Low MAGI3 expression promotes Wnt/β-catenin signaling to mediate metastasis and rapalogs resistance of clear cell renal cell carcinoma

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Abstract

Metastatic renal cell carcinoma (mRCC) is considered a fatal malignancy with poor overall survival, but the efficacy of the currently available strategies is limited. Hence, a better understanding of the detailed mechanisms behind the pathogenesis of RCC and more effective treatment strategies were urgently required. In this study, we found that MAGI3 was low-expressed, and associated with metastasis of ccRCC by screening and validating from multi-central cohorts. Low MAGI3 level was correlated with advance stage, grade and poor survival of ccRCC in several independent cohorts, revealing that MAGI3 might be employed as a biomarker in predicting prognosis of ccRCC. By both ectopic expression and knockdown of MAGI3 assays, we found that MAGI3 could inhibit the migration, invasion, and metastasis of ccRCC cells in vitro and in vivo. Mechanistically, we revealed that the C-tail of β-catenin binding with MAGI3 facilitated its N-tail to release from intramolecular interaction of β-catenin, and reinforced the association between β-catenin and GSK-3β, thereby, promoted GSK3β-mediated β-catenin phosphorylation, and ubiquitin-proteasome degradation, subsequently attenuates Wnt/β-catenin activation in ccRCC. Our results also demonstrated that MAGI3 conferred ccRCC cell sensitivity to Everolimus through Wnt/β-catenin pathway, and the combination of Wnt inhibitors synergistically increased the sensitivity of ccRCC cells to rapalogs. Therefore, this is a first report of a synthetic lethal interaction between inhibition of mTOR/AKT and Wnt/β-catenin pathway in ccRCC, revealing a new therapeutic approach for the treatment of advanced ccRCC patients by combined therapy of mTOR inhibitors (rapalogs) with Wnt inhibitors or upregulation of MAGI3 expression.

Introduction

Clear cell renal cell carcinoma (ccRCC) is the most lethal and the third most common urological disease, accounting for approximately 80% of diagnosed RCCs[1]. It exhibits an increased incidence in recent years. For early stage or localized ccRCC patients, surgical nephrectomy is an effective treatment. However, almost 17% of patients with RCC have distant metastases at the time of diagnosis[2], and about 25% will develop metastases after local therapy of localized ccRCC patients[3]. Therefore, up to 50% of RCC patients will die as the disease progresses[3]. Most patients with advanced or metastatic ccRCC (mRCC) have low sensitivity to chemotherapy or radiotherapy, and only a few patients have response to cytokine treatment with IL-2 or IFN-α[4].

In recent years, studies on the pathogenesis of RCC has promoted the development of targeted drug therapy for mRCC. The VHL-HIF-HRG pathway has been found to play an important role in the development of ccRCC. Inactivation of VHL by mutation is the key driver mutation in more than 90% of ccRCC[5]. VHL promotes HIF1-α degradation through the ubiquitin-proteasome pathway, therefore VHL inactivation results in a significant increase in the level of HIF1-α, leading to increase the expression of angiogenic factors, and then promotes tumor growth by activating RAS/RAF/MAPK and PI3K/AKT/mTOR signaling pathways[6]. Thus, the inhibition of tyrosine kinase and mTOR are attractive strategies to treat mRCC. Currently, the FDA-approved targeted drugs are classified into two categories, belonging tyrosine kinase inhibitors (TKIs) or mTOR inhibitors as rapamycin and its analogs (rapalogs).

Apply these targeted drugs has improved the therapeutic efficacy of mRCC to prolong the median survival of patients to 28 ~ 29 months[7]. However, up to 20–30% of mRCC patients do not respond to treatment with these drugs[8], and for all other patients usually develop drug resistance after 6–15 months of treatment. Nowadays, after targeted therapy treated with TKI or rapalogs, the five-year survival rate of mRCC patients is is only 12%[7], therefore, the studies on the pathology mechanisms are urgently required to develop more effective strategies for mRCC targeted therapy.

Recently, it has been reported that Wnt/β-catenin signaling plays an important role in tumorigenesis and progression in various cancers including ccRCC[9]. Genetic silencing β-catenin or inhibition of the Wnt/β-catenin pathway in cancer cell lines has been demonstrated to restrain cancer cell growth, trigger apoptosis activity and prevent chemoresistance[10]. Cytoplasmic β-catenin accumulation is a risk factor for cancer-specific survival[11]. No elevated levels of β-catenin mRNA[12] and rare gene mutations[13] of β-catenin was observed in ccRCC, therefore, the elevated levels of β-catenin protein is mainly responsible for activation of Wnt pathway in ccRCC. It is well known that β-catenin is phosphorylated by GSK3β, and then degraded by the destruction complex contains APC[14]. APC mutation is a major cause of β-catenin protein accumulation and drive human colorectal cancer development[15]. Due to the absence of APC mutation, the accumulation of β-catenin protein in ccRCC has a different mechanism from that in colorectal cancer, so far, the mechanisms underlying the accumulation of β-catenin protein in ccRCC remain elusive.

In this paper, differentially expressed genes between renal epithelial tissues and ccRCC, or between primary and mRCC, were screened by analyzing mRNA expression profiles downloaded from TCGA and GEO datasets. MAGI3 was identified to be significantly downregulated in mRCC. Loss of MAGI3 expression was correlated with poor prognostic ccRCC. MAGI3 was verified as a novel tumor suppressor in ccRCC and demonstrated its inhibition on cell migration and invasion. Mechanistically, by regulation of intramolecular conformation of β-catenin, MAGI3 promoted GSK-3β-mediated β-catenin phosphorylation and degradation, as well as inhibited Wnt signaling activation, and suppresses invasion, migration and metastasis of renal cancer cells. More interestingly, MAGI3 could increase the sensitivity of ccRCC cells to mTOR inhibitor Everolimus via inhibiting Wnt/β-catenin signaling, and the combination of Wnt small molecule inhibitor could synergistically increase the sensitivity of ccRCC cells to mTOR inhibitors. These results reveal a synthetic lethal interaction between mTOR/AKT and Wnt/β-catenin pathway, and suggest enhancing MAGI3 expression may have potential therapeutic value to enhance the sensitivity to rapalogs through targeted inhibition of the Wnt/β-catenin pathway.

Results

MAGI3 is low-expressed in ccRCC, and correlates with poor prognosis of ccRCC. Metastasis is responsible for more than 90% of cancer-associated deaths in RCC patients[7]. To screen key genes and pathways associated with progression and metastasis in ccRCC, we compared the gene expression profiles of ccRCC tissues with the adjacent normal tissues (GSE12606), and metastatic ccRCC tissues with non-metastatic ccRCC samples (GSE31232) respectively. GEO2R analysis found 1827 differential expression

genes (DEGs) in ccRCC and 40 DEGs in mRCC (Fig S1A), with five common DEGs identified, including MAGI3, EGF, ADH1C, EYA4 and C3orf52 (Fig S1B). These genes were further verified in TCGA-KIRC dataset, four genes including EGF, ADH1C, EYA4 and C3orf52 were significantly reduced in ccRCC tissues (Fig. S2A), however, none of them had statistically different between metastatic and non-metastatic ccRCC samples (Fig. S2B).

MAGI3 was also investigated with TCGA and another independent cohort, and it was shown that MAGI3 expression was robustly downregulated in ccRCC tissues at both mRNA (Fig. 1A) and protein levels (Fig. 1B, 1C). Compared with non-metastatic ccRCC samples, MAGI3 expression was further significantly reduced in metastatic ccRCC samples at either mRNA (Fig. 1D) or protein levels (Fig. 1E) in TCGA-KIRC datasets or clinical specimen respectively. These results indicated that MAGI3 was a novel gene identified to associate with metastasis of ccRCC. Taken together, all of these data indicated that MAGI3 was low-expressed in ccRCC, and correlated with metastasis of RCC.

In order to explore the clinical relevance of MAGI3 in ccRCC, the correlations of MAGI3 level with ccRCC stage, grade and prognosis were evaluated with several independent ccRCC patient cohorts of TCGA-KIRC or GEO. The results showed that low level of MAGI3 was correlated with advanced stage (Fig. 1F), high grade (Fig. 1G) and poor prognosis (Fig. 1H). Cox univariate and multivariate analysis indicated that MAGI3 expression level was an independent prognostic factor for OS and PFS of ccRCC patients (Table 1). These results suggested that MAGI3 might be employed as a biomarker in predicting prognosis of ccRCC.

MAGI3 inhibits migration, invasion and metastasis of ccRCC. To further verify the correlation of ccRCC metastasis with MAGI3 expression level, Gene Set Enrichment Analysis (GSEA) from TCGA-KIRC or GSE36895 dataset was conducted, the results showed that the gene signatures of metastasis-related upregulated gene-set were enriched in ccRCC specimens with MAGI3 low expression group (Fig. 2A). The metastatic process is a result of tumor cell migration and invasion. To investigate the biological impact of MAGI3 on metastasis of ccRCC, migration and invasion were examined by transwell migration and invasion assays as well as wound healing assays. Overexpression or knockdown of MAGI3 in 769-P or 786-O cells was confirmed by western blotting (Fig. 2B). Ectopic expression of MAGI3 significantly suppressed migration and invasion of 786-O and 769-P cells (Fig. 2C&E), while knockdown of MAGI3 markedly promoted migration and invasion in the both ccRCC cells (Fig. 2D&F). To verify that MAGI3 inhibits ccRCC cell metastasis in vivo, a xenograft tumor model was established by injection of 786-O/shMAGI3 or 786-O/shCtrl cells into the tail vein of NOD/SCID mice. A higher ratio of lung metastasis was observed in the 786-O/shMAGI3 group as compared with the control group, indicating that knock down of MAGI3 expression significantly enhanced 786-O cell metastasis in vivo (Fig. 2G). Taken together, these data indicated that MAGI3 could inhibit the migration, invasion, and metastasis of ccRCC cells.

MAGI3 promotes β-catenin protein phosphorylation and ubiquitin-proteasome degradation. To gain insight into the molecular mechanism of MAGI3 expression on control cell functions, we used KIRC database in TCGA to screen the gene set closely related to MAGI3 expression in ccRCC through UALCAN

website, and then conducted the pathway enrichment analysis for MAGI3-related gene set by DAVID. The results indicated that the function of MAGI3 related genes was mainly enriched in "ubiquitination dependent protein degradation" and "Wnt signaling pathway"(Fig. S3), reminding MAGI3 expression may influence Wnt signaling pathway activation. To verify the effects of MAGI3 expression on Wnt signaling pathway, the GSEA was performed. The results showed that the gene signatures of Wnt/β-catenin activation were enriched in patients with MAGI3 lower levels in TCGA KIRC and GSE36895 datasets (Fig. 3Aa), which is consistent with our previously report that MAGI3 inhibited malignant phenotypes of glioma and cervical cancer through negatively regulating Wnt/β-catenin signaling[16, 17]. However, the underlying molecular mechanisms was not yet elucidated. Meanwhile, we noticed that there were no correlations between MAGI3 and β-catenin at mRNA level (Fig. 3Ab), but MAGI3 significantly negatively correlated with β-catenin at protein level in clinical specimens (Fig. 3Ac). Moreover, overexpression MAGI3 could significantly reduce the levels of β-catenin protein in ccRCC cells (Fig. 3B). On the contrary, knockdown of MAGI3 increased the levels of β-catenin protein (Fig. 3C). It is well known that β-catenin is a short half-life protein and rapidly turned over by proteasomal degradation[14]. To explore whether MAGI3 regulates β-catenin stability, we next determined the half-life of β-catenin protein by overexpression MAGI3. As shown in Fig. 3D, Overexpression of MAGI3 significantly accelerated degradation of the wild type β-catenin, but not β-catenin-T779A mutant (Fig. 3D) as T779A mutant of β-catenin can abolish their interaction[16], suggesting that MAGI3 decreased β-catenin stability dependent on the interaction between MAGI3 and β-catenin. Studies have shown that β-catenin is mainly degraded by ubiquitin-proteasome pathway[18]. Therefore, we next investigated whether MAGI3 promoted β-catenin degradation by enhancing β-catenin ubiquitination. Also, the results showed that MAGI3 indeed significantly promoted ubiquitination of the wild type β-catenin (Fig. 3E) but not β-catenin-ΔCT (β-catenin deleted C-terminus) (Fig. 3F). Taken together, these results indicated that MAGI3 promoted β-catenin ubiquitination and degradation via interaction with β-catenin.

It is well known that phosphorylation of β-catenin at Ser33/Ser37/Thr41 by glycogen synthase kinase 3β (GSK3β) targets β-catenin for ubiquitination and degradation[14]. Therefore, we next examined whether MAGI3 promoted the phosphorylation of β-catenin, and our results showed that MAGI3 overexpression increased the levels of phospho-β-catenin (Ser33/Ser37/Thr41) (Fig. 4A), while MAGI3 knockdown reduced the phospho-β-catenin (Ser33/Ser37/Thr41) in ccRCC cells (Fig. 4B). For the regulatory mechanism of MAGI3 on β-catenin phosphorylation, we proposed that MAGI3 acts as a scaffold protein to facilitate the phosphorylation of β-catenin by GSK3β. This hypothesis was further confirmed by the results of Fig. 4C&D that MAGI3 enhanced the association of GSK3β with wild type β-catenin, but not with β-catenin-ΔCT, indicating that MAGI3 promotes β-catenin phosphorylation by facilitating the association of GSK3β with β-catenin, and the C-terminus of β-catenin plays an indispensable role during this process. β-catenin protein composed three domains, including N-tail, armadillo repeat domain (ARM) and C-tail (Fig. 5A). We found that the N-tail could associate with ARM domain of β-catenin, β-catenin-ΔNT (β-catenin deleted N-tail) and β-catenin-wt. Moreover, the N-tail had a stronger binding affinity with β-catenin-ΔNT (β-catenin deleted N-tail) than β-catenin-wt (Fig. 5B lane 2&3), indicating that the intramolecular N-tail of β-catenin was able to associate with ARM domain, thereby disturb the binding between extra

molecular N-tail with β-catenin-wt. Also, we found that the N-tail had a stronger binding affinity with β-catenin-ΔNT than the ARM (Fig. 5B lane 3&4), indicating that the C-tail facilitated the interaction of the N-tail with its Arm domain. Meanwhile, overexpression MAGI3 could reduce extra molecular Flag-N-tail binding with GST-ΔNT (Fig. 5C). However, when C-terminus was deleted from β-catenin-ΔNT, MAGI3 had no effect on the interaction of extra molecular Flag-N-tail binding with GST-ΔNT-ΔCT (Fig. 5D), indicating that the C-tail of β-catenin associated with MAGI3 retarded the interaction of the intramolecular N-tail with its Arm domain of β-catenin, and the free C-tail facilitated the interaction of the N-tail with its Arm domain of β-catenin to form an intramolecular conformation, such as a loop structure. Therefore, when MAGI3 bound to the C-tail, the intramolecular N-tail could be released from the ARM domain to reinforced the association between β-catenin and GSK3β, and promoted GSK3β-mediated β-catenin phosphorylation (Fig. 5E).

MAGI3 suppresses metastasis and overcomes rapalogs resistance of ccRCC cells via inhibition of β-catenin signaling. We further investigate whether MAGI3 inhibits invasion and migration of ccRCC cells through suppressing Wnt/β-catenin pathway. The results showed that knockdown of MAGI3 increased β-catenin protein level (Fig. 6A, lane 2), and promoted invasion and migration of ccRCC cells (Fig. 6B). To rescue the level of β-catenin protein with knockdown β-catenin (Fig. 6A, lane 4), the invasion and migration induced by MAGI3 knockdown of ccRCC cells were inhibited (Fig. 6B). Consistent with these results, when treated with Wnt/β-catenin inhibitor IWR-1-endo, the invasion and migration induced by MAGI3 knockdown of ccRCC cells were also inhibited (Fig. 6C). Taken together, these data suggest that MAGI3 inhibits migration and invasion in ccRCC cells through suppressing the Wnt/β-catenin pathway.

Evidences suggest that both Wnt/β-catenin and PI3K/Akt/mTOR pathways contribute to progression and poor prognosis of ccRCC, and inhibition of Wnt/β-catenin and PI3K/Akt/mTOR pathways is regarded as a promising innovative therapeutic approach for cancer treatment[19]. Surprisingly, the potential therapeutic effect of simultaneous targeting of PI3K/Akt/mTOR and Wnt/β-catenin pathways has never been explored in RCC preclinical models. Therefore, we next to investigate the effects of MAGI3 expression on the sensitivity of ccRCC cells to rapalogs (Everolimus). As shown in Fig. 7, the assay of cell viability in 786-O or 769-P cells showed that overexpression of MAGI3 to suppress Wnt/β-catenin signaling could significantly increase the sensitivity of ccRCC cells to Everolimus, while knockdown of MAGI3 to activate Wnt/β-catenin signaling could robustly decreased the sensitivity of ccRCC cells to Everolimus (Fig. 7A). Meanwhile, when activated Wnt/β-catenin signaling by CHIR, the drug sensitivity-enhanced by MAGI3 over expression was totally abolished (Fig. 7Ba, right panel); while blocked Wnt/β-catenin signaling by XAV-939, an inhibitor of Wnt/β-catenin pathway, the drug sensitivity-reduced by knockdown of MAGI3 was also rescued (Fig. 7Bb, right panel). These results demonstrated that MAGI3 conferred ccRCC cell sensitivity to Everolimus through Wnt/β-catenin pathway. Moreover, the sensitivity of ccRCC cells to Everolimus was dose-dependently increased when treated with XAV-939 (Fig. 7C). Furthermore, simultaneous targeting of PI3K/Akt/mTOR and Wnt/β-catenin pathways by using Wnt inhibitor XAV-939, and rapalogs such as Everolimus could synergistically inhibited clone formation (Fig. 7D), and the cell migration (Fig. 7E) of ccRCC respectively. Altogether, these data indicate that

inhibition of Wnt/β-catenin pathway by with Wnt inhibitor or MAGI3 overexpression could synergistically potentiates the sensitivity of ccRCC cells to rapalogs.

In summary, this study provide first lines of evidences that Low MAGI3 expression promotes Wnt/β-catenin signaling activation to mediate metastasis and rapalogs resistance of clear cell renal cell carcinoma; while high MAGI3 expression suppresses Wnt/β-catenin signaling via facilitating GSK3β-mediated β-catenin phosphorylation and subsequently ubiquitination and degradation, leading to inhibit ccRCC metastasis. MAGI3 overexpression or application of Wnt inhibitor synergistically potentiates the sensitivity of ccRCC cells to rapalogs (Fig. 7F).

Discussion

Advanced metastatic renal cell carcinoma (mRCC) is a life-threatening disease with a poor overall survival (5-year OS about 12%), but the efficacy of the currently available strategies is limited. Hence, a better understanding of the detailed mechanisms behind the pathogenesis of RCC and more effective treatment strategies were urgently required.

In the present study, we found that the expression level of MAGI3 correlated with poor prognostic of RCC by screening and validating from multi-central cohorts (Fig. 1). Low level of MAGI3 was identified as a risk factor of poor survival, and a predictor of ccRCC metastasis independent on pathological features (Table 1). Decreased expression levels of MAGI3 was also reported to associate with inflammation in colonic IBD, and targeted by several microRNAs such as miR-20b-5p[20], miR-34c-3p[21] and miR-5692[22] to down regulate MAGI3 expression. Inflammation and its related microRNAs is a critical component of ccRCC progression[23, 24]. Therefore, it is possible that systemic inflammation or some miRNAs may reduce MAGI3 expression in RCC. However, the underlying molecular mechanism need to further explored.

Studies in ccRCC cells revealed that MAGI3 expression inhibited cell migration and invasion by wound healing and transwell assays (Fig. 2C&D, E&F). These results were further confirmed by our in vivo studies of xenograft tumor (Fig. 2G). Thereby, these findings demonstrated that MAGI3 played an important role in ccRRC progression. Herein, we further proved that dysregulated low expression of MAGI3 in ccRCC associated with Wnt/β-catenin activation and the progression of ccRCC. As shown in Fig. 6, knockdown of MAGI3 activated Wnt/β-catenin pathway via increasing the expression of β-catenin in protein level (Fig. 6A), and promoted the invasion & migration of ccRCC cells (Fig. 6B). The results were further confirmed with our rescue experiments by knockdown β-catenin expression to suppress Wnt/β-catenin pathway, which is consistent with our previous report that MAGI3 inhibited malignant phenotypes of glioma[16] and cervical cancer[17] through negatively regulating Wnt/β-catenin signaling, but the underlying molecular mechanisms mechanism was not yet elucidated.

In this study, MAGI3 was found to promote β-catenin protein phosphorylation and ubiquitin-proteasome degradation, subsequently attenuates Wnt/β-catenin activation (Fig. 3&4). The N-tail of β-catenin is the key region for its stability by phosphorylation and ubiquitin-proteasome degradation, and

phosphorylation of β-catenin at the N-tail Ser33, Ser37, Thr41, and Ser45 mediated by GSK3β and CK1, promotes its ubiquitination and degradation[14]. Herein, we found that MAGI3 facilitated the association of GSK3β with β-catenin, and promoted β-catenin phosphorylation at Ser33/Ser37/Thr41 depending on MAGI3 interaction with the carboxyl terminus of β-catenin (Fig. 4). β-catenin protein is composed of three domains: N-tail, ARM and C-tail (Fig. 5A). In this study, we revealed that C-tail of β-catenin interaction with MAGI3, could enhance the phosphorylation and ubiquitination of β-catenin in its N-tail. The ARM domain of β-catenin was able to associate with its N-tail and C-tail to form an intramolecular conformation, such as a loop structure (Fig. 5B). When MAGI3 bound to the C-tail, the N-tail could be released from the ARM domain as the C-tail facilitated the interaction of the N-tail with its Arm domain (Fig. 5C&D). The released free N-tail of β-catenin might reinforce the association between β-catenin and GSK-3β, and promoted GSK3β-mediated β-catenin phosphorylation, and phosphorylation-dependent ubiquitination and degradation by proteasome. Thus, MAGI3 suppressed Wnt/β-catenin signaling by promoting phosphorylation and degradation of β-catenin (Fig. 5E).

In the current study, our results demonstrated that MAGI3 conferred ccRCC cell sensitivity to Everolimus through Wnt/β-catenin pathway. As shown in Fig. 7, MAGI3 robustly increased sensitivity of ccRCC cells to Everolimus through suppressing Wnt/β-catenin pathway (Fig. 7A&B). Consistently, simultaneous targeting of PI3K/Akt/mTOR and Wnt/β-catenin pathways by using Wnt inhibitor, and rapalogs such as Everolimus could synergistically inhibit the cell survive (Fig. 7C), proliferation (Fig. 7D), and migration (Fig. 7E) of ccRCC. These studies reminded a potential synthetic lethal interaction between inhibition of PI3K/AKT/mTOR and Wnt/β-catenin pathways. It is worth noting that there is a crosstalk between Wnt/β-catenin signaling and PI3K/AKT/mTOR pathway, the concomitant activation of compensatory pathway of Wnt/β-catenin signaling is observed when PI3K/AKT/mTOR pathway is suppressed with treatment with PI3K inhibitor[25]. While over-expression of β-catenin could antagonize cell apoptosis-induced by inhibition of PI3K/AKT/mTOR signaling[25]. Targeted inhibition any one of these pathways may have clinical significance for the treatment of ccRCC, but it only has a limited efficacy[26, 27]. Therefore, this is a first report of a synthetic lethal interaction between inhibition of mTOR/AKT and Wnt/β-catenin pathway in ccRCC, revealing a new therapeutic approach for the treatment of advanced ccRCC patients by simultaneous combination therapy with mTOR inhibitors with Wnt inhibitors.

Wnt/β-catenin signaling pathway controls many normal developmental and cellular processes. Thereby, although Wnt inhibitors might be applicable for targeted therapy, the inhibitors targeting this pathway may have many side effects that are not desired to the treatment. Thus, finding specific molecules targeting the Wnt pathway would be essential to achieve a successful targeted therapy. Herein, we demonstrated that aberrant low expression of MAGI3 in ccRCC promoted Wnt/β-catenin activation and the progression of ccRCC. Therefore, MAGI3 is a potential drug target to selectively inhibited Wnt signaling, may have potential therapeutic value for the therapy of ccRCC.

In summary, the present study has identified MAGI3 as a novel tumor suppressor in ccRCC and demonstrated its inhibition on migration and invasion by targeting β-catenin. We provide the convincing evidence for the first time that MAGI3 promote β-catenin protein phosphorylation and ubiquitin-

proteasome degradation, subsequently attenuates Wnt/β-catenin activation in ccRCC. The loss of MAGI3 correlates with an unfavorable prognosis, resistance to rapalogs-based therapy to mRCC patients. In addition, simultaneous inhibition of these two pathways could achieve robust clinical efficacy for mRCC and warrant further clinical investigation.

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Declarations

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Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions

Junqi He designed, managed and supervised the research. Hua Liu designed research, analyzed data and prepared the manuscript. Siyu Gu performed most of the experiments and prepared the manuscript. Lijie Zhang, Pengyan Fa and Wenxiu Lu performed partial experiments. Yang Yang, Xuedi Cao and Lian Huang contributed to the computational analysis and the statistical analysis. Ying Yang, Haibo Wang, Qiong Qin, and Xiaomei Yang reviewed and revised the manuscript. Chunjuan Zhao, Duiping Feng provided human RCC samples and revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Consent for publication

All subjects have written informed consent.

Availability of data and materials

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

Supplementary information

Additional file:

Additional file 1: Materials and methods, References, Supplementary figure legends, Fig. S1- Fig. S4 and Supplemental methods table 1.

Tables

Table 1 is in the supplementary files section.

Figures

Fig. 1

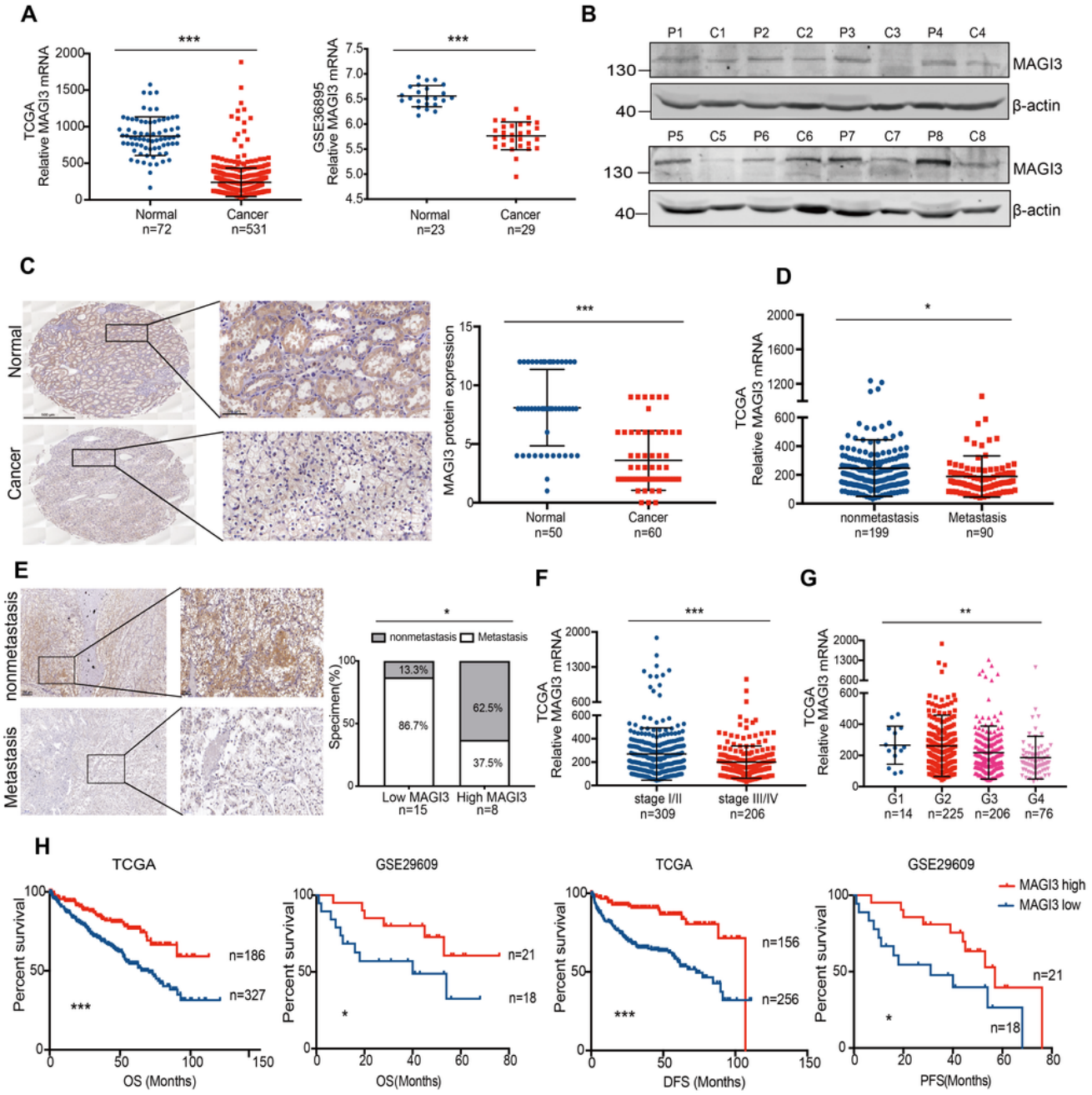

Figure 1

MAGI3 is low-expressed, and negatively associated with metastasis and predicts good prognosis of ccRCC. (**A**) The differential expression of MAGI3 mRNA in ccRCC and tumor-adjacent normal tissues. Scatter plots of the relative MAGI3 mRNA expression in ccRCC and adjacent normal tissues from TCGA or from GSE36895. *** $p < 0.001$, Independent sample *t*-test. (**B**) MAGI3 protein levels are reduced in human ccRCC. MAGI3 expression was detected by western blot. (**C**) Low expression of MAGI3 in ccRCC was

measured by immunohistochemical staining (IHC). ccRCC (lower panel) and its adjacent normal tissue (upper panel) in tissue microarray (TMA) were analyzed by immunohistochemical (IHC) staining. Scale bars, 500 μm and 50 μm respectively. *** $p < 0.001$, the independent sample *t*-test (right panel). (**D**) Lower level of MAGI3 mRNA was associated with metastasis of ccRCC, the RNAseq data were retrieved from TCGA (the independent sample *t*-test, * $p < 0.05$). (**E**) Lower level of MAGI3 protein was associated with metastasis of ccRCC, Representative images of IHC staining of MAGI3 in nonmetastatic or metastatic ccRCC specimens. Scale bars: 200 μm and 50 μm (left panel). Statistical analysis of the percentage of nonmetastatic and metastatic ccRCC specimens between MAGI3 high or low expression groups by Fisher's exact test (right panel). (**F-G**) MAGI3 mRNA level was downregulated in advanced stages (F) or high grades (G) of ccRCC tissues, the data were retrieved from TCGA (*t*-test, or one-way ANOVA, ** $p < 0.01$, *** $p < 0.001$). (**H**) Low expression of MAGI3 was correlated poor survival outcome in ccRCC. Kaplan-Meier Survival Analysis was used to compare OS or DFS/PFS in the indicated multiple ccRCC cohorts dichotomized as MAGI3 high or low based on the average value of MAGI3 mRNA expression.

Fig. 2

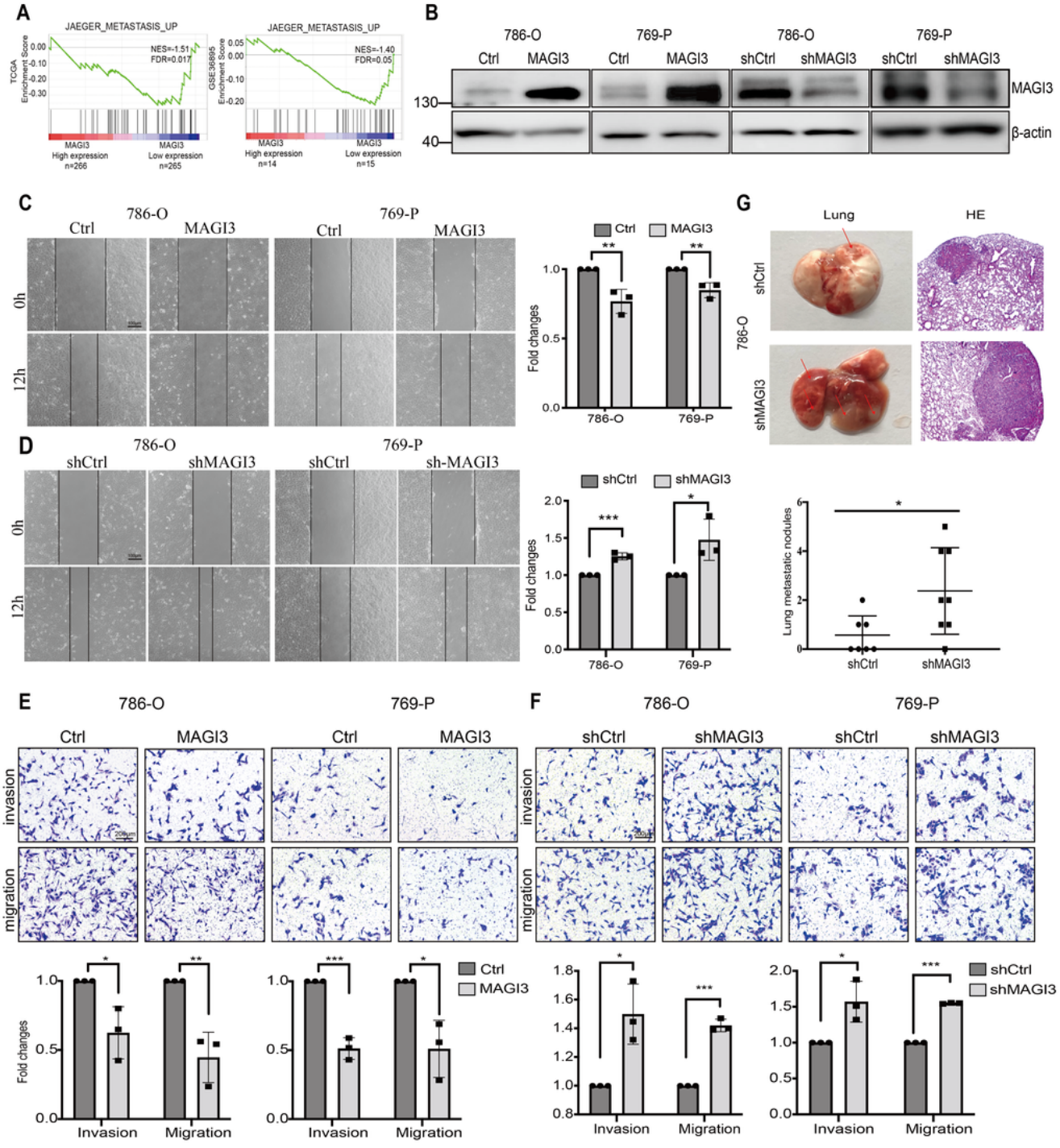

Figure 2

MAGI3 inhibits the migration, invasion and metastasis of ccRCC cells. (**A**) The gene signatures of metastasis-related upregulated gene-set were enriched in ccRCC specimens with MAGI3 low expression group. The data of MAGI3 mRNA expression were from TCGA (left panel), or GSE36895 (right panel) were analyzed by GSEA. **(B)** Stable overexpression or knockdown of MAGI3 in 786-O and 769-P cells was verified by western blotting. (**C**) Ectopic expression of MAGI3 suppressed migration of 786-O and 769-P

cells. (**D**) Knockdown of MAGI3 promoted migration of 786-O and 769-P cells. The in vitro scratch assay was conducted to study wound healing cell migration. Representative images of wound healing cell migration were shown in the left panel. Values were presented as fold change of the migration shown in the right panel (*t* test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Scale bars: 100 μm. (**E**) Overexpression of MAGI3 inhibited migration and invasion of 786-O and 769 cells. **(F)** Knockdown of MAGI3 promoted migration and invasion of 786-O and 769 cells. Transwell assays were conducted to study migration and invasion, respectively. Representative images of cell migration or invasion were shown in the up panel, and values were presented as fold change of migration or invasion shown in the low panel (*t* test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Scale bars, 200 μm. (**G**) Knockdown of MAGI3 expression promoted lung metastasis in a ccRCC xenograft model. 786-O/shCtrl or 786-O/shMAGI3 cells were injected into SCID/NOD mice (n=15) via tail vein. The mice were sacrificed after 3 months, and the number of pulmonary metastatic nodules was statistically analyzed (*t* test, * $p < 0.05$).

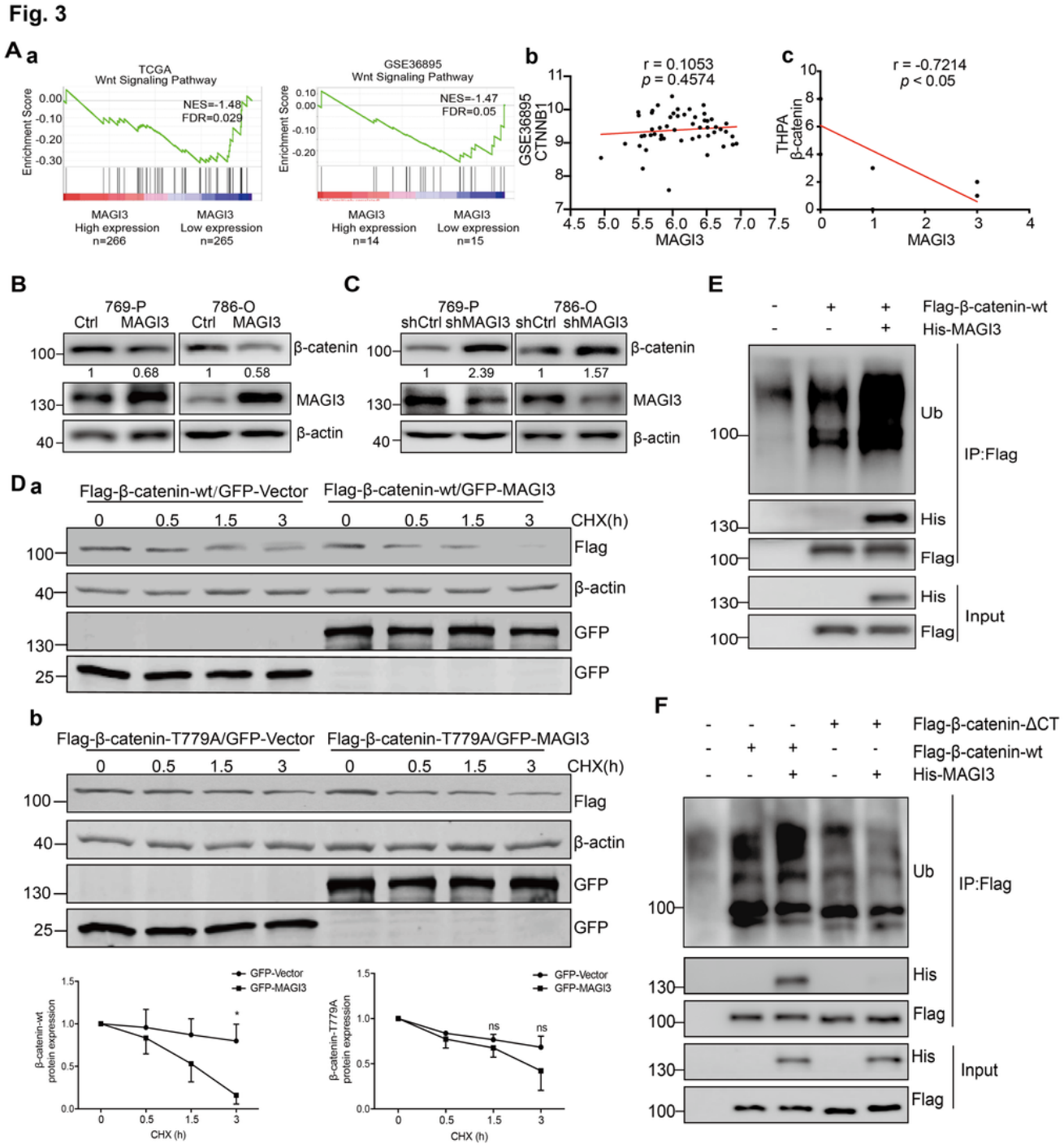

Figure 3

MAGI3 reduces β-catenin protein level through β-catenin ubiquitination and degradation. (A) MAGI3 was negatively correlated with β-catenin protein level and activation of Wnt signaling in ccRCC specimens. (a) Low MAGI3 level was associated with activation of Wnt signaling in ccRCC. The gene signatures of Wnt/β-catenin activation were enriched in patients with MAGI3 lower levels in TCGA KIRC and GSE36895 datasets. (b) MAGI3 did not correlate with β-catenin at mRNA levels in clinical specimens. (c) MAGI3

negatively correlated with β-catenin at protein levels in THPA KIRC datasets. (**B**) MAGI3 overexpression reduced β-catenin protein level in 786-O or 769-P cells. (**C**) Knockdown of MAGI3 increased β-catenin protein level in 786-O or 769-P cells. Cells stably transfected with MAGI3 or Ctrl (B), or knockdown with shMAGI3 or shCtrl (C) constructs respectively, were subjected to western blotting analysis to measure the levels of β-catenin, MAGI3 and β-actin protein. (**D**) Overexpression of MAGI3 decreased the half-lives of β-catenin protein via its interaction with MAGI3. (a) MAGI3 decreased the half-lives of β-catenin protein. (b) By interaction with β-catenin, MAGI3 decreased the half-lives of β-catenin protein. HEK293 cells were transient transfected with Flag-β-catenin-wt (a) or Flag-β-catenin-T779A (b) in absence or presence of GFP-MAGI3 respectively. Cells were treated with CHX (25 μg/mL) as the indicated time before cell harvest for western blotting. The band density was quantified using Image J software. Data were representatives of three independent experiments (n=3). * $p < 0.05$; ns, no significant difference (*t* test). (**E**) Overexpression of MAGI3 promoted β-catenin ubiquitination. (**F**) The interaction with β-catenin was essential for MAGI3 to promote β-catenin ubiquitination. HEK293 cells were transient transfected with Flag-β-catenin-wt (E) or Flag-β-catenin-ΔCT (F) in absence or presence of His-MAGI3 respectively, and treated with MG132 for 10 h. Cell lysates were subjected to IP with anti-Flag antibody-coupled beads. The precipitated complexes were probed with anti-ubiquitin antibody to detect ubiquitinated Flag-β-catenin.

Fig. 4

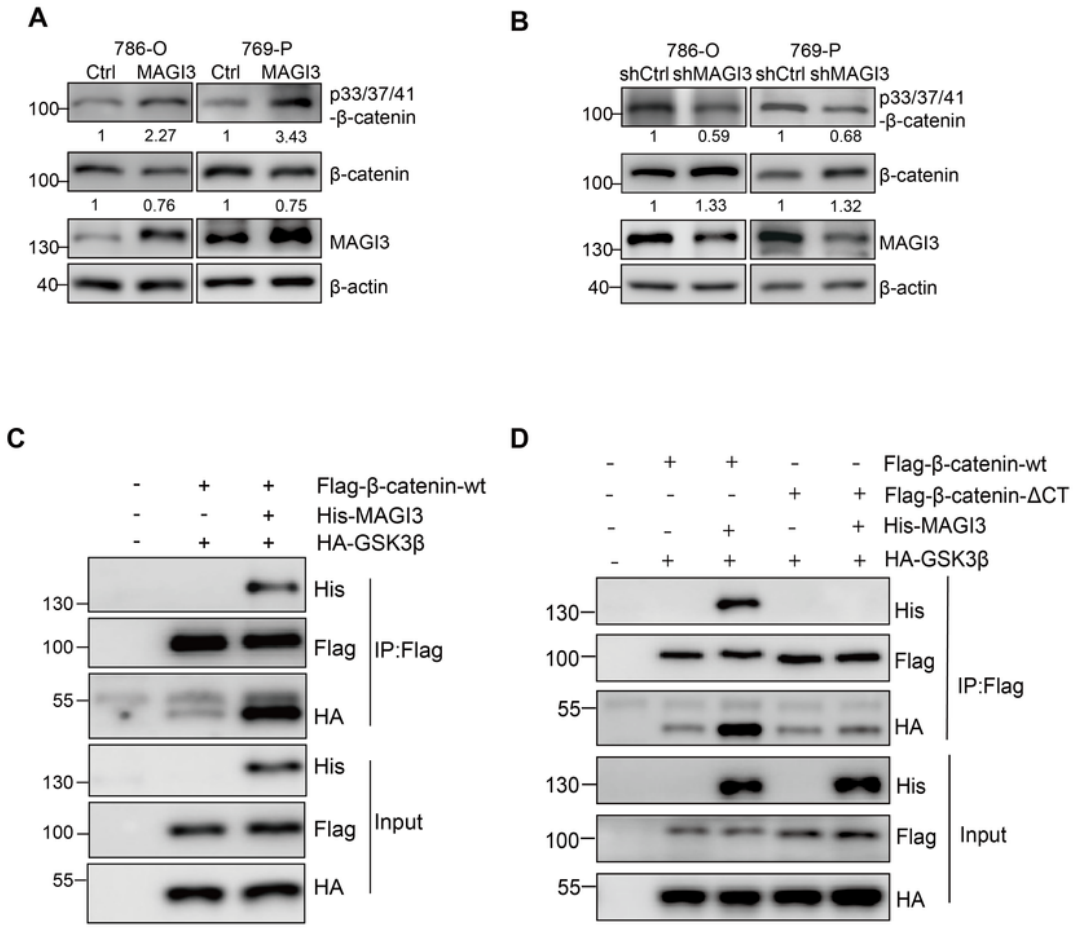

Figure 4

MAGI3 acts as a scaffold protein to facilitate the phosphorylation of β-catenin by GSK3β. (**A**) MAGI3 overexpression enhanced β-catenin phosphorylation and reduced β-catenin protein level in 786-O or 769-P cells. (**B**) MAGI3 kncokdown reduced β-catenin phosphorylation and increased β-catenin protein level in 786-O or 769-P cells. Cells stably transfected with MAGI3 or Ctrl (A), or knockdown with shMAGI3 or shCtrl (B) constructs respectively, were subjected to western blotting analysis. Phosphorylation of β-

catenin at Ser33/Ser37/Thr41 was detected with anti-phospho-β-catenin (Ser33/Ser37/Thr41) antibody (**C**) MAGI3 acted as a scaffold protein to facilitate the interaction between GSK3β and β-catenin. (**D**) The C-terminus of β-catenin plays an indispensable role for MAGI3 in improving the association of GSK3β with β-catenin. HEK293 cells were transient transfected Flag-β-catenin-wt or Flag-β-catenin-ΔCT with HA-GSK3β in presence or absence of His-MAGI3, lysates were precipitated with an anti-Flag antibody and blotted with anti-His, anti-Flag or anti-HA antibody respectively.

Fig. 5

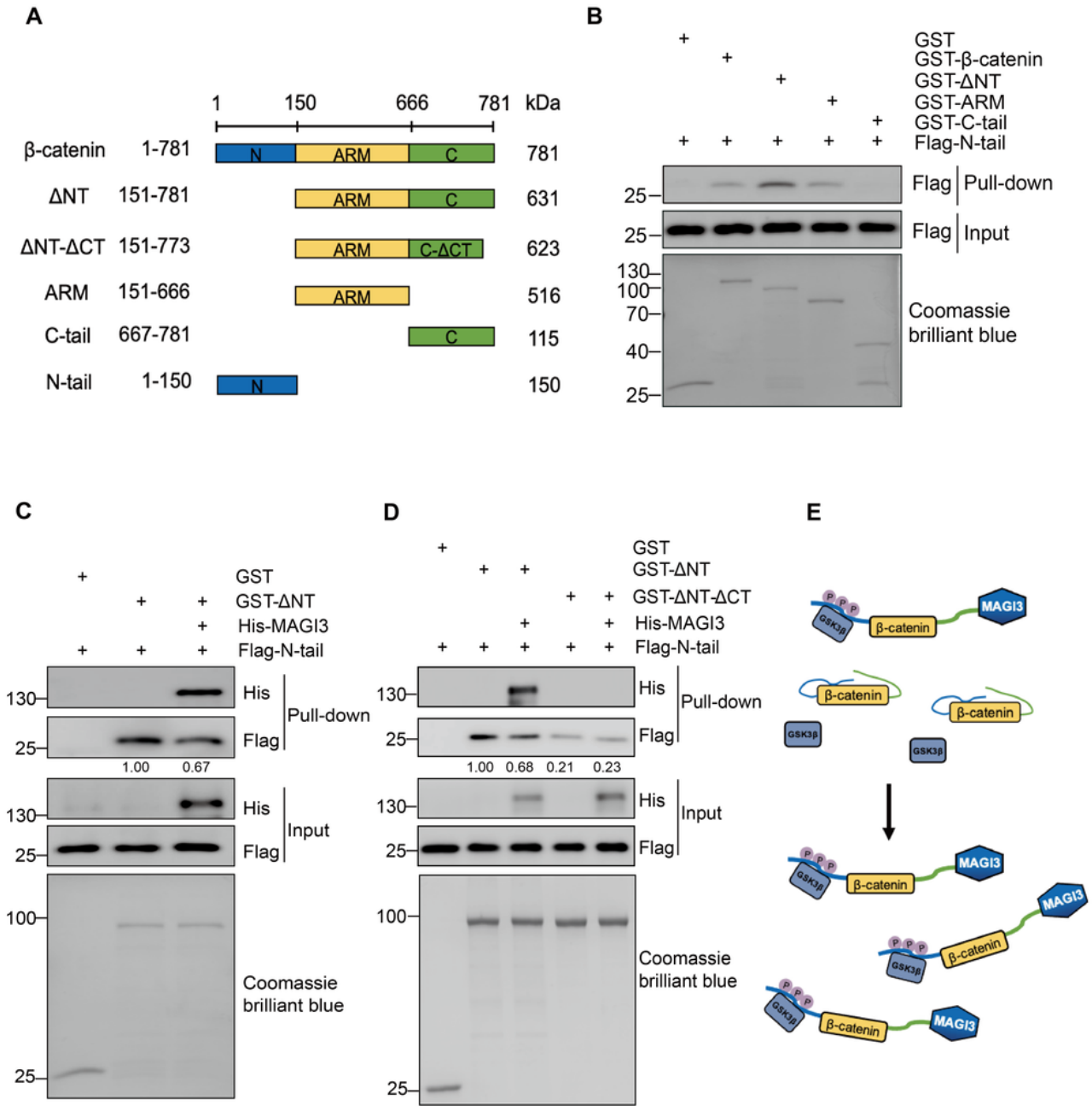

Figure 5

The C-tail of β-catenin binding with MAGI3 facilitated its N-tail to release from intramolecular interaction of β-catenin. **(A)** The diagram of β-catenin GST fusion proteins, showing the full-length β-catenin (WT), N-tail, armadillo domain (ARM), C-tail, ΔNT (β-catenin deleted N-tail) and ΔNT-ΔCT (β-catenin deleted N-tail and C-terminus) of β-catenin. (**B**) β-catenin N-tail associated with its ARM domain to form β-catenin a loop structure by intramolecular interaction. The β-catenin GST fusion proteins containing the indicated domains were used to pull down cell lysates of HEK293 transfected with Flag-N-tail, and the pull downed complexes were detected by western blotting. (**C**) MAGI3 significantly reduced extra molecular Flag-N-tail binding with β-catenin. (**D**) The C-terminus of β-catenin was indispensable for MAGI3 reducing the interaction of extra molecular Flag-N-tail with β-catenin. The β-catenin GST fusion proteins containing the indicated domains were used to pull down cell lysates of HEK293 transfected with Flag-N-tail w/o His-MAGI3, and the pull downed complexes were detected by western blotting. (**E**) The schematic illustration of the mechanism that MAGI3 facilitated β-catenin N-tail to release from its intramolecular interaction.

Fig. 6

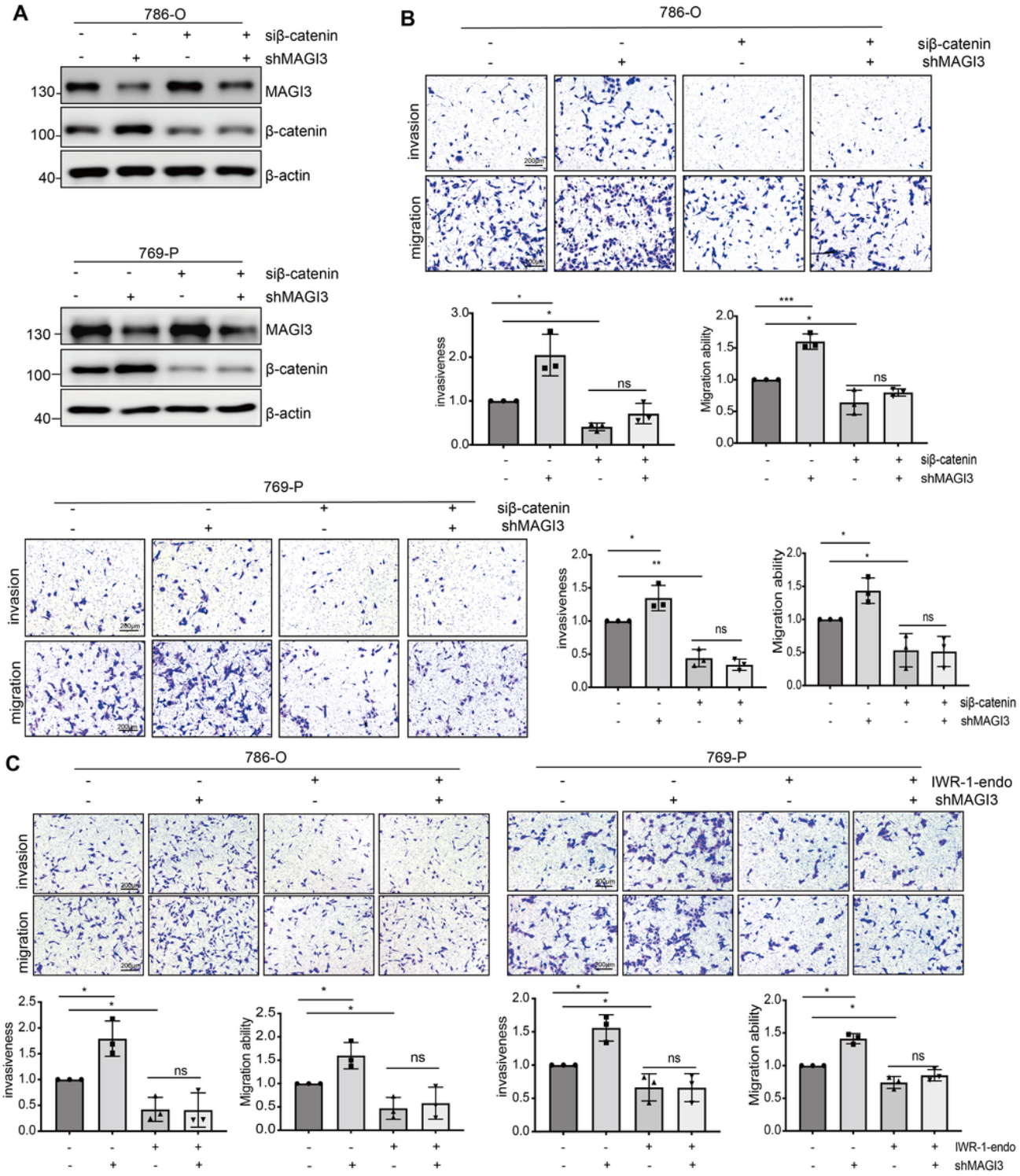

Figure 6

MAGI3 suppresses ccRCC cell migration and invasion through inhibition of Wnt/β-catenin signaling. (**A**) Knockdown of β-catenin rescused β-catenin protein level increased by MAGI3 knockdown. (**B**). Knockdown of β-catenin recused the cell migration and invasion increased by knockdown of MAGI3. 786-O or 769-P cells were transfected with shMAGI3 alone, or in presence with β-catenin siRNA, cell lysates were analyzed by western blotting with indicated antibodies. The migration and invasion were detected

by transwell assays. (**C**) Blocking Wnt/β-catenin signaling recused the cell migration and invasion increased by knockdown of MAGI3. 786-O or 769-P cells were transfected with shMAGI3 alone, or in presence with IWR-1-endo, an inhibitor of Wnt/β-catenin pathway. The migration and invasion were detected by transwell assays (*t* test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, no significant difference; values represent mean ± SD, n = 3).

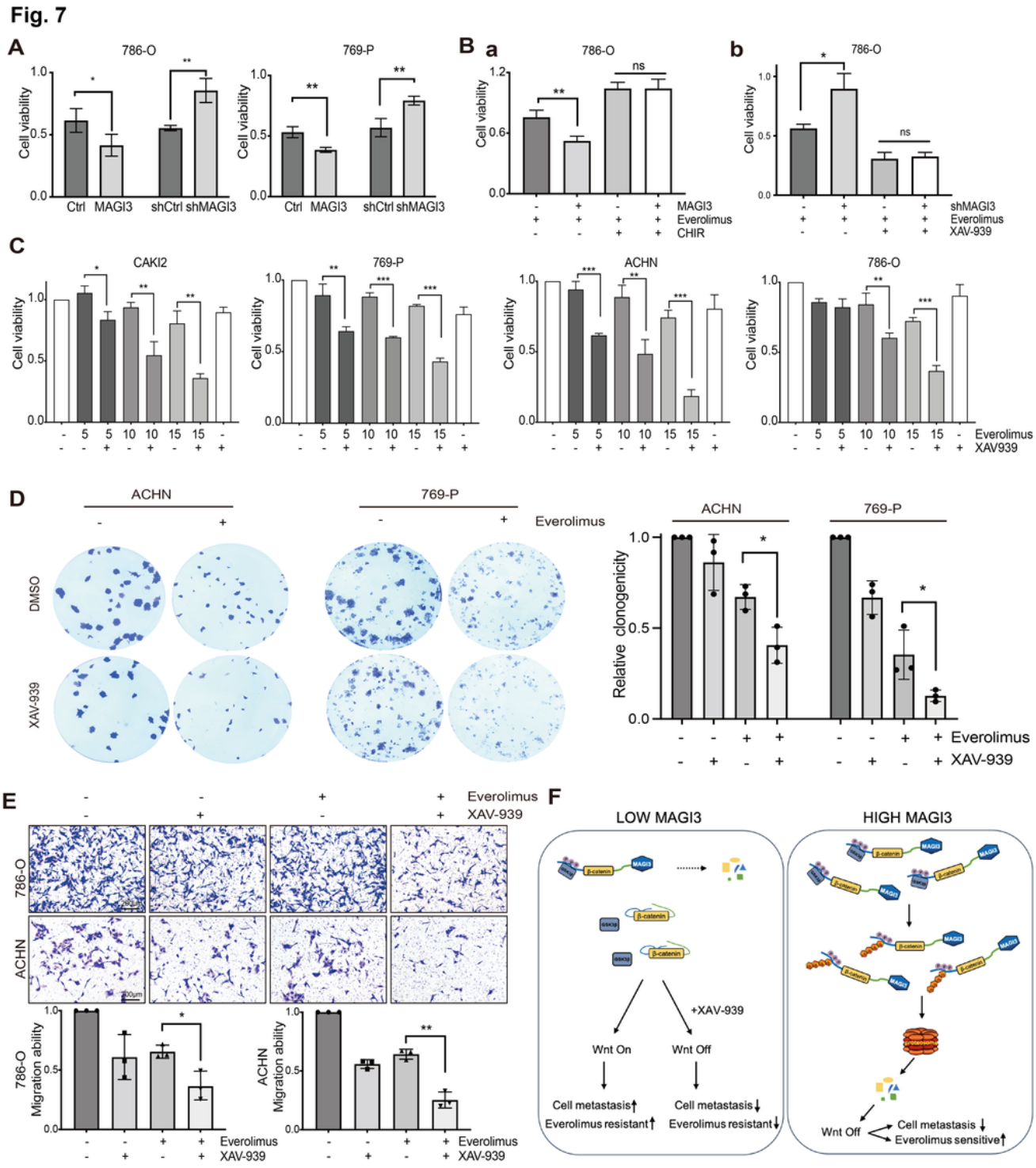

Figure 7

MAGI3 confers sensitivity to rapalogs in ccRCC via inhibition of Wnt/β-catenin signaling. (**A**) MAGI3 expression promoted sensitivity to rapalogs in ccRCC. Overexpression of MAGI3 inceased sensitivity of ccRCC cells to Everolimus, and knockdown of MAGI3 decreased sensitivity of ccRCC cells to Everolimus. Cell viability of 786-O or 769-P was determined using CCK-8 assays. (**B)** MAGI3 expression promoted sensitivity to rapalogs in ccRCC through Wnt/ β-catenin signaling. (Ba) The sensitivity of Everolimus to 786-O cells increased by MAGI3 over expression was abolished when the cell treated with CHIR (0.1 μM) to activate Wnt/β-catenin pathway. (Bb) The drug sensitivity decreased by MAGI3 knockdown was reversed when the cell treated with XAV-939 (50 μM), an inhibitor of Wnt/β-catenin pathway, to suppress Wnt/β-catenin pathway. Cell viability of 786-O was determined using CCK-8 assays. (**C**) The sensitivity of ccRCC cells to Everolimus was dose-dependently increased when treated with XAV-939 (50 μM). Cell viability of ccRCC cells treated with Everolimus (5~15 μM for 24 h respectively) was determined using CCK-8 assays. The values are depicted as the relative viability to the DMSO treatment. (**D**) Combination treatment with XAV-939, Everolimus could synergistically inhibited cell viability of ccRCC.
Colony formation assays were conducted in ACHN or 769-P cells treated with Everolimus in absence or presence of XAV-939 for 10-14 days, ACHN (Everolimus 0.001 μM, XAV-939 10 μM) or 769-P (Everolimus 0.01 μM, XAV-939 1 μM). (**E**) Combination treatment with XAV-939, Everolimus could synergistically inhibited cell migration of ccRCC. Transwell migration assays were conducted in ACHN or 786-O cells treated with Everolimus (1 μM) in absence or presence of XAV-939 (10 μM) for 24 h or 17 h respectively. (*t* test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, no significant difference; values represent mean ± SD, n = 3). (F) Schematic illustration of the mechanisms underlying the inhibitive effects of MAGI3 expression on metastasis and rapalogs resistance of ccRCC via retarding Wnt/β-catenin signaling.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementalinformation.pdf
- MAGI3cox.xls