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Order of Authors:	Xinming Hu
	Jie Yang
	Pengfei Wang
	Jiangtao Zhan
	Mengqi Long
	Xusong Meng
	Mei Xie
	Xianping Che
	Desheng Li
Additional Information:	
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Knockdown of lncRNA XIST suppresses epithelial-mesenchymal transition, migration and invasion by targeting miR-141-3p in TGF-β-induced renal cancer cells

Subtitle: lncRNA XIST in renal cancer

Xinming Hu1#, Jie Yang1#, Pengfei Wang2, Jiangtao Zhan1, Mengqi Long3, Xusong Meng2, Mei Xie4, Xianping Che1, Desheng Li1*

1 Department of Urology, The Second Affiliated Hospital of Hainan Medical University, Haikou, 570100, PR China

2 Hainan Medical University, Haikou, 570100, PR China

3 Department of anesthesia, The Second Affiliated Hospital of Hainan Medical University, Haikou, 570100, PR China

4 Department of otolaryngology, The Second Affiliated Hospital of Hainan Medical University, Haikou, 570100, PR China

#Authors contribute to equal work.

*Corresponding author

Desheng Li

Department of Urology, The Second Affiliated Hospital of Hainan Medical University, No. 48, bai shui tang road, long hua district, Haikou, 570100, PR China

Phone: +86089866808281

E-mail: adhyl995@126.com

Abstract

Long non-coding RNA (lncRNA) X inactive-specific transcript (XIST) associates with multiple tumor metastases. While the action and mechanism of lncRNA XIST in renal cancer (RC) have not been clearly elucidated. This study is aim to explore the action and mechanism of lncRNA XIST in RC. After TGF-β treatment of 786-0 and Caki-1 cells, the cell morphology was observed by microscopy and the expression of lncRNA XIST was monitored by RT-qPCR. Then the targeting effect of lncRNA XIST on miR-141-3p was identified with Dual luciferase reporter assay. After processing with si-XIST alone or in combination with miR-141-3p inhibitor, XIST and miR-141-3p expressions, cell morphology, E-cadherin expression, migration, and invasion were tested using RT-qPCR, light microscope, immunofluorescence staining, and Transwell, respectively. Results indicated that cells changed from epithelial-like carcinoma cells to shuttle-shaped after TGF-β treatment, indicating that TGF-β could induce epithelial-mesenchymal transition (EMT) differentiation of RC cells. Moreover, TGF-β upregulated XIST in RC cells, and XIST silencing could prevent EMT, migration and invasion of TGF-β-mediated RC cells. Besides, XIST can target miR-141-3p, and inhibition of miR-141-3p could attenuate the blocking role of XIST silencing on TGF-β-induced RC cell processes. Taken together, XIST/miR-141-3p axis regulated TGF-β-induced EMT and metastasis in RC cells.

Keywords: long noncoding RNA X inactive-specific transcript; renal cancer; miR-141-3p; transforming growth factor-beta; epithelial-mesenchymal transition

Introduction

Renal cancer (RC) is one of the hackneyed malignant tumors of the urinary system, and about 90% of them occur in the renal parenchyma[1]. The incidence of RC worldwide is yearly increasing, and smoking, age, obesity and hypertension are all known to be major risk factors for RC[2]. And RC is often non-specific in its early stages due to its insidious onset[3]. Currently, the adjuvant examinations of RC mainly consist of ultrasound, urine examination, CT, tumor markers and other modalities[4]. While the ideal early diagnosis and screening modality remain undefined[5]. And RC therapy is still based on early surgical resection and late targeted drugs, while the high rate of postoperative metastasis and recurrence affects the survival rate of patients[6]. Therefore, exploring the key molecules in the RC progression might provide new directions for the clinical diagnosis and therapy of RC.

Transforming growth factor-beta (TGF-β) is an anti-inflammatory immune factor that can regulate cell growth and differentiation[7]. In the body, a variety of cells can secrete TGF-β in an inactive state[8]. Study testified that TGF-β in tumor microcirculation can initiate the metastatic process in kidney cells[9]. Besides, TGF-β can also affect RC cell proliferation, neovascularization, and immunosuppression, etc[10]. Therefore, TGF-β can induce the RC progression, especially metastasis. In the present study, we further investigated the influence of TGF-β on RC cell metastasis.

As a class of non-coding RNAs, long non-coding RNAs (lncRNAs) are defined as greater than 200 nucleotides in length[11]. Among them, lncRNA XIST (XIST), a transcription product of chromosome Xq13.2, can affect the activation of X chromosome-associated genes[12]. Studies confirmed that XIST, as a biologically functional lncRNA, can participate in multiple cancer processes[13, 14]. And recent studies manifested that XIST is connected with the prognosis of RC patients[15]. However, the function and mechanism of XIST in the RC process has not been clearly elucidated.

The competitive endogenous RNA (ceRNA) hypothesis suggests that lncRNAs can serve as microRNA (miRNA) sponges to regulate target gene expression and participate in cancer

progression[16, 17]. To further verify whether the XIST can be involved in RC cellular processes as ceRNA, this study utilized bioinformatics to predict miRNAs that can bind XIST. Through analysis, we discovered that miR-141-3p had binding sites to XIST. And miR-141-3p has also been reported to block the malignant behavior of cancers, such as oral squamous cell carcinoma[18], clear cell renal cell carcinoma[19], and colon cancer, etc. However, whether XIST can interact with miR-141-3p to regulate the RC process remains to be further explored.

In our study, we further investigated the influences of XIST on RC cells metastasis induced by TGF-β, and the regulatory role of XIST on miR-141-3p expression in RC. And the aim is to provide new directions for the clinical therapy of RC.

Materials and Methods

Cell culture

786-0 cells (#CC1503) and Caki-1 cells (#CC1501) were purchased from CellCook (Guangzhou, China). 293T cells were purchased from ATCC. 786-0 cells were grown in RPMI1640 with 2mM L-glutamine and 10% fetal bovine serum (Gibco, USA). Caki-1 cells were cultured in McCoy's 5A with 10% FBS. 293T cells were incubated in DMEM (Gibco). All cells were incubated at 37°C with 5% CO2.

Cell treatment

786-0 and Caki-1 cells were first treated with 10 ng/mL TGF-β (#RP00161; ABCLONAL) for 0, 7, 14, and 21 days. XIST siRNAs (si-XIST), its control (NC), miR-141-3p inhibitor, and miR-141-3p mimics were provided by Gene-Seed (Guangzhou, China). The sequences of siRNAs were presented as follows: si-XIST-1: 5'-TCAGGTGTCCATATTTGCAGCTATT-3'; si_control_1: 5'-TCATGTACCTATGTTGACTCGGATT-3'; si-XIST-2: 5'-CAAGGCCCTTTCTCTTGGACTTAAA-3'; si_control_2: 5'-CAACCCTTTCTCGTTCAGTTGGAAA-3'; si-XIST-3: 5'-CCCTTTCTCTTGGACTTAAACAATT-3'; si_control_3: 5'-CCCTCTCTTGGACTTAAACATTATT-3'.

786-0 and Caki-1 cells were (density about 70%) in 6-well plate were transfected with the oligonucleotides above with Lipofectamine 3000 (Invitrogen) for 48 h in line with the instructions.

RT-qPCR (reverse transcription-quantitative polymerase chain reaction)

Total RNA was isolated from each group of cells using TriQuick Reagent (Solarbio, R1100). And reverse transcription was conducted by applying HiScript III RT SuperMix (Novozymes Biotechnology, China, R323-01) with total RNA as template based on the instructions. Then qPCR assay was carried out through ChamQ Universal SYBR qPCR Master Mix (Novozymes, Q711-02). The relative level of the target gene was calculated with $2^{-\Delta\Delta Ct}$ method. Primer sequences were displayed in Table 1.

Immunofluorescence (IF) staining

The processed 786-0 and Caki-1 cells were inoculated on the cover glasses covering polylysine and cultured at 37°C for 24 h. Then the cells were fixed with 4% paraformaldehyde (PFA) at 4℃ for 40 min, and addressed with 0.1% Triton-X-100 for 2 times, 5 min/time. Cells were closed with 3% bovine serum albumin (BSA) (in PBS) at room temperature (RT) for 1 h. After 3 washes with PBS, cells were added with the corresponding primary antibody (anti-E-cadherin) (in 3% BSA) and incubated overnight at 4°C. After 3 washes with PBS, cells were titrated with fluorescently labeled secondary antibody (Abcam) at RT for 1h and protected from light. After washing with PBS, cells were stained with DAPI for 10 min avoiding light and blocked with Mounting Medium. After solidification, a fluorescence microscopy was applied for observation.

Transwell

For migration, groups of cells were harvested and made into cell suspensions. After counting, the cell concentration was adjusted to 1×10^6/ml with basal medium. Then 100 μl cell suspension was positioned to the upper chamber of Transwell (8 μm, Corning) and 600 μl of complete medium was supplemented to the lower chamber. After incubation for 48 h at 37°C, the cells in the upper chamber were gently wiped off. Then the cells were fixed in 4% PFA for 15 min, and stained with 1% crystal violet for 10 min. After washing the cells were observed and photographed using a light microscope. And the number of cells penetrated was counted. For invasion, Matrigel (EMD

Millipore; Cat. No. 356234) was placed in 4°C overnight to dissolve and diluted with pre-cooling basal medium at a ratio of Matrigel: medium = 1:3. Then the diluted Matrigel (40 μl) was added to Transwell chamber for 2 h at 37°C to solidify. The remaining steps were consistent with the migration.

Dual luciferase reporter assay

The potential binding genes for XIST and miR-141-3p were first predicted though RNA22. XIST 3'-UTR were first amplified by PCR, and wild-type (WT) XIST vector (5'-GCCTCTTTGCTGGGTAATGTTG-3') was constructed with pmirGLO. Then the mutants (Mut) XIST vector (5'-GCCTGTTCCTTGAGTGATGTTG-3') were constructed using point mutation kits. The 293T cells were transfected with miR-141-3p mimics and recombinant plasmids through Lipofectamine 3000 (Invitrogen). The luciferase activity was then tested by TransDetect® Double-Luciferase Reporter Assay Kit (Takara).

Statistical Analysis

The experiment of this study was independently repeated three times. SPSS 23.0 software (Inc., Chicago, IL) was utilized for data statistics, and data were represented as mean ± SD. Student's t-test and one-way ANOVA were adopted for comparison between two groups and multiple groups, respectively. $P<0.05$ signified that the difference is statistically significant.

Results

TGF-β induces epithelial-mesenchymal transition (EMT) differentiation and upregulates XIST in RC cells

We first cultured RC cells (786-0 and Caki-1) in vitro, and all cells grew in good condition. To observe the impact of TGF-β on RC cells, we treated 786-0 and Caki-1 cells with 10 ng/mL TGF-β. In the control group (day 0), the cells were polygonal, closely linked to each other and tended to grow in clusters; in the TGF-β groups, the cells shifted from epithelioid carcinoma cell morphology to shuttle shape, intercellular became loose, and the cells grew in a dispersed manner. And the proportion of EMT differentiated cells gradually increased with the increase of TGF-β treatment time (Figure 1A). Besides, RT-qPCR data manifested that the expression level of XIST

gradually elevated with the processing time of TGF-β, especially for 21 days (Figure 1B). These data testified that TGF-β could induce the development of EMT and upregulate XIST in RC cells.

XIST silencing reverses TGF-β-mediated induction of EMT, migration and invasion of RC cells

Based on the above study, TGF-β could upregulate XIST in RC cells. Therefore, we speculated that XIST has a major role in TGF-β-induced EMT and metastasis of RC cells. After 21 days of 10 ng/mL TGF-β treatment, 786-0 and Caki-1 cells were transfected with si-XIST-1, si-XIST-2, and si-XIST-3, respectively. As denoted in Figure 2A, relative to the si-NC group, the expression level of XIST was memorably lowered in the si-XIST groups, and the silencing effect of XIST was the best in the si-XIST-3 group. Thus si-XIST-3 was applied to silence XIST in TGF-β-mediated 786-0 and Caki-1 cells in subsequent experiments. We first found that cells in NC group were tightly arranged and grow in clusters; TGF-β-treated cells were shuttle-shaped, with loss of polarity and dispersed growth; after XIST silencing, the proportion of EMT differentiated cells was markedly lowered (Figure 2B). This suggested that XIST silencing may prevent RC cells induced by TGF-β from undergoing EMT. Next, IF staining results signified that addition of TGF-β could downregulate E-cadherin in RC cells, which also could be observably reversed by XIST silencing (Figure 2C). And the Transwell results also exhibited that the number of cell migration and invasion was dramatically increased in the TGF-β-treated group relative to the NC group; while this increase mediated by TGF-β could also be prominently attenuated by XIST silencing in RC cells (Figure 2D and 2E). Overall, our results certified that TGF-β could induce the EMT, migration and invasion of RC cells by upregulating XIST.

XIST can target and regulate miR-141-3p

Subsequently, we predicted the miRNAs possibly regulated by XIST through bioinformatics software, and miR-141-3p was confirmed by screening. As displayed in Figure 3A, there were the potential binding sites between XIST and miR-141-3p. and our data revealed that XIST silencing could downregulate XIST and upregulate miR-141-3p in TGF-β-treated 786-0 and Caki-1 cells (Figure 3B). Also, dual luciferase reporter results indicated that miR-141-3p overexpression

markedly decreased the luciferase activity of WT-XIST, while there was no change in luciferase activity of Mut-XIST (Figure 3C). So, we suggested that XIST can target miR-141-3p.

Inhibition of miR-141-3p attenuates the suppression of EMT, migration and invasion mediated by XIST silencing in TGF-β-induced RC cells

On account of the relationship between XIST and miR-141-3p, we further investigated the roles of both in the metastasis process of TGF-β-induced RC cells. si-XIST or/and miR-141-3p inhibitor were utilized to transfect TGF-β-processed 786-0 and Caki-1 cells. And RT-qPCR results represented that XIST silencing observably upregulated miR-141-3p in TGF-β-treated RC cells, and inhibition of miR-141-3p could signally attenuate the upregulation of miR-141-3p mediated by XIST silencing (Figure 4A). Functionally, we first discovered by microscopic observation that XIST silencing notably reduced the proportion of EMT differentiated cells in TGF-β-treated RC cells, which also could be dramatically reversed by miR-141-3p inhibitor (Figure 4B). And IF staining results denoted that E-cadherin expression could be memorably upregulated by XIST silencing in TGF-β-induced RC cells, while this upregulation of E-cadherin expression mediated by XIST silencing could also be observably weakened by miR-141-3p inhibitor (Figure 4C). Additionally, Transwell data manifested that inhibition of miR-141-3p also could notably induce the migration and invasion capacities of TGF-β-induced RC cells, which were mediated by XIST silencing (Figure 4D and 4E). In short, we uncovered that silence of XIST could prevent the EMT, migration and invasion of TGF-β-induced RC cells by inhibiting miR-141-3p expression.

Discussion

RC has the highest mortality rate among urological tumors, and about 1/3 of RC patients will die due to tumor metastasis[20]. Further elucidation of the molecular mechanisms of RC metastasis has positive implications for improving RC therapeutic effect[21]. TGF-β is one of the key components of the tumor microenvironment and can be secreted by multiple cells, including tumor cells and mesenchymal cells[22]. Earlier studies manifested that TGF-β is highly expressed in tumors such as RC, and the TGF-β levels in serum and urinary are negatively correlated with the prognosis of RC patients[23]. Additionally, several researches testified that TGF-β has a crucial role in RC metastasis, and the mechanisms mainly contain induction of EMT[24]. EMT refers to

the transformation of epithelial cells to mesenchymal cells under specific physiological and pathological conditions[25]. The absence of epithelial phenotype (e.g., E-cadherin) and the acquisition of mesenchymal properties are the main features of EMT[26]. And previous study proved that EMT is associated with the development and metastasis of RC[27]. while the specific molecular mechanisms of EMT in RC are not fully understood. In our study, our results uncovered that TGF-β also could induce the EMT, migration and invasion of RC cells.

LncRNAs can regulate gene expression through complex molecular mechanisms due to structural and base specificity[28]. They have multiple functions, such as interfering with transcription, regulating protein activity, inducing chromosome remodeling, and altering cell or protein structure[29]. Furthermore, recent researches confirmed that lncRNA can affect the proliferation, apoptosis and invasion of tumor cells, which can also be serve as a marker to judge the biological behavior of tumor cells[30]. Among them, lncRNA XIST is derived from X-nonspecific transcription factors, and plays a role in diseases such as tumors[31], Alzheimer's disease[32], and cardiac hypertrophy[33]. Based on the previous literatures, lncRNAXIST can act as both oncogenes and oncogenes in different tumors: XIST can play an oncogenic role in colorectal cancer by regulating cell metastasis and EMT[34]; XIST can also participate in bladder cancer development process by regulating TET1[35]; XIST can block in breast cancer cell proliferation and metastasis by regulating miR-155/CDX1[36]. The present study found that TGF-β could markedly upregulate lncRNA XIST in RC cells, and XIST silencing could weaken TGF-β-mediated induction of EMT, migration and invasion of RC cells. This suggests that lncRNA XIST plays a pro-oncogene role in TGF-β-induced RC. However, the mechanism by which XIST silencing blocks the RC process is not fully understood.

MiRNAs are short endogenous non-coding molecules that can regulate gene expression by binding to the 3'-UTR of target genes[37]. And miRNAs can act as oncogenes or suppressor genes during tumor development[38]. Study confirmed that miRNAs are downstream target genes for lncRNAs to exert biological effects, and lncRNAs can exert multiple effects by binding to miRNAs[17]. In our study, we revealed that miR-141-3p is a target gene of XIST, which suggests that the mechanism of XIST in RC is related to miR-141-3p. And our cellular experiments also

proved that silencing of lncRNA XIST could prevent proliferation, EMT and metastasis of TGF-β-mediated RC cells by regulating miR-141-3p. MiR-141-3p is also a well-studied miRNA in tumors. And the most vital mechanism of miR-141-3p is to block cancer cell proliferation, EMT and metastasis[39, 40], which also is consistent with our study in that miR-141-3p has a significant blocking effect on RC metastasis. However, this study has not yet addressed the target gene of miR-141-3p, which will be explored in depth in subsequent experiments.

In summary, this study discovered that lncRNA XIST could accelerate the proliferation and metastasis of RC cells induced by TGF-β, and miR-141-3p is the key mechanism for lncRNA XIST to induce the RC progression mediated by TGF-β. Therefore, lncRNA XIST and miR-141-3p have the potential to be targets for RC diagnosis and therapy.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Figure legends

Figure 1. TGF-β induces EMT differentiation and upregulates long coding RNA XIST (XIST) in renal cancer cells. 786-0 and Caki-1 cells were addressed with 10 ng/mL TGF-β for 0, 7, 14, and 21 days. (A) Light microscope observation of cell morphology in each group. (B) Reverse transcription-quantitative polymerase chain reaction results displayed the expression change of XIST gene in the processed 786-0 and Caki-1 cells. *, $P < 0.05$.

Figure 2. Long non-coding RNA XIST (XIST) silencing reverses TGF-β-mediated induction of EMT, migration and invasion of renal cancer (RC) cells. (A) 786-0 and Caki-1 cells were processed with 10 ng/mL TGF-β for 21 days, and then transfected with si-XIST-1, si-XIST-2, and si-XIST-3. And the silencing effect of XIST in TGF-β-treated RC cells was verified through reverse transcription-quantitative polymerase chain reaction. (B) Morphology of TGF-β-induced RC cells was observed with a microscope after interfering with XIST. (C) Changes in expression of E-cadherin protein were identified using immunofluorescence staining in TGF-β and si-XIST-treated RC cells. Magnification, 200×. (D) Cell migration was identified by Transwell assay in TGF-β-induced RC cells after XIST silencing. Magnification, 200×. (D) The influence of XIST silencing on the invasive capacity of TGF-β-induced RC cells was tested by Transwell assay. Magnification, 200×. *, $P < 0.05$.

Figure 3. Long non-coding RNA XIST (XIST) can target and regulate miR-141-3p. (A) Potential binding sites between XIST and miR-141-3p was predicted by RNA22. (B) After XIST silencing, the levels of XIST and miR-141-3p were analyzed by applying reverse transcription-quantitative polymerase chain reaction in TGF-β-treated 786-0 and Caki-1 cells. (C) Dual luciferase reporter was applied to determine the changes in luciferase activity of WT (wild type)- or Mut (mutant type)-XIST in miR-141-3p-overeexoressed 293T cells. *, $P < 0.05$.

Figure 4. Inhibition of miR-141-3p attenuates the suppression of EMT, migration and invasion mediated by long non-coding RNA XIST (XIST) silencing in TGF-β-induced renal cancer (RC) cells. TGF-β-processed 786-0 and Caki-1 cells were transfected with si-XIST or/and miR-141-3p inhibitor, respectively. (A) reverse transcription-quantitative polymerase chain

reaction presented the change in miR-141-3p expression. (B) The morphology of RC cells was observed by light microscopy. (C) E-cadherin expression was monitored by applying immunofluorescence staining. Magnification, 200×. (D-E) Transwell assay was adopted to test the number of migrating (D) and invading (E) cells. Magnification, 200×. *, $P < 0.05$.

Table 1. Primer sequences in reverse transcription-quantitative polymerase chain reaction analysis.

Gene name	Primer sequences (5'-3')
miR-141-3p RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACcccatct
miR-141-3p-F	TAACACTGTCTGGTAA
Universe-R	GTGCAGGGTCCGAGGT
H-TGF-β-F：	CTCCGAAAATGCCATCCCGC
H-TGF-β-R：	GCTCAATCCGTTGTTCAGGC
XIST-F：	ATGCTGACTACCCAAAGCCC
XIST-R：	GCACCAACACACCAAAGTGG
H-GAPDH-F	GAGTCAACGGATTTGGTCGT
H-GAPDH-R	GACAAGCTTCCCGTTCTCAG
hsa-U6-F	CTCGCTTCGGCAGCACA
hsa-U6-R	AACGCTTCACGAATTTGCGT

Figure 1

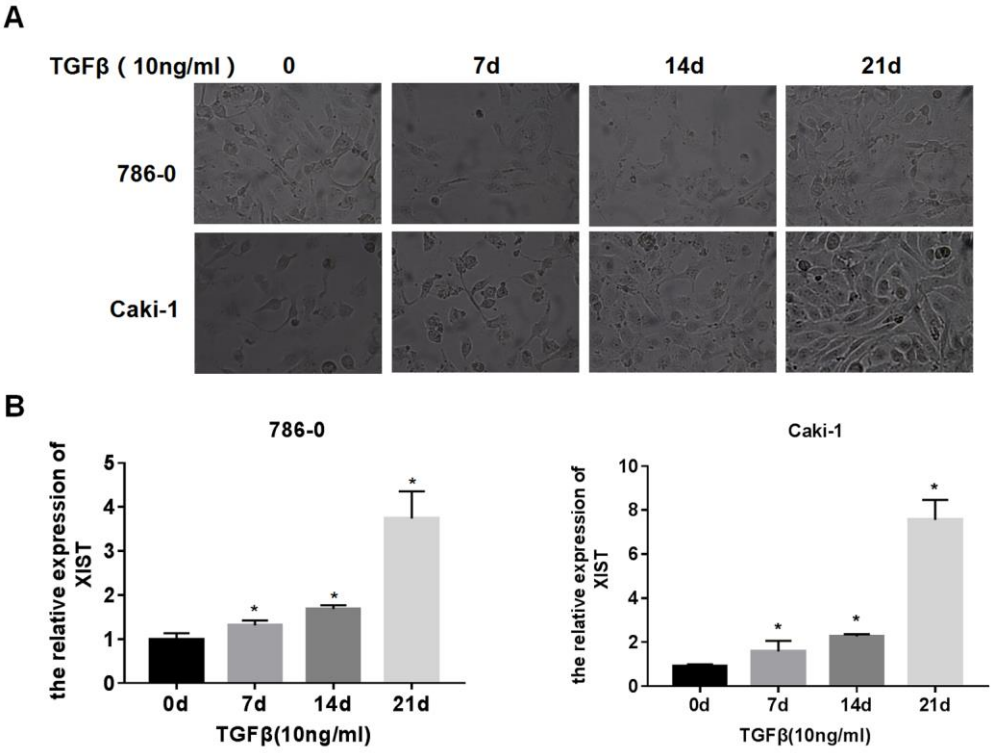

A
TGFβ (10ng/ml)
0
7d
14d
21d
786-0
Caki-1
B
786-0
the relative expression of XIST
0d
7d
14d
21d
TGFβ(10ng/ml)
Caki-1
the relative expression of XIST
0d
7d
14d
21d
TGFβ(10ng/ml)

Figure 2

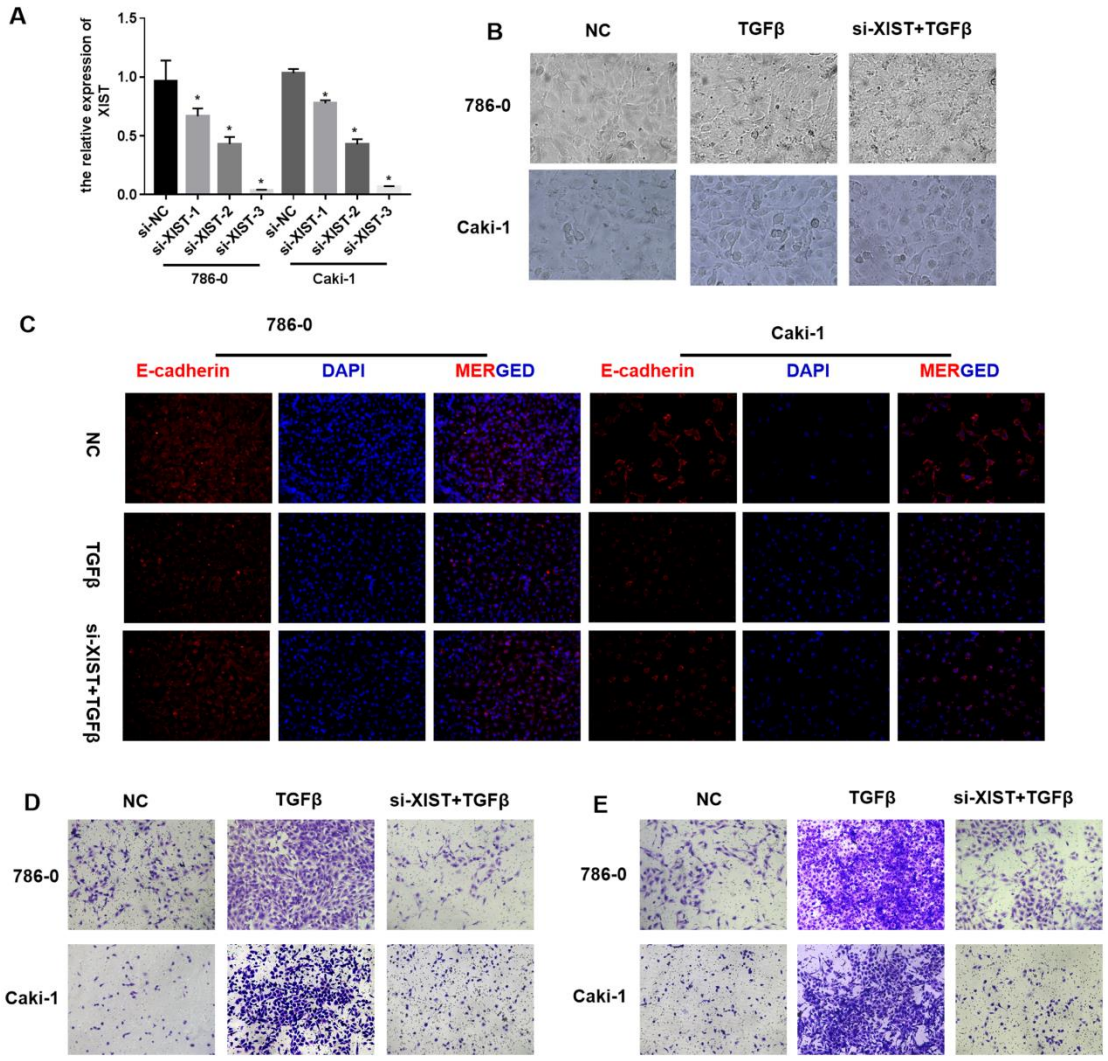

A
the relative expression of XIST
1.5
1.0
0.5
0.0
si-NC
si-XIST-1
si-XIST-2
si-XIST-3
si-NC
si-XIST-1
si-XIST-2
si-XIST-3
786-0
Caki-1
B
NC
TGFβ
si-XIST+TGFβ
786-0
Caki-1
C
786-0
Caki-1
E-cadherin
DAPI
MERGED
E-cadherin
DAPI
MERGED
NC
TGFβ
si-XIST+TGFβ
D
NC
TGFβ
si-XIST+TGFβ
786-0
Caki-1
E
NC
TGFβ
si-XIST+TGFβ
786-0
Caki-1

Figure 3

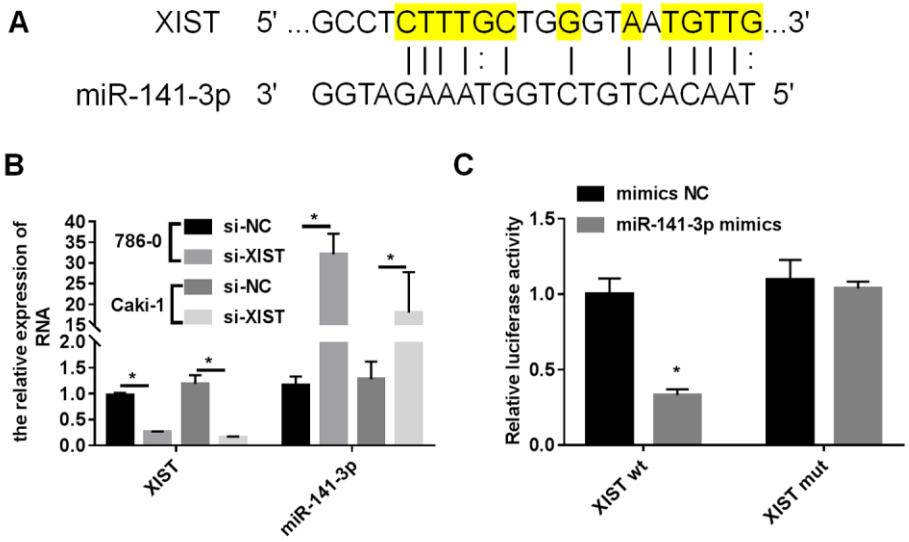
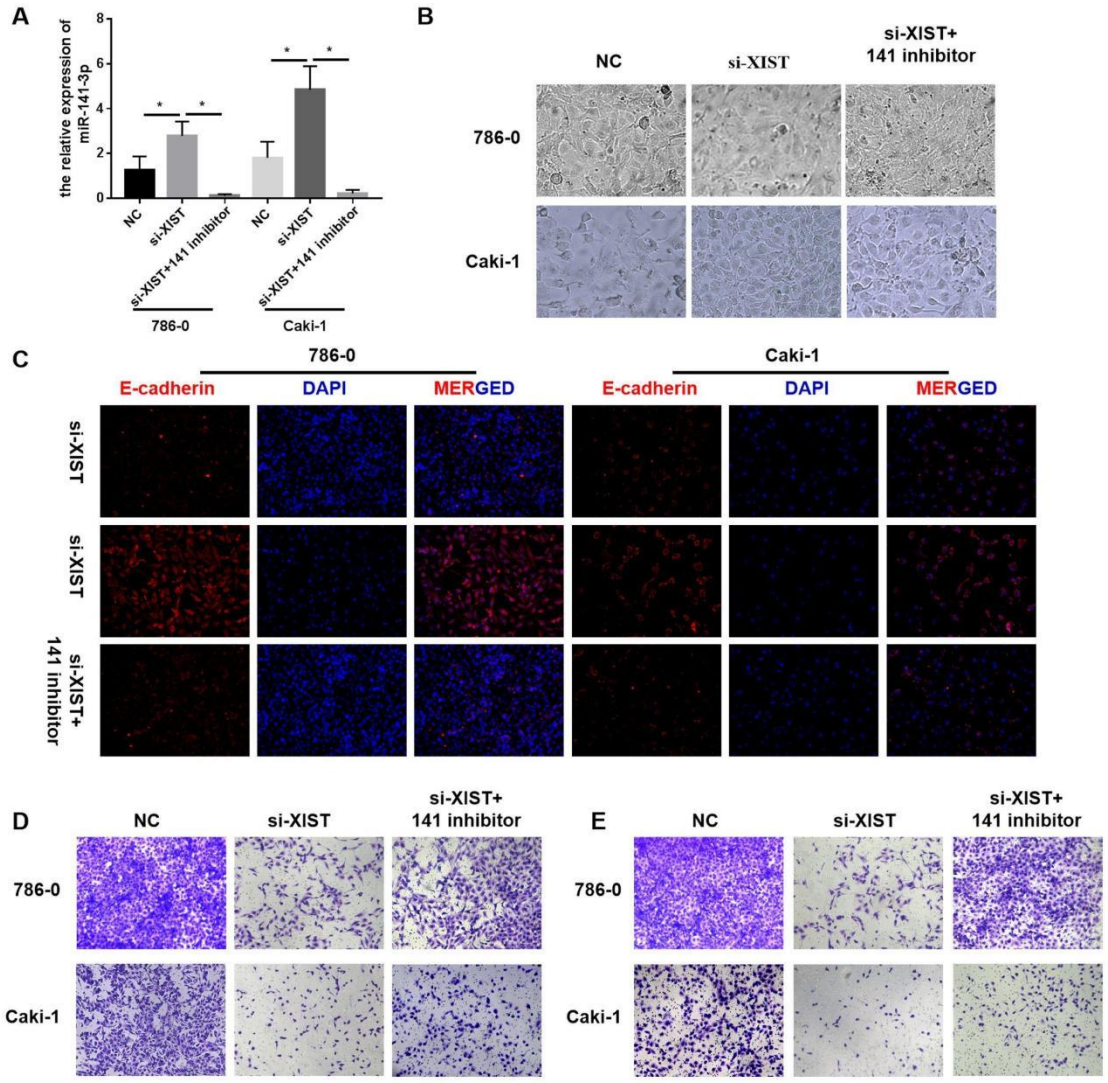

A
XIST 5' ...GCCTCTTTGCTGGGTAATGTTG...3'
miR-141-3p 3' GGTAGAAATGGTCTGTCACAAT 5'
B
the relative expression of RNA
786-0
si-NC
si-XIST
Caki-1
si-NC
si-XIST
XIST
miR-141-3p
C
mimics NC
miR-141-3p mimics
Relative luciferase activity
XIST wt
XIST mut

Figure 4


A
the relative expression of miR-141-3p
NC
si-XIST
si-XIST+141 inhibitor
786-0
Caki-1
B
NC
si-XIST
si-XIST+ 141 inhibitor
786-0
Caki-1
C
786-0
Caki-1
E-cadherin
DAPI
MERGED
si-XIST
si-XIST
si-XIST+ 141 inhibitor
D
E
NC
si-XIST
si-XIST+ 141 inhibitor
786-0
Caki-1