

Original Paper

Long Non-Coding RNA BANCR Promotes Endometrial Cancer Cell Proliferation and Invasion by Regulating MMP2 and MMP1 via ERK/MAPK Signaling Pathway

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Key Words

BANCR • Type 1 endometrial cancer • lncRNA • ERK/MAPK • MMP2 • MMP1

Abstract

Background/Aims: Microarray screening had found BRAF-activated non-coding RNA (BANCR) was significantly upregulated in type 1 endometrial cancer (EC). This study aimed to assess the potential role of long non-coding RNA (lncRNA) BANCR in the pathogenesis and progression of type 1 EC. **Methods:** Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to confirm the expression of BANCR in type 1 EC tissue, and analyze its clinical significance. *In vitro*, RNA interference (siRNA) was used to investigate the biological role of BANCR in type 1 EC. **Results:** qRT-PCR revealed that the expression of lncRNA BANCR was higher in type 1 EC ($P < 0.01$). BANCR expression was significantly correlated with FIGO stage, pathological grade, myometrial invasion, and lymph node metastasis. The expression of BANCR was significantly correlated with that of MMP2/MMP1. *In vitro*, knockdown of BANCR significantly suppressed proliferation, migration, and invasion of Ishikawa and HEC-1A cells, and significantly inhibited the ERK/MAPK signaling pathway that decreased MMP2 and MMP1 expression. **Conclusion:** BANCR is highly expressed in type 1 EC tissue and promotes EC-cell proliferation, migration, and invasion by activating ERK/MAPK signaling pathway that regulates MMP2/MMP1 expression. BANCR is expected to become a prognostic marker and therapeutic target in type 1 EC.

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Introduction

Endometrial cancer (EC) is a prevalent female reproductive system tumor, and the third leading cause of gynecological cancer death. In western countries, it has the highest incidence of cancers affecting women [1]. The incidence of EC has significantly increased recently because of the expansion of high-risk groups, such as women with high leptin or

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adiponectin levels [2, 3]. In 1983, EC was divided into two types by its micro-morphological, clinical and epidemiological characteristics by Bokhman [4]. Although the incidence of type 1 EC is significantly higher than that of type 2, its pathogenesis remains unclear. The identification of new prognostic indicators and novel treatments is highly desired.

Potential medical applications of microRNAs (miRNAs) [5], long non-coding RNAs (lncRNAs) have been received extensive attention. lncRNAs are longer than 200 nucleotides that do not have protein-coding function or encode insufficient lengths of open reading frames, but participate in biologically important activities including X-chromosome silencing [6], chromatin modification [7], transcriptional activation or interference [8]. The transcription of lncRNA can influence transcription of a nearby gene by inhibiting the binding of transcription factors or changing the chromosomal conformation [9]. lncRNAs can also interact with miRNA as a competitive endogenous RNA to regulate the expression of target genes [10]. Emerging evidence indicates that lncRNAs play a critical role in the development and progression of various cancers. For example, Leucci et al. showed that lncRNA SAMMSON modulated mitochondrial metabolism in trans by targeting p32 to the mitochondria in melanoma [11]. H19 was highly expressed in bladder carcinoma, and over-expression of H19 promoted metastasis by upregulating E-cadherin via combination with EZH2 [12]. Over expression of MEG3 suppressed the proliferation of hepatoma cells and induced apoptosis [13]. MALAT1 promoted the proliferation and metastasis of gall bladder cancer cells via the extracellular signal-regulated kinase (ERK) / mitogen-activated protein kinase (MAPK) signaling pathway [14]. HOTAIR was highly expressed in epithelial ovarian cancer and promoted migration by regulating matrix metalloproteinases (MMPs) and the expression of genes involved in epithelial-mesenchymal transition [15]. Studies show that lncRNAs have the potential to become new diagnostic tools, and markers of prognosis, metastasis, and treatment response in cancers. However, little is known about the biological role of lncRNAs in EC.

We previously found that BRAF^{V600E}-Activated Non-Protein-Coding RNA (BANCR) is significantly upregulated in type 1 EC by Arraystar Human lncRNA V3.0 microarray screening (Data not shown). BANCR is a 693 bp RNA on chromosome 9, and it was first reported by Nicola [16] and Ross [17] by RNA-seq screening for transcripts affected by the expression of the oncogene BRAF^{V600E}. BANCR promoted melanoma cell migration and played an important role in the development and progression of gastric cancer [18], colorectal cancer [19], and lung cancer [20]. Little is known about the role of BANCR in EC. But by coding and non-coding network (CNC) bioinformatics analysis [21], we found a potential co-expression relationship of BANCR with MMP2 and MMP1. MMPs are the main enzymes participating in extracellular matrix degradation and remodeling. Both MMP1 [22] and MMP2 [23] were over-expressed in EC and are crucial for metastasis and invasion of surrounding tissue. BRAF mutation could up-regulate MMP1 via activating ERK pathway in invasive melanoma cells [24]. The ERK pathway is a key branch of MAPK and has been shown to take part in cell proliferation, differentiation, apoptosis, migration and invasion [25]. Blocking ERK/MAPK signaling has been shown to suppress cancer progression via down-regulating MMP expression [26, 27]. In this study, we found that BANCR was over-expressed in type 1 EC, and analyzed the correlation with MMP2 and MMP1 expression. We studied the effect of BANCR on the proliferation, migration, and invasion of type 1 EC cells by regulating ERK/MAPK signaling pathway that regulated MMP1 and MMP2 expression *in vitro*.

Materials and Methods

Tissue sample collection

After obtaining informed consent, malignant and benign endometrial tissue specimens were collected from 30 surgical patients with type 1 EC and 20 surgical patients with myoma of uterus. All diagnoses were pathologically confirmed. Patients who were free of other surgical, endocrine, immune, metabolic, and malignant diseases and had not received hormone treatment 3 months before this surgery were eligible. Fresh, sterile specimens were frozen in liquid nitrogen, and stored at -80°C. We recorded clinicopathological

data including age, menstruation status, FIGO stage, tumor histological grade, and myometrial invasion and lymphatic metastasis for each. The collection of specimens for use in this study was approved by the hospital ethics committee.

Cell lines and cell culture

The highly differentiated human endometrial adenocarcinoma Ishikawa cell line and moderately differentiated HEC-1A cell line were purchased from The Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences, Shanghai, China. The cells were cultured in RPMI-1640 (Hyclone, Utah, USA) and DMEM high glucose (Hyclone) media supplemented 10% fetal bovine serum (FBS; PAN-Biotech, Adenbach, Germany) in a sterile incubator at 37°C and 5% CO₂.

Isolation of total RNA, reverse transcription, and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from tissues and cells using TRIzol reagent (9108, Invitrogen, Carlsbad, CA, USA). All steps were carried out under RNase-free condition. RNA concentration and optical density (OD) value were read by a UV spectrophotometer (Bio-Rad, California, USA). The reverse transcription reactions were performed by using a PrimeScript RT reagent kit (RR047A, TaKaRa Bio Inc., Japan). QRT-PCRs were performed with a SYBR PremixEx Taq kit (RR420A, TaKaRa) and a LightCycler 480 (Roche Applied Science) following the manufacturers' protocols. Relative lncRNA and mRNA expression levels (means ± SD) were calculated using the 2^{-ΔΔCt} method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control. Each sample was assayed in triplicate. The qRT-PCR primers were designed by PrimerPremier5.0 software (Premier, Canada), and their sequences were shown in Table 1.

Small interfering RNA (siRNA) transfection

lncRNA BANCR siRNA (si-BANCR) and a negative control (siRNA-NC) were purchased from GenePharma (Shanghai, China). The sequences of these siRNAs are shown in Table 2. si-BANCR and siRNA-NC were transfected into HEC-1A and Ishikawa cells cultured in six-well plates by using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. There are two types of controls in our study. First control is cell transfected with siRNA negative control (siRNA-NC) as showed in Table 2. And the other control is cell without any siRNA-transfection, called mock control (Control). The knock-down effect of si-BANCR was assayed by qRT-PCR after transfected for 48h as the manufacturer's instructions.

Cell proliferation assay

Cell proliferation rate was determined by using a Cell Counting Kit-8 (CCK-8; CK04, Dojindo Molecular Technologies, Osaka, Japan). Briefly, HEC-1A or Ishikawa cells were seeded into 96-well plates and transfected with siRNA. After transfection for another 24, 48, 72, or 96 h, 10 μl CCK-8 reagent was added to each well. Cell was incubated for another 3 h before measuring the absorbance at 450 nm with an enzyme-linked immunosorbent assay plate reader (Bio-Rad). The cell proliferation rate was calculated as the ratio of the OD values of the experimental and control groups. All assays were performed in triplicate.

Wound healing assay

HEC-1A and Ishikawa cells were transfected with siRNA in

Table 1. Primer sequences used in qRT-PCR

RNA	Primers	Length(bp)
BANCR	Forward: 5'-ACAGGACTCCATGGCAAACG-3'	80
	Reverse: 5'-ATGAAGAAAGCCTGGTGCAGT-3'	
MMP2	Forward: 5'-CACACCACATCTTCCGTC-3'	142
	Reverse: 5'-TATGGCTTCTGCCCTGAGAC-3'	
MMP1	Forward: 5'-AACATCACTTCTCCCCGAAT-3'	103
	Reverse: 5'-GTTCCCAAATCCTGTCCA-3'	
GAPDH	Forward: 5'-GCACCGTCAAGGCTGAGAAC-3'	138
	Reverse: 5'-TGGTGAAGACGCCAGTGA-3'	

Table 2. siRNA sequences

siRNA	Sequence
si-BANCR-1	sense: 5'-GGACUCCAUGGCAAACGUUTT-3'
	antisense: 5'-AACGUUUGCCAUGGAGUCCTT-3'
si-BANCR-2	sense: 5'-GGAGUGGCGACUAUAGCAATT-3'
	antisense: 5'-UUGCUAUAGUCGCCACUCCTT-3'
siRNA-NC	sense: 5'-UUCUCCGAACGUGUCACGUTT-3'
	antisense: 5'-ACGUGACACGUUCGGAGAATT-3'

six-well plates and cultured until reaching 90% confluence. A wound was created with a pipette tip, the cells were washed twice, and cultured in medium without FBS. The wound was observed and photographed at 0 h and 24 h using an inverted microscope (Nikon, Japan). Cell migration ability was described as the number of cells that migrated into the wound. All assays were performed in triplicate.

Transwell migration assay and transwell invasion assay

Matrigel (BD Biosciences, New York, USA) was diluted with cold serum-free medium and incubated for 4 h until it solidified (for transwell invasion assay). After being starved for 24 h, 200 μ l siRNA transfected HEC-1A or Ishikawa cells (5×10^4) cultured in serum-free medium were seeded into the upper chambers of Transwell plates (8 μ m pores; Corning, New York, USA), and 600 μ l 20% FBS medium was added to the lower chamber as a chemoattractant. After the chambers were incubated at 37°C for 18 h (for migration assay) and 26 h (for invasion assay), cells could be seen in lower the chamber. Cells remaining on the top of the transwell chamber were scraped off with a cotton swab. Migration cells and invasive cells were fixed with 4% paraformaldehyde for 30 min, stained with 0.1% crystal violet for 30 min, and observed, photographed and counted using a light microscope. All assays were performed in triplicate.

Cell cycle assay

siRNA transfected HEC-1A and Ishikawa cells (1×10^6) were fixed with cold 70% absolute ethyl alcohol overnight at 4°C. Cells was added 100 μ l RNase A reagent (Keygen Biotech, Nanjing, China), incubated at 37°C for 30 min, and stained in 400 μ l propidium iodide reagent (Keygen Biotech) for another 30 min. DNA content was assayed at 488 nm by a FACSCalibur flow cytometer (Becton Dickinson) and analyzed by ModFit LT software (Verity Software House). All assays were performed in triplicate.

Western blot assay

HEC-1A and Ishikawa cells were harvested, washed twice and lysed in RIPA buffer (Keygen Biotech), containing protease inhibitors and phosphatase inhibitors after transfection for 48-72 h. Protein concentration was determined using a bicinchoninic acid assay (BCA) kit (Thermo Scientific, MA, USA). Total protein was separated by 10% polyacrylamide gel electrophoresis and transferred onto 0.45 μ m polyvinylidene fluoride membranes (Millipore, MA, USA). After blocking for 1 h, the membranes were incubated with specific primary antibodies overnight at 4°C followed by secondary antibodies for 2 h at room temperature. The intensity of the protein bands was measured with C300 imaging system (Azure Biosystem, USA) by enhanced chemiluminescence (ECL; Thermo Scientific) and relative concentration was calculated using Quantity One software (Bio-Rad). Primary antibodies included P-ERK1/2 (1:2000, Catalog number 4370), P-MEK (1:1000, Number 9154) and MEK (1:1000, Number 8727) from Cell Signaling Technology, CA, USA. And ERK1/2 (1:500, Catalog number 16443-1-AP), MMP2 (1:200, 10373-2-AP), MMP1 (1:200, 10371-2-AP), Cyclin D 1 (1:200, 60186-1-Ig), Bcl-2 (1:200, 60178-1-Ig) and GAPDH endogenous control (1:10000, 10494-1-AP) were from Proteintech, Chicago, USA. Horseradish peroxidase (HRP)-conjugated secondary antibody (1:2500, Catalog number ZB-2301 and ZB-2305) was from ZSGB-Bio Origene, Beijing, China. U0126 (KGR0075) was from Keygen Biotech. All assays were performed in triplicate.

Statistical analysis

All data were expressed as means \pm standard deviation (SD). SPSS 17.0 software (SSPS Inc., Chicago, USA) was used for the statistical tests. Student's t-test was used to determine the significance of two group differences. One-way analysis of variance analysis was used for multiple comparison. Chi-square tests were used to compare lncRNA BANCR expression and clinicopathological features. The Pearson correlation coefficient (r) was used for correlation analysis. $P < 0.05$ (two-tailed) was considered to be significant.

Results

Expression of lncRNA BANCR in type 1 EC tissues

QRT-PCR (Fig. 1A) revealed that the expression of lncRNA BANCR was significantly higher in type 1 EC tissues (9.002 ± 1.303) than that in normal endometrium tissues (2.279 ± 0.6072) ($P < 0.01$). According our previous differential expression profile of lncRNAs and mRNAs of EC (Data not shown), CNC bioinformatics analysis that was done by Kangchen, Shanghai, China (Fig. 1B) suggested the possibility of co-expression of BANCR with MMP2

and MMP1. QRT-PCR showed that expression of MMP2 and MMP1 were higher in type 1 EC tissues than normal endometrium tissues ($P < 0.05$; Fig. 1C). There was highly significant positive correlation between MMP2 mRNA and BANCR expression in type 1 EC tissues ($r = 0.785$, $P < 0.001$; Fig. 1D). There was also positive correlation of MMP1 mRNA and BANCR expression ($r = 0.488$, $P = 0.0062$; Fig. 1E).

Relationship of lncRNA BANCR expression and patients clinicopathological variables

According to the literature method [28], 30 type 1 EC tissues were divided into high- and low-expression groups of 15 each using of the median BANCR expression level ($Y = 9.115$) (Fig. 1F). The level of BANCR expression was not correlated with age or menstruation status (both $P > 0.05$), but it was significantly correlated with FIGO stage ($P = 0.010$), pathological

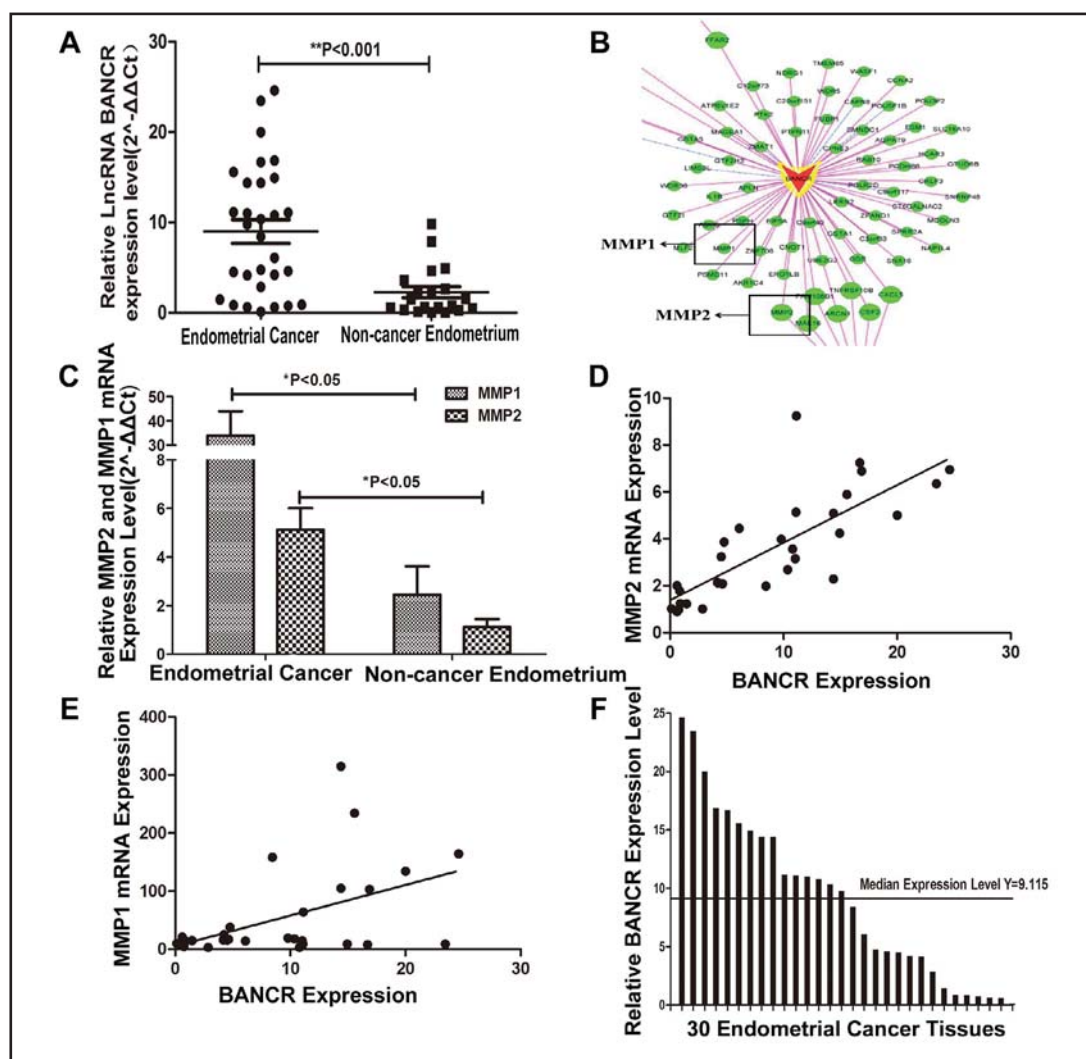
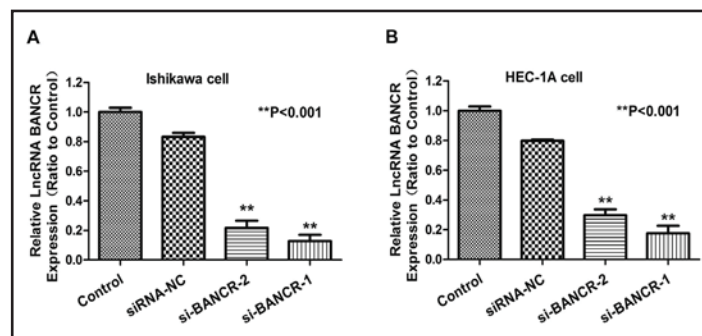


Fig. 1. LncRNA BANCR and MMP2/MMP1 expression in type 1 EC tissues. (A) The expression level of BANCR in type 1 EC tissues was higher than that in normal endometrium tissues by qRT-PCR (** $P < 0.01$). (B) CNC bioinformatics analysis suggested the possibility of co-expression of BANCR with MMP2 and MMP1 (Pearson's correlation coefficient, $r = 0.990$ and 0.988 , $*P < 0.05$). (C) The expression level of MMP2 and MMP1 in type 1 EC tissues were higher than that in normal endometrium tissues by qRT-PCR ($*P < 0.05$). (D) The expression levels of MMP2 mRNA and LncRNA BANCR were strongly positive correlation ($r = 0.785$, $p < 0.001$) in type 1 EC tissues. (E) The expression levels of MMP1 mRNA and BANCR were positive correlation ($r = 0.488$, $p = 0.0062$). (F) The 30 type 1 EC tissues were divided into high- and a low-expression groups of 15 each using the median BANCR expression level ($Y = 9.115$).

Table 3. Relationship of lncRNA BANCR expression and patients clinicopathological variables. * $P < 0.05$, the difference is significant

Parameters Group	Total Number (Total n=30)	LncRNA BANCR expression		P Value
		High(%)	Low(%)	
Age(Years)				
<50	6	5(83.33)	1(16.67)	0.058
≥50	24	10(41.67)	14(58.33)	
Menstruation				
Non-menopause	9	6(66.67)	3(33.33)	0.229
Menopause	21	9(42.86)	12(57.14)	
FIGO stage				
I-II	22	8(36.36)	14(63.64)	0.010*
III-IV	8	7(87.50)	1(12.50)	
Histological grade				
G1	12	3(25.00)	9(75.00)	0.023*
G2+G3	18	12(66.67)	6(33.33)	
Myometrial invasion				
<1/2	6	1(16.67)	5(83.33)	0.049*
≥1/2	24	14(58.33)	10(41.67)	
Lymphatic metastasis				
Yes	7	6(85.71)	1(14.29)	0.025*
No	23	9(39.13)	14(60.87)	

Fig. 2. The interference effect of si-BANCR in Ishikawa and HEC-1A cell lines. The expression levels of BANCR in Ishikawa (A) and HEC-1A (B) cells transfected with si-BANCR-1 and si-BANCR-2 were obviously decreased compared with the control and siRNA-NC groups (** $P < 0.01$).



grade ($P = 0.023$), myometrial invasion ($P = 0.049$) and lymph node metastasis ($P = 0.025$) (Table 3).

Interference effect of BANCR siRNA in type 1 EC cells

The expression of BANCR in Ishikawa and HEC-1A cells was assayed by qRT-PCR after transfection of siRNA for 24h-48h. Compared with siRNA-NC transfected cells and control cells, BANCR expression in Ishikawa and HEC-1A cells transfected with si-BANCR-1 and si-BANCR-2 was decreased ($P < 0.01$). The interference effect of si-BANCR was 70% to 90% (Fig. 2A-B).

Influence of BANCR on the proliferation of EC cell lines in vitro

The CCK-8 assay was used to determine whether lncRNA BANCR had an effect on the proliferation of Ishikawa and HEC-1A cells *in vitro*. The proliferation of both Ishikawa and HEC-1A cells decreased significantly after transfection with si-BANCR-1 and si-BANCR-2 compared with siRNA-NC-transfected cells and control cells ($P < 0.05$; Fig. 3A-B).

Influence of BANCR on the cell cycle of EC cell lines in vitro

To investigate whether BANCR influence the cell cycle distribution of Ishikawa and HEC-1A cells *in vitro*, flow cytometry was used to detect the percentage of cells in G0/G1, S, and G2/M. The percentage changes of cells in every phase were shown in Table 4. The percentage of G0/G1 cells increased and the percentage of S decreased, significantly after transfection with si-BANCR-1 and si-BANCR-2, compared with siRNA-NC cells and control cells ($P < 0.05$; Fig. 3C-F).

Cyclin D1 is a kind of cell cycle protein, and its content changes periodically as cell cycle. Over-expression of cyclin D1 can form a complex with CDK4/6 to promote cell cycle

Fig. 3. Influence of BANCR on the proliferation and cell cycle of EC cell lines *in vitro*. (A-B) The proliferation abilities of Ishikawa and HEC-1A cells decreased significantly after transfected with si-BANCR-1 and si-BANCR-2 compared to control and siRNA-NC groups ($P < 0.05$). (C-D) In cell cycle assay, flow cytometry was used to detect the percentage of G0/G1, S and G2/M. (E-F) The percentage of G0/G1 increased and the percentage of S decreased significantly after transfected with si-BANCR-1 and si-BANCR-2, compared to control and siRNA-NC groups ($P < 0.05$).

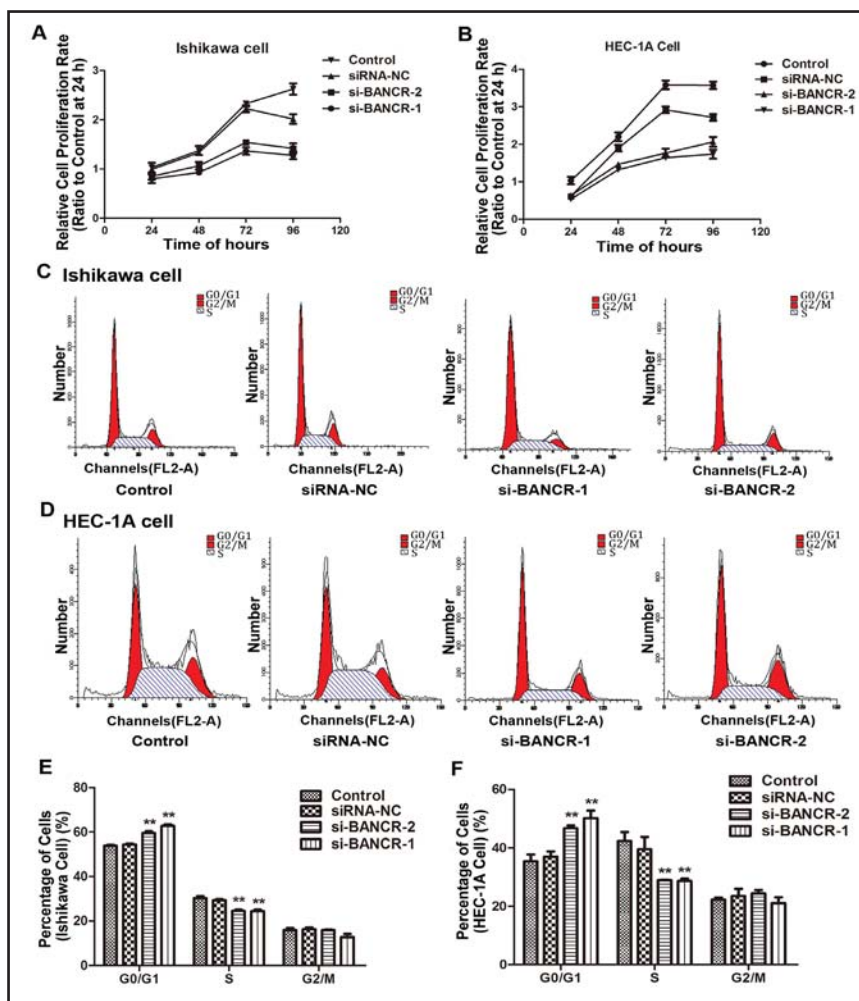


Table 4. The changes of Ishikawa and HEC-1A cells in cell cycle after transfected with si-BANCR compared with siRNA-NC and control cells. ** $P < 0.01$, * $P < 0.05$, the difference is significant

Cell	Cell Cycle	Control	siRNA-NC	si-BANCR-1	si-BANCR-2	P Value
Ishikawa	G0/G1(%)	53.8±0.4	54.4±0.4	59.5±0.8	62.8±0.7	<0.001**
	S(%)	30.3±0.9	29.3±0.6	24.6±0.5	24.4±0.7	<0.001**
	G2/M(%)	15.9±0.9	16.3±0.9	15.9±0.4	12.8±1.4	0.1017
HEC-1A	G0/G1(%)	35.4±2.4	37.0±1.8	46.7±1.0	50.2±2.5	0.0019**
	S(%)	42.3±3.0	39.5±4.2	28.9±0.2	28.7±0.7	0.0111*
	G2/M(%)	22.3±0.8	23.5±0.4	24.4±1.1	21.2±1.0	0.5797

progression from G1 to S phase, which can promote the proliferation of cells [29, 30]. Bcl-2 is a kind of anti-apoptosis protein that inhibit cell cycle transforming of G1/S, which promote cell survival and inhibit cell apoptosis [31]. To better understand the influence of BANCR on the cell cycle, the expression of Cyclin D1 and Bcl-2 protein were assayed by western blot (Fig. 4A-B). The expression of Cyclin D1 and Bcl-2 protein were significantly reduced after transfection with si-BANCR, compared with siRNA-NC-transfected cells and control cells ($P < 0.05$; Fig. 4C-D).

The effects of BANCR on Ishikawa and HEC-1A cell migration and invasion

The effects of BANCR on the migration and invasion of Ishikawa and HEC-1A cells, were tested by wound healing, transwell migration and invasion assays. In the wound healing assay, the migration of both Ishikawa and HEC-1A cells was significantly decreased after si-BANCR-1 and si-BANCR-2 transfection compared with siRNA-NC and control ($P < 0.01$;

Fig. 4. (A-B) The expression of Cyclin D1 and Bcl-2 protein were tested by western blot. (C-D) The protein level of CyclinD1 and Bcl-2 were obviously reduced after transfected with si-BANCR-1 and si-BANCR-2, compared to control and siRNA-NC groups (* $P < 0.05$, ** $P < 0.01$).

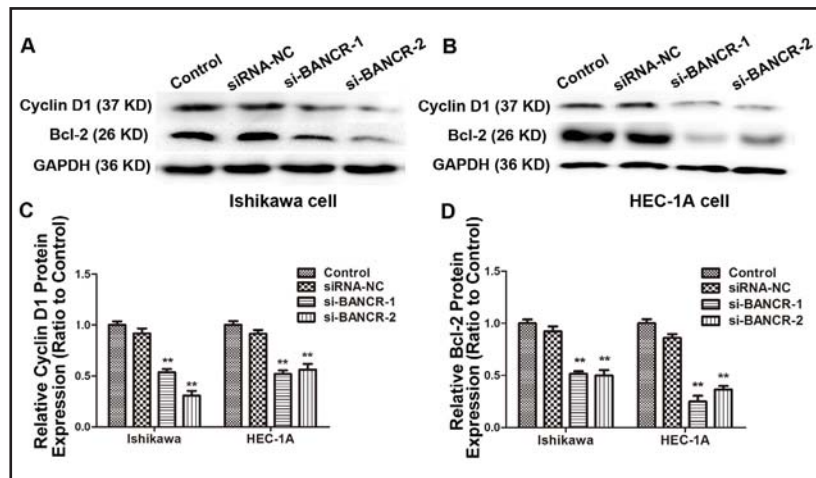


Fig. 5. The effects of BANCR on Ishikawa and HEC-1A cell migration and invasion *in vitro*. (A-B) In wound healing assay, the cell migration ability of Ishikawa and HEC-1A cells were significantly decreased after transfected with si-BANCR-1 and si-BANCR-2, compared to control and siRNA-NC group (** $P < 0.01$). (C-D) In the transwell migration assay, Ishikawa and HEC-1A cells migrated into lower chamber were obviously reduced with si-BANCR, compared to control and siRNA-NC (** $P < 0.01$). (E-F) In the transwell invasion assay, Ishikawa and HEC-1A cells invaded into lower chamber were notably decreased with si-BANCR, compared to control and siRNA-NC (** $P < 0.01$).

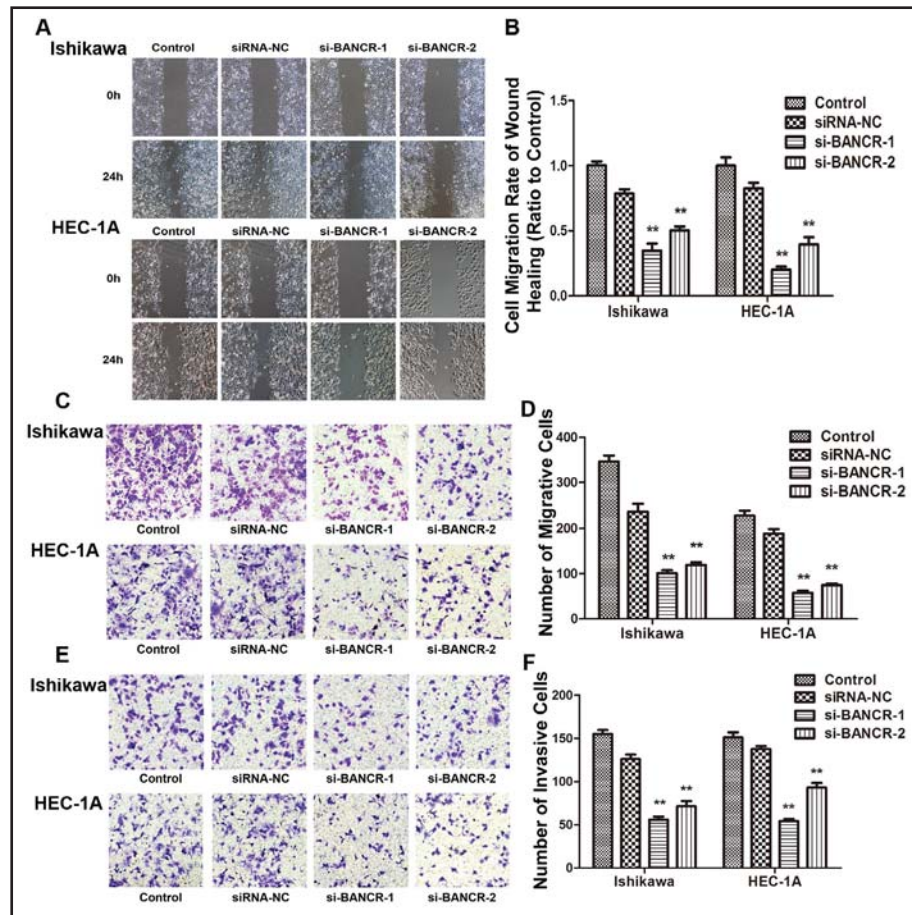


Fig. 5A-B). In the transwell migration assay, the number of both Ishikawa and HEC-1A cells that migrated into the lower chamber was significantly reduced by si-BANCR-transfection ($P < 0.01$; Fig. 5C-D). In the transwell invasion assay, the number of Ishikawa and HEC-1A cells that invaded into the lower chamber was also significantly decreased by si-BANCR-transfection ($P < 0.01$; Fig. 5E-F).

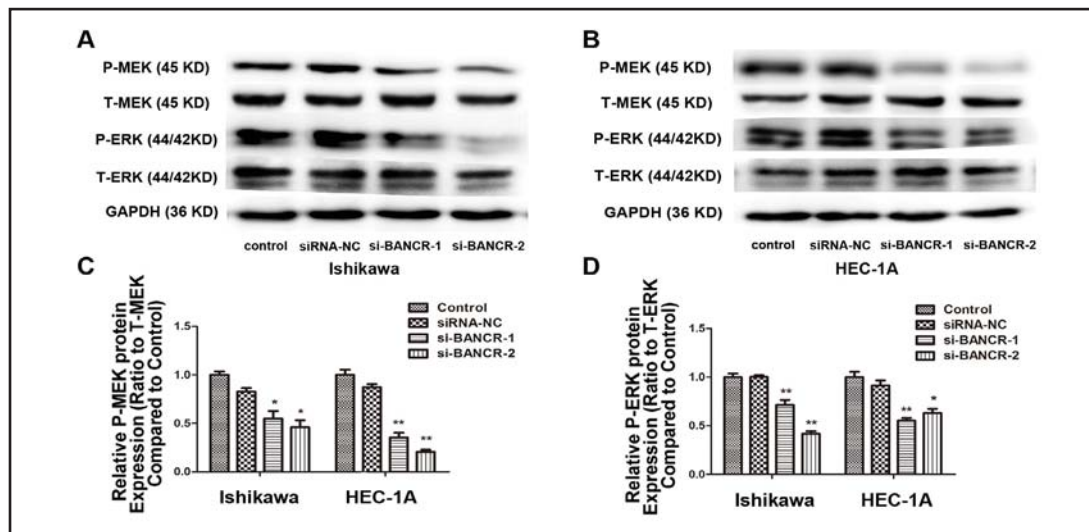


Fig. 6. (A-B) Influence of lncRNA BANCR on the ERK/MAPK signaling pathway. (C-D) Compared to control and siRNA-NC group, P-MEK and P-ERK1/2 protein expression levels of Ishikawa and HEC-1A cells in si-BANCR-1 and si-BANCR-2 group were obviously decreased (* $P < 0.05$, ** $P < 0.01$), while T-MEK and T-ERK1/2 protein have no changed.

Influence of BANCR on the ERK/MAPK signaling pathway and expression of MMP2 and MMP1

Western blot assays were used to detect the influence of BANCR on the ERK/MAPK signaling pathway (Fig. 6A-B). Compared with control cells and siRNA-NC-transfected cells, the expression of phosphorylated-MEK (P-MEK) and phosphorylated-ERK1/2 (P-ERK1/2) protein of Ishikawa and HEC-1A si-BANCR-transfected cells were significantly decreased ($P < 0.05$); total-MEK (T-MEK) and total-ERK1/2 (T-ERK1/2) protein expression was not changed (Fig. 6C-D).

QRT-PCR and western blots were used to assay the expression of MMP2 and MMP1 mRNA and protein after transfected with si-BANCR and treated with U0126, an inhibitor of MEK/ERK/MAPK, inhibiting of ERK/MAPK signaling specially. Compared with control cells and siRNA-NC-transfected cells, the expression of MMP2 and MMP1 mRNA (Fig. 7A-B) and protein (Fig. 7C-F) in Ishikawa and HEC-1A cells transfected with si-BANCR or treated with U0126 were significantly reduced ($P < 0.05$). Between cells transfected with si-BANCR and treated with U0126, there were no significant differences in the expression level of MMP2 ($P > 0.05$). However, between cells transfected with si-BANCR and added U0126, there was significant difference in MMP1 expression ($P < 0.05$, Table 5). The results showed that lncRNA BANCR promoted EC-cell proliferation, migration and invasion by at least in part directly activating ERK/MAPK signaling pathway then to regulate MMP2/MMP1 expression.

Discussion

The worldwide incidence of EC has been increasing, and the pathogenesis of EC has not been well understood yet [1]. The pathological grade of type 1 EC tends to be high or moderate rather than low, but some patients still have a poor prognosis because of distant metastasis or lymph node invasion. Therefore, studying the pathogenesis of type 1 EC may help in prevention, diagnosis, treatment and the improvement of prognosis.

High-throughput microarray has been used to screen and identify specific lncRNAs as new markers of some cancers. Selective silencing lncRNA therapy mediated by RNA interference might become a treatment option for some cancers. According to our previous Arraystar Human LncRNA V3.0 microarray (Data not shown), we found that lncRNA BANCR

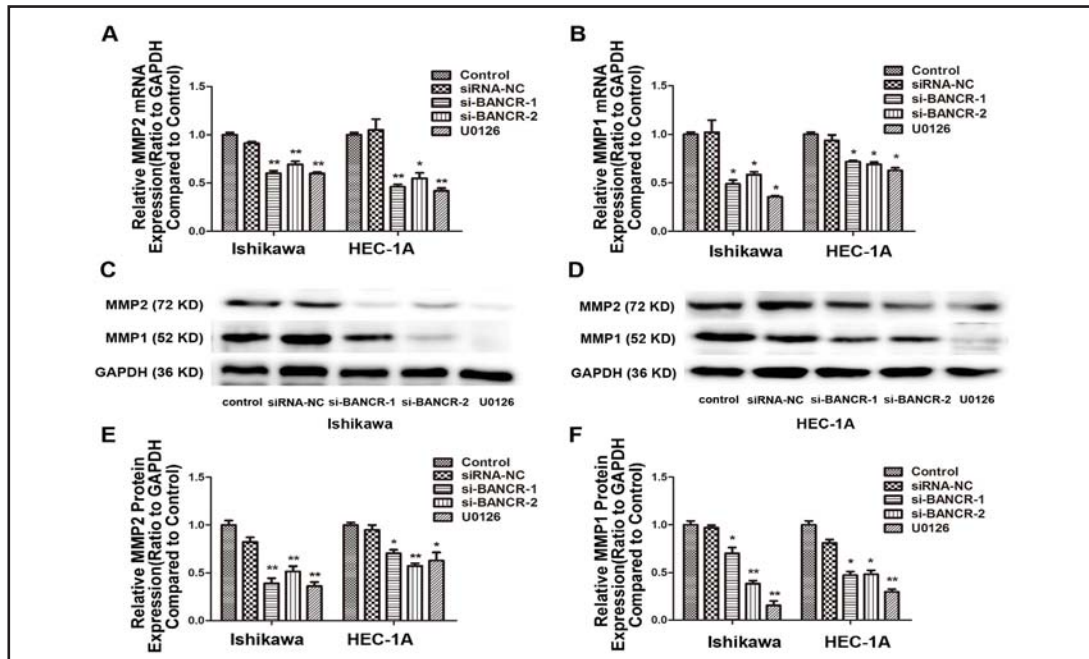


Fig. 7. (A-B) Compared to control and siRNA-NC, MMP2 and MMP1 mRNA levels were significantly reduced in Ishikawa and HEC-1A cells that transfected with si-BANCR-1 and si-BANCR-2 or added U0126 (* $P < 0.05$, ** $P < 0.01$). (C-D) Influence of MMP2 and MMP1 protein after si-BANCR-transfected or treated with U0126. (E-F) Compared to control and siRNA-NC groups, MMP2 and MMP1 protein expression levels were significantly reduced in Ishikawa and HEC-1A cells that transfected with si-BANCR-1, si-BANCR-2 or added U0126 (* $P < 0.05$, ** $P < 0.01$).

Table 5. Compare the mRNA and protein levels of MMP2 and MMP1 between si-BANCR-transfection and U0126-added in Ishikawa and HEC-1A cells. ** $P < 0.01$, * $P < 0.05$, the difference is significant

Cell	Relative expression level compared to Control				P Value (si-BANCR-1 vs.U0126)	P Value (si-BANCR-2 vs.U0126)
	Control	si-BANCR-1	si-BANCR-2	U0126		
Ishikawa						
MMP2 mRNA	1.00±0.02	0.60±0.03	0.69±0.03	0.60±0.01	0.9890	0.0549
MMP2 protein	1.00±0.05	0.39±0.05	0.52±0.05	0.36±0.04	0.7030	0.0898
MMP1 mRNA	1.00±0.02	0.49±0.04	0.58±0.03	0.36±0.01	0.0368*	0.0019**
MMP1 protein	1.00±0.04	0.70±0.06	0.38±0.03	0.15±0.05	0.0021**	0.0160*
HEC-1A						
MMP2 mRNA	1.00±0.02	0.46±0.02	0.55±0.06	0.42±0.03	0.3145	0.1076
MMP2 protein	1.00±0.03	0.70±0.04	0.57±0.03	0.63±0.08	0.4631	0.5574
MMP1 mRNA	1.00±0.02	0.72±0.01	0.69±0.04	0.63±0.03	0.0446*	0.1476
MMP1 protein	1.00±0.04	0.47±0.04	0.48±0.04	0.30±0.03	0.0200*	0.0216*

is one of significantly upregulated lncRNAs in type 1 EC. In our study, we confirmed that the expression level of BANCR in type 1 EC tissues was higher than that in normal endometrium tissues by qRT-PCR. And BANCR expression level was significantly correlated with FIGO stage, pathological grade, myometrial invasion, and lymph node metastasis. The results indicated that high expression of BANCR might prompt the progression of type 1 EC.

In vitro, knockdown of lncRNA BANCR suppressed the proliferation of Ishikawa and HEC-1A cells, arrested the cell cycle at G0/G1 and significantly decreased the percentage of cells in S phase. The levels of Cyclin D1 and Bcl-2 proteins were significantly reduced after BANCR-knockdown, showing that BANCR may promote proliferation and inhibit apoptosis of EC cells through up-regulating Cyclin D1 and Bcl-2. The migration and invasion of Ishikawa and HEC-1A cells were significantly decreased after si-BANCR transfection in wound healing, transwell migration and invasion assays. These results indicate that BANCR had a significant influence on proliferation, migration, and invasion of type 1 EC cells.

In 2014, Li et al. [32] reported that BANCR promoted melanoma proliferation by activating the ERK1/2 and JNK MAPK signaling pathway. BANCR was highly expressed in colorectal cancer, and participated in EMT via the MEK/ERK pathway [19]. BANCR promoted proliferation and migration of lung cancer cells through inactivating P38 and JNK MAPK [20]. The MAPK signaling pathway, especially ERK/MAPK was highly activated, correlated with the EC FIGO stage, and influenced proliferation and apoptosis of EC cells [33]. Study suggested that ERK1/2 activation occurs in a KRAS- and BRAF-independent manner in endometrial cancer [34]. The question was whether BANCR promoted EC-cell proliferation, migration, and invasion through the ERK/MAPK signaling pathway.

According to the method in the study [35], CNC network bioinformatics analysis by differential expression profiles of lncRNAs and mRNAs was used to analysis the function of lncRNA through co-expression mRNA. For example, a report suggested a role for the lncRNA TK170605 (AK079380) in oligodendrocyte lineage commitment [36]. In the CNC network, TK170605 was co-expressed with Map6d1, a member of the STOP family that is responsible for the stabilization of neuronal microtubules [37]. CNC bioinformatics analysis found a potential co-expression relationship of BANCR with MMP2 and MMP1, which suggested us lncRNA BANCR might have similar function or regulative relation to MMP2/MMP1. Dysregulation of MMP may promote migration and invasion of tumor cells [38]. In line with previous studies, MMP2 and MMP1 were highly expressed in type 1 EC tissues. And MMP2/MMP1 mRNA were positively correlated with lncRNA BANCR expression. Previous studies have reported that blocking the ERK/MAPK pathway inhibited colorectal cancer cell migration and invasion by down-regulating the expression of MMP [26]. We thus speculate that lncRNA BANCR can activate ERK/MAPK pathway that regulates MMP2 and MMP1 expression then to influence EC cell biological effects.

In this study, P-MEK and P-ERK1/2 protein expression were significantly decreased in Ishikawa and HEC-1A cells transfected with si-BANCR, while T-MEK and T-ERK1/2 protein expression had not changed. However, expression of MMP2 and MMP1 mRNA and protein in Ishikawa and HEC-1A cells transfected with si-BANCR was significantly inhibited by U0126. These results showed that, in EC cells, the ERK/MAPK pathway played an important role in the regulation of MMP2 and MMP1 by lncRNA BANCR. lncRNA BANCR stimulated ERK/MAPK pathway, and then upregulated MMP2 and MMP1 expression, which promoted the proliferation, migration, and invasion of Ishikawa and HEC-1A cells. It is interesting that, between cells transfected with si-BANCR and treated with U0126, we found no difference in MMP2 mRNA and protein levels. But there was significant difference in MMP1 mRNA and protein expression. This suggested that lncRNA BANCR regulated MMP2 more specifically than MMP1 via ERK/MAPK pathway or that other upstream genes regulated MMP1 in type 1 EC cells in addition to BANCR, and which need to be further researched. And whether BANCR can directly regulate MMP2 and MMP1 expression also need to be further studied.

In conclusion, lncRNA BANCR was highly expressed in type 1 EC tissues, and took part in the progression of EC. Knockdown of BANCR blocked the ERK/MAPK signaling pathway that reduced MMP2 and MMP1 levels, which inhibited cell proliferation, migration, and invasion of EC cells. More animal studies and bioinformatics evaluations will help determine the mechanism of BANCR in type 1 EC. Because BANCR was found by RNA-seq screening for transcripts affected by the expression of the oncogene BRAF^{V600E}, the potential regulating relationship of the expression of BRAF or BRAF mutation and lncRNA BANCR in EC will be assessed in our future studies. Study of type 2 EC tissue will reveal differences in BANCR expression in type1 and type 2 EC that will help identify roles of lncRNA BANCR in the diagnosis, treatment and prognosis of EC.

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Disclosure Statement

All authors declare there are no conflicts of interest.

References

- 1 Siegel R L, Miller K D, Jemal A: Cancer statistics, 2016. *CA Cancer J Clin* 2016;66:10-29.
- 2 Wang PP, He XY, Wang R, Wang Z, Wang YG: High leptin level is an independent risk factor of endometrial cancer: a meta-analysis. *Cell Physiol Biochem* 2014;34:1477-1484.
- 3 Zeng F, Shi J, Long Y, Tian H, Li X, Zhao AZ, Li RF, Chen T: Adiponectin and Endometrial Cancer: A Systematic Review and Meta-Analysis. *Cell Physiol Biochem* 2015;36:1670-1678.
- 4 Bokhman J V: Two pathogenetic types of endometrial carcinoma. *Gynecol Oncol* 1983;15:10-17.
- 5 Alowayed N, Salker MS, Zeng N, Singh Y, Lang F. LEFTY2 Controls Migration of Human Endometrial Cancer Cells via Focal Adhesion Kinase Activity (FAK) and miRNA-200a. *Cell Physiol Biochem*. 2016;39(3):815-826.
- 6 Kanduri C, Whitehead J, Mohammad F: The long and the short of it: RNA-directed chromatin asymmetry in mammalian X-chromosome inactivation. *Febs Lett* 2009;583:857-864.
- 7 Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Bruggmann SA, Goodnough LH, Helms JA, Farnham PJ, Segal E, Chang HY: Functional Demarcation of Active and Silent Chromatin Domains in Human HOX, Loci by Noncoding RNAs. *Cell* 2007;129:1311-1323.
- 8 Feng J, Bi C, Clark B S, Mady R, Shah P, Kohtz J D: The Evf-2 noncoding RNA is transcribed from the Dlx-5/6 ultraconserved region and functions as a Dlx-2 transcriptional coactivator. *Gene Dev* 2006;20:1470-1484.
- 9 Martianov I, Ramadass A, Serra B A, Chow N, Akoulitchev A: Repression of the human dihydrofolate reductase gene by a non-coding interfering transcript. *Nature* 2007;445:666-670.
- 10 Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, Tramontano A, Bozzoni L: A Long Noncoding RNA Controls Muscle Differentiation by Functioning as a Competing Endogenous RNA. *Cell* 2011;147:358-369
- 11 Leucci E, Vendramin R, Spinazzi M, Laurette P, Fiers M, Wouters J, Radaelli E, Eyckerman S, Leonelli C, Vanderheyden K, Rogiers A, Hermans E, Baatsen P, Aerts S, Amant F, Van A S, Oord J, Strooper B, Davidson I, Lafontaine DL, Gevaert K, Vandesompele J, Mestdagh P, Marine JC: Melanoma addiction to the long non-coding RNA SAMMSON. *Nature* 2016;531:518-522.
- 12 Ming L, Li Z, Wei W, Zeng Y, Liu Z, Qiu J: Long non-coding rna h19 increases bladder cancer metastasis by associating with ezh2 and inhibiting e-cadherin expression. *Cancer Lett* 2013;333:213-221.
- 13 Braconi C, Kogure T, Valeri N, Huang N, Nuovo G, Costinean S, Negrini M, Miotto E, Croce CM, and Patel T: microRNA-29 can regulate expression of the long non-coding RNA gene MEG3 in hepatocellular cancer. *Oncogene* 2011;30:4750-4756.
- 14 Wu XS, Wang XA, Wu WG, Hu YP, Li ML, Ding Q, Weng H, Shu YJ, Liu TY, Jiang L, Cao Y, Bao RF, Mu JS, Tan ZJ, Tao F, Liu YB: MALAT1 promotes the proliferation and metastasis of gallbladder cancer cells by activating the ERK/MAPK pathway. *Cancer Biol Ther* 2014;15:806-814.
- 15 Qiu JJ, Lin YY, Ye LC, Ding JX, Feng WW, Jin HY, Zhang Y, Li Q, Hua KQ: Overexpression of long non-coding RNA HOTAIR predicts poor patient prognosis and promotes tumor metastasis in epithelial ovarian cancer. *Gynecol Oncol* 2014;134:121-128.
- 16 Mccarthy N: Epigenetics. Going places with BANCR. *Nat Rev Cancer* 2012;12:454-454.
- 17 Flockhart R J, Webster D E, Qu K, Flockhart RJ, Webster DE, Qu K, Mascarenhas N, Kovalski J, Kretz M: BRAFV600E remodels the melanocyte transcriptome and induces BANCR to regulate melanoma cell migration. *Genome Res* 2012;22:1006-1014.
- 18 Zhang Z X, Liu Z Q, Jiang B, Lu X Y, Ning X F, Yuan C T, Wang A L: BRAF activated non-coding RNA (BANCR) promoting gastric cancer cells proliferation via regulation of NF-κB1. *Biochem Bioph Res Co* 2015;465:225-231.
- 19 Guo Q, Zhao Y, Chen J, Hu J, Wang S, Zhang D: BRAF-activated long non-coding RNA contributes to colorectal cancer migration by inducing epithelial-mesenchymal transition. *Oncology Lett* 2014;8:869-875.

- 20 Jiang W, Zhang D, Xu B, Wu Z, Liu S, Zhang L, Tian Y, Han X, Tian D: Long non-coding RNA BANCR promotes proliferation and migration of lung carcinoma via MAPK pathways. *Biomed Pharmacoth* 2015;69:90-95.
- 21 Liao Q, Liu C, Yuan X, Kang S, Miao R, Xiao H, Zhao G, Luo H, Bu D, Zhao H, Skogerb G, Wu Z, Zhao Y: Large-scale prediction of long non-coding RNA functions in a coding-non-coding gene co-expression network. *Nucleic Acids Res* 2011;39:3864-3878.
- 22 Lin C Y, Chao A, Wang T H, Hsueh S, Lee YS, Wu TI: A dual tyrosine kinase inhibitor lapatinib suppresses overexpression of matrix metalloproteinase 1 (MMP1) in endometrial cancer. *J Mol Med* 2014;92:969-981.
- 23 Honkavuoritoivola M, Santala M, Soini Y, Turpeenniemi-Hujanen T, Talvensaari-Mattila A: Combination of strong MMP-2 and weak TIMP-2 immunostainings is a significant prognostic factor in endometrial carcinoma. *Dis Markers* 2013;35:261-266.
- 24 Huntington J T, Shields J M, Der C J, Wyatt C A, Benbow U, Slingluff C L Jr, Brinckerhoff C E: Overexpression of Collagenase 1 (MMP-1) Is Mediated by the ERK Pathway in Invasive Melanoma Cells: Role of BRAF mutation and Fibroblast growth factor signaling. *J Biol Chem* 2004;279:33168-33176.
- 25 Chang L, Karin M: Mammalian MAP kinase signalling cascades. *Nature* 2001;410:37-40.
- 26 Deng W, Hua S, Wang Q, He N, Duan CY, Han L, Li Q, Lu M, Lv SQ: A Chinese herbal formula, Yi-Qi-Fu-Sheng, inhibits migration/invasion of colorectal cancer by down-regulating MMP-2/9 via inhibiting the activation of ERK/MAPK signaling pathways. *Bmc Complement Altern* 2013;13:1-10.
- 27 Li Y, Xie Y, Cui D, Ma Y, Sui L, Zhu C, Kong H, Kong Y: Osteopontin Promotes Invasion, Migration and Epithelial-Mesenchymal Transition of Human Endometrial Carcinoma Cell HEC-1A Through AKT and ERK1/2 Signaling. *Cell Physiol Biochem* 2015;37:1503-1512.
- 28 Motoyama K, Inoue H, Nakamura Y, Uetake H, Sugihara K, Mori M: Clinical Significance of High Mobility Group A2 in Human Gastric Cancer and Its Relationship to let-7 MicroRNA Family. *Clin Cancer Res* 2008;14:2334-2340.
- 29 Zhou J X, Niehans G A, Shar A, Rubins J B, Frizelle S P, Kratzke R A: Mechanisms of G1 checkpoint loss in resected early stage non-small cell lung cancer. *Lung Cancer* 2001;32:27-38.
- 30 Jiang HL, Xu D, Yu H, Ma X, Lin GF, Ma DY, Jin JZ: DAX-1 inhibits hepatocellular carcinoma proliferation by inhibiting β -catenin transcriptional activity. *Cell Physiol Biochem* 2014;34:734-742.
- 31 Vaillant F, Merino D, Lee L, Breslin K, Pal B, Ritchie M E, Smyth G K, Christie M, Phillipson L J, Burns C J, Mann G B, Visvader J E, Lindeman G J: Targeting BCL-2 with the BH3 Mimetic ABT-199 in Estrogen Receptor-Positive Breast Cancer. *Cancer Cell* 2013;24:120-129.
- 32 Li R, Zhang L, Jia L, Duan Y, Li Y, Bao L, Sha N: Long non-coding RNA BANCR promotes proliferation in malignant melanoma by regulating MAPK pathway activation. *Plos One* 2014;9:e100893.
- 33 Zhou L, Cai B, Bao W, He YY, Chen XY, Yang YX, Liu XL, Wan XP: Crosstalk between estrogen receptor and mitogen-activated protein kinase signaling in the development and progression of endometrial cancer. *Int J Gynecol Cancer* 2011;21:1357-1365.
- 34 Mizumoto Y, Kyo S, Mori N, Sakaguchi J, Ohno S, Maida Y, Hashimoto M, Takakura M, Inoue M: Activation of ERK1/2 occurs independently of KRAS, or BRAF, status in endometrial cancer and is associated with favorable prognosis. *Cancer Sci* 2007;98:652-658.
- 35 Liao Q, Xiao H, Bu D, Xie C, Miao R, Luo H, Zhao G, Yu K, Zhao H, Skogerb G, Chen R, Wu Z, Liu C, Zhao Y: ncFANS: a web server for functional annotation of long non-coding RNAs. *Nucleic Acids Res* 2011;39:118-124.
- 36 Mercer T R, Qureshi I A, Gokhan S, Dinger M E, Li G, Mattick J S, Mehler M F: Long noncoding RNAs in neuronal-glial fate specification and oligodendrocyte lineage maturation. *Bmc Neurosci* 2010;11:14.
- 37 Goryfauré S, Windscheid V, Bosc C, Peris L, Proietto D, Franck R, Denarier E, Job D, Andrieux A: STOP-like protein 21 is a novel member of the STOP family, revealing a Golgi localization of STOP proteins. *J Biol Chem* 2006;281:28387-28396.
- 38 Tubergen E A V, Banerjee R, Liu M, Vander B R, Light E, Kuo S, Feinberg S E, Willis A L, Wolf G, Carey T, Bradford C, Prince M, Worden F P, Kirkwood K L, D'Silva N J: Inactivation or loss of TTP promotes invasion in head and neck cancer via transcript stabilization and secretion of MMP9, MMP2 and IL-6. *Clin Cancer Res* 2013;19:1169-1179.