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A standardized suppresses breast cancer cell proliferation by regulating the expression of EphA2 antisense RNA-mRNA axis independently of micro RNA

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Submission Date: July 25th, 2019. **Acceptance Date:** September 27th, 2019. **Publication Date:** September 30th, 2019.

Citation: Sakamoto R., Kumagai K., Odaka T., Okuyama T., Nishizawa M., and Kimura T.. A standardized suppresses breast cancer cell proliferation by regulating the expression of EphA2 antisense RNA-mRNA axis independently of micro RNA. *Bioactive Compounds in Health and Disease* 2019; 2(9): 191-205. DOI: https://doi.org/10.31989/bchd.v2i9.642

ABSTRACT

Background: A standardized extract of cultured *Lentinula edodes* mycelia (ECLM), an extract from cultured *Lentinula edodes*, has been reported to suppress breast cancer stem cell proliferation by regulating microRNA (miR) expression. Natural antisense RNAs (ASs), a type of protein non-coding RNA, can regulate the expression of protein-coding genes by acting as a competing endogenous RNA (ceRNA) that adsorbs miRNAs, resulting in the prevention of mRNA degradation, and can also form a transient RNA duplex with mRNA. EphA2, a receptor tyrosine kinase, is typically expressed at low levels in normal epithelial cells, whereas its overexpression

has been widely observed in numerous solid tumors and is associated with cell transformation, primary tumor initiation, and tumor progression.

Objective: This study aimed to investigate the effect of ECLM on the expression of both EphA2 mRNA and endogenous AS to this mRNA, which could negatively affect human breast carcinoma cell proliferation.

Methods: We used MCF7 and MDA-MB-231 human breast carcinoma cells, which were sub-cultured three times in the presence of optimized concentrations of ECLM. The effect of ECLM on the expression of EphA2 AS and mRNA was analyzed by RT-qPCR. miRNAs targeting both EphA2 AS and EphA2 mRNA and their RNA miR response elements (MREs) were predicted and analyzed by RT-qPCR and luciferase reporter assays.

Results: ECLM suppressed the proliferation of MCF7 and MDA-MB-231 cells in a dose-dependent manner. In cells for which proliferation was negatively affected by ECLM, EphA2 AS and mRNA expression was also significantly inhibited by ECLM. Although neutralization of miR-335 led to the de-repression of both EphA2 AS and mRNA, results did not fully support the possibility that EphA2 AS might function as a ceRNA to regulate EphA2 mRNA levels.

Conclusion: ECLM suppressed the proliferation of breast carcinoma cells in a specific dose-dependent manner. This suppressive effect was associated with a concordant reduction in both EphA2 AS and mRNA expression. These effects were not thought to occur via the reported ceRNA effect. Therefore, these results suggest that ECLM suppresses the expression of EphA2 mRNA, which is regulated by EphA2 AS in a ceRNA-independent system, by suppressing the expression of EphA2 AS.

Keywords: ECLM, regulatory RNA, antisense RNA, microRNA, EphA2

INTRODUCTION

A standardized extract of cultured *Lentinula edodes* mycelia (ECLM) is an extract from cultured *Lentinula edodes* that reportedly exerts immune-protective effects against many types of cancer, including liver, breast, colon, and prostate [1-4]. In a recent study, ECLM was also reported to suppress breast cancer stem cell proliferation by regulating the expression of microRNAs (miRNAs) [5, 6]. These miRNAs are controlling genes involved in cellular processes such as inflammation, cell cycle regulation, stress response, differentiation, apoptosis, and migration [7].

Natural antisense RNAs (ASs), comprising a type of protein non-coding RNA, have been reported to regulate the expression of protein-coding genes by acting as a competing endogenous RNA (ceRNA) that adsorbs miRNAs resulting in the prevention of mRNA degradation; further, they can also form a transient RNA duplex [8-10].

EphA2, a receptor tyrosine kinase, is typically expressed at low levels in normal epithelial cells, whereas its overexpression has been widely observed in numerous solid tumors and is associated with cell transformation, primary tumor initiation, and tumor progression [11]. Therefore, we verified the possibility that ECLM regulated the expression of EphA2 mRNA by regulating EphA2 AS expression and suppresses breast cancer cell proliferation. We also verified the possibility that AS could function as a ceRNA for EphA2 as well.

MATERIALS AND METHODS

Cell culture

Human MDA-MB-231 cells (originating from mammary adenocarcinoma; ATCC HTB-26) were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (R10 medium). Human MCF7 cells (originating from mammary adenocarcinoma; ATCC HTB-22) were maintained in Dulbecco's Modified Eagle's Medium (D-MEM) containing 10% FCS, 0.1 mM non-essential amino acids, and 1 mM sodium pyruvate (D10 medium).

To examine the effect(s) of ECLM (Amino Up Chemical, Sapporo, Japan) on EphA2 AS and mRNA expression levels and the potential effects as a ceRNA, MDA-MB-231 cells were maintained in R10 medium supplemented with this compound at 1.25 mg/ml, 0.625 mg/ml and 0 mg/ml; cells were sub-cultured three times. MCF7 cells were maintained in D10 medium supplemented with this compound at 0.625 mg/ml and 0 mg/ml, and cells were also sub-cultured three times.

Measurement of lactate dehydrogenase (LDH) activity

As an indicator of cytotoxicity, LDH activity in the medium was measured in triplicate using LDH Cytotoxicity Detection Kits (Takara Bio, Otsu, Japan), according to the manufacturer's instructions.

Oligonucleotides

Unconjugated locked nucleic acid (LNA)-modified anti-miR-126, anti-miR-335, and anti-miR-4267 oligonucleotides were synthesized with a complete phosphorothioate backbone (Gene-Design, Osaka, Japan). The sequence of antimiR-126, anti-miR-335, and anti-miR-4267 were

complementary to the seed region of mature miR-126, miR-335, and miR-4267 (nucleotides 2–9), respectively. The LNA mismatch control was designed according to the miR-126 seed region sequence.

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

DNase I-treated total cellular RNA was annealed with strand-specific primers. cDNA was synthesized in both the presence and absence of 100 U ReverTra Ace (TOYOBO, Osaka, Japan) at 50 °C for 30 min. The cDNAs were then amplified by PCR using rTaq DNA Polymerase (TOYOBO) in a Thermal Cycler Dice Real Time System II (TaKaRa Bio, Shiga, Japan) using the following default thermocycler program: 1 min of pre-incubation at 95 °C followed by 22–38 cycles of 15 s at 95 °C, 1 min at 72 °C (reducing the annealing temperature 0.3 °C per cycle), and 30 s at 72 °C. The primers used for RT and PCR of AS, mRNA, and 18S rRNA are shown in Table 1.

Table 1. List of primers used for RT-PCR

For detection of EphA2 AS and mRNA
RT primer for EphA2 AS: F, 5′-CGACATCAAGAGGATTGGGGTGCGG-3′ (EphA2 mRNA NM_004431 nt 2981–3005)
RT primer for EphA2 AS: R, 5′-CGGTTTGAATCATCTGCAACTTTATTCC-3′ (EphA2 mRNA nt 3963–3936)
PCR primer pair: F, 5'-CGACATCAAGAGGATTGGGGTGCGG-3' (EphA2 mRNA nt: 2981 -3005) / R, 5'- CAGCATCCCTGGTCATCTCCTCAGTTC-3' (EphA2 mRNA nt: 3271-3245)
For detection of 18S rRNA
RT primer for 18S rRNA: a nona-deoxyribonucleotide random primer mixture (Takara Bio)
PCR primer pair: F, 5′-CTTAGAGGGACAAGTGGCG-3′ (human 18S rRNA nt1443–1461) / R, 5′-ACGCTGAGCCAGTCAGTGTA-3′ (human 18S rRNA nt 1549–1529)

RNA preparation and RNA-seq

Total cellular RNA was isolated from MDA-MB-231 and MCF7 cells using Sepasol®-RNA I Super G (Nacalai tesque, Kyoto, Japan). RNA samples were then treated with proteinase K (Wako, Osaka, Japan) and TURBO DNA-free DNase I kit (Applied Biosystems, Carlsbad, USA) to remove RNase and genomic DNA.

For RNA-seq, ribosomal RNA was removed using the Ribo-Zero rRNA Removal Kit

(Illumina, San Diego, USA), according to the manufacturer's instructions.

Sequence libraries were generated using TruSeq Stranded Total RNA Sample Prep Kit (Illumina). The libraries were then sequenced on an Illumina Hiseq 2500 platform, and 100-bp pair-end reads were generated. Sequence reads were analyzed for expression levels using the CLC genomics workbench (QIAGEN, Hilden, Germany).

Plasmid construction

To construct the pEF-*luc*-EphA2 AS reporter plasmid, EphA2 AS was amplified by PCR using pCG-EphA2 AS (accompanying paper) as a template. EphA2 AS was digested with *Xba*I and ligated into the *Xba*I site of the pEF-*luc*-SVpA [12] reporter vector.

To construct the pEF-*luc*-EphA2 AS miR response element (MRE)-126 seed revertant (SR), MRE-335SR, and MRE-4267SR, PCR and the Gibson assembly system were employed using the Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, USA) and Gibson Assembly Master Mix (New England Biolabs, Ipswich, USA), according to the manufacturer's instructions. Primers for construction of the above vectors are shown in Table 2. These MRE revertants were changed MRE sequences to these complementary sequences.

The stem loop sequences of *Homo sapiens* miR-126, miR-335, and miR-4267 (DDBJ/EMBL/GenBank Accession Numbers: MI0000471, MI0000816, and MI0015871, respectively) were retrieved from the miRBase database. DNA fragments containing 100 bases of both upstream and downstream native flank sequence of each miRNA stem loop were then amplified by PCR and cloned into the *Bam*HI and *Nhe*I sites of the pEGFP-miR expression vector (Cell Biolabs, San Diego, CA, USA) to create pEGFP-miR-126, pEGFP-miR-335, and pEGFP-miR-4267. pLKO-miR-126, pLKO-miR-335, and pLKO-miR-4267 lentiviral vectors were then constructed by replacing the blunted *Xho*I and *Kpn*I fragments of shTORC2 containing the U6 promoter-shTORC2 complementary DNA (cDNA) with a blunted *Cla*I and *Kpn*I fragment of pEGFP-miR-126, pEGFP-miR-335, and pEGFP-miR-4267 containing EF-1α promoter-miRNA-126, EF-1α promoter-miR-335, and EF-1α promoter-miR-4267 or the same fragment from the pEGFP-miR-null control vector (Cell Biolabs). Recombinant lentiviruses encoding miR-126, miR-335, miR-4267, and miR-null were generated in HEK293T cells (ATCC CRL-1126) through the co-transfection of pLKO-miR-126, pLKO-miR-335, and pLKO-miR-4267 or pLKO-null with the packaging constructs pCMVΔR8.2 and pVSV-G.

Transfection and lentiviral transduction

MDA-MB-231 cells were transfected using Lipofectamine®3000 reagent. 2.2 μg of plasmids were mixed with P3000 regent and Lipofectamine® 3000 reagent was added. After a 15-min incubation,

the mixed regent was added to MDA-MB-231 cells, which were transferred to a CO_2 incubator for 24 h at 37 ℃.

For transduction with lentiviral vectors, viral supernatants were collected 48 h after transfecting HEK293T cells with pLKO-miR-126, pLKO-miR-335, and pLKO-miR-4267 or pLKO-null and MDA-MB-231 cells were transduced with the recombinant lentiviruses. The transduced cells were then selected with puromycin (2 μg/ml; Sigma-Aldrich, St. Louis, MO, USA). For the transfection of anti-miR, 1 μg of anti-miR was transfected as described.

Table 2. Nucleotide sequences of primers for the construction of miR response element mutant reporters

For construction of pEF-*luc*-EphA2 AS
F, 5′-gctctagaGAGGATGGGGCCCGAGGG-3′ (EphA2 mRNA nt 3589–3572)
R, 5′-gctctagaCCCACACCCATGGACTGCCC-3′ (EphA2 mRNA nt 2664–2683)
For construction of pEF-*luc*-EphA2 AS MRE-126 SR
Fragment1
F, 5′-TCAGAGAGATCCTCATAAAGGCCAA-3′ (U47295.2 Cloning vector pGL3-Basic nt1691-1715)
R, 5′-GCAGcatggcaCACGTCCCAGCATCCCTG-3′ (EphA2 mRNA nt3317-3345)
Fragment2
F, 5′-GCTGGGACGTGtgccatgCTGCTAAGTGCTCAGCT-3′ (EphA2 mRNA nt3338-3304)
R, 5′-CATTCTAGTTGTGGTTTGTCCAAACTC-3′ (U47295.2 Cloning vector pGL3-Basic nt1821-1795)
For construction of pEF-*luc*-EphA2 AS MRE-335 SR
Fragment1
F, 5′-TCAGAGAGATCCTCATAAAGGCCAA-3′ (U47295.2 Cloning vector pGL3-Basic nt1691-1715)
R, 5′-CGACAagttctcGATTGGGGTGCGGCTGCC-3′ (EphA2 mRNA nt2981-3009)
Fragment2
F, 5′-GCACCCCAATCgagaactTGTCGTCGTTGGTCATC-3′ (EphA2 mRNA nt3003-2969)
R, 5′-CATTCTAGTTGTGGTTTGTCCAAACTC-3′ (U47295.2 Cloning vector pGL3-Basic nt1821-1795)
For construction of pEF-*luc*-EphA2 AS MRE-4267 SR
Fragment1
F, 5′-TCAGAGAGATCCTCATAAAGGCCAA-3′ (U47295.2 Cloning vector pGL3-Basic nt1691-1715)
R, 5′-CATCTAggtcgagATGATGCAGTGCTGGCAGCAG-3′ (EphA2 mRNA nt 2690-2723)
Fragment2
F, 5′-CACTGCATCATctcgaccTAGATGGCGGAGGGGCA-3′ (EphA2 mRNA nt 2713-2679)
R, 5′-CATTCTAGTTGTGGTTTGTCCAAACTC-3′ (U47295.2 Cloning vector pGL3-Basic nt1821-1795)

Luciferase reporter assays

To analyze the effect of miRNA and ECLM on EphA2 AS and mRNA expression, we performed luciferase reporter assays. For reporter gene assays, 6.2×10^5 MDA-MB-231 cells/well were transfected with a luciferase reporter plasmid (0.3 μg/well) using Lipofectamine 3000 (Invitrogen, Carlsbad, USA). Cells were co-transfected with the pRL-RSV plasmid encoding *Renilla* luciferase (0.8 μg/well), to control for differences in transfection efficiency.

Firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instruction. Protein concentrations were measured using the Protein Assay BCA Kit (Wako, Osaka, Japan). Firefly luciferase activity was normalized to *Renilla* luciferase activity and the amount of total protein.

Statistical analysis and informatics

Differences presented in the figures were analyzed by performing a Student's *t* test.

RESULTS

ECLM suppresses the proliferation of human breast cancer cells

We first investigated whether ECLM inhibits proliferation in breast cancer cells by cell counting. ECLM significantly suppressed proliferation at concentrations of 0.625 mg/ml for MCF7 cells (48 hr) and 1.25 mg/ml for MDA-MB-231 cells (48-72 hr). These results indicated that ECLM functions during periods of the low confluence with poor cell adhesion. In addition, ECLM at a concentration of 1.25 mg / ml completely inhibited the growth of MCF7 cells (data not shown). The cytotoxicity of ECLM toward MDA-MB-231 cells was observed 24 h after sub-culture, but not after 48 h of sub-culture, at which time cell proliferation was suppressed (Fig.1).

Identification of a naturally occurring antisense transcript overlapping human *EPHA2*

To determine whether EphA2 AS is expressed in MDA-MB-231 cells we employed RNA-seq. This revealed the presence of reads corresponding to the opposite strand of *EPHA2*, which were widely distributed in the region overlapping mRNA exon 1–17, and included the overlapping EphA2 mRNA3′UTR. (Table 3).

Effect of ECLM on EphA2 antisense and mRNA levels

We next examined the effect of ECLM on the expression levels of EphA2 AS and mRNA in MDA-MB-231 and MCF7 cells. Levels of EphA2 AS and mRNA were concordantly suppressed by ECLM in MDA-MB-231 (accompanying paper) and MCF7 cells (Fig. 2).

EphA2 antisense regulates the miRNA-independent expression of EphA2 mRNA

A recent study showed that AS sequences sharing miRNA response elements (MREs) with coding transcripts can be similarly targeted, resulting in the sequestering of miRNAs to prevent them from acting on mRNAs. Bioinformatics analysis (RegRNA [13], RNAhybrid [14], miRDB [15], and PITA [16]) was performed to predict the MREs that are shared by EphA2 AS and EphA2 mRNA 3′UTR, and these were predicted to share several MREs (MRE-126, MRE-335, and MRE-4267; Fig.3).

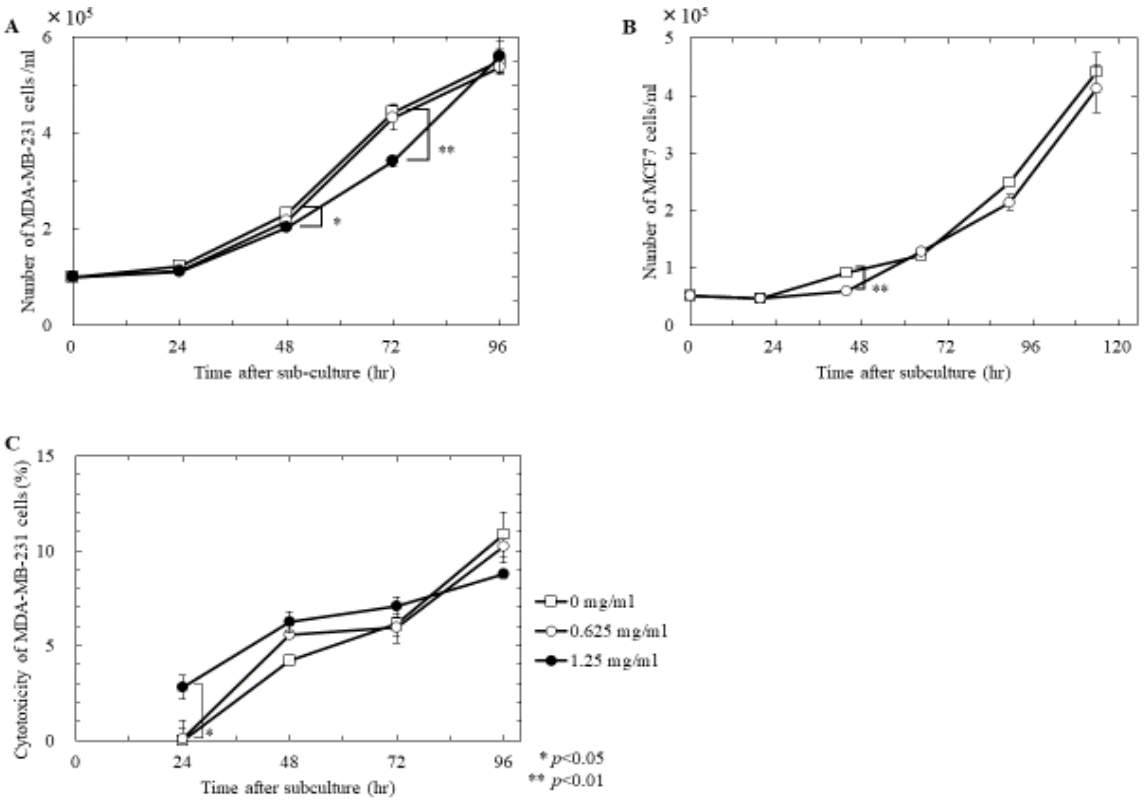

Figure 1. The effects of ECLM on breast cancer cell proliferation and cytotoxicity. A, B. Growth curves of MDA-MB-231 (A) and MCF7 (B) cells, as assessed by cell counting. C. Cytotoxicity of ECLM toward MDA-MB-231 cells, as assessed by LDH assays.

Validation of miR-126, miR-335, and miR-4267 MREs in EphA2 AS and mRNA

To validate the presence of miR-126, miR-335, and miR-4267 MREs in EphA2 AS and mRNA, we assessed the effect of endogenous miR-126, miR-335, and miR-4267 expression on EphA2 AS and mRNA levels by employing an anti-miR approach to inhibit endogenous miR expression[17]. For this knockdown strategy, we designed 8-mer anti-miR-126, miR-335, and miR-4267 sequences, using a fully LNA-modified phosphorothioate oligonucleotide complementary to the seed region of these miRNAs. We then tested their effects on EphA2 AS and mRNA expression in

non-ECLM treated MDA-MB-231 cells. Silencing endogenous miR-335 increased EphA2 AS and mRNA levels by 36- and 28-fold, respectively, compared to expression with an LNA mismatch control (Fig. 4).

Table 3. Positions of EphA2 antisense reads in *EPHA2*

Position of EphA2 AS reads in *EPHA2*			Position of EphA2 AS reads in *EphA2* mRNA			
31,650	-	31,538	3,863	-	3,751	exon17
31,188	-	31,088	3,401	-	3,301	exon17
30,416	-	30,362				intron16
26,654	-	26,568	2,980	-	2,894	exon16
24,249	-	23,898				exon14-13
23,891	-	23,679				exon13-12
22,796	-	22,203	2,096	-	1,868	exon11-9
20,975	-	20,389	1,660	-	1,539	exon7-6
20,621	-	20,537				intron6
18,213	-	17,983	1,445	-	1,215	exon5
16,004	-	15,749				intron3
15,567	-	15,480				intron3
14,110	-	13,998				intron3
11,740	-	11,494				intron3
11,134	-	11,024				intron3
10,291	-	10,184				intron3
10,125	-	10,027				intron3
9,850	-	9,703				intron3
9,382	-	9,251				intron3
9,123	-	8,961				intron3
8,864	-	8,681				intron3
8,575	-	8,383				intron3
7,358	-	7,194	626	-	462	exon3
4,268	-	4,123				intron1
4,103	-	3,995				intron1
3,761	-	3,637				intron1
3,350	-	3,154				intron1
2,920	-	2,782				intron1
1,829	-	1,648				intron1
944	-	290				intron1
198	-	25	198	-	25	exon1

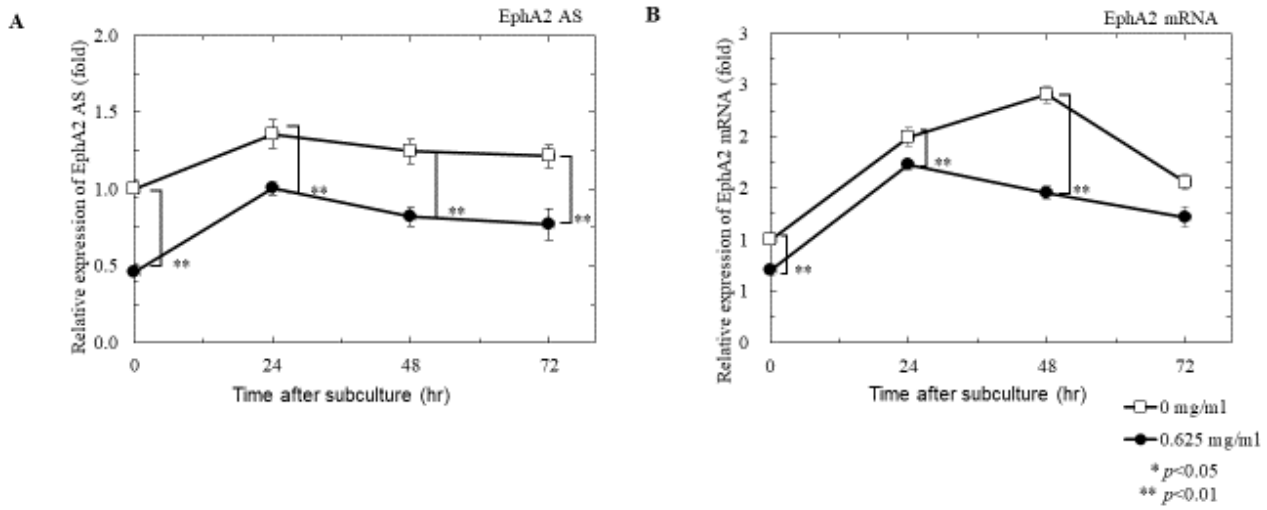

Figure 2. Effect of ECLM on EphA2 antisense (AS) and mRNA levels in MCF7 cells. Shown are expression levels of EphA2 AS (A) and EphA2 mRNA (B).

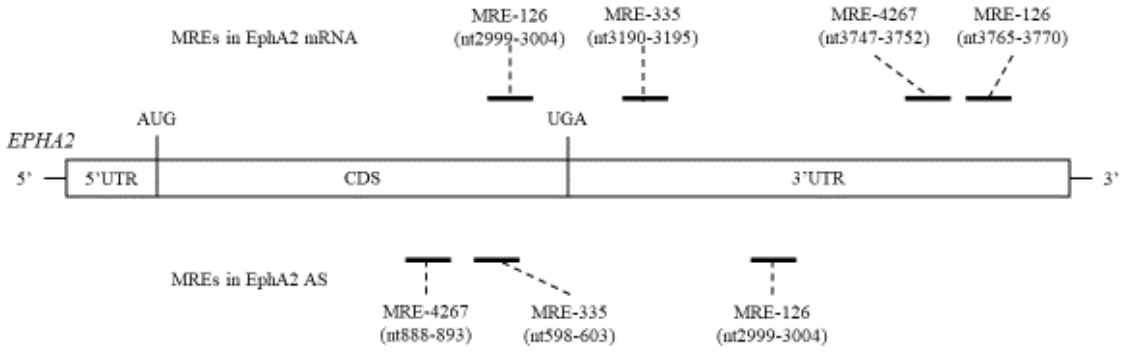

Figure 3. Predicted miRNA response elements (MREs) shared by EphA2 antisense (AS) and EphA2 mRNA 3′UTR. Several MREs (MRE-126, -MRE335, and MRE-4267) in EphA2 AS (reverse strand of mRNA) and EphA2 mRNA 3′UTR were predicted.

We next examined the effect of miR-126, miR-335, and miR-4267 on EphA2 AS and mRNA levels by transducing MDA-MB-231 cells with the virus to overexpress these miRNAs. Contrary to the results of the miR-335-neutralization experiment, suppression of EphA2 AS and mRNA after transfection of the predicted miRNA overexpression construct was not observed by RT-qPCR and luciferase reporter assays (Fig. 5).

To verify specific MRE–miRNA interactions, we used MRE-inverted luciferase reporters. Inverse MRE-126, MRE-335, and MRE-4267 sequences in EphA2 AS were inserted downstream of the luciferase reporter gene and their effects were tested on luciferase reporter activity using pLKO-miR-126, pLKO-miR-335, and pLKO-miR-4267 virus-transduced cells (Fig. 6). These

transduced cells were overexpressed these miRs (data not shown). MiR-126, -335, and -4267 MRE-inverted luciferase reporters in the corresponding miRNA transduced cell, there are no increase in luciferase activity was observed, indicating that these miRNAs did not affect EphA2 AS expression. In addition, similar results was obtained for miR-126, -335, and -4267 MRE triple-inverted luciferase reporter in the parental cell.

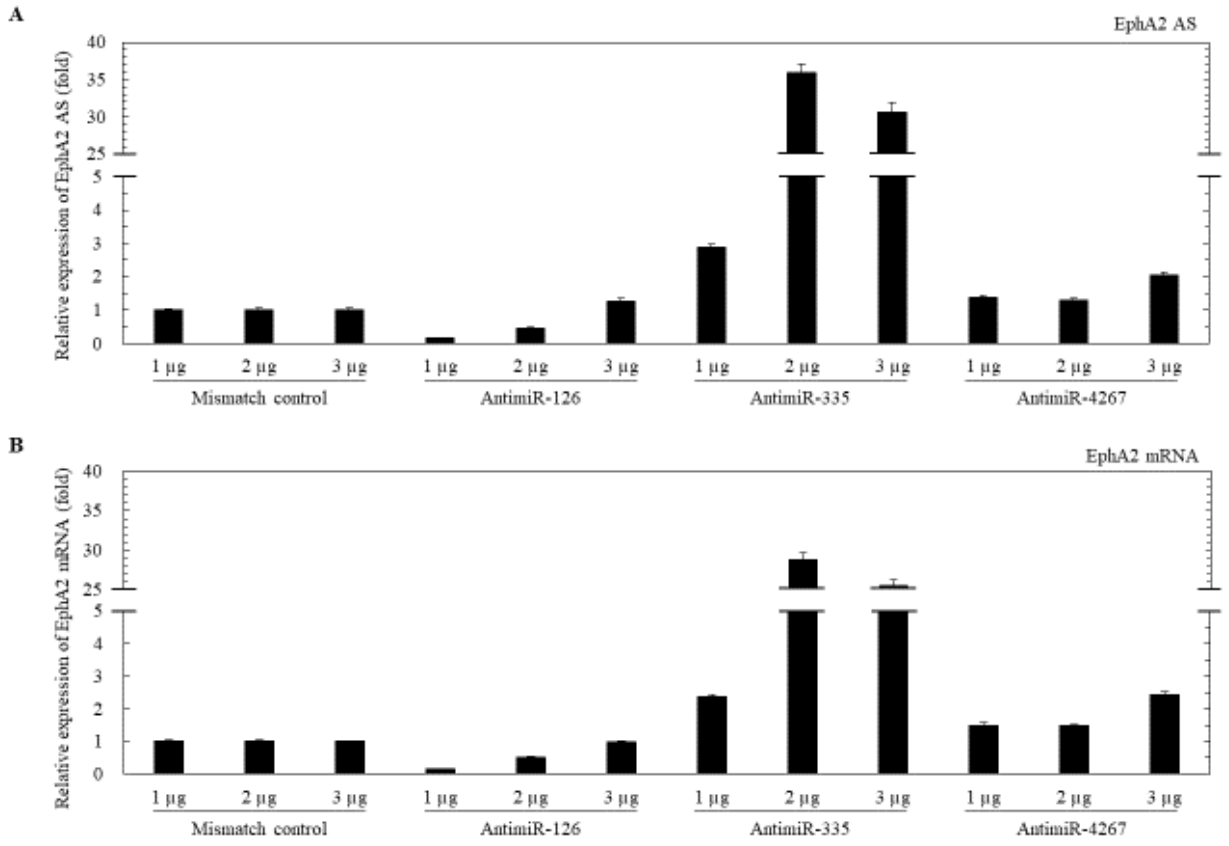

Figure 4. Effect of miR-126, miR-335, and miR-4267 neutralization on EphA2 antisense (AS)/mRNA levels. LNA-modified anti-miR-126, anti-miR-335, and anti-miR-4267 were transfected into MDA-MB-231 cells, which were harvested for the RT-qPCR analysis of relative EphA2 AS (A) and EphA2 mRNA (B) expression. The relative expression was presented as the fold-change in AS and mRNA levels relative to those obtained in LNA mismatch control-transfected cells.

DISCUSSION

ECLM was previously reported to have various anti-cancer effects via an immune-protective effect and through the regulation of miRNA expression [1-5]. ECLM is also known to reduce the risk of adverse neutrophil events in breast cancer patients who received adjuvant chemotherapy at 3 g/day

oral administration [2]. These reports suggested that ECLM is useful for both the treatment of breast cancer and the prevention of chemotherapy side effects.

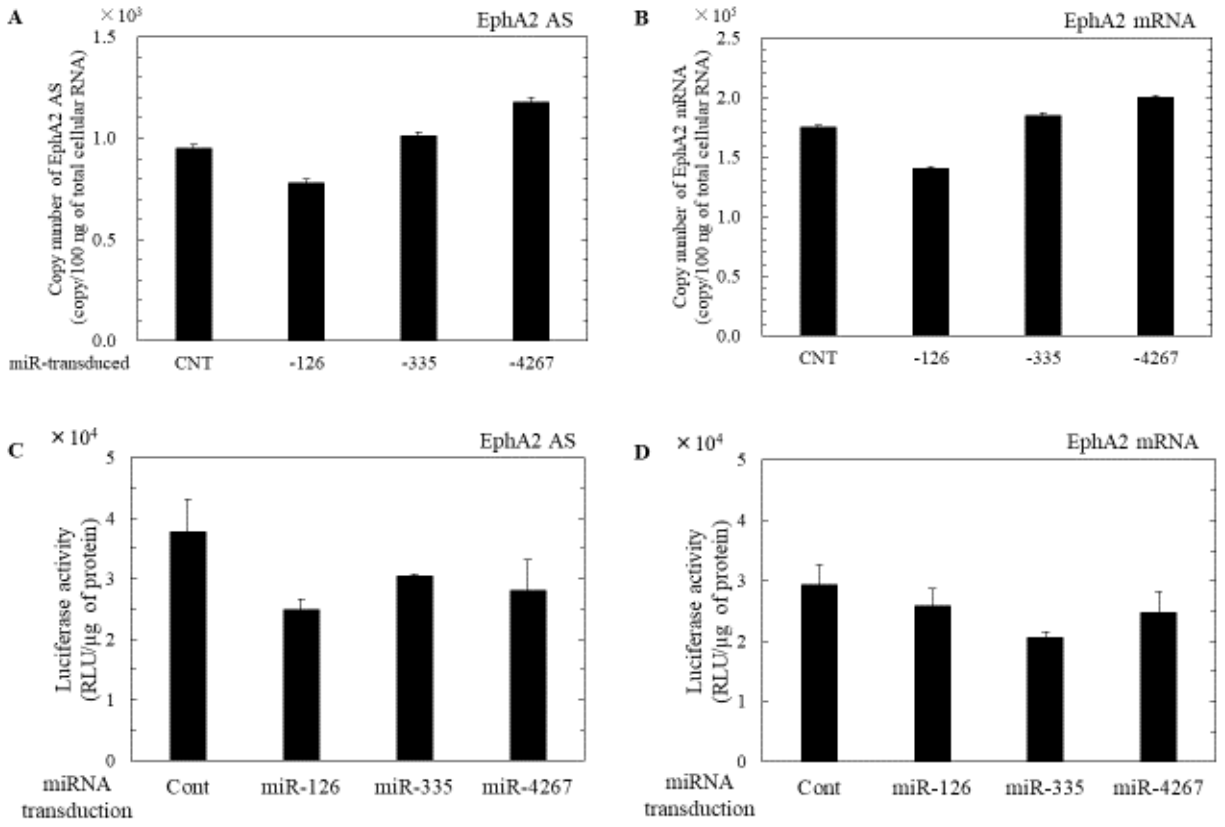

Figure 5. Validation of the effect of miR-126, miR-335, and miR-4267 on EphA2 antisense (AS)/mRNA levels. Effects miR-126, miR-335, and miR-4267 overexpression on EphA2 AS and mRNA expression were examined after transduction into MDA-MB-231 cells. The cells were then harvested for the RT-qPCR analysis of relative EphA2 AS (A) and EphA2 mRNA (B); luciferase reporter assays were also performed for EphA2 AS (C) and EphA2 mRNA (D) activity.

EphA2, which is highly expressed in triple-negative breast cancer, forms a heterodimer with EGFR and is involved in cell proliferation [18]. Accordingly, the delivery of EphrinA1 to tumor tissues, which is involved in the suppression of EphA2, as well as methods using siRNA against EphA2 and antibodies have been examined [19-21]. Compared to these methods, the suppression of breast cancer cell proliferation by inhibiting of EphA2 AS and mRNA expression via ECLM administration is advantageous in that the oral administration of this compound is and safe.

The possibility that EphA2 AS functions as a ceRNA to protect mRNA could be confirmed by miR-335 neutralization experiments; however, the negative results obtained herein did not support this effect. Therefore, it is thought that there is a regulating mechanism by non-ceRNA effect

including mRNA stabilization through sense-antisense duplex formation reported [8]..

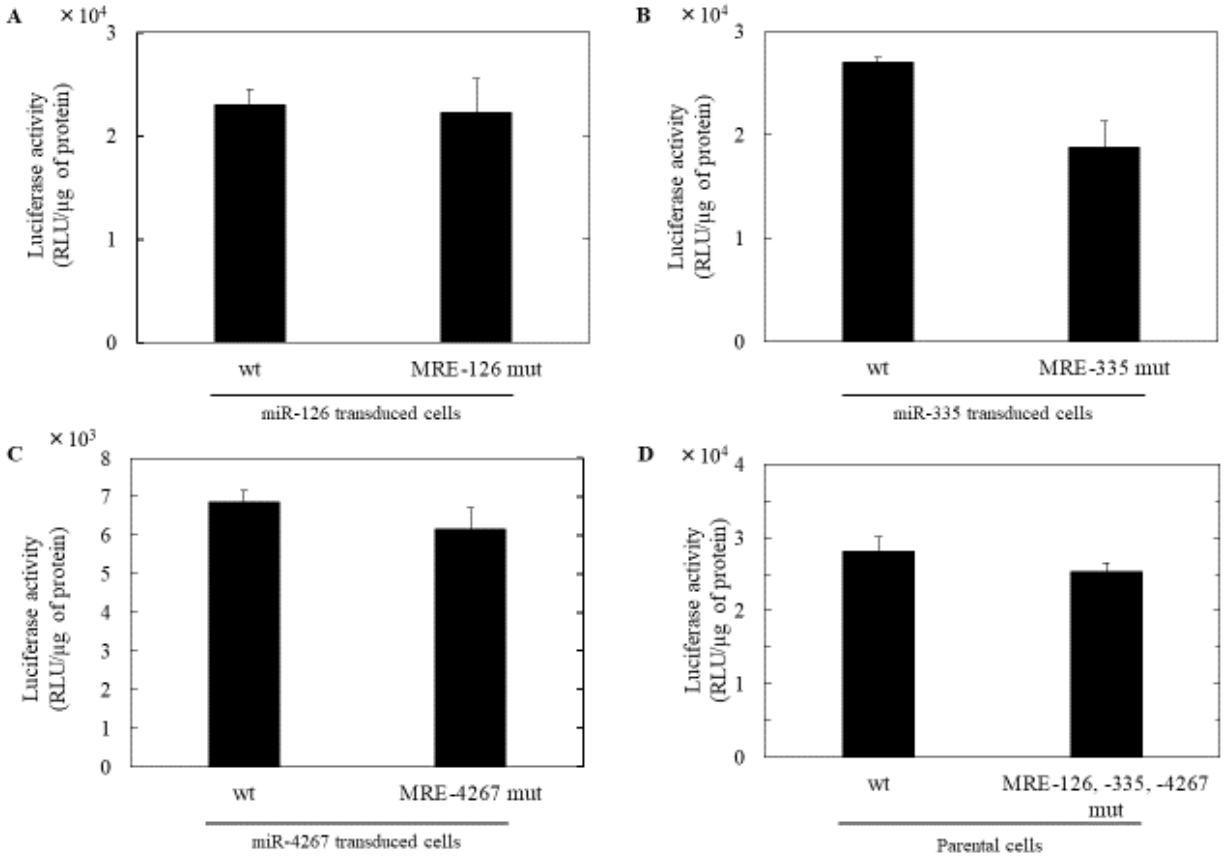

Figure 6. Validation of the effect of inverse miRNA response element (MRE)-126, MRE-335, and MRE-4267 on EphA2 antisense (AS). The effects of miRNA-MRE on EphA2 AS levels were analyzed using an MRE-126 seed revertant (SR) in miR-126-transduced cells (A), an MRE-335 SR in miR-335-transduced cells (B), an MRE-4267 SR in miR-4267-transduced cells (C), and MRE-126, MRE-335, and MRE-4267 SRs in parental cells (D).

CONCLUSION

ECLM suppresses the proliferation of breast carcinoma cells in a specific dose-dependent manner and inhibits both EphA2 AS and mRNA expression. However, this regulatory event did not appear to occur through a ceRNA effect. These results thus suggest that ECLM could regulate EphA2 AS and mRNA expression by not ceRNA effect, thereby stabilizing EphA2 mRNA. Therefore, it is necessary to further examine this regulation mechanism.

Acknowledgements

We are grateful to Noriyuki Yoshida who participated in early stages of this project and to all the present members of the Kimura laboratory for stimulating discussions.

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