MicroRNA-3648 Is Upregulated to Suppress TCF21, Resulting in Promotion of Invasion and Metastasis of Human Bladder Cancer

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Although microRNAs (miRNAs) are well-known for their potential in cancer, the function and mechanisms of miR-3648 have barely been explored in any type of cancer. We show here that miR-3648 is upregulated in human BC tissues in comparison with adjacent non-tumor tissues. Functional studies showed that inhibition of miR-3648 expression in the human invasive BC UMUC3 and T24T cell lines decreased migration and invasion in vitro and suppressed lung metastasis in vivo, whereas miR-3648 overexpression promoted BC cell migration and invasion. A bioinformatics screen and mRNA 3' UTR luciferase reporter assay showed that transcription factor 21 (TCF21) was a direct target of miR-3648, and the results obtained from using a miR-3648 inhibitor revealed that miR-3648 inhibited TCF21 protein expression by reduction of its mRNA stability. Further, Kisspeptin 1 (KISS1) was identified as a TCF21 downstream effector responsible for miR-3648-mediated BC invasion and lung metastasis. Collectively, the present results suggest that miR-3648 is overexpressed and plays an oncogenic role in mediation of BC invasion and metastasis through directing the TCF21/KISS1 axis, revealing miR-3648 as a potential biomarker for BC prognosis and a target for BC therapy.

INTRODUCTION

Bladder cancer (BC) is a complex disease with high morbidity and mortality.1,2 It is the fourth most common malignancy in the world and the second most common cause of death among urinary tumors.3,4 BCs are divided into two categories: non-muscle-invasive BC (NMIBC) and muscle-invasive BC (MIBC). MIBC is associated with a higher degree of malignancy and a greater rate of metastasis and is responsible for 100% of deaths of BC patients.5,6 Despite advances in surgical intervention and adjuvant chemotherapy, some patients with NMIBC experience recurrence and progress to MIBC.7,8 Therefore, identification of biomarkers of metastasis is urgently needed to improve the treatment of patients with BC.

MicroRNAs (miRNAs) are small, non-coding, single-stranded RNAs approximately 22 nt in length that are closely related to the development of cancer.9,10 miRNAs regulate mRNA expression by recognizing and binding to the 3' UTR of target mRNAs, which affects their stability or inhibits their protein translation.11-13 miRNAs have been implicated in a variety of physiological processes, including cell differentiation, the cell cycle, apoptosis, and metastasis.14,15 The function of miRNAs as tumor suppressors or oncogenes depends on the functions of their target mRNAs and stages of cancer.16 Abnormal expression of miRNAs is considered a biomarker for the diagnosis, prognosis, and treatment of cancer.17,18 However, the potential alteration and biological function of miR-3648 in cancer, especially in BC, remains unexplored, to the best of our knowledge. We therefore examined the alteration of miR-3648 in BC tissues in comparison with their paired adjacent non-tumor bladder tissues or in BC cell lines versus normal urothelial cell lines and evaluated the biological role and molecular mechanism underpinning miR-3648 action in BC invasion and metastasis.

Transcription factor 21 (TCF21), which is located on chromosome 6q23, belongs to the basic helix-loop-helix family. It is downregulated...
in several malignancies, including lung, breast, colon, and gastric cancer.\textsuperscript{19,20} TCF21 is associated with the occurrence, invasion, and metastasis of tumors and might be an important mediator involved in tumor progression.\textsuperscript{21} However, the mechanisms underlying the downregulation of TCF21 in cancer and its tumor suppressor properties are unclear.\textsuperscript{22} In the present study, we identified TCF21 as a direct target of miR-3648 and that it is downregulated by miR-3648 at the mRNA degradation level, in turn affecting KISS1 expression and BC invasion and metastasis.

RESULTS

\textbf{miR-3648 Is Upregulated in Human BC Tissues and Cell Lines}

miRNAs may serve as biomarkers for cancer prognosis and treatment.\textsuperscript{23,24} However, the potential alteration and role of miR-3648 in cancer remains unclear. To confirm the function of miR-3648 in BC, miR-3648 expression was analyzed by real-time PCR in 33 pairs of fresh human BC tissues and adjacent non-tumor bladder tissues (3 cm away from tumor lesion margin), and the results showed that miR-3648 was remarkably upregulated in human BC tissues (n = 33, \( p < 0.05 \)) (Figure 1A), suggesting that miR-3648 is overexpressed in BC tissues. This notion was greatly supported by the results obtained from the bioinformatics analysis of all of 19 pairs of BC patients from The Cancer Genome Atlas (TCGA) database, showing that miR-3648 expression is significantly higher in BC patients than in normal controls (Figure S1). The expression of miR-3648 was also analyzed by real-time PCR in BC cell lines (5637, T24, T24T, UMUC3, and TCCSUP) in comparison with two immortalized human urothelial cell lines (SV-HUC-1 and UROtsa). The results indicated that miR-3648 expression is higher in BC cells than in SV-HUC-1 cells and UROtsa cells (Figure 1B). These results indicate that miR-3648 is markedly upregulated and might play a role in BC development.

\textbf{Inhibition of miR-3648 Decreases BC Cell Migration and Invasion In Vitro}

To explore the function of miR-3648 in BC cells, a specific miR-3648 sponge inhibitor and control vector were transfected into UMUC3 and T24T cells (Materials and Methods) to establish stable transfectants. Real-time PCR showed that miR-3648 was successfully downregulated in T24T and UMUC3 cells compared with control cells (Figures 1C and 1D). To examine the effect of miR-3648 on migration and invasion, Transwell assays were performed using UMUC3(miR-3648i), T24T(miR-3648i), and the corresponding control cells. The
results indicated that the number of migrated and invaded cells was lower in T24T(miR-3648i) and UMUC3(miR-3648i) than in vector control cells (Figures 1E–1H), indicating that overexpressed miR-3648 has a positive effect on the migration and invasion of UMUC3 and T24T cells in vitro, which may be correlated with BC metastasis.

Ectopic Expression of miR-3648 Promotes BC Cell Migration and Invasion

To confirm the involvement of miR-3648 in BC, miR-3648-overexpressing stable transfectants were established in T24T and UMUC3 cells (Materials and Methods). The efficiency of miR-3648 overexpression was confirmed by real-time PCR (Figures 2A and 2D). A wound healing assay was performed to measure the migration of BC cells. The results showed that, 36 h after the monolayer scratch, the wound area was smaller in T24T(miR-3648) cells than in control cells, suggesting that miR-3648 overexpression promotes the migration of BC cells in vitro (Figures S2A and S2B). Migration and invasion assays using T24T(miR-3648), UMUC3(miR-3648), and the respective control cells showed that miR-3648 promotes the invasive ability of BC cells compared with controls (Figures 2B and 2E). These results reveal that miR-3648 contributes to BC cell migration and invasion and may play a role in BC metastasis (Figures 2C and 2F).

miR-3648 Targets the 3’ UTR of TCF21 mRNA to Destabilize Its mRNA

To examine the mechanism underlying the regulation of BC cell migration and invasion by miR-3648 in relation to the suppression of target gene expression mediated by binding to the 3’ UTR of target genes, potential miR-3648 target genes were screened using TargetScan (http://www.targetscan.org). The screening revealed that HOXA3 and TCF21 might be miR-3648 candidate target genes that could interact with the 5’ end of miR-3648 (Figure 3A). Western blot analysis was performed to determine whether miR-3648 regulates the expression of HOXA3 and TCF21 in BC cells. MiR-3648 inhibition had no consistent effect on HOXA3 expression in T24T and UMUC3 cells (Figure 3B), whereas TCF21 was markedly upregulated in both T24T(miR-3648i) and UMUC3(miR-3648i) cells. Conversely, miR-3648 overexpression downregulated TCF21 in both T24T(miR-3648) and UMUC3(miR-3648) cells without showing a consistent effect on HOXA3 (Figure 3B). These results suggest that miR-3648 downregulates TCF21 protein expression in BC cells. To confirm that TCF21 is a direct target of miR-3648, TCF21 mRNA 3’ UTR luciferase reporters were employed, and the results showed that inhibition of miR-3648 resulted in activation of the TCF21 mRNA 3’ UTR-wild type (WT) reporter, whereas such activation was completely abolished in the miR-3648 binding site-mutated reporter (Figure 3C). These results demonstrate that miR-3648 binds directly to the TCF21 mRNA 3’ UTR to regulate TCF21 expression. To further examine the mechanism underlying miR-3648 regulation of TCF21 expression, real-time PCR detection of TCF21 mRNA levels was performed in T24T(miR-3648i) and UMUC3(miR-3648i) cells. The results showed that TCF21 mRNA levels were significantly higher in T24T(miR-3648i) and UMUC3(miR-3648i) cells than in vector control cells (Figure 3D). This was consistent with TCF21 protein expression levels, suggesting that miR-3648 downregulates TCF21 at the mRNA level. The effect of miR-3648 on inducing TCF21 mRNA degradation was confirmed using TCF21 mRNA stability assays, and the decay rate of TCF21 mRNA was assessed by semiquantitative PCR. As shown in Figure 3E, the rate of TCF21 degradation in response to actinomycin D (Act D; 10 μM) treatment for 12 h was lower in UMUC3(Vector) cells than in UMUC3(miR-3648i) cells, revealing that TCF21 mRNA stability increases in a time-dependent manner in UMUC3(miR-3648i) cells and that miR-3648 plays a crucial role in TCF21 mRNA degradation in BC cells.
defined. Therefore, we further explored the role of TCF21 in BC cells. The results of H&E staining, immunohistochemistry (IHC), and real-time PCR indicated that TCF21 expression was significantly downregulated in BC tissues relative to normal bladder tissues at the protein (Figures 4A and 4B) and mRNA levels (Figure 4C). The results from analyses of the TCGA database also showed that TCF21 was dramatically downregulated in all available 19 paired (with normal tissue) BC patients (Figure 4D). These results indicated that TCF21 may act as a tumor suppressor in BC. This notion was verified in the functional determination of TCF21 in BC by overexpressing TCF21 in T24T and UMUC3 cells (Figures 4E and 4F), showing that TCF21 overexpression inhibits BC cell migration and invasion (Figures 4F–4J). Collectively, our results demonstrate that TCF21 is overexpressed in BC and that the overexpressed TCF21 inhibits BC cell migration and invasion.

TCF21 Is a miR-3648 Downstream Negative Mediator Responsible for miR-3648 Promotion of BC Migration and Invasion

The role of TCF21 in miR-3648 inhibition-mediated suppression of BC invasion and migration was investigated by short hairpin RNA (shRNA)-mediated silencing of TCF21 in UMUC3(miR-3648i) and T24T(miR-3648i) cells. Knockdown of TCF21 in T24T(miR-3648i/shTCF21#1), T24T(miR-3648i/shTCF21#4), UMUC3(miR-3648i/shTCF21#1), and UMUC3(miR-3648i/shTCF21#4) cells could reverse the inhibitory effect of miR-3648i on inhibition of cell migration and invasion (Figures 5A–5F). These results suggest that TCF21 is a direct target and downstream mediator of miR-3648 for cell migration and invasion in BC cells.

KISS1 Is a miR-3648/TCF21 Downstream Effector for Promoting BC Cell Migration and Invasion

Matrix metalloproteinase-2 (MMP2) is a member of the matrix metalloproteinase (MMP) family, which is involved in breakdown of the extracellular matrix in normal physiological processes and tumor invasion and metastasis.25 The KISS1 gene was initially discovered to be a tumor metastasis suppressor gene of melanoma cells in nude mice without affecting their tumorigenicity.26 To explore miR-3648 and TCF21 downstream effector(s) in promoting BC cell migration and invasion, the effects of miR-3648 and miR-3648i on invasion/migration-related molecules, such as MMP2 and KISS1, were evaluated in T24T(miR-3648), T24T(miR-3648i), UMUC3 (miR-3648), and UMUC3 (miR-3648i) in comparison with their respective vector transfectants. The results showed that there was no significant change in MMP2 expression, whereas KISS1 was remarkably upregulated, which was correlated with TCF21 upregulation (Figure 6A). This observation suggests that KISS1 could be a downstream effector of TCF21 in mediating BC invasion because of miR-3648 overexpression. The results obtained from western blot detection of KISS1 in T24T(miR-3648i/shTCF21#1) and T24T(miR-3648i/shTCF21#4)

![Figure 3. miR-3648 Regulates the Direct Target TCF21 at the mRNA Degradation Level in BC Cells](image-url)
Inhibit migration and invasion in BC cells.

Direct target of miR-3648 and positively regulates KISS1 expression to effect of shTCF21 on promoting BC cell migration and invasion (Figure 3). Attenuation of miR-3648 by its inhibitor suppresses mouse lung metastasis in vivo. (A) H&E staining and IHC staining were performed to evaluate morphology and TCF21 expression in 33 pairs of BC tissues. IHC images were captured using the Nikon Eclipse Ni microsystem (Materials and Methods). (B) The TCF21 protein expression levels were analyzed by calculating the integrated IOD per area. Student’s t test was used to determine the p value (p < 0.05). (C) Real-time PCR was used to determine the expression level of TCF21 mRNA in BC tissues, and GAPDH was used as the internal control. The results are expressed as the mean ± SD, and the asterisk indicates significant downregulation relative to the control group (p < 0.05). (D) The fold change of TCF21 in BC tissues obtained from analysis of the TCGA database (n = 19), (E and F) TCF21 overexpression in T24T (G) and UMUC3 (H) cells, GAPDH was used as the internal control. (G and H) The migration and invasion abilities of T24T(Vector) (G) or UMUC3 (H) transfected cells were determined (Materials and Methods). (H and I) Migrated and invaded T24T (H) and UMUC3 (J) transfected cells were counted. The results are expressed as the mean ± SD. Student’s t test was used to determine the p value, and the asterisk indicates a significant decrease (p < 0.05).

Cells and their respective control cells showed that TCF21 knockdown in T24T(miR-3648i) cells dramatically attenuated KISS1 levels (Figure 6B), greatly supporting our notion that KISS1 is the downstream target of TCF21 in our cell model. Because KISS1 acts as a tumor invasion suppressor, we examined its potential tumor suppressor role in BC cell invasion with miR-3648 downregulation. KISS1 overexpression in T24T(miR-3648i/shTCF21#1) cells (Figure 6C) restored the effect of shTCF21 on promoting BC cell migration and invasion (Figures 6D and 6E). Taken together, these results suggest that TCF21 is a direct target of miR-3648 and positively regulates KISS1 expression to inhibit migration and invasion in BC cells.

Attenuation of miR-3648 by Its Inhibitor Suppresses Mouse Lung Metastasis of Human BC Cells In Vivo

To extend our findings in vivo, a lung metastasis model was established in nude mice via tail vein injection of T24T(Vector), T24T(miR-3648i), UMUC3(Vector), and UMUC3(miR-3648i) cells. After 2 months of injection, the lung tissue of nude mice was surgically excised and fixed with picric acid for 24 h, and metastasis was analyzed by imaging the lung tissue using a stereoscopic microscope. miR-3648 inhibition by its inhibitor resulted in suppression of BC lung metastasis in both T24T cells and UMUC3 cells (Figures 7A–7D), consistent with the in vitro results. A total of 128 and 16 lung metastatic tumors were observed in mice injected with T24T(Vector) and UMUC3(Vector) cells, respectively, which were remarkably reduced to 19 and 6 lung metastatic tumors in mice injected with T24T(miR-3648i) and UMUC3(miR-3648i) (Tables 1 and 2). Taken together, these results indicate that miR-3648 has an oncogenic role in BC cell migration and invasion in vitro and metastasis in vivo, as diagrammed in Figure 7E, suggesting that miR-3648 has potential as a target for BC treatment.

DISCUSSION

BC is a heterogeneous disease, with 30% presenting as muscle-invasive disease associated with a high risk of death from distant metastases.27-29 Invasion through the basement membrane is the hallmark of malignancy; therefore, improving our understanding of the migration and invasion processes in BC would be useful to develop new treatment strategies.30-32 In the present study, we showed that miR–3648 was markedly upregulated in BC tissues in comparison with adjacent non-tumor bladder tissues, and this finding was also greatly supported by the results obtained from analysis of the TCGA database. The results of the present...
study demonstrate that miR-3648 has a positive regulatory effect on BC migration and invasion in vitro and lung metastasis in vivo. Of two predicted targets of miR-3648, TCF21 was identified as a direct target of miR-3648 in BC cells. We show that miR-3648 promotes BC migration and invasion by downregulating the expression of the tumor suppressor TCF21 at the mRNA level, downregulating the TCF21 protein, which positively regulates KISS1 (Figure 7E). KISS1 is a well-known protein with an important role in inhibition of BC invasion,33 and our results reveal the specific function of miR-3648 in regulating BC migration and invasion and metastasis without affecting BC cell growth (Figure S3), suggesting that miR-3648 could serve as a novel target for the treatment of metastatic BC. A previous study suggested that miR-3648 has roles in promoting HBV-positive HCC cell proliferation.34 Endoplasmic reticulum (ER) stress mediates induction of miR-3648 in human HEK293T cells, which then downregulates adenomatous polyposis coli 2 (APC2) to increase cell proliferation.35 miR-3648 has also been found to be upregulated in several diseases, such as renal cell carcinoma (RCC), hepatocellular carcinoma (HCC), and upper tract urothelial carcinoma (UT-UC);34,36,37 however, the functions and objective mechanisms of miR-3648 in these diseases has not been clarified. In this study, we found that miR-3648 is upregulated in BC tissues and cell lines, which specifically positively regulates the ability of BC migration and invasion without any observed effect on BC cell growth, revealing that overexpressed miR-3648 has a novel function in human BC cells that is distinct from its role in other cancers. We further discovered that TCF21 is a direct target of miR-3648 and that miR-3648 suppresses TCF21 levels and increases BC cell migration and invasion in vitro and lung metastasis in vivo.

The function of miRNA is mainly to bind to the 3' UTR or 5' UTR of its target mRNA to regulate mRNA transcription, stability, and protein translation.38,39 miR-3648 has been predicted to bind to the hoxa3 mRNA 3' UTR and regulate the expression of HOXA3, a member of the homeobox gene cluster reported to be involved in modulation of cell proliferation and migration.40 HOXA3 is required for cell proliferation and differentiation in the third pharyngeal arch mesenchyme.41 HOXA3 promotes endothelial cell migration in adult tissues during wound repair42 and promotes endothelial cell migration and angiogenesis in vivo.43 However, our results show that overexpression of miR-3648 in BC has no overt effect on HOXA3 expression, and it was therefore excluded as a direct target of miR-3648 in BC cells. The transcription factor TCF21 is expressed in numerous tissues,44 and recent studies reveal that TCF21 acts as a tumor suppressor and that its downregulation increases the number of undifferentiated mesenchymal cells and lung cancer cell migration; however, the underlying mechanism remains to be elucidated.45,46 Consistent with these observations, forced expression of TCF21 in BC cells suppresses BC cell migration and invasion. Loss or reduced expression of TCF21 occurs in different cancers, including BC,47 although the underlying mechanism is unclear.48,49 The results obtained from the current studies indicate that miR-3648 is able to bind to the 3' UTR of tcf21 mRNA to reduce mRNA stability, inhibiting TCF21 expression. Given our results showing that miR-3648 is overexpressed in human
Metastasis is a complex process that involves impairment of cell-cell adhesion, invasion into adjacent tissue, and the spread of cancer cells through the lymphatic and vascular routes. It is originally described as a suppressor of metastasis in malignant melanoma, and further studies showed that KISS1 suppresses metastasis in various cancers, including esophageal, breast, pancreatic, gastric, ovarian, prostate, and bladder cancer, through regulation of cell migration and invasion. It has been reported that TCF21 is downregulated in prostate, and bladder cancer, through regulation of cell migration and invasion. These findings provide insight into the upstream regulator being responsible for TCF21 downregulation and further mediating downregulation of the downstream effector KISS1 of miR-3648 for promotion of migration and invasion in human BCs.

In summary, the results of the present study indicate that miR-3648 functions as an oncogene by directly targeting and inhibiting expression of the mRNA stability of the tumor suppressor TCF21, which leads to a reduction in KISS1 expression, promoting BC cell migration and invasion. These findings provide insight into the upstream regulator responsible for TCF21 downregulation and further mediating downregulation of the downstream effector KISS1 of miR-3648 for promotion of migration and invasion in human BCs, further suggesting that miR-3648 could be a potential target for the treatment of invasive and metastatic BCs.

MATERIALS AND METHODS

Plasmids, Antibodies, and Reagents
The miR-3648 sponge inhibitor (miR-3648i), miR-3648 overexpression constructs, and control vector were purchased from GenePharma (CS819, Shanghai, China). The human TCF21 mRNA 3’ UTR was cloned into the pMIR luciferase reporter vector obtained from Applied Biosystems (AM5795, Foster City, CA, USA). The TCF21 mRNA 3’ UTR point mutation was amplified from the WT template by overlapping PCR using the following primers: forward, 5’-CCC CAG CGC AGC CGG GCC GGG ATG CGG CAG A-3’; reverse, 5’-TCT GGC GCA TCG GCC CGG CCG GGC GGC ATG GC-3’. The set of shRNAs for TCF21 was purchased from Genecopoeia (RHS4531-EG6943, Lafayette, CO, USA). The antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (AB0037) and tubulin (AB0012) were bought from Abways (Shanghai, China). The antibody against TCF21 was purchased from GeneTex (GTX52981, Irvine, CA, USA). The antibodies against KISS1 (sc-101246), HOXA3 (sc-RHS4531-EG6943, Lafayette, CO, USA). The antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (AB0037) and tubulin (AB0012) were bought from Abways (Shanghai, China). The antibody against TCF21 was purchased from GeneTex (GTX52981, Irvine, CA, USA).

Cell Culture and Transfection
Human BC cell lines (5637, T24, T24T, UMUC3, and TCCSUP) and immortalized normal human urothelial cell lines (SV-HUC-1 and immortalized normal human urothelial cell lines (SV-HUC-1 and
UROtsa) were used in the study. UMUC3 cells were maintained at 37°C in a 5% CO2 incubator in DMEM (11995-065, Gibco, USA) supplemented with 10% fetal bovine serum (FBS; 1750114, Gibco). T24 and T24T cells were cultured with 5% FBS and DMEM-F12 medium (10565-018, Gibco). UROtsa and 5637 cells were cultured in RPMI 1640 medium (11875-093, Gibco) containing 10% FBS. TCCSUP cells were cultured in 10% minimum essential medium (MEM; 11095-080, Gibco). SV-HUC-1 cells were cultured with 10% F-12K (21127-022, Gibco). Stable transfections were performed with specific cDNA constructs using PolyJet DNA in vitro transfection reagent (SL100688, SignaGen Laboratories, MD, USA) according to the manufacturer’s instructions. Stable transfectants were selected by puromycin, hygromycin, or G418 for 4–6 weeks. The shRNA against TCF21(shTCF21) was introduced into BC cells by lentivirus infection, and stable transfectants were screened with hygromycin for 3–4 weeks.55

Lentivirus Infection

For lentiviral packaging, 293T cells were used following the manufacturer’s instructions, and transfections were performed for 48 h. The supernatant was collected and filtered using a 0.45-µm-pore sterile filter after centrifugation at 2,500 rpm for 30 min. A certain amount of the filtered supernatant was used to infect target cells, followed by culture for 48–72 h. Then the culture medium was removed and replaced with 2 mL of complete medium with 300 µg/mL hygromycin, and cells were incubated at 37°C with 5% CO2 until control cells (without infections) died completely (2–3 days).

Real-Time PCR

Total miRNA was isolated from 5 × 10⁶ cells with the miRNeasy Mini Kit (QIAGEN), and real-time PCR was performed with the Q6 real-time PCR system (Applied Biosystems, CA, USA). The primer for miR-3648 (5’-AGC CGC GGG GAT CGC CGA G-3’) was synthesized by Sunny Biotechnology (Shanghai, China), and U6 was used as an internal loading control.56

Semiquantitative PCR

The TCF21 PCR amplification included an initial “hot start” 5-min denaturation by incubation at 94°C, followed by 30 cycles of denaturation (94°C, 30 s), annealing (58°C, 30 s), and elongation (72°C, 30 s). An additional 72°C final extension step was performed for 10 min. All procedures were performed using an Eppendorf instrument (22331, Hamburg, Germany). Annealing temperatures were selected according to the melting temperature (Tm) of TCF21 primers, which were as follows: TCF21 forward (5’-CCC AAG CTT ATG TCC ACC CGG TCC CTC AGC-3’) and reverse (5’-CGC TCT GAG TCA GGA TCA CCG GTC-3’).
CGC GGT GGT TCC ACA TA-3′). Amplifications were typically performed in volumes of 20 μL per template and primer combination, with 1 μL each of 30 μM TCF21 forward and reverse primer stock solutions, 1 μL of 1 μM GAPDH forward and reverse primer mix stock solutions, 10 μL of 2× Taq Polymerase PCR Master Mix (PC0902, Aidlab Biotechnologies, Beijing, China), and 1 μL of cDNA (cDNAs were synthesized from 2.5 μL stock solutions, 10 μM solutions, 1 μL) with 1 μL of 0.2–12% SDS-polyacrylamide gel and then wet-electro-transferred to PVDF membranes (Immobilon, USA). The cDNAs were synthesized from 2.5 μL stock solutions, 10 μM solutions, 1 μL of cDNA (cDNAs were synthesized from 2.5 μg of total RNA with the ThermoScript RT-PCR system). Double-distilled water was added to the reactions to reach a volume of 20 μL.

**Wound Healing Assay**

Cells (5 × 10^5) were plated in 6-well plates, wounded with a sterile pipette tip, and washed twice with PBS after reaching 95%–100% confluence. Then fresh complete medium was added, and the cells were incubated for 36 h. Photographs of the same wound position were taken at the indicated times until the wounds healed. The wound area was measured and quantified. Experiments were performed in triplicate.

**Migration and Invasion Assay**

For the migration assay, 3 × 10^4 cells were seeded in 0.1% FBS medium into Transwell chambers (353097, Corning, USA) and allowed to migrate for 24 h. Invasion assays were performed according to the manufacturer’s instructions (Corning Biocoat Matrigel invasion chambers, 354480). Cells were seeded into the upper chamber at a density of 3 × 10^4 cells/mL in 400 μL of 0.1% medium, and 700 μL of complete medium was placed in the lower chamber. Cells were allowed to invade for 24 h. Then the cells in and outside of the chamber were fixed with 3.7% formalin for 5 min at room temperature, washed twice with PBS, and transferred to 100% methanol for 20 min. The cells were then washed twice with PBS and stained using Giemsa (1:20 diluted in PBS) at room temperature (RT) for 15 min in the dark. After an additional two washes, cells were scraped off from the upper surface of the membranes with a cotton swab (PBS-wetted) 3–4 times, and stained cells were counted in five randomly chosen fields under a light microscope (DMi1, Germany). The experiment was repeated three times.

**Western Blotting**

Equivalent amounts of total protein (40 μg) were separated using a 12% SDS-polyacrylamide gel and then wet-electro-transferred to 0.2-μm polyvinylidene fluoride (PVDF) membranes (Immobilon, USA). The blots were incubated overnight at 4°C with primary antibodies (rabbit anti-TCF21, 1:1,000; rabbit anti-GAPDH, 1:4,000; mouse anti-MMP2, 1:200; mouse anti-KISS1, 1:500; and goat anti-HOXA3, 1:1,000), followed by incubation with alkaline phosphatase-conjugated secondary antibody (1:2,000) for 3 h at 4°C. Signals were detected using enhanced chemiluminescence (ECL) substrate (RPN5787, GE Healthcare, PA, USA) and a Typhoon FLA 7000 imager (GE Healthcare).

**mRNA Stability Assay**

Cells were seeded into 6-well plates and grown to approximately 80% confluence. Then cells were starved for 12 h with 0.1 FBS medium, followed by incubation with 0.1% FBS medium containing Act D (10 μM) for 0, 6, and 12 h. Total RNA was isolated from cells at the indicated time points (0, 6, and 12 h) and reverse-transcribed into cDNA to perform semiquantitative PCR analysis to determine the TCF21 degradation kinetics of each mRNA.

**Animals and In Vivo Experiments**

Five-week-old specific pathogen-free male BALB/c nude mice were purchased from Shanghai Sihai Experimental Animal Company (license number SCXK, Shanghai 2010-0002). The mice were randomly divided into four groups and subcutaneously implanted with 0.1 mL of T24T(miR-3648i), T24T(Vector), UMUC3(miR-3648i), or UMUC3(Vector) cells (2 × 10^6 suspended in 100 μL of PBS) by lateral tail vein injection. Metastatic progression was monitored weekly, and after 2 months, the mice were sacrificed and the lungs were removed surgically and cleared using PBS. Lung tissues were fixed with picric acid for 24–48 h and stained with H&E. All animal studies were performed in the animal institute of Wenzhou Medical University according to protocols approved by the Medical Experimental Animal Care Commission of Wenzhou Medical University.

**H&E Staining**

The fixed lung tissues were dehydrated using an alcohol gradient and then paraffin-embedded. For H&E staining, sections were deparaffinized in xylene with two changes of 10 min each. Then sections were rehydrated in two changes of absolute alcohol for 5 min each, followed by 95% alcohol for 2 min, 70% alcohol for 2 min, and a brief wash in distilled water. After staining in hematoxylin solution for
10 min, sections were washed in running tap water for 5 min, incubated in 1% acetic alcohol for 30 s, and washed in running tap water for 1 min. Bluing was performed in 0.2% ammonia water for 30 s to 1 min, followed by a wash in running tap water for 5 min and rinsing in 95% alcohol (10 dips). Sections were counterstained in eosin-phloxine solution for 30 s to 1 min and dehydrated in 95% alcohol, followed by two changes of absolute alcohol for 5 min each. Then sections were cleared with two changes of xylene for 5 min each and mounted with a xylene-based mounting medium, neutral balsam.

**Immunohistochemistry Paraffin**

Antibodies specific against TCF21 were used for IHC staining as described in our previous publication. The resulting immunostaining images were captured using the Nikon Eclipse Ni microsystem (Nikon DS-Ri2, Japan). Protein expression levels were determined by calculating the integrated optical density (IOD) per stained area using Image-Pro Plus version 6.0 (Media Cybernetics), as described in our previous publication.

**Cell Viability Assay**

A total of 400 viable cells suspended in 200 μL of complete medium were added to each well of 96-well plates. After adherence, cells were synchronized by replacing the medium with 0.1% FBS medium for 24 h and cultured with complete medium for 1, 3, or 5 days. The cell proliferation index was determined using the Cell Titer-Glo Luminescent Cell Viability Assay Kit (G7572, Promega) with a luminometer (Centro LB 960, Berthold).65

**Clinical Tissue Samples**

BC tissues and adjacent normal bladder tissues were collected as described previously. Two pathologists independently reviewed the H&E-stained tissue sections to identify the tumor stage and histological grade for each specimen. RNA was extracted immediately from tissues, reverse-transcribed into cDNA, and stored at −80°C.

**Statistical Analysis**

Student’s t test was used to determine the significance of differences between groups. The differences were considered significant at p < 0.05.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2019.04.006.

**AUTHOR CONTRIBUTIONS**

H.H. and C.H. conceived and designed the study. W.S., H.J., Y.Y., and X.H. carried out the studies related to cell biological function, conducted the real-time PCR assays, carried out the Transwell and invasion assays, wound-healing assay, and western blot and luciferase reporter assays, and performed the statistical analysis. S.W. and Z.T. carried out the animal studies, H&E assays, and IHC staining assays. S.L., H.Z., and G.J. performed pathological analyses of human bladder cancers. W.S., H.H., Q.X., and C.H. drafted the manuscript. All authors read and approved the final manuscript.

**CONFLICTS OF INTEREST**

The authors declare no competing interests.

**ACKNOWLEDGMENTS**

The results published here are partially based on data generated by the TCGA Research Network: (https://www.cancer.gov/about-ncci/organization/ccg/research/structural-genomics/tcga). We thank the participants, specimen donors, and research groups who developed the TCGA bladder cancer dataset resource for their contributions to database construction, and we also thank Eryn Information Technology Co., Ltd. (Shanghai, China) for assisting us with data analysis. This work was partially supported by the Natural Science Foundation of China (NSFC81872587, NSFC81702530, NSFC81601849, and NSFC81773391), the Wenzhou Science and Technology Bureau (Y20170028, Y20160075, and Y20180109), the Zhejiang Medical and Health Science and Technology Project (2019RC217), Key Discipline of Zhejiang Province in Medical Technology (First Class, Category A), the Key Project of Science and Technology Innovation Team of Zhejiang Province (2013TD10), and Xinniao Talent Program of Zhejiang Province (2018R413068).

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