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# A functional role of LEFTY during progesterone therapy for endometrial carcinoma

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## Abstract

**Background:** The left-right determination factor (LEFTY) is a novel member of the TGF- $\beta$ /Smad2 pathway and belongs to the premenstrual/menstrual repertoire in human endometrium, but little is known about its functional role in endometrial carcinomas (Em Cas). Herein, we focused on LEFTY expression and its association with progesterone therapy in Em Cas.

**Methods:** Regulation and function of LEFTY, as well as its associated molecules including Smad2, ovarian hormone receptors, GSK-3 $\beta$ , and cell cycle-related factors, were assessed using clinical samples and cell lines of Em Cas.

**Results:** In clinical samples, LEFTY expression was positively correlated with estrogen receptor- $\alpha$ , but not progesterone receptor (PR), status, and was inversely related to phosphorylated (p) Smad2, cyclin A2, and Ki-67 levels. During progesterone therapy, expression of LEFTY, pSmad2, and pGSK-3 $\beta$  showed stepwise increases, with significant correlations to morphological changes toward secretory features and decreased Ki-67 values. In Ishikawa cells, an Em Ca cell line that expresses PR, progesterone treatment reduced proliferation and induced increased expression of LEFTY and pGSK-3 $\beta$ , although *LEFTY* promoter regions were inhibited by transfection of PR. Moreover, inhibition of GSK-3 $\beta$  resulted in increased LEFTY expression through a decrease in its ubiquitinated form, suggesting posttranslational regulation of LEFTY protein via GSK-3 $\beta$  suppression in response to progesterone. In addition, overexpression or knockdown of LEFTY led to suppression or enhancement of Smad2-dependent cyclin A2 expression, respectively.

**Conclusion:** Upregulation of LEFTY may serve as a useful clinical marker for the therapeutic effects of progesterone for Em Cas, leading to inhibition of tumor cell proliferation through alteration in Smad2-dependent transcription of *cyclin A2*.

**Keywords:** Lefty, Endometrial carcinoma, Progesterone therapy, TGF- $\beta$ , Smad, Cyclin a

## Background

Endometrial carcinoma (Em Ca) is the most common gynecologic malignancy. Although most patients are postmenopausal, approximately 5% of women with this disease are diagnosed before 40 years of age [1, 2]. This paradigm shift to younger ages may be due to an increase in the risk

factors including marked obesity, diabetes mellitus, and hypertension [3, 4]. In these younger patients who want to have children in the future, hormone therapy using high-dose progesterone is frequently performed for the preservation of fertility [3–5].

Transforming growth factor  $\beta$  (TGF- $\beta$ ) is a member of the large family of structurally related cytokines that play an important role in controlling cell proliferation, differentiation, migration, and apoptosis [6–9]. Upon ligand binding, TGF- $\beta$  receptor I recruits and subsequently induces an intracellular downstream signaling cascade involving

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Smad proteins which in turn act as transcription factors [6–9]. In human endometrium, TGF- $\beta$ 1 expression is up-regulated during periods of increasing plasma progesterone concentrations and down-regulated during its withdrawal, along with significant correlations to manifestation of various abnormalities including endometriosis, abnormal uterine bleeding, and Em Ca [10–15].

The left-right determination factor (LEFTY) is a novel member of the TGF- $\beta$  superfamily, consisting of LEFTY1 and LEFTY2. They are both 366 amino acids long, and differ at only 16 residues, thus they are 97% identical and share 350 identical residues [16, 17]. LEFTY1/2 serve as repressors for TGF- $\beta$  signaling by inhibiting the phosphorylation of Smad2 after activation of the TGF- $\beta$  receptor [18]. LEFTY2, also known as endometrial bleeding-associated factor (ebaf), is controlled by ovarian steroids, and its expression correlates chronologically and spatially with that of bleeding-associated matrix metalloproteinases, indicating that LEFTY2 is a good candidate to locally modulate progesterone action on these proteinases within the endometrium [19–21].

In this study, we set out to first clarify the expression and the regulation of LEFTY1/2 in normal and malignant endometrium. Next, change in LEFTY1/2 expression in patients with Em Cas undergoing progesterone therapy was investigated. Finally, we examined the functional roles of LEFTY1/2 in TGF- $\beta$ /Smad signaling pathways in Em Ca patients receiving the therapy.

## Methods

### Clinical cases

Histological findings were reviewed in hysterectomy specimens of endometrioid-type Em Cas from the case records of Kitasato University Hospital during the period from 2007 to 2015, according to the criteria of the 2014 World Health Organization classification. [22] Each case was also staged according to the 2009 International Federation of Gynecology and Obstetrics (FIGO) staging system. [23] A total of 120 cases of Em Cas, including 49 of grade (G)1, 14 of G2, and 57 of G3, as well as 22 samples of complex hyperplasia with or without atypia, were investigated. For the carcinoma cases, the mean age of the patients was 54.5 years (range from 30 to 84), and 53 were postmenopausal. In addition, our cases included 74 subcategorized as clinical FIGO stage I and 29 as stage II-IV, 27 with upper ( $\leq 1/2$ ) myometrial invasion and 39 with lower ( $> 1/2$ ) myometrial invasion, as well as 18 that were positive for nodal metastasis and 67 that were negative.

A total of 32 patients with G1 Em Cas who received progesterone therapy were also selected (Table 1). These patients were diagnosed as stage I based on the findings of both physical and radiographic examinations, and their mean age was 30.3 years (range from 22 to 38). All patients received 400 to 800 mg of medroxyprogesterone

acetate (MPA) via daily oral administration. Biopsy samples were taken by diagnostic curettage or hysteroscopy before and during progesterone therapy, and all contained sufficient tumor elements to allow examination of sequential morphological changes.

Ninety-six biopsy specimens of normal endometrial tissues including 24 in the proliferative phase, 52 in the secretory phase (10 early and 20 middle and late), and 20 in the menstrual phase were also investigated. All tissues were routinely fixed in 10% formalin and processed for embedding in paraffin. In addition, 40 fresh Em Ca samples (20 G1, 7 G2, and 13 G3), as well as 22 normal endometrial tissues were applied.

Histopathological analysis of endometrial tumors during progesterone therapy.

Evaluation of morphological changes that occurred during progesterone therapy was performed in accordance with methods described previously [24, 25]. Briefly, the sections obtained from tumors were examined in terms of the following four parameters: 1) cellularity, 2) nuclear rearrangement, 3) eosinophilia in the cytoplasm, and 4) the nuclear / cytoplasmic ratio. Therapeutic efficacy (TE) was graded by counting the numbers of altered parameters. Cases were subdivided into two categories, as follows: TE grade 0, 1, or 2 is defined as poor response; TE grade 3 or 4 is defined as good response.

### Antibodies and reagents

Anti-LEFTY, anti-Smad2, and anti-phospho(p)-Smad2 at serine 255 (pSmad2) antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-p27<sup>Kip1</sup> and anti-glycogen synthase kinase (GSK)-3 $\beta$  antibodies were bought from BD Biosciences (San Jose, CA, USA). Anti-p21<sup>waf1</sup>, anti-cyclin D1, and anti-Ki-67 antibodies were purchased from Dako (Copenhagen, Denmark). Anti-cyclin A2, anti-estrogen receptor  $\alpha$  (ER $\alpha$ ), and anti-progesterone receptor (PR) antibodies were from Novocastra (Newcastle, UK). Anti-pGSK-3 $\beta$  at Ser9 (pGSK-3 $\beta$ ), anti-Akt, anti-pAkt at serine 473 (pAkt), and anti-ubiquitin antibodies were from Cell Signaling Technology (Danvers, MA, USA). Anti- $\beta$ -actin antibody, nocodazole, 17 $\beta$ -estradiol (E2), medroxyprogesterone 17-acetate (MPA), MG132, and lithium chloride (LiCl) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Rapamycin and aphidicolin were obtained from Calbiochem (Cambridge, MA, USA). Recombinant TGF- $\beta$ 1 was purchased from R&D Systems (Minneapolis, MN, USA).

### Immunohistochemistry (IHC)

IHC was performed using a combination of microwave heating and Histofine Simple Stain MAX-PO (MULTI) (Nichirei Biosciences, Tokyo, Japan) methods. For evaluation of IHC findings, scoring of nuclear and/or cytoplasmic immunoreactivity for LEFTY, pSmad2,

**Table 1** Summary of Em Ca patients receiving progesterone therapy

Case No	Age (years)	When tissues are taken	Histology	MPA dosage/day	TE grade	IHC markers			
						LEFTY score	pSmad2 score	Ki-67 LI (%)	pGSK-3β score
1	29	before Tx	G1 Em Ca <sup>a</sup>	<sup>a</sup>	<sup>a</sup>	4	6	30.6	4
		3 m	G1 Em Ca	600	3	6	8	18	*
		6 m	G1 Em Ca	600	4	12	12	4	4
2	30	before Tx	G1 Em Ca <sup>a</sup>	<sup>a</sup>	<sup>a</sup>	6	4	2.2	6
		3 m	G1 Em Ca/AH	600	2	6	6	2.6	8
		6 m	G1 Em Ca/AH	600	3	12	8	1	<sup>a</sup>
		9 m	G1 Em Ca/AH	600	3	12	8	15.2	8
3	35	before Tx	G1 Em Ca <sup>a</sup>	<sup>a</sup>	<sup>a</sup>	3	6	60.6	
		6 m	G1 Em Ca	600	2	3	6	22.2	8
4	35	before Tx	G1 Em Ca <sup>a</sup>	<sup>a</sup>	<sup>a</sup>	3	9	26.4	4
		3 m	G1 Em Ca/AH	600	3	6	12	2	8
		9 m	G1 Em Ca/AH	600	3	12	12	11.4	6
5	34	before Tx	G1 Em Ca <sup>a</sup>	<sup>a</sup>	<sup>a</sup>	0	6	3	8
		3 m	G1 Em Ca/AH	600	3	3	4	13	12
		before Tx	G1 Em Ca <sup>a</sup>	<sup>a</sup>	<sup>a</sup>	9	3	60.5	4
		3 m	G1 Em Ca	600	4	12	6	8.4	6
6	35	before Tx	G1 Em Ca <sup>a</sup>	<sup>a</sup>	<sup>a</sup>	0	8	19.8	4
		3 m	G1 Em Ca	600	3	0	8	17.6	8
		6 m	G1 Em Ca	600	4	3	6	6.2	8
7	29	before Tx	G1 Em Ca <sup>a</sup>	<sup>a</sup>	<sup>a</sup>	4	8	50	6
		3 m	G1 Em Ca/AH	600	4	9	8	1	12
		9 m	G1 Em Ca/AH	600	4	12	12	5.5	12
8	37	before Tx	G1 Em Ca <sup>a</sup>	<sup>a</sup>	<sup>a</sup>	6	12	20.2	6
		3 m	G1 Em Ca/AH	600	4	12	6	0	9
		6 m	G1 Em Ca	600	4	9	8	2.2	12
		9 m	G1 Em Ca/AH	600	4	8	4	3.5	12
9	24	before Tx	G1 Em Ca <sup>a</sup>	<sup>a</sup>	<sup>a</sup>	9	8	33.4	9
		3 m	G1 Em Ca/AH	600	3	12	8	18.6	9
		6 m	G1 Em Ca/AH	600	3	12	12	48.2	9
10	29	before Tx	G1 Em Ca <sup>a</sup>	<sup>a</sup>	<sup>a</sup>	4	4	52.5	9
		3 m	G1 Em Ca/AH	600	4	12	4	5	8
11	34	before Tx	G1 Em Ca <sup>a</sup>	<sup>a</sup>	<sup>a</sup>	3	8	34.3	9
		3 m	G1 Em Ca/AH	600	4	6	8	2	12
		6 m	G1 Em Ca/AH	600	4	6	8	5	9
12	38	before Tx	G1 Em Ca <sup>a</sup>	<sup>a</sup>	<sup>a</sup>	3	4	2	4
		3 m	G1 Em Ca/AH	800	4	6	6	1.3	8
		6 m	G1 Em Ca/AH	800	3	9	9	3	12
		9 m	G1 Em Ca/AH	800	4	12	6	0	12
13	30	before Tx	G1 Em Ca <sup>a</sup>	<sup>a</sup>	<sup>a</sup>	4	9	34.5	6
		3 m	G1 Em Ca/AH	600	3	8	12	8.2	12
		6 m	G1 Em Ca	600	4	9	12	10.5	12
		9 m	G1 Em Ca/AH	600	4	8	12	20.7	12

**Table 1** Summary of Em Ca patients receiving progesterone therapy (Continued)

Case No	Age (years)	When tissues are taken	Histology	MPA dosage/day	TE grade	IHC markers			
						LEFTY score	pSmad2 score	Ki-67 LI (%)	pGSK-3β score
14	29	before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	3	8	55.8	8
		3 m	G1 Em Ca/AH	600	3	3	8	1	8
		6 m	G1 Em Ca/AH	600	3	4	9	2	12
15	22	before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	3	4	60.8	6
		3 m	G1 Em Ca	600	1	6	8	35.4	9
		6 m	G1 Em Ca	600	3	6	8	13.5	12
16	38	before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	4	8	18.8	8
		9 m	G1 Em Ca/AH	600	0	4	6	40.8	12
17	30	before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	2	4	48.6	3
		3 m	G1 Em Ca	600	2	4	2	35.5	9
18	34	before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	1	6	40	2
		3 m	G1 Em Ca	600	4	4	4	5.5	6
		6 m	G1 Em Ca	600	4	6	6	5	6
		9 m	G1 Em Ca/AH	600	4	4	9	4.6	8
19	26	before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	4	9	17.8	
		3 m	G1 Em Ca	600	4	6	12	0	8
		6 m	G1 Em Ca	600	3	9	9	0	8
		9 m	G1 Em Ca/AH	600	4	9	9	9.6	12
20	28	before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	4	6	30.5	6
		6 m	G1 Em Ca	600	3	6	4	7.6	12
21	30	before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	2	4	52	3
		3 m	G1 Em Ca	400	3	9	9	6.5	9
22	32	before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	6	8	43.6	9
		3 m	G1 Em Ca	400	4	8	4	20	12
		6 m	G1 Em Ca	600	4	12	12	3	8
23	28	before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	6	12	43.5	8
		6 m	G1 Em Ca	600	4	9	12	8	12
24	25	before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	1	8	18.8	4
		before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	12	12	40.6	6
		before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	6	6	67	8
		3 m	G1 Em Ca	400	4	9	9	7	8
		9 m	G1 Em Ca/AH	600	3	9	6	8.5	8
25	29	before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	4	12	60.4	9
		before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	4	4	14.2	4
		3 m	G1 Em Ca	400 mg/d	0	8	4	5	12
		6 m	G1 Em Ca	400	0	4	4	20.8	12
		9 m	G1 Em Ca	400	0	9	12	24	6
26	30	before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	4	8	29.8	8
		3 m	G1 Em Ca	400	0	4	8	39	8
		6 m	G1 Em Ca	400	1	6	8	28.8	6
27	32	before	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	9	9	18	9
		3 m	G1 Em Ca	400	2	8	8	3.3	12

**Table 1** Summary of Em Ca patients receiving progesterone therapy (Continued)

Case No	Age (years)	When tissues are taken	Histology	MPA dosage/day	TE grade	IHC markers			
						LEFTY score	pSmad2 score	Ki-67 LI (%)	pGSK-3β score
28	29	before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	6	6	15	6
		before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	6	8	20	*
		3 m	G1 Em Ca	400	3	8	8	1	*
29	22	before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	6	8	48	12
		3 m	G1 Em Ca	600	3	8	4	30.5	8
		3 m	G1 Em Ca	600	2	12	4	5.5	6
		6 m	G1 Em Ca	600	0	9	2	22.4	6
30	24	before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	6	8	46	3
		3 m	G1 Em Ca	600	3	12	12	6	6
		6 m	G1 Em Ca	600	3	12	12	1	6
31	29	before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	6	8	55.6	12
		3 m	G1 Em Ca	400	0	4	8	3.4	12
		9 m	G1 Em Ca/AH	400	2	9	8	7	12
32	30	before Tx	G1 Em Ca	<sup>a</sup>	<sup>a</sup>	3	8	63.8	3
		3 m	G1 Em Ca	600	0	8	8	18.5	8
		9 m	G1 Em Ca/AH	600	2	4	6	30.5	6

TE therapeutic efficacy, Tx therapy, m month, G1 Em Ca grade 1 endometrial carcinoma, AH atypical hyperplasia  
<sup>a</sup>not examined

ERα, and PR was performed as described previously [26, 27]. Briefly, the proportion of immunopositive cells among the total number of counted cells was subdivided into five categories as follows: 0, all negative; 1, <10%; 2, 10–30%; 3, 30–50%; and 4, >50% positive cells. The immunointensity was also subclassified into four groups: 0, negative; 1, weak; 2, moderate; and 3, strong immunointensity. IHC scores were generated by multiplication of the values of the two parameters. Nuclear immunopositivity for Ki-67 and cyclin A2 was also counted in at least 1000 cells in five randomly selected fields. Labeling indices (LIs) were then calculated as number per 100 cells, as described previously [26, 27].

**Plasmids and cell lines**

The human *cyclin A2* promoter (GenBank accession number AF518006) between -1467 and +26 bp and the human *p27<sup>kip1</sup>* promoter (NG016341) encompassing -1565 to -12 bp (where +1 represents the transcription start site) were amplified by polymerase chain reaction (PCR) and were cloned into the pGL-3B vector (Promega, Madison WI, USA). Site-mutagenesis in putative GSK-3β phosphorylation motifs in the LEFTY1 protein (pcDNA3.1-LEFTY1 mutant type (mt) 5SA-HA) was also carried out using the PrimeSTAR Mutagenesis Basal kit (Takara Bio, Shiga, Japan) and pcDNA3.1-LEFTY1-wild-type (wt)-HA tag construct. The identity of all constructs was confirmed by sequencing prior to use. The sequences of PCR primers employed in this study are

listed in Additional file 1: Table S1. pcDNA3.1-LEFTY1, pcDNA3.1-Smad2, pSG5-HEG0(ERα), pSG5-hPRB, pGL3B(-3533) LEFTY1 luc, pGL3B(-4805) LEFTY2 luc, pGL3B-(2338) p21<sup>waf1</sup> luc, pGL3B(-963) CD1 luc, and pSIREN-RetroQ- short hairpin (sh) LEFTY1 were used as described previously [26–29, 32].

Eight Em Ca cell lines, including Ishikawa, Hec1A, Hec6, Hec88, Hec108, Hec155, Hec180, and Hec251 cells, were used as described previously [30, 31]. Ovarian clear cell carcinoma cell line, ES-2, was obtained from the American Type Culture Collection (Manassas, VA, USA). ES-2 cells stably overexpressing LEFTY1 were also used as described previously [32]. In addition, ES-2 cells stably overexpressing LEFTY1wt-HA and LEFTY1mt 5SA-HA, and Ishikawa cells with knockdown of LEFTY1 by shRNA were also established.

**Transfection**

Transfection was carried out using LipofectAMINE PLUS (Invitrogen, Carlsbad, CA, USA), in duplicate or triplicate, and luciferase activity was assayed as described previously [26, 27]. The siRNA against LEFTY1 or the negative control was transfected using the siPort NeoFx transfection agent (Ambion, Austin, TX, USA), according to the manufacturer’s instructions.

**RT-PCR (reverse transcription-PCR)**

cDNA was synthesized from 2 μg of total RNA. Amplification by RT-PCR was carried out in the exponential

phase to allow comparison among cDNAs synthesized from identical reactions using specific primers for LEFTY1/2 as described previously [32]. Primer sequences for both ER $\alpha$  and progesterone receptor B (PRB) are listed in Additional file 1: Table S1.

#### Western blot assay and immunoprecipitation

Cells were lysed using RIPA buffer [50 mM/L, Tris-HCl (pH 7.2), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate] and clinical samples were lysed directly with 2 $\times$  Laemmli sample buffer [65 mM/L Tris-HCl (pH 6.8), 5% 2-mercaptoethanol, 3% sodium dodecyl sulfate, 10% glycerol]. Aliquots of the proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and probed with primary antibodies coupled to the ECL detection system (Amersham Pharmacia Biotechnology, Tokyo, Japan). The intensity of individual signals was measured using ImageJ software version 1.41 (NIH, Bethesda, MD, USA).

For immunoprecipitation, cells stably overexpressing LEFTY1 treated with LiCl and/or MG132 were lysed with TNE buffer [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1 mM EDTA]. Cell lysates were cleared and incubated with anti-LEFTY antibody, followed by incubation with Protein G-Sepharose (Amersham Pharmacia Biotechnology). Western blot assay was subsequently performed with anti-LEFTY and anti-ubiquitin antibodies.

#### Methylation analysis of the *LEFTY1* gene

The methylation status of the *LEFTY1* gene was analyzed as described previously [33]. Briefly, genomic DNA extracted from cell lines using Wizard Genomic DNA Purification kit (Promega) was treated with bisulfate using an EZ DNA Methylation-Gold kit (ZYMO Research, Orange, CA, USA). Bisulfate-treated DNA was amplified by PCR using specific primers as described previously. Amplicons were ligated into a pCR2.1 vector using the TA cloning kit (Invitrogen). Four positive clones were identified and sequenced from each sample. The DNA sequence generated was subjected to *in silico* analysis to describe the CpG methylation status of these clones.

#### In situ hybridization (ISH)

Riboprobes for LEFTY1 and LEFTY2 were generated by *in vitro* transcription, and ISH assays were performed using the GenPoint Tyramide Signal Amplification System (Dako), as described previously [32, 34]. The ISH signal score was determined on the basis of the percentage of ISH signal-positive cells (1, less than 10% positive cells; 2, 10–30%; 3, 30–50%; 4, more than 50%) and the ISH signal intensity (0, none; 1, weak; 2, moderate; 3, strong) with the multiplication of the values of the two parameters.

#### Flow cytometry

Cells were fixed using 70% ethanol and stained with propidium iodide (Sigma-Aldrich Chemicals) for cell cycle analysis. The prepared cells were analyzed by flow cytometry using BD FACS Calibur flow cytometer (BD Biosciences) and CellQuest Pro software (BD Biosciences).

#### Statistics

Comparative data were analyzed using the Mann-Whitney *U*-test, chi-square test, and the Spearman's correlation coefficient, whichever was appropriate. The cutoff for statistical significance was set as  $p < 0.05$ .

## Results

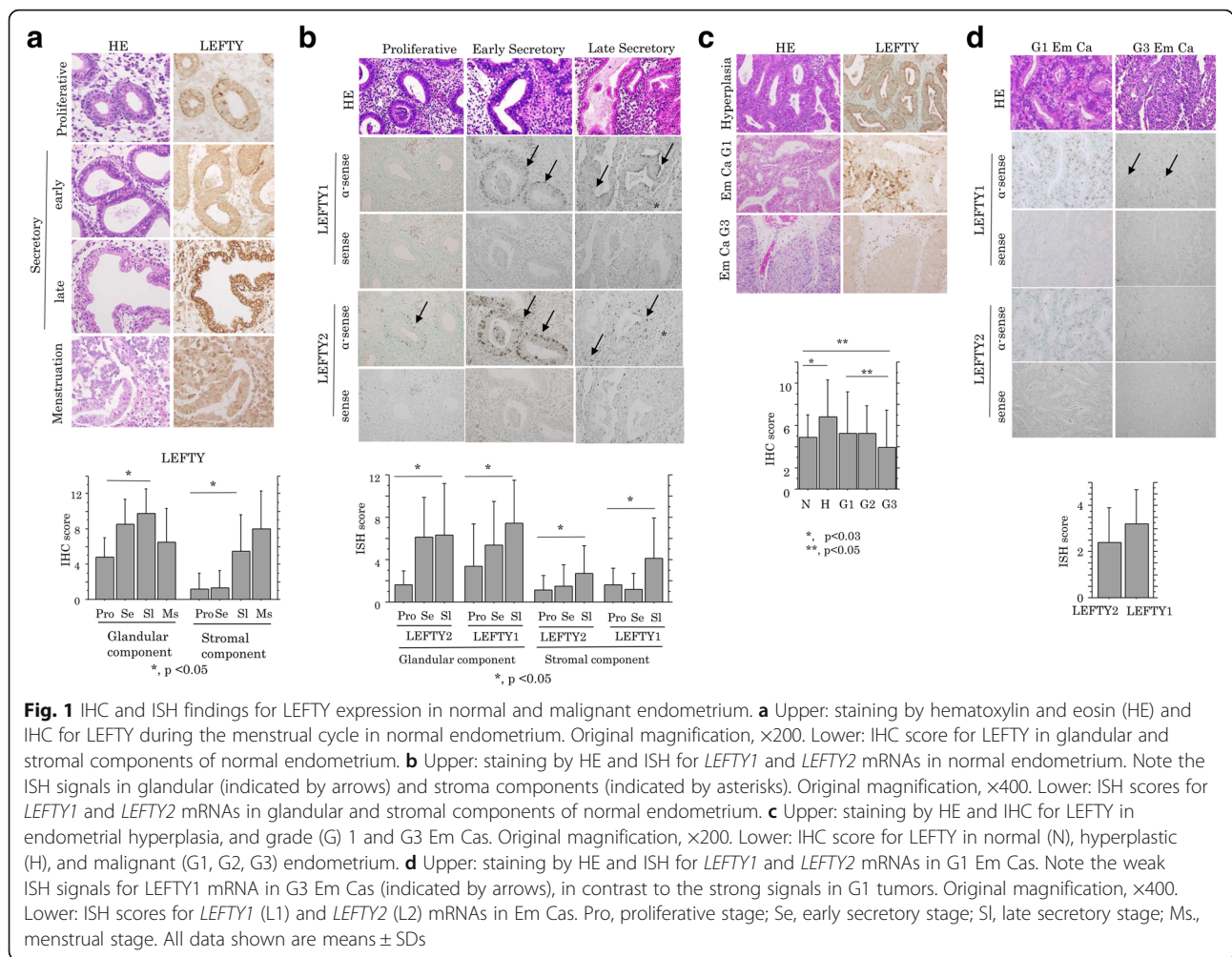
### LEFTY1/2 expression in normal and malignant endometrium

Representative images of IHC findings for LEFTY during the menstrual cycle in normal endometrium are illustrated in Fig. 1a (upper panels). The anti-LEFTY antibody used in this study was able to react with both LEFTY1/2 as described previously [32]. Cytoplasmic LEFTY immunoreactivity was frequently observed in both glandular and stromal components. Average LEFTY score showed significant stepwise increase from the proliferative phase to late secretory or menstrual stage in both components (Fig. 1A, lower graph). Both *LEFTY1* and *LEFTY2* mRNA expression also showed similar cyclic changes, with no difference in the expression values between the two (Fig. 1b and Additional file 2: Figure S1A). In contrast, there were no differences in pSmad2 scores during the menstrual cycle (Additional file 2: Figure S1B).

Representative images of LEFTY immunoreaction in hyperplastic and malignant endometrium are also illustrated in Fig. 1c (upper panels). Average LEFTY score was significantly higher in hyperplastic lesions as compared to normal endometrium and showed significant decrease from hyperplasia through G1 to G3 Em Cas. The score was also significantly lower in G3 tumors as compared to normal lesions, but such associations were not evident between normal and either G1 or G2 tumor tissues (Fig. 1c, lower graph). No difference in the mRNA expression levels between *LEFTY1* and *LEFTY2* was also observed in Em Cas (Fig. 1d and Additional file 3: Figure S2A). Clinicopathologically, LEFTY score was significantly associated with advanced clinical stages and deep myometrial invasion, but not lymph node status (Additional file 3: Figure S2B).

Because active demethylation is essential for regulating a subset of TGF- $\beta$ 1-dependent genes [35], we further investigated changes in the CpG island status of the *LEFTY1* gene by sodium bisulfate sequencing. Although *LEFTY1*





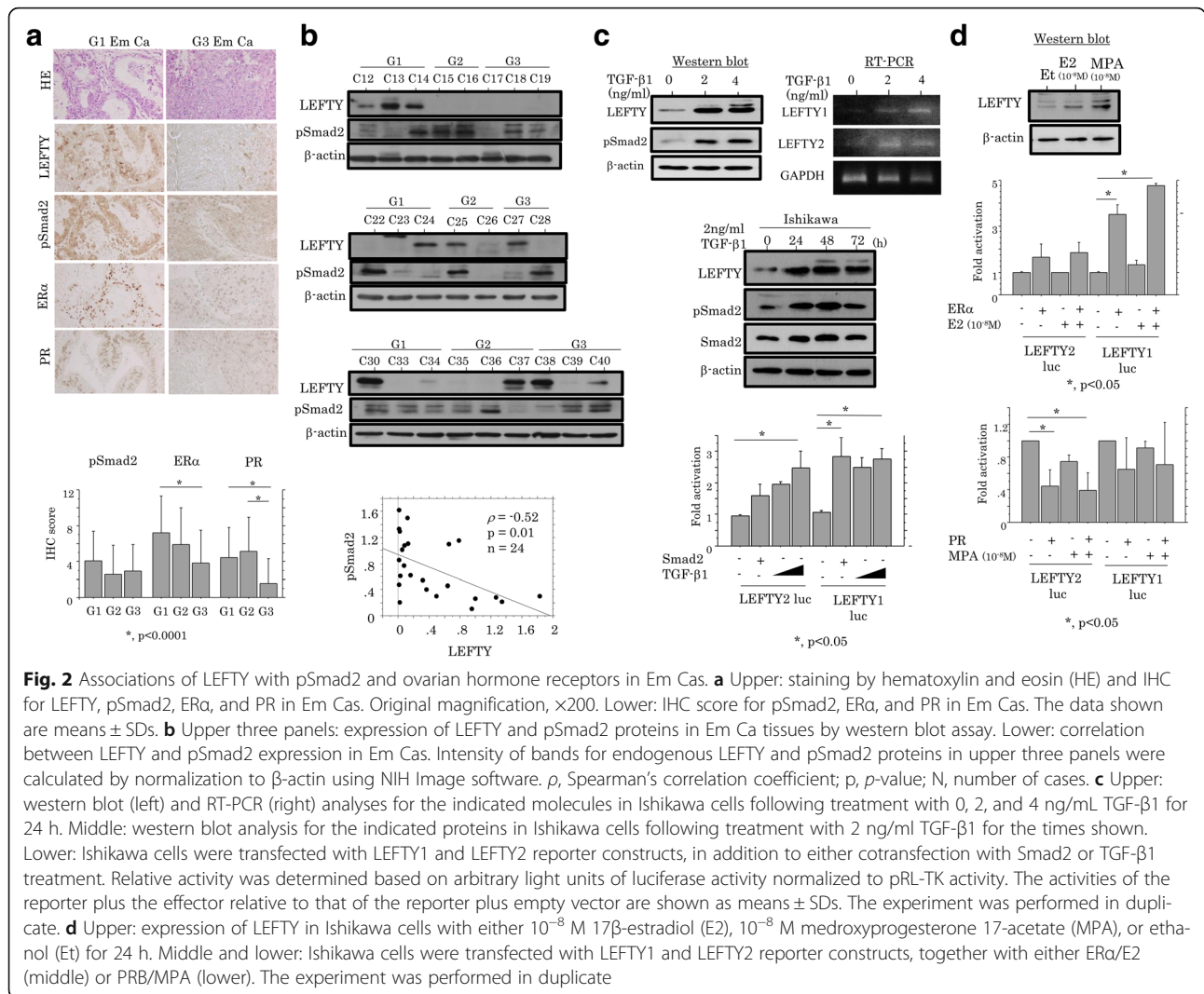
contained a region of CpG islands that was highly methylated in both Em Ca and normal tissues, the methylation status was not associated with LEFTY mRNA and protein expression (Additional file 4: Figure S3). These findings suggest that LEFTY1/2 were frequently expressed in normal and malignant endometrial tissues, independent of the gene methylation status.

#### Associations of LEFTY with pSmad2 and ovarian hormone receptors in Em Cas

Based on the above findings, we first investigated associations of LEFTY with the TGF- $\beta$ /pSmad2 pathway and ovarian hormone receptor status in Em Ca tissues. Representative images of immunohistochemistry for LEFTY, pSmad2, ER $\alpha$ , and PR in Em Ca tissues are illustrated in Fig. 2a (upper panels). Nuclear/cytoplasmic immunoreactivity for pSmad2 was observed in Em Cas, independent of the histological malignancy, while distinct nuclear stainings for both ER $\alpha$  and PR showed significant stepwise decrease from G1 to G3 tumors (Fig. 2a, lower graph). The LEFTY score was positively correlated with ER $\alpha$ ,

but not PR and pSmad2, score (Table 2). In contrast, western blot analysis revealed an inverse association between LEFTY and pSmad2 expression (Fig. 2b and Additional file 5: Table S2).

In Ishikawa cells, which express both ER $\alpha$  and PR (Additional file 6: Figure S4A), treatment with TGF- $\beta$ 1 resulted in increased LEFTY expression at both mRNA and protein levels, along with increased pSmad2 expression (Fig. 2c, upper panels). A time-course analysis of TGF- $\beta$ 1 treatment revealed that both pSmad2 and LEFTY expression increased within 48 h (h), and the former returned to basal levels after 72 h, in contrast to the continuous high expression of the latter (Fig. 2c, middle panels). Both *LEFTY1* and *LEFTY2* promoters were activated by either Smad2 transfection or TGF- $\beta$ 1 treatment (Fig. 2c, lower graph). LEFTY expression was also increased by either E2 or MPA treatment (Fig. 2d, upper panels). Both *LEFTY* promoters were activated by ER $\alpha$  transfection with or without E2 treatment, although we could not find any putative ER $\alpha$ -binding elements within both promoter regions (Fig. 2d, middle graph). In contrast, the promoter activities



**Table 2** Correlations among IHC markers investigated in endometrial carcinomas

	LEFTY	pSmad2	ER $\alpha$	PR	Cyclin A2
	$\rho$ (p)	$\rho$ (p)	$\rho$ (p)	$\rho$ (p)	$\rho$ (p)
pSmad2	0.14 (0.15)	* (0.15)	* (0.15)	* (0.15)	* (0.15)
ER $\alpha$	0.37 ( $<0.0001$ )	0.12 (0.2)	* (0.2)	* (0.2)	* (0.2)
PR	0.3 (0.002)	0.16 (0.08)	0.61 ( $<0.0001$ )	* (0.0001)	* (0.0001)
Cyclin A2	-0.4 ( $<0.0001$ )	-0.19 (0.05)	-0.4 ( $<0.0001$ )	-0.44 ( $<0.0001$ )	* ( $<0.0001$ )
Ki-67	-0.37 ( $<0.0001$ )	-0.24 (0.01)	-0.25 (0.0009)	-0.22 (0.18)	0.75 ( $<0.0001$ )

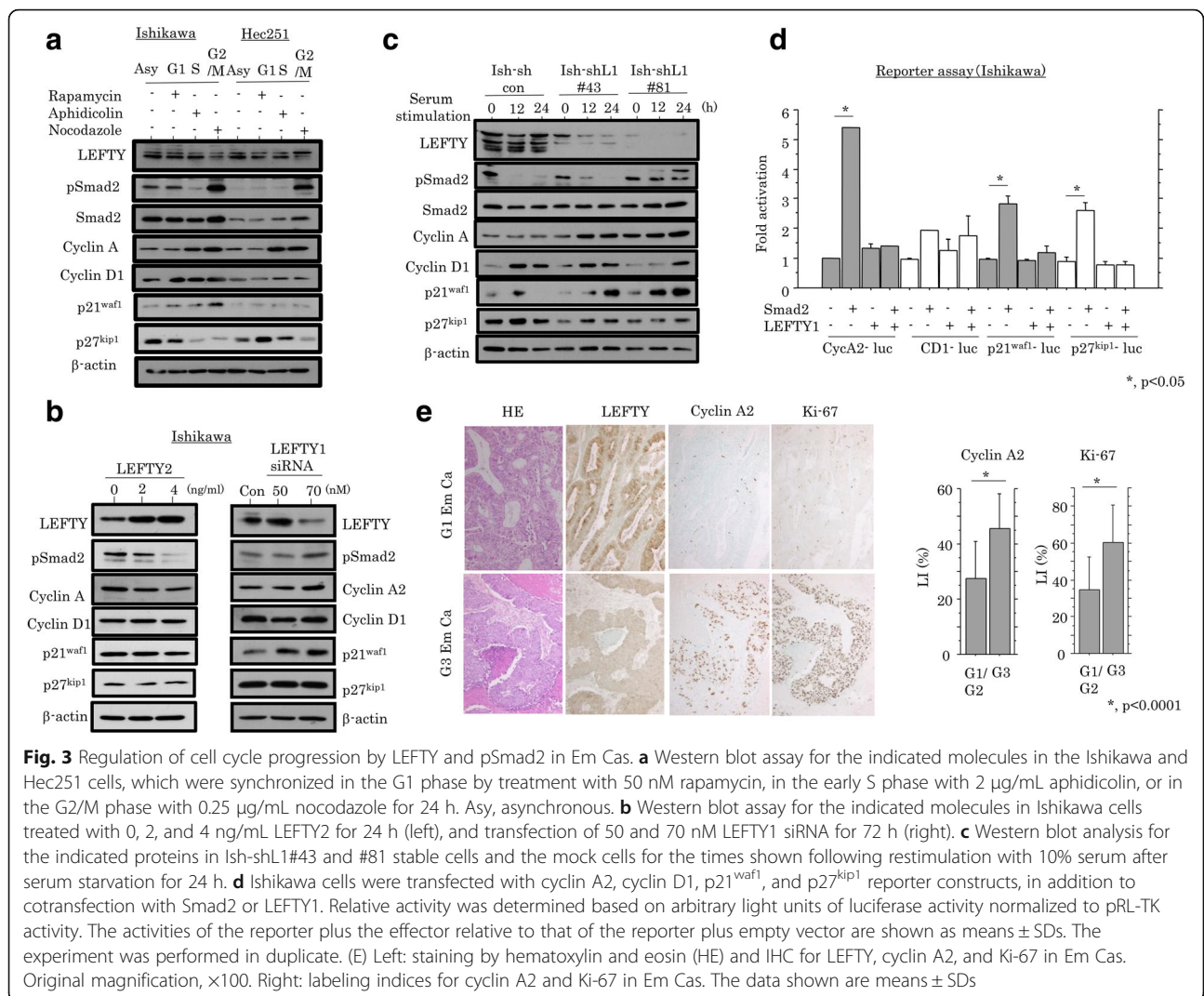
$\rho$  Spearman's correlation coefficient, IHC immunohistochemistry, \*, not examined

were suppressed by transfection of PRB, independent of MPA status (Fig. 2d, lower graph).

**Regulation of cell cycle progression by LEFTY in Em ca cells**

Since LEFTY2 can inhibit cell proliferation and suppress growth in some tumors [36], we examined an association between LEFTY expression and cell cycle progression using Ishikawa and Hec251 cells. In synchronized cells (Additional file 7: Figure S5A), LEFTY and p27<sup>kip1</sup> expression was substantially decreased in G2/M-arrested cells, in contrast to increased expression of pSmad2, cyclin A2, and p21<sup>waf1</sup> (Fig. 3a). Treatment of Ishikawa cells with recombinant LEFTY2 led to decreased expression of pSmad2, cyclin A2, and p21<sup>waf1</sup>, while LEFTY1 knockdown by siRNA resulted in increased expression of pSmad2, cyclin A2, and p21<sup>waf1</sup> (Fig. 3b). To further examine an association between LEFTY1 and alterations in expression of several cell cycle-related molecules during cell growth, we established two independent

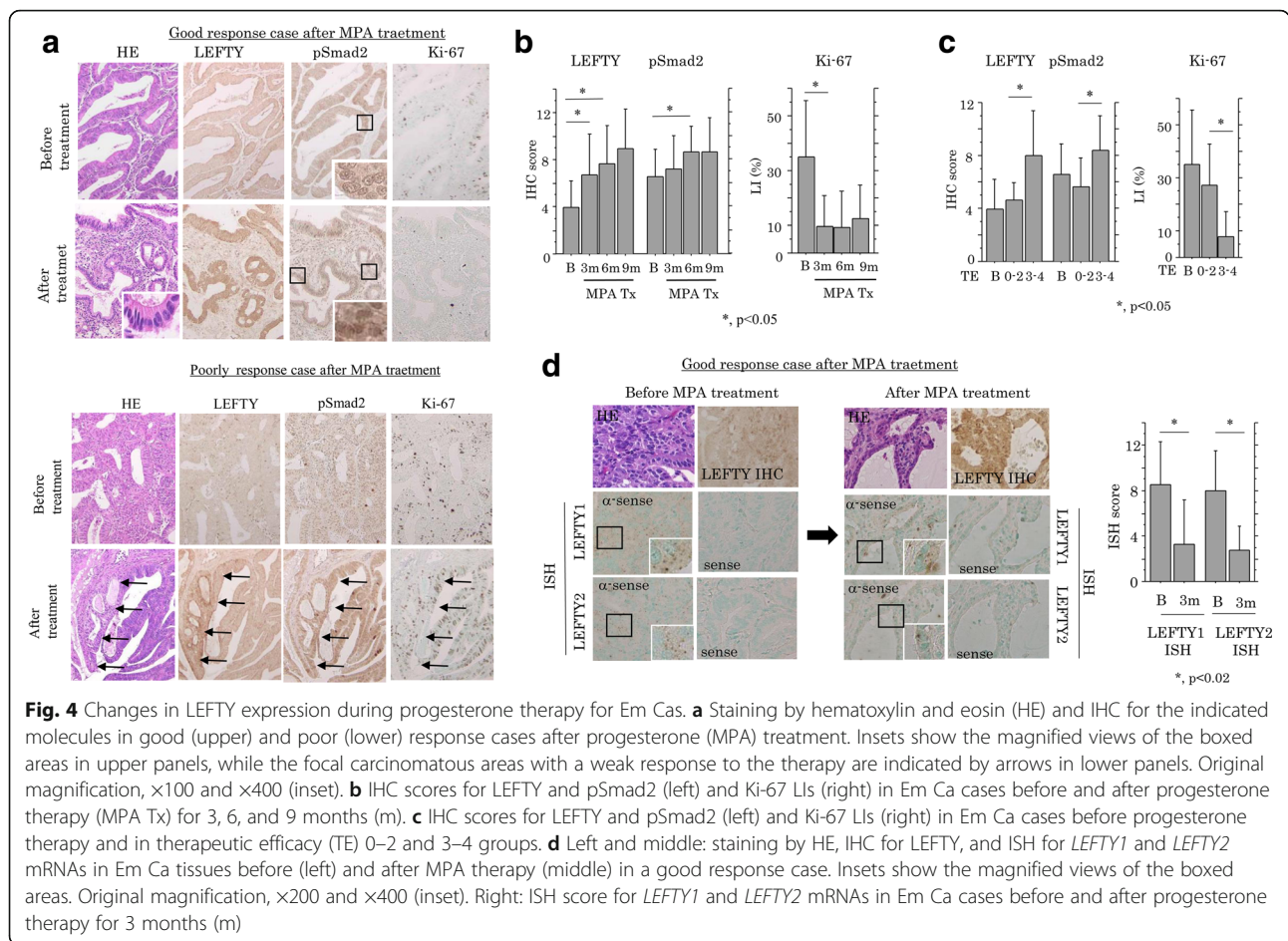




Ishikawa cell lines with LEFTY expression blocked by a LEFTY1 specific shRNA. The cell lines were rendered quiescent by serum starvation and were subsequently stimulated with serum. At 12 and 24 h after release in the cell cycle, expression levels of pSmad2, cyclin A2, and p21<sup>waf1</sup> were substantially increased relative to the mock cells, in contrast to the decreased p27<sup>kip1</sup> expression (Fig. 3c). Transfection with Smad2 led to increased promoter activity of *cyclin A2*, as well as *p21waf1* and *p27kip1* genes, while these effects were abrogated by cotransfection with LEFTY1 (Fig. 3d, Additional file 7: Figure S5B). In clinical samples, both cyclin A2 and Ki-67 LI values were significantly higher in G3 Em Cas as compared to those of G1 tumors (Fig. 3e), in addition to an inverse correlation to LEFTY score (Table 2). These findings suggest that LEFTY participates in modulation of cell cycle progression through suppression of pSmad2-dependent transcription of the genes including *cyclin A2* in Em Cas.

### Changes in LEFTY expression during progesterone therapy for Em Cas

Since our data showed that LEFTY can be upregulated by E2 and MPA, we examined the impact of progesterone therapy on LEFTY expression using clinical Em Ca samples from patients receiving the therapy (Table 1). In the good response group (TE ≥ 3), tumor cells showed marked changes in their morphological appearance, often becoming closer in resemblance to normal endometrial glandular components in the secretory phase, in contrast to the poor response group (TE ≤ 2) (Fig. 4a). Average IHC scores for LEFTY and pSmad2 showed stepwise increases during progesterone therapy in Em Ca patients, which were significantly correlated with TE grading, while Ki-67 LIs were significantly decreased (Fig. 4b and c). In 10 cases selected from the good response group, *LEFTY1/2* mRNA expression was significantly decreased during the therapy, in contrast to the



increased protein expression (Fig. 4d). In Ishikawa cells treated with MPA, there was a tendency towards a lower proliferation rate, along with increased expression of LEFTY and pSmad2, as well as p21<sup>waf1</sup> and p27<sup>kip1</sup> (Additional file 8: Figure S6). These findings suggest that upregulation of LEFTY and pSmad2 occurred during progesterone therapy in Em Cas, in line with changes in the morphology toward secretory features and lower cell proliferation.

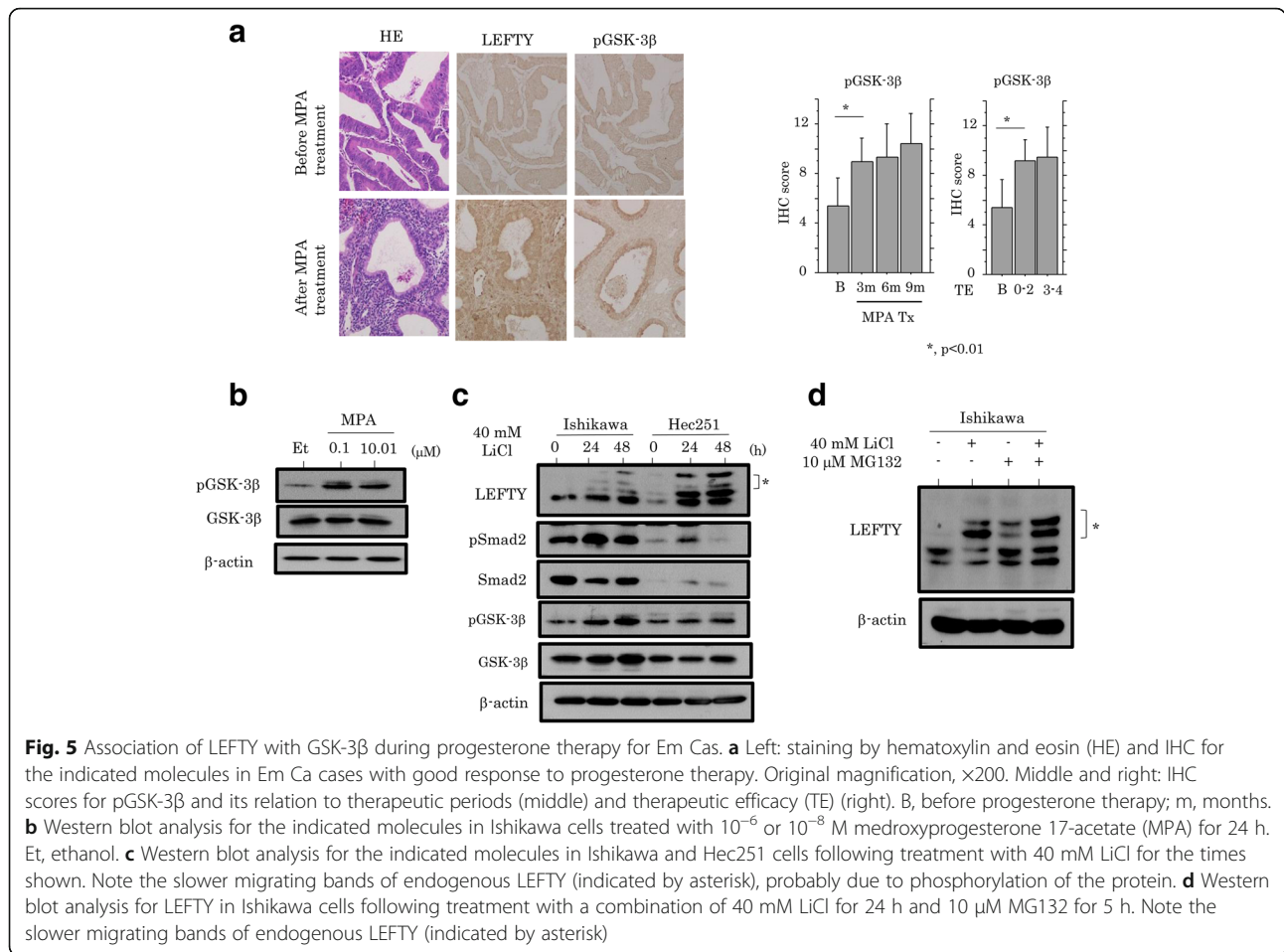
#### Association between LEFTY and GSK-3 $\beta$ during progesterone therapy for Em Cas

Since GSK-3 $\beta$  is a major target of ovarian steroid hormones [37], we investigated changes in GSK-3 $\beta$  expression during progesterone therapy in Em Ca patients. The inactive pGSK-3 $\beta$  score was significantly increased during the therapy, independent of TE grading (Fig. 5a), in line with the findings from Ishikawa cells treated with MPA (Fig. 5b). Treatment of Ishikawa and Hec251 cells with LiCl, an inhibitor of GSK-3 $\beta$  revealed slower migrating bands of endogenous LEFTY, probably due to phosphorylation of the protein (Fig. 5c). To further examine whether LEFTY is tightly regulated by the

ubiquitin-proteasome pathway, Ishikawa cells were also treated with a combination of LiCl and proteasome inhibitor, MG132. As shown in Fig. 5d, similar slow migration of LEFTY was also observed by treatment of MG132 with or without LiCl compared with those without the pretreatment.

Next, we noticed that LEFTY contains five putative GSK-3 $\beta$  phosphorylation motifs in the LEFTY1/2 protein (Fig. 6a). To examine whether LEFTY can be phosphorylated by GSK-3 $\beta$  and rapidly degraded through the ubiquitin-proteasome pathway, ES-2 cells stably overexpressing LEFTY1 were treated with LiCl and MG132, and LEFTY was immunoprecipitated. As shown in Fig. 6b, the level of ubiquitinated LEFTY1 was decreased by inhibition of GSK-3 $\beta$  activity following treatment with both LiCl and MG132, as compared to the treatment with MG132 alone. Changes in LEFTY expression by LiCl treatment were abrogated by mutations in the putative GSK-3 $\beta$  phosphorylation motifs (5SA) in the protein (Fig. 6c).

Finally, treatment of Ishikawa and Hec251 cells with TGF- $\beta$ 1 resulted in increased expression of pGSK-3 $\beta$ , as well as pAkt (Additional file 9: Figure S7). These findings



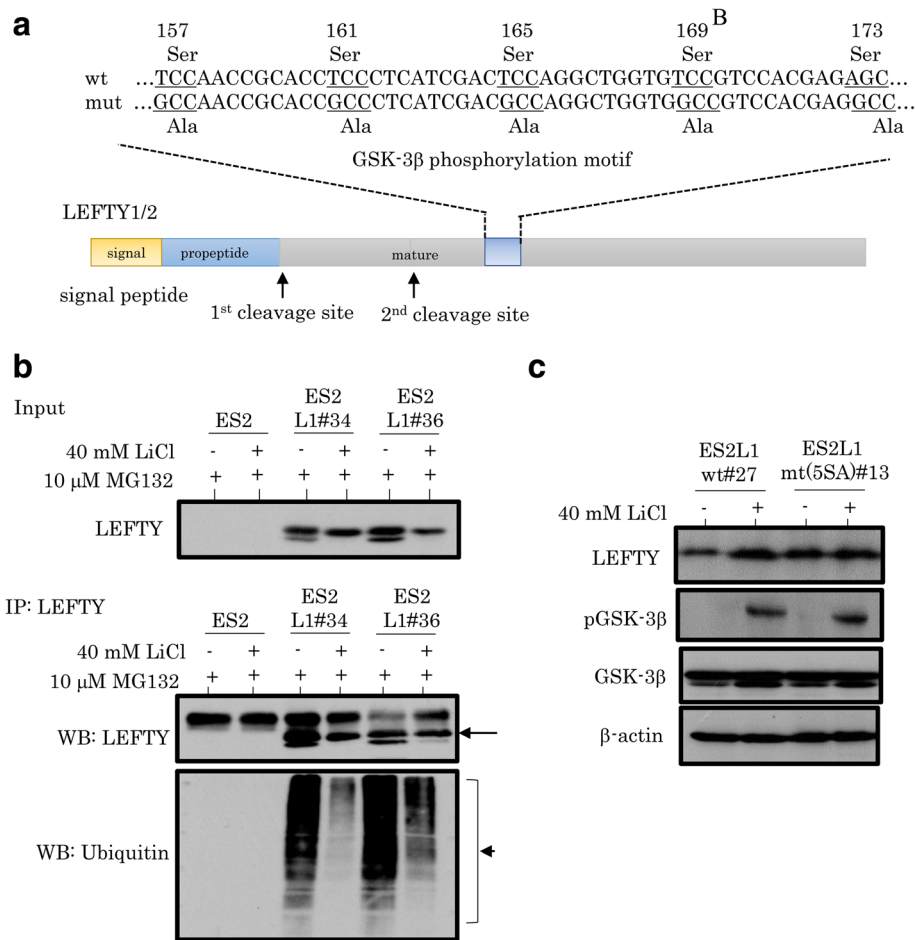
suggest that the LEFTY protein is also posttranslationally regulated through alteration in GSK-3β activity in response to progesterone and TGF-β1.

### Discussion

The present study clearly provided evidence that LEFTY1/2 mRNA as well as protein showed a cyclic expression in both glandular and stromal components in normal endometrium, demonstrating that the expression was significantly higher in late secretory and menstrual stages. In contrast, it has been reported that the mRNA expression of LEFTY2/ebaf occurs primarily in stromal, but not epithelial, cells that have been predicedualized, indicating that it is a member of the premenstrual/menstrual molecular repertoire in human endometrium [19, 20]. One possible reason for the discrepant results may be the difference in sensitivity of mRNA detection. Given our previous study showing that LEFTY has biological effects in altering cell proliferation and cellular susceptibility to apoptosis in ovarian clear cell carcinoma [32], it appears that the molecule may also play an important role in the establishment and maintenance of homeostasis in normal endometrium

through modulation of the terminal cell differentiation and the cell kinetics.

Although it has been reported that epigenetic factors participate in a mechanism underlying the regulation of LEFTY expression mediated by TGF-β1 [33], we found no association between LEFTY1 gene methylation and its protein expression in normal and malignant endometrial tissues. In contrast, LEFTY1/2 genes were transcriptionally upregulated by TGF-β1/Smad2 signaling in Ishikawa cells, while western blot analysis using Em Ca tissues revealed an inverse relation between LEFTY and pSmad2 expression. In addition, TGF-β1 treatment induced an increase in both pSmad2 and LEFTY expression within 48 h, and the former returned to basal levels after 72 h, in contrast to the continuous high expression of the latter. These findings allow us to speculate the existence of negative feedback loops among LEFTY, TGF-β, and Smad2, since LEFTY exerts significant inhibition of receptor-regulated Smad phosphorylation by TGF-β [18]. Moreover, TGF-β action has been demonstrated to be tightly controlled by a number of negative feedback mechanisms [38–40].



**Fig. 6** LEFTY is a GSK-3β substrate. **a** Structure of LEFTY1/2 protein showing the positions of five putative GSK-3β phosphorylation motifs. The corresponding constructs that were used in this study are also shown. wt, wild-type; mut, mutant type. **b** After treatment of ES-2 cells stably overexpressing LEFTY1 with 10 μM MG132 for 5 h with or without 40 mM LiCl for 24 h (upper: input), LEFTY (indicated by arrow) was immunoprecipitated (middle), and then ubiquitination of LEFTY (indicated by arrowhead) was examined (lower). **c** Western blot analysis for the indicated proteins after treatment of ES-2 cells stably overexpressing LEFTY1wt-HA or LEFTY1 mt (5SA)-HA with 40 mM LiCl for 24 h

Interestingly, we found that LEFTY protein can be posttranslationally regulated by progesterone through alteration in GSK-3β activity in Em Cas from the following evidence: i) LEFTY protein contains five putative GSK-3β phosphorylation motifs and can be degraded through the ubiquitin-proteasome pathway; ii) inhibition of GSK-3β by LiCl resulted in increased expression of LEFTY, while the effects were abrogated by mutations in the five putative GSK-3β phosphorylation motifs in the protein; iii) pGSK-3β score was significantly increased during progesterone therapy in Em Ca tissues, in line with the findings demonstrating an increase in pGSK-3β expression by progesterone in Ishikawa cells; and iv) *LEFTY1/2* mRNA expression levels were significantly decreased during the therapy, probably through transcriptional repression of the promoter activities by progesterone signals, despite the increased LEFTY protein expression.

We also found that LEFTY was positively associated with ERα status in Em Ca tissues and *LEFTY1/2* expression was transcriptionally regulated by estrogen and ERα in Ishikawa cells. In addition, LEFTY expression was significantly higher in hyperplastic endometrial tissues as compared to those of both normal and malignant lesions, in line with the notion that endometrial hyperplasia is induced by continuous exposure to estrogen unopposed by progesterone [41]. However, it seems likely that the up-regulation may be indirectly affected by estrogen-related signaling, since both *LEFTY1/2* promoter regions lack any putative ERα-binding elements. Whatever the case, these findings suggest that ovarian hormones may be major regulators of LEFTY expression at transcriptional and post-translational levels in Em Cas.

Several lines of evidence from the present study support the conclusion that LEFTY expression is associated with lower cell proliferation in Em Cas, probably through

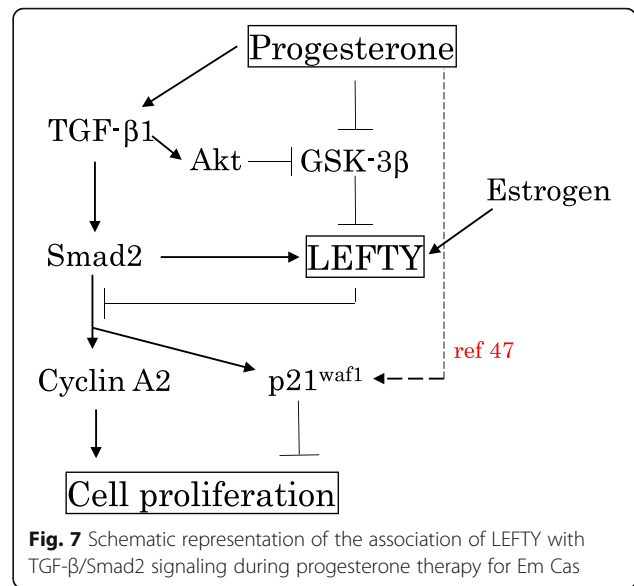


suppression of Smad2-dependent cyclin A2 expression. First, the concordant upregulation of pSmad2 and cyclin A2 expression in the G2/M phase was correlated with decreased LEFTY expression during cell cycle progression. Second, overexpression of LEFTY2 or knock-down of LEFTY1 resulted in decreased or increased expression, respectively, of both pSmad2 and cyclin A2. Given that LEFTY1/2 are both 366 amino acids long, and they are 97% identical and share 350 identical residues [16, 17], it seems likely that both LEFTY1/2 may have similar effects on cell cycle progression. Further studies to investigate this are clearly warranted. Third, LEFTY inhibited Smad2-mediated promoter activation of *cyclin A2*. Finally, LEFTY score was negatively correlated with both cyclin A2 and Ki-67 IIs in Em Cas. Together with our results that demonstrated high LEFTY expression in Em Cas of advanced stages and with deep myometrial invasion, it appears that LEFTY may have anti-tumor effects through suppression of aggressive tumor growth by blocking TGF- $\beta$ /Smad2 action in Em Cas. This conclusion is supported by the evidence showing a promotion of late-stage tumor progression, invasion, and metastasis by TGF- $\beta$  and a reduction of tumor growth in nude mice by introduction of LEFTY to tumor cells [42–45].

An important finding in this study was that LEFTY expression was significantly increased during progesterone therapy for Em Cas, in line with changes in the morphology toward secretory features and the inhibition of cell proliferation. The pSmad2 score also showed a significant increase during the therapy, despite increased LEFTY expression. This may be due to an increase in secretion of TGF- $\beta$ 1 by progesterone [46], which in turn resulted in further activation of TGF- $\beta$ 1/Smad2-dependent transcription of *LEFTY1/2* genes. Moreover, treatment of Ishikawa cells with TGF- $\beta$  induced an increase in signaling through the pAkt/pGSK-3 $\beta$  axis, which likely reflected LEFTY stability, indicating the existence of complex regulatory mechanisms for LEFTY expression during progesterone therapy in Em Cas.

### Conclusion

Our observations suggest some novel functional roles of LEFTY in Em Ca cases receiving progesterone therapy (Fig. 7). Prolonged progesterone therapy induces upregulation of LEFTY through TGF- $\beta$ -dependent or -independent GSK-3 $\beta$  inactivation. Estrogenic stimulation may be also due to transcriptional upregulation of LEFTY. The increased LEFTY expression leads to inhibition of cell proliferation, probably through changes in Smad2-mediated transcription of genes including *cyclin A2*, in line with changes in their morphological appearances toward secretory features. Activation of



other signal pathways such as the  $\beta$ -catenin/p53/p21<sup>waf1</sup> axis in response to progesterone therapy also contributes to the suppression of cell cycle progression [47]. Thus, LEFTY may serve as a useful clinical marker for the therapeutic effects of progesterone for Em Cas.

### Additional files

**Additional file 1: Table S1.** Primer sequences used in this study. (XLSX 10 kb)

**Additional file 2: Figure S1.** Expression of LEFTY1/2 mRNA and pSmad2 protein in normal endometrium. (TIFF 4603 kb)

**Additional file 3: Figure S2.** LEFTY1/2 mRNA expression and associations of LEFTY protein expression with clinicopathological factors in endometrial carcinomas. (TIFF 1138 kb)

**Additional file 4: Figure S3.** Relationship between *LEFTY1* methylation and its expression levels in normal and malignant endometrial tissues. (TIFF 3843 kb)

**Additional file 5: Table S2.** Relationship between LEFTY and pSmad2 expression as detected by western blot assay in endometrial carcinomas. (XLSX 9 kb)

**Additional file 6: Figure S4.** mRNA expression of ovarian hormone receptors in endometrial carcinoma cell lines. (TIFF 1467 kb)

**Additional file 7: Figure S5.** Cell cycle analysis and reporter assay for several cell cycle-related genes. (TIFF 786 kb)

**Additional file 8: Figure S6.** Changes in cell growth and expression of cell cycle-related molecules in Ishikawa cells in response to MPA treatment. (TIFF 1007 kb)

**Additional file 9: Figure S7.** Association between TGF- $\beta$  and Akt/GSK-3 $\beta$  pathways in endometrial carcinoma cells. (TIFF 1083 kb)

### Abbreviations

E2: 17 $\beta$ -estradiol; Em Ca: endometrial carcinoma; ER $\alpha$ : estrogen receptor- $\alpha$ ; GSK-3 $\beta$ : glycogen synthase kinase-3 $\beta$ ; IHC: immunohistochemistry; ISH: in situ hybridization; LEFTY: left-right determination factor; LiCl: lithium chloride; MPA: medroxyprogesterone 17-acetate; PR: progesterone receptor; PRB: progesterone receptor B; TGF- $\beta$ 1: transforming growth factor- $\beta$ 1



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**Availability of data and materials**

Data and materials will be shared.

**Authors' contributions**

WF, DK, MH and MS carried out the majority of the experiments, analyzed the data, and wrote the manuscript. They were helped by SK, TM, MH, YO. All authors reviewed and approved the final manuscript.

**Ethics approval and consent to participate**

Approval for this study was given by the Ethics Committee of the Kitasato University School of Medicine (B17–168).

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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