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Upregulation of Fibroblast Growth Factors Caused by Heart and Neural Crest Derivatives Expressed 2 Suppression in Endometriotic Cells: A Possible Therapeutic Target in Endometriosis

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Abstract

Several features exist that distinguish endometriotic cells from eutopic endometrial cells. Progesterone resistance is one of the main distinguishing features, although how progesterone resistance affects the phenotype of endometriotic cells is not fully elucidated. Heart and neural crest derivatives expressed 2 (HAND2) is a transcriptional factor that plays an important role in maintaining endometrial function in a progesterone-dependent manner. Therefore, we explored whether progesterone-dependent HAND2 is implicated in the progression of endometriosis. HAND2 was less expressed by endometriotic tissues compared to endometrial tissues. Suppression of HAND2 expression induced fibroblast growth factor 1 (FGF1), FGF2, and FGF9 in endometriotic stromal cells and consequently enhanced migration and invasion capacity. AZD4547, a FGF receptor inhibitor, diminished the migration and invasion of endometriotic cells in vitro. In the murine model of endometriosis, AZD4547 showed suppressive effects on the development of endometriotic lesions at a relatively low concentration. In conclusion, we demonstrated that FGF1, FGF2, and FGF9 are downstream effectors of HAND2 in endometriotic cells. Since HAND2-dependent FGFs play roles in enhancing invasive capacity of endometriotic cells, our results suggest that FGF receptor inhibitors, such as AZD4547, can be promising therapeutic targets for endometriosis.

Keywords

AZD4547, endometriosis, FGF, HAND2

Introduction

Endometriosis is a common gynecologic disease causing dysmenorrhea, chronic pelvic pain, and infertility; it affects approximately 10% of reproductive-age women, and 30% to 50% of cases result in infertility.[1-4] Endometriosis is defined by the existence of endometrium-like glands and stroma outside uterus, such as the peritoneal surface (peritoneal lesion). Endometriotic ovarian cysts are one of the common lesions of endometriosis (endometrioma), while the lesions sometimes infiltrate deeply into the peritoneum, bowel, urinary bladder, and so on (called deep infiltrating endometriosis).[5]

Etiology and pathophysiology of endometriosis is currently not fully elucidated. Although different types of endometriosis might arise from different etiologies, implantation of endometrial cells in retrograde menstruation is a widely accepted

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hypothesis. However, several differences distinguish endometriotic cells from eutopic endometrial cells. Disruption of sex steroidal regulation may be one of the significant pathologies in endometriosis. Numerous reports support that progesterone resistance in endometriotic cells is implicated in the development of endometriosis.[6-8] Endometriotic stromal cells show less decidualization response to simultaneous stimulation with estradiol and progesterone.[9,10] In addition, a number of genes induced or suppressed by progesterone are dysregulated.[8,11] These data suggest that endometriotic cells are characterized by impairment in the progesterone-induced differentiation.

Heart and neural crest derivatives expressed 2 (HAND2) has been discovered as a significant transcriptional factor for the development of the heart and limb buds.[12] It has been recently reported that in the uterine stroma, HAND2 is regulated by progesterone and inhibits estrogen-dependent epithelial cell proliferation and therefore acts as a mediator of progesterone to maintain uterine receptivity.[13] Moreover, it was also demonstrated that progestin-induced HAND2 expression is significantly involved in the regulation of decidualization in endometrial stromal cells.[14]

Fibroblast growth factors (FGFs) play a wide range of roles in physiological and pathological conditions, such as embryonic development and angiogenesis. Among over 20 FGF members, FGF1 and FGF2 induce proliferation of endothelial cells and consequently promote angiogenesis.[15] Fibroblast growth factors 1 and FGF2 also have activity as transforming factors in tumor progression.[16,17] Fibroblast growth factors 9 is another FGF family member that has mitogenic activity in endometrial cells.[18] It has been demonstrated that HAND2 suppresses the expression of FGF1, FGF2, and FGF9 in the uterine stroma.[13]

In the current study, we explored the possible role of HAND2 in progesterone resistance of endometriosis. We demonstrated that HAND2 was less expressed in endometriotic stromal cells compared to endometrial stromal cells and that the suppression of HAND2 expression plays a role in developing endometriotic lesion. We also identified FGF1, FGF2, and FGF9 as downstream effectors of HAND2 in the endometrium/endometriotic tissues and that they can be therapeutic targets in endometriosis.

Materials and Methods

Patients and Immunohistochemistry

Patients referred between January 2010 and January 2016 to the fertility clinic at Nagoya University Hospital with endometriomas and uterine leiomyomas were enrolled in this study. This study was approved by the ethical committee of the Nagoya University Graduate School of Medicine, and informed consent was obtained from all participants. All patients underwent laparoscopic surgery as described previously.[19] Surgically excised specimens of uterus and endometriomas were fixed in 10 mmol/L phosphate-buffered saline (PBS), 10% formalin, and embedded in paraffin. Immunohistochemical staining was conducted using the avidin–biotin immunoperoxidase method and Histofine SAB-PO kit (Nichirei Biosciences Inc, Tokyo, Japan) according to the manufacturer's protocol. Endogenous peroxidase activity was blocked by 3% H_2O_2 in methanol for 20 minutes, and nonspecific immunoglobulin binding was blocked by incubation for 15 minutes in 10% normal serum in PBS with the corresponding species of the secondary antibody. Tissue sections were incubated at 4°C overnight with antibodies against the HAND2 protein (ab60037, 1:100 dilution, abcam, Cambridge, Massachusetts) and further incubated with (horseradish peroxidase)-conjugated streptavidin. For the negative controls, the primary antibody was replaced by a nonspecific immunoglobulin G at the same dilution. The immunoreactive intensity of HAND2-positive stromal cells was graded as 0 = faint or very weak, 1 = weak, 2 = moderate, and 3 = strong. The tissue expression of HAND2 was evaluated semiquantitatively. Photographic images of the prepared slides were taken using AXIO Imager A1 (Carl Zeiss Microscopy Co, Ltd, Tokyo, Japan).

Culture of Endometrial Stromal Cells and Endometriotic Cyst Stromal Cells

The stromal cells were taken from the eutopic uterine endometrium of patients without endometriosis (endometrial stromal cells without endometriosis [ESC] 1-3, primary cultured cells), and the eutopic uterine endometrium of patients with endometriosis (endometrial stromal cells with endometriosis [eESC] 1-3, primary cultured cells) and the endometriotic cyst linings of the ovaries (cyst stromal cells [CSC] 1-3, primary cultured cells) of patients with endometriosis collected as described previously.[20] The ESCs, eESCs, and CSCs collected from the lower receptacle were suspended and plated onto 60-mm collagen 1-coated dishes and cultured in Dulbecco-modified eagle medium (DMEM; Sigma, St Louis, Michigan) containing 10% fetal bovine serum (FBS; Sigma), 100 IU/mL penicillin, and 100 μg/mL streptomycin. The purity of the ESCs, eESCs, and CSCs was assessed by morphological determination using light microscopy. Each cell population was routinely 98% pure as assessed by phase-contrast microscopy. We also used immortalized endometrial stromal cells (ESC 4 and 5; eESC 4 and 5) and endometriotic cyst stromal cells (CSC 4 and 5) established as described previously.[21]

Quantitative Reverse Transcription (RT) Polymerase Chain Reaction

The ESCs, eESCs, and CSCs were cultured at 37°C in a humidified atmosphere of 5% CO2. Total RNA was isolated from cells in 35-mm dishes using an RNeasy Mini Kit (Qiagen Inc, Valencia, California) following the manufacturer's protocol. A reverse transcription (RT) reaction with 1 μg of total RNA was carried out with a first-strand complementary DNA (cDNA) synthesis kit (ReverTra Ace-α-; Toyobo Co, Ltd, Osaka, Japan). Thereafter, real-time polymerase chain reaction (PCR) was performed in 96-well, 0.2-mL thin-wall PCR plates using the Thermal Cycler Dice (Takara Bio Inc, Tokyo, Japan). The

Table 1. Primers for Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) (5′-3′).

	Forward	Reverse
FGF1	ACACCGACGGGCTTTTATACG	CCCATTCTTCTTGAGGCCAAC
FGF2	CGACCCTCACATCAAGCTACAA	ACTGCCCAGTTCGTTTCAGT
FGF9	AGCTCATGGGTTTGCATCCAG	ACCAACAAGACAGTGGTTTGGCTA
HAND2	CCACCAGCTACATCGCCTACCT	TCGTTGCTGCTCACTGTGCTT
GAPDH	CAGCCTCAAGATCATCAGCA	GTCTTCTGGGTGGCAGTGAT

real-time PCR mixture contained KOD SYBR qPCR Mix (Toyobo Co, Ltd.), 0.2 μmol/L of PCR primers, and 1 μg of cDNA in a total volume of 25 μL. The oligonucleotide primer sets for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), HAND2, FGF1, FGF2, and FGF9 are shown in Table 1. The PCR profile consisted of an initial incubation at 98°C for 2 minutes followed by 40 cycles of denaturation at 98°C for 10 seconds, annealing at 60°C for 10 seconds, and extension at 68°C for 30 seconds. Quantification was performed by calculating the ratio to GAPDH mRNA using the comparative Ct method.

Small-Interfering RNA

A small interfering RNA (siRNA) for HAND2 (5'-UUCUU-GUCGUUGCUGCUCACUGUGC-3') and Stealth RNAi siRNA Negative Control as a negative control (no. 12935114, Life Technologies) were purchased from Invitrogen Technologies (Thermo Fisher Scientific Inc., Waltham, MA). The immortalized endometrial (ESC 4) and endometriotic stromal cells (CSC 5) were transfected with the siRNA using Lipofectamine RNAiMax (Life Technologies/Invitrogen, Carlsbad, California), according to the manufacturer's instructions. For siRNA experiments, the transfected cells were used 48 hours after transfection.

Migration and Invasion Assay

We used 24-well transwell chambers (6.5 mm filter; 8 μm pore size; Corning Costar, Inc, Corning, New York) to evaluate motility and invasive ability. For the migration assay, CSCs transfected with nonsense siRNA as a control (siControl) or siHAND2 (100 mL of suspension containing 1.5×10^4 cells) were seeded to each of the upper compartments in DMEM without FBS. The cells were then treated with or without 0.1 μmol/L AZD4547 (Selleck Chemicals, Houston, Massachusetts). The lower chamber was filled with DMEM containing 10% FBS. All assays were performed at 37°C in humidified air with 5% CO_2 for a period of 16 hours, and then, cells in the upper surface of the filter were removed by wiping with a cotton swab. The cells on the lower surface of the membrane were fixed by methanol and stained by Giemsa Stain Solution (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan). The stained cells on the lower surface were visualized and photographed using AXIO Imager A1 (ZEISS, Oberkochen, Germany). The numbers of migrated cells were counted at least 3 different visual fields of each chamber at the magnification 4×. For the invasion assay, we used a similar experimental procedure to that of the cell migration assay, except for precoating of the transwell membranes with 5 μg/well Matrigel (Becton Dickinson, San Jose, California). The cells were incubated at 37°C for 16 hours in 5% CO_2 atmosphere. The cells in the lower surface were measured by the same method as the cell migration assay. The individual experiments were performed in triplicate.

Cell Cycle Analysis

After siControl and siHAND2 CSC 5 were cultured in 60-mm collagen 1-coated dishes for 48 hours in DMEM, the cells were treated with or without 0.1 μmol/L AZD4547 and incubated for 24 hours. The cells were trypsinized, centrifuged, and fixed in 70% ethanol/PBS on ice for 30 minutes. The cells were then treated with RNase A (0.25 mg/mL, Thermo Fisher Scientific, Inc., Waltham, MA) and stained with propidium iodide (50 μg/mL, Sigma-Aldrich) on ice for 30 minutes in the dark. Fluorescence intensity was analyzed using Attune Acoustic Focusing Cytometer (Thermo Fisher Scientific, Inc).

Animal Experiments

We used a murine endometriosis model as described previously,[22] and the experimental protocol is summarized in Figure 4A. All animal experiments were approved by The Animal Experimental Committee of Nagoya University Graduate School of Medicine. Before starting the experiments, animals were allowed to acclimatize for 7 days at 23°C to 25°C with a 12-hour dark/light cycle and were given standard chow and water. Donor female mice were ovariectomized through a 1-cm longitudinal skin incision and were injected subcutaneously with estradiol (E_2; 100 μg/kg per mouse per week; Fuji Pharma, Tokyo, Japan) in olive oil once a week. After 2 weeks, the donor uterus was removed en bloc after euthanasia and was cleaned of supplementary tissue in saline. The uterus was cut longitudinally with a linear incision and minced ($\sim$0.5 mm in diameter) with scissors. A total of 33 recipient mice (9 weeks of age) were anesthetized using Sevoflurane and were ovariectomized. Thereafter, recipient mice received a 0.5-cm subabdominal midline incision, and minced donor uterine tissue in 500 μL saline was injected into the peritoneal cavity of each recipient with the equivalent of tissue from half of the uterus, and the abdomen was sutured closed. Recipient mice were then

injected subcutaneously with E_2 (100 μg/kg per mouse per week) in olive oil once per week for 4 weeks until the end of the experiment (13 weeks of age) when the final autopsy evaluation was performed. Twenty-five micro gram of FGF-receptor inhibitor (AZD4547) was injected 3 times per week into the peritoneal cavity until end of the experiment (13 weeks of age).

Histological Analysis

We counted the number of murine endometriotic cysts and measured the cyst diameters. Murine endometriotic cysts were fixed with 10% phosphate-buffered formalin and immunohistochemical staining was performed as described previously.[22] The primary antibody for Ki67 (clone MK167, 1:100, NB600-1252; Novus Billogicals) was used. Proliferative activity was evaluated by counting Ki67-positive and -negative nuclei of all epithelial cells at 200× magnification.

Statistical Analysis

All data sets are presented as mean (standard deviation [SD]). Statistical analyses were performed by the use of SPSS Software (IBM SPSS Statistics Version 24, Armonk, NY). Student *t* tests or Mann-Whitney *U* tests were used to compare the variables between 2 groups. A *P* Value of <.05 was considered to be statistically significant.

Results

Expression of HAND2 in Clinical Samples and Cells of Endometrium and Endometriotic Lesions

We first explored expression of HAND2 in eutopic uterine endometrium and endometriotic ovarian cysts. Immunohistochemistry showed moderate and strong expression of HAND2 in proliferative and secretory phase eutopic endometrial stromal cells, respectively (Figure 1A and C). Expression of HAND2 in stromal cells of endometriotic ovarian cysts in proliferative and secretory phases was much weaker than eutopic endometrial stromal cells in proliferative phase (Figure 1B and D). Semiquantitative analysis showed significant differences between the normal eutopic endometrium and endometriotic tissues from ovarian cysts in both the proliferative phase and the secretory phase (Figure 1E). We then compared expression levels of *HAND2* in primary and immortalized cultured stromal cells from eutopic endometrium (ESC and eESC) and endometriotic ovarian cysts (CSC). Quantitative RT-PCR showed that *HAND2* expression in ESCs and eESCs was approximately 2- to 5-fold higher than that of CSCs. Expression levels of *HAND2* were almost similar in ESCs and eESCs (Figure 1F).

Expression of FGFs Were Upregulated With HAND2 Suppression

We next explored the expression of FGFs and found that expression of *FGF1, FGF2,* and *FGF9* was higher in CSCs compared to ESCs. Suppression of HAND2 with siRNA induced significant upregulation of *FGF1, FGF2,* and *FGF9* (Figure 2).

Phenotypic Alterations Induced by HAND2 Suppression Were Attenuated by FGF Inhibitor

We then investigated whether the phenotypic changes are mediated by FGFs of which expression could be altered by HAND2 suppression. Increases in migration and invasion capacity of CSCs induced with HAND2 siRNA were partially inhibited with an antagonist of FGF receptors, AZD4547 (Figure 3A and B). We also found similar tendencies using ESCs and eESCs (data not shown). Cell cycle analyses revealed that the proportion of CSCs in G2/M phases were increased by suppression of HAND2 (Figure 3C). AZD4547 did not cause significant changes in the proportion of cell cycles.

Fibroblast Growth Factors Receptor Antagonist Prevents Progression of Murine Endometriotic Lesions

The experimental protocol using the murine model of endometriosis is summarized in Figure 4A. After treatment of AZD4547 for 4 weeks following implantation with minced murine uterine tissues, the total number of lesions (5.3 ± 1.2 vs 3.4 ± 0.5 per mouse) was significantly reduced (Figure 4B, $P < .05$), and the surface area of lesions (259.0 ± 53.2 vs 163.6 ± 34.1 mm^2 per mouse) was significantly decreased ($P < .05$, Figure 4C). To evaluate the proliferative activity of endometriotic lesions, the ratio of Ki67-positive cells was calculated. Ki67-positive proliferating epithelial cells in the endometriotic lesions were also significantly decreased after treatment of AZD4547 ($32.8 \pm 5.4\%$ vs $13.4 \pm 2.6\%$; $P < .01$, Figure 4D and E).

Discussion

Sampson's retrograde menstruation theory has been widely accepted as the strongest hypothesis for the pathogenesis of endometriosis.[23] Although endometriotic cells are presumed to be derived from endometrial cells in this theory, numerous studies have demonstrated that there are distinguishing characteristics in endometriotic cells when compared to eutopic endometrial cells. These differences include reduced progesterone responsiveness,[8,24] increased resistance to apoptosis,[25] as well as increased cell adhesion/migration/invasion capacity.[26] Among them, progesterone resistance is the most fundamental feature that may influence other phenotypes of endometriotic cells. However, the mechanism of how effectors cause phenotypic alterations downstream of the progesterone resistance are yet to be fully elucidated. These effectors could potentially become new specific therapeutic targets for endometriosis, since progestin is now one of the most efficacious medications for endometriosis.

In this current study, we found that a progesterone-dependent transcriptional factor, HAND2, is less expressed

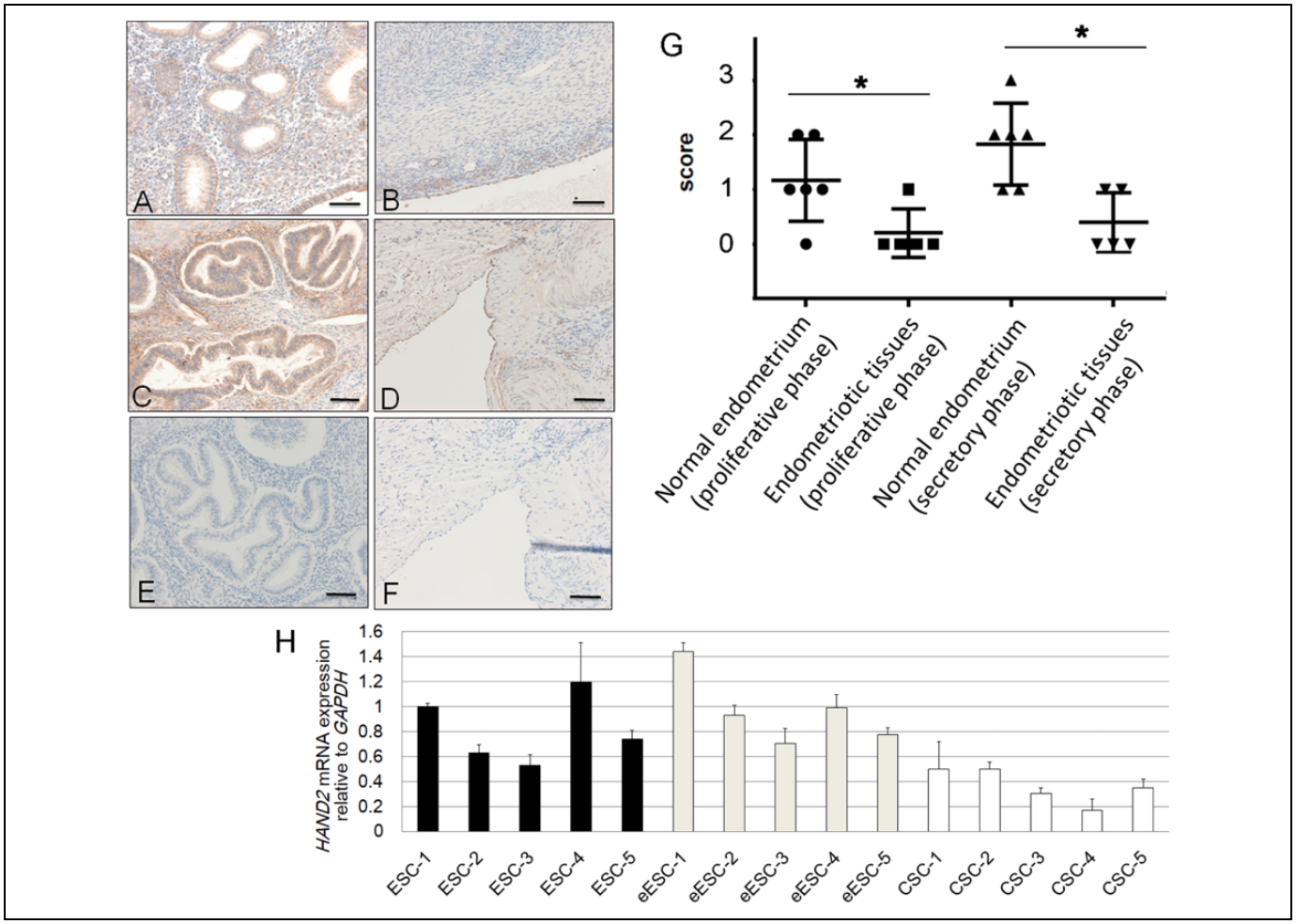

Figure 1. Immunohistochemical Localization of Heart and neural crest derivatives expressed 2 (HAND2) in (A) proliferative-phase normal eutopic endometrium, (B) proliferative-phase endometriotic ovarian cyst, (C) secretory-phase normal eutopic endometrium, and (D) secretory-phase endometriotic ovarian cyst. Negative controls using (E) secretory-phase normal eutopic endometrium and (F) secretory-phase endometriotic ovarian cyst. HAND2 is Expressed in Epithelial Cells and Stromal Cells. The Scale Bars Represent 50 μm. (G) The staining intensity for stromal expression of HAND2 in proliferative-phase normal endometrium (n = 6), proliferative-phase endometriotic tissues from ovarian cysts (n = 5), secretory-phase normal endometrium (n = 6), and secretory-phase endometriotic tissues from ovarian cysts (n = 5). The bars indicate 25, 50, and 75 percentile; *$P < .05$. (H) Expression levels of *HAND2* by quantitative RT polymerase chain reaction (PCR) in in vitro cultured stromal cells from endometrium and endometriotic cysts. 1-3, Primary cultured cells; 4 and 5, immortalized cells; Mean (standard deviation). CSC indicates cyst stromal cells; eESC, endometrial stromal cells with endometriosis; ESC, endometrial stromal cells without endometriosis.

by endometriotic stromal cells compared to endometrial stromal cells. We also demonstrated that FGF1, FGF2, and FGF9 in the endometrial and endometriotic stromal cells are upregulated in an HAND2-dependent manner and that the upregulation of FGFs induces the migration and invasion of endometriotic stromal cells. Moreover, animal experiments support that FGFs and the FGF receptor system can be therapeutic targets for endometriosis.

AZD4547 is a potent inhibitor of FGF receptor-1, 2, and 3 and inhibits proliferation in several cancer cells, especially in cells overexpressing FGF receptors.[27-29] AZD4547 is now under clinical trial for treating advanced-stage cancers.[30,31] We demonstrated that AZD4547 treatments at relatively low doses inhibited in vitro migration and invasion of endometriotic stromal cells as well as the development of endometriotic lesions in the mouse model. We failed to show the suppressive effects of AZD4547 on cell proliferation in vitro, while Ki67 immunostaining in mouse endometriotic lesions was significantly decreased by AZD4547 treatment. Further detailed evaluation will be required to fully elucidate the effects of AZD4547 as a therapeutic agent for endometriosis.

We previously reported that the expression of another progesterone-dependent molecule, CD10 (a marker for the decidualization of endometrial stromal cells induced by progestins), is attenuated in endometriotic stromal cells.[10] Decreased expression of CD10 in endometriotic cells was involved in the enhancement of CD44-mediated cell adhesions. Since the decidualization of endometrial stromal cells is one of

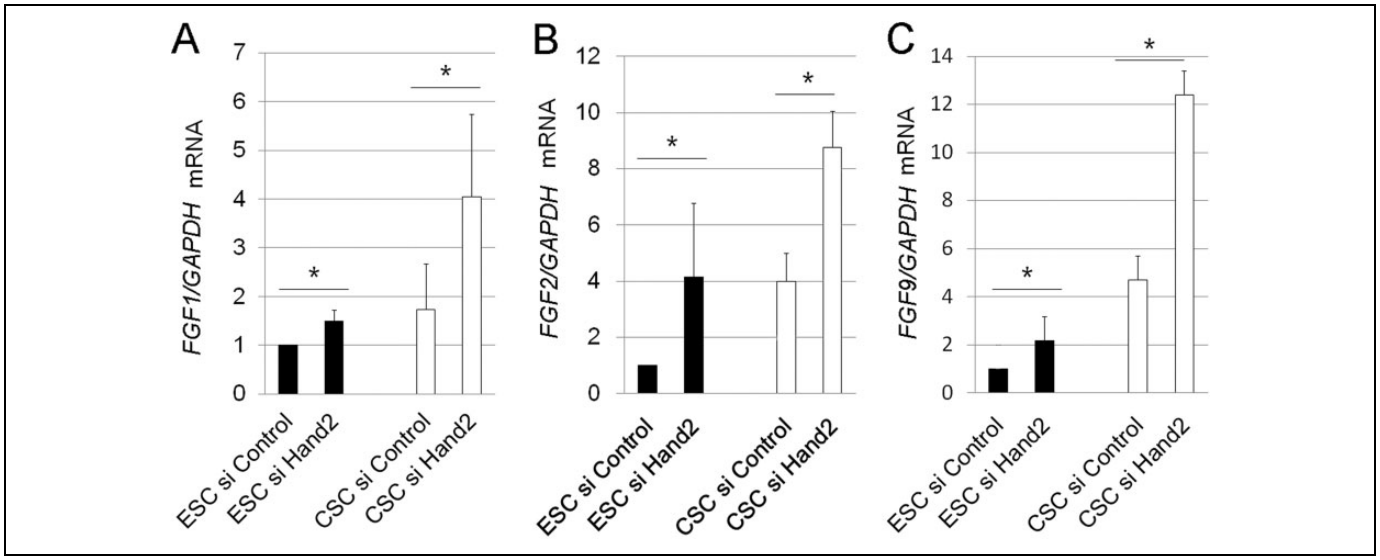

Figure 2. Expression levels of (A) *FGF1*, (B) *FGF2*, and (C) *FGF9* in ESCs and CSCs with or without small-interfering RNA (siRNA) for Heart and neural crest derivatives expressed 2 (HAND2). Suppression of HAND2 using siRNA significantly upregulated expression of *FGF1*, *FGF2*, and *FGF9* in ESC 3 and CSC 4. Mean (standard deviation). *$P < .05$. CSC indicates cyst stromal cells; ESC, endometrial stromal cells without endometriosis; FGF, fibroblast growth factor; HAND, heart and neural crest derivatives expressed 2.

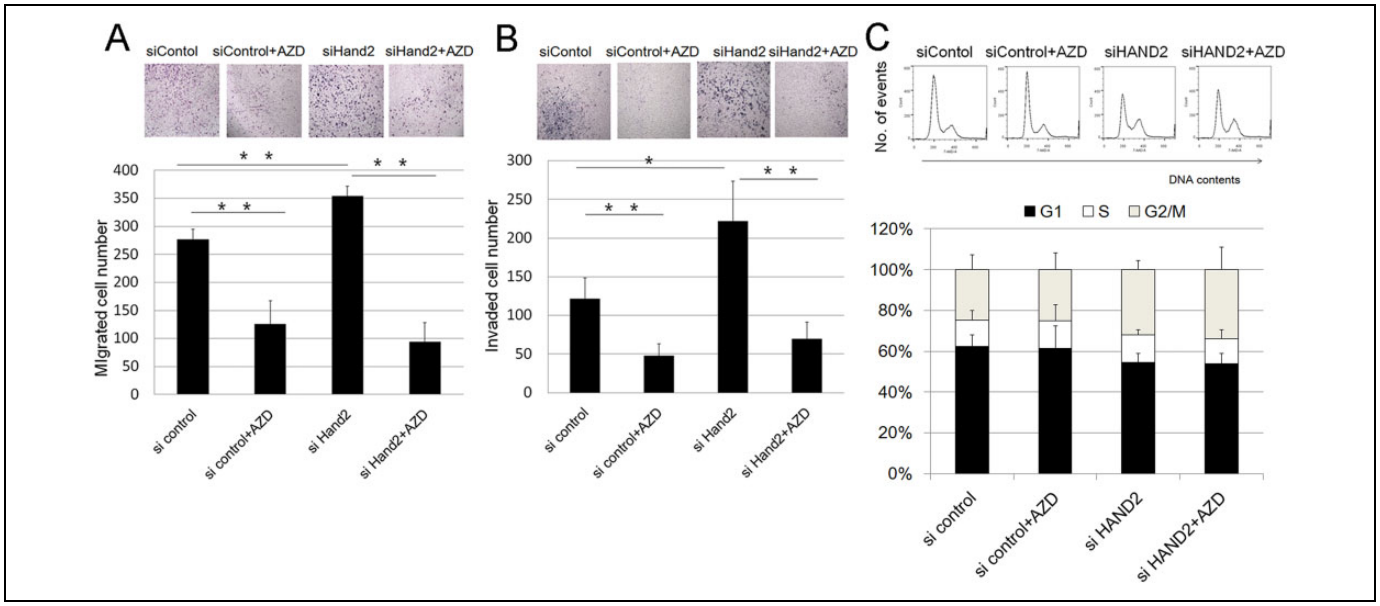

Figure 3. A, Migration assay of CSC 4. Suppression of HAND2 using small-interfering RNA (siRNA) significantly increased the number of migrated cells. AZD4547 (AZD), an FGF receptor inhibitor, significantly inhibited migration of CSCs. B, Invasion assay of CSCs. Suppression of HAND2 Using siRNA significantly increased the number of invaded cells. AZD4547 (AZD) significantly inhibited invasion of CSCs. Mean (standard deviation). *$P < .05$, **$P < .01$. (C) Effects of HAND2 siRNA and AZD4547 on cell cycle status. Cell cycle distribution in in CSCs treated with HAND2 siRNAs and/or AZD4547. DNA content was measured by propidium iodide staining 24 hours after AZD4547 treatment and cell cycle stage determined by flow cytometry. Mean (standard deviation) of 3 independent experiments. CSC indicates cyst stromal cells; FGF, fibroblast growth factor; HAND, heart and neural crest derivatives expressed.

the steps in mesenchymal–epithelial transition, reduced expression of CD10, signifying less decidualization, may be involved in cell motility and the development of endometriotic lesions.

Deep infiltrating endometriosis is pathologically defined by the existence of endometriotic lesions at a depth of more than 5 mm beneath the peritoneal surface.[32] Very recently, it has been demonstrated that deep infiltrating endometriosis is associated with a certain type of adenomyosis.[33] Deep infiltrating endometriosis is considered to be caused by aberrant enhancement of cell motility and invasion ability. Progesterone resistance might contribute to the progression of deep infiltrating endometriosis through downregulation of CD10 and HAND2.

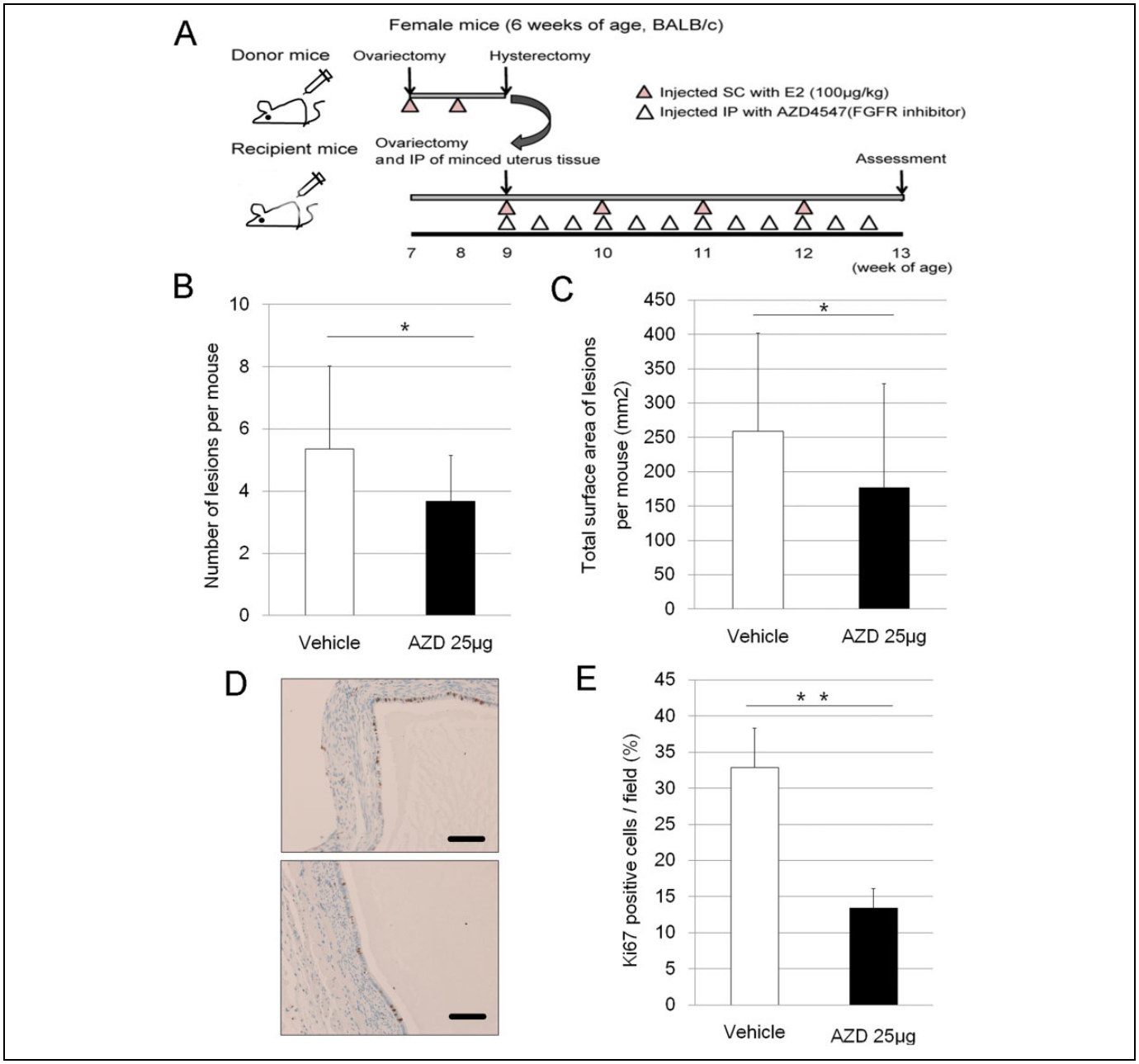

Figure 4. (A) Schematic illustration of AZD4547-treatment for endometriosis-model mice and their evaluation. (B) The number of endometriotic lesions per mouse and (C) the total surface area of endometriotic lesions per mouse significantly decreased with AZD4547 (AZD)-treatment (n = 19) compared to vehicles (n = 14). (D) Immunohistochemistry for Ki67 in endometriotic lesions from vehicle (upper panel) and AZD4547-treated mice (lower panel). (E) The number of Ki67-positive cells in each specimen of endometriotic lesions was significantly decreased with AZD4547 (AZD)-treatment. Mean (standard deviation). *$P < .05$, **$P < .01$. The scale bars represent 50 μm

One of the limitations of the current study is that we did not find significant differences in the expression levels of HAND2 between control endometrium without endometriosis and eutopic endometrium with endometriosis in vitro or in vivo. In Sampson retrograde menstruation theory, it has been generally assumed that endometriotic cells are derived from eutopic endometrial cells. There are several research demonstrating that progesterone resistance can be seen in eutopic endometrium of women with endometriosis to some extent.[6,8] In this point, further experiments will be required to reveal how and when downregulation of HAND2 in endometriotic cells occurs. Another limitation is that we did not indicate whether induction of HAND2 in endometriotic cells results in downregulation of FGFs. Although the main purpose of our current study is to demonstrate FGFs, a possible therapeutic target as an effector of downregulation of HAND2 in endometriotic cells, we may need further experiments to get more reliable evidence of HAND2–FGF sequence in endometriotic cells.

In conclusion, we demonstrated that FGF1, FGF2, and FGF9 are downstream effectors of the progesterone-dependent transcriptional factor, HAND2, and are consequently upregulated with suppression of HAND2 in the endometriotic cells. Since HAND2-dependent FGFs play roles in enhancing invasive capacity of endometriotic cells, our results suggest that FGF receptor inhibitors could be promising therapeutic agents for endometriosis.

Authors' Note

Nao Kato is the principal investigator and wrote the manuscript. Akira Iwase designed the study and guided the experiments. Chiharu Ishida, Takashi Nagai, and Masahiko Mori performed the major part of this project in collaboration with Nao Kato. Bayasula, Umida Ganiyeva, Ying Qin and Rika Miki participated in in vitro experiments and collected the data. Tomoko Nakamura and Satoko Osuka contributed to the scientific discussion and the manuscript preparation. Rika Miki and Fumitaka Kikkawa revised the manuscript.

Declaration of Conflicting Interests

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