—Original Article—

Runx3 Expression and Its Roles in Mouse Endometrial Cells

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Abstract. Runx3 is a transcription factor that belongs to the Runx family. We studied the localization of *Runx3* mRNA in the mouse uterus, and its function in the mouse endometrium using Runx3 knockout (*Runx3*^{-/-}) mice. *Runx3* mRNA was detected in the endometrial luminal epithelial cells, glandular epithelial cells and stromal cells below the epithelial cell layer on the luminal side. The uteri of *Runx3*^{-/-} mice were smaller than those of wt mice. The endometrial layer and uterine glands of *Runx3*^{-/-} mice were less developed than those of wild-type mice, and the endometrial stromal layer was thinner. Transforming growth factor β 1 and β 3 (TGF β 1 and β 3) mRNA levels in endometrial stromal cells of *Runx3*^{-/-} mice were low compared with those of wild-type mice. Estradiol-17 β (E2) increased *Tgfb2* mRNA levels in endometrial stromal cells of *Runx3*^{-/-} mice, but not in those of wild-type mice. E2 increased epidermal growth factor (EGF) mRNA levels in endometrial stromal cells of wild-type mice, but did not increase those of *Runx3*^{-/-} mice. The diminished *Tgfb1* and *Tgfb3* mRNA expressions may lead to the reduced proliferation of endometrial stromal cells. Alterations of E2-associated expressions of *Tgfb2* and *Egf* mRNA in endometrial stromal cells of *Runx3*^{-/-} mice may be associated with suppression of E2-dependent endometrial epithelial cell proliferation in *Runx3*^{-/-} mice. Thus, Runx3 is likely to be a regulatory factor responsible for endometrial growth.

Key words: Estrogen, Growth factor, Mouse, Runx3, Uterus

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The mammalian protein runt-related transcription factor 3 (Runx3) belongs to a family of runt domain transcription factors and is also called the polyomavirus enhancer binding protein 2 (PEBP2)/core binding factor (CBF). PEBP2 is heterodimeric complex composed of α and β subunits [1]. The α subunit-encoding gene is homologous to the *Drosophila* gene *runt*, and the encoded protein contains a conserved Runt domain, which is required for dimerization with the β subunit and binds with the consensus DNA sequence. Three runt-related genes, *Runx1*, *Runx2* and *Runx3*, have been identified in mammals. These Runx members interact with Smad2 or Smad3, and are involved in transforming growth factor β (TGF β) superfamily signaling [2].

Endometrial epithelial cell proliferation is induced by estradiol-17 β (E2), while proliferation of endometrial stromal cells is induced by the combination of estrogen and progesterone [3]. The uterus of *Runx3*^{-/-} mice is atrophic, and the endometrial layer is less developed than that of wild-type (wt) mice. E2-induced endometrial epithelial cell proliferation is not detected in *Runx3*^{-/-} mice [4]. Therefore, Runx3 may be involved in the regulation of E2-induced endometrial cell proliferation. Endometrial cell proliferation is regulated by growth factors produced in the uterus [5–7]. Insulin-like growth factor-1 (IGF1), and epidermal growth factor (EGF) family member, EGF and transforming growth factor- α (TGF α), are well known to stimulate proliferation of endometrial epithelial and stromal cells

[8–13]. TGF β family members are also involved in the regulation of proliferation of endometrial cells [6, 14]. It is probable that Runx3 may play roles in the regulation of growth factor production.

In the present study, we aimed to know *Runx3* mRNA expression in the mouse uterus, and the mechanism by which E2-induced endometrial cell proliferation does not occur in *Runx3*^{-/-} mice. We analyzed *Runx3* mRNA expression in uteri by *in situ* hybridization. To investigate endometrial proliferation in *Runx3*^{-/-} mice, we used a primary endometrial epithelial and stromal cell culture system. We studied the mRNA expressions of the estrogen receptor α (ER α) gene (*Esr1*) and growth factors regulating endometrial cell proliferation (IGF1, EGF, TGF α , TGF β 1, TGF β 2 and TGF β 3).

Materials and Methods

Animals

Runx3 knockout (*Runx3*^{-/-}) mice on a BALB/c genetic background were generated as described previously [15]. The animals were kept in a temperature-controlled animal room, and were fed CA-1 (CLEA Japan, Tokyo, Japan), and tap water *ad libitum*. All animal care and experimental procedures were performed in accordance with Guidelines for the Animal Experimentation of Okayama University, Japan.

Genotyping

Heterozygous mice (*Runx3*^{+/-}) were intercrossed. The offsprings were genotyped as described previously [16]. Polymerase chain reaction (PCR) was performed using Blend Taq (Toyobo, Osaka, Japan) and the following primers: 1) 5'-GAC TGT GCA TGC ACC TTT CAC CAA-3', 2) 5'-ATGAAACGCCGAGTTAACGCCATCA-3'

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and 3) 5'-TAGGGCTCAGTAGCACTTACGTCG-3'. The conditions comprised a denaturation step at 95 C for 5 min; 12 cycles at 95 C for 35 sec, 58 C for 30 sec, 72 C for 45 sec; 25 cycles at 95 C for 35 sec, 64 C for 45 sec, 72 C for 45 sec; and then a final extension step at 72 C for 10 min. PCR products were electrophoresed on 2.0% agarose gels, and stained with ethidium bromide. The presence of a 170-base pair (bp) band indicated that the mice were wt. The presence of a 550-bp fragment indicated that the mice were homozygous for the *Runx3* deletion, whereas the presence of both the 170- and 550-bp bands indicated that the mice were heterozygous for the *Runx3* deletion.

Endometrial epithelial and stromal cell isolation

Endometrial epithelial and stromal cells were isolated from 3-week-old wt mice and 9-week-old *Runx3*^{-/-} mice for culture, and from 8 week-old wt and *Runx3*^{-/-} mice for RNA extraction according to a previously described method of Ross *et al.* [17]. Briefly, the uteri were dissected, and the uterine horns were longitudinally cut to expose the endometrial luminal surface. The tissue fragments were treated with 0.1% trypsin (Sigma-Aldrich, St. Louis, MO, USA) at 4 C for 1 h and then at 37 C for 55 min. Trypsin was inactivated by 0.1% soybean trypsin inhibitor (STI, Sigma-Aldrich) at 37 C for 10 min. The epithelial tissues were separated from the stromal tissues with forceps under a stereoscopic microscope. The collected epithelial tissue fragments were further divided into smaller fragments by gentle pipetting. The endometrial stromal and myometrial tissues separated from the epithelial tissues were incubated with 0.1% trypsin and 1 mM EDTA at 37 C for 1 h. Then they were treated with 0.1% STI at 37 C for 10 min. After gentle pipetting and DNase I (Sigma-Aldrich) treatment, the stromal cells separated from the myometrium were collected by centrifugation. The cell viability was assessed by the trypan blue dye exclusion test (usually more than 95% in each study). The purities of the epithelial and stromal cells were more than 90%, as verified by our previously described method [8, 13]. All chemicals were dissolved in Ca²⁺- and Mg²⁺-free Hanks' solution containing 20 mM HEPES and 0.3% bovine serum albumin (BSA, Sigma-Aldrich).

Endometrial epithelial and stromal cell culture

The isolated endometrial epithelial and stromal cells were seeded on poly-L-lysine-coated culture wells at a density of approximately 5×10⁴ or 6×10⁴ cells/cm², respectively. Stromal cells were first cultured for 1 day in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium without phenol red (DME/F12; Sigma-Aldrich) containing 2% dextran-coated charcoal-treated fetal bovine serum for 1 day. After the 1-day pre-culture, the cells were cultured in serum-free DME/F12 supplemented with BSA (1 g/l), hydrocortisone (100 µg/l), triiodothyronine (400 ng/l), transferrin (10 mg/l), glucagon (10 ng/l), parathormone (200 ng/l), sodium selenite (5 µg/l) and insulin (100 µg/l). The plates were incubated at 37 C in an atmosphere of a 5% CO₂.

E2 (Sigma-Aldrich) was initially dissolved in ethanol to yield a 10 µM stock solutions (0.03% ethanol). The stock solution was diluted with culture medium, and the final ethanol concentration was less than 0.01%.

RNA extraction and reverse transcription (RT)

Total RNA was extracted using TRIpure reagent (BIOLINE, London, UK). Five hundred nanograms of RNA were used for RT using the ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Random hexamers were used for the RT reactions.

RT-PCR

PCR was carried out in a 15-µl final volume containing the following reagents: autoclaved water up to 15 µl, 0.375 U of Blend Taq (Toyobo), 1×PCR Buffer for Blend Taq, 0.2 µM each of forward and reverse primer and 1.2 µl of 1:8 diluted cDNA. For *Runx3* RT-PCR, 0.5 µM of each primer was used. PCR reactions were performed using a Gene Amp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). The PCR conditions were 30 sec at 94 C; an appropriate number of cycles of 94 C for 30 sec, a primer-appropriate temperature for 30 sec and 72 C for 30 sec; and finally 10 min at 72 C. The primers and PCR conditions are listed in Table 1. A 10-µl aliquot of each reaction was electrophoresed on 2% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light.

Western blotting

The uteri and ovaries of 8-week-old wt mice were frozen and homogenized in cold lysis buffer (50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 0.1% Nonidet P-40, 2 mM EDTA (pH 8.0), 10% glycerol) containing 1% protease inhibitor cocktail (Sigma-Aldrich). After sonication, protein lysates were separated by centrifugation. Lysates containing 80 µg of protein were subjected to 8% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). The membranes were blocked for 1 h with 10% skim milk dissolved in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). Then the membranes were incubated sequentially with 0.2 µg/ml mouse anti-Runx3 antibody (R3-1E10; Medical & Biological Laboratories, Nagoya, Japan) diluted with phosphate-buffered saline (PBS, pH 7.2) and with EnVision+ Dual Link rabbit/mouse (1:50, Dako, Glostrup, Denmark) diluted with TBS-T containing 1% skim milk, each for 1 h at room temperature. Peroxidase activity was detected by using luminol (Sigma-Aldrich) and a hydrogen peroxide reaction. β-actin was used as an internal control, and was detected using anti-β-actin antibody (GeneTex, Irvine, CA, USA).

Riboprobes

A mouse *Runx3* riboprobe was generated from a *Runx3* fragment (NM_019732: nucleotides 1244-2344) subcloned into pBluescript II KS(-), according to a previously described method [18]. The plasmid DNA was linearized using restriction enzymes, and RNA probes were synthesized using a T3 and T7 polymerase system (Promega, Madison, WI, USA) according to the manufacturer's instructions. The probe was labeled with digoxigenin (DIG; Roche Diagnostics, Mannheim, Germany).

In situ hybridization analysis

The dissected uteri of 8-week-old wt mice were immediately embedded in O.C.T. Compound (Sakura Finetek Japan, Tokyo, Japan)

Table 1. PCR primer sequences

Target gene		Sequence	Length
<i>Runx3</i>	FP	AAGTGCCTCGATGGTGGACG	369
	RP	CAGTGACCTTGATGGCTCGGT	
<i>Krt19</i>	FP	GTGTCTGATGGGCTGCTGTCT	539
	RP	CTCAGGATCTTGGCTAGGTCG	
<i>Vim</i>	FP	GGCCGAGGAATGGTACAAGTC	320
	RP	GGGCCATCTTAACATTGAGCAG	
<i>Esr1</i>	FP	GGCTGGAGATTCTGATGATTGG	55
	RP	TCCCCGGGTGTTCCAT	
<i>Igf1</i>	FP	AAAGCAGCCCCTCTATCC	57
	RP	CTTCTGAGTCTTGGCATGTCA	
<i>Igfbp3</i>	FP	AAGCACCTACCTCCCTCCCAA	98
	RP	TGCTGGGGACAACCTGGCTTTC	
<i>Egf</i>	FP	CTACGGGCAGAAGCATGACA	97
	RP	CCTGTAGTAGTAAGTCCCCCACATC	
<i>Tgfa</i>	FP	ATCCTGTTAGCTGTGTGCCA	105
	RP	GGAATCTGGGCACTTGTGA	
<i>Tgfb1</i>	FP	CTGGAGTTGTACGGCAGTG	98
	RP	GTGGAGTTTGTATCTTTGCTG	
<i>Tgfb2</i>	FP	GCTTGCAAAACCCCAAAGC	90
	RP	GCTGGGTGGGAGATGTTAAGTCT	
<i>Tgfb3</i>	FP	GATCCATAAATTCGACATGATCCA	60
	RP	GGCAGACGGCCAGTTC	
<i>Rpl19</i>	FP	CCGCAGCCATGAGTATGCT	60
	RP	CGCAGCGGAGGACACTAGA	

and then frozen in liquid nitrogen. Sections at 10- μ m thickness were cut horizontally and stored at -80 C until use.

The sections were completely dried, processed using 0.5 μ g/ml proteinase K (Nacalai Tesque, Kyoto, Japan) at 37 C for 10 min, treated at room temperature with 4% paraformaldehyde in PBS for 10 min and 0.2% glycine in PBS for 20 min, and then acetylated with 0.15 M acetic anhydride in 0.1 M triethanolamine for 10 min. The sections were then prehybridized in 4 $\times$ SSPE, 1 $\times$ Denhardt's solution, 10% dextran sulfate, 50% deionized formamide, and yeast tRNA (50 μ g/slide) at room temperature for 30 min. The sections were then hybridized with DIG-labeled antisense or sense *Runx3* riboprobes (50 ng/slide) in prehybridization solution, and were placed in a moist chamber, overnight at 70 C.

After hybridization for 16 h, the uterine tissue slides were washed in 1 $\times$ SSC in 30% formamide at room temperature for 10 min, at 53 C for 15 min and then in 0.5 $\times$ SSC in 30% formamide at 53 C for 15 min. Next, the sections were blocked in 0.5% Blocking Reagent (Roche Diagnostics) dissolved in TBS-T at room temperature for 30 min, and then were incubated with anti-DIG-alkaline phosphatase (AP), Fab fragments (Roche Diagnostics) in blocking solution overnight at 4 C.

For color development, the slides were washed in TBS-T and AP buffer containing 0.1 M NaCl, 0.05 M MgCl₂, 0.1 M Tris-HCl (pH9.5), 0.1% Tween-20, 2 mM (-)-tetramisole hydrochloride (Sigma-Aldrich). Hybridization signals were detected in AP buffer containing 35 μ g/ml nitro blue tetrazolium chloride (Wako Pure Chemical Industries, Osaka, Japan) and 17.5 μ g/ml 5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt (Wako Pure Chemical Industries).

Morphological observation

Runx3^{-/-} and wt mice were sacrificed under CO₂ anesthesia, and their uteri were fixed in Bouin's fixative, dehydrated and embedded in paraffin. Seven-micrometer-thick sections were cut and stained with hematoxylin and eosin.

Quantitative real-time RT-PCR analysis

Quantitative real-time PCR was conducted in a 15- μ l final volume containing the following reagents: autoclaved water up to 15 μ l, 1 $\times$ SYBR Premix Ex Taq (Takara Bio, Otsu, Japan), 0.4 μ M each of forward and reverse primers, 1 $\times$ ROX Reference Dye and 1.5 μ l of cDNA appropriately diluted for each gene. A 7300 Fast Real-Time PCR System (Applied Biosystems) was used for PCR with the following conditions: 10 sec at 95 C; 40 cycles of 95 C for 5 sec and 60 C for 31 sec; 95 C for 15 sec, 60 C for 1 min, 95 C for 15 sec, and 60 C for 15 sec. The primers are listed in Table 1. The relative expression levels of each sample were calculated based on the cycle threshold and growth curves. PCR amplification curves were evaluated by determining the fluorescence of the double-stranded DNA-specific dye SYBR Green in the test product vs. the amount in standardized PCR products. The expression level of each target mRNA was normalized to that of ribosomal protein L19 (*Rpl19*) mRNA.

Analysis of cell proliferation

DNA-synthesizing cells of endometrial epithelial cells were determined by bromodeoxyuridine (BrdU) incorporation using a Cell Proliferation ELISA, BrdU kit (Roche Diagnostics). Cultured uterine epithelial cells were treated with epidermal growth factor (EGF; 10 ng/ml), TGF α (10 ng/ml) and TGF β family proteins (1 ng/ml) for 12 h or IGF1 (100 ng/ml) for 24 h. Four hours prior to measurement, BrdU was added to each well to a final concentration of 10 μ M. The cells were fixed, and their DNA was denatured using the FixDenat supplied with the ELISA kit (Roche Diagnostics). Cells were incubated with anti-BrdU-POD at room temperature for 90 min. Then, the cells were washed and added to tetramethylbenzidine as a substrate. The substrate reaction was stopped by adding 1 M sulfuric acid. The absorbance of the samples was measured at a wavelength of 450 nm in a model 680 Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Data are shown as the mean $\pm$ standard error. Differences among groups were analyzed by analysis of variance followed by Tukey's test. Differences in the means between two groups were analyzed by the Student's *t*-test. Differences were considered significant at *P*<0.05.

Results

Runx3 mRNA and *Runx3* protein expressions in mouse uteri and ovaries

RT-PCR analysis of *Runx3* mRNA expression showed that *Runx3* mRNA was present in the uteri and ovaries of 8-week-old mice (Fig. 1A). *Runx3* protein expression in the mouse uteri and ovaries was detected by Western blotting with an anti-*Runx3* antibody (R3-1E10). The major band at 47 kDa corresponded to *Runx3* (calculated molecular weight 44,980) (Fig. 1B).

Fig. 1. Runx3 expressions in the uterus and ovaries from 8-week-old mice. A: RT-PCR analysis of *Runx3* mRNA expression; *Rpl19* mRNA expression was used as a control. M: molecular marker. B: Western blotting of Runx3 expression. An immunoreactive band (47 kDa) was detected using an anti-Runx3 antibody (R3-1E10). β -actin expression was used as a control. C: RT-PCR analysis of *Runx3* mRNA expression in endometrial epithelial and stromal cells isolated from the uteri of immature mice. *Krt19*: keratin 19, a marker of epithelial cells. *Vim*: vimentin, a marker of stromal cells.

Endometrial epithelial and stromal cells were isolated from the uteri of immature mice, and were confirmed by detection of keratin or vimentin mRNA expression (Fig. 1C). *Runx3* mRNA was detected in both cell types.

In situ hybridization analysis of *Runx3* mRNA expression in mouse uteri

The expression of *Runx3* mRNA in the uteri of 8-week-old mice was examined using DIG-labeled riboprobes. The uteri comprised endometrial luminal epithelial and stromal cells, endometrial glandular epithelial cells, myometrial cells, and cells of the outer layers (Fig. 2A, D). Hybridization signals from DIG-labeled antisense riboprobes were localized in endometrial luminal epithelial cells, glandular epithelial cells, and stromal cells below the epithelial cell layer on the luminal side (Fig. 2B). There was no signal from the control sense riboprobe (Fig. 2C).

Morphological observation of *Runx3*^{-/-} mouse uterus

The uteri of *Runx3*^{-/-} mice were smaller than those of wt mice. The endometrial layer and uterine glands of *Runx3*^{-/-} mice were less developed than those of wt mice (Fig. 3), and the endometrial stromal layer was thinner.

Effect of E2 on estrogen receptor and growth factor mRNA expressions in endometrial stromal cells of wt and *Runx3*^{-/-} mice

To investigate the effect of E2 on the production of growth factors in the uterus of *Runx3*^{-/-} mice, isolated endometrial stromal cells from immature mice were cultured, and treated with E2 (10^{-8} M) for 2 and 12 h. *Esr1*, *Igf1*, *Igfbp3*, *Egf*, *Tgfa*, *Tgfb1*, *Tgfb2* and *Tgfb3* mRNA levels were analyzed by quantitative real-time PCR (Fig. 4).

Esr1: *Esr1* mRNA levels in *Runx3*^{-/-} stromal cells of the control

Fig. 2. *In situ* hybridization analysis of *Runx3* mRNA in mouse uteri. Uterine sections were obtained from 8-week-old mice. A–C: *In situ* hybridization with DIG-labeled *Runx3* antisense (A, B) and sense (C) RNA probes. The boxed area in A corresponds to the area shown in B. *Runx3* mRNA signals were detected in endometrial luminal epithelial cells (arrow), glandular epithelial cells (arrowheads), and stromal cells of the luminal side (asterisk). No signals were detected with the sense probe (C). D: Hematoxylin and eosin-stained section. Bars = 50 μ m.

Fig. 3. Morphological analysis of the uteri of *Runx3*^{-/-} mice. Representative cross sections of uteri from 8-week-old wt (A, C) and *Runx3*^{-/-} (B, D) mice are shown. Bars = 100 μ m.

group were lower than in wt stromal cells. E2 decreased *Esr1* mRNA levels in wt and *Runx3*^{-/-} stromal cells at 12 h.

Igf1: E2 tended to increase *Igf1* mRNA levels at 2 h, but statistical significant differences were not detected in wt and *Runx3*^{-/-} stromal cells. E2 increased *Igf1* mRNA levels at 12 h in *Runx3*^{-/-} stromal cells.

Fig. 4. Effect of E2 on ER α and growth factor mRNA expression in the endometrial stromal cells of wt and *Runx3*^{-/-} mice. Endometrial stromal cells were treated with E2 (10⁻⁸ M). Cells were collected at 2 and 12 h after the hormone treatment. Real-time RT-PCR was performed for quantitative analysis of *Esr1*, *Igf1*, *Igfbp3*, *Egf*, *Tgfa*, *Tgfb1*, *Tgfb2* and *Tgfb3* mRNA expression. The amount of each mRNA in wt and *Runx3*^{-/-} mice was normalized against that of *Rpl19* mRNA. Each group consisted of triplicate wells, and the data are expressed as the mean $\pm$ standard error. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from control of wt mice. # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$; significantly different from the control.

***Igfbp3*:** *Igfbp3* mRNA levels in *Runx3*^{-/-} stromal cells of the control group were lower than in wt stromal cells. E2 decreased *Igfbp3* mRNA levels at 12 h in wt and *Runx3*^{-/-} stromal cells.

***Egf*:** E2 increased *Egf* mRNA levels at 2 h in wt stromal cells, but not in *Runx3*^{-/-} stromal cells.

***Tgfa*:** E2 increased *Tgfa* mRNA levels at 2 h in wt and *Runx3*^{-/-} stromal cells, but statistically significant differences were not obtained, while high *Tgfa* mRNA levels remained at 12 h in *Runx3*^{-/-} stromal cells.

***Tgfb1*:** *Tgfb1* mRNA levels in *Runx3*^{-/-} stromal cells were lower than in wt stromal cells. E2 decreased *Tgfb1* mRNA levels at 12 h in wt and *Runx3*^{-/-} stromal cells.

Fig. 5. Effect of growth factors on DNA synthesis in cultured endometrial epithelial cells. The endometrial epithelial cells were treated with IGF1 for 48 h (A) or with EGF, TGF α , TGF β 1, TGF β 2 or TGF β 3 for 12 h (B). DNA synthesis was measured by BrdU incorporation. The results are expressed as a percentage taking the BrdU incorporation of the controls as 100%. * $P < 0.05$; ** $P < 0.01$; significantly different from the control.

***Tgfb2*:** E2 increased *Tgfb2* mRNA levels at 2 and 12 h in *Runx3*^{-/-} stromal cells.

***Tgfb3*:** *Tgfb3* mRNA levels in *Runx3*^{-/-} stromal cells were lower than those in wt stromal cells. E2 decreased *Tgfb3* mRNA levels in wt and *Runx3*^{-/-} stromal cells.

Effects of growth factors on proliferation of endometrial epithelial and stromal cells in *Runx3*^{-/-} mice and wt mice

We examined the effect of growth factors on proliferation of endometrial epithelial cells by analyzing the BrdU uptake into the nucleus.

Endometrial epithelial cells: In endometrial epithelial cells of wt and *Runx3*^{-/-} mice, IGF1 treatment significantly increased BrdU incorporation (Fig. 5A), whereas TGF β 1, TGF β 2 and TGF β 3 significantly decreased BrdU incorporation (Fig. 5B). EGF and TGF α increased BrdU incorporation in endometrial epithelial cells of *Runx3*^{-/-} mice only.

Endometrial stromal cells: IGF1 treatment did not change BrdU incorporation in the stromal cells of wt mice but did increase it in the stromal cells of *Runx3*^{-/-} mice (Fig. 6A). On the other hand, EGF, TGF α and the TGF β family proteins increased BrdU incorporation in stromal cells of wt and *Runx3*^{-/-} mice (Fig. 6B).

Discussion

Transcription factor *Runx3*-knockout mice are infertile, and their uteri are atrophied, suggesting that *Runx3* is involved in the regulation of reproductive function. Accordingly, we demonstrated the presence of *Runx3* mRNA and protein in the mouse uterus. By *in situ* hybridization, we detected *Runx3* mRNA in the endometrial luminal epithelial cells, glandular epithelial cells, and stromal cells below the epithelial cell layer on the luminal side. RT-PCR analysis confirmed that *Runx3* mRNA was expressed in the endometrial epithelial and stromal cells. *Runx3* mRNA expression within the mouse endometrium strongly suggests that *Runx3* plays important roles in the regulation of uterine functions. In addition, *Tgfb1* and *Tgfb3* mRNA expression, and E2-induced synthesis of EGF and TGF β 2 was altered in the endometrial stromal cells of *Runx3*^{-/-} mice. These alterations may be involved in the atrophy of uteri and loss of estrogen-induced proliferation of endometrial epithelial cells in *Runx3*^{-/-} mice.

Morphological observations showed that the endometrial layer and uterine glands of *Runx3*^{-/-} mice were less developed than those of wt mice. The growth of endometrial epithelial and stromal cells is regulated by estrogen and progesterone [3]. The ovaries of *Runx3*^{-/-} mice contained fewer antral follicles, which mainly secrete estrogen, and no corpora lutea, which secrete progesterone, suggesting reduced secretion of estrogen and progesterone. Recently, we found that E2 did not promote the proliferation of the endometrial epithelial cells in *Runx3*^{-/-} mice [4]. This indicates that the growth and function of endometrial cells were retarded in *Runx3*^{-/-} mice, and that the atrophy of *Runx3*^{-/-} mouse uteri was not only because of reduced estrogen and progesterone secretion but also because of a diminished response of endometrial epithelial cells to estrogen in terms of cell proliferation. Thus, it is highly probable that *Runx3* in endometrial cells is involved in the regulation of their proliferation.

ER α expression in the uterus of *Runx3*^{-/-} mice was not altered, because uterine *Esr1* mRNA levels did not differ between wt and *Runx3*^{-/-} mice [4]. However, *Esr1* mRNA levels in endometrial stromal cells of *Runx3*^{-/-} mice were significantly lower than those in wt mice. Hence, ER α expression in endometrial stromal cells may be reduced by *Runx3* knockout, although we did not specifically determine ER α levels. *Esr1* mRNA expression was down-regulated by E2 treatment in both endometrial stromal cells of both wt and *Runx3*^{-/-} mice. Thus, it is still not clear whether *Runx3* is involved in the regulation of ER expression.

The proliferation of endometrial epithelial and stromal cells is regulated by growth factors produced in endometrial cells [6, 7, 13, 19–21]. Among the growth factors produced in endometrial cells, IGF1 and the EGF family members stimulate proliferation and the TGF β family members inhibited proliferation of endometrial epithelial cells [8, 9, 11–13, 22, 23]. On the other hand, IGF1, the EGF family members and the TGF β family members stimulate DNA synthesis of endometrial stromal cells [5, 10]. In the present study, *Tgfb1* and *Tgfb3* mRNA expression in endometrial stromal cells of *Runx3*^{-/-} mice was reduced compared with that in control wt mice, implying that the decrease in synthesis of these growth factors in *Runx3*^{-/-} mouse uteri was probably associated with retarded growth of *Runx3*^{-/-} mouse uteri. *Runx3* may be involved in the proliferation

Fig. 6. Effect of growth factors on DNA synthesis in cultured endometrial stromal cells. The endometrial stromal cells were treated with IGF1 for 48 h (A), or with EGF, TGF α , TGF β 1, TGF β 2, or TGF β 3 for 12 h (B). DNA synthesis was measured by BrdU incorporation. The results were expressed as a percentage taking the BrdU incorporation of controls as 100%. *P<0.05; **P<0.01; significantly different from the control.

of endometrial cells through the production of growth factors.

The production of growth factors in endometrial stromal cells is regulated by estrogen [6, 24–26]. The TGF β family growth factors control cell proliferation [23]. The TGF β family growth factors inhibited proliferation of endometrial epithelial cells and stimulated that of endometrial stromal cells. The EGF family growth factors stimulate both endometrial epithelial and stromal cells. E2 treatment resulted in an increase in *Tgfb2* mRNA expression at 2 h in endometrial stromal cells of *Runx3*^{-/-} mice, but did not in those of wild-type mice. On the other hand, an E2-induced increase in *Egf* mRNA at 2 h was detected in endometrial stromal cells of wt mice, but not in those of *Runx3*^{-/-} mice. Therefore, it is probable that the failure of E2-induced proliferation of endometrial epithelial cells in *Runx3*^{-/-} mice occurs because of the enhanced release of TGF β 2 induced by estrogen and the reduced release of EGF.

IGF1 promotes the proliferation of endometrial epithelial and stromal cells [8–10, 27]. Moreover, the expression of *Igf1* is regulated by estrogen [24, 28–30]. IGF binding protein-3 (IGFBP-3) is known to inhibit IGF1 action and extend its half-life by binding to it [31, 32], and is considered to regulate endometrial cell proliferation [33]. Therefore, we examined the E2-induced mRNA expression of *Igf1* and *Igfbp3*. E2 treatment appeared to increase *Igf1* mRNA levels at 2 h in endometrial stromal cells of both the wt and *Runx3*^{-/-} uterus, although a statistically significant difference was not obtained because of large variations of mRNA levels of E2-treated endometrial cells. E2 treatment inhibited *Igfbp3* mRNA expression at 12 h in endometrial stromal cells of both wt and *Runx3*^{-/-} uterus. This may

suggest that Runx3 is not involved in IGF1-mediated regulation of endometrial cell proliferation.

We assessed the proliferation of endometrial epithelial and stromal cells by detection BrdU incorporation into the nucleus. In endometrial epithelial cells, IGF1 increased BrdU uptake in wt and *Runx3*^{-/-} mice, but TGFβ1, TGFβ2 and TGFβ3 decreased it. EGF and TGFα increased BrdU uptake in the endometrial epithelial cells of *Runx3*^{-/-} mice only. The endometrial stromal cells of *Runx3*^{-/-} mice responded to TGFβ1, TGFβ2 and TGFβ3, resulting in increases in DNA synthesis, while IGF1 increased BrdU uptake in the endometrial cells of *Runx3*^{-/-} mice. The discrepancy between the present study and previous studies [10, 12] may be due to differences in the culture conditions and growth factor treatments employed. However, these findings imply that endometrial epithelial and stromal cells of *Runx3*^{-/-} mice had the ability to respond to these growth factors like those of wt mice.

In conclusion, we detected local expression of *Runx3* mRNA in the endometrial luminal epithelial cells, glandular epithelial cells, and stromal cells below the epithelial cell layer on the luminal side. *Tgfb1* and *Tgfb3* mRNA expression in endometrial stromal cells of *Runx3*^{-/-} mice was reduced, probably leading to hypoplasia of the endometrium. E2 increased *Tgfb2* mRNA expression but not *Egf* mRNA expression in the endometrial stromal cells of *Runx3*^{-/-} mice. It is possible that these alterations caused by *Runx3* deletion are associated with loss of E2-dependent endometrial epithelial cell proliferation in *Runx3*^{-/-} mice. Runx3 is likely a regulatory factor responsible for endometrial growth.

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