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ADAMTS-1: a novel target gene of an estrogen-induced transcription factor, EGR1, critical for embryo implantation in the mouse uterus

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Abstract

Background: Recently, we demonstrated that estrogen (E₂) induces early growth response 1 (Egr1) to mediate its actions on the uterine epithelium by controlling progesterone receptor signaling for successful embryo implantation. EGR1 is a transcription factor that regulates the spectrum of target genes in many different tissues, including the uterus. E₂-induced EGR1 regulates a set of genes involved in epithelial cell remodeling during embryo implantation in the uterus. However, only few target genes of EGR1 in the uterus have been identified.

Result: The expression of ADAM metallopeptidase with thrombospondin type 1 motif 1 (Adamts-1) was significantly downregulated in the uteri of E₂-treated ovariectomized (OVX) Egr1(−/-) mice. Immunostaining of ADAMTS-1 revealed its exclusive expression in the uterine epithelium of OVX wild-type but not Egr1(−/-) mice treated with E₂. The expression profiles of Adamts-1 and Egr1 were similar in the uteri of E₂-treated OVX mice at various time points tested. Pre-treatment with ICI 182, 780, a nuclear estrogen receptor (ER) antagonist, effectively inhibited the E₂-dependent induction of Egr1 and Adamts-1. Pharmacologic inhibition of E₂-induced ERK1/2 or p38 phosphorylation interfered with the induction of EGR1 and ADAMTS-1. Furthermore, ADAMTS-1, as well as EGR1, was induced in stroma cells surrounding the implanting blastocyst during embryo implantation. Transient transfection with EGR1 expression vectors significantly induced the expression of ADAMTS-1. Luciferase activity of the Adamts-1 promoter containing Egr1 binding sites (EBSs) was increased by EGR1 in a dose-dependent manner, suggesting functional regulation of Adamts-1 transcription by EGR1. Site-directed mutagenesis of EBS on the Adamts-1 promoter demonstrated that EGR1 directly binds to the EBS at -1151/-1134 among four putative EBSs.

Conclusions: Collectively, we have demonstrated that Adamts-1 is a novel target gene of E₂-ER-MAPK-EGR1, which is critical for embryo implantation in the mouse uterus during early pregnancy.

Keywords: Estrogen, EGR1, ADAMTS-1, Transcription, Uterus

Background

Early growth response 1 (Egr1) was initially known as an immediate-early response gene that is induced by various stress signals, including cytokines, growth factors, hormones, and DNA-damaging agents [1]. EGR1 as a transcription factor recognizes a highly conserved GC-rich promoter consensus motif on its target genes, such as Tnf-a, Pten, and Socs-1 [2, 3]. It
functions either as a tumor suppressor or oncogene, depending on the cell type and environmental conditions [4]. Several growth factors, such as Igf-II, Pdgf-A, and Tgf-β1, have been identified as direct targets of EGR1 in various tissues and pathological contexts including cancers [5–8]. Under normal conditions, EGR1 participates in the transcriptional regulation of several clock genes, such as Per1, Per2, and Bmal1 [2], and is required for the transcriptional activation of Mmp1 during damaged tissue remodeling [9]. Collectively, the pleiotropic actions of EGR1 are brought about through many target genes that act as key factors in various physiological and pathological conditions. However, only few target genes of EGR1 in the uterus have been identified.

The balanced function of ovarian estrogen (E2) and progesterone (P4) is critical for successful pregnancy following embryo implantation [10]. Uncontrolled estrogenic activity could be a major cause of endometrial disorders, including infertility and cancers; however, only a handful of the downstream signaling factors of E2 have been identified in the uterus [11–13]. E2 induces the transcription factor EGR1 to fine-tune its functions that are responsible for uterine receptivity during embryo implantation [12, 14–16]. In addition, E2 promotes ERK1/2-dependent activation of ELK-1 to induce EGR1 in the MCF-7 breast cancer cells [17, 18]. While Egr1(−/−) mice showed normal ovulation, fertilization, and embryo development if exogenous gonadotrophins were administered, they completely failed in embryo implantation [12, 19]. To understand the function(s) of EGR1 as a key mediator of E2 in the uterus, the identification of the genes directly targeted by EGR1 is critical. By performing multi-step in silico promoter analyses using mRNA microarray data, we identified some of the potential target genes of EGR1, such as Egr2, c-Kit, and Gadd45g, in the mouse uterus [20]. We recently demonstrated that EGR1 transcriptionally regulates c-Kit expression to maintain uterine receptivity for embryo implantation in the mouse uterus [21]. A disintegrin and metalloproteinase with thrombospondin motifs 1 (Adams-1), a new member of the ADAM-related proteins family, plays an key role in normal growth, organogenesis, and fertility [22, 23]. Although Adams-1 is a well-known target of P4-progesterone receptor (PR) signaling in the ovary during ovulation [24], the molecular mechanisms by which ovarian steroids regulate the transcription of Adams-1 in the uterus are poorly understood. Using multiple molecular and histological approaches, we have shown that EGR1, under the control of E2, transcriptionally regulates the expression of Adams-1 in the mouse uterus.

Results

Expression of Adams-1 is dependent on E2-induced EGR1 in the uterine epithelium

Previously, in silico promoter analyses of the microarray data from Egr1(+/+) and Egr1(−/−) mice uteri revealed putative EGR1 binding sites (EBSs) in the Adams-1 promoter [20]. Thus, we examined if Adams-1 was a direct target gene of the transcription factor, EGR1, in the uterus. The results of reverse transcription (RT)-PCR and real-time RT-PCR showed that the expression of Adams-1 was significantly reduced in the uteri of Egr1(−/−) mice treated with E2 for 3 h (Fig. 1a). Western blot analyses also showed that the expression levels of both EGR1 and ADAMTS-1 were significantly reduced in Egr1(−/−) mice (Fig. 1b). Immunofluorescence staining of ADAMTS-1 showed its localization in the uterine epithelial cells of wild-type mice, but not Egr1(−/−) mice, at 3 h post E2 treatment (Fig. 1c). To investigate the expression of Adams-1 in uterine cell type, we isolate the epithelial and stromal cells. The results of RT-PCR and real-time RT-PCR showed that the expression of Adams-1 was significantly increased in the epithelial cells of the uteri treated with E2 for 3 h (Fig. 1d).

Adams-1 and Egr1 are rapidly and transiently induced by E2 in the mouse uterus

To further examine the mechanisms underlying the regulation of Adams-1 expression by E2-induced EGR1 in the uterus, we investigated the spatiotemporal expression patterns of Adams-1 in the uteri of ovariectomized (OVX) mice treated with E2 for 0, 3, 6, or 24 h following hormone treatment. RT-PCR and real-time RT-PCR analyses demonstrated that the expression pattern of Adams-1 was similar to that of Egr1 in the uterus (Fig. 2a). Both Egr1 and Adams-1 were transiently and rapidly induced by E2, with a peak at 3 h post E2 treatment. Western blot analyses revealed the unique expression pattern of ADAMTS-1 induced by E2 (Fig. 2b). Immunofluorescence staining showed that ADAMTS-1 was predominantly localized in the epithelial cells of the uterus (arrowheads in Fig. 2c).

To investigate the effects of P4 on Adams-1 expression in the mouse uterus, P4 alone or a combination of E2 + P4 was administered to mice OVX at various time points following hormone treatment. We found that P4 alone did not induce the expression of Adams-1 in the uteri of OVX mice (Fig. 2d). However, when a combination of E2 + P4 was used, P4 effectively inhibited
the E2-induced expression of Adamts-1 in the uterus (Fig. 2e).

**Activation of ERK1/2 and p38 MAPK by the E2-ER(s) pathways is required for the EGR1-dependent induction of ADAMTS-1 in the uterus**

To determine whether E2 induces Egr1 and Adamts-1 expression via the activation of its nuclear estrogen receptor (ER)α and ERβ, in the mouse uterus, we examined the expression levels of Adamts-1 in OVX mice pre-treated with ICI 182,780 (an ER antagonist), 30 min before E2 injection. At 2 and 4 h post-E2 injection, the E2-dependent expression of Adamts-1 and Egr1 mRNAs was profoundly reduced in the uteri of OVX mice pre-treated with ICI 182,780 (Fig. 3a). E2 exerts its functions through both genomic and non-genomic pathways, such as MAPK pathways, which regulate EGR1 expression in various cell types [16, 25, 26]. Thus, to determine if the non-genomic function(s) of E2 is/are required for the induction of ADAMTS-1 in the uterus, we evaluated the phosphorylation patterns of the MAPK and AKT pathways in the uteri of OVX mice pre-treated with ICI 182,780 (Fig. 3b). E2 gradually increased the phosphorylation of AKT and JNK, ERK1/2 and p38 were rapidly activated, with a peak at 2 h post-E2 treatment (Fig. 3b). ICI 182,780 significantly inhibited the E2-induced phosphorylation of AKT, ERK1/2, and p38. Pre-treatment with the pharmacological inhibitors of each kinase showed that the E2-ER-dependent phosphorylation of p38 and ERK1/2 was required for the induction of EGR1 and ADAMTS-1 in the uterus (Fig. 3c, d). These results suggest that the transcription of Adamts-1 could be regulated by the E2-ER-ERK/p38-EGR1 pathway in the mouse uterus.
ADAMTS-1 and EGR1 are induced in the stromal cells surrounding the implanting blastocyst during embryo implantation

During early pregnancy, we found that the expression level of *Adamts-1* mRNA increased on the day 4 of pregnancy (D4) (Fig. 4a). Previously, we had demonstrated that EGR1 was exclusively induced in the stromal cells surrounding the implanting blastocyst (Bl) during mice embryo implantation [12]. Immunostaining of EGR1 and ADAMTS-1 in the uterus containing the implantation sites on D5 showed that both EGR1 and ADAMTS-1 were induced in the decidualizing stromal cells during embryo implantation (right panels in Fig. 4b). Furthermore, ADAMTS-1 was also found in the trophoblasts of the Bl (yellow dots in Bl). However, ADAMTS-1, but not EGR1, was found in a subset of decidualizing cells in the primary decidual zone (PDZ) on D6 and D8 (Fig. 4c).

The effective EBS is present within the distal region of the *Adamts-1* promoter

To further understand the molecular interaction(s) of EGR1 with the *Adamts-1* promoter, we performed a series of luciferase promoter-reporter assays. First, we examined whether the forced expression of EGR1 induced ADAMTS-1 expression in 293 T cells. As shown in Fig. 5a, Western blot analyses clearly showed that transfection with an EGR1 overexpression vector significantly increased the expression of ADAMTS-1 and EGR1 in a time-dependent manner. We then performed luciferase assays on a construct with a region of the *Adamts-1* promoter region (−1705 to +90) containing four putative EBS. We found that the luciferase activity of the *Adamts-1* promoter was increased by EGR1 expression vectors in a dose-dependent manner (Fig. 5b), suggesting the presence of EBS in the *Adamts-1* promoter. Furthermore, we found that the distal 1 kb region of the *Adamts-1* promoter (−1705 to −978 from the transcription start site) contained one EBS (−1151/−1134) that was sufficient for the EGR1-dependent activation of the *Adamts-1* promoter (Fig. 5c). To further examine the function of the EBS at the distal region of the *Adamts-1* promoter, luciferase assays were performed on a mutant EBS containing the *Adamts-1* promoter. A mutation (mut) at the −1151/−1134 of the EBS completely destroyed the transcriptional activity of the plasmid construct (Fig. 5d). We also found that EGR1 did not activate the distal 1 kb region of the *Adamts-1* promoter containing a −1151/−1134 mut, although the wild-type distal promoter region responded to EGR1 in a dose-dependent manner (Fig. 5e).

Discussion

Previously, we had demonstrated that E2 induces the transcription factor EGR1, to fine-tune its major effects on the uterine epithelium during embryo implantation [12]. EGR1 governs the expression of an array of genes regulated by E2 in the uterus. Our in silico analyses suggested that EGR1 physically binds to the *Adamts-1* promoter and induces its expression in the uterus [20]. Using multiple approaches, we have shown that EGR1 regulates the expression of *Adamts-1* at the transcriptional level in the uterus. EGR1 is rapidly and transiently induced by the E2-induced phosphorylation of p38 and ERK1/2 in the uterus [21]. It is also induced in the ovary immediately after ovulation by a surge of luteinizing hormone (LH) [27]. A recent study suggested that LH-dependent ERK1/2 signaling promotes the simultaneous induction of Egr1 and *Adamts-1* in bovine granulosa cells [28]. Interestingly, while the expression of *Adamts-1* was significantly reduced in the uterus of Egr1 (−/−) mice (Fig. 1), its expression was not altered in the ovary of Egr1 (−/−) mice following the LH surge (unpublished observation). In addition, we found that the expression of *Adamts-1* coincided with that of Egr1 in the uterus of mice treated with E2 (Fig. 2). However, further studies are warranted to understand the different regulatory mechanisms by which EGR1 interacts with the *Adamts-1*.
Fig. 2 (See legend on previous page.)
promoter in different cellular contexts in the uterus and ovary of mice.

ADAMTS-1 is a protease that mediates follicular rupture [29]. At the time of ovulation, it cleaves the extracellular matrix of the thecal cell layer, thereby allowing the release of the cumulus oocyte complexes from the ovary [30]. In addition, it facilitates the hormonal response of granulosa cells by cleaving the proteoglycans that inhibit the binding of gonadotrophins to their receptors [23, 29]. Although Adamts-1 is a well-known target of the P4-PR signaling pathway in the ovary, during ovulation, its expression in the uterus is strictly regulated by E2, and not by P4. We found that the P4-PR signaling pathway alone did not affect the expression of Adamts-1 in the uterus (Fig. 2d). Rather, it interfered with the effects of E2 on Egr1 expression, leading to a reduction in Adamts-1 expression in the uterus (Fig. 2e). Consistent with a previous study [31], we found that the expression of Adamts-1 increased during the proestrus and estrus stages when E2 is the dominant hormone (data not shown), suggesting that Adamts-1 is mainly regulated by E2 in the uterus. However, a previous report showed that the expression
of Adamts-1 mRNA was not significantly altered in the bovine endometrium during the estrous cycle [32]. In addition, other studies have revealed an increase in the expression of Adamts-1 mRNA by P4 in cultured bovine and human stromal cells [32, 33]. Furthermore, E2 attenuated the positive effects of P4 on Adamts-1 in a concentration-dependent manner [33]. This discrepancy could be derived from species differences and/or different cellular/physiological contexts.

Endometrial stromal cells undergo extensive remodeling during decidualization [34, 35]. Decidualization response is partially impaired in Egr1(−/−) mice [12], and EGR1 transcript levels are downregulated in the endometrium of patients with repeated implantation failure [36]. Furthermore, EGR1 is required to transcriptionally program pre-decidual human endometrial stromal cells for decidualization and its expression levels are required to be reduced to enable decidualization [36, 37]. This down-regulation of EGR1 in human decidualized stromal cells is consistent with our results that the expression of EGR1 is reduced in the PDZ of mice on D6 and D8 (Fig. 4c). However, ADAMTS-1 was persistently maintained in these cells on D6 and D8, suggesting the presence of other regulatory mechanisms that affect ADAMTS-1 expression during this event. ADAMTS-1, which is responsible for extracellular matrix remodeling,
is known to play a crucial role in initiating and successfully maintaining decidualization [31–33]. However, Adamts-1 (−/−) mice appear normal with respect to embryo implantation and decidualization [22, 23, 38]. This could be due to the redundant expression of other metalloproteases in the uterus during embryo implantation and decidualization [39–41]. In fact, not only ADAMTS-1 but also ADAMTS-5 proteins were identified in human decidualized stromal cells [40].

Using in silico analyses, we found four putative EBS within −2 kb of the Adamts-1 promoter (Fig. 5b). Previous studies, including ours, have shown that EBS are enriched within −500 of the promoters of genes whose expressions are influenced by EGR1 [20, 42, 43]. For example, in many species, EBS is found within −500 of the LH-β subunit promoter [19]. Moreover, EGR1 directly interacts with an EBS in the promoter regions of MMP9 (−569/−553) and MMP1 (−137/−119) in HeLa

**Fig. 5** The functional EGR1 binding site is located in the distal region of the Adamts-1 promoter. **a** Western blot analyses of the expression profiles of ADAMTS-1 and EGR1 after transfection of the EGR1 expression vector. GAPDH represents the loading control. **b** Adamts-1 luciferase vector (−1705/+90) was co-transfected with increasing concentrations of the EGR1 expression vector (pRES dsRED2/EGR1) in 293 T cells, as indicated. At 48 h post transfection, the cells were collected, and the luciferase activity was measured. **c** EGR1 expression vector was transiently co-transfected with one of three different Adamts-1 promoter constructs in 293 T cells. The luciferase activity was measured at 48 h post transfection. The firefly activity was normalized to the activity of Renilla luciferase, and the luciferase activity of the untreated cells was designated as one relative value. **d** EGR1 expression vector was co-transfected in 293 T cells along with one of two different Adamts-1 promoter constructs containing mutations in EGR1 binding sites. The firefly activity was measured at 48 h post transfection. The luciferase activity was calculated relative to the expression of the pGL4.10 basic vector, which served as the negative control. *P < 0.05, **P < 0.01. **f** Chromatin immunoprecipitation (ChIP)-PCR and real-time ChIP-PCR for four putative EBS in the Adamts-1 promoter. The EGR1-MYC-FLAG expression vector was transfected into 293 T cells and genomic DNA was used for ChIP-PCR. A schematic cartoon (right panel in **e**) to show locations of four different primers on the Adamts-1 promoter to amplify genomic DNAs precipitated by FLAG antibody. Note that the genomic DNA containing −1151/−1134 of the Adamts-1 promoter was significantly enriched. *P < 0.05.
cells [9, 44]. Although a putative EBS at −133/−122 of the Adamts-1 promoter is conserved among many species, including humans, mice, and rats [20], we found that EGR1 does not interact with this EBS. Instead, we found that EGR1 binds to the EBS at the −1151/−1134 region of the Adamts-1 promoter, the mutation of which exclusively abrogated the transcriptional activity of EGR1 (Fig. 5). While we cannot exclude that the other EBS present within −1 kb of the Adamts-1 promoter may play a role in the EGR1-dependent transcription of Adamts-1, our results suggest that EGR1 physically interacts with the Adamts-1 promoter at the −1151/−1134 region and enhances its expression.

Conclusion
We have demonstrated that the transcription of Adamts-1 is regulated exclusively by the E2-dependent EGR1 transcription factor in the uterus, whereas by the P4 signaling pathway in the ovary in mice. Collectively, Adamts-1, whose expression is localized in the uterine epithelium, is a novel target gene of E2-ER-MAPK-EGR1, critical for embryo implantation in the mouse uterus during early pregnancy.

Methods
Reagents and chemicals
E2 (17β-estradiol; Sigma-Aldrich, St. Louis, MO, USA), P4 (Sigma-Aldrich), ICI 182,780 (Sigma-Aldrich), U0126 (MEK 1/2 inhibitor; Cell Signaling Technology, Danvers, MA, USA), wortmannin (AKT inhibitor; Cell signaling Technology), and SB203580 (p38 inhibitor; Selleck Chemicals, Houston, TX, USA) were used in this study.

Animals
All mice were housed following the institutional guidelines for laboratory animals (Animal Care Facility of CHA University). This study was approved by the Institutional Animal Care and Use Committee (IACUC, Approval Number: 190168). Adult ICR mice (8-week-old), provided by KOATECH (Pyeontaek, Gyeonggi, Korea) containing 1X phosphatase inhibitor (Roche Applied Sciences, Indianapolis, IN, USA) by lysing the cells. The protein extracts were separated by SDS-PAGE.
(8–10%) and transferred to a nitrocellulose membrane (Bio-Rad). Subsequently, the membranes were subjected to Western blot analyses with anti-ADAMTS-1 (Abcam, Cambridge, UK, ab39194, 1:1000), anti-EGR1 (Cell Signaling, #4153, 1:1000), anti-pAKT (Cell Signaling, #9271, 1:1000), anti-AKT (Cell signaling, #9272, 1:1000), anti-pERK1/2 (Cell Signaling, #9101, 1:1000), anti-ERK1/2 (Cell Signaling, #9102, 1:1000), anti-p-p38 (Santa Cruz, sc-17852, 1:1000), anti-p38 (Ab frontier, LF-MA0126, 1:1000), anti-p-JNK1/2 (Santa Cruz, 1:1000), and anti-GAPDH (Cell Signaling, #2118, 1:2000) antibodies. The secondary antibodies were HRP-conjugated goat anti-rabbit or mouse (Invitrogen, Carlsbad, CA, USA, 1:3000). Immunoreactive bands were detected using the Immune-Star Western Chemiluminescence Kit (Bio-Rad). The chemiluminescence signal was detected using the ChemiDOC™ XRS + System (Bio-Rad).

**Immunofluorescence**

To determine the cell-type specific localization of ADAMTS-1 following E2 treatment, the uteri were fixed in 4% paraformaldehyde and embedded in paraplast (Leica Biosystems, St. Louis LLC, Diemen, Netherlands). Uterine Sects. (5 µm) were de-paraffinized and rehydrated, and the endogenous peroxidase was inactivated using 3% H2O2. The tissue sections were subjected to antigen retrieval by immersing in sodium citrate buffer (10 mM, pH 6.0) for 20 min. Serum as a protein block antigen retrieval by immersing in sodium citrate buffer (8–10%) and transferred to a nitrocellulose membrane (Bio-Rad). Subsequently, the membranes were subjected to Western blot analyses with anti-ADAMTS-1 (Abcam, Cambridge, UK, ab39194, 1:1000), anti-EGR1 (Cell Signaling, #4153, 1:1000), anti-pAKT (Cell Signaling, #9271, 1:1000), anti-AKT (Cell signaling, #9272, 1:1000), anti-pERK1/2 (Cell Signaling, #9101, 1:1000), anti-ERK1/2 (Cell Signaling, #9102, 1:1000), anti-p-p38 (Santa Cruz, sc-17852, 1:1000), anti-p38 (Ab frontier, LF-MA0126, 1:1000), anti-p-JNK1/2 (Santa Cruz, 1:1000), and anti-GAPDH (Cell Signaling, #2118, 1:2000) antibodies. The secondary antibodies were HRP-conjugated goat anti-rabbit or mouse (Invitrogen, Carlsbad, CA, USA, 1:3000). Immunoreactive bands were detected using the Immune-Star Western Chemiluminescence Kit (Bio-Rad). The chemiluminescence signal was detected using the ChemiDOC™ XRS + System (Bio-Rad).

**Chromatin immunoprecipitation (ChIP)**

Human EGR1 was amplified and cloned into the pCMV6-ACIRES-GFP-Puro vector to tag EGR1 with Myc and FLAG (Origene Technologies, Rockville, MD, USA). 293 T cells were transfected with an EGR1-MYC-FLAG expression plasmid for 48 h, washed once with PBS, and added to 10 ml of PBS containing 1% formaldehyde to covalently cross-link any DNA–protein complexes at room temperature for 10 min. The beads were added to the samples, rotated for 30 min, and collected by centrifugation at 12,000×g for 1 min. The elution buffers were added to the samples and the supernatants were transferred to clean microcentrifuge tubes. The DNA samples were used for PCR reactions with the appropriate primers.

**Statistics**

Each experiment was performed at least three times. Data were presented as mean ± S.D. GraphPad Prism ver.
8 software (GraphPad Software, La Jolla, CA, USA) was used to perform statistical analyses for the Mann–Whitney U test. Statistical significance was set at \( P < 0.05 \).

**Abbreviations**

E:
- Estrogen; P:
- Progesterone; ER:
- Estrogen receptor; PR:
- Progesterone receptor; OVX:
- Ovariectomized; Egr:
- Early growth response; ADAMS:
- ADAM-metallopeptidase with thrombospondin type 1 motif; SC:
- Stromal cell; EC:
- Epithelial cell; EBS:
- EGR1 binding site; RT:
- Reverse transcription; LE:
- Luminal epithelial cells; GE:
- Glandular epithelial cells; S:
- Stromal cells; Em:
- Embryonic; Bl:
- Blastocyst; PDZ:
- Primary decidual zone; D4:
- Day 4 of pregnancy; ChiP:
- Chromatin immunoprecipitation.

**Acknowledgements**

Not applicable.

**Authors’ contributions**

HJL and HS conceived and designed the experiments in the manuscript. MP and SHP performed the formal analysis. MP and SHP performed the experiments and analyzed data. MP, SHP, HP, and HRK performed the data visualization. HRK, HJL, and HS supervised this study. MP and HS wrote the original draft. MP, HJL, and HS reviewed and edited the manuscript. All authors read and approved the final manuscript.

**Funding**

This work was supported in part by a Grant of the National Research Foundation of Korea (NRF-2019R1A6A1A03032888, 2020R1A2C1004122), and 2020R1A2C20035012.

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

The content of the manuscript has been approved by all the authors.

**Competing interests**

The authors declare that they have no competing interests.

**Received:** 5 April 2021  **Accepted:** 28 July 2021  **Published online:** 04 August 2021

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