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Esophageal cancer related gene-4 inhibits the migration and proliferation of oral squamous cell carcinoma through BC200 lncRNA/MMP-9 and -13 signaling pathway

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ABSTRACT

Esophageal cancer related gene-4 (ECRG4) inhibits the malignant phenotype of oral squamous cell carcinoma. However, the molecular mechanisms remain to be explored. Using the tongue carcinoma cell line, TCA8113 as a cell model, we showed that forced expression of ECRG4 down-regulated the expression of the BC200 long non-coding RNA (lncRNA) and matrix metalloproteinases (MMP-9 and MMP-13). Restoration of BC200 lncRNA rescued ECRG4-mediated down-regulation of MMP-9 and -13. Furthermore, over-expression of Ecrg4 inhibited cell proliferation and migration, which was abolished by forced expression of BC200 lncRNA in TCA8113 cells. Our results indicate that ECRG4 inhibits the malignant phenotype of TCA8113 cells most likely through suppression of BC200 lncRNA/MMPs signaling pathway, rationalizing that BC200 lncRNA may be a potential target for oral squamous cell carcinoma (OSCC) therapy.

1. Introduction

Oral squamous cell carcinoma (OSCC) is the most common squamous cell carcinoma of the head and neck (SCCHN) [1,2]. Despite the continuous improvement in surgical procedures and introduction of newer radio- and chemo-therapeutics, the overall 5-year survival rate for head and neck cancer patients remains at 40–50% in the past 30 years [3,4]. This is mainly attributed to the poor understanding of the molecular mechanisms driving the malignant behaviors of SCCHN.

Esophageal cancer related gene-4 (ECRG4) was originally cloned from normal esophageal epithelium by mRNA differential display comparing normal versus esophageal squamous cell carcinoma [5,6]. It has been shown that Ecrg4 is constitutively expressed in many quiescent tissues and decreased in various cancers including SCCHN [7–9]. This decreased Ecrg4 expression, mediated mainly by promoter hypermethylation, was directly correlated with increased regional lymph node metastasis, enlarged primary tumor size, advanced tumor stage, and poor prognosis [10–15]. Consistent with the decreased expression of Ecrg4 in tumorigenesis, restoration of Ecrg4 through forced expression, administration of recombinant Ecrg4, and application of 5-Azacytidine (a de-methylation reagent) significantly inhibited the malignant phenotype of tumor cells in vitro, and suppressed tumor growth in vivo in xenograft mouse models [16–19]. Unlike other tumor suppressors that are usually membrane or intracellular proteins, Ecrg4 is a pre-pro-peptide that can be processed proteolytically, in a tissue-dependent manner, into about a dozen of small peptides possessing various biological activities [6,20]. Accordingly, the molecular mechanisms underlying the tumor suppressive effect of Ecrg4 may be mediated by full-length Ecrg4, processed small peptides, or a combination of both, and remain open to debate [6]. Using tumor cell lines, restoration of Ecrg4 has been shown to inhibit NF-kb activation [21], arrest cell cycle [17,18,22,23], sensitize tumor cells to chemotherapeutics [8], and promote apoptosis [9,24]. In 2016, Moriguchi et al. demonstrated that Ecrg4 null glioma-initiating cell (GIC) line consistently formed tumors in the brain of an immunocompetent mouse model, whereas the transplanted Ecrg4 wild type-GIC line was frequently eliminated. This antitumor effect of Ecrg4 depends on its ability to enhance host immunity via an intact type-I IFN signaling pathway [25]. Recently,

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lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) was identified as Ecrg4 receptor that specifically binds Ecrg4 peptide (AA71–132) and facilitate its internalization, leading to a MyD88-dependent NF-kB activation in microglia [26]. This discovery is in line with the finding that Ecrg4 interacts with innate immune complex (TLR4, MD2, and CD14) in leukocytes, corroborating that Ecrg4 functions through modulation of immune system [25–28].

Emerging evidence has shown that long non-coding RNAs (lncRNAs) can function as either oncogenes or tumor suppressor genes, which has been implicated in tumor cell proliferation, invasion, and metastasis [29]. Here we provide evidence to show that Ecrg4 suppressed the malignant phenotype of oral squamous cell carcinoma most likely through BC200 lncRNA/MMPs signaling pathway.

2. Methods

2.1. Cell culture

The human tongue squamous carcinoma cell line, TCA8113 purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China), was cultured with RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin and in humidified air with 5% CO_2 at 37 °C. PC3 cells, a human prostate adenocarcinoma cell line, were originally purchased from ATCC (Manassas, VA, USA) and propagated in RPMI-1640 supplemented with 10% FCS as that for TCA8113 cells. Cells in logarithmic phase of growth were harvested for all experiments. All disposable plastic wares for cell culture were the products of Corning, NY, USA.

2.1.1. Construction of expression plasmids

ECEG4 open reading frame (ORF) were amplified by PCR using pcDNA3.1-hEcrg4 (a gift from Dr. Andrew Baird, Department of Surgery, University of California in San Diego School of Medicine) as template, with sense primer containing an *Xho*I site and anti-sense primer containing a BamHI site (primers were listed in Table 1). The PCR products were purified and fused in-frame to the N-terminus of GFP in pEGFP-N1, generating plasmids pEGFP-N1-Ecrg4 (Ecrg4), which was sequenced (Qingke, Chengdu) to confirm its identity and in-frame fusion with GFP. The sequencing of the plasmid and the plasmid are available upon reasonable request.

Table 1
List of all primers for cloning and real-time PCR.

Name	Sequence
Cloning	
ECRG4	
For.	5′-AGATCTCGAGCCGCCATGGCTGCCTCCCCC-3′
Rev.	5′-CGGTGGATCCGCGTAGTCATCGTAGTTGACGC-3′
BC200	
200Ec	5′-ACTAGAATTCGGCCGGGCGCGGTGGCTCAC-3′
200Ba:	5′-CATCGGATCCAAAGGGGGGGGGGGGTTGTT-3′
Real-time PCR	
ECRG4	
For.	5′-ACTAAGACTAAAGTGGCCGTTG- 3′
Rev.	5′-AATTTCGCTTCGTCAAAGCCC-3′
BC200	
For.	5′-CTGGGCAATATAGCGAGAC-3′
Rev.	5′-TGCTTTGAGGGAAGTTACG-3′
MMP9	
For.	5′-GGGACGCAGACATCGTCATC-3′
Rev.	5′- TCGTCATCGTCGAAATGGGC-3′
MMP13	
For.	5′- CCAGACTTCACGATGGCATTG-3′
Rev.	5′- GGCATCTCCTCCATAATTTGGC-3′
MIEN1	
For.	5′-*CAGTGCTGTGGAGCAGT*-3′
Rev.	5′-*GACGGCTGTTGGTGATCTTT*-3′
GAPDH	
For.	5′-GAGTCCACTGGCGTCTTCA-3′
Rev.	5′-TCTTGAGGCTGTTGTCATACTTC-3′

For human long non-coding RNA BC200 (GenBank: NR_001568.1) cloning, total RNA from HEK293 cells was prepared by Trizol following the manufacturer's instructions. One μg of total RNA was reverse transcribed in a 20 μl volume. Of the 20 μl cDNA, 1 μl was used to amplify BC200 lncRNA coding sequence with forward primer containing an *Eco*RI site and reverse primer containing a BamHI site (primers were listed in Table 1). The PCR products were purified and cloned into pcDNA3.1 (+), and the identity of the resulting plasmid, pcDNA3.1-hBC200 (BC200) was confirmed by DNA sequencing (Qingke, Chengdu). The sequencing of the plasmid and the plasmid are available upon reasonable request.

2.2. Establishment of TCA8113 cells stably expressing Ecrg4

TCA8113 cells were seeded in 60 mm dishes at the density of 6×10^5 per dish the day before lentivirus transduction. Lentivirus of vector (pLVX-IRES-ZsGreen-1) (Clontech) or Ecrg4 (pLVX-IRES-ZsGreen-1-hEcrg4) was packaged using Lenti-X Packaging Single Shots (VSV-G) system (Takara) as described previously [20]. The viruses were tittered by transduction of HEK293 cells with serially diluted viral stock for 48 h, followed by counting GFP-positive cells by flow cytometry. Vector or Ecrg4 lentivirus was added into separate dish at multiplicity of infection (MOI) of 3 for 36 h. GFP positive cells were selected twice by flow cytometry, generating TCA8113-Vector (TCA-Vec) and TCA8113-Ecrg4 (TCA-Ecrg4) stables cell lines that were expanded for cell proliferation and migration, and BC200 RNA rescue experiments.

2.3. Subcellular localization of ECRG4-GFP by fluorescence microscopy

TCA8113 cells were seeded in a 6-well plate (CLS3516, Corning Costar) containing a lysine-treated glass coverslip at the density of 5×10^5 per well the day before transient transfection. Cells were then transiently transfected with pEGFP-N1 or pEGFP-N1-hEcrg4 using Lipofectamine 2000 as described previously [20]. After 36 h of incubation, cells on the coverslips were fixed with 4% (w/v) Paraformaldehyde (PFA) for 10 min at room temperature, permeabilized, and incubated with anti-Ecrg4 antibody (NBP2–00599, NOVUS BIOLOGICAL), followed by alexa fluo 594 labeled goat anti-rabbit IgG (R37177, Invitrogen) as described previously [28]. The slides were then counterstained with the DAPI solution at 0.5 μg/ml (CST, 1:50000) for 15 min at room temperature. After rinsing with PBS, the coverslips were mounted with mounting medium, and the localization of Ecrg4 was visualized by fluorescence microscopy (IX83, Olympus).

2.4. RNA isolation and qRT-PCR

TCA8113 cells were seeded in a 6-well plate the day before the experiments. Cells were transfected with 3 μg of vector, ECRG4, or ECRG4 and BC200 for 36 h. Total RNA was prepared using Trizol (Invitrogen). One μg of total RNA was reverse transcribed in a total volume of 10 μl with ReverTra Ace qPCR RT Master Mix kit (TOYOBO) following manufacturer's instructions. cDNA was diluted 3 times, and 2 μl was used for real-time PCR in a 20 μl reaction using SYBR Green Real Time PCR Mix (Qiagen). The PCR conditions were 95 °C for 2 min, followed by 40 cycles of 95 °C for 20″ and 60 °C for 10″. PCR efficiency was derived from standard curve and calculated using Thermo Fisher Scientific's qPCR Efficiency Calculator. The efficiency for each primer pair was 95–105%. Primers used were listed in Table 1.

2.5. CCK-8 cell viability assay

TCA8113 stable cell lines expressing either vector or Ecrg4 were seeded at 3×10^3 cells per well in a 96-well plate and cultured for overnight. At 24, 48, and 72 h after plating, CCK8 assay was conducted.

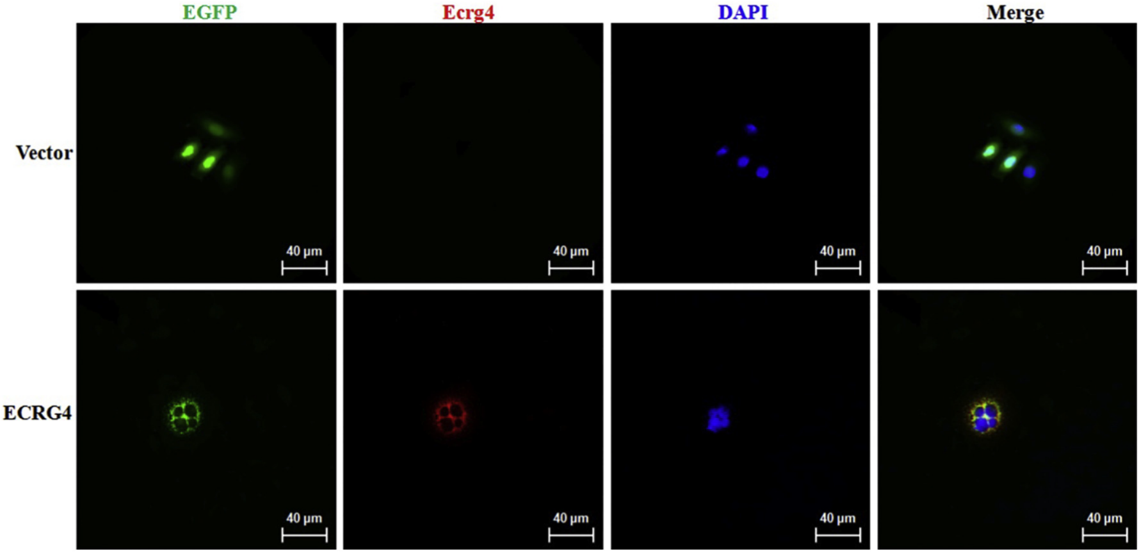

Fig. 1. Ecrg4 is localized perinucleus. Plasmid encoding human full-length ECRG4 fused to the N-terminus of EGFP was transiently transfected into TCA8113 cells for 36 h. Recombinant Ecrg4 is localized perinucleus, with some on cell membrane (bottom panel, from left to right: Ecrg4-EGFP in green, Ecrg4 in red, nuclei in blue, and merged image) compared to a marked nuclear localization of EGFP alone and no detectable endogenous Ecrg4 in Vector transfected cells (Top panel). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

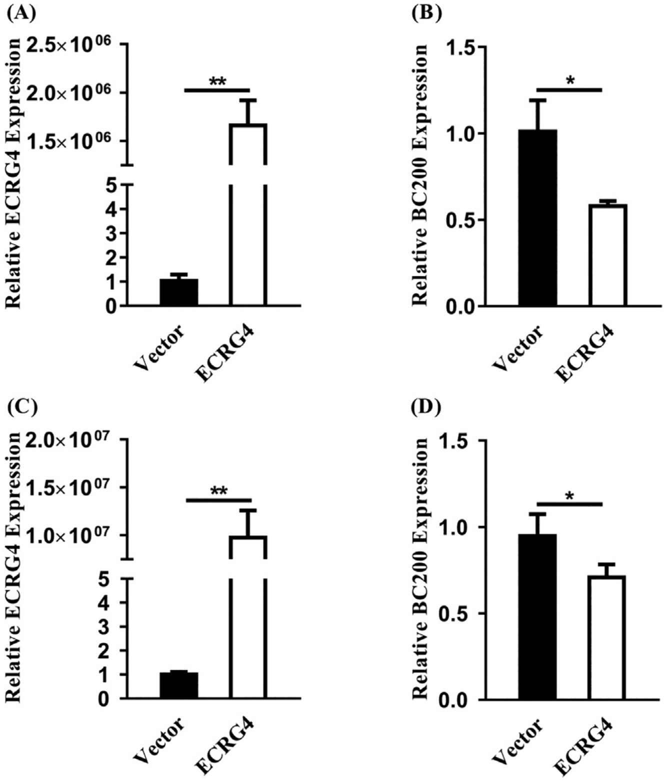

Fig. 2. Forced expression of ECRG4 decreases BC200 lncRNA expression. Plasmid encoding human ECRG4 was transiently transfected, and the increased ECRG4 mRNA expression was confirmed by real-time PCR in TCA8113 (2A, open bar) and PC3 (2C, open bar) cells compared to vector transfected cells (2A and 2C, solid bars), respectively. Over-expression of ECRG4 significantly decreased the expression of BC200 lncRNA in both TCA8113 (2B) and PC3 (2D) cells, respectively, compared to that of Vector transfected cells (2B and 2D, solid bar) (n = 3). The expression of ECRG4 mRNA and BC200 lncRNA was normalized to that of GAPDH. All experiments were in triplicate and repeated at least three times. Data presented as 'Mean ± SD', with * denotes $p < .05$, and ** denotes $p < .01$.

Cell Counting Kit-8 (CCK-8) uses the highly water-soluble tetrazolium salt, WST-8[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]. It produces a water-soluble orange colored formazan dye upon reduction in the presence of dehydrogenases in the cells, which is directly proportional to the number of living cells. Briefly, 10 μl of CCK8 solution was added to each well with 100 μl of medium and continued to incubate for 4 h at 37 °C. The optical density was determined at an absorbance of 450 nm using a microplate reader (Synergy2, BioTek). The experiments were repeated at least three times and with each in octuplicate.

2.6. *ELISA*

Stable cell lines, TCA-Vec and TCA-Ecrg4 were seeded in a 6-well plate at the density of 5.0×10^5 per well the day before transient transfection. One μg of pcDNA3.1 plasmids were transiently transfected into TCA-Vec and TCA-Ecrg4 respectively, whereas 1.0 μg of pcDNA3.1-BC200 (BC200) plasmids were transfected into TCA-Ecrg4 stable cells. The supernatants were used for quantitation of the activated forms of MMP-9 and MMP-13 using sandwich-type ELISA kits (4A Biotech Co, Ltd., Beijing, China), and the cells were used for real time PCR as mentioned above. For ELISA assay, a monoclonal coating antibody was adsorbed onto polystyrene 96 microwells to bind the target protein. A biotin-conjugated monoclonal antibody was used to bind the target molecules captured by the first antibody. Horseradish-peroxidase-conjugated avidin was used to amplify the signals. Then a substrate solution specific to horseradish peroxidase was added to produce a color reaction proportional to the amount of MMP-9 and MMP-13. A microplate reader (Synergy2, BioTek) was employed to detect the OD value at the wavelength of 450 nm; the concentrations of MMP-9 and MMP-13 were finally calculated according to the standard curve.

2.7. *Transwell cell invasion assay*

Transwell cell culture inserts with 8-μm pores (Corning) were used to assess the capability of cell invasion. 1.0×10^5 TCA-Vec or TCA–Ecrg4 stable cells in 0.1 ml RPMI-1640 free of serum were seeded into the top chambers pre-coated with 200 mg/ml of Matrigel (BD Biosciences). 0.65 ml of RPMI-1640 medium containing 20% bovine serum was filled into lower chamber serving as a chemotactic gradient.

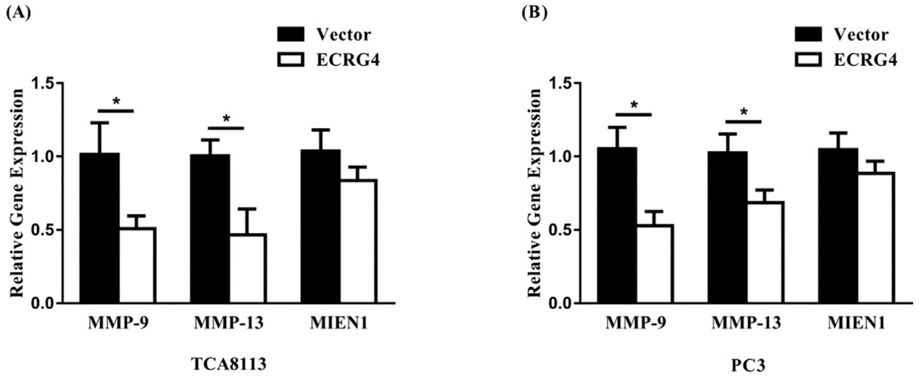

Fig. 3. Forced expression of Ecrg4 decreases MMP-9 and MMP-13 expression but not MIEN1. Overexpression of Ecrg4 significantly decreased the expression of MMP-9 (left, open bar) and MMP-13 (middle, open bar) but not MIEN1 (right, open bar) in both TCA8113 (3A) and PC3 (3B) cells compared to controls (solid bars) (n = 3). The expression of MMPs and MIEN1 was normalized to that of GAPDH. All experiments were in triplicate and repeated at least three times. Data presented as 'Mean ± SD', with * denotes $p < .05$.

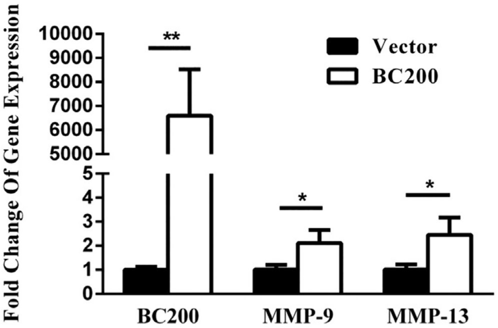

Fig. 4. Forced expression of BC200 lncRNA increases MMP-9 and -13 expression. Plasmid encoding human BC200 RNA was transiently transfected, and the increased BC200 lncRNA expression was confirmed by real-time PCR in TCA8113 cells (open bar, far left). Overexpression of BC200 lncRNA increased the expression of MMP-9 (open bar, middle) and MMP-13 (open bar, far right) compared to vector transfected cells (solid bars) in TCA8113 cells (n = 3). The expression of BC200 lncRNA and MMPs was normalized to that of GAPDH. All experiments were in triplicate and repeated at least three times. Data presented as 'Mean ± SD', with * denotes p < .05, and ** denotes $p < .01$.

After 24 h incubation at 37 °C, the top side of the membrane was wiped with Q-tips to remove medium and any remaining cells that have not transmigrated to the bottom of the membrane, and the transmigrated cells on the bottom side was fixed by using 100% methanol for 10 min, then dried under room temperate and stained with 0.1% crystal violet for 20 min. The number of cells was counted under a microscopy with 100× magnification. Experiments were repeated three times with each in triplicate.

2.8. Cell scratch assay

The two TCA8113 stable cell lines were seeded in a 6-well plate at the density of 5.0×10^5 per well the day before transient transfection. One μg of pcDNA3.1 plasmids were transiently transfected into TCA-Vec and TCA-Ecrg4 respectively, whereas 1 μg of BC200 plasmids were transfected into TCA-Ecrg4 stable cells, and cells were continued to incubate until they reach 80–85% confluence. Wounds were inflicted by scraping central area of the cell monolayers with a 200 μl pipette tip. Photographs immediately after scraping and the cells were continued to culture for 48 h. The wound areas were photographed again and the images were quantitated (percentage of the scratched area decreased at 48 h compared to 0 h) using the Image J software.

2.9. Statistical analysis

Experimental data are presented as mean ± SD with at least three repeats. Between groups comparisons were performed by using one-way analysis of variance (one-way ANOVA). $p < .05$ was considered significant and < 0.01 was considered very significant.

3. Results

3.1. Over-expression of ECRG4 down-regulates the expression of BC200 lncRNA and MMP-9 and -13

Ecrg4 has been shown to inhibit the growth and metastasis of SCCHN cells [7]. In our effort to characterize the pathophysiological role of Ecrg4 in heart, we found that restoration of Ecrg4 in HL1 cell, a cardiac muscle cell line, significantly down-regulated the expression of BC200 lncRNA in our RNAseq analysis (unpublished data). Given that BC200 lncRNA, a neuronal restricted lncRNA at physiological state, is well-documented to be re-activated in various cancers and its levels of expression are inversely correlated with the prognosis [30–33], we explored the correlation between Ecrg4 and BC200 lncRNA in SCCHN. When Ecrg4-EGFP fusion protein was over-expressed in a highly invasive tongue squamous cell carcinoma cell line, TCA8113 cells and stained with anti-Ecrg4 antibody. As what reported from literatures that Ecrg4 is a membrane tethered/secreted protein [20,34,35], the perfectly co-localized GFP and Ecrg4 (red) was mainly distributed peri-nucleus and ER-like, with some on plasma membrane (Fig. 1, bottom panel) compared to the EGFP control showing no Ecrg4 expression and diffused GFP localized mainly in nucleus (Fig. 1, top panel) [20]. Consistent with what was observed in HL1 cells in RNAseq, forced expression of Ecrg4 (Fig. 2A, open bar) significantly down-regulated the expression of BC200 lncRNA (Fig. 2B, open bar) compared to Vector control (solid bars). These discoveries were also validated in a prostate cancer cell line, PC3 cells (Fig. 2C and D). In contrast, when BC200 was overexpressed in TCA8113 cells, it tended to increase the expression of ECRG4 at higher amount of BC200 plasmid (4 μg) used in transfection ($p > .05$) (Supplement Fig. 1).

3.2. BC200 lncRNA mediates Ecrg4-induced down-regulation of MMP-9 and -13 in TCA8113 cells

Since BC200 lncRNA has been reported to be an upstream regulator of MMPs in NSCLC [33], we evaluated the expression of MMP-9 and -13 and migration and invasion enhancer 1 (MIEN1) that are commonly involved in tumor metastasis in TCA8113 (Fig. 3A) and PC3 cells (Fig. 3B) respectively. Forced expression of Ecrg4 (solid bar) significantly decreased MMP-9 and -13 expression compared to vector control (open bars) in both cell lines. However, forced expression of Ecrg4 did not change the expression of MIEN1 significantly despite showing a decreased tendency. To see whether BC200 lncRNA regulates MMPs in our cell model, plasmids encoding BC200 lncRNA were transfected into TCA8113 cells, and the expression of MMP-9 and -13 were analyzed. As shown in Fig. 4, when BC200 lncRNA was over-expressed (open bar, far left) the expression of MMP-9 and -13 (open bars, middle and far right respectively) were significantly up-regulated compared to vector control (solid bars). Unfortunately, attempts to knockdown the expression of BC200 lncRNA using either lentivirus or

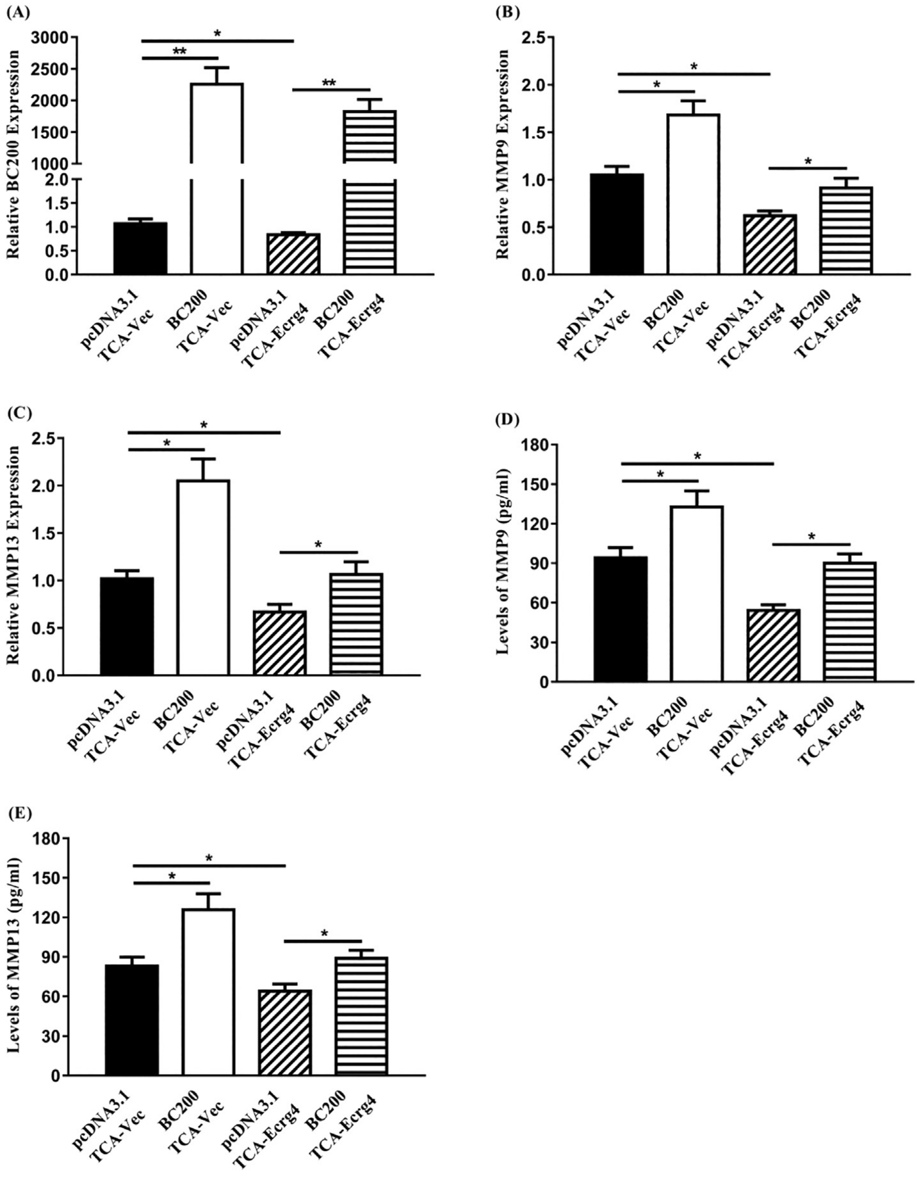

Fig. 5. BC200 lncRNA increases the levels, and partially reverses Ecrg4-mediated down-regulation, of MMPs. TCA-Vec and TCA-Ecrg4 were transiently transfected with either 1.0 μg of pcDNA3.1 or pcDNA3.1-hBC200 (BC200) respectively. (A) TCA-Ecrg4 (bar filled with slanted lines) expressed lower level of BC200 than that of TCA-Vec (solid bar), and BC200 significantly increased the levels of BC200 lncRNA in TCA-Vec (open bar) and TCA-Ecrg4 (bar filled with horizontal lines) compared to pcDNA3.1 transfection respectively (n = 3). (B) and (C) TCA-Ecrg4 (bars filled with slanted lines) suppressed the levels of MMP-9 and -13 mRNAs than that of TCA-Vec (solid bars), BC200 significantly increased the levels of MMP-9 and -13 mRNAs (open bars), and partially reversed Ecrg4-induced down-regulation of MMP-9 and -13 mRNA levels (bars filled with horizontal lines) (n = 3). (D) and (E) TCA-Ecrg4 (bars filled with slanted lines) suppressed the levels of the activated MMP-9 and -13 in the conditioned medium compared to TCA-Vec (solid bars), BC200 significantly increased the levels of the activated MMP-9 and -13 (open bars), and partially reversed Ecrg4-induced down-regulation of MMP-9 and -13 (bars filled with horizontal lines) (n = 5). The expression of BC200 lncRNA and MMPs was normalized to that of GAPDH. All experiments were in triplicate and repeated at least three times. Data presented as 'Mean ± SD', with * denotes $p < .05$, and ** denotes $p < .01$.

synthetic oligos reported from literatures [30,36] was not successful in our hands in TCA8113 cells.

3.3. *BC200 lncRNA increases the levels, and partially reverses Ecrg4-mediated down-regulation, of MMP-9 and -13 in TCA8113 cells*

We have shown that over-expression of Ecrg4 down-regulated, and forced expression of BC200 lncRNA up-regulated, the expression of MMP-9 and -13 in TCA8113 cells. To further confirm that BC200 lncRNA mediates the tumor suppressive effect of Ecrg4, TCA8113 cells were transduced with a bicistronic lentivirus encoding full-length Ecrg4 or vector alone, and GFP positive cells were selected twice by flow cytometry, establishing two TCA8113 stable cell lines, TCA8113-Vector (TCA-Vec) and TCA8113–Ecrg4 (TCA-Ecrg4) [19], where the role of BC200 lncRNA on Ecrg4-induced down-regulation of MMPs was tested. As shown in Fig. 5, the level of BC200 lncRNA was lower in TCA-Ecrg4 (5A, bar filled with slanted lines) than TCA-Vec (5A, solid bar), and forced expression of BC200 significantly increased the levels of BC200 lncRNA in TCA-Vec (5A, open bar) and TCA-Ecrg4 (5A, bar filled with horizontal lines) compared to pcDNA3.1 transfection respectively. TCA-Ecrg4 suppressed the levels of MMP-9 and -13 mRNAs (5B and 5C, bars filled with slanted lines) compared to TCA-Vec (5B and 5C, solid bars), BC200 significantly increased the levels of MMP-9 and -13 mRNAs in TCA-Vec (5B and 5C, open bars) compared to TCA-Vec transfected with pcDNA3.1(solid bars), and partially reversed Ecrg4-induced down-regulation of MMP-9 and -13 mRNA levels (5B and 5C, bars filled with horizontal lines). Similar results were obtained when the levels of activated MMPs in the conditioned medium were analyzed by ELISA (5D and 5E). TCA-Ecrg4 (bars filled with slanted lines) suppressed the levels of activated MMP-9 and -13 compared to TCA-Vec (solid bars), BC200 significantly increased the levels of activated MMP-9 and -13 (open bars), and partially reversed Ecrg4-induced down-regulation of activated MMP-9 and -13 (bars filled with horizontal lines).

3.4. *Over-expression of BC200 lncRNA rescued Ecrg4-mediated suppression of the malignant phenotype of TCA8113 cells*

To analyze the effect of BC200 on cell proliferation and migration, TCA-Vec and TCA-Ecrg4 were transiently transfected with pcDNA3.1 and BC200 respectively. When cell proliferation was analyzed (Fig. 6), TCA-Ecrg4 (open bars) showed significantly inhibited cell proliferation compared TCA-Vec (solid bars) at 24, 48, and 72 h regardless of pcDNA3.1 transfection. Forced expression of BC200 lncRNA significantly increased cell proliferation of TCA-Vec (bars with slanted

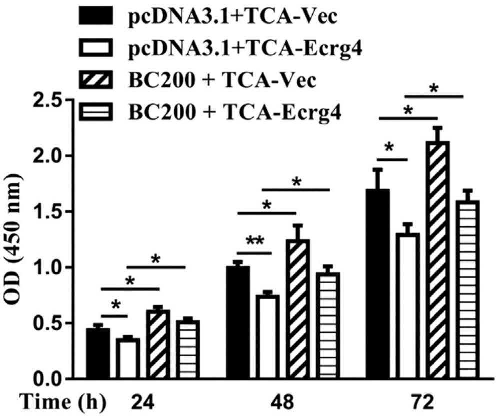

Fig. 6. Forced expression of BC200 RNA cancels out Ecrg4-mediated inhibition of cell proliferation. One μg of either pcDNA3.1 or plasmid expressing BC200 lncRNA were transfected into TCA-Vec or TCA-Ecrg4 cells respectively for 36 h. Cell proliferation was significantly inhibited in 'pcDNA3.1 + TCA-Ecrg4' (open bars) compared to 'pcDNA3.1 + TCA-Vec' (solid bars) cells, whereas forced expression of BC200 lncRNA significantly increased the proliferation of TCA-Vec cells (bars with slanted lines) and partially reversed the inhibited cell proliferation observed in 'BC200 + TCA-Ecrg4' cells (stripped bars) at 24, 48 and 72 h respectively ($n = 3$). Experiments were repeated three times with each treatment in octuplicate. Data presented as 'Mean ± SD', with * denotes $p < .05$, and ** denotes $p < .01$.

lines) and rescued the Ecrg4-mediated inhibition of cell proliferation (bars with horizontal lines), at 24, 48 and 72 h. Wound healing assay was then performed to evaluate cell migration. Representative images of cell scratch were shown in Fig. 6A , and quantification of the percentage of healed area (from 0 h to 48 h) (Fig. 6B) showed that the area was much smaller in TCA-Ecrg4 (open bar) than that in TCA-Vec (solid bar), and forced expression of BC200 partially abrogated Ecrg4-mediated inhibition of wound healing (stripped bar). When cell invasive ability was analyzed using trans-wells (Fig. 7C), TCA-Ecrg4 cells showed significantly less cell trans-migration (Fig. 7D, open bar) compared to TCA-Vec cells (Fig. 7D, solid bar), whereas forced expression of BC200 lncRNA cancelled out, at least partially, the Ecrg4-mediated suppression of cell trans-migration (Fig. 7D, striped bar).

4. Discussion

Different from other known tumor suppressors that loss-of-function mutations are common during tumorigenesis, Ecrg4 mutations have not been reported [6]. In addition, Ecrg4 is a pre-pro-peptide that can be processed proteolytically in a tissue-dependent manner into several small peptides with different, even opposing biological functions [20,37]. Accordingly, the molecular mechanisms of the tumor suppressive function of Ecrg4 are multiple. Here, we expanded the list by showing that Ecrg4 inhibits the expression of BC200 lncRNA that most likely through targeting MMP-9 and -13 suppresses the malignant phenotype of TCA8113 cells.

Since the first report that ECRG4 is a novel tumor suppressor gene in esophageal squamous cell carcinoma, numerous reports have demonstrated the tumor suppressive role of Ecrg4 in cancers of many other organs including SCCHN [6]. In nasopharyngeal carcinoma (NPC) patients, the lower levels of Ecrg4 expression were observed in tumor tissues, which were closely associated with lymph node metastasis and poor prognosis [11]. Restoration of Ecrg4 strongly inhibited the growth and invasive capacities of NPC cell lines, and enhanced their chemosensitivity to cisplatin through autophagy induction [8]. In a highly

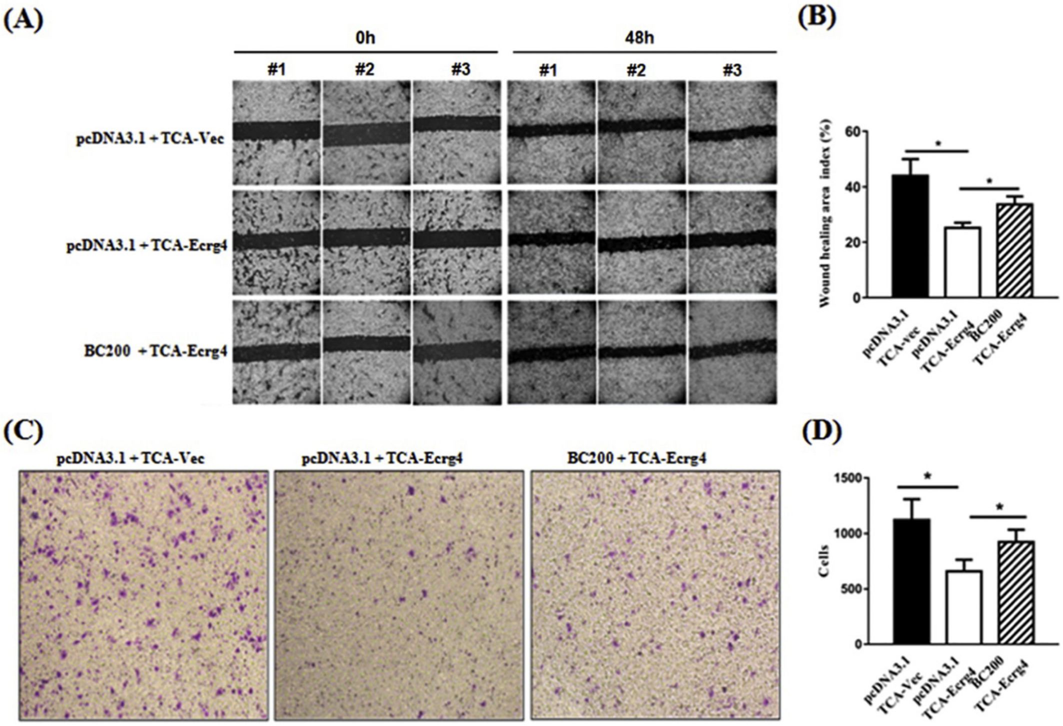

Fig. 7. Forced expression of BC200 lncRNA partially abolished Ecrg4-mediated inhibition of cell transmigration. One μg of pcDNA3.1 was transiently transfected into TCA-Vec stable cells, and 1.0 μg of either pcDNA3.1 or plasmid expressing BC200 lncRNA were transfected into TCA-Ecrg4 stable cells respectively for 36 h. Representative images showing cell scratch assay at 0 h (7A, left panel) and 48 h (7A, right panel), and quantification (7B) showing that the percentage of area decreased (at 0 versus 48 h) was significantly lower in TCA-Ecrg4 (open bar) than TCA-Vec (solid bar), and BC200 partially cancelled out Ecrg4-mediated decreased cell invasion (stripped bar) ($n = 4$). Representative images showing cell migration in trans-well assay (7C), and quantification (7D) showing that invaded cells were significant less in TCA-Ecrg4 (open bar) than TCA-Vec (solid bar), and BC200 partially cancelled out Ecrg4-mediated inhibition of cell invasion (stripped bar) (n = 4). Experiments were repeated three times with each treatment in triplicate. Data presented as 'Mean ± SD', with * denotes $p < .05$.

invasive M2 cell line of SCCHN, forced expression of Ecrg4 significantly inhibited cell proliferation, invasion, migration, and clonogenicity, and promoted cell cycle arrest and apoptosis in vitro; and markedly suppressed tumor growth and metastasis in a xenograft M2 cell mouse model [7]. Consistent with the roles of Ecrg4 in SCCHN, forced expression of Ecrg4 suppressed the proliferation (Fig. 6) and cell migration (Fig. 7) in TCA8113 cells.

lncRNAs have been documented to affect many aspects of the development and progression of OSCC including cell proliferation, apoptosis, cell cycle, invasion, stem cell differentiation, and hypoxia [38]. Up to now, nine lncRNAs including MEG3, Hotair, Malat1, Ccat2, Uca1, Has2-As1, Tug1, Hifcar, and linc-RoR have been implicated in the pathogenesis of OSCC by targeting their downstream molecules through *cis* and *trans* regulation of gene expression, scaffold, decoy, sponging, or inducing degradation of target proteins [38,39]. BC200 has been shown to be activated in tumors [40], forced expression of ECRG4 significantly down-regulated the levels of BC200 in TCA8113 cells (Fig. 2), suggesting the potential roles of BC200 in the pathogenesis of OSCC.

BC200 gene is also known as BCYRN1 (Brain Cytoplasmic RNA1) gene that is mapped on 2p16 and transcribed by RNA polymerase III [41]. BC200 gene contains an internal promoter that may contribute to its neuron-restricted expression and a 5′ promoter that could be transactivated by c-MYC and estrogen receptor α [29,33]. It is believed that epigenetic regulatory mechanisms also play a critical role in the tight control of BC200 gene expression. We showed that Ecrg4 suppressed BC200 lncRNA expression in our RNAseq in HL1 cells (unpublished data) and in the two cell lines presented here. However, how Ecrg4, a membrane and/or secreted protein, inhibits BC200 lncRNA expression remains to be explored.

Normally, BC200 lncRNA regulates neuronal plasticity through targeting components of translation initiation complex [42]. During tumorigenesis of non-neuronal tissues, BC200 gene becomes re-activated and has been shown to contribute to the development of carcinomas of many organs through different molecular mechanisms [31,32]. These include promotion of cell proliferation and tumor sphere formation through up-regulation of cyclin related genes and stemness markers [43], stabilization of S100A11 mRNA to promote cell migration and invasion [30], induction of epithelial-mesenchymal transition by activation of signal transducer and activator of transcription 3 (STAT3) [44], down-regulation of tumor suppressive microRNA138 (miRNA138) [40], and suppression of apoptosis [29,36,45]. In addition, BC200 lncRNA is shown to be highly expressed in invasive cancers, where it promoted the metastasis through up-regulation of MMP-9 and -13 in lung cancer [33], and MMP-2 and -9 in colon cancer [44]. In agreement with these late reports, we showed that forced expression of ECRG4 suppressed the level of BC200 lncRNA in OSCC. Interestingly, when BC200 was over-expressed, it tended to increase ECRG4 expression at higher dosage (Supplement fig. 1), suggesting a self-protection mechanism of cells that keeps the tumorigenic BC200 level in check. However, how ECRG4 and BC200, both localized on chromosome 2, cross-regulates each other remains a mystery. Worthy to mention is that although Ecrg4 decreased the expression of BC200 and MMPs (Fig. 2), and forced expression of BC200 partially abrogated Ecrg4-mediated down-regulation of MMPs (Fig. 5) and suppression of cell proliferation and migration (Fig. 7), the loss of function of BC200 is warranted.

5. Conclusions

We, for the first time, demonstrated that BC200 lncRNA mediated the Ecrg4-induced inhibition of cell proliferation and trans-migration of TCA8113 cells most likely through targeting MMP-9 and -13 signaling pathway. This discovery suggests that BC200 lncRNA may be a potential target for OSCC therapy.

Declarations

- All authors have read the manuscript, and agreed to publish.
- The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
- Authors have no conflict of interest to disclose.
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- The research was conceived by X. D. and C. D., experiments were performed and data were analyzed by W. H., R. Z., and L. M., and manuscript was drafted by X. D. and edited by C. D. and R. Z.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.05.012.

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