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Inhibition of endothelin converting enzyme-1 activity or expression ameliorates angiotensin II-induced myocardial hypertrophy in cultured cardiomyocytes

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Angiotensin II (Ang II)-induced hypertrophy response in cultured cardiomyocytes is partially mediated by endothelin-1 (ET-1). Endothelin converting enzyme-1 (ECE-1) is the rate limiting enzyme in the process of ET-1 production. In this study, two peptides which have significant inhibitory effect to the activity of rat ECE-1 purified from stable rat ECE-1-expressed CHO lines, were selected from 13 big ET-1 analogues. We found that treatment of P8 or P9 reversed the increase of hypertrophy genetic markers and cell surface area in primary cultured neonatal rat cardiomyocytes stimulated by Ang II. Besides, depletion of ECE-1 by RNA interference also revealed similar results as P8 or P9 treatment. These results confirmed that ECE-1 plays a key role in regulating Ang II-induced hypertrophy response in cultured cardiomyocytes.

1. Introduction

Endothelin-1 (ET-1) is a 21-amino acid peptide with potent vasoconstriction activity which was first discovered in the culture medium of porcine aortic endothelial cells (Yanagisawa et al. 1988). Biosynthesis of ET-1 begins with the actions of a furin-like protease on prepro-ET-1 to form big ET-1 (Gonzalez-Santiago et al. 2002), which is further cleaved at Trp²¹-Val²² to form biologically active ET-1 by endothelin converting enzyme-1 (ECE-1) (Harrison et al. 1995). ECE-1 is the rate limiting enzyme in the ET-1 biosynthetic process. Therefore, the ET-1 level in myocardial interstitial is relative to cardiomyocytes ECE-1 activity and expression (Ergul et al. 2000). Accumulating evidence has revealed that ET-1 contributes to the development of myocardial hypertrophy both *in vitro* and *in vivo* (Ito 1997; Yamazaki et al. 1996).

Myocardial hypertrophy is characterized by the increase of cardiomyocytes size, protein synthesis, the contractile protein content of individual cardiomyocyte and expressions of specific embryo genes including atrial natriuretic factor (ANF) and β -myosin heavy chain (β -MHC) (Barry et al. 2008; Knowlton et al. 1991). It is an adaptive response of cardiomyocytes to chronic pressure or volume overload, and is thought to be one of the crucial risk factors leading to various cardiovascular diseases including heart failure (Levy 1991). Angiotensin II (Ang II), as a Gq stimulator of myocardial hypertrophy (Selvetella et al. 2004), has been implicated in causing hypertrophy response in several models (Crowley et al. 2006; Hu et al. 2004). The hypertrophy effect of Ang II in cultured neonatal rat cardiomyocytes has been confirmed partially through ET-1

(Alexander et al. 2001; Maki et al. 1998; Wollert and Drexler 1999). Nevertheless, the direct relationship between ECE-1 and Ang II-induced cardiomyocyte hypertrophy is scarcely reported.

The aim of this study, therefore, was to determine whether inhibiting ECE-1 activity or down-regulating ECE-1 expression could ameliorate Ang II-induced hypertrophy response in cultured cardiomyocytes. For this purpose, rat ECE-1 was expressed in Chinese Hamster Ovary (CHO) cells and purified for enzyme activity assay. 13 Peptides (P1-P13) were synthesized in forward or reverse order of C terminal region of rat big ET-1 (21–36) and their inhibitory effect against the activity of expressed rat ECE-1 were tested. Two peptides (P8 and P9), which have significant inhibitory effect to rat ECE-1 activity, were selected. Treatment of P8 or P9 and RNA interference of ECE-1 were performed in primary cultured neonatal rat cardiomyocytes (NCM).

2. Investigations and results

2.1. Screening of synthetic peptides for inhibitory effect to rat ECE-1 activity

The Michaelis constant (K_m) for rat big ET-1 of expressed rat ECE-1 protein was $3.9 \pm 0.8 \mu\text{M}$, which was consistent with the previous findings (Liu et al. 1997). Rat ECE-1 protein was incubated with various concentrations of 13 peptides (0, 30, 50 or 75 μM) in the presence of 0.2, 0.4, 1, 2.5, 5, and 10 μM rat big ET-1 respectively, the production rates of the ET-1 and rat big ET-1 concentrations were reciprocally plotted. IC_{50} of all peptides on the

Table 1: Inhibitory effects to rat ECE-1 activity of big ET-1 analogues

Number	Big ET-1 analogue	Peptide sequence	IC ₅₀ (μM)
P1	big ET-1(18–25)	DIIWVNTP	>200
P2	big ET-1(24–19)	TNVWII	>200
P3	big ET-1(25–18)	PTNVWIID	137.2
P4	big ET-1(34–27)	GLGYPVVR	69.7
P5	big ET-1(34–21)	GLGYPVVREPTNVW	47.1
P6	[Phe ²¹]big ET-1(34–21)	GLGYPVVREPTNVF	129.6
P7	[Phe ²¹ , Leu ²²]big ET-1(34–21)	GLGYPVVREPTNLF	52.7
P8	[Leu ²²]big ET-1(36–21)	PSGLGYPVVREPTNLW	19.6
P9	[Leu ²²]big ET-1(34–21)	GLGYPVVREPTNLW	33.8
P10	[Leu ²²]big ET-1(32–21)	GYPVVREPTNLW	55.1
P11	[Leu ²²]big ET-1(30–21)	PVVREPTNLW	76.1
P12	[Leu ²²]big ET-1(27–21)	REPTNLW	89.4
P13	[Leu ²²]big ET-1(25–21)	PTNLW	110.7

inhibition of rat ECE-1 were calculated (Table 1). Among the various peptides examined, two peptides: [Leu²²]big ET-1(36–21) (P8) and [Leu²²]big ET-1(34–21) (P9), showed significant inhibitory effect to rat ECE-1, with IC₅₀ = 19.6 μM and 33.8 μM respectively. They were selected for further experiments.

2.2. Effects of P8 and P9 on cell viability of NCM

The cell viability effects of P8 and P9 at 10, 50 and 100 μM on NCM were assessed by MTT assay. There was no significant difference in absorbance at 570 nm between groups treated with various concentrations of P8 or P9 and control group ($p > 0.05$), showing that P8 or P9 at 10, 50 and 100 μM did not affect the viability of NCM (Fig. 1).

2.3. P8 and P9 inhibited Ang II-induced increase of β-MHC mRNA and cell surface area

After serum starvation for 24 h, NCM were treated with 0, 10, 30 and 50 μM of P8 or P9 for 1 h, followed by co-cubation with or without 0.1 μM Ang II for 24 h. The mRNA level of β-MHC and surface area of NCM were assessed. We found that β-MHC mRNA level in NCM treated with 50, 30 and 10 μM P8 was reduced in a concentration-dependent manner to 65.7% ($p < 0.001$), 71.4% ($p < 0.001$) and 89.8% ($p < 0.05$) of no treatment Ang II alone group respectively. Similar reduction was also observed in NCM treated by 50 and 30 μM, but not 10 μM of P9 (68.9%, $p < 0.001$ and 80.5%, $p < 0.01$ respectively, Fig. 2a and b).

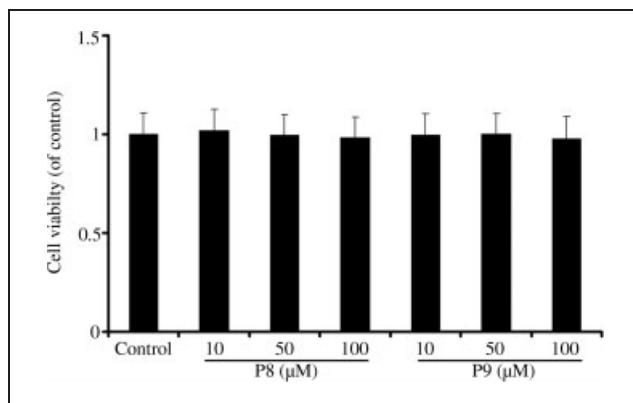


Fig. 1: Cell viability effects of various concentrations of P8 or P9 on NCM. Cell viability effects of 10, 50 and 100 μM of P8 or P9 on NCM were assessed. The data are the summary of five independent experiments

The effect of P8 or P9 on Ang II-induced hypertrophy response was also observed by microscopy (Fig. 2c). Quantification of cell surface area revealed a significant decrease in the NCM treated by 50, 30 and 10 μM P8 in a concentration-dependent manner (65.5%, $p < 0.001$, 74.9%, $p < 0.01$ and 84%, $p < 0.05$ of no treatment Ang II alone group respectively, Fig. 2d). Similar reduction was

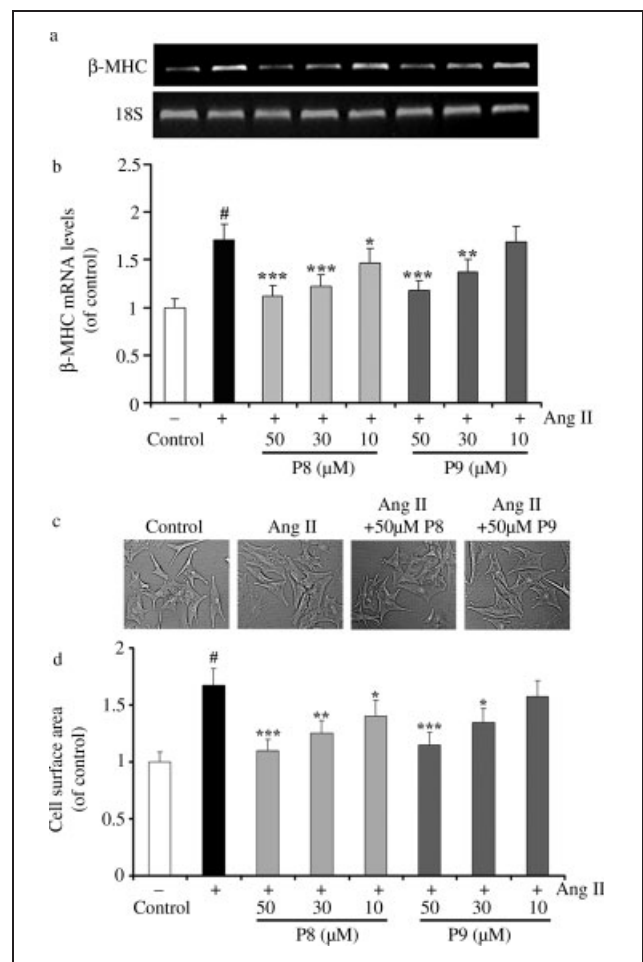


Fig. 2: P8 and P9 inhibited Ang II-induced increase of beta-MHC mRNA and cell surface area in NCM. beta-MHC mRNA levels in Ang II stimulated NCM which were treated with P8 or P9 were analyzed (a) and quantified by densitometry (b). Cell surface area of Ang II stimulated NCM which were treated with P8 or P9 were observed by using phase contrast micrographs at a magnification of 200 (c) and quantified (d). The data are the summary from three independent experiments. # $p < 0.001$ vs control group; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs Ang II alone treatment group

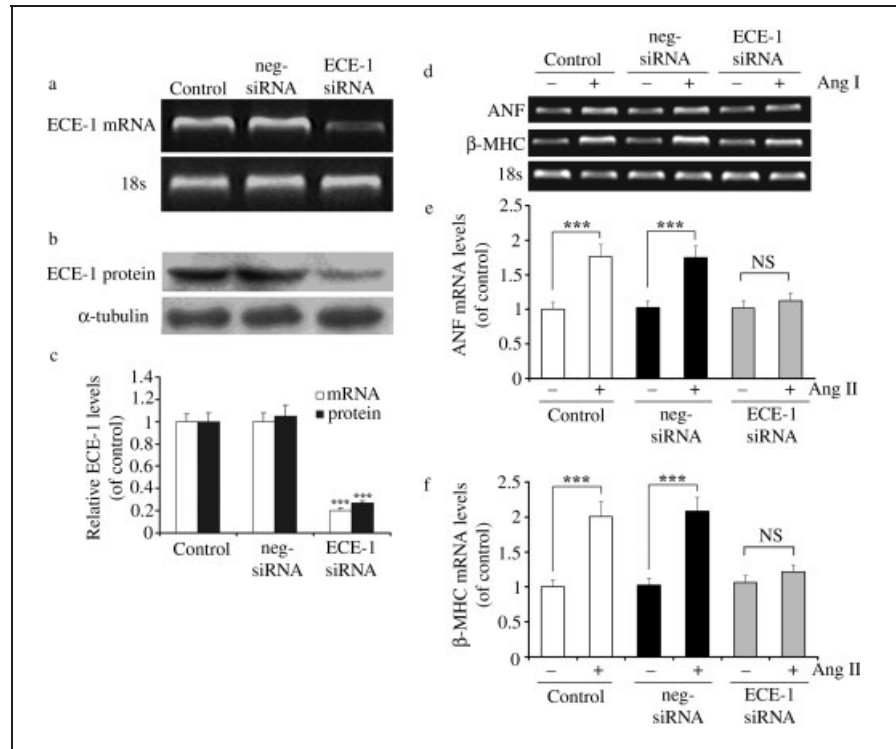


Fig. 3: RNA interference of ECE-1 inhibited Ang II-induced increase of ANF, beta-MHC mRNA. Levels of ECE-1 mRNA (a) or protein (b) in NCM transfected without (control), or with negative siRNA or ECE-1 siRNA were assessed and quantified (c). ANF and beta-MHC mRNA levels in Ang II stimulated NCM which were transfected without (control) or with negative siRNA or ECE-1 siRNA were analyzed (d) and quantified by densitometry (e and f). The data are the summary from three independent experiments. *** $p < 0.001$ vs control group

also found in NCM treated by 50 and 30 μM , but not 10 μM of P9 (68.8%, $p < 0.001$ and 80.6%, $p < 0.05$ of no treatment Ang II alone group respectively, Fig. 2d).

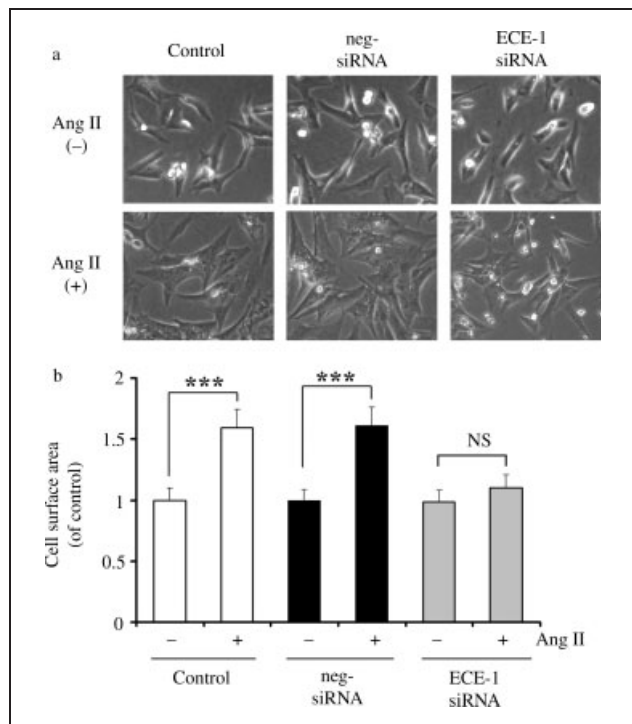


Fig. 4: RNA interference of ECE-1 inhibited Ang II-induced increase of cell surface area of NCM. Cell surface area of Ang II stimulated NCM which were transfected without (control) or with negative siRNA or ECE-1 siRNA were observed by using phase contrast micrographs at a magnification of 200 (a) and quantified (b). The data are the summary from three independent experiments. *** $p < 0.001$ vs control group

2.4. RNA interference of ECE-1 inhibited Ang II-induced increase of ANF, β -MHC mRNA and cell surface area

Expression of ECE-1 was silenced by siRNA RSS331827 in NCM. Both mRNA (Fig. 3a) and protein level (Fig. 3b) of ECE-1 were decreased to $21 \pm 2\%$ ($p < 0.001$) and $27 \pm 2.5\%$ ($p < 0.001$) of control respectively (Fig. 3c). The mRNA levels of ANF were increased 1.7-fold ($p < 0.001$) in control and negative siRNA treated groups after 0.1 μM Ang II stimulation for 24 h. The mRNA levels of β -MHC were increased 2-fold ($p < 0.001$) in control and negative siRNA treated groups after 0.1 μM Ang II stimulation for 24 h. These changes were abolished in the ECE-1 siRNA treated groups ($p > 0.05$, Fig. 3d, e and f). The effect of ECE-1 RNA interference on Ang II-induced hypertrophy response was also observed by microscopy (Fig. 4a). NCM surface area were both increased 1.6-fold ($p < 0.001$) in control and negative siRNA treated groups after 0.1 μM Ang II stimulation for 24 h. These changes were abolished in the ECE-1 siRNA treated groups ($p > 0.05$, Fig. 4b).

3. Discussion

Recent studies have suggested that ECE-1 is correlated with cardiovascular pathology. For instance, RO0687629 which is an ECE inhibitor provided protection against end-organ damage in rats harboring both human renin and angiotensinogen genes (Muller et al. 2002); CGS 26303 which is an ECE/NEP dual inhibitor, ameliorated LV perivascular fibrosis, and decreased the heart weight/body weight ratio in hypertensive rats (Emoto et al. 2005). It was also reported that Ece1 $-/-$ mice and a patient with a mutation in ECE-1 gene displayed a complex phenotype including cardiovascular malformations, craniofacial and neuronal (Hofstra et al. 1999; Yanagisawa et al. 1998). In the current study, inhibition of ECE-1 activity with P8 or

P9 significantly reversed Ang II-induced hypertrophy response in NCM. Additionally, down-regulation of ECE-1 expression by RNA interference ameliorated cardiomyocyte hypertrophy stimulated by Ang II as well. These results showed that ECE-1 has direct relationship with Ang II-induced cardiomyocyte hypertrophy.

Among the big ET-1 analogues tested in the present article, P8 and P9 displayed the most pronounced inhibitory activity against expressed rat ECE-1. Substitution of Trp²¹ to Phe²¹ caused a large decrease of the inhibitory activity, while replacement of Val²² to Leu²² greatly increased the inhibitory activity suggesting that the presence of Trp²¹ is important to inhibitory activity, and amino acid residue with larger bulk is necessary for inhibitory activity at position 22. Other peptides which lacked of the fragment from Arg²⁷ to Pro³⁶ showed weaker inhibitory activity indicating that the region of Arg²⁷ to Pro³⁶ could be a critical site for the interaction of big ET-1 and ECE-1. These data were partly consistent with the findings obtained in the expressed human ECE-1 (Liu et al. 1997).

Our data are the first to show that both inhibition of ECE-1 activity and down-regulation of ECE-1 expression ameliorate Ang II-induced hypertrophy response in NCM. These results confirmed the important role of ECE-1 in regulating Ang II-induced cardiomyocyte hypertrophy and indicated that ECE-1 might be a potential drug target in the treatment of Ang II-related myocardial hypertrophy.

4. Experimental

4.1. Cell culture

NCM were prepared as described previously (Fu et al. 2005) and cultured in DMEM medium containing 10% fetal bovine serum (FBS). CHO cells were cultured in monolayers in DMEM/F12 medium supplemented with 10% FBS.

4.2. Clone and expression of rat ECE-1 in CHO cells

Rat ECE-1 cDNA was cloned from adult Sprague-Dawley rat common carotid artery using the oligonucleotides with sequences of 5'-TAGAATTCATG ATGTCATCCTACAAG-3' and 5'-TACTCGAGCCAGACTTCG-CATTTGTGGC-3' as sense and anti-sense primers, respectively. Recombinant plasmid pCDNA3.1-ECE-1 was constructed and transfected into CHO cells. Stable lines were selected under the pressure of 0.8 mg/ml G418. Expressed rat ECE-1 protein was extracted by CytoBuster protein extraction reagent (Novagen, Carlsbad, CA, USA) and purified by Ni²⁺-NTA His bind Resins (Novagen) according to the manufacturer's instructions. Purified protein was collected for enzyme activity assay.

4.3. Peptide synthesis

Peptides (P1-P13) were synthesized by solid phase synthesis techniques on a PreludeTM parallel peptide synthesizer (PTI, USA). Crude peptides were purified by preparative reversed-phase HPLC using a C18 reversed-phase column (1×20 cm) with monitoring at 214 nm and elution with a linear gradient of 0.1% aqueous trifluoroacetic acid (TFA) with increasing concentrations of acetonitrile in 0.1% (v/v) TFA. Purity of all peptides used was >95% (214 nm HPLC).

4.4. ECE-1 activity assay

ECE-1 activity was determined by the production rate of ET-1 from big ET-1 (rat, 1–39; Sigma-Aldrich, USA). Purified ECE-1 protein (100 ng) were incubated in 100 µl buffer A (50 mM Tris-HCl, 150 mM NaCl and 0.1 mM ZnCl₂, pH = 7.0), containing various concentrations of big ET-1 in the presence or absence of various concentrations of peptides for 60 min at 37 °C. Then the reaction was terminated by the addition of 100 µl 5 mM EDTA and the amount of formed ET-1 were measured using an ET-1 specific enzyme-linked immunoassay (ELISA) kit (IBL, Gunma, Japan) according to the manufacture's instruction. The cross-reactivity of this kit was as follows: ET-1, 100%; ET-2, <0.1%; ET-3, <0.1%, rat big ET-1 (1–39), <0.1% and rat big ET-1 (22–39), <0.1%.

4.5. Cell viability assay

NCM were seeded in 96-well plates to achieve 60% confluence and cultured for 12 h, followed by treatment with the various concentrations of P8 or P9 for 12 h. Then methyl thiazolyl tetrazolium (MTT, Sigma-Aldrich) at 5 mg/ml was added into the wells and incubated for 4 h at 37 °C. The MTT/medium in each well was carefully removed and 150 µl DMSO was added into each well. The plates were incubated at 37 °C for 10 min with horizontally shaking, and then absorbance at 570 nm was measured with an automated microplate reader (Bio-Tek, Winooski, VT, USA).

4.6. RNA Interference and Ang II stimulation

NCM were cultured in DMEM with 10% FBS without antibiotics. ECE-1 Stealth RNAi RSS331827 or Stealth RNAi Negative Control Low GC (Invitrogen, Carlsbad, CA, USA) was transfected into NCM with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 48 Hours after transfection, reverse transcription-PCR (RT-PCR) and western-blot were used to detect silencing efficiency of RSS331827. In parallel experiments, 6 h after transfection, the culture medium was replaced by DMEM with 10% FBS. 18 h later, the NCM were serum starved for 24 h, followed by treatment with or without 0.1 µM Ang II for 24 h. RT-PCR was used to assess the mRNA levels of ANF and β-MHC. The surface area of NCM was also assessed.

4.7. RT-PCR, western-blot and cell surface area assay

Total RNA extracted from cells using Trizol Reagent (Invitrogen) was reverse-transcribed, and PCR amplification was performed as described previously (Zhang et al. 2007). Conditions for all PCR reactions were chosen at the linear part of the amplification curve of the density of PCR product. The primers used in this article are shown in Table 2. For the semi-quantification, images of the gels were captured and the intensity of the bands was analyzed by Labworks software. 18S was used as internal control to normalize the RNA loading.

Whole cell protein extract and western-blot assay was performed as previously described (Zhang et al. 2007). Rabbit polyclonal antibody against ECE-1 (1:1000 dilution, Santa Cruz Biotechnology, USA) and antibody against α-tubulin (1:10000 dilution, Sigma-Aldrich) were used for immunoblotting. Protein bands were quantified by densitometry (Labworks software). α-Tubulin was used as internal control to normalize the protein loading.

The cell surface area were observed by using phase contrast micrographs at a magnification of 200 and quantified by imaging to the complete boundary of 100–120 individual cells using Labworks software.

4.8. Statistical analysis

Data are expressed as means ± S.D. Statistical analysis of the differences between multiple groups were performed using one-way ANOVA employing the post hoc Dunnett's test. The SPSS 16.0 program was used for the calculations.

Table 2: Primer sequence and PCR product size

Gene	Product Size	Primers
ANF	320bp	Sense: 5'-CTGCTAGACCACCTGGAGGA-3' AntiSense: 5'-AAGCTGTTGCAGCCTAGTCC-3'
β-MHC	546bp	Sense: 5'-CACTCAACGCCAGGA-3' AntiSense: 5'-TTGACAGAACGCTGTGTCTCTCC-3'
18S	419bp	Sense: 5'-GTCCCCCAACTTCTTAGAG-3' AntiSense: 5'-CACCTACGGAACCTTGTTAC-3'
ECE-1	666bp	Sense: 5'-CATCAACAGCACCCGACAAA-3' AntiSense: 5'-ACCCCGATACCACCAAAG-3'

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