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Estrogen induces cell proliferation by promoting ABCG2-mediated efflux in endometrial cancer cells

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A B S T R A C T

Recently, it has reported that overeating of lipid-food has led to increase the amount of estrogen in vivo and the incidence of endometrial carcinomas. It is well-known that ATP-binding cassette transporter sub-family G2 (ABCG2) is highly expressed in cancer stem cells (CSCs). CSCs possess the ability for differentiation, tumorigenesis, stem cell self-renewal, and the efflux of anti-cancer drug and these abilities affect malignancy of cancer cells. However, little is known about the relationship between the expression of ABCG2 and malignancy of cancer cells. The present study aimed at understanding the regulatory mechanism underlying 17-β-estradiol (E_2)-induced cell proliferation under the control of ABCG2. E_2 increased cell viability with a peak at 1 µM and facilitated ABCG2 mRNA expression followed by the increase of ABCG2 expression level at plasma membrane. E_2-induced cell proliferation was inhibited by reserpine, an inhibitor of ABCG2, and the ABCG2 siRNA treatment. Thus, these results imply that ABCG2 plays an important role in the promotion of E_2-induced cell proliferation in Ishikawa cells.

1. Introduction

One of the most important transporters for proteins, lipids, and ions efflux is ATP-binding cassette (ABC) transporter superfamily and these transporters are widely expressed in organelles such as plasma membrane, vesicle, and mitochondria. ABC transporters function as active transporters for multiple substrates across the cellular membrane by ATP hydrolysis [1]. Recently, it has reported that cancer stem cells (CSCs) are thought to play an important role in tumor recurrence and drug resistance, which result in therapy failure and tumor relapse [2]. Furthermore, CSCs possess the ability for self-renewal, differentiation, and tumorigenesis [3]. It is well-known that P-glycoprotein (ABCB1) [4], multidrug resistance-associated protein 4 (MRP4, ABCC4) [5] and ATP-binding cassette transporter sub-family G2 (ABCG2) are expressed in CSCs. Especially, ABCG2 is highly expressed in CSCs. CSCs acquire anti-cancer drug resistance ability and ABCG2 is used as marker of collecting CSCs from cancer cells [6,7].

Endometrial adenocarcinomas can be divided into two types: Type I, Ishikawa cells, has a hormone-derived etiology such as estrogen, while Type II is more aggressive and estrogen-independent [8]. Type I occurs more frequently in young people and develops via endometrial hyperplasia due to overexposure of estrogen. Phosphatase and tensin homolog (PTEN) and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) mutations in Type I [9–12], and Tp53 mutation in Type II [13,14] play an important role in the carcinogenic process of endometrial cancer, however, the relationship between genetic mutations and the mechanism of the onset of endometrial carcinoma is not well understood.

In Ishikawa cells, it is not fully understood the mechanisms underlying E_2-induced cell proliferation. The present study focused upon ABCG2 functions and investigated the role of ABCG2 on E_2-induced cell proliferation. The results showed that 17-β-estradiol (E_2) (1 µM) increases the number of Ki-67 positive cells, and upregulated the expression level of ABCG2 at plasma membrane. The E_2 effect was inhibited by reserpine and the ABCG2 siRNA, thus, ABCG2 activated by E_2 promoted unnecessary substances efflux and led to accelerate malignancy of Ishikawa cells.

2. Materials and methods

2.1. Cell culture

Ishikawa cells were obtained from Japan Health Sciences Foundation (Tokyo, Japan). Cells in DMEM supplemented with 10% heat-inactivated fetal bovine serum without phenol red were cultured at 37 °C in 5% CO_2 incubator.

2.2. Cell viability

Cell viability was measured using 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay (Dojindo, kumamoto, Japan). MTT-reactive cells were quantified at an absorbance of 570 nm using a micro-plate reader Model 680 (Bio Rad, CA, USA), and percentage of independent basal levels was calculated.

2.3. Western blotting

Lysates from Ishikawa cells were loaded onto 10% (w/v) or 12% (w/v) sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF). Blotting membranes were blocked by TBST [150 mM NaCl, 0.1% (v/v) Tween20, and 20 mM Tris] containing 5% (v/v) bovine serum albumin (BSA) and reacted with an anti-ABCG2 antibody (1:250, Santa Cruz Biotechnology, YX, USA) or an anti-β-actin antibody (1:1000, Sigma-Aldrich, Tokyo, Japan) followed by Histofine Simple Stain MAX-PO (Nichirei Bioscience, Tokyo, Japan). Immunoreactivity was detected with a diaminobenzidine (DAB).

2.4. Immunohistochemistry

Cells were fixed with 4% paraformaldehyde and were reacted with Ki-67 (1:20, Dako, CA, USA), ABCG2 (1:250), or SYTO 59 Red Fluorescent Nucleic Acid Stain (Thermo Fisher Science, MA, USA). Following an anti-Ki-67 antibody, cells were reacted with Histofine Simple Stain MAX-PO followed by the detection with a DAB. Cells stained by DAB were observed with a light microscope (Nikon, Tokyo, Japan) at 20 × objection lens. Cells were reacted with an anti-ABCG2 antibody followed by the reaction of Alexa Fluor 488 (Thermo Fisher Science, MA, USA). Fluorescence signals were obtained with Olympus Fluoview FV300 confocal microscope (Olympus, Tokyo, Japan) at 20 × objection lens.

2.5. RNA interference

Small interfering RNAs (siRNAs) silencing the ABCG2 gene: sense, 5′-UGAGGAUGUUACCAAGUAU-3′ and anti-sense, 5′-AUACUUGGUAA CAUCCUCA-3′; and sense, 5′-ACAGGGUCAUUCAAGAGUU-3′ and anti-sense, 5′-AACUCUUGAAUGACCCUGU-3′; and sense, 5′-GUAUAGGAA UGGAGCUUAU-3′ and snit-sense, 5′-AUAAGCUCCAUUCCUAUAC-3′, and negative control (NC) siRNA were purchased from Nippon gene (Toyama, Japan). Each siRNA was transfected by Lipofectamine 3000 (Invitrogen, CA, USA).

2.6. RT-PCR

Total RNAs were purified from Ishikawa cells using TRIZol reagent (Thermo Fisher Scientific, Kanagawa, Japan). Each cDNA (1 μL) was amplified with rTaq polymerase (TOYOBO, Osaka, Japan) as follows: the first step, 94 °C for 2 min and the ensuring 30–35 cycles, 94 °C for 1 s, 62 °C for 15 s, and 72 °C for 30 s. We carried out using primers as follows: 5′- TTATGATGGTGGCTTATTCAGCCAGTTCC-3′ and 5′- TTCG CCAGTACATGTTGCATAGTTACAAGG-3′ for ABCG2; 5′- GAGGTGAAG AAGGGCCAGACGCTGGC-3′ and 5′- GGCTTTGTCCAGGGCTTCTTGGA CAACC-3′ for ABCB1; 5′- TGACAATGTGAACTTCATGTACAGTCC
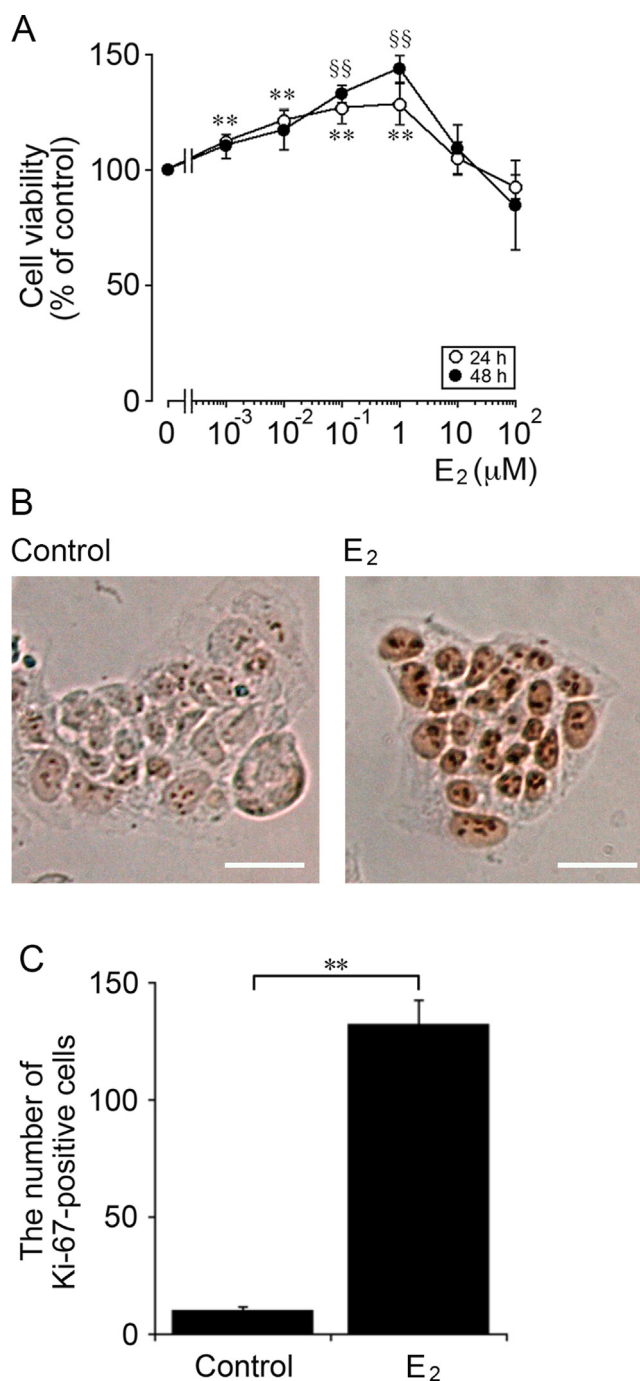
Fig. 1. E_2 increases cell viability in Ishikawa cells. A: Ishikawa cells were treated with E_2 at concentration as indicated for 24 and 48 h, and cell viability was measured by MTT assay. Data showed the mean (± SEM) percentage of basal level (n = 5–8 independent experiments). **, §§ $P < 0.01$ as compared with control, unpaired *t*-test. B: Immunohistochemistry was carried out in the presence or absence of E_2. After 24-h treatment, cells were reacted with an anti-Ki67 antibody followed by Histofine Simple Stain MAX-PO and detected with DAB. In the graph, indicative bar showed 50 μm. Results are from n = 3 independent experiments. C: The number of Ki-67 positive cells were counted in the consistent area (680 μm x 900 μm). In the graph, each value represents the mean (± SEM) number of Ki-67 positive cells (n = 3 independent experiments). **$P < 0.01$, unpaired *t*-test.

AGG-3′ and 5′- CCAGTTGTCTTTGTCCAACACTAAAATTGG-3′ for ABCC4; 5′-GACTTCAACAGCGACACCCACTCC-3′ and 5′-AGGTCCACCA CCCTGTTGCTGTAG-3′ for GAPDH. PCR products were separated by an electrophoresis on 2% (v/v) agarose gel and stained with ethidium

bromide followed by visualization using UV Transilluminator (Funakoshi, Tokyo, Japan).

2.7. Statistical analysis

Statistical analysis was performed by using unpaired t-test with the Stat View software. A value of $P < 0.05$ was considered significant.

3. Results

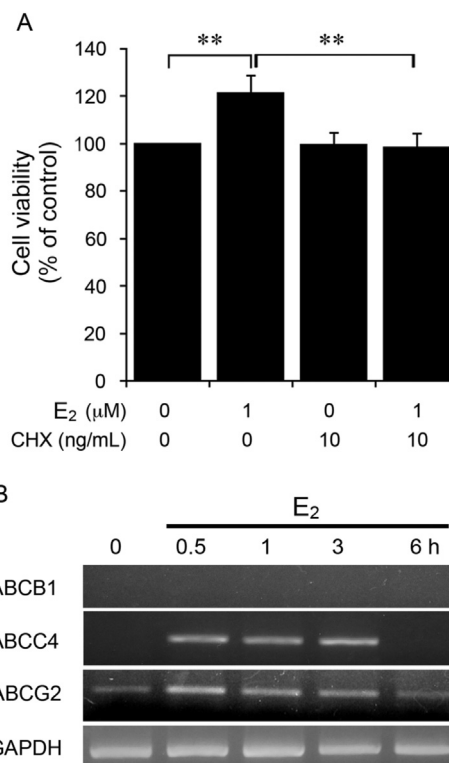### 3.1. E_2 increases cell viability in Ishikawa cells

We examined the effect of E_2 on cell viability in Ishikawa cells. In survival analysis, cell viability induced by E_2 was increased in a bell shape at 24, 48 h, each reaching $129 \pm 10\%$ and $144 \pm 7\%$ with a peak at $1\,\mu M$, respectively (Fig. 1A). Subsequently, we examined whether E_2 induced cell proliferation in Ishikawa cells. E_2 significantly increased the number of Ki-67 positive cells as compared with the control after 24-h treatment (Fig. 1B, C). These results suggest that E_2 induces cell proliferation in Ishikawa cells.

3.2. E_2 upregulated the expression level of ABCG2 followed by leading to facilitate cell proliferation

It is well-known that E_2 binds to estrogen receptors (ERs) followed by leading to transcribe E_2/ER-targeted genes. To investigate whether E_2 facilitated de novo protein synthesis, we used cycloheximide (CHX), an inhibitor of protein synthesis. E_2-induced cell proliferation was significantly inhibited by CHX ($10\,ng/mL$) (Fig. 2A). This result suggests that E_2 facilitated cell proliferation via de novo protein synthesis. We next asked a question as to what kind of gene expression underlie the E_2 action. We focused upon investigating the expression of ABC transporters such as ABCB1, ABCC4 and ABCG2. It is well-known that these transporters were widely expressed in various cancer cells and facilitated the efflux of exogenous toxins or anti-cancer drugs. So, we examined the expression level of these transporters using RT-PCR. E_2 did not affect the expression of ABCB1 mRNA, however, increased the expression of ABCC4 and ABCG2 mRNA transiently at 30-min treatment (Fig. 2B). In the present study, we focused upon ABCG2 expression, because ABCG2 was widely expressed in CSCs and was thought to play an important role in malignancy of cancer cells. E_2 increased ABCG2 protein expression level (Fig. 3A) and the expression level of ABCG2 at plasma membrane after 24-h treatment (Fig. 3B). These results imply that E_2 upregulates the expression level of ABCG2 at plasma membrane and activates ABCG2 function followed by promoting cell proliferation.

3.3. ABCG2 inhibitor and the ABCG2 siRNA attenuate E_2-induced cell proliferation

We finally examined whether ABCG2 exhibits a promotive effect against cell proliferation. Reserpine, an inhibitor of ABCG2, significantly attenuated E_2-induced cell proliferation as compared with E_2-treated cell group at 24 h (Fig. 4B). Furthermore, we constructed the siRNA silencing the ABCG2 gene. The expression of ABCG2 mRNA was clearly suppressed for cells transfected with the ABCG2 siRNA as compared with the expression for cells transfected with the negative control (NC) siRNA (Fig. 4A). E_2-induced cell proliferation was significantly blocked by the ABCG2 siRNA treatment (Fig. 4A). These results suggest that ABCG2 plays an important role in E_2-induced cell proliferation. Overall, these results indicate that E_2 increases the expression level of ABCG2 at plasma membrane followed by activating ABCG2 function and increases cell proliferation via the facilitation of ABCG2-mdeiated efflux.
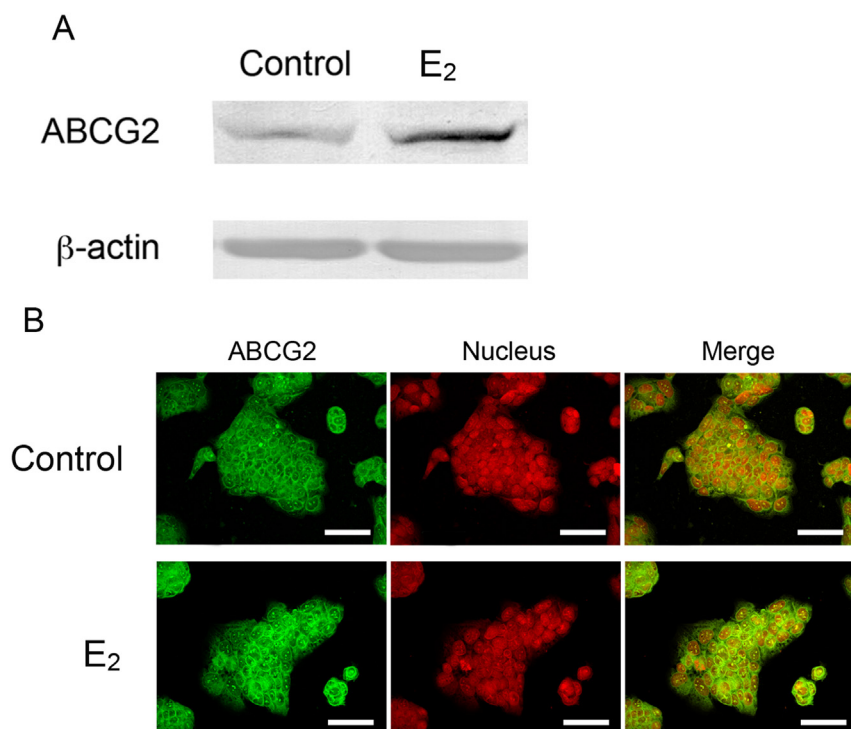

Fig. 2. E_2 increases the expression level of ABC transporters in Ishikawa cells. A: Cells were treated with $1\,\mu M$ of E_2 in the presence or absence of CHX ($10\,ng/mL$) for 24 h, and cell viability was measured by MTT assay. Data showed the mean ($\pm$ SEM) percentage of basal level (n = 6–8 independent experiments). $^{**}P < 0.01$ as compared with control, unpaired t-test. B: Cells were treated with E_2 ($1\,\mu M$) for period time as indicated. Total mRNAs were extracted from Ishikawa cells and ABC transporter mRNAs (ABCB1, ABCC4, and ABCG2) were detected using RT-PCR. Note that a similar result was obtained with 3–5 independent experiments.

4. Discussion

The results of the present study demonstrated that $1\,\mu M$ E_2 facilitated cell proliferation via the new protein synthesis. Generally, it was reported that E_2 bound to ERs followed by entering the nucleus and E_2/ERs complex transcribed various target genes, such as PTEN [15], IL-20 [16], and p21-activated kinase 4 (PAK4) [17]. Especially, PAK4 is a family of serine/threonine kinases that is first identified as effectors for Rho-family GTPases and are divided into group I (PAK1–3) and group II (PAK4–6) in humans [18,19]. PAK4 regulates cytoskeleton reorganization, directional motility, embryonic development, survival, invasion, metastasis, and gene regulation [20]. PAK4-signaling dependent cellular functions regulate disease processes such as cancer as PAK4 is overexpressed and/or hyper-stimulated in human cancer [21]. Thus, E_2/ER-signaling pathways might play crucial roles in malignancy of cancer cells.

ABC transporters are widely expressed in CSCs and promote the efflux of proteins, lipids, ions, and anti-cancer drug. The superfamily of ABC transporters consists of 48 members and ABCB1, ABCC4 and ABCG2 hold a major key in malignancy of cancers by mediating the efflux of many anti-cancer drug [22]. ABCG2 are also widely detected in CSCs and used as a surface marker of CSCs. We hypothesized that ABC transporters expressed in CSCs played an important role in keeping intracellular environment clear by facilitating the efflux of the unnecessary substances and might lead to induce cell proliferation. In Ishikawa cells, E_2 upregulated ABCC4 and ABCG2 mRNA, but not ABCB1 mRNA. These results imply that not only ABCG2 but also ABCC4 plays an important role in E_2-induced cell proliferation. The present

Fig. 3. E_2 increases the expression level of ABCG2 in Ishikawa cells. A: After 24-h treatment with E_2, proteins were collected from Ishikawa cells and Western blotting was carried out. The blotting membranes were reacted with an anti-ABCG2 antibody or an anti-β-actin antibody followed by Histofine Simple Stain MAX-PO and detected with DAB. B: After 24-h treatment with E_2, immunohistochemistry was carried out. Ishikawa cells were fixed with 4% paraformaldehyde and were reacted with an anti-ABCG2 antibody followed by Alexa Fluor 488 (green). Subsequently, cells were stained with SYTO 59 Red Fluorescent Nucleic Acid Stain (red). Results are from 3 to 5 independent experiments (A, B).
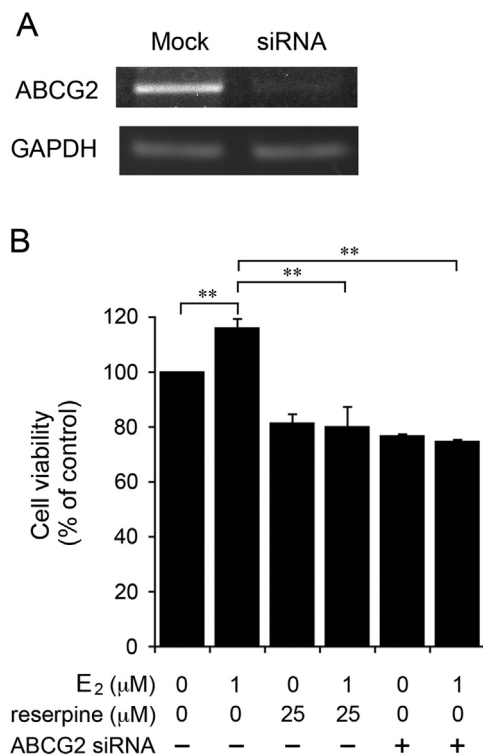
Fig. 4. ABCG2 inhibitor and the ABCG2 siRNA attenuate E_2-induced cell proliferation. A: Cells were transfected with the NC siRNA (Mock) or the ABCG2 siRNA. After 24-h treatment, total RNAs were purified and RT-PCR was carried out (n = 3 independent experiments). Note that no signal band for ABCG2 mRNA is visible in cells transfected with the ABCG2 siRNA. B: Cells were treated and untreated with reserpine (25 μM) or were transfected with the NC siRNA or the ABCG2 siRNA. These cells were treated and untreated with E_2 (1 μM) for 24 h followed by MTT assay. Data showed the mean (± SEM) percentage of basal level (n = 6–8 independent experiments). **$P < 0.01$ as compared with control, unpaired t-test.

study focused upon ABCG2 function, however it will be necessary that we examine the function of ABCC4 for E_2-induced cell proliferation in future. According to the previous report, the expression of ABCG2 was controlled via SIRT1/CREB signaling pathway [23]. SIRT1, a member of sirtuin family, acts as an anti-aging regulator for adult stem cells. SIRT1 regulates AMPK and mTORC1 signaling pathways as well as gene expression, and the activities of genes maintaining stem cell functions and delays cellular senescence [24], thus, ABCG2 regulated by SIRT1 also implies the possibility of functioning as an anti-aging regulator. However, it remains unknown what kind of transcription factors are induced ABCG2 expression by E_2 stimulation in Ishikawa cells. To examine the mechanism underlying ABCG2 expression by E_2 stimulation, we are currently carrying out the promoter assay.

We used reserpine, an inhibitor of ABCG2, and ABCG2 siRNA due to investigate the role of ABCG2 on E_2-induced cell proliferation in Ishikawa cells. Reserpine and ABCG2 siRNA significantly inhibited E_2-induced cell proliferation. ABCG2 acts as a part of a self-defense mechanism for the organism [25]. E_2 might regulate the intracellular environment by promoting the unnecessary substances efflux from intracellular space via ABCG2, thus, accelerate malignancy of Ishikawa cells.

In conclusion, E_2 increased ABCG2 mRNA expression and facilitated the expression level of ABCG2 at plasma membrane. Furthermore, ABCG2 promoted the unnecessary substances efflux followed by increasing cell viability of Ishikawa cells. ABCG2 plays an important role in malignancy of Ishikawa cells and is considered to be useful as a target for cancer treatment.

Author contribution

1) Conceptualization: T.Y.; 2) Data curation: T.Y.; 3) Formal analysis: T.O. and T.Y.; 4) Funding acquisition: I have no fund.; 5)

Investigation: T.O. and T.Y.; 6) Methodology: T.O. and T.Y.; 7) Project administration: T.Y.; 8) Resources: T.Y.; 9) Software: T.Y.; 10) Supervision:T.Y.; 11) Validation: T.Y.; 12) Visualization: T.Y.; 13) Writing - original draft: T.Y.; 14) Writing - review & editing: T.Y.

Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2018.10.005.

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