

KLF2 inhibits colorectal cancer progression and metastasis by inducing ferroptosis via the PI3K/AKT signaling pathway

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Abstract

Krüppel-like factor 2 (KLF2) belongs to the zinc finger family and is thought to be a tumor suppressor gene due to its low expression in various cancer types. However, its functional role and molecular pathway involvement in colorectal cancer (CRC) are not well defined. Herein, we investigated the potential mechanism of KLF2 in CRC cell invasion, migration, and epithelial–mesenchymal transition (EMT). We utilized the TCGA and GEPIA databases to analyze the expression of KLF2 in CRC patients and its correlation with different CRC stages and CRC prognosis. RT-PCR, western blot, and immunohistochemistry assays were used to measure KLF2 expression. Gain-of-function assays were performed to evaluate the role of KLF2 in CRC progression. Moreover, mechanistic experiments were conducted to investigate the molecular mechanism and involved signaling pathways regulated by KLF2. Additionally, we also conducted a xenograft tumor assay to evaluate the role of KLF2 in tumorigenesis. KLF2 expression was low in CRC patient tissues and cell lines, and low expression of KLF2 was associated with poor CRC prognosis. Remarkably, overexpressing KLF2 significantly inhibited the invasion, migration, and EMT capabilities of CRC cells, and tumor growth in xenografts. Mechanistically, KLF2 overexpression induced ferroptosis in CRC cells by regulating glutathione peroxidase 4 expression. Moreover, this KLF2-dependent ferroptosis in CRC cells was mediated by inhibiting the PI3K/AKT signaling pathway that resulted in the suppression of invasion, migration, and EMT of CRC cells. We report for the first time that KLF2 acts as a tumor suppressor in CRC by inducing ferroptosis via inhibiting the PI3K/AKT signaling pathway, thus providing a new direction for CRC prognosis assessment and targeted therapy.

Keywords: colorectal cancer; KLF2; ferroptosis; metastasis; pathway

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Introduction

Colorectal cancer (CRC) is a common malignant tumor of the gastrointestinal tract. In recent years, the incidence of CRC has increased gradually, and it is the third most common tumor in the world [1]. In PR China, the incidence and mortality of CRC are on the rise, ranking it third in all malignant tumors [2,3], seriously endangering people's health. At present, the treatment of CRC is mainly surgery and postoperative radiotherapy, and chemotherapy. However, these treatments may cause adverse reactions and complications, and reduce the quality of life of patients [4,5]. Although various surgical resection methods have been developed to deal with CRC, the average 5-year survival rate of patients with

different stages is less than 50% and about 30% of patients still have recurrence after surgery [6]. Recurrence presents a challenge to physicians when treating patients with CRC. The mechanism of recurrence is still unclear and therapeutic options are limited, so exploring mechanisms on how to prevent tumor recurrence is an important direction to improve the postoperative survival rate and quality of life of patients.

Krüppel-like factor 2 (KLF2) is one of a family of transcription factors that contain conserved zinc finger domains. Many primary studies have demonstrated that KLF2 plays an important role in lung development, erythropoiesis, hemodynamic regulation, lymphocyte development, and T-cell survival [7,8]. KLF2 is especially intriguing in the context of cancer prevention.

Recent studies have shown that KLF2 is downregulated in lung cancer [9], gastric cancer [10], and breast cancer [11] and high expression of KLF2 can inhibit the further development of CRC [1]. Other studies have shown that KLF2 inhibits cell proliferation, migration, and metastasis while promoting cell apoptosis [12,13]. Similarly, Qin *et al* [14] demonstrated low KLF2 expression in oral squamous cell carcinoma (OSCC) and that KLF2 overexpression could markedly reduce cell proliferation, migration, and invasion in OSCC. In line with these studies, another report showed that miR-572 promotes non-small lung cancer development by targeting KLF2 [15].

Ferroptosis is a new oxidative and nonapoptotic programmed cell death mechanism discovered in recent years, which is different from apoptosis, autophagy, necrosis, and other cell death modes in morphology, genetics, and molecular biology [16,17], and is characterized by lipid peroxidation and iron accumulation [18]. The sensitivity of cells to ferroptosis is mainly regulated by glutathione peroxidase 4 (GPX4) [19]. GPX4 is a selenocysteine enzyme that prevents ferroptosis by reducing toxic lipid reactive oxygen species (ROS) to nontoxic lipid alcohols in the presence of glutathione. GPX4, as a key enzyme in lipid ROS clearance, is essential for maintaining cell activity. Studies have found that KLF2 can bind to GPX4, a key protective protein of ferroptosis, and promote GPX4 transcriptional activation, thereby promoting the development of ferroptosis [20]. However, the mechanism of KLF2 regulation of ferroptosis in CRC remains unclear.

The primary objective of our study was to explore the relationship between KLF2 and ferroptosis and the molecular mechanism by which KLF2 regulates ferroptosis in CRC. Our data show that KLF2 expression was low in CRC, and was associated with poor prognosis in CRC patients. Furthermore, we found that overexpression of KLF2 could inhibit the invasion, migration, epithelial–mesenchymal transition (EMT), and tumor growth of CRC cells. Mechanistically, we observed that KLF2 induced ferroptosis in CRC cells via GPX4 by inhibiting the PI3K/AKT signaling pathway, thereby suppressing the invasion, migration, and EMT of CRC cells.

Materials and methods

Clinical samples

In this study, 25 primary CRC tissues and the matched corresponding adjacent normal colorectal tissues were collected from stage III/IV CRC patients who had undergone tumor resection without neoadjuvant

therapy at the Department of Gastrointestinal Surgery, First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, PR China.

The studies on patient samples and data were conducted in accordance with the Declaration of Helsinki. All participants provided written consent for the anonymous use of their data for research purposes, and the study was approved by the Ethics Committee of our institution. Colorectal tissue samples were collected, liquid nitrogen-frozen, and stored at -80°C until needed.

Bioinformatics

Gene expression data of *KLF2* in 152 CRC patients and healthy controls were obtained from The Cancer Genome Atlas (TCGA) database (portal.gdc.cancer.gov). The correlation of *KLF2* with different stages of CRC was obtained from the Gene Expression Profiling Interactive Analysis (GEPIA) database. The survival analysis of *KLF2* in CRC was predicted using the TCGA database (TCGA portal, <http://tumorsurvival.org/index.html>).

Cell culture

All cell lines used in this study were purchased from the American Type Culture Collection and validated by short tandem repeat analysis. HCoEpiC, FHC, and RKO cells were cultured in DMEM medium (Gibco, C11995500BT, Tianhe, Guangzhou, China) with 10% fetal bovine serum (FBS) (ExCell Bio, FSD500, Tianhe, Guangzhou, China). SW480 cells were cultured in RPMI-1640 medium (Gibco, C11875500BT) with 10% FBS (ExCell Bio, FSD500). All cells were maintained at 37°C in an incubator with 5% CO_2 .

Cell transfection

For target gene overexpression experiments, SW480 and RKO cells were transfected with pcDNA3.1 empty vector, pcDNA3.1/*KLF2*, pcDNA3.1/vector-Fer1, and pcDNA3.1/*KLF2*+Fer1 recombinant plasmids. The plasmids were designed and generated by GenScript Co. Ltd. (Nanjing, PR China). Transfections of CRC cell lines were conducted using a Lipofectamine[®] 2000 Reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. Cells were harvested at 48 h after transfection for assays.

Immunohistochemistry

KLF2 protein expression was assessed using immunohistochemistry (IHC) in both CRC and adjacent normal tissues. The tissue samples were fixed in 4%

paraformaldehyde, embedded in paraffin, and cut into 5–8 μm thick sections. The sections were deparaffinized, rehydrated, and heated using a pressure cooker for 2.5 min in EDTA for antigen retrieval. Endogenous peroxidase activity was inactivated by adding an endogenous peroxidase blocker (OriGene, Beijing, PR China) for 15 min at room temperature. Sections were then incubated with the KLF2 antibody (Abcam, ab194486, Cambridge, UK) overnight at 4 °C. Next, the sections were washed three times with 1 $\times$ PBS and incubated with secondary antibody (OriGene) at room temperature for 20 min. After treatment with diaminobenzidine (DAB; Sigma, Darmstadt, Germany) solution, sections were counterstained with 0.5% hematoxylin, dehydrated with graded concentrations of ethanol for 3 min each, mounted on slides, and observed under a microscope (Olympus IX73, Life Science Solutions, MA, USA).

RT-PCR

KLF2 expression in CRC tissue samples was detected by quantitative real-time PCR (RT-PCR). TRIzol reagent (Invitrogen) was used to extract the RNA following the manufacturer's instructions. PrimerScript RT Master Mix (Takara, Dalian, PR China) was used to reverse transcribe 1 μg of total RNA in a final volume of 20 μl . The primer sequences used for PCR amplification are provided in Table 1. KLF2 gene expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method and was normalized to β -actin as an endogenous control.

Western blotting

Cells were lysed by RIPA (radioimmunoprecipitation assay) buffer supplemented with 1 $\times$ proteinase inhibitor cocktail (MCE, HY-K0010, MedChemExpress, NJ, USA). Protein quantification was determined by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, 23225, Waltham, MA, USA). Approximately 25 μg of protein in each lane was loaded onto 10% SDS-PAGE gels and then transferred to a polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% milk in 1 $\times$ PBST (phosphate-buffered saline with Tween 20) for 1 h at room temperature. Subsequently, the membranes were incubated with primary antibodies [anti-KLF2

(1:2,000, cat. no. MA5-26825, Invitrogen), anti-GPX4 (1:1,000, cat. no. MA5-32827, Invitrogen), anti-p-AKT (1:1,000, cat. no. 44-621G, Invitrogen), anti-p-PI3K (1:1,000, cat. no. PA5-17387, Invitrogen), anti-AKT (1:1,000, cat. no. MA5-14916, Invitrogen), anti-PI3K (1:1,000, cat. no. MA1-74183, Invitrogen), anti-E-cadherin (1:1,000, cat. no. 14-3249-82, Invitrogen), anti-N-cadherin (1:500, cat. no. MA1-91128, Invitrogen), anti-vimentin (1:1,000, cat. no. MA5-11883, Invitrogen), and anti- β -actin (1:5,000, cat. no. MA1-140, Invitrogen)], overnight at 4 °C. Next morning, after washing the membranes four times with TBS-T, corresponding secondary antibodies were incubated for 1 h at room temperature. Finally, ECL detection reagent (Cytiva RPN2232, Fisher Scientific, MA, USA) was used to detect the target protein bands using ImageQuant LAS 4000 System. The blots were analyzed using ImageJ software.

Transwell migration and invasion assays

Migration and invasion experiments were performed using Boyden chambers consisting of transwell membrane filter inserts (Corning Costar, cat. no. 3422, Life Sciences, New York, USA) covered in Matrigel. In brief, 5×10^4 SW480 and RKO transfected cells were seeded into each 24-well transwell chamber (8- μm pore size) for the migration assay, or into chambers coated with Matrigel for the invasion assay with serum-free medium in the upper chamber and medium with 10% FBS was added to the lower chamber following 24 h of incubation, respectively. Cells that did not penetrate the filter were wiped off with a cotton swab, while cells on the lower surface of the filter were stained with 0.4% crystal violet. The numbers of migrating or invading cells were observed under an inverted microscope (Olympus, Tokyo, Japan) from five fields in a single chamber and counted using ImageJ software. For migration assay, images were taken at 0 and 24 h. The relative change of the area at 24 h was normalized to the area at 0 h using the area at 0 h as the frame of reference.

Xenograft tumor model

Six- to seven-week-old female nude mice were purchased from SJA Laboratory Animal Co., Ltd. (Changsha, Hunan, PR China). The animal protocol was approved by the animal ethics committee of our institution. A total of 10 nude mice were subcutaneously injected into the third pair of mammary gland fad pads with SW480 cells (2×10^6 cells) transfected with pcDNA3.1-vector or pcDNA3.1-KLF2 ($n = 5$ mice each). Tumor size was measured every 4 days, and the tumor volume was calculated using the

Table 1. Primer sequences used in the study

Primer name	Sequence (5'–3')
KLF2 (forward)	CTGCACATGAAACGGCAGCAT
KLF2 (reverse)	CAGTCACAGTTTGGGAGGGG
β -Actin (forward)	CACCCACTCTCCACCT TTG
β -Actin (reverse)	CCACCACCCTGTTGCTGTAG

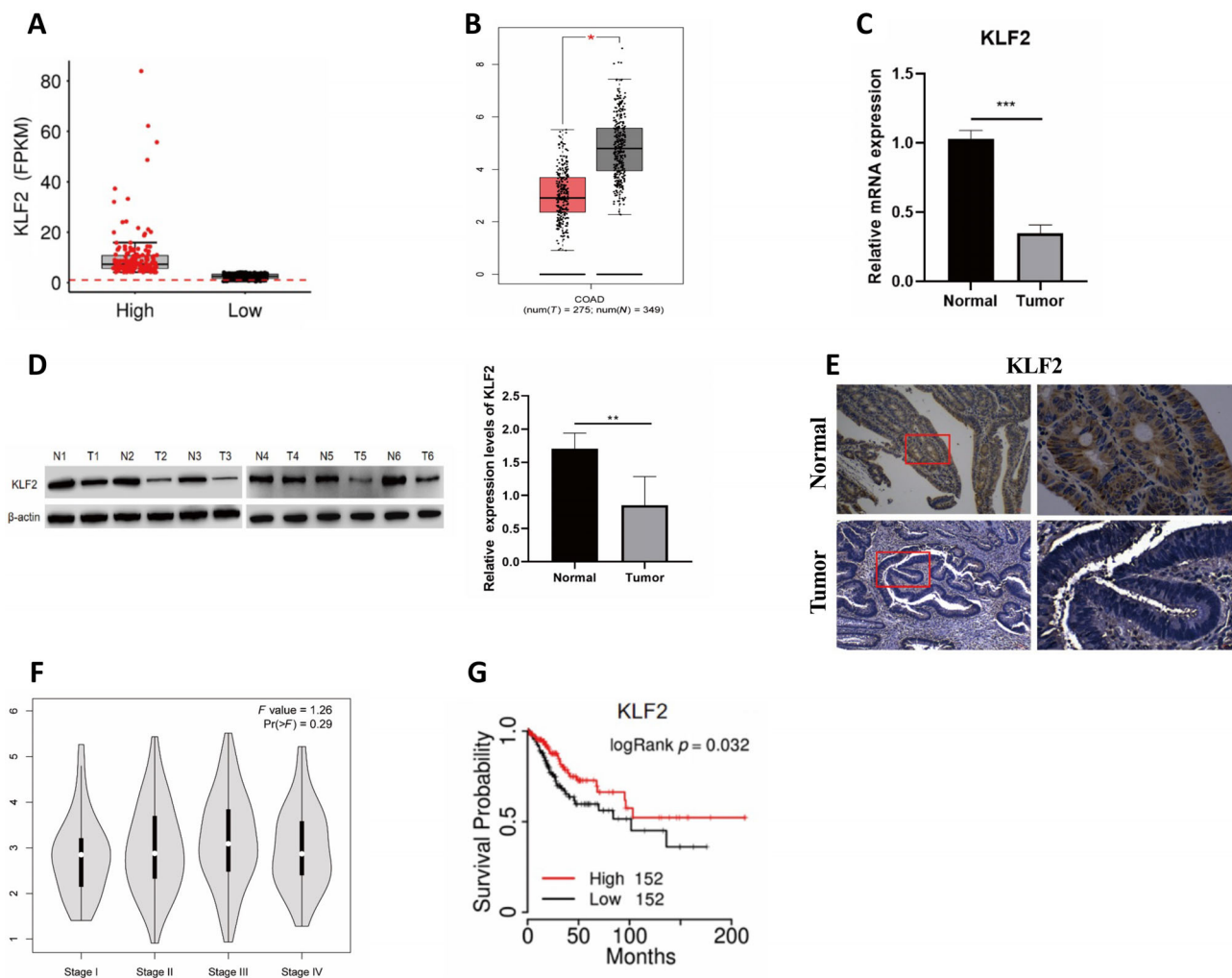


Figure 1. KLF2 Expression in CRC tissues and its correlation with CRC prognosis. (A and B) Expression of *KLF2* in CRC patients utilizing the TCGA and GEPIA databases. (C) *KLF2* expression was detected by RT-PCR in CRC and adjacent normal tissue samples. (D) *KLF2* expression was detected by western blot analysis in normal and CRC tissue samples. β -Actin was used as an internal control. N, normal; T, tumor. (E) IHC analysis of *KLF2* expression in CRC and normal human tissue samples. Scale bar: 100 μ m. (F) The correlation of *KLF2* expression with different stages of CRC was analyzed using the GEPIA database. (G) The correlation of *KLF2* expression with CRC prognosis was determined using Kaplan–Meier analysis. *** $P < 0.001$.

following equation: $\text{volume} = (\text{length} \times \text{width}^2)/2$. After 24 days of injections, all the mice were euthanized, and the tumors were weighted.

Statistical analysis

Data were analyzed using GraphPad Prism software and all experiments were repeated at least three times. Data were shown as mean $\pm$ SD. Significant differences between the two groups were calculated using Student's *t* test. Multiple comparisons were analyzed by one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Results

KLF2 expression is low in human CRC tissues and correlates with poor prognosis

In order to explore the role of *KLF2* in CRC, we first analyzed the expression of *KLF2* in CRC patients utilizing the TCGA and GEPIA databases and found that the expression of *KLF2* was low in CRC patients (Figure 1A,B). To validate the bioinformatics results, we used RT-PCR to detect the level of *KLF2* in 50 paired CRC tissues and adjacent normal tissues, showing that *KLF2* expression was low in CRC tissues while it was

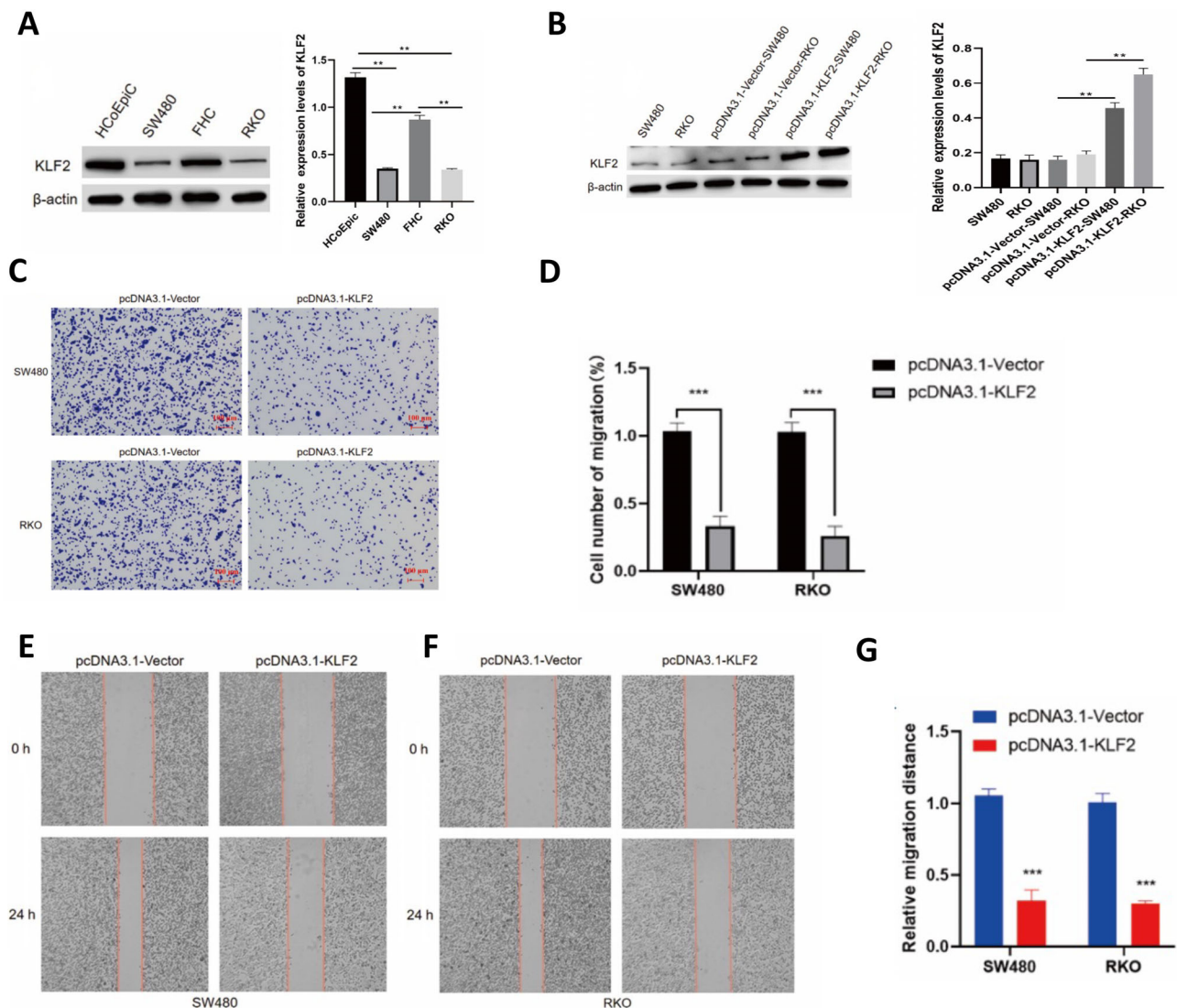


Figure 2. KLF2 overexpression suppresses the metastasis of CRC cells. (A) Expression of KLF2 in HCoEpiC, FHC, SW480, and RKO cell lines was detected by western blot assay. (B) Western blotting assessment of KLF2 overexpression in SW480 and RKO cell lines. (C) The invasion activity of transfected SW480 and RKO cells was detected by transwell assay. Scale bar: 100 μ m. (D) Statistical representation of the transwell assay shown in (C). (E) The migration activity of transfected SW480 cells was detected by wound healing assay. Scale bar: 100 μ m. (F) The migration activity of transfected RKO cells was detected by wound healing assay. Scale bar: 100 μ m. (G) Statistical representation of wound healing assays shown in (E) and (F). * $P < 0.05$; ** $P < 0.01$.

high in adjacent normal tissues (Figure 1C). In addition, we also detected KLF2 expression in CRC human tissues by western blot and found that KLF2 expression was low in CRC samples compared to the adjacent normal tissues (Figure 1D). These results were further validated by IHC analysis, and KLF2 expression was prominently low in CRC tissues samples while high in adjacent normal tissues (Figure 1E), further supporting the above results. In addition, we also analyzed the correlation of KLF2 expression with different stages of

CRC through the GEPIA database and found that there was no significant difference in the expression of KLF2 from stage I to IV (Figure 1F). Furthermore, we used Kaplan–Meier analysis to evaluate the effects of KLF2 expression on survival probability. Upon analysis, we found that KLF2 was correlated with a poor prognosis of CRC patients ($p = 0.032$) (Figure 1G). Taken together, these data indicate that KLF2 expression is low in CRC patients, and its expression is correlated with poor CRC prognosis.

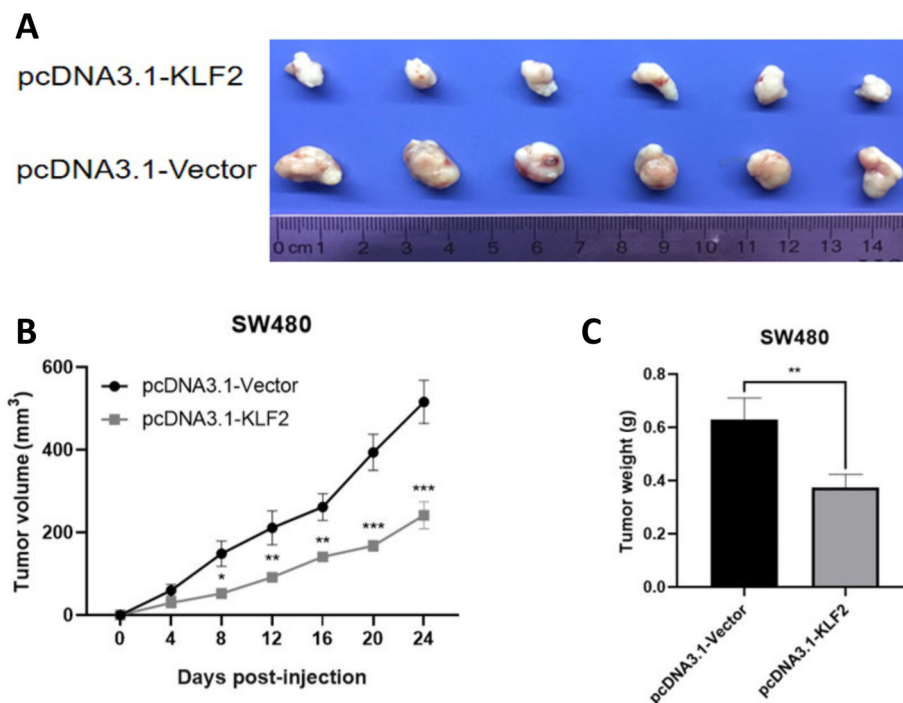


Figure 3. KLF2 overexpression suppresses tumor growth in the xenograft mouse model. The *in vivo* assays were performed using the stable KLF2 overexpressed SW480 cells. (A) Representative images of tumors collected from mice injected with SW480 cells transfected with empty vector or KLF2 plasmid. (B) The tumor volume of xenograft tumors in nude mice was measured every fourth day. (C) The tumor weights were measured on the 24th day after injection. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

KLF2 overexpression inhibits the invasion and migration of CRC cells

We next investigated the functional role of KLF2 in CRC progression. We first examined the expression of KLF2 in several normal colorectal epithelial and mucosal cells and CRC cell lines and found that KLF2 was highly expressed in normal colorectal cell lines such as HCoEpiC and FHC (Figure 2A). However, the expression of KLF2 was significantly lower in CRC cell lines SW480 and RKO (Figure 2A), which is consistent with above results. To explore the functional role of KLF2 in CRC, we constructed a KLF2 plasmid which was then transfected into CRC cells SW480 and RKO to overexpress KLF2. Western blot analysis revealed that KLF2 was highly expressed in SW480, and RKO cells transfected with KLF2 plasmid in contrast to nontransfected and/or cells transfected with empty vector (Figure 2B). After successfully overexpressing KLF2, we then evaluated its effect on CRC invasion and migration capabilities. Our data demonstrated that KLF2 overexpression resulted in decreased cell migration and invasion of SW480 and RKO cells (Figure 2C–G). These results demonstrate

that KLF2 overexpression could inhibit the invasion and migration of CRC cells.

KLF2 overexpression prevents tumor growth *in vivo*

To confirm the role of KLF2 in CRC progression *in vivo*, xenograft tumor experiments were performed. After inoculation of SW480 cells transfected with empty vector or KLF2 plasmid into nude mice, we discovered that tumor growth was slower in nude mice injected with KLF2 overexpression cells than in the control group (Figure 3A). In addition, we also analyzed the volume and weight of xenograft tumors in nude mice, which showed that overexpression of KLF2 could inhibit the tumor volume and weight (Figure 3B,C). These results suggest that overexpression of KLF2 could suppress CRC development *in vivo*.

KLF2 overexpression suppresses CRC development by inducing ferroptosis through GPX4

It has been reported that KLF2 can bind to GPX4, a key protective protein of ferroptosis, and promotes GPX4 transcriptional activation [20], which suggests

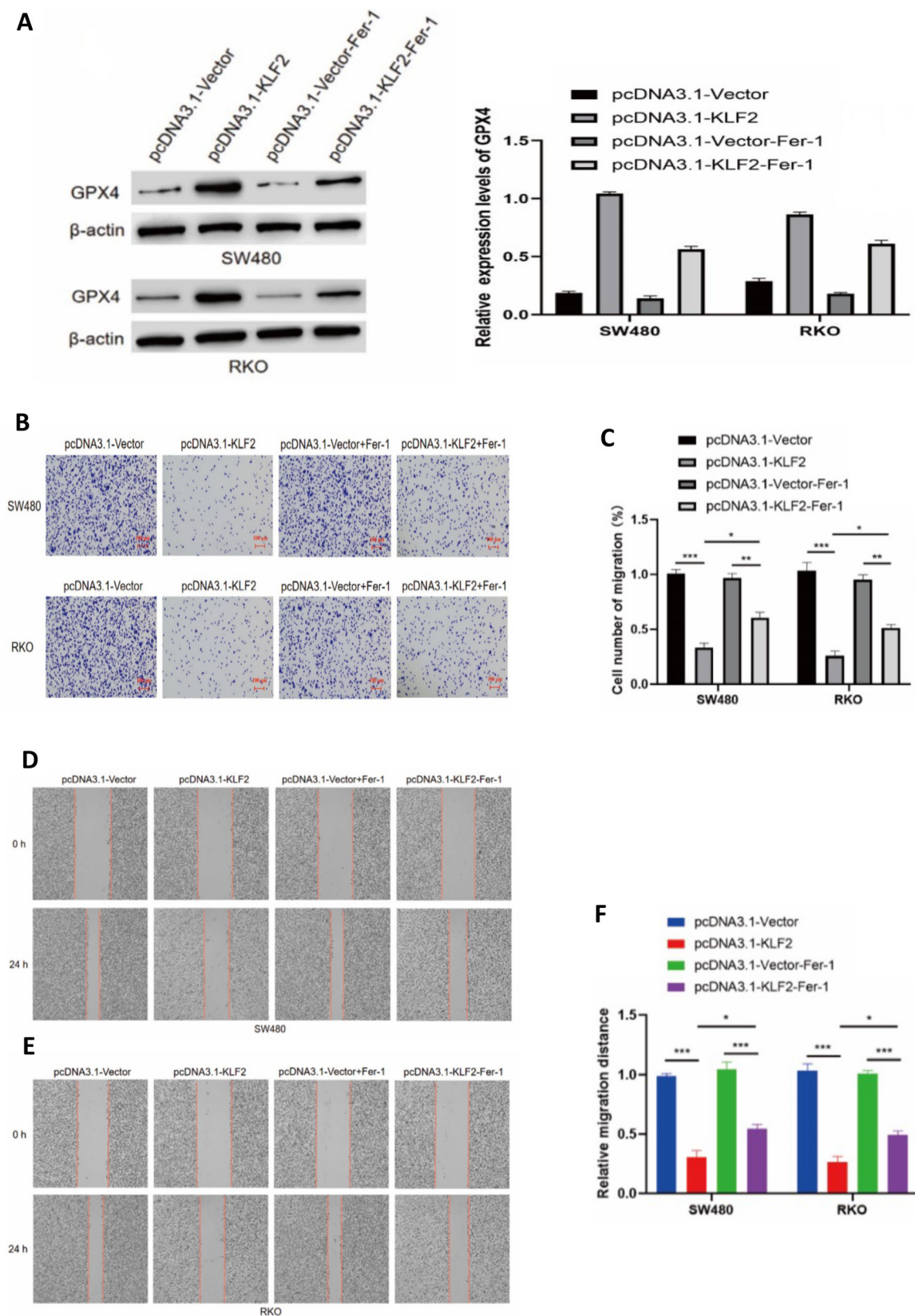


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that the anticancer effect of KLF2 may be related to the upregulation of GPX4 to activate ferroptosis in tumor cells. Therefore, we next evaluated the effect of KLF2 overexpression on the regulation of GPX4 expression. Our results demonstrated that KLF2 overexpression significantly promoted GPX4 expression compared with the control (Figure 4A). However, GPX4 expression significantly reduced when KLF2 overexpressed cells were treated with ferrostatin-1 (Fer-1), a ferroptosis inhibitor, indicating that KLF2 controls ferroptosis via regulating GPX4 expression. We then hypothesized that KLF2 might regulate the metastasis of CRC cells through ferroptosis. We found that inhibiting ferroptosis significantly reduced the invasion and migration capabilities of CRC cells (Figure 4B–F). Collectively, these results indicate that KLF2 inhibits CRC cell progression by inducing ferroptosis via GPX4.

KLF2 triggers ferroptosis in CRC cells by inhibiting the PI3K/AKT pathway

Previously, it has been found that KLF2 could inhibit the PI3K/AKT signaling pathway in gastric cancer [21,22]. We therefore speculated that KLF2 might trigger ferroptosis by inhibiting the PI3K/AKT pathway. The data showed that overexpression of KLF2 significantly inhibited the phosphorylation of AKT and PI3K, suggesting that KLF2 can inhibit the PI3K/AKT signaling pathway in CRC cells (Figure 5A,B). Notably, inhibiting ferroptosis prominently reversed this phenomenon even in KLF2 overexpressing cells (Figure 5A,B). In addition, we found that KLF2 overexpression could promote GPX4 expression, but GPX4 expression could not be well recovered when SW480 and RKO cells were co-transfected with KLF2 and PI3K plasmids (Figure 5C). These results suggest that KLF2 induces ferroptosis in CRC cells by inhibiting the PI3K/AKT signaling pathway.

KLF2 hinders EMT in CRC cells by preventing PI3K/AKT activation

EMT is an important biological process for epithelial-derived malignant tumor cells to acquire the ability of

migration and invasion [23,24]. To explore the relationship between KLF2 and EMT, we first evaluated the expression of EMT biomarkers. Overexpression of KLF2 significantly promoted the expression of E-cadherin, and significantly inhibited the expression of N-cadherin and vimentin, suggesting that KLF2 could inhibit EMT (Figure 6A,B). In order to further explore the mechanism of KLF2 inhibiting EMT, we overexpressed PI3K together with KLF2 and found that PI3K activator could inhibit the expression of E-cadherin and significantly promote the expression of N-cadherin and vimentin (Figure 6A,B). Taken together, these results indicate that KLF2 inhibits EMT in CRC cells by inhibiting the PI3K/AKT signaling pathway.

Discussion

In the current study, we identified KLF2 as one of the regulators preventing CRC metastasis. Mechanistically, KLF2 overexpression promotes GPX4 transcriptional activation thereby inducing ferroptosis and, in turn, inhibiting EMT and metastasis of CRC.

Members of the KLF family are zinc finger proteins that bind to DNA transcription regions, thereby playing a crucial role in transcriptional regulation [25]. Studies have revealed that KLF2 plays an essential role in various cancer types. KLF2 expression has been shown to be downregulated in many human cancers, including ovarian and lung cancer [26,27]. Another study showed that KLF2 expression was significantly lower in both gastric cancer human tissue specimens and cell lines, and that it was negatively correlated with patient survival [28]. Although research suggests that KLF2 inhibits the malignant progression of CRC [1,29], the exact mechanism by which KLF2 influences CRC migration, invasion, and metastasis is not well understood, especially the mechanisms on the relationship between KLF2 and ferroptosis in CRC. In the present study, we found that KLF2 expression was low in CRC, and this low expression of KLF2 was associated with poor prognosis. *In vitro* experiments confirmed that overexpressing KLF2 inhibited the invasion and migration of CRC cells. Furthermore, *in vivo* data revealed that overexpressing

Figure 4. Overexpression of KLF2 inhibits CRC metastasis by inducing ferroptosis via GPX4. (A) Transfected cells were treated with/without ferroptosis inhibitor Fer-1 (2 μ M) for 24 h and then GPX4 expression was detected by western blot assay. (B) The invasion activity of transfected SW480 and RKO cells was detected by transwell assay. Scale bar: 100 μ m. (C) Statistical representation of transwell assay shown in (B). (D) The migration activity of transfected SW480 cells was detected by wound healing assay. Scale bar: 100 μ m. (E) The migration activity of transfected RKO cells was detected by wound healing assay. Scale bar: 100 μ m. (F) Statistical representation of transwell assay shown in (D) and (E). * P < 0.05; ** P < 0.01; *** P < 0.001.

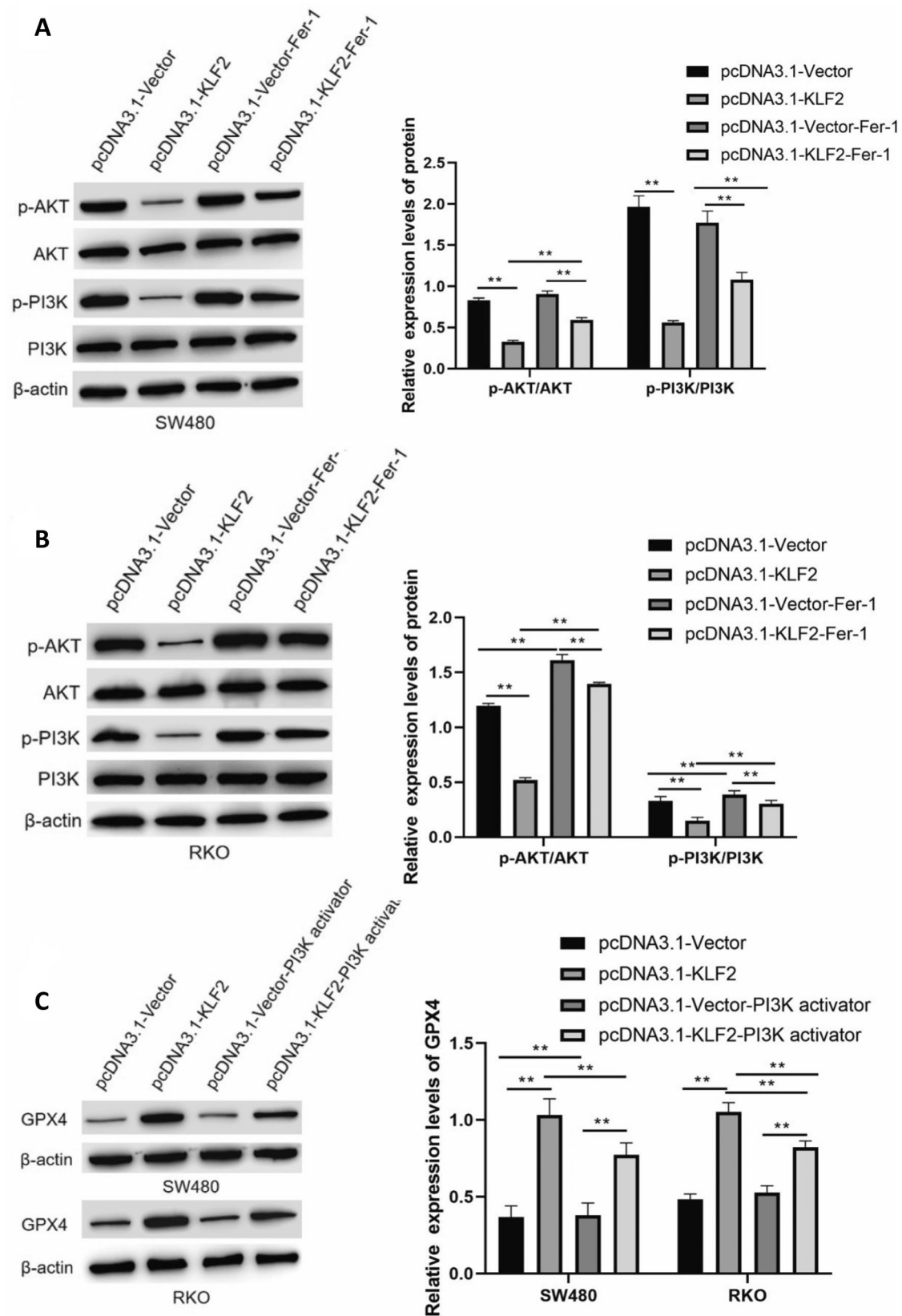


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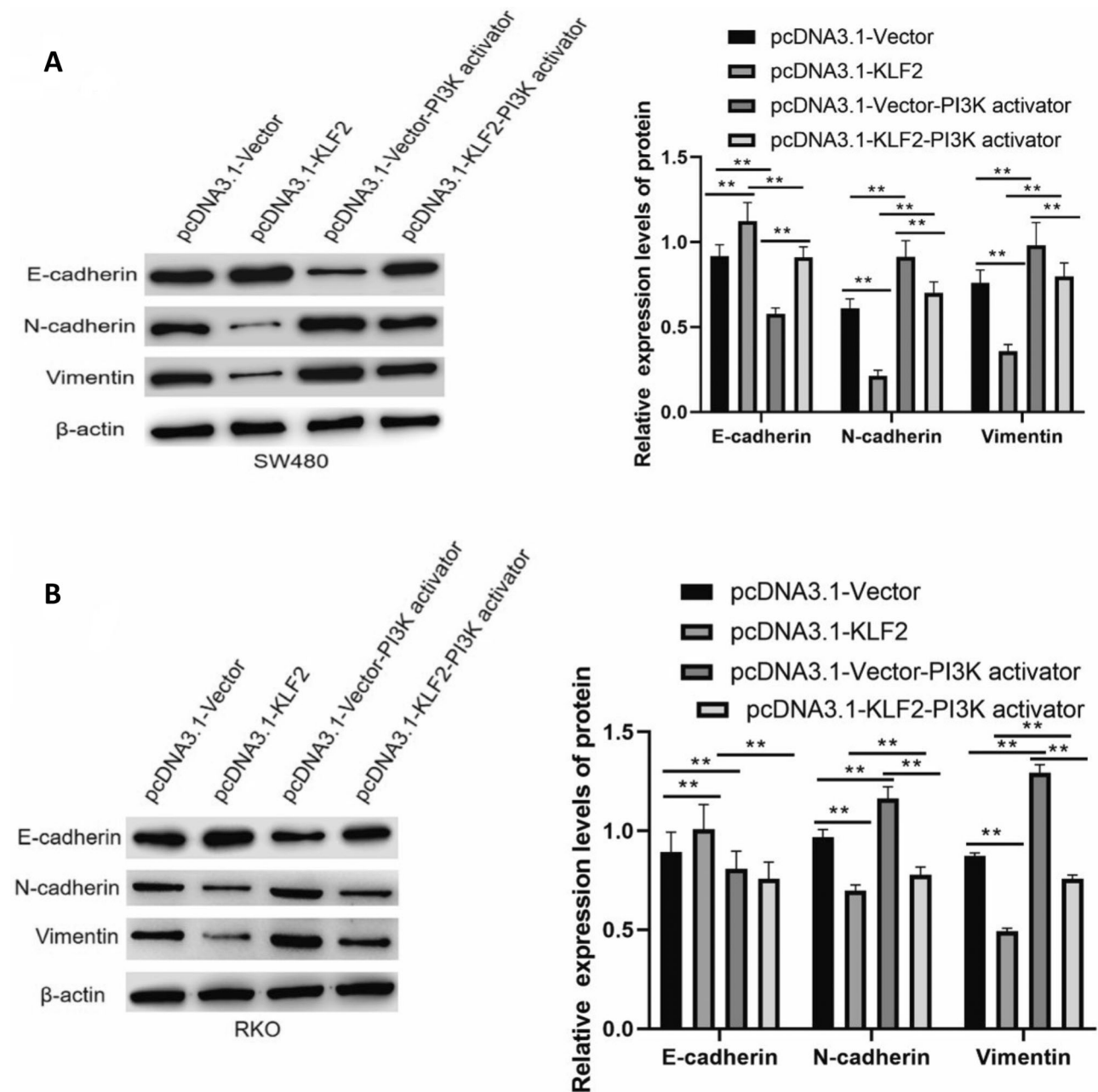


Figure 6. KLF2 represses EMT by inhibiting the PI3K/AKT signaling pathway. (A) EMT markers (E-cadherin, N-cadherin, and vimentin) were detected by western blot in transfected SW480 cells. (B) The EMT markers (E-cadherin, N-cadherin, and vimentin) were detected by western blot in transfected RKO cells. * $P < 0.05$; ** $P < 0.01$.

Figure 5. The PI3K/AKT signaling pathway is involved in KLF2-mediated ferroptosis. (A) SW480 transfected cells were treated with/without ferroptosis inhibitor Fer-1 (2 μM) for 24 h and then PI3K/AKT signaling pathway markers (p-AKT, AKT, p-PI3K, and PI3K) were detected by western blot assay. (B) RKO transfected cells were treated with/without ferroptosis inhibitor Fer-1 (2 μM) for 24 h and then PI3K/AKT signaling pathway markers (p-AKT, AKT, p-PI3K, and PI3K) were detected by western blot assay. (C) GPX4 expression was detected by western blot in transfected SW480 and RKO cells. * $P < 0.05$; ** $P < 0.01$.

KLF2 significantly suppressed CRC tumor growth. Our results are in agreement with the aforementioned studies.

Ferroptosis is a new type of cell death that is characterized by lipid peroxidation and iron accumulation [17,18]. The occurrence of ferroptosis involves the expression and regulation of many genes and different signaling pathways, resulting in a series of complex biochemical reactions. Ferroptosis has been shown to play a crucial role in tumor metastasis. In one study, lymphoid tissue protected tumor cells and promoted melanoma metastasis by inhibiting ferroptosis [30]. Moreover, ferroptosis induction inhibits tumor brain metastasis in a spontaneous mouse model of HER2-positive breast cancer [31]. Emerging evidence has shown that GPX4 promotes cancer behavior by controlling ferroptosis. Previous research has demonstrated that GPX4 is highly expressed in metastatic cancers and is closely associated with tumor development [19,32]. Lu *et al* found that GPX4-mediated ferroptosis regulation by KLF2 prevents renal cell carcinoma cell migration and invasion [20]. Consistent with these studies, our results demonstrate that KLF2 overexpression significantly promotes GPX4 expression. Moreover, when KLF2 overexpressed cells were treated with Fer-1, a ferroptosis inhibitor, GPX4 expression was significantly reduced, indicating that KLF2 controls ferroptosis by regulating GPX4 expression.

To explore the underlying molecular mechanisms of KLF2 involvement in regulating ferroptosis, we studied the PI3K/AKT pathway. Previously, it has been shown that the PI3K/AKT signaling pathway regulates cancer cell proliferation, growth, invasion, metabolism, and motility [33–35]. A growing body of research indicates that this pathway is also involved in CRC progression and its activation is reported in 60–70% of CRC cases [36]. In addition, it has been found that KLF2 can activate and thereby regulate the PI3K/AKT pathway in gastric cancer [21]. However, it is not clear whether KLF2 regulates this pathway in CRC and whether KLF2-induced ferroptosis is a PI3K/AKT-dependent process. In this research, we constructed KLF2 overexpression and found that overexpressing KLF2 significantly inhibited the PI3K/AKT pathway in CRC which is in line with the results of previous reports [21,28]. Mechanistically, treatment of KLF2 overexpressing cells with a ferroptosis inhibitor downregulated PI3K/AKT pathway related proteins in contrast to the controls. Moreover, activating this pathway resulted in reduced GPX4 expression. Our data suggest that KLF2 induces ferroptosis via GPX4 by deactivating the PI3K/AKT pathway in CRC.

EMT is another important process that contributes to colon cancer metastasis [37]. It occurs during tumor growth and confers invasive and metastatic properties on

cancer cells, and is closely related to tumor recurrence [38,39]. EMT is characterized by reduced expression of cell adhesion molecule E-cadherin, with increased expression of N-cadherin, high expression of vimentin, and transition to mesenchymal cell morphology [23,24]. Interestingly, it has been demonstrated that activation of the PI3K/AKT signaling pathway is a key regulatory mechanism controlling EMT in a variety of cancers [34,40]. An abnormally activated PI3K/AKT pathway can suppress E-cadherin expression, and upregulate N-cadherin and vimentin expression, thus promoting tumor cell EMT and metastasis progression [41,42]. In accordance with these studies, we also found that enforced KLF2 expression upregulated E-cadherin expression, while downregulating N-cadherin and vimentin expression. However, activating PI3K signaling in these cells prominently reverses this phenomenon, thus promoting EMT in CRC cells.

Our investigation had some limitations. In this study, we did not explore the downstream signaling axis of the PI3K/AKT pathway, which needs further exploration. Moreover, we only studied the relationship of KLF2 with ferroptosis but not with oxidative stress and lipid metabolism as ferroptosis is reportedly mediated by these processes, which warrants further research [43,44].

The results from the present study underpin a novel role for KLF2 in CRC progression. We found that KLF2 functions as a tumor suppressor in CRC by inducing ferroptosis possibly via mediating the PI3K/AKT signaling axis, thereby inhibiting the invasion, migration, and EMT of CRC cells. These results indicate that KLF2 might be an effective therapeutic target for the treatment of CRC.

Author contributions statement

JL and WQL designed the study. JL and JLI conducted the experiments. YMC performed the data analyses. JL wrote the draft manuscript. WQL critically revised the manuscript. All authors read and approved the final manuscript.

Ethics statement

All experiments and procedures were conducted in compliance with the ethical principles of First Affiliated Hospital of Guangzhou University of Chinese Medicine and received ethical approval from the Animal Ethics Committee of Guangzhou University of Chinese Medicine.

Informed consent

All patients agreed and signed informed consent.

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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