KLF6 Suppresses Metastasis of Clear Cell Renal Cell Carcinoma via Transcriptional Repression of E2F1

Yu Gao1,2, Hongzhao Li1,2, Xin Ma1,2, Yang Fan1,2, Dong Ni1,2, Yu Zhang1,2, Qingbo Huang1,2, Kan Liu1,2, Xintao Li1,2, Lei Wang1,2, Liangyou Gu1,2, Yuanxin Yao1,2, Qing Ai1,2, Qingshan Du1,2, Erlin Song3, and Xu Zhang1,2

Abstract

The transcription factor KLF6 has an essential role in the development and metastasis of multiple human cancers. Paradoxically, KLF6 expression was found to be attenuated in primary metastatic clear cell renal cell carcinoma (ccRCC), such that it is unclear how KLF6 affects malignant progression in this setting. In this study, we demonstrate that KLF6 attenuation in renal cells is sufficient to promote E2F1-mediated epithelial-mesenchymal transition and metastatic prowess. In a mouse xenograft model of human ccRCC, silencing KLF6 increased tumor cell proliferation and malignant character, whereas E2F1 silencing reversed these properties. These effects were corroborated in a metastatic model system, where we observed a greater number of pulmonary metastatic lesions formed by ccRCC cells where KLF6 was silenced and E2F1 enforced. Analysis of clinical specimens of ccRCC revealed that low levels of KLF6 and high levels of E2F1 correlated closely with ccRCC development. Overall, our results established the significance of activating the KLF6–E2F1 axis in aggressive ccRCC, defining a novel critical signaling mechanism that drives human ccRCC invasion and metastasis. Cancer Res; 77(2); 330–42. ©2016 AACR.

Introduction

Renal cell carcinoma (RCC) is the second leading cause of death in patients with urological malignant tumors and accounts for 4.2% of all adult malignancies (1). Clear cell RCC (ccRCC) is the most commonly observed of the 5 major subtypes of RCCs: the clear cell, papillary, chromophobe, collecting duct, and the unclassified types. Metastasis occurs in the later stages and predicts very poor clinical outcome (2). The 5-year survival rate of metastatic RCC is only 10% because of resistance to chemotherapy and radiation therapy (3). Cytokine therapy with IFNα and IL2 was introduced in the past decades, but this approach does not function well in late-stage disease management (4). Molecular targeting drugs, such as sunitinib and temsirolimus, have been recently approved and widely used for advanced metastatic and radiation therapy (3). Cytokine therapy with IFNα and IL2 remained poor (5). Thus, the characterization of genetic alterations underlying the key steps in the metastatic progression of ccRCC is crucial.

The Krüppel-like factor 6 (KLF6) gene is a tumor suppressor that is deregulated and inactivated by gene loss or somatic mutation in various cancers, such as colorectal cancer (6), prostate cancer (7), and glioblastoma (8). Our previous tissue profiling results indicated that KLF6 was significantly downregulated in metastatic specimens. Gene set enrichment analysis (GSEA) suggested that the aberrant expression of E2F1 is a key regulator of the entire metastatic process. Bioinformatics predicted the strong linkage between KLF6 attenuation and E2F1 activation in metastatic processes. E2F1 plays an important role in regulating development, differentiation, proliferation, cellular signal transduction, and apoptosis (9–12). Certain studies reported that E2F1 disrupts a range of pathways in numerous cancers and is closely correlated with clinical parameters (13–15). We had also previously identified that E2F1 is upregulated in ccRCC progression (16).

In this study, we illustrated that KLF6 downregulation promoted epithelial–mesenchymal transition (EMT) and metastatic transformation. On the basis of bioinformatics analysis, the present work focused on the combined effects of KLF6 and E2F1 in the migration and invasion of ccRCC cells in vitro and in vivo, as well as their mechanistic relationship and clinical relevance. A cooperative requirement between KLF6 and E2F1 in metastasis was suggested by the stimulation of EMT-related process, thereby causing enhanced tumor progression and distant organ colonization. Therefore, the functional significance of the KLF6 and E2F1 in the development and metastasis of ccRCC was investigated.

Materials and Methods

Ethics statement

Written informed consent was obtained from each individual who underwent nephrectomy prior to sample collection. The
KLF6 Transcriptionally Represses E2F1 in ccRCC Metastasis

study was approved by the Protection of Human Subjects Committee of the Chinese People’s Liberation Army (PLA) General Hospital. All animal studies were conducted according to the guidelines of the Institutional Animal Care and Use Committee of the Chinese PLA General Hospital.

Patients and clinical materials
The study cohort included 114 patients with ccRCC admitted to the Urology Department of the Chinese People’s Liberation Army (PLA) General Hospital from January 2005 to December 2010. Cancerous tissues were collected and immunohistochemically analyzed. All tissue samples were clinically and pathologically confirmed to be of the clear cell type and classified according to the 2011 Union for International Cancer Control TNM classification of malignant tumors. The nuclear grade was determined with the Fuhrman nuclear grading system.

RNA isolation and quantitative real-time PCR
Total RNA was extracted with the TRIzol reagent (ComWin Biotech). cDNA synthesis of regular genes was performed with a TransScript First-Strand cDNA Synthesis SuperMix Kit (TransGen Biotech). Afterward, quantitative real-time PCR (qRT-PCR) was performed with qPCR SYBR Green SuperMix (TransGen Biotech). Relative mRNA expressions were normalized to peptidylprolyl isomerase A (PPIA) with the 2−ΔΔCt method. The primer sequences used are listed in Supplementary Table S1.

Methylation-specific PCR
Genomic DNA from ccRCC tissues and cell lines was modified by sodium bisulfite treatment as previously described (17). CpG island spanning the KLF6 gene was found (Supplementary Fig. S1). Methylation-specific PCR (MSP) primers for KLF6 were designed and synthesized (Invitrogen). For detecting the methylated DNA (M) and unmethylated DNA (U), the primers were designed and the sequences used are listed in Supplementary Table S1.

Gene expression profile and GSEA analysis
Our previous study investigated the genes that promoted ccRCC metastasis in 4 primary metastatic and 5 nonmetastatic tumor samples. The U133 plus 2.0 Array was used to identify the differently expressed genes between the primary metastatic and nonmetastatic ccRCC samples. The microarray data were deposited with the Gene Expression Omnibus (GEO) as a microarray dataset (GSE47352). The dataset is publicly available and accessible through the NCBI GEO website. A heatmap was generated with part of the clustered genes on the data set (Supplementary Fig. S2). GSEA was applied to run the data for the microarray according to the manufacturer’s protocol. The C2cft (transcript factor target) calculation was applied and the top factors were normalized and conducted.

Immunohistochemistry
Immunohistochemistry (IHC) was performed on renal cancer samples and xenograft tumors. As previously described, staining intensity was scored 0 (negative), 1 (low), 2 (moderate), and 3 (high). Staining range was scored 0 (0% stained), 1 (1%–25% stained), 2 (26%–50% stained), and 3 (51%–100% stained). The final score was obtained by multiplying the intensity scores with staining range, and the results ranged from 0 to 9. For the final scores (intensity score × percentage score), scores less than 2 were considered as negative staining, 2–3 indicated weak staining, 4–6 was moderate staining, and >6 was strong staining (18). For the in vivo angiogenesis experiments, staining with CD34 was quantified on the basis of the intensity of microvessel staining in selected fields. The primary antibody sources are listed in Supplementary Table S1.

Cell line and cell culture
The 786-O, OS-RC-2, 769-P, and Caki-2 human ccRCC cell lines and human umbilical vein endothelial cells (HUVEC) were acquired from the National Platform of Experimental Cell Resources for Sci-Tech. SN12-PM6 cells were preserved in our laboratory, and all cell lines were obtained in 2015 and authenticated in September 2015 by the short tandem repeat analysis method. 786-O, OS-RC-2, 769-P, Caki-2, and SN12-PM6 cells were cultured in DMEM (HyClone), McCoy 5A medium (HyClone), DMEM/F12 (HyClone), and high glucose-DMEM with 10% FBS (Gibco), respectively.

Tube formation assay
Growth factor–reduced basement membrane extract (BME; Trevigen) was added to each well of a 96-well plate and prepared for 1 hour at 37°C. HUVECs cocultured with ccRCC cells from different groups for 48 hours were pre-incubated with serum-free DMEM for 1 hour and seeded into the BME-coated wells.

Western blot assay
Western blot assays were performed as previously described (19). The information on all primary antibodies is listed in Supplementary Table S1.

MTS assay
The cells in different groups were seeded into 96-well plates (1,000 cells per well). Absorbance was measured at 24, 48, 72, and 96 hours after seeding. All experiments were performed triplicate.

Cell migration and invasion assay
Twenty-four–well plates were applied with Boyden chambers containing Transwell membrane filter (8 μm). For the invasion assay, diluted Matrigel (BD Biosciences) was used. The migration and invasion assays were performed as previously described (20).

Wound-healing assay
Images of wounds were captured at 0 and 12 hours after scratching with a sterile 200 μl pipette. The coverage of the scratched area was measured at three different positions. All experiments were performed in triplicates.

Immunofluorescence
Cells of different groups were seeded and grown on glass slides at 24 hours prior to the proper experiment. After fixation with 4% paraformaldehyde/PBS for 15 minutes, cells were washed once with PBS and permeabilized with 0.5% Triton X-100. Coverslips were stained with the primary antibodies (KLF6 and E2F1) at 37°C for 1 hour, respectively. Nuclei were stained by application of 0.2 mg/mL DAPI.
siRNA and plasmid constructs

siRNAs against KLF6 and E2F1 were designed (Supplementary Table S1) and synthesized by GenePharma. A fragment of the KLF6 coding sequence was inserted into the lentiviral vector pLV-EGFP-(2A)Puro (Invogen Tech. Co.). XhoI and EcoRI were used to generate pLV-EGFP-KLF6. Lentivirus-encoding DNA was packaged as previously described (21). For knockdown of KLF6 and E2F1, siKLF6#2 and siE2F1 sequences were cloned into the pLVshRNA-EGFP-(2A)Puro vector. The primer sequences are listed in Supplementary Table S1. Extensions containing 2 restriction sites for KpnI and HindIII were designed in each primer to facilitate the cloning of this fragment into the pGL3-basic luciferase vector (Promega).

Bioinformatics of the E2F1 promoter

The human promoter sequence of E2F1 was retrieved from NCBI and defined as a putative promoter located in a 700-bp region spanning −700 to +1 bp relative to the transcriptional start site. The sequence was amplified from cell line 293T. Genomatix (www.genomatix.de/en/index.html) was used to identify transcription factor–binding sites for KLF6 within the human E2F1 promoter. The mutation construct was created by Genewiz. The mutation was confirmed by sequencing.

Luciferase assay

293T cells were plated at a density of 5 × 10^4 cells per well in 6-well plates prior to transfection. Transfection started when cell density reached approximately 60% confluency. The pGL3-E2F1 vector, a plasmid construct containing the Renilla luciferase reporter, and a pcDNA3.1 control or increasing doses (0.5, 1, and 2 μg) of the pcDNA3.1-KLF6 expression vector were cotransfected into 293 cells. After 48 hours, luciferase activity was measured with the Dual Luciferase Reporter Assay System (Promega). The value of firefly luciferase activity was normalized to that of the Renilla luciferase activity.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed on 808-O and Caki-2 cells with an antibody for KLF6, with anti-rabbit IgG as the negative control. The interaction with predicted promoters was detected with qRT-PCR. The detailed primer sequences are shown in Supplementary Table S1. The whole procedure was performed according to the protocol provided by the ChIP Assay Kit (Upstate EZ-ChIP, 17-371). Lysates were immunoprecipitated with an antibody specific for KLF6, normal rabbit IgG, and a positive control.

In vivo orthotopic xenograft tumor and metastatic model

The cells (1 × 10^6 in 0.1 mL of sterilized PBS) in respective groups were injected into the left kidneys of 4- to 5-week-old male nude mice (10 mice per group). Lesions, including the orthotopic xenograft tumors and lung metastatic nodules, were monitored in vivo with a molecular imaging system (NightOWL II LB983; ref. 22). The signal intensity of luc-labeled cells from lung tissues represented the amount of lung metastatic lesions. For the metastatic seeding model, approximately 5 × 10^3 786-O cells were injected into the tail vein of each nude mouse. For SN12-PMe established model (6 mice per group), 3 months later, the images were taken.

Statistical analysis

Statistical analysis was performed with SPSS 18.0 (SPSS Inc.). Normally distributed data were expressed as mean ± SD, and comparisons were performed with Student t test. Categorical data were analyzed with either Fisher exact test or the χ^2 test. Correlations of gene expression were determined with the Pearson coefficient. Kaplan–Meier and log-rank tests were used for the overall survival analysis.

Results

Downregulation of KLF6 in primary metastatic ccRCC tissues

To investigate the potential mechanism that promotes distant metastasis of ccRCC, mRNA expression profiling was performed with 4 primary metastatic and 5 primary nonmetastatic ccRCC tissue samples. The tissue profiles indicated that KLF6 mRNA levels are significantly reduced in primary metastatic ccRCC as compared with primary nonmetastatic ccRCC (Fig. 1A). Subsequent validation was performed on the differential gene expression between the two groups. Freshly collected specimens were tested after matching according to age and gender. Similar to the profiling data, the mRNA and protein expression levels of KLF6 also significantly declined upon ccRCC metastasis (Fig. 1B and C). As expected, KLF2 expression was attenuated in primary metastatic ccRCC. However, KLF9 expression was not consistent with the profiling results (Fig. 1C). Furthermore, IHC illustrated that the KLF6 protein was mainly localized in the nuclei of cancer cells from primary metastatic and primary nonmetastatic samples. As shown in Supplementary Table S2, a total of 114 ccRCC cases were analyzed. The nonmetastatic group included 79 cases, and the metastatic group contained 35 cases. In the nonmetastatic group, absent/weak immunostaining was observed in 33% (26 of 79) of the tumors, moderate staining in 29% (23 of 79) of the tumors, and strong staining in 38% (30 of 79) of the tumors. In comparison, absent/weak immunostaining was observed in 69% (24 of 35) of the tumors, moderate staining in 17% (6 of 35) of the tumors, and strong staining in 14% (5 of 35) of the tumors. KLF6 expression was significantly decreased in primary tumors when metastasis occurred.

In 12 pairs of comparative tissues, complete and partial methylation of the KLF6 promoter region were found in metastatic samples, whereas in 4 cell lines, the methylation status was also evaluated (Fig. 1E). Untargeted analysis of KLF6 expression levels with ccRCC metastasis, the clinicopathologic parameters and KLF6 mRNA levels with metastatic incidence in 87 ccRCC tissue specimens were analyzed with univariate and multivariable logistic regression models. Patient characteristics are provided in Supplementary Table S3. We ranked the relative KLF6 mRNA expression in sequence and selected the median (0.279%) of the entire dataset as the cutoff point between the high KLF6 group and the low KLF6 group (Fig. 1F). Univariate analysis indicated that a higher clinical T stage (OR, 6.359; 95% CI, 1.782–22.690; P = 0.004) and lower KLF6 expression (OR, 2.367; 95% CI, 1.239–5.426; P = 0.004) were correlated with the ccRCC progression related to other clinicopathologic parameters, including gender, age, body mass index (BMI), and the Fuhrman grade (Table 1).
Downregulation of KLF6 in primary metastatic ccRCC tissues. A, Heatmap illustration of KLF6, KLF2, and KLF9 transcript abundance determined by tissue microarrays between the primary metastatic and nonmetastatic ccRCC groups. Blue shading, low; red shading, high expression of mRNA. B, Protein expressions of KLF6 in clinical samples was determined by Western blot assay in patients with primary metastatic and nonmetastatic ccRCC. C, Differentiated mRNA levels of KLF6, KLF2, and KLF9 in primary metastatic and nonmetastatic ccRCC tissues. D, ccRCC tissues were elucidated by KLF6 antibody, being classified into the absent/weak, moderate, and strong groups. KLF6 protein exhibited distinguished expressions between primary metastatic and nonmetastatic ccRCC (P = 0.002). E, Representative MSP results in ccRCC tissues and cell lines. IVD, in vitro methylated DNA; M, methylated alleles; NL, normal blood lymphocyte DNA; U, unmethylated alleles, F, Of the enrolled 87 cases, the relative mRNA levels were tested in convenience of the logistic regression analysis. *P < 0.05; **P < 0.01; ***P < 0.001.
Table 1. Univariate and multivariable logistic regression models comparing patients developing metastatic disease with patients free from metastatic incidence

<table>
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<th>Variables</th>
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<th>Multivariate analysis</th>
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<td>Female</td>
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<tr>
<td>≥25</td>
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<tr>
<td>T1 + T2</td>
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<tr>
<td>T3 + T4</td>
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<td>Fuhrman grade</td>
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<tr>
<td>1 + 2</td>
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<tr>
<td>3 + 4</td>
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<td>Low</td>
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NOTE: TNM staging: TNM stage grouping was assigned according to the 2009 TNM staging classification system.

Overall, the results illustrated that KLF6 downregulation is highly associated with ccRCC metastasis.

Loss of KLF6 promotes ccRCC tumorigenesis in vitro

To corroborate the above-mentioned data derived from human ccRCC samples and to validate the function of KLF6 in cancer cells, KLF6 expression was measured in a panel of ccRCC cell lines with different malignant behaviors. As shown in Fig. 2A, KLF6 was expressed at a relatively high level in 786-O and Caki-2 cells but at a relatively low level in 769-P and OS-RC-2 cells. The reduction of KLF6 protein levels was observed in the 786-O and Caki-2 ccRCC cell lines after treatment with 3 siRNA sequences. In addition, the lentiviral plasmid introduction of KLF6 in OS-RC-2 and 769-P cell lines obviously increased the KLF6 expression compared with the empty vector (EV; Fig. 2A).

After confirming the knockdown efficacy of siRNA transient transfection by the Western blot assay, the MTS assay was performed and 786-O and Caki-2 cells transfected with siKLF6#2 showed remarkably enhanced growth relative to cells in the siNC group (Fig. 2B). Conversely, KLF6 overexpression in OS-RC-2 and 769-P cells significantly inhibited cell proliferation as compared with the EV group.

Transwell with or without a Matrigel coating were applied for the invasion and migration assays of ccRCC cells. 786-O and Caki-2 cells treated with siKLF6#2 showed enhanced invasive abilities compared with cells treated with siNC. The invasive and migratory abilities were both significantly attenuated in KLF6-infected OS-RC-2 and 769-P cells (Fig. 2C). In the wound-healing assay, KLF6 knockdown augmented 786-O and Caki-2 cell migration at 12 hours after scratching; 769-P and OS-RC-2 cells transfected with KLF6 lentiviral particles displayed decreased migratory capacity compared with the controls (Fig. 2D). A series of markers related to the EMT, including N-cadherin, E-cadherin, and vimentin, were detected by immunofluorescent staining, thereby suggesting the malignant shift in marker expression. As shown in Fig. 2E, impaired KLF6 expression in 786-O and Caki-2 cells transformed the cell morphology from a rounded, epithelial-like contour to a spindle shape. In contrast, the polarized morphology of 769-P and OS-RC-2 cells was disturbed after KLF6 overexpression, causing a rounded shape with cytoskeletal remodeling. Visualizing cells with confocal microscopy showed that KLF6 gene silencing in 786-O and Caki-2 cells elevated the expression of N-cadherin and vimentin but decreased E-cadherin expression. KLF6 overexpression in 769-P and OS-RC-2 cells conferred a phenotype with the reversed expression of N-cadherin, E-cadherin, and vimentin. The above results, taken collectively, suggested that KLF6 might suppress EMT of ccRCC cells and thereby inhibit metastasis.

E2F1 is aberrantly activated in ccRCC and is a direct target of KLF6

GSEA was used to analyze microarray data (GSE47352). As previously illustrated, the profiling sheet included 4 primary metastatic and 5 primary nonmetastatic samples. The C2ft calculation of gene clusters containing the matched binding motifs (NRTTCCGCGCS) was conducted (Fig. 3B and Supplementary Fig. S2). Results revealed that E2F1 is the top gene signature that was enriched in differentially expressed genes (Fig. 3A). To gain more reliable outcomes, we combined several reference parameters, including the normalized enrichment score (NES), P value, and fold change. The ranking flowchart is shown in Supplementary Fig. S2. E2F1 was significantly found to be the key upregulated factor in the primary metastatic group. This result was consistent with our previously published findings. The validation of E2F1 expression in a larger sample size was subsequently conducted by qRT-PCR and Western blot analysis (Fig. 3C). The correlation between the KLF6 and E2F1 mRNA levels in ccRCC tissue samples (n = 45) was further analyzed by linear regression analysis. As shown in Fig. 3D, E2F1 expression was progressively reduced (r² = 0.3546, P < 0.0001) compared with the associated elevation of KLF6 expression. The 769-P and OS-RC-2 cell lines showed higher E2F1 expression and low levels of KLF6 expression, whereas the 786-O and Caki-2 cell lines showed decreased E2F1 expression and high levels of KLF6 expression (Fig. 3E). A double-color immunofluorescent test showed that endogenous KLF6 and E2F1 proteins were localized in the nuclei of most 769-P cells. A merged signal suggested the colocalization of both proteins (Fig. 3F). Reduced KLF6 expression was associated with a strong induction of E2F1 mRNA expression in 786-O cells.
Figure 2.
KLF6 is a tumor suppressor in ccRCC. A, Relative KLF6 protein levels in different ccRCC cell lines (left) and the efficiency of knockdown and overexpression were validated in related cell lines (right). B, MTS assay showed that KLF6 knockdown promoted the tumor growth in 786-O and Caki-2 cells, whereas KLF6 overexpression in OS-RC-2 and 769-P reduced the proliferation velocity. C, KLF6 attenuation significantly increased migration and invasion in 786-O and Caki-2. On the contrary, KLF6 overexpression suppressed tumor aggressiveness in vitro in OS-RC-2 and 769-P (P < 0.001). D, For wound-healing assay, in 786-O and Caki-2 cells, KLF6 knockdown significantly increased the number of viable cells (P < 0.001); in 769-P and OS-RC-2 cells, KLF6 introduction inhibited the cell viability (P < 0.001). E, Immunofluorescent staining was used to explore the EMT-related protein alteration after KLF6 knockdown or upregulation in different cell lines. Magnification, ×600. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
E-cadherin expression was found in Caki-2 cells. After the introduction of KLF6 knockdown, whereas impaired cell migration and invasion, thereby suggesting that KLF6 downregulates E2F1 expression measured for a luciferase construct where the core binding region was explored. The sequence upstream of the transcriptional initiation site by Genomatix (www.genomatix.de/en/index.html) was found to contain putative KLF6-binding sites at −485/−468, −421/−404, −291/−272, −230/−212, and −176/−114. The putative binding sequences are listed in the box on the right, with the core element labeled in red (Fig. 3H). To determine whether endogenous KLF6 binds to the E2F1 promoter region in vivo, ChIP assays were performed on 786-O and Caki-2 cell lysates. Three primers were designed to capture the precipitated DNA fragment. A DNA fragment containing the KLF6-binding site (−485/−468) was significantly enriched in chromatin precipitated with an anti-KLF6 antibody, but no bands were evident in the immunoprecipitates of the other possible binding sequences (Fig. 3I). Our findings implied that KLF6 may directly interact with E2F1 by binding with the promoter. Given the relatively low transient transfection efficiency in renal cancer cell lines, 293T cells were used to perform a luciferase assay. When transfected into 293T cells, the dose-dependent luciferase activity of the pGL3-E2F1 reporter treated with pcDNA3.1-empty or pcDNA3.1-KLF6 (0.5, 1, and 2 µg) was significantly attenuated compared with that of pGL3-basic (Fig. 3J). The activity was also measured for a luciferase construct where the core binding element, GGTC (−485/−468), was replaced with CCAC. The MUT reporter activity was markedly reversed in 293T cells, thereby suggesting that KLF6 downregulates E2F1 expression by binding to the site (−485/−468) in the E2F1 promoter region (Fig. 3K).

**KLF6 downregulation is required for E2F1-mediated EMT and the metastatic phenotype**

To investigate the combined biologic effects of KLF6 and E2F1, a Transwell assay was performed. As shown in Fig. 4A, the suppressed KLF6 expression caused a robust increase in Caki-2 cell migration and invasion. ShE2F1 introduction significantly abrogated the invasive capacity of cancer cells transfected with shKLF6. The E2F1 plasmid was ectopically transected in OS-RC-2. Subsequently, KLF6 overexpression reversed the positive effect of E2F1 on cell migration and invasion, thereby suggesting that KLF6 was involved in E2F1-mediated cell aggressiveness. The abovementioned process was further examined by expression analysis of E-cadherin, N-cadherin, ZEB1, and vimentin, which are potential vital genes that indicate the high malignancy of cancer cells. N-cadherin, vimentin, and ZEB1 expression was greatly upregulated after KLF6 knockdown, whereas impaired E-cadherin expression was found in Caki-2 cells. After the introduction of anti-E2F1 plasmids, the cells with elevated invasion suppressed N-cadherin, ZEB1, and vimentin expression. Therefore, KLF6 mitigation significantly stimulated E2F1 expression and EMT-related gene expression levels (Fig. 4B).

The morphologic changes in different cell lines were observed. Compared with the shNC group cells, stable cells with KLF6 knockdown displayed a spindle-like, fibroblastic morphology, whereas shE2F1 employment can partially reverse the EMT process (Fig. 4C).

Angiogenesis is important in malignancy and metastasis; thus, the functional consequence of KLF6-mediated regulation on E2F1 expression was explored by testing the function of HUVECs. KLF6 knockdown in Caki-2 increased the expression of E2F1 and boosted the formation of endothelial tubule-like structures. This effect was completely abolished by shE2F1 transfection, thereby demonstrating that tubule formation is mediated by E2F1 (Fig. 4D, left). In contrast, KLF6 overexpression in OS-RC-2 cells considerably reduced tubule formation (Fig. 4D, right). Taken together, these data indicated that ccRCC progression was promoted by a key signaling axis involving KLF6-mediated E2F1 regulation.

**KLF6 downregulation promotes E2F1-mediated cell proliferation and metastasis in vivo**

To investigate the comprehensive function of KLF6 and E2F1 in vivo, 786-O cells of different groups were subsequently injected into the left kidney of a nude mouse. At 8 weeks after injection, bioluminescent signals in the kidneys were significantly higher in the shKLF6 group than in the shNC group; the effect of KLF6 was further mitigated by E2F1 knockdown (Fig. 5A). All mice were sacrificed at 8 weeks after injection, and the kidneys were harvested. Orthotopic implantation was identified and measured (Fig. 5B, left). Orthotopic xenograft tumor volumes of the shKLF6 group significantly increased as compared with the shNC group (P < 0.001), whereas tumor volumes after E2F1 knockdown in the shNC group were greatly reduced (Fig. 5B, right). Histopathologic analysis of tumors clearly revealed the interface between the tumor and normal kidney tissue (Fig. 5C).

The development of lung metastasis was evaluated after 786-O cells were injected through the mouse tail vein in the different treatment groups. In this model, KLF6 knockdown significantly enhanced lung metastasis at 4 and 8 weeks after injection. ShE2F1 may counteract the metastasis-elevating effect of curtailed KLF6 expression (Fig. 5D). All mice were sacrificed, and gross lung specimens were collected for bioluminescence imaging (Fig. 5E). Furthermore, the presence of metastatic colonies in the lungs was validated by histologic analysis. The number of metastatic nodules in each group agreed with the bioluminescence intensity detected in vivo (Fig. 5F). SN12-PM6 was introduced to establish...
E2F1 is critical for KLF6-mediated ccRCC migration and invasion. A, E2F1 knockdown significantly reduced migration and invasion of Caki-2 cells transfected with shKLF6. On the contrary, KLF6 overexpression suppressed E2F1-mediated aggressiveness in OS-RC-2 cells. B, KLF6, E2F1, and EMT-related gene expressions were evaluated by Western blot assay in ccRCC cell lines. C, Morphologic alteration of cells transfected with the vectors as indicated. D, KLF6 knockdown in Caki-2 significantly increased the amount of forming tubules. The angiogenic effect could be reversed by subsequent E2F1 abrogation. For OS-RC-2, inhibitory effect of KLF6 on microvessel forming could be reversed by E2F1 (P < 0.001). *P < 0.05; **P < 0.01; ***P < 0.001.
Figure 5.
Loss of KLF6 elevates metastatic potential of ccRCC cells in vivo by modulating E2F1. **A,** Representative bioluminescent pictures of nude mice that underwent orthotopic implantation with Luc-labeling 786-O cells stably transfected by indicated vectors (P < 0.001). **B,** Representative gross sample retrieved from the nude mice (left). The tumors were resected and measured (right). **C,** Hematoxylin and eosin staining of section from the orthotopic specimen. **D,** Representative bioluminescent images of nude mice injected via vein with Luc-labeling 786-O cells stably transfected by indicated vectors in 4 and 8 weeks, respectively (left). Measurement of bioluminescent signals of different groups (right). *** P < 0.001. **E,** Representative gross view of the lungs with metastatic nodules of different groups (black arrows, metastatic nodules; left). The amount of the pulmonary metastatic lesions was counted in individual group (right). *** P < 0.001. **F,** Hematoxylin and eosin stained slides of lungs dissected from nude mice from different groups. **G,** Representative bioluminescent images of nude mice bearing orthotopic implantation of Luc-labeling SN12-PM6 cells stably transfected by indicated plasmids in 3 months (left). Measurement of bioluminescent signals of different groups (right). *** P < 0.001. **H,** IHC staining showed the changes in tubule formation in vivo following different vector transfection (**, P < 0.05).
the orthotopic and metastatic model, due to its pulmonary metastatic property (22). ShE2F1 group mice exhibited a strong reversed effect (Fig. 5G). The in vitro assay proved that KLF6 and E2F1 affected tube formation, IHC staining was performed with the capillary-specific antibody CD34. Metastatic lesions of the shKLF6 group presented more CD34 staining than the shNC group, whereas E2F1 depletion largely prevented tube formation (Fig. 5H). Therefore, the in vivo studies demonstrated that KLF6 repressed the metastatic properties of ccRCC cells by modulating E2F1.

KLF6 expression is negatively correlated with E2F1 in ccRCC tissues and may predict overall survival

The observed serial sections showed that KLF6 and E2F1 are mainly distributed in nuclei of the same cancer cells (Fig. 6A). We analyzed the expression of both proteins by IHC with serial sections from the same tissue microarrays. Among the 114 ccRCC specimens, strong KLF6 and E2F1 immunostaining was found in 30.7% (35 of 114) and 65.8% (75 of 114) of the tumors, respectively. High levels of KLF6 expression were found in 61.5% (24 of 39) of the tumors with low E2F1 staining, whereas KLF6 presented low staining in 85.3% (64 of 75) of the tumors with high E2F1 expression. Statistical analysis with the Pearson χ² test revealed the significant negative correlation between E2F1 and KLF6 expression (P < 0.001, Fig. 6B). The quantification of protein expressions was confirmed in the platform of TissueGnostics GmbH (Supplementary Fig. S3). CD34 staining was correlated closely with KLF6 and E2F1 staining in cancer tissues (Fig. 6C). The Kaplan–Meier analysis demonstrated that patients with low KLF6 levels have poorer overall survival than those with high KLF6 levels (log-rank test; P = 0.0079; Fig. 6D, left). For samples grouped by the inverse expression of both proteins, patients with low KLF6 and high E2F1 had poorer overall survival than those with high KLF6 and low E2F1 levels (log-rank test; P = 0.0003; Fig. 6D, right), with more significant differentiation. The coexpression and negative correlation of KLF6 and E2F1 in ccRCC tissues were observed and may predict overall survival.

Discussion

Cancer metastasis is a multistep process, wherein malignant cells escape from the primary tumor to colonize distant sites (23). The overall mortality rates remain relatively constant because the cancer was already at a late stage when the disease was diagnosed, even with distant metastasis (24). However, the metastatic changes during disease progression are relatively unknown.

Previous tissue profiling results suggested that KLF6 down-regulation and E2F1 activation are pivotal events in distant metastasis of ccRCC. Loss of KLF6 in a hepatocellular carcinoma mouse model results in increased carcinogenesis, promoted metastasis to the lungs, and decreased survival (25). KLF6-SV1, an oncogenic splice variant of KLF6, was reported to increase
metastatic potential in patients with lymph node–negative breast cancer. KLF6-SV1 overexpression in mammary epithelial cells drove EMT and resulted in aggressive multiorgan metastasis in vivo (26). The metastatic potential of KLF6-initiated metastasis in ccRCC relied on a novel function of the classic transcription factor E2F1. KLF6 directly modulated E2F1, which acted as an EMT inducer. In addition, KLF6 depletion linked E2F1 activity to the integrin EMT signaling pathway associated with cellular morphologic change and enhanced tumor cell malignancy. According to clinical data, a negative correlation was observed between the mRNA and protein expression of KLF6 and E2F1 in ccRCC specimens.

KLF6 expression is downregulated in various cancers, including non–small cell lung cancer (27), prostate cancer (7), and hepatocellular carcinomas (28). In addition, recent studies have associated the loss of KLF6 expression with poor clinical outcomes, cancer recurrence, and chemotherapeutic resistance (29–31). KLF6 has been previously suggested in OS-RC-2 cell line (ccRCC-derived) showing that stable expression of KLF6 lead to reduced cell proliferation and cell-cycle arrest at G0–G1, correlating with an upregulation of p21 expression. It was the first study showing the effect of KLF6 on proliferation of ccRCC-derived cells (32). Ectopic KLF6 expression led to a G1 phase cell-cycle arrest, thereby attenuating the cell proliferation. These findings highlighted KLF6 as a tumor suppressor.

E2F1 prompts oncocgenic and metastatic programs aside from its proliferative and apoptotic functions (33). E2F1 upregulation in mammary cell lines causes carcinogenesis and aggressive distant metastasis in multiple cancers (34–36). E2F1 may enhance metastatic behavior in melanomas by directly binding with VEGFC and the corresponding receptor VEGFR-3 for transactivation (37). The oncogenic properties of E2F1 have been associated with high EMT transition (38). However, the forces that drive E2F1 gene expression in ccRCC remain unclear.

The in vitro study revealed that KLF6 mRNA and protein levels were upregulated, with a concomitant reduction of E2F1 expression upon KLF6 transfection. KLF6 upregulation abolished E2F1 expression, whereas KLF6 downregulation significantly increased E2F1 expression. The previous document demonstrated that E2F1 and KLF6 cooperated in activating the DAPK2 promoter (39). In our study, promoter luciferase reporter and ChIP assays illustrated that KLF6 directly binds to the human E2F1 gene promoter and inactivates it. In addition, the siRNA-mediated repression of E2F1 in stable shKLF6-transfected cells reversed cell migration and metastatic phenotypes. E2F1 downregulation in vivo decreased the shKLF6-mediated proliferation according to the orthotopic implantation model. We tested the role of KLF6 in ccRCC metastasis with a nude mouse lung seeding assay. Compared with the control shNC 786-O cells, the shKLF6-expressing cells developed more lung metastases, which were completely diminished by E2F1 knockdown. Therefore, targeting E2F1 may be a promising approach to avoid KLF6-mediated metastatic dissemination.

In summary, a novel genetic event was associated with ccRCC metastasis. We identified KLF6 as a direct repressor of E2F1 and demonstrated the novel function of KLF6–E2F1 in augmenting EMT-related induction. The biologic significance of enhanced ccRCC progression and metastasis was highlighted by KLF6 downregulation, which leads to the induction of E2F1 expression. E2F1 can antagonize the tumor-suppressive function of KLF6 by inhibiting cell proliferation, migration, invasion, and in vivo tumorigenesis. Sections of metastasized lung nodules were stained by CD34 to establish whether KLF6 depletion in the presence of high levels of E2F1 enhances the ability of the ccRCC cell line to induce angiogenesis. As expected, the shKLF6-infected 786-O cells significantly enhanced tubule formation, whereas cells in the shKLF6 + shE2F1 group prevented microvessel formation as compared with the shKLF6 group. This result suggested that E2F1 prometastatic activity is strengthened when KLF6 expression is abrogated. Unneglected, putative underlying mechanism may occur considering that some tumors without EMT phenotype exhibited metastasis, further investigation is required to exclude the possibility that EMT may not be direct but an indirect factor associated with invasion or metastasis.

In total, we elucidated that the KLF6-mediated transcriptional control of E2F1 expression maintains the invasive potential of cancer, which plays a pivotal role in the malignant progression of human ccRCC and carries therapeutic implications.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: Y. Zhang, Q. Huang, X. Zhang

Development of methodology: Y. Zhang, Q. Huang, L. Wang, Y. Yao, X. Zhang

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Gao, Y. Fan, D. Ni, X. Li, X. Zhang

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Gao, X. Li, L. Gu, Y. Yao, Q. Ai, X. Zhang

Writing, review, and/or revision of the manuscript: Y. Gao, X. Ma, X. Li, Y. Yao, Q. Du, X. Zhang

Administrative, technical, or material support (i.e., reporting and organizing data, constructing databases): Y. Gao, X. Ma, D. Ni, Q. Huang, K. Liu, E. Song, X. Zhang

Study supervision: H. Li, X. Ma, Y. Zhang, X. Zhang

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KLF6 Suppresses Metastasis of Clear Cell Renal Cell Carcinoma via Transcriptional Repression of E2F1

Yu Gao, Hongzhao Li, Xin Ma, et al.


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