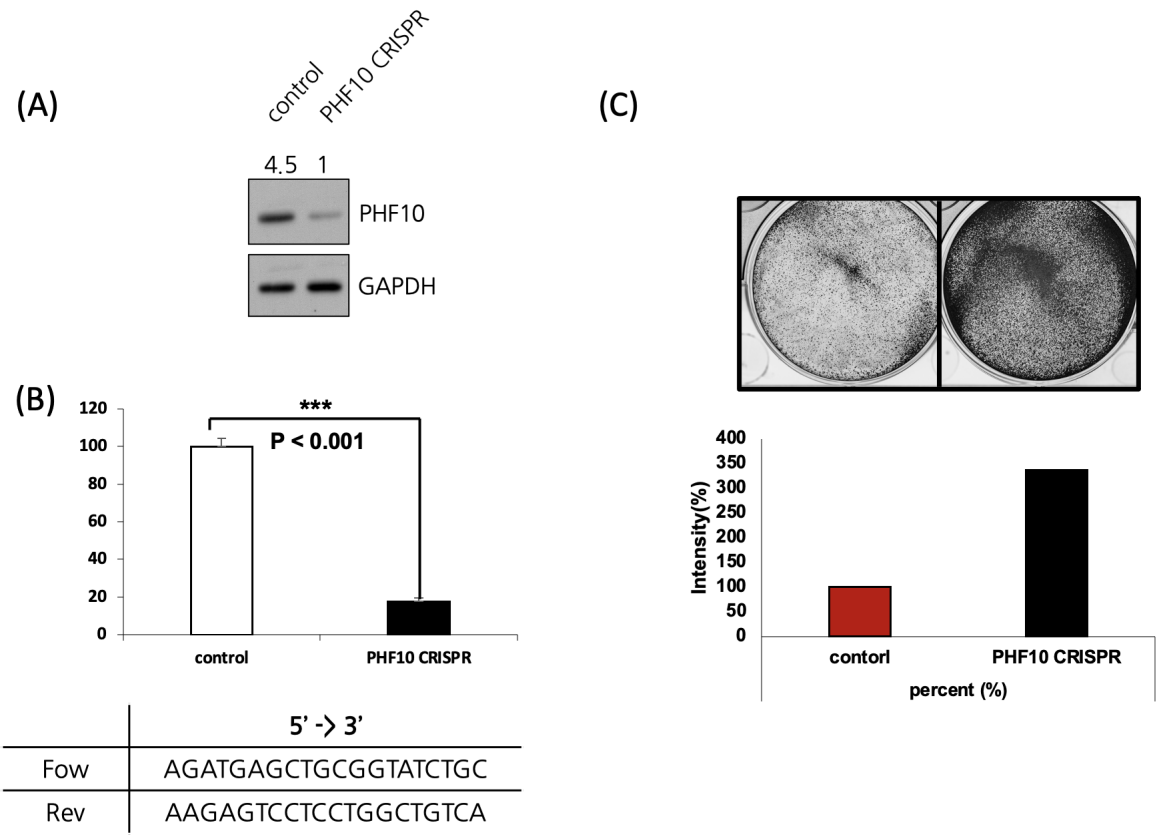

Fig. 2 PHF10 knockdown upregulates proliferation of HCT116 cells
(A) Western blot analyses for PHF10 expression in control and PHF10 CRISPR in HCT116 cells
(B) RT-qPCR analysis of the expression level of PHF10 in control and PHF10 CRISPR in HCT116 cells
(C) Clonogenic assay shown that PHF10 knockout on HCT116 cell (top) and quantification by image J (bottom)

the enhanced proliferation and potentially aggressive phenotype observed in CRC.

Figure 3 demonstrates that PHF10 knockdown promotes epithelial-mesenchymal transition (EMT), as well as increases in migration and invasion of colorectal cancer (CRC) cells.

Migration and invasion are pivotal early steps in cancer progression that facilitate metastasis[8]. We investigated whether the effect of PHF10 knockout (KO) on cancer cell proliferation was associated with changes in migration and invasion. Following PHF10 KO, we observed an expected increase in the expression of mesenchymal-associated markers such as Vimentin (VIM), Vascular Cell Adhesion Molecule (VCAM), and Transforming Growth Factor Beta Receptor 1 (TGFBR1) using quantitative RT-PCR (Figure 3a).

Additionally, both transwell migration assays (Figure 3b) and invasion assays (Figure 3c) showed significant increases, indicating enhanced migratory and invasive capabilities of the cells post-KO. Our findings demonstrate that knocking out PHF10, which has tumor suppressive properties, leads to increased invasiveness and migration in CRC cells, thereby promoting tumor proliferation. This suggests that PHF10 downregulation may play a crucial role in the progression of CRC by facilitating the activation of pathways that lead to EMT and metastatic behavior.

Figure 4 investigates the role of the PHF10-mediated mTOR pathway in regulating migration and invasion in colorectal cancer (CRC) cells.

Previous studies have indicated that the mTOR pathway regulates processes such as epithelial-mesenchymal transition (EMT) and metastasis in cancer cells[7]. To explore this further in the context of PHF10 knockout (KO), we examined changes in mTOR protein levels using Western blot analysis in our PHF10 KO samples. Surprisingly, we observed an increase in mTOR1 protein following PHF10 KO (Figure 4a).

To assess the functional significance of this increase, we treated cells with rapamycin, a well-known inhibitor of the mTOR pathway. The results showed that PHF10 KO cells were

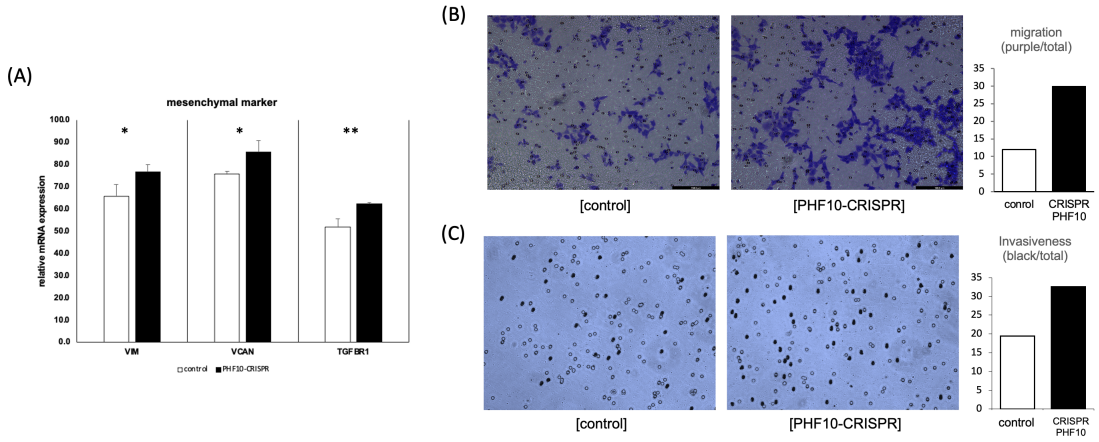

Fig. 3 PHF10 knockdown promotes EMT, migration and invasion
(A) RT-qPCR analysis of the expression level of VIM, VCAM and TGFBR1 in control and PHF10 CRISPR in HCT116 cells (*P<0.05, **P<0.01)
(B) Transwell migration assay was carried out with HCT116 control and PHF10 CRISPR (left) and its quantification by Image J (right)
(C) Transwell invasion assay was carried out with HCT116 control and PHF10 CRIPSR (left) and its quantification by Image J (right)

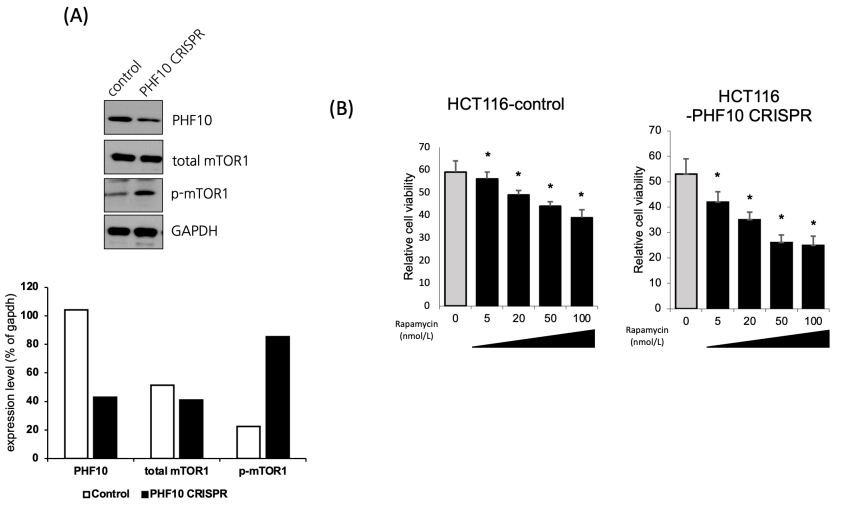

Fig. 4 PHF10 mediated mTOR pathway can regulate migration and invasion
(A) Immunoblot analysis for PHF10, mTOR and phospho-mTOR in HCT116 cells with PHF10 CRISPR (top) and quantification by image J (bottom)
(B) Cell viability was measured using ATP treated with 0, 5, 20, 50 and 100nmol/L rapamycin in HCT 116 control and PHF10 CRISPR cells for 24hours (*P<0.05)

more sensitive to rapamycin compared to control cells (Figure 4b).

These results suggest that in CRC, PHF10 regulates cell migration and invasion through the mTOR pathway. The heightened sensitivity to rapamycin in PHF10 KO cells underscores the critical role of this pathway in mediating the cellular behaviors associated with tumor progression and response to therapy.

Figure 5 explores whether the effects of loss of the tumor suppressive PHF10 can be reversed by re-expression. We performed a rescue experiment of PHF10 to re-express PHF10 (Figure 5a). When we rescued PHF10 knockout cells by re-expressing CRISPR-resistant PHF10, we found that cell proliferation, which was increased by PHF10 knockout, decreased again (Figure 2c, 5b). Furthermore, the data also showed that

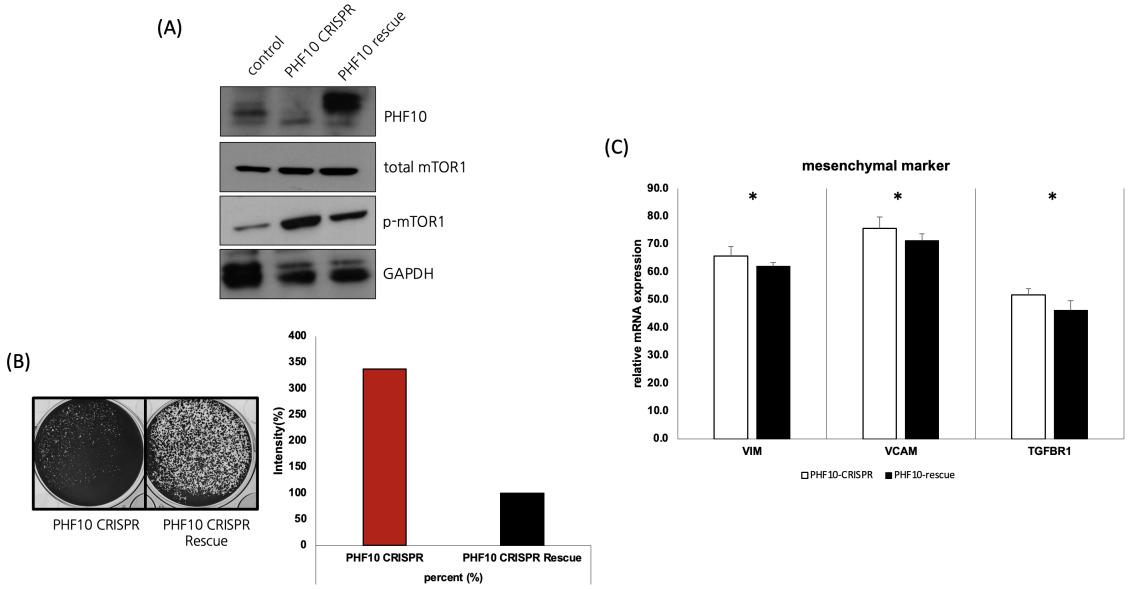

Fig. 5 PHF10 re-expression downregulate cell proliferation and EMT
(A) Immunoblot analysis for PHF10, mTOR and phospho-mTOR in HCT116 cells with PHF10 CRISPR and PHF10 rescue **(B)** Clonogenic assay shown that PHF10 knockout and re-expression on HCT116 cell (left) and quantification by image J (right) **(C)** RT-qPCR analysis of the expression level of VIM, VCAM and TGFBR1 in PHF10 CRISPR and PHF10 rescue in HCT116 cells (*P<0.05)

the mTOR pathway upregulated by PHF10 knockout was also reduced (Figure 5a), leading to a decrease in mesenchymal-associated markers (Figure 5c). To sum up, PHF10 could regulate mesenchymal state through the mTOR pathway.

Discussion

Multiple studies have focused on the role of PHF10 as a chromatin remodeler[6]. However, its direct impact on the progression of colorectal cancer (CRC) through low expression levels has not been well established. This study aimed to explore the mechanisms by which PHF10 influences tumorigenesis in CRC.

Observations of downregulated PHF10 expression in CRC patients led us to hypothesize that PHF10 may perform a tumor suppressive function. Utilizing the CRISPR associated nuclease 9 (CRISPR/Cas9) system, first discovered in 1987 by Japanese scientists and later developed into a powerful genetic editing tool,[9] we knocked out PHF10. This knockout resulted in increased proliferation of CRC cells.

Our goal was to ascertain the origin of the increased proliferation observed with PHF10 knockout. Given that migration and invasion are critical steps in the initial progression of cancer that facilitate metastasis[10], we employed transwell migration and invasion assays to measure these processes post-knockout. PHF10 knockout not only increased proliferation but also enhanced migration and invasion, and elevated the expression of mesenchymal markers, thus identifying PHF10 as a novel tumor suppressor in CRC.

Furthermore, citing Gulhati et al.,[7] it is revealed that the mTOR pathway regulates epithelial-mesenchymal transition (EMT) and metastasis in cancer cells. Our study investigated whether the EMT and invasiveness regulated by PHF10 are also associated with the mTOR pathway. Upon PHF10 knockout, we observed activation of the mTOR pathway. Treatment with rapamycin, a well-known mTOR pathway inhibitor, showed a more pronounced response in PHF10 knockout samples compared to controls.

However, this study has limitations. Although this study reveals a tumor suppressive role of PHF10, it does not prove whether this has clinical relevance in colon cancer patients. Therefore, it will be necessary to examine the relationship between PHF10 expression and prognosis in patients. In addition, in vivo experiments in mice may increase the reliability of our results. Nevertheless, this study is the first to report that the PHF10-mediated mTOR pathway regulates cell migration and invasion in CRC. Our findings not only reveal the critical role of PHF10 expression in regulating colon cell migration and invasion but also suggest that treatment with mTOR inhibitors like rapamycin could be a potential therapeutic target for patients with low PHF10 expression.

Methods

Cell Culture

The human colorectal cancer cell line HCT116 was purchased from the Korean Cell Line Bank (Seoul, Korea). HCT116 cells were cultured in Gibco's RPMI-1640 media containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin, at 37°C in a 5% CO2 atmosphere.

Protein Isolation and Western Blot

Cells were lysed in RIPA lysis buffer containing a protease and phosphatase inhibitor cocktail (Thermo Scientific) for 20 min on ice and were centrifuged at 13,000 $\times$ g at 4oC for 20 min. Using a BCA protein assay kit (Thermo Scientific, Rockford, USA), the protein concentration was determined after the supernatant was collected. Using the appropriate antibodies, the protein levels were examined by Western blotting, and the blots were then normalized by probing them with a GAPDH antibody. Santa Cruz Biotechnology supplied the antibodies against GAPDH (sc-365062, Santa Cruz, CA, USA). Abcam supplied the antibodies against PHF10 (ab259946) and p-mTOR(sc-291333). Bethyl Laboratory supplied the antibodies against mTOR (Bethyl A300-503)

Clonogenic Survival Assay

Briefly, 2.0 * 10^4 cells were seeded in 10cm dishes and the media were replaced regularly and all cultures were incubated for 7 days until the colonies were large enough to be clearly discerned. After 7 days, surviving colonies were fixed, stained with crystal violet, and counted.

Quantitative RT-PCR

Quantitative real-time PCR (qRT-PCR) analysis was carried out. A cDNAs derived from total RNA in HCT116 was extracted. Data from the array were normalized to 18S rRNA.

Transwell Migration Assay

A Corning chamber migration assay with collagen-coated Transwells was executed with HCT116 for 18 hours, respectively. The chemoattractant used was 10% FBS. Cells were counted with an inverted microscope and quantified by Image J.

Transwell Invasion Assay

A Corning chamber invasion assay with Matrigel-coated Transwell chambers was conducted with HCT116 over 48 hours. The chemoattractant used was 10% FBS. Cells were counted with an inverted microscope and quantified by Image J.

Cell Viability Assay

Equal numbers of cells were treated with 0, 5, 20, 50 and 100 nmol/L rapamycin or dimethyl sulfoxide (DMSO; control) for 24 hours. Cell viability was quantitated using CellTiter-Glo (Promega, USA) as detailed in the manufacturer's instructions.

Knockout of The PHF10 Gene in HCT116 Cells

A lentiviral-based CRISPR/Cas9 system was used to knockout PHF10 in HCT116 cells. Oligonucleotides were designed to encode a sgRNA that would direct Cas9 endonuclease activity to sequences within the human NRP-1 gene (sense: TCCTTTAAAAGGAAATATCC; anti-sense: GGATATTTCCTTTTAAAGGA) and EGFP gene (sense : GGGCGAGGAGCTGTTCACCG; anti-sense : CGGTGAACAGCTCCTCGCCC). These were cloned into the LentiCRISRPv2 vector (Addgene 52961) and used to generate lentivirus, as previously described[11]. Lentivirus-transduced HCT116 cells were selected for puromycin resistance. The selected null knockout (KO) clones were transduced with pWPXLd (AddGene plasmid 12258) lentivirus-encoding a human rescue PHF10 cDNA with a silent mutation in the sgRNA target sequence. This mutation was introduced into the PHF10 cDNA using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's instructions with the following mutagenic primer. This mutation was introduced into the PHF10 cDNA using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's instructions with the following mutagenic primer (GGG TGT GAC CTC CTC TAA AAG GAA ATA TCC AGG TAC TAG T), where the bolded C residue corresponds to a mutation of the CTT to a CTC. This silent mutation protected the rescue cDNA from CRISPR-Cas9-targeted disruption. Paired knockout PHF10 CRISPR (KO) and rescue PHF10 CRISPR rescue cell lines were used for in vitro.

Statistical Analysis

The means $\pm$ standard deviations of the means (SD) are used to represent all the results. A one-way ANOVA test for parametric data was used to do statistical analysis on the means of technical triplicates of at least three independently repeated experiments. If the p-value was less than 0.05, the differences were deemed significant. The significant p values (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) are shown as stars on the graphs.

Acknowledgment

I'm so grateful to every teachers and professors who helped and gave me a chance to participate in CRISPR research.

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